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Mageswaran
Memorial Oration - 2009

Sri Lankan Lichens: Their Medicinal & Ecological Potential

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Department of Chemistry,
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18th December 2009

UNIVERSITY OF JAFFNA

SRILANKA

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Professor Sivapathasuntharam Mageswaran
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Vice-Chancellor's Message

I am delighted to welcome all the participants to this memorable event.

Cherishing the memories of our Pioneers give us strength and guidance.

Prof. Mageswaran played a key role in building our institution as a centre of excellence in learning. His contribution to the shaping of the Faculty of Science and enhancing quality education of Chemistry being valued by the university community and the world of chemistry

We are proud to have with us Prof. Veranja Karunaratne, Professor in Chemistry, University of Peradeniya who earned many awards for his scientific excellence to deliver the memorial oration of our Prof. Mageswaran.

Knowledge and dissemination of knowledge become more and more valuable when it contributes to the human and social development. In this context the chosen topic for today 'Sri Lankan Lichens: Their Medicinal and Ecological Potential' is very much relevant in mobilizing the natural resources to the betterment of human living.

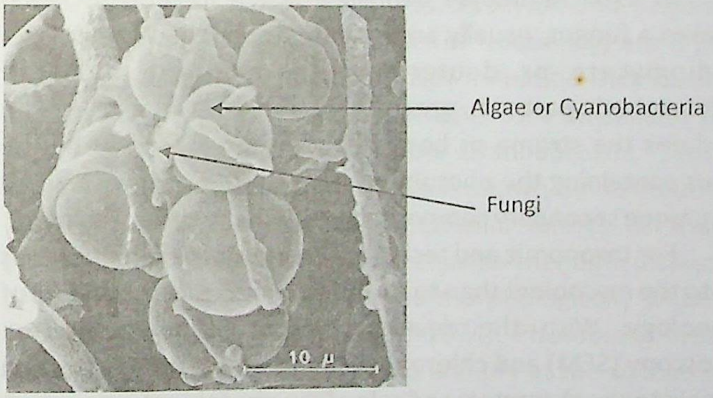
I extend my sincere thanks and wishes to Prof. Veranja for his contribution to this event and his future achievements of creating new knowledge.

Prof. N. Shanmugalingam
Vice-Chancellor
University of Jaffna.
18/12/2009.

Sri Lankan Lichens: Their Ecological & Medicinal Potential

INTRODUCTION

Lichens are the symbiotic phenotype of nutritionally specialised fungi that live as ecologically obligate biotrophs in symbiosis with algal and/or cyanobacterial photobionts (Honegger, 1991). Although Schwendener (1896) viewed this as a parasitic relationship with the algal partner as a slave to the fungus, it is now regarded as a symbiosis in which both partners benefit. Despite this, the algal partner is never found in a sexual state, and fungal partner is dependent on nutrients from photosynthesis or nitrogen fixation of the photobiont partner.



- This symbiosis between simple haploid organisms with low genetic diversity has developed morphological, anatomical and chemical characters of great complexity producing stable organisms that are able to survive and reproduce in a range of extreme conditions of temperature and moisture.
- About 8% of terrestrial ecosystems are dominated by lichens in situations where vascular plants are at their physiological

limits, and where poikilohydrous lichen thalli survive unharmed in a state of dormancy.

- The taxonomy of lichens is based on the characteristics of the sexually reproducing partner the fungus, yet many lichens reproduce asexually from propagules containing both symbionts and are not known in a sexual state.
- Lichenisation has probably occurred many times over long periods of time in different groups of fungi, so that lichens represent a polyphyletic group.
- Lichens are distinguished from most non-lichenised ascomycetes by the longevity of their thalli as well as their ascocarps (Poelt, 1994).

Symbionts

In 1993 Ahmadjan defined a lichen as an association between a fungus, usually an ascomycete, but in a few cases a basidiomycete or deuteromycete, and one or more photosynthetic partners, green algae and/or cyanobacteria. In all lichens the stroma or body of the lichen is formed by the fungus containing the photobiont. Many lichens are associated with unique secondary compounds produced by the mycobiont.

For taxonomic and technical reasons more attention was paid to the mycobiont than to the photobiont in the early days of lichenology. With the development of Scanning Electron Microscopy (SEM) and chlorophyll fluorescence more attention was paid to the characters and role of the photobiont.

Mycobiont

Mycobiont is the fungus. Lichenized fungi are widespread in nature and represent about 20% of all fungi species (Hawksworth, 1988). The lichen thallus shape is mainly determined by the fungus comprising of two classes. In the

majority of lichen taxa (over 95%), the mycobiont is an ascomycete, while in a few taxa it is a basdiomyccete (2%). Poelt (1970) estimated 14,370 species of lichenised fungi. One fifth of fungal species are lichenised.

Photobiont

There are two major types of photobionts, namely Chlorophyceae (green algae) and Cyanophyceae (blue-green cyanobacteria). Chorophyceae are filamentous orange algae or Trentepohlia (found in shaded moist habitats) and Trebouxioid or unicellular green algae (found in well-lit habitats). Other green algal photobionts include *Coccomyxa* and *Dictyochloropsis.* Altogether about 25 genera of algae are involved in lichen symbiosis.

Cyanophyceae including *Nostoc, Scytonema, Stigonema, Gloeocapsa* and *Calothrix* (in order of frequency) are nitrogen fixers which are dependent on high humidity. Distribution of algae in about 14,370 lichen species is 61% unicellular green algae, 31% Trentepohlioid and 8% Cyanobacteria. That this diversity has ecological significance is shown by distribution of lichen algae in evergreen and seasonal forests of Thailand (Wolseley, 1997). Taxonomy of the algal partner difficult because of plasticity, which may be altered morphologically within the lichen thallus (Green and Smith, 1974). Photobiont must be separated and cultured in order to identify at the species level. *Trebouxia* are not known to exist in free-living state and *Trentepohlia* and cyanobacteria are found free living.

How many partners?

Fungal partner is specific to lichen and is the accepted taxonomic marker, but the same fungus may be associated with up to 5 different algae in the same thallus or may produce an

entirely different morphotype, even on the same host plant (James & Henssen, 1993). The extraction of DNA from photosymbiodemes has demonstrated their specific character (Armaleo & Clerc, 1991). However great care must be taken in the extraction of fungal hyphae as there are many associated parasitic and free-living fungi under tropical conditions.

Recent work has shown that mycobionts are strongly selective towards their photobionts (Friedl, 1989) and that they may exchange one photobiont for another. An experiment on the isolated partners of the basidiolichen *Omphalina ericetorum* demonstrated the alteration in behaviour of the fungal hyphae in the presence of the photobiont *Coccomyxa* with increased growth and division of algal cells following capture (Langenstein & Oberwinkler -IAL3).

The Phenotype

Despite low genetic diversity of relatively simple organisms of fungi, algae and cyanobacteria, the combination of these organisms in a lichen includes a surprising range of physiological and morphological adaptations that has enabled lichens to invade and dominate a range of extreme environments across the world.

Although most systematic groups of organisms show strong morphological affinities, the phenotype of lichens is an expression of the symbiosis, whereas the classification is based on the characters of the mycobiont, including structure and ontogony of the fruiting body. Lichen families may contain genera with widely different life forms e.g. Teloschistaceae, Roccellaceae.

Life form

There are different types of life forms of lichens. These forms include foliose, fruticose, crustose, squamulose,

leprarioid and placodioid. Crustose thalli cannot be separated from the substrate. May form a continuous crust with a cortex and algal layer, or as powdery leprarioid thallus with no obvious structure. Fruticose lichens are branched and shrub-like, with or without a central chondroid tissue as in *Usnea.* Foliose lichens are flattened and leaf like with an clear upper and lower surface, and usually with obvious organs of attachment. Placodioid lichens have closely attached lobes radiating from a crust-like centre without distinct organs of attachment e.g. *Dirinaria* spp. These lichens are common in seasonally dry habitats. Squamulose thalli such as *Phyllopsora* are formed of thalline bodies on a web of brown, white or black fungal hyphae. This is called a hypothallus and precedes the formation of the lichen thallus in most crustose and squamulose lichens where it can be seen at the margins of each lichen thallus, often forming a contrasting pattern of individual thalli of the same species as in *Rhizocarpon geographicum*.

Crustose

Foliose

Fruticose

Thallus structure

Whatever the life form of the lichen the functional arrangement of the mycobiont and photobiont partners is critical to the success of the phenotype. In the majority of lichens the photobiont occurs within a tissue formed by the mycobiont, but in *Dictyonema* the algal cells are packed inside the fungal hyphae.

Within the thallus photobiont and mycobiont, cells must be placed in an intra cellular environment which provides access

to air and water and allows transport of substances between the partners. The relationship of fungal hyphae to photobiont and to air spaces and moisture within the thallus will vary greatly according to the partner species.

Homoiomerous thalli - the photobiont is distributed throughout the lichen thallus. This is a pattern associated with Nostoc and other cyanobacteria which are only active in a moisture saturated environment, but are often tolerant of low light intensities as in species of *Leptogium* and *Collema*.

Heteromerous thalli - have a distinct upper and lower surface with the photobiont layer concentrated in the upper layers where they are exposed to maximum light. This arrangement is characteristic of foliose species with *Trebouxia*. These species are often tolerant of extreme conditions of drought and high light intensities including UV radiation in situations where the photobiont can not survive. In the fruticose lichens the algal layer is arranged around a central core of chondroid tissue that may be solid or hollow.

The arrangement of the photobiont can be detected in the field by scratching or cutting through the thallus to show the position of photobiont layer or of cephalodia and medulla. The photobiont can also often be identified at a macro level by wetting the cut surface when cyanobacteria become blue-grey and chloroccoid algae are either green (Trebouxioid) or yellow (Trentepohlia).

Water uptake takes place mainly through the surface of the thallus, and there are several specialised features within lichens that are associated with this:

Cortex

The cortex is made up of rather specialised hyphal cells so that cortex thickness and structure is now used to define genera

of lichens. The use of this character has been extended by the use of SEM to investigate surface and cortex structure.

The surface may be smooth, scabrid or pruinose, or it may have pseudocyphellae - pore-like or crack-like aeration structures on the upper or lower surface. The cortex may be absent as in some homoiomerous crusts and foliose species such as *Collema* and *Physma* allowing rapid water uptake or loss from the unprotected surface. This is associated with the rapid swelling up of both genera when water is applied in the field distinguishing them from similar *Leptogium* species with a cortex on upper and lower surfaces that do not swell and become gelatinous.

The cortex may be one to several layers thick and influences the rate of water uptake in all species. In the Parmelioid genera, the cortex is well-developed on both surfaces, whereas in the Peltigeraceae, Cladoniaceae and foliose Physciaceae the lower cortex is frequently absent. In closely appressed or terricolous species this may allow water absorption directly from the substrate surface, so allowing rapid resumption of photosynthesis following wetting.

The structure of the cortex provides characters at all levels of taxonomy from family to species. It may be paraplectenchymatous with isodiametric cells as in Parmeliaceae or prosoplechtenchymatous with periclinal hyphae lying in the same plane as the surface as in *Heterodermia* (cut sections along length of lobe).

Internal transport of substances

Using techniques of cryofixation which immobilise thallus contents within milliseconds, water movements and drought tolerance of lichens have been investigated. Low Temperature SEM has demonstrated that extreme drought

tolerance of many lichen species is associated with the development of a hydrophobic cell wall in the aerial hyphae of the mycobiont and to the crystallisation of hydrophobic secondary compounds on the surface of the hyphae. Within Parmeliaceae the mycobiont hyphae contact juvenile *Trebouxia* cells at an early stage with the development of these substances around the *Trebouxia* cells protecting them from water loss and maintaining the gas-filled thalline interior where CO_2 uptake occurs via the cortex or via aeration pores (pseudocyphellae) (Honegger, 1991).

Rates of photosynthesis have been investigated using chlorophyll fluoresencence. Results show that *Trebouxia* containing thalli may be active at low water content and high relative humidity and that photosynthesis may be depressed in a water saturated thallus due to exclusion of air from interior spaces. Translocation of carbohydrates from the algal cells to the mycobiont has been investigated using inhibition techniques (Lawrey, 1986). This demonstrated that most material was translocated directly through the haustoria and not through the extracellular spaces of the thallus. Within lichens containing cyanobacteria, haustoria are not developed, but a gelatinous matrix develops around the fungal hyphae (Honegger, 1991).

Chemistry

Although the identification of secondary metabolites is now an established taxonomic tool in lichen systematics, the development of techniques for the identification of these substances has taken 60 years.

The use of lichens for dyeing cloth had been developed by the Greeks, and in the tropics *Parmotrema tinctorum* was widely collected for this purpose, but the chemical basis for this was not used in lichen taxonomy until Nylander developed the

now widely used spot tests using hypochlorite (C) and Potassium hydroxide (K), and distinguishing between reactions in the cortex and in the medulla of lichens (1865-66). Asahina introduced *para* phenylenediamine (PD) into spot tests (1934) and developed microcrystal tests to identify the chemical compounds found in lichen thalli (1936-1944). By 1954 Asahina and Shibata had demonstrated the structure and biosynthetic pathways of these substances.

The development of thin layer chromatography (TLC) by William and Chicita Culberson using 3 solvent systems and 2 internal controls, atranorin and norstictic acid is well known. Portions of the whole lichen were macerated in acetone and the solution spotted on glass or silica gel plates and placed in the solvent. Spots were identified by their R_f class and by additional characteristics using fluoresence under long or short wave ultraviolet light and spots developed using sulphuric acid charring. Further separation of identical spots was obtained using other solvents and 2-way chromatography. These methods are described in White and James (1985) with a table of lichen substances. The number of identified compounds grew rapidly and in 1988 Elix et al., produced a computer programme to identify known lichen compounds using six solvent systems and eight control compounds. This is now available as Mactabolites-2 (1993), and as a printed version of about 630 known compounds.

Lichen products are also suitable for analysis by high performance liquid chromatography (HPLC), which allows very accurate detection of substances using a UV detector. This method can also be used to detect absolute or relative concentrations of lichen compounds, because the peak intensity is proportional to the concentration. This has led to the discovery of many new compounds and has been used to more accurately

define specific characteristics within difficult genera in the tropics, such as *Pertusaria*.

Biosynthetic pathways

The majority of lichen substances are derived by the acetate-malonate pathway, including depsides, depsidones and dibenzofurans. These are produced by oxidative coupling of simple phenolic units. Pigmented compounds such as usnic acids, anthraquinones, xanthones and chronomes are also produced by this pathway but these are formed from intramolecular condensation of long folded polyketide chains. They are widespread throughout lichen families, particulary well developed in those with Trebouxioid photobiont.

The shikimic acid pathway produces two major groups of pigmented compounds- the pulvinic acid derivatives and the terphenylquinones. The requirement of nitrogen is unique among lichen chemical substances.

The mevalonic acid pathway produces terpenes and steroids. These include compounds that are unique to lichens or many shared with higher plants.

Location of compounds in the thallus

Cortical components include atranorin, usnic acid, vulpinic, parietin, lichexanthone and medullary components mainly include depsides and depsidones. The location of crystals on the medullary hyphae was first observed by SEM. The presence of secondary metabolites in apothecia was also observed e.g. *Haematomma*.

Ecological role of secondary metabolites

The majority of lichen substances are peculiar to lichens, and are not found in free-living fungi or algae indicating that they have a role in the symbiosis. Possible roles include 1. Light screen

compounds, 2. Hydrophobic compounds, 3. Allelopathic compounds including antibiotics, 4. Anti-herbivore defense compounds and 5. Chemical weathering compounds.

Light screen compounds

Lichens may be found in extreme conditions of temperature and UV radiation outside the range of free living photobiont cells. The presence of sunscreen substances in the cortex and medulla produced by the mycobiont can protect the photobiont cells from damage by high light irradiation. It has been shown that a reduction of light irradiance benefits *Trebouxia* which grows best at lower light intensities. It would appear that the development of chemical sunscreen compounds has enabled lichens to live outside the normal range of Trebouxioid algae. These sunscreen compounds are widely found in the cortex and in associated pigments (Rundel, 1978). The prevalence of lichexanthone and the anthraquinones in lichens of high altitudes indicates that there is also protection against UV irradiation.

Concentrations of light screening compounds such as usnic acid have been shown to vary linearly along light gradients. This may have a significant effect on the colour of the lichen so that those with more sun screen compounds may be yellower than their shaded counterparts (Rundel, 1978). Lichens frequently have long-lived apothecia that produce spores over several seasons. The frequency of red-fruited specimens with anthraquinones in the seasonally dry habitats of the tropics may protect the hymenium during seasonal droughts (Wolseley, 1997).

Hydrophobic compounds

Most of the secondary metabolites found in lichens are insoluble in water and are hydrophobic. Their deposition as

crystals on the aerial hyphae of the medulla may be important in maintaining air spaces within the medulla and preventing water saturation in the internal spaces (Honegger, 1991). It is well known that many leprarioid crusts that are not highly developed structurally contain hydrophobic compounds. In the field the hydrophobic character is obvious when water is applied to the thallus.

Allelopathic compounds

Lichen substances that are known to have an antibiotic affect include usnic acids, lichesterinic fatty acids and several orcinol depsides and depsidones. These substances have been shown to inhibit bacterial and pathogenic fungal infection (Härmälä et al., 1992), but also may play a role in many crusts in preventing over growth by other species of lichen or flowering plant. The inhibition of germination of flowering plant seedlings by gyrophoric, lecanoric and usnic acids has shown that the maintenance of lichen health may be a more active process than previously thought. The presence of terpenes in many species of water saturated conditions may reduce susceptibility to pathogenic fungal infection (Rundel, 1978).

Herbivory

Although lichens have not formed a major source of nutrients for larger animals other than caribou, there are numerous records of molluscs, larval stages of lepidoptera, and mites feeding on lichens. Pulvinic acid derivatives are known to be toxic e.g. vulpinic acid in *Letharia vulpina* has been used for poisoning animals as large as wolves. These compounds are found in the cortex where their protective role against grazing

herbivores would be effective. Other products which are known to inhibit predation include the terpenes and depsides such as protocetraric acid.

Chemical weathering

Lichens have been shown to be agents in metal complex formation associated with biochemical weathering. Compounds of copper with norstictic acid and psoromic acid have been ifdentified in lichens (Purvis, 1987) and a review of this subject is covered by Purvis (1996).

Reproduction of lichens

Sexual reproduction in lichens occurs only in the mycobiont. The spore produced by this process must germinate on a suitable substrate and acquire a photobiont at an early stage. This is a very risky operation for all symbiotic species and has not been demonstrated in nature for lichens. In contrast vegetative propagules are a mixture of both symbionts and allow rapid colonisation of suitable substrates.

Vegetative propagules

Soredia -spherical aggregations of hyphal and algal cells 20-100 m in diameter produced within soralia.
Isidia - finger-like outgrowth from the cortex.
Phyllidia / schizidia - leaf-like outgrowths from the thallus.

It was assumed that sexual form is primitive and that recombination cannot take place in asexual forms, and therefore that genetic diversification including chemical pathways and products must be established in sexual forms prior to

development of asexual form, or as a mutant (Poelt, 1970; Culberson et al., 1973).

ngbmAncestral forms using fungal sexual mechanism with same chemistry as asexual forms with vegetative diaspores have found that asexual states would have evolved once and are monophyletic. Recent work on ITS sequences of DNA of *Dendrographa leucophaea* and *D. alectoroides* has shown that asexual forms are widely different and not monophyletic, suggesting that the sterile clones were formed from sexual specimens at different places and times in the evolutionary history (Lohtander et al., 1998).

Lichenological Studies in Sri Lanka

Sri Lanka has very rich plant diversity and includes plants that belong a variety of taxonomic groups. Out of these, lichens are a highly specialized and ubiquitous group as they have the ability to adapt to extreme environmental conditions, which enabled them to become pioneers of vegetation. However, little work has been done on lichens in Sri Lanka and thus knowledge with regard to diversity and distribution in Sri Lanka is rather incomplete.

G. H. K. Thwaites, superintendent and later director at the Botanical Garden at Peradeniya, made the first collection of lichens in central highlands of Sri Lanka between 1849 to 1880. This collection was studied and described by Leighton (1869) who was able to identify 196 lichen species. Of them 44 species were new to science including many Graphidaceae and Thelotremataceae. Then Almquist, the famous Swedish explorer collected lichens at Peradeniya area in 1879. This collection was sent to Nylander for identification on the basis of "Lichens Ceylonenses" in 1900. A.G.H. Alston collected few lichens in 19261931. He wrote a Kandy flora, a supplement to Trimen's

handbook, and unpublished taxonomic treatments of Ceylon Bryophytes, Algae and Lichens. Kurokawa and Mineta collected mainly in montane forest in 1966/68 contributing to *Anaptychia* (Kurokawa, 1973) and family Parmeliaceae (Kurokawa & Mineta, 1973). Another lichen collection has been done incidentally under the Smithsonian Institution Flora of Ceylon Project from 19701976, mostly by the Louis Wheeler in drier lowland areas. Then Rolf Santesson and Roland Moberg visited to Sri Lanka in 1975, and those together collected about 10 specimens of Thelotremataceae, mostly in the Horton Plains area.

During 19761978, Hale collected lichens from canopies of virgin Dipterocarp trees being logged in Sinharaja forest. At that time he was able to add 76 species to the family Thelotremataceae and 4 additional species of *Relicina*. Following a botanical excursion from the University of Vienna in 1984, Brunbauer compiled an account of the literature on lichens in Sri Lanka in 15 articles (Brunbauer 19841987), including 546 species and their synonymy at that time. This includes 550 species belonging to 122 genera and 48 families.

In 1986, Moberg describe a new lichen genus *Rolfidium* in the family Bacidiaceae. Further publications by Singh (1990), Awasthi (1991), Makhil and Patwaradhan (1992), described some microlichen genera including *Buellia* and *Diplotomma*, Macolichens and *Trypethelium* species respectively. In 1997, Breub described 54 new lichen species reporting from central and Southern part of Sri Lanka. In the same year, Vezda described 53 folicolous lichens from Sri Lanka. Among them, 32 species were new to Sri Lanka. Above all the literatures have brought the lichen number up to 659 species recorded to Sri Lanka.

Then in 1999, preliminary survey of lichen conducted during first lichen workshop at Peradeniya University and participants were able to collect 98 of different types of lichen

specimens (unpublished data). All these specimens are kept in the National Herbarium, Peradeniya. Other than that Jayasooriya recorded 17 species of lichen during his study of the flora of Ritigala and those specimens also kept at the National herbarium (Jayasooriya, 1984).

During 19992003, Chandrani Wijerathne surveyed lichens flora at Ritigala Mountains and its vicinity. During their survey, they were able to describe 35 new lichen species to Sri Lanka (unpublished dataNSF report 2004). In 2001, Orange et al. described two new additional lepraioid lichens to Sri Lankan lichen flora. In 2003, Nayanakantha and Gajameragedara described about 50 lichen species collected from Kandy municipal region.

In 2006, M.K. Karunarathne carried out a survey in five ecoregions in Sri Lanka and they were able to find out 23 genera of lichens. In our survey at Horton Plains National Park Sri Lanka during 2004 to 2006, total of 1515 specimens of macrolichens belonging to 13 families 48 genera and 293 species were identified. Amongst them, 4 genera were new to Sri Lankan lichen flora. According to the literature regarding the Sri Lankan lichens, 696 species are already recorded. However, this number should be exceeded 1500 according to the unpublished data and ongoing research findings.

Lichenological and air sampling studies in Horton Plains National Park

A total 379 lichen species belonging to 67 genera and 24 families were identified. This study documented several new lichen taxa for Sri Lanka that consist of 11 genera and 102 species. The 12 new genera were composed of 7 folioses (*Fuscopannaria, Leioderma, Anzia, Canomaculina, Cetrelia, Canoparmelia, Menegazzia*), 4 crustoses (*Punctelia,*

Pleurotrema, Parathelium, Pleurotheliopsis) and 01 fruticose (*Polichidium*) lichen genera. Similarly the 102 species were composed of 11 species of crustose, 89 species of foliose and 03 species of fruticose. A principal co-ordinate analysis (PCA) of site data, tree data and lichen cover shows that most of the lichens are highly correlated with the bark pH and light demanding crustoses such as Thelotremataceae, Pyrenulaceae are positively correlated with the high light intensities. Most of the lichens present in the study plots preferred relatively high pH range (5.0-6.0). The most dominant lichen species (314 species) within this pH range were macrolichens like *Lobaria retigera, Pseudocyphellaria beccarii, Heterodermia microphylla* and microlichens like *Graphis sp. Myriotrema sp.* like microlichens. The number of lichen colonies increase with increase diameter class from 5-10 cm to 11-20 cm. The highest number of lichen colonies was found on hosts of 11-20 cm diameter class in both forest types. It was clearly observed that the number of lichen colonies decrease with increasing diameter size of the lichen host species. According to the observations smooth bark type had the highest diversity of lichens in both forest types and this was followed by smooth to rough bark type. The least lichen diversity was observed on deeply furrowed bark type in the continuous forest, while in the forest islands, the least diversity was observed on flaky bark type.

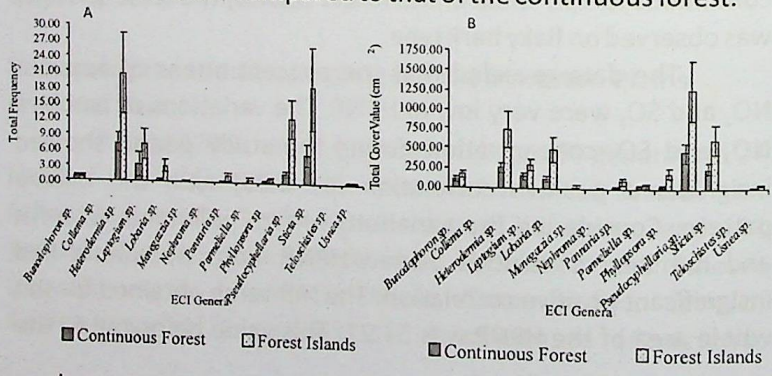
The data revealed that the concentrations of ambient NO_2 and SO_2 were very low in HPNP. The variations of ambient NO_2 and SO_2 concentration during the study period showed insignificant positive correlation ($p \geq 0.05$) with the rainfall pattern. Considering the variations of two pollutants with RH and number of vehicles visited HPNP, both pollutants had insignificant positive correlation. The IAP value obtained for the whole area of the HPNP was 54.22. This value belonged to the

quality level 5 which represent the 'very low' pollution level. The results including lichen data and air quality data could confirm that the ambient air quality at HPNP is very high.

Ecological continuity of Horton Plains National Park

Ecological continuity (EC) means the time span a forest habitat requires to reach the successional stage of dynamic equilibrium (Wu and Loucks, 1995). In the tropics, lichens have been used as bioindicators to predict the environmental changes in forests of Thailand (Wolseley and Aguirre-Hudson, 1997a, 1997b). Peterken (1974) and Rose (1974) have found that presence of some vascular plants and lichens in recent woods are different from those present in ancient woods which are continuously wooded since 1600 A.D. In considering the Horton Plains National Park (HPNP), the forest area declines ending around 3,600 cal yr BP. and the growth of HPNP fluctuated from that time onward (Premathilake and Risberg, 2003).

Total frequency of Ecological Continuity Indicator (ECI) genera in continuous forest and forest islands were shown in figure 1A. All the ECI genera occurred at a higher total frequency in forest islands. Total frequency values of *Heterodermia*, *Lobaria*, *Pseudocyphellaria* and *Sticta* were significantly higher in the forest islands compared to that of the continuous forest.



A) Total frequency B) Total cover value, of Ecological Continuity Indicator (ECI) genera in continuous forest and forest islands in HPNP.

Total cover value of ECI genera in continuous forest and forest islands were shown in figure B. All the ECI genera occurred at a higher cover value in forest islands as well (figure B). The genus *Pseudocyphellaria* showed the highest total cover value in the forest islands as well as the continuous forest. More than 50% of the ECI genera showed significantly higher cover values in the forest islands. Although the cover values of remaining genera were not significant they were considerably higher when compared to that of ECI genera present in the continuous forest.

Parameters such as average host tree height, average host tree diameter and average light intensities were not significantly different between the two forest types. However, host bark pH was significantly different ($p \leq 0.05$) between both forest types. Macrolichen species, *Parmeliella* sp.1, *Parmeliella papillata*, *Phyllopsora buettneri*, *Leptogium* sp. 4 and *Pseudocyphellaria beccarii* were found as possible ECI species in HPNP.

Continuous forest supports fewer macrolichen genera at lower frequency and cover than forest islands. The higher values of such parameters indicated that, ecological continuity is better preserved in forest islands than the continuous forest. According to the Wolseley and Aguirre-Hudson (1997a), use of lichen taxa frequency allows them to assess rates of change when occurring environmental continuity and deterioration in tropical forests. They also found that species rich and fire tolerant lichen communities associated with ancient forest of the Dry Dipterocarp Forest in Northern Thailand.

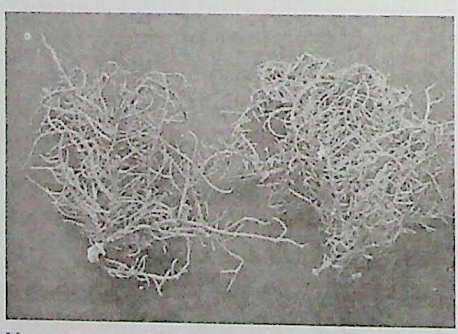
Considering the hosts bark pH, it may temporary increase from 6.3 to 6.7 within one year due to the severe burning in some forest of Thailand. However, the increased pH

may be reversed during the rainy season (Wolseley and Aguirre-Hudson (1997b). This phenomenon may not be applicable for the host plants present in forest islands due to the lack of forest fires during last few decades. This may be a result of less amount of leaching experienced by trees with large diameter having larger crowns. When the crown is large, it facilitates draining the rain away from main trunk (Kermit *et al.*, 2001).

The ECI lichen species determined in this study will be useful in predicting the ecological continuity of forest in HPNP. According to the Kooch *et al.*, (2008) this type of ecological analysis have been carried out in different countries. Considering all the above facts, we can conclude that the ecological continuity is highly preserved in forest islands of the HPNP and five macrolichen species were identified as ECI species from the studied area.

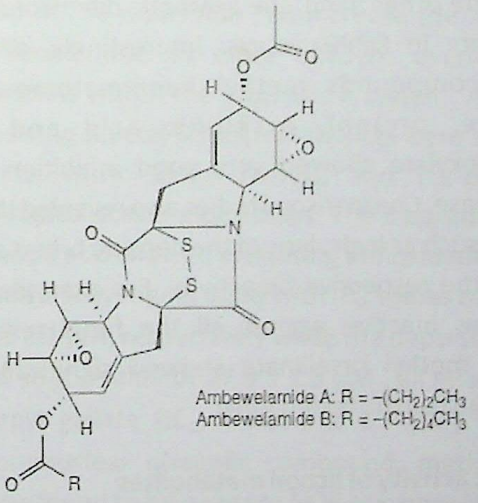
SEARCH for drugs

While biologists are primarily interested in studying the natural habitat and its organisms, chemists have their eyes on the pharmaceutical value of the organic compounds isolated from natural organisms. In our search for anti-cancer compounds we investigated lichens growing in a mountainous region of the central province of Sri Lanka, which is an area rich in plant life. In Ambewela (1898 m above sea level) we encountered the lichen *Usnea sp.* growing on the decaying branches of an acacia tree.



Usnea, a type of lichen growing on the dead bark of an acacia tree (*Acacia decurrans*).

Samples of the lichen were collected, washed with water, air-dried and then dichloromethane (CH_2Cl_2) was used to extract compounds from the lichen. The extract was tested for antifungal activity against the fungus *Cladosporium cladosporioides*. We also examined the effect of the extract on the larvae of yellow fever mosquitoes (*Aedes aegypti*), which carry the viruses of several devastating human diseases. We were able to isolate two active compounds responsible, which we named ambewelamide A and ambewelamide B which are new members of a family of highly modified diketopiperazines and constitute the first examples of this family of compounds isolated from a lichen (Williams et al., 1998). Ambewelamide A was found possess potent in vitro cytotoxicity (murine leukemia P388: IC50 8.6 ng/ml) and significant in vivo antineoplastic activity (P388: %T/C 140 @ 160 mg/Kg). This is the most potent anticancer compound isolated from any Sri Lankan source.



Structure of ambewelamide A and ambewelamide B

Semisynthesis and bioactivities of lichen substances

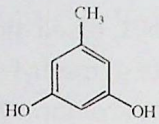
In an attempt to isolate compounds from natural sources, three lichens, namely *Parmotrema grayana*, *Cladonia* sp. and *Heterodermia obscurata* were chemically investigated. Isolated compounds were subjected to various bioassays. Chemical investigation of these lichens led to the isolation of atranorin , usnic acid, divaricatic acid, methyl haemmatommate, methyl orsellinate, orcinol, orsellinic acid, lecanoric acid, zeorin, methyl-β-orcinolcarboxylate, lobaric acid, and sekikaic acid. Structure-reactivity relationship of lichen compounds in various bioassays revealed that depsides and depsidones showed good antioxidant activity in SOI due to the extended conjugation of such compounds. Thus lichens have natural mechanisms or components to combat oxidative stress, which is probably why they we have shown very promising antioxidant activities in SOI assay. On the other hand, the synthetic dibenzofurans showed good activity in DPPH assays. Interestingly, all the simple aromatic compounds methyl haemmatommate, methyl orsellinate, orcinol, orsellinic acid and methyl-β-orcinolcarboxylate showed very good inhibition against the enzyme urease. Comparison studies also revealed that by simple conversion such as hydrolysis of the depside brings about drastic change to the respective bioactivity. For example, compound erythrin was inactive against all the fungi tested whereas compound methyl orsellinate showed significant antifungal activity against all the tested fungi.

Antioxidant activity of lichen metabolites

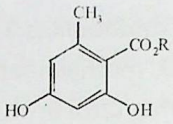
In continuation of our search for biologically active compounds from tropical lichens, the comprehensive

antioxidant activity of the metabolites **1-14** (orcinol **1**, orsellinic acid **2**, methyl orsellinate **3**, methyl haematommate **4**, methyl--orcinolcarboxylate **5**, montagnetol **6**, *para*-depsides atranorin **7** lecanoric acid **8**, divericatic acid **9**, erythrin **10**, a *meta*-depside sekikiac acid **11**, the depsidone lobaric acid **12**, the ubiquitous dibenzofuran (+)- usnic acid **13** and a triterpenoid, zeorin **14)** representing several classes of low molecular weight lichen substances isolated from four lichen species namely ***Parmotrema grayana*** *Hue*, ***Cladonia sp., Heterodermia obscurata*** (Nyl.) Trevisan**, and *Roccella montagnei*** *Bel.* ***and*** two derivatives of compounds **8** and **10** ***are reported.***

Owing to the chemical structures of these *p*-substituted polyphenolic compounds **1-14** they would be interesting candidates for their evaluation of antioxidant potentials, as it is been reported by Burton *et al.* (1985), that bridging of the phenolic group in the *p*-position can increase the antioxidant activity of phenols due to more efficient overlap of the substituent orbitals within the aromatic π system. Antioxidant activity of following several lichen metabolites were assessed in the superoxide, nitric oxide radical, and 2,2-diphenyl-1-picrylhydrazil radical scavenging assays. The despsides sekikaic acid and lecanoric acid showed promising antioxidant activity in superoxide radical scavenging assay with IC_{50} values of 82.0 ± 0.3 μM and 91. 5 ± 2.1 μM, respectively while the depsidone lobaric acid exhibited an IC_{50} value of 97.9 ± 1.6 μM , all relative to the standard, propyl gallate (IC_{50} = 106.0 ± 1.7 μM). The most abundant mononuclear phenolic compound, methyl--orcinol carboxylate was found to be a potent NOR scavenger (IC_{50} = 84.7 ± 0.1 μM), compared to the standard rutin (IC_{50} = 86.8±1.9 μM**).**



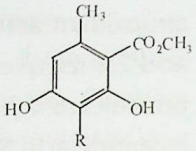
1



R = H **2**

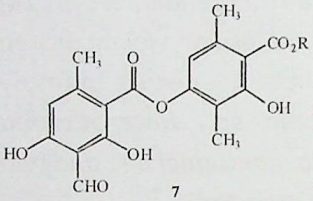
R = CH_3 **3**

R = $CH_2CH(OH)CH(OH)CH_2OH$ **6**



R = CHO **4**

R = CH_3 **5**



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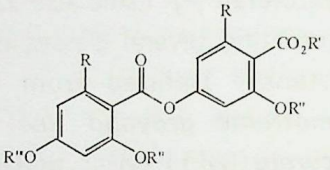


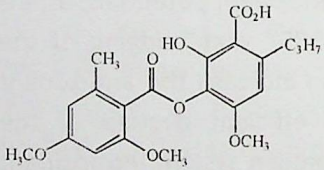
R = CH_3; R'=R''=R'''= H **8**

R = R' = R'' = R''' = CH_3 **15**

R = C_3H_7; R'=R'''= H; R''=CH_3 **9**

R = CH_3; R' = $CH_2CH(OH)CH(OH)CH_2OH$; R'' = R''' = H **10**

R = CH_3; R' = $CH_2CH(OH)CH(OH)CH_2OH$; R''' = R'' = CH_3 **16**



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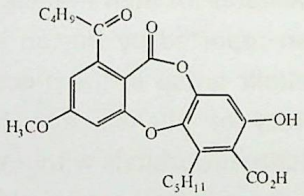

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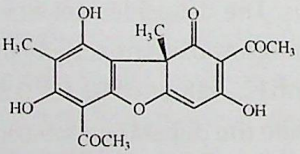

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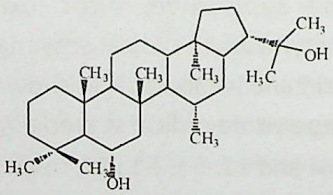


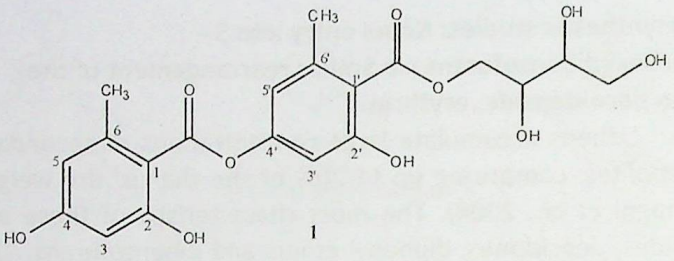
14

Semisynthestic studies: Novel entry into 5-decarboxydibenzofurans via Smiles rearrangement of the lichen *para*-depside, erythrin

Lichens accumulate large concentrations of secondary metabolites, comprising up to 20% of the thallus' dry weight (Romagni *et al.*, 2004). The most characteristic of these are depsides, depsidones, diphenyl ethers and dibenzofurans. The depsides and to some extent the depsidones are isolated as major constituents. However, diphenyl ethers and dibenzofurans is relatively rare (Huneck and Yoshimura, 1996). Biosynthetically, depsides are considered to be precursors of dibenzofurans through the intermediacy of diphenyl ethers (Elix *et al.*, 1987) Following this biosynthetic hypothesis, the *para* depside erythrin **1**, isolated in 7.6% yield from *Roccella montagnei* Bel., was successfully converted to its diphenyl ether **2** *via* Smiles rearrangement 9 (Truce *et al.*, 1970). Smiles rearrangement had been used previously on synthetic *para* depsides, assembled from simple aromatic units, where one such attempt led to a total synthesis of pannaric acid (Elix and Parker, 1987). However, prior to our studies, Smiles rearrangement had not been tested on naturally occurring *para*-depsides nor on depsides with unprotected 4-hydroxy group.

Compound **2** underwent transesterification upon refluxing in methanol and base to form the diphenyl ether **3** which, on permethylation with methyl iodide in presence of NaOH in DMSO, yielded the diphenyl ether **4**.

Interestingly and importantly, Palladium (II) acetate mediated oxidative coupling (Shiotani and Itatani, 1976) of diphenyl ethers **2** and **3** containing a free carboxylic acid at C-1 provided the dibenzofurans **5** and **6**, (65% and 77 %, respectively), where the structural evidence (^{13}C and Mass) indicated that the carboxyl group had been lost during the coupling.



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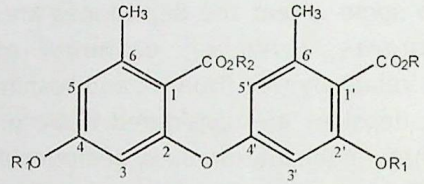
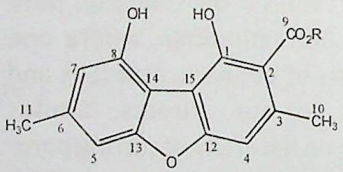
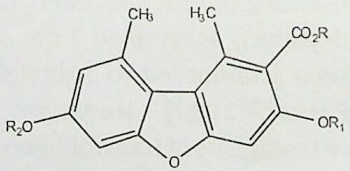


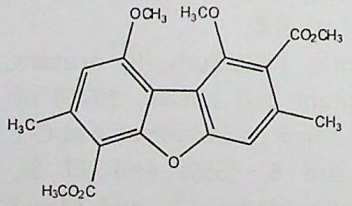
R = -$CH_2CHOHCHOHCH_2OH$, $R_1 = R_2 = H$ **2**
R = CH_3, $R_1 = R_2 = H$ **3**
$R = R_1 = R_2 = CH_3$ **4**



R = -$CH_2CHOHCHOHCHOHCH_2OH$ **5**
R = CH_3 **6**



$R = R_1 = R_2 = H$ **7**
$R = R_1 = H, R_2 = CH_3$ **8**
$R = H, R_1 = R_2 = CH_3$ **9**
$R = R_1 = R_2 = CH_3$ **10**



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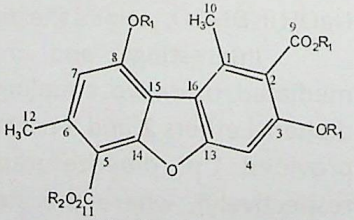
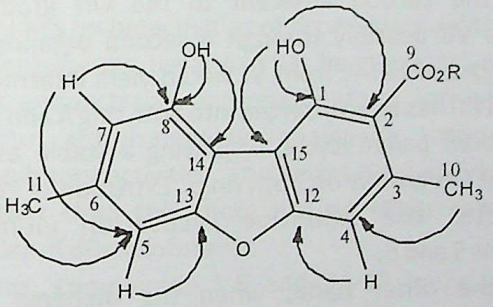


$R_1 = R_2 = CH_3$ **12**
$R_1 = R_2 = H$ **13**

Structures of compounds 1-13

Four structural analogues of compounds **5** and **6**, namely hypostrepsilic acid **7**, 6-*O*-methylnorascomatic acid **8**, ascomatic acid **9** and methyl ascomate **10**, were reported in 1994 by Elix *et al.* as minor constituents from the lichen *Bundophoron patagonicum*, where, C-1 and C-8 carried CH_3 groups whereas C-3 and C-6 had OH groups. These are the only examples of naturally occurring 5-decarboxydibenzofurans isolated from lichens. Additionally, Tanahasi *et al.*(2001) have reported four other decarboxylated dibenzofurans including several chlorinated ones isolated from cultured micobionts of the lichen *Lecanora cinereocarnea*. The NMR data and HMBC of compounds **5** and **6** confirmed the reversal of the CH_3/OH substitution pattern in compounds **7**-**10**, where the two OH groups were at C-1 and C-8 and CH_3 groups were at C-3 and C-6 thus establishing that they are members of a new class of dibenzofurans.

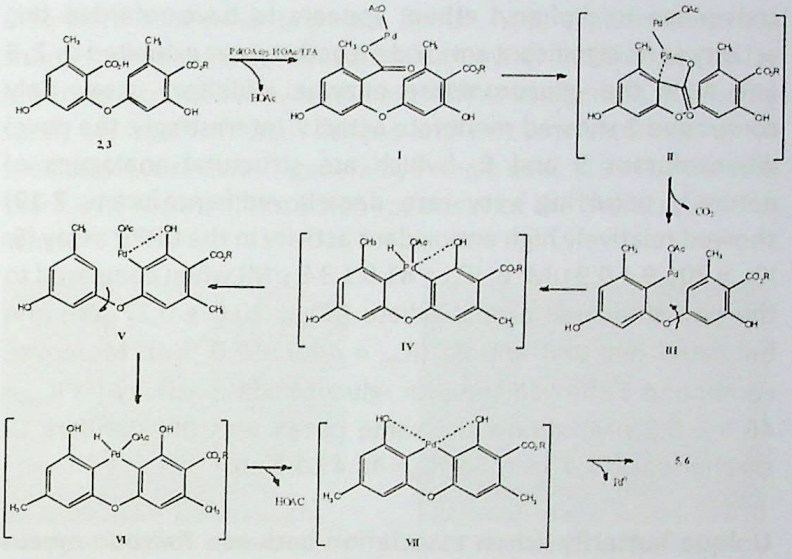


Selected HMBC of 5 (R = -$CH_2CHOHCHOHCH_2OH$), and 6 (R = CH_3)

This novel Pd (II) acetate mediated decarboxylation followed by two through-space palladium migrations prior to oxidative coupling is represented in scheme 1. Invoking a model proposed recently by Tanka *et al.*(2005) for Pd (II) assisted

decarboxylation in simple aromatic systems, we propose that the decarboxylative palladation of the acetato palladium (II) species **I** proceeds via the formation of a four-membered palladacyclic intermediate **II** (Tanka et al.,2005) in which the electrophilic Pd (II) is bonded to the carboxylate oxygen and the *ipso*-carbon of the aromatic ring. Loss of carbon dioxide follows the insertion of palladium to the aromatic ring, giving intermediate **III** which would then undergo Pd migration from ring A to B at the carbon adjacent to the OH group, possibly through an organopalladium (IV) hydride **IV** (Canty, 1992; Zhao and Larock, 2006) giving rise to the intermediate **V**. Although such an Pd (IV) intermediate has not been reported, other organopalladium (IV) species are well known (Tanka et al., 2005) These fairly general through-space migrations of Pd have been reported between vinylic to aryl (Larock and Tian, 2001), aryl to aryl (Campo *et al.*, 2003), alkyl to aryl (Huang et al., 2004), and vinylic to aryl to allylic (Zhao et al., 2005). Intermediate **V** then might undergo a second Pd through- space migration back to ring A (at the carbon adjacent to the OH group) giving intermediate **VII** possibly through a second organopalladium hydride **VI**. Loss of HOAc from **VI** would yield intermediate **VII** where the Pd (II) has been inserted into both ring A and B forming a six-membered palladocycle, displaying a stable association with adjacent OH groups on both rings. Extrusion of Pd (0) from **VII** completes the oxidative cyclisation yielding the dibenzofurans **5** and **6.**

On the other hand, when the diphenyl ether **4**, containing an ester group at C-1,was treated with Pd (II) acetate, it led to a mixture of dibenzofurans **11** and **12** (corresponding to equally facile coupling between C-3 of ring A and C-3' or C-5' of ring B) in 1:1 ratio. The latter dibenzofuran **12,** obtained in 12% overall yield from erythrin **1** in four steps, has been converted to pannaric acid **13** (Elix and Parker, 1987).



Possible mechanistic pathway of the decarboxylatve oxidative cyclisations of 2 and 3

Studies on bioactivities of lichen metabolites are scarce. As for dibenzofurans, usnic acid has been extensively studied. It possesses antibacterial, anti-proliferative, anti-inflammattory, anti-tumour, anti-mutagenic, analgesic, anti-pyretic plant growth inhibitory activities, and insecticidal activities (Huneck, 1999). **Other than this there are no other reports on the bioactivities of dibenzofurans.**

Thus, compounds **1-6** and **11-12** were subjected to antioxidant activity in the Super Oxide inhibition (SOI) assay and DPPH radical scavenging assay and enzyme inhibitory activity against -Glucuronidase.

Bioassay results showed that erythrin **1,** was a good SOI inhibitor with a IC_{50} value of 127.1 ± 0.1 µM, comparable to the standard propyl gallate (IC_{50} = 106.0 ± 1.70 µM). However, its

conversion to diphenyl ethers appears to have retarded this activity as no significant antioxidant activity was exhibited by **2**, **3** and **4**. In the -glucuronidase enzyme inhibitory assay, only compound **2** showed moderate activity. Interestingly, the novel dibenzofurans **5** and **6**, (which are structural analogues of naturally occurring very rare decarboxydibenzofurans **7-10**) showed relatively high antioxidant activity in the DPPH assay (**5**: IC_{50} = 201.9 ± 0.9 µM; **6**: IC_{50} = 81.9 ± 3.8 µM) when compared to the two standards propyl gallate (IC_{50} = 30.0 ± 0.27 µM) and butylated hydroxyl anisole (IC_{50} = 44.0 ± 2.0 µM). Moreover, compound **5** showed superior -glucuronidase activity (**5**: IC_{50} = 46.9 ± 0.2 µM) almost the same potency as the standard D-saccharic acid 1,4- lactone (IC_{50}= 48.4 ± 1.3 µM).

Unique butterfly-lichen association between *Talicada nyseus nyseus* And *Leproloma sipmanianum*

Many species of invertebrates live on and among lichens, using them for concealment, shelter and/or food (Gerson & Seaward, 1977). Other organisms known to consume lichens include orbit mites (Syed & Seaward, 1984) and terrestrial gastropods (Baur et al. 1992). Moths of the family *Arctiidae* are well known lichen feeders and Hesbacher *et al.* (1995) have shown that lichen phenolics such as parietin, atranorin and the hydrolytic cleavage product of atranorin (methyl-2,4-dihydroxy-3,6-methylbenzoate) were detected in 11 different species. However, Pöykkö *et al.* (2003) showed that members of the Arctiidae preferrentially grazed on lichens that did not contain polyphenolic substances, and that these substances affected growth rate and survival of the larvae. Removal of secondary metabolites from the lichen also affects the food choice and survival of lichenivorous moth larvae (Pöykkö et al. 2005). Interestingly, females and larvae of the geometrid moth

Cleorodes lichenaria prefer a lichen host that assures the shortest larval period (Pöykkö, 2006). Gowan and Dickson (1971) reported 6 species of Lycaenid butterflies in 4 genera as feeding solely on 'rock' or 'tree' lichens and this was extended to 14 species of Lycaenid in 7 genera by Pringle et al. (1994). However there is no record of the lichen species or of the chemistry of the lichen.

T. nyseus on lichen *L.sipmanianum*

T. nyseus on *K. pinnata* leaf

Recent research in Sri Lanka has shown, for the first time, an association of a widespread butterfly *Talicada nyseus nyseus* Guerin-Meneville (Red Pierrot) with a leprose lichen *Leproloma sipmanianum* Kummerling & Leukert at Beragala, (80º 54' 30" E, 6º 45' 30" N), growing on rock below Horton Plains, Uva Province, where the natural food plant of the butterfly is *Kalanchoe pinnata* (Lam) Pers. The larvae have been found feeding on the host plant and on lichen growing on extensive adjacent rock surfaces. Karunaratne *et al.* (2002) have demonstrated that lichen products found in *Leproloma sipmanianum* are also present in the wild caught imagines, including zeorin **1**, β-sitosterol **2**, the fatty acid ester tritetracontylpentanoate **3**, atranorin **4** and (+)-usnic acid **5**.

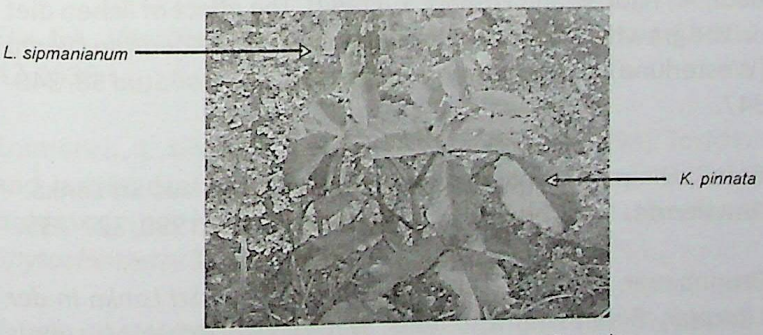
In our present study, we conducted a series of experiments to confirm the presence of lichen compounds in a population of the butterfly *T. nyseus nyseus*, by High Performance Liquid Chromatography (HPLC). In order to

determine the stage of entry of lichen compounds into the butterfly, adults, larvae, pupae and larval waste were subjected to chemical analysis.

In order to determine the effects of lichen compounds on the life cycle of the butterfly and of the progeny, in separate experiments, wild caught adults were caged in pairs in the presence of the host plant and their larvae reared under three different feeding regimes including the host plant *K. pinnata* and the lichen *L. sipmanianum*.

The butterfly *T. nyseus nyseus* is widespread throughout Sri Lanka, being equally abundant in gardens and in the wild (Woodhouse, 1952) where the food plant *K. pinnata* is also widely available and frequently planted. The adults of *T. nyseus nyseus* feed on nectar from the bushes of *Lantana camara* (Singh, 2005) growing freely in the same location. In contrast, *L. sipmanianum* has only been recorded at altitudes above 1000m in Uva Province (Orange *et al.* 2001), so is not available to the butterfly over most of its range. As such, when reared at lower altitudes on *Kalanchoe* species the larvae and butterfly do not contain lichen products. However the experiments on feeding regimes and supplementary diet show that the life cycle and growth rate of *T. nyseus nyseus* is strongly affected by the nutritional value of the diet, as demonstrated for moths by Pöykkö et al. (2003) where growth rate and survival are adversely affected by the presence of lichen compounds. Pöykkö et al. also showed that the effect is proportional to the amount of lichen compound ingested. During the period of population sampling at Beragala the butterflies sampled all contained the specific lichen products atranorin, usnic acid, 3,6-dimethyl-2-hydroxy-4-methoxybenzoic acid and zeorin, suggesting that this population of *T. nyseus nyseus* were regularly feeding on *L.* sipmanianum in combination with *K. pinnata*. Both atranorin

and usnic acid have been shown to have toxic and adverse effects on the growth and development of generalist herbivores (Emmerich *et al.*1993, Lawrey 1986, Romagni *et al.* 2004). While this paper has shown that lichen compounds in *L. sipmannianum* had deleterious effects on larvae fed only on the lichen, larvae fed on *L. sipmanianum* together with the food plant were healthy. Lichen products pass through the larvae of *T. nyseus nyseus* and enter the adult butterflies with no apparent adverse effect on their life cycle, lending credence to the beneficial effects of a host plant/lichen diet. Regular sampling of the population throughout the butterfly season at Beragala showed that lichen compounds were present in all butterflies sampled at this site, suggesting that the larvae are regularly feeding on *L. sipmannianum* together with *K. pinnata*. It is well known that Monarch butterflies (Nymphalidae) accumulate toxic cardiac glycosides in milkweeds, which are used by the adults to deter predators (Reichstein *et al.* 1968), and we suggest that the presence of toxic products in the larvae and adult butterfly of *T. nysseus nyseus* may deter predators. Further observations on predators of this population of *T. nysseus nyseus* in upland Sri Lanka are required to elucidate this hypothesis.



K. pinnata growing beside *L. sipmanianum* in Beragala.

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