American Journal of Bioscience and Bioengineering
2017; 5(6): 113-120
http://www.sciencepublishinggroup.com/j/bio
doi: 10.11648/j.bio.20170506.11
ISSN: 2328-5885 (Print); ISSN: 2328-5893 (Online)

Screening and Characterization of Fungal Taxol from Leaf Spot Fungi

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To cite this article:
Bose Chitra Kani, Senthuran Suresh Kumar, Mohan Pandi. Screening and Characterization of Fungal Taxol from Leaf Spot Fungi. *American Journal of Bioscience and Bioengineering*. Vol. 5, No. 6, 2017, pp. 113-120. doi: 10.11648/j.bio.20170506.11

Received: November 1, 2017; **Accepted**: November 9, 2017; **Published**: December 6, 2017

Abstract: Plants are the tremendous source for the discovery of new biotherapeutic products with medicinal importance in drug development. Paclitaxel (Taxol) is a plant-derived natural product that exhibits potential anticancer activity. Taxol binding specifically with β-tubulin and prevents depolymerisation during the process of cell division in cancer cells as anticancer property. This billion dollar drug was first isolated in trace amounts (1kg taxol recovered from 13,500kg of bark) from the bark of Yew tree (*Taxus brevifolia*), but huge drawback is production rate was low and shortage of yew trees. Currently, endophytic fungi and pathogenic fungi are the best alternative source for the taxol production. In this study, twenty-seven different plants were used for infected leaves collection from Madurai Kamaraj University (MKU) campus. Fungi were isolated from infected leaf spots on PDA plates using hyphal tip method. All the isolated fungi were subjected to pathogenicity tests, and positive pathogenic fungi were re-isolated. Totally eighteen fungal plant pathogens were isolated. The positive isolates identified using morphological and molecular characters. All the positive pathogens are screened preliminarily for taxol production. Among the eighteen isolates, three fungi were produced taxol. The test fungus *Phoma moricola*, isolated from *Bauhinia tomentosa* was the first report for Taxol production. The amount of taxol produced by *Phoma moricola* was quantified by HPLC. The maximum amount of taxol produced was found to be 302µg/L.

Keywords: Paclitaxel (Taxol), Leaf Spot Fungus, *Phoma moricola*

1. Introduction

Taxol is a diterpenoid antineoplastic drug widely used in the treatment of various cancers. In 1992, taxol was first approved a drug by food and Drug Administration (FDA). It inhibits the taxol mitotic cell division by preventing the depolymerization of tubulin during mitosis. Taxol was first isolated from the barks of pacific yew, *T. brevifolia* [1], to produce 1kg of taxol it requires 13,500 kg of host plant which results in deforestation and high cost due to their increased demand in a global market. To overcome these problems scientists searched for an alternative source for taxol production. Over the last 30 year, endophytic fungi isolated from the yews and the gymnosperm plant species were given sole attention for taxol production. An alternative approach was made to screen taxol from the leaf fungus *Phyllosticta discoreae* and *P. citricarpa* [2-3].

Leaf spot is a common descriptive term applied to a number of diseases affecting the foliage. The majority of leaf spots are caused by fungi, but some are caused by bacteria and virus. Leaf spots may result in some defoliation of the plant. Leaf spots initially start as small, water-soaked lesions that then turn various shades of yellow, gray, reddish-brown, brown, black etc., These lesions are usually surrounded by a halo or ring of tissue in a different colour. Disease manifestations have been noticed by the presence of Leaf spots, which can increase in number or size in large portions of leaf blade. The most common fungal leaf spot disease is caused by *Phyllosticta fraxinicola*. The fungus causes large, yellowish brown lesions with small black spots developed on the lower side of the infected leaves. Currently, 90,000 species of fungi described [4] 1,700 new species described

each year [5]. However very limited studies were reported for fungal taxol from leaf spot fungi, therefore more studies are needed in this direction.

2. Materials and Methods

2.1. Isolation of Leaf Spot Fungi

Totally 27 infected leaf samples were collected from different locations of Madurai Kamaraj University campus. The leaf samples were sealed and transferred to the laboratory in a sterile polythene bags. The infected leaves samples were washed with running tap water in order to remove dirt and debris. The infected region of leaf sample was cut approximately 0.5cm. The excised leaf segments were surface sterilized by sequential washes in 70% ethanol for 5 seconds, 4% sodium hypochlorite for 90 seconds and in sterile distilled water for 10 seconds. The sterilized leaf segments were spaced in petridishes containing sterile tissue paper to remove excess moisture under aseptic condition. Then the surface sterilized leaf segment was embedded in petridishes containing potato dextrose agar medium, with 50 mg/L streptomycin sulfate added to prevent bacterial growth. Then, the plates were incubated at 22±2°C with 12h light/12h dark cycle [5-6]. The petridishes were observed every day check for fungal growth from the leaf segments. Hyphal tips growing out the plated segments were immediately transferred in to new PDA plates. All the isolates were evaluated for their Pathogenicity (Figure-1). The isolates producing leaf spot on the leaves were selected for further study.

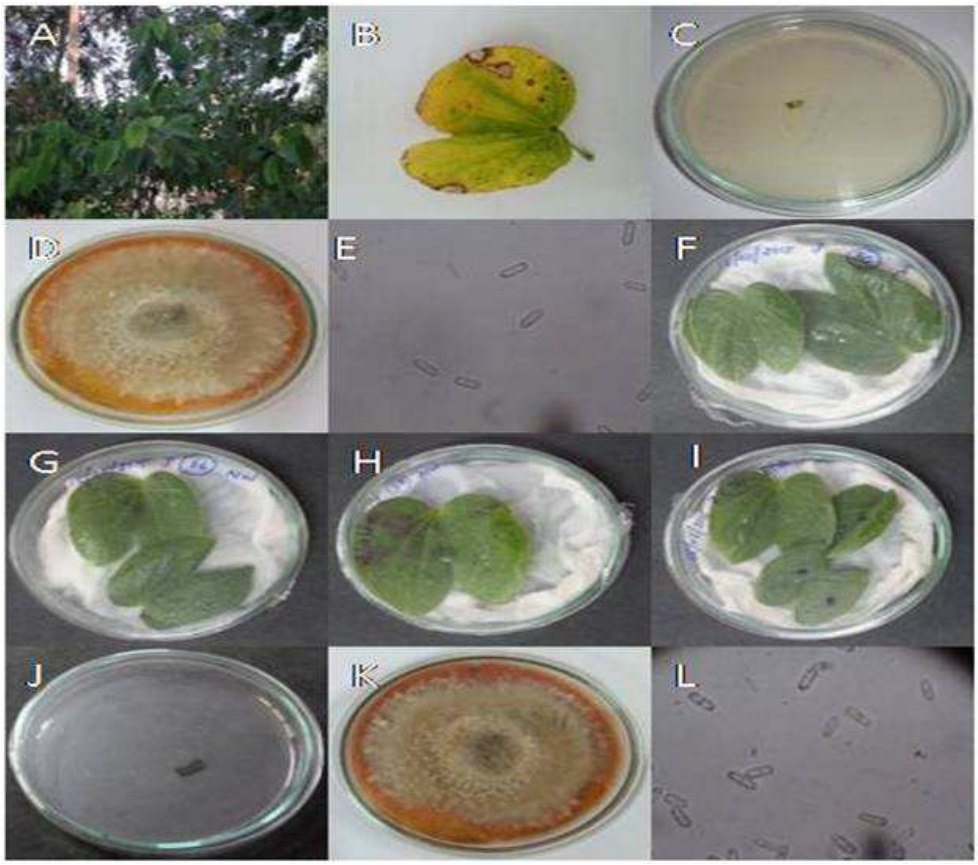


Figure 1. *Isolation: (A) Plant (B) infected leaf. (C) Isolation of fungi from initial leaf spot segment (D) Culture morphology (E) Spore. Pathogenicity test of leaf spot: (F) control, (G) spore suspension inoculated on Without wound leaves, (H) Midrib wound leaves, (I) PDA plugs inoculated on without wound leaves. The Conformation of leaf spot: (J) Isolation of fungi from reinoculated leaf spot segment,(K) Culture morphology and (L) Spore.*

2.2. Identification of Leaf Spot Fungi

Amplification of ITS region and sequencing

The partial nucleotide internal transcribed spacer (ITS) rDNA region was amplified from the fungal genomic DNA using the polymerase chain reaction by ITS1 forward primer (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 reverse primers (5'TCC-TCC-GCT-TAT-TGA-TAT-GC3') [7]. The PCR amplification was performed in a Bio-RAD instrument with a total 20µl reaction comprised of genomic DNA template, 10X buffer with 10mM DNTP's, 25mM $MgCl_2$, 2U of Taq DNA polymerase and 10 p mol of each primer (All molecular chemicals were purchased from Sigma - Aldrich). The following reaction conditions were used: 4 min at 94°C for denaturation, 30 cycles each of 30 seconds at 94°C for denaturation, 1min at 58.2°C for annealing, 2 min at 72°C for extension followed by the final extension at 72°C for 7 mins. The amplified DNA fragments were analyzed by 1% agarose gel electrophoresis with a 100bp ladder purchased from Bangalore genei (Catalogue No. 3231s) and

the amplicons were visualized using a gel documentation system (Gel logic 2200 PRO). A non-template control was included in each run. Further, the amplified products were sequenced by Euro fins Private Limited, Bangalore, India.

2.3. Extraction of Fungal Taxol

On the positive fungal pathogens, were grown in 1000 ml conical flasks containing 250 ml of MID medium supplemented with soyatone. Agar plugs (5mm diameter) containing sterile mycelia were used as the inoculums. The isolates were grown for 21 days in 25°C(± 25°C) at 12 hours light/12 hours dark cycle under immobile conditions. After three weeks of still culture at 25°C, the culture fluid was passed through four layers of cheesecloth to remove solid materials, and the extracellular fungal compounds in the liquid medium were extracted with organic solvent dichloromethane (DCM) with ratio 1:2 V:V (Fungal extract: DCM). The organic phase was collected after the solvent was removed by evaporation under reduced pressure at 40°C using rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol for the subsequent separation and extracts were analyzed by chromatographic separation analysis [8, 9].

2.4. Thin Layer Chromatography

Thin-layer Chromatographic (TLC) analysis was carried out on 1mm (20 x 20cm) silica gel pre-coated plates, developed in the following solvent: chloroform/methanol (7:0:35V/V). The presence of fungal taxol in the extract was confirmed by thin layer chromatographic analysis (TLC) which possess Rf value were congruent with authentic taxol. The presence of taxol was detected, and then the area of the plate containing putative taxol was removed by scraping off the silica gel at the appropriate Rf value and exhaustively eluted with methanol [9].

2.5. Spectroscopic Analysis

Ultra-violet (UV) and Infra –red (IR) analysis:

The partial purified sample of fungal taxol was analyzed by UV absorption spectrum, dissolved in 100% methanol at 220 nm and 270 nm and compared with standard taxol. The IR spectra of the sample were recorded on Shimadzu FT–IR 8000 series instrument in USIC-Madurai Kamaraj University. The purified taxol was ground with IR grade with KBr pellet (1:10) pressed into discs under vacuum using spectra lab and compared with authentic taxol (Sigma). The IR spectrum was recorded in the region between 4000 - 400 cm^{-1} [2, 10, 11].

HPLC analysis:

High - performance liquid chromatography (HPLC) analysis of Taxol was analyzed by HPLC using a reverse phase C18 column with UV detector [10-11]. A C18 column was used for determining the character of the fungal compound by high- performance liquid chromatography (HPLC). A 20µl of the sample was injected each time and detected at 270nm. The mobile phase was methanol/water (65:35) at 1ml min^{-1}. The fungal compound and the mobile phase were filtered before entering the column. Taxol was quantified by comparing the peaks of the sample with that of the authentic taxol.

3. Results

In the present study totally twenty-seven isolates were obtained from leaf segments of the twenty different plants located in the Madurai Kamaraj University (MKU) campus, among the isolates, only eighteen isolates were well supported for pathogenicity test, and there remaining nine isolates failed to produce leaf spot (Table-1). All eighteen positive pathogens are identified based on the molecular phylogenetic analysis. In this study totally eighteen fungi were identified, they are seven different genera i.e. *Colletotrichum-10, Phoma-1, Alternaria-1, Phomopsis-1, Diaporthe-1, Nigrospora-3, Guignardia-1*. Based on the preliminary screening we confirmed that among these isolates *Colletotrichum truncatum, Phoma moricola, Colletotrichum gloeosporioides* having the potential to produce taxol. The band appears as bluish color spot when visualized under UV (Figure-3). Then the sample containing taxol was scrapped off from the silica gel and eluted with methanol and subjected to UV spectroscopy, IR and HPLC. The UV absorption spectrums of *Colletotrichum truncatum, Phoma moricola and Colletotrichum gloeosporioides* fractions are absorbed at the range of 230 & 270 nm and they are similar to authentic taxol (Figure-3). In IR spectrum of compound extracted from *Phoma moricola, Colletotrichum truncatum, Colletotrichum gloeosporioides* showed various functional group peaks and compared with the standard taxol, the peaks were observed at ranges from 400-4,000cm^{-1} (Figure-4 a, b, c, & d). In all three compounds, a broad peak at 3365-3394 cm^{-1} showed the presence of OH stretching, while a peak at 2945cm^{-1} revealed the presence of CH stretching and peaks at`2364 and 1454cm^{-1} depict the NH stretching. The COO stretching peaks were observed at 1383 and 671cm^{-1} while peaks in the 1114-1028cm^{-1} were predominantly due to the presence of aromatic C and H bonds. These peaks have identical to that of standard taxol which gives a promising proof of the presence of taxol in the Leaf spot fungi. Among the three taxol producing fungi, the test fungus *Phoma moricola*., isolated from *Bauhinia tomentosa* is the first report for Taxol production. The amount of taxol produced by *Phoma moricola*., was quantified by HPLC (Figure-5a & b). The maximum amount of taxol produced was found to be 302µg/L.

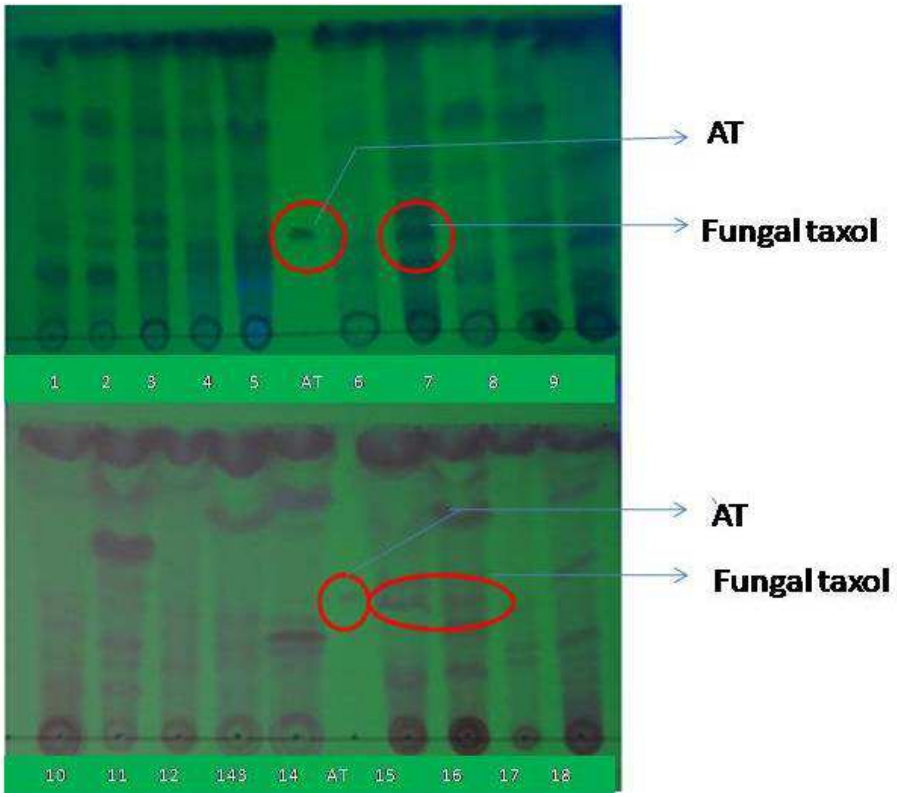


Figure 2. *TLC analysis of standard taxol and Fungal taxol.*

TLC analysis of Taxol. AT- Authentic Taxol and 7,15 and 16 is fungal Taxol *Phoma moricola, Colletotrichum truncatum, and Colletotrichum gloeosporioides*. RF value - 0.52.

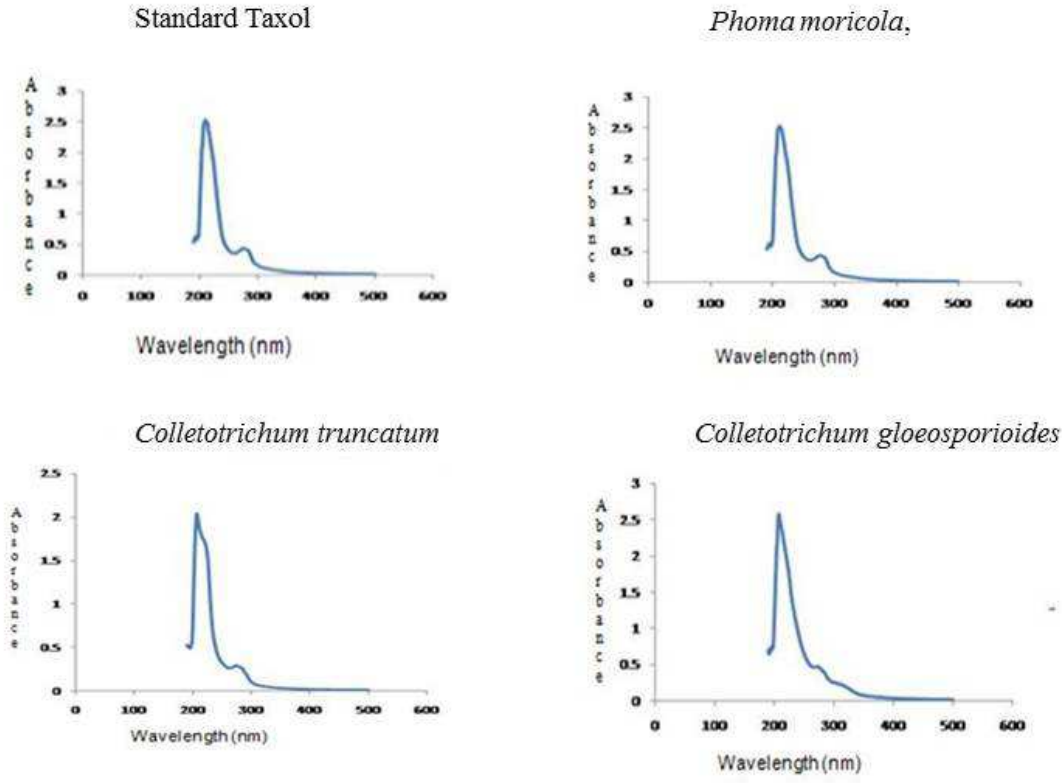
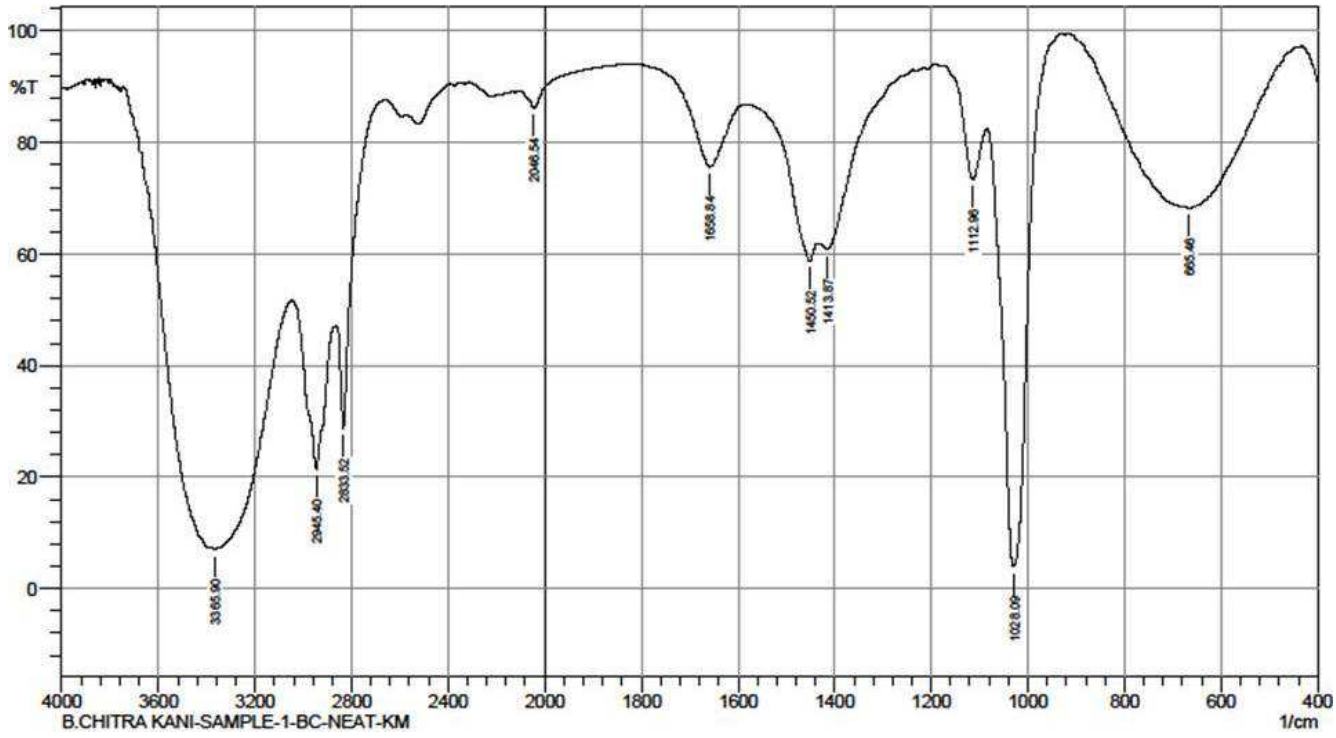
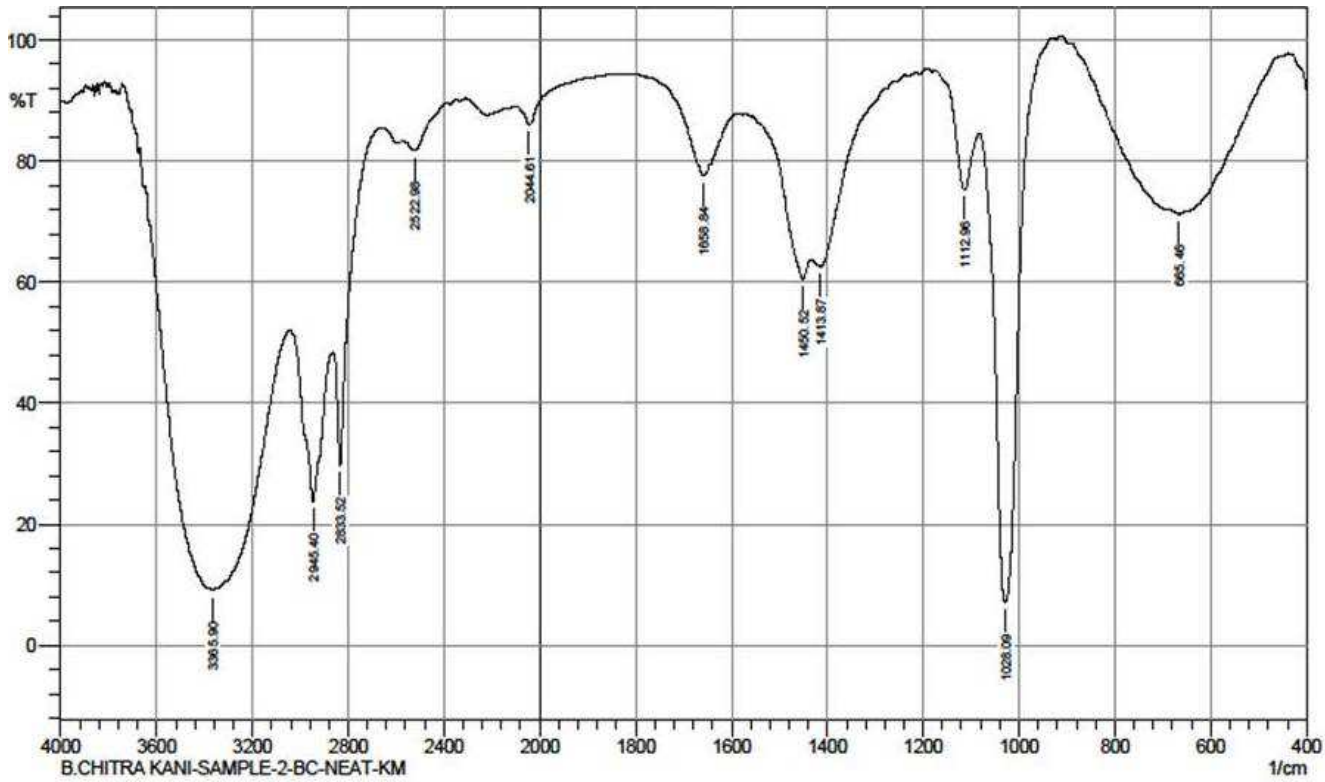


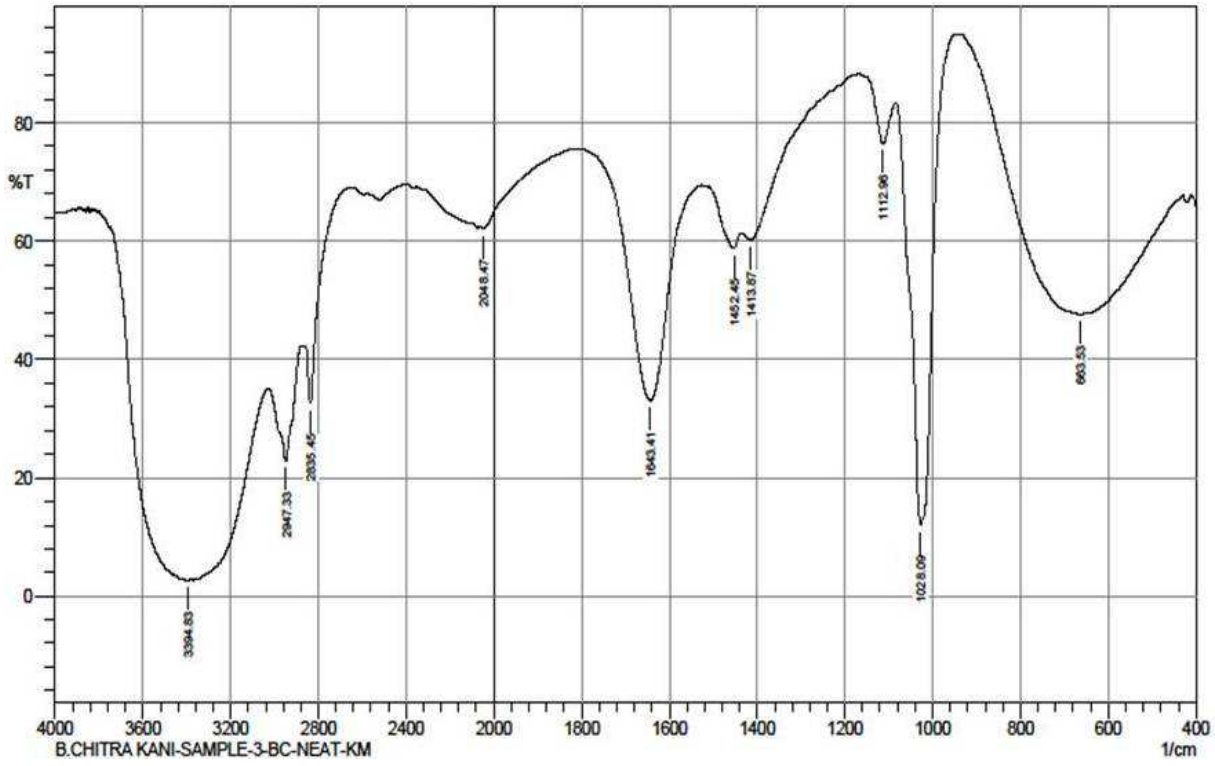
Figure 3. *UV absorption spectrum of fungal taxol and standard taxol.*



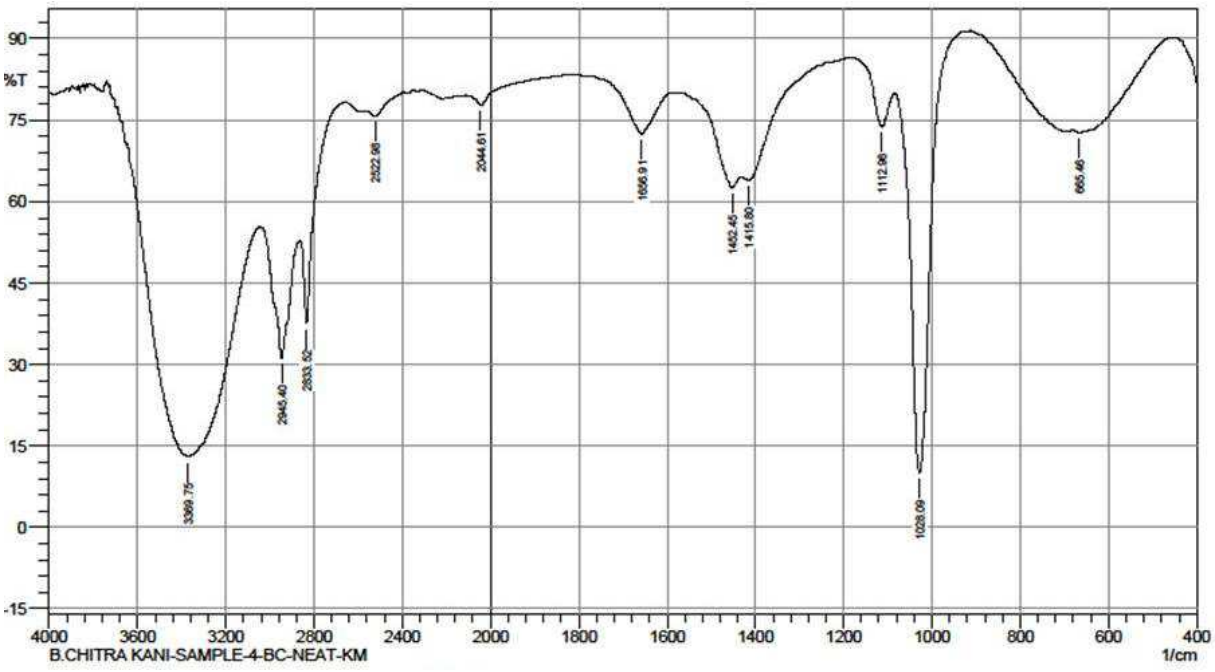
a. *Standard taxol*.



b. *Phoma moricola*,



c. *Colletotrichum truncatum*



d. *Colletotrichum gloeosporioides*

Figure 4. *FT-IR Spectrum of the fungal taxol and the Standard taxol.*

Table 1. *Peaks observed in the authentic taxol and fungal taxol (Phoma moricola, Colletotrichum truncatum, and Colletotrichum gloeosporioides.,).*

POSITIVE ISOLATES	OH	NH	CH	C=C
Authentic Taxol	3365.90	2945.40	665.46	1028.09
Phoma moricola	3365.90	2945.40	665.46	1028.09
Colletotrichum truncatum	3394.63	2947.33	665.46	1028.09
Colletotrichum gleosporioides	3369.75	2945.40	665.46	1028.09

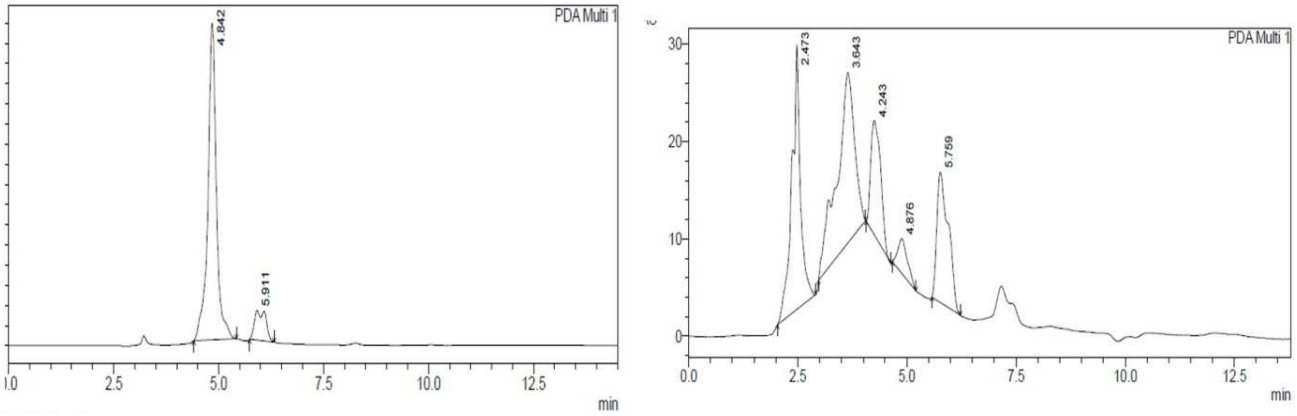


Figure 5. *HPLC analysis of Standard taxol and Fungal Taxol. 5a - Standard taxol and 5b - Fungal Taxol – Phoma moricola.*

4. Discussion

Leaf spot diseases are the one of the most common diseases in an agricultural and medicinal plant. Fungi cause the most diseases. In this present study, we isolated twenty-seven fungi from different plants and they were subjected to pathogenicity test. Among the fungi eighteen were positive and they were characterized based on molecular characterization. The molecular phylogeny supported research has greatly illustrated the fungal systematic, ecological and diversity studies as robust computational algorithms with high statistical support and advancement sinse quenching technology continue to evolve [7]. Paclitaxel (taxol) is a highly functionalized diterpene, active against a broad range of human tumors, including ovarian and breast carcinoma [12-13]. It was originally isolated and characterized from the inner bark of pacific yew, *Taxus brevifolia* (9). In the present study taxol from all pathogenic fungi was extracted and the presence of taxol was prelimary screening using TLC. Among the eighteen three had similar Rf values identical to that of authentic taxol. Further these three fungi subjected to UV, IR, and HPLC analysis.

The UV absorption spectrum of fungal compounds yielded similar absorption to authentic Taxol with a minimum absorption at 235 nm and 270 nm. The appearance of bands in IR spectra convincingly illustrates the identical chemical nature of the extracted Taxol from the fungus with that of authentic taxol. The fungal sample was analyzed by HPLC to confirm the presence of Taxol and gave a peak when eluting from are verse phase C18 column, with about the similar retention time as authentic Taxol. The quantity of Taxol produced by the fungus was calculated and it was estimated to be 302µg/L. Recently several taxol producing endophytic fungi and pathogenic fungi have been identified such as *Bartalinia robillardoides Tassi, Pestalotiopsis terminaliae, Colletotrichum gleosporioides, Fusariumsp., Periconia sp.,* and *Nodulisporium sylviforme* [1, 2, 3, 14, 15, 16, 17]. The spectroscopic and chromatographic analyses of these fungal taxol found to beare identical to that of authentic taxol [18-21]. The techniques like UV, TLC, IR, and HPLC are the tools applied in the confirmation test for the antitumor compound taxol isolated from fungi and are supported by many workers [1, 2, 3, 14, 15, 16, and 17]. The production of taxol from a microbial source has many advantages over other sources. Industrial production of bioactive compounds like taxol requires reproducible, dependable productivity. If a fungus is the source organism, it can be grown in tank fermenters to produce an in exhaustible supply of taxol. The added advantage is that the fungi usually respond favorably to routine cultural techniques.

Acknowledgements

B. Chitra kani wishes to thank University Grant Commission (UGC) Non-net fellowship for financial support and DST-PURSE for the instrument facility.

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