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Isolation and Identification of Taxol, an Anticancer Drug from *Phyllosticta melochiae* Yates, an Endophytic Fungus of *Melochia corchorifolia* L.

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Abstract *Phyllosticta melochiae*, an endophytic fungus isolated from the healthy leaves of *Melochia corchorifolia*, was screened for the production of an anticancer drug, taxol on modified liquid medium and potato dextrose broth medium in culture for the first time. The presence of taxol was confirmed by spectroscopic and chromatographic methods of analysis. The amount of taxol produced by this fungus was quantified by high performance liquid chromatography. The maximum amount of fungal taxol production was recorded as 274 μg/L. The production rate was increased to 5.5×1,000 fold than that found in the culture broth of earlier reported fungus, *Taxomyces andreanae*. The fungal taxol extracted also showed a strong cytotoxic activity in the *in vitro* culture of tested human cancer cells by apoptotic assay. The results designate that the fungal endophyte, *P. melochiae* is an excellent candidate for an alternate source of taxol supply and can serve as a potential species for genetic engineering to enhance the production of taxol to a higher level.

Keywords: anticancer drug, fungal endophyte, *in vitro* cytotoxicity test, *Phyllosticta melochiae*, taxol production

Introduction

Taxol is a highly functionalized and expensive diterpene, anticancer drug widely used in hospitals and clinics. It is especially targeted to treat breast, lung, and ovarian cancers and was originally isolated from the bark of the Pacific yew, *Taxus brevifolia* (1). Its mode of action is revealed by the fact that the taxol binds specifically with tubulin-β and prevents its depolymerization during the process of cell division (2). Taxol is found extremely in low amounts in the needles, bark, and roots of *Taxus* spp. Ultimately, to lower the price of taxol and make it more available, a fermentation process involving microorganisms would be the most desirable and alternate source of supply (3). The search for novel sources of taxol from trees has lead to the isolation of an endophytic fungus, *Taxomyces andreanae* colonizing the inner bark of the yew tree which is capable of producing taxol and other taxanes *de novo* when grown in semi-synthetic medium (4). A diversity of endophytic fungal species isolated from *Taxus* spp. has also been reported to produce taxol (3-5). However, the yield of taxol and taxanes were found to be low. Over the last 15 years, there has been a great deal of interest in finding endophytic fungi isolated from *Taxus* spp. and a few other gymnosperm plant species (3-14). Specifically, these species alone were given importance towards the isolation of fungal taxol. In the present study, an attempt was made to screen taxol from the endophytic fungus, *Phyllosticta melochiae* associated with the leaves of *Melochia corchorifolia*, an angiosperm for the first time.

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Materials and Methods

Fungal isolation and identification *Phyllosticta melochiae* Yates was isolated from the healthy leaves of *M. corchorifolia* L. (Chocolate-weed, Family: Sterculiaceae) collected from the Kodaikanal region of Tamilnadu, India. The leaf samples were thoroughly washed in running tap water and cut into small fragments (approximately 0.5 cm^2 pieces) with the aid of a flame-sterilized razor blade. Then the leaf fragments were surface sterilized by immersion in 70% ethanol for 5 sec, followed by 4% sodium hypochlorite for 60 sec and then rinsed thrice in sterile distilled water for 10 sec each. The excess moisture was blotted on a sterile filter paper. The surface sterilized leaf segments were evenly spaced in petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium (amended with chloramphenicol 150 mg/L). The petri dishes were sealed using Parafilm™ and incubated at 24±2°C in a light chamber with 16 hr of light, followed by 8 hr of dark cycles. The petri dishes were monitored every day to check the growth of fungal colonies from the leaf segments. The hyphal tips, which grew out from the leaf segments were isolated and sub-cultured onto PDA and brought into pure culture. Photomicrographs of conidia were taken with the help of Carl Zeiss Axiostar plus-Photomicroscope (phase contrast) with Nikon FM 10 Camera using Konica film. The isolated fungus was identified as *P. melochiae* by using standard monograph (15). The fungal culture was deposited at Madras University Botany Laboratory culture collection, University of Madras (MUBL). The Colelomycetous fungus, *P. melochiae* (MUBL No. 614), was screened for taxol production.

Extraction of taxol The fungus was grown in a 3-L Erlenmeyer flask containing 1,000 mL of modified M1D

medium supplemented with soytone (16). The potato dextrose broth (PDB) was also prepared for screening the test fungus for taxol production. The discs of 3 agar plugs (5 mm diameter) containing mycelia were used as inoculum. The organisms were grown at 24±2°C under still condition for 3 weeks in a light chamber with 16 hr of light, followed by 8 hr of dark cycles. The blank cultures (uninoculated sterile medium) were also maintained. After 3 weeks, the culture fluid was passed through 4 layers of cheese cloth to remove solids. Extracellular taxol was extracted from the culture medium by using dichloromethane (7). The solvent was then removed by evaporation under reduced pressure at 35°C with a rotary vacuum evaporator. The solid residue was dissolved in 1 mL of dichloromethane and placed on a 1.5×30 cm column of silica gel (40 μm). Elution of the column was performed in a stepwise manner starting with 70 mL of 100% dichloromethane, followed by methylene chloride:ethylacetate at different proportions (viz., 20:1 $_{v/v}$, 10:1 $_{v/v}$, 6:1 $_{v/v}$, 3:1 $_{v/v}$, 1:1 $_{v/v}$). Fractions with same mobility as the standard taxol were combined and evaporated to dryness. The residue was subjected to chromatographic and spectroscopic analyses. The solvents used for the analyses were high performance liquid chromatography (HPLC) grade and standard taxol (Paclitaxel) used for reference purposes was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Thin layer chromatography (TLC), ultra violet (UV), and infrared (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 (17). It appeared as a bluish spot fading to dark grey after 24 hr. 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 (1) in a Beckman DU-40 spectrophotometer. Samples were ground with IR grade KBr pressed into discs under vacuum using spectra lab pelletiser and the spectrum was recorded in a Bruker Optics Vertex 80v FT-IR spectrometer.

HPLC, liquid chromatography-mass spectroscopy (LC-MS), and proton nuclear magnetic resonance (^{1}H NMR) spectroscopic analyses A HPLC study was conducted on HP1100 series using C_{18} reverse phase column (Bondapak, 300 mm×3.9 mm×10 μm) with an isocratic mobile phase consisting of methanol:water (80:20, v/v) 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 ^{1}H 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 $CDCl_3$ (Sigma-Aldrich) were used for the analysis. Proton spectrums were assigned by comparison of chemical shifts and by coupling

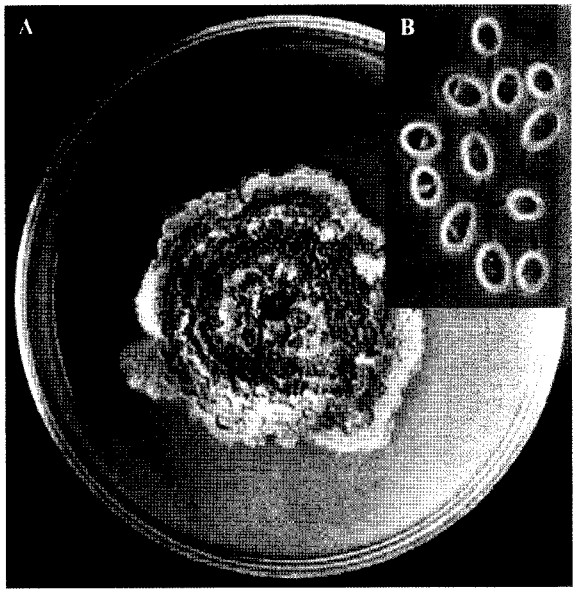

Fig. 1. Culture morphology of the taxol producing fungus, *P. melochiae*. A. Twelve day old fungal colony on PDA medium showing the mycelial growth with the formation of globular fruit bodies called pycnidia. B. Inserted figure: Conidial cells (400×) viewed under phase contrast microscopy showing apical appendage.

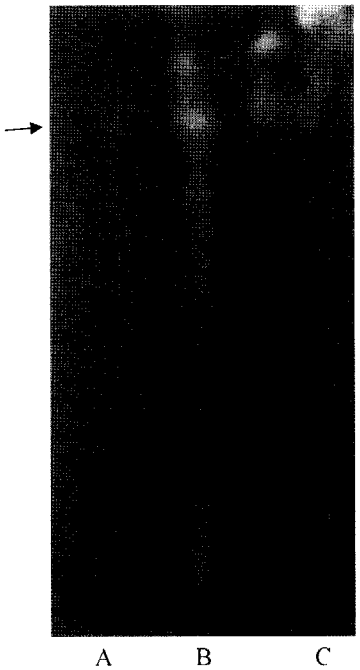

Fig. 2. TLC analysis of standard taxol (A) and fungal taxol from M1D (B), and PDB (C) in silica gel pre-coated aluminium sheet. Arrow mark indicates the presence of taxol spot in all the 3 samples illuminated with UV at 254 nm.

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 for taxol Cytotoxicity effect of fungal taxol isolated from the culture filtrate of M1D and PDB, were detected and quantified by using *in vitro* apoptotic method of assay (18) on various cancer cells, at various concentrations. The human cancer cell lines (HLK 210, H116, Int 407, HL 251, and BT 220) used in the present study, were procured from the National Centre for Cell Sciences (NCCS), Pune, India. 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 hr. The cells were then stained (DNA staining) with 0.5 mg/mL propidium iodide in phosphate buffered saline (PBS) for 15 min and destained in PBS solution. After treatment with different concentrations of fungal taxol, the cell morphology was determined by light microscopy. In all, 5 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 3 independent experiments. The cells in the apoptosis were calculated by using the following formula

$$\% \text{ Apoptotic cells} = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells}} \times 100$$

Results and Discussion

Morphological characterization of the fungus *Phyllosticta melochiae* is an endophytic Coelomycetous fungus. Colonies grew well in PDA and good sporulation was obtained in about 10-15 days of incubation (Fig. 1A). Pycnidia were single or growing together in groups, unilocular, 40-100 μm in diameter with a sub-circular ostiole of 6-14 μm diameter in size. Conidia were one-celled, hyaline, obovoidal, ovoidal to almost globose, 7.5-12×6-7.5 μm, enclosed by slime with an apical appendage, 4-5 μm long (Fig. 1B). Van der Aa (15) reported the species on the

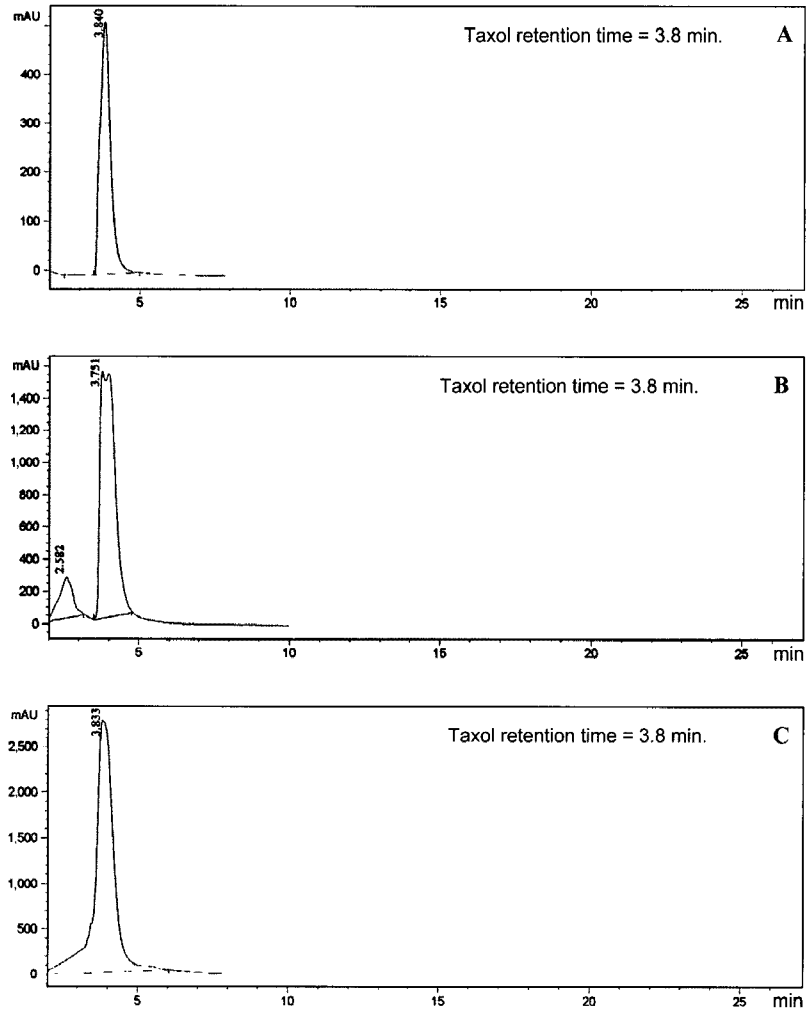

Fig. 3. HPLC analysis of standard taxol (A), and fungal taxol from M1D (B) and PDB (C). The mobile phase was methanol/water (80:20, v/v), with the flow rate of 1.0 mL/min. The registration of peak and retention time was recorded on UV at 254 nm. Fungal samples showing a peak with retention time 3.8 min, which were found to be identical in comparison with standard taxol.

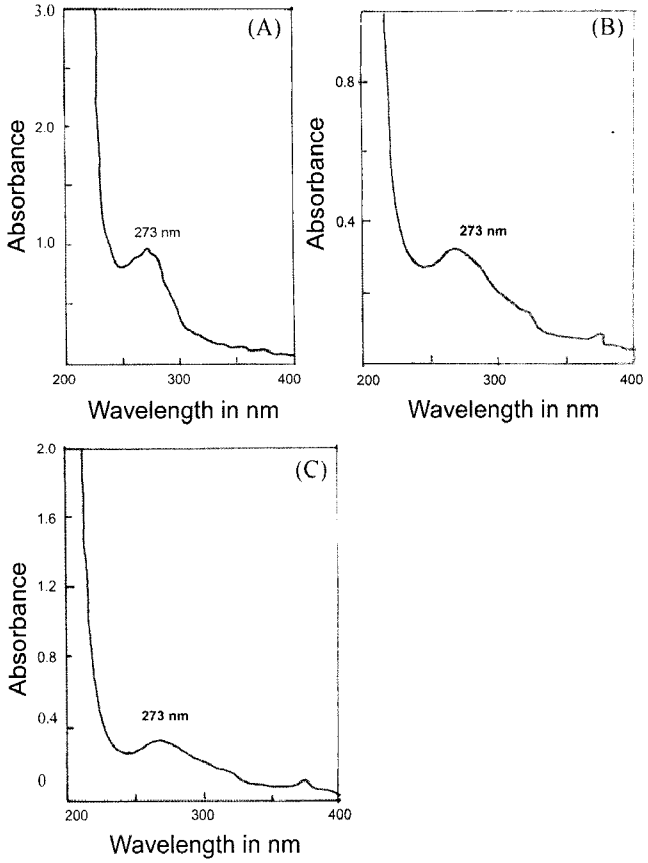

Fig. 4. UV spectrophotometric analysis of standard taxol (A), fungal taxol obtained from M1D (B) and PDB (C) showing the maximum absorption at a wave length of 273 nm in methanol.

leaves of *Melochia* sp. as a pathogen, whereas in the present study it was isolated as an endophyte from the fresh uninfected leaves of *M. corchorifolia.* This species was reported for the first time in India as a new record not before reported to the scientific community.

Chromatographic study Presence of taxol in the fungal samples were confirmed by TLC, displaying taxol band under UV illumination at 254 nm (Fig. 2) showing a blue-gray color reaction with the vanillin/sulfuric acid reagent. The compounds displayed chromatographic properties similar to that of standard taxol, giving color reaction with the spray reagent and exhibiting a Rf value identical with the taxol standard (17). Results of HPLC analysis showed the presence of taxol by showing a peak with a retention time of 3.8 min (Fig. 3A-C). *P. melochiae* produced a high content of taxol in M1D (274 μg/L) when compared with PDB (126 μg/L). Taxol detection was not observed in the blank culture samples, where they showed negative results in all analyses. The fungal taxol yield was easily quantified with HPLC analysis since the production was found to be higher (in μg). Whereas, in the earlier reports it was quantified with the aid of immunoassay since the yield level was recorded to be low (in ng) (5,6,8). The biggest problem of using fungi in fermentation is the low level of yield accompanied by unstable taxol production. Taxol yield of such reported fungi diverge from 24 to 70 ng/L (4,5). Although, the amount of taxol produced by the

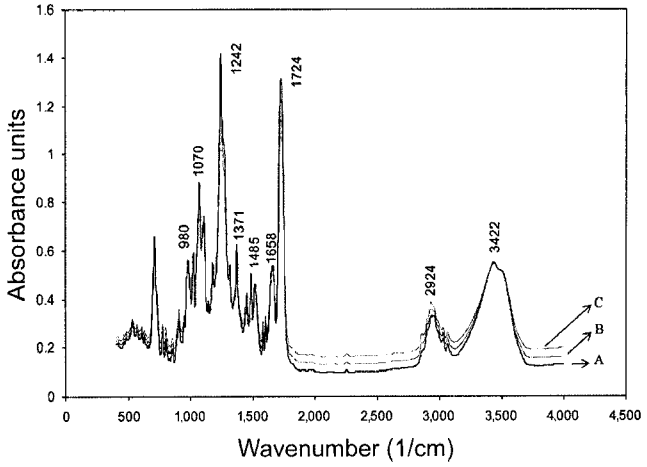

Fig. 5. IR spectrum of standard taxol (A), fungal taxol obtained from M1D (B) and PDB (C) showing finger print region between 980 and 3,500/cm.

endophytic fungi associated with yew trees is relatively small, when compared with the host trees, the short generation time, and high growth rate of fungi will make it worthwhile to continue our investigation on *P. melochiae* isolated from *M. corchorifolia.*

Spectroscopic investigation The UV absorption at 273 nm in the spectrophotometer (Fig. 4A-C) also authenticates the presence of taxol in the fungal samples, in comparison with standard taxol (1). The IR spectrum showed a broad peak at 3,422/cm, which was assigned for the presence of O-group in the parent compound, and it was evident by its OH stretch. The aliphatic CH stretch was observed at 2,924/cm. The C=O (keto group) stretch was positioned at 1,724 and 1,658/cm. The registration peak observed at 1,485/cm was due to NH stretching frequency. The COO stretching frequency was observed at 1,371 and 1,242/cm. The peaks in the range between 1,070 and 980/cm were due to the presence of aromatic C and H bends. Fungal taxol was further confirmed by IR finger prints recorded between 980 and 3,500/cm, which was also identical in comparison with standard taxol (Fig. 5A-C). Therefore, it was evident that this fungus showed positive results for taxol production in both M1D and PDB medium.

In ^{1}H NMR spectroscopic analysis, almost all signals were well resolved and distributed in the region between 1.0 and 8.5 ppm (Fig. 6A-C). The strong 3-proton signals caused by the methyl and acetyl groups lie in the region between 1.0 and 2.5 ppm, together with multiplets caused by certain methylene groups. Most of the protons in the taxane skeleton and the side chain were observed in the region between 2.5 and 7.0 ppm, and the aromatic proton signals caused by C-2 benzoate, C-3 phenyl, and C-3 benzamide groups appeared between 7.0 and 8.3 ppm. The ^{1}H NMR spectrum of the fungal taxol was found to be identical in comparison with standard taxol. The characteristic chemical shifts of taxol are shown in Fig. 6. The taxol assignments obtained in the present investigation were also confirmed with an earlier report (19). Further convincing evidence for the identity of taxol was obtained by LC-MS spectroscopic analysis (Fig. 7A-C). Characteristically, standard taxol yielded both a $(M+H)^+$ peak at a molecular

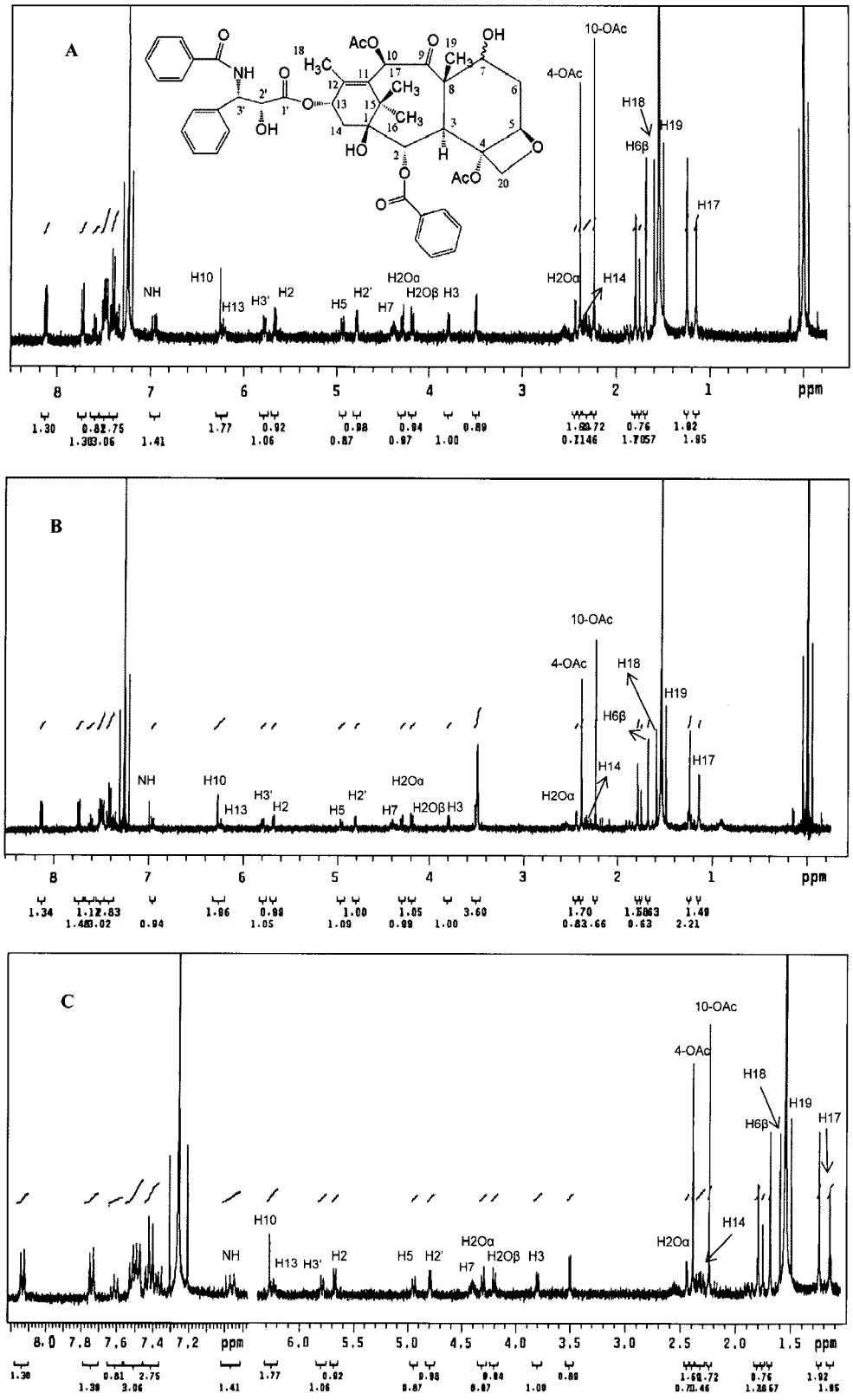

Fig. 6. ^{1}H NMR spectrum of standard taxol (A), fungal taxol from M1D (B) and PDB (C) in $CDCl_3$ at 400 MHz. All the signals were well resolved and distributed in the region between 1.0 and 8.5 ppm. The chemical shifts in ppm high frequenzy from TMS. The structure of taxol is shown as an insert (A). In comparison with standard taxol, fungal taxol also produced an identical spectrum.

weight of 854 and a $(M+Na)^+$ peak at a molecular weight of 876. On comparison, 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 3 categories, which represent the major portion

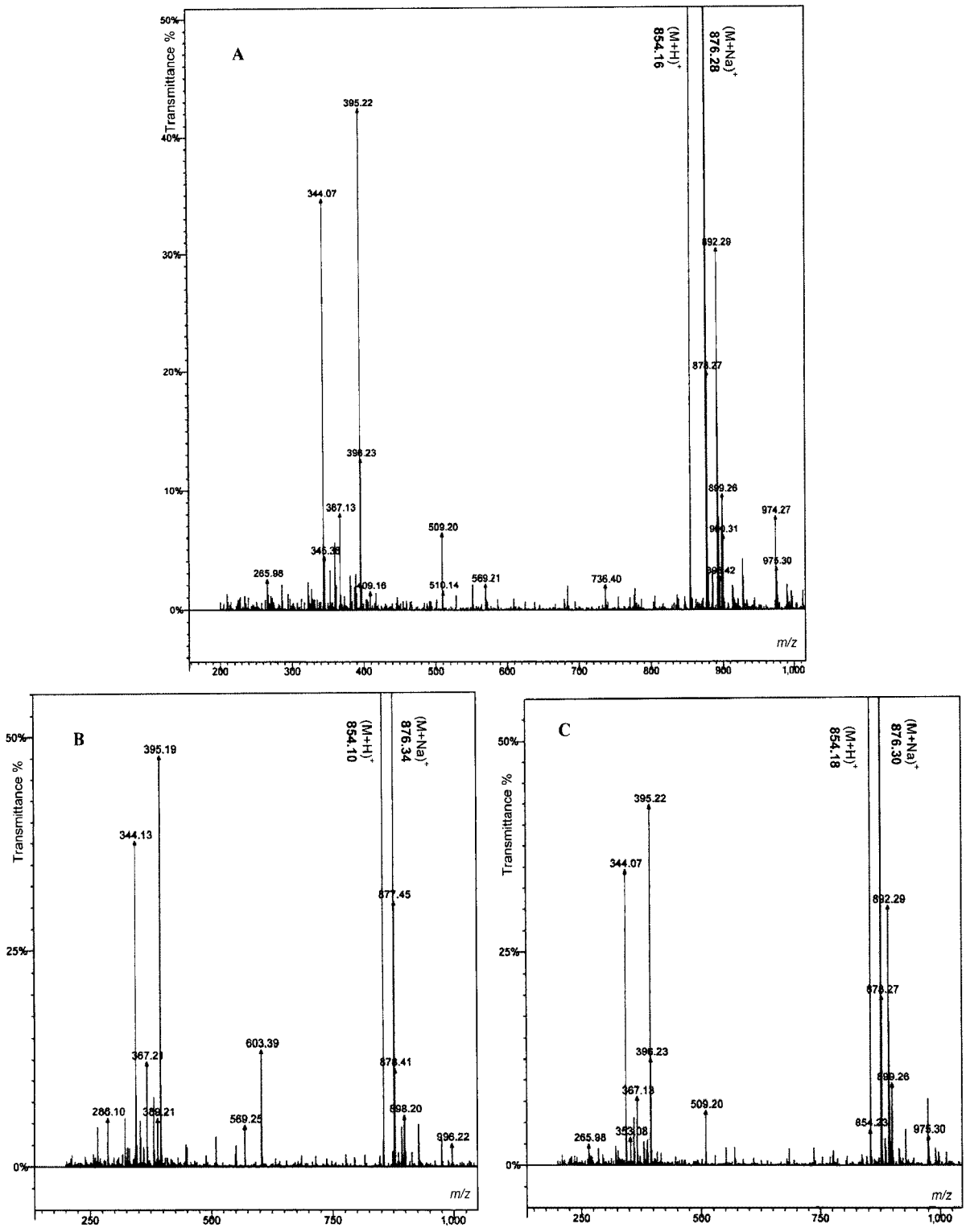

Fig. 7. LC-MS analysis of standard taxol (A), fungal taxol from M1D (B) and PDB (C). Mass spectrum of the fungal extracts showing a $(M+H)^+$ peak at molecular weight of 854 and a $(M+Na)^+$ peak at molecular weight of 876 was identical in comparison with standard taxol. Sample was dissolved in methanol/water (9:1, v/v) and injected with a spray flow of 2 μL/min with a spray voltage of 2.2 kV.

of the taxol molecule (20). The peaks analogous to taxol exhibited mass-to-charge (*m/z*) ratios corresponding to the molecular ions $(M+H)^+$ of the 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 is 854, corresponding to the molecular formula of $C_{47}H_{51}NO_{14}$ as reported earlier (20).

Analytical methods acquired clearly suggested that the fungal compound is taxol, which was produced in both M1D and PDB in comparison with standard taxol. The blank cultures did not detect the presence of taxol. The total amount of taxol produced/L in M1D, was 274 μg, and found to be the highest in the fungi reported so far (Table

Table 1. Production of taxol evidenced from different fungal species and its hosts

Fungal endophytes	Host plant	Plant group	Taxol yield/L	References
Taxomyces andeanae	*Taxus brevifolia*	Gymnosperm	24-50 ng	Stierle *et al.* (4)
Monochaetia sp.			102 ng	
Fusarium lateritium	*T. baccata*	Gymnosperm	130 ng	
Pestalotia bicilia			1081 ng	
Alternaria sp.			157 ng	Strobel *et al.* (5)
Pestalotiopsis microspora	*T. cuspidata*	Gymnosperm	268 ng	
P. microspora	*T. wallachiana*	Gymnosperm	500 ng	
Pithomyces sp.	*T. sumatrana*	Gymnosperm	95 ng	
P. microspora	*Taxodium distichum*	Gymnosperm	14-1487 ng	Li *et al.* (6)
P. microspora	*T. walachiana*	Gymnosperm	60-70 μg	Strobel *et al.* (7)
P. guepinii			485 ng	
Pestalotiopsis sp.1	*Wollemia nobilis*	Gymnosperm	172 ng	Strobel *et al.* (8)
Pestalotiopsis sp.3			127 ng	
Periconia sp.	*Torreya grandifolia*	Gymnosperm	30-831 ng	Li *et al.* (9)
P. microspora	*T. walachiana*	Gymnosperm	0.312-1.8 μg	Li *et al.* (10)
Alternaria sp.	*Ginkgo biloba*	Gymnosperm	115-260 ng	Kim *et al.* (11)
Pestalotia heterocornis	from yew forest soil	-	31 μg	Noh *et al.* (12)
Ozonium sp. (BT2)	*T. chinensis*	Gymnosperm	50-1487 ng	Guo *et al.* (13)
Phyllosticta spinarum	*Cupressus* sp.	Gymnosperm	235 μg	Kumaran *et al.* (14)
Phyllosticta melochia	*Melochia corchorifolia*	Angiosperm-Flowering plant	126 & 274 μg	Present study

1). This was 3 orders of magnitude (i.e., 5.5×1,000 fold) more than that produced by *T. andreanae* (4). The taxol production rate was increased to 4-fold than that found in the culture filtrate of *Pestalotiopsis microspora* (7). Most of the earlier reported fungal taxol producers are isolated as endophytic forms especially from yews (*Taxus* spp.) rather than other plant groups. Where as, in the present study, *P. melochiae* is a endophytic fungus isolated from the healthy leaves of an angiosperm plant, *M. corchorifolia* exhibited extracellular taxol production in M1D and PDB medium for the first time. Thus, taxol-producing fungi may be found not only in yews, but from other plant species that share the same environmental requirements as the yew. The genetic origin of fungal taxol production has been speculated to have arisen by horizontal gene transfer from host plant to its endophytes (7,8). Little documentation exists for gene transfer from a higher plant to an endophyte or parasite. However, fungi may be an independently evolved system for taxol production (5). Fungi are, obviously, a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential.

Cytotoxic evaluation of taxol on human cancer cells

Cytotoxicity effect of fungal taxol from *P. melochiae* was further tested using apoptotic assay on various human cancer cells viz. leukemia cell HLK 210, lung cell HL 251, intestine cell Int 407, colon cell H 116, breast cell BT 220. It is indicated that with the increase of taxol concentration from 0.005 to 0.05 μM, taxol induced increased cell death through apoptosis. With further increase of taxol concentration from 0.05 to 0.5 μM, the taxol-induced cell death through apoptosis only increased slightly. When the taxol concentration

Table 2. Taxol-induced apoptosis by the endophytic fungi, *P. melochiae* in various human cancer cell lines

Sl.No.	Cell lines	Taxol conc. (μM)	% Apoptotic cell	
			Taxol from M1D	Taxol from PDP
1	HLK 210	0	0	0
	(Leukemia)	0.005	24.2±1.32	23.7±1.44
		0.05	63.7±3.58	62.8±2.74
		0.5	78.5±4.68	77.4±4.62
		5	34.7±3.24	34.2±2.98
2	HL 251	0	0	0
	(Lung)	0.005	18.5±1.23	18.2±0.94
		0.05	65.7±2.96	63.6±3.26
		0.5	77.4±4.22	76.3±3.88
		5	35.6±2.34	35.2±2.15
3	Int 407	0	0	0
	(Intestine)	0.005	19.3±1.15	18.8±0.92
		0.05	65.4±2.85	64.5±2.27
		0.5	76.7±4.34	74.9±3.74
		5	22.5±1.56	21.7±1.38
4	H 116	0	0	
	(Colon)	0.005	15.3±0.67	15.2±0.88
		0.05	60.2±2.85	58.6±1.95
		0.5	74.6±3.54	73.5±4.45
		5	32.8±1.80	32.4±1.57
5	BT 220	0	0	0
	(Breast)	0.005	18.6±0.85	18.2±0.58
		0.05	78.4±3.37	77.6±2.95
		0.5	83.8±4.44	81.7±3.91
		5	24.7±1.20	24.3±1.48

was increased from 0.5 to 5 μM, the taxol-induced cell death through apoptosis decreased significantly (Table 2). In the present investigation, it was observed at low to medium concentration (0.005 to 5 μM), the efficacy of fungal taxol was relatively dependent on the specific cell type. This is in concurrence with the results of earlier report (21). It has been reported that taxol at low concentrations (nM) induces cell apoptosis and the efficacy of taxol is fairly dependent on the specific cell type. This also supports the earlier findings of other groups that at low concentration, taxol inhibits cell proliferation by blocking mitosis (18,21).

Taxol production from the fungi reported so far are isolated as endophytic forms from gymnosperm plants. Where as, in this investigation, production of taxol was recorded from an endophytic fungus, *P. melochiae*, isolated from *M. corchorifolia* an angiosperm plant, for the first time. In the present study, it is confidently evidenced that the spectroscopic and chromatographic estimates are close to reality given the fact that the fungal taxol and standard taxol yielded identical results. It also indicates that the formation of taxol by the fungus, *P. melochiae*, was found to be the highest, and suggests that the fungus can serve as a potential species for genetic engineering to enhance the production of taxol.

Acknowledgments

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