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Revelation on antimicrobial activity of Lichen extract

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Abstract

Aim: Lichen is an association of fungus and alga which intertwined together to form a thallus. The genus Usnea longissima (Parmeliaceae), is a fruticose lichen with numerous biological activities like antimicrobial, antifungal and inhibitory activities for plant and human pathogens. In present work we aimed to reveal the antimicrobial characteristics of some lichens. Taxonomically it is well distinguished by pale greenish to yellowish green colour and pendulous thallus with dense branches usually emerging from the main stem. Its fungal partner releases an extra cellular compound called Usnic acid, a derivative of Dibenzofuran which is a naturally occurring secondary metabolite.

Methods and Results: The present study deals with the potential antimicrobial activity of Usnea longissima in different extraction solvents. The crude extracts were prepared in methanol, ethanol, ethyl acetate, acetone and different concentrations (2.5,5,10,15,20 mg/ml) and prepared extracts were tested for antimicrobial activity against Staphylococcus aureus, *Escherichia coli, Pseudomonas aeruginosa and Fusarium oxysporum* through agar well diffusion method. The maximum zone of inhibition was in the 15 mg/ml methanolic extract for *Escherichia coli* (34 mm), 10 mg/ml methanolic extract for Staphylococcus *aureus (30 mm), 10 mg/ml ethyl acetate for Pseudomonas aeruginosa (16 mm)* and 0.5 mg/ml ethanolic extract for Fusarium oxysporium (14 mm).

Conclusions: This analysis represent good antibacterial and antifungal activity of Usnea longissima. The study encourages exploring novel antimicrobial biomolecules within lichen biodiversity. These lichens further can be used to treat various human diseases on the basis of research findings.

Significance and Impact of Study: Lichen are now being considered as one the major food supplement. This study will be helpful to find out the unique antimicrobial properties of Lichen and their benefits for human being. Lichen are very economic and easily can be produce by basis researchers that may enhance the lichen investigations towards human welfare.

Keywords: Usnea longissima, thallus extract, *Usnic acid, antimicrobial, lichen*

Introduction

A great variety of secondary metabolites are synthesized by lichens with distinct biological properties [1,2]. These (aliphatic and aromatic) lichen metabolites have relatively low molecular weight [3]. These metabolites are complex, but predominantly small molecules, which comprise up to 20% of lichen's dry weight. Lichen secondary metabolites comprise many classes of compounds including amino acid derivatives, sugar alcohols, aliphatic acids, mucolytic lactones, monocyclic aromatic compounds, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers. Mycobiont produce the secondary metabolites, and accumulate these compounds in the cortex (such as atranorin, parietin, usnic acid, fungal melanins) or in the medullary layer (such as physodic acid, physodalic acid, protocetraric acid) in the form of extracellular tiny crystals on the outer surface of the hyphae.

The secondary metabolism of the mycobiont also has some influence by the photobiont. Along with this, these lichens have also shown significant role in estimating air pollution [4].

Suitable culture conditions (such as nutrient medium, added sugars or polyols, pH, temperature, light, stress) are required for production of specific secondary metabolites. In many cases, lichen "tissue" cultures, can produce secondary substances, but the chemistry is different from the secondary metabolite of the corresponding natural lichen. Usnic acid is a natural lichen compound which is used in pharmaceutical preparation [5]. It is active against microorganisms and viruses as well as analgesics and antipyretics as well as useful in the treatment of hyperproliferative skin diseases, such as psoriasis as well as parasitic infestations. Pulvinic acid extracted from several lichens and higher fungi represents bright yellow and orange pigments with antioxidant properties. Boldine is a natural lichen metabolite having antioxidant activity, anti-inflammatory effects, as well as photoprotector capacity [6]. Antibacterial and antifungal activity reported that some of the lichen metabolites such as atranorin, fumarprotocetraric acid, gyrophoric acid, lecanoric acid, physodic acid protocetraric acid, stictic acid and usnic acid showed relatively strong antimicrobial effects against six bacteria and ten fungi, among which were human, animal

and plant pathogens, mycotoxin-producers and food spoilage organisms [7]. Plaudel has demonstrated [8, 9] methanol extracts of five lichens from Antarctica (Caloplaca regalis, Caloplaca spp, Ramalina terebrata, Stereocaulon alpinum) and its target-specific antibacterial activity, against Gram-positive bacteria. Usnic acid isolated from lichens from south Spain has high antibacterial activity against Gram-positive bacteria. Hirtusneanoside isolated from Usnea hirta showed growth inhibitory activities against Gram-positive bacteria. Investigation has [10] reported that dichloromethane and methanol extracts of Protousnea poeppigii possess strong antifungal effects against some fungal pathogens (Microsporum gypseum, Trichophyton mentagrophytes and T. rubrum) as well as against the yeasts Candida albicans, C. tropicalis, Saccharomyces cerevisiae. and the filamentous fungi Aspergillus niger, A. flavus and A. fumigates. Protousnea poeppigii contains metabolites like isodivaricatic acid, divaricatinic acid and usnic acid also showed antifungal activity against Microsporum gypseum, Trichophyton mentagrophytes and T. rubrum. Ascospore germination of Sordaria fimicola was significantly inhibited by evernic and vulpinic acids, anthraquinone isolated from methanol extract of Caloplaca cerina has been reported to have significant antifungal [11] isolated some bioactive compounds from Usnea longissima and highlighted its anti-inflammatory activity. Staphylococcus aureus was also found as an important cause of disease in human [12, 13].

Lichen, Usnea longissimi and Cetrelia braunsiana are among the crucial Lichens which are being considered for antibacterial purposes [14]. Usnea longissimi is very sensitive to air pollution. Very few U. longissima lichens have been seen with spore-producing structures and are considered very rare. In India U. longissima is known to occur in Eastern Himalayas out of which 6 species were endemic to the region whereas Western Ghats in the southern part of the country represent the occurrence of 40 species out of which 11 species were endemic [15]. Cetrelia braunsiana shows the characteristics of the broad lobe, presence of laminal pseudocyphella, and production of aromatic compounds such as orcinol-type depsides or depsidones. Previous researches have represented the impactful response of U. longissimi and Cetrelia braunsiana against some human diseases but still its wide application in human health yet to be explored [16, 17] and more attention needs to be given to exploration of biological activities of these two lichens. Therefore, the present work focused to investigate the antibacterial properties on Usnea longissimi and Cetrelia braunsiana. The findings may facilitate to utilize these lichens for human health.

Material and methods

Lichen material

The fresh lichen samples of Usnea longissima and Cetrelia braunsiana were given by Lichenology Laboratory of CSIR-National Botanical Research Institute (NBRI) which was collected previously from Himalaya.

Chemical analysis of the lichen thallus

TLC (Thin Layer Chromatography) is more accurate and very common technique for detecting and identifying lichen substances. Optimal condition has been developed in the laboratory of Culberson [18] and are employed as standard procedure for TLC. The lichen substances were extracted in acetone and then loaded on merck silica gel 60 F254 plates and chromatogram was developed in standard solvent system Taluene : Dioxane : Acetic acid - 180: 60 : 8). The spots on the chromatography were visualized in normal light and short/long wave length ultraviolet (UV) light. Parmelinella wallichiana with the two common lichen substances salazinic acid (Rf class 2) and Atranorin (Rf class7) were used as control for relating Rf values and Rf class of the unknown's substances.

Extraction of Lichen Material

Initially, the samples of Usnea longissimia and Cetrelia braunsiana were washed 5-6 times properly with tap water. Subsequent washing was done using distilled water and Polysorbate (Tween) 80 only once followed by frequent washing (4 times) with distilled water. Washing was done to minimize the contamination during activity process. The washed sample was dried at room temperature (about 73-750 F). After drying, about 200 gm of the material was crushed using HL 1606 500-Watt Mixer Grinder. Five cycles were run to minimize the size of the sample particles.

The powdered lichen (10 gm) was wrapped in 8 x 6 cm cylindrical pouch made up of Whatmann filter paper grade 1 and kept inside the extractor arm of Soxhlet apparatus [19]. 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 period of 48 hrs for the complete extraction of secondary compounds.

After soxhlet, the samples were evaporated using Rotary evaporation (Rotavap).

Preparation of Culture media for growth of microorganism

Nutrient agar plates were prepared by plating the media on petri dishes. Suspend 18.5 gm of nutrient agar powder in 1 litre of distilled water. Heat the mixture while stirring to dissolve all components thoroughly. Resultant mixture of media was autoclaved and poured in petri plate.

PDA plates were prepared by using potato dextrose. Suspended 39.0 gm of potato dextrose agar powder in 1 litre of distilled water. To dissolve it, media was heated and autoclaved. Sterilized media was poured to prepare plates for culture.

Microorganisms

Total of five bacterial cultures (Pseudomonas aeruginosa, Agrobacterium tumefaciens, Escherichia coli, Streptococcus mutans and Staphylococcus aureus) and three fungal culture of Aspergillus niger, Candida albicans, Fusarium oxysporum were

used in experimentations and screening process. All the cultures were obtained from the Pharmacological Laboratory, National Botanical Research Institute (NBRI), Lucknow. The cultures were stored at 40 C and for further analysis, concerned strains were revive from the same stock in solid and semisolid nutrient agar slants.

Determination of Antimicrobial Activity

Antimicrobial activity was investigated using well-diffusion method [20]. The Nutrient Agar medium was transferred into one fourth volume of Petri plates for antibacterial activity. Potato Dextrose Agar medium was transferred into one fourth volume of Petri plates 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 Petri plate. 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 along with DMSO as negative control and Streptomycin (in antibacterial testing) and Ketoconazole (in antifungal testing) as positive control. The plates were labelled and incubated for 24 hrs at 37 degrees Celsius

Results

Chemical analysis of the lichen thallus

Extracted components from the Lichen thallus was further identified by thin layer chromatography and its analysis of the separated bands reveal the presence of various compounds present in lichen thallus. The presence of varied components in the different extracts of Lichen thallus is demonstrated in Figure 1 (Table 1). This is notable from the different colour changes depicted by individual compounds due to their reaction with the spray reagent used. In TLC, Rf (retardation factor) value played an important factor to quantify the movement of a compound on a stationary phase e.g. silica with a certain solvent system and is the ratio of the distance moved by the compound from its origin to the movement of the solvent from the origin. In case of Usnea longissimi, the resultant plate revealed the presence of usnic acids in natural thallus and evernic acid, barbatic acid, diffractaic acid, usnic acid in thallus extract. For Cetrelia braunsiana, natural thallus showed the alectoronic acid whereas thallus extract showed the presence of alectoronic acid and collatolic acid (Table 1).

Antimicrobial activity of lichens sample against microorganisms

We found that the Usnea longissimi and Cetrelia braunsiana extracts showed antimicrobial activity against most of the tested micro-organism. Zone diameters were found varied for each extract of Usnea longissimi and Cetrelia braunsiana that shows the effectiveness and amount of present antimicrobial compounds in extract. Different concentration (2.5mg/ml, 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml) of extracts from Usnea longissima and Cetralia braunsiana were employed as well as for the extraction different solvent were used namely, methanol, ethanol, ethyl acetate and acetone in order to get the best results against eight pathogens. The maximum inhibitory effect was recorded in the methanolic extract of Usnea longissima against E. Coli. However, the minimum inhibitory effect was observed in the acetone extract against Fusarium oxysporium (Figure 2 & 3)

After the analysis of the results, the methanolic extract of the Usnea longissima showed the largest inhibitory zone among the other extracts against the microbes of which their growth is inhibited. The ethanol, ethyl acetate and acetone extracts were slightly less active compared to that of the methanol extract. For the methanol extract, the antimicrobial was detected at a concentration a 15mg/ml against E. coli. On the basis of these results we may conclude that Methanol could be the more suitable solvent for the extraction than ethanol, ethyl acetate and acetone in case of U. longissima.

In the present study methanolic extract of Usnea longissima with the concentration of 15mg/ml showed maximum inhibitory concentration having zone of 34 mm against E. coli [Table S1(iv) and Table S1(v)] whereas and minimum inhibitory concentration zone of 10 mm against Fusarium oxiporum was shown by acetone extract [Table S1(iii) and Table S1(v)]. In case of Cetrelia braunsiana, ethanolic extract showed maximum inhibitory concentration zone of 30 mm against Candida albicans having concentration of 20mg/ml [Table S2(v)]. Aspergillus niger represents the minimum inhibitory zone of 12 mm by ethanolic extract of C. braunsiana [Table S2(iii), Table S2(iv), Table S2(v)].

Remarkably, no zone has been observed against Pseudomonas aeruginosa by thallus extracts of the lichens at the concentration of 2.5mg/ml and 5mg/ml in any of the solvents. [Table S1 and Table S2)] that indicates the effective concertation limit of the thallus extract against P. aeruginosa.

In case of Cetralia braunsiana the maximum inhibitory effect was observed in the ethanolic extract (Figure 5) against Candida albicans, while minimum inhibitory effect was shown in the ethyl extract against Aspergillus niger.

These results clearly indicate that the lichen extract has strong potential to act as secondary metabolites in lichen which causes its antimicrobial activity (Figure 4).

Discussion

The aim of the research is to explore the overall antimicrobial activity of thallus extracts of lichens, specifically Usnea longissima and Cetrelia braunsiana. After the studies, a certain correlation was established between the antimicrobial activity and the concentration of thallus extract in various solvents. In previous studies some work has been done on antibacterial activity of lichen [8] but still further investigation needs to be done in order to implement novel properties of lichen towards human health and diseases. This study and out comes on lichen have shed a light on its antimicrobial properties that indicates the need of lichens and its harvesting on large scale to benefit mankind. In this investigation we found that the methanol and

ethanol extract of Usnea longissima and Cetrelia braunsiana have high potential as an antimicrobial and antifungal compound. From the result obtained in the present study, it can be speculated that U. longissima and C. braunsiana have very broad antimicrobial activity with the ethanol and methanol extract. On the basis of results, it is suggested that the extracts of U. longissimaand C.braunsiana can be used as source of natural antimicrobial component for food supplement or in medicinal or pharmaceutical industry. The study encourages to explore more to novel antimicrobial biomolecules within lichen biodiversity. Some of the bioactive phenolic compound from medicinal lichens have been characterized by researchers [14] that shows its wide application in human welfare.

Another major aspect of lichen that showed the presence of secondary metabolites in its thallus which are responsible for their antimicrobial activity. But in my studies, the lichen species taken during this experiment did not show strong inhibitory effect against pathogenic fungi. There could be some reason behind this property of lichen shown by its thallus extract as explained here. i) The acids present in these lichen species do not have any antimicrobial effect. ii) The acids present in these species do not properly get dissolved in acetone. 3) The secondary metabolites extract used in this experiment may be of weaker concentration and hence forth in effective against fungal pathogen. 4) The combination of acids present in lichen species is not effective with each other. Definitely, further investigations on the antimicrobial activity as well as the economical and efficient isolation process of the metabolite from the lichens are needed.

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