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Antimicrobial Activity of *Usnea ghattensis* G. Awasthi and *Usnea undulata* Stirt

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ABSTRACT

Microorganisms, in particular bacteria and fungi, are known to cause a number of diseases in plants and animals. Many pathogenic microorganisms have developed resistance against commonly used antibiotics and synthetic fungicides. Lichens and their metabolites are shown to be promising alternatives for synthetic chemicals. The lichen genus Usnea (Parmeliaceae) is characterized by pendulous growth and the presence of usnic acid. The present study was conducted to determine antimicrobial activity two Usnea species namely U. ghattensis G. Awasthi and U. undulata Stirt. The lichens were identified on the basis of morphological, anatomical and chemical tests. Extraction was carried out by maceration using methanol as solvent. Antibacterial activity was determined against a panel of 8 test bacteria by Agar well diffusion assay. Antifungal activity was assessed by Poisoned food technique against three phytopathogenic fungi. TLC revealed the presence of Usnic acid in both lichens. Both lichens inhibited test bacteria in a concentration dependent manner. Inhibition of Klebsiella pneumoniae was marked when compared to other bacteria. Extracts of both lichens inhibited test fungi dose dependently. Diameter of fungal colonies in poisoned plates was considerably lesser when compared to control plates. Inhibitory activity was highest against Bipolaris sorokiniana followed by Colletotrichum capsici and Fusarium oxysporum. The promising inhibitory activity of both Usnea species could be ascribed to the presence of secondary metabolites mainly usnic acid. These lichens appear to be promising candidates for the development of antimicrobial agents. Further studies on isolation of active principles from lichen extracts and their bioactivity determinations are to be carried out.

Keywords*:* Lichens; *Usnea*; Agar well diffusion; Minimum inhibitory concentration; Poisoned food technique

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INTRODUCTION

Discovery of antibiotic seems to be one of the greatest discoveries in the field of medicine. The large scale production and subsequent use of these antibiotics resulted in dramatic decrease in mortality and morbidity. However, the use of these wonder drugs faced a serious problem of development of resistance in pathogenic bacteria in community as well as hospital settings. The dissemination of resistance gene from resistant pathogens to susceptible strains makes the situation more complicated. These resistant microbes make the therapy more difficult. Besides, the use of antibiotics suffers from other drawbacks such as high cost and adverse effects on the health of individual [1-6]. Fungi cause more number of diseases in crops and results in devastating effects on crop productivity leading to economic loss to farmers. In severe cases, a crop loss of >50% occurs. The incidence of fungal diseases is usually retarded by the use of chemicals. The use of these chemical agents is associated with drawbacks such as high cost, residual effects on environment, effects on non-target organisms including humans and more importantly the emergence of fungicide resistant fungal pathogens. Searching antimicrobial agents from natural sources seems to be an important strategy for treatment of bacterial infections and controlling phytopathogenic fungi [5,7-10]. Lichens and their metabolites are shown to be effective against a variety of pathogenic bacteria (including drug resistant strains) and phytopathogenic fungi [6,11-13].

Lichens represent the most fascinating and widely distributed organisms on earth. They are composite organisms being represented by a photosynthetic partner (phycobiont or photobiont, an alga or a cyanobacterium) and a fungal partner (mycobiont) exhibiting mutualistic interaction with each other. Lichens exhibit high tolerance to drought and cold by virtue of their peculiar structure and physiology. Lichens are capable of growing in the

diverse regions and on any substratum that provides a convenient foothold to them. This may be terricolous (soil), humicolous (humus), saxicolous (rocks), follicolous (leaves), corticolous (tree trunk), lignicolous (decaying wood). Besides, lichens also grow on monuments and other man-made substrata such as iron pipes, asbestos sheet, lime or cement plaster and glass panes. Lichens have different growth forms such as foliose (leaf like), crustose (firmly attached to substratum) and fruticose (hanging and bushy). In many parts of the world, lichens have been used traditionally as medicine by various indigenous communities for treating several ailments. Lichens have been used in various systems of medicine such as Ayurveda and Unani. Lichens have been utilized in the preparation of food and in the manufacture of perfumes and dyes. Lichens form the primary food for reindeer and caribou especially during winter where there is scarcity of food. Lichens are considered as one of the best indicators of air pollution. Lichens synthesize a range of secondary metabolites by metabolic pathways namely Acetyl-polymalonate pathway, Shikimic acid pathway and Mevalonic acid pathway. These metabolites are of taxonomic importance and are known to exhibit diverse bioactivities. India is one of the countries with rich lichen flora. About 10% of the total lichen species are found in India. Western Ghats and Himalayas are known to harbor a rich diversity of lichens. The lichens are widely distributed in tropical, subtropical, temperate and alpine regions of India [14-18].

Among various genera of lichens, the 'beard-like' genus *Usnea* Dill. ex Adans. (Parmeliaceae) seems to be a beloved genus for beginners in lichenology. Usnea species are macrolichens, cosmopolitan and are characterized by a shrubby to pendant thallus (fruticose), pale yellowish green branches with radial symmetry and having cartilaginous central axis and usnic acid present in the cortex. The species of *Usnea* are commonly found hanging from tree barks and rocks [19,20,21]. By occurrence the lichen genus *Usnea* represents ca. 300 species in the world and India represents 60 species [21]. Species of *Usnea* are used in folklore medicine for treatment of several diseases or disorders. The lichens of *Usnea* genus contain chemical constituents such as mono-substituted phenyl rings, depsides, anthraquinones, dibenzofurans, steroids, terpenes, fatty acids and polysaccharides. Extracts and purified metabolites from *Usnea* species have shown to exhibit various bioactivities such as antimicrobial, antioxidant, anticancer, antiviral, antiprotozoal, anti-inflammatory, analgesic, antiherbivore, insecticidal properties [14,22-26].

U. ghattensis G. Awasthi is characterized by its erect bushy thallus. The surface of the thallus is papillate and pseudocyphellate when seen under microscope. The apothecia are terminal with ciliate margins. It is distributed in Karnataka and Maharashtra [27,28]. *U. undulata* Stirt. is shrubby, sub–pendent to pendulous; branching subdichotomous to sympodial, branches articulated and inflated; tuberculate and isidiate; papillae and soredia absent; cortex thick, medulla thin and compact. *U. undulata* is distributed in Arunachal Pradesh, Meghalaya, Nagaland, Sikkim, Uttarakhand and West–Bengal hills in Himalayas and Karnataka, Kerala and Tamil Nadu in south [21,28]. The present investigation was carried out to determine antimicrobial activity of two *Usnea* species namely *U. ghattensis* and *U. undulata* collected from Chikmagalur, Karnataka, India.

MATERIALS AND METHODS

Collection and identification of lichens

In the present review, information regarding medicinal properties and biochemical properties of *Pleiocarpa pycnantha* was gathered via searching books and scientific databases including PubMed, Elsevier, google scholar, Springer, etc.

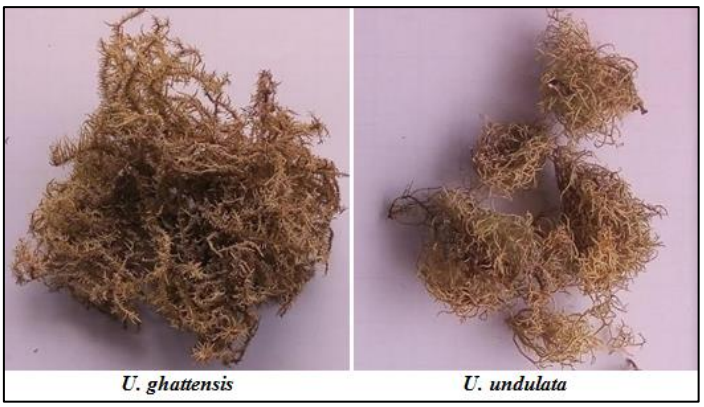

Figure 1: *Usnea* species used in this study

Extraction

Maceration process was employed for extraction of lichen materials. Here, 10g of each of the powdered lichen material was transferred into a flask and 100ml methanol was added to the flask. The flasks were plugged, shaken well, left for 48 hours and stirred occasionally. The contents of flasks were filtered through clean muslin

cloth followed by Whatman filter paper No. 1. The filtrates were then evaporated to dryness at 40oC in oven. The extracts thus obtained were stored in refrigerator until use [5].

Antibacterial activity of lichen extracts

A total of 8 bacteria comprising of 5 Gram negative bacteria viz., Salmonella typhi, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa and Ralstonia solanacearum and 3 Gram positive bacteria viz., Staphylococcus aureus, Bacillus subtilis and B. coagulans were used in this study. The test bacteria were inoculated aseptically into tubes containing sterile Mueller-Hinton (M-H) broth (HiMedia, Mumbai). The tubes were incubated overnight at 37oC. The broth cultures of test bacteria were used determine their susceptibility to lichen extracts by Agar well diffusion assay. Inoculation of test bacteria (0.5 McFarlands) was done on sterile M-H agar plates using sterile cotton swabs. A cork borer was sterilized by dipping in alcohol followed by flaming and was used to punch wells of 6mm diameter in the inoculated plates. 100µl of extract (10 and 20mg extract/ml of dimethyl sulfoxide [DMSO]), chloramphenicol (1mg/ml of sterile distilled water) and DMSO were transferred into respective wells using micropipette. The plates were incubated in upright position for 24 hours at 37oC. The zones of inhibition formed were measured using a ruler [5].

Minimum inhibitory concentration (MIC) of lichen extracts

Broth dilution method was performed to determine MIC of lichen extracts. The extract dilutions (ranging from 20 to 0.0mg/ml) were tested against B. subtilis and K. pneumoniae. Two-fold dilutions of lichen extracts were prepared in sterile M-H broth tubes. The tubes were inoculated with 100µl of broth culture of test bacteria and incubated at 37oC for 24 hours. The tubes were observed for the visible growth of the test bacteria after incubation. The dilution showing no visible growth of bacteria was considered as the MIC of lichen extracts [31].

Antifungal activity of lichen extracts

Three field fungi namely Colletotrichum capsici, Fusarium oxysporum f.sp. zingiberi and Bipolaris sorokiniana which were maintained on Potato dextrose agar slants were selected to assess their susceptibility to lichen extracts. In order to determine the antifungal effect of lichen extracts, we employed Poisoned food technique. In brief, well sporulated cultures of the test fungi were aseptically inoculated at the centre of control and poisoned (0.5mg lichen extract/ml of medium) followed by incubating the plates in upright position for 120 hours at room temperature. After incubation, the diameter of fungal colonies was measured in mutual perpendicular directions and the antifungal activity (in terms of inhibition of growth) was calculated using the formula:

Inhibition of mycelial growth (%) = (C - T / C) × 100, where C and T refers to diameter of fungal colonies on control and poisoned plates respectively [5].

RESULTS AND DISCUSSION

Extract of both lichens was yellowish brown in color. Extract yield was more in *U. ghattensis* (4.10%) when compared to *U. undulata* (3.55%). Table 1 shows the result of color test and the secondary metabolites detected in *Usnea* species. Usnic acid was detected in both lichens whereas galbinic acid, norstictic acid and salazinic acid were detected only in *U. undulata*.

Table 1: Result of color test and secondary metabolites of selected lichens

Lichen	Color test	Secondary metabolites	Voucher number
U. ghattensis	K-, P-, I-	Usnic acid	LHKFGC0020
U. undulata	K-, P-, I-	Usnic acid, Galbinic acid, Norstictic acid, Salazinic acid	LHKFGC0005

The result of antibacterial activity of both lichens is shown in Table 2. Both lichens were effective in inhibiting all test bacteria, however, the extent of inhibition of test bacteria varied from one bacterium to another. The extracts caused dose dependent suppression of bacterial growth as evidenced by the sizes of inhibition zones.

The extent of inhibition shown by both lichens was more or less similar. No inhibitory effect was observed against *R.solanacearum*. Overall, when compared to all other bacteria, *K. pneumoniae* was shown to exhibit higher susceptibility to both extracts.

Least inhibitory effect of extracts against Gram negative bacteria was against *S. typhi*. In previous studies, extract of various species of *Usnea* such as *U. rubrotincta* [18], U. pictoides [25], *U. ghattensis* [32,33], *U. steineri* [34] and *U. barbata* [35] have shown antibacterial activity against various bacteria.

Table 2: Antibacterial activity of U. ghattensis and U. undulate

Test bacteria	Zone of inhibition in cm					
	U. ghattensis		*U. undulata*		Antibiotic	DMSO
	10mg/ml	20mg/ml	10mg/ml	20mg/ml	(1mg/ml)	
S. typhi	1.5	1.7	1.4	1.6	2.6	0
K. pneumoniae	2.3	2.8	2.3	2.5	3	0
E. coli	1.8	2.1	1.9	2.1	3.1	0
P. aeruginosa	1.9	2.1	2	2.1	2.8	0
R. solanacearum	0	0	0	0	2.6	0
S. aureus	1.8	1.9	1.8	2	3.4	0
B. subtilis	2.3	2.5	2.1	2.3	3.4	0
B. coagulans	1.7	1.8	1.8	2	3.3	0

The term MIC represents the minimum concentration of extract which is required to inhibit the growth of bacteria. In the present study, the MIC of lichen extracts was determined against two bacteria namely B. subtilis and K. pneumoniae and the result is shown in Table 3. Comparatively, extract of U. ghattensis inhibited test bacteria at lower concentration when compared to U. undulata. The MIC of extracts was lower in case of K. pneumoniae when compared to B. subtilis. The earlier studies by Madamombe and Afolayan [35] and Srivastava et al. [33] showed lesser MIC value of lichen extracts against Gram positive bacteria.

Table 3: MIC of lichen extracts

Lichen	MIC (mg/ml)	
	B. subtilis	***K. pneumoniae***
U. ghattensis	0.625	0.312
U. undulata	1.25	0.625

Lichens produce a wide range of secondary metabolites (rightly called lichen substances) that seldom occur in other organisms. Detection of these compounds seems to be important in the identification of the lichens. In the present study, the TLC of both lichens showed the presence of usnic acid. Usnic acid is the yellowish signature pigment present in the genus Usnea but is also present in other lichens that are phylogenetically distant [36]. It is positively correlated with the antimicrobial activity.

In a study by Cansaran et al. [20], an increase in the concentration of Usnic acid was positively correlated with increased antimicrobial activity of Usnea species as the *Usnea* species containing high usnic acid displayed marked antimicrobial activity. In a previous study, 2'-O-methylhypostictic acid and (+) Usnic acid isolated from *U. undulata* exhibited marked antibacterial activity against Gram positive and Gram negative bacteria [24]. When compared to acetone extract, the usnic acid isolated from U. steineri inhibited Mycobacterium species with low MIC values [34].

More recently, Usnic acid, Barbatic acid and 8-Hydroxybarbatic acid isolated from *U. rubrotincta* were shown to exhibit antibacterial activity with maximum activity displayed by usnic acid [18].

The result of antifungal activity exhibited by two Usnea species against phytopathogenic fungi is shown in Table 4 and Figure 2. The extracts were appreciably effective in inhibiting the radial growth of all test fungi. The inhibitory activity observed was concentration dependent. An inhibition of >40% of mycelial growth of all test fungi was shown by both lichens at 0.5mg/ml concentration. At 1mg/ml concentration, both lichens inhibited test fungi to more than 50%.

Overall, inhibitory activity was highest against B. sorokiniana followed by *C. capsici* and *F. oxysporum*. *U. ghattensis* was more effective against *F. oxysporum* and *B. sorokiniana* while *C. capsici* was inhibited to high extent by *U. undulata*. In a similar study, Madamombe and Afolayan [35] showed concentration dependent inhibitory activity of solvent extracts of *U. barbata* against a panel of phytopathogenic fungi. Solvent extracts of Usnea species from Sri Lanka exhibited antifungal activity against *Cladosporium cladosporioides* [37]. Methanol and acetone extract of *Usnea* sp. from Karnataka were shown to exhibit inhibitory activity against *Fusarium oxysporum* f.sp. *capsici* [38].

Vinayaka et al. [39] showed dose dependent inhibitory activity of extract of *U. pictoides* against *F. oxysporum* f.sp. zingiberi and *Pythium aphanidermataum*, causal agents of rhizome rot of ginger. Aqueous extract of *U. ghattensis* is shown to inhibit *F. oxysporum* and *Aspergillus niger* [40]. More recently, the usnic acid derivatives isolated from *U. longissima* were shown to inhibit the fungus Trichophyton rubrum with an MIC value of 41.0μM [41].

Table 4: Antifungal activity of *U. ghattensis* and *U. undulata*

Extract/Control	Colony diameter in cm		
	C. capsici	***F. oxysporum***	***B. sorokiniana***
Control	3.4	4.9	3.6
U.ghattensis(0.5mg/ml)	1.2	2.5	0.8
U.ghattensis(1.0mg/ml)	1	2.2	0.4
U. undulata (0.5mg/ml)	1.1	2.7	0.9
U. undulata (1.0mg/ml)	0.8	2.4	0.7

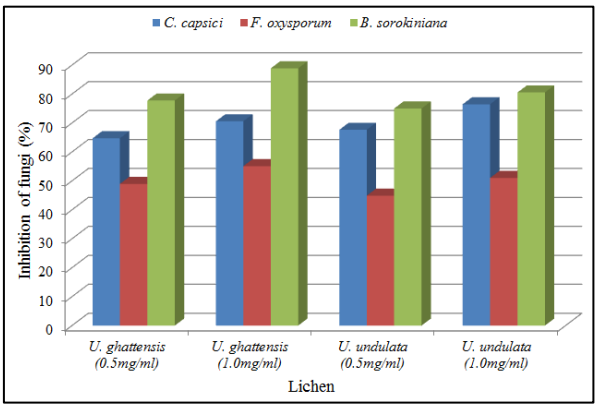

Figure 2: Extent of inhibition of test fungi by lichen extracts

CONCLUSION

In conclusion, the extract of *U. ghattensis* and *U. undulata* displayed antimicrobial activity which might be attributed to the presence of secondary metabolites in particular usnic acid. These lichens can be used against pathogenic bacteria and fungi and can contribute to the development of new and safe agents for inclusion in antimicrobial regimes.

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