# GC-MS analysis of endolichenic fungus isolated from *Hypotrachyna infirma* (Kurok.)Hale

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***Abstract***
*Lichen is a stable, ecologically obligate, self-supporting mutualism between an exhabitant fungus and one or more inhabitant extracellularly located unicellular or filamentous photoautotrophic partners. Endolichenic fungi are an emerging group of endosymbiotic microorganisms that live within the lichen thalli. Natural products from endolichenic fungi isolated from variety of different lichen species, have been attracting increased attention for their potential to produce bioactive metabolites. The bioactive metabolite produced by endolichenic fungi originate from multiple biosynthetic pathways and occupy different chemical structure classes which including steroids, quinones, terpenoids, peptides, xanthones etc. With this background, ethyl acetate extract of endolichenic fungus (Nigrospora oryzae (Berk and Broome)) isolated from Hypotrachyna infirma was subjected to Gas chromatography-mass spectrometry (GC-MS) analysis using Perkin-Erlenmer Gas chromatography-Mass spectrometry.*

*The GC-MS analysis showed the presence of different phytochemical compounds in the extract of the endolichenic fungus 30 compounds were identified with valuable biological activity in the above extracts. From the results, it is evident that endolichenic fungus contain various phyto components and can be recommended as the fungus of phytopharmaceutical importance.*

**Keywords:** Endolichenic fungi, *Hypotrachyna infirma*, antiviral, phytocomponents, phytopharmaceutical.

## Introduction

Lichens are symbiotic organisms consisting of a fungus partner and a photosynthetic organism, either an alga or cyanobacteria[1,11]. They are ubiquitous on barks, stems, leaves and in soil but often grow in habitats that are less favourable for higher plants[39]. Kirk et al[19] showed 97,330 species of described fungi at the "Numbers of fungi" entry in "The Dictionary of Fungi". Till date a total of 99,000 species have been described by Kirk et al[19]. The term "endophyte" was coined by de Bary[7] for the organisms which live inside plants without producing any symptoms. Lichens are generally thought about as asymbiotic association between algae and fungi. Fungi are called as endophytes and these are generally termed as endolichenic fungi. The term 'Endolichenic fungi' was coined by Miadlikowsha et al[23] for the fungi that live within lichen thalli without producing any apparent disease symptoms.

Amongst all the known organisms, microorganisms are the crucial source of bioactive compounds with colossal prospective to unearth novel molecules for drug discovery, industrial use and agricultural applications[8,18,27,37]. The metabolites derived from microbes have shown to be more amenable to biosynthetic studies in comparison to natural products obtained from higher plants partly because no seasonal problems have been reported in the production of metabolites and also the incorporation of labeled precursors is quite high[13].

In 1990 the first study was undertaken by Petrini et al[26] to isolate an endolichenic fungi where filamentous fungi were isolated from sterilized segments of fruticose lichens. Surveys to isolate endolichenic fungi are over a decade old[10,26,38], even though studies focused on isolation of secondary metabolites are more recent.The first study to carry out on secondary metabolites isolated from endolichenic fungi was carried out a decade ago[25].

The mycobiont reportedly produces around 1000 chemically variegated lichen substances[3], most of which are specific to lichens, with only a small amount occurring in other fungi, algae and higher plants[32]. These metabolites were found to showcase significant bioactivities including antibacterial[15], Di(2-ethylhexyl) phthalate (DEHP) was isolated from the fungus and screened for its *in vitro* cytotoxic activity against the human tumor cell lines, human liver carcinoma cells (HEPG2), human colon carcinoma cells (HCT 116), human breast carcinoma cells (MCF7) and human cervix carcinoma cells (HELA)[22].

Like endophytic fungi, they are also a valuable source of bioactive products, although only a limited number of strains have been chemically explored[20]. Several novel metabolites including cucurbitarins, chaetoglobosin, terpenoids, naphthalene derivatives, heptaketides**,** diphenylethers, polyketides, alkaloids, pyridoxatin, variecolortide, tricycloalternarenes, thiopyranchromenone and chromone derivatives revealing interesting bioactivities have been synthesized from endolichenic fungi.[4-6,14,16,17,21,31,41-46]

The biological potential of many lichens and endolichenic fungi has largely remained unexplored. Considering the medicinal activity, traditional information and various experimental evidence, the present study aimed at evaluating the phytochemical constituents of endolichenic fungi

isolated from *Hypotrachyna infirma* (Kurok.) Hale by Gas chromatography-mass spectrometry (GC-MS) analysis.

## Material and Methods

**Collection of Plant Material:** *Hypotrachyna infirma* (kurok.) Hale was collected from Sholaiyar, Anamalai hills, Coimbatore district, Tamilnadu, India during the monsoon season. The lichen was duly identified by Dr.Sanjeeva Nayaka, Senior Principal Scientist, Lichenology Laboratory, CSIR-National Botanical Research Institute, Lucknow, India and was deposited at herbarium of LWG with accession no. 36008.

**Isolation and identification of endolichenic fungi:** The surface sterilized samples were placed on Petri plates containing PDA (Potato Dextrose Agar) and sealed using parafilm. These plates were incubated at 25±1°C until fungal growth was initiated. After the growth of the mycelial tip, the culture was transferred to new PDA plates.

Then these pure cultures were examined periodically. After 15 days the endophyte was identified based on morphology at National Fungal Culture Collection of India (NFCCI) –a national facility, Pune, India.

**Fungal Extraction** The endolichenic fungal strain was cultured on plates on Potato Dextrose Agar at 25°C for 7 days. The fungal mycelia were inoculated in 250 mL Erlenmeyer flasks, each containing 100 mL of Potato Dextrose Broth (PDB).

These flasks were incubated at room temperature for 28 days. After a period of time, the fungal extract was filtered using Whatmann filter paper no. 1. From this filtrate, compounds were extracted by solvent extraction method using ethyl acetate as a solvent.

To this filtrate, equal volume of ethyl acetate was added in separating funnel and mixed well for 10 minutes. After few minutes the two clear immiscible layers were formed. From this, upper layer of solvent containing the compounds was separated. The collected solvent was evaporated using rotator vacuum evaporator to yield the crude extract[2]. Then the crude extract was dissolved in ethyl acetate at 1mg/mL of concentration and used for GC-MS analysis.

**Gas chromatography –MS analysis:** GC-MS analysis was performed at the TUV SUD South Asia Pvt. Ltd., Tirupur, India. 5mL of ethyl acetate extract was evaporated to desiccation and made into 2 mL ethyl acetate. Then the extract was subjected to Gas chromatography – MS analysis. The chromatographic separation was finished with CE GC 8000 top MSMD 8000 Fyson instrument with Db 35mr column ($10x10^3$mm x 0.5 mm, 0.25 mm film thickness). In split mode (1:50) with a flow rate of 1 mL/min heating programs were executed from 100-250ºC at 3 minutes by using helium as a carrier gas. 2 mL of aliquot oil was injected into the column with the injector heater at 250ºC.

**Analytical conditions:** The following temperature was kept for this analysis. Injection temperature - 250ºC, interface temperature - 200ºC, quadruple temperature - 150ºC and ion source temperature - 230ºC were maintained. In split less mode, the injection was performed.

**Identification of components (Data analysis):** The mass spectra of compounds in sample was obtained by electron ionization (EI) at 70 eV and the detector operated in scan mode from 20 to 600 atomic mass units (AMU). Identification was based on the molecular mass, calculated fragmentations and molecular structure. By using the standard mass spectral database of WILEY and NIST, resolved spectrum was identified for phytochemicals.[9,24]

**Identification of components:** Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The name, molecular weight and structure of the components of the test material was determined.

By comparing its average peak area to the total area, the relative percentage amount of each component was calculated. The unknown component's spectrum was compared with the spectrum of the component stored in the NIST library version (2005) software, Turbomas 5.2.

## Results and Discussion

The endolichenic fungus was isolated from *Hypotrachyna infirma* (Kurok.) Hale. The fungus was mass cultured and extracted using the method described above. The prominent compounds present in the endolichenic fungal culture was identified by GC-MS analysis. This is the best technique to identify the constituents of volatile matter, long chain, branched chain hydrocarbons, alcohols, acids, esters etc. Peak area, retention time and molecular formula are used for the confirmation of phytochemical compounds.[30] Applications of GC-MS include drug detection, environmental analysis, explosives investigation and identification of unknown samples[29].

The ethyl acetate extract of the endolichenic fungus revealed the existence of various compounds and the results are shown in table 1 and fig. 1. The GC-MS analysis of *Nigrospora oryzae*(Berk and Broome) extract revealed the presence of 30 compounds (phytochemical constituents) (Figure 1) that could contribute to the medicinal properties.

The first compound identified with less retention time (4.201min) was acetic acid, pentyl ester, while methyl tris (trimethylsiloxy)silane had long retention time (26.334 min). These compounds were recognized by relating their peak retention time, peak area (%), height (%) and mass spectral fragmentation patterns (Table 1).

Alkane hydrocarbons such as tridecan, pentadecane, octadecane, nonadecane, heptadecane, dodecane, eicosane, decane and hexadecane have antimicrobial activity[33].

**Table 1**
**GC-MS analysis of *Nigrospora oryzae* extract**

| S.N. | Retention Time | Name of the compound | Molecular formula | Area % |
|---|---|---|---|---|
| 1 | 4.201 | Acetic acid, pentyl ester | $C_{17}H_{14}O_2$ | 0.54 |
| 2 | 8.264 | Cyclopentasiloxane, decamethyl | $C_{10}H_{30}O_5Si_5$ | 0.41 |
| 3 | 9.334 | Azulene | $C_{10}H_8$ | 1.00 |
| 4 | 10.375 | Tetradecane | $C_{14}H_{30}$ | 0.43 |
| 5 | 10.696 | Cyclohexasiloxane, dodecamethyl | $C_{10}H_{32}O6Si_6$ | 0.68 |
| 6 | 11.989 | 1-Tetradecene | $C_{14}H_{28}$ | 0.66 |
| 7. | 12.086 | Carbonic acid, decyl vinyl ester | $C_{13}H_{24}O_3$ | 0.92 |
| 8 | 13.208 | Hentriacontane | $C_{31}H_{64}$ | 0.61 |
| 9 | 13.768 | Hentriacontane | $C_{31}H64$ | 0.53 |
| 10 | 14.484 | Pentafluoropropionic acid, tetra... | $C_3HF_5O_2$ | 2.36 |
| 11 | 14.570 | Hexadecane | $C_{16}H_{34}$ | 0.64 |
| 12 | 16.189 | Pentacosane | $C_{25}H_{52}$ | 0.58 |
| 13 | 16.607 | 1H-Pyrazole, 3-ethyl-4,5-dihydro... | $C_5H_{10}N_2$ | 1.71 |
| 14 | 16.727 | 1 1-Octadecene | $C_{18}H_{36}$ | 4.54 |
| 15 | 16.795 | 1-Dodecanol, 2-hexyl- | $C_{18}H_{38}O$ | 1.48 |
| 16 | 17.207 | 3-pyridinamine, 2-[(4-methyl-4H-.. | $C_9H_{10}N_2$ | 1.59 |
| 17 | 17.442 | Phthalic acid, butyl tetradecyl ... | $C_{26}H_{42}O_4$ | 0.90 |
| 18 | 17.923 | 2-Nitro-4-(trifluoromethyl)pheno | $C_{17}H_4F_3NO_3$ | 0.58 |
| 19 | 18.415 | Phthalic acid, 3-(2-methoxyethyl... | $C_{23}H_{36}O_5$ | 0.54 |
| 20 | 18.764 | Carbonic acid, octadecyl 2,2,2-t... | $C_{21}H_{39}CI_3O_3$ | 1.71 |
| 21 | 18.827 | Eicosane | $C_{20}H_{42}$ | 0.72 |
| 22 | 20.623 | 1-Docosene | $C_{22}H_{44}$ | 2.67 |
| 23 | 20.681 | 1,2-Bis(trimethylsilyl)benzene | $C_{12}H_{22}Si_2$ | 1.29 |
| 24 | 21.837 | Silicic acid, diethyl bis(trimet.. | $C_{10}H_{28}O_4Si_3$ | 0.81 |
| 25 | 22.151 | Methyltris(trimethylsiloxy)silane | $C_{10}H_{30}O_3Si_4$ | 0.41 |
| 26 | 22.334 | Cyclotrisiloxane, hexamethyl- | $C_6H_{18}O_3Si_3$ | 0.76 |
| 27 | 22.924 | 4-(4-Hydroxyphenyl)-4-methyl-2-p | $C_{12}H_{16}O_2$ | 0.84 |
| 28 | 23.422 | Di(2-ethylhexyl) phthalate | $C_{24}H_{38}O_4$ | 52.67 |
| 29 | 23.948 | 2, 4, 6-Cycloheptatrien-1-one, 3, 5... | $C_6H_{22}OSi_2$ | 1.08 |
| 30 | 26.334 | Methyltris(trimethylsiloxy)silane | $C_{10}H_{30}O_3Si_4$ | 16.34 |

In this study also *Nigrospora oryzae* conforms the antimicrobial activity due to the presence of octadecane and dodecane compounds. Di(2-ethylhexyl) phthalate (DEHP) was previously recorded with premium biological activities such as antiviral activity and antioxidant activity in addition to antitumor[35,36].

Zota et al[47] reported that Di(2-ethylhexyl) phthalate (DEHP) has potent dose dependent antitumor activity against Ehrlich cells *in vivo* while that DEHP has antitumor activity against Ehrlich cells *in vitro* also. *Bacillus subtilis* AD35 synthesized DEHP compounds have antibacterial activity against various gram positive and gram negative strain such as *Salmonella typhimurium*, MRSA, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli and Pseudomonas aeruginosa*.[12,28]

From the above discussion it is proved that Di(2-ethylhexyl) phthalate (DEHP) has antimicrobial, antioxidant, antiviral and antitumor activities. From the present study, it can be concluded that DEHP compound is present at the high peak in the ethyl acetate extract of *Nigrospora oryzae* from which further isolation of this compound and pharmacological studies can be carried out.

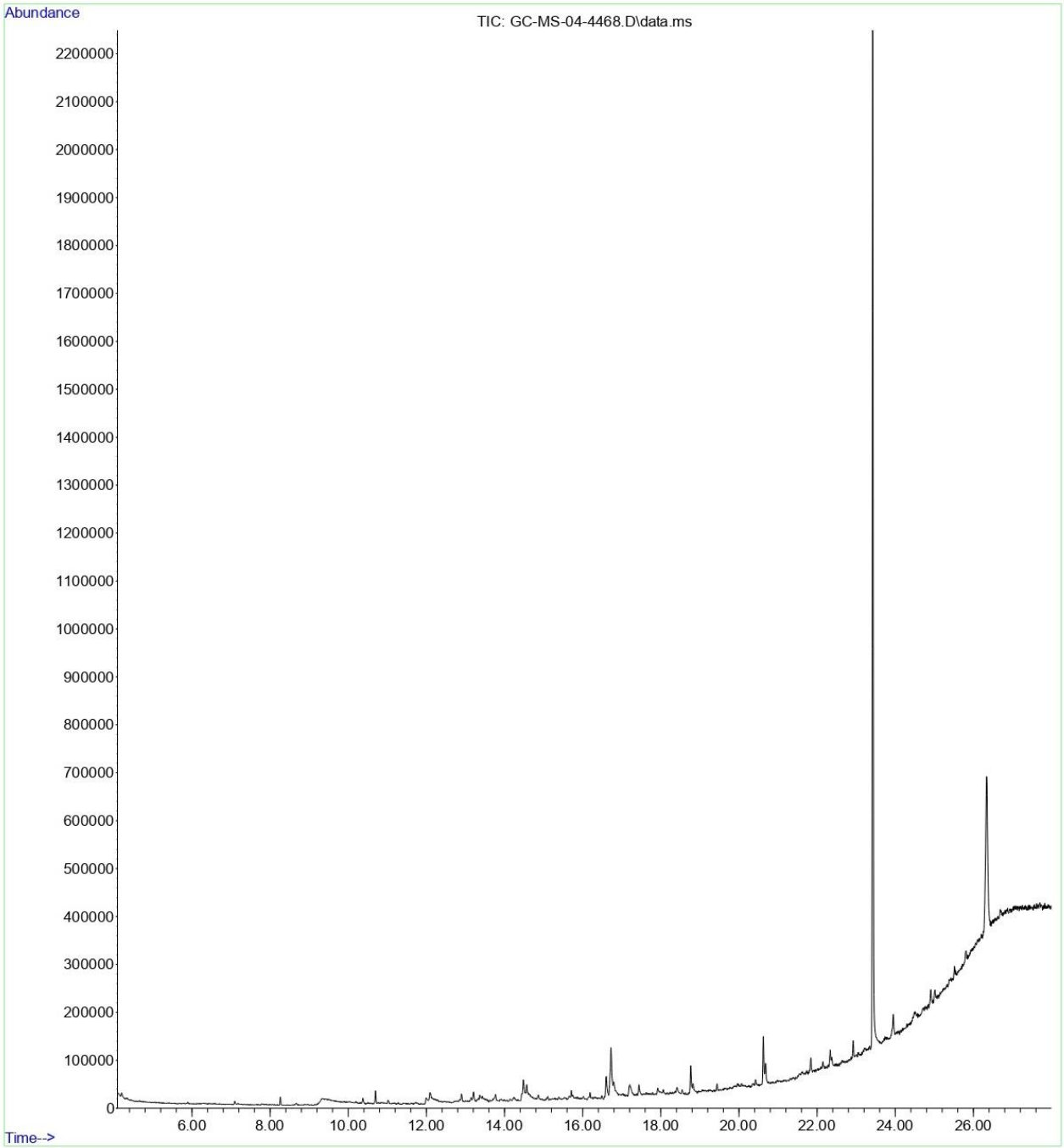


**Fig. 1: GC-MS spectral chromatogram analysis of *Nigrospora oryzae* extract**

## Conclusion

The present investigation focused on identification of various bioactive compounds from the *Nigrospora oryzae* (Berk and Broome) extract of Ethylacetate by GC–MS analysis. These compounds are responsible for the different therapeutic and pharmacological properties. Further investigations to determine its bioactivity, toxicity profile and clinical studies are necessary for broad-spectrum drug discovery.

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