Efficiency of fungal taxol on human liver carcinoma cell lines

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Abstract

Plectranthus amboinicus is a medicinal plant that possesses anticancer activity. An endophytic fungus, *Pestalotiopsis microspora* EF01, was isolated from the fresh healthy leaves and was screened for the production of taxol, an anticancer drug, in artificial culture medium. The presence of taxol was confirmed by chromatographic and spectroscopic experiments with authentic taxol. The quantity of taxol produced by the fungus was calculated and estimated to be 204.7 µg/l. The fungal taxol extracted from an organic extract of the fungal culture had strong cytotoxic activity towards human liver carcinoma cell line (Hep G2) *in vitro* when tested using an apoptosis assay.

Keywords: anticancer drug, 18S rRNA gene, Hep G2, *Pestalotiopsis microspora*

{**Citation:** Lavanya Rajendran, Kalyanaraman Rajagopal, Karthikeyan Subbarayan*, Kamatchi Ulagappan, Ashwini Sampath and Gowri Karthik. Efficiency of fungal taxol on human liver carcinoma cell lines. American Journal of Research Communication, 2013, 1(6): 112-121} www.usa-journals.com, ISSN: 2325-4076.

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, a powerful anti-mitotic agent with the excellent activity against a range of cancers, was originally isolated from *Taxus brevifolia* (Wani *et al*., 1971). Presently, all taxol in the world's market has originated from *Taxus* spp. Since the first taxol-producing fungus *Taxomyces andreanae* was isolated in 1993 (Stierle *et al*. 1993, 1994), there have been a few reports on the isolation of taxol-producing endophytic fungi (Li *et al*., 1996; Strobel *et al*., 1996; Gangadevi and Muthumary, 2007; Kumaran *et al.,* 2012), demonstrating that organisms other than *Taxus* sp. could produce taxol. The term 'Entophytes' was introduced by de Bary in 1866, and applied to organisms that within plant tissues (Taylor *et al*., 2000). The term includes all organisms that live symptomless within plant tissues at some period of their life cycle (Petrini 1991; Wilson, 1995). The entophytes may produce a plethora of substances of potential use to modern medicine, agriculture, anti mycotics, immune suppressants, and anticancer compounds (Strobel and Daisy, 2003; Mitchell *et al*., 2008). Recent studies of endophytic fungi from tropical and temperate forests support the high estimates of species diversity (Kumar and Hyde, 2004; Santamaria and Bayman, 2005). Virtually very few reports are available on screening taxol-producing endophytic fungi from tropical medicinal plant species (Kumaran *et al*., 2008; Gangadevi and Muthumary, 2009; Kathiravan and Vithiyanathan, 2010). In this investigation, an attempt was made for the first time to screen the taxol from an endophytic fungus, *Pestalotiopsis microspora* associated with leaves of *Plectranthus amboinicus*, a medicinal plant.

Material and Methods

Isolation of entophytic fungi

Leaves (petiole and lamina) of *Plectranthus amboinicus* were collected from the University Herbal Garden, Tamil Nadu, India. Samples were transported in closed sterile polythene bags and processed within 24 hours of collection (Fisher and Petrini, 1987). Midrib portion of lamina segments (0.5 cm^2) cut from the middle portion of healthy leaves and segments (0.5 cm^2) cut from the basal part of the petiole, were dipped in 70 % ethanol for 5 seconds, immersed in 4 % NaOCl for 1 minute and rinsed in sterile distilled water for 10 seconds. Five hundred segments of lamina and petiole from each plant species were placed on Potato Dextrose Agar amended

with 150 mg/l Chloramphenicol (Rajagopal *et al*., 2010). The incubation temperature was 27 ºC ± 1 ºC.

Identification of endophytic fungi

The endophytic fungi were identified was identified using standard morphological keys (Udaya Prakash, 2004) and confirmed at molecular level by 18 rRNA gene sequencing methods. Fungal DNA was isolated from fungal mycelium based on protocol prescribed in Gonzalez-Mendoza, 2010. The resulting genomic DNA was used as template to amplify the fungal 18S rRNA gene fragment using the primers ITS1 (5-TCCGTAGGTGAACCTGCG-3′)/ITS4 (5-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Sequencing analysis was performed on an ABI 3730 XL (Applied Biosystems) automated sequencer. Sequences of fungal 18S rRNA genes and ITS region (Figure 1) sequences were compared to those in GenBank database by BLAST algorithm to identify sequences similarity.

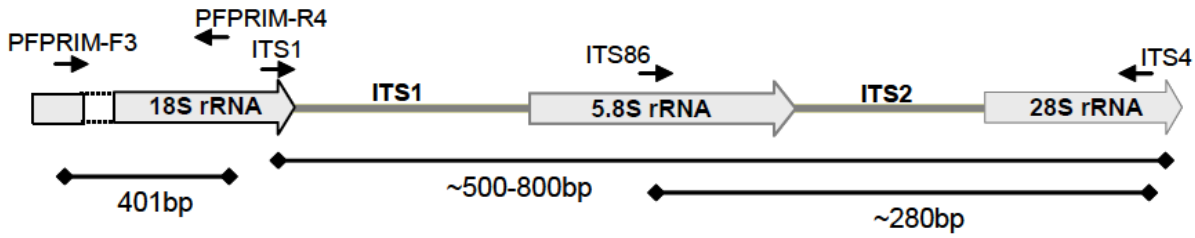

Figure 1. Schematic representation of the fungal ribosomal 18S rRNA gene and ITS regions with primer binding locations. ITS- Internal transcribed spacer region (Embong *et al.* 2008).

Extraction of taxol

For, taxol screening the *Lasiodiplodia theobromae* EF03 and *Pestalotiopsis microspora* EF01 was grown in two different 3 L flasks that contained 1500 ml of M1D medium supplemented with 1g soytone. The extraction procedure for taxol was made by using to the methods of Strobel *et al*. (1996). The culture filtrate was extracted with equal volumes of 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.

UV –spectroscopic analysis:

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 fungal extract was dissolved in 100% methanol, analyzed by Shimadzu (UV – 1601) UV spectrophotometer and compared with standard taxol.

Taxol detection and anti-cancer activity

TLC analysis was carried out on silica gel plates. The plates were developed by the solvent system as reported (Strobel *et al*., 1996). 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 hours. 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 UV spectroscopic analysis. The purified sample of taxol was dissolved in 100% methanol, analyzed by Shimadzu UV – 1601 UV spectrophotometer and compared with standard taxol (Cadila Pharmaceuticals Limited, India).

The cytotoxic effect of fungal taxol was tested by the MTT assay on Hep G2 (human liver carcinoma cell line) (Mosmann, 1983). The cell viability is presented as percentage of apoptotic cells (Kumaran *et al.,* 2012).

Results

Based on standard morphological keys (Udayaprakash, 2004), the endophytic fungus EF01 was identified as *Pestalotiopsis microspora*. The identification was confirmed at molecular level

using 18S rRNA gene sequence and BLAST algorithm. The sequence analysis of EF01 strain showed 98% identity with the 18S rRNA gene sequences of *Pestalotiopsis microspora* isolate DN-S-1 (GenBank accession no. GU441597) and *Pestalotiopsis microspora* strain 03-s-90 (GenBank accession no. JF487784). This result suggests that strain EF01 should be identified as *Pestalotiopsis microspora*. Further the partial 18S rRNA gene sequence of *Pestalotiopsis microspora* stored in NCBI-GenBank under the accession number JF754490. Likewise *Lasiodiplodia theobromae* EF03 was also confirmed at molecular level 99% similarly was noticed with *Lasiodiplodia theobromae* strain Ss57.

The presence of fungal taxol isolated from *Pestalotiopsis microspora* EF01 and *Lasiodiplodia theobromae* EF03 was detected using a spray reagent consisting of 1% (w/v) vanillin in sulfuric acid after gentle heating. The presence of taxol in the fungal extract was confirmed by the appearance of a bluish spot fading to dark grey. The crude extract was dissolved in 1 ml of methylene chloride and loaded on to a 1.5 cm × 30 cm column of silica gel and eluted in a series of solvent systems (volume 70 ml): 1, 100 % (v/v). The partially purified taxol was further analyzed by TLC. The fractions had Rf values identical with those of standard taxol.

The fungal taxol showed the Rf value at 0.25 which was found to be identical when compared with standard taxol. Further detection of taxol in the fungal sample was authenticated by TLC, displaying taxol band under UV illumination at 235 nm and showing a blue grey color reaction with the vanillin/sulfuric acid reagent. 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. This purified fungal taxol showed similar value as that of standard taxol at 235 nm.

Determination of anti-cancer activity using human liver carcinoma cell line:

Cytotoxic effect of fungal taxol of from *Pestalotiopsis microspora* EF01 and *Lasiodiplodia theobromae* EF03 was tested by apoptotic assay on human liver carcinoma cells viz. Hep G2 lines. The assay was tested with different dilutions of fungal taxol with reference to standard taxol. The dilutions were made up with DMSO. Initially DMSO has confirmed for its non-inhibitory effects on Hep G2 cell lines. An increasing scenario of taxol-induced apoptosis level is recorded with increasing level of standard taxol concentration from 0.005 to 0.05 µM. At the

same time, when the standard taxol concentration increased higher from 0.5 to 5 µM, the taxol-induced cell death through apoptosis decreased significantly (Table 1). Similar pattern was observed with the fungal taxol, although the inhibitory effects of fungal taxol showed slightly lesser inhibitory effects than standard taxol. Among the taxol derived from fungal stains, *Pestalotiopsis microspora* (Figure 2A) shows the maximum inhibitory activity than that of *Lasiodiplodia theobromae* (Figure 2B).

Table 1. Taxol-induced apoptosis by in human liver carcinoma cell line

S/N	Taxol source	Concentration (µM)	Percentage of apoptotic cell
1	Standard taxol	0	0
		0.005	26.57 ± 1.13
		0.05	62.33 ± 3.85
		0.5	79.6 ± 3.26
		5	34.37 ± 1.19
2	*Pestalotiopsis microspora*	0	0
		0.005	23.13 ± 3.48
		0.05	57.27 ± 5.64
		0.5	74.85 ± 6.14
		5	35.94 ± 4.57
3	*Lasiodiplodia theobromae*	0	0
		0.005	26.57 ± 1.13
		0.05	51.12 ± 5.22
		0.5	69.41 ± 4.71
		5	34.86 ± 2.84

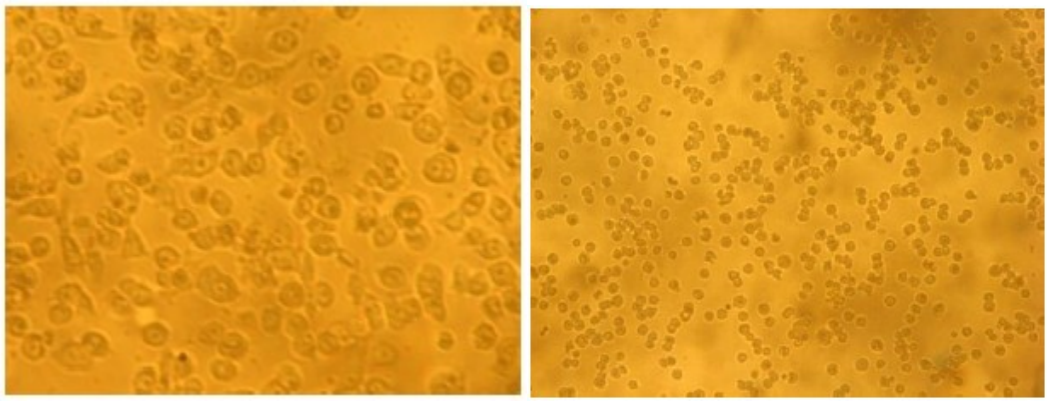

Figure 2: Inhibitory activity of 0.5 mM fungal taxol on human liver carcinoma cell lines, Hep G2. *A, Pestalotiopsis microspora*; *B, Lasiodiplodia theobromae*

Discussion

Applying molecular detection and identification techniques, especially in the case of the production of medicinal compounds, substantially improves identification not only of sterile fungal strains but also of sporulating fungi that are generally considered easy to identify (Breitkopf *et al*., 2005). This study was investigated the extraction, detection and efficacy of taxol from endophytic fungal strains such as *Lasiodiplodia theobromae* and *Pestalotiopsis microspora* from the medicinal plant, *Plectranthus amboinicus*. There are previous reports of taxol production obtained from fungi isolated from diverse plant species (Strobel *et al*., 1997; Kumaran *et al.,* 2012). *Pestalotiopsis breviseta* was reported to produces taxol in the amount of 0.211 mg/l (Gangadevi and Muthumary, 2009) which is slightly higher than the *Pestalotiopsis microspora* (0. 204 mg/l). Literature reveals that based on the results of the UV and IR analysis, taxol can be detected (Wani *et al*., 1971; Kumaran *et al*., 2008). In this study UV analysis was used as analytical tool to confirm the presence of taxol. Fungal taxol is a proven anticancer agent that has been tested in various human carcinoma cell lines such as MCF-7 (Pandi *et al*., 2011; Kumaran *et al.,* 2012), BT220 (Gangadevi and Muthumary, 2007; Kumaran *et al.,* 2012), HL251 (Kumaran *et al.,* 2012). This study determines the inhibitory effects of taxol on Hep G2 cell lines. The scenario of taxol-inhibitory effects was also concurrent with the earlier reports (Yeung et al., 1999), thus supporting the earlier findings that at low concentration taxol inhibit cell proliferation by blocking mitosis (Gangadevi and Muthumary, 2007; Kumaran *et al.,* 2012). This was confidently evidenced that the fungal taxol and standard taxol yielded identical results. In conclusion, fungal endophytes are gaining importance because of their enormous potential to produce novel bioactive compounds of medicinal importance.

Acknowledges

We are thankful to the Management of VELS University, Chennai, Tamilnadu, India, for providing the infrastructure for the present study.

References

- Breitkopf C, Hammel D, Scheld HH, Peters G, Becker K (2005). Impact of a Molecular Approach to Improve the Microbiological Diagnosis of Infective Heart Valve Endocarditis. Circulation. 2005; 111: 1415-1421.
- Croom EM (1995). Taxol: science and application, in Taxus for taxol and taxoids (ed.) M. Suffness, Boca Raton- CRC press, pp. 37-70.
- Fisher P.J., Petrini O. (1987). Location of fungal Endophytes in tissues of Suaeda fruticosa: A preliminary study. –Transactions British Mycological Society. 89. 246-249.
- Gangadevi V and Muthumary J (2007). Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines. African Journal of Biotechnology 6 (12): 1382-1386.
- Gangadevi V, Muthumary J (2009). Taxol production by *Pestalotiopsis terminaliae*, an endophytic fungus of *Terminalia arjuna*. Biotechnol.Appl. Biochem. 52: 9-15.
- Kathiravan.G, Vithiyanathan S.V., 2010. In vitro taxol production, by *Pestalotiopsis breviseta, Fitoterapia* 81 (2010) 557–564.
- Kumar, D.S.S. and Hyde, K.D. (2004). Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal Diversity 17: 69-90.
- Kumaran RS, Choi Y, Lee S, Jeon HJ, Jung H and Kim HJ (2012). Isolation of taxol, an anticancer drug produced by the endophytic fungus, Phoma betae. African Journal of Biotechnology 11 (4): 950-960.
- Kumaran. R. S., Muthumary. J. and B. K. Hur 2008 Taxol from *Phyllosticta citricarpa,* a leaf spot fungus of the angiosperm *Citrus medica. J. Biosci. Bioeng.* 106: 103-106
- Kumaran. R. S., Muthumary. J. and B. K. Hur 2008 Taxol from *Phyllosticta citricarpa,* a leaf spot fungus of the angiosperm *Citrus medica. J. Biosci. Bioeng.* 106: 103-106
- Li, J.Y., Strobel, G.A., Sidhu, R.S., Hess, W.M., Ford, E.J. 1996. Endophytic taxol producing fungi from bald cypress, *Taxodium distichum. Microbiology*, 142: 2223-2226
- Mitchell AM, Strobel GA, Hess WM, Vargas PN, Ezra D (2008) Muscodor crispans, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. Fungal Divers 31:37–43
- Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.

- Pandi.M, Senthil Kumaran.R, Yong-Keun Choi , Hyung.C , Muthumary.J Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia*,2011, *African Journal of Biotechnology* 10, 1428-1435
- Petrini O., Fungal Endophytes in Tree Leaves; in Andrews J.H. and Hirano S.S.,eds., *Microbial Ecology of Leaves*, Springer, New York, 1991: 179-197.
- Rajagopal K, Kalavathy S, Kokila S, Karthikeyan S, Kathiravan G and Prasad R, Balasubraminan (2010) Diversity of Fungal Endophytes in Few Medicinal Herbs of South India. Asian J. Exp. Biol. Sci. 1 (2): 415- 418.
- Rowinsky EK, Cazenave LA, Donehower RC (1990). Taxol: a novel investigational antimicrotuble agent. J. Natl. Cancer Inst. 82: 1247-1259.
- Santamaria, J. and Bayman, P. (2005). Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). Microbial Ecology 50: 1-8
- Stierle A, Strobel G, Stierle D (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew.Science, 260: 214-216.
- Strobel G, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502.
- Strobel GA, Hess WM, Li JY, Sears J, Sidhu RS, Summerell B (1997). Pestalotiopsis guepinii, a taxol producing endophyte of wollemi pine, Wollemia nobilis. Aust. J. Bot. 45: 1073-1082.
- Strobel GA, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM (1996). Taxol from Pestalotiopsis microspora, an endophytic fungus of Taxus wallachiana. Microbiology, 142: 435-440.
- Taylor J.E, Hyde K.D. and Jones E.B.G., The biogeographical distribution of microfungi associated with three palm species from tropical and temperate habitats. *J.Biogeogr*., 2000 ;27:297-310.
- Udaya Prakash NK (2004). Indoor molds: isolation and identification, Color wings (M) Pvt. Ltd. Chennai, India.
- Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971). 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.

- Wani. M. C., Taylor. W. H., Wall. M. E., Coggon. P. and McPhail. A. T. Plant antitumor agents. VI. 1997. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc.* 93: 2325–2327.
- Wilson, D. (1995). Endophyte the evolution of a term, and clarification of its use and definition. Oikos 73: 274-276.