



***In-vitro* evaluation of some Indian lichens against human pathogenic bacteria**

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Abstract

Antimicrobial activity of the acetone, methanol and ethanol extracts of some common lichen species such as *Usnea longissima* Ach., *Everniastrum cirrhatum* (Fr.) Hale, *Peltigera polydactylon* (Neck.) Hoffm. and *Sulcaria sulcata* (Lév.) Bystr. ex Brodo & D. Hawksw., were screened *in vitro* against six clinically important pathogenic bacteria, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Escherichia coli* by Kirby-Bauer technique of disc diffusion method. Minimum inhibitory concentration was taken out by Broth micro dilution method according to the NCCLS guidelines. Acetone, methanol and ethanol extracts of the investigated lichens showed relatively strong antimicrobial activity against all the gram positive bacteria and two gram negative bacteria. It was found that the inhibition zone of tested bacteria against lichen extracts varied between 7.6 – 30.7 mm diameters. The lowest MIC value was observed to be as low as 6.25 µg/ml against *B. cereus* of *U. longissima*. Generally the lichen extracts tested demonstrated antimicrobial effect which suggests a possibility of their use in treatment of various diseases caused by these and similar microorganisms.

Key words – Kirby-Bauer method – Lichenized fungi – MIC – pathogenic bacteria.

Introduction

Lichens are composite organisms consisting of symbiotic association of a fungus (the mycobiont) with a photosynthetic partner (the photobiont or phycobiont), usually either a green alga or cyanobacterium. Lichens are unique in nature and physiology because they look and behave quite differently from their component organisms. Lichens produce a wide range of organic compounds that can be grouped as primary metabolites and secondary metabolites (Elix 1996). Primary metabolites such as proteins, lipids, carbohydrates, and some other organic compounds are produced by both the partners and are needed for the lichen's metabolism and structure. Secondary metabolites are produced by the fungus alone and secreted onto the surface of lichen's hyphae either in amorphous forms or as crystals. The secondary metabolites include aliphatic,

cycloaliphatic, aromatic and terpenic components are extra cellular products of relatively low molecular weight usually insoluble in water and can be extracted into organic solvents (Ötzürk et al. 1999). They make even more than 30% of the dry mass of thalus (Galun 1988). Up till now, about 350 biologically active secondary metabolites of lichens have been discovered and approximately 200 have been characterized (Chand et al. 2009, Tay et al. 2004). Lichen secondary metabolites have been investigated mostly for chemotaxonomic purposes and in connection with their potential as phytomedicines and natural biopesticides (Dayan and Romagni, 2001). Many lichens have been used for human or animal nutrition and used as dyes, perfumes, in preparation of alcohol and in the pharmaceutical industries (Kirmizigül et al. 2003, Richardson 1988, Romagni 2002). Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, antiinflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects (Muller K. 2001). A number of lichen species are listed as having use as in folk medicine for treatment of stomach diseases, diabetes, whooping cough, pulmonary tuberculosis, cancer treatment and skin diseases (Baytop 1999, Huneck 1999, Richardson 1991). Medicinal activities of some lichens and their components are known, such as: antiviral, anti-tumor, anti-inflammatory, analgesic, antipyretic, antiproliferative and antiprotozoal (Lawrey 1986, Halama and Van Haluvin 2004 and Huneck 1999). However, only very limited numbers of lichen substances have been screened only very limited numbers of lichen substances have been screened for their biological activities and their therapeutic potential in medicine. This is certainly due to the difficulties encountered in identification of the species, collection of bulk quantities, and the isolation of pure substances for structure determination and testing activity. Recently, possibility for by passing some of these former difficulties have arisen by the introduction of new techniques. This includes axenic cultivation for production of the genuine compounds or new ones, extraction of focused compounds, or synthesis of natural products or their derivatives for testing (Boustie and Grube 2005).

According to one estimate, 50% of all lichens have antibiotic properties (Sharnoff 1997). The development and spread of microbial resistance to the available antibiotics has prompted investigators to study antimicrobial substances from other sources. India being a mega diversity country exhibit rich diversity and luxuriance of lichens together with other group of plants. A number of vascular plants from the country are known having their potential medicinal use; however, so far the studies and enumeration of medicinal non vascular plants are not available from the country. In India, few studies regarding use of lichens against plant pathogen are available (Tiwari et al. 2011a, Tiwari et al. 2012b). Since, there has not been much work done on the antimicrobial activity of Indian lichens, especially against human pathogens. Thus the aim of the present study was to conduct *in vitro* evaluation of antibacterial activity with acetone, ethanol and aqueous extracts from the lichens against human pathogenic bacteria.

Materials & Methods

Collection and identification of lichens

The lichen species were collected from different parts of the Himalayas in Uttarakhand and Himachal Pradesh. Lichen specimens were air dried at room temperature and identified by studying their morphology, anatomy and chemistry and authenticated using the standard literatures (Awasthi 2007, Orange et al. 2001). The voucher specimens are deposited in herbarium of National Botanical Research Institute, Lucknow (LWG).

Extraction

For extraction, air-dried lichen samples were ground, then 10 g portions were taken and added to 100 ml of solvents of acetone, ethanol and methanol. The mixtures were sonicated for 30 minutes, then left at room temperature for seven days and flasks were sealed with parafilm so that the solvent cannot evaporate. The extracts were filtered over Whatman No 1 filter paper, and the filtrates were sterilized by membrane filtration using 0.45 μ m pore size filters. The extracts were

then evaporated to dryness under reduced pressure and re-dissolved in respective solvents to attain the required concentration 0.2 mg/ml for antibacterial screening. These extracts were kept at 4°C till used.

Specimens used in the present study

Fig.1

1. *Everniastrum cirrhatum* (Fr.) Hale.

India – Uttarakhand, Pithoragarh district, Narayan Swami Ashram. 02.11.2009, D.K. Upreti & al., 09-013411-LWG.

2. *Peltigera polydactylon* (Neck.) Hoffm.

India – Uttarakhand, Pithoragarh district, Munsiyari, Khuliya top. 31.10.2009. D.K. Upreti & al., 09-023666-LWG.

3. *Sulcaria sulcata* (Lév.) Bystr. ex Brodo

India – Uttarakhand, Pithoragarh district, Munsiyari, Khuliya Top, 17.11.2006. Y. Joshi & R. Bajpai. 06-006935-LWG.

4. *Usnea longissima* Ach.

India – Uttarakhand, Pithoragarh district, Narayan Swami Ashram. 02.11.2009. D.K. Upreti & al., 09-012179-LWG.



Fig. 1 – 1 *Everniastrum cirrhatum* (Fr.) Hale; 2 *Peltigera polydactylon* (Neck.) Hoffm.; 3 *Sulcaria sulcata* (Lév.) Bystr. ex Brodo; 4 *Usnea longissima* Ach.

Identification of lichen acids

To identify the lichen substances present, the extracted lichen crude extracts were dissolved in acetone to a final concentration of 1 mg/ml. The crude extracts were then spotted on silica gel thin layer chromatography (TLC) plates (silica gel 60 F254 aluminum plates, Merck) and run in solvent systems A (36:9:1 toluene/dioxane/glacial acetic acid). The plates were kept for air drying at room temperature. Before spraying, the dried plates were examined in day light for pigments and for fluorescence or quenching under short and long wave length ultraviolet (U.V) light. Subsequently, each TLC plate was then sprayed with 10% sulfuric acid and heated at 110°C for 10 minutes to visualize the lichen substances (Santos and Mondragon 1969). The R_f values for each spot were determined and compared with selected lichen compound standards: (1) norstictic acid, (2) usnic acid, (3) salazinic acid, (4) caperatic : usnic acid, 25:1, (5) protocetraric acid, (6) stictic : constictic acid, 2:1, (7) diffractaic acid, (8) barbatic acid, and (9) galbinic acid. The lichen acids standards were generously provided by Prof. Dr. Jack A. Elix, Australian National University, Canberra, Australia.

Antibacterial activity of lichen extracts

The antimicrobial activity of lichen extracts against tested bacteria was determined employing Kirby-Bauer technique of disk diffusion method (Bauer et al. 1966, NCCLS 1993) The lichen crude extracts were then tested for their inhibitory activities against representative test bacterial gram-positive bacteria such as *Staphylococcus aureus* (ATCC 25923), *Streptococcus faecalis* (ATCC 33186) and *Bacillus cereus* (ATCC 14579); gram-negative bacteria such as *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 29853), *Salmonella typhimurium* (ATCC 13311). Bacterial cell suspension was prepared from a 24-hour old culture, adjusted to 0.5 McFarland standard, and swabbed on petri plates pre-filled with 25 ml Mueller-Hinton Agar (MHA, Hi-Media). Antibiotic disks (Whatmann) measuring 6 mm in diameter were then placed onto the inoculated MHA plates (two disks per plate). To determine the sensitivity of lichen crude extracts, to each paper disk 10 µl of 0.2 mg/ml concentration of lichen crude extract was added. The positive control were Gentamycin (for gram positive bacteria) and Ceftriaxone (for gram negative bacteria) and the negative control were the solvents acetone, methanol, ethanol respectively. Plates were then incubated at 37°C for 18-24 hrs. Following incubation, zones of inhibition including paper disks were then measured with a ruler and recorded.

The minimal inhibitory concentration (MIC) of the crude extract was determined by micro dilution techniques in Mueller-Hinton Broth (MHB), according to National Committee for Clinical Laboratory Standard, USA guidelines (NCCLS, 2002). A series of two fold dilutions with concentrations ranging from 200 µg/ml to 0.195 µg/ml of extract was used in the experiment against *S. aureus*, *S. faecalis* and *B. cereus*, *P. aeruginosa*, and *E. coli*. The starting solutions of extracts and component were obtained by measuring a certain quantity of extract and dissolving it in dimethylsulphoxide (DMSO). Two-fold dilutions of extracts and components were prepared in Mueller-Hinton broth (MHB) for bacterial cultures. The inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard colony forming units, and diluted 1:10 for the broth micro dilution procedure. Then 100 µl of diluted extracts and 100 µl of bacterial suspensions were dispensed in 96 well sterile microtitre plate. The microtitre plates were incubated at 37°C and MIC was determined after 24 h of incubation. The MIC was determined by establishing visible growth of the microorganisms. The boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration. Untreated bacteria were taken as Positive control and MHB was taken as negative control. All experiments were performed in triplicate.

The results of the antibacterial screening for disc diffusion assay are expressed as mean ± SD of three replicates in each test and for MIC, the boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration.

Results

Identification of lichen compounds

The results of thin layer chromatography showed that lichen substances were present in all the four lichens used for the study. *Usnea longissima* showed presence of four compounds; stictic acid complex at *Rf* class 3, barbatic acid at *Rf* class 4, diffractic acid at *Rf* class 4 and usnic acid at *Rf* class 6. *Everniastrum cirrhatum* shown salazinic acid at *Rf* class 2, protolichesterinic acid between *Rf* classes 3-4, atranorin at *Rf* class 7. In *Peltigera polydactyla*, two compounds were detected such as gyrophoric acid at *Rf* class 3 and tenuiorin at *Rf* class 7. In lichen *Sulcaria sulcata* has pulvinic acid at *Rf* class 1, virensic acid at *Rf* class 3-4, psoromic acid at *Rf* class 4 and vulpinic acid at *Rf* class 6-7 were present.

Antibacterial activity of extracts

The present study confirmed the presence of antibacterial substance in all the extracts of tested lichens and the results were presented (Table 1). The majority of acetone, methanol and ethanol extracts of *U. longissima* exhibited activity against the gram positive *Staphylococcus aureus*, *Streptococcus faecalis* and *Bacillus cereus*. Importantly the ethanol extract showed activity against two gram negative bacteria, *Pseudomonas aeruginosa* and *E. coli*. No activity was recorded against *S. typhimurium*. Against *S. aureus*, the methanol extract was most active with a mean zone of 18.4 ± 1.4 mm diameters. Against *S. faecalis* and *B. cereus*, again the zone of methanol extract was better than acetone and ethanol extract and the mean zone of inhibition was 22.6 ± 1.4 mm and 30.7 ± 0.9 mm diameters respectively. The activity of ethanol extract against *P. aeruginosa* and *E. coli* was 16.3 ± 0.5 mm and 18.8 ± 0.8 mm diameters respectively. The inhibitory effect of solvent alone on microorganisms was nil.

E. cirrhatum extracts were active against all the gram positive and a single gram negative bacteria. Against *S. aureus*, the activity of ethanol extract was greater than acetone and methanol extract and the calculated zone of inhibition against ethanol extract was 20.2 ± 1.1 mm diameters. The zone of inhibition noted for ethanol extract against *S. faecalis* and *B. cereus* was 16.3 ± 0.5 mm and 24.1 ± 0.6 mm diameters respectively. Against *P. aeruginosa*, only ethanol extract was effective and the calculated zone of inhibition was 19.3 ± 1.1 mm.

Ethanol extract of *P. polydactylon* showed activity against *S. aureus* with a mean zone of 10.8 ± 0.8 mm diameters. Both the acetone and ethanol extract showed activity against *S. faecalis* with a mean zone of 9.6 ± 0.5 mm and 11 ± 1.1 mm diameters respectively. Against *B. cereus*, only ethanol extract was effective with a mean zone of 15.1 ± 0.7 mm. Only acetone extract shown activity against *P. aeruginosa* with a zone of 7.6 ± 0.5 mm.

All the three extracts of *S. sulcata* showed activity against *S. aureus* and *B. cereus*. Against *S. aureus*, the acetone extract was most effective and the calculated zone was 17.1 ± 0.8 mm diameters while against *B. cereus*, the zone of acetone extract (8.7 ± 1.2 mm) was more than methanol and ethanol extract. Marked activity was shown by ethanol against *P. aeruginosa* and the mean zone of inhibition was 7.6 ± 0.5 mm diameters. No activity was noted against *S. faecalis*, *E. coli* and *S. typhimurium*

Minimum Inhibitory Concentration

The results of minimum inhibitory concentration of lichen *U. longissima* ranged within the range of 3.125-200 $\mu\text{g/ml}$. The least MIC value was calculated against *B. cereus* (6.25 $\mu\text{g/ml}$). The MIC results of *E. cirrhatum* were varying between 3.125-200 $\mu\text{g/ml}$. Lowest MIC was observed against *S. faecalis* (12.5 $\mu\text{g/ml}$). The MIC value noted for *P. polydactylon* against all the tested bacteria was 200 $\mu\text{g/ml}$. The MIC value calculated for the extract of *S. sulcata* against all the tested bacteria was 200 $\mu\text{g/ml}$ (Table 2).

Table1 Results of zone of inhibition (mm) of extracts of *Usnea longissima*, *Everniastrum cirrhatum*, *Peltigera polydactylon* and *Sulcaria sulcata* against tested microorganisms.

S. No.	Bacteria	<i>Usnea longissima</i>			<i>Everniastrum cirrhatum</i>			<i>Peltigera polydactylon</i>			<i>Sulcaria sulcata</i>		
		Ac. extract	Me. extract	Et. extract	Ac. extract	Me. extract	Et. extract	Ac. extract	Me. extract	Et. extract	Ac. extract	Me. extract	Et. extract
1.	<i>Staphylococcus aureus</i>	13.2 ± 0.7	18.4 ± 1.4	17.6 ± 1.1	18.5 ± 0.8	12.8 ± 1.6	20.2 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	10.8 ± 0.8	17.1 ± 0.8	13.4 ± 0.5	14.9 ± 0.9
2.	<i>Streptococcus faecalis</i>	21.5 ± 0.9	22.6 ± 1.4	20.3 ± 0.5	14.4 ± 0.7	15.4 ± 0.8	16.3 ± 0.5	9.6 ± 0.5	0.0 ± 0.0	11 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.	<i>Bacillus cereus</i>	19.4 ± 0.7	30.7 ± 0.9	20.4 ± 0.7	28.3 ± 1.5	21.4 ± 1	24.1 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	15.1 ± 0.7	8.7 ± 1.2	8.6 ± 1.2	8.3 ± 0.5
4.	<i>Escherichia coli</i>	0.0 ± 0.0	0.0 ± 0.0	16.3 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5.	<i>Pseudomonas aeruginosa</i>	8.4 ± 0.6	8.7 ± 0.3	18.8 ± 0.5	0.0 ± 0.0	10.6 ± 0.5	19.3 ± 1.1	7.6 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.6 ± 0.5
6.	<i>Salmonella typhimurium</i>	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

(*values are in mean ± Standard deviation, n=3)
(Ac. = Acetone, Me. = Methanol, Et. = Ethanol)

Table 2 Results of Minimum Inhibitory Concentration (MIC) of extracts of *Usnea longissima*, *Everniastrum cirrhatum*, *Peltigera polydactylon* and *Sulcaria sulcata* against tested microorganisms.

S.No	Bacterial pathogen	Lichen Species (MIC in µg/ml)			
		<i>Usnea longissima</i>	<i>Everniastrum cirrhatum</i>	<i>Peltigera polydactylon</i>	<i>Sulcaria sulcata</i>
1.	<i>Staphylococcus aureus</i>	3.125	3.125	200	200
2.	<i>Streptococcus faecalis</i>	25	12.5	200	NA
3.	<i>Bacillus cereus</i>	6.25	3.125	200	200
4.	<i>Pseudomonas aeruginosa</i>	200	200	200	200
5.	<i>Escherichia coli</i>	200	NA	NA	NA

NA= No Activity

Discussion

Lichens produce antibiotic secondary metabolites that provide defense against most of the pathogens in nature (Molnar and Farkas 2010). The tested lichen extracts showed a relatively strong antimicrobial activity. The screening results showed that nearly all the extracts of the tested lichens showed antibacterial activity against gram positive as well as gram negative bacteria. Antibacterial activity observed in this present investigation depended on the sort of the extract, its concentration and the tested microorganisms. Similar results were also noticed by other investigators (Rankovic et al. 2007), there is no antibacterial activity of the extracts against *S. typhimurium* was detected. Strong antibacterial activity was given by all the tested lichens against *S. aureus*, but extracts of *E. cirrhatum* showed larger zone of inhibition against this bacterium. Dülger et al. (1997, 1998) have found that although 4 different extracts of a macrofungus have inhibition effect against *B. subtilis* ATCC 6633 and other some gram positive and negative bacteria, and they do not have effect against *E. coli* ATCC 11230, *S. epidermidis* NRRL B-4377, *S. aureus* ATCC 6538P (Dülger et al. 1998). Activity against *S. faecalis* was given by all the lichens except *S. sulcata*. Among all the tested lichens, *U. longissima* was most effective against this bacterium. Rowe et al. (1999) reported that the lichens from Turkey, *Evernia prunastri*, *Pseudovernia furfuracea* and *Alectoria capillaris* were active against gram-positive bacteria and *Candida albicans*.

U. longissima showed the maximum activity against *B. cereus*. Similar to *U. longissima*, extracts of *E. cirrhatum* and *P. polydactylon* were also showing better activity against *B. cereus*. It has been determined that lichens have shown the inhibition effect against a lot of bacteria such as *Bacillus*, *Pseudomonas*, *E. coli*, *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Mycobacterium* (Esimone and Adikwn 1999, Perry et al. 1999). However, *S. sulcata* showed poor activity against *B. cereus*. The previous studies carried out by Burkholder et al. (1944), Rowe et al. (1989) and Silva et al. (1986) indicated that the lichens inhibit mostly gram positive bacteria. Even though most of the lichens have been reported to be active against gram positive bacteria, but it is of great interest to note that the extracts of *U. longissima*, *E. cirrhatum*, *P. polydactylon* and *S. sulcata* inhibited the growth of gram negative bacteria together with gram positive bacteria. All the extracts of four lichens showed activity against *P. aeruginosa* except *U. longissima* which showed activity against both gram negative bacteria such as *P. aeruginosa* and *E. coli*.

All the three extracts of *U. longissima*, methanol extract was found to have larger zone of inhibitions while according to Gulluce et al. (2006) and the methanol extract of the lichen *Parmelia saxatilis* had a strong antimicrobial influence. Results of *U. longissima* are comparable to Thippeswamy et al.(2011) where Ethanol extract of *Usnea Longissima* exhibited significant antibacterial activity and antifungal activity with 1mg/ml Agar well diffusion method against the Gram positive and Gram negative bacteria. Ethanol extracts of *E. cirrhatum* showed maximum inhibition as compared to acetone and methanol extracts while in the studies done by Swathi et al. (2010), Methanol extract of *E. cirrhatum* caused more inhibition of *Streptococcus epidermidis* than other bacteria whereas *P. aeruginosa* was least inhibited. The MIC was found to be least for *S. epidermidis*. Similar to *Parmelia kamstchandalis* tested using disc diffusion method by Mazid et al. (1999). Ethanol extract of both *P. polydactyla* and *S. sulcata* show inhibitory activity against large number of bacteria. According to Burkholder et al. (1944), out of 100 species of American lichens, only 52% of lichens showed activity against gram positive bacteria. Silva et al. (1986) also observed that most of the Brazilian lichens were active against gram positive bacteria.

In the present study, the extracts showed activity with different MIC values against the same microorganisms. Although the obtained MIC values for all extracts were varying between 6.25-200 µg/ml and for each microorganism. Madamombe and Afolayan (2003) reported significant activity of *Usnea barbata* against gram positive bacteria with MIC as low as 100 µg/ml on *B. subtilis*, *S. faecalis*, *M. viridans* and *S. aureus*. However in our study, the MIC value calculated against *Usnea longissima* was as low as 6.25 µg/ml against *B. cereus* and *S. aureus* respectively.

Both *U. longissima* and *E. cirrhatum* have a higher activity against gram positive as well as gram negative bacteria. However, *P. polydactylon* and *S. sulcata* were also effective against tested bacteria but the zones of inhibitions were quite smaller. Owing to pronounced antimicrobial activity of some of their secondary metabolites, lichens (along with algae, micro fungi and higher plants) are attracting much attention among researchers as significant new sources of bioactive substance. (Hostettmann et. al. 1997, Ingoldsdottir et al. 1997). Lichen compounds do have antimicrobial property that must be investigated to determine that what compounds are useful and how best to extract them in order to provide some legitimacy to a potential medical goldmine. Hence, there is an interest in the potential uses of antibiotics derived from lichens for the pharmaceutical industry in the future. In addition, the data may also suggest that the extracts of lichen species tested possess compounds with antibacterial properties, which require further studies to determine antimicrobial agents for therapy of infectious diseases in humans.

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