

An association between the butterfly *Talicauda nyseus* and the lichen *Leproloma sipmanianum* as evidenced from chemical studies

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Lichens produce a variety of lichen products having a wide range of biological activities. Not surprisingly, it has been found that snails and some insects, particularly moths (Lepidoptera) of the family Arctiidae use lichen phenolics for defence purposes. On the other hand, members of the butterfly family, Lycaenidae (e.g. *Talicauda nyseus*) are considered plant feeders. Monarch butterflies (Nymphalidae) accumulate toxic cardiac glycosides in milkweeds, which are used by the adults to deter predators. In this communication we report the occurrence of common lichen products, including phenolics in the butterfly *T. nyseus*.

LICHENS are symbiotic organisms of fungi (mycobionts) and algae (photobionts) comprising about 17,000 species recorded worldwide¹. They commonly grow on rock surfaces, poorly developed soils such as those found in arid lands and boreal–arctic regions, and as epiphytes on trees and shrubs². In habitats where the amount of available nutrients is limited, lichens may become the dominant flora, thus providing an important potential source of food for herbivores. Lichens accumulate large concentrations of products, particularly aromatic phenolic compounds, not found in other plants (sometimes exceeding 20% of dry weight). The majority of these compounds originate from the fungal symbiont. The general resistance of lichens to insects and microbial attack is attributed to the presence of lichen products³.

Recent experiments using polyphagous larvae of the insect *Spodoptera littoralis* (Noctuidae) have shown acute toxicity and feeding deterrence for common lichen compounds such as (+)- and (–)-usnic acid and vulpinic acid at concentrations found in lichens³. When added to the larval diet of *S. littoralis*, lichen compounds such as oxyphysodic acid and fumarprotocetraric acid prolonged

the larval period and elicited malformations of imagines⁴. Although lichen products show antifeedant and toxic effects toward generalist herbivores, there are specialized lichen feeders such as oribatid mites⁵ and terrestrial gastropods⁶. Hesbacher *et al.*⁷ have shown that lichen phenolics such as parietin and atranorin are accumulated by 11 moth species of the family Arctiidae (Lepidoptera), and they may be used in defence. The same two phenolics are stored by the lichen-feeding snails, *Balea perversa* and *Chondrina clienta*⁸.

In the present study, we report sequestration of lichen products belonging to the crustaceous lichen *Leproloma sipmanianum* Kümmerl & Leuckert by the butterfly *Talicauda nyseus* Guérin (red pierrot) (Lycaenidae) in Beragala (80°54'30"E, 6°45'30"N), Uva Province, Sri Lanka. *T. nyseus* was found flying close to the extensive lichen thallus, growing on roadside proterozoic rocks of gneiss and quartz schist which are exposed at road edges, and roosting on it periodically (Figure 1). Pupae of the butterflies were also located anchored to the thallus surface.

T. nyseus is distributed in Sri Lanka and India. The Sri Lankan population belongs to the nominotypical race, which flies throughout the island⁹. In *T. nyseus* the sexes are similar, the female being slightly larger and paler, with rounder wings. The butterfly is found close to human settlements as well as forested areas. The larvae of *T. nyseus* are onisciform, pale pink, with a row of dorso-lateral black spots and covered all over with short hairs. The natural food source of the larvae is *Bryophyllum calycinum* (Sinhala: akkapana; Tamil: malai-kalli), where the larvae live within its juicy leaves (Figure 2). The female butterflies oviposit on the plant. Eggs hatch and the larvae eat their way into the laminae of the leaf. The pupae, which emerge, are indistinguishable from the larvae, but are fixed by tail and girdle.

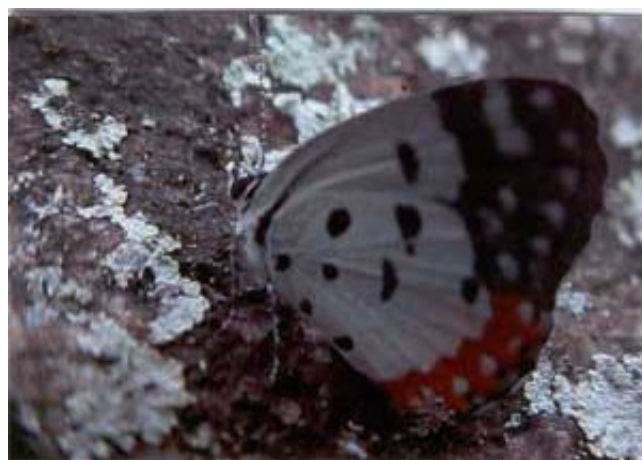


Figure 1. *Talicauda nyseus* butterfly landing on *Leproloma sipmanianum* lichen.

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L. sipmanianum is a persistently sterile leprose lichen belonging to the genus *Lepraria*, which often forms the conspicuous taxon in temperate regions in microhabitats sheltered from direct rain. It is restricted to montane habitats and is rare in lowland tropical rainforests^{10,11}. The algal partner of *L. sipmanianum* is green and cells are more or less spherical. The lichen does not produce ascomata and conidiomata. *L. sipmanianum* has previously been reported from South Africa, Columbia and Brazil¹². The lichen was first reported from Beragala in the Asian region¹¹.

L. sipmanianum was collected in plastic bags by scraping the rock surface with a blade. Four adult butterflies and four pupae of *T. nyseus* were also collected from the above site in September 1996. A small portion of the lichen thallus was extracted into dichloromethane. The adult butterflies, and also those which emerged from the pupae (after five days in the laboratory), were extracted into dichloromethane. Following drying under reduced pressure, all extracts were subjected to Thin Layer Chromatography (TLC, eluent: dichloromethane); the extract of *L. sipmanianum* showed five major spots. Upon comparison, the extracts of *T. nyseus* (both collected adults and those emerging from pupae) contained the same compounds. In order to isolate and characterize these compounds, the air-dried lichen *L. sipmanianum* (130 g) was sequentially extracted into *n*-hexane (2.5 l) and dichloromethane (2 l) at 27°C for 24 h and concentrated under reduced pressure. The *n*-hexane extraction yielded a brownish, gummy extract (0.53 g) and dichloromethane extraction gave a yellow, gummy extract (2.0 g).

The *n*-hexane extract upon Medium Pressure Liquid Chromatography (MPLC) on silica gel (eluent: step gradient from *n*-hexane to 20% methanol/dichloromethane) provided several fractions. The ninth fraction (0.3 g) was subjected to flash chromatography (eluent: 2% methanol/dichloromethane); the third and fourth resultant fractions were pooled (0.20 g) and subjected to flash chromatogra-



Figure 2. *T. nyseus* on *Bryophyllum calycinum*.

phy (eluent: step gradient from dichloromethane to 50% dichloromethane/ethyl acetate) to yield zeorin (**1**) (0.036 g)¹³. This is the most widespread triterpene in lichens. The tenth fraction when subjected to flash chromatography (eluent: 5% methanol/dichloromethane) yielded colourless crystals of **bsitosterol** (**2**)¹⁴ (recrystallized in 0.5% methanol/dichloromethane). It is a common plant sterol that has been identified in the macrolichens *Anaptychia fusca* and *Cladonia boryi*¹⁵, and more recently from crustose species *Lecanora dispersa*¹⁶. These widely divergent lichens all contain chlorococcoid photobionts, which are likely to be the source of **bsitosterol** and are of no taxonomic significance.

The dichloromethane extract upon MPLC (step gradient: *n*-hexane to 20% methanol/dichloromethane) provided several fractions; the first fraction (0.032 g) when subjected to two flash chromatographic separations (eluent: 50% dichloromethane/*n*-hexane; 25% *n*-hexane/dichloromethane) yielded the long-chain fatty acid ester (**3**) (0.010 g) as a colourless solid. The structure of this compound was elucidated by: (a) base hydrolysis to give the C₄₃ straight-chain alcohol and (b) high-resolution mass spectroscopy, infrared and extensive nuclear magnetic resonance studies. To the best of our knowledge, this compound has not been previously reported from a lichen. Straight-chain fatty acid esters are rare in lichens, although they elaborate a variety of lactone carboxylic acids¹⁶.

The fourth fraction upon MPLC (eluent: step gradient from 1 to 10% methanol/dichloromethane) yielded atra-



Figure 3. Lane 1, Butterfly extract; lane 2, Hexane extract of *L. sipmanianum*; lane 3, CH₂Cl₂ extract of *L. sipmanianum*.

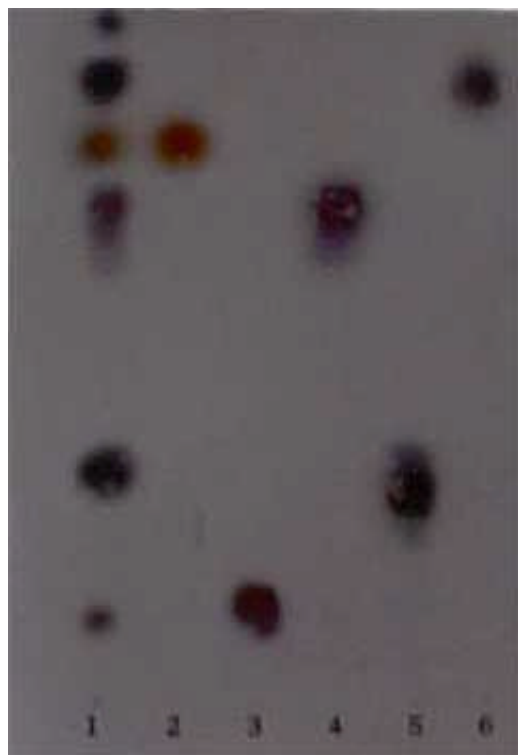


Figure 4. Lane 1, CH₂Cl₂ extract of *T. nyseus*; lane 2, Atranorin; lane 3, Zeorin; lane 4, Usnic acid; lane 5, **b**-Sitosterol; and lane 6, Long-chain fatty acid ester.

Table 1. Lichen products present in adult *T. nyseus*

Collection date	Compound present ^a
21 September 1996 ^{b,c}	(1) [0.65] ^d , (2) [0.95], (3) [2.35], (4) [2.17], (5) [1.85]
12 December 1996 ^c	(1)–(5)
30 June 1997 ^c	(1)–(4)
12 December 1998 ^c	(1)–(3)
12 December 1998 ^c	(2)
11 March 1999 ^{b,c}	(1)–(5)
20 November 1999 ^f	(2), (4)
7 July 1999 ^g	(2), (5)
8 June 2000 ^c	(1)–(3)
10 August 2000 ^h	(2)
10 December 2000 ^c	(1)–(3)
March 2001 ^c	(1)–(4)

^aCompounds (1)–(5) were common to both *T. nyseus* and *L. sipmanianum*; ^bBoth the collected adult butterflies and the ones which emerged from pupae contained compounds; ^cCollected from Beragala, Uva Province; (1)–(5); ^dR_f values of each compound in CH₂Cl₂ are given in parentheses; ^eCollected from the premises of Department of Botany, University of Peradeniya, Central Province; ^fCollected from Ukgalkalthota, Uva Province; ^gCollected from the foothills of Adam's Peak, Central Province; ^hCollected from a domestic garden, in Kandy, Central Province.

norin (4) (0.082 g), which is a common aromatic phenolic compound isolated from many lichen species¹⁷. It is antifungal and moderately cytotoxic¹⁸, and is the only colourless, cortical compound found in lichens.

The sixth and seventh fractions yielded (+)-usnic acid (5) (0.087 g). Usnic acid is a yellow, cortical compound which controls light radiation reaching the algal layer³. This ubiquitous dibenzofuran derivative is strongly cytotoxic as well as antimicrobial¹⁹. Zeorin, **b**sitosterol, atranorin and usnic acid exhibited spectral properties (¹H and ¹³C NMR) identical with those reported for the compounds¹⁶.

In Beragala, adult *T. nyseus* were collected between the period from September 1996 and March 2001 (Table 1). Compounds (1)–(5) isolated as described above from *L. sipmanianum*, were compared by TLC and co-TLC (see Table 1 for R_f values) with those in the butterfly, and they were found to be identical. Since only minute quantities of the compounds were present in the butterfly, in order to confirm the presence of compounds (1)–(5), the following protocol was adopted for all butterfly extracts, except for the September and December 1996 collections which were compared only by TLC analysis: butterflies were extracted into dichloromethane (typically the weight of two adult butterflies = 0.020 g; the weight of the extract = 0.0050 g). The butterfly extract was partially separated by preparative TLC (eluent: 20% *n*-hexane/dichloromethane).

Two broad bands containing zeorin, **b**sitosterol, fatty acid ester, atranorin and usnic acid were scraped and extracted into dichloromethane. Concentration (0.0010 g) followed by GC-MS analysis revealed molecular ions belonging to usnic acid (*m/z* 344), atranorin (*m/z* 374), **b**sitosterol (*m/z* 414), zeorin (*m/z* 444), and fatty acid ester (*m/z* 676 and 620), thus confirming their presence in the butterfly extract.

Out of the seven butterfly collections made from Beragala, all five compounds were present in adult butterflies on three occasions (Table 1). Importantly, on two occasions, the butterflies which emerged from laboratory-reared pupae also contained compounds (1)–(5). (+)-Usnic acid was present only on three occasions and atranorin on four occasions. *T. nyseus* collected during all the field visits contained zeorin, **b**sitosterol and the fatty acid ester.

In order to expand the scope of the study and investigate the presence of lichen products in *T. nyseus* in other areas of the island, butterflies were collected from four other locations: foothills of Adam's Peak in Central Province (80°30'50"E, 6°50'N); Peradeniya University premises (80°35'30"E, 7°15'N), and a domestic garden in Aniewatta, Kandy (80°38'E, 7°17'30"N), both in the Central Province, and in Ukgalkalthota (80°52'30"E, 6°39'30"N) in the Uva Province. Butterflies were subjected to the same analysis protocol as described above, using both TLC and GC-MS. Interestingly, the butterflies found at the University of Peradeniya and Kandy contained only **b**sitosterol with no trace of other compounds. However, those collected in Ukgalkalthota contained atranorin and **b**sitosterol while butterflies at

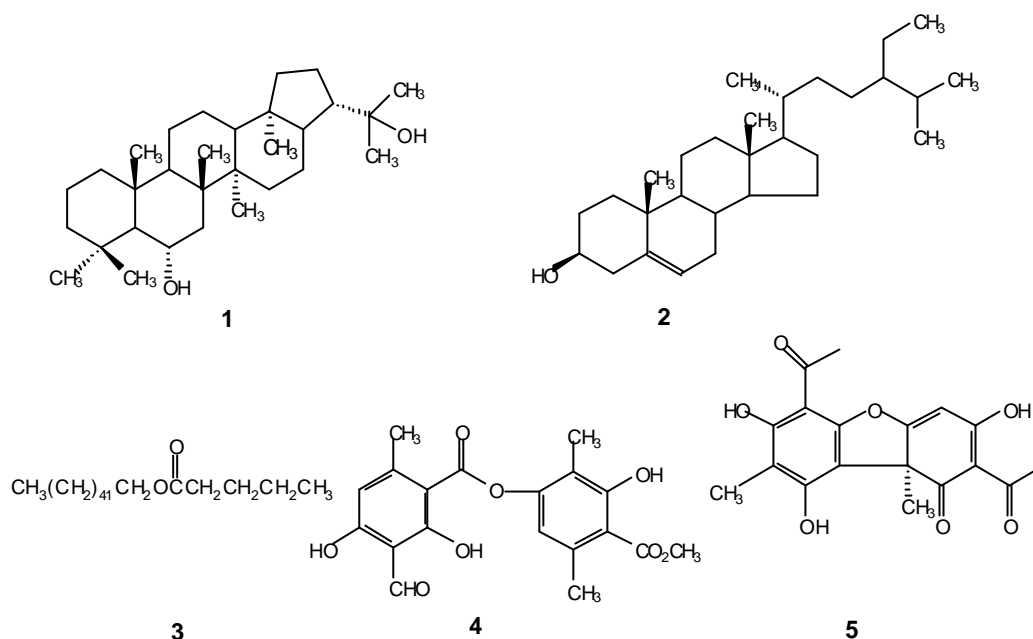


Figure 5. Structure of lichen compounds present in *L. sipmanianum* and *T. nyseus*: **1**, Zeorin; **2**, *b*Sitosterol; **3**, C₄₃ Fatty acid ester; **4**, Atranorin; **5**, (+)-Usnic acid.

the foothills of Adam's Peak contained (+)-usnic acid along with *b*sitosterol. Examination of the extracts (hexane, dichloromethane and methanol) of air-dried plant material (140 g) of *B. calycinum* by TLC and MPLC did not reveal presence of lichen compounds (**1**), (**3**), (**4**) and (**5**). However, the dichloromethane extract (1.50 g) whose TLC showed the presence of *b*sitosterol upon MPLC on silica gel (eluent: step gradient from 40% dichloromethane/hexane to 1.25% methanol/dichloromethane) yielded *b*sitosterol as a colourless, crystalline solid (0.021 g), confirming that *B. calycinum* contains *b*sitosterol and it could be the source of the butterfly.

As early as 1968, Reichstein *et al.*²⁰ showed that toxic cardiac glycosides (cardenolides) synthesized by milkweeds (*Asclepias* spp.) on which the larvae of the monarch butterfly, *Danaus plexipus* feed, could also be detected in the insect. Monarch butterflies, whose larvae had been raised on a cardenolide diet, caused the blue jay (*Cyanocitta cristata*) to vomit and avoid future encounters with butterflies of similar appearance²¹. Furthermore, *D. plexipus* adult butterflies take in pyrrolizidine alkaloids, which facilitate mating, from plants of Boraginaceae and Compositae by sucking the exudates from withered leaves²².

It may be concluded that the presence of lichen products found in *L. sipmanianum* in adult *T. nyseus* butterfly indicates that its larvae can feed on the lichen, although its natural food source is *B. calycinum*. In the case of *T. nyseus* individuals which contained only *b*sitosterol (Peradeniya and Kandy), the larvae probably used the

natural food source as their only diet. On the other hand, the presence of atranorin and usnic acid, compounds which are found only in lichens, in the butterflies in Ukgalkalthota and at the foothills of Adam's Peak respectively, lends credence to the evidence accumulated in Beragala that the larvae of *T. nyseus* may indeed sequester lichen products. Further research is in progress to investigate the biological role of the sequestered lichen products in *T. nyseus*.

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Microbial detoxification of *Colletotrichum falcatum* toxin

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A study was conducted on the possible detoxification of phytotoxin produced by the sugarcane red-rot pathogen *Colletotrichum falcatum* Went by antagonistic fungal and bacterial strains. Eleven *Pseudomonas fluorescens* strains and two *Trichoderma harzianum* strains were grown on a medium containing the pathogen toxin. Later, the treated toxin was tested for its phytotoxic activity using symptom bioassay, electrolytic leakage and spectral analysis. In symptom bioassay, the phytotoxin incubated with *P. fluorescens* strains FP 7 and VPT 4 and *T. harzianum* strain T-5 did not exhibit any symptom on susceptible sugarcane leaves. These treatments caused reduction in electrolyte leakage compared to other treatments and untreated toxin. Also, spectral analysis showed varied spectral patterns by different treatments. The results revealed that certain strains of the biocontrol agents cause detoxification of the pathogen toxin, which is one of the major pathogenicity determinants of the red-rot pathogen.

DETOXIFICATION or inactivation of the phytotoxin reduces the toxicity of metabolite produced by plant

pathogens. This process leads to development of resistant reaction or acts as a defence mechanism in susceptible plants to protect them from pathogen infection. Toxin resistance genes can be isolated from the pathogen itself, as well as from other microbes. Microorganisms form an exotic source of enzymes which are capable of inactivating synthetic chemicals that are potentially phytotoxic¹⁻³. Studies are now being carried out on transformation of plants utilizing phytotoxin resistance genes of microbial origin.

The red-rot pathogen *Colletotrichum falcatum* Went is known to produce a phytotoxic metabolite identified as an anthraquinone compound⁴. It has been established that the toxic metabolite is host-specific and produces part of the disease symptoms⁵. Both fungal and bacterial antagonists are being studied for the possible protection of sugarcane against the red-rot pathogen. Recently, strains of *Pseudomonas* spp. and *Trichoderma harzianum* effective against the pathogen have been identified^{6,7}. In this context, further studies were made on the possible inactivation of the pathogen toxin by these antagonistic strains.

The antagonistic strains of biocontrol agents, including two isolates of *T. harzianum* (T-5 and T-62) and 11 isolates of *Pseudomonas fluorescens* (ARR 1G, ARR 2, ARR 10, CHAO, EP 1, FP 7, KKM 2, Pfl, VPT 2, VPT 4 and VPT 10) isolated from sugarcane rhizosphere were employed for this purpose^{6,7}. Regarding the phytotoxin, the partially purified toxin from the *C. falcatum* pathotype Cf 671 was obtained and utilized in this study^{5,8}.

For growing the antagonists in the toxin-amended medium, the fluorescent pseudomonads and *T. harzianum* isolates were inoculated in synthetic medium (K₂HPO₄, 1.50 g; MgSO₄, 1.50 g; NaNO₃, 2.00 g; FeSO₄, 0.01 g; distilled water, 1000 ml incorporated with the toxin @1000 ppm) and incubated for eight days at room temperature. All the isolates were inoculated at equal concentration in 100 ml of medium and incubated as stationary cultures. The cultures were centrifuged, supernatants filter-sterilized and used for bioassay, absorption spectral analysis and for the estimation of electrolyte leakage (EC). The pellets were used for the estimation of cell concentration.

After centrifugation, pellets of all the *P. fluorescens* isolates were suspended in a known quantity of distilled water and cell concentration was estimated by examining the turbidity at 595 nm with a spectrophotometer⁹. Growth of *T. harzianum* isolates was observed visually.

Bioassay was carried out on sugarcane leaves as symptom production using antagonists treated and untreated *C. falcatum* toxin. The highly susceptible sugarcane cultivars, viz. CoC 86062 and CoC 92061 were used for this study. The youngest, fully expanded leaves were chosen and four leaf segments from four different plants were used for each treatment. Twenty µl of phytotoxin solution was placed over each spot on segments of the sugarcane leaves injured with fine pinpricks and incubated at

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