

**Characterization of an unexplored lichen *Hypotrachyna infirma*
(Kurok.) Hale and its selected endolichenic fungi for
biological properties**

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Summary

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The word 'Lichen' has a Greek origin, which denotes the superficial growth on the bark of olive trees. A number of lichen species are repeatedly used in traditional or folk medicines, sold as condiments in the Indian markets, cooked as vegetable curry by the tribal people of Sikkim, Himalayas particularly during the scarcity of food and are even utilized as common livestock fodder in some places of South India. Lichens are known to produce variety of biological compounds as their primary and secondary metabolites. The lichens also have been well known as valuable plant resources in the ancient time and are still used as medicine, food, fodder, perfume, spices and dyes. Recently the lichenological studies initiated in India are mostly related to biodeterioration, bioprospection, air pollution and mycobiont culture.

In lichen thallus (body), the mycobiont predominates with 90% of the thallus volume and provides shape, structure and colour to the lichen with partial contribution from the photobiont. The estimated global lichen diversity is about 20,000 and only a small number of lichen species have been screened for harvesting the endolichenic fungi with the potential to offer bioactive metabolites, hence one can assume the magnitude of prospective lichen diversity which is waiting to be unveiled.

In the present study, the lichen was collected from Sholaiyar hills, Valparai, Coimbatore District, Tamilnadu, India, from the tree trunk of silver wood in the month of June 2019. The lichen was taken from the tree trunks with the help of knife and chisel without damaging tree trunks. The lichen was identified and authenticated by Dr. Sanjeeva Nayaka, Senior Scientist, CSIR—National Botanical Research Institute, Lucknow, India. Herbarium specimen (36008) was prepared and deposited at the herbarium of the Institute.

For extraction, lichen sample was shade dried for one to two weeks. After drying, it was ground and stored in an airtight container. Lichen was extracted with increasing polarity of solvents such as petroleum ether, chloroform, ethyl acetate, ethanol, methanol and water. The results suggested that methanol extract showed maximum extractive value than the other extract.

For the endolichenic fungal isolation surface sterilized samples in different media, pH and temperature were used. From these, PDA media gave good result compared to other medium. Similarly, pH 7 and temperature 35°C gave more number

of fungal isolation compare to other pH and temperature. A total of 13 endolichenic fungi were isolated.

For fungal extraction, a fraction of fungal isolates was transferred into conical flasks containing Potato Dextrose Broth (PDB) by aseptically scraping using an inoculation loop. The culture liquid was filtered and extracted with solvents such as chloroform and ethyl acetate. The result suggested that ethyl acetate extract showed maximum extractive value than the chloroform extract.

The presence of different phytochemicals in lichen and their endolichenic fungi was assessed by qualitative analysis. From the qualitative analysis result, lichen showed the presence of alkaloids, flavonoids, phenols, tannins, saponins, steroids, glycosides, terpenoids, carbohydrates and proteins in methanol extract. Similarly, qualitative analysis of endolichenic fungi extract showed the presence of most of the phytochemical constituents in ethyl acetate extract of *Nigrospora oryzae*, *Aspergillus niger*, *Aspergillus oryzae*, *Geotrichum candidum* and *Scytalidium lignicola*.

The amount of phytochemicals such as phenolic compounds, flavonoids, tannins, alkaloids and saponins was assessed using quantitative analysis. From the result, it was revealed that the methanolic extract showed the maximum amount of tested phytochemicals than the other solvent extracts. Similarly, endolichenic fungi extract showed the maximum amount of tested phytochemical constituents present in ethyl acetate extract of *Nigrospora oryzae*, *Aspergillus niger*, *Aspergillus oryzae*, *Geotrichum candidum* and *Scytalidium lignicola*.

The GC-MS analysis revealed the presence of 40 phytochemical constituents in the methanol extract of *Hypotrachyna infirma* and 30 phytochemical constituents in the ethyl acetate extracts of *Nigrospora oryzae*, *Aspergillus niger*, *Aspergillus oryzae*, *Geotrichum candidum* and *Scytalidium lignicola* based on the peak area, retention time and molecular formula.

Antimicrobial activity of the methanolic extract of *Hypotrachyna infirma* showed good inhibition against tested microorganisms *Escherichia coli*, *Streptococcus sp.*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. Ethyl acetate extract of *Nigrospora oryzae* showed significant activity against all the bacteria as well as fungi when compared to that of other endolichenic fungi.

Antioxidant activity of lichen and their endolichenic fungi was investigated by using various assays such as DPPH free radical scavenging assay, Superoxide radical scavenging assay, Nitric oxide radical scavenging assay, Hydrogen peroxide scavenging assay and Total antioxidant assay. Free radical scavenging method was evaluated to be increasing with increase in concentration of the extracts from 20 µg/ml to 100 µg/ml. Antioxidant activity of the extracts was expressed in terms of IC₅₀ value and low IC₅₀ value corresponds to a high antioxidant activity. The results revealed that the methanolic extract of lichen showed low IC₅₀ values in all antioxidant assays. Similarly, ethyl acetate extract of *Nigrospora oryzae* showed low IC₅₀ value in all assays compared to other endolichenic fungi.

In inhibition of albumin denaturation, Aspirin was used as standard drug that showed the activity with the value of 80.19±0.30 µg/ml which was higher than the lichen and endolichenic fungus. The highest inhibition percentage of endolichenic fungus *Nigrospora oryzae* (68.28 ± 0.05 µg/ml) and the lichen (75.14± 0.81 µg/ml) was showed in 500 µg/ml concentration.

In the membrane stabilization assay, the standard drug Aspirin possessed the activity with the value of 82.36±0.18 µg/ml which was higher than the lichen and endolichenic fungus. The highest inhibition percentage of endolichenic fungus *Nigrospora oryzae* (76.26± 0.20 µg/ml) and the lichen (74.33±0.15 µg/ml) was showed in 500 µg/ml concentration.

Docking aims to accurately predict the structure of a ligand within the constraints of a receptor binding site and to correctly estimate the strength of binding. The receptors for the docking were retrieved from Protein Data Bank (Berman *et al.*, 2000). The ligands in the crystal structure of the retrieved receptors were removed so as to enable binding of the required ligands. *In silico* studies have reported the potency of Bis (2-ethylhexyl) phthalate as an alternative to treat liver cancer.

The methanolic extracts of *H. infirma* and ethyl acetate extract of endolichenic fungus *Nigrospora oryzae*, were screened to test their cytotoxicity activity against liver cancer cell line Hep G2 using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and inhibitory concentration value (IC₅₀) was evaluated. Cells were grown (1×10⁴ cells/well) in a 96-well plate for 48 h into 80% confluence. Positive control was Doxorubicin. Both the methanolic extracts of *H. infirma* (IC₅₀ 19±1.2µg/ml) and ethyl acetate extract of endolichenic fungus *Nigrospora oryzae* (IC₅₀ 24±0.5 µg/ml) had remarkable cytotoxic activity

The formation of Au nanoparticles was visually confirmed by the colour change. Spectral analysis showed that the UV–VIS peaked at 600 nm typically observed for AuNPs that increased as a function of time without altering the peak position. In the FT-IR analysis various functional groups were observed in the methanol extract of lichen and ethyl acetate extract of endolichenic fungus. Spectral bands wave numbers in lichen were 3332.99, 2947.23, 2831.50, 2036.83 cm^{-1} and endolichenic fungus 3379.29, 1627.92, 1296.16 and 1149.57 cm^{-1} . The SEM micrographs clearly indicated that the biogenic nanoparticles were cylindrical and rectangular shaped.

Conclusion:

To conclude, the chemical constituents and medicinal properties of *Hypotrachyna infirma* and its selected endolichenic fungus *Nigrospora oryzae* such as antimicrobial, antioxidant, antiinflammatory, cytotoxicity along with molecular docking and bio nanoparticle synthesis. Both the extracts contained various bioactive compounds which are responsible for their medicinal properties. Our study strongly suggests that both the lichen and endolichenic fungi can be further explored for their use as medicine in the treatment of various diseases.