

## ***In-vitro* Antimicrobial and Antioxidant activities of Himalayan Lichen *Heterodermia obscurata* (Nyl.) Trevis**

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### **ABSTRACT**

The lichen *Heterodermia obscurata* is a foliose lichen, collected from the Munsyari Hills of the Himalayan region. In the present study, we investigated the antimicrobial, and antioxidant activities of this macro lichen. The acetone, chloroform, ethyl acetate, and methanol extracts of lichen were subjected to antimicrobial activities at 5mg/ml (10µl/disc) concentration by disc diffusion assay. Methanol extract of *H. obscurata* showed more inhibition against *A. baumannii* with zone of inhibition (ZOI) 13.2±0.5mm than other microorganisms. In contrast, the significant minimum inhibitory concentration (MIC) observed against *K. pneumoniae* was 120µg/ml. Methanol extracts showed more inhibition of *F. oxysporum* than *A. niger*. The ZOI of antimicrobial activities also summarised by Principal Component Analysis (PCA). The antioxidant activities of methanol extract are the most active than the other extracts at 1 mg/ml concentrations. The maximum free radicals scavenging activities was 68.45±0.6% calculated in methanol extract. The lichen thallus forms a potential candidate for drug discovery and development. Further studies on the isolation of active principles from the lichens and their bioactivities are under investigation.

**Keywords:** Lichen, Secondary metabolites, Antimicrobials, MDR, PCA

### **INTRODUCTION**

The lichens are symbiotic organisms composed of mycobionts and photobionts, which may be either green algae/cyanobacteria (Nash, 1996). Lichens usually grow on the surface of the bark of trees and leaves as epiphytic lichens. Various secondary metabolites synthesized from the fungal components of the lichens are unique and called "lichen compounds". These metabolites are also useful in the lichen chemotaxonomy. Lichen secondary metabolites are organized into several distinct chemical classes such as depsides, depsidones, dibenzofurans, xanthenes, and terpenes (Manojlović *et al.*, 2012) derivatives. Lichen metabolites have several properties viz. allelopathy, photoprotection, antioxidant, antimicrobial, antituberculosis (Molnár and Farkas, 2010; Cantrell *et al.*, 2001), antiherbivore, cytotoxic, analgesic, wound healing, anti-inflammatory and antitermite (Perry *et al.*, 1999; Ul Haq *et al.*, 2012; Pavithra *et al.*, 2013; Taechowisan *et al.*, 2014; Shukla *et al.*, 2014). Burkholder *et al.* (1944) reported first time the presence of antibiotic substances in lichens. These organisms have historically been used as food, and dyes, in the production of alcohol and

perfume industry (Elkhateeb *et al.*, 2022). This lichen has also been used in traditional medicine, such as in plaster material, in cuts, injury, and wounds healing (Saklani and Upreti, 1992; Tiwari *et al.*, 2011), and used as antiseptic, free radical scavenging, antilipoxygenase, antibacterial and antifungal (Balasubramanian and Nirmala, 2014). Lichen crude extracts and isolated metabolites can show to have high potential in comparison to synthetic chemicals.

Further, multidrug resistance (MDR) of pathogens is a major problem worldwide, which can cause catastrophic events in the world (Frieden, 2013). MDR is defined as the ability of insensitivity against administered antimicrobial medicine (RITM and DOH, 2014). Natural sources such as plants and fungi can provide valuable molecules to fight MDR. At present time, it is suspected that much usage of synthetic antioxidants has toxic and carcinogenic effects. Consequently, there is a growing interest in finding new antioxidants from natural resources without any undesirable effects. The present work is an attempt to investigate the antibiotic and antioxidant potential of the Himalayan lichen in this context.

## MATERIALS AND METHODS

### *Lichen collection and identification*

The lichen *H. obscurata* was selected based on their medicinal importance from traditional uses and published biological activities, the lichen was collected from the Munsiyari, District Pithoragarh, Uttarakhand (N 30° 03' 40.51", E 80° 13' 05.75" alti. 2745m) on 04<sup>th</sup> Nov. 2019. The sample was dried at room temperature for 48 hours. Collected lichen was identified at Lichenology Laboratory, CSIR-NBRI, Lucknow, based on their morphological, anatomical, and chemical characters by following methodology (Awasthi, 2007). The morphological characters were identified under a dissection microscope. Colour and spot tests were done by using chemical reagents viz., an aqueous solution of potassium hydroxide (K), aqueous calcium hypochlorite (C), and paraphenylenediamine (P). Lichen secondary metabolites were detected by thin layer chromatography using solvent system A (toluene: 1-4 dioxane: glacial acetic acid in the ratio of 180: 90: 8ml) (Culberson and Kristinsson, 1970; Walker and James, 1980). The demonstration samples are preserved in the facilities of the PDSH division of LWG herbarium.

### *Extract preparation*

The cleaned shade air-dried (Santiago *et al.*, 2010) lichen sample was ground into fine powder and subjected to cold percolation extraction by using four different organic solvents acetone (Ac), chloroform (Ch), ethyl acetate (Ea) and methanol (Me) at 100ml/10gm of solvent and lichen powder. Extraction duration were varied from 3-4 days. The solvent extract was collected and filtered through Whatman No.1 filter paper and the solvent was further concentrated under reduced pressure in the rotary evaporator system to completely remove the solvent. Before storage at -18°C (Kosanić *et al.*, 2016), the extract samples were calculated to know the percent yield by following equations.

$$\text{Percent yield (\%)} = \left[ \frac{\text{(Dry weight of crude extracts)}}{\text{(Dry weight of dry samples)}} \right] \times 100$$

### *Microorganisms*

The pathogenic microorganisms were procured from Hi-Media. The normal pathogens are *A. tumefaciens* (1D1609), *Bacillus subtilis* (ATCC6051)

and the MDR pathogens are *Acinetobacter baumannii* (ATCC19606), Methicillin-resistant *Staphylococcus aureus* MRSA (ATCC43300), *Klebsiella pneumonia* (ATCC1705). The fungus *Aspergillus niger* (ATCC 16880) and *Fusarium oxysporum* (ATCC 62705) are plant pathogens utilized for antifungal tests. The bacterial culture was maintained at Mueller Hinton (MH) agar while the fungal culture was maintained at Potato Dextrose (PD) Agar medium.

### *Antimicrobial activities*

The antimicrobial activities were tested at 5mg/ml concentration dissolved into 1ml (20% DMSO) with distilled water. Microbial inoculum was obtained from cultures medium and maintained the dilution according to the 0.5 McFarland technique to approximately 10<sup>8</sup> CFU/ml in case of bacteria and fungus 10<sup>6</sup> CFU/ml. Turbidity of microbial culture were determined by spectrophotometer at 530 nm recommended by NCCLS (1998). The antimicrobial activities of the extracts were done by the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1966) at 10 µl/disc against selected pathogenic bacteria and fungi and the extract was loaded on disc of 6 mm diameter. Streptomycin (S<sup>10</sup>) and Ketoconazole (KT<sup>10</sup>) are used as positive control against selected bacteria and fungi. Experiments proceeded in triplicates and the zone of inhibition was measured in millimetres. The MIC was tested at the range between 10 to 0.001 mg/ml by the broth microdilution assay using 96-well microtiter plates (Sarker *et al.*, 2007). The MIC was determined by the visible growth of microorganisms after incubation at 37°C for bacteria and 25°C for fungus. The boundary dilution without any visible growth in well defined as the values of MIC for the tested microorganism at the given concentration. Principal Component Analysis (PCA) was used to summarise the most effective ZOI from the lichen extracts against bacteria and fungi. The tested lichen extracts were compared with the positive control. PCA was done by utilizing a correlation matrix and multivariate option in PAST 4.03 (Rubnawaz *et al.*, 2021).

### *Antioxidant activity*

#### *DPPH radicals scavenging activities*

The free radical scavenging activity of lichen extracts was investigated with 1,1 diphenyl 2-picryl-hydrazine (DPPH) by following methodology (Gadow *et al.*, 1997; Dorman *et al.*, 2004) with little modification. Prepared the 0.05

mg/ml concentration of DPPH in 2 ml of methanol solvent, with 1 to 0.5 mg of lichen extracts separately and ascorbic acid was used as standard. The prepared stock solution was shaken vigorously and left for 30 min at room temperature. finally, 1 ml of the stock solution was put into the cuvettes and free radicals scavenging activities were tested with a spectrophotometer by taking absorbance at 517 nm (Model: Lambda 35, Perkin Elmer). The following equation was used to calculate the DPPH radical scavenging activities.

[DPPH radical scavenging (%) =  $(A_0 - A_1/A_0) \times 100$ ]. A1 is the absorbance of the reaction mixture or standards and A0 is the absorbance value of the negative control.

## RESULTS

### Characteristics of lichen

The lichen thallus are corticolous and upper side greyish darker while lower side yellow-brown (Figure 1). Various chemical constituents were detected by colour test during microscopy, which explained the medulla of thallus K<sup>+</sup> yellow. In the TLC, compounds atranorin, chloroatranorin and zeorin were detected (Figure 2).



Figure 1. *Heterodermia obscurata* (Nyl.) Trevis

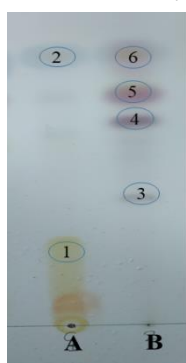


Figure 2. Chemical analysis by TLC, 'A'-control of *Parmelinella wallichiana* (Taylor) Elix & Hale and 'B' selected lichen specimen

### Lichen extracts

Out of the four solvents system used for the extraction, methanol solvent exhibits maximum yield (0.76%) of extract followed by the extracts of acetone, ethyl acetate and chloroform. Percent yields were calculated in milligram (Figure 3).

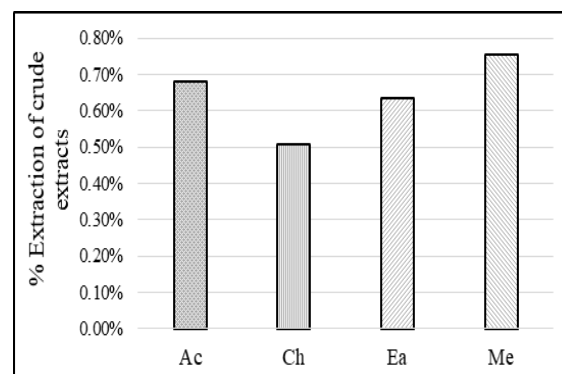
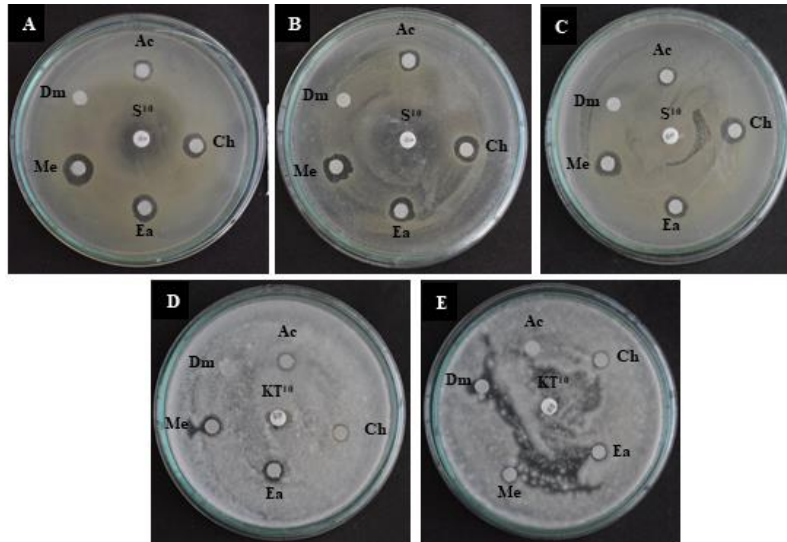


Figure 3. Percent yields of crude extracts in various solvents

### Antimicrobial screening

Antimicrobial activities of various extracts of *H. obscurata* were tested against bacteria and fungi. Methanol extracts of lichen showed better antimicrobial activities followed by the acetone and other extracts (Figure 4). *Acinetobacter baumannii* was much more affected with ZOI of  $13.2 \pm 0.5$  mm from methanol extract. The other pathogenic strains are significantly inhibited and ZOI range from (>10-12mm). At the same time, a significant MIC was observed at 120 µg/ml against *K. pneumoniae*. Acetone extracts also showed significant antimicrobials activities against all selected pathogens while *S. mutans* are much more affected with  $10.8 \pm 0.5$  mm ZOI. In contrast, the significant and effective MIC was 240 µg/ml observed against *B. subtilis*. Extracts of ethyl acetate and chloroform showed little antimicrobial activity even *K. pneumoniae* was inhibited with  $9.4 \pm 0.3$  to  $9.3 \pm 0.4$  mm ZOI from ethyl acetate and chloroform extracts respectively. We have found that the fungal strains are almost less sensitive for the lichen extracts, while methanol and ethyl acetate showed better antifungal activities  $9.8 \pm 0.4$  and  $8.4 \pm 0.4$  mm ZOI against *A. niger*. The lichen extracts are mostly comparable to antibiotics at the given concentration while the MIC values are more significant than the lichen extracts (Table 1).



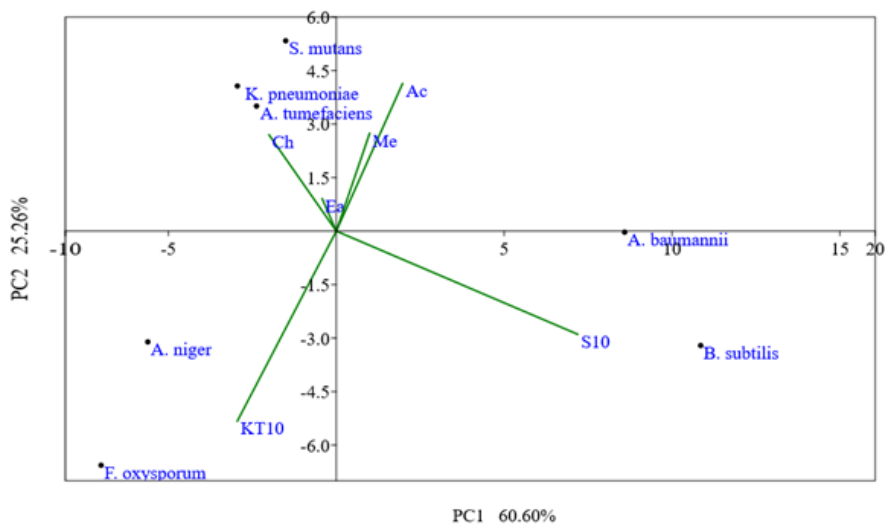
**Figure 4.** Antimicrobial activities of *Heterodermia obscurata*. A=*A. baumannii*; B=*A. tumefaciens*; C=*K. pneumoniae*; D=*A. niger*; E=*F. oxysporum*; Ac-acetone; Ch-chloroform; Ea-ethyl acetate; Me-methanol; Dm-Dimethyl sulfoxide; S<sup>10</sup>-streptomycin; KT<sup>10</sup>-Ketoconazole.

Principal component analysis has been used to summarise the most effective ZOI and potential extracts against pathogens. Acetone extracts showed higher antibacterial activities only against *S. mutans*. While the methanol extracts showed a high diversity of antimicrobial activities with higher ZOI grouped with *K. pneumoniae*, *A.*

*tumefaciens*, *A. baumannii*, and *S. mutans*. Ethyl acetate and chloroform are the least effective. The bacteria *A. baumannii* and *B. subtilis* make a separate group and get similar effects with streptomycin similarly, *A. niger* and *F. oxysporum* were very less affected with ketoconazole (**Figure 5**).

**Table 1.** Antimicrobial activities of lichens extracts; ZOI measured in mm after disc diffusion, and MIC tested in 96 well plates

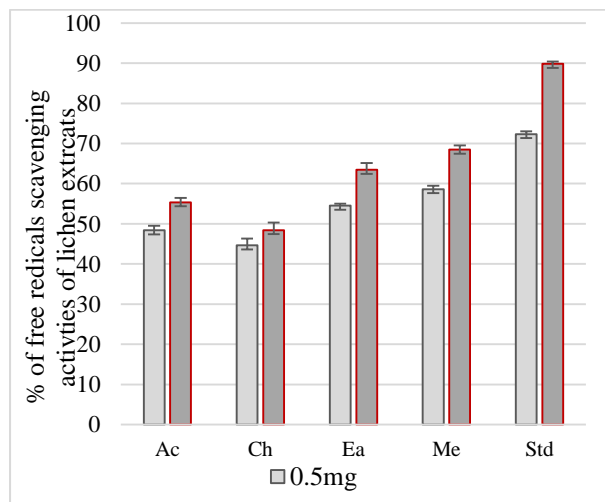
S. N.	Microorganism	Ac		Ch		Ea		Me		S10		KT10		DMSO	
		ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC	ZOI	MIC
1	<i>A. baumannii</i>	7.4±0.4 5	440	8.4±0.4	870	8.3±0.3	760	13.2±0.5	124	12.7±0.5	0.053	--	--	00	00
2	<i>A. tumefaciens</i>	7.2±0.2	320	7.4±0.4	950	8.7±0.2	580	11.7±0.2	320	00	nt	--	--	00	00
3	<i>B. subtilis</i>	8.8±0.5	240	00	nt	6.8±0.5	640	10.4±0.5	220	12.9±0.5	0.045	--	--	00	00
4	<i>S. mutans</i>	10.8±0.5 5	530	7.6	nt	7.9	nt	11.9±0.5	340	00	nt	--	--	00	00
5	<i>K. pneumoniae</i>	6.5±0.2	320	9.3±0.4	840	9.4±0.3	314	12.4±0.5	120	00	nt	--	--	00	00
6	<i>A. niger</i>	6.8±0.4	840	6.8±0.5	742	8.4±0.4	230	9.8±0.4	520	--	--	8.7	0.072	00	00
7	<i>F. oxysporum</i>	00	nt	6.7±0.5	640	7.5±0.4	456	7.8±0.3	543	--	--	7.6	0.042	00	00



**Figure 5.** Principal component analysis to summarise the ZOI of antimicrobial activities in various extracts

### DPPH radical scavenging

The free radicals scavenging activities of lichen extracts increased at increasing concentrations of extracts. More than 50% radical scavenging activities were observed in all tested extracts except



**Figure 6.** Free radicals scavenging activities of lichen extracts

### DISCUSSION

The discovery of various drugs and antibiotics is considered one of the major milestones in pharmaceuticals and represents a fundamental triumph of science in medicine. However, inappropriate use of these antibiotics and genetic evolution in microorganisms resulted in the development of resistance against pathogens. In different countries, the use of various drugs such as aminoglycosides, penicillin, and cephalosporin is no longer effective against certain pathogenic microorganism. The resistance development in microorganisms increases the potential to cause diseases and poses a serious health problem. The Reactive Oxygen Species (ROS) viz. hydrogen peroxide, hydroxyl radical and superoxide anion, peroxy nitrite, and nitric oxide plays a significant role in maintaining the biochemical processes and cells maturation (Halliwell, 1996). Occasionally free radicals are involved in undesirable activity in the cell and damage of tissue, and biomolecules such as protein, nucleic acid, and fatty acid. Thus further onset of various diseases, such as Atherosclerosis, Alzheimer's, Hemochromatosis, Cancer and aging process (Young and Woodside, 2001; Huda-Faujan *et al.*, 2009; Marante *et al.*, 2003).

the chloroform extract (48.41%) at 1mg concentration. Methanol extracts showed the highest 68.45% of radical scavenging activities. Ethyl acetate is the second strongest (63.46%) free radicals scavenging extract at the tested concentration (**Figure 6**).

Today's variety of natural compounds reported in the plants, augment with phytochemicals play a vital role in human and animal bodies and safeguarding against a spectrum of diseases (Rayavarapu *et al.*, 2011). The methanol extracts of tested lichens showed higher antimicrobial and antioxidant activities. The present study also supported by Kekuda *et al.* (2015) tested the antimicrobial activities in *H. obscurata* against various gram-positive and gram-negative bacteria among them the interesting ZOI 2.3, 2.2, and 2.1cm observed against *Klebsiella pneumoniae*, *Bacillus coagulans* and *Staphylococcus aureus* respectively, almost similar activities also observed in positive controls, authors also reported the secondary metabolites in TLC such as atranorin, chloroatranorin and zeorin. Raghavendra *et al.* (2017) also reported antimicrobial activities in methanol extracts of *Heterodermia incana* against *Bacillus cereus* and *Pseudomonas aeruginosa* with ZOI 2.26±0.05 and 1.76±0.05cm, and antifungal activities reported at 1mg concentration against *Fusarium sp.*, *Curvularia sp.*, and *Alternaria* species. The various biological activities of lichen compounds and extracts also validated by Kumar and Müller (1999); Hidalgo *et al.* (1994). Türk *et al.* (2006) isolated the compounds atranorin, chloroatranorin, olivetoric acid and physodic acid from *Pseudevernia furfuracea* (L.) Zopf, showed diverse antimicrobials activities. The compounds atranorin and physodic acid isolated from various species of lichens exhibits anti-neurodegenerative, antioxidant, antidiabetic and anticancer activities (Reddy *et al.*, 2016; Thadhani, 2013). The compound zeorin showed the antimicrobials, antioxidant, and antidiabetic activities reported by Karunaratne *et al.* (2014) and Behera *et al.* (2005). Pereira *et al.* (2010) identified and characterized the compounds glucomannan from the *H. obscurata* might have the potential to reduce leukocyte migration and anti-inflammatory, antinociceptive activities. Gupta *et al.* (2007) found antimycobacterial activity against a virulent strain of H37Rv susceptible at MIC 250 mg/ml from ethanol extract of related species of *H.*

*leucomela*. Prabhu *et al.* (2019) made the nanoparticles from the methanol extracts of *H. boryi* and tested the antimicrobial activities and got better results against *A. baumannii*, *Viridans streptococci* and MRSA-Methicillin

Resistant *Staphylococcus aureus* with ZOI 22±2.0, 14±0.0 and 11±0.0mm respectively. In our study the methanol extract of lichens showed higher antioxidant activities, similarly, Behera *et al.* (2016) presented positive results of DPPH radical scavenging (52%) and lipoxygenase inhibition from ethyl acetate extracts of *H. pседospeciosa*.

## CONCLUSION

The selected lichen species of *Heterodermia* had shown mild to moderate antimicrobial activities in acetone, ethyl acetate, and chloroform extracts against bacteria and fungi. On the other hand, the methanol extracts showed the highest antimicrobial activities. It may be due to the presence of various secondary metabolites in the lichen thallus that are easily dissolved in the methanol. Because of interesting antimicrobials and antioxidant activities, further studies would be required to isolate and characterize the constituents possessed by the lichen species that should be tested. Probably this could explain the mode of action of bioactivities and usefulness of the lichen metabolites.

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**Data availability** All data and materials are available upon request.

**Ethical approval is not applicable.**

**Competing interests:** The authors declare no competing interests.

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