Screening of Taxol, an Anticancer Drug produced from *Pestaloptiopsis stellata* isolated from *Ficus infectoria*

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Abstract: A taxol producing fungus from *Pestalotiopsis stellata* was isolated from *Ficus infectoria* and screened for the production of Taxol. The Taxol isolated from these fungi is biologically active against cancer cell lines and is also spectroscopically identical to authentic Taxol. In order to lower the price of Taxol and market it more available, a fermentation process involving a microorganism would be the most desirable means of supply. The fungus was identified based on the morphology and spore characterization. Secondary metabolites were extracted using Dichloromethane. Taxol extracted from secondary metabolites was characterized by spectral analysis and chromatographic techniques. The λmax for the authentic Taxol is observed at 274 nm with the absorbance at 3.8nm, similarly λmax for the sample Taxol found at 274 nm range with variation in the absorbance at 1.8nm. Spectral data confirms the presence of alkyl groups attached to the aromatic ring with the side chain containing unsaturation hydrocarbons like alkene/ alkyne groups and High Performance Liquid Chromatography (HPLC) analysis showed the yield of 0.044µg/L amount of Taxol produced from the *Pestalotiopsis stellata*. The bio prospecting of endophytic fungus *Pestalotiopsis stellata* isolated from *Ficus infectoria* is discussed may serve as a potential material for the production of Taxol.

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1. Introduction

The Taxanes are a group of drugs that includes Paclitaxel (Taxol) and Docetaxel (Taxotere), which are used in the treatment of cancer. Paclitaxel is a complex diterpenoid alkaloid, and the structure of this novel compound was reported first by Wani *et al*., 1971 [1]. The molecular formula is C47 H51 NO14, and it contains 11 stereo centers. Paclitaxel is a compound that was originally isolated from the bark of pacific Yew tree (Taxus brevifolia) more than two decade ago and has proved to possess anticancer activity [2]. Early research using Paclitaxel was limited due to difficulties in obtaining the drug. The amount of paclitaxel in yew bark is small, and extracting it is a complicated and expensive process. However, a complete treatment for the patient requires approximately 2g of Taxol administered several times over many months. To obtain 1kg of Taxol it requires 10,000kg of bark [3]. The occurrence of taxol in other endophytes such as *Taxomyces andreanae, Pestalotia sp, Pestalotiopsis sp, Fusarium sp, Alternaria sp*, *Pithomyces sp* and *Monochaetia sp* was also recorded [4]. The culture amended viz lowering of phosphate and addition of sodium benzoate increases the Taxol production. The Taxol isolated from these fungi is biologically active against cancer cell lines and is also spectroscopically identical to authentic Taxol. In order to lower the price of Taxol and market it more available, a fermentation process involving a microorganism would be the most desirable means of supply. The present study prompted us to continue the search for Taxol from *Pestalotiopsis stellata* screening. The culture was initially screened for the ability to produce Taxol using chromatographic methods.

2. Material and Methods

2.1. Isolation and identification of endophytic fungi

The fungus was isolated from the leaves of *Ficus infectoria* Linn by following the standard method [5]. The isolated fungus was then identified and was grown in 2 L Erlenmayer flasks containing 500ml of M1D medium supplemented with 1g of soyatone L-1 [6] for Taxol production. Mycelial agar plugs (0.5 cm) were used as inoculums. The organism was grown under 24±2°C statistically for 21 days.

2.2. Extraction of Taxol

Extraction of Taxol was performed according to [7] after 21 days of incubation the culture filtrate was filtered through four layered cheesecloth. In order to avoid fatty acid contamination of Taxol, 0.25g of Na_2CO_3 was added with frequent shaking to the filtrate. The culture filtrate is extracted with two equal volume of Dichloromethane and the organic phase was evaporated to dryness under reduced pressure at 35°C.

The dry solid residue is re-dissolved in methanol for the subsequent separation.

2.4. Ultra Violet (UV) Spectroscopic Analysis

The purified sample of Taxol was analysed by UV absorption, dissolved in 100% methanol at 274nm [1] and compared with authentic Taxol.

2.4. Infra Red (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 compared with standard Taxol. The IR spectrum was recorded (3329-430 cm-1nm).

2.5. High Performance Liquid Chromatography Analysis

HPLC is to further confirm the presence of Taxol in the fungal extract. The fungal sample was subjected to HPLC using a reverse phase C18 column. The sample solution of *Pestalotiopsis stellata* for HPLC analysis was filtered through a 0.2µm membrane before injection. Though the mobile phase was methanol/acetonitrile/water (25:35:40 v/v). The flow rate was 1.0 ml min-1. The concentration of Taxol was calculated as follows:

$$\text{Con. Of Taxol} = \frac{\text{Total sample area X Dilution of Standard X Purity of Standard}}{\text{Total Standard area X Dilution of Sample}} \text{ X100}$$

Cell proliferation assay

The cytotoxic effect of fungal taxol was tested by the MTT assay on PC-3 (Prostate cancer cell line) and A549 (Human Lung Cancer cell line). Cancer cell lines were purchased. The cells were thawed in a water bath at 37°C for approximately one to two min and added to 10 ml of prewarmed (37°C) antibiotic free RPMI 1640 (sigma) growth medium containing 10% Foetal Calf serum (FCS) (Sigma) in order to dilute out the DMSO. The cells were centrifuge (500xg, 5min, 25°C) in order to obtain a cell pellet and the supernatant discarded. The pellet was suspended in 10ml pre-warmed total growth medium and transferred to a cell culture dish. The cells were incubated in a humified CO_2 incubator (5% Co_2) at 37°C until the monolayer was sub confluent (Rose and Connolly, 1990). The cultures were incubated with the conditions as mentioned earlier. After 24 and 48 hours incubation,100 µl of 0.5 mg ml-1 MTT solution was added to each well and the culture were further incubated for 4 h and then, 100 µl of 20% SDS in 50% dimethyl formamide (DMF) was added. A micro plate reader was used to measure the absorbance at 650 nm foreach well [8]. Growth inhibition rate was calculated as follows: % cell viability = A570 of treated cells / A570 of control cells × 100%.

The cell viability was calculated as percentage of viable cells and then plotted on a graph.

3. Result

The *Pestalotiopsis stellata* culture grown in liquid medium was screened for Taxol production and the extract was examined for the presence of Taxol by Chromatographic and Spectroscopic analysis

3.1. Morphological characters of *Pestalotiopsis stellate*

Conidia are five celled, not constricted at septa, erect or slightly curved, (19-24)µm × (6.5-7.5) µm. intermediate cells are coloured, 14-16µm long, the upper two umber, lowest olivaceous, the walls are darker, broader, broadest at septa dividing two upper coloured cells. Exterior hyaline cells are conspicuous, apical cell is broad-conic, turbinate, bearing 3 setulae, 2131µm long, filiform widely diverged. Conidiamata are acervular, amphigenous black scattered (Plate.1a, b).

3.4. Infra Red analysis

The appearance of band in IR data of fungal taxol from *Pestalotiopsis stellata* showed a broad peak in the region 3329.602 cm-1 was described to hydroxyl group (O-H) and amide (NH) group's stretch. The methyl group is an alkyl derived from methane containing one carbon atom to three hydrogen atoms which is stretched at 2943.983 cm-1. The stronger the bond, the higher the vibration frequency makes up the finger print region at 2288.108. The intensity of the IR peak is proportional to change the dipole moment that a bond undergoes during a vibration. Benzene derivative is witnessed by peaks at 1656.625 and 1408.529 cm-1. The IR spectrums of *Pestalotiopsis stellata* were composed on that of authentic taxol (fig.2a, b).

3.2. Taxol extract and Analysis

The culture filtrate was extracted and extracted with Dichloromethane. The solvent was then removed by evaporation under vacuum and the dry solid residue is re-dissolved in methanol. The extract was then subjected to chromatographic and spectroscopic analysis by UV, IR and HPLC.

Plate 1a. *Pestalotiopsis stellata*

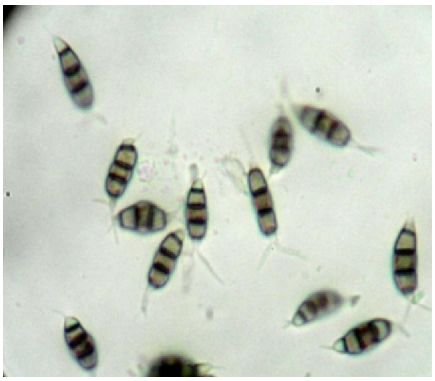


Plate.1b Conidial Morphology of *Pestalotiopsis stellata* 40X

3.3. Ultra Violet Absorbance

The UV absorption of the fungal compound isolated from *Ficus infectoria* yield similar absorption to authentic Taxol. The authentic Taxol observed at 274nm with the absorbance of 2.1 were as the sample observed 274nm with the absorbance at 1.6 (fig.1a, b).

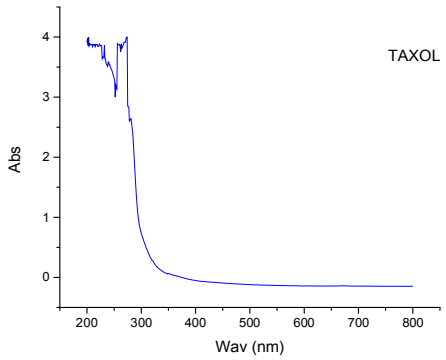


Fig.1a) UV Absorbance of Authentic Taxol

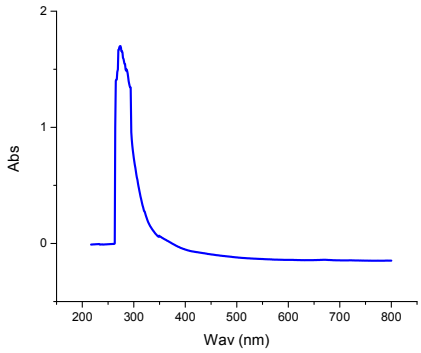


Fig.1b) UV Absorbance of *Pestalotiopsis stellata*

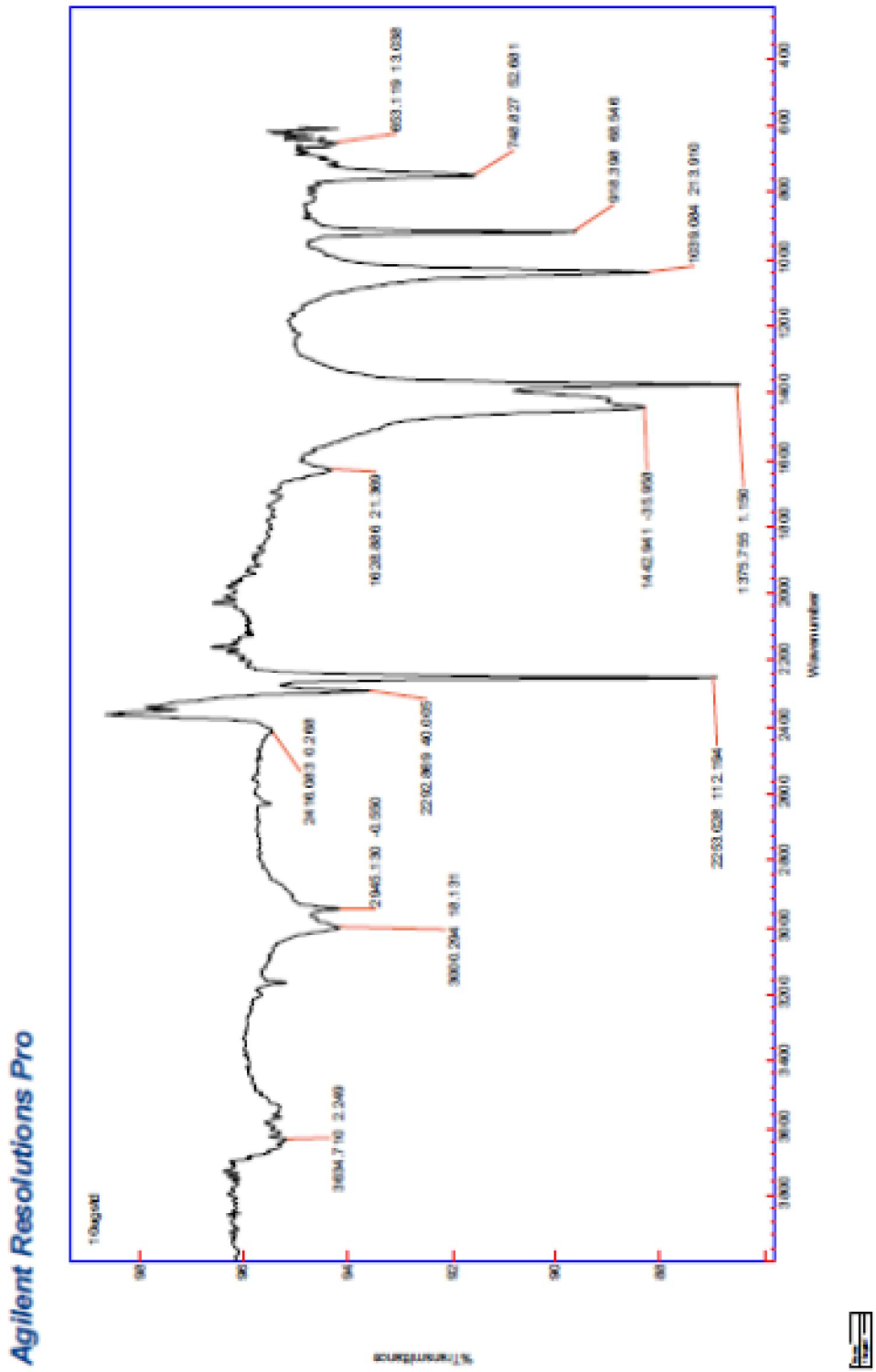
3.5. High Performance Liquid Chromatography

To further confirm the presence of Taxol from *Pestalotiopsis stellata* was analyzed HPLC which gives a peak when eluted with the reverse phase C18 column with the retention time of 2.8 min and the mobile phase consists of methanol: acetonitrile: water 25:35:40 v/v. before injection the sample was filtered through 0.2µm membrane. It was found to be similar in comparison with standard Taxol (himedia) (fig.3a, b).

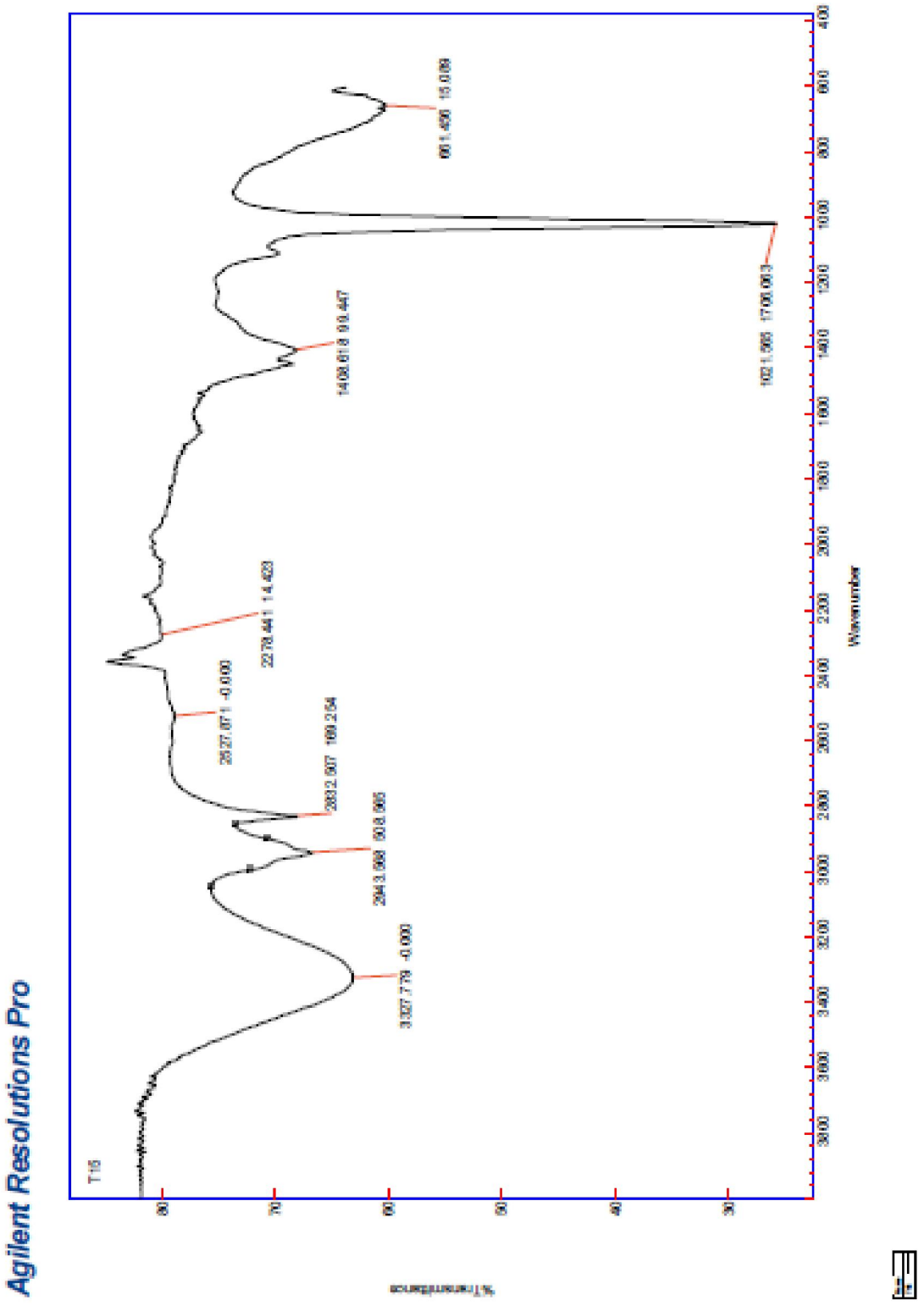
3.6. *In vitro* study

Anticancer study of Taxol against Lung Cancer and Prostate Cancer Cell Lines

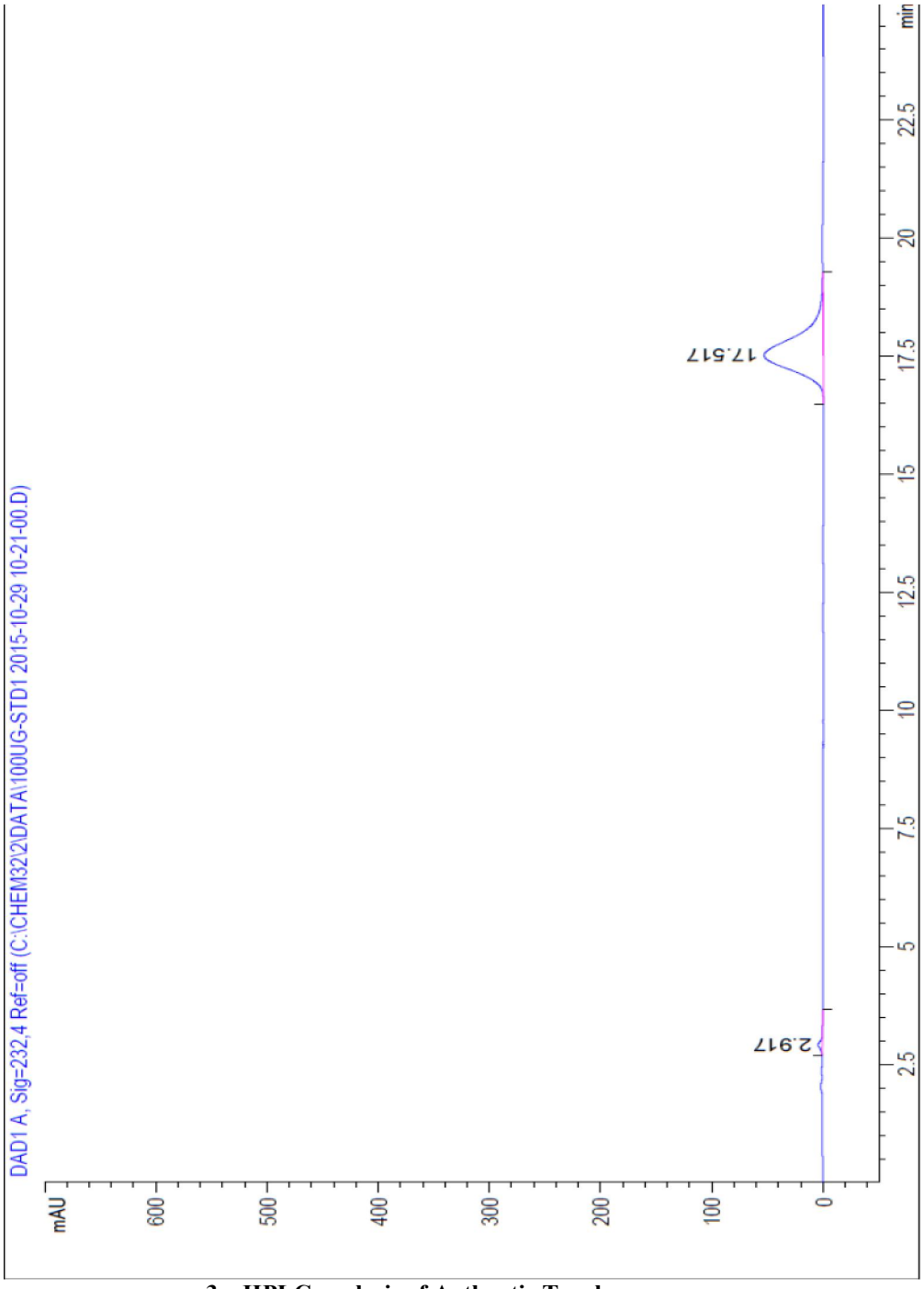
The effectiveness of anticancer drugs can be calculated based on its ability to eradicate the cancer cells. Thus, the inhibitory effect of Taxol on A549 lung adenocarcinoma cells and PC-3 Prostate Cancer Cells was assessed by culturing the cells with different concentrations of the compound for 24hrs and 48hrs. Depict the graphical representation of percentage of cell survival determined by cell proliferation assay (MTT assay). A549 lung adenocarcinoma cells and PC-3 Prostate Cancer Cells were evaluated to investigate the cytotoxicity activity of Taxol. MTT assay is a sensitive, simple and reliable practice which measure cell viability. The average values of the percentage of viable cells were included in Table 1a, b which clearly shows that Taxol exhibited concentration dependent cytotoxicity in A549 and PC-3 cells. When cells treated with 60µM of Taxol for 24hrs, 50% reduction in cell viability was observed 49.87 in A549 and 44.87 in PC-3 and it further decreased upon higher concentration. At the highest Concentration of *Pestalotiopsis stellata* (100µM) the percentage of viable cells for 24hrs and 48hrs were 48.87±3.13 and 33.92±4.35 respectively. Cell viability of androgen-independent Prostate cancer PC-3 cells and adenocarcinoma cells A459 on treatment with Taxol was assessed using MTT assay. A significant decrease in the percentage of viable cells was observed with increase in concentration of Taxol after 24h and 48hrs. Based on the viability trend obtained the IC50 value of Taxol in A549 cells and PC- 3 was calculated as 60µM for 24hrs fig 4.



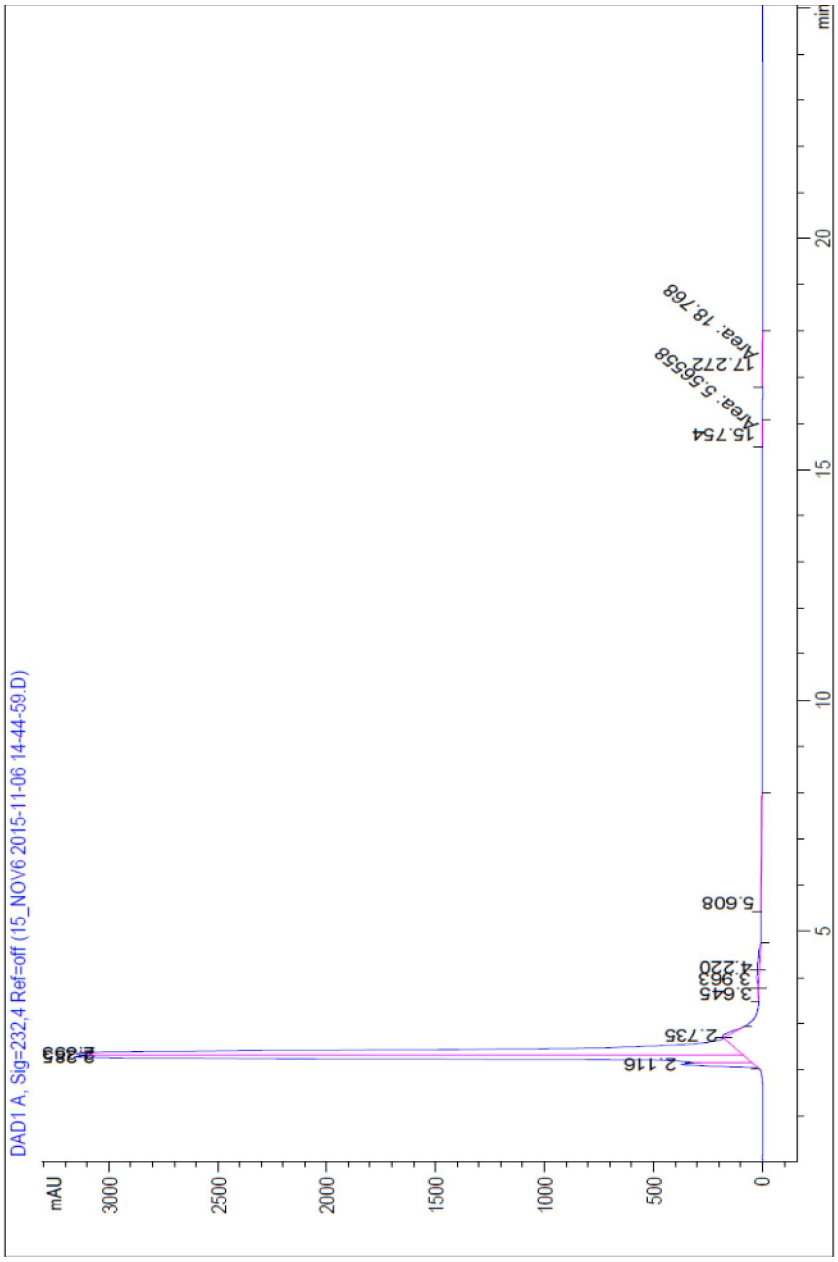
2a. FT-IR Spectra of Authentic taxol



2b.FT-IR Spectra of *Pestalotiopsis stellata*



3a. HPLC analysis of Authentic Taxol



3a. HPLC analysis of *Pestalotiopsis stellate*

Vero Cell Line A549 PC- 3

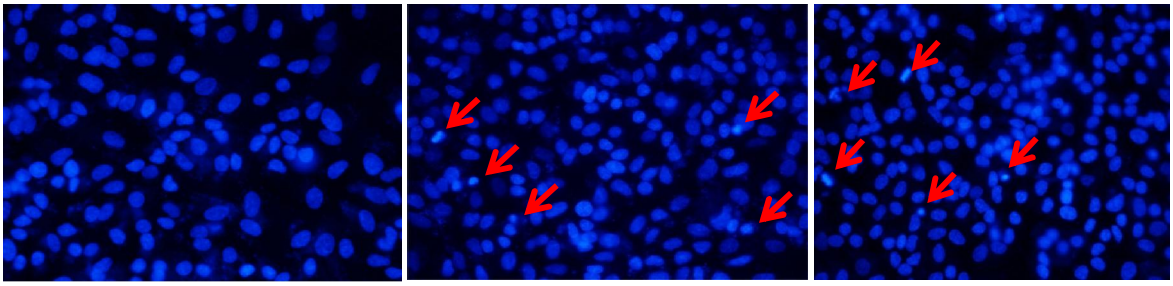


Fig4. Cytotoxicity of *Pestalotiopsis stelleta* against Vero, A549 and PC-3

4. Discussion

Steyaert, R.L (1949) *Bull. Jard. Bot. Burx*. **19:** 171, Berkeley, M.J. and Curtis, G. (1874) *Grevillea* **2:** 155, Saccardo, P.A. (1884) *Syll. Fung*. **3:** 787. Guba, E.F. (1961) *Har. Uni. Pre*. 171

Spots pale, definite, acervuli stellata, black, punctiform, sparse, amphigenous, flat, 140-280 µ in diam, seated on definite pale, circular spots or irregular shaped dead areas with black borders. Conidia 5- celled, straight or sometimes slightly curved, broad- fusiform or braod- elliptic, only slightly constricted at septa, 1-20 µm; 3 intermediate coloured cells cask- shaped, guttulate, 12- 16 × 6.5- 9 µm. The 2 upper umber, the lowest olivaceous; exterior hyaline cells small short- conic, the apical cells braring a crest of 3, broadly divergent setulae, 4-11 µm or some what longer; pedicels attenuated downwards.

Endophytes may contribute to their host plant by producing plethora substance, which provides protection and also the survival of plant. The endophytic fungi from Coelomycetes viz *Chaetomell raphige Colletotrichum falcatum Fusicoccum sp* and *Pestalotiopsis stellata* isolated from medicinal plants were screened for the production of [9, 10]. The toxicity of Taxus plant have been studied thoroughly and attributed to taxine, a complex mixture that was first isolated from the leaves of Taxus in 1856. However taxines are relatively abundant in plants and they serve as alternative starting material for semi-synthetic production of Paclitaxel [11]. *Pestalotiospsis stellata* isolated from the plant *Ficus infectoria* were screened for the production of Taxol, which yielded the UV absorption spectra that is identical to Taxol standard. The absorption of UV absorbance was 274nm and the peak observed at 1.6 (fig2.b). The IR spectra showed the identical appearance with the standard Paclitaxel (hi-media). Further the Taxol was confirmed by using HPLC. Based on the standard Paclitaxel the quantification of Taxol produced by fungi was done. The total amount of Taxol produced per L in M1D was 0.045µg. Taxol production was too low to be exploited commercially at present but improved culturing techniques; in addition of activators and application of genetic engineering method may ultimately permit fungus commercialization [6, 7]. Since the production of taxol was very low in *Pestalotiopsis breviseta* have a great potential for commercial exploitation in future for better Taxol production by enhancing the cultural conditions [11]. In this study certain endophytic fungi is able produce Taxol, has brought the possibility that a cheaper and more widely available product may eventually be available via industrial fermentation. The biggest problem of using fermentation to produce taxol is its very low yield and unstable production. The short generation time and high growth rate of fungi make it worthwhile to continue our investigation of this *Pestalotiopsis stellata*.

Cytotoxic evaluation of taxol on human cancer cells

Taxol producing fungi *Pestalotiopsis stellate* is further studied for the cytotoxic studies. Cytotoxic effect of fungal Taxol was treated on 2 different human cancer cells viz. PC-3 and A549. The assay indicated an increased Taxol concentration from 0.005 to 0.05µM. With further increase in taxol concentration, the taxol induced cell death increased slightly. When the Taxol concentration increased from 0.5 to 5 µM, the taxol induced cell death decreased significantly. Hence, the efficacy of fungal taxol was relatively dependent on the specific cell type. This was also concurrent with the results of earlier report [12], thus supporting the earlier findings that a low concentration Taxol inhibit cell proliferation by blocking mitosis. In conclusion, fungal endophytes are gaining importance because of their enormous potential to produce novel bioactive compounds for medicinal and agricultural importance. Taxol production from the fungi reported so far from yews demonstrated low level of taxol yield, whereas in this study the Taxol production was high in *Pestalotiopsis*.

5. Conflict of Interest Statement:

Nil

Reference

1. Wani MC. Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents VI. Ent from Taxus brevifolia. *Journal of the American Chemical Society* 1971; 93:2325-2327.
2. Georg GI, Chen TT, Ojima I, Vyas DM, editors. Taxane anticancer agent (Basic science Current status Ameriacn Chemical Society, Symposium series) Washinton. D.C. *American Chemical Society* 1994; p.583.
3. Strobe GA, Hess WM, Ford E Sidhu RS, Yang X. Taxol from fungal endophytes and the issue of biodiversity. *Journal of Indian Microbiology* 1996a; 17: 417-423
4. Strobel GA, Hess WM, Ford E, Sidhu RS, Yang X. Taxol from fungal endophytes and the issue of biodiversity. *Journal of Indian Microbiology* 1996b; 17:417-423
5. Kumarran RS, Muthumary J, Hur BK. Production of taxol from *Phyllosticta spinarum*, an endophytic fungus of Cupressus sp. *Engineering in Life science* 2008a; 8:438-446
6. Meitz AM, Haddad A, Worapong J, Long Dm, Ford E, Hess WM and strobe GA. Induction of the sexual stage of *Pestalotiopsis microspora*, a

Taxol producing fungus. *Microbiology* 2000; 146:2079-89.
7. Stierle A, Strobel GA, Stierle D. *Science* 1993;260:214-6
8. Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.
9. Venkatahalam R, Subban K, Paul MJ. Taxol from *Botriyodiplodia theobromae* (BT 115) an endophytic fungus of Taxus baccata. *Jouranl of Biotechnology* 2008; doi:101016/j.jbiotec.2008.07.1823
10. Muthumary J, Sashirekha S. detection of taxol, an anticancer drug, from selected Coelomycetes fungi. *Indian Journal of Science and Technology* 2007; vol 1,no1
11. Kathiravan G and Sri Raman V. In vitro Taxol production, by *Pestalotiopsis breviseta*- A first report. *Fitoterapia* 2010; 81: 557-564
12. Yeung TK, Germond C, Chen X, Wang Z (1999). The mode of action of taxol: Apoptosis at low concentration and necrosis at high concentration. *Biochemical and Biophysical Research Communication*. 263: 398-404.

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