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Evaluation of Antibacterial Potential of some *Lichen* Species of Eastern Himalayan Region

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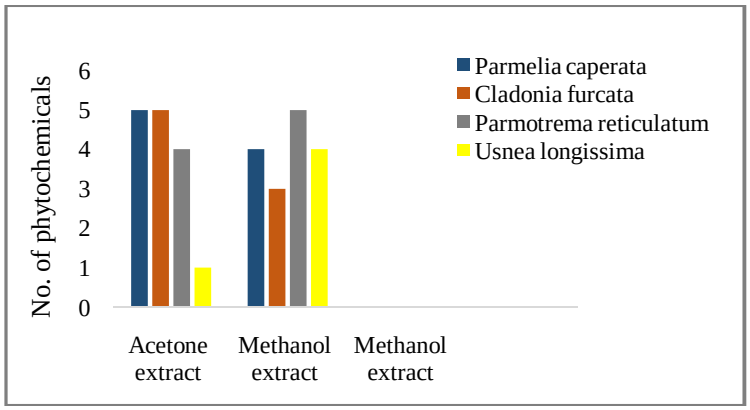
ABSTRACT

Antibacterial activity of aqueous, methanol and acetone extract prepared from four lichen species were evaluated against five bacterial strains viz. Staphylococcus aureus, Escherichia coli, Vibrio cholerae, Shigella dysenteriae and Shigella flexneri The aqueous, methanol and acetone extracts exhibited a variable range of antibacterial activity to bacterial strains. The acetone and methanol extracts of all investigated lichens showed antibacterial activity. The methanol extracts were most active against the test organisms. Aqueous extracts of all investigated lichens were found to be inactive against all tested bacteria. All the four lichen species showed the presence of alkaloids, tannins, saponins, cardiac glycosides, flavonoids, anthraquinones, terpenoids and Steroids. The results of antioxidant activity indicated a concentration dependent activity.

Graphical Abstract



Qualitative test for extracts

Keywords: Lichen, Antibacterial activity, Phytochemicals, Eastern Himalaya.

INTRODUCTION

An antibacterial is a substance that kills or inhibits the growth of bacteria. Infectious diseases account for about one third of all deaths worldwide. The spread of drug resistant strains of bacteria make it

necessary to discover antimicrobial compounds that inhibit these resistance mechanisms [**1**]. Thus discovery of antibiotics seems to be an wonderful discovery in the field of medicine and antibiotic resistance has become a global concern. In search of new groups of antibiotics, Lichens are being used on the basis of the traditional uses of lichens. The term '*Lichen*' was coined by Theophrastus, the Father of Botany, during 300 BC. Lichens are self-supporting symbiotic association between a photobiont (i.e. the Algal partner) and mycobiont (the fungal partner). The vegetative bodies are called thalli in which the mycobiont dominated over the photobiont and contains a wide range of organic compounds that can be grouped as primary metabolites (produced by both the partners) and secondary metabolites (produced by fungus alone).The secondary metabolites include aliphatic, areomatic and terpenic compounds which have relatively low molecular weight and usually insoluble in water and can be extracted into organic solvents. Up till now, about 350 biologically active secondary metabolites of lichen have been discovered [**2**]. Lichen secondary metabolites have a variety of biological actions including antibiotic, antiinflammatory, antipyretic, antiproliferative etc. According to Sharnoff (1997), 50% of all lichens have antibiotic properties [**3**].

Thus the aim of the present study was to evaluation of antibacterial activity of acetone, methanol and Aqueous extracts of four selected lichen species against five bacterial strain viz. *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholerae*, *Shigella flexneri* and *Shigella dysenteriae*.

MATERIALS AND METHODS

Collection and Identification of Lichens: The lichen species were collected from different parts of the eastern Himalaya during March, 2018. Lichen specimens were air dried at room temperature and identified by studying their morphology, anatomy and Chemistry [**4-7**].

Lichen samples used in the present study: *Parmelia caperata* (L.) Ach. *Cladoniafurcata* (Huds.) Schrad, *Parmotremareticulatum* (Taylor.) Choisy, *Usnea longissima* Ach.

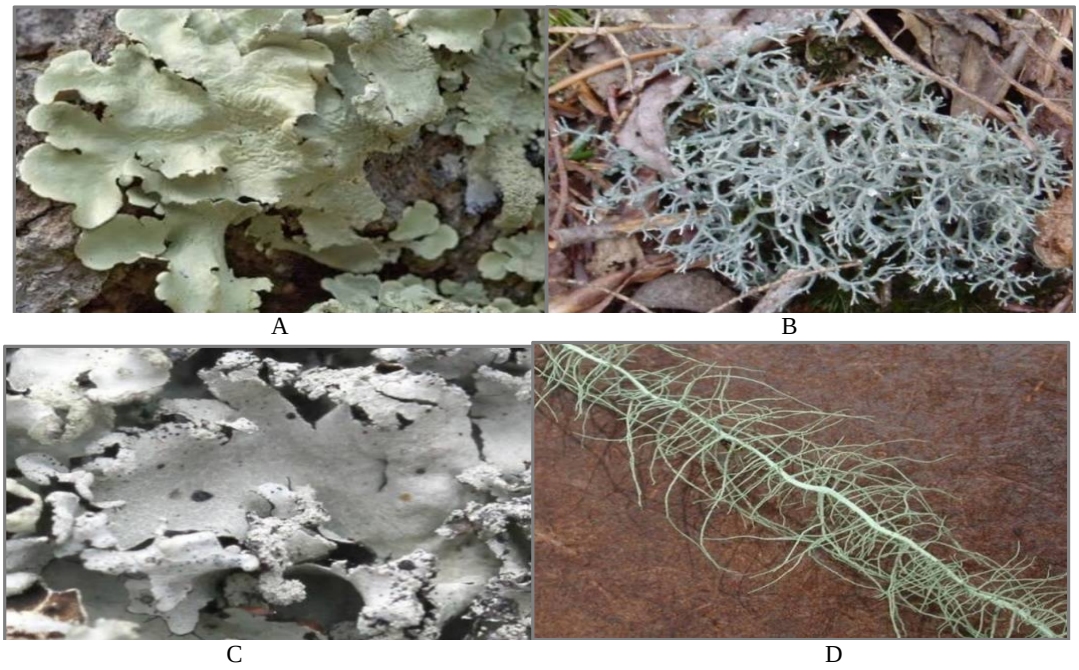


Figure 1. Collected Lichen Samples from the Eastern Himalaya. A. ***Parmelia caperata*** (L.) Ach, B. ***Cladoniafurcata*** (Huds.) Schrad, C. ***Parmotremareticulatum*** (Taylor.) Choisy, D. ***Usnea longissima*** Ach.

Preparation of Lichen extracts: For extraction, air-dried lichen thalli were ground, and then 10 g portions were taken and added to 100 mL of solvents of acetone, methanol and Water. The sample mixtures were sonicated for 30 min then kept at room temperature for 7 days and flasks were sealed so that the solvent cannot evaporate. The extracts were filtered by Whatman No 1 filter paper, and the filtrates were sterilized by membrane filtration using 0.45 µm 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 which used in antibacterial screening. These lichen extracts were kept at 4°C till used.

Qualitative chemical evaluation: The different extracts thus obtained were qualitatively tested for the presence of various phytochemical constituents [**8**].

Test for Tannins: Ferric chloride Test; 2 mL of Crude extract was mixed with a few drops of 5% ferric chloride soln. Formation of blue colour indicated the presence of hydrolysable tannins in the lichen extract.

Test for Alkaloids: Dragendroff's test; 2 mL of crude extract is added to 1% HCl and steam for 10 min. To this add 6 drops of Dragendroff's reagent. A reddish-brown precipitation indicates the presence of alkaloids.

Test for Saponins: Frothing test; 2 mL of Crude extract was mixed with 5 mL of distilled water in a test tube and it was shaken vigorously. The formation of stable foam at the surface indicates the presence of saponins.

Test for glycosides: Keller-Kalani test; 2 mL of Crude extract was mixed with 2mL of glacial acetic acid containing 2 drops of 2% solution of $FeCl_3$. The mixture was taken into another test tube which contain 2 mL of concentrated H_2SO_4. A brown coloured ring at the interphase indicates the presence of cardiac glycosides.
Test for Flavonoids: NaOH solution test; 2 mL of crude extract is added to 2 mL of10% NaOH solution. The yellow to orange colour indicates the presence of flavonoids.

Test for Proteins: Xanthoproteic test; 2 mL of crude extract is added to 2 mL ofHNO_3, boil in a water bath. The orange co-lour indicates the presence of proteins.

Test for Triterpenoids: Salkowski Test; 2 mL of crude extract is shaken with 1 mL of chloroform and a few drops of concentrated sulphuric acid were added along the side of the test tube. A red brown colour at the interface indicates the test as positive for triterpenoids.

Test for carbohydrates: Benedict's test; 2 mL of Crude extract when mixed with 2 mL of Benedict's reagent and boiled, a reddish-brown precipitate formed which indicated the presence of the carbohydrates.

Test for Steroids: Liebermann-Burchard reaction; 2 mL of crude extract is mixed to 2 mL acetic anhydride and a few drops of conc. H_2SO_4 is added. A blue-green ring indicates the presence of steroids.

Antibacterial activity of lichen extracts: The antimicrobial activity of collected lichen extracts against tested bacteria was determined by Kirby-Bauer technique of disk diffusion method (Bauer *et al*. 1966 [**9**], NCCLS 1993[**10**]) The lichen crude extracts were then tested for their inhibitory activities against representative test bacterial gram-positive bacteria such as *Staphylococcus aureus* gram-negative bacteria such as *Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae*, *Shigella flexneri*. Bacterial cell suspension was prepared from a 24 h old culture, adjusted to 0.5 McFarland standard, and swabbed on petri plates pre-filled with 25 mL Muellerhinton Agar (Hi-Media). Then

antibiotic diskso (6 mm in diameter) were placed onto the inoculated MHA plates. Then to each paper disk 10 µL of 0.2 mg mL^{-1} concentration of lichen crude extract was added to determine the sensitivity of lichen extracts. The positive control were Ceftriaxone (for gram negative bacteria) and Gentamycin (for gram positive bacteria) and the negative control were the solvents acetone, methanol, water respectively [**11, 12**]. Plates were then incubated at 37°C for 18-24 h. After incubation, the zones of inhibition including antibiotic disks were then measured with a ruler and recorded.

RESULTS AND DISCUSSION

In the present investigation, collection, identification, extraction and phytochemical evaluation of various lichen extracts. In this study, 4 lichen species were collected from which 12 extracts were prepared using Acetone, Methanol and water solvents. The preliminary phytochemical screening was carried out for determining the presence of Tannins, Alkaloids, Saponins, Glycosides, Flavonoids, Proteins, Triterpenes, Carbohydrates and Steroids. Important phytochemicals like Tannins, Proteins, Carbohydrates and Steroids were present in most of the sample extracts tested. Water extracts did not show any phytochemicals. The extracts from *Parmelia caperata* showed most of the constituents like Tannins from methanolic extract, Flavonoids from Acetone and Methanolic extract, Proteins from acetone and methanolic extract, Triterpenes from acetone extract, steroids from acetone and methanolic extracts (Table 1).

Table 1. Preliminary phytochemical constituents of lichen species with various solvent extracts

S.No	Lichen	Tannins			Alkaloids			Saponins			Glycosides			Flavonoids			Proteins			Triterpenes			Carbohydrates			Steroids		
		A	M	W	A	M	W	A	M	W	A	M	W	A	M	W	A	M	W	A	M	W	A	M	W	A	M	W
1	*Parmelia caperata*	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	-	-	-	-	+	+	-
2	*Cladonia furcata*	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	+	+	-
3	*Parmotrema reticulatum*	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+	-	-	-	-
4	*Usnea longissima*	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-

+ : Presence of compound, - : Absence of compound, A – Acetone extract , M – Methanol extract , W – Methanol extract

The present study also confirmed the presence of antibacterial substance in all the extracts of tested lichens and the results were presented (Table 2). The majority of acetone and methanol extracts of *Parmelia caperata* exhibited activity against the gram positive *Staphylococcus aureus*. Importantly the mehanol extract showed activity against one gram negative bacteria, *Shigella dysenteriae*. No activity was recorded against *Escherichia coli*, *Vibrio cholerae*, and *Shigella flexneri*, *S. typhimurium*. Against *S. aureus*, the methanol extract was most active with a mean zone of 18.4 mm diameters. Against *Shigella dysenteriae*, the methanol extract was better than acetone and water extract and the mean zone of inhibition was 8.7 mm diameter. The inhibitory effect of solvent alone on microorganisms was blank.

Against *Staphylococcus aureus*, the activity of methanol extracts of *Cladonia furcate* was greater than acetone and water extract and the calculated zone of inhibition against ethanol extract 18.0 mm diameters. The zone of inhibition noted for methanol and acetone extract against *Escherichia coli* was 10 mm and 9.4 mm diameters respectively. Against *Shigella dysenteriae* only methanol extract was effective and the calculated zone of inhibition was 10.6mm.

Ethanol extract of *Parmotrema reticulatum* showed activity against *Staphylococcus aureus and Vibrio cholerae* with a mean zone of 10.8 mm and 7.6 mm diameters. Both the acetone and methanol extract showed activity against *Escherichia coli* with a mean zone of 9.6 mm and 11 mm diameters respectively.

Both the acetone and methanol extract of *Usnea longissima* showed activity against *Staphylococcus aureus* with a mean zone of 13.4 mm and 17.1 mm diameters respectively and *Escherichia coli* with a mean zone of 8.6 mm and 16.3 mm diameters respectively. Against *Vibrio cholera*, only methanol extract was effective and the calculated zone of inhibition was 7.6 mm.

Table 2. Results of zone of inhibition (mm) of extracts of *Parmelia caperata, Cladoniafurcata, Parmotrema reticulatum, Usnea longissima* against tested microorganisms.

S. No	Bacteria	*Parmelia caperata*			*Cladoniafurcata*			*Parmotrema reticulatum*			*Usnea longissima*		
		Ac	Me	Aq	Ac	Me	Aq	Ac	Me	Aq	Ac	Me	Aq
1	*Staphylococcus aureus*	**13.2**	**18.4**	0.0	**12.8**	**18.0**	0.0	0.0	**10.8**	0.0	**13.4**	**17.1**	0.0
2	*Escherichia coli*	0.0	0.0	0.0	**9.4**	**10**	0.0	**9.6**	**11**	0.0	**8.6**	**16.3**	0.0
3	*Vibrio cholerae*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**7.6**	0.0	0.0	**7.6**	0.0
4	*Shigella dysenteriae*	0.0	**8.7**	0.0	0.0	**10.6**	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	*Shigella flexneri*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Ac – Acetone extract , Me – Methanol extract , Aq – Aqueous extract

Phytochemical analysis conducted on the lichen extracts revealed the presence of constituents which are known to exhibit medicinal as well as physiological activities. The utility of lichens is due to producing a range of secondary compounds by them. Lichen substances display a large-scale diversity of biological effects, including antimicrobial, antiinflammatory, antipyretic and antiproliferative and cytotoxic activities, and thus it provides a growing interest in the medicinal properties of secondary compounds obtained from lichens [**13-15**]. Our present study shows the presence of Tannins, Proteins, Carbohydrates and Steroids which were present in most of the lichen extracts. The different solvents of different polarity are used to separate phytochemicals based on their solubility in the extraction solvent. This extraction method was performed under continuous moving and short term, is used to extract the maximum number of bioactive components and prevent their modification.

The other results obtained in this study indicate differences in antibacterial activity between extracts depending on the species of lichen and type of extracting solvent.

APPLICATION

The presence of these phytochemicals from the lichen extracts can be used as a new group of antibiotics in the field of Pharmaceutical Industry.

CONCLUSION

The present study may be useful to supplement the information with regard to its standardization and identification and in carrying out further research as a significant new source for novel bioactive substances. These compounds could be Eco friendly, environmentally safe and be replaced by fungicides or insecticides, also be used as a biodegradable product and also be safe alternative to treat infectious diseases due to the presence of different components with strong antibacterial activity.

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