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RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF ANTICANCER COMPOUND, TAXOL FROM AN ENDOPHYTIC FUNGUS *PHOMOPSIS LONGICOLLA*

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ABSTRACT

Taxol is that the most renowned and best antineoplastic agent belonging to the massive family of taxane diterpenoids. To stare for the taxol manufacturing endophytic fungi, nearly seventy seven Coelomycetes fungi were isolated as associate endophytes from five medicinal plants *viz*., *Anisomeles malabarica* (L.) R. Br., *Mesua ferrea* L., *Piper nigrum* L., *Rauvolfia tetraphylla* L. and *Vitex negundo* L. procured from Aliyar, situated in Anamalai foothills, Western Ghats, Southern India, Tamilnadu. Seventy seven endophytic coelomycetous fungi obtained from the plants were subjected to screening for the production of taxol. Presence of taxol was detected by chromatographic and spectrometric analysis. High Performance Liquid Chromatography showed that the amount of taxol produced by *P. longicolla* was 381µg/L. The present work accomplish that the formation of taxol by endophytic fungi can serve as a potential species in genetic engineering for improving the production of taxol to overcome the urgent need in clinics.

INTRODUCTION

Cancer is a growing public health menace and has been an austere killer disease in most of the countries. Taxol, a malignant tumor drug is a compound, effective within the treatment of varied sorts of cancer. Taxol was isolated from the bark of the Pacific yew (*Taxus brevifolia*) and its structure was elucidated (**Wani *et al*., 1971**). The endophytic fungi was found to be a good source of biologically active secondary metabolites (**Pandey *et al*., 2014; Liang *et al*., 2015; Pinheiro *et al*., 2013; Li *et al*., 2012**). An endophytic fungus was isolated from *Taxus chinensis* var. *mairei*, and it was observed to produce taxol (**Guo *et al*., 2006**). The role of taxol depolymerizing microtubules in the ultimate growth of cancer cells is by means that the cell division could not occur. The foremost common source of taxol is that the bark of trees belonging to the Taxus family as well as Yew trees. Unfortunately, these trees tend to be rare, apathetic growing, and an outsized amount of bark ought to be processed to appetite paltry bulk of the drug. Over the last decade, there has been an excellent deal of interest in finding different fungi that end up taxol by endophytic fungi (**Zhou *et al*., 2001**). With the discovery that certain endophytic fungi are able to produce taxol has brought the possibility that a cheaper and more widely available product may eventually be available via industrial fermentation. *Pestalotiopsis guepini* was isolated from extraordinarily rare and antecedently thought to be extinct, Wollemi pine (*Wollemia nobilis*), a non-Taxus plant that has been proved to provide taxol (**Strobel *et al*., 1997**) and additionally *Seimatoantlerium tepuiense* isolated from a Madderwort, (*Maguireothamnus speciosus*) produced taxol (**Strobel *et al*., 1999**). Therefore, the use of endophytic fungi opens up new areas of biotechnological exploitations, thus emphasizing the necessity to isolate and cultivate these organisms.

The endophytic fungus *Phomopsis* sp. exhibited glorious antimicrobial and antioxidant activity (**Jayanthi *et al*., 2011**). To date, numerous bioactive compounds have been isolated from the genus *Phomopsis* (**Prachya *et al*., 2007; Vatcharin *et al*., 2008**). The need for new and useful compounds to accommodate attrition and abatement in all aspects of human conditions is overgrowing. Furthermore, it is accustomed that anticancer compound from the microbial source is of high value product and may be easier and added economical to aftermath effectively, thereby reducing its market price. Hence, a maiden attempt was fabricated to abstraction the absolute endophyte assortment of coelomycetes for their potential in bearing anticancer compound.

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MATERIALS AND METHODS

Collection of plant samples

The samples from medicinal plants *viz*., *Anisomeles malabarica* (L.) R.Br., *Mesua ferrea* L., *Piper nigrum* L., *Rauvolfia tetraphylla* L. and *Vitex negundo* L. were collected from Azhiyar, Western ghats, Tamil Nadu, Southern India. The samples gathered in sterile polythene bags were taken to the laboratory and treated within 24 h of collection (**Fisher and Petrini, 1987**).

Growth and biomass of endophytic fungi

The samples were processed following the protocol (**Dobranic *et al*., 1995**) with slight alterations. The fungal endophytes were isolated and studied (**Dobranic *et al*., 1995; Fisher *et al*., 1993**). Seventy seven endophytic coelomycetous fungi were grown in 100 ml saline bottles containing 25 ml of M-1D medium enriched with 1.0 g of soytone per liter and incubated at 23 ± 2°C under static condition for 21 days (Pinkerton and Strobel, 1976) and M-1D medium was maintained as a control (**Strobel *et al*., 1996a**). The mycelial dry weight (MDW) was determined gravimetrically by passing 10 ml of each broth samples through a pre-weighed filter paper (Whatman No.1) and rinsed meticulously by using distilled water. The filtered mycelia were blot dried using blotting paper to remove the surplus wetness and the mycelial fresh weight was determined. Subsequently the mycelia was kept in hot air oven for 24 h at 70ºC for the determination of mycelial dry weight (**Bitew *et al*., 2010**).

Extraction of taxol

The extraction of taxol from all the coelomycetous fungus was done according to the strategy described by **Strobel *et al.* (1996a)**. After twenty one days of incubation time span, the cultures were filtered by using four layers of cheese cloth to remove mycelia. To the culture filtrate 0.25 g $NaCO_3$ was added and extracted with two equal volumes of dichloromethane. The solvent was then condensed by evaporation under reduced pressure at 35°C using rotary vacuum evaporator. The obtained crude extract was assessed diagnose for the existence of taxol by chromatographic separation and spectroscopic analyses.

Thin Layer Chromatography (TLC)

The crude extracts of 77 coelomycetous fungal samples was screened for the production of taxol by performimg on 0.25 mm (10 x 20 cm) aluminium precoated silica gel TLC plates (Merck). Samples were spotted including authentic taxol (Paclitaxel-SIGMA Grade) as internal standard and the plates were developed in various solvent systems viz., Chloroform/Methanol 7:1, v/v, Chloroform/Acetonitrile 7:3, Ethylacetate/2-Propanol 95:5, v/v, Dichloromethane/ Tetrahydrofuran 6:2, v/v, Dichloromethane/Methanol/ Dimethylformamide 90:9:1, v/v/v. The existence of taxol was diagnosed with 1% vanillin/sulphuric acid spray reagent (w/v) after gentle heating (**Cardellina, 1991**). It appears as a bluish spot of fading to dark gray after 24 hours. The R_f values of the samples were calculated and compared with authentic taxol.

Radial growth of *P. longicolla*

Radial growth of *P. longicolla* was analyzed using five different media PDA, PDYEA, M1DA, CDA and MEA. The mycelial discs were placed inversely at the center of each Petri Plate containing the specific medium and incubated for 10 days at different temperature such as 15°C, 25°C and 35°C. The diameter of the mycelial growth was evaluated at every 48 h intervals.

Statistical analysis

Every experiment was performed in triplicate manner and the data obtained were represented as mean ± standard deviations that were interpreted using Microsoft Office Excel 2007.

Silica gel column chromatography and Preparative Thin Layer Chromatography (PTLC)

The extracted *P. longicolla* was purified through silica gel column chromatography. The crude extact was dissolved in 1 ml dichloromethane and packed on a 1.5 x 30 cm column of silica gel and eluted in a step-wise manner with dichloromethane and ethyl acetate with an increase in the solvent ratio. The fractions having same mobility as the authentic taxol were combined and evaporated to dryness and subjected to PTLC. The partially purified taxol was loaded on the TLC plate by using capillary tube and developed with a solvent system containing 90:10, (v/v) chloroform/methanol. The area of the TLC plate containing putative taxol was carefully removed by scraping off the silica gel at the appropriate R_f value and eluting it with methanol and further analyzed.

Ultra Violet (UV) and Infra-Red (IR) spectroscopic analysis

The partially purified taxol was dissolved in 100% methanol at 230 nm and analyzed in a Beckman DU-40 Spectrophotometer and compared with authentic taxol (Paclitaxel-SIGMA Grade). The IR spectra of the compound were recorded on Shimadzu FT IR 8000 series instrument. The partially purified fungal taxol was ground with IR grade potassium bromide (KBr) (1:10) pressed into discs under vacuum using spectra lab pelletiser and compared with authentic taxol. The spectra were recorded within the region 4000-500 cm^{-1}.

High performance liquid chromatography (HPLC) and Mass spectral analyses

To further affirm the presence of taxol, the dichloromethane extract was injected into the HPLC (Schimadzu 9A model) (Liu *et al*., 2009). The HPLC was performed with reverse phase C18 column with UV detector. Twenty microlitre of each sample was injected and the detection was examined at 232 nm. Methanol/water (65:35, v/v) at 1 ml $mins^{-1}$ was acclimated as mobile phase.

Samples and the solvents were filtered through 0.2 µm PVDF filter before the performance of HPLC. Taxol was quantified by the peak area of the samples compared with the authentic taxol by using the formula.

$$\text{Amount of taxol} = \frac{\text{Standard concentration} \times \text{Total area of sample peak}}{\text{Total area of the authentic peak.}}$$

The FAB (Fast Atom Bombardment) Mass spectra were record using JEOL SX 102/DA-6000 mass spectrometer/Data System using Argon/Xenon (6 kV, 10 mA). The accelerating voltage was 10 kV and the spectra were recorded at room temperature.

RESULTS AND DISCUSSION

The production of taxol by the plants as well as by the endophytic fungi suggests that some mechanism for genetic exchange between trees and fungus might exist. Most studies have focused on the paclitaxel-producing capacity of a particular fungus, but not on the endophytic population associated with *Taxus* species (**Chafre *et al*., 2011**).

Keeping this in view, the present work was focused on the screening of the seventy seven morphospecies of endophytic coelomycetous fungal population from leaves and stems of five medicinal plants *viz*., *A. malabarica*, *M. ferrea*, *R. tetraphylla*, *P. nigrum* and *V. negundo* for the potential in taxol production. It is worth screening the medicinal plants for their endophytes, which could produce taxol. Representative fungal isolates (n=107) were cultured in 250ml Erlenmeyer flasks containing 50ml M1D medium (**Chafre *et al*., 2011; Pinkerton *et al*., 1976**). In the present study, all the seventy seven morphospecies of endophytic coelomycetous fungal cultures were grown in M1D medium for 21 days and it was also found that the fungal mats possessed maximum mycelial dry weight in M1D media.

This specifies that the M1D medium enhances the taxol production of the fungus in the media. It is apparent from Table 1 that the seventy seven endophytic fungal mats were harvested and the mycelial dry weight was determined.

Table 1. Screening of Coelomycetous fungi for growth (Mycelial Dry Weight) and taxol production in M-1D medium

S. No	Culture No	MDW/25 ml	Taxol	S. No	Culture No	MDW/25 ml	Taxol
1	1201	0.39±0.12	-	40	1240	0.25±0.21	-
2	1202	0.24±0.22	-	41	1241	0.29±0.11	-
3	1203	0.29±0.14	-	42	1242	0.27±0.19	-
4	1204	0.30±0.18	-	43	1243	0.15±0.22	-
5	1205	0.34±0.13	-	44	1244	0.26±0.16	-
6	1206	0.26±0.21	-	45	1245	0.28±0.26	-
7	1207	0.71±0.15	*	46	1246	0.31±0.17	-
8	1208	0.24±0.27	-	47	1247	0.17±0.14	-
9	1209	0.20±0.22	-	48	1248	0.28±0.20	-
10	1210	0.15±0.19	-	49	1249	0.22±0.25	-
11	1211	0.14±0.11	-	50	1250	0.24±0.11	-
12	1212	0.23±0.17	-	51	1251	0.16±0.21	-
13	1213	0.17±0.13	-	52	1252	0.22±0.24	-
14	1214	0.29±0.18	-	53	1253	0.11±0.12	-
15	1215	0.28±0.23	-	54	1254	0.26±0.18	-
16	1216	0.31±0.21	-	55	1255	0.10±0.24	-
17	1217	0.40±0.12	-	56	1256	0.19±0.16	-
18	1218	0.21±0.14	-	57	1257	0.34±0.26	-
19	1219	0.27±0.16	-	58	1258	0.27±0.12	-
20	1220	0.13±0.19	-	59	1259	0.14±0.18	-
21	1221	0.17±0.24	-	60	1260	0.17±0.20	-
22	1222	0.29±0.28	-	61	1261	0.22±0.21	-
23	1223	0.24±0.25	-	62	1262	0.13±0.14	-
24	1224	0.17±0.11	-	63	1263	0.26±0.11	-
25	1225	0.10±0.17	-	64	1264	0.21±0.23	-
26	1226	0.21±0.14	-	65	1265	0.12±0.16	-
27	1227	0.17±0.18	-	66	1266	0.27±0.19	-
28	1228	0.19±0.20	-	67	1267	0.31±0.22	-
29	1229	0.26±0.11	-	68	1268	0.17±0.12	-
30	1230	0.15±0.19	-	69	1269	0.21±0.27	-
31	1231	0.16±0.13	-	70	1270	0.01±0.18	-
32	1232	0.67±0.15	-	71	1271	0.16±0.15	-
33	1233	0.30±0.19	-	72	1272	0.28±0.20	-
34	1234	0.26±0.12	-	73	1273	0.51±0.28	-
35	1235	0.19±0.28	-	74	1274	0.42±0.14	-
36	1236	0.28±0.22	-	75	1275	0.37±0.26	-
37	1237	0.31±0.14	-	76	1276	0.54±0.17	-
38	1238	0.26±0.11	-	77	1277	0.28±0.23	-
39	1239	0.24±0.23	-	-	-	-	-

Values are the mean ± SD with three replicates
`(*) Presence of taxol
(-) Absence of taxol
MDW-Mycelial dry weight

Table 2. Radial growth of *P. longicolla* in different medium at different temperature

Days		*Phomopsis* sp. GJJM1207		
	Medium	15°C	25°C	35°C
2	PDA	–	0.59±0.20	0.19±0.22
4		0.28±0.14	1.98±0.13	0.68±0.28
6		1.18±0.19	2.79±0.22	1.55±0.14
8		1.97±0.32	3.86±0.12	2.79±0.10
10		2.12±0.21	4.40±0.24	3.97±0.18
2	PDYEA	–	0.97±0.18	0.54±0.12
4		0.19±0.32	1.26±0.24	1.17±0.16
6		0.88±0.18	2.74±0.10	1.98±0.24
8		1.07±0.12	3.97±0.18	2.85±0.18
10		1.25±0.20	4.05±0.22	3.45±0.15
2	CDA	–	0.75±0.16	0.19±0.12
4		0.12±0.42	1.16±0.28	0.84±0.16
6		0.45±0.15	2.37±0.14	1.55±0.21
8		0.97±0.18	3.04±0.12	2.16±0.18
10		1.12±0.32	3.85±0.22	3.21±0.26
2	MEA	–	0.61±0.20	0.13±0.22
4		0.08±0.10	1.84±0.16	0.68±0.28
6		0.27±0.19	2.77±0.14	1.45±0.18
8		0.84±0.20	3.34±0.17	2.61±0.14
10		1.05±0.12	3.91±0.22	3.01±0.20
2	M1DA	–	0.59±0.20	0.19±0.32
4		0.28±0.12	1.98±0.14	0.68±0.23
6		1.18±0.18	2.79±0.22	1.55±0.17
8		1.97±0.25	3.86±0.11	2.79±0.11
10		2.12±0.20	4.40±0.12	3.97±0.14

Values are the mean ± SD with three replicates, PDA: Potato Dextrose Agar, PDYEA: Potato Dextrose Yeast extract Agar, M-1DA: Modified medium-1 Agar, MEA: Malt Extract Agar, CDA: Czapex-Dox Agar

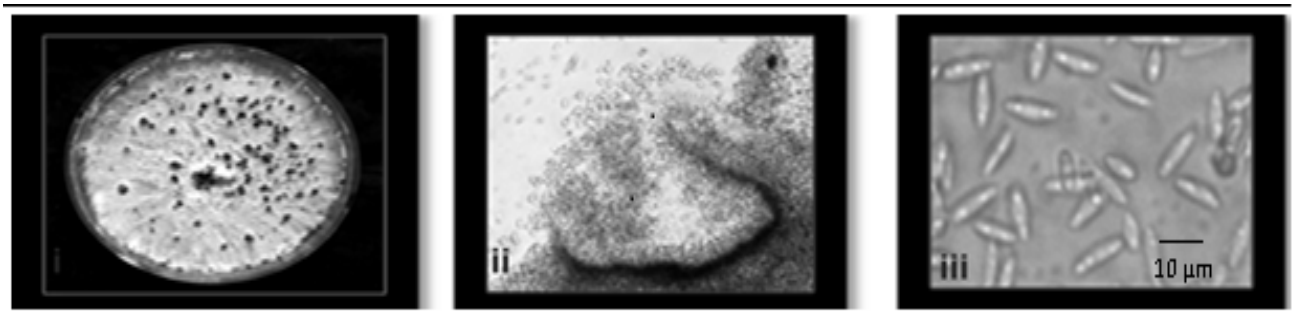

Figure 1. i. Culture plate, ii. conidiomata (300X) and iii. conidia (700X) of *P. longicolla*

Taxol is positively identified via its co-chromatographic mobilities with authentic taxol in a multitude of thin layer chromatographic systems (**Stierle *et al*., 1993; Strobel *et al*., 1996b**). In connection with TLC, it was evident that out of 77 fungi screened, only one particular strain *P. longicolla* expressed a similar metabolite profile compared to authentic taxol at 254 nm. The lingering 76 isolates showed a negative result for taxol production was publicized in Table 1. In TLC, the occurrence of taxol was diagnosed with 1% vanillin sulfuric acid (w/v) spray reagent. The incident of fungal taxol in the fungal extracts was proved by the appearance of a bluish spot fading to dark gray after 24 h (**Cardellina, 1991**). Consequently, the present study coincides with the previous report that the strain *P. longicolla* isolated from *M. ferrea* showed positive results for taxol production with R_f values 0.72 identical to that of authentic taxol was diagnosed with 1% vanillin sulfuric acid (w/v) spray reagent followed by the appearance of a bluish spot fading to dark gray after 24 h.

The fungal taxol were isolated from the organic extract of endophytic fungus *Phomopsis arnoldiae* (**Muthumary *et al*., 2007**). In the present study it was confirmed that among 77 morphospecies of endophytic coelomycetous fungi, *P. longicolla* possesses the capability of taxol production.

The fungus exhibited positive reactivity for taxol production and thereby, *P. longicolla* was targeted for the further study (Fig. 1). Amongst the liquid media, M-1DB medium enhanced the maximum growth of *P. longicolla* (1.57±0.10) followed by PDB, PDYEB, CDB and MEB (**Jayanthi *et al*., 2011**). In the present study, Table 2 clearly depicts that among all the solid media, *P. longicolla*, established maximum radial growth of about 4.40±0.12 cm at 25□C in M-1DA medium on 10th day followed by PDA, PDYEA, MEA and CDA. The minimum radial growth was observed at 15°C and 35°C temperatures. The *Phyllosticta tabernaemontanae* produced a high amount of taxol in M1D medium than PDB medium (**Senthil Kumaran *et al*., 2009**). Therefore, *P. longicolla* was subjected to mass cultivation in M1DB medium at 25□C.

In Column chromatography, the sample was eluted with dichloromethane/ethyl acetate and the fraction was collected sequentially. Each fraction was tested for the presence of taxol by TLC. The partially purified taxol obtained from PTLC was processed and subjected to spectrometric analyses. UV absorption of paclitaxel was found to be 230 nm (**Kesarwani *et al*., 2011**). The UV spectrum of the isolated compound had a maximum absorbance at 225 nm (**Muthumary *et al*., 2007**) and 227 nm (**Chakravarthi *et al*., 2008**) similar to authentic taxol.

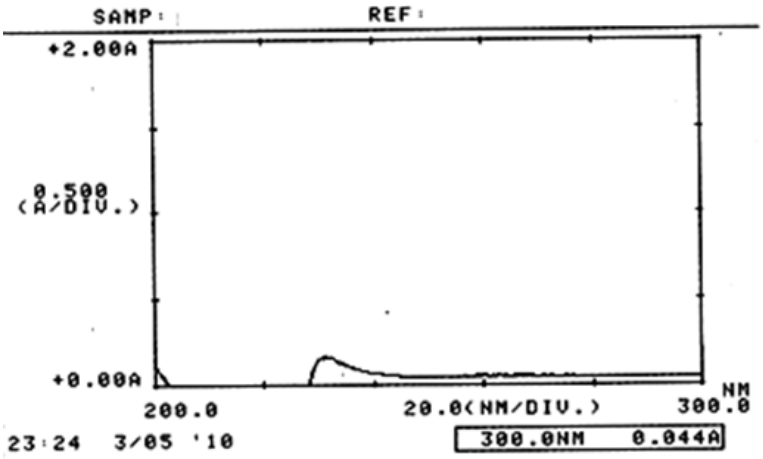

Figure 2A . UV spectrum of authentic taxol

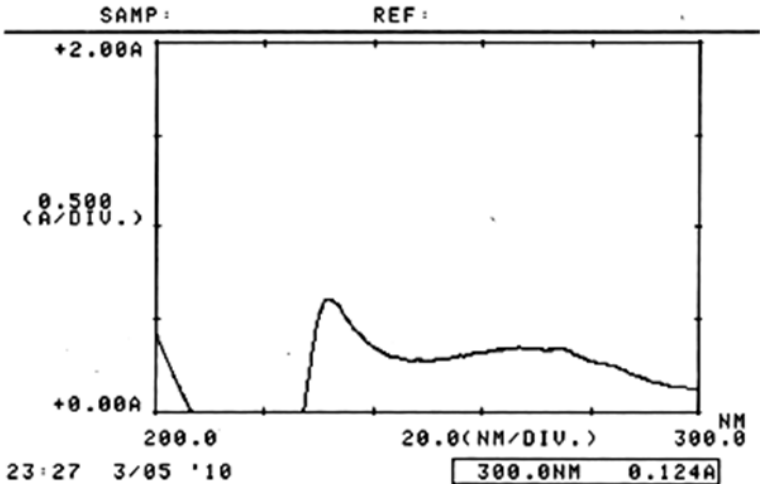

Figure 2B. UV spectrum of partially purified taxol from *P. longicolla*

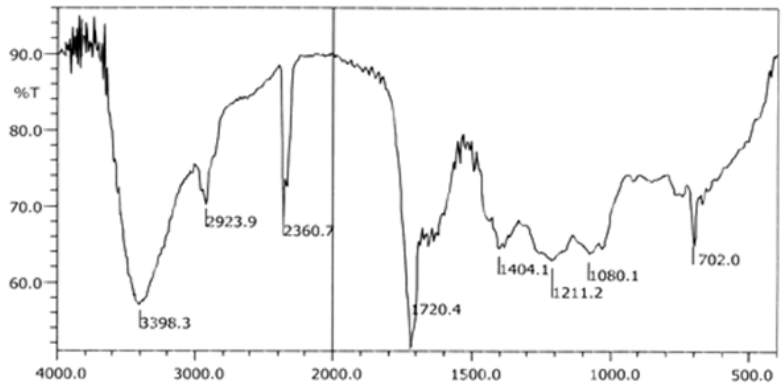

Figure 3A. FT-IR spectrum of authentic taxol

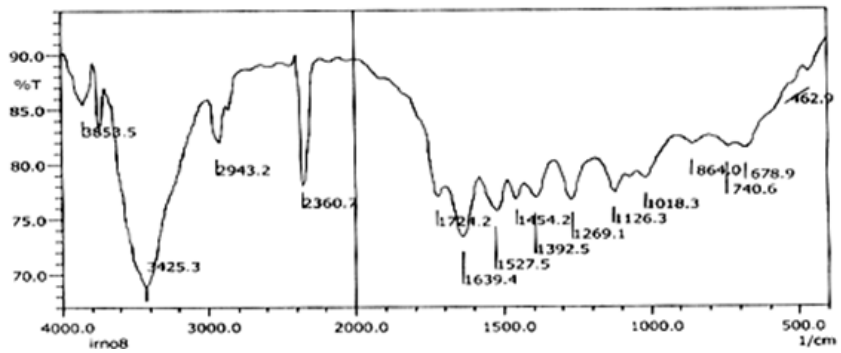

Figure 3B. FT-IR spectrum of partially purified taxol from *P. longicolla*

Similarly, in the present study, the UV spectral analysis of fungal taxol was established at 230 nm and was found to be identical to that of authentic taxol (Figure 2A and 2B). It is apparent from Figure 3A and 3B, that the appearance of bands in IR spectra persuasively illustrates the identical feature of the extracted fungal sample with authentic taxol. In IR spectrum, a extensive peak in the range of 3336 to 3436 cm^{-1} was observed due to hydroxyl (-OH) groups stretch. The aromatic ring (C=C) stretching frequency was observed in the range of 1590 to 1735 cm^{-1}.

The IR spectra of the fungal samples were superimposed on the spectrum of authentic taxol. In HPLC, the retention time for taxol was 15.1 respectively (**Sadeghi-aliabadi *et al*., 2009**). Similarly, in the present study, the retention time of authentic taxol was 15.28 and retention time of *P. longicolla* was 15.76. Consequently, fungal sample showed a peak with similar retention time as that of authentic taxol. Fungal paclitaxel yields are low when compared to those of yew cell cultures (**Zhong *et al*., 2002**). The maximum amount of fungal taxol production was recorded as 418 µg/L in the strain *Phomopsis* sp. BKH 27 (**Kumaran *et al*., 2009**).

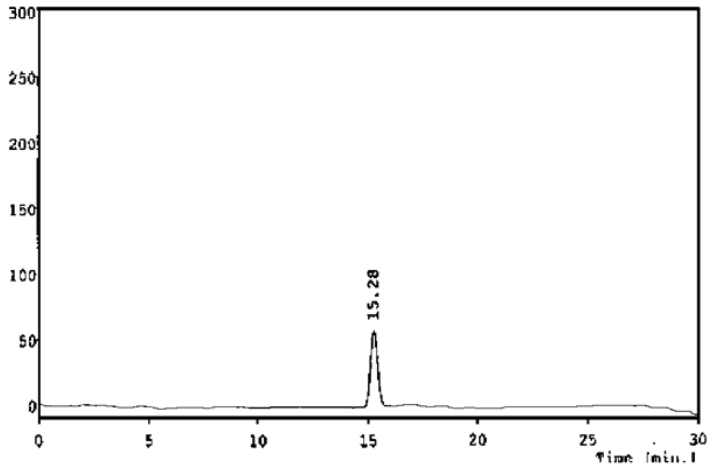

Figure 4A. HPLC of authentic taxol

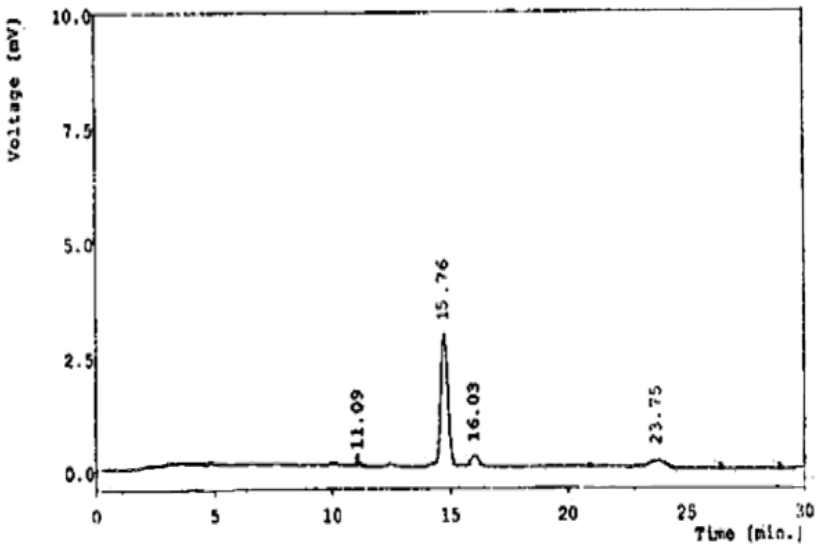

Figure 4B. HPLC of partially purified taxol from *P. longicolla*.

The record of the peaks was also observed in the range of 1045 to 1068 cm^{-1} was due to the presence of aromatic C and H bends (**Pandi *et al*., 2011**). Subsequently, in the present study, the appearance of bands in IR spectra persuasively illustrates the identical feature of the extracted fungal sample with authentic taxol. Due to hydroxyl (-OH) and amide (-NH) groups stretch, a broad peak was identified in the range of 3425-3858 cm^{-1}. The aromatic ring (C=C) stretching frequency was observed in the range of 1527-1724 cm^{-1}. Due to the presence of aromatic C, H bends, the registration of peak was found in the range of 1018-1126 cm^{-1}.

Accordingly, in the present study, *P. longicolla* produced 381 µg/L of fungal taxol as shown in Figure 4A and 4B. Thus the taxol production from the fungus was high compared to the *Phomopsis* sp. BKH 27. The retention time of authentic taxol was found to be 15.28. The retention time of *P. longicolla* was recorded at 15.76 and the fungus produced 381 µg/L of fungal taxol. FAB mass spectroscopy yielded a characteristic (M+H) + peak for authentic taxol at 854.5 and 876.5 m/z. On comparison the fungal taxol also yielded a (M+H) + at 877.6. The other prominent peaks at 563, 924, 813 and 986 m/z, which could be classified as taxoid derivatives (**Georgios *et al*., 1999**).

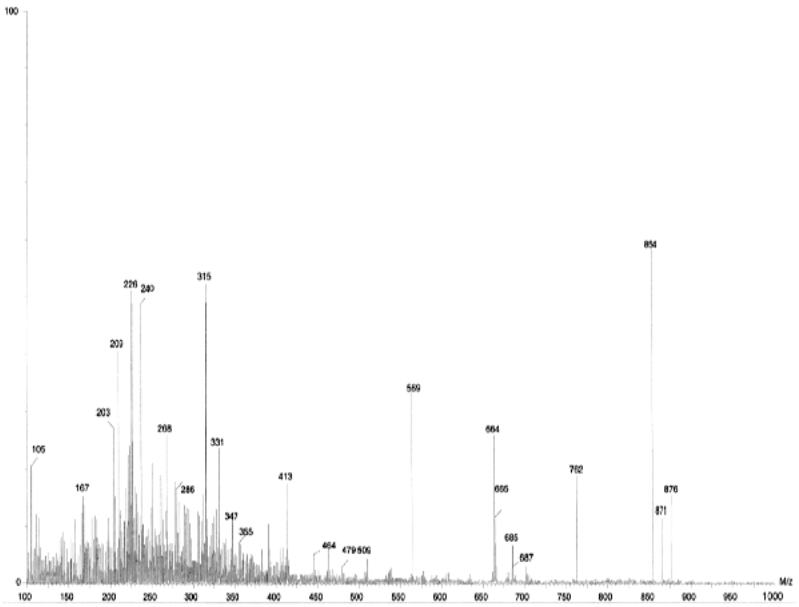

Figure 5A. Mass spectrum of authentic taxol

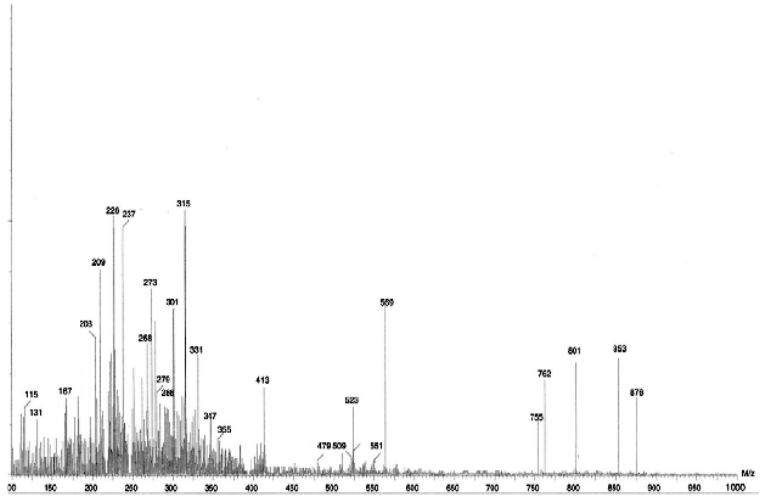

Figure 5B. Mass spectrum of partially purified taxol from *P. longicolla*

In the present study, it was evident from Figure 5A and 5B, that high resolution mass spectrometry divulge that the taxol is more complex with an empirical formula $C_{47}H_{51}NO_{14}$ corresponding to a molecular weight of 854. Characteristically, standard taxol was also yielded a peak 854 m/z with characteristic fragment peaks at 509, 569, 464, 286 and 268 of major fragment ions observed in the mass spectrum. The fungal taxol yielded the peak at 853 m/z and some related peak such as 509, 523, 569, 551, 268 and 286 was observed with an empirical formula $C_{45}H_{49}NO_{13}$ corresponding to the molecular weight.

Conclusion

The limited supply of the drug has prompted efforts to find alternative sources of paclitaxel. Knowledge of the biosynthesis of paclitaxel in fungi is necessary for determining the conditions for fermentation and metabolic engineering of the strains for overproduction of paclitaxel.

From the present investigation, it is implicit that the fungi *P. longicolla* isolated from the host *M. ferrea* plays a vital role in the production of taxol. The study concludes that the improved culturing techniques and the application of genetic engineering may improve paclitaxel production by *P. longicolla*.

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