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Research Paper

Assessment of antimicrobial efficacy of secondary metabolites of lichen species from Uttarakhand temperate Himalayas, India

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ABSTRACT

Our investigation deals with the assessment of two different lichen *Peltigera* sp. (Sample A) and *Cladomia* sp. (Sample B) extracted in two separate solvent methanol (ME) and water (AQ). Their antimicrobial efficacies were assessed against various pathogenic bacteria and fungal agents. The MIC value of both these samples varied within the range of 0.7-27mg/ml. Quantitative and qualitative phytochemical analysis such as total phenol, polysaccharide concentration, TBA, reducing capacity, antioxidant and free radical scavenging ability were also determined. Higher total phenol concentration was observed in sample A in both the extract ME and AQ i.e. 15.6mg GA/g and 14.3 respectively while the AQ extract in both the samples exhibited significant inhibitory activity towards lipid peroxidation by TBA method. Consequently, reducing power also increases with the concentration of extract. Moreover, AQ extract of both the samples were observed with appreciable amount of polysaccharides comparatively to ME extracts. Lichen samples also exhibited remarkable antioxidant capacity in AQ extract of sample A i.e.5.4µg AA/g. In addition to this, in ME extracts of both samples showed comparable color change in TLC chromatogram. Therefore, our results clearly gave insight that lichen extract in water and organic solvent contain medicinally important bioactive compounds and which justifies their use in the traditional medicine.

Keywords: Lichens; Antimicrobial activity; MIC; Phytochemical properties.

INTRODUCTION

Medicinal plants are well-known natural sources for the treatment of various diseases since ancient times. Among them lichens are the most fascinating organisms on this planet as they have symbiotic association of fungi and a photosynthetic partner that can be an alga or a cyanobacterium (Ahmadijan, 1993). They usually grow on rocks,

non-fertile ground, as well as epiphytes on the trees and leaves (Taylor, et al., 1995). In current scenario, the challenge for today's pharmaceutical industry lies in the discovery and development of new pharmacological active molecules (Behera, et al., 2005). However, lichens components and its secondary metabolites mainly depsides, depsidones, dibenzofurans, xanthones and terpene derivatives are reported to act as antiviral, anti-tumor, anti-inflammatory, analgetic, antipyretic, antiproliferative, antiprotozoal agent (Halama, et al., 2004; Barnes, 2000). Besides, lichens are valuable plant resources and are used as medicines, food, fodder, dyes perfume, spice, and for miscellaneous purposes (Kirmizigul, et al., 2003). Although about 8% of the terrestrial ecosystem consists of lichens and more than 20,000 lichen species are distributed throughout the world, their biological activities and biologically active compounds remain unexplored to a great extent (Toma, et al., 2001)

Moreover, lichens substances have strong antioxidant activity and have high ability to scavenge toxic free radicals due to the presence of phenolic metabolites such as usnic acid, anthraquinone (Marcano, et al., 1999) and phenolic content (Shahi, et al., 2001). Even though with such manifold activities of lichens, their therapeutic potential has not yet been fully explored and thus remains pharmaceutically unexploited. Today's intensive use of antibiotics facilitated the spread of multiple drug resistant microorganisms, therefore, investigators seek for new antimicrobial substance from different sources so new sources of bioactive substances have been searched for such as medicinal herbs and lichens, mainly collected from Himalayan region, due to this a wide array of climatic zones, which favor the luxuriant growth of diversified and rich vegetation (Stocker-Wörgötter, 2008). As evident from the available literature previous record on the antibacterial activity of lichen are in scarce therefore the present investigation was undertaken to study the antimicrobial efficacy of lichen extracted in different solvent against various pathogens of animal and plants along with its *in vitro* assessment of phytochemical activity.

MATERIALS AND METHODS

***Collection and identification of lichen samples*:** By observing the lot of infestations in our university fruit field mainly by crustose and foliose like in appearance. Therefore, only these two samples were collected in a sterile polybags during September, 2014, they were designated as sample A and B and maintained in our Department of Molecular Biology and Biotechnology for further processing. Then, samples were air dried at room temperature for 48h and identified by studying their morphology using the standard literatures (Awasthi, 2007; Orange, et al., 2001). Although, various flora reference books were used for their identification (Aslan, 2000; 2001; 2002; Yazici, et al., 2004).

Extraction of lichen material in different solvent: Two different samples were air dried and pulverized into powdered form (10g) each followed by extraction in different solvents i. e. water and methanol respectively. For extraction in water (AQ), 5g powdered lichen were poured in 200ml double distilled water (DDW) and heating was done with continuous stirring at 30– 40°C for 20min. Then the water extract was filtered through whatman No 1 filter paper and the filtrates were sterilized by membrane filtration using 0.45µm pore size filters. Similarly, in methanol (ME) extraction 20gm powdered lichen material was taken and extracted with 250ml methanol using soxhlet extraction. Continuous process of extraction was performed up

to 24h, till the solvent in siphon tube of an extract become colorless. After this extract was taken in a beaker and heating was done at 30-40°**C** till the solvent got evaporated. Dried extract from both the solvent was kept in refrigerator at 4°**C** for their future use for antibacterial screening and phytochemical analysis (Tiwari, et al., 1992). While the yield percentage of the respective extract was calculated by using the formulae given below:

$$\text{Percentage yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of sample}} \times 100$$

Efficacy of antimicrobial activity of lichen extracts

Procurement of standard cultures: The microorganism used as test organisms in this study were both bacteria and fungus which are as follows: *Staphylococcus aureus* (9886), *Streptococcus mutans* (890), *Streptococcus pneumonia* (2672), *Vibro cholera* (3906), *Pseudomonas aeroginosa* (6458), *Clostridium tetani* (449) and Fungus cultures were *Aspergillus niger* (872) and *Fusarium* sp. (2671). All these standard microbial cultures were procured form Microbial type culture collection center (MTCC), IMTECH, Chandigarh. Agar well diffusion technique used to determine the antimicrobial efficacy of extracted lichen compound (Bauer, et al., 1966).

Determination of antimicrobial activity and minimum inhibitory concentration (MIC): Briefly, for this 24h and 168h old culture for bacteria and fungus were grown in Luria (LB) and potato dextrose (PD) broth respectively. Then, suspension of microbial cultures were spreaded on sterile Mueller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA) for bacteria and fungi respectively by using sterile spreader followed by placing the filter paper disks (6mm in diameter) on the surface of the inoculated plates using flame sterilized forceps. Simultaneously, streptomycin for bacteria and flucanazole in the case of fungi standard antibiotic disk was also placed as control. Subsequently, using sterile micropipette 10µl (0.002mg) sample of lichen compounds from both extract were poured onto the disk, and the plates were incubated at 37°C and 27°C respectively, for both bacteria and fungus. The zone of inhibition was measured as described by Subramanyam et al. (2013). The MIC of crude extract was also estimated by tube dilution method. In our experiment, concentrations of dilution ranged from 50 to 0.05mg/ml was used for each extract against each microorganism. Microdillution of each extract content was prepared in dimethyl sulpho-oxide (DMSO). The MIC was determined by establishing the visible growth of the each microorganism in their respective cultures. The dilution without any visible growth was defined as the MIC for the tested microorganism at the given lichen extracts concentration. Experiments were carried in triplicates and data were recorded statistically.

Quantitative analysis of the total phenolics, thiobarbituric acid (TBA), reducing power and polysaccharide assay: The total phenolics content in each extract was determined using Folin–Ciocalteu (FC) method. Supernatant of lichen compounds were diluted to the concentration of 1 mg/ml, and aliquots of 0.5ml were mixed with 2.5ml of FC reagent and 2ml of NaH_2CO_3 (7.5%). After 15min., absorbance was measured spectrophotometrically at 765nm. In our experiment, total phenols were determined as gallic acid equivalents (mg GA/g extract). For estimation of thiobarbituric acid (TBA), 2ml of lichen compound was mixed with 1ml of 20% aqueous trichloroacetic acid and 2ml of 0.67% aqueous thiobarbituric acid. After boiling for 10min, the samples were cooled and then centrifuged at 3,000 rpm for 30min. Control samples

was prepared containing only extract. Readings were taken spectophotometrically at 532nm (Saha, et al., 2004).The activity was calculated by percentage of inhibition of this method is follows:

$$\%\text{inhibition} = 100 - [(A1 - A0) \times 100]$$

- Where A_0 is the absorbance of the control and A_1 is the absorbance of the lichen sample.

Determination of reducing power of lichen extract was measured by method followed by Oyaizu (1986**).** For this, extract concentration of both the samples ranged from 50µg to 500µg/ml. The polysaccharide content in lichen extract was analyzed by using the phenol sulphuric acid method (Dubois, et al., 1956).

Determination of total antioxidant capacity and free radical scavenging: The total antioxidant capacity of the lichen samples was evaluated by the method with slight modification as described by Prieto et al. (1999). Methanol in the place of extract was used as blank. Ascorbic acid (AA) was used as standard and the total antioxidant capacity is expressed as milligram of ascorbic acid per gram of the dry thalli. Qualitative analysis of free radical scavenging was conducted by thin layer chromatography (TLC) using the mobile phase methanol: chloroform (95:5, v/v). After completion whole plates was sprayed with DPPH (0.15% w/v) solution using an atomizer. Development of pink color was considered as positive for antioxidant substance (Takao, et al., 1994).

All the experimental data were compiled as Mean±SD (standard deviation) and analysed by one way ANOVA with $P<0.05$ was regarded as significance and $P<0.01$as very significant.

RESULTS

***Identification and extraction yield*:** Based on the morphological appearance, the collected lichen samples were identified as *Peltigera* sp. (Sample A) and *Cladomia* sp. (Sample B). Their morphological features are shown in Table 1. With respect to AQ extract the yield of both the samples are higher in ME i.e. 3.8 and 2.6 for sample A and B respectively (Table 1).

Antimicrobial activity and MIC: The antimicrobial activity of lichen extracts was investigated against Gram positive, Gram negative bacteria as well as fungus. The diameter of inhibition zones around each well with lichen compound is represented in Table 2. It clearly depicts that the sample A content in ME showed a very strong influence against both bacteria as well as fungi while weak inhibition was recorded in AQ extract for both the samples undertaken. The MIC of lichen extracts of both the samples related to the tested bacteria and fungi were found within the range 0.7-27 mg/ml (Table 3). The highest antibacterial and antifungal activity was found in sample A, which inhibited the tested bacteria in a very low concentration (0.7 mg/ml).

Determination of phenolics, polysaccharide assay, reducing power, antioxidant and free radical scavenging activity: The results for estimation of total phenolic, polysaccharide content and antioxidant were shown in Table 4. More total phenolic concentration was observed in sample A in both the extract ME and AQ i.e. 15.6 mg GA/g and 14.3 respectively. Polysaccharide content in lichen extracts was also evaluated, which indicates that the AQ extract of both the sample shows more concentrations of polysaccharides when compared to ME extract. Additionally, reducing power of both extracts suggest that with the successive increase in the

concentration of extracts i.e. 50 to 500µg/ml, the optical density (O.D) also increases (Table 5) and it values varied from 0.001 to 0.54. Among the lichen sample A exhibited greater reducing power in ME; although the maximum reducing power showed by control containing pure ascorbic acid. Although, extractions from these lichens samples showed very potent total antioxidant capacity. The more total antioxidant capacity was found in AQ extract of sample A i.e.5.4µg AA/g. The qualitative result for determination of free radical scavenging ability, it showed from the TLC chromatogram that ME extract of both the sample were observed with significant color change than the AQ extract.

Determination of thiobarbituric acid (TBA): Moreover, lipid proxidation by TBA method demonstrated that both the extracts of lichen samples exhibited significant inhibitory activity towards lipid peroxidation. The absorbance values of extraction in AQ showed significantly higher values than the extraction in ME for both samples (Table 6). Irrespective to that the inhibition is more in sample A than sample B**.**

DISCUSSION

Various bioactive molecules are produced by the plants which have more beneficial effects on organism as compared to synthetic drugs (Vartia, 1973). Also considering the multi-drug resistant ability of pathogens towards synthetic drugs therefore this study was carried out to screen the antimicrobial agents including its phytochemical analysis of lichen. Earlier, it was reported that lichen comprise different compounds including amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthones, dibenzofuranes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers (Clix, et al., 1984). Nevertheless our present investigation also revealed many possible advantage of our studied lichen samples i.e. *Peltigera* sp (Sample A) and *Cladomia* sp. (Sample B) (Table 1). The intensity of the antimicrobial effect is mainly depending upon the kind of extraction, as in our study extraction were conducted in ME and AQ while in Table 1, their yield percentage are also shown. In comparison to ME the yield in AQ was found less that's probably because the active components produced by lichens can be little diluted in water (Kinoshita, et al., 1994).

The antimicrobial potential of lichens i.e. *Peltigera* sp (Sample A) and *Cladomia* sp. (Sample B) extract against both bacterial and fungal pathogens has also been presented in this work. The results obtained in this study showed strong antimicrobial action on these test organisms, which usually depends on the species of lichen, the type of extracting solvent used and the concentration of lichen extract. Similarly findings about antimicrobial actions by lichens were also observed by numbers of workers (Rankovic, et al., 2007; Yilmaz, et al., 2005). Previously, Chauhan and Abraham (2013) reported that aqueous extracts manifested no activity in relation to the microorganisms tested but our result also showed inhibitory effect in aqueous extract and the antibacterial effect is stronger compared to the antifungal (Table 2). These results could be expected considering the fact that numerous tests proved that bacteria are more sensitive to the antibiotic compared with fungi (Hugo and Russell, 1983).The reason for different sensitivity between the fungi and bacteria can be found in different transparency of the cell wall (Yang and Anderson, 1999). The antimicrobial activities were produced in different extents by the various fractions of the two different lichens studied. According to the result, it is shown that

the antimicrobial inhibition vary with the different types of the lichens, the solvent used for extraction. Although the obtained MIC values for these extracts were varying between the ranges of 0.7-27 mg/ml (Table 3). Madamombe and Afolayan, (2003) reported significant activity of lichens against 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 was as low as 0.7µg/ml against bacteria.

Earlier, Rankovic et al. (2007) stated that lichen contain phytoconstituents like flavonoids due to which it exhibited wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties. Our studies also deal with quantitative and qualitative analysis of phytochemical assay of lichen like phenolic, polysaccharides, free radical scavenging, thiobarbituric acid (TBA) and reducing power. Phenolic compounds have been reported to be associated with antioxidative action in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching single and triplet oxygen, or decomposing peroxides (Saha, et al., 2008). Currently studied lichens samples showed appreciable extent of phenolic and inhibition of lipid peroxidation by TBA (Table 4 and 6**).** It also revealed high absorbance of extracts samples indicates a potent reducing power with increased concentration of extracts (Table 5). Our result are found in accordance with the findings reported by Rahman (2011) that reducing power of their studied lichen also increased with increasing concentration of the sample. Though with such activity of lichen confirms that it can be also be used for wounds treatment which includes severe burns and to arrests bleeding and might be further explored as noble medicine.

Particularly polysaccharide and free radicals play a vital role in many chemical processes inside the cells, but particularly free radicals they also had detrimental effects such cell damage etc. However, synthetic antioxidants against these damages are often carcinogenic therefore finding substitutes from natural origin are of great interest. Therefore, our studied lichens have been found to contain a variety of metabolites, which have polysaccharide concentration and strong antioxidant properties (Table 4). Although, potent antioxidant activity of our sample is more similar to that of measured standard antioxidant ascorbic acid. Similarly, Amo de Paz et al. (2010) showed that methanol extracts of *Xanthoparmelia camtschadalis* and *X. conspersa*, as well as their separated lichen compounds (salazinic acid, stictic acid, and usnic acid) through HPLC technique protected biological cells from a very reactive oxidizing agent i.e. hydrogen peroxide as they also proved that lichenin compounds could act as antioxidant agents in those neurodegenerative disorders associated with oxidative damage (e.g. Alzheimer's disease and Parkinson's disease).

CONCLUSION

Among these two lichen sp. *Peltigera* sp. had profound results against most of test conducted with their antimicrobial and phytochemical activities. Under *in vitro* condition, it significantly inhibited the tested microorganisms, the majority of which are pathogens of man, animals and plants. The obtained results also showed that the tested lichen extracts had remarkable antioxidant capability, reducing power, free radical scavenging, TBA, phenolic and polysaccharide content which can be useful in treatment of multiple disease. Moreover, these lichen sp. are also used as fodder for

animals and in spices for human consumption where it could play major role for possible formulation of new drug to fight against pathogens.

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Table- 1: Identification and extraction yield of lichen in different solvent.

Designation	Morphological features*	Extraction yield in different solvent		Identification
		Methanol	Water	
Sample A#	Foliose type, with broad lobed thalli.	3.80	3.0	*Peltigera* sp
Sample B@	Mat-forming lichens, cup-bearing lichens	2.6	1.8	*Cladomia* sp.

- *- features were taken from flora books.

Table-2: Antimicrobial activity of lichen extract (50mg/ml) against test organism by AWD method.

Test Organism	Sample A		Sample B	
	Zone of inhibition (mm) in ME@	Zone of inhibition (mm) in AQ#	Zone of inhibition (mm) in ME@	Zone of inhibition (mm) in AQ#
Staphylococcus aureus	14.2±2.34	8.94±3.56	6.78±1.89	4.25±2.11
Streptococcus mutans	11.34±1.19	2.45±0.45	-	-
Vibro cholera	8.74±3.47	4.56±1.77	-	-
Pseudomonas aeroginosa	13.4±4.20	5.67±0.57	4.89±2.60	3.78±0.45
Clostridium tetani	7.20±2.56	3.24±1.36	-	-
Aspergillus niger	5.5±1.65	2.87±2.34	-	-
Fusarium sp.	2.7±0.34	1.78±0.45	1.50±0.27	-

- @= methanol extract; #= aqueous extract; -=no zone of inhibition; ±= standard deviation; AWD= agar well diffusion method.

Table-3: MIC concentration (µg/ml) of lichen extract against pathogenic strains.

Lichen extract	Standard pathogenic strains						
	S. aureus	*S. mutans*	*V. cholera*	*P. aeroginosa*	*C. tetani*	*A. niger*	*Fusarium sp.*
Samples A	*27*	*9*	*1.2*	*14*	*0.7*	*5*	9
Samples B	*15*	-#	-#	*7.8*	-#	-#	4.6

- #- = no inhibition were observed against these pathogenic strains.

Table-4: Estimation of total phenol content, polysaccharide concentration and antioxidant activity of lichen extracted in aqueous (AQ) and methanol (ME) solvent.

Lichen extracts	Total Phenol (mg GA/g extract)		Polysaccharides concentration (mg/ml)		Antioxidant activity (µg AA/g)		Standard Ascorbic acid@
	ME	AQ	ME	AQ	ME	AQ	9.6
Sample A	15.6	14.3	16	32	3.4	5.4	
Sample B	10.5	8.7	12	24	2.6	3.1	

- @= Standard ascorbic acid was used for antioxidant activity only.

Table-5: Qualitative analysis of reductive capabilities of lichen extracts.

Concentration (µg/ml)	Optical density (OD_{700} nm)			
	Sample A		Samples B	
	ME	AQ	ME	AQ
50	0.02	0.01	0.005	0.001
100	0.08	0.04	0.008	0.005
150	0.1	0.08	0.03	0.01
200	0.15	0.1	0.07	0.05
250	0.24	0.13	0.1	0.09
300	0.34	0.17	0.13	0.1
350	0.41	0.23	0.16	0.11
400	0.49	0.31	0.21	0.14
450	0.52	0.39	0.24	0.18
500	0.54	0.44	0.29	0.2

Table-6: Quantitative inhibition of lipid peroxidase enzyme by TBA methods.

Lichen extracts	Inhibition %	
	ME	AQ
Sample A	54	63
Sample B	48	51

