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Isolation of taxol, an anticancer drug produced by the endophytic fungus, *Phoma betae*

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Phoma betae, an endophytic fungus, was isolated from the healthy leaves of *Ginkgo biloba*. The fungus was screened for the production of taxol on a modified liquid medium for the first time. The fungal species were identified by their characteristic culture morphology and molecular analysis. The presence of taxol was confirmed by spectroscopic: ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR), liquid chromatography–mass spectrometry (LC-MS); and chromatographic: thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) methods of analysis. The taxol production was quantified by HPLC analysis. The maximum amount of taxol production was recorded as 795 µg/L. The production rate was 15,900-fold more than that found in the culture broth of earlier reported fungus, *Taxomyces andreanae*. The extracted fungal taxol demonstrate a strong cytotoxic activity in the *in vitro* culture of tested human cancer cells by apoptotic assay. This indicates that the increase in taxol concentration induced an increased cell death. A polymerase chain reaction (PCR) based screening for *taxadiene synthase (ts)*, a unique gene in the formation of the taxane skeleton was confirmed as a molecular blueprint for taxol biosynthesis. These results designate that the fungus, *P. betae* is an excellent candidate for taxol supply and can serve as a potential species for genetic engineering to enhance the production of taxol to a higher level.

Key words: Taxol production, *Phoma betae*, analytical methods, cytotoxicity assay, taxadiene synthase.

INTRODUCTION

Taxol, a diterpene and a highly functionalized anticancer drug is used widely in hospitals and clinics. Taxol was originally isolated from the inner bark of Pacific yew, *Taxus brevifolia* (Wani et al., 1971). It is specifically targeted to treat breast, lung and ovarian cancers. Taxol is the world's first billion dollar anticancer drug used to treat a number of human tissue-proliferating diseases. Taxol differs from other cytotoxic drugs by its unique mode of

action. It acts by interfering with cell division by way of manipulating molecular regulation of cell cycle. Taxol primarily targets the microtubule, which is vital for mitosis, mortality, secretion and proliferation. Taxol also assembles tubulin and stabilizes the resulting microtubules (Horwitz et al., 1993). Thus, taxol interrupts division of the cell in two equal daughter cells (Horwitz, 1992; Jordan et al., 1993).

Taxol is found extremely in low amount in the needles, bark and roots of yews (*Taxus* spp.). Presently, taxol in the world's market has originated from *Taxus* spp (Malik et al., 2011). Although complete chemical synthesis of taxol has been achieved, the process is too expensive for commercialization. The cost makes it unavailable to many people worldwide. Ultimately, to lower the cost of taxol, a fermentation route through microorganisms would be the most desirable source of supply (Yuan et al., 2006). The search for novel sources of taxol from the tree has led to the isolation of an endophytic fungus,

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Abbreviations: UV, Ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; LC-MS, liquid chromatography-mass spectrometry; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction.

Taxomyces andreanae. *T. andreanae* colonize the inner bark of the yew tree capable of producing taxol and other taxanes *de novo* when grown in semi-synthetic medium (Stierle et al., 1993). Over the last 18 years, there has been a great deal of interest in finding fungi isolated from the yews (Ruiz-Sanchez et al., 2010; Yuan et al., 2006; Strobel et al., 1996a, b; Guo et al., 2006; Stierle et al., 1993) and few other plant species (Pandi et al., 2011; Kumaran et al., 2008a, b, 2009, 2011). So far, there are more than 30 species isolated from plants tested for the production of taxol.

Though, the separation of fungi from plant materials is comparatively a simple process, the detection of taxol producing fungus is laborious (Zhou et al., 2007). On the other hand, genes encoding for taxol biosynthetic enzymes have been used as a molecular marker for screening taxol producing fungi.

In this study, we used the gene encoding for *taxadiene synthase (ts)*, as a molecular marker to screen the fungus (*Phoma betae*) for taxol production. *P. betae* was observed to be the com-mon and dominant fungal endophyte isolated from *Ginkgo biloba*. In a previous report, the endophytic species of *Phoma* isolated from plants demonstrated the production of bioactive metabolites (Heinig and Jennewein, 2009). Whereas, in this investigation, an attempt was made to screen taxol from the endophytic fungus, *P. betae* associated with the plant, *G. biloba* for the first time.

MATERIALS AND METHODS

Fungal isolation

Fresh and healthy leaves of *G. biloba* L. (maiden hair tree) were collected from the Konkuk University campus and processed within 24 h of collection. The fungal endophytes were isolated from the stalk part of *G. biloba* leaves (petiole region) using standard *in vitro* culture techniques (Kumaran et al., 2008a). The hyphal tips, which grew out from the plant segments were isolated and sub-cultured onto PDA and brought into pure culture. Among the fungi isolated, *P. betae* (from leaves) were recorded as the most frequent and common in all samples cultured. Photomicrographs of conidia were taken with the help of Nikon Optiphot trinocular microscope (bright field) with Nikon Coolpix900 digital camera. The isolated fungal endophytes were identified as *P. betae* on the basis of their morphological (Boerema et al., 2004) and molecular analysis.

The analysis of 18S ribosome RNA sequence of the isolates was achieved using polymerase chain reaction (PCR). DNA extraction, PCR amplification and sequencing were carried out by standard methods (Kumaran et al., 2011). The rRNA sequences of the test fungus were compared with the NCBI site database. Nucleotide BLAST program of Gene Bank were used to estimate the percentage of similarity/homology among the available fungal endophytes. The test fungus (no. RSK-21) was deposited at the laboratory culture collection, Konkuk University.

Tagging of taxol producing fungi: Extraction of fungal DNA and PCR amplification

The discs of 2 agar plugs (5 mm diameter) from the margins of actively growing hypha of the test fungus was inoculated into a 150

ml Erlenmeyer flask containing 20 ml of PD broth medium. Culture was maintained on a rotary shaker at 120 rpm at $25 \pm 2^\circ\text{C}$ for 5 days and harvested by centrifugation at 5000 g for 10 min. After being washed twice with water, the mycelia were collected by centrifugation at 3000 g for 15 min. Then 1 g of mycelia was ground into powder in liquid nitrogen using pre-chilled mortar and pestle. Extraction of the fungal DNA and PCR amplification were carried out using standard methods (Kumaran et al., 2011). The subsequent step was screening of taxol-producing fungi based on PCR amplification. Based on the conserved sequence of the *ts* gene (GenBank no: AY364469), the specific primers *ts-F* (3'-CTTAACGTCTCGGTTTAGACTCCCGG-5') and *ts-R* (3'-CTACTCGACAGGGCCTAAAGCTTTAT-5') were designed and synthesized. PCR amplification was performed in a thermal cycler (Techgene) for 32 cycles (95°C for 30 s, 60°C for 60 s and 72°C for 60 s) followed by extension for 3 min at 72°C . The PCR products were purified using the Gel extraction mini kit (Qiagen), ligated to pMD18-T vectors (TaKaRa Biotech, South Korea), transformed into *Escherichia coli* strain DH5, and then sequenced with the DYEnamic Direct dGTP Sequencing Kit (Amersham Biosciences, UK) and a 373A DNA sequencer.

Extraction of taxol

Each fungal species was grown in a 3-L Erlenmeyer flask containing 1000 ml of chemically modified liquid medium, M1D, supplemented with soytone. The discs of 3 agar plugs (5 mm diameter) containing mycelia were used as inoculum. The organisms were initially grown for 7 days under still condition and later kept in a shaking incubator (80 rpm rotation speed) for 7 days at $25 \pm 2^\circ\text{C}$ with the treatment of 16 h of light, followed by 8 h of dark cycles. The blank cultures (uninoculated sterile medium) were also maintained. After 2 weeks, the culture fluid was passed through four layers of cheese cloth to remove solids. Extra-cellular taxol was extracted and purified using different solvents (Kumaran et al., 2008b). The purified sample was subjected to chromatographic and spectroscopic analyses. The solvents used for the analyses were of high performance liquid chromatography (HPLC) grade and standard taxol (Paclitaxel) used for reference purposes was purchased from SIGMA (St. Louis, MO, USA).

Thin layer chromatography (TLC), ultra violet (UV) spectroscopy, and infra red (IR) analyses

Analysis of TLC was carried out on Merck 1 mm (20 × 20 cm) silica gel pre-coated 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); solvent E, methylene chloride: methanol: dimethylformamide (90:9:1, v/v/v) respectively. Taxol was detected with 1% w/v vanillin in sulphuric acid reagent after gentle heating (Cardellina, 1991). It appeared as a bluish spot fading to dark grey after 24 h. The area of the plate containing putative taxol was carefully removed by scraping off the silica and eluted with acetonitrile. The UV absorption of the samples was carried out with methanol at 273 nm (Wani et al., 1971) in a Backman DU-40 spectrophotometer. Samples were ground with IR grade potassium bromide pressed into discs under vacuum using spectra lab pelletiser and the spectrum was recorded in a Bruker Optics Vertex 80v FT-IR spectrometer.

High performance liquid chromatography (HPLC), liquid chromatography-mass spectroscopy (LC-MS), and proton nuclear magnetic resonance (^1H NMR) spectroscopic analyses

An HPLC study was conducted on Younglin Instrument using C^{18}

reverse phase column (Alltech Econosil, Deerfield, IL, USA; 250 mm × 4.4 mm × 10 μM) with an isocratic mobile phase consisting of methanol: water (80:20) at flow rate of 1 ml/min. Each sample of 10 μL was injected with the help of a micro syringe. Registration of peak and retention time was recorded on UV at 254 nm. Based on the HPLC analysis, fungal taxol was quantified by comparing the peak area of the samples with that of the taxol standard. Analysis of LC-MS was also carried out on samples dissolved in methanol: water (9:1 v/v). Each sample was injected in Varian LC/MS 1200L Single Quadrupole MS with a spray flow of 2 μL/min and a spray voltage of 2.2 kV by using the loop injection method. The ¹H NMR spectroscopic analysis was also performed in order to confirm the taxol structure by using Varian Unity Inova Spectrometer at 23°C (operating at 400 MHz with 16 scans and 298 real points). Samples dissolved in CDCl₃ (SIGMA) were used for the analysis. Proton spectrums were assigned by comparison of chemical shifts and by coupling constants with those of related compounds. Chemical shifts were reported as δ-values relative to tetramethylsilane (TMS) as internal reference and coupling constants were reported in Hertz.

Cytotoxicity test

Cytotoxicity effect of fungal taxol isolated from the culture filtrate of the *P. betae* was detected and quantified by using *in vitro* apoptotic method of assay (Ruckdeschel et al., 1997) on cancer cells at various concentrations. The human cancer cell lines were derived from epithelial cells of breast cancer cells (MCF-7, ATCC HTB-22), lung adenocarcinoma cells (A549, ATCC CCL-185), and glioblastoma cells (T98G, ATCC CRL-1690). The cell lines between 20 and 30 of the subculture passages were used as adherent cells. The morphological changes of the cancer cells which were treated with different concentrations of fungal taxol ranging between 0.005 and 5 μM were incubated for 48 h. The cells were then stained (DNA staining) with 0.5 mg/ml propidium iodide in PBS for 15 min and destained in phosphate buffered saline (PBS) solution. After treatment with different concentrations of fungal taxol, the cell morphology was determined by light microscopy. In all, five different fields were randomly selected for counting at least 500 cells. The percentage of apoptotic cells was calculated for each experiment. Cells designated as apoptotic were those that displayed the characteristic morphological features of apoptosis, including cell volume shrinkage, chromatin condensation and the presence of membrane bound apoptotic bodies. For each experiment, 500 cells were counted. All cytotoxicity data shown are the means of at least three independent experiments. The percentage of apoptotic cells in apoptosis were calculated by dividing the number of apoptotic cells by total number of cells × 100.

RESULTS AND DISCUSSION

Morphological and molecular characterization of the fungus

P. betae is a common plant pathogenic Coelomycetous fungus causing disease in the leaves of agricultural, horticultural and forest plants in nature. In the present study, *P. betae* was the most common and frequently isolated endophytic fungus from the selected host plant materials. Colonies grew well in PDA (5 to 7 cm diameter after 7 days) and good sporulations were obtained in about 7 to 10 days of incubation (Figure 1). The colony appeared regular to slightly irregular, grey olivaceous with an aerial mycelium. The isolated endophytic fungus

was identified with salient features of the culture morphology, conidial characters (Boerema et al., 2004) and molecular analysis. The conidia in the culture were single celled, aseptate and globose to subglobose measuring about 100 to 200 μM diameter with densely covered mycelial hairs (non-papillate pore). Overall, the conidial cells measured about 4 - 6.5 × 2.5 - 4 μM in size. Based on molecular analysis, 18s ribosomal RNA gene consisted 1020-bp fragment amplified from the DNA of *P. betae*. This sequence was similar with other endophytic fungus, *P. betae* (GenBank no: NG_016542) as revealed by nucleotide BLAST search. The similarity between the strains was found to be 99%. This close relationship between the strains confirms the fungal identification.

ts gene - molecular marker for taxol producing fungus

Specific primers were designed based on the conserved region of *ts* gene. The synthesized primers were amplified for core DNA fragment of *ts* from the test fungus, *P. betae*. The PCR amplification yielded a 637-bp product that was further sequenced. This 637-bp DNA fragment demonstrated high homology with *ts* gene in a BLAST search (Figure S1). Interestingly, the test fungus of the present study showed positive sign towards PCR for the conserved sequence of the *ts* gene. This study confirms the presence of *ts* gene in test fungus, *P. betae*, one of the most important gene actively involved in the biosynthesis of taxol. This statement is supported by the evidence from earlier report on the study of *ts* gene demonstrating taxol production under *in vitro* condition (Kumaran et al., 2011). Taxadiene synthase is one among the 12 genes specifically involved in taxol biosynthesis. It catalyses the cyclization of geranylgeranyl diphosphate to taxa-4(5), 11(12)-diene, and constructs the unique taxane skeleton, the first step in taxol biosynthesis (Chow et al., 2007; Walker and Croteau, 2001). In an earlier report, heterologous overexpression of cDNA encoding *ts* in *E. coli* was done using thioredoxin fusion expression system which increased the solubility of expressed protein (Huang et al., 1998). Another study demonstrated that *ts* was the first rate-limited enzyme in the taxol biosynthetic pathway in *Taxus* (Walker and Croteau, 2001). Subsequently, other studies also demonstrated that the purified recombinant *ts* fusion protein was similar to native protein with respect to its steady-state kinetic parameters and electrophoresis (Williams et al., 2000). Hence, the *ts* gene in taxol-producing fungi, isolated from *Taxus chinensis* has been demonstrated to be a rate-limiting gene in the fungal taxol biosynthetic pathway (Zhou et al., 2007).

In the present study, we confirmed the presence of *ts* gene in the fungal genome demonstrating a positive sign in the *de nova* biosynthesis of taxol in the artificial medium. A PCR based screening was done for *ts* to confirm the blueprint for paclitaxel biosynthesis. However,

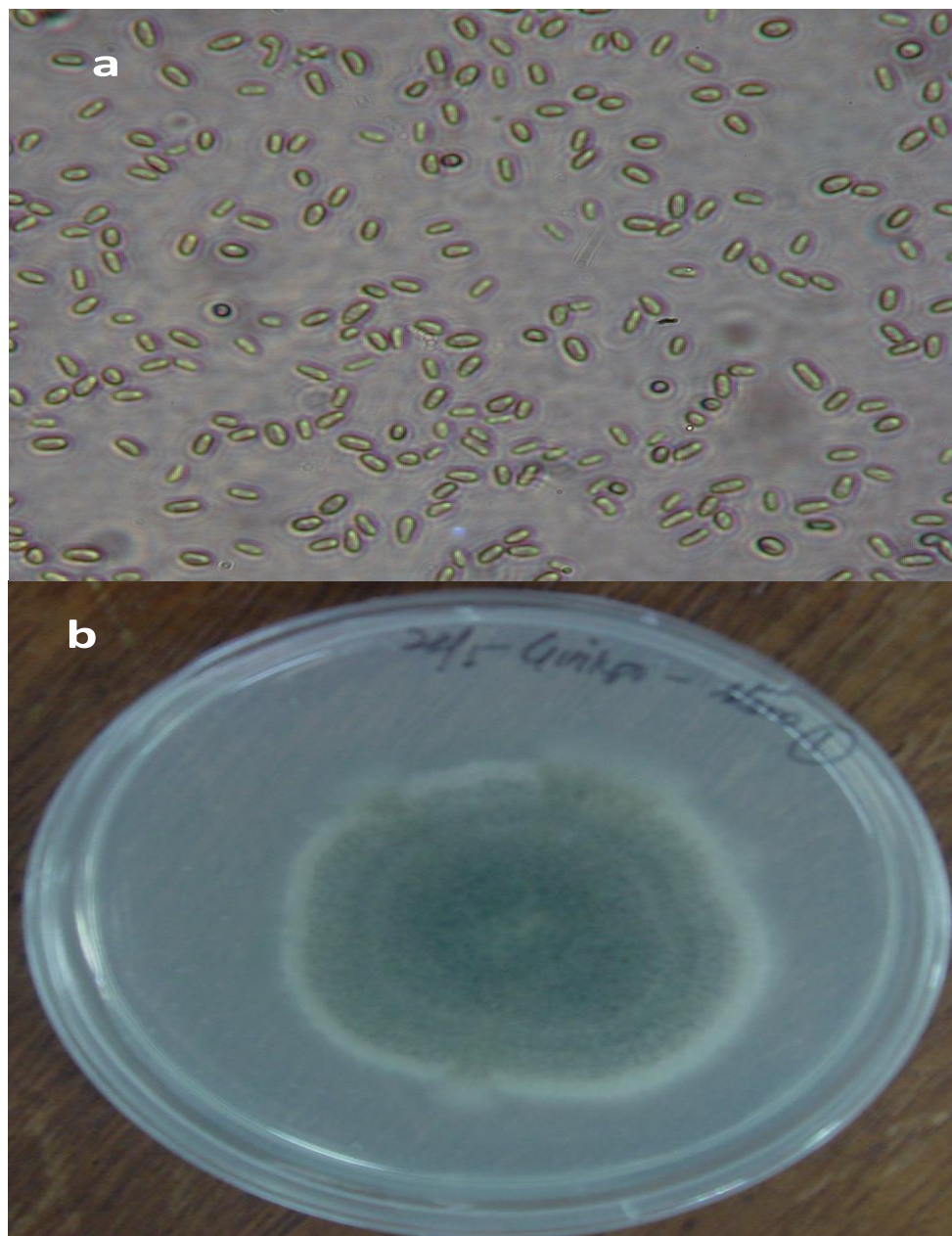


Figure 1. Asexual conidia (400 X) of *P. betae* (a) were viewed under phase contrast microscopy, and 5 day old fungal culture showing the mycelia radial growth on PDA medium (b).

in addition to *ts* gene, other genes encoding for taxol biosynthesis includes, 10-deacetylbaaccatin III-10-O-acetyl transferase and C-13 phenylpropanoid side chain-CoA acyltransferase that are used as molecular markers for screening taxol producing endophytic fungi from yews (Zhang et al., 2008).

Chromatographic study

Taxol in the fungal samples was confirmed by TLC,

displaying the taxol band under UV illumination (254 nm) and demonstrating a blue-gray colour reaction with he vanillin/sulfuric acid reagent. The taxol band displayed chromatographic properties and Rf value were similar to that of standard taxol. HPLC analysis demonstrated the presence of taxol with a peak retention time of 4.7 min (Figure 2). Also, the endophytic fungus produced a higher content of taxol in M1D (795 µg/L). Taxol detection was not observed in the blank culture samples, thereby indicating a negative result in all the analyses. In this study, the fungal taxol yield was easily quantified with

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Query 1 GAATTGCAGAGCCAAATCTGAGGGCCAAATGATGTGGGTTTGTCTCCAAATCAGGGCGAAC 60
|
Sbjct 72 GAATTGCAGAGCCAAATCTGAGGGCCAAATGATGTGGGTTTGTCTCCAAATCAGGGCGAAC 131
Query 61 CAGAGTAAAAATGTTCGAGAGGAAGTGGTGGTCTGTCCTGTCGTAATGATGAGCAGTAG 120
|
Sbjct 132 CAGAGTAAAAATGTTCGAGAGGAAGTGGTGGTCTGTCCTGTCGTAATGATGAGCAGTAG 191
Query 121 CACTGGCACTAGCAAGGTGGTTTCCGAGACTTCCAGTACCATTGTGGATGATATCCCTCG 180
|
Sbjct 192 CACTGGCACTAGCAAGGTGGTTTCCGAGACTTCCAGTACCATTGTGGATGATATCCCTCG 251
Query 181 ACTCTCCGCCAATTATCATGGCGATCTGTGGCACCACAATGTTATACAAACTCTGGAGAC 240
|
Sbjct 252 ACTCTCCGCCAATTATCATGGCGATCTGTGGCACCACAATGTTATACAAACTCTGGAGAC 311
Query 241 ACCATTTTCGTGAGAGTTCTACTTACCAAGAACGGGCAGATGAGCTGGTTGTGAAAATTAA 300
|
Sbjct 312 ACCATTTTCGTGAGAGTTCTACTTACCAAGAACGGGCAGATGAGCTGGTTGTGAAAATTAA 371
Query 301 AGATATGTTCAATGCGCTCGGAGACGGAGATATCAGTCCGTCTGCATACGACACTGCGTG 360
|
Sbjct 372 AGATATGTTCAATGCGCTCGGAGACGGAGATATCAGTCCGTCTGCATACGACACTGCGTG 431
Query 361 GGTGGCGAGGGTGGCGACCATTTCTCTGATGGATCTGAGAAGCCACGGTTTCTCAGGC 420
|
Sbjct 432 GGTGGCGAGGGTGGCGACCATTTCTCTGATGGATCTGAGAAGCCACGGTTTCTCAGGC 491
Query 421 CCTCAACTGGGTTTTCAACAACCAGCTCCAGGATGGATCGTGGGGTATCGAATCGCACTT 480
|
Sbjct 492 CCTCAACTGGGTTTTCAACAACCAGCTCCAGGATGGATCGTGGGGTATCGAATCGCACTT 551
Query 481 TAGTTTATGCGATCGATTGCTTAACACGACCAATTCTGTTATCGCCCTCTCGGTTTGAA 540
|
Sbjct 552 TAGTTTATGCGATCGATTGCTTAACACGACCAATTCTGTTATCGCCCTCTCGGTTTGAA 611
Query 541 AACAGGGCACAGCCAAGTAGAACAAGGTACTGAGTTTATTGCAGAGAATCTAAGATTACT 600
|
Sbjct 612 AACAGGGCACAGCCAAGTAGAACAAGGTACTGAGTTTATTGCAGAGAATCTAAGATTACT 671
Query 601 CAATGAGGAAGATGAGCTGTCCCCGGATTTTCGAAATA 637
|
Sbjct 672 CAATGAGGAAGATGAGCTGTCCCCGGATTTTCGAAATA 708

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Figure S1. The blast homology sequence of *ts* gene of the fungus *P. betae* with *ts* gene (AY364469.1) of the NCBI gene bank (Score = 1177 bits (637), homology/identities = 637/637 (100%), gaps = 0/637 (0%).

HPLC analysis since the production was high (in micrograms). Whereas, in earlier reports it was quantified with the aid of immunoassay since the yield level was low (in nanograms) (Strobel et al., 1996a, 1997; Li et al., 1996). The biggest problem of using fungi in fermentation is the low level of yield with unstable taxol production. Taxol yield of such reported fungi range from 24 to 70 ng/L (Stierle et al., 1993, 1996a). Consequently, the amount of taxol produced by the endophytic fungi from yew trees was relatively small, when compared with the host trees. However, the short generation time and high growth rate of fungi will make it worthwhile to continue our investigation on the endophytic fungal, *P. betae*.

Spectroscopic investigation

The presence of taxol in the fungal sample was confirmed using a spectrophotometer demonstrating UV absorption at 273 nm when compared with standard taxol (Kumaran et al., 2009). The IR spectrum demonstrated a broad peak at 3422 cm^{-1} due to the presence of O-group in the parent compound, which was evident by its OH stretch. The aliphatic CH stretch was observed at 2924 cm^{-1} . The C=O (keto group) stretch was observed at 1724 and 1658 cm^{-1} . The registration peak was observed at 1485 cm^{-1} due to NH stretching frequency. The COO stretching frequency was observed at 1371 and 1242 cm^{-1} .

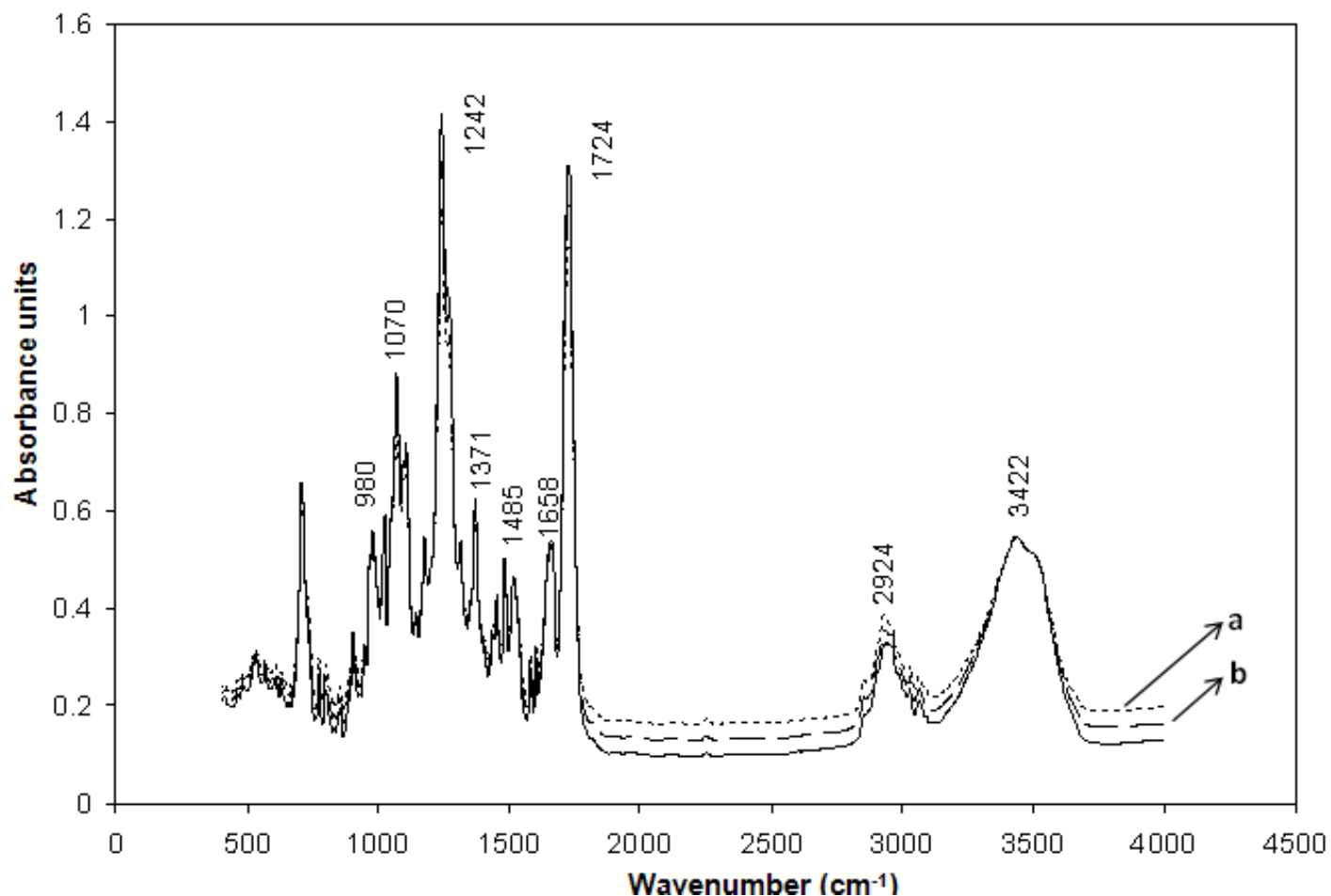


Figure 3. IR spectrum of standard taxol (a) and fungal taxol obtained from *P. betae* (b) were showing finger print region between 980 and 3500 cm^{-1} .

¹. The peaks in the range between 1070 and 980 cm^{-1} were due to the presence of aromatic C and H bonds. The fungal taxol was further confirmed by IR finger prints observed between 980 and 3500 cm^{-1} , which was similar when compared with standard taxol (Figure 3). Therefore, it was evident that the fungal samples showed positive results for taxol production in M1D medium.

In ¹H NMR spectroscopic analysis, almost all signals were well resolved and distributed in the region between 1.0 and 8.0 ppm (Figure 4). The strong three-proton signals produced by the methyl and acetyl groups lie in the region between 1.0 and 2.5 ppm with multiplets due to certain methylene groups. Most of the protons in the taxane skeleton and side-chain were observed in the region between 2.5 and 7.0 ppm. The aromatic proton signals produced by C-2' benzoate, C-3' phenyl and C-3' benzamide groups appeared between 7.0 and 8.0 ppm. The ¹H NMR spectrum of the fungal taxol was identical when compared with standard taxol. The characteristic chemical shifts of taxol are shown in Figure 4. The taxol assignments obtained in the present investigation

were also confirmed with an earlier report (Chmurny et al., 1992). Furthermore, convincing evidence for the identity of taxol was also obtained by LC-MS spectroscopic analysis (Figure 5). Characteristically, standard taxol yielded both a $(M+H)^+$ peak at a molecular weight of 854 and a $(M+Na)^+$ peak at a molecular weight of 876. Similarly, fungal taxol also produced peaks $(M+H)^+$ at m/z 854 and $(M+Na)^+$ at m/z 876 with characteristic fragment peaks at 344, 367 and 395. Major fragment ions observed in the mass spectrum of taxol are placed into three categories, representing the major portion of the taxol molecule (McClure and Schram, 1992). The peaks analogous 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. It was evident that the diterpene taxol was much more complex since its molecular weight from high-resolution mass spectrometry was 854, equivalent to the molecular formula of $C_{47}H_{51}NO_{14}$ as earlier reported (McClure and Schram, 1992).

Analytical methods acquired clearly suggested that the

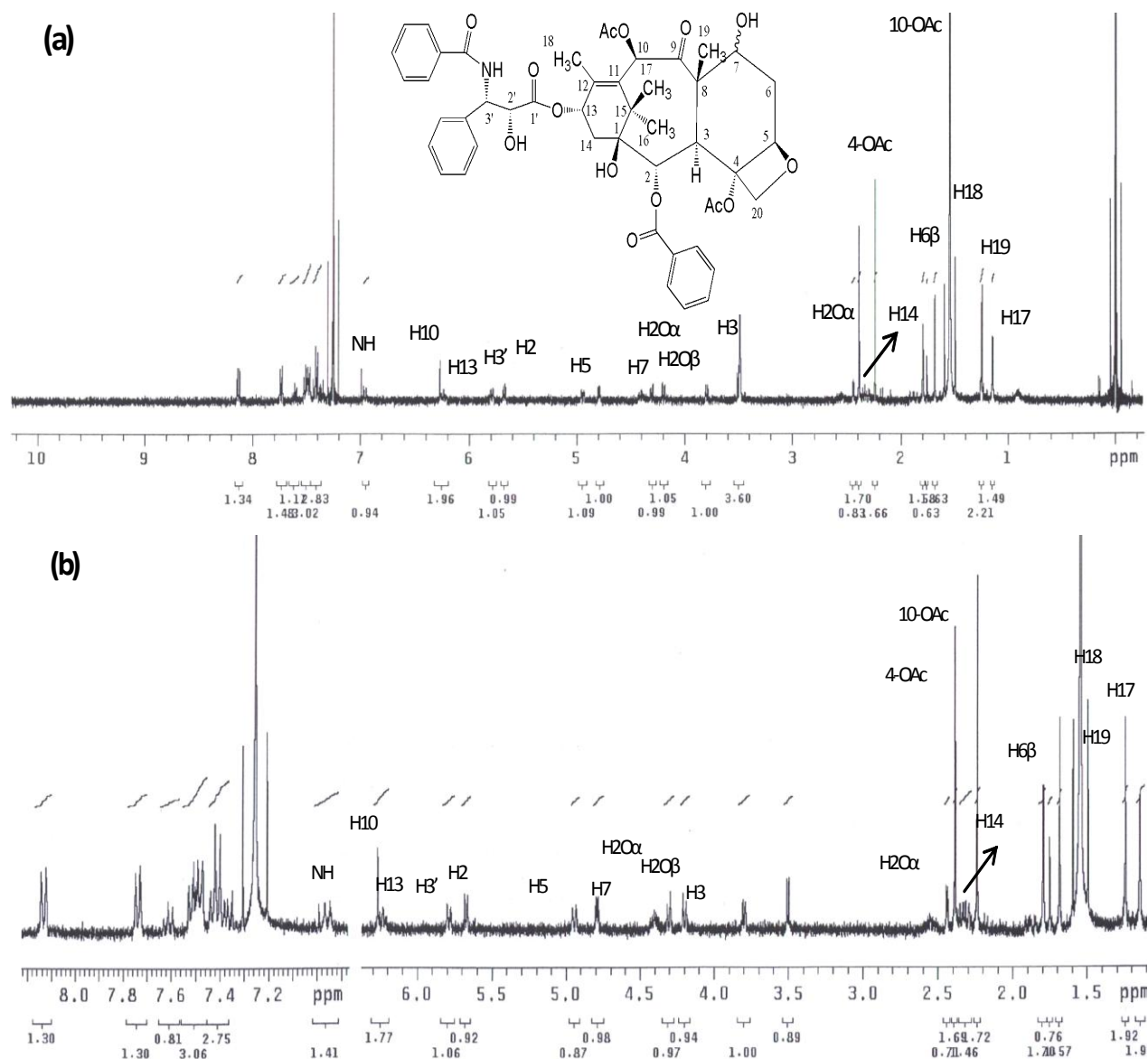


Figure 4. ¹H NMR spectrum of standard taxol (a) and fungal taxol isolated from *P. betae* (b) in CDCl₃ at 400 MHz. All the signals were well resolved and distributed in the region between 1.0 and 8.0 ppm. The chemical shifts in ppm high frequency from TMS. The structure of taxol is shown as an insert (a).

fungal compound is taxol, which was produced in M1D in comparison with standard taxol. The blank cultures did not detect the presence of taxol. The total amount of taxol produced per liter in M1D medium was 795 µg/L (*P. betae*) and found to be the highest in the fungi reported so far. The production rate was three orders of magnitude (15,900-fold) more than that produced by *T. andreanae* (Stierle et al., 1993). The taxol production rate was increased between 11 to 12-fold than that found in the culture filtrate of *Pestalotiopsis microspora* (Strobel et al., 1996b). Most of the earlier reported fungal taxol producers isolated from yews (*Taxus* spp.) demonstrated

only limited yield of taxol whereas, in the present study, *P. betae* showed a higher yield on taxol production recorded for the first time. The genetic origin of fungal taxol production has been speculated to have arisen by horizontal gene transfer from host plant to its pathogen (Strobel et al., 1996a, 1997). Little documentation exists for gene transfer from a higher plant to microorganisms.

Cytotoxic evaluation of taxol on human cancer cells

Cytotoxic effect of fungal taxol was tested by apoptotic

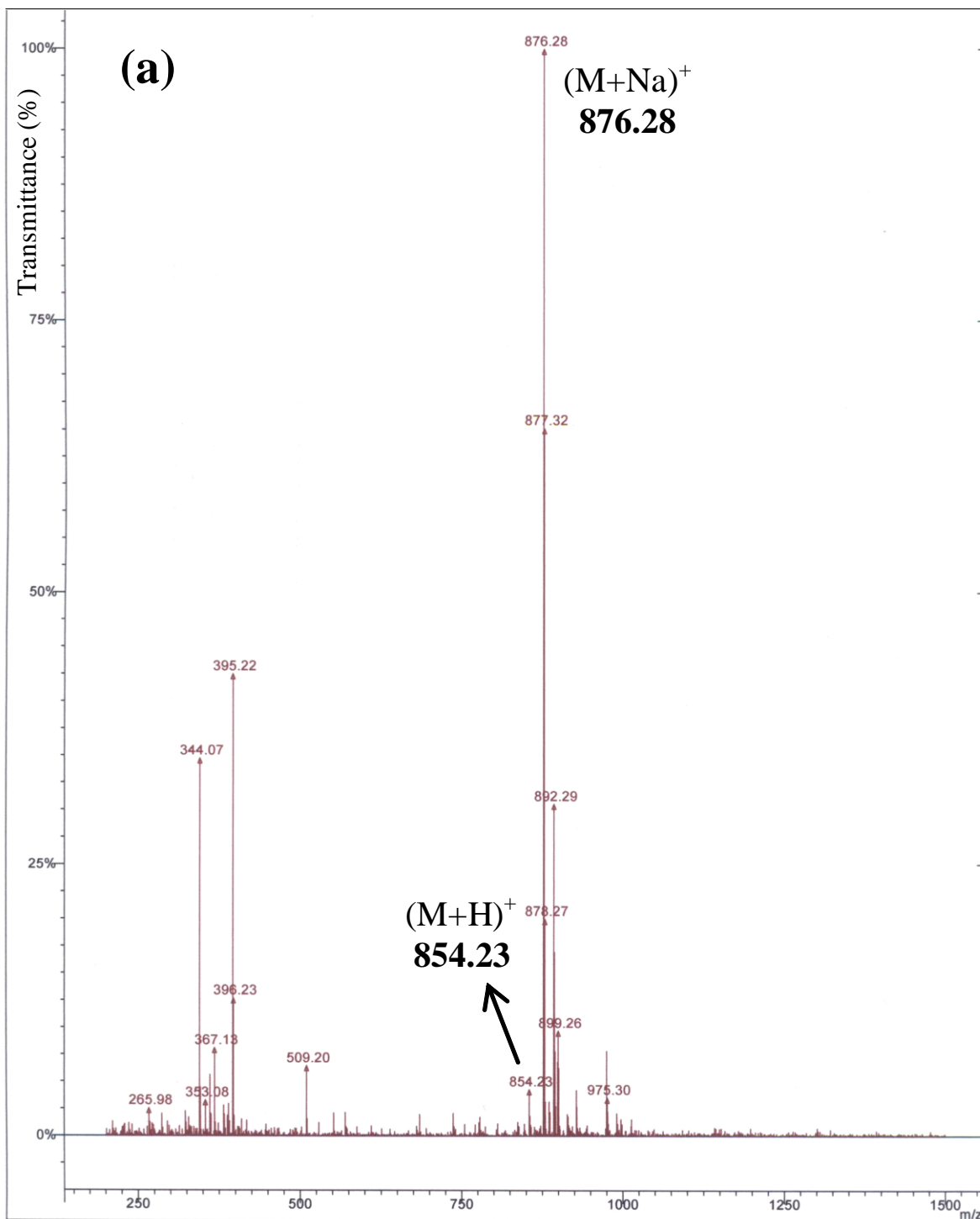


Figure 5. LC-MS analysis of standard taxol (a) and fungal taxol obtained from *P. betae* (b). Mass spectrum of the fungal extracts showing a $(M+H)^+$ peak at molecular weight of 854 and a $(M+Na)^+$ peak at molecular weight of 876.

assay on 3 different human cancer cells viz. breast cell–MCF-7, lung cell–A549 and glioblastoma cell–T98G. The assay indicated an increased taxol concentration from 0.005 to 0.05 μM . With further increase in taxol

concentration, the taxol-induced cell death through apoptosis increased slightly. When the taxol concentration increased from 0.5 to 5 μM , the taxol-induced cell death through apoptosis decreased significantly

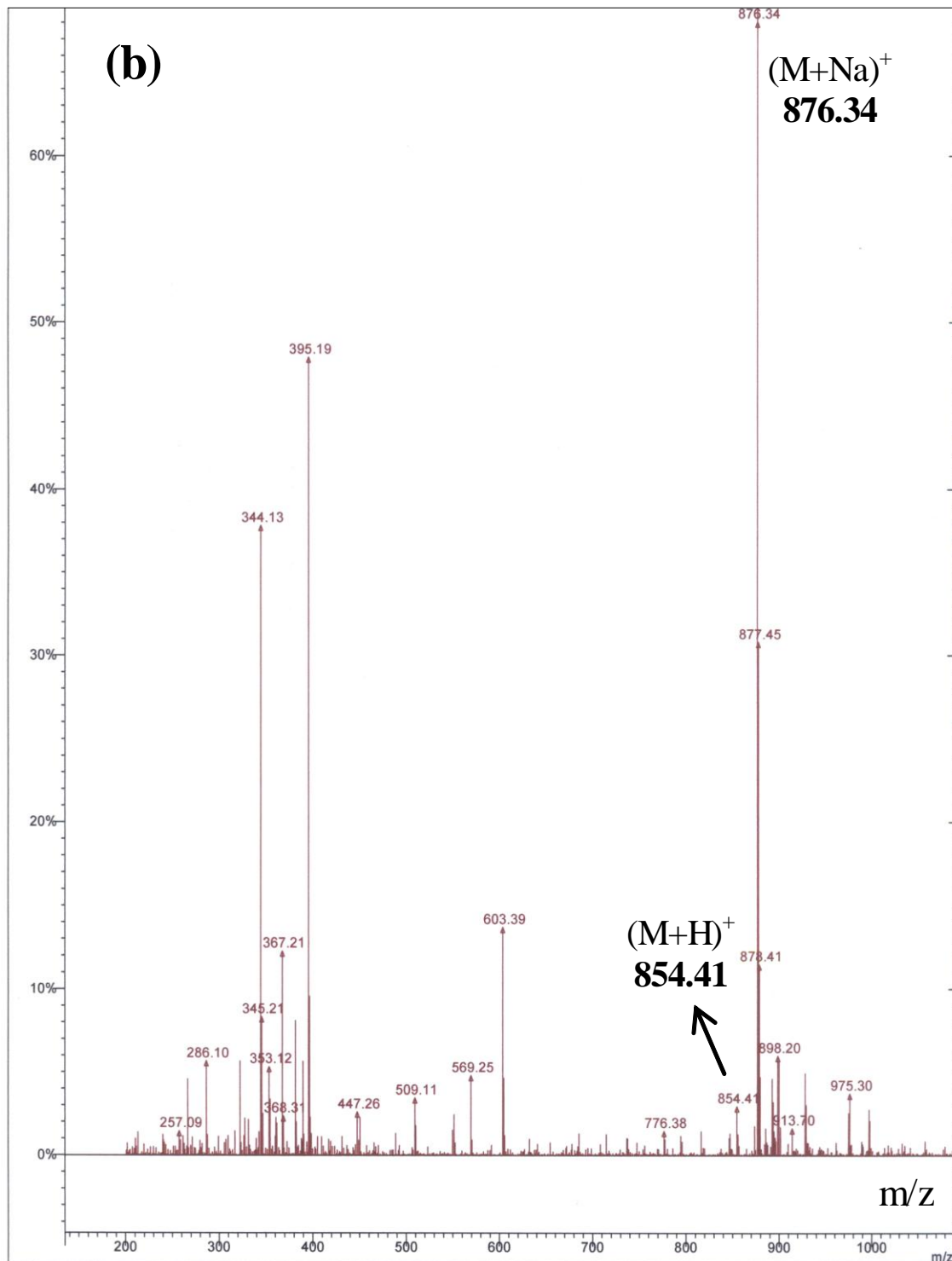


Figure 5. Contd.

(Table 1). Hence, the efficacy of fungal taxol was relatively dependent on the specific cell type. This was also concurrent with the results of earlier report (Yeung et al., 1999), thus supporting the earlier findings that at 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 of medicinal and

Table 1. Taxol-induced apoptosis by in various human cancer cell lines

S/N	Cell line	Taxol Concentration (μM)	Percentage of apoptotic cell	
			Taxol standard	Fungal taxol
1	MCF-7 (Breast cells)	0	0	0
		0.005	25.22 \pm 1.38	24.57 \pm 2.54
		0.05	62.72 \pm 4.48	60.87 \pm 4.45
		0.5	79.51 \pm 5.68	80.35 \pm 5.98
		5	35.74 \pm 3.24	35.94 \pm 2.34
2	BT 220 (Lung cells)	0	0	0
		0.005	25.22 \pm 1.38	13.65 \pm 1.55
		0.05	62.72 \pm 4.48	52.62 \pm 2.59
		0.5	79.51 \pm 5.68	72.75 \pm 5.49
		5	35.74 \pm 3.24	29.85 \pm 2.86
3	HL 251 (glioblastoma cells)	0	0	0
		0.005	15.37 \pm 1.67	14.73 \pm 1.654
		0.05	60.27 \pm 2.85	61.54 (\pm 3.23)
		0.5	74.64 \pm 3.54	70.07 \pm 5.35
		5	32.84 \pm 1.80	35.15 \pm 3.45

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 higher in *P. betae*. In the present study, it was confidently evidenced that the spectroscopic and chromatographic estimates are close to authenticity given the fact that the fungal taxol and standard taxol yielded identical results. As an alternate method, gene encoding for *ts* (taxol biosynthetic enzyme) have been used as a molecular marker for screening taxol producing fungal endophytes. It was also indicated that the formation of taxol by the fungus, *P. betae* was found to be the highest, suggesting that the fungus can serve as a potential species for genetic engineering to enhance the production of taxol that is currently underway.

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