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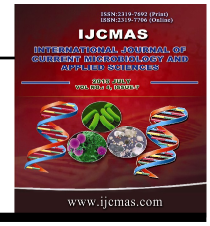

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Original Research Article

Assessment of Antibacterial Activity of Usnea Species of Shimla Hills

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A B S T R A C T

An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi and protozoan. Antimicrobial drugs either kill microbes or prevent the growth of microbes. The aim of this study has been attempted to determine the antibacterial activity of Usnea sp. extracts against some selective pathogenic bacterial strains. Lichen was collected from Mashobra, Shimla Hills Himachal Pradesh brought to Microbiology Laboratory of Shoolini institute of life sciences and business management, Solan (H.P).The specimens were identified as Usnea sp. with the help of morphological and microscopic characters, Lichen was washed to remove debris, dried, ground to powder and stored in a sterile glass bottle in the refrigerator. The 5g portions of powder was added to 50 ml of solvents (ethanol and methanol), sonicated for 30 min and left overnight at room temperature. The extracts were prepared by decanting and filtered with Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate was evaporated to obtain 10 ml of concentrated extracts. Sterilized filtrate was stored in airtight containers in the refrigerator. Usneasp. shows antibacterial activity against pathogenic organisms such as (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhiand Escherichia coli*). Ethanolic and Methanolic extract of Usnea sp. shows zone of inhibition against some pathogenic bacterial strains. It is therefore proposed that further investigation required for develop new drugs.

Keywords

Antimicrobial, Antibiotic, lichen, Bacteria, Usnea

Introduction

An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi and protozoan. Antimicrobial drugs either kill microbes or prevent the growth of microbes (Levi *et al*., 1994).Infectious diseases account for one third of all deaths worldwide. The spread of multidrug-resistant strains of microbes make it necessary to discover new classes of antimicrobial and compounds that inhibit these resistance mechanisms (Maria *et al*., 2008).Antibiotic resistance has become a global concern. The clinical efficacy of many existing antibiotics being threatened by the emergence of multidrug resistant pathogens. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microorganisms has led to the screening of several medicinal plants for their antimicrobial activity (Westh *et al*., 2004; Bandow *et al*., 2003; Colombo and Bolsisio, 1996; Iwu *et al*., 1999).Whole world is frantically in search of new antibiotics due to their inappropriate and indiscriminate use. In search of new antibiotics, herbs and plants are being used (Iftekher *et a*l., 2011).Lichens also show antibacterial activity against a wide range of pathogenic microbial species. Lichens are mutualistic symbiosis between algal and fungal components. These components are known as the phycobiont and mycobiont respectively. The phycobiont is the photosynthetic component of the lichen. The mycobiont almost always dictates form of the lichen thalli (Paulo *et al.*, 2003). Sometimes Cyanobacteria (prokaryotic algae) are the photosynthetic component known as the cynobiont (Hodkinson *et al.*, 2012). When Cyanobacteria are used, lichen do not select for particular species of cyanobionts, as an adaptation to distribution in harsh environments rather than to metabolic mechanisms (Honegger *et al.*, 1998).

Lichen is not a single organism the way most other living things are, but rather it is combination of two organisms which live together intimately. The fungus forms a thallus or lichenized stroma that may contain characteristic secondary metabolites in all lichen (Rankovic *et al.*, 2012). The lichen flora is rather poor in the vicinity of industrial areas and big cities as lichens are very sensitive to various air pollutions. Thus these organisms are used as air pollution monitors (Jezierski *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 and Karlen, 1973).Lichens are valuable plant resources and are used as medicines, food, fodder, dyes, perfume, spices and for miscellaneous purposes. Lichens have been used for medicinal purposes throughout the ages, such as *Cetrariais landica*, *Lobaria pulmonaria*. According to a report issued by the World Health Organization (WHO), plant species that are currently used for medicinal purposes are about 20,000. Hoffm were reported to be effective in the treatment of pulmonary tuberculosis (Vartia, 1973).The use of lichen 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 (Lauterwein *et al.*, 1995). 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, anti-proliferative and cytotoxic effects (Molnar and Farkas, 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.*Usnea* species is endemic fruticose lichen that grows on different trees and shrubs in Northen 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 infection or external ulcers. It is still used today in Traditional Chinese Medicine (TCM) in liquid extract and tincture to treat tuberculosis lymphadenitis (Malhotra *et al.,* 2007). Usnic acid has been used as a human papilloma virus (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* species were investigated in vitro in relation to test microorganisms where some of them promote diseases in human, animal and plant and even produce toxins and provoke food deterioration. Lichen synthesize numerous metabolites called lichen substances including aliphatic, cycloaliphatic, aromatic and terpenic components. These metabolites exert a wide variety of biological action including antibiotic, immunomodulatory, antioxidant, cytotoxic, antiherbivore and antitumour effects (Bucar *et al.,* 2004). Lichen forming fungi produce antimicrobial secondary metabolites that protect many animals from pathogenic microorganism. The first study of antibiotic properties of lichen was carried out by (Burkholder, 1944). Vartia reported antimicrobial properties of several lichens and other researchers have since then studied antimicrobial activity of several lichens against gram-positive, gram-negative bacteria as well as several fungi.

The search for novel natural bioactive compounds as a foundation to new drug discovery is receiving attention as previously reliable standard drugs become less effective against the emerging new strains of multiple drug resistant pathogens (Muller, 2001). India is a rich center of biodiversity contributing nearly 15% of the 13,500 species of lichens. Many lichen species of the Himalayan region are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders and many disorders of blood and heart. Even though manifold activities of lichen metabolites have now been recognized their therapeutic potential has yet not been fully explored and thus remains pharmaceutically unexploited (Taylor *et al.,* 1996).Lichens are good sources of biologically active secondary metabolites. They have been used as medicine in treating wounds, stomach diseases and whooping cough in America and in Europe (Rankovic *et al.,* 2007). They are also reported to produce secondary metabolites with antimicrobial and anticancer activities (Rankovic *et al.,* 2007). However, in spite of their potential as sources of drugs, the biological activities of lichens remain less studied. Quisumbing (1951) earlier reported the medicinal properties of fruticose lichen *Usnea philippina.* Manojlovic and coworker (2010) tested the biological activities of these lichens and other fruticose lichens e.g. *Usnea sp., Ramalina sp.,* and *Stereocaulon sp.,* and reported their inhibitory activities against Gram-positive bacteria such as *Micrococcus pyogenes, Bacillus subtilis* and *acid-fast bacilli*. Interestingly, the latter is known to have acquired resistance against major anti-TB drugs due to incomplete or partial treatment and necessitates treatment with new antibiotics (WHO, 2009). The search for novel bioactive secondary metabolites is of primary concern since infectious diseases are continuously emerging and re-emerging. For e.g. *Mycobacterium tuberculosis* infects approximately 9 million new individuals every year with 1.7 million deaths annually (WHO, 2009). Since lichens offer alternative sources of bioactive metabolites, study explores the antibacterial activities of fruticose lichens belonging to the *Cladonia*, *Ramalina*, *Stereocaulon* and *Usnea* collected from selected provinces. It is hoped that the lichen acids extracted from these species may be potentially novel and biologically active against emerging and re-emerging diseases (Chandra and Singh, 1971).Simon Parietin, anthraquinone isolated from methanol extract of *Caloplaca* *cerina* has been reported to have significant antifungal activity (Manojlovic *et al*., 2005) Extracts of Andean lichens Protousnea poeppigii Vain.

Parmeliaceae and *Usnea florida* Acharius demonstrated antimicrobial activity against the pathogenic fungi. Usnicacid were identified as antifungal agents (Schmeda *et al*., 2007).Lichens have been used for medicinal purposes throughout the ages, such as *Cetrariais landica* (Parmeliaceae), *Lobaria pulmonaria* were reported to be effective in the treatment of pulmonary tuberculosis. 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 anti- microbial 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*., 2013). *Usnea longissima* is an epiphyte species of lichen belongs to the family Parmeliaceae. Lichenic acids isolated from *Usnea longissima* are growth inhibitors. *Usnea longissima* was used a dermatological aid for wounds in the pecific North West. The ethanol extract of *Usnea longissima* were screened for potential antibacterial activity and antifungal activity by using Agar well diffusion method. *Longissima* was screened their level of antimicrobial potential (Thippeswamy *et al.,* 2011). *Usnea ghattensis* endemic fruticose lichen found growing luxuriantly in Northern Western Ghats of India. It also contains Usnic acid as a major chemical and tested against some human pathogenic bacteria. *In vitro* antimicrobial activity was tested initially by *Kirby-Bauer* technique of disc diffusion method and was confirmed by minimum inhibitory concentration using broth microdilution method according to the NCCLS guidelines. The present study demonstrates the relatively higher activity of this lichen against gram positive but significantly also against gram negative bacteria. Following objectives were taken for screening of antibacterial activity of *Usnea* sp. To Prepare ethanolic and methanolic extracts of *Usnea* sp. Screening of extracts for antibacterial activity.

Material and Methods

Collection of the sample:Lichen was collected from Mashobra, Shimla Hills. The specimens were identified as *Usnea* sp. with the help of morphological and microscopic characters as mentioned in the literature (Sochting, 1999). Lichen was collected from Mashobra, Shimla Hills. The specimens were identified as *Usnea* sp. with the help of morphological and microscopic characters as mentioned in the literature (Sochting, 1999). Test Organisms: Five bacterial cultures were procured from Indira Gandhi Medical College (IGMC) Shimla. *a.Staphylococcus aureus., b. Pseudomonas aeruginosac. Klebsiella pneumoniae. Salmonella typhi. Escherichia coli.* Lichen was washed to remove debris, dried, ground to powder and stored in a sterile glass bottle in the refrigerator. The 5g portions of powder was added to 50 ml of solvents (ethanol and methanol), sonicated for 30 min and left overnight at room temperature. The extracts were prepared by decanting and filtered with Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate was evaporated to obtain 10 ml of concentrated extracts. Sterilized filtrate was stored in airtight containers in the refrigerator (Thippeswamy *et al.,* 2011).Disc diffusion method (Baur *et al.,* 1966) was followed for screening of antibacterial activity. Overnight grown bacterial cultures were spreaded on Muller Hinton agar plates to achieve semi confluent growth. Sterile filter paper discs was soaked in extracts, allowed to dry between the applications and placed on plates which were then incubated at 37°C for 24 hrs. Tetracycline was used as positive control and distilled water as negative control. Growth was evaluated and inhibition zone was measured. The bacterial isolates (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi and Klebsiella pneumoniae)* were sub-cultured on media and morphological and microscopic characters were studied. All the experiments were repeated twice and data presented are average of three replications.

Results and Discussion

The results of inhibitory activity of extracts of *Usnea* sp. were observed on the growth of various clinical isolates such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi and Klebsiella pneumonia*e. The extracts of *Usnea* sp. had inhibitory activity against all pathogens. Data presented in table (2) depicted that maximum zone of inhibition was found against *Staphylococcus aureus* (16mm) (Fig-8) followed by *Pseudomonas aeruginosa* (15mm) and *Echerichia coli* (15mm) and minimum against *Klebsiella pneumoniae* (10mm) (Fig-11). Present findings are in accordance with the work of the (Thippeswamy *et al.*, 2011), (Mandamombe *et al.*, 2003) and (Srivastava *et al.*, 2013) who reported inhibitory activity of ethanolic extract *Usnea longissima* and *Usneabarbata* against gram positive and gram negative bacteria. Data presented in table (3) depicts that maximum zone of inhibition was against *Salmonella typhi*(13mm) (Fig-9) followed by *Staphylococcus aureus* (12mm) (Fig-8) and minimum against *Echerichia coli* (7mm) (Fig-12). Present findings are in accordance with the work of (Madamombe *et al.*, 2003), (Srivastava *et al.*, 2013) and (Idamokoro *et al.*, 2013) who reported inhibitory activity of methanolic extract *Usnea barbata* against gram positive and gram negative bacteria.

The major phenolic compounds in these extracts were norstictic acid (*T. candida*) and usnic acid (*U. barbata*). Antioxidant activity was evaluated by free radical scavenging, superoxide anion radical scavenging, reducing power and determination of total phenolic compounds. Results of the study proved that norstictic acid had the largest antioxidant activity. The total content of phenols in the extracts was determined as the pyrocatechol equivalent. The antimicrobial activity was estimated by determination of the minimal inhibitory concentration using the broth microdilution method.

The most active was usnic acid with minimum inhibitory concentration values ranging from 0.0008 to 0.5 mg/ml. Anticancer activity was tested against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines using the microculture tetrazolium test. Usnic acid was found to have the strongest anticancer activity towards both cell lines with IC50 values of 12.72 and 15.66 μg/ml. *Usnea longissima* is an epiphyte species of lichen belongs to the family Parmeliaceae. Lichenic acids isolated from *Usnea longissima* are growth inhibitors. *Usnea longissima* was used a dermatological aid for wounds in the pecific North West. The ethanol extract of *Usnea longissima* were screened for potential antibacterial activity and antifungal activity by using Agar well diffusion method against six infectious strains and two dermatophytic fungi (*Trichodermav iride* and *Candida albicans)*. Ethanol extract of *Usnea longissima* exhibited significant antibacterial activity and antifungal activity with 1mg/ml Agar well diffusion method against the Gram positive *Staphylococcus aureus* (26 ± 0.5), and Gram negative *Pseudomonas* *aeruginosa* (18 ± 0.5), *Klebsiella pneumonia* (21 ± 0.5), *Shigella dysenteriae* (10 ± 0.3), *Salmonella typhi* (14 ± 0.5), *Escherichia coli* (-) and two dermatophytic fungi *Trichoderma viride* (14 ± 0.5) and *Candida albicans* (11 ± 0.5). This study is justified the traditional use and the effect of ethanol extract of lichen *Usnea longissima* was screened their level of antimicrobial potential *Usnea ghattensis* endemic fruticose lichen found growing luxuriantly in Northern Western Ghats of India. It also contains Usnic acid as a major chemical and tested against some human pathogenic bacteria. *In vitro* antimicrobial activity was tested initially by *Kirby-Bauer* technique of disc diffusion method and was confirmed by minimum inhibitory concentration using broth microdilution 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 were 3.125 μg/ml and 200 μg/ml respectively. The present study demonstrates the relatively higher activity of this lichen against gram positive but significantly also against gram negative bacteria. This indicates that this lichen might be a rich source of effective antimicrobial agents (Srivastava *et al.*, 2013). Various solvent extracts of the lichen *Usnea ghattensis* showed good antioxidant activity. A methanol extract prevented lipid peroxidation by 87% followed by 65% in Troloxat 20 μg/ml. It also showed superoxide anion scavenging activity and free radical scavenging activity 56% and 73% respectively. The known antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and quercetin at similar concentrations showed superoxide anion scavenging activity of 68, 59 and 47% and free radical scavenging activity 83, 77 and 69%, respectively. In addition, these extracts were inhibitory against *Bacillus licheniformis, Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus aureus* with MIC values of 5–10 μg/ml (Behera*et al*., 2005).*In-vitro* screening of the methanolic and ethyl-acetate extracts of *U. barbata* were evaluated to determine their antimicrobial activity against thirteen different *Staphylococcus* species. The selected organisms were isolated from raw bovine milk by several biochemical tests. The antimicrobial activity of extracts were evaluated using both the agar well diffusion method and the broth micro-dilution technique to determine the mean zone of inhibition and the minimum inhibitory concentration (MIC) respectively. The minimum bactericidal concentrations (MBC) of the extracts were also evaluated. Both the methanolic and ethyl-acetate extract showed variable antimicrobial activity against the *Staphylococcus* species with mean zones of inhibition ranging from 0 - 34 mm in diameter. Susceptibility by the *Staphylococcus* species tested in the methanol and the ethyl-acetate extract was 92.31% and 53.85% respectively. The MIC result for the methanol extract ranged from 0.0390 to10 mg/ml while that of the ethyl-acetate extract ranged from 0.15625 to 5 mg/ml.

Table.2 Zones of inhibition (in mm) of ethanolic extract of Usnea sp.

S. No	**Name of organisms**	**Zones of inhibition (mm) Ethanolic extract**	**Negative control (mm) (Distilled water**	**Positive control (mm) (Tetracycline)**
1.	*Staphylococcus aureus*	**16**	**-**	**26**
2.	*Pseudomonas aeruginosa*	**15**	**-**	**22**
3.	*Klebsiellapneumoniae*	**10**	**-**	**18**
4.	*Salmonella typhi*	**14**	**-**	**25**
5.	*Echerichia coli*	**15**	**-**	**26**

- = No activity

Table.3 Zones of inhibition (in mm) of methanolic extract of Usnea sp.

S. No	**Name of organisms**	**Zones of inhibition (mm) methanolic extract**	**Negative control (mm) Distilled water**	**Positive control (mm) (Tetracycline)**
1.	*Staphylococcus aureus*	**12**	**-**	**26**
2.	*Salmonella typhi*	**13**	**-**	**25**
3.	*Pseudomonas aeruginosa*	**10**	**-**	**22**
4.	*Klebsiellapneumoniae*	**8**	**-**	**18**
5.	*E. coli*	**7**	**-**	**26**

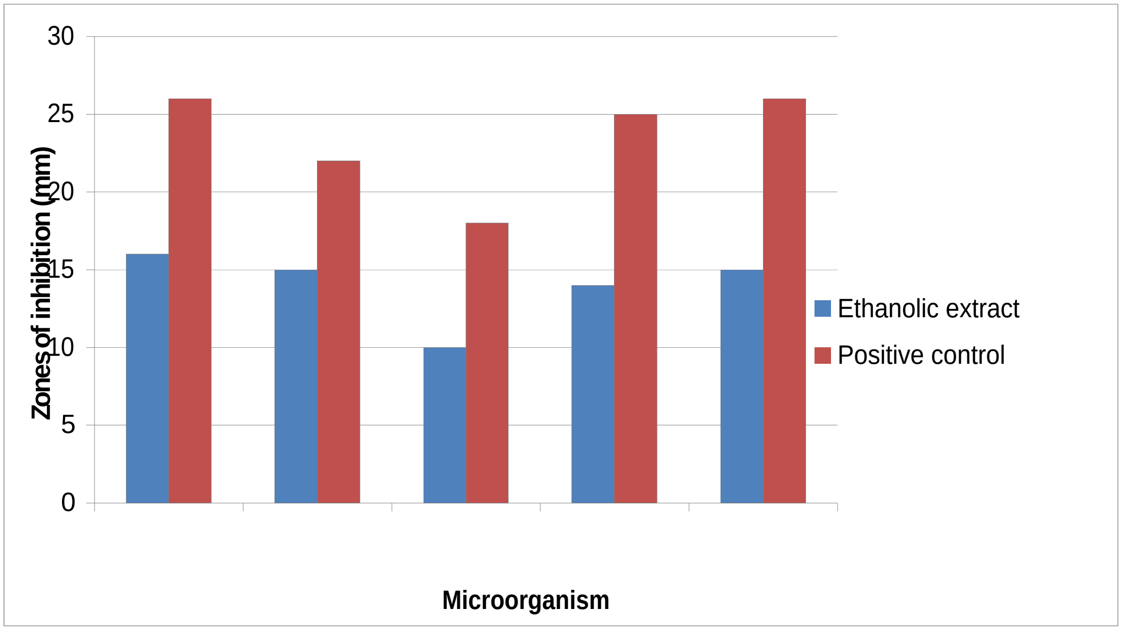
- = No activity

Table.4 Comparative analysis of ethanolic and methanolic extracts of Usnea sp.

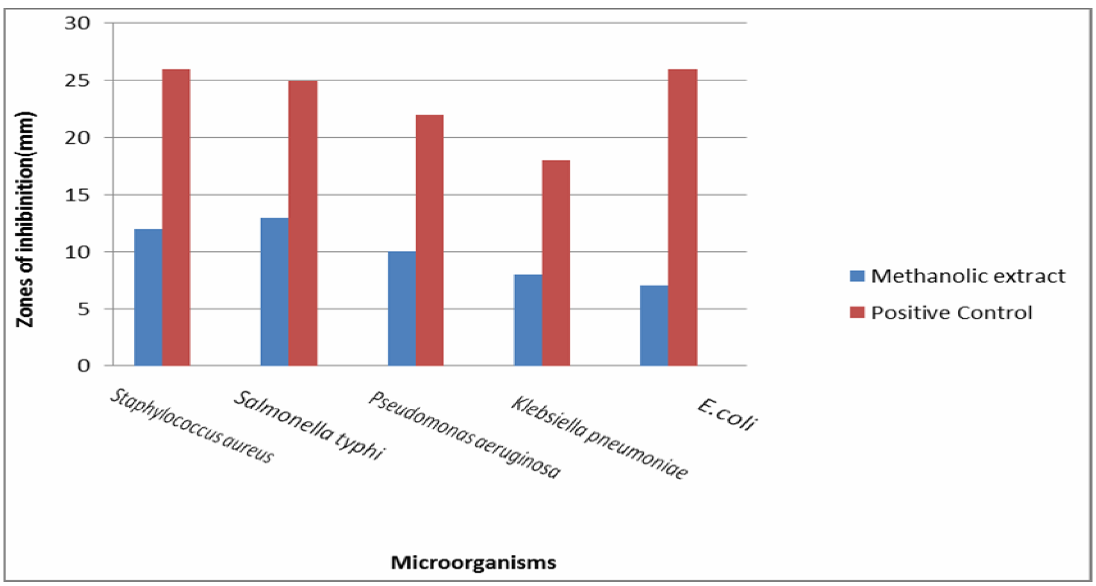
Sr. No	Name of organism	Zones of inhibition (mm) ethanolic extract	Zones of inhibition (mm) methanolic extract	Negative control (mm) Distilled water	Positive control (mm) Tetracycline
1	*Staphylococcus aureus*	16	12	-	26
2	*Pseudomonas aeruginosa*	15	10	-	22
3	*Klebsiella pneumoniae*	10	8	-	18
4	*Salmonella typhi*	14	13	-	25
5	*Esherichia coli*	15	7	-	26

- = No activity

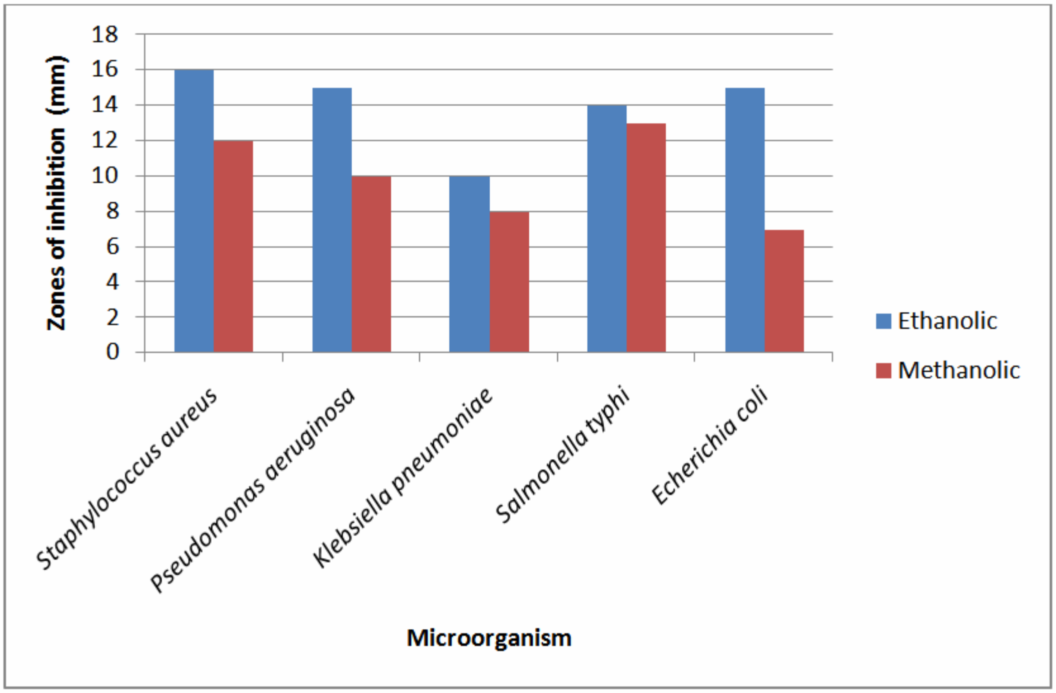
Graph.1 Graphical representation of ethanolic extract of Usnea sp.



Graph.2 Graphical representations of methanolic extract of Usnea sp.



Graph.3 Graphical representation of comparative analysis of ethanolic and methanolic extracts



Microscopic view of Usnea speciesUsnea species

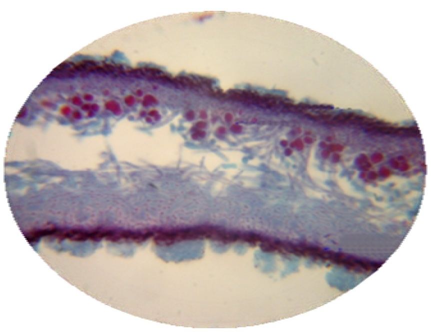
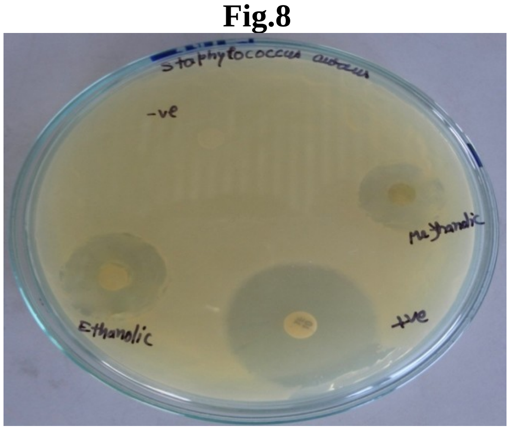


Fig.8 **Fig.9**



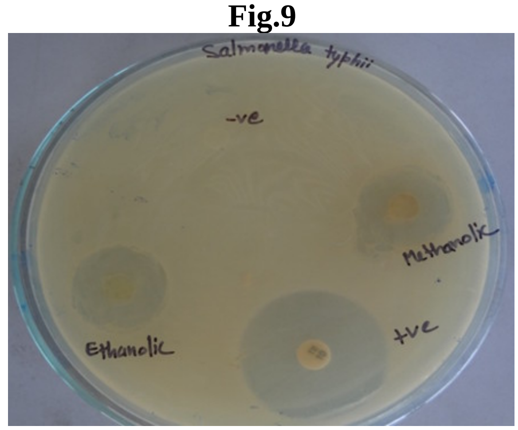


Fig.10

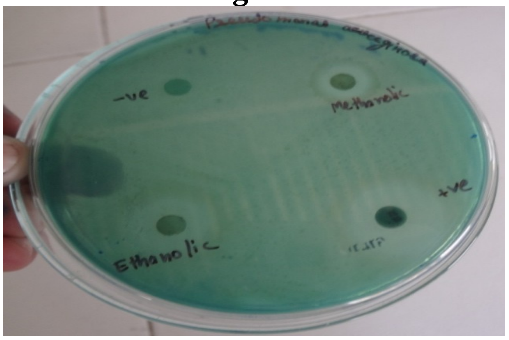


Fig.11

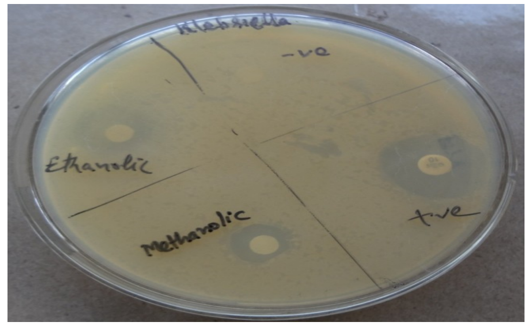
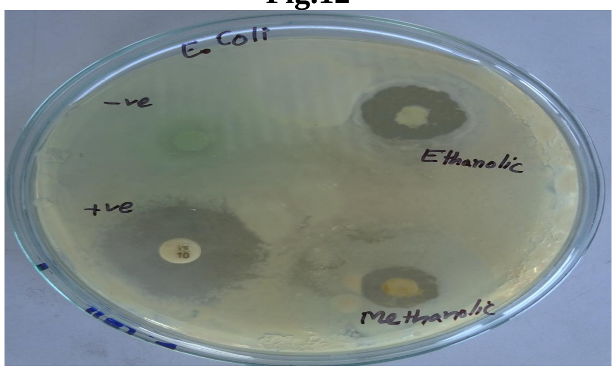


Fig.12



The MBC's were in the range of 40 to > 160 mg/ml and 80 to > 160 mg/ml for the methanol and the ethyl-acetate extracts respectively.

Results from this study revealed the *in vitro* antimicrobial activity of *Usnea barbata* lichen and therefore validate the use of the plant in traditional medicine. Lichens represent a unique division in the plant kingdom. They have been used in Traditional systems of medicine including Traditional Indian Medicine (TIM), Traditional Chinese Medicine (TCM), Homeopathic and Western Medical Herbals. Lichens have been used in the treatment of diverse diseases like arthritis, alopecia, constipation, kidney diseases, leprosy and pharyngitis.

Usnea sp. showed antibacterial activity against pathogenic organisms (*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhi* and *Escherichia coli).* Ethanolic extract of *Usnea sp.* showed highest zone of inhibition against *Staphylococcus aureus* (16mm) followed by *Pseudomonas aeruginosa* (15mm) and *Escherichia coli* (15mm) and minimum against *Klebsiella pneumoniae* (10mm). Methanolic extract showed highest zone of inhibition against *Salmonella typhi*(13mm) followed by *Staphylococcus aureus* (12mm) and minimum against *Escherichia coli (7mm)*.The medicinal utility of lichens is regarded to presence of secondary compounds like of usnic acid and atranorin. Animal investigations on lichens have demonstrated antimicrobial, antitumor and immunomodulaor activity. One of the reasons for exploring biological compounds in lichens is the potential for medical use. It would be advantageous to standardize the methods of extraction and in vivo testing so that the search could be more systematic and it may facilitate to control the pathogenic microorganisms. However much work remains to link medical effects with specific lichen species. It is therefore proposed that further investigation required for developing new drugs.

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