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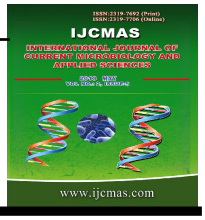

Original Research Article

In-vitro evaluation of the antimicrobial activities of lichen *Usnea ghattensis*

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A B S T R A C T

Usnea ghattensis G. Awasthi (Usneaceae) an endemic fruticose lichen found growing luxuriantly in northern western ghats of India, also contain Usnic acid as a major chemical and tested against some human pathogenic bacteria. To investigate antimicrobial properties of *Usnea ghattensis* against some human pathogenic bacteria. The lichen was extracted in acetone, methanol and ethanol. *In vitro* antimicrobial activity was tested initially by Kirby-Bauer technique of disc diffusion method and was confirmed by Minimum inhibitory concentration using Broth micro dilution method according to the NCCLS guidelines. Ethanol extract was most effective against *Bacillus cereus* and *Pseudomonas aeruginosa* with a zone of inhibition 29.8 ± 0.6 mm and 12.3 ± 0.5 mm diameters at a concentration of 0.2 mg/ml. Acetone and methanol extract demonstrated almost similar activity against *Staphylococcus aureus* and the zone of inhibition was 24.6 ± 0.5 and 24.7 ± 0.4 mm. Only methanol extract was showing activity against *Streptococcus faecalis* with a 13.5 ± 0.8 mm zone. MIC value noted against *Staphylococcus aureus* and *Streptococcus faecalis* was 6.25 µg/ml and 25 µg/ml, whereas against *Bacillus cereus* and *Pseudomonas aeruginosa*, MIC calculated was 3.125 µg/ml and 200 µg/ml. The present study demonstrates the relatively higher activity of this lichen against not only Gram (+) but significantly also against Gram (-) bacteria. This indicates that this lichen might be a rich source of effective antimicrobial agents.

Introduction

Medicinal plants are well known natural sources for the treatment of various diseases since ancient times. According to a report issued by the World Health Organization (WHO), plant species that are currently used for medicinal purposes are

about 20 000. Lichens are among the most fascinating organisms on this planet. Lichen is not a single organism the way most other living things are, but rather it is a combination of two organisms which live together intimately. The fungus forms a thallus or lichenized stroma that may contain characteristic secondary metabolites in all lichens (Ahmadjian, 1993). Lichens are valuable plant resources and are used as medicines, food, fodder, dyes perfume, spice, and for miscellaneous purposes. The lichen flora is rather poor in the vicinity of industrial areas and big cities (Hegnauer, 1962), as lichens are very sensitive to various air pollutions. Thus these organisms are used as air pollution monitors (Jezeirski *et al.*, 1999). The specific, even extreme, conditions of their existence, slow growth and long duration (maximum lifetime spans to several thousand years) are consistent with their abundance in protective metabolites against different physical and biological influences (Denton & Karlen, 1973). Lichens have been used for medicinal purposes throughout the ages, such as *Cetraria islandica* (L.) Ach. (Parmeliaceae), *Lobaria pulmonaria* (Schreb.) Hoffm. (Lobariaceae) were reported to be effective in the treatment of pulmonary tuberculosis (Vartia, 1973).

The use of lichens in medicine is based on the fact that they contain unique and varied biologically active substances, mainly with antimicrobial actions. Because of marked antimicrobial activity of secondary metabolites, lichens, macro fungi, and vascular plants attract great attention of investigators as new significant sources of bioactive substances (Mitscher *et al*., 1987; Ingólfsdottir *et al*., 1997; Hostettman *et al*., 1997; Karaman *et al*., 2003). The intensive use of antibiotics has selected for antibiotic resistance factors and facilitated the spread of multiply resistant microorganisms. Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects (Molnár and Farkas, 2010; Huneck, 1999; Manojlovic *et al.*, 2002; 2010 a, b; Shukla *et al.*, 2010). Although about 8% of the terrestrial ecosystem consists of lichens, and more than 20 000 lichen species are distributed throughout the world, but their biological activities and biologically active compounds remain unexplored in great extent (Toma *et al*., 2001).

Usnea ghattensis, is an endemic fruticose lichen that grows on different trees and shrubs in Northern Western Ghats of India . Most of the lichen species of the genus Usnea containing Usnic acid as the major chemical constituent used traditionally in upper respiratory infections, and applied on the skin to treat surface infections or external ulcers. It is still used today in traditional Chinese medicine (TCM) in liquid extract and tincture form to treat tuberculosis lymphadenitis. Usnic acid has been used as a human papillomavirus (HPV) treatment and as an oral hygiene agent, with limited effectiveness. In accordance with these facts, in this study, the antimicrobial activity of acetone, methanol, and ethanol extracts of *Usnea ghattensis* were investigated *in vitro* in relation to test microorganisms, where some of them promote diseases in humans, animals and plants, and even produce toxins and provoke food deterioration.

Materials and Methods

Microorganisms

Total six bacteria, three gram positive (*Staphylococcus aureus* (ATCC 25923), *Streptococcus faecalis* (ATCC 33186),

Bacillus cereus (ATCC 14579)) and three gram negative (*Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 29853), *Salmonella typhi* (ATCC 13311)) were used to assess the antimicrobial properties of the test samples. These Bacteria were kept on nutrient agar plates at 4^0C, respectively. For use in experiments, the organisms were sub-cultured in their respective selective medium.

Lichen material

The plant material of *Usnea ghattensis* was collected during Dec. 2009 from Lingmala Forest area, Mahabaleshwar, Satara district, Maharashtra, India the northern western Ghat area of India between altitudes of 1200-1340m. One voucher specimen preserved in the herbarium of National Botanical Research Institute, Lucknow (LWG), India.

Extraction of lichen material

The lichen sample was washed to remove debris; air dried and pulverized to powder, and was stored in a sterile glass bottle in the refrigerator. 10 g portions of sieved powder was added to 100 ml of solvents (acetone, ethanol, and methanol) and left for three days at room temperature. The crude extract was prepared by decanting, followed by filtration through muslin cloth, and further filtered with Whatman No. 1 filter paper to obtain a clear filtrate. 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 redissolved in respective solvents to attain the required concentrations of 0.1mg/ml and 0.2mg/mL for antibacterial screening. These extracts were kept at 4^0C till used.

Determination of Antimicrobial Activity

Antimicrobial susceptibility test of the selected pathogens was done by Disc diffusion method using Kirby-Bauer technique (Bauer *et al*., 1966) and as per recommendation of NCCLS (NCCLS, 1997). All the tests were performed on Mueller Hinton agar plates. Suspension of microbial cultures (0.5 Mc Farlands) was inoculated on the entire surface of the Mueller Hinton agar media in a Petri plate using sterile swab sticks. The sterile discs of diameter 6mm were impregnated with lichen extract solutions (0.1 mg/ml and 0.2 mg/ml) and placed onto the cultured Mueller Hinton agar plates. Inoculated plates were incubated at 37^0C for 24 hrs.

On the second day, plates were read by taking measurement of zone of inhibition around each disc. The diameter of zone of inhibition of bacteria was recorded in millimeters. Pure acetone, methanol and ethanol were taken as negative control whereas commercial Gentamicin and Ceftriaxone were used as positive control. Gentamicin was taken as positive control for gram positive bacteria and Ceftriaxone was used for gram negative bacteria. The assay was done in triplicates and checked with the control plate. To determine the effectivity of lichen crude extracts at different volumes, two different concentrations of lichen crude extracts were taken on each paper disc, on every Petri plate.

Minimum Inhibitory Concentration

The minimal inhibitory concentration (MIC) of the crude extract was determined by micro dilution techniques in Mueller Hinton Broth (MHB), according to National Committee for Clinical Laboratory Standard, USA guidelines

(NCCLS, 2002). A series of two fold dilutions with concentrations ranging from100 µg/ml to 0.195 µg/ml for methanol extract was used in the experiment against *S. aureus, S. faecalis* and *B. cereus*.

The starting solutions of extracts and component were obtained by measuring a certain quantity of extract and dissolving it in dimethylsulphoxide (DMSO). Two-fold dilutions of extracts and components were prepared in Mueller -Hinton broth (MHB) for bacterial cultures. The inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard colony forming units, and diluted 1:10 for the broth micro dilution procedure .Then 100 µl of diluted extracts and 100 µl of bacterial suspensions were dispensed in 96 well sterile microtitre plate. The microtitre plates were incubated at 37°C and MIC was determined after 24 h of incubation. The MIC was determined by establishing visible growth of the microorganisms. The boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration. Untreated bacteria were taken as Positive control and MHB was taken as negative control. All experiments were performed in triplicate.

Results and Discussion

Disc diffusion assays

After the treatment had been applied and the inoculated plates were allowed to grow for 24 hours, the acetone extract and ethanol extract of *U. ghattensis were* showing activity against *Staphylococcus aureus, Bacillus cereus* and *Pseudomonas aeruginosa* while no activity was found against *Streptococcus faecalis, Escherichia coli* and *Salmonella typhi*.

Both the concentration of methanol extract was showing activity against all the gram positive bacteria and one gram negative bacteria. No activity was recorded against *Escherichia coli* and *Salmonella typhi*. The acetone extract inhibited growth *B.cereus* with a mean zone of 23.9 ± 1.1 mm(0.2mg/ml conc.) while ethanol extract of the lichen had the greatest effect on plates inoculated with *Bacillus cereus* with a mean zone of inhibition of 29.8 ± 0.6 mm at 0.2 mg/ml concentration. The acetone and methanol extract were showing equal inhibitory effect on *S. aureus* with a mean zone of inhibition 24.6 ± 0.5 mm and 24.7 ± 0.4 mm at 0.2 mg/ml concentration respectively.

Crude methanol extract showed poor activity against *S. faecalis* with a zone of inhibition 8.3 ± 0.5 mm at a concentration of 0.1 mg/ml while the concentration 0.2 mg/ml was showing a zone of inhibition of 13.5 ± 0.8 mm. Ethanol extract showed greater effect on *P. aeruginosa* with a zone of inhibition of 12.3 ± 0.5 mm at a concentration of 0.2 µg/ml in comparison to acetone (8.4 ± 0.6 mm dia. Zone) methanolic extract (8.7 ± 0.4 mm) Although the extracts were not as effective as the commercial antibiotics Gentamicin and Ceftriaxone but they have potent antibacterial activity.

Minimum Inhibitory Concentration

The MIC values of the extract related to the tested bacterial strains varied between 25-3.125 µg/ml in case of gram positive bacteria. The measured MIC value for the extract against *Staphylococcus aureus* was 6.25 µg/ml while the MIC value against *Bacillus cereus* was found to be 3.125 µg/ml. *Streptococcus faecalis* was also showing 25 µg/ml MIC value. Against *Pseudomonas aeruginosa*, the MIC value noted was 200 µg/ml. Positive control was

Table.1 Results of zone of inhibition of acetone, methanol, ethanol extracts of *Usnea ghattensis* against tested microorganisms.

S. No.	bacterial pathogen	bacterial strain no.	acetone extract		methanol extract		ethanol extract		positive control	negative control
			0.1 mg/ml	0.2 mg/ml	0.1 mg/ml	0.2 mg/ml	0.1 mg/ml	0.2 mg/ml		
1.	*Staphlyococcus aureus*	ATCC 25923	13.8 ± 0.7	24.6 ± 0.5	15.2 ± 0.9	24.7 ± 0.4	8.8 ± 0.8	19.1 ± 0.8	25.6 ± 0.7	0.0 ±0.0
2.	*Streptococcus faecalis*	ATCC 33186	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 0.5	13.5 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	24.8 ± 0.6	0.0 ±0.0
3.	*Bacillus cereus*	ATCC 14579	20.6 ± 0.5	23.9 ± 1.1	19.1 ± 1.1	23.6 ± 0.5	20.4 ± 0.5	29.8 ± 0.6	29.1 ±1.1	0.0 ± 0.0
4.	*Escherichia coli*	ATCC 25922	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	25 ± 0.4	0.0 ± 0.0
5.	*Pseudomonas aeruginosa*	ATCC 29853	0.0 ± 0.0	8.4 ± 0.6	0.0 ± 0.0	8.7 ± 0.4	0.0 ± 0.0	12.3 ± 0.5	26.6 ± 0.7	0.0 ± 0.0
6.	*Salmonella typhi*	ATCC 13311	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	24 ± 0.6	0.0 ± 0.0

*values are in Arithmetic mean ± Standard error

showing growth of bacteria and negative control was clear and not showing any growth of bacteria.

The results of disc diffusion assay are expressed as mean ± SD of three replicates in each test, given in table 1 and results of MIC are given in table 2.

Table.2 Minimum inhibitory concentration (MIC) of *Usnea ghattensis* against tested microorganisms

S.No.	Bacterial pathogen	*Usnea ghattensis*
1.	*Staphlyococcus aureus*	6.25
2	*Streptococcus faecalis*	25
2.	*Bacillus cereus*	3.125
3.	*Pseudomonas aeruginosa*	200

The intensity of the antimicrobial effect depended on the type of extract, its concentration, and the tested microorganisms. The tested concentrations of all the three extracts were showing activity against all bacteria except *S. faecalis,* for which only methanol extract was showing trace activity. Against *P. aeruginosa*, 0.2 mg/ml concentration was showing activity while the concentration 0.1mg/ml was ineffective.

Acetone and methanol extract was showing almost equal activity against *S .aureus* whereas ethanol extract was found to be more effective against *B. cereus* and *P. aeruginosa.* The reason for different sensitivity of bacteria can be found in different transparency of the cell wall (Yang *et al.,* 1999). The cell wall of the gram-positive bacteria consists of peptidoglycan (mureins) and teichoic acids; the cell wall of the gram-negative cells consists of lipo polysaccharides, and lipopoliproteins (Hugenholtz, 2002; Heijenoort, 2001). Most of the Parmelloid lichens exhibit strong antimicrobial activity (Gulluce *et al.,* (2006), Candan *et al.,* (2007), Rankovic *et al.,* (2007b).

According to Burkholder *et al*., (1944), Rowe *et al*., (1989) and Silva *et al.,* (1986), the lichens inhibit mostly Gram positive bacteria, but it is of great interest to note that the extracts of *U. ghattensis* inhibited the growth of both Gram positive bacteria and one gram negative bacteria in the present study.

U. ghattensis showed that MIC values were varying between 25-3.125 µg/ml. Similar to other *Usnea* species *U.ghattensis* also showed equal MIC values (Madamombe and Afolayan, 2003). Lichens and their metabolites have manifold biological activity: - antiviral, antibiotic, enzyme inhibitory, allergenic. Behera (2005b) reported that the acetone, methanol and light petroleum extracts of lichen were effective against *Bacillus licheniformis, B.megatarium* and *S. aureus.* Ali karagoz *et al.,* (2009) reported antibacterial activity of aqueous and ethnolic extracts lichens like *Lecanora muralis, Peltigera polydactyla, Ramalina farinacea, Xanthria elegans.*

The acetone, methanol and ethanol extracts of *U.ghattensis* have a potential towards antibacterial activity. The obtained results showed that the tested lichen extracts showed a significant antimicrobial activity relative to the tested bacteria, which could be of significance in human therapy, animal and plant diseases. Further investigations on the antibacterial activity as well as the economical and fast isolation of the metabolite from the lichen

are needed. Consequently, the antibacterial effect of plants tested can be explained with new studies by using different solvents for extraction and other bacteria, accurately.

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Ethical approval

Approval was obtained by the authors from Research ethical committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS).

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