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RESEARCH ARTICLE

Antimicrobial and toxicological activities of some depsides and depsidones

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Abstract: Four depsides, namely, atranorin, lecanoric acid, erythrin, sekikaic acid and the depsidone lobaric acid were screened for their microbial activity against six bacterial species and six fungal species with respect to standards. The depside sekikaic acid and the depsidone lobaric acid showed potent activity against *Escherichia coli, Bacillus subtilis* and *Salmonella typhi* compared to the standard imipenum. Although the depsides atranorin, lecanoric acid and erythrin showed insignificant antimicrobial activity, their hydrolytic products such as methyl orsellinate and methyl-β-orcinolcarboxylate showed significant antifungal activity. Toxicological studies of these compounds were carried out using brine shrimp cytotoxicity assay.

Keywords: Antimicrobial activity, depsides, depsidones, lichen substances.

INTRODUCTION

The challenges for today's pharmaceutical industry lies in discovery and development of new pharmacologically active molecules. Lichens represent taxonomically and physiologically a very diverse group that consists of a symbiotic association between members of three different plant kingdoms (fungi, algae and / or cyanobacteria). Similar to higher plants lichens have been used since antiquity as medicines. Herbal medicinal texts include accounts of several species of lichens such as *Cladonia*, *Rocella*, *Evernia*, *Lobaria*, *Parmelia*, *Peltigra*, *Pertusaria*, *Physica*, *Usnea* and *Xanthoria* (Malhotra *et al.,* 2008). Lichens are well known for their diversity of secondary metabolites, which are characteristic and unique compounds restricted to the lichenized state and are not found in higher plants. The medicinal utility of lichens is ascribed to the presence of these secondary compounds (Boustie & Grube, 2005). Depsides and depsidones are the most common secondary products uniquely produced in lichens by the fungal symbiont, amounting to as large as 5–10% of thallus' dry weight. The core structural motif of this acetyl polymalonyl-derived family of about 700 depsides and depsidones is uniform across thousands of lichen species and generally consists of two phenolic rings with various substituents, joined by ester and/or ether linkages. Depsides and depsidones have manifold properties, such as antioxidant, antiviral, antibiotic, antitumor, allergenic, plant growth inhibitory, antiherbivore and enzyme inhibitory activities. There are various reports on the antimicrobial activity of crude lichen extracts. However, studies on antimicrobial activity of lichen compounds are scarce and scattered. A correlation between phenolic constituents and antimicrobial activity has been established (Vartia, 1973; Ingolfsdottir *et al*., 1998; Gulluce *et al*., 2006; Ranković *et al*., 2007 a, b). The antimicrobial activity of some common depsides, namely, atranorin (1), lecanoric acid (2), erythrin (3), the *meta*-depside sekikaic acid (4), the depsidone lobaric acid (5) and mononuclear aromatic compounds methyl orsellinate (6) and methyl-*b*-orcinolcarboxylate (7) (Figure 1) were tested against six human and plant pathogenic bacteria and fungi *in vitro* to evaluate their medical efficacy as pharmacophores. In addition, toxicological studies of lichen metabolites (1) – (7) (Table 1) were carried out using brine shrimp cytotoxicity assay.

METHODS AND MATERIALS

***General experimental*:** Medium pressure liquid chromatography (MPLC) was employed with accelerating

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gradient elution on SEPARO column packed with Merck Kieselgel (230 – 400 mesh ASTM) and metering pump (FM1-pump), model QD OSSY and column chromatography used silica gel 60 (230 – 400 mesh). Analytical thin layer chromatography (TLC) was carried out on Kieselgel 60 pre-coated aluminum foil plates. The spots on the TLC plates were detected under UV light (wavelengths 254 and 365 nm) by spraying anisaldehyde followed by heating. Purity of the compounds were confirmed using Waters 2690 high performance liquid chromatography (HPLC) coupled to Waters 996 UV Photodiode Array Detector using a Novapack C_{18} reversed phase column. All two dimensional nuclear magnetic resonance (NMR) experiments were carried out using standard and in-house modified Bruker pulse sequences. ESIMS were recorded on a Fisons VG Autospec mass spectrometer operating at 70 eV (direct insertion). HRESIMS were recorded on a Micromass LCT spectrometer. Identification of the compounds was carried out using ^{1}D, ^{2}D, and mass spectral data. Spectroscopic (^{1}H and ^{13}C) data were consistent with those of literature reports or authentic samples.

***Lichen material*:** Four lichen species, which have been reported to show antimicrobial activity at least up to the genus level (Karthikaidevi *et al.,* 2009) were selected for screening, which included *Parmotrema grayana* Hue, *Cladonia* sp., *Heterodermia obscurata* (Nyl.) Trevisan and *Roccella montagnei* Bel. Lichens were collected from the following locations: *P. grayana* from the stem bark of palm tree [*Roystonea regia* (Kunth) O.F. Cook (syn. *R. elata*, *R. floridana*)] at the University of Peradeniya; *Cladonia* sp. from the rocks in the Labukelle region, Central Province; *H. obscurata* from the rocks in the Beragala region, Central Province; and *R. montagnei* from coconut palm trees (*Coccus nucifera* L.) at the Coconut Research Institute in Kurunegala District, North Western Province. Lichen specimens were identified and deposited at the National Herbarium, Peradeniya.

***Isolation of compounds*:** Manually cleaned, air-dried and crushed lichens were sequentially extracted with CH_2Cl_2, followed by MeOH. The crude CH_2Cl_2 and MeOH extracts were fractionated *via* chromatography. *Para*-depsides atranorin (1) (Culberson & Kristinsson, 1969), lecanoric acid (2) (Nicollier *et al*., 1979), erythrin (3) (Culberson, 1969), *meta*-depside sekikaic acid (4) (Elix & Norfolk, 1975) and depsidone lobaric acid (5) (Hunneck, 1991) were isolated in considerable yields (Table 1). Methanolyis of lecanoric acid yielded the mononuclear aromatic compound methyl orsellinate (6) (Witiak *et al*., 1967) whereas atranorin gave methyl-*b*-orcinolcarboxylate (7) (Takenata *et al*., 1972), which were also isolated from the above lichens as minor metabolites (Table 1).

Table 1: Lichen sources and isolated substances

Source	Compound*
Cladonia sp.	**1** (0.29), **5** (0.37), **6** (0.02), **7** (0.27)
H. obscurata	**1** (0.68), **4** (1.07), **6** (0.86), **7** (0.08)
P. grayana	**1** (0.11), **2** (3.20), **6** (0.21)
R. montagnei	**1** (0.15), **3** (7.60)

*Yields are given within brackets

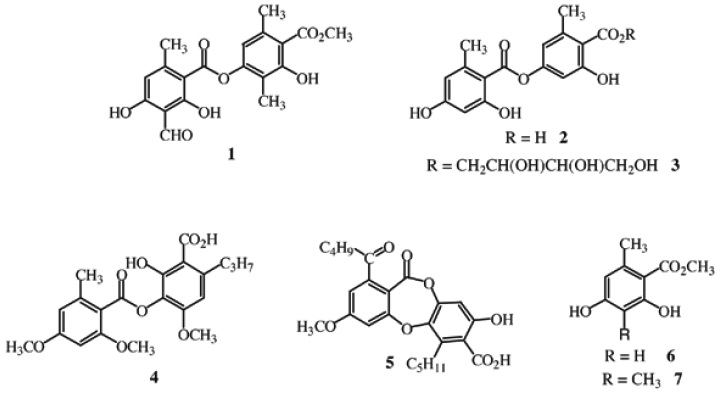

Figure 1: Structures of compounds isolated from lichens

***Antibacterial assay*:** Antibacterial screening of lichen compounds were carried out using agar well diffusion method. Fresh bacterial cultures (10 µl) of *Escherichia coli, Bacillus subtilis, Shigella flexenari, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella typhi* were added to melted soft agar and poured into nutrient agar plates to solidify. Six millimeter diameter wells were made in the agar and test samples 1 – 7 (100 µL, 1 mg/ml) were added and incubated at 30 °C for 24 h. Dimethyl sulfoxide (DMSO) was used as the negative control whereas the reference antibacterial drug imipenum (100 µg) served as the positive control. The inhibition zone of each well was measured in mm after 18–24 h (Bennett *et al*., 1966).

***Antifungal assay*:** Sabouraud dextrose agar (SDA) slants were used as the medium for the growth of fungi. Test compounds 1 – 7 (200 µg/mL) were loaded to non solidified SDA at 50 °C and allowed to solidify. Each tube was then inoculated with a 4 mm diameter piece of inoculum of the following fungi: *Trichophyton longifusis, Aspergillus flavus, Microsporum canis, Fusarium solani, Candida albicans* and *Candida glabrata*. DMSO was used as the negative control whereas the reference antifungal drug miconazole and amphotericin-B were used as positive controls. The tubes were then incubated at 27–29 °C for 7 ds. Growth in the compound amended media was determined by measuring linear growth (mm) and growth inhibition was calculated with reference to the negative control (Berhge & Vlietinck, 1991).

***Brine shrimp assay*:** Brine shrimp cytotoxicity assay was performed for the test compounds using *Artemia salina*. Shrimp eggs (50 mg), were incubated at 37 ºC in brine solution (38 g sea salt in 1 L of distilled water, pH 7.4) for 48–72 h to provide large numbers of larvae. After 2 ds of hatching and maturation, 30 larvae were placed (using pasture pipette) in each vial, containing 5, 50 and 500 µL of test compounds 1–7 (10 mg/ml MeOH). Total volume was brought to 5 mL with seawater and incubated at 28 ± 1 °C for 24 h under illumination. The vials supplemented with MeOH, and reference cytotoxic drug etoposide served as negative and positive controls, respectively (Meyer *et al*., 1982). The Finney computer programme (Finney, 1976) was used to determine LD_{50} values with 95% confidence intervals. The LD_{50} value of the standard drug etoposide was 7.43 μgmL^{-1}. The percentage growth regulation was analysed with reference to the negative control (Mc Laughlin *et al*., 1991).

RESULTS AND DISCUSSION

Antibacterial activity

From the 6 types of bacteria used in this study, the lichen compounds only showed antibacterial activity against *E.coli*, *B. subtilis* and *S. typhi* (Table 2). All the tested compounds 1-7 were inactive against *S. flexenari, S. aureus* and the anaerobic bacteria *P. aeruginosa.*

From the tested compounds, *meta*-depside sekikaic acid (4) and lobaric acid (5) showed significant antibacterial activity (Table 2). However prior to this study there have been no reports on antibacterial activity of lobaric acid (5) or sekikaic acid (4). These compounds showed antibacterial activity against the Gram negative (*E.coli, S. typhi*) and Gram positive bacterial strains (*B. subtilis*) that were tested.

The current study revealed that lecanoric acid (2) did not show antibacterial activity at 100 µg/mL against the six types of plant and animal phathogens tested. However, there have been a previous report of lecanoric acid (2) showing antibacterial activity against *E.coli* and

Table 2: Antibacterial activity of lichen compounds

Microorganism & inhibition zone (mm)	Inhibition zone (mm) of the compounds at 100 µg/mL						
	1	2	3	4	5	6	7
E. coli (25)*	13	0	0	16	17	0	0
B. subtillis (26)	12	0	0	16	16	0	0
S. typhi (21)	12	0	0	16	18	0	0
S. flexenari (25)	0	0	0	0	0	0	0
S. aureus (25)	0	0	0	0	0	0	0
P. aeruginosa (24)	0	0	0	0	0	0	0

* Inhibition zone of the standard imipenum at 100 µg/mL is given inside parentheses.
Criteria: Inhibition zone, 13 –15: low activity, 16 –18: significant activity

S. aureus at a higher concentrations of above 1000 µg/mL (Gomes *et al.*, 2003). Even when the free carboxylic acid of compound (2) possesses an ester group containing erythritol, as in erythrin (3), it showed no response to any of the bacterial cultures.

However, the ubiquitous lichen depside, atranorin (1), exhibited relatively low antibacterial activity at 100 µg/mL concentration tested. Prior to this study antibacterial activity of atranorin has been reported at a higher concentration of 500 µg/mL against *E.coli, B. subtilis* and *S. aureus* (Rankovic *et al*., 2008; Yilmaz *et al*., 2004).

Antifungal activity

The ubiquitous dibenzofuran usnic acid is a well known antifungal lichen compound (Ingolfsdottir, 2002). However, other than usnic acid there are only a few reports on the antifungal activity of lichen metabolites. In this study antifungal assay was performed on six reference fungal strains, namely, *T. longifusis, A. flavus, M. canis, F. solani, C. albicans* and *C. glabrata.*

None of the depsides and depsidones tested showed significant broad spectrum activity. Lobaric acid (5) showed significant antifungal activity only against *A. flavus,* whereas, lecanoric acid (2) showed moderate activity only against *F. solani*. Sekikaic acid (4) was active against both *A. flavus,* and *F. solani.* The ubiquitous depside atranorin (1) and erythrin (3) showed no antifungal activity against any of the fungi tested.

The two simple aromatic compounds, namely, methyl-*β*-orcinol carboxylate (7) and methyl orsellinate (6) showed significant overall antifungal activity. Methyl orsellinate (6) showed significant activity against *T. longifusus*, *A. flavus*, *M. canis* and *F. solani,* whereas of all the compounds tested methyl-*β*-orcinol carboxylate (7) showed the widest range of antifungal activity, being active against all species (Table 3).

Toxicological studies of test compounds 1-7, were carried out using brine shrimp lethality assay. The brine shrimp (*Artemia salina*) lethality assay is considered a useful tool for preliminary assessment of toxicity as a positive correlation exists between brine shrimp lethality and human carcinoma. The procedure, which is rapid and reliable, determines lethal concentrations of active compounds in brine medium. The brine shrimp lethality test was conducted on each of the compounds 1-7 at three concentrations, 10, 100 and 1000 µg/mL. In this assay none of the depsides and depsidones tested showed cytotoxicity even at 1000 µg/mL concentration. However, methyl β-orcinol carboxylate (7), the most significant antifungal mononuclear aromatic lichen compound (Table 3), which can also be obtained by hydrolysis of atranorin (1), showed toxicity with shrimp death

Table 3: Antifungal activity of lichen compounds

Microorganisms and Standards**	% Inhibition of test compounds at 200 µg/ml*						
	1	2	3	4	5	6	7
T. longifusus Micanozole (70 µg/mL) **	0	40	0	70	0	80	80
A. flavus Amphotericin (20 µg/mL) **	0	40	0	0	90	80	90
M. canis Micanozole (99 µg/mL) **	0	0	0	60	0	80	80
F. solani Micanozole (74 µg/mL) **	0	50	0	0	0	70	90
C. glabrata Micanozole (111 µg/mL) **	0	0	0	0	0	0	90
C. albicans Micanozole (111 µg/mL) **	0	0	0	0	0	0	90

* % Inhibition of test compounds are given at 200 µg/mL

** Concentration of the standards used to give 100% inhibition against the respective fungi

Criteria: % Inhibition, 30 – 40 : low antifungal activity, 50 – 60 : moderate antifungal activity, 60 –70 : good antifungal activity, >70 : significant antifungal activity

between 50 –70 % at 1000 µg/mL as well as 100 µg/mL concentrations. The LD_{50} valued obtained for compound (7) was 17.07 µg/mL when compared to the standard etopside (LD_{50} 7.43 µg/mL).

Methyl orsellinate (6), which showed significant antifungal activity, as well as sekikaic acid (4) and lobaric acid (5), which showed potent antibacterial activity did not show any cytotoxicity even at 1000 µg/mL concentrations. The ubiquitous depside atranorin (1), with moderate antibacterial activity also showed no cytotoxic effects. Significantly, the absence of cytotoxicity in depsides and depsidone highlights their medicinal importance.

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REFERENCES

1. Bennett J.V., Brodie J.L., Benner E.J. & Kirby W.M. (1966). Simplified accurate method for antibiotic assay of clinical specimens. *Applied and Environmental Microbiology* **14**(2): 170 –177.
2. Berhge D. A. V. & Vlietinck A. J. (1991). *Methods in Plant Biochemistry Assays for Bioactivity* (ed. K. Hostettmann), volume 6, pp. 47 – 69. Academic Press, London, UK.
3. Boustie J. & Grube M. (2005). Lichens - a promising source of bioactive secondary metabolites. *Plant Genetic Resources* **3**:273–278.
4. Culberson C.F. (1969). *Chemical and Botanical Guide to Lichen Products*, p. 628. University of North Carolina Press, Chapel Hill, North Carolina, USA.
5. Culberson C.F. & Kristinsson H. (1969). Studies on the *Cladonia chlorophaea* group: a new species, a new *m*-depside, and the identity of "novochlorophaeic acid". *Bryologist* **72**: 431– 443.
6. Elix J. A. & Norfolk S. (1975). Synthesis of *meta*-divarinol and olivetol depsides. *Australian Journal of Chemistry* **28**(5): 1113–1124.
7. Finney D.J. (1976). A computer program for parallel line bioassays. *Pharmacology and Experimental Therapeutics* **198**(2): 497–506.
8. Gomes A.T., Smania A., Seidle C., Smania E.F.A., Honda N.K., Roeses F.M. & Muzzi R.M. (2003). Antibacterial activity of orsellinates. *Brazilian Journal of Microbiology* **34**(3): 194 –196.
9. Gulluce M., Aslan A., Sokmen M., Sahin F., Adiguzel A., Agar G. & Sokmen A. (2006). Screening the antioxidant and antimicrobial properties of the lichens *Parmelia saxatilis, Platismatia glauca, Ramalina pollinaria, Ramalina polymorpha* and *Umbilicaria nylanderiana. Phytomedicine* **13**(7): 515–521.
10. Hunneck S. (1991). New results in the chemistry of lichens. *Symbiosis* **11**: 225–248.
11. Ingolfsdottir K. (2002). Usnic acid. *Phytochemistry* **61** (7): 729–36.
12. Ingolfsdottir K., Chung G.A.C., Vilhjalmur G.S., Gissurarson S.F. & Vilhelmsdottir M. (1998). Antimycrobacterial activity of lichen metabolites *in vitro*. *European Journal of Pharmaceutical Sciences* **6**: 141–144.
13. Karthikaidevi G., Thirumaran K., Manivannan P., Anantharaman K.K. & Balasubramanian T. (2009). Screening of antibacterial properties of lichen *Rocella belangeriana* (Awasthi) from Pichavaran mangrove (*Rhizophora* sp.). *Advances in Biological Research* **3**(3 – 4): 127–131.
14. Malhotra S., Subban R. & Singh A.P. (2008). Lichens- role in traditional medicine and drug discovery. *The Internet Journal of Alternative Medicine* **5**(2).
15. McLaughlin J.L., Chang C.J. & Smith D.L. (1991). "Bench-Top" bioassays for the discovery of bioactive natural products. *Studies in Natural Product Chemistry* (ed. Atta-ur-Rahman), volume 6, pp. 385-409. Elsevier Science Publishers, The Netherlands.
16. Meyer B.N., Ferrigni N.R., Putnam J.E., Jacobsen L.B., Nichols D.E. & Mclaughlin J.L. (1982). Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Medica* **45**(5): 31– 34.
17. Nicollier G., Rebetez M., Tabacchi R. (1979). Identification et synthese de nouveaux depsides isoles de la mousse de chene [*Evernia prunastri* (L.) Ach.]. *Helvetica Chimica Acta* **62**: 711–717.
18. Rankovic B., Misic M. & Sukdolak S. (2007a). Antimicrobial activity of extracts of the lichens *Cladonia furcata, Parmelia caperata, Parmelia pertusa, Hypogymnia physodes* and *Umbilicaria polyphylla. British Journal of Biomedical Sciences* **64**(4): 143–148.
19. Rankovic B., Misic M. & Sukdolak S. (2007b). Evaluation of antimicrobial activity of the lichens *Lasia pustulata, Parmelia sulcata, Umbilicaria crustulosa* and *Umbilicaria cylindrica. Mikrobiologiia* (Engl. Trans.) **76**(6): 723–727.
20. Rankovic B., Misic M. & Sukdolak S. (2008). The antimicrobial activity of substances derived from lichens *Physcia aipolia*, *Umbilicaria polyphylla*, *Parmelia caperata* and *Hypogymnia physodes*. *World Journal of Microbiology and Biotechnology* **24**(7): 1239 –1242.
21. Ranković B., Mišić M., Sukdolak S. & Milosavljević D. (2007). Antimicrobial activity of the lichens *Aspicilia cinerea, Collema cristatum, Ochrolechia androgyna, Physcia aipolia* and *Physcia caesia. Italian Journal of Food Sciences* **19**(4): 461– 469.
22. Takenata S., Ojima N. & Seto S. (1972). The isolation

of 2,4-dihydroxy, 3-6-dimethylbenzoic acid (3-methyl orsellinic acid) from a culture of *Aspergillus terreus*. *Chemical Communications* 391–392.

23. Vartia K.O. (1973). Antibiotics in lichens. *The Lichens* (eds. V. Ahmadjian & M. E. Hale), pp. 547–561. Academic Press, New York, USA.
24. Witiak D., Patel D. & Lin Y. (1967). Nuclear magnetic resonance. Influence of substituents on the long-range spin-spin coupling constant between benzylic and ring protons in the orcinol series. *Journal of American Chemical Society* **89**(8): 1908–1911.
25. Yilmaz Y., Turk A.O., Tay T. & Kivanc M. (2004). The antimicrobial activity of extracts of the lichen *Cladonia foliacea* and its (-)-usinic acid, atranorin and fumarprotocetraric acid consitituents. *Zeitschrift fur Naturforschung* **59c**: 249 – 254.