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Original Research Article

Pharmaceutically relevant metabolites from Lichens of Kodaikanal, India

K.Ramya[1] and T.Thirunalasundari[2]*

[1]Merck Millipore India Pvt. Ltd, No. 6, 6th Main Road, BDA Industrial Suburb, Near SRS Road, Peenya, Bangalore - 560 058, Karnataka, India
[2]Department of Industrial Biotechnology, Bharathidasan University, Tiruchirappalli - 620 024, Tamil Nadu, India
**Corresponding author*

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A B S T R A C T

Lichens are a world wide spread consortium of self-supporting associations between fungi and algae. In nature they encompass a complex and diverse assemblage of life forms, which occur throughout Kodaikanal on rocks, soil and on trees. Kodaikanal is at a high altitude and has a temperate climate. The existing environment here is pollution free, which favours the growth of Lichens, hence can be rightly described as the Biomonitors. Lichens are renowned for their extraordinary diversity of primary and secondary metabolites. Because of the physiochemical environment and biological interaction, many lichen species have evolved secondary metabolic pathway. Thus, promising a wealth of bioactive substance, many as yet unknown with novel structure and activities. Metabolic activities mainly respiration and photosynthesis, frequently result in production of free radicals or reactive oxygen species (ROS). Certain antioxidant enzymes produced by lichens like superoxide dismutase, catalase, glutathione reductase, etc., scavenge the effect of free radicals. Such bioactive compounds produced by lichens should be brought to the knowledge of Chemists and Pharmacologists. Having this in mind, extracts of *Ramalina sp*., *Usnea complanata, Usnea fischeri, Physcia dilatata, Parmotrema austrosinensis, Parmelia andinum* and *Parmelia sulcata* were subjected to analysis of macromolecules, phytochemicals and antibacterial activity testing on clinical isolates. Macromolecular quantification showed the presence of considerable amount of carbohydrates, proteins and fats in all the lichen species. It was found that all the extracts had antibacterial activity against one or the other clinical isolates.

Introduction

Whenever we discuss the probability that life exists in the harsh environment of the planet, lichens enter the conservation, which occupy the most forbidding environment on earth. They are diverse and ubiquitous group of lower plants, occupying 8% of the Earth' surface. About 20,000 species of lichens exists worldwide with India sharing 12.25

species (Sanjeeva Nayaka *et al.*, 2003). They do not possess roots or waxy cuticles and depend mainly on the atmospheric input of water and mineral nutrients. Consequently the entire thallus area of lichens is susceptible to penetration and accumulation of airborne elements, some essential for the proper functioning of lichen but others being toxic. These features combined with their ability to grow at a wide geographical range rank lichens among the best bioindicators of air pollution (Garty, 2001). Lichens produce various groups of metabolic byproducts. These include esters and organic acids (Julia Levy and Jack, 1973), which are produced by both the partners either individually or together (Ho lm – Hansen, 1968). Lichen metabolites exert a wide variety of biological actions including antibiotic, antiinflammatory, analgesic, antipyretic and cytotoxic effects. Metabolic activities mainly respiration and photosynthesis frequently result in production of reactive oxygen species (ROS) (Fridovich, 1999**,** Kohen and Nyska, 2002). These are enhanced during stresses such as nutrition limitation, exposure to xenobiotics or dessication and/or rehydration. To evade the damaging effects of ROS, cells have evolved protection mechanisms including antioxidant enzymes such as SOD, catalase, peroxidases and low molecular weight antioxidants. Even though these manifold activities of lichen metabolites have now been recognized, their therapeutic potential has not yet been fully explored and thus remains pharmaceutically unexploited (Muller, 2001). Among all the lichen metabolites, usnic acid is the most studied and used compound. After 1980's interest in such metabolites was renewed because of increasing experience of multidrug resistance caused by over usage of synthetic antibiotics (Cocchietto *et al.*, 2002). Hence it is important to discover newer sources of bioactive natural products to treat infectious diseases. One such resource being the lichens can be explored for the potential as producers of biomedically relevant metabolites. Hence the present study was aimed to characterize the bioactive compounds from lichens of Kodaikanal in India.

Materials and Methods

Study material: Different types of Lichen grown on plum tree.

Collection of the study material: All the lichen samples chosen for the study were collected from the plum tree; *Prunus salcinia.*

Nutritive value of lichens:

- **Preparation of extract:** Known quantity of lichen samples were taken and extracted with acetone in a pestle and mortar. The extract was centrifuged at 10,000 rpm for 5 mins in a centrifuge and the supernatant collected was used for macromolecular analysis. Carbohydrate was estimated by DNS method; protein by Lowry's method (Lowry *et al.,* 1951). Lipids were analysed by making use of Cholesterol kit supplied by M/S Biosystems.

Antibacterial activity testing:

- **Organism chosen for antibacterial activity testing**

  Gram negative organisms like *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium* and Gram positive organisms like *Staphylococcus aureus* and *Streptococcus faecalis* were used for this study.

- **Preparation of sample**

  Stock solutions of the samples were prepared by dissolving known concentrations of the crude lichen extracts in a known volume of sterile distilled water. Various concentrations

like 50µg, 100µg, 150µg and 200µg were taken from the stock solution and were used for antibacterial activity testing.

- **Assay method.**
  Antibacterial activity of different extracts was done by Kirby Bauyer method (Bauer, 1966).

Spot analysis of chemical constituents of lichens (Nylandervand Flora, 1856 & Asahina, 1934)

For the preliminary identification of the chemical compounds present in lichens, spot analysis was done by applying appropriate reagents to the lichen fragments by means of a syringe and the color formed was observed. The results were tabulated immediately. The test being C test (aqueous calcium hypo chlorite); K test (10 – 25% aqueous potassium hydroxide); PD test (P – phenylenediamine); KC test (first K test reagent and then C test reagent) and the other color tests using 5% aqueous solution of chloramine T was also done.

Thin layer Chromatography (Culberson, 1972)

Thin layer chromatography was performed to identify the secondary metabolites.

a. **Preparation of extract:**
   Known quantity of lichens was extracted with acetone and the supernatant was used for TLC.

b. **Solvent used:**
   Mixture containing toluene, ethyl acetate and formic acid in the ratio of 139:83:8 was used to develop the chromatogram.

Phytochemical analysis of secondary metabolites

Chemical tests were carried out on the aqueous extract and on the powdered samples using standard protocols to identify the constituents as described by Sofowora (1993), Trease and Evans (1989) and Harborne (1973).

Enzyme assay

The presence of two antioxidant enzymes; superoxide dismutase (SOD) and catalase were assayed in the lichens, which were freshly collected from the plum tree.

SOD: Enzymatic assay of superoxide dismutase was carried out by Winterbourn *et al.* (1975) and is based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide.

Catalase: Enzymatic assay of catalase was performed by following the method of Beer and Sizer (1952) and Stern (1937).

Results and Discussion

Seven lichen samples sharing different niches of the same tree; *Prunus salcinia* were collected from Attuvampatty campus, Mother Teresa Women's University, Kodaikanal. These samples were identified and categorized as fruticose, foliose and crustose (Table 1).

Macromolecular analysis showed the presence of considerable quantity of carbohydrates, proteins and fats in the samples analysed. *Parmelia sulcata* had high content of carbohydrate (900 µg/gm of sample), protein (930 µg/gm of sample) and fat (26.07 µg/gm of sample) (Table 2).

To have the knowledge on secondary metabolites of lichens, which may be a cause for the antibacterial activity, spot

analysis and TLC, were also performed. Spot analysis of color tests revealed certain aromatic aldehydes and other related compounds like depsides, dibenzofuranes, usnic acid, etc., (Table 3). TLC data obtained gave a picture about the presence of phenolic acids and other compounds in lichens (Table 4a,b).

Screening for antibacterial activity of the Lichen extracts against different clinical pathogens showed that all the lichen samples exhibited antibacterial activity against some of the clinical isolates tested. It revealed that none of the samples were active against *E. coli, Pseudomonas aeruginosa and Klebsiella pneumoniae* (Table 5). *Ramalina sp*., *Parmelia andinum* and *Parmelia sulcata* showed activity against *Proteus mirabilis* (Table 5). *Salmonella typhimurium* was the most susceptible among the Gram negative organisms tested with four species; *Ramalina sp., Usnea complanata*, *Physcia dilatata* and *Parmotrema austrosinensis* showing positive results (Table 5).

With respect to the activity of the samples against Gram-positive organisms, it was found that all the lichen samples except for *Parmotrema austrosinensis* were susceptible to *Staphylococcus aureus* (Table 5) while only *Ramalina sp., Usnea complanata*, and *Parmelia sulcata*, had very less activity against S*treptococcus faecalis* (Table 5).

Phytochemical screening test portrayed the presence of flavanoids, saponins, alkaloids, tannins, phlobotannins, steroids and glycosides (Table 6). Tannin and saponins were present in all the lichen samples tested. PL 2 & PL3 had all the secondary metabolites tested (Table 6). Enzyme assay performed detected the presence of SOD and catalase activity. *Usnea sp*., and *Ramalina sp.,* had more activity when compared to traces of the enzyme found in the other lichens (Table 7). PL 2 had high catalase activity (10.27U/mg) followed by PL 1 & PL 7 (8.96U/mg & 8.1U/mg) and PL 2 had high SOD activity (6.01U/mg) followed by PL 1, PL 2 & PL 4 (4.8U/mg, 3.96U/mg & 3.9U/mg). Interestingly there was no catalase activity in PL 4 & no SOD activity in PL 5 (Table 7 and Fig. 1).

In the present work, seven lichen species were analyzed for the presence of different metabolites in them. Considerable amount of the macromolecules present in lichens indicate their high nutritive value (Table 2). Many researchers have reported the presence of polysaccharides in lichens (Perez-Llano and Lichens, 1944).

People have traditionally used various preparative methods like boiling and steaming to make the macrolichens edible by removing the lichen secondary compounds and hydrolyzing lichen polysaccharides to yield glucose and other digestible simple sugars (Lal and Ranganatha Rao, 1956). The lichen extracts were subjected for antibacterial activity against Gram positive and Gram negative clinical isolates. Even though, all the extracts showed the presence of antibacterial activity, it was found interesting that four of the lichen species sp., had high activity against the Gram negative *Salmonella typhimurium* (Table 5).

On the contrary, *Staphylococcus aureus* used in this study was found to be the most susceptible organism of all the isolates used for testing and it was evidenced by a total of six samples of lichen extracts that inhibited the growth of the bacterium (Table 5). Also, the crude extracts were found to be potential sources of new broad-spectrum antibacterial compounds (Table 5 & Table 6).

Table.1 List of lichens chosen for the study

S. No.	Code no.	Source	Category/Nature	***Binomial Name of the lichen***
01	PL1	Plum tree	Fruticose	*Ramalina sp.,*
02	PL 2	Plum tree	Fruticose	*Usnea complanata*
03	PL 3	Plum tree	Fruticose	*Usnea fisheri*
04	PL 4	Plum tree	Crustose	*Physcia dilatata*
05	PL 5	Plum tree	Foliose	*Parmotrema austrosinensis*
06	PL 6	Plum tree	Foliose	*Parmelia andinum*
07	PL 7	Plum tree	Foliose	*Parmelia sulcata*

Table.2 Quantitative of macromolecules

S. No.	Binomial Name of the lichen	Carbohydrates μg / gm of the sample	Proteins μg / gm of the sample	Lipid μg / gm of sample
1	*Ramalina sp.,*	380	520	19.4
2	*Usnea complanata*	690	850	15.14
3	*Usnea fisheri*	300	260	18.76
4	*Physcia dilatata*	240	380	13.11
5	*Parmotrema austrosinensis*	740	780	18.16
6	*Parmelia andinum*	720	150	22.10
7	*Parmelia sulcata*	900	930	26.07

Table.3 Spot analysis of lichen phytochemicals

S. No.	Code No.	Color tests				Other test chloromine T	Probable compound
		C Test	K Test	KC Test	PD Test		
01	PL 1	-	-	+	-	+	Divaricatic acid, sekikaic acid
02	PL 2	-	-	+	+	+	Usnic acid, stictic acid
03	PL3	-	-	+	+	+	Usnic acid, stictic acid
04	PL4	-	+	-	+	+	Depsides- atranorin
05	PL5	+	-	-	-	-	Didymic acid,Pannaric acid
06	PL6	+	-	-	+	+	Lecanoric acid
07	PL7	-	+	-	-	+	Salazinic acid

Table.4a Phytochemicals of lichens based on TLC

Colour produced after acid spray & heating	Rf value	Probable compound
Slategy	0.68	**A**-protocetratic
Orange brown	0.26	**B**-Stictic
Dark brown	0.55	**C**-Pannarin
Light green	0.99	**D**-Usnic acid
Brownish orange	0.74	**E**-Salazinic acid
Grey	0.78	**F**-Pannaric acid
Pale pink	0.53	**G**-Sekikaic acid

Table.4b Compounds present in lichens

Sl No.	Code No.	Compounds present in lichens						
		A	**B**	**C**	**D**	**E**	**F**	**G**
01	PL 1	+	-	+	+	-	-	+
02	PL 2	-	+	-	+	-	-	-
03	PL3	-	+	-	+	-	-	-
04	PL4	-	+	-	+	-	+	+
05	PL5	-	+	+	-	-	+	-
06	PL6	+	+	-	+	+	+	-
07	PL7	-	+	-	-	+	+	-

Table.5 Antibacterial activity of samples

S. No	Name of the lichen	Activity against *E. coli / P. aeuroginosa / K. pneumoniae*	Activity against *P. mirabilis*				Activity against *S. typhimurium*				Activity against *S. aureus*				Activity against *S. faecalis*			
		Up 200μg	**50 μg**	**100 μg**	**150 μg**	**200 μg**	**50 μg**	**100 μg**	**150 μg**	**200 μg**	**50 μg**	**100 μg**	**150 μg**	**200 μg**	**50 μg**	**100 μg**	**150 μg**	**200 μg**
01	*Ramalina sp.,*	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+
02	*Usnea complanata*	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+
03	Usnea fisheri	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
04	Physcia dilatata	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-
05	Parmotrema austrosinensis	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-	-
06	Parmelia andinum	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
07	Parmelia sulcata	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+

Note: '+ '= presence of activity '-' = no activity

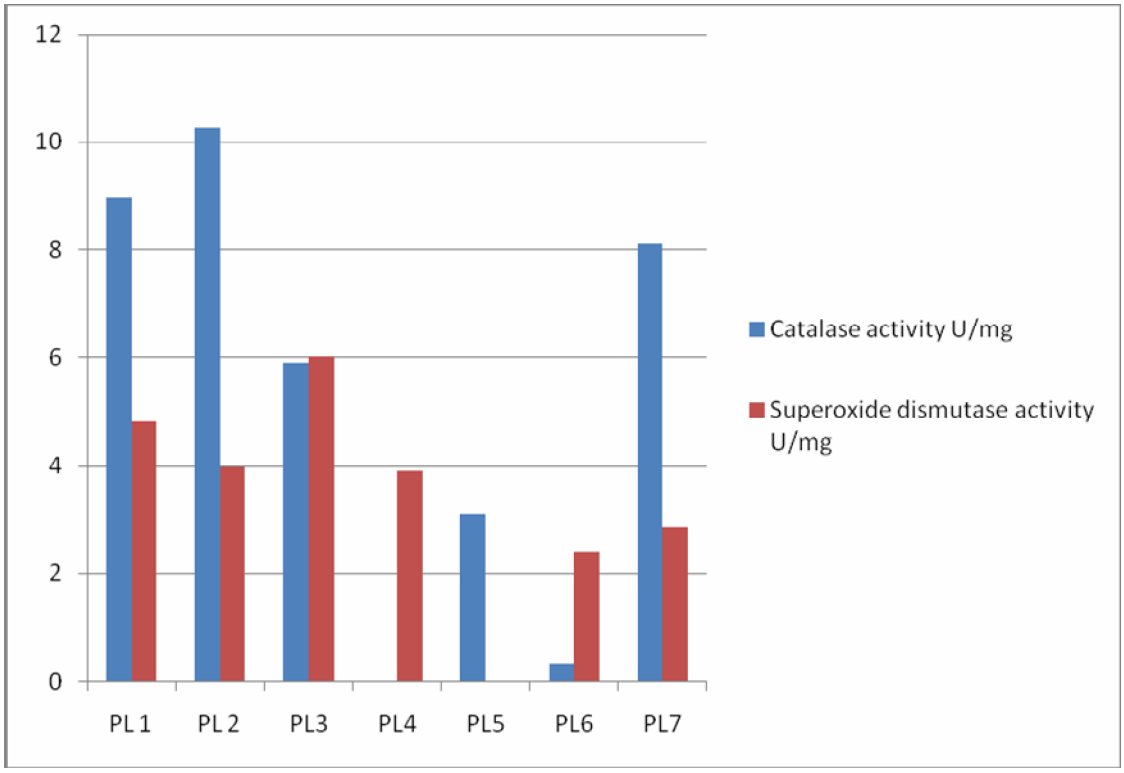
Table.6 Phytochemical analysis of lichens

Sl No.	Code No.	Flavanoid	Saponins	Alkaloids	Tannins	Phlobotannins	Steroids	Glycosides
01	PL 1	-	+	-	+	-	-	+
02	PL 2	+	+	+	+	+	+	+
03	PL3	+	+	+	+	+	+	+
04	PL4	+	+	-	+	+	-	-
05	PL5	-	+	+	+	-	-	-
06	PL6	-	+	-	+	-	+	+
07	PL7	+	+	+	+	+	-	+

Table.7 Antioxidant enzyme activity in lichens

Sl No.	Code No.	Catalase activity U/mg	Superoxide dismutase activity U/mg
01	PL 1	**8.96**	**4.8**
02	PL 2	**10.27**	**3.96**
03	PL3	**5.9**	**6.01**
04	PL4	**-ve**	**3.9**
05	PL5	**3.1**	**-ve**
06	PL6	**0.32**	**2.4**
07	PL7	**8.1**	**2.86**

Fig.1 Antioxidant enzyme activity in lichens



Reportedly, lichens produce over 800 secondary metabolites comprising many classes of compounds including amino acid derivatives, aromatic & aliphatic acids, depsides, depsidones, terpenoids, steroids, xanthones, and many more (Huneck, 1999). Usnic acid, a compound derived from *Usnea sp.,* which is found to possess very high antibacterial activity. *Usnea* is also the most common source material for antibiotic and antifungal lichen acids (Hobbs and Usnea, 1986). Catalase and superoxide dismutase activity indicates that lichens are a good source of antioxidants also (Table 7). This is however supported by the superoxide scavenging activity and free radical scavenging activity of *Usnea sp*., (Behera *et al.,* 2005). A report on enzymatic antioxidants of *Ramalina lacera* states that this species is a very good source for four Fe-SOD and four MN-SOD synthesized by the algal partner as well as a Cu-SOD and a Mn-SOD that are products of the fungal partner (Lior Weissman *et al.,* 2005). Hence, the various metabolites present in the 'Lichens' indicate that they can be effectively used in the pharmaceutical companies for the production of antibiotics.

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