BIODIVERSITY - BIOTECHNOLOGY: GATEWAY TO DISCOVERIES, SUSTAINABLE UTILIZATION AND WEALTH CREATION

Proceedings of the International Symposium held in Kuching Sarawak, Malaysia 19th – 21st November 2008

EDITORS

Rita Manurung
Zaliha C. Abdullah
Fasihuddin Badruddin Ahmad
Clem Kuek

SARAWAK MAJU DALAM MALAYSIA

BIOTECHCORP

Sarawak Biodiversity Centre

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PREFACE

The Sarawak Biodiversity Centre (SBC) was established in 1998 following the enactment of the Sarawak Biodiversity Centre Ordinance 1997 and the Sarawak Biodiversity Regulations 1998. In the early years, SBC's role leaned heavily towards inventory and conservation of biological resources. After the Sarawak Biodiversity Centre (Amendment) Ordinance, 2003 was passed, the role of SBC changed significantly. Now SBC's task is to initiate intensive biotechnology based research and development on Sarawak's biodiversity, especially genetic resources linked to Traditional Knowledge, in order to harness its commercial potential.

With a launching grant of RM10 million on a 50 acre plot, SBC now stands as an institution with modern facilities for carrying out Traditional Knowledge Documentation, R&D for biodiscovery, a herbarium and an ethnobotanic garden for biodiversity awareness and appreciation.

The Biodiversity – Biotechnology Symposium was jointly organized by SBC and the Malaysian Biotechnology Corporation (Biotechcorp). The theme 'Biodiversity-Biotechnology: Gateway to Discoveries, Sustainable Utilisation and Wealth Creation' was chosen as it reflected SBC's role as stipulated in the Ordinance and the overall goal for biodiversity biotechnology research nationally.

The symposium was divided into six sessions and for each session prominent speakers delivered inspiring plenary and keynote addresses. Each session was supported by a number of oral and poster presentations. The symposium brought together speakers and participants from USA, England, Scotland, China, India, Denmark, Australia, Singapore and Malaysia covering a wide array of professions such as lawyers, academicians, business entrepreneurs, policy makers, researchers and undergraduate students.

This proceeding is a compilation of some of the selected papers and posters presented in the symposium. For a complete reference of the papers and posters, please refer to the programme booklet distributed at the symposium.

As chairman of the organizing committee, I would like to thank Biotechcorp and all speakers and poster presenters for participating in SBC's first symposium. We received many requests from students and scientists from universities within the region to present their findings. Unfortunately the organizing committee had to disappoint some of them. I hope they will continue to support other conferences that SBC will organize in future.

The organizing committee, symposium advisors and all staff of SBC worked tirelessly for many weeks. Although this was not the first time that the Centre organized an international conference, it was not without much trepidation that I agreed to take up the challenge of organizing a major event and be involved in all aspects from planning until preparation of manuscripts for the proceedings. It was a rewarding experience.

Finally, one of my personal objectives in organizing this symposium was to develop organizational skills among SBC's officers and staff. They demonstrated not only that they have organizational skills but also delivered quality service, teamwork and commitment.

Thank You.

Dr. Rita Manurung
Chairman, Organising Committee
COO, Sarawak Biodiversity Centre

Session 1
Value of Biodiversity

Biodiversity Conservation and Utilisation in Sarawak

Cranbrook, Earl of

International Trust for Zoological Nomenclature, Natural History Museum, London SW7 5BD, and Great Glemham Farms, Saxmundham IP17 1LP, UK
lordcranbrook@greatglemhamfarms.co.uk

Abstract

Sponsored by the United Nations Environment Programme (UNEP), the 1992 Convention on Biological Diversity (CBD) was the first international instrument to regulate the management of ecosystems, habitats and species within a holistic framework. Adherent states have both responsibilities and opportunities for sustainable utilisation of the global biodiversity resource. Situated within one of the most important global biodiversity hotspots, these complementary factors are especially relevant for Sarawak. Since 2003, faced with the growing world biodiversity crisis, UNEP has promoted Planetary Biodiversity Inventories (PBI). These include both global programmes to discover, identify and list all species of selected taxonomic groups (e.g., amphibians, catfish, flies) and also national inventories of biota (e.g., the Swedish All Taxa Initiative). These and other examples of CBD-related actions will be examined, in order to provide a background against which Sarawak's own initiatives can be evaluated during the Symposium.

Introduction

In 1992, sponsored by the United Nations Environment Programme (UNEP), the Convention on Biological Diversity (CBD) agreed at Rio was the first international instrument to regulate the management of ecosystems, habitats and species within a holistic framework1. A decade later, in April 2002, facing up to the world biodiversity crisis, the Parties to the CBD committed themselves to achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national level (COP Decision VII/30). Subsequently endorsed by the UN Summit on Sustainable Development, this target was incorporated in the UN Millennium Development Goals. It was then realised that there existed a substantial "taxonomic impediment" to the achievement of this goal. From 2003, CBD has therefore promoted a Global Taxonomic Initiative (GTI). Related to this initiative, Planetary Biodiversity Inventories (PBI) have been launched, to include both global programmes to discover, identify and list all species of selected clades2, and also 'all taxa' initiatives (ATI) to create inventories of biota within the territorial bounds of states or nations.

Reflecting Sarawak's constitutional position, which gives considerable autonomy in matters relating to land, water and wildlife, the Malaysian delegation at Rio 1992 included the Chief Minister, supported by a professional team. In subsequent CBD meetings, Sarawak representatives have attended as part of the Malaysian delegation and with other Contracting Parties, Sarawak has thereby accepted the obligation, under Article 6 of CBD (see Appendix), to develop "strategies, plans or programmes for the conservation and sustainable use of biological diversity …".

To initiate discussion on a core theme of this conference, this presentation will offer a preliminary assessment of Sarawak's achievements in biodiversity conservation and utilisation, in the context of the goals of CBD, with particular attention to Articles 6, 7 and 8. It could also be helpful to consider further measures that might be adopted towards the sustainable utilisation of the biodiversity resource as encouraged in Article 10, and touch on dissemination and education, Article 13. Direct proposals towards these objectives made in this Plenary Address are numbered in bold**, (1) – (12).**

It is the role of a Plenary Address to stimulate constructive discussion and debate. I am grateful to several Malaysian colleagues for points of information, but the opinions and proposals in this paper are solely personal. As an outside observer, it is not unlikely that I have overlooked relevant implementations successfully undertaken by the Sarawak State Government or institutions. I

[1] Key articles from the Convention are repeated in the Appendix to this presentation.
[2] A clade may be defined as a related group of organisms with a common ancestor.

apologise in advance for any such oversights, and I hope they will be corrected in discussion so that any final version can be free of such inadequacies. Equally, some of my suggestions may seem impracticable to participants faced with the recurrent problems of financial management, the pressures of development and other important constraints that prevail in Sarawak. None the less, I hope that these ideas may lead to the emergence of positive consequences from this Symposium.

A global hotspot

As we all know, the huge island of Borneo (~ 575,000 km^2), transected by the equator, lies at the heart of western Malesia, one of three major world biogeographical regions of perhumid evergreen tropical rainforests noted for their rich biodiversity. Borneo shares many species with the other main landmasses of west Malesia, i.e., Sumatra, Java and the Thai-Malay peninsula, but also hosts a high proportion of endemics. Nowadays, in books, learned journals or, increasingly, on websites, there is a substantial and accessible resource treating the biodiversity heritage of Borneo. The totality of references is large; selected examples will suffice for present purposes. Among these, an impressive series of reference books has been published by Natural History Publications (Borneo).

The flora of Borneo amounts to 10,000 – 15, 000 species, compared with 8500 for Peninsular Malaysia (Fatawa and Mori, 2000). Dipterocarps are especially numerous, with 267 species recorded of which 155 are endemic. Species area curves for trees from Borneo sites are exceeded only by a plot from Peru (Morley, 2000). Early work at the LTER plot at Lambir FR, Sarawak, identified 1083 tree species (278 genera, in 72 families) in the 52 ha plot (Chai *et al.,* 1995*).* On a larger scale, Mulu National Park (544 km^2) is estimated to support 3500 plant species (about 1500 being trees) and 8000 fungi (Hazebrook and Abang Kashim 2001). Ongoing specialist studies include a multi-volume *Orchids of Borneo* (ed. P. J. Cribb) published by the Sabah Society in conjunction with the Royal Botanic Gardens, Kew.

Animal diversity is also exceptional. For example, a preliminary list of mammals of Borneo posted on Wikipedia by Mohd Tajuddin Abdullah3 comprises 288 terrestrial species, of which 102 are bats and 61 rodents, plus 91 marine species within territorial waters, i.e., about 7.2% of the global total of ~5400 species. Among terrestrial mammals, the world checklist identifies 50 species as Borneo endemics (Wilson and Reeder, 2005). Davison (1999) listed 622 bird species, Das and Ghazally (2001, 2002) 105 lizards, plus crocodiles and turtles, and Inger and Stuebing (2005) 150 frogs. Invertebrate groups are strikingly numerous: the 'macro' moths alone amount to ~4500 species (Holloway, 1985 – 2008), the phasmids exceed 350 species (Bragg, 2001), and the dragonflies 275 species (Orr, 2003). 550 species of one beetle family (Cerambycidae) were found in ~1000 ha study plot in E. Kalimantan (Fatawa and Mori, 2000).

CBD Preamble: "Conscious of the intrinsic value of biological diversity and of the ecological, genetic, social, economic, scientific, educational, cultural, recreational and aesthetic values of biological diversity and its components, …"

There can be no doubt about the economic value of Sarawak's biodiversity resource. For instance, timber extraction from natural forests has been a huge contributor to the State revenue. The importance of wild animal meat in rural diets has been highlighted by Bennett *et al*., 2000), and the (unquantified) contribution of freshwater fisheries is significant (Latiff and Zakri, 1998). Managed exploitation of natural colonies of edible-nest swiftlets can lead to personal prosperity (Lim and Cranbrook, 2000). Many other biodiversity resources, and their untilisation in Borneo, are listed by MacKinnon *et al.* (1996).

The formulation of CBD in 1992 was preceded by the report of the World Commission on Environment and Development, entitled *Our Common Future* (1987). This roused international debate on the concept of "sustainable" utilisation of natural resources. The hot issue of sustainability of tropical forest management (Poore, 1989) prompted the Chief Minister's invitation to the ITTO Mission to Sarawak of 1988-89. The Mission report concluded that current logging practices were not sustainable, but could become so with certain specific actions. The debate on sustainable logging has

[3] See http://en.wikipedia.org/wiki/Mammals_of_Borneo

now moved on with the establishment of the Forest Stewardship Council and Malaysian Timber Certification Council, which includes representation from Sarawak. The regional debate on many other aspects of nature conservation and sustainability rumbles on (Wu, 2007). Without ignoring the importance of this issue, for positive discussion at this conference let us look at some key articles of the CBD and their implications for Sarawak.

CBD Article 7. Identification and monitoring

(a) A state inventory of biodiversity

Although many of the original data may have derived from Sarawak, the bulk of published sources are pan-Bornean in treatment. More closely Sarawak-oriented inventories are needed in order to conform with CBD Art. 7, and to provide the baseline from which to assess progress towards the Millennium Goal of reduction in the rate of biodiversity loss by 2010. The production of such inventories is the stated objective of Sarawak Forestry4. Relevant achievements include publication of the 3-volume *Tree Flora of Sabah and Sarawak*, establishment of the Sarawak Herbarium, and the accumulation of information on plants used by local communities for food, medicines, handicraft and other traditional uses The publication of pocket guides to Sarawak flora, such as pitcher plants (Clarke and Chi'en Lee, 2004) or gingers (Poulsen, 2006), contributes to this objective, while also meeting requirements for public education. More cogently, the publication of field guides, and the dissemination of the means of identification by other means (including web sites) laudably encourages the assimilation of volunteers and "parataxonomists" into the official drive towards CBD objectives.

Animal biodiversity is much higher than that of plants, so that the task of compiling Sarawak State inventories is more demanding. Atack's (2006) field guide to fishes of Kuching rivers is commendable, but a comprehensive guide to Sarawak's rich freshwater fish fauna is lacking. Watson (1986) provided a list of Sarawak mammals, but his source is now out of date and a revised compilation needs to draw on new knowledge. With evident deficiencies for planning purposes, it is reasonable to propose that effort should be dedicated to **(1) abstraction of Sarawak inventories of animal species from existing pan-Borneo literature and other published sources.**

The zoological collections (off display) of the Sarawak Museum (SM) are used for teaching to a limited extent, and for reference by visitors and members of the voluntary community such as the Kuching branch of the Malaysian Nature Society. Whether remaining at SM, or transferred to another holder, these historically important specimens remain an under-used and poorly known resource. Many museums, world wide, have by now digitised their catalogues and many of these electronic versions are publicly accessible via websites. Production of a digitised catalogue would be fully compatible with the aspirations of the UNDP (Malaysia) project to set up a national natural history museum (NHM) (Ng, 2008). Regardless of progress on development of the national NHM, **(2) a digitised catalogue of the zoological collections of the Sarawak Museum, together with other collections in secure repositories elsewhere** (e.g., the entomology section of SFC, or Unimas), will enhance the State's capacity to meet the objectives of GTI. The exercise would also build upon local expertise, potentially draw from international experience through technological transfer, while creating a digital centre for communication, education and public awareness of biodiversity (CBD Art. 13).

The inability to identify (or obtain identifications of) specimens is a major component of the taxonomic impediment. The GTI recognises that, while resources for the identification (or description, if new) of species of large organisms, e.g., trees or vertebrate animals, are generally available, the most biodiverse groups are invertebrates, fungi and micro-organisms. These require expert skills for correct identification and, indeed, most of them have not been categorized or given formal scientific names. The increasing deployment of molecular technologies has provided a novel tool to investigate taxonomic problems. It has still been estimated that, while only 10% of vertebrates remain to be described, greater than 50% of terrestrial arthropods and up to 95% of nematodes and protozoa are undescribed.

One approach to tackling this deficiency, through global inventories of large or small clades (PBIs), is likely to produce many species that are undescribed or otherwise require major revision to

[4] See *forestry.Sarawak.gov.my/forweb/research/frc/facility/botany.htm*.

complete their taxonomy. New techniques can assist. Biotechnology has enhanced the researcher's capability to define taxonomic boundaries and, sometimes, to identify cryptic species. Molecular evidence can also assist the understanding of evolutionary relationships (Ghazally Ismail, 1998; Campbell *et al.,* 2004; Jayaraj and Abdullah, under review; Kho *et al.,* in prep).

Some PBIs are supported by public money: in 2003, four awards were made by the US National Science Foundation for the study of plant bugs (Miridae), slime moulds (Eumycetozoa), the genus *Solanum* (containing nightshades, tomatoes, potatoes and related plants) and catfishes (Siluriformes). Comparable initiatives have also arisen by coordinated action of specialists. A botanical example is the Species Plantarum Project, set up in 1995 under the International Organisation for Plant Information, affiliated to the International Union of Biological Sciences (IUBS). Instructions to contributors and accounts of five relatively small families have already been published in hard copy, and accounts of two further families totalling nearly 1000 species in press (Brummitt *et al.,* 2001*)*, perhaps now published. A zoological parallel is the BioSystematic Database of World Diptera, of which the current version 10 contains nomenclatural data for an estimated 98% of all named flies, amounting to 156,599 species, in 11,671 genera and 154 families (Evenhuis *et al.,* 2008). To accomplish the huge task of a global inventory of a large clade, each PBI must engage a multinational team of taxonomic experts and involve institutions with biological research collections. In building up the State biodiversity database, it would be beneficial for Sarawak (3) to develop active awareness of relevant PBIs and to offer partnership, if only as a location for associated field studies.

Of alternative approaches, the first is habitat based. For example, freshwater animal diversity is astonishingly rich and at the macro-biotic level exceeds marine biodiversity. The global freshwater animal diversity assessment (FADA), involving a consortium of more than 100 taxonomists, has compiled world data on the diversity and distribution of vertebrates, insects, crustaceans and a suite of minor phyla (Balian *et al.*, 2008). Individual Sarawak river systems have been sampled, mainly for fish and fisheries (e.g., Anon, 1986), but **a State-wide synthesis of the freshwater biodiversity resource is needed (4).** Other habitats of importance that could be treated in a State inventory include estuarine waters and mangrove.

A second alternative approach is the local, regional or national "all taxa" initiative (ATI). An example at the national scale is the Swedish Taxonomy Initiative to create a total inventory and develop keys for the identification of all multicellular organisms of Sweden. Started in 2002, the work has since discovered so many new species (for instance, 2000 previously unknown insects, about one-third of which are new to science) that the time scale will certainly be extended (Ronquist, 2008); the estimated 20-year cost runs to US$ 200 million.

Such huge sums of money appear daunting but, if spread over time and through existing institutional resources, the investment is not unattainable. Within annual research budgets, Sarawak Forestry has promoted multi-taxa inventories (MTI) of the State's National Parks, published in the house journal *Hornbill* and elsewhere. External funding supported the first coordinated project of SBC, the survey of the Bau limestone area (Yong *et al.,* 2004). This was a good example of a local MTI applied to a limited area of distinctive topography and land settlement. The human effort and overall budget of this exercise provide a model for similar studies under Sarawak conditions, such as Sarawak Forestry's current study on the Pedawan/Serian limestone area. The results of these, and comparable studies by others, can be collated. **(5) An overview of the local inventories already achieved could then lead to a coordinated programme towards a full State inventory.**

Art 7. (b) Monitoring

Nature is dynamic and, when people intervene, for instance through hunting, agriculture, forestry or mining, natural systems are disrupted, species populations altered and the balance of communities shifted. Article 7 (2) of CBD requires monitoring "through sampling and other techniques". The techniques required by a monitoring are likely to be the same as those useful in sampling. Fifty years ago, the mist-net revolutionised knowledge of Sarawak birds (particularly understorey passerines), and monel-metal cattle ear-tags (provided by Great Glemham Farms) were used to initiate the tagging of turtles on Talang-talang and Satang. New technologies once again offer new insights into the distribution and behaviour of animal species: for instance, harp nets for bats, implanted transponders for small animals, satellite tracking by gps and new, compact, long-lasting and weatherproof cameras for photo-trapping, with or without lures, still and video, by natural light or by infra red. Already, photo-

trapping has been used to monitor wildlife in Sarawak planted forests (Giman *et al*., 2007) and to investigate the distribution of the rare bay cat *Catopuma badia* (Mohd-Azlan and Sanderson, 2007). **(6) For cross-comparisons between place and time, monitoring sites should be selected and methods and protocols need to be standardised.**

Art 8. *In-situ* conservation

(a) Protected areas: selection, management and protection

Since the first National Park at Bako in 1958, Sarawak has undertaken a progressive policy in the selection and designation of Totally Protected Areas (TPA), as National Parks, Wildlife Reserves and other designations. The programme is still unrolling and it is encouraging that local communities are increasingly involved. In the case of the Bungoh range, decisive support for a new NP came from the Bidayuh communities of the Krokong area (pers. obs.). A long-term target for TPAs had been 10% of the State area; the present achievement is ~ 700,000 ha, i.e about 5.4% of the State area of 13 M ha. However, the natural richness of the tropical biota creates special difficulties in Sarawak. Many of the components of the extraordinarily rich biodiversity are rare, or localised in distribution, creating special problems for in-situ conservation. For rare organisms (whether forest trees or herbaceous plants, or vertebrate or invertebrate animals) exceptionally large areas may be needed for effective conservation of viable populations (7) It is important to keep a perspective on the programme of TPA designation, in the light of the known distribution, population densities and ecological requirements of individual species.

The management of TPAs and protection of biodiversity within their boundaries may require effective management of people outside the pay-roll of protection and enforcement agencies. Some Sarawak TPAs with open boundaries, easily accessible from adjoining land, are particularly vulnerable to illegal intrusion and consequent depletion of the biodiversity resource. For instance, I myself have witnessed wholesale illegal extraction of timber from Samunsam. Regulations encouraging the appointment of Honorary Wildlife Rangers have been introduced, and indicate one route whereby local community involvement can be enlisted. Education and out-reach programmes are important aspects of the management and protection roles. But, experience elsewhere in the world indicates that **(8) enduring biodiversity protection is dependent on the widespread acceptance by people, at all levels of society, of the values of natural biodiversity and the objectives of protective legislation (CBD, Art. 13)**.

Art. 8 (b) Rehabilitation, restoration and recovery

Sarawak is a large State. The ordinary visitor, especially when travelling along established routes such as the main highways, is struck by the extent of areas of apparently degraded forest land. Of course there are recognised ownership and agronomic factors involved, and social, economic, regulatory and other reasons why reforestation is not at all easy. From the perspective of scientific ecology, however, projects and programmes to replant native forest species are a first step towards biodiversity restoration, for instance, Samajaya at Setutong. Native tree plantations, even of small size, are likely to attract and maintain populations of dependent or semi-dependent small animal species, invertebrate or vertebrate, and are being encouraged by the “Trees for life” project (MNS and SF). **(9) Encouragement of the re-establishment (on any scale) of native timber trees could improve the prospects of sustainability of ecologically linked animal communities.**

Nineteenth-century travellers’ accounts, past tales of hunting prowess, and other anecdotal sources together point out the losses of large vertebrate diversity that have occurred in Sarawak in historic times. Archaeology has exposed significant changes in the large mammal fauna over a longer time scale. The principal site in Sarawak, at Niah, is well known; other sites at Jambusan (Bau district) and Sireh (Serian district), and at Madai cave, Sabah, have yielded important confirming data. Identification of the animal remains recovered from these excavation have shown that the past distributions of orang-utan, Sumatran rhinoceros and banteng were much wider than historic records suggest. Moreover, three large mammals are shown to have been present in Sarawak, in each case possibly within the past 1000 years and even within the past couple of centuries: Javan rhinoceros, Malay tapir and tiger (Cranbrook and Piper, 2007). Piper and Cranbrook (2007) have offered a worked proposition for the reintroduction of the Malay tapir in the Grand Perfect concession. **(10) The**

possibility of managed re-introductions of Malay tapir from Peninsular Malaysia (or Sumatra), or Javan rhino from Java, deserve professional evaluation.

Art. 8 (c) Prevent the introduction of, control, or eradicate invasive species

Alien herbaceous or bushy plant species abound in Sarawak, particularly in cultivated or disturbed areas including roadsides, paths and gardens (e.g., sensitive plant, Siam weed, the smothering creeper *Mikania*). Among bird-dispersed tree species, the alien 'ceri' *Muntingia*, is widespread in waste land at the edges of cultivation and, because of its edible fruits, often in fact encouraged by rural people or urban householders. So far as I know, these plant introductions have not invaded undisturbed natural vegetation. The expansion of *Acacia mangium* plantations, however, merits a watch. As yet, this alien tree has not penetrated closed forest but it is clearly spreading in disturbed or secondary habitat.

The aquatic habitat is especially at risk. The ITTO Mission's report identified the impacts of logging on freshwater habitats as being severe, and the greatest impediment to sustainability. The heightened turbidity of the great rivers of Sarawak, so visible from the air, remains witness to persistent environmental deterioration. The additional impacts of introduced species on the native aquatic fauna are unquantified. The introduced American bullfrog is thought to displace native amphibians. The fish faunas of ditches, ponds and small streams have been altered by escapes from fish ponds, such as *Tilapia* varieties, and releases of exotic aliens from aquariums, such as members of the carnivorous South American piranha group. The extent of affected waters has not been surveyed in detail. **(11) The distributions of alien aquatic animals in the freshwater environment need to be surveyed, and regulations to prevent further releases of alien species need to be enforced.**

Urban bird communities have changed dramatically in past decades. The first appearance of the tree sparrow *Passer montanus* was monitored by Harrisson (1974), and the species is now pervasive. Mynas *Acridotheres tristis* and *A. cinereus* also appeared from the 1970s, and have multiplied in the Kuching area and elsewhere (Davison, 1999; pers. obs.); their nest parasite, the koel *Eudynamis scolopacea* will surely increase accordingly. These introduced species seem to have ousted others of similar habits, including the earlier introduction, Java sparrow *Padda oryzivora.* More to be feared is the house crow *Corvus splendens*, an unspecialised predator that has spread phenomenally in Peninsular Malaysia. **(12) Vigilance is necessary to detect the arrival of further alien birds; the house crow presents a high risk to local biodiversity and, if it appears, should be eradicated at once.**

CBD Article 10. Sustainable Use of Components of Biological Diversity
Integrate consideration of the conservation and sustainable use of biological resources into national decision-making;

CBD Article 18. Technical and Scientific Cooperation
Promote international technical and scientific cooperation in the field of conservation and sustainable use of biological diversity.

A dozen suggestions for action have been made, above. In many cases, cooperative engagement with another local body, or a foreign institution or international agency, could assist in implementation. Whether or not these suggestions are adopted, the test of fulfilment of Articles 10 and 18 requires clarification. In short, which Minister, Department or agency should grasp the varied and complex issues involved in a strategic overview of the sustainable use of the components of biological diversity in Sarawak, and integrate consideration of these into State decision-making?

The first Sarawak Biodiversity Centre Ordinance, 1977, set up a grand vision of SBC with the defined roles (under Section 5), not only of providing Government and other approved institutions with "accurate (!) information or data" on the State's biodiversity, but also *inter alia* to determine policies and guidelines for the use of biological resources, to keep records, hold collections, identify research priorities, establish linkages within Malaysia or outside the country, and generally to promote education and understanding of biodiversity.

For a variety of reasons, this broad, all-embracing, powerful and independent new agency encountered obstacles and objections that led to the curtailment of its remit. The Sarawak Biodiversity Centre (Amendment) Ordinance, 2003, enacted a heavily revised S. 5 which led to the four current functions of maintaining a library of extracts, undertaking research into traditional usage of biodiversity among Sarawak communities, providing screening facilities for bioactive compounds, and establishing partnerships (within Malaysia or overseas) to develop products from the biological resources of the State.

In addition to the new SBC, at its foundation in 1997, there already existed long-established and well-resourced State agencies with functions that partly met the requirements of CBD, including departments of forestry (and SFC), agriculture, fisheries, the museum, and the growing number of university biology departments. Each of these contributes to the target, but it is not evident to me that all of them mutually cooperate towards the objectives of CBD. Nor is it evident that theses bodies have a coordinated approach to international funding, or a shared awareness of opportunities for international cooperation with initiatives such as the UK Natural Environment Research Council's strategic biodiversity theme (Peck, 2008), or the British Ecological Society's Tropical Ecology Group (Gosling, 2008).

With the intention, as I have already said, of promoting (or provoking) discussion, I make a final proposal, that, in order to fulfil the obligations of a Contracting Party to CBD to integrate consideration of the conservation and sustainable use of biological resources into national decision-making, and to promote international technical and scientific cooperation in the field of conservation and sustainable use of biological diversity, thereby releasing for the benefit of the people of Sarawak the full value of the State's biodiversity resources, there needs to be a single over-arching body, with an adequate budget, that will bring together, on an equal footing, all available expertise from the diversity of relevant State departments, agencies, institutions, universities and research centres, together with experienced individuals and voluntary conservation bodies.

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Appendix

Article 6. General measures for conservation and sustainable use

Each Contracting Party shall, in accordance with its particular conditions and capabilities:

(a) Develop national strategies, plans or programmes for the conservation and sustainable use of biological diversity …; and

(b) Integrate, as far as possible and as appropriate, the conservation and sustainable use of biological diversity into relevant sectoral or cross-sectoral plans, programmes and policies.

Article 7. Identification and monitoring

Each Contracting Party shall, as far as possible and as appropriate, in particular for the purposes of Articles 8 to 10:

(a) Identify components of biological diversity important for its conservation and sustainable use having regard to the indicative list of categories set down in Annex I;

(b) Monitor, through sampling and other techniques, the components of biological diversity identified pursuant to subparagraph (a) above, paying particular attention to those requiring urgent conservation measures and those which offer the greatest potential for sustainable use;

(c) Identify processes and categories of activities which have or are likely to have significant adverse impacts on the conservation and sustainable use of biological diversity, and monitor their effects through sampling and other techniques; and

(d) Maintain and organize, by any mechanism, data derived from identification and monitoring activities pursuant to subparagraphs (a), (b) and (c) above.

Article 8. *In-situ* conservation

Each Contracting Party shall, as far as possible and as appropriate:

(a) Establish a system of protected areas or areas where special measures need to be taken to conserve biological diversity;

(b) Develop, where necessary, guidelines for the selection, establishment and management of protected areas or areas where special measures need to be taken to conserve biological diversity;

(c) Regulate or manage biological resources important for the conservation of biological diversity whether within or outside protected areas, with a view to ensuring their conservation and sustainable use;

(d) Promote the protection of ecosystems, natural habitats and the maintenance of viable populations of species in natural surroundings;

(e) Promote environmentally sound and sustainable development in areas adjacent to protected areas with a view to furthering protection of these areas;

(f) Rehabilitate and restore degraded ecosystems and promote the recovery of threatened species, inter alia, through the development and implementation of plans or other management strategies;

(g) Establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health;

(h) Prevent the introduction of, control or eradicate those alien species which threaten ecosystems, habitats or species;

(i) Endeavour to provide the conditions needed for compatibility between present uses and the conservation of biological diversity and the sustainable use of its components;

(j) Subject to its national legislation, respect, preserve and maintain knowledge, innovations and practices of indigenous and local communities embodying traditional lifestyles relevant for the conservation and sustainable use of biological diversity and promote

their wider application with the approval and involvement of the holders of such knowledge, innovations and practices and encourage the equitable sharing of the benefits arising from the utilization of such knowledge, innovations and practices;

(k) Develop or maintain necessary legislation and/or other regulatory provisions for the protection of threatened species and populations;

(l) Where a significant adverse effect on biological diversity has been determined pursuant to Article 7, regulate or manage the relevant processes and categories of activities; and

(m) Cooperate in providing financial and other support for in-situ conservation outlined in subparagraphs (a) to (l) above, particularly to developing countries.

Article 10. Sustainable use of components of biological diversity

Each Contracting Party shall, as far as possible and as appropriate:

(a) Integrate consideration of the conservation and sustainable use of biological resources into national decision-making;

(b) Adopt measures relating to the use of biological resources to avoid or minimize adverse impacts on biological diversity;

(c) Protect and encourage customary use of biological resources in accordance with traditional cultural practices that are compatible with conservation or sustainable use requirements;

(d) Support local populations to develop and implement remedial action in degraded areas where biological diversity has been reduced; and

(e) Encourage cooperation between its governmental authorities and its private sector in developing methods for sustainable use of biological resources.

Article 13. Public education and awareness

The Contracting Parties shall:

(a) Promote and encourage understanding of the importance of, and the measures required for, the conservation of biological diversity, as well as its propagation through media, and the inclusion of these topics in educational programmes; and

(b) Cooperate, as appropriate, with other States and international organizations in developing educational and public awareness programmes, with respect to conservation and sustainable use of biological diversity.

Article 18. Technical and scientific cooperation

The Contracting Parties shall promote international technical and scientific cooperation in the field of conservation and sustainable use of biological diversity, where necessary, through the appropriate international and national institutions.

Biodiversity in Malaysia: The Most Undervalued Natural Asset

Latiff, A.

Faculty of Science and Technology, Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor, Malaysia
latiff@ukm.my

Abstract

Malaysia is well endowed with rich and diverse natural asset which has been in the custodian of indigenous communities for ages. Efforts to conserve and sustainably utilize the diverse ecosystem, rich flora and fauna and the possible use of genetic materials have been taken through the national and state efforts in establishing National Parks, State Parks, Wildlife Sanctuaries, Virgin Jungle Reserves and other protected forests. To-day a total of 1.39 million ha (about 7.6%) of the forest of all types have been set aside for in situ conservation of biodiversity. It is hoped that these protected areas have captured most of the diverse and useful species of plants and animals found in various ecosystems. Biodiversity affects the lives of rural Malaysians more than it does to the more affluent urban communities. Much of the advances in agro-industries, forestry, bioprospecting, supply of clean water and electricity, flood control and maintaining of ecological functions and balance had impacted the structure, organization, traditional practices and culture of rural communities that had contributed to impoverishment of their life style. Indigenous culture especially the use of medicinal plants is often kept out of the definition of physical and social development. There are evidences that diversity has become tenets of great religions, traditions and beliefs. There are also examples both at global and local levels where the diverse culture, through uses of resources have contributed to our understanding of human's dependence on the complexity of biodiversity. However, developing and marketing biodiversity to create new wealth can be a complex and frustrating task. It is often said by those with experience that, “Developing biodiversity is more difficult than conserving it!”. There are many reasons for this, and some are less obvious. Underlying all of them is the need to recognise the science value chain and the different techniques and competencies required to manage and package biodiversity for strategic planning and management programmes. The science value chain can broadly be described in the following sequence : idea, proof of concept, working prototype, early customisation and installation of products and commercialization. The traditional sellers and international buyers of biodiversity usually operate in different paradigms with different view points of the same subject. This paper attempts to illustrate a framework within which biodiversity can support commercialization of traditional knowledge such as in biodiversity prospecting and eco-tourism.

Introduction

Malaysia is one of the 12 megadiversity countries of the world. These 12 countries altogether contain at least 60% of the world’s known species of living organisms. In Asia among the biodiversity rich countries are China, India, Indonesia, Papua New Guinea, Malaysia, Thailand, The Philippines and several others. Malaysia is situated in the western part of Malesia and between two great floras and faunas, the Asiatic flora and fauna in the north and the Australian flora and fauna in the south. The flora and fauna of Malaysia is exceedingly rich and had been conservatively estimated to consist about 15,000 species of flowering plants (Bidin and Latiff, 1995) and more than 1,170 species of ferns (Parris and Latiff, 1997). For Peninsular Malaysia alone, more than 8,500 species of seed plants have been catalogued (Turner, 1995). The tree taxa were well treated taxonomically through the Tree Flora of Malaya project (Whitmore, 1972, 1973; Ng 1978, 1989) and the Tree Flora of Sabah and Sarawak (Soepadmo and Wong, 1995; Soepadmo *et al.*, 2004). For Peninsular Malaysia there are more than 2830 tree species were enumerated and over 26% of these tree species are endemic (Ng *et al.,* 1990). However, the fate of non-tree taxa is not well documented as well as the tree taxa, except for orchids, grasses and palms, begonias and several other groups.

The fate of the Malaysian fauna is no better as the country still lack a comprehensive fauna. Except for the avifauna, fish fauna, herpetofauna, mammals and several other groups, most of the

insects are unknown. Many more species are yet to be described but their habitats are consistently being destroyed and degraded.

Malaysia has undergone and is still undergoing tremendous physical and socio-economic development, especially in the last 20 years or so, paving the way for future industrialisation by the year 2020. In the process of achieving that end through exploitation of natural resources some forms of endangerment to the environment and biodiversity was inevitable. It is quite natural to expect a continuous process of habitat transformation, forest fragmentation, conversion of forest lands to agriculture and destruction of rural landscape which have taken great and significant toll of our rich biodiversity to leave a tremendous impact on our environment. Although this concomitant loss of habitat and also species has not been quantified scientifically, by and large the scientists, public and the administrators in Malaysia recognised the gradual but steady loss of biodiversity. These losses are real and rationally it should be addressed before it is too late.

A brief Introduction to biodiversity

Biodiversity may be introduced to the public at three levels of organisation, namely the ecosystem or community, species and genetic diversity. From the coasts to the mountains there is a spectrum of natural ecosystems, each contains a very specific community of plants and animals, including the microbes. These biotic components together with the abiotic components have made the ecosystem functioning very well not only for their inhabitants but also for human populations through ecosystem services. The ecosystem services have been free and yet humans were not grateful to them. The species diversity has been realized by numerous taxonomic studies either at family or genus level. It also may be realized through the study of taxonomic composition of the ecosystem. However, genetic diversity is still in its infancy stage.

Over the years there had been tremendous environmental degradation and forest loss due to socio-economic development that was planned and implemented both by the state and federal governments. The irony is that much of the biodiversity resources, especially those of species and genetic diversity have yet to be understood, studied and documented, and hence would definitely impede our efforts to better utilise them sustainably for our own benefits and those of generations to come.

The diversity of biological resources provides direct economic benefits such as timber products and some non-timber goods in the forestry sector, such as rattans, bamboos, fruits and others. Biodiversity provides food security for the nation and industrial crops for the agricultural sector, and food in the fisheries sector. Certain indigenous plants, animals and their derivatives have long been used in traditional medicine by various ethnics in Malaysia. Many plants, not presently used in traditional medicine, and some have also been found to contain biologically active compounds that are likely to be starting materials for a large number of drugs. A case in point is the recent discovery of chemically active compounds in the bark of *Callophylum lanigerum* and *C. teysmanii* which have positive reactions against the HIV virus. On the basis of the above, there is tremendous prospect to promote the development of natural products as an industry in this country.

Biodiversity for biotechnology

The world community also recognizes the sovereignty of our natural resources and they also recognized some forms of equitable sharing of the biodiversity's monetary and non-monetary values, to spur biotechnology cooperation, and to establish mechanisms (such as GEF) to finance some investments in maintaining and utilizing biodiversity, especially in the biodiversity-rich countries, like ours.

Over the years, we have heard a number of foreign scientists who talked about the potential of using biotechnology to realize the commercial potential of food crop productivity, fisheries harvest, producing fine chemicals and even in waste management. And I envy such high hopes and dreams of those learned scientists! These scientists knew what they were talking about, and many of them based on their personal experience. More relevant to all of us when I realized that Malaysia could offer such resources to realize the biotechnological dreams of those scientists from the developed countries, working together with the local scientists. More relevant is, for a start, such a rich resource could be

harness by the local scientists either working locally or in collaboration with foreign scientists to develop certain commercial products through biotechnology.

However, we were also told that many small and smart start-up companies in western countries suffered from backlash of investments in biotechnology. Most venture capital companies also realized that biotechnology is expensive and time-consuming. That is why we felt a little perturbed and sometimes emotionally upset when the local scientists, policy makers and science managers envisaged biodiversity promises many potential for local biotechnology. Many people tend to equate our potential with those that exist in many laboratories of the developed countries. As a scientist I have visited many laboratories in Japan, South Korea, Germany and England, and we can say, without fear, that our laboratories are 50 years behind them. Thus, it poses several questions on our real and perceived capacity to address these issues. For thought, we have a couple of questions to ask these prophets of science. How many bacterial and fungal isolates have we made in our own laboratories in the last 10 years that promise certain break-through in biotechnology? We know some local scientists worked with their counterparts in developed countries, but we did not know of their progress. How many of them that shows promises in fermentation or industrial technology and medical technology that could be developed by local industries? We were informed that there are no local industries with adequate venture capital which dare take the plunge in this biotechnology.

How many genetic transformations of food crops have we produced for our agriculture? We also knew some of our scientists had made break-though in this field in our oil palm and rubber biotechnology. More relevant this is now that, the Honourable Prime Minister has wanted Malaysia to realize our food security through intensive agriculture. We are sure many budding, small and medium industries in agriculture will mushroom our industrial landscape in the next 10 years. Recently, we read in the local paper that a prominent Chinese rice breeder has been invited to develop certain rich cultivars in our own paddy fields. Is this a way forward?

Many more questions follow. How many fine chemicals have we isolated and characterized in the last 10 years to harness pharmaceutical industries? All we heard that foreign chemists had done so from our plants with the help of the locals. How many of these will make to the pharmacy shelf in 10 years from now? Of course, I believed that somewhere out there, there are answers that I do not think of, as of now. Perhaps the advent of high-throughput screening for secondary metabolites will deeply affect the field of natural products and chemical modeling and simulation. One foreseeable consequences of this new scientific context, where massive data are produced exclusively at the molecular level, is an increased emphasis on constructive modeling approaches, starting from molecules to produce pharmaceutical products.

In the medical science, on the other hand, the completed human genome project may also hints some answers to some of the world worst enemies such as cancer, etc. As a plant taxonomist who professes some humble philosophy of enhancing local scientific capacity in biodiversity, much is yet to be done to biodiversity before we could talk about using biotechnology to realize our dreams that are product-driven. We need to know the mechanisms of harnessing ecosystem services to assist biotechnology and we need to know how discoveries of new strains of bacteria and fungi in our local ecosystems could directly help biotechnology. Sometimes, we also asked ourselves do we have the human resources and the laboratory capacity to address such issues in modern biotechnology. Perhaps the various research institutes that are planned for the country's Biovalley have some answers.

Economic benefits

In the past the country had relied heavily on timber from the most productive lowland dipterocarp forests and the two most important imported agricultural crops, rubber and oil palm as foreign exchange earners. The logged forests lands gave rise to agro-plantations, oil palm and rubber plantations as well as other commodity crops. These are the three natural major earners, in addition to pineapples, pepper, cocoa, etc. In the light of depleting hard-wood and medium-wood timber resources, the country has gone to agro-plantations. Millions of hectares are grown with *Acacia mangium, Gmelina arborea* and some other exotic species for the supply of soft wood products. However, much of the present day foreign exchange has been derived from non-timber resources such as rubber-wood, rattans, palms, resins, fruits, medicinal plants and orchids (de Beer and MacDermott, 1996). The genetic diversity of these non-timber resources are being threatened by

genetic erosion as a by-product of excessive logging. In particular the advancement in floriculture and biotechnology is going to be dependent on indigenous biodiversity. Lately biotechnology has helped tremendously in the introduction of Timber-Latex Clone for rubber, excellent breed of oil palms and other commodity crops. These augurs very well for agro-industrialisation of the country in light of depletion petroleum.

Food security

The genetic diversity of our staple food (*Oryza sativa*) and commodity crops such as rubber has been consistently eroded by changes in climatic and water regimes both in the country as well as in other agricultural countries. Elsewhere genetic engineering and biotechnology are being utilized to improve the current cultivars and hence produce more rice for the world. However, such an undertaking will not go easy with the more conservative consumers. The supply of edible fruits and nuts from the forests and village orchards has been irregular and threatened. This has affected the local village market. Realising the population is increasing steadily annually, the country must ensure that food security problems are addressed so that the future generations will not go hungry and malnutrition prevails.

Value of traditional knowledge

In 1992 the Convention of Biological Diversity (CBD) was held at Rio de Janeiro, Brazil, in conjunction with the United Nations Conference on Environment and Development (UNCED). The CBD is the most important document discussed and negotiated by many countries and parties and subsequently it paved the way for establishing the Global Environmental Facility, a global-scale funding mechanism, Conference of Parties (COP), a global negotiation forum, among others. As of February 1995, a total of 168 countries have signed this Convention, while in April 1995, already 118 countries had ratified it. Malaysia signed it on 29th December 1993, thus becomes a party to it. The CBD has been considered as one of the most significant and far-reaching environmental treaties ever developed. However, from the on-set of negotiation of CBD until to-day there is an underlying dissension between countries of the North and of the South which lies on the fact that for the former biodiversity is essentially a global issue, thus belonging to the world community. Conversely, developing countries of the South tend to show a strong 'country-driven' approach in order to make use of their biodiversity for economical and developmental benefits and to ensure equity in the transfer of appropriate technology for application in agriculture, forestry and industry.

Malaysia, having ratified the CBD is working towards incorporating into its national policies and planning a set of commitment under the treaty. Thus it is facing this global challenge with a few initiatives at national and institutional levels. By far the most significant steps taken by the country is to initiate a Biodiversity Country Study and to formulate the national policy on Biological diversity. One of the important aspect of the Biodiversity country study is to present the state-of-the art of the socio-economic factors affecting biodiversity. With increasing population there will be increasing demands for infrastructure development, lands and resources. And cultural factors can play a significant role in use of biodiversity and the conversion of forest land to agriculture. This conversion may have a major effect upon the appearance of the landscape especially in rural areas.

The national policy on biological diversity includes a policy statement, "to conserve Malaysia's biodiversity and to ensure that its components are utilised in a sustainable manner for the continued progress and socio-economic development of the nation". This motherhood statement is formulated in such a manner that it observes the ethics and the inherent right to existence of all living forms which is deeply rooted in the religious and cultural values of all Malaysians. One of its objectives is to enhance scientific and technological knowledge and educational, social, cultural and aesthetic values of biodiversity.

Biodiversity for ecotourism

To-day we are engaged in speeding up losses in species and their ecosystems, some knowingly and others unknowingly. Our understanding of the value of ecosystems, species and genes is so low that we sacrificed most of the components that may ensure our survival. Until the industrial revolution, the effects of human activities were local, or at worst regional, rather than global. All the great civilisations

of the past have cleared land for cultivation, introduced plants and animals from elsewhere, and caused lasting change. The consequence of such industrial revolution include population growth, huge growth in consumption of the resources and saturation of its sinks, notably the river systems and oceans. Higher standards of living inevitably involve higher consumption of food and more waste. Higher consumption of resources in rich countries and heavy pressure in poor ones had already changed its face. Similarly, at the local level higher consumption in towns put pressure on rural areas. Demand for more water and energy give similar effect on the rural populations. These are the issues that have the social and political implications.

Ethical - Do the exploiters have the right to exterminate plants, animals fungi and planktons, etc. that are used in many traditional societies. This has the basis that the capitalist man see humans as separate from the rest of nature. But respect for life as such has always been a central tenet of Buddhism, Taoism, Christianity and Islam, among other systems of belief.

Aesthetic - The rural populations are used to the beautiful greeneries and landscapes. With certain development activities these rural landscapes may be changed forever and representing a total loss. Examples of such loss are the construction of roads and highways across the rural villages, factories in rural areas, new townships, etc. In reality these kind of development affect the livelihood of traditional communities.

Direct economic - Malaysians have realised the direct economic importance of biodiversity, especially plant and animal species diversity. The exploitation of various timber species as source of hard tropical wood is a testimony of our understanding of the potential of diversity. More and more lowland dipterocarps forest are being logged to obtain foreign exchange needed to develop the nation. In such as development in many instances, the traditional communities are sidelined. The question of sustainability does not arise because we believe we still have plenty of the resources. Many people still do not comprehend the concept of susutainable development, let alone the sustainability and renewal of natural resources.

As well as conserving biodiversity at the level of species and ecosystems through in situ conservation, we also need to cherish the genetic diversity that occurs within them. The wild relatives of useful strains of our economic crops, fruit trees, vegetables, domesticated animals etc. are often loss when natural habitats are converted for other land uses (Osman *et al.*, 1995). Without a large natural genetic reservoir, we make our food supplies vulnerable to diseases.

The pristine tropical forests and corals are a major attractions of the modern ecotourists. Malaysia is sitting both in the centre of the most diverse and oldest tropical rain forests in the world. The likes of Orang utans, proboscis monkeys, hornbills, *Rafflesia*, orchids among others are still the iconic biodiversity that attract the tourists from the Far East, Middle East and Europe. These tourists would pay substantially to observe some of these floras and fauna, in addition to traveling in the green tropical forests. This is a great “green” asset for the country. Malaysia also is sitting in the centre of Coral Triangle with a great diversity of corals and tropical fishes in the vicinity of many off-shore islands. Mabul and Sipadan Islands in Sabah and Pulau Langkawi and Pulau Tioman in Peninsular Malaysia have been hosts to divers from all over the world.

Issues for effective management of biodiversity assets

IUCN (1997) recognized six categories of threatened plants, namely extinct (Ex), Extinct/Endangered (Ex/E), Endangered (E), Vulnerable (V), Rare (R) and Indeterminate (I). These categories are clearly and extensively defined. However, Lim and Whitmore (2001) argued that for ease of application in the field, complex classification of status as proposed by IUCN can be replaced by a simple index for monitoring observable endangerment. Four categories were subsequently proposed, graded in ascending degree of survival or perceived survivability, also in order of urgency for rechecking extant populations. Category 1 is for already rare, which calls for urgent protection and regular surveillance of sites; category 2 for not yet rare, rapid reduction in population anticipated due to habitat loss; category 3 is for sporadic distribution and adequate surviving population to be identified and category 4 is for relatively common and widespread but local protection is desireable.

There are several causes of rarity of species in Peninsular Malaysia and these include: The plant specimens are present in the wild but were not collected by the collectors over time and these species are not quite known locally. The populations are isolated in distribution and probably the population is on the verge of extinction. Some species have suffered from over-collected or over-exploited and some taxa probably are newly evolved.

An endangered species is a species in serious risk of disappearing from the wild within one or two decades if present land use and other causal factors continue to operate (Cropper, 1993). Such species usually represent the rare and unusual end of the spectrum of biodiversity. Rarity may arise from the fact that the species has a highly specialized and unusual habitat requirement which is extremely restricted in distribution. In other words rare species are not necessarily endangered species unless there are threats to their habitats. At population level some of the criteria that are worth noting for conservation efforts include:

- Minimum Viable Population. An estimate of the number, density, frequency of the viable population must be made
- Minimum Dynamic Area. Similarly, an estimate of its reproductive range should be ascertained
- Loss of genetic Variability. Through DNA studies an estimate of genetic variability could be made
- Inbreeding Depression. As estimate by population and genetic studies
- Outbreeding depression. Similarly an estimate by population and genetic studies could be initiated
- Demographic variation. As estimate by population studies in various habitats such as Virgin Jungle Reserve may be considered.
- Environmental Variation. As estimate the effect of environmental factors on populations
- Extinction Vortex. if any.

Some conservation measures may be adopted. These include:

- Study population natural history as far as known. This is lacking for the endemic species, especially for those which are considered very rare and rare.
- Monitoring populations from time to time. As proposed by Lim and Whitmore (2001) such threatened populations must be monitored frequently.
    - (i) Demographic studies covering adults, saplings, regenerants. Ecologists should now consider some demographic studies involving not only adults but saplings and a series of regenerants.
    - (ii) Establishment of new population. If nee arises, new population may be established from seeds or through tissue culture of species which are at the brink of extinction
    - (iii) Improve the scientific knowledge base of endemic species. Our scientific knowledge about endemic species is yet to be realised. There is great progress with the tree taxa but not with the non-tree species.
    - (iv) Develop a centre of excellence in threatened plants. Some cooperative research between research institutes and universities may be explored to address this issue.
    - (v) Strengthen the institutional framework for biodiversity management. There should be an agency to coordinate all biodiversity management to strengthen the current activities regarding endemic species, including threatened species. This also could strengthen inter-institutional frameworks and networking.
    - (vi) Strengthen and integrate conservation programmes. Some conservation programmes in the country is in place under the Department of Wildlife and National Parks. However, much is desired to address the issue of capacity strength within that department and also integration of all efforts towards sustainable utilisation and conservation.
    - (vii) Enhance skill, capabilities and competence. The national critical mass in biodiversity conservation is still far from being realised. At present there are independent research groups with small number of ecologists in various

universities and research institutes but the number is still small considering the mammoth tasks of elucidating the strategic management of threatened taxa.

(viii) Encourage private sector participation. The private industries are encouraged to participate actively in biodiversity conservation efforts. Currently Malaysian Nature Society and WWF Malaysia have taken some active parts in promoting conservation.

(ix) Reviews legislation to reflect biodiversity needs. At present the current legislations are more than adequate to address the issue.

(x) Minimise impacts of human activities on biodiversity. As long as unplanned or ill-planned developments are allowed to operate there is no guarantee that minimisation is going to occur.

(xi) Enhance institutional and public awareness. All parties should address this issue at all levels, starting at primary school level to university and also the communities at large.

(xii) Promote international co-operation and collaboration. This is in place as many local scientists have worked with their overseas counterparts from Japan, United Kingdom, Germany, France, United States and other countries on government to government arrangements.

(xiii) Promote exchange of information. In the current iinformation technology scenario, exchange of information via the electronic media should be addressed, in addition to the conventional methods.

(xiv) Establish funding mechanisms. At present the IRPA mechanism is adequate, however, much is to be desired with respect to addressing biodiversity research and biodiversity conservation as a national agenda for the coming years.

Malaysia ratified the Convention on Biological Diversity in June 1994, which set the platform of commitments under the treaty and reaffirms the sovereign rights of states over their biodiversity and the responsibility to conserve and utilise the resources in a sustainable manner. As most of the countries are a party to the Convention of International Trade in Endangered species of wild fauna and flora (CITES) and also a member of the International Union of Conservation of nature and natural resources (IUCN), as well other international networks, the responsibility is even more important. Ironically, Malaysia is yet to prepare our Red Data Book for her biodiversity.

It is a common knowledge that protection of the environment is the central issue in the preservation and conservation of biodiversity, especially the threatened plant species. It is widely accepted that Malaysia has enough concurrent laws and regulations to protect her environment, but there is no single legislation, which relates to biodiversity conservation and management. Much of the present legislations are sector-based, for instance, the National Forestry Act 1984 deals with the management and utilisation of forests and forest products alone. However, what are more pertinent are not the number of laws and regulations but the mechanism of strict implementation and enforcement of such legislations to achieve the required expectations. In order for the legislation to be more effective, not only the government agencies should adhere to them but also the public must participate actively to ensure its accountability. In this context the communities, and in particular the indigenous communities who are directly associated with the protected areas should be aware that they are not only the users of the resources but also the custodian of the biodiversity. The legislation relevant to this include:

National Legislation

- Protection of Wildlife Act 1972
- National Park Act 1980
- National Forestry Act 1984

State Legislation

- National Parks (Johor) Corporation Ordinance 1989
- Taman Negara (Pahang) Enactment 1939
- Taman Negara (Kelantan) Enactment 1938
- Taman Negara (Terengganu) Enactment 1938

Of the existing national policies and regulations in Malaysia, the national Forest Policy 1978 is one of the important policies in ensuring the survival of forest ecosystems and species. However, the National Forest Policy 1978 is only applicable in Peninsular Malaysia, whereas the National Forestry Act 1984, as a federal legislation is deemed to apply nationwide only provides the classification of the forests. As land is a state matter, gazettement and degazettement of forest reserves are within the power of the state legislatives. The process of degazettement of the protected forests must be made with prior consultation with the Federal Government. However, there are other Federal legislations which are supposed to complement and support the above policies. These include Land Conservation Act 1960, Protection of Wildlife Act 1972, National Park Act 1980 and Environmental Quality Act 1974.

Biodiversity has both the positive and negative aspects. When we look as the diversity of life within and between countries, we are struck by huge inequalities. The developed countries by and large have lost their biodiversity through industrialisation and the developing countries are still rich with their natural endowment. The former now focused their endeavors in bioprospecting in the developing countries which need the financial resources to develop their countries. These inequalities make it very difficult to preserve biodiversity because of the rate of consumption and of pollution of the privileged minority on Earth have been made burden of the developing world. How can one ethically give first priority to biodiversity as long as the problem of poverty has not been solved? In the world about 1500 million people live in a state of absolute poverty, have no drinkable water or electricity and are illiterate. On the other hand we find 20% of the world population earning 150 times more than the poorest 20%. There are more than 1100 million persons who earn less than 2 ringgit per day. We just wonder for how long can the ethics of biodiversity endure the consequences of inequitable economic diversity within and between countries?

The challenge ahead

From the above deliberation it becomes apparent that it is important to bring in the cultural perspectives, especially the traditional knowledge in biodiversity issues. The indigenous culture is more important in conserving and protecting the environment, whether locally or nationally. On of the major challenge is to incorporate the cultural and ecological aspects in the planning for the development of natural resources. Optimal economic development has to be integrated with cultural and ecological consideration and understanding to enable sustainability to be achieved (de Beer and McDermott, 1996). It is felt that in future scientists should play a more positive role in planning and management processes affecting the environment and biodiversity, in particular, rather than withdraw from all the planning and bureaucracy. Dassmann *et al.* (1974) has long called for a partnership between the scientists and policy makers in working towards a sustainable development, especially in issues affecting the traditional societies.

Another challenge lies in effective communication between all concerned with the environment. For example, in the management of forest, there are many sectors involved in influencing the decision. Developmental, agricultural, forestry, industrial sectors and public also play a role in determining the quality of forest, it is not the sole responsibility of the forest department anymore. An awareness towards the relationship of a healthy environment and the well-being of all communities need to be enhanced, so that each and every party realises that we need one another, working together, to conserve the environment.

Sustainable utilization of biodiversity

Man has been appointed the custodian for the earth. He is allowed to use all other species, plants and animals, for his benefits in a sustainable manner. Prior to Industrial Revolution, most things apparently were in harmony and equilibrium.. Since the Industrial Revolution, man has succeeded in accelerating the rate of producing products for consumption in a massive form that has threatened food security and health. As a result an important ecological resource has suffered in the pursuit of economic advancement is the forest (Mannion and Bolwby, 1994) .Much of the degazetted forests have been converted to agriculture and when agriculture proved less competitive these lands are converted to industrial or housing estates. Each step of conversion would result in some losses of

biodiversity, especially the vegetation types, plant species and animal populations. Some are replaceable but most are not.

Human activities affecting the sustainability of the biosphere was well discussed by Lubchenko *et al*. (1991) and some of the pertinent components that affect the traditional communities and knowledge are shown (Table 1).

Table 1. Relationship between human activities and ecological functions

Activities	**Intended results**	**Improved quality of life**
Land clearing Agriculture Forestry Fisheries Water diversion Mineral extraction Fuel consumption Industrialisation Urbanisation Recreation	Food production Shelter Consumer goods Culture	Fibre production Water supply Enjoyment Knowledge
	Unintended results	**Environment costs**
	Habitat destruction Deforestation Acid precipitation Climate change	Soil degradation Pollution Eutrophication Loss of biotic Diversity

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Biodiversity Conservation in Sarawak

Demies, M.[1], Lading, E.[2] and Silang, S.[1]

[1]Sarawak Forestry Corporation, Sarawak, Malaysia
[2]Sarawak Forest Department, Sarawak, Malaysia
malcom@sarawakforestry.com

Abstract

The tropical rainforest of Sarawak is well gifted with some of the world's richest forests. Its forest is not only rich in terms of numbers and uniqueness of species but also diversity of habitats and ecosystems. The total forest cover in Sarawak is about 70% of its land area which include Permanent Forest Estate, State land and Totally Protected Areas (TPAs). Of this total, 700,727 hectares are within TPAs. TPAs are legally gazetted Forest Reserves, managed sustainably for economic, social and environmental values. Large forest areas had been cleared to give way to plantation and others development purposes, at the same time there was a significant increase in the gazettement of TPAs. In 1950s, the total area gazette as TPAs was 2,727 hectares and it was increased to 700,727 hectares in 2007. The Sarawak Government is targeting at least one million hectares (10% of the state land area) to be gazette as TPA and also committed in forest and biodiversity conservation, where effort is taken to ensure the *in situ* and *ex situ* conservation. This paper discusses on the efforts taken by Sarawak Government on conserving its biodiversity.

Introduction

Biological diversity or popularly termed as biodiversity, is the total variety of life on earth. Article 2 of the Convention on Biological Diversity (CBD) defines Biodiversity as "the variability among living organisms from all sources including terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of the ecosystems".

Malaysia's biodiversity is our precious treasure, as a source of food, fuel, medicine and materials for shelter and livelihood. It will continue to play an important role in the country's socioeconomic development and environmental conservation. Besides the thousands of plants species, the forests are also habitats for the animal life found within the forests. Malaysia is one of the world's 12 "megadiverse" countries due to the biodiversity hotspot. The World Development Indicators stated that Malaysia has only 20% of the world's land mass, but its diversity of flora and fauna species makes it one of the richest countries in the world in terms of biodiversity per unit area. The number of species in Malaysia is not known with certainty, but it is estimated that there are more than 170,000 species in Malaysia. Malaysia homes to over 15,000 species of flowering plants and trees, 600 species of birds, 286 species of mammal, 140 species of snake and 80 species of lizards (MPI, 2004).

Forest types in Sarawak

The total forest cover in Sarawak is amounted to 10 million ha or about 70% of the land area. These forests area comprises of the inland forests, wetland, planted forest and secondary forest which include Permanent Forest Estate, State land and Totally Protected Areas (TPAs). Of the total forested area in 2008, 700,727 hectares has been earmarked as TPAs.

The TPAs comprises of wildlife sanctuaries, national parks and nature reserves. The main objective of TPAs is to conserve the biodiversity (Ngui, 1991; Kassim and Gumal, 1995; Gumal and Ahmad, 1995). The wildlife sanctuaries are strictly for conservation and research, so public access is strictly limited. The national parks and nature reserves are open for public recreation and for tourism.

A large proportion of Sarawak's animals are unique to Borneo and do not occur in mainland South-east Asia. These include approximately 19% of the mammals, 6% of the birds, 20% of the snakes and 32% of the lizards; and these species are largely found in TPAs (FDS 2009). The vascular flora of Sarawak comprises more than 8,000 species. Over 2,000 tree species have been enumerated

whereas orchid would number more than 1000 species, ferns account for 757 species and palm make up another 260 species (FDS 2009).

It is an undeniable fact that much of Sarawak's endangered species is under threat of disappearing forever to extinction and this degeneration must be redressed. To conserve all species and habitats in perpetuity, Sarawak has adopted the International Union for the Conservation of Nature (IUCN) recommended action that at least 10 per cent of its land area should be in TPAs.

In 1996, the Sarawak Government commissioned a Master Plan for Wildlife, which comprised a strategy to balance wildlife conservation with development in the State. Following its recommendations, the Government passed a new law, the Wild Life Protection Ordinance 1998, which banned all commercial sales of wildlife and wildlife products taken from the wild. It recognised that rural communities depend on wild meat and thus did not ban hunting. The new law was strictly enforced in urban areas followed by a widespread publicity and education campaign. Under this Ordinance, some of Sarawak's wildlife which are endangered or rare are categorised as Totally Protected and Protected Animals. Sarawak has also implemented the total ban or trade of wildlife.

Wildlife in Sarawak

In Wild Life Protection Ordinance, 1998, wild life is defined to include all species of wild animal or wild plant. Wild animals mean all species of animal and include mammals, birds, reptiles, amphibians, fish, or invertebrates that exist or occur in the wild state in Sarawak or elsewhere in the world. Wild plants mean all species of plants which exist or occur in the wild state in Sarawak or elsewhere in the world. All wildlife in Sarawak is legally assumed to be the property of the State.

Totally Protected Animals

Totally Protected Animals are defined as animal species which are in danger of extinction due to hunting and habitat destruction. Most of these species are now extremely rare (Appendix 1). Thus, any person who hunts, kills, captures, sells, offer for sale or claim to be offering for sale, imports, reports or in possession of Totally Protected and Protected species, or its part thereof without written permission of the Controller for scientific or educational purposes and conservation of the species shall be guilty of an offence as stipulated in Section 29 (1) of the Wild Life Protection Ordinance, 1998.

Protected animals, on the other hand, are species having potential to be endangered if hunting and its habitat destructions are not properly controlled. Keeping, hunting, importing and exporting any of protected animal species require license from the Controller of Wild Life.

Protected animals

Protected animals are those listed in Part II of the First Schedule of the Wild Life Protection Ordinance, 1998 (Appendix 2). These animals are protected in Sarawak because they are now rare, due to hunting and habitat destruction. Protected animal may however be hunted, killed, imported, exported or held in possession under the terms and conditions of a licence issued under the above mentioned Ordinance. License are needed to keep them as pets, hunt, kill, capture, sell import or export them, or possess any recognizable part of these animals. Issuing of licenses is at the discretion of the Forest Department.

Other animals

Any other animal other than a totally Protected and Protected species are those that also received some degree of protection in Sarawak. It is illegal to trade, breed or be in possession, for own consumption or use, unless licensed under the Wild Life Protection Ordinance. Only natives residing within a Native Area Land or Native Customary Land can keep, consume or use this category of wild animals and its recognisable parts.

Totally Protected Plants

Totally protected plants are those categorised of wild plants listed in Part I of the Second Schedule of the Wild Life Protection Ordinance, 1998 (Appendix 3). No person shall collect, cultivate, cut, trim, remove, burn, poison, injure, sell, offer for sale, import, export or be in possession of any totally plant or recognisable part or derivative thereof, except with the written permission of the Controller of Wild Life and only for scientific or educational purposes or for the protection and conservation of such wild plant.

Protected plants

Protected plants are those category of wild plant listed in Part II of the Second Schedule of the Wild Life Protection Ordinance, 1998 (Appendix 3). Protected plants may however be collected, cultivated, sold, imported, exported, or held in possession under the terms and conditions of a licence issued under the above mentioned Ordinance.

Other plants

Any wild plants other than a totally protected or protected species are those wild plants that need a licence for the purpose of export out or import into Sarawak.

Constitutional provisions, policy and legislations

Under Article 72(2) of the Malaysian Constitution, forestry comes under the jurisdiction of the respective State Governments. As such, each state in Malaysia is empowered to enact laws on forestry and to formulate forest policy independently. The executive authority of the Federal Government only extends to the provision of the maintenance of the experimental and demonstration stations, training and in the conduct of research.

Malaysia has formulated laws and legislations to protect the environment and the diverse biological resources. However, there is no single legislation related to biodiversity conservation and management at the national level except for the National Biodiversity Policy. As for Sarawak, the Sarawak Biodiversity Centre Ordinance, 1997 (amended 2003) is the only ordinance specifically prepare to protect biodiversity in Sarawak.

Apart from SBC Ordinance, 1997 (amended 2003), other ordinances related or relevant to forestry industry in Sarawak are Sarawak Forestry Corporation Ordinance 1995; the Forest Ordinance, 1958; the National Parks and Nature Reserves Ordinance, 1998 and the Wild Life Protection Ordinance, 1998 (amended 2003). Its functions include:

- Sustainable forest management and conservation
- Reforestation and rehabilitation
- Management of protected and totally protected areas
- Enforcement of forestry and forestry-related legislation
- Conducting scientific research on Sarawak's rainforests and its products
- Training and education of employees, stakeholders and the general public
- Providing customers of Sarawak's forest products with reliable information and support

Management and conservation of forest biological resources

Forest management in Sarawak has started since the Colonials era in early 1900s. Over the years, forest management has evolved and subjected to constant review and refinement. Since its start it has undergone a number of changes in its major trend, i.e. from the supply of timber to a strategic role in the maintenance of environmental quality, protection of water quality and supply, moderation of local climate and conservation of biological resources.

Sarawak has adopted a Sustainable Forest Management (SFM) practice to achieve a continuous flow of desired forest products and services without any socially unacceptable, environmental or social impact or reduction of its inherent value and potential future performance. Selective logging practice is carried out in the Permanent Forest Estates (PFEs) whereby only the

matured and over matured trees are felled in a single operation, while at the same time, leaving behind a residual stand with sufficient number of trees in the intermediate diameter classes to form the next crop.

The forest management practices is also being assessed under the forest certification programme using the Malaysian Criteria and Indicators (MC&I), the essence of which is to conduct good forest practices socially, economically and environmentally. To date two timber companies in Sarawak have been certified and awarded with the certificates by the Malaysian Timber Certification Scheme after being assessed by independent assessors.

As a signatory to the Convention on Biological Diversity's Conference of Parties, Malaysia (including Sarawak) is committed to implement its obligation in biodiversity conservation. In this regard, Malaysia has established the National Bio-Diversity and Bio-Technology Council in 2001 to further elaborate on related issues and provide strategy and direction for the conservation of biodiversity and development of bio-technology in the country.

Conservation effort by Sarawak Government

The National Policy 1978 (revised in 1992) provides Sarawak Government direction in implementing conservation programme towards achieving SFM.

In response, Sarawak has designated 1 million hectare or 10% of its forests as TPAs for in situ conservation of its biological and genetic resources. Rare and threatened plant species are also conserved on the ex situ basis, in Botanical Research Centre, Kuching. Small-scale ex situ conservation of wildlife is carried out in Semengoh Wildlife Rehabilitation Centre, Matang Wildlife Centre and in a number of turtle islands near Kuching where turtle eggs deposited near the shorelines are removed to hatcheries.

In-situ conservation at Totally Protected Area

As of now, Totally Protected Areas are the best place for in-situ conservation. In-situ conservation, among other things, provides protection of flora and fauna in its natural habitats. Currently, Sarawak has a total of 29 Totally Protected Areas (TPAs), comprising of 20 National Parks, 4 Wildlife Sanctuaries and 5 Nature Reserves, covering a total area of 700, 727 ha.

Totally Protected Area (TPA) in Sarawak refers to forest lands so designated and established under the provisions of the National Parks Ordinance, 1998 and Wild Life Protection Ordinance, 1998.

i. National Parks

At present there are 20 National Parks in Sarawak. Among those, Bako National Park, with a total area of 2727 ha, is the smallest and the first National Park. It was gazetted in 1957. On the other hand, the largest National park in Sarawak is the Pulong Tau National Park, gazetted on 24 March 2005, and covers an area of 59,817ha. Stated below are lists of National Parks in Sarawak as of November 2007;

- Bako National Park
- Gunung Mulu National Park
- Niah National Park
- Lambir Hills National Park
- Similajau National Park
- Gunung Gading National Park
- Kubah National Park
- Batang Ai National Park
- Loagan Bunut National Park
- Tanjung Datu National Park
- Talang-Satang National Park
- Bukit Tiban National Park
- Maludam National Park

- Rajang Mangrove National Park
- Gunung Buda National Park
- Kuching Wetland National Park
- Pulong Tau National Park
- Usun Apau National Park.
- Miri-Sibuti Coral Reefs National Park
- Santubong National Park

ii. Nature Reserves

Nature Reserves are similar to National Parks. However, the sizes of each one of the 5 Nature Reserves we are having thus far is smaller (less than 1000 ha) than that for National Parks. By definition, a Nature Reserve is constituted to conserve the flora, fauna and the aesthetic value of the whole area either for education, research or recreation.

Nature Reserve has contributed a total of 945.07 ha of the TPAs in Sarawak. Wind Cave Nature Reserve is the oldest nature reserve in Sarawak which was gazetted on 4 November 1999 covering an area of 6.16 ha. Five Nature Reserves in Sarawak are as follows;

1. Wind Cave Nature Reserve
2. Sama Jaya Nature Reserve
3. Semenggoh Nature Reserve
4. Bukit Hitam Nature Reserve
5. Bukit Sembiling Nature Reserve

iii. Wildlife sanctuaries

Currently there are 4 wildlife sanctuaries been gazetted since 1978, with a total area of 192,235.4 hectares. The oldest one is the Samusam Wildlife Sanctuary, with a total area of 6,092 ha. It was gazetted on 1 July 1978. Its first Extension, with an area of 16,706 ha, was gazetted in May 2000. The primary objective for its gazettment is to provide protection to a population of proboscis monkeys (Nasalis larvatus) which are endemic to Borneo. Below are the lists of gazetted Wildlife Sanctuaries in Sarawak;

1. Samusam Wildlife Sanctuary
2. Pulau Tukong Ara/Banun Wildlife Sanctuary
3. Lanjak Entimau Wildlife Sanctuary
4. Sibuti Wildlife Sanctuary

iv. Arboretum

The Semengoh Arboretum in the Semengoh Forest Reserve was established in 1951 to preserve in perpetuity a small area of easily accessible, natural primary lowland forest. It covers an area of approximately 14 hectares and is one of the few least disturbed lowland dipterocarp forests near Kuching city. The Arboretum is floristically rich with about 2,500 tree species from 60 plant families and has attracted a large number of researchers and naturalists.

b. Ex situ conservation for forest biodiversity

Ex-situ conservation is an important tool to complement the function of in-situ conservation. The Botanical Research Centre (BRC), Semengoh, was established in 1976 within an area of 22 hectares. BRC lies in Semenggoh Forest Reserve which is the first and oldest Forest Reserve in Sarawak. Semenggoh FR with a total area of 653 ha approximately was gazetted on 16 November 1920, a year after the Forest Department was established. BRC consists of 9 specialized gardens. At present it is developed as Wild Orchid Garden, Nepenthes Garden, Mixed Planting Garden, Palms Garden, Ethnobotany Garden, Aroids Garden, Bamboo Garden, Fernarium Garden and Wild Fruits Orchard within an area of 22 hectares.

c. Conservation measures in Permanent Forest Estate (PFE)

Environmental protection and conservation measures have also been instituted within the PFE. No logging is allowed on steep slope more than 40 degrees. Harvesting operation has to subscribe to approved Forest Harvesting Guidelines and Forest Engineering Plan. Tree felling during harvesting operation is carefully done to ensure minimal impact to the environment. The cutting limits prescriptions allow only the selected and correct trees to be harvested thus reducing damage, conserving diversity and the environment.

Other measures include retention of trees for protection, buffer zones along rivers and streams, timber tagging and directional felling as well as leaving behind buffer zones to protect the water resources and minimise soil erosion. Reduce Impact Logging (RIL) is another milestone innovation with the objective of reducing the negative impact of timber harvesting to environment and damage to residual stand.

Forest Management Certification

Forest Management Certification involves an independent assessment of forest management operation, according to specific economic, social, environmental and ecological criteria, indicators, activities and management specifications. This forest evaluation typically includes an evaluation of economic viability of the operation, social and environmental impact of the forest management activities and ecological health of the forest.

Since the establishment of Malaysia Timber Certification Scheme (MTCS), it has been involved in a number of internal consultative processes to formulate and revise the MC&I. It involved government departments and agencies, environmental non-governmental organisations (NGOs), forest licensees, manufactures of wood and panel products and trade unions. To date, a total of 162,769 ha of PFE in Sarawak covering the state Forest Management Units (FMUs) had been given MTCS' Certificate for Forest Management.

The MS ISO 9000, in brief is a series of standards for quality management and quality assurance system. The adoption of MS ISO 9000 series will ensure the establishment of quality systems, products ands services. The MS ISO 9000 processes can help to attain sustainable forest management because the processes will ensure activities are carried out according to the standards. SFC is certified with ISO 9001:2000 and also awarded the ISO 14001:2004 and OHSAS 18001:1999 from the Department of Standards Malaysia, United Kingdom Accreditation Service and Moody International Certification.

International agenda

Malaysia is signatory to international agreements, having a direct bearing on forest management, such as the Convention on the International Trade in Endangered Species of Wild Fauna and Flora (CITES), Convention on Biological Diversity (CBD), the United Nations Conference on Environment and Development (UNCED), the Kyoto Protocol, the RAMSAR Convention and Trade agreements and United Nation Framework Convention on Climate Change (UNFCCC). This is Malaysia's commitment and contribution to the world in the conservation of the nation's rich and diverse biological resources.

Issues and challenges

Biodiversity conservation issue is very complex and recently has become one of the prime issues in forest management. The issue of biodiversity has changed the whole scenario of forest values and their development potential. The increasing pressure for conserving of biodiversity has influenced our way to manage the forest. The need to reduce deforestation and forest degradation; and to protect, conserve and ensure the sustainable use of the genetic resources, remained as a widespread concern requiring actions. These are the uphill challenge to foresters.

Our knowledge on conservation is comparatively lagged as it cannot work without adequate knowledge and information. There are lot of gaps to be filled in terms of knowledge and information about our forest biodiversity. Although some works have been done on the valuation of goods and services that the forest provided, more work need to be done to enhance their full valuation.

Mechanisms and methodologies for valuation of the various goods and services need to further develop. Among the essential focus are the development of rapid assessment techniques for a complete appraisal of the biological resources of the forest, methodologies for ascribing economic values to them, the formulation of nationally agreed biogeographic area for the conservation of biological diversity and the completeness of representations of the various forest ecosystems.

The need to develop methodologies and technique for recovery and rehabilitation of species and habitats to avoid their loss or extinction is also crucial. Present legislation may need review, whether they are comprehensive enough to ensure effective conservation, and whether the endangered, rare and threatened species are sufficiently protected.

Knowledge on relating plants and animals and their uses in medicine, food and cultural need to be recorded to enhance knowledge in forest biodiversity conservation. Non-timber forest products and environmental functions of forest will be accorded as higher priority. Forest based recreation is likely to become even more important as per capita productivity rises and leisure time increases.

There has been an increasing concern over bio-piracy and bio prospecting. Samples of a diverse range of target organisms were taken for screening, bio-assaying or chemical elucidation. These searches for new bioactive compounds have to be looked upon with caution. In most cases, it has not been benefiting the source country. Sarawak has the advantage in terms of its rich biological resources, but the latest technologies are with the developed countries. It is only through an equitable partnership between parties concerned that biodiversity could be utilised for the benefits of the source country as well as the developed nations.

The way forward

The need for effective forest management and conservation must be given priority, not only to ensure a sustained supply of wood and non-wood forest products but also to maintain forest health for environmental stability, to provide sanctuary for wildlife and to serve as an invaluable storehouse of genetic resource useful for indigenous tree species, agricultural crops and livestock. This renewal asset will continue to be managed in accordance with national objectives and priorities so that the country will continue to enjoy the benefits generated from the forests and forest industries.

The total extent of PFEs in Sarawak has not changed much, while the TPAs showed an increasing trend. This is clear indication of Sarawak government's effort in giving more emphasis to conservation. Forest certification and timber labelling are expected to become an integral component of forest resource management and development. It is envisaged that more intensive forest management will be carried out in the coming years, particularly with the increasing emphasis now being placed on sustainable management, conservation and development.

Latest technology will be used for rapid appraisal of the physical and some biological resources of the forest such as Airborne Hyperspectral Imaging Technique and Geographical Information System. These techniques will provide reliable data and information which may take months to complete with other convectional method.

Finally, as the effects of global climate change become more visible, the roles of forests as both carbon sinks and moderators of climate disturbance (such as flooding) will take a new meaning.

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Appendix 1

English name	Scientific name	Local name
A. MAMMALS		
Slow loris	*Nycticebus coucang*	Ukang; bengkang (I)
Western tarsier	*Tarsius bancanus*	Kera hantu; ingkata (I)
Silvered langur	*Presbytis cristata*	Lotong
Hose's langur	*Presbytis hosei*	Berangad
White-fronted langur	*Presbytis frontata*	Puan
Banded langur	*Presbytis melalophos*	Penetat
Maroon langur	*Presbytis rubicunda*	Lotong merah; jelu merah (I)
Proboscis monkey	*Nasalis larvatus*	Orang belanda; rasong (I)
Bornean gibbon	*Hylobates muelleri*	Wak-wak; empeliau (I)
Orang utan	*Pongo pygmaeus*	Mawas; maias (I)
Giant squirrel	*Ratufa affinis*	Tupai kerawak
Tufted ground squirrel	*Rheithrosciurus macrotis*	Tupai
Clouded leopard	*Neofelis nebulosa*	Rimau dahan; engkuli (I)
Bay cat	*Felis badia*	Kucing merah
Marbled cat	*Felis marmorata*	Kucing dahan
Flat-headed cat	*Felis planiceps*	Kucing hutan
All whales, dolphins and porpoises	All species of *Cetacea*	Paus; lumba lumbi
Dugong	*Dugong dugon*	Dugong; duyong (I)
Rhinocerous	*Decerorhinus sumatrensis*	Badak
Wild cattle	*Bos javanicus*	Tembadau
Naked bat	*Cheiromeles torquatus*	
B. BIRDS		
Oriental darter	*Anhinga melanogaster*	
Pacific reef egret	*Egretta sacra*	Ujoh laut
Cattle egret	*Bubulcus ibis*	Burung apuh; burung lima ringgit
Storm's stork	*Ciconia stormi*	Bangau
Lesser adjutant stork	*Leptoptilos javanicus*	Bangau
White-bellied fish eagle	*Haliaeetus leucogaster*	Lang laut
Grey-headed fish eagle	*Ichthyophaga ichthyaetus*	Lang laut
Bornean peacock pheasant	*Polyplectron schleiermacheri*	Ruai
Argus pheasant	*Argusianus argus*	Ruai
Bulwer's pheasant	*Lophura bulweri*	Bekia
Black-naped tern	*Sterna sumatrana*	Burung laut
Bridled/brown-winged tern	*Sterna anaethetus*	Burung laut; entala puteh (I)
All phalaropes	*Phalaropus spp.*	Kedidi
All imperial pigeons	*Ducula spp.*	Rawa
Silvery (grey) wood pigeon	*Columbia argentina*	Pergam
White-crowned hornbill	*Aceros comatus*	Sentuku (I)
Bushy-crested hornbill	*Anorrhinus galeritus*	Kakalau (I)
Wrinkled hornbill	*Aceros corrugatus*	Alau buloh
Wreathed hornbill	*Aceros undulatus*	Alau sangoh
Asian black hornbill	*Anthracoceros malayanus*	Alau babi; gagak/rengak (I)
Oriental pied hornbill	*Anthracoceros albirostris*	Alau pedada; bruie (I)
Rhinoceros hornbill	*Buceros rhinoceros*	Kenyalang (I)
Helmeted hornbill	*Buceros vigil*	Tajai (I)
All pittas	*Pitta spp.*	Burung pacat
Straw-headed bulbul	*Pycnonotus zeylanicus*	Barau-barau
Bornean bristle headed	*Pityriasis gymnocephala*	
C. REPTILES		
All marine turtles	All species of *Chelonidae and Dermochelyidae*	Penyu-penyu laut
Painted terrapin	*Callagur borneoensis*	Beluku
Terrapin	*Orlitia borneensis*	Beluku
Niah cave gecko	*Cyrtodactylus cavernicolus*	Cicak Gua Niah
Earless monitor lizard	*Lanthanotus borneensis*	Cicak purba

*I – Iban name

Appendix 2

English name	Scientific name	Local name
A. MAMMALS		
All treeshrews	All species of *Tupaiidae*	
All bats	All species of *Chiroptera* excluding those already listed in Part I	
All primates	All species of *Primates* excluding those already listed in Part I	
Flying lemur/colugo	*Cynocephalus variegatus*	Kubung
Pangolin	*Manis javanica*	Tenggiling
All flying squirrels	All species of *Petuaristinae*	Tupai terbang
Porcupines	All species of *Hystricidae*	Landak
Sun bear	*Helarctos malayanus*	Beruang
Bear cat	*Arctitis binturong*	Binturung
All civets and mongooses	All species of *Viverridae*	Musang
All otters	All species of *Lutra and Aonyx*	Memerang
All cats	All species of *Felidae* excluding those already listed in Part I	Kucing hutan
B. BIRDS		
Christmas frigatebirds	*Fregata andrewsi*	
All herons, egrets and bitterns, excluding those already listed in Part I	All species of *Ardeidae*	
All storks, excluding those already listed in Part I	All species of *Ciconiidae*	
Osprey	*Pandion haliaetus*	Lang; menaul
All falcons	All species of *Falconidae*	Rajawali; menaul
All scrubfowl, partridges and pheasants, excluding those already listed in Part I	All species of *Phasianidae*	
All waders, excluding those already listed in Part I	All species of *Charadiiformes*	
Metallic pigeon	*Columbia vitiensis*	Pergam
Nicobar pigeon	*Caloenas nicobarica*	Pergam
All owls	All species of *Tytonidae* and *Strigidae*	Burung hantu
All swiflets	All species of *Aerodramus, Hydrochous and Collocalia*	Burung layang
All kingfishers	All species of *Alcedinidae*	Pekaka
All woodpeckers	All species of *Picidae*	Belatok
Asian paradise flycatcher	*Terpsiphone paradisi*	Burung sambar ekor panjang
Crackle or hill myna	*Gracula religiosa*	Burung tiong
All parrots and parakeets	All species of *Psittacidae*	Bayan
White-rumped shama	*Copyschus malabaricus*	
C. REPTILES		
Asian Giant Tortoise	*Manouria emys*	Baning
All soft-shelled turtles	All species of *Tryonychidea*	Labi-labi
Falso ghairal	*Tomistoma schleglii*	Buaya jujulong
Estuarine crocodile	*Crocodylus porosus*	Buaya katak
All monitor lizards	*All species of Varanus*	Biawak
King cobra	*Ophiophagus hannah*	Ular tedung
Common cobra	*Naja naja*	Ular tedung
All pythons	*All species of Phyton*	Ular sawa
D. FISH		
Arowana (dragonfish)	All species of *Osteoglossidae*	Ikan seruk; ikan siluk
E. INVERTEBRATES		
All hard and soft corals	All species of *Hydrozoa and Anthozoa (Actinozoan)*	Batu karang
Raja Brooke's birdwing	*Troides brookiana*	
F. ADDITIONAL SPECIES		
All species of animal listed in Appendics I and II of the convention of International Trade in Endangered Species of Wild Flora and Fauna (CITES) excluding those already listed in Part I.		

Appendix 3

Totally Protected Plants

No.	Scientific name	Common name
1.	*All Rafflesia species*	Bunga pakma
2.	*Dipterocarpus obloglofolius*	Ensurai

Protected Plants

No.	Scientific name	Common name
1	*Shaorea macrophylla*	Engkabang jantong
2	*Shorea splendida*	Engkabang bintang
3	*Shorea helmsleyana*	Engkabang gading
4	*Shorea siminis*	Engkabang terendak
5	*Shorea palembanica*	Engkabang asu
6	*Shorea stenoptera*	Engkabang rusa
7	*Shorea pinanga*	Engkabang langai bukit
8	*Shorea ochracea*	Raru
9	*All Ficus Species*	Pokok ara
10	*Sonneratia alba*	Perepat
11	*Sonneratia caseolaris*	Pedada
12	*Avincennia alba*	Api-api hitam
13	*Avincennia lanata*	Api-api
14	*Avincennia marina*	Api-api merah
15	*Avincennia officinalis*	Api-api sudu
16	*Lumnizera littorea*	Terentum merah
17	*Koompassia excelsa*	Tapang
18	*Koompassia malaccensis*	Menggris
19	*Aetoxylon sympetalum*	Kayu gahru
20	*Aquilaria beccariana*	Kayu gahru, engkaras (I)
21	*Aquilaria malaccensis*	Kayu gahru
22	*Aquilaria microcarpa*	Kayu gahru
23	*Didesmandra aspera*	
24	*Casuarina equisetifolia*	Rhu laut
25	*All Rhodedendron species*	
26	*All Nepenthes species*	Periok kera
27	*All Orchidaceae spesies*	
28	*Salacca magnifica*	
29	*Johannesteysmannia altifrons*	Ekor buaya
30	*Areca triadra*	Pinang
31	*Areca jugahpunya*	Pinang
32	*Pinanga mirabilis*	Pinang
33	*Areca subcaulis*	Pinang
34	*Licaula orbicularis*	Biris
35	*Eurycoma longifolia*	Tongkat ali, sengkayap
36	*Goniothalamus velutinus*	Kayu hujan panas
37	*All Monophyllaea species*	
38	*Antiaris toxicaria*	Ipoh
39	*All peat swamp species of Madhuca*	Ketiau
40	*Calophyllum lanigerum*	Bintangor
41	*Calphyllum teysmanii*	Bintangor
42	*Cycas rumphii*	Paku gajah, paku laut
43	*All epiphytic Lycopodium species*	Ekor tupai
44	*All Begonia species*	Riang, telinga gajah
45	*All Aeschynanthus species*	
46	*All Cyrtandra, Didymorcarpus species*	Melebab
47	*All species of plants listed in Appendices I and II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES), excluding those already in Part I.*	

Taxonomy, Taxonomists and Biodiversity

Lim, L.H.S.[1] and Gibson, D.I.[2]

[1]Institute of Biological Sciences, University of Malaya, Kuala Lumpur, 50603, Malaysia
[2]Department of Zoology, Natural History Museum, London SW7 5BD, UK
susan@um.edu.my

Abstract

Malaysia is one of the mega-biodiversity centres, with many species still waiting to be described. The number of taxonomists in Malaysia is small and they are fast dwindling for a variety of reasons. The lack of young taxonomists to replace them is worrying. Universities in this country are churning out students with little or no knowledge of how taxonomy is carried out. Despite the fact that identification is fundamental to all aspects of biological science, most organisations, students and funding bodies regard such endeavours as unimportant, and there are no national initiatives to train taxonomists. Taxonomists are needed to identify organisms for biodiversity, ecological, medical and economic reasons, for future food security and bioprospecting, and for a proper understanding of ecosystems. As long as man is dependent on biological resources for food and other essential products and is still under threat from pathogens, taxonomy will remain relevant. The challenges faced by many concerned taxonomists are to train interested young researchers and to equip them with relevant skills. Future taxonomists will need to master morphological, molecular and other techniques, develop verbal and writing skills to communicate and disseminate information, acquire computational skills and stimulate an interest in taxonomy in organisations and funding bodies. The current lack of interested candidates results from the low esteem of the discipline, the lack of job opportunities and the fact that future taxonomists will need to utilise a wide range of technologies and work on a range animal groups with little opportunity to specialise. Since there is currently no viable alternative to traditional taxonomy, what can be done to solve this problem?

Introduction

Taxonomy is the prerequisite for all biological endeavours and the key to the exploitation of biodiversity for biotechnology. Currently, although taxonomy has many advocates and most biologists agree that is an important discipline, it is still given very little attention in the universities and even less by funding agencies. This growing national and global trend is worrying.

We would like to use this opportunity to present the current state of taxonomy in the global arena, deliberate on its importance, highlight the need and plight of taxonomist and discuss what can be done to improve our knowledge of our national biodiversity and enhance our taxonomic expertise. The value and importance of biodiversity has been well elaborated by several presenters at the present symposium, particularly in the sessions on Values of Biodiversity and the Policies and Law on Biodiversity. The current status and the importance of biodiversity as the source of material for biotechnological endeavours had been very effectively dealt with by Latiff (2008) and Ng and Saw (2008), while Cranbrook (2008) presented the global initiatives for safe-guarding and to documenting biodiversity resources of ecosystems. Hence, it will not be necessary for us to elaborate further on aspects biodiversity and, in this presentation, we will be concentrating on taxonomy and taxonomists.

Current state of taxonomy: is it still relevant?

Taxonomy (taxo-, *arrangement* (Greek **taxis**) + -nomy, *method* or *law* (Greek **nomia**) deals with the scientific classification of organisms into named groups based either on shared characteristics or on evolutionary relationships as inferred from the fossil record or established by genetic analysis (Wikipedia). Taxonomy therefore involves collecting, identifying, describing, naming and classifying organisms.

250 years ago Carl Linnaeus provided scientists with the binomial system of nomenclature for plants and animals. This important milestone in the history of biology essentially revolutionised science and has inspired and influenced many generations of scientists, such as Darwin and Wallace.

The name of a species is important because it unites all the information and knowledge accumulated on that particular group of individual organisms, such as physical attributes, reproductive biology, ecology, distribution patterns and other life functions. The accumulated knowledge is useful for a variety of reasons, particularly for the production of plants and animals for food, medicine and shelter. Such knowledge is also necessary for the proper maintenance of our environment and for the strategic and effective control of parasitic diseases.

After 250 years of research since Linnaeus' innovative binomial system of nomenclature, it is generally estimated that only a fraction of the world's species have been identified, named and documented. This has been noted by various researchers, such as E. Wilson, an eminent ecologist, who stated that "we have still only classified as few as 10 percent of the organisms living on Earth" (AFP, 2008). He also stated that there are an estimated 1.5 million species of fungi, but only 60,000 are known. Nematodes, including parasitic pinworms and hookworms, comprise the most abundant phylum in the animal kingdom - probably four or five million strong - but only 80,000 have been classified (AFP, 2008). Both Cranbrook and Ng and Saw (this symposium) have independently provided information on our knowledge of the status of vertebrates in Malaysia, essentially noting that we only know about 10-20% of our existing fauna. Data from parasitology, and particularly from the global helminthological records (Table 1) and estimates of monogenean diversity from biodiversity hotspots (Table 2), support the fact that there are still many species waiting to be discovered and named. Malaysia has been independent for more than 50 years, yet we have not even begun to close the gaps in the knowledge of our biodiversity, and, given the rapid rate of environmental degradation, much of this biodiversity will disappear before taxonomists have the chance to document it for posterity.

The number of the helminth species described to date is given in Table 1. However, the figures presented are certainly well below their actual diversity, given the facts that the diversity of parasite species usually outnumbers that of host species by a conservative estimate of three or more times and that in many cases these parasites are host specific (Lim, 1998). This is magnified when one considers that many of the hosts have yet to be examined for parasites.

Table 1. Estimates on global helminth diversity

Helminth group	Number of species known
Nematodes	70,000
Trematodes	20,000
Monogeneans	8,000
Cestodes	5,000
Acanthocephalans	800

Table 2. Known and estimated monogenean diversity in three biodiversity hot-spots

Regions	Percentage documented to date	Estimated total diversity	Source of data
Malaysia	c.10%	1,883–3,039 (2–4 spp. /host) (freshwater and marine)	Lim, 2007
New Caledonia	c.1%	1,000 (marine)	Justine, 2007
South America	c.2.5%	16,000 (freshwater)	D.C. Kritsky, pers. comm. (from Kritsky, 2007)

During 2007 at a conference in Viterbo, Italy, three researchers independently provided estimates of the numbers of monogenean species in three biodiversity hotspots (Malaysia, South America and New Caledonia) (Table 2). At best only 10% of the monogenean species available on fishes are known, and, in the case of New Caledonia, only c.1% of the estimated number of species has been described. Monogenean diversity was estimated by multiplying the host diversity by the number of monogenean species per host species (Lim, 1998).

Thus, 250 years after the introduction of binomial nomenclature, taxonomy is still necessary and relevant, because much of the world's fauna and flora are still unknown. Do we need to identify

all of the unknown organisms? Being aware of the species present in ecosystems will enable us to develop effective strategic plans for the conservation and management of these ecosystems and the species therein. In some areas, the process of species identification and cataloguing are really only now beginning. These areas are usually the most vulnerable in terms of biodiversity loss. Will we have the time to document the taxa before they are lost? Do we have enough man-power (taxonomists) to undertake the enormous task of describing, naming and documenting the unknown species of the fauna and flora? Do we have good taxonomists available to do the necessary training to ensure the replacement of their expertise? Who is responsible to ensure that such training is on-going and sustained? *These are some of the important questions that we need to consider!*

Taxonomists – the number in Malaysia

Currently there are no statistics on the actual number of taxonomists and their expertise in Malaysia. Attempts to determine this number by using publications in ISI-indexed systematic journals will be inaccurate, since some international journals do not publish papers on species which they consider to be of local interest only. However, such statistics are necessary for estimating the number and types of taxonomists we need in the future for documenting our biodiversity.

Taxonomists - are they still needed?

Taxonomists are needed to examine the specimens, identify the species and, if the species is new, to describe it, name it, publish the description and deposit the name-bearing specimens in a suitable museum. The known species diversity within an ecosystem is dependent on taxonomic activity, which, in turn, is dependent on the availability of taxonomists. This is well illustrated by the fact that, prior to the 1980s, practically nothing was known of the monogenean fauna of Malaysia; however, between 1984 and 2008 about 200 species have been recorded from fishes in and around Peninsular Malaysia (Lim, unpublished information).

Taxonomy is more than just the identification and description of new species and taxonomists are more than the custodians of biodiversity. Specialist taxonomists also have the task of identifying taxa for academic, economic, ecological, medical and veterinary purposes. They are also needed to rationalise and classify known taxa. So long as man is still dependent on biodiversity-related resources for food, medicine and shelter, and as long as man, his domesticated animals and other food resources suffer from disease, particularly parasitic diseases, we will need taxonomists. We also need taxonomists to identify our natural food resources for other reasons, but especially for the maintenance of genetic diversity.

Are taxonomists still available?

There are growing global concerns over declining number of taxonomists. Is this concern warranted? We would like to address this issue by assessing the health of taxonomy as suggested by taxonomic activity in terms of the number of taxonomic publications on over a 15 years period, and also to indicate how long the currently active taxonomists will be around to do the work by estimating the average age of the current crop of active taxonomists both as authors and as reviewers (specialist experts).

The global trends, particularly in the number of submissions by taxonomic groups (1991-2006) to *Systematic Parasitology* [SYPA] (Fig. 1) and the number of new species described for different taxonomic groups since 1988 (Fig. 2), indicate that taxonomy is still an active discipline and that experts on the different groups are still available.

Fig. 3 and 4 show the average age of authors and reviewers of the same systematic journal to be 46.2 and 54.5 years, respectively, in 2006. The ages of authors increased during the 1990s, but they have levelled off apparently due to young students writing papers under supervision, especially in the US and South America (Fig. 3); unfortunately many of these students fail to find positions to continue their work.

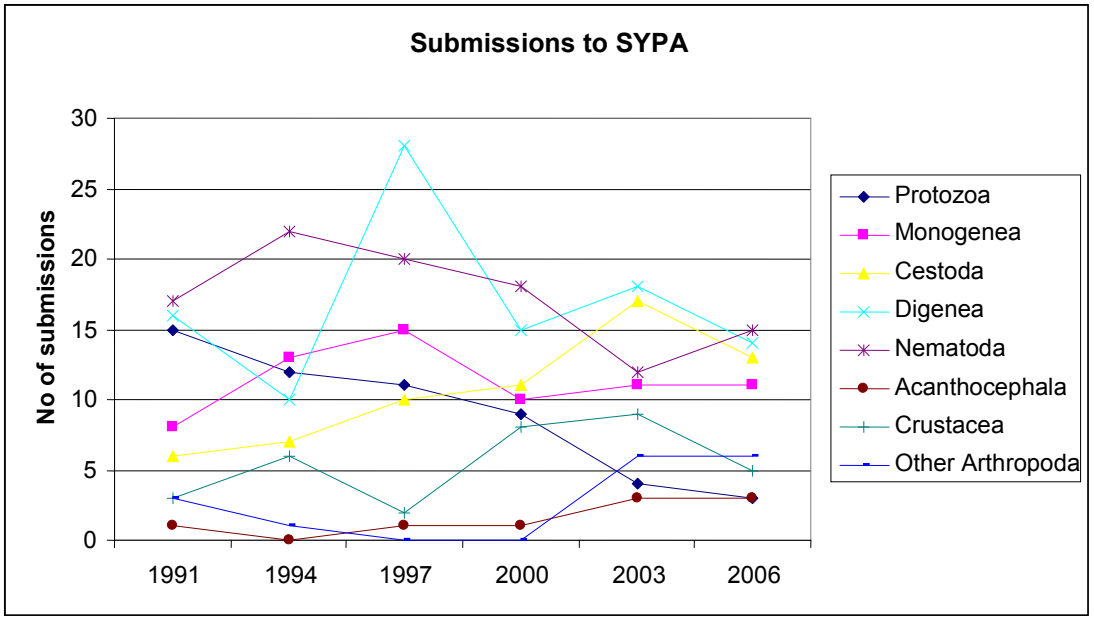

Fig. 1. Trends in the number of submissions on different taxonomic groups (1991-2006).

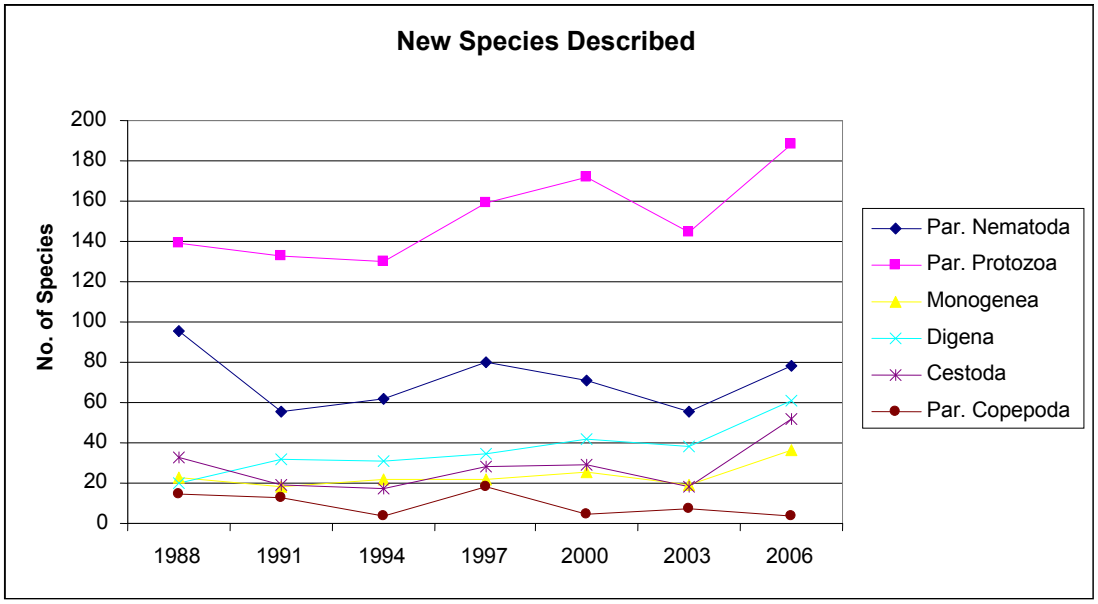

Fig. 2. New species for different taxonomic groups described since 1988.

Although, generally speaking, specialists are still currently available, they are aging and their numbers are declining. It seems that, unless something is done to prevent it, this decline will inevitably continue. Fig. 4 indicates that the average age of reviewers of one international taxonomic journal is 55 years. Considering that many retirements are inevitable within the next 20 years, particularly in areas where the retirement age is still primitively 55-58, this average age is unlikely to drop. There are far too many species to describe and document, and, in the case of some faunal groups, there are no taxonomic specialists. Trained taxonomists or taxonomic specialists are very important, particularly in biodiversity hotspots where they are often in very short supply. To make matters worse, the centres of

mega-biodiversity are usually in countries prone to poor environmental management and conservation strategies, with high rates of extinction of both known and unknown species. There is little effort to replace or retain retired taxonomists by most universities in these countries.

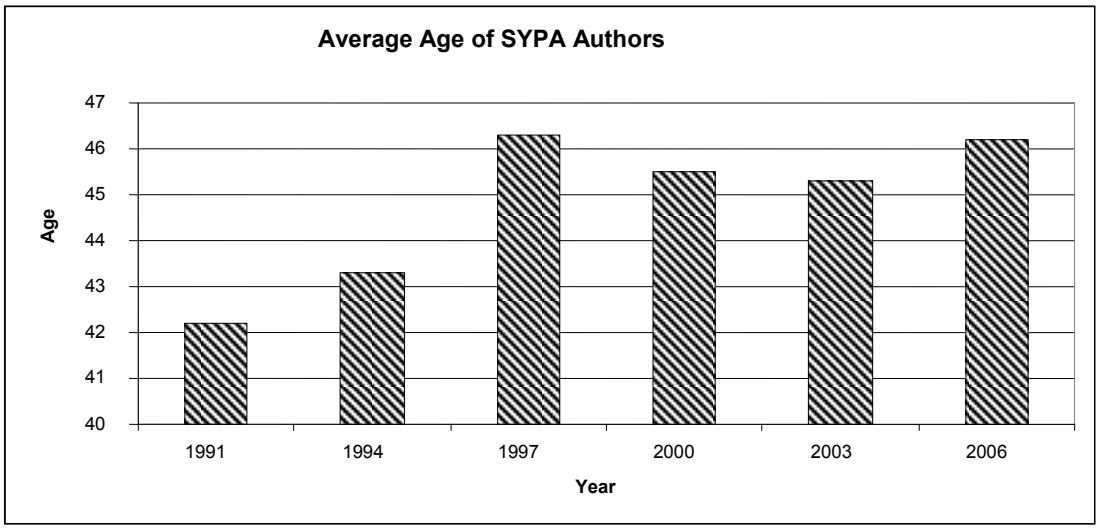

Fig. 3 Estimated average age of first authors.

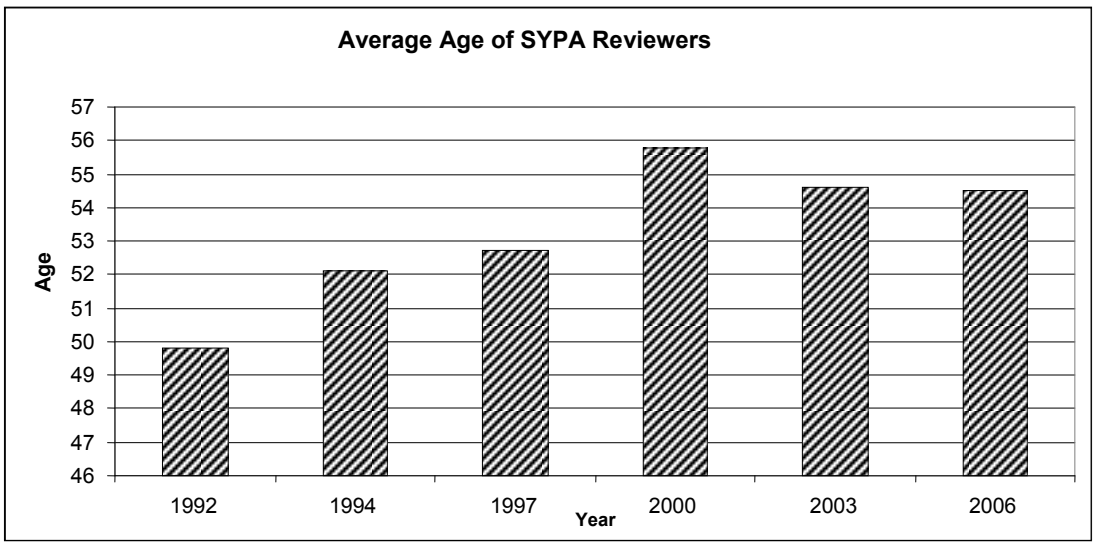

Fig. 4 Estimated average age of reviewers.

There are currently still taxonomists available to do at least part of the task of documenting biodiversity and to start to train future specialists to replace their declining numbers. However, will this 'old guard' be around long enough, taking into account the fact that acquiring specialist taxonomic skills takes a long time, for a complete replacement of their expertise (both regionally and globally)?

Do we have the resources or the will-power to face the challenges in order to sustain and further enhance taxonomy? As indicated above, in many areas we do currently still have the expertise, but, in order to sustain it, a new generation of taxonomists has to be found, or we will need to find alternatives to current taxonomic practices.

Can we find the right candidates to train?

Students are generally **not** keen on taxonomy because many of them consider it to be dull and old-fashioned, and this perception is also held by some biologists. Nothing has been done to promote taxonomy, whereas biotechnology is always in the media and is usually well funded. To foster a new generation of taxonomists, it is necessary for the present group of taxonomists to:

a. encourage research in taxonomy,
b. advertise the value of the discipline, and
c. increase the general perception of the value of taxonomy.

If we delve more deeply into the life of taxonomists in order to provide would-be taxonomists with an honest view of what they are up against, we find that, generally speaking, professional taxonomists are unappreciated (even in taxonomic institutes), lack funds, publish in journals with lower impact factors and have a low personal h-index. Characteristics of the professional life of taxonomists include:

a. Poor salary
b. Not glamorous
c. Long training period
d. Poor working conditions
e. Long working hours
f. Poor job prospects: institutions traditionally hiring taxonomists are now hiring generalist molecular biologists who are more effective in attracting funds
g. Lack of support from the universities/institutions in terms of facilities and funds
h. Promotion prospects are dismal in view of the lack of funding and poor citation rates, especially since universities are using citation indices as criteria for promotions. (Taxonomic journals usually have low impact factors, and taxonomic papers are poorly cited because journals do not require authors to cite the authorities for taxonomic names as references, resulting in a lack of recognition by colleagues from other disciplines).

This essentially means that most taxonomic works are so specialised that they are not well-cited, at least in the short term. Taxonomic (systematic) journals usually have lower impact factors than other journals. For example, compare *Systematic Parasitology* with an impact factor 0.786 with that of *Trends in Parasitology* at 4.528 (Table 3: data for 2006). Even very good papers on obscure group may not be cited for 10-20 years or more. Although taxonomic papers do not have the immediacy of papers in other disciplines, they may continue to be cited for up to 100 years or more. Unfortunately, the half-life of a paper is calculated over the period of only a few years. Perhaps some of the new ways suggested for the calculation of impact factors and other indices might assist in raising the profiles of taxonomists.

Table 3. Impact factors (2006) of some parasitological and taxonomic journals

Journal	Impact factor
Taxonomy/Systematics	
Systematic Parasitology	0.786
Journal of Natural History	0.694
Comparative Parasitology	0.615
Zootaxa	0.612
Zoosystema	0.462
Systematics and Biodiversity	Unrated
Review	
Trends in Parasitology	4.526
Advances in Parasitology	3.154
Molecular	
Molecular and Biochemical Parasitology	2.733

Journal	Impact factor
General Parasitological Journals	
International Journal for Parasitology	3.346
Parasitology	1.703
Journal of Fish Diseases	1.661
Journal of Parasitology	1.524
Experimental Parasitology	1.306
Parasitology International	1.280
Parasitology Research	1.226
Folio Parasitologica	1.138
Acta Parasitologica	0.617
Journal of Helminthology	0.581
Helminthologica	0.480

Other ISI indices include the Immediacy index, Half-life index and recently the h-index. Characteristically taxonomic papers have a low Immediacy index, i.e. the average citation of an article per year. The Half-life index is the number of publication years from the current year which account for 50% of current citations received. Theoretically taxonomic papers should rate high in the Half-life index, but they do not because this ISI index only takes into account the last few years and many taxonomic publications are still cited after 50 years or more. A new index, which is being used for both scientists and journals, is the h-index or Hirsch-index (Fig. 5). This index is useful because it discounts the disproportionate weight of highly cited papers or papers that have not yet been cited and essentially averages out the effect of highly cited and uncited papers.

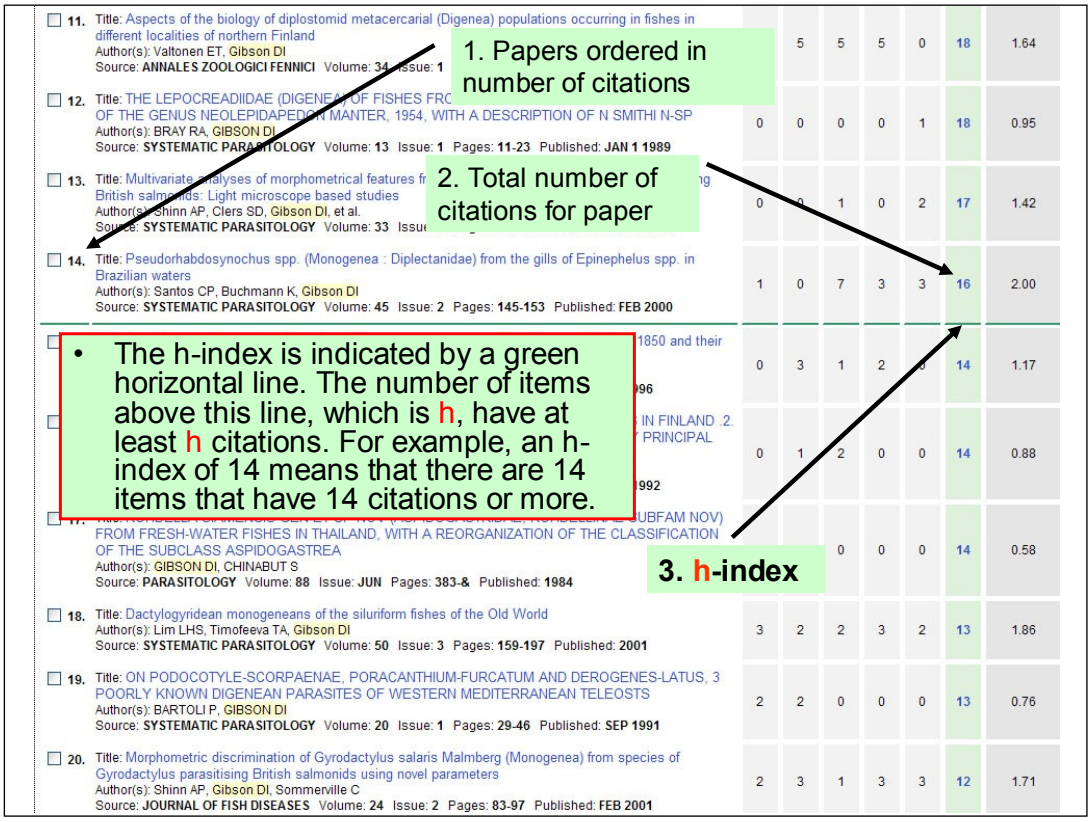

Fig. 5. Example of the h-index of a generalist taxonomist.

Table 4. H-indices of parasitologists according to research and geographical areas

Expertise	h-index
Senior specialist taxonomic expert in a restricted geographical area	8
Senior generalist taxonomic expert – specialising in more than one helminth group	14
Senior ecologist	17
Senior generalist expert working in a range of disciplines	18
Young molecular biologist	10

Table 4 is a summary of the h-indices of several parasitologists in different disciplines, regions and periods of their careers. The h-index of a taxonomist will tend to be low if that person is very specialised or works in a corner of the world of little interest to others, e.g. the first example, where the expert works on an obscure group in a restricted geographical area. This compares unfavourably with a taxonomist specialising more widely on several groups, an ecologist, a generalist and even a young molecular biologist early in his career.

Skills needed by a young taxonomist

Assuming that there are young researchers willing to do taxonomic work, in order to be relevant new taxonomists need to acquire multiple skills. This is because on most occasions they will have to work alone and will need to incorporate other aspects into their investigations, particular those of molecular biology and computerised analyses. Hence, the new generation of taxonomists will need to master skills varying from the basic techniques of specimen preparation to advanced molecular and computational techniques. Future taxonomists might also need to acquire knowledge such that they are able to work with several groups rather than being able to specialise on a single group of organisms, learn to work with other specialists and be able to extend the boundaries of their research field. Skills of the new generation of taxonomists include:

a. Basic morphology using standard techniques and instruments
b. Molecular techniques
c. Use of computational tools for capturing images and for image recognition
d. Use of illustration gadgetry
e. Accessing global databases
f. Communication and writing
g. Involvement with international projects, such as Barcoding and the Encyclopedia of Life initiatives
h. Being aware of funding sources and performance indicators, such as impact factors and other indices
i. Specialising in more groups and acquiring a global expertise

Alternatives to traditional taxonomy

Current taxonomists have also to consider the possibility that there will be few or no candidates wanting to be the taxonomists of the future. If so, we have then to think of alternatives to ensure that the knowledge acquired thus far will be retained by using whatever technologies are available to us. The most likely alternatives to replace the human taxonomist are the use of DNA markers and computer-aided image recognition systems linked to artificial neural networks. However, such alternatives are not currently practicable, nor are they likely to be for some considerable time.

The process of determining the molecular markers necessary for identification is currently expensive and time-consuming and the necessary facilities and expertise are often not readily available in the poor, developing countries which harbour the biodiversity hotspots. Furthermore, in order to determine the markers, we need first to identify the specimens. The Bar-coding Initiative likely has this aim in mind, but it will be many, many years before one can determine the identity of an organism automatically within a few hours from a piece of tissue.

Will the computerised alternatives be better? It also takes time to develop the software capable of using images, learning and identifying species, and the initial phase of the development of such recognition will also required the active involvement of taxonomists, as the organisms will still, at least initially, need to be identified. The infrastructure and high performance computers will be needed to

store and process the information. This alternative is very much in its infancy, with few investigators interested in this aspect of image analysis, and existing image databases are currently far too small. However, developed countries are now looking at the use of computation to 'curate' biodiversity data (Howe *et al*., 2008), and in Malaysia such an initiative started in 2004 (Sarinder *et al*., 2008), but the rate of development of this database is slow.

These alternatives can only assist but not replace the human taxonomist - at least not in the near future, because time is needed to develop such alternative tools. Unless the technology has advanced to a stage where the artificial intelligence has the ability to work like a human brain and take over decision making, we still need trained human taxonomists to point the way and to confirm results.

Who is responsible for training new taxonomists?

So, if we need candidates for training in taxonomy, who is responsible for attracting and training them? The various governments, universities, research institutions, journals, funding agencies and, of course, current taxonomists are all responsible, at least to some extent, to ensure that young taxonomists are trained and nurtured (Table 5). But, most importantly, they also need to ensure that there are career opportunities to allow them to continue doing taxonomic work once they are trained.

We really need funding agencies, whether within or outside the government systems, for funds to train taxonomists. All taxonomic and biodiversity projects should have a strong training element. The PEET initiative (Partnerships for Enhancing Expertise in Taxonomy) in the USA has shown that such research programmes do yield good results, as suggested by an increase in the numbers of taxonomic papers in parasitology from the US after 1984, the year PEET started (Fig. 6). However, such an initiative is not evident in developing countries, as exemplified by Malaysia (one of the mega-diversity centres). In Malaysia most taxonomists appear to work 'in disguise' just to obtain funding to feed their taxonomic activities and, in doing so, jeopardise their taxonomic research.

Even if there are students trained in taxonomy, there is not enough done to ensure that these young experts have positions suitable to continue the work which they are trained to do. The Government and universities have the moral responsibility to safe-guard our biodiversity and the environment and, hence, should ensure that such career opportunities are available.

Although most scientists and biotechnologists agree that taxonomy is important and necessary for the development of biotechnology in Malaysia, there are no visible signs of a revival of taxonomic investigations in this country, nor are there any changes in university curricula to incorporate taxonomy. There needs to be an increase in funding for fundamental science and, in particular, in taxonomy; currently the monies allocated for fundamental science is very much smaller than for biotechnology, and this amount has to be shared by all branches of science. However, the need to train young taxonomists to replace older ones is a global problem as well as a national one.

Table 5. Agencies responsible for nurturing new generations of taxonomists

Agency	Role and function
Governments	Funding, facilities and employment
Funding agencies	Funding research with a strong training element, e.g. PEET (Partnerships for Enhancing Expertise in Taxonomy)
Universities	Facilities, buildings, employment and training; also education through the basic science curriculum (it is the duty of the university to provide the curriculum)
Institutions – Research, Museums and Non-Government Organisations	Champion the cause; funding, facilities and employment
Journals	Help in general, even if only in self interest
Concerned taxonomists	Promote taxonomy in general; train future taxonomists such that their own expertise does not die with them; ensure the continuous existence of scientists capable of expert identification

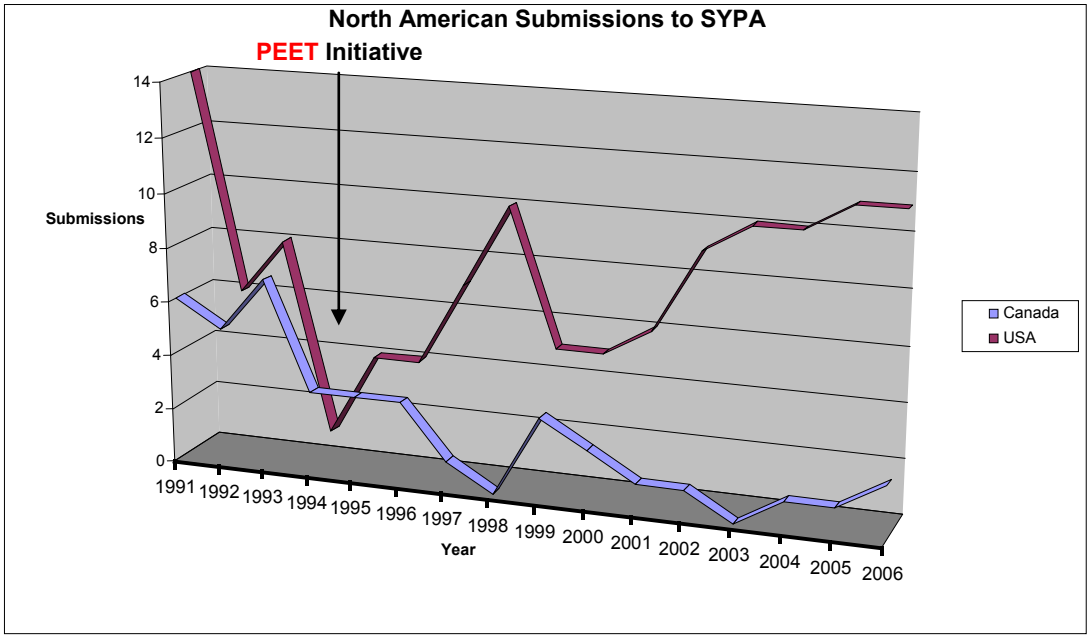

Fig. 6. The apparent affect of the PEET Initiative on taxonomic submissions to one parasitological journal.

Malaysia is not making any significant effort to keep taxonomy active, nor is there any nation-wide initiative to train taxonomists capable of identifying and curating the flora and fauna. Biodiversity has been given a new lease in life by biotechnology, but this information seems to be well hidden from students by well-meaning educationists, especially in developing countries, who believe that students should be trained for jobs rather than to acquire knowledge, which is leading to the rush to acquire technological expertise.

There is a need to change the current perception that biotechnology can exist without a proper knowledge of biodiversity. To tap natural resources in a sustainable manner, we need taxonomists to identify the species and ecologists to ascertain their environmental needs. Without the taxonomists providing the key to biodiversity, biotechnological endeavours may come to a stand-still. Malaysia should realise that in order to keep her biotechnological hopes alive, competent taxonomists are needed to identify the available biodiversity resources.

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Arbuscular Mycorrhizal Fungi Associated with *Calophyllum* sp. in Universiti Putra Malaysia Bintulu Campus Forests, Sarawak, Malaysia

Lee, C.S., Ong, K.H. and Chubo, J.K.

Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus
PO Box 396, 97008 Bintulu, Sarawak
claire_cshing@hotmail.com

Abstract

Arbuscular mycorrhiza fungi are soil fungi that form mutualistic symbiosis with the roots of plant. The objectives of this study were to investigate the diversity of arbuscular mycorrhiza and to identify isolated spores associated with *Calophyllum* sp. This study was carried out in a replanted forest and a logged-over forest in Universiti Putra Malaysia Bintulu Campus (UPMBC). Samples of top soils that coexist with the selected host plant, *Calophyllum* sp. were collected. Total abundance of spores, species richness (*S*), Shannon-wiener Diversity Index (*H'*) and evenness (E_H) were calculated. A total of 13 species of arbuscular mycorrhiza were isolated where 10 species were identified at the genus level. The replanted forest showed higher arbuscular mycorrhiza spores diversity as compared to the logged-over forest. On the other hand, the logged-over forest recorded higher evenness value as compared to the replanted forest. This was probably due to the fact that the replanted forest had the highest number of the *Glomus* species spores when compared to other genera. The present study suggested that proper logging practices have to be imposed in order to enhance the abundances and activities of AM fungi. The impact of harvesting on soil or site must be taken into consideration especially in maintaining soil productivity. The present study also proposed that the number of spores in the soil might act as one of the indicator to detect the level of disturbances in the forest.

Introduction

Mycorrhizal fungi are critical component of most plants which are intimately associated with plant roots. They improve plant health and the development of plants by enhancing nutrition uptake, modifying physiological functions of plants, reducing plant response to environmental stresses, and modifying the chemistry and biology of the rhizosphere in ways that alter nutrient cycling and suppress activity of root pathogens (Read, 1999).

Several *Calophyllum* spp. have been recorded in Malaysia. *Calophyllum inophyllum* is the most widespread and has superior timber qualities (Wong, 1982). However, plantation of *Calophyllum* sp. is rare in Malaysia. There is a potential to cultivate this plant species as it is getting well-known especially in the field of research due to its anti-HIV active compounds. Thus, should such claim be true the mass planting of *Calophyllum* spp. will be of great interest.

The conversion of the primary forest into plantations had caused great loss of nutrients in the soil (Lee, 1999). Thus, the regeneration of trees is extremely slow due to the depletion of nutrients. As such, mycorrhizae with their ability to enhance nutrient uptake and drought tolerance are expected to play a vital role in the success of reforestation effort. Studies by Jasper *et al.* (1987), Amaranthus and Steinfeld (2005), and Gehring and Connell (2006) suggested that the inoculation of tree seedlings with mycorrhiza prior to planting will be good in promoting the growth of the tree. Therefore, this study was conducted to investigate the diversity of arbuscular mycorrhiza (AM) related to *Callophylum* spp. in replanted forest and logged-over forest.

Materials and methods

Site description

This study was carried out at two different forest areas in Universiti Putra Malaysia Bintulu Campus (UPMBC), Bintulu, Sarawak, namely replanted forest and logged-over forest. The forests differ in their planting method and management practices. Both forest areas receive an annual rainfall of 350 cm and have an average annual temperature of 28°C. The replanted forest is situated in a Malaysia

Tropical Forest Regeneration Experimental Project launched by Mistubishi Corporation in 1990. This project was based on the idea of recreating native forests with indigenous trees, on the concept of potential latent natural vegetation. On the other hand, the logged-over forest which is known as Nirvana Forest, was once belonged to the State Government before it was transfer to UPMBC in 1985. In 1977, this forest had been selectively logged. When UPMBC was temporarily closed in the 1994, illegal logging was committed by the locals where majority of trees with diameter of more than 40 cm were logged. Now, the logged-over forest is used for teaching and research purposes.

Collection of soil samples

Approximately 500 g of soil in close proximity to the roots of the host plant, *Calophyllum* spp., was taken from a depth of 10 – 15 cm after removing the top litter layer (0 – 10 cm). Random sampling of soils was collected in triplicate. The soil samples were brought to the laboratory and stored at 4°C.

Extraction and identification of arbuscular mycorrhiza spores

Extraction of AM spores was done by wet-sieving and 50% sucrose centrifugation method (Brundrett *et al*., 1996; Chaurasia *et al.,* 2005). The soil sample was decanted through a series of sieves (53 µm, 150 µm and 710 µm). Spores were separated into groups based on their colour and size (Brundrett *et al.,* 1996) under a dissecting microscope (×30 magnification). Spores were then mounted on glass slides in polyvinylalcohol–lacto–glycerol (PVLG) + Melzer's reagent (Shi *et al.,* 2006) for the purpose of identification. Identification was done up to the genus level according to Morton (1998). The characteristics of spores such as size, shape, cell wall, subtending hyphal wall and other spore structures were carefully recorded and analyzed.

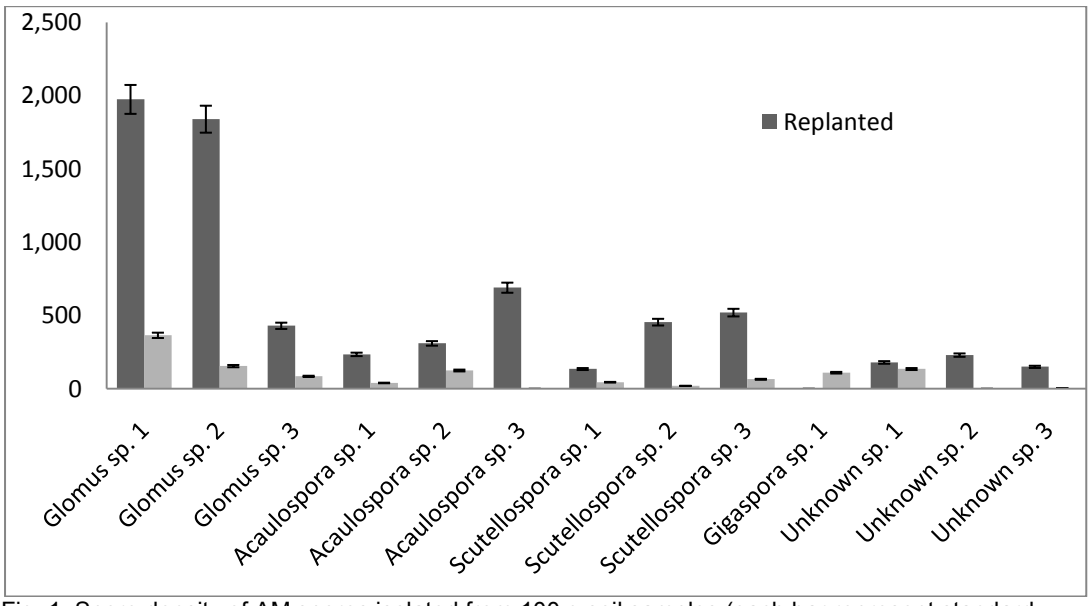

Fig. 1. Spore density of AM spores isolated from 100 g soil samples (each bar represent standard error with 5% value).

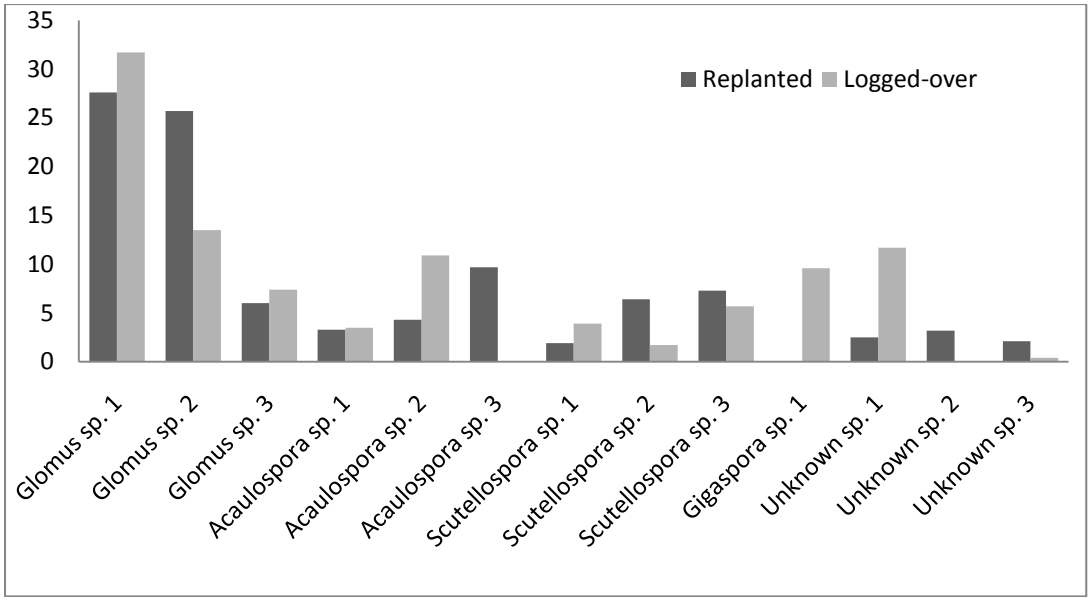

Fig. 2. Relative abundance of AM spores isolated from 100 g soil samples.

Data analysis

Total abundance of spores, species richness (*S*), Shannon-Wiener Diversity Index (*H'*) and evenness (E_H) were calculated in order to evaluate the distribution and diversity of AM. Mean differences were tested using the ANOVA with unequal number of samples. The Duncan Multiple Range Test was used for mean separation within the mycorrhizal species.

Results

Spores were isolated from 11 soil samples collected in close proximity to the host plant. The isolated spores showed clear occurence pattern between the two different forest sites. In the replanted forest, a total of 7,150 spores were isolated in contrast to the low number of spores (1,150) isolated from the logged-over forest. The spore densities for each species in both forests ranged from 5 to 1,975 per 100 g soil with *Glomus* spp. 1 recording the highest number in both forests (Fig. 1).

Fig. 2 shows the relative abundance of all the AM species found in this study. In the replanted forest, *Glomus* sp. 1 and *Glomus* sp. 2 gave the highest relative abundance of 27.6% and 25.7% respectively. Likewise, in the logged-over forest, *Glomus* sp. 1 and *Glomus* sp. 2 also recorded the highest relative abundance of 31.7% and 13.5% respectively.

Samples taken from the replanted forest showed higher *H'* than the logged-over forest (Table 1). However, the logged-over forest showed a higher E_H with individual species in the samples distributed more equally. The low E_H indicated that higher species dominance occurred in the replanted forest with *Glomus* sp. 1 and *Glomus* sp. 2 numbers tremendously high.

Table 1. Arbuscular mycorrhizal fungi diversity indexes of replanted forest and logged-over forest in UPMBC

Index	Replanted Forest	Logged-over Forest
Shannon-Wiener Diversity (*H'*)	2.0729	2.0442
Evenness (E_H)	0.8342	0.8525

Discussion

The species number of AM between the replanted forest and the logged-over forest indicated an unambiguous dominance of the AM spores in the replanted forest. According to Allen *et al.* (2002), the populations of mycorrhizae can be affected by any form of land management that involves tillage, timber harvesting, vegetation clearing, or other forms of disturbance.

In the present study, the replanted forest was ploughed to a depth of 1 m before the plantation was established, thus soil structure was loose and had high soil porosity. In addition, seedlings planted in the replanted forest were raised using topsoil collected from the natural forest. Hence, existing mycorrhiza in the soil are able to propagate and thus increased the number of spores. A low number of spores in the logged-over forest may due to high intensity logging activity carried out in 1994. Soil was compacted due to the heavy machinery used. Besides that, a lot of skid trials and logging roads were established during such logging operations. Entry *et al.* (2002) also reported that soil compaction is among the factors that influenced the formation and function of AM. This is consistent with the logged-over forest in the present study where spore numbers did not show recovery over time. It also demonstrated that soil ecosystem impacts caused by heavy machinery might not be easily reverted.

The abundance of AM in soil varies in response to disturbance. This is because viable spores might not persist long enough after the disturbance. However, regeneration or rehabilitation will boost the populations of spores. Abbott and Gazey (1994) reported that AM spores can colonized disturbed soils but the impoverished populations of mycorrhiza could be constrain to subsequent rehabilitation of such disturbed sites.

Glomus recorded the highest number of spores in both sites. This suggests that *Glomus* is tolerant to a broad range of ecological adaptation in relation to floristic composition and soil characteristics. Many studies have reported similar observation which includes Gaur *et al.* (1998), Franke-Synder *et al.* (2001), Chaurasia *et al.* (2005), and Shi *et al.* (2006).

There were three species that were unidentified and were labeled as Unknown sp. 1, Unknown sp. 2, and Unknown sp. 3. The failure to identify these species was due to poor spore development where the defining characteristics were not obvious. This includes spore wall layers which were not obvious well as the absence of swollen and bulbous subtending hyphae.

Overall, samples taken from the replanted forest showed higher abundance of spores and species richness than the logged-over forest. Similar pattern was also observed for the species diversity. Chaurasia *et al*. (2005) reported similar finding for diversity indices. However, evenness of the replanted forest was lower compared to the logged-over forest. This could be due to the higher species dominance of *Glomus* sp. 1 and *Glomus* sp. 2 in the replanted forest which affected the distribution of individuals in the samples among the species.

The present study suggested that proper logging practices have to be imposed in order to enhance the abundances and activities of AM fungi. The impact of harvesting on soil or site must be taken into consideration especially in maintaining soil productivity. The present study also proposed that the number of spores in the soil might act as one of the indicator to detect the level of disturbances in the forest.

Conclusions

The number of spores recorded in the replanted forest was six times higher than the logged-over forest. Higher biodiversity was also found in the replanted forest. From the comparison of index value of evenness, results for the replanted forest indicated a dominance of the *Glomus* species which led to a slightly less diverse in condition when compared to the logged-over forest. This suggests that logging practices could influence the abundances and activities of AM fungi.

Acknowledgements

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Attitudes, Beliefs and Perceptions on Complementary Indigenous Malay Therapies: A Terengganu Perspective

Lua, P.L., Rohayu Izanwati, M.R., Suffian, M.T., Norlida, M. and Ahmad Zubaidi, A.L.

Faculty of Medicine and Health Sciences, Universiti Darul Iman Malaysia,
Kuala Terengganu, Terengganu, Malaysia
izanwati@udm.edu.my

Abstract

The practice and usage of Complementary Indigenous Malay Therapies (CIMT) continues to flourish in Malaysia in spite of the availability of modern treatments. The objectives of this study were to 1) identify common types of CIMT used/practiced and 2) compare attitudes, beliefs and perceptions (ABPs) on CIMT between urban and rural users. A prospective, cross-sectional recruitment of adult volunteers were conducted in pre-identified areas in Kuala Terengganu, Dungun, Kemaman and Besut (including Redang Island). A newly-adapted instrument assessing ABPs was administered to consenting respondents (response scale: 1: strongly disagree – 5: strongly agree). Data analysis employed descriptive and non-parametric statistics. Altogether, 130 respondents were enrolled (median age = 48 years old; age range = 17-90 years; female = 56.9%, married = 77.7%; Malay = 96.2%). Sixty percent were CIMT users (urban = 28.2%; rural = 71.8%) with a median usage duration of 6 years. Majority preferred traditional massage (74.4%), followed by herbs (66.7%), sea cucumber products (65.4%), roots (64.1%) and tuku (55.1%). Rural residents were significantly more confident with regard to CIMT's quality of care ($p = 0.018$), its unnecessary scientific studies ($p = 0.042$) or discussion with healthcare providers ($p = 0.010$) but were significantly more negative on CIMT's effectiveness when used concurrently with modern medicine ($p < 0.001$). These outcomes indicate the need for on-going efforts from healthcare authorities and providers alike to consistently disseminate awareness and knowledge on CIMT so that public self-treatment is always accompanied by sound understanding of its benefits and consequences.

Introduction

Since thousands of years ago, complementary and other alternative medicines (CAM) have been used for curing purposes and to generally improve the quality of human health. Complementary indigenous Malay therapies (CIMT) represent an example of such existing practice in the Malay community today. Several varieties of CIMT are still widely practiced throughout Malaysia, for instance herbal, gamat ointments, traditional massage, homeopathy, cupping and flower bath (mandi bunga). CIMT is also well received among Chinese and Indian communities nowadays and this has led to the belief that CIMT is closely related to an individual's confidence, belief and level of life. It also believed that the people especially from the rural areas are more inclined to use CIMT due to strong confidence in traditional medicine passed down over generations. Nonetheless, without on-going scientific research CIMT, its usage trend as well as their true attitudes, beliefs and perceptions (ABPs) in this practice cannot be consistently gauged. To- date, there is little documented data on such practices particularly in rural Terengganu, despite CIMT's popularity in the state. The current study seeks to explore the common types of CIMT practiced in Terengganu generally and to make a comparison between urban and rural users with regard to their ABPs toward CIMT.

Materials and methods

A prospective, cross-sectional survey was conducted in pre-identified areas in Kuala Terengganu, Dungun, Kemaman and Besut (including Redang Island). In terms of "rural" designation, the areas were identified according to guidelines from Jabatan Perangkaan Malaysia Terengganu, which also provided details on the distribution of residents within the area. With the exception of Kuala Terengganu district, the rest of the samples were all enrolled from rural areas in Dungun, Kemaman, Besut and Redang Island. Adult respondents living in each pre-determined village within the four districts were randomly approached by a team of researchers. A specific survey modified from

another similar study (Lim *et al*., 2005) was administered to respondents who agreed to participate. Items were largely exploring the respondents' demographic characteristics and the types of CIMT currently used or have used in the past (respondents were allowed to tick more than one selection), duration of usage, reasons for usage and their ABPs towards it. For the purpose of this study, CIMT practices include any form of medicinal, spiritual or practicebased traditional Malay treatments. For the items on ABPs, Likert responses were used (1 = strongly disagree; 5 = strongly agree) and higher scores indicate more favourable opinion. Findings were analysed using SPSS 14. Demographic data was presented descriptively while non-parametric statistics were employed for group comparisons.

Results

A total of 130 respondents were recruited. Fifteen respondents (11.5%) were recruited from Dungun or Kemaman, 41.5% from Kuala Terengganu and nearly half were from Besut or Redang Island (46.9%). Their median age was 48 years, ranging from 18 years to 90 years. Females comprised of 56.9% of the sample and 77.7% of respondents were already married. Malays were the majority with 96.2%. A large proportion had at least primary school education and 24.6% were supportive workers. Table 1 provides a more detailed description.

Sixty percent were CIMT users, majority from rural areas (71.8%). Urban dwellers who were CIMT users consisted of only 28.2% of the sample. The median usage duration for all respondents was 6 years, with range as short as from 3 weeks to as long as 50 years. Many preferred traditional massage (74.4%). Usage of herbs came next (66.7%), indicating its continuous popularity. Sea cucumber products, either formulated as tonic or ointment were also well-liked at 65.4%, followed closely by roots (64.1%) and tuku (55.1%).

Table 1. Demographic characteristics of respondents (n=130)

Variable	Frequency / Percentage	p-value*
Gender		< 0.001
Female	74 (56.9%)	
Male	56 (43.1%)	
Marital Status		
Married	101(77.7%)	0.114
Single	15 (11.5%)	
Widowed	10 (7.7%)	
Educational level		
None	19 (14.6%)	< 0.001
Primary school	26 (20.0%)	
SRP/PMR	13 (10.0%)	
SPM	37 (28.5%)	
STPM	7 (5.4%)	
Diploma	3 (2.3%)	
Degree	5 (3.8%)	
Master and above	19 (14.6%)	

* Chi-square test for goodness of fit.

Table 2. Comparisons of ABPs toward CIMT: Rural vs Urban

Items	Respondent Type	n	Mean rank	p-value*
Traditional Malay therapies work well with modern therapies.	Rural	50	30.83	< 0.001
	Urban	22	49.39	
Traditional Malay therapies are better in terms of quality of care and services.	Rural	48	39.18	0.018
	Urban	22	27.48	
Traditional Malay therapies do not require more scientific research.	Rural	48	38.17	0.042
	Urban	21	27.76	
The practice of traditional Malay therapies need not be discussed with doctor/pharmacist/nurse.	Rural	50	39.94	0.010
	Urban	21	26.62	

* Non-parametric Mann-Whitney U test.

In terms of ABPs, for the majority of the items asked, a trend involving rural residents expressing more positive views was observed. Mean ranks for rural respondents were consistently higher than urban dwellers with regard to CIMT being more effective in healthcare, better in quality of care, better in providing value for money, a superior choice of treatment, a highly recommended choice to friends/family, proud of its availability, unnecessary to undergo extensive scientific studies, more focused on human's well-being and unnecessary to discuss its usage with the healthcare providers. Specifically, rural residents were significantly more confident with regard to its better quality of care ($p=0.018$), no necessity for extensive scientific studies ($p=0.042$) or its need for discussion with healthcare providers ($p=0.010$)(Table 3). On the other hand, they were more negative about CIMT's effectiveness when used concurrently with modern medicine ($p < 0.001$), its absence of interaction with modern medicine ($p>0.05$) or that it should be promoted more extensively ($p>0.05$).

Discussion

This study among both rural and urban residents in Terengganu has revealed interesting trends in the prevalence and pattern of CIMT use. It was clear that CIMT use was rather widespread in Terengganu, especially among rural folks as compared to urban population. A study in Singapore reported that 76% of the respondents were CAM users (Lim *et al.*, 2005) while Ceullar *et al.*, (2003) has shown that approximately 48% of the African American and Caucasian American rural communities were CAM practitioners. The popularity of traditional massage, an undying practice which has also been a common service offered in many health centres today, has also been normally reported in studies involving 24 women in the treatment of premenstrual dysphoric disorder (PDD) by massage theraphy (Hernandez *et al.*, 2000). The premenstrual symptoms are relieved with decreases in anxiety, depressed mood and pain immediately after the massage. Usage of herbs and their related products is also expected, as in a US study of 267 respondents in Kansas City whereby 21% respondents were currently taking the at least one herbal product (Marinac *et al.*, 2007). Roots such as *Eurycoma longifolia* are well-known in Malaysia and its widespread use is expected. Tuku, a heated ball-like metal object, wrapped with cloth or paper and then gently rolled over the abdomen to promote good blood circulation is especially common during the post-partum period among mothers. Our sample respondents who used both roots and tuku also gave beauty and maintaining body structure as the reasons for use, particularly during first pregnancy. Overall, it is believed staying far from the healthcare services and practicing CIMT over the generations (and hence strong faith in its effectiveness) had created a norm for these residents to rely more on CIMT. Furthermore, this geographical isolation means that they depended heavily on natural resources from both the sea and agriculture products as their main socioeconomic activities (majority were fishermen or farmers). For example, sea cucumber represents a source of both medicine and supplement to maintain the good healthy living.

Despite the findings, our present study has suffered from limitations concerning sample size and ethnic proportion. A larger sample of respondents throughout Terengganu is vital in order to

provide a more generalised picture of CIMT usage. Because other minority ethnic groups also reside in the state, their data would reinforce the outcomes generated. It has even been suggested that cultural and clinical factors are highly influential in CAM practices among asthmatic patients (Ng *et al*., 2003). Overall, these outcomes indicate the need for on-going efforts from healthcare authorities and providers alike to consistently disseminate awareness and knowledge on CIMT so that public self-treatment is always accompanied by sound understanding of its benefits and consequences.

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Biodiversity of Phytoplankton: A Micro Floral Study in Aliyar Reservoir, Pollachi, Tamilanadu, India

Ezhili, N.[1] and Thirumathal, K.[2]

[1]Department of Zoology, PSGR Krishnammal College for Women, Coimbatore - 641004, India
[2]Department of Zoology, Arulmigu PalaniAndavar College for Women, Palani - 624615, India
ezhilinatarajan@yahoo.com

Abstract

Reservoirs play a key role in balancing the hydrological cycle and in providing water for drinking, municipal, industrial, irrigation and consumption. Since all biological reactions occur in water, its pollution becomes significant as it affects the biological system. The maintenance of the healthy aquatic ecosystem depends on the physical and chemical properties of water as also on biological diversity of the ecosystems Agarwal *et al*. (2000). Lentic ecosystem is the treasure house of varied biotic communities. The biodiversity provide and maintain ecosystem stability and is the very basis for sustainable development for the aquatic systems. This provides a model for the development of the biodiversity strategies. Plankton ascertain the historical background by their existence.the rivers are the best home for the flora being an index of water status. Aliyar reservoir is situated in Aliyar township just below the Anaimalai Hill ranges, at a distance of 20 km from Pollachi town (10° 20' N 76° 57' E). This receives water from Aliyar Basin with a catchment area of 122 m^2 the streams contributing to this being Aliyar, Chinnar and Sithar. A feeder channel also brings the water from Thunakkadavu (Parambikkulam) to aliyar reservoir to augment its supplies.The maximum water spread is 41 m (135') and the mean depth 16.8 m (55'). Aliyar reservoir is studied for its diverse nature of the biotic components such as phytoplankton. Qualitative analysis of the micro and macro communities of the reservoir is undertaken, which revealed the varied forms of species composition. The present investigation has been undertaken to identify the nature of biodiversity which is one of the complex characteristics of the reservoirs. It also acts as a valuble tool for the aquatic ecologists to study and to ascertain the dynamics and functional status. The phytoplankton recorded in the present study revealed the presence of Cyanophyceae, Chlorophyceae, Bacillariophyceae and Euglenophyceae.

Introduction

The Aliyar Reservoir is one of the hot spots of South India and it has the catchment area of 198 km^2. Aliyar reservoir is situated in Aliyar township just below the Anaimalai Hill ranges of Western Ghats, Tamilndu, India at a distance of 25 km from Pollachi town (10° 20' N 76° 57' E). It receives water from Aliyar Basin with a catchment area of 198 m^2. Aliyar, Chinnar and Sithar stream supply water to the Aliyar reservoir. A feeder channel also brings the water from Thunakkadavu (Parambikkulam) to Aliyar reservoir to augment its supplies. The maximum water spread is 41 m^2 (135') and the mean depth 16.8 m (55').

Importance of reservoirs in maintaining a healthy as well as a prosperous nation in a healthy environment is amply understood from the very existence of the civilization on this globe. The healthy functioning of the biosphere of our planet, of life, including all human life, depends entirely on the water flow and steady state phenomenon. Reservoir water is primarily used to satisfy the daily needs of the living world in and around them.

Reservoir water offers a very promising source of water for domestic, industrial and agricultural utilization. The aquatic systems have also been used as a convenient means of disposal of wastes and waste waters. Due to reckless exploitation and misuse, reservoir water gets polluted. Our cities could not function without water; our forest and agricultural crops would die; and the wheels of our industry would come to a grinding halt.

Aquatic ecosystems are the treasure house of varied forms of biotic communities. The biodiversity provide and maintain ecosystem stability and is the very basis for sustainable development for the aquatic systems. This provide a model for the development of the biodiversity

strategies. Plankton ascertain the historical background by their existence. The reservoirs are the best home for the flora being an index of water status.

A body of water harbours a fascinating world of myriads of living organisms including microscopic phytoplanktom which function as a dynamic integrated system. Hence, understanding of biological environment is necessary for comprehending the nature and the behaviour of the aquatic ecosystem (Wright, 1958). The biological information can be of great significance in water quality monitoring, which allow the pollutants to wash away leaving behind only the affected biota, in turn reflect the conditions existing in the environment.

The use of agrochemicals is resulting in the increased contamination of water sources by addition of nitrates, phosphates, iron and pesticides (Ghose *et al.,* 2000). Fertilizers increase the levels of nutrients leading to eutrophication. Depletion of dissolved oxygen caused by phosphate induced algal growth leads to death of fishes and other aquatic forms. Nitrates too bring about excess growth of vegetation, often leads to accumulation of nitrates in water. Water gets deteriorated due to ionic imbalance, high acidity and alkalinity. In enclosed bodies of water such as lakes and reservoirs, high concentrations of pollutants can cause a sudden increase in the quantity of algae, which will turn the water into a turbid, depleting its oxygen content (Pandey *et al.,* 1995).

In India, all the fourteen major rivers get attached to the important reservoirs which have been polluted beyond their self-purification limits. Many parts of the reservoirs do not seem to have any dissolved oxygen and fail to support the growth of desirable aquatic flora and fauna (Jha *et al.,* 1997). Many of the water bodies have become darkened, smelly and chocked with excessive growth of algae.

A comprehensive study with multidimensional approach will pave way for uplifting the environmental status of reservoirs by which the ecobalance can be maintained, thereby the problem of resource depletion and environmental deterioration can be reduced. Hence, the present investigation on biological qualities in terms of phytoplankton in the Aliyar reservoir has been undertaken to ascertain the ecological status of the reservoir.

Materials and methods

The Aliyar reservoir water has been studied for a period of two years from January, 2006 to December, 2007, to identify its phytoplanktonic compositions, based on the study of biological characteristics in three sampling stations. So, composite surface water samples were collected from three stations (North, South and East). The samples for plankton analysis was collected early in the morning by plankton net of silk bolting clothe size of 25μ and preserved in Lugol's Iodine solution for further analysis. The samples were analysed as per the methods of Trivedy and Goel (1986) and APHA (1995).

The identification of plankton was carried out by Standard Research Monocular Microscope, Getner make No.GR-5 and Universal Trinocular Research Microscope –Asahi Pentex Camera Model GMX – UE.

Results and discussion

Different phytoplankton genera were identified, in which Cyanophyceae comprised of 6 genera, 10 genera belonged to Chlorophyceae, 1 genus under Euglenophyceae, 18 genera in Bacillariophyceae and Dinophyceae comprised of only 1 genus. The species composition is shown in Fig. 1. Photographs of the species identified are given in Fig. 2–6. Species Diversity Index and Polmer',s Generic Index had been calculated to identify the pollution status (Menhinick, 1964 and Palmer, 1969).

Plankton ascertain the historical background of the aquatic environments by their existence. Reservoirs are the best home for the phytoplankton. Cyanophyceae, Chlorophyceae and Bacillariophyceae were found to be common in the reservoir as also observed by Paramasivam and Sreenivasan (1982) and Shukla *et al.* (1991). The sudden appearance of blue green algae as blooms at the surface of the productive water has been commented on their frequency and is usually taken as a sign of chemical enrichment as stated by Jeejibai and Rajendra (1980) and Pearl (1988). During November and December (rainy months) the phytoplankton population is at minimum due to increased water discharge (Kristiansen, 1998). In the present study, the heterogenity have been observed, showing the maximum with pollution tolerant species. Many pollution tolerant species have been

identified in the present study and viz., *Microcystis* sp., *Oscillatoria* sp., *Phormidium* sp., *Chlorella* sp., *Pediastrum* sp., *Closterium* sp., *Coelastrum* sp., *Scenedesmus* sp., *Phacus* sp., *Melosira* sp., *Synedra* sp., *Fragillaria* sp., *Achnanthes* sp., *Rhopalodia* sp., *Nitzschia* sp., *Surirella* sp., *Navicula* sp., *Pinnularia* sp., *Gomphonema* sp., *Anabaena* sp. and *Cymbella* sp. Presence of diatoms chiefly indicates that they are the tolerant to polluted waters and the prevailing environmental conditions evidenced in the present study is in accordance with the findings of Lam (1971) in the Waiketo river, New Zealand; Gunale and Balakrishnan (1981) in the Pavana, Mula and Mutha rivers of Poona and Nandan and Patel (1985) analyzed the different polluted water bodies in relation to pollution. The flow of pollutants cause, a rise in pollutant tolerant organisms in the river (Ray and David, 1966; Sinha *et al.,* 2002). The presence of tolerant species indicates the organic pollution due to the mixing of sewage and anthropogenic activities. Being an index of trophic status, phytoplankton reflects the overall polluted conditions of the systems in all the three stations and it reflects the reduced water quality (Lepistoe, 1999). Regular monitoring of phytoplankton leads to the identification of pollution status. Summer maximum (March, April and May) is shown by *Melosira* sp., due to the increased exploitation of favorable chemical pollution. In the present study stagnant environments in summer and a fall in reservoir levels show fluctuated population levels as observed Reynolds and Descy (1996) and De Ceballoius *et al.* (1998). Species diversity index and Palmer's pollution index indicated the moderately eutrophic status of pollution, by the identification of species diversity.

Population explosion and urbanization in India pose the problem of waste disposal and in turn the wastes are indiscriminately dumped into the nearby aquatic bodies like rivers, reservoirs, ponds and lakes, due to which they are polluted apart from their selfpurification limits. As a result of overexploitation of rivers and reservoirs, the ecobiology of the systems get altered. Aliyar reservoir is one of the important freshwater sources which got polluted by the upstream activities, dumping of carcasses, agricultural run off, sand quarrying, bathing and other human activities. These pollutants cause major stress to the aquatic organisms and in turn lead to ill effects in human beings.

Conclusion

Variations in species have been used to summarize the information on community structure. In the river studies it is used to evaluate the effects of environment on species composition.

The perusal of the above investigations of the Aliyar reservoir, at three sampling sites for a period of two years, reveals that the water is polluted to a near eutrophic condition. Small open drainage from the bank opening into the reservoir is numerous which can be considered as non-point sources of pollution. Various upstream activities are the regular features. Further indiscriminate agricultural washouts with pesticides, fertilizers, and some anthropogenic stresses which were encountered in this stretch are symptomatic to the pollution. Presence of pollution tolerant species of plankton and supports the poor quality of the Aliyar water. The results observed reveal the essential regular pollution monitoring in order to safeguard the health of the reservoir. If, alternate disposal systems are not adopted in near future, the pollution load will jeopardize the ecological balance completely and will lead to the extinction of the resources in near future.

Nowadays, the water pollution scenario is quite frightening. All major reservoirs in the country is extremely deteriorated beyond normal use. The horrifying fact is that all government efforts to rejuvenate the water bodies have come to naught. If, indeed, we are serious about saving our rivers the public at large, people who rivet our reservoirs, have to take on the challenge of keeping them clean. Public participation and awareness is the prime factor to reduce the pollution. Non-Governmental agencies should be involved for periodical monitoring of the river.

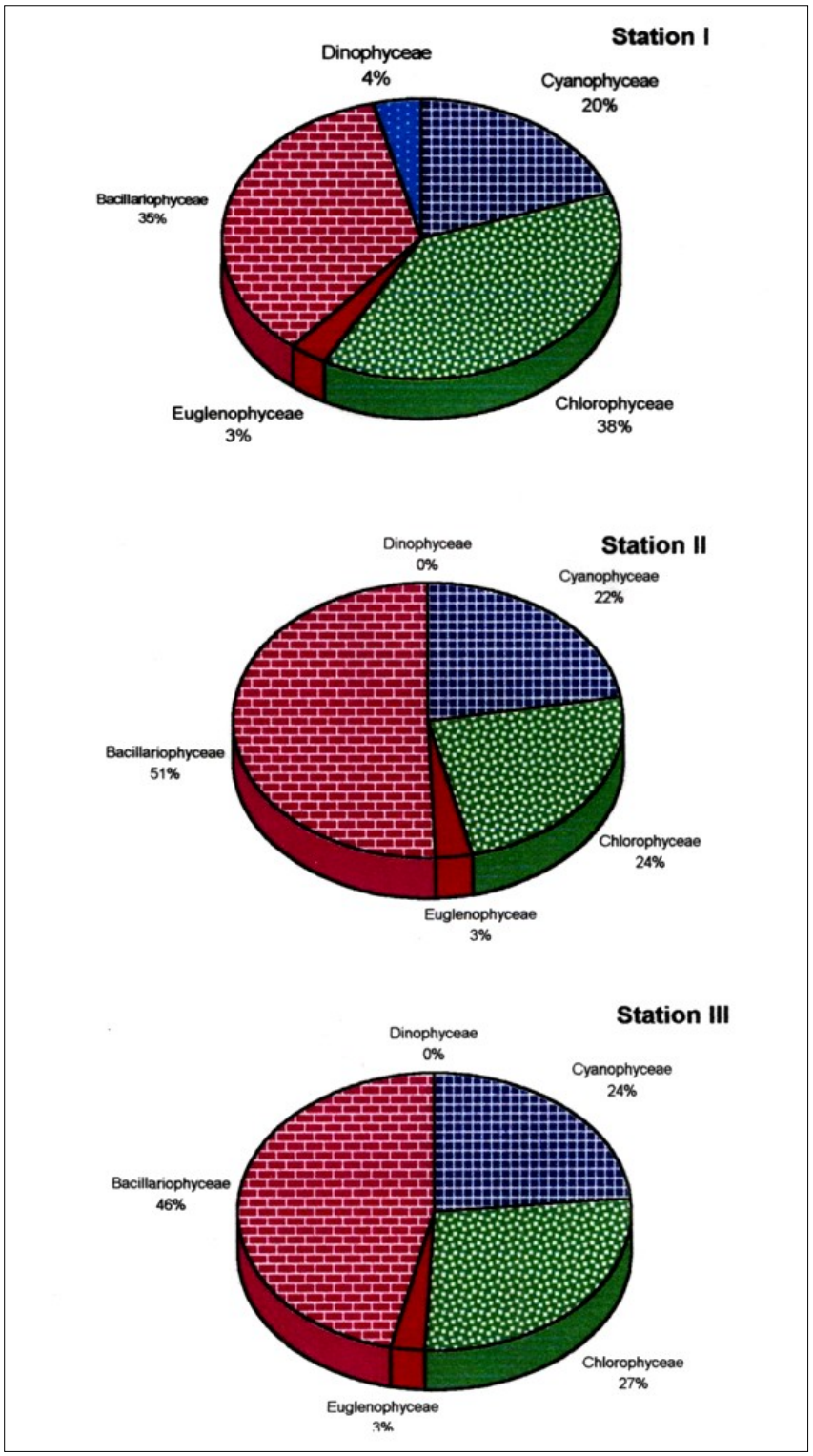
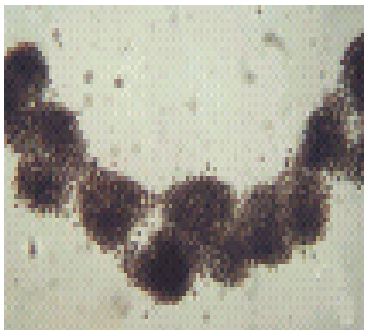
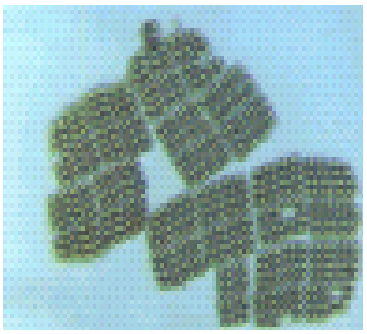


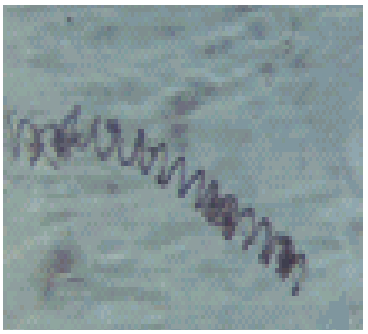
Fig.1. Phytoplankton species composition in Aliyar reservoir.



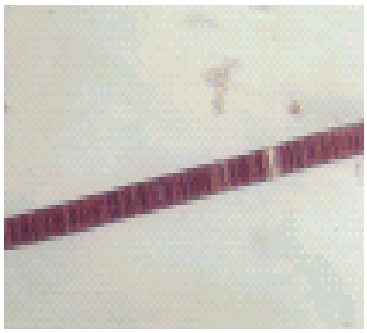
Microcystis aeurginosa



Merismopedia glauca

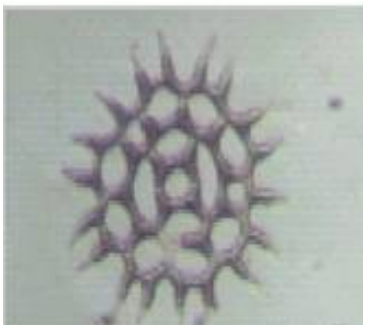


Spirulilna sp

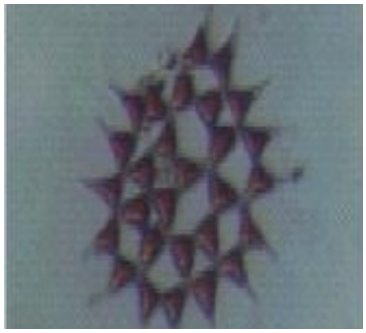


Oscillatoria

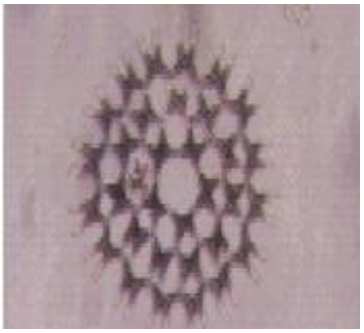
Pediastrum boryanam



Pediastrum duplex



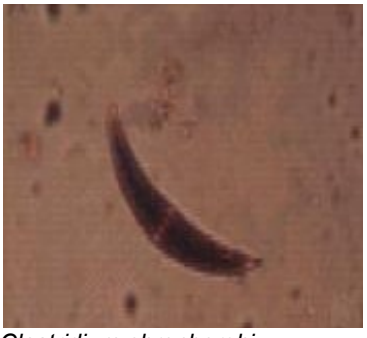
Pediastrum simplex



Pediastrum tetras

Spirogyra indica

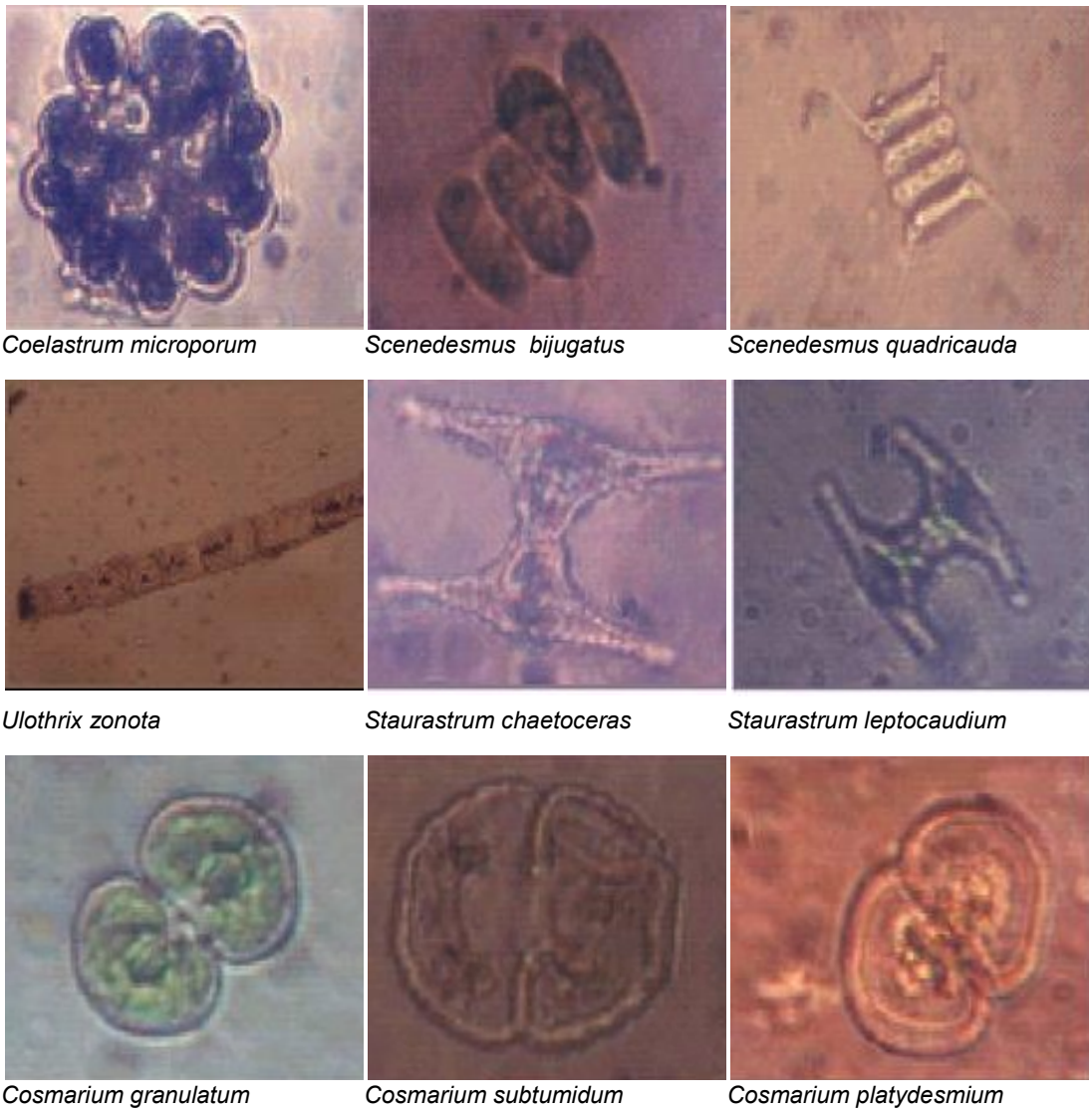
Clostridium acerosum



Clostridium ehrenberghi

Clostridium tumidum

Fig. 2. Cyanophyceae recorded in the Aliyar Resevoir.



Coelastrum microporum *Scenedesmus bijugatus* *Scenedesmus quadricauda*

Ulothrix zonota *Staurastrum chaetoceras* *Staurastrum leptocaudium*

Cosmarium granulatum *Cosmarium subtumidum* *Cosmarium platydesmium*

Fig. 3. Chlorophyceae recorded in the Aliyar Resevoir.

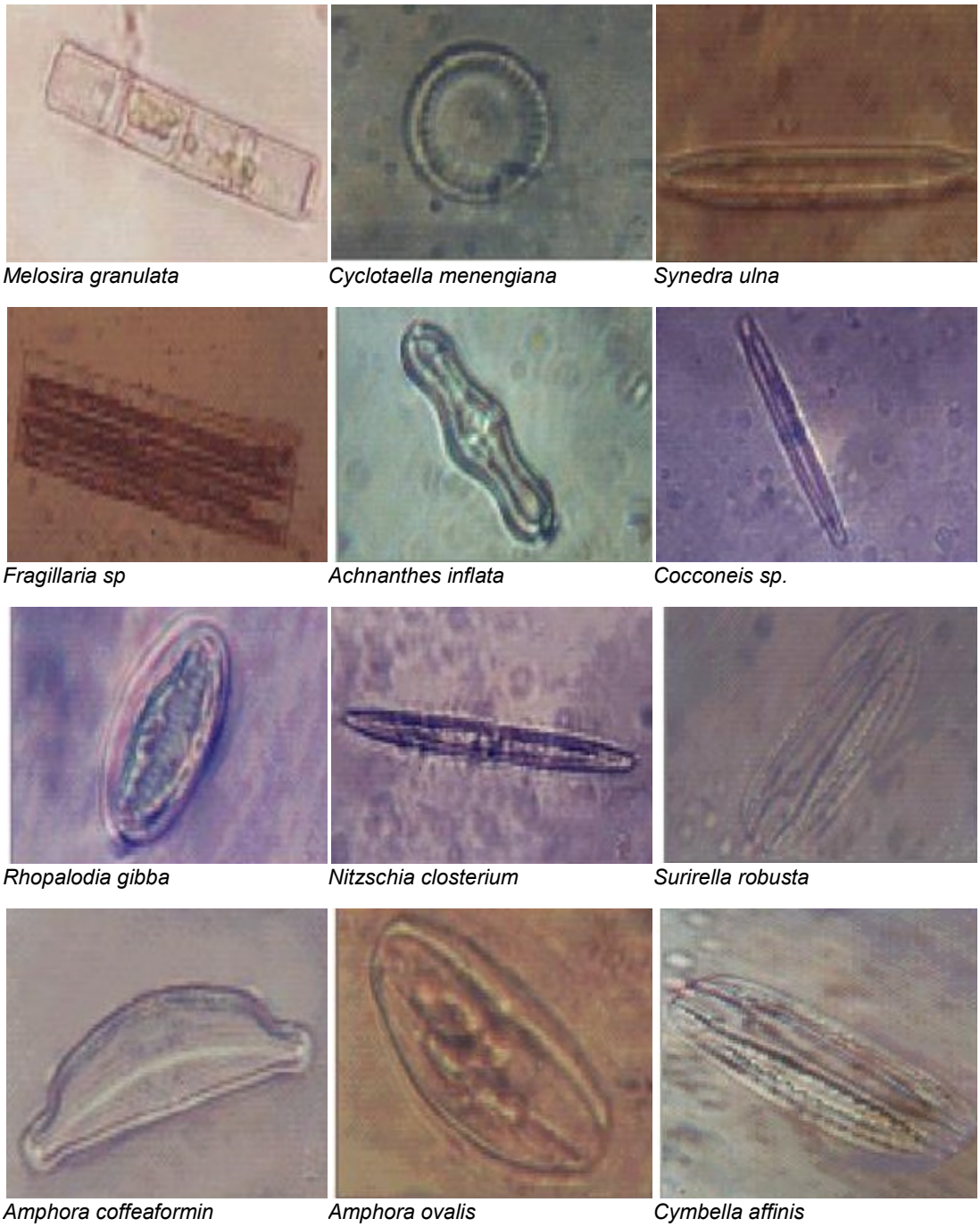
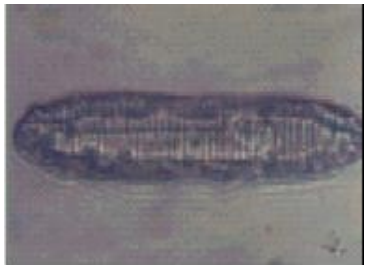


Fig. 4. Bacillariophyceae recorded in the Aliyar Reservoir


Gyrosigma acuminatum


Cymbals cymbiforms


Frustulia sp.


Caloneis bacillus


Gomphonema sphearophorum


Gomphonema herculeanum

Fig. 4. Bacillariophyceae recorded in the Aliyar Reservoir (cont.)


Ceratium hirudinella

Fig. 5. Dinophyceae recorded in the Aliyar Reservoir

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Biodiversity Conservation in Malaysia: Application of DNA Marker Technology for Precise Genotype Identification of Plants

Nurul, A.H.[1] and Shah, F.H.[2]

[1]Molecular Biology Division, Melaka Institute of Biotechnology, Lot 7, Melaka International Trade Center City, Ayer Keroh, Melaka, Malaysia
[2]Novel Plants Sdn. Bhd., 27C Jln Petaling Utama 12 , 7.5miles Old Klang Road, 46000 Petaling Jaya, Selangor, Malaysia
nurul@mib.gov.my

Abstract

A DNA marker database has been developed for compliance and management of biodiversity genetic resources in Malaysia through population genetic polymorphism studies of plant species that needs protection and taxonomic level studies to clarify the evolutional history of species. A combination of random amplified polymorphic DNA (RAPD-PCR) and microsatellites-primed PCR (MP-PCR) techniques were employed as tools for precise genotype identification. These methods are based on polymerase DNA chain reaction involving arbitrarily small oligonucleotides and microsatellites primers which involves the study of DNA loci with the aid of selected single primers that identify complementary sites in both DNA chains. The methods allow analysis of not only the unique, but also the non-coding DNA portion. The products of these applications were highly polymorphic band patterns that could be used as genetic fingerprints to identify plants precisely for their commercial exploitation as well as conservation. It could also identify the taxonomy of species and characterize the genetic structure of populations including the rare and unknown plant species. Realizing the importance to protect the genetic resources in plants, this project was initiated to cover herbal pants in Malaysia. This project has established optimized DNA Marker protocols to be used as a tool in plants identification. We have successfully identified 688 plants accessions from DNA markers and population studies. The distinctive DNA band patterns provide important baseline data for conservation and collection strategies for plants. Our study showed that both techniques were rapid and reliable for precise identification of plants. DNA-based marker profiles were developed for a total number of 688 plants accessions and 12 SCAR fragments (sequence characterizes amplified region based-RAPD). Out of these, 169 plants varieties are from 46 species, and a total number of 395 species with 347 genera. This project helps in, i) documentation of plants which have high economic value, ii) providing molecular descriptors for species/variety/cultivar/accessions identification, iii) utilization as national reference for variety/species protection, iv) building database references which is useful in plants forensics investigation and vi) classification and IP protection for plants, vii) help to fight biopiracy issues, viii) proper documentation of natural resources at DNA level. The outcome of this study indicated that both RAPD-PCR and MP-PCR combined techniques is useful tool for identification of germplasm analysis and genetic relationship between and within the plant species. The reasonable diversity observed in this study may be exploited for further genetic improvement and biodiversity conservation of our natural resources and protection of its genetic resources.

Introduction

Random amplification of polymorphic DNA is a technique that utilizes short synthetic oligonucleotides (10 bases) long of random sequences as primers to amplify nanogram amounts of total genomic DNA under low temperature by PCR. RAPD produces DNA profiles of varying complexity, depending on the primer and template used (Welsh and McCelland, 1990; William *et al*., 1990). Each amplification product is expected to result from the existence of two annealing sites in inverted orientations, 3' ends facing each other, within amplifiable distance (William *et al*., 1990). Polymorphisms could be caused by differences in nucleotide sequences or by structural arrangements (William *et al*., 1990).

Several approaches of using microsatellites primers for PCR also have been used. This technique involves PCR amplification with oligonucleotide primers that are complementary to specific SSRs (Meyer *et al.,* 1993, Perring *et al.,* 1993, Gupta *et al.,* 1994). In this approach, synthetic oligonucleotides, each representing a specific SSR, are used as single PCR primers, so that if two inversely oriented microsatellites are present within an amplifiable distance from each other, the inter-

repeat sequence is amplified. The amplified products generate distinct banding patterns that can be resolved on low-resolution agarose gels using EtBr staining (Sharma *et al.,* 1995, Weising *et al.,* 1995).

The purpose of this work is to investigate the genetic variability and identification by RAPD and MP-PCR and to report on the DNA sequences of selected RAPD fragments in selected highly medicinal Malay herbs. The identification of plants genotype and its genetic variability are important in plant taxonomic study to conserve endangered rare species. It is part of plant genetic resources are a biological basis of food and other essential uses. It is part of the biodiversity that nurture people and encompass the diversity of genetic materials in traditional varieties, modern cultivars, wild relatives of crop plants and other natural plant species which is used as food. Genetic resources also are the main source of raw materials for modern medicines such as antibiotics and natural drugs. Realizing the importance to protect the genetic resources of medicinal and aromatic plants, this research is initiated to identify the medicinal and aromatic plants in Malaysia.

The output from this research is establishment of DNA Marker protocols which can be used as a tool in plants identification. This DNA marker technique can be exploited for genotype identification. Identification and correlation of genotype for bioactive compounds of each plant varieties collected from forests and fields, it is important to have DNA barcode/s to precisely identify the plants. In herbal medicine industry, identification of correct medicinal plants is essential as many plant species come in with different varieties, which have similar appearances as well as similar usages in traditional medicine.

The use of molecular markers in herbal research are increasingly used for screening of germplasm to study genetic diversity, identify redundancies in the collections, test accessions stability and integrity, and resolve taxonomic relationship. This molecular technology also expands the scope of genetic resources utilization.

Materials and methods

Plant materials sampling and morphological based study

Plant materials were collected from the fields representing each targeted plants. The collected plants were maintained in our collection as a reference. Morphological based studies were conducted to document the characteristics of collected plants. The fresh leaf were stored at -80°C and used for genomic DNA extraction.

Genomic DNA extraction from plant tissues

Young leaves were washed in 70% ethanol for 5 minute and in sterile deionized water for 2 minute to avoid surface contamination. After being air-dried, the samples were ground separately into powder with the help of liquid nitrogen using mortar-pestle. Total genomic DNA was isolated from fresh leaves using a method described by [8] with minor modifications. 2 gram of the powdered leaves was added to 15 ml extraction buffer [0.1 M Tris HCl, 0.05 M EDTA, 0.5 M NaCl, 1% PVP, 1.4% SDS, 10 mM 2-mercaptoethanol]. The suspension was incubated in a water bath at 37 °C for an hour. Potassium acetate was added to each tube and again incubated on ice for an hour. The phases were separated by centrifugation and isopropanol was added to the new suspension. The mixtures were then allowed to precipitate for an hour at a temperature of -20°C. After centrifugation, pellets were dissolved in TES. RNase (10 mg/mL) was added to each tube and incubated for an hour at 37°C. The mixture was extracted by phenol/chloroform/isoamyl-alcohol twice, followed by precipitation with isopropanol and sodium acetate. After centrifugation, pellet was washed with 70% ethanol twice. Then, dissolved in 1X TE. The mixer was used for RAPD reaction.

RAPD-PCR fingerprinting and agarose gel electrophoresis.

PCR amplification were carried out for RAPD fingerprinting using sixty arbitrary decamer oligonucleotides primers (Kit A, D, and E). The RAPD-PCR reactions were performed in a total volume of 25 µL reaction consisting of 25 ng template genomic DNA, 1X PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTP mix, 2.5 U of Taq polymerase and 0.6 µM primer. The amplification was done in a

programmable thermal cycler. PCR cycles were as follows; 50 cycles of 95.0 ºC for 30 sec, 38.5 ºC for 1 min, and 74.0 ºC for 1 min. A final step of extension was carried out at 72.0 ºC for 10 min. 15 µL of RAPD products were analysed by electrophoresis in 1% agarose gel electrophoresis with TBE buffer and stained with ethidium bromide.

MP-PCR fingerprinting and agarose gel electrophoresis

For MP-PCR fingerprinting, PCR amplification were tested using 11 microsatellites primers; $(GGAT)_4$,$(GACA)_4$, $(CA)_8$, $(GT)_8$, $(GAA)_6$, $(GTG)_5$, $(GAC)_5$, T3, T7, M13, and $(GATA)_4$. The reactions were performed in a total volume of 25 µL reaction consisting 25 ng template genomic DNA, 1X PCR buffer, 4 mM $MgCl_2$, 0.4 mM dNTP mix, 2.5 U of Taq polymerase and 0.9 µM primer. The amplification was done in a programmable thermal cycler. PCR cycles were as follows; 40 cycles of 94.0ºC for 20 sec, vary from 43.5-60.8ºC for 1 min, and 72.0ºC for 20 sec. A final step of extension was carried out at 72.0 º C for 5 min. 10 µL of PCR products were analysed by electrophoresis in 1.8% agarose gel electrophoresis with TAE buffer and stained with ethidium bromide.

Cloning and sequencing of RAPD fragments

RAPD band pattern were analyzed in order to get the most prominent bands for cloning. RAPD fragments were excised from the agarose gel and were purified using Qiagen Gel Extraction Kit (Qiagen, Germany). Purified RAPD DNA fragments then was clone in pGEMt-Easy vector (Promega) in the *E. coli* strains. Plasmid DNA was isolated using DNA purification Kit (Promega). In order to confirm the insert length, pDNA samples were digested with restriction enzymes, EcoRI. After confirmation of insert length, bacterial cells were cultivated for respective recombinant clones and purification of the pDNAs was carried out. Sequencing of the RAPD fragments was carried out from both ends by using SP6 and T7 primers.

Data collection, data analysis, and database development

DNA marker data were collected from PCR band profiles generated through analysis of gel-electrophoresis images. From DNA fingerprint profiles, PCR band will be scored (1) for the presence or (0) for the absence of a band-pairwise distance (similarity matrices). It was computed based on Jaccard's coefficient of similarity, using NTSYS-pc version 2.0 software. Dendogram were constructed using unweighted pair group method with arithmetric averages (UPGMA) cluster analysis using NTYSYS-pc version 2.0 software. The informative markers of generated RAPD bands was selected and marked manually. These markers will be used as DNA markers to differentiate between plants species or varieties. To identify and quantify the DNA markers, data will be collected from sequenced RAPD fragments. Sequences from RAPD fragments were compared with other organism in plant database. Annotation of the sequences was carried out using online/offline softwares such as BLASTn, BLASTx of NCBI and other online modules available at justbio.com. All above-mentioned data, as well as botanical source and characteristics were included into new-based database for future references.

Results and discussion

Authentic identification of taxa is necessary to ensure protection of intellectual property right (IPR) and also for consumers, propagators and plant breeders. The traditional method for identifying species by morphological characters is now being replaced by DNA profiling because of some limitations of morphological data (Virk *et al.,* 1995). Evidently, DNA marker technique is a rapid and sensitive, which can be used to estimate relationship between closely, and more distantly related species and group. The primer screening step resulted in 10 decamer primers which detected good polymorphisms. PCR amplification was carried out in triplicate to make sure all bands are reliable and consistent.

Development of DNA marker database

The development of PCR procedures using single, arbitrarily small oligonucleotides, known as Random Amplified Polymorphic DNA (RAPD) and microsatellites primers, known as Microsatellites-primed PCR (MPPCR), allows the amplification of DNA sequences throughout the genome. The products of these amplifications are highly polymorphic band patterns that can be used as genetic fingerprints to identify the medicinal plants precisely for their commercial exploitation as well as conservation. RAPD-based and microsatellites-based DNA marker profiles have been developed for a total number of 688 plants accessions and 12 RAPD fragments sequences from *Ficus deltoidea* sp and *Labisia pumila* sp. Out of this; 169 plants varieties are from 46 species. While, total number of species 395 with 347 genus (Table 1). The DNA fingerprint database of herbal plants is a collection of PCR band size data. The database is divided into sections according to plant species and primers utilized in the PCR reaction. Database contains information about local name, scientific name, morphological variations, localities, DNA concentrations (µg/µL), primers used, primer sequences, gel images, schematic diagrams, informative markers, bands size (bp), DNA sequences, and botanical characteristics. From distinctive DNA band patterns, it provides important baseline data for conservation and collection strategies for this plant/s. The study showed that RAPD and MP-PCR is rapid and reliable method for identification of plants precisely.

Table 1. Total number of plants in DNA Marker database

Taxonomy	**Number of plant species**
Accessions	688
Varieties	169 var. from 46 sp.
Species	395
Genus	347

DNA sequences of RAPD fragments of Ficus deltoidea sp.

The Mistletoe Fig (*F. deltoidea*) RAPD amplification, generated by primer OPD-10 and OPD-11, can be classified into two groups: variable (polymorphic) and constant (non-polymorphic). Fragments F5-1, F6B and F10-3 were generated by primer OPD-10. While, fragments D11-1-2, D11-2-1, D11-3-1, D11-5-2 and D11-6-3 were generated by primer OPD-11 (Fig. 1). Constant fragments are diagnostic for a genus, and operationally identify members of a certain genus exclusively if the fragment is a unique polymorphism in a comparison of genera, genus specific band or character. Similarly, fragment polymorphic at the species level will operationally identify members of a given species if the fragment is constant among all members of the species, species specific bands or characters (Millan *et al.,* 1996).

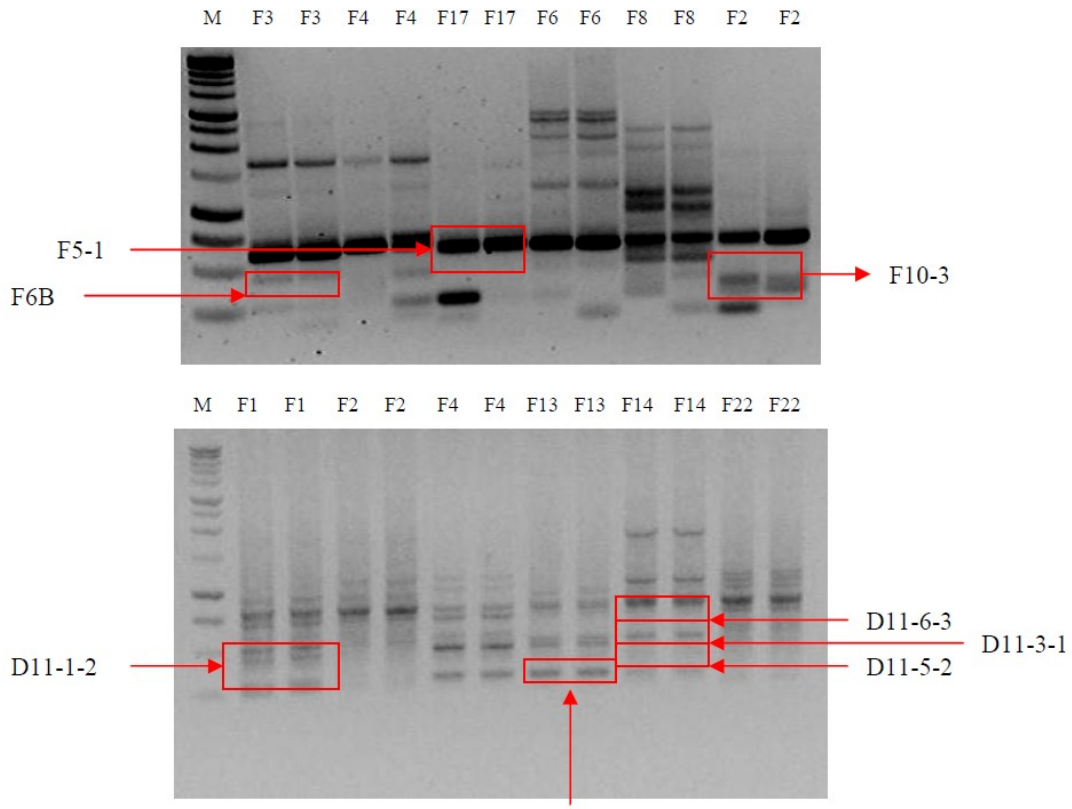

Fig. 1. RAPD products from six *Ficus deltoidea* accessions. (A) generated by random primer OPD-10 (B) generated by random primer OPD-11. Sequenced fragments are marked by arrows.

Nine RAPD fragments representing polymorphic and non polymorphic bands were investigated at the molecular level using DNA sequence analysis. Complete DNA sequence of the aforementioned RAPD fragments were analyzed and deposited in the NCBI nucleotide sequence database, GenBank as shown in Table 2.

Analysis by with nucleotide-nucleotide BLASTS using NCBI database information revealed that the clone F5-1 produced DNA sequence homology of 22% identical with *Oryza sativa* (japonica cultivar-group) chromosome 11 clone OSJNBb0018H03, complete sequence (ACCESSION AC151599), *Oryza sativa* (japonica cultivar-group) chromosome 5 clone OSJNBb0111O13, complete sequence (ACCESSION AC137621), and *Oryza sativa* chromosome 3 BAC OSJNBb0096H12 genomic sequence, complete sequence (ACCESSION AC135227). Polymorphic DNA sequence for F6B, showed homology of 22% with Homo sapiens genomic DNA chromosome 11 clone: RP11-795E18, complete sequence (ACCESSION AP002760). Interestingly, DNA sequences for F10-3 shows has 98% identity with *Labisia pothoina* clone L1A RAPD marker (ACCESSION DQ825505). Similarly, polymorphic fragments D11-2-1 shows high homology (99%) to *F. deltoidea* var. *trengganuensis* clone D11-2-2 RAPD marker genomic sequence (ACCESSION EF029043). D11-3-1 shared a 22% homology to *Mus musculus* BAC clone RP24-149K9 from chromosome 6, complete Sequence (ACCESSION AC161448). For RAPD fragment D11-5-2, it shows high identity homology (99%) with *Escherichia coli* K12 MG1655, complete genome (ACCESSION U00096 AE000111-AE000510). It is known that repetitive DNA has often been occurred and detected in RAPD fragments (Powell *et al.,* 1996). Our result also shows that 3 out of 8 studied RAPD fragments represent highly repetitive DNA sequences. RAPD comparative purpose relies on the similarity of fragment size is a dependable indicator of homology (Bretting *et al.,*1995). To test the validity, homology on non polymorphic RAPD DNA fragment was carried out between two different varieties for *F. deltoidea*

Jack, fragments D11-1-2 and D11-6-3 (Fig. 2). Comparative analysis using CLUSTALW program revealed that these two sequences do have similar between each other with alignment score of 1275. It shows that similarity of fragments size is an indicator of homology between species or varieties. It is known that DNA sequence analysis provides the most specific and specific method for detecting homology (Paran and Michaelmore, 1993)

Table 2. Molecular characterization of Ficus deltoidea RAPD DNA fragments and their Genbank accession numbers

Clone ID	Name of varieties	Polymorphic (P) / non polymorphic (N)	Sequence length (bp)	Genbank accessions number	Used primer
F5-1	*F. deltoidea* Jack. var. motleyana	N	610	EFO29045	OPD-10
F6B	*F. deltoidea* Jack. var. trengganuensis	P	427	DQ825506	OPD-10
F10-3	*F. deltoidea* Jack. var. bilobata	P	400	EFO29047	OPD-10
D11-1-2	*F. deltoidea* Jack var. bilobata	N	558	EFO29044	OPD-11
D11-2-1	*F. deltoidea* Jack var. trengganuensis	P	300	EFO29042	OPD-11
D11-3-1	*F. deltoidea* Jack var. angustifolia	P	599	EFO29039	OPD-11
D11-5-2	*F. deltoidea* Jack. var. motleyana	P	507	EFO29041	OPD-11
D11-6-3	*F. deltoidea* Jack. var. kunstleri king	N	558	EFO29046	OPD-11

DNA sequences of RAPD fragments from Labisia pumila.

The Kacip Fatimah (*L. pumila*) RAPD amplification, generated by primer OPA-2, can be classified into two groups: variable (polymorphic) and constant (non-polymorphic) (Fig. 3). Constant fragments are diagnostic for a genus, and operationally identify members of a certain genus exclusively if the fragment is a unique polymorphism in a comparison of genera, genus specific band or character. Similarly, fragment polymorphic at the species level will operationally identify members of a given species if the fragment is constant among all members of the species, species specific bands or characters (Rieseberg 1996, Franca *et al.* 2002).

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D11-1-2 AGCGCCATTGCC-TATTAGTTGGCCTTTCGAGTCAAACGATACTTGAATTCGTTCTCC-- 57
D11-6-3 AGCGCCATTGTTATATCAGAAATTTTTGGCAGATCACTAGGACTCCATTTCAGACGCCAT 60
        **********   *** **       **   **   *     ***  * ***   * **

D11-1-2 TCTCGACC--GGACAACGTGCACTCG--TTTCAGTAGGGTTGCTCTTCTTTTTCCATCCT 113
D11-6-3 TCTCAACGAAGGACCACCGTTTTTCGGACTTCCACAGTGTCTCTTAGCACCCACCACACA 120
        **** **   **** **       ***   ***   ** **  **   *     ***  *

D11-1-2 CCTCGGCATTT--AATCTGT-----CAGTGTTGGTCCTTTTGGGTTTTCCAACATGAGAT 166
D11-6-3 TCCGGTAAGTTGTAATGTATACGTATAATGCTGAGCGTTTCGGATTTCTTAAAATTTTTT 180
        *  *  * **  *** * *      * ** **  * *** ** ***   ** **    *

D11-1-2 TGGGTCAACG--TGGTCTCGGCGTTCTCGTTCGAGATT-GTG-AATCAATCTATCCGACA 222
D11-6-3 GGCTTCTGTGAATAACCTATTCGGGTTAGGGTGGGATTTGTGTAATTTTTTTAAGGGAAA 240
        *  **   * *   **   **   * *   * **** *** ***   * **   ** *

D11-1-2 AAACACAAAATCTCAGTATAATCCTAAAATTTACCTAACAACGGACATAATCTCACCTCT 282
D11-6-3 AACCAATAGACTGTTAT-TAATGATTGTATGTAAATAAGAGGTGAGATTATGTCCGTTGT 299
        ** **  * *      * ****  *   ** **  *** *   ** ** ** **   * *

D11-1-2 TATTTACATACAATCATTA-ATAACAGTCTATTGGTTTTTCCCTTAAAAAAATTACACAA 341
D11-6-3 TAGGTAAATTTTAGGATTATACTGAGATTTTGTGTTTTGTCGGATAGATTGATT-CACAA 358
        **  ** **   *  **** *      * *  ** *** **   ** *   *** *****
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D11-1-2 ATCCCACCCTAACCCGAATAGGTTATTCACAGAAGCCAAAAAATTTTAAGAAATCCGAAA 401
D11-6-3 -TCTCGAACGAGAACGCCGAGACCA--CGTTGACCCAATCTCATGTTGGAAAACCCAAAA 415
        ** *   * *   **   **   *  *   **  * *    ** **   *** ** ***
D11-1-2 CGCTCAGCATTATACGTATACATTACAACTTACCGGATGTGTGGTGGGCGCTAAGAGACA 461
D11-6-3 GGACCAACACTG-----ACAGATTAAA--TGCCGAGGAGGATGGAAAAAGAAGAGCAACG 468
        *  ** ** *      * * **** *  *  *  *  *  ***     *   **  **
D11-1-2 CTGTGGAAGTCCGAAAAACGGTGGTCCTTCGTTGAGAATGGCGTCTGAAATGGAGTCCTA 521
D11-6-3 CTACTGAAA--CGAGTGCACGTTGTCA--GGTCGAGA--GGAGAACGAATTCAAGTATCG 522
        **   ***   ***      ** ***   **  ****  ** *   *** *  ***
D11-1-2 GTGATCTGCCAAAAATTTCTGATATAACAATGGCGCT 558
D11-6-3 TTTGACTCGAAAGGCCAACTAATA-GGCAATGGCGCT 558
        *   **   **      ** ***   **********
```

Fig. 2. Comparative DNA sequence analysis of non polymorphic RAPD marker (NP) for D11-1-2 and D11-6-3 using CLUSTALW.

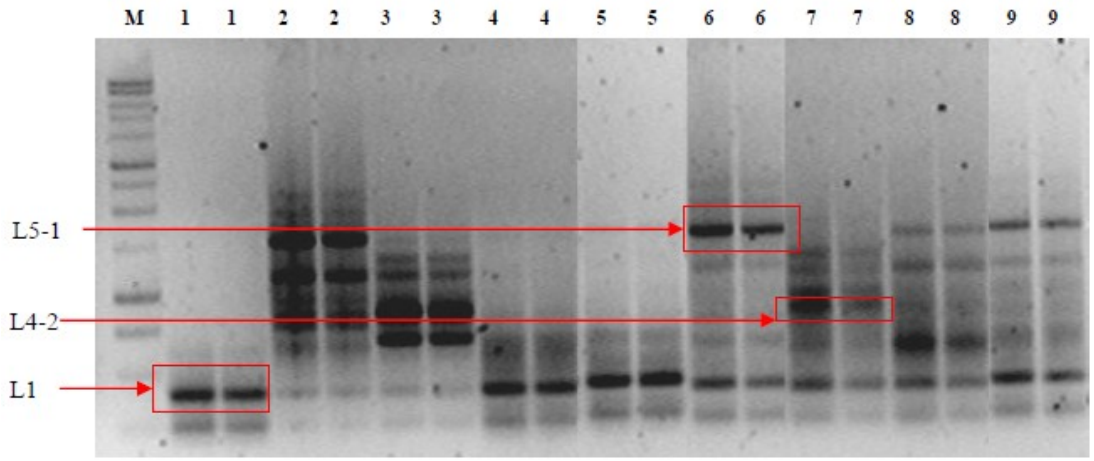

Fig. 3. RAPD products from eight accession of *Labisa pumila* generated by random primer OPA-2. Lane M; 1 kb ladder, number 1-5; *L. pumila* var. *lanceolata*, number 6 *L. pumila* var. *alata*; number 7-9 *L. pumila* var. *pumila*. Sequenced fragments are marked by arrows. Noted all amplication are repeated twice.

Three RAPD fragments representing each varieties were further investigated at the molecular level using DNA sequences analysis (Table 3). Polymorphic fragments L1A, showed highly homology 98% to *F. deltoidea* var. *bilobata* clone F10-3 RAPD marker genomic sequence (ACCESSION EF029047). DNA sequence of L4-2 showed 25% homology to *Homo sapiens* 12p12 BAC RPCI11-501E24 (Roswell Park Cancer Institute Human BAC Library) complete sequence (ACCESSION AC006559).

Table 3. Molecular characterization of L. pumila varieties RAPD DNA fragments and their Genbank accession numbers

Clone	Name of varieties	Molecular Weight (bp)	Genbank accessions number
L1A	*L. pumila* var. *lanceolata*	400	DQ825505
L4-2	*L. pumila* var. *pumila*	750	EFO29038
L5-1	*L. pumila* var. *alata*	1,500	EFO29039

Conclusion

Results from this study indicate that the RAPD and MP-PCR techniques are useful tool for identification of germplasm analysis and genetic relationship between and within the plants species. Documentation of the plants will be much helpful in i) biodiversity conservation, ii) fight biopiracy issues, iii) help in plant forensic investigation, iv) proper documentation of natural resources at DNA level. Nevertheless, the reasonable diversity observed in this study may be exploited for further genetic improvement and biodiversity conservation.

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Seasonal Occurrence of Butterfly Fauna in Cubbon Park, Bangalore, India

Shashikumar, L. and Venkatesha, M.G.

Department of Zoology, Bangalore University, Bangalore-560 056, India
venkatmelally@gmail.com

Abstract

A study was conducted on the occurrence of various species of butterfly and their abundance in the park during 2004-06. A total number of 50 species of butterflies belonging to seven families were recorded during the study period. Of which, four species were abundant, 12 were common, seven were occasional and 27 were rare. Thirty one species of butterflies in winter, 24 species in summer and 40 species in monsoon were recorded. Seventeen species of butterflies were found throughout the year. Of the seven recorded species of Papilionidae, two species were rare in the park. Eleven species were recorded under Pieridae, of them one species was abundant, two species less common and remaining were rare in the area. Interestingly only one species recorded from the family Acraeidae which was common in the park. Only two species were recorded under the family Hesperiidae, of which, one was less common and other was rare. Four butterfly species were recorded from the family Danaidae, of which, two species were common, one was abundant and one was less common. The family Nymphalidae was the largest family represented 17 butterfly species. Out of them two species were abundant, four common and others were rare in the area. Eight species of lycaenid butterflies were recorded. Among them two were common, one less common and five were rare. The diversity and ecology of various species of butterflies are discussed.

Introduction

Cubbon Park is in the heart of Bangalore city with lush green vegetations consisting of many indigenous and exotic varieties. It is verdant area for various species of insects especially for butterflies. The butterflies are the potential umbrella group for biodiversity conservation (New, 1997). They serve as food for predators at various tropic levels. The importance of biologically active butterfly communities of Cubbon Park cannot be neglected in the interest of conservation of biodiversity and environment. Hence, the study was undertaken on the occurrence of butterfly species of Cubbon Park during 2004-2006.

Materials and methods

Regular surveys were conducted in 334 acres of cubbon park region at different locations to record various butterflies present in the area. Recording of butterflies were made on weekly basis in different locations. Seasonal activities/abundance of different butterflies was studied by visual counting. Minimal number of insects was collected and preserved for valid identification. The occurrence and seasonal activities of various butterfly was conducted using presence – absence scoring method (Bholodia *et al.*, 2002). The linear transect method (Pollard, 1977; 1982) was employed for sampling of butterfly. Biology and behaviour of the important and rare species was studied both in the field and laboratory by field observations as well rearing the insects in the laboratory. Presence of different species of amphibians, reptiles, birds and mammals, which feed on butterfly stages in the field were identified and recorded. Host plants of butterfly larval stages and nectar plants of adults were identified. Most of the field collected insect developmental stages (eggs, larvae and pupae) were maintained in the laboratory for possible emergence of parasitoids. Insect parasites and predators of butterflies in the field are being collected and their interactions are studied by rearing them in the laboratory. Photographs of butterfly, nectar providing flowers, etc. are taken for preparing a valid document. Indices of evenness, frequency, abundance, dominance, richness and species diversity of butterfly species were calculated. Weather factors such as temp., humidity and precipitation and plant phenology in the study locations are being recorded throughout the year to correlate with the activities and population levels of butterfly fauna.

Results and discussion

Fifty species of butterflies consisting of seven families were recorded in the study area. A total of 84 species of butterflies belonging to eight families were collected during this study period in different locations of Bangalore. Out of 50 species, four species were abundant, 12 were common, seven were occasional and 27 were rare. Maximum number of species collected belonged to the families Nymphalidae, Pieridae and Lycaenidae. The families Papilionidae, Danaidae, Hesperiidae and Acraeidae showed minimum number of species in this region. Similar findings were observed in Bangalore region. The study revealed that the occurrence and abundance of various species of butterflies were related to different seasons. Of the seven recorded species of Papilionidae, two species were rare in the park. Interestingly, only one species recorded from the family Acraeidae which was common in the park. Only two species were recorded under the family Hesperiidae, of which, one was less common and other was rare. Four butterfly species were recorded from the family Danaidae, of which, two species were common, one was abundant and one was less common. The family Nymphalidae was the largest family represented with 17 butterfly species. Out of them, two species were abundant, four common and others were rare in the area. The nectar plants, food sources, parasites and predators of butterfly were also recorded. The weather parameters such as temperature, humidity and rainfall appeared to have a strong influence on specific groups of butterflies. In addition to the above observations, behaviour of the most of the butterfly species was studied in the field. A few parasitoids on Common Jay, Lime Butterfly, Common Rose, Plain Tiger and Common Indian Crow butterflies were recorded in the field. Some predatory spiders were also recorded in the study area. Besides parasitoids and spiders, seven species of insectivorous birds were also recorded in the study areas.

The analysis of species richness index indicated that among seven families, Nymphalidae was the richest (2.14) followed by Pieridae (1.43) and Lycaenidae (1.09)(Fig.1). Whereas in overall Bangalore region, the family Lycaenidae was the richest (2.84). Among various butterfly families, Nymphalidae was the most abundant (29.96%) family and Hesperiidae was the least (1.17%)(Fig. 2). Similar findings were observed in Bangalore region.

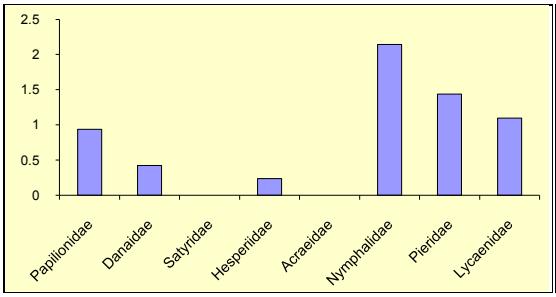

Fig. 1. Richness index of butterfly families in the Cubbon Park (2004-2006).

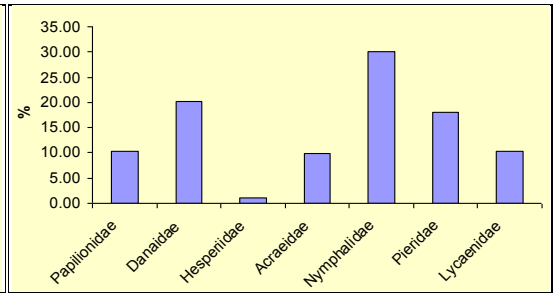

Fig. 2. Abundance (%) of butterfly families at Cubbon Park (2004-2006).

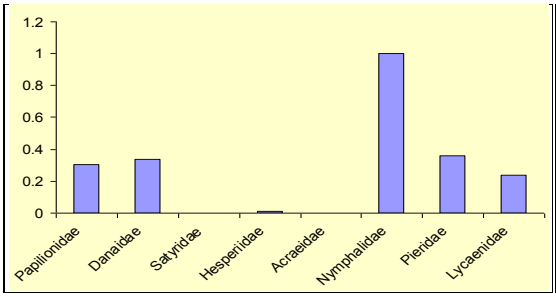

Fig. 3. Evenness index of butterfly families in the Cubbon Park (2004-2006).

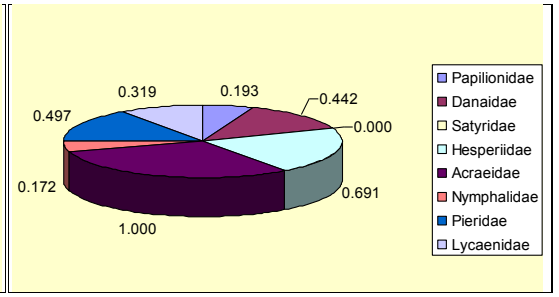

Fig. 5. Dominance index of butterfly families in the Cubbon Park (2004-2006).

Shannon-Weiner index was applied to measure the diversity of a community. Evenness index was used for calculation of evenness of insect populations. The Diversity (H') and Evenness (E_1) indices of butterfly species in the study areas for three complete years (2004-06) was significantly different (t (H') =3.11, t (E_1) =1.00) (Fig. 3). Similar findings were observed in Bangalore region.

Frequency index for different butterflies species at Cubbon Park is given in Fig. 4. Mottled Emigrant (*Catopsilia pyranthae*) showed highest percent frequency (66.48), whereas Nilgiri Clouded Yellow (*Colias nilgiriensis*) was observed less frequently (0.73) during the study period (Fig. 4).
The dominance index for different families of butterflies in Cubbon Park is shown in Fig. 5.. Acraeidae was the most dominant family (1.00%), whereas Nymphalidae was the least one (0.17%).

The correlation of various weather factors such as maximum temperature, minimum temperature, rainfall and humidity including different butterflies species as well as individuals in the Cubbon Park studied are presented. The individuals ($p<0.05$) and species ($P<0.01$) showed significant positive correlation with minimum temperature. Whereas, the individuals and species showed no significant correlation ($p>0.05$) with maximum temperature, rainfall and humidity (Table. 1).

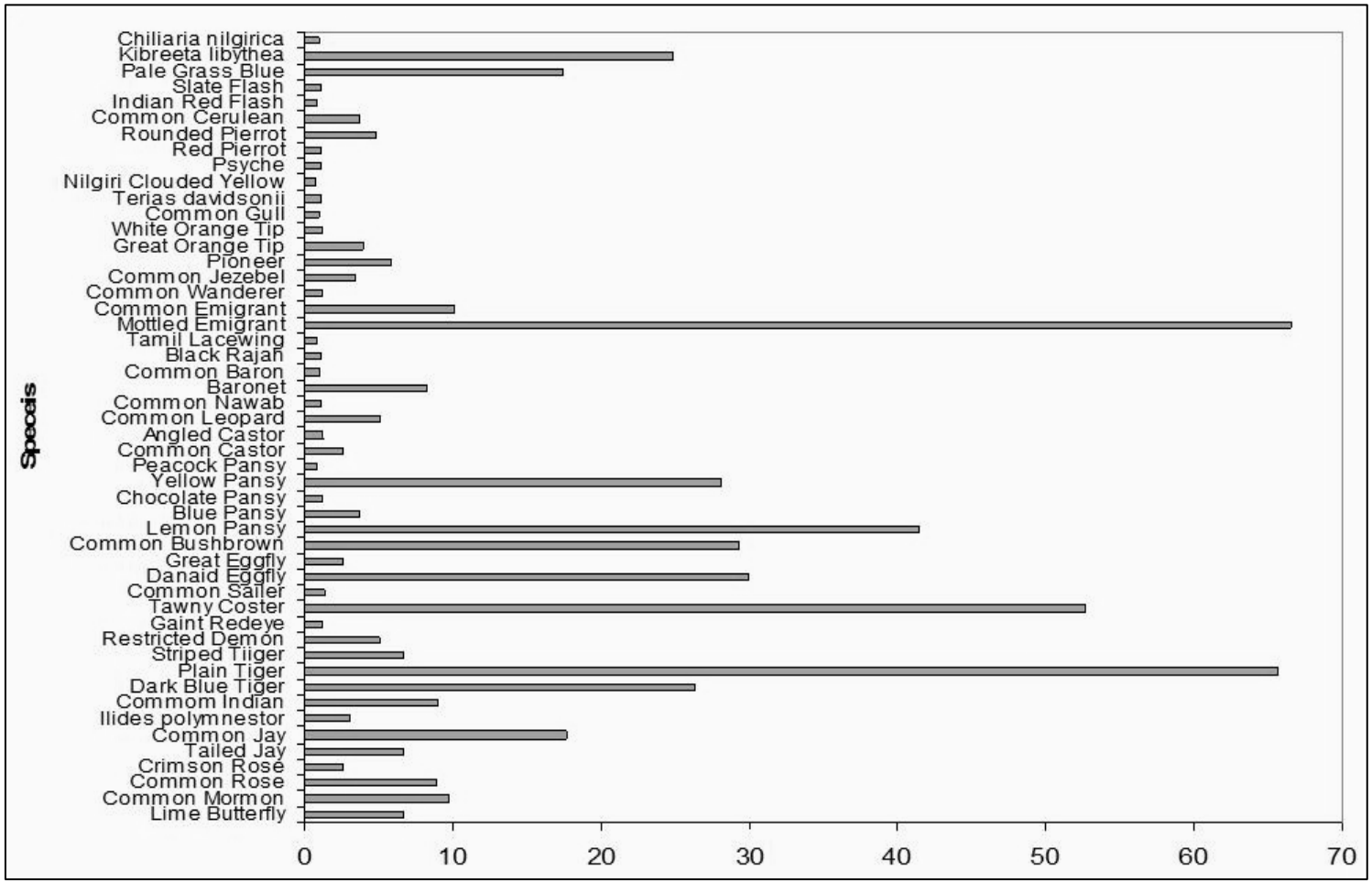

Fig. 4. Soyer's frequency index (%) of different species at Cubbon Park.

Table 1. Pearson's correlation matrix showing relationhip between various weather factors and butterfly populations in Cubbon Park (2004-2006)

Correlations	Temp. Min.	Temp. Max.	Rainfall	Humidity	Cubbon individuals	Cubbon species
Temp. Min.	1.000	0.393(*)	-0.162	-0.633(**)	0.367(*)	0.486(**)
Temp. Max.	0.393(*)	1.000	0.323	0.300	0.161	0.250
Rainfall	-0.162	0.323	1.000	0.624(**)	0.143	0.188
Humidity	-0.633(**)	0.300	0.624(**)	1.000	-0.151	-0.218
Cubbon individuals	0.367(*)	0.161	0.143	-0.151	1.000	0.609(**)
Cubbon species	0.486(**)	0.250	0.188	-0.218	0.609(**)	1.000

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

Conclusion

By regulating humidity and temperature, it is possible to manipulate local populations of butterflies. Establishment of butterfly gardens helps to maximize butterfly diversity and abundance in urban and suburban areas. By careful selection of host plants and restoration of habitats, a diverse assemblage of butterflies could be sustained in our surroundings. The control of grazing and high anthropogenic activities in the protected areas may be the best steps taken to enhance or maintain the diversity of butterfly fauna.

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Biodiversity of Butterflies of the Bangalore Region, India

Shashikumar, L. and Venkatesha, M.G.

Department of Zoology, Jnana Bharathi, Bangalore University, Bangalore-560 056, India
**venkatmelally@gmail.com*

Abstract

The common butterfly communities of the Bangalore region occurring in various landscapes were studied during 2004-06. A total of 84 species of butterflies belonging to different families, viz., Acraeidae, Danaidae, Hesperiidae, Lycaenidae, Nymphalidae, Papilionidae, Pieridae and Satyridae were recorded. Of them, maximum number of species were from Lycaenidae (24) followed by Nymphalidae (21), Pieridae (19), Papilionidae (11), Danaidae (4), Satyridae (2), Hesperiidae (2) and Acraeidae (1). The occurrence and abundance of various species of butterflies were related to different seasons. Overall investigation showed that the highest numbers of butterfly species occur during summer and monsoon periods, whereas they were sparse during winter period. Among eight families, Lycaenidae was the richest family. The percent abundance of all species of butterfly populations during summer, monsoon and winter seasons was 41.8, 40.2 and 18.0 respectively. Of all the recorded species, *Madais fausta* (Pieridae) was the most dominant species, whereas *Graphium nomius* (Papilionidae) was the least one. Seasonal occurrence of butterfly species correlated with weather factors showed that weather has a strong influence on the occurrence of specific groups of butterflies. The cluster analysis has revealed the interrelationships of various groups of butterfly species in tandem with bioclimatological factors. Behaviour of the most of the butterfly species was studied in the field. A few parasitoids of some species of butterflies were recorded in the field. A few predatory spiders and about 25 species of insectivorous birds were also recorded in the study area. The findings will be discussed in detail with a note on conservation methods.

Introduction

Bangalore is considered to be climatologically a well-favoured city situated in the heart of the South Deccan of Peninsular India. It is verdant area for various species of insects especially butterflies. The occurrence and diversity of butterflies are considered to be a good indicator of the status of any given terrestrial habitat (Kunte, 2000; Raju and Rao, 2002). Like any other species, their extinction could narrow the species gene pool considerably (Khatri, 1998).The importance of biologically active butterfly communities of Bangalore cannot be neglected in the interest of conservation of butterfly biodiversity and environment. Hence, the study was undertaken to understand the diversity and occurence of butterfly species in the Bangalore region.

Materials and methods

Regular surveys were conducted in 365 square miles of Bangalore region especially in parks, gardens and forest areas to record various butterflies present in the area. Recording of species were made on weekly basis in different locations. Seasonal activities/abundance of different butterflies were being studied by visual counting. Minimal number of insects was collected and preserved for valid identification. The occurrence and seasonal activities of various butterfly species was conducted using presence – absence scoring method (Bholodia *et al*., 2002). The linear transect method (Pollard, 1977; 1982) was employed for sampling of butterfly species. Based on the average number of all the three years (2004 – 2006) the score classes used were 1-15 = Rare (+), 16-50 = Common (++), 51 & above = Abundant (+++). Biology and bahaviour of the important and rare species were studied both in the field and laboratory. Presence of different species of amphibians, reptiles, birds and mammals, which feed on butterfly stages in the field were identified and recorded. Host plants of butterfly larval stages and nectar plants of adults were identified. Indices of evenness, frequency, abundance, dominance, richness and species diversity of butterfly species were calculated. Weather factors such as temperature, humidity and precipitation and plant phenology in the study locations were recorded throughout the year to correlate with the activities and population levels of butterflies.

Results and discussion

About 84 species of butterflies belonging to eight families were recorded during this study period in different locations of Bangalore. Maximum number of species recorded belonged to the families Lycaenidae (24), Nymphalidae (21) and Pieridae (19). The families Papilionidae (11), Danaidae (04), Satyridae (02), Hesperiidae (02) and Acraeidae (01) showed minimum number of species in this region (Table 1). Butterflies as most other Lepidoptera show distinct patterns of habitat associations. The study revealed that the occurrence and abundance of various species of butterflies were related to different seasons. Of the various species of butterflies of Papilionidae, Crimson Rose (*Pachliopta hector)* was abundant during summer and was rare during winter, whereas Southern Birdwing (*Troides minos)* was rare during summer and was absent in remaining periods. These butterflies mainly feed on nectar of *Lantana camara* flowers. Whereas Tailed Jay (*Graphium agamemnon*) and Common Rose (*Pachliopta hecto*r) were found feeding on nectar of *Ixora* flowers. Among Pierids, Mottled Emigrant (*Catopsilia pyranthe*) was abundant throughout the year. It is distributed throughout India and also occurs in Eastern China and as far as Australia southwards (Talbot, 1939; 1947). Similarly, from Acraeidae, Tawny Coster (*Acraea violae*) was abundant throughout the year and usually it was found feeding on nectar of *Tridax* flowers. The Common Indian Crow (*Euploea core*) and Plain Tiger (*Danaus Chrysippus)* of Danaidae was abundant during both summer and monsoon seasons and found feeding on many types of flowers. The Plain Tiger (*Danaus Chrysippus)* is one the most wide spread and well known plain butterflies (Bholodia *et al*. 2002).

In Nymphalidae, Lemon Pansy (*Junonia lemonias*), Danaid Eggfly (*Hypolimnas misippus*), Yellow Pansy (*Junonia hierta*) and Common Bushbrown (*Mycalesis perseus*) were abundant during monsoon period. These butterflies were more attracted to flowers of *Lantana camara.* The Nymphalidae is one of the largest butterfly family in the Western Ghats (Sreekumar and Balakrishnan, 2001).

Among Lycaenids, Pale Grass Blue (*Zizeeria maha ossa*) was abundant during winter and monsoon periods and rare during summer period whereas, Slate Flash (*Rapala manea*) was rare in winter and absent in remaining periods. From Hesperiidae, Gaint Redeye (*Gangara thyrsis*) was rare during summer and monsoon seasons and totally absent in winter period. Under Satyridae, Common Palmfly (*Elymnias hypermenstra*) Common Evening Brown (*Melanitis leda)* were rare during winter and summer and both were common during monsoon periods (Table 1). The highest numbers of butterfly species were recorded during summer (March-May) and monsoon (June-November) periods in three years of observations. During winter period (December-February) the populations of butterflies tended to be sparse.

Seasonal occurrence of butterfly species correlated with weather factors (temperature, humidity and precipitation) showed that weather has a strong influence on the occurrence of specific groups of butterflies.

The analysis of species richness index indicated that among eight families, Lycaenidae was the richest (2.84) followed by Nymphalidae (2.29) and Pieridae (2.14). (Fig.1). Among various recorded butterfly families, Nymphalidae was the most abundant (24.0%) and Hesperiidae was the least abundant (0.63%). (Fig.2).

The Dominance index for different families of butterflies of overall Bangalore was presented. Acraeidae (1.00%) was the dominant, whereas Papilionidae (0.12%) was the least one (Fig. 3).

Shannon-Weiner index was applied to measure the diversity of a community (Fig. 4). Evenness index was used for calculation of evenness of insect populations (Fig. 5). The Diversity (H') and Evenness (E_1) indices of butterfly species in the study areas for three complete years (2004 - 2006) was significantly different (t (H') =3.50, t (E_1) =1.00).

The correlation of various weather factors such as maximum temperature, minimum temperature, rainfall and humidity including different butterflies species as well as individuals in the Bangalore region studied are presented. The individuals in the overall Bangalore showed significant positive correlation with maximum temperature (Table. 2)

Conclusion

To conserve various butterfly species in the city, primarily different species of host and nectar plants of butterflies should be maintained and conserved in wide areas of the city. In addition, high anthropogenic activities should be avoided in various landscapes of the city. More trees and water source should be maintained for regulating humidity and temperature. Alternative host plants should also be maintained to maintain diversity of butterflies in different seasons.

Table 1. Butterflies diversity and its status in the Bangalore Region

Family	Scientific Name (Common name)	Seasonal occurrence*		
		W	S	M
Papilionidae	*Papilio demoleus* L. (Lime Butterfly)	+	+++	+++
	Papilio polytes L. (Common Mormon)	++	++	++
	Papilo helenus L. (Red Helen)	+	+	++
	Papilio buddha Westwood (Malabar Banded Peacock)	+	+	++
	Pachliopta artistolochiae F. (Common Rose)	+	++	++
	Pachliopta hector L. (Crimson Rose)	+	+++	++
	Graphium agamemnon L. (Tailed Jay)	++	++	++
	Graphium doson C & R Felder (Common Joy)	+	+++	+++
	Graphium nomius Esper (Spot Swordtail)	+	++	+
	*Iliades polymnestor C*ramer (Blue Mormon)	+	++	++
	Troides minos Cramer (Southern Birdwing)	-	+	-
Pieridae	*Catopsilia pyranthe* L. (Mottled Emigrant)	+++	+++	+++
	Catopsilia pomona F. (Common Emigrant)	++	+	++
	Catopsilia crocale Cramer Lemon Emigrant)	++	+	++
	Pareronia valeria Cramer (Common Wanderer)	-	-	+
	Pareronia hippia Cramer	+	+	++
	Delias eucharis Drury (Common Jezebel)	++	+	++
	Anaphaeis aurota F. (Pioneer)	++	+	++
	Hebomoia glaucippe L. (Great Orange Tip)	++	++	+
	Ixias marianne Cramer (White Orange Tip)	+	+	+
	Ixias pyrene L. (Yellow Orange Tip)	-	+	-
	Capora nerissa F. (Common Gull)	+	+	+
	Calotis fausta Olivior (Salman Arab)	-	-	+
	Calotis dulcis Swinhoe	-	+	+
	Calotis eucharis (Plane Orange Tip)	-	+	-
	Terias davidsonii	-	+	+++
	Huphina coronis	-	+	-
	Colias nilagiriensis C & R Felder (Nilgiri Clouded Yellow)	-	+	-
	Leptosia xiphia	+	+	+
	Leptosia nina F. (Psyche)	+	-	-

Table 1. Butterflies diversity and its status in the Bangalore Region (cont.)

Family	Scientific Name (Common name)	Seasonal occurrence*		
		W	S	M
Acraeidae	*Acraea violae* F. (Tawny Coster)	+++	+++	+++
Danaidae	*Euploea core* Cramer (Common Indian Crow)	++	+++	+++
	Tirumala septentrionis dravidarum Frushtofer (Dark Blue Tiger)	++	+++	+++
	Danaus chrysippus L. (Plain Tiger)	++	+++	+++
	Danaus genutia Cramer (Striped Tiger)	+	++	++
Nymphalidae	*Neptis hylas* Moore (Common Sailer)	+	++	+
	Hypolimnas misippus L. (Danaid Eggfly)	++	++	+++
	Hypolimnas bolina Jacintha Drury (Great Eggfly)	+	+	+
	Mycalesis perseus F. (Common Bushbrown)	+++	++	+++
	Precis lemonias L. (Lemon Pansy)	+++	+++	+++
	Precis orithya L. (Blue Pansy)	+	+	++
	Precis iphita iphita Cramer (Chocolate Pansy)	+	+	+
	Precis hierta F. (Yellow Pansy)	++	++	+++
	Precis almana L. (Peacock Pansy)	-	+	+
	Ariadne merione Cramer (Common Castor)	+	++	++
	Ariadne ariadne Cramer (Angled Castor)	-	-	+
	Phalantha phalantha Drury (Common Leopard)	-	++	++
	Cynthia cardui L. (Painted Lady)	-	-	+
	Eriboea athamas Drury (Common Nawab)	-	-	+
	Euthalia nais Forster (Baronet)	++	++	+++
	Euthalia aconthea Cramer (Common Baron)	+	+	+
	Charaxes fabius F. (Black Rajah)	-	-	+
	Calysisme visala	+	-	-
	Cethosia nietneri C & R Felder (Tamil Lacewing)	-	+	-
	Papilio dravidarum Wood Mason (Malabar Raven)	+	+	+
	Ythima boldus F. (Common Fivering)	++	+	+++

Table 1. Butterflies diversity and its status in the Bangalore Region (cont.)

Family	Scientific Name (Common name)	Seasonal occurrence*		
		W	S	M
Lycaenidae	*Talicada nyseus* Guerin meneville (Red Pierrot)	-	+	+
	Castalius rosimon F. (Common Pierrot)	+++	+	++
	Tarucus nara Kollar (Rounded Pierrot)	-	-	++
	Jamides celeno Cramer (Common Cerulean)	+	+	+
	Jamides elpis	-	-	+
	Jamides boeticus L. (Pea Blue)	+	-	-
	Jamides bochus Cramer (Dark Cerulean)	+	+	++
	Rapala Jarbas F. (Indian Redflash)	-	-	+
	Rapala manea Hewitson (Slate Flash)	+	-	-
	Zizeeria maha ossa Swinhoe (Pale Grass Blue)	+++	+	+++
	Pithauriopsis marcena	+	-	-
	Taractrocera danna (Himalayan Grass Dark)	+	+	-
	Augiades subhyalina	++	-	-
	Kibreeta libythea (Small Blue Grecian)	+++	++	++
	Lycaenopsis oreana (Holly Blue)	+	-	+
	Lycaenopsis Jynteana (Hedge Blue)	+	-	-
	Chrysophanus sp. Evans (Purple Gold Fire)	+	+	+
	Chilades laius Cramer (Lime Blue)	++	-	++
	Tarucus theophrastus (Pointed Pierrot)	++	-	+
	Actolepis liliacea Hampson (Hampson's Hedge Blue)	-	-	+
	Lycaena omphisa Moore (Dusky Green Underwing)	-	++	++
	Neopithecops Zalmora Butler (Quaker)	+	+	++
	Curetis thetis Drury (Indian Sunbeam)	+	-	-
	Chiliaria nilgirica (Common Ringlet)	+	-	+
Satyridae	*Melanitis leda* L. (Common Evening Brown)	+	+	++
	Elymnias hypermenstra L. (Common Palmfly)	+	+	++
Hesperiidae	*Notocrypta curvifascia* C & R Felder (Restricted Demon)	+	-	++
	Gangara thyrsis F. (Gaint Redeye)	-	+	+

*W = Winter, S= Summer, M = Monsoon, - absent, + rare, ++ common, +++ abundant

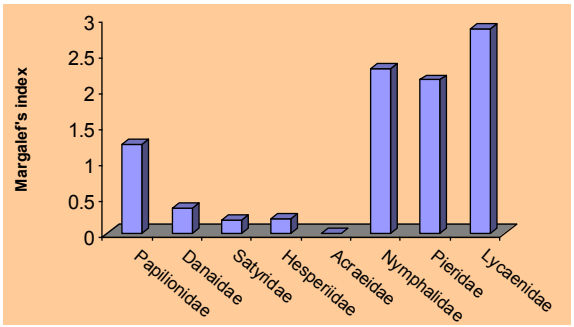

Fig. 1. Richness indes of butterfly families in the Bangalore region (2004-2006).

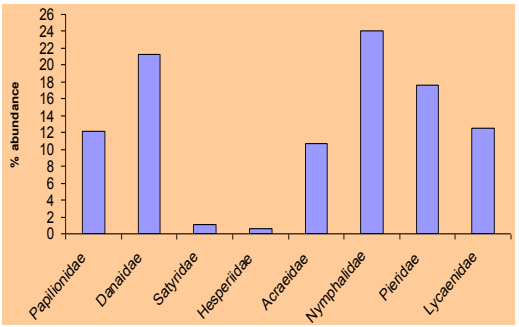

Fig. 2. Abundance (%) of butterflies in Bangalore region (2004-2006).

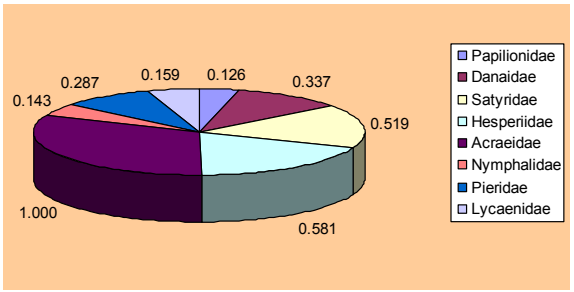

Fig. 3. Simpson's dominance index of different butterfly families.

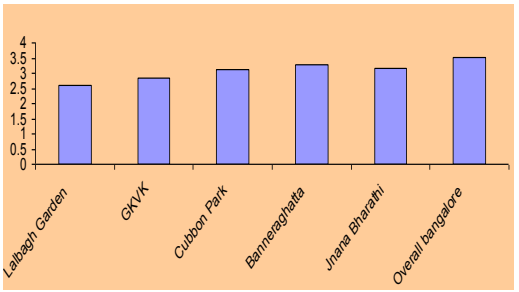

Fig. 4. Diversity of butterflies in different parts of Bangalore.

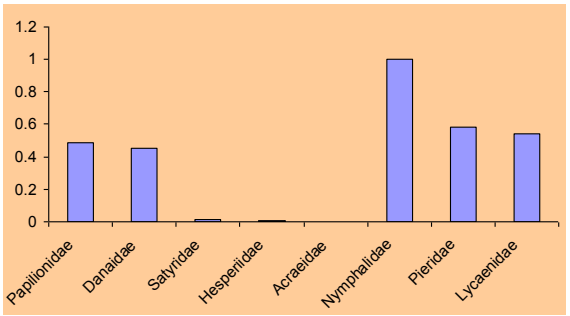

Fig. 5. Pielou's Evenness Index of different butterfly families.

Table 2. Correlation of various weather factors with butterfly populations

Correlations	Temp Min	Temo Max	Rainfall	Humidity	Lalbagh individuals	Lalbagh species	GKVK individuals	GKVK species	Cubbon individuals	Cubbon species	Bannera ghatta individuals	Bannera ghatta species	Jnana Bharathi individuals	Jnana Bharathi species	Overall Bangalore individuals
Temp Min	1.000	0.393(*)	-0.162	-0.633(**)	0.341(*)	-0.410(*)	0.700(**)	0.705(**)	0.367(*)	0.486(**)	-0.504(**)	-0.723(**)	0.639(**)	0.823(**)	0.090
Temp Max	0.393(*)	1.000	0.323	0.300	0.494(**)	0.059	0.361(*)	0.251	0.161	0.250	0.318	-0.166	0.557(**)	0.488(**)	0.383(*)
Rainfall	-0.162	0.323	1.000	0.624(**)	0.303	0.194	-0.038	-0.105	0.143	0.188	0.434(**)	0.286	0.281	-0.006	0.195
Humidity	-0.633(**)	0.300	.624(**)	1.000	0.128	0.457(**)	-0.425(**)	-0.497(**)	-0.151	-0.218	0.812(**)	0.613(**)	-0.121	-0.419(*)	0.220
Bangalore individuals	0.090	0.383(*)	0.195	0.220	0.708(**)	0.054	-0.058	-0.244	0.227	-0.059	0.063	-0.186	0.282	0.088	1.000
Bangalore Species	0.100	0.424(**)	0.242	0.201	0.096	0.311	-0.057	0.071	-0.228	-0.169	0.355(*)	0.218	0.25	0.255	0.197

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

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Biodiversity of Odonates of the Bangalore region, India

Dineshkumar, B.P., Venkatesha, M.G. and Ramakrishna, S.

Department of Zoology, Bangalore University,Jnana Bahrathi, Bangalore 560 056, India
venkatmelally@gmail.com

Abstract

Biodiversity of odonates in the Bangalore region was studied during 2003-2006. The study was conducted in selected six major lakes in and around Bangalore. During the study period, 20 species of dragonflies under 15 genera and six species of damselflies under four genera were recorded. Of the 20 species of dragonflies, populations of *Brachythemis contaminata*, *Crocothemis servilia servilia*, *Orthetrum sabina sabina* and *Trithemis aurora* were abundant almost throughout the year. However, other species viz., *Tramea limbata*, *Ictinogomphus rapax*, *Orthetrum pruinosum neglectum*, *Anax parthenope parthenope* and *Brachydiplax sorbina* were less in number and occur throughout the year. In addition, a few species such as *Acisoma panorpoides panorpoides*, *Aethriamanta brevipennis*, *Rhyothemis variegata variegata*, *Tramea basilaris*, *Trithemis kirbyi kirbyi* and *Trithemis pallidinervis* were found only during some seasons. Of all the dragonflies, *Pantala flavenscens* was the most abundant species throughout the Bangalore region during September-April. Among the six species of damselflies, *Ceriagrion coromandelianum* and *Ischnura senegalensis* were abundant almost throughout the year. However, *Lestes elatus*, *Lestes praemorous*, *Pseudagrion rubriceps rubriceps* and *Pseudagrion microcephalum* were rarely seen only during some seasons of the year in specific lakes. The diversity and ecology of odonates are discussed.

Introduction

Odonates are exceptionally vulnerable to urban and agricultural expansion, and sensitive to human disturbance (Samways, 1989). They play fundamental role in fresh water ecosystems as they are important food sources for fish and birds and play significant role in nutrient cycling and organic material processing. Odonates are characterized as excellent habitat indicators (Clark and Samways, 1996; Samways and Steytler, 1996; Stewart and Samways, 1998). Information on the odonate communities of the Bangalore region is totally lacking. In view of this, it was proposed to conduct detailed studies on the occurrence and biodiversity of various species of odonate communities in the Bangalore region.

Materials and methods

Bangalore is situated in the south-eastern corner of Karnataka state with a geographical area of about 2,191 sq km and an average elevation of 900 m MSL. The climate of the city enjoys an agreeable temperature range from the highest mean maximum of 36.20^0C in April to lowest mean minimum of 11.40^0C in January. The mean value of the rainfall is about 900 mm per year. Over 6.52 million people inhabit the metropolitan area.

Insect samplings were made around water bodies once a fortnight from October 2003 to September 2006. Walked at a slow pace around the lakes and counted odonate adults from the visual radius of 10 feet. Adult odonates sampling was standardized by direct counts made while observing habitats on hourly basis in the early, mid and late hours of the day, during suitable flight conditions (i.e. low winds, warm, sunny). Time spent for sampling depends on the area of the lake. Direct counts are considered a conservative measurement of odonate abundance (Conard *et al*., 1999). Biodiversity indices such as frequency index (f%), Dominance index (Di%), and species richness (R2) were used to estimate the populations of various species of odonates.

Results and discussion

Table 1. Odonate species recorded in the Bangalore region during 2003-06

Order/ sub-order	Family	Scientific name	Common name
Odonata : Anisoptera	Libellulidae	*Acisoma panorpoides* Ramb.	Trumpet Tail
		Aethriamanta brevipennis (Ramb.)	Scarlet Marsh Hawk
		Brachydiplax sobrina (Ramb.)	-
		Brachythemis contaminata (F.)	Ditch Jewel
		Crochothemis servilia (Drury)	Ruddy Marsh Skimmer
		Orthetrum pruinosum (Ramb.)	Crimson-tailed Marsh Hawk
		Orthetrum sabina (Drury)	Green Marsh Hawk
		Rhyothemis variegata (Linn.)	Common Picture Wing
		Tramea basilaris Kirby	Red Marsh Trotter
		Tramea limbata (Ramb.)	Black Marsh Trotter
		Trithemis aurora (Burm.)	Crimson Marsh Glider
		*Trithemis pallidinervis (*Kirby)	Long-legged Marsh Glider
	Aeshnidae	*Anax parthenope* (Selys)	-
	Gomphidae	*Ictinogomphus rapax* (Ramb.)	Common Club Tail
Zygoptera	Coenagrionidae	*Ceriagrion coromandelianum* (F.)	Coromandsl Marsh Dart
		Ishnura senegalensis (Ramb.)	Senegal Golden Dartlet
		Pseudagrion microcephalum (Ramb.)	Blue Grass Dartlet
		Pseudagrion rubriceps (Selys)	Saffron faced Blue Dart
	Lestidae	*Lestes elatus* Hagen	Emerald Spread Wing

Seasonal occurrence

A total number of 19 odonate species were recorded (Table 1). Of which, 14 species were anisopterans and five were zygopterans. The seasonal occurrence of anisopterans and zygopterans is given in Fig. 1 and 2. Of the various species, *Brachythemis contaminata* (Anisoptera) and *Ishnura senegalensis* and *Ceriagrion coromandelianum* (Zygoptera) were present throughout the year with high incidence during June-August, whereas rest of the species observed infrequently in the field. Among anisopterans, *B. contaminata* was abundant, while *Tramea limbata* was observed only about five months with meager numbers during the study period. Among zygopterans, *Ceriagrion coromandelianum* and *Ischnura senegalensis* were abundant, while *Pseudagrion rubriceps, P. microcephalum* and *Lestes elatus* were observed infrequently with less numbers during the study period. The various odonates recorded in Bangalore are widely distributed in India and other parts of the world (Fraser, 1933; 1934; 1936; Emiliyamma *et al*., 2005; Subramaninan, 2005).

Frequency index

Frequency index for different species of odonates is shown in Fig. 3. *Brachythemis contaminata* (Anisoptera) *Ishnura senegalensis* and *Ceriagrion coromandelianum* (Zygoptera) showed high frequency, whereas *Aethriamanta brevipennis, Tramea busilaris, Tramea limbata, Trithemis aurora, Anax parthenope* and *Pseudogrion microcephalum* were observed less frequently during the study period.

Dominance index

In Anisoptera, *Brachydiplax sobrina* was the most dominant species and *Athriamanta brevipennis* was the least, whereas in Zygoptera, *Ishnura senegalensis* was the most dominant species and *Pseudogrion microcephalum* was the least dominant species (Fig. 4 and 5).

Richness index

Zygoptera was the richest suborder, while Coenagrionidae (Zygoptera) and Gomphidae (Anisoptera) were the richest families (Fig. 6). Adult odonate richness has been shown to correlate with macrophyte richness (Hornung and Rile, 2003). Decline in odonate richness has been linked to activities that trample and remove vegetations from littoral zone, including intensive sport fishing (Muller *et al*., 2003), and buffalo trampling (Stewart and Samways, 1998). These features were most commonly observed in the present investigations.

Conclusion

The study revealed that most of the odnoates (dragonflies and damselflies) are occurring around both polluted and unpolluted water bodies. Therefore, based on the activities of odonates it is difficult to assess the quality of water. However, of the 13 species of dragonflies recorded in the Bangalore region, the two dragonfly species viz., *Aethriamanta brevipennis* and *Trithemis pallidinervis* were active only around highly polluted lakes. Hence, the activities of these two dragonfly species around a particular water body may be considered to assess the quality of water. Therefore, these two dragonfly species perhaps serve as indicator species of water quality. But these observations need further in-depth investigation.

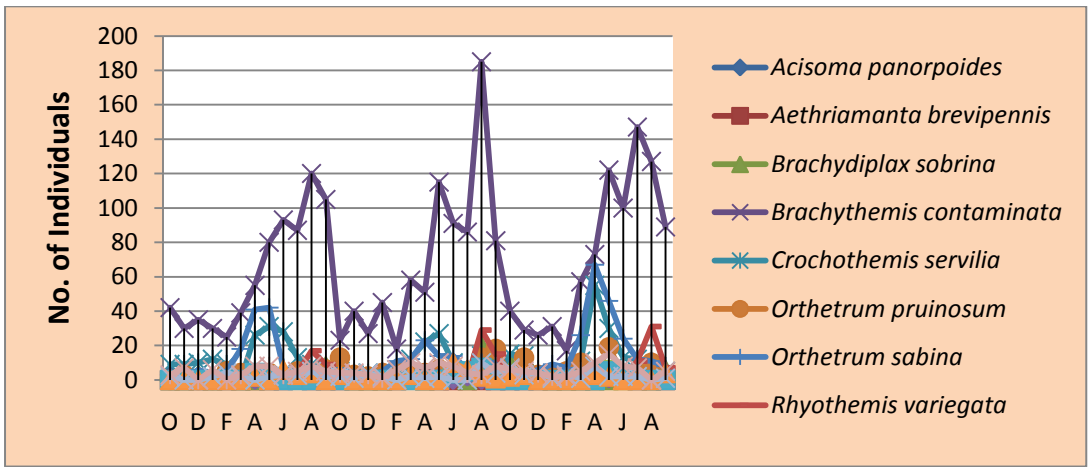

Fig. 1. Seasonal occurence of Anisopterans (Odonata) during 2003-2006.

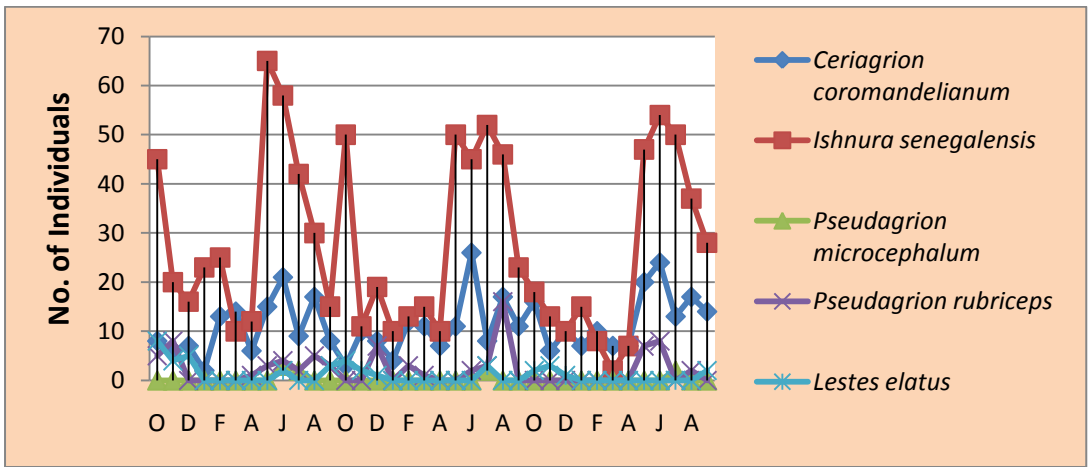

Fig.2. Seasonal occurence of Zygopterans (Odonata) during 2003-2006.

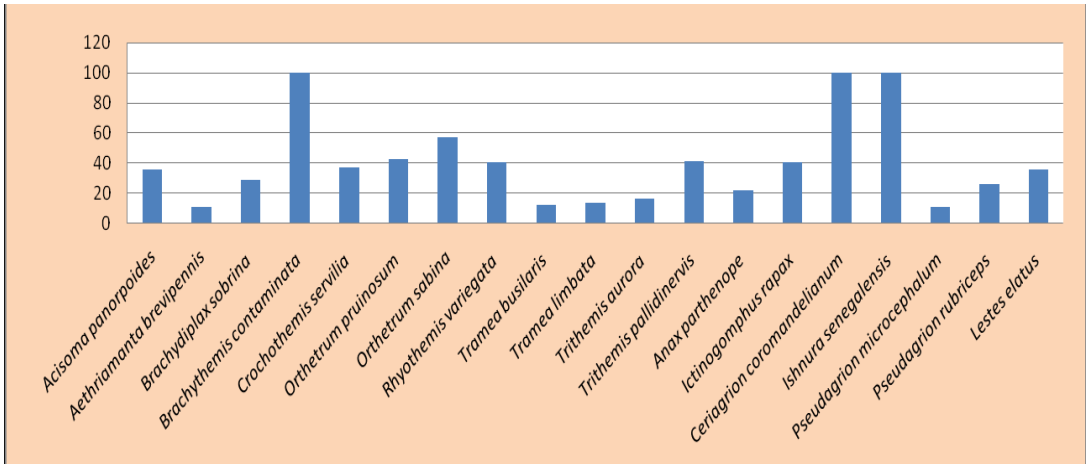

Fig. 3. Per cent frequency of odonates (2003-2004).

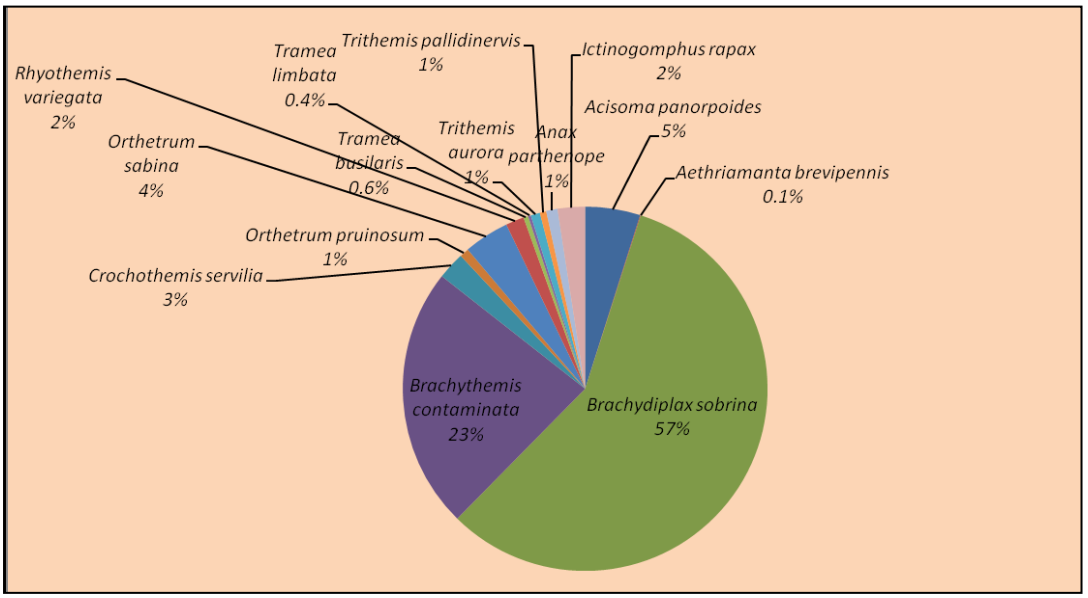

Fig. 4. Per cent dominance of Anisopterans (2003-2006).

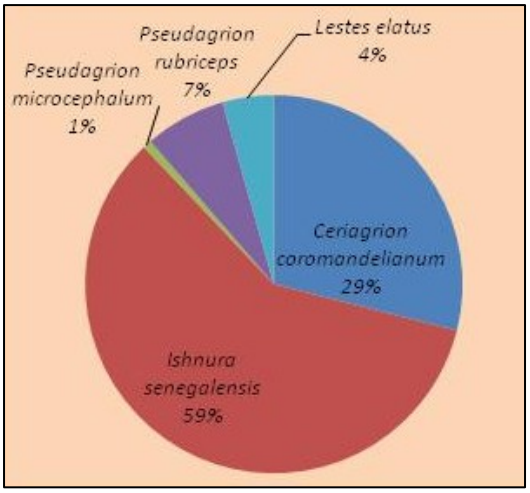

Fig. 5. Per cent dominance of Zygopterans (2003-2006).

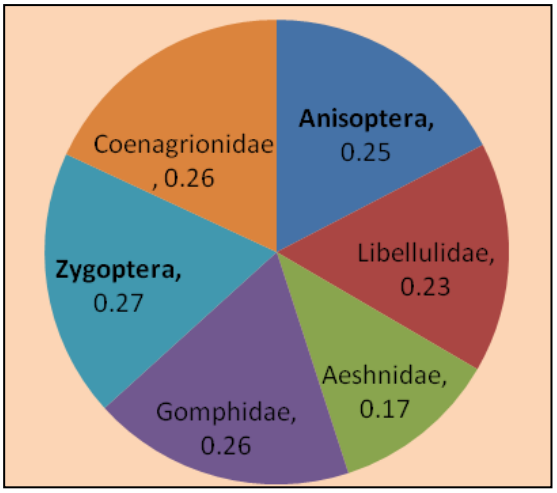

Fig. 6. Richness index of Odonata.

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Bioindication of Atmospheric Heavy Metal Pollution using *Trismegistia* sp. (Musci) at Mount Kinabalu, Sabah, Malaysia

Khairul-Nizam, Y.[1], Monica, S.M.[1] and Suhaimi, Y.[2]

[1]Institute for Tropical Biology and Conservation, [2]School of Science and Technology, Universiti Malaysia Sabah, Locked Bag No. 2073, 88999 Kota Kinabalu, Sabah, Malaysia
khairulny@gmail.com

Abstract

This study was conducted to determine the level of atmospheric heavy metal pollution by using local mosses at Mount Kinabalu, Sabah, Malaysia. As for the records, this is the first study of its kind in South East Asia. The objectives of this study were: (a) to use moss as an active biomonitoring organisms as a part of a monitoring study to assess the impact of metals associated with ambient particles on mosses at roadsides., (b) to investigate the accumulation of the metals on moss over a period of exposure, and (c) to determine the effectiveness of moss as biological indicator. The moss species that studied were *Trismegistia calderensis* (Sull.) Broth. and *Trismegistia panduriformis* (C.H. Wright) Broth. Moss bags techniques were used in this experiments. The moss samples were exposed at 3 difference zones (roadside, 2 km and 8 km) over a period of 0, 4, 8 and 12 weeks for 3 months for subsequent analysis of metal concentration (Cd, Cr, Cu, Pb and Zn). The concentrations of heavy metals in the desiccated apical shoots of mosses were determined by atomic absorption spectrophotometry (AAS). Chlorophyll concentrations were also analysed to measure the stress response of the moss. The results confirm that mosses would be very effective bioindicators of environmental metal concentrations because the concentration of metal in the moss rapidly and directly reflects the metal concentrations in the ambient air.

Introduction

Moss, which also known as bryophytes has been widely used for biomonitoring for the last four decades. It has mainly been applied for surveys on an international level in many ways (Zechmeister *et al.*, 2004). There are a large number of studies being carried on this kind of fields that support its applicability. Mosses have several advantages compared with higher plants as a biomonitoring organism (Čerburnis and Valiulis, 1999; Giordano *et al.*, 2005; Adamo et al., 2008). The lack of a thick cuticle promotes the migration of heavy metals and other elements to the free cation exchange sites located on the walls of the cells in mosses (Poikolainen *et al.*, 2004). The use of mosses as biomonitors is a very convenient method for determining the total levels of atmospheric deposition of contaminants (Fernández *et al.*, 2000).

Mosses have been used as active and passive biomonitors to estimate the deposition of contaminants in the polluted areas (Fernández *et al.*, 2000). Transplants techniques have been used in this study as active biomonitors to estimate and assess the impact of metals associated with ambient particles on mosses at the contaminated areas. The 'moss bags' technique, originally introduced by Goodman and Robert (1971) and later being modified from time to time. The transplants technique appears to be very useful, because of its ability to accumulate metals of high concentrations and thus facilitate analysis. There is a huge variety of sources that contribute to trace metals in the atmosphere, such as combustion processes, metal industries and mining. Traffic plays the major local source of metal pollution, especially in urban areas (Tremper *et al.*, 2004).

Mosses require small amounts of heavy metals for their metabolic functioning (Poikolainen *et al.*, 2004). But it still can accumulate the heavy metals more than the amount of its uses. The accumulation of metals in mosses depends on many factors such as the availability of the elements, the characteristics of the mosses (such as species, age, state of health and type of reproduction), and other such parameters as temperature, moisture availability and substratum characteristics (Conti and Cecchetti, 2001). This reflects the environmental conditions of the areas as the result of the equilibrium process of biota compound intake/discharge from and into the surrounding environment.

Mount Kinabalu is the highest mountain in Southeast Asia, which located in North Borneo (Sabah, Malaysia). Its summit height is 4,095 metres (13,435 ft) above sea level. It is located at

Kinabalu Park, Kundasang, Sabah. As recorded by Frahm *et al.*, (1990), Mount Kinabalu is considered as mossy forest. About 582 species of mosses recorded for Sabah, and more than half of them are found in Mount Kinabalu. Kinabalu Park is nearby a main road, which linked the west coast to east coast of Sabah, and exposed to the air pollution. In this study, we would like to report atmospheric heavy metals pollution level on Mount Kinabalu using mosses.

Materials and methods

Field sampling

As the preliminary phase, a survey was carried out on Mount Kinabalu to find out the moss species, which dominantly survived surrounding the areas. As the results, two different local mosses from the genus of *Trismegistia* were selected. It is *Trismegistia calderensis* (Sull.) Broth. and *Trismegistia panduriformis* (C.H. Wright) Broth. The samples was acclimatised in a controlled room for a week. Then, the apical segments was placed in a nylon bags which known as moss bags. The bags was washed before use with dilute nitric acid and rinsed with distilled water.

Transplants

Samples of *T. calderensis* and *T. panduriformis* were transplanted at 3 differences exposure zones (Zone A – nearby roadside; Zone B – 2 km from roadside; 8 km from roadside). Samples was exposed at the sites and collected over a period of 0, 30, 60 and 90 days for subsequent analysis of metal concentration (Cd, Cr, Cu, Pb and Zn). Three replicates were placed for each species at each zones. At the end of each exposures period, the moss samples were removed and transported to the laboratory for analysis.

Chemical analysis

Prior to analysis, the samples was oven dried at 65^0C for 48 hours and grinded into powder. Then, each samples (~0.5g) were digested by using wet digestion extraction. Approximately 5 ml of HNO_3 : HCl (4:1) was added into the samples for digestion. The digestion was carried out by using a digester at 180^0C for 2 hours. Then, the samples was made up to a final volume of 25 ml in double distilled water. The concentration of each heavy metals was determined by using atomic absorption spectrophotometry (AAS Perkin Elmer). This method was modified from Wietieska *et al.*, (1996).

Besides, chlorophyll analysis was also been carried out to investigate the stress response of heavy metals to the chlorophyll *a* and *b* in the samples. Chlorophyll extraction was done by using dimethyl sulfoxide (DMSO). 20-25 mg of samples was added with 5 ml of DMSO into the boiling tubes which was covered with aluminium foil and been left in a covered water bath at 80°C for 8 hours (Raeymaekers and Longwith, 1987; Tremper *et al.*, 2004). The absorbance was measured by using a UV/VIS spectrophotometer at the wavelengths of 648, 665 and 750 nm and the chlorophyll *a* and *b* concentration was calculated according to Barnes *et al.*, (1992).

Data analysis

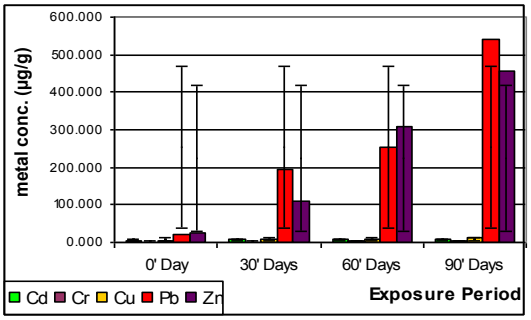
All the data from the heavy metals analysis are expressed in parts per million (ppm) units. For the data analysis purposes, all the data are converted into a µg/g dry weight basis. In the statistical and graphical data treatment, all values below the detection level were not used in the further analysis.

Determinations of standard deviation (SD) for intracellular metals concentrations as well as chlorophyll absorbance were performed with MS Excel 6.0. Data normality was assessed by a Shapiro and Wilk test ($n < 50$). Statistical software (SPSS) was used to asses the correlation and regression values.

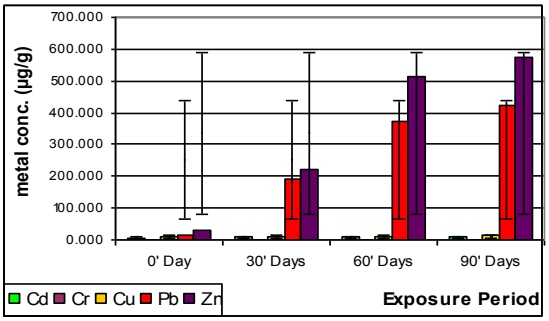
Results and discussion

The concentrations of heavy metals (cadmium, chromium, copper, lead and zinc) in *T. calderensis* and *T. panduriformis* at three difference zones over a period of three months exposure are shown in Fig. 1.

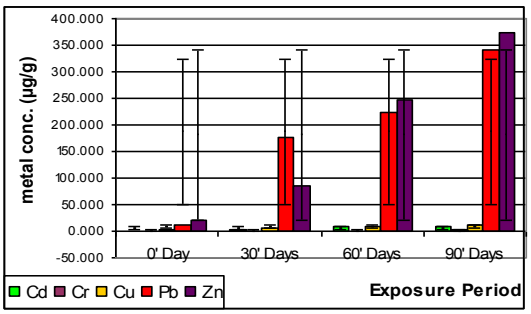
The initials concentrations of the heavy metals in the moss samples increased over the exposure period. It showed a significant increased from the first day of exposure until after 90 days of exposure. The final concentrations of the heavy metals, was higher at Zone A which is nearby the main roadside compared to Zone B and Zone C. All the heavy metals studied which are Cd, Cr, Cu, Pb and Zn, showed similar pattern of increase. Pb and Zn concentrations were higher over the exposure period at all study zones.



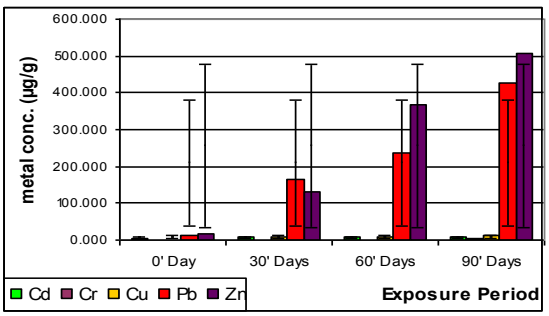
A) *T. calderensis* (Zone A)



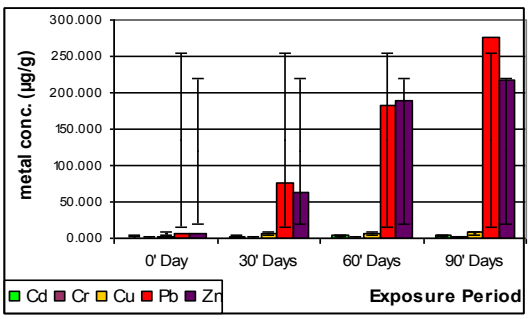
B) T. panduriformis (Zone A)



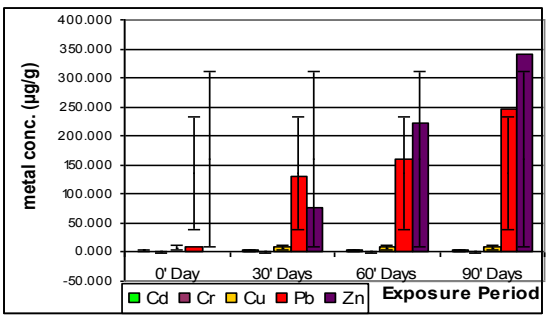
C) T. calderensis (Zone B)



D) T. panduriformis (Zone B)

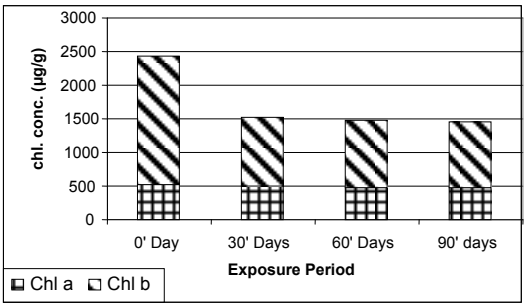


E) T. calderensis (Zone C)

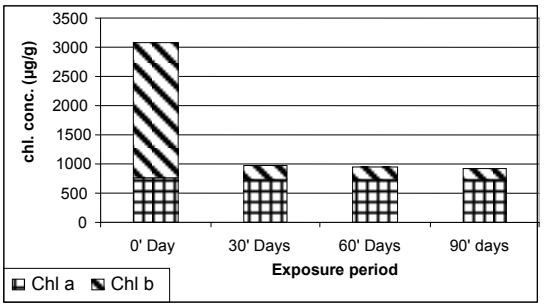


F) T. panduriformis (Zone C)

Fig. 1. Metal concentration (µg/g dry weight) in the moss samples after three months of exposure.



A) T. calderensis



B) T. panduriformis

Fig. 2. Chlorophyll *a* (Chl a) and Chlorophyll *b* (Chl b) concentrations (µg/g dry weight) in the moss samples after three months field exposure.

In the case of *T. calderensis*, the metal concentrations of all five metals increased over the three month period (Fig. 1). This increase was more pronounced in Zone A. Furthermore, the final concentrations of Pb were higher compared to *T. panduriformis*. It is about 539.8 µg/g after 90 days.

For *T. panduriformis*, the metal concentrations of all five metals showed similar pattern over the three month period (Fig. 1). Similar to *T. calderensis*, when the moss samples are located far away from the sources of emission, the metals concentrations decreased. Zn and Pb also recorded the highest concentrations compared to the other metals at all of the zones. *T. panduriformis* absorbed the highest Pb concentrations at Zone A after the three month exposure which was 457.1 µg/g. This shows that the level of Pb pollution nearby the roadside at Kinabalu Park is high and may harm the people there.

The concentrations of Cd, Cr, Pb and Zn were positively correlated ($p=0.01$) in the moss *T. calderensis* and *T. panduriformis* over the exposure period, whereas low correlation could be found for Cu concentration in the two moss species. Tremper *et al.* (2004) reported similar result.

Regression between concentrations of similar elements in the different zones was also carried out to examine the strengths of the relationship. All showed that regression values were strong. The minimum value was 0.833 and the maximum value was 0.9931.

To examine whether metal concentrations had an impact on the mosses, the chlorophyll *a* and *b* concentrations were measured. The same samples batch as for metal concentrations analysis was used in the chlorophyll concentrations analysis. The chlorophyll *a* and *b* concentration in *T. calderensis* and *T. panduriformis* decreased over the first week exposure. After that, only small changes occur in chlorophyll concentrations. However, the correlations between the metal concentrations and chlorophyll concentration are not strongly correlated. It is due to small decrease between the exposure periods. It is not negatively affected by the metals concentrations by the moss species (Fig. 2).

Conclusions

The main objectives of the study were achieved as planned except for the Cu in both moss species. The metal (Cd, Cr, Pb and Zn) concentrations in both moss species increased with increasing exposure time. The metal concentrations decreased over the distance from the source of emission. The chlorophyll concentrations of the mosses exposed in the field was negatively affected by the metals analyzed.

Both mosses was greater in reflecting at the roadside zone exposure and the both mosses also showed similar behavior in the metals uptake. For this reasons, it can be concluded that *T. calderensis* and *T. panduriformis* are very effective in indicating the atmospheric pollution at highland areas, because it could rapidly and directly reflects the metal concentrations in the ambient air

Acknowledgements

We would like to thank Mr. Handry Mujih and Sabah Parks Trustee for their excellent assistance in running this project at Mount Kinabalu, Kinabalu Park, Sabah. We gratefully acknowledge all individuals who involved and made the research possible. This work was supported by Sabah Parks Trustees and Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah.

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Comparative Morphology of Carnivorous Plants, *Utricularia minutissima* and *U. caerulae* from Bintulu, Sarawak

Noorhawanie Izura, A.I., Japar Sidik, B. and Muta Harah, Z.

Department of Animals Sciences and Fisheries, Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus, Nyabau Road, P.O. Box 396, 97008 Bintulu, Sarawak, Malaysia
hawanie_izura@yahoo.com

Abstract

The species of *Utricularia* are among the carnivorous plants which have complexity on their body morphology because there is no clear distinction between roots, stems and leaves. There is some confusion regarding this genus especially in *Utricularia minutissima* and *U. caerulae* because their morphological appearances are extremely similar. These plants can be divided into two sections; vegetative parts are usually on the ground and reproductive parts are very much visible above the ground. *U. minutissima* and *U. caerulae* showed similarities in their leaves, being green and aroused from the stolon which bears the bladder traps. These plants have no roots; instead, they have rhizoids which affixed the plant to the soil substrate. *U. minutissima* possessed lilac flowers and comprised of upper lip, lower lip, spur and calyx lobes. Fruit present in capsule and oblong-ovoid in shape with seeds are non-endospermic and have reddish reticulations on their surfaces. In contrast, *U. caerulae* possessed white flowers along inflorescence stalk in raceme fashion carrying one to six flowers. Fruit is a capsule globose in shape and seeds are brown with black edges, ellipsoidal in shape and have elongated reticulation at the surface.

Introduction

Carnivorous plants are defined as plants that absorb nutrients from dead animals next to their surfaces and thus obtain increased fitness and have some morphological, physiological, or behavioral features to attract, capture, and digest preys (Juniper *et al*., 1989). All carnivorous plants absorbed metabolites from preys and then utilized these metabolites for their growth and development (Lloyd, 1942). These unique plants are belonging to three unrelated families, Nepenthaceae, Droseraceae and Lentibulariaceae (Barthlott *et al*., 2004). Local species of carnivorous plants, belonging to Nepenthaceae are common, while those of Droseraceae and Lentibulariaceae are rare. Casual observation on two species of *Utricularia* collected from Bintulu, *U. minutissima* and *U. caerulae* indicated they have similarity in morphology. In this present study details morphology of vegetative (stolons, rhizoids, leaves, bladder traps,) and reproductive (inflorescences, flowers, fruits and seeds) structures were compared to enable one to differentiate the two species.

Materials and methods

U. minutissima and *U. caerulae* were collected from September 2007 to January 2008 from wetland areas around Bintulu Old Airport, Bintulu, Sarawak and Universiti Putra Malaysia Bintulu Campus. At Bintulu Old Airport, both species were found affixed to clay substrate growing partially submerged near the water margin in ditches with stagnant water. However, at Universiti Putra Malaysia Bintulu Campus, both species were growing among grasses in seasonally-wet ground. The morphology of vegetative (stolon, rhizoids, leaves, bladder traps) and reproductive (inflorescences, flowers, fruits, seeds) characteristics were observed, measured and recorded. Various components mentioned above were observed under the dissecting microscope Zeiss Stemi SV II and PixeLink and LEICA DMLS light microscope and images were recorded.

Results and discussion

General morphology description

Non-flowering plants of *U. minutissima* and *U. caerulae* were similar in morphology, possessing vegetative structures comprising modified stems, the stolons with rhizoids (structure which functioned as roots that allow the plants affixed to the substrate) and minute leaves bearing bladders. In addition to leaves, the stolons also bear bladders. These structures are unique from other plants as they were formed below the surface of substrate. Bladders are trap devices with trigger hairs along their trap aperture which are very sensitive to touch (Slack, 1979; Soepadmo, 1998; Uno *et al*., 2001). In reproductive or flowering plants, part of the stolons are elongated into vertical or inflorescence axes of variable heights, 27 to 33 mm in *U. minutissima* and 58 to 80 mm in *U. caerulae* that bore flowers. With the present of vertical or inflorescence axis, *U. minutissima* is described as a minute capillary plant while *U. caerulae* is a herbaceous plant often branched.

Specific morphology description

Vegetative structures

Non-flowering plants of *U. minutissima* and *U. caerulae* or those with flowering shoot bearing flowers were similar in appearance and morphology and the description of both plant species were in agreement with those described by Ridley (1924) and Taylor (1977). Although superficially similar in morphology, they differed in certain vegetative structure characteristics that could be used to distinguish the two species. In *U. minutissima,* beside the stolons, the stalks of the bladders were also covered with trichomes (Fig. 1A). In addition, the bladders were not only distributed on the stolons but they were also found attached to the petioles of the leaves (Fig. 1B). *Utricularia caerulae* lacks trichomes covering the stalk of bladders and petioles of leaves were devoid of bladders. These characteristics were not reported either by Ridley (1924), Taylor (1977) and Tan (1997). The bladder morphology is unique for each of the species. In *U. minutissima,* the bladders are broadly ovoid, devoid of carinate beak but possess a solitary multicellular subulate appendage (Fig. 1C) at the upper lip and radiating rows of basally connate obliquely gland tipped hair on lower lip. In contrast, bladders of *U. caerulae* are ovoid, with two types of bladders; a bladder with short carinate beak and a bladder with extended tubular projection or carinate beak (Fig. 1D). The bladder functioned as capturing device and digesting small animals to gain extra nitrogenous nutrients. Bladders do not function as floating device.

Reproductive structures

Reproductive structures of *U. minutissima* and *U. caerulae* grew above the ground which is an inflorescence that produces flowers during flowering season. After flowering, fruits are formed followed by seeds dispersal.

The similarity in characteristic of *U. minutissima* and *U. caerulae* are that both possess racemose inflorescence, i.e. flower stalk continues to produce new flower buds resulting in the youngest flowers at the top and the oldest flowers are at the base of the stalk. Each flower has a corolla with three petals namely lower lip, upper lip and spur (Fig. 1E). The distinct reproductive characteristics that distinguish *U. minutissima* from *U. caerulae* are flowers, fruit and seeds. *U. minutissima* flower has lilac corolla, hairless upper and lower lips and, two yellow spots present at the lower lip (Fig. 1F). The flower is subtended by a reddish calyx. *U. caerulae* flower has white corolla, hairy upper and lower lips and a yellow patch at the lower lip (Fig. 1G) and the calyx is yellow in colour. Both species produced fruits (oblong-ovoid in *U. minutissima* and globose in *U. caerulae*) dehiscing by longitudinal ventral slits on their underside from capsule base to tip (Macfarlane and Coleman, 2007). *U. minutissima* seeds are round to oblong ovoid, reddish-brown with a smooth testa that has hexagonal reticulation. In contrast, *U. caerulae* seeds are oblong-ellipsoid, reddish-brown with rough testa that has elongated reticulation (Fig. 1H).

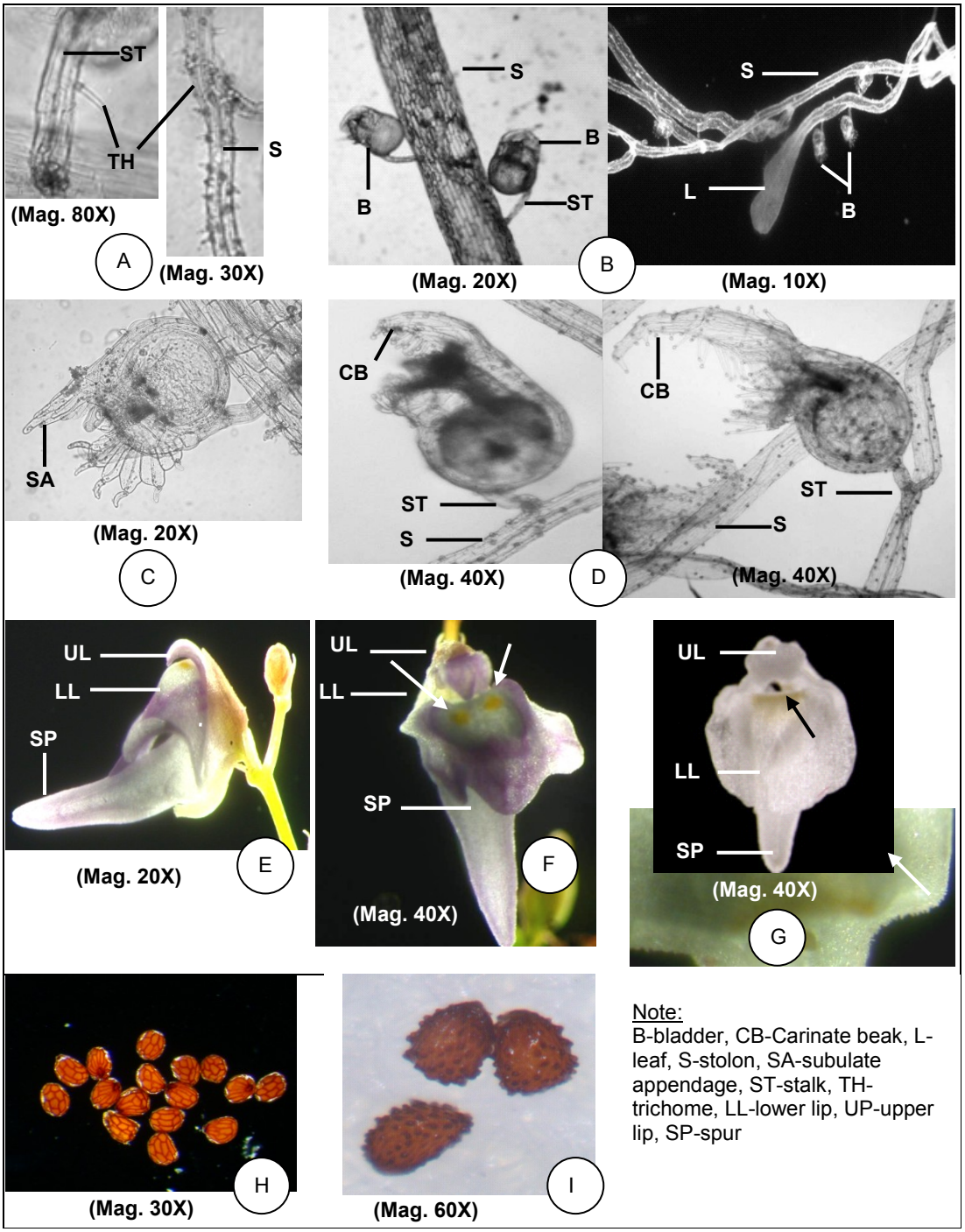

Fig. 1. The characteristics of *Utricularia minutissima*: A- Stalk of bladder and stolon have trichomes, B- Stalked bladders attached to the stolon and petiole of the leaves, C- Upper lip has a solitary multicellular subulate appendage, E-the flower with the three petals, upper lip, lower lip and spur, F- the lower lip has two yellow dots (indicated by the two arrows), H-seeds are round to oblong ovoid with smooth testa and hexagonal reticulations. The characteristics of *Utricularia caerulae*: D- A bladder

with short carinate beak and a bladder with tubular projection or carinate beak that is curved down side, G- the flower showing upper lip, lower lip and spur, and the lower lip has a yellow patch (indicated by an arrow) and lower and upper lips are hairy (indicated by an arrow), I- seeds are round to oblong ellipsoid with rough testa.

Conclusions

In summary, both non-flowering and flowering plants of *U. minutissima* and *U. caerulae* can be distinguished by their morphology of vegetative or reproductive structures respectively.

Acknowledgements

We would like to thank Vice-Chancellor, Universiti Putra Malaysia, for encouragement and facilities. This research is funded by the Ministry of Higher Education Malaysia under the Research Cluster of Natural Sciences and Natural Heritage entitled "Biology and Diversity of Carnivorous Plants, *Utricularia* Species from Wetland Habitats".

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Isolation of Fungi from some Diseased Wild Ginger Species in Bintangor, Sarikei, Sarawak – An Update Study

Rizuan, S.M. and Sepiah, M.

Faculty of Resource Science and Technology, Universiti Malaysia Sarawak,
94300 Kota Samarahan, Sarawak, Malaysia
rizuan99@yahoo.com

Abstract

Preliminary isolation of foliar disease study has been conducted on wild ginger diseases in Sarawak. All of ginger samples were collected in various locations in Sarawak for further species identification based on morphology appearances or symptoms formation which is caused by fungi. Seventeen of ginger samples were studied namely *Scaphochlamys* sp., *Etlingera elatior, Boesenburgia* sp., *Hedyehium coronarium, Globba bracyanthera, Alpinia galanga, Alpinia latilabris, Globba atrosanguinea, Elattariopsis kerbyi, Plagiostachyhs borneensis, Plagiostachys* sp*, Hornstedtia schypifera, Hornstedtia reticulate, Etlingera punicea, Alpinia ligulata, Geocharis rubra and Zingiber longipenduculata*. The ginger samples later were examined under microscopic observation for the fungi identification. Fourteen of genera of foliar diseased wild ginger were identified caused by fungi.

Introduction

Native plants such as wild ginger are found widely distributed across the geographical map of Sarawak. Some of them have potential for ornamental, producing unique flowers and aroma. Although it has a specific own of values, it has known to be non-protected from several disease. Little publications were reported about Sarawak native ginger diseases and future challenges facing them against rapid land development despite infestation from insect pests. However, steps to protect the inheritance of native ginger for future biological assets excluding the legal authorisation, scientific approaches had been taken to identify a common ginger disease. According to Karjane (2005), most common soil borne of ginger diseases in India affected by bacterial wilt infected by *Ralstonia solanacearum* and United State Department of Agriculture, USDA (2006) has found phytopathogenic fungi namely *Rizoctonia solanacearus* and *Phytopthora* sp. and *Fusarium* sp. to caused diseases. Foliar disorders such as rust, leaf spots and blight are common diseases amongst ginger. In this study, the collected diseased ginger leaves samples were isolated and identified the pathogens.

Materials and methods

Source of samples

The ginger species of samples were taken from various locations of Sarawak. Seventeen of them were confirmed species identification and all of them were subsequently kept for sample preparation.

Samples preparation

The diseased samples especially leaves of ginger were cut into square 5x5 mm where the square containing the infection zone apart of healthy tissue and dead tissue as shown in Fig. 1. The surface sterilisation solution were prepared earlier i.e., 100 ml of 30% v/v of NaOCl, 100 ml sterilised distilled water and sterilised of filtered paper for square cut surface drying. The malt extract agar (MEA) and water agar (WA) were prepared in Petri plates for the mycelia germination (Fig. 2).

Isolation and identification of pathogen

The square cut of ginger leaves were divided into surface sterilisation and non-surface sterilisation treatments. The non-surface sterilisation were done by direct plating the square cut into MEA and WA in Petri plate and incubated at ambient temperature 25$\pm$2°C for 5 days for the formation of reproductive structure for the identification. The surface sterilisation treatment, square cut were

immersed into 30% v/v of NaOCl for 10 minutes and subsequently transferred into sterilised distilled water for a minute before surface drying using sterilised filter paper. Dried square cut were transferred into MEA and WA in Petri plate and incubated at ambient temperature 25±2°C for 5 days. The emerging mycelia from the square cut were observed microscopically for the identification and isolation of the pathogen.

Results and discussion

All of the seventeen treated samples were done to obtain and identify the foliar pathogen on ginger. The identifications and characteristics of fungi were using the Burner's method (1984) and Agrios (1997). Table 1 shows the ginger species and the foliar pathogens but *Alpinia ligulata*, *Etlingera punica* and *Hornstedtia schypifera* were found not yet detected as causal pathogen and the identification on these fungi are being studied. Fig. 1 and 2 shows the foliar symptoms occurred on disease ginger.

Conclusion

The isolation of wild ginger foliar species were diseased by the isolated fungi had been successfully done. Some of the fungi are enable to infect another species of ginger for the broad spectrum of infection and some species of ginger were not yet detected as diseased fungi and still under investigation. From this obtained study done, further investigation should be focus especially to the soil borne fungi in near future as a record and update study for some disease incidence of wild ginger species in Sarawak.

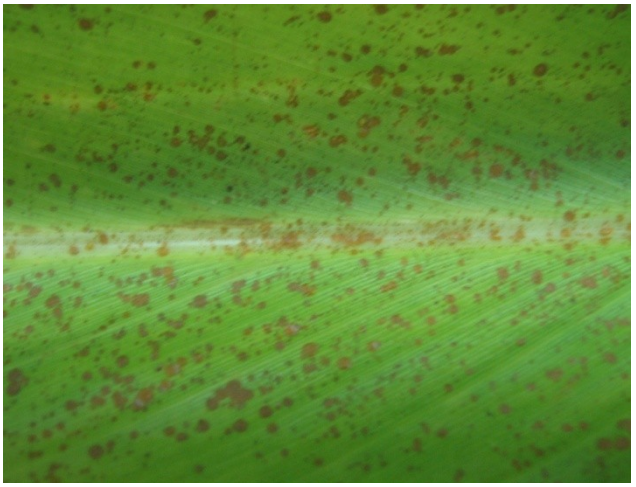

Fig. 2. Symptom: leaf blast; distribution at the frond towards the edge of leaf. Disease infecting at the fourth leaf and older, seldom found on younger leaves of *Alpinia galanga*. Pathogen: *Septocylindrium* sp. Mode of infection: mycelia expanding along the peripheral vein of leaf.

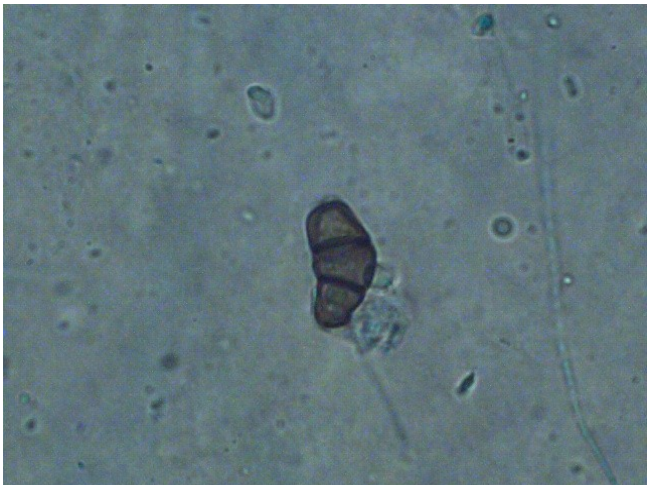

Fig. 3. Microscopically observation *Culvularia* sp. on the magnification 100X (immersion oil).

Table 1. Phyto pathogens on ginger leaves

No	Ginger species	Pathogen
1	*Alpinia galangal*	*Septocylindrium* sp.
2	*Alpinia latilabris*	*Diplococcium* sp
3	*Alpinia ligulata*	Undetected
4	*Boesenburgia* sp.	*Mucor* sp.
5	*Elattariopsis kerbyi*	*Pleiochaeta* sp.
6	*Etlingera elatior*	*Periconiella* sp.
7	*Etlingera punicea*	*Mucor* sp.
8	*Geocharis rubra*	*Albugo* sp.
9	*Globba atrosanguinea*	*Culvularia* sp
10	*Globba bracyanthera*	*Pleiochaeta* sp.
11	*Hedyehium coronarium*	*Mucor* sp.
12	*Hornstedtia reticulate*	*Culvularia* sp
13	*Hornstedtia schypifera*	*Mucor* sp.
14	*Plagiostachyhs borneensis*	*Pleiochaeta* sp.
15	*Plagiostachys* sp	*Pleiochaeta* sp.
16	*Scaphochlamys* sp.	*Pleiochaeta* sp.*, Dresclera* sp.
17	*Zingiber longipenduculata*	*Colletotrichum capsici*

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Macroalgal Communities of Intertidal Rocky Shores around Bintulu, Sarawak

Wong, S.C.[1], Muta Harah, Z.[1], Japar Sidik, B.[1], Arshad, A.[2] and Ogawa, H.[3]

[1]Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus, Nyabau Road, P.O.Box 396, 97008 Bintulu, Sarawak, Malaysia
[2]Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Faculty of Fisheries, Kitasato University, Sanriku-cho, Ohofunato City, Iwate 022–0101, Japan
wongsiawchia@gmail.com

Abstract

Macroalgae are unique compared to seagrasses in that they are adapted to colonise range of soft to hard substrates from sand, boulders, rocks to coral reefs. Based on previous observations for examples around Similajau National Park, Bintulu macroalgae communities tend to utilize hard substrates as their striving environments. Only few species such as *Caulerpa* and *Halimeda* colonize and thrive in sandy environments. As an extension to the previous study, this present study is perform to evaluate the macroalgae community structure and their species distribution in relation to their environments, the rocky shores around Bintulu, Sarawak. Five study sites were sampled monthly during January to August 2008, in the intertidal rocky shores around Bintulu, Sarawak, Malaysia. A total of 19 families and 35 species of intertidal macroalgae were determined comprising the three divisions, Chlorophyta, Phaeophyta and Rhodophyta. Of these, Rhodophyta has the diverse representative of macroalgae with 18 species from 10 families. Chlorophyta with 12 species from seven families and Phaeopyta with five species from two families. The Family Rhodomelaceae is represented with five species namely *Acanthophora spicifera, Bostrychia* sp., *Laurencia papillosa, Laurencia* sp. and *Leveillea jungermannioides.* Dictyotaceae with four species, Ulvaceae and Corallinaceae with three species. Caulerpaceae, Cladophoraceae, Udoteaceae, Gelidiaceae, Gracilariaceae with two species, while the rest such as Anadyomenaceae, Polyphysaceae, Valoniaceae, Sargassaceae, Bangiaceae, Ceramiaceae, Champiaceae, Garaxauraceae, Hypneaceae and Rhodymeniaceae with one species respectively. The macroalgae community structure, the coexistence of the species in relation to their environments is described in this paper.

Introduction

Rocky shore can be defined as the part of the coastline that extends from the lowest tide mark uncovered by the tides up to the highest tide mark splashed by the waves at highest tides. Inter-tidal rocky area is astonishingly rich in marine plants such as macroalgae which is one of the most important components of primary producers in the marine ecosystem that substantially contribute to the food chain and habitat of a variety of vertebrates and invertebrates. Other than dominating the sub-tidal area, macroalgae tends to grow on the wave-washed rocky shore habitats. Studies and publications on macroalgae on rocky shores area are scarce especially in Bintulu. Previous studies at certain areas of Bintulu were performed by Fisheries Research Centre of Sarawak (http://www.fri.gov.my/friswak/seaweed.htm) and Muta Harah *et al.* (2006 and 2007). The present study was conducted at various rocky shores around Bintulu to investigate the current status of macroalgal diversity at the selected areas.

Materials and methods

Macroalgae were collected from five sites around Bintulu in the inter-tidal rocky zone during low tide: Telecom Beach, Tanjung Batu Rocky Shore, Kg. Kuala Nyalau Rocky Shore, Kuala Similajau Rocky Shore and Similajau National Park Rocky Shore (Table 1) from January to August 2008. The zones (high intertidal, mid intertidal and low intertidal) were determined at the beginning of the sampling. Data on species composition, abundance in terms of percentage cover (Saito and Atobe, 1970), species assemblages were recorded from 20 randomly 50 cm x 50 cm quadrat at each zone monthly. Representative samples of macroalgae were preserved in 5% saline formalin solution for taxonomical identification following the references of Lewmanomont and Ogawa (1995), Trono (1997 and 2004) and Tsutsui *et al.* (2005).

Table 1. Relative substratum observed in the rocky intertidal zone of the five sites as observed during low-tide periods

Sites	Description
Telecom Beach (3° 18' 57.4" N, 113° 06' 59.3" E)	Wave splash area, rocks, boulders, gradual slope and sandy shores with tide pools, inhabited by epifauna such as sea urchins, barnacles, mussels, snails and oysters.
Tg. Batu (3° 12' 28.3" N, 113° 02' 38.4" E)	Wave splash area, rock, boulders, gradual slope with tide pools, inhabited by epifauna such as mussels, barnacles, oysters and snails.
Kg. Kuala Nyalau (3° 37' 50.8" N, 113° 22' 16.1" E)	Broad and flat rocky area, boulders and pebbles, with some part frequently receive splash of wave, various size of tide pools, inhabited by large numbers of oysters, snails, sea cucumbers, sea slugs, sea urchins and mussels. Little sandy-muddy shores towards landward.
Kuala Similajau (3° 22' 13.9" N, 113° 17' 39.1" E)	Rocky area, boulders, pebbles and sandy-muddy shores with less wave splash, inhabited by various epifauna such as sea urchins, sea slugs, sea cucumbers, snails, oysters. A small lagoon was observed inhibited by seagrasses.
Similajau National Park (3° 21' 13.6" N, 113° 09' 21.4 E)	Broad rocky area, boulders and sandy shores with various size of tide pools, inhabited by various epifauna such as sea urchins, sea slugs, mussels, snails and oysters.

Results and discussion

Species composition

A total of 35 species of macroalgae were recorded from 5 study sites (12 Chlorophyta, 5 Phaeophyta and 18 Rhodophyta) (Table 2). Composition of macroalgae varied with localities where 26 macroagae were observed in Kg. Kuala Nyalau, followed by 24 macroalgal species and two species of seagrasses in Kuala Similajau, 20 species in Telecom Beach, 19 species in Similajau National Park and 14 species in Tg. Batu (Table 3). Locations that received less wave splash were dominated by macroalgae especially Rhodophyta (Table 1 and 3). Waves and currents play an important role in influencing distribution and abundance of macroalgae (Ahmad and Go, 1992). The high diversity of Rhodophyta in the sites is attributed to the hard substrates availability for attachment. In other parts of the world, for example shallow warm marine environments of tropical and sub-tropical areas, many of the macroscopic marine algae are members of Rhodophyta and they are especially distributed in both intertidal and sub-littoral zones (Pritchard and Bradt, 1984).

Table 2. Number of taxa by major taxonomic groups

Major Groups	Number of Taxa Recorded
Chlorophyta	12
Phaeophyta	5
Rhodophyta	18
Spermatophyta (seagrasses)	2
Total	**35**

Table 3. Number of taxa at the five rocky shores sites

Major Groups	Rocky Shore Sites				
	Telecom Beach	Tg. Batu	Kg. Kuala Nyalau	Kuala Similajau	Similajau National Park
Chlorophyta	7	4	9	7	6
Phaeophyta	4	2	5	5	3
Rhodophyta	9	8	12	12	10
Spermatophyta (seagrasses)				2	
Total	20	14	26	26	19

Nine species were common to all sites and have been identified as *Cladophora prolifera, Ulva intestinalis, Padina minor, Sargassum* sp.*, Gracilaria salicornia, Hydropuntia edulis, Acanthophora spicifera, Laurencia papillosa* and *Laurencia* sp. (Table 4). The families with most species were Rhodomelaceae represented by five species, Dictyotaceae four species, Ulvaceae and Corallinaceae three species, Caulerpaceae, Cladophoraceae, Udoteaceae, Gelidiaceae, Gracilariaceae two species, while the rest such as Anadyomenaceae, Polyphysaceae, Valoniaceae, Sargassaceae, Bangiaceae, Ceramiaceae, Champiaceae, Garaxauraceae, Hypneaceae and Rhodymeniaceae one species respectively (Table 4). Study by Fisheries Research Centre Sarawak (http://www.fri.gov.my/friswak/seaweed.htm) reported 31 species (10 Chlorophyta, 5 Phaeophyta and 16 Rhodophyta) in Bintulu area. Phang (2006) reported there were 27 species (2 Chlorophyta, 8 Phaeophyta and 17 Rhodophyta) of macroalgae found in Sarawak. However, Muta Harah *et al.* (2006) noted there were 35 species (10 Chlorophyta, 6 Phaeophyta and 19 Rhodophyta) associated with coral reef at Golden Beach, Similajau National Park of Bintulu. In addition, Muta Harah *et al.* (2007) also observed 35 species (12 Chlorophyta, 8 Phaeophyta and 15 Rhodophyta) of inter-tidal macroalgae along the stretch of Tg. Batu Kudu to Tg. Lubok Padok of Similajau National Park. With respect to species composition, Rhodophyta is always with the most number of species irrespective of the geographical locations as suggested by the species composition by the previous work of the authors mentioned above.

Abundances of macroalgae

As light and space are believed to be the primary limiting resources in rocky inter-tidal habitats, cover measurement is use in this study that represent the most meaningful method of quantifying abundances of macroalgae. Based on percentage cover, macrolagae abundances were high at Kg. Kuala Nyalau and low at Tg. Batu (Fig. 1). Kg. Kuala Nyalau as well as Kg. Similajau landscapes are both broad and flat rocky areas with a variety of macrohabitats, stable substrates including boulders, pebbles and tide pools (Table 1) that afford suitable attachments for macroalgae, while, Telecom Beach and Tg. Batu are two areas with short inter-tidal areas hence less favourable for macroalgae attachment. Other factors that also contributed to the higher coverage at any one site are the size and life form of the macrolagae. An appropriate example to illustrate these is macroalgae with large thallus and in clusters e.g. *Sargassum* will definitely give a larger cover per unit area as compared to small and individual macroalgae e.g. *Avrainvillea*. With respect to the zones of the rocky shores, the low and mid intertidal zones have relatively higher abundance (percentage cover) as compared to the high intertidal zone (Fig. 2). This observation is attributed to more species composition occurred in the two zones as well as favourable environmental condition (usually covered by water during most tidal periods) as compared to the less species and drier condition, often exposed to air in the high intertidal zone.

Species assemblages and inter-tidal zonation

The population data obtained for the individual quadrats were subjected to classification analysis to determine the species assemblages related to the zonation pattern in all investigated sites. Macroalgae from the three divisions (Chlorophyta, Phaeophyta and Rhodophyta) did not show any distinct zonation. Mixed divisions of macroalgae assemblages were observed in the five sites.

Diverse assemblages of macroalgae were observed in the mid and low intertidal zones which are near to the sea as compared to the high intertidal zone. This is due to the variation of the environmental characteristic (Trono, 1997; 2004).

Table 4. Division, Family, Species of Macroalgae of Rocky Shores Around Bintulu

No.	Division/Family	Species
CHLOROPHYTA		
1	Anadyomenaceae	*Anadyomene plicata* C. Agardh
2	Caulerpaceae	*Caulerpa racemosa* var. *peltata* (Lamouroux) Eubank
3		*Caulerpa sertularioides* (Gmelin) Howe
4	Cladophoraceae	*Chaetomorpha antennina* (Bory) Kutzing
5		*Cladophora prolifera* (Roth) Kutzing*
6	Polyphysaceae	*Acetabularia major* G. Martens
7	Udoteaceae	*Avrainvillea obscura* (C. Agardh) J. Agardh
8		*Halimeda macroloba* Decaisne
9	Ulvaceae	*Ulva clathrata* (Roth) Greville
10		*Ulva intestinalis* (Linnaeus) Nees*
11		*Ulva prolifera* (Muller) J. Agardh
12	Valoniaceae	*Valonia aegagropila* C. Agardh
PHAEOPHYTA		
13	Dictyotaceae	*Dictyota* sp.
14		*Lobophora variegata* (Lamouroux) Wolmsley
15		*Padina australis* Hauck
16		*Padina minor* Yamada*
17	Sargassaceae	*Sargassum* sp.*
RHODOPHYTA		
18	Bangiaceae	*Porphyra* sp.
19	Ceramiaceae	*Ceramium* sp.
20	Champiaceae	*Champia* sp.
21	Corallinaceae	*Amphiroa fragilissima* (Linnaeus) Lamouroux
22		*Cheilosporum acutilobum* (Decaisne) Piccone
23		*Sporolithon* sp.
24	Garaxauraceae	*Galaxaura oblongata* (Ellis and Solander) Lamouroux
25	Gelidiaceae	*Gelidiella acerosa* (Forsskal) Feldmann and Hamel
26		*Pterocladia* sp.
27	Gracilariaceae	*Gracilaria salicornia* (C. Agardh) Dawson*
28		*Hydropuntia edulis* (S.G. Gmelin) P.C. Silva*
29	Hypneaceae	*Hypnea cervicornis* J. Agardh
30	Rhodomelaceae	*Acanthophora spicifera* (Vahl) Borgesen*
31		*Bostrychia* sp.
32		*Laurencia papillosa* (C. Agardh) Grevillle*
33		*Laurencia* sp.*
34		*Leveillea jungermannioides* Hering et Martens) Harvey
35	Rhodymeniaceae	*Gelidiopsis intricata* (C. Agardh) Vickers

*Species common to all sites.

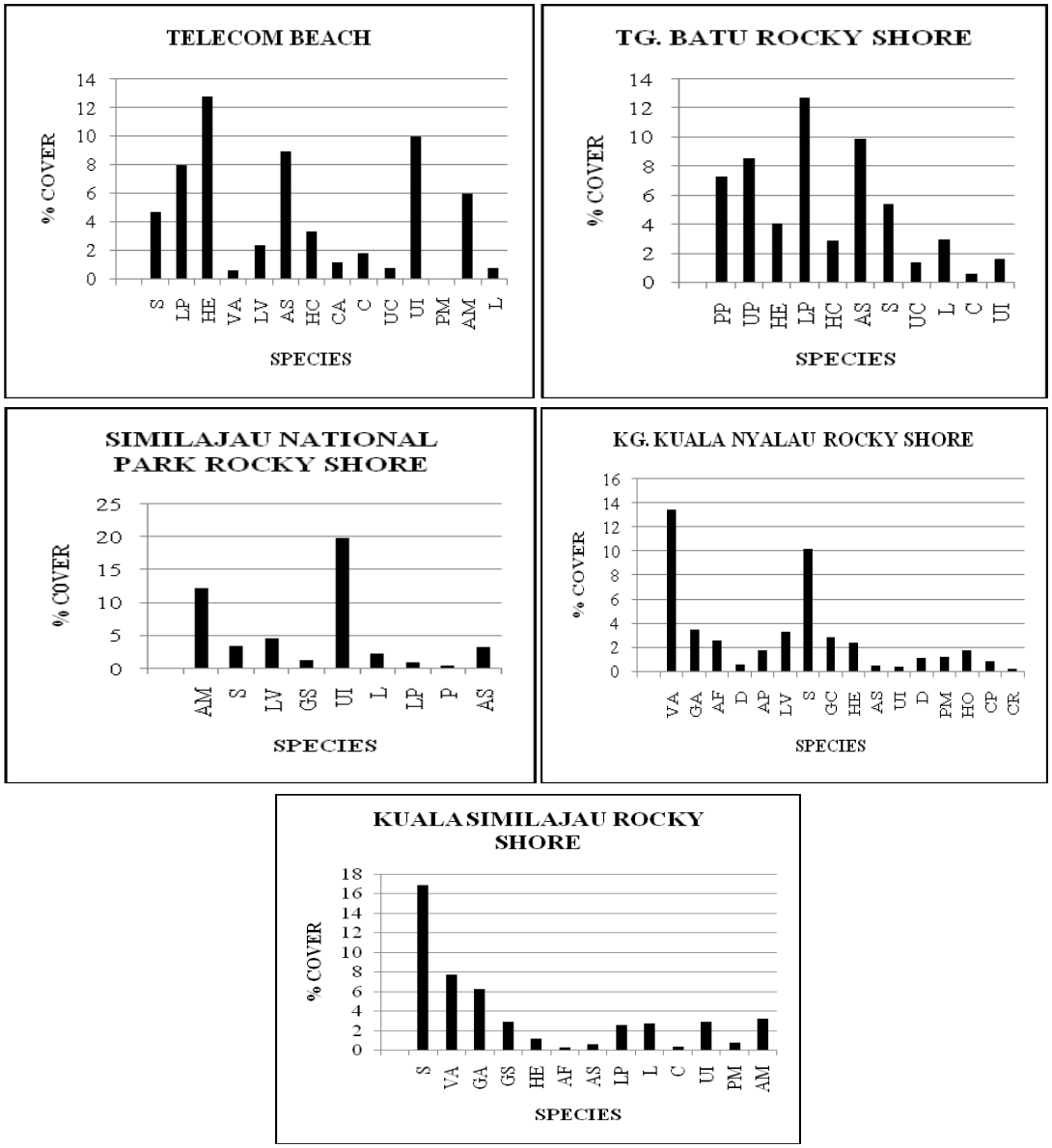

Fig. 1. Percentage cover of dominant and sub-dominant macroalgae species from different study sites. S-*Sargassum* sp., LP-*Laurencia papillosa*, HE-*Hydropuntia edulis*, VA-*Valonia aegagropila*, LV-*Lobophora variegata*, AS-*Acanthophora spicifera*, HC-*Hypnea cervicornis*, CA-*Chaetomorpha antennina*, C-*Ceramium* sp., UC-*Ulva clathrata*, UI-*Ulva intestinalis*, PM-*Padina minor*, AM-*Acetabularia major*, L-*Laurencia* sp., PP-*Porphyra* sp., UP-*Ulva prolifera*, GS-*Gracilaria salicornia*, P-*Pterocladia* sp., GA-*Gelidiella acerosa*, AF-*Amphiroa fragilissima*, D-*Dictyota* sp., AP-*Anadyomene plicata*, HO-*Halimeda macroloba*, CP-*Cladophora prolifera*, CR-*Caulerpa racemosa* var. *peltata.*

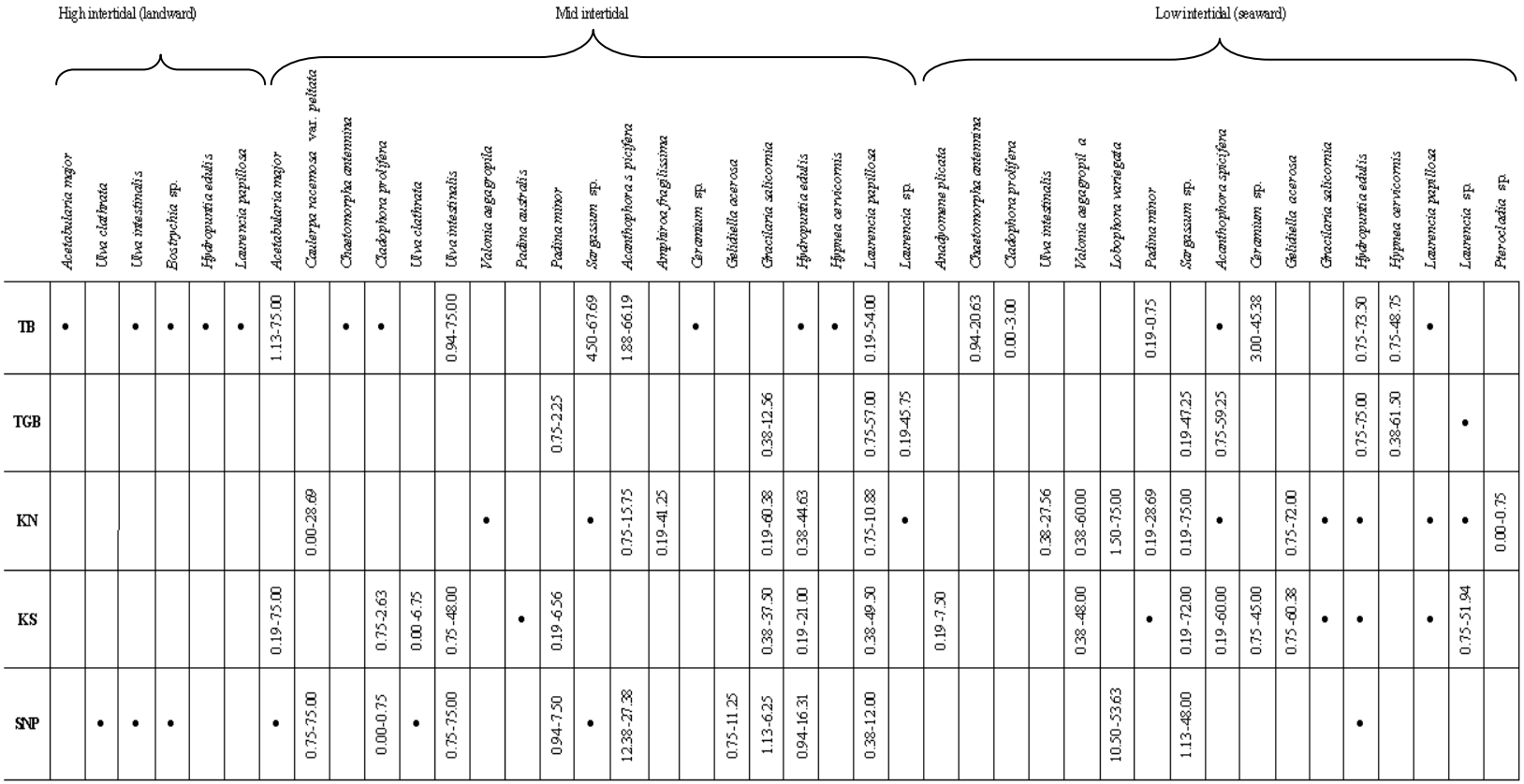

Fig. 2. Distributional patterns, species assemblages and range of percentage cover values of dominant species in relation to the three zones. z• indicates the presence of the species but with low percentage coverage. TB – Telecom Beach; TGB – Tg. Batu; KN – Kg. Kuala Nyalau; KS – Kuala Similajau and SNP – Similajau National Park.

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Morphological Diversity of *Capsicum frutescens* in Malaysia

Muhammad Shafie, M.S. and Mohd Shukor, N.

Strategic Resource Research Centre,
Malaysian Agriculture Research and Development Institute (MARDI),
Persiaran MARDI-UPM, GPO 12301, 50774 Kuala Lumpur, Malaysia
shafiems@mardi.gov.my

Abstract

Capsicum spp. or commonly known as chilli is one of the most important vegetable crops among Malaysians. It has been recognized in decades as an important menu in every dish. It is also being used as spices. There are 2 indigenous species cultivated in Malaysia i.e. *Capsicum annum* L. (lada besar) and *Capsicum frutescens* L. (cili api). *C. frutescens* is the most popular among Malays, Chinese and even Indian due to hot taste. Moreover some varieties of *C. frutescens* have been widely used in ornamental purposes for their attractive characteristics. *C. frutescens* is a herbaceous annual plant that can be found in lowland area and planted at backyard. Their characteristics can be visibly differentiated on their fruits and vegetative characters such as their size, colours and shape. In this study the variation of *C. frutescens* accessions collected from various location in Malaysia were analyzed using their morphological characteristics based on descriptor provided by AVRDC.

Introduction

Capsicum spp. has been reported as the number one important vegetable in Malaysia (Berke, 2002). It is an important ingredient in every dish. There are two species of *Capsicum* cultivated in Malaysia i.e. *Capsicum annum* L. (lada besar) and *Capsicum frutescens* L. (cili api) mainly for food purposes. There are few varieties of *Capsicum* spp. used as an ornamental because of their attractive characteristics (Stommel and Bosland, 2006). *Capsicum frutescens* or hot peppers is the most popular among Malaysians and are commonly used in sauces, soup, stew and also as a flavouring. It is also mixed with other spices in curry making. *C. frutescens* is a herbaceous annual plant that can be found in lowland area and planted mostly at backyard. Their characteristics can be visibly differentiate based on their fruits, plant and leaf due to size, colour and shape. In this study the variation of *C. frutescens* collected from various location in Malaysia were analyzed based on their morphological characteristics using descriptor list provided by International Plant Genetic Resource Institute (IPGRI).

Materials and methods

Chillies plant collected from various location in Negeri Sembilan, Pahang, Perak, Melaka and Kelantan were planted in the field using RCBD with three replication. The accessions were characterized for their morphological characters. The characterization activity was done at Stesen MARDI Jelebu, Negeri Sembilan using a descriptor list provided by IPGRI. All regenerated seeds were collected and conserved at seed gene bank in Stesen MARDI Serdang.

Fig. 1. Variation of fruit colour.

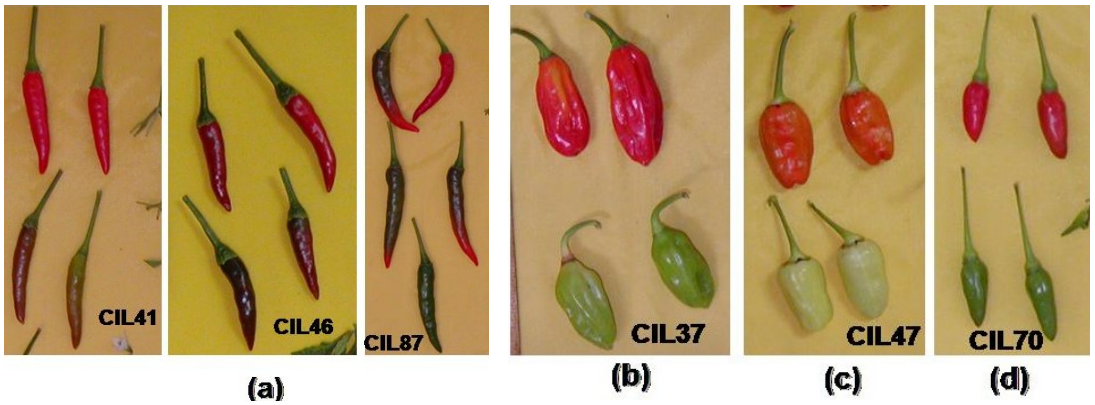

Fig. 2. Variation of fruit shape: (a) elongate, (b) campanulate, (c) blocky and (d) triangular.

Fig. 3. Variation of leaf shape (*from left to right*); CIL31 (deltoid), CIL85 (lanceolate) and CIL54 (ovate).

Results and discussion

A total of 60 accessions were characterized. Analysis of variance revealed a moderate variation for most characters with a Coefficient of Variation (CV) values ranged from 18.3 – 39.4% (Table 1). Leaf length, leaf width, fruit length and fruit width showed wide variation with CV values of more than 20%. While plant height (19.1%) and canopy width (18.3%) showed a moderate variations. There are also some other morphological characteristics that distinct particularly on their fruit shape and colour at different maturity. The fruit colour at immature stage ranges from white, pale orange and pale orange-yellow while at mature stage the colour change to either brown, orange or orange-yellow colour. For accession CIL53, it immature fruit colour is purple. During ripening stage, fruits of all accessions turn to red. Their fruit shape either elongate, campanulate or blocky (Fig. 2). Leaf shape also showed some variation (Fig. 3).

Table 1. Mean, Range and Coefficient of Variation in six Quantitative Characters of *C. frutescens* collections in Malaysia

Characters	Mean	Range	CV(%)
Plant Height (cm)	66.68	115.33 – 43.67	19.1
Canopy Width (cm)	66.61	99.33 – 39.67	18.3
Leaf Length (cm)	7.77	12.33 – 4.0	23.9
Leaf Width (cm)	1.30	7.17 – 1.0	30.2
Fruit Length (cm)	3.22	6.0 – 2.0	30.2
Fruit Width (cm)	0.84	2.23 – 0.53	39.4

Conclusions

Some variations in morphological traits were observed in *Capsicum frutescens* planted in Malaysia. Fruit and vegetative characters can be used as markers to identify a genetic diversity in hot pepper.

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Sarawak's Experience – Documenting Traditional Knowledge of Useful Plants among Sarawak's Indigenous Communities

Tu, C.L., Naming, M. and Jovita, E.R.

Sarawak Biodiversity Centre, KM 20 Jalan Borneo Heights, Semengoh,
Locked Bag No. 3032, 93990 Kuching, Sarawak, Malaysia
chulee@sbc.org.my

Abstract

Under the Sarawak Biodiversity Centre (Amendment) Ordinance 2003, Sarawak Biodiversity Centre was empowered to facilitate the preservation and documentation of Traditional Knowledge (TK) through proper recording or documenting techniques among the local indigenous communities in the State. With the rush for natural products development and bioprospecting from the world's natural resources, it has become even more important to document Traditional Knowledge to ensure that the indigenous communities who have practiced such knowledge over the centuries are duly acknowledged in the event that their knowledge is used and contributed to the development of commercial products or biodiscovery. This paper shares the experience of the Sarawak Biodiversity Centre (SBC) in documenting TK and its outcomes which highlighted the value of TK amongst the indigenous communities in Sarawak.

Introduction

The word of 'Tradition' based on Oxford Dictionary is referring to passing of beliefs or customs from one generation to the next generation by oral or without writing. Traditional knowledge (TK) is the information of the matured long-standing traditions and practices or indigenous communities. Traditional knowledge also includes the wisdom, knowledge and teachings of these communities. In many cases, such knowledge is often passed down through generations orally and seldom put in any formal form of documentation. TK associated with biological resources is an intangible component of the resource itself. TK has the potential of being translated into commercial benefits by providing leads for development of useful products and processes. While many of the older generation in these communities still retain traditional knowledge, there is a concern about the loss of knowledge as a result of changing lifestyles, priorities, the availability of modern amenities and the diminishing dependence of indigenous communities on natural resources.

Sarawak with over 30 different indigenous groups (Chai, 2006) has inherited a rich array of traditional knowledge from their ancestors, much of which has not been documented. Sarawak Biodiversity Centre has taken the initiative to help the local community to document the plant usage and preserve the data in SBC database.

Methodology

The Traditional Knowledge Documentation programme is implemented by the Sarawak Biodiversity Centre (SBC) through the Traditional Knowledge Methodology Journal. This method was developed together with Dr Paul Quek of Bioversity International.

Before starting the Traditional Knowledge Documentation Programme, SBC will contact the Resident Office of a Division or the District Office of a District to arrange for a consultative meeting with the various ethnic community leaders within the area. The ethnic community leaders will be invited to the meeting through the Resident Office or District Office. During the consultative meeting, community leaders are briefed on the purpose and activities of the Traditional Knowledge Documentation Programme.

After the consultative meeting, the community leaders will discuss with their community members on their participation in the programme. Once the project idea is agreeable by the villagers, the community leaders would contact SBC either directly or through the Resident or District Office. SBC will then arrange for a date to conduct the Capacity Building Workshop with the community.

The Capacity Building Workshop is usually held over a period of three days. This involves the training of community participants in documenting their traditional knowledge through recording with a tape recorder, recording through a written form in the field notebook and capturing the images of the useful plants through the use of a digital camera. The workshop also provides guidelines in collecting plant specimens for herbarium, for propagation and as raw materials for R&D. Under the TK Doc project, SBC provides all the necessary equipments and tools for carrying out the documentation of traditional knowledge.

During the documentation process, the following information based on the field notebook is recorded:

- Field Collection No. e.g. SABC 0001
- Location
- Date
- Name of Plant (Local Name)
- Language
- Family
- Scientific Name
- Collector's Name
- Type of Specimen – Propagation/Herbarium/R&D
- Habitat
- Habit of Plant
- Characteristic of Plant
  - Colour of fruit
  - Colour of flower
  - Colour of latex
- Uses
- Parts Use
- Method of Preparation
- GPS

Information recorded in the field is verified during the Plant Review Session which is conducted in the evenings and involves the men, women and children. During this session, spelling of the plant local name, its uses and method of preparation is recorded in detail. This is an important session as there is a lot of interaction among the communities and learning of plant uses among the communities are in progress especially between the younger generation and the older generation.

As a follow-up to the Capacity Building Workshop, SBC will conduct follow-up field work among the participating communities. During the field work, SBC will collect and document new plants or collect additional amount of plants with the community. SBC will also collect fertile collection for herbarium specimens where possible.

Besides conducting follow-up field work, SBC will also conduct annual/biannual seminar on traditional knowledge documentation. Representatives from the various participating communities will be invited to participate in this seminar. SBC will also organize workshop such as handmade soap workshop, landscape and plant maintenance workshop which involves community participants. Fig. 1 summarizes the Journal Methodology used in carrying out the TK Documentation Project.

Results and discussion

During the Traditional Knowledge Documentation Programme, SBC has conducted Consultative Meetings in 32 locations throughout Sarawak and started 48 collection and documentation of useful plants among eight (8) ethnic communities in 20 locations. The result of our study shows that a total of 2122 plants were documented from these locations. The locations of where SBC has conduct Consultative Meetings and started collection and documentation of useful plants are shown in Fig. 2. Among these, the Bidayuh community from Kampung Kiding recorded the most plants (304 plants), followed by Penan community at Long Iman (260 plants)(Fig. 3).

Among the various ethnic communities, the Bidayuh community recorded the highest plant usage in Sarawak with 551 plants and the Penan community documented the second highest number of useful plants with 461 (Fig. 4). The high number of plants documented among the Bidayuh and Penan could be due to the frequency in collection as compared to the other locations.

The various uses of plants are recorded in Fig. 5 where the highest no. of plants documented is for medicine (46.4%). This is because when SBC started the Traditional Knowledge Documentation Programme, the documentation was focused on medicinal plants.

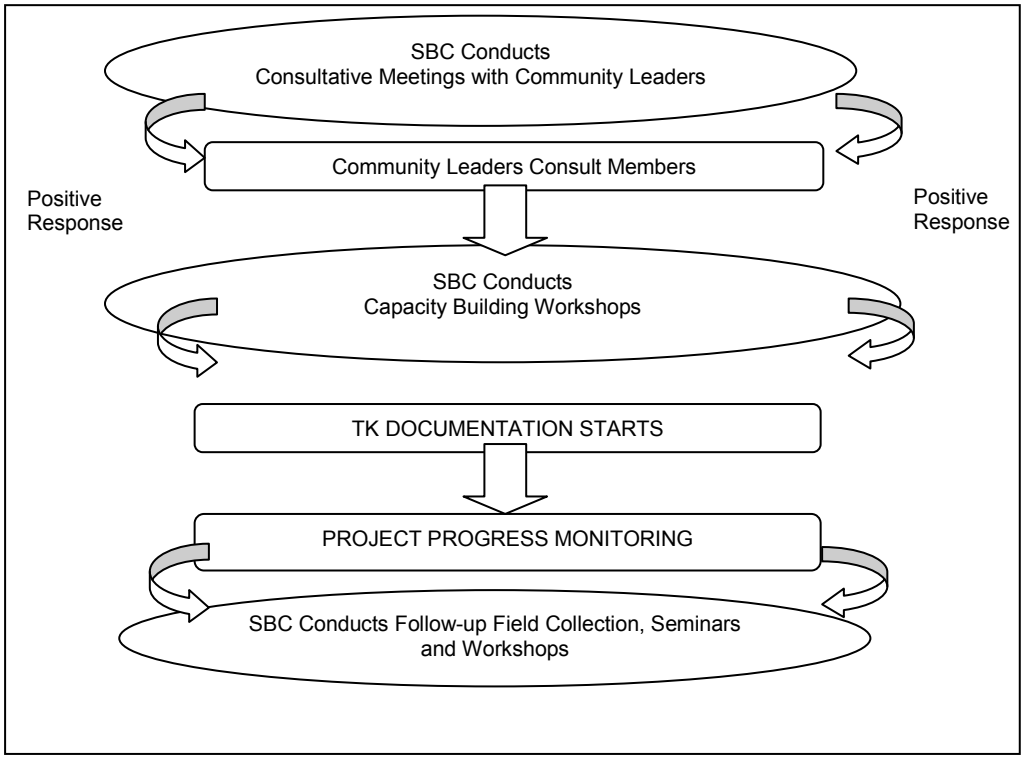

Fig. 1. Journal Methodology used in carrying out the TK Documentation Project.

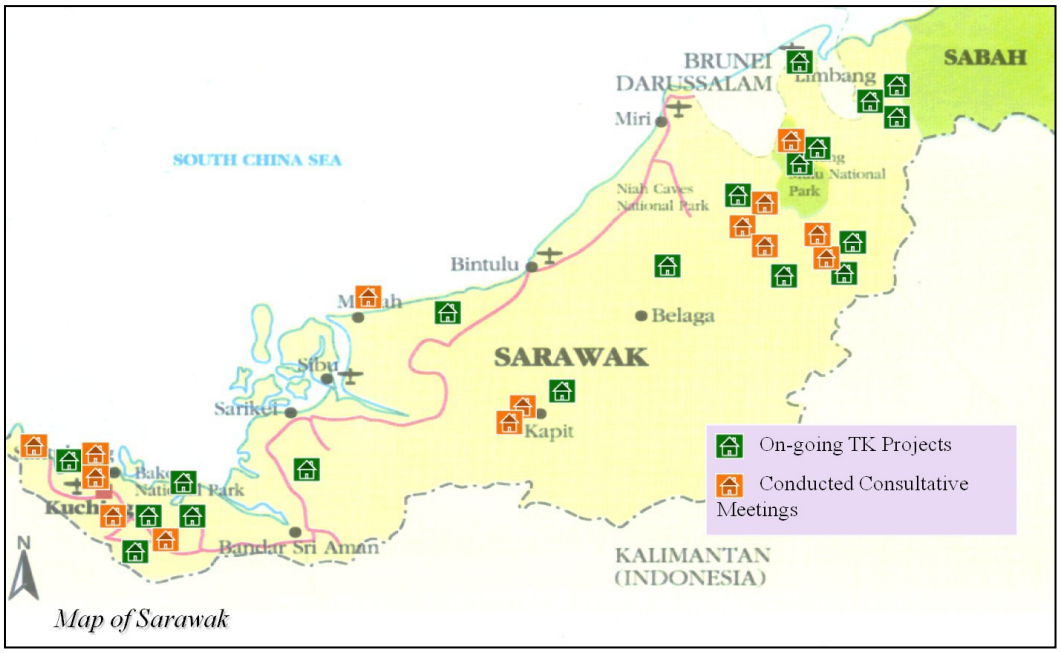

Fig. 2. The locations of where SBC has conduct Consultative Meetings and started collection and documentation of useful plants.

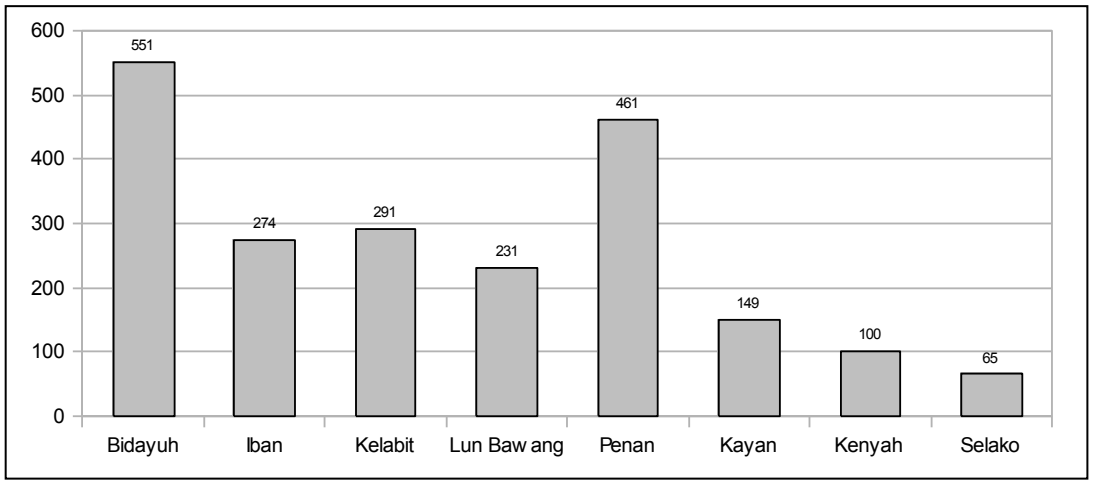

Fig. 3. Plant usage among the different ethnic communities in SBC's TK Documentation Project.

COMMUNITY LOCATION	NO OF PLANTS
Bidayuh Kpg Semadang	165
Bidayuh Kpg Kiding	304
Bidayuh Kpg Semban	65
Bidayuh Kawasan Serin	17
Iban Rh Lulut, Kapit	55
Iban Rh Skatap, Betong	166
Iban Rh Nyambong, Selangau	53
Kelabit Pa Ukat	108
Kelabit Pa Lungan	171
Kelabit Pa Derung	12
Lun Bawang Long Telingan	92
Lun Bawang Long Kerebangan	139
Penan Long Latei	6
Penan Long Seridan	8
Penan Batu Bungan, Mulu	187
Penan Long Iman, Mulu	260
Kayan Long Bedian, Baram	68
Kayan Sg Asap	81
Kenyah Lg Pelutan	100
Selako Kpg Pueh	65
TOTAL	**2122**

Fig. 4. No. of plants collected according to community locations in SBC's TK Documentation Project.

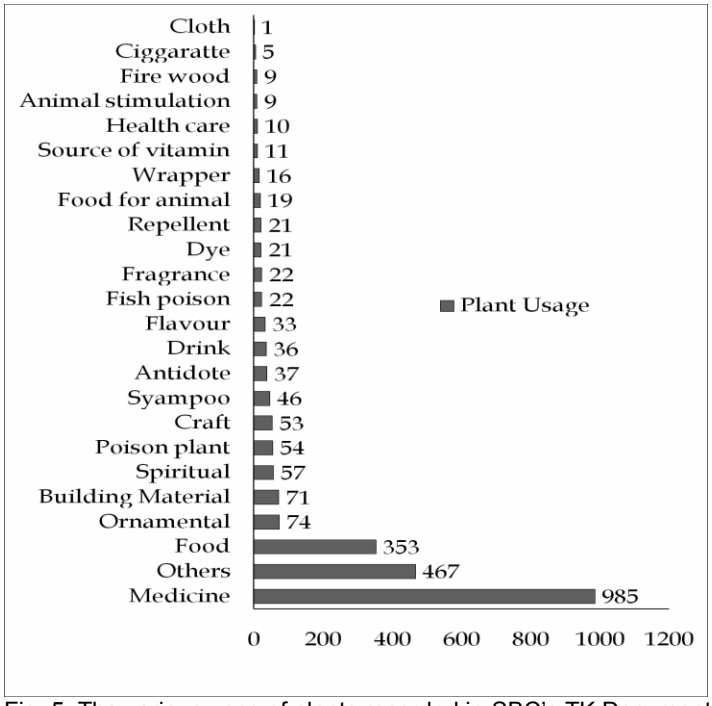

Fig. 5. The various uses of plants recorded in SBC's TK Documentation Project.

Conclusions

SBC's TK Documentation project methodology was workable and well received by ethnic communities in Sarawak. Plant information are documented and compiled into a database. The database was kept at SBC. *Ex-situ* conservation of living useful plants is to ensure useful plant can be sustained for future generation. Present TK Committee in the villages can be used to document other TKs such as folklore, rituals and handicraft. Community gained new knowledge and benefit from interactions with other communities, SBC, political leaders, visiting scientists and through participations in Workshops/ Seminars/ Conferences. Community can develop ecotourism projects and small cottage industries from TK activities. SBC's R&D can assist to identify viable projects for product development such as personal care product and health supplements. SBC's R&D also aims for bio-prospecting (drug discovery) from plant material in a long run finding.

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Some Observations on the Morphological Characteristics of *Labisia pumila* (Kacip Fatimah) at Pasoh Forest Reserve, Negeri Sembilan, Malaysia

Tan, A.L.[1], Sum, S.P.[2] and Chang, Y.S.[1]

[1]Medicinal Plants Programme, Forest Research Institute Malaysia (FRIM),
52109 Kepong, Selangor, Malaysia
[2]Faculty of Science and Technology, Universiti Malaysia Terengganu (UMT),
21030 Kuala Terengganu, Terengganu, Malaysia
tanal@frim.gov.my

Abstract

Labisia pumila, locally known as *kacip fatimah*, is a popular herb used in traditional Malay medicine for women. At least four varieties of *L. pumila* are found in Malaysia and all of them are called *kacip fatimah*. Plant characterisation will help to establish the range of variation in a taxon. A study with the aim to establish the range of variation within each of the varieties of *L. pumila* was carried out in Pasoh Forest Reserve, Negeri Sembilan, Peninsular Malaysia. Two sites, namely, the Nature Trail and Arboretum, were selected for this study. Selected samples of all varieties of *L. pumila* encountered were collected and the leaf morphology examined. The distribution of *L. pumila* varieties in the Arboretum and along about 2 km of Nature Trail was also studied. From the results, it was found that three varieties of *L. pumila* occurred at the Pasoh Forest Reserve, namely, *L. pumila* var. *pumila*, *L. pumila* var. *alata* and *L. pumila* var. *lanceolata*. In accordance to the descriptions, the main character that differentiated the three varieties was the petiole. The marginate wing on the petiole delimited *L. pumila* var. *pumila* from *L. pumila* var. *alata* with broad wing. The terete petiole (no wing) of *L. pumila* var. *lanceolata* easily separated it from the rest. *Labisia pumila* var. *alata* was most dominant, occupying about 61% of the *L. pumila* population while *L. pumila* var. *lanceolata* was relatively rare consisting of only 3% at the study sites. It was also noted that *L. pumila* was a shade tolerant plant found in heavily to partially shaded areas with a preference to high humidity and non-water logged area.

Introduction

Labisia pumila (Synonym: *L. pothoina*), a family member of Myrsinaceae is a herbaceous plant that commonly grow in shady lowland primary forest on humus-rich soils. *L. pumila* or locally known as Kacip Fatimah have been widely used among Malay women in Malaysia as a remedy before childbirth to expedite labour and after delivery to regain strength. Besides that, *L. pumila* have also been used in the treatment of dysmenorrhoea, gonorrhoea and venereal diseases (Burkill, 1935). Sunarno (2005) on his latest revision of the genus *Labisia* recognised 8 varieties of *L. pumila* seven in Indonesia and one, *L. pumila* var. *gladiata* from North Borneo. Out of the 8 varieties, only 4 had been recorded in Peninsular Malaysia, namely *L. pumila* var. *pumila*, *L. pumila* var. *alata*, *L. pumila* var. *lanceolata* and the newly described *L. pumila* var. *malintangensis* (Sunarno, 2005). *L. pumila* var. *malintangensis* is very much alike var. *alata*, but differs in the broader wings, 8-12 mm of the petioles and the shape and size of the flower parts. The study aims to determine the variety of *L. pumila* occurring at Pasoh Forest Reserve and to find good characters that could be used to differentiate these varieties. It also aims to find the most abundance variety at the study sites.

Materials and methods

Two study sites, namely, the Nature Trail and Arboretum at Pasoh Forest Reserve, Negeri Sembilan were selected. Samples of all varieties of *L. pumila* encountered were collected and their morphological characters examined. In addition, the abundance of *L. pumila* varieties was also studied for a stretch of 2 km of Nature Trail and Arboretum. Data obtained were compiled and analyzed.

Results and discussion

Three varieties of *L. pumila* occurred in Pasoh Forest Reserve, namely *L. pumila* var. *pumila*, *L. pumila* var. *alata* and *L. pumila* var. *lanceolata*. Seventeen samples were collected and their morphology were examined. Generally, *L. pumila* in Pasoh Forest Reserve were small, erect or creeping undershrub with brown to reddish brown roots. They had rounded, brown (dark) to brownish green stem with dark brown basal. Stems often marked with horseshoe's scar from fallen leaves. Meanwhile, *L. pumila* had simple leaf that was alternately arrange. The leaves vary in shapes from elliptic to lanceolate or ovate to elliptic, usually grass green with entire to subserrate or serrate margin (rarely subdentate). Leaf apex of *L. pumila* was acute to acuminate while leaf base was cuneate or acute. In addition, the texture of the leaf was leathery to subcoriaceous.

Apart from that, *L. pumila* had reticulodromus veins. Both surfaces of leaf covered densely with brown peltate scales on the midrib but lesser towards the margin. Furthermore, *L. pumila* had petioles that range from short to long with various colourizations; i.e. reddish-brown to reddish-green or brownish-green to green, often with brown to dark brown basal and evenly distributed brown peltate scales. *Labisia pumila* had axillary elongated inflorescence or infructescence with the occurrence of 2 bracts and brownish red or reddish brown racemes. The post-flowering state of *L. pumila* had subglobose ovary that was reddish brown attached with subulate style. Meanwhile, flower of *L. pumila* had 5 connate sepals and petals with epipetalous stamens enclosed by petals and basifixed anthers. The flower characters are typical for the genus of *Labisia* that distinguish it from others in Myrisinaceae (Sunarno, 2005). In addition, the fruits were baccate (berry-like), globose, green with brown stripes when young and turning red with brown stripes when ripen. Each fruit contained one globose seed.

The petiole characteristics play an important role in delimiting the three varieties of *L. pumila* occurring in Pasoh Forest Reserve. Table 1 and Fig. 1 highlight the characters that differentiate the three varieties.

L. pumila var. *pumila* can be easily recognised by having marginate wing petiole with protective flaps at basal and its wing was rather stiff and not flexible compared to *L. pumila* var. *alata*. On the other hand, *L. pumila* var. *alata* had broader wing petiole that was softer and flexible to bend. Meanwhile, *L. pumila* var. *lanceolata* can be distinguished from the others by having terete petiole without any wing. Several leaves from the same plant have to be examined before making the finalization for the varieties, as some new leaves may be misleading. In addition, the wing of *L. pumila* var. *alata* also were in many forms that differ from petiole wing of equal size all the way to the basal and those that becoming marginate towards basal.

Table 1. Diagnostic characters that delimit the 3 varieties of *L. pumila*

Character	**Type of**		
	var *pumila*	**var *alata***	**var *lanceolata***
Height (cm)	16.0 – 45.0	20.0 – 45.0	32.0
Leaf index (total leaf number)	5 – 18	8 – 12	7
Leaf length (cm)	17.9 – 31.0	16.4 – 27.0	14.0
Leaf width (cm)	4.9 – 7.2	4.3 – 7.3	3.0
Petiole length (cm)	2.2 – 8.1	1.0 – 5.9	5
Petiole wing width (mm)	0.9 – 3.0	1.0 – 3.6	-
Type of petiole	Marginate wing with protective flaps at basal	Broad wing	Terete without wing
Flexibility of wings	Stiff, not flexible	Soft, flexible to bend	-

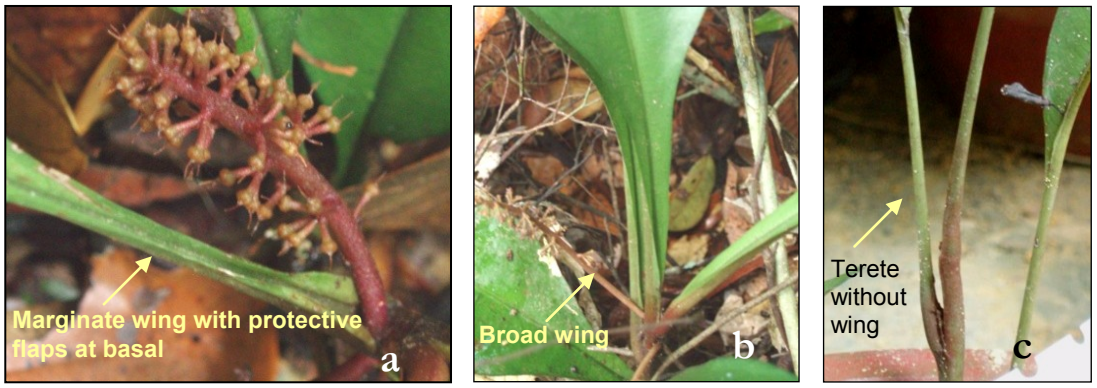

Fig. 1. Petiole characteristics that distinguish the varieties of *L. pumila*. a) *L. pumila* var. *pumila*, b) *L. pumila* var. *alata* and c) *L. pumila* var. *lanceolata.*

These findings are in accordance to the descriptions from Ridley (1923), Stone (1988) and Sunarno (2005). However, there is a slight difference for the range of wing width of *L. pumila* var. *alata* noted by Sunarno (2005); 3-5 mm which are wider than the one recorded in Pasoh. In addition, the shape and size of the flowering parts that are used as diagnostic characters by Sunarno (2005) are not used here as most of the plants in Pasoh during the study period were in the post-flowering state.

From a total of 99 individuals observed and recorded along 2 km stretch of Nature Trail and Arboretum, it was found that *Labisia pumila* var. *alata* was most dominant (61%) while *L. pumila* var. *lanceolata* was relatively rare (3%) (Table 2). On the other hand, *L. pumila* var. *pumila* occupied 26% of the population. The remaining 10% were the amount of unrecognised seedlings, as the petiole characteristics had not formed to enable the determination of variety it belongs to.

Table 2. Abundance of *L. pumila* varieties at Nature Trail (~2 km) and Arboretum

Variety	**Nature Trail**	**Arboretum**
L.pumila var. *alata*	26	34
L.pumila var. *pumila*	21	5
L.pumila var. *lanceolata*	1	2
Unrecognised seedling	9	1

From the survey performed within the study sites, it was observed that young seedling of *L. pumila* had magenta to dark green leaves with crenate to dentate margin and were often compact (Fig. 2). However, the leaves change colour into green, the stem will start to elongate and the petiole will start to form accordingly to the varieties when it approached the mature state. Besides that, it was also noted that *L. pumila* often grows in small clusters and there can be a mixture of varieties among the clusters (Fig. 2). Stone (1998) also noted the similar situation. It was also noted that *L. pumila* was a shade tolerant plant found in heavily to partially shaded areas with a preference to high humidity and non-water logged area. The allied plants of *L. pumila* seen on study sites are palms such as 'bertam', rattan and fan palms apart from *Molineria latifolia* and ferns.

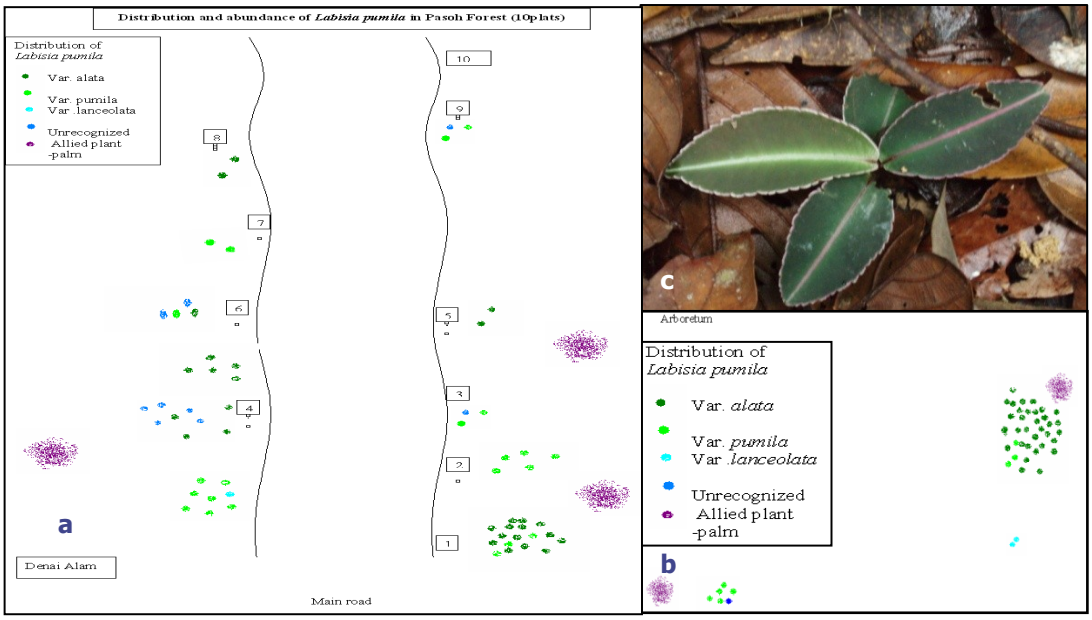

Fig. 2. Distribution of *L. pumila* varieties at a) Nature Trail and b) Arboretum; c) Seedling of *L. pumila.*

Conclusions

In conclusion, petiole characteristics are very useful in delimiting the varieties of *L. pumila* and they are useful field characters. Three varieties of *L. pumila* occurred in Pasoh Forest Reserve, namely, *L. pumila* var. *pumila*, *L. pumila* var. *alata* and *L. pumila* var. *lanceolata*. The most dominant variety at the study sites is *L. pumila* var. *alata*.

Acknowledgements

The authors wish to thank all the staff in Pasoh Forest Reserve and to all that has contributed in this study.

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Sources of Powdery Mildew Resistance in Pea (*Pisum sativum* L.*)* Varieties in Sabah

Poili, E. and Atong, M.

School of Science and Technology, Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia
evenni.poili@sabah.gov.my

Abstract

Powdery mildew is a serious disease in pea cultivation. It is essential to plant resistant varieties in a commercial scale to produce pesticide-free vegetable. The trial evaluated seven pea varieties for resistance to powdery mildew in a randomized complete block design replicated four times. The plants were exposed to the disease under natural field infection and bombardment from infector rows. The experiment found one variety highly tolerant to the disease with zero infection under both disease exposures. The rest of the varieties were susceptible to powdery mildew infections with a DSI of between 79% and 98%. The agronomic performance and characteristics of the resistant variety were presented and discussed.

Introduction

Pea (*Pisum sativum* L.) is a leguminous plant that belongs to the family Pea (*Pisum sativum* L.) is a leguminous plant that belongs to the family Papilionaceae. This plant is used as vegetable such as the green peas (fresh, frozen, preserved) and mature peas (cooked for soup, broth) and also as forage crop, for hay, pasturage or silage. Powdery mildew caused by *Erysiphe pisi* (syn. *E. polygoni*) (CABI CPC, 2002) is one of the serious diseases affecting the pea cropping. It infects the leaves, stems and pods and easily recognized by the formation of white dust or powder on the infected plants. Powdery mildews or Erysiphaceae are fungi with white superficial hyphae on the aerial parts of living plants, with large one-celled conidia produced terminally on the cells of their hosts (Yarwood, 1978). Other than the leaves, the stems and pods may be infected causing death of vine, withering of foliage and occasional plant death (Dixon, 1978). Rathi and Tripathi (1994) showed that the disease caused significant reduction in plant growth and yield parameters in both artificial and natural conditions. A maximum of 31.1% and 28.6% reduction in the number of pods per plant was recorded at 82.8% disease intensity under artificial condition and 100% disease intensity under natural condition respectively. The powdery mildews not only common it also cause serious diseases in cool and warm, humid areas and in warm, dry climate. Prolonged warm, dry daytime conditions and nights that are cool enough for dew formation make powdery mildew difficult to control (Hagedorn, 1973). This happens because their spores can be released, germinate, and cause infection even when there is no film of water on the plant surfaces as long as the relative humidity in the air is fairly high. Once infection has begun, the mycelium continues to spread on the plant surface regardless of the moisture conditions in the atmosphere.

Chemical application is the most preferred method of controlling the powdery mildews (McGrath, 2007; Janousek *et al*., 2006; Gupta and Sharma, 2005; Gammon et al., 2001; Olsen *et al*. 2001; Hansen, 2000; Matheron and Porchas 1999; Chase 1999) as it is practical and easy to use. Systemic fungicides such as ethirimol, triadimenol and triflorine are used as seed treatments and prochloraz, triadimefon, tridemorph and triflorine and others used as foliar sprays though elemental sulphur (Williams and Cooper 2004, Gammon et al., 2001) and dinocap (Agrios, 1997) have been and still are used extensively and effectively. However, chemical residual toxicity and the high cost involved made this method less attractive in a long run. Furthermore development of fungicide resistance and consequent control failure is always a concern with systemic fungicides. Strains of powdery mildew fungus resistant or insensitive to such fungicide have been found in vegetable crops (McGrath, 2001).

In Sabah, the inappropriate use of pesticide caused great concern for the environment and food safety and resulted in adverse economic impact of the pea crop production. Samplings of pea in Kundasang in 1993-1997 for fungicide residues revealed that the crop was tainted with ethylene *bis*-dithio-carbamate (EBDC) above the maximum residual limit (MRL) (Department of Agriculture Sabah,

1996 and 1997). Consequently Brunei and Sarawak banned the import of this vegetable from Sabah (Jipanin *et al*., 2000 and 2001). As a result Sabah export of peas fell from 42.26 t in 1995 (Department of Agriculture Sabah, 1998) to 13.95 t in 2002 (Department of Agriculture Sabah, 2004).

Good agriculture practices and crop rotation have little effect on powdery mildew incidence and development even though Sharma (1992) stated that healthy, vigorous leaves and stems (less lush pea stand) are less prone to infection. In many countries powdery mildews are economically impractical to control in other ways; the use of resistant varieties provides a means of producing acceptable yields without pesticides. Growing pea varieties with resistance to the disease is the least expensive, safest, and one of the most effective means of controlling the disease (Thakur *et al*,1996; Dang *et al* ,1994 and Kuo, 1998) and recommended during periods of high powdery mildew incidence. Resistance is expressed as the ability to survive high level of inoculums in environment favourable for the disease. Resistant varieties could be obtained through selection of the host species from wide genetic resources. Thus field experiments were conducted to evaluate pea varieties for resistance to powdery mildew. This paper presents the results of the field trials carried out.

Materials and methods

The trials were carried out at the Department of Agriculture (DOA) Research Station, Mesilou, Kundasang situated at 6^{o} 00' 17" N, 116^{o} 36' 12" E and 1700 m above sea level. The experimental area was on a flat ground of the station. The field was rotovated to give a fine tilt then planting beds were constructed at a size of 0.5 m x 3.0 m. Organic manure was incorporated in the soil at a rate of 5-10 t/ha. The fertilizer rates used were 90 kg N, 100 kg P_2O_5 and 120 kg K_2O. Half rate of the N, P_2O_5 and K_2O were applied as basal dressing; the other half rate of the N was applied twice as top dressing (22.5 kg/ha) at two weeks interval while the P_2O_5 (50 kg/ha) and K_2O (60 kg/ha) at flowering stage. Insect pests were controlled using Cypermethrin and *Bacillus thuringiensis* at weekly interval commencing at 14 days after planting (DAS) to flowering stage. Subsequently sticky yellow traps were erected based on the plants height at every 3.8 m distance to control the leaf miner. Diseases such as damping off, root and/or collar rot were controlled by soil drenching with Thiram or Benlate a week before and after planting. Pea varieties were planted in a row at 30 cm distance. Wooden poles were erected into the soil and raffia strings tied across to give support to the pea vine, which could climb to 1-2 m high. Axillary shoots were pruned or picked to avoid bushy growth of the vine.

Pea varieties coded as S1, S2, S3, S4, S5, S6, and S7 obtained from the local vegetable seed suppliers were evaluated for powdery mildew infection in three sets of experiment conducted in 2004 and 2005. The Randomised Complete Block Design (RCBD) with four replications of 10 plants per plot was used in the experiment. The pea varieties were exposed to powdery mildew under natural field infection (Experiment 1) and bombardment from infector plants established prior to sowing of the test plants (Experiment 2 and Experiment 3). This is to observe the responses of the pea varieties to infection of the powdery mildews under different inoculums exposures. All test varieties were planted together in each trial plot in Experiment 1 whereas in Experiment 2 and Experiment 3 the varieties were split into two groups due to limited space as part of the area used to establish the infector plants. Variety S7 was used in both Experiment 2 and Experiment 3 as a check variety based on its performance in Experiment 1. This variety was also reported to have high tolerant to powdery mildew under field infection in Kundasang environment (Department of Agriculture Sabah, 2001, 2002 and 2003).

Infection of powdery mildew was recorded at the first sign of the disease symptom using a 0-9 disease severity scale adopted from Singh (1991) as cited by Dang *et al*. (1994). Recordings were stopped when the scale reached 9. Disease severity index (DSI) was calculated as the sum of severity scores of the 10 plants evaluated divided by the maximum severity score x 100.

$$DSI = \frac{\sum \text{sum of severity score}}{\text{No.of plant evaluated x max. severity score}} \times 100$$

Meanwhile the monthly temperature (^{o}C), relative humidity (%RH) and rainfall (mm) at the Station in the duration of the experiments were obtained to relate the effect of weather to the powdery mildew infection on the pea plants.

The agronomic performance and plant parameters of the resistant variety were determined by field planting on a 55.44 m^2 plot using a planting distance of 7.5cm (3") which is the normal practice of the pea growers in Kundasang and replicated 3 times. The plant parameters were recorded based on number of leaf branch, number of days to flower, colour of the flower, pod size in length (cm) and width (cm) and weight (g), number of pods per plant and yield estimate for documentation. Insect pests were controlled using Cypermethrin and *Bacillus thuringiensis* at weekly interval commencing at 14 days after planting (DAS) to flowering stage. Subsequently sticky yellow traps were erected based on the plants height at every 3.8 m distance to control the leaf miner. Diseases such as damping off, root and/or collar rot were controlled by soil drenching with Thiram or Benlate a week before and after planting.

Results and discussion

The weather during the course of the experiments is shown in Figure 1 and Figure 2. The monthly temperature, RH and total rainfall in 2004 and 2005 are ranged from 18.7^{o}C to 20.7^{o}C, 65.3% to 87.0%, 58.6 mm to 374.6 mm and 18.9^{o}C to 20.7^{o}C, 69.3% to 81.9%, 4.8 mm to 141.6 mm respectively. There were not much difference in the temperature and RH during the experiment periods in both years but the rainfalls were. More rainfalls were recorded in Experiment 1 followed by Experiment 3 and the least rainfall in Experiment 2. The graphs showed that more rainfalls occurred in the second half of the year. This may explained the disease occurrences that were much earlier in Experiment 1 and Experiment 3 which were 40 DAS and 35 DAS respectively compared to Experiment 2 at 50 DAS. The powdery mildew can caused similar infection on the susceptible varieties be it under natural field infection or inoculums bombardment as showed by the results of the experiments in Table 1, Table 2 and Table 3. Variety S7 was consistently resistant to the disease with zero infection. The rest of the varieties were susceptible with a DSI of between 75% and 98%. There was no significant difference among all the susceptible varieties with regards to powdery mildew infections in most of the plant growth periods for all experiments. The DSI for each of the variety under inoculums bombardment is at 86.77%, 98.33%, 90.80%, 96.30%, 79.45%, 98.15% and 0% for S1, S2, S3, S4, S5, S6 and S7 respectively whereas under natural field infection is at 82.22%, 86.83%, 75.25%, 78.89%, 87.26%, 80.56% and 0% respectively. The fact that S7 is not infected in all of the experiments confirmed its resistance to powdery mildew infection. This plant flowers at about 41 DAS and has flat pod, 8.92 cm in length and 2.36 cm in width with one pod per fruit stalk weighing 4.76 g each. A total of 27 pods were produced per cropping and the plant could grow to a height of 1.5–1.8 m. The total estimated yield of this variety is about 7.2 tha^{-1}. Table 4 showed the plant parameters and the extrapolated yield of this variety. The distinctive character of this variety was the purple pink colour on the flower and on the base of the stipule. Yarnell (1962) reported that the purple flower colour was linked to the tolerance factor of pinkish-purple sweet pea to powdery mildew.

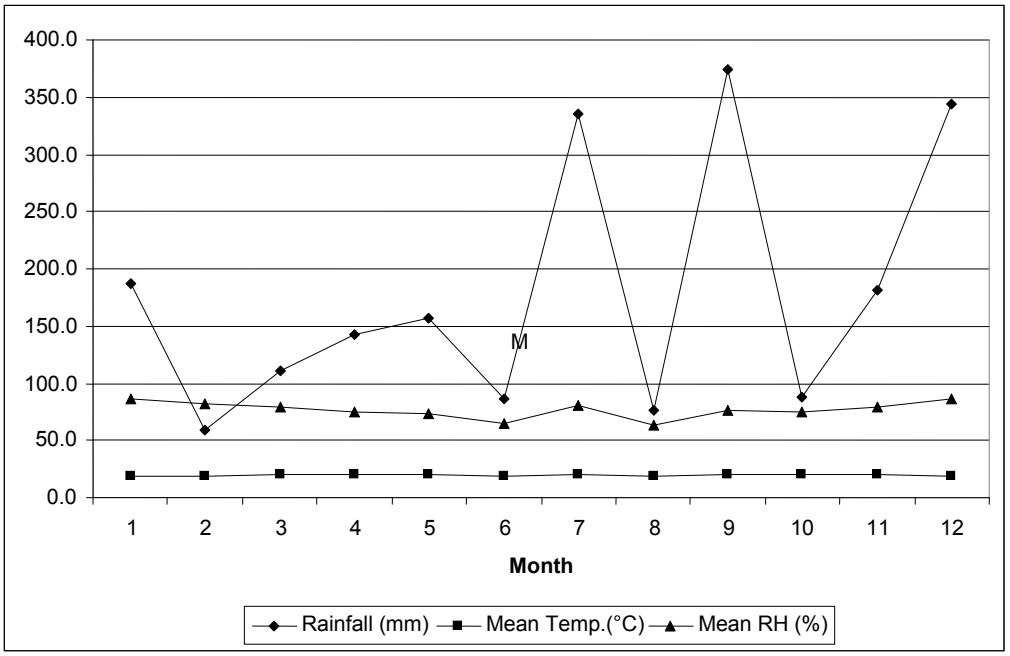

Fig. 1. Mean Temperature (°C), RH (%) and total rainfall (mm) in 2004 at Department of Agriculture Research Station, Mesilou

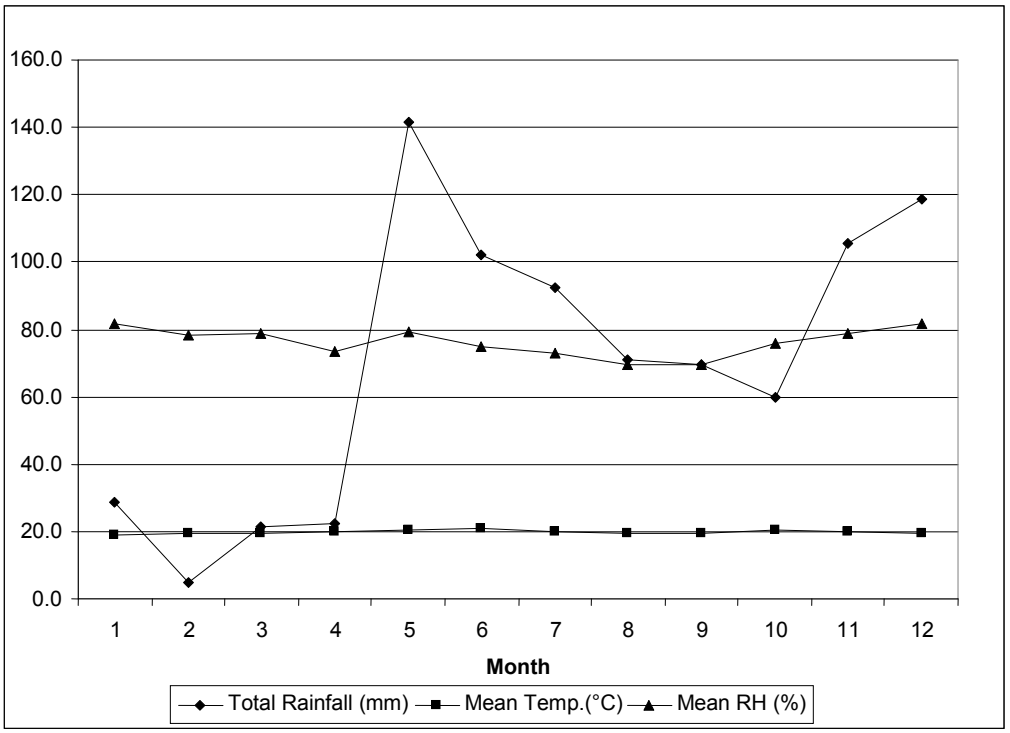

Fig. 2. Mean temperature (°C), RH (%) and total rainfall (mm) in 2005 at Department of Agriculture Research Station, Mesilou.

Table 1. Powdery Mildew Infection (DSI%) on Pea Varieties in Experiment 1 (6 July – 23 September 2004) at Department of Agriculture Research Station, Mesilou

Variety	Days After Sowing										
	40	**44**	**47**	**50**	**57**	**61**	**64**	**68**	**72**	**75**	**78**
SP001	11.11	11.11	21.48	31.25	33.33 b	65.95	74.90 a	79.22	62.74	79.63	82.22
SP002	11.11	11.11	33.33	33.33	42.66 a	75.56	77.78 a	86.89	68.58	80.79	86.83
SP003	8.33	11.11	28.70	33.33	33.33 b	58.89	60.78 b	74.99	62.90	69.57	75.25
SP004	11.11	12.03	23.75	33.33	33.33 b	62.28	76.38 a	77.78	63.46	69.42	78.89
SP005	8.33	5.56	15.52	33.33	33.33 b	60.56	77.16 a	82.27	68.94	82.49	87.26
SP006	11.11	11.11	26.70	33.33	34.37 b	70.45	71.03 a	77.78	72.57	77.78	80.56
SP007	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CV(%)	27.81	22.83	24.71	3.72	8.31	12.29	10.26	8.97	15.01	18.04	9.29
s.e	0.39	0.32	0.54	0.54	1.30	3.61	3.35	3.20	4.49	6.18	3.40
(at d.f)	15	15	15	15	15	15	15	15	15	15	14
F Test	ns	ns	ns	ns	**	ns	*	ns	ns	ns	ns

Note:
Figures in the same column having the same alphabet are not significantly different based on the Duncan Multiple Range Test
(DMRT); SP007 with zero value was not included in the analysis; **- significantly different at 1% level; *- significantly different
at 5% level; ns – not significant

Table 2. Powdery Mildew Infection (DSI%) on Pea Varieties in Experiment 1 (16 January – 18 April 2005) at Department of Agriculture Research Station, Mesilou

Variety	Days After Sowing							
	50	**54**	**57**	**61**	**64**	**68**	**71**	**75**
S1	31.25	55.76	73.34	77.77	75.83	82.36	85.58	86.77
S2	31.66	63.89	76.66	76.77	77.77	84.97	91.29	98.33
S3	32.08	59.31	73.64	76.06	78.33	78.00	82.22	90.80
S7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CV	13.17	18.14	6.08	2.32	5.49	7.72	8.52	13.18
s.e (at d.f=11)	2.95	7.65	3.21	1.03	3.00	4.50	5.21	8.57
F Test	ns	ns	ns	ns	ns	ns	ns	ns

Note:

S7 with zero value was not included in the analysis; ns – not significant

Table 3. Powdery Mildew Infection (DSI%) on Pea Varieties in Experiment 2 (3 August – 10 October 2005) at Department of Agriculture Research Station, Mesilou

Variety	Days After Sowing								
	35	**39**	**42**	**46**	**49**	**53**	**56**	**60**	**67**
S4	16.41	33.93	33.90	55.50	47.26	55.60	58.20	72.69 a	96.30
S5	16.68	33.33	32.48	55.03	55.60	55.60	55.60	59.77 b	79.45
S6	15.56	33.30	34.43	54.43	55.60	55.60	58.75	74.25 a	98.15
S7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CV	40.64	5.97	5.97	6.34	2.89	0.00	4.79	9.20	10.56
s.e (at d.f=11)	4.66	1.42	1.42	1.51	0.92	0.00	1.95	4.48	6.82
F Test	ns	ns	ns	ns	ns		ns	**	ns

Note:
Figures in the same column having the same alphabet are not significantly different based on the Duncan Multiple Range Test (DMRT); SP007 with zero value was not included in the analysis; **-highly significant ; ns – not significant

Table 4. Characters and Yield Component of Resistant Pea Variety (S7)

	Rep 1	**Rep 2**	**Rep 3**	**Mean**
Pod harvest (Kg/ha)	7198.11	7641.51	6685.54	7175.05
No. of pod/pod stalk[a]	1	1	1	1
No. of pod/plant[a]	26.20	27.40	27.00	26.87
Single pod weight (gm)[b]	5.18	4.24	4.85	4.76
Single pod width (cm)[b]	2.05	2.34	2.69	2.36
Single pod length (cm)[b]	8.89	9.18	8.68	8.92
Pod character	Flat	Flat	Flat	
Days of flower	41	41	41	41
Flower colour	Purple-pink	Purple-pink	Purple-pink	
Distinctive feature	Purple colour at the base of the petiole			
Plant height	1.5-1.8 m			

a= means of 10 plants; b=means of 30 pods

Conclusion

The powdery mildew resistant variety (S7) identified in the trial can be used for planting in Kundasang to overcome the problem caused by the disease. Planting of this accession together with the adoption of insect pest control techniques could produce an IPM package for pea cultivation in Sabah. S7 is easily distinguishable from other pea accessions as it has distinctive pink-purple colour of the flower and the base of the stipule. When proper crop management is practiced this accession could produce a high yield.

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Comparative Distribution and Abundance of Bats in Selected Localities in Sarawak

Jayaraj, V.K.[1,2], Ketol, B.[2], Marni, W.[2], Sait, I.[2], Anwarali, F.A.A.K.[2,3], Fong, P.H.[1,2], Abdullah, M.T.[2]

[1]UCSI University, No. 1, Jalan Menara Gading, UCSI Heights,56000 Kuala Lumpur, Malaysia
[2]Department of Zoology, Faculty of Resource Science and Technology,
Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia
[3]Department of Biological Sciences, Texas Tech University,
Main and Flint, Lubbock, TX 79409-3131, USA
jayaraj_vijayakumaran@yahoo.com

Abstract

Borneo is the third largest island in the world and holds a big variety of flora and fauna including bats. The diversity of bats in this island has been progressively documented by several authors but most of these data are outdated thus prompting the attempt to document current and previous chiropteran diversity in Sarawak using mist-nets and harp traps and cross reference check on previous literature available. The results shows that protected areas in Sarawak are rich and diverse in terms of chiropteran diversity, and submontane areas that are unexplored previously do hold distinct chiroptera composition. Severely logged areas do pose a threat to the overall diversity of bats, with generalist are more viable to survive than those with specialized habitat requirements. Overall the vegetation in terms of forest types, elevation and level of disturbance is a factor determining the diversity of bats in a particular area.

Introduction

Borneo has a very diverse chiropteran diversity. Payne *et al.* (1985) reported that there are 94 species from eight families of chiroptera in Borneo. The knowledge on Bornean chiropteran diversity has been reported by various authors (Hall *et al.,* 2004). Davis (1958), Medway (nd ; 1958 ; 1978), Lim (1965), Lim *et al.* (1972), Francis *et al.* (1984), Francis (1989; 1990; 1994) and Hall (1996) pioneering studies have provided an early understanding of the chiropteran diversity in Borneo. Subsequent studies by Start (1975), Nor (1996; 1997), Tuen *et al.* (2002a; 2002b) and Mohd. Azlan *et al.* (2003) have enhanced our knowledge on bat diversity and distribution. Recent surveys in Sarawak by Abdullah and Hall (1997), Abdullah *et al.* (1997a; 1997b), Abdullah *et al*. (2000), Hall *et al*. (2002; 2004), Abdullah (2003), Abdullah *et al*. (2003), Karim *et al*. (2004), Tuen *et al*. (2004), Abdullah *et al*. (2005), Mohd. Azlan *et al.* (2005), Jayaraj *et al*. (2005), Struebig and Suyanto (2005) and Anwarali *et al*. (2006a) have recorded many new bat distributions in Sarawak. Although the chiropteran diversity in various localities in Borneo have been studied, there is still a need to document and update new information on bats in Borneo particularly Sarawak. Thus the aim of this study is to document bat diversity and its distribution in selected localities in Sarawak.

Materials and methods

Ten to 20 mist nets and one to three harp traps were deployed at various locations for at least three consecutive nights. Bats were identified using Payne *et al.* (1985) information. Sampling sites are as follow: Tubau camp at Bintulu, Batang Ai National Park, Bako National Park, Mount Penrisen at Padawan, Kubah National Park, Kubah National Park, Similajau National Park, Lambir Hills National Park and Mount Murud.

Results

Sampling effort totaling of 691 net-nights was done to document bat diversity in the selected localities (Table 1). There were also many new records for Sarawak and the sampling areas. There were four were new records to Sarawak (*Arielulus cuprosus, Murina rozendaali, Kerivoula intermedia* and *K. minuta*) and there were 68 new records for the sampled areas.

Table 1. Summarized results of sampling at eight sampling locations with comparison with previous literature

	TBC	LHNP	SNP	MM	BANP	KNP	MPP	BNP
Pteropodidae	3/3	2/8	4/5	7/7	5/5	4/6	10/10	4/5
Emballunuridae	0/0	0/0	0/0	0/0	0/0	0/0	0/1	3/4
Megadermatidae	0/0	0/1	0/0	0/0	0/0	0/0	0/0	1/1
Nycteridae	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1/1
Rhinolophidae	1/1	3/3	3/3	0/2	0/0	4/5	2/2	2/3
Hipposideridae	1/1	5/5	3/3	1/2	0/0	5/5	1/1	8/9
Vespertilionidae	1/1	7/11	5/5	2/2	1/1	3/7	2/2	4/8
Total No. Family	4/4	4/5	4/4	3/4	2/2	4/4	4/5	7/7
Total No. Species	6/6	17/28	15/16	10/13	6/6	16/23	15/16	23/31
Effort (nets x night)	14	84	168	85	17	52	45	226
No. Individuals	25	123	53	112	83	83	108	219
Capture rate (100 net-nights)	179	146	32	132	488	160	240	131
New records for Sarawak	0	0	1	1	0	0	2	0
Total new records for sampling area	5	7	15	7	5	6	15	8

*Within table: left values indicate sampling results of this study and right values indicate total results of this study and available literature; TBC= Tubau camp, Bintulu, LHNP= Lambir Hills National Park, SNP= Similajau National Park, MM= Mount Murud, BANP= Batang Ai National Park, KNP= Kubah National Park, MPP= Mount Penrisen, Padawan, BNP= Bako National Park

Fig. 1. Four new records for Sarawak. *Kerivoula intermedia* (top left) and *K. minuta* (top right) were netted at the golf course within Borneo Heights, Mount Penrisen. *Arielulus cuprosus* (bottom left) which was recorded at Similajau National Park is a rare species and was previously known to be confined to Sabah (Payne *et al.*, 1985). *Murina rozendaali* (bottom right) which was recorded at Mount Murud was previously known to be only found in Poring, Tepadong and Gomantong in Sabah (Payne *et al.,* 1985).

Fig. 2. Fruit bats (Pteropodinae) netted in this survey. *Cynopterus brachyotis* (top left), *Balionycteris maculata* (top right), *Dycopterus spadiceus* (bottom left) and *Aethalops aequalis* (bottom right).

Fig. 3. Nectar bats (Macroglossinae) netted in this survey. *Eonycteris major* (left) and *Macroglossus minimus* (right).

Fig. 4. *Hipposideros larvatus* (left) is the largest Hipposiderid in Borneo, *Rhinolophus trifoliatus* (middle) netted at Kubah National Park and *H. dyacorum* (right) from Bako National Park.

Discussion

The four new records for Sarawak indicate chiropteran diversity is still under represented in Sarawak. All new records were previously recorded in Sabah, indicating that most available references (Payne *et al.,* 1985; Corbet and Hill, 1992) were concentrated on Sabah.

For species diversity; Bako National Park has the highest probably due to high number of vegetation types available from that area. Mount Murud and Mount Penrisen have a very distinct chiropteran composition due to submontane climate. Batang Ai National Park and Tubau camp are disturbed areas and these areas have been known to cater only several species of bats that are more tolerant to human presence (Mickleburgh *et al.,* 1992). In general the disturbances, distribution of resources in the canopy are the main factors that affect the bats diversity in an area. Other influences that might affect the results of the study include disturbance in the area, climatic conditions, fruiting and flowering season and the deployment of nets in the understorey.

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Session 2
Policies and Laws on Biodiversity

Laws and Policy on Biodiversity In Malaysia

Shaik Md Noor Alam, S.M.H.[1] and Wan Izatul Asma, W.T.[2]

[1]Professor of Law, Universiti Putra Malaysia, Malaysia
[2]Associate Professor of Law, Universiti Malaysia Terengganu, Malaysia
shaik@putra.upm.edu.my

Abstract

This paper deals with international regimes on biodiversity impacting Malaysia's position as a biodiversity rich country. The Convention on Biodiversity (CBD) recognizes the sovereign rights of states to the exclusive use and control over their biodiversity resources. The CBD too expects access to these resources should be made available to others on mutually agreed terms (MAT) subject to prior informed consent (PIC) and on the basis of benefits sharing (BS). Thus ABS (access and benefit sharing) becomes a cornerstone of an international understanding on equitable sharing of the world resources between the technology rich nations and resource rich nations. Concerns have been raised by some that the ABS mechanism may be subverted by TRIPs patentability requirements. The DOHA Declaration and call by some states for inclusion of CBD into TRIPs including disclosure requirement in patent application under TRIPs are central to the current debates over the CBD-TRIPs interface. The Biosafety aspect of biotechnology essentially covered under the Cartagena Protocol obligates member countries in respect of biosafety. The Malaysia Biosafety Act exemplifies this obligation. All these issues are examined in the context of Malaysia's NBSAPS (National Biodiversity Strategies and Action Plan 1998).

Introduction

This paper examines Malaysia's efforts towards meeting her obligations under the Convention on Biological Diversity (CBD) and the Cartagena Protocol on Biosafety through the launching of the National Policy of Biological Diversity and the subsequent enactment of the Biosafety Act 2007. This paper is divided into three main parts. Part 1 deals with the salient features of the CBD and the Cartagena Protocol. Part 2 deals with the Malaysian laws and policy on biodiversity in particular the National Policy of Biological Diversity and the Biosafety Act 2007 while Part 3 highlights on other domestic legislations on biodiversity conservation and management. This paper concludes with some observations on the shortcomings of both the Policy and the Act and some recommendations to deal with the shortcomings.

1.0 The Convention and the Protocol

1.1 The Convention on Biodiversity

The Convention on Biodiversity (CBD) entered into force in December of 1993 with the first meeting of the Conference of the Parties (COP) in November 1994. The most fundamental objective of the Convention is the provision of a broad universally accepted regime in the realm of genetic resources. Today it stands as the most important, comprehensive and holistic international agreement addressing biodiversity issues. It sets out a comprehensive approach to the conservation of biological resources and diversity, sustainable use of natural resources and the fair equitable sharing of benefits derived from the use of such resources. The essence of the Convention appears to be the need to achieve a balance between the full deployment of the potential of biotechnology and the need in so doing to develop appropriate legislative, administrative and policy measures to enhance the safety of biotechnology in the context of the Convention's overall goal of reducing all potential threats to biological diversity, environment and human health. The essential components of CBD fundamental to achieving the Conventions objectives and expectations can be identified under five core fundamentals, namely:

(i) Recognition of the sovereign rights of States to their natural resources and the authority to determine access to genetic resources according to national legislation.
(ii) Obligation of each Contracting Party to endeavor to facilitate access to genetic resources for environmentally sound uses by other Contracting Parties and must not impose restrictions inconsistent with the objectives of the Convention. Access to genetic resources shall be on the basis of prior informed consent (PIC) of the party providing such resources and on mutually agreed terms (MAT).
(iii) Obligations of each Contracting Party to facilitate access for and transfer to other Contracting Parties of technologies relevant to the conservation and sustainable use of biological diversity.
(iv) Obligation of each Contracting Party to provide for the active participation in biotechnological research and to promote priority access on a fair and equitable basis to the result and benefits of such research to Contracting Parties providing the genetic resources for such research.
(v) Obligation to consider the need for a protocol, including advance informed agreement (AIA), for the safe transfer, handling and use of any living modified organism produced by biotechnology that may have adverse effect on conservation and sustainable use of biological diversity.

1.1.1 Sovereign rights and ABS

Article 15 of the CBD is a compromise between two opposing propositions. One group, representing mainly developed countries argued for the recognition of biodiversity resources of the world as 'common heritage of mankind' and should be freely available without exclusive claim of ownership by any country. The other group, representing mostly developing countries where most of these resources are located argued for the recognition of the sovereign rights of States over their natural resources. The text and substance of Article 15 offers a very amicable compromise in that while recognizing the sovereign rights of States to their natural resources, it imposes on these States the obligation to endeavor to create, conditions to facilitate access to genetic resources[5] and not to impose restrictions contrary to the objectives of the Convention. Access shall be on mutually agreed terms (MAT) and subject to prior informed consent (PIC). While mandating facilitation of access to genetic resources, the Convention further obligates each Contracting Party to take legislative, administrative and policy measures (LAP) for the sharing in a fair and equitable way the result of research and development and benefits arising from the commercial and other utilization of the genetic resources with the Party providing such resources.

However Article 15 is not a stand-alone provision. It is further buffered by the provisions of Article 8 and Article 16. In particular Article 8(j) deserves special mention in that each Contracting Party is mandated, "subject to its national legislation, to respect, preserve and maintain knowledge, innovations and practices of indigenous and local communities embodying traditional lifestyles relevant for the conservation and sustainable use of biological diversity and promote their wider application with the approval and involvement of holders of such knowledge, innovations and practices and encourage the equitable sharing of the benefits arising from the utilization of such knowledge, innovations and practices. It must be borne in mind that the qualifying words used in Article 8 are, "shall, as far as possible and as appropriate" and similar qualifying words in para (j) "subject to its national legislation" appear to allow Contracting Parties to offer excuses for non compliance on the basis of perceived inappropriateness and unsuitability. Be that as it may, the full dimension of Article 15 provisions read together with Article 8(j) offer a regime of Access Benefit Sharing (ABS) with proper respect being given to the involvement of owners of traditional knowledge in both the promotion of wider application of such knowledge and the equitable sharing of benefits derived from use of such

[5] Article 2 of CBD defines 'genetic resources' to include "any material of plant, animal and micro-organism communities and their non-living environment interacting as a functional unit."

knowledge[6]. It is obvious that the full benefits of Article 15 can only be realized within a framework of LAP measures that address the main components of ABS, namely:

(i) Establishing and identifying the authorities responsible for granting prior informed consent.[7]
(ii) Establishing an appropriate benefit sharing arrangements and procedures.
(iii) Inventory of existing traditional knowledge and a register of owners of such knowledge from among the indigenous population.

1.1.1 Centralised national authority for PIC requirement

This matter, as far as Malaysia is concerned has to be resolved in the context of the federal arrangement on ownership and control over natural resources. It is more appropriate, in the meantime to leave this particular issue for deliberation in another forum.

1.1.2 The ABS Measures

Apart from the limited pioneering experience of a handful of countries in establishing the ABS regimes[8,] ABS remains essentially an uncharted territory. In an attempt to provide a guiding mechanism which could act as a precursor to a more comprehensive ABS mechanism, the Bonn Guidelines on Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization were formulated and adopted in 2002 by member countries. While work on more appropriate regime on ABS was in progress in many member countries, concerns have been raised in many quarters, mainly diversity rich developing nations, that the full realization of the objectives of Article 15 may effectively be undermined by the Agreement on Trade Related Aspects of Intellectual Property Rights (TRIPS) of the World Trade Organisation. It has been argued that the CBD and WTO/TRIPS "are in conflict with one another"[9]. The debates on this perceived conflict center primarily on the lack of recognition of the objectives of the CBD by some members and that they are encouraged and emboldened in this respect by the text of Article 27.3(b) of the TRIPS agreement itself which appear to give precedence to private right over public rights and allows the recognition of patents and other IPRs using genetic resources and traditional knowledge without prior informed consent and benefit sharing and without the due recognition given to owners of such traditional knowledge. Article 27.3(b) of TRIPS reads:

> "Article 27 Patentable Subject Matter
> 3. Members may also exclude from patentability:
> (b) plants and animals other than microorganisms, and essentially biological processes for the production of plants or animals other than non-biological and microbiological processes. However, members shall provide for the protection of plant varieties either by patents or by an effective *sui generis* system or by any combination thereof. The provisions of this paragraph shall be reviewed four years after the date of entry into force of the WTO Agreement".

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[6] This has been categorized as 'yet another layer' to the ABS regime of Article 15: Discussion Paper, ICC, Document. nº212/12 29 October 2004.
[7] In a federal governmental set-up in Malaysia, this may prove to be a contentious issue.
[8] The ABS arrangement between government of Costa Rica and Merck, also the arrangement between Tropical Botanic Gardens Institute of Kerala, South India and the Kani Tribes over the use of Aarogyapacha (*Trichopas zeylanicus*).
[9] Suman Sahai, "The Relationship Between TRIPS and CBD" Li Enheng, "Promotion of for a Mutually Supportive and Complimentary Relationship Between TRIPS Agreement and CBD" presented at WIPO/ESCAP High Level Policy Forum on Intellectual Property Rights and Trade, 23-2005, July, Macao, China. Also the views expressed in Anil K. Gupta, "CBD and TRIPS: Empowering Knowledge Rich, economically poor people through IPR reforms", paper presented at National Seminar on TRIPS-CBD and Subsidiary Issues", at WTO, 25 August 2005, New Delhi.

Owing to continued tussle between the developed and developing nations, the review of Article 27.3(h) mandated by the Article itself did not take place in 1999 but started a year later in 2000. However, it was in December 2001 at the DOHA Ministerial of the WTO that a far reaching dimension of the review was agreed in the form of para 19 of the DOHA Declaration. Member countries made the following decision,

> "We instruct the Council of TRIPS, in pursuing its work programme including under the review of Article 27.3(b), the review of the implementation of the TRIPS Agreement under Article 71.1 and the work foreseen pursuant to paragraph 12 of this declaration, to examine, inter alia, the relationship between the TRIPS Agreement and the Convention of Biological Diversity, the protection of traditional knowledge and forklore, and other relevant new developments raised by members pursuant to Article 71.1. In undertaking this work, the TRIPS Council shall be guided by the objectives and principles set out in Article 7 and 8 of the TRIPS Agreement and shall take fully into account the development dimension."

However, despite several negotiations under this mandate views continue to show a sharp divergence oscillating towards two extreme positions. On one of the extreme is the African group who has taken the position against the patent on life forms and has urged that Article 27.3(b) should be revised to prohibit patents on plants, microorganism and essentially biological processes for the production of plants and animals, including non-biological processes. On the other extreme is a group of developed countries led by United States of America, Australia and Japan, supported by Korea and Singapore which believe that there is no conflict between TRIPS and CBD and that the exceptions to patentability in Article 27.3(b) were unnecessary. The group is also of the view that in the interest of scientific advancement, transparency and technology transfer, it is necessary to provide patents on plants and animals. The group argued against the use of TRIPS to enforce ABS.

In between these two extremes are the groups of developing countries led by Brazil and India and including Bolivia, Columbia, Cuba, Dominican Republic, Equador, Peru and Thailand and the European Union group. The India-led group submitted two proposals; the first calling for "a mandatory disclosure of the source of origin of the genetic resources and associated TK"[10]. In a later proposal the group urged for a patent application based on genetic resources and TK to show evidence of benefit sharing with the country of origin and non compliance should result in the patent application being stopped or withdrawn.[11] Moreover the group demanded that where non-compliance is discovered after the granting of the patent, the patent should be revoked.

Representing a more conciliatory stand is the EU group who agrees with the inclusion of the disclosure requirement in TRIPS but argues that failure to disclose, prior to or after the grant of patent shall not affect the grant of patent or the validity of patent already granted.[12] The two main diverging views remain poles apart to this day, although there have been numerous attempts to negotiate mutually acceptable compromises. Significantly SAWTEE[13] in its Policy Brief has offered several conciliatory options[14], particularly to allow for patents on life forms of genetically modified plants, animals, organisms and associated non-biological processes and such patent to cover real inventions and not mere discoveries and the right of Member States to reject patent on life forms on grounds of ethical, religions, environmental and development concerns.

The ICC (International Chamber of Commerce) meanwhile views the making of disclosure requirement a condition of patentability as a disturbing development because it tends to turn it into an enforcement tool rather than a tool to encourage access to innovation.[15] It also raises the logistic problems of identifying with reasonable degree of legal certainty the holders of TK. With due respect, if disclosure requirement, once included in the TRIPS Agreement is perceived as a tool of

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[10] IP/C/W/429/Rev.I.
[11] IP/C/W/438.
[12] IP/C/W/383.
[13] South Asia Watch on Trade, Economic and Environment.
[14] Policy Brief on Review of Article 27.3(b) TRIPS Agreement No. 14, 2007.
[15] ICC, Discussion Paper: Access and Benefit-Sharing for Genetic Resources: Document nº212/12. 29 Okt. 2004.

enforcement, the opposite is equally true: its absence without TRIPS will allow TRIPS to be used as a tool to avoid the meaningful implementation of the objectives of CBD, in particular ABS and PIC.[16]

1.2 Biosafety: The Cartagena Protocol

One of the several principal issues addressed by the CBD is biosafety. Biosafety refers to the need to protect human health and the environment from any adverse effect of biotechnology and its products. The Convention is quite explicit on the measures that each Parties are obliged to take in respect of biosafety. At the national level, parties are obliged to regulate, manage or control the risk associated with the use and release of living modified organisms (LMOs) resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, also taking into account the risk to human health. [Article 8(g) CBD] while at the international level the Parties shall consider the need for and modalities of setting out appropriate procedures, including and in particular, advance informed agreement, in the field of the safe transfer, handling and use of any living modified organism resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity.

Work on protocol on biosafety was finally completed in 2000 when at an extra-ordinary meeting of the Conference of the Parties, the Protocol, known as the Cartagena Protocol on Biosafety (CPB) to the Convention on Biological Diversity was adopted, focusing specifically on trans-boundary movement of any living modified organism. Article 1 of the Protocol states its objectives:

> In accordance with the precautionary approach contained in Principle 15 of the RIO Declaration on Environment and Development, the objective of this Protocol is to contribute to ensuring an adequate level of protection in the field of safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risk to human health, and specifically focusing on trans-boundary movements.

Article 4 meanwhile defines the scope of the Protocol by providing that:

> This Protocol shall apply to the trans-boundary movement, transit, handling and use of all living modified organisms that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health.

However the actual scope can only be understood by looking at Article 5 and 6 which exclude the requirements of advance informed agreement procedure in respect of LMOs which are pharmaceuticals for humans LMOs in transit and LMOs for contained use.

Essentially the Protocol outlines the rights and obligations of Parties in respect of trans-boundary movement, transit, handling and use of LMOs in a manner to avoid any adverse effects on conservation and sustainable use of biological diversity including risk to human health. The Protocol calls for the enactment of legislation and the formulation of policy and administrative measures to ensure biosafety consistent with the objectives of the Protocol. In this respect domestic legislation on biosafety needs to deal with the matters provided in the Protocol, namely:

(i) Risk Assessment standards and procedures.[17]
(ii) Risk Management standards and procedures.[18]
(iii) Prior Informed Agreement standards and procedures.[19]
(iv) Standards for contained use.[20]

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[16] The CBD obligations have become the subject matter of several proposals in other international forums including International Union for the Protection of New Varieties of Plants (UPOV), the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGR) of the UNFAO.
[17] Article 15.
[18] Article 16.
[19] Article 7.

(v) Procedures of import of LMOs for direct use as food.[21]
(vi) Handling, Transportation, Purchasing and Identification[22] [Parties to take necessary measures (including but not necessarily limited to legislative measure)] of LMOs.
(vii) Prevention and Punishment for illegal trans-boundary movement.[23]

It must be noted that the Protocol deals with LMOs not their products and Parties are encouraged[24] to take cognizance of available expertise, instrument and work undertaken in international forums with competence in the area of risk to human health. The design of any domestic biosafety law, to be consistent with the Protocol must surely take into account of both the above matters. More importantly the Protocol allows the formulation of standards or the taking of actions that are more protective of the conservation and sustainable use of biological diversity than that demanded under the Protocol, PROVIDED THAT, they are consistent with the objective and provisions of this protocol and Party's other obligations under international law[25]. Paragraph 5 of Article 2 uses the term 'encouraged', thus implying a non mandatory requirement but in the light of the spirit of the Protocol and the CBD, the paragraph must be deemed to be a strong ethical exhortation bordering virtually on the mandatory. It would tantamount to lack of good faith to ignore it. Similarly paragraph 4 of Article 2 does not grant the right to arbitrarily set a more stringent standard of biosafety than those prescribed in the Protocol. Parties desiring to set these more protective measures must ensure that they do not conflict with the Party's other obligations under international law and the Protocol.

While the main thrust of the Protocol appears consistently focused on conservation and sustainable use of biological diversity and risks to human health, socio economic considerations may be taken into account when considering the impact of living modified organism on the conservation and sustainable use of such resources, especially with respect to the value of biological diversity to indigenous and local communities.[26]

1.2.1 Liability and redress

Despite the observance of the safety procedures of the Protocol or domestic regulatory requirements of the country of import, damage arising from TBM of LMOs may still occur. In such an event liability and redress provisions become an integral part of the Protocol, almost a condition for its effective observance. Hence Article 27 provides:

> The Conference of the Parties serving as the meeting of the Parties to the Protocol shall, at its first meeting, adopt a process with respect to the appropriate elaboration of international rules and procedures in the field of liability and redress for damage resulting from trans-boundary movements of living modified organisms, analyzing and taking into account of the ongoing processes in international law on these matter and shall endeavor to complete the process within four years.

At the first meeting of the Conference of the Parties serving as meeting of the Parties to the Protocol (COP-MOP) an Open-Ended Ad Hoc Working Group of Legal and Technical Experts on Liability and Redress was established to carry out the mandate under Article 27. Since then several

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[20] Article 6.
[21] Article 11.
[22] Article 18.
[23] Article 25.
[24] Article 2(5).
[25] Article 2(4). See Sabrina Safrin, 'The Relationship with other Agreements', in the Categena Protocol on Biosafety: Reconciling Trade in Biotechnology with Environment and Development? Bail, Falkner and Marquand (eds), Earthscan, London, (2002). What about WTO Agreement on the Application of Sanitary and Phytosanitary Measures – any possible conflict?
[26] Article 26.

meetings had taken place and in May 2008 the COP-MOP 4 considered the final report of the Working Committee.[27]

The contents of the Final Report are encouraging as they point towards a real likelihood of consensus in the near future negotiations. Of great significance are matters dealing with State responsibility, both primary and residual, scope of L&R rules and procedures, definition of damage and operators, standards of liability and computation and definition of damage, extended local standi, compensation scheme and the pivotal role of domestic law in giving effect to the rules and procedures of L&R once finally accepted by the members at the COP-MOP.[28]

1.2.2 State responsibility: Primary and residual

The Final Report of the Open-Ended Ad Hoc Committee in both its operational text and the Preambular text categorically states that the rules and procedures shall not and would not affect the rights and obligations of States under the rules of general international law with respect to the responsibility of State for internationally wrongful acts. It is interesting to note that the question of what constitutes 'internationally wrongful act' is not a simple straight forward matter. The International Law Commission's Articles on State Responsibility define international wrongful act as conduct not in conformity with an international obligation and attributable to a State and for which the State should be responsible. Thus State responsibility covers both the internationally wrongful acts of a State or of private actors whose acts can be attributed to that particular State, which generally means a State agency, or non-State agency acting under the direction, control and instructions of that state or which carry on aspects of governmental authority. This rule of attribution operates even retroactively by making a State responsible for prior conduct by private parties if the States "acknowledges and adopts the conduct as its own"[29]. It has been pointed out that in most cases this responsibility arises as a result of primary rules – to prevent or limit particular types of private conduct.[30]

Though in discussion leading to the Final Report of the Working Committee, many countries opposed the inclusion of primary state responsibility, the idea of residual state responsibility appeared less objectionable. This principle of state residual responsibility entails the obligation of a state to assume liability to pay when an operator of that state who is liable to pay compensation for damages for harm arising from the trans-boundary movement of LMOs becomes unable to meet the full sum claimed. The state to which the operator belong is liable to meet the difference.

While the mechanism of state residual responsibility serves a useful propose of ensuring that the claimant will be appropriately and fully compensated, the rational of making the state residually liable for the act of a private actor (the operator) without any determinants of attribution may be questionable. As pointed out by Boyle, sovereign states are less involved in the production and transport of LMOs although they have some power to control the industry.[31] Moreover the inclusion of residual state liability may result in 'hesitation for state to ratify this regime'[32].

1.2.2.1 Scope of L&R rules and procedures

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[27] UNEP/CBD/BS/COP-MOP/4/11.

[28] Groups of the Friends of the Co-Chair (established vide Decision BS-IV-12) are mandated to further negotiate international rules and procedures in the field of liability and redress for damage resulting from trans-boundary movement of LMOs in the context of Cartagena Protocol on Biosafety on the basis of UNEP/CBD/BS/COP_MOP/4/11 and Add. I).

[29] Daniel Bodansky and John R. Crook, "Symposium: The ILC's State responsibility Articles: Introduction and Overview" 96 AJIL 773 (2002). In the discussion leading to the Final Report of the Ad Hoc Working Committee, many countries apposed the inclusion of primary state responsibility: Earth Negotiation Bulletin Vol.-9, No. 400, 23 Okt. 2007.

[30] Gordon A. Christenson, "Attributing Acts of Omission to the State." 12 Mich. J. INT'L.L. 312 (1991).

[31] A. E. Boyle, "Globalizing Environmental Liability: The Interplay of National and International Law", 17 J. ENVTL.L, (2005) 6-7.

[32] Katherine E. Kohm. "Shortcomings of the Cartagena Protocol: Resolving the Liability Loophole at an International Level", Kohm, University of Richmond School of Law, Aug. 18, 2008.

The Final report offers four operational texts to deal with the scope of the L&R rules and procedures. Operational Text 3 appears to be the most comprehensive in that it proposes that the rules and procedures will apply to "shipments, transit, handling and use of living modified organism, provided that these activities find their origin in a trans-boundary movements". The text continues to provide that these rules and procedures will apply to both intentional as well as unintentional trans-boundary movement of LMOs and also to the trans-boundary movement of goods in contravention of domestic measures to implement the Protocol. The text emphasizes that with respect to intentional trans-boundary movements, the rules apply to damage resulting from any authorized use of the LMOs, that is where the LMOs are intended for direct use as food and feed or for processing, for contained use or for intentional introduction into the environment including the use in violation of the authorization.
Text No. 1 however proposes to expand the scope to include damage arising from the trans-boundary movement of LMOs and products thereof.

1.2.2.2 Definition of critical terms

The Final Report appears to have excluded the principle of primary state responsibility but has instead; consistent with the *polluter pays* principle, attached primary liability for damage arising from the trans-boundary movement of LMOs on the operator. Hence it is necessary to clearly define the term 'operator' and the 'damage' for which he is to be made primarily liable to.

(a) **Damage**

Operational text 6 provides the full ambit of the damage to be covered by the rules and procedures. It states that the rules and procedures apply to "damage to the conservation and sustainable use of biological diversity, taking also into account damage/risk to human health resulting from the trans-boundary movement of living modified organism" and damage to the conservation and sustainable use of biological diversity means an adverse or negative effect on biological diversity that is measurable or observable taking into account available scientifically established base-lines recognized by a competent national authority taking into account any other human or natural induced variation and be significant in nature. A 'significant'[33] adverse or negative effects is to be determined on the basis of factors such as:

(a) The long term or permanent change, to be understood as change that will not be redressed through national recovery within a reasonable period of time.
(b) (which is the (b) and (c) as altered). A qualitative and quantitative reduction of components of biodiversity and their potential to provide goods and services.[34]
(c) The extent of any adverse or negative effects of the conservation and sustainable use of biological diversity on human health. [(d) as altered].

(b) **Operator**

These are two options on the options on the definition of operator in operational text 13:

'Operator' means the developer, producer, notifier, exporter, importer, carrier or supplier' that is almost every possible actor in the chain of trans-boundary movement.

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[33] Note the definition of significant damage in ILC by stating that this should mean something more than detectable or appreciable, but not necessarily serious or sustainable. This definition is "*a de minimis* threshold" C. Voight, "State Responsibility for Climate Change Damages', Nordin, Journal International Law 77 (2008) 1-22 at p.9.
[34] To quantify biodiversity or any perceived loss in biodiversity is not an easy matter. "for it is essentially impossible to indicate where one ecosystem stops and another begins." Anthony J. Conner, et al. 'The Release of Genetically Modified Crops into the Environment: Pt. 11. Overview of ecological risk assessment." The Plant Journal (2003) 33. 19-46 at p.33.

Also:

'Operator' means any person in operational control of the activity at the time of the incident and causing damage resulting from the trans-boundary movement of living modified organisms.

1.2.2.3 Standards of liability

Perhaps among the most debated issues in the preparation of the L&R rules and procedures for trans-boundary movements of LMOs is the question of the most appropriate standard of liability. Some countries advocate the adoption of strict liability standards, including Malaysia while others proposed a fault-based standard of liability. The COP-MOP 4 Decision BS-IV/12 provides three options for standards of liability: strict liability, mitigated strict liability and fault-based liability.

The general trend in other international treaties and convention appears to favour strict liability regime for trans-boundary harm to the environment. According to Boyle, "the choice of strict, or in exceptional cases absolute, liability is an invariable feature of all international liability conventions".[35] Be that as it may, unlike other strict liability imposing Protocols, the Ad Hoc Working Group does not provide a cap on amount of damages that a party is liable for.[36]

Another option considered by the Working Group is mitigated strict liability, that is the switching of fault-based liability to strict liability in cases where risk assessment has identified a living modified organisms as ultra-hazardous and/or acts or omissions in violation of national law have occurred or violation of the written conditions of any approval has occurred.[37] Of course the other option considered is fault-based liability regime which requires proof of intentional, reckless or negligent conduct on the part of the defendant. Both strict liability and fault based liability proposals channel liability to the person proven to have caused the damage.

1.2.2.4 Choice of instrument

As with other issues, parties were divided over the choice of instrument for the liability and redress regime. One group called for legally binding instrument, while another group, particularly the EC favoured a kind of model law upon which parties may enact their domestic regime. The choice of instrument was left for further deliberations.[38]

1.2.2.5 Extended locus standi

One of the most significant proposals under the COP-MO 4 Decision is the introduction of an extended *locus standi* to include non-governmental organizations having an interest in the conservation and sustainable use of biological diversity including access to remedies in the courts of the exporting state: This proposal however is made subject to the domestic law of any member state. This qualification is unfortunate because domestic law may frustrate the essence of this significant proposal by imposing unreasonable conditions and restrictions.[39]

2.0 The Malaysian position

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[35] A. E. Boyle, supra note at 13. Strict liability a "compensatory automatism"?

[36] Though domestic law may provide for financial limits for strict liability but it shall not be less than (2) drawing rights. COP-MOP 4.

[37] Operational Text 7, COP-MOP 4 Decision BS-IV/12.

[38] See generally, Guardial Singh Nijar, *et al*. "Liability and redress Under the Cartegena Protocol on Biosafety", CEBLAW, 2008.

[39] See Operational Text 3 in Section C of the Decision.

Malaysia is a party to the CBD and that being so has undertaken to abide by what has been stipulated by the Convention and to endeavour to provide legislative, administrative and policy measures towards achieving the objectives and expectation of the Convention.

Among the first measure taken by Malaysia was the conduct of a country survey on biological diversity cumulating in the "Assessment of Biological Diversity in Malaysia" in 1997.This was almost immediately followed by the launching of the National Policy on Biological Diversity on 16th July 1998. To provide legislative implementation of the broad objectives of the Policy, three specific legislation are to be put in place, namely the Biosafety Act 2007 (Act 678) the Access to Genetic Resources Act and the National Biodiversity Council Act (both of which are still in the bill stage).

2.1 The National Policy on Biological Diversity

The Malaysian National Policy on Biological Diversity was officially declared on April 16 1998 and its declared vision is,

> "To transform Malaysia into a world center of excellence in conservation, research and utilization of tropical biological diversity by the year 2020".

The Policy Statement reads:

> "To conserve Malaysia's biological diversity and to ensure that its components are utilized in a sustainable manner for the continued progress and socio-economic development of the nation"

Both the vision statement and the Policy statement undeniably emphasize Malaysia's commitment towards the conservation and sustainable use of her biological diversity heritage for the sustainable progress of the nation. However the direction of legislative, administrative and other measures effectuating both the vision and policy statements of principles and six heads of objectives.

Four of the 11 statements of principles warrant mention here, namely:

> Principle (VI): It is the duty of the Government to formulate and implement the policy framework for sustainable management and utilization of biological diversity in close cooperation with scientists, the business community and the public".
>
> Principle (VII) : "The role of local communities in the conservation, management and utilization of biological diversity must be recognized and their rightful share of benefits should be ensured."
>
> Principle (IX) : "The interdependence of nations on biological diversity and in the utilization of its components for the well being of mankind is recognized. International cooperation and collaboration is vital for fair and equitable sharing of biological resources, as well as access to and transfer of relevant technology".
>
> Principle (XI) : "In the utilization of biological diversity, including the development of biotechnology, the principles and practice of biosafety should be adhered to."

Among the six heads of objectives, three heads merit further deliberation. These are:

> Objective (i) : "to optimize economic benefits from sustainable utilization of the components of biological diversity".
>
> Objective (iv) : "to ensure preservation of the unique biological heritage of the nation for the benefit of present and future generations".
>
> Objective (vi) : "To emphasize biosafety considerations in the development and application of biotechnology".

2.1.1 Governments' duty towards sustainable management and utilization of biological diversity: Public trust doctrine.

Clearly Principle (VI) categorically declares the duty of the government in a manner that can achieve sustainable development of biological diversity in Malaysia. In this regard the government is mandated to work in close cooperation with scientists, the business community and the public. This duty if read together with Objective (iv), that is, preserving the unique biological diversity for the benefit of present and future generations must necessarily imply the existence of a broader duty in the nature of public trust. This public trust doctrine, though never expressly stated in any of the many natural resource legislation can and should be judicially recognized as an implied duty imposed on the government by virtue of the combined operations of Principle (VI) and Objectives (iv) of the National Biodiversity.

2.1.2 Traditional knowledge and rights of indigenous communities

Quite related to the principle of sustainable management and use of biological diversity is the need to recognize, protect and enforce the rights of indigenous communities to have continued access to biological resources not only for the continued sustenance of their culture[40] but also to protect their knowledge, acquired over thousand of years of experimentation and experience, about the uses biological resources can be put to, particularly in medicinal and pharmaceutical preparations. This knowledge now popularly termed as traditional knowledge is required by the CBD to be duly protected. Principle (VII) takes cognizance of this but in a rather oblique way. There is no specific mention of 'traditional knowledge' among the 15 strategies for effective management of biological diversity outlined in the Policy, none either directly or indirectly refers to traditional knowledge. Even Strategy 9, that is to undertake "review and update existing legislation" to reflect biological diversity needs is silent on TK. The Action Plan proposed for Strategy 1 calls for the establishment of an inventory of traditional knowledge on the use of species and genetic diversity: similarly the Action Plan for Strategy 9 calls for identification of areas where new legislation or enhancement of present legislation are needed, among others, for "intellectual property and other ownership rights".

Protection of traditional knowledge requires more than just a policy declaration. It requires legislative recognition which shall form the basis for the establishment of the features necessary for its protection such as National Data Base, Registration of Ownership, Dispute Resolution Mechanism to resolve conflicting claims, procedures for participation in decision making, especially with regard to ABS. The absence of very explicit strategic and action commitment on TK in the Policy is quite perplexing.[41]

The rather inadequate provisions in the Policy regarding the recognition and protection of traditional knowledge in further compounded by the lack of real proprietary interest in land. It appears that for the indigenous peoples[42] of Peninsular Malaysia obtaining tenure has been extremely tenuous. As pointed out by Rachagan[43], the special privileges accorded to the Malays of Peninsular Malaysia and the natives of Sabah and Sarawak under the doctrine of affirmative action in Article 153 and 161A of the Federal Constitution do not extend to the Orang Asli. The only mechanism for extending a similar affirmative action to the Orang Asli can be found in Article 8(5)(c) which is a mere enabling

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[40] A. Latiff and A.H. Zaki, "Biodiversity and Traditional Knowledge?" The Malaysian Experience. UNCTAD Expert Meeting on Systems and National Experience, Geneva, 30 Oct - 1 Nov 2000.

[41] Perplexing because Malaysia was one of the active initiators of the Charter of Indigenous Tribal People of the Tropical Forest 1992 which was signed in Kuala Lumpur and the Sabah Declaration 1995 both of which advocated the strong protection of indigenous rights. See also Srividya Ragavan, "Protection of Traditional Knowledge" Minnesota Intellectual Property Law review, Vol. 2, No. 2, 2001. Also the enlightening "Rights Regime" proposed by Nijar GS (1995) in AH Zaki (Ed.).'Prospects in Biodiversity Prospecting' Universiti Kebangsaan Malaysia: 225-265.

[42] 'Orang Asli' a generic term to refer to three tribal groups, Negritos, Senois and Proto-Malays.

[43] S. Sothi Rachagan, "Sustainable Forest Management in Malaysia – Guidelines for Conflict Resolution". http://www.iqes.or.jp/en/fc/phase1/ir98-2-9 PDF 7.10.08.

provision without being mandatory. Even the enactment of the Aboriginal People Act in 1954 (Revised in 1974) does not put the rights of the aborigines beyond legislative and administrative derogation.

The unique feature of Malaysian federal legislative arrangement is that forests are separate from land, though physically they are the same terrestrial resources. This artificial distinction can have significant impact on the rights of indigenous people. Absence of proprietary tenurial right over their 'forested land' can mean that theses lands may subsequently end up becoming part of a reserved forest under the relevant forest laws of the states and the rights of indigenous people to their forested lands will only subsist as a common law right of usufruct, i.e. their right is the right to "live from the produce of the land itself but not to the land itself".[44]

It is obvious that recognizing, protecting and ensuring the rightful place of traditional knowledge within the broader framework of sustainable use and conservation of biological diversity requires a total review of existing legislation pertaining to the rights of indigenous people beyond formal declarations. After all, as succinctly put by Nina L. Etkin, "More recently these communities (indigenous communities) have come to be appreciated as repositories of not only of knowledge but also of biological diversity itself".[45]

2.1.2.1 Biosafety Act 2007

Malaysia's ratification of the Cartagena Protocol mandated the performance of several obligations, one of which was the enactment of domestic law along the lines of the Protocol to dial with the safety aspect of transfer, handling and use of LMOs. Malaysia accordingly enacted the Biosafety Act 2007. Reactions to the Act had been mixed. There are those who view the Act as being too lenient, almost 'throwing precaution to the wind'[46], while others blame the Act for being too prohibited and not business friendly[47]. In most legislation dealing with standards of safety, it is quite common that views tend to oscillate between two extremes. Be that as it may, for the non-partisan bystanders but very much concerned with the issue of safety of LMOs a detailed discussion of several important provisions of the Act can be useful.

2.1.2.2 Organization of the Act

The Act is divided into 7 parts. Part I deals with Preliminary matters such as the name by which it is to be cited, date of coming into force and the usual interpretation provisions. Part II deals with the establishment, composition and powers and functions of the National Advisory Board, the Genetic Modification Advisory Committee and other committees and sub-committees. The office of a Director General of Biosafety and the power of the Minister to give directions to the Board and the Advisory Committee are provided under this Part.

Part III deals with the difficult question of approval for release and import. Basically this Part embraces the spirit of the AIA regime of the Protocol but not in its entirety[48]. There are significant divergences.

Part IV is dedicated to notification for export, contained use and importation of living modified organism for contained use activities. The risk assessment, risk management and emergency response plan are covered in Part V while Part VI deals with enforcement and Part VII, as in most legislation contains miscellaneous provisions.

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[44] Per Mokhtar Sidin JCA in *Adong bin Kuwana and Ors v Kerajaan Johor and Anor* [1997] 1 MLJ 418 at pp 429-430.

[45] Nina L. Etkin, "Indigenous patterns of conserving biodiversity: pharmacological implication', Journal of Ethno pharmacology, Vol. 63, Issue 3, December 1998, pp 233 – 245. Note: The State of Sarawak has announced plans to document ethnic knowledge from among the ethnic communities in the State in order to preserve them and to explore the potential for commercialization : The Star, 4.10.2008.

[46] Kwan Khai Hee, "Malaysia's Biosafety Bill and NDASH : Throwing Precaution to the wind?" [2007]5 CLJi

[47] See Yee Ai, 'Malaysia : An ambitions start toward the formulationof Biosafety Law' Crop Biotech Net October 2001.

[48] Section 35 of the Act embracing Article 10(6) of the Cartagena Protocol.

The three fundamental parts of the Act are Part III, IV and V as they form 'substantive interpretation' of biosafety measures as mandated by the Protocol and as cautioned by the Precautionary Principle.

Part III – Release Activity and Importation:

Section II of Part III of the Act declares that this Part shall apply to release activities and import activities involving living modified organisms. 'Release activity' is defined to mean "any intentional introduction of living modified organisms or products of such organisms into the environment through activities or for the purposes specified in the second schedule". The Advanced Informed Agreement procedures of Part III therefore applies to all LMOs intended for direct introduction into the environment, including products of such LMOs. Apparently this provisions appears to conflict with Article 7(1) and (2) of the Protocol which excludes the AIA procedures regarding first trans-boundary movement of LMOs for intentional introduction into the environment of the importing state where the LMOs are intended for direct use as food or feed, or for processing.[49] It may be argued that these apparently stricter measures can be justified by the words of Article 2(4) of the Protocol, which in essence recognize the right of a Party to take a more protective action in respect of the conservation and sustainable use of biological diversity than that called for in the Protocol "so long as such action is consistent with the objective of the Protocol and that Party's other obligations under international law." Clearly Malaysia's stricter measures in Part III can only be justified in the context of these measures being consistent with the Protocol and Malaysia's other international obligations.

2.1.2.3 Precautionary principle

The 'precautionary principle', which underlines the Cartagena Protocol, appears to similarly characterize the essence of the Act, though its presence in the Act takes various levels of observance.

Precaution has always played an essential role in regulating environmental risks. As a factor influencing decision about anything uncertain precaution is the deeply ingrained cultural inclination towards being safe rather than sorry but as a principle guiding the difficult decision choices in environmental matters, the principle finds its origin in the 1970s in the former West Germany in the concept of 'vorsorge' or foresight which later developed into the vorsorgeprinzip (or the precautionary principle) and which became the underlying justification for the new West German vigorous policies to tackle environmental issues such as acid rain, global warming and the North Sea Pollution.[50] The impact of this principle in environmental regulation and management has been remarkable and it made its most significant appearance in the Rio Declarations in 1992.

What does this principle exactly mean? 'At its core lies the intuitively simple idea that decision makers should act in advance of scientific certainty to protect the environment from incurring harm'.[51] There is no single universally accepted statement of the principle and both proponents and opponents are inclined to move towards two opposing extremes, that is prohibitive action and reactionary action.[52]

The prohibitive action claims justification on the rationale that lack of scientific certainty as to the threats to the environment shall not hinder them from prohibiting a particular action while the reactionary action believes that no action should be prevented unless there is scientific certainty as to its threats to the environment.

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[49] Though the Minister may exempt these LMOs from any or all provisions of this Act: section 68 of the Act.

[50] Andrew Jordan and Timothy O' Riordan, "The Precautionary Principle in Contemporary Environmental Policy and Politics" paper presented at Wingspread Conference on 'Implementing the Precautionary Principle' 23 – 25 Jan 1998, Racine, Winconsin.

[51] Ibid.

[52] David Kriebel *et al*. The Precautionary Principle in Environmental Science, Env. Health Perspectives, Vol. 109 No. 9, Sept. 2001: also at http://www.ehponline.org/members/2001/109p871-87kriebel/knebel -full.html.

Even the two famous formulations of the Principle, namely the Rio Declaration and the Wingspread Statement appear to differ in critical aspects.[53]

The Rio Declaration (1992):

> "When there are threats of serious and irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost effective measures to prevent environmental degradation."

The Wingspread Statement (1998)

> "When any activity raises threats of harm to human health or the environment, precautionary measures should be taken even if some cause and effect relationships are not fully established scientifically."

Marchant points out that the Rio Declaration aims at environmental degradation while the Wingspread Statement is wider to include harm to human health. More importantly the Rio Declaration is phrased as a guiding principle while Wingspread appears to impose an obligation to act.[54]

The lack of definitive formulation of the Principle has led to inconsistent application of the Principle even to the point that it can become a tool for inaction or arbitrary action. Bodansky argues that the Principle should not require a technology proponent to prove that its products has zero risks,[55] but this is exactly what can happen when the parameters of the Principle are not fully formulated. Such inconsistency is also clearly evident in Malaysian Biosafety Act 2007.

It is now evident that Part III of the Act carries the precautionary principle to its most extreme interpretation by requiring the AIA procedure for importation of LMOs intended for direct use as food, feed or processing when indeed such procedure is exempted for such products under the Protocol which is itself a collective solemnization of the Precautionary Principle by the Parties, including Malaysia. However while maintaining what seemingly is a more stringent application of the PP, the decision norm in the entire regime of import and export regulation under the Act appears to be a derogation of the PP of the Protocol; compare the PP in the Act and the same principle in the Protocol:

Section 35 of the Act:

> "The Board or Minister shall not be prevented from taking a decision, as appropriate, under Part III or IV, where there is lack of scientific certainty due to insufficient relevant scientific information and knowledge regarding the extent of the potential adverse effects of living modified organisms or products of such organisms on human, plant and animal health, the environment and biological diversity and may also take into account socio-economic considerations."

Under the Protocol, Article 10(6):

> Lack of scientific certainty due to insufficient relevant scientific information and knowledge regarding the extent of the potential adverse effects of a living modified organism on the conservation and sustainable use of the biological diversity in the Party of import, taking also into account risks to human health, shall not prevent that party from taking a decision, as

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[53] Gary E. Marchant, "From general Policy to Legal Rule: Aspirations and Limitations of the Precautionary Principle", Environmental Health Perspectives: Vol. III, No. 14, Nov. 2003.
[54] Marchant, at 1800.
[55] Bodansky, D. 'The precautionary Principle in US environmental law'. In Interpreting the Precautionary Principle: (O'Riordan T. Cameron J. eds) London: Earthson Publications, 1994, pp 31-60.

appropriate, with regard to the import of the living modified organism in question as referred to in paragraph 3 above, in order to avoid or minimize such potential adverse effects."

Comparing the two statements of PP above, several significant divergences become apparent: Firstly, the PP statement of the Act makes no mention of 'sustainable use' which is the very essence of biological diversity. Secondly, the Protocol statement allows a Party to either prohibit importation of LMOs (risk avoidance) or permit importation subject to appropriate measures being taken to minimize risks (risk management). Hence a Party may oscillate between the two extremes of absolute prohibition of import or conditional permission of import, either of the two decisions to be subject to the "overriding aim" of "avoiding or minimizing such potential adverse effects". The PP under Act however does not mention this 'overriding aim' and a decision to allow importation (if considered appropriate) can be made purely on "socio economic considerations". It is therefore not surprising, and it is not entirely wrong for Kwan to claim that the Act "has thrown precaution to the wind"[56]. However a closer examination of the PP under the Act may well suggest a plausible contrary. The Board or Minister may decide to prohibit import of LMOs or the product thereof if such importation may adversely affect the socio economic activities and interests in Malaysia, but this is only a plausibility not a possibility given the fact that the 'overriding aim' is absent.

It must be appreciated that the text of the PP in most, if not all, environmental related agreements and Protocols are worded in cautionary, albeit, prohibitive terms. As aptly commented by Rebecca Bratspies, "…. the uncertainty relates not to the possibility of harm but to the degree and care needed to prevent the harm."[57]

2.1.2.4 Labeling of LMOs and products

Apart from the crucial role of the precautionary principle in ensuring biosafety in matters relating the LMOs and products of such organism the Act appears to add another dimension of control in the form of labeling. Section 61 of the Act makes it mandatory for all living modified organism, items containing living modified organism and products of such organisms to be clearly identified and labeled in a manner to be prescribed (by regulations). Labeling presupposes consent for importation has already been approved thus implying that risks have been satisfactorily assessed and there is either no risk or no unacceptable level of risk for such importation. Hence the importation of subsequent labeling cannot be relevant to the safety of the LMOs or their products and being a non-safety issue it should not find its way in the Act.[58] Of course it is admitted that one of the most controversial public policy issues surrounding GM foods is whether such food products should be labeled to allow consumers to make informed decision[59], and labeling has no role to play in safety issue. Existing legislation on labeling of food products can sufficiently provide ample protection for consumer preference. Additional labeling, in particular directed at GM foods under a 'biosafety' law "may cause unnecessary fears over products that most scientists have found to be as safe as their conventional counterparts."[60] Even there are renable arguments in favor of labeling of GM foods, such labeling should be mandated under

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[56] Kwan Khai Hee, Malaysia's Biosafety [2007]5 CLJ i.

[57] Rebecca Bratspies, "The Illusion of Care: Regulation, Uncertainty, and Genetically Modified Food Crops," N.Y.U. Environmental Law Journal, Vol 10, 2002, p.320.

[58] The booklet published by the Malaysia's Ministry of Natural Resources and Environment (NRE) Malaysia entitles "The Biosafety Act of Malaysia: Dispelling the Myth" is quite unconvincing on this point, in fact it almost agrees that labeling should not be in the Act. See answer to question no. 26. p. 27.

[59] See generally Lawren Zeichner, "Product v Process: Two Labeling Regimes for genetically Engineered Foods and How They Relate To Consumer Preference" 27 Environs Envtl. L. and Pol'y. J. 467.

[60] Micheal Rodemeyer in PR Newswire, How Consumers Process Information at Heart of Debate Over Labeling of Genetically Modified Foods: International and Economic Issues Around Biotech Crop. June 27, 2002: Requiring labeling og GM foods is surely a rejection of the "principle of substantial equivalence" See John Fagan, "The Failings of the Principle of Sustantial Equivalence in Regulating Transgenic Foods" http://www.organicconn.sumers.org/subeqniv.html. 3.11.2008.

the generic legislation on labeling and not under a statute dealing specifically with biosafety because this will segregate the GM foods in a disadvantaged marketing position when other foods only required to be labeled under a generic labeling law.[61] Apart from issue of consumer sovereignty inherent in the arguments of pro-mandatory labeling advocates, labeling also raises the issue of non-tariff barriers to trade, quite repugnant to the spirit of the WTO rules[62], because "the use of labels to inform consumers in one country may be viewed by another country as trade protectionism."[63]

3.0 Other legislations on biodiversity

As a state party to CBD, Malaysia is obligated to develop national strategies, plans and programmes by taking legislative, administrative and policy measures for the conservation and sustainable use of biological resources and diversity.[64] Although CBD is silent on the meaning of "conservation", it can be generally understood as preventing a species from loss, waste or change.[65] For a country like Malaysia, which is one of the 12 mega-biodiversity countries of the world[66], an integrated approach to conservation is necessary to develop cornerstone biodiversity conservation. One of the most important approaches is to have adequate laws for that purpose.

At present, there is not a single legislation in Malaysia that comprehensively provides for biodiversity conservation and management as a whole,[67] where most of the existing legislations are sector-based. For instance, the Protection of Wildlife Act 1972 deals specifically with protection of wild life, Fisheries Act 1985 deals mainly with the conservation and management of fisheries resources and National Forestry Act 1982 deals with the utilisation and management of forests. Some of these piecemeal laws, whether at federal or state levels, were passed without specific considerations on the issues of biodiversity conservation and management. Most of these laws were passed years before biological diversity began to take center stage and when awareness of the pertinence of preserving the global ecosystems, especially amongst the developing countries like Malaysia, was still very low.

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[61] The Food Act 1983. section 34(a) allows for labeling regulations to be made under the Act to cover matters including "Other property of any food or any ingredient or component thereof." And the Trade Descriptions Act, 1972 which makes it an offence to apply false, misleading trade descriptions to goods.

[62] See: Julie A. Caswell, "Labeling Policy for GMOs: To each His Own?" The Journal of Agrobiotechnology Management and Economics. Vol. 3, No. 1, Article 8.

[63] Peter W.B. Phillips and Grant Isaac, "GMO Labeling: Threat or Opportunity?" Journal of Agrobiotechnology Management and Economic, Vol. 1, No. 1/ Article 7.

[64] There are three kinds of biological diversity necessary for the preservation of the global ecosystem namely (i) Genetic diversity describing the variation of genes within a species; (ii) species diversity describes the number of various organisms within individual communities or ecosystems; and (iii) ecological diversity which assesses a biological community's richness and complexity. See http://www.nre.gov.my/opencms/NRE/EN/Services/Biodiversity (15/7/08). See also Cunningham, W. P. and Cunningham, M.A. (2007), Environmental Science Global Concern, Mc Graw Hill:New York (cited in Sulaiman, S.S. and Md. Khalid, R, *"Biodiversity Conservation in Taman Negara –Legal and Planning Issues",* International Conference on Environmental Conference on Environmental Research and Technology (2008), Penang.

[65] See Abdul Haseed Ansari, *"Future Directions in Conservation of Biological Diversity: An Interdisciplinary Approach".* Michael I. Jeffery et. al, Biodiversity and Conservation: Bridging in North and South Divide. 2008. Cambridge University Press: London. There are wide array of techniques for conservation from species management program in the wild to off-site protection in biotic gardens, zoos, genebanks and aquaria etc.

[66] Secretary General of the Ministry of Natural Resources speech at the International Conference on Biodiversity: Science and Governance at UNESCO Headquarters, Paris on January 24-28, 2005. Malaysia is estimated to have 15,000 plants species, 4,000 fish species and 150,000 species of insects and invertebrates.

[67] *"Status of Biological Diversity Management in Malaysia"* http://www.frim.gov.my/CHM/IFBiologicalDiversityStatus.html (15/10/2008)

In order to implement the obligations under CBD, Malaysia may face difficulty in formulating legislative as well as executive measures. Under the principle of federalism, Parliament's powers to make laws are subject to the distribution of powers and jurisdiction between federal and the States as enshrined in the Federal Constitution. Under Article 73, Parliament may make laws for the whole or any part of the Federation as well laws having effect outside as well as within the Federation while the State Legislature may make laws for the whole or any part of that particular State only. Thus, in order to realise the covenants under CBD, which was signed by the Federal Government and not by the individual states in Malaysia, it is Parliament, which is obliged to make laws in line with CBD. However, Article 74, which provides that, Parliament may only make laws with respect of any of the matters in the Ninth Schedule that are under the Federal List (First List) and Concurrent List (Third List). The State Legislature, on the other hand, may make laws with respect of any of the matters in the Ninth Schedule under the State List (Second List), which covers land matters as well as most other natural resources. Article 75 provides that in the event of inconsistency between a Federal and a State law, the Federal law shall prevail and the State law, only to the extent of inconsistency, shall be void.

However, despite the clear distribution of legislative powers between the Parliament and State Legislatures, there are still exceptional instances where the Parliament can still legislate on state matters. These exceptions will ensure that the Federal Government, can be empowered to honour their covenants under international treaties or convention such as CBD. These exceptions, as provided under Article 76 of the Federal Constitution, empower the Parliament to legislate for States in certain cases and when it involves the obligation under CBD, these exceptions are especially useful when most of the natural resources are within the States' jurisdiction. Clause 1 of Article 76 allows Parliament to make laws under State List under three instances:-

- For the purpose of implementing firstly any treaty, agreement or convention between the Federation and any other country, which includes CBD and secondly, any decision of an international organisation of which the Federation is a member.
- For the purpose of promoting uniformity of the laws of two or more States.
- If so requested by the State Legislature Assembly of any state.

If a law is enacted by the Parliament for paragraph (a), the Federal Government must first consult the government of the state concerned if it relates to Islamic Law, Malay Customs and any matters of native law or custom in Sabah and Sarawak.[68] Subject to Clause (4), any law made pursuant to paragraph (b) or (c) above cannot be enforced in any state unless adopted by a law made by the State Legislature Assembly of that state. After such adoption, the federal law shall become a state law and may accordingly be amended or repealed by a law made by the State Legislature Assembly.[69] Examples are the National Forestry Act 1982 and Fisheries Act 1985, which are both enacted under Article 76(1)(b).

The uniformity of laws as targeted under Clause 1(b), however, may not happen easily now. This is because before the 12th General Election on 8th March 2008, almost all the states were controlled by Barisan Nasional (National Front), which was also the ruling Federal Government. Therefore, party allegiance would ensure that the states would not amend any of the Federal Laws adopted. However, the position at present may be different when four major states in the West Coast are now ruled by the Opposition. Hence, there are possibilities that they will be amendments to Federal laws that have been adopted by the states and uniformity of laws throughout the whole Federation may no longer be present. Nonetheless, the Federal Government can still overcome the problem by using the excuse of Article 76(1) (a), where only the Federal Government can pass law to honour an international treaty and under such situation, the state government cannot amend the law.[70]

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[68] Clause 2 of Article 76 of the Federal Constitution.

[69] Clause 3 of Article 76 of the Federal Constitution.

[70] A law made under Article 76(1)(b) will come into force in all States upon its enactment. See also the suggestion by Abdul Haseeb Ansari in *"Legal Issues in Forest Management in India and Malaysia: A Critical Appraisal"* [2004] MLJ xxi, this mode is especially useful in ensuring a uniformed law in the country. See also Shaik Md. Noor Alam, 'Uniform Forest Code – Whose Dilemma?" Malaysian Forester, Vol. 46, No. 3, 1983.

Clause 4 of Article 76 further provides that for purpose only of ensuring uniformity of law and policy throughout the whole Federation, Parliament may make laws with respect to:-

i) Land tenure
ii) Relations of landlord and tenant
iii) Registration of titles and deeds relating to land, transfer of land, mortgages, leases and charges in respect of land
iv) Easements and other rights and interests in land
v) Compulsory acquisition of land
vi) Rating and valuation of land
vii) Local government

Clause 1(b) and 3 of Article 76 do not apply to these laws. One good example is the National Land Code 1965, which remains a Federal law and applies throughout the Peninsular Malaysia without having to be adopted by each State Legislature.

Besides the recent Biosafety Act 2007, amongst other federal laws that relate to the conservation and management of biological diversity are as follows:-

(i) National Forestry Act 1982
(ii) Malaysian Forestry Research and Development Board 1985
(iii) Wood-based Industries (State Legislatures Competency) Act 1984
(iv) National Parks Act 1980
(v) Protection of Wild Life Act 1972
(vi) Environmental Quality Act 1974
(vii) National Land Code 1965
(viii) Land Conservation Act 1956 (revised 1991).
(ix) Pesticides Act 1974
(x) Plant Quarantine Act 1976
(xi) Waters Act 1920 (Revised 1989)
(xii) Fisheries Act 1985
(xiii) Exclusive Economic Zone Act 1984
(xiv) Continental Shelf Act 1966
(xv) Customs (Prohibition of Exports Amendment No.4) Order 1993
(xvi) Aboriginal Peoples Act 1954
(xvii) Protection of New Plant Varieties Act 2004
(xviii) Abattoirs (Privatisation) Act 1993

The role played by forests is pertinent in the socio-economic and industrial development of a country. Apart from that, forests also contribute significantly in maintaining environmental stability including the protection of water resources, biological diversity and the flora and fauna.[71] The National Forestry Act 1982, stands as one of the principal legislations in the conservation and management of biodiversity in this country since forests play a major role in regulating the climatic and physical conditions of the country, safeguarding water supplies, ensuring environmental stability as well as minimising damage to agricultural lands. Tropical rainforest, as we have in Malaysia, is one the most complex ecosystems in the world.[72] Since forests fall under the jurisdiction of States under the State List[73], the National Forestry Act was formulated to uniformise and update the various state forests legislations, which were considered as deficient and weak in areas of forest conservation and management planning and in forest renewal operations, which are vital for sustainable forest

[71] See supra no.2 where according to Ansari, amongst all kinds of ecosystems, tropical rainforests ecosystem and wetland and mangrove ecosystem, both of which constitute the Malaysian forests, are the most species-rich. Although these ecosystems only cover 10% of the world's land area, they have 90% of world species.
[72] See http://www.mtc.com.my/issues/index.php (15/10/2008)
[73] Second Schedule of Article 74 Federal Constitution.

management.[74] This Act was enacted under Article 76(1) (b) that is to provide uniformity in the States of Malaysia by providing for the administration, management and conservation of forestry and forestry development throughout Malaysia.[75] This Act provides for the constitution and classification of Permanent Reserved Forests as well as excision therefrom[76] and for Forests Management and Development[77], which among others deals with granting of licence for logging of timber.[78]

In addition to the National Forestry Act 1982, the Malaysian Forestry Research and Development Board 1985 was subsequently enacted for the purpose of establishing a forest research and development institute, in the name of Forest Research Institute Malaysia (FRIM) and also for the administration of research fund in forestry. Amongst the main functions of FRIM is to conduct research into "forest development", which involves the management and development policies as well as activities for all natural and man-made forests, based on sound ecological and economic principles. In doing so, FRIM is also subjected to oversee the achievement of the expressed purposes not only of the production of forest produce but more importantly of the protection of the environment. Apart from this Act, another Federal law relating to forest is the Wood-based Industries (State Legislatures Competency) Act 1984, which confers authority on the State Legislatures to pass laws with respect to the establishment and operation of wood-based industries in their respective states.

The National Parks Act 1980 provides for the establishment and control of National Parks in Malaysia. Although all National Parks in Malaysia are located in the States, this Federal legislation applies throughout Malaysia except in the states of Sabah and Sarawak. This Act is also not applicable to the State Parks of Kelantan, Pahang and Terengganu, which collectively constitute the *Taman Negara*.[79] In Malaysia, National Parks are established to preserve and protect the wild life as well as plant life in the designated areas.[80] Apart from that, the conservation of objects of geological, archaeological, historical and ethnological and other scientific and scenic interest are also aimed to be achieved through the establishment of these National Parks.

The Protection of Wild Life Act 1972 was passed to consolidate laws relating to the protection of wildlife and to further make laws for the purpose of protecting wildlife in Peninsular Malaysia. This specific law prohibits certain activities relating to wild life without licence, permit or special permit.[81] Unlicensed persons are prohibited from the following activities:-

(i) Shooting, killing or taking any protected wild animal or wild bird or the nest or egg;
(ii) Carrying on the business of a dealer of wild animal or wild bird;
(iii) Carrying on the business of a taxidermist;

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[74] *"Policy and Legislations".* http://www.forestry.gov.my/ePolicy.html (15/10/08)

[75] See supra no. 8 at p. li where Ansari proposed that since forest is a State subject, states have to manage their forests and for that purpose, should develop their own management plan compatible with the rules of sustainable forest management as developed by the International Tropical Timber Organisation (ITTO). It is further proposed that this matter must be jointly tackled by both the Federal and States Authorities where the Forest Department should develop action plans suited to each state and the states should follow them.

[76] Part III (Sections 7-13).

[77] Chapter IV (Sections 14-60).

[78] See Section 16 where timber rights in both Permanent Forest Estates and State land Forests may be transferred by the State Authority in any of the following three ways namely tendering, negotiation or other processes such as grant and status.

[79] As described in the Schedule to the Taman Negara (Kelantan) Enactment 1938 and First Schedules to the Taman Negara (Pahang) Enactment 1939 and Taman Negara (Terengganu) Enactment 1958. The State of Perlis has its own State Parks enacted under state law.

[80] See Section 4.

[81] See Section 29. Under Section 3, a "licensed hunter" is someone who is granted a license under this act to shoot, kill or take a protected wild animal or wild bird excluding immature wild animal or immature wild bird. "Protected wild animal" are listed under Schedule Two and Schedule Five. "Wild bird" is listed under Schedule Four. However, no license can be granted to shoot, kill, take or hold in possession "totally protected wild animal" or "totally protected wild bird" as listed under Schedule One and Schedule Three respectively.

(iv) Housing, confining or breeding a protected wild animal or wild bird other than as a dealer or a taxidermist;
(v) Importing or exporting from Peninsular Malaysia any protected wild animal or wild bird or part thereof;
(vi) Keeping a trophy of any protected wild animal or wild bird[82]; and
(vii) Entering a wild life sanctuary or a wild life reserve.

Under this Act, a State Ruler or Yang di-Pertua Negeri is allowed to declare any state land to be a wild life reserve or a wild life sanctuary.[83] Entry to these wild life reserves or wild life sanctuaries is prohibited unless a written permit is first obtained from the Director for Wild Life and national Parks. Even then, the law is very clear that those capable to apply for the permit must either be a licensed hunter or someone who satisfies the Director in writing that his entry into the wild life reserve or wild life sanctuary is for the any of the purposes of art, science and recreation. For the former, his entry is limited only to a wild life reserve because in a wild life sanctuary, the acts of shooting, killing or taking any animal or bird and taking or disturbing the nest of egg of any animal or bird are totally prohibited.

Another principal federal legislation for the conservation and management of biodiversity is the Environmental Quality Act 1974, which mainly relates to the prevention, abatement and control of environmental pollution as well as the advancement of environment. Part IV of the Act deals specifically with prohibition and control of pollution, which include restrictions on pollution of the atmosphere[84], the soil[85] and inland waters.[86] This Act also prohibits the discharge of oil and wastes into Malaysian waters[87] and open burning.[88] The amendment to this Act in 1985 to include Environmental Impact Assessment (EIA) was an illustration of Malaysia's commitment to conserve its biodiversity through protecting the environment. This amendment prescribes for activities that involve forest lands including:

(i) Land development schemes converting an area of 500 hectares or more of forest land into a different land use;
(ii) Drainage of wetland, wildlife habitat or virgin forest covering an area of 100 hectares or more;
(iii) Land-based aquaculture projects accompanied by clearing of mangrove forests covering an area of 50 hectares or more;
(iv) Conversion of hill forest land to other land use covering an area of 50 hectares or more;
(v) Logging or conversion of forest land to other land-use within the catchment area or reservoirs used for municipal water supply, irrigation or hydro-power generation or areas adjacent to state and national parks, and national marine parks;
(vi) Logging covering an area of 500 hectares or more;
(vii) Conversion of mangrove forests for industrial, housing or agricultural use covering an area of 50 hectares or more;
(viii) Clearing of mangrove forests on islands adjacent to national marine parks; and
(ix) Other activities, which may affect forest, such as coastal reclamation, and hydro-power projects.

Another important federal legislation relating to the conservation and management of biodiversity is the Fisheries Act 1985. Fishing is one of the main sources of economic growth in Malaysia relying mostly on the natural resources either from the sea or rivers and also aquaculture, which is becoming more popular. The Fisheries Act 1985 deals with fisheries including the

[82] Whether in the form of skins or feathers of wild animal or wild bird, stuffed or mounted wild animal or wild bird or any horn, tusk, tooth, nail or scale.
[83] Part IV (Section 47-50).
[84] Section 22.
[85] Section 24.
[86] Section 25.
[87] Section 27 and 28.
[88] Section 29A.

conservation, management and development of maritime and estuarine fishing and fisheries in Malaysian fisheries waters as well as to turtles and riverine fishing in Malaysia. This Act applies throughout Malaysia since fisheries fall under the Federal List and for Sabah and Sarawak, fisheries fall under the Concurrent List.[89] The Fisheries Act prohibits the use of explosives, poisons or pollutants, or any electrified apparatus for fishing by imposing a fine not exceeding RM50,000/= on the violators.[90] In the realisation that marine fisheries resources in Malaysia are depleting and that the importance of coral reefs areas as critical habitats zone, the Fisheries (Prohibited Area) Regulations was enacted under the then Fisheries Act 1963 and Pulau Redang, Terengganu was declared as the first Fisheries Prohibited Area (FPA).[91] When the present Fisheries Act was enacted in 1985 to replace the Fisheries Act 1963, another three islands on the coast of Sarawak were declared as FPA.

Part IX of the Fisheries Act 1985 provides for the establishment of Marine Parks in Malaysia.[92] The main purpose of establishing Marine Parks in the country is to protect, conserve and manage in perpetuity the significant representatives of marine ecosystems, particularly coral reefs and their associated flora and fauna. Marine parks or marine reserves may be gazetted to provide special protection to the aquatic flora and fauna and to protect, preserve and manage the natural breeding grounds and habitat of aquatic life particularly of the endangered species. Other objectives are to allow for natural regeneration of depleting aquatic life, to promote scientific research, to preserve and enhance the pristine state and productivity and most importantly, to regulate recreational and other activities in such areas in order to avoid irreversible damage to its environment. The passing of Marine Parks Malaysia Order 1994[93] firmly entrenched 40 islands as protected areas, which consist of the following:-

(i) Pulau Redang Archipelago and Pulau Perhentian Archipelago off the Terengganu waters;
(ii) Pulau Payar Archipelago, off the Kedah waters;
(iii) Pulau Tioman Archipelago, off the Pahang waters;
(iv) Pulau Tinggi Archipelago, off the Johor; and
(v) The Federal Territory of Labuan Archipelago.

Other maritime-related legislations concerning biodiversity conservation are the Exclusive Economic Zone Act 1984 and the Continental Shelf Act 1966. The Exclusive Economic Zone Act 1984 regulates the activities in the Malaysian exclusive economic zone and certain parts on the continental shelf. The Malaysian Government has sovereign rights to explore and exploit, conserve and manage the natural resources[94] in the exclusive economic zone.[95] The rights to economically explore and exploit the exclusive economic zone include activities such as production of renewable energy from the water, current and winds whilst the rights to conserve and manage the natural resources, both living and non living, extend to the sea-bed and subsoil as well as superjacent water. Malaysia also has jurisdiction with regard to the establishment and use of artificial islands, installations

[89] See Article 74(1). However, since turtles and riverine fishing falls under the State List, the provision under Article 76(1)(b) applies where Parliament may make laws for the purpose of promoting uniformity of the laws or two or more States.
[90] See Section 26 and 25(1)(b).
[91] http://www.dmpm.nre.gov.my/jtl/content.php/lang. (26/10/08). Waters stretching 3 km from shore and surrounding 22 islands in the states of Kedah, Terengganu, Pahang and Johor were declared as FPA under the then Fisheries Act of 1963.
[92] Section 41 to 45.
[93] See the First Schedule of , “the limit of any area or part of an area established as a marine park shall be at a distance of two nautical miles seaward from the outermost points of the islands specified”.
[94] Natural resources are not specifically defined under this Act while under the Continental Shelf Act 1966, natural resources are specifically defined to exclude fish and turtle. The silence under the Exclusive Economic Zone Act 1984 may raise a question whether natural resources under this Act also includes fisheries resources and turtles.
[95] Section 4.

and structures[96], marine scientific research and the protection and preservation of the marine environment in the exclusive economic zone.[97] Part IV provides for the protection and preservation of the marine environment by giving Malaysia the right to exploit its natural resources in the exclusive economic zone pursuant to its environmental policies and in accordance with its duties to protect and preserve the marine environment in the zone.[98]

The Continental Shelf Act 1966 regulates the Malaysian continental shelf as well as the exploration and exploitation of its natural resources. "Continental shelf" is defined as the sea-bed or subsoil of submarine areas adjacent to the Malaysian coast but beyond the limits of the territorial waters of the States.[99] This Act defines natural resources as the mineral and non-living resources from the sea bed and subsoil as well as living organisms belonging to sedentary species.[100] Mining within the continental shelf is expressly prohibited except under the Petroleum Mining Act 1966.[101]

Waters Act 1920 (Revised 1989), which provides for the control of rivers and streams certain States in Peninsular Malaysia[102], is another important legislation relating to natural resources. The importance of water is irrefutable especially when it comes to the issues of biodiversity conservation and management. This Act expressly prohibits pollution of rivers in these States by providing that no person is allowed, except under licence, to discharge into any river the following matters:-

(a) Any poisonous, noxious or polluting matter that will render such river as harmful to public health, animal, vegetation or its other beneficial users;
(b) Any matter with temperature, chemical or biological content or effect in discolouring the water makes such river as potentially dangerous to public health, animal, vegetation or its other beneficial users;
(c) Any matter, which due its physical nature or its effect in discolouring the water makes such water difficult to treat; or
(d) Oil of any nature irrespective of whether it is used, waste or otherwise.

There are two main Federal legislations relating to land. Firstly, the National Land Code 1965, which generally provides for the registration of title to land and dealings of lands and the Land Conservation Act 1956 (revised 1991), which relates to the conservation of hill land and the protection from erosion of soil and inroad of silt. The National Land Code was enacted pursuant to Article 76(4) and shall apply only to the States of Malaya[103] and basically reflects the Malaysian Torrens System.[104]

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[96] Which may include oil rigs.
[97] See Section 4(b). Under Section 5, the following activities in the exclusive economic zone are prohibited, unless authorised:- (i) exploration or exploitation of any natural resources, whether living or non-living; (ii) carrying out of research, excavation or drilling operations; (iii) conducting any marine scientific research; and (iv) construction of or authorising and regulating the construction and use of any artificial island, any installation or structure for the purposes under Section 4 or for any other economic purposes or any installation or structure, which may interfere with the exercise of sovereign rights under Section 4.
[98] See Section 10, which provides for offences in respect of discharge or escape of certain substances into the exclusive economic zone from any vessel, land-based source, installation, device or aircraft (from or through the atmosphere or by dumping). The maximum fine imposable is RM1 million.
[99] Section 2. The surface of the sea bed or subsoil must not be greater than 200 metres below the sea surface.
[100] Ibid. "Sedentary species" means organisms which are either immobile on or under the sea-bed or are unable to move at the harvestable stage. This definition clearly excludes fish and turtle, which are both covered under the Fisheries Act 1985.
[101] Section 4. Licence must first be granted before a person can explore, prospect or bore for or carry on operations to get minerals (other than petroleum) in the sea-bed or subsoil of the continental shelf.
[102] Section 1(2), this Act shall only apply to the States of Negeri Sembilan, Pahang, Perak, Selangor, Malacca, Penang and Federal Territory. These states are formerly the Federated Malay States and the Straits Settlements. Note that the Unfederated Malay States of Kedah, Perlis, Kelantan, Terengganu and Johore are not included in this Act, which was first enacted before Independence.
[103] Section 2. States of Malaya refers to the states in Peninsular Malaysia.

The Land Conservation Act 1956, meanwhile, is more relevant to the issues of biodiversity conservation and management. This Act was enacted pursuant to Article 76(3), which requires to be adopted by the States in order to be applicable of those States.[105] Part II provides for the control of hill land where it expressly prohibits plantation of short-term crops on any hill land except under permit, which can only be issued by the Land Administrator if he is satisfied that such cultivation will not cause soil erosion.[106] Clearings and cultivation of hill land are also prohibited provided there is permit by the Land Administrator.

The Aboriginal Peoples Act 1954 provides for the protection, well-being and advancement of the aboriginal people in Peninsular Malaysia. The Act provides that the State Authority may gazette any area exclusively inhabited by aborigines as an aboriginal reserve.[107] Any area predominantly or exclusively inhabited by aborigines may also be gazetted by the State Authority as aboriginal area[108], within which there shall not be any land declared as a Malay Reservation[109] or a wild sanctuary or reserve.[110] Similarly, no land within aboriginal area shall be alienated, granted, leased or disposed to persons who are not aborigines and no licence shall be issued for collection of forest produce within an aboriginal area.

Another federal legislation related to biodiversity conservation is the Protection of New Plant Varieties Act 2004, which provides protection for farmers and plant breeders, including indigenous people, rights in conserving, improving and providing genetic resources for the cultivation of new plant varieties as well as to encourage investment and development of breeding new plant varieties. Two other relevant legislations are the Plant Quarantine Act 1976, which was enacted to amend and consolidate the laws relating to the control, prevention and eradication of agricultural pests, noxious plants and plant diseases and to extend co-operation in the control of pests movement in international trade and the Pesticides Act 1974, which regulates the use of pesticides containing active ingredients in Malaysia.

4.0 Conclusion

Malaysia has amply shown her commitment towards environmental issues and the conservation of biological resources both through effective domestic legislative, administrative and policy measures as well as active involvement in and speedy ratification of international agreements and protocols on environmental biodiversity and safe and sustainable use of resources. However despite this positive attitude towards sustainable use of resources, there are many policy and legislative aspects of safe and sustainable use of resources that require urgent revisit, especially having in mind Malaysia's obligation to comply with international protocols.

As of now, there is no single unified and comprehensive federal legislation to deal with the management, safe and sustainable use of biological resources. This is quite understandable in view of the fact that resources are under the jurisdiction of individual states. This notwithstanding there are provisions in the Federal Constitution to enable such a broad comprehensive federal law on biodiversity to be enacted. Such a law may provide for the much needed central direction and central authority to meet the many demands of the CBD and the CPB.

The present BSA 2007 cannot be faulted for appearing more stringent than the CPB, because this divergence is permitted by the CPB itself, though Malaysia needs to be wary of claims that the Act contradicts the spirit of the Protocol and may even be anti-trade in nature, or at the very last, a disguised non-tariff barrier.

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[104] Which deals in land and land tenure, the registration of title and dealings to land and collection of revenue from land.
[105] 11 States in Peninsular Malaysia have adopted this Act.
[106] Section 5.
[107] Section 7.
[108] Section 6.
[109] Under any written law relating to Malay Reservations.
[110] Section 6(2)(i)and(ii).

With or without the comprehensive federal legislation, there is indeed a dire need to immediate formulate a suitable ABS mechanism, incorporating explicit recognition and participation of owners of traditional knowledge.

Presently, the precautionary principle enacted into Section 35 of the Act has generated some debates and concern. It may well be in Malaysia's interest to review the formulation of that principle because in the ultimate analysis, the overzealous desire to be safe rather than sorry may eventually induce a culture of biotech phobia; and that of course is not the road that Malaysia's National Biodiversity Policy wants to take.

Bio-Diversity, Traditional Knowledge and Intellectual Property Is s*ui generis* the Way Forward For Malaysia?

Ida Madieha, A.

Private Law Department, International Islamic University Malaysia, Malaysia
imadieha@iiu.edu.my

Abstract

One of the obligations set under the Convention on Biological Diversity is the protection of the rights and knowledge of indigenous and local communities. This has been the issue that has been explored at various international forums such as CBD COP, WIPO'S IGC and WTO, FAO, UNCTAD, UNHCHR and WHO. Issues being investigated include access to genetic resources; benefit sharing options, biodiversity, traditional medicine and rights of the indigenous peoples. Generally, the existing discourse on traditional knowledge could be broadly classified into two major options; positive and defensive protection. The positive protection comes in a variety of forms: property rights and liability rules, customary law based regime, registration and documentation system and access and benefit sharing. Whilst the proposal for defensive mechanism is developed based on misappropriation doctrine and is heavily influenced by the rules of unfair competition. Some national countries have developed their own *sui generis* system for the protection of traditional knowledge. A quick glance on the provisions of the statutory provisions reveals that the mechanism and framework of protection differs. One model is having a regulatory framework for the protection of local communities within its intricate relationship with farmers and plant breeders. A second model is premised upon a framework that promotes conservation of biodiversity like Costa Rica. A third model is predicated upon the sovereign right of the indigenous people over their innovative contribution and development and conservation of genetic resources and biological diversities as its core preoccupation like the Philippines. The fourth model is one that sets the registration system for traditional medicine such as Thailand and China. All these models differ in terms of scope of protection and the rights conferred. This paper explores the sui generis options available and offers some recommendations on which options would be suitable to Malaysia. As the discussion at WIPO's IGC constitutes the most extensive discussion so far, this paper surveys the various policy options explored by the WIPO's IGC. As we sift through the official documents of the Intergovernmental Committee, it would transpire that all of these mechanisms are explored and representations are sought from national countries and indigenous people's group. To date, the WIPO IGC has come up with a model law on the protection of traditional knowledge and it has divided traditional knowledge into two broad knowledge system; traditional knowledge and traditional cultural expression. For that purpose, this paper would focus more on traditional knowledge as this would be beneficial to SBC and its current initiatives in documenting traditional knowledge.

Traditional knowledge within the confines of the Convention of Biological Diversity

Malaysia is an active member of the Convention of Biological Diversity[111] (the CBD) as well as the World Trade Organisation.[112] One of the obligations imposed by the CBD is the recognition and promotion of traditional knowledge.[113]

The most important provision is Article 8 (j) which contains three essential elements:

- Respect, preserve and maintain knowledge, innovations and practices or indigenous and local communities embodying life styles relevant for the conservation and sustainable use of biological diversity;
- Promote their wider application; and
- Encourage the sharing of benefits arising from its utilization.

Article 10(c) of the same convention reiterates further the objective of protection of traditional knowledge to that of 'protecting and encouraging customary use of biological resources in accordance with traditional cultural practices that are compatible with conservation and sustainable use of biological resources'. Article 17(2), reinforces this further by obliging the duty to facilitate the exchange of information relating to indigenous and traditional knowledge. Article 18(4) further declares the need to encourage and develop methods of cooperation for the development and use of indigenous and traditional technologies.

The most important observation that one could draw from Article 8 (j) is that the term traditional knowledge is understood in a narrow sense, i.e. bio-diversity related knowledge, and not traditional knowledge in its holistic sense (i.e. knowledge, innovations and practices of indigenous peoples and local communities). As made clear under from Article 8(j) of the Convention On Biological Diversity that refers 'traditional knowledge' to that of:

> 'knowledge, innovations and practices of indigenous and local communities embodying traditional lifestyles **relevant for the conservation and sustainable use of biological diversity'**.

This conclusion is supported from the preamble of the Convention that seeks to focus on the conservation of the biological diversity.[114] The corollary effect is that the Convention, does not in any way impact on the promotion of traditional knowledge in a broad sense, but purposely narrows down the scope of its ambit to those of biodiversity related traditional knowledge.

The linkage between traditional knowledge and practices to bio-diversity highlights another major point. And that is the central reason why this knowledge must be respected, preserved and conserved is because it is relevant to biodiversity conservation and sustainability and not necessarily because it is of economic value or of industrial application.

Read this way, our first impression is that Article 8(j) does not in any way require the introduction of property like or exclusive rights over traditional knowledge and practices. On this point,

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[111] United Nations Convention on Biological Diversity, June 5, 1993.

[112] The most important treaty on intellectual property concluded under the aegis of WTO is the Agreement on Trade-related Aspects of Intellectual Property Rights (TRIPS Agreement).

[113] For a more detailed exposition of the international discourse on traditional knowledge, see Charles Mc Manis, Intellectual Property, Genetic Resources and Traditional Knowledge Protection: Thinking Globally, Acting Locally, Cardozo Journal of International and Comparative Law, Vol. 11, No.2, Summer 2003, 547-583.

[114] The preamble states: "the **conservation** of biological diversity, the **sustainable use** of its components and the **fair and equitable sharing** of the benefits arising out of the utilization of genetic resources, including by appropriate **access to genetic resources** and by appropriate **transfer of relevant technologies** taking into account all rights over those resources and technologies, and by appropriate funding'.

Dutfield is of the view that Article 8(j) seems to affirm, that the holders have rights over their knowledge, innovations and practices, whether or not they are capable of being protected by IPRs[115].

Costa e Silva, on the other hand, interprets the provision as conferring rights to traditional communities over their traditional knowledge as companies have over their inventions. According to her, article 8 (j) in effect elevates the position of traditional knowledge to be at par with modern knowledge[116].

The logical conclusion is that from a reading of Article 8 (j), IPRs should not be the sole framework in which TK is being recognized, but more of in general sense, such as when TK is safeguarded, preserved or collected to ensure its continued existence.[117] Thus, the protection of TK could be achieved in a number of ways, such as through contracts and licences, or national laws governing such issues such as environmental protection, cultural heritage or the interests of the indigenous peoples. In other words, the three specific objectives set under Article 8(j) could be pursued through non IP legal mechanisms.

As stated in the IGC Report, 'the distinction between IP protection and general notions of protection of TK is highlighted by the fact that the CBD tends to refer to respect, preservation, maintenance or use of *knowledge,* rather than respect, preservation, maintenance or use of *rights in knowledge.*[118]

Another more fundamental reason why IP should not be the sole framework for which TK is to be recognized is the fact that TK is not originally developed with a commercial goal and is not intended to be commercialized in its traditional form[119]. In fact, it is the misappropriation of TK that triggers concerns that TK should be given some form of recognition.

The context and scope of traditional knowledge within the obligations of the CBD is fundamental in our effort to arrive at a proper framework of traditional knowledge in Malaysia. This point would be further explained later in this paper.

Conceptual problems with definition

The WIPO Intergovernmental Committee on Intellectual Property and Genetic Resources, Traditional Knowledge and Folklore offers a broader and more comprehensive definition of traditional knowledge. The term 'traditional knowledge' according to WIPO Document is:

Traditional knowledge that is generated, preserved and transmitted in a traditional and intergenerational context, which is distinctively associated with a community which preserves and transmits it between generations and is an integral to the cultural identity of the community which is recognized as holding the knowledge.[120]

The definition comprises of three main elements:

(i) Generated, preserved and transmitted in a traditional and intergenerational context;

(ii) Distinctively associated with a traditional or indigenous community or people which preserves and transmits it between generations; and

(iii) Integral to the cultural identity of an indigenous or traditional community or people which is recognized as holding the knowledge through a form of custodianship, guardianship, collective ownership or cultural responsibility. This relationship may be expressed formally or informally by customary or traditional practices, protocols or laws.[121]

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[115] Graham Dutfield, Intellectual Property Rights, Trade and Biodiversity, (2000), International Union for Conservation of Nature and Natural Resources, London, at p. 35.
[116] As quoted by Graham Dutfield in Intellectual Property Rights, Trade and Biodiversity, (2000), International Union for Conservation of Nature and Natural Resources, London, at p. 35.
[117] WIPO, Intergovernmental Committee on Intellectual Property and Genetic Resources, Traditional Knowledge and Folklore, Fifth Session, Geneva, July 7 to 15, 2003, WIPO/GRTKF/IC/5/8
[118] Ibid, p. 7.
[119] Ibid, p. 12.
[120] Source: WIPO/GRTKF/IC/8/5
[121] Source: WIPO/GRTKF/IC/8/5

The reason why the three elements are considered to be essential is to ensure only knowledge that has a subjective association within that community and form part of the community's self identity would be worthy of description as traditional knowledge.[122]

This definition is wider than the notion of bio-diversity related traditional knowledge as that envisaged in the Convention of Biological Diversity. It is worth noting at this juncture that having a clear definition of TK is important otherwise it does not capture the full diversity of TK and knowledge systems, and TK holding communities. The diversity in the understanding what constitute traditional knowledge would determine the scope of its protection, its main beneficiaries and the purpose of the protection.

There are, however, some major constraints to the WIPO working definition. From the IGC Report itself, these constraints have been identified:

- The notion is restricted to innovation that is cumulative and collective over generations within the one community. It does not extend to cumulative, collectively held and intergenerational traditional knowledge, unless it meets criteria for undisclosed or confidential information.
- It does not recognize collective or community ownership, custodianship or other forms of authority or entitlement over their knowledge, or distinct elements of the knowledge.[123]

A quick comparison between the WIPO model and the national country approaches in the protection of TK reveals that most of these countries have bio-diversity related knowledge to be its main focus. That is the case with Brazil, Peru and Portugal. The Panama law focuses in its law indigenous knowledge rather than traditional knowledge. Indigenous knowledge is narrower than TK and constitutes a sub set of TK. The Peru law meanwhile describes traditional knowledge as collective, cumulative and trans-generational knowledge created by indigenous peoples and communities. Portugal recognizes that traditional knowledge could also be developed individual members of the community but yet do not discard its traditional character. Such extension of traditional knowledge to individual knowledge is relevant as in some traditional communities, often knowledge is held within individuals of certain ranks, such as shamans. This type of knowledge is still passed down from generation to generation but kept secret and confidential within selected individuals in the community. One important constraint with the Brazilian law is that traditional knowledge must have real or potential value to be protectable. The ensuing question is how would this value be quantified? Would it be confined to only monetary value? Would emotional attachment, moral and spiritual value be equally considered?

Table 1 sums up the different notions of TK in the WIPO model and the national countries that have introduced specific *sui generis* laws on TK.

The WIPO definition was reached upon by drawing distinctions between two types of traditional knowledge:

(a) 'traditional knowledge' as a broad description of subject matter, generally the intellectual and intangible cultural heritage, practices and knowledge systems of traditional communities, including indigenous and local communities (TK in a general sense or *lato sensu*), and

(b) 'tradition knowledge' as the specific subject of rights and entitlements, with a more precise focus on the content and substance of knowledge as such (TK in a precise sense or strict sensu) to be distinguished from traditional cultural expressions (TCEs)/ expressions of folklore.[124]

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[122] Draft Gap Analysis on the Protection of Traditional Knowledge, WIPO Intergovernmental Committee on Intellectual Property and Genetic Resources, Traditional Knowledge and Folklore, May 2008, at p.21.

[123] Draft Gap Analysis on the Protection of Traditional Knowledge, WIPO Intergovernmental Committee on Intellectual Property and Genetic Resources, Traditional Knowledge and Folklore, May 2008, at p.23 and 24.

[124] IGC Report, WIPO/GRTKF/IC/5/8 at p. 8

Table 1. Differences in the definition of 'traditional knowledge'

	WIPO Model	**Brazilian**	**Panama**	**Peru**	**Portugal**
Elements	(i) Generated, preserved and transmitted in a traditional and intergenerational context; (ii) Distinctively associated with a traditional or indigenous community or people which preserves and transmits it between generations; and (iii) Integral to the cultural identity of an indigenous or traditional community or people which is recognized as holding the knowledge through a form of custodianship, guardianship, collective ownership or cultural responsibility. This relationship may be expressed formally or informally by customary or traditional practices, protocols or laws.	(i) Associated traditional knowledge about the uses of genetic resources (ii) Knowledge be either created or in control of indigenous and local communities (iii) Knowledge should have real or potential value	(i)TK that is owned by indigenous communities (ii) Capable of commercial use.	(i) Collective (ii) Accumulated and transgenerational (iii) Created by indigenous peoples and communities (iv) Concerning properties, uses and characteristics of biodiversity components	(i) TK that is associated to local plant varieties (ii) may either be of collective or an individual nature (iii) its creation must be traditional in the sense that it is non systematic and inserted in the cultural and spiritual tradition of the traditional communities

Table 1. Differences in the definition of 'traditional knowledge' (cont.)

	WIPO Model	Brazilian	Panama	Peru	Portugal
Constraints	- Restricted to innovation that is cumulative and collective over generations within the one community. Does not extend to cumulative, collectively held and intergenerational traditional knowledge, unless it meets criteria for undisclosed or confidential information. - It does not recognize collective or community ownership, custodianship or other forms of authority or entitlement over their knowledge, or distinct elements of the knowledge.	- restricted to knowledge that is associated to the Brazilian genetic heritage	Must be capable of commercial use	Restricted to bio-diversity related knowledge	- restricted to TK associated to local plant varieties

Differences in the scope of protection between national countries' *sui gen*eris legislation

One important characteristic of national *sui generis* approaches to protection of traditional knowledge is that they differ in terms of:

- Definition of subject matter
- Requirements of protection
- Extent of rights to be conferred
- Title-holders
- Modes of acquisition, including registration
- Duration of protection
- Enforcement measures

The differences are important as they relate to the different social needs of the country. In general there are four distinct frameworks of traditional knowledge protection:

(a) Agro-based TK
(b) indigenous people's right
(c) traditional medicine
(d) biodiversity laws

Protection of TK within agricultural industry setting

This model provides a regulatory framework for the protection of local communities within its intricate relationship with farmers and plant breeders. The main focus of this model is to set a balance of rights between the three groups involved in agriculture; elevating the position of ethno-botany within the commercial usage of plant varieties.

The African Model Legislation for the Protection of the Rights of Local Communities, Farmers and Breeders, and for the Regulation of Access to Biological Resources (2000) aims not only to recognize, protect and support the inalienable rights of local communities as well as the rights of farmers. The scope of subject matter to which the legislation applies includes biological resources, their derivatives, and community knowledge and technologies. Community knowledge here is confined to biological diversity related knowledge. The Model legislation regulates access to community knowledge and benefit sharing. Local communities have a right to refuse access to their TK if access is detrimental to the integrity of their natural or cultural heritage. The model law declares that the intellectual property rights of the local communities are inalienable. It also establishes the setting up of databases on the knowledge and technologies of local communities. In relation to benefit sharing, the model law provides that at least 50 per cent of benefits provided should be channeled to the concerned local community.

Indigenous people's right

This model is predicated upon the sovereign right of the indigenous people over their innovative contribution and development and conservation of genetic resources and biological diversities as its core preoccupation.

Philippines

One good example of such model is the Philippines' Indigenous People's Rights Act of 1997 which provides a comprehensive protection for indigenous communities' rights in general, including their rights in traditional knowledge and the rights to limit the access of researchers into their ancestral domains/ land or territories.

The Indigenous People's Rights Act of 1997 establish the rights of the indigenous people over their ancestral domains which include the right to use, manage, protect and conserve sustainable traditional resource right.[125] The term sustainable traditional resource right has been defined to include areas of economic, ceremonial and aesthetic value in accordance with the indigenous knowledge, beliefs, systems and practices[126]. The concept of ancestral lands/domains include such concepts as territories which cover not only the physical environment but the total environment including the spiritual and cultural bonds to the areas which the indigenous people possess, occupy and use to which they have claims of ownership.[127] The right over indigenous knowledge is considered to be community property that belongs to all generations and therefore cannot be sold, disposed or destroyed.[128]

Tied to the right to their ancestral domain, the indigenous peoples are given the sole right over their community intellectual rights. This includes the right to practice and revitalize their own cultural traditions and customs[129] as well as rights to religious, cultural sites and ceremonies.

Included in the concept of community intellectual rights is ethno-botanical knowledge and traditional medicine. This is made clear in Section 34 of the Act that confers the right to control, develop and protect their sciences, technologies and cultural manifestations, including human and other genetic resources, seeds including derivatives of these resources, traditional medicines and health practices, vital medicinal plants, animals and minerals, indigenous knowledge systems and practices, knowledge of the properties of fauna and flora, oral traditions, literature, designs, and visual and performing arts.

In addition, the Act further recognizes the right of the indigenous peoples in controlling access to biological and genetic resources and sustainable agro-technical development. Section 35 of the Act provides that access to biological and genetic resources and to indigenous knowledge related to the conservation, utilization and enhancement of these resources within ancestral lands belongs to the

[125] Section 3 (a)
[126] Section 3 (o)
[127] Section 4
[128] Section 5
[129] Section 32

indigenous people. Whilst, Section 36 recognizes the right of the indigenous people to a sustainable agro-technological development.

Peru

The Peru Law N. 27,811 of 2002 provides a legal framework for the promotion, respect for and the protection, preservation, wider application and development of the collective knowledge of indigenous peoples. The scope of protection is however is confined those related to biological diversity. Article 13 sets four conditions for the protection of traditional knowledge; (i) collective nature (ii) related to biological diversity (iii) developed by indigenous people (iv) not in the public domain.

As mentioned such knowledge must be collective and should not belong to individuals.[130] "Collective knowledge" means the accumulated, trans-generational knowledge evolved by indigenous peoples and communities concerning the properties, uses and characteristics of biological diversity.[131] The Law provides a framework in which access to collective knowledge is made possible. The conditions set are:

(i) such access must be subject to prior informed consent;[132]
(ii) there is an additional obligation that there must be respect for the indigenous people's spiritual values or religious beliefs.[133]
(iii) Access is also conditional on license agreement carrying terms on access and benefit sharing.[134]

One highlight of the law is that it imposes 10 per cent of the value, before tax, of the gross sales resulting from the marketing of the goods developed on the basis of collective knowledge be set aside for the Fund of the Development of Indigenous Peoples[135].

The Law categorically creates a specific positive right of the indigenous people over their traditional knowledge which is described as inalienable and indefeasible.[136] In situations where the knowledge has passed into the public domain within the previous 20 years, a percentage of the value, before tax, of the gross sales resulting from the marketing of the goods developed on the basis of the knowledge must still be set aside for the Fund for the Development of Indigenous Peoples.[137] This means that if a particular intellectual property could still be traced back to the Peru traditional knowledge, a percentage of the value of sales could still be collected.

The Law also introduces a registration system for TK, which could be registered in three types of register:

(a) Public National Register of Collective Knowledge of Indigenous Peoples;
(b) Confidential National Register of Collective Knowledge of Indigenous Peoples;
(c) Local Registers of Collective Knowledge of Indigenous Peoples.[138]

The setting up of a specific fund for the development of indigenous people is commendable as it demonstrates the seriousness of the state in recognizing the contribution of the indigenous people in the conservation of biodiversity. The fund could be used for the purpose of comprehensive development of the indigenous peoples through the financing of projects and other activities.[139]

In addition to the positive right, the law also creates negative right against the disclosure, acquisition or use of that collective knowledge without their consent and in an improper manner.[140]

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[130] Article 10
[131] Article 2(b)
[132] Article 6
[133] Article 6
[134] Article 7
[135] Article 8
[136] Article 12
[137] Article 13
[138] Article 15
[139] Article 37
[140] Article 42

The indigenous people could also bring an action claiming ownership of a specific collective knowledge and seek indemnification for the unlawful use of their collective knowledge.[141]

The Philippines and Peru approach corresponds to obligations set under the United Nations Declaration of the Rights of Indigenous Peoples that stipulates:

Indigenous peoples have the right to maintain, control, protect and develop their traditional knowledge as well as the manifestations of their sciences, technologies and cultures, including human and genetic resources, seeds, medicines, knowledge of the properties of fauna and flora, oral traditions, literatures, designs, sports and traditional games and visual and performing arts. They also have the right to maintain, control and protect and develop their intellectual property over such traditional knowledge.

In sum, an indigenous people law model, in its most ideal form, should have the following characteristics:

- Communal ownership/custodianship of traditional knowledge;
- Traditional knowledge that is not reduced to a material form;
- Duration of protection that is perpetual.

Whilst it would be ideal to have this model, however, it's effectiveness would depend substantially on the economic and legal status of the people *vis a vis* the modern world. The fact remains that many of the indigenous people's group suffer from lack of economic self-sufficiency and unequal power relations between them and the corporate world.

Traditional medicine

This model sets the registration system for traditional medicine such as Thailand and China.

China

The patent Law of the People's Republic of China of 2000 and Regulations on the Protection of Varieties of Chinese Traditional Medicine establishes semi patent like positive rights to traditional medicine. Unlike patents, the exclusive right over traditional medicine is only restricted to protect the production of the protected species. Under this law, product, method and use of traditional medicines are patentable. There is no requirement of novelty to be eligible for protection but traditional medicines should pass a quality inspection. The protection of traditional medicines is limited to medicines produced only in China and without patent protection. The period of protection is between 7- 30 years but could be renewed. The Law also provides for the use of TK databases for defensive purposes, i.e. during substantive examination of TK-related patent applications.

Thailand

The Thai system, through the Act on Protection and Promotion of Traditional Thai Medicinal Intelligence, B.E. 2542, confers protection on 'formulas of traditional Thai drugs' and 'texts on traditional Thai medicine'.[142] The term 'text on traditional Thai medicine' is defined as "the technical knowledge concerned with traditional Thai medicine or recorded in Thai books, palm leaf, stone inscription or other materials or that have not been recorded but passed on from generation to generation[143]. Whilst the term 'formulas of traditional Thai drugs' is defined as "a formula stated as the production process and ingredients which contain Thai traditional drugs, no matter what form the ingredients are."[144] The Act contains the qualification that the medicinal procedures must be based on knowledge that has been passed on from generation to generation. The registration functions on a first to file basis. The medicinal procedures that are registrable are wider than patents as it includes procedures for diagnosis, therapy, treatment or human and animals. The Act classify traditional

[141] Article 45
[142] Section 3 of the Act
[143] Section 3 of the Act
[144] Section 3 of the Act

medicinal procedures to three: (1) the national formula of traditional Thai drugs or the national text on traditional Thai medicine; (2) the general formula of traditional Thai drugs or general traditional Thai medicine document; and (3) the personal formula of traditional Thai drugs or personal text on traditional Thai medicine. The Act confers the right holder "the sole ownership on the production of the drug and the sole right over the research, distribution, improvement or development of formulas on traditional Thai drugs or intellectual property rights of traditional Thai medicine under the registered text on traditional Thai medicine.[145] The intellectual property right on traditional medicine is not transferable to others except for the case in which it is passed on by succession[146]. The IP right on traditional Thai medicine is valid for the life time of the holder and extends another 50 years after his decease.[147]

Biodiversity laws

This model promotes conservation of biodiversity like Costa Rica Biodiversity Law No. 7788, the Brazilian Provisional Measure N.2186-16 of August 2001 and Decree-Law No 118/2002 of Peru. The main premise of this model is that indigenous people and local community serve as guardians of their immediate environment and that they also possess specific knowledge of conservation and sustainable use mechanisms that can be of much use to us. The contributions of TK in the conservation of biodiversity comes in many forms – ranging from systems of indigenous management, seed selection and preservation and breeding of animals to other methods of hunting, fishing or agriculture.[148]

Costa Rica

The Costa Rica Biodiversity Law No. 7788, provides two main scopes of TK subject matter; i.e. the scope of TK to which the Law regulates access and second, the scope of TK for which the Law provides exclusive rights (i.e. community intellectual rights). TK is recognized as an intangible component within the term 'biodiversity'. Article 2 defines that "intangible components, which are: the knowledge, innovations and practices, be they traditional, individual or collective with real or potential value associated with biochemical or genetic resources, whether these are protected or not by systems of intellectual property or by *sui generis* registration systems."[149] The concept of TK is thus restricted to the knowledge, practices and innovations of indigenous peoples and local communities related to the use of components of biodiversity and associated knowledge.[150] More fundamentally, the law creates a *sui generis* community intellectual right, which is described in the law as exists and is legally recognized by the mere existence of the cultural practice or knowledge related to genetic resources and biochemicals; it does not require prior declaration, explicit recognition nor official registration.[151] The Law regulates access to biological resources and empowers the indigenous people the power to control access[152]. The law also imposes fines for exploration and bio-prospecting without proper authorization of the authorities[153]. Further, the law establishes an inventory for the registration of TK. Registration of TK, is, however, not compulsory, only voluntary[154]. In relation to access to TK, the law categorically mandates prior informed consent[155] and equitable distribution of

[145] Section 34 of the Act
[146] Section 35 of the Act
[147] Section 33 of the Act
[148] Padmashree Gehl Sampath, Regulating Bioprospecting: Institutions for Drug Research, Access and Benefit Sharing, (2005, United Nations University, USA) at p. 120.
[149] Article 7.2
[150] Article 82
[151] Article 82
[152] Article 66
[153] Article 111
[154] Article 84
[155] Article 63

benefits[156]. The law also has a defensive component in it. It is categorically declared that patents, trade secrets, plant breeders' rights, *sui gene*ris community intellectual rights and farmers' rights shall not apply to inventions essentially derived from knowledge which is associated with traditional or cultural biological practices in the public domain.[157]

India

The Indian Biological Diversity Act of 2002 provides for the conservation of biological diversity, sustainable use of its components and fair and equitable sharing of the benefits arising out of the use of biological resources and knowledge. The Act, however, only recognize traditional knowledge related to biological diversity and held by local people.[158] In that context, the term 'biological diversity' has been defined as 'the variability among living organisms from all sources and the ecological complexes of which they are a part, and includes diversity within species or between species and of eco-systems."[159] The Act pre-conditions access to TK to the attainment of approval from a National Biodiversity Authority.[160] It sets a registration system of traditional knowledge at the local, State and national levels[161] but does not expressly condition recognition only upon registration. It also provides for benefit sharing for innovations and practices associated with the use of accessed biological resources. The benefit sharing can be in the form of joint ownership of IP rights to the National Biodiversity Authority or benefit claimers.[162] The money generated from the benefit sharing is to be deposited in the National Biodiversity Fund. The money could also be paid directly to persons/ groups responsible for the access to the TK.[163] The National Biodiversity Authority is also mandated under the Act to take measures to oppose the grant of IPR in any country outside India on any biological resource obtained from India or knowledge associated with such biological resource, which is derived from India.[164]

Brazil

One permutation of the biodiversity related model is that of Brazil. The Provisional Measure N.2186-16 of August 2001 regulates access to genetic heritage and the fair and equitable sharing of the benefits deriving from exploitation of associated traditional knowledge. Access to associated traditional knowledge is defined as the acquisition of information pertaining to knowledge or individual or collective practices, associated with the genetic heritage, of an indigenous or local community for purposes of scientific research, technological development or biological prospection, with a view of its application in industry or elsewhere.[165] The Provisional Measure recognizes TK but it must relate to the genetic heritage, belong to an indigenous or local community and have real or potential value.[166] Indigenous or local communities are guaranteed the rights on the condition that they created, developed, held or preserved the TK.[167] In many ways, the rights of the indigenous people are recognized but only to the extent of their contribution in the conservation of bio-diversity. The Provisional Measure confers a number of exclusive rights:

(i) To have the origin of access to TK mentioned in all publications, uses, exploitation and disclosures;
(ii) To prevent unauthorized third parties from:

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[156] Article 63(3)
[157] Article 78
[158] Article 36(5)
[159] Article 2(b)
[160] Article 20(1)
[161] Article 36(5)
[162] Article 21(1)(a)
[163] Article 21(3)
[164] Article 18(4)
[165] Article 7 (V)
[166] Article 7.II and 8
[167] Article 9

(a) Using or carrying out tests, research or investigations relating to associated TK;
(b) Disclosing, broadcasting or rebroadcasting data or information that incorporate or constitute associated TK
(c) To derive profit from economic exploitation by third parties of associated TK the rights in which are owned by the community.[168]

The Provisional Measure declares that any associated traditional knowledge may be owned by the community, even if only single member of the community holds that knowledge.[169] The Measure sets up an indemnity system whereby any unauthorized economic exploitation of TK would be subjected to an indemnity of 20% of the gross invoiced amount obtained through the marketing of the product or the royalties obtained from third parties as a result of the licensing of the products.[170] The Measure also incorporates prior informed consent, disclosure requirements and benefit sharing within its framework.[171]

Portugal

Portugal, through Decree-Law No 118/2002, confers sovereign rights of States over their genetic resources and the fair and equitable allocation of benefits arising from their use as specified in the Convention on Biological Diversity. The Decree establishes the legal regime for the registration, conservation, legal safeguarding and transfer of autochthonous plant material.[172]

Traditional knowledge is defined under the Decree to comprise all intangible elements associated with the commercial or industrial utilization of local varieties and other autochthonous material developed in a non-systematic manner by local populations, either collectively or individually, which form part of the cultural and spiritual traditions of those populations. That includes, but it not limited to, knowledge of methods, processes, products and designations with applications in agriculture, food and industrial activities in general, including traditional crafts, commerce and service informally associated with the use and preservation of local varieties and other spontaneously occurring autochthonous material.[173] The Decree set a registration system for TK known as the Register of Plant Genetic Resource (RRGV). The benefits of registration are that it affords its owners the following rights:

(i) The right to object to its direct or indirect reproduction, imitation and/or use by unauthorized third parties for commercial purposes;
(ii) The right to assign, transfer or licence the rights in the traditional knowledge including transfer by succession;
(iii) The right to exclude from protection any traditional knowledge that may be covered by specific industrial property registrations.[174]

The registration of traditional knowledge is effective for a period of 50 years from the date of application.[175] The Decree sets the condition in which access to germ plasm is allowed; i.e. subject to prior authorization by the authorities.

SWOT analysis of the various *Sui Generis* approaches to protection of TK

(i) The Brazilian model is the most comprehensive in terms of protection of bio-diversity. It recognizes the communal property rights over its traditional knowledge. It has within its framework broad and strong exclusive rights conferred to both local community and

[168] Article 9
[169] Article 9
[170] Article 26
[171] Article 16
[172] Article 1
[173] Article 3(1)
[174] Article 3(4)
[175] Article 3(6)

indigenous people. It also provides for access and benefit sharing and prior informed consent. It sets up a database system but does not condition the protection of TK on registration. It also recognizes individual ownership of knowledge within the confines of traditional community.

(ii) The Indian biodiversity law is the closest to spirits of the Convention of Biological Diversity. Knowledge of local people is only recognized if it relates to biological diversity. The Indian law also provides for access and benefit sharing. It goes further than the CBD by establishing a national register of TK for defensive purposes. The Act establishes a National Biodiversity Authority, the body responsible for the monitoring of access to TK as well as the usage of Indian TK overseas.

(iii) The traditional medicine model would best work in national countries where the usage of traditional medicine is considered to be a mainstream treatment. In China registration confers exclusive rights to produce medicines in China. It does not have a norm of preventing others from misappropriating traditional medicinal knowledge. The establishment of the traditional medicine database, however, acts as defensive protection against unlawful patenting of Chinese traditional medicinal practices than others. It is commendable as it elevates the status of Chinese traditional medicine to be at par with modern medicine.

(iv) The indigenous people system takes the rights of the indigenous people as its central pre-occupation. However, not all knowledge of the indigenous people is recognized, only those related to bio-diversity conservation. The Peru model goes further to alienate individual knowledge. The restriction of traditional knowledge to only cumulative and collective knowledge ignores the fact that within the domain of traditional knowledge system, there is three contested domains, (i) knowledge in the public domain; (ii) community knowledge and (iii) individual knowledge, innovations and/or practices.[176] For example, there may be some individuals that have inherited some knowledge from their forefathers and may have practiced it faithfully with or without modification. It is also often the case with traditional healers or shamans that have knowledge of herbal plants and how to calibrate the dose and combination of herbal drugs according to the condition of the patient. This knowledge is commonly kept closed and not freely shared within traditional system.

TK and IP: The gap

One approach to accommodate TK within the existing legal framework is through the extension of the existing intellectual property right concepts.[177] Some aspects of TK could be protected through various types of intellectual property as summarized in Table 2.

Proponents for the extension of IPRs cite a number of reasons why an IP approach is the best mode of conferring rights over traditional knowledge:

- IP could empower TK holders against unauthorized commodification of their TK. The presence of well-defined, enforceable property rights would pre-condition any access to TK. With IPRs, the traditional community and indigenous people would have a more entrenched position to bargain for higher benefit and compensation for the usage of their TK. Whilst the same effect could be achieved through contractual negotiation, the absence of any legal claim over TK would reduce the value that could have otherwise been claimed from the usage of the TK. In worst case scenario, it may reduce their entitlement to TK to zero if not at a very minimal sum.
- The promise or intellectual property rights on traditional medicinal knowledge is recognition and benefit sharing for communities in drug discovery and research, apart from incentive generation for biodiversity conservation. TK could be a major contributor in the economic development of the indigenous community and alleviate poverty.

[176] See Anil Gupta, WIPO-UNEP Study on the Role of Intellectual Property Rights in the Sharing of Benefits Arising From The Use of Biological Resources and Traditional Knowledge.

[177] For a more detailed discussion on this, see Carlos M Correa, Traditional Knowledge and Intellectual Property: Issues and Options Surrounding the Protection of Traditional Knowledge: A Discussion Paper, Quaker United Nations Office, Geneva, November 2001.

Table 2. IPR related TK protection

Type of IPR	Subject matter of protection	Conditions for protection
Patent	Specific innovations developed within traditional context	(i) novel (ii)involves inventive step (iii)useful or industrially applicable
Distinctive signs	Conventional trade marks Certification and collective marks Geographical indications	Distinctive
Industrial design	Traditional design	New Not applicable to functional designs
Copyright	All forms of expression including traditional cultural expression	original
Unfair competition		Acts that create confusion with the establishment, goods or industrial or commercial activities of a competitor False allegations in the course of trade to discredit a competitor's establishment, goods, or industrial or commercial activities Indications or allegations liable to mislead the public as to the nature, the manufacturing process, the characteristics, the suitability for their purpose, or the quantity, of goods
Trade secrets	Undisclosed TK	Be secret; not generally known among or readily accessible to persons within the circle Have commercial value Reasonable steps taken to ensure the secrecy of the information

Source: Draft Gap Analysis on the Protection of Traditional Knowledge, WIPO Intergovernmental Committee on Intellectual Property and Genetic Resources, Traditional Knowledge and Folklore, May 2008

Admittedly, many national countries have explored the applicability of IPRS to TK. WIPO has studies how countries use intellectual property tools for promoting TK and traditional cultural expression (TCEs). The use of existing IPR tools to TK by national countries could be said to better explored than the *sui generis* approach. Countries like Australia, Canada, New Zealand have illustrated how useful IPRs could be in the promotion of TK and TCEs. In Australia for example, the courts have adopted IPR to give redress for unlawful appropriation of TK in cases like *Foster v Mountford,*[178] *Milpurrurru v Indofurn Pty Ltd*[179], *Bulun Bulun and Milpurrur v R. and T. Textiles Pty. Ltd.*[180] and *Bulun Bulun v Flash Screenprinters.*[181] In New Zealand, both geographical indication and certification marks are used to preserve the authenticity of traditional products.[182] Marks which may offend indigenous people would also be rejected. Industrial design has also been sought for traditional designs as has been practiced in Australia, Costa Rica, Kazakhstan, the Russian Federation, Tonga,

[178] (1976) 29 FLR 233.
[179] (1995) 30 IPR 209.
[180] (1998) 41 IPR 513
[181] See also the discussion in (1980) EIPR Vol. 2, pp. 346-355.
[182] For further discussion, see Bernard O'Connor, Protecting Traditional Knowledge: An Overview of a Developing Area of Intellectual Property Law, The Journal of World Intellectual Property, Vol. 6 No. 5, September 2003, at p. 677.

and Uruguay. The reliance on trade secrets law is also common in some countries such as Canada, Hungary, Indonesia and the United States of America.[183]

IPRs is amenable to cover TK in three specific ways; content, expression and reputation as expressed in Fig. 1.

Fig. 1. TK related-IP protection

(a)	Protection extended to the content, substance, or idea of knowledge and culture (such as traditional know how about the medicinal use of a plant, or traditional ecological management practices)
(b)	Protection extended to the form, expression or representation of traditional cultures (such as traditional song, performance, oral narrative or graphic design)
(c)	Protection extended to the reputation and distinctive character of signs, symbols, indications, patterns and styles associated with traditional cultures, including the suppression of misleading, deceptive and offensive use of the subject matter

Source: WIPO/GRTKF/IC/5/12

In addition, IPR could be extended to cover TK in both positive and defensive sense. The term 'positive protection' means the conferral of exclusive rights to the TK holders if it falls within the existing subject matter of IPR. The term "defensive protection" when applied to TK and genetic resources, refers to measures aimed at preventing the acquisition of intellectual property rights over TK or genetic resources by parties other than the customary custodians of the knowledge or resources.[184]

Table 3. Accommodation of TK within IPR

Positive IP protection	**Defensive IP Protection**
Existing IP systems applied to TK subject matter	Focus is to prevent others from asserting or acquiring IP rights over TK subject matter
Adaptations and *sui generis* elements of existing IP systems to ensure their application to TK subject matter (for instance, the incorporation of TK subject matter in the IPC	Making information available to patent and trade mark examiners for examination purposes
Stand alone *sui generis* IP systems, whether for the protection of the content of TK as such, for the protection of TCEs or expressions of folklore, or for both content and expression.	Recognition of certain official traditional signs or marks
	The development of distinct classes or sub-classes for TK in the International patent classification
	Specific disclosure obligations in relations to patents for inventions derived from genetic resources and associated TK

Source: WIPO IGC on IP and Genetic Resources, TK and Folklore, Draft Gap Analysis on the Protection of TK

However, IP alone could not satisfy all the interests that need to be fulfilled. The promotion of TK through IP could lead to unmet needs as there are gaps in the protection of TK through IP. These gaps could be classified into four groups:

[183] For a whole list of countries that have explored IP mechanisms to protect TK, see the WIPO Report, Intergovernmental Committee on Intellectual Property and Genetic Resources, Traditional Knowledge and Folklore, Fifth Session, Geneva, July 7 to 15, 2003, WIPO/GRTKF/IC/5/8.
[184] For details, see WIPO/GRTKF/IC/6/8.

(i) Subject matter not covered under existing IP law;
(ii) Beneficiaries or right holders not recognized;
(iii) Forms of use and other actions that cannot be prevented;
(iv) Absence of entitlement to obtain remuneration or other benefits.[185]

What is the type of TK subject matter that would not be covered by existing forms of IP protection? Several of them have been identified which include:

- TK which is considered not novel, by virtue of having being disclosed to the public in a relevant manner;
- TK which is considered obvious, including obvious to practitioners or holders of traditional knowledge as persons skilled in the relevant art, with reference to other knowledge already available to the relevant public;
- TK which has been publicly disclosed and otherwise does not meet the criteria for protection of confidential information, trade secrets, or undisclosed information;
- Protection does not extend to cumulative, collectively held and inter-generational traditional knowledge, unless it meets criteria for undisclosed or confidential information;
- Protection does not extend to an integrated traditional knowledge system as such, and at best existing protection may only extend to certain, isolated elements of knowledge within a TK system;
- Recognition of the rights of an indigenous or local community as such as having rights, authority, custodianship or other entitlements over knowledge within a traditional knowledge system that is distinctively associated with them;
- There is no express norm against illegitimate patenting of TK;
- There is no specific disclosure requirement relating to TK;
- There is no explicit requirement to suppress unjust enrichment from or misappropriation of TK;
- There is no express principle of free prior informed consent over TK held by a recognized indigenous or local community;
- There is no right to object to the use of TK without explicit acknowledgement of the community which is the source of the knowledge;
- There is no right to object to the use of TK when the use creates cultural or spiritual offence, or otherwise impairs its integrity;
- Absence of entitlement to obtain remuneration or other benefits.[186]

In Malaysia, however, no serious effort has been taken to incorporate TK related provisions into the current IPR legislative framework. Fig. 2 illustrates the gap analysis in terms of subject matter of protection alone.

Fig. 2. IPRs and TK: Gap Analysis of the Malaysian IPR legislative framework.

Patent
Absence of provisions requiring prior informed consent and evidence of benefit sharing, procedure for examining TK related patent application

Trade Mark
Absence of provisions on traditional distinctive marks. Lack of promotion on the use of geographical indications and certification marks for traditional products.

Copyright

[185] WIPO IGC on IP and Genetic Resources, TK and Folklore, Draft Gap Analysis on the Protection of TK, at p. 23.
[186] WIPO IGC on IP and Genetic Resources, TK and Folklore, Draft Gap Analysis on the Protection of TK, pgs 23-28.

Absence of provisions on traditional cultural expression.

Industrial Design
Absence of provisions on traditional designs

Despite that, Malaysia has progressed in the area of plant varieties. The New Protection of Plant Varieties Act 2004, has commendably given expression to the Convention of Biological Diversity with its express recognition of traditional variety, elevation of local community and farmer's interest, mandatory disclosure of genetic origin requirement and mandatory disclosure of access and benefit sharing in cases of varieties developed from traditional variety. Malaysia has also made significant progress in documenting traditional knowledge from the efforts of certain institutions at state level like the Sarawak Biodiversity Centre.

Fig. 3. Bridging the gap: The Milestones.

The New Protection of Plant Varieties Act 2004
Manage the balance between four groups of people involved in the conservation, development and commercialization of plants; i.e. local community, farmers, indigenous people and commercial breeders.

Documenting traditional knowledge
Efforts have been taken at institutional and state level to document traditional knowledge to ensure that the knowledge is not lost and for future commercialization.

Policy options for Malaysia

There is much work to be done in Malaysia. Despite being an active member of the CBD, we are taking too long in debating which route to take. As I see it, there are three main options;

i) To have a full blown biodiversity law with elements of access of benefit sharing, prior informed consent and protection of TK such as India;
ii) To have a full blown indigenous people's protection law recognizing their communal property over their TK; or
iii) To revise the existing IPRs to make them more amenable to TK, with both positive and negative rights incorporated within its legal framework.

Whatever option we take, either (i) or (ii), it does not take away the need to work on (iii). The short term solution is by revising the IPR framework. Perhaps work on (i) and (ii) would take time but option (iii) would be considered as most immediate. Though IPR protection is patchy and covers only some products based on traditional knowledge and is not considered as protection of the knowledge *per se*, it's importance should not be dismissed lightly.

It is futile to introduce a full blown indigenous rights system based on human rights perspective if there is no proper recognition of their rights over their landraces and proper understanding about how traditional communities generate, use, manage and control their knowledge. This model would require the conferral of special rights to indigenous people. The law could only be enforced effectively if there are strong groups representing the indigenous people's interest. This in turn depends on how entrenched the position of the indigenous people's are, whether they are legally recognized to have self determination and whether their native customary rights and cultural heritage is properly given attribution. The fate of an indigenous system's model depends substantially on communal rights on land. In reality, it requires the existence of customary rules relating to ownership, custodianship, entitlement and equitable interests.

Secondly, such model confers rights exclusively to indigenous people. It is also possible that TK may be held by other local and cultural communities who are not recognized as being indigenous. In fact, indigenous knowledge is only a sub set of traditional knowledge.

This is one area in which one size does not fill all. It is imperative that we weigh our options carefully and take decision based on what is appropriate to our national context. What are our main concerns?

Whatever option that we take, either (i) or (ii), there must be explicit acknowledgement of source of TK-based creations and innovations, and the control of their use and exploitation of TK and benefit-sharing. This would have been accommodated satisfactorily through a suitably framed access and benefit sharing legislation. The design of a *sui generis* legal framework would require close collaboration with the TK holders and communities. It is not an over statement to state that framing an effective *sui generis* framework would require nation-wide consensus building.

The other concern is whether we want to address TK in its holistic sense, including ethical, environmental and socio-economic concerns. As shown in some of the *sui generis* models, TK has been restricted to either agricultural related or biodiversity related. Is this what we want?

Another paramount constraint of the *sui generis* legal framework is that it does not have any extra territorial effect. Would this be effective in curbing piracy and misappropriation of TK that is occurring across territorial constraints?

Another option that has not been discussed here is the misappropriation model as proposed by the WIPO. However, it is felt that the misappropriation model would not work if the communities are not given the right to control access to their land.[187]

It has often been stressed at WIPO that TK should be given a comprehensive protection, using both positive and defensive sense. Concerns have been raised as to the documentation effort of oral TK for defensive purpose, could in turn, accelerate or facilitate its misappropriation, including its commercial use by third parties without the prior informed consent of the holders of the TK.[188]

Whatever legal options that we choose, it must contain the following legal norms:

(i) Recognition of TK holder's right over their TK and the intrinsic value of TK;
(ii) Right to control access to the TK
(iii) Right to prevent misappropriation
(iv) Right to preclude the granting of unauthorized IP rights
(v) Seek to ensure prior informed consent and exchanges based on mutually agreed terms
(vi) Promote equitable benefit-sharing
(vii) Promote conservation and sustainable use.

Fig. 4. Policy options for Malaysia. Gaps that need to be addressed.

1. Misappropriation model: an express norm against illegitimate patenting of TK
   Protection against unjust enrichment or misappropriation of TK
2. Access and Benefit Sharing
   Equitable sharing of the benefits, particularly when the use entails financial or commercial gain as a collective right
   There is a need to strengthen the framework of contractual obligations governing access to TK under national law to require disclosure and other conditions of access to TK
3. Biodiversity laws
4. A specific disclosure requirement on TK
5. Prior informed consent
6. Community IPR, including the right of acknowledgement and integrity

[187] For a lengthier discussion on this, see, Graham Dutfield, 'Protecting Traditional Knowledge: Pathways to the Future, ICTSD Issue paper No. 16, (2006) ICTSD, Geneva.
[188] For details, see WIPO/GRTKF/IC/6/8.

National Policy on Biological Diversity

Noor Haliza, A.H.

Ministry of Natural Resources and Environment, Malaysia
noorhaliza@nre.gov.my

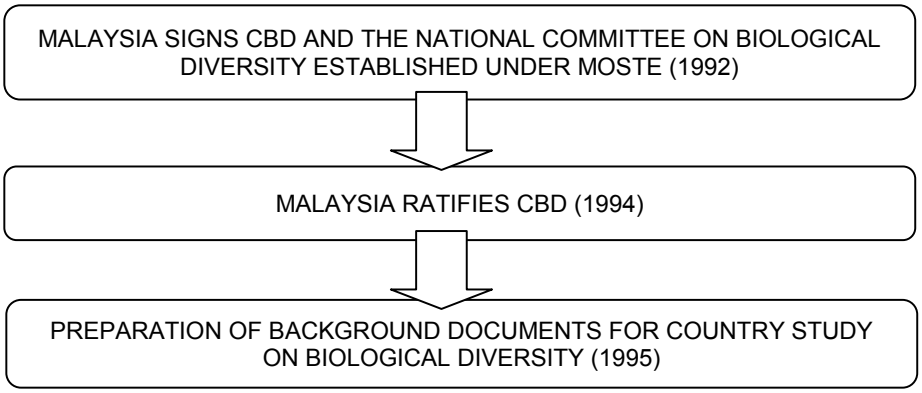
Abstract

As one of the 12 megadiverse countries, Malaysia takes biodiversity conservation and management very seriously. Malaysia ratified the Convention on Biological Diversity (CBD) in the year 1994 as a commitment to manage and sustainably utilize her biodiversity. To further strengthen domestic implementation and to fulfil our obligations under CBD, the National Policy on Biological Diversity was launched in 1998. The policy outlines 15 strategies and 87 plan of action in order to fulfill its vision to transform Malaysia into a world centre of excellence in conservation research of biodiversity by year 2020. Numerous activities have been implemented since the Policy is adopted. Nevertheless the most significant activities in terms of biodiversity management and conservation implemented so far are the establishment of National Council of Biodiversity-Biotechnology in 2002 and the set up of Ministry of Natural Resources and Environment in 2004. Cooperation and participation from all the stakeholders are vital to ensure the successful implementation of the activities planned towards realizing the vision enshrined the Policy.

Introduction

Malaysia is one of the 12 `Megadiversity' countries of the world. Megadiversity countries are the 12 nations with the greatest biological diversity and species endemism. These nations together hold about 60 to 70 percent of the world's biodiversity. Malaysia ratified the Convention on Biological Diversity (CBD) on 24th June 1994 and it entered into force 3 months later. To date, 191 countries have signed the convention. As a party to the convention, Malaysia is required to develop national biodiversity strategies and action plans and to integrate these into broader national plans for environment and development. In this regard, Malaysia has established a National Committee on Biological Diversity to plan, coordinate and implement actions as required under the Convention and also to address national issues on biodiversity

The National Committee on Biodiversity formulated the National Policy on Biological Diversity (NPBD) in 1998 to provide direction and strategies to all government agencies, including the state governments on the conservation and management of biological diversity in the country. The NPBD sets the vision of transforming Malaysia into a world centre of excellence in conservation, research and use of tropical biology by the year 2020. To accomplish this vision, **15 strategies and 87 action plans** outlined in the NPBD have been carefully selected and prioritized taking into consideration the importance to balance development with conservation and protection of Malaysia's biological resources and the environment. Fig. 1 clearly demonstrates the chronology for establishment of NPBD.



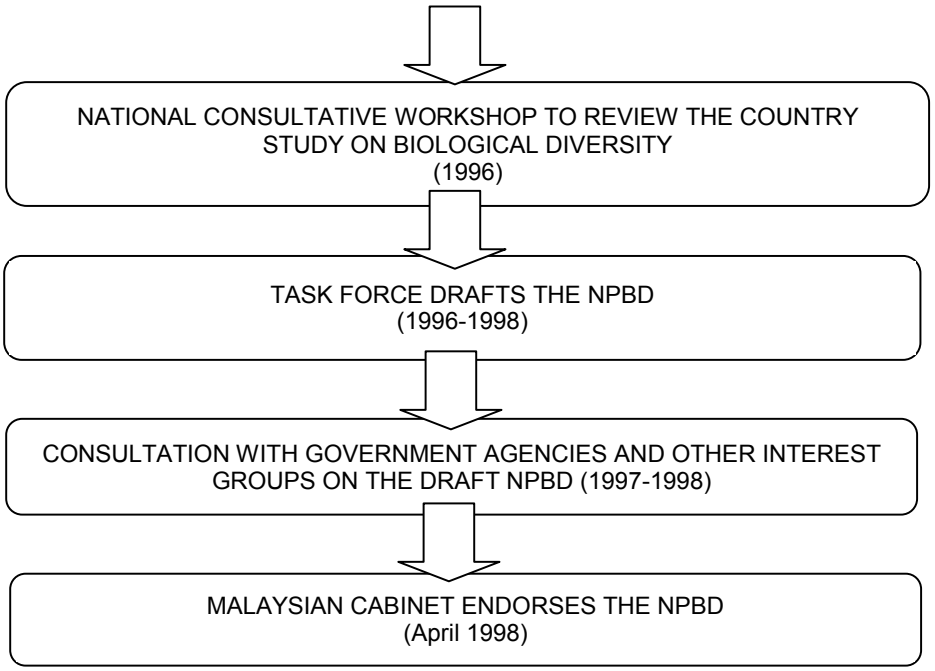


Fig. 1. Chronology for establishment of NPBD.

Strategies in National Policy on Biological Diversity

The strategies in the policy were ranked based on relevance and priority for implementation. Each of the strategies is further refined into 87 action plans which are to be implemented to achieve the goals of the Policy. These action plans will be implemented by various sectoral agencies according to their mandate and ability. NRE takes the lead in implementing actions that require cross-sectoral coordination, as well as those which do not fall under the purview of any one sectoral agency. The 15 strategies are listed as below;

Strategy I	Improve the scientific knowledge base
Strategy II	Enhance sustainable utilization of the components of biological diversity
Strategy III	Develop a Centre of Excellence in industrial research in tropical biological diversity
Strategy IV	Strengthen the institutional framework for biological diversity management
Strategy V	Strengthen and integrate conservation programmes
Strategy VI	Integrate biological diversity consideration into sectoral planning strategy
Strategy VII	Enhance skill, capacities and competence
Strategy VIII	Encourage private sector participation
Strategy IX	Review legislation to reflect biological diversity needs
Strategy X	Minimize impacts of human activities on biological diversity
Strategy XI	Develop policies, regulations, laws and capacity building on biosafety
Strategy XII	Enhance institutional and public awareness
Strategy XIII	Promote international cooperation and collaboration
Strategy XIV	Exchange of information
Strategy XV	Establishing funding mechanism

Implementation status of National Policy on Biological Diversity (NPBD)

Conservation of biodiversity is cross-sectoral in nature and the implementation of the policy involves many federal ministries, state governments and other stakeholders. Implementation of the policy is

mostly done based on sectors, with some agencies playing an important role without necessarily realising it. Issues also arise due to the fact that under the Malaysian Constitution, land (including forest) and water lie under State jurisdiction and therefore, some decisions face challenges in implementation. Since NPBD was launched, various activities have been implemented. However, the two most significant milestones thus far in terms of biodiversity management are the establishment of The National Biodiversity-Biotechnology Council (MBBN) in 2002 and the set up of The Ministry of Natural Resources and Environment (NRE) in 2004.

The National Council on Biodiversity and Biotechnology (MBBN) was established in 2002 to strengthen biodiversity management in Malaysia. The council which comprises of all key federal ministers and 13 state chief ministers is the highest body in decision making for biodiversity management in Malaysia. The Council is chaired by the Prime Minister himself. The thrust of this council is to set policy and implementation direction in managing, conserving and sustainable use of biodiversity. This council also serves as a driving force for the national biotechnology agenda by capitalizing on Malaysia's rich biodiversity. Since its establishment, the council has met 5 times and among decisions made at the last council held in 29 September 2006 were to table the much awaited and highly debated of Biosafety Bill in Parliament and to sow the seed for the establishment of the Natural History Museum (NHM). The council also directed all states to improve existing conservation programmes of marine coastal, river basin and mangroves areas. All these decisions are in line with Malaysia's commitment to CBD and the NPBD.

The establishment of The Ministry of Natural Resources and Environment (NRE) in April 2004 reflects a strong signal from the Government in sharing her commitment on biodiversity conservation and management. Under the new set-up, all the biodiversity related agencies and departments such as Department of Forestry, Wildlife Department, Marine Park Section, Drainage and Irrigation Department and the Department of Environment were for the first time brought under one ministry. The formation of NRE allows for a more integrated approach on biodiversity conservation and management in Malaysia. Listed below is a summary of some programmes and activities taken up by the stakeholders pertaining to the implementation of the policy.

Table 1. Summary of some activities implemented with regards to the NPBD's strategies

Strategy	Implementation
Strategy I	• listing of literature on vascular and non vascular plants, fungi, vertebrates (estimate: 12,500 species), and also establishment of the Rainforest Tropical Centre at Forest Research by FRIM • Biodiversity inventory by FRIM. • Establishment of Natural History Museum
Strategy II	• Sustainable Forest Management • Introduction of Environmentally Sensitive Areas in planning via National Physical Plan • National Parks and Reserve Management Plans by Wildlife Department
Strategy III	• All departments and government agencies to have regular capacity building programmes, education and training programmes for staff members
Strategy IV	• Establishment of MBBN (2001) • Establishment of NRE (2004)

Strategy V	• A number of parks have been gazetted in the last five years such as;
	  • Penang National Park
	  • Royal Belum State Park
	  • Selangor State Park
	  • Sarawak RAMSAR site
	  • Lower Kinabatangan-Segama RAMSAR site

Strategy VI	• The National Physical Plan (NPP) is established in 2005 to include conservation of natural resources and environment as a major element in the national physical development.

Table 1. Summary of some activities implemented with regards to the NPBD's strategies (cont.)

Strategy	Implementation
Strategy VII	• Malaysia is taking steps to increase the number of young graduates to be trained in the fields of botany, entomology, zoology and biotechnology at local universities. • Collaboration with International Organizations to enhance skill, knowledge and capacity in all sectors.
Strategy VIII	• The government is currently reviewing the Wildlife Act 1972 to place more emphasis on biodiversity conservation. Malaysia is also currently preparing legislation to regulate the access to biological resources, including forest resources, and the sharing of benefits resulting from the utilization of the resources.
Strategy X	• The establishment of Genetic Modification Committee (GMAC) in 1998 provided administrative and monitoring mechanism on transboundary movement of GMO. • EIA provisions on assessment of fauna and flora for some specific development projects.
Strategy XI	• The Biosafety Bill has been passed by the Parliament in 2007 and the regulations are now in the final stage to be enforced.
Strategy XII	• NRE are engaged in organizing seminars and conferences including international conferences to exchange knowledge and expertise on biodiversity research and management. Media coverage on biological diversity issues is also enhanced.

Strategy XIII	• Malaysia established collaboration and cooperation with several developed countries such as:
	  • Bornean Biodiversity and Ecosystem Conservation (BBEC) project (with the Japanese government)
	  • Danish government (DANIDA)
	• Malaysia collaborates with international organizations such as;
	  • Global Environment Facilities (GEF)
	  • International Timber Tropical Organization (ITTO)
	  • International Treaty Plant Genetic Resources for Food and Agriculture (ITPGRFA)
	• The Heart of Borneo conservation project is also an initiative to strengthen collaboration of three countries namely Malaysia, Indonesia and Brunei in biodiversity conservation

Strategy XV	• RMK 9 and Global Environment Facility (GEF) Fund

The way forward

The Malaysian government recognizes that there is still much to do in order to sustainably manage its biodiversity. Therefore, we have recognized several issues that need to be overcome in order to strengthen the biodiversity management in Malaysia;
Legislation on emerging issues in biodiversity management such as IP, ABS and biosafety need to be strengthened.
Further develop skills, capability and competency especially in the area of taxonomy. The government needs to inspire more young Malaysians to enroll in taxonomy.
Strengthening collaboration among stakeholders. The federal and state relation posed difficulties in managing biodiversity as land, water, forestry are under state jurisdiction. There is need to have strong cooperation, understanding and collaboration between different stakeholders mainly the federal government and state governments.

Conclusion

Malaysia is committed to the conservation and environmental management of her invaluable biological resources. This commitment is clearly envisaged in the NPBD based on the outline strategies and plan of action. Nevertheless, the NPBD cannot be implemented in isolation. Cooperation and complete participation from all the stakeholders are necessary to ensure the activities planned are implemented successfully.

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The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA): Global Rules for Food Security

Mohd Shukor, N, Zulhairil, H., Mohd Shafie, M.S. and Mohd Norfaizal, M.Z.

Biological Resources Management and Utilization Program,
Strategic Resource Research Center, MARDI Headquarters,
P.O. Box 12301, General Post Office,50774 Kuala Lumpur, Malaysia
dino@mardi.gov.my

Abstract

Never before in the history of modern Malaysia has the general population at all levels been threatened by food security. Food is becoming less available and more expensive. Many factors, both long- and short-term, have contributed to the shortage. At a global level, available data seems to indicate that we have been consuming more than we produce. Then came the shortfall in global rice production caused by the impact of climate change such as the widespread drought in India and China in 2002, typhoons in the Philippines in 2006, and the major flooding in Bangladesh in 2007. This was followed by the return of pests such as plant hoppers, and the various virus diseases transmitted particularly at regions with growing seasons having abnormally higher temperatures caused by climate change. Since the crisis started, scientists, particularly breeders all over the world were requested to strengthen and upgrade tbreeding programs and facilities for the development of new varieties with increased tolerance to drought, flooding, and salinity as well resistance to insects and diseases. An important pre-requisite for such activities is the free flow of plant genetic resources for food and agriculture (*pgrfa*). The adoption of the Convention on Biological Diversity (CBD) has drastically slowed down the movement of many plant genetic resources and this has threatened the future supply of food. The International Treaty on Plant Genetic Resource for Food and Agriculture (ITPGRFA or the Treaty) is a global reaction to the rising tide of measures taken by many governments as a result CBD to extend their sovereign control over genetic resources. Many countries felt that those measures are inappropriate for food and agricultural crop genetic resources. The Treaty on the other hand recognizes that access and benefit sharing for agricultural biodiversity must be treated differently from the way it is generally treated under CBD. This paper discusses some of the key points and provisions from the treaty and some issues arising from its negotiation and future implementation in Malaysia.

Introduction

The availability of new and better varieties is important to food security and to develop those varieties, plant breeders need genetic resources. Most countries, including Malaysia, are dependent for at least 60% of their genetic resources from other countries. In the past, the required genetic resources used to be shared or exchanged freely among farmers and countries. After the adoption and enforcement of the Convention on Biological Diversity (CBD), the situation has changed. CBD has brought with it various measures that extend private or sovereign control over genetic resources for food and agriculture. Many felt that access and benefit sharing for agricultural biodiversity especially *pgrfa* should be treated differently from the way it is treated under the CBD. CBD relies heavily on legal procedures in many of its dealings and this has caused a **sharp decline in** the **flow of genetic resources** worldwide and this in turn has seriously threatened varietal improvement work and subsequently food security. The decline in supply of germplasm materials as a result of CBD is also being experienced by the international genebanks. The rice genebank of IRRI for instance found it increasingly difficult to fill the gaps in their germplasm collections. Accessions of several crops which are currently being conserved in some International genebanks remained unused for several years because the regulations for exchange such as who had the authority to release the samples, were not yet clear.

The development of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) is seen as the global reaction to the rising tide of measures that slowed down the movement of plant genetic resources for food and agriculture (*pgrfa*) following the adoption of CBD

(Halewood and Nnadozie, 2008). The Treaty was adopted by the FAO Conference and entered into force in June 2004, after almost seven years of negotiations. As of now, 120 Parties have signed (approved or acceded to) the Treaty.

Negotiating the Treaty

The negotiations of the Treaty text took more than six years. The scope of materials to be included in the MLS was one of the most contentious negotiating issues before negotiators agreed upon a list of 35 crops and 29 forage genera to be included in the MLS (Halewood and Nnadozie, 2008). These are popularly referred to as 'Annex I' crops or materials since they are included in Annex I to the Treaty. Other contentious issues include the actual terms of benefit sharing, and IPRs... The text of the Treaty was finally adopted in November 2001 by the FAO Conference and entered into force in June 2004. At the same time parties also negotiated the Standard Material Transfer Agreement (SMTA) to be used for all transfers of materials under the multilateral system (MLS). The SMTA sets out the legal conditions that apply to both suppliers and recipients and establishes procedures for dispute resolution. The process for the development of the SMTA was spread out over almost four years before it was finally adopted in the First Session of the Governing Body in June 2006.

The Treaty is divided into seven parts consisting of a total of 35 articles. This paper will only highlight articles that touch on the subject of multilateral system, Standard Material Transfer Agreement and the conservation and sustainable use of *pgrfa*, the three most important components of implementing or enforcing the Treaty in Malaysia.

The Multilateral Systems (MLS) and Standard Material Transfer Agreements (SMTA) – The core of the Treaty

The Treaty creates a 'multilateral system of access and benefit sharing' (MLS) within which members, in exercising their sovereignty, provide free (or almost free) access to each other's plant genetic resources for research, breeding, conservation and training. The purpose does not include chemical, pharmaceutical and/or other nonfood/feed industrial uses. Of course, we can make materials available for these other purposes if we choose to, but we are not obliged to under the Treaty. It does not matter how many accessions of different species we bring with us into the MLS; as long as we agree to share what we have, we can get access to all the other members' materials for our own use.

The MLS does not in any way restrict the sovereignty of countries over our resources. The preamble to the Treaty explicitly recognizes that 'in the exercise of states sovereign rights over their plant genetic resources for food and agriculture, states may mutually benefit from the creation of an effective multilateral system for facilitated access to a negotiated selection of these resources and for the fair and equitable sharing of the benefits arising from their use'. Parties first exercised their sovereignty by participating in the negotiations of the Treaty and the creation of the MLS, and then by choosing to become a member of it. Furthermore, they can, of course, withdraw from membership in the Treaty if they wish.

Access to materials within the MLS comes largely without strings attached, and the strings that do exist are there to maintain the spirit of the Treaty. For example, one cannot take out intellectual property rights (IPRs) that prohibit others receiving them in the same form from the multilateral system. And if recipients choose to prohibit others from using, for their own research and breeding, any product they develop using materials they got from the MLS, they must share a percentage of their sales of that product with the international community through a conservation fund to be established.

The MLS does not include all *pgrfa* (Moore and Tymowsky, 2005). Furthermore, not all instances of Annex I crops in a country are automatically included in the MLS, though those which **'are under the management and control of the Contracting Parties and in the public domain' certainly are**. Beyond those materials, governments, individuals and organizations are encouraged to voluntarily include additional materials. Furthermore, international organizations are also encouraged to place their collections under the Treaty by signing agreements with the Governing Body. Eleven CGIAR Centres, CATIE and Coconut Genetic Resources Network (COGENT) have already placed their collections under the Treaty.

All materials in the MLS will be distributed under the Standard Materials Transfer Agreement (SMTA). The Treaty makes clear that materials for use for food and agriculture will be made available

for free, or for the minimal costs involved. If recipients use the materials for something else, they will be in violation of the SMTA. They are, however, allowed to use materials received to develop improved materials. In such cases, if they commercialize a final product that is itself a *pgrfa* and restrict others from using it for research and breeding, they must pay 1.1 per cent of sales of the product into a common fund created under the Treaty. If the *pgrfa* product is available for further research and breeding, no payment is necessary, although it is still encouraged. The monetary benefits go back to the MLS, not to any particular supplier and this is where the MLS differs from the kind of bilateral regulatory arrangements that many countries have created (or are creating) pursuant to the CBD. The funds generated will be used to support conservation and sustainable use in developing countries. The fact that monetary benefits go to an international fund, and not to the supplier, however, raises questions about enforcement. Stated bluntly, if suppliers do not receive direct benefits back in the form of royalty payments, they will not have an incentive to pursue recipients who violate the terms of the SMTA. To deal with those questions, an Article which will lead to the creation of legally authorized `third party beneficiary' has already been included in the Treaty.

Conservation and sustainable use of PGRFA - The ultimate goal of the Treaty

The MLS received the most attention during the negotiations of the Treaty. Now, the Governing Body has shifted its attention to sustainable use under the Treaty. Sustainable use of *pgrfa* is the ultimate goal. The MLS is not an end itself; it exists to support sustainable use. Moreover, apart from those parts of the Treaty concerning the MLS, the Treaty actually applies to all *pgrfa* (in other words well beyond the Annex I list). Article 5, concerning conservation of all *pgrfa*, encourages countries, to survey existing inventories, collect materials under threat, support farmers to conserve on-farm, promote *in-situ* conservation of wild crop relatives and wild plants, and document, characterize, regenerate and evaluate *pgrfa*. Subsequently, Article 6 obliges member countries to develop policy and legal measures to promote maintenance of diverse farming systems, research that maximizes variation for farmers' benefit, broadening the genetic base of crops available to farmers, and expanding use of local and locally adapted crops and underutilized species. Malaysia, in particular MOA through Mardi has already initiated the development of such policy, strategy and action plans which use the two articles as framework.

National implementation of the Treaty

Many issues remain to be ironed out in the course of implementing and enforcing the Treaty in Malaysia including how the country is going to approach implementing herparticipation in the MLS, both as suppliers and receivers of materials. To date, there have been very few examples of other countries' implementation to look to as examples. There is clearly a need for in-country consultation to be made and assistance to be made available to national focal point and relevant agencies responsible to oversee its implementation

Conclusion

Conservation of plant genetic resources is a global responsibility and countries have to work hand in hand to ensure the resources are conserved and continue to be available now and in the future. ITPGRFA is the best framework to achieve this goal of having the *pgrfa* conserved, sustainably utilized, and whatever benefits derived from its commercial utilization are shared in an equitable manner.

Aknowledgement

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Biodiversity-Biotechnology in the Context of Knowledge Generation and Management

Ng, F.S.P. and Saw, L.G.

Forest Research Institute Malaysia, Kepong, 52109 Kuala Lumpur, Malaysia
fng@pc.jaring.my

Abstract

Knowledge of biodiversity consists of traditional knowledge, open-access knowledge and proprietary knowledge. The mechanisms for generating and managing knowledge in all these categories are under stress. To support development based on biodiversity, a better climate for investment has to be created, based on a better understanding of the different and complementary roles of the private and public sectors in generating and managing knowledge.

Introduction

Knowledge of biodiversity consists of what is known about plants and animals and their environments. Such knowledge can be classified under three systems of knowledge: traditional, open-access and proprietary. The aim of this paper is to highlight the different properties of these three systems, the stresses they are under, and the need to manage them appropriately for the benefit of society. Knowledge, to the 'knowledge economy' (K economy) should be like water to agriculture. There must be plenty of it, readily available, and efficiently being renewed at source.

Traditional knowledge

Traditional or folk knowledge is the knowledge of the peoples who live close to nature. It includes knowledge of what is edible and drinkable; what is poisonous; how to search, gather, hunt or trap food and other necessities; how to treat sicknesses; and how to use local materials for housing, clothing and crafts. Folk knowledge is what human society started with, and until about 100 years ago it formed the bulk of the world's knowledge of biodiversity. Knowledgeable individuals were respected within their communities, and they passed on their knowledge to other members of their community as a communal duty.

The traditional knowledge system is under stress because the need for it has declined drastically. Most people now live in urban and semi-urban environments and their needs are met by the other two knowledge systems: open-access and proprietary. In another generation, traditional knowledge will disappear except for what is documented, published and thereby transferred to the open-access system of knowledge. The problem with traditional knowledge is how to get the documentation done and preserved quickly and effectively.

For Malaysia, the greatest compendium of traditional knowledge is Burkill's (1936) two-volume Economic Products of the Malay Peninsula. Burkill did not set out to document traditional knowledge only. His purpose was to document all the known uses and properties of plants, animals and minerals of the Malay Peninsula. He compiled all the information he could obtain, including information from scientific publications, from personal observations, and from people he interviewed. A brief account of how he did his work has been published by Holttum (1965).

The best recent example in the documentation of traditional knowledge in Malaysia is perhaps Christensen's (2002) record of the plants used by the Iban and Kelabit communities in Sarawak. Christensen's approach differs from Burkill in the linkage of each plant and its usage to the life of the community. Christensen also includes many photos whereas there are no illustrations at all in Burkill. Other notable contributions to the documentation of traditional knowledge in Malaysia have been made by Chin (1985), Werner (2002) and Chai (2006). A study in Kayan Mentarang (Kalimantan, Indonesia) by Sorensen and Morris (1997) is also applicable to Malaysia.

The Sarawak Biodiversity Centre (SBC) has for the past several years been documenting plant knowledge and usage by different communities in Sarawak. One of us (Ng) has been involved in some of the documentation activities, specifically to provide advice on botanical documentation. The teams doing the documentation have made it a standard procedure to collect, label, photograph, and

preserve specimens of every plant they document. This is vital because the scientific identity of a plant rests on the specimens collected as vouchers. The voucher specimens are preserved and kept at the SBC and there are usually two duplicates available for distribution to other scientific centres.

Botanical documentation is only one part of what should be regarded as a three-part documentation effort. The second part is the recording what the plant means to the community. The documentation teams focus on communities that are willing to participate in the documentation, hence there is no lack of willingness in documentation. The documenters are urbanized young Malaysians and the people being interviewed are forest community elders. This carries a danger of mistranslation of concepts, but the danger is avoided by adopting a simple and standard interview format that focuses on actual usage of the plants and not on concepts. Before the documentation team leaves, the community is gathered together to view a multimedia presentation in which the photographs and notes for each plant are projected on a screen and the entire audience takes part in editing the record. The result is a record approved by the community. This is a 'bare-bones' record. Fleshing out the record will require interviewers to actively probe for information beyond what the basic format allows.

To take a simple example, the team went to one Bidayuh community where the chewing of betel is still practiced by the elders. It is difficult for young urban people to imagine how important and widespread this activity was in the past, when it was normal for everyone to chew betel and polite to offer it to friends and visitors. Throughout tropical Asia, from Pakistan and Southern China to Southeast Asia and into the Pacific, the chewing of betel was the most important of all social activities. The primary ingredient is the nut of the betel nut palm (*Areca catechu*). Slices of betel nut are placed in the centre of a fresh betel leaf (*Piper betel*) together with a small amount of slaked lime (calcium hydroxide) and a small amount of leaves of gambir (*Uncaria gambir*). The mixture of ingredients is folded into a wad for chewing. There are descriptions of the various components by Burkill (1936), but details of the practice vary from place to place. In the Bidayuh community, the team was shown a plant that the community called *gambir Sarawak*. Because Ng was familiar with the betel practice in many countries, he realized immediately that *gambir Sarawak* was not the true gambir, *Uncaria gambir*, which is a liana in the family Rubiaceae. The g*ambir Sarawak* demonstrated was from a tree belonging to some other plant family. We have not yet been able to identify it botanically. After some discussion, the elders agreed that there was another plant that 'others' used as 'gambir'. By a fantastic stroke of luck, a specimen of that other gambir, complete with flowers and fruits, had been made that day. This turned out to be the true *Uncaria gambir*. It was interesting that this community had access to the true gambir, yet preferred another plant as substitute. Where and when did the substitute first come into use? This can only be discovered by comparative study and active interviewing of the various communities in Sarawak. The team was also offered the interesting observation that nobody in this community had ever developed cancer of the mouth from betel chewing.

This example illustrates how traditional or folk knowledge of a community is usually part of a widespread and diffuse pattern of knowledge across communities, tribes and countries, but with local differences between them. *Gambir Sarawak* was only one of over 20 species documented in one day. To comprehensively probe and document the traditional knowledge of Sarawak will require many trained persons deployed for the next 20 years. After that, it will be too late.

The third part of documentation is the preservation of traditional knowledge. History provides ample evidence that the only knowledge that survives through time is knowledge available to the public, i.e. knowledge 'in the public domain'. All surviving documents from the past are documents that have been available for copying and dissemination. Documents not available for copying and dissemination have been lost and we have no idea of the scale of loss except when a loss itself has been documented, e.g. the deliberate destruction by a government official in Ming Dynasty China of the records of the maritime expeditions undertaken by Admiral Cheng He in the early 1400s (Landes, 1998). In striking contrast, the logs of Christopher Columbus' four expeditions to the Americas, undertaken almost a century later, were made available by the Spanish government for study, and eventually published. A modern English translation of the logs and dispatches of Columbus (by J.M. Cohen, 1969) is available as a Penguin Classics paperback. The lesson from history is that the best way to ensure the survival of knowledge is to make it public. We know from the logs and dispatches of Columbus that he took planting materials of grapes, sugar cane, oranges, lemons, citrons and melons to the New World and brought back to Europe chilli, maize and pineapple. However, the first European explorers to reach Panama came across a small population of the coconut, a long way from its home

range in tropical Asia. Also, the first European missionaries in China came across a small population of cultivated maize in China, a long way from its home range in America. It is a mystery how these two species could have crossed the Pacific Ocean before the arrival of the Europeans. The only other possible agent would have been the Chinese fleet commanded by Cheng He (Menzies, 2002).

The bulk of traditional knowledge in the tropics has to do with day-to-day living in the jungle. The responsibility for documentation and preservation of traditional knowledge now rests with governments, but the instinct of government bureaucracies is to keep information in their files as state secrets. The situation has been further complicated by fears about traditional knowledge being 'patented' by predatory commercial interests. Recent patent decisions in the US have added to these fears. In this connection, it is interesting that in India, the publication of traditional knowledge has been seen as a means for "safeguarding hundreds of Indian medicinal plants to prevent patenting against Indian interests…" (Mohan Ram, 2005).

For traditional knowledge, secrecy cannot ensure its survival, while patenting would be expensive and pointless. A patent has a short life span and upon expiry, its information content passes into the public domain, so it is better to put the information into the public domain without delay through publication. The idea that communities can profit by selling secret traditional knowledge is a mischievous one that cannot be implemented in practice and can only lead to conflicts between communities.

Open-access knowledge

The term 'open-access' is most frequently associated with information accessible freely on the Internet. In this paper we use the term in a broader sense, for all published information. In principle, the act of publication incorporates the intent to make information available to the whole world. In this sense, open-access is the knowledge system of modern science.

Open-access is based on the idea that reliable knowledge of nature is best built up by well-reasoned interpretations of evidence obtained by careful observation. Who is to judge what is 'well-reasoned' and what is 'careful'? In modern science, the judge is the global scientific community now and in the future, and the instrument is publication, through which interpretations, evidence, and methods of obtaining evidence, are all placed on public record. New interpretations can be made and old evidence reinterpreted whenever new evidence is obtained (e.g. with better instruments, under more critical experimental conditions, or using new technologies such as those for reading DNA) or when new concepts are proposed. Open-access is a process that allows anybody, anywhere, to participate. Open-access took root in Western Europe in the 1600s due to the efforts of pioneers like Galileo Galilei (1564 - 1642) in Italy and William Harvey (1578 - 1657) in England, who discovered the benefits of open discussion with like-minded colleagues and in publishing to reach a wider audience.

Open-access goes against the idea that it is advantageous, elitist or clever to hoard information. In the early decades of modern science, not all scientists were convinced of the benefits of open access. The eminent scientist Isaac Newton (1642 - 1727) was unconvinced—until he got embroiled in a feud with Gottfried Leibniz (1646 - 1716) over the development of calculus. Modern scholars believe that Leibniz had independently developed calculus, but Newton had developed it first and kept it as a secret that he shared only with close associates. After Liebniz published his version of calculus, a shocked Newton, realizing his loss, accused Liebniz of stealing his idea. Using his position as President of the Royal Society, Newton ran a sustained and ugly campaign against Leibniz (White, 1997). Nowadays, there would be little sympathy for a Newton.

Table 1. Comparison of the three systems of knowledge with respect to biodiversity

	Traditional or Folk	**Open-access**	**Proprietary**
Origin	Undocumented	Originators are identified by authorship of publications	(a) Originators are identified in patent documents (b) Originators are holders of secrets known to themselves and trusted associates

	Traditional or Folk	Open-access	Proprietary
Generation of knowledge	Informal and not sustained	Formal (programmed) and sustained	Variable
Ownership	Communal ownership but not critically or legally defined	Moral ownership in the form of peer/public recognition for the originators	(a) Legal ownership defined under patent law (b) secret holders protected by secrecy
Rate of growth	Stagnant	Growing exponentially, sustained by large and growing body of publicly-funded scientists in the world	Rapid turnover due to expiry of old patents, loss of relevance of secrets, and development of new patents and new secrets
Propagation of knowledge	Word of mouth within the community	Publication in scientific journals and books; dissemination in conferences and exchanges between scientists; taught in universities	Declaration in patent documents. Publication of scientific papers if allowed by corporate policy
Motivation for knowledge practitioners	Respect within the community	Peer recognition by fellow scientists, and career advancement	Commercial success
Future	Critically endangered unless published	Dependent on public funding and management of public institutions	Dependent on market success and management of corporations

Open-access changed the way knowledge was generated and disseminated, and its benefits were to become evident in the stream of technological advances that powered the Industrial Revolution in western Europe in the 1700s. Self-correcting and self-renewing, open-assess knowledge has been growing exponentially, as indicated by the rate of increase of scientific publications. It is available through scientific journals and books. More and more of it is being made available on the Internet. Most importantly, open-access has provided the intellectual platform for modern university development and the justification for public expenditure on scientific research now adopted by all countries.

Active scientists who contribute to the open-access system are the best persons to access it. When the senior author (Ng) was head of the office for Forestry Research, Education and Training at the Food and Agriculture Organization of the United Nations from 1991 – 1994, his office received requests from forestry colleges in many developing countries for assistance to revise their teaching curriculums. He was initially puzzled why these colleges could not revise their curriculums themselves. It turned out that these colleges had been established with the help of FAO and donor funding in the 1960s and 1970s. Their staff had been sent to universities in the West for training and on their return they took over the teaching. After 20 or 30 years they were still teaching the same things, until it had become apparent to everybody that they were out of date. Students who could afford it were avoiding the local universities in favour of universities in the West. The staff trained by FAO now wanted to be sent overseas again for 'refresher' training, but the real problem was that the staff had become obsolete because they had not participated in research and publication as would have been required in universities in the West.

The activity of discovering, naming and classifying all species of the world, known as 'taxonomy', is part of the aim of modern science. Inspired by the vision of the Swedish biologist Carolus Linnaeus (1707 - 1778) scientists have, in 250 years of effort, named and described 1.8 million species. This accumulated knowledge of biodiversity, combined with associated advances in ecology, physiology, and molecular biology, has laid the foundations for modern biotechnology and driven biology to the forefront of science in the 21st Century.

In addition to the 1.8 million known species there is a vast 'reserve' of unknown species, estimated at between 5 million and 100 million. Even at the lowest estimate of 5 million species, the majority of species are still waiting to be discovered. Malaysia, ranked 12th in wealth of species according to the National Biodiversity Index (Anon., 2001) hopes to turn its biodiversity advantage to good use.

However the mechanism for species discovery, documentation and classification is in deep trouble. After the concept of national ownership of biodiversity was adopted under the Convention on Biological Diversity in 1993, there has been a marked decline in the number of young scientists in the developed countries taking up taxonomic research. Most of the global experts are close to retirement or already retired. Funding for such research, especially in Europe, has declined steeply. These trends are highlighted in the 5th Report of Session 2007-2008 of the Science and Technology of the House of Lords of Great Britain, (Anon. 2008) from which the following quotes are obtained:

> "There are no, effectively, no fungal systematists employed in UK universities" (European Mycological Association).
>
> "There are no lichen taxonomists left in British Universities" (British Mycological Society).
>
> "(There is) near-elimination of taxonomists from the university sector in the UK" (Systematics Association).
>
> "Whole sets of skills and expertise to maintain the international standards for identification is disappearing rapidly from the UK" (Research Councils UK).
>
> "There is a lack of taxonomical expertise that is accessible to government, conservationists and education establishments" (Plantlife International).
>
> "(A 2002 study of UK insect taxonomists) shows a clear decline in numbers of both amateur and professional taxonomists, and our own difficulties confirm that the decline is continuing" (Royal Entomological Society).

Tropical countries, the owners of a wealth of biodiversity, face a looming shortage of experts to work out what this wealth consists of. Almost all the taxonomic knowledge of tropical biodiversity has been created by scientists in the developed countries under the open-access system. This arrangement is coming to an end, but the public institutions in tropical countries responsible for biodiversity management are not ready to step into the breach. Existing public institutions in Malaysia face a number of impediments. They were established to serve forestry, agriculture, fisheries, wildlife, health, and other established interests. Biodiversity that falls outside established interests has been ignored. Hence there are no national experts on snails, nematodes, fungi, earthworms, lichens etc. which may be of significance in biotechnology. There is no overall biodiversity institution dealing with all biodiversity that can fill the gaps or maintain cross-sectoral linkages. Existing public institutions are also parochial. To identify a new species, we nearly always need help from overseas because the critical reference specimens are not available in Malaysia. This is not only because many of the critical Malaysian specimens are in overseas museums but also because a specimen critical to Malaysia may be a specimen from another country e.g. Indonesia, Thailand, Philippines or even further away. Expertise involves the ability to see the bigger picture, but existing local institutions have never been challenged to see the bigger picture.

To develop Malaysia's potential as a mega-biodiversity country we think it is necessary to establish an institution for biodiversity that will concentrate on the discovery, documentation and scientific classification of biodiversity and the dissemination of biodiversity information, free from

current limitations. Nearly all countries have such an institution, known as a natural history museum, and new ones are being currently being planned in Singapore and Vietnam. A similar planning exercise has just been completed in Malaysia and the report of the consultants (Anon., 2008) has been published. The plan for Malaysia envisages that the Natural History Museum Malaysia will view Malaysia as its centre but its vision should extend beyond national borders. This will ensure that species discovery and inventory in Malaysia will proceed faster than if left to chance. Locally based corporations will benefit most from proximity to world class identification and diagnostic services and to easily accessible expertise. A world-class museum will provide a bridge to the museum resources of the world. It will help Malaysian universities to develop training programmes in biodiversity that are of global relevance.

Proprietary knowledge

Proprietary knowledge is the knowledge that business enterprises consider to be their intellectual property. Proprietary knowledge consists of trade secrets and patents. The keeping of trade secrets for economic advantage is as old as commerce, but a secret can only be effectively kept by a small number of people. If many people share a secret, it is no longer secret. Once a secret is leaked, there is no remedy.

The modern approach in protecting commercial interests is to seek legal protection for proprietary knowledge in the form of patents. To qualify for a patent, the essential details of such knowledge have to be original and novel (not already known to others) and sufficiently well defined to survive examination by the patents office and to defend in court. Such details are proprietary but not secret. The life span of a patent is fixed at about 20 years, according on the law of the country. Upon expiry, the contents of patents become part of open-access knowledge.

Patenting involves a heavy cost. There is the cost of research to develop something patentable, the cost of obtaining the patent, and the cost of surveillance to detect cases of patent infringement. If there is a suspected case of infringement, the patent holder has to negotiate a settlement or bring the infringer to court, and there is no guarantee that the patent holder will win the case. Corporations that depend on patents have to maintain a stream of new patents and strong legal departments. The patent system has become expensive and cumbersome for all except the most profitable corporations, and even these may eventually find the cost too high. The alternative is to maintain a secretive environment, which itself is not sustainable. Secrets become obsolete, and for pharmaceuticals, public health regulations demand full disclosure of ingredients. Consumers increasingly demand full disclosure for other products as well.

The interface between proprietary and open-access knowledge

The relationship between proprietary and open-access knowledge may be visualized as a thin layer of proprietary knowledge resting on an exponentially rising base of open-access knowledge. As their patent portfolios run down, many well-known pharmaceutical corporations are facing a crisis because their own research cannot fill the developing gaps. They have to buy successful biotech companies, but the successful biotech companies are themselves only a fraction of the start-up companies, most of which run out of capital before their research produces marketable products. This shows that the proprietary knowledge system can only work as an appendage to a robust publicly-financed open-access system in which the cost of generating basic knowledge is spread out across society. For corporations in the private sector, the best deal is to tap open-access knowledge and to limit their own R&D to bridge small gaps between open-access knowledge and marketable products. This requires the public sector research institutions to stay true to their role in generation and dissemination of public-access knowledge. We have an instructive example in Malaysia of what can happen when the public sector deviates from its public role.

In the early and mid 20th Century, Malaysia became the world's top producer of natural rubber and this was due to the role of Malaysia's Rubber Research Institute (RRIM) in developing improved *Hevea* germplasm and making it freely available to the public. Due to the open and helpful attitude of RRIM, free availability of materials for people to test and gain confidence in, and the high level of public participation in the development and dissemination of improved clones, the country prospered and RRIM and its scientists enjoyed high public respect.

The wood of *Hevea* trees, as an almost worthless by-product of the rubber industry, was disposed of as firewood in Malaysia until the 1980s, when the Forest Research Institute Malaysia (FRIM) began to promote it as a furniture timber. FRIM's research on the properties of *Hevea* wood was made freely available to the public. In just over 10 years, *Hevea* wood became the favoured material for booming furniture and particle board industries.

However, the supply of *Hevea* wood was threatened by the increasing trend to convert rubber plantations to oil palm. To increase the value of rubber plantations, the RRIM began a research project in about 1985 to develop rubber clones that would be high-yielding not only for latex but also for timber. However, this time, the policy of RRIM had changed. Research findings were treated as corporate property, to be commercialized by the RRIM. The rubber-growing public was not invited to take part in the development of new clones. Interested growers faced secretive scientists when they tried to get access to materials to test for themselves. After 20 years, this programme has ground to a halt. The researchers have retired, first-hand knowledge has been lost, and the experimental and demonstration plots have been given up. The supply of *Hevea* wood for the furniture and particle board industries is close to exhaustion and this particular segment of the national economy is headed for extinction.

The Malaysian oil palm industry is now concerned about the Malaysian Palm Oil Board (MPOB) heading in the same direction with its 'corporatization' of research (Hanim, 2008). There is good reason to be concerned, not only with the direction of MPOB, but also with all public institutions entrusted with research missions and financed by public funds. When research is not published and openly available, the normal mechanisms that ensure quality in research are suspended. And since the salaries of public sector scientists are not linked to market performance, the market forces that keep the private sector efficient cannot come into play. There is therefore need for a critical review of the concept of corporatization of public sector research.

Wealth in biodiversity should result in diversification and enlargement of the economy, but this will not happen unless information is generated and made readily available. For biodiversity-biotechnology to be an effective gateway to discoveries, sustainable utilization and wealth creation (the theme of this symposium), the government should create a climate in which the private sector can expect information, materials and other support services from knowledgeable and helpful public sector institutions. This will be in line with the declared vision of Malaysia's national policy on biological diversity (Anon., 1998) "To transform Malaysia into a world centre of excellence in conservation, research and utilization of tropical biodiversity by the year 2020".

The public and private sectors have different but complementary roles in the knowledge economy (K economy), of which biotechnology is a part. The private sector takes on the financial risk of bringing new products to market. The role of government and universities, as public institutions, should be to support the private sector by generating and providing open-access knowledge efficiently, and by providing supporting scientific services based on open-access such as

- services for the identification and discovery of biological species
- information on biological resources and their properties
- laboratory diagnostic and other tests
- consultants for short term hire
- libraries and databases
- graduates and other skilled workers, trained under up-to-date curriculums

The dismal scientific publication output of developing (Third World) countries documented by Gibbs (1995), can be interpreted in various ways, but it is clear that developing countries, including Malaysia, have mostly been passive hangers-on in the world's system of knowledge generation and dissemination. For biodiversity wealth to be translated into tangible wealth, we will need to be far more active in knowledge generation and dissemination than at present.

In biotechnology, the aggressiveness of the private sector in promoting and glorifying its short term interests has produced a dangerous backlash in which public sector institutions everywhere are pressured to behave like private sector corporations. We have provided examples to show that this is detrimental to national interests and to the interests of the biotechnology industry itself.

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Interpretation of the Agreement on Access to, and Benefit Sharing from, *Teff* – An Ethiopian Plant Genetic Resource

Gebreselassie, A.

Århus School of Business, Århus University, Funglesangs Alle 4, 8210 Århus V, Denmark
abge@asb.dk

Abstract

Plant genetic resources are any material of plant origin containing functional units of heredity, which have an actual or potential value. A plant genetic resource may serve to satisfy human needs for food, pharmaceuticals, clothing and so forth. *Teff* is one example of a plant genetic resource of Ethiopian origin with many identified values for food such as pancakes, bread, soup, and drinks. It may have unidentified or potential values. Furthermore, flour from *Teff* is gluten-free, and thus it can be used for the preparation of diet food for example, for peoples with gluten intolerance.Some of the poor countries of the world are rich in plant genetic resources. Ensuring a sustainable protection of plant genetic resources in such countries requires the strengthening of the financial capacities of the country. Affirming the sovereign right of States over their plant genetic resources, the FAO-Treaty and the CBD provide a comprehensive framework on how access to and benefit sharing from, plant genetic resources is to be carried out. Benefit sharing is one of the tools adopted the CBD and the FAO-Treaty to increase the financial capacity of poor countries to invest in sustainability of plant genetic resources. The paper will discuss the international legal frame work for access and benefit sharing by taking as an example the agreement between the Ethiopian government, and the Netherlands company Health and performance Food International bv.(HPFI) on access to, and benefit sharing from, *Teff* Genetic Resources. Moreover, the paper interprets the implications of the agreement for the sustainability of plant genetic resources.

Introduction

Plant genetic resources are any material of plant origin containing functional units of heredity, which have an actual or potential value. A plant genetic resource may serve to satisfy human needs for food, pharmaceuticals, clothing and so forth. *Teff* is one example of a plant genetic resource of Ethiopian origin with many identified values for food such as pancakes, bread, soup, and drinks. Furthermore, flour from *Teff* is gluten-free, and thus it can be used for the preparation of diet food for example, for people with gluten intolerance. For such immense advantages, sustainability of plant genetic resource should be ensured.

Some of the poor countries of the world are rich in plant genetic resources. Ensuring a sustainable protection of plant genetic resources in such countries can be pictured as a three legged stool. Each leg of the stool represent different yet interrelated requirement: 'conservation and sustainable use of plant genetic resources' (leg one); 'availability of opportunities for States to exercise their right to determine access to plant genetic resources' (leg two); 'fair and equitable sharing of the benefits obtained from the utilization of genetic resources' (leg three). If one of the legs is missing, the stool cannot stand.

The three legs of the stool are the three objectives of the Convention on Biological Diversity (hereinafter the CBD) and the FAO International Treaty on Plant Genetic Resources for Food and Agriculture (hereinafter the FAO-Treaty) which aim to keep all the three legs of the stool in place, so that the stool can serve both the present and the future generation. Such legal instruments enable genetic resource providing countries to share the benefits and strengthen their capability to invest in sustainability of plant genetic resources. The purpose of this Article is to discuss the agreement between the Ethiopian Government (Provider) and the Dutch company, Health and Performance Food International B.V. (User) concerning access to and benefit sharing from *Teff* Genetic Resources to illustrate the CBD, the FAO-Treaty and the Bonn Guidelines on access to genetic resources and fair and equitable sharing of the benefits arising from their utilization (hereinafter the Bonn Guidelines).

Materials and methods

The materials used for this work includes international legal instruments such as the CBD, FAO-Treaty, and Bonn Guidelines. Besides, the agreement between the Ethiopian Government and the Dutch company, Health and Performance Food International B.V concerning access to and benefit sharing from *Teff Genetic Resources* (hereinafter the *Teff* Agreement) is taken as an example to show how access and benefit sharing agreements are designed under the international legal instruments.

Results and discussion

Access to plant genetic resources

Access determined by national authorities, is subject to prior informed consent. Prior informed consent is the first step when the User Company or country has to bring the providing country to negotiation. Prior informed consent among other things may include specification of the intended use of the actual plant genetic resource and the expected results (Bonn Guidelines, Provision 36(f&k)0. Besides, contracting states are required to control the use of plant genetic resources obtained without prior informed consent.

It follows from the Bonn Guidelines that States are required to facilitate access to plant genetic resources. Access has to be facilitated at minimum cost and also, any restriction on access should be based on legal grounds. Moreover, arbitrary restrictions on access, which are counter to the objectives of the CBD, should be avoided. Without facilitated access, the utilization of plant genetic resources could not be enhanced.

However, access is not subject to negotiation costs when the plant genetic resource is covered under the Multilateral System of the FAO-Treaty. Again by way of example, as Teff is not covered under the Multilateral System, the Dutch company accessed twelve varieties of Teff by negotiating with the Ethiopian government (The Teff Agreement, Preamble 2(8) and for the lists of varieties, see Annex I of the Agreement).

Mutually agreed terms

Except for plant genetic resources which are covered under the Multilateral System of the FAO-Treaty, the CBD and the Bonn-Guidelines provide a framework concerning the subjects of the mutual agreement. According to these frameworks, some of the many subjects of the agreement include type and quantity of plant genetic resources, specification of use and benefit sharing terms.

Besides, parties add different terms which are considered relevant and important for that specific agreement. As an example of terms in this regard the Dutch company agreed not to claim intellectual property rights over genetic resources of *Teff* or over any components of *Teff* genetic resources (The *Teff* Agreement, Provision 4(1)). Broadly speaking this term allows the government of Ethiopia to continue exercising the right to determine access to *Teff* genetic resources. Furthermore, the company has consented to acknowledge Ethiopia as the source of *Teff* genetic resources in its application for the registration of new varieties and applications for intellectual property rights over the products from *Teff* (The *Teff* Agreement, Preamble 2(2)).

Specification of use

As mentioned before, plant genetic resources have actual or potential values. Such value can be used for 'taxonomy, collection, research, and commercialization'. (Bonn Guidelines, Provision 42.b (e)). It is based on these values and uses that a providing country and user country (or company) agree on terms including types of uses and limitation on the possible uses of the material (Bonn Guidelines, Provision 44(b)). Access from the Multilateral System of the FAO-Treaty is limited for the purpose of utilization and conservation, for research, breeding and training for food and agriculture which exclude non-food/feed industries (FAO- Treaty Art. 12.3(a)).

Users are required to use plant genetic resources in consistence with the agreed purposes (Bonn Guidelines, Provision 16.b (IV)). Any change of use even if the use is unforeseen requires a new application for prior informed consent and mutually agreed terms (Bonn Guidelines, Provision 34).

Moreover, according to the Bonn-Guidelines, States may monitor whether the use of plant genetic resources is in compliance with the terms of access and benefit sharing. The monitoring can be on applications for intellectual property rights relating to the material supplied (Bonn Guidelines, Provision 55).

According to the *Teff* Agreement, the Dutch company can 'use the genetic resource of *Teff* only for the purpose of developing non-traditional *Teff* based food and beverage products that are listed in Annex 3 of the agreement' (The *Teff* Agreement). The lists of products in Annex 3 of the Agreement include *Teff* flour (gluten free flour which can also be premix, and bread mix with *Teff*) and seeds (which includes gluten free beverages such as beer and distilled drinks). The Agreement prohibits the company from using *Teff* genetic resources for any unspecified uses including chemical and pharmaceutical uses.

Following the specification of use, the Ethiopian government is bound with two terms. The *first* term states that the Ethiopian government shall not grant access to *Teff* genetic resources to any other party for the purpose of producing the products of the company listed in Annex 3, unless the Ethiopian government secures the consent of the company (the *Teff* Agreement, Provision 3(4)). The *second* term binds Ethiopia not to export *Teff* seeds if the importer or anyone wants to use *Teff* for products listed in Annex 3 (The Teff Agreement, Provision 6(4)).

The implication of the two terms is however that Ethiopia on the one hand permitted the use of *Teff* genetic resources to produce *Teff* seed and *Teff* flour; on the other hand, limited the use of *Teff* plant genetic resources to non-traditional *Teff* based food and beverages. Despite the fact that *Teff* is traditionally used for developing *Teff* based food (including pancake and bread), the same uses are mentioned as non-traditional uses (or as new applications of *Teff*). Thus, these two terms can be the subject of further critics.

Briefly, the right to determine access to plant genetic resources is an important step to negotiate on terms for the sharing of benefits. It is the shared benefit, which in turn, will strengthen the capabilities of Ethiopia to invest back in the sustainable protection of plant genetic resources. Thus, the right to determine access is not by itself the end; it has to be exercised to bring benefit sharing clauses. Such exercise of the right, is possible with the existence of opportunities *i.e.* others demanding to access *Teff* plant genetic resources. Besides, others will seek to access *Teff* genetic resources for its values and uses. Therefore, the 'monopolization' of the possible existing uses of *Teff* under the guise of claiming these uses as the new uses of *Teff*, will negatively affect the enhancement of the utilization of *Teff* genetic resources.

The two terms in the Agreement compromised other parties' interest in accessing *Teff* genetic resources and Ethiopia's opportunities to exercise its right to determine access to *Teff* genetic resources. Any new negotiation with other companies on access to *Teff* genetic resources can attract additional benefit sharing. Thus, if the benefit sharing is considered as not comparable to the 'monopolization' of the use of *Teff* genetic resources, the critics can go further.

Benefit sharing

The fair and equitable sharing of the benefits arising from the utilization of plant genetic resources is the third objective of the CBD and the FAO-Treaty. Sharing of benefits will enable States to have the capacity and to get an incentive for sustainable protection of plant genetic resources. In his legal writing Gulatit (2001) noted that 'benefit sharing is not based on charity but as recognition for States' investment and the principle that States should not have to bear the entire burden of subsidizing global public goods necessary for human survival.' The benefit sharing obtained from the utilization of plant genetic resources, which are covered under the Multilateral System of the FAO-Treaty is to be made to the mechanisms established to collect benefit sharing (FAO- Treaty, Article 13(2)).

The benefit sharing has to be in a fair and equitable manner, which depends on the existence of different factors (Bonn Guidelines, Provision 45). From the *Teff* example, one of the factors can be the limits which the agreement imposed upon the country's opportunity to negotiate on access and benefit sharing for those uses, which the company 'monopolizes'.

Benefit sharing can be both monetary and non-monetary. For example, regarding monetary benefits, the *Teff* Agreement, states for a payment of a lump sum calculated on gross net income of years (one percent of the average gross net income of year 2007, 2008 and 2009) and annual payment of thirty percent of the profit obtained from the sale of basic and certified seeds.

As part of the non-monetary benefits, States can negotiate on the possibility of jointly owning intellectual property rights with users of plant genetic resources (Bonn Guidelines, Provision 44). As shown in the *Teff* example, The Ethiopian government and the Dutch company, Health and Performance Food International B.V agreed to jointly own new *Teff* varieties which are developed by the user company (the *Teff* Agreement, Provision 4(2)). Besides, the Agreement includes sharing of research results, knowledge and technologies developed using *Teff* (the *Teff* Agreement, Provision 7(6)). On this point, the FAO-Treaty considers as the benefit sharing requirement fulfilled if the product is available for further breeding to others without restriction (FAO- Treaty, Article 13.d(2)).

It is interesting to mention that the *Teff* Agreement includes a term to establish a Financial Resource Support for *Teff* (FiST). For that, the company agreed to contribute five percent of its net annual profit which cannot be less than 20,000 Euro. The purpose of the FiST is to improve the living conditions of local farming communities and to develop *Teff* business in Ethiopia (the *Teff* Agreement, Provision 7(4) and 7(5)).

In summary, States have the right to determine access to their plant genetic resources. States have to get the opportunity to exercise the right to determine access to negotiate on benefit sharing arising from the utilization of plant genetic resources. The benefit sharing strengthens States capabilities to invest in sustainable protection of plant genetic resources. Thus, in order to have a sustainable protection of plant genetic resources, States opportunity to exercise the right to determine access, followed by benefit sharing is important. Therefore, logically with the existence of benefit sharing, it is financially possible for states to invest back in conservation. Hence the three legs which pictured the three interrelated objectives of the CBD and the FAO-Treaty have to remain together to support the sustainable protection of plant genetic resources.

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The Convention on Biological Diversity.

The FAO International Treaty on Plant Genetic Resources for Food and Agriculture.

Session 3
Natural Product and Drug Discovery

Back to the Future: The Continuing Value of Natural Products for Drug Discovery

Harvey, A.L.

Strathclyde Innovations in Drug Research, University of Strathclyde, Glasgow G4 0NR, UK
a.l.harvey@strath.ac.uk

Abstract

This presentation will address the question "How can a globally significant drug discovery operation be created in Malaysia?" There are good reasons, both scientific and commercial, to focus on natural product-based drug discovery. Natural products have been the most successful source of medicines throughout history and they continue to provide leads for major classes of drugs, including antibiotics, anti-cancers, immunosuppressants and neurological agents. Many natural products have physicochemical properties suitable for drug development and their unmatched structural diversity allows them to interact with many different types of therapeutic targets. Surprisingly, major pharmaceutical companies have abandoned their use of natural products in drug discovery; also surprisingly, the vast majority of natural products have never been tested for relevant biological activity. There are clear opportunities for specialist ventures to exploit natural chemical diversity to discover novel leads that can be developed into valuable new medicines. The technologies required for natural product chemistry and for drug discovery bioassaying are becoming cheaper and more accessible. The key requirements for successful drug discovery are (1) outstanding chemical diversity in a sample collection that is in an "assay-ready" format and (2) sensible selection of therapeutic targets that are relevant to the diseases of interest, whether these are previously neglected illnesses endemic to the region or unmet therapeutic needs of large commercial markets. Given Malaysia's rich biodiversity in land and sea and the quality of Malaysian scientific infrastructure, a drug discovery initiative based on natural products would be expected to produce valuable leads for significant new medicines.

Introduction

Both from a scientific and a commercial perspective, there are good reasons to focus on drug discovery based on natural products: the historical record of success is obvious; recent examples include major classes of new medicines; they have provided the first members of new classes of drugs; and there are continuing developments, as shown by the numbers of natural product-related medicines currently in clinical trials (for more details, see the reviews by Butler, 2008, and Harvey, 2008). As well as providing breakthrough drugs in important therapeutic areas (e.g., the ACE inhibitors, the statins, the immunosuppressants), the majority of leads for new anti-cancer and anti-infective agents are derived from natural products (Newman and Cragg, 2007).

Why should natural products be such a successful route for drug discovery? In general, the chemical diversity from nature provides a better chance of "hitting" a biological target that is relevant to a disease process. Small molecules from a variety of natural sources tend to be more drug-like in their physicochemical properties than collections of synthetic compounds (Feher and Schmidt, 2003; Ganesan, 2008). Since much of the world's biodiversity has not yet been tested for pharmaceutically relevant bioactivity, it would appear that there should be great opportunities for further drug discoveries from nature. There would also appear to be a great opportunity for countries like Malaysia which have high endemic levels of biodiversity.

Perceptions about natural products for drug discovery

Currently, natural products are very much out of favour with most companies active in drug discovery. Why should this be?

Various problems undoubtedly exist with natural products, but the rewards for overcoming them would seem to justify the effort required, and technical solutions are being described in the literature. For example, purification and identification of natural products are believed to be difficult and slow: high throughput separation methods coupled with sensitive analytical techniques can resolve this

(Appleton *et* al., 2007; Bugni *et al.,* 2008; Wagenaar, 2008). Natural products are chemically complex: comparisons of the chemical properties of collections of natural products show that they more closely match the "chemical space" of successful drugs than collections of synthetic chemicals (Grabowski and Schneider 2007; Ganesan 2008). Natural products give too many false positives on modern screening assays, but phenotypic assays are becoming more and more popular and it has been suggested that natural products, with their drug-like properties, are well-matched to such cell-based approaches, and extracts of natural products can be processed to remove reactive compounds or even convert them into novel drug-like structures (Rishton 2008). Natural products may only be available in small amounts: techniques for direct synthesis (Sunazuka *et al*., 2008) or production by molecular biology (Kennedy 2008) have been rapidly developing.

An opportunity for Malaysia

I believe that creating a significant natural product-based drug discovery activity in Malaysia is technically feasible and financially reasonable. The biodiversity of Malaysia is staggering: plants, animals and microbes; terrestrial and marine.

What is needed is a comprehensive and focused development of the resources needed for relevant chemistry and biology. The chemical resource will require broad collections to provide the widest possible chemical diversity; many collections already exist, and there may be opportunities to share such resources. The samples of natural products have to be extracted with solvents so that their constituents can be tested for biological activity. Such samples for screening need to be prepared in a standardized "assay-ready" format. In addition, there needs to be facilities for the rapid analysis and scale-up of samples that are found to contain activities of interest. In the past, the inherent slowness of the follow-up of initial discoveries with natural products has been one of the biggest drawbacks, but there are suitable modern technologies to overcome these problems (Bugni *et al.,* 2008).

In addition to having the necessary chemistry resources, there must also be suitable resources in biology that will allow the rapid testing of samples for activities that are relevant to unmet therapeutic needs. There is no shortage of such needs (both medical and commercial) but the key is to have robust assays that detect meaningful activities and then to have the ability to assess the active chemicals for their potential to turn into a compound that is likely to progress successfully through toxicology tests and then clinical trials in order to reach the market.

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Realizing the Value of Rainforests: The Role of Chemistry

Lajis, N.H.

Laboratory of Natural Products Institute of Bioscience
University Putra Malaysia, Serdang 43400, Selangor, Malaysia
nhlajis@ibs.upm.edu.my

Abstract

Malaysia, being located in the bio-diverse region, is endowed with rich and diverse bioresources. In addition, the rich cultural and ethnic diversity of its people has enriched the knowledge on the use of these resources in their lives. Thus, combination of these factors is expected to increase the chance of discovering new and highly valuable bioactive natural products. The value of the abounding chemical store-house in the form of tropical rain forests will not be realized unless their uses are not recognized. The primary task towards realizing the value of the rain forests is therefore to identify which among the millions of constituents stored in this store-house possess the desired properties. The search for novel bioactive candidates for lead compounds require highly integrated fields of expertise to avoid unnecessary costly and fruitless experiments due to inappropriate decision. Chemistry has contributed not only in characterizing the active constituents but also in other aspects of the process of discovery and developing of these constituents including understanding how it affects the biological system, improvement of their efficacy and safety, method development extraction and isolation as well as standardization of herbal drugs and development of new approaches in drug discovery. Recently, the interest on herbal products and preparations has also reemerged strongly among the public as alternative to conventional treatment to well-known or new diseases. There is a tremendous need for the natural products chemist now to reassess and reconsider their role in this new dimension of science related to the use of natural products in human health. In this presentation, the author will discuss the conventional aspects in drug discovery, the current development in its methodology, instrumentations and new approaches in standardization and evaluation of physiological effects and toxicity using metabolomics. Logical approaches and challenges to achieve the objective of realizing the value of rain forests relevant to Malaysian scenario and context and its challenges will also be discussed.

Introduction

Malaysia is blessed with rich and diverse flora and fauna. It is estimated that there are about 12,000 plant species available in Malaysia. They are indeed a priceless everlasting wealth if they are managed and the benefits realized wisely. Aside from the source of materials for the livelihood of the people for shelter, food and tools, these resources have also the agent of other spiritual needs. Due its long and close association human has also utilized plant and animal as the source medicines. In addition to the diversity of its biological resources, Malaysia is also blessed with rich and diverse culture originating from the multiethnic component of its population. This mix of cultural origin has further enriched the knowledge on the use of plant and animal for health and medicinal purposes.

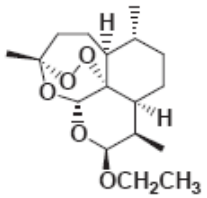
Microorganism and plant have been the source of antibiotics and medicines, either in their natural forms or their derivatives, and developed into drugs. Although the search of bioactive compounds from biological resources has been pursued since the last 200 years, new chemical leads and drug candidates are still being discovered and pushed into clinical trial and further into their development pipeline. Thus, even if the organizational set up of the business of discovery and development may have changed over the years or centuries, the general belief that biological resource is the best potential source of new candidate, whether they are new chemical entities with desirable biological activity or known chemical entity with new biological activity still persists. This is despite the recent strong devotion and expectation vested into the new trendy and stylish approaches, such as genomics, combinatorial chemistry, *in silico* etc., in drug discovery. Fig. 1 shows some examples of recently developed drugs derived from plants.

Due to its lushness and diversity of its biological resources, it is also natural to expect that this wealth should be a source of new economic/revenue generator. This based on the idea that diverse

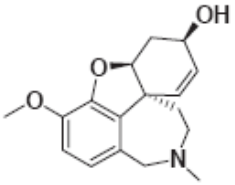
biological resources will offer diverse chemical entity not known to organic synthesis, which may demonstrate unique biological activity relevant to specific human disease. The abundance in bioresource coupled with the its rich cultural heritage is hoped to offer the better chance of discovery of new and highly valuable bioactive natural products.

The value of this bountiful chemical store-house in the form of tropical rain forests will not be realized unless their uses are not recognized. Therefore, the primary task towards realizing the value of the rain forests is to identify which among the millions of constituents stored in this store-house possess the desired properties. The search for novel bioactive candidates for lead compounds require highly integrated fields of expertise such as rapid selection of highly likely structures with unique pharmacology and provide a good chance of *drugability*. Experience and in-depth knowledge in identifying these candidates are necessary to avoid unnecessary costly and fruitless experiments due to inappropriate decision.

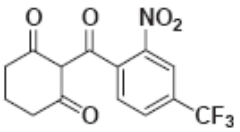
To assist the improvement of chance of success several approaches have been adopted by different organizations depending on their nature and the available resource capacity. Chemotaxonomic approach was used by a French group in discovering and developing taxotere as an alternative anticancer agent. A precursor for taxotere was discovered from the leaves of *Taxus brevifolius* rather than obtaining taxol from the bark of *T. baccatus.* Thus the need to cut-down the whole tree was avoided. The contribution of ethnomedicinal knowledge has been recognized to in leading to the discovery and development of numerous drugs currently in the market. Due to this, particular attention on the value and intellectual the rights of indigenous knowledge on the use of biological resources has been inscribed in the United Nation's Convention of Biological Diversity. Since only small percentage of the total number of species that are used or recorded in the various pharmacopoeias, there is potentially a great number of unique chemical entities which are at all related to ethno-medicinal uses. On this assumption, bio- evaluation of extracts from randomly collected sample is relevant. In view of the limitation on time and cost such activity, this approach may only be appropriate for high thru-put screenings. In most of cases however, combination of these elements and literature knowledge are still very highly necessary.



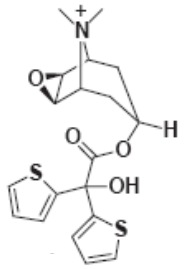
arteether (antimalarial, *Artemisia annua*)



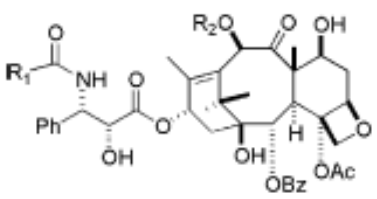
galantamine (AChE inhibitor, *Galanthus woronowii)*



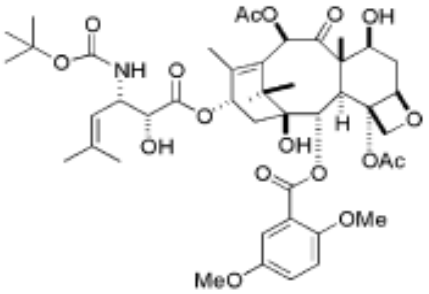
nitisinone (tyrosinaemia, *Callistemon citrinus*)



tiotropium (pulmonary disease, *Atropa belladona*)



taxol



taxotere

Fig. 1. Some recently developed drugs derived from plant.

Recently, the interest on herbal products and preparations has reemerged strongly among the public as alternative to conventional treatment to well-known or new diseases. There is a formidable need for the natural products chemist now to reassess and reconsider their role in this new dimension of science related to the use natural products in human life. The main reasons leading to this trend including the rising cost of medical treatment and the belief that products derived from natural sources are safe. The rising popularity of herbal medicines has led to the growth of the industry, and the lack of stringent regulations relating to the use has raised concern with regards to the efficacy and safety of the products. Several examples on the detrimental effects of herbal preparations have been reported due to intended or unintended mistakes. Several other medical issues include the compatibility of one herbal preparation with other drugs or herbal preparations (when used concurrently), dosage as well its true medical values since very few comprehensive clinical evaluation were done on these products.

Chemistry has been one of the crucial knowledge in all of the issues mentioned above. The field has contributed not only in characterizing the active constituents but also in other aspects of the process of discovery and developing of these constituents including understanding how it affects the biological system, improvement of their efficacy and safety, method development extraction and isolation as well as standardization of herbal drugs and development of new approaches in drug discovery. This is only logical since biological, physiological, and even emotional outcomes manifested in living organisms are the results of chemical interactions in that system.

Role of chemistry in drug discovery

The conventional approach in drug discovery program involves the isolation of pure constituents from the sources (biological specimens), which are supposed to possess the desired bioactivity. This activity normally involves continuous application of the combined separation process with bio-/pharmacological activity assessment on constituents from a mixture. The chemical constituent with unambiguously resolved structure, which possesses a good potential of being developed into drug is termed the *drug lead*. The process involved in drug discovery is shown in Fig. 2.

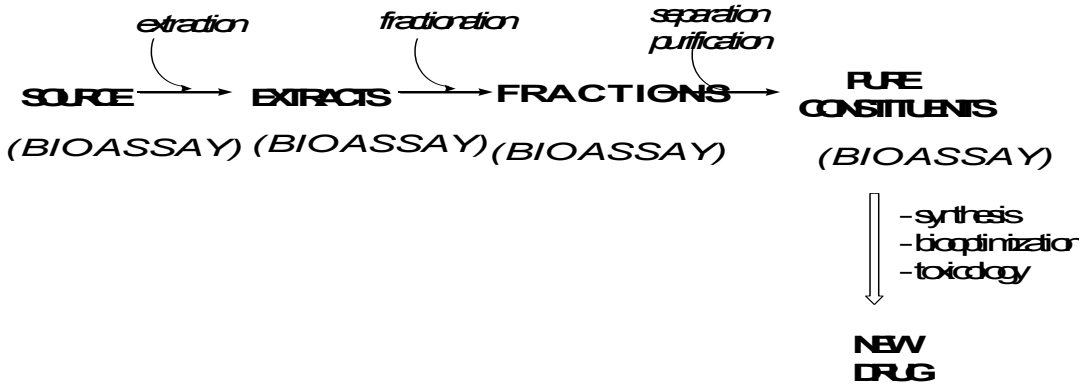


Fig. 2. Flow chart of conventional approach in drug discovery program.

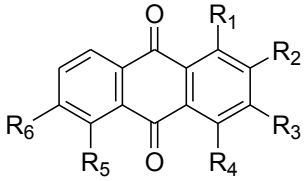
Beginning with extraction, the knowledge on chemical phenomenon is applied by the basic concept of “like dissolves like”. The knowledge in all fields of chemistry is consistently utilized to solve the problems in isolation and purification of constituents. Upon isolation of pure constituents the identity of each are determined based on spectroscopic interpretation, a very special field of relating the physicochemical signals or data with chemical structures. Knowing the structures of the active compound allows researchers to understand how the molecule might interact with the biological receptor through structure-activity relationship (SAR) analysis, which also rely very much on physical chemistry discipline. Organic Synthesis has been useful in the preparation of various analogues of the drug leads in the course of establishing SAR. Organic synthesis has also been the most sought approach to producing drug commercially, if obtaining it from its biological origin proves to be impractical.

The study on bioactive constituents from Morinda elliptica

Morinda elliptica was selected based on preliminary screening using brine shrimp lethality test, antimicrobial, antiviral and cytotoxicity assays, on which the crude methanol extract showed strong activity. The plant is a shrub or small tree and traditionally used to remedy loss of appetite, headache, cholera, diarrhea, fever, enlarged spleen and wounds as well as hemorrhoids. The leaves are also taken after childbirth.

Large scale extraction and isolation of the constituents from the root part of this species was conducted resulting in the identification of eleven substituted anthraquinones, which are presented in Table 1. Both the hydroxylated and and non hydroxylated ring A of the anthraquinones were isolated from the species.

Table 1. The substituted anthraquinones isolated from the root of *M. elliptica*



Compounds	R_1	R_2	R_3	R_5	R_6
1-Hydroxy-2-methylanthraquinone	OH	CH_3	H	H	H
2-Formyl-1-hydroxyanthraquinone	OH	CHO	H	H	H
Nordamnacanthal	OH	CHO	OH	H	H
Damnacanthal	OCH_3	CHO	OH	H	H
Lucidin-ω-methyl ether	OH	CH_2OCH_3	OH	H	H
Rubiadin	OH	CH_3	OH	H	H
Soranjidiol	OH	CH_3	H	H	OH
Morindone	OH	CH_3	H	OH	OH
Rubiadin-1-methyl ether	OCH_3	CH_3	OH	H	H
Morindone-5-methyl ether	OH	CH_3	H	OCH_3	OH
Alizarin-1-methyl ether	OCH_3	OH	H	H	H

The cytotoxic concentration at 50% toxicity (CC_{50}) of the isolates was evaluated against three cancer cell-lines using 3-(4,5-dimetnylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method, and the results are presented in Table 2. Three reference compounds were used in the cytotoxic assay; doxorubicin, a natural product compound which intercalates between DNA base pairs; tamoxifen, an estrogen antagonist which binds to estrogen receptor, and colchicine, a natural product mitotic inhibitor which disrupts microtubullar protein. The CEM-SS cell line was very sensitive towards colchicine and doxorubicin (CC_{50} = 0.02-0.1 μg/ml) whereas the HeLa and MCF-7 cell lines were sensitive towards tamoxifen (CC_{50} = 7 μg/ml). Among the three cell lines tested, CEM-SS was the most sensitive towards nordamnacanthal (CC_{50} = 1.7 μg/ml), lucidin-w-methyl ether (CC_{50} = 3 μg/ml) and rubiadin (CC_{50} = 3 μg/ml). Other anthraquinones showed moderate activity (CC_{50} = 10 μg/ml) towards the:CEM-SS cell line, except alizarin-l-methyl ether, which gave a CC_{50} value of 30 μg/ml. The MCF-7 cell line was very sensitive towards damnacanathal and lucidin-ω-methyl ether (CC_{50} = 3 μg/ml). Most of the anthraquninones were weakly cytotoxic towards the HeLa cell line except for damnacanthal and morindone, both of which showed moderate cytotoxic activity. Structurally, all the four anthraquinones that showed strong cytotoxicity (nordamnacnathal, damnacanthal, lucidin-ω-methyl ether and rubiadin) were hydroxylated at carbon number three and possessed a hydroxyl at carbon number one and/or a *arrha-formyl* group at carbon number two.

Table 2. Cytotoxic activity of anthraquinones from *M. elliptica* (IC_{50} in μg/ml)

Test compounds	Cell-line		
	HeLa	CEM-SS	MCF-7
l-Hydroxy-2-methylanthraquinone	30	10	30
2-Formyl-l-hydroxyanthraquinone	30	10	10
Nordamnacanthal	30	1.7	10
Damnacanthal	10	10	3
Lucidin-ω-methyl ether	>30	3	3
Rubiadin	>30	3	10
Soranjidiol	>30	10	10
Morindone	10	10	10
Rubiadin-1-methyl ether	30	10	30
Morindone-5-methyl ether	>30	10	30
Alizarin-1-methyl ether	>30	30	10

Reference compounds			
Doxorubicin	11	0.1	29
Tamoxifen	7	36	7
Colchicine	21	0.02	21

HeLa - cervical adenocarcinoma; CEM-SS - T-lymphoblastic leukemia; MCF7 - breast carcinoma

Antimicrobial activity assay on the same set of compounds found three anthraquinones, including nordamnacanthal, damnacanthal and alizarin-1-methyl ether, showed strong antimicrobial activity when tested against one bacterium and three fungi (Table 3). Only 2-formyl-hydroxyanthraquinone and damnacanthal were active against *P. aeruginosa* with MID values of 80 and 10 μg/disk, respectively. For antifungal activity, damnacanthal and nordamnacanthal showed very strong activity against all the tested fungi with MID values of 10 to 20 μg/disk. Morindone, however, showed very strong antifungal activity only against *C. lipolytica* (MID = 20 μg/disk). Three anthraquinones showed significant antimicrobial activity was most probably due to the presence of a formyl group at carbon number two (nordamnacanthal and damnacanthal) and I,2-dihydroxyl groups (morindone).

Table 3. Antimicrobial activity (MID in μg/disc) of anthraquinones from *M. elliptica*

Test compounds	***P. aeruginosa***	***A. ochraceus***	***A. niger***	**C. *lipolytica***
1-Hydroxy-2-methylanthraquinone	-	-	>80	-
2-Formyl-l-hydroxyanthraquinone	80	>80	>80	>80
Nordamnacanthal	-	10	20	20
Damnacanthal	10	20	20	20
Lucidin-ω-methyl ether	-	-	-	-
Rubiadin	-	>80	-	-
Soranjidiol	-	-	-	-
Morindone	-	-	-	20
Rubiadin-1-methyl ether	-	-	-	-
Morindone-5-methyl ether	-	-	-	-
Alizarin-1-methyl ether	-	>80	>80	>80

Note: Standard antibiotic; gentamycin (10 μg/disc) was used against P. aeruginosa (24 mm diameter inhibition zone); nystatin (100 unit/disk) was used against fungi (25 mm diameter inhibition zone); - = no activity.

The two formylated anthraquinones, damnacanthal and nordamnacanthal were found to exhibit strong activities against most of the cancer cell-lines. 2-Formyl-1-hydroxyanthraquinone, nordamnacanthal, damnacanthal, morindone, rubiadin-1-methyl ether and alizarin-l-methyl ether were also tested for anti-viral activity based on the XTT cytoprotection assay. Only damnacanthal showed moderate activity with 72.4% cytoprotection against HIV. The 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) values were 3.43 and 9.11 μg/ml, respectively. The control compound, dextran sulfate, which gave 100% cytoprotective effect with EC_{50} of 0.53 μg/ml and CC_{50} of more than 9.5 μg/ml.

Antioxidative properties of the anthraquinone derivatives, isolated from the roots of *M. elliptica* and four from synthetic origin were evaluated using thin layer chromatography (TLC), ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. Damnacanthal, nordamnacanthal, 2-formyl-1-hydroxyanthraquinone, morindone and alizarin were stronger antioxidant than of α-tocopherol. Alizarin was in fact comparable to butylated hydroxytoluene (BHT), a commercially used synthetic antioxidant. Structurally, alizarin and morindone possessed two hydroxyl groups arranged in *ortho* position. Both compounds exhibited strong antioxidant activity. In morindone-5-methyl ether, the 5-hydroxyl group of morindone had been methylated thus destroying the *ortho* arrangement of hydroxyl groups. Morindone-5-methyl ether did not show antioxidant activity, so were alizarin-1-methyl ether and alizarin-2-methyl ether, confirming the necessity of having two *ortho* arranged hydroxyl groups. In 2-formyl-1-hydroxyanthraquinone, a hydroxyl group and a formyl group are arranged in *ortho* position.

This compound also exhibited antioxidant activity, although it was weaker than alizarin and morindone but significantly stronger than α-tocopherol. Nordamnacanthal and damnacanthal which possess the same feature also exhibited good antioxidant activity. From these observations, it can be concluded that the formyl group next to a hydroxyl in anthraquinone derivatives also plays an important role in antioxidant activity. Rubiadin, an anthraquinone which possesses 1,3-dihydroxy structure did not show antioxidative activity. Lucidin-ω-methyl ether, another 1,3-dihydroxyanthraquinone, on the other hand showed only weak antioxidative activity. These observations showed that hydroxyl groups arranged in *meta* positions are less crucial for antioxidative property of anthraquinones. The mono-hydroxyanthraquinones did not show antioxidant activity as expected.

Table 4. Antioxidant Activity of Anthraquinones Measured by TBA Method

Test Compounds	**Absorbance at 532 nm (8^{th} day)**
Control	3.03
BHT (Butylated hydroxy toluene)	0.02
Vitamin E (α-Tocopherol)	0.38
1-Hydroxy-2-methylanthraquinone	3.62
2-Formyl-1-hydroxyanthraquinone	0.15
Nordamnacanthal	0.16
Damnacanthal	0.23
Lucidin-ω-methyl ether	0.95
Rubiadin	2.43
Soranjidiol	2.43
Morindone	0.07
Rubiadin-1-methyl ether	2.80
Morindone-5-methyl ether	2.34
Alizarin-1-methyl ether	1.04
Alizarin-2-methyl ether	2.66
1-Hydroxyanthraquinone	4.04
4-Bromo-1-hydroxyanthraquinone	3.72
Alizarin	0.01

The synthesis of damnacanthal and nordamnacanthal was pursued to evaluate further their potential as new bioactive anthraquinone analogues based on this lead. Several synthetic approaches were explored but the route based on Friedel-Crafts bis-condensation of phthalic anhydride and dihydroxybezenes was successfully adopted to achieve this.

Fourteen anthraquinone analogues were synthesized as the outcome from this pursuit. These compounds were subjected to cytotoxicity assay on five cell-lines which showed increased activity of some analogues. Of particular interest is the methyl ether analogue damnacanthal, damnacanthal-3-*O*-methyl ether, which exhibited mower selestivity towards MES-SA (uterus) cell-line. Other analogues showing stronger or more selective activity are lower cytotoxicity on specific cell 3-acetoxy-2-bromomethyl-1-methoxyanthraquinone, 2-bromomethyl-1-hydroxy-3-methoxyanthraquinone and 2-bromomethyl-1,3-dimethoxyanthraquinone (Table 5).

Table 5. Anthroquinones derivatives derived from *M. elliptica*

Compounds	MCF7 *(breast)*	MES-SA *(uterus)*	MES-SA/DX5	DU145 *(prostate)*	H460 *(lung)*
4-Bromo-1-hydroxyanthraquinone	36	14	20	27	20
Rubiadin	56	38	35	32	42
1,3-Dihydroxyanthraquinone	55	30	21	29	23
1-Bromo-3-hydroxyanthraquinone	27	56	29	44	43
4-Bromo-1,3-dihydroxy-2-methylanthraquinone	28	20	30	31	19
1-Methoxy-4-methylanthraquinone	55	19	-	48	54
2,2-Dibromomethyl-1,3-dimethoxyanthraquinone	27	10	7	28	23
2-Bromomethyl-1,3-dimethoxyanthraquinone	8	2	2	4	5
2-Bromomethyl-1-hydroxy-3-methoxyanthraquinone	34	4	-	26	30
3-Acetoxy-2-bromomethyl-1-methoxyanthraquinone	6	-	-	5	26
4-Bromo-2-formyl-3-hydroxy-1-methoxyanthraquinone	19	-	-	34	32
Nordamnacanthal	36	18	-	42	40
Damnacanthal-3-*O*-methyl ether	55	5	-	32	65
Damnacanthal	11	-	-	26	25

The availability of high speed and high capacity computers have allowed rapid digital processing of data, thus large volume information can be obtained in a rather short time. Various soft-wares on QSARs are now available to assist researchers in this field. Prediction of intermolecular drug-ligand interaction can also be accomplished using such facility based on molecular dynamic calculations and thus an approach of drug design based on this was developed and called molecular modeling.

Cell culture studies of Morinda elliptica

The production of anthraquinones from the cell culture of *M. elliptica* was also studied. The chemical investigation on the cell suspension culture has identified eight anthraquinones, two of which, anthragallol-1,2-dimethyl ether and purpurin-1-methyl ether, have not been isolated from the original plant. Other compounds isolated include nordamnacanthal, alizarin-1-methyl ether, rubiadin, soranjidiol, lucidin-ω-methyl ether, and morindone (Table 6). The structures of anthraquinones were established based on spectral studies.

Table 6. Occurrence of the anthraquinones in the suspension cell culture and in the plant

Compounds	Substituents						Occurrence of Compounds	
	R_1	R_2	R_3	R_4	R_5	R_6	Original plants	Cell suspension
Alizarin-1-methyl ether	OCH_3	OH	H	H	H	H	minor	Minor
Anthragallol-1,2-dimethyl ether	OCH_3	OCH_3	OH	H	H	H	-	Moderate
Damnacanthal	OCH_3	CHO	OH	H	H	H	moderate	-
1-Hydroxy-2-methyl-anthraquinone	OH	CH_3	H	H	H	H	minor	-
1-Hydroxy-2-formyl-Anthraquinone	OH	CHO	H	H	H	H	minor	-
Lucidin-ω-methyl ether	OH	CH_2OCH_3	OH	H	H	H	minor	Major
Morindone	OH	CH_3	H	H	OH	OH	major	Moderate
Morindone-5-methyl ether	OH	CH_3	H	H	OCH_3	OH	minor	-
Nordamnacanthal	OH	CHO	OH	H	H	H	major	Major
Purpurin-1-methyl ether	OCH_3	OH	H	OH	H	H	-	Minor
Rubiadin	OH	CH_3	OH	H	H	H	moderate	Moderate
Rubiadin-1-methyl ether	OCH_3	CH_3	OH	H	H	H	major	-
Soranjidiol	OH	CH_3	H	H	H	OH	moderate	Moderate

Comparison of the constituents isolated from the cell suspension culture and the original plant showed that most of the constituents present in the plant were also present in the cell suspension culture although the abundance may differ between them. Nordamnacanthal was the major constituent in both samples but damnacanthal was absent in the cell culture. Two compounds, anthragallol-1,2-dimethyl ether and purpurin-1-methyl ether, which were not detected in the plant present in the cell culture.

Further investigations on cell culture technology of *M. elliptica* showed that synergistic effect of sucrose as well as nitrogen levels, phosphate and myo-inositol. Three types of medium strategies were tested including the maintenance (M), growth (G) and production (P) media. In the M medium, incorporation of NO_3- between 30 mM and 67.7 mM, was vital for optimum growth and anthraquinone (AQ) production. NO_3 – level of up to 95.9 mM did not affect cell growth although AQ may be slightly reduced. At NO_3-level of 77.1 mM, 8% (w/v) sucrose in G medium produced optimum cell growth and AQ content but cell growth was reduced, whilst AQ content was unaffected in P medium. At 10% (w/v) sucrose in both G and P media, both cell growth and AQ content were reduced. Phosphate was more a growth-limiting nutrient than an AQ-limiting nutrient. The absence of phosphate retarded cell growth in M medium but no significant difference on cell growth and AQ yield was observed with 5-fold increase in phosphate in M medium at 8% (w/v) and 10% (w/v) sucrose in G medium; and at 8% (w/v) sucrose in P medium. At 10% (w/v) sucrose in P medium, the AQ yield was reduced by almost 50% (w/v). Nitrogen and phosphate toxicity was not only a function of both individual and collective level of ammonium, nitrate and phosphate, but also the level of sucrose and medium strategies. It was also established that myo-inositol was not an absolute requirement in *M. elliptica* cell suspension culture.

The effect of medium strategy by the number of impellers, aeration mode, and mode of operation on *M. elliptica* cell suspension cultures in a stirred-tank bioreactor was also studied. A lower number of impellers and continuous aeration contributed toward high cell growth rate, whereas a higher number of impellers reduced cell growth rate, although not anthraquinone yield. The semicontinuous mode could indirectly imitate the larger scale version of production medium strategy and improved anthraquinone production even with 0.012% (v/v) antifoam addition. Production medium promoted both growth (maximum dry cell weight of 24.6 g/L) and anthraquinone formation (maximum content of 19.5 mg/g of dry cell weight), without any necessity for antifoam addition. Cultures in production medium or with higher growth rate and anthraquinone production were less acidic than cultures in growth medium or with lower growth rate and anthraquinone production. Using the best operating variables, growth of *M. elliptica* cells (24.6 g/L) and anthraquinone yield (0.25 g/L) were 45%

and 140%, respectively, lower than those using a shake flask culture after 12 days of cultivation. Based on this study it was concluded that growth and AQ formation of *M. elliptica* cell cultures were greatly affected by changes in the operating variables of the stirred-tank bioreactor. Continuous aeration may assist mixing by providing greater turbulence than intermittent air supply, although continuous high air flow rate may bring the culture into its early deceleration phase. A lower number of impellers could lead to higher growth rate, but a higher number of impellers could reduce only cell growth rate but not the AQ content and yield. Cultures in production medium or with higher growth rate and AQ production were less acidic than cultures in growth medium or with lower growth rate and AQ production. Despite some improvement with suitable bioreactor operating variables, DCW and AQ yield in the bioreactor were *ca.* 45% and *ca.* 140%, respectively, lower than in the shake flask.

The cell cultures in an intermediary-G medium treated with jasmonic acid (JA) elicitor, was used as a model system to understand the effects of elicitation on cell growth, anthraquinone production, stress levels [lipid peroxidation and hydrogen peroxide (H_2O_2)], enzymic [catalase (CAT)], ascorbate peroxidase (APO) and glutathione reductase (GR), and non-enzymic antioxidant responses (total carotenoids, vitamin C and E). Cultures were elicitor-treated on day 12 with 50 or 100 μM JA, and harvested after 3 and 6 days of elicitation. The highest anthraquinone content (23.6 mg/g dry weight), which was 2-fold to level of control but with cell growth (35 g/l) 15% lower, was achieved with 100 μM of JA. Prolonged elicitor treatment resulted in the highest level of lipid peroxidation [total malondialdehyde of 9 nmol/g of fresh weight (FW)] and H_2O_2 level (5.5 μmol/g FW). Total carotenoids, vitamin C and E were induced 200, 10 and 50%, respectively, after 6 days of elicitation. Although APO was induced between 100–300%, it was only half the level in control, while GR was induced less than 100%. CAT on the other hand was reduced by 100%. This study showed that JA elicitation could induce AQ production but with a slight reduction in dry cell weight, higher oxidative stress levels, and increased antioxidant activities in *M. elliptica* cell cultures in an intermediary medium. JA elicitation could enhance total carotenoids and vitamin E to the level comparable to P medium strategy and the leaf. The reduction of both CAT and APO activities in elicited cultures after 6 days may indicate that the antioxidative roles were played more prominently by AQ, tocopherols and carotenoids. On the other hand, vitamin C in elicited culture was higher than control, which corresponds well with low APO but high GR activities, suggesting the need to maintain GSH level. The induction or suppression of antioxidant activities provides evidence for the occurrence of oxidative burst in elicited cell cultures and the versatility of plant secondary compounds.

Rapid characterization of extracts and fractions

In view of the difficulty in elucidating the complete map of all the events taking place in pharmacological manifestation including that of potential polyvalent pharmacological and synergistic mechanisms in herbal medicines, the most fundamental idea to address this is to identify all the constituents present in the herbal concoction. The techniques to accomplish this task, termed as metabolomic profiling, have been developed utilizing combination of separation and spectroscopic procedures as mentioned previously such as the hyphenated (LC-PDA-MS-NMR) or semi-hyphenated (LC-PDA-MS-{SPE}-NMR) procedures.

LC-MS-DAD analysis on two medicinal ginger extracts

Several products derived from these two ginger species (*Z. zerumbet* and *C. xanthorrhiza*) have appeared in the market in the form of spray dried powder of the water extract, or sachets containing the dried powdered rhizomes. Antioxidant activity was selected as the initial biopotential assessment, since antioxidants have been strongly associated with the defence mechanisms of living cells against oxidative damage. Several classes of plant-derived compounds such as flavonoids, phenolics and alkaloids, have been reported to exhibit antioxidant properties. In view of their popularity among the locals, it is important that the chemical profiles of these two species be established as the marker compounds, as well as to validate the rationale for their efficacies.

The water extracts of the individual species were fractionated into hexane, ethyl acetate and butanol, and the antioxidant activity of the fractions was measured. The ethyl acetate fractions from both species exhibited strongest activity in the assays performed. On-line high performance liquid chromatography, coupled with diode array detection and electrospray ion trap tandem mass

spectroscopy (HPLC–DAD–ESI/MS^n), was successfully used to analyze the components in the antioxidant-active fractions from the rhizomes of these species (Fig. 3). Three components were identified from *C. xanthorrhiza*, including bisdemethoxycurcumin , demethoxycurcumin and curcumin (Fig. 4). The active fraction from *Z. zerumbet* consisted of five components, including kaempferol 3-*O*-rhamnoside, kaempferol 3-*O*-(2''-*O*-acetyl)rhamnoside or kaempferol 3-*O*-(3''-*O*-acetyl)rhamnoside, kaempferol 3-*O*-(4''-*O*-acetyl)rhamnoside , kaempferol 3-*O*-(3'',4''-*O*-diacetyl)rhamnoside and kaempferol 3-*O*-(2'',4''-*O*-diacetyl)rhamnoside (Fig. 5). To confirm their identities, the components from *Z. zerumbet* were isolated conventionally and were analyzed by spectroscopic techniques as well as by comparison with literature data.

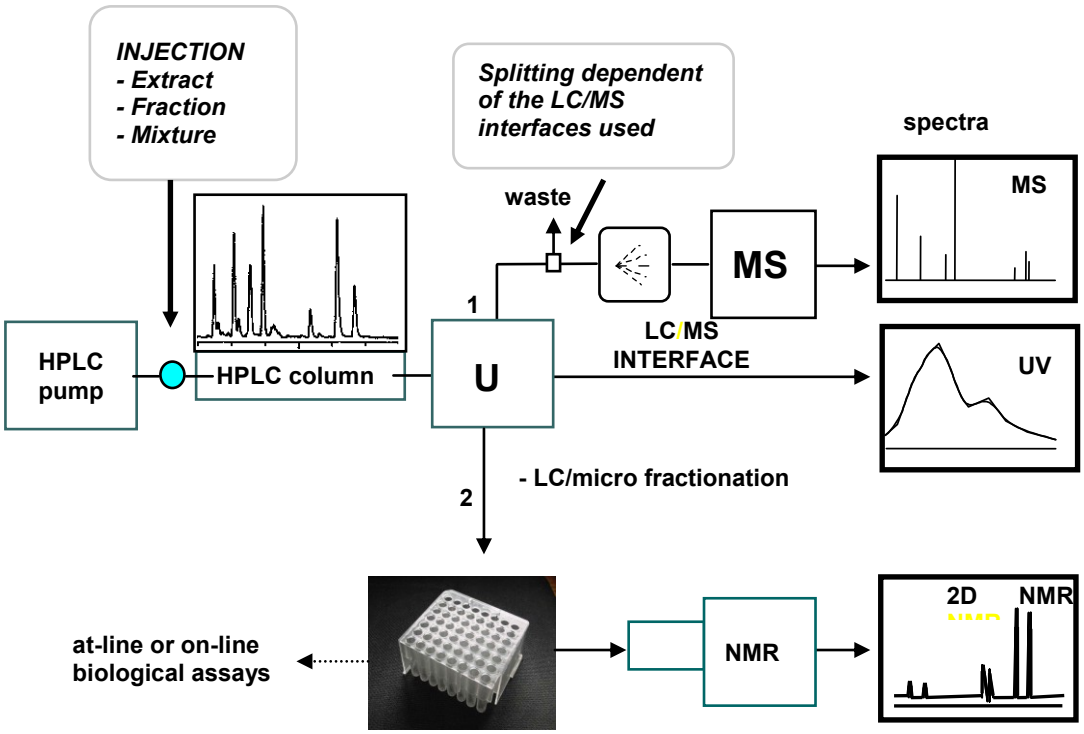
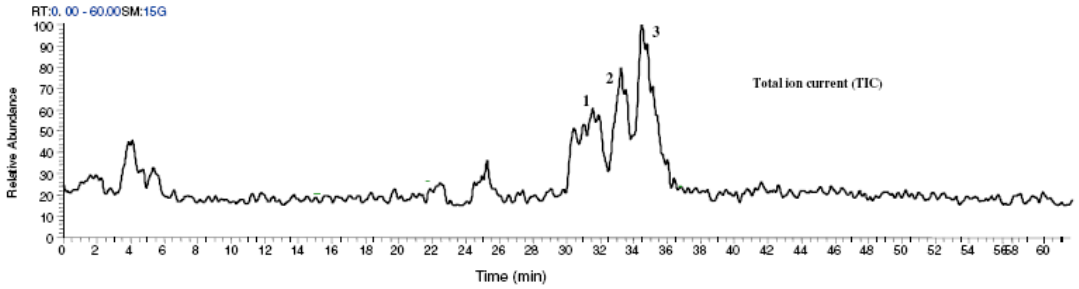
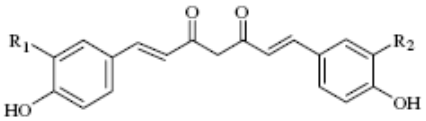


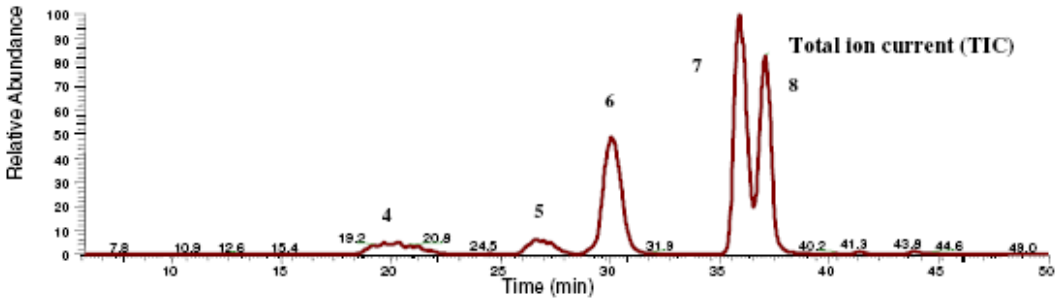
Fig. 3. Flow chart of rapid analysis of mixtures.

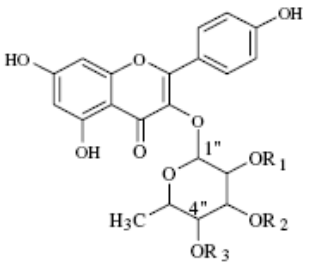




1-Bisdemethoxycurcumin: R_1=R_2=H,
2-Demethoxycurcumin: R_1=OMe, R_2 =H,
3-Curcumin: R_1=R_2=OMe

Fig. 4. Total ion profile of the active fraction (ethyl acetate) from the water extract of *Curcuma xanthorrhiza.*





	R_1	R_2	R_3
4-Kaempferol-3-*O*-rhamnoside	H	H	H
5-Kaempferol-3-*O*-(2”- or 3”- acetyl) rhamnoside	H	Ac	H (not isolated)
6-Kaempferol-3-*O*-(4”- acetyl) rhamnoside	H	H	Ac
7-Kaempferol-3-*O*-(3”,4”- diacetyl) rhamnoside	H	Ac	Ac
8-Kaempferol-3-*O*-(2”,4”- diacetyl) rhamnoside	Ac	H	Ac

Fig. 5. Total ion profile of the active fraction (ethyl acetate) from the water extract of *Zingiber zerumbet.*

Such approach of identifying the constituents at initial stage of discovery, before a large scale separation need to be carried out, is also known as *hyphenated technique* or *on-line analyses* of mixture. Using this approach a researcher can decide at an early stage whether the subsequent large scale extraction and extraction is worthwhile doing or not based on the knowledge of the nature of the constituents. This process, which is termed as *dereplication*, can save enormous financial and time-costs in the discovery program.

New challenges in natural products research – herbal preparations

Challenges

The health care costs are continuously rising at an alarming rate throughout the world. On the other hand, the world market for phytopharmaceuticals grows steadily. The turnover of these products in 1995 was estimated US$ 12.4 billion. In 2005 the herbal industry has a turnover of about US$ 62 billion with a strong growth potential. The World Bank reports state that trade in medicinal plants, botanical drug products and raw materials are growing at an annual growth rate between 5% and 15%. In addition to the cost, resurgence of interest in going back to herbal was partly due to the fact that more than 25% of the modern drugs currently used, including our common aspirin can be traced back to originate from plant. Furthermore, the general public tends to perceive that anything that comes from nature (plant or animal) is safe and cause less side effects.

It is noted that initially during the early 1990s, the market-driven growth of the business was in double digits, namely 17–20%. However, the market-driven enterprise is undergoing a fundamental change to a business of science-based products that will be driven by the results of clinical research. This is reflected in the recent market growth projection by the Nutrition Business journal to 6–8%. Several news items and articles in leading national newspapers point to the fact several marketed products do not live up to their label claim. Recent surveys reported in American news media indicate that a large percentage of the public would like to see products backed by science, which means products supported by clinical research. This means consumers are increasingly demanding products of known quality. A transparent process involving the manufacturers, government agencies, academia, research institutions and other interested parties might be involved in establishing public standards. Such standards assure the consumers proper identity, safety and quality of the products. It is clear then that, there is a strong demand and necessity to accelerate the research in phytomedicine .

The validation of efficacy of phytomedicines and the understanding of their mode of action remain the permanent challenges for evidence-based therapy, and the need to enhance this knowledge is becoming even more evident. In the recent years the use of genomic and proteomic based technology platforms in addressing these issues have been explored. These are high-throughput technologies, which allow substantially high number of proteins and/or genes to be detected simultaneously, thus having a great potential to relate complex mixtures to complex effects in the form of gene and/or protein expression profiles. Provided that the specific signatures of herbal preparation in the form of gene and/or protein expression profiles can be developed, these technologies will be useful for the chemical and pharmacological standardization and the proof of the toxicological potential of a plant extract. Such techniques may eventually economize the proof of efficacy, the determination of the mode of action of herbal medicines and allow the investigation of herbal extracts having no prominent active principle(s) or having to know the effect of each individual component. The application of these techniques has already revealed that gene expression profiles induced by single individual drugs and that induced by the combination of the same drugs can be entirely different. The result from this study has actually made the information on the mode of action of isolated active substances in herbal preparations become redundant. The development of gene expression signatures for extracts would allow a fast screening and would simultaneously generate the potential to cover synergistic effects of the plant extracts. Application of microarray analysis further demonstrated that the combination of both acted transiently synergistic on certain gene expressions (defensin and thionin, betaglucuronidase) in tobacco plants when both were applied at low concentrations, but antagonistic when more prolonged treatment or at higher concentrations were applied. The application of the ‘‘-omic’’ technologies may lead to a change of paradigms towards the application of complex mixtures in medicine. These technologies however, are still too expensive, less easily available in most laboratories, and are still at validation stage

Metabolomics in standardization

In the real practice this approach is quite tedious due the presence of minor constituents which sometime are too small to allow identification and quantitation. The more recent approach is called metabolomic finger-printing. This approach does not consider the individual constituent, but taking into account the total constituents exist in the extract through the spectrometric signals generated by all the constituents exist the concoction. The patterns of the total signals are recognized based on statistical pattern recognition analyses namely, the Principle Component Analysis (PCA) and other such as the Simulated Independent Modeling of Class Analogy (SIMCA) algorithms. NMR spectrometry in combination with these chemometric techniques, has been recognized as a very powerful tool to classify samples according to their total chemical composition. The low molecular weight metabolite contents of cells, organs or organisms can contain a significant amount of information on the physiological status of the cell, organ or organism. High field ^{1}H-NMR has crucial advantages since every organic molecule contains hydrogen atoms and hydrogen is the only atom where the isotope with the highest natural abundance giving highly resolved NMR signals. Due to its sensitivity, high quality spectra can be generally obtained in less than 5 minutes. The amount of information it can provide much is higher than of other fingerprinting technologies as NIR or HPLC providing a maximum resolution, non-reductive fingerprint of the total chemical composition of a sample.

Further development of this approach has successfully been used to characterize the metabolite patterns of apple juices, *Strychnos* species and tobacco. The use of this technique in herbal products consistency is done by narrowly defining a particular target quality of a medicinal herb with known and/or desired properties and differentiates it from non-target qualities of the same species and non-target species. This is, to create a “fuzzy” definition of quality to serve as specification for quality control purposes. This target quality specification is in the ideal case, a perfect representation of the intrinsic variability of the total chemical composition of a particular accession of a medicinal herb from a plantation effort where all factors of “avoidable” variability such as genetic variability, soil conditions and management protocols have been eliminated or under control. To differentiate and separate target quality from unwanted non-target qualities, the specification data set has to include representations of non-target qualities such as different accessions of the same species grown under the same conditions and the same accession grown under different conditions, wild collections of the species, closely related species as well as common misidentifications or substitutes.

Metabolomics in health diagnosis

An interesting development in metabolomics is in an effort directed toward using this technique in health/disease diagnosis. Metabolomics offers a complementary approach to alternative “omics” platforms such as genomics, transcriptomics, and proteomics, which involve the study of genetic complement, gene expression, and protein synthesis, respectively. Various physiological factors affect the metabolic composition of biological matrices including diet, state of health, diurnal cycles, genetic drift, stress and strain differences, and it is necessary to characterise these differences in order to distinguish between physiological and pathophysiological responses in animal models. An efficient nuclear magnetic resonance (NMR)-based metabolomic approach has been developed and introduced to understanding pathophysiological and toxicological processes, in which multivariate metabolic events over a period of time can be interrogated using pattern recognition analysis methods. ^{1}H NMR spectroscopy of biological matrices, coupled with appropriate pattern recognition (PR) and multivariate statistical analysis methods offers a novel in vivo approach to the investigation of drug toxicity. Applications of this metabolomic technology include the identification of biomarkers of toxicity and disease, monitoring of sequential metabolic perturbations in biofluids and tissues following toxic insult and metabolic characterization of physiological variance in humans under mild physiological stress. This approach has also been applied in determination of genetic variation in experimental animals and man .

The metabolic status of the organism is reflected in the spectral profile of a biofluid and there are species differences observable in the NMR spectrum of a biofluid such as blood plasma. The complexity of these information-rich NMR spectral profiles provides a significant analytical challenge,

as does the relationship between the profile and biological interpretation. However, information recovery can be maximised by applying multivariate statistical tools to the analysis of these NMR data. This metabotype concept could be widely applicable to define the metabolic consequences of a genetic modification or disease process that may not be directly observable from gross examination of the phenotype and allows statistical comparisons of genetic modification on physiological status.

It was also suggested that a further advantage of NMR-based metabolomics of biofluids is the capability of obtaining a sequential uninterrupted set of biofluid samples collected non-invasively over time, thereby allowing continuous analysis of the metabolic profile, unlike genomic and proteomic analysis which can only provide a `snapshot' picture of the status of a particular cell type. Since the development of disease or toxicity is generally a dynamic process, often with several phases each represented by a suite of unique biochemical markers, the ability to monitor dynamic metabolic profiles is useful.

^{1}H NMR on the tissue has also been applied for metabolomic fingerprinting to accommodate for the possibility of incomplete extraction of metabolite to be used in solution NMR spectrometry. Due to the lack of isotropic molecular motion in biological tissues, ^{1}H NMR spectra of tissues suffer from major line-broadening contributions when acquired by conventional solution-state NMR spectroscopy. These limitations can be overcome by using high-resolution magic-angle spinning (MAS) ^{1}H NMR spectroscopy of tissues, which produce spectra of resolution comparable to that of solution-state NMR spectroscopy. These observations provided complementary information to that obtained from analysis of urine and plasma from hydrazine-treated rats and hence increase understanding of the mechanism of hydrazine toxicity.

The first clinical studies in the application of a metabolomic strategy showed that a clear differentiation of metabolite profiles before and after Chamomile tea drinking can be obtained although strong extrinsic physiological variations were observed. About 14 volunteers had ingested chamomile tea for a period of 2 weeks. Urine samples before, during and after chamomile ingestion were analyzed. Chamomile tea ingestion was shown to lead to an increased urinary excretion of hippurate and glycine with depleted creatinine concentrations. This study highlights the potential for the metabolomic technology in the assessment of ‘‘small’’ interventions despite a high degree of variation from genetic and environmental sources. Variations in diet or local environment can become important confounding factors when metabolic responses to nutritional or minor interventions are studied.

Challenges in pursuing drug discovery and natural product research In Malaysia

Natural products research is relatively new in Malaysia. The earliest investigation on Malaysian plants can be traced back to the 1950’s, by the works of Nakanishi and Amarisingham. The establishment of University Malaya in Kuala Lumpur in 1960, saw the continuation of investigation of Malaysian plants by K.C. Chan and S. W. Goh and others. The more vigorous advancement in natural products chemistry was evidently observed in the 80’s, after the return of a large number of foreign trained post-graduates to several universities and research institutions in Malaysia to pick up their respective jobs in these institutions. Various organizations and institutions have contributed in many different ways in the progress of the chemistry of natural products including such as, UNESCO (through the Regional Network for the Chemistry of Natural Products in Southeast Asia), NCBNP (initiative of the Australian Development Bureau), JSPS (Japanese Society for the Promotion of Science), IFS (through research grants).

The popularization of bioscience as the new promising wealth generating knowledge based industry, especially those perceived to fall into biotechnology, the conventional approaches to the search and discovery of bioactive natural products have undergone some shifts. The interest in natural products has also attracted non-chemists to undertake this chemically oriented research discipline with the main interest of identifying the active components from medicinal plants. Although such strong interest may contribute toward enhancement of the outcomes from natural product research, the lack of collaborative effort among the researchers from the different disciplines did not result in the expectation. This may have resulted from the failure in recognizing and harnessing the expertise of each individual co-researcher involved in the projects. These failures may also have contributed to the lack in research atmosphere and culture in universities and research institutions.

Being a relatively young nation, Malaysia is still rather juvenile in research endeavors. As such, there is still a wide gap need to be filled before a truly highly acclaimed research status could be achieved. These critical requirements include:

- Improved facilities, funds, infrastructure (electrical, mechanical, technical support, etc.)
- Improved research management (reduce bureaucracy, timely dependable disbursement of research fund, making available specific fund for instruments maintenance, approval and monitoring of projects, etc.)
- Wise political insight, appreciation and clear direction in scientific endeavors (true scientific value or mere window dressings, quality research or quantity of research, pressure to generate merchandize, etc).
- Continuous improvement of university education system and its quality, especially in more difficult fundamental areas such as chemistry and physics and mathematics.
- Establish clear and unambiguous mission of specific organizations such as universities, research institutions, and other government departments.
- Increase the number of research chemists in most organizations.
- Encourage relevant industry to support research, expertise to follow through the findings.

Recommendations and conclusion

Biological resources have been and will continue to become the important source of drugs and other beneficial chemical constituents to humankind. Malaysia being blessed with rich and diverse biological resources can benefit from them if proper and wise strategic efforts are undertaken to realize their good values.

Considering the limited human and capital resources available to us, Malaysia needs to be prudent in establishing its direction and strategizing its efforts in enhancing its capacity in science to be at par with other developed nations. These efforts must be in line with the local conditions, the necessity and availability of the resources. We need to adopt the right appropriate strategic plan and actions based on proper understanding of the research elements and activities essential to the mission.

Chemistry is the central and controlling ingredient in life. Thus, its role in understanding the action of specific chemical entity onto a biological system is unmistakable. Furthermore, since any physical materials are constructed of chemical elements, the knowledge in this field is crucial to isolation efforts of these materials, identification of the chemical structures, and construction of certain desired chemical compounds. In view of its important role in realizing and enhancing the value of natural products derived constituents, the need to increase the critical mass of active chemists strongly recommended.

Recently, combination of multivariate analysis and spectrometry has been explored and developed to address the non-conventional single drug – single ligand concept in understanding and rationalizing a physiology action taking place in a body system. This approach, now known as metabolomics is expected to be important as a new alternative in non-destructive disease diagnosis, bioassay or efficacy evaluation of a single or multi-components system. Again, the knowledge of chemistry/biochemistry, spectrometry and statistics are crucial in the future development of this area. There is also a formidable need for the natural products chemist now to reassess and reconsider their role in this new dimension of science related to the use natural products in human life.

In view of the increasing importance of chemistry to the well-being and survival of human life, IUPAC has proposed to the United Nations that the year 2011 be adopted as the International Year of Chemistry. This intention is reflected by the following statements, which sets the proposal in motion:

a. Recognizing that humankind's understanding of the material nature of our world is grounded in our knowledge of chemistry.
b. Stressing that education in and about chemistry is critical in addressing challenges such as global climate change, in providing sustainable sources of clean water, food and energy, and in maintaining a wholesome environment for the well-being of all people.
c. Considering that the science and application of chemistry produces medicines, fuels, metals and virtually all other manufactured products.

d. Taking note that the ongoing United Nations initiatives in industrial best practices.
e. Welcomes the unanimous resolution of the International Union of Pure and Applied Chemistry, at its 2007 Council meeting, to declare 2011 as the International Year of Chemistry and to play a lead role in coordinating and promoting chemistry activities at the national and regional levels around the world.
f. Invites the Director General to support all effort leading the United Nations General Assembly to declare 2011 as the International Year of Chemistry.

The specific benefits of this proclamation include:

- Enhancement of international cooperation
- Improvement of the understanding and appreciation of chemistry among the public
- Promotion of the role of chemistry in solving global problems.
- Capacity building by engaging young people with scientific method of analysis developed by hypothesis, experiment, analysis and conclusion.

This move is timely considering the diminishing popularity of chemistry among the students and lack of recognition among the public and policy makers on the importance of this knowledge area. As a chemist, I am looking forward to the positive response by the government and NGOs positive towards this move, and implementation of appropriate measures to deal with the concerns on the need to upgrade the chemical elements of our scientific pursuit. This will eventually to lead improved well-being of the people, their lively-hood, as well as economic gains.

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Addressing the Challenges of Natural Products Drug Discovery

Appleton, D.R.[1], Tan, S.[2], Mustafa, M.R.[1], Mustafa, A.M.[1], Hadi, A.H.A[1] and Buss, A.D.[2]

[1]Centre for Natural Products Research and Drug Discovery (CENAR), Department of Pharmacology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, Malaysia
[2]MerLion Pharmaceuticals Pte Ltd, 1 Science Park Road, #05-01 The Capricorn, Singapore 117528
david@um.edu.my

Abstract

Natural products research has a clear role to play in the discovery of new pharmaceuticals with around 50% of marketed drugs being derived from natural sources. After a significant scaling back of natural products research by large pharma in the 1990's, it is this continuing trend that led to a revival of interest in the past 5-8 years. Despite this renewed interest in natural products drug discovery, there are still not many success stories. However, natural products research clearly has a place in the world within pharmaceutical research and, more widely in other fields such as nutraceuticals and agrochemicals. We must continually develop new tools and technologies to address the challenges of natural products research and look for ways to use them outside of traditional drug discovery activities. Chemical Fingerprinting of natural products extracts, prefractionation and LCMS dereplication-biological profiling are such tools that improve the natural products drug discovery process and can also be applied in many other areas. Examples of these tools and their applications along with discussion on the future of natural products research will be presented.

Introduction

Natural products (NP) drug discovery has had its ups and downs over the past few decades. Of particular note, are the significant scaling back of natural products research activities by large pharma in the 1990's and the subsequent resurgence of NP drug discovery efforts as new compound isolation and elucidation technologies allowed the application of high throughput screening to NP libraries (Butler, 2004; Baker and Chu, 2007). Natural product templates still feature strongly in new drug launches (Butler, 2005; Newman and Cragg, 2007; Ganisan, 2008) and stand as a clear reason why we should continue to look to nature for new medicines. However, the road to launching a drug is long and hard, so we must continually address the challenges and bottlenecks of NP drug discovery, as well as look for new applications of our assets and technologies in order to ensure that NP research continues to contribute to the discovery of new medicinal treatments.

Three of the major challenges of NP drug discovery that remain today are; 1) ensuring a novel and diverse extract/organism collection, 2) issues related to the complexity and unknown nature of extracts, and 3) the efficiency of isolation and elucidation of novel active components from extracts. The challenges involved with assay selection, interference and progression will not be dealt here, however other reviews are available (Butler, 2004; Shoichet, 2006). Chemical fingerprinting, automated extract prefractionation and LC-MS/MS-bioassay dereplication are technologies that have been recently developed to address these challenges by giving in depth understanding of NP extracts early in the screening process, simplifying and enriching extracts prior to screening and lead to a more efficient isolation process. This paper introduces proprietary chemical fingerprinting technologies as well as extract prefractionation and compound dereplication tools developed at MerLion Pharmaceuticals in Singapore and demonstrates their application to NP drug discovery and beyond.

Materials and methods

Chemical fingerprinting raw data were acquired using a Waters UPLC system equipped with a PDA detector, ELSD and Micromass ZQ mass spectrometer. Data processing algorithms were developed to export data, normalize compound retention times and detector response as well as for background subtraction, noise reduction and peak validation. Inter-sample similarity, extract productivity and chemical diversity calculations were then performed using the processed data. All data were stored in an in-house developed database for flexible data queries and further processing via a user interface.

Prefractionation of crude extracts was accomplished using 400-500 mg of crude extract dissolved in 1 mL of methanol and separated into 4 fractions using a Gilson 322/215/156 preparative HPLC and Waters Xterra RPC18 30x19 mm column as described elsewhere (Appleton *et al.*, 2007). Fractions were transferred into a Genevac HT8 centrifugal evaporator for drying before re-suspension and transfer into screening plates.

Extract dereplication was performed using an Agilent HP1100 HPLC with 90% flow directed to a microtitre plate fraction collector and 10% flow to a Bruker Esquire ion trap MS after the PDA detector. A standard gradient was employed on a C18 150x4.6 column and 40 fractions were collected during the gradient. Fractions were dried and submitted for biological assay, allowing alignment of biological activity profiles and LC-MS/MS chemical profiles.

Results and discussion

Chemical fingerprinting

The extract fingerprinting programme at MerLion Pharmaceuticals was initiated to address the need for an understanding of extract components. Chemical fingerprinting tools were designed to provide measures of extract productivity, diversity and similarity, which can be combined to give an overall indicator of extract and library quality. These measures can then guide a wide range of organism growth/secondary metabolite production, extraction and sample handling experiments, as well as assist with assay hit profiling. In addition, the processed chromatographic data for each extract are stored in a searchable database so that any specific data can be easily retrieved for many other purposes.

Fig. 1 shows an example of how the chemical diversity of a group of extracts can be viewed as a compound array and provides a clear picture of productivity in terms of compound concentration, diversity and uniqueness. This representation can also be used for several other functions, such as viewing chemical space coverage, identification of common compounds/biomarkers, guiding extract preparation/storage experiments and extract library quality calculation.

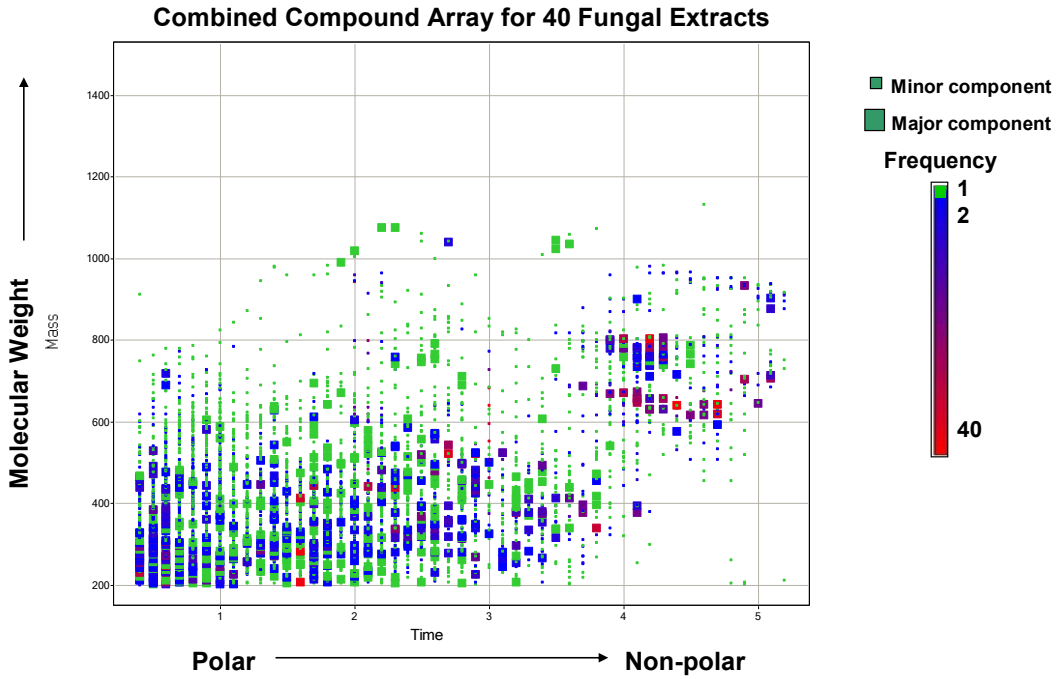

Fig. 1. Viewing the chemical diversity of a group of extracts, mass versus retention time for several components.

Compound arrays can be used to see clear differences between extracts of different organism types. Extract sets that show evidence of low productivity and/or diversity can be identified for further research, for example; growth conditions/media, extract concentration or prefractionation/enrichment of extracts prior to screening.

Extract productivity, in terms of relative mass of compounds can be determined using fingerprinting data by calculating the area under the chromatograms for UV, MS and ELSD data.

Finally, the similarity of two extracts can be calculated by comparing the multidimensional arrays of UV and MS data (both positive and negative ionization modes). The similarity between many samples pairs can be represented as single numbers that can then be used to build similarity heat maps or dendrogram trees.

Chemical fingerprinting opens up many possibilities by giving us a clearer understanding of what extracts contain and therefore the producing organisms themselves, including, microbial fermentation optimization for compound production, extract library quality control, dereplication of known compounds and similar extracts early in the screening process, chemotaxonomy and metabolomics.

Prefractionation

Prefractionation can be defined as the fractionation of crude extracts before primary screening and serves the purpose of enriching and simplifying the mixtures of metabolites in the primary assay samples. This enables detection of active compounds at much lower concentration and those whose activity is masked in some way in the crude extract. Several methods of prefractionation have been developed, from simple solvent extraction methods and SPE to more complex methods involving CCC HPLC and fully automated hyphenated techniques that generate many fractions that contain less than five compounds each (Appleton *et al*., 2007). MerLion Pharmaceuticals developed a simple automated method using preparative scale C18 HPLC to generate 4 fractions from 500 mg of crude extract with a cycle time of 15 min. Overnight usage of a single HPLC resulted in the generation of 48k fractions per year in 5 copies of mother screening plates, quickly producing a substantial and diverse library of prefractionated samples. Screening results demonstrated a five times increase in hit rates from prefractionated samples and revealed that 80% of the hits had inactive crude extracts before prefractionation.

Dereplication

Dereplication of natural product samples involves the identification of active samples that contain known active compounds that account for the whole extract activity, as well as the grouping of similar samples prior to chemical isolation efforts. Dereplication is a necessary step in the hit progression pathway since the isolation of active components from active extracts is traditionally the bottleneck of NP drug discovery and samples progressing to this point must be unique. In addition, the dereplication profiles of samples that are progressed can provide a valuable head-start to the isolation process by identifying the polarity and possible masses of the active components. Dereplication can be achieved by splitting the flow from an analytical HPLC to a microtitre plate fraction collector and mass spectrometer (and other detectors such as NMR (Corcoran and Spraul, 2003). The fractions can be submitted for biological assay and the resulting profile can be aligned with the LC-MS profiles to identify the active regions. Fractions are assayed at equivalent dose to the crude extract, which allows an assessment as to whether the active component/s account for the whole crude activity. Multiple regions of activity can be used to identify the presence of multiple active components.

We must continually develop new tools and technologies that address the challenges of NP drug discovery. Chemical fingerprinting combined with dereplication technologies serve to take away some of the ‘mystery’ of natural products extracts before significant resource has been committed to identify the active components and hence, significantly improves the NP drug discovery process from organism growth to extract preparation and screening hit selection. Combined with prefractionation to simplify and enrich extracts, these technologies will ensure NP research remains at the forefront of pharmaceutical discovery. In addition, these technologies have many applications outside of drug discovery including herbal and traditional medicines QC and metabolite profiling.

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Antimicrobial Compounds from Australian Traditional Medicines

McRae, J.M.[1,2], Yang, Q.[2], Crawford, R.J.[1] and Palombo, E.A.[1]

[1]Environment and Biotechnology Centre, Faculty of Life and Social Sciences, Swinburne University of Technology, Hawthorn, VIC, Australia
[2]CSIRO Molecular and Health Technologies, Clayton, VIC, Australia
epalombo@swin.edu.au

Abstract

The leaves of the tropical tree, *Planchonia careya* (Lecythidaceae), are used traditionally in the treatment of wounds and ulcers by indigenous communities in northern Australia. The purpose of this investigation was to isolate and identify some antibacterial compounds from the leaves of *P. careya* to validate the use of this species as a wound healing remedy, and also to evaluate the isolated active compounds for potential use as chemotherapeutic or topical antibacterial agents. The comprising compounds of the crude aqueous and methanol leaf extracts were separated using activity-guided fractionation and the structures of the isolated antibacterial compounds were elucidated using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. The biological activity of each compound was assessed using minimum inhibitory concentration (MIC) assays against a range of bacteria, and with MTT cell viability assays against monkey kidney epithelial (MA104) cells. Two known antibacterial compounds, gallocatechin-(4→8)-gallocatechin (**1**) and (+)-gallocatechin (**2**), were isolated from the aqueous extract. The methanol extract yielded 9(*S*)-hydroxyoctadeca-10*E*,12*Z,15Z*trienoic acid (**3**), 9(*S*)-hydroxyoctadeca-10*E*,12*Z*-dienoic acid (**4**), 2α,3β,24-trihydroxyolean-12-en-28-oic acid (**5**), and a mixture of the *cis* and *trans* isomers of 3-*O*-*p*-coumaroyl-2α,19α-dihydroxyurs-12-en-28-oic acid (**6ab**). The mixture of **6ab** demonstrated the greatest antibacterial activity and prokaryotic selectivity of the isolated compounds with a MIC against vancomycin resistant enterococci (VRE) of 59 µg/mL. The isolation of known antibacterial compounds **1**-**5** as well as **6ab** from the crude leaf extracts of *P. careya* validates the use of this species as a treatment for sores and ulcers.

Introduction

Traditional wound-healing remedies generally contain antibacterial compounds in greater concentrations than other plants, particularly compounds with activity against Gram positive bacteria, which are known skin pathogens. For this reason, the investigation of plants used in the treatment of wounds can reveal potentially novel antibacterial compounds. The antimicrobial compounds of the Australian traditional medicinal plant, *Planchonia careya*, were investigated to determine the types of compounds responsible for the observed healing properties.

Materials and methods

General experimental procedures

NMR spectra were obtained using a Bruker DRX500 NMR spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C using the Topspin software package and with CD_3OD as the solvent. Gradient versions of ^{1}H-^{1}H DQF-COSY, ^{1}H-^{13}C HSQC and HMBC experiments were carried out using the standard pulse sequences as supplied by Bruker. HPLC analysis was achieved with a Waters 600 instrument using an Alltech Alltima 5µm RP-18 column (150 x 4.6 mm) with MeOH/H_2O, 5:95 (v/v) for the aqueous compounds and 70:30 (v/v) for the methanolic compounds. Peak detection was made with a Waters 996 Photodiode Array UV Detector. Positive and negative ion electrospray mass spectra were acquired with a VG Platform mass spectrometer using a cone voltage of 50 V and the source was maintained at 80°C. The solvent system used was MeOH with a flow rate of 0.04 mL/min. Optical rotations were measured with an Optical Activity Ltd PolAAr3005 automatic polarimeter, and melting points were determined with a micro melting point apparatus and were uncorrected.

Plant material

The collection of leaves from *Planchonia careya* (F. Muell.) R. Knuth was authenticated by Dr. Andrew Ford, CSIRO Tropical Forest Research Centre, Queensland, Australia, and voucher specimens were lodged at the Australian National Herbarium (A.Ford4328). Leaves were stored at -10°C before extraction.

Extraction and isolation

The chopped fresh leaves of *Planchonia careya* (4.74 kg) were initially extracted with water using the immersion technique. The main antibacterial compounds were isolated from the crude aqueous extract (460.1 g) using repeated column chromatography with Amberlite XAD-16 resin, Chromatorex® C18 media (100-200 mesh), Sephadex™ LH-20 gel and preparative HPLC columns (5 µm). At each stage fractions were tested using plate-hole diffusion assays against *Bacillus cereus* and methicillin-resistant *Staphylococcus aureus* (MRSA). This isolation gave 6.5 mg (0.001%) of **1** and 12 mg (0.003%) of **2**. After the aqueous extraction, the leaf material was then extracted with methanol using the immersion technique. The combined extract (496.6 g) was partitioned using water and dichloromethane (DCM), and the DCM-soluble fraction was fractionated with silica gel (100-200 mesh, Merck), MPLC (Delta-Pak™ column with Waters PrepPak® 15 µm C18 cartridges) and preparative HPLC (Alltima RP-C18, 5 µm). This gave compounds **3 (**21.7 mg, 0.004%), **4 (**15.0 mg, 0.003%), and a mixture containing a 9:1 ratio of compounds **5** and **6** (2.1 mg).

Biological activity assays

Isolated compounds were tested for antimicrobial activity against *B. cereus* (American Type Culture Collection (ATCC) 11778), *S. aureus* (ATCC 25923), and *Escherichia coli* (ATCC 25922), clinical isolates of MRSA (M99320) and vancomycin-resistant *Enterococcus* (VRE, 193272) and *Mycobacterium smegmatis*, and *M. fortuitum*. Plate-hole diffusion assays (PHDAs) with *B. cereus* and MRSA were used in the activity-guided fractionation with methanol as a negative control and tetracycline as a positive control. PHDAs using duplicate two-fold serial dilutions of the test compounds and crude extract were used to assess the minimum inhibitory concentrations (MIC). The MIC of the crude extracts and some compounds against the fast-growing strains of mycobacteria were assessed with broth dilution assays in Middlebrook 7H9 broth (Difco) supplemented with glycerol and enrichment broth (supplied by BD) using a 96 well microtitre plate. Rifampicin was used as the positive control and methanol was again used as a negative control.

The cytotoxicity of the isolated compounds and crude extracts was assessed against monkey epithelial (MA104). The cell viability after exposure to the test sample was assessed with MTT assays as reported by Mosmann (1983). The inhibitory rate (IR) was calculated as: $IR = (A_{SC} - A_{TC})/ A_{SC} \times 100$, where A_{TC} is the average absorbance for each concentration of test compound and A_{SC} is the average absorbance of the methanol control. Absorbance readings were first corrected for the media blank.

Results and discussion

The chopped fresh leaves of *Planchonia careya* were extracted with distilled water followed by methanol, and both extracts showed antibacterial activity against Gram positive bacteria and fast-growing strains of mycobacteria. The compounds responsible for the observed activity of the crude extract were separated by HPLC-piloted activity-guided fractionation. The isolated compounds (Fig. 1) were characterized using 1D and 2D NMR spectroscopy, as well as ESIMS. The melting point and optical rotation of each compound was also determined and compared to the literature values where possible.

Compounds **1** and **2** were identified as the known (+)-gallocatechin and gallocatechin-(4α→8)-gallocatechin, respectively, and the ^{13}C NMR chemical shifts and other spectral and physical properties concurred with those reported in the literature (Sun *et al*., 1987). Compound **3** was identified as the known 9(S)-hydroxy-10*E*,12*Z*-octadecadienoic acid (α-dimorphecolic acid) (Martini and Buono, 1996) and Compound **4** was the known triterpene, 2α,3β,24-trihydroxyolean-12-en-28-oic

acid (hyptatic acid-A) (Yamagishi *et al*., 1988). The spectral and physical properties of **3** and **4** concurred with the data reported in the literature (Yamagishi *et al*.,1988; Martini and Buono, 1996). The mixture of compounds **5** and **6** was found to consist of two acylated triterpene isomers, and were identified as the *cis* and *trans* isomers of 3β-*O*-*p*-coumaroyltormentic acid, respectively (Taniguchi *et al*., 2002).

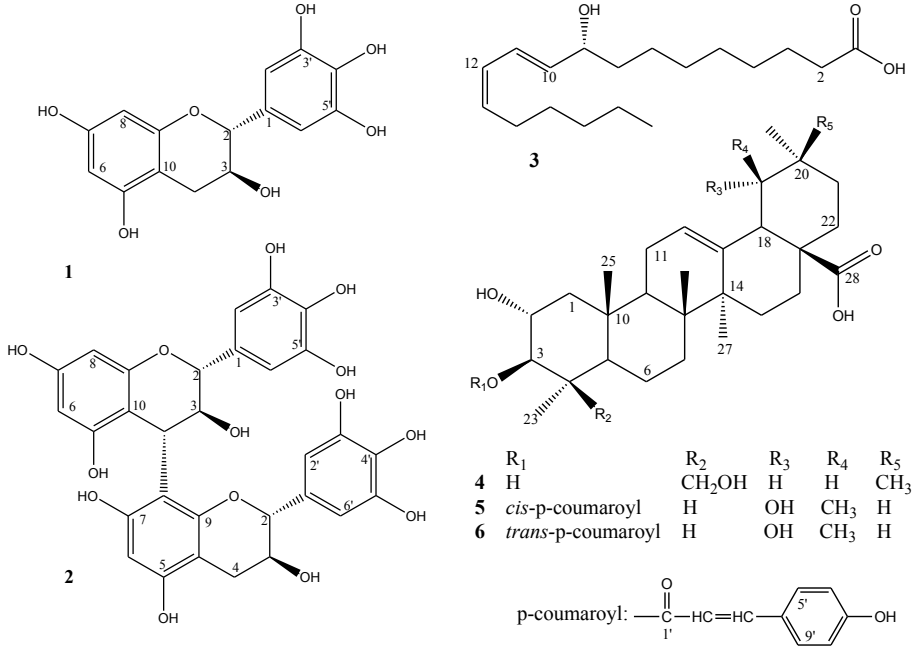

Fig. 1. Compounds isolated from the leaf extracts of *P. careya*.

The antibacterial activity of compounds **1**-**6** and the crude extracts was evaluated against a range of bacteria with MIC assays. Tests were performed in duplicate, and the average MIC for each compound and crude extract against each tested bacteria strain is given in Table 1. Each of the isolated compounds and the crude extracts demonstrated activity against Gram positive bacteria, and therefore the traditional use of *P. careya* leaves in treating sores and abrasions is valid. The mixture of **5** and **6** produced the lowest MIC of the isolated compounds against *S. aureus* and VRE, with concentrations of 0.118 and 0.059 mg/mL, respectively.

The selectivity of each compound for prokaryotic cells relative to eukaryotic cells (T.I.) was assessed by comparing the MIC against bacteria with the 50% inhibitory concentration (IC_{50}) against MA104 cells (T.I. = IC_{50}/ MIC). In general, the isolated compounds were much more selective for the eukaryotic cells than the prokaryotic cells with T.I.s of < 0.05 for compounds **1**-**4**. These compounds were therefore considered to be unsuitable for use as oral antibacterial agents. The isomeric mixture of **5** and **6**, however, produced a T.I. of 1.2 against VRE, suggesting that these compounds were weakly selective for this antibiotic-resistant strain. Further research into **5** and **6** including the relative biological activity of the isolated compounds is therefore warranted.

The isolation of antibacterial compounds from *P. careya* leaves validated the traditional use of this species in the treatment of wounds, and the weak selectivity of **5** and **6** for VRE highlights the importance of investigating wound-healing remedies for antibacterial agents.

Table 1. The MIC of the isolated compounds and crude extracts against bacteria (mg/mL), and the IC_{50} of each compound against MA104 cells (µg/mL)

Compound	MIC							IC_{50}
or extract	MRSA[a]	VRE	*Bc*	*Sa*	*Ec*	*Mf*	*Ms*	MA104
Aqueous	9.0	>36	9.0	9.0	>36	9.0	4.5	-[b]
1	6.0	>12	6.0	6.0	>12	>24	24	220.9
2	6.0	>12	6.0	6.0	>12	24	12	145.4
Methanol	9.0	>36	9.0	9.0	>36	9.0	9.0	-
3	1.0	1.0	-	0.50	>16	-	2.0	35.0
4	0.80	0.40	-	-	-	-	-	19.2
5, **6**	-	0.059	-	0.118	-	-	-	72.0
Tetracycline	0.16	0.08	-	0.001	-	-	-	153.6
Rifampicin	-	-	-	-	-	0.02	0.03	-

[a] MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin-resistant *Enterococcus*; *Bc*, *Bacillus cereus*; *Sa*, *S. aureus*; *Ec*, *Escherichia coli*; *Mf*, *Mycobacterium fortuitum*; *Ms*, *M. smegmatis;* [b] – Results were not obtained for these samples

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Silvestrol: An Emerging Compound for Cancer Therapy

Yeo, T.C.

Sarawak Biodiversity Centre, KM 20 Jalan Borneo Heights, Semengoh,
Locked Bag No. 3032, 93990 Kuching, Sarawak, Malaysia
cyeo@sbc.org.my

Abstract

Silvestrol is a rocaglate derivative that was isolated from the bark, fruits and twigs of *Aglaia* sp. commonly found in Malaysia and Indonesia. This compound showed potent *in-vitro* cytotoxic activity against several human cancer cell lines including lung, prostrate and breast cancer. Its activity is comparable to the well known cancer drug, Paclitaxel (Taxol). Silvestrol is composed of a cyclopentabenzofuran core which is phytochemically confined to the genus *Aglaia*. It is attached to an unusual dioxyanyloxy group critical for its potent cytotoxic activity. The compound was active in P388 murine leukemia model and induced cell death through an apoptotic response in hormone-dependent human prostrate cancer cell line. Total synthesis of silvestrol and its enantiomer, epi-silvestrol, has been achieved by two different chemistry research groups. Recently, silvestrol was reported to be selective in inhibiting B cells compared to T cells in chronic lymphocytic leukemia (CLL) patients' blood. This compound is currently being developed for CLL therapy by National Cancer Institute and Ohio State University Medical Centre, USA. This paper provides a review of the related work as well as research initiated by the Sarawak Biodiversity Centre and its collaborators to develop the patent of the compound owned by the Government of Sarawak.

Introduction

Silvestrol is a derivative of rocaglate isolated from the bark, fruits and twigs of the genus *Aglaia* (Meliaceae) trees which are mainly found distributed in South East Asia region. This compound has a unique molecular structure which is unprecedented in nature and possesses cytotoxic activity comparable to known anti-cancer compounds such as paclitaxel and camptothecin (Mi *et al*., 2006). The significance of this compound as an anti-cancer agent is supported by key discoveries of several research groups which showed that silvestrol is both highly active *in vitro* and *in vivo* studies against a variety of human cancer cell lines (breast, prostate, lung) and mouse tumor models (P388, Eμ-myc/PTEN, Eμ-Tcl-1) (Hwang *et al*., 2004, Bordeleau *et al*., 2008, Lucas *et al*., 2009). Interestingly, Bordeleau *et al*. reported that silvestrol enhances the chemosensitivity of cancer cells to in a mouse lymphoma cancer model (Bordeleau *et al*., 2008). Silvestrol is exceptionally potent (ED_{50} 1.2-1.5 nM range) but it exists in low concentrations in its natural source; about 0.002% w/w in the leaves, 0.01% w/w in the fruit, 0.0085% w/w in the twigs and 0.02% w/w in the bark (Mi *et al*., 2006, Salim *et al*. 2007). This paper reviews the discovery work published by various groups on silvestrol and report on the initial screening and analysis of *Aglaia* trees undertaken by the Sarawak Biodiversity Centre in collaboration with other governmental agencies.

Discovery of Silvestrol

In 2004, Dr. A. Douglas Kinghorn and his group of multi-disciplinary scientists, published a paper in the Journal of Organic Chemistry describing a novel compound called silvestrol (Hwang *et al*., 2004). The compound was isolated from a tree, *Aglaia silvestris*, collected from Kalimantan, Indonesia. The taxonomical identification of the plant was later corrected to *Aglaia foveolata* (Hwang *et al.* erratum, 2004). Kinghorn's group resolved the structure and stereochemistry of the silvestrol and its diastereomer, episilvestrol, including documenting their highly potent cytotoxic activity against a number of human cancer cell lines. Since then, a series of publications reporting the unique biological activity of silvestrol in cancer models, the mechanisms of action and synthesis of the compound were published.

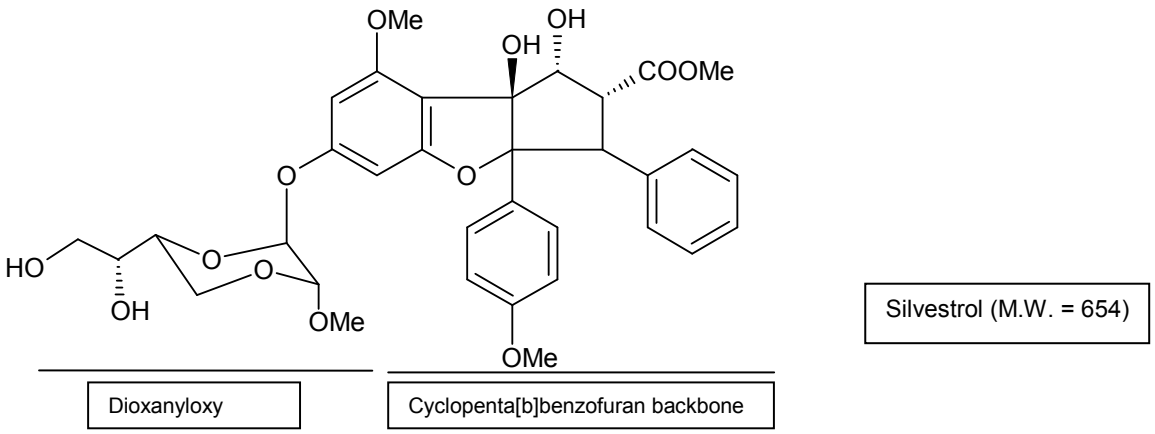

Fig. 1. Molecular structure of silvestrol.

Traditional uses of *Aglaia*

Aglaia spp. have been traditionally used in several South East Asian countries. In Vietnam, the crude extracts from the leaves and flowers of *Aglaia* spp are used to treat inflammatory skin diseases and allergic disorders such as asthma (Proksch *et al*., 2005). The flowers of *Aglaia odorata* were used as perfume in China and in Indonesia, *Aglaia* plants are used to treat fever and as preparation in leprosy treatment by using the latex from the plant to smear on the affect parts of the skin disease (Christensen H, 2002). There are also report of rocaglamide derivatives that are biologically active as anti-inflammatory and anti-leukemic agent (Baumann *et al*., 2002)

At around the same time when Kinghorn and colleagues published their work on silvestrol, an Australian biotech company called Cerylid Biosciences Ltd, engaging in discovery of novel lead compounds for development of new medicines, filed a worldwide patent on a molecule identical to the chemical structure of silvestrol. The patent was derived from a research collaboration between the Sarawak Government and Cerylid Biosciences Ltd to screen and identify bioactive compounds from Sarawak's biodiversity for pharmaceutical development. In 2007, the patent was assigned to the Sarawak Government for patent maintenance and commercial development when Cerylid Biosciences Ltd ceased its operation.

The scope of the patent invention covers the description of two compounds (compound A and B) which uniquely possess a dioxanyloxy sidechain at 6-position of the cyclopenta[*b*]benzofuran core as well as their potential applications in cancer therapy (Fig. 1)(Meurer-Grimes et al., 2004). The project name for compounds A and B in the patent are CBL316 and CBL330 respectively. CBL316 and CBL330 are functionally distinct diastereomers (epimers at 5'''). These compounds were isolated from a tree called *Aglaia leptantha,* Miq. (Meliaceae). The species name was later corrected to *Aglaia stellatopilosa* (Pannell) (Soepadmo, 2007). The effectiveness and potency of these compounds were demonstrated using various human cancer cell lines *in vitro*, and *in vivo*, with mouse xenograft models. CBL316 and CBL330 are synonymous in chemical composition and structure with silvestrol and episilvestrol, respectively. They appear to exhibit similar biological activities and chromatographic profiles.

***In-vitro* and *in vivo* activity of silvestrol**

Cyclopenta[*b*]benzofuran core of compounds and derivatives from the genus *Aglaia* (Meliaceae) are a very interesting group of compounds for providing unique carbon skeleton lead structures with anti-cancer and immune-modulating activities (Kim *et al*, 2007). Cyclopenta[*b*]benzofurans' anti-proliferative and cytostatic activity against human cancer cell lines have been well documented. They block protein synthesis and induce cell-cycle arrest at the G2/M transition in human monocytic leukemia cell lines (King *et al*., 1982, Bohnenstangel *et al*., 1999). These compounds (including rocaglate and rocaglamides) are effective in reducing inflammation by acting as a specific inhibitor of

NF-AT activation which is involved in the triggering of selective inflammatory cytokine expression, (Proskch *et al*., 2001, Fahrig *et al*., 2005).

Silvestrol, a rocaglate derivative, shows very potent cytotoxic *in vitro* activity against various human cell lines. The potency (ED_{50} 1.2to 1.5 nM) is comparable to well-known anticancer drugs, paclitaxel (Taxol) (ED_{50} 0.7 to 4.7 nM) and camptothecin (ED_{50} 30nM) (Table 1). When compared to methyl rocaglate, the dioxyanyloxy side chain of silvestrol was identified as a key determinant of the potency (Hwang *et al*., 2004, Rivero-cruz *et al*., 2004). Compound CBL316 (silvestrol) had been tested on panel of cancer cell lines from National Cancer Institute and the overall data showed significant *in vitro* cytotoxic activity against a wide range of tumor cell lines representing different major types of cancers including leukemia, lung, colon, brain, melanoma, ovarian, renal, prostate and breast tumours (Meurer-Grimes *et al*., 2004).

Table 1. *In vitro* cytotoxic activity in human cell lines

Compound	**Cell line**[a]			
	Lu1	**LNCaP**	**MCF-7**	**HUVEC**
Silvestrol	1.2	1.5	1.5	4.6
Methyl rocaglate	163	325	Not determined	203
Paclitaxel	2.3	4.7	0.7	105.5
Camptothecin	28.7	28.7	28.7	258.6

[a] Results are expressed as ED_{50} values (nM). Lu1 (human cancer); LNCaP (hormone-dependent human prostate cancer); MCF-7 (human breast cancer); HUVEC (human umbilical vein endothelial cells) (Hwang et al., 2004)

The *in vivo* data on silvestrol is as impressive and promising. In hollow fiber assay test developed at the U.S. National Cancer Institute, silvestrol was capable of inhibiting proliferation of several cancer cell lines with the most prominent result against LNCaP (83%) at doses up to 5mg/kg with no detectable gross toxicity in the animals tested (Hwang et al., 2004). The compound was also tested in P388 murine leukemia model and achieved an increased lifespan in treated mice of 150% (maximum value) compared to controls of at doses of 2.5mg/kg which were given as five daily *i.p.* injections (Hwang *et al*., 2004). In a mouse xenograft model using PC3 (prostate cancer) as the tumour tranplanted into athymic mice, treatment with Compound A (silvestrol) at 3mg/kg *i.p.* at 3 times per week reduced the tumour size by about ~60% with no significant body weight loss in treated controls (Meurer-Grimes, 2004). These data, not only suggests that silvestrol is effective *in vivo* in mouse cancer models against various types of tumor challenge but it is also relatively non-toxic at the effective dose used.

Molecular target

An attempt to define the molecular target(s) of silvestrol was carried out using gene expression microarray technology which is useful for assessing overall global effects on gene expression in a biological system (Mi *et al*., 2006). The results showed that silvestrol affected the expression of 20 apoptosis and cell cycle genes in human prostate cancer cells (LNCaP) including p53. p53 down-regulation was the most interestingly, with a concomitant down-regulation of MDM2, a protein that targets p53 for proteosome degradation. The effect is not preventable by inhibition of proteosome activity, suggesting that silvestrol acts on p53 degradation via a proteosome-independent pathway. Further work by Lucas *et al*. showed that silvestrol is capable of activating cell apoptosis in p53-negative human chronic lymphocytic leukemia cells. Hence, it supported earlier observation and provided for a possible treatment in drug resistant leukemia patients acquiring p53 deletions (Lucas *et al*., 2009).

Chronic Lymphocytic Leukemia (CLL)

Chronic lymphocytic leukemia is one of the most common types of adult leukemia registering over 10,000 cases in USA each year. It has a wide variable clinical course with survival rates ranging from just months to over a decade. The cell type mostly affected is B cells and they are highly characterized by chromosomal abnormality including deletion of the short arm of the chromosome 17 (del 17p) where the p53 gene is located. CLL is incurable and the patients most of time ended up developing resistance to the treatments that were initially active. The therapeutic options for drug resistant CLL are extremely limited. Death in the patients usually occurs from both the disease and the treatment because there is a risk of infections, immune disorders and secondary cancers (Lucas *et al*., 2009).

David Lucas and his colleagues at Ohio State University explored the pre-clinical efficacy of silvestrol in CLL and found that silvestrol is more cytotoxic towards B cells than T cells in isolated and whole blood cells from normal and CLL patients. There is no difference in sensitivity in cells from patients with or without del (17p3.1) abnormality suggesting that silvestrol is effective towards abnormal cancer cells that could confer drug resistance. Furthermore, the Ohio research group showed that the compound maintains B cell selectivity *in vivo* in Eμ-Tcl-1 murine CLL model. These findings indicated that silvestrol affects different cell-type and could be used as a treatment for other kind of B cell malignancies as well. The initial results are promising and it convinced the National Cancer Institute Developmental Drug Therapeutics Program's Drug Development Group (USA) to fund pre-clinical studies at Stage IIA level to develop the compound further in clinical trials (Lucas *et al*., 2009).

Total synthesis of silvestrol

The yield of silvestrol and epi-silvestrol from the natural source is extremely low (Salim *et al*., 2007). Consequently, there is a strong interest among organic chemists to work towards an efficient synthesis route for both compounds. Typical synthesis routes from simple precursors will generate analogues for biological evaluation and possibly, define the lead compound. To date, only two groups have successfully synthesized silvestrol/ epi-silvestrol. Gerard *et al.* demonstrated that the synthesized compounds derive from an enantioselective process maintained their biological activity which is greatly influenced by the stereochemistry of the dioxanyl moiety (Gerard *et al*., 2007). Another group, El Sous *et al*., reported the total synthesis of epi-silvestrol in 21 steps from available precursors and the stereo-conversion to silvestrol (El Sous *et al.*, 2007). In summary, the total synthesis of silvestrol is successful but current attempts could not achieve the efficiency and scalability to obtain more compounds for further research into its anti-cancer properties.

Collection and phytochemical screening of *Aglaia* trees

The Sarawak Biodiversity Centre (SBC) conducted a collection and phytochemical analysis of *Aglaia* samples Sarawak in collaboration with Forest Department and Department of Agriculture. The objective of the study is to identify *Aglaia* trees that express silvestrol using a variety of methods; cytotoxic screen on cell lines, HPLC and mass spectrometry analyses. This collection and screening of *Aglaia* samples is important to identify the trees for further scale-up isolation of the compound because there is yet a viable method to synthetically make sufficient amount of silvestrol for clinical studies. Furthermore, the collection allows us to characterize the *Aglaia* trees in Sarawak and identify primary materials for propagation.

In our preliminary screen, we identified a total of 22/99 *Aglaia* samples with significant cytotoxic activity on two human cancer cell lines, MCF-7 (breast cancer) and NCI-H460 (lung cancer). A total of 12/140 extracted leave samples show target peaks with mass fragmentation pattern similar to silvestrol and epi-silvestrol. This preliminary survey indicates that there is a strong likelihood that our collection will yield silvestrol-positive plants for further studies. Currently, SBC is working towards the completion of this work and is compiling a database on the *Aglaia* collection which will include its taxonomic identification as well as the phytochemical and biological data. This information will be useful for a key

international collaboration with the National Cancer Institute and Ohio State University, USA, to advance silvestrol through pre-clinical trials for chronic lymphocytic leukemia.

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Endophytic Fungi in Malaysian Medicinal Plants: Evaluation of Antimicrobial Potential

Santiago, C.[1], Refaei, J.[1], Jalil, J.[2] and Santhanam, J.[1]

[1]Department of Biomedical Science, Faculty of Allied Health Sciences
[2]Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300 Kuala Lumpur, Malaysia
jacinta@medic.ukm.my

Abstract

Endophytic fungi, which asymptomatically inhabit plants, are known to produce bioactive secondary metabolites which often have pharmacological potential. Tropical rainforests with high biodiversity are expected to harbour diverse microorganisms producing more active metabolites. In this study, endophytic fungi were isolated from seventeen Malaysian medicinal plants to be evaluated for bioactive metabolites with antimicrobial activity. The selected plant species included *Alseodaphne* sp., *Calophyllum* sp., *Alpinia* sp., *Alstonia* sp., *Artocarpus* sp., *Tetracera* sp., *Rafflesia* sp., *Goniothalamus* sp., *Dillenia* sp., *Solanum* sp., *Tetrastigma* sp., *Catharanthus* sp., and *Polyalthia* sp. These plants are used traditionally to treat fever, abrasions, wounds, tooth ache, diarrhea, malaria, herpes, ulcers, bronchitis and other ailments. Previous research on these plants revealed that the plant extracts are mostly rich in compounds like alkaloids, flavonoids and triterpenes. The actual antimicrobial activities may have been contributed by the endophytes within the plants. The plants were sampled from forest reserves in peninsular Malaysia. A total of 288 endophytic fungal isolates were obtained and categorized as166 morphotypes. Of these, 16 isolates showed promising antifungal and antibacterial activity. The range of their activities varied from strong to weak inhibition of microbial growth. Among the inhibited pathogenic fungi were *Aspergillus niger*, *Aspergillus fumigatus, Candida albicans, Fusarium solani* and *Trichoderma viridae.* Meanwhile for antibacterial activity, inhibition was observed for *Bacillus subtilis, Staphylococcus aureus* and *Pseudomonas aeruginosa.* Methanol extracts from active isolates have also shown similar antimicrobial activity. This study shows endophytic fungi isolated from traditional Malaysian medicinal plants have promising antimicrobial activity and can be further researched to identity the active secondary metabolites.

Introduction

Endophytic fungi live within plant tissue (intracellular or intercellular) without causing apparent injury or symptoms to the host plant (Maheswari, 2006). Some of these endophytes provide protection to their host plant from tissue invading pathogens (Carroll, 1986; Bettucci and Saravay, 1994). Endophytes are also known to produce secondary metabolites which have pharmacologically importance (Strobel *et al*., 2004; Maheswari, 2006).

Bioactive natural products from endophytes have been reported to have antibiotic, antiviral, antidiabetic, and antiiflammatory properties (Brady and Clardy, 2000, Guo *et al*., 2000; Zhang *et al*., 1999; Weber *et al*., 2004). For instance, a *Phomopsis* species fungal isolate from *Erythrina cristagalli*, a medicinal plant, produces Phomol, a novel compound that is antibacterial and anti-inflammatory (Weber *et al*., 2004). One of the most significant findings is the anticancer drug, Taxol, first discovered in the yew tree (*Taxus* sp.) and years later, found in the endophytic fungi *Taxomyces andreanae* isolated from a yew tree (Strobel *et al*.,1993). Previous research on endophytic fungi isolated from Malaysian medicinal plants demonstrated antimicrobial and antitumor activity (Radu and Cheah, 2002; Tomita, 2003). These examples give credibility to the assumption that endophytic fungi isolated from medicinal plants are likely to have some pharmacological activity which may be similar to that of their host plant.

Endemic plants growing in tropical rainforests have a higher potential to yield novel endophytes and a greater number of natural products due to the dense population of diverse plants which create an enormous competition for resources (Strobel, 2002). In our research endophytic fungi were isolated from several medicinal plants and evaluated for their antimicrobial activity.

Materials and methods

Plant sampling

Selected plant samples (shown in Table 1) were collected from Hutan Lipur Cali and Menchali Forest Reserve in Rompin, Pahang, Endau-Rompin National Park in Kuala Rompin, Pahang and Pos Dipang, Kuala Kangsar, Perak. Different parts of the plant such as leaf, bark, stem, flower and fruit were collected and stored in a zipper bags and transported to the laboratory.

Plant surface sterilization

Plant samples were cut into small pieces, approximately 1cm x 1cm prior to surface sterilization process. Samples were washed with tap water, rinsed with 70% ethanol, followed by rinsing with 50% commercial Clorox® and finally washed with sterile distilled water. The outer surface of the bark was removed with a sterile blade and the leaves were cut into small pieces, approximately 0.5cm x 0.5 cm. The samples were placed on potato dextrose agar (PDA) and water agar and incubated at room temperature (RT).

Isolation of endophytic fungi

The agar plates were observed everyday for a month. New fungal hyphal tips emerging from the samples were immediately subcultured on PDA and coded accordingly. Fungal cultures were observed macroscopically and microscopically to determine their morphotypes.

Evaluation of antimicrobial activity

Plate based assay: Fungal isolate was inoculated in the middle of a PDA plate. Panel organisms were streaked perpendicularly to original growth of the fungus.
Well-diffusion assay: Fungal broth cultures were tested against the panel organisms. 20µl of extract or broth culture were filled in a hole punched in PDA. The agar plates were streaked with the panel organism at a concentration of 0.5 OD.
Evaluation of antimicrobial activity was made based on growth inhibition of the panel organisms. The nine panel organisms used in this study were *Staphylococcus aureus, Bacillus subtilus, Eschericha coli, Pseudomonas aeruginosa, Candida albicans, and Aspergillus niger, Aspergillus fumigatus*, *Fusarium solani* and *Trichoderma viridae.*

Extraction of fungal secondary metabolites

Fungal isolates showing antimicrobial activity were cultured in 200 ml of potato dextrose broth with shaking, for two weeks. The culture broth and mycelia were separated by filtration where the broth was extracted with equal amount of ethyl acetate and the mycelia were crushed with 100 ml of methanol. Both mycelia and broth were extracted with the solvents overnight. These extracts were concentrated with a rotary evaporator. The crude extracts were tested against the panel organisms with the well-diffusion assay.

Table 1. The medicinal importance of selected plant species

Plant species	Medicinal Importance
Calophyllum sp.	Treat depression, infectious disease and heal wound (Wiart, 2002)
Rhodomyrtus tomentosa	Treat bloody vomits (Kulip *et al*., 2007)
Alseodaphne perakensis	Alkaloids with morphine-like effect (Lajis *et al*., 1989)
Tetracera indica	Ease cough, treat fever and poisonous bite (Wiart, 2002)
Artocarpus heterophyllus	Treat wound, ulcers, abscesses, herpes and expel intestinal worms (Wiart, 2002)
Alstonia sp.	Reduce fever, expel intestinal worms, treat malaria and passive congestion of liver, and to alleviate toothache (Burkhill, 1966; Perry and Metzger, 1980)
Alpinia sp.	Treat intestinal and fungal infection, bronchitis, type II diabetes, and rheumatism (Wiart, 2002)
Cinnamomum sp.	Treatment of colic and diarrhoea, postpartum (Taher *et al*., 2006)
Rafflesia cantleyi	Fed to pregnant females as a strength-giver (Noor *et al*. 2006)
Goniothalamus sp.	Post-partum decoction or in abortion (Wiart *et al*. 2007)
Dillenia sp.	Treat wounds and stop bleeding (Ahmad and Raji, 1991)
Solanum torvum	Treat toothache, crack on the feet (Ahmad and Raji, 1991)
Tetrastigma sp.	Lumbago, high fever and bronchitis (Ahmad and Raji, 1991)
Polyathia sp	Treat rheumatism and skin infection (Ahmad and Raji 1991)

Table 2. Endophytic fungi isolated and their antimicrobial activity

Plant sample	Fungal isolates (#)	Morphotypes (#)	Active isolates (code name)	Microrganism(s) inhibited
Calophyllum sp.	36	15	CS1L003	*Staphylococcus aureus, Bacillus subtilis*
			CS1B001	*Aspergillus niger, Aspergillus fumigatus, Trichoderma viridae, Fusarium solani*
Rhodomyrtus tomentosa	15	8	-	-
Tetracera indica	11	8	KL 002	*Staphylococcus aureus, Bacillus subtilis*
Artocarpus heterophyllus	18	12	ARSB 002	*Aspergillus niger, Aspergillus fumigatus, Trichoderma viridae, Fusarium solani*
			ARSB 003	*Escherichia coli*
Alseodaphne perakensis	20	9	-	-
Alstonia sp.	13	8	-	-
Alpinia sp.	9	3	-	-
Rafflesia cantleyi	8	7	RP-3	*Candida albicans*
			RP-4	*Candida albicans*
			RP-6	*Candida albicans*
Cinnamomum sp.	62	16	CL 016(WA)	*Aspergillus niger, Aspergillus fumigatus, Trichoderma viridae*
			CL 011	*Escherichia coli, Aspergillus niger, Staplylococcus aureus, Fusarium solani*
			CB 007(WA)	*Bacillus subtilis, Escherichia coli, Aspergillus niger, Aspergillus fumigates*
			CL-8(WA)	*Aspergillus niger , Trichoderma viridae,*
			CL- 12(WA)	*Candida albicans, Bacillus subtillis*
Goniothalamus sp.	42	26	GL-15	*Aspergillus niger, Aspergillus fumigatus, Staphylococcus aureus*
Dillenia sp.	9	5	-	-
Solanum torvum	36	25	SB-2	*Fusarium solani*
Tetrastigma sp.	25	15	TL-6(WA)	*Fusarium solani,*
			TS-1(WA)	*Aspergillus fumigatus, Staphylococcus aureus*

Plant sample	Fungal isolates (#)	Morphotypes (#)	Active isolates (code name)	Microrganism(s) inhibited
Polyalthia sp.	32	21	PL-4	*Candida albicans, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa*

Results and discussion

A total of 288 endophytic fungal isolates were obtained and categorized as 166 morphotypes. Of these, 16 isolates showed promising antifungal and antibacterial activity. The range of their activities varied from strong to weak inhibition of microbial growth. The panel organisms are commonly occurring fungal and bacterial pathogens. Table 2 shows the fungal isolation and antimicrobial assay results.

Methanolic extracts of fungal isolates demonstrated better activity compared to ethyl acetate extracts (results not shown). Methanol and ethyl acetate extracts from isolates coded as CL 016(WA) and CB 007(WA) showed antifungal and antibacterial activities. Meanwhile, only methanol extracts from KL 002 and CS1L 003 had activity. Better activities observed in methanol extracts may indicate that the bioactive compounds are strongly polar.

Isolates from *Cinnamomum* spp., *Artocarpus* spp., *Calophyllum* spp., *Goniothalamus* spp. and *Polyalthia* spp. demonstrated antimicrobial activity against a broad range of organisms. Extracts from these plant spp. are known to have medicinal importance. It is reported that *Cinnamomum* spp. produces essential oil which has insecticidal (Samarasekere *et al*., 2006) and antifungal (Moharm *et al*., 2005) properties. *Calophyllum* spp. is another plant which has been widely studied due to the presence HIV-inhibitory compound in the leaf extracts. Besides, *Calophyllum* spp. produces a phenolic compound with activity against pathogenic bacteria and yeast (Reyes *et al*., 2004). This plant is used to treat infectious disease (Wiart, 2002) and endophytes within the plant may also contribute to its activity. Our research has also shown that endophytes isolated from medicinal plant have a similar activity to the plant extracts. This indicates that there may be a correlation between a plant's medicinal values and its associated endophytes.

Conclusion

Medicinal plants in Malaysian rainforests harbour a wide range of endophytic fungi, some of which have significant antimicrobial activity. Currently our research is focused on identifying the bioactive compounds and characterizing the endophytic fungi. The Malaysian forest biodiversity offers a vast resource for science and mankind which ought to be researched and preserved.

Acknowledgements

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A Study on the Hepatoprotective Effect of *Andrographis paniculata* on Mice

Sutha, D., Jegathambigai, R and Sudhakaran, S.

Department of Biotechnology, Faculty of Applied Sciences,
AIMST University, Semeling, Kedah, Malaysia
sutha133@ yahoo.com

Abstract

Andrographis paniculata is a well known medicinal plant of Ayurveda with various pharmacological as well as medicinal properties. The present study determined the hepatoprotective effect of crude methanolic extracts of *A. paniculata* on mice. The phytochemical screening of the crude methanolic extracts of *A. paniculata* plant was also determined followed by the confirmation of the active compound using Thin Layer Chromatography. The hepatoprotective activity of methanolic extracts of *A. paniculata* was evaluated against paracetamol induced (500mg/kg) hepatic damage in mice. The extracts at doses of 10mg/kg and 100mg/kg were orally administered at 24 and 72 hours time interval in each group. Histological analysis of the liver and the liver protein content was determined. The results of the study indicated that the crude extracts of *A. paniculata* at both doses exhibited a significant protective effect in the liver morphology of the paracetamol induced hepatotoxicity in mice. There was also a significant decrease ($P<0.05$) the liver protein content of the hepatotoxic mice after the treatments. The crude methanolic extracts of *A. paniculata* indicated the presence of phytochemical constituents such as flavonoid, alkaloid, tannin and terpenoids. Finally, Thin Layer Chromatography confirmed the presence of active compound, diterpene lactone or andrographolide which has contributed to the hepatoprotective activity of *A. paniculata*. Hence, it can be concluded that treatment of low and high doses of *A. paniculata* with time period revealed the hepatoprotective effect in mice.

Introduction

Andrographis paniculata is one of the medicinal plants that seem promising found throughout Southeast Asia. Basically, the taste of *A. paniculata* is very bitter. This bitterness is related with its various pharmacological properties such as antibiotic, antiviral, antimicrobial, anti inflammatory, antivenom and immunostimmulatory, anticancer, anti-HIV, anti-allergic, and hypoglycemic activity (Bone and Mills, 2001). These properties are due to the presence of distinct lactones and flavones.

A. paniculata is employed extensively as hepatoprotective agent proven by some research studies. This is functional since liver diseases appear to be a serious problem for the past few years .In general; liver plays an astonishing array of vital functions in the maintenance and performance of the body such as for metabolism, storage, biosynthesis and detoxification. Unfortunately, the liver is often abused by environmental toxins, alcohol and over-the-counter drug use (xenobiotics) which can damage the liver and eventually lead to hepatitis, cirrhosis and liver diseases.

Therefore, conventional medicine is now pursuing the exploitation of natural products such as herbs to provide the support that liver needs on a daily basis. One such herb is *A. paniculata* which encompasses a long history of traditional use in revitalizing the liver and treating liver dysfunction and diseases due to the presence of the main active compound, 'andrographolide' or diterpene lactone which contributes to the hepatoprotective activity (Trivedi and Rawal, 2001).

The present research project was undertaken in order to determine the hepatoprotective activity of the crude methanolic extracts of *A. paniculata* on the paracetamol-induced hepatotoxicity in mice. Along with the phytochemical screening and Thin Layer Chromatography analysis of the crude methanolic extracts of *A.paniculata* was also performed.

Materials and methods

Plant material

Andrographis paniculata was collected at various locations around Gurun, Kedah. The leaves were used for the study.

Animals

The animals used were adult female mice of albino *Mus musculus* weighing between 25 to 30 g obtained from Universiti Sains Malaysia. The mice were housed in a clean and well ventilated experimental unit of animal house and they were fed with clean water and diet ad libitum.

Experimental design

18 adult female mice were divided into 6 groups and treated as shown in table 1:

Table 1. Grouping of mice and treatment.18 adult mice were divided into 3 main groups with 2 different time intervals (n=3)

No.	Groups	Treatment
1.	Control(Hepatotoxic) (C1)-24 hours	500 mg/kg of paracetamol body w.t
2.	Control(Hepatotoxic) (C2)-72 hours	500 mg/kg of paracetamol body w.t
3.	Low dose (L1)-24 hours	10 mg/kg of AP extract body w.t
4.	Low dose (L2)-72 hours	10 mg/kg of AP extract body w.t
5.	High dose (H1)-24 hours	100 mg/kg of AP extract body w.t
6.	High dose (H2)-72 hours	100 mg/kg of AP extract body w.t

All the mice were given paracetamol intraperitoneally at a dose of 500 mg/kg to induce hepatotoxicity. After 24 hrs, the mice were treated orally with crude extracts of *A. paniculata* at low and high doses (10 mg/kg and 100 mg/kg) respectively for a period of 24 and 72 hours except the control group which was given the vehicle only. Along with normal controls were also used for comparison.

The mice were sacrificed after the last treatment. The liver was removed carefully through dissection and weighed accurately. Simultaneously, a small portion of the liver, 0.3 g was stored at -80^{o}C for protein quantification using Biuret Assay. While, the remaining portion of the liver were fixed in 10% neutral buffered formalin at room temperature for histological analysis.

Phytochemical qualitative tests of the crude methanolic extracts of *A. paniculata* was conducted such as Benedict test, frothing test, Borntrager test, flavonoid test, tannin test, alkaloid and terpenoid tests as well as moisture content determination by using *A. paniculata* methanolic extracts at a concentration of 20 g in 20 ml. Thin layer chromatography (TLC) was also performed using silica gel pre-coated aluminium backed TLC plates with 40 % ethyl acetate and 60% methanol as the mobile phase. A spray solution of potassium permanganate (1 % in methanol) was used for the TLC plates, and then dried and observed under UV at 254 and 360 nm (James, 1963).

Results and discussion

Liver morphology

Histologically, the normal liver tissue (Fig. 1A) pointed out the presence of normal hepatocytes which are polyhedral in shape and distinct nuclei with one or two prominent nucleoli. The liver tissues of control group at 24 hours (Fig. 1B) specified the presence of distorted shaped hepatocytes with undefined cell lining as well as vacuolated hepatocytes and vacuolated nucleus. The hepatotoxicity control group (72 hrs) demonstrated the degeneration of hepatocytes with more prominent and increased number of vacuoles in the hepatocytes and multinucleated giant-like cells (Fig. 1C). The *A. paniculata* treated liver (10 mg/kg) at 24 hours (Fig. 1D) and 72 hours (Fig. 1E) indicated the presence of hepatocyte which almost recovered to the normal polyhedral shape with the development of clear

cell lining and reduced vacuolated nuclei. While liver tissue treated with 100mg/kg of *A. paniculata* extracts at both 24 hours (Fig. 1F) and 72 hours (Fig. 1G) displayed the complete recovery of the hepatocytes with normal hepatocytes, reduced vacuolations, clear cell lining as well as absence of multinucleated giant cells.

Liver protein content

The liver protein content were significantly ($p<0.05$)) decreased in the both *A.paniculata* treated of low dose (10 mg/kg) and high dose (100 mg/kg) groups at 24 and 72 hours compared to the hepatotoxic control group as mentioned in Table 2 and Fig. 2. This can be explained using a number of reasons. At normal therapeutic dosage of paracetamol, the liver is able to perform its normal function such as the protein metabolism. However, when overdosage of paracetamol is induced, the liver is not capable to perform its normal functions where the protein metabolism is affected. Therefore, deamination of amino acids in order to breakdown the protein fails to arise resulting in the protein accumulation in the liver (Mitchell *et al*., 1981).

Besides, an obvious sign of hepatic injury is the leaking of cellular enzymes into the plasma (Schmidt *et al*., 1975) due to the disturbance caused in the transport functions of hepatocytes (Zimmermanand , 1970). When liver cell plasma is damaged, a variety of enzymes located normally in cytosol is released into the blood, thereby causing increased enzyme levels in the serum. Such enzymes are the serum ALT and AST which significantly increase in the paracetamol treated control group (Sadasivan *et al*., 2006). Since all enzymes are proteins, the enzyme increase is related to protein increase as well in the liver.

Table 2. Liver protein content of the control and *A. paniculata* treated (low and high dose) groups

Treatment	**Amount of protein (mean ± s.d)**
Control 1	730.50 ± 58.34
Control 2	858.00 ± 58.34
Low dose 1	399.25 ± 6.61*
Low dose 2	369.25 ± 7.50*
High dose 1	282.58 ± 65.26*
High dose 2	126.75 ± 28.28*

Phytochemical creening and TLC analysis of crude extracts

Phytochemical screening indicated the presence of flavonoid, tannin, alkaloid and terpenoid in the crude extracts of *A. paniculata* (Table 3).

Table 3. Phytochemical Screening data of *A. paniculata*

Phytochemical Tests	**Results**
Benedict test	-
Frothing test	-
Borntrager test	-
Flavonoid test	+
Tannin test	+
Alkaloid test	+
*Terpenoid test	+
Moisture content	82.17 %

Absent (-); present (+)

The presence of terpenoid is highlighted because andrographolide, the main active compound of the *A. paniculata* extracts is a terpenoid (Sharma *et al*., 1992). The positive result of the terpenoid test indicates the presence of other compounds as well in the AP extracts such as deoxyandrographolide, neo-andrographolide, homoandrographolide, and andrographan which happen to be in terpenoid category as well (Siripong *et al*., 1992). All these compounds are responsible for the

pharmacological properties of the *A.paniculata* extracts. TLC analysis confirmed the presence of Andrographolide (Fig. 3).

A dark violet spot was observed on the silica gel column at 360 nm under UV radiation as in the Fig. 3. Based on the previous studies, Thai Herbal Pharmacopoeia, 1995, dark violet colored spot indicates the 'andrographolide' compound after the separation of the *A.paniculata* methanolic extracts. This confirms that the andrographolide which is the main active compound is present in the *A. paniculata* plant extract.

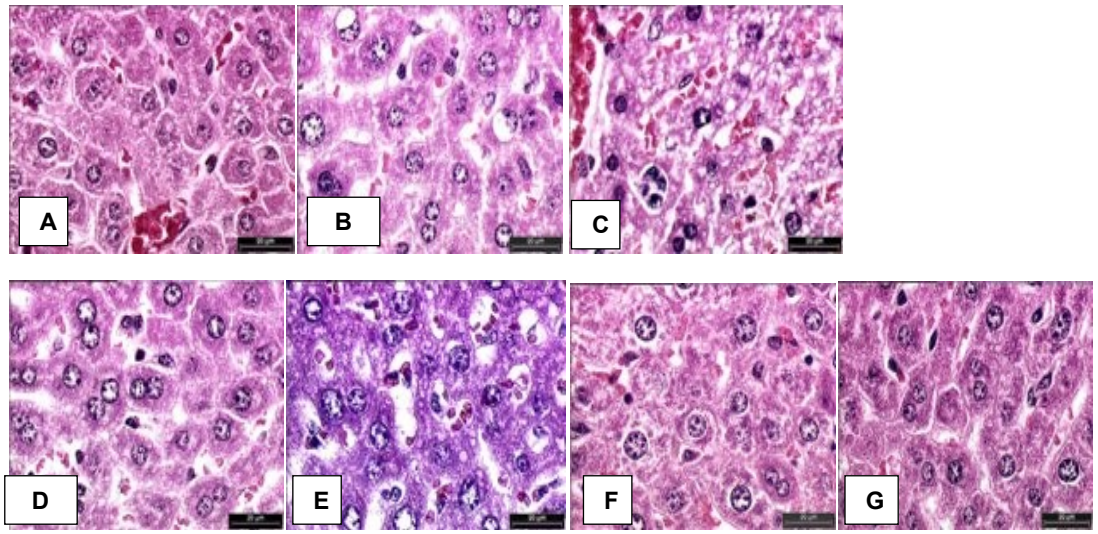

Fig. 1A-G: Histology of Liver Tissue Section.

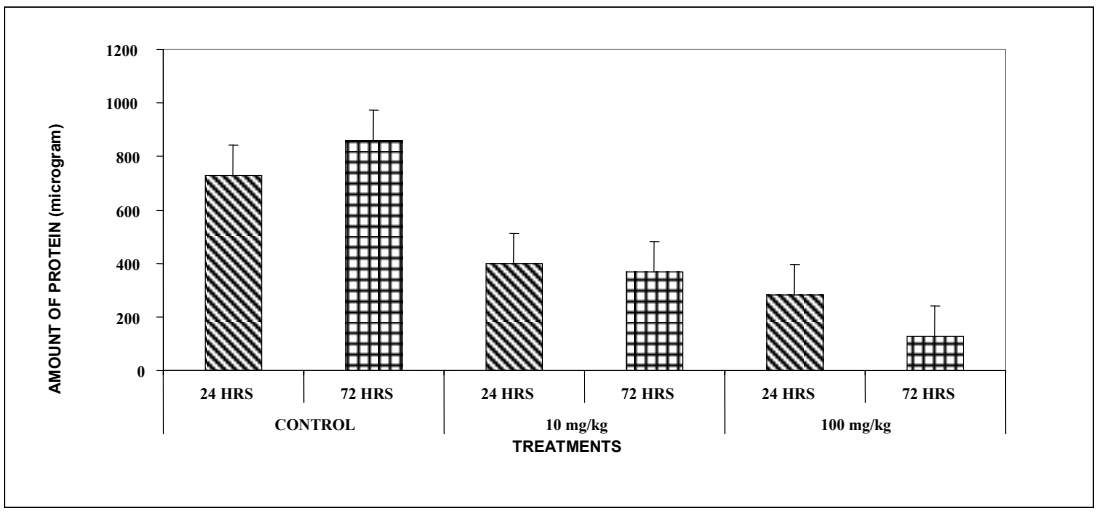

Fig. 2. Graph of the liver protein content of the control and *A.paniculata* treated (low and high dose) groups. Each bar represents mean values .The error bar indicates the standard deviation of the mean.

Fig. 3. TLC analysis of *A.paniculata* extract showing the presence of andrographolide (dark violet spot).

Andrographolide is the primary medicinal component and the main active compound of the *A. paniculata* plant. Andrographolide is also called as 'diterpene lactone', a chemical name that describes its ring like structure. It has a very bitter taste which is the basis for the various pharmacological properties in it. Besides, it is a non-protein compound, colorless and crystalline in appearance (Siripong *et al*., 1992). The leaves contain the highest amount of andrographolide (2.39%), the most medicinally active phytochemical in the plant, while the seeds contain the lowest (Sharma *et al*., 1992).

Andrographolide is the one which contributes to the hepatoprotective effect of the *A. paniculata* extract. For instance, in a study, andrographolide from *A.paniculata* was shown to produce a significant increase in bile flow (Shukla *et al*., 1992). Bile is produced in the liver and stored in the gallbladder and aids in digestion. When the chemical, paracetamol, was given to animals pretreated with andrographolide, the usual decrease in bile production seen with this chemical was prevented. Thus, this proves that andrographolide contributes to the hepatoprotective activity of the *A. paniculata* extract.

Conclusion

The present study shows the hepatoprotective activity of the crude methanolic extracts of the *A. paniculata*. This is concluded using the results obtained from the histological studies as well as the liver protein content. The hepatoprotective activity of *A. paniculata* is due to the presence of the active compound, andrographolide which is a diterpene lactone.

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The Anti-Candida Activities of *Etlingera coccinea* (*Tuhau*), *Curcuma longa* (Turmeric) and A*lpinia galanga* (Greater Galangal)

Mohamad Hafiz, A.T., Rozlianah, F.S., Siti Sarayati, A.M. and Julenah, Ag.N.

Faculty of Applied Science, Universiti Teknologi MARA Sabah, Locked Bag 71, 88997 Kota Kinabalu, Sabah, Malaysia
moham585@sabah.uitm.edu.my

Abstract

The methanolic and hexane extracts of the aerial parts of *Curcuma longa* L. (turmeric), *Alpinia galanga* (greater galangal) and *Etlingera coccinea* (*tuhau*) were investigated for the evaluation of the antifungal properties towards *Candida albicans* strain. Three of the extracts (methanol extracts of galangal stalks, tuhau leaves and turmeric leaves respectively) exhibited weak anti-candida activity against *C. albicans*.

Introduction

Candida albicans are predominantly emerged as clinically significant pathogens which is hazardous to human health. Although numerous antibiotics have been produced, it has resulted to several drawbacks (Gur *et al.*, 2006). In contrast, the antifungal/antimicrobial compounds of medicinal plants possess fewer side-effects, better patient tolerance, naturally renewable and relatively cheaper than the current drugs and/or antibiotics used (Vermani *et al.*, 2002). All these facts highlight the need for new alternative, natural, yet effective drugs regimens.

The Zingirberaceae is among the plant families which are widely distributed throughout the Southeast Asia (Habsah *et al.*, 2000). In Sabah, although more than 60 unique species have been documented by Julius (1997) and Andersen *et al.* (2003), only few plants has been used regularly in daily practices; mainly for cooking and medicinal purposes.

In this report, three species of gingers under the family of Zingiberaceae have been studied; *E. coccinea* (*tuhau*), *C. longa* (turmeric) and *A. galanga* (greater galanga). Preliminary studies indicate the gingers have microbial activities (Jayaprakasha *et al.*, 2005; Oonmetta-aree *et al.*, 2006). The gingers plants extracts were tested against *C. albicans* strain in order to evaluate the potential anti-candida properties.

Materials and methods

Collection of samples

Herbarium specimens of the gingers were prepared and deposited at the herbarium of Faculty of Applied Sciences UiTM Sabah.

The fresh samples were collected from Kiawayan, Tambunan Sabah. The experiment was carried out at the Chemistry and Plant Pathology Laboratory, Faculty of Applied Sciences, UiTM Sabah.

Sample preparation

The fresh samples of *E. coccinea* (*tuhau*), *C. longa* (turmeric) and *A. galanga* (greater galanga) were divided into leaf, stalk and rhizome cleans and cut into parts. Only the non-edible parts of the plants were studied; the leaves for turmeric and *tuhau* and the stalks for galangal and *tuhau*.

Extraction

Exactly 500 g of samples were soaked for 4 days in 1 L of analytical grade (AR) methanol (Merck, Germany). The methanol extracts were filtered and concentrated *in vacuo* (Heidolph Laborota 4001, Germany). The extracts were partitioned between hexane (AR, Merck, Germany) and distilled water.

The pooled hexane extracts were dried using sodium sulphate anhydrous (Merck, Germany). The extracts were then filtered and concentrated *in vacuo* before weighing. The extracts concentration was 50 µg/L.

Anti-candida activity assay

The experiment employed yeast strain which was acquired from the Pathology Department, Queen Elizabeth Hospital, Kota Kinabalu; *C. albicans* (ref. strain: csv-y-2004-01). The test was run exactly as an antibacterial activity test as follows: -

A microbial density of 0.5, according to McFarland Standard, was achieved from the precultured yeast culture. 0.1 ml of the adjusted culture was used to seed 3% NaCl nutrient agar (NA) plates. It was spread on the agar's surface using 'hockey stick' method. 4 mm paper discs were then impregnated with hexane and methanol crude extracts of respective gingers at 50 µg/disc concentration before placed on the seeded agar plates. The plates were then incubated for 24 hours at 37^{o}C. At the end of the period, plates were observed for signs of inhibition. The diameter of the inhibitory zones was measured to each positive observation.

Results and discussion

The results showed that the extract A, B and D have significant anti-candida activities towards *C. albicans* in the NA media (Table 1). These observations, however, is different from our previous study (Mohamad *et al.*, 2008) which focuses on the edible parts of the ginger plants. The uses of different parts of the plants (edible and non-edible parts) may explain the different outcomes on literatures reviewed by Chrubasik *et al.* (2005).

Although extract A, B and D were notable as inhibitors, they are not as effective as the current fungicide used; fluconazole and ketoconazole (Pattayanak *et al.*, 2008). These extract (A, B and D) are not only requiring higher concentration (50µg/ml) to activate the anti-candida properties, they also produce inferior results against the commonly used fungicide, thus limiting its potential to become an alternative for antifungal compounds. In addition, based on data provided by Ficker *et al.* (2003), it can be assumed that the plant extracts which only have weak effects towards the anti-microbial activities, will possess least, if not any anti-candida properties. The hexane extracts of these gingers, however, do not exhibit any significant anti-candida activities compared to the methanol extracts.

Table 1: Anti-candida activity of methanol and hexane extracts of gingers against *C. albicans*

Extract[a]	Description	Inhibition zone (mm)
Control	No extracts	0.0
Methanol extracts		
A	*A. galanga* (stalks)	6.0
B	*E. coccinea* (leaves)	5.0
C	*E. coccinea* (stalks)	0.0
D	*C. longa* (leaves)	5.0
Standard fungicide	Fluconazole (30µg/disc)	30.3[b]
	Ketoconazole (10µg/disc)	32.0[b]

[a]Tested against the *C. albicans* in the nutrient agar (NA) media
[b]Pattanayak *et al.* (2008)

Conclusion

Only the methanolic extracts of the *A. galangal* stalks, *E. coccinea* leaves and *C. longa* leaves at 50 µg/ml exhibited the anti-candida properties against the *C. albicans* strain.

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Anti-diabetic Activities of *Hibiscus cannabinus* Extracts Leaves and Roots *in vitro* Study

Zuraida, M.K.[1], Zalinah, A.[1,3], Muhajir, H.[2] and Maznah, I.[1,3]

[1]Institute of Bioscience, [2]Faculty of Biotechnology and Biomolecular Sciences,
[3]Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Malaysia
uminoyaniruka@yahoo.com

Abstract

Hibiscus cannabinus (Kenaf) is an annual herbaceous crop of the Malvaceae family. This plant is composed of various active components including tannins, saponins, polyphenolic, alkaloid, essential oil and steroid, has long been prescribed in traditional folk medicine in India and Africa. The aqueous leaf extract of this plant has been demonstrated to be haematically active and anti-oxidative. The underlying mechanisms that account for Kenaf pharmacology and active components however remain to be elucidated. This experiment was conducted to identify the active compounds of *H.cannabinus* extracts which had an anti-diabetic activity. There were yields of *Hibiscus cannabinus* extract which are 28.33% (80% ethanol leaves extract) and 42.23% (hot water leaves extract), 6.62% (80% ethanol extract) and 11.95% (hot water extract). *H.cannabinus* extracts enhanced insulin secretion in BRIN BD11 cells higher than Glibenclamide (100uM) except 80% ethanol *H. cannabinus* leaves extract at concentration 1000 ug/ml.

Introduction

Hibiscus cannabinus (Kenaf) is an annual herbaceous crop of the malvaceae family. This plant has long been used as a folk medicine in India and Africa for the treatment of many disorders (Lawton, 2004). The seed were used externally to treat aches and bruise and they were also considered to be fattening (Lawton, 2004). *H. cannabinus* leaves were also applicable in treating dysentery, blood and throat disorder (Lawton, 2004). Although this plant has been used in various symptoms and disease, however, few pharmacological studies have been reported. In addition, it has heen reported that the aqueous leaf extract of *H. cannabinus* displays hepatoprotective activity against carbon tetrachloro abd paracetamol induced liver damage in rats (Agbor *et al.,* 2005). These reports seem to link recent findings to ethnopharmacological properties such as anti-inflammation (fever and pain) and anti-blood disorder (anemia). Nonetheless, the underlying mechanisms that account for *H. cannabinus* pharmacology and active components however remain to be elucidated. The presented study was conducted to utilize the BRIN BD11 cell lines to provide an assessment of the effects of *H. cannabinus* treatment on the function and responsiveness of insulin secreting cells and to measure the glucose uptake activities on 3T3-L1 adipocytes and L6 myotubes.

Materials and methods

Materials

3T3-L1 fibroblast and L6 myotube cells were purchased from American Type Culture Collection (Manassas, VA). BRIN BD11 cells were kindly provided by Professor Peter R. Flatt (University of Ulster Coleraine, UK). MTS assay reagent was purchased from Promega Co. (Madison, USA) Rat insulin ELISA kit was purchased from Mercodia, Sweden. Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich Chemical Co.(St Louis, MO). RPMI-1640 was purchased from InVitrogen, GIBCO co. Trypsin-EDTA, penicillin-streptomycin and Foetal bovine serum were purchased from PAA laboratories (Pasching, Austria).

Hot water extraction

To prepare aqueous extract of *H. cannabinus* leaves and roots, 20 g of dried for each *H. cannabinus* leaves and roots were needed. The leaves and the roots washed before used. Then, the leaves were dried in the oven overnight and the roots were cut into pieces. The roots were grinded. The dried

leaves and the grinded roots were soaked in boiling (distilled) water into different container. Homogenized the mixture using homogenizer for 15 minutes. Then, filter the homogenized mixture using whatman no.1 filter paper. The filtrate was collected and dried by using rotary vacuum evaporator. The dried sample was kept at -80^{o}C.

80% ethanol extraction

To prepare 80% ethanol extract of *H. cannabinus* leaves and roots, 20g of fresh for each *H. cannabinus* leaves and roots were needed. The leaves and the roots washed before used. Then, the leaves were dried in the oven overnight and the roots were cut into pieces. The roots were grinded. The dried leaves and the grinded roots soaked in 80% ethanol into different container. Homogenized the mixture using homogenizer for 15 minutes. Then, sonicated the mixture with sonicator. Filter the mixture with whatman no.1 filter paper. The filtrate was collected and dried by using rotary vacuum evaporator. The dried sample was kept at -80^{o}C.

Cell cultures

3T3-L1 and L6 cells were maintained in high glucose DMEM containing 10% FBS and 1% penicillin-streptomycin in two different sterilize flask at 37°C in humidified atmosphere at 5% CO_2. Both types of cell were sub-cultured every 3-4 days at approximately 90% confluence. BRIN-BD11 cells were maintained in RPMI-1640 containing 10% FBS and 1% penicillin-streptomycin in a sterilize flask at 37°C in humidified atmosphere at 5% CO_2. The BRIN BD11 cells were sub-cultured every 3-4 days at approximately 90% confluence.

Insulin secretion assay

Glucose responsive clonal insulin secreting cell lines (BRIN-BD11) was used to evaluate insulin secretion. Cells were seeded in 24-well plate's culture at a concentration of 2.5 X 10^5 cells/well in RPMI-1640 medium. RPMI-1640 medium containing 1.1 mM glucose, 10 % fetal bovine serum and 1% of antibiotic (penicillin-streptomycin)-allow attachment overnight prior to acute tests. The cells were washed thrice with KRH buffer (KRH; 115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 10 mM HEPES-free acid, 1 g/L serum albumin, 1.1 mM glucose; pH 7.4). This was followed by pre-incubation with 1 ml of KRH buffer for ~40 minutes. Then followed by incubation with different concentration and different type of *H. cannabinus* extracts for ~40 minutes. From each well, aliquots 1 ml of cell insulin extracts and stored at -20°C for insulin assay.

Results and discussion

The results are presented in Fig. 1 as percentages insulin released of *H.cannabinus* extracts on BRIN BD11 cells after 40 minutes incubation. (1000ug/ml (A1), 2000ug/ml (A2) and 3000ug/ml (A3), 80% ethanol leaves extracts, 1000ug/ml (B1), 2000ug/ml (B2) and 3000ug/ml (B3), hot water leaves extracts, 1000ug/ml (C1), 2000ug/ml (C2) and 3000ug/ml (C3), 80% ethanol roots extracts, and 1000ug/ml (D1), 2000ug/ml (D2) and 3000ug/ml (D3), hot water roots extracts, Glibenclamide (GLA) and KRH buffer (KRH).

H. cannabinus leaves have higher yield compare to roots which are 28.33% (80% ethanol leaves extract) and 42.23% (hot water leaves extract). The yield of roots extracts which are 6.62% (80% ethanol extract) and 11.95% (hot water extract). Hot water extraction of *H. cannabinus* has higher yield than 80% ethanol extraction.

In insulin secretion assay, 2000 ug/ml of extract *H. cannabinus* hot water roots have the highest percentage of insulin release (308.44%) compared to Glibenclamide (anti-diabetic drug) and others extract. Most of the extracts showed high activity of insulin release at concentration 2000 ug/ml. The effect of insulin secretion in *H.cannabinus* leaves extract not as good as *H. cannabinus* roots extracts. KRH buffer (negative control) showed better insulin secretion effects compared to 80% ethanol *H. cannabinus* leaves extract probably because of KRH buffer contain glucose which are the main component that triggering the insulin released by BRIN BD11 cell lines. The insulinotropic sulphonylureas glibenclamide is well established as a therapeutic tool for the treatment of type 2

diabetes (Groop, 1997). Like other sulphonylureas, glibenclamide increase insulin secretion by directly closing ATP-sensitive K+ (KATP) channels in pancreatic beta cells (Cook *et al.,* 1998), causing membrane depolarization, opening of voltage-dependent Ca^{2+} channels leading to influx and elevation of intracellular Ca^{2+}, triggering exocytosis of insulin (Hellman *et al.,* 1994). The glibenclamide induced desensitization followed a progressive concentration-dependnt pattern, with maximal effects at 100-200 uM and the decline in basal and sulphonylureas-induced insulin release was associated with a 34% decrease in cellular content. This corresponds with known inability of glibenclamide to promote insulin biosynthesis (Grodsky *et al.,* 1977; Schatz *et al.,* 1977, 1978).

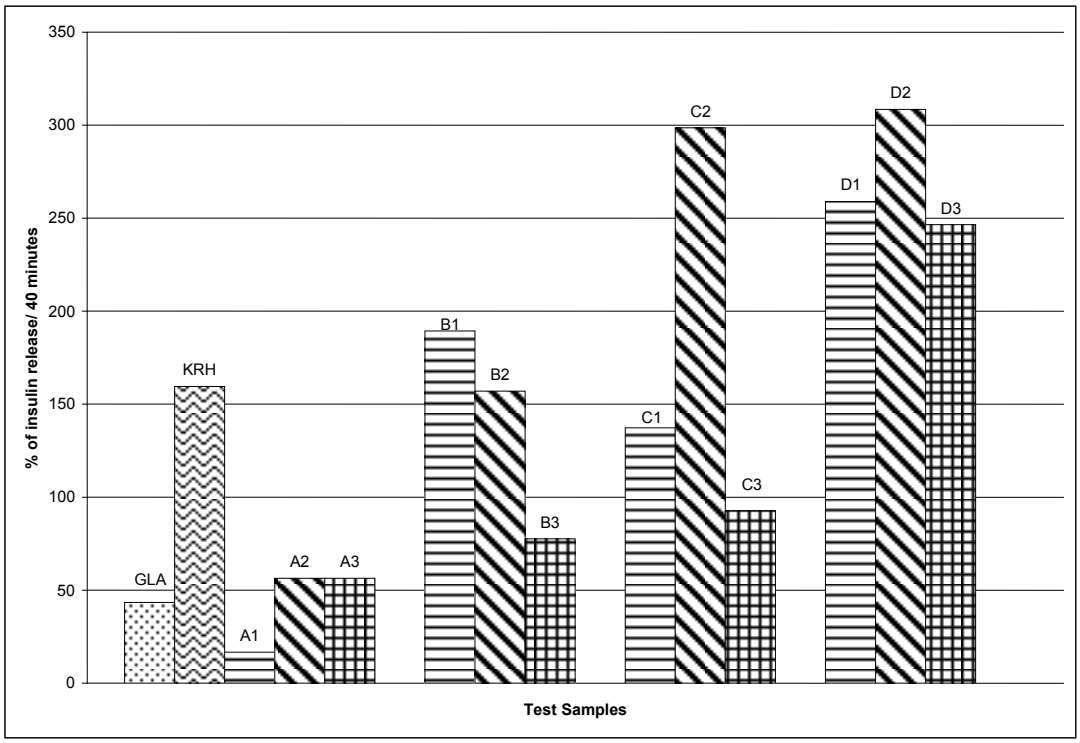

Fig. 1. Effects of *H.cannabinus* extracts on insulin secretion by BRIN BD11 cells.

In conclusion, the yield of hot water extract was higher compared to 80% ethanol extract of *Hibiscus cannabinus* leaves and roots. Compared to roots extracts, yield for leaves was significantly higher. Most *H. cannabinus* extracts showed insulin released effects on BRIN BD11 cell lines higher than glibenclamide.

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Anti-human Papillomavirus (Anti-HPV) Type 16 Activity of Selected Polyporales

Law, I.K., Nurhayati, Z.A. and Noorlidah, A.

Institute of Biological Sciences, Faculty of Science, University of Malaya,
50603 Kuala Lumpur, Malaysia
albireo29@hotmail.com

Abstract

Cervical cancer is the second most common cancer in women worldwide. Specific types of human papillomaviruses (HPV) such as HPV 16 are closely associated with the development of cervical cancer. The transforming properties of high-risk HPV 16 primarily reside in E6 gene which encodes for an oncoprotein directly involved in cellular transformation of the cervical epithelia leading to neoplasm. The E6 protein is present in cervical cancer precursor lesions and is consistently expressed in HPV-positive cervical cancer and derived cell lines. Therefore, this study was undertaken to screen for natural products which suppress the expression of E6 protein. *Polyporales* which are *Pycnoporus sanguineus* (KUM60501), *Trametes menziesii* (KUM60503) and *Trametes versicolor* (KUM61109) were extracted from mycelia biomass using dichloromethane (DCM), except for *T. versicolor* which was also extracted using methanol. CaSki cells were incubated with each of the extracts prepared in six different concentrations and were subsequently analyzed qualitatively for anti-HPV 16 E6 activity using immunocytochemistry technique. The presence of reddish-brown product in the nuclei and/ or cytoplasm of the CaSki cells indicate the presence of HPV 16 E6 oncoprotein. The selected Polyporales extracts showed reduction in the expression of E6 protein at the different concentrations suggesting the presence of active compounds which suppress the HPV 16 E6 protein. The DCM extract of *T. menziesii* produced a better suppression of E6 protein at lower concentrations compared to the other extracts which showed the suppression at the higher concentration. The DCM extract of *T. menziesii* therefore holds a potential to be used as prevention or treatment for cervical cancer.

Introduction

Cervical cancer remains the second most common cause of cancer deaths in women (Parkin *et al*., 1999). Human papillomaviruses (HPVs) are non-enveloped double-stranded DNA viruses. The International Agency for Research on Cancer (IARC) has classified high risk types 16 and 18 as definite carcinogens in humans (Lyon, 1995), with HPV 16 recognized as the most common high risk HPV type in most countries (Wallboomers *et al*., 1999). Continued expression of the HPV E6 and E7 genes is responsible over the progression of cervical cancer, a major subset of neoplasia in women worldwide (Gissmann and Hausen, 1976).

The viral E6 and E7 oncoproteins can alter the keratinocyte terminal differentiation phenotype and abrogate negative cell cycle controls to induce cells to enter S-phase (Hudson *et al*., 1990 and Song *et al*., 1998). In doing so the E6 and E7 proteins are establishing conditions in which viral DNA synthesis can occur in the differentiating epithelial cells. Cells constitutively expressing E6 and E7 proteins can bypass normal cell cycle checkpoints resulting in the accumulation of genetic damage which could ultimately result in malignant progression.

The p53 is a transcription factor in nucleus cell and cytoplasmic which played role in cell cycle and division regulation and apoptosis (Bonnez, 1997). Combination of E6 protein with p53 causing the degradation of p53 and abrogating its function. This leads to cellular transformation of the cervical epithelia leading to neoplasm (Lechner *et al*., 1994).

Realizing the importance of the E6 oncoprotein in the development and progression of cervical cancer, this study was undertaken to screen for natural products which suppress the expression of E6 protein.

The polypores are among the most common, widespread, and easily identifiable groups of wild mushrooms, with some excellent edible species and no poisonous ones. Mushrooms are often used as adaptogens and immunostimulants. Cellular components and secondary metabolites of many mushrooms have been shown to affect host immune systems and might be used to treat a variety of

diseases including cancer, immunodeficiency disease, and immunosuppression after drug treatment (Wasser, 2002).

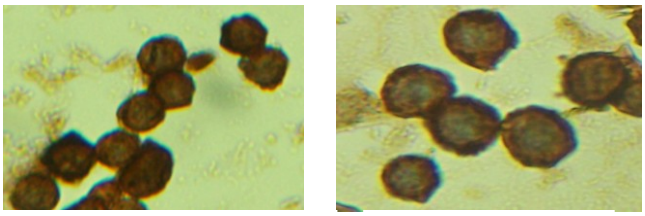
Polyporales extracts comprising of dichloromethane extract of *Trametes menziesii*, dichloromethane (DCM) extract of *Trametes versicolor*, methanolic extract of *Trametes versicolor* and dichloromethane extract of *Pycnoporus sanguineus* were analysed qualitatively for anti-HPV 16 E6 activity using immunocytochemistry technique.

Materials and methods

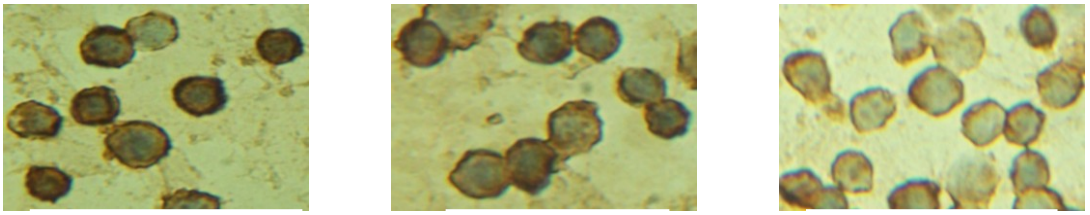
Mycelial biomass was obtained by submerged fermentation of Polypore species in liquid Glucose-Yeast-Malt-Peptone media for 14 days. The freeze-dried mycelia were then extracted with methanol and dichloromethane (DCM) by soaking for 48 hours. Each extract of Polypore species was tested at six concentrations which are 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml were then added to cervical cancer-derived CaSki cells in 10% RPMI 1640 supplemented media and incubated under 5% CO2 at 37°C for 72 hours. The presence of HPV 16 E6 oncoprotein, in the treated CaSki cells were subsequently analyzed using the immunocytochemistry method (Dodson, 2002). Result for anti- HPV 16 E6 activity was based on the staining intensity of the cells and compared to the cells not treated with extract (positive control)(Marc, 2006).

Results and discussion

The presence of reddish-brown product in the nuclei and/or cytoplasm of the CaSki cells indicate the presence of the E6 oncoprotein. High intensity of the reddish-brown product indicates abundant E6 oncoprotein while low intensity of reddish-brown product indicates lower amount of E6 oncoprotein indicating the suppression by the extracts. The staining intensity was classified as: very weak stain (+), weak stain (++), moderate stain (+++), strong stain (++++) and very strong stain (+++++) as illustrated in Fig. 1. The suppressing effect of the testing extract is considered as weak for (+++++), moderate for (+++) and high for (+).



Very strong (+++++) Strong (++++)



Moderate (+++) Weak (++) Very weak (+)

Fig. 1. Classification of staining intensity.

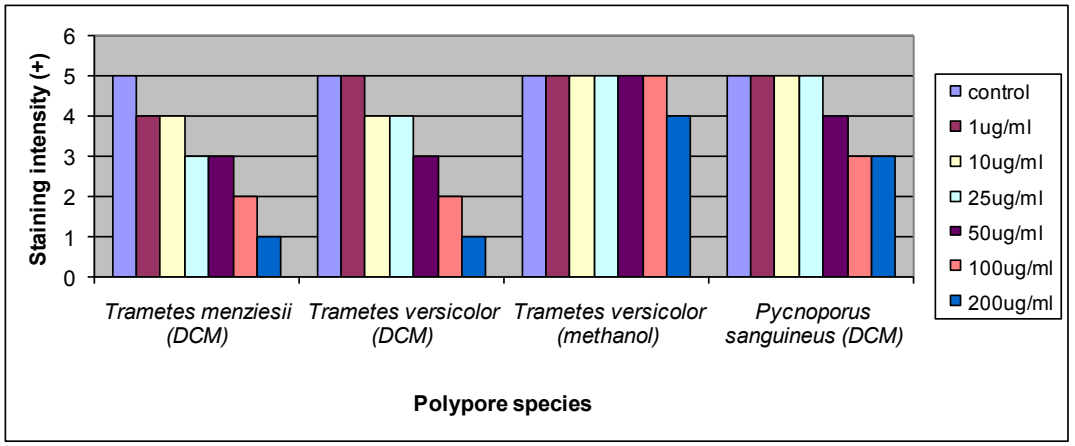
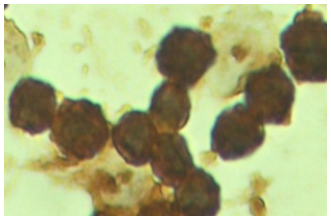


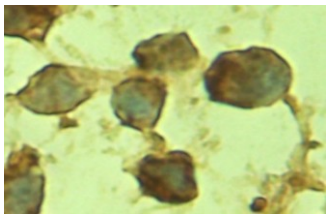
Fig. 2. Comparison of staining intensities of CaSki cells among four Polyporales extracts tested at six different concentrations.

Fig. 2 shows the comparison of staining intensities of CaSki cells among the dichloromethane extract of *T. menziesii*, dichloromethane extract of *T. versicolor*, methanolic extract of *T. versicolor* and dichloromethane extract of *Pycnoporus sanguineus*. Each extract was tested at six different concentrations which were 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. The extracts showed reduction in the expression of E6 protein in CaSki cells at the different concentrations suggesting the presence of active compounds which suppress the HPV 16 E6 protein. The extract showing the most potential in HPV 16 E6 oncoprotein suppression activity is dichloromethane extract of *T. menziesii* followed by the dichloromethane extract of *T. versicolor*, dichloromethane extract of *Pycnoporus sanguineus* and methanolic extract of *T. versicolor*.

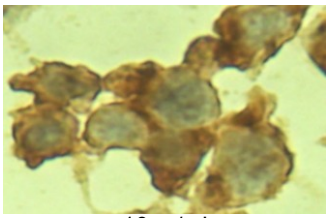
The dichloromethane extract of *T. menziesii* showed excellent anti-HPV 16 E6 oncoprotein trend whereby the relative intensity of the reddish-brown stain decreased as the concentration of the extract increased gradually from 1 µg/ml to 200µg/ml. This indicates that anti-HPV 16 activity was increasingly being suppressed with higher concentration of the extract. Cell lysis was seen to increase from low to higher concentration of dichloromethane extract of *T. menziesii* (Fig. 3). This suggests that dichloromethane extract of *T. menziesii* contain active compounds which promote cell lysis and suppress the HPV 16 E6 protein in CaSki cells. Chemical characterization of anti-HPV compounds will be carried out in future studies.



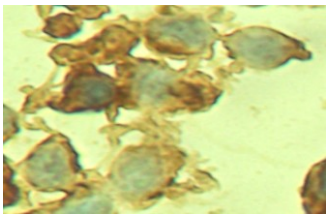
Positive control
(+++++)



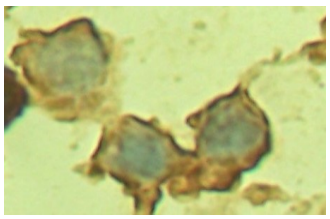
1µg/ml
(++++)



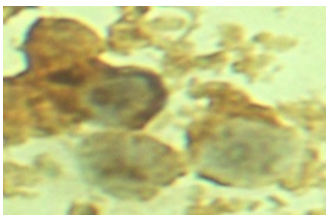
10µg/ml
(++++)



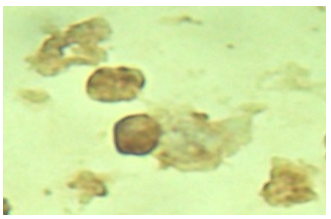
25µg/ml
(+++)



50µg/ml
(+++)



100µg/ml
(++)



200µg/ml
(+)

Fig. 3. Staining intensities of CaSki cells after treatment with varying concentrations of dichloromethane extract of *T. menziesii*.

Conclusion

Among the four extracts, dichloromethane extract of *T. menziesii* produced a better suppression of E6 protein and therefore holds a potential to be used in chemoprevention or treatment for cervical cancer.

Acknowledgements

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Anti-human Papillomavirus (HPV) Type 16 E6 Activities in *Ganoderma lucidum*

Lai, L.K., Nurhayati, Z.A. and Noorlidah, A.

Institute of Biological Sciences, Faculty of Science, Universiti Malaya,
50603 Kuala Lumpur, Malaysia
likuan1308@hotmail.com

Abstract

The development of cervical cancer is reported to be associated with human papillomavirus type 16 (HPV 16). The E6 region of HPV 16 encodes for the E6 oncoprotein which is involved in cellular transformation of the cervical epithelia leading to neoplasm. The E6 oncoprotein is abundant in cervical cancer and cervical cancer-derived cell lines. Realising the importance of the E6 oncoprotein in the development of cervical cancer, the present study was undertaken to screen for *Ganoderma lucidum* extracts for the suppression of the E6 oncoprotein. In this study, dichloromethane, ethanol, water and polysaccharide extracts of *G. lucidum* were analysed for anti-HPV 16 E6 activity in the HPV 16-containing cervical cancer-derived cell line, CaSki. The extracts were first incubated with the CaSki cells for 72 hours. A polymer-based immunocytochemistry technique was then performed on the cells to analyse the expression of the E6 oncoprotein. Qualitative observation showed that expression of E6 oncoproteins reduced in CaSki cells incubated with the extracts of *G. lucidum* indicating anti-HPV 16 E6 activities in these extracts. When compared, the dichloromethane extract demonstrated the strongest anti-HPV 16 E6 activity. The dichloromethane extract was subsequently subjected to thin layer chromatography (TLC) to evaluate the profile of secondary metabolites present in the extract. When evaluated, the profiling showed the presence of terpenoids and alkaloids. This study indicated that *G. lucidum* is a potential treatment for cervical cancer.

Introduction

In Malaysia, cervical cancer is the second most common cancer affecting females with the first being breast cancer (Cheah and Looi, 1999; Rosemawati and Sallehudin, 2001). Human papillomavirus (HPV) were found to contribute directly to the development of cervical cancer, specifically HPV 16 and HPV 18 strains (Qiu *et al.*, 2007). Both HPV 16 and 18 produce E6 and E7 oncoproteins respectively. Oncoproteins cause abnormalities in cell cycles, causing uncontrolled proliferation of cells leading to tumour. E6 and E7 suppress p53 and Rb proteins respectively (Sdek *et al.*, 2006). p53 is a tumour suppressor protein that regulates cell cycles. Inactivation of p53 causes disruption of the normal cell cycle, leading to inability of cell proliferation control. E6 inhibits p53 by binding itself to it. Rb is a suppressor protein that inhibits production of growth factors in cells. Inhibition of Rb causes cell growth factors to multiply, resulting in uncontrolled cell proliferation. The E7 oncoprotein inhibits Rb by binding itself to it (Sherman *et al.*, 1997). It is therefore important to suppress the detrimental effects caused by E6 and E7 oncoproteins as a combat against cervical cancer.

Mushrooms of the *Ganoderma* genus were claimed to promote longevity and were able to treat various symptoms including migraine, hypertension, hepatitis and cancer (Gao and Zhou, 2003; Paterson, 2006). This suggests that *Ganoderma* may possess other medicinal properties which have not been tested. The present study shows that extracts of *Ganoderma lucidum* possessed anti-HPV activity in cervical cancer-derived CaSki cell lines. A CaSki cell contains 500-600 copies of HPV 16 genome. Walboomers *et al.* (1999) found that in 99.7% of cervical cancer samples obtained worldwide, strains of HPV 16 were prevalent. HPV 16 is a causal agent for more cervical cancer cases than HPV 18 even though the latter caused more invasive cervical cancer cases than cervical intraepithelial neoplasia (CIN) cases (Arends *et al.*, 1998). Because this research focused on combating HPV 16-causing cervical cancers, CaSki cells were used.

The Ninth Malaysia Plan will develop new ways to deal with cancer, which include prevention, diagnosis and treatment as well as usage of natural products and complementary therapies (Lim, 2006). Therefore the in depth research on *G. lucidum* compounds with anti-HPV activities is indeed a move to discover natural products for cervical cancer treatment.

Materials and methods

Screening of anti-HPV 16 E6 active extracts of Ganoderma lucidum against CaSki cell lines

Dichloromethane, ethanol, water and polysaccharide extracts of *G. lucidum* were prepared. The dichloromethane, ethanol and polysaccharide extracts were weighed (0.02 g) and diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA)(1 ml) as stock solution. The water extract was weighed (0.02 g) and diluted in distilled water (1 ml). The stock solutions were subsequently diluted to the following concentrations – 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. CaSki cells were incubated with the extracts at all the concentrations for 72 hours. The cells were harvested and fixed on 12-well Teflon-coated slides (Marienfeld-Superior, Germany). A polymer-based immunocytochemistry protocol i.e. EnVision™+ (Dako, Denmark) was performed on the cells to evaluate anti-HPV 16 E6 activities. All tests were performed in triplicates. Qualitative observation was photographed to determine the most active crude extracts of *G. lucidum*. Cells not treated with any extract served as negative controls.

Thin layer chromatography (TLC) profiling on the active Ganoderma lucidum extract

The active crude extract was weighed (0.02 g) and diluted with dichloromethane (1 ml) as extract stock. Thin layer chromatography (TLC) plates consisting of silica gel 60 on aluminum sheets (Merck, Germany) was cut to 10 × 10 cm. The plate was divided into four longitudinal sections. A short line (approximately 4 mm) of extract stock was repeatedly spotted across the baseline of the TLC plate in each section. Chloroform:ethanol (93:7) was used as the developing solvent in the TLC chamber (Sigma, USA). The TLC plate was developed and left to dry in the fume cupboard. The four sections were cut and treated with the following viewing agents – UV_{254}, vanillin-sulfuric acid (Sigma-Aldrich, USA), iodine vapour (Sigma-Aldrich, USA), and Dragendorff's reagent (Sigma-Aldrich, USA).

Results and discussion

Screening of anti-HPV 16 E6 active extracts of Ganoderma lucidum against CaSki cell lines

Fig. 1 shows the highest intensity of brown products in untreated CaSki cells. This corresponded to abundant expression of HPV 16 E6 oncoprotein. The intensity of brown products were observed to have decreased (HPV 16 E6 expression decreased) with higher concentration *G. lucidum* extracts. Cells which were treated with polysaccharide extract showed the highest intensity of brown products, followed by CaSki cells treated with ethanol, water and dichloromethane extracts of *G. lucidum*.

Treatment with the dichloromethane extract showed the lowest intensity of brown products. The present study showed that the dichloromethane extract was the most active for anti-HPV activity. TLC profiling was subsequently carried out to analyse the compounds present in the extract.

Thin layer chromatography (TLC) profiling on the active Ganoderma lucidum extract

The dichloromethane extract of *G. lucidum* was further subjected to TLC profiling. Fig. 2 shows that a total of 18 bands were detected. The marked bands showed positive reaction towards various viewing reagents. Houghton and Raman (1998) stated that TLC bands which contain conjugated double bonds detected by UV_{254} expressed fluorescence, bands that contain double bonds expressed yellow or brown zones by exposure to iodine vapour and bands which contain alkaloids expressed orange zones on yellow background when detected with Dragendorff's reagent. Bands that contain terpenoids showed positive reaction to vanillin-sulfuric acid reagent (Pelander *et al.*, 2000). The TLC profiling soundly concluded that the dichloromethane extract of *G. lucidum* contains terpenoids and alkaloids. Further fractionation of the extract will be done to identify the compounds which were active of anti-HPV 16 E6 activity.

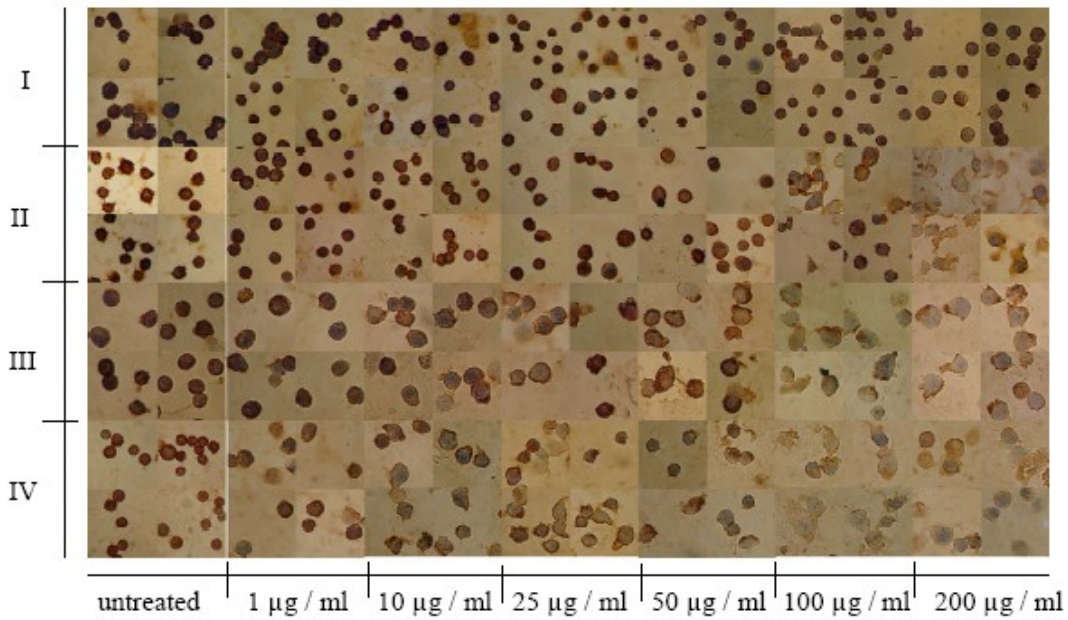

Fig. 1. Comparison of untreated CaSki cells and CaSki cells treated with crude polysaccharide (I), ethanol (II), water (III) and dichloromethane (IV) extracts of *G. lucidum* at different concentrations. The brown products represent the distribution of HPV 16 E6 oncoprotein whilst the intensity of the brown products represent the expression of HPV 16 E6 oncoprotein.

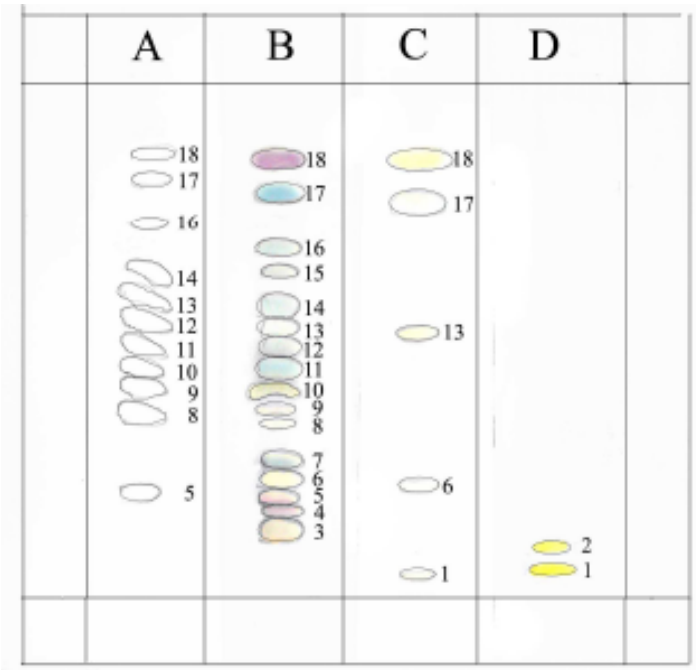

Fig. 2. Depiction of TLC profile on the dichloromethane extract of *G. lucidum*. The developed TLC plate was cut into sections and subjected to UV_{254} exposure (A), sprayed with vanillin-sulfuric acid (B), exposed to iodine vapour (C) and sprayed with Dragendorff's reagent (D).

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Anti-hypercholesterolemic Property of *Phaleria macrocarpa* Fruit Aqueous Extract in Hypercholesterolemia-Induced Rats

Chong, S.C. and Dollah, M.A.

Department of Biomedical Sciences, Faculty of Medicine and Health Sciences,
University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
mohdaziz@medic.upm.edu.my

Abstract

Phaleria macocarpa is a herb that been used in traditional medicine and has been claimed that *P. macocarpa* could cure hypercholesterolemia, diabetes, cancer and others. The scientific information on the therapeutic effects of this herb however is rather limited. In this study, anti-hypercholesterolemic property of various concentration of Pm aqueous fruit extract and the extend of its effects compared with Simvastatin was determined. In the study, 36 female rats were divided into 6 groups equally. Five groups were fed with 3% pure cholesterol during pre treatment (2 weeks) and treatment periods (4 weeks). Supplementation of 1 ml of the *P. macocarpa* extract (0, 67, 134 and 183 mg/kg bw) and Simvastatin (0.33 mg/kg bw) was given during treatment period. Blood samples were collected before and after the supplementation periods via cardiac puncture to analyze the total cholesterol (TG), triglycerides (TRG), high density lipid (HDL) and low density lipid (LDL) levels. The results shows that the mean difference of the total cholesterol after and before supplementation of *P. macocarpa* for negative control, 0, 67, 134, 183 mg/kg bw and simvastatin was 0.05±0.49, 0.87±1.56, -0.95±1.74, -0.66±1.66, -0.59±0.48 and -0.48±0.61 mmol/L. The mean difference for Triglycerides was -0.73±0.67, 0.28±1.74, -3.14±1.68, -1.24±1.16, -1.22±0.94 and 0.16±0.18. The mean difference for the HDL was 0.28±0.35, 0.13±0.50, 0.41±0.44, 0.11±0.45, 0.06±0.19 and -0.42±1.35 while the mean difference of the LDL was 0.07±0.08, 0.35±0.53, 0.22±1.28, -0.15±1.15, -0.48±0.75 and -0.05±0.54. The study concludes that *P. macocarpa* significantly reduced triglycerides level ($P<0.05$) and showed a tendency to reduce other lipid profiles. The effect of *P. macocarpa* supplementation was dose-dependant and *P. macocarpa* at the concentration of 44 mg/kg bw showed the same cholesterol reducing effect as simvastatin. Thus, *P. macocarpa* has anti hypercholesterolemic effect on hypercholesterolemic-induced rats

Introduction

Cholesterol is an essential body chemical that is needed to synthesize bile acids, vitamin D, sex hormones and so forth. Cholesterol is a mixture of compounds which include high density lipoproteins (HDL), low density lipoproteins (LDL), intermediate density lipoprotein (IDL) and very low density lipoprotein (VLDL). A normal or desirable cholesterol level in human is less than 200 mg/dL and hypercholesterolemia is when the level has reached 240 mg/dL or above (Fallon, 2002). The common cause of high cholesterol is high fat diet, lack of exercise, smoking and etc. Hypercholesterolemia increases the risk of atherosclerosis whereby it can cause heart diseases. Due to this reason, cholesterol-lowering medicine is needed especially if the total cholesterol level remains higher than 240mg/dL and already has cardiovascular disease. Most of anti-hypecholesterolemic drugs used are statins such as simvastatin which is HMG-CoA reductase inhibitors. Other drugs used are nicotinic acid and bile acid resin. Alternative medicine to treat hypercholesterolemia is to use herbs such as *Phaleria macrocarpa* (Scheff) Boerl. The fruit contain active ingredients that had been claimed to be able to reduce cholesterol level, treat cancer, diabetic mellitus, hypertension and heart problem. In the current study, anti-hypercholesterolemic effect and the dose-effect relationship of *P. macrocarpa* fruit aqueous extract in hypercholesterolemic-induced rats was investigated. The extent of *P. macrocarpa*'s anti-hypercholesterolemic property as compared to the standard drug, simvastatin was also studied.

Materials and methods

Preparation of the cholesterol pellet

Cholesterol pellet containing 4% of 95 % pure cholesterol was prepared by mixing grounded rat feed chloroform and cholesterol. The starch flour was mixed with distilled water and added to the mixture of pellet and cholesterol. The mixture was baked in an oven at 70^0C overnight.

Preparation of the *Phaleria macrocarpa* extract

Dried *P. macrocarpa* slices were grind, mixed with distilled water and boiled until its volume about one third of its initial volume. After the mixture was cold, it was filtered, centrifuged and filtered again. The supernatant was freeze-dried. The stock solution of *P. macrocarpa* was prepared by dissolving the *P. macrocarpa* powder with distilled water and the stock solution was used to prepare different concentration of the working solution of 67, 134 and 183mg/kg bw respectively. The simvastatin (20 mg human dose) working solution was prepared.

Experimental animals

In this study, 36 female *Sprague dawley*, 7-8 months olds, weight 255-300 gm were divided equally into six groups with 5 groups fed with 4 % pure cholesterol and supplemented with increasing amount of *P. macrocarpa* and Simvastatin and one group fed with normal feed. This experiment last for 6 weeks and consisted of two periods, pre-supplementation and supplementation periods. Body weight of the rats was measured once every fortnight. At the end of the experimental period, the blood samples were collected by cardiac puncture. The blood samples were centrifuge and the plasma was analyzed for the lipid profile (Total Cholesterol, Triglycerides, LDL and HDL). All the data obtain was analyzed using ANOVA and Duncan Multiple Range test with $P<0.05$ as the limit of significance.

Results and discussion

Phaleria macrocarpa is a traditional herb that is used to treat various diseases such as cancer, diabetic, hypercholesterolemia and etc. Its fruit aqueous extract yield 10.99 % of crude extract.

Lipid profile of the rats that were fed with normal feeding was normal. The normal range for total cholesterol (TC) was less than 2.12 mmol/L, triglycerides (TG) less than 1.51 mmol/L, HDL less than 0.776 mmol/L and LDL level less than 0.712 mmol/L (Nanthini, 2004; Selvaratnam, 2004; Norfariza, 2003). The level of the TC, TG, HDL and LDL of the negative control rats during pre-supplementation and supplementation periods were similar among the periods. Feeding with 4 % of cholesterol diet before supplementation of Pm significantly increased the lipid profile 2.3 to 3.7 times for TC, 2 to 5.3 times for TG, 15 times to 21 times for LDL but no change for HDL level. Supplementation of Pm extract at increasing concentration showed decreasing effects on the lipid profile although the declining effects were not significant. The similar effect also shown by the lipid profile of the rats that supplement with simvastatin. It was possible that the supplementation period of 29 days was not significant enough to have significant depression effect for both simvastatin and Pm extract. This was because Simvastatin is a HMG-CoA reductase inhibitor. It act by competitively inhibit the HMG-CoA reductase from catalyses the conversion of HMG-CoA to mevalonate. Hence, causes a reduction in the hepatocytes cholesterol synthesis and this leads to increment in the LDL receptor expression on the hepatocytes cell surface, which results in the extraction of LDL-c from the blood and decrease the circulating LDL-c (Schachter, 2005). The expression of LDL receptor on the hepatocytes cells require some times and it is possible that the 29 days of treatment period might not be enough for the cell to express the LDL receptor or some of the rats might not respond to the Simvastatin treatment as reported by Gylling (2004). So, these might contribute to the low reduction in the LDL. Simvastatin at the dose of 20mg are able to reduce Total Cholesterol by 28%, 38% for Triglycerides and 19% for LDL-c and an increment of 8% of HDL-c by week 6 as shown in the pamphlet manufactured by Cadila Healthcare Limited (2006). Thus, the proportion of reduction for Triglyceride and the total cholesterol in this experiment is approximately the same as shown by the company when converted to 4 weeks of consuming simvastatin (Table 1).

Rats that were supplemented with simvastatin had a reduction of 0.05 mmol/L for LDL in 4 weeks. Thus, if the treatment period was prolonged to 6 weeks, it might had a reduction of 0.075 mmol/L which is equivalent to 44.78 mg/kg bw of *P. macrocarpa*. However, in this experiment, *P. macrocarpa* at the concentration of 67.17 mg/kg show an increment in the LDL level. This may be due to the shortage of TG level in the serum as a result of increment in the LPL activity, whereby it increased in hydrolyzing the TG in the chylomicron, VLDL and IDL. Hence LDL level was increased. This may explained the reason why LDL increased in this experiment.

Table 1. Mean and solution (±) of lipid profile (Total Cholesterol, Triglycerides, HDL and LDL) of the rats at pre-supplementation and supplementation periods

Day/ treatment	No Cholesterol No (control)	Cholesterol (4%)					Total
		Phaleria macrocarpa (mg/kg)				Simvastatin (0.33 mg/kg)	
		0	67	134	183		
Total Cholesterol (mmol/L)							
Pre-supplementation	1.39 ± 0.29^{c}	3.19 ± 0.70^{b}	5.16 ± 0.67^{a}	3.71 ± 1.08^{ab}	3.24 ± 0.58^{b}	3.21 ± 0.58^{b}	3.37±1.33
Supplementation	1.43 ± 0.54^{c}	4.05 ± 1.58^{ab}	4.21 ± 2.06^{ab}	3.05 ± 2.06^{b}	2.65 ± 0.76^{bc}	2.73 ± 0.78^{bc}	3.06±1.66
Total	1.41 ± 0.41^{C}	3.62 ± 1.24^{B}	4.68 ± 1.54^{A}	3.38 ± 1.60^{B}	2.95 ± 0.71^{B}	2.97 ± 0.69^{B}	
*Changes	0.05 ± 0.49^{AB}	0.87 ± 1.56^{B}	-0.95 ± 1.74^{A}	-0.66 ± 1.66^{AB}	-0.59 ± 0.48^{AB}	-0.48 ± 0.61^{AB}	
Triglycerides (mmol/L)							
Pre-supplementation	0.81 ± 0.09^{c}	1.60 ± 1.12^{bc}	4.27 ± 1.11^{a}	2.15 ± 1.11^{b}	1.75 ± 0.89^{bc}	2.01 ± 0.79^{bc}	2.32±1.32
Supplementation	0.9 ± 0.15^{c}	1.88 ± 0.99^{bc}	1.11 ± 0.97^{bc}	0.91 ± 0.28^{c}	1.58 ± 1.15^{bc}	0.80 ± 0.33^{c}	1.18±0.80
Total	0.86 ± 0.06^{B}	1.74 ± 1.00^{AB}	2.69 ± 1.92^{A}	1.53 ± 1.01^{AB}	1.67 ± 0.98^{AB}	1.41 ± 0.86^{AB}	
*Changes	-0.09 ± 0.01^{B}	0.28 ± 1.74^{B}	-3.14 ± 1.68^{A}	-1.24 ± 1.16^{B}	-0.17 ± 1.32^{B}	-1.22 ± 0.94^{B}	
High Density Lipoprotein (mmol/L)							
Pre-supplementation	0.87 ± 0.14^{b}	1.08 ± 0.32^{b}	1.03 ± 0.42^{b}	1.11 ± 0.51^{b}	1.18 ± 0.22^{b}	1.23 ± 0.39^{a}	1.07±0.35
Supplementation	1.50 ± 0.38^{b}	1.21 ± 0.21^{ab}	1.43 ± 0.15^{ab}	1.22 ± 0.27^{ab}	1.24 ± 0.14^{ab}	1.39 ± 0.25^{ab}	1.28±0.26
Total	1.19 ± 0.31^{B}	1.15 ± 0.27^{B}	1.23 ± 0.37^{AB}	1.17 ± 0.39^{B}	1.21 ± 0.18^{AB}	1.31 ± 0.32^{A}	
*Changes	0.28 ± 0.35^{A}	0.13 ± 0.50^{A}	0.41 ± 0.44^{A}	0.11 ± 0.45^{A}	0.06 ± 0.19^{A}	0.16 ± 0.18^{A}	
Low Density Lipoprotein (mmol/L)							
Pre-supplementation	0.09 ± 0.05^{b}	1.43 ± 0.53^{a}	1.90 ± 0.59^{a}	1.67 ± 0.57^{a}	1.42 ± 0.41^{a}	1.18 ± 0.76^{ab}	1.29±0.79
Supplementation	0.16 ± 0.11^{b}	1.78 ± 0.99^{a}	2.12 ± 1.61^{a}	1.52 ± 1.46^{a}	0.94 ± 0.81^{ab}	1.12 ± 0.89^{ab}	1.29±1.23
Total	0.12 ± 0.09^{C}	1.60 ± 0.77^{AB}	2.01 ± 1.17^{A}	1.59 ± 1.06^{AB}	1.18 ± 0.66^{B}	1.15 ± 0.78^{B}	
*Changes	0.07 ± 0.08^{A}	0.35 ± 0.53^{A}	0.22 ± 1.28^{A}	-0.15 ± 1.15^{A}	-0.48 ± 0.75^{A}	-0.05 ± 0.54^{A}	

* supplementation- presupplementation

abcd : comparison of the mean between column and between row significant at $P<0.05$

ABCD: comparison of the means between column significant at $P<0.05$

Conclusion

From the study, it showed that supplementation of cholesterol–rich diet increased the total cholesterol, triglycerides and LDL level but not HDL level. Treatment with the *P. macrocarpa* had the potential in reducing the total cholesterol and LDL level and increased HDL level in the blood but significantly reduced triglycerides. Similarly, simvastatin had the tendency to reduce total cholesterol, triglycerides and LDL level and increased HDL level. *P. macrocarpa* at the concentration of 44.78 mg/kg bw had the similar effect on lipid profile as in simavastatin.

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Anti-hyperglycemic Effect of *Phaleria macrocarpa* Fruit Aqueous Extract in Type I Diabetic Mellitus-induced Rats

Dollah, M.A. and Siti Raudzah, M.K.

Department of Biomedical Sciences, Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
mohdaziz@medic.upm.edu.my

Abstract

Phaleria macrocarpa is used traditionally to treat various diseases. Its fruits are claimed to give anti-hyperglycemic effect to those people suffering from diabetes mellitus. The scientific information on the hypoglycemic properties however is not conclusive. For this reason, an experiment was conducted to evaluate the anti-hyperglycemic properties of *P. macrocarpa* fruit aqueous extract in diabetic-induced rats. A two by three randomized experimental design was used with two periods of supplementation (14 and 28 days) and three concentration levels (0, 90 and 180 mg / kg) of *P. macrocarpa* fruit aqueous extract as the factors. Thirty six diabetic-induced Sprague Drawly female rats were divided into six groups and each group was allocated to one of the treatment. Rats in each group were administered with 0.2 ml of *P. macrocarpa* fruit aqueous extract orally once a day. The blood glucose level was measured twice a week while the bodyweight of the animals were monitored once a week. The means of body weight of diabetic-induced rats for 28 days of *P. macrocarpa* supplementation at 0, 90 and 180 mg/kg were 202.67±32.77, 186.69±32.41 and 225.53±20.65 g respectively. The means of body weight of diabetic-induced rats for 14 days of PM supplementation were 188.47±44.21, 192.36±30.29 and 218.89±25.37 g for 0, 90 and 180 mg / kg of *P. macrocarpa* supplementation respectively. The means of blood glucose level of diabetic-induced rats for 28 days of *P. macrocarpa* supplementation at 0, 90 and 180 mg/kg were 19.26±4.47, 12.94±7.58 and 10.23±4.07 mmol/L respectively. The means of blood glucose level of diabetic-induced rats for 14 days of PM supplementation at 0, 90 and 180 mg/kg were 20.73±6.48, 12.80±6.21 and 10.89±5.79 mmol/L respectively. The results showed that *P. macrocarpa* has anti hyperglycemic activity by reducing blood glucose level in diabetic-induced rats and the effect was found to be dose dependent. Consumption of *P. macrocarpa* fruit aqueous extract for a minimum of 17 days at the dose of at least 90 mg/kg was found to be sufficiently reduced the blood glucose level to the normal level in diabetic-induced rats.

Introduction

Diabetes mellitus is a disease as a result of an endocrine disorder which characterize by persistent of high blood glucose level in blood (hyperglycemia). Diabetes mellitus type I is resulting from absent or inadequate insulin secretion, without impairment of insulin action (Bertram, 2004). Diabetes mellitus type II is closely linked to the insulin resistance associated with adiposity. Insulin increases the liver uptake of glucose and accelerates glucose uptake of muscle and fat cells. The effect of insulin therefore is to lower the blood glucose level. When insulin production is insufficient or cells become resistant or respond poorly to insulin, the blood glucose level in the body then will rise.

Both Type I and Type II diabetes are accompanied by long-term microvascular or macrovascular complications that can result in serious morbidity and premature mortility. The symptoms of these complications develop years after the emergence of overt hyperglycemia (Foster *et al.*, 1991). Diabetic microangiopathy, could lead to disruption of local circulation in the kidney, retina and pheripheral and autonomic nerves (McMillan, 1997). Consequently, it will develop retinopathy and nephropathy and neuropathy.

P. macrocarpa is used traditionally to treat various diseases such as diabetes mellitus, cancer, cardiovascular disease, rheumatic, high blood pressure, acne and insect bites (Sri Sugiwati *et al*., 2006). Unfortunately, there is not enough scientific information or data available to claim its therapeutic effect. For this reason, this experiment was conducted to evaluate the anti-hyperglycemic properties of *P. macrocarpa* fruit extract in type I diabetic-induced rats.

Materials and methods

Preparation of plant extraction

Aqueous *P. macrocarpa* fruit extract was prepared by boiling the sun dried *P. macrocarpa* fruit slices in distilled water. After cooling, the mixture was filtered and then freeze dried. The powder of the extract was weighed and reconstituted with distilled water to prepare different concentration of working solutions.

Experimental design

A two by three factorial experimental design was used with two different periods of supplementation (14 and 28 days) and three levels of *P. macrocarpa* fruit extract concentration (0, 90 and 180 mg/kg body weight).

Experimental animals

36 Sprague Dawley female rats, two months old and 200–250 g weight were injected intraperitoneally with Streptozotocin (STZ) solution (50 mg/kg body weight). These rats were then randomly divided into six groups and allocated to one of the six combination treatments mentioned above.

The rats were kept in groups and housed under 12 to 12 hours of dark/light cycle in animal house. They were fed with standard rat pellet once a day in the morning. Drinking water was given *ad libitum*.

All groups were force fed with 0.2 ml of aqueous *P. macrocarpa* fruit extract once a day throughout the experimental period.

Blood glucose level determination

Blood glucose level was determined by ACCU-CHEK® Advantage II glucose test strips (Roche Diagnostics Gmbh, Germany) using the whole blood collected at tip of the rat's tail. The measurement was done twice a week throughout the experimental period.

Results and discussion

Diabetes mellitus is one of the major public health problems of transition period between the 20th and 21st centuries. The proportion of diabetes mellitus in lifestyle diseases has increased remarkably in recent years (Ishihara et. al, 2003). More than four hundred herbal remedies have been documented for managing diabetes mellitus worldwide but only a few have been scientifically evaluated. Therefore, this study discussed the therapeutic effect of *P. macrocarpa* fruit aqueous extract in reducing blood glucose level of diabetic-induced rats.

The mean values of blood glucose level in diabetic-induced rats treated with various concentrations of *P. macrocarpa* fruit aqueous extract at two different periods are shown in Table 1. The changes of blood glucose level though out the experimental periods are illustrated graphically in Fig. 1.

Injection of STZ successfully caused the rats to become diabetic. Blood glucose level (19.73 mmol/L) increased 3 times higher to the normal level (6.5 mmol/L) as reported earlier (Zuraida, 2007). Streptozotocin is a glucosamine-nitrosourea compound, similar to glucose which is transported into the cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. Streptozotocin causes toxicity to beta cells, since these cells have relatively high levels of GLUT2 (*Wang and Gleichmann, 1998)*. The B-cells appear degranulated, but not necrotic, approximately four days after and hence secretion of insulin is impaired. The glucose level therefore increased and reach to the diabetic type I state at about 4 days post STZ injection.

Analysis of variance of the blood glucose level measured in this study indicated that the doses of *P. macrocarpa* fruit aqueous extract supplementation influenced significantly ($P<0.001$) the level of blood glucose level of diabetic-induced rats and the responses were negatively correlated with

dose of *P. macrocarpa*. Although the means of blood glucose did not influence ($p>0.05$) by the period of supplementation, however it was observed that the level of blood glucose at the end of supplementation period was lower ($P>0.05$) for the long term period (5.2 mmol/L) than the short term supplementation (10.0 mmol/L).

As shown in Fig. 1, *P. macrocarpa* supplementation significantly reduced the blood glucose level of the diabetic-induced rats. 14 days of supplementation of *P. macrocarpa* reduced the blood glucose to the hyperglycemic state (10 mmol/L). Extending the period of supplementation was further reduced the blood glucose level and reached the plateau at normal level (3.9 – 6.1 mmol/L) after 17 days of supplementation. This plateau normal level was achieved due to the counter action of glucagon which was secreted when the blood glucose is lower than the normal range. Glucagon was secreted by α cells of the pancreas and these cells were not destroyed by the injection of STZ.

Table 1. Mean of blood glucose level (mmol/L) of diabetic induced rats supplemented for different periods with various doses of *P. macrocarpa* fruit extract

Supplementation periods	**Doses of *Pm* fruit aqueous extract (mg / kg bw)**			**Total Mean**
	0	**90**	**180**	
Long Term (28 days)	19.26 ± 4.47^{a}	12.94 ± 7.58^{b}	10.23 ± 4.07^{c}	13.20 ± 6.94
Short Term (14 days)	20.73 ± 6.48^{a}	12.80 ± 6.21^{b}	10.89 ± 5.79^{c}	14.80 ± 7.44
Total Mean	19.73 ± 5.23^{A}	11.74 ± 6.92^{B}	9.73 ± 4.71^{C}	

Means with different superscripts are significantly different at $p<0.05$.
± : standard deviation.
^{abc} : comparison of means between columns and between rows significant at $p<0.05$.
^{ABC} : comparison of means between columns within rows significant at $p<0.05$

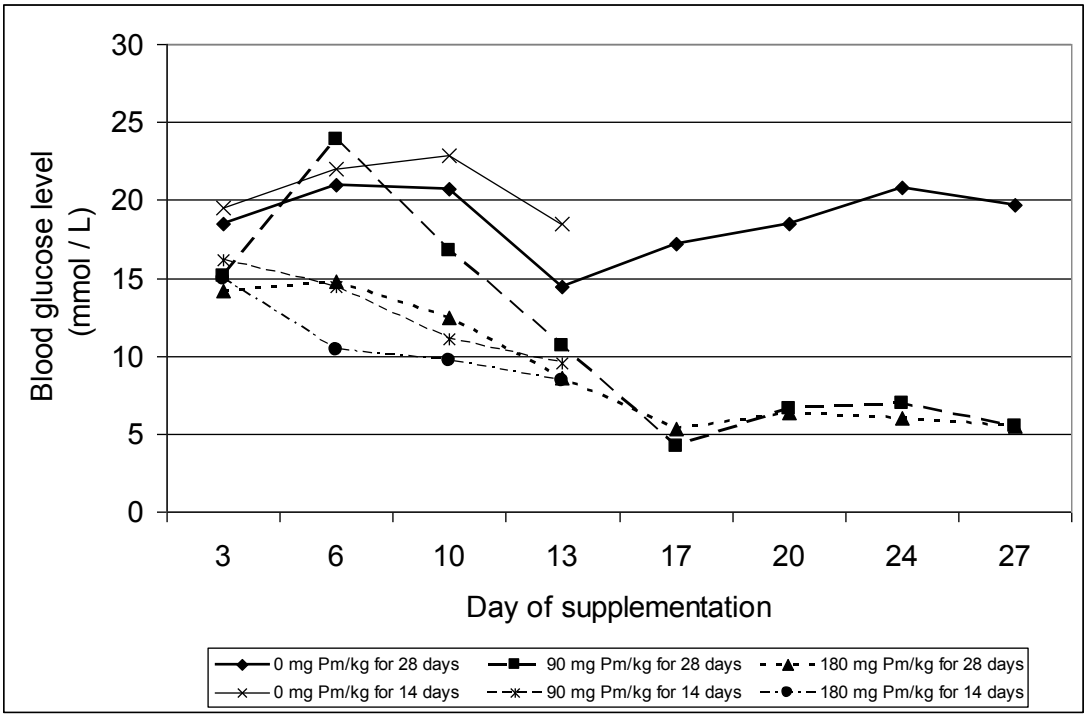

Fig. 1. Changes of the mean of blood glucose levels of diabetic induced rats supplemented with various doses of *P. macrocarpa* fruit aqueous extract for different periods.

These findings indicated that *P. macrocarpa* was able to treat diabetes mellitus and the action was slow which took about 17 days to fully recovered from diabetic type I. The mechanism of *P. macrocarpa* action is not fully understood although there was suggested that *P. macrocarpa* might inhibit the glucose absorption by the digestive system (Sri Sugiwati *et al*., 2006). The slow action of *P. macrocarpa,* which required 17 days for fully recovered from diabetic mellitus, possibly indicated that the effect was the result of improving glucose uptake and utilization by the cell through the improvement of GLUT2 action and synthesis of enzymes involved in glucogeonesis in the cells. Mahmood *et al*. (2003) have shown that the induction of hepatic glucokinase can be promoted by quercertin. The hepatic glucokinase is an enzyme that causes initial phosphorylation of glucose after it diffused into the liver cell. Once phosphorylated, the glucose temporarily trapped inside the liver cells because phosphorylated glucose cannot diffuse back through the cell membrane to the circulating blood (Guyton and Hall, 2000). Thus, it is possible that *P. macrocarpa* fruit extract intervened with the insulin action on glucokinese enzyme to reduce the blood glucose level. Further study however needs to be done to determine the exact mechanism of anti-diabetic *P. macrocarpa* actions.

In summary, it was found that *P. macrocarpa* fruit aqueous extract had anti-hyperglycemic effect in type 1 diabetic mellitus-induced rats. The effect is negatively correlated with the dose. A 17 days of supplementation at the dose of 90 mg/kg of extract is required to fully treat diabetes mellitus in rats and possibly in human as well.

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Antimicrobial Properties and Brine Shrimp Toxicity of Anti-Cancer Herbs : *Pereskia bleo, Pereskia grandifolia* and *Strobilanthes crispus*

Siew, K.Y. and Wong, S.K.

Department of Animal and Fishery Sciences, Faculty of Agricultuare and Food Sciences
Universiti Putra Malaysia (Bintulu Campus), P.O.Box 396, 97008 Bintulu, Sarawak, Malaysia
wongsk@putra.upm.edu.my

Abstract

Pereskia bleo, Pereskia grandifolia and *Strobilanthes crispus* are widely recognized as anti-cancer herbs among local community. The antimicrobial properties and toxicity of these herbs were analyzed. Aqueous extraction using both blending and decoction methods were used to obtain the herbal crude extracts. Antibacterial susceptible testing of the extracts was done using disc diffusion and minimum inhibition concentration (MIC) techniques. Toxicity test against brine shrimps was performed to determine the lethal dose (LD_{50}) of those plant extracts. All the crude extracts of *P. bleo, P. grandifolia* and *S. crispus* did not show any antibacterial activities against *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa* at amount up to 1000 ppm for MIC and 200 µg in disc diffusion. *P. bleo* and *S. crispus* showed LC_{50} values beyond 1000 ppm. The LC_{50} value of blended and decocted extracts of *P. grandifolia* was 13.34 µg/ml and 66.07 µg/ml respectively.

Introduction

Cancer was reported as third principal cause of in death in the government hospitals in 2005 that claimed over 4,000 lives or 10.11% of the total death recorded. In the same year, 55,572 hospitalization cases in government hospitals were due to malignant neoplasm (Health Facts, 2005). Treatment of cancer normally involves surgery, radiotherapy, chemotherapy, hormonal therapy and immune therapy. Besides these treatment methods, alternative or supportive treatment means using herbs have become increasingly popular. Anticancer herbs are widely accepted because they are relatively cheap and abundant as well as generally regarded as safe without giving side effects. In Malaysia, *Pereskia bleo, Pereskia grandifolia* and *Strobilanthes crispus* are traditional herbs well known for their medical importance among the local community. Studies have revealed some anticancer properties of these herbal plants (Tan *et al.,* 2005; Hafzan *et al.,* 2005; Rahmat *et al.,* 2006). However, there is still insufficient information on the toxicity of these plants whether misuse or over dosage would lead to poisoning or undesired effects. Furthermore, the antimicrobial activities of the mentioned herbs were evaluated against four pathogenic bacteria: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Materials and methods

Preparation of extracts

Plant extracts were obtained through decoction and blending methods. For the decoction method, 50 g of leave samples were cut into tiny pieces prior to boiling in 1 L of distilled water at for 2 h. The extract solutions were left to cool down and filtered through a cotton coffee filter. The resulting filtrates were then freeze-dried. For the blending method, 100 g of leaves were blended using electrical blender in 200 ml of distilled water. The juice was filtered with a cotton coffee filter and the filtrate was freeze-dried.

Antimicrobial tests

The bacterial cultures: *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* used in this study were obtained from Sarikei General Hospital. Antibacterial tests were carried out through disc diffusion and minimum inhibitory concentration (MIC) assays respectively adapted from Andrews (2007) and Lalitha

(2004). In the disc diffusion assay, sterile paper discs containing 200 µg of herbal extracts were placed on surface of nutrient agar prespread with overnight cultures of bacteria. The agar plates were incubated overnight at 35°C. The MIC assay was performed in series of two-fold dilution of plant extracts in nutrient broth ranging from 2000 ppm down to 0.39 ppm. Each concentration of the extracts was inoculated with 10 µl of overnight bacterial cultures and incubated for 18 h at 35°C with agitation at 200 rpm. The antibiotic levofloxacin served as positive control while sterile distilled water as negative control.

Brine shrimp toxicity test

Brine shrimp test was modified from MacLaughlin and Rogers (1998). The cysts were hatched in well-aerated artificial seawater (38 g sea salt in 1L distilled water) at room temperature (25°C). After 24-hour, the larvae were attracted by a light source and collected with pipette. Series of ten-fold dilutions (2000 ppm to 0.2 ppm) of herbal extracts were prepared in artificial seawater. Ten nauplii in a volume of 1 ml were added to 1 ml of diluted extracts to give concentrations ranging from 0.1 to 1000 ppm in total volumes of 2 ml. All samples were incubated at room temperature for 24 h. The mortality rate of the shrimp was determined by counting the number of dead larvae. The experiment was done in triplicates. Copper sulfate ($CuSO_4$) solution (1,000 ppm) was used as positive control while artificial seawater as negative control.

Results and discussion

Antimicrobial tests

Both disc diffusion and MIC assays revealed that all the blended and decocted extracts of *P. bleo, P. grandifolia* and *S. crispus* did not show any inhibitory effect against the tested bacteria: *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. The result for *P. bleo* is consistent with the antimicrobial works reported by Rüegg *et al*. (2006). According to Rüegg *et al.* (2006), the methanol or dicloromethane extracts of *P. bleo* did not exhibit any antimicrobial activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. In other research, *S. crispus* was reported to inhibit *Streptococcus sobrinus* and *Streptococcus mutans* (Rahmat and Abu Bakar, 2005).

Brine shrimp toxicity test

Brine shrimp toxicity test is a useful tool for preliminary assessment of toxicity. It has been used for the detection of toxicity of various samples including plant extracts (McLauglin *et al.*, 1991) Acute toxicity is usually expressed as 50% lethal concentration (LC_{50}). The LC_{50} value was determined by fitting the 50 % mortality of brine shrimps at the best fit line derived from the mean of mortality of each treatment.

Only the dose response curves of *P. grandifolia* extracts resembled typical sigmoid graphs (Fig. 1). The LC_{50} values of blended and decocted extracts of *P. grandifolia* were derived as 13.34 ppm and 66.07 ppm respectively. However, *P. bleo* and *S. crispus* extracts caused brine shrimp mortality rates rarely beyond 10% even at the highest extract concentrations at 1,000 ppm. Furthermore, the mortality rates for the *P. bleo* decocted extract were 0% for all the tested concentrations (Table 1). As a result, the decocted and blended extracts of *P. bleo* and *S. crispus* exhibited LC_{50} greater than 1000 ppm which was the top limit of the tested extract concentrations.

The results suggest that *P. bleo* and *S. crispus* might be consumed at higher amount, in raw or decoction form without the worry of toxicity. Traditionally, *P. bleo* is taken raw as “ulam” while *S. crispus* as herbal infusions. It is widely believed among the local community that the purple-flowered “jarum tujuh bilah” (*P. grandifolia*) is stronger than its species with red flowers (*P. bleo*), and it is not advisable to consume more than two raw leaves of either type of “jarum tujuh bilah” each day. Our findings show that both blended and decocted extracts of *P. grandifolia* are stronger in terms of the toxicity than their counterparts of *P. bleo*. Nevertheless, we observed that both blended and decocted extracts of *P. bleo* as well as the decocted extract of *P. grandifolia* are considered safe with LC_{50} values greater than 20 µg/ml, the cutoff point for cytotoxic activity (Geran *et al*., 1972). The toxicity of the decocted extract of *P. grandifolia* was found approximately five times lower than its blended

extract. It is thought that the boiling process could have led to degradation of certain heat sensitive active compounds. Therefore, it is reasonable to restrict the dosage of raw *P. grandifolia* leaves as recommended by the traditional medicine practitioners.

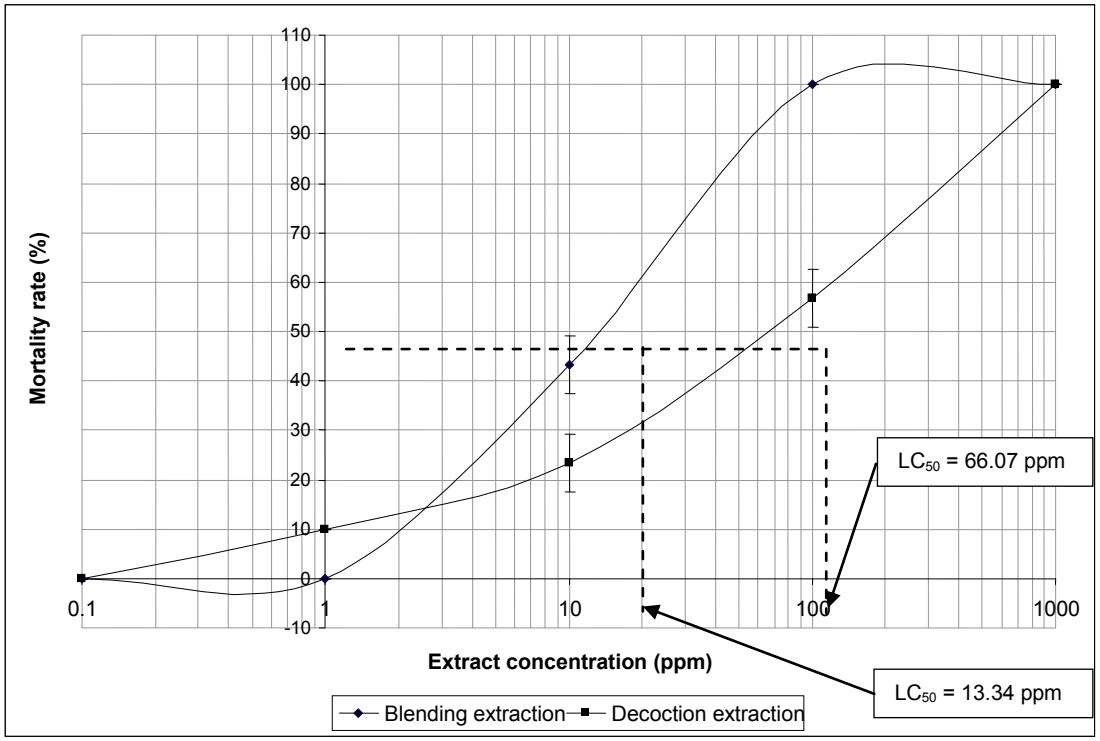

Fig. 1. Brine shrimp toxicity of *Pereskia grandifolia* extracts

Table 1. Mortality of brine shrimp against plant extracts

Extract Concentration (ppm)	Brine Shrimp Mortality Rate (%)					
	P. bleo		*P. grandifolia*		*S. crispus*	
	blend	decoction	blend	decoction	blend	decoction
1000	10.00	0	100.00	100.00	9.6±0.6	10.00
100	10.00	0	100.00	56.7±5.7	10.00	10.3±0.6
10	0	0	43.3±5.7	23.3±5.7	0	0
1	0	0	0	10.00	0	0
0.1	0	0	0	0	0	0
Artificial seawater	0	0	0	0	0	0
$CuSO_4$ (1000 ppm)	100.00	100.00	100.00	100.00	100.00	100.00

Conclusion

All the blended and decocted extracts of *P. bleo, P. grandifolia* and *S. crispus* did not show any antibacterial activities against *E. coli, K. pneumoniae, S. aureus,* and *P. aeruginosa*. Extracts of *P. bleo* and *S. crispus* did not indicate any toxicity with their LC_{50} >1000 ppm. *P. grandifolia* demonstrated some toxicity at LC_{50} of 13.34 µg/ml and 66.07 µg/ml respectively for extracts obtained through blending and decoction methods.

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Antioxidant Activity of Methanol Extracts of Different Parts of *Lantana camara*

Badakhshan, M.P.[1], Sasidharan, S.[1,2], Naidu, J.R.[1] and Ramanathan, S.[3]

[1]Department of Biotechnology, Faculty of Applied Sciences, AIMST University, 08100 Semeling- Bedong, Kedah, Malaysia
[2]Institute for Research in Molecular Medicine (INFORM),[3]Centre for Drug Research, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia
srisasidharan@yahoo.com

Abstract

In this study, the antioxidant properties of *Lantana camara* (root, stem, leaf, flower and fruit) extracts were evaluated through DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging activity and xanthine oxidase inhibition assay. *L. camara* leaf revealed the best antioxidant property, presenting much lower IC_{50} values (16.02 ± 0.94 µg/ml for DPPH assay and 17.54±1.87 µg/ml for xanthine oxidase assay). Furthermore, the highest antioxidant contents (polyphenols) were found for this extract (245.50 ± 3.54 mg GAEs/g extract). This study suggests that possible mechanism of antioxidant activity of methanolic extracts of this plant may be due to free radical-scavenging which may be due to the presence of polyphenols in the extracts.

Introduction

There has been interest in the contribution of free radical reaction participating in reactive oxygen species to the overall metabolic perturbation that results in tissue injury and disease. Reactive oxygen such as superoxide anion, hydrogen peroxide, and hydroxyradical are generated in specific organelles of cells (mitochondria and microsomes) under normal physiological condition. These reactive oxygen species can damage DNA, so as to cause mutation and chromosomal damage, oxidize cellular thiols and abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Halliwell and Gutteridge, 1985; Ames *et al.*, 1993). Recently, various phytochemicals and their effect on health, especially the suppression of active oxygen species by natural antioxidant from tea, spices and herbs, have been intensively studied (Sies and Stahl, 1995; Elmastas *et al.,* 2006). In this work we studied the antioxidant potential of different extracts of *Lantana camara*.

Materials and methods

Plant

Samples were collected from Sungai Petani, Kedah, Malaysia, on January of 2008. Plants were identified by a botanist of Department of Biotechnology, AIMST, University, Kedah, Malaysia. Root, stem, leaf, flower and fruit of selected plants were dried (60 °C) and powdered with a mortar. 100 g of dried and powdered plant material were extracted at room temperature with 500 ml of methanol with constant shaking for 24 h. After filtration, the methanolic solutions were evaporated to dryness in a rotary evaporator for the antioxidant assays.

DPPH radical-scavenging activity

Various concentrations of *L. camara* extracts (50.0 µl) were mixed with 5.0 ml of methanolic solution containing DPPH radicals (0.004% w/v). The mixture was shaken vigorously and left to stand for 30 min in the dark (until stable absorbance values were obtained). The reduction of the DPPH radical was determined by reading the absorbance at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration, using the equation: % RSA = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract is added at a particular level and A_{DPPH} is the absorbance of the DPPH solution (Oktay *et al.*, 2003). The extract concentration providing 50% of radical scavenging activity (IC_{50}) was calculated from the graph of RSA percentage against extract concentration. Vitamin C was used as standard.

Folin-Ciocalteu method

The total phenolic content of extracts was determined according to the Folin-Ciocalteu spectrophotometric method (Singleton and Rossi, 1965). The test was done in triplicate. The results were expressed as gallic acid equivalents (GAE, mg/g) of *L. camara* extract.

Xanthine oxidase inhibition assay

The assay was conducted as described by Cos *et al.,* (1998) and Owen and Johns (1999) methods. The uric acid production was calculated according to the increasing absorbance at 290 nm. Test solutions were prepared by adding 400 µl xanthine (final concentration 2 mM), 50 µl hydroxylamine (final concentration 0.2 mM), 50 µl EDTA (final concentration 0.1 mM), and 4 µl, 10 µl, 20 µl and 40 µl extract (final concentrations 2, 5, 10 and 20 µg/ml, respectively). The reaction was started by adding 50 µl of xanthine oxidase (final concentration 50 mU/ml). The mixture (total 5 ml) was incubated for 30 min at 25°C. Prior to the measurement of uric acid production by measuring the UV absorbance at 290 nm, the reaction was stopped by adding 200 µl of HCl 0.58 N. Xanthine oxidase inhibition activity was expressed as the percentage inhibition of xanthine oxidase in the above assay system, calculated as:

$$\text{Xanthine Oxidase Inhibition (\%)} = \left\{\frac{(A-B)-(C-D)}{(A-B)}\right\} \times 100$$

where A is the activity of enzyme without test extract, B the control of A without test extract and enzyme, C and D are the activities of the test extract with and without xanthine oxidase. Allopurinol, a known inhibitor of xanthine oxidase was used as a positive control. The test was done in triplicate. The results were expressed as IC_{50} values for each part of plant.

Results and discussion

Table 1 presents the antioxidant activities and polyphenols contents, obtained for all the *L. camara* extracts. IC_{50} values for DPPH radical scavenging activity of the methanol extracts of root, stem, leaf, flower and fruit of *L. camara* are comparable with known antioxidant vitamin C. Overall, *L. camara* leaf revealed the best antioxidant properties (significantly lower IC_{50} values = 16.02±0.94 µg/ml; $p<0.05$) and the fruit revealed a very poor antioxidant activity (significantly higher IC_{50} values = 90.11±0.57 µg/ml; $p<0.05$). In the xanthine oxidase system, the IC_{50} values of all extracts were found to range from 17.54 to 30.20 µg/ml. The obtained results are in agreement with the phenol contents determined for each sample.

Table 1. Antioxidant activities of *Lantana camara* extracts

Sample	IC_{50} (µg/ml) ± SD (Xanthine Oxidase Inhibition)	IC_{50} (µg/ml) ± SD (DPPH)	GAE (mg/g) of *L. camara* Extract ± SD
Vitamin C	ND	6.21 ± 0.04	ND
Allopurinol	7.98 ± 0.38	ND	ND
Root	21.49 ± 0.63	31.52 ± 0.74	92.00 ± 8.00
Stem	19.24 ± 1.32	46.96 ± 2.51	85.33 ± 4.16
Leaf	17.54 ± 1.87	16.02 ± 0.94	245.50 ± 3.54
Flower	30.20 ± 2.87	28.92 ± 0.19	212.33 ± 6.81
Fruit	28.53 ± 2.44	90.11 ± 0.57	77.50 ± 3.53

ND - Not determined

Conclusion

As far as we know, this is the first report concerning the antioxidant activity of root, stem, leaf, flower and fruit extracts of *L. camara*. The work herein indicates that the leaf of *L. camara* presents the highest antioxidant activity.

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Anti-proliferative Activity of Zingiberaceae Methanol Extract on MDA-MB-231 Human Breast Cancer Cells

Yap, V.A.[1], Nurhayati, Z.A.[1] and Norhanom, A.W.[2]

[1]Institute of Biological Sciences, Faculty of Science, [2]Institute of Graduate Studies, University of Malaya 50603 Kuala Lumpur, Malaysia
veron.alicia@gmail.com.

Abstract

Breast cancer has been, and still is the leading cause of death among women both locally and worldwide. The lack of cure for this has encouraged the search for anticancer treatments in natural products. Species of the Zingiberaceae family have long been utilized for both culinary and medicinal use and therefore has made them potential candidates in the search for new chemotherapeutic agents. In this study, methanolic extracts of five selected Zingiberaceae species namely *Curcuma domestica, Languas galanga, Zingiber zerumbet, Zingiber officinale* and *Zingiber montanum* were investigated for possible anti-proliferative activity using MTT assay against human breast cancer (MDA-MB-231) cell line. The cells were treated with crude methanol extracts at different concentrations ranging from 1 µg/ml to 100 µg/ml for 72 hours. All extracts showed dose-dependant anti-proliferative activity but at different intensities. Out of the five species tested, three of the methanolic extracts namely *L. galanga , C. domestica,* and *Z. zerumbet* exhibited good anti-proliferative activities against MDA-MB-231 cells with ED_{50} values of 4.7 µg/ml, 7.2 µg/ml and 10.5 µg/ml respectively. ED_{50} denotes the concentration of extract that inhibits the growth of 50% of the cells. Crude extracts that give ED_{50} values lesser than or equal to 20 µg/ml is considered active. Thus, these findings suggest that Zingerbers have potential in the role of tumor chemoprevention or as a potential tumor therapeutic agent.

Introduction

Many studies have correlated diet and cancer prevention (Greenwald *et al.,* 2001) that has opened doors for nutritional sciences beyond the traditional approach to fields of research encompassing nutraceuticals and phytochemicals in search of an ideal treatment or chemoprevention of cancers.

Plants of the Zingiberaceae or better known as the ginger family have long been known for its culinary and medicinal use in treating various illnesses including cancers (Halijah and Ahmad, 1988; Vimala *et al.*, 1999). The cancer hemoprevention potential of gingers has been shown to reduce cancer risks and its extracts have been shown to exhibit anti-tumor activities (Lee and Houghton, 2005).

In previous studies, Zingiberaceae species have been found to exert inhibitory activity towards Epstein-Barr virus activation in Raji cells (Murakami *et al.*, 2000*).* Recent studies have also successfully identified several anti-tumor compounds from the Zingeberaceae such as paradol, curcumin, and Zerumbone (Chung *et al*., 2001; Li *et al*., 2002; Kinrana Grame *et al.*, 2003). Researches have extended to the production of synthetic derivatives of curcumin to evaluate their chemopreventive potential. However none of these compounds proved to be more potent that the natural occurring curcumin (Gafner *et al*., 2004).

Several researches have documented on the anti-tumor potential of Zingiberaceae extracts. Possible mechanisms linking to the inhibition of tumerogenesis reported include the induction of apoptosis, and its anti-angiogenic capacity (Ito *et al.,* 2004; Kim *et al.*, 2005). Therefore Zingiberaceae are very promising candidates for the discovery as source of biologically active substances.

In this study, we investigate the effect of five methanol crude extract from the Zingiberaceae on cell proliferation particularly against human breast cancer cell line, MDA-MB-231 using 3-[4,5-dimethlythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Materials and methods

Materials

Fresh rhizomes of *Curcuma domestica, Alpinia galanga, Zingiber zerumbet, Zingiber officinale* and *Zingiber montanum* were soaked in methanol for 72 hours, then concentrated in a rotary evaporator under reduced pressure at 50°C. The concentrated methanol extracts were then reconstituted in dimethyl sulfoxide (DMSO). All samples were prepared aseptically and kept at -20°C until use.

Cell culture

MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC) and cultured under standard condition of 5% CO2-95% air at 37°C in Dubelcco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), Penicillin/streptomycin and Amphotericin B.

Anti-proliferative activity assay

MDA-MB-231 cells were plated at the density of 3 x 104 cells per ml into 96-well flat bottom plates. The cells were then maintained for one day in complete culture medium to allow attachment. After 24 hours, cells were incubated with the addition of extracts at different concentrations ranging from 1, 10, 25, 50, 75 and 100 µg/ml for 72 hours. Negative controls comprise of cells not treated with extracts. The final volume for each well was made up to 200 µl with the media containing less then 3% DMSO in each well. The experiments were performed in triplicates. Cell growth inhibition was evaluated using MTT assay (Scudiero *et al.*, 1988) with slight modifications. 5 mg/ml stock solution of MTT in phosphate buffered saline (PBS) was added to each well. After 4 hours of incubation at 37°C, the residual MTT was carefully removed from each well and DMSO was added to dissolve the MTT-formazan product. Absorbance was measured with a microplate reader at 540 nm with 650 nm as the reference wavelength.

Determination of ED_{50}

Inhibition of cell proliferation is calculated as (% of inhibition= [OD Control –OD540-650 / OD Control] x 100%). ED_{50} is the concentration of extract resulting in 50% inhibition of cell proliferation and was obtained from plots of percentage of inhibition versus concentration of extract.

Results and discussion

A total of five methanol extracts were investigated for their anti-proliferative activity against MDA-MB-231 cells. Fig. 1 shows the Dose response curves representing the percentage of inhibition of proliferation on MDA-MB-231 cell lines versus concentration of Zingiberaceae extract, whereby all extracts have suppressive effects but at varying degrees of inhibition. ED_{50} were extrapolated from the curves as shown in Table 1. A crude extract is generally considered to have active *in vitro* antiproliferative activity if the ED_{50} is at least less than or equal to 20 µg/ml (Boik, 2001). In this present study, the methanolic extracts of *A. galanga, C. domestica and Z. zerumbet* exhibited the most promising anti-proliferative activity. This could be due to the cell membrane affinity of the potentially active substance(s).

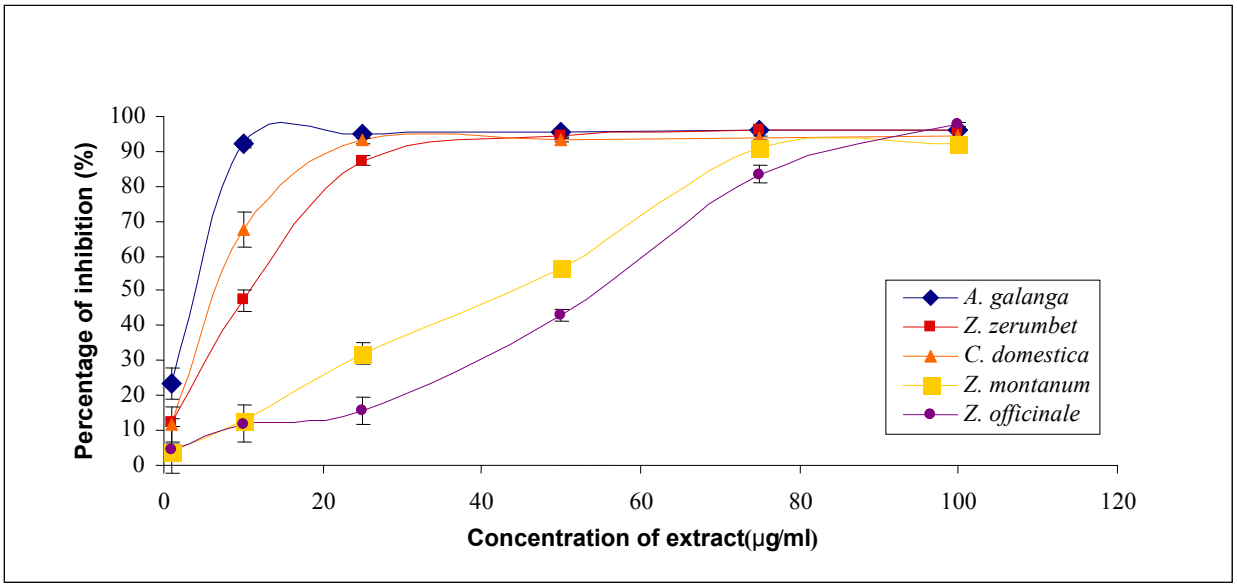

Fig. 1 Anti-proliferative profile of five Zingiberaceae methanol extracts on MDA-MB-231 cell line using MTT assay. Results are expressed as mean ± SD (n = 3).

Table 1 The ED_{50} values of Zingiberaceae methanolic extracts against MDA-MB-231 human breast cancer cells

Samples	**ED_{50} (µg/ml)**
Zingiber montanum	43.5
Zingiber zerumbet	10.5
Zingiber officinale	54.0
Languas galangal	4.7
Curcuma domestica	7.2

The methanolic extract of *A. galanga* which showed the lowest ED_{50} value among the five samples investigated has also been documented for it strong toxicity *in vitro* towards breast cancer cell line, MCF 7 (Lee and Houghton, 2005). However the ED_{50} obtained in this study was slightly lower than reported by Lee and Houghton (2005).

In this study, *Z. officinale* was found to be inactive against MDA-MB- 231 cell line as opposed to previous documentations of its major active compound gingerol, as an apoptotic inducer (Wei *et al.*, 2005) and anti-metastatic agent (Hyun
et al., 2008).

The extracts of *C. domestica, A. galanga, Z. zerumbet, Z. officinale, Z. montanum* possess anti-cancer activities that may provide further insights to the development of new therapeutic agents. Long-term consumption of these species could also be promoted for cancer chemoprevention. The sensitivity of MDA-MB-231 cells towards these extracts is important as this breast cancer cell line expresses growth factor receptors which is evident for the process of metastasis therefore inhibition of the proliferation of this cell line could result in the inhibition of migration potential of these malignant cells.

Acknowledgements

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Phytochemical Studies of *Goniothalamus uvarioides* and *Goniothalamus velutinus*

Wong, M.H.

Faculty of Applied Science, Universiti Teknologi MARA, Samarahan campus, Sarawak branch, 94300 Kota Samarahan, Sarawak, Malaysia
wongmh@sarawak.uitm.edu.my

Abstract

Goniothalamus uvarioides and *Goniothalamus velutinus* belonging to the Annonaceae family have been used in traditional medicine for the treatment of giddy, injury, diarrhea, aphrodisiac, body pain, cold, stomachache, swollen, headache, food poisoning, to maintain body health and as mosquito repellent. The phytochemical studies of *G. uvarioides and G. velutinus* were carried out. Two alkaloids, one styryllactone (styrylhydropyrone) and two flavonoids have been isolated. These compounds were characterized by spectroscopic analysis, chemical methods and by comparison with the published and data. The identified compounds were velutinum, aristolactam-BII from *G. velutinus*, and pinocembrin, goniothalamin as well as 5,7,4'-trihydroxyflavanone (naringenin) from *G. uvarioides*.

Introduction

Members of Annonaceae family have been investigated as interesting and potential sources of biologically active natural products. Many of them are used for the treatment of various human diseases in folk medicine and exhibited anti-tumor activities (Yu, 1999). *Goniothalamus*, the largest genus of the Annonaceae family comprises approximately 120 species of shrubs, climbers and trees, is growing widely in Asia, Oceania, South-eastern Asia and throughout Malaysia (Airy-Shaw, 1966; Burkill, 1966; Corner, 1988; Saunders, 2003). About 18 species are distributed widely in West Malaysia (Leboeuf *et al*., 1982; Saunders, 2003). Around 30 species of *Goniothalamus* can be found in Borneo Island (Kamarudin, 1993). In Sarawak, approximately 14 species of *Goniothalamus* were recorded (Andersons, 1980). Ethnobotanical uses of several *Goniothalamus* species are well known in Malaysia. *Goniothalamus* species are widely used in traditional medicine, alone or as part of herbal mixtures, as post-partum protective remedies, abortifacients, rheumatism, as insect repellents, to treat some diseases such as skin disease, snake bite, edema, fever, skin pain, rheumatism, febrifuge, cholera and malaria (Burkill, 1966; Perry and Metzger, 1980). *Goniothalamus uvarioides* and *Goniothalamus velutinus* have been used in traditional medicine for the treatment of diarrhea, aphrodisiac, body pain, cold, stomachache, swollen, headache, food poisoning, to maintain body health and as mosquito repellent (Burkill, 1966; Omar *et al.*, 1992; Fasihuddin and Hasmah, 1993). *G. uvarioides* is known as selukai amat and selukai daun besar putih (Burkill, 1935; Ridley, 1967; Andersons, 1980). *G. uvarioides* are found throughout Malaysia (Burkill, 1935; Ridley, 1967). *G. velutinus* is known as kayu hujan panas, limpanas, lakum, tungkat, langgau, kerikut kayu tas, limpanas hitam and found throughout Sarawak (Andersons, 1980) and endemic to Borneo (Omar *et al.*, 1992). It is believed to have magical power by the locals and has medicinal values. It is used to protect and to scare away ghosts or evil spirits by hanging the stem on doorways or burning the bark to produce a strong smell. It possessed anti-tumor properties (Burkill, 1966; Omar *et al.*, 1992; Fasihuddin and Hasmah, 1993). Phytochemical studies on the *Goniothalamus* species resulted in the isolation on natural compounds with significant cytotoxic, antitumor, pesticidal, insecticidal, antimicrobial, abortfacient, teratogenic, embryotoxic and teratogenic activities (Razak *et al.*, 1984; Wiart, 2000). The predominant isolates of bioactive secondary metabolites have been acetogenins (Zafra-Polo *et al.*, 1998), styryllactones (Bermejo *et al.*, 1998), styrylpyrone, alkaloids (Omar *et al.*, 1992), flavonoids and their derivatives.

Materials and methods

General experimental procedures

Column chromatography was carried out with silica gel (Merck, 230-400 mesh). MPs were recorded on a melting point apparatus Electrothermal 1A9100 Operation. IR spectra were measured with KBr on either a Shimadzu 8012PC infrared spectrometer (KBr or film) or a Perkin Elmer Spectrum GX spectrometer (dried sample powder) in a frequency range from 400 cm^{-1} to 4000 cm^{-1}. MS spectra were obtained on a Shimadzu GC-17A/MS QP-5000 equipped with capillary column DB-5 (J&W Scientific) with the moderate polarity (30 m length x 0.25 mm internal diameter; 0.25 µm film thickness) under ionization energy of 70 eV. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on either a Bruker Avance300 spectrometer or a NMR Varian Unity INOVA 500 MHz. Chemical shifts were reported in parts per million (δ) with solvent signal as the internal reference. Tetra-methylsilane (TMS) was used as the internal standard for ^{1}H NMR; $CDCl_3$ and MeOD were used as reference chemical shifts for ^{13}C NMR respectively. All 1D (DEPT-135) and 2D (COSY-H-H Correlation Spectroscopy, NOESY, HSQC and HMBC) acquisition were accomplished with standard Bruker pulse programs. Analytical TLC was performed on Merck F_{254} silica gel plates (0.25 mm thickness). Alkaloids were detected with Mayer test on the plant samples as well as by spraying with Dragendorff reagent to visualize yellowish-orange spots. Styryllactone and flavonoid were detected by spraying with vanillin-sulfuric acid, followed by heating the plates at 100°C for 5-10 min until the appearance of a spot.

Plant materials

The stem barks and roots of *G. uvarioides* and *G. velutinus* were collected from Limbang and Lawas, Sarawak. Identification (leaves, stem, fruit, shoot) was performed by comparison with the herbarium specimens in UNIMAS.

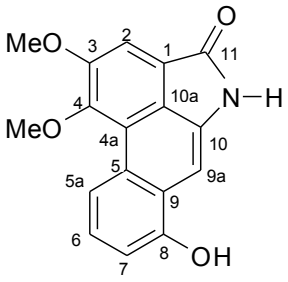
Extraction and isolation

Dried and ground stem barks of *G. velutinus* (2.64 kg), roots of *G. velutinus* (0.81 kg), stem barks of *G. uvarioides* (1.00 kg) and roots of *G. uvarioides* (0.80 kg) were repeatedly and successively extracted with n-hexane, ethyl acetate and then methanol at room temperature. The solvents were evaporated and removed. The combined extracts were concentrated under reduced pressure to yield brownish viscous materials: hexane extract (1.24 g), ethyl acetate extract (56.34 g) and methanol extract (131.64 g) of *G. velutinus* stem barks; hexane extract (1.75 g), ethyl acetate extract (77.79 g) and methanol extract (77.09 g) of *G. velutinus* roots; hexane extract (4.36 g), ethyl acetate extract (27.25 g) and methanol extract (17.14 g) of *G. uvarioides* stem barks; hexane extract (5.06 g), ethyl acetate extract (64.43 g) and methanol extract (12.07 g) of *G. uvarioides* roots. The brown crude extracts were applied on to the column chromatography of silica gel and eluted with n-hexane containing gradually increasing polarity and amounts of ethyl acetate then methanol. Fractions and impure components were obtained and combined according to their similar appearance on TLC analysis, and these were again subjected to repeated chromatography (gradients or solvent systems of n-hexane-ethyl acetate mixtures of increasing polarity) to yield velutinum (**1**) (24.7 mg) from the methanol extracts of *G. velutinus* stem barks and roots; aristolactam-BII (**2**) (5.0 mg) from the methanol extract of *G. velutinus* stem barks; pinocembrin (**3**) (5028.2 mg) from the ethyl acetate and hexane extracts of *G. uvarioides* stem barks and roots; goniothalamin (**4**) (888.1 mg) from the ethyl acetate and hexane extracts of *G. uvarioides* roots; 5,7,4'-trihydroxyflavanone, naringenin (**5**) (20.0 mg) from the ethyl acetate extract of *G. uvarioides* stem barks.

Results and discussion

Alkaloid screening using Mayer reagent showed the presence of alkaloid in all of the samples are moderate in quantity. Five pure compounds (**1**-**5**): two alkaloids, one styryllactone and two flavonoids have been isolated. These isolated compounds were characterized and elucidated by spectroscopic analysis (FTIR, UV, GCMS, NMR), and by comparison with the published data. The identified

compounds were velutinum (10-amino-8-hydroxy-3,4-dimethoxyphenanthrene-1-carboxylic acid lactam **1**) and aristolactam-BII (10-amino-3,4-dimethoxyphenanthrene-1-carboxylic acid lactam **2**) from *G. velutinus*; pinocembrin (5,7-dihydroxyflavanone **3**), goniothalamin ((+)-(5s)-delta-lactone of 5-hydroxy-7-phenylhepta-2,6-dienoic acid **4**) and naringenin (5,7,4'-trihydroxyflavanone **5**) from *G. uvarioides*. Compound **1** and **2** have been isolated from the roots of *G. griffithii* (Zhang *et al.*, 1999a, b). Compound **3** have been isolated from *G. macrophyllus* (Chew *et al.*, 1985), stem of *G. borneensis* (Cao *et al.*, 1998), *G. grifithii* (Talapatra *et al.*, 1985) and *G. boesenbergia pandurata* (Hasnah *et al.*, 1995). Compound **4** have been isolated from stem barks of *G. andersonii* (Salmaan *et al.*, 1999; Tanaka *et al.*, 2001), *Borneensis* (Cao *et al.*, 1998), *G. dolichocarpus*, *G. cardiopetalus* (Hisham *et al.*, 2003) and roots of *G. griffithii* (Zhang *et al.*, 1999b). Compound **5** was the first time isolated from *G. uvarioides*. Biological activities of the samples were also carried out and will be discussed in the future.



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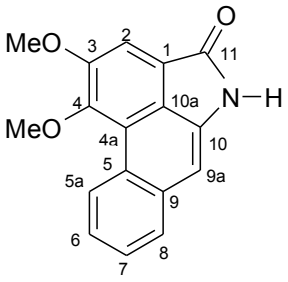

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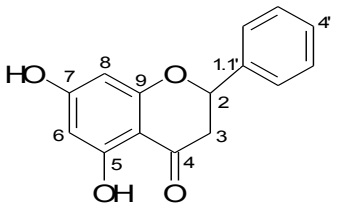

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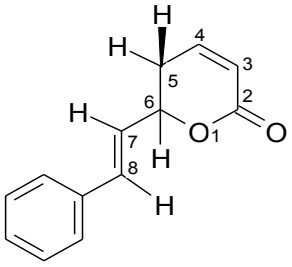

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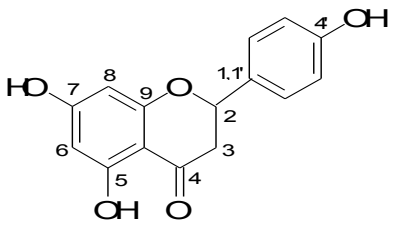

5

Velutinum (1): Yellowish, orange amorphous powder (24.7 mg; MeOH). Mp 299.0-299.2 °C. IR υ_{max} cm^{-1}: 3120-2900 (N-H; -OH), 1670.04 (C=O), 1350-1300 (C-O; C-N or –CONH-), 1600.00-1400.00 (C=C or aromatic group). ^{1}H NMR (500 MHz; MeOD) δ ppm: 4.11 (3H, s; OMe-C-4), 4.87 (s, 3H; OMe-C-3), 8.77 (s, 1H; -CONH-), 8.76 (d, J=8.0; 1H; H-5 on aromatic ring), 7.88 (1H, s; H-2), 7.41 (t, J=8.0; 1H; H-6 on aromatic ring), 7.06 (d, J=7.0. 1H; H-7), 7.65 (s, 1H; H-9). ^{13}C NMR (125 MHz; MeOD) δ ppm 17 C: 190.83 (C-O; C-N or 11–CONH-), 59.51 and 56.34 (two C of methoxy group), 152.02 (C-4-OMe), 154.40 (C-3-OMe), 125.96 (C-1), 100.62 (C-2), 124.40 (C-4a), 111.92 (C-5a), 128.40 (C-6), 118.74 (C-7), 146.31 (C-8), 109.62 (C-9), 154.02 (C-9a), 128.01 (C-10), 124.20 (C-10a). MS (70 eV; CH_2Cl_2/MeOH) for $C_{17}H_{13}O_4N$ $(M+H)^+$ 295 *m/z* (rel. int., %): 280 (7.50), 252 (22.54), 237 (12.68), 225 (12.68), 207 (14.08), 197 (12.68), 181 (7.50), 153 (8.75), 140 (14.08), 126 (15.49), 90 (19.72), 83 (7.50), 63 (19.72), 57 (14.08), 43 (18.31). Comparison with the reported data (Omar *et al.*, 1992; Cao *et al.*, 1998; Zhang *et al.*, 1999a).

Aristolactam-BII (2): Brownish, orange amorphous powder (5.0 mg; MeOH). Mp 257.0-258.0 °C. IR (KBr) υ_{max} cm^{-1}: 3120-2900 (N-H), 1690 (C=O), 1350-1300 (C-O; C-N or –CONH-), 1600.00-1400.00 (C=C or aromatic group). UV (MeOH) λ_{max} nm: 208 (aromatic ring), 231 (C-O), 262 (N-H), 275 (C=O), 286 (-CONH-). ^{1}H NMR (300 MHz; $CDCl_3$) δ ppm: two C of methoxy group 4.10 (3H, s; OMe-C-4), 4.16 (s, 3H; OMe-C-3), 9.28 (s, 1H; -CONH-), 9.26 (m, J=3.3, 1H; H-5 on aromatic ring), 7.84 (s, 1H; H-2), 7.85 (m, J=2.7, 9.6, 1H; H-8), 7.85 (m, J=9.3, 1H; H-8), 7.62 (1H, m; H-6 on aromatic ring), 7.11 (s, 1H; H-9). ^{13}C NMR (75 MHz; $CDCl_3$) δ ppm 17 C: 170.20 (C-O; C-N or 11–CONH-), 56.92 and 60.38 (two C of methoxy group), 154.52 (C-3-OMe), 151.80 (C-4-OMe), 125.20 (C-1), 109.49 (C-2), 122.80 (C-4a), 127.58 (C-5), 127.02 (C-5a), 126.00 (C-6), 127.60 (C-7), 129.04 (C-8), 105.60 (C-9), 133.89 (C-9a), 134.71 (C-10), 121.60 (C-10a). MS (70 eV; CH_2Cl_2/MeOH) for $C_{17}H_{13}O_3N$ $(M+H)^+$ 279 *m/z* (rel. int., %): 264 (17.81), 236 (24.66), 221 (19.18), 193 (21.92), 164 (23.29), 140 (19.18), 82 (44.83), 69 (19.54). Comparison with the reported data (Omar *et al.*, 1992; Cao *et al.*, 1998; Zhang *et al.*, 1999a).

Pinocembrin (3): Yellowish, white powder, solid (5028.2 mg; MeOH). Mp 194.7-194.9 °C. IR υ_{max} cm^{-1}: 3200-2800 (-OH), 1629.53 (C=O), 1602.66 and 1486.74 (C=C or aromatic group). ^{1}H NMR (300 MHz; $CDCl_3$) δ ppm: 5.47 (dd, J=12.9, 3.3, 1H; H-2), 3.14 (dd, 1Ha, H-3a, $J_{2,3a}$=17.1, 12.6-12.9 Hz, *trans*), 2.75 (dd, 1Hb, H-3b, $J_{2,3b}$=17.1, 3.3 Hz, *cis*), 5.95 (d, J=2.1, 1H; H-6), 5.92 (d, J=2.1, 1H; H-8), 7.34-7.52 (m, 5H). ^{13}C NMR (75 MHz; MeOD) δ ppm: 138.99 (C-1'), 128.32 (C-4'), 128.25 (C-3' and 5'), 125.96 (C-2' and 6'), 79.04 (C-2), 42.79 (C-3), 195.92 (C-4), 164.07 (C-5), 95.81 (C-6), 167.04 (C-7), 94.87 (C-8), 163.25 (C-9), 101.97 (C-10). MS (70 eV; CH_2Cl_2/MeOH) for $C_{15}H_{12}O_4$ $(M+H)^+$ 256 *m/z* (rel. int., %): 238 (8.64), 179 (86.42), 165 (1.23), 152 (98.00), 136 (1.23), 124 (62.96). Comparison with the reported data (Talapatra *et al.*, 1985; Zakaria *et al.*, 1998; Seidel *et al.*, 2000).

Goniothalamin (4): Yellowish, white needle, prism, crystals (888.1 mg; DCM). Mp 72.9-73.1 °C. IR υ_{max} cm^{-1}: 2918.15 (C-H), 1703.11 (C=O), 1638.00, 1577.64 and 1493.76 (C=C or aromatic group), 1239.86 (C-O). ^{1}H NMR (300 MHz; $CDCl_3$) δ ppm: 7.35 or 7.27-7.43 (m, 5H; aromatic), 6.26 (dd, J=15.9, 6.3, 1H; H-7), 6.72 (dd, J=15.9, 1.3, 1H; H-8), 6.09 (dt, J=9.9, 2.1, 1H; H-3), 6.91 (dd, J=9.6, 4.8, 1H; H-4), 2.54 (m, 2H; H-5), 5.09 (m or ddd, J=7.2, 6.6, 1.2, 1H; H-6). ^{13}C NMR (75 MHz; $CDCl_3$) δ ppm 13 C: 163.91 (C-2), 126.71 (C-10), 128.71 (C-11), 128.37 (C-12), 128.71 (C-13), 126.71 (C-14) on aromatic ring, 29.89 (2H at C-5), 121.68 (C-3), 144.66 (C-4), 77.96 (C-6), 126.71 (C-7), 133.15 (C-8), 135.77 (C-9). MS (70 eV; CH_2Cl_2) for C_{13} H_{12} O_2 $(M+H)^+$ 200 *m/z* (rel. int., %): 172 (3.80), 131 (7.59) 115 (7.59), 104 (41.77), 77 (15.19) dan 68 (100.00). Comparison with the reported data (Hasan *et al.*, 1994; Goh *et al.*, 1995; Anjee, 1999).

Naringenin (5): Greenish needle crystals (20.0 mg; MeOH). Mp 226.8-228.2 °C. IR υ_{max} cm^{-1}: 3500-2000 (-OH), 1631.33 (C=O), 1598.20 and 1458.06 (C=C or aromatic group). ^{1}H NMR (500 MHz; $CDCl_3$) δ ppm: 5.32 (dd, J=13.0, 2.5, 1H; H-2), 3.12 (dd, 1Ha; H-3a, $J_{2,3a}$=17.0, 13.0-13.5 Hz, *trans*; $J_{2,3b}$=17.0, 2.5-3.0 Hz, *cis*), 5.90 (s, 1H; s, 1H; H-6 and H-8), 6.84 (d, J=8.5, 1H), 7.31 (d, J=8.0, 1H). ^{13}C NMR (125 MHz; MeOD) δ ppm: 129.93 (C-1,1'), 157.82 (C-4'), 127.91 (C-3' and 5'), 115.20 (C-2' and 6'), 196.61 (C-4), 79.29 (C-2), 42.84 (C-3), 164.28 (C-5), 95.95 (C-6), 167.15 (C-7), 95.06 (C-8), 163.71 (C-9), 102.22 (C-10). MS (70 eV; CH_2Cl_2) for $C_{15}H_{12}O_5$ $(M+H)^+$ 272 *m/z* (rel. int., %): 254 (3.97), 180 (4.76), 166 (19.84), 153 (84.13), 124 (19.05), 120 (50.79), 69 (100.00), 57 (88.89), 43 (89.25). Comparison with the reported data (Seidel *et al.*, 2000).

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Bioactive Constituents from Lengkuas Ranting (*Alpinia conchigera*)

Sukari, M.A.[1], Tang, S.W.[1], Rahmani, M.[1], Lajis, N.L.[1] and Ali, A.M.[2]

[1]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Faculty of Agriculture and Biotechnology, Universiti Darul Iman Malaysia, 20400 Kuala Terengganu, Terengganu, Malaysia
aspollah@science.upm.edu.my

Abstract

Zingiberaceae is one of the most important herbaceous species found in tropical forests which comprises about 52 genera with 1500 species in the world. Isolation work on crude extracts of *Alpinia conchigera* yielded *p*-hydroxycinnamaldehyde and β-sitosterol-3-*O*-β-D-glucopyranoside which are new to the species besides β-sitosterol. Extracts and *p*-hydroxycinnamaldehyde were subjected to cytotoxic testing against different cancer cell lines (HL-60, MCF-7, HeLa and HT-29) as well as antimicrobial testing. Crude hexane, chloroform and ethyl acetate extracts were strongly active against *Salmonella choleraesuis* in antimicrobial assay. In antifungal screening test, hexane extract showed slight inhibition on *Aspergillus ochraceaus* and *Saccharomyces cerevisiae* while ethyl acetate extract was active towards *S. cerevisiae*. All crude extracts except methanol extract exhibited very strong activity in cytotoxic screening test against HL-60 cell line (human promyelocytic leukemia) with IC_{50} values < 5 µg/mL. *p*- Hydroxycinnamaldehyde is cytotoxic against all cancerous cell lines tested and strongly active in antimicrobial testing.

Introduction

Zingiberaceae is one of the largest families of the plant kingdom (Sirirugsa, 1998). Zingiberaceae plants are well-known for its usage as food, spices, medicines, dyes, perfume, as well as ornamental. *Alpinia* is a genus of about 200 species of ginger-scented, rhizomatous perennials natives to Asia and Australia. *Alpinia conchigera* is one of the *Alpinia* species which is also known as joint-whip ginger, *lengkuas ranting*, *lengkuas genting, lengkuas kecil* or *lengkuas* padi. It is a species of herb about 2 to 5 feet tall found in eastern Bengal and southwards to the Malay Peninsula and Sumatra. The rhizome of *A. conchigera* is used for flavoring rice-spirit and food, while its fruits are eaten and medicinal (Burkill, 1966). The rhizome is stimulating, diaphoretic, regulating in uterine hemorrhage. External uses of this plant are used to treat rheumatism, arthritis, and as a poultrice after confinement (Sirirugsa, 1998). Hence, this research is intended to report on the isolation of bioactive constituents from the extracts of *A. conchigera*.

Materials and methods

General

Melting points (uncorrected) were determined on Electrothermal IA 9000 series digital melting point equipment. Infrared spectra were recorded on Perkin Elmer FTIR model 1725X spectrophotometer using potassium bromide (KBr) discs. Mass spectra were recorded on equipped Shimadzu model QP5050A at 70 eV. ^{1}H and ^{13}C NMR spectra were obtained on JEOL FTNMR 400 and 100 MHz spectrometer, respectively with tetramethylsilane (TMS) as internal standard. Chemical shifts are given in δ (ppm) values relative to that of the solvents used. Column chromatography was carried using silica gel (Merck 7749 and 9385) and Merck DC Alufolien F_{254} was used for TLC analysis.

Plant material

The rhizomes of *A. conchigera* Griff. were collected from Kelantan in 2006. The plant was identified by Mr. Shamsul Khamis from Institute of Bioscience, Universiti Putra Malaysia.

Extraction

Finely ground air-dried rhizomes (1.35 kg) were extracted successively with hexane, chloroform, ethyl acetate and methanol, respectively. The extracts were filtered and concentrated under reduced pressure using rotary evaporator to give hexane extract (33.43 g), chloroform extract (54.07 g), ethyl acetate extract (15.00 g) and methanol extract (31.70 g). The crude extracts were further subjected to chromatographic isolation and bioactivity testing. Brown crystalline ethyl acetate extract (14.00 g) were subjected to vacuum column chromatography which were eluted with increasing polarity of hexane, hexane/ethyl acetate, ethyl acetate and ethyl acetate/methanol and methanol to give 100 fractions. Fraction 35-38 were combined and recrystallized with chloroform to give pale yellowish needles shaped-crystals of *p*-hydroxycinnamaldehyde **(1)** (0.136 g), $C_9H_8O_2$, m.p. 140.0-141.0 °C (lit. 140 °C (Naidoo *et al*., 1992)). Fraction 89-90 afforded β-sitosterol-3-*O*-β-D-glucopyranoside **(2)** (0.015 g) as white amorphous powder from the same column after washing the fractions (0.373 g) with acetone.

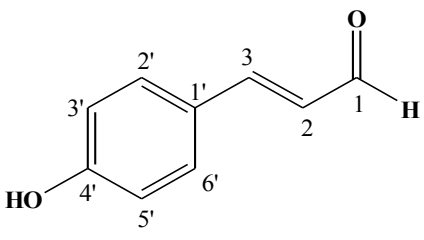
Bioassay

The HL-60 (human promyelocytic leukemia), MCF-7 (human breast cancer), HT-29 (human colon cancer) and HeLa (human cervical cancer) cell lines were procured from the American Type Cell Culture Collection (ATCC), USA. The concentration of samples were prepared in 100 mg/mL (crude extracts) and 10 mg/mL (pure compounds) for antimicrobial testing while solution of extracts in concentration of 10 mg/mL (crude extracts) and 1 mg/mL (pure compounds) was used for cytotoxic screening. Absolute ethanol was used in the dilution of crude extracts. Cytotoxic testing was carried out using MTT assay as described in Mosmann, 1983 while antimicrobial testing was done based on disc diffusion method in literature (Bauer *et al*., 1997).

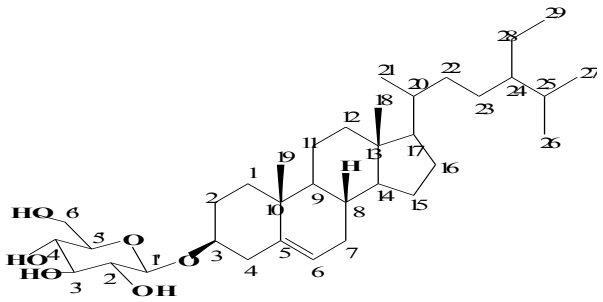
Results and discussion

Compound **(1)** was isolated as pale yellowish needles shaped-crystals from ethyl acetate and chloroform extracts of *A. conchigera* with melting point of 140.0-141.0 °C (lit. 140 °C (Naidoo *et al*., 1992)). The EI-MS spectrum gave a molecular ion peak at *m/z* 148 which correspond to the molecular formula of $C_9H_8O_2$. The ^1H and ^{13}C NMR spectral data together with the molecular formula have identified the compound as *p*-hydroxycinnamaldehyde, previously isolated from *A. galanga* (Barik *et al*., 1987) from the same genus.

Compound **(2)** was obtained as white amorphous powder and correspond to molecular formula of $C_{35}H_{60}O_6$. The molecular ion peak for compound **(2)** which is suggested to be at *m/z* 576 could not be identified in the EI-MS spectrum. However, the fragment ions at *m/z* 414 $[M\text{-}C_6H_{10}O_5]^+$, 396 $[M\text{-}C_6H_{10}O_5\text{-}H_2O]^+$, 255 $[M\text{-}C_6H_{10}O_5\text{-}H_2O\text{-}C_{10}H_{21}$ (side chain)$]^+$ and *m/z* 127, 73, and 57 which were obtained from the sugar moiety suggested that compound **(2)** may be monoglycoside with a C_{29}-sterol (*m/z* 414) aglycone moiety. From the evidences in spectral data and comparison with reported data (Mizushina *et al*., 2006), compound **(2)** was therefore established as β-sitosterol-3-*O*-β-D-glucopyranoside.



(1)



(2)

All crude extracts except methanol extract exhibited very strong inhibitory activity in cytotoxic screening test against HL-60 cell (human promyelocytic leukemic) with IC_{50} values <5 µg/mL. Hexanes extract showing the most outstanding activity with IC_{50} 0.9 µg/mL, followed by chloroform and ethyl acetate extracts with 1.9 and 3.0 µg/mL, respectively. *p*-Hydroxycinnamaldehyde **(1)** exhibited interesting cytotoxic activity against four cancer cell lines tested, with most significant inhibition against HL-60 cell line (IC_{50} value = 5.2 µg/mL), followed by HT-29 (IC_{50} = 12.7 µg/mL), HeLa (IC_{50} = 12.8 µg/mL) and MCF-7 (IC_{50} = 22.3 µg/mL). Similarly, β-sitosterol-3-*O*-β-D-glucopyranoside **(2)** also demonstrated wide range of cytotoxic activity, with most pronounced activity against HeLa cell line (human cervical cancer), having IC_{50} value of 0.6 µg/mL comparable to tamoxifen (standard). This compound was also cytotoxic against MCF-7 and HL-60 cell lines, with IC_{50} values of 9.4 and 26.5 µg/mL, respectively. Extracts or isolated compounds which gives IC_{50} value <10 µg/mL, were considered to have significant cytotoxic activity (Mosmann, 1983). It can be suggested that compounds **(1)** and **(2)** are bioactive constituents which showed synergistic mechanism towards the activity of extracts. Goniothalamin, tamoxifen and 5-fluorouracil were used as positive control for the respective cancerous cell line in the cytotoxic testing.

The crude extracts and pure compound of *A. conchigera* Griff. were subjected to antimicrobial test against four bacteria: Methicillin Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Salmonella choleraesuis*, *Bacillus subtilis*, with streptomycin and ampicillin as positive control. Crude hexane, chloroform and ethyl acetate extracts of *A. conchigera* showed very strong inhibition against *Salmonella choleraesuis* with inhibition zones of 21, 25 and 20 mm, respectively. However, the extracts were not active against three other microbes tested, except for ethyl acetate extract which showed moderate inhibition against *P. aeruginosa*. On the other hand, methanol extract did not show any significant activity in antimicrobial testing. As for antifungal screening result, crude hexane extract of *A. conchigera* was mildly active against *Aspergillus ochraceaus* and *Saccharomyces cerevisiae*, with inhibition zones of 6 mm and 9 mm respectively, whereas crude ethyl acetate extract of *A. conchigera* was moderately active against *Saccharomyces cerevisiae* with inhibition zone of 11 mm. None of the extracts were active towards inhibition of *Candida albicans*. Chloroform and methanol extracts were not active in antifungal testing in this study. Nystatin was used as positive control for antifungal testing. *p*-Hydroxycinnamaldehyde **(1)** demonstrated strong inhibition against all bacteria tested with inhibition zone > 22 mm but not active in antifungal testing. Hence, compound **(1)** is suggested to be contributing to the bioactive principles of the extracts of *A. conchigera*.

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Biological Promise of Bioactive Endophytic Streptomyces from Ethnobotanical Plants of the Malay Peninsula

Zin, N.M. and Norazli, G.

Department of Biomedical Science, Faculty of Allied Health Sciences,
Universiti Kebangsaan Malaysia, 50300 Kuala Lumpur, Malaysia
nora@medic.ukm.my

Abstract

Endophytic streptomycetes have been isolated and identified from ethno botanical plants of the Malay Peninsula. At least six plants species from the eastern and southern part harbored seven endophytic streptomycetes. The isolates were cultured on Starch casein agar supplemented with the antibiotic nalidixic acid and anti fungal Cycloheximide and Nystatin. All isolates were Gram positive with branching filaments. All isolates as viewed by scanning electron microscopy, have small hyphae, some produce typical barrel-shaped spores in culture and each has some unique hyphal surface structures. Molecular biological studies on the 16S rRNA gene sequence of each isolate revealed that SUK 01, SUK 02 and SUK 03 were 96%-98% similar while SUK 04, 05, and 06 were 98% similar to *Streptomycetes* in the Gene Bank. SUK 02 was antagonistic to *Pseudomonas aeruginosa* ATCC 27853, *Enterococus fecalis* and *Pleisiomonas shigelloide*. Nevertheless, SUK 06 was inhibitory to *Pseudomonas aeruginosa* ATCC 27853, *Staphylococus aureus* ATCC 25923, *Pleisiomonas shigelloides*, *Bacillus subtilis*, MRSA ATCC 700699 and *B. cereus* ATCC 6464. The anti bacterial activity of SUK 06 was extractable in various organic solvents and three fractions were acquired. The MIC values for each fraction A, B and C was 0.075, 1.25 and 0.625 mg/ml, respectively.

Introduction

Endophyte is any microorganisms live in the intercellular spaces of plant tissues. Most commonly found was fungi and actinomycetes family which some of them do influence the resistance of host toward infection. The Malay peninsula, a great biodiversity in the world might contribute huge potential for new endophytes finding. The advent of drug resistance in most bacterial pathogens and the current increase in the number of fungal infections has caused a resurgence of interest in finding other reserves of biologically active compounds from the streptomycetes, in which nearly 80% of all of the world's antibiotics produced by this bacteria (Cao *et al*., 2004). A search reveals that all of these organisms have their origins in the soil. One biologically important niche that has been overlooked as a source of novel streptomycetes is the plant kingdom (Castillo *et al*., 2006). Presumably, the simplest biological arrangement between these organisms is that the plant provides nutrition for the microbe and the microbe provides some form of protection for the plant. Also, since the endophytic streptomycete is associated with a eukaryotic organism, the possibilities of it making products that are not toxic to its host organism seem more likely. Thus, one of the major concerns in drug discovery, relating to the toxicity of a drug candidate, may be averted by dealing with endophytic streptomycetes and their biologically active products. In this study, the isolation of endophytic Streptomyces from selected medicinal plants were carried out. The Scanning electron microscope and 16S rDNA analysis were developed for identification. In addition, antimicrobial activity of the isolates were assessed against the pathogenic bacteria.

Materials and methods

Plant collection

Plant samples were selected for isolation of endophytic *Streptomyces sp.* based on traditional medicine usage such as for post partum tonic, curing fever and malarial infection. The plants species collected from eastern and southern part of the Malay peninsula were *Thottea grandiflora*, *Mapania* spp., *Polyalthia* spp., *Pyllanthus amarus*, *Senna alata* and *Melastoma malabathricum*. Stems with length of approximately 10 cm was cut, tagged and stored in a clean plastic bag.

***Isolation and storage of endophytic Streptomyces* sp.**

The surface of each stem was surface sterilized by sequential immersion in 70% alcohol for 5 min, 0.9% NaOCl for 10 min and 95% alcohol for 30 s (Zin *et al*., 2007). The bark of the stems was removed aseptically and the inner tissues were cut into small pieces (0.5x0.5 cm). The tissue was then placed onto starch casein agar (SCA) supplemented with 50 µg/ml cycloheximide, 50 µg/ml nyastatin and 20 µg/ml nalidixic acid. The Petri dish was sealed and incubated at 28ºC and then recultured on International Streptomyces Project medium (ISP 2) agar. The organisms tentatively identified as actinomycetes, produced an earthy odor in culture, are potentially Streptomycetes. They were then cultured on nutrient agar and stored in sterile 15% glycerol in water at -70ºC.

Results and discussion

Based on identification of 16S rDNA we conclude that the isolated endophytic organisms were closely related with *Streptomyces* sp. It is shown that the SUK 01 has 94% similarity with *Streptomyces fulvoviolaceus,* SUK 02 93% similar to *Streptomyces caelestis*, SUK 03 96% similar with *Streptomyces coelicolor,* SUK 04 was 98% similar with *Streptomyces albus,* SUK 05 98% similar with *Streptomyces albidoflavus,* SUK 06 has 98% similarity to *Streptomyces misawanensis* and SUK 07 has 99% similarity with *Streptomyces albus.* Interestingly, although the plants used for isolation of *Streptomyces* SUK 04 and SUK 07 was different, they seemed to harbor similar streptomycetes (Table 1)

The antibacterial activity was done to all the isolates but only SUK 02 and SUK 06 have some antibacterial activities (Table 2). The SUK 02 did have activities towards both Gram positive and negative bacterial. While SUK 06 inhibited all Gram positive bacteria and not the Gram negatives with exception of *Pleisiomonas shigelloides*. Other isolates did not display antibacterial activities but in our previous work SUK01, SUK 02 and SUK 03 did have some activities towards plant pathogenic fungi (Zin *et al*. 2007b). The MICs value for each fraction A, B and C were obtained as 0.075, 1.25 and 0.625 mg/mL, respectively. With current information, we could not declare the relationship between medicinal properties of the plant with the presence of these bioactive streptomycetes. However this work does give us some information about the bio activity of endophytic streptomycetes and it may serve as biological control or as new drugs to be used in medicine (Ezra *et al*. 2004; Castillo *et al*. 2006).

Table 1. Identification of isolated Streptomyces based on partial sequencing of the 750 bp region of 16S rDNA gene sequence

Streptomyces isolate	Closely related streptomycetes	% similarity to rRNA gene sequence	Score bit
SUK 01	*Streptomyces fulvoviolaceus*	94	655
SUK 02	*Streptomyces caelestis*	93	648
SUK 03	*Streptomyces coelicolor*	96	669
SUK 04	*Streptomyces albus*	98	1026
SUK 05	*Streptomyces albidoflavus*	98	1203
SUK 06	*Streptomyces misawanensis*	98	2503
SUK 07	*Streptomyces albus*	99	1269

Table 2 Antibacterial assay of *Streptomyces* SUK 02 and SUK 06 towards human pathogenic bacteria

Test bacteria	Diameter of inhibition zone (mm)	
	SUK02	SUK 06
Pseudomonas aeruginosa ATCC 27853	18	20
Staphylococcus aureus ATCC 25923	0	23
Bacillus subtilis	0	18
Bacillus cereus ATCC 6464	N/A	30
Enterococcus fecalis	18	0
Salmonella aberdeen NCTC 74	0	0
Escherichia coli NCTC 11061	0	0
Plesiomonas shigelloides	24	22
Methichilin resistant Staphylococcus aureus ATCC 700699	0	18

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Cytotoxic and Anti-human Papillomavirus Activities of Methanol and Dichloromethane Extracts from Selected Polyporales

Nurhayati, Z.A.[1], Noorlidah, A.[1], Razif, D.[1], Lau, C.C.[1], Sabaratnam, V.[1] and Lai, L.K.[1]

[1]Institute of Biological Sciences, Faculty of Science, University of Malaya,
50603 Kuala Lumpur, Malaysia
dr.nurhayati@gmail.com

Abstract

In Malaysia, cervical cancer ranks as the second most common neoplasia in women. Human papillomavirus (HPV) type 16 is the major causative agent for cervical cancer. The E6 region of the HPV 16 genome encodes for an oncoprotein directly involved in the carcinogenesis of the disease. The E6 protein is abundant in cervical cancer and cervical cancer-derived cell lines. Realizing such importance of the E6 oncoprotein, this study was undertaken to screen for natural products which show cytotoxic activity against cervical cancer-derived cell lines – CaSki and HeLa – and suppress the expression of HPV 16 E6 oncoprotein in CaSki. Methanol and dichloromethane crude extracts from the mycelia of selected Polyporales, namely *Polyporus grammocephalus* (KUM60500), *Coriolus* sp. (KUM50012) and *Trametes* sp. (KUM50035) were prepared. The cell lines were first treated with crude extracts at varying concentrations. *In vitro* neutral red (NR) cytotoxicity assay showed that only dichloromethane extract of *P. grammocephalus* (KUM60500) showed potent activity against HeLa with an ED_{50} value of 10 µg/ml. ED_{50} denotes the concentration of extract that inhibits the growth of 50% of the cells. Crude extracts with ED_{50} values ≤ 20 µg/ml are considered active. Immunocytochemistry technique was performed to analyze the expression of the HPV 16 E6 oncoprotein in CaSki cells pretreated with the Polyporales extracts. Reduction in the expression of E6 protein was observed, with the best suppression activity seen in cells treated with dichloromethane extract of *P. grammocephalus* (KUM60500). These findings suggest that *P. grammocephalus* (KUM60500), *Coriolus* sp. (KUM50012) and *Trametes* sp. (KUM50035) have promising potentials to be used as therapy for cervical cancer.

Introduction

Cervical cancer ranks as the second most common female cancer worldwide (Qiu *et al.*, 2007) and in Malaysia (Lim and Halimah, 2004). Human papillomavirus (HPV), particularly subtypes HPV 16 and HPV 18 is well established as the prevailing cause of cervical cancer and its precursor lesions (zur Hausen, 1989).

The E6 region of these high risk HPV genomes encodes for the E6 oncoprotein which is directly involved in cellular transformations of the cervical epithelia leading to neoplasm (Chan *et al.*, 1989). The E6 oncoprotein is highly expressed in cervical cancer and HPV-containing, cervical cancer-derived cell lines such as HeLa and CaSki (Pfister, 1987). The E6 oncoprotein mediate cell transformations by forming complexes with the cellular tumor suppressor protein, p53 (Werness *et al.*, 1990). When functioning normally, the p53 protein negatively regulates cell growth. Complex formation of the E6 protein and p53 stimulates rapid degradation of the latter, abrogating its function in the nucleus leading to uncontrolled cellular proliferation, chromosomal instability and aneuploidy (Scheffner *et al.*, 1990; Lechner *et al.*, 1992).

Mushrooms have been used in folk medicine since ancient times. Their medicinal properties have been increasingly recognized through scientific research. The family Polyporaceae contains many secondary metabolites which have been isolated and tested positive for medicinal properties (Quang *et al.*, 2006). Mushrooms of the genus *Polyporus* has been shown to possess antioxidative activities (Sekiya *et al.*, 2005). Sun and Liu (2008) have shown that *Polyporus albicans* possess immunomodulatory activities. Another mushroom identified as *Coriolus versicolor* also expressed similar physiological effects i.e. immunomodulating activities (Cui and Chisti, 2003) and anti-proliferative effects against several liver and breast cancer cell lines (Zhou *et al.*, 2007). Majcherczyk *et al.* (1998) found that laccase enzyme in *Trametes versicolor* possess oxidative activities towards

polycyclic aromatic hydrocarbons (PAH), a well known chemical carcinogen. Previous findings as such calls for more exploration on other bioactivities which members of the Polyporales may possess.

Realizing the importance of HPV 16 and HPV 18 in the development and progression of cervical cancer, the present study was undertaken to screen natural products from selected Polyporales, namely *P. grammocephalus* (KUM60500), *Coriolus* sp. (KUM 50012) and *Trametes* sp. (KUM 50035) which (a) exhibit cytotoxic activity against HPV-containing, cervical cancer-derived cell lines, CaSki and HeLa and (b) suppress the expression of the HPV 16 E6 oncoprotein in CaSki cells.

Materials and methods

Preparation of extracts

Mycelium cultures of selected Polyporales - *P. grammocephalus* (KUM60500), *Coriolus* sp. (KUM 50012) and *Trametes* sp. (KUM 50035) – were grown under static condition in glucose-yeast-malt-peptone (GYMP) liquid medium for 14 days at 25^{o}C. The mycelial culture and broth were then freeze-dried and soaked in methanol for 2 days. The contents were filtered and residue further soaked in dichloromethane for 2 days. The filtrates were dried using rotary evaporation. Extract stocks were prepared, consisting of 0.02 g of each extract diluted in 1 ml dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

Neutral Red (NR) cytotoxicity assay against CaSki and HeLa cell lines

The NR cytotoxicity assay was conducted based on the method developed by Borenfruend and Puerner (1984). CaSki and HeLa cells [American Type Culture Collection (ATCC)] were cultured in 10% Roswell Park Memorial Institute (RPMI) 1640 media (Sigma-Aldrich) and 10% Dulbecco's Modified Eagle's Media (DMEM) (Sigma-Aldrich), respectively. Cells were placed in wells of 96-well microtiter plates (Nunclon) for 3 hours. Extracts were then added to the cells to yield a final concentration 20 µg/ml. Incubation was carried out for 72 hours at 37ºC. Cells not treated with any extract served as the negative control. After the incubation period, the culture media was replaced with NR dye (Sigma-Aldrich) (200 µl per well) and further incubated for 3 hours at 37ºC to allow maximum uptake of the dye by viable cells. Subsequently, the cells were washed with NR washing solution. The NR dye was extracted from viable cells with NR extraction solution (200 µl per well) and the microtiter plate was left to stand for 30 minutes at room temperature with gentle agitation. Optical density (OD) was determined spectrophotometrically at 540 nm using an ELISA reader. All tests were performed in triplicates. The average data were expressed in terms of percentage of killing relative to negative controls. Extracts giving percentage of killing of at least 50% was further evaluated for cytotoxic activity at varying concentrations of 1 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml.

Anti-human papillomavirus (HPV) 16 E6 oncoprotein assay in CaSki cell lines using polymer-based immunocytochemistry technique

CaSki cells were cultured using 10% RPMI 1640 media. The extract stocks were serially diluted with sterile distilled water. The diluted extract stocks were added to CaSki cell suspension in tissue culture flasks to give final concentrations of 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml. Incubation proceeded for 72 hours at 37ºC. CaSki cells not treated with any extract served as the negative control. The cells were harvested and fixed on 12-well teflon-coated slides. Expression of HPV 16 E6 oncoprotein in these cells were analyzed immunocytochemically using the anti-HPV 16 E6 monoclonal antibody (Chemicon). A polymer-based immunocytochemistry protocol – EnVision™+ (Dako) staining method was employed. All tests were done in triplicates.

Results and discussion

Neutral Red (NR) cytotoxicity assay against CaSki and HeLa cell lines

Crude methanol and dichloromethane extracts of the *P. grammocephalus* (KUM60500), *Coriolus* sp. (KUM 50012) and *Trametes* sp. (KUM 50035) were evaluated for their cytotoxic activities against

CaSki and HeLa cell lines using the *in vitro* NR cytotoxicity assay. The average data from triplicates were expressed in terms of percentage of killing relative to negative controls.

Table 1. Percentages of killing of CaSki and HeLa cell lines by 6 Polyporales extracts at 20 µg/ml

Extract	% of killing on HeLa cells	% of killing on CaSki cells
Methanol *P. grammocephalus* (KUM60500)	25.6	9.9
Methanol *Coriolus* sp. (KUM 50012)	23.8	13.5
Methanol *Trametes* sp. (KUM 50035)	45.3	25.1
Dichloromethane *P. grammocephalus* (KUM60500)	58.3	33.0
Dichloromethane *Coriolus* sp. (KUM 50012)	22.0	14.2
Dichloromethane *Trametes* sp. (KUM 50035)	41.3	21.9

All crude extracts tested showed varying degrees of cytotoxic effects on CaSki and HeLa cells at 20 µg/ml (Table 1). However, percentage of killing ≥ 50% was observed only for dichloromethane extract of *P. grammocephalus* (KUM60500) on HeLa cells. This extract was further evaluated for its cytotoxic activity at varying concentrations of 1 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml. The dose-response curve for the extract was then plotted to obtain the ED_{50} value (Fig. 1). ED_{50} value refers to the concentration of extract (µg/ml) that inhibits 50% of cell growth.

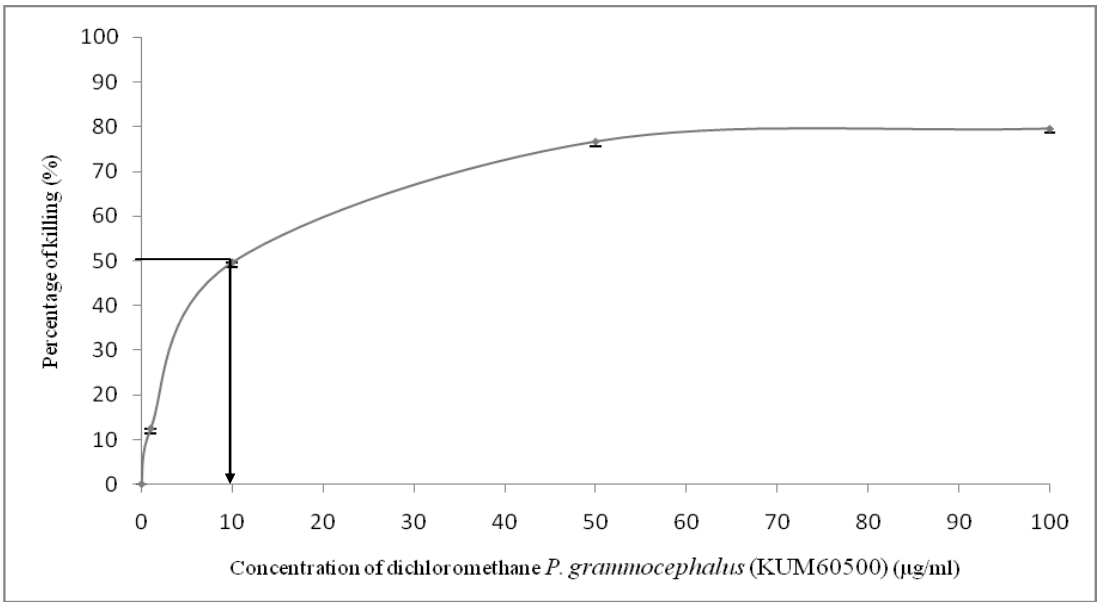

Fig. 1. Concentration of the dichloromethane *P. grammocephalus* (KUM 60500) extract against the percentage of killing of HeLa cells. The values show the percentage of inhibition ± SEM. ED_{50} was extrapolated from the graph.

An extract that show an ED_{50} value ≤ 20 µg/ml is deemed actively cytotoxic (Geran *et al.*, 1972). The ED_{50} value for the dichloromethane extract of *P. grammocephalus* (KUM60500) was extrapolated at 10 µg/ml. This extract is therefore regarded as actively cytotoxic against HeLa cells. The trend observed was that the percentage of killing increased with increasing concentrations of the extract. However, the mechanism of action has not been investigated in the present study. Limited publications were found to connect *P. grammocephalus* (KUM60500) to bioactivities, although – ergone, a secondary metabolite extracted from crude methanol extract of *Polyporus umbellatus* expressed an ED_{50} value of 26.3 ± 0.93 µg/ml when tested against HeLa cells (Lee *et al.*, 2005).

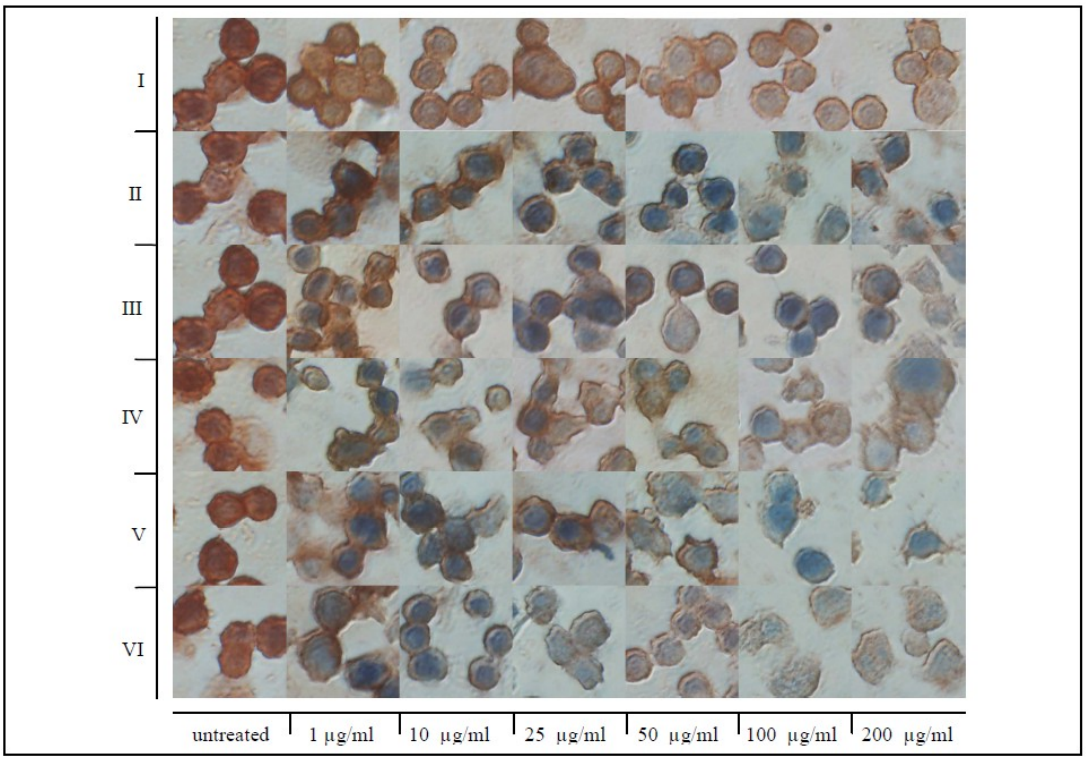

Fig. 2: Appearance of untreated CaSki cells and CaSki cells treated with methanol extract of *P. grammocephalus* (KUM60500) (I), dichloromethane extract of *Trametes* sp. (KUM 50035) (II), methanol extract of *Trametes* sp. (KUM 50035) (III), methanol extract of *Coriolus* sp. (KUM 50012) (IV), dichloromethane extract of *Coriolus* sp. (KUM 50012) (V) and dichloromethane extract of *P. grammocephalus* (KUM60500) (VI).

Anti-human papillomavirus (HPV) 16 E6 oncoprotein assay in CaSki cell lines using polymer-based immunocytochemistry technique

The CaSki cells treated separately with various concentrations of crude methanol and dichloromethane extracts of *P. grammocephalus* (KUM60500), *Coriolus* sp. (KUM 50012) and *Trametes* sp. (KUM 50035) were analyzed for the expression of HPV 16 E6 oncoprotein. The E6 oncoprotein was recorded as present when reddish-brown stain was clearly observed in the nuclear and/or cytoplasmic regions. The appearance of CaSki cells before and after treatment with the extracts are shown in Fig. 2.

In general, reduction of the intensity of the reddish brown stain was observed in cells treated with all extracts, indicating suppression of the HPV 16 E6 oncoprotein. The suppression of the E6 oncoprotein seem to increase with increased concentrations of the extracts. All extracts except the methanol extract of *P. grammocephalus* (KUM60500) produced good suppression of the HPV 16 E6

oncoprotein. Observations showed that the dichloromethane extract of *P. grammocephalus* (KUM60500) exhibited the best HPV 16 E6 suppression activity, followed by dichloromethane extract of *Coriolus* sp. (KUM 50012), methanol extract of *Coriolus* sp. (KUM 50012), methanol extract of *Trametes* sp. (KUM50035) and dichloromethane extract of *Trametes* sp. (KUM50035).

Conclusions

In the present study, the dichloromethane extract of *P. grammocephalus* (KUM60500) has shown excellent cytotoxic activity against HeLa cells and HPV 16 E6 suppression activity in CaSki cells. Good suppression of HPV 16 E6 oncoprotein was also exhibited by methanol and dichloromethane extracts of *Coriolus* sp. (KUM 50012) and *Trametes* sp. (KUM 50035). Since the E6 oncoprotein has been found to play such an important role in cellular transformation of the cervical epithelia leading to neoplasm, suppressing it is crucial in order to slow down, if not halt the development and progression of cervical cancer. These findings show that *P. grammocephalus* (KUM60500), *Coriolus* sp. (KUM 50012) and *Trametes* sp. (KUM 50035) indeed have promising potentials as therapy for cervical cancer.

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Sustainable Anti-MRSA *Gracilaria changii* Extract through Molecular Biotechnology Approach

Mariana Nor, S.[1,2], Haslinda Ayu, M.Y.[2] and Habsah, M.[3]

1Department of Medical Microbiology and Parasitology,
Faculty of Medicine and Health Sciences,
2Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
3Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia
haslindaayu_my@yahoo.com

Abstract

Global bacterial infection management has a universal trend involving the fight against antibiotic resistant strains. The pressing microbial resistance problem incurred high infectious disease burden and cost constraints, requiring application of newer, more expensive agents. An approach for treatment against an antibiotic resistant pathogen, MRSA, involving a combination therapy is investigated. In the present study, synergy was determined by the checkerboard assay. The effect in combination of commercial antibiotic and seaweed extract were screened on MRSA strain. Following the disc diffusion test, the checkerboard assay, giving the FIC index of 0.93 indicate that the seaweed extract works in synergy with commercial antibiotics and it has the potential as a resistance modulator. In view of the fact that there was positive effect of the extract, further study were conducted molecularly on several genes to study the effect of the extract in combination with antibiotic toward the selected genes through RT-PCR analysis as compared to effect of the extract alone. The synergy effect on resistant genes of MRSA, namely mecA and mecI showed different nucleotide sequence changes as compared to untreated. The promising application of local *G. changii* as an antiMRSA agent is an added value to mega-diversity tropical flora.

Introduction

Staphylococcus aureus is a major pathogen that causes a wide spectrum of clinical manifestation such as wound infection, septicemia, and endocarditis. It is treatable by the use of antibiotic. However, these pathogens have shown the ability to develop resistance to commonly used antibiotics and resulted in the emergence of antibiotic resistance strains which are more virulent. The phenomenon leads to the increment of morbidity and mortality rate as well as healthcare cost due to treatment failure. In Malaysia the prevalence of MRSA infection is about 0.23% from 5% to 10% of total cases of nosocomial infection upon admission it is predicted to increase due to the emergence of new strains that resistant against other antimicrobial agents including vancomycin that is now the drug of choice in treating MRSA infection. The resistance trend has alarmed the realization of the need to find new antibiotics against this pathogen. Other than that, the development of new antibacterial agent is needed in order to produce antibiotics, which are inexpensive and safe for human use. As a result, there is high demand from the world biodiversity resources in the screening program seeking for the therapeutic drugs from natural products including the marine organism (Del Val *et al.,* 2001). Diverse marine tropical seaweeds could provide alternative compounds that have antibacterial potentials through application of molecular biotechnology approach. The aim of this study is to assess the antimicrobial effects of crude extracts of local seaweed on MRSA strains as well as to evaluate its effect in combination with antibiotic in order to full fill the high demand of alternative treatment for improving drug development outcome.

Materials and methods

Antimicrobial agents

A standard laboratory powder of penicillin was used in this study. The seaweed extract (*G. changii*) was obtained from Universiti Malaysia Terengganu. Stock solutions of antimicrobial agents were prepared by dissolving the commercial antibiotics in sterile water and in 40% methanol for seaweed extract. All the stock solutions were stored in 4˚C and diluted prior to use.

Susceptibility testing

Antimicrobial effect of seaweed extract was screened through the disc diffusion test. Commercial antibiotic disc as well as the solvent, 40% methanol were used as control. The test was followed by the MIC determination of antimicrobial agents by broth microdilution technique (Tamakou *et al.,* 2008).

Synergy testing

The evaluation of synergy effect between commercial antibiotic and seaweed extract were obtained by several techniques (Balke *et al*., 2006). The techniques include disc diffusion and checkerboard test. In the disc diffusion test, the synergy effect was evaluated by comparing the size of the inhibition zones in plates containing seaweed extract and in control plates without seaweed extract. In the checkerboard test, 96 wells microtiter plate was filled with penicillin at various concentrations ranging from 40 mg/µl to 0.625 mg/µl and seaweed extract at concentrations that ranged from 4x MIC to 1/16x MIC. Each microtiter well was then inoculated with 100 µl of a bacterial inoculum of approximately $5x10^5$ CFU/ml, which was verified by colony counts. Both the microtitter and the agar plates were incubated at 37°C for 24 h. The interaction between antimicrobial agents was measured by the fractional inhibitory concentration index (FIC) by the formula FIC index = (MIC of penicillin in combination/MIC of penicillin alone) + (MIC of the seaweed extract in combination/ MIC of the seaweed extract alone). Synergism was defined as an FIC index of ≤0.5, additivity was defined as an FIC index of >0.5 ≤ 1, indifference was defined as an FIC index >1 ≤2 and antagonism was defined as an FIC index of >2 (Balke *et al*., 2006).

RT-PCR

Partial expression of bacterial target genes after treatment was determined using RT-PCR analysis. Total RNA was extracted from treated and untreated MRSA using RNA Extraction kit (Master Pure, Epicenter). The total RNA was then converted to cDNA using Monster Script (Epicenter) according to manufacturer's instruction. PCR were carried out in a total volume of 25 µL based on methods outlined by Katayama *et al* (2001). The RT-PCR product was run on 1.2% agarose gels and stain with ethidium bromide and viewed under UV light (Image Analyzer, Image Master). The bands of interest were extracted with QIAquick gel extraction kit (Qiagen) and sent for commercial sequencing

Results and discussion

Susceptibility testing

The disc diffusion test showed present of inhibition activity, which was indicated by the clear zones surrounding the discs that were impregnated with the seaweed extract. The MICs of penicillin was at 20mg/ml. As for the seaweed extract, the MICs were at 90mg/ml. The finding suggests that the seaweed extracts used in this study had antimicrobial effect towards MRSA even though the MIC dosage is higher than penicillin.

Synergy

In the combination treatment, the synergy effect was seen when clear zones around penicillin disc-seaweed impregnated in MRSA plates was observed. Therefore, checkerboard test was performed to test for combinations of two antimicrobial agents at different concentrations to determine whether the combination is more or less active than either drug given alone. The viable count after 24 h after treatment revealed the FIC index of 0.93 which is defined as having an additive effect (Balke *et al*., 2006). However the interpretation of drug interactions according to Berenbaum (1978), which has often been cited in dental investigations defines synergy to occur when FIC <1.0 and antagonism when FIC >1.0, additivity occurs when FIC = 1.0. When these evaluation criteria were applied to the present results, the antibacterial combinations showed synergy.

RT-PCR

mecA and mecI gene were successfully amplified as shown in Fig. 1. The sequencing results showed changes in the nucleotide sequences of mecA and mecI genes of treated bacterial strains (Fig. 2). The changes was greatly seen in C3 which was treated with high concentration of both penicillin and seaweed extract. These findings indicate that the combination treatment of seaweed extract and antibiotic have the ability to alter the transcription of resistance genes in MRSA. Further evaluation of synergy mechanism between antibiotic and seaweed extract will be perform *in vivo.* The effect of inhibition suggests the valuable potential in combination therapy in treatment of MRSA.

Fig. 1. PCR products of (A) mecA gene-588bps and (B) mecI gene 481bps.

```
mecA_G2          TGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAACAA
mecA_B7          TGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAACAA
mecA_C1          TGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAACAA
mecA_untreated   TGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCAACAA
mecA_genebank    TGGAAGAAAAATATTATTTCCAAAGAAAATATCAATCTATTAACTGATGGTATGCCACAA
mecA_C3          TGGAAGAAAAATATTATTTCCAAAAAAAAAAGCTTCCAATGATTGGTTGATGTGCAGCAA
                 ************************ **** * *   * ** *   * ** * ***  ***

mecI_C3_         TCGTAATAAAGACAATAAAATTTTTCAATATTACTCTCTTGTAGAAGAAAGTGATATAAA
mecI_C1          TCGTAAAAAAGACAATAAAATTTTTTAATATTACTCTCTTGTAGAAGAAAGTGATATAAA
mecI_G2_         TCGTAAAAAAGACAATAAAATTTTTTAATATTACTCTCTTGTAGAAGAAAGTGATATAAA
mecI_genebank    TCGTAAAAAAGACAATAAAATTTTTTAATATTACTCTCTTGTAGAAGAAAGTGATATAAA
mecI_B7_         TCGTAAAAAAGACAATAAAATTTTTTAATATTACTCTCTTGTAGAAGAAAGTGATATAAA
                 ****** ****************** **********************************
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Fig. 2. Alignment of sequencing results of treated *S. aureus* and gene bank sequence of mecA and mecI genes.

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We would like to thank to all who are involved directly or indirectly with this project especially the Microbiology Research Laboratory members, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia and NBD IRPA grant, 54818

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Isolation and Characterization of Angiotensin Converting Enzyme (ACE) Inhibitory Compound derived from *Tacca integrifolia*

Farah, L. and Jamaludin, M.

Institute of Biological Sciences, Faculty of Science, University Malaya, 50603 Kuala Lumpur, Malaysia
jamal@um.edu.my

Abstract

Tacca integrifolia from the family of Taccaceae locally known as *belimbing tanah* has been used traditionally to treat high blood pressure related disease. However, no scientific research has been carried out to evaluate its effectiveness for antihypertension treatments. The bioactive compounds from the leaves and rhizomes of *T. integrifolia* were extracted with hexane, petroleum ether, methanol, chloroform and water. The present of phenols, terpenoids and alkaloids were detected with phenol, vanillin and dragendorrfs reagents respectively. The crude extract of leaves and rhizomes shows more than 50% ACE inhibition. These findings provide evidence that the crude extract contains antihypertensive active compounds. Further research is still in progress to evaluate for its antihypertensive action by oral administration in spontaneously hypertensive rats (SHR).

Introduction

Hypertension is one of the main causes for cardiovascular disease. However, it can be controlled by taking captopril that specifically inhibits Angiotensin Converting Enzyme (ACE), the major regulators of blood pressure. It acts mainly via the Renin Angiotensin System with Angiotensin I and bradykinin as the natural substrates. Angiotensin I is cleaved by ACE into Angiotensin II a potent vasoconstrictor, whereas bradykinin, which is a vasodilator is inactivated by ACE. Inhibition of ACE thus leads to decrease of blood pressure.

Inhibition of Angiotensin Converting Enzyme (ACE) is currently considered to be a useful therafeutic approach in the treatment of high blood pressure. It is also effective screening method in the search for new antihypertensive agents (Wagner, 1993, Hansen *et al*., 1996a,1996b, Somanadhan *et al*., 1999). By using an in vitro ACE-inhibition assay (Elbl and Wagner,1991; Wagner and Elbl,1992) anti-hypertension effect of *Tacca integrifolis* was studied using ACE extracted from rabbit lung and N-Hippuryl-His-Leu tetrahydrate (HHL) as specific substrate (Cushman and Cheung, 1971; Guan-Hong *et al*., 2005).

The genus *Tacca* consists of long-lived, short stemmed, rhizomatous or tuberous herbaceous plants. Asian *Tacca* have attractive, entire leaves, vertical growth habit, and strange whisker-like (filiform) bracts below the flowers. *T. integrifolia* has white bracts hovering over the nodding flowers. The bracts are beautifully veined with purple. The rhizome of this species grows vertically and the crown of large, attractive leaves emerge from the top of the rhizome. It has been used traditionally as preventing and treatment of high blood pressure.

Materials and methods

Plant material

The leaves and rhizomes of *T. integrifolia* was collected at the Field Study Center (FSC), Gombak, Selangor. The captopril was purchased from Merck; Hippuryl-L-Histidyl-L-Leucine (HHL) was purchased from Fisher Scientific. ACE was extracted from rats' lung. All chemical and solvents were of analytical grade and were purchased from Fisher Scientific.

Extraction and isolation

Oven dried and ground sample were extracted with hexane, petroleum ether, chloroform, methanol and water. The plant chemical compounds were detected using Thin Layer Chromatography (TLC) with chloroform and chloroform-ethanol (9.7:0.3) as solvent.

Determination of ACE activity

ACE inhibitory activity was determined by a modification of the method of Cushman and Cheung, 1971. The reaction mixture contain 0.25ml test sample solution, 0.25 ml 100 mM phosphate buffer pH 8.3, 0.25 ml 300 mM NaCl, 0.25 ml 5 mM HHL and 0.25 ml crude enzyme and incubated for 60 min at 37^{o}C. The reaction was terminated by adding 0.25 ml 1 N HCl. The hippuric acid formed by the action of ACE was extracted into 3ml of ethyl acetate and transferred into crucible. It was then evaporated to dryness at 80^{o}C. The residue was dissolved in 3ml of distilled water and the absorbance was measured at 228nm against distilled water.

The percentage of ACE inhibition was calculated as follows:

$$\% \text{ ACE inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

Result and discussion

All of the crude extracts from leaves and rhizomes (Table 1) show that more than 50% ACE inhibitions which suggested that it contains bioactive compound for lowering high blood pressure and be able to maintain normal level of blood pressure.

The presence of bioactive compounds such as phenols, terpenoids and alkaloids were detected with phenol, vanillin and dragendorffs reagents respectively. The Isolated bands from hexane, petroleum ether and chloroform of leaves crude extracts exhibit ACE inhibition but not with the methanol extract (Table 2). Whereas the isolated bands of rhizomes crude extracts shows ACE inhibition in hexane, petroleum ether, chloroform and methanol extraction (Table 3). This indicated that rhizomes is more effective for ACE inhibition rather than the leaves of *T. integrifolia*. However, the crude extracts of leaves shows slightly higher ACE inhibition compared with rhizomes (Fig. 1). This could be due to the antagonists' effects of the chemical compounds presents in the crude extracts that give high ACE inhibition. Research is still in progress to investigate further the effectiveness of *T. integrifolia* as antihypertension agents.

Table 1. Percentage of ACE inhibition from leaves and rhizomes crude extract of *T. integrifolia*

Plant parts	Hexane	Pet-ether	Chloroform	Methanol	Water
Leaves	76.48	68.6	74.48	74.79	68.79
Rhizomes	68.57	69.88	59.02	66.98	70.52

Table 2. Percentage of ACE inhibition of isolated compounds from leaves crude extract of *T. integrifolia*

Isolated Compounds	Hexane	Pet-ether	Chloroform	Methanol
P1	13.54	5.23	-	-
P2	-	-	-	-
P3	-	74.31	76.46	26.31
P4	12.46	-	5.08	-
P5	78	78.31	-	-
P6	-	9.38	-	-
P7	76	5.38	-	-
P8	53.77	39.85	-	-
P9	72	-	56.92	-
P10	-	-	30.77	-

Table 3. Percentage of ACE inhibition of isolated compounds from rhizomes crude extract of *T. integrifolia*

Isolated Compounds	Hexane	Pet-ether	Chloroform	Methanol
H1	66.15	59.08	77.38	25.85
H2	31.69	13.69	16	22.46
H3	83.54	9.08	43.34	7.38
H4	81.08	23.69	-	26.15
H5	-	6.92	-	-
H6	-	29.38	64.77	9.69
H7	-	-	-	0.46
H8	-	-	-	-
H9	-	-	-	-
H10	-	-	2.15	-

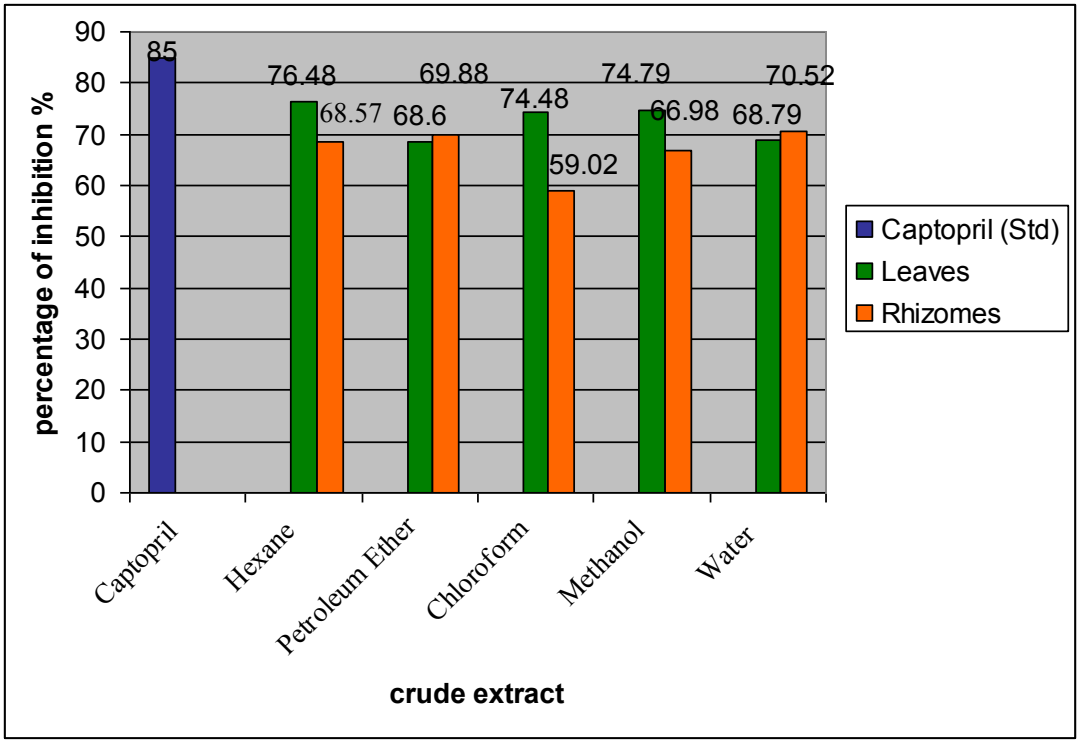

Fig. 1. Percentage of ACE inhibition of leaves and rhizomes crude extracts of *Tacca integrifolia.*

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Isolation, Structural Elucidation and Antimicrobial Activity of the Chemical Constituents of *Scaevola spinescens*

Mejin, M. and Pyke, S.M.

School of Chemistry and Physics, University of Adelaide, Adelaide, SA, 5005, Australia
michele@sbc.org.my

Abstract

Scaevola spinescens, an Australian indigenous plant has been used by the Aboriginal people in their traditional medicines for treating colds, stomach ache, urinary problems and pain in the alimentary tract, skin rashes, boils and sores. The purpose of this research is to isolate the chemical constituents of *S. spinescens* and to investigate their antimicrobial activity. The dried aerial parts were extracted sequentially in a Soxhlet apparatus with hexane, ethyl acetate, methanol and water. A water decoction was prepared as well. So far, six compounds have been isolated, three of which are known. The structures of these compounds were determined using 1D and 2D NMR, UV-Visible spectrometry, FTIR, mass spectrometry and optical rotation. The structures of the remaining three isolated compounds are yet to be determined. Organic and aqueous crude extracts of *Scaevola spinescens* were screened for antimicrobial activity against Gram-positive (*Staphylococcus aureus* ATCC25923 and *Streptococcus pyogenes* ATCC 10389) and Gram-negative (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) bacteria. Hexane, ethyl acetate, methanol and aqueous extracts were further investigated for antimicrobial activity. One of the isolated compounds has antimicrobial activity against Gram-positive bacteria with a minimum inhibitory concentration (MIC) ranging from 1.875 to 7.5 µg/ml and minimum bactericidal concentration (MBC) ranging from 3.75 to 15 µg/ml. No activity was observed against Gram-negative bacteria. Further investigation of the aqueous extract on the antimicrobial activity will be of interest as it may validate the remedy used by the Aboriginal people.

Introduction

Scaevola spinescens, an Australian indigenous plant, has been used by the Aboriginal people in their traditional medicines for treating colds, stomach ache, urinary problems and pain in the alimentary tract, skin rashes, boils and sores (Lassak; McCarthy, 1993). This plant is mostly found on sandy loams and hills in inland regions of Central and Western Australia as well as New South Wales (Carolin; Morrison; Rajput, 1992).

S. spinescens has been tested for anti-viral activity against a range of DNA and RNA virus (Semple; Reynolds, 1998). An extract of *S. spinescens* was found to be active against human cytomegalovirus (HCMV), a DNA virus (Semple; Reynolds, 1998).

An infusion of leaves and twigs of *S. spinescens* and *Codonocarpus cotinifolius* has been reputed to cure cancer. To verify the activity in the remedy used by the Aboriginal people, the Chemistry Centre of Western Australia (CCWA) provided aqueous extracts of the plant as a treatment for terminally ill cancer patients from 1957 to 1991 (Chemistry Centre of Western Australia, 1999-2000). In 1999, this activity was further investigated using a crown gall tumor assay where it was shown that both the CCWA extract and a number of compounds isolated from methanol extracts showed significant anti-tumor activity (Kerr; Longmore; Yench, 1999). In 2000, the crude methanol fractions of *Scaevola spinescens* were tested on a range of cancer cell lines for cytotoxic activity (Nobbs, 2001). The results were inconclusive as some of the fractions showed massive stimulatory effect at higher concentrations.

S. spinescens has also been tested for antimicrobial activity. However, the results were inconclusive as some of the fractions tested have the ability to enhance the growth of bacteria (Kerr; Longmore; Yench, 1999). In 2000, this activity was further investigated by Nobbs (2001). Some of the crude fractions isolated from the methanol extract were found to have significant antimicrobial activity (Nobbs, 2001).

The chemical constituents that have been isolated previously from *S. spinescens* were myricadiol (Kerr; Longmore; Betts, 1996), taraxerol (Kerr; Longmore; Betts, 1996; Nobbs, 2001),

taraxerone (Kerr; Longmore; Betts, 1996), taraxerol acetate (Kerr; Longmore; Betts; 1996), ursolic acid (Nobbs, 2001), Judarrylol (Nobbs, 2001), Lupenone (Kerr; Longmore; Betts, 1996), β-sitosterol (Kerr; Longmore; Betts, 1996), scaevoloside (Nobbs, 2001), scaevoloside dimethyl acetal (Nobbs, 2001), homoscaevoloside (Nobbs, 2001), loganin (Nobbs, 2001), luteolin-7-*O*-glucoside (Nobbs, 2001; Nissler; Grebhardt; Berger, 2004; Lu; Foo, 2000; Agrawal, 1988; Feeny; Sachdev; Rosenberry; Carter, 1988), luteolin-7-*O*-glucuronide methyl ester (Nobbs, 2001), emmarin (Nobbs, 2001), nodakenetin (Kerr; Longmore; Betts, 1996), xanthyletin (Kerr; Longmore; Betts, 1996), 2-deoxy-D-chiro-inositol ((Nobbs, 2001; Gultekin; Celik; Turkut; Tanyeli; Balci, 2004; Buckingham; Donaghy, 1982) and decursinol (Abyshev; Gindin; Semenov; Agaev; Abdulla-zade; Guseinov, 2006; Abyshev; Zmeikov; Sidorova, 1982).

Materials and methods

Plant material

The aerial parts of *S. spinescens* were collected from a site 10 km west of Morgan, on the Morgan-Eudunda Road, South Australia. A voucher specimen was deposited at the State Herbarium of South Australia – Voucher Number AD99702040.

Extraction

The dried aerial parts (0.96 kg) of *S. spinescens* was divided into nine portions (each ~ 100 g). Each portion was extracted sequentially in a Soxhlet apparatus with hexane (2.5 L), ethyl acetate (2.5 L), methanol (2.5 L) and water (2.5 L) (approximately 11 hours extraction for each solvent). The solvents were evaporated in vacuo to afford a concentrated hexane, ethyl acetate, methanol and aqueous extract. A separate water decoction was also prepared where the dried aerial parts (0.48 kg) of *S. spinescens* were soaked twice in warm water for 30 minutes, heated to boiling, then simmered for about 45 minutes. The sample was then filtered using a Buchner funnel and the water from the filtrate was evaporated in vacuo to afford a concentrated aqueous extract.

Fractionation

The hexane, ethyl acetate and methanol extracts were each subjected to silica gel (40-63 micron) flash column chromatography and eluted with increasing polarity gradient mixtures of hexane-dichloromethane, hexane-ethyl acetate and ethyl acetate-methanol. The aqueous extracts were subjected to C18 reversed-phase silica gel (55-105 micron) flash column chromatography and eluted with decreasing polarity gradient mixtures of methanol-water.

Structural elucidation

The structures of the isolated compounds were determined using 1D and 2D NMR techniques (Varian INOVA 600 MHz spectrometer), UV-visible spectroscopy (SP-8001 Metertech UV/Vis spectrophotometer), FTIR spectroscopy (Perkin Elmer BX FT-IR system), mass spectrometry (Kratos Concept ISQ magnetic sector mass spectrometer and LTQ Orbitrap^{TM} mass spectrometer) and optical rotation (AP-100 automatic polarimeter).

Antimicrobial assay

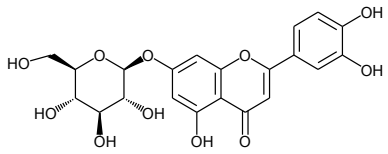
Organic and aqueous crude extracts of *Scaevola spinescens* were screened for antimicrobial activity against Gram-positive (*Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC 10389) and Gram-negative (*Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) bacteria using a broth micro-dilution assay to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Ampicilin was used as standard positive control for Gram-positive strains where as gentamicin was used as standard positive control for Gram-negative strains. The minimum inhibitory concentration (MIC) of each extract was determined as the lowest concentration at which no growth was observed in the duplicate wells. Plates were set up to a

previously suggested format (Cos, 2006). The minimum bactericidal concentration (MBC) was then determined for the extracts with MIC value of 2000 µg/mL and below using a method published previously (Ndi, 2007).

Results and discussion

Extraction and fractionation

In this work, six pure compounds have been isolated from *S. spinescens* extracts. Three of these are known as luteolin-7-*O*-glucoside **(1)**, 2-*deoxy*-D-*chiro*-inositol **(2)** and decursinol **(3)**. The structures of these known compounds were reconfirmed by comparing their physical and spectroscopic data ($[\alpha]_D$, ^{1}H NMR, ^{13}C NMR) with those of published values. Compound **(1)** and **(2)** have been previously isolated (Nobbs, 2001). The structures of the three remaining isolated compounds are yet to be determined.



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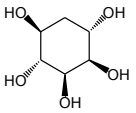

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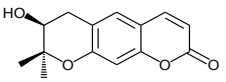

(3)

Luteolin-7-O-glucoside (1)
Pale yellow, amorphous solid. **MP**: 260 – 265 °C. **IR**: ν_{max} (nujol) 3495 cm^{-1} (OH), 3451 cm^{-1} (OH), 1659 cm^{-1} (C=O), 1609 cm^{-1} (C=C), 1597 cm^{-1} (C=C). **UV**: λ_{max} (methanol) 352 nm. **^{1}H NMR** (600 MHz, CD_3OD); δ_H: 3.41 [1H, m, H(4")], 3.48 [1H, m, H(2")], 3.49 [1H, m, H(3")], 3.54 [1H, ddd, $J_{5",6"\beta}$ = 2.3 Hz, $J_{5",6"\alpha,}$ = 5.6 Hz, $J_{4",5"}$ = 9.7 Hz, H(5")], 3.71 [1H, dd, $J_{5",6"\alpha}$ = 5.6 Hz, $J_{6"\beta,6"\alpha}$ = 12.0 Hz, H(6"α)], 3.92 [1H, dd, $J_{5",6"\beta}$ = 2.3 Hz, $J_{6"\alpha,6"\beta}$ = 12.0 Hz, H(6"β)], 5.06 [1H, d, $J_{1",2"}$ = 7.2 Hz, H(1")], 6.47 [1H, d, $J_{6,8}$ = 2.4 Hz, H(6)], 6.56 [1H, s, H(3)], 6.68 [1H, d, $J_{5',6'}$ = 8.4 Hz, H(5')], 6.80 [1H, d, $J_{6,8}$ = 2.4 Hz, H(8)], 7.16 [1H, d, $J_{2',6'}$ = 2.4 Hz, H(2')], 7.36 [1H, dd, $J_{2',6'}$ = 2.4 Hz, H(6')]. **^{13}C NMR** (600 MHz, CD_3OD); δ_C: 62.8 [C(6")], 71.7 [C(4")], 75.2 [C(2")], 78.3 [C(3")], 78.8 [C(5")], 96.5 [C(8)], 101.1 [C(6)], 102.1 [C(1")], 103.5 [C(3)], 106.9 [C(2')], 107.5 [C(4a)], 109.9 [C(5')], 120.6 [C(6')], 121.6 [C(1'), 154.8 [C(3')], 159.46 [C(4'*)], 159.48 [C(8a*)], 163.3 [C(5)], 165.1 [C(7)], 168.9 [C(2)], 184.4 [C(4)]. **APCI MS** (Negative ion) *m/z*: 447.0936 (calculated for $C_{21}H_{20}O_{11}$ – H, 447.0927), 285.0406 (100%).
* Assignments may be reversed.

2-deoxy-D-chiro-Inositol (2)
Colourless crystals. **MP**: 230 – 233°C. **$[\alpha]_D$:** +23.6° (c 0.22, water). **IR**: ν_{max} (nujol) 3306 cm^{-1} (OH). **^{1}H NMR** (600 MHz, D_2O); δ_H: 1.86 [1H, ddd, $J_{2,6\alpha}$ = 3.6 Hz, $J_{5,6\alpha}$ = 11.5 Hz, $J_{6\beta,6\alpha}$ = 14.4 Hz, H(6α)], 2.04 [1H, dddd, $J_{2,6\beta}$ = 1.2 Hz, $J_{2,6\beta}$ = 3.6 Hz, $J_{5,6\beta}$ = 4.8 Hz, $J_{6\alpha,6\beta}$ = 14.4 Hz, H(6β)], 3.62 [1H, t, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$ = 9.6 Hz, H(4)], 3.76 [1H, ddd, $J_{3,5}$ = 0.6 Hz, $J_{1,3}$ = 3.6 Hz, $J_{3,4}$ = 9.6 Hz, H(3), 3.80 [1H, dddd, $J_{3,5}$ = 0.6 Hz, $J_{5,6\beta}$ = 4.8 Hz, $J_{4,5}$ = 9.6 Hz, $J_{5,6\alpha}$ = 11.5 Hz, H(5)], 3.98 [1H, td, $J_{2,6\beta}$ = 1.2 Hz, $J_{2,6\beta}$ = 3.6 Hz, H(2)], 4.07 [1H, q, $J_{1,3}$ = 3.6 Hz, H(1)]. **^{13}C NMR** (600 MHz, D_2O); δ_C: 35.2 [C(6)], 70.5 [C(1)], 70.8 [C(5)], 72.9 [C(3)], 74.1 [C(2)], 76.4 [C(4)]. **ESI-MS** *m/z*: 166.0751 (calculated for $C_6H_{12}O_5$ + H, 165.0762).

Decursinol (3)
Pale yellow, amorphous solid. **MP**: 170°C. **IR**: ν_{max} (nujol) 3412 cm^{-1} (OH), 1708 cm^{-1} (C=O), 1598 cm^{-1} (C=C). **^{1}H NMR** (600 MHz, d_6-DMSO); δ_H: 1.12 [3H, s, Me(12)], 1.13 [3H, s, Me(11)], 3.15 [2H, s, H(6)], 4.69 [1H, t, $J_{6,7}$ = 9.0 Hz, H(7)], 6.20 [1H, d, $J_{3,4}$ = 9.0 Hz, H(3)], 6.78 [1H, s, H(10)], 7.47 [1H, s, H(5)], 7.92 [1H, d, $J_{3,4}$ = 9.0 Hz, H(4)]. **^{13}C NMR** (600 MHz, d_6-DMSO); δ_C: 24.8 [C(12)], 25.8 [C(11)], 28.6 [C(6)], 69.9 [C(8)], 90.9 [C(7)], 96.7 [C(10)], 111.1 [C(3)], 112.0 [C(4a)], 123.8 [C(5)], 125.5 [C(5a)], 144.7 [C(4)], 155.0 [C(10a)], 160.5 [C(2)], 163.3 [C(9a)]. **LREIMS** *m/z* 246 [M$^+$, 35%], 213 [25%], 187 [100%], 175 [14%], 160 [27%], 131 [15%]. **HREIMS** *m/z*: 246.0889 (calculated for $C_{14}H_{14}O_4$, 246.0892).

Antimicrobial assay

The hexane, ethyl acetate and methanol crude extracts showed antimicrobial activity against Gram-positive bacteria, especially *S. pyogenes* ATCC 10389 with minimum inhibitory concentration (MIC) ranging from 500 to 4000 µg/ml and minimum bactericidal concentration (MBC) ranging from 500 to 1000 µg/ml. No antimicrobial activity was observed against Gram-negative bacteria. DMSO medium control was carried out in each assay to show that the concentration used to dissolve the extracts is not responsible for the killing effect. Therefore, any inhibition or killing effects observed are solely due to the active compounds in the extracts.

The aqueous crude extracts did not show any antimicrobial activity against Gram-positive bacteria at a minimum concentration of 4000 µg/ml. However, significant antimicrobial activity against *S. aureus* ATCC 25923 was observed in the aqueous fractions with MIC ranging from 31.25 to 4000 µg/ml.

One of the isolated compounds showed antimicrobial activity against *S. pyogenes* ATCC 10389 with an MIC from 3.75 to 7.5 µg/ml and MBC from 7.5 to 15 µg/ml. The compound also showed antimicrobial activity against *S. aureus* ATCC 25923 with MIC from 1.87 to 3.75 µg/ml and MBC from 3.75 to 7.5 µg/ml. However, the structure of this compound has not yet been determined.

Conclusion

Coumarins, terpenoids, iridoids and flavonoids have previously been isolated from *S. spinescens*. Active compounds isolated from *S. spinescens* might be potential lead compounds for the development of new anti-microbial therapeutics. Further investigation into the anti-microbial activity of the aqueous extract may be of interest as it may validate the remedy used by the Aboriginal people.

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Development of Natural Products Library

Su, N.N., Ng, L.T. and Toh, K.L.

Sarawak Biodiversity Centre, KM 20 Jalan Borneo Heights, Semengoh,
Locked Bag No. 3032, 93990 Kuching, Sarawak, Malaysia
molbio@sbc.org.my

Abstract

The Sarawak Biodiversity Centre (SBC) is one of the leading state government agencies that had been set up to carry out R&D on the rich biological resources of Sarawak. The Centre started its activities in 1998, initially to carry out taxonomic inventory of Sarawak's biodiversity but its role was amended to focus on Sarawak's Traditional Knowledge (TK) Documentation and Natural Products Discovery. The goal is to discover novel therapeutic/useful compounds from Sarawak's unique biodiversity for healthcare, pharmaceutical and relevant industrial applications. A natural products library was established by SBC consisting of extracts derived from useful plants identified by the indigenous communities through our Traditional Knowledge (TK) Documentation Programme. The Library also contains extracts from fermentation products of biodiverse microbial isolates (actinomycetes and fungi) isolated from various sites in Sarawak. Currently, the Library holds a total of about 50,034 plants extracts (in triplicates) from approximately 3,362 plant species. A majority of the extracts were derived from ethno-plants (~60%) while the rest were randomly collected. The microbial collection with recent figure of about 11,185 isolates of actinomycetes and 2,130 isolates of fungi is a representative sampling of Sarawak's microbial diversity which also have been tentatively classified based on their morphological characteristics. This Library is the main resource for SBC's & R&D and also, for collaborative work with other institutions and pharmaceutical companies. SBC's Natural Products Discovery Programme carried out bioassays for screening the Library for lead compounds to be developed as pharmaceutical candidates or therapeutic applications. To date, the Centre is working on several potential plants and microbial strains that provided leads for cancer therapy, antibiotics and antifungal, and inhibitors for neurodegenerative diseases. SBC's other R&D activities includes chemical characterization and fingerprinting of biologically active natural products by high performance liquid chromatography (HPLC), fourier transformed infrared (FTIR) and mass spectrometry. These methods in combination with Nuclear Magnetic Resonance (NMR) technology will provide analytical solutions to elucidate novel compounds from Sarawak's rich biodiversity.

Introduction

The Sarawak Biodiversity Centre (SBC) is a Government Statutory Body set up under the provision of the Sarawak Biodiversity Centre Ordinance (1997). It is one of the leading state government agencies within the Ministry of Planning and Resource Management that had been set up to carry out R&D on the rich biological resources of Sarawak. The Centre started its activities in Year 1998, initially to carry out taxonomic inventory of Sarawak's biodiversity but its focus was changed to documentation of Sarawak's traditional knowledge and conduct R&D on biological resources in terms of its medicinal or therapeutic properties and for the establishment of a Natural Products Discovery Library. This library consists of extracts from biological resources for research, study or screening for their bioactive compounds or pharmaceutical, medicinal, therapeutic or nutritional properties, or for agricultural purposes and also for maintaining records of and databases for biological resources that are found in the Sarawak State [Sarawak Biodiversity Centre (Amendment) Ordinance, 2003].

In concordance with the centre's functions, SBC established a Natural Product Library (NPL). Natural Product Library or NPL is a collection of extracts from biological resources that are utilize for bioassay screening. Inclusive in the library is the linkage of the extracts' information with regards to its usages as medicinal, food, poison, or ornamental, geographical origin, barcode, bioassay results, type of extract sources, sources local and scientific name, etc that are compiled in a database. Recently, there are about 200 000 natural compounds known (Füllbeck *et al.,* 2006). However, only about 5-15% are from higher plants whereas the majority of the compounds are derived from actinomycetes. Today, researchers have paid more attention on the marine natural products apart from natural

products extracted from plants and animals. Marine natural products have yielded a considerable number of drug candidates (Haefner, 2003).

Natural product databases

Generally, there are few molecular databases which publicly available online (Table 1). These databases comprise, for instance, structures, names, synonyms, CAS Registry Numbers, chemical properties, information about the usage of a compound, patents and corresponding literature references (Füllbeck *et al.,* 2006).

Table 1. Public databases that containing natural compounds (Adapted from Füllbeck *et al.,* 2006)

Name	Homepage http://	No. of compounds	No. of Natural compounds
ChEBI	www.ebi.ac.uk/chebi	>6800	~3500
ChemBank	chembank.broad.harvard.edu	>1 100 000	N/A
ChemID	chem.sis.nlm.nih.gov/chemidplus	>379 000	~2000
NCI	cactus.nci.nih.gov/ncidb3/download_ncidb3.html	>260 000	N/A
PubChem	pubchem.ncbi.nlm.nih.gov	5 000 000	N/A

N/A: Not available.

Apart from that, commercial databases are also available which provide similar search options as public databases, but with fee-based (Table 2). There are also databases from suppliers (Gaia Chemical Corporation, Life Pharms Inc., Mera Pharmaceutical, Inc.) and manufacturers (e.g. SuperNatural database) who are specialized in the discovery and development of new natural products from the various biological habitats (Füllbeck *et al.,* 2006).

Table 2. Commercial natural compound databases that are available (Adapted from Füllbeck *et al.,* 2006)

Name	Homepage http://...	No. of compounds	No. of Natural compounds	Price
AntiBase	eu.wiley.com/WileyCDA	>31 000	~31000	$8250 £4714 €6875
Chapman and Hall	www.crcpress.com/	>190 000 (CD-ROM) >185 000 (Web)	>190 000 (CD-ROM) >185 000 (Web)	$6600 (CD-ROM) $3995 (Web)
Chemical Abstracts	www.cas.org	>27 000 000	N/A	$28 400 (printed version) $31 500 (CD-ROM)
RÖMPP Online	www.roempp.com/thieme-chemistry/np/info	~6000	~6000	On inquiry
The Beilstein Database	www.beilsteininstitut.de/englisch/1024/chemie/index.php 3 Available at: www.mdli.com/products/knowledge/crossfire_beilstein	>9 300 000	>142 000	On inquiry
The Merck	www.merckbooks.com	>10 000	N/A	$65 (printed

Name	Homepage http://...	No. of compounds	No. of Natural compounds	Price
Index				version); prices on request (CD-ROM); on subscription basis (ONLINE versions)

N/A: Not available.

SBC Natural Product Library

SBC NPL consists of extracts derived from useful plants identified by the Sarawak indigenous communities through our Traditional Knowledge (TK) Documentation Programme. To date, SBC NPL holds a total of about 50,034 plants extracts (in triplicates) with approximately 3,362 plant species. A majority of the extracts were derived from ethno-plants (~60%) while the rest were randomly collected. Moreover, the Library also contains extracts from fermentation products of biodiverse microbial isolates (actinomycetes and fungi) that were isolated from various locations within Sarawak. Currently, the microbial collection, with recent figure of about 11,185 isolates of actinomycetes and 2,130 isolates of fungi is a representative sampling of Sarawak's microbial diversity, which also have been tentatively classified based on their morphological characteristics.

This Library serves as the main resource for SBC's R&D and also, triggers collaborative works with other institutions and pharmaceutical companies. SBC's Natural Products Discovery Programme carried out bioassays such as Anticancer Compound Screening and Photodynamic Therapy (PDT) assay on different cancer cells by screening through the Library for lead compounds to be developed as pharmaceutical candidates or therapeutic applications. To date, the Centre is working on several potential plants and microbial strains that provided leads for cancer therapy, antibiotics and antifungal, as well as inhibitors for neurodegenerative diseases.

Conclusion

SBC NPL is still in the infant stage as more collections and data depositions needed to be done. Furthermore, the Library database will be upgraded to accommodate robust applications by the researchers.

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Photodynamic Activity of Plant Extracts from Sarawak

Jong, W.W.[1], Kamarulzaman, F.A.[2], Thomas, V.M.Q[1] and Lee, H.B.[2]

[1] Sarawak Biodiversity Centre, KM 20 Jalan Borneo Heights, Semengoh, Locked Bag No. 3032, 93990 Kuching, Sarawak, Malaysia
[2]Cancer Research Initiatives Foundation (CARIF), Subang Jaya Medical Centre, 1, Jalan SS12/1A, 47500 Subang Jaya, Selangor, Malaysia
wwjong@sbc.org.my

Abstract

Photodynamic Therapy (PDT) is a relatively new cancer treatment that involves the selective uptake and retention of a photosensitizer in cancerous cells, followed by irradiation with light of a particular wavelength to activate the photosensitizer to kill the cells. To date, only a small number of photosensitizers have been clinically approved for PDT and researchers continue to look for new molecules that may have more desirable properties for clinical applications. Natural products have long been important sources of pharmaceuticals and there is great potential for discovery of novel photosensitizers from Sarawak's rich biodiversity. In this study, we screened for photosensitizing activity from extracts of plants collected from various locations in the state. Organic extracts from different part of the plants were tested on HL60 and K562 human leukemic cell lines for PDT activity at 20 µg/ml. Extracts that reduced cell viability to lower than 50% after light treatment but not in the dark were considered active. Of the 2400 extracts coming from 888 plant species tested, 14.6% or 351 extracts showed PDT activity with 50 extracts from 37 plants showed at least five-fold more photo-cytoxicity than general cytotoxicity in the dark. The active samples included extracts derived from plants that are common to Sarawak such as Kacang Ma (*Leonurus sibiricus*), Sembong (*Blumea balsamifera*) and Entemu (*Curcuma zedoaria*). Work is underway to fractionate the extracts to identify active components that may be further developed into PDT agents.

Introduction

Photodynamic Therapy (PDT) is a relatively new cancer treatment that involves the selective uptake and retention of a photosensitizer in cancerous cells, followed by irradiation with light of a particular wavelength to activate the photosensitizer to kill the cells (Ahmad, *et al.,* 1998; Nyman and Hynninen, 2004). Fig. 1 summarizes the process of treating cancer using PDT. To date, only a small number of photosensitizers have been developed for PDT and researchers continue to look for new molecules that may have more desirable properties for clinical applications (Castano *et al*., 2005). Natural products have long been important sources of pharmaceuticals and there is great potential for discovery of novel photosensitizers from Sarawak's rich biodiversity. In this study, we screened for photosensitizing activity from extracts of plants collected in Sarawak.

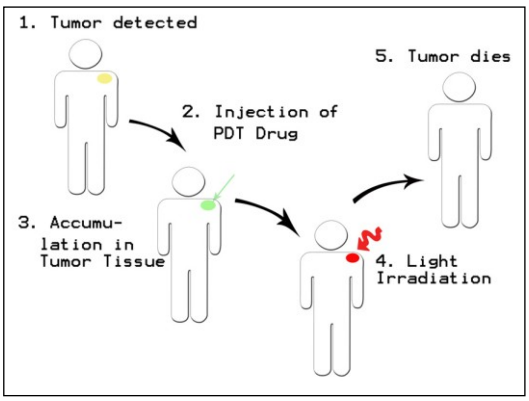

Fig. 1. PDT for cancer treatment

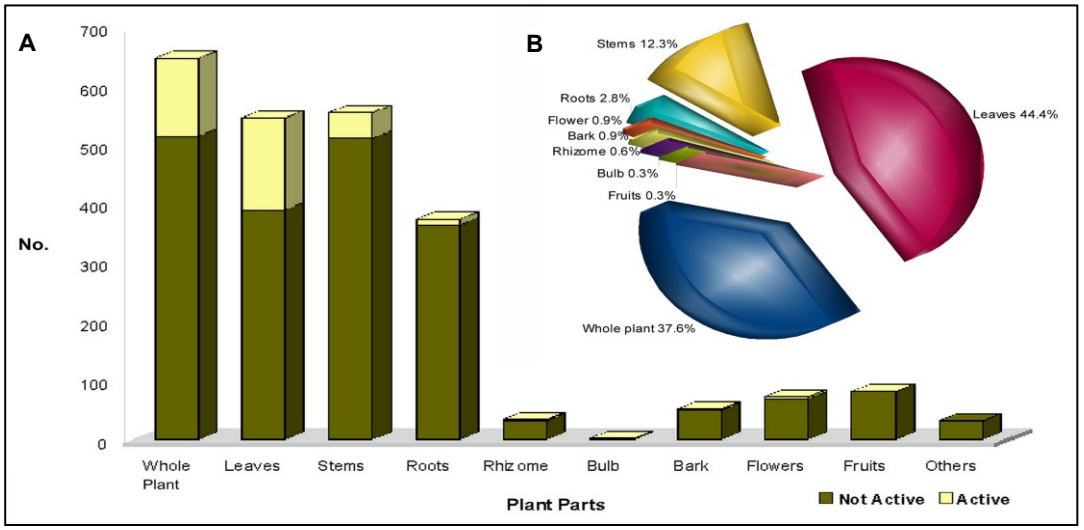

Fig. 2. PDT activity in extracts of different plant parts. A: The number of active plant parts out of the total extracts (2400) tested. B: Percentage of different plant parts in total number of active extracts (351).

Materials and methods

Plants were collected from various locations in Sarawak. The samples were rinsed with water, sorted into different parts and cut into small pieces. These were then dried in an oven at 40^{o}C and ground. Extraction was done by overnight soaking (10% w/v) in dichloromethane:methanol (1:1) on a orbital shaker at room temperature. Extracts were dried using a rotary evaporator and stored at 4^{o}C.

For the photocytotoxicity assay, HL60 and K562 human leukemic cell lines were incubated with 20 µg/ml of extract at 30,000 cells/well for 2 hours at 37^{o}C in 5% CO_2. The 96-well plates were then irradiated with a 300 W Tungsten lamp for 10 min, which equals to a light dose of 9.6 J/cm^2. The plates were further incubated overnight before cell viability was quantified using a modified MTT assay (Mosmann, 1983). 20 µl of a stock solution of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide) was added to each well in the plates and the plates were incubated for 4 hrs at 37^{o}C in 5% CO_2. 80 µl of the supernatant was then removed from each well and 100 µl of DMSO added to dissolve the formazan crystals. Absorbance was then read at 570 nm and cell viability was calculated.

Results and discussion

Extracts that reduced cell viability to lower than 50% after light treatment but not in the dark were considered active. A total of 2400 extracts from 888 plants were screened. 351 extracts or 14.6% of these extracts that were derived from various plant parts showed PDT activity (Fig. 2A). Photo-cytotoxicity was significantly high in the leaves, followed by whole plant and stems (Fig. 2B). This could be the result of an abundance of chlorophylls and their degradative products that are photosensitizers with cyclic tetrapyrollic structure.

50 extracts from 37 plants showed at least five-fold more photo-cytotoxicity than general cytotoxicity in the dark. Most of these plants are common to our region and some are well known medicinal plants used by Sarawak's communities. A fraction of plants that were identified for further study, with their uses, is shown in Table 1 (Burkill, 2002; Chai, 2006).

Table 1. PDT-Active Plants and Their Known Uses

Local Name	Scientific Name	Uses
Kacang Ma	*Leonurus sibiricus*	As food during confinement.
Sembong	*Blumea balsamifera*	Used in post-natal baths and for treating fever.
Entemu	*Curcuma zedoaria*	Used to warm the body after childbirth.
Tuba	*Derris elliptica*	As fish poison and for treating diabetes.
Gendarusa Putih	*Justicia betonica*	For treating swellings with massage.
Lengkuas	*Alpinia galanga*	For treating fever and rheumatism.
Daun Kadok	*Piper sarmentosum*	Used in cooking and for cuts and wounds.

Conclusions

The PDT activity that we detected in the extracts of plants found in Sarawak appears promising. Work is underway to fractionate the extracts to identify active components that may be further developed into PDT agents.

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Phytochemical Analysis and Antimicrobial Screening of Selected Medicinal Herbs Used in Malaysian Diet

Vikneswaran, V., Jegathambigai, R., and Sasidharan, S.

Department of Biotechnology, AIMST Univrsity, Jalan Bedong, Semeling, Kedah, Malaysia
vicky_g8@hotmail.com

Abstract

Drug development to combat infectious disease has gain popularity in many researches at present time. The undesirable side effects of certain antibiotics and the emergence of previously uncommon infections have forced scientists to look for new antimicrobial substances from various sources like medicinal plants. Antimicrobial screening and phytochemical analysis was done for seven different plant spcies including *Cymbopogon citratus* (Lemon grass), *Citrus hystrix* (Kaffir lime leaf), *Pandanus amaryllifolius* (Pandan), *Murraya koenigii* (Curry leaf), *Allium ascalonicum* (Spring onion), *Apium graveolens* (Celery) and *Mentha spicata* (Pudina) ethanolic extract were analyzed initially. Disc diffusion assay was done by using four different bacterial strain, *Staphylococcus aereus, Escherichia coli, Shigella sonnei* and *Salmonella choleraciuis.* Further investigation including Minimum Inhibitory Concentration (MIC) and Thin Layer Chromatography (TLC) was determined for positive sample in antimicrobial screening. Phytochemical analysis indicated that glycoside was present in ethanolic extract of *A. graveolens* and *M. spicata* while flavonoid was present only in *C.citratus*. Tannins and saponins were present in *P. amaryllifoluis, M. koenigii, C. hystrix* and *A.ascalonicum* while *A. graveolens* only had tannins. Presence of anthroquinone was detected only in ethanolic extract of *C.citratus*. The antimicrobial screening results of ethanolic extract of *C.citratus* inhibited growth of only *S.aereus* bacterial strain with an inhibition zone of 0.87$\pm$0.12cm while the other plants did not show any antimicrobial activity. Ethanolic extract of six other samples showed negative effect on all four strains of bacteria. The MIC value of *S.aereus* against *C. citratus* is 12.5mg/ml. Hence, *C. citratus* has potential antimicrobial activity which can be further studied for active compound.

Introduction

The initial task in drug research is to identify new lead compounds with desired biological activities. These can be obtained by chemically modifying known drugs which are already available in market. Yet the production of modified drugs or synthetic drugs is a rather tedious and expensive process. The undesirable side effects of certain antibiotics and the emergence of previously uncommon infections (Marchese and Shito, 2001; Poole, 2001; Zampini *et al*., 2005) have forced scientist into looking for new antimicrobial substance from various sources like medicinal plants. Therefore, it was decided that this search be narrowed to plants with known therapeutic activities used in traditional remedies. Various medicinal plants have been used for years in daily life for curing diseases all over the world. Plants are basic source of knowledge of modern medicine. Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Illness resulting from consumption of food contaminated with pathogenic bacteria and/or their toxins is a priority concern to public health. The factors contributing to the food borne disease are changes in human demographics and behavior, technology and industry, international travel and commerce, microbial adaptation including antimicrobial resistance to antibiotics and other products (Altekruse *et al*., 1997). Most common food borne illnesses are caused by *Shigella sp., Salmonellae sp., Staphylococcus sp.* and *Escherichia coli.* The present study focused on the evaluation of pyhtochemical constituents and antimicrobial activity of seven different plants ethanolic crude extract including *Cymbopogon citratus* (Lemon grass), *Citrus hystrix* (Kaffir lime leaf), *Pandanus amaryllifolius* (Pandan), *Murraya koenigii* (Curry leaf), *Allium ascalonicum* (Spring onion), *Apium graveolens* (Celery) and *Mentha spicata* (Pudina) and further investigation via Minimum Inhibitory Concentration (MIC) analysis and Thin Layer Chromatography (TLC) screening on the plant pronouncing prominent antimicrobial activity against tested bacterial strains.

Materials and methods

C. citratus (Lemon Grass), *Citrus hystrix* (Kaffir Lime leaf), *Pandanus amaryllifolius* (Pandan), *Murraya koenigii* (Curry Leaf), *Allium ascalonicum* (Spring Onion), *Apium graveolens* (Celery) and *Mentha spicata* (Pudina), 95% Ethanol, 10% Ethanol, 70% ethanol10% DMSO, Distilled water, Ferric chloride reagent, Diluted hydrochloric acid, Benedict solution, 40% Ammonia, Sodium hydroxide, 10% Sulphuric acid, Copper sulphate, Sodium bicarbonate, Chloroform, Fehling solution, 1% Aluminium chloride, Potassium hydroxide solution, Muller-Hinton agar and Nutrient broth.

Extraction

Plant samples are washed clean, dried and ground to fine powder. Ethanol was used for crude extraction preparation. The amount of ethanol used was an estimation of three times the weight of ground plant materials used. The mixtures were stirred with sterile glass rod. The mixture was stored in a dark area for seventy two hours with stirring. After seventy two hours, mixtures were filtered using Whatman No.1 filter papers. Each extract was concentrated into a paste form by using rotary evaporator (EYELA, AS). A stock solution of 0.15 g/ml of each crude extract was prepared. From this stock, desired concentrations of working solution were prepared for other tests.

Phytochemical screening

Seven phytochemical tests were conducted on each plant extracts which were Benedicts test to determine glycoside presences, Flavanoid test, Tannins test, Borntranger's test to determine presences of anthraquinone, Saponins test, Alkaloid test as well as moisture content determination done separately on plant samples that were not extracted via ethanol extraction to determine quantitatively amount of moisture present in plant sample. The screening of chemical constituents was carried out with the ethanol extracts using chemical methods according to the methodology of Wagner and Bladt (1996).

Antimicrobial screening

Four test microorganisms were used, three gram negative strain; *Escherichia coli*, *Salmonella choleraciuis* and *Shigella sonnei* and one Gram positive strain; *Staphylococcus aureus*. Disc Diffusion Method was used (Wagner and Bladt,1996). to determine the antimicrobial activity of all seven plant ethanol extract against the four bacterial strains tested. The turbidity of the bacterial culture used for seeding on Muller Hinton agar plates was adjusted equivalent to No.0.5 tube of the McFarland scale. A sterile swab stick was used to seed the culture onto Muller Hinton agar plates. The sterilized discs were impregnated on the seeded culture plates using sterile forceps. 20 µl of each plant extracts from 50 mg/ml working solution were dropped on the sterile discs. Commercial antibiotic disc, Ceftriaxone was used as positive control while 10% of ethanol was used as negative control. The plates were incubated for 24 hours at 37^0C. The zone of inhibition were measured and recorded.

Minimal Inhibitory Concentration (MIC) and Thin Layer Chromatography (TLC)

Both tests were preformed on plant samples that show positive results in antimicrobial screening. MIC follows a two fold serial dilution method proposed by Sahin *et al.* (2003). TLC was performed to fractionate active compound of positively tested samples. Silica coated TLC plates are used for fractionation. Only one solvent system was used as mobile phase which was composed of ethyl acetate: methanol (20:80). The plates were observed under UV light. The separated spots were marked and the Rf value was calculated.

Results and discussion

The phytochemical analysis reveals that glycoside is present in ethanol crude extract of *A. graveolens* and *P. amaryllifolius,* while flavonoid is present only in ethanolic crude extract of *C. citratus*. Tannins and saponins were found to be present in *P. amaryllifolius, C. hystrix, M. koenigii* and *A. ascalonicum.*

M. spicata ethanol crude extract was found to contain tannins only. Anthraquinone was found to be present only in *C. hystrix*. Alkaloid was not detected in any crude extract. Moisture content determination shows that all seven plant samples has high amount of moisture (Table 1). The antimicrobial screening via disc diffusion method conferred positive results with an average of 8.67mm diameter zone of inhibition only for *C. citratus* ethanol crude extract against *S. aereus* bacterial strain (Fig. 1), while rest of the ethanol crude extract samples did not exhibit any antibacterial activity. As only *C. citratus* ethanolic crude extract conferred a positive result in antimicrobial screening, therefore the same plant extract was chosen for MIC and TLC test. MIC of this extract was 12.5 mg/ml, below this concentration the broth containing the extracts were turbid. The TLC test showed two separated spots, one bright yellow in colour with an R_f of 0.54 and another dark brown in colour with a R_f of 0.43 when observed under UV light (Fig.2.0 b). While observation under visible light showed only one pale brown spot with an R_f of 0.43 (Fig. 1).

Table 1. Phytochemical analysis of plant ethanolic crude extract

Test	Inference						
	C.citratus	***A.graveolens***	***M.spicata***	***P.amaryllifolius***	***C.hystrix***	***M.koenigii***	***A.ascalonicum***
Benedict test (glycoside)	-	+	-	+	-	-	-
Flavonoid test	+	-	-	-	-	-	-
Tannins test	-	-	+	+	+	+	+
Borntranger's test (anthraquinone)	-	-	-	-	+	-	-
Saponins test	-	-	-	+	+	+	+
Alkaloid test	-	-	-	-	-	-	-
Moisture Content (%)	80.4	88.0	95.5	87.2	63.2	68.2	94.4

* +: present; -: absent

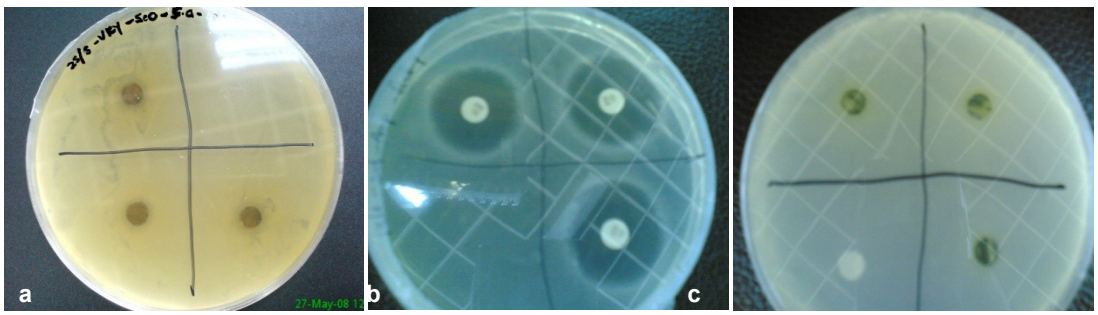

Fig. 1. Screening of C. citratus ethanol extract on S. aureus. shows inhibition zone for 3 replicates. b. Screening of Ceftriaxone antibiotic disc on S. aureus. indicates inhibition zones on bacterial strains which are susceptible to the antibiotic. c. Screening of negative control disc on S. aureus. indicates that 10% ethanol (negative control) has no effect on bacterial strain.

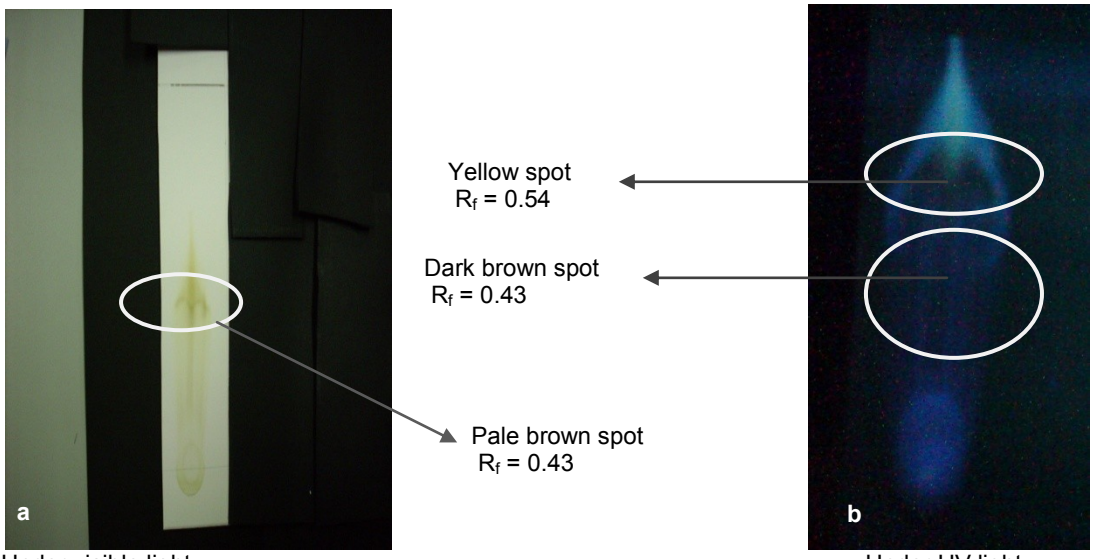

Fig. 2. TLC results under visible light and UV light observation with corresponding R_f values.

The results obtained meet the goal of this study and the scientific information that is now documented can be used to enhance the overall knowledge of the plant species under investigation and their role in patient care, also to healthcare professionals to improve human health. The first test to be conducted was phytochemical analysis of each plant samples ethanolic crude extracts. The analysis method employed was a qualitative one. It can be inferred from this analysis that ethanol was capable of extracting flavonoid, saponins, tannins, glycoside and anthraquinone. This result coincides with previous study by Cota *et al*. (2003) that ethanol is the best extraction solvent for conventional extracting method of flavonoid, tannins, saponins and glycoside constituents of a plant at room temperature. On the other hand, anthraquinone is extracted less effectively by ethanol extraction method at room temperature (Hemwimon *et al*., 2005).

The agar disc diffusion methods is ideal for screening antimicrobial activity, however, it does not give any qualitative information about the active components present in the ethanolic crude extracts. *C. citratus* ethanolic crude extract showed an average of 8.67mm in diameter zone of inhibition while rest of the ethanolic crude extracts showed negative results. It can be inferred that samples showing negative results maybe due to absences of antimicrobial compounds in the ethanolic extract. This inference can be justified by referring to previous study done which proved that alkaloid isolated from *Murraya koenigii* exhibited strong antimicrobial activity (Rahman and Gray, 2005). As observed from Table 1.0, there were absences of alkaloid not only in ethanolic extract of *M. koenigii* but also in other samples ethanolic crude extracts. Hence, indicating that compounds extracted via ethanol extraction did not have antimicrobial capability except for *C. citratus* ethanolic crude extract.

As antimicrobial screening and phytochemical analysis showed that ethanolic extract of plant samples do not contain compounds which elucidates antimicrobial activity except for *C. citratus* ethanolic extract which contains flavonoid, hence it was further tested by TLC to identify the active compound of flavonoid that maybe present in *C. citratus* ethanolic extract which imposed its antimicrobial activity. The TLC separation showed a yellow spot and also a dark brown spot under UV light observation.

As a standard flavonoid solution was not available for direct comparison, reference comparison on colour basis, which again is a qualitative analysis, was made with previous study (Harborne and Williams, 1972) in a published book, Phytochemicals. In reference to this, the bright yellow spot observed under UV light was identified as a flavonol and inferred to be quercetin, kaempherol, myricetin or isohamnetin. With reference to another study conducted, whereby a flavonoid compound called quercetagetin which gave a dark brown spot under UV light observation and that this compound

exhibited a strong antimicrobial activity against *S.aureus* bacterial strain (Tereschuk *et al*., 1997), it could be inferred that the dark brown spot found in present study may also be quercetagetin.

Conclusions

Ethanolic extraction is proven to be capable of extracting flavonoid, saponins, tannins and also glycoside based on the phytochemical analysis done. Antimicrobial screening of ethanolic extracts of plant samples showed antimicrobial activity in extract of *C. citratus* on *S. aureus* bacterial strain with MIC value of 12.5 mg/ml. TLC screening of the ethanolic crude extract of *C. citratus* revealed a bright yellow and a dark brown spot which is believed to be a flavonol compound which may as well acts as an antimicrobial substance. This study provides new leads regarding ongoing search for novel antimicrobial drugs. However, further studies on the isolation and characterization of the active compounds as well as bioautography and efficacy study on this medicinal herb, *C. citratus* may well provide us with novel antimicrobial agents for chronic infections.

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Preliminary Screening of Antagonistic Activity of Six Strains of *Monascus purpureus*

Sukirah, A.R., Musaalbakri, A.M., Siti Norbaya, K., Zuraidah, Z., Mohamad Husni, O. and Mohd Azman, A.

Biotechnology Research Centre, Malaysian Agriculture Research and Development Institute, Kuala Lumpur, Malaysia
bakri@mardi.gov.my

Abstract

This study was conducted to test the potential antimicrobial activity produce by six strains of *Monascus purpureus* against pathogens. *Monascus purpureus* 1379, 1604, 5354, 5357, 5391 and 5460 were preliminary screened for antagonistic activity by agar plug test against 9 test microorganisms; *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Listeria monocytogen, Salmonella typhi*, *Shigella dysentri, Pseudomonas aeruginosa, Candida albicans* and *Candida krusei.* All strains showed antagonistic effect against *Bacillus subtilis*. *Monascus purpureus* 5460 and 5391 also inhibit *Bacillus cereus, Pseudomonas aeruginosa* and *Shigella dysentri* while *Monascus purpureus* 1379 also inhibit *Pseudomonas aeruginosa*. The study exhibited zone inhibitions produced by the fungus due to the antimicrobial action towards some gram positive and gram negative bacteria, further study will be proceed base on this result to enhance the antimicrobial activity of the fungus.

Introduction

Monascus spp. belongs to the group of *Ascomycetes* and particularly to the family of *Monascaceae*. The genus *Monascus* can be divided into four species: *M. pilosus*, *M. purpureus*, *M. ruber* and *M. floridanus*, which account for the majority of strains isolated from traditional oriental food (Blanc *et al*., 1994; Juzlova *et al*., 1996). The common names of this fungal product are red yeast rice, red rice, angkak, red leaven, beni-koji (Japanese), hung-chu, hong qu, zhitai (Chinese), rotschimmelreis (Europe), red mould (USA). *Monascus* has been used as a traditional fermented food and its metabolic products have also been utilized as a food pigment or biological agents in oriental countries for centuries (Lee *et al*., 2008). It has been used extensively as a natural food colorant in fish, chinese cheese, red wine and sausages. It is an effective natural dietary supplement for controlling serum cholesterol. It contains Monacolin, a blood-reducing cholesterol substance, which inhibit the production of cholesterol by blocking HMG-CoA reductase that activates cholesterol synthesis (Pattanagul *et al*., 2007). *Monascus* spp. produce angkak or red fermented rice, which can convert starchy substrates into several metabolites such as alcohols, antibiotic agents, antihypertensive, enzymes, fatty acids, flavor compounds, flocculants, ketones, organic acids, pigments and vitamins (Yongsmith *et al*., 2000). Thus, this study investigates the implementation of *Monascus* pigment as a coloring agent in food may provide additional advantage such as performing antimicrobial actions against pathogens.

Materials and methods

Six strains of *Monascus spp.* FTCC 1379, 1604, 5354, 5357, 5391 and 5460 maintained in potato dextrose agar slant were obtained from local and international sources and the test organisms were obtained from Institute for Medical Research, Kuala Lumpur. All of the strains were streaked on nutrient agar and incubated for seven days, at 32ºC. For a primary screening, plugs of agar (approximately 5 mm in diameters) of each strain from areas of dense growth on the streak plates was removed and deposited onto nutrient agar plates seeded with 18 hours old test organisms. Antimicrobial activity was detected with the formation of a clear zone around the agar plug and the diameters of the inhibition zones were measured.

Table 1. Antimicrobial activity of *Monascus* strains against several pathogenic microorganisms

Pathogens	***Monascus* strains**					
	FTCC 1379	**FTCC 1604**	**FTCC 5354**	**FTCC 5357**	**FTCC 5391**	**FTCC 5469**
B, subtilis	√	√	√	√	√	√
B.cereus					√	√
L. monocytogens						
S. aureus						
P. aeruginosa	√				√	√
S. typhimurium						
S. dysentri	√				√	√
C. albicans						
C. krusei						

√ Zone inhibition detected

* Range of inhibition zone detected were 1mm-2mm in diameter from all strains

Results and discussion

Monascus was selected because it is safe and widely used traditional food microorganisms. Beni-koji, a rice koji prepared by fermentation with *Monascus* spp., is a traditional food in Asian countries with beneficial health properties (Wang *et al*., 2002). *Monascus* spp. is also for pigment production and antibacterial properties of *Monascus* were first reported by Wong and Bau, 1978. The so-called monascidin A was effective against *Bacillus*, *Streptococcus* and *Pseudomonas*. It was shown that this molecule was citrinin (Blanc *et al*., 1995b) and its production by various *Monascus* species was studied using different culture media and conditions (Blanc *et al*., 1995a). The antimicrobial activities of the strains from our studies are presented in Table 1. The zone of inhibition (diameter) was recorded in each case. All strains showed antimicrobial activity against *B. subtilis.* with 1 mm to 2 mm range of inhibition zone. *M. purpureus* 5460 and 5391 were able to inhibit *B. cereus, P. aeruginosa* and *S. dysentri* with 1 mm to 2 mm inhibition zone while *M. purpureus* 1379 also inhibited *P. aeruginosa* at 1mm of inhibition zone. The agar plugs method elucidated that the fungus shows antimicrobial action towards some Gram positive and Gram negative bacteria.

Even though the inhibition zones were small but optimization process and several methods can be carried out to enhance the antimicrobial activity. The optimization of cultural conditions such as carbon and nitrogen sources had shown significance effect on growth and secondary metabolites production by fungus as well as temperature and pH (Gogoi *et al*., 2008). Inoculating pool of autoclaved bacteria into the fungus culture during fermentation significantly increased the antimicrobial activity from 50 % to 100% (Furtado *et al*., 2002).

Conclusion

Monascus was selected because it is a safe and widely used traditional food microorganisms. The fungus *Monascus* were found to inhibit the growth of some pathogenic bacterial and it is therefore a potential candidate to become a natural antibiotic source. Further research will continue to analyse the growth enhancing compounds in the *Monascus*-fermented based products.

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Session 4
Commercialisation

Transcribed Speech

Technology Commercialization

Tan, C.L.

Biomedical Strategy Consultants Pte. Ltd., Singapore
sunnytan@biomedstrategy.com

My talk is about the commercialization of biomedical technologies.

Technology commercialization is defined as the process by which information, knowledge or technology, which is collectively known as Intellectual Property or IP, gets converted into a product or service for customers and consumers. In this context, technology means potential business opportunities that are at various stages of R&D, but are generally not yet at the point of being productized and available to consumers in the marketplace. This will set the context for the term "technology" that I'll be using throughout this talk.

What are some of the origins of technology that I am going to talk about? Well, with very few exceptions, most basic biomedical research is performed at universities and is funded by the government. University-conducted research takes places very early in the technology development timeline. Universities play a crucial role in the development and commercialization of new medical technologies.

Dr David Owen was at one time the CEO of the Medical Research Council in UK. He said that "Although there are many challenges facing technology commercialization, active, planned tech transfer can and does work if there is a Win-Win Partnership between the tech transfer professionals and the inventors in the labs." That basically sums up the challenges in early stage technology commercialization.

Challenges in commercializing early stage technologies are many and varied. Firstly, commercialization gaps exist between the discovery process and the commercial opportunity. There is a high failure rate if companies are spun out too early, ie the technologies are commercialized too early. This is often due to the fact that the concept has not been proven and validated. It could also be that the technology is too early in the development cycle.

Tech transfer professionals also need very attractive technology with which to work. We're all humans and we are motivated by interesting projects and this is one of the key challenges in tech transfer work. With only a very few exceptions, the researchers rarely understand the range of matters essential to advance technology from publicly funded labs into the commercial marketplace. There is a need to meet those expectations and to disseminate the knowledge and build the rapport and to basically create awareness of the whole process.

The financial crisis has put a great strain in the way that businesses are being done these days. Early stage technology commercialization is not exempted. The majority of VCs have moved away from seed funding. The appetite for early stage technology has quickly diminished. Angels, family and friends are not enough to fill in the early biomedical company seed rounds of financing probably because their net wealth has gone down at least 40 percent. That is a challenge.

What are some of the challenges facing academic technology commercialization? The often raw and early stage inventions are often prophetic inventions. There are no products developed as yet. We have to look beyond them to embrace technology platforms which have got multiple applications. We also have to be aware that most of the interesting opportunities often occur at the intersection of different disciplines. That's when multidisciplinary research comes in, eg at the interface of engineering and biomedical sciences.

How do we commercialize early stage technologies? What should be the right commercialization models to look out for? Often in the academic environment, Licensing and Spin-Outs are the two most common commercialization routes. How do we decide if the technology is suitable for Licensing or a Spin-Out? Licensing is invariably appropriate if the technology offers the prospect of a single product and if there is suitable licensing partner with both the competence and the commitment to advance the opportunity. Spin-Outs can usually pursued if the initial IP might provide a platform for exploitation across a wide variety of sectors and products, and the realization of the IP

value relies on the investment of financial and technical resources which are often quite substantial and beyond the control of the university.

For licensing, one has to review the intellectual property to make sure that it has the freedom to operate and one's intellectual property does not infringe upon others. We need to do a market analysis to define what exactly is the product, how is it going to be positioned and the market to which it is targeted. We also need to add value to further develop the technology at the bench to ensure more value creation so that it could be licensed or spun out at a higher value. Accurate valuation is always a challenge, we need to have a reality check. The actual operating environment, approaches such as the cost approach and comparables approach, are all considered. It is also very important for the universities, together with industry, to have a prototype development fund, to help to advance the technology further along the bench.

Spin-Outs are often constrained by the lack of pre-seed and seed venture capital. It will be very helpful if there are incubator facilities in certain research parks to be able to give the entrepreneurs a very conducive environment for technology validation before commercialization. Finding the entrepreneur management talent is another challenge. It is often useful to stay virtual as long as possible, so as to reduce the burn rate. A business plan for developing the technology is of course also important. One of the biggest benefits and arguments in favor of Spin-Out companies is that it creates jobs and economic development. That is how a lot of biotech companies in San Francisco and Boston have spurred the development of the local economies there.

Entrepreneurs need a product development and commercialization plan. Firstly, we need to understand the market positioning of the technology that it is intended to serve, within the context of the industry that we are operating in. We need to have strategic positioning within the industrial value chain. This is known as Technology Intelligence and Comparative Intelligence (TICI). We need to look at the market needs and size. We need to look at how the product should be characterized in terms of performance, reliability, modifiability, cost of use and product support.

The IP strategy for market protection and commercialization is critical. For product development, the proof of concept validation is important. If you are developing a device to measure hypertension, for example, you want to have some animal models to proof that the concept is validated in these animal models before you move on to spend further funds to commercialize it further through product reliability testing and validation.

Regulatory strategy is also important. If your product is going to be a drug, the regulatory strategy is different from a medical device product. Other considerations include the planning of manufacturing productions, marketing, sales, distribution, and product reimbursement and payment.

I now like to highlight three Singaporean examples of fairly successful early stage technology commercialization. Veredus Laboratories produces molecular diagnostics assays for tropical infectious diseases. Their lead product is the Avian Influenza H5N1 Molecular Diagnostic test kit which detects a gene specific to the H5N1 strain under four hours. When I was a technology commercialization professional at the Agency for Science, Technology and Research (A*Star) in Singapore, our scientists Lisa Ng and Ren Ee Chee developed some Avian flu primers. We filed a provisional patent application on 10 June 2004 and converted that to PCT a year later. This provisional filing strategy allows you to log in your priority date and file "as it is" and gives you a one year timeframe to validate the technology further before deciding on further commercialization steps. If after one year, you cannot market the technology, you just don't convert it into a regular patent application as it probably has no commercial value. A*Star successfully found Veredus Laboratories, started by Dr Rosemary Tan, a former researcher in one of our institutes, The Institute of Molecular and Cell Biology, who decided to become an entrepreneur. We successfully validated the primers with Veredus, with the use of some tissue samples from infected birds from one of our neighboring countries affected by the bird flu pandemic. That helped Veredus to develop the world's first validated H5N1 bird flu diagnostic test kit, which has been evaluated by 6 independent countries, endorsed by WHO and has been sold and distributed worldwide. Timing is everything, and we were also lucky to find an entrepreneur who was motivated to work with us. If they are successful, we are successful. We also helped to publicize the product as it has our technology in it. The Avian Flu kits were eventually given as part of Singapore's Bird Flu Aid Package to Indonesia in Dec 2005. And that again created a lot of awareness for the company and was also one of the key factors why the company went on to clinch more contracts.

ProTherapeutics is a Spin-Out as opposed to licensing deal because it has a platform technology to generate a pipeline of products. It utilizes a patented prediction method, called the Proline Technology, to engineer small therapeutics peptides derived from the venom of poisonous snakes such as the King Cobra, for oral (sublingual) delivery. It also has expertise in optimizing the functional sites of those peptides to enhance efficacy and reduce toxicity. Initial funding came from Bio 1 Capital (Singapore Economic Development Board, EDB investment fund) and A*Star in Jan 2005. It also has expertise in optimizing the functional sites of those peptides to enhance efficacy and reduce toxicity. We filed a provisional patent application in June '04 and converted to PCT in June '05. The company was formed shortly after we have $2m of funding from EDB and $1m from A*Star. Through TICI and IP mapping, we found another complimentary technology from the Virginia Medical College in US and licensed that technology into the company. The intellectual property of the company was the result of extramural research funding from A*Star to NUS, where the ownership/title of the intellectual property belongs to NUS but A*Star has the first rights to commercialize that technology because of A*Star's research funding support. In return for the IP, NUS was rewarded with equity in the company.

Amaranth Medical is a Spin-Out of the Nanyang Technological University (NTU) in Singapore. It is a medical device company producing a biodegradable stent for peripheral and coronary vascular applications. Why was this considered an innovation? This is because Amaranth's stent is totally biodegradable unlike typical drug eluting stents which are basically springs covered with drug eluting polymers. The whole device is a polymer. How then can the stent be put in place? Where is the real innovation? It is not in the fact that the polymers are novel (in fact the polymers PLA and PLGA), but that the manufacturing method is novel. The polymers are laid down in multiple layers, and embedded with different drugs within those multiple layers. In addition, the unique structural and thermodynamic properties such as flexibility and temperature sensitivity, constitute in totality an innovative product. The company was founded by 2 professors at the NTU - Freddy Boey and Subbu. Both of them are material scientists. Dr Boey is a seasoned entrepreneur, whilst Subbu worked in a couple of Biotech companies in US, which gave him the entrepreneurial spirit. The Amaranth technology was developed over 5 years of R&D at NTU. The two founders pumped in their own money for the first year and tried to raise funds, before the university licensed the technology to the company and began real operations. Finally, in January 2006, the first $1m came in from EDB (Singapore Economic Development Board)'s Bio1 Capital, after which NTU decided to grant them a license for the technology. In September 2006, Amaranth managed to close a USD7.5 million Series A Financing led by Bio1 of EDB Singapore and a US VC called Charter Life Sciences in California. And with that money, they managed to recruit a full time CEO, Guy Heathers. Dr Guy Heathers had been the commercialization manager for UK's Cancer Research Campaign Technology which was the tech transfer office for the Cancer Research Campaign, the biggest medical charity in the UK. He was the person who wrote the first business plan for Cyclacel, a cancer therapeutics company formed by Sir David Lane, who discovered the p53 tumour suppressor gene. In 2003, Guy had moved from the UK to Singapore to help the National Cancer Centre set up its technology transfer office. So Amaranth had Guy Heathers to manage its money, its board and all the various contracts that are outsourced by the company to external contract researchers. He also had manage the company founders. Amaranth's very first business plan was focussed on commercializing coronary stents. When they got the first million from Bio1 Capital, they were told that there was a bad need for urethric stents, which led them to switch development into urethric stents. Eventually when Charter Life Sciences invested, a proper market study was conducted, in which feedback was sought from surgeons, and cardiologists. This gave the company a sense of what the market is really lacking. The answer that came back indicated that peripheral vascular stents are what is needed, and that is where the company is focused on currently. Therefore the takeaway lesson is that for early stage technologies, commercialization much depends on the market, investors and the stage of development of the technologies.

With that, I conclude my talk. Thank you for your attention.

Commercializing Traditional Knowledge and Biodiversity through Science

Miller, M.

Director, Project Management and Product Development, Biotropics Malaysia Berhad, Malaysia
info@biotropics-malaysia.com

Abstract

Malaysia is one of the most biodiversity-rich countries in the world. The richness of Malaysia's flora makes them unparalleled 'national treasures'. Further research holds the potential to transform them into commercialised high-value medicinal products and dietary supplements to enhance the quality of life. In addition to its rich biodiversity, Malaysia is also home to various ethnic groups, over generations they have acquired intimate knowledge of plants and practiced different forms of traditional medicine for healing and health maintenance. This presentation covers on the up surging trends on natural products and traditional medicines globally and the approach to develop new ingredients from the Malaysia's rich natural resources and heritage as well as promoting community development and sustainability.

Introduction

Since the ancient times, human have used plants to heal and cures diseases and improving health and wellbeing. Despite ancient nature of the tradition, medicinal plants still form the basis of traditional or indigenous health systems and still been used by the majority of the populations in most developing countries. Medicinal plants play a significant role in meeting the demands of the traditional medicine markets which are found both domestically in producing and in overseas markets. The evolution of healthcare - from nature as a cure, mankind moved to magic potions and onwards to the allopathic and synthetic medicine after World War II. There was even the belief that it is just a matter of "some years" to find a cure for all human ailments. But this promise of chemistry has failed, and when the pipeline of the big pharma companies becomes dry, the researchers turn back to natural products as a source to treat diseases and it become an important factor in overcoming current problems in healthcare.

Herbal products: multiple sub sectors

Herbal products industry has multiple sub sectors that include (Fig. 1):

- Ethical products - either unchanged natural molecules or precursors for synthesis of chemical mono substances. Examples would be the Vinca Alkaloids, Taxol, Lovastatine from fermented red rice as models for the statins, or Tamiflu against Bird flu, synthesized out of shikimic acid from star anise.
- OTC products – examples would be medicines for cough and cold, mild sleep aids like valerian, liver support like milk thistle or saw palmetto extracts for prostate ailments. The dietary supplements are quite similar to the OTC products, and depending on the various regulations in different countries, there is a degree of overlap.
- The culinary sector is another big contributor, and even exotic spices have a good market prospect due to the success of "ethnic foods". Asian cuisine is an example in becoming a part of a "normal" diet in Europe and the US.
- Nutraceuticals and functional foods, an area where every day new products are on sale, starting from herbal drinks with Ginkgo, Guarana or Ginseng to coffee with Tongkat Ali *(Eurycoma longifolia)* - which seems to be such a success story in Malaysia that now even multi nationals have put Tongkat Ali coffee on the shelves. Another example is the low calorie sweetener Stevia for in a renowned carbonated drink, where the plant extract is optimized with a biotechnology step to reduce the slight bitter taste of natural Stevia extracts.
- Cosmetics and cosmeceuticals; flavours and fragrance and natural colourance are another huge market for natural products with a very high growth rate.

Global herbal-based products market

Based on data of various sources it can be estimated that the total global market for herbals has a value of 83 billion annually for botanicals and 3 major contributors – the pharmaceutical industry, the nutrition industry and the cosmetics industry.

The pharmaceutical Industry is the biggest contributor, accounting for approximately 44 billion of this volume. Sales of drugs from herbal precursors are the main segment, accounting for an over 30 billion annually. The other contribution of the pharmaceuticals industries are 13 billion sales of registered herbal medicines - examples for that category would be the ginkgo and St. Johns Wort products on the German market or the Japanese Kampo medicines.

The second largest contribution with 25 billion comes from the nutritional industry, around 9-10 billion thereof are sales of herbal dietary supplements. Under this category the majority of botanical products is sold in the US, while the nutrition industry sells functional foods based on herbals with an even a bigger volume of about 15-16 billion retail value. Examples would be phytosterols for blood lipid lowering in shortenings or herbal drinks that contain Guarana, Ginko, Aloe, as well as the Malaysian Tongkat Ali coffee.

Finally the cosmetic and cosmeceutical industry also sells a wide range of products based on herbals, not only topical products for "beauty from outside" but also increasingly products similar to dietary supplements and aimed at beauty from inside, i.e. supplements to improve skin condition. This accounts for the remaining 14 billion of the global herbal market.

According to Nutrition Business Journal data from 2008, the "classic" herbs like Ginseng, Ginkgo, Echinacea, garlic, soy and St. Johns Wort have lost some of ground in the US, but others have made tremendous successes: On the winning side we find green tea extracts - sales propelled due to good scientific data on cancer prevention and also due to the use in weight loss and slimming formulas.

A very high growth rate was found for mangosteen, on one side due to a MLM marketing approach, but science has contributed as well and confirmed the antioxidant properties. High Antioxidant claims, well understood by the consumer nowadays, have helped another super fruit newcomer, Goji or wolfberry to come from zero to a 100 million business in the US.

An example of a traditionally used product is Maca extract from Peru, and it could secure a market share of 70 million USD within a few years.

Commercializing Malaysia's biodiversity and health traditions

Blessed with an abundance of bio-resources, Malaysia is among the world's top mega biodiversity-rich countries and has the oldest rainforest in the world. Malaysia also home to various ethnic groups and over generations they have acquired intimate knowledge of plants and practice different form of traditional medicine for healing and health maintenance.

A second unique situation comes from the confluence of cultures - Malaysia as a Melting Pot; – The Inhabitants of the Malays, Indian, Chinese, Arabian, Malay and Indigenous people have contributed to rich traditions which is especially true for the healing traditions found in Malaysia and have been utilized and valued in many different ways. Medicinal and aromatic plants (MAP) in Malaysia are often still used in traditional way and offer alternatives to expensive pharmaceutical products. At present, many of the raw plants materials are being gathered or collected from the wild forest, and some are grown in small farms.

Traditional knowledge provides the underpinning for successful ways of subsisting in what are often hostile natural environments. Indeed, there is growing recognition that traditional knowledge, technologies and cultural expressions are not just old, absolute and maladaptive. They can be highly evolutionary, adaptive, creative and even novel. Moreover, as a body of knowledge, customs, belief and cultural works and expressions handed down from generation to the next generations that strengthen the cultural identity.

Once we have identified interesting plants, based on pointers from traditional use, we start our "lead to shelve" process with the help of our strategic partners and their world-class technology platform that allows for rapid fractionation.

Once novel compounds are identified, very early in the development process we look at IP protection, if possible to draw from in silico data , (i.e. to claim novel chemical / at least for that species) possibly adding initial confirmation with bioassays.

Then we follow a pretty classical scientific way of development, and science is nowadays not only required in drug development but also to bring a new ingredient or supplement to international markets (Fig. 2).

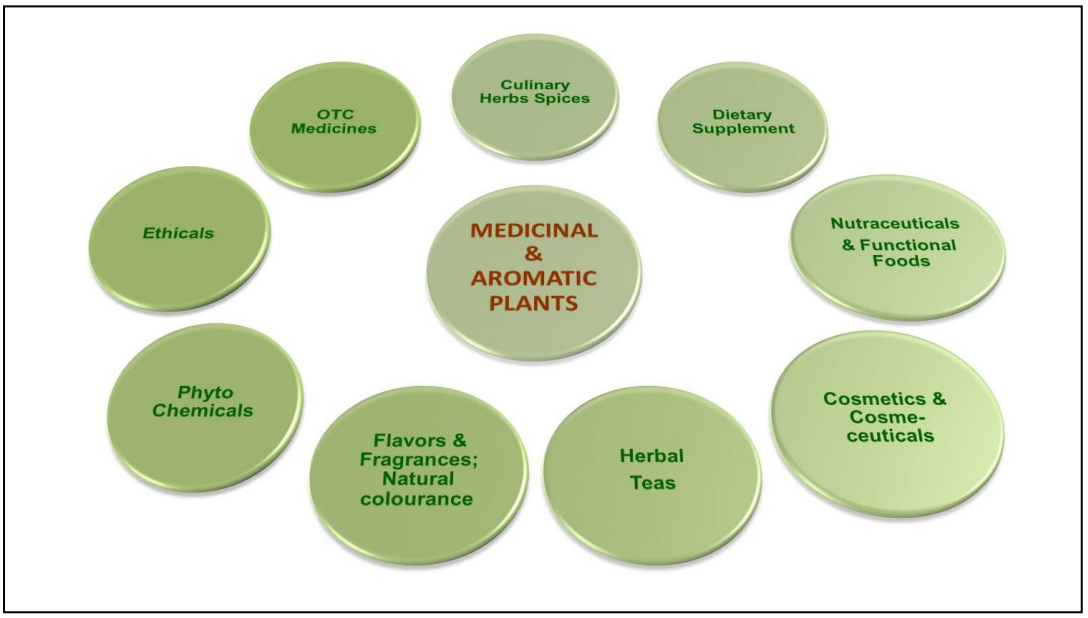

Fig. 1. Multiple sub sectors of herbal products

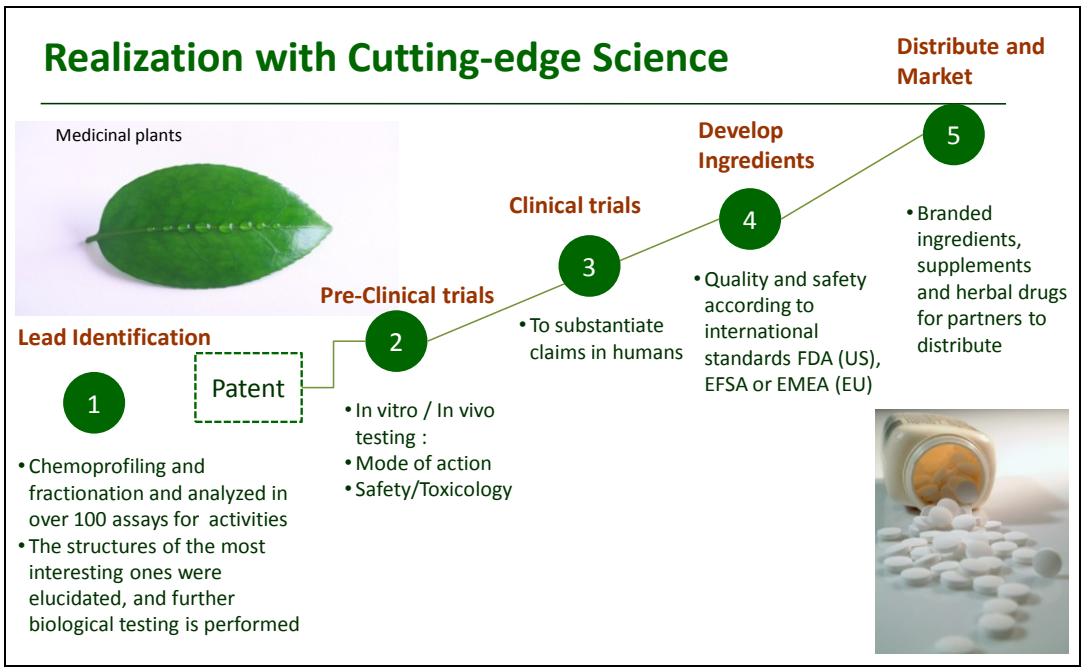

Fig. 2. Commercializing and Realization through Science

Community project with Orang Asli

As we derive many pointers and hints from health traditions, it is also important to return a fair share to the communities.

Although our first products are reaching the international market only by next year, we have already initiated a project with an Orang Asli community in peninsular Malaysia, as part of our commitment towards conserving the traditional knowledge and bio resources.

We put a special emphasis in participation of the younger generations, in order to contribute to the conservation and handing down of the traditional knowledge. And we also worked with lawyers and consultants to make sure that there will be a fair and transparent benefit sharing framework in place

The importance of science

The dynamic and demanding market of herbal products is always looking for:-

- Innovative ingredients that really work
- Safety, quality and traceability for credibility
- Proper files and documentation for claim substantiation
- Reliable supply and good quality"

According to a recent survey conducted, the studies found that advertising and scientific evidence were the main important factors for a successful launch of nutraceutical ingredients to market.

The success stories of cinnamon, green tea and mangosteen were several examples which became a block buster products were based on an in-depth scientific research in providing proof for its efficacy.

Conclusion

As a conclusion, today's trend towards natural ingredients has been driving the herbal products industry to the next level. Thanks to the increasing of scientific evidence and support from the state-of-the-art science research with a wide range of opportunities- from medicine, through functional foods to cosmetics and personal care. This scenario also helps to boost up Malaysia's herbal and its traditional knowledge to be recognized among the global market players as well as promoting community development and sustainability at the same time.

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Session 5
Food / Flavour Biotechnology

Genomics: Recent Breakthrough and Its Impacts

Yang, H.M.

Beijing Genomics Institute, Chaoyang District, Beijing, China
yanghm@genomics.org.cn

Abstract

Recent breakthrough in genomics, especially the next-generation sequencing technology with significantly higher throughput and lower cost, will revolutionise the whole field of life sciences, medicine and bioindustry by providing huge amount of basic and upstream information of genes and genomes. The new application of sequencing technology indicates that sequencing should be understood as an approach for mutation detection, structural analysis, digital gene expression profiling, epigenomics and metagenomics, as well as gene regulation studies. Beijing Genomics Institute (BGI) has just published the complete genome sequence of the first Asian individual (*Nature, Nov. 6, 2008*), and announced the working drafts of giant panda by *de novo* sequencing wholly with the new sequencers, and that of cucumber by a proper combination of Sanger and new sequencing technologies (Shenzhen, Oct. 11, 2008), as well as the pilot phase of the "International 1000 Genome Project", following its previous contributions to the genome sequences of rice, chicken, silkworm, pig and others, as well as numerous microbes such as SARS virus, AIV, *Streptococcus suis*, etc. Like any others in technology in history, the recent break-through in genomics provides the developing countries the opportunities to catch up and to apply it to their own biological research and bioindustries. We call for international collaboration of developing countries on genomics of species of scientific, economic and ecologic significance.

Congratulations

First of all, I would like to congratulate Sarawak Biodiversity Centre (SBC) on YOUR 10th anniversary. I am very honored to be invited to attend this historic event, the wonderful ceremony, and the excellent symposium! I wish YOU become a world known and globally influential biodiversity and life science centre! I am confident that YOU will!

The gift I brought here is something from China. It is well grown and harvested in China, even it was originated from the USA and Europe. I brought here a seed, once growing in China. I am confident the seed will grow even better in the bright sunshine on the rich soil, and grow into a big tree in Sarawak, in Malaysia. The seed I brought here as a gift is GENOMICS.

Breakthroughs in 2007

The Year of 2007 witnessed the global energy crisis, the global food crisis, and the global financial and then economic crisis. However, it is also a year of hope and opportunity, because we have learnt from history that technological revolution could save the world from such global crises, and we have found the right tools this time: innovative sequencing technology.

The Year 2007 was credited by *Newsweek* as 'A year of Miracles', comparable with the Year 1905 when Albert Einstein's findings laid the foundation for the development of physics in the 20th century. This is a year for biology reborn with breakthroughs in genetic sciences. The top three are next-generation sequencing technology, identification of variants for common diseases, and 'man-made' life.

Dr. Lee Silver, a professor of molecular biology at Princeton, writes in that article: "These findings are just a prelude to what's shaping up as a true conceptual and technological revolution. Just as physics shocked the world in the 20th century, it is now clear that the life sciences will shake up the world in the 21st."

According to Dr. Eric Topol, the head of the Scripps Translational Science Institute, "The study of the human genome and its variations has advanced to the point where there were more breakthroughs last year than in several of the past decades combined". This advanced understanding

of genetic variations provoked us to realize that we have moved from asking what in our DNA makes us human to striving to know what in my DNA makes ME.

Two pillars of genomics

Let's start with genomics and sequencing technology. In those years, the fast development of genomics attribute to two fundamental findings. The first is 'LIFE IS OF SEQUENCE'. In other word, genetic information is stored in and carried by sequence. This was asserted early in 1953, by Dr. James Watson and Dr. Francis Crick, "the precise sequence of the bases is the code which carries the genetic information." Although the discovery of DNA and its double helix structure empowered geneticists to analyze life at the molecular level, it is the sequencing technology that makes deciphering genetic codes possible. Despite the fact genetic factors interacting with environmental factors and jointly impacting on life and its evolution, the sequence itself has the primary fundamental influence on shaping who we are and who we are not.

The second is 'LIFE IS DIGITAL' as quoted from Dr. John Sulston, "the instructions for making a life from one generation to the next is digital, not analogue …". This understanding bridges life science and information science and makes computers powerful tools for decoding life. Thus, keeping those two principles in mind, it is predictable that the revolution in sequencing technology may accelerate every aspect of life sciences. One cannot help but ask 'What does it mean if a human genome sequence costs less than $1000?'

Once this was a question one dared to imagine, but thanks to the biotechnology breakthrough, it is highly likely that the bold assertion will be fulfilled in the couple of years. Dated back in 2003, the U.S. National Institute of Health launched the '$1,000 sequencing grants' to advocate researchers to invent new methods to decrease the price of sequencing a human genome down to $100,000 and $1,000. Scientists predicted that goal could be achieved around 2010.

The next-generation sequencing technologies, such as sequencing-by-synthesis and pyrosequencing, have resulted in successful products as Roche/454, Illumina/Solexa, and ABI/SOLiD sequencing machines. In the '$1000 Genome' competition, those three companies, Roche, Ilumna and ABI, are the first winners. However, their competitors, such as PacBio, Complete Genomics, VisiGen Biotechnologies and others, have been innovating single-molecule sequencing technology, nanopore sequencing technology and optical trapping technology, and will promote the 'next-next' sequencing machines on the market soon. It is no longer a dream of biologists, but a biotechnology revolution that has just got started. Obviously, the inevitable new round market competition will ultimately benefit genetic research.

The second biotechnology breakthrough, Human Genetic Variation, occurs in biomedical research, and was selected by *Science* magazine as 'BREAKTHROUGH OF THE YEAR in 2007. Employing the next-generation sequencing technology, and the 'SNPs' (single nucleotide polymorphisms) generated by the International HapMap Project, scientists developed the Genome Wide Association Study (GWAS) to capture genetic variations that specifically associated with certain diseases. Dr. Francis Collins once used a vivid metaphor to illustrate this approach, and I recall here: trying to identify a genetic factor in a disease is like searching for a lost key alongside the road in the dark, and the SNPs are the street lamps. The success to find the key depends on the lamp available. But now it is much easier to locate this key under bright lights from all street lamps. This method helps scientists identify a series of genetic alternations in a broad range of common diseases, including type 1 and type 2 diabetes, schizophrenia, bipolar disorder, glaucoma, inflammatory bowel disease, rheumatoid arthritis, hypertension, restless legs syndrome, susceptibility to gallstone formation, lupus, multiple sclerosis, coronary heart disease, colorectal, prostate and breast cancers, and susceptibility to HIV infection.

In fact, as early as in 2006, at the International Conference on Genome in Hangzhou, China, Dr. Collins predicated the day's coming, and urged biologists to employ sequencing technology to creatively conduct their researches. More importantly, 'these findings are being replicated and validated'. Therefore scientists can confidently claim that after nearly two decades hard work in human genome studies, we eventually start harvesting the fruits, and an era of 'hunt for genetic gold' arrives.

Moreover, a 'man-made' life was created at Dr. Craig Venter's laboratory in July 2007. At the early stage, this synthesized viral genome was made to function similarly to the naturally occurring viruses. The researchers then worked on identifying and synthesizing 'minimal' genome, a genome

contains no 'irrelevant' genes thus can most efficiently use each base of its sequence. The final step is to add new genes to the minimal genome, and make the cell produce proteins like biofuels and vaccines. The progress of synthetic biology proves again the two perceptions of life and the two pillars of genomics, 'LIFE IS OF SEQUENCE' and 'LIFE IS DIGITAL'. This third breakthrough in biology, however, raises not only new scientific questions, but also profound philosophical and ethical questions. In the near future, scientists, policy makers, and the general public shall work together to address the emerging questions, such as is it the time for us to 'rewrite the program of life', and 'life is what we make it'.

Besides the three significant breakthroughs, new applications of sequencing also vitalize bioscience research, in the fields of digital karyotyping, epigenomics, digital transcriptomics, and gene regulation studies. Pair-end sequencing makes detect copy number variations (CNV), such as deletions, duplications, translocations, inversions and other chromosomal rearrangements, effective and efficient. It challenges all the conventional techniques for karotyping such as chromosome-banding, FISH, even microarrays. Applying sequencing technology to gene expression studies can characterize new transcripts, discover alternative splicing, identify rare transcripts, distinguish gene family members, and at the same time, make a quantitative/digital profile for gene expression. Similarly, the utilization of sequencing in DNA methylation and DNA-protein interaction studies has advanced epigenomics and gene regulation research, respectively.

BGI's achievements in genomics

As much as we acknowledge the recent advances in genomics research, we cannot wait but to actively participate in this biosciences revolution. Ranked as the third biggest sequencing centre in the world, my institute, Beijing Genomics Institute, has co-initiated and participates in multiple-national and international projects. These include the International 1000 Genomes Project, the International Cancer Genome Project, the Metagenomics Projects, the Pathogen Genome Project, and the Tree of Life Project aiming at sequencing dozens of thousands of organisms..

After contributing to the International Human Genome Project by sequencing 1% of human genome, and to the International HapMap Project by 10%, BGI now takes full responsibility for the 'Asian Part' of the International 1000 Genomes Project. Its pilot project promises to sequence 180 individuals at the genome coverage of 4, 2 trios of 20, and deeply sequence 1,000 genes for more than 1,000 individuals. Till October 2008, the Pilot Project has generated sequence data of 3,838.57 gigabases (Gb), or more than 1,000 equivalents of the human genome size. Holding the HGP sprit of 'OWNED BY ALL, DONE BY ALL, SHARED BY ALL', all data generated by the international project are now freely available via ftp://ftp.1000genomes.ebi.ac.uk, and ftp://ftp-trace.ncbi.nih.gov/1000genomes/. On November 14, 2008, the Steering Committee announced the target of the next plan: by winter 2009, the International 1000 Genomes Project will sequence 1,200 people with the genome coverage of 4, and release high quality data accordingly.

Sensing the new race of human genome study, in 2007, BGI initiated the 'Yanhuang Project', aiming at sequencing 100 Chinese. On October 12th, 2007, the researchers in BGI-SZ successfully decoded the first sequence of an Asian individual, and completed Yanhuang I Project. This was the third sequenced individual human genome, after Drs. James Watson's and Craig Venter's genomes. Researchers also conducted variation analysis, ancestry analysis, and identified possible phenotype relevance. The work was published in *Nature* on November 6th, 2008. The first African genome was also published in the same issue.

To achieve the potential of the study of individual genome diversity, scientists need to sequence more and more individuals. Such initiatives have been taken, echoing the call of 'Genomes For All' made by Dr. George Church in 2005, "[The] new sequencing technology's potential to revolutionize research and bring about the era of truly personalized medicine means the time to start preparing is now." Dr. Church lunched the Personal Genome Project in 2005, and in June 2007, 10 participants voluntarily consented to put their DNA sequences, medical records and other personal information in a public database, and share the information with general public. This ambitious project has won support from the internet giant Google, and ultimately will recruit 100,000 American citizens in the study. Similarly, the J. Craig Venter Institute initiated a study to sequence approximately 10,000 genomes within the next ten years.

The second giant project that BGI devotes to is the International Cancer Genome Project. It has been widely accepted that human cancer is a genetic disease. On February 16, 2005, the NCAB Working Group on Biomedical Technology submitted a Report to National Cancer Advisory Board, to propose a 'Human Cancer Genome Project'. Consequently, the U.S. major federal funding agency NIH 'announced a $100 million down payment on a project expected to cost $1.5 billion over a decade', which was considered by *Science* magazine 'A big-biology moon shot'. This initiative is later known as the Cancer Genome Atlas (TCGA). I consider science should unify developing countries and developed countries, rather than increasing the gap between them. Since 2005, soon after NCAB published the report, I have been actively promoting globalization of the cancer genome project. In 2006, China announced the Chinese Initiative on Cancer Genomics, and on April 29, 2008, the International Cancer Genome Consortium/Project was launched by researchers from Australia, Canada, China, Europe, India, Japan, Singapore, Spain, the United Kingdom and the United States. It is estimated that more than 26 countries are applying to join this internationally cooperative research. This global cooperation aims at solving the mysterious correlations between genotype and phenotype in cancer, by analyzing 50 types of cancers. 500 samples will be sophisticatedly collected for each cancer's study. Researchers will analyze sequence variations, digital karyotypings, gene expression regulation, and proteomics/GWAS. The results will release rapidly on a public database managed by the Consortium.

The third project I would like to introduce is an EU FP7 large collaborative project: MetHIT Project. It is also known as 'the 2nd Human Genome Project'. Human microbiota, is the microorganisms that live inside (in cavities of the human body and even within human cells and on humans (surfaces). The human microbiome (metagenome) is the collective genomes of human microbiota, including the genomes of bacteria (the majority), archaea, yeasts, and viruses. Since the human microbiota have at least ten times more cells than in normal human body, the human microbiome can contain at least one hundred times unique genes than in human genome. Therefore analyzing human metagenome is an effective approach to detect environmental factors that contribute to human diseases. At this stage, the consortium plans to sequence the genomes of reference microbes and to generate metagenomic sequence data of samples from the human gut.

The last two major research projects undergoing at BGI are the Pathogen Genomes Project, and the 'Tree of Life', or 'the 100,000 Species' Genomes Project. In the last five years, BGI has released rapidly the genomes of vicious bacteria, like SARS-Cov, HPAIV-H5 and *Streptococcus suis,* soon after they triggered the epidemics. The work is a part of National Biosafety Project, and now proceeds to international collaborations on TB, AIV, and HPV research.

The Tree of Life Project is inspired by comparative studies of human genome project. As *Nature* editor remarked early on September 6th, 2007, 'a better understanding of DNA function will come only from generating data from diverse genomes'. Till 2008, GenBank has collected genomes of different species that equals to 80 human genomes. Working with other Chinese and international colleagues, BGI has sequenced and analyzed genomes of rice, silkworm, chicken, and panda. We have also joined the international plant-genome project Alberta Project, which aims to analyze genomes of 1,000 plants.

Currently, the Tree of Life Project at BGI includes genome projects of species like pig, oyster, duck, cucumber, maize and wheat. Those projects have not only deciphered the sequences of the organisms' genomes, but also answered scientific questions like 'why panda has an extremely small new-born rate and size'. The fundamental knowledge of the genomes of these animals and plants help biologists better understand their biological functions, and further contribute to the protection, cultivation and utilizing our bio-resources.

Opportunities for developing countries

The reasons that I am confident in the future of developing countries' genomic studies is that till now what geneticists have learned are from 'genes' (nobody has 'created' a gene yet), and that nature favors developing countries and gives us rich genetic resources. By building capacity in genomic research, the developing countries like Malaysia can develop their own bioindustries quickly. Once *Genome Technology* asked Dr. Béatrice Séguin at the McLaughlin-Rotman Centre for Global Health, 'why should developing countries invest in genomics', and she answered, 'with any technology that can improve health, if you don't invest in it, the divide between developed and developing countries

will only get bigger.' If you ask me the same question today, I would like to say that the rich resource of biodiversity can only be fully explored by genomics and by the researchers in their own countries.

Further more, if the genomic era is now a reality, just like Dr. Francis Collins claims, it should be characterized by using genome data in every bio-laboratory in the world. Quoted from Dr. James Watson, "all biology in the future will start with the knowledge of genomes and proceed hopefully.", I provoke here let us developing countries actively devote to the second wave of genomics and harvest the fruits of bioscience in the 21st century.

As Chinese proverbs say 'when you drink from the well, don't forget who helped dig it!', and that 'a help as small as a drop of water should be returned with a pond of spring.'

I would like to thank all the funding agencies that support my institute, and all our supporters, collaborators, international advisors, colleagues and friends, and all my young staff.

Genomics cannot be done alone, and I look forward to working with the researchers and scientists from Malaysia! Let us work together!

Biotechnology Applications to Ensure Food Security

Teng, P.S.

National Institute of Education, Nanyang Technological University,
Nanyang Walk, Singapore 637616
paul.teng@nie.edu.sg

Abstract

Global demand for 30 major food crops is increasing due to population growth and the demand for animal feed. New solutions are needed to grow more on less land, with less water and labor, and with reduced inputs like fertilizer and pesticides. The new knowledge from the "gene revolution", and embedded in the "Biology Century" provides much potential to utilize biodiversity and biotechnology to address the supply-side issues. Biotechnology applications to ensure food security by increasing the supply of crop-based foodstuffs include – Improving conventional breeding using marker aided selection, Diagnostic and early detection tools, Increasing knowledge of genetics and ecology (Biodiversity management), and Genetic engineering for improved traits using transgenes (genetically-modified or "GM" crops). Marker-aided selection requires that background information on the species genome be available and to date, several crop genomes, e.g. rice, have been mapped. Diagnostic tools developed using hybridoma technology and molecular knowledge have allowed early detection of pathogens and the production of clean seed stock for crops such as potato. It has also enabled early warning systems for disease management, such as for rice blast. Biodiversity management using biotechnology is best exemplified by the rice variety deployment system practiced in many provinces in China. GM, biotech crops have grown in area to > 114 Million ha worldwide in 2007, been adopted by > 12 Million farmers in 23 countries, and have generated proven benefits to farmers and consumers, and the larger environment. Fifty-three countries have formally approved over 670 food/feed products from GM crops after rigorous safety assessments. Global GM crop area is projected to increase to > 200 Million ha in > 40 countries by 2015. This technology is likely to continue making a vital contribution towards meeting the future demands for food, feed, fiber and fuel and concurrently reducing the need for more farm land.

Introduction

Global demand for 30 major food crops is increasing due to population growth and the demand for animal feed. New solutions are needed to grow more on less land, with less water and labor, and with reduced inputs like fertilizer and pesticides. The new knowledge from the "gene revolution", and embedded in the "Biology Century" provides much potential to utilize biodiversity and biotechnology to address the supply-side issues. A central issue in increasing the supply of food is crop yield. Genetic diversity within species have been exploited by plant breeders to increase the yield per hectare of the major food crops of the world, while knowledge on genotype by environment interactions has enabled the development of proper crop management technology. However, many of the world's staple crops like rice and wheat have experienced a decrease in growth rates of yield per hectare due to multiple reasons, after the initial yield gains of the "Green Revolution" (Teng, 2008, Ch. 10). This, together with declining land and water resources, have resulted in global shortfalls in recent years of major food crops, thereby threatening food security. Modern science and technology, among which is biotechnology, provide some solution to this problem. Biotechnology applications ensure food security by increasing the supply of crop-based foodstuffs and include a) Improving conventional breeding using marker aided selection, b) Diagnostic and early detection tools for reducing losses caused by pests and diseases, c) Increasing the knowledge of genetics and ecology for managing yield and losses (Biodiversity management), and d) Genetic engineering for improved yield and pest resistance traits using transgenes (genetically-modified or "GM" crops).

Marker-aided selection (MAS)

MAS requires that background information on the species genome be available and to date, several crop genomes, e.g. rice, have been mapped. The development of new varieties using MAS, while more accurate than the conventional approach of selecting using morphological or phenotype traits, is still a tedious and slow process. The International Rice Research Institute (IRRI) has developed pyramided rice lines carrying the genes for resistance to bacterial blight disease -- *Xa4, xa5, xa13, Xa21*. These rice lines have been used by breeders in The Philippines, India and Indonesia to transfer the resistance into local varieties before they were released to farmers. The IRRI has also successfully transferred *Sub*1 gene (submergence tolerance) through MAS into several varieties which are under evaluation at farmer's field in South and South East Asia (Darshan Brar, *pers comm*., IRRI). More recently, MAS was used at IRRI to transfer the beta carotene gene (a "golden rice" trait) into IR63 and BR29 lines as part of a large multi-country project.

Diagnostic tools

Plant diseases cause major crop losses, estimated to average 10-20% of potential production each year before crop harvest. Early detection of the disease agents (bacteria, fungi and/ or viruses), coupled with correct diagnosis, can help to reduce these potential losses with appropriate action. Diagnostic tools developed using hybridoma technology and molecular knowledge have allowed early detection of pathogens and the production of clean seed stock for crops such as potato (Teng, 2008). It has also enabled early warning systems for disease management, such as for rice blast. The development of diagnostic kits for plant diseases and detection kits for Genetically Modified Food (GMO) has been considered a subset of the field of molecular biology.

The era of modern diagnostic technology in plant disease management began in 1976 with the first application of the Enzyme-Linked Immunosorbent Assay (ELISA) technique to detect plant viruses. This was soon followed by the highly specific monoclonal antibodies (MAbs) to detect most classes of plant pathogenic microorganisms. Combining the speed and sensitivity of ELISA with the specificity of MAbs, the rapid and accurate diagnosis of plant diseases and detection of pathogens in a variety of substrates was made possible. During the mid-1980s, it became possible to detect plant pathogens in a very short time (i.e. 10 minutes). The molecular kits which enabled such quick detection could usually perform molecular diagnosis in a few simple steps. They could also be used by people who did not have prior specialized training in molecular diagnosis techniques. Diagnostics technology for plant pathogens uses chemicals, antibodies or enzymes to detect certain DNA/ RNA chain sequences in bacteria, fungi and other plant pathogens. Practical use of nucleic acid-based detection technologies in plant pathology was limited until the introduction of the polymerase chain reaction (PCR) assay in the late 1980s.

A decade later, development of more advanced technology resulted in assays that could be completed ten times faster. These assays were targeted at the detection of plant pathogenic fungi, including species of *Phytophthora*, *Pythium*, *Rhizoctonia* and *Septoria*, as a diagnostic method and for decision making. Information provided by the kits was used to select the appropriate fungicide and also indicate the appropriate time of application.

The use of such kits contributed to reducing the environmental impact of fungicides and is an important component of modern-day precision agriculture. However, the cost of developing such tests has been relatively high, making them available for only a relatively small number of applications in the developed countries, whereas most of the potential beneficiaries are in the developing countries with the most plant disease problems in their agriculture.

Biodiversity management

Biodiversity management using biotechnology is best exemplified by the rice variety deployment system practiced in many provinces in China. The literature shows that crop monocultures using a narrow genetic base in crop varieties renders these monocultures susceptible to disease pathotypes which have evolved to overcome the host plant resistance. Before the advent of molecular characterization tools, it was difficult and tedious to develop genetic maps of the host or the pathogen.

Marker technology has now enabled the mapping of gene populations in geographic areas, and allowed the deployment of host plant resistance genes in specific crop varieties, as a response to the prevailing pathogen virulence genes. This form of "managed co-evolution" is an example of using biodiversity for disease management, as shown by the rice blast disease variety deployment system developed in Yunnan Province and now used in other provinces of China. This work has received much international attention and recognition (Zhu *et al.,* 2000).

Rice blast commonly causes yield losses of 25-36% in Glutinous Rice and 26-50% in Upland Rice in China. In the first experiment jointly conducted by a team from the Yunnan Agricultural University and International Rice Research Institute, crop heterogeneity was introduced by planting genetically diversified rice crops in five townships in 1998 and 10 townships in 1999 in Yunnan Province, China. Monoculture control plots replicated within the diversified townships allowed the causal effect of diversity on severity of rice blast to be determined. Blast was 94% less severe on susceptible rice varieties planted in mixtures with resistant varieties than when grown in monocultures, and fungicidal sprays were no longer applied by the end of the two-year program. On average, approximately 1.18 hectares of monoculture rice would need to be planted to provide the same amount of grain as one hectare of a mixed population. In subsequent years, as more information of the genetic diversity of blast pathogen populations and the rice varieties became even better understood, it was possible to formulate more mixtures of rice crops (Zhu, YouYong *pers comm*., Yunnan Agricultural University). In 2000, 43,000 ha of mixture-plantings were done in 41 counties of Yunnan. In 2001, 107,400 ha of mixture-plantings were in 62 counties of China. In 2002, there were 314,368 ha of mixture planting in 102 counties, and in 2003, 543,151 ha in 201 counties. The area reached 606.671 ha in 224 counties in 2004. Over the seasons, varieties had 83-94% less blast when they were grown in mixtures of monoculture compared to single variety monocultures. Rice yield increased by 636.3 to 1119.3 kg/ha. A beneficial side effect too was that the conservation of traditional varieties increased with an increased number of such traditions being used in the rice mixtures, thereby also promoting biodiversity conservation.

Since 2000, a total area of 1.577 million ha have been planted with such mixtures in Yunnan, Sichuan and other provinces. The survey results from 202 counties showed control efficiency of blast between 57% to 86%, reduction of fungicide application between 56% to 62.5%, yield increased by 10.53 billion Kg of rice, and farmers' income by 13.17 billion yuan. The diversification concept has also been extended to control diseases and insect pests of other major crops in Yunnan, particularly wheat, barley, and broad bean.

GM biotech crops

The most impactful application of biotechnology in agriculture has been the development and deployment of genetically modified (GM or biotech) crops with improved traits for pest and herbicide resistance. Biotech crops are those with traits which are derived either using recombinant DNA technology. High quality seed of crop cultivars with the desirable genetic background still form the foundation for farming. Biotech crops have the capability, through new traits, to both raise yields as well as reduce losses (Teng, 2008).

There has been an exponential, double digit percent per annum growth in the global area of biotech crops since the first plantings on a commercial scale in 1996. GM biotech crops have been grown in area to > 114 Million ha worldwide in 2007, been adopted by > 12 Million farmers in 23 countries, and have generated proven benefits to farmers and consumers, and the larger environment (James, 2007). Commercial crop biotech products consists of different crop varieties possessing specific traits and in 2007, the global area grown with biotech crops was made up primarily of four crops – soybean, maize, cotton and canola (James, 2007).

Countries which have commercialized biotech crops in Asia have developed regulatory frameworks to review and approve such crops for planting and for their subsequent use as food, feed, or fiber. Although only four Asian countries *grow* a significant area of biotech crops, many more *import* biotech crop products for processing into food items. Fifty-three countries have formally approved over 670 food/feed products from GM crops after rigorous safety assessments. In Asia, countries which have formal regulations to approve the importation of biotech products are Japan, Australia, Korea, Philippines, China, Taiwan, Thailand, India, Indonesia, Malaysia, and Singapore. Many more countries import biotech food products but do not have the formal regulations. Also, in Asia, the public

sector investment in agribiotech R&D outweighs the private sector investment, and is estimated to be over US$6 billion per year.

Global GM crop area is projected to increase to > 200 Million ha in > 40 countries by 2015. The growth in demand for biotech seeds, as monitored by ISAAA, and reflected in the area planted to biotech seeds, has globally been in the double digit percentages per year since 1996. Farmers find value in biotech seeds as the traits reduce input costs (cost of insecticides, cost of labor for applying insecticides, cost of fuel to drive spraying equipment), increase yield and provide more flexible timing of spray operations (as with herbicide application). The global value of biotech seeds was estimated at about US$ 6.2 Billion for 2006, representing 16% of the $38.5 billion global crop protection market in 2006 and 21% of the $30 billion global commercial seed market (James, 2007). This technology is likely to continue making a vital contribution towards meeting the future demands for food, feed, fiber and fuel and concurrently reducing the need for more farm land. To date, only a small number of traits in five crops have been commercialized while the R&D pipeline shows hundreds of traits and many plant species.

Conclusion

Ensuring food security in the years ahead is a challenge for scientists worldwide as global human population is predicted to grow to >9 Billion by 2050. In Asia, food demand is predicted to exceed supply by 2010 unless measures are taken to significantly increase productivity (yield per hectare) and total production. Biotechnology on its own will not be able to ensure food security unless accompanied by efforts in crop, water and land management using other new technologies and experiential knowledge. There is no “silver bullet” solution and an integrated approach is needed. The inherent biodiversity in the tropical Asian landscape offers much opportunity to be exploited for this integrated approach.

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Fragrance Compounds of Pomelo (*Citrus grandis*) Blossom Flowers from Various Extraction Techniques

Ishak, M.A.M., Zakaria, S., Nordin, A.R. and Zakaria, Z.[1]

Faculty of Applied Sciences, Universiti Teknologi MARA Perlis, 02600 Arau, Perlis, Malaysia
[1]School of Bioprocess Engineering, Universiti Malaysia Perlis, Malaysia
UniMAP's Academic Complex-Jejawi 3, 02600 Arau, Perlis, Malaysia
azlanishak@perlis.uitm.edu.my

Abstract

Citrus grandis or pomelo flowers possess a characteristic of strong blended of jasmine- and orange-like fragrance. In this study, three different types of extraction techniques namely Solid Phase Micro-Extraction (SPME), Soxhlet extraction and hydrodistillation technique were used to extract the fragrance compounds from the flowers. Determination of the fragrance constituents was done by gas chromatography-mass spectroscopy (GC-MS). In the SPME method, a fiber equipped with 65 µm poly(dimethylsiloxane-divinylbenzene) was used and samples were permitted to achieve an equilibrium state for 20 minutes prior to fiber adsorption for 15 min at room temperature followed by desorption time of 10 min. The analysis showed the presence of volatile compounds such as 3.89% of β-myrcene, 61.69% of limonene, 13.89% of ocimene, 5.76% of linalool and 1.53% of naphthalene as the major constituents. In the Soxhlet extraction method however, the major compounds detected were limonene (9.41%), ocimene (1.62%), linalool (1.93%), cyclohexasiloxane (1.17%) and benzoic acid (2.92%). From the hydrodistillation technique, the major compounds found in blossom were linalool (15.93%), benzoic acid (10.02%), 1,6,10-dodecatrien-3-ol (6.80%), geraniol (6.43%), 2-furanmethanol (4.24%), indole (4.03%) and limonene (3.11%). This preliminary study shows that SPME offers better analytes extraction, simplicity and rapidness, and could be used directly without organic solvents.

Introduction

Citrus grandis or pomelo is the largest citrus fruit with thick peel that Malaysians usually consume as fresh fruits, juices or in cultural festivals. So far, fruit is the main production of pomelo plantation and listed among 16 major fruits for commercial plantation in Malaysia (DOA, 2008). There are other parts of the pomelo trees that can be utilized, for example the flowers. Pomelo trees have large creamy white flowers that consist of 3-7 thick petals. The advantage is those flowers are strongly scented and described as having a floral-, jasmine- and orange-like fragrance that people did not realize before. *Citrus* spp. has obtained great interest among researchers on their valuable compounds especially for flavanoid and monoterpenoid production. The monoterpenoids are the major component of many essential oils and as such, have economic importance as flavours and perfumes. No study had been reported on *C. grandis* flower compounds. As part of our interest on its fragrance, the compounds of this flower were examined. Demand for essential oils keeps on increasing for the utilization and consequent industrial processing that widely used in fragrances, flavours, cosmetics and pharmaceuticals. Most essential oils are obtained directly from the plant materials in small amount whose availability may be limited (Vanisree and Hsin, 2005). The use of plant materials however, cannot ensure the continuous supply due to shortage of land for plantation and increasing costs in agriculture sector.

Method of extraction is crucial in the study of natural products composition and the selection of the methods is usually based on how far the compounds can be maintained prior to analysis. Solvent extraction is among commonly used especially from the flowers compared to steam distillation. Hot steam usually would alter and destroy the floral accords that determine the characteristic of the flowers. SPME is a fast and easily conducted sampling device as described from many studies (Flamini *et al*., 2003). It is a solventless technique that is based on the partitioning of volatiles between the sample matrixes, the headspace above the sample, and a stationary phase coated on a fused-silica fiber. Furthermore, SPME permits the sampling of samples in considerably smaller amounts.

Materials and methods

Sample preparation

Flowers of *C. grandis* were picked from an established plantation and immediately submitted to volatile collection. Two different samples were prepared: (i) first sample which consisted of the whole flowers (including sepals, petals, stamen and gynoecium) and are referred to as blossoms. Three flowers were collected after flower opening and introduced into a 40 ml septum-cap vial and allowed to equilibrate for 20 min at room temperature before sampling; (ii) second sample which consisted a total of five buds was placed in a vial and allowed to equilibrate as described above.

SPME procedure

Volatiles from each sample were collected by means of the SPME technique. The SPME fibre holder and 100 µm PDMS were purchased from Supelco and fibre was conditioned in a hot GC injection port at 250^{o}C for 30-60 min prior to sample extraction. The SPME fiber was then introduced into the vial and exposed to the sample headspace for 15 min at room temperature. Once sampling was finished, the fiber was withdrawn into the needle and inserted to the injection port the GC and thermally desorbed with desorption time of 10 min at 250^{o}C using the splitless injection mode.

Soxhlet extraction

Fresh samples (about 20 g) were introduced into a cellulose cartridge, and extracted in a soxhlet apparatus with 200 ml of dichloromethane for 3 hr. After completed, solvent was rota-evaporated at 40^{o}C, then the extract was further dried overnight in an oven at 50^{o}C and left to cool to room temperature.

Hydrodistillation extraction

About 50 gram of fresh flowers (buds or blossoms) was placed in 250 ml round bottom flask which contained 100 ml distilled water. The mixture was heated up to 100ºC and the distillate was then extracted using three times 15 ml portions of dichloromethane. The organic layer was collected and drawn into a beaker which contained anhydrous magnesium sulphate for drying. The concentrated extracts were later analyzed using GC-MS system for compounds identification.

Gas chromatography-mass spectroscopy analysis

GC-MS analysis was performed on an Agilent Gas Chromatography model 6890N coupled to a mass selective detector 5973 inert. Compounds were separated on a cross-linked fused silica capillary column HP5-MS (30 m x 0.25 mm x 0.25 µm). The head pressure of the carrier gas helium (high purity) was 50 kPa. The temperature programme was set at an initial 40^{o}C, followed by an increase by 4^{o}C/min to 180^{o}C and lastly increase by 10^{o}C/min to 250^{o}C and held for 5 min. For component identification, fragrance compounds were identified by matching their mass spectra with the spectral library (NIST 02) with a resemblance percentage of above 90%.

Results and discussion

Fig. 1 represents GC-MS chromatogram of the fragrance compounds isolated by hydrodistillation and Soxhlet extraction from *C. grandis* flowers. This chromatogram illustrates that each technique was quite similar in the profile of the bud and blossom respectively. Using hydrodistillation, it appears that linalool (15.93%), benzoic acid (10.02%) and ocimene (7.37%) were analyzed higher in blossom compared to the bud. In buds, the higher compounds were geraniol (13.35%), farnesol isomer a (9.14%) and indole (8.20%). In Soxhlet extraction, limonene showed the highest percent in both bud (9.41%) and blossom (27.27%) followed by heptacosane (8.07-16.62%) and 1-hexacosene (2.06-2.79%). Besides, ocimene, linalool, indole, benzoic acid and 1,6,10-dodecatrien-3-ol also were among the high volatile compounds detected by both extraction techniques. Heptane, cyclotetrasiloxane,

cyclopentasiloxane and cyclohexasiloxane could only be detected in both bud and blossom by Soxhlet extraction. This situation possibly due to the dicloromethane that act as a solvent which is able to isolate terpenes from plant material and dissolves a wide range of organic compounds (Castro *et al*., 1999). Beside that, hydrolysis, thermal degradation, molecular rearrangements and loss of components due to harsh conditions during the distillation process might have occurred in hydrodistillation extraction (Kim and Lee, 2002). There are slightly fewer compounds present in the chromatograms of *C. grandis* flowers extracted by Soxhlet extraction compared with those obtained by hydrodistillation. Water is a polar solvent, which accelerates many reactions, especially reactions via carbocation as intermediates. In addition, the avoidance of water in Soxhlet extraction avoids the degradation of compounds by hydrolysis, *tran*-esterification, or oxidation, and hence there are fewer degradation products noted in the analysis (Lucchesi *et al*, 2004). In this study, the objective of using different kind of extraction techniques is to justify which technique is most suitable for the analysis of *C. grandis* flowers. This justification was made based on which technique can sample the highest concentrations of major fragrance compounds contain in the buds as well in the blossoms. Justification was also made based on the total compounds that can be analyzed. The concentrations of the major compounds were taken as a percentage of a peak area after analyzed with the GC-MS system. Upon using SPME for compounds sampling, it was found that β-myrcene, limonene, ocimene, linalool and caryophyllene were the major compounds detected. Limonene was the most prominent compound found in highest concentration both in buds and blossoms. This statement is also true for the other compounds such as β-myrcene, ocimene, caryophyllene and several others. The 100 µm PDMS fiber was reported as the most efficient fiber for sampling of volatile compound especially in flowers because of the good analyte detection. Furthermore, the fiber has limited production of artifacts and produced highest recoveries with oxygenated sesquiterpenes compounds (Kim and Lee, 2002).

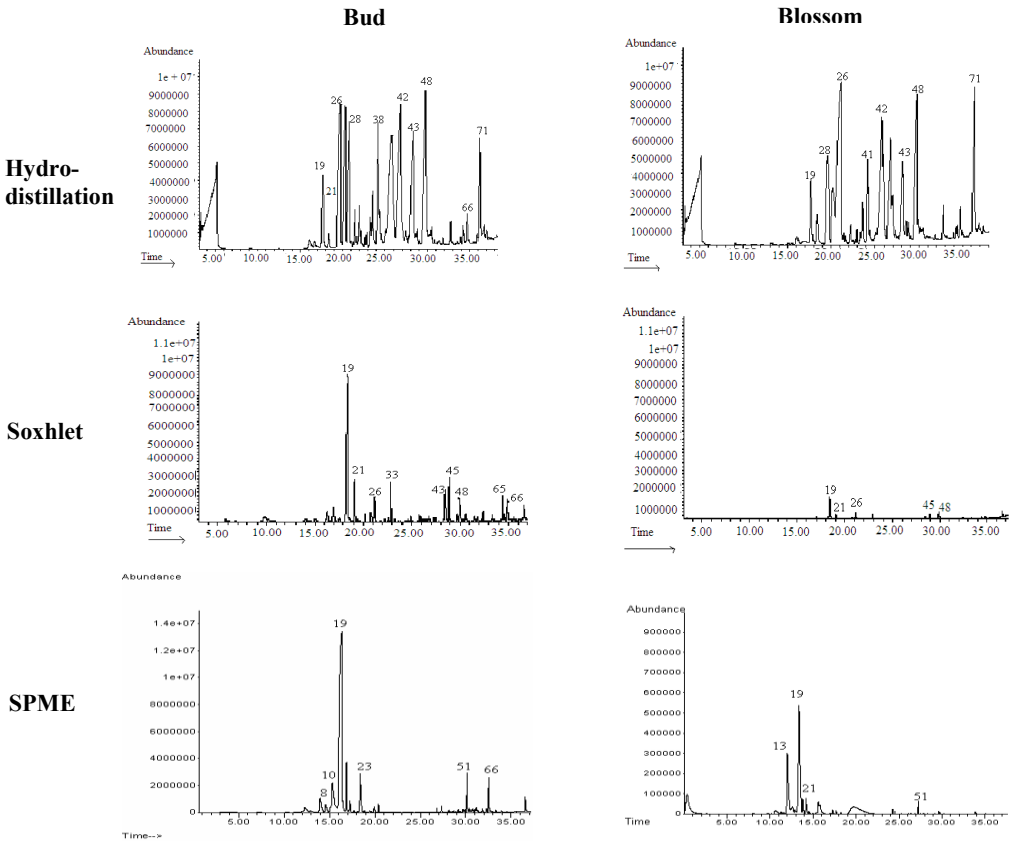

Fig. 1. GC-MS chromatogram of bud and blossom of *C. grandis* flowers using hydrodistillation, Soxhlet extraction and SPME extraction techniques.

Conclusion

As a conclusion, the use of different methods for the extraction of *C. grandis* flowers results in almost similar major compounds expected to contribute to the production of fragrance in the flowers. In terms of efficiency, SPME coupled with GC-MS represents a simple, time saving, highly sensitive and solvent-free, which has great prospects for the future use as an alternative to conventional techniques for the analysis of volatile compounds.

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The Effect of Processing Parameters on the Yield of Inulin in Sweet Potato (*Ipomoea batatas*)

Noor Azwani, Z., Nur Mahirah, S. and Mohamad, R.S.

Chemical Engineering Pilot Plant (CEPP), Faculty of Chemical and Natural Resource Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia
azwani@cepp.utm.my

Abstract

Inulin is a group of oligosaccharide (OS) that belongs to a class of carbohydrates known as fructan. Inulin is widely used as food ingredient in food industries due to its unique functionality. In western region, inulin is isolated from Jerusalem artichoke, chicory and dahlia plants. In Malaysia, there are a number of potential sources of inulin. In this research, sweet potato was used to obtain inulin. The inulin was extracted from sweet potato using hot water at 65^{o}C and 95^{o}C followed by fractionation step using ethanol-water at 35%, 65% and 95% (w/w). A series experiments were conducted to determine the effect of temperature and solvent concentration on the yield of inulin. The highest yield of inulin obtained from sweet potato was 6.02%w/w at concentration of 8.85 mg/mL. The optimum temperature for extraction was at 95^{o}C followed by using 95% ethanol-water. The results showed that a higher yield of inulin was achieved at the highest extraction temperature permitted using a higher ethanol concentration.

Introduction

Inulin, fructo-oligosacharides and oligofructose are fructans. These fructans are differentiated by their degree of polymerization (DP) which is defined as the number of fructosyl units linked to the terminal glucose (Sarkar, 2007). Generally, fructans are classified being either inulin (DP_n > 10) or oligofructose (DP_n < 10) (Waltz *et al*. 2005).

Both inulin and oligofructose are used either for their nutritional value or technical properties. Generally, they are often applied to improve organoleptic quality and a better-balanced nutritional composition (Franck, 2002). The variation in DP between inulin and oligofructose accounts for their different functional attributes. The functional attributes are prebiotic activity, indigestibility, energy value and potential in promoting health (Moerman, van Leeuwen and Delcour, 2004). Thus, the DP value plays an important role in contributing the unique functionality which benefited the food industries.

Inulin is less soluble due to the long chain length thus it helps to form micro crystals that form a smooth and creamy texture and provide a fat-like mouth feel. Due to its gelling characteristic, inulin is also suitable in application of low fat products without compromising the texture and taste (Franck, 2000). Others technological properties that inulin provides are as a fat replacement and as sweetening synergies (Causey *et al*., 2000). The effective water management, affect rheology and improve texture in food with synergistic action on high water binding hydrocolloids has allowed inulin to be used in wide range of food products application areas (Crow, 2000).

Besides providing technological properties, inulin also exhibits some nutritional properties. During digestion, inulin act as prebiotic, contributing the growth of bifidobacteria which may lead to better gastrointestinal health of the host. Due to these properties, inulin has been employed in food and pharmaceutical industries and the production of inulin has been associated to functional food and in nutritional composites. (Franck 2002).

They are a wide range of sources for inulin. Commonly, inulin is extracted from fruits, legumes and whole grains, soya bean, banana, onion, Jerusalem artichoke, chicory root and raw oats. For industrial production, sources of inulin come from chicory. In western region, inulin was isolated from Jerusalem artichoke, chicory and dahlia plants. In Malaysia, the use of local sources as inulin is not established even though there are a number of potential sources to be used. Hence, sweet potato is used in this research as the local source of inulin.

The objective of this research was to investigate the effects of extraction temperature and solvent concentration in maximizing the yield of inulin from sweet potato. In present, several methods for inulin extraction from Jerusalem artichoke have been developed. Many researchers have studied

the effects of different types of solvent and generally have used commercial standard grade inulin (Moerman *et al*., 2004). However, those parameters are mainly used to extract the inulin from Jerusalem artichoke, chicory and dahlia. Thus, several parameters are selected to determine yield of inulin from sweet potato. Based on previous work, temperature and pH of extraction medium, extraction time, solvent:solid ratio were considered to be important factors affecting the extraction yield and inulin quality (Lingyun *et al*., 2007).

Materials and methods

Materials and standard

Sweet potato used in this study was obtained from the local market at Taman Universiti. Three different concentrations of ethanol which are 35%, 65% and 95% were prepared from pure ethanol. Standard inulin was purchased from Sigma Chemical Co.

Inulin concentrated solution

To extract inulin, 400 g of ground sweet potato was added to 400ml deionized water. The sample was then divided to two portions and boiled at 65°C and 95°C for 30minutes. The resulting extracts were filtered through muslin cloth then each portion was divided to three parts that contained approximately 100 ml each. Different ethanol/water ratios (35%, 65%and 95%) were added slowly to each fraction respectively. The solutions were kept overnight in a chiller at temperature of 4°C. The resulting solutions were concentrated by evaporation using rotary evaporator. The concentrated solutions were stored in the chiller at 4°C for quantitative analysis.

Determination of inulin content

To determine the inulin content, high performance chromatography was used. The concentrated solution was filtered via Whatman syringe filter 0.2 µm nylon membrane. The concentrated solution was then diluted 10 times before analysis. The concentrated solution was analyzed by using high performance chromatography with RI detector (Waters 2410) using a Shodex series KS-806 column. The mobile phase used was 0.005M Sodium Nitrate using a flow rate of 0.80ml/min. Peak areas were converted into concentration using the inulin standards. A series of inulin solutions of known concentration were used to establish a standard curve.

Results and discussion

Inulin concentrated solution

The concentration of the extracted inulin is a function of the temperature and solvent used. The precipitation layer was in a larger amount for sample treated with 95°C and 95% ethanol. The sample treated with 65°C and 35% ethanol showed the lowest precipitation layer. According to Silva (1996), inulin is soluble in water and the solubility is temperature dependent. Kim, Faqih and Wang (2001) reported that at 25°C, inulin was almost insoluble in water, but its solubility increased significantly with increased in temperature. Ethanol was used as the extraction solvent due to it food grade status. Another research by Simon (2002), acetonitrile and acetone precipitated more inulin compared to ethanol and propanol. However, due to safety reasons and food purposes, ethanol was evaluated to be the best choice for precipitation (Moerman, 2004). Ethanol also result in high recovery yields (Simon, 2002). Based on previous study by Yuoh *et al.* (2003), lower percentage of ethanol used for extraction will give low or none precipitation depend on the molecular weight distributions of the various inulin.

Determination of inulin

High performance liquid chromatography was used to determine the inulin content in the extracted. The result is shown in Table 1. The percent inulin yield was calculated based on the equation 1 as follows:

Yield (%w/w) [redacted] (Equation 1)

On the whole, the highest and the lowest inulin yield was 6.02%w/w and 0.88%w/w.

Table 1. Inulin extraction concentration at different condition

Sample		Area (mV.s)	Concentration Inulin (mg/mL)	Yield (%w/w)
Temperature (^{o}C)	Solvent concentration			
65	35%	131.3108	1.3	1.21
	65%	354.8663	2.4	1.62
	95%	30.1147	0.8	0.88
95	35%	479.0936	2.9	2.39
	65%	932.0133	5.2	3.43
	95%	1678.1882	8.8	6.02

Effects of extraction temperature on percent inulin yield

The graph in Fig. 1 shows the increment in inulin yield with the increase of extraction temperature for different ethanol/water concentration. The sample extracted at 95^{o}C with 95% ethanol result the highest yield (6.02%w/w). For 35% and 65% ethanol, there are observable increase in the yield obtained. The best extraction condition was at 95^{o}C at 95% ethanol-water. This result is similar with a research conducted by Singh in 2005. An increased in temperature provide a higher recovery yields.

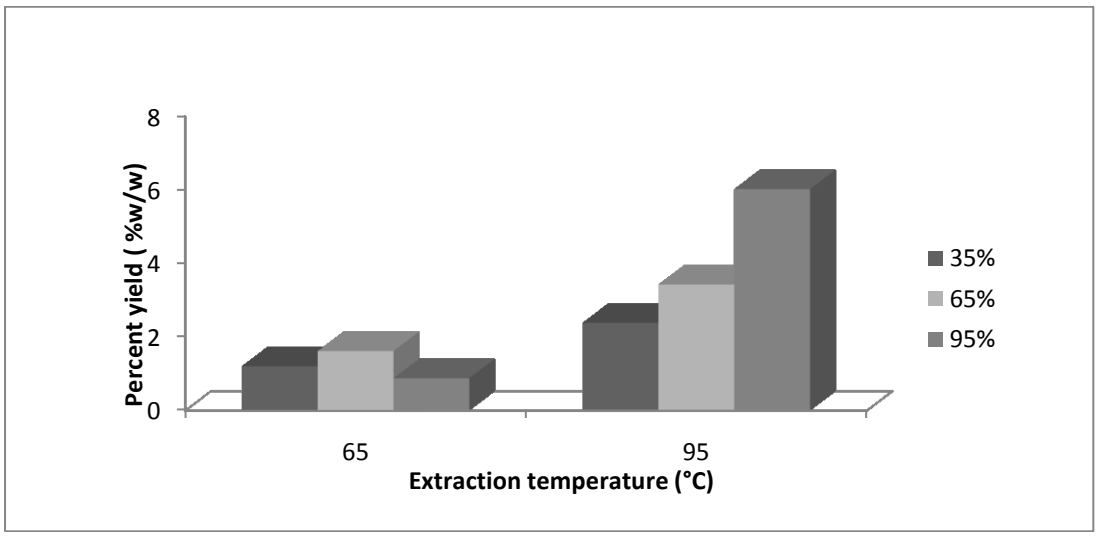

Fig. 1. Effect of extraction temperature on percent inulin yield

Effect of solvent on percent inulin yield

Fig. 2 describes the effect of ethanol concentration on yield of inulin. Generally, percent yield of inulin increases with the increase of ethanol concentration for both temperatures. At 95°C for 95% ethanol the maximum yield of inulin was obtained (6.02%w/w). However, at 65°C for 95% ethanol, a lower inulin yield was obtained. The reduction was caused by the minimum precipitation thus resulting in minimum inulin recovery. According to Simon (2002), the short chains with low degree of polymerization (DP) values may remained in solution while larger molecules were precipitated. At 35% ethanol and 95°C, the lowest yield of inulin (2.39%w/w) was obtained.

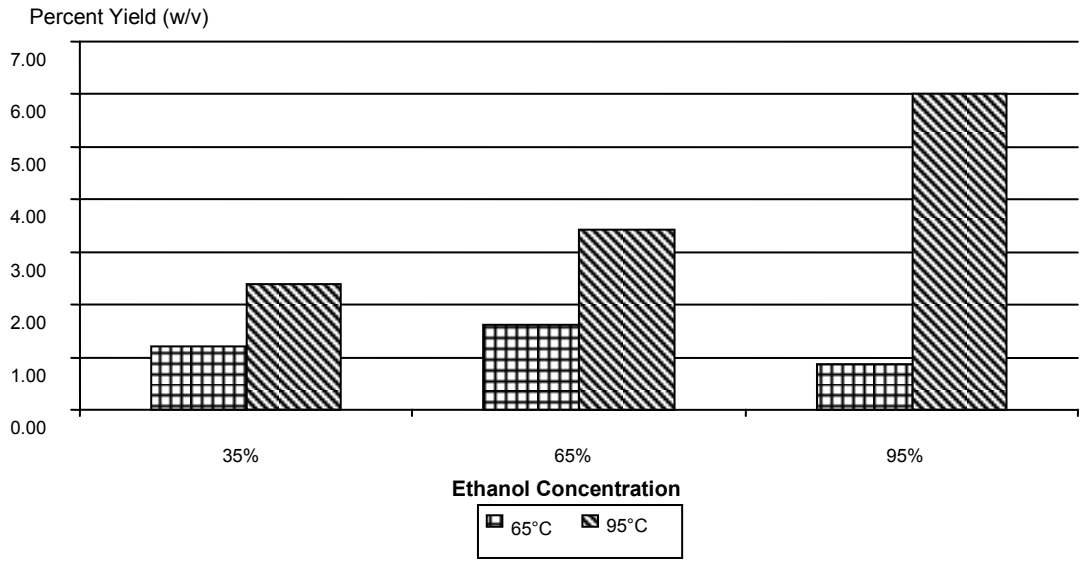

Fig. 2. Effect of ethanol concentration (%) on percent inulin yield.

Conclusion

The result obtained showed that the extraction temperature and solvent used in extraction and precipitation are effecting the inulin yield. Sweet potato provide 6% wt/wt inulin form which obtained from the parameter used. The value is comparable to the value obtained from Artichoke. Thus, sweet potato is one of potential sources for inulin.

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Traditional Knowledge Documentation in Sarawak: Benefit towards Food, Flavours and Fragrance Research

Naming, M., Tu, C.L. and Jovita, E.R.

Sarawak Biodiversity Centre, KM 20 Jalan Borneo Heights, Semengoh,
Locked Bag No. 3032, 93990 Kuching, Sarawak, Malaysia
tk@sbc.org.my

Abstract

This paper shares the experiences of the Sarawak Biodiversity Centre (SBC) in documenting traditional knowledge (TK) and highlights a significant number of indigenous plants that are used as food, flavours and fragrance by the local ethnic communities of Sarawak. Traditional knowledge of useful plants from eight ethnic communities in 20 locations participated in the programme, and their knowledge on uses of plants was documented. The ethnic communities comprised of Bidayuh, Iban, Penan, Kelabit, Lun Bawang, Kayan, Kenyah and Selako. To-date, a total of 353 plants were documented for food, 33 plants were documented for flavours and 22 plants were documented for fragrance. As this is a preliminary documentation of useful plants, further scientific study of these plants will be carried out to identify, analyze and determine the beneficial constituents in the plants used for food and also, identify the active components in the flavours and fragrance plants.

Introduction

The term “Traditional knowledge (TK)” refers to knowledge possessed by indigenous people, in one or more societies and in one or more forms, including, but not limited to, art, dance and music, medicines and folk remedies, folk culture, biodiversity, knowledge and protection of plant varieties, handicrafts, designs and literature (Ragavan, 2001). Traditional knowledge of a community is in complete synchronization with their way of life. It is a dynamic entity constantly changing and evolving with generations and time. In many cases, such knowledge is often passed down through generations orally and seldom put in any formal form of documentation.

Traditional knowledge (TK) associated with biological resources is an intangible component of the resource itself. Over the past decade or so, biotechnology, pharmaceutical and human healthcare industries have increased their interest in natural products as sources of new biochemical compounds for drug, chemical and agro-products development (Mugabe, 1999). The decade has also witnessed a great interest in traditional knowledge. This interest has been stimulated by the importance of traditional knowledge as a lead in new product development.

It is estimated that between 25,000 and 75,000 plant species were used for traditional medicine globally but only 1% is known by scientists and is accepted for commercial purposes (Kartal, 2006). Of the 119 drugs developed from higher plants and on the world market today, it is estimated that 74% were discovered from a pool of traditional herbal medicine (Mugabe, 1999). The world market for herbal medicine has been estimated at US$60-US$80 billion and a great part of the modern pharmaceutical industry has been developed on the bases of medicinal plants discovered by indigenous communities (Chai, 2006; Kartal, 2006).

In Sarawak, the Sarawak Biodiversity Centre has been given the mandate to facilitate the preservation and documentation of traditional knowledge through proper recording or documenting techniques among the local indigenous communities in the State. This was spelt out in the Sarawak Biodiversity Centre (Amendment) Ordinance 2003.

There is great concern that many of the older generation in the ethnic communities in Sarawak who still retain traditional knowledge may lose the knowledge as a result of changing lifestyles, priorities, the availability of modern amenities and the diminishing dependence of indigenous communities on natural resources. This has made it increasingly important that traditional knowledge is documented by the respective indigenous communities and retained as heritage so that it will not be lost. Traditional Knowledge is a legacy to be handed down through generations that cannot be matched by any known material or monetary means.

This paper presents the experience of the Sarawak Biodiversity Centre in documenting Traditional Knowledge and highlights a significant number of indigenous plants that are used as food, flavours and fragrance by the local ethnic communities of Sarawak.

Materials and methods

SBC implements the Traditional Knowledge Documentation programme through the Journal Methodology depicted in Fig. 1. Before SBC starts the documentation process, SBC conducts awareness on the importance of TK among the community leaders and communities. This step was carried out by organizing a consultative meeting with the community leaders and/or the communities though the District Office or the Resident Office. After the community leaders had consulted and discussed with the other community members and there is a positive response from the community either through verbal or written communication, SBC would set a date to organize a Capacity Building Workshop with the Community. It is important to understand that a community must give permission for access to the knowledge.

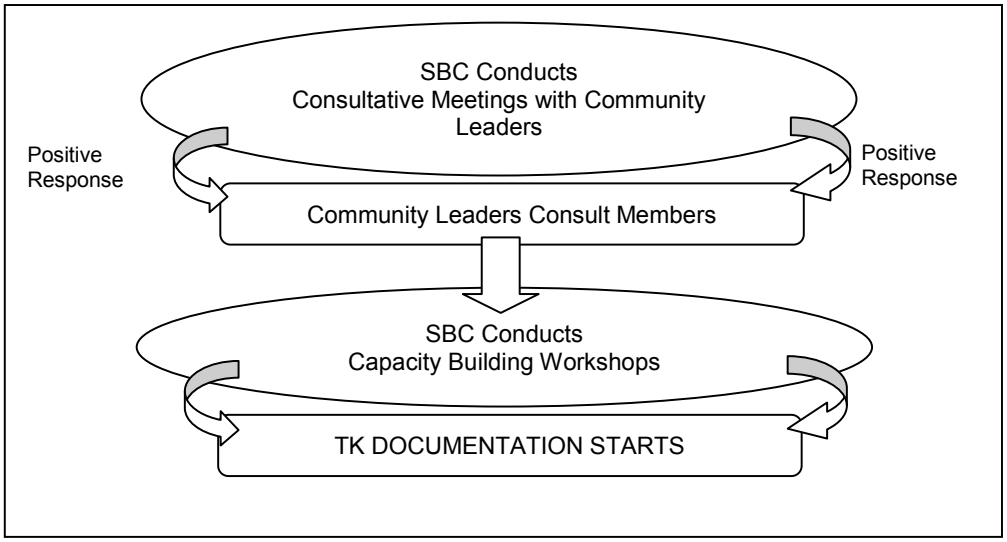
The Capacity Building Workshop, which is usually held over a period of three days involves the training of community participants in documenting their traditional knowledge through recording with a tape recorder, in written form in the field notebook and capturing the images of the useful plants with a digital camera. The workshop also provides guidelines in collecting plant specimens for herbarium specimen, for propagation and as raw materials for R&D. SBC also provides all the necessary equipment and tools for carrying out the documentation of traditional knowledge.

During the documentation process, the information listed in the table below is recorded:

Field Collection No. e.g. SABC 0001	Habitat
Location	Habit of Plant
Date	Characteristic of Plant – Height, Diameter at Breast Height, leaf, fruit, flower and others (depending on plant type)
Name of Plant (Local Name)	
Language	
Family	Uses
Scientific Name	Parts Use
Collector's Name	Method of Preparation
Type of Specimen – Propagation/Herbarium/R&D	GPS

Information recorded in the field was verified during the Plant Review Session. During this Session, spelling of the plant local name, its uses and method of preparation were recorded in detail.

Fig. 1. below summarizes the Journal Methodology used in carrying out the TK Documentation Project:

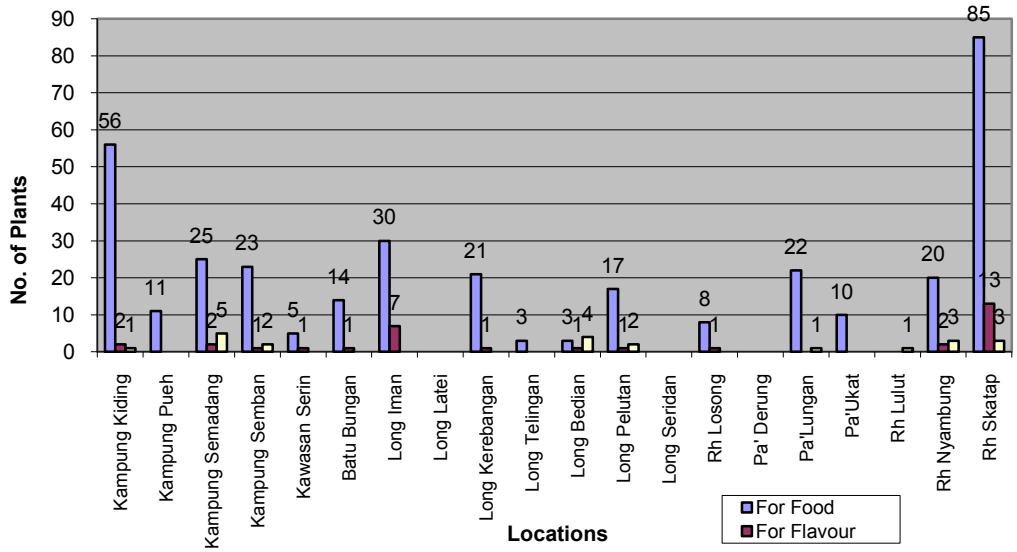


Results and discussion

The results of our study indicated that a total of 353 plants were used as food, 33 plants for flavours and 22 plants for fragrance. To-date, a total of 126 species have been identified for food, 7 species for flavours and 5 species for fragrance. Identification of plants is on-going and confirmation with experts would be undertaken by SBC.

The documentation of plants for food, flavours and fragrance was recorded from eight ethnic communities in 20 locations. The ethnic communities comprised of - Bidayuh, Iban, Penan, Kelabit, Lun Bawang, Kayan, Kenyah and Selako. Fig. 2 shows the number of plants collected from the various locations.

Fig. 2. Distribution of food, flavor and fragrance plants among the various locations in Sarawak.



The documentation of plants by SBC has shown that the Bidayuh community has documented the highest number of plants for food, totalling 34% (120 plants) and the Lun Bawang community has documented the lowest number of plants for food (7% - 24 plants). However, if we compare the documentation of plants used for food based on individual location, the record shows that Rumah Skatap, Betong which is an Iban community recorded a very high number of plants (85 plants) for food. The high number of plants documented for food among the Iban community is also reported in Christensen (2002). Christensen documented 184 species of plants for fruits, 54 species of plants with edible seeds and 214 species of plants use for vegetables. Fig. 3 shows the no. of plants documented for food among various communities.

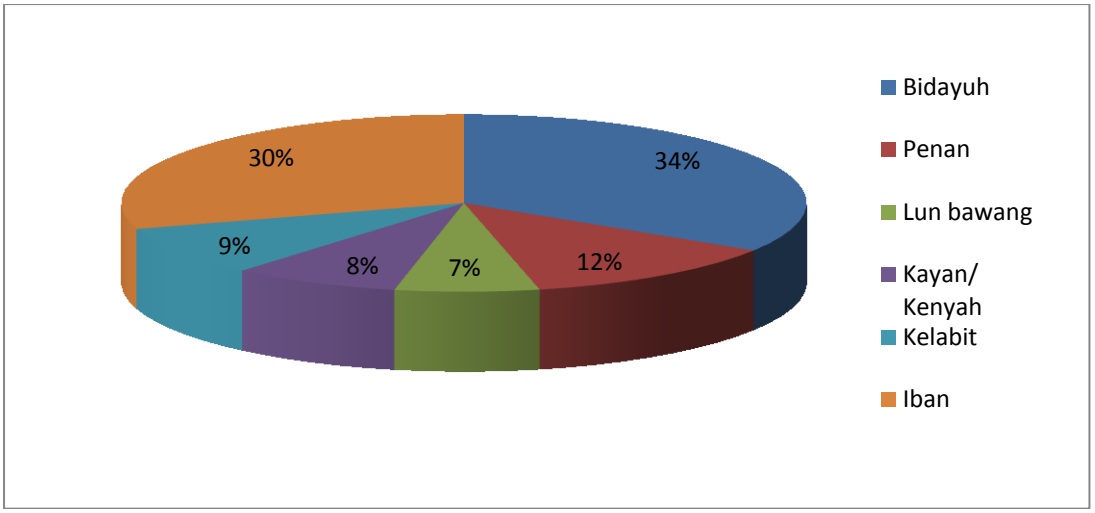


Fig. 3. Plants documented for food among the various ethnic communities in Sarawak.

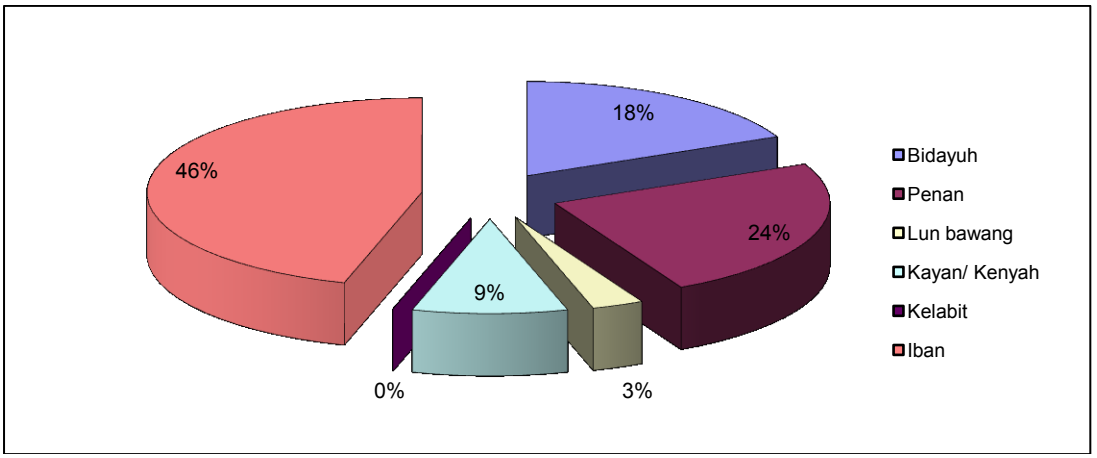


Fig. 4. Plants documented for flavor among the various ethnic communities in Sarawak.

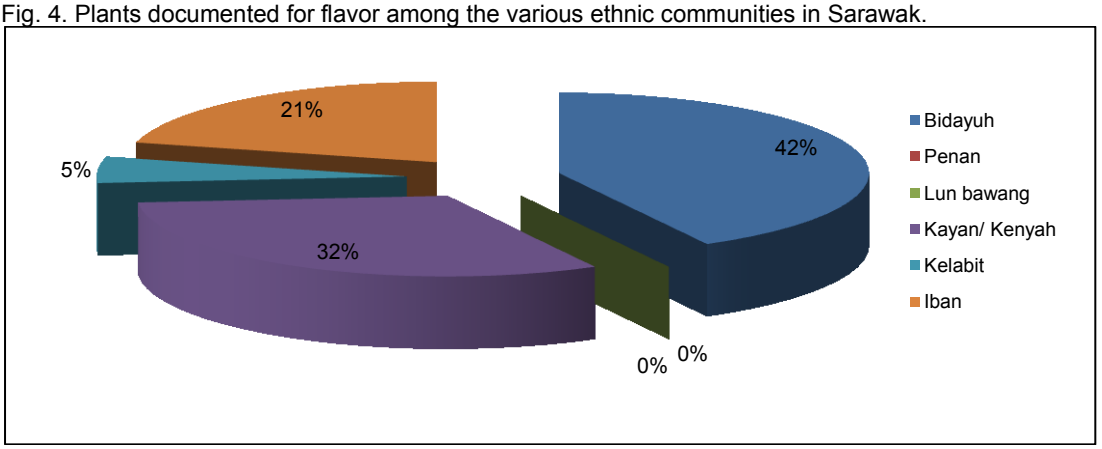


Fig. 5. shows plants documented for fragrance among the various ethnic communities in Sarawak

On plants for flavour, the Iban community documented the highest number of plants which is 46% (15 plants) of the total number of plants documented for flavour and the Lun Bawang only documented 1 type of plant for flavour and there was no record of plants used for flavour from the Kelabit. Christensen (2002) also recorded a high number of plants used for flavour by the Iban community (61 species) as compared to the Kelabit community (34 species). Fig. 4 shows the number of plants documented for flavour among various communities.

For plants used as fragrance, the Iban also documented the highest number of plants which is 42% (19 plants) of the total number of plants used as fragrance as compared to the Kelabit which has only documented 1 type of plant for fragrance. There was no record of plants use for fragrance from the Penan and Lun Bawang communities. Christensen (2002) also recorded 30 species of plants used by the Iban community for shampoo, soap and perfumes as compared to the Kelabit community where only 5 species of plants were used for perfume. Fig. 5 shows the number of plants documented for fragrance among various communities.

Based on our preliminary R&D work in SBC, we have identified various plants which could have potential for further R&D work and could be potentially developed as healthcare products.

Conclusion

As this project started with the documentation of medicinal plants and advanced with documentation of various uses of plants such as for food, flavour and fragrance, data recorded from the communities are not comprehensive. The amount of field work conducted at each site is also not consistent.

However, the data we have presented showed that consuming and utilizing wild plants for food, flavour and fragrance are still important activities among the ethnic communities of Sarawak. During this study, it was realized that Sarawak ethnic people's knowledge of the older generation is not transmitted to the younger generation. Therefore, if not properly documented, the knowledge would also face deterioration and 'extinction'.

Our study also strongly indicates that similar documentation must be conducted among the various locations and ethnic communities in Sarawak. Information from various locations and among different ethnic communities may show similar plants used in different communities, similar plants but with different uses and specific plants used in only that community.

As the preliminary research work has identified potential projects for product development, SBC plans to continue to identify, analyze and determine the various beneficial constituents and active compounds in all the plants within its depository and this could be potentially developed as healthcare products.

Acknowledgements

We are grateful for the continuous support and active participation of all communities under the Traditional Knowledge Documentation programme. We thank the technical assistance of Jugah Tagi and officers from Sarawak Forestry Corporation and Sarawak Forestry Department in plant identification work and for the use of the Sarawak Herbarium. We also appreciate the hard work contributed by all staff of the Sarawak Biodiversity Centre.

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Development of Bio-Health Products from Rice Bran Ferulates

Gururaj, B.[1], Maznah, I.[1,2], Ho, K,L,[1] and Nur Hazwani, R.[1]

[1]Laboratory of Molecular BioMedicine, Insitute of Bioscience,
[2]Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
gururaja78@gmail.com

Abstract

Rice bran, the major byproduct of the rice milling industry is also the source of high quality vegetable oil viz. rice bran oil (RBO). Rice bran oil has attracted much medical attention due to its strong hypocholesterolemic properties primarily attributable to its balanced fatty acid composition and high levels of antioxidant phytochemicals such as oryzanols, tocopherols and tocotrienols. Rice bran and rice bran oil have particularly been under investigation recently because of their potential benefits. Rice bran oil is also rich source of phytochemicals with nutraceutical and antioxidant properties and product development could certainly add value to these bio-resources that are available in abundance. Nevertheless, there is yet a study on the biological properties and identification of stabilized rice bran phytochemical rich fractions and biotransformation products from rice bran oil. The phytochemical rich fractions from RBO were successfully extracted using SFE and sequential extraction methods. Ferulate esters were isolated and purified using R-HPLC and Prep-HPLC and analyzed using LC-MS (Q-TOF). Antioxidant activity of ferulate rich fractions from RBO has been determined using DPPH method and anticancer activity of ferulate derivatives from RBO has been determined using MTS assay against HT-29 cancer cell lines. The extracted ferulic acid and its derivatives (campesteryl ferulate (**1**) and sitosterol ferulate (**2**)) were tested against human colon cancer (HT-29) cell line using MTS cytotoxic assay. Ferulic acid derivatives (campesteryl ferulate (**1**) and sitosterol ferulate (**2**)) exhibited significant cytotoxic activity against the tested cancer cell line with the IC_{50} values of 420 and 465 *μ*g/ml, respectively. These enriched fractions and phtytochemicals from rice bran could find application as antitumour agents and natural antioxidants for processed foods or as dietary supplements.

Introduction

Rice bran is the outer layer of brown rice kernel that can be obtained when it is removed from starchy endosperm during milling process. Rice bran is a rich source of vitamin E (0.1-0.14%) and has high concentration of oryzanols (0.9-2.9%)(Xu and Godber, 1999). Rice bran contains a unique complex of naturally occurring antioxidant compounds such as γ-oryzanol, ferulic acid esters and tocopherols and tocotrienols (Duve and White, 1991). These antioxidant compounds are beneficial in lowering cholesterol as well as preventing cardiovascular diseases (Lloyd *et al*., 2000). So far, there are limited research has been done on structural properties and bioactivities of fraction and ferulic acid esters in rice bran. Preliminary studies conducted in our laboratory showed that the commercial solvent extracted rice bran contained substantial quantities of phytochemicals such as oryzanols, tocols, and ferulic acid and its derivatives that has prompted us to develop an extraction protocol of these phytochemicals (ferulic acid and its derivatives) and enrich them through fractionation and confirm the chemical structures employing LC-MS analysis. Such enriched phytochemicals could find application as natural antioxidants and anticancer agents.

Materials and methods

Materials

Rice bran samples were obtained from a rice milling factory (BERNAS Sdn. Bhd, Malaysia). Standard compounds of ferulic acid, and ferulates were obtained from Sigma Chemical Co. (USA). Commonly used solvents for extraction, purification and HPLC grade solvents were purchased from Fisher Scientific UK.

Analysis of ferulic acid esters

The analysis was performed using LC-MS (Q-tof) system (Waters). Agilent Technologies Alltima-C18 column (150 x 4.6 mm, 5 µm) was used in the reversed-phase with isocratic mobile phase 5 mM ammonium acetate in acetonitrile at a flow rate of 0.25 ml/min. The UV detector was set at 320 nm. Compounds were diluted with the mobile phase and directly analyzed by LC-MS/MS with a 40-µL injection. Peak identification was based on comparison of RT values with authentic standards of ferulic acid esters.

Antioxidant activities of ferulic acid esters (DPPH radical scavenging activities)

Antioxidant activity was determined by the 2, 2,-diphenyl-2-picryl-hydrazyl (DPPH) method with some modifications. Antioxidant activity was expressed as percentage inhibition of the DPPH radical and was determined by the following equation:

$$AA\ (\%) = \frac{Abs_{control} - Abs_{sample} \times 100}{Abs_{control}}$$

Cytotoxicity assay

MTS cytotoxic assay was used to determine the anticancer effect of ferulic acid and its derivatives against colon cancer cell lines. The absorbance was determined at 550 nm using a 96-well microplate reader. The percentage of cell viability is calculated by using the formula:

$$\%\ \text{of Viability} = \frac{\text{Abs of Sample} - \text{Abs of Blank (media only)}}{\text{Abs of Control} - \text{Abs of Blank (media only)}} \times 100$$

Dose-response curves (% of viability *vs* concentration) were constructed to obtain the 50% inhibitory concentrations (IC_{50}).

Results and discussion

Confirmation of ferulic acid and its derivatives in rice bran by LC-MS (Q-TOF) analysis

TLC (Thin layer chromatography), and PTLC (Preparative thin layer chromatography) have been used to isolate and purify the ferulic acid and its derivatives from rice bran extracts. Ferulic acid and ferulates monitored at 320nm eluting at 20-55 min were detected. Ferulic acid (**1**) displayed its protonated molecular ion at *m*/*z* 195 ($[M + H]^+$) and lost one water ($[M + H - H_2O]^+$) to obtain its major daughter ion at *m*/*z* 177. Using negative-ion ESI-MS, campesterol ferulate (2) and sitosterol ferulate (**3**) yielded base peaks for the deprotonated molecular ions, and indeed there were no other significant peaks in their mass spectra. The ¢7-isomers of two major ferulic acid esters, campesterol ferulate (**2**) and sitosterol ferulate (**3**), have been identified at *m*/*z* 575, and 589 in rice bran extract and found to have shorter retention times than their ¢5-isomers on the C18 reverse-phase HPLC (Xu and Godber, 1999).

Antioxidant activities of ferulic acid esters of γ-oryzanol fraction from stabilized rice bran

In this study, γ-oryzanol components possessed significantly higher antioxidant activity than synthetic γ-oryzanol standard components and vitamin E components. The scavenging abilities on DPPH radicals were 24-methylenecycloartanyl ferulate (37.45 %), campesteryl ferulate (23.10 %), sitosteryl ferulate (21.23 %) and cycloartenyl ferulate (18.22 %). So the scavenging ability on DPPH radicals for the components of γ-oryzanol fraction from stabilized rice bran was effective in the order: 24-methylenecycloartanyl ferulate > campesteryl ferulate > sitosteryl ferulate > cycloartenyl ferulate. The

higher antioxidant activities of γ-oryzanol components may be due to their structure, which is very similar to that of cholesterol. The unique structure of 24-methylenecycloartanyl ferulate that is different from cycloartenyl ferulate, sitosteryl ferulate and campesteryl ferulate is a methylene group on C-24. The methylene group is attached to two alkyl groups, which may confer greater antioxidant activity than the alkene on the C-24 of cycloartenyl ferulate in the emulsion system.

Anti-cancer activity of ferulic acid and its derivatives

The isolated purified ferulic acid and its derivatives (campesterol ferulate (**2**) and sitosterol ferulate (**3**)) were subjected to MTS cell viability assay to identify compounds with cytotoxic activities against colon cancer cell lines (HT-29). Campesterol ferulate (**2**) and sitosterol ferulate (**3**) exhibited a significant activity against HT-29 cells with an IC_{50} value of 420 mg/ml and 465 mg/ml, respectively. However ferulic acid at a highest concentration of 500 mg/ml failed to exhibit cytotoxic effect against HT-29 cell lines. Failure of the ferulic acid to exert significant cytotoxic activity against HT-29 cell lines suggested that the absence of big moieties (campesterol and sitosterol) attached to ferulic acid side chain may have reduced its cytotoxic activity. It is noteworthy that the big moieties are important to enhance the cytotoxic activity (Puapairoj *et al.,* 2005) against the cancer cell lines. This indicates that the presence of campesterol and sitosterol moieties in the campesterol ferulate (**2**) and sitosterol ferulate (**3**) is important for expression of cytotoxic activity.

Conclusion

There is good potential for further value addition to rice bran through separation of high purity ferulic acid and ferulic acid derivatives from the extracts and fractions obtained from the process. It may therefore be concluded that rich ferulic acid derivatives and its fractions from rice bran could be a good application as natural antioxidants and anticancer agents reported here.

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Innovation in Producing Virgin Coconut Oil through Integrated Wet Process

Hamid, M.A., Sarmidi, M.R., Mokhtar, T.H., Rashid, N.M. and Aziz, R.A.

Chemical Engineering Pilot Plant, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia
mariani@cepp.utm.my

Abstract

Virgin coconut oil (VCO) is the new millennium discovery on usage of the ever multifunctional coconut. VCO is rich in the medium chain fatty acids (MCFAs) that have been shown to speed up the metabolism (Nandi *et al.* 2004). Almost 50% of the fatty acid in virgin coconut oil is lauric acid which is known as anti-viral, antibacterial, anti microbial and anti-fungal (Enig, 1998). The fatty acid profile in coconut oil shares a similar characteristics with breast milk. The MCFAs in coconut oil are not stored in the body cells but rather processed directly in the liver where they are converted immediately into energy (Berit and Jurgen, 2006). It therefore puts less strain on other organs of the body (liver, pancreas and digestive system), allowing them to function more effectively (Rethinam, 2008). The paper describes the process in producing virgin coconut oil through integrated wet process. The novel features of this process is the production of virgin coconut oil itself which can minimize the time, cost, energy and man power as well as can maximize the yield and improve the quality of coconut oil. The VCO obtained by this process contribute about 30-35% wt/wt of yield. The physical characteristics of VCO along this process shows that the VCO is colorless, retain fresh coconut aroma and sweet coconut taste with the highest content of lauric acid; 49.85%. Besides that, the result also indicates that vitamin E present in VCO. In general, the overall results for sensory analysis were acceptable in terms of aroma and taste of the product. The score is between 6.30 and 7.90 in hedonic scale which is like slightly and like very much. On the other hand, the results obtained from microbiological testing shows that the product is free from microorganisms. The performance of the product was also evaluated using several tests such as moisture content, proximate analysis and heavy metal content testing and was found comparable to the Malaysian and Philippine National Standard (PNS).

Introduction

The Philippine National Standards (PNS) defines virgin coconut oil (VCO) as the oil obtained from the fresh and mature kernel of the coconut by mechanical or natural means without the use of heat and chemical refining. PNS also describes VCO as colorless with natural coconut scent and free from all flavor and odor. The moisture in VCO must be at least 0.1% or less. VCO also should contain minor components such as tocopherols and tocotrienol, polyphenols, sterol and others (Kabara *et al*., 2008).

In the production of VCO, the yield and the retention of minor components are very important. In general, there are two types of process to obtain VCO; the dry and wet process. At Chemical Engineering Pilot Plant (CEPP), an integrated wet process has been used to produce high quality VCO. The integrated wet process offers shorter production time and higher yield oil retention of the minor components in the final product.

Materials and methods

Samples

Fresh *coconut meat* was procured from local supplier. The process begins with the mechanical pressing of coconut grated to obtain coconut milk. The coconut milk was then chilled to 10°C to break the emulsion for easier water and coconut butter separation. The chilled coconut milk was transferred to a mixing vessel where it was churned until coconut butter was heated to 45°C followed by centrifugation to separate the non-oil fraction from the product VCO. Finally the product was filtered to remove any suspended solid.

Integrated wet process

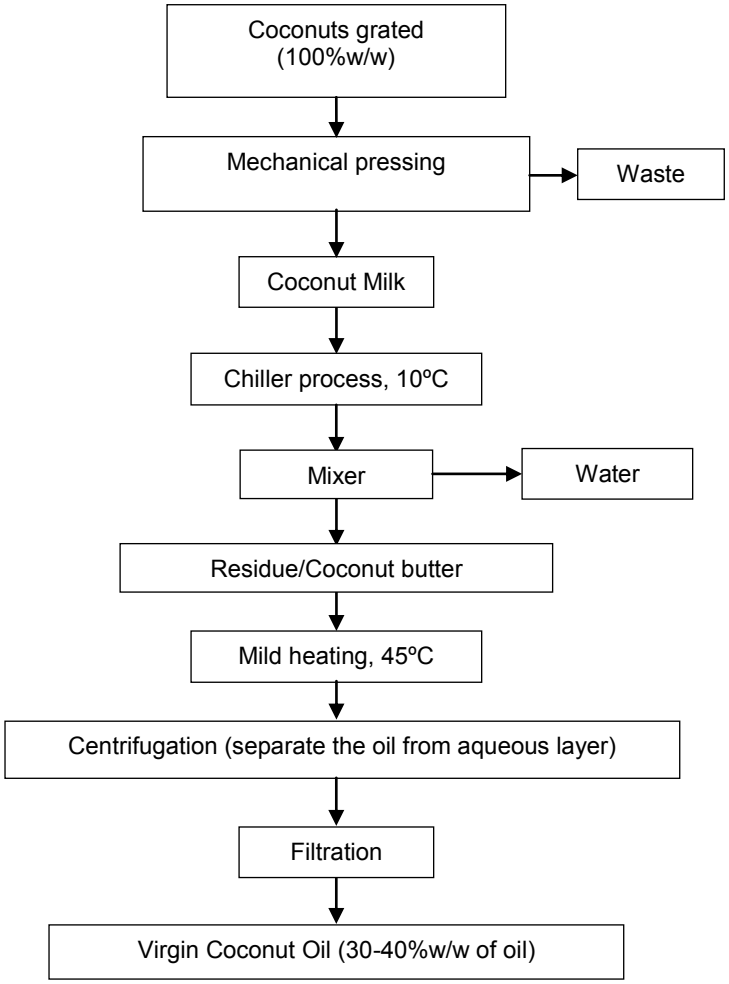

Fig. 1. Flow Diagram of Integrated Wet Process

Results and discussion

According to the fatty acids composition analysied, lauric acid (12:0) is the highest composition in the virgin coconut oil at 48.92%, which is much higher than fermentation process (46%). The result obtain from proximate analysis shows that VCO is an ideal food to be consumed due to the highest energy value of food. On the other hand, the microbiology analysis, indicate that there was no microbe or fungi in the virgin coconut oil. This is due to the effectiveness of the process which is clean and shorter time compared to the current practice.

Sensory evaluation test which was conducted at Chemical Engineering Pilot Plant, Faculty of Chemical Engineering and Natural Resources Engineering, Universiti Teknologi Malaysia shows that the VCO obtain from this process is acceptable and comparable with the commercial sample. For the aroma, VCO obtained from integrated wet process score about 7.90 which is “like very much” and 6.30 for aroma which is “like slightly”. The integrated wet process contributes to the fresh and sweet coconut aroma due to the shorter time in processing compared to the fermentation process. Table 1 shows the comparison data between integrated wet process and fermentation process of VCO.

Table 1. Comparison Data of Integrated Wet Process and Fermentation

	Integrated wet process	Fermentation process
Colour	Colourless	White to Yellowish
Aroma	Fresh coconut aroma	Sour coconut aroma
Taste	Sweet coconut taste	Natural coconut taste
Lauric Acid	48.92%	39.0%

Proximate analysis

Proximate analysis of the virgin coconut oil are shawn in Table 2.

Table 2. Proximate Analysis

Proximate Result	Percentage, %
Ash	0.05
Fibre	0
Protein	0.33
Fat	98
Moisture	0.1
Carbohydrate	1.34
Energy value of food	888.68 kcal/100g

Fatty acid content

Fatty acid composition of the virgin oil are shown in Table 3.

Table 3. Fatty Acid Composition

Parameter	Unit	Test Method	Test Result
C6:0 Caproic	% w/w	HPLC	0.47
C8:0 Caprylic	% w/w	HPLC	7.32
C10:0 Capric	% w/w	HPLC	6.29
C11:0 Undecanoic	% w/w	HPLC	0.02
C12:0 Lauric	% w/w	HPLC	48.92
C13:0 Tridecanoic	% w/w	HPLC	0.03
C14:0 Myristic	% w/w	HPLC	18.30
C16:0 Palmitic	% w/w	HPLC	8.62
C18:0 Stearic	% w/w	HPLC	3.23
C18:1n9C Oleic	% w/w	HPLC	5.65
C18:2n6C Linoleic	% w/w	HPLC	0.94
C18:3n6 Linolenic	% w/w	HPLC	0.10
C20:1 Eicosenoic	% w/w	HPLC	0.04
C20:3n6 cis-8, 11, 14-Picosatricnoic	% w/w	HPLC	0.08

Mineral content determination

Mineral content of the virgin oil are shown in Table 4.

Table 4. ICP-MS Analysis

ICP-MS Result	
Sample test result	
Lead, Pb (ppm)	ND
Mercury, Hg (ppm)	ND
Copper, Cu (ppm)	0.001
Zinc, Zn (ppm)	0.043
Arsenic, As (ppm)	ND
Iron, Fe (ppm)	0.047
Magnesium, Mg (ppm)	0.008
Nikel, Ni (ppm)	0.001
Cadmium, Cd (ppm)	ND
Chromium, Cr (ppm)	0.001
Cobalt, Co (ppm)	ND
Calcium, Ca (ppm)	0.116
Sodium, Na (ppm)	0.052
Phosphorus, P (ppm)	0.012
Selenium, Se (ppm)	ND
Aluminum, Al (ppm)	0.002
Silver, Ag (ppm)	0.002
Barium, Ba (ppm)	0.001
Potassium, K (ppm)	0.236

Microbiology analysis

Table 5 shows the data on microbiological analysis.

Table 5. Microbiology Analysis

Test Parameters	**Units**	**Results**	**Method References**
Aerobic Plate Count	cfu/g	ND (<10)	AOAC 990.12
Escherichia coli Count		ND (<10)	AOAC 991.14
Total Yeast and Mold Count		ND (<10)	AOAC 997.02
Salmonella	Absent/Present	Absent	FDA BAM Chapter 5
Staphylococcus aureus	cfu/g	ND (<10)	FDA BAM Chapter 12
Enterobacteria		ND (<10)	BP 2002

Conclusion

The virgin coconut oil obtained from integrated wet process give much more benefits especially in saving the time, energy and cost as well as improving the quality of virgin coconut oil. Furthermore, the use of a virgin coconut oil nowadays is becoming increasingly important in the developed countries due to health benefit and it's nutritionally wholesome. This research will lead to a new finding on developing the process in producing virgin coconut oil.

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Isolation of Acetic Acid Bacteria from Vinegar and Its Application for Over-Production of Natural Vinegar from Pineapple

Malek, R.A., Othman, N., Ramli, M., Mohd. Noor, N., Latif, S.I., Sarmidi, M.R. and Abd Aziz, R.

Chemical Engineering pilot Plant (CEPP), Faculty of Chemical and Natural Resources Engineering,
Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia
zalina@cepp.utm.my

Abstract

Vinegar is one of the oldest known fermentation products. Nowadays, vinegar application is not limited for culinary use but also find many applications in medical, nutraceutical and cosmeceutical industries. The production of vinegar is carried out either by natural fermentation of alcohol by acetic acid bacteria. The present work focuses on the production of natural vinegar from pineapple using newly isolated bacterial strain. The first part of this study was focused on the isolation of local strains from natural vinegar. Four strains of acetic acid bacteria were isolated and identified using biochemical identification method (API 120E) and APILAB Plus software (V.3.2.2). All strains isolated were found belong to *Acetobacter aceti*. The most promising isolate was deposited at CEPP culture collection under the name *Acetobacter aceti* WICC B-01. The performance of this new strain for acetic acid production was compared to the standard strain *A. aceti* DSMZ 3508. After 6 days fermentation, *A. aceti* WICC B-01 produced 3.97% acetic acid compared to only 2.95% produced by standard *A. aceti* DSMZ 3508 under a similar cultivation conditions. Moreover, the vinegar produced by the newly isolated strain was characterized by a pleasant fruity pineapple aroma.

Introduction

Pineapple originated from South America but is now widely distributed in the tropics and sub tropics. Notable producing countries are the Philippines, Thailand, Hawaii, Kenya, South Africa, Malaysia, Taiwan and Australia. There are three general groups of pineapple of economic importance, i.e., Cayenne, Queen and Spanish. In Malaysia the pineapple is mainly grown in Johore, Perak, Selangor, Terengganu and Sarawak.

In production of natural pineapple vinegar involved two distinct biochemical processes. The first process is brought about by the action of yeasts (*Saccharomyces cerevisiae*), which change natural sugars to alcohol under controlled conditions. This is called the alcoholic fermentation. The second process results from the action of a group of bacteria (*Acetobacter*) upon the alcohol portion, converting it to acid. This is the acetic or acid fermentation that forms vinegar. Proper bacterial cultures and timing are important and fermentation should be carefully controlled. As mention by Zahoor *et al*., (2006) there is a need to develop pure vinegar cultures for vinegar production instead of using a mixed culture due to uncontrolled conditions and contamination.

Although, there are two genera of acetic acid are known in the production of vinegar which is *Acetobacter* and *Gluconobacter. Acetobacter* bacteria oxidize ethanol more strongly compared to *Gluconobacter* bacteria (Tesfaye *et al*., 1999). Acetobacter commonly known as acetic acid bacteria that can oxidize ethanol to acetic acid. Commonly *Acetobacter* may cause root disease in apples, pears and pineapple, however its non-pathogenic towards humans. Sievers *et al*., (1992) and Entani *et al*., (1985) found that *Acetobacter* species, especially *A. aceti, A. Pasteuriansus, A. Polyoxogenes* and *A. europaneus* are commonly used in vinegar production as their oxidation for ethanol is better and they do not attack acetic acid later. The aims of the present study were to isolate a pure culture of *Acetobacter aceti* from local pineapple vinegar and utilization in production of natural pineapple vinegar. The comparison production of acetic acid between *Acetobacter aceti* DSM 3508 an *Acetobacter aceti* WICC B-01 isolated from local pineapple vinegar was also studied.

Material and methods

Isolation of acetic acid bacteria

The bacteria were isolated from local natural pineapple vinegar. The isolation medium composition: 20 g/l glucose, 10 g/l yeast extract, 20 g/l agar.

Morphological, physiological and biochemical properties

The morphological, physiological and biochemical characteristics were examined according to Bergey's Manual (Holt *et al*., 1994; Lu, 1999). The following tests were performed: overoxidation of ethanol; catalase production; growth in Hoyer's medium; oxidase test, indole test and formation of brown-soluble pigments on GYC agar.

Microorganisms used as referances strains

Acetobacter aceti DSMZ 3508 was used as reference strains.

Materials and strains for vinegar production

Pineapple was processed as follows. Pineapple's outer skin was peeled, after that cut into small pieces and pressed in a mechanical juicer. Pineapple juice was packed in a heat-sealed pouch and was promptly cooked in 90°C hot water. The cooked juice was then cooled, stored at -20°C. Prior to the experiments, the defrosted juice was filtered and then autoclaved at 120°C. Flocculating yeast *S. cerevisiae* was used for the alcohol fermentation in anaerobic condition. When final ethanol concentration was reached, yeast was removed by filtration followed by inoculation with aerobic fermentation for conversion of alcohol to acetic acid by *A. aceti* DSMZ 3508 and *Acetobacter sp* that was isolated from local natural vinegar. Stirred and aerated acetic acid fermentation was carried out at 30°C and 150 rpm in 500mL flask. The pH was not controlled.

Analytical procedures

Acetic acid analysis by using HPLC: Samples from the shake flask were centrifuged at 4°C, 6000 x g for 15 minutes. Supernatant was subsequently filtered through a 0.45 µm nylon filter into a glass vial. Then, samples were analysed using an Aminex HPX-87H column for 30 minutes, with a Reflective Index Detector using sulphuric acid 0.00125M as a mobile phase and a sample injection volume of 20 µL. Eluent flow was set to 0.7 mL/min.

Result and discussion

Morphological, physiological and biochemical properties

Fig. 1 shows the colonies of *A. aceti*.

Fig. 1. Colonies of *A. aceti* WICC B-01 on GYC agar

Based on the result of SIM test (Table 1), there is no *Gluconobacter* isolated from any sample as bacteria from this genus can produce hydrogen sulphide from the amino acid cysteine. However, the results for the bacteria $Y10^{-1}B$, $Z10^{-3}D$ and $W10^{-3}E$ correlated well with the characteristics of *Acetobacter* as outlined in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). For further confirmation, colonies of $Y10^{-1}B$, $Z10^{-3}D$ and $W10^{-3}E$ were each inoculated on Hoyer's medium and GYC agar plates. Theoretically, *Acetobacter* can grow on Hoyer's medium as it can metabolize ethanol, the selective component of the medium. Furthermore, according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994), *Acetobacter* do not produce brown water-soluble pigments on GYC agar. However, *Acetobacter* can easily distinguished such that clear zone or ring forms around the colonies on GYC medium indicating that calcium carbonate in the medium was dissolved by acetic acid produced by the bacteria as shown in Fig. 1. Therefore, as only colonies of $Y10^{-1}B$ had these characteristics, it can be concluded that the colonies belong to the *A. aceti* when analysis the biochemical test (API120E) using APILAB PLUS (V3.2.2) with 99.9% acceptance and this strain known as *A. aceti* WICC B-01.

Table 1. Result of biochemical tests for determination of *Acetobacter* and *Gluconobacter*

Characteristic	**DSMZ *A.aceti* 3508**	**$X10^{-1}$**	**$Y10^{-1}B$**	**$Z10^{-3}D$**	**$W10^{-3}B$**
Gram staining	-	-	-	-	-
Cell Shape					
Rods or coccobacilli	+	+	+	+	+
Straight or curved cocci	-	-	-	-	-
Overoxidation of ethanol	+	+	+	-	+
Catalase test	+	+	+	+	-
Oxidase test	-	-	-	-	-
Indole test	-	-	-	-	+
TSI Test					
Fermentation/ gas production	+/+	+/-	+/+	+/-	-/-
Acid Formation	+	+	+	+	-
Formation of brown-soluble pigments on GYC agar	-		-		
SIM Test					
Motility	-	-	-	-	+
H_2S formation	-	-	-	-	-

Notes: + = positives result; - = negatives result

Acetic acid production

The first step in the production of pineapple vinegar is the alcoholic fermentation of pineapple with *S. cerevisiae.* Cell growth, ethanol consumption, acetic acid production are shown in Fig. 2 and 3. The acetic acid bacteria, isolated from the local natural pineapple vinegar, were classified as *A.aceti*. The strain was catalase-positive, grows in Hoyer's medium and exhibited overoxidation of ethanol. The conversion of ethanol to acetic acid during the fermentation of alcoholic product obtained from the fermentation of pineapple juice by *A. aceti* WICC B-01 and standard culture of *A. aceti* DSMZ 3508 was compared and shown in Fig. 2 and 3. Fig. 2 shows that acetic acid began to produced immediately after the shift to aerobic condition. For both strains, ethanol was metabolised in five days of fermentation.

Acetic acid fermentation by *A. aceti* DSMZ 3508 in the alcoholic product of pineapple juice was compared with the fermentation of *A. aceti* that isolated from the natural pineapple vinegar. Fig. 2 clearly demonstrated that, high concentration of acetic acid was reached after three days of fermentation with 2.95% of acetic acid. The conversion of the alcoholic pineapple juice by *A. aceti* DSMZ 3508 to acetic acid was faster compared to fermentation by *A. aceti* WICC B-01 approximately three days with the product yield from the substrate and the specific growth rate (μ_m) was about 0.017g/l and 0.074 h^{-1} respectively. However the concentration of the acetic acid was lower compared to production of acetic acid by *A. aceti* WICC B-01.

The cultivation of *A. aceti* WICC B-01, in the alcoholic product of pineapple juice, the acetic acid concentration was increased gradually. The acetic acid concentration continued to increase progressively until around day 5 after inoculation and the ethanol concentration decreased but the cell density of the strain was maintained during 6 days of the fermentation process (Fig. 3). The highest production of the acetic acid was observed after 5 days of the process approximately 4.0% of acetic acid. The USFDA required that vinegar for commercialization should contain at least 4% acetic acid. Its clearly demonstrated from this research, *A. aceti* WICC B-01 is a potential as a strain for production of high quality of vinegar with the product yield ($Y_{P/S}$) 0.007 g/l and the specific growth rate (μ_m) was about 0.18 h^{-1} respectively.

Mineral contents

Table 2 compares the main mineral contents of the pineapple juices fermented by *A. aceti* WICC B-01 and *A. aceti* DSMZ 3508. The pineapple vinegar fermented by *A.aceti* strain was shown to have an extremely high potassium content, while the amount of sodium was rather low in comparison with malt and rice vinegar. The calcium, iron and magnesium contents were also higher compare to other types of vinegars. In conclusion, it was found that pineapple vinegar could be successfully produced by *A. aceti* WICC B-01 comparable with the fermentation of the alcoholic pineapple juice by *A. aceti* DSMZ 3508. This suggests that pineapple vinegar could be used as a nutraceutical food as suggested by Horiuchi *et al.* (2003).

Table 2. Mineral concentrations in pineapple vinegar and other vinegars (mg/l)

	Na	**Ca**	**Fe**	**K**	**Mg**	**Zn**	**Ref.**
Vinegar cultured A. aceti WICC B-01	102.1	27.0	7.1	632.0	113.4	0.9	-
Vinegar cultured with A. aceti DSMZ 3508	96	25.3	7.8	802.9	118.3	0.55	-
Malt vinegar	3100	20	1.0	80	10	0.6	Horiuchi *et al*., 1999
Rice vinegar	2900	20	1.0	60	50	1.9	Horiuchi *et al*., 1999

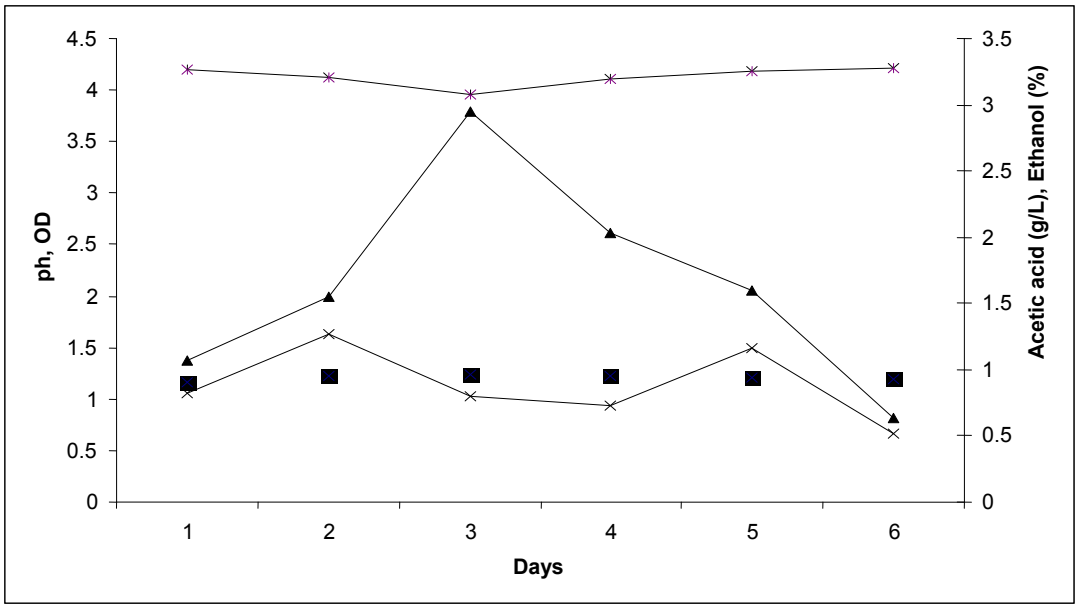

Fig. 2. Acetic acid (×), ethanol (▲) and cell density (▄) of *A. aceti* DSMZ 3508 during acetic acid fermentation of pineapple juice at 30°C.

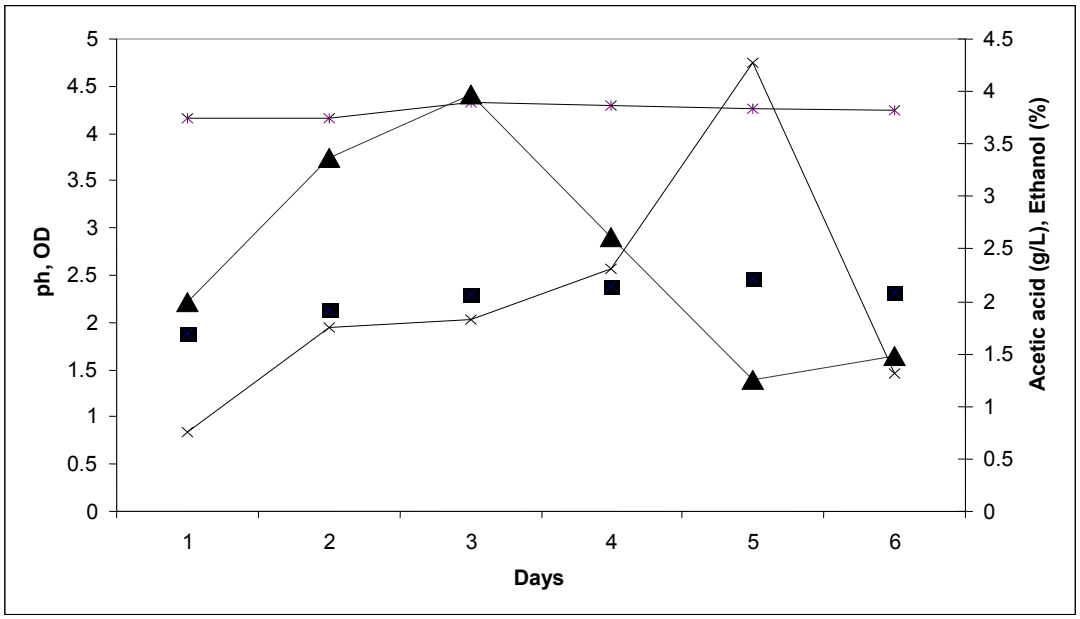

Fig. 3. Acetic acid (×), ethanol (▲) and cell density (▄) of *A. aceti* WICC B-01 during acetic acid fermentation of pineapple juice at 30°C.

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The Growth and Interaction of Yeast and Lactic Acid Bacteria in Dragon Fruit Fermentation

Azlina, M., Rosmawati, O., Hazniza, A., Sukirah, A.R., Musawirah, A.G. and M.S. Nizam, H.

Biotechnology Research Centre, MARDI Headquarters, Serdang, Selangor, Malaysia
dikna@mardi.gov.my

Abstract

From seven microbial strains isolated from naturally fermented dragon fruit, two strains of each lactic acid bacteria and yeast were selected as starter cultures for dragon fruit fermentation. The lactic acid bacteria (LAB), identified as *Leuconostoc mesenteroides* subsp *mesenteroides* and yeast were tested singly or in combination for their ability to enhance antioxidant activity in fermented dragon fruit. All types of fermentation showed an increase in the total colony forming unit (cfu) during the fermentation period. Highest number of total cfu was observed in fermentation of dragon fruit using yeast in co-culture with lactic acid bacteria ($4x10^8$cfu/ml); followed by yeast used singly ($3.5x10^8$ cfu/ml). The total number of microbial population in fermentation using lactic acid bacteria and in control was about the same with $2.49x10^8$ and $2.43x10^8$ cfu/ml, respectively. Two log cycles were observed during the fermentation of dragon fruit inoculated with co-culture of yeast and LAB which prolonged until day 14 of the fermentation. The increase of microbial growth in all types of fermentation was parallel with the pH changes that decreased to pH 3.8 due to lactic acid production. Better microbial growth observed in fermentation of co-culture of yeast and LAB, indicated positive interaction between yeast and LAB, which may enhance the production of metabolites such as antioxidants in the fermented dragon fruit.

Introduction

The red dragon fruit (*Hylocereus polyrhizus*), is most common among the dragon fruit cacti. They are well known all over the world for their high nutritional value especially in health benefits. Some of their great nutritional benefits are high in antioxidant amount and vitamin C levels, besides being low in calories due to their richness in dietary fiber. However, it cannot be kept long, has to be marketed and consumed in short period upon maturity. Therefore, many people have ventured into the various possibilities with this cactus, coming up with new ways to savour its various products like wine, health drinks and supplements.

For thousand of years fermentation has been used in most parts of the world as a low-cost and effective way of preserving food. Apart from its primary role, fermentation adds value and enhances nutritional quality and food properties such as improving its taste, flavour, texture nutritional value and shelf life (Gocheva *et al*., 2000). The biochemical modification of food products was the result of the activity of microorganisms and their enzymes which are present naturally (spontaneous fermentation) or inoculated (added with starter cultures) into the raw material.

Lactic acid bacteria and yeast are common predominant microorganisms that are present in most fermented food. Stable co-metabolism between LAB and yeasts was reported in many foods, which enabled them to utilize complex substrate and increase their adaptability to the food ecosystems. It was suggested that the proliferation of yeast is favoured by the acidic environment created by LAB while the growth of bacteria is stimulated by the presence of yeast which may provide growth factors, such as vitamins and amino acids (Mugula *et al*., 2002). The incorporation of certain LAB or yeast as starter cultures plays an important role in preventing the growth of undesirable microorganisms and contributes to desirable organoleptic properties due to the production of metabolite compounds.

Therefore, the aim of this work was to study the effect of using single LAB or yeast pure cultures, or yeast and LAB as co-cultures as starter cultures on the microbial growth and their interaction in the dragon fruit fermentation.

Materials and methods

Microbial identification

Samples isolated from natural pitaya fermentation of previous work were identified. Identification of LAB was carried out by examination of morphological and biochemical characteristics. The colonies were tested for Gram stain and catalase production. A commercial identification kit API50 CHL (BioMerieux, France) was then employed for identification of LAB, while API 20 AUX was used for yeast identification purpose. Methods of preparation were as described at the identification kit. Reactions were observed both after 24 and 48 hours. The biochemical profiles obtained were then compared with the identification table provided

Preparation of fruit sample

The commercially matured fresh red dragon fruit (*H. polyrhizus*) used in this study was purchased from a farm in Sepang, Selangor, Malaysia. Fruits were soaked for 30 min, washed using tap water to remove dirt and finally sterilized using disinfection tablets for 30 minutes. The fruits were then left air-dried on a sieve. The skin was peeled off and flesh was cut into 4 pieces before being blended into a fine slurry.

Fermentation condition

A volume of the blended pitaya (4L) was placed into 5L sterilised conical flask. Glacial acetic acid (1%, w/v) was added into the mixture and homogenised slowly. The fruit sample preparations were subjected to four different fermentation conditions; natural fermentation (Control/C) whereby no inoculum was added and the other 3 were subjected to inoculation of different starter cultures i.e. yeast (Y), lactic acid bacteria (LAB) (B) and mixture of yeast and LAB (1:1, v/v)(YB). The mixture was then incubated at 30°C for 30days. Sampling was done at time intervals to determine the microbial population, pH changes and organic acids produced during the fermentation.

Enumeration of LAB and yeast

Samples of fermented red dragon fruit (1ml) were suspended into 9 ml of sterile distilled water. A serial dilution was carried out and a 100μL of appropriate diluted samples were spread onto the MRS agar plates and incubated at 30°C for 48 hours and the total colony count was enumerated.

Chemical analysis

The pH was determined using a calibrated portable pH spear model Oaklon. The lactic acid content was analysed using method of AOAC (2000), based on sample weight (g/100g).

Results and discussion

Identification of LAB and yeast strain

Among the strains isolated from previous natural fermentation work (Sukirah *et al*., 2007), strain No. 4 was suspected to be a lactic acid bacteria strain which has showed a coccobacilli shape, gram positive and catalase negative. Comparison of the biochemical profile of strain No. 4 using API 50 CHL kit with the identification table provided by the manufacturer (Bio-Merieux) was matched with *Leuconostoc mesenteroides* subsp. *mesenteroides.* On the other hand, morphological observation of strain No. 2 showed oval shape yeast; always occur in clusters and budding laterally. The yeast was subjected for further identification using API20C AUX (Bio Merieux). Comparison of the reaction with the identification table did not 100% match with any of the yeast listed but nearest to *Candida kefyr*. Strain No. 2. will be sent to CABI, UK for further identification.

Enumeration of total microbial count and pH changes

There was an increase in total microbial plate count or colony forming unit (cfu) in all four fermentations during the fermentation period. Fig. 1 shows that the highest number of total cfu ($4.0X10^8 cfuml^{-1}$) was obtained in dragon fruit fermentation inoculated with co-culture of yeast and LAB. Second higher total cfu was obtained in dragon fruit fermentation inoculated with yeast ($3.54X10^8 cfuml^{-1}$) after 2 days of fermentation. While fermentation inoculated with LAB and in natural fermentation exhibited insignificant in total number of cfu with $2.49x10^8$ and $2.43x10^8 cfuml^{-1}$, respectively.

Two log cycles were observed in fermentation of dragon fruit with co-cultures which prolonged until 14 days of dragon fruit fermentation. The increase of microbial growth in all types of fermentation was parallel to the pH changes. The final pH in all fermentation flasks was reduced from pH 4.9 to pH 3.4-4.2 (Fig. 2), due to lactic acid production. The pH reduced concomitantly with the increase of lactic acid (data not shown). Despite the notable changes in fermentation pH observed in co-cultures, no significant pH change was observed in natural and LAB fermentation.

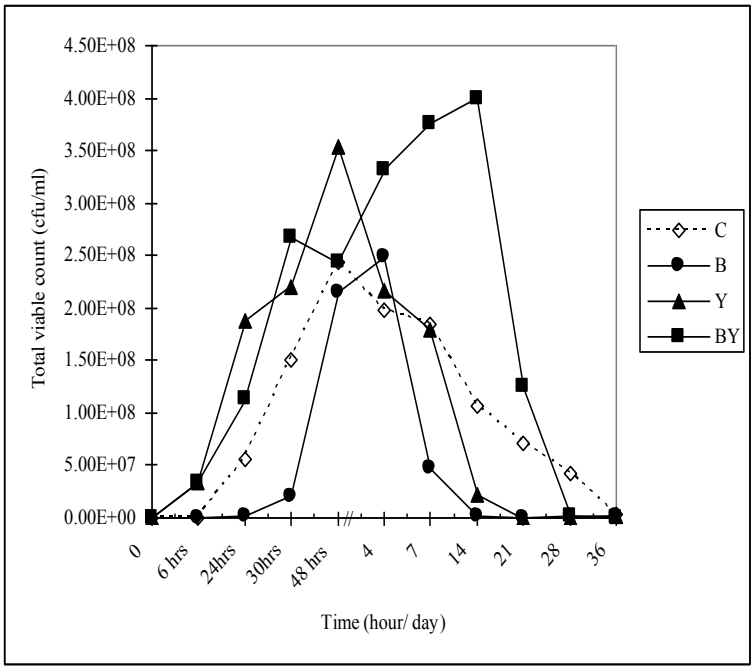

Fig 1. Total viable count during the fermentation of pitaya.

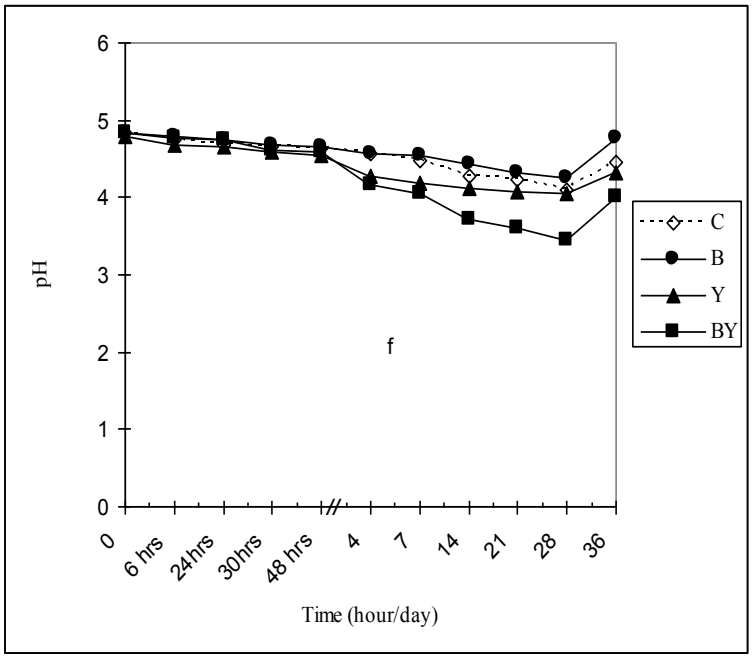

Fig 2. Changes of pH during the fermentation of pitaya.

The higher number of total microbial population and more lactic acid produced by co-cultures than the single yeast or LAB cultures, suggests some form of interaction between yeast and LAB. Lactic acid bacteria are known to be auxotrophic for some amino acids. It was suggested that the yeasts could provide vitamins and essential amino acids and enhanced the bacterial growth, while the bacterial end products could be used by the yeasts as an energy source (Leroi and Pidoux, 1993).

Conclusion

This study showed that the introduction of co-culture had enhanced the growth and microbial population in dragon fruit fermentation. The final pH and metabolite content were also affected in the co-culture. The higher population of microbes and enhanced production of metabolite compounds such as lactic acid in co-cultures of yeast and LAB were deduced as an indication of beneficial effect of the yeast on the LAB. The positive interaction in co-culture of yeast and LAB is believed to enhance the production of other metabolites as well such as antioxidants compounds in the fermented dragon fruit.

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Session 6
Industrial Biotechnology

Transcribed Speech

Industrial Biomanufacturing in Asia

Tan, K.S.

Springhill Management Ltd, Surrey, UK
office@springhilluk.com

Well. Good afternoon. It is a pleasure to be back. Thank you to the SBC for this kind invitation, a particular pleasure in that I was involved in a small way with a strategic paper that resulted in a laboratory being established. And I was just thrilled early on talking to some of the people here about the number of plants that have now been collected, classified and screened as well. It is particularly exciting each year I come back, to see the library getting bigger and bigger.

I will concentrate a little on what the SBC has asked me to speak about which is essentially bio-manufacturing which is one area I am engaged in and then say a little about the different ways that we see the industrial bio-manufacturing taking place, particularly from venture capitalist's point of view. And finally to say a little about the two facilities that we have, one in Penang on the Science Park and the other a company called Reliance in India with facilities in both Bombay and in Ireland. And these are the two bio-manufacturing plants that I am involved with.

First, we take a look at some of the trends that are going on within the biotech industry. And we have time today just to look at this particular one - the issue of R&D and manufacturing cost. Cost is a key factor driving the thinking on how to do bio-manufacturing. Any kind of manufacturing in our sector has to address at least three different issues. The first, incredible high volumes are needed. We are talking about gram quantity of very large complex protein being administered to patients. The task of producing kilograms quantity of pure proteins is extremely challenging.

At the same time we will also have to address the issue of cost. Currently some of our top selling antibody drugs are in the region of 15 to 30 thousand US dollar per patient. Multiply that across numerous patients and very quickly healthcare budget of most countries will simply go bust. And so there is terrific pressure on us in the industry to think of ways to reduce cost, to think of new technologies that can manufacture this high volume at lower cost all the time.

Thirdly, there is the matter of safety we are talking about complex protein that are injected rather ingested. And injection carries with it a whole series of extra stringencies that you wouldn't get with orally administered drug. So it is a big challenge for us in this field. Just to give you a sense of how much is going on. There are currently a thousand recombinant proteins in development. And when I say recombinant proteins we are talking about proteins that are produced in *E. coli*, yeast and particularly in CHO cells. There are a thousand of these in development, 500 of these in clinical trial. Approximately 140 are approved products now, and about 50 are waiting approval.

All these numerous number of products needs manufacturing. And the overlying lesson and message is there is a capacity constraint currently in the industry globally to produce these kinds of proteins. Obviously, *E. coli* is much easier to use for the simpler proteins. They grow a lot faster. The big challenges for us are with the big antibody based molecules. They are extremely challenging to produce and require mammalian cell systems. Global market is expected to be around 70 to 90 billions dollars and growing steadily and the projection is by that 2010, 50 percent of all new products launched will be biotech in nature.

So there is an ever increasing need and we don't have enough capacity to produce all these molecules. Looking at the projection here for new compounds coming through in 2010 - because of genomics and because of all all the new things that are going on in the field of science, we are expecting that something like three and half thousand new compounds would be discovered. All of these molecules need manufacturing. And so capacity constraint is probably the overlying message currently that we have because of the sheer complexity of manufacturing these molecules.

And one of the other trends now is to outsource. We are seeing a growing number of contract manufacturing organisations (CMOs) that are being set up around the world and increasingly in Asia. This is why the bio-manufacturing sector is a very interesting area for us in Asia. This is an area that is lower risk but one that addresses the international market.

These are some biomanufacturing facilities that have been being built in Asia in recent years. Quite a number of these are in Singapore. A-Bio is well known as a contract manufacturing organisation for clinical trial materials. Lonza has a 80-thousand-litre facilities that complements their other facilities in Europe and United States. GSK has large facilities and so has Scherling Plough. Just to give you idea on cost of building these facilities. In the region of 100 to 150 millions US dollars you wouldn't get any change out of this. There are other facilities going up. In Malaysia there are two facilities Inno Biologic, a small contract manufacturing house and Alpha Biologics. And I will say more about Alpha towards the end. In India, I will say a little about Reliance because I am an advisor to the board there. There are) Glenmark, Wockhardt, Biocon and Shanta Biotech and many others now. These slides are out of date. Many more plants are being built to address the capacity constraints that now exist globally.

One of the efforts going on is trying to see how we can extract more proteins out of the existing capacity, to look at enhancing technology that can amplify expression of these proteins in a fermentation system. And by far, the most established is the GS (Glutamine Synthetase) system patented by Celltech and passed on to Lonza. That has a very powerful increment in term of expression. Standard mammalian system produces anywhere from 0.2 to 1 gram per litre. With the GS system, consistently one sees 2 to gram per litre. So you can see this kind of increased expression. There are a number of these systems now being made available for licensing to try to increase expression levels so that we can extract more proteins out of existing capacity.

There are others who are working at the other end which is trying to stabilize the protein itself. Part of the problem that we have obviously is while we may have a good yield in fermentation tanks, nevertheless when it comes to purification we lose an awful lot of materials. And a lot of the loss is because the protein itself is not stable. There are now companies looking at ways to stabilize protein prior to downstream processes and seeing increase in the yield that they are getting.

Beyond that, people are now looking at alternative manufacturing systems. At this time, something like 90 to 95 of all of these proteins are produced by fermentation. Be it *E. coli*, be it yeast or mammalian cells. 90 to 95 of all proteins are currently are produced by fermentation. But I think we are going to see increased number of alternative manufacturing systems and I just want to run through a number of these that could be of interest.

Because we are in Sarawak and this is Sarawak Biodiversity Centre's conference, we need to say something about plants and there have been efforts over a number of years now to use genetically modified plants to produce proteins. Then, there is a very clever technology what we call 'semi-synthetic system' where they are using *E. coli* in some very novel way and we will say a little about that. In recent years, transgenic milk particularly has now been looked at as an alternative system as well as transgenic eggs. And we will see a little about each one of those.

We now know for instance that plants can be used very effectively as factories to produce proteins. We haven't seen any approved products yet. There are still challenges but without a doubt we now know we can express full antibody molecules and antibody fragments in tobacco. corn and rice. So, plants could become efficient protein factories in years to come. And there are all kinds of refinements going on in terms of cellular targeting - where to target the gene, where you target to minimise hydrolysis that will take place invariably and the whole issue of glycosylation - that is probably the biggest issue for us in thinking of protein for human use. Because obviously, glycosylation in plants is very different from glycosylation in mammalian system. So that is probably the biggest challenge. So we have Plantibodies, one of the leading companies in the space producing plant antibodies. It has to be said that quite a number of these companies have been backed by venture capital and have failed so far. I can think of at least one company in Cambridge that was trying to produce vaccines, plantized vaccine in tomato and for a number of reasons it has not been successful. This is a promising manufacturing platform but it remains to be seen how efficient and effective this particular manufacturing system will be. There are a number of ways of introducing DNA into plants but by far, according to literature, the agrobacterium is the method of choice currently for efficient transformation.

Another clever little manufacturing system is a semi-synthetic one. This is a typical pathway for the synthesis of isoprenoids, terpenes and whole lot of other molecules. This is what plants do efficiently and effectively but not as quickly as we would need to be able to manufacture. Also we cannot have a good control environment as we can with a fermentation system. What scientists have done is to take the gene that encode for enzymes for this pathway, put them into the right sequence

and put them into a *E. coli* to produce a whole lot of what they called universal (isoprenoid) precursors, that can be then be extracted and using one of two chemical steps to convert to the molecule of choice. And the feed stock is pretty standard, sugar cane and cellulose and water. The output can be these intermediaries here or what they are really gunning for is biodiesel. The whole series of hydrocarbons are produced in a *E. coli* system and produced in batch and in stirred tank in a very controlled environment.

So here is a very interesting technology and one of big targets that scientists are going after is Artemisinin as a treatment for Malaria. And so here is an interesting thought for us who are interested in the whole area of plant extracts. Using this technology what the scientists are saying is that what we need to do is to identify the vector, find the gene that are involved in the synthesis and we can clone the gene into *E. coli* system and produce them in fermentation batches. In other words, we don't need acres and acres to grow crops and plants of interest and do classical extraction. Here is an interesting technology to think about. And Berkeley is certainly very active in this and so are a bunch of Japanese universities. And from intermediaries you can see a whole range of molecules can be synthesized in this kind of way. So Artemisinin, Retinol and Taxol, we know about, and a whole lot of lycopenes here. So this is an interesting and novel way of producing molecules.

Transgenic goats have been with us for quite a number of years, and here is another novel manufacturing technology with very high yields, particularly antibody molecules. You take a gene of interest. You do microinjection into eggs. You transfer them into recipient females. They produce offspring. You screen the offsprings that give you the highest expression. You breed them and you get a whole flock of goats and you can milk them, and from the milk, purify proteins. And we now know that with transgenic milk, we get some very very high expression levels. So perhaps in the future this may be another route of synthesizing proteins.

Another interesting technology that we were involved with is transgenic eggs. Here the eggs are injected with the genes of choice to produce chimeric chickens that are then screened, and you breed them out, and when they are mature they are able lay eggs and from the yolk you then purify the antibody. Another way of introducing the gene is directly into the testes and allows breeding to take place. You then screen the climeric chicks that hatched and you select the one that you want and breed those for eggs and extract those proteins from egg. We know the technology works but the problem here is that the expression levels are low. And in term of transgenics, this is probably a better route to go than goat. It is much faster and takes a shorter time to have a whole flock of chicken and you know chicken lays an egg a day and you just need 10 thousands bird in a chicken coop and you will have ten thousands eggs per day. The problem here is that the yield remains extremely low but someone will crack it literally, and one day we may be able to produce protein this way.

Finally, just want to show a couple of slides on the facilities that we have built. First in Penang, this is Alpha Biologics a 5000 sq. m facility with two 500-L reactors and a couple of 150-L tanks. And the process development is done in Cambridge in UK. So the model here is that is that the head office is based in UK because it needs to be near its customers in Europe and US. And then once the process development is done in Cambridge, it is transferred to Penang for the scale up. And this facility can produce all the way up to phase III clinical trial material. And phase III, we are looking here at around kilogramme quantities, which is challenging. I will just show you some slides, for those who never been inside one of these cGMP faclities. There are huge clean water and buffer systems. Here is one of the tanks. And finally, this is Reliance GeneMedix's facility in Ireland. That is built about 4 to 5 years ago, producing EPO. They also have a plant in Bombay producing GCSF, and other bio-similars. Again this is fairly large scale manufacturing. These are pretty expensive plants and you can see the buffer system there and finally part of the downstream processing of erythropoietin.

So in conclusion, we have big challenges because we have lots of molecules that need manufacturing facilities. We have a global capacity constraint. We need to address high volumes. We need to address the issue of manufacturing cost. And all along the way, we need to address the safety issue for all these biologics. And we need better ways, more efficient systems, alternative manufacturing systems and this is where I think Asia has an opportunity. We are still by comparison lower cost in terms of our labour and manufacturing. And this is an area that I hope to see more and more Asian economies moving into.

Thank you very much.

The Discovery of Rainforest Endophytes with Enormous Biological Promise

Strobel, G.A.

Department of Plant Sciences, Montana State University,
Bozeman, Montana, 59717, USA.
uplgs@montana.edu

Abstract

A dramatic increase in the number of people in the world having health problems caused by certain cancers, drug resistant bacteria, parasitic protozoans, and fungi has caused alarm. An intensive search for newer and more effective agents to deal with these problems is now underway. Endophytes are a potential source of novel chemistry and biology to assist in helping solve not only human health, but plant and animal health problems also. Endophytes reside in the tissues between living plant cells. The relationship that they establish with the plant varies from symbiotic to bordering on pathogenic. Of all of the world's plants, it seems that only a few grass species have had their complete complement of endophytes studied. As a result, the opportunity to find new and interesting endophytes among the myriad of plants is great. Sometimes extremely unusual and valuable organic substances are produced by these endophytes. These compounds may contribute to the host – microbe relationship. The initial step, in dealing with endophytic microorganisms, is the selection of a proper and promising plant for study. Then, the endophytes may be successfully isolated from plant materials. After a correct growth medium is selected, the isolation and characterization of bioactive substances from culture filtrates is done using bioassay guided fractionation and spectroscopic methods. Some of the more interesting compounds produced by endophytic microbes, with which we have dealt, are taxol, cryptocin, cryptocandin, jesterone, oocydin, isopestacin, the munumbicins, kakadumycin, the pseudomycins and ambuic acid. This seminar and short course will deal with novel and interesting rainforest microbes, their biology, biochemistry and potential promise for solving problems in health, agriculture and industry.

Microbes for humankind

Microorganisms have long served mankind by virtue of the myriad of the enzymes and secondary products that they make (Demain, 1981). Interestingly, however, is the fact that only a relatively small number of microbes are used directly in human applications i.e. bread, cheese, and alcoholic beverage making. It seems that a more comprehensive search of the earth's niches may reveal novel microbes having other direct utilities to human societies. Of course, the advantages of direct use are numerous including reduced economic and regulatory factors while at the same time successfully completing a useful task. The diversity of microbial life is enormous and the niches in which microbes live is truly amazing ranging from deep ocean sediments to the earth's thermal pools (Bull, 2004). A relatively untapped source of microbial diversity is the world's rainforests. Each plant supports a suite of microorganisms known as endophytes (Strobel and Daisy, 2003). These organisms cause no overt symptoms on the plants in which they live (Bacon and White, 2000). Furthermore, since so little work on these endophytes has been done it is suspected that untold numbers of novel fungal and bacterial genera exist as plant-associated microbes (Hawksworth and Rossman, 1987). The rationale for sampling rainforest species is the fact that high plant biodiversity existing in the world's rainforest areas may also be accompanied by high microbial diversity (Mittermeier *et al*. 1999; Strobel and Daisy, 2003). Thus, we have begun a concerted search for novel endophytic microbes and the prospects that they may produce novel bioactive products as well as processes that may prove useful at the organismal level. This report concentrates mostly on the discovery of one novel endophytic fungal genus – Muscodor and the systematic study of its VOC production and its future for human use and biological discovery.

The discovery of *Muscodor albus*

In the late 90's I was on a collecting trip in the jungles near to the Caribbean coast of Honduras. I had selected this area to visit because Central America is one of the world's "hot spots of biodiversity" (Mittermeier *et al*. 1999). One modestly sized tree, not native to the new world, was introduced to me as *Cinnamomum zeylanicum*. Small limb specimens were taken and placed in a plastic bag and brought back to Montana and the sample was processed according to standard isolation procedures for endophytes (Strobel and Daisy, 2003). However, we had been plagued with microscopic phytophagous mites in the lab for many months. This is not uncommon for labs in which plant materials are being brought on a regular basis. Mites infest the bench tops and invade parafilm sealed Petri plates containing agar in which they take up residence. Thus, in order to eliminate this persistent mite problem we decided to place the Petri plates, with plant tissues, in a large plastic box having a firmly fitting lid. This maneuver would make it difficult for the tiny animals to find their way from the bench surfaces to the inside of the box. After a few days most plant specimens had sported endophytic fungal growth. Eventually the plates were removed and the individual hyphae transferred to fresh plates of potato dextrose agar. After a day or two of incubation we noted that no transferred endophyte grew except one. Had the placement of the endophytes in the large plastic box killed the endophytes by limiting oxygen availability? Soon it became obvious that the one endophytic fungus (designated isolate 620) remaining alive was producing volatile antibiotics or volatile organic compounds (VOCs). The hypothesis that an endophyte can make volatile antibiotic substances with a wide range of biological activity was born. It was quickly learned that although many wood inhabiting fungi make volatile substances, none of these possessed the biological activity of isolate 620 (McFee and Taylor, 1999). However some early data supported the observations that *Trichoderma* sp. produced some VOCs, however, with only modest biological activity and no attempt was made to identify the VOCs of this organism (Dennis and Webster, 1971). Later, another report on Trichoderma showed the identity of the VOCs and pointed out that inhibitory activity was associated with these compounds (Wheatly *et al*. 1997).

Muscodor albus and its VOCs

Isolate 620 is a sterile (not producing spores) endophytic fungus possessing some interesting hyphal characteristics including coiling, ropyness, and right angle branching. The mycelia of the fungus on most media are whitish and suppressed. In fact, the mycelia commonly make undulations on the agar surface. However, it has a tendency to produce aerial discrete organized mycelia yielding what appear to be synemma, however closer inspection reveals that they too are sterile. All attempts to initiate spore formation have failed. Isolate 620 grows nicely on a number of agar media including potato dextrose agar and other media containing plant extracts or various media made with wood shavings supplemented with starch since it appears that a rich carbohydrate source is critical to VOC production by this organism (Woropong *et al*., 2001).

Therefore, in order to taxonomically characterize this organism, the partial regions of the internal transcribed spacer region and the 5.8S of the nuclear ribosomal DNA operon (ITS- 5.8 rDNA) were isolated, sequenced, and deposited in GenBank as **AF 324336** and **AF 324337** (Woropong *et al*., 2001). It turns out that isolate 620 is unique and has 82-92% sequence similarity to several *Xylaria* spp. (Woropong *et al*., 2001). The GC/MS analysis of the fungal VOCs showed the presence of at least 28 VOCs (Table 1)(Strobel *et al*., 2001). These compounds represented at least five general classes of organic substance (lipids, esters, alcohol, ketones, and acids). Final identification of the volatile compounds was done by GC/MS of authentic compounds obtained from commercial sources or synthesized by us or others and compared directly by GC/MS to the VOCs of the fungus (Strobel *et al.,* 2001). With this chemical information in hand, along with the DNA sequence data, we felt secure in proposing a binomial for this fungus derived from the Latin – *Muscodor* (stinky) *albus* (white)(Woropong *et al*., 2001)(Fig. 1).

Ultimately, artificial mixtures of the compounds were used in a biological assay system to demonstrate the relative activity of individual compounds (Strobel *et al*., 2001). Although over 80% of the volatiles could be identified, this seemed to be adequate to achieve an excellent reproduction of the lethal- antibiotic effects of the VOCs that were being produced by the fungus (Strobel *et al*., 2001). The bioassay test was used to examine the five general classes of VOCs. Each class possessed

some inhibitory activity with the esters being the most active (Strobel *et al*., 2001). Of these the most active individual compound was 1-butanol, 3 methyl- and acetate. However, it is to be strongly stressed that no individual compound or class of compounds was lethal to any of the test microbes which consisted of representative plant pathogenic fungi, Gram positive and Gram negative bacteria, and others (Strobel *et al*., 2001). Obviously, the antibiotic effect of the VOCs of *M. albus* is strictly related to the synergistic activity of the compounds in the gas phase. Very little is known about the mode of action of these compounds on the test microbes thus, this represents an interesting academic avenue to pursue in the future.

Table 1. GC/MS analysis of the volatile compounds produced by *M. albus.* Several minor peaks and the breakthrough peak were omitted from the total analysis since they represent only 1% of the total area. Compounds found in the control PDA plate are not included in this table

RT	Total Area (%)	M/z	Possible compound	MW
3:45	0.33	114	Octane	114
4:19	0.93	58	Acetone	58
4:37	0.68	74	Methyl acetate	74
5:56	7.63	88	Ethyl acetate	88
6:51	0.31	102	Propanoic acid, 2-methyl, methyl ester	102
7:16	6.24	*	Ethanol	46
8:03	2.07	116	Propanoic acid, 2-methyl-ethyl ester	116
11:45	0.58	*	Propanoic acid, 2-methyl 2-methylpropyl ester	144
12:05	2.06	74	1-Propanol, 2-methyl-	74
12:50	22.24	*	1-Butanol, 3-methyl, acetate	130
14:57	1.53	*	Propanoic acid, 2-methyl, 3-methylbutyl ester	158
15:28	22.99	*	1-Butanol, 3-methyl-	88
16:08	0.29	138	#Furan, 2-pentyl-	138
18:53	0.29	142	#4-Nonanone	142
20:38	0.41	142	2-Nonanone	142
21:07	0.30	204	# Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR-trans)-	204
22:54	1.51	204	# Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-,[1S-(1.alpha.,4.alpha.,7.alpha.)]	204
23:16	0.94	204	# Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	204
25:20	3.63	204	# 1H-3a,7-methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8 tetramethyl-, [3R-(3.alpha., 3a.beta.,7.beta.,8a.alpha.)]	204
25:30	6.08	88	Propanoic acid, 2-methyl	88
26:04	0.48	204	Caryophyllene	204
27:55	0.34	204	# Naphthalene,1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1R-(1.alpha., 4a.alpha.,8a.alpha.)]	204
28:34	0.36	204	# Spiro[5.5]undec-2-ene,3,7,7-trimethyl-11-methylene-	204
28:50	1.07	204	Azulene, 1,2,3,5,6,7,8, 8a-octahydro-1, 4-dimethyl-7- (1-methylethyenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)] Common Name: Bulnesene	204
28:57	3.24	204	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-,[1R-(1.alpha.,7.beta.,8a.alpha.)] Common Name: Valencene	204
31:12	1.74	*	Acetic acid,2-phenylethyl ester	164
33:17	1.06	122	Phenylethyl alcohol	122
39:00	9.76	204	# Unknown	204

* No molecular-ion peak was observed in the spectrum of either the standard compound or the compound undergoing the analysis.

# Denotes that a spectrum and retention time of this component was observed and the substance matched to the most likely compound in the NIST data base, but the data have not been confirmed by use of an appropriate identical standard compound by either retention time or MS. These compounds were not placed in the artificial mixture in the bioassay test.

Table 2. The effects of the volatile compounds of *M. albus* and an artificial mixture of *M. albus* compounds on a group of test fungi and bacteria. After exposure to *M. albus* gasses, the test organism was evaluated for its growth and viability after removal from the gases. The artificial atmosphere consisted of the compounds identified after analysis of the *M. albus* gases*. The growth of the test organisms in the artificial atmosphere was measured after exposure to the artificial mixture of compounds at 3.2- 90 µl/50CC in order to obtain IC_{50}'s. The % growth over the control and viability were measured after exposure to 60 µl/50CC. Viability was determined after the removal of the compounds at 3 days

Test Microbe	**% Growth over control after a 2 day exposure to *M. albus***	**Viability after 3 days exposure to *M. albus* culture**	**IC_{50} in artificial atmosphere for 2days (µl/CC)**	**% Growth (mm) over control in artifical atmosphere**	**Viability after 3 days exposure artificial atmosphere**
Pythium ultimum	0	Dead	0.48±0.01	0	Dead
Phytophthora cinnamoni	0	Dead	0.29±0.06	0	Dead
Rhizoctonia solani	0	Dead	0.08±0.02	0	Dead
Ustilago hordei	0	Dead	0.31±0.09	0	Dead
Stagnospora nodorum	0	Dead	0.15±0	0	Dead
Sclerotinia sclerotiorum	0	Dead	0.17±0.05	0	Alive
Aspergillus fumigatus	0	Dead	0.41±0.05	0	Alive
Fusarium solani	19.4±0.28	Alive	1.13±0.07	42.0±2	Alive
Verticillum dahlia	0	Dead	0.3±0	0	Dead
Cercospora beticola	17.5± 3.5	Alive	0.12±0.15	8±2	Alive
Tapesia yallundae	0	Dead	0.64±0	0	Dead
Xylaria sp.	25±0	Alive	0.41±0.03	0	Alive
Muscodor albus	100±0	Alive	0.6±0	17.5±7.5	Alive
Escherichia coli	0	Dead	#	0	Dead
Staphlococcus aureus	0	Dead	#	0	Dead
Micrococcus luteus	0	Dead	#	0	Dead
Candida albicans	0	Dead	#	trace	Alive
Bacillus subtilus	0	Alive	#	0	Alive

* The amount of each positively identified compound used in the artificial mixture was obtained by applying the electron ionization cross section (% of the total area) of the compound obtained in the GC/MS analysis (Table 1). The artifical mixtures were subsequently tested by placing them in a presterilized microcup (4x6 mm) located in the center of a test Petri plate containing PDA. Agar plugs containing freshly growing test microbes (or streaked microbes) were positioned about 2-3 cm from the center microcup. Then the plate was wrapped with 2 layers of parafilm and incubated for two or more days at 23°C. Measurements of linear mycelial growth were made from the edge of the inoculum agar plug to the edge of the mycelial colony.
# Not measured in this experimental design.

Using the bioassay test system it was possible to calculate the 50% inhibitory concentration (IC_{50}) for each test microbe and compare the killing ability of the artificial mixture with that of the *M. albus* VOCs (Strobel *et al*., 2001). One of the most sensitive fungi was *Rhizoctonia solani* and one of the least sensitive was *Fusarium solani*. In the later case, *F. solani* is only inhibited by both the artificial VOC mixture as well as the VOCs of the fungus. Finally, the viability of all test organisms was also determined after exposure to either the fungus or to its artificial mixture of VOCs (Tables 1 and 2) (Strobel *et al*., 2001). The artificial mixture generally mimicked the effects of the fungus itself (Table 2)(Strobel *et al*., 2001). Not all compounds in the fungal VOCs are necessary for biological activity (Strobel *et al*., 2001).

Other isolates and species of Muscodor

Recently, seven new *M. albus* isolates were retrieved from the Northern Territory of Australia (Ezra *et al*., 2004). These organisms were obtained by using *M. albus* (isolate 620) as a selection tool in Petri plates containing agar in the presence of the plant tissues containing endophytic fungi (Ezra *et al*.,

2004). The host plants of these isolates were *Terminalia prostrata* (Combretaceae), *Kennedia nigriscans* (Leguminosae) and *Grevillea pterifolia* (Proteaceae). Each isolate was biologically active, produced some but not all of the VOCs made by *M. albus* 620, and had between 96 % and 99% ITS-5.8S rDNA sequence similarity to *M. albus* 620 (Ezra *et al*., 2004).

Another isolate of *M. albus* has been recovered as an endophyte from nutmeg (*Myristica nutans*- family Myristaceae) in Thailand (Sopalun *et al*. 2003). This isolate has 98% sequence similarity to the ITS-5.8 rDNA of *M. albus* and produces many of the same volatiles as isolate 620 and it also possesses antibiotic properties. Furthermore, again using the selection technique, *M. albus* isolate 1-41-3s was obtained from an unidentified vine in the Sumatran jungle of Tesso Nilo in Indonesia, and it possessed 98% ITS-5.8SrDNA sequence similarity to *M. albus* 620 (Atmosukarto *et al*., 2005). This isolate possesses unusual hyphae, a slime layer, and some VOCs not observed before in other *M. albus* isolates including tetrahydrofuran, 2-methyl furan; 2-butanone; aciphyllene, and large amounts of an unusual azulene derivative (Atmosukarto *et al*., 2005). Discovery of these new isolates of *M. albus* confirms that this organism may be a *bona fide* novel endophytic fungal genus rather than the original 620 isolate occurring as a localized phenomenon in nature. The discovery also shows that several plant families other than Lauraceae (*Cinnamomum* sp.) can serve as a host for *M. albus*.

Two other species of Muscodor have recently been found including *M. roseus* (endophytic on *Grevillea pteridifolia*, Australia) and *M. vitigenus* (endophytic on *Paullinia paullinoides*, Peru)(Daisy *et al*. 2002a; Worapong, *et al*., 2002). These isolates are also closely genetically related (ITS-5.8S rDNA sequences) to the Australian isolates of *M. albus* at the 96%-99% level (Ezra *et al*., 2004). The later organism makes only naphthalene as a VOC and its repellency towards a plant associated insect has been demonstrated (Daisy *et al*., 2002b). Finally, many temperate areas of the world have been explored and examined for the presence of *Muscodor* spp., but with no success except for an isolate of *Muscodor* sp. from *Prosopis* sp. in the little karoo of South Africa.

The newest addition to the list of *Muscodor* spp is *Muscodor crispans* (Mitchell *et al*., 2008). This endophytic fungus resides within the stem tissues of *Ananas ananassoides*, a wild pineapple in the Bolivian Amazon Basin. This strain is characterized by the production of a pinkish felt-like mycelium on potato dextrose agar (PDA) and other media under lighted conditions, but developing a whitish mycelium in the dark. The fungus produces no fruiting structures or spores of any kind when incubated on multiple synthetic or natural media. On PDA and other common laboratory media, its hyphae develop into regular undulating patterns and associated with them are cauliflower-like structures (3.5-14 µm). Analysis of the volatile organic compounds done by GC/MS showed that *M. crispans* primarily produces a number of esters, alcohols, and small molecular weight acids but no naphthalene or azulene derivatives as per other members of this genus. The volatiles possess antibiotic properties making this organism potentially useful in a number of venues. A molecular genetic analysis of the ITS1, 5.8S rDNA, and ITS2 regions showed 100% similarity to *Muscodor albus*. Justification for the designation of a new species is primarily based on its novel phenotypic characters including its peculiar hyphal growth patterns (undulating hyphae), its reddish pigment production in the light, the odd cauliflower-like structures associated with its hyphae, and its unusual gaseous products. In spite of its 100% genetic similarity to the rDNA regions of *M. albus*, this organism is considered distinct because of the number and kind of its unusual phenotypic characteristics.

Finally, another new isolate of *M. albus* (E-6), with unusual biochemical and biological properties, has been obtained from the branches of a mature *Guazuma ulmifolia* (Sterculiaceae) tree growing in a dry tropical forest in SW Ecuador (19). This unique organism produces many VOC's not previously observed in other *M. albus* isolates including: butanoic acid, 2-methyl-; butanoic acid, 3-methyl-;2-butenal, 2-methyl-; butanoic acid, 3-methylbutyl ester; 3-buten-1-ol, 3-methyl; guaiol; 1-octene, 3-ethyl-, formamide,N-(1-methylpropyl), and along with certain azulene and naphthalene derivatives. On the other hand, some compounds usually seen in *M. albus* isolates also appeared in the VOCs of isolate E-6 including: caryophyllene; phenylethyl alcohol; acetic acid, 2-phenylethyl ester; bulnesene; and various propanoic acid, 2-methyl- derivatives. Scanning electron micrographs of the mycelium showed substantial intertwining of the hyphal strands. These strands seemed to be held together by an extracellular matrix accounting for the strong mat-like nature of the mycelium which easily lifts off the agar surface upon transfer unlike any other isolate of this fungus. The ITS -5.8S rDNA partial sequence data showed 99% similarity to the original *M. albus* strain- 620. Now, for the first time, a successful establishment of *M. albus* into its natural host, followed by recovery of the

fungus, was accomplished in seedlings of *G. ulmifolia*. The biological activity of the VOCs of E-6 appears different from the original isolate of this fungus - 620 since a Gram positive bacterium was killed and *Sclerotinia sclerotiorum* along with *Rhizoctonia solani* were not.

Physiological aspects of VOC production

The composition of the medium greatly influences the quality and effectiveness of the VOCs emitted by *M. albus* (Ezra and Strobel, 2003). For instance, a sucrose enriched medium primarily yields methyl isobutylketone and acetic acid, butyl ester as the primary volatiles, yet neither of these compounds appeared in any other medium. Furthermore, the VOCs under these conditions, were limited in their bioactivity. More enriched media were more effective in inhibiting a suite of plant pathogens used as test microbes (Ezra and Strobel, 2003).

Although only qualitative methodology was initially used to gather information on the fungal VOCs there was a need to obtain quantitative data on VOC production. A relatively new technology called proton transfer reaction-mass spectrometry (PTR-MS) was used to monitor the concentration of VOCs emitted by *M. albus* (Ezra *et al*., 2004). PTR-MS is a particularly useful technique because it can be run on line while continuously yielding data on the concentrations of specific ions of interest. Data gathered from a long term *M. albus* culture in a carboy by PTR-MS indicated that the production of VOCs is temperature-dependent with diurnal fluctuations in gas production occurring as the temperature varied (Ezra *et al*., 2004). Furthermore, continuous monitoring after 3 weeks revealed a slow, but steady decline in VOC production which is probably a reflection of the depletion of the carbohydrate sources in the potato dextrose agar. This is consistent with the observations showing VOC production is related to the presence of a carbohydrate source (Ezra and Strobel, 2003).

The PTR-MS technique was also applied to soils containing *M. albus* along with the plant pathogen *Pythium ultimum* and it was possible to show the production of VOCs from *M. albus in situ*. An estimation of the range of concentrations of total VOCs being produced by *M. albus* is in the order of 100-300 ppb based on the determination of the concentration of propanoic acid, 2-methyl, methyl ester.

Interestingly, since *M. albus* has not been artificially established in any host or non- host plant, no data are available on the *in planta* production of VOCs by endophytically established *M. albus.* This is an important phenomenon awaiting observation and the PTR-MS technique seems like the most useful method to make such observations. Establishing a successful *M. albus*/plant relationship is critical in doing these *in planta* mass spectral studies.

"Mycofumigation" with *Muscodor* spp.

The VOCs of *M. albus* kill many of the pathogens that affect plants, people and even buildings (Strobel *et al*. 2001; ATmosukarto *et al*., 2005)(Table 2). The term "mycofumigation" has been applied to the practical aspects of this fungus (Strobel *et al*., 2001). The first practical demonstration of its effects against a pathogen was the mycofumigation of covered smut infected barley seeds for a few days. The seeds were eventually planted and the resulting plants, in contrast to the untreated control group, produced no infected heads (Strobel *et al*., 2001). Mycofumigation is also important for the treatment of fruits in storage and transit (Mercer and Jimenez, 2004). Soil treatments have also been effectively used in both field and greenhouse situations (Stinson *et al*., 2003a; Jacobsen *et al*. 2004; Mercier and Manker, 2005). In these cases, soils are pretreated with a *M. albus* formulation in order to preclude the development of infected seedlings. *M. albus* is now being produced, by solid state fermentation, by the ton in order for its use in many practical applications.

AgraQuest, an agricultural biotech company, of Davis, Calif., is developing *M. albus* for numerous agricultural applications with a soon anticipated release of products. The concept of mycofumigation, for a multitude of uses, has the potential to replace hazardous substances that are currently applied to humans, food, soil and buildings. The most notable of which is methyl bromide for soil sterilization which use will cease in 2007 because of its toxicity and negative influences on the world's ozone layer. On the other hand, it turns out that the VOCs of *M. albus* appear safe, effective and environmentally friendly and may serve as a replacement soil treatment (Stinson *et al*., 2003a; Jacobsen *et al*. 2004; Mercier and Manker, 2005). A general new-like review on the practical applications of *M. albus* has recently appeared (Anon, 2007).

M. albus is used as a tool for selecting other fungi of biotechnical interest

Using *M. albus* as a selection tool, *Gliocladium* sp. was isolated from *Eucryphia cordifolia* in Patagonia and this organism is phylogenetically related to *Ascocoryne* (Stinson *et al.*, 2003b; Strobel *et al*, 2008). Initially it produced [8]annulene, as its main recoverable VOC, a compound not previously found as a natural product. However, upon storage there was a general change in the caste of VOCs produced by this organism (Strobel *et al*, 2008). In fact, it produced a series of volatile hydrocarbons and hydrocarbon derivatives on an oatmeal- based agar under microaerophilic conditions as analyzed by SPME-GC/MS. In the VOC mixture was an extensive series of the acetic acid esters of straight chained alkanes including those of pentyl, hexyl, heptyl, octyl, sec-octyl and decyl alcohols. Other hydrocarbons were also produced by this organism including: undecane, 2, 6-dimethyl; decane, 3, 3, 5-trimethyl; cyclohexene, 4-methyl; decane, 3,3,6-trimethyl; undecane ,4,4-dimethyl. Volatile hydrocarbons were also produced on a cellulose- based medium including heptane, octane, benzene, and some branched hydrocarbons. An extract of the host plant, *Eucryphia cordifolia,* supported the growth and hydrocarbon production of this fungus. Quantification of volatile organic compounds, as measured by proton transfer mass spectrometry (PTR-MS), produced a level of detectable organic substances in the air space of the oatmeal agar medium in the order of 80 ppmv (parts per million by volume) in a 18 day old culture. Scaling the PTR-MS profile the acetic acid heptyl ester was quantified (at 500 ppbv) and subsequently the amount of each compound in the GC/MS profile could be estimated and all yielded a total value of ca. 4.0 ppmv. The hydrocarbon profile of *G. roseum* contains a number of compounds normally associated with diesel fuel and so the volatiles of this fungus have been dubbed - "**myco-diesel.**" Extraction of liquid cultures of the fungus revealed the presence of numerous fatty acids and other lipids. All of these findings have implications in energy production and utilization.

Concluding remarks and future perspectives

Obviously, because of the impressive biological activity of *M. albus* and its introduction into practical agriculture/industry, it seems that the fungus should be more fully studied relative to its location and role in nature. Overall, the most pressing question regarding *M. albus* is the mode of action of a multitude of volatile compounds and how they act synergistically to cause the death of microbes.

Certainly, knowledge of its host preferences and those factors controlling host preference may eventually allow for the use and development of this organism for hosts that it does not naturally frequent. Such information may result in still more applications and direct uses of *M. albus*. Further, we need to learn if *Muscodor* isolates can be directly inoculated into agricultural and forest species in order to provide protection against invading pests and pathogens. In this regard, it has been recently learned that *M. albus* has activity against certain plant pathogenic nematodes. Other studies are in progress to test its biological activity against plant insect pests.

It seems that there may be some factors that may limit the use of *M. albus* for biological control. One of the most important of which is the inability of *M. albus* to kill, and only inhibit isolates of *Fusarium* spp. This seems to be a universal observation with all isolates of Muscodor (Strobel *et al*., 2001; Ezra *et al*., 2004; Atmosukarno *et al*., 2005). The fusaria species are some of the most important plant pathogens occurring on a wide range of agricultural and forest species. Furthermore, unless adequate sources of carbohydrates are available, *M. albus* ceases producing its VOCs (Strobel *et al*., 2007; Mitchell *et al*., 2008). This may be present a problem in situations in which VOC production stops even though unwanted organisms persist.

In addition, it is extremely important to possess information on the distribution, life cycle and other aspects of the chemistry of this organism. This will aid in expanding its utility as a biological control agent. Hopefully, the development of information on *M. albus* will have broad implications for the discovery and development of other rainforest microbes.

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Production of Taxol and Other Taxanes from Selected Cell Lines of *Taxus* spp. Cell Suspension Cultures

Khairiah, A.K.[1] and Mavituna, F.[2]

[1]School of Chemical Engineering, Universiti Sains Malaysia, Engineering Campus, 14300 Nibong Tebal, Penang, Malaysia
[2]School of Chemical Engineering and Analytical Science, The University of Manchester, Manchester, M60 1QD, UK
chkhairiah@eng.usm.my

Abstract

In this study, callus cultures from three different *Taxus* spp. were used as the starting materials, which were induced from different tissue explants, i.e.: both needles and stems of *Taxus cuspidata* and *Taxus cuspidata* nana and seedlings of *Taxus baccata*. Callus and cell suspension cultures were successfully initiated from the young seedlings of *T. baccata*. The addition of 1.5 % (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and use of half strength of picloram in callus maintenance medium reduced the problem of cell darkening considerably. In cell suspension cultures, the use of non-ionic XAD-4 adsorbent has helped to overcome the same problem. The best carbohydrate combination, 15 g/L fructose + 5 g/L glucose, was significantly better than all other combinations of carbohydrates tested to support the growth of *T. baccata* cell suspension cultures. Fructose was found the best carbon source compared to sucrose and glucose. The addition of fructose (10 g/L) on day 8 and methyl jasmonate (100 µM) on day 10 after incubation has increased the total Taxol and cephalomannine production to 17 and 29 mg/L, respectively, which was achieved from the suspension cultures initiated from needle explants of the seedlings. The presence of Taxol and cephalomannine was detected by high performance liquid chromatography (HPLC).

Introduction

Taxol, the novel and complex diterpene alkaloid, was originally found and isolated from the bark of pacific yew tree (*Taxus brevifolia* Nutt.) (Suffness and Wall 1995). Taxol has been the focus of great scientific interest since 1970's because of its unique mode of action to promote tubulin polymerization and stabilize microtubules against depolymerization (Schiff *et al.*, 1979). The US Food and Drug Administration (FDA) has approved this drug for the treatment of ovarian, lung and breast cancer, as well as AIDS-related Kaposi's sarcoma and lymphoma (Pezzuto, 1996). Nevertheless, the limited supply of taxol has been the hindrance to the rapid development of this anticancer drug.

The yew tree is a limited resource and the collection and extraction of bark are difficult and expensive. Chemical synthesis is not a feasible and economical process because of the complex chemical structure of taxol (Yukimune *et al.* 1996). For this reason, a variety of approaches have been explored to find the alternative sources for the production of taxol and other taxanes. Plant cell culture is considered as one of the most promising methods to provide sufficient quantities of taxol for commercial supply.

The problem of growth rate reduction and cell darkening in callus and suspension cultures are major concerns for establishing good characteristics and healthy cell cultures. The use of insoluble polyvinylpolypyrrolidone (PVPP) and half strength of picloram have been investigated in callus maintenance medium, while the non-ionic XAD-4 adsorbent was added in suspension cultures. The effects of different cell lines, carbohydrate combination and methyl jasmonate (MeJA) and fructose addition on dependent parameters such as growth rate, taxol and other taxanes production rate was studied in cell suspension cultures.

Materials and methods

Cell cultures were initiated from three different *Taxus* spp., i.e.: both needles and stems of *Taxus cuspidata* and *Taxus cuspidata* nana and seedlings of *Taxus baccata.* Cells were maintained as callus and suspension using Gamborg's B5 medium in the dark at 23 ± 1^{o}C. Subculturing was at least every 2 weeks for the suspension culture and 4 weeks for the callus culture. Suspension cultures were

cultivated in Erlenmeyer flasks and agitated on a rotary shaker at 120 rpm in the dark. 1.5% (w/v) of phenolic-binding compound polyvinylpolypyrrolidone (PVPP) (Fett Neto *et al.*, 1992 and 1993) was added to the callus maintenance (CM) medium with exclusion of ascorbic acid to reduce the excretion of phenolic compounds in callus cultures. The effect of phenolic-binding compounds on cell suspensions cultures was investigated by adding Amberlite XAD-4, the non-ionic resin (Sigma) in order to remove the dark colours/pigments from the medium. The dry Amberlite XAD-4 resins (0.5 – 1.0 g) were wrapped in small bags made from nylon/cotton cloth and tightened with a long cotton thread to facilitate the removal process of XAD-4 from the medium. To study the influence of carbohydrate on the growth of *T. baccata* cell suspension cultures, seven different combinations and concentrations of carbohydrate were assessed (Table 1). 10 g/L (1% w/v) of fructose was added in to 8-day-old cell cultures (after inoculation), and further elicited with 100 µM MeJA 2 days later.

Medium and cell samples were prepared by filtration of culture broth. The medium samples were extracted with methylene chloride. The water phase was removed and the methylene chloride phase was evaporated using rotary evaporator. The residue was resuspended in methanol and filtered through a 0.2 µm membrane filter prior to analysis. Biomass were weighed and dried in an oven to obtain dry weight for growth data. Determination of taxol and other taxanes in the sample was performed by HPLC (Phenomenex, Curosil PFP-type column) analysis using photo diode array (PDA) detector at 228 nm. The mobile phase consisted of acetonitrile and water used in gradient mode and at the flow rate of 1 ml/min. The residual sugars were also detected by HPLC using the Hypersil NH_2 (APS-2) column, operated at isocratic mode with acetonitrile:water (80:20) as a mobile phase. The samples were separated and detected by the Evaporative Light Scattering Detector (ELSD).

Results and discussion

Callus and cell suspension cultures were successfully initiated from the young seedlings of *T. baccata*. At the initial stages of callus maintenance, cell darkening occurred. The addition of 50 mg/L ascorbic acid (Hirasuna *et al*., 1996) did not help prevent the darkening problem. Therefore, in this study, 1.5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) was incorporated to the CM medium as the phenolic-binding compound in order to overcome this problem and to secure the cells. PVPP seems effective and helped reduce the problem of darkening, although the recovery of culture viability was very slow. Therefore, modification of CM medium was done by using half strength of picloram (a type of plant growth regulator). The growth rate of callus cultures was improved and problem of cell darkening was considerably reduced. The “red-coloured” exudates produced in the *T. baccata* cell suspension cultures were successfully adsorbed and removed from the suspension cultures after 2-3 change of resins in 2-week intervals.

Fig. 1 shows the relationship between cell growth and sugar consumption in *T. baccata* cell suspension cultures. Sucrose was completely hydrolyzed to glucose and fructose and depleted from the suspension medium 3 days after inoculation. The cells utilized glucose preferentially compared to fructose for growth, as fructose gradually accumulated in the medium until day 8. All glucose was fully consumed during the second week and cells started to use fructose until day 20 when no sugars remained in the medium.

The influence of carbohydrate was examined based on the fresh and dry weight analysis, and reported in growth index (Fig. 2). The best carbohydrate combination, 15 g/L fructose + 5 g/L glucose (Set B, Table 1), was significantly better than all other combinations of carbohydrates tested to support the growth of *T. baccata* cell suspension cultures. The presence of a high amount of fructose in the medium (Set A and B) produced significant amount of cells, suggesting that fructose was favoured by cells as the sole carbohydrate (Ketchum and Gibson, 1996).

T. baccata cell culture was able to produce other taxanes in higher level than taxol (Table 2). The addition of fructose and MeJA has increased the total (intracellular and extracellular) taxol and cephalomannine production to 17 and 29 mg/L, respectively, which was achieved from the suspension cultures initiated from needle explants of the seedlings.

Conclusion

We have demonstrated that taxol and other taxane compounds could be produced from the seedlings of *T. baccata*. Elicitation and fructose addition gave synergistic effects to the production of taxol and other taxanes. Most of taxane compounds were excreted into the medium but some were only found in the cell. Problem of cell darkening could be reduced and cell growth was significantly improved by manipulating culture medium.

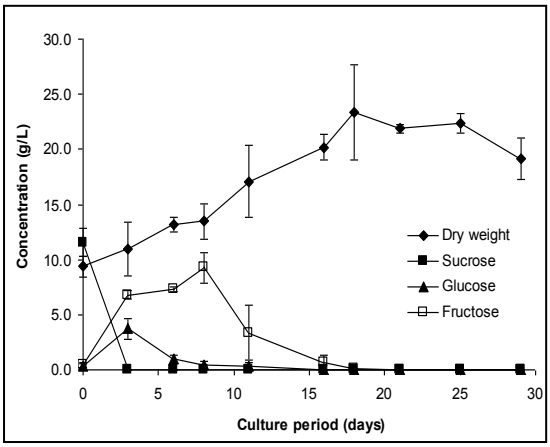

Fig. 1. Cell growth and sugar consumption in *T. baccata* cell suspension cultures.

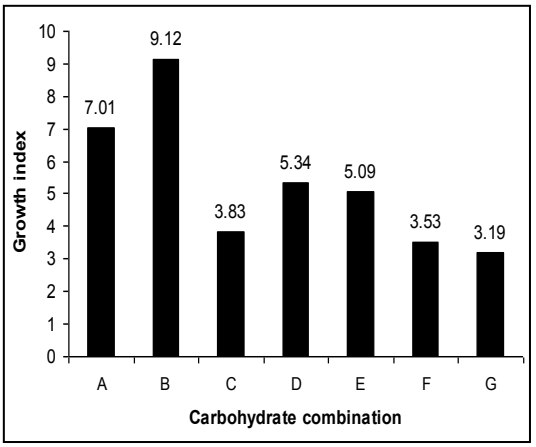

Fig. 2. Effect of different carbohydrate combinations on growth of *T. baccata* cell cultures.

Table 1. Combination of carbohydrate tested for optimal cell growth

Set	Combination*	Set	Combination*
A	20 F	E	10 F + 10 S
B	5 G + 15 F	F	15 G + 5 F
C	10 G + 10 F	G	6.7 G + 6.7 F + 6.7 S
D	20 G		

*F = fructose, G = glucose, S =sucrose. Unit in g/L.

Table 2. The maximum concentration of total taxol and other taxanes produced (in mg/L) by *T. baccata* cell cultures derived from different parts of seedlings

Source*	Taxol	Cephalomannine	7-Epi-10-Deacetyltaxol
ISD	13.330	21.448	9.074
SSD	12.390	21.469	10.323
NSD	17.071	29.200	11.593

*ISD: injured seeds; SSD: stem explants of seedlings; NSD: needle explants of seedlings.

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Cloning and Expression of Industrial Important Thermostable Amylase Gene

Moohamad Ropaning, S.[1], Lokman, S.[1], Abu Bakar, S.[2], Raja Noor Zaliha, R.A.R.[2] and Mahiran, B.[3]

[1]Faculty of Biotechnology and Life Sciences, Universiti Industri Selangor,
40000 UNISEL Shah Alam, Selangor, Malaysia
[2]Faculty of Biotechnology and Biomolecular Sciences, [3]Faculty of Science, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
sulong2002@yahoo.com

Abstract

Production of thermostable amylases was achieved using local strains. These strains were isolated from various hot springs with water temperatures ranging from 65°C to 90°C. However, a quantitative test of the thermostable amylases using dinitrosalicylic acid (DNSA) method revealed inadequate level of the enzyme production (0.36 U/ml). Therefore, molecular approaches such as cloning and expression were adopted to increase the amount of amylases. The thermostable amylase gene with approaximately 1.6 kb was successfully isolated. The gene was cloned into pET-32b vector and then transformed into *E. coli* BL21 expression host. The formation of clear zone on starch agar plate proved the presence of the recombinant amylases and thus indicated the success in cloning and expression of thermostable amylase gene. The level of the recombinant thermostable amylase after 24 h of induction time was higher as compared to the wild type (2.3 U/ml). Studies on the optimization of different concentrations of inducer (IPTG) as well as the post induction time are in progress.

Introduction

Amylases (EC 3.2.1.1) are digestive enzyme that cleaves a long chain carbohydrate such as starch into maltose and subsequently degraded it into glucose. There are three types of amylases, namely, α-amylase, β-amylase, and gluco-amylase. The α-amylase randomly cleaves the glycosidic linkage of amylose to yield dextrin, maltose or glucose molecules. The β-amylase catalyzes the hydrolysis of a-1,4 glycosidic bonds but only from the non-reducing end to yield maltose molecules whilst gluco-amylase cleaves α- (1-4) and α (1-6) glycosidic linkage of amylose and amylopectin to yield glucose molecules. Diverse in source, amylases are found in human, animals, plants, and microbes. However, the microbial amylases have been paid much attention. Microorganisms such as *Bacillus* sp. KCA102 (Agrawal *et al*., 2005), *Aspergillus niger* (Mitidieri *et al*., 2006) and *Streptococcus bovis* 148 (Satoh *et al*., 1997) have been reported to be good amylase proucers. Several amylases are used in industrial applications such as in the apple juice processing (Ceci and Lozano, 2002), in beverages, baby foods, and pharmaceutical manufacturing industries (Rao *et al*., 2005). α-amylases from fungal and bacterial have dominated applications in industrial sector (Pandey *et al*., 2000). β-amylases (Qi *et al*., 2006) and glucoamylases (Zhao *et al*., 2000) are also used industrially.

Materials and methods

Isolation of amylase producing bacteria

Amylase producing bacteria was isolated around hot spring areas at Sungai Klah and Slim River in Perak as well as Sungai Dusun Tua, Hulu Langat in Selangor where temperatures ranged from 64°C to 90°C. The isolates were grown on a selective media comprised of Nutrient Agar with 1% starch at temperatures at the original environment for 24 h with agitation rate of 150 rpm after which screening for amylase was carried out.

Qualitative and quantitative amylase assay

Screening for amylase was carried out using starch-iodine reaction concept. In this method, positive starch-iodine reaction will appear as blue colour, whereas negative starch-iodine reaction will produce halo or clear zone due to the absence of starch that has been hydrolyzed by the amylase from the

isolate. On the other hand, quantitative amylase assay was carried out using dinitrosalicylic acid (DNSA) method (Bernfeld, 1955), unless otherwise stated. One unit of amylase activity is defined as the the rate of production of 1 µmol of reducing sugar from 1% soluble starch as substrate in 1 min at 37°C at pH 7.0.

Genomic DNA extraction and purification and 16s rDNA gene amplification

Genomic DNA of the microbial isolate was extracted using AquaPure GenomicDNA Isolation Kit (Bio-Rad, USA). The extracted DNA was then purified using Axyprep bacterial genomic DNA Miniprep Kit purchased from (Axygen Biosciences, USA). The extracted DNA was amplified using polymerase chain reaction (PCR) technology and the universal 16S rDNA universal primers. Amplification and sequencing, (First Base Laboratories Sdn Bhd), of the amplified product were done. The 16S rDNA sequences obtained were compared with those available in the GenBank through National Centre for Biotechnology Information (NCBI) free software for identification of the isolated bacteria.

Cloning of the amylase gene into pET-32b

A DNA fragment encoding 1650 bp of the amylase gene was amplified by PCR with forward primers *Hind*III (5'-aag **aag ctt** act tat cca gcc ttg agc atc acc-3') and reverse primer is *XhoI (* 5'-tcg **ctc gag** cca agg cca tgc cac caa ccg tgg -3'). This PCR product was then cloned into *E. coli* BL21 expression host, using pET-32b as the vector. This cloning procedure was done by using kits.

Results and discussion

Qualitative and quantitative amylase assay

The qualitative assay of the isolated samples showed the formation of clear zone or halo around the colony indicating the presence of amylase (Fig. 1). The quantitative assay of different isolates using dinitrosalicylic acid (DNSA) method (Bernfeld, 1955), revealed that the isolate SK2 produced highest amylase activity (~ 0.54 $U.mL^{-1}$) after 72h of incubation at 150 rpm compared to the isolate SK1 (Fig. 2).

Genomic DNA extraction, purification and 16s rDNA gene amplification

The genomic DNA of the isolate SK2 was successfully extracted using the AquaPure GenomicDNA Isolation Kit (Bio-Rad, USA). The 16S rDNA obtained from the isolate (Fig. 3) then sequenced and compared with those available in the GenBank through National Centre for Biotechnology Information (NCBI) free software for identification of the isolated bacteria and showed that the strain had 98% similarity with *Geobacillus stearothermophilus.*

Cloning of the amylase gene into pET-32b

The amylase gene was successfully transformed into *E. coli* BL21 expression host by using pET-32b plasmid as the vector. The recombinant amylase was been screened on starch agar plate and shows positive result to the expression of the thermostable amylase gene.

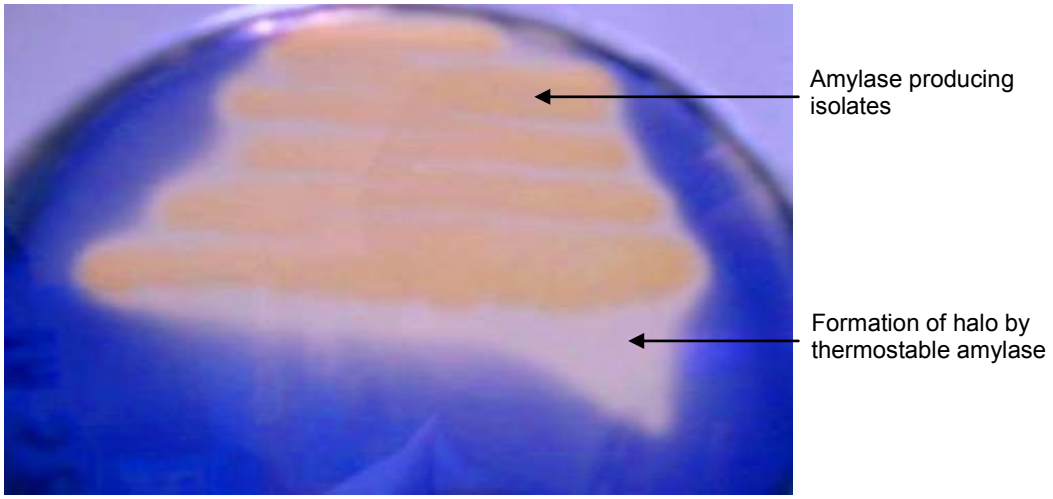

Fig. 1. Qualitative assay of the thermostable amylase.

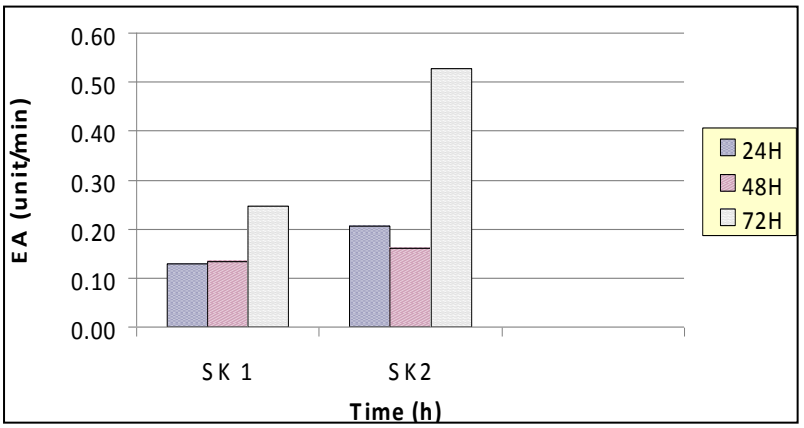

Fig. 2. Quantitative assay of the thermostable amylase.

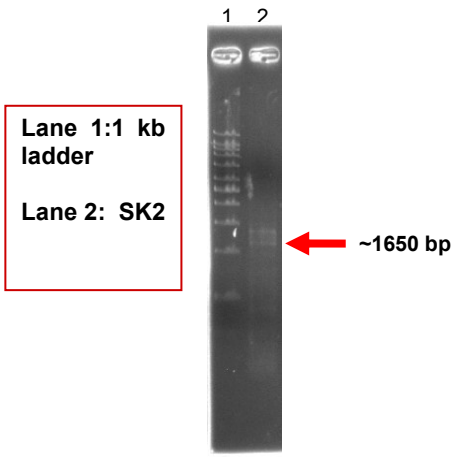

Fig. 3: The amylase gene of the isolates amplified by PCR.

Conclusion

In conclusion, the presence of halo on LB-ampicillin agar plate supplemented with 1% starch has proved the presence of the recombinant amylase and thus indicated the success in cloning and expression of the thermostable amylase gene. The optimization of different parameters such as different production media, different concentrations of inducer (IPTG) and the post induction time are in progress.

Acknowledgments

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Adaptation of Compact Callus of Pomelo (*Citrus Grandis*) to Liquid Media as Potential Approach to Increase Growth

Zakaria, Z.[1], Nazri, N.A.[1], Ali, A.M.[1], Ismail, K.S.K.[1] and Ishak, M.A.M.[2]

[1]School of Bioprocess Engineering, Universiti Malaysia Perlis,
UniMAP's Academic Complex-Jejawi 3, 02600 Arau, Perlis, Malaysia
[2]Faculty of Applied Sciences, Universiti Teknologi MARA Perlis, 02600 Arau, Perlis, Malaysia
zarinaz@unimap.edu.my

Abstract

There were various attempts to establish suspension cultures of *Citrus grandis* from compact callus that proved futile. Suspension cultures provide more benefits over callus culture as cells in suspension divide faster and controllable. Tissue culture work on pomelo is aiming for ascorbic acid production independent of raw material from plant parts. An adaptation approach by reducing agar in solid media from 100% to 75, 50 and 25% was used in the study. Callus grown on less percentage of agar substance for 4 weeks showed promotion to the growth and the lowest percentage of agar used, 25%, showed highest increment that is double in the fresh weight. Later, the treated callus showed the ability to maintain their viability in liquid medium in longer duration (11 days) compared to untreated callus (7 days). To study the changes in cells structure and arrangement, an investigation through scanning electron microscope (SEM) was carried out.

Introduction

Citrus grandis or pomelo is a local Malaysian fruit that is found contain high antioxidant compounds such as ascorbic acid and flavonoids. Therefore, the use of young pomelo fruit as explants sources in tissue culture approach for *in vitro* production of natural antioxidant compounds might sound promising. The study of compounds such as limonene and linalool has been carried out with successful production of those particular compounds in callus culture (Nik Norulaini *et al*., 2003). Callus culture of young pomelo fruits was successfully established and the growth was favorable from the explants originated from 5-week old fruits cultured on modified Murashige and Skoog (MS) media (Murashige and Skoog, 1962) supplemented with 510 mg/l phosphate, 3 mg/l each of 2,4-D and kinetin and 0.2 mg/l ABA (Jenimar, 2001).

There were various attempts to culture the pomelo tissues in liquid media but with no success. First, the callus was initiated on solidified media and transferred into the liquid media after 4 weeks in culture. After about 7 days in liquid media, the callus turned brown and eventually would die. Suspension cultures offer same advantages over callus cultures in terms of better mixing and improved product synthesis. Few researchers modified hormone concentration as an approach to obtain soft callus that easily disperse in the liquid media. This kind of study is time consuming and need a lot of effort to find the correct combination of hormone. A simpler approach introduced in this study is to adapt the cells to their preferable surrounding at a slow pace for a certain period.

Materials and methods

Preparation of culture on agar media

Young *C. grandis* fruits about 4-5 cm in diameter was obtained from an established plantation. Sterilisation was by immersing the whole fruit into 20% Clorox® for 2 hours prior to peeling and cutting. Callus was initiated on MS salts with 3X phosphate and supplemented with 30 g/l sucrose, 7 g/l agar, 3 mg/l 2,4-D, 3 mg/l kinetin and 0.2 mg/l ABA based on Jenimar (2001) for optimum callus growth. Cultures were incubated in the dark for 24 hours at room temperature (25 ± 2^{o}C) for about a month before any callus was ready to be used for agar reduction study.

Variation of agar content in the culture media

For this study the medium used, MS medium, was similar in the composition but with reduced agar content. Agar content was reduced to 75%, 50% and 25% (100% = 7g) that was used for the initiation of callus. The media were poured into disposable Petri dishes for the study of callus growth. Callus weighing 0.1 g from a month old culture were transferred onto media with each percentage of agar content. The cultures were incubated in the dark, at room temperature for a month before harvesting for growth weight measurement. One gram of the callus was chopped into small pieces and transferred into 40 ml liquid media in 250 ml Erlenmeyer flask. The liquid media was MS media used before without addition of agar. The suspension was incubated on an orbital shaker and was observed for the indication of browing.

Surface structure analysis

The surface structure changes were analyzed using analytical scanning electron microscope (SEM, Model JEOL JSM-6460L4, Japan). The callus applied on an aluminium stub using double-sided adhesive tape and the callus was coated with gold powder to avoid charging under the electron beam after the acetone was volatilized.

Results and discussion

A study on agar reduction

Fig. 1 shows the growth of callus when cultured on MS media which have been reduced the agar content. Generally the growth of the adapted callus was improved when the media was less solidified compared to the control. The reduction of 75% and 50% agar content demonstrated no difference in the growth compared to 25% where the weight was more than double of the control. The agar cannot be reduced to less than 25% as the callus turned brown and died as they are suspended in the media. Therefore, 25% is the lowest percentage of agar content that can maintain the floating of callus on the media. It was expected that the failure of growth when suspended because of the lack of oxygen, even though the uptake of nutrient was more effective. Xing *et al*. (2001) showed that the transfer of oxygen inside the callus was the main limiting factor for the slow callus growth. Therefore one way to sustain oxygen supply is to keep callus floating while increasing the efficiency of nutrient uptake by reducing the solidness of the medium. It was proven that nutrient uptake was better as shown by the increase in the callus weight. In addition, viability and survival of the adapted callus in the suspension was well improved as callus maintained healthy up to 14 days (Fig. 2).

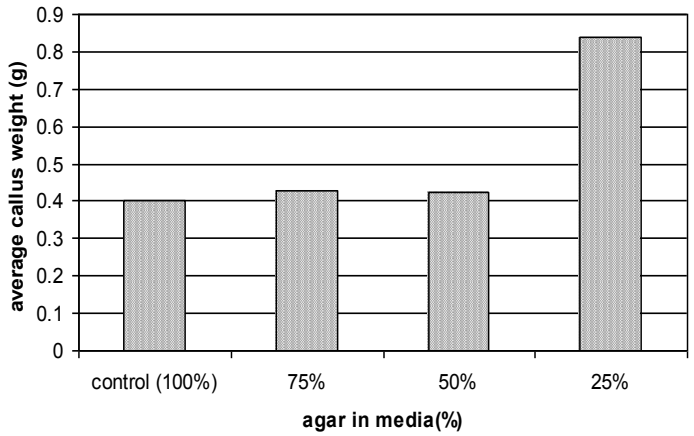


Fig. 1. Results on the average callus weight produced with the reduction of agar content in growth media.

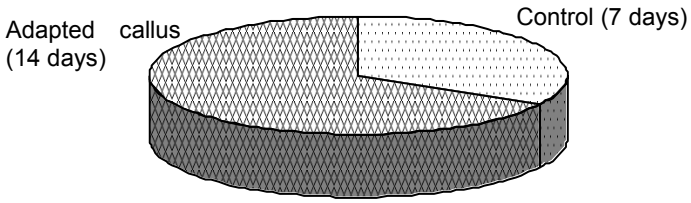
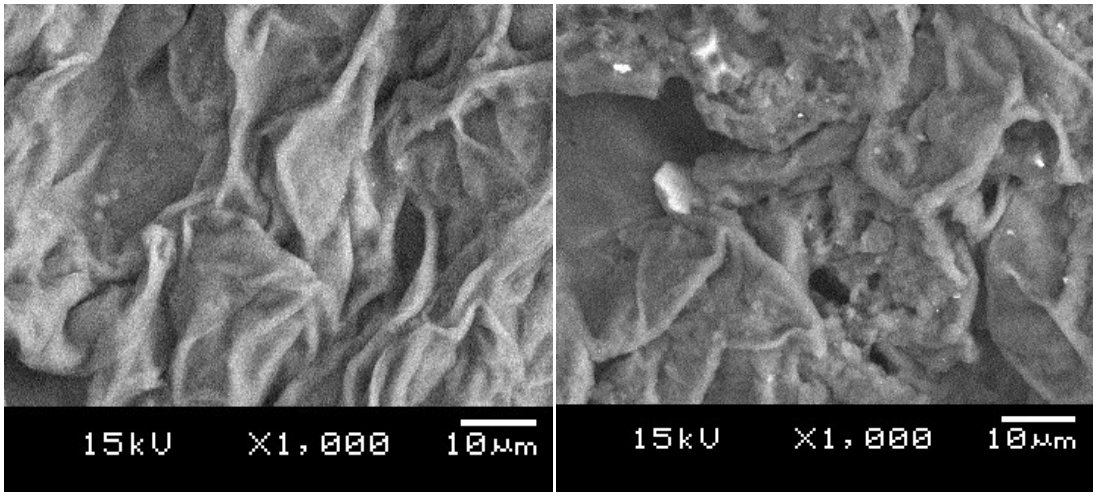


Fig. 2. Days of browning development in suspension.



Adapted Callus Control

Fig. 3. SEM microscopy of adapted callus and control at 1000 x magnification.

Investigation on cell structures change using SEM

To prove that there were some changes in the surface structure of the callus, an investigation was carried out by SEM (Fig. 3). The microscopy of adapted callus shows more folds that line very close to each other. The surface of control callus has less uneven folds. The close up of the surface of the adapted callus shows a deeper hole was developed. It was concluded that the changes in the surface structure of the adapted callus has increased the surface area and therefore increased the efficiency of oxygen and nutrients uptake.

Acknowledgements

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Microbial Diversity of Spontaneous *Bambangan* (*Mangifera pajang*) Fermentation, a Traditional Fermented Fruit from Northern Borneo

Chin, S.Y.[1], Sim, K.Y.[1], Chye, F.Y.[2] and Anton, A.[1]

[1]Biotechnology Research Institute, [2] School of Food Science and Nutrition,
Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia
sengyee2881@hotmail.com

Abstract

The main purpose of this study was to identify the microorganisms that involved in the spontaneous fermentation of *bambangan* (M*angifera pajang*). Enumeration and isolation of lactic acid bacteria (LAB), halophilic bacteria, mesophilic bacteria, and yeast were carried out during the fermentation process and isolates were identified using Biolog Microstation™ Reader Release 4.2 based on their phenotypic characteristics. Hydrolytic activities and technological properties of the isolates including enzymatic activities, acid resistance, bile tolerance, and antimicrobial activities were also performed. A microbial succession was observed during fermentation which dominated by LAB in the initial stage with the mean numbers of 6.44 log CFU/g corresponding to the reduction of pH from 3.60 to 3.21. Lactic and citric acid were the major organic acids detected during the process. Yeasts became dominant after 10 days of fermentation and remained predominant throughout the process. Microorganisms isolated include *Lactobacillus plantarum, Pediococcus pentosaceus* and *Candida krusei* which showed excellent pectinolytic and cellulolytic activities facilitated the hydrolysis of pectin and cellulose rich substrate. *Candida Krusei* demonstrated antimicrobial activity against *Salmonella typhimurium, vibrio cholerae* and *Staphylococcus aureus* whereas *Lactobacillus plantarum* gave significant inhibitory against *S.typhimurium* and *Listeria monocytogenes*. The results revealed that *bambangan* fermentation is primarily lactic acid fermentation with the ubiquitous presence of *Lactobacillus plantarum* and *Pediococcus pentosaceus* while *Candida krusei* and *Kloeckera apis* were the dominant character at the later stage of fermentation. Further study on the development of potential starter cultures should be carried out to upgrade the fermentation process with controllable parameters.

Introduction

Indigenous fermented foods make up an important contribution to the human diet in many developing countries (Murty and Kumar, 1995; Steinkraus, 1996). Some of these foods formed the essential diet components of certain populations and are consumed either as main dishes or as condiments (Steinkraus, 1996). Studies on indigenous fermented fruits has been carried out in many regions and include tempoyak (Leisner *et al*., 2001), Masau fruit (Loveness *et al*., 2007), table olive (Alejandro *et al*., 2007), cocoa (Nielsen *et al*., 2007), grape (Christine *et al*., 2006) and apple must (Emmanuel *et al*., 2006).

Bambangan (*Mangifera pajang*) which belongs to the family Anacardiaceae is an indigenous fruit with brownish skin and yellow colour flesh grows wild in the forest of Borneo Island (Rukayah, 1999). This mango-like fruit can be eaten fresh or to be processed into fermented products that retain its high degree of sensory qualities with soft and tender texture, mango-like fragrance, pale yellow colour, salty and sour taste. It can be eaten with rice, as pickle or used as a condiment with fish and it's usually served by the locals as their traditional ethnic food to the tourists. Fermented *bambangan* are made based on the empirical knowledge by mixing the bambangan cubes and its grated seed together with salt and left to ferment at ambient temperature (28ºC - 30ºC) in a tightly closed container for 7-10 days. The fermentation of bambangan remains as a cottage industry in Northern Borneo and its method of processing may vary among producers. In addition, poor hygiene practice during production of bambangan usually results in spoilage of final product and poor keeping quality. Hence, it is important to assess the microbiological and biochemical changes of indigenous fermented foods with the intention to improve the fermentation process and product acceptability. Therefore, this study was undertaken to identify the types of microorganisms that involved in the bambangan fermentation.

Materials and methods

Sample collection and preparation

Ten kg of freshly made substrate (mixture of *bambangan* fruit and salts) was obtained from a consumer preferred local and kept in ceramic jars for fermentation at conditions similar to that of the producer. Samples were withdrawn and analyzed every two day interval for the first two weeks and subsequently every week during the study.

Proximate analysis

Proximate analyses included moisture content, protein, fat, ash and fiber were done according to the AOAC methods (AOAC, 2000).

Chemical analysis

Salt content, pH, titratable acidity, total soluble solid and alcohol content were done according to AOAC methods (AOAC, 2000). Analysis of sugar composition and organic acids were done according to the method described by Flavio *et al*. (2005) and Beatriz *et al*. (2001).

Microbiological analysis

Enumeration of total aerobic, lactic acid bacteria (LAB), yeast and mould, halophilic bacteria and enterobacteriaceae counts were done. Isolation and identification of isolates were done according to their morphological and biochemical characteristics. The morphological properties of the different colony types were recorded and counts made for each type. Five typical colonies from each different morphology were picked from the higher countable dilution plates and subjected for identification. The isolates were identified based on the phenotypic properties such as carbon dioxide production from glucose, growth at different temperatures and the ability to grow in different concentrations of sodium chloride and pH in nutrient broth. The yeast isolates were identified by using the Simplified Identification Method (SIM) described by Deak and Beuchat (1996), with additional standard taxonomical methods (Kurtzmen, 1998; Meyer *et al*., 1998). Further identification for bacteria, LAB and yeast were subjected for their ability to utilized 95 single carbon sources by using Biolog bacterial identification system (Biolog Inc., USA). The metabolism of the carbon sources produced a specific "metabolic fingerprint" for each strain to be compared with the data from Biolog MicroLog database software Release 4.2 (Biolog Inc.).

Hydrolytic and probiotic test

Pectinolytic, cellulolytic, proteolytic, amylolytic and lipolytic were done according to Hankin *et al.,* 1971; Ruiz, Pastor and Diaz, 2005; Harrigan, 1998; Mauriello *et al.,* 2004 whereas acid resistance (pH 1.5, 2.0, 3.0), bile tolerance (0.1% – 0.5%), growth at 37°C, 45°C and antimicrobial activity were done according to Psomas *et al*., 2001 and Schillinger and Lucke, 1989.

Results and discussion

There were no significant changes of the proximate content during fermentation except ash and protein content. The increment of ash and protein content during fermentation process is commonly practice found in some fermented food (Azokpota *et al*., 2006; Giami, 2004; Onyango *et al*., 2004). Table 1 shows the chemical properties of the bambangan during fermentation. The pH decreased from 3.60 to 3.21 within 10 days of fermentation. There was significant negative correlation in between pH and titratable acidity for the substrate. This phenomenon is in agreement with the study reported previously for other fermented foods such as *kimchi, ogi*, cassava, yogurt, rice vinegar, and finger millet (Sulma *et al*., 1991; Choi *et al*., 1994; Dziedzoaze *et al*., 1996, Haruta *et al*., 2006; Viander *et al*., 2003). The increment of total acidity might be due to the raised organic acids which is showed in Fig. 1. Lactic acid and citric acid were the main organic acids in fermented *bambangan*. Maleic acid, acetic

acid, quinnic acid, fumaric acid, succinic acid and alpha-ketoglutaric acid were not found in the fermented sample. The salt content was in ranged from 3.43 – 3.50. A steep drop was seen in the concentration of reducing sugar from 17 mg/g to 0.29 mg/g. Total soluble solids in fermented bambangan decreased with fermentation time from 16.2 ºBrix to 12.2 ºBrix seeing as the amount of sugar depleted gradually when the fermentation progressed. Glucose was the main sugar followed by sucrose and fructose in bambangan. During *bambangan* fermentation, glucose content dropped sharply from 38.4 mg/g to an undetectable level after 14 days presumably caused by the increasing microbial population since it was the major carbon source to support the growth of microorganisms.

LAB count increased in the early stages of fermentation from 4.65 log cfu/g to 6.44 log cfu/g followed by a steady fall in the count after 6 days as shown in Fig. 2. The yeast number increased continuously from initial 3.90 log cfu/g to reach the highest level at 6.09 log cfu/g on day 12 before dropping gradually in the later stage of fermentation. Halotolerant count increased about 1 log cycle from 3.6 log cfu/g to 4.73 log cfu/g on day 8 and remained at this level for the following 6 days. None of the halophilic and enterobacteriace group was found in fermented *bambangan*.

Hundred thirty-nine isolates belonging to 8 genera (*Lactobacillus, pediococcus, Candida, kloeckera, Pichia, Micrococcus, Staphylococcus* and *Corynebacterium*) were obtained from different stages of indigenous fermentation of bambangan (Table 2). *Lactobacillus plantarum* and *Pediococcus pentosaceus* were found to be the predominant bacteria at the initial stage of the fermentation since their counts exceeded the other populations such as *Lactobacillus delbrueckii spp delbrueckii* and *Lactobacillus paracasei spp paracasei 1* which are commonly found in *kimchi* fermentation (Ha and Cha, 1994; Kang *et al*., 1995; Kim *et al*., 1998). However the numbers dropped significantly after day 6 of fermentation to a barely discernible level at day 14.

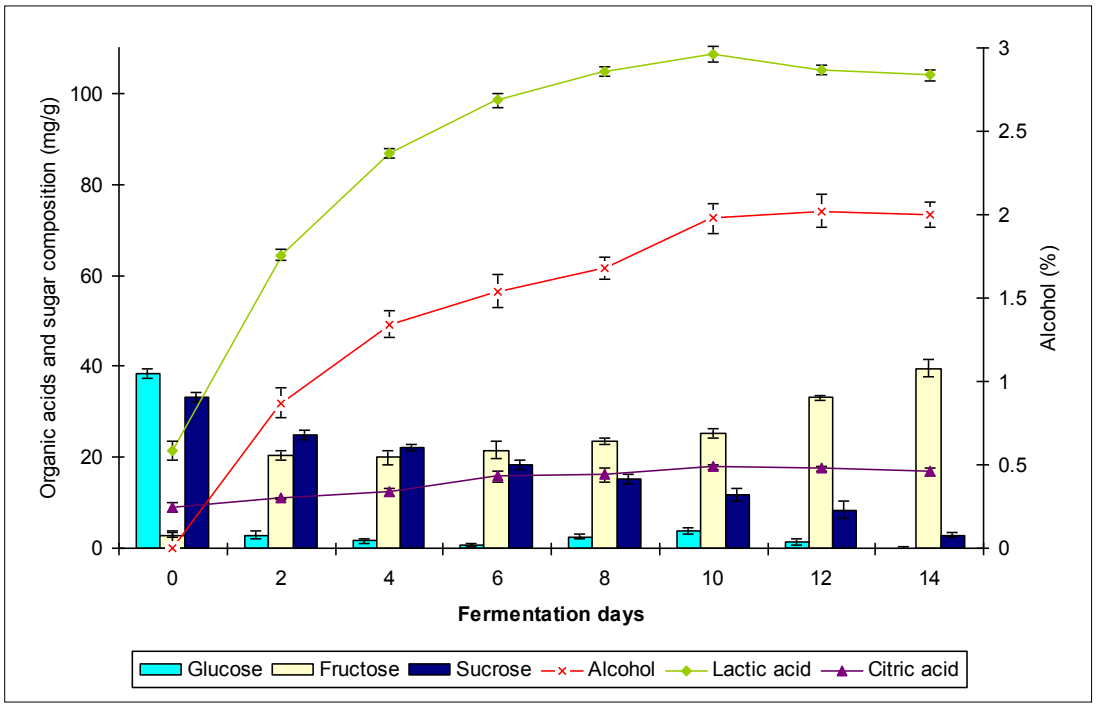
Yeasts population increased throughout the fermentation but the types of yeasts present differed with time. *Candida krusei* and *kloeckera apis* were the only yeasts that appeared throughout the fermentation process. *kloeckera spp* has been widely documented in cocoa, cider and wine fermentation as the major species found in cocoa bean and fresh must (Morrissey *et al*., 2004). They are capable to producing several enzymes that play important roles in variety of aroma compounds (Charoenchai *et al*; 1997). Therefore, *Kloeckera apis* might have similar contribution in bambangan fermentation. *Candida krusei* was present with the highest percentage at the end of fermentation. It is found to be dominant in several different types of indigenous fermented foods and beverages such as maize dough, milk, cassava dough, cocoa bean and sorghum beer (Lore, Mbugua and Wangoh, 2005, Oyewole, 2001, Jespersan, 2003). This yeast also has been demonstrated to be involved in the typical odor and flavour development during fufu and cocoa fermentation (Omemu, Oyewole and Bankole, 2007). It may be possible that *Candida krusei* plays a similar role in *bambangan* fermentation.

Based on the results, *bambangan* fermentation is primarily a lactic acid fermentation with the ubiquitous presence of *Lactobacillus plantarum* and *Pediococcus pentosaceus* while *Candida krusei* and *Kloeckera apis* were the overriding microorganisms at the later stage of fermentation. The identification of the dominant microorganisms and its performance at each stage of bambangan fermentation contributes to a better understanding of the fermentation process which might used for further developments aimed at improving the control of bambangan fermentation for example the development of a potential starter culture to improve the traditional fermentation. The development of potential starter culture is important for the production of fermented bambangan at commercial and small industrial scale.

Table 1. Chemical analysis in bambangan fermentation

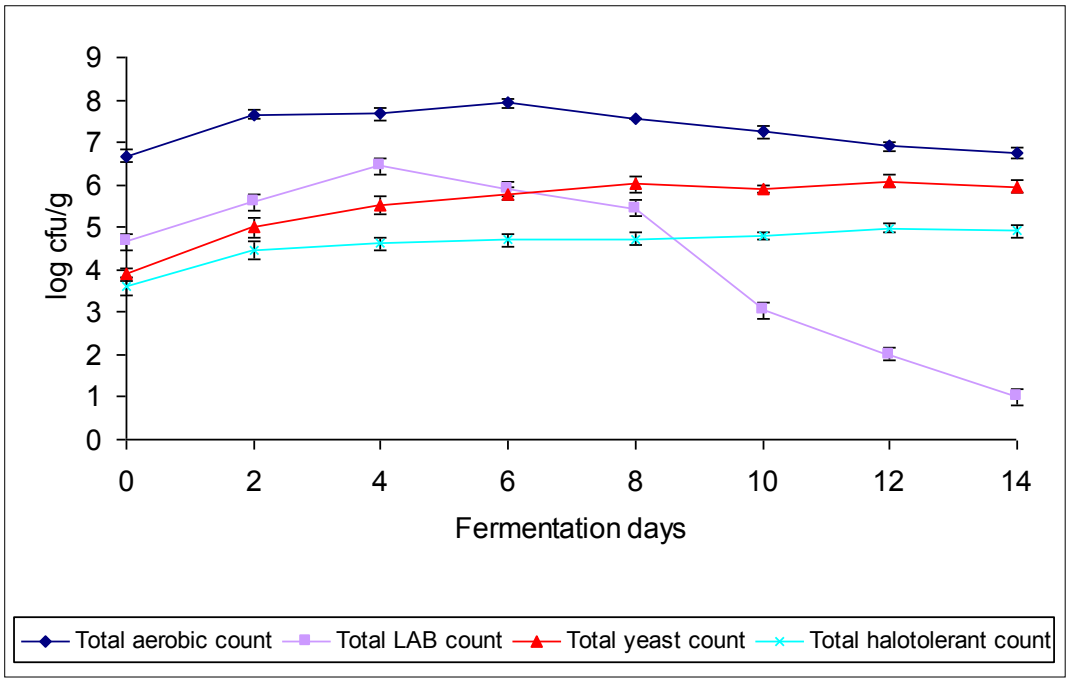
Fermentation time (day)	pH	Acidity (%)	Total soluble solid (ºBrix)	Reducing sugar (mg/g)
0	3.60^{a}±0.10	1.51^{f}±0.10	16.2^{a}±0.30	17.00^{a}±1.50
2	3.45^{b}±0.05	1.78^{e}±0.21	15.6^{a}±1.00	10.40^{b}±1.04
4	3.26^{c}±0.06	1.97^{d}±0.17	14.8^{b}±0.86	6.23^{c}±0.97
6	3.21^{cd}±0.09	2.52^{c}±0.38	14.8^{b}±1.50	2.54^{d}±0.21
8	3.23^{cd}±0.10	2.50^{b}±0.28	14.6^{b}±0.50	1.77^{e}±0.09
10	3.21^{cd}±0.10	2.53^{a}±0.20	12.2^{c}±0.80	0.95^{f}±0.06
12	3.23^{cd}±0.05	2.49^{b}±0.15	13.8^{c}±1.51	0.48^{g}±0.02
14	3.30^{d}±0.08	2.25^{b}±0.20	13.8^{c}±0.50	0.29^{g}±0.10

*Data are express as mean ± standard deviation; all assay done in triplicates
*The mean followed by different letters in the same column indicate a significant difference ($p<0.05$).



*Maleic acid, acetic acid, quinnic acid, fumaric acid, succinic acid and alpha-ketoglutaric acid were not found in the sample.

Fig. 1. Organic acids, sugar composition and alcohol content in *bambangan* fermentation.



*Enterobacteriace and halophilic count (15% NaCl) were not detected along the fermentation process
Fig. 2. Microbial load of bambangan fermentation.

Table 2. Microbial population dynamics in bambangan fermentation

Species	Fermentation period (day)							
	0	2	4	6	8	10	12	14
Lactic acid bacteria								
Lactobacillus plantarum 2	7[a] (14.6)[b]	8 (24.2)	8 (32.0)	6 (33.3)	7 (30.4)	5 (23.8)	2 (14.3)	1 (7.7)
Pediococcus pentosaceus	8 (16.6)	6 (18.2)	6 (24.0)	5 (27.8)	5 (2.7)	2 (9.5)	1 (7.1)	0
Lactobacillus delbrueckii spp delbrueckii	0	3 (9.1)	2 (8.0)	1 (5.6)	0	0	0	0
Lactobacillus paracasei spp paracasei 1	4 (8.3)	1 (3.0)	1 (4.0)	0	0	0	0	0
Yeast								
Kloeckera apis	3 (6.3)	4 (12.1)	3 (12.0)	3 (16.7)	4 (17.4)	5 (23.8)	3 (21.4)	2 (15.4)
Candida krusei	0	0	2 (8.0)	2 (11.1)	7 (30.4)	9 (42.9)	8 (57.1)	10 (76.9)
Candida lusitaniae	4 (8.3)	2 (6.1)	1 (4.0)	0	0	0	0	0
Candida guilliermondii	1 (2.1)	0	0	0	0	0	0	0
Candida parapsilosis	4 (8.3)	1 (3.0)	0	0	0	0	0	0
Candida diversa	0	1 (3.0)	0	0	0	0	0	0
Pichia fluxuum	3 (6.3)	2 (6.1)	1 (4.0)	0	0	0	0	0
Bacteria								
Micrococcus luteus	5(10.4)	2 (6.1)	0	0	0	0	0	0
Staphylococcus xylosus	2 (4.2)	1 (3.0)	0	0	0	0	0	0
Staphylococcus arlettae	1 (2.1)	0	0	0	0	0	0	0
Corynebacterium nitrilo	2 (4.2)	0	0	0	0	0	0	0
Unidentified								
Unknown (closest relative *Lactobacillus acidophilus*)	1 (2.1)	0	0	0	0	0	0	0
Unknown (closest relative *Candida utilis*)	1 (2.1)	1 (3.0)	0	0	0	0	0	0
Unknown (closest relative *Candida glabata*)	1 (2.1)	0	0	0	0	0	0	0
Unknown (closest relative *Candida boidinii*)	0	1 (3.0)	0	0	0	0	0	0
Unknown (closest relative *Issatchenkia scululata var exigua*)	0	0	1 (4.0)	1 (5.6)	0	0	0	0
Unknown	1 (2.1)	0	0	0	0	0	0	0
Total isolates	48	33	25	18	23	21	14	13

* [a] Number of isolate
* [b] Value in bracket represented the percentage of isolates in each fermentation day
* 0 = not detected

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Prolyl Endopeptidase (PEP) Inhibitory Activity From an Endophytic Fungus Isolated in Sarawak

Ng, L.T., Noreha, M., Jamilah, H. and Tan, L.T.

Sarawak Biodiversity Centre, KM 20 Jalan Borneo Heights, Semengoh,
Locked Bag No. 3032, 93990 Kuching, Sarawak, Malaysia
leetze@sbc.org.my

Abstract

Prolyl endopeptidases (PEPs), are proline-specific proteases which are actively involved in every phase of metabolism in plants and animals. They have been implicated in neurological disorders such as Alzheimer's Disease, amnesia, depression, and schizophrenia. Hence, inhibitors of PEP could play a role in the control of these diseases. Through our microbial bioprospecting programme, an endophytic fungus with potential PEP inbibitory activity was isolated from yam flower in the Padawan area, Kuching, Sarawak. This fungus, F0274 was isolated and maintained on Potato Dextrose Agar. Based on morphological examination, the fungus is preliminary classified as a *Fusarium* sp. To study the production of PEP inhibitors, the fungus was first grown statically in flasks with 20 mL Potato Dextrose Broth at 28°C until mycelia covered up the surface, and then shaken overnight at 200 rpm. Butanol crude extracts were prepared from the culture broths in a 1:1 ratio. PEP inhibitory assay revealed that 200 µL of crude extract was able to inhibit the activity of 0.0416 Units of PEP by 90%. Neither sonication of culture broth, nor removal of mycelia before solvent extraction appeared to affect the inhibitory activity, suggesting that the active compound is likely to be an extracellular metabolite. Optimization studies of submerge aerobic fungus culture is currently being undertaken.

Introduction

Sarawak is rich with natural resources that can be exploited for many purposes. Under the Sarawak Biodiversity Centre's (SBC) Microbiology programme, as of September 2008, 1,080 endophytic fungal isolates have been collected from various plants in Sarawak. Endophytic fungi live symbiotically with plants and it is asymptomatic within the plant tissues (Faeth and Fagan, 2002). The isolated fungi are being tested at the SBC for their ability to synthesize bioactive compounds including those which can inhibit prolyl endopeptidase (PEP). Current research has associated the neurological disorders Alzheimer and schizophrenia with the degradation of neuropeptides by PEP in the brain (Garcia-Horsman *et al.,* 2006). Thus, there is interest in bioprospecting for compounds which can inhibit PEP.

Peptides that inhibit PEP have been isolated from red wine made from Cabernet Sauvignon grapes (Yanai *et al.,* 2002). Lee and workers (2004) found that leaves of *Ginkgo biloba* showed significant PEP inhibition and concluded that it is potentially a source of new types of PEP inhibitors. Hexane extracts of the leaves of *Syzygium samarangense* (Blume) Merr. and L. M. Perry have also been found to contain PEP inhibitors (Amor *et al.,* 2004). There are few reports of PEP inhibitors being found in fungi. Thus, the SBC's endophytic fungi collection was screened for PEP inhibitory activity. We report our preliminary findings on a fungus isolated near Kuching, Sarawak. In flask liquid culture, it exhibits ability to synthesize compounds which inhibit PEP activity in assay.

Materials and methods

Isolation of F0274

Strain F0274 was isolated from the flower of a yam plant collected in the Padawan area 30 km from Kuching, Sarawak. The inner part of the yam flower was excised and cut into several 0.5 cm^2 blocks. The blocks surface was sterilized by sequential washes in 70% ethanol, 0.8% NaOCl and rinsed with sterile reverse osmosis water. The sterilized blocks was dried under aseptic condition and placed on Potato Dextrose Agar (PDA) in Petri dishes. All plates were incubated at room temperature for at least seven days. Fungi growing out from the plant tissue were inoculated on new PDA plates. After checking for purity, each isolate was stored in sterile 25% glycerol at 4°C and -80°C respectively.

Morphological description of F0274

a) Colony Morphology. Seven-day old agar plugs containing mycelium and spores were one- and three-point inoculated on PDA. The plates were incubated at room temperatures for 14 days. The growth characteristics (growth rates, aerial mycelium and growth pattern) and colony colour (surface and reverse) were recorded and photographed. b) Micro-morphology. The micro-morphological structures of mycelium, spores and accessories structures were observed microscopically. The characteristics were detail attention are: i) colour of sporodochia; ii) shape, size, septation, basal shape, conidiophores and colour of macroconidia; iii) shape, size, present of septation, abundance, conidiophores and colour of microconidia; iv) shape, arrangement, colour and present of chlamydospores; v) shape, size, pattern and colour of hyphae (Booth, 1971).

Fermentation

For liquid culture, an agar plug contained mycelium and spores of a 7-day old fungal colony was placed into a 125 ml conical flask containing 20 ml Potato Dextrose Broth (PDB). Flasks were incubated at 28^{o}C until mycelium covered the broth surface. The flasks were then shaken at 200 rpm overnight. The procedure for large cultures was the same except that 4L conical flasks containing 2 L of PDB were used, and the inoculum comprised 8 agar plugs.

Extraction

At the end of the culture, the mycelium was ultrasonicated. Butanol was added to each flask in a1:1 ratio with culture volume. The mixture was then shaken for 2 h after which it was centrifuged at 10,000 rpm, 4^{o}C for 10 minutes and the supernatant (butanol extract) was retained for further analysis. The first crude type of extract was prepared for PEP inhibition assay by completely drying 200 µl of butanol extract prepared as described above, at room temperature. The dried residue was re-dissolved in 50 µl of 10% DMSO. A later form of crude extract was prepared using 4 mL of butanol extract which was similarly dried and the weight of the residue determined. Then, crude extracts containing 0.2, 0.1, and 0.05 mg mL^{-1} of dried residues were prepared by re-dissolution in 10% DMSO.

Prolyl endopeptidase (PEP) inhibition assay

Into each well of a microtitre plate was added, 78 µl of Phosphate buffer (100 mM, pH 7.0), 10 µl of PEP (from *Flavobacterium meningosepticum*; MP Biomedicals, Inc.) solution (0.5 U mL^{-1} giving 0.0416 units well^{-1}), and 12 µl of crude extract. At this stage the culture broth is 0.4 times its original concentration (referred to as C). The plate was then equilibrated at 30^{o}C for 15 minutes. The reaction was initiated with addition of 20 µl of the substrate carbobenzyloxyglycyl-L-prolyl-ρ-nitroanilide (2 mM in 40% dioxane-100 mM phosphate buffer, pH 7.0) and incubated for 15 minutes at 30^{o}C. The reaction was stopped by adding an equal volume of stopper (2 M acetate buffer, pH4.0 with 10% Triton-X). The released ρ-nitroanilide was determined colorimetrically with a microtitre plate reader (Sunrise, TECAN) at 414 nm. Percentage inhibition was calculated thus:

% inhibition = $1 - \left(\frac{\Delta OD_{sample}}{\Delta OD_{Blank}}\right)$ where ΔOD = change in OD during the assay.

For the determination of the Minimum Inhibitory Concentration of the fungal compounds found in liquid culture required to inhibit the activity of PEP by 90% ($MIC_{90\%}$), assay was performed with crude extract at serially diluted strength to give 0.4C, 0.2C, 0.1C, 0.05C, and 0.025C. $MIC_{90\%}$ was also determined using crude extracts containing 0.2, 0.1, and 0.05 mg mL^{-1} of dried residue.

Results and discussion

Examination of isolate F0274

On PDA, 14-day old culture turned pink to pale red (Fig. 1). The hyphae is cylindrical, septate, colourless and <1-2 µm wide. Oval to oblong shaped microconidia with diameters <10 µm and hyaline in colour are produced (Fig. 2) as are chain chlamydospores (Fig. 3), and colourless crescent shaped of macroconidia with 3-6 septa, 24-48 µm wide (Fig. 4).

Fig. 1. Isolate F0274 after 14 days on PDA at room temperature.

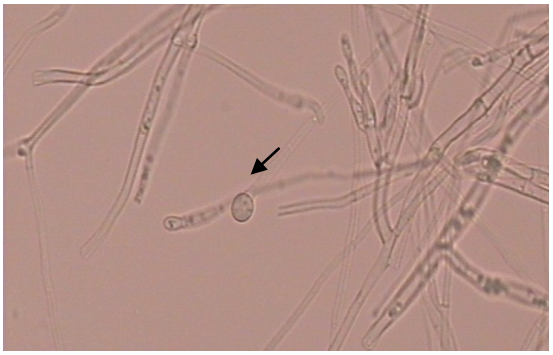

Fig. 2. Oval to oblong microconidia (→) on biphialides produced by Isolate F0274 on PDA.

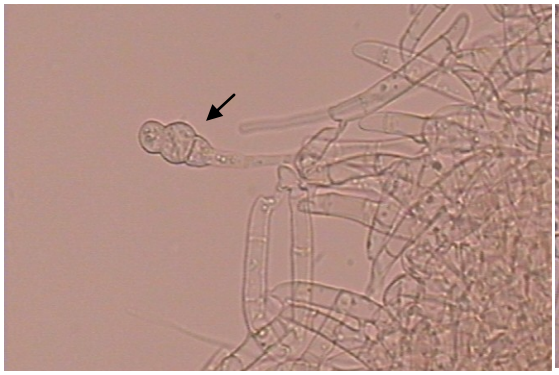

Fig. 3. Chlamydospores (→) produced in chains by Isolate F0274 on PDA.

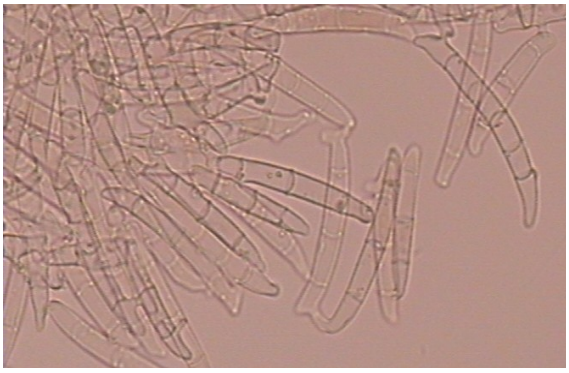

Fig. 4. Crescent shaped macroconidia of Isolate F0274 on PDA.

PEP inhibitory activity

Crude extract from 20 mL flask cultures at Standard Strength had high potency against PEP (98% inhibition of the activity of 0.0416 Units) (Table 1). The $MIC_{90\%}$ was reached when the crude extracts were one-tenth the concentration of the original culture broth (91% inhibition).

In a different experiment with 20 mL flask cultures, the fungus appeared to maximize the synthesis of inhibitor compounds by about 14 days into the incubation and the concentration of these compounds did not lower within 28 days (Table 2). Diluting the recovered residue from butanol extraction four-fold (to 0.05 mg mL^{-1}) did not change potency in inhibiting PEP. $MIC_{90\%}$ is associated with a dried residue concentration of less than 0.05 mg mL^{-1}.

When culture volumes were increased from 20 mL to 2 L, the synthesis of inhibitor compounds was clearly lower (Table 3). Assay with 0.2 mg mL^{-1} of dried residue indicates that inhibitor compounds at Day 7 of the 2 L culture were only 30% of that found after the same period of

incubation in the 20 mL culture. $MIC_{90\%}$ was not attained from any of the crude extracts from the 2 L cultures.

Table 1. The inhibition of prolyl endopeptidase by various strengths of crude extracts from 20 mL liquid culture of Isolate F0274

Strength of crude extract	**0.4C**	**0.2**	**0.1**	**0.005**	**0.025**
% inhibition	98	95	91	86	70

Table 2. The inhibition of prolyl endopeptidase by various strengths of crude extracts from 20 mL liquid culture of Isolate F0274 incubated for various periods

Length of flask culture (days)	**% inhibition by crude extracts***		
	0.2 mg mL^{-1}	**0.1 mg mL^{-1}**	**0.05 mL^{-1}**
7	92	78	54
14	98	101	97
21	97	97	96
28	97	98	97

*the concentrations (mg mL^{-1}) are of dried residues from butanol extraction, re-dissolved in 10%DMSO

Table 3. The inhibition of prolyl endopeptidase by various strengths of crude extracts from 2 L liquid cultures of Isolate F0274 incubated for different periods

Length of flask culture (days)	**% inhibition by crude extracts***		
	0.2 mg mL^{-1}	**0.1 mg mL^{-1}**	**0.05 mg mL^{-1}**
7	28	21	12
14	23	16	8
21	31	16	6
28	15	14	2

*the concentrations (mg mL^{-1}) are of dried residues from butanol extraction, re-dissolved in 10%DMSO

Macro- and microscopic examination reveal key morphological characters that allow a preliminary identification of Isolate F0274 as a *Fusarium* sp. Further confirmatory tests including genetic profiling will be undertaken.

Butanol extracts of liquid cultured F0274 yields inhibitor compounds of high potency. Further assay will be conducted to determine the $MIC_{90\%}$ in mg mL^{-1}, now known to be less than 0.05.

Large scale liquid cultures are necessary in order to yield sufficient quantities of material for analysis. It is clear that optimization studies on the growth of Isolate F0274 in submerged aerobic culture and the associated synthesis of inhibitor compounds will be required as the yields with the 2 L cultures are only about a third of that from 20 mL cultures. The parameters that can be gainfully investigated include the inoculum protocol (form and size), shaking speed, flask to working volume ratio, and type of flask closure.

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Callus Induction from Mature Endosperm and Cotyledon Tissues of *Jatropha curcas*

Siow, T.S., Ting, S.Y.A. and Tee, C.S.

Faculty of Engineering and Science, Universiti Tunku Abdul Rahman,
Jalan Genting Kelang, Setapak, 53000 Kuala Lumpur, Malaysia
teecs@mail.utar.edu.my

Abstract

Jatropha curcas (Euphorbiaceae) is a perennial, oil-bearing and multipurpose shrub, native to the tropics and sub-tropics of Asia and Africa. To date, the non-edible oil in Jatropha seeds is discovered to be suitable and has good value for biofuel production. However, the genetic variation in seed traits was found to significantly affect the seed germination and oil yields. Therefore, in vitro plant propagation provides an alternative to mass propagate the plants. In this study, we aim to induce callus from mature endosperm and cotyledon explants in order to facilitate propagation studies and oil content analysis. Callus induction study was carried out using the MS basal medium supplemented with a single auxin (NAA, IAA, IBA, 2,4-D, picloram or dicamba) with concentrations of 0 to 8 µM. Results showed that picloram, dicamba and 2,4-D were more suitable auxins for callus induction using the endosperm tissues. Of these, 4 µM picloram gave the highest callus induction percentage (53.33±6.67%), followed by 4 µM dicamba (51.11±5.88%) and 6 µM 2,4-D (40.00±5.77%). As for the cotyledons, 4 µM 2,4-D and dicamba were suitable for callus induction with 31.11±10.61% and 28.89±4.84% of callus induction, respectively. The induced calli were further maintained and multiplied on new MS basal supplemented with combination of a single cytokinin (BAP, kinetin, TDZ or zeatin) and suitable callus-inducing auxin as our preliminary observation indicated that induction medium was unsuitable. Further study to induce embryogenic callus is now being investigated for regeneration purpose and oil content analysis.

Introduction

Jatropha curcas is a deciduous and perennial shrub, belonging to the Euphorbiaceae family, and has a 50-year productive life-span (Openshaw 2000; Kulkarni *et a*l. 2005). It is native to the tropical America and the tropics and now thrives in sub-tropics of Asia and Africa. It is considered as a multipurpose plant or miracle tree, attributed to its enormous usages and contributions in domesticity, agricultural and economical sectors (Benge 2006). In India, *J. curcas* has been planted and used for conventional purposes for a few decades. In rural area, Jatropha is grown as live hedges, and also used in the reclamation of wastelands and for medicinal purposes. Besides, jatropha oil is widely used as source of light and soap making (Openshaw 2000). At present, due to the rapid exhaustion of the fossil fuel, the high oil content in jatropha seeds is attracting worldwide attention for biofuel production (Chhetri *et al*., 2008).

Currently, large-scale of jatropha plantation has been initiated in many countries for income-generating activities by harvesting jatropha seeds (Eijck and Romijn 2008). Conventional cultivation of jatropha is normally through seeds or vegetative propagations. However, plants from cuttings have lower longevity and less drought and disease resistance (Heller, 1996), while survival rate of plant geminated from seed is dependent on sowing time and depth of sowing (Kumar and Sharma, 2008). Devastation of pests which causes flower fall, fruit abortion and malformation of seed is another problem (Shanker and Dhyani, 2006). Hence, specialized planting practices and managements are required in seed propagation (Openshaw, 2000). On the other hand, recent study revealed that genetic variation in seed traits significantly affected the seed morphology, seed germination rate and seedling growth parameters and oil content of seed (Ginwal *et al*., 2005; Kaushik, 2007). Besides, seed yields varied from 0.1 to 15 tonnes per ha per year in different countries and regions (Heller, 1996; Jones and Miller, 1993). These critical limitations had created a tough challenge to the growers and investors.

As an alternative, *in vitro* propagation was introduced in cultivation of *J. curcas* to overcome the limitations from conventional breeding. In this manner, true-to-type plants could be regenerated from mother plant which have elite desired traits and the multiplication rate of plant could be speeded up as

well. Besides, it is also economically reasonable in terms of space requirements and labour costs (George *et al*., 2007). Early studies showed that plants were able to be regenerated from leaf and hypocotyls tissues (Sujatha and Mukta, 1996). Further studies were targeted on direct plant regeneration from epicotyl, axillary nodes, leaf and shoot tip (Wei *et al*., 2004; Sujatha *et al*., 2005; Rajore and Batra, 2005; Datta *et al*., 2007; Deore and Johnson, 2008). Other than that, plant regeneration from somatic embryogenesis was successfully developed using leaf tissues (Jha *et al*., 2007).

Plant regeneration system from those studies used MS basal medium supplemented with combinations of cytokinin and auxin. However, the oil content study of in vitro tissues of *J. curcas* has not been documented. It is important as *in vitro* tissue might provide another source of crude oil from *J. curcas* other than from seeds. In addition, studies also revealed that plants could be regenerated from mature endosperm and cotyledons tissues (Selvaraj *et al*., 2007; Thomas and Chaturvedi, 2008). Hence, in this study, mature endosperm and cotyledons tissues were being used for callus induction using single auxin media for further facilitate indirect plant regeneration and oil content analysis.

Materials and methods

Explant source

Two to three month-old mature seeds were bought from a commercial company (Global Bonanza Sdn. Bhd.). Surface sterilisation was initiated by washing the seeds three times with detergent for 1 min and then slightly flamed for 3 sec. Seeds were dehulled and surface sterilized with 30% Clorox® for 15 min. Dehulled seeds were rinsed twice with sterile distilled water thoroughly for 2 min. Surface sterilisation was repeated of using 20% Clorox® for 10 min and 2 min rinsing was repeated twice as well. Dehulled seeds were further disinfected with absolute alcohol for 1 min and rinsed for 2 min twice. Endosperm and cotyledons tissues were removed from disinfected seeds and cut into 5x5 mm and 3x3 mm in size, respectively.

Callus induction and conditions

Explants were cultured on phytohormone-free MS basal medium prior to culture on callus induction media which containing MS basal medium supplemented with single auxin (NAA, IAA, IBA, 2,4-D, picloram or dicamba) within 0 to 8 µM. Viable explants were selected from one-week-old culture and cultured on callus induction media. Cultures were maintained at 25±1^{o}C under 16 h photoperiod illuminated in fluorescent lamps at light intensity about 1000 lux.

Medium and culture conditions

MS basal medium containing vitamins and 3% (w/v) sucrose in combinations with plant growth regulators was used. All media was adjusted to pH 8.0 using 1M NaOH or 1M HCl and solidified with 0.28% (w/v) gelrite powder prior to autoclave at 121^{o}C for 15 min.

Data collection and analysis

Three replicates were carried out. Callusing percentage was recorded for endosperm and cotyledons explants after 14 days and 4 weeks of culturing, respectively. Data in term of callusing percentage from different auxins with various concentrations (0-8 µM) which obtained from three replications were statistically analysed using Oneway ANOVA (Tukey's test). Mean values which grouped in same letter and colour were not significantly different at significant level at 0.05 ($p > 0.05$).

Callus maintainence

Induced calli derived from endosperm and cotyledons were further maintained in callus maintenance media containing MS basal medium supplemented in combination of a single cytokinin (BAP, kinetin, TDZ or zeatin) and suitable callus-induction auxins. Two-week-old endosperm-derived calli induced from picloram (4 µM), dicamba (4 µM) and 2,4-D (6 µM) were subcultured individually into callus

maintenance media which containing MS basal media supplemented with 6 µM of BAP, kinetin, TDZ or zeatin combined with 4 µM picloram or 4 µM dicamba or 6 µM 2,4-D. As for cotyledon-derived calli induced from dicamba (4 µM) and 2,4-D (4 µM), the calli were subcultured individually into callus maintenance media which contain MS basal media supplemented with 6 µM of BAP, kinetin, TDZ or zeatin combined with 4 µM picloram or 4 µM dicamba. Callus growth was measured in terms of fresh weight weekly.

Results and discussion

Callogenesis of explants

Callus was induced within 2 to 4 days for the endosperm explants on all the callus induction media and control. Initially, the explants were swollen followed by the appearance of friable or yellowish callus depending on the type of the auxin used. However, the degree of callus proliferation was found to be different in different induction media. Generally, 2,4-D, picloram and dicamba were found to be more effective auxins in inducing intensive callus proliferation compared to NAA, IAA, IBA for the endosperm explants. Besides, colour and morphological differences of the induced calli was observed among the different media used for callus induction after 2 weeks. Colour of calli which induced from the medium containing NAA, IAA, IBA and the control medium turned from yellowish to brownish and eventually seemed to be non-viable (Fig. 1a, b, c, g). However, the induced calli from the medium containing 2,4-D, picloram and dicamba were found to be soft, friable and yellowish (Fig. 1d, e, f).

On the other hand, callogenesis from the cotyledon explants was found to be slower, requiring about 3 weeks. Morphological change of the explants was observed within 2 weeks of culture. All the explants were swollen or doubled in size and turned from white to green colour prior to callusing. This phenomenon was also found in callogenesis of cotyledons cultures in *Feronia limonia* and *Macadamia tetraphylla* L. (Hossain *et al*. 1994; Mulwa and Bhalla 2006). The callus was emerged from the wound sites of tissues. Results indicated that the cotyledon explants was not responsive to the the media containing NAA and IAA (Fig. 2a, b, c) after 4 weeks. Callus was only observed in the MS medium containing IBA, 2,4-D, picloram and dicamba. The morphological appearance of the induced calli were different from medium containing IBA to 2,4-D, picloram and dicamba. The induced calli from the IBA was white, compact and nodular (Fig. 2d). However, the induced calli from 2,4- D, picloram and dicamba were greenish and friable (Fig. 2e, f, g).

Effects of various concentrations of auxins on callus induction of endosperm and cotyledons

Results indicated that the callus induction of endosperm was found to be intensive in the MS medium supplemented with picloram, dicamba or 2,4-D, regardless of the concentration used. The various auxin concentrations (2-8 µM) studied in this study showed mild effect on the callus induction percentage, except dicamba (Fig. 5). Nevertheless, the highest callus induction percentage was found in the MS supplemented with 4 µM picloram (53.33±6.67 %), followed by 4 µM dicamba (51.11±5.88 %) and 6 µM 2,4-D (40.00±5.77 %), respectively. The efficiency of the callus induction was comparable to the immature endosperm callusing study of Neem (53.0±1.0 %) which was induced in the MS medium supplemented with 5 µM 2,4-D, 2 µM BAP and 500 mg/l casein hydrolysate (Chaturvedi et al. 2003). Callus induction of the endosperm tissues without associating with the embryo was also reported from *Achras sapota, Santalum album, Emblica officinalis* and *Juglans regia* (Thomas & Chaturvedi 2008). In callus initiation of *Acacia nilotica* endosperm cultures, the degree of callusing was found to be higher when the endosperm was cultured without the embryo (Garg *et al*. 1996). Other than that, the callus induction percentage found in picloram, dicamba and 2,4-D in this study was higher than the callus induced from the endosperm of *Morus alba* L. (25.7%) on MS medium supplemented with 5 µM BAP and 1 µM IBA (Thomas et al. 2000). However, the induced calli were not sustainable in callus induction media after 2 weeks of culture. Induced calli turned brown and eventually non-viable. This showed single auxin alone might be unsuitable for sustaining induced calli. In *Cupressus macrocarpa* and *arizonica*, single auxin IAA was also found not suitable for sustaining callus initiation and maintaining induced calli (Lorenzo and Giovanni 2004).

For callus induction of cotyledons in this study, dicamba and 2,4-D were found to be more suitable, regardless of the concentration used. NAA and IAA were not able to induce callus from the

cotyledon tissues and the capability of inducing callus was limited in the MS medium supplemented with IBA and picloram. Among various concentrations studied, 4 µM was found to be more suitable for dicamba and 2,4-D in inducing callus, 31.11±10.61% and 28.89±4.84%, respectively (Fig. 6). Similarly for the endosperm tissues, our study found the auxin concentration used (2-8 µM) did not significantly affect callus induction percentage, except dicamba. Higher 2,4-D and picloram (4-8 µM) concentrations were required for callus induction. In conclusion, dicamba was found to be more the effective auxin in inducing callus formation in cotyledon explants among auxins tried, presumably because dicamba is metabolized quickly in callus formation in certain species and the concentration required (0.06-4.0 µM) is generally less than that necessary for other auxins (George et al. 2007).

Callus maintenance

The induced calli derived from endosperm and cotyledons did not maintain well in the callus induction medium. Thus, the study of a suitable callus maintenance medium is required to be established and formulated. Preliminary observations indicated that the induced calli (Fig. 3 and 4) were able to be maintained and multiplied in the callus maintenance medium (data not shown). Previous studies revealed that many species require supplementary cytokinin along with auxin for optimum callusing response and in some cases to prevent necrosis of callus (Roy and Banerjee, 2003). Similarly, callus maintenance media were developed for long-term callus maintenance for induced calli from zygotic embryo of *Alstroemeria pelegrina* and *Alstroemeria psittacina* (Hutchinson *et al*., 1994).

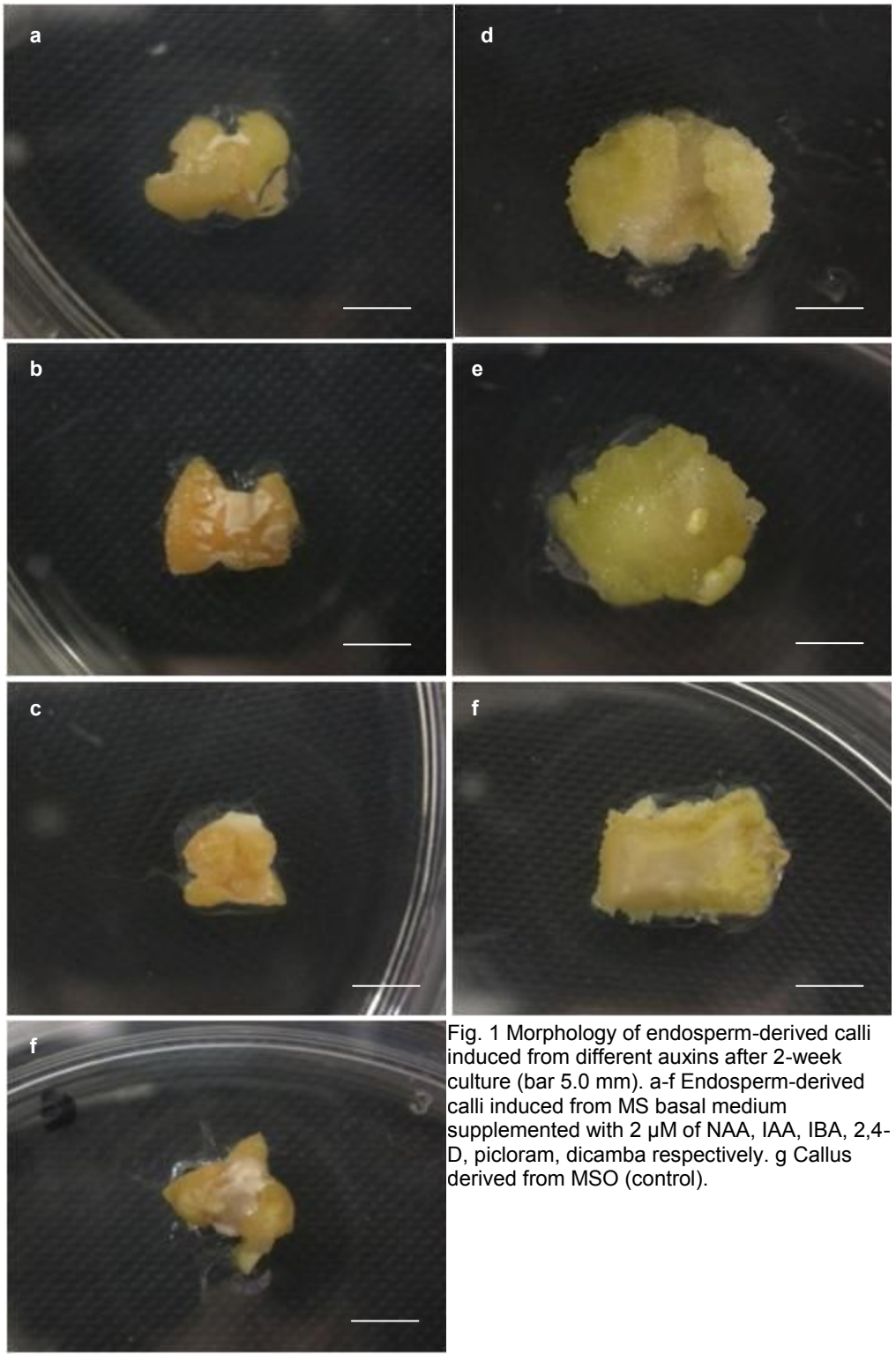

Fig. 1 Morphology of endosperm-derived calli induced from different auxins after 2-week culture (bar 5.0 mm). a-f Endosperm-derived calli induced from MS basal medium supplemented with 2 μM of NAA, IAA, IBA, 2,4-D, picloram, dicamba respectively. g Callus derived from MSO (control).

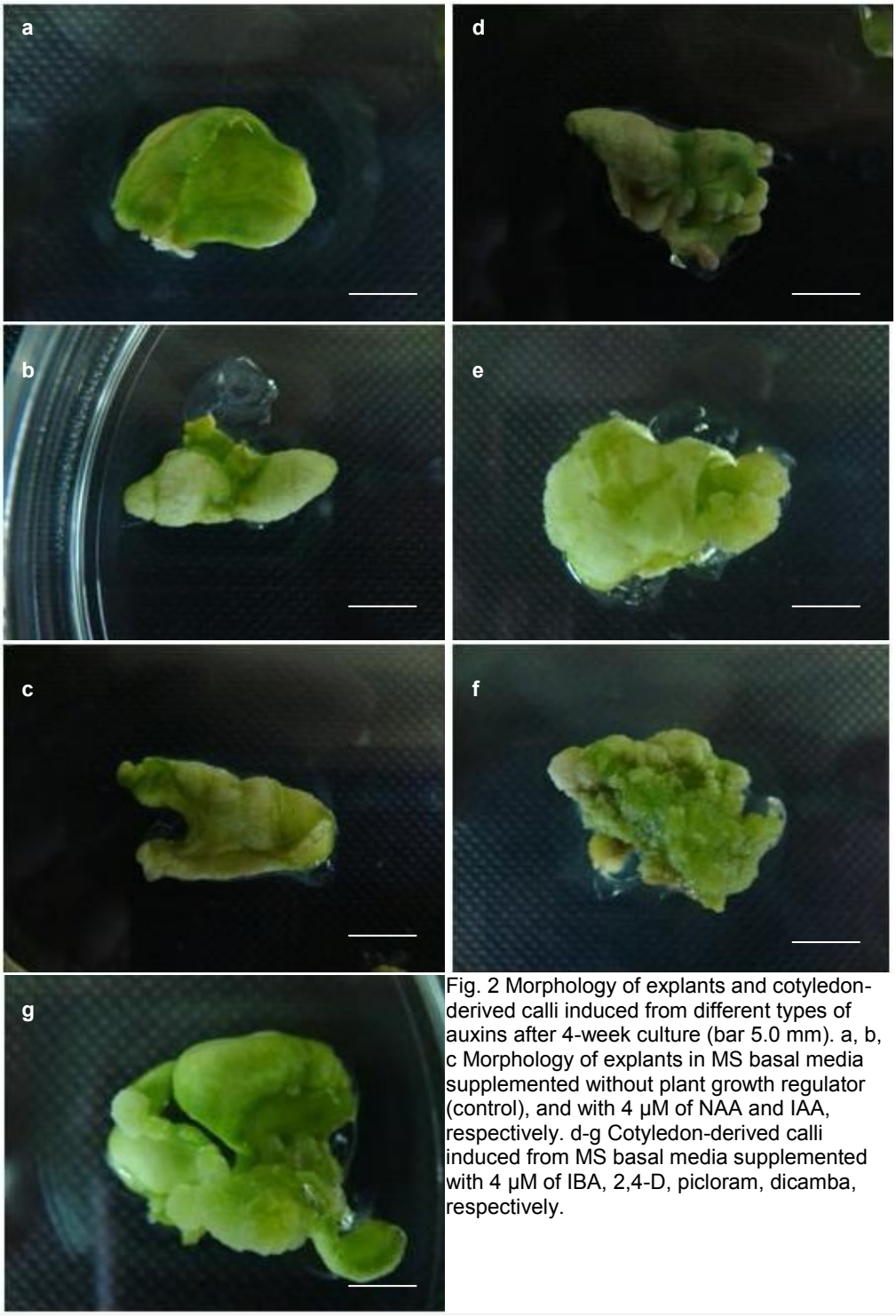

Fig. 2 Morphology of explants and cotyledon-derived calli induced from different types of auxins after 4-week culture (bar 5.0 mm). a, b, c Morphology of explants in MS basal media supplemented without plant growth regulator (control), and with 4 μM of NAA and IAA, respectively. d-g Cotyledon-derived calli induced from MS basal media supplemented with 4 μM of IBA, 2,4-D, picloram, dicamba, respectively.

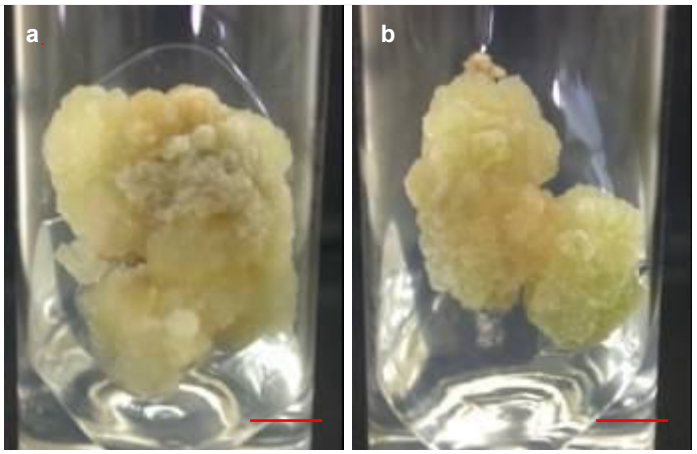

Fig. 3 a,b. Morphology of endosperm-derived calli (4-week-old culture) maintained in MS basal medium supplemented with TDZ (6 µM) and dicamba (4 µM), and TDZ (6 µM) and (4 µM) picloram (bar 5.0 mm).

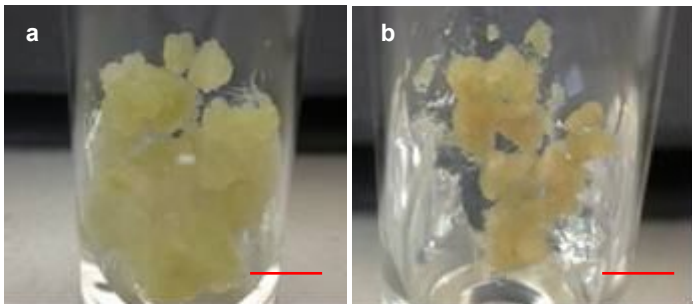

Fig. 4 a, b. Morphology of cotyledon-derived calli (4-week-old culture) maintained in MS basal medium supplemented with TDZ (6 µM) and dicamba (4 µM), and TDZ (6 µM) and (4 µM) 2,4-D (bar 10.0 mm).

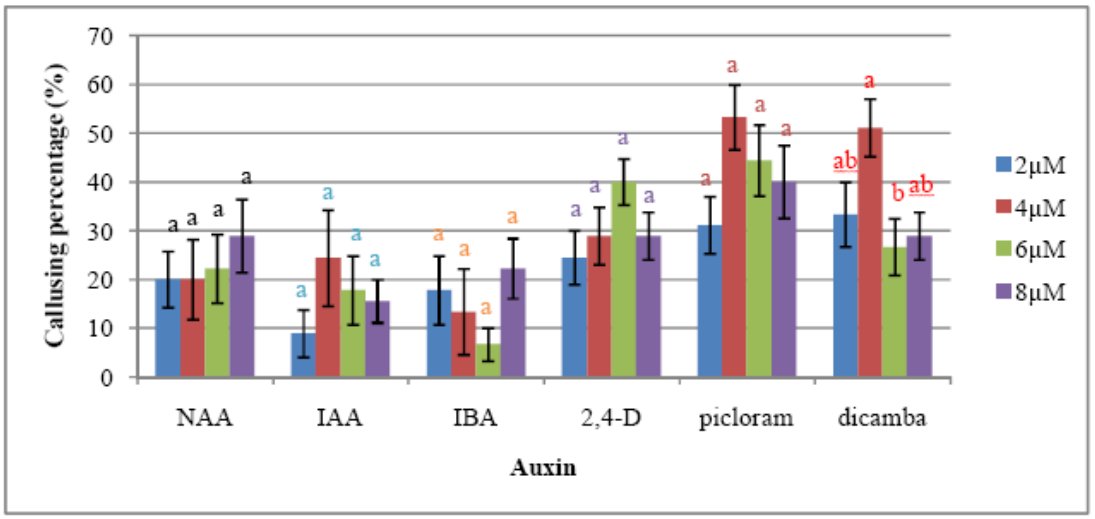

Fig. 5. Effects of various concentrations of auxins on callus induction of endosperm. Data were recorded after 14-day of culture and mean values (±S.E.) followed by same letter and colour are not significantly different according to Tukey test at a = 0.05.

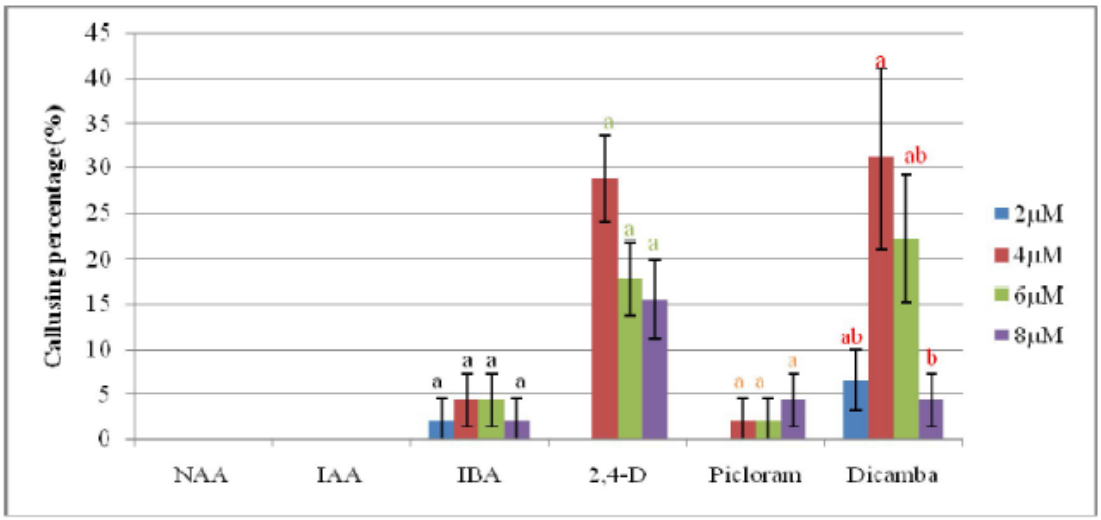

Fig. 6. Effects of various concentrations of auxins on callus induction of cotyledons. Data were recorded after 4-week of culture and mean values (±S.E.) followed by same letter and colour are not significantly different according to Tukey test at a = 0.05.

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Induction of Callus and Protocorm-like Bodies (PLBs) in *Phalaenopsis gigantea*

Azadeh, N.[1], Kadir, M.A.[1], Kadzimin, S.B.[2] and Abdullah, N.A.[2]

[1]Department of Agriculture Technology, [2]Department of Crop Science,
Faculty of Agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
azadehnik@yahoo.com

Abstract

An efficient and reproducible method for large-scale propagation of *Phalaenopsis gigantea* using leaf sections has been developed. Leaf sections from young plants were cultured on New Dogashima medium (NDM) supplemented with cytokinins (6-Benzylaminopurine (BAP), thidiazuron (TDZ), and kinetin (KIN), each at 0.01, 0.1, 0.5 and 1.0 mgL^{-1}) alone and in combinations with (auxins a-naphthaleneacetic acid (NAA), at 0.01, 0.1, 0.5 and 1.0 mgL^{-1}). The explants developed calli and protocorm-like-bodies (PLBs) within 8 weeks of culture. Treatment TDZ in combination with auxins was found to be best for the induction of callus and PLBs.

Introduction

Tissue culture techniques have contributed to large scale production of plants of economic importance such as orchids and other ornamentals. The culture of meristems and other tissue culture techniques have produced plants that are virus free.The techniqnes are based on the principle of totipotency (Purohit, 2005). In orchids, the techniques have been widely used for mass production of plants serving as an *ex situ* conservation strategy of species (Tokuhara and Mii; 1993). Many protocols have been developed including for *Cymbidium*, *Vanda*, *Phaphiopedilum* and *Phalaenopsis*)(Arditti and Ernst, 1993). Protocols for *Phalaenopsis* utilizing flower stalk buds (Arditti, 1977; Tanaka and Sakanishi, 1978; Tokuhara and Mii, 1993, 2001; Kosir *et al*., 2004) entire shoots, shoot tips, stem nodes (Griesbach, 1983), leaf tissues/segment (Tanaka and Sakanishi, 1980; Park *et al*., 2002) or root tip culture (Tanaka *et al*., 1976; Park *et al.,* 2003) have been reported. Unfortunately, these methods have been reported to be very difficult and inefficient (Yam *et al*. 1991 and Chen *et al*. 2000). The present paper reports on work accomplished in the induction of callus and protocorms-like bodies (PLBs) in *Phalaenopsis gigantea*, an important and endangered species of Sabah, Malaysia.

Materials and methods

Young leaves from *in vitro* grown seedlings were used as explants. Leaf tip segments (about 1 cm in length) were taken from donor plants and cultured on New Dogashima (NDM) medium supplemented with cytokinins [6-Benzylaminopurine (BAP), thidiazuron (TDZ), and kinetin (KIN), each at 0.01, 0.1, 0.5 and 1.0 mgL^{-1}] alone and in combination with auxins [naphthalene acetic acid (NAA), at 0.01, 0.1, 0.5 and 1.0 mgL^{-1}]. Protocorms-like bodies (PLBs) developed from leaf segment culture were sub cultured every 6 weeks. The experimental design used was Randomized Complete Block Design (RCBD). Data were analyzed using analysis of variance (ANOVA) and Duncan New Multiple Range Test (DNMRT).

Table 1. Influence of different growth regulators on development of Calli and PLBs from leaf explants of *Phalaenopsis gigantean*

Growth regulator (mgL^{-1})	PLB and Calli (%)
BAP	
0	0b
0.01	0b
0.1	3.12ab
0.5	6.25ab
1	14.06a
TDZ	
0	0b
0.01	0b
0.1	6.25b
0.5	14.6b
1	39.06a

Table 2. Influence of combinations of TDZ and NAA concentrations

Plant growth regulator		Mean percentage of PLBs and callus	Code
NAA (mgL^{-1})	TDZ (mgL^{-1})		
0	0	0f	N0T0
0.01	0	0f	N0.01T0
0.1	0	0f	N0.1T0
0.5	0	0f	N0.5T0
1	0	0f	N1T0
0	0.01	0f	N0T0.01
0.01	0.01	0f	N0.01T0.01
0.1	0.01	0f	N0.1T0.01
0.5	0.01	6.25f	N0.5T0.01
1	0.01	6.25f	N1T0.01
0	0.1	6.25f	N0T0.1
0.01	0.1	6.25f	N0.01T0.1
0.1	0.1	6.25f	N0.1T0.1
0.5	0.1	25de	N0.5T0.1
1	0.1	100a	N1T0.1
0	0.5	14.06ef	N0T0.5
0.01	0.5	14.06ef	N0.01T0.5
0.1	0.5	14.06ef	N0.1T0.5
0.5	0.5	14.06ef	N0.5T0.5
1	0.5	76.56b	N1T0.5
0	1	39.06cd	N0T1
0.01	1	39.06cd	N0.01T1
0.1	1	39.06cd	N0.1T1
0.5	1	39.06cd	N0.5T1
1	1	56.25c	N1T1

Results

There are few reports on the induction of callus in *Phalaenopsis* especially in *P. gigantea*. In earlier studies basic media as ½ MS, VW and NDM with different concentrations of Cytokinin and Axuin were used, but the explants were necrotic and no callus or PLBs developed. In this present study callus induction and protocorm-like bodies (PLBs) from leaf segments was observed on basic NDM medium supplemented with TDZ and NAA within 6-8 weeks. The highest percentage of callus formation (100%) was obtained on treatment containing 1 mgL^{-1} (w/v) of NAA with 0.1 mgL^{-1} (w/v) of TDZ (N1T0.1) followed by treatment supplemented with 1 mgL^{-1} (w/v) of NAA with 0.5 mgL^{-1} (w/v) of NAA (N1T0.5) (76.56%). Of the three cytokinin tested, TDZ was more effective in inducing PLBs and calli from leaf sections. All leaf explants responded and differentiated an optimum number of PLBs and calli on medium supplemented with TDZ in combination with NAA. Arditti and Ernst, (1993), Tisserat and Jones, (1999) reported frequent callus formation as an intermediary phase just before shoot regeneration. In our study, Thidiazuron (0.1 mg/L^{-1}) proved to be beneficial in multiple productions of *Phalaenopsis* from leaf tip.

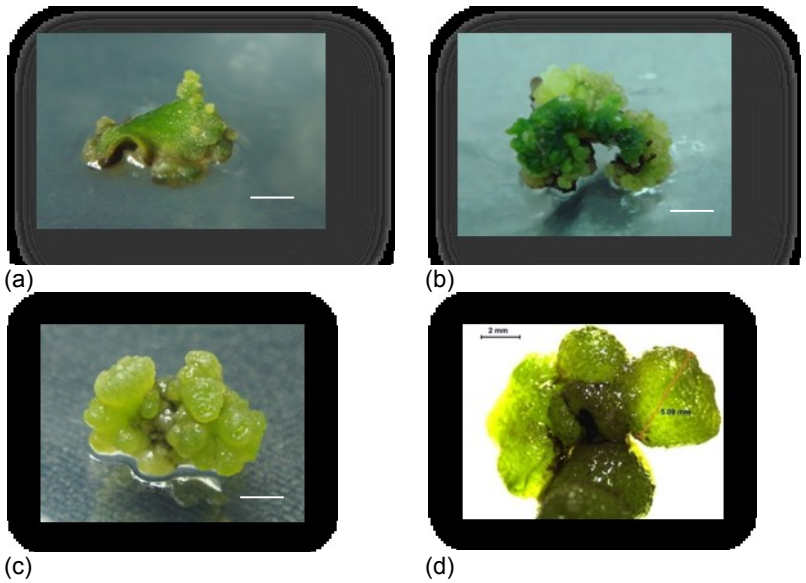

Fig. 1. (a) Leaf culture in NDM medium supplemented with TDZ and NAA after 2 weeks of culture (b) PLB and callus from leaf culture after 5 weeks of culture (bar =5.5 mm). (c) Develop PLBs from leaf section (bar=5.5 mm) (d) PLBs on microscope.

Discussion

The type, concentration and combination of growth regulators plays an important role during *in vitro* propagation of many orchid species (Arditti and Ernst, 1993). In the present studies, leaf sections developed PLBs and calli on medium supplemented with cytokinins alone and in combination with auxin. The best result was obtained when leaf sections were used as explants on medium supplemented with TDZ in combination with NAA. Among the various cytokinins tested (BAP, TDZ, KIN), TDZ was found to be more efficient in PLB and callus induction from leaf sections. BAP has also been found to play an important role in tissue cultures of other orchid's species (Lin, 1986; Tanaka *et al*., 1988; Sheelavanthmath *et al*., 2000; Murthy and Pyati, 2001). KIN was not efficient in inducing PLB and callus induction from leaf sections. TDZ has also been reported to be effective in the regeneration for a number of orchid species (Ernst, 1994; Chen and Piluek, 1995; Nayak *et al*., 1997; Chang and Chang, 1998). TDZ when used alone was more efficient than BAP in orchids such as *Phalaenopsis* and *Doritaenopsis* (Chang and Chang, 1998; Ernst, 1994). The combined effect of

cytokinin and auxin proved to be useful in PLB and callus induction in *Vanda* spp. (Vij and Pathak, 1990), *Rhynchostylis gigantea* (Bui von Le *et al*., 1999). NAA is frequently used in combination with BAP or TDZ in many orchid species like *Vanda* and *Phalaenopsis* (Goh and Wong, 1990, Park *et al*., 2002). In the present studies, TDZ ($1mgL^{-1}$) resulted in optimum PLB and callus induction. However, these results were found to be very low compared to the TDZ ($0.1\ mgL^{-1}$) with NAA ($1mgL^{-1}$) treatment.

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Effect of Crosslink Chemicals on the Mechanical Properties of Injection Molded Rice Straw/HDPE Biocomposite

Wan Aizan, W.A.R, Nurnadia, A. and Roshafima, R.A.

Polymer Engineering Department, Faculty of Chemical and Natural Resources Engineering,
University Technology Malaysia, 81310 UTM Skudai, Johor, Malaysia
w.aizan@fkkksa.utm.my

Abstract

A formulation was designed to produce silane crosslinkable rice straw/HDPE (RSPE) compound suitable for injection molding process. The formulations consist of HDPE as the base polymer, rice straw as the filler, processing aids and a mixture of crosslink chemicals. Crosslink chemicals consist of vinyltrimethoxysilane (VTMO) as crosslinking agent, dicumyl peroxide (DCP) as the initiator, dibutyltin dilaurate (DBTL) as the condensation catalyst. Lignocellulosic material, rice straw was oven dried at 70ºC for 24 hours, grinded and sieved. A counter rotating twin shaft high speed mixer was utilized to mix the rice straw, HDPE and the processing aids. Blends were then compounded on co-rotating and intermeshing twin screw extruder. Prior to injection compounded biocomposite were injection molded into test specimens followed by curing in an oven at 90ºC. FTIR was used to determine the chemical groups involved in the crosslinking reaction that occur in samples. Degree of crosslinking of the silane crosslinked sample was measured by determining their gel content after crosslinking reactions. The degree of crosslinking in RSPE increases with an increased in VTMO and DCP concentration. The results from FTIR showed the presence of Si-O-Si bond indicative of crosslinking occurring during curing process.

Introduction

Polymer composites have been a subject of research and utilization for some decades. The need for materials having specific characteristics for specific purposes, while at the same time being non-toxic and environmentally friendly, is increasing due to the lack of resources and increasing environmental pollution. All this issues have induced researchers to look for alternatives. Thus, an interest arises toward polymer composites filled with natural organic fillers. This class of composites (sometimes indicated as “green composites”) shows interesting features (La Mantia and Morreale, 2006).

Industries are developing and manufacturing “greener” materials; government is encouraging bio-based product research while the academicians are searching for eco-friendly materials; and the public is coming to value the benefit of environment friendly products and processes, but at affordable prices. Previously, most researchers have used wood flour, rice husk and empty fruit bunch as reinforcement in the various polymers such as polyethylene, polyvinyl chloride and polypropylene (Abu Bakar *et al*., 2005; Chen, 2006).

In spite of all the advantages of natural fiber, there are also drawbacks in using natural fiber as reinforcement in thermoplastics. The main drawbacks are the difficulties in achieving good dispersion and strong interfacial adhesion between the hydrophilic fiber and the hydrophobic polymer (Bengston and Oksman, 2005). Poor adhesion leads to composites with rather poor durability and toughness. This is the main reason for using silane technology in crosslinking polyethylene-rice straw composites. Silanes are used as coupling agents to adhere fibers to a polymer matrix, stabilizing the composite material. In general, the use of this coupling agent significantly improves the mechanical properties of the composites (Bengston and Oksman, 2005). This study will be carried out to develop an optimum formulation for the silane crosslinkable rice straw/HDPE compound grade for injection molding.

Materials and methods

Materials

The raw materials used in this research are rice straw and high density polyethylene injection grade, where HDPE as a matrix while rice straw as filler or fiber reinforcement in this composite.

Vinyltrimethoxysilane (VTMO) is used as a crosslinking agent, dicumyl peroxide is used as radical initiator and dibutyltin dilaurate (DBTL) catalyst is used to promote hydrolysis and condensation reaction.

Methods

Filler preparation

The rice straw were placed on a pan and dried in Carbolite oven at 80ºC for 24 hours to eliminate the moisture content, followed by grinding. Grinding was done on the dried straw to form fine particles. The particles were sieved to obtain the required range sizes of the rice straw particles. The particle size used was 500-75 µm.

Compounding of fibre formulation

HDPE and rice straw were first premixing in a counter rotating twin shaft high speed mixer. Then the mixture was compounded using a co-rotating and intermeshing twin extruder with a screw speed of 90 rpm at a temperature range of 180–190ºC. The HDPE/ rice straw pellets were then placed in a dry, sealable cylindrical mixer, filling the mixer to about three-quarters of its capacity. The resin was then injected with silane chemical liquid mixture by using a syringe and blended on a laboratory scale Fielder mixer for 3 minutes. At the end of this time, silane chemical mixture is expected to be uniformly coated onto the HDPE/rice straw pellets. The coated pellets are allowed to rest for 2-3 hours, the time necessary for complete absorption of the silane chemicals liquid mixture. These pellets were then injection molded into ASTM standard specimens for mechanical testing. Machine settings used were: 150ºC first zones, 160ºC zone two, 170ºC zone three and 180ºC for zone four. Cooling time was 60 s, injection hold pressure time 3 s, injection time 1.8 s and injection pressure 80 kgf/cm^2.

Characterization

Degree of crosslinking

Degree of crosslinking of the silane crosslinked RSPE sample was measured by determining their gel content after crosslinking reactions. The degree of grafting is regulated both by the silane and the peroxide concentration (Chen, 2006).

The determination of the gel content test was performed by extracting the soluble component with decahyronapthalene with refluxing at 200ºC for 6 hours according to ASTM D 2765-1995 Method B.

Material performance test

(a) Tensile test

The tensile test was performed on Lloyd machine according to ASTM D 638. Shape of dumb-bell specimens were obtained from the injection moulding process. 50 mm/min of crosshead speed was used and the test was performed at room temperature.

(b) Izod Impact test

Izod impact is the kinetic energy needed to initiate fracture and continue the fracture until the specimen is broken and used to determine the impact properties of the materials. Notching at the middle of the specimen carried out using the notching tool. The notch depth fixed at 2.5+ 0.02 mm in accordance to ASTM D-256.

Results and discussion

Material characterization

Gel content test

Fig. 1 shows the effect of vinylmethoxysilane (VTMO) concentration on the gel content of crosslinked rice straw/HDPE biocomposite. With increasing VTMO concentration, the gel content of the composite increases slowly from 17.14% to about 20.10% at low crosslinker concentrations (1 to 2 phr), and the gel percentage increases rapidly at high crosslinker concentrations (2.5 to 3 phr). A dosage of low crosslinker concentration does not significantly improve the crosslinking results. In order to iniatiate the crosslinking reaction, dicumylperoxide (DCP) is used in this study. The degree of crosslinking of crosslinked RSPE biocomposite increases with increasing concentration of DCP initiator. It was observed that the gel content increases with an increased in DCP initiator concentration. Fig. 2 shows that gel content is highest at 0.4 to 0.5 phr of DCP concentration. When the amount is less than 0.1 phr, the silane grafting seems not to proceed sufficiently. Investigation done on crosslinking systems without a catalyst show that a much lower degree of crosslinking is obtained than in systems containing a catalyst. From Fig. 3, increasing the dosage at 0.005 to 0.015 significantly increased the amount of gel content.

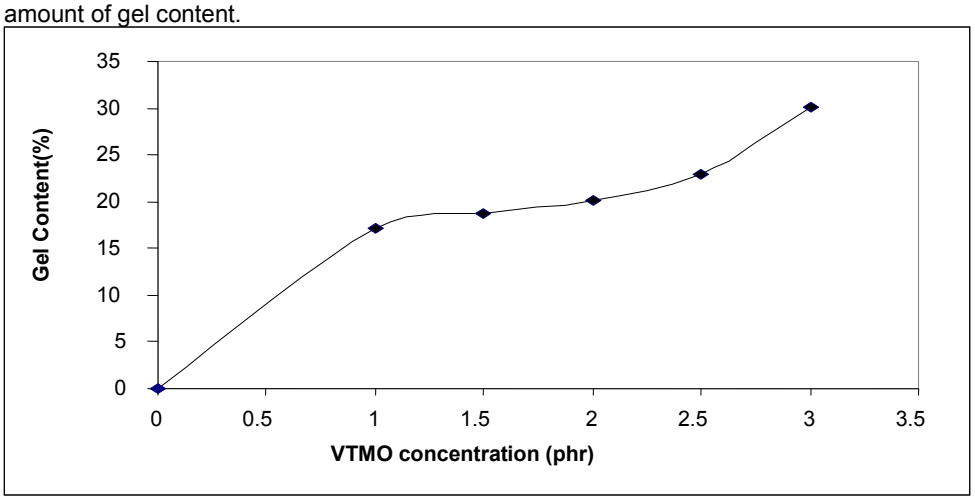

Fig. 1. The Effect of the VTMO concentration on gel content of RSPE biocomposite.

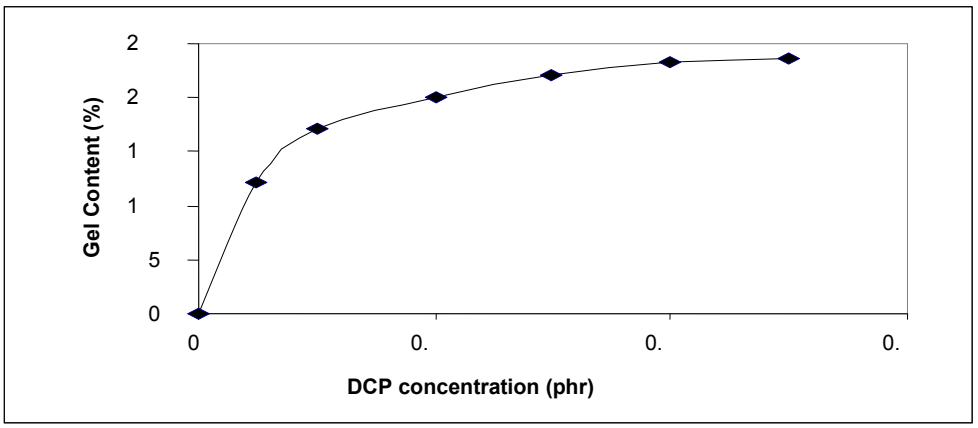

Fig. 2. The Effect of the DCP concentration on gel content of RSPE biocomposite.

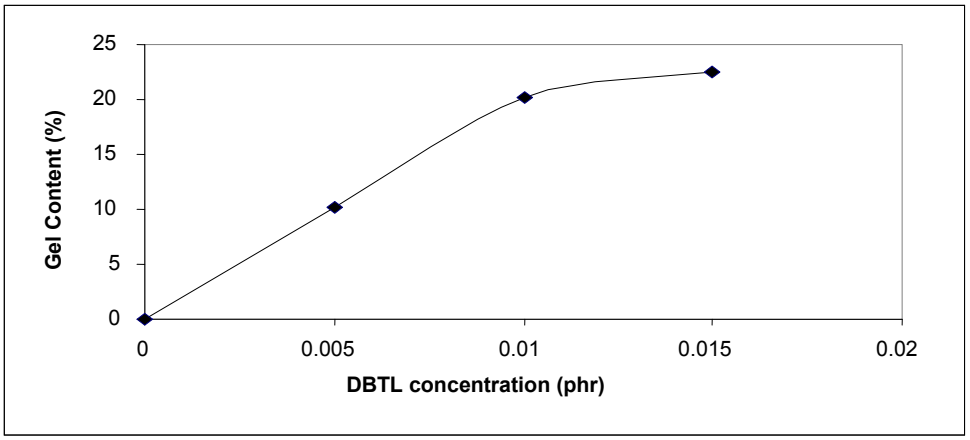

Fig. 3. The Effect of the DBTL concentration on gel content of RSPE biocomposite.

Material performance test

Tensile test

The tensile strength of crosslinked rice straw/HDPE biocomposite with various formulations of VTMO (Vinylmethoxysilane) is shown in Fig. 4. According to previous researches, the tensile strength of the composite decreased with the addition of fillers (Yang *et al*., 2004). The tensile strength of crosslinked rice straw/HDPE biocomposite is correlated with gel content as reported by Toh (2005) which is shown in Fig. 1. The graph indicates, at low gel values (up to 17%), the tensile strength is higher compared to non-crosslinked rice straw/HDPE biocomposite. At higher values of the gel (30%), the tensile strength seems to be comparable to non-crosslink virgin HDPE. This shows that VTMO have significantly increased the tensile strength of RSPE biocomposite. The concentration of DCP also enhanced the tensile strength of RSPE biocomposite. DCP act as an initiator and produce free radical sites on the backbone of polyethylene. This free radical acts as point for silane grafting. Fig. 5 shows that satisfactory mechanical properties can be obtained at low dosage. At higher concentrations of DCP, tensile strength of RSPE is decreased. Previous researches also state similar results whereby mechanical properties are reduced at high DCP concentration (Kang and Ha, 1999). The crosslinking catalyst also plays an important role respect to the degree of crosslinking that is achieved. Fig. 3 shows that gel content is improved with the addition of catalyst. It gave higher tensile strength to RSPE biocomposite as can be seen in Fig. 6. This proves that DBTL plays an important role in improving the degree of crosslinking with respect to tensile strength.

Impact strength

Impact strength of the composites was one of the major criteria used besides rheological properties. Previous researches show that the impact strength of pure HDPE decreases with the incorporation of fillers (Kang and Ha, 1999). The results for the impact strength test at various VTMO concentration is shown in Fig. 7. As can be seen from the figure, with silane at 2.5 to 3 phr VTMO concentration, biocomposites with 30% rice straw have at least 35% to 47% higher impact strength than uncrosslinked RSPE biocomposite. Fig. 8 and 9, illustrate the graph of impact strength at various DBTL and DCP compositions, which do not show significant improvements in impact strength. Generally, the incorporation of rice straw into the composite results in the decrease in impact strength. But with the presence of crosslinks in the biocomposite impact strength is still high. Thus, crosslinking of the matrix is one method of improving the toughness of the polymer matrix. There is also indication of improved adhesion between the rice straw and the polyethylene matrix in the crosslinked composites. Therefore, crosslinking is a method of improving and enhancing the adhesion between rice straw and polyethylene matrix which is also reported by Magnus (Bengston, and Oksman, 2005) on another filler system.

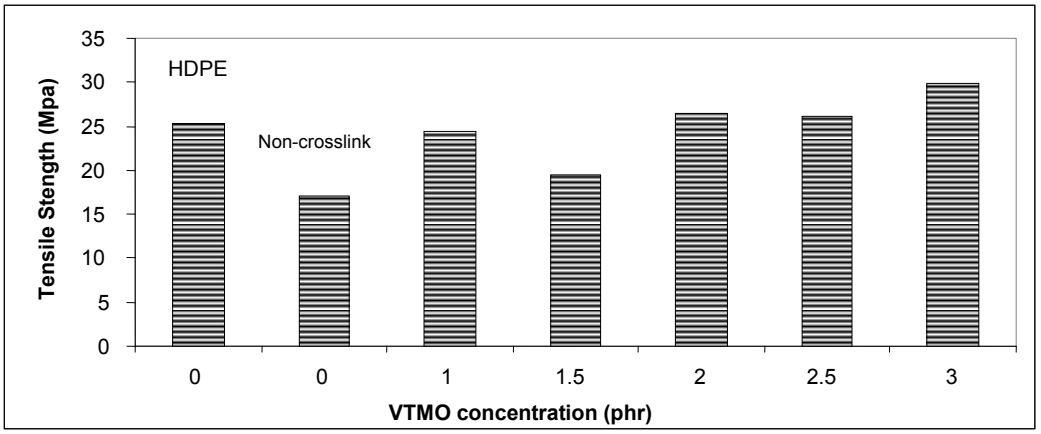

Fig. 4. Effect of VTMO concentration on tensile strength of RSPE biocomposite.

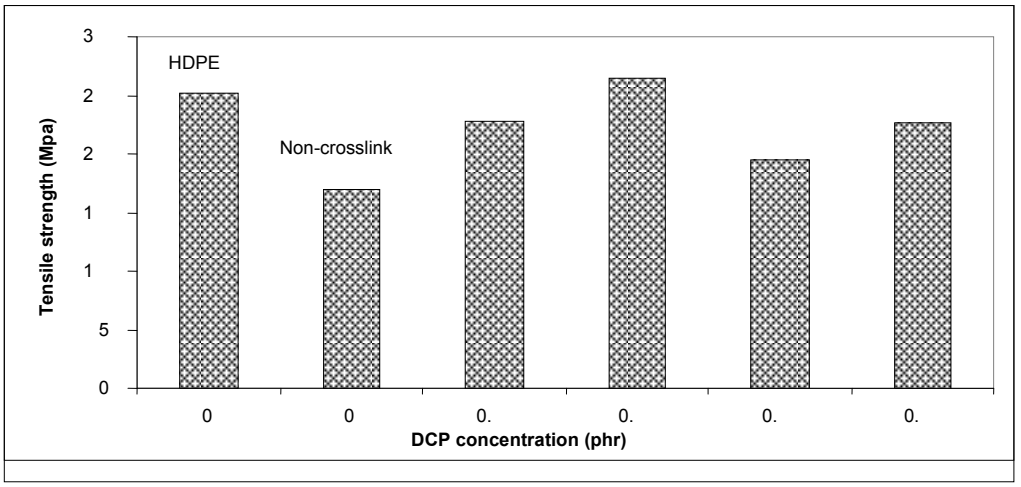

Fig. 5. Effect of DCP concentration on tensile strength of RSPE biocomposite.

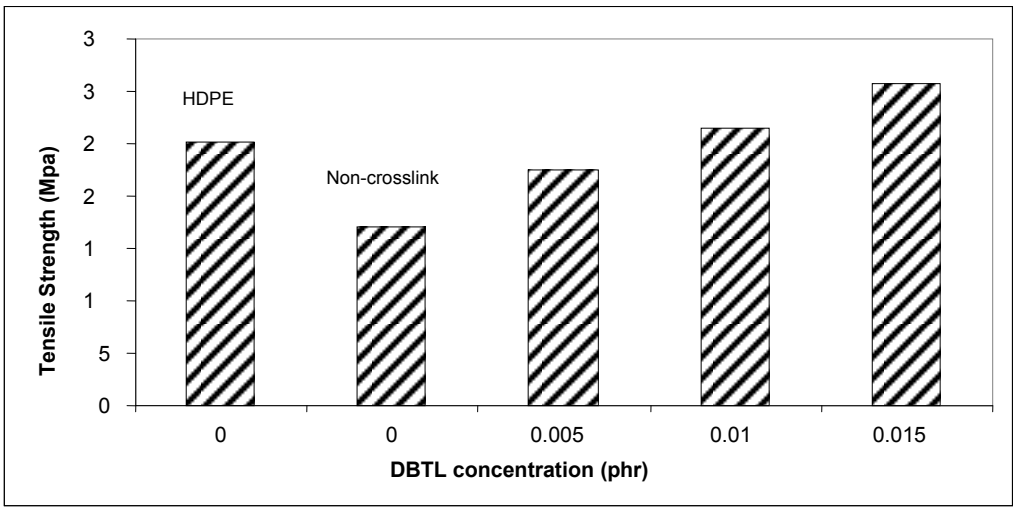

Fig. 6. Effect of DBTL concentration on tensile strength of RSPE biocomposite.

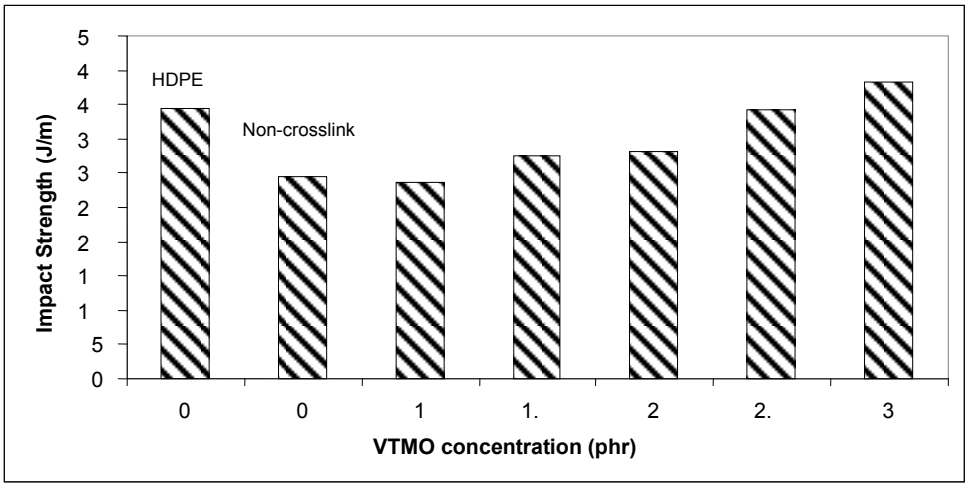

Fig. 7. Effect of VTMO concentration on impact strength of RSPE biocomposite.

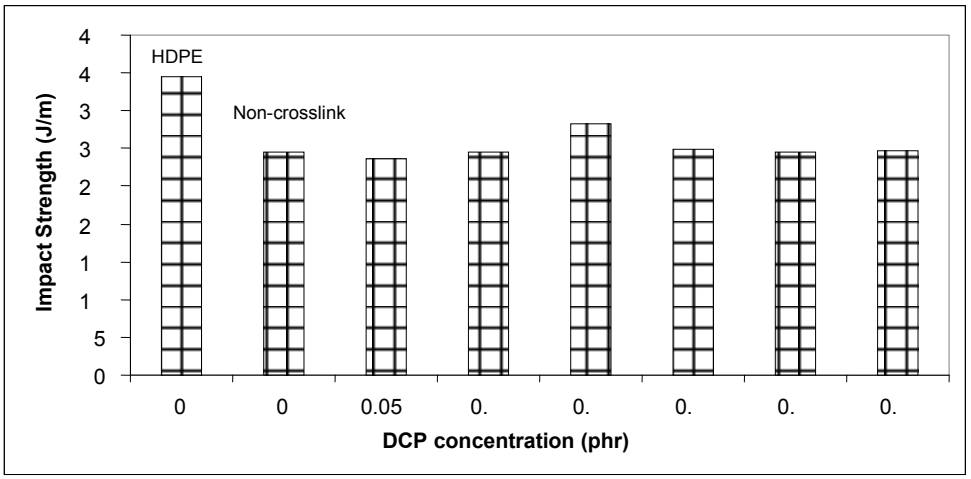

Fig. 8. Effect of DCP concentration on impact strength of RSPE biocomposite.

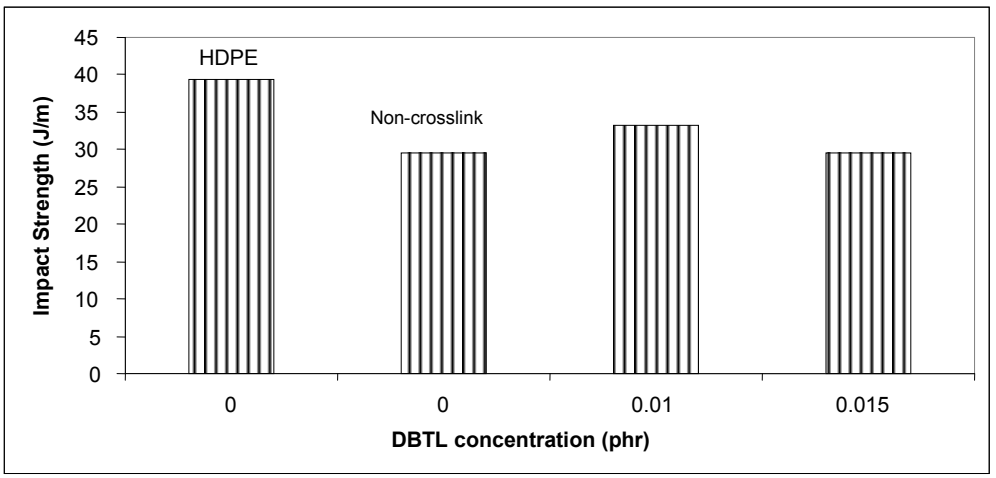

Fig. 9. Effect of DBTL concentration on impact strength of RSPE biocomposite.

Conclusions

Silane crosslinked composites were successfully produced by injection molding process. Three chemical components that are used in the crosslinking process are VTMO as the crosslinking agent, DCP as initiator and DBTL as catalyst. Mechanical properties of RSPE biocomposite showed improvement in tensile strength and impact strength as a result of strong interfacial bonding between the fiber and polymer matrix. Crosslinked RSPE biocomposites show satisfactory mechanical properties compared to non-crosslinked ones. Tensile strength of crosslinked rice straw/HDPE biocomposite is correlated with gel content. At high percentage of gel content, tensile strength is increased. The crosslinked composites also showed significant impact strength due to enhanced adhesion between the phases, but is also related to the strengthening of matrix upon crosslinking.

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Effect of Fermentation Time on Some Nutritive Properties of Red Dragon Fruit (*Hylocereus polyrhizus*)

Hazniza, A., Azlina, M., Rosmawati, O., M.S. Nizam, H. and Musawirah, A.G.

Biotechnology Research Centre, MARDI Headquarters,
Persiaran MARDI-UPM, Serdang, Selangor, Malaysia
hazniza@mardi.gov.my

Abstract

The red dragon fruit (*Hylocereus polyhrizus)* have recently drawn much attention of growers worldwide. This vine cacti were from subfamily Cactoideae contains many varieties such as red, white and yellow with edible fruits. Amongst all the red variety is more preferable to be cultivated and studied due to its attractive red-purple colour and economic value as food products. In this study the red dragon fruit was undergo natural fermentation in a controlled environment. Thus, this study was conducted to determine the effect of different fermentation time on some nutritive properties of fermented red dragon fruit namely, 30, 40 and 50 days. Results indicated that the fermented red dragon fruit in day 30 were high in lactic acid, total soluble solid (TSS) and protein contents compared with control. Results also indicated a trend of TSS and protein contents between each fermentation time. The TSS and protein contents were initially increased at day 30 and slowly decreased at day 40, however increased again at 50 days of fermentation. The fermentation trend was contributed by the activities of endogenous microorganisms in the red dragon fruit during the natural fermentation process.

Introduction

The vine cacti of *Hylocereus polyrhizus* is known as dragon fruit due to it bracts of 'scales' on the fruit skin (Merten, 2003). There are three varieties that are commonly cultivated, *Hylocereus polyrhizus* (red), *Hylocereus undatus* (white) and *Selenicerius megalanthus* (yellow) as reported by Zainudin (2005). All the *H. polyrhizus* are reported to have a high economic value as food products due to its antioxidative activity from the betacyanin contents (Wybraniec and Mizrahi, 2002), phenolic and ascorbic acid compounds (Mahattanatawee *et al*., 2006). Fermented dragon fruit is a new product and currently no studies have been reported elsewhere. Fermentation of dairy products have been widely reported but only limited studies have been done on non-dairy product such as fruit (Luckow and Delahunty, 2004) and oat (Martesson *et al*., 2000). Fruit is positioned as a healthy food product and does not contain any dairy allergens such as lactose. Many documented technical challenges involved with the formulation of non-dairy foods were reported (Mattila-sandholm *et al*., 2002) and fruit was suggested as a good medium for functional ingredients like probiotics (Tuorila and Cardello, 2002). Therefore, the aim of this study was to determine the effect of fermentation time on some nutritive content of fermented red dragon fruit using different incubation period. Information gained from this study will be used to conduct more research and technology development in production of fermented food products from the red dragon fruit.

Materials and methods

Preparation of fruit sample

The fresh red dragon fruit (*Hylocereus polyrhizus*) used in this study was of commercial maturity and purchased from a commercial farm in Sepang, Selangor, Malaysia. Fruits were washed and soaked with tap water for 30 min to remove the dirt and sterilized using disinfection tablets for another 30 minutes. The fruits were sieved and air dried on a metal sieve. The fruit was then cut into 4 pieces, the peel was discarded and the flesh was blended until fine.

Fermentation condition

A portion of blended flesh was weighed and placed in a sterilised conical flask. Glacial acetic acid (1%, w/v) was added into the mixture and homogenised slowly. The mixture then was incubated at 32°C for 30, 40 and 50 days. At the end of each incubation time a dark red-purple liquid (fermented juice) was collected and analysed for its nutritional contents.

Nutritional analyses

The nutritional analyses done were determination of crude protein, total soluble solids, pH and lactic acid (LA) content. The pH was determined using a calibrated portable pH spear model Oaklon. The total Soluble Solids (TSS) content was determined using pocket refractometer (0~53% Brix, Atago PAL-1, Japan). The protein content (%) was estimated using a calibrated Kjeltec™ protein analyzer unit model 2300, Denmark. The lactic acid and dietary fiber were analysed using method of AOAC International (2000). In this study, the evaluations of lactic acid and crude were based on fresh weight (g/100 g) of sample. The fermented juice collected in each incubation period was represented as the fermented product of red dragon fruit.

Results and discussion

The pH value and lactic acid (LA) content in different incubation periods of fermented red dragon fruit is shown as in Fig. 1. The pH of fermented juice in each fermentation time is more acidic than the control. The figure shows that pH was correlated to each other with the LA content in the control and fermented juice.

Fermentation of dragon fruit thus reduced the pH and increased the LA content in the fermented juice. LA in fermented juice increased up to 0.48 % at 30 days of incubation day, however it decreased gradually to 0.15 and 0.13 % at 40 and 50 days of incubation day, respectively. The content of total soluble solids (TSS) in the control and fermented juice of red dragon fruit is shown in Fig. 2. The fermented juice was high in TSS contents compared with control. The microorganisms in the fermented pitaya possibly converted the starch into a carbohydrate and sugars, thus increased the TSS content in the fermented juice. The TSS content initially increased after 30 days but slightly decreased at 40 days and increased again at 50 days.

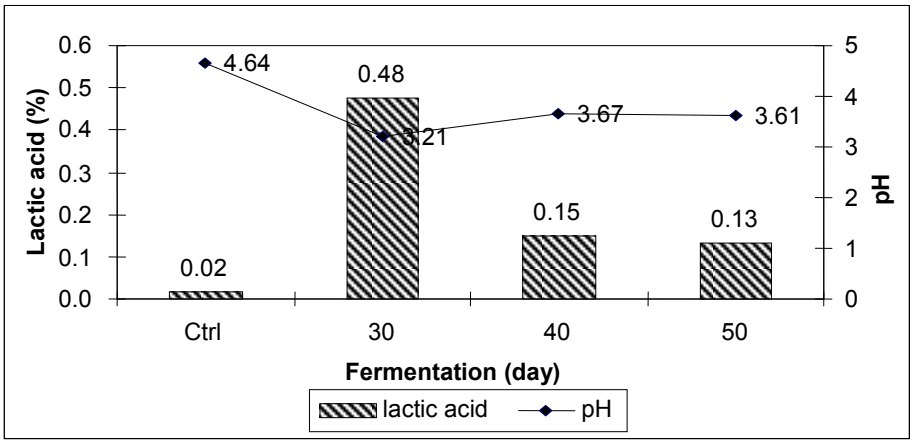

Fig. 1. The pH and lactic acid content in fermented red dragon fruit at different incubation time.

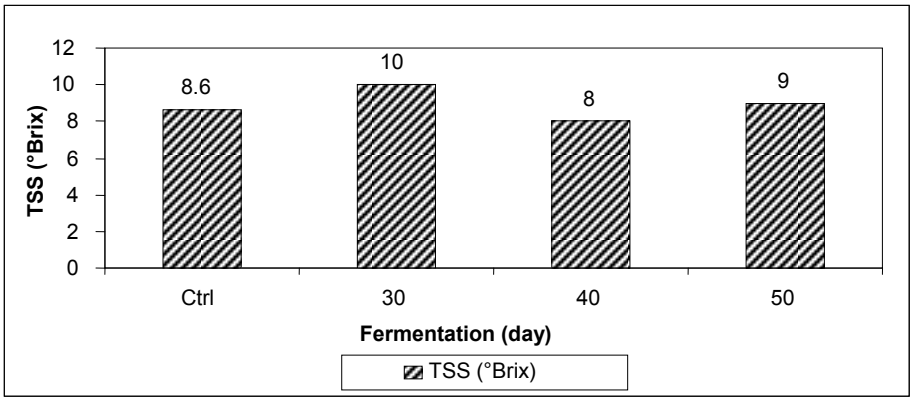

Fig. 2. The total soluble solid (TSS) in fermented red dragon fruit at different incubation time.

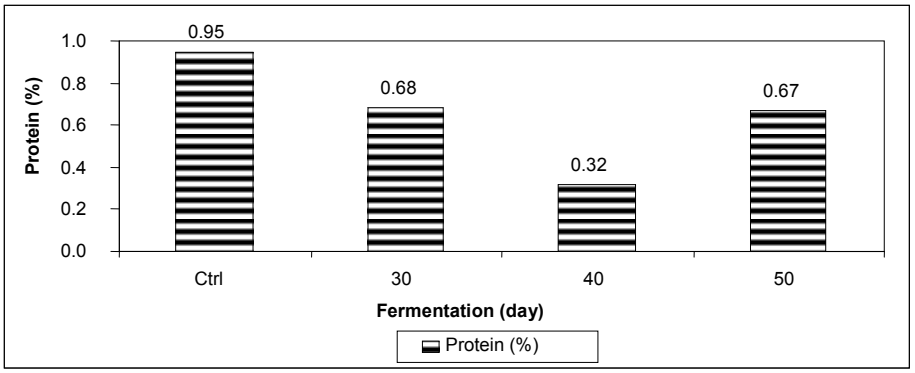

Fig. 3. The protein content in fermented red dragon fruit at different incubation time.

The contents of protein in the control and fermented juice of red dragon fruit is shown in Fig. 3. Protein content in the fermented juice was low compared with control. The protein content decreased from 0.95% in the control to 0.68% in 30 days, 0.32% at 40 days and increased again to 0.67% at 50 days. This indicated that fermentation of more than 40 days can enhance the protein content in the fermented red dragon fruit. The decrease in protein content from 30 to 40 days maybe due to protein consumption by the microorganisms during the fermentation. The increase of protein content from 40 to 50 days maybe contributed by the microorganisms produced the protein during the fermentation process.

Conclusion

Prolonged incubation of 30 to 50 days in fermentation of red dragon fruit decreased lactic acid content and increased total soluble solid and protein contents compared with control. In this study, 30 days was found to be the best fermentation time for the red dragon fruit. Different fermentation times affected some nutritive properties in the fermented products of red dragon fruit and can make it more nutritious as food product.

Acknowledgements

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Effect of TDZ and BAP on Shoot Proliferation from *in vitro* Shoot Tips of Strawberry

Haddadi, F.[1], Maheran, A.A.[1], Ghizan, S.[2], Azmi, A.R.[1] and Kamaladini, H.[1]

[1]Department of Agriculture Technology, [2]Department of Crop Science,
Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
tinyvitis@yahoo.com

Abstract

An experiment was conducted to induce shoot proliferation from shoot tips derived from runner tips of strawberry cv. Camarosa. The shoot tips were cultured on Murashige and Skoog (MS) medium supplemented with TDZ and BAP, either alone or in combination. The concentrations used were 0 to 8 µM TDZ and 0 to 27 µM BAP. MS medium supplemented with a moderate concentration of 4 µM BAP in combination with 2 µM TDZ was optimum for strawberry shoot proliferation from shoot tips derived from runner tips. In this combination of cytokinins, a 100 % shoot proliferation with a mean number of 14.6 shoots per explant was produced. This regeneration protocol is suitable for mass propagation of planting materials for commercial cultivation of strawberry and for the recovery of transgenic strawberry plants following genetic transformation.

Introduction

Strawberries belong to the *Rosaceae* family and is the third economically important cultivated crop (Oosumi *et al*., 2006). The most common strawberries grown commercially are cultivars of the Garden Strawberry (Fragaria × ananassa). One of the *Fragaria* x *ananassa* Duchesne cultivars is Camarosa which was developed by University of California in 1988. In Malaysia, local production of strawberry is limited by small area. Strawberry is grown on the highlands especially in Cameron Highlands (Sharif *et al*., 2006). *In vitro* culture system is an efficient method for shoot regeneration and proliferation from various parts of strawberry plants to ensure abundant supply of this plant material for commercial cultivation. Since the first report on *in vitro* strawberry propagation by Boxus (1974), there have been many reports of investigations on different types of media and plant growth regulators based on factors like genotype and type of explant. To date BAP is one of the cytokinins that is mostly used for shoot proliferation of strawberry plants (Barcelo et al., 1998). In contrast, TDZ is the cytokinin of more recent use for shoot regeneration in a restricted number of *Fragaria x ananassa* cultivars (Landi and Mezzetti, 2006; Passey *et al*., 2003) either alone (Mohamed *et al*., 2007) or in combination with auxins like IBA (Landi and Mezzetti, 2006). However, there is no report so far on effect of TDZ in combination with another cytokinin on shoot regeneration of strawberry. In the present study, the effect of TDZ in combination with BAP on shoot regeneration from shoot tips derived from runner tips of strawberry cv. Camarosa was investigated.

Materials and methods

Individual shoot tips of strawberry cv. Camarosa, measuring 5 - 8 mm, which were proliferated *in vitro* from runner tips, were used (Fig. 1a). Combinations of different concentrations of BAP and TDZ were incorporated into MS medium. The BAP concentrations were 0, 4, 9, 18 and 27 µM, while the TDZ concentrations were 0, 2, 4 and 8 µM. All media were autoclaved after adjustment to pH 5.7-5.8 with NaOH 1 M. All cultures were incubated in the culture room at 25 ± 2 ºC, under 16 h photoperiod with light intensity of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. The percentage of shoot formation and number of shoots produced per explant were recorded after 12 weeks of culture, but growth characteristics were observed every week. The experiment was conducted in a Randomized Complete Block Design (RCBD). All data were analyzed using the Analysis of Variance (ANOVA) and treatment means were separated by Duncan New Multiple Range Test (DNMRT) at α = 5 %.

Results and discussion

In most of the treatments, shoots started to proliferate after one week of culture (Fig. 1b). Media containing both BAP and TDZ tended to produced more shoots than the control (B0T0). Moderate concentration of 4 µM BAP in combination with 2 µM of TDZ (B4T2) produced the highest number of shoots per explant with a mean number of 14.6 after 12 weeks of culture (Fig. 1c and 2). This mean number was very high in comparison with the control with a mean number of 2.3 (Fig. 2). Highest percentage of shoot formation was 100 % which was also obtained on MS medium containing 4 µM BAP and 2 µM TDZ (B4T2) after 12 weeks of culture, followed by 93.33 % in both treatments B0T2 (0 µM BAP and 2 µM TDZ) and B18T2 (18 µM BAP and 2 µM TDZ) (Fig. 3). These treatments were not significantly different from each other. However treatment B4T2 differed significantly from the rest of the treatments. Increasing the concentration of BAP reduced the percentage of shoot formation and number of shoots formed per explant. In addition, some abnormal responses such as shoot fasciation and inhibition of shoot elongation were observed in some treatments. However after subculturing from MS medium containing BAP and TDZ to hormone free MS medium, shoots elongated with normal appearance (Fig. 1d). According to Huetteman and Preece (1993), to overcome the inhibition of shoot elongation, shoots can be transferred to less active cytokinin for shoot elongation in some cases. One of the key factors that can affect the *in vitro* culture of strawberries is hormonal balance (Landi and Mezzetti, 2005; Passey *et al*., 2003). Combination of low TDZ concentration (2 µM) with moderate BAP concentration (4 µM), promoted highest percentage of shoot proliferation and number of shoots per explants from shoot tips derived from runner tips of cv. Camarosa. It showed that by using a low concentration of TDZ, which has a high cytokinin activity, less amount of BAP is needed. Meanwhile, for micropropagation from nodal segments of strawberry, Sakila *et al*. (2007) reported using a high concentration of 7 µM BAP in media with 0.5-2 µM kinetin, since the kinetin is less active than TDZ.

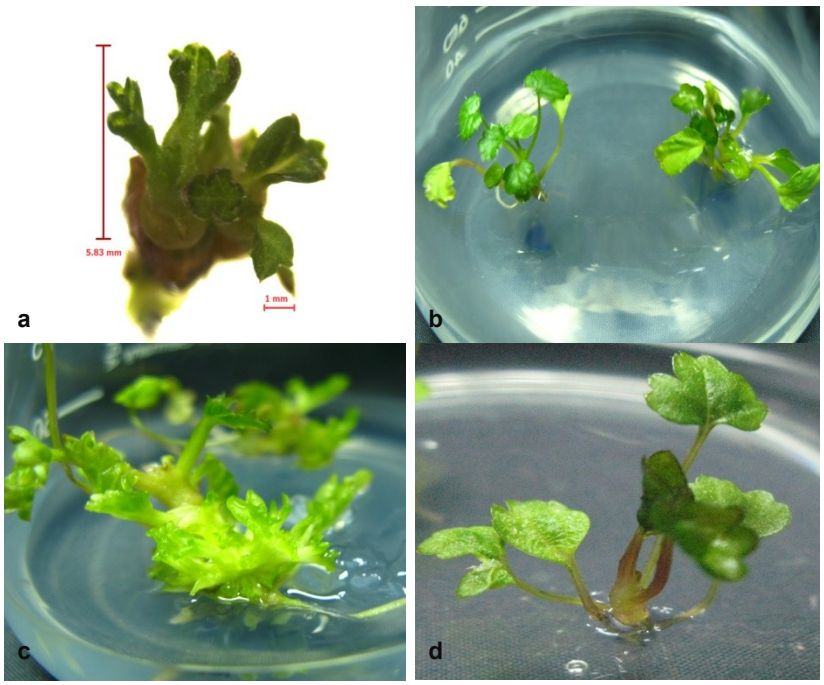

Fig. 1. Shoot regeneration from shoot tips of strawberry; a: Shoot tips derived from runner tips; b: Shoot beginning to proliferate after one week of culture on MS medium containing 4 µM BAP and 2 µM TDZ (B4T2), (Bar = 0.5 cm); c: Shoot formation on MS medium containing 4 µM BAP and 2 µM TDZ after 12 weeks of culture (B4T2) (Bars = 1 cm); d: Shoot elongation on hormone free MS medium (Bar = 0.6 cm).

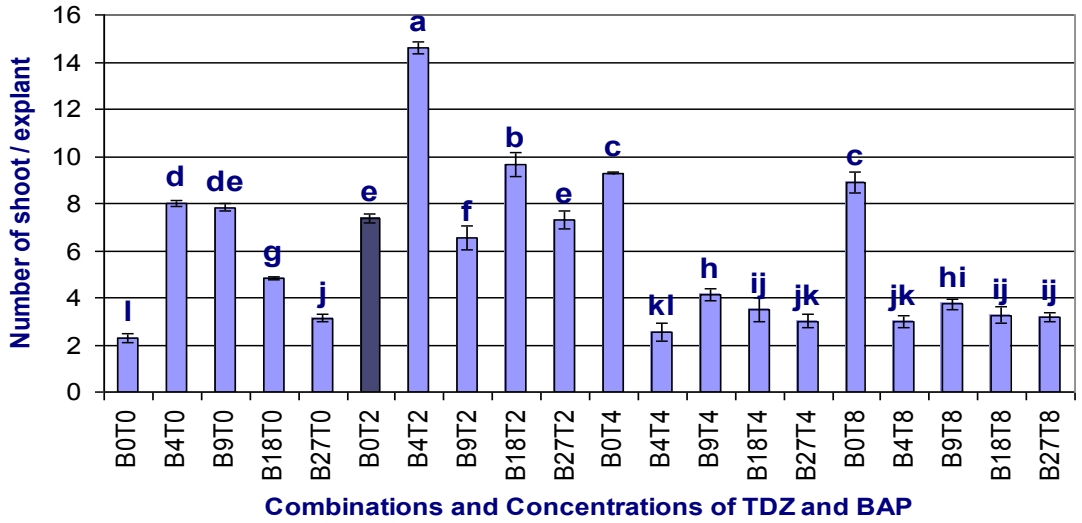

Fig. 2. Effect of different concentrations of BAP in combination with TDZ on number of shoot induced from shoot tips of strawberry after 12 weeks of culture. B0T0: 0 µM BAP + 0µM TDZ; B4T0: 4 µM BAP + 0 µM TDZ; B9T0: 9 µM BAP + 0 µM TDZ; B18T0: 18 µM BAP + 0 µM TDZ; B27T0: 27 µM BAP + 0 µM TDZ; B0T2: 0 µM BAP + 2 µM TDZ; B4T2: 4 µM BAP + 2 µM TDZ; B9T2: 9 µM BAP + 2 µM TDZ; B18T2: 18 µM BAP + 2 µM TDZ; B27T2: 27 µM BAP + 2 µM TDZ; B0T4: 0 µM BAP + 4 µM TDZ; B4T4: 4 µM BAP + 4 µM TDZ; B9T4: 9 µM BAP + 4 µM TDZ; B18T4: 18 µM BAP + 4 µM TDZ; B27T4: 27 µM BAP + 4 µM TDZ; B0T8: 0 µM BAP + 8 µM TDZ; B4T8: 4 µM BAP + 8 µM TDZ; B9T8: 9 µM BAP + 8 µM TDZ; B18T8: 18 µM BAP + 8 µM TDZ; B27T8: 27 µM BAP + 8 µM TDZ.

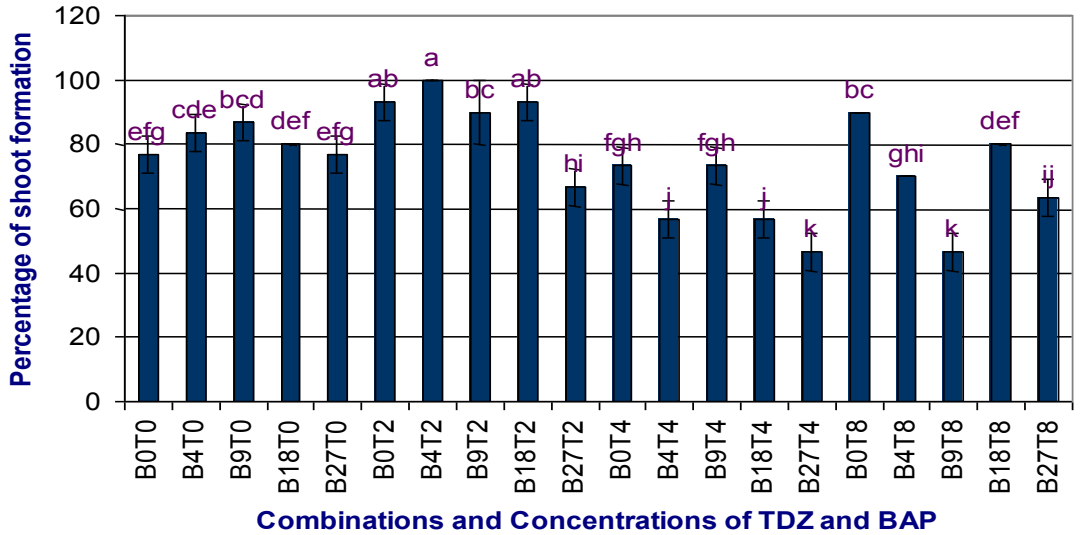

Fig. 3. Effect of different concentrations of BAP in combination with TDZ on percentage of shoot formation from shoot tips of strawberry after 12 weeks of culture. B0T0: 0 µM BAP + 0µM TDZ; B4T0: 4 µM BAP + 0 µM TDZ; B9T0: 9 µM BAP + 0 µM TDZ; B18T0: 18 µM BAP + 0 µM TDZ; B27T0: 27 µM BAP + 0 µM TDZ; B0T2: 0 µM BAP + 2 µM TDZ; B4T2: 4 µM BAP + 2 µM TDZ; B9T2: 9 µM BAP + 2 µM TDZ; B18T2: 18 µM BAP + 2 µM TDZ; B27T2: 27 µM BAP + 2 µM TDZ; B0T4: 0 µM BAP + 4 µM TDZ; B4T4: 4 µM BAP + 4 µM TDZ; B9T4: 9 µM BAP + 4 µM TDZ; B18T4: 18 µM BAP + 4 µM TDZ;

B27T4: 27 µM BAP + 4 µM TDZ; B0T8: 0 µM BAP + 8 µM TDZ; B4T8: 4 µM BAP + 8 µM TDZ; B9T8: 9 µM BAP + 8 µM TDZ; B18T8: 18 µM BAP + 8 µM TDZ; B27T8: 27 µM BAP + 8 µM TDZ.

Conclusion

MS medium supplemented with a moderate concentration of BAP in combination with TDZ (4 µM BAP and 2 µM TDZ) was optimum for strawberry shoots proliferation. This *in vitro* regeneration system can be assessed for the purpose of micropropagation as well as for plantlet recovery following *Agrobaceterium*–mediated transformation.

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Fermentation of *Jatropha curcas* Solid Waste with *Aspergillus niger*

Khairul, F.K., Nurul Iwani, A., Mahmad, N.J. and Mohamed Zulkali, M.D.

School of Bioprocess Engineering, Universiti Malaysia Perlis, Perlis, Malaysia
khairulfarihan@unimap.edu.my

Abstract

An experiment has been carried out by using *Jatropha curcas* solid waste as substrate for fermentation with *Aspergillus niger*. Enzyme production from the fermentation activities and the toxicity level i.e. phorbol ester contents of the biomass were determined. The results suggested that the utilization of Jatropha solid waste as substrate for enzyme production could be an alternative in replacing its traditional used as fodder or fertilizer especially when there is no practical way for its detoxification.

Introduction

J. curcas has great potential for biodiesel production as an alternative to fossil fuel. Thus, it will generate huge quantities of residual jatropha solid waste. However, this solid waste while containing nutrients cannot be used as a feed supplement for livestock because of its toxicity. The toxicity of *J. curcas* is based on several components such as phorbol esters, curcins, trypsin inhibitors, lectin and others (Martinez Herrera *et al.,* 2006). Phorbol esters have been identified as one of the toxic substance in *J. curcas* solid waste. The high concentrations of phorbol esters make complete detoxification a complicated process (Makkar and Becker, 1997). The detoxification process can be carried out by physical, chemicals and microbial methods but physical and chemical methods are expensive although they achieve a good results. Our study was focused on the use of microbes in submerged fermentation to detoxify the solid waste and also to check enzyme production.

According to Akintayo 2004, *J. curcas* contains approximately 47% of crude fat, 25% of crude protein, 10% of crude fibre, 5% of moisture and 8% of carbohydrate. Thus, jatropha solid waste can be used as substrate for fermentation because its composition is appropriate to support good microbial growth and the production of enzymes.

Materials and methods

Plant material

The matured seed of *J. curcas* used was collected from Perlis. The seed was ground and defatted in Soxhelt apparatus by using petroleum ether. The solid waste was kept overnight in an oven at 60^{o}C to remove any remaining petroleum ether.

Preparation of inoculum

The strain of *A. niger* was taken from Microbiology Laboratory, Universiti Malaysia Perlis. The culture was maintained on potato dextrose agar at 4^{o}C and subcultured every 15 days.

Fermentation process

Fermentation of jatropha solid waste was carried out by cultivating the solid waste in 150 ml water with *A. niger* at 240 rpm, 30°C and 65 % humidity for 78 hours.

Enzyme extraction and assay

Enzyme extraction was carried out by the method of Ramachandran *et al*. (2004). Crude enzyme was extracted by mixing the fermented substrate with 1.0 ml of 0.1 M Tris HCl buffer pH 8.0 and then

shaking in an orbital shaker. The suspension then was centrifuged at 12,000 rpm for 10 min and was used for enzyme assay.

Protease activity was determined as described by Shimogaki *et al*. (1991) using casein as substrate and lipase activity was determined as described by Kilcawley *et al*. (2002)

Phorbol esters detection by using TLC

The detection of phorbol esters was done by using Thin Layer Chromatography method. It was performed by using Merck Silica 60 F_{254} plate, CH_2Cl_2/acetone (3:1) as eluent and vanillin/sulphuric acid for colour reaction.

Results and discussion

The objective of this project is to detoxify the jatropha solid waste through submerged fermentation by using *A. niger*. It was also to check the suitability of jatropha solid waste as a substrate for production of enzymes. From the study, it was revealed that the jatropha solid waste was able to provide all the necessary nutrients for the growth of *A. niger*.

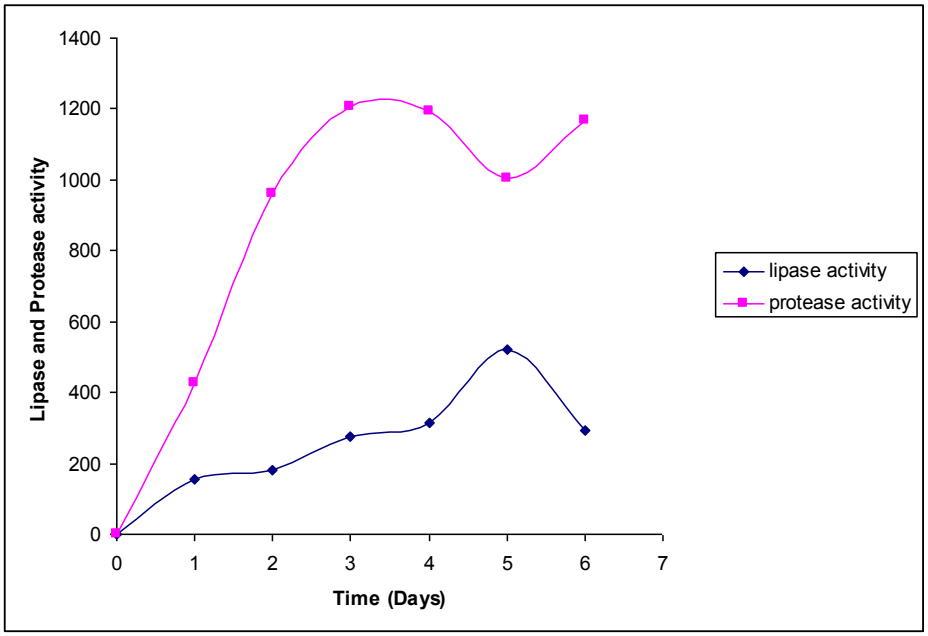

Fig. 1. Protease and lipase production in submerge fermentation by using jatropha solid waste as substrate.

Fig. 1 shows the fermentation profile of Jatropha solid waste by using *A. niger*. The protease activity was found to be maximum on the 3rd – 4th day after the inoculation. This is almost similar to the finding reported by Kaur *et al*. (2001). Kaur *et al* (2001) also reported that the reason why the maximum protease production on the 3rd day is due to the depletion of the nutrients or denaturation of the enzyme caused by the interaction with other components in the medium or change in the pH of the medium. Lipase activity was found to be maximum on the 5th day after inoculation. This finding also similar to the trend for lipase production that was obtained by Mahadik *et al*. (2001) and Mahanta *et al*. (2008).

From the TLC profile of the methanol extract of the fermented materials (biomass) fermentation with *A. niger* did not destroy the phorbol esters. This finding agrees with Goel *et al*. (2007) and Makkar and Becker (1997).

Conclusions

This preliminary study indicated that jatropha solid waste could be a potential substrate in submerged fermentation for enzyme production by *A. niger*. Evidently it provided necessary nutrients for bacteria to grow and synthesize enzymes. So that, *J. curcas* solid waste can be one of substrate for production of other industrial enzyme from different microbes. Further study will be carried out to optimize the parameters for submerged fermentation, in stabilizing the enzyme production as well as the detoxification of the biomass.

Acknowledgements

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In vitro Shoot Regeneration from Cotyledonary Leaf Explant of Tomato Variety MT1

Kamaladini, H., Siti Nor, A.A. and Maheran, A.A.

Department of Agriculture Technology, Faculty of Agriculture, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia
hu_kamaladiny@yahoo.com.au

Abstract

Development of an efficient regeneration system for commercial tomato cultivars is a necessary precondition for genetic manipulation. Regeneration system of tomato variety MT1 was developed. Seeds were cultured on half strength Murashig and Skoog (MS) medium. The 12-14 days old cotyledon leaf explants were cut and cultured on full strength MS medium supplemented with 1 mg/l NAA and 1 mg/l BAP. After 48 h these explants were transferred to regeneration medium containing two types of cytokinin which were 2ip and zeatin. The concentrations used were 0, 1, 2, 3, 4, 5 mg/l for both 2ip and zeatin. High frequency of shoot regeneration by using different concentrations of 2ip was achieved on MS medium with 4 mg/l giving the best results. Meanwhile MS medium containing 2 mg/l zeatin showed highest percentage and number of shoots formed per explant. Results showed that more callus formation occurred on medium containing 2ip. Individual shoots derived from cotyledon were used for root induction. Root induction medium was MS containing 5 mg/l IAA and shoots maintained on this media for three weeks until roots were well developed. These systems would provide the means for recovering genetically modified plants after subjecting explants to *Agrobacterium*-mediated transformation or particle bombardment.

Introduction

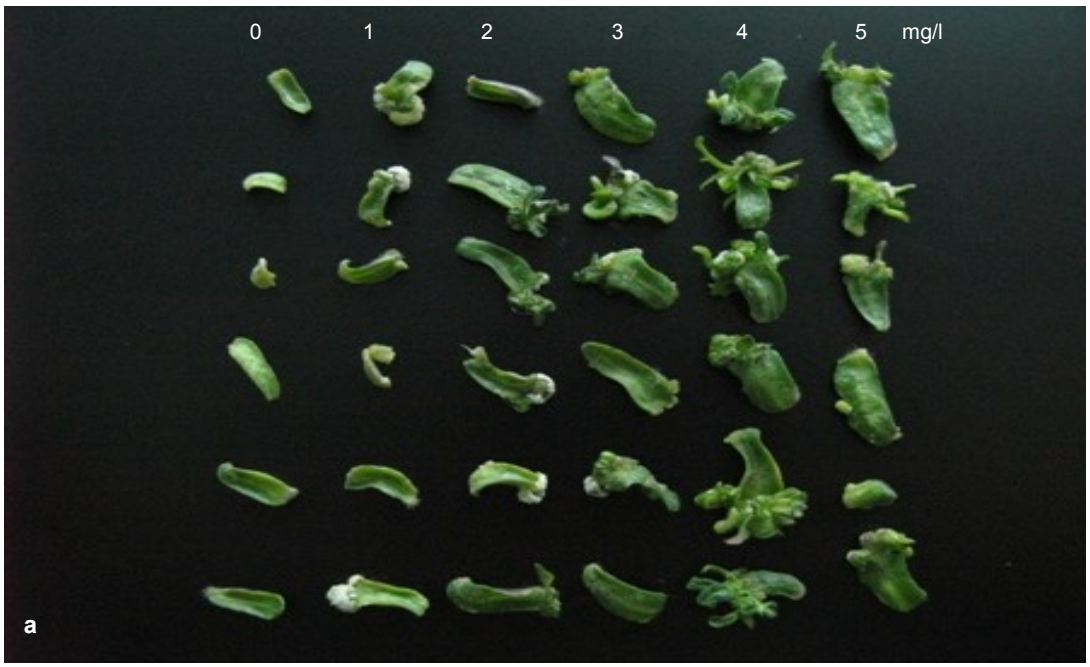
Tomato (*Lycopersicon esculentum)* is now grown worldwide for its edible fruits. Thousands of cultivars have been selected for optimum growth in different growing conditions. Tomatoes are rich in vitamins including A, K, and C, Calcium, potassium as well as fiber. They are rich in the antioxidant Lycopene. Tomato is a favorite model plant system for studying gene expression. Availability of efficient regeneration system is critical for genetic manipulation. There are many reports on *in vitro* culture of tomato using different types of media, different variety and various concentrations of plant growth regulators (PGRs). Cortina *et al.* (2004) reported that optimal shoot regeneration rate from Cotyledon explants of *L. esculentum* var. UC82B was obtained on N6 medium using 0.5 mg/l IAA and 0.5 mg/l zeatin riboside. Khan *et al*. (2006) reported using MS medium supplemented with 0.1mg/L NAA, 1mg/L BAP for shoot regeneration from leaf explants of tomato. More recent, Majoul *et al.* (2007) investigated the effect of IK basal medium with zeatin, BAP and IAA on shoot regeneration from leaf explant of tomato var. Justar and Nemador. There have no reports been found on *in vitro* regeneration system of tomato variety MTI (MARDI Tomoto of Malaysia) using 2ip and zeatin.

Materials and methods

Seeds of tomato var. MT1 were used. Tomato seeds were surface sterilized by placing under running tap water for one hour followed by immersion and shaking in 2 % teepol for 10 min and then immersion and shaking in 70 % ethanol for one min. Seeds immersed and shacked in 20 % Clorox plus a few drops of Tween 20 for 16 min. Finally the seeds were rinsed with sterile distilled water three to five times. Sterilized seeds were cultured on half strength MS medium. Cotyledonary leaves of 12-14 days old seedling were cut and transferred on MS medium supplemented with 1 mg/l NAA and 1 mg/l BAP. After 48 h these explants were transferred to regeneration medium containing 0, 1, 2, 3, 4, 5 mg/l of both 2ip and zeatin. The percentage of shoot regeneration and number of shoots produced per explant were recorded after 4 weeks of culture, but growth characteristics were observed every week. The experiment was conducted in a Randomized Completely Block Design (RCBD). All data were analyzed using the Analysis of Variance (ANOVA) and treatment means were separated by Duncan New Multiple Range Test (DNMRT) at α = 5 %. Shoots produced from cotyledonary leaves were transferred to root induction medium containing 5 mg/l IAA.

Results and discussion

In MS medium containing zeatin, shoot regeneration started after two weeks of culture. On the medium containing 2ip, shoot regeneration started after three weeks of culture. More callus formation occurred on medium containing 2ip. Moreover root formation without shoot regeneration occurred after five weeks of culture on MS medium containing 1 mg/l 2ip (Fig. 1d). Significant differences were found on the mean number of shoots formed per explants on the medium containing zeatin and 2ip (Fig. 2). In shoot regeneration using zeatin, the highest number of shoots produced per explants(18.91) on MS medium containing 2 mg/l zeatin (Z2)(Fig. 1b and 1c). This concentration is significantly different from the control and the rest of the treatments. However, shoot regeneration using 2ip occurred on MS medium containing 4 mg/l 2ip (i2) with a mean number of 14.21(Fig. 1a). The highest percentage of explants with shoot proliferation was 100% for those treatment containing 1, 2, 3, 4 mg/l zeatin and 3, 4, 5, mg/l 2ip (Fig. 3). The lowest mean number of shoots produced per explant (0) and lowest mean percentage of explants with shoot proliferation (0%) occurred on control which was free of both of zeatin and 2ip (z0 and i0). Some of the factors that can affect regeneration responses are the genotype-PGR interactions (Nehra *et al*., 1990), age of tissue (Nehra *et al*., 1990), different concentration of plant growth regulators (PGRs) and type of explants (Debnath, 2005). Majoul *et al*. (2007) reported using 1 mg/l Zeatin for shoot regeneration from seedling leaf explants of 18–21 days old for var. Justar and Nemador. In addition they used 0.1 mg/l IAA for root induction. However in this study, 2 mg/l zeatin is optimum to promote the shoot regeneration from 12-14 days old cotyledonary leaf explants for var. MT1. Also the individual shoots produced well developed root in medium containing 5 mg/l IAA. The advantage of zeatin is promotion of entire plant regeneration (Shtereva *et al*., 1998) but 2ip resulted in more callus formation.



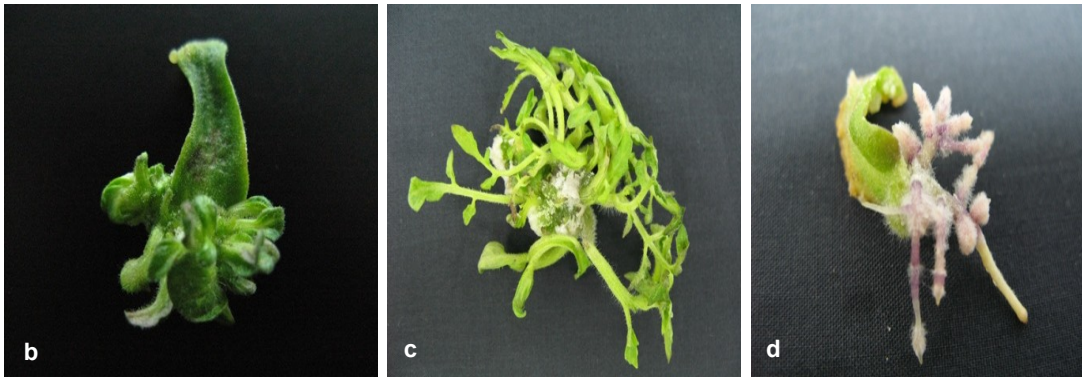


Fig. 1. Effect of shoot regeneration from cotyledonary leaf explants of tomato on MS medium containing; a: different concentration of 2ip (mg/l) after three weeks of culture; b, 2 mg/l zeatin after three weeks of culture; c: 2 mg/l zeatin after five weeks of culture; d: 1 mg/l 2ip after five weeks of culture.

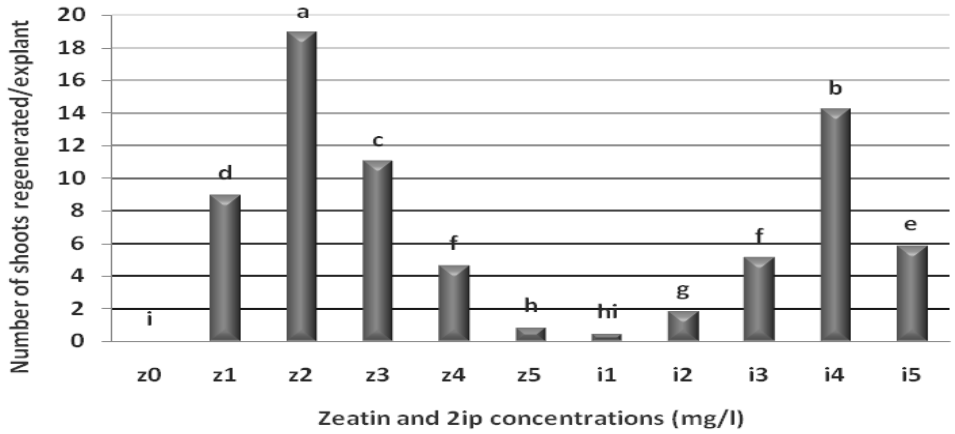


Fig. 2. Effects of different concentrations of zeatin and 2ip on shoot regeneration from cotyledonary leaf explants of tomato.

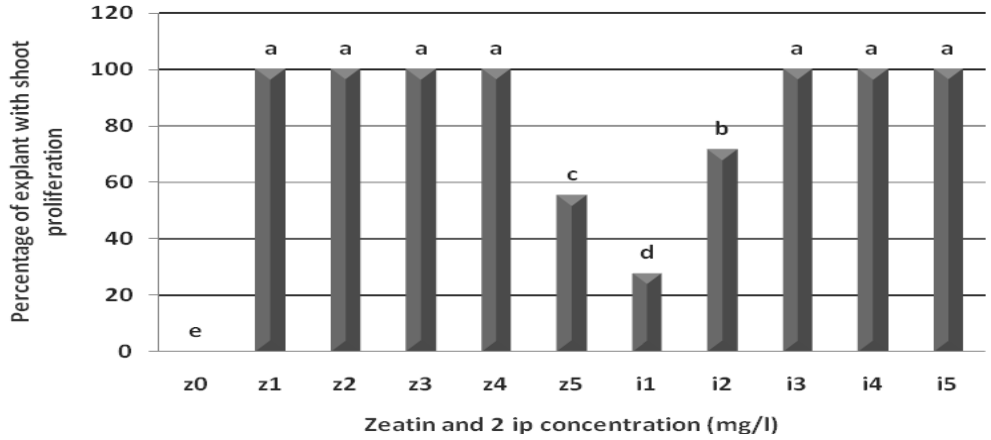


Fig. 3. Effects of different concentrations of zeatin and 2ip on percentage of explants with shoot proliferation from cotyledonry leaf explants of tomato.

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Immobilization of Lipase Enzyme from *Aspergillus Niger* by Various Techniques on Different Support Materials

Norhafizi, H., Anusha, N., Mohd Amiron, E., Lim, C.M., Miftahu Salihin, M., Bazli, S.A. and Hirzun, M.Y.

Bioprocessing Technology Department, Sime Darby Technology Centre,
2 Jalan Tandang, 46050 Petaling Jaya, Malaysia
norhafizi.hashim @simedarby.com

Abstract

Three support materials; Amberlite IRC-50, Silica Gel and Kielserguhr were selected among nine carriers due to the highest activity obtained. The nine carriers that has been studied were inorganic materials (sea sand, silica gel, kielserguhr, Al2O3, Zeolite), inorganic salts (CaCO3, CaSO4), ion-exchange resins (Amberlite IRC-50) and synthetic polymer (polypropylene). Immobilization of lipase enzyme from *Aspergillus niger* was carried out by simple adsorption, adsorption on ion exchange resins, combined adsorption and precipitation and pure precipitation. The suitability of the support materials and techniques for the immobilization of lipase enzyme was evaluated by determining the enzyme activity, protein loading, immobilization efficiency and lypolytic activity of the immobilizates before and after freeze drying. Most of the immobilizates exhibited low enzyme activity during the hydrolytic reaction. Only those prepared by ionic adsorption on Amberlite IRC-50 and simple adsorption technique on Silica Gel and Kielserguhr showed good activity after freeze drying process i.e. 35.50 U/g, 21.00 U/g and 11.50 U/g, respectively.

Introduction

The steadily growing interest in lipases over the last two decades stems from their biotechnological versatility and the ability of this enzyme to catalyze a broad spectrum of bioconversion reactions with tremendous potential in various areas such as in food technology, biomedical sciences and chemical industries. Many of these applications are performed with immobilizated lipases. The immobilization is an advantageous method that improves the stability of the biocatalyst and provides for its repeated use and the easy separation of the catalyst from the reaction medium (Vilma *et al.*, 2004).

Various techniques and even more support materials have been studied and consequently many immobilized preparations with a wide range of efficiency, stability and activity have been offered. Lipases have been covalently bound to activated poly (vinyl chloride), nylon or to controlled pore silica (Soares *et al.*, 1995). Adsorption on hydrophobic or hydrophilic supports, as a simple method, still attracts attention. Among the various supports, celite, cellulose, ethyl cellulose, carbon and synthetic polymer, as well as rice straw and alumina beads, have served as carriers for this purpose (Padmini *et al.*, 1993). Recently, a novel form of lipase covalently immobilized on reversibly soluble polymers was proposed (Amridkar *et al.*, 2002). Another novel technique for immobilization of lipase on colloidal gas aphrons, which is on spherical microbubbles, was described (Connel *et al.*, 2001).

The supports used for immobilizing enzyme should posses' mechanical strength, microbial resistance, thermostability, chemical durability, chemical functionality, low cost, hydrophilicity, regenerability and a high capacity of enzyme (Kilara, 1981). Immobilization of enzymes through physical method is still the most commonly used because it is the easiest to perform and the least expensive. In this method, the forces between a support and the enzymes include hydrogen bonding, Van der Wall forces and hydrophobic interactions (Burns, 1986).

Obviously, the problem of selecting the support material and the proper technique are very important and therefore the pursuit for suitable materials has not yet ceased. Joining the search for suitable and low cost materials, in this study, nine inexpensive materials were examined as supports using several immobilization techniques, among them simple adsorption, adsorption on ion exchange resins, combined adsorption and precipitation and pure precipitation were evaluated and analyzed. The selection criteria were based on the enzyme loading onto or in the support, immobilization efficiency and lypolytic activity of the immobilizates before and after freeze drying.

Materials and methods

Microorganism and cultivation

Aspergillus niger culture purchased from DSMZ Culture Collection, Germany was used in this study. The stock cultures of the strain were maintained in glycerol (20%v/v) stored at -20°C.

Inoculation preparation and solid state cultivation of palm kernel cake

10 mL of sterilized water was poured onto the surface of potato dextrose agar (PDA) containing *A. niger* culture and gently scrapped off using a sterilized glass rod. The spore suspensions of *A. niger* were filtered and counted by using hemacytometer to determine its concentration in spores per mL. The spores were inoculated in Palm Kernel Cake (PKC). The medium was then fermented for 7 days. The fermented PKC was extracted with 0.02 M Sodium Phosphate Buffer pH 7.0 and shaken at 16°C, 200 rpm for 30 minutes.

A tangential flow filtration system (Millipore Labscale^{TM} TFF) was later used to concentrate lipases from the crude extract. Ten-fold concentration was done using 5kDa MWCO TFF membrane at 4°C. The retentate obtained from TFF was assayed for lipase activity.

Lipolytic activity assay

2.5 mL of olive oil emulsion and 2.0mL of 0.02M Sodium Phosphate Buffer pH 7 were added into 25 mL conical flasks. The flasks were incubated in at 37°C, 200rpm for 5 minutes. Then, 0.5 mL of samples were added into conical flasks and continued to be shaken for 30 minutes. 5 mL of acetone: ethanol (1:1) was added into the flasks to stop the reaction. The samples were titrated with 0.05M NaOH using an autotitrator (Mettler Toledo T50) and the lipase activity was calculated. One unit of lipase activity was defined as the amount of enzyme which liberated 1 µmol equivalent of fatty acid at 37°C and pH 7.0 in one minute.

Total protein assay (Bradford)

160 µL of each samples solution was pipetted into separate microtiter plate wells. 40 µL of Bradford dye reagent was added to each well. The samples and reagents were mixed thoroughly. The sample was incubated at room temperature for 5 minutes and was measured at 595 nm for absorbance.

Immobilization of lipase

Unless otherwise stated, 200 mL of enzyme was added to 20 g of each support materials. The enzyme solution was prepared by dissolving 1 g of freeze drying crude lipase powder in 200 mL distilled water or buffer. The activity of this solution was 8.50 µmoles/min.g. The immobilization was carried out in 500 mL beaker placed on a magnetic stirring plate (200 rpm) at room temperature.

Immobilization by simple adsorption

This procedure was employed with four carriers, three of inorganic origin (silica gel, sea sand and kielserguhr) and one synthetic polymer (polypropylene). 20 g of stated support materials and 200mL of crude lipase from *A. niger* were incubated for 3 h on stirring plate at room temperature. After immobilization, the preparations were vacuum filtered through a Buchner funnel, rinsed 3 times with distilled water and freeze dried. The sea sand and kielserguhr were rinsed with sodium phosphate buffer (0.02M, pH 7.0), vacuum filtered and then the suspension were stirred for 2 h the above mentioned conditions. In these cases the freeze drying crude lipase powder was dissolved in sodium phosphate buffer (0.02M, pH 7.0). The immobilized preparations on polypropylene carrier were obtained by a similar procedure, except that the carrier was preactivated with 2.5 ml of ethanol per gram of polypropylene. The immobilized lipase was then lyophilized in a freeze dryer.

Immobilization by adsorption on ion exchange resins

20 g of Amberlite IRC 50 was first equilibrated in 200 mL of sodium phosphate buffer (0.02M, pH 7) at room temperature overnight. After vacuum filtration, the resin was incubated with the freeze dried crude lipase powder dissolved in sodium phosphate buffer (0.02M, pH 7.0) at 4ºC for 1 h. Then the resin was vacuum filtered through a Buchner funnel and freeze dried.

Immobilization by combined adsorption and precipitation

20 g of aluminum oxide, calcium carbonate and calcium sulphate were incubated with 200 mL of lipase solution from *A. niger* on stirring plate for 3 h respectively. Then 100 mL of chilled acetone was added to the mixture and immediately vacuum filtered. The immobilizates were kept in dessicator for 24 h to evaporate the acetone and later the immobilizates were freeze dried.

Immobilization by precipitation

20 g of Zeolite was incubated with 200mL of lipase solution from *A. niger* on stirring plate for 1 h. Then, 50 mL of chilled acetone was poured into the suspension and the precipitation was allowed to proceed for additional 1hour at 4°C. The acetone was removed by keeping the immobilizates in dessicator for 4 h. Then the immobilizates were freeze dried.

Evaluation of the immobilization techniques

Each of the techniques were determined by protein loading (mg protein/g immobilizates), lipase activity before and after freeze drying and immobilization efficiency.

The protein loading, *P*, was determined as follows:

$$P = [(c_0V_0 - c_fV_f) / W_g]$$

where the concentration of protein in lipase solution before (c_0) and after immobilization (c_f) are given in mg/ml, while the volumes of the solution, V_0 and V_f are in mL. W_g is the weight of the wet immobilizates in gram (g).

The efficiency of the immobilization techniques, η was determined by calculating the lypolytic activity of the lipase solution before (E_0) and after immobilization (E_f), using the relation:

$$\eta = [(E_0V_0 - E_fV_f)/V_0E_0] \times 100\%$$

The activities are given in U/mL, and the volumes are in mL.

Results

In order to select an inexpensive support for optimal lipase immobilization, nine carriers belonging to 2 types of support materials i.e. adsorbents and ion exchange resins were used. According to the chemical nature, they belong to silicates, aluminosilicates, oxides, carbonates, sulfates, natural biopolymer of polysaccharide type and synthetic polymer composed of propylene and acrylic monomers. Lipase immobilization on these materials occurred by various binding mechanisms, from simple adsorption by van der Walls to ionic forces interaction. The results of determination of the immobilization techniques and the support materials are given in Table I.

Table 1. Activity and efficiency of the immobilizates prepared by various techniques

Support Materials/ Techniques	Lipolytic activity of IM support before freeze drying (U/g)	Lipolytic activity of IM support after freeze drying (U/g)	Protein loading (mg/g)	Immobilization Efficiency, %
1. *Simple adsorption*				
Silica gel	5.26	21.00	0.779	35.98
Kielserguhr	6.29	11.50	0.865	32.13
Sea Sand	3.59	5.33	0.166	27.52
Polypropelene	2.67	2.50	0.048	3.44
2.Adsorption on resins				
Amberlite IRC 50	12.50	35.50	0.277	5.45
3. Adsorption followed by precipitation.				
Al2O3	5.33	3.17	0.828	21.57
CaCO3	2.50	1.80	0.807	9.95
CaSO4	6.00	3.50	1.283	22.73
4. *Precipitation*				
Zeolite	7.84	6.00	1.134	42.72

Discussion

Simple adsorption

Silica gel and kielserguhr demonstrated high lypolytic activity after freeze drying (21.00 U/g and 11.50 U/g respectively). The protein loading ranged from 0.048 mg/g to 0.865 mg/g from an initial 1.387 mg/g protein. Silica gel and kielserguhr retained 56.2% and 62.5% of total protein with percentage of immobilization efficiency were 35.98% and 32.13% respectively. Polypropylene showed insignificant lypolytic activity before and after freeze drying with the amount of protein loaded to the support being 0.048 mg/g. Silica gel which due to its small crystalline particles, displayed a very good capacity of adsorption of 80%, while Kielserguhr demonstrated a moderate capacity (Rose *et al*, 1993). This supports the results of Rose *et al*. (1993) where silica gel and kielserguhr exhibited a higher adsorption capacity because its fine powder structure produced a larger adsorption area.

Adsorption on resin by ionic binding

The experiment was done by following the theory that the ability of the support materials to bind the enzyme by ionic interaction. Amberlite IRC-50 was used in this technique of immobilization because it has ionic charge that can bind to lipase. The protein loaded into the Amberlite IRC-50 was only 0.277 mg/g with 19.97% of protein retained and immobilization efficiency was 5.45%. Nevertheless, the lypolytic activity after freeze drying was the highest (35.50 U/g support). Amberlite IRC -50 is a weak cation acidic resin. It has carboxyl end group functionality which can give highest activity compared to strongly acidic cation exchange resins. It was observed that end group functionality and structure of the matrices plays an important role in obtaining highest activity yield (Muthy *et al.*, 1998). Therefore, the functionality of the Amberlite IRC-50 might be the reason that gave Amberlite IRC-50 the highest lypolytic activity.

Adsorption followed by precipitation

All the three support materials used in this study did not give good result. The calcium sulphate ($CaSO_4$) retained 92.5% of total protein with 22.73% immobilization efficiency. Its lypolytic activity after freeze drying was only 3.50 U/g support and did not directly correlate to the protein loading. Aluminum oxide (Al_2O_3) and calcium carbonate ($CaCO_3$) also gave lower lypolytic activity after freeze drying;

3.17 U/g and 1.18 U/g enzyme respectively. According to Saxena *et al*, the solid inorganic salts, $CaSO_4$ and $CaCO_3$ exhibited moderate adsorptive properties while Al_2O_3 demonstrated very poor adsorption.

Immobilization by precipitation

Zeolite was a poor carrier for the enzyme when used as adsorbents, as well as when the enzyme was precipitated onto it. The protein loading was 1.134 mg/g which retained 81.8% of total protein. However, it does not correlate to the amount of the protein loaded which gave the lypolytic activity of 6.00 U/g after freeze drying and 42.70% of immobilization efficiency. Lie and Molin working with Zeolite found that it exhibited very low lypolytic activity which was quite comparable to our results.

Conclusion

As conclusion, among nine numerous support materials and several techniques used in this study; Amberlite IRC-50, Silica gel and Kielserguhr were found to have good activity after freeze drying process i.e. 35.50 U/g, 21.00 U/g and 11.50 U/g for respectively.

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