SHORT COMMUNICATION

Analysis of Antimicrobial Properties of Lichen Taxa *Usnea eumitrioides* Motyka against Plant Pathogens

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Abstract

Fresh samples of fruticose lichen (*Usnea eumitrioides*) were collected from a *Quercus* Forest of a temperate region, Almora, Uttarakhand Himalaya. This lichen species abundantly occurred from temperate to alpine regions. The lichen genus *Usnea* is a good source of secondary metabolites and is applied in the treatment of various diseases. In the present investigation, *U. eumitrioides* was studied for its antimicrobial activity. It was obtained against five bacterial and two fungal pathogens, respectively, in a solvent extract of ethanol and ethyl acetate. It was observed that it shows antimicrobial activity against all the tested plant pathogens. The ethyl acetate extract shows significant inhibitory effects on the growth of *Pseudomonas syringae* with a mean area of inhibition of 7.47 ± 0.057 mm at a concentration of 0.2 mg/mL. The ethanol extract obtained from lichen exhibited higher inhibitory activity against *P. syringae* and it showed poor activity against *P. aeruginosa*. The study concluded that lichens have great potential to treat and manage the diseases affecting humans, animals, and plants.

Keywords: Antimicrobial activity, Fruticose lichen, Plant pathogens, Temperate forests.

Highlights

- The lichen genus *Usnea* is well-explored for its secondary metabolites. However, very few studies were found on the antimicrobial activity of *U. eumitrioides*.
- This lichen is abundant in the Indian Himalayan regions particularly in temperate to alpine areas of Uttarakhand, where the present study was done.
- It is our original work that contributes to plant science, particularly in the field of lichens.
- The datasets generated during and/ or analyzed during the current study are available from the corresponding author upon reasonable request.

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Introduction

Lichen comprises a unique group of plants that consists of two unrelated organisms, a mycobiont and a phycobiont, growing together in a close symbiotic association. In this way, the fungal partner provides most of the structure and mass of the composite organism. It includes numerous fungal, bacterial and other microscopic organisms and is always grouped with the lichens (Hawksworth and Grube, 2020). More than 2000 species of lichens represent our country and, which is about 10% of the world's lichen diversity. In India, particularly temperate to alpine regions of 12 Himalayan states contribute maximum diversity and biomass of lichens (Kumar *et al*., 2009). Lichen provides a valuable source for preparing medicines, condiments, perfumes, food, fodder and other miscellaneous items. Many foliose lichens, such as *Everniastrum* and *Pramotrema* species, are widely used by the tribal communities to prepare spices. The fruticose lichens- usnioid and ramalinoid forms are also used to prepare traditional medicines. Some species of lichens are also applied in the preparation of ethnic dyes and as stuffing material (Upreti *et al*., 2005). *Evernia furfuracea* was recorded in an Egyptian pot belonging to 18th Dynasty (1700-1600 BC) to preserve the smell of spices used in the preservation of mummies (Llano, 1948). Besides, lichens also play a significant role in ecological biomonitoring and lichenometry studies (Maser *et al*., 1985; Pike, 1978; Berryman & McCune, 2006)). About 800 secondary metabolites are produced by lichens, which are useful for the development of antibiotics, analgesics, antioxidants, antiviral, antimicrobial, anti-inflammatory, antipyretic, antiproliferative, and cytotoxic activities (Huneck and Yoshimura, 1996; Boustie and Grube 2005).

The lichen genus *Usnea* is well explored for its secondary metabolites known as usnic acid, which has pharmaceutical importance. The genus *Usnea* comprises more than 300 species worldwide (Ohmura, 2012). Out of which India represents 60 species (Singh and Sinha, 2010). It is a fruticose, long hair-like branched lichen hanging from a tree trunk, branches and twigs in high altitude forests of the Himalayas. Various researchers have done many antimicrobial activities of lichens in the past. However, less exploration of the antimicrobial activity of *U. eumitrioides* was observed. The genus *Usnea* is regularly fallen lichen taxa along with some other species of lichens in a brown

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oak forest (Kumar, *et al*., 2009). The genus has also been found to be an excellent contributor to lichen biomass in the area. Therefore, the present investigation has been done on the antimicrobial properties of *U. eumitrioides*.

Material and methods

Lichen sample collection for their antimicrobial activity

Fresh samples of *U. eumitrioides* were collected from Mornaula *Quercus* mixed forest district Almora (Uttarakhand), India. The area falls in 29°30'N to 30°20'N latitudes and 79°20' E to 80°20'E longitudes and the elevation range from 1800 to 2200 m amsl. The collected samples were studied morphology by using chemical and anatomical techniques at the Lichenology Laboratory, CSIR-NBRI Lucknow (India) (Joseph *et al*., 2018). The voucher specimens (herbarium sample No. 12) were preserved at the Biodiversity Conservation Laboratory, Department of Botany, Soban Singh Jeena University Campus at Almora (Uttarakhand).

Preparing lichen extract

The dried powder of *U. eumitrioides* was ground carefully by using a sterile mortar and pestle. Now, 10 g of this powder was soaked in 100 mL of soluble solvent of ethanol and ethyl acetate for 72 hours at normal temperature. Extracts were filtered and under reduced pressure, the filtrate was evaporated. Then, extracts were tested for their antimicrobial activity.

Antibacterial activity

Microorganism

Total five bacterial strains, including four gram-negative such as *Pseudomonas aeruginosa* (MTCC-1934), *Salmonella typhi* (MTCC-734), *Xanthomonas compestris* (MTCC-2286) and *P. syringae* (MTCC-1604) and one gram-positive *Staphylococcus aureus* (MTCC-4734) were used to test. Besides, two fungal pathogens (*Fusarium oxysporum* and *Alternaria alternate*) were also taken to evaluate the antimicrobial capacity of the test lichen thallus. These Bacterial strains were put on nutrient agar plates at 4°C.

Agar well diffusion assay

The lichen extracts were dissolved in respective solvents with 0.1 and 0.2 mg concentrations. The extracts were monitored by using ciprofloxacin as a positive control and selected solvents as a negative control. To obtain fresh cultures, an appropriate suspension of the test bacterium was inoculated and incubated at 37°C for 16 to 18 hours. The petri dishes were sterilized and poured with 20 mL of LB agar media. About 100 µL of fresh inoculum was coated and kept for 10 minutes to make it dry. Four wells of 6 mm diameter were prepared on the agar plates. For this purpose, an agar punch was applied. The selected two concentrations 0.1 and 0.2 mg, along with control standards, were added to each labeled well. The three sets of petri dishes were made and incubated for 16 to 18 hours at 37°C. The antibacterial property of every compound was estimated by taking the diameter (mm) of the inhibition area (Barrow and Feltham, 1993; Thippeswamy *et al*., 2011; Srivastava *et al*., 2013).

Antifungal activity

The agar well diffusion technique was taken to examine the antifungal property of lichen species (*U. eumitrioides*) against fungal pathogen- *Fusarium oxysporum* and *Alternaria alternata*. Again, the same quantity of extracts and concentrations were taken. In this way, the extracts were monitored with fluconazole as positive control and the solvents as negative control. To obtain fresh cultures, an appropriate suspension of the test fungal pathogen was also inoculated and incubated at 37°C for 16 to 18 hours. Sterilized petri dishes were again poured with PDA agar media. The antifungal property of every compound was estimated by measuring the diameter of the inhibition area, too (Thippeswamy *et al*., 2011).

Statistical Analysis

All the experiments were conducted in triplicate and results were shown in mean values with ± standard error. Data was calculated in a two-way analysis of variance (ANOVA) and $p < 0.05$ was considered significant.

Result

Differential antimicrobial activity was obtained against five bacteria and two fungal pathogens, respectively, in a solvent extract of ethanol and ethyl acetate. Both concentrations of the ethanol and ethyl acetate extract, 0.1 and 0.2 mg/mL, respectively, exhibit antimicrobial properties against all tested gram-negative bacteria strains, as well as exhibiting efficacy against one gram-positive bacterial strain. The ethyl acetate extract demonstrated significant strong impacts on the growth of *Pseudomonas syringae* with a mean area of inhibition of 7.47 ± 0.057 mm at a concentration of 0.2 mg/mL. The ethanol extract obtained from lichen exhibited the most potent inhibitory activity against *P. syringae,* with a mean area of inhibition measuring 13.50 ± 0.057 mm at the same concentration of 0.2 mg/mL. The ethyl acetate extract showed poor effect against *P. aeruginosa* with area of inhibition 2.50 ± 0.057 and 3.50 ± 0.057 at 0.1 mg/mL followed by 0.2 mg/mL concentration, respectively. However, ethanol extract displayed the most significant effect with area of inhibition of 11.03 ± 0.088 (0.1 mg/mL); and 12.07 ± 0.033 (0.2 mg/mL) (Table 1 & Figs 1 and 2).

The ethanolic extract obtained from *U. eumitrioides* demonstrated significant antibacterial activity, leading to the formation of a considerable zone of inhibition when subjected

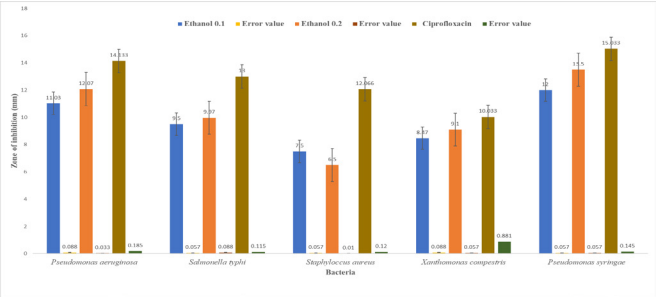

Fig. 1: Graph showing zone of inhibition of *U. eumitrioides* extracts in different solvent at different concentration against bacteria; Ethanol 0.1 Ethanol 0.2

Table 1: Area of inhibition (mm) of extracts of *U. eumitrioides* against tested microorganisms

Microorganism	*Bacterial strain No.*	*Ethanol*		*Ethyl acetate*		*Control*
Bacteria		*0.1*	*0.2*	*0.1*	*0.2*	*Ciprofloxacin (3µg/mL)*
P. aeruginosa	MTCC-1934	11.03 ± 0.088	12.07 ± 0.033	2.50 ± 0.057	3.50 ± 0.057	14.133 ± 0.185
S. typhi	MTCC-734	9.50 ± 0.057	9.97 ± 0.088	10.51 ± 0.057	10.03 ± 0.088	13 ± 0.115
S. aureus	MTCC-4734	7.50 ± 0.057	6.50 ± 0.01	5.47 ± 0.088	6.53 ± 0.088	12.066 ± 0.120
X. compestris	MTCC-2286	8.47 ± 0.088	9.10 ± 0.057	9.00 ± 0.057	8.47 ± 0.088	10.033 ± 0.881
P. syringae	MTCC-1604	12.00 ± 0.057	13.50 ± 0.057	6.00 ± 0.011	7.47 ± 0.057	15.033 ± 0.145
Fungus						Fluconazole (3 µg/mL)
F. oxysporum		9.1 ± 0.057	8.1 ± 0.057	8.7 ± 0.057	7.6 ± 0.057	10.9 ± 0.057
A. alternata		4.4 ± 0.057	5.1 ± 0.057	5.4 ± 0.057	5.2 ± 0.057	9.86 ± 0.120

(Values are in mean ± Standard error)

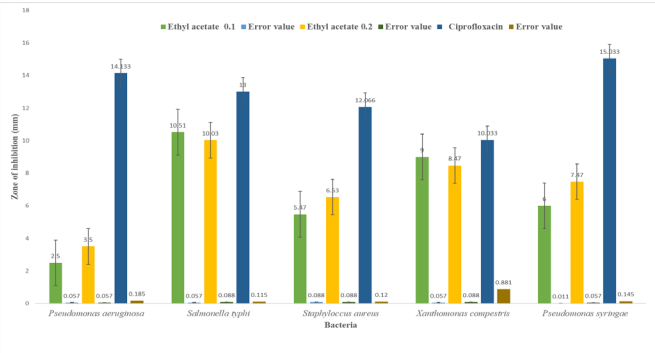

Fig. 2: Graph showing zone of inhibitions of ***U. eumitrioides*** extracts in different solvent at different concentration against bacteria; Ethyl acetate 0.1 Ethyl acetate 0.2

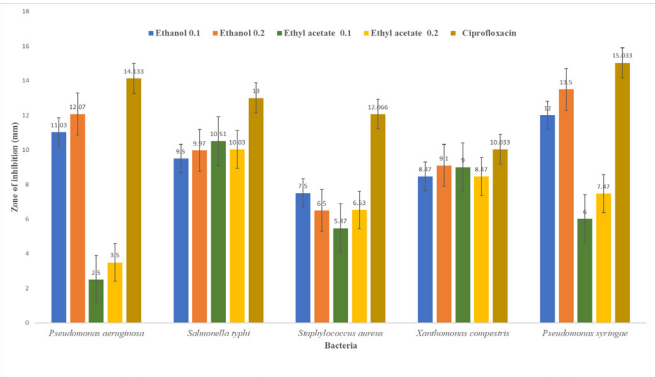

Fig. 3: Graphical representation of comparative analysis of *U. eumitrioides* ethanolic and ethyl acetate extracts at different concentration against bacteria; Ethanol and Ethyl acetate

to testing against *P. syringae* followed by *P. aeruginosa*, *S. typhi*, *X. compestris* and *S. aureus* (Table 1 & Figs 1-3).

Ethyl acetate extract of *U. eumitrioides* showed a high area of inhibition against *S. typhi* and poor activity against *Pseudomonas aeruginosa*. The effectiveness of all samples is shown in a dose-dependent manner, where 0.2 mg/mL is shown as an inhibition area compared to 0.1 mg/mL.

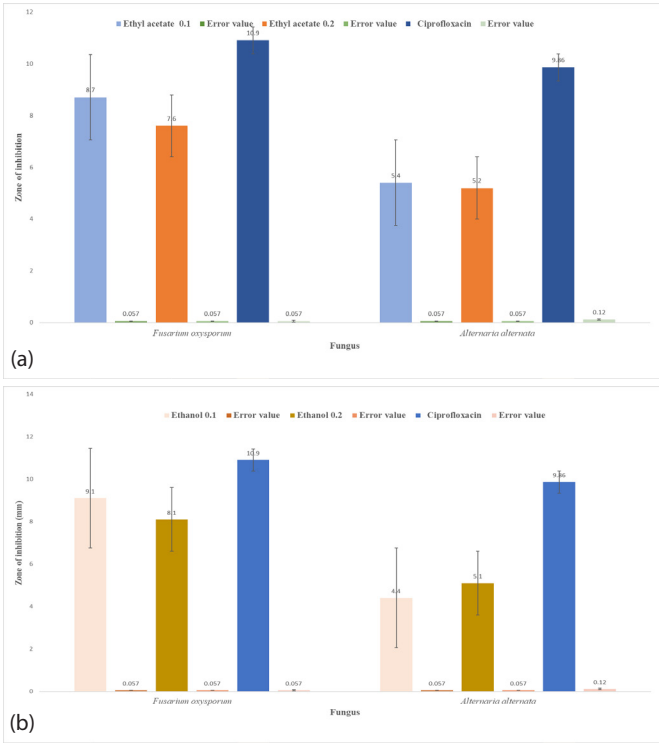

Fig. 4: Graphical representation of *U. eumitrioides* ethanolic and ethyl acetate extracts at different concentration against fungal pathogen; Ethyl acetate; Ethanol

Among fungi, both *F. oxysporum* and *A. alternata* extracts of *U. eumitrioides* displayed significant antifungal effects. The ethanol extract was marked with 9.1 ± 0.057 at 0.1 mg/mL maximum zone of inhibition against *F. oxysporum*. However, low activity against *A. alternata* with 4.4 ± 0.057 at 0.1 mg/mL. *U. eumitrioides* exhibited the highest area of inhibition against *F. oxysporum* (8.7 ± 0.057 in 0.1 mg/mL). *A. alternata* showed less area of inhibition (5.4 ± 0.057 in 0.1 mg/mL) with the ethyl acetate extract (Table 1 & Fig. 4).

Discussion

The experiment revealed that the screened extracts of lichen thallus exhibited an important and high antimicrobial

properties. The screening analysis demonstrated that all of the examined lichen extracts possess antibacterial attributes against both bacterial strains. However, the observed antibacterial activity in the study depended upon several factors, including the specific type of lichen material utilized, its engrossment and the particular strains of microbes under examination. Lichens produce antibiotic secondary metabolites that serve as a natural defense mechanism against a wide range of pathogens found in the environment (Molnar and Farkas, 2010). The antibacterial activity of ethanolic extracts against gram-negative bacteria *P. aeruginosa* was also studied by (Srivastava *et al*., 2013; Rauf *et al*., 2011; Kamal *et al*., 2015 Kumar *et al*., 2017)**.** A notable antibacterial activity against *P. aeruginosa* in the ethanolic extract in contrast to the ethyl acetate, was also observed in the present study.

Toksoz *et al*., (2022) evaluated the antibacterial potential of used lichens against the growth of *S. aureus* at different concentrations and no significant antibacterial effect on *Salmonella* sp. was found. However, in the present investigation, *U. eumitrioides* extracts showed significant activity against *Salmonella*. Besides *U. eumitrioides* extracts, ethanol extract of *U. longissima* also shows an important antibacterial effect against all the plant pathogens used in the present study (Thippeswamy *et al*., 2011). The acetone and ethanol extracts of *U. ghattensis* were also studied and it showed activity against *S. aureus* and *P. aeruginosa* pathogens (Srivastava *et al*., 2013). Likewise, extracts of *U. eumitrioides* demonstrated significant antimicrobial activity against the same bacterial pathogens. The antifungal property of *Usnea* sp. against *F. oxysporum* was also studied, in which microdilution assay showed that methanol acetone extracts had inhibition zone diameters of 11.3 and 12.6 mm (Shivanna and Garampalli, 2014). No inhibition zone was observed in ethyl acetate extract. In the present study, ethyl acetate extract of *U. eumitrioides* shows a noticeable area of inhibition in response to its evaluation against the same fungal pathogen, i.e., *F. oxysporum*.

Conclusion

In the current study, it is evident that *U. eumitrioides* exhibits antimicrobial properties and shows efficacy against both gram-positive as well as gram-negative bacteria. It provides evidence of the potential use of lichens in treating various diseases caused by pathogens. Additional research is needed on the antimicrobial activity of the metabolite derived from the lichens. Consequently, lichens are a safe and benign natural antimicrobial agent, offering promising applications in the management of diseases affecting humans, animals and plants. Future research will search for the exploration of novel lichen metabolites through diverse solvent extraction methodologies. Furthermore, the future researcher will contribute on mechanism of action of lichen compounds in more detail and might synthesize new and possibly more potent derivatives for prospective applications.

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Author's contribution

The research scholar (Ms. Ritika Tamta, Co-author) registered for her Ph.D. at our university, SSJ University Almora (India). She had an objective in her research synopsis on biomass estimation of fallen lichens (lichen litter). In this context, she collected more than 100 samples of lichens from the wild (study area). Indian Himalayan Region (IHR) is the abode of many plant species. So, the researchers simply collect plant samples from the forest for their research purpose without causing any harm to the forest. The high-altitude forests particularly oak forests, possess rich lichen diversity and biomass. The candidate had collected lichen samples along with *U. eumitrioides* Motyka directly from the wild for further identification. She also prepared the manuscript under the direction of her Ph D supervisor (Dr. Balwant Kumar).

Dr. Balwant Kumar and Dr. Sanjeeva Nayaka have revised the manuscript.

Conflict of Interest

The authors have no conflict of interest.

Statements and Declaration

We have not submitted this paper to any other journal.

There is no plagiarism in the provided paper and all the data and ideas are original.

Authors are responsible for the correctness of the statements provided in the manuscript.

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