



## ASSESSMENT OF ANTIMICROBIAL AND PHYTOCHEMICAL POTENTIALS OF HIGH ALTITUDINAL NEPALESE LICHENS

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### ABSTRACT

Lichens and lichen products have been used in traditional medicines for centuries. The lichens of the high altitudinal meadow of MCA (Manaslu Conservation Area) have remained unexplored for which this research has been conducted with an aim of unveiling the phytochemical and antimicrobial properties of lichens present there. Four densely populated lichen species namely *Usnea longifolia*, *Cetraria* spp, *Parmotrema reticulatum* and *Evernastrium nepalense* were chosen for the study. The extracts of these species were obtained in 6 different solvents viz. hexane, chloroform, ethyl acetate, acetone, methanol and water by soxhlet extraction method and the antimicrobial assay was carried out by agar well diffusion method. The extract yield varied from 0.07 -29.4%. The extracts obtained showed the presence of volatile oil, saponins, coumarins and quinines, flavonic glycosides and carotenoids. The ethyl acetate fraction of *E. nepalense* and *U. longifolia* were found to be most effective against all the 8 clinical bacterial pathogens and 5 phytopathogenic fungi tested. The extracts of *Cetraria* spp and *P. milghenensis* were found to be specifically inhibiting the fungal pathogens compared to the bacterial pathogens. 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.

**Keywords:** antibacterial, antifungal, phytochemicals, zone of inhibition, lichens

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## INTRODUCTION

The challenge for today's pharmaceutical industry lies in the discovery and development of new pharmacological active molecules due to microbial resistance to available antibiotics (**Bahera et al., 2005**). Since long back, plants have provided a source of inspiration for novel drug compounds, as plant derived medicine have made large contributions to human health and well being by becoming the natural blue print for drug discovery and the development of phytomedicines to cure diseases (**Iwu, 1993**). Similar to higher plants, lichens were used since antiquity as natural drugs (**Barner, 2000**).

Lichen is a symbiotic organism consisting of a fungus (mycobiont) and a photosynthetic partner (photobiont) which can be either an alga or a cyanobacterium (**Ahmadian, 1993**). Lichens produce a diverse range of primary and secondary metabolites (**Hale, 1983**). Slow growth and often harsh living conditions make production of protective metabolites a necessity to lichens and many secondary constituents are believed to serve as antigrowth, antimicrobial or antiherbivore agents (**Hale, 1983; Rankovic et al., 2008**).

Lichen synthesizes a variety of secondary metabolites "lichen substances", mostly from fungal metabolism (**Brenan et al., 2009**). Lichen substances include aliphatic, cycloaliphatic, aromatic and terpenic components (**Chand et al., 2009**). They are extracellular products of relatively low molecular weight crystallized on the hyphal cell walls. Also, they are usually insoluble in water and can be extracted into organic solvents (**Otzurk et al., 1999**).

Screening the lichens has revealed the frequent occurrence of metabolites with antibiotics, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic properties. 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, 2002**). The present study unveils the phytochemicals present and the antibacterial and antifungal activity of the four different lichens from high altitude of Manaslu Conservation Area.

## MATERIAL AND METHODS

### Reagents, chemicals and solvents

Research grade media (Mueller Hinton Agar, MHA and Potato Dextrose Agar, PDA) were purchased from HiMedia Laboratories Pvt. Ltd, India. The chemicals were purchased from Salvi Chemical Industries Ltd., India, reagents from Bengal Chemicals and Pharmaceuticals Limited (BCPL), Calcutta, India and the solvents purchased from Triveni Interchem P. Ltd, Pancharatna Char Rasta, G.I.D.C., Vapi, Gujarat,-396 195 India.

### Bacterial and fungal culture strains

The standard bacterial cultures were obtained from Central Laboratory, Teku, Kathmandu, Nepal and the fungal cultures from Nepal Agricultural Research Council (NARC), Khumaltar, Lalitpur, Nepal.

### Study site and sample collection

The lichen specimens *Usnea longifolia*, *Cetraria* spp, *Parmelia milghenensis* and *Evernastrium nepalense* were harvested from alpine meadows of Manaslu Conservation Area in the autumn of year 2010. Various flora books and experts of the sample field were consulted for identification of samples (Dobson, 2000; Poelt, 1981; Purvis et al., 1992; Wirth, 1995). Voucher samples were prepared and deposited in the Tribhuvan University Central Herbarium (TUTH) for reference.

### Extraction

The harvested samples were air dried and were pulverized to powder. Each powdered samples were soxhlet extracted in different solvents, vis., hexane, chloroform, ethyl acetate, acetone, methanol and water differing in polarity. The extracts obtained were concentrated to dryness using Rotary vacuum evaporator (Tiwari et al., 1992). The yield of respective extract was calculated as:

$$\text{Percentage yield (\%)} = (\text{dry weight of extract/dry weight of samples}) \times 100$$

### Phytochemical screening

The presence of several chemical compounds in the various fractions of extracts obtained from different lichen species were screened by the chemical tests (Harborne, 1998).

**Antimicrobial assay**

The antimicrobial assay was performed using agar well diffusion method (Dingle et al., 1953). The antibacterial testing was carried out against 8 different human pathogenic bacteria i.e. *Staphylococcus aureus*, *Escherichia coli*, *Salmonella* Typhi, *Salmonella* Paratyphi, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Schigella* sp., and *Klebsiella pneumoniae* on Muller Hinton Agar. The standard culture inoculum for each bacterial species comparable to Mac Farland 0.5 standard was prepared for the test. Similarly, the antifungal testing was done against 5 different plant pathogenic fungi i.e. *Fusarium oxysporum*, *F. moniliforme*, *F. erdifforme*, *F. porliferatum* and *Exserhiliium turticum* on Potato Dextrose Agar. The standard culture inoculum for each fungal species was prepared on Potato Dextrose Broth with adjusting to a range of  $1 \times 10^6$  -  $5 \times 10^6$  spores.mL<sup>-1</sup> (Aberkene et al., 2002).

The antimicrobial activity was evaluated by measuring the inhibition zone diameter observed after incubating the media plates (at 37<sup>0</sup>C for 24 h and 27<sup>0</sup>C for 3-4 days for bacteria and fungi resp.) swabbed with the respective microbial strains and loading the different fractions of the extracts (50 µl of 100 mg.mL<sup>-1</sup>) into the bored wells in media. To every sample tested, a set of control was run parallel.

**RESULTS AND DISCUSSION**

The yield percentage of the various fractions of the lichen extracts has been shown in table 1. The extract yield varied from 0.07-29.4%. The phytochemical constituent in the tested lichens has been summarized in table 2. The volatile oil, saponins, coumarins and quinines were present in all the lichens tested. Flavonic glycosides was present in all the lichens except *Cetraria* spp while carotenoids was absent in *E. nepalense*.

The antimicrobial activity of the various fractions of the four different lichen species against the tested microorganisms was estimated on the basis of presence or absence of inhibitory zones and the results are depicted in the tables 3 and 4. Different fractions of the various lichen extracts exhibited different degrees of antibacterial and antifungal activity.

**Tab 1** Yield percentage of the different fractions of various lichen extracts

Lichen species	Extracts obtained in solvents (%)					
	Hexane	Chloroform	Ethyl acetate	Methanol	Acetone	Water
<i>E. nepalense</i>	13.8	0.07	1.5	9.7	0.9	2.2
<i>U. longifolia</i>	29.4	3.4	2.2	17.2	1.03	25.5
<i>Cetraria</i> spp.	12.2	2.3	1.1	16.5	0.8	3.9
<i>P. milghenensis</i>	10.8	2.9	1.1	5.6	1.02	9.05

**Tab 2** Qualitative phytochemical screening of methanolic plant extracts

Phytochemicals	Lichen species studied			
	<i>E. nepalense</i>	<i>U. longifolia</i>	<i>Cetraria</i> spp	<i>P. milghenensis</i>
Volatile oil	+	+	+	+
Sterol and triterpenes	-	-	+	+
Carotenoids	-	+	+	+
Fatty acids	-	-	-	+
Polyoses	+	-	-	+
Saponins	+	+	+	+
Polyphenols	-	-	-	-
Red. compound	-	-	-	-
Alkaloids	-	-	-	-
Glycosides	-	-	-	-
Quinones	+	+	+	+
Anthocyanosides	-	-	-	-
Anthracyanosides	-	-	-	-
Flavonic glycosides	+	+	-	+
Coumarins	+	+	+	+

The ethyl acetate fractions of *E. nepalense* and *U. longifolia* were found to be most effective against all the tested 8 bacterial pathogens. The inhibition zones of tested bacterial strains *E. nepalense* have been found as 9-21 mm and that of *U. longifolia* as 7-14 mm. the extracts of *Cetraria* spp were found to be at least effective inhibiting only the 4 tested bacteria while the extracts of *P. milghenensis* showed the activity against all the tested bacteria except *Salmonella typhimurium*. All the fungal pathogens tested were inhibited by one or more fractions of the lichens extracts tested and the inhibition zone ranged from 7-23 mm.

**Tab 3** *In-vitro* growth of Inhibition zones of some bacteria in crude extracts of different lichens

Lichens	Sol. used	Diameters of ZOI of microorganisms (mm) (100 mg.mL <sup>-1</sup> )*							
		<i>S. aureus</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>	<i>S. Paratyphi</i>	<i>S. Typhi</i>	<i>S. typhimurium</i>	<i>Schigella sp.</i>
<i>E. nepalense</i>	Hex	ND	9	ND	ND	ND	ND	ND	ND
	Chl	8	ND	8	ND	ND	ND	ND	11
	EtoAc	20	11	18	21	17	19	16	19
	Met	ND	19	ND	ND	ND	ND	ND	ND
	Ace	ND	ND	ND	ND	ND	ND	ND	ND
	Aq	ND	9	ND	ND	ND	ND	ND	ND
<i>U. longifolia</i>	Hex	7	ND	7	9	ND	ND	ND	ND
	Chl	9	7	8	10	7	9	9	7
	EtoAc	12	14	10	13	10	13	11	10
	Met	8	ND	7	8	7	8	8	8
	Ace	ND	ND	8	8	ND	7	7	7
	Aq	ND	ND	ND	8	ND	7	7	7
<i>Cetraria spp.</i>	Hex	ND	ND	ND	ND	ND	ND	ND	6
	Chl	7	ND	ND	ND	ND	ND	ND	6
	EtoAc	7	ND	ND	ND	ND	ND	ND	7
	Met	6	ND	ND	ND	ND	ND	ND	ND
	Ace	7	6	6	ND	ND	ND	ND	ND
	Aq	ND	ND	ND	ND	ND	ND	ND	ND
<i>P. milghenensis</i>	Hex	6	ND	10	ND	6	8	ND	ND
	Chl	6	ND	8	7	6	8	ND	ND
	EtoAc	8	8	8	8	8	6	ND	8
	Met	ND	ND	ND	ND	ND	ND	ND	ND
	Ace	7	6	ND	6	10	ND	ND	9
	Aq	ND	ND	ND	ND	ND	ND	ND	ND

Legend: Hex: Hexane- Chl : Chloroform- EtoAc: Ethyl Acetate- Met: Methanol- Ace: Acetone- Aq: Aqueous, ND: not determine, \*  $\bar{x} \pm SD$  (n = 4)

The ethyl acetate fraction of *E. nepalense* and *U. longifolia* were found to be effective towards the fungal pathogens compared to the other fractions. The extracts of *Cetraria* spp and *P. milghenensis* were found to be specifically inhibiting the fungal pathogens compared to the bacterial pathogens. The different fractions of the lichens except the aqueous showed the broad antimycotic activity against the tested fungal pathogens. The ability of the extracts to inhibit the growth of several bacterial and fungal species is an indicator of the broad spectrum antimicrobial potential of different lichens which makes them a candidate for bioprospecting for antibiotic and antifungal drugs.

**Tab 4** *In-vitro* growth of Inhibition zones of some fungi in crude extracts of different lichens

Lichens	Solvents used	Diameters of ZOI of microorganisms (mm), (100 mg.mL <sup>-1</sup> )*				
		<i>E. truticum</i>	<i>F. erdiforme</i>	<i>F. oxysporum</i>	<i>F. moniliforme</i>	<i>F. proliferatum</i>
<i>E. nepalense</i>	Hex	ND	ND	ND	ND	ND
	Chl	ND	10	11	11	15
	EtoAc	14	19	13	13	17
	Met	12	ND	12	12	13
	Ace	9	ND	ND	ND	12
	Aq	ND	ND	ND	ND	ND
<i>U. longifolia</i>	Hex	ND	ND	8	11	ND
	Chl	ND	ND	15	9	14
	EtoAc	14	19	23	14	20
	Met	ND	9	14	11	13
	Ace	ND	ND	9	13	ND
	Aq	ND	ND	ND	ND	ND
<i>Cetraria spp</i>	Hex	8	16	ND	11	16
	Chl	ND	14	8	14	13
	EtoAc	ND	ND	ND	9	8
	Met	ND	ND	ND	ND	ND
	Ace	ND	19	ND	9	12
	Aq	ND	ND	ND	ND	9
<i>P. milghenensis</i>	Hex	ND	12	11	9	7
	Chl	10	12	12	14	9
	EtoAc	9	18	14	19	13
	Met	8	12	8	9	ND
	Ace	8	21	18	17	11
	Aq	ND	ND	ND	ND	ND

Legend: Hex: Hexane- Chl : Chloroform- EtoAc: Ethyl Acetate- Met: Methanol- Ace: Acetone- Aq: Aqueous, ND: not determine, \*  $\bar{x} \pm SD$  (n = 4)

Plants are known to produce certain bioactive molecules which react with other organism in the environment including bacterial or fungal growth. Lichens which are the symbiotic organisms of fungi and algae synthesize numerous metabolites, the lichen substances which comprise amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinones, chromones, xanthonones, dibenzofuranes, depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers (Clix et al., 1984).

In the study, the lichens *Usnea longifolia*, *Cetraria spp*, *Parmelia milghenensis* and *Evernastrium nepalense* were extracted in the six solvents viz. hexane, chloroform, ethyl acetate, methanol, acetone and water. There was a variation in the extract yield of the different lichens in the various solvents which indicated the variation in their chemical constituents that was shown by the phytochemical tests. The antimicrobial activities were produced in different extents by the various fractions of the four 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 and the microbes tested. The ethyl acetate fraction was found to be more effective against the pathogens tested which was similar as compared to the result obtained by **Chao et al. (2010)**. Flavonoids, a phytoconstituent in most lichens exhibit a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties (**Rankovic et al., 2007**).

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 *E. nepalense* and *U. longifolia* equally inhibited the growth of both Gram negative and positive bacteria. Numerous tests proved that the bacteria are more sensitive to antibiotics compared to fungi. The reason of difference in sensitivity between bacteria and fungi can be found in different transparency of the cell wall (**Yang et al., 1999**). The study done by **Marijiana et al. (2010)** reported the fungi to be more resistant towards lichen extracts than bacteria. But the present study revealed that the lichen extracts showed the strong antifungal activity. The extracts of *Cetraria* spp and *P. milghenensis* showed the specific antifungal activity while least or ineffective toward bacterial pathogens. **Kumar et al. (2010)** and **Ullah et al. (2009)** also demonstrated antifungal activity of lichens. Isodivaricatic acid, 5-propylresorcinol, divaricatinic acid and usnic acid have been identified as antifungal agents (**Schmeda-Hirschmann et al., 2008**).

Earlier studies revealed that the aqueous extracts of tested lichens did not show any antimicrobial activity (**Marijiana et al., 2010; Rankovic et al., 2008; Tay et al., 2004; Yilmaz et al., 2004**). This is probably because the active components produced by lichens are either insoluble or poorly soluble in water.

## CONCLUSION

The obtained results showed that the tested lichen extracts demonstrated a significant antimicrobial activity relative to the tested bacteria and fungi, which could be of significant in therapy of human and plant diseases. The wide variety of biological activities of lichens is generally correlated with their special ecological niche and with the production of metabolites that are involved in their antimicrobial actions. Further investigations on the antimicrobial activity as well as the economical and fast isolation of the metabolite from the lichens are



needed. Consequently, the antimicrobial effect of plants tested can be explained with new studies by testing other microbes and conducting the pharmacological tests. Further, research for search and isolation of the lichen metabolites, greater detail investigation in the action of lichen substances for their application is essential.

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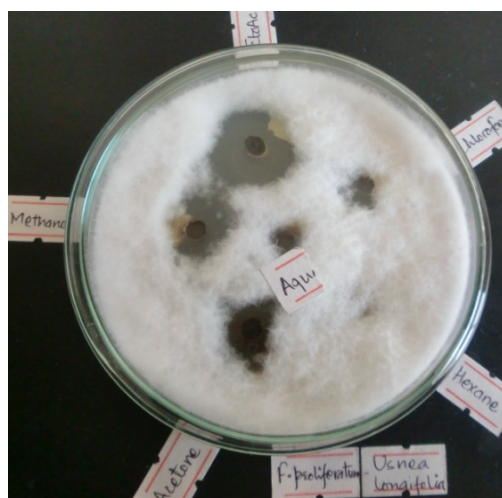
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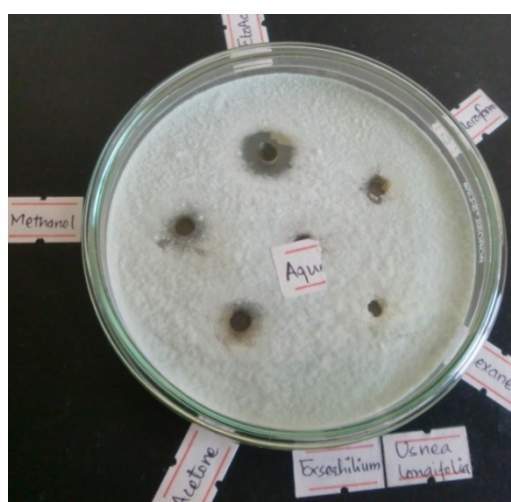
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SUPPLEMENTARY MATERIALS



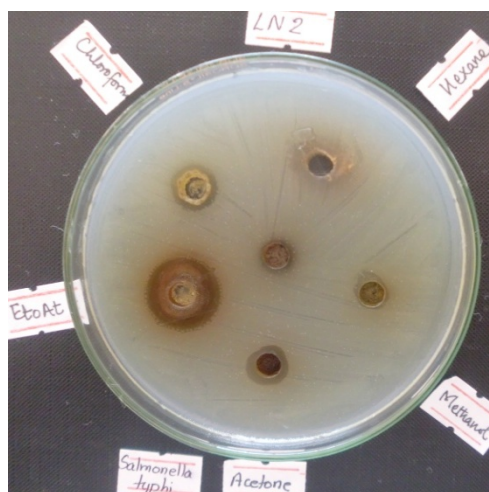
**Fig 1** Plate showing antifungal activity against *F. proliferatum* by extracts of *Usnea longifolia* after incubation at 27°C for 4 days



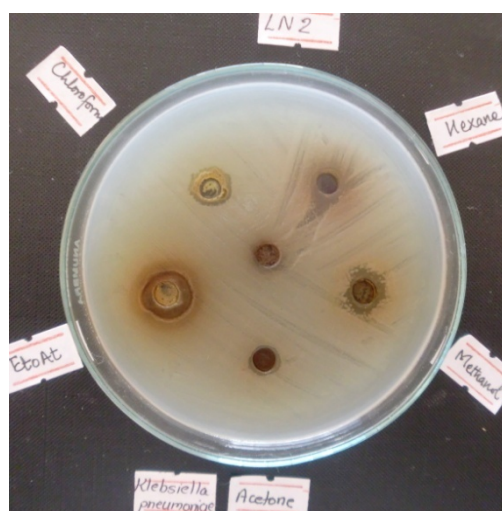
**Fig 2** Plate showing antifungal activity against *E. turticum* by extracts of *Usnea longifolia* after incubation at 27°C for 4 days



**Fig 3** Plate showing antifungal activity against *F. oxysporum* by extracts of *Usnea longifolia* after incubation at 27<sup>0</sup>C for 4 days



**Fig 4** Plate showing antibacterial activity against *S. Typhi* by extracts of *Usnea longifolia* after incubation at 37<sup>0</sup>C for 24 h



**Fig 5** Plate showing antibacterial activity against *K.pneumoniae* by extracts of *Usnea longifolia* after incubation at 37°C for 24 h



**Fig 6** Plate showing antibacterial activity against *P. aeruginosa* by extracts of *Usnea longifolia* after incubation at 37°C for 24 h