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RESEARCH ARTICLE

Endophytic *Chaetomium* sp. from *Michelia champaca* L. and its taxol production

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Abstract

The study of naturally occurring compounds from plants has progressed to its present state of development by passing through a long time. Fungi have an excellent record in producing novel bioactive compounds and many of these presently have important medicinal properties. We subsequently isolated few endophytic fungi from plant parts and tested for the production of taxol like compound. Medicinal plants produce bioactive constituents capable of preventing and curing illnesses. They also provide a unique environment for endophytes. Diverse fungal endophytes exist within plant tissues, with a global estimate of upto a million species. These endophytes presumably occur in a mutualistic association with the host plants, such as enhancing hosts competitive abilities by excreting some bioactive metabolites. Fungal endophytes residing in medicinal plants have been recognized to be a likely source of novel metabolites of pharmaceutical importance. However, these special fungi have not been systematically characterized. In this paper, an endophytic *Chaetomium* sp. from *Michelia champaca* L. was investigated for taxol, an anti-cancer compound and its molecular characterization.

Keywords: Endophytic fungi, taxol, fungal endophytes, medicinal plants, anti-cancer compound.

Introduction

Fungi are being used as a source for antibiotics and various enzymes from fungi such as cellulase, pectinase, and protease are widely used in various industries. An array of natural products have been characterized from endophytes which includes anti-cancer, anti-fungal, anti-bacterial, anti-viral, anti-oxidant, anti-insecticidal and immunosuppressant's (Strobel and Daisy, 2003). It is more common to find natural products with restricted taxonomic distributions in plants. For example taxol appears to be restricted to Yews (viz., eleven species of the genus *Taxus*) but interestingly, it has also been found in a number of different genera of fungal endophytes from yew sources viz; *Taxodium distichum* (Pinkerton and Strobel, 1976). The genetic origin of fungal taxol production has been speculated to have arisen to horizontal gene transfer from *Taxus* spp. to its endophytes (Cardellina, 1991).

A number of antimicrobial compounds have been isolated from various endophytes, which include *Chaetomium* sp., *Colletotrichum* sp. and *Fusarium* sp. Developing a productive microbial source for anti-cancer, immunosupressants and anti-microbials not only would lower the cost but also would help to make it more widely available. Endophytic organism in culture can produce secondary metabolites in high yield, particularly when subjected to strain improvement program (Penalva *et al*., 1998).

Moreover, the metabolites they produce are largely generated by enzymatic pathways that have the potential to biosynthetically link existing structures to chemical adjuncts in a reproducible manner at yield that are acceptable for industrial use (Verdine, 1996). In this sense, natural products generated as a microbial secondary metabolites exhibit a number of properties that make them excellent candidates for industrial processes.

Fig.1. *Michelia champaca* L.

Paclitaxel (taxol) is the most effective antitumor agent developed in past three decades. It has been used for effective treatment of a variety of cancers including refractory ovarian cancer, breast cancer, non-small cell lung cancer, AIDS related Kaposi's sarcoma, head and neck carcinoma and other cancers (Wani *et al.*, 1971; Wall *et al*., 1976; Croom, 1995). Taxol inhibits cell proliferation by promoting the stabilization of microtubules at the G2-M phase of the cell cycle by which depolymerization of microtubules to soluble tubulin is blocked (Stierle *et al*., 1993; Pinkerton and Strobel, 1976; Cardellina, 1991). *Michelia champaca* L. known as champaca, belong to the family of *Magnoliaceae*. There are three species of *Michelia* available. They are *Michelia alba* (white chempaka), *Michelia champaca* (orange chempaka) and *Michelia figo* (dwarf chempaka) with *M. champaca* and *M. alba* being the most popular species within the family (Ibrahim *et al.*, 2005). It has commercial value from almost every parts of the plant especially the flower. The flower has a number of other cosmetic, medicinal and economic uses. Fresh flowers can be taken as natural fragrant and also can be extracted into perfumes and medicinal products such as cure for coughs and rheumatism. Some cosmetic products such as Joy, J'adore and Dior contain *M. champaca* fragrant extracts in their ingredient composition (Warren, 1998). Aromatic and medicinal flowers are reported to be used in dyspepsia, nausea, fever and also useful as a diuretic in renal diseases. Flower oil used in cephalagia. Bark is a stimulant, diuretic and febrifuge. Dry roots are reported to be used as a purgative and emmounogogue.

Investigating the metabolites of endophytic fungi can increase the chance of finding novel compounds (Tan and Zou, 2001; Hawksworth and Rossman, 1987). In this paper, *Chaetomium* is isolated which is a dematiaceous filamentous fungus found in soil, air and plant debris. Some species are thermophilic and neurotropic in nature. *Chaetomium* colonies are rapidly growing, cottony and white in colour initially (De Hoog *et al*., 2000). *Chaetomium* have been isolated because on one hand, these endophytes can produce similar or the same biologically active constituents as its host, such as an endophytic fungus producing Taxol. On the other hand, these endophytes are a prolific source of metabolites with significant biological activities. Many important anticancer, antifungal and antibacterial hemotherapeutics are either microbial metabolites or their semi-synthetic derivatives. In this study, we report an endophytic *Chaetomium* sp. from *Michelia champaca* L. for taxol production and its molecular characterization.

Materials and methods

Isolation of endophytic fungi

The fungus that has been used in this study is one of the nine endophytic fungi isolated from the leaves of medicinal plants in Chennai, India. The leaf samples were surface sterilized according to Suryanarayanan *et al*. (1998). An endophytic fungus was isolated from *Michelia champaca* L. leaves was sampled for the investigation (Fig. 1). The plant material was rinsed gently in running water to remove dust and debris. After proper washing, leaves were selected for further processing under aseptic condition. Highly sterile condition was maintained for the isolation of endophytes. The isolation of endophytic fungi was done according to the modified method described by Dobranic *et al.* (1995). Potato Dextrose Agar (PDA) medium was supplemented with antibiotics penicillin G (100 U/mL) and streptomycin (100 µg/mL) concentrations. The Petri dishes were sealed with paraffin and incubated at 27 ± 2°C for different days. Isolation from the master plates was done by the transfer of hyphal tips to fresh PDA plates with addition of antibiotics to obtain pure cultures for identification. The fungi in the pure culture were preserved on the slant and preserved at 8°C with proper labeling.

Production of taxol

Production of taxol was carried out by Pinkerton and Strobel method (1976). The test fungus was grown in 500 mL Erlenmeyer flasks containing 250 mL of M1D medium supplemented with 0.25 g of soytone for the production of taxol. The extraction procedure was performed according to the method of Strobel and Daisy (2003). After 21 d of incubation period, the cultures were filtered through 4 layers of cheese-cloth to remove mycelia. Then, to the culture filtrate 0.25 g $NaCO_3$ was added with frequent shaking in order to reduce the amount of fatty acids that may contaminate taxol in culture. Then, the culture filtrate was extracted with two equal volumes of dichloromethane. The organic phase was collected and then removed by evaporation under reduced pressure at 350℃ using rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol for the subsequent separation and crude extracts were analyzed by chromatographic separation and spectroscopic analysis.

Thin Layer Chromatography (TLC)

Thin layer chromatography for the fungal sample containing taxol was carried out on 0.25 mm (10 cm × 20 cm) aluminium pre-coated silica gel plates (Merck) by Cardellina (1991) method. Samples were spotted along with authentic taxol (Paclitaxel, Sigma grade) as internal standard and the plates are developed in different following solvent system successively.

1. Chloroform/methanol; 7:1, v/v
2. Chloroform:acetonitrile; 7:3, v/v
3. Ethylacetate/2 propanol; 95:5, v/v
4. Methylene chloride/tetrahydrofuran; 6:2, v/v
5. Methylene chloride/methanol/dimethyl formamicle; 90:9:1, v/v/v.

The presence of taxol was detected with 1% w/v vanillin/sulphuric acid reagent after gentle heating (Cardellina, 1991). Later it appears as a bluish spot of fading to dark gray after 24 h. The RF values of the samples were calculated and compared with the authentic taxol.

High Performance Liquid Chromatography (HPLC)
Taxol was analyzed by HPLC (Shimatzu 94 model) using a reverse phase C18 column with a UV detector. A C18 column chromatography was used for determining the behaviour of the fungal compound by HPLC. A 20 mL of sample was injected each time and detected at 232 nm. The mobile phase was methanol/acetonitride/water (25:35:40, v/v/v) at 1.0 mL min^{-1}.

Ultraviolet (UV) spectroscopic analysis
After chromatography, the area of plate containing putative taxol was carefully removed by scrapping off the silica at the appropriate R_f and exhaustively absorption dissolved in 100% methanol at 272 nm (Wani *et al*., 1971) in a Beckman DU-40 spectrophotometer and eluting it with methanol. The purified sample of taxol was analyzed by UV absorption dissolved in 100% methanol at 272 nm in a Beckman DU-40 spectrophotometer and compared with authentic taxol (Paclitaxel, Sigma grade).

Morphological identification
Morphological identification of endophytic fungal strain was based on the morphology of the fungal colony or hyphae, the characteristics of the spores, and reproductive structures (Wei and Xu, 2007; Carmichael *et al.,* 1982; Barnett and Hunter, 1998). The identities of some major groups were subsequently verified with molecular methods. For inducing sporulation, each of isolated fungal strains was separately inoculated on PDA in petri dishes. Measurements of all fungal characters were made in water mounts and the slides were subsequently mounted in lactophenol and sealed with nail vanish. All experiments and observations were repeated at least twice. Those cultures which failed to sporulate were named as mycelia sterilia, and divided into different morphospecies according to their cultural characteristics.

Molecular identification
Test strain was grown in yeast peptone sucrose broth (Sette *et al*., 2006). Graham *et al.* (1994) method is quick and easy isolation method of genomic DNA from fungal mat. The isolates were grown in liquid shake culture (130 rpm) in PD broth media for 7 days at 25℃. Mycelia were collected from the cultures by filtration and transferred to sterile plastic tubes. These samples were frozen at −70℃ for minimum 1 h and then freeze-dried and sto red in hygrometer. Mycelia were ground in 1.5 Eppendorf tubes. Extraction buffer 200 mM Tris–HCl (pH 8.0), 200 mM NaCl, 30 mM EDTA, 0.5% SDS and proteinase K were added to each tube and incubated at 37℃ for 1 h. Samples were extracted with 2×CTAB solution (2% CTAB (w/v), 100 mM Tris–HCL (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% PVP (Polyvinylpyrrolidone) and chloroform: isoamyl alcohol (24:1), followed by centrifugation (10 min). Genomic DNA was washed with 70% ethanol, dried and re-suspended in 50 µL sterile distilled water. RNase enzyme added to each sample to remove RNA, followed by incubation at 37℃ for 2 h. The amount of DNA was examined by electrophoresis through 0.7% agarose gel in TBE buffer stained with ethidium bromide and visualized with UV light. DNA was stored at 4℃ until used.

DNA of the fungal sample was amplified by polymerase chain reaction (PCR) using 18S rRNA, ITS 1, 5.8S rRNA, ITS 2 and 28S rRNA. Partial and complete gene marker was carried out based on the methodology of Lacap and Hyde (2003) in Thermocycler (PTC-100 TM Programmable Thermal Controller, USA). Amplification reactions was performed using primers ITS1 (5´-TCC GTA GGT GAA CCT TGC GG-3´) and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) in a total volume of 50 µL containing 10× buffer; 5 µL, 10x BSA buffer; 5 µL, Dntp; 5 µL, 1 µL of each primer, 0.3 µL of Taq polymerase, 1 µL of genomic DNA and 31.7 µL of distilled water. PCR amplification was carried out in Eppendorf Mastercycler (5330) for 30 cycles of 94℃ for 1 min denaturing, 55℃ for 1 min annealing and 72℃ for 1.30 min extension. Initial denaturing at 94℃ was extended to 5 min and the final extension was for 10 min at 72℃. The Amplified product was separated by electrophoresis in gels containing 1.0% agarose. The electrophoresis was run in 0.5×TBE buffer and the amplification products were visualized by ethidium bromide staining under UV light. The lengths of the amplification products were estimated by comparing to 1500 bp DNA ladder. The PCR products were purified using the wizard PCR prep. DNA. The gene sequencing was carried out using ABI 3130 Genetic Analyzer. The amplified products were cleaned up using QIAQuick (Qiagen) Spin column. The cycle sequencing was carried out using Big Dye Terminator version 3.1 Cycle sequencing kit. The PCR product was sequenced bi-directionally using the forward, reverse primers. The unbound dye was removed and nucleotide from cycle sequenced product was carried out using DyeEx spin columns (Qiagen). The purified samples were sequenced by ABI 3130 Genetic Analyzer. Sequence similarity searches were performed for each fungal sequence against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST–Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov). The ITS1 5.8S-ITS2 sequences of fungal strains were aligned with the sequences of available nucleotides data in NCBI. The sequence data have been submitted and deposited in NCBI gene bank (Accession number JN400594). We named the isolated strain NMK6. Phylogenetic analysis was conducted based on both the ITS and 5.8S gene data using neighbor joining (NJ) approaches. In the present study a phylogenetic tree was constructed using BLAST tree tool.

Results and discussion

Michelia champaca is a member of family *Magnoliaceae*. It is well-known and widely used in traditional medicine for fever, colic, leprosy, post partumprotection (Perry, 1980) and many more. This plant was claimed to possess various pharmacological properties such as

antipyretic, anti-inflammatory, insecticidal, antimicrobial and etc (Vimala *et al.,* 1997; Li *et al.,* 2007). Furthermore, Atjanasupp-at *et al*. (2009) reported that this plant can be used as a remedy of antiuretic, carminative and antidinic. Several compounds of this plant were also characterized and identified such as alkaloids, saponins, tannins, sterols, flavonoids and triterpenoids (Khan *et al.,* 2002). In the present investigation the endophytic fungus is isolated from the leaf part of medicinal plant *Michelia champaca* (Fig. 1). The isolated endophytic fungus is screened for taxol production in M1D medium. The extract of fungal culture was examined for the presence of taxol by chromatographic and spectroscopic analysis.

Fig. 2. Photographic representation of TLC.

Fig. 3. Photographic representation of HPLC.

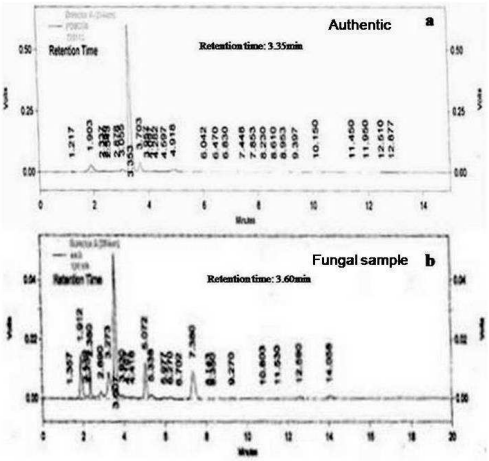
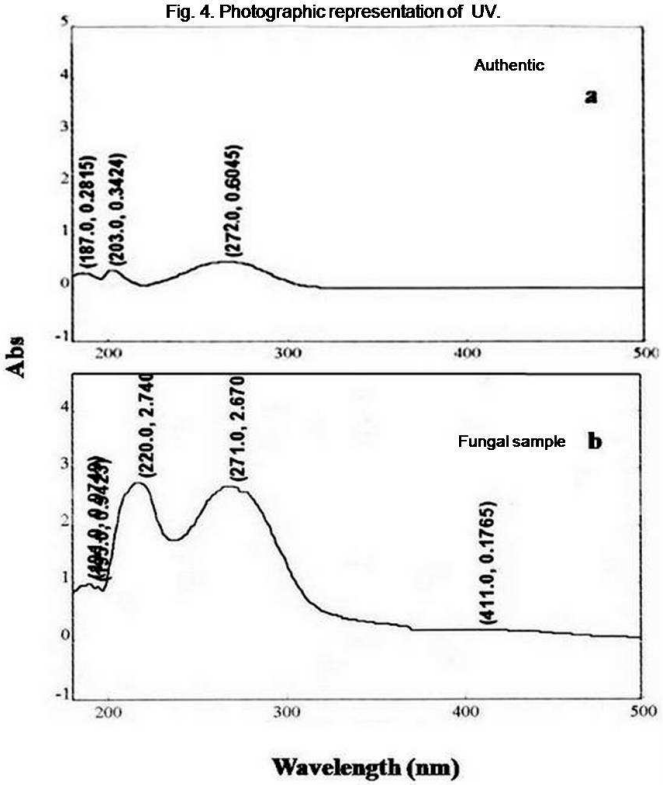


Fig. 4. Photographic representation of UV.



Thin layer Chromatographic analyses were carried out on Merck 0.25 mm silica gel plates developed in different solvent system. Taxol was detected using a spray of 1% Vanillin (w/v) in sulphuric acid after gentle heating. The presence of taxol in the fungal extract was confirmed by the appearance of a bluish spot fading to dark gray after 24 h. The compound has chromatographic properties identical to authentic taxol in solvent systems. They had RF values identical to that of standard taxol. Therefore, it was evident that this fungus showed positive results for taxol production (Fig. 2). Presence of taxol was further confirmed by HPLC analysis. The fungal extracts gave a peak when eluting from a reverse phase C18 column, with about the similar retention time as standard taxol. The quantity of taxol produced by fungi was calculated based on the area of the sample peak, concentration and peak area of authentic taxol (Fig. 3). The test fungus recorded about 77.23 µg/L of taxol in the liquid culture. The presence of taxol in the fungal extract was further confirmed by UV Spectroscopy. The UV spectral analysis of the fungal extract was examined and the spectrum was superimposed on that of the authentic taxol at 272 nm (Fig. 4).

The microscopical and cultural characteristics of the concerned *Chaetomium* strain fit the identity of *Chaetomium* but to confirm, molecular identification is must for species level determination. In addition to the morphological characterization, molecular methods were carried out to confirm the identification of fungus strain isolated from *Michelia champaca*. The ITS1-5.8S-ITS2 sequences of these strain was compared to fungal sequences in the database. Taxol is known to be produced by a number of endophytic fungi, including the following species or genera from the current literature (Zhao et al., 2008b): *Taxomycesandreanae*, *Taxomyces* sp., *Trichoderma*, *Tubercularia* sp., *Monochaetia* sp., *Fusarium lateritium*, *Pestalotiopsis microspora*, *Pestalotiopsis guepinii*, *Pithomyces* sp., *Pestalotiabicilia*, *Papulaspora* sp.1, *Pseudomonas aureofaciens*, *Pleurocytospora taxi*, *Cephalosporium* spp., *Chaetomium* sp., *Martensiomyces* spp., *Mycelia sterilia*, *Nodulisporiumm sylviforme*, *Rhizoctonia* sp., *Penicillium*, *Alternaria* sp., *Alternaria taxi*, *Ectostroma* sp.1, *Botrytis* sp.1, and *Botrytis taxi* (Zhao *et al.,* 2008a).

The Taxol producing endophytic fungus, was ascertained its systematic position based on 18S rRNA, ITS 1, 5.8S rRNA, ITS 2, 28S rRNA, partial and complete sequence analysis and with the aid of computational programme, BLAST homology analysis was also carried out to compare with other 18S rRNA, ITS 1, 5.8S rRNA, ITS 2, 28S rRNA, partial and complete sequences available in the GenBank of NCBI and it revealed that the fungi sequence of *Chaetomium* sp. strain NMK6 shows the maximum homology (100%) with other *Chaetomium* sp. strains from nucleotide database of NCBI. Therefore, our present study reports the isolation, characterization and identification of a new variant of the *Chaetomium* sp. strain NMK6 from *Michelia champaca* L.

Conclusion

An endophytic *Chaetomium* sp. isolated from *Michelia champaca* L. was investigated for taxol production. The presence of taxol in the fungal extract was confirmed by TLC, HPLC and UV analysis. Nucleotide sequences of *Chaetomium* sp. NMK6 showed maximum homology (100%) with other *Chaetomium* sp. from nucleotide database of NCBI. The findings on taxol production may be further exploited after careful investigations in future to develop it as an effective anti-cancerous drug.

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