ISSN 0975-6299 Vol.1/Issue-3/Jul-Sep.2010

International Journal of Pharma and Bio Sciences

TAXOL (ANTICANCER DRUG) PRODUCING ENDOPHYTIC FUNGI: AN OVERVIEW

SONAIMUTHU VISALAKCHI* AND JOHNPAUL MUTHUMARY

Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai - 600 025, Tamil Nadu, India.

Corresponding Authors svisalakchi@gmail.com/mm_j@rediffmail.com

ABSTRACT

In this review, we mainly focused on characterization of the taxol producing endophytic fungi from medicinal plants. The different endophytic fungi were isolated and characterized for its potential in taxol production in past two decades. All the strains were identified based on the morphology of the fungal culture and the characteristics of the spores and with specific molecular techniques. The endophytic strains were grown in MID liquid medium for the taxol production. The qualitative and quantitative measures were analyzed to confirm the presence of taxol. The amounts of taxol production were quantified by high performance liquid chromatography which suggests that the fungus can serve as a potential material for genetic engineering to improve the taxol production. Further, the endophytic fungal taxol have strong cytotoxic activity on different human cancer cells *in vitro*, tested by apoptotic assay

KEY WORDS

Anticancer drug, Cancer cell lines, Endophytic fungi, Medicinal plant, Taxol, Taxus.

INTRODUCTION

Medicinal plants

A medicinal plant is any plant which in one or more of the organ contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs. Plants are one of the most important sources of medicine in the recent years. Plant products have been a source of medicinal agent since time immemorial. From the dawn of civilization, human have been utilizing the important biological properties of various plants for treat different types of the diseases. Even today, plants are the most exclusive source of drugs for the majority of world's population and plant products constitute about 25% of prescribed medicines[5]. Medicinal plants have their values in the substance(s) present in various plant tissues. These produce specific physiological action in the human body. The more important of these substances are alkaloids, compounds of carbon, hydrogen, oxygen and nitrogen. These substances glucosides, essential fatty oils, resins, gums, mucilage, tannins and secondary metabolites etc. are also of large use. These active principles may be present in the storage organs of the plant.

Endophytes

Endophytes, microorganisms that reside in the internal tissues of living plants without causing any immediate overt negative effects have been found in every plant species examined to date and recognized as the potential sources of novel natural products for exploitation in medicine, agriculture and industry with more bioactive natural products isolated from the microorganisms[1,27]. Endophytes are ubiquitous with rich biodiversity, which have been found in every plant species examined to date. It is noteworthy that, of the nearly 3,00,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes[27]. In this view of the special colonization in certain hosts, it is estimated that there may be as many as 1 million different endophyte species. However, only a handful of them have been described[19], which means the opportunity to find new and targeting natural products from interesting endophytic microorganisms among myriads of plants in different niches and ecosystems is great. Some of the endophytes are the chemical synthesizers in inside the plants[18]. Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against human pathogens and some of these compounds have been proven useful for novel drug discovery. Recent studies have reported hundreds of natural products including substance of alkaloids, terpenoids, flavonoids, steroids, etc. from endophytes. Up to now, most of the natural products from endophytes are antibiotics, anticancer agents, biological control agents and other bioactive compounds by their different functional roles.

Endophytic fungus

Endophytic fungi are increasingly recognized as sources of novel bioactive compounds and secondary metabolites for biological control. 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

Taxol is an important anticancer drug used widely in the clinical field. Taxol is a diterpenoids, which was first extracted in the bark of yew (*Taxus brevifolia*)[33]. It can kill tumor cells by enhancing the assembly of microtubules and inhibiting their depolymerisation[24]. This compound is the world's first billion dollar anticancer drug and it is used to treat breast, lung, ovarian cancer and other human tissue proliferating disease[31]. Taxol is a white to off white crystalline power contains 11 sterocenters and with the empirical formula $C_{47}H_{51}NO_{14}$ (molecular weight 853.9). It is highly lipophillic, insoluble in water and melts at around 216-217ºC. Structurally, it can be viewed as the N-benzoyl-β-phenylisoserine ester of diterpenoid bacentia III with a very characteristic oxetane ring. Kingson[10] have completely studied the chemical structure of the taxol which is classified as taxane diterpenoids or taxoid. Taxol has a unique mode of action when compared to other anticancer drug. It acts as a promoter of tubulin polymerization and stabilizes microtubules to depolymerization by different agents, both *in vitro* and *in vivo*[24]. Taxol has been well established and approved by Food and Drug Administration (FDA) as a very important effective chemotherapeutic agent against a wide range of tumors since 1992[11]. However, the supply of Taxol has been very limited since the discovery of this natural product and with increasing the applications in chemotherapy, the availability and cost of the drug will remain an important issue[12].

Taxol used in cancer chemotherapy and scientific research is isolated from yew tree or semisynthesized from its precursors such as

baccatin III and 10-deacetylbaccatin III which are all isolated from this natural plant[4]. However, this natural resource is being threatened day by day due to the destructive collection of *Taxus* bark for Taxol. In order to protect *Taxus* in the world and lighten the pressure of Taxol sourcing, other approaches to obtain Taxol have been under investigation and some progresses have been made. Besides semi-synthesis and isolation from plant, there are several other possible routes to industrialize Taxol production: tissue or cell culture[3,9], total chemical synthesis[8,16,17], endophytic fungal fermentation[14,26,29,32] and a potential way of engineering for Taxol production.

TAXOL FROM ENDOPHYTIC FUNGI

The first Taxol producing fungus *Taxomyces andreanae* was reported[26]. Although the yield of Taxol is only as low as 24-50 ng/L, this finding causes scientists' great interest. Ever since, there have been a few reports on the isolation of Taxol producing endophytic fungi[14,29,32], demonstrating that organisms other than *Taxus* species could produce Taxol. Thus, the Taxol producing microorganisms may be an alternative promising way to produce using fermentation process. Meanwhile, the biggest problem of using fungi fermentation to produce Taxol is its very poor yield and unstable production. The Taxol yield of such reported fungi varies from 24 ng to 70 µg/L of culture[26,29]. One strain of *Pestalotiopsis microspora* CP-4 produces Taxol varying from 50 to 1487 ng/L, indicating that it is genetically unstable[14]. To solve such a problem, current studies mainly focuses on the tedious work of finding and isolating fungi with high, stable yield of Taxol, as well as optimization of fermenting conditions. The genetic origin of fungal taxol production has been speculated to have arisen by horizontal gene transfer from host plant to its endophytes. Little documentation exists with regards to gene transfer from a higher plant to an endophyte or parasite. Alternatively, fungi might be an independently evolved system for taxol production. Some endophytic fungi belonging to different genera such as *T. andreanae, Pestalotiopsis microspora, Alternaria alternata, Periconia* sp., *Pithomyces* sp., *Chaetomella raphigera*, *Monochaetia* sp. and *Seimatoantlerium nepalense* are reported to produce Taxol.

Recently, our research group has isolated the several endophytic fungal strains (more than 100 strains) from varoius medicinal plants in India for the taxol production[6]. The higher taxol producing endophytic fungal strains such as *Botryodiplodia theobromae* from *Taxus baccata* and leaf spot strain, *Phyllosticta citricarpa* from *Citrus medica* were reported[22,25]. In addition, a simple and rapid method for the determination of taxol produced by fungal endophytes from medicinal plants using high performance thin layer chromatography has been well established. The optimization of fermenting conditions on the taxol producing endophytic fungal starins is under progress. An endophytic fungus, *Pestalotiopsis terminaliae* was isolated from the *Terminalia arjuna* plant was produced the highest amount of Taxol[7].

Extraction of taxol

The standard extraction procedure[29] was followed for the taxol isolation from endophytic fungal strains. All the strains were grown in 2 L Erlenmeyer flasks containing 500 mL of standard liquid medium for taxol production supplemented with 1 g/L soytone. The fungi were individually inoculated and incubated for 21 days. After the incubation, the cultures were filtered through four layers of cheesecloth to remove the mycelia. 0.25 g Na_2CO_3 was added to the culture filtrate with frequent shaking, 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 35ºC using a rotary vacuum evaporator. The dry solid residue was re-dissolved in methanol for subsequent separation. The crude extracts were analyzed by chromatographic separation and spectroscopic analyses.

Thin layer chromatographic (TLC) analysis

The TLC analysis was carried out on 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, methylenechloride:Tetrahydrofuran (6:2, v/v) and solvent E, methylenechloride:methanol:dimethylformamide (90:9:1, v/v/v). Taxol was detected with 1% w/v vanillin in sulphuric acid reagent after gentle heating. The fungal taxol was identified by comparison with the authentic taxol. The fungal taxol had identical and positive reactivity with the spray reagent, which yielded a blue spot and turned to dark gray after 24 hours. The endophytic fungal compounds are with the same chromatographic mobility as the authentic taxol were recorded and they showed the R_f values ranged between 0.22–0.24. Some of the endophytic fungal strains did not show evidence of taxol production.

Ultraviolet (UV) spectroscopic analysis

The presence of taxol in the fungal extract was further confirmed by UV spectroscopy. After the TLC method, 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. After the elution, the crude taxol was performed for the qualitative and quantitative analyses. The taxol samples were analyzed by UV absorption (Beckman DU-40 Spectrophotometer), dissolved in 100% methanol and compared with authentic taxol (Paclitaxel, Sigma Grade). They had a characteristic absorption peak at 235–273 nm, similar to those previous reports.

High performance thin layer chromatography (HPTLC) analysis

The prepurified fungal taxol samples obtained in TLC were further subjected to HPTLC (CAMAG-Planar HPTLC, Anchrom). With the automatic TLC Sampler, all the extracted taxol samples and authentic taxol (5 µL for each samples) were injected into their respective tracks separately, with the data pair technique, with the band length being 5 mm, the distance from lower edge 10 mm, the distance from the side 20 mm and the track distance 6.4 mm, on a pre-coated silica gel 60 F254 (20 cm × 10 cm) plate (Merck, Germany). After loading, the plate was dried for 30 s using a plate heater. After loading, the TLC plate was developed in a CAMAG pre-saturated flat bottom developing chamber with chloroform-methanol (9:1, v/v) for 20 min. Documentation of the TLC plate was performed under a shorter wavelength (254 nm) UV lamp and a longer wavelength (366 nm) UV lamp, prior to derivatization. The presence of the taxol was visualized after it was sprayed with 1% (w/v) vanillin sulfuric acid and heated gently for 2 min. After derivatization with the spray reagent, documentation was performed under the UV lamp at 366 nm and white light, which was used to visualize the colored substance after derivatization. In HPTLC, the amount of taxol produced by the fungi was calculated through comparing the area and the height of peaks observed in fungal taxol with that of authentic taxol. The results obtained in HPTLC technique can be used for rapid separation and high throughput screening for taxol producing endophytic fungi and used for quantification of the amount of taxol. The best results were achieved using visualization after derivatization by spraying a reagent and by identifying with the help of the fingerprint technique. This validated method was found to be simple, reliable and convenient for routine analysis. In HPTLC, the principal requirement for the documentation was the visibility of the chromatogram with or without derivatization. The substances with the absorbance of UV light at 254 nm were visualized as dark zones on the plates, with a fluorescence indicator, which was excited to emit green light under a shorter wavelength (254 nm) UV lamp. A longer wavelength (366 nm) UV lamp was used to excite substances that were able to fluoresce. White light was used to visualize colored

substances. The documentation of the TLC plate was performed under a shorter wavelength (254 nm) UV lamp.

Infrared (IR) spectroscopic analysis

IR spectrum of the fungal taxol was 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. Further, the extracted fungal taxol were analysed by IR spectrum for the confirmation. The IR spectrum showed a broad peak at 3434.99/cm which was assigned for the presence of the O-group in the parent compound, as evidenced by its OH stretch. The aliphatic CH stretch was observed at 2927.74/cm. The C=O (keto group) stretch was positioned at 1724.24 and 1656.74/cm. The registration peak observed at 1485.08 and 1450.37/cm was due to the NH stretching frequency. The COO stretching frequency was observed at 1371.29 and 1242.07/cm. The peaks in the range between 1070.42 and 979.77/cm were due to the presence of aromatic C and H bends. Fungal taxol was further confirmed by IR fingerprints recorded between 1000 and 3500/cm, which were also identical in comparison to the standard taxol. Therefore, it was evident that the endophytic fungus showed positive results for taxol production in M1D medium. For example, the IR spectral data of fungal Taxol from *C. raphigera* showed a broad peak in the region 3417.6/cm was described 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. The aromatic ring (C=C) stretching frequency was observed in the region 1,658.7/cm. A peak observed in the region 1026.1/cm is due to the presence of aromatic C, H bends.

High performance liquid chromatography (HPLC) analysis

Taxol was analyzed by HPLC (Shimadzu 9A model) using a reverse phase C18 column with UV detector. Twenty microliters of the sample was injected each time and detected at 232 - 275 nm. The mobile phase was methanol/acetonitrile/water (25:35:40, by v/v/v) at 1.0 ml/min. 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. Results of HPLC analysis showed the presence of taxol by recording a peak with a specific retention time 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 taxol in liquid culture. For example, HPLC analysis showed the presence of taxol by showing a peak with a retention time of 4.7 min. *P. tabernaemontanae* produced a high content of taxol in M1D (461 µg/L) when compared with PDB (150 µg/L). Taxol detection was not observed in the blank culture samples, in which they showed negative results in all the analyses. The fungal taxol yield was easily quantified with HPLC analysis since the production was found to be higher (in micrograms). Where as, in the earlier reports it was quantified with the aid of immunoassay since the yield level was recorded to be low (in nanograms)[14,28,29]. The biggest problem of using fungi in fermentation is the low level of yield with unstable taxol production. Although the amount of taxol produced by the 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 investigation on endophytic fungi isolated from the medicinal plants

LC-MS analysis

Further evidence confirming the identity of taxol was obtained by LC-MS spectroscopic analysis. LC-MS analysis was carried out on all the 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 via the loop injection method. Characteristically, standard taxol yielded both a $(M+H)^+$ peak at a molecular weight of 854 m/z and a $(M+Na)^+$ peak at a molecular weight of 876 m/z. 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 m/z. Major fragment ions observed in the mass spectrum of taxol are divided into three categories, which represent the major portion of the respective taxol molecule. The peaks analogous to taxol exhibited mass-to-charge (m/z) ratios corresponding to the molecular ions $(M+H)^+$ of the standard taxol (854 m/z), confirming the presence of endophytic taxol. It was evident that the diterpene taxol was much more complex since its molecular weight from high resolution MS is 854, corresponding to the molecular formula of $C_{47}H_{51}NO_{14}$ as reported earlier. By comparison, fungal taxol also yielded a peak MH^+ at m/z 854 with characteristic fragment peaks at 569, 551, 509, 464, 286 and 268. Major fragment ions observed in the mass spectrum of taxol can be placed into three categories which represents major portions of the molecule[15]. The peaks corresponding to taxol, exhibited mass-to-charge (m/z) ratios corresponding to the molecular ions $(M+H)^+$ of standard taxol (854) confirming the presence of taxol in the fungal extracts. As detailed report[33], the esterified position was found to be the allylic C^{13} hydroxyl moiety.

Nuclear magnetic resonance (NMR) spectroscopic analysis

^1H NMR spectra were recorded to confirm the structure of fungal taxol at 23℃ 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 δ values relative to tetramethylsilane (TMS) as internal reference and coupling constants were reported in Hertz. Samples dissolved in $CDCl_3$ (Sigma) were used for the analysis. Proton spectrums were assigned by comparison of chemical shifts and coupling constants with those of related compounds. Chemical shifts were reported as δ-values relative to TMS as an internal reference and coupling constants were reported in Hertz. In ^1H NMR spectroscopic analysis, almost all signals were well-resolved and distributed in the region between 1.0 and 8.5 ppm. The strong three proton signals caused by the methyl and acetate groups lie in the region between 1.0 and 2.5 ppm (H17, H19, H18, H6β, 10-OAc, H14, 4-OAc and H2Oα), 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 (H3, H2Oβ, H2Oα, H7, H2 ′, H5, H2, H3′, H13, H10 and NH) 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 ^1H NMR spectrum of the fungal taxol was identical in comparison with standard taxol. The taxol assignments obtained was confirmed with the earlier reports[2]. The method has been established for isolation, identification and characterization of a novel fungal endophyte (*Trametes hirsute*) that produces aryl tetralin lignans detected by HPLC, LC-MS, LC/MSMS and NMR[21].

ANTICANCER AGENTS FROM ENDOPHYTES

The cytotoxic effect of fungal taxol isolated from the culture filtrate of M1D and PDB, was detected and quantified by using in vitro apoptotic method of assay[23] on various cancer cells, at various concentrations. The human cancer cell lines (HLK210, H116, Int407, HL251 and BT220) 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 µM and 5 µM were incubated for 48 h. The cells were then stained (DNA staining) with 0.5 mg/ml propidium iodide in phosphate buffered saline (PBS) for 15 min and de-stained in PBS solution. After

treatment with different concentrations of fungal taxol, the cell morphology was determined by light microscopy. In all, five different fields were randomly selected for counting at least 500 cells. The percentage of apoptotic cells was calculated for each experiment. Cells designated as apoptotic were those that displayed the characteristic morphological features of apoptosis, including cell volume shrinkage, chromatin condensation and the presence of membrane bound apoptotic bodies. The cells in the apoptosis were calculated by using the following formula.

Percentage of apoptotic cells = Number of apoptotic cells/ Total number of cells × 100

Cytotoxicity effect of fungal taxol isolated from the endophytic fungus was detected and quantified using apoptotic assay[23] on various cancer cellsThe taxol-producing endophytic fungi had previously tested for their cytotoxic activity via an apoptotic assay against different cancer cell lines. They showed strong cytotoxic activity in the presence of BT220, H116, Int407, HL251 and HLK210 human cancer cells *in vitro*. Previous report of the endophytic fungus showed the strong cytotoxic activity towards BT 220, H116, Int 407, HL 251 and HLK 210 human cancer cells *in vitro*, tested by Apoptotic assay[13,30]. Recently, Taxol was tested using an in vitro cytotoxicity assay against human cancer cell lines (A-549 for lung cancer, HEP-2 for liver cancer, OVCAR-5 for ovarian cancer) in comparison with the standard authentic example, resulting in comparable activities[20].

Cytotoxicity effect of fungal taxol from medicinal plants were further tested using apoptotic assay on various cancer cells viz., human breast cell BT220, human colon H116, human intestine Int407, human lung HL251 and human leukemia HLK 210. It is indicated that with the increase of taxol concentration from 0.005 µM to 0.05 µM, taxol induced increased cell death through apoptosis. With further increase of taxol concentration from 0.05 µM to 0.5 µM, the taxol-induced cell death through apoptosis only increased slightly. When the taxol concentration was increased from 0.5 µM to 5 µM, the taxol-induced cell death through apoptosis decreased dramatically. It was observed at low to medium concentration (0.005–5 µM), the efficacy of fungal taxol was quite dependent on the specific cell type. It has been reported that taxol at low concentrations (nM) induces cell apoptosis and the efficacy of taxol is quite dependent on the specific cell type. This also supports the previous findings of other groups that at low concentration, taxol inhibits cell proliferation by blocking mitosis.

CONCLUSION

The aim of this review clearly mention about the isolation and characterization of taxol producing endophytic fungi from medicinal plants. In the review, it is confidently evident that the spectroscopic and chromatographic estimates are close to reality, given fact that the fungal taxol and standard taxol give identical results. It also indicates that the formation of taxol by endophytic fungus 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. The significance in the discovery of fungi that produce taxol indicate that there are abundant resources of fungi that produce taxol. 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. To meet the urgent need of clinic and scientific research, besides Taxus supply, other approaches to obtain Taxol have also been discussed here.

ACKNOWLEDGEMENTS

We are gratefully acknowledged Prof. R. Rengaswamy, Director, CAS in Botany, Chennai, India.

REFERENCES

1. Bacon CW and White JF, Microbial Endophytes, New York, Marcel Dekker: 341–388, (2000).
2. Chmurny GN, Hilton BD, Brobst S, Look SA, Witherup KN, Beutler JA, 1H- and 13C-NMR assignments for Taxol, 7-epi-Taxol and Cephalomannine. J Nat Prod, 55:414–423, (1992).
3. Christen AA, Bland J, Gibson DM, Cell cultures as a means to produce Taxol. Proc.Am. Assoc. Cancer Res, 30: 566, (1989).
4. Denis JN, Greene AE, Guenard D, Guéritte-Voegelein F, Mangatal L, Potier P, Highly efficient practical approach to natural Taxol. J. Am. Chem. Soc, 110: 5917-5919, (1988).
5. Fransworth MR and Bingel AS, New natural products and plants drug with pharmacological, biological or therapeutical activity, Springer: New York, 61-73, (1977).
6. Gangadevi V, Muthumary J, A simple and rapid method for the determination of taxol produced by fungal endophytes from medicinal plants using high performance thin layer chromatography. Chinese Journal of Chromatography, 26 (1): 50–55, (2008).
7. Gangadevi V, Muthumary J, Taxol production by *Pestalotiopsis terminaliae*, an endophytic fungus of *Terminalia arjuna* (arjun tree). Biotechnology and Applied Biochemistry, 52: 9–15, (2009).
8. Holton RA, Somoza C, Kim HB, Liang F, Biediger RJ, Boatman PD, Shindo M, Smith CC, Kim S, Nadizadeh H, Suzuki Y, Tao C, Yu P, Tang S, Zhang P, Murthi KK, Gentile LN, Liu JH, First total synthesis of Taxol. J. Am. Chem. Soc, 116: 1597-1600, (1994).
9. Hu YM, Gan FY, Lu CH, Ding HS, Shen YM, Production of Taxol and related taxanes by cell suspension cultures of *Taxus yunnanensis*. Acta. Bot. Sin, 45: 373-378, (2003).
10. Kingston DGI, ACS Symposium Series 583, *Taxane Anticancer Agents*, 203, Georg, G.I. et al.,eds, Chap. 15, American Chemical Society, (1995).
11. Kohler J, Goldspiel BR, Evaluation of new drug Paclitaxel (Taxol). Pharmacotherapy, 14: 3-34, (1994).
12. Kwon IC, Yoo YJ, Lee JH, Hyun JO, Enhancement of Taxol production by in situ recovery of product. Process Biochem, 33: 701-707, (1998).
13. Lee JC, Strobel GA, Lobkovsky E, Clardy JC, J. Org. Chem. 61: 3232–3233, (1996).
14. Li JY, Stroble GA, Sidhu R, Hess WM, Ford EJ, Endophytic Taxol producing fungi from bald cypress, *Taxodium distichum*. Microbiology, 142: 2223-2226, (1996).
15. McClure TD, KH Schram, The mass spectrometry of taxol. J. Am. Soc. Mass Spectrom. 3: 672-679, (1992).
16. Morihira K, Hara R, Kawahara S, Nishimori T, Nakamura N, Kusama H, Kuwajima I, Enantio-selective total synthesis of Taxol. J. Am. Chem. Soc, 120: 12980-12981, (1998).
17. Nicolaou KC, Yang Z, Liu JJ, Ueno H, Nantermet PG, Guy RK, Claiborne CF, Renaud J, Couladouros EA, Paulvannan K, Sorensen EJ, Total synthesis of Taxol. Nature, 367: 630-634, (1994).
18. Owen NL, Hundley N, Endophytes-the chemical synthesizers inside plants. Sci. Prog, 87: 79–99, (2004).
19. Petrini O, Microbial Ecology of Leaves, New York, Springer-Verlag:179–197, (1991).
20. Puri SC, Verma V, Amna T, Qazi GN, Spiteller MJ, Nat. Prod. 68: 1717–1719, (2005).
21. Puri SC, Nazir A, Chawla R., Arora R, Amna T, Ahmed B, Verma V, Singh S, Sagar R, Sharma A, Kumar R, Sharma RK, Qazi GN, J. Biotechnol. 122: 494–510, (2006).
22. Raja V, Kamalraj S, Muthumary J, Taxol from *Botryodiplodia theobromae* (BT 115) - AN endophytic fungus of *Taxus baccata.* Journal of Biotechnology, 136: 187–197, (2008).
23. Ruckdeschel K, Roggenkamp A, Lafont V, Mangeat P, Hessmann J, Rouot B, Interaction of *Yersinia enterocolitica* with macrophages

leads to macrophage cell death through apoptosis. Infect Immun, 65:4813–4821, (1997).
24. Schiff PB, Fant J, Horowitz SB, Promotion of microtubule assembly in vitro by taxol. Nature, 277:665–667, (1979).
25. Senthil Kumaran R, Muthumary J, Hur BK, Taxol from *Phyllosticta citricarpa*, a Leaf Spot Fungus of the Angiosperm *Citrus medica*. Journal of BioScience and BioEngineering, 106(1): 103-106, (2008).
26. Stierle A, Strobel GA, Stierle D, Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacifi c yew. Science, 260: 214–216, (1993).
27. Strobel GA, Daisy B, Bioprospecting for Microbial Endophytes and Their Natural Products. Microbiol. Mol. Biol. Rev, 67: 491–502, (2003).
28. Strobel GA, Hess WM, Li JY, Ford E, Sears J, Sidhu RS, Summerell B, *Pestalotiopsis guepinii* a taxol producing endophyte of Wollemi Pine - *Wollemi nobilis* . Aust. J. Bot., 45:1073–1082, (1997).
29. Strobel GA, WM Hess, E Ford, RS Sidhu, X Yang, Taxol from fungal endophytes and the issue of biodiversity. J. Ind. Microbiol, 17: 417-423, (1996).
30. Wagenaar M, Corwin J, Strobel GA, Clardy J, Three new chytochalasins produced by an endophytic fungus in the genus *Rhinocladiella*. J. Nat. Prod, 63, 1692–1695, (2000).
31. Wall ME, Wani MC, Cooke CE, Palmer KT, McPhail AT, Sim GA, J. Am. Chem. Soc, 88: 3888–3890, (1966).
32. Wang JF, Li GL, Lu HY, Zheng ZH, Huang YJ, Su WJ, Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus maire*i. FEMS Microbiol. Lett, 193: 249-253, (2000).
33. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail A, Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J. Am. Chem. Soc, 93: 2325-2327, (1971).
34. Yeung TK, Germond C, Chen X, Wang Z, The mode of action of taxol: apoptosis at low concentration and necrosis at high concentration. Biochem Biophy Res Comm, 263:398–404, (1999).