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RESEARCH ARTICLE

IDENTIFICATION AND PHYTOCHEMICAL STUDIES ON *HYPOGYMNIA SP*

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ABSTRACT

The present study investigates the lichen species identification by using morphological and chemical analysis. There were six different lichen species were collected identified based on different standard procedures and analyzed for its bio-potentials. Anatomically, they were examined for their growth type and thallus color. Spot test, and micro-crystallography were analyzed, and chemical analysis were applied to lichen fragment and their extracts for the species identification. To characterize the lichen in terms of secondary metabolites. The results showed the presence of several types of phenolic compounds; namely tannins, flavonoids and other secondary metabolites such as alkaloids, sterols and terpenes, essential oils, saponins and quinones. Thin Layer Chromatography (TLC) was performed to confirm the qualitative characterization of photochemical. To separate different molecules of each secondary metabolite showed the diversity of metabolite in extracts. The TLC result was reported in the form of spots and frontal report was expressed in Rf value. It reflects the purity of the component molecules in lichen. This diversity of secondary metabolites can be at the origin of the widespread medicinal properties and therapeutic uses of the tested lichen.

Key words: Lichen, Spot test, Micro-crystallography,Secondary metabolites, Phytochemicals, Phenolic compounds,TLC, Usnic acid.

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INTRODUCTION

Lichens are symbiotic organisms composed of a fungus and an algae. They produce characteristic secondary metabolites "lichen substances" that seldom occur in other organisms. Lichens are symbiotic organisms resulting from the association between a fungus, called the Mycobiont. The lichens thallus is a support for other microorganisms living inside and outside the thallus, including endo- and epi-lichenic fungi and bacteria. Interactions exist within this complex ecosystem and many of the molecules that make up this chemical environment are involved in interactions between the community members influencing overall community homeostasis and survival. This complex community is a potential source of new pharmaceutical drugs. Lichens are symbiotic systems that arise as self-supporting mutualistic association between a fungal partner and one or more photosynthetic partners (unicellular green algae and/or cyanobacteria). They are unique phenotypes (holobionts) in which complex interactions enable the emergence of new structural and functional characteristics representing evolutionary innovations. These novel properties allow the colonization of diverse habitats and survival under extreme environmental and climatic conditions.

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Lichen mycobionts, as other fungi, could therefore be a potential source in pharmaceutical chemicals. Lichens and their metabolites have many biological activities such as antimicrobial, antiviral, antiprotozoal, anti-proliferative, anti-oxidant, anti-inflammatory and analgesic activity. Inspite of the wide spectrum of biological activities shown by the lichens, they have long been neglected by mycologists and overlooked by pharmaceutical industry because of its slow growth in nature and difficulties in the artificial cultivation of the organisms. Hence the large-scale industrial productions of the lichen metabolites have never been accomplished. Lichen extracts and their metabolites have been widely studied for their antimicrobial properties, but their anti-bioflim potential is still poorly explore. Some of these studies, particularly on cultured mycobionts, tried to improve the culture conditions to trigger and enhance the synthesis of secondary metabolites, to reveal the factors involved in this process, to understand the role of each of the partners in the synthesis of the lichens substances, or to find sources of therapeutic agents. In some cases the asymbiotic fungal strains can able to synthesize the same secondary metabolites as naturally available in liche. Some mycobionts produce secondary metabolites which are different from those present as major compounds in the symbiotic state, including novel molecules such as the graphislactones, graphenone and xanthones. Some of the primary metabolites are produced by fungi and some by algae. Most of these metabolites are non-specific and also may occur

in free-living fungi, algae and higher plants. In general, the amount of nitrogen compounds between 1.6 and 11.4% dry weight of the lichen thallus. Among vitamins, lichen contains ascorbic acid, biotin, α-tocopherol, nicotinic acid, pantothenic acid, riboflavin, thiamine and folic acid. Vitamins were identified as metabolic products which bio-synthesis algae, while the mushrooms are poor sources of these compounds.

More than 800 secondary metabolites are known from lichens, most are unique to these organisms and only a small quantity occurs in the other fungi or higher plants. All the secondary substances in lichens are of fungal origin. These substances are the crystals deposited on the surface of the hyphae, which are poorly soluble in water, and usually can be isolated from the lichens by organic solvent.

MATERIALS AND METHODS

Collection of lichen

Lichen samples were collected from the Nilgiris Mountain, Tamil Nadu, India at December 2017. Crustose, Squamulose lichens are couldn't present. Foliose and Fruticose lichens are mostly grow in that areas. So mostly collected the foliose like lichens were collected. The collected samples were packed in the acid free packets and stored in the $4^{o}C$ for the further experiments and studies.

Identification of lichens

Lichen samples were identified on the base of their morphology and chemical characteristics.

- Lichens were screened based on their morphological characterization like growth type, presence or absence of vegetative parts (Rhizines, Cilia) and the colour of Thallus.
- Basically lichens are occurring in one of four growth forms. Crustose, Squamulose lichens are couldn't present in the Nilgiris area. Foliose and Fruticose lichens are mostly growing in that areas.
- Spot test is one of the chemical methods. Chemicals were applied on the lichen fragments and produce color reactions.

"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 hyphochlorite (NaOCl) was applied a 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 was used by a 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 a 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 few drops of acetone and leave to evaporate remove the thallus fragment.Add few drops of crystallization agent like GAW (Glycerol: Ethanol: Water in 1:1:1 ratio) to the slide. Keep the slide on a warm place.Place the cover slip and observe it under the microscope and capture the images, it will shows the result as crystal formation.

Cultivation

The cultures of lichen were started within 7 days after the collection. The thalli of lichen were cut into 1cm square pieces, washed with tap water for over-night 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 $18^{o}C$ 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

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 motor and pestle. The powdered material (50g) was subjected to Soxhlet extraction using hexane, followed by ethyl acetate and finally with the methanol (500ml) for each at 6 hours. The solvent was evaporated in *vacuo* and the dried residues obtained were stored at $4^{o}C$ for further studies and biological screenings.

Phytochemical Activity

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 indicates 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, boil 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, it indicates the presence of Triterpenoids.

Test for Carbohydrates: 2ml of crude extract is 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.

RESULTS AND DISCUSSION

Collection of Lichen

The Nilgiris district of TamilNadu is located between 10 - 38 and 11- 49 North Latitude and between 76.0 and 77.15 East Longitude. The district covers an area of 2452.50 Sq. Km. Six different lichens were collected from Nilgiris (Coonor and Kotagiri) hills station and selected based upon the colour reaction from spot test. Characterization of collected lichen species was done based on morphology and chemical characteristics. Lichens were identified based upon the morphological structure, presence and absence of vegetative part, reproduction parts, colour and texture of thallus.
Chemical characteristics of lichen is another important identification method, which was accomplish by identification of their secondary metabolites and their chemical nature using micro-crystallization.

Spot test

'K' test: Addition of 10% aqueous solution of Potassium hydroxide (KOH) is react with the lichen thallus (Cortex and Medulla), shows the reddish yellow colour change reaction. It indicates the positive result, which is due to presence of certain phytochemicals.

'I' test: The mixture of iodine and potassium iodide is react with the lichen thallus, shows the green colour (Fig:3.4 and 3.5) change reaction. It will be indicates the positive result, which is due to presence of certain phytochemicals in the lichens.

Before applying of chemical **After applying of chemical**

Figure. 3.1

Before applying of chemical **After applying of chemical**

Figure. 3.2

Before applying of chemical **After applying of chemical**

Figure. 3.3

Before applying of chemical **After applying of chemical**

Figure. 3.4

Before applying of chemical **After applying of chemical**

Figure. 3.5

Table .3.1.Colour reaction of Lichens

Test and reagents	Positive reactions	Examples	Result
'K' test Reagent: KOH or NaOH	Yellow (dirty yellow, bright yellow, reddish yellow), red, purple colour reactions are appear. Some turn slowly red forming microscopic crystals.	*Phlyctis sp.*, (red with crystals) *Pertusaria corallina* (bright yellow) *Hypogymnia sp.*, (reddish yellow) *Cladonia sp.*, (yellow) *Xanthoria sp.*, (red or purple)	Reddish yellow colour will be appearance. *Hypogymnia sp.*,
'I' test Reagent: Iodine + Potassium iodide	Blue, green, violet and rarely red	*Porpidia tuberculosa* and *Lecidea lacteal* (both are violet in their medulla) *Hypogymnia physodes* (bright green) *Parmelia sulcata* (orange)	Green colour will be appearance. *Hypogymnia physodes*

Based on the morphological characteristics such as structure, vegetative parts reproductive partsand spot test *Hypo*gymnia sp., was identified and used for further studies (Table:3.1).

Identification of Lichens

Identification of lichens from their morphology. In those areas, mostly Foliose (leafy) like lichens are identified.

Figure. 3.6. Leafy lichen (Foliose)

Micro-Crystallograpy test

It is a microscopic test. The GAW (crystallization agent) is react with the lichen thallus and shows the results in the crystal forms. And the algal part of lichen will be observed (Fig:3.7).

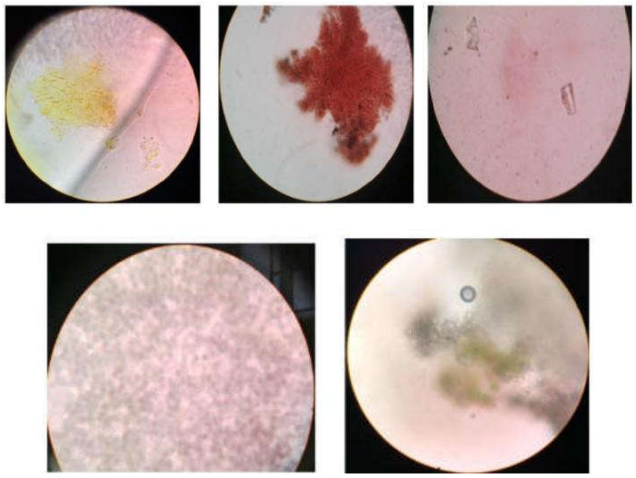

Figure. 3.7. Microscopic observations

Cultivation

Mycobionts and Photobionts can be cultured separately or together. Mycobionts can be obtained from hyphal fragment, spores or even conidia, in the Malt Yeast Extract Broth (MYE) and Lilly Barnett's Broth (LBB). In, the Malt Yeast Extract Broth (MYE) the hyphal fragments of lichen will be grow on the broth media, after the 15 days of the incubation at 4^{o}C with light source. It will show fluorescent under UV light (Fig: 3.9). Following the morphological identification, spot test identification, micro-crystallograpy test and cultivation process, the foliose lichen sample is identified as the *Hypogymnia sp.,* the confirmation of *Hypogymnia* is based on the spot test.

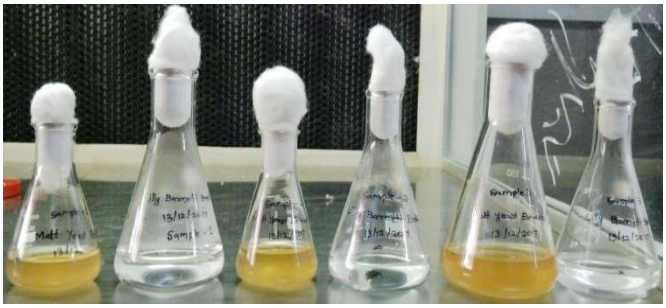

Figure. 3.8. Broth media (MYE and Lilly Barnett's)

Under Bright light **Under UV light**

Figure. 3.9. Mass Cultivation of lichen

Extraction of Lichen

The *Hypogymnia sp.,*was extracted by Soxhlet apparatus method using of organic solvents Hexane, Ethyl Acetate and Methanol (500mL each solvent). 50g of powered sample was loaded in the apparatus with each solvents for 6 hours. The extract was evaporated in rotatory shaker. The crude extract was stored at 4^{o}C.

Phytochemical analysis

The crude extracts from different solvents such as hexane, ethyl acetate, methanol were used to identify the phytochemical properties of the *Hypogymnia* sp The phytochemical properties of the *Hypogymnia* sp shows the positive result for the tannin, alkaloids, glycosides, carbohydrates and negative result for the saponins, flavonoids, protein, Triterpenoids for the solvent hexane and positive result for the glycosides, saponins, flavonoids, protein, Triterpenoids, steroids and negative result for the tannins, alkaloids and carbohydrates for the solvent ethyl acetate, for methanol it shows the positive results for alkaloids and shows negative result for the tannin, glycosides, saponins, flavonoids, protein, Triterpenoids, carbohydrates and steroids. Hence due to the higher amount of phytochemicals present in the ethyl acetate extract. The ethyl acetate extract was taken for the further studies. Results of preliminary phytochemical screening were summarized in the table 3.3. This investigation reveals the presence of various important phytochemical in different extracts of this lichen (*Hypogymnia* sp.,).

Table. 3.3. Phytochemical test result

Phytochemicals	Test	Observation	Hexane extract	Ethyl acetate extract	Methanol extract
Tannin	Ferric chlorite test	Blue colour	+	-	-
Alkaloids	Dragondroff's test	Reddish brown colour	+	-	+
Glycosides	Keller-Kilani test	Brown ring	+	+	-
Saponins	Forthern test	Stable foam	-	+	-
Flavonoids	NaOH solution test	Yellow to Orange	+	+	-
Proteins	Xanthoproteic test	Orange colour	-	+	-
Triterpenoids	Salkowski test	Red brown colour	-	+	-
Carbohydrates	Benedict's test	Reddish brown	+	-	-
Steroids	Liebermann-Burchard test	Blue green ring	-	+	-

Table:3.4. Solvent system for TLC

Phytochemicals	Solvent	Spray reagent
Alkaloids	Ethyl acetate: Chloroform: Water (5:3:1)	Mayer's reagent
Flavonoids	N-Butanol: Ethyl acetate: Water (5:10:15)	3% boric acid + 10% oxalic acid
Tannins	Chloroform: Water (6:4)	$FeCl_3$
Phenolic compounds	Methanol: Water (6:3)	$FeCl_3$

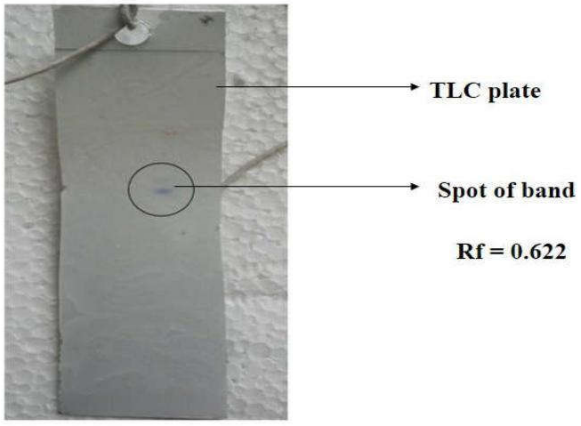

Figure. 3.10. TLC result

TLC Analysis

The Ethyl acetate extract of *Hypogymnia* sp was taken for TLC analysis. Because of the ethyl acetate extract contains more phytochemical compounds.

$$\text{solute Rf value} = \frac{\text{Distance from baseline travelled by}}{\text{Distance from baseline travelled by solvent}}$$

$$= \frac{5.6}{9}$$

$$Rf = 0.622$$

The percentage yield of phytochemicals of *Hypogymnia* sp., in ethyl acetate extracts were shown in figure 3.0 showed that Rf value indicates the presence of Flavonoids in ethyl acetate extract. Characterization of collected lichen species was done based on morphology and chemical characteristics. Lichens were identified based upon the morphological structure, presence and absence of vegetative part, reproduction parts, colour and texture of thallus.

A chemical characteristic of lichen sample is another important identification method, which was accomplished by the identification of their secondary metabolites and their chemical nature using micro-crystallization. Phytochemical analysis conducted on the lichen extracts that reveals the presence of constituents which are exhibit a medicinal importance. Secondary metabolite of lichen exhibited a great diversity of biological effects including antimicrobial, anti-inflammatory, anti-proliferation and cytotoxic activities (Huneck, 1999). The presence study shows the presence of phytochmicals 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. (Vinayaka *et al*., 2009).

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