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RESEARCH ARTICLE

Isolation and Characterization of Taxol Producing Endophytic *Phoma* sp. from *Calotropis gigantea* and its Anti-proliferative Studies

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Abstract
The fungal population in the leaves of the medicinal plant, *Calotropis gigantea* was enumerated and screened for taxol production. In the quantitative screening of taxol production process, the efficient taxol-producing fungal strain was isolated and identified as *Phoma* sp. using Internal transcribed spacer (ITS) region analysis. The amount of taxol produced by the *Phoma* sp. was estimated quantitatively and was found to be 76.13 µg. The endophytic fungus was cultured artificially for producing taxol. The presence of taxol in the fungal extract was authenticated using chromatographic and spectroscopic techniques with reference to commercial taxol. The taxol extracted from the fungal culture showed strong anti-proliferative activity against MCF 7 cell lines *in vitro*. The findings evidenced that the endophytic fungus *Phoma* sp. isolated from *Calotropis gigantea* can act as a potential candidate for taxol production laying a foundation for further research heading towards the scale-up studies related to taxol production.

Keywords: *Calotropis gigantea*, endopytic fungi, taxol, *Phoma* sp., MCF 7, ITS region.

Introduction
Taxol is an anti-cancer drug used for the treatment of various types of cancers including breast, ovarian, lung cancer, etc. (Rowinsky *et al*., 1990; Croom, 1995). Taxol promotes the stabilization of microtubules at G2 to M phase of cell cycle thereby inhibiting the cell proliferation (Walker and Croteau, 2001; Sambantham *et al*., 2013). At first, taxol was commercially extracted from the barks of the yew tree, *Taxus brevifolia* (Wani *et al*., 1971). These conventional methods possess various disadvantages such as slow growth of the source tree and cause of severe environmental issues (Strobel *et al*., 1996). As an alternative technique, plant cell culture-based taxol production was utilized to facilitate taxol production. However, due to various technical reasons subjecting to low yield, the plant cell culture has not been established as a successful technique for the production of commercial taxol, 'Paclitaxel'. In order to meet the increasing demand for taxol, an alternative source to produce taxol in large quantities became essential. Owing to its demand, many researchers in the past decades used the endophytic fungi as the potential source for taxol production (Gangadevi and Muthumary, 2009; Kathiravan and Vithiyanathan, 2010). Endophytic fungi are ubiquitous, ecologically specialized group growing on the aerial parts of plant tissues such as stems, leaves, etc. and are believed to be neutral i.e. neither harmful nor beneficial to the host plant. Various studies were evidenced that these endophytes protect the host plant against various pathogens and predators (Azevedo *et al*., 2000). Most of the endophytic candidates were evidentially proven to produce a variety of secondary metabolites with medical importance including taxol. It is true that majority of the fungal distribution remains unexplored and needs to be explored for further medical applications. *Calotropis gigantea* or milk-weed is one among such plants, widely distributed in India, which is used as a traditional medicinal plant (Kumar and Roy, 2007). This plant is grown as a shrub with milky stem possessing with various medicinal properties and used for the treatment of elephantiasis, leprosy, skin diseases and ulcer. Against these backdrops, the present study was attempted to screen the taxol-producing ability of the endophytic fungus, *Phoma* sp. isolated from the leaves of *Calotropis gigantea*. The produced taxol was quantitatively estimated, characterised, and its anti-proliferative activity was determined towards human liver carcinoma cell line (MCF 7) (Zhang *et al*., 2014).

Materials and methods
Plant collection and isolation of endophytic fungi: For the present investigation, leaf samples of *Calotropis gigantea* were randomly collected from Chennai, TN, India and surface-sterilized through sequential washing with ethanol and sodium hypochlorite; later rinsed with sterile distilled water and dried on a sterile surface. With the help of sterile blade, the leaves were cut into small pieces (appropriate dia of 0.5 cm). The sliced leaf segments were then placed on potato dextrose agar (PDA) supplemented with chloramphenicol and the plates were incubated for two weeks at 25°C to observe

the emergence of hyphae from the leaf segments. The hyphal tips of the endophytic fungi grown on PDA plates were sub-cultured on PDA without antibiotics for further purification. Each pure fungal isolate was maintained on PDA plates and slants at 4ºC.

Screening for taxol: The isolated endophytic fungal strains were inoculated in Erlenmeyer flasks containing 500 mL of modified liquid medium (MID) medium supplemented with 1 g soytone. The flasks were then incubated in shaker for another 21 d. After incubation, the fungal mycelium was removed from the medium by filtering through four-layered cheese cloth. To the culture filtrate, 0.25 g of sodium carbonate was added under shaken condition to reduce the amount of fatty acids so as to maintain the purity of taxol. The culture filtrate was then extracted twice with equal volumes of dichloromethane solvent. After extraction, the solvent was removed by evaporation under reduced pressure at 35°C using rotary vacuum evaporator and the organic phase was collected as dry solid residue. The residue was re-dissolved in methanol and was examined for the presence of taxol.

Characterization: Presence of taxol in the culture filtrate was confirmed applying Thin Layer Chromatography, High Performance Liquid Chromatography, UV-Visible Spectroscopy and Infra-Red Spectroscopy and with the help of standard taxol. From the obtained results, the best taxol-producing fungal strain was selected and subjected to identification.

Molecular identification of fungi: Fungal strains showing better taxol production were identified using 18 rRNA ITS gene sequence. The genomic DNA was isolated from fungal mycelium and was amplified using PCR with the primers ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplified PCR product was with the help of automated sequencer (Chromous Biotech, Chennai). Prior to identifying the fungal strain, the sequence similarity search was performed by analyzing the sequence using online search tool BLAST. The unknown fungal strain was identified using the organism showing maximum alignment via BLAST search.

Anti-proliferative activity: The anti-proliferative activity of the taxol extracted from the experimental fungus was examined by the MTT assay against MCF-7 (human breast cancer cell line). The MCF-7 cell line was procured from the National Centre for Cell Sciences (NCCS), India and the cells were seeded in a 96 microwell plate. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and incubated for 24 h at 37°C. After incubation, the cells were washed with Phosphate buffered saline (PBS) and added with serum free medium (SFM).

Two sets of cells were maintained such as control and test, wherein control set contains SFM alone and test set was treated with SFM and different concentration of fungal taxol (10-100 µg/mL). After 72 h incubation, 100 µL of MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h. The wells were then added with 100 µL of 20% SDS dissolved in 50% dimethyl formamide (DMF) and the rate of absorbance was read at 650 nm for each well using micro plate reader. The inhibition rate was calculated using the formula:

$$\text{Growth inhibition} = \frac{\text{A650/nm of the cells after test}}{\text{A650/nm of the control cells}} \times 100\%$$

Results and discussion

The role of medicinal plants is invincible due to their applications in the drug discovery process for various diseases. In addition, recent development of new technologies offers various novel opportunities for the discovery and implementation of drug molecules from natural sources. This can be achieved by series of steps including product sourcing, focusing reliable source, targeting and utilization of microorganism as the renewable source of drug molecules for potential pharmaceutical applications (Wan *et al*., 2013). As a preliminary attempt, in the present study, taxol-producing endophytic fungi was isolated from leaves of *Calotrophis gigantea* and the anti-proliferative activity of the isolated fungi was tested against MCF-7 cell lines.

The healthy leaves were cleaned and enumerated for the fungal diversity in PDA plates. From the leaf samples, about 9 different fungal strains were isolated. The fungal strains were distinguished based on their colony and spore morphology under microscopic examination. The strains were purified by sub-culturing in fresh PDA plates and maintained for further analysis. The spore morphology revealed that the fungal strains belonged to the species *Aspergillus, Phoma* and *Penicillium*. Although different genera of fungi were found, *Aspergillus* and *Phoma* were found to be predominant. From the previous reports, it has been evidenced that the isolated genera of fungi were generally epiphytic and known to grow endophytically (Schulthess and Faeth, 1998). In a similar work involving biodiversity studies on endophytic fungi from *Calotrophis gigantea*, Srimathi *et al*. (2011) found that the plant was a rich source of fungal population and the predominant fungal species grown included *Aspergillus, Fusarium* and *Phoma* sp. In order to screen the ability of the isolated fungal strains in producing taxol, the strains were inoculated into MID medium supplemented with 1 g soytone and allowed to grow under shaken condition at room temperature. The culture medium after fungal growth was filtered using cheese cloth and extracted with the help of dichloromethane solvent. The culture filtrate was further screened and authenticated for the presence of taxol. The main problem in the usage of fungi in fermentation was the production of unstable taxol with low-level yield.

Fig. 1. Thin layer chromatography of the isolated taxol.

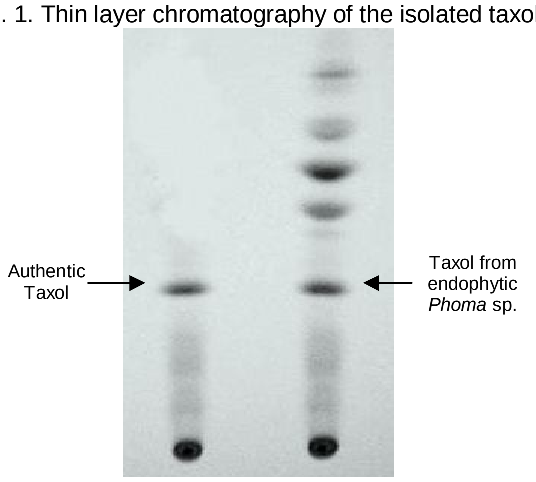
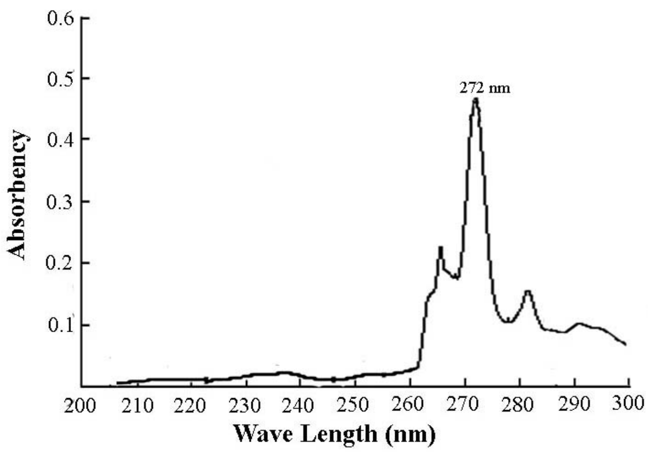


Fig. 2. UV-Visible spectrum of taxol from endophytic *Phoma* sp.



However, high growth rate and short generation time of the fungi helped the researchers to continue the investigation on taxol production (Kumaran *et al*., 2008; 2011). The partially purified taxol was analyzed using thin layer chromatography (Fig. 1) and was visualized under UV illumination at 235 nm (Fig. 2). After running, the presence of taxol in the plate was detected by adding 1% vanillin in sulfuric acid to the culture filtrate. The appearance of bluish spot in the beginning and later changing to dark grey in the culture filtrate is the indication for the presence of taxol. The Rf value of the partially purified taxol was found to be identical with that of the standard authentic taxol (0.25). The presence of taxol was further authenticated using HPLC (Fig. 3a and 3b) and FTIR (Fig. 4a and 4b). The formation of peak corresponding to the retention time of 2.8 min similar to the standard taxol indicates the presence of taxol in the sample. The quantitative estimation reveals that from 500 mL of the MID medium, the amount of taxol produced by the fungi equals 76.13 µg. The amount of taxol produced by means of experimental fungi was found to be higher than that of previously obtained reports of endophytic fungi (Stierle *et al*., 1993; Strubel *et al*., 1996; Gangadevi and Muthumary, 2009).

Fig. 3a. HPLC profile of standard taxol.

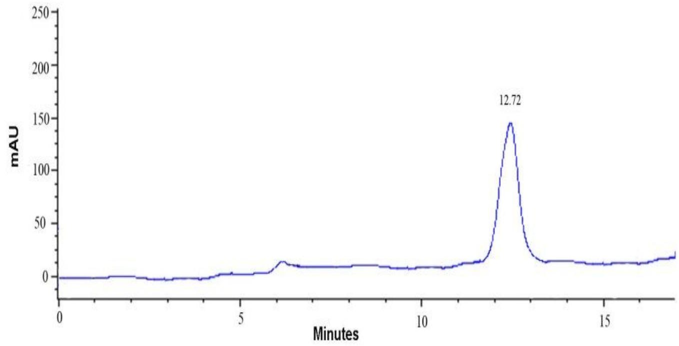


Fig. 3b. HPLC profile of taxol from *Phoma* sp.

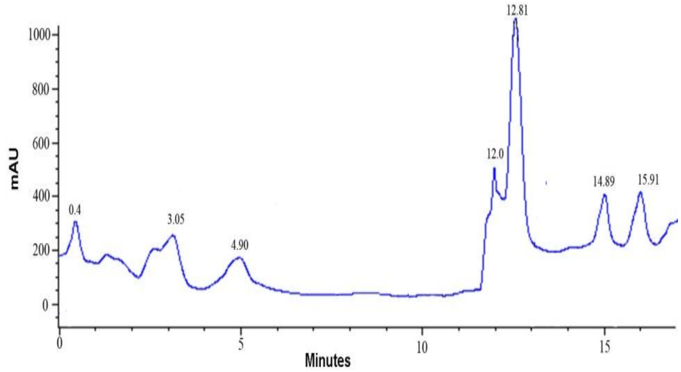


Fig. 4a. FTIR profile of standard taxol.

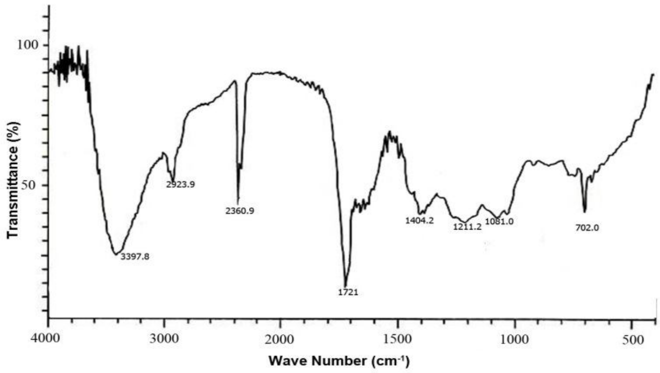


Fig. 4b. FTIR profile of taxol taxol from *Phoma* sp.

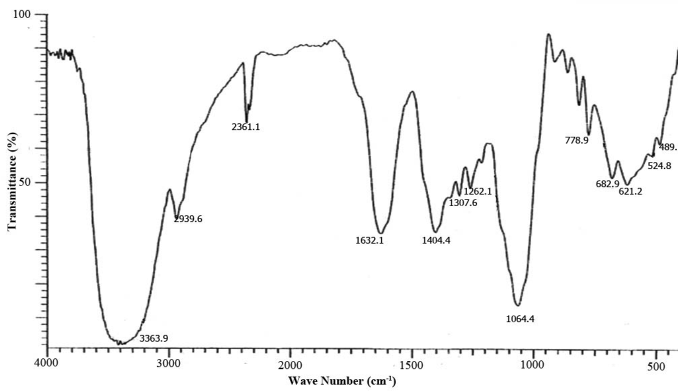


Fig. 5. Amplified 28S ITS region electrophoresed on agarose gel.

The UV absorption spectrum of the partially purified taxol shows a maximum absorption at 235 nm similar to the authentic taxol. The similar IR bands also indicate identical chemical property of both the authentic and extracted taxol. The isolated endophytic fungal strain CTP 05 showing better production of taxol was characterized morphologically and further authenticated by ITS region analysis. The size of the 28S ITS region was determined by running the amplified gene on agarose gel electrophoresis with DNA ladder. The size of the amplified 28S ITS region was found to be 0.5 kb (Fig. 5). The 28S ITS region of the fungi was sequenced and compared with the similar sequences available in Genbank using BLASTn search. The obtained result revealed that the ITS sequence of the isolated fungal strain showed maximum similarity (100%) with the available *Phoma* sp. Hence, the isolate was identified and authenticated as *Phoma* sp. previously reported by many researchers as endophytic fungal species. The 28S ITS sequence of *Phoma* sp. strain CTP 05 was then published online in Genbank with the accession number KP317204.

The anti-proliferative activity of taxol extracted from endophytic *Phoma* sp. was analyzed using MTT assay. The effect of extracted taxol on the viability of MCF-7 cell lines was tested at different time intervals. The concentration of the taxol was found to vary between 100-1000 µg/mL. Fungal taxol concentration of 500 µg/mL showed 50% cell viability in 48 h of incubation and hence, the calculated IC_{50} value was 50 µg/mL. From the findings, it has been revealed that as the concentration of fungal taxol increases, there will be increase in cell death due to apoptosis. The results found were in concurrence with the results obtained in previous reports involving standard taxol (Ruckdeschel *et al*., 1997; Gangadevi and Muthumary, 2007). The rate of cell death might differ due to various factors including, taxol concentration, incubation time, cell specificity, etc.(Yeung *et al*., 1999; Kumaran *et al*., 2011; Zhang *et al*., 2014).

Conclusion

From this study, it is revealed that identical results exist between the fungal taxol and commercial taxol in all the respective spectroscopic, chromatographic and cytotoxic estimates. It is also found that the taxol produced by the fungus *Phoma* sp. CTP 05 was found to be promising and can serve as a potential source for further scale-up process by increasing taxol production.

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