

Research Article



GC-MS Analysis of Metabolites from Endophytic Fungus *Colletotrichum gloeosporioides* Isolated from *Phlogacanthus thyrsoiflorus* Nees

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ABSTRACT

Endophytes are those organisms that colonize the living internal tissues of their hosts without causing detectable symptoms. Several fungal endophytes have been isolated from a variety of plant species which have proved as a rich source of secondary metabolites. For the first time *Phlogacanthus thyrsoiflorus* Nees, a well known medicinal plant in Manipur has been explored for its endophytes. In this present work a total of 6 fungal endophytes were isolated from *Phlogacanthus thyrsoiflorus* Nees. *Colletotrichum gloeosporioides* was the main isolate and were screened for the production of secondary metabolites. The fungus was identified based on the morphological and molecular characterization. Volatile metabolites in the ethyl acetate extract of the *Colletotrichum gloeosporioides* were determined using Gas chromatography– Mass spectrometry (GC–MS). GCMS analysis revealed the presence of various metabolites. The main constituents were Phenol,2,4-bis (1,1-dimethylethyl), 1-Hexadecene, 1-Hexadecanol, Hexadecanoic acid, octadecanoic acid methyl ester and 1-Nonadecene. The compounds produced by endophytic fungus *Colletotrichum gloeosporioides* could be an alternative source for human welfare. This study is an important step in investigating the endophytic fungal diversity and to explore the novel bioactive compounds from medicinal plants of Manipur.

Keywords: *Colletotrichum gloeosporioides*, Fungal endophytes, GCMS, Medicinal plant, *Phlogacanthus thyrsoiflorus* Nees.

INTRODUCTION

Phlogacanthus thyrsoiflorus Nees locally known as Nongmangkha in Manipuri is a gregarious shrub of Acanthaceae family, common in the Manipur valley. This plant has long orange-red tubular flowers, appearing in upright spikes at the end of branches. Leaves are ovoid to lance-like, with smooth margins. The plant possesses immense medicinal property. Both the leaves and flower are used for the treatment of various diseases¹. Endophytic fungi have been associated with various plants. Because of the high diversity of endophytic fungi, the study of endophytic fungi has become one of the main research area². Endophytic fungi produces a variety of novel bioactive metabolites for exploitation in modern medicines. Endophytes are recognized as rich sources of secondary metabolites of immense importance^{3,4}.

Previous works have reported that the endophytes isolated from medicinal plants are excellent producers of strong fungicidal, bactericidal and cytotoxic metabolites⁵. There is a need to exploits fungal endophytes from medicinal plants because it has been hypothesized that medicinal plants harbour distinct and rare microbes that mimic the chemistry of their respective hosts and synthesize identical bioactive products or derivatives that are more bioactive than the one produced by the host. The endophytes are mostly unexplored in Manipur. Thus, an investigation was carried out to isolate and characterize the endophytic fungi from medicinal plant *Phlogacanthus thyrsoiflorus* Nees to investigate a repository and explore their bioactive potential.

MATERIALS AND METHODS

Plant materials and fungal isolates

Healthy symptomless leaf samples were randomly collected from Imphal, Manipur. Samples were washed under running tap water and surface sterilization was carried out by immersion in 70% ethanol for 1 min, 5% sodium hypochlorite for 5 min, 70 % ethanol for 1 min and sterile distilled water for 1 min for two times, then allowed to dry under sterile conditions⁶.

The leaf samples were then inoculated in PDA amended with streptomycin 150mg/l¹ concentration. The petridishes were incubated at 28° C and continuously monitored for fungal growth. Individual fungal colonies were transferred onto PDA plates. The identifications of the endophytic fungi were based on their morphology and mechanism of spore production. The promising fungus was identified by using molecular techniques.

Statistical analysis

The endophytic fungal isolates from each host plant tissue segment were analyzed based on the Percentage of density of colonization frequency⁷, Relative percentage occurrence of different groups of fungi⁸.

Colonization frequency

$$CF (\%) = \frac{\text{Number of species isolated}}{\text{Number segments screened}} \times 100$$



Identification of Endophytic Fungi

DNA extraction, PCR and sequencing

Total genomic DNA was extracted from fresh mycelia of the endophytic fungus grown on PDA medium⁹. A pair of primer Fungal forward: 5'-TCC GTA GGT GAA CCT GCG G-3' and Fungal reverse: 5'-TCC TCC GCT TAT TGA TAT GC-3 were used to amplify the highly specific sequence for endophytic fungi targeting the gene encoding for 18S rRNA¹⁰. The PCR amplification cycle consisted of 30 s at 94°C, 1 min at 50 °C, and 1 min at 72 °C. PCR products were sequenced by using an instrument ABI 3130, Amersham Biosciences United Kingdom using the same primers as for the amplification. The sequence of the fungal strain was deposited in the NCBI GenBank database with the help of BankIT system. Sequence similarity search was performed for the fungal sequence. The sequence was compared to the sequences in GenBank database with BLAST algorithm to identify known closely related sequences.

Cultivation and metabolite extraction

The fungus was cultivated on potato dextrose broth by placing agar blocks of pure culture of actively growing culture in 500ml Erlenmeyer flask containing 200ml of the medium. The flask was incubated with periodic shaking at 150 rpm for 30 days at 28± 2°C. The fermentation broth of the endophyte was filtered through Whatmann filter paper to remove the mycelial mats. The filtrate was extracted thrice with ethyl acetate at room temperature. Ethyl acetate collected after extraction was evaporated and the resultant compound was dried in vacuum evaporator using MgSO₄ to yield the crude metabolite. After evaporation a brown coloured crude extract was obtained.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The crude extract of fungus was analyzed by FTIR (FTIR BRUKER ALPHA-E) to know the different functional groups present in the fungal extracts. The dried crude extract was subjected to analyze in FTIR in which the diffuse reflectance technique was followed. The crude dried sample was mixed with potassium bromide (KBr) to form a very fine powder. This powder was then compressed into a thin pellet which was analyzed. KBr was also transparent in infra red light.

The sample was irradiated by a broad spectrum of infra red light and the level of absorbance at a particular frequency was plotted after Fourier transformation of the data. The resulting spectrum was characteristic of the organic molecule present in the sample. The absorbance was measured at 400- 600 nm for the identification and quantification of organic species. Compounds contained in the extract were identified according to established criteria of¹¹.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The fungal extract was subjected to GC MS analysis to identify the bioactive compound. The sample was analysed in GC Clarus 500 Perkin Elmer by using software Turbomass 5.2 equipped with mass detector Turbo mass gold Perkin Elmer. 2µl sample was introduced via an all-glass injector working in the split mode, with He as the carrier gas with a linear velocity of 32 cm/s. The HP-5 fused silica capillary column (Length – 30 m; Film thickness- 25 µm I.D - 0.2 mm) was used. The identification of components was accomplished using computer searches in NIST version 2005.

RESULTS AND DISCUSSION

A total of 6 endophytes were isolated from *Phlogacanthus thyrsoiflorus* Nees only one fungus that showed promising result was selected for analysis. The medicinal plants are known to harbour the endophytic fungi which have the capability to produce the bioactive compounds, which can be used for pharmaceutical application¹². *Phlogacanthus thyrsoiflorus* Nees has been used for various treatments¹³. We used conidia and mycelia morphology to identify the endophyte, and then confirmed the identification by rDNA sequences analysis. The colonization frequency was found to be 62%. Molecular characterization was carried out and it revealed that the fungus sequence showed 100% homology with *Colletotrichum gloeosporioides* from nucleotide database of NCBI. This sequence is submitted in genebank as *Colletotrichum gloeosporioides* strain NP13 and can be accessed with the Accession number KC662170. *Colletotrichum gloeosporioides* has been reported as an endophytes from other plants¹⁴⁻¹⁶.

A novel endophytic fungus, *Colletotrichum gloeosporioides*, was isolated from the medicinal plant *Vitex negundo* L. and extracts of *C. gloeosporioides* exhibited an effective antimicrobial activity against bacterial and fungal strains¹⁷. There are many studies on endophytic fungi from various plants; this is the first report on endophytic fungi from *Phlogacanthus thyrsoiflorus* Nees. The combined use of molecular diagnostic tools along with traditional morphological techniques is at present an appropriate approach for studying *Colletotrichum* species¹⁸.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FT-IR spectral analysis showed the wave number 878.88cm⁻¹(CH alkene group), 938.62 cm⁻¹ (OH group), 699.18 cm⁻¹(C-H bond of Aromatic group), 1035 cm⁻¹(C-N stretch Aliphatic amine group), 1647.92 cm⁻¹ (C=C,stretch, alkene group), 2929.00 cm⁻¹ (C-H, stretch, alkyl group), 3371.50 cm⁻¹ (N-H,stretch, Amine group) and 1409.68 cm⁻¹(C-C,stretch, aromatic group) as in Figure 1.

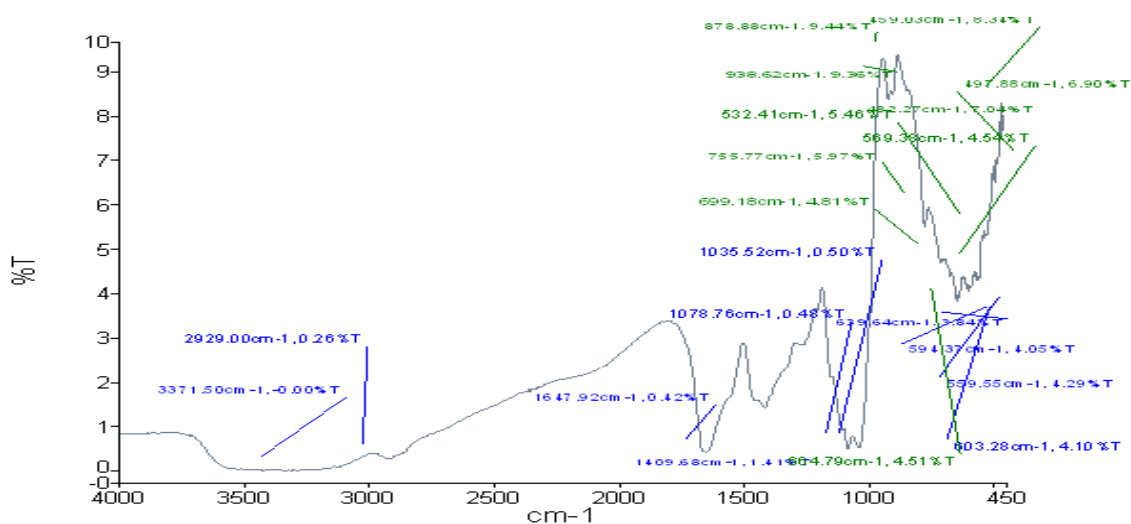


Figure 1: FTIR result for ethyl acetate extract of *Colletotrichum gloeosporioides* NP13

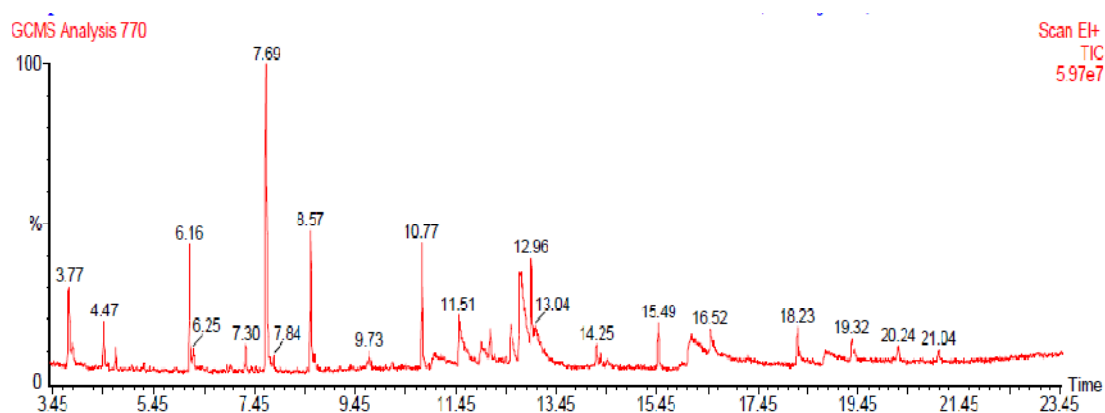


Figure 2: GC/MS Chromatogram of volatile compounds from *Colletotrichum gloeosporioides* NP13 extract.

GCMS analysis

GCMS analysis indicated that the fungus *Colletotrichum gloeosporioides* NP13 produced bioactive compounds. The compounds were identified based on the NIST database by virtue of comparisons made of the actual mass spectral data acquired shown in Figure 2. The chromatogram predict the presence of 3-Dodecene, Benzene,1,3-bis(1,1-dimethylethyl), Dodecane,2,6,10-trimethyl-, 1-Undecanol, Phenol,2,4-bis(1,1-dimethylethyl)-, 1-Hexadecene, 1-Hexadecanol, Octahydro-2H-pyrido(1,2- α)pyrimidin-2-one, Pyrrolo[1,2- α]pyrazine-1,4-dione, hexahydro-3(2-methylpropyl)-, 1-Nonadecene, 9,12-Octadecadienoic acid, methyl ester(E,E)-, 9-Octadecanoic acid(Z)-methyl ester, 3-Eicosene(E)-, Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester, 10-Heneicosene(c,t), Octadecanoic acid,3-hydroxypropyl ester, 1,2-Benzenedicarboxylic acid, diisooctylester. Hexadecanoic acid and octadecanoic acid methyl ester were mainly found in endophytic fungi obtained from five Thai medicinal plants which are commonly used in Thai traditional medicines^{19, 20}. Dodecene was obtained as a major compound from an endophytic fungus *Fusarium solani* isolated from *Taxus baccata* bark²¹. 1-Hexadecene was identified as an extract of fungi *Monochaetia*

kansensis. Phenol, 2, 4-bis (1,1- dimethyl ethyl) and eicosene was reported in ethyl acetate fraction of the fungi *Monochaetia kansensis* as a main compounds identified by GCMS²². Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl) has good antioxidant activity²³. (9Z)-octadecenoic acid methyl ester and (9Z,12Z)-octadecadienoic acid methyl ester that displayed cytotoxic activity against different cancer cell lines were reported from the fungal endophyte *Fusarium oxysporum* SS46 isolated from the medicinal plant *Smallanthus sonchifolius* (Poepp.)²⁴. It was reported that various antimicrobial compounds were isolated from *Colletotrichum gloeosporioides*²⁵.

CONCLUSION

In this study 6 endophytic fungi from medicinal plant *Phlogacanthus thyriflorus* Nees were isolated and one promising fungus *Colletotrichum gloeosporioides* was studied. This preliminary work is for exploration of the endophytic fungal strain for their novel bioactive compounds. The compounds were identified by using FTIR and GCMS. In conclusion, the identification of the metabolites from the culture of *Colletotrichum gloeosporioides* predict that this endophyte is capable of producing bioactive compounds. Further work is to be carried out to confirm the bioactivities of these metabolites.

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REFERENCES

- Kalita, D and Bora, R. L. Some folk medicines from Lakhimpur district, Assam. *Indian J. Traditional Knowledge* 7(3),2008, 414 - 416.
- Arnold AE, Lutzoni F. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* 88, 2007,541–549.
- Tan RX, Zou WX. Endophytes: a rich source of functional metabolites. *Natural Product Report* 18,2001, 448–459.
- Strobel GA, Daisy B. Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Review* 67, 2003, 491–502.
- Wang FW, Jiao RH, Cheng AB, Tan SH, Song YC. Antimicrobial potentials of endophytic fungi residing in *Quercus variabilis* and brefeldin A obtained from *Cladosporium* sp. *World Journal of Microbiology and Biotechnology* 23,2007, 79–83.
- Erza D, Hess WH, Strobel GA. New endophytic isolates of *M. albus*, a volatile antibiotic producing fungus. *Microbiology* 150, 2004, 4023-4031.
- Suryanarayanan TS, Senthilarasu G, Muruganandam. 'Endophytic fungi from *Cuscuta rejelexa* and its host plants'. *Fungal Diversity*, 4, 2000, 117-123.
- Suryanarayanan TS, Thennarasan S. 'Temporal variation in endophyt assemblages of *Plumeria rubra* leaves'. *Fungal Diversity*, 15, 2004, 197-204.
- Cai L, Jeewon R, Hyde KD. Phylogenetic investigation of *Sordaria* - ceae based on multiple gene sequences and morphology. *Mycological Research* 110, 2006, 137–150.
- White T.J., Bruns T.D., Lee S. And Taylor J.W., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics; in Innis M.A., Gelfand D.H., Sninsky J.S. and White T.J., ed., *PCR Protocols: Applications*, Academic Press, New york, 1990: 315-322.
- Nyquist RA, Kagel O .The handbook of infrared and Raman Spectra of inorganic compounds and organic salts.2nd Edition,1997, San Diego:Academic Press.
- Zhang HW, Song YC, Tan RX (2006). Biology and Chemistry of endophytes. *Nat. Pro.Rep.* 23, 2006, 753-771.
- Choudhury MD, Bawari M, Singh LS, Some antipyretic ethnomedicinal plants of Manipuri community of Barak Valley, Assam, India. *Ethnobotanical Leaflets* 14, 2010, 21-28.
- Zou WX, Meng JC, Lu H, Chen GX, Shi GX, Zhang TY and Tan RX. Metabolites of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*. *J. Nat. Prod.* 63(11), 2000, 1529-1530.
- Larran SC, Monaco, Alippi. Endophytic fungi in leaves of *Lycopersicon esculentum* Mill. *World J. Microbiol. Biotechnol* 17, 2001, 181-184.
- Photita W, Lumyong S, Lumyong P, Hyde KD, Endophytic fungi of wild banana (*Musa acuminata*) at Doi Suthep Pui National Park, Thailand. *Mycol. Res.* 105, 2001, 1508-1513.
- Arivudainambi USE, Anand TD, Shanmugaiah V, Karunakaran C, Rajendran A. Novel bioactive metabolites producing endophytic fungus *Colletotrichum gloeosporioides* against multidrug-resistant *Staphylococcus aureus*. *FEMS Immunology & Medical Microbiology*, 61(3), 2011, 340-345.
- Cai L, Wu CD. Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens. *Journal of Natural Products* 59(10), 1996, 987-990.
- Theantana T, Kanjanapothi D, Lumyong S. *In vitro* Inhibition of Lipid Peroxidation and the Antioxidant System of Endophytic Fungi from Thai Medicinal Plants. *Chiang Mai J. Sci.* 39(3), 2012, 429-444.
- Guo L, Wu JZ, Han T, Cao T, Rahman K, Qin LP. Chemical composition, antifungal and antitumor properties of ether extracts of *Scapania verrucosa* Heeg. and its endophytic fungus *Chaetomium fusiforme*. *Molecules*. 13(9), 2008, 2114-2125.
- Tayung K, Barik BP, Jha DK, Deka DC. Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani* isolated from bark of Himalayan yew. *Mycosphere* 2(3), 2011, 203–213.
- Yogeswari S, Ramalakshmi S, Neelavathy R, Muthumary J, Identification and Comparative Studies of Different Volatile Fractions from *Monochaetia kansensis* by GCMS *Global J Pharm*, 2012; 6 (2): 65- 71.
- Ajayi GO, Olagunju JA, Ademuyiwa O and Martins OC.. Gas chromatography-mass spectrometry analysis and phytochemical screening of ethanolic root extract of *Plumbago zeylanica* Linn. *Journal of Medicinal Plants Research* 5(9), 2011, 1756-1761.
- Nascimento AM, Conti R, Turatti ICC, Cavalcanti BC, Costa-Lotufo LV, Pessoa C, Mores MO, Manfrim V, Toledo, JS, Cruz AK, Pupo MT. Bioactive extracts and chemical constituents of two endophytic strains of *Fusarium oxysporum*. *Revista Brasileira de Farmacognosia Brazilian Journal of Pharmacognosy*. 22(6), 2012, 1276-1281.
- Aoyagi A, Ito-Kobayashi M, Ono Y, Furukawa Y, Takahashi M, Muramatsu Y, Umetani M, Takatsu T. Colletic Acid, a Novel 11 β -Hydroxysteroid Dehydrogenase Type 1 Inhibitor from *Colletotrichum gloeosporioides* SANK 21404. *J. Antibiot.* 61, 2008, 136–141.

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