INTRODUCTION

Lichen is a stable self-supporting association of a mycobiont (fungus) and a photobiont (algae). Also, houses fungi as endophytes and these are generally termed as endophytic fungi. The term ‘endophytic fungi’ was coined by Dr. Sanjeeva Nayaka, Principal Scientist, CSIR-National Botanical Research Institute, Coimbatore District, Tamilnadu. The lichen sample was identified by Dr. Sanjeeva Nayaka, Principal Scientist, CSIR-National Botanical Research Institute, Lucknow, India. The collected lichen material was identified as Hypotrachyna infirma (Kurok.) Hale. This lichen was deposited at CSIR-National Botanical Research Institute, Lucknow, India with the Voucher number 36008.

The use of lichens in medicine is due to their secondary metabolites varied activity in response to external environmental conditions. These secondary metabolites are to be owned to different compounds [3] that are synthesized via various metabolic pathways [4]. These secondary metabolites are to be owned to different structural groups i.e., aliphatics, alkaloids, cytochalasines, depsipeptides, furanones, isocumarines, phenols, quinines, steroids, terpenoids and xanthones, have been commercially utilized for pharmaceutical, medical and agricultural purposes [4-14].

The endophytic fungal species investigated till date for the isolation of bioactive secondary metabolites belong to several geographical locations. The estimated global lichen diversity is about 20,000 [15] from this only a small number of lichen species have been screened for harvesting the endophytic fungi with the potential to offer bioactive metabolites. Therefore one can assume the magnitude of prospective lichen diversity which is waiting to be unveiled. The test for the analysis of phytochemical compounds bring up the way to determine therapeutic drugs progressively [16].

Many researches were undergone based on endophytic fungi but less investigation was made on endophytic fungi and their bioactive compounds. The aim of this work is to identify and analyse the phytochemistry of endophytic fungi with two different solvent extract.

MATERIALS AND METHODS

Collection of lichen

Lichen sample was collected from Sholaiyar, Valparai Hills, Coimbatore District, Tamilnadu. The lichen sample was identified by Dr. Sanjeeva Nayaka, Principal Scientist, CSIR-National Botanical Research Institute, Lucknow, India. The collected lichen material was identified as Hypotrachyna infirma (Kurok.) Hale. This lichen was deposited at CSIR-National Botanical Research Institute, Lucknow, India with the Voucher number 36008.

Chemicals and reagents

Dextrose, Agar, Dimethyl sulfoxide, Sodium carbonate, Vanillin Chloroform, Ethyl acetate, gallic acid, Phosphomolybdic acid, Folin-ciocalteau reagent, Catechin, aluminium chloride, Sulphuric acid, Hydrogen peroxide, Sodium hypochlorite (NaClO), Ethanol were purchased from Hi-media.

Surface sterilization of lichen

Different types of protocols are available for surface sterilization. For this study modified protocol of [17] was used.

Healthy-looking macro lichen thallus was washed in tap water to remove all debris. Then the lichen was subjected to repeated washing in double distilled water to remove bryophytes/mosses and all other visible contamination. After this the washed samples were subjected to chemical surface sterilization by dipping them in 30% Hydrogen peroxide (H2O2) for 30 seconds, followed by 4% Sodium hypochlorite (NaClO) for 30 seconds and finally immersing them in 75% ethanol for 30 seconds.

After the chemical surface sterilization, the samples were rinsed in sterile double distilled water twice and dried under aseptic conditions and were cut into small segments (0.5 cm × 0.5 cm).
Isolation of endolichenic fungi

The sterilized lichen samples were placed on petriplates containing Potato Dextrose Agar (PDA) media and sealed using paraffin.

These petriplates were incubated in the culture room at 25±1 °C until fungal growth was initiated. After initiation, the growing fungal mycelia tips were transferred to new PDA plates for obtaining pure culture. After 15 d, these endophytes that had grown on the PDA media were identified based on the morphology at National Fungal Culture Collection of India (NFCCI)-A National Facility, Pune, India.

Preliminary phytochemical studies of endolichenic fungi

Fungal extraction

A fraction of fungal isolates was transferred into Potato Dextrose Broth (PDB) by aseptically scraping using an inoculation loop. The isolates were transferred into conical flasks containing the Potato Dextrose Broth. These conical flasks were incubated in room temperature over a period of time. After 28 d, culture liquid was diluted to a concentration of 1 mg/ml of protein. After 10 min and kept for 5 min. Till the two clear immiscible layers were formed. The upper layer of the solvent containing the extracted compounds was separated using separating funnel. Solvent was evaporated and the compound was dried using a rotary vacuum evaporator to yield the crude metabolite [18]. The crude extract was then dissolved in Dimethyl sulphoxide at 1 mg/ml of concentration for further phytochemical studies.

Total flavonoid content

Total flavonoid content was determined by the aluminium chloride colorimetric method [26]. 1 ml of extract and standard solution of catechin (100 mg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled water. To this 0.3 ml of 5% sodium nitrate was added. After 5 min, 0.3 ml of 10% aluminium chloride was added. Then after 1 minute, 2 ml of 1M sodium hydroxide was added and the total volume was made up to 10 ml with distilled water. The solution was mixed and the absorbance was measured against prepared reagent blank at 510 nm.

Total saponin content

The vanillin-sulphuric acid assay [27] for determining the TSC of the sample materials is usually done by incubating 0.25 ml of sample extracts, standards or reagent blank with 0.25 ml of 8% (w/v) vanillin in ethanol and 2.50 ml of 72% (v/v) sulphuric acid in water for 15 min at 60 °C in a shaking water bath, with the standards and the reagent blank made up with the solvent used for extracting the plant samples (extraction solvent). After cooling in water at the ambient temperature for 5 min, the absorbance of the standards and extracts are measured at 560 nm using a Cary 50 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) after zeroing it with the reagent blank. The TSC of the samples is then expressed in mg of standard equivalents per gram of plant sample (mg SE g⁻¹).

RESULTS

In the present study, a total of 13 endolichenic fungi were isolated from the lichen species Hypotrachyna infirma (Kurok.) Hale. Phytochemical studies including qualitative (Tannins, Flavanoids, Saponins, Steroids, Carbohydrates, Glycosides, Alkaloids, Proteins and Phenols) and quantitative (Tannins, Flavanoids, Saponins and Phenols) were done in two solvents ethyl acetate and chloroform for the isolated endolichenic fungi. From these two solvents, ethyl acetate gave best result compared to chloroform. Based on the results obtained from the qualitative analysis, the quantitative analysis was carried out.

Quantitative phytochemical screening

Table 1 shows qualitative analysis of 13 endolichenic fungal extracts in two solvents (ethyl acetate and Chloroform). The results of the phytochemical analysis of ethyl acetate extracts of Geotrichum candidum Link, Scytalidium lignicola Pesante, Aspergillus niger Gr, Aspergillus oryzae(Alb), Nigrospora oryzae(Berk and Broome) Petch have high content of tannin, flavonoids, saponin and phenols, less content of Terpenoids, Steroids, Carbohydrates, Glycosides, Alkaloids, proteins. But Terpenoids were absent in Aspergillus oryzae(Alb), cohn, and Glycosides was absent in Geotrichum candidum Link.

Quantitative phytochemical screening

Quantitative phytochemical analysis is performed in two solvents ethyl acetate and chloroform. Graph 1 to 13 showed the presence and the amount of certain phytochemicals in the fungal extracts.

Table 1: Preliminary phytochemical constituents of a fungus with two different extracts

| Fungal cultures | Tannins | Flavanoids | Terpenoids | Saponins | Steroids | Carbohydrates | Glycosides | Alkaloids | Proteins | Phenols |
|-----------------|---------|------------|------------|----------|----------|---------------|------------|-----------|----------|---------|
| Ethyl acetate   | ++      | +++        | +          | -        | -        | -             | -          | -         | -        | ++      |
| Chloroform      | -       | -          | -          | -        | -        | -             | -          | -         | -        | -       |
| Ethyl acetate   | +       | ++         | ++         | -        | -        | -             | -          | -         | -        | ++      |
| Chloroform      | -       | -          | -          | -        | -        | -             | -          | -         | -        | -       |
| Ethyl acetate   | ++      | ++         | +          | -        | -        | -             | -          | -         | -        | ++      |
| Chloroform      | -       | -          | -          | -        | -        | -             | -          | -         | -        | -       |
| Ethyl acetate   | +       | ++         | ++         | -        | -        | -             | -          | -         | -        | ++      |
| Chloroform      | -       | -          | -          | -        | -        | -             | -          | -         | -        | -       |
| Ethyl acetate   | ++      | ++         | +          | -        | -        | -             | -          | -         | -        | ++      |
| Chloroform      | -       | -          | -          | -        | -        | -             | -          | -         | -        | -       |

Notes: ++ = High, + = Moderate, - = Low
The secondary metabolites are different in various organic extracts of all the fungal culture. Their qualitative analysis revealed their appearance whereas their quantitative analysis gave almost approximate idea for their quantity present. Endolichenic fungi were discovered when attempts were being made to isolate the lichen forming mycobiont into pure culture [28, 29]. These fungi are similar to the endophytic fungi [sometimes also referred to as endophyte-like fungi] [30, 31], which reside within healthy tissues of plants and are phylogenetically and ecologically diverse without causing any disease symptoms [32, 33].

The overlap between endolichenic and endophytic metabolites is consistent with their biological similarities; there exists considerable overlap in the taxa represented in endolichenic and endophytic fungal strains, and they are believed to perform similar ecological roles for the host organism [34]. However, endolichenic fungal metabolites remain relatively distinct from the natural products produced by lichens individually [35, 36].

In the living systems, alkaloids are most significant compounds that play a metabolic role and are involved in the protective function in animals. *Nigrospora oryzae* (Berkand Broome) Petch and *Geotrichum candidum* (Berkland Broome) Petch gave good results. The results showed the high amount of Flavonoids and phenols present in *Nigrospora oryzae* (Berkand Broome) Petch > *Geotrichum candidum*

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### Table 2: Quantitative phytochemical analysis of endolichenic fungi

| Fungal cultures               | Tannins                      | Flavonoids          | Saponins                       | Phenols                      |
|-------------------------------|------------------------------|---------------------|--------------------------------|------------------------------|
|                               | Ethyl acetate (mg) | Chloroform (mg) | Ethyl acetate (mg) | Chloroform (mg) | Ethyl acetate (mg) | Chloroform (mg) | Ethyl acetate (mg) | Chloroform (mg) |
| Trichoderma piliferum J.       | 7.67±0.27                    | 5.23±1.10          | 4.70±0.08                    | 3.07±0.10                    | 4.77±0.01                    | 1.53±0.07                    | 6.40±0.16                    | 4.13±0.05                    |
| Trichoderma harzianum Rfai.    | 8.23±0.11                    | 4.63±0.11          | 5.63±0.14                    | 4.53±0.01                    | 2.23±0.01                    | 2.30±0.00                    | 3.40±0.12                    | 2.57±0.01                    |
| Scytalidium lignicola pesante  | 10.53±0.29                   | 7.37±0.11          | 7.37±0.11                    | 5.10±0.08                    | 5.40±0.12                    | 3.40±0.04                    | 22.77±0.01                   | 17.23±0.14                   |
| Geotrichum candidum Link       | 17.77±0.10                   | 14.23±0.10         | 9.63±0.07                    | 7.40±0.16                    | 3.17±0.10                    | 3.07±0.01                    | 26.97±0.19                   | 20.60±0.24                   |
| Aspergillus stellatus curzi    | 7.77±0.30                    | 5.07±0.10          | 4.17±0.07                    | 2.20±0.08                    | 4.67±0.05                    | 2.67±0.07                    | 13.20±0.08                   | 8.03±0.10                    |
| Aspergillus niger Gr           | 14.57±0.15                   | 11.90±0.16         | 6.30±0.12                    | 4.23±0.10                    | 9.13±0.11                    | 3.77±0.05                    | 13.87±0.27                   | 6.07±0.07                    |
| Aspergillus oryzae             | 16.00±0.08                   | 13.47±0.19         | 8.30±0.08                    | 4.97±0.11                    | 8.43±0.07                    | 4.37±0.05                    | 12.10±0.08                   | 5.73±0.18                    |
| (Abh. &coh)                    |                             |                     |                               |                             |                             |                             | 19.67±0.23                   |                             |
| Aspergillus flavus link         | 10.83±1.56                   | 10.80±0.12         | 5.53±0.18                    | 2.57±0.14                    | 5.47±0.07                    | 2.20±0.04                    | 8.27±0.11                    | 4.00±0.04                    |
| Nigrospora oryzae (Berk and Broome)Petch | 30.20±0.78   | 22.10±0.12         | 14.50±0.08                   | 9.23±0.03                    | 17.63±0.01                   | 12.67±0.07                   | 30.13±0.01                   | 19.67±0.23                   |
| Nodulisporium gregarium        | 6.77±0.23                    | 5.40±0.16          | 6.07±0.03                    | 4.73±0.07                    | 4.23±0.14                    | 2.67±0.05                    | 8.47±0.19                    | 6.73±0.11                    |
| (Berkand A. Curtis) A. Mey     |                             |                     |                               |                             |                             |                             |                             |                             |
| Microascus cirrussus Curzi     | 5.80±0.12                    | 3.70±0.08          | 5.53±0.14                    | 2.37±0.10                    | 6.53±0.03                    | 1.47±0.05                    | 7.17±0.11                    | 3.23±0.10                    |
| Trichoderma sp.                | 8.80±0.08                    | 6.27±0.11          | 3.27±0.03                    | 2.13±0.01                    | 2.43±0.14                    | 1.20±0.08                    | 5.00±0.04                    | 2.47±0.03                    |
| Mucor sp.                      | 7.53±0.07                    | 5.47±0.18          | 4.63±0.11                    | 1.33±0.01                    | 3.43±0.05                    | 0.97±0.00                    | 3.70±0.09                    | 2.07±0.10                    |

**Note:** +++: Strong intensity reaction, ++: Medium intensity reaction, +: Weak intensity reaction, −: Non detected

**Maximum amount of tannin present in the ethyl acetate extract of Nigrospora oryzae (Berk and Broome)Petch [30.20±0.78 mg/ml], Geotrichum candidum Link [17.77±0.10 mg/ml], Aspergillus oryzae(Ahb.)cohn (16.00±0.08 mg/ml).**

**Maximum content of phenols present in the ethylacetate extract of Nigrospora oryzae (Berk and Broome)Petch [30.20±0.78 mg/ml], Geotrichum candidum Link (26.97±0.19 mg/ml), Scytalidium lignicola pesante (22.77±0.01 mg/ml).**

**High amount of flavonoids present in the ethyl acetate extract of Nigrospora oryzae (Berk and Broome)Petch [14.50±0.08 mg/ml], Geotrichum candidum Link (9.63±0.07 mg/ml), Aspergillus oryzae(Ahb.)cohn (8.30±0.08 mg/ml).**

**Maximum amount of saponin present in the ethyl acetate extract of Nigrospora oryzae (Berk and Broome) Petch [17.63±0.01 mg/ml], Aspergillus niger Gr [9.13±0.11 mg/ml], Aspergillus oryzae(Ahb.)cohn [8.43±0.07 mg/ml].**
Link>Scytalidium lignicola</Link> pesante, <Link>Aspergillus oryzae</Link> (Ahbl.) <Link>Aspergillus niger</Link> Gr. Flavonoids inhibit the promotion of growth and progression of tumors and also used against the cancer-causing tumors [37]. In plants phenols when mixed with the flavonoid compounds it show multiple activities like antioxidant, antinociceptogenic, anti-inflammatory, etc. [39]. Singh and Bhat (2003) [39] studied flavonoids are responsible for the antimicrobial activity associated with some ethno medicinal plants.

The presence of tannins in extracts inhibits the pathogenic fungi and showed better antimicrobial activity [40]. The presence of tannins in diets for livestock have been showed to have antinutritional and toxic effects including fed intake, growth, feed efficiency and net metabolizable energy [41]. *Nigrospora oryzae* (Berk and Broome) Petch contain high amount of tannin compared to the other endolichenic fungus.

The natural compounds have an effective dosage response and minimum side effects when compared to synthetic compounds. The plant screened for phytochemical constituents seemed to have the potential function as a source of beneficial drugs and also to improve the health status of the consumers as a result of the presence of various compounds that are vital for good health [42].

**CONCLUSION**

From this study *Nigrospora oryzae* (Berk and Broome) Petch gave the best results in both qualitative and quantitative compared to other endolichenic fungi. *Geotrichum candidum* (Berkand Broome) Petch, *Scytalidium lignicola* Pesante gave the moderate results. *Aspergillus niger* Gr, *Aspergillus oryzae* (Ahbl.) cohn gave less results compared to other organisms. Based on the phytochemical studies, further research can be carried out to isolate the particular phytochemical compounds in the endolichenic fungi.

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**AUTHORS CONTRIBUTIONS**

All authors’ contributions are equal.

**CONFLICT OF INTERESTS**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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