
Screening and Characterization of Fungal Taxol from Leaf Spot Fungi

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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 13,500 kg of bark) from the bark of Yew tree (*Taxus brevifolia*), but huge drawback in 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 infected plant leaves were collected 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. All the positive pathogens are screened preliminarily for taxol production. Among the eighteen isolates, three fungi produced taxol. The test fungus *Phoma sp.*, isolated from *Bauhinia tomentosa* was the first report for taxol production. The amount of taxol produced by *Phoma sp.*, was quantified by HPLC. The maximum amount of taxol produced was found to be 302 μ g/L.

Keywords: Paclitaxel (Taxol), Leaf spot fungus, *Phoma sp.*

1. Introduction

Taxol is a diterpenoid antineoplastic drug widely used in the treatment of various cancers. In 1992, taxol was first approved as 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 into 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 0.5 cm. The excised leaf segments were surface sterilized by sequential washes in 70% ethanol for 5 seconds, 4% sodium hypo chlorite for 90 seconds and

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 $22\pm 2^{\circ}\text{C}$ with 12 hlight/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 test by three different methods [7] (Figure-1).

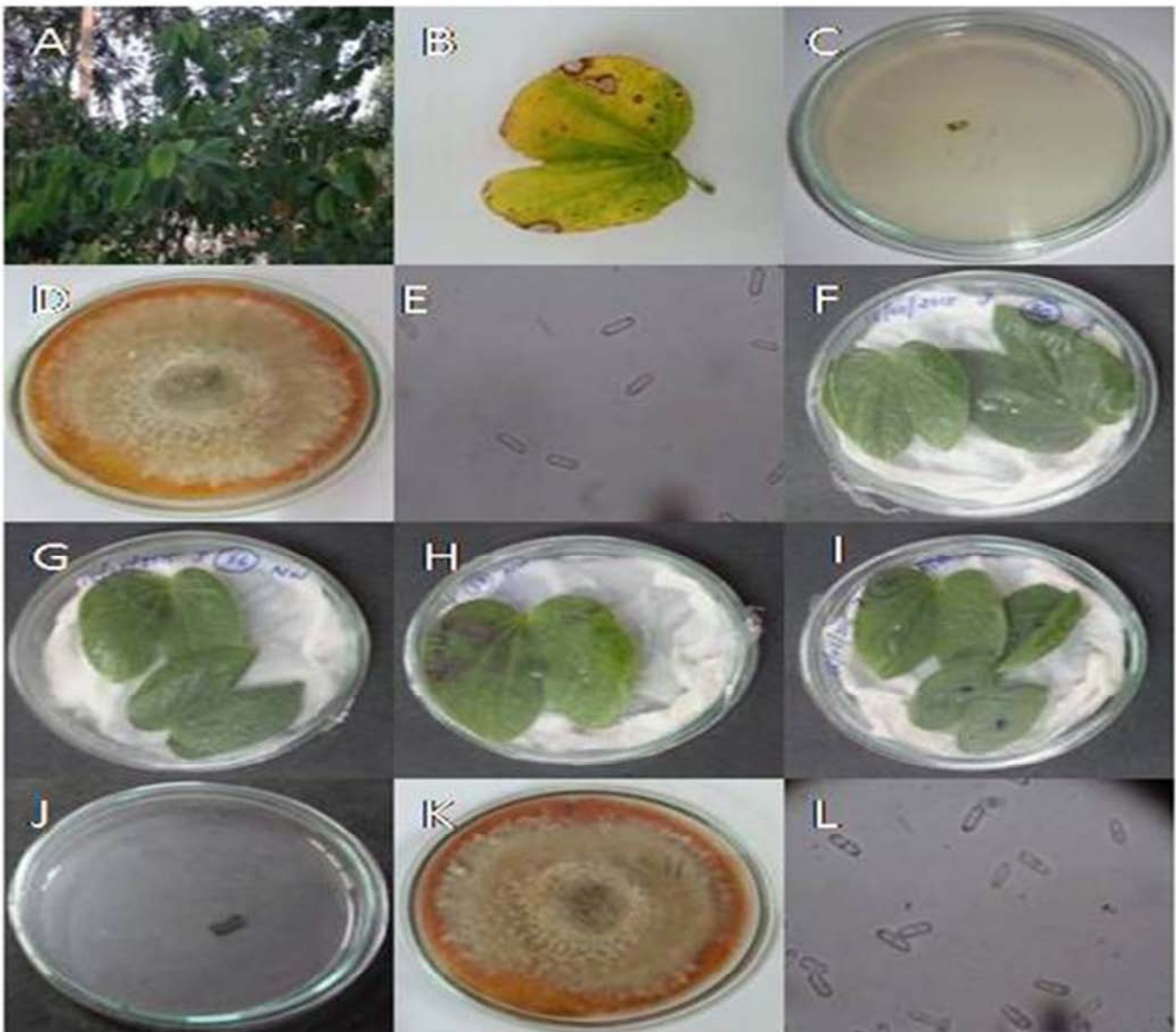


Figure 1. Isolation: (A) Plant (B) infected leaf. (C) Isolation of fungi from initial leaf spot segment (D) Culture morphology (E) Spore. Pathogenicity test of leaf spot: (F) control, (G) spore suspension inoculated on Without wound leaves, (H) Midrib wound leaves, (I) PDA plugs inoculated on without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

2.2. Extraction of Fungal Taxol

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 ($\pm 25^\circ\text{C}$) at 12hours light/12hours dark cycle under immobile conditions. After three weeks of still culture at 25°C, the culture fluid was passed through four layers of cheese cloth 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].

2.3. Thin Layer Chromatography

Thin-layer Chromatographic (TLC) analysis was carried out on 1mm (20x20cm) 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 possesses R_f 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 R_f value and exhaustively eluted with methanol [9].

2.4. 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 220nm and 270nm and compared with standard taxol. The IR spectra of the sample were recorded on Shimadzu FT-IR 8000 series instrument in USC-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 20 μl of the sample was injected each time and detected at 270nm. The mobile phase was methanol/water (65:35) at 1 ml 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, eighteen isolates were well supported for pathogenicity test, and there remaining nine isolates failed to produce leaf spot. All the eighteen fungal pathogens were preliminarily screened for taxol production. In Thin Layer chromatography the eighteen isolates - authentic taxol, *Phoma sp.*, (7), *Colletotrichum sp.*, (15), and *Colletotrichum sp.*, (16) band appears as bluish color spot when visualized under UV (Figure-2). 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 sp.*, *Phoma sp.*, and *Colletotrichum sp.*, 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 sp.*, *Colletotrichum sp.*, *Colletotrichum sp.*, showed various functional group peaks and compared with the standard taxol, the peaks were observed at ranges from 400-4,000 cm^{-1} (Figure-4 a, b, c, & d) and table-1. In all three compounds, a broad peak at 3365-3394 cm^{-1} showed the presence of OH stretching, while a peak at 2945 cm^{-1} revealed the presence of CH stretching and peaks at 2364 and 1454 cm^{-1} depict the NH stretching. The COO stretching peaks were observed at 1383 and 671 cm^{-1} while peaks in the 1114-1028 cm^{-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 sp.*, isolated from *Bauhinia tomentosa* is the first report for Taxol production. The amount of taxol produced by *Phoma sp.*, was quantified by HPLC (Figure-5a & b). The maximum amount of taxol produced was found to be 302 $\mu\text{g/L}$.

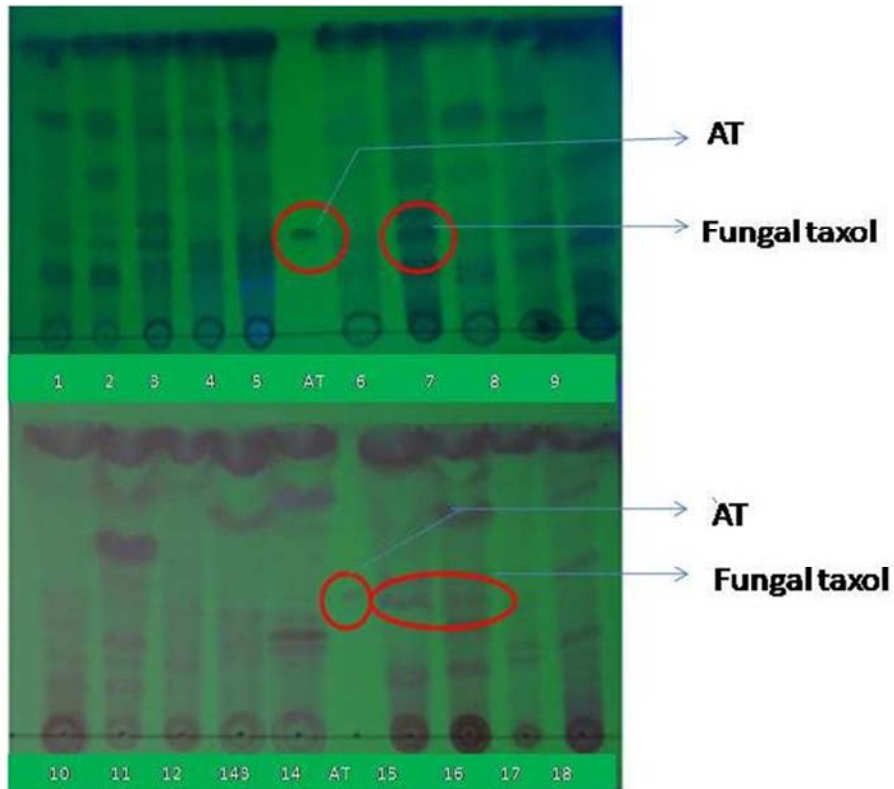


Figure 2. TLC analysis of standard taxol and Fungal taxol.

TLC analysis of Taxol. AT Authentic Taxol and 7, 15 and 16 is fungal Taxol from *Phoma* sp., *Colletotrichum* sp., and *Colletotrichum* sp.

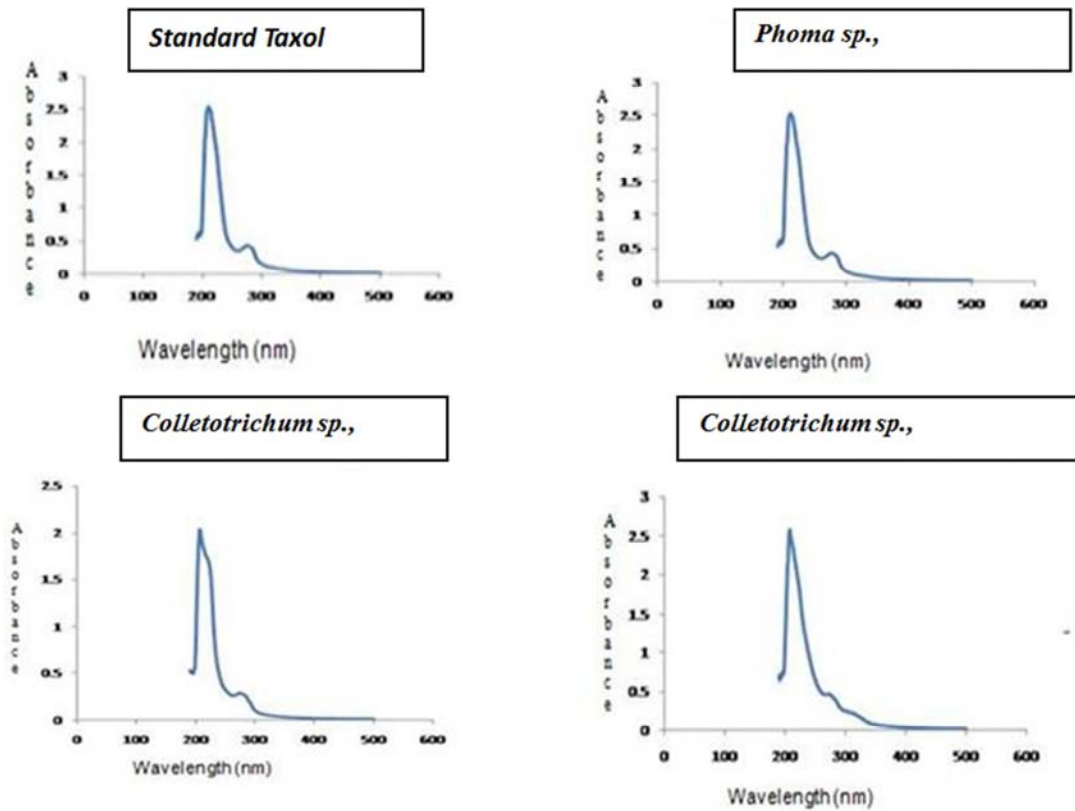
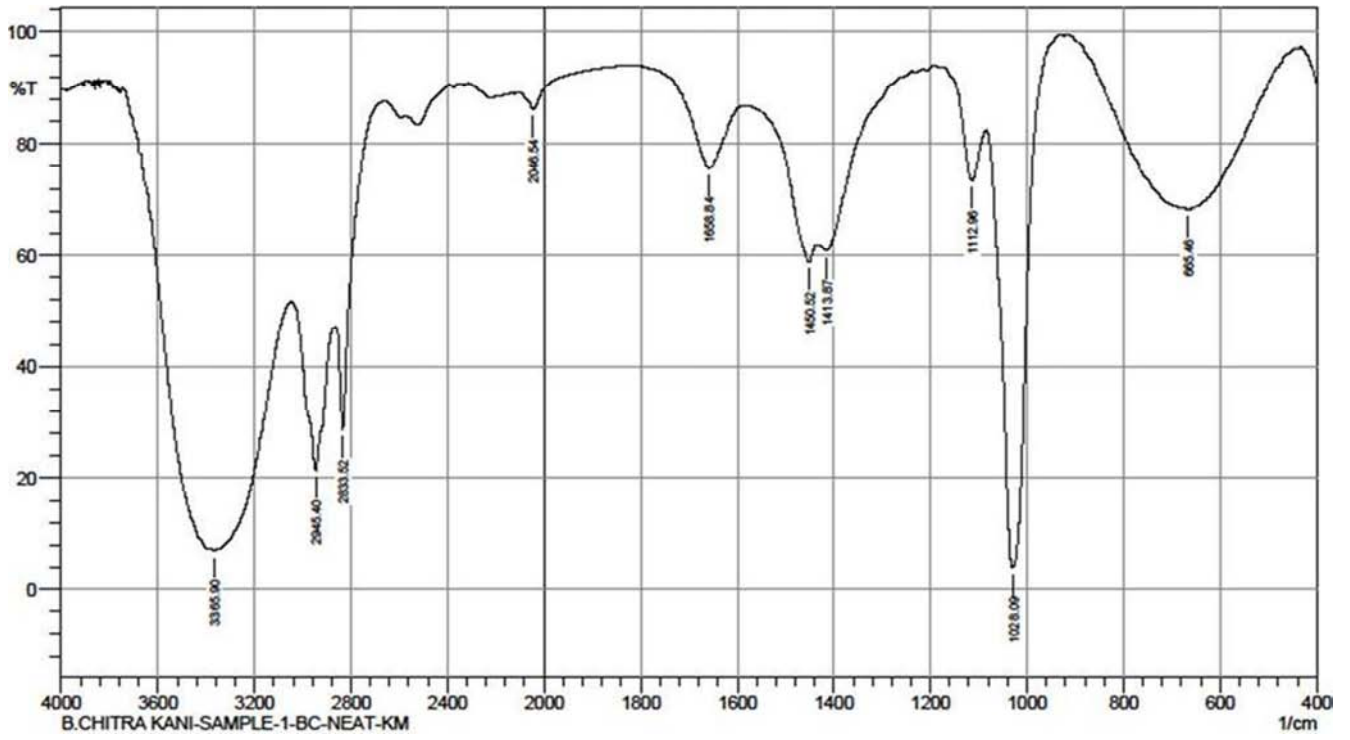
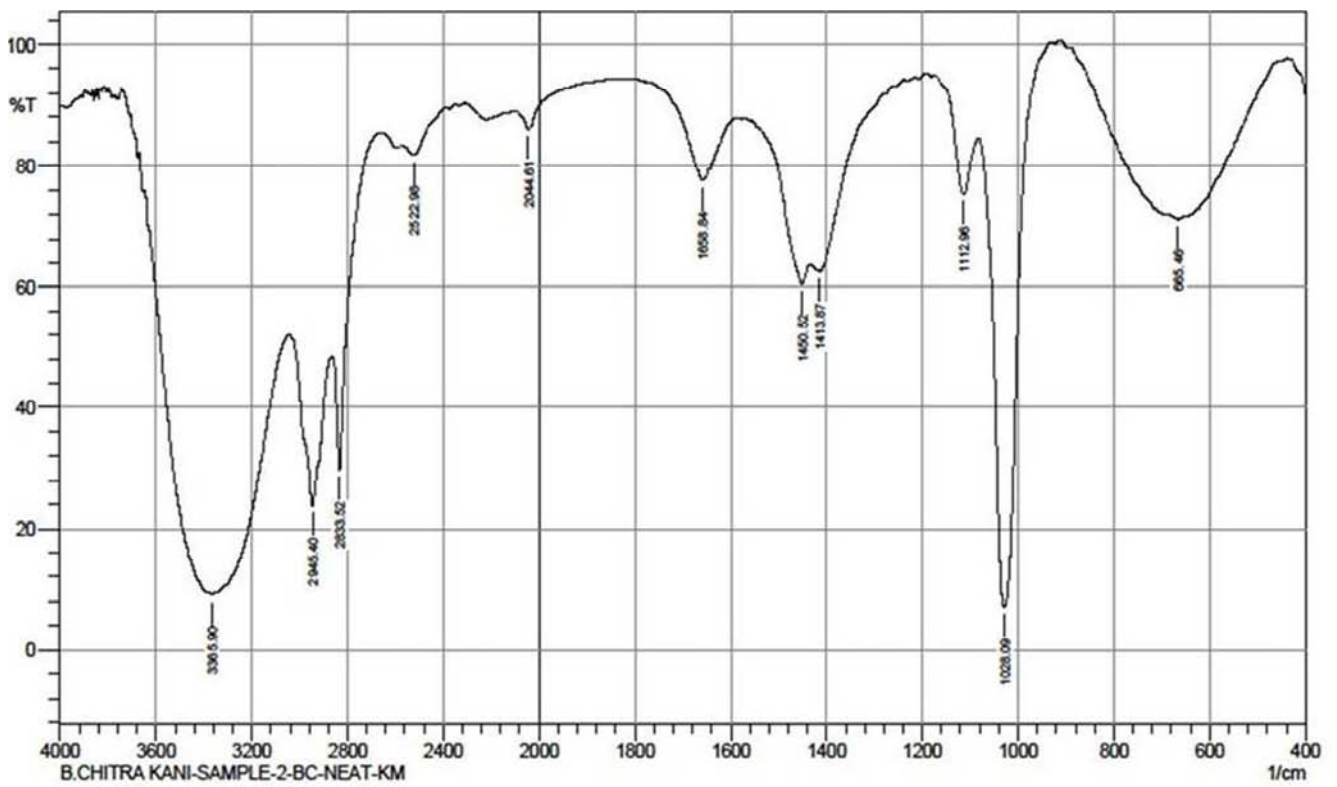


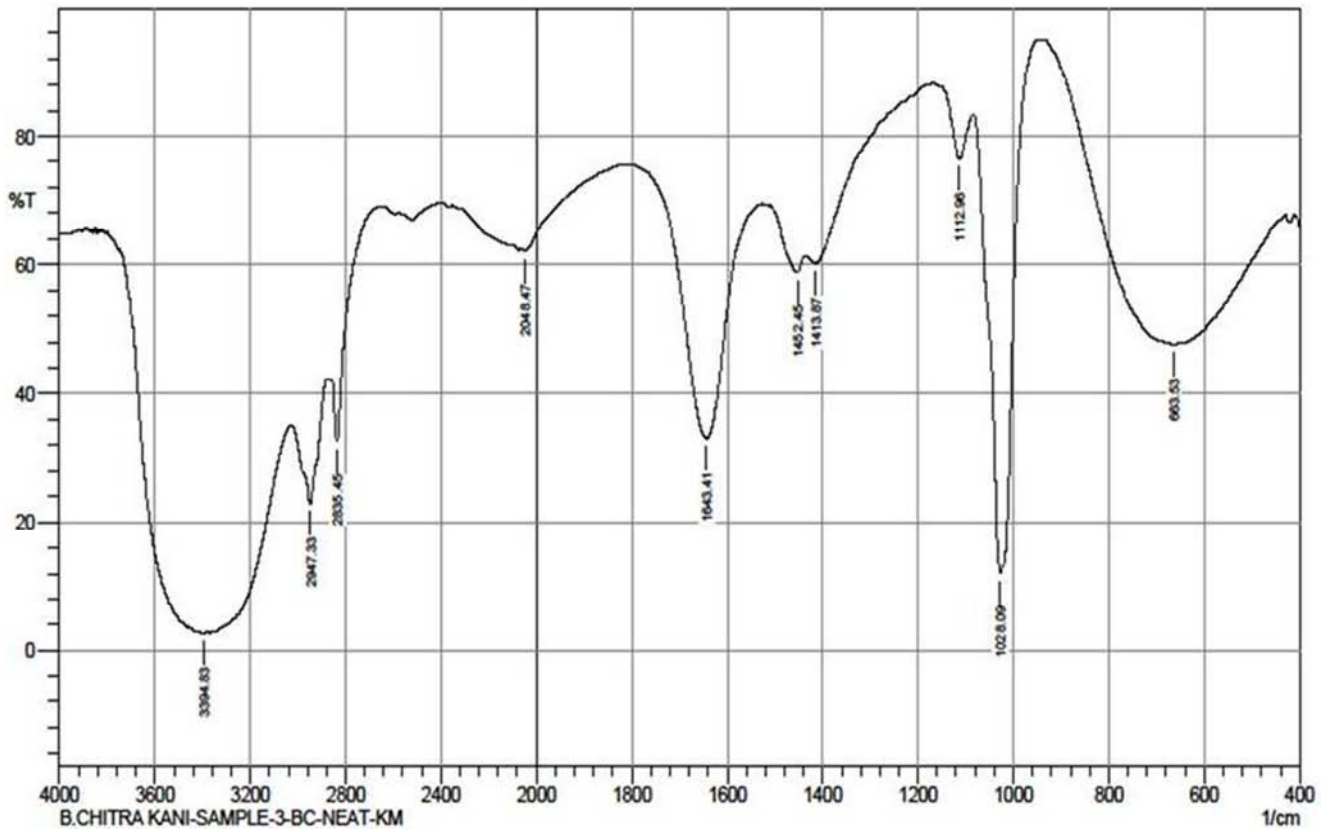
Figure 3. UV absorption spectrum of fungal taxol and standard taxol.



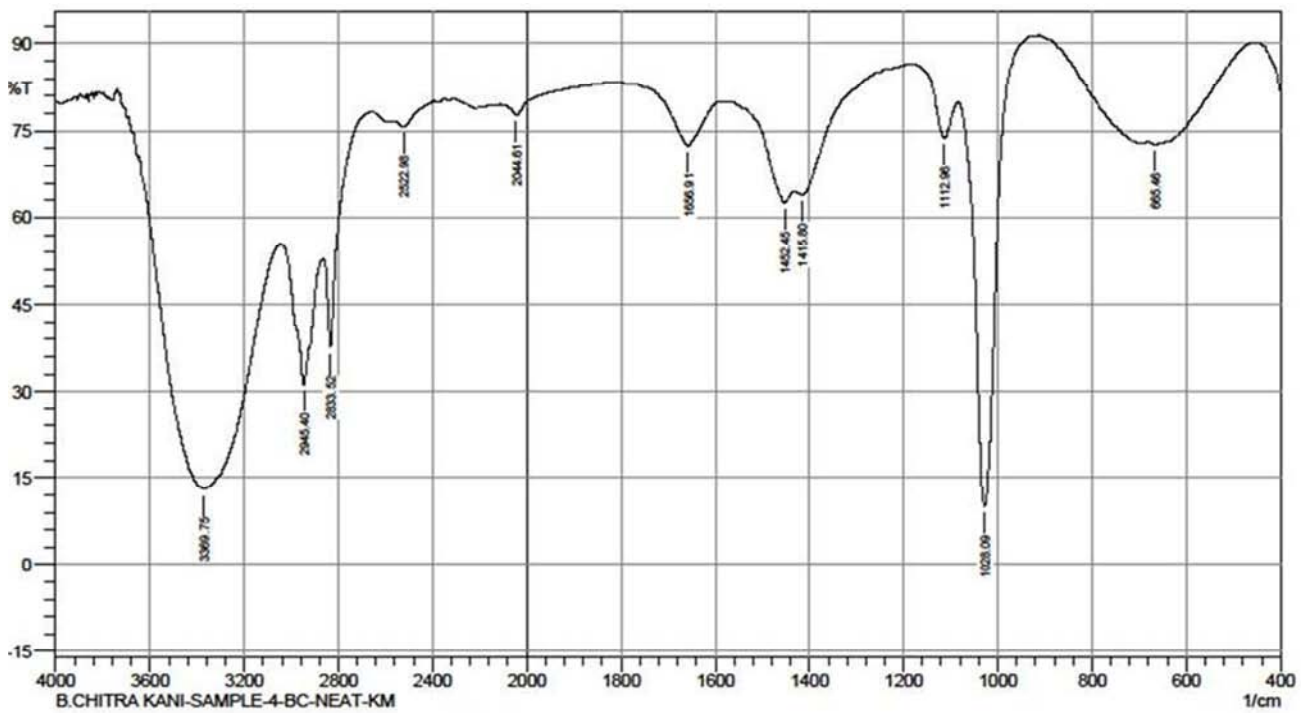
a. Standard taxol.



b. *Phoma* sp.,



c. *Colletotrichum* sp.,

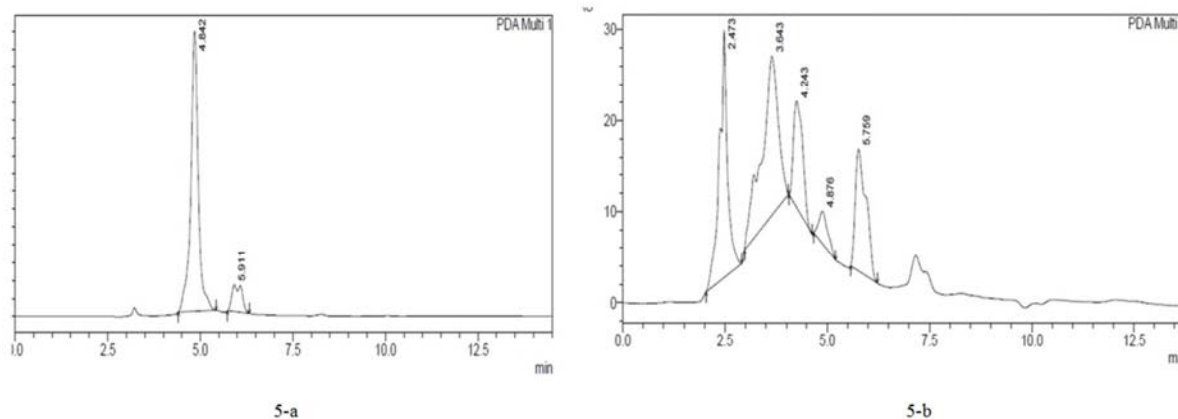


d. *Colletotrichum* sp.,

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 sp.*, *Colletotrichum sp.*, and *Colletotrichum sp.*).

POSITIVE ISOLATES	OH	NH	CH	C=C
Authentic Taxol	3365.90	2945.40	665.46	1028.09
<i>Phoma sp.</i> ,	3365.90	2945.40	665.46	1028.09
<i>Colletotrichum sp.</i> ,	3394.63	2947.33	665.46	1028.09
<i>Colletotrichum sp.</i> ,	3369.75	2945.40	665.46	1028.09

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

4. Discussion

Fungi cause more pathogenic diseases. Leaf spot diseases are one of the most common fungal diseases in agricultural and medicinal plant. In this present study, we isolated twenty-seven fungi from different plants and they were subjected to pathogenicity test. Among the twenty-seven fungi eighteen were positive and they were used for taxol screening. 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 was extracted from all the pathogenic fungi and the presence of taxol was preliminary screened using TLC. Among the eighteen isolates, three isolates had similar Rf values identical to that of authentic taxol. Further these three fungi were 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 a reverse phase C18 column, with 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*, *Fusarium sp.*, *Periconia sp.*, and *Nodulisporium sylviforme* [1-3, 14-17]. The spectroscopic and chromatographic analyses of these fungal taxol are found to be identical to that of authentic taxol [18-

21]. The techniques like TLC, UV, 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 [1-3, 14-17]. 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 fermentors to produce an in exhaustible supply of taxol. The added advantage is that the fungi usually respond favourably to the routine cultural techniques.

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