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Morphological and Bio-potential investigation of Fruticose lichens from Nilgiri mountain

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Abstract

Lichens are catching the interest of experts all around the world as new substantial sources of bioactive compounds in this area. A symbiotic organism known as lichen is made up of a fungal partner (Mycobiont) along with one or more photosynthetic partners (photobiont). They frequently grow as epiphytes on trees and shrubs as well as on rock surfaces, poorly developed soils as those in desert zones and boreal polar regions. Because cultivating lichens is challenging, experimental lichenology has lagged significantly behind descriptive investigations. This analysis of research goal is to identify different lichen species using morphological and chemical investigations. The fruticose-lichen species were collected from the Nilgiris Mountain in Tamil Nadu, India, and were first identified using a variety of traditional methods before having their bio-potentials assessed. In order to ascertain their growth type and thallus colour, they underwent anatomical dissection. Spot tests, micro-crystallography, chemical analysis of lichen extracts, and micro-crystallography were all employed to identify the species. Finding the lichen's secondary metabolites is the objective. The results showed the presence of essential oils, saponins, quinones, alkaloids, sterols, terpenes, tannins, flavonoids, and other secondary metabolites. Thin layer chromatography (TLC) was employed to verify the phytochemicals' qualitative characterisation. The diversity of metabolites in extracts was demonstrated by separating distinct molecules of each secondary metabolite. Spots represented the TLC result, and an Rf value represented the frontal report. The well diffusion test was used to analyse the antimicrobial components.

Key words: Lichens, fruticose, *Usnea* sp., secondary metabolites and antimicrobial compounds.

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The symbiotic lichens are made up of one or more photosynthetic (photobiont) partners and a fungal partner (Mycobiont)[22]. They live on rock surfaces, such those found in desert areas and the boreal arctic, and as epiphytes on trees and plants. Most of the Endo and epi-lichen fungi and bacteria, living inside and outside the lichens thallus, rely on the lichens thallus for support. Within this complex ecosystem, this chemical environment is involved in interactions between community members, which have an impact on overall community homeostasis and survival. This diversified population might be a good place to look for novel pharmaceuticals[2]. Carbon-based secondary compounds (CBSCs) are produced by several lichens, the majority of which are weak phenolic acid derivatives. Antibiotic secondary metabolites are produced by lichen-forming fungi, which give protection against pathogenic microorganisms. In comparison to plant metabolites, their metabolites are structurally distinct and extremely active. Lichen metabolites have been demonstrated to be poisonous to insect larvae and to prevent them from feeding. Numerous lichen extracts have been utilised in traditional medicine for various cures, and metabolites with antibacterial, anti-mycobacterial, antiviral, anticancer, analgesic, and antipyretic effects have been found often in lichens[17]. They've been ignored by mycologists and the pharmaceutical business for a long time due to their slow growth and problems in artificially cultivating the organisms. As a result, there has never been large-scale industrial manufacturing of lichen metabolites. Many lichenologists have tried to culture complete lichens as well as their individual or recombined symbionts[6,18,19]. Lichen-derived cultures come in a variety of forms. Mycobionts and Photobionts can be grown alone or in combination. The colour test is carried out by putting a specified reagent on a tiny lichen sample for identification[7]. Separate tests should be performed on the cortex and the medulla. The presence of specific phyto-chemicals is thought to be responsible for the change in colour of the spotted region caused by each solvent[19]. Lichens are best source of secondary metabolites[13].They are chemical groups that have been shown to have therapeutic properties against a variety of human ailments, which may explain why medicinal plants have traditionally been used to treat certain illnesses. Chemical compounds with complicated structures and more limited distribution than primary metabolites exist (phenolic compounds, alkaloids, terpenoids, steroids, quinones, saponins, and *etc*.,). They are not required for the survival of the plant in which they are found; at least, their metabolic activities have yet to be discovered[14]. Several studies have shown that lichens have the potential to be effective anti-diabetic medicines. Their hypoglycemic action has been measured in a variety of ways, including inhibitory activity of carbohydrate hydrolyzing enzymes (– amylase and – glucosidase) or protein tyrosine phosphatase 1B (PTP1B), which is known as the major negative regulator in insulin signalling, or anti-oxidative effect, which is involved in the restoration of insulin-secreting pancreatic cells[10,15].

Lichens appear to be a viable source of phenolic chemicals that aren't found in higher plants or other free-living fungi. The antioxidant capabilities of these phenolic compounds, as well as their crude extracts,

have been comprehensively evaluated through "in vitro and in vivo research." There is a lot of evidence that lichens are a good source of antioxidants[5]. As a result, the search for better and safer anti-oxidants is receiving a lot of attention. Many lichenologists have attempted to culture whole lichens and the separate or recombined symbionts[18,19]. There are several kinds of lichen derived cultures. Mycobionts and Photobionts can be cultured separately or together. Mycobionts can be obtained from hyphal fragments, spores or even conidia[21]. In most cases, lichen tissue culture and cultures derived by recombining symbionts that have been cultured separately remain as undifferentiated cell aggregates, but such aggregates are very useful for many biological experiments. Lichens appear to be a promising source of unique phenolic compounds, which do not occur in higher plants and other free-living fungi. The antioxidant properties of these phenolic compounds, as well as their crude extracts, have been thoroughly assessed using both "in vitro and in vivo studies'. Ample data exist to prove lichens as a reliable source of antioxidants[5]. Main emphasis of this study is to collect lichen specimens, cut them into pieces for identification, cultivation and the sort of extraction procedure for analysis. Thus, major attention is being to search for better and safer efficiency novel pharmaceutical components.

Collection of specimens :

Lichen samples were collected from the Nilgiris Mountain, Tamil Nadu, India in December 2019. Foliose and Fruticose lichens are mostly growing in those areas. The fruticose samples were collected and packed in the acid free packets and stored in the 4^{o}C for the further experiments and studies.

Identification of Lichens:
Morphology:

Lichen samples were identified on the basis of their morphology and chemical characteristics. Lichens were screened for their morphology based on their growth type, presence or absence of vegetative parts (Rhizines, Cilia) and the colour of thallus.

Chemical characterization :

Spot test : is a chemical method, the chemicals were applied on the lichen fragments and their secondary metabolites were to help the identification of the lichen species.

"K" test : 10% aqueous solution of potassium hydroxide (KOH) or 10% aqueous solution of the sodium hydroxide (NaOH), the solution was applied on the lichen fragments. Quinonoid lichen pigments react to this solution as dark red colour.

"C" test : 5-25% solution of sodium hypochlorite (NaOCl) was applied drop by drop on the lichen fragments. Aromatic compounds with two free -OH- *Meta* group's react to this solution by a red colour on the lichen thallus.

"I" test : also used 1.5% of potassium iodide and 0.5% of iodine, the mixed solution which reacts with certain polysaccharides in lichen. Cortex and medulla of the lichen should be tested separately. Change in colour of spotted area by each solvent is assumed as

positive results, which is due to presence of the certain phytochemicals.

Micro-crystallography (Microscopic identification) test :

Place a small fragment of the lichen thallus over the slide. Add a few drops of acetone and leave to evaporate and remove the thallus fragment. Add a few drops of crystallization agent like GAW (Glycerol: Ethanol: Water in 1:1:1 ratio) to the slide. Keep the slide in a warm place. Place the cover slip and observe it under the microscope and capture the images, it will show the result as crystal formation.

Cultivation of lichen in laboratory :

The cultures of lichen were started within 7 days after the collection. The thalli of lichen were cut into 1 cm square pieces, washed with tap water overnight and homogenized with 5ml of distilled water under sterilized conditions. Small segments from the lichen thalli were picked up with sterilized stainless-steel loop and were inoculated onto the broth media. The inoculums were grown at 18°C and with alternating photoperiod of 8 hours light (400 lux)/16 hours dark in the culture room for a period of 2 months. The following culture media (broth) were used: Malt-Yeast Extract (MYE) and Lilly Barnett (LB), adjusted the broth to pH range of 5.0.

Solvent extraction of lichen material :

The lichen thalli were washed thoroughly with water to remove dust particles and dried at room temperature. The air dried lichen thallus was powdered with a mortar and pestle. The powdered material (50g) was subjected to Soxhlet extraction using ethyl acetate (500ml) for 6 hours. The solvent was evaporated in *vacuo* and the dried residues obtained were stored at 4°C for further studies and biological screenings.

Phytochemical screening :

Chemical tests for the screening and identification of bioactive chemical compounds like alkaloids, carbohydrates, glycosides, Saponins, phenolic compounds, sterols, proteins, amino acids, flavonoids and tannins. In the lichens under study were carried out in extracts by using standard procedure.

Test for Tannins : 2ml of crude extract was mixed with a few drops of 5% ferric chloride solution. Formation of blue colour indicates the presence of tannins.

Test for Alkaloids : 2ml of crude extract is added to 1% HCl, steam it for 10 minutes. To this add 6 drops of Dragondroff's reagent. Reddish brown precipitate indicates the presence of alkaloids.

Test for Saponins : 2ml of crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of Saponins.

Test for Glycosides : 2ml of crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 2% solution of $FeCl_3$. The mixture was then poured into another test tube containing 2ml of concentrated H_2SO_4. A brown ring at the interphase indicated the presence of Glycosides.

Test for Flavonoids : 2ml of crude extract is added to 2ml of 10% NaOH solution. Yellow to orange colour indicates the presence of flavonoids.

Test for proteins : 2ml of crude extract is added to 2ml of HNO_3, boiled in a water bath. Orange colour indicates the presence of proteins.

Test for Triterpenoids : 2ml of crude extract is shaken with 1ml of chloroform and a few drops of concentrated H_2SO_4 were added along the side of the test tube. A red brown colour formed indicates the presence of Triterpenoids.

Test for Carbohydrates : 2ml of crude extract is when mixed with 2ml of Benedict's reagent and boiled, a reddish brown precipitate formed which indicates the presence of Carbohydrates.

Test for Steroids : 2ml of crude extract is added to 2ml of acetic anhydride and a few drops of concentrated H_2SO_4 is added. Blue-green ring indicates the presence of steroids.

Thin Layer Chromatography (TLC) :[14]

Pre-oated aluminum silica gel plates are cut into a size of 20x15 cm. A pencil mark is made at a distance of 2 cm from bottom end and points made at appropriate distance through width of plate based on number of samples. Small fragments of thallus are kept in small test tubes and lichen substances are extracted with acetone. With the help of capillaries extracts are spotted on the silica gel plates. Spots are kept as small as possible. The first, middle and last markers are made control. Spotted plates are kept in rectangular TLC jar, containing solvent A, B or C. After completion of run, plates are taken out, and dried in air. A 10% aqueous solution of H_2SO_4, is sprayed over the plate and then placed in a pre- heated oven at temperature of until 110°C the colored spots are developed clearly. The developed plate is divided in to approximately 7 equal parts which are called as Rf classes. Identification of lichen substances are made on the basis of the position and colour of the spots by comparison with the charts published by Orange *et.al.*,[12]. The solvent used here is HEF - Hexane: diethyl ether: formic acid; 130:100: 20 ml

Screening for Antimicrobial Activity :

The lichen crude extract was prepared from Soxhlet apparatus by ethyl acetate solvent. The extracts were stored in 4°C. The crude extracts were then dissolved in 15% Dimethyl sulfoxide (DMSO) for antimicrobial bioassay. Antimicrobial activities of the crude metabolites isolated from the Endolichenic fungi were determined by agar diffusion method. This method worked against human bacterial and fungal pathogens. The bacterial pathogens are *Staphylococcus* sp, *Pseudomonas* sp, *Proteus* sp, *Klebsiella* sp, *E. coli*. Muller Hinton agar (MHA) plates were inoculated with overnight culture of each bacterial suspension. The plates with inoculated organisms were evenly spread out with sterile cotton swabs. Agar cups were prepared by scooping out the media with a sterile cork borer (7mm diameter). The cups were then filled with 20µl, 40µl, 60µl and 80µl concentration of the crude metabolites that were dissolved in DMSO to get a concentration

of 1mg/ml. The MHA plates were incubated at 37°C for 24 to 48 hours. Antimicrobial activity was determined as growth inhibition of the target organism around agar cup as appearance of clear zone.

Collection of lichen sample :

"Queen of Hill Stations" is Ooty, one of the oldest mountain ranges, located at the tri-junction of Tamil Nadu, Kerala and Karnataka were situated between 10 and 38 degrees north latitude and 76.0- and 77.15-degrees east longitude. The district has a total size of 2452.50 square kilometers. Lichens were obtained from the Nilgiris (Coonor and Kotagiri) mountain station and chosen based on spot test colour reactivity. The morphology and chemical features of the collected lichen species were used to characterize them. The presence and absence of vegetative parts, reproductive parts, texture and colour of the thallus were all used to identify lichens. The chemical properties of lichen are another essential identification approach, which was accomplished via micro-crystallization to identify their secondary metabolites and chemical composition.

Identification of Lichens by spot test :

Identification of lichens from their morphology. The structural features of Rhizines root are squarrous 1mm composed of mycobionts. In those areas, mostly fruticose, cylindrical shaped old man's beards like mini shrubs on tree trunks were yellow green in colour. It makes a sound when it breaks. The chemical spot test of K test, C Test and KC test results shows that positive yellowish red colour conformed the *Usnea* sp.,

Fig. 1. Fruticose lichen

Micro-crystallography test : It's a microscopic examination. The GAW (crystallisation agent) reacts with the lichen thallus and produces crystal formations as a result. In addition, the algal component of lichen will be examined. In 1936 Asahina[1] introduced micro-crystallization as the first generally applicable method for tentative identification of lichens on a micro scale. The method has been used extensively for chemical studies in connection with taxonomic work. It is controlled by recrystallization of extracted lichen substances and observation of these crystals under microscope and can distinguish substances more accurately. Components of lichens may react with certain test chemicals to give colour reactions which assist in the identification of a species. The best guide to the use of chemicals and to appropriate precautions and procedures is micro-chemical methods for the identification of lichens[16].

Isolation of fungal mycobiont :

Mycobionts and Photobionts can be cultured separately or together. Mycobionts can be obtained from hyphal fragments, spores or even conidia, in the Malt Yeast Extract Broth (MYE) and Lilly Barnett's Broth (LBB). In

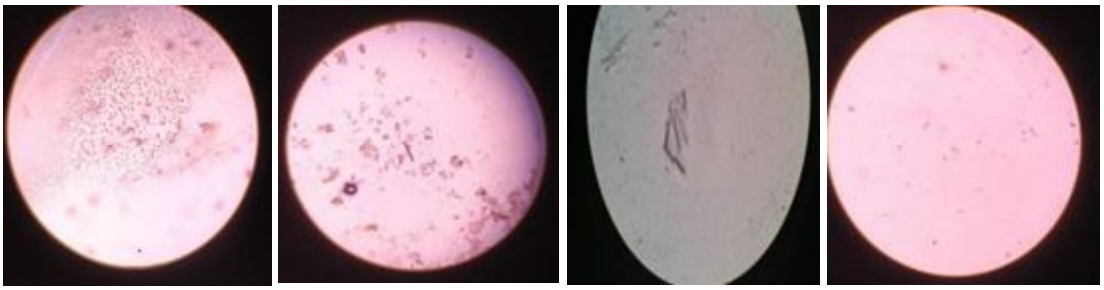

Fig. 2. Microscopic examination of lichen crystals

the Malt Yeast Extract Broth (MYE) the hyphal fragments of lichen will be grown on the broth media, after the 15 days of the incubation at 4°C with light source. It will show fluorescence under UV. Based on the morphological identification, spot test, micro-crystallography test and cultivation process, the fruticose lichen sample is identified as the *Usnea* sp.,

Fig. 3. Cultivation of Lichens

Microscopic observation of fungal mycobiont:

The microscopic observation revealed the fruticose thallus of cup shaped fruiting bodies.

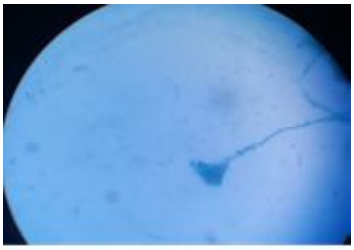

Fig. 4. Microscopic examination of thallus

Phytochemical analysis :

The crude extracts from solvent of ethyl acetate were used to identify the phytochemical properties of the *Usnae* sp., shows the positive result for the tannin, alkaloids, glycosides, carbohydrates and saponins, flavonoids, protein, Triterpenoids. Phytochemical analysis conducted on the lichen extracts reveals the presence of constituents which exhibit a medicinal importance. Secondary metabolites of lichen exhibited a great diversity of biological effects including antimicrobial, anti-inflammatory, anti-proliferation and cytotoxic activities[8]. The presence study shows the presence of phytochemicals like glycosides, saponins, flavonoids, Triterpenoids, proteins and steroids. Whereas presence of tannin and Triterpenoids. E*verniastrum cirrhatum* (fr) hale revealed the presence of alkaloids, saponins, tannin and triterpenoids[20].

Screening of antimicrobial activity :

The lichen forming fungi produce antibiotic secondary metabolites and provide protection from animals and pathogenic microorganisms. Their metabolites are structurally unique and highly active in

Fig. 5. Phytochemical analysis of ethyl acetate

comparison to plant metabolites. Lichen metabolites have been shown to be toxic and a feeding deterrent to insect larvae. Several lichen extracts have been used for various remedies in folk medicine and screening tests with lichens have shown the frequent occurrence of metabolites with antibiotic, anti-mycobacterial, antiviral, antitumor, analgesic and antipyretic properties[17]. Usnic acid is one of the most widely studied lichen secondary metabolites[4,9]. The antimicrobial activities of lichen extracts and their specific compounds including usnic acid have been studied for many years[11]. It was reported that usnic acid inhibits the growth of numerous gram positive bacteria, while it has no effect on Gram negative species[9].

Table-2. Zone of inhibition - Antibacterial assay

S.No	Pathogens	Zone of Inhibition (mm)			
		20µl	40µl	60µl	80µl
1	*Staphylococcus* sp.,	25	28	31	36
2	*Pseudomonas* sp.,	22	25	26	29
3	*Proteus* sp.,	23	26	28	31
4	*Klebsiella* sp.,	21	25	27	28
5	*E. coli*	24	27	27	30

Fig. 6. Antibacterial activity of extract in diffusion method

Thin layer chromatography :

The Ethyl acetate extract of *Usnea* sp., of fruticose lichens were used for TLC analysis. The result shows that extracts contain more phytochemical compounds. Further study needs to find out the components.

Modern wool and fabric dyers usually emphasise conservation when gathering lichens. The following components are found in deodorants and perfumes: Lichen components are frequently used in deodorants and perfumes due to their pleasant aroma.

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