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RESEARCH ARTICLE

Taxol production by endophytic *Fusarium solani* LCPANCF01 from *Tylophora indica*

J. Nomila Merlin[1], I.V.S. Nimal Christhudas[2], P. Praveen Kumar[2], M. Kumar[3] and P. Agastian[2]
[1]Dept.of Biotechnology, Bharathiar University, Coimbatore-641046, TN, India
[2]Dept. of Plant Biology and Biotechnology, Loyola College, Chennai-600034, TN, India
[3]Dept. of Plant Biology and Plant Biotechnology, Madras Christian College, Tambaram, Chennai-600059, TN, India
agastian@loyolacollege.edu; +91 9444433117

Abstract

Twenty five endophytic fungal isolates were obtained from the roots of *Tylophora indica* (Burm. f) and screened for the presence of an anticancer drug, taxol. Among the 25 isolates, *Fusarium solani* LCPANCF01 was identified based on the micro morphology, cultural characteristics and sequence analysis using internal transcribed spacer (ITS1 and ITS4). *Fusarium solani* LCPANCF01 strain was grown in M1D liquid medium for 21 d and extracted with dichloromethane. The presence of taxol was confirmed by TLC, HPLC, UV, IR, and ESI-MS spectroscopy analysis by comparing with the standard drug.

Keywords: *Tylophora indica, Fusarium solani,* dichloromethane, taxol, anticancer drug.

Introduction

Taxol is isolated from the barks of pacific yew tree (*Taxus brevifolia*) (George *et al*., 1994; Dewick, 2009) and the most important antimitotic agent which is active against lung, ovarian, breast, head-neck cancer and advanced forms of Kaposi's sarcoma. Taxol inhibits cell proliferation by binding to the β-subunit of the tubulin heterodimers, thus promoting its polymerization (Kovacs *et al*., 2007). However, a complete course of treatment per patient may requires 2 g of taxol administered several times over many months. To obtain 1 kg of taxol, it requires 3000 yew trees (10,000 kg of bark) and current demand of taxol is 250 kg per annum (Dewick, 2009). This insufficiency of taxol and environmental issue of harvesting from trees demands researcher to discover the alternate technique for the production of taxol.

Taxol is now obtained by several methods, e.g. total chemical synthesis (Nicolaou *et al*., 1994), semi-synthesis from its precursor (Commercn *et al*., 1995), plant tissue or cell culture (Hu *et al*., 2003). Endophytic fungus from various taxus species produces taxol although the amounts are very small. First taxol producing endophytic fungi *T. andreanae* is from *Taxus brevifolia* (Stierle *et al*., 1993). Since then, many other endophytic fungi from other plant species have been successfully reported to produce taxol (Strobel *et al*., 1996; Li *et al*., 1996). *Tylophora indica* (Burm. f) (Asclepiadaceae) is a climbing perennial plant that grows in India, commonly called as antmool in Ayurveda. The leaves of *Tylophora* have been traditionally used as a folk medicine. It has been used for respiratory problems such as asthma, allergies, bronchitis and common cold. The roots and leaves contain 0.2 to 0.46% of therapeutically important alkaloids namely tylophorine, tylophorinine and tylophrinidine.

Major alkaloid tylophorine has immunosuppressive, anti-inflammatory (Gopalakrishnan *et al*., 1979), anti-tumor (Donaldson *et al*., 1968) and anti-amoebic (Bhutani *et al*., 1987) properties. Since, *T. indica* is an endemic plant an attempt is made to conserve the medicinal plant through exploration of endophytic fungi and screened for its taxol production.

Materials and methods

Plant collection: Tylophora indica fresh plants were collected from Kerala Forest Research Institute (KFRI), Trissur, Kerala, South India during the month of January 2012 (Fig. 1). The taxonomical identity of the plant was confirmed by Dr. D. Narashiman, Department of Botany, Madras Christian College, Tambaram, South India.

Fig 1. *Tylophora indica.*

Isolation and Identification of endophytic fungi: The fungus used in this study is one of the 25 endophytic fungi isolated from the root/transition zone of medicinal plants in *T. indica*. The root/transition zone samples were surface sterilized by the modified method of Dobranic *et al*. (1995). The root/transition were thoroughly washed in running tap water and small pieces of approximately 0.5 to 1 cm diameter were cut and then the pieces were surface sterilized by immersion in 70% ethanol for 5 sec, followed by 4% sodium hypochlorite for 90 sec and then rinsed in sterile distilled water for 10 sec. The excess moisture was blotted in a sterile filter paper. The surface sterilized samples was aseptically dissected to expose cortex region and plated onto petri dishes (9 cm dia) containing potato dextrose agar (PDA) medium (amended with chloramphenicol 150 mg L^{-1}). The petri dishes were sealed using Parafilm TM and incubated at 26 ± 1°C in a light chamber with 12 h light/ dark cycles. The petri dishes were monitored every day to check the growth of endophytic fungal colonies from the root segments. The hyphal tips, which grew out from sample segments were isolated and sub-cultured onto PDA and brought into pure culture. The isolated endophytic fungi were identified based on colony character and morphology of spore.

Molecular identification: The total genomic DNA was extracted using CTAB-Method. The endophytic fungal DNA fragments were amplified using Universal primers ITS1F, ITS4R and the PCR reactions were standardised as initial denaturation at 94°C for 4 min, followed by 32 cycle of 4 min at 94°C, 50°C for 1 min, 72°C for 2 min and a final extension at 72°C for 8-10 min. The recation was stopped at 4°C for 1 h. The PCR products were stored at 4°C and visualized by DNA gel electrophoresis. The amplified product was purified and sequenced with primers, ITS1F (5` AGT TTG ATC CTG GCT CAG 3`) and ITS4R (5` ACG GCT ACC TTG TTA CGA CTT 3`) and the sequences obtained were submitted to GenBank for homology search with Blast. The immediate concern is to find one or more fungi that produce more taxol. An endophytic fungus *Fusarium solani* LCPANCF01 strain was identified and tested for taxol production.

Preparation of fungal extracts: The test isolate was grown in 2 L Erlenmeyer flasks containing 500 mL of M1D medium supplemented with soytone (Pinkerton and Strobel, 1976) and incubated for 21 d. After 3 weeks of still culture at 26°C, the culture fluid was passed through 4 layers of cheese cloth to remove solids and extracted with organic solvent. The extraction and isolation procedure was followed according to Strobel *et al*. (1996). After methylene chloride extraction, the organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 35°C using rotary vacuum evaporator. The dry solid residue was redissolved in methanol for the subsequent separation and extracts were analyzed by chromatographic separation and spectroscopic analyses, confirmed with standard taxol (Paclitaxel) purchased from Sigma, USA.

Thin layer chromatography analysis: TLC analysis was carried out on Merck 1 mm (20 × 20 cm) silica gel plate developed in solvent A (chloroform : methanol, 7:1 v/v) followed by solvent B (chloroform : acetonitrile, 7:3 v/v), solvent C (Ethyl acetate: 2-propanol, 95:5, v/v), solvent D (methylene chloride: tetrahydrofuran, 6:2 v/v) and solvent E (methylene chloride:methanol : dimethylformamide, 90:9:1, v/v/v) respectively. The area of the plate containing putative taxol was carefully removed by scraping off the silica at the appropriate R_f and eluted with acetonitrile. Taxol was detected with 1% w/v vanillin/sulphuric acid reagent after gentle heating. It appeared as a bluish spot that faded to dark grey after 24 h (Cardellina *et al*., 1991).

High performance liquid chromatography: Further confirmation for the presence of taxol was performed by HPLC. The separation was eluting from a RP-C18 (4.6 x 150 mm, 5 µm) reverse phase column with ultraviolet detector. The sample (20 µL) was injected each time detected at 270 nm. The mobile phase was methanol/acetonitrile/water (25:35:40, by v/v/v) at 1.0 mL min^{-1}. The sample and mobile phase were filtered through 0.2 µm PVDF filter before entering the column. Taxol was quantified using the formula: $M=M_o x V1 x 10^6/V2$. M(µgL^{-1})-taxol content in fermentation liquid, M_o (mg/mL)-taxol content of methanol solution; V1 (mL)-volume of methanol used in redissolving of residues; V2 (mL)-volume of fermentation broth for extraction.

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 value and exhaustively eluting it with methanol. The purified sample of taxol was analysed by UV absorption, dissolved in 100% methanol at 273 λmax in a UV spectrophotometer (Hitachi) and compared with the authentic taxol.

IR spectroscopic analysis: The purified taxol was ground with IR quality potassium bromide (1:10), pressed into discs under vacuum using spectra lab pelletiser and the spectrum was recorded (4000-5000 cm^{-1} nm) in a Burker 17S 85 FTIR spectrophotometer.

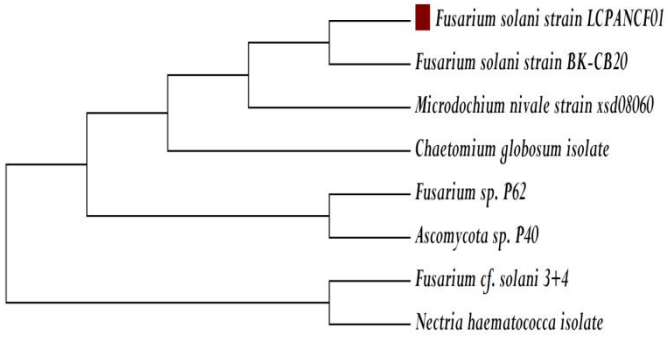
ESI-MS analysis: Taxol was identified by MS analysis using the Electro Spray Ionization (ESI) technique with an Agilent 1100 LC/ MSD trap. The nebulizer gas flow rate of the sample was 2 µL min^{-1} and the capillary voltage was 2.2 kV.

Results

Isolation and identification of the endophytic fungi: The strain LCPANCF01 was isolated from the root/transition zone of *Tylophora indica* (Fig. 2). The isolated endophyte typically possessed small hyphae, as a white colony mycelium, when young. The mycelia are thread-like, branched, septate and slow-growing; spores were cylindrical to oval resembled *Fusarium solani*.

Fig. 2. Strain LCPANCF01 isolated from *T. indica.*

Fig. 3. Phylogenetic tree of *Fusarium solani* LCPANCF01.

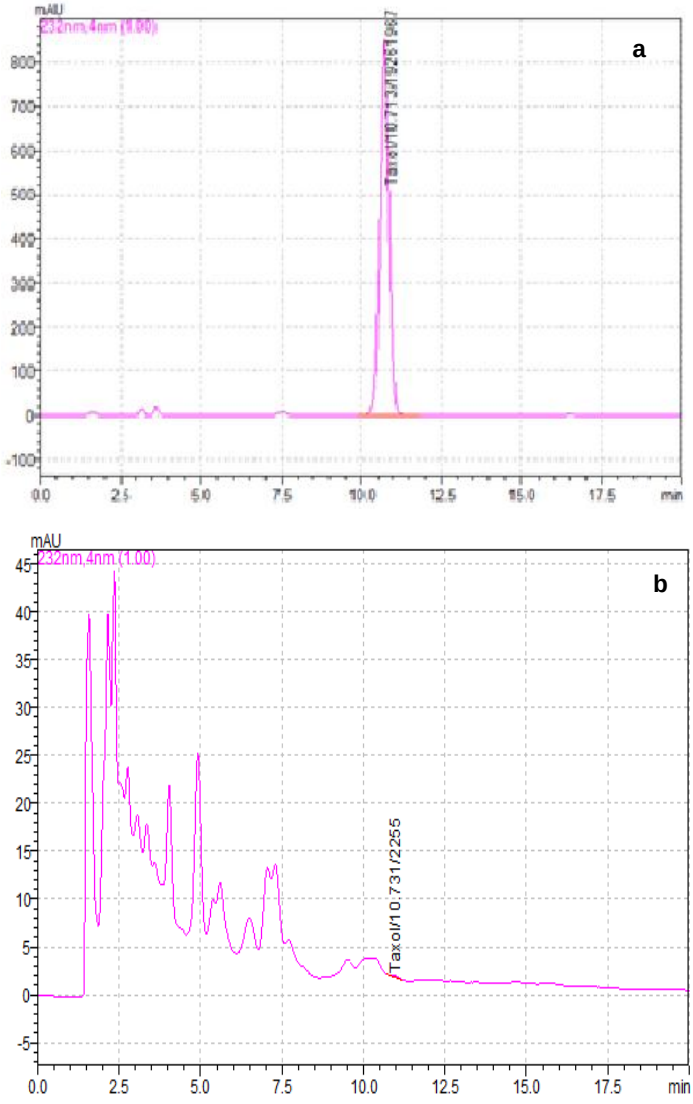


The ITS rDNA sequence data, whereby the closest match (99% similarity) in the NCBI Gen Bank database was found to be *Fusarium solani* LCPANCF01. The phylogenetic tree was obtained by applying the neighbor-joining method (Fig. 3). The ITS rDNA sequence of the strain has been deposited in the NCBI Gen Bank database (accession number-JN786598).

Screening of taxol: The extract of the test fungal culture was examined for the presence of taxol by chromatographic and spectroscopic analyses. Taxol, produced by the fungus was detected using a spray reagent consisting of 1% vanillin (w/v) in sulfuric acid after gentle heating; it appeared as a bluish spot fading to dark gray after 24 h. The compound has chromatographic properties identical to standard taxol in solvent systems A-E and gave colour reaction with the spray reagent and they had R_f value of 0.58 identical to that of standard taxol.

High pressure liquid chromatography: In HPLC analysis, the fungal extract gave a peak when eluting from a reverse phase C18 column, with a similar retention time as standard taxol (Fig. 4a and b). The amount of taxol produced by this fungus in liquid culture was 157.38 µg/L.

Fig. 4. HPLC analysis of authentic taxol; (a) Fungal taxol from *F. solani* LCPANCF01; (b) The mobile phase was methanol/acetonitrile/water (25:35:40, v/v/v), flow rate at 1.0 mL/min; retention time of authentic taxol: 10.71 min; retention time of fungal taxol: 10.73.



UV spectroscopic analysis: The UV spectral analysis of the fungal taxol is given in Fig. 5a and b, the spectrum was superimposed on that of authentic taxol.

IR spectroscopic and ESI MS analysis: The IR spectral data of fungal taxol from *Fusarium solani* LCPANCF01 showed broad peak in the region 3447.19 cm^{-1} was ascribed to hydroxyl (-OH) and amide (-NH) groups stretch. The aromatic ring (C=C) group stretch was observed in the region of 1632.15 cm^{-1}. A peak observed in the region 1065.73 cm^{-1} is due to the presence of aromatic C, H bends. The IR spectrum of DCM extract of the test isolate showed similar stretching frequency as that of authentic taxol (Fig. 6a and b). The ESI mass spectrum of fungal taxol isolated from *Fusarium solani* LCPANCF01 is given in Fig. 7a and b.

Fig. 5(a) UV spectra absorption of authentic taxol; (b) Fungal taxol from *F. solani* LCPANCF01.

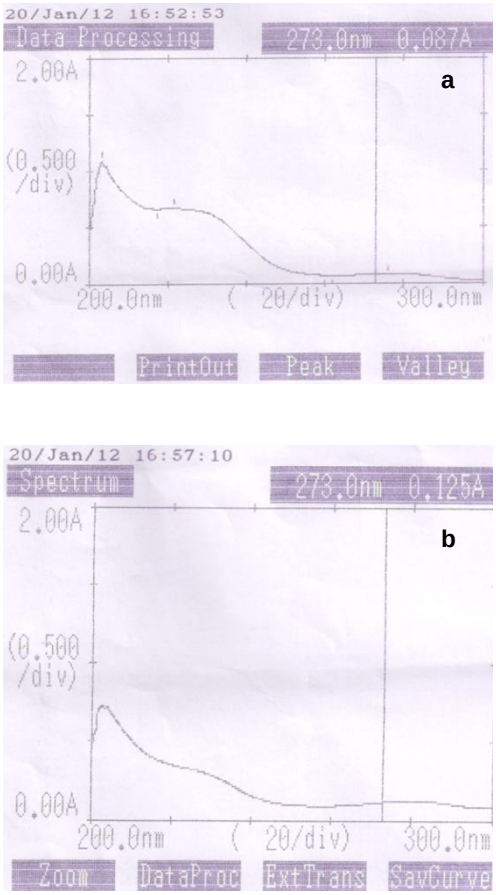


Fig. 6(a) IR spectral analysis of authentic taxol; (b) Fungal taxol from *F. solani* LCPANCF01.

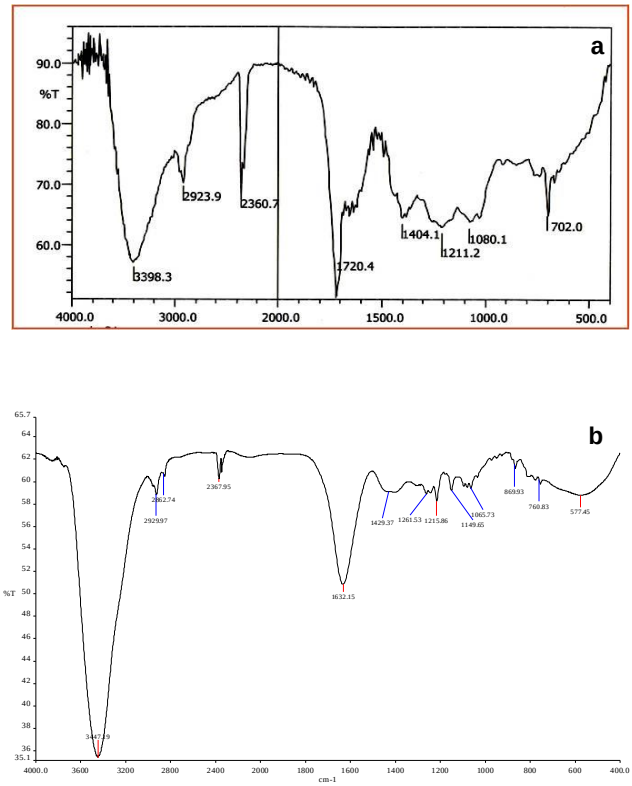
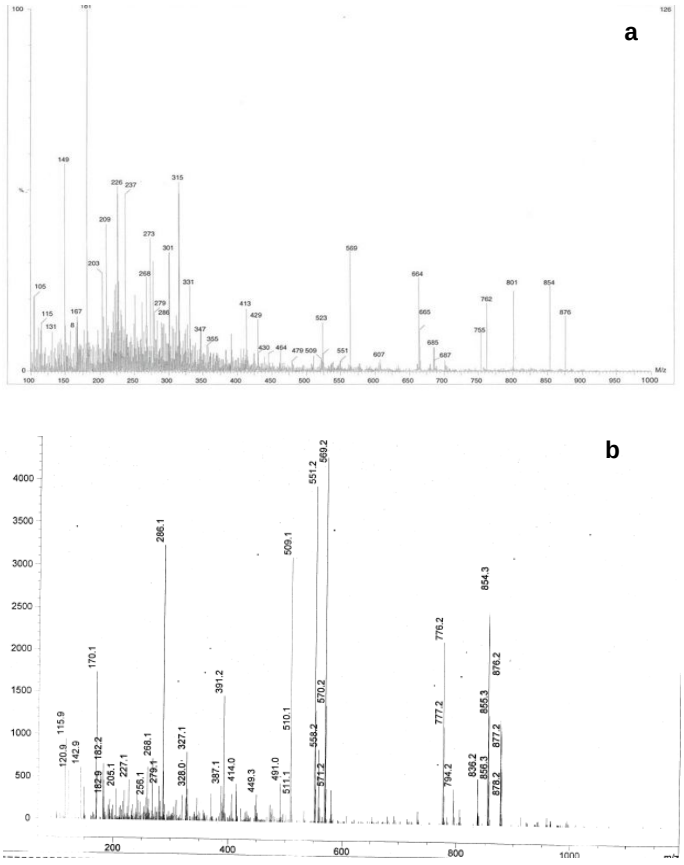


Fig. 7(a) ESI mass spectrum of authentic taxol; (b) Fungal taxol from *F. solani* LCPANCF01.



Discussion

The anticancer drugs which are available at present possess enormous side effects and are not effective against many forms of cancer. Taxol has a unique mode of action when compared with other anti-cancer compounds. The aim of this present study is to isolate and identify the taxol-producing endophytic fungi from *Tylophora indica*. Therefore, it was evident that this fungus showed positive results for taxol production. Taxol is positively identified via its co-chromatographic mobilities with authentic taxol in a multitude of thin layer chromatographic systems (Stierle *et al*., 1993; Strobel *et al*., 1996). The biggest problem of using fungi in fermentation was less production of taxol, its very low yield and unstable production. The taxol yield of such reported fungi varies from 24 ng to 70 ng per litre of culture medium (Stierle *et al*., 1993; Strobel *et al*., 1996). Although, the amount of taxol produced by most of the endophytic fungi associated with taxus trees is relatively small when compared with that of the trees, the short generation time and high growth rate of fungi make it worthwhile to continue our investigation of these species. In general, taxol represents 0.01%-0.02% of the weight of dry bark. The trace amount of compounds other than taxol may perturb the absorbance reading, giving weight estimates for taxol greater than the content which was actually present.

It was evident that the diterpenoid taxol was much more complex since its molecular weight from high-resolution mass spectrometry is $C_{47}H_{51}NO_{14}$, corresponding to a molecular weight of 853. Characteristically, standard taxol yielded $+H^+$ at m/z 854 and $+Na^+$ m/z at 876. By comparison, fungal taxol also yielded a peak $+H^+$ at m/z 854.3and $+Na^+$ m/z at 876.2 with characteristic fragment peaks at 569, 551, 509, 286 and 268. Major fragment ions observed in the mass spectrum of taxol can be placed into 3 categories which represents major portions of the molecule (McClure and Schram, 1992). The peaks corresponding to taxol, exhibited mass-to-charge (m/z) ratios corresponding to the molecular ions $(M+H)^+$ of standard taxol (854) confirming the presence of taxol in the fungal extracts.

Conclusion

As the taxol producing plant has become endangered, this endophyte can be used as an alternative source for taxol production. *Fusarium solani* LCPANCF01 produces taxol which is confirmed by TLC, HPLC, IR and Mass spectrum. The isolated fungi can be a good source of taxol for large scale production by pharmaceutical industries in near future.

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