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Full Length Research paper

Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines

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Taxol is an important anticancer drug used widely in the clinical field. Some endophytic fungi were isolated from selected medicinal plants and screened for the production of taxol. The effect of cytotoxicity of fungal taxol isolated from fungal endophytes was investigated by apoptosis method. The presence of taxol in the culture filtrate of endophytic fungi was determined by thin layer chromatography. The fungal taxol isolated from the organic extract of six fungal cultures, had strong cytotoxic activity towards BT 220, H116, Int 407, HL 251 and HLK 210 human cancer cells *in vitro*.

Key words: Endophytic fungi, medicinal plants, taxol, cytotoxic activity, apoptosis.

INTRODUCTION

An increase in the number of people in the world having health problems caused by various bacteria, parasitic protozoans and fungi is a cause for alarm. Millions of people alive today have had some type of cancer. Attempts are now being made to find plant products, which may economically be useful for commercial exploitation and more relevant to the need of the society. Paclitaxel, a taxol, has a unique mechanism of action when compared with other anti-cancer compounds. The drug has been approved for the treatment of ovarian cancer by the U.S. Food and Drug Administration in 1992. By binding to polymerized tubulin proteins, paclitaxel promotes microtubular stabilization and thus interferes with their normal function. As a result, actively dividing cells are blocked in the G2-mitotic phase of the cell cycle.

Taxol, a highly functionalised diterpene, was first discovered in the bark of western yew (*Taxus brevifolia*), a relatively rare and slow growing tree, by Wani et al. (1971) and the yield is very low (0.01 - 0.03%). Efforts were, therefore, made by several research groups to address the supply problem of taxol in a variety of ways. If the fungus can be manipulated to increase the production of taxol by some technique, it can provide an inexhaustible source of taxol and the supply problem of this drug can be solved for ever. After several years of effort, a novel taxol producing endophytic fungus, *Taxomyces andreanae*, was discovered in *T. brevifolia* by Strobel et al. (1993). The most critical line of evidence for the presence of taxol in the culture fluids of this fungus, among others, was the electrospray mass spectrum of the putative taxol isolated from *T. andreanae* as well as ^{14}C labelling studies, which irrefutably showed the presence of fungal-derived taxol in the culture (Stierle et al., 1993), though, the yields of taxol and taxanes have been low. However, taxol has been reported in a novel endophytic fungus *T. andreanae* but also has been demonstrated to occur in a number of unrelated fungal endophytes including *Pestalotia, Pestalotiopsis, Fusarium, Alternaria, Pithomyces, Monochaetia, Periconia* and others (Strobel, 2003).

The endophytes of medicinal plants provide a good source for compounds of biological activity and endophytes are an untapped reservoir of potentially novel, effective drugs (Stierle et al., 1995). In addition to that, Shrestha et al. (2001) showed new report in the production of paclitaxel from three different endophytic fungi isolated from the Himalayan yew *Taxus Wallichiana* which including *Sporormia minima*, *Trichothecium* sp. and an unidentified dimorphic fungus were confirmed by different analytical and immunoassay methods. It appears that fungi more commonly produce taxol than higher

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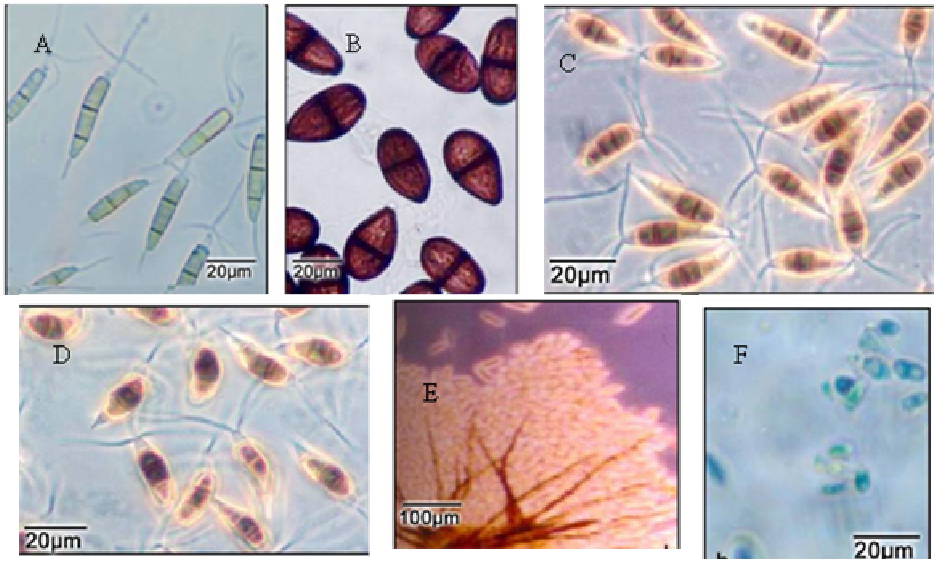

Figure 1. A. Photomicrographs of conidia of endophytic fungi. (A) *Bartalinia robillardoides,* (B) *Botryodiplodia theobromae,* (C) *Colletotrichum gloeosporioides,* (D) *Pestalotiopsis pauciseta,* (E) *P. terminaliae* and (F) *Phomopsis arnoldiae*.

plants and the distribution of those fungi making taxol is worldwide and not confined to endophytes of yews.

By studying taxol, biologists also revealed a unique mechanism of antitumor activity. Taxol is a widely used anti-cancer agent with significant anti-mitotic and apoptotic activity. Apoptosis has now established its importance in numerous areas of biology and it recently received due attention as an important area related to the development and treatment of disease. The aim of this present investigation was to isolate and identify the taxol-producing endophytic fungi from selected medicinal plants and study their anticancer activities. The anticancer activity of taxol isolated from the potential endophytic fungi towards five types of human cancer cell lines was examined by apoptosis method.

MATERIALS AND METHODS

Fungal culturing and taxol isolation

Since there is only little information available on endophytic fungi from the leaves of medicinal plants, endophytic fungi were isolated from some medicinal plants collected from Chennai city, India. 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. Endophytic fungi such as *Bartalinia robillardoides* Tassi, isolated from *Aegle marmelos* Correa ex Roxb., *Botryodiplodia theobromae* Pat., and *Pestalotiopsis pauciseta* Sacc., from *Cardiospermum helicacabum* Linn., *Colletotrichum gloeosporioides* (Penz.) Sacc., from *Justicia gendarussa* Burm.f., *Pestalotiopsis terminaliae* Agarwal and Hasija, from *Terminalia arjuna* (Roxb.) Wight and Arn., and *Phomopsis arnoldiae* Sutton, from *Rauvolfia tetraphylla* Linn. were selected for taxol isolation and grown in 2 l Erlenmeyer flasks containing 500 ml of MID medium supplemented with soytone (Pinkerton and Strobel, 1976) and incubated for 21 days. After 3 weeks of still culture at 26°C, the culture fluid was passed through four layers of cheesecloth to remove solids and extracted with organic solvent. The extraction and isolation procedure followed was that of Strobel et al. (1996). 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 re-dissolved in methanol for the subsequent separation and the crude extracts were analyzed by thin layer chromatography. The authentic taxol (Paclitaxel) was purchased from Sigma.

Thin layer chromatography (TLC)

All comparative TLC analyses were carried out on Merck 0.25 mm silica gel plates developed in the following solvents: a. chloroform/methanol (7:1 v/v); b. chloroform/acetonitrile (7:3 v/v); c. ethylacetate/2-propanol (95:5 v/v); d. methylene chloride/tetrahydrofuran (6:2 v/v); e. methylene chloride/methanol/dimethylformamide (90:9:1v/v/v). The presence of taxol was detected with 1% w/v vanillin/sulphuric acid reagent after gentle heating. Appearance of a bluish spot of fading to dark gray after 24 h indicates the presence of taxol.

Cytotoxic effect of fungal taxol on various cancer cells

Cytotoxicity effect of fungal taxol isolated from six different species of fungal endophytes viz., *B. robillardoides, B. theobromae, C. gloeosporioides, P. pauciseta, P. terminaliae* and *P. arnoldiae* (Figure 1) were 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 at different concentrations. All the cell lines used in this study were obtained from National Centre for Cell Sciences (NCCS), Pune, India. Apoptosis cell death induced by fungal taxol was detected

and quantified according to the method of Ruckdeschel et al. (1997). The dry solid partially purified fungal taxol with known concentrations were dissolved in 0.1% dimethyl sulphoxide (DMSO) and different concentrations of 0.005, 0.05, 0.5 and 5 µM were used for the cytotoxicity assay.

The various cancer cells (1 x 10^5 cells) in 35 mm sterile disposable petriplates were seeded and grown to confluency at 37°C in 5% Minimal Essential Medium (MEM), supplied with different concentration of fungal taxol (0.005, 0.05, 0.5 and 5 µM) and control (without fungal taxol) and incubated at 37°C for 24, 48 and 72 h. After the incubation period, the medium was decanted and the cells were washed with phosphate buffered saline (PBS) at pH 7.4 twice and fixed in 70% methanol. The cell DNA was stained with 0.5 mg/ml propidium iodide in PBS for 15 min and destained in PBS. Percentage of cell viability was determined.

Morphology of apoptotic cells

After treatment with different concentrations of fungal taxol, the cell morphology was determined by light microcopy. 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 apoptosis were those that displayed the characteristic morphological features of apoptosis, including cell volume shrinkage, chromatin condensation and the presence of membrane-bound apoptotic bodies. For each experiment, 500 cells were counted. The cells in apoptosis were calculated by the following formula: % of cells = (number of apoptotic cells) / total number of cells x 100.

RESULTS AND DISCUSSION

Taxol continues to have an increasing role in the treatment of human malignancies, particularly ovarian and breast cancer (DeVita et al., 1997). Recent accumulating reports have demonstrated that taxol-induced microtubular bundling and mitotic arrest of human leukemia cells are followed by DNA fragmentation and morphological features of apoptosis (Lieu et al., 1997; Moos and Fitzpatrick, 1998; Diaz et al., 2000). All comparative thin layer chromatographic analyses were carried out on Merck 0.25 mm silica gel plates developed in different solvent systems. Taxol was detected using a spray reagent consisting of 1% vanillin (w/v) in sulfuric acid after gentle heating (Cardellina, 1991). The presence of taxol in the fungal extract was confirmed by the appearance of a bluish spot fading to dark gray after 24 h. The compound having chromatographic properties identical to authentic taxol in solvent systems A-E, and giving colour reaction with the spray reagent was consistently isolated from *B. robillardoides*, *B. theobromae*, *C. gloeosporioides*, *P. pauciseta*, *P. terminaliae* and *P. arnoldiae*. They had R_f values identical to that of authentic taxol.

The morphological changes of the cancer cells treated with different concentrations of fungal taxol ranging from 0.005 to 5 µM were studied at different time incubation period for 24, 48 and 72 h. All the six fungal taxol samples showed cytotoxic activity positively. The five different types of cancer cells at 1 x 10^5 cells were incubated with different concentrations of partially purified fungal taxol for different times. The growth of all the cancer cells were markedly inhibited by fungal taxol at 0.05, 0.5 concentrations (Table 1). At 0.05 and 0.5 µM taxol, most of the cells were arrested during cell division and the cell nuclei became condensed and segmented after 24 h incubation which is the indication of apoptosis (Figure 2). The results from light microscope displayed morphological abnormality of the cells after treatment with fungal taxol. On the contrary, the untreated cells (control) did not show these apoptotic characteristics. Most of the condensed and segmented nuclei degraded after 72 h incubation (Figure 2).

Apoptosis is an energy-dependent mode of cell death requiring active participation of the target cell (Wyllie and Duvall, 1992). Apoptotic cells are readily seen in developing tissues and in tumors and can be found at low rates in adult tissues (Kerr et al., 1972). The sequence of changes in cellular morphology of apoptosis can be summed up as;

(1) Cell shrinkage.
(2) Condensation, margination and fragmentation of chromatin.
(3) Retention of cytoplasmic organelle structure, but loss of positional interrelationships of organelles (Wyllie et al., 1980; Kerr and Harmon, 1981; Wyllie, 1992).

It is indicated that with the increase of taxol concentration from 0.005 to 0.05 µM, taxol induced increased cell death through apoptosis. With further increase of taxol concentration from 0.05 to 0.5 µM, the taxol-induced cell death through apoptosis only increased slightly. When the taxol concentration was increased from 0.5 to 5 µM, the taxol-induced cell death through apoptosis decreased dramatically (Table 1). In the present study, we observed at low to medium concentration (0.005 - 5 µM), the efficacy of fungal taxol isolated from different species of fungal endophytes was quite dependent on the specific cell type.

Moreover, the cytotoxicity of fungal taxol was concentration dependent. The time effect analysis of fungal taxol showed that its cytotoxicity against human cancer cells was in a time dependent manner. With increasing concentrations, there is a gradual increase in the formation of bundles of microtubules. Consequently, at a very low concentration (0.005 µM), taxol only block a small portion of cells in the G2/M phase by inducing the formation of ball-shaped aggregations of condensed chromosomes containing one or more asters of microtubules. With the increase of taxol concentration to 0.05 µM, the portion of ball-shaped spindles increased dramatically. This is coincident with the enhanced inhibition on DNA synthesis, the increase of the cell apoptosis and the first phase

Table 1. Taxol-induced apoptosis by the endophytic fungi in various cancer cell lines

Cell lines	Taxol content (µM)	Apoptotic cell (%)					
		Bartalinia robillardoides	***Botryodiplodia theobromae***	***Colletotrichum gloeosporioides***	***Pestalotiopsis pauciseta***	***Pestalotiopsis terminaliae***	***Phomopsis arnoldiae***
BT 220 (Breast)	0	0	0	0	0	0	0
	0.005	15.8	16.6	16.8	16.3	16.8	16.5
	0.05	75.5	78.5	78.3	79.6	78.6	75.8
	0.5	80.6	81.3	81.6	80.3	81.5	80.3
	5	21.3	26.8	25.6	27.5	28.3	27.6
H 116 (Human colon)	0	0	0	0	0	0	0
	0.005	13.6	13.5	13.8	15.3	16.3	8.8
	0.05	65.8	65.6	63.5	65.8	70.6	31.3
	0.5	76.3	73.8	70.6	75.6	78.5	63.5
	5	36.3	37.5	38.3	36.8	36.8	37.6
Int 407 (Human intestine)	0	0	0	0	0	0	0
	0.005	15.8	15.3	14.9	14.6	16.5	15.3
	0.05	68.6	70.3	68.8	69.5	70.6	68.8
	0.5	79.5	78.9	79.6	78.3	79.8	76.5
	5	18.8	20.8	20.3	20.9	23.6	20.6
HL 251 (Human lung)	0	0	0	0	0	0	0
	0.005	12.8	12.6	12.6	11.3	12.3	12.6
	0.05	69.3	68.3	68.8	67.8	68.6	67.8
	0.5	71.8	72.5	68.9	70.5	72.5	70.6
	5	32.9	33.8	30.8	33.5	32.3	29.3
HLK 210 (Human leukemia)	0	0	0	0	0	0	0
	0.005	21.6	23.6	23.6	25.6	24.5	22.6
	0.05	66.5	67.5	65.6	68.6	68.3	67.5
	0.5	76.3	76.8	75.3	75.3	79.6	78.5
	5	30.8	30.2	32.5	33.8	36.5	32.8

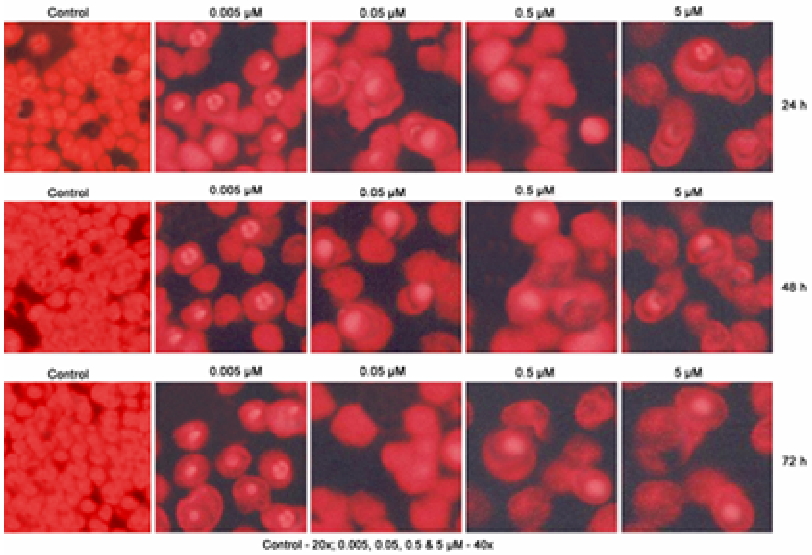

Figure 2. Cytotoxicity tests using fungal taxol on cancer cell lines (BT 220) at different time intervals and concentrations.

of viable cell decrease. In fact, all of the apoptotic cells are cells blocked at G2/M phase. With the further increase of the taxol concentration from 0.05 to 0.5 μM, the proportion of the ball-shaped spindles only increased slightly. This is the coincident with the unchanged percentages of the cells in apoptosis. However, at 5 μM concentration range, most of the cells was prevented from entering S phase and cell necrosis increased dramatically (Figure 2). We observed that the efficacy of fungal taxol isolated from different species of fungal endophytes at low to medium concentration (0.005 - 5 μM) was quite dependent on the specific cell type. This is in agreement with the results of Yeung et al. (1999) and 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. While supporting the previous findings of other groups that at low concentration, taxol inhibits cell proliferation by blocking mitosis.

Further experiments to understand the molecular mechanisms underlining the differences would be greatly important to guide the clinical application of taxol. Additional studies are required to understand the molecular basis for this differential response to enhance the effectiveness of taxol in the treatment of patients with malignant disease.

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REFERENCES

Cardellina JH (1991). HPLC separation of taxol and cephalomannine. J. Chromatogr 14: 659-665

DeVita VT Jr, Hellman S, Rosenberg SA (1997). Cancer. Principles and Practice of Oncology. Philadelphia, PA: Lippincott-Raven Publishers, 5th edn. pp. 103-119.

Diaz JF, Strobel R, Engelborghs Y, Souto AA, Andreu JM (2000). Molecular recognition of taxol by microtubules. Kinetics and thermodyamics of binding of fluorescent taxol derivatives to an exposed site. J. Biol. Chem. 275: 26265-26276.

Kerr JFR, Harmon BR (1991). Definition and incidence of apoptosis: a historical perspective. In: Current Communications in cell and molecular biology. Vol.3, Apoptosis: the molecular basis of cell death, (Ed, LD Torrei, FO Cope), Coldspring Harbor Laboratory Press, New York. pp. 5-29.

Kerr JFR, Wyllie AH, Currie AR (1972). Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br. J. Cancer 26: 239-257.

Lieu CH, Chang YN, Lai YK (1997). Dual cytotoxic mechanisms of submicromolar taxol on human leukemia HL-60 cells. Biochem. Pharmacol. 53: 1587-1596.

Moos PJ, Fitzpatrick PA (1998). Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. Cell Growth Differ. 9: 687-697.

Pinkerton R, Strobel G (1976). Serinol as an activator of toxin production in attenuated cultures of *H. sacchari*. Proc. Natl. Acad. Sci. USA 73: 4007-4011.

Ruckdeschel K, Roggenkamp A, Lafont V, Mangeat P, Hessmann J, Rouot B (1997). Interaction of *Yersina enterocolitica* with macrophage leads to macrophage cell death through apoptosis. Infect. Immun. 65: 4813-4821.

Shrestha K, Strobel GA, Shrivastava SP, Gewali MB (2001). Evidence for paclitaxel from three new endophytic fungi of Himalayan Yew of Nepal. Pl. Med. 67: 374-376.

Stierle A, Strobel GA, Stierle D (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific Yew. Science 260: 214-216.

Stierle A, Strobel GA, Stierle D (1995). The search for a taxol producing microorganism among the endophytic fungi of the Pacific Yew, *Taxus brevifolia*. J. Nat. Prod. 58: 1315-1324.

Strobel GA, Stierle A, Stierle D, Hess WM (1993). *Taxomyces andreanae* a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific yew. Mycotaxon, 47: 71-78.

Strobel GA, Yang X, Sears J, Kramer R, Sidhu RS, Hess WM (1996). Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiol. 142: 435-440.

Strobel GA (2003) Endophytes as sources of bioactive products. Microbes Infect. 5: 535-544.

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.

Wyllie AH, Duvall E (1992). In: Oxford Text book of Pathology, Eds. Job McGees, PG Issacson and NA Wright, Oxford University Press, Oxford. pp. 141-157.

Wyllie AH, Kerr JFR, Currie AR (1980). Cell death the significance of apoptosis. Int. Rev. Cytol. 68: 251-307.

Wyllie AH (1992). Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer Metastasis Rev. 11: 95-103.

Yeung TK, Germond C, Chen X, Wang Z (1999). The Mode of Action of Taxol: Apoptosis at Low Concentration and Necrosis at High Concentration. Biochem. Biophys. Res. Comm. 263: 398-404.