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EVALUATION OF SOME TRADITIONAL THERAPEUTIC PROPERTIES OF *USNEA LONGISSIMA* (ASCOMYCOTA, LICHENIZED FUNGI): ANTIMICROBIAL, ANTIQUORUM AND ANTIOXIDANT

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ABSTRACT

Usnea longissima, fruticose lichen growing in the temperate region, is well known traditional medicinal herb and a reservoir of some unique secondary metabolites. Traditionally it has been commonly used as an antimicrobial agent for the treatment of ailments like tuberculosis, ulcers and skin diseases, etc. In our study, we evaluate antimicrobial, antiquorum sensing (QS) and antioxidant activity of *Usnea longissima* secondary metabolites extracted in acetone, methanol, and 70% hydroalcoholic solvents. Antimicrobial activity of extracts was evaluated against fungus (*Aspergillus niger*, *Candida albicans)* and six bacterial (*Agrobacterium tumefaciens, Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aerogenosa*, *Staphylococcus aureus*, *Streptococcus mutans)* strains. Whereas anti-QS activity was tested against biosensor strain *Chromobacterium violaceum* (CV) MCC 2290 and antibiofilm activity was checked in two gram-negative bacteria (*P. aeruginosa* and *E. coli*), two gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and fungus (*Candida albicans*). Results of experimental data indicated that methanolic extract exhibited maximum activity against *A. tumefaciens* (24±0.2 mm) and *C. albicans* (30±0.3 mm) with the minimum inhibitory concentration of 300µg/mL and 200µg/mL respectively. Interestingly, we also observed that all extracts effectively inhibited quorum sensing in *C. violaceum* and biofilm formation in *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aerogenosa*, *Staphylococcus aureus*, at the concentration (100-300 µg/mL), for *Candida albicans* (50-150 µg/mL). *Usnea longissima* extract showed strong reducing power and hydroxyl radical activity and low DPPH scavenging activity. The present study suggested that *U. longissima* possesses potential antimicrobial and antiquorum sensing activity. These biological properties could be attributed to its significant antioxidant potential which validates its traditional use as an antimicrobial herbal source.

Keywords: Antibiofilm; Infectious disease; Yeast; Antioxidant

INTRODUCTION

Lichens represent the most successful symbiotic relationship between fungi (90%) and algae (10%), sometimes cyanobacteria, survive in extreme environmental conditions and geographically distributed from tropical to the polar region, covering almost 8% of the earth's surface **(Alahmadi, 2017)**. Lichens have a wide array of unique secondary metabolites such as fatty acid polysaccharides, terpene, flavonoid depsides, depsidones, di-benzofurans, depsones which confer protection against biological attack and physical stress **(Ullah *et al.*, 2003)**. Lichens are used in traditional medicines as well as incontemporary homeopathic, naturopathic medicines **(Madamombe and Afolayan, 2003)**. The most common uses of lichens are; for treating wounds, ulcers, respiratory and gastrointestinal issues. In the last three decades, studies explored antimicrobial, antiviral, antiprotozoal, antipyretic, antitumor, antiproliferative, anti-inflammatory, analgesic, antioxidant, and photoprotective activities of different lichens from different regions of the world **(Behera *et al.*, 2013; Bao *et al.*, 2012; Correche *et al.*, 2004; Paliya *et al.*, 2016; Nishanth *et al.*, 2015)**. In India, though the folklore and ethnic use of lichen as medicine were known from ancient times, however more systematic validation of such species initiated recently. Some of the macrolichens, commonly occurring in India such as species of *Heterodermia, Parmotrema, Sticta, Ramalina* and *Usnea* exploited for their preliminary biological screening against humans and plants pathogen **(Shahi *et al.*, 2001; Shivanna and Garampalli, 2014)**. Among the different lichen texa, the species of fruticose lichen, *Usnea* has been found growing luxuriantly in the Indian Himalayan region at around 3000 to 3500 meters. The species having usnic acid is well explored for its medicinal potential throughout the world **(Paliya *et al.*, 2016)**.

Most easily recognizable fruticose lichen, *Usnea longissima* Ach. (*Dolichousnea longissima*(Ach.) Articus) is also known as an old man's beard, belongs to the family *Parmeliaceae*. It is pale-green or light yellowish color usually grows on the bark of the old trees. The world's longest lichen *U. longissima* is a common inhabitant Western of Ghats and the Indian Himalaya region. *U. longissima* shows diversity in metabolite production and thereby getting attention from researchers; who have isolated and explored their hidden unique metabolite activities in different areas; such metabolites are usnic acid, diffractaic acid, evernic acid, salazinic acid, fumarprotocetraric acid, ramalinolic acid, squamatic acid, orcinol, and atranorin **(Ullah *et al.*, 2003; Dandapat and Paul, 2019)**. Other anti-inflammatory phenolic compounds are glutinol, longissiminone **(Choudhary and Jalil, 2005)**. *Usnea longissima is* natural antioxidant and used to cure bone fractures, the leg and loin injuries, as an expectorant, immune strengtheners **(Atalay *et al.*, 2011; Rauf *et al.*, 2011)**, to treat surface infection, *Tuberculosis lymphadenitis* **(Blumenthal *et al.*, 2000)**. American natives use *U. longissima* as a natural skin drug **(Sudarwanti *et al.*, 2018)**; significantly inhibit bacterial neuraminidase, saprolegniasis **(Ullah *et al.*, 2003; Guo, 2017)**. *Usnea* increases lactation in breastfeeding women **(Verma *et al.*, 2008)**.

Quorum sensing (QS) in bacteria is a cell communication process in which intercellular biochemical signal releases when cell density reaches high concentration **(Girard and Bloemberg, 2008; Sifri, 2008; Solano *et al.*, 2014; Gokalsin and Sesal, 2016)**. These signals aid to modify the gene expression and cascade the release of different virulence factors, biofilm formation **(Sifri 2008; Al Wrafy *et al.*, 2017)**. Biofilm and QS both are interdependent mechanisms and develop a new antibiotic-resistant lifestyle in bacteria. Such types of pathogenic bacteria are very dangerous for the host, for example, *Pseudomonas aeruginosa*, *Candida* **(Sifri, 2008; Solano *et al.*, 2014; Millot *et al.*, 2017)** cause severe infection in an immune-compromised patient due to biofilm formation that confers resistance to antibiotics and hosts immune responses **(Guo, 2017; Flemming and Wuertz, 2019; Shah *et al.*, 2019)** and gives an open challenge to herbal medicine. Usnic acid, a major metabolite in *U. longissima* (first time isolated by Knop in 1844) **(Yu X *et al.*, 2016)**, plays an important role in inhibiting biofilm formation in *S. aureus* (isolated from *Cystic fibrosis* patient)

(Nithyanand *et al*., 2015) and *P. aeruginosa* by interfering with DNA, RNA synthesis and signaling pathway **(Maciag-Dorszynska *et al*., 2014).** Since QS determines the intensity of microbial infection, therefore inhibition of QS could be a better way or therapeutic practice against microbial disease without exerting selective pressure on microbe (the main cause of drug-resistant in bacteria).
In the present study, an attempt has been made to evaluate the antimicrobial, antioxidant activity and investigate antiquorum sensing activity of *U. longissima* extracts through biofilm inhibition against some pathogenic microorganisms and validate its traditional use as herbal medicine.

MATERIAL AND METHODS

Media, Chemicals and Microorganisms used

TLC silica gel $60F_{254}$ aluminum-backed plate, Streptomycin and ketoconazole, nutrient culture media and muller Hilton agar (MHA), potato dextrose agar (PDA), potato dextrose broth (PDB), nutrient broth (NB), DPPH, ascorbic acid, sodium nitrite, Aluminium chloride, sodium hydroxide, potassium ferri-cyanide, ferric chloride, hydrogen peroxide, trichloroacetic acid (TCA), TBA (thiobarbituric acid), sodium dodecyl sulphate (SDS), n-butanol were obtained from the Hi-Media Laboratories, Mumbai, India. Microorganism strains used in this study – *Agrobacterium tumefaciens, Aspergillus niger, Candida albicans, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans, Bacillus cereus, Chromobacterium violaceum* (MCC 2290) obtained from NBRI and BBAU, Lucknow, India.

Collection of Sample

The lichen sample was collected from the Uttarkashi district of Uttarakhand, India. The samples were segregated and identified by studying their morphology, anatomy, and chemistry. Voucher samples of the lichens were utilized and deposited in the herbarium of CSIR-National Botanical Research Institute, Lucknow, India.

Preparation of lichen extract

The lichen samples were washed in tap water, tween 80 detergent was used to remove dirt and unwanted materials and then samples were scattered on sterile filter paper and left at room temperature till dry. The dried lichen sample (30.6 gm) was frozen with liquid nitrogen and crushed. The crushed material was divided into three equal amounts and extracted in soxhlets using three different solvents acetone, methanol, 70% hydroalcohol. The evaporation of the solvent in the extract was carried out in a rota vapour machine at a definite temperature for particular solvents to avoid any metabolite degradation. The final dried crude extract was weighed (Acetone 330.7 mg, 70% hydroalcohol 453 mg and Methanol 280.4 mg) and stored in Eppendorf tubes at 4ºC for further preparation of the stock solution.

Thin-layer chromatography

Thin-layer chromatography is a simple fast sensitive and inexpensive solid-liquid analytical technique. TLC has been used to identify lichen compounds and study the specific group of lichen (mycobiont) secondary metabolites **(Bendz *et al*., 1967; Molnar and Farkas, 2010)**. The fraction of *U. longissima* was immersed in acetone solvent and left for 15-20 minutes. Further, spot-on silica gel precoated $60F_{254}$ aluminium sheet and then the TLC plate was developed in solvent system A, containing a mixture of toluene: dioxane: acetic acid (ratio180/60/08 v/v/v) solution used for spot running **(Yılmaz *et al*., 2004)**.

Antioxidant activity assay

Determination of total phenolic compounds

The total phenolic content of methanolic extract (ME) and 70% hydroalcoholic extract (HE) of *U. longissima* was evaluated by the Folin-Ciocalteu method **(Singleton and Rossi, 1965)**. Different doses of each sample were prepared in distilled water (DW), mixed with 1 mL of Folin-Ciocalteu reagent and 1 mL of 20 % Na_2CO_3 diluted mixture with 7 mL DW and kept in dark for 90 min. and absorbance was measured at 760 nm. Results were expressed as gallic acid equivalents (GAE), which represent the amount of phenolic content in 1 mg of sample.

Determination of total flavonoid content (TFC)

The TFC of *U. longissima* extracts was measured by the calorimetric method as described earlier **(Aydin *et al*., 2018)** with some modification. Briefly, samples were prepared at a concentration of 500µg in 6 mL with DW. Then 300 µL of 5% $NaNO_2$ and 300 µL of Aluminium chloride (10 % $AlCl_3$) were added followed by incubation of 6 min room temperature (RT). Then 2 mL of 1M NaOH was added and the mixture was diluted with 2.5 mL of DW. The TFC of the extract was calculated by plotting a standard curve of rutin with five different concentration ranges (20-100 µg) and expressed as quercetin equivalents (mgQE/g).

DPPH (1, 1-diphenyl-2-picryl-hydrazyl) free radical Scavenging Assay

The quantitative free radical scavenging effects of *U. longissima* extract were measured by DPPH assay, the method described by **(Xu W *et al*., 2009)** with few changes. Extract was prepared at different concentrations (100-500 µg/mL) and mixed with 2 mL of 0.1mM solution of DPPH in 95 % solutions. The solution was shaken vigorously and incubated at room temperature for 20 min. the percentage of radical scavenging was determined by observing the change in color from dark purple to pale yellow, which can be measured by taking absorbance at 517 nm. Ascorbic acid was used as a positive control.

Ferric Reducing Power Assay

The reducing power of *U. longissima* extract was determined by the method **(Yen *et al*., 2005)** with slight modification. Briefly, 1 mL of *Usnea longissima* extract were prepared at each dose of 100-500 µg/mL and mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.8), 2.5 mL of 1% potassium ferri-cyanide and incubated at 50°C for 20 min., 2.5 mL of 10% TCA was added to stop the reaction. After centrifuged to 1000 x g for 10 min, 2.5 mL of the upper layer was taken, mixed with 2.5 mL of DW and 0.5 mL of 0.1 % ferric chloride ($FeCl_3$). The absorbance of the final mixture was measured at 700 nm. Higher absorbance indicates greater reducing power.

Hydroxyl radical scavenging activity

Usnea longissima extract samples (100-500 µg/mL) in 1 mL DW respectively, were added to 1 mM 0.1 mL EDTA, 0.01 mL of 10mM $FeCl_3$, 0.1 mL of 10mM H_2O_2, 0.36 mL of 2.8 mM deoxyribose, 0.33 mL of phosphate buffer (50mM) and 0.1 mL of ascorbic acid (1 mM). This mixture was incubated at 37ºC for 1 hour. After incubation, 1 mL of 10%, TCA and 1 mL of 1% TBA were added then the mixture was boiled at 90ºC for 30 min. The color change was measured by taking absorbance at 532 nm **(Pal *et al*., 2010).**

Assessment of antimicrobial activity

The antimicrobial activity of *U. longissima* extract was determined through agar well diffusion assay against bacterial and fungal pathogens. The test microorganism was revived on nutrient agar medium overnight and the cell suspension was prepared by suspended bacteria in sterile saline water (0.85% saline) and adjusted the inoculum density according to 0.5 McFarland turbidity standard i.e.1×10^5 to 2×10^5 CFU/mL. Similarly, fungus culture was grown on PDA for 48 to 72 hrs and fungal filaments were used to inoculate in sabouraud dextrose broth. 100µL of each suspended microorganism culture equally spread on sterile MHA and PDA Petri plates. Sterile cork borer was used to create wells of size 6mm diameter. Each sample was prepared for 15 mg/mL concentration in 20% DMSO. Each well was properly labeled and supplemented with extracts sample of concentration 600 µg/mL. For positive control, Streptomycin was used for bacterial pathogens and Ketoconazole was used for fungal pathogens, both at 10 µg/mL concentration. Whereas, for negative control 20% DMSO was used. Bacterial Petri plates were incubated at ±37ºC for 24hrs and the fungal Petri plate was incubated at ±25ºC for 48 hrs. The extract's sensitivity against microorganisms was determined by measured the diameter of the zone of inhibition (ZOI, in mm) formed around each well. All the antimicrobial activities were performed in triplicate.

Minimum inhibitory concentration (MIC)

It represents the minimum concentration of crude extract of lichen at which no bacterial population can survive in a particular medium. The micro-dilution technique was used to determine the MIC of lichen extract against test pathogens. Lichen crude extracts serially diluted to concentration range 100-600µg/mL. For positive control untreated bacterial and fungal pathogens were used. The test was performed in 96 well plates, 100µL Luria broth for bacteria and Potato dextrose broth for fungus were inoculated with 20µL suspended pathogens in each well. *Usnea longissima* crude extract was added at different concentrations of 100-600 µg/mL and incubated at ±37ºC for 24 hrs (bacteria) and ± 25ºC for 48 hrs (fungus). 0.1 mM Alamar Blue reagent was add in each well and incubate culture for the predetermined time in an incubator at 37ºC. After the incubation, absorbance was taken at 570nm and 600nm wavelengths. Alamar blue is nontoxic and enters into the cell then the blue color turns pink due to the reduction of resazurin dye. Viable cells continuously reduce resazurin that gives pink color.

Antiquorum sensing and antibiofilm activity

Antiquorum sensing activity

Antiquorum sensing activity was determined by the standard overlay method. A quorum-sensing test was performed on the biomonitor strain of *C. violaceum*, bacteria produce quorum sensing regulated violet color pigment called violacein **(Truchado *et al.*, 2009)**. First, the inoculum was prepared in normal saline and maintaining a cell density of 0.5 OD at 600 nm. LB agar was prepared with 0.3% agar and inoculum was added after cooling to 40ºC, before solidifying. This 3% LB agar was used to overlay on previously prepared LB agar plates to form a layer on it. When agar was solidifying, the disc was placed and different concentrations of extract solution (100, 200 and 300 µg disk^{-1}) were dropped on the disc. 50% of DMSO was used for negative control. Plates were incubated at 30ºC overnight.

Quantification of violacein production

Inhibitory activity of *U. longissima* extract against violacein production in *C. violaceum* was performed according to the method **(Singh *et al.*, 2009)**. A culture of *C. violaceum* was grown in Luria broth in test tubes containing 5 mL media and treated with extract at three different concentration ranges from 100-300 µg/mL. Bacterial cells were incubated for 24 hours at 30ºC and lysed by adding 10% SDS. Violacein was extracted by adding n-butanol and vortex it properly and centrifuge at 10,000 rpm for 10 min. The upper butanol layer was isolated and absorbance was taken at 585 nm.

Biofilm Inhibition assay

Effect of *U. longissima* extract on biofilm inhibition was tested against *B. cereus*, *C. albican*, *E. coli*, *P. aeruginosa*, *S. aureus*, according to the previously described method **(Singh *et al.*, 2012)**. An overnight grown microbial culture was used to inoculate (1% v/v) media in the petri dish (55 x 12) and sterile glass cover-slips are also placed in a petri dish. These cultures were treated with methanolic extract in three different concentrations 100-300 µg/mL, for bacterial strains and 50-150 µg/mL, for fungal strain, followed by incubation of 24 hours at 37±2°C. After incubation media were discarded carefully so that formed biofilm was not getting disturbed and washed with phosphate buffer (PBS). The biofilms that were got adhere to a glass coverslip were stained with 10% crystal violet dye and washed again with PBS. Inhibition in biofilm formation was examined under a microscope (Leica DM 2500) in a bright field.

RESULTS

Total phenol and Flavonoid content

Usnea longissima is rich in phenolic compounds, the total phenolic content of *U. longissima* in HE and ME was found to be 24.04±0.05µg and 20.73±0.01µg gallic acid equivalents, phenols content in 1 mg of *U. longissima* extract. However, flavonoid content was found in a negligible amount in both extracts of *U. longissima*.

Major chemical compounds

Two compounds identified in the TLC result that is- usnic acid and very low barbatic acid (Figure 1A). The characterization of the compounds was based on the calculated R_f value.

Determination of antioxidant activity

The DPPH free radical scavenging activity of extracts was increased in a concentration-dependent manner (Figure 1B). But, the overall scavenging property of *U. longissima* is low (5-10%) in HE and (10-20%) in ME at 100-500 µg/mL, as compared to standard ascorbic acid with (50-94%) radical scavenging activity at 10-50 µg/mL concentration.

Table 1 IC_{50} value of *Usnea longissima* extracts.

	IC_{50} concentration (µg/mL)		
Antioxidant assays	70% hydroalcoholic extract	Methanolic extract	Ascorbic acid
DPPH	321±22	334±18	10.7±0.35
Ferric reducing power	133±15	141±12	4.2±0.25
Hydroxyl radical scavenging	58±05	63±08	3.8±0.23

* Data represented as mean ± SD of three independent readings.

In reducing power assay, results demonstrate a dose-dependent activity in both extracts (Figure 1C). The ME showed higher activity than HE at the concentration range (100-500 µg/mL). Reducing power ability of *U. longissima* extracts is half potential when compared to standard ascorbic acid at 10-50 µg/mL concentration. Hydroxyl radical scavenging activity of *U. longissima* extract showed concentration-dependent increased in scavenging activity (Figure1D). Around 50% of radical scavenging was observed in both extracts at 500 µg/mL concentration. While ascorbic acid showed 80-85% Hydroxyl radical scavenging at a concentration of 50 µg/mL. These antioxidant results demonstrated that *U. longissima* metabolite expresses potential reducing power and hydroxyl radical scavenging properties. Results of the IC_{50} value of *U. longissima* extract of the different assays are represented in (Table 1).

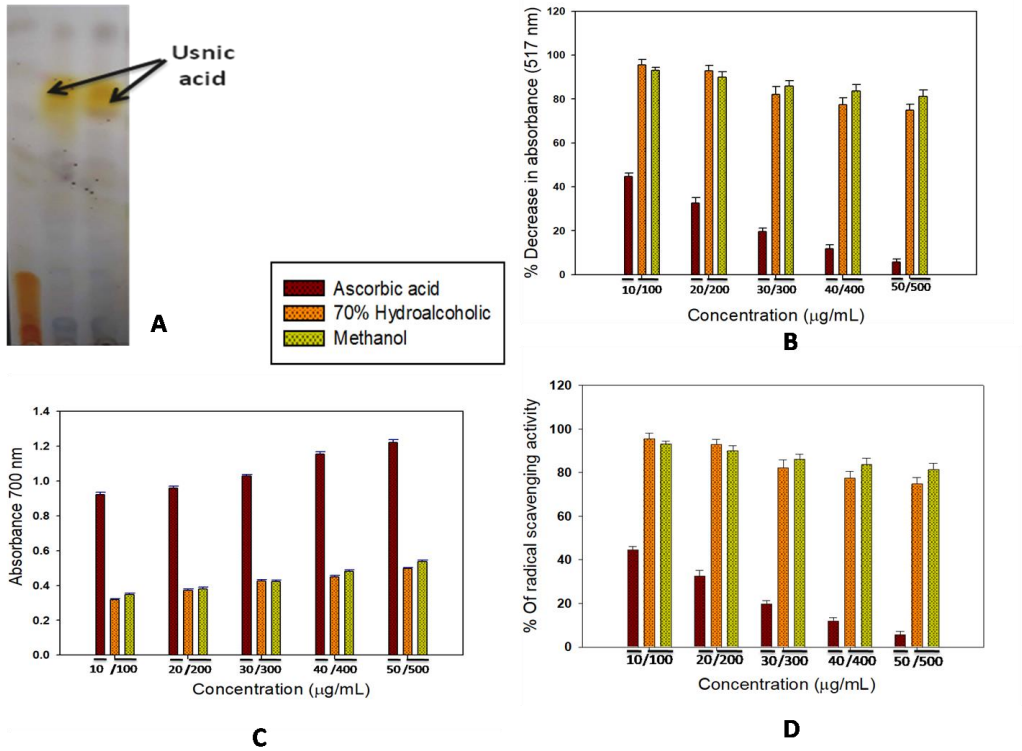

Figure 1 TLC plate image (yellow spots represent Usnic acid) **(A)** DPPH assay **(B)** Reducing power assay **(C)** Hydroxyl radical scavenging assay (**D)** of *U. longissima* extracts in different solvents. Results are represented in terms of change in absorbance and radical scavenging Percentage. The test was performed in triplicates and all values were considered as mean ± SD of three independent experiments with P > 0.05.

Antimicrobial activity

Antimicrobial assay results revealed that lichen crude extract showed a varied range of antimicrobial activity against the tested microorganism (Table 2). ME showed broad-spectrum antimicrobial activity against all pathogens (except *A. niger*). Among them, the most significant activity was reported against *C. albicans* (30±0.3mm) and *A. tumifaciens* (24±0.2mm).

The MIC of all lichen extracts was tested against respective human and plant pathogenic bacteria and fungus using Alamar blue assay in 96 well microtiter plates (Table 2). Methanolic and acetone extracts showed significant antimicrobial activity against *C. albicans* with MIC 200 µg/mL and 400 µg/mL respectively. The MIC of *A. tumifaciens* was 400 µg/mL for acetone extract, 300µg/mL in methanol and 70% hydroalcoholic extract. *P. aeruginosa* showed complete inhibition at 600 µg/mL in all extracts of *U. longissima*. Similarly, the MIC range for *S. mutans* was 400-500 µg/mL for different extracts.

Table 2 Zone of inhibition (ZOI) and MIC value of *Usnea longissima* in a crude extract of acetone, methanol, and 70% hydroalcoholic.

Microorganisms	Acetone	MIC µg/mL	Methanol	MIC µg/mL	70% Hydroalchoholic	MIC µg/mL	+ve control	-ve control
Bacteria								
Agrobactreium tumefaciens	22±0.7	400	24±0.2	300	20±0.5	300	25±0.5	00
Escherichia coli	10±0.3	500	11±0.2	400	07±0.3	600	23±0.2	00
Klebsiella pneumoniae	13±0.2	600	15±0.3	500	11±0.2	600	26±0.2	00
Pseudomonas aeruginosa	15±0.1	600	18±0.2	600	10±0.2	600	28±00	00
Stephyllococcus aureus	20±0.2	500	23±0.2	500	20±0.3	500	28±00	00
Streptococcus mutans	19±0.2	500	22±0.2	400	16±0.5	500	23±00	00
Fungus								
Candida albicans	25±0.2	400	30±0.3	200	22±0.2	400	38±0.2	00
Aspergillus niger	12±0.2	500	10±0.2	600	09±0.2	600	25±0.2	00

* The average value of three calculations is presented as mean ± S.D. (standard deviation) Positive control: Streptomycin, ketoconazole 10 µg/mL; Negative control: 20% DMSO Included diameter of well 6 mm.

Antiquorum sensing activity

Antiqurom sensing results were determined by observing the halo zone (without violacein pigment) around the disc (Figure 2B). The overlay disc diffusion assay on *C. violaceum* showed a clear halo zone around the disc treated with samples. All the extracts were showing antiquorum sensing activity against *C. violaceum* as the bacterial cells were grown without the production of violacein pigment. Most significan activities were reported in ME and HE, while acetone extract showed moderate activity and no activities were reported in the negative control.

The effect of *U. longissima* extracts in the production of violacein pigment in *C. violaceum* (Figure 2A). Significant inhibition of violacein production by *U. longissima* in a dose-dependent manner was seen (Figure 2C). The most significant inhibition was observed in ME (81%), HE (75%) at 300 µg/mL. whereas more than 50% inhibition was observed in each extract at 200 µg/mL concentration.

Biofilm Inhibition

Inhibition of biofilm formation in *B. cereus*, *C. albican*, *E. coli*, *P. aeruginosa*, *S. aureus*, by *U. longissima* extract was performed by crystal violet staining method. Biofilm inhibition results were observed under a microscope (Lieca DM 2500) and represented in the form of images (Figure 2D) further quantitative data revealed that *U. longissima* extract causes destruction of biofilm in a concentration-dependent manner. 50% biofilm inhibition was observed in bacterial pathogenic strains at 200 µg/mL methanolic extract while at 50 µg/mL for *C. albicans.*

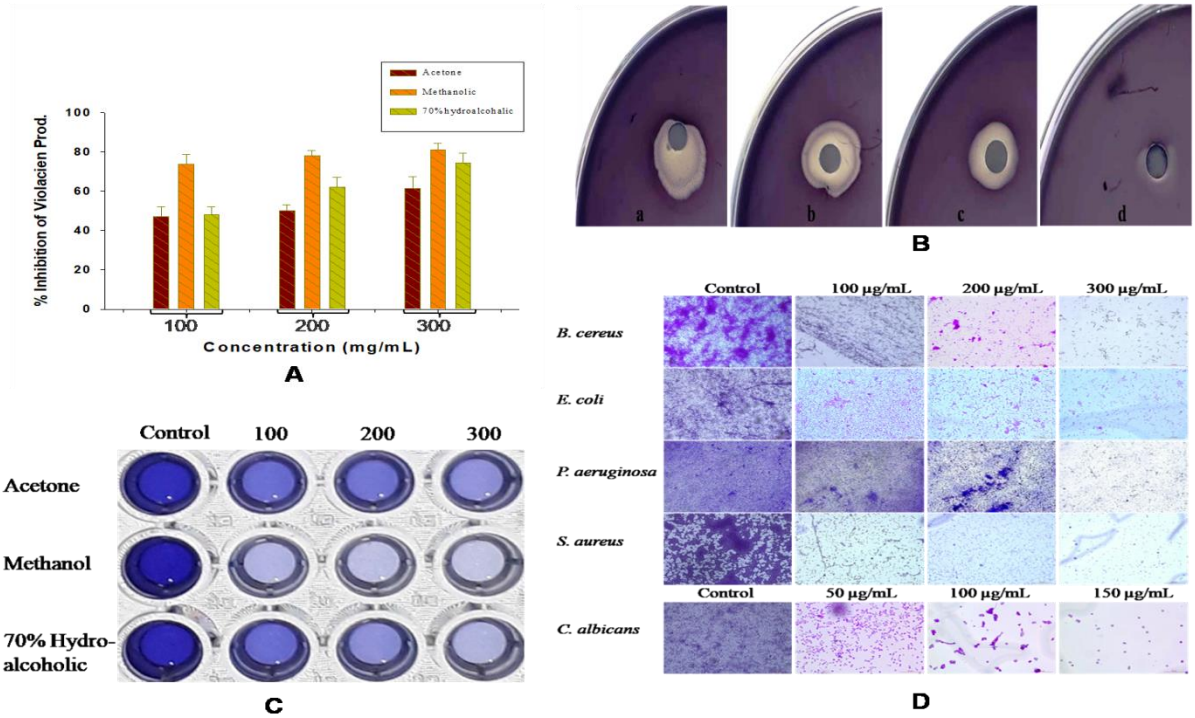

Figure 2 Quantification of violacein production, results are shown as percentage inhibition in violacein production. Data represented in mean value ± SD **(A)**. Antiquorum sensing activity (a) Methanolic (b) 70% hydroalcoholic (c) Acetone extract (d) 50% DMSO. The halo zone around the disc represents inhibition of quorum sensing in biomonitor strain *C. violaceum* **(B)**. Qualitative Representation of inhibition in violacein production w.r.t. control in all extracts **(C)**. Show inhibition of biofilm Formation in *B.cereus, E. coli, P.aeruginosa, S. aureus, C. albicans.* Complete eradication of biofilm was observed at 300 µg/mL and 150µg/mL (*Candida albicans)* concentration w.r.t. to the non-treated control **(D)**.

DISCUSSION

In the light of the present study, we extracted *U. longissima* secondary metabolites in different solvents to extract the maximum possible compounds of different polarities and verify antimicrobial and antiquorum sensing activity of different extracts. Results of experiments validate the traditional uses of *U. longissima* by showing a varied range of activity with different extracts in concentrations range between (100-600µg/mL). The most prominent antimicrobial activity was reported in ME against *C. albicans* at MIC value 200µg/mL, these results are corresponding to earlier reports where the significant anticandidal activity of *U. longissima* in the ethanolic extract was reported **(Rauf *et al*., 2011; Thippeswamy *et al*., 2011)**. While in another study **(Sudarwanti *et al*., 2018)** identified acetone extract of *U. longissima* promising against *C. albicans*. However, few studies have been done in detail for the anticandidal activity of *U. longissima*. **(Pavithra *et al*., 2013)** reported anticandidal activity in *Usnea pictoides* chloroform, ethyl acetate and methanolic extract at 2 mg/mL concentration. Their study suggested usnic acid the major bioactive compound responsible for antimicrobial activity. Usnic acid isolated from *Cladonia foliacea* has shown inhibition of a panel of bacteria and fungi that included *S. aureus* and *C. albicans* **(Yilmaz *et al*., 2004)**. We reported moderate to non-significant activity against *E. coli*, previous studies also reported *U. longissima* extracts non-effective against *E. coli* and significant activity of HE, ME and ethyl acetate extracts against *K. pneumoniae* in **(Rauf *et al*., 2011; Kamal *et al*., 2015; Kumar *et al*., 2017)**. *P. aeruginosa* is a Gram-negative opportunistic pathogen capable of infecting humans with compromised natural defenses and causing severe pulmonary disease. This bacterium has the remarkable property of biofilm formation which allows them to become resistant to many drugs **(Alhazmi, 2015)**. We found significant activity against *P. aeruginosa* in ME as compared to HE and acetone extract. The antibacterial potential of methanolic and hydroalcoholic extracts against *P. aeruginosa* was also reported in separate studies. **(Rauf *et al*., 2011; Srivastava *et al*., 2013; Kamal *et al*., 2015; Kumar *et al*., 2017)**. All the extracts in the present study showed strong antibacterial activity against *S. aureus* and *S. mutans* corresponds to other studies where they reported moderate activity in 50% alcoholic extract **(Rauf *et al*., 2011; Srivastava *et al*., 2013)** and in acetone extract (10.65±0.4 mm) which was found to be half potential activity as compared to present results **(Sudarwanti *et al*., 2018)**. When Usnic acid, a major compound present in *Usnea* species, tested against *P. aeruginosa, S. aureus and E. coli* gives significant antimicrobial activity **(Ananthi *et al*., 2016)**. However, all the results of the present study are not as eminent when compared to standard streptomycin.

Antioxidant activity of *U. longissima* extract suggested significant radical scavenging activity in reducing power and hydroxyl radical scavenging assay except for DPPH. Despite the presence of phenolic compounds in *Usnea*, its extract was showing low DPPH activity. Previous studies reported that the *U. longissima* compound was not scavenged DPPH significantly **(Kumar and Muller, 1999; Atalay *et al*., 2011)**. However, **Verma *et al*. (2017)** reported significant DPPH radical scavenging activity in the ethanolic extract, higher than standard ascorbic acid. Varying results of antimicrobial and antioxidant scavenging activity may be due to a geographical difference in the area of collected samples **(Deduke *et al*., 2012)** or enantiomer character of usnic acid. Usnic acid has two enantiomer structure and each enantiomer have different biological activity **(Singh *et al*., 2012).**

We evaluated the antiquorum sensing potential of *U. longissima* extracts in bacterial strain *C. violaceum*. Results showed inhibition in quorum sensing at all ranges of treatment doses. A similar effect was reported in the biofilm inhibition experiment against *B. cereus, C. albican, E. coli, P. aeruginosa, S. aureus*. Previously, it was suggested that usnic acid, a major component of *U. longissima* causes interference with signaling pathways in pathogens **(Maciag-Dorszynska *et al*., 2014; Milot *et al*., 2017; Nithyanand *et al*., 2015; Ozyigitoglu and Acikgoz, 2017)** and could be a possible mechanism of antibiofilm and antiquorum activity of *U. longissima*.

CONCLUSIONS

It is clear from the present study that the usnic acid of *U. longissima* is acting as a potential antimicrobial agent against human as well as plant pathogens. Among different fractions, the methanolic extract is more potential, specifically against *C. albicans* and plant pathogen *A. tumefaciens* (Gram-negative bacteria). Its extract contains a unique phenolics compound that shows significant antioxidant activity. Moreover, *U. longissima* also acts as a potential antiquorum sensing agent and could be an alternative source of antibacterial agents. Owing to the unique metabolites and potent antimicrobial, antiquorum and antioxidant activity *U. longissima* metabolites could be significantly used further for the control of urinary tract infection or as food-borne and immune-suppressed diseases thus provide a huge reservoir for the production of antibiotics therapy that cures humans and plants infectious and antibiotic-resistant diseases in the future.

Abbreviation ME=Methanolic extract, HE=70% hydroalcoholic extract, DW= Distilled water.

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