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# A Novel Endophytic Taxol-Producing Fungus *Chaetomella raphigera* Isolated From a Medicinal Plant, *Terminalia arjuna*

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**Abstract** Taxol is the most important member of the clinically useful natural anticancer drug. An endophytic fungus *Chaetomella raphigera* (strain TAC-15) was isolated from a medicinal plant *Terminalia arjuna* and screened for its potential in Taxol production. The fungus was identified based on the morphology of the fungal culture and the characteristics of the spores. This fungus was grown in MID liquid medium and analyzed by chromatographically and spectrometrically for the presence of Taxol. The amount of Taxol produced by this endophytic fungus was quantified by HPLC which showed that it produced 79.6 μg/L, and further confirmative analyses were done by using UV, IR, FAB mass spectroscopy, and NMR spectroscopy. Thus, the fungus can serve as a potential material for fungus engineering to improve the production of Taxol.

**Keywords** Endophytic fungus · *Chaetomella raphigera* · Medicinal plant · Taxol · Anticancer drug.

## Introduction

Endophytic fungi are increasingly recognized as sources of novel bioactive compounds and secondary metabolites for biological control [1]. In addition to studying the distribution and ecology of fungal endophytes from medicinal plants, special attention should be given to screening them for potent metabolites. The endophytic fungi are of biotechnological importance as new pharmaceutical compounds, secondary metabolites, agents of biological control, and other useful characteristics could be found by further exploration of endophytes. Therefore, the use of endophytic fungi opens up new areas of biotechnological exploitations, which leads to the necessity of isolation and cultivation of these organisms. Taxol is a

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complicated diterpenoid compound with anticancer properties, which was first isolated from *Taxus brevifolia* [2]. Its mode of action is unique in that it prevents the depolymerization of tubulin during the processes of cell division [3]. The most common source of Taxol is the bark of trees belonging to the *Taxus* family including Yew trees. Unfortunately, these trees tend to be rare, slow growing, and a large amount of bark may have to be processed to obtain a small amount of the drug. The amount of Taxol found in yews is relatively small, ca 0.01–0.03% dry weight, and this has been a major factor contributing to its high price. With the discovery that certain endophytic fungi are able to produce, Taxol has become the possibility that a cheaper and more widely available product may eventually be available via industrial fermentation [4, 5]. Over the last decade, there has been a great deal of interest in finding other fungi that produce Taxol.

The first choice was to search for Taxol from other parts of *T. brevifolia* or other *Taxus* species. Taxol and related taxanes were found in *Taxus cuspiata*, *Taxus baccata* [2], *Taxus wallachiana* [6], *Taxus canadensis*, *Taxus floridana*, *Taxus chinensis*, and *Taxus media* also and Taxol levels exceeding those reported for *T. brevifolia* are present in shoots of individual trees from most taxa studied [7]. The problem is that the amount of the taxanes needed for each cancer treatment may require kilograms of bark tissue. Therefore, other ways to exploit the value of this chemical have to be found. The structure of Taxol is very complex and for a long time, this impeded the chemical synthesis, an alternative to using the natural source. It has recently been accomplished [8]. Yet, it is very time-consuming and difficult, requiring 23 steps and, thus, is not practical, at present, as an alternative to the natural source.

The discovery of *Taxomyces andreanae* was the first demonstration that any organism other than *Taxus* spp. could produce Taxol [9]. Recent work carried out reported that plants other than yew have endophytic fungi associated with them that also make this drug. Some endophytic fungi belonging to different genera such as *T. andreanae*, *Pestalotiopsis microspora*, *Alternaria alternata*, *Periconia* sp., *Pithomyces* sp., *Monochaetia* sp., *Seimatoantlerium nepalense* [10–12] are reported to produce Taxol. Thus, if a microbial source of the drug would be available, it would eliminate the need to harvest and extract the slow growing and relatively rare yew trees for this drug. The price for the drug would then be reduced. Therefore, the main objective of the present invention is to provide a novel endophytic fungus *Chaetomella raphigera* isolated from a medicinal plant, *Terminalia arjuna*. Virtually, very few reports are available on screening Taxol-producing endophytic fungi from tropical medicinal plant species. Therefore, this study provides first report on Taxol-producing endophytic fungus *C. raphigera* isolated from a medicinal plant from Southern India.

## Materials and Methods

### Isolation and Identification of Endophytic Fungi

The fungus used in this study was isolated as endophyte from the leaves of medicinal plant in Chennai City, India. The leaf samples were surface sterilized [13]. The surface-sterilized leaf segments were evenly spaced in Petri dishes (9 cm diameter) containing potato dextrose agar medium (amended with chloramphenicol 150 mg/l). The Petri dishes were sealed using Parafilm$^{TM}$ and incubated at 26±1 °C in a light chamber with 12 h of light followed by 12 h of dark cycles. The Petri dishes were monitored everyday to check the growth of endophytic fungal colonies from the leaf segments. The hyphal tips, which grew out from leaf segments, were isolated and identified using standard monographs. The identified fungal cultures were deposited at the Madras University Botany Laboratory

(MUBL), CAS in Botany, University of Madras, Chennai—600 025. The immediate concern is to find one or more fungi that produce more Taxol. This endophytic fungus *C. raphigera* (strain TAC-15) (MUBL No. 665), was screened for Taxol production. Photomicrographs of conidia were taken with the help of Carl Zeiss Axiostar plus-Photomicroscope (phase contrast) with Nikon FM 10 Camera and Nikon HFX Labophot (bright field) with Nicon FX-35A by using Konica films.

## Preparation of Fungal Extracts

The endophytic fungus was grown in 2-l Erlenmeyer flasks containing 500 ml of MID medium supplemented with soytone (14) and incubated for 21 days. After 3 weeks of still culture at 26 °C, the culture fluid was passed through four layers of cheese cloth to remove solids and extracted with organic solvent. The extraction and isolation procedure followed was that of Strobel et al. [15]. After methylene chloride extraction, 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 redissolved in methanol for the subsequent separation, and extracts were analyzed by chromatographic separation and spectroscopic analyses. The standard Taxol (Paclitaxel) was purchased from SIGMA.

## High Performance Liquid Chromatography Analysis

To further confirm the presence of Taxol, the fungal samples were subjected to high performance liquid chromatography (HPLC). Taxol was analyzed by HPLC (Shimadzu 9A model) using a reverse phase $C_{18}$ column with an ultraviolet (UV) detector. Twenty microliters of the sample was injected each time and detected at 232 nm. The mobile phase was methanol/acetonitrile/water (25:35:40, by *v*/*v*/*v*) at 1.0 ml $min^{-1}$. The sample and the mobile phase were filtered through 0.2 μm PVDF filter before entering the column. Taxol was quantified by comparing the peak area of the samples with that of the standard Taxol.

## Ultraviolet Spectroscopic Analysis

After chromatography, the area of plate containing putative Taxol was carefully removed by scrapping off the silica at the appropriate $R_f$ and exhaustively eluting it with methanol. The purified sample of Taxol was analyzed by UV absorption, dissolved in 100% methanol at 273 nm in a Beckman DU-40 Spectrophotometer and compared with authentic Taxol (Paclitaxel—SIGMA Grade).

## Infrared Spectroscopic Analysis

The infrared (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 Pelletizer and compared with authentic Taxol. The IR spectra were recorded in the region 4000–500 $cm^{-1}$.

## Fast Atom Bombardment (FAB) Mass Spectroscopic Analysis

The fast atom bombardment (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.

Nuclear Magnetic Resonance Spectroscopic Analysis

$^1$H nuclear magnetic resonance (NMR) spectra were recorded to confirm the structure of fungal Taxol 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 $\delta$ values relative to tetramethylsilane (TMS) as internal reference, and coupling constants were reported in Hertz.

## Results and Discussion

Endophytic microorganisms that reside in the tissues of living plants are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture, and industry. It is worthy to note that of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes. The purpose of this work was to identify a Taxol-producing endophytic fungus from the selected medicinal plant and study its anticancer activities. Therefore, the use of endophytic fungi opens up new areas of biotechnological exploitations, which leads to the necessity of isolation and cultivation of these organisms. The aim of this present study is to isolate and identify the Taxol-producing endophytic fungi from medicinal plants so that the fungus can serve as a potential material for fungus engineering to improve the production of Taxol. Based on the morphology of the mycelial colony as well as the characteristics of the conidia, the endophytic fungus was identified as *C. raphigera*. Colonies are brown, septate, branched mycelium. Conidiomata are pycnidial, separate, globose but opening widely, very shortly stipitate, dark brown to black, thick-walled, 200–350×100–250 μm, Ostiole single, longitudinal. Setae brown, smooth, thick-walled, septate, unbranched, apices hooked 50–100×2.5–5 μm. Conidiophores hyaline, branched, filiform, septate, and smooth. Conidiogenous cells enteroblastic, phialidic, determinate, integrated, filiform, hyaline, smooth. Conidia hyaline, aseptate, cymbiform to allantoid, 3.75–6.25×1.25–2.5 μm (Fig. 1). This fungus was screened for Taxol production and the extract of the fungal culture was examined for the presence of Taxol by chromatographic and spectroscopic analyses. The compound has chromatographic properties identical to standard Taxol in solvent systems AE and gives color reaction with the spray reagent, and it appeared as a bluish spot fading to dark gray after 24 h [16]. They had $R_f$ values identical to that of standard Taxol. Therefore, it was evident that this fungus showed positive results for Taxol production. Taxol is positively identified via its co-chromatographic mobilities with authentic Taxol in a multitude of thin layer chromatographic systems [4, 5]. Strobel et al. isolated *P. microspora* from the inner bark of *T. wallachiana*, which was shown to produce Taxol in mycelial culture and the total amount of Taxol produced per liter was about 60–70 μg [15]. In the present investigation, HPLC analysis of the fungal extract gave a peak when eluting from a reverse phase $C_{18}$ column, with about the similar retention time as authentic Taxol and the fungus produced 79.6 μg/L of Taxol (Fig. 2) in liquid culture. The presence of Taxol in the fungal extract was further confirmed by UV spectroscopy. The UV spectral analysis of the fungal Taxol is given in Fig. 3, and the spectrum was superimposed on that of authentic Taxol. Commonly, Taxol represents 0.01–0.02% of the weight of dry bark, and the Taxol content of 1 l of *P. microspora* culture is about 0.001% of the total dry weight of the culture contents. Trace amounts of compounds other than Taxol may perturb the absorbance reading, giving weight estimates for Taxol greater than are actually present.

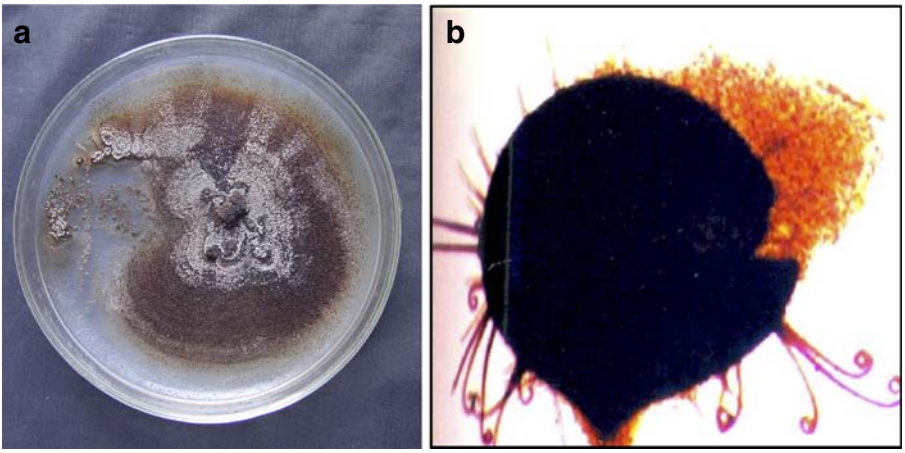


**Fig. 1** **a** Morphological observation of the fungus *C. raphigera* and **b** magnification of spores: ×40

An endophytic fungus *Pestalotiopsis terminaliae* was isolated from the same medicinal plant was produced Taxol about 211.1 μg/l, and the fungal Taxol extracted from the organic extract of this fungal culture, had strong cytotoxic activity towards BT 220, H116, Int 407, HL 251 and HLK 210 human cancer cells in vitro, tested by Apoptotic assay [17].

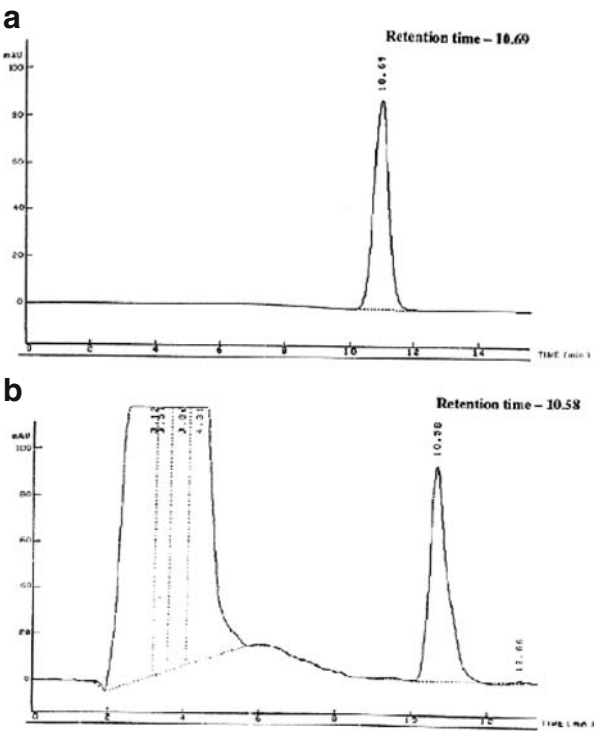


**Fig. 2** High performance liquid chromatogram of authentic Taxol (**a**) and fungal Taxol from *C. raphigera* (**b**); the mobile phase was methanol/acetonitrile/water (25:35:40, *v*/*v*/*v*), flow rate at 1.0 ml/min; Retention time of authentic Taxol: 10.69 min; retention time of fungal Taxol: 10.58

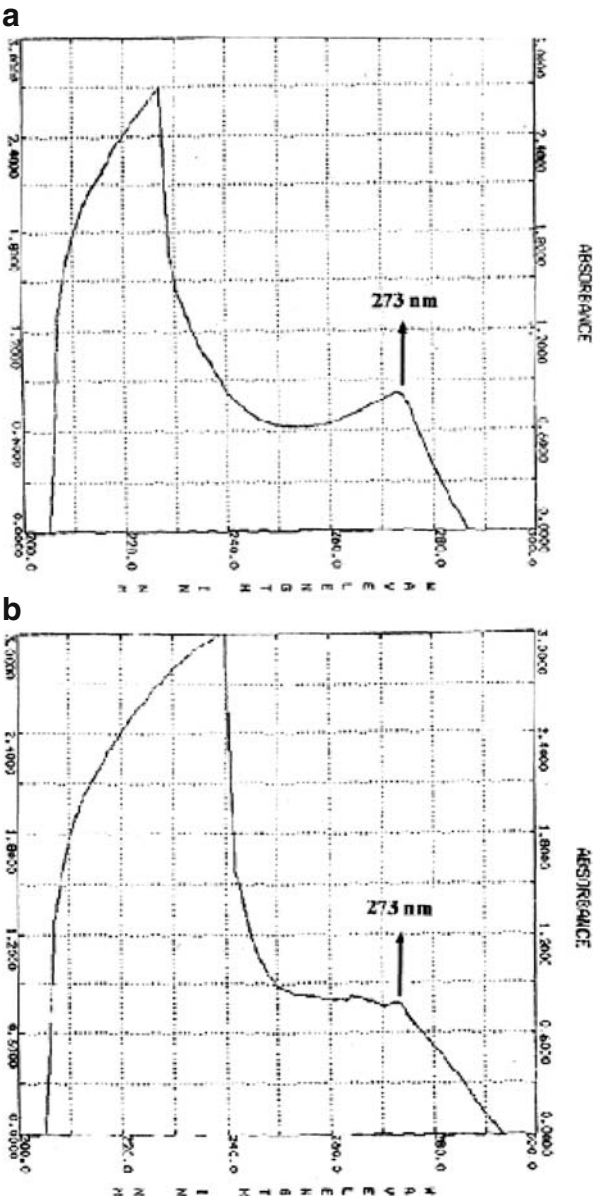


**Fig. 3** Ultraviolet Absorption Spectra of authentic Taxol (**a**) and fungal Taxol from *C. raphigera* (**b**) was recorded in methanol at 273 nm

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. The biggest problem of using fungi fermentation to produce Taxol is its very low yield and unstable production. The Taxol yield of such reported fungi varies from 24 ng to 70 μg/l culture [4, 6, 15].

The IR spectral data of fungal Taxol from *C. raphigera* showed a broad peak in the region 3417.6 $cm^{-1}$ was ascribed to hydroxyl (–OH) and amide (–NH) groups stretch. The

esters and ketone (C=O) groups stretch was observed in the region of 1,724.2 $cm^{-1}$. The aromatic ring (C=C) stretching frequency was observed in the region 1,658.7 $cm^{-1}$. A peak observed in the region 1026.1 $cm^{-1}$ is due to the presence of aromatic C, H bends. The IR spectrum of *C. raphigera* was superimposed on that of authentic Taxol (Fig. 4).

The FAB mass spectrum of *C. raphigera* was identical to that of authentic Taxol (Fig. 5). Characteristically, authentic Taxol yielded $[M+H]^+$ at *m/z* 854. By comparison, fungal Taxol also yielded a peak $[M+H]^+$ at *m/z* 854 with characteristic fragment peaks at 569, 551, 509, 464, 286, and 268. It was evident that the diterpenoid Taxol was much more complex since its molecular weight from high resolution mass spectrometry is $C_{47}H_{51}NO_{14}$, corresponding to a molecular weight of 853. Characteristically, authentic Taxol yielded $[M+H]^+$ at *m/z* 854. Major fragment ions observed in the mass spectrum of Taxol can be placed into three categories representative of the major portions of the molecule [18]. The peaks corresponding to Taxol, exhibited mass-to-charge (*m/z*) ratios corresponding to the molecular ions $[M+H]^+$ of authentic Taxol (854) confirming the presence of Taxol. As reported in detail by Wani et al. [2], the esterified position was found to be the allylic $C_{13}$ hydroxyl moiety. The FAB mass spectrum of the fungal sample not only corroborated the molecular formula, $C_{47}H_{51}NO_{14}$ but was also found to be identical with FAB-MS of authentic Taxol. Taxol continues to have an increasing role in the treatment of human malignancies, particularly ovarian and breast cancer [19]. Recent accumulating reports have demonstrated that Taxol-induced microtubular bundling and mitotic arrest of human

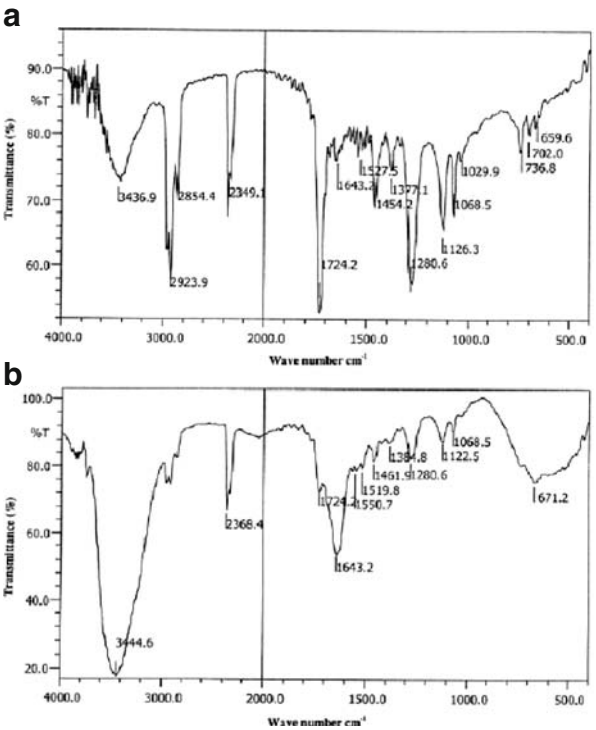


**Fig. 4** Infrared spectroscopic analysis of authentic Taxol (**a**), fungal Taxol from *C. raphigera* (**b**)

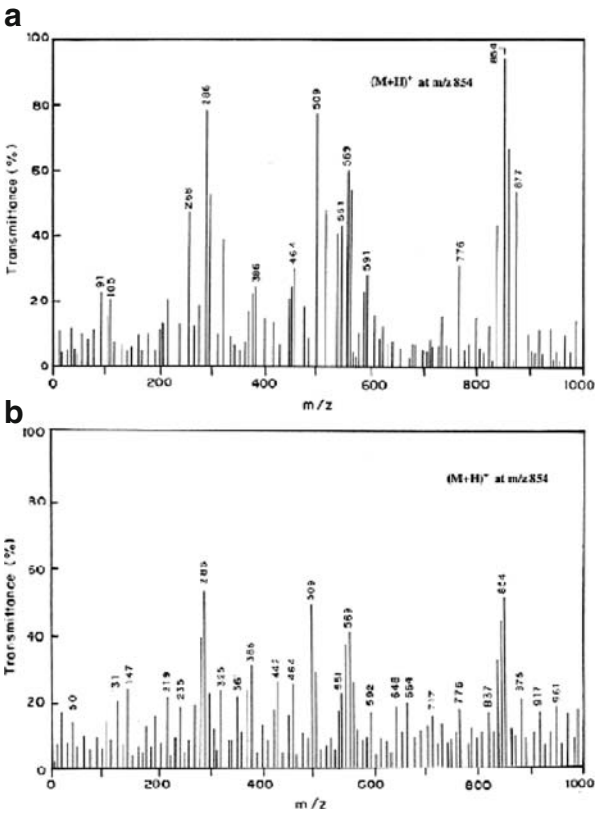


**Fig. 5** Fast atom bombardment mass spectrum of authentic Taxol (**a**) fungal Taxol from *C. raphigera* (**b**). The accelerating voltage was 10 kV and recorded at room temperature

leukemia cells are followed by DNA fragmentation and morphological features of apoptosis [20].

In the $^{1}$H NMR spectra, almost all of the signals are well resolved and are distributed in the region from 1.0 to 8.5 ppm (Fig. 6). The strong three-proton signals caused by the methyl and acetate 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 are observed in the region between 2.5 and 7.0 ppm, and the aromatic proton signals caused by the C-2 benzoate, C-3′ phenyl, and C-3′ benzamide groups appear between 7.0 and 8.3 ppm. The Taxol assignments obtained was confirmed with earlier reports [21].

Although the amount of Taxol produced by most endophytic fungi associated with *Taxus* trees is relatively small when compared with that of the trees, the short generation time and high growth rate of fungi make it worthwhile to continue our investigation of these species. Some endophytic fungi isolated from medicinal plants in India, produced considerable amount of Taxol. The results of HPTLC showed that the endophytic fungal species isolated from some medicinal plants had identical UV characteristics, positive reactivity with a spray reagent, yielding a blue spot, which turned to dark gray after 24 h, and had $R_f$ values identical to that of the authentic Taxol. The amount of Taxol was also quantified by comparing the peak area and the peak height of the fungal samples with those of authentic Taxol [22–24].

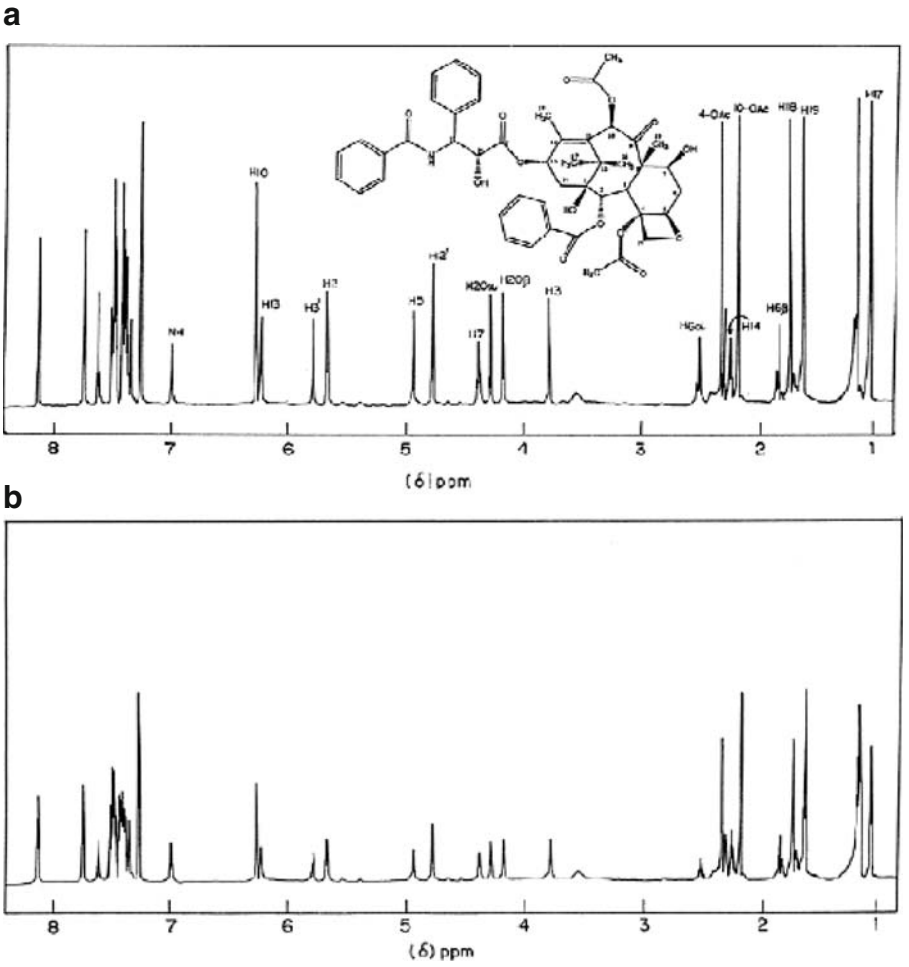


**Fig. 6** $^{1}$H NMR spectrum of authentic Taxol (**a**) and fungal Taxol isolated from *C. raphigera* (**b**) in $CDCl_3$ at 500 MHz. The structure of Taxol is shown as an *insert*. The chemical shifts in ppm high frequency from TMS. The structure of Taxol is shown as an *insert*

This study also suggests that improved culture techniques, addition of activators or elicitors, and application of genetic engineering methods may result in enhanced production of Taxol by these microbes and permit commercialization of *C. raphigera* for Taxol production. A background understanding that involves some specific examples and rationale of the presence of endophytic microorganisms in higher plants will aid in the development of a drug discovery program involving these organisms. A search for a rare and, thus, expensive product such as Taxol may be facilitated by examining the endophytic microorganisms of certain plants for their ability to make this drug [1].

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