Antimicrobial role of *Usnea longissima* against pathogenic microorganisms

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Abstract

An attempt was made to study the antimicrobial activity of fruticose lichen *Usnea longissima* under invitro conditions. The antimicrobial activities of Methanol, Ethanol, Ethyl Acetate and Acetone extracts of *Usnea longissima* were assayed against nine pathogenic microorganisms using standard well diffusion method. The Methanolic extract was found most effective antibacterial as well as antifungal against most of organisms. The maximum inhibition zone was recorded in *E.coli* with inhibition zone 34 mm. Fungal pathogens showed their inhibition zones in varying levels as 18 mm in *Candida albicans* and *Fusarium oxysporium.* The present study reveals that extracts obtained from *U. longissima* have potential compounds that can lead to control human pathogenic microorganisms in future.

Keywords :Lichens; well diffusion ; inhibition zone ; antimicrobial ; *Usnea longissima*

Introduction

Lichens are symbiotic associations of fungi, green algae, or cyanobacteria. Lichens have been used by various ethnic groups from the time of early civilization. Irrespective of the advances in medical sciences, tribal people still utilize these plants. The lichens are utilized for different purposes depending on their nutritive, medicinal, decorative brewing, distilling, dyeing, cosmetic, and perfumery properties. These different uses are substantiated by the complex lichen secondary metabolism producing many polyketidederived compounds such as depsides, depsidones, and dibenzofurans, most of which are not known from other groups of plants. Lichens have been appreciated in traditional medicine, but their importance has largely been

ignored by the modern pharmaceutical industry because of the difficulties in establishing axenic cultures and conditions for rapid growth. Lichens are considered as potential resource since these compounds function as chemical defence against biotic and abiotic stresses and they are antibacterial (Lawrey,1986)., anticancer (Williams et al.,1998), anti HIV (Huneck and Yoshimura,1996), analgesic and antipyretic (Muller, 2001). It has been documented that more than 1050 secondary metabolites were found so far (Huneck and Yoshimura, 1996) and among them 550 are unique in lichens.Lichens produce large number of primary and secondary metabolites. Lichen extracts have been used for various remedies in folk medicines and screening of compounds has shown potentiality as antimicrobial, anticancer, antioxidant, antitumour and analgesic. India is among the richest biodiversity centres contributing about 15% of 13,500 species of the world (Negi, 2000). Total of 2450 species of lichens were present in India and were abundant in temperate and alpine regions of Peninsular India (Nayaka et al, 2010).

Usnea is a genus of mostly pale grayish-green fruticose lichens that grow like leafless mini-shrubs or tassels anchored on bark or twigs. The genus is in the family Parmeliaceae. It grows all over the world. Members of the genus are commonly called **old man's beard**, or **beard papa**. It resembles *Evernia*, which is also called **tree moss**. Like other lichens it is a symbiosis of a fungus and an alga. *Usnea* appears as a shrub-like growth on host trees. The growth rate of lichens in nature is slow, but the growth rate has been sped up in laboratory conditions where *Usnea* is being cultured. *Usnea* looks very similar to the plant Spanish moss. *Usnea* is very sensitive to air pollution, especially sulfur dioxide. Under bad conditions they may grow no larger than a few millimetres, if they survive at all. Where the air is unpolluted, they can grow to 10–20 cm long. It can sometimes be used as a bioindicator, because it tends to only grow in those regions where the air is clean and of high quality.The usnic acid in *Usnea* is effective against gram positive bacteria such as *Streptococcus* and *Staphylococcus*, making *Usnea* a valuable addition to herbal formulas for sore throats and skin infections. It is also effective against a bacterium that commonly causes pneumonia.

The present study reports the extraction of secondary compounds from *U. longissima* using solvents Methanol, Ethanol, Ethyl Acetate and Acetone, subjected to test the antimicrobial activity using bacterial and fungal pathogens under laboratory conditions.

Materials and Methods

Fig 1. *U. longissima* sample was crushed to powdered form.

Lichen material : *U. longissima* thalli was collected in the month of November from Sikkim and was identified based on standard literature (Awasthi, 1988; Swinscow and Krog,1988). The collected material was washed thoroughly with distilled water followed by tween 80 and made air dried. The dried material was weighed and made into powdered form.

Extraction of Lichen Material : The powdered lichen (10gms) was wrapped in 8 x 6 cm cylindrical pouch made of Whatmann filter paper grade 1 and kept inside the extracter arm of Soxhlet apparatus(Balaji, 2005). A series of solvents as Methanol, Ethanol, Ethyl acetate and Acetone were used for extraction based on their polarity and each extraction was carried out at the specific boiling temperature for a priod of 48 hrs for the complete extraction of secondary compounds. The final filtrate of each of the extraction obtained was concentrated using Rotatory Evaporator or Rotavapour.

Culture Media : Nutrient Agar (NA) and Potato Dextrose Agar (PDA) medium were used to culture pathogens and for bacterial and fungal susceptibility test (Balaji,2005).

Microorganisms source : Total of Six bacterial cultures (*Pseudomonas aeruginosa, Agrobacterium tumefaciens, Escherichia coli, Streptococcus mutans, Staphylococcus aureus and Klebsiella pneumoniae*) and three fungal cultures of *Aspergillus niger , Candida albicans and Fusarium oxysporium* was used in this testing and screening process. All the cultures were obtained from the Pharmacological Laboratory, National Botanical Research Institute (NBRI), Lucknow. The cultures were maintained at 4 degrees Celsius and subcultured in solid and semisolid nutrient agar slants.

Determination of Antimicrobial Activity : Antimicrobial activity was tested using well-diffusion method (Bauer et al.,1966). The Nutrient Agar medium was transferred into one fourth volume of petriplates for antibacterial activity. Potato Dextrose Agar medium was transferred into one fourth volume of petriplates for antifungal activity. Inoculation of cultures (100 mg/ml) to this medium was carried out uniformly using glass spreader. Five wells were made in each petriplate. Different concentration of crude extracts of Methanol, Ethanol, Ethyl acetate and Acetone (i.e. 2.5%, 5%, 10%,15% and 20%) were prepared as individual stock solutions by mixing Dimethyl Sulfoxide (DMSO) and Distilled Water. These stock solutions of different concentrations were filled in their respective wells alongwith DMSO as negative control and Streptomycin (in antibacterial testing) and Ketoconazole (in antifungal testing) as positive control. The plates were labeled and incubated for 24 hrs at 37 degree Celsius in BOD.

Results and Discussion

The inhibitory zones were recorded and measured with the help of Hi-Antibiotic Zone Scale. The results of antimicrobial activity of extracts are given in Table.1. Among the four different extracts, Methanolic and Ethanolic extract exhibited growth inhibition on *Agrobacterium tumefaciens, Staphylococcus aureus and E.coli* and no inhibition agains *Streptococcus mutans* and *Klebsiella pneumonia.* There was least inhibitory activity for Ethyl acetate extract against *Staphylococcus aureus, Escherichia coli* and *Aspergillus niger* .

The Methanolic extract inhibited the growth of all the organisms tested and specially exhibited 30-34 mm zones of inhibition against *Staphylococcus aureus, Streptococcus mutans, Escherichia coli, Candida albicans and Fusarium oxysporium.* The various concentrations (5-20%) of Methanolic extracts exhibited more effective zone of inhibition compared to the antibiotic

standard Streptomycin (25-28 mm) against *Staphylococcus aureus* and *Escherichia coli (*28-32 mm) also compared to the antifungal standard Ketoconazole (14-15 mm) against *Aspergillus niger*, *Candida albicans and Fusarium oxysporium* (16-18 mm).

The antimicrobial potential of Methanolic extracts of *U. longissima* is much more than the ethanolic and acetonic extracts of *U. longissima*.

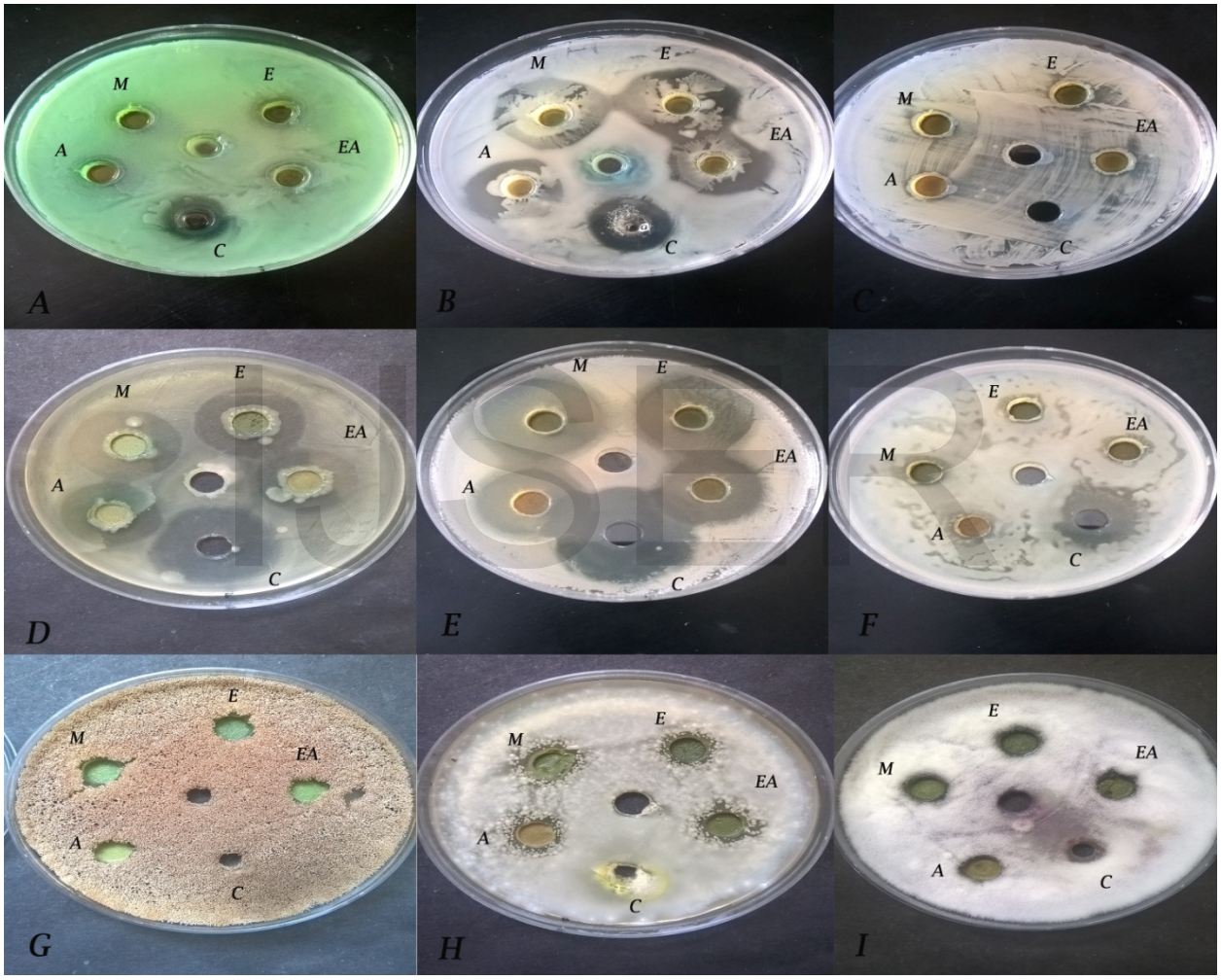


Fig 2. Inhibition zones after Antimicrobial test activity where, A. *Pseudomonas aeruginosa* B. *Staphylococcus aureus* C. *Streptococcus mutans* D. *Agrobacterium tumefaciens* E. *Escherichia coli* F. *Klebsiella pneumonia* G. *Aspergillus niger* H. *Candida albicans* and I. *Fusarium oxysporium.*

In the figure 2. M denotes Methanolic Extract, E denotes Ethanolic extract and A denotes Acetonic extract. C is the positive control used as Streptomycin for antibacterial and Ketoconazole for antifungal testing. In the middle, DMSO or DimethylSulphoxide has been used as negative control in antibacterial as well as antifungal testing.

The results of antimicrobial activities of extracts are given in table 1.

	BACTERIAL and FUNGAL PATHOGENS	DIAMETER OF INHIBITION ZONES (mm)				
			Solvent systems			
		Control(+)	Methanol	Ethanol	Acetone	Ethyl Acetate
1.	*Pseudomonas aeruginosa*	18 ± 0.0	16 ± 0.7	16 ± 0.7	15 ± 0.7	15 ± 1.4
2.	*Staphylococcus aureus*	25 ± 1.4	32 ± 0.7	30 ± 1.4	28 ± 1.4	27 ± 0.7
3.	*Streptococcus mutans*	14 ± 0.7	0.0 ± 0.0	0.0 ± 0.7	0.0 ± 0.0	0.0 ± 0.7
4.	*Agrobacterium tumefaciens*	28 ± 1.4	30 ± 0.7	28 ± 1.4	27 ± 0.7	25 ± 2.1
5.	*Escherichia coli*	28 ± 1.4	34 ± 0.7	32 ± 1.4	30 ± 1.4	28 ± 0.7
6.	*Klebsiella pneumonia*	16 ± 0.0	14 ± 0.7	14 ± 0.7	12 ± 0.0	14 ± 0.0
7.	*Candida albicans*	15 ± 0.7	18 ± 1.4	16 ± 0.7	14 ± 1.4	14 ± 0.7
8.	*Aspergillus niger*	14 ± 0.7	15 ± 0.0	15 ± 1.4	0.0 ± 0.0	0.0 ± 0.7
9.	*Fusarium oxysporium*	14 ± 1.4	18 ± 2.1	16 ± 1.4	12 ± 0.7	14 ± 1.4

Table 1. Inhibitory zones of extracts of lichen

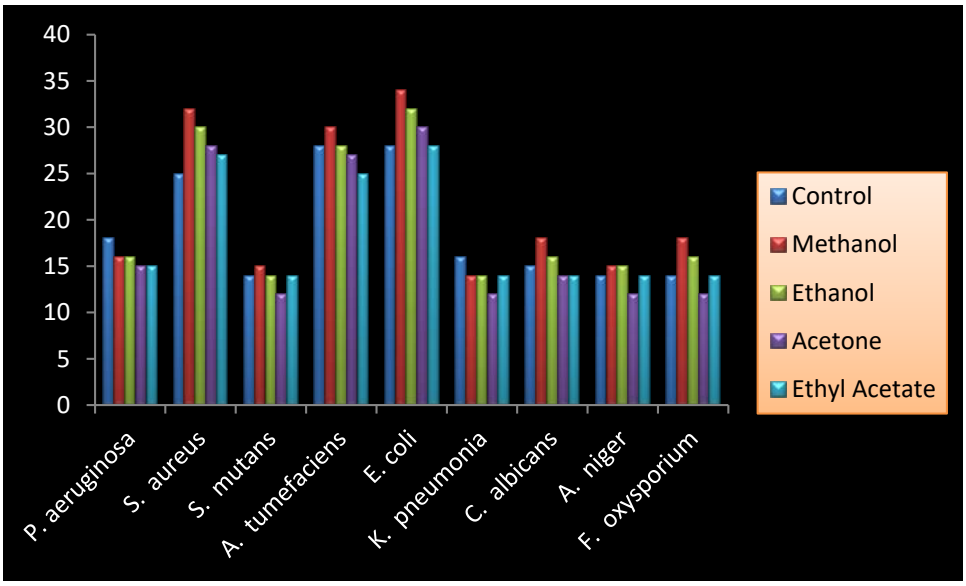
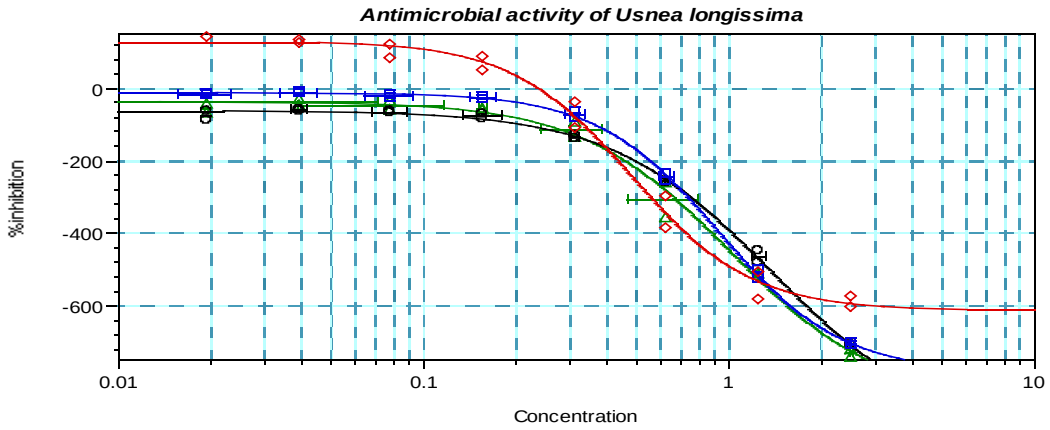


Fig.3 . Yield of concentration of different lichen extracts against 9 pathogens

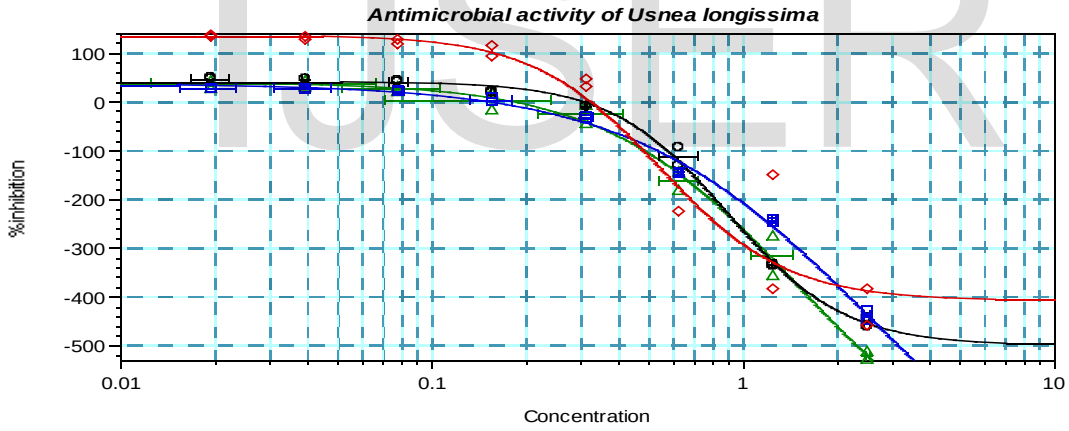
Assayed microtitre plates incubated at 35 + 2 degree Celsius for 24 hours. After incubation, Optical density or OD taken at 492 nm from Spectramax Plus 384 spectro for growth inhibition and quantitative data, in form of IC and MIC (mg/ml). All the results in form of Standard deviation error calculated by Softmax Pro-5 software. Lichens studied for antibacterial against observation on basis of antibacterial susceptibility assay of lichen belongs to family Roccellaceae with acids Roccellic acid, Lecanoric acid, Lepraric acid and Pulvinic acid as Lichen acids. Tested against Bacteria as well as fungi. Lichen was found to have activity only against *Pseudomonas aeruginosa, Staphylococcus aureus, Agrobacterium tumefacienss, Escherichia coli, Candida albicans, Fusarium oxysporium* with MIC values percentage growth inhibition at various concentrations and graph for growth inhibitory activity as in figures 4, 5 and 6. Absorbance data depicts colour of the drug can be a factor hindering spectrophotometer means of quantitative analysis.



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
△ Against Pseudomonas aeruginosa (Group#1: Conce...	-38	1.77	1.02	-865	0.99
○ Against Agrobacterium tumefaciens (Group#2: Conc...	-62.5	1.64	1.39	-954	0.998
□ Against Streptococcus mutans (Group#3: Concentra...	-13	2.17	0.937	-786	0.999
◇ Plot#4 (group#4: Concentration vs %inhibition)	129	2.2	0.483	-615	0.991

Curve Fit Option - Fixed Weight Value

h Fig.4. MIC against bacterial pathogens



4-P Fit: y = (A - D)/(1 + (x/C)^B) + D:	A	B	C	D	R^2
△ Against Staphylococcus aureus (Group#1: Concentr...	39.7	1.42	1.47	-781	0.988
○ Against Escherichia coli (Group#2: Concentration v...	41	2.32	0.899	-500	0.997
□ Against Klebsiella pneumoniae (Group#3: Concentr...	37	1.13	2.66	-941	0.995
◇ Plot#4 (group#4: Concentration vs %inhibition)	137	2.13	0.543	-408	0.935

Curve Fit Option - Fixed Weight Value

h Fig.5. MIC against bacterial pathogens

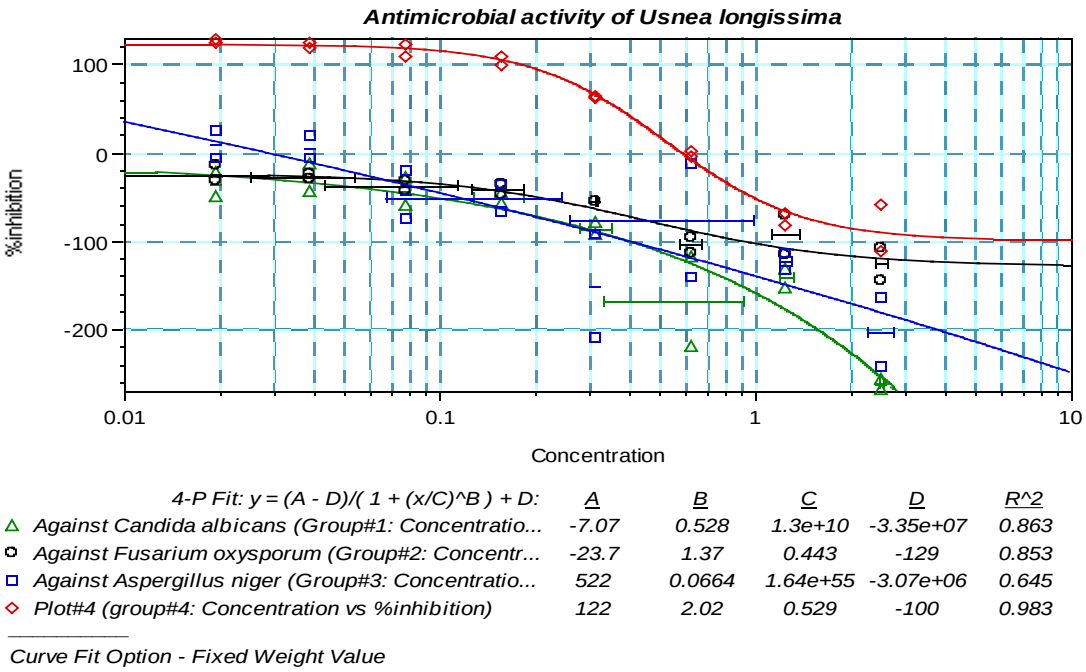


Fig.6. MIC against fungal pathogens

Conclusions

The ethyl acetate extract (Standard Lichenological Procedure) showed minimum inhibitory effect or activity against the pathogens tested. However, The ethanolic extracts showed significant antimicrobial activity while the Methanolic extract showed maximum inhibitory zones against the pathogens tested. Therefore, this study proves the antimicrobial potential of methanolic extracts of *U.longissima* and in the discovery of the novel potential biomolecules from lichens , application of different solvents in combination with extraction procedures. Further processing and investigation into fractionation and purification of ethanolic extract may result in the isolation of viable alternate source to the presently available antibiotics. Lichens hold great potential that needs to be fully explored and utilized for the benefit of human health and our society. This will definitely provide a new base and ray of light for the future perspectives and highlight the need for further studies of this promising source to harvest more beneficial in the field of bioprospection. This work is intended to contribute in the current research and development trends in the bioprospection of lichens and their bioactive compounds in the applications of commercial interest as well.

Acknowledgements

The authors are grateful to the Director (CSIR-NBRI) for constant support and encouragement. Also, I would like to thank my seniors Shweta Bharti and Balwant Singh at NBRI for their guidance and immense support throughout my work.

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