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Full Length Research Paper

Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia*

Mohan Pandi[1], Rangarajulu Senthil Kumaran[2]*, Yong-Keun Choi[2], Hyung Joo Kim[2] and Johnpaul Muthumary[3]

[1]Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai, India.
[2]Department of Microbial Engineering, Konkuk University, 1 Hwayang-Dong, Gwangjin-Gu, Seoul - 143701, South Korea.
[3]Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai 600025, India.

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To determine the production of taxol from an endophytic fungus, *Lasiodiplodia theobromae* isolated from the medicinal plant *Morinda citrifolia* and also, to evaluate its cytotoxicity against human breast cancer cell line, taxol produced by the test fungus in MID culture medium was isolated for its characterization. The presence of taxol was confirmed by different chromatographic and spectroscopic analyses. The quantity of taxol produced by the fungus was calculated and estimated to be 245 µg/l. The fungal taxol was tested for its bioactivity against human cancer cell line (MCF-7) and the results showed that, the taxol possessed anticancer activity. The production of taxol was achieved from an endophytic fungus, *L. theobromae*. The screened taxol showed a potential toxicity against breast cancer cell lines. Fungal based production of taxol from an endophytic fungus would be the most desirable and alternate source of supply. This study proved that the fungal endophyte *L. theobromae* is an excellent candidate for an alternate source of taxol supply. Confirmations of the *in vitro* activity of taxol against human breast cancer cell lines should encourage further research. Furthermore, it is the first report of the screening of taxol from the fungus *L. theobromae*, isolated from the medicinal plant *M. citrifolia*.

Key words: Endophytic fungus, *Lasiodiplodia theobromae*, anticancer drug, taxol production, cytotoxicity test.

INTRODUCTION

Taxol is the most effective antitumor agent developed in the past three decades. It has been used for effective treatment of a variety of cancers including refractory ovarian cancer, breast cancer, non-small cell lung cancer, AIDS related Kaposi's sarcoma, head and neck carcinoma and other cancer types (Wani et al.,1971; Rowinsky et al., 1990; Croom, 1995). Taxol inhibits cell proliferation by promoting the stabilization of microtubules at the G_2-M phase of the cell cycle, by which depolymerization of microtubules to soluble tubulin is blocked (Nicolaou et al., 1994; Jennewein and Croteau, 2001).

Taxol was originally isolated from the bark of Pacific yew, *Taxus brevifolia* (Wani et al., 1971). The limited availability of mature yew trees, slow growth rate of cultivated plants and the low yield of the taxol has resulted in its high cost and also, has raised the concerns due to environmental damage from excessive exploitation of wild trees. This makes taxol a financial burden for many patients. Plant cell culture has been suggested as

*Corresponding author. E-mail: kumaran.ran@gmail.com. Tel: 82 2 2049 6112. Fax: 82 2 3437 8360.

an attractive alternative technique that could overcome the limitation of extracting useful metabolites from natural resources. However, the commercial paclitaxel production by plant cell culture methods has not been successful, though many reports from various groups are published. The major obstacle to commercialization has been the low yield of paclitaxel from plant cell culture. Recently, many researchers reported about taxol-producing endophytic fungus isolated from phloem of yews (Stierle et al., 1993; Strobel et al., 1996a, 1996b; Li et al., 1998). The observation on taxol producing endophytic fungi, *Pestalotiopsis terminaliae*, *Colletotrichum gleosporioides*, *Phyllosticta spinarum* and *Phyllosticta citricarpa*, has been reported and also demonstrated that, the organisms other than *Taxus* spp. could produce taxol (Kumaran et al., 2008a, 2008b; Gangadevi and Muthumary, 2008, 2009). Thus, the endophytic fungi can produce taxol as a cheaper and more widely available product, eventually via industrial fermentation. In this investigation, an attempt was made for the first time to screen the taxol from an endophytic fungus, *Lasiodiplodia theobromae* associated with leaves of *Morinda citrifolia*, a medicinal plant. The production and bioactivity of fungal taxol were evaluated by different analytical methods.

MATERIALS AND METHODS

Fungal isolation

The endophytic fungus, *L. theobromae* (Pat.) Griffon & Maubl was isolated from the leaves of the medicinal plant, *M. citrifolia* Linn. by following the standard method (Kumaran et al., 2008a). It was then, identified and deposited at the Madras University Botany Laboratory (MUBL) culture collection (Accession No. MUBL-BT1), University of Madras, India. The fungus was sub-cultured and maintained on potato dextrose agar (PDA) medium. For taxol screening, the test fungus was grown in 3 L Hopkins flasks that contained 1500 ml of MID medium supplemented with 1 g soytone l^{-1}. The cultures were incubated for 22 days. The standard taxol was purchased from Sigma chemical company (St. Louis, USA).

Extraction of taxol

The extraction procedure for taxol was made by using to the methods of Strobel et al. (1996a & 1996b). After the incubation period, the cultures were filtered through four layers of cheesecloth to remove mycelia. To the culture filtrate, 0.25 g Na_2CO_3 was added with frequent shaking in order to reduce the amount of fatty acids that may contaminate taxol in the culture. Then, the culture filtrate was extracted with two equal volumes of solvent dichloromethane. The organic phase was collected and the solvent was then removed by evaporation under reduced pressure at 35°C using rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol for the subsequent separation. The crude extracts were analyzed by chromatographic and spectroscopic methods.

Thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC) and high performance liquid chromatographic (HPLC) analyses

TLC analysis was carried out on Merck 1 mm (20 x 20 cm) silica gel pre-coated plates. The plates were developed by the solvent system as reported (Strobel et al., 1996b). The taxol was detected with 1% vanillin in sulfuric acid (w/v) by gentle heating. It appeared as a bluish spot that faded to dark grey after 24 h. Then, the area of the plate containing putative taxol was carefully removed by scraping off the silica at the appropriate Rf value and eluted with methanol. The partially purified fungal taxol samples obtained through TLC were further subjected to HPTLC (CAMAG-Planer HPTLC, Anch-rom). The TLC plate containing fungal taxol was scanned using TLC scanner-3 with winCATS software. Documentation of the TLC plate was performed under a shortwave (254 nm) UV lamp. The presence of taxol was visualized by spraying 1% vanillin in sulfuric acid (w/v) with gentle heating for 2 min. The presence of taxol was identified by comparison with standard taxol. The peak area and peak height of the standard taxol and fungal taxol were evaluated and the Rf values were also evaluated and compared with standard taxol. The fungal extract was subjected to HPLC (Shimatzu 9A model) for further confirmation using a reverse phase C_{18} column with a UV detector. Each time, samples of 20 µl was injected and detected at 232 nm. The mobile phase was methanol/ acetonitrile/water (25:35:40, v/v) at a flow rate of 1.0 ml min^{-1}. The sample and the mobile phase were filtered through 0.2 µm PVDF filter before injecting into the column. Taxol was confirmed by comparing the peak area of the fungal samples with standard taxol.

Ultra violet (UV), infra-red (IR), proton nuclear magnetic resonance (^{1}H NMR) and fast atom bombardment mass (FAB-MS) spectroscopic analyses

After chromatography, the area of the TLC plate containing putative taxol was carefully removed by scrapping off the silica at the appropriate Rf and exhaustively eluting it with methanol. The purified sample of taxol was dissolved in 100% methanol, analyzed by Beckman DU-40 UV spectrophotometer and compared with standard taxol. The IR spectra of the compound were recorded on Shimadzu FT-IR 8000 series instrument. The purified taxol was ground with IR grade KBr (1:10) pressed into discs under vacuum using spectra lab pelletiser and compared with standard taxol. The IR spectrum was recorded in the region between 4000 and 5000 cm^{-1}. ^{1}H NMR spectra were recorded at 23°C in $CDCl_3$ using a JEOL GSX 500 spectrometer (operating at 499.65 MHz) and were assigned by comparison of chemical shifts and coupling constants with those of related compounds. Chemical shifts were reported as values δ-relative to tetramethylsilane (TMS) as internal reference and the coupling constant were reported in hertz. The FAB mass spectra were recorded on a JEOL SX 102/DA-6000 mass spectrometer/data system using Argon/Xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature.

Cell proliferation assay

The cytotoxic effect of fungal taxol was tested by the MTT assay on MCF-7 (human breast cancer cell line). The cell line MCF-7 used in the study was procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were seeded in a 96 micro well at the concentration of 5 x 10^4 cells/ml with Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin (10000 IU/ml) - streptomycin (10000 µg/ml). The cells were incubated for 24 h under 5% CO_2, 95% O_2 at 37°C. Then, the serum medium was removed and washed with PBS and then, the fresh serum free medium (SFM) was added and kept for 1 h in the CO_2 incubator. Then, SFM was removed and the control plates received SFM and treatment plates received 100 to 600 µg ml^{-1} of fungal taxol containing medium. The cultures were incubated

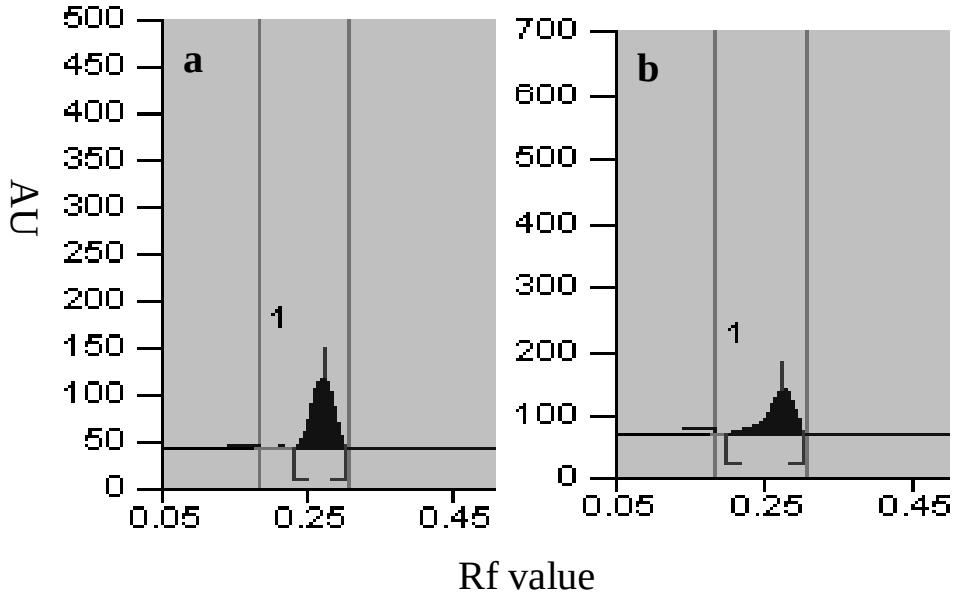

Figure 1. HPTLC analysis of standard taxol (a) and fungal taxol (b) obtained from the cultures of *L. theobromae*. The Rf value (0.25) of the fungal sample was identical in comparison with standard taxol.

with the conditions as mentioned earlier. After 24, 48 and 72 h 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% dimethylformamide (DMF) was added. A micro plate reader was used to measure the absorbance at 650 nm for each well (Mosmann, 1983). Growth inhibition rate was calculated as follows:

$$\text{Growth inhibition} = \frac{A_{650/nm} \text{ of treated cells}}{A_{650/nm} \text{ of control cells}} \times 100\%$$

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

RESULTS AND DISCUSSION

Chromatographic separations

In the study, the taxol was extracted from the fungus and its presence was detected using HPTLC. The fungal taxol showed the Rf value at 0.25 which was found to be identical when compared with standard taxol (Figure 1a, b). Further detection of taxol in the fungal sample was authenticated by TLC, displaying taxol band under UV illumination at 235 nm (results not shown) and 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 spray reagent in TLC and exhibiting a Rf value as in HPTLC (Cardellina, 1991).

To confirm the presence of taxol, the fungal sample was analyzed by HPLC which gave a peak when eluted from a reverse phase C_{18} column with the retention time of 2.8 min. It was found to be similar in comparison with standard taxol (Figure 2a, b). The total amount of taxol produced/L in MID was 245 µg and was found to be higher with three orders of magnitude (4.9 x1000 fold) more than that produced by *Taxomyces andreanae* (Stierle et al., 1993). The fungal taxol in this investigation 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) (Strobel et al., 1996a, 1997; Li et al., 1996). The biggest problem of using fungi in fer-mentation is the low level yield accompanied by unstable taxol production. Taxol yield of such reported fungi diverged from 24 to 70 ng/l (Stierle et al., 1993; Strobel et al., 1996a). However, the amount of taxol produced by the endophytic fungi associated with yew trees was rela-tively small, when compared with the host trees. The short generation time and high growth rate of the fungi will make it worthwhile to continue the investigation on *L. theobromae* isolated from *M. citrifolia*.

Spectroscopic analyses

The UV absorption spectrum of the fungal compound isolated from *L. theobromae* yielded similar absorption to standard taxol with a maximum absorption at 235 nm (Figure 3a, b). The appearance of bands in IR spectra convincingly illustrated the identical chemical nature of the extracted taxol from the fungus with that of standard taxol.

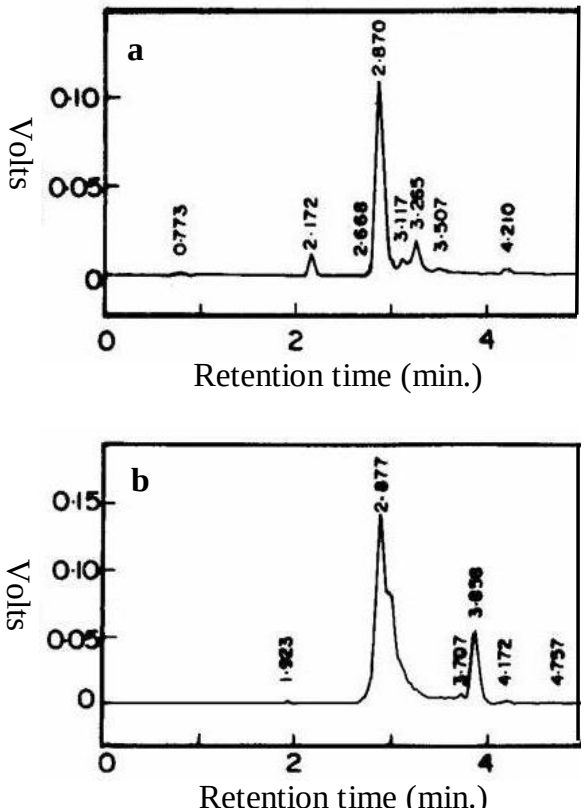

Figure 2. HPLC spectrum of the standard taxol (a) and fungal taxol (B). The registration peak and retention time was recorded on UV at 232 nm. Fungal sample showing a peak with retention time 3.8 min was identical in comparison with standard taxol.

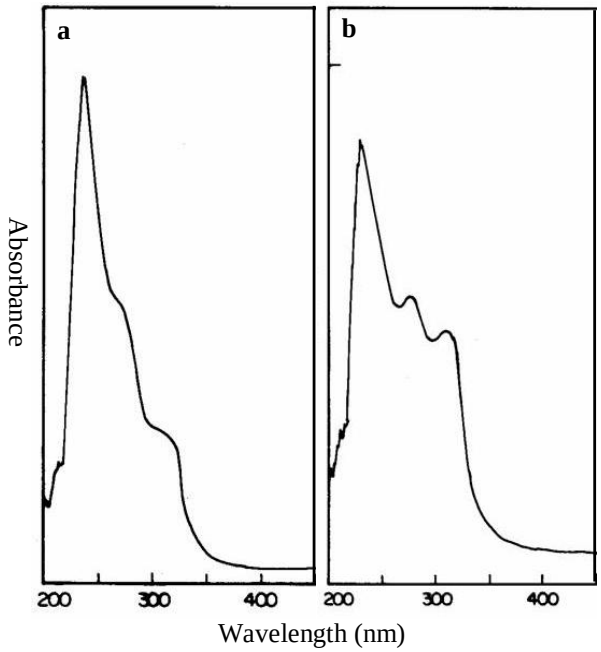

Figure 3. UV absorption spectrum of standard taxol (a) and fungal taxol (b) showing the maximum absorbance at a wavelength of 235 nm in methanol.

A broad peak in the range of 3336 to 3436 cm^{-1} was observed due to hydroxyl (-OH) groups stretch. The aliphatic CH stretch was observed in the range of 2920 to 2939 cm^{-1}. The registration peak observed in the range of 2356 to 2364 cm^{-1} was due to amine (NH) group stretching frequency. The aromatic ring (C=C) stretching frequency was observed in the range of 1590 to 1735 cm^{-1}. The registration of the peak observed in the range of 1045 to 1068 cm^{-1} was due to the presence of aromatic C and H bends. The IR spectra of the fungal samples were superimposed on the spectrum of standard taxol (Figure 4a, b). Based on the results of the UV and IR analysis, it was found that this fungus showed positive sign for the production of taxol in the culture medium, which was evidenced in the earlier reports (Wani et al., 1971; Kumaran et al., 2008a, 2008b).

The fungal compound produced an identical FAB-MS spectrum as the standard taxol. Characteristically, the standard paclitaxel yielded both an (M + H)+ peak at 854 and an $(M + Na)^+$ peak at 876. On comparison, fungal taxol also produced peaks (M + H)+ at *m/z* 854 and $(M +Na)^+$ at *m/z* 876 (Figure 5a, b) with characteristic fragment peaks at *m/z* 819, 820, 855, 871, 872 and 877. The major fragment ions observed in the mass spectrum of taxol are placed into three categories, which represent the major portion of the taxol molecule (McClure and Schram, 1992). The peaks were analogous to the 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 acquired from high resolution mass spectroscopy was 854, corresponding to the molecular formula as reported earlier (McClure and Schram, 1992). In ^{1}H-NMR analysis, almost all the signals were resolved and distributed in the regions between 1.0 and 8.5 ppm (Figure 6a, b). The strong three protons 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 sample was found to be identical in all respects to that of the standard taxol and is also supported by the early literature (Falzone et al., 1992). The characteristic chemical shifts of taxol assignments obtained in the investigations were also confirmed with an earlier report (Chmurny et al., 1992). Analytical methods acquired clearly suggested that, the fungal compound is a taxol, which was produced in MID medium in comparison with standard taxol. The techniques like UV, TLC, IR, HPLC, the high resolution ^{1}H- and ^{13}C-NMR NMR and MS are the tools applied in

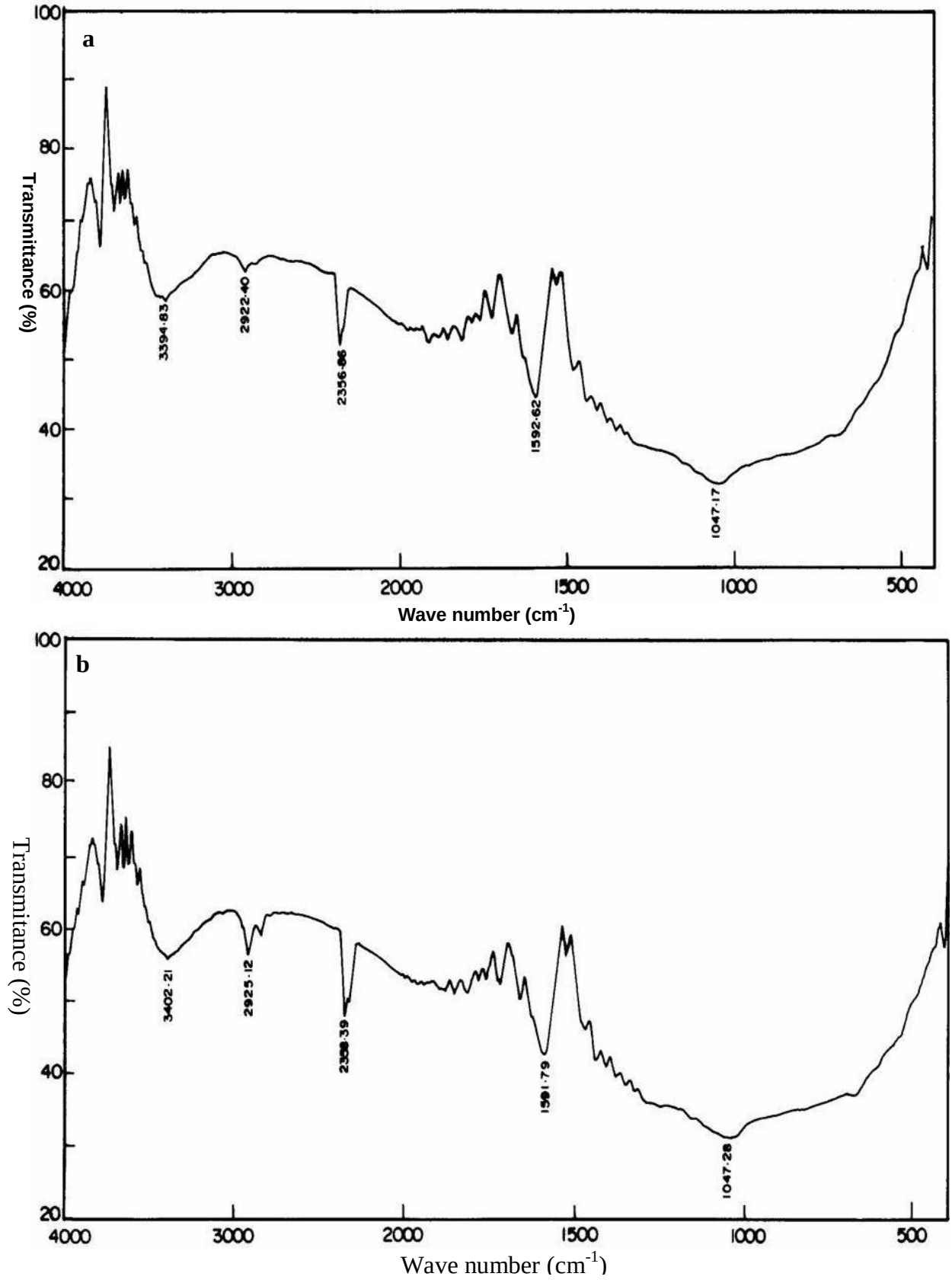

Figure 4. IR spectrum of standard taxol (a) and fungal taxol (b) showing finger print region between 1000 and 4000 cm^{-1}.

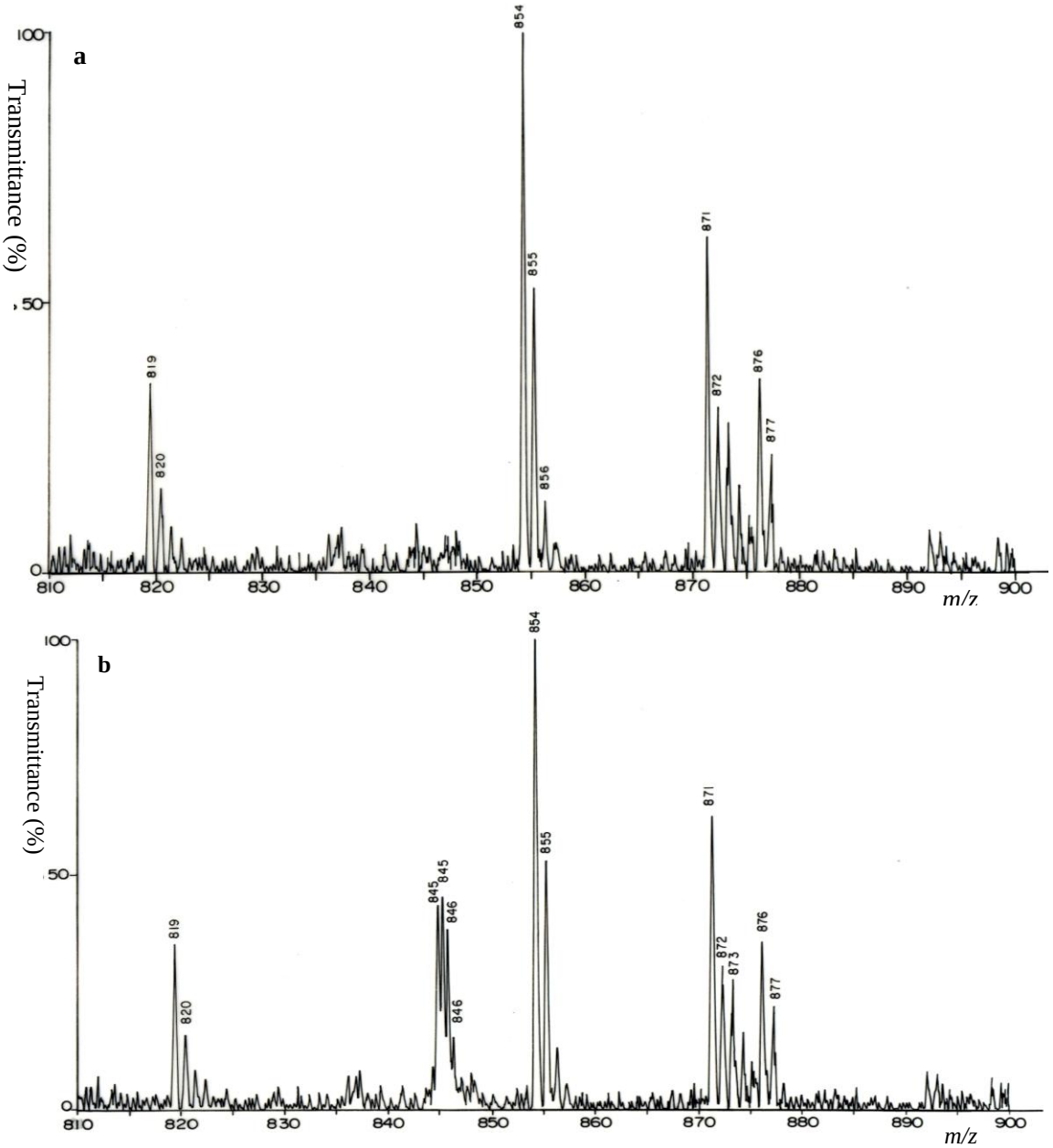

Figure 5. FAB-MS spectrum of standard taxol (a) and fungal taxol (b). Mass spectrum of the fungal extracts showing a $(M+H)^+$ peak at molecular weight 854 and a $(M+Na)^+$ peak at molecular weight of 876 was identical in comparison with standard taxol.

the confirmation test for the antitumor compound taxol isolated from fungi, which are also supported by many workers (Stierle et al., 1993; Strobel et al., 1996a, 1996b; Kumaran et al., 2008a).

Cyotoxicity test

The cytotoxic effect of fungal taxol was tested by the MTT assay, which showed the effect of fungal taxol on the cell viability in MCF-7 cell line for 24, 48 and 72 h. The cells treated with fungal taxol of concentration ranging between 100 and 600 µg showed a significant decrease in the cell viability (Table 1). Treatment at 300 µg of fungal taxol showed that only 50% of the cells were viable in 48 h incubation. The IC_{50} value of fungal taxol was calculated as 300 µg. The MTT cell viability assay indicated that, with the increase in taxol concentration from 300 to 600 µg, taxol induced an increased cell death through apoptosis. In this investigation, it was observed that at higher concentration and incubation time, the efficacy of fungal taxol was relatively dependent on the specific cell

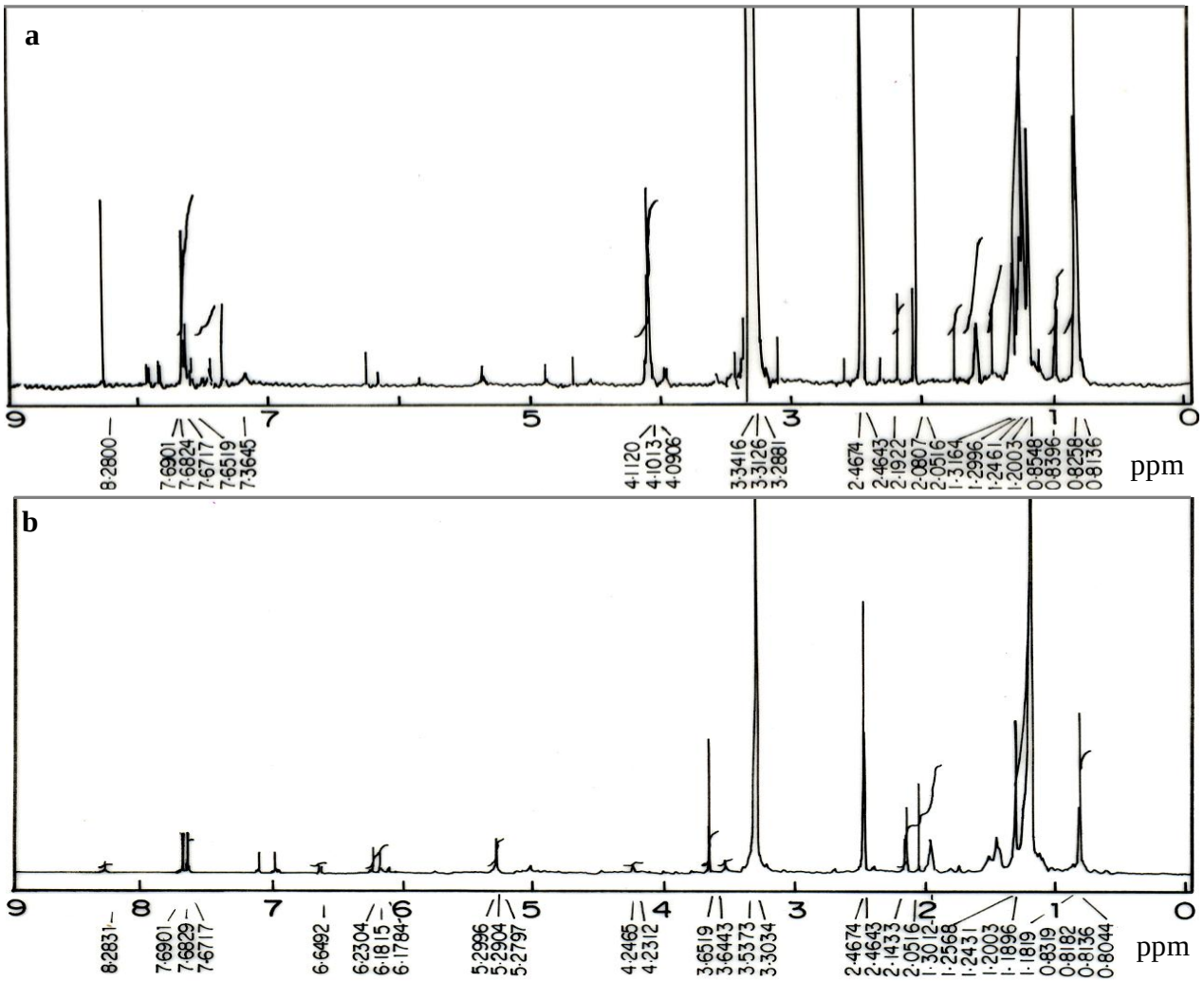

Figure 6. ^{1}H NMR spectrum of standard taxol (a) and fungal sample (b) in $CDCl_3$ at 500 MHz. All signals were resolved and distributed in the region between 1.0 and 8.5 ppm. The chemical shifts in ppm with high frequency from TMS. In comparison with standard taxol, fungal taxol also produced an identical spectrum.

type (MCF-7). This is in concurrence with the results of earlier report (Ruckdeschel et al., 1997). It has been reported that taxol at particular concentrations with higher incubation period induces cell apoptosis and the efficacy of taxol is fairly dependent on the specificity of the cell type. This also supports the earlier findings of other groups that at specific concentration, taxol inhibits cell proliferation by blocking mitosis (Ruckdeschel et al., 1997; Yeung et al., 1999).

Most of the earlier reported fungal taxol producers were isolated from yews (*Taxus* spp.) rather than other groups whereas, in this study, *L. theobromae* was an endophytic form of fungus isolated from the healthy leaves of an angiosperm medicinal plant, *M. citrifolia* exhibiting extracellular production of taxol in MID medium. 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 from horizontal gene transfer from the host plant to its endophytes (Strobel et al., 1996b, 1997). 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 (Strobel et al., 2006a). Fungi are obviously, a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential.

In the recent years, the quest for isolation of new compounds from medicinal plants has become a fascinating area of research. Plants with ethno-pharmaceutical importance are being exploited because of their healing properties. However, large scale harvesting of medicinal plants has already become a major threat to biodiversity. As an alternative, microbes that live inside the plants (endophytes) may be a potential source of therapeutic compounds. Hence, this study was investigated for the

Table 1. Cell viability of MCF-7 cancer cell lines treated with different concentration of fungal taxol isolated from the fungus, *L. theobromae*.

Taxol concentration (µg/ml)	Cell viability (%)		
	24 h	48 h	72 h
0	100	100	100
50	96	90	84
100	92	88	75
150	85	81	72
200	79	73	63
250	65	57	42
300	62	49	38
350	49	42	36
400	45	38	31
450	38	32	29
500	30	26	24
550	32	22	21
600	28	23	19

isolation and identification of taxol from an endophytic fungus *L. theobromae*.

In conclusion, it is evidenced that the spectroscopic, chromatographic and cytotoxic estimates are close to the reality given fact that the fungal taxol and standard taxol yielded identical results. It also indicates that, the formation of taxol by the fungus *L. theobromae* was found to be the higher and suggests that the fungus can serve as a potential species for genetic engineering to enhance the production of taxol.

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