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IN-VITRO ANTIBACTERIAL SCREENING OF EXTRACTS OF *USNEA LONGISSIMA* LICHEN

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ABSTRACT: Objective: To evaluate the antibacterial activity of the hydroalcoholic and ethanolic extracts of the *Usnea longissima* lichen.

Material and methods: Kirby Bauer's disk diffusion method and broth serial dilution tests according to CLSI Guidelines (2000) were used, to find out the antibacterial effect of the 50% Hydro-ethanolic and 90% ethanolic extracts of the selected lichen. The efficacy of the extracts were measured after the period of incubation as Zone of Inhibition (ZOI) in mm compared with the Standard drug used i.e. Ciprofloxacin for Gram positive and Gentamicin for Gram negative bacterial strains. MIC was further tested for susceptible organisms. DMSO was used as control. All the experiments were conducted in triplicates and in proper sterilized condition

Results: It was found that *Usnea longissima* has a significant activity towards *Bacillus cereus* (ZOI 25-26 mm as compared to Ciprofloxacin ZOI-23 mm) MIC-0.625 mg/ml, *Pseudomonas aeruginosa* (ZOI 20-27 mm as compared to Gentamicin ZOI-14 mm) MIC- 1.25 mg/ml and *Proteus vulgaris* (ZOI of 13 - 16 mm where as Gentamicin produced ZOI as 14 mm) MIC 2.5 mg/ml. Moderate activity was shown towards *Staphylococcus aureus*, *Corynebacterium xerosis*, *Escherichia coli* and *Klebseilla pneuomoniae* while no activity towards *S. epidermidis* and *S. pyrogenes*. Moreover it was seen that 50% Hydro-ethanolic extracts produced more significant ZOI than ethanolic extract in all tested strains.

Conclusions: *U. longissima* contains potent chemical constituents as Usnic acid which can halt infection and is effective against various gram-positive and gram-negative bacterial species. It can be concluded that due to their antimicrobial effects extracts of the lichen can be used for the infectious diseases caused by these microbes. This study provides an in-vitro proof of the antibacterial activity of *Usnea longissima*.

Keywords: *Usnea longissima*, Zone of Inhibition, Antibacterial efficacy.

INTRODUCTION

Various lichens are used in many traditional cultures like Chinese, Pacific Island, New Zealand, North America and in Unani and Homeopathy. *Usnea longissima* is one of them which belongs to Parmealiaceae family and identified in Unani System of Medicine as Ushna and Shaibat-al– Ajooz (Antaki, 1924; Ghani, 1921). It is also known as "Methuselah's beard" that is large hanging hair lichen, 15-35 cm or more in length, pale yellowish green consisting of a single, unbranched central strand and numerous short lateral branchlets. It grows on oak and pines and other perennial trees growing in the forests. It is distributed at temperate Himalayas, Nilgiris and Pilani hills in South India (Rastogi and Mehrotra, 1969). Chopra (1979) reported that Usnea being inflammable easily catches fire causing forest fires. Interestingly he pointed out that some authors mentioned it as radio-active.

Ushna is reported in Unani literature as astringent, antidote, analgesic, cardiotonic, resolvent and stomachic (Husain, 1874; Attar, 1888; Antaki, 1924; Ibn Sina, 1931; Azam, 1987; Majoosi, 1869 and Razi, 1967). It also acts as healing agent on the wound and increasing the lactation in women if applied locally as paste on breast (Attar, 1888; Ghani, 1921; Baitar, 1986; Azam, 1987). This drug contains a yellow pigment usnic acid in cortex. The usnic acid inhibits Gram positive bacteria such as *Streptococcus*, *Staphylococcus pneuomoniae* in adults. Recently Usnic acid has been tested for positivity as antitumour activity. Since Usnic acid is the chief constituent of *Usnea longissima* used in Unani System of Medicine to treat wounds and inflammatory diseases and presently reported as antibacterial, expectorant and immune strengthener. Usnea appears to kill bacteria by distrupting their metabolic functions; especially it uncouples oxidative phosphorylation by acting on the inner mitochondrial membrane and also has ATPase activity. Unlike bacterial cells, human cells are less permeable to Usnic acid and are not adversely affected (Dube, 1983; Brodd 1984; Vashishta, 2002). It is necessitated to carry out the antimicrobial effect of Usnea longissima to validate the claims of Unani physicians.

MATERIAL AND METHODS

Plant collection

Usnea longissima, the whole herb was procured from the local market Baradari of Aligarh city, U.P (INDIA) during summer (August) 2010 and was authenticated by the Department of Botany, Aligarh Muslim University, Aligarh and the botanical literature available. Sample of the test drug was kept in Advia museum, Department of Ilmul Advia, AMU, Aligarh for future references (SC-0020/10).

Market Sample of *Usnea longissima*

Preparation of plant extracts

The test drug was dried at room temperature in a ventilated room, milled to a fine powder and stored in a closed container in dark until use.

50 % Hydro alcoholic extract: 10 gm of the crude drug powder and 150 ml of 50% Hydro alcoholic solvent were put into a soxhlet apparatus. The solvent was boiled at 400C and refluxed for a period of 150 min. The extract was filtered and evaporated to dryness under reduced pressure in the Lyophilizer (MacroScientific, Delhi). For experiment extract was redissolved in DMSO to the desired concentration.

90% ethanolic extract: 10 gm of crude drug powder and 150 ml of 90% ethanol were put into a soxhlet apparatus. The solvent was boiled at 400C and refluxed for a period of 150 min. The extract was filtered and evaporated to dryness under reduced pressure in the Lyophilizer (MacroScientific, Delhi).. For experiment extract was redissolved in DMSO to the desired concentration.

Microorganisms Used

Clinical strains of Gram positive and Gram negative bacteria were obtained from the Department of Microbiology, Jawaharlal Nehru Medical College & Hospital and Department of Biotechnology, Interdisciplinary Unit, Aligarh Muslim University, Aligarh. Bacterial Control strains investigated were *S.aureus* (ATCC 29213), S.pyrogenes (ATCC 14289), *S.mutans* (ATCC 700610), *S.epidermidis* (ATCC 155), *B.cereus* (ATCC 11778), *E.coli* (ATCC 25922), *K.pneuomoniae* (ATCC 15380), *P aeruginosa* (ATCC 25619), *C.xerosis (ATCC 373), P.vulgaris* (ATCC 6380). *S.mutans* were grown in Brain Heart Infusion (BHI) broth (LQ210 Himedia Labs, Mumbai, India), rest of the strains were grown in Nutrient Broth (M002 Himedia Labs, Mumbai, India) and incubated at 370C for 24 hours followed by frequent sub culturing to fresh media and were used as test bacteria and the bacterial cultures were checked to confirm the presence of sufficient number of bacterial cells on nutrient broth and maintained on nutrient agar slant.

Antimicrobial susceptibility testing

Antibacterial tests were performed as CLSI Guidelines (2000). 50% Hydro-alcoholic and 90% Ethanolic extract of Usnea longissima were used for their antimicrobial activities using Agar well diffusion on solid media. Brain Heart Infusion (BHI) Agar (SM 211 Himedia Labs, Mumbai, India) was used for *S.mutans* while Mueller Hinton Agar No.2 (M1084 Hi media Labs,
India) & Nutrient Agar (Himedia Labs, Mumbai, India) for preparing plates for rest of the bacterial strains. The solid Agar was punched with 6mm diameter wells. The inoculums (1.5×10^8 CFU/ml) were spread on to their respective agar plates using sterile swabs (PW041 Himedia Labs, Mumbai, India) and then filled with 40 µl extract. The concentration of the extract employed was 0.02 g/ml. All the plates were incubated at 37^0C for 24 hours. Ciprofloxacin disk (SD-142, Himedia labs, Mumbai, India) was used as standard drug for Gram positive while Gentamicin (SD170 Hi media Labs, Mumbai, India) for Gram negative bacteria. Wells containing respective solvent (DMSO) served as control.

Growth Inhibition was recorded by measuring the diameter of the Inhibitory Zones after the period of incubation. Triplicates were maintained and the experiment was repeated thrice and the Mean values along with Standard error (Mean +S.E.M) were calculated as shown in table-1& 2.

Table-1 Zone of Inhibition (in mm) of *U.longissima* against Gram-positive bacterial strains

S.No.	Strains	Usnea longissimia		Standard	Control
		50% Hydro-alcoholic extract	90% Ethanolic extract	Ciprofloxacin (30µgm)	DMSO (50µl)
1.	*Staphylococcus aureus*	13.33 ± 0.33*	12.6 ± 0.33*	22*	6
2.	*Streptococcus mutans*	12.66 ± 0.33*	_	21*	6
3.	*Streptococcus epidermidis*	_	_	23*	6
4.	*Staphylococcus pyrogenes*	_	_	22*	6
5.	*Corynebacterium xerosis*	14.33 ± 0.33*	13.33 ± 0.33*	21*	6
6.	*Bacillus cereus*	26.33 ± 0.33*	25.33 ± 0.33*	23*	6

-Results are expressed as Mean ± Standard error of Mean Probability error*p-value>0.001

Table-2 Zone of Inhibition (in mm) of *U.longissima* against Gram-negative bacterial strains

S.No.	Strains	*Usnea longissimia*		*Standard*	*Control*
		50% Hydro-alcoholic extract	90%Ethanolic extract	Gentamicin (30µgm)	DMSO (50µl)
1.	*Escherichia coli*	14.33 ± 0.33*	11.33 ± 0.33*	15*	6
2.	*Pseudomonas aeruginosa*	27.33 ± 0.33*	20.6 ± 0.33*	14*	6
3.	*Proteus vulgaris*	16.66 ± 0.33**	13 ± 0.57**	14*	6
4.	*Klebsiella pneuomoniae*	13.33 ± 0.33*	11.33 ± 0.33*	15*	6

-Results are expressed as Mean ± Standard error of Mean Probability error*p-value > 0.001**p-value > 0.05

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration of various extracts against tested microorganisms was determined by broth dilution method (Jennifer, 2001). 96-well microtitre plates were used, 50 µl of standardized suspension of a strain (10^6cfu/ml) was added to each tube containing extracts at various concentrations in the range of 5×10^{-3} mg/ml to 0.16×10^{-3} mg/ml. The plates were incubated at 37°C for 24h and observed for visible growth. The minimum inhibitory concentration (MIC) is taken as the lowest concentration of the extracts at which there is turbidity after incubation.

RESULTS AND DISCUSSION

The results of antimicrobial activity of *Usnea longissima* extracts showed a wide range of antibacterial activity against Gram positive and Gram negative bacteria. Significant antibacterial activity was seen towards *B.cereus*, where test drug hydro-ethanolic extract produces a large ZOI of 26.33±0.33 as compared to its 90% ethanolic extract ZOI – 25.33±0.33 (MIC-0.625 mg/ml) while the Standard drug Ciprofloxacin produces ZOI- of 23mm. Against *C.xerosis* also hydro-ethanolic extract produces a moderate antibacterial effect ZOI of 14.33±0.33 mm as compared to ethanolic extract ZOI of 13.33±0.33 mm (MIC-5.0 mg/ml) but it was lower than ZOI by Ciprofloxacin 21 mm. Similarly in *S.aureus* a moderate effect by extract was shown ZOI of 13.33±0.33 by hydro ethanolic and 12.6±0.33 mm by ethanolic extract (MIC-2.5 mg/ml) which was lower than Standard drug ZOI 22 mm. In case of *S.mutans* only hydro ethanolic extract produces activity 12.66±0.33 mm where as ethanolic extract produces no activity. There was no activity seen towards *S.epidermidis* and *S.pyrogenes* by either extract.

Among the Gram negative strains of bacteria selected for the study, a significant activity was seen towards *P.aeruginosa* where test drug hydro ethanolic extract produced ZOI of 27.33±0.33 mm and ethanolic extract produces ZOI of 20.33±0.33 mm (MIC- 1.25 mg/ml) which was much greater than ZOI produced by the Standard drug used- Gentamicin ZOI of 14 mm, antibacterial activity towards *P.vulgaris* was also more as compared to Gentamicin where hydro ethanolic extract produced ZOI of 16.66±0.33 and ethanolic extract of 13±0.57 mm (MIC-2.5 mg/ml) and Gentamicin ZOI was 14mm. There was also a considerable activity towards *E.coli* where hydro ethanolic extract ZOI was 14.33±0.33 mm and ethanolic extract as 11.33±0.33 mm (MIC-5.0 mg/ml) which was lower than ZOI by Gentamicin 15mm and same was seen towards *K.pneuomoniae* where ZOI by test drug hydro ethanolic extract was 13.33±0.33 mm and by ethanolic extract was 11.33±0.33 mm (MIC-5.0 mg/ml) which was also lower than Gentamicin ZOI 15 mm, (Fig 1 and 2).

In all the cases it was seen that 50% hydro-ethanolic extract has more antibacterial effect as compared to 90% ethanolic extract.

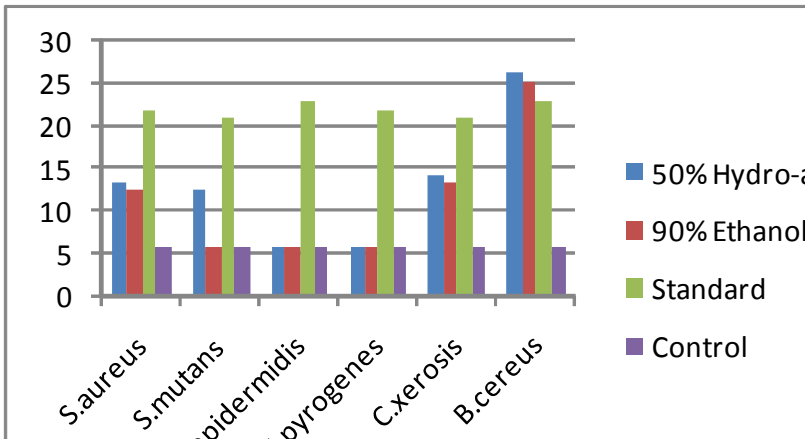

Fig-1: Zone of Inhibition (in mm) against Gram positive bacterial strains

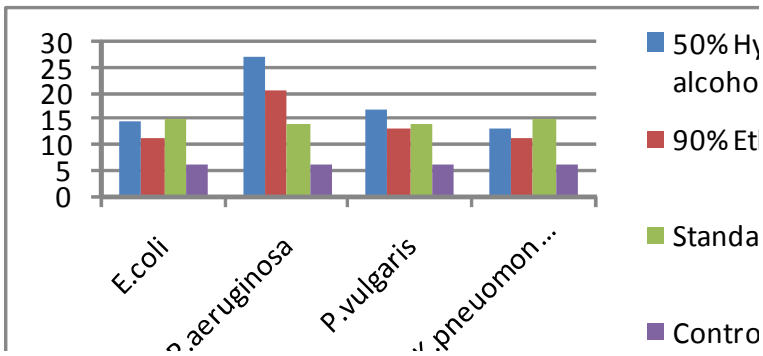

Fig-2: Zone of Inhibition (in mm) against Gram negative bacterial strains

CONCLUSION

The study concludes that *Usnea longissima* has a potent antibacterial activity against various pathogenic bacteria. The chemical constituents present in the lichen like Usnic acid may be responsible for its antibacterial effect as it is seen that Usnea contains a yellow pigment Usnic
acid in its cortex, which is reported to inhibit bacterial pathogens such as *Staphylococcus pneuomoniae* in adults. Usnea appears to kill bacteria by disrupting their metabolic functions (Brodo, 1984; Dube, 1983). So, it can be concluded that after further exploring its pharmacological details, as this is just an in-vitro proof of its existing antibacterial efficacy, this drug can be used to drive antimicrobial agents to fight against the number of infectious diseases mainly against *B.cereus*, *P.vulgaris and P.aeruginosa.*

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