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Effect of taxol from *Pestalotiopsis mangiferae* on A549 cells-In vitro study

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ABSTRACT: *Pestalotiopsis mangiferae* Coelomycete fungi were used to examine the production of taxol. The taxol isolated from this fungus is biologically active against cancer cell lines were investigated for its antiproliferative activity in human Non Small Cell Lung Cancer A549 cells. The results showed that the methylene chloride extraction of *Pestalotiopsis mangiferae* inhibited the proliferation of A 549 cells as measured by MTT and Trypan blue assay. Flow cytometric analysis showed that methylene chloride extraction of *Pestalotiopsis mangiferae* blocked cell cycle progression in G0/G1 phase. In addition fungal taxol induced A549 cell apoptosis as determined by propidium iodide staining. Further the percentage of LDH release was increased at increasing concentrations which is a measure of cell death. The levels of sialic acid levels and DNA, RNA and protein levels were decreased after treatment with methylene chloride extraction of *Pestalotiopsis mangiferae*. We suggests that methylene chloride extraction of *Pestalotiopsis mangiferae* might be considered for future therapeutic application with further studies against lung cancer.

KEYWORDS:

Pestalotiopsis mangiferae;
taxol;
MTT; Trypan blue;
G0/G1; LDH;
apoptosis

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INTRODUCTION

Lung cancer is the leading cause of cancer death in the world and Non Small Cell Lung Carcinoma (NSCLC) accounts for approximately 75–85% of these cancers. Non small cell lung cancers commonly develop resistance to radiation and chemotherapy, and they often present at stages beyond surgical remedy. Since current treatment modalities are inadequate, novel therapies are necessary to reduce the effects of the increasing incidence in pulmonary neoplasm (Chen *et al.*, 1995; Park *et al.*, 2001).

Taxol, a diterpene was originally isolated from the bark of Pacific Yew tree (*Taxus brevifolia*) more than two decades ago and has proved to possess an anticancer activity. The US National Cancer Institute, in collaboration with Bristol Myers Squibb Co and other workers have demonstrated the efficacy

of taxol against certain human cancer. Its mode of action is unique in that it inhibits mitosis through enhancement of polymerization of tubulin and consequent stabilization of microtubules during the process of cell division. However, a complete treatment for the patient requires 2 grams of taxol, administered several times and many months. To obtain 1kg of taxol requires about 10,000 kg of bark, and several thousand tresses must be cut to procure this quantity of bark. This scarcity of taxol and the ecological impact of harvesting it encouraged scientists to find alternative methods using microorganisms. A hypomycetous fungus namely *Taxomyces andreanae* on *Taxus* sp., could produce taxol. A coelomycetous fungus, *pestalotiopsis microspora*, an entophyte from inner bark of *Taxus wallachiana* produced taxol in culture. Keeping in this mind, an attempt has been made to examine the production of taxol by some other coelomycetous fungus as well. The taxol isolated from these fungi is biologically active against can-

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cer cell lines. In order to the price of taxol and make it more available, a fermentation process involving a microorganisms would be the most desirable means supply. It was first discovered by Strobel *et al* (1997)., that the fungus *Taxomyces andreanae* could produce taxol, through the yield was low. (Strobel *et al.*, 1996) showed that *Pestalotiopsis microspore* isolated from the bark of *Taxus wallachiana* produced taxol in mycelial culture. This work prompted us to continue the search for the taxol production from fungal sources.

Apoptosis has been characterized as a fundamental cellular activity to maintain the physiological balance of the organism. It is also involved in immune defense machinery (Hengartner, 2000) and plays a necessary role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells proliferated owing to various chemical agents induction (Brown and Wooters, 1999). Emerging evidence has demonstrated that the anti cancer activities of certain chemotherapeutic agents are involved in induction of apoptosis, which is regarded as the preferred way to manage cancer.

MATERIALS AND METHODS

Source of chemicals

Fetal bovine serum (FBS), Penicillin G, Streptomycin and amphotericin B were obtained from Hi Media. Dimethylsulfoxide (DMSO) and RPMI-1640 were purchased from King Institute of Preventive Medicine. MTT and propidium iodide was purchased from SRL, Laboratories India. All other chemicals used were of analytical grade.

Fungal material and extract preparation

The general laboratory techniques followed in the course of the present investigation were as outlined by Booth (1971). The test fungi used in the present study were grown in Erlenmeyer flasks containing 500 ml MID medium supplemented with 1 gram of soytone L⁻¹ (Pinkerton and Strobel, 1976) for taxol production. Three mycelial agar plugs (0.5 cm) were used as inoculums. The organisms were grown at 24±2°C statistically for 3-4 weeks.

Culture media (Strobel, 1996)

MID medium was supplemented with soytone, sucrose -30.0 g/l, (CHOH.COONH₄)₂-5.0 g, Yeast extract -0.5g, Soytone-1.0g, Ca₂(NO₃)₃-280

mg, KNO₃-80 mg, KCl-60 mg, MgSO₄-360 mg, NaH₂PO₄-20 mg, H₃BO₃-1.4 mg, MnSO₄-5.0 mg, ZnSO₄-2.5 mg and KI-0.7 mg.

Extraction of taxol

Extraction of taxol was performed according to Strobel *et al.* (1996). After incubating the fungal culture for 3-4 weeks, the culture filtrate was passed through four layered cheesecloth. In order to avoid fatty acid contamination of taxol, 0.25g of NaCO₃ was added to the filtrate. The culture fluid was extracted with two equal volumes of methylene chloride and the organic phase was evaporated to dryness under reduced pressure at 35°C.

2.2. Cell culture

A549 was obtained from NCCS Pune, India. A 549 cells were cultured in Ham's F12k medium containing 10% new born calf serum containing 100µl/ml of penicillin and streptomycin 100µl/ml. Cells were maintained in a humidified atmosphere of 5% CO₂ incubator at 37°C, until confluency stage is attained. The medium is replaced every two days and maintenance is in strictly accordance with the standard methods. The cells were dissociated with TPVG in Phosphate buffered solution.

Light microscopy

After 48 hours incubation with methylene chloride extract of fungal taxol, the A-549 cells were washed with PBS. The cells were observed for morphological changes under light microscopy at 100X and photographed.

Cell viability assay

Inhibition of cell proliferation by methylene chloride extract of fungal taxol, was measured by MTT assay as described (Mossmann, 1983). Briefly, cells were plated in 24 well culture plates (1x10⁶cells/well). After 24 hrs incubation, cells were treated with crude extract (10µgs, 25 and 50 µgs) for 48 hrs. Fifty microlitres of MTT was added and the reading was taken at 570nm after lysing in isopropanol.

Trypan blue exclusion studies

The viability of cells were assessed by trypan blue exclusion studies for various concentrations (10, 25 and 50µgs/ml) at different time intervals 12, 24 and 48 hrs by the standard method (Moldeus, 1978).

Assay of lactate dehydrogenase (EC 1.1.1.27)

The activity of lactate dehydrogenase was assayed by the method described (King, 1965 a).

Assay of sialic acid levels

Sialic acid level were determined as according to the method described (Warren, 1959).

Estimation of macromolecules

Protein was estimated by the method of Lowry et al (1951). DNA was estimated as according to the method of Burton (1956). RNA was estimated by the method of Rawal et al (1977).

Measurement of apoptosis by propidium iodide staining

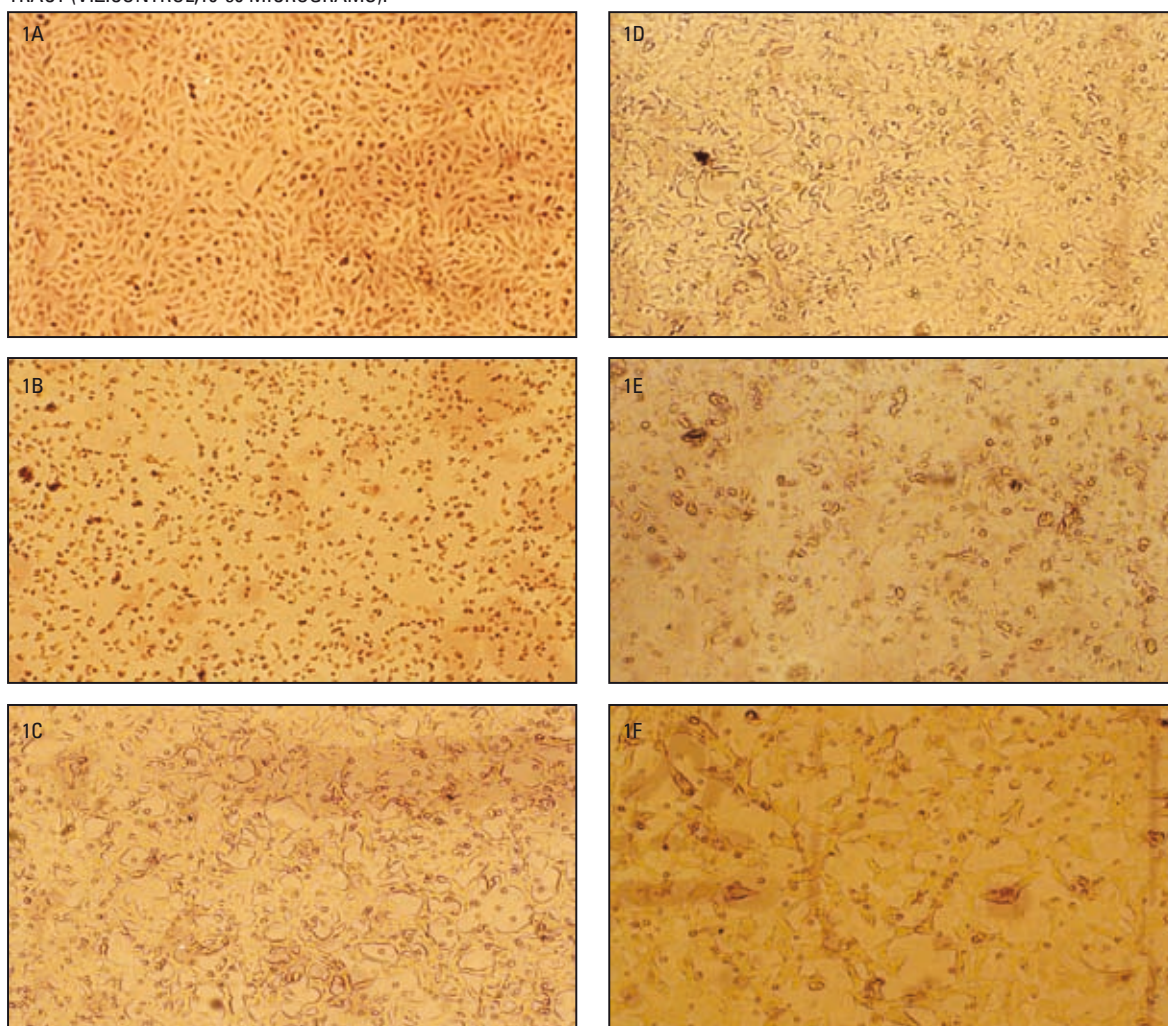
The induction of apoptosis by methylene chloride extract of fungal taxol was assayed by the propidium

iodide staining. For determination of apoptosis by propidium iodide staining, the A549 cells were treated with methylene chloride extract of fungal taxol and stained with propidium iodide (50 µg/ml). This is then viewed under the fluorescent microscope.

Flow cytometry

This analysis was carried according to the method described by Nicotelli *et al* (1991). 10 lakh cells of A549 were cultured in 6 well plates and incubated for 48 hours to obtain a monolayer. To this culture, the drug was added at 50µg concentrations and incubated for a period of 24 hours. The cellular morphology was viewed once every few hours. After the incubation period, the media was removed and the cells gently washed with PBS. The cells were then trypsinised. The suspension was then centrifuged at 3000 rpm for 5 minutes and supernatant was dis-

FIGURE 1A-1F: SHOWING THE MORPHOLOGICAL FEATURES OF A 549 CELLS TREATED WITH VARIOUS CONCENTRATIONS OF THE EXTRACT (VIZ.CONTROL,10-50 MICROGRAMS).

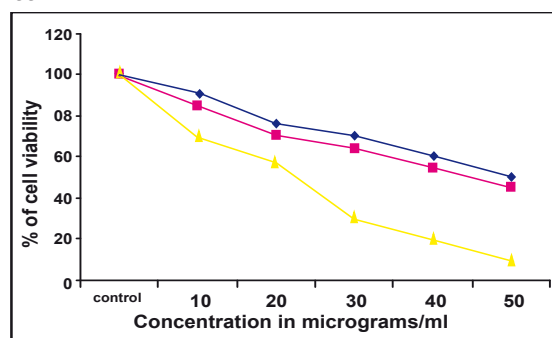


carded. The pellet obtained was gently washed with ice cold PBS and resuspended. To this 40 μ l of 2mg/ml propidium iodide was added and kept for 4 hours of incubation at 4 $^{\circ}$ C. This was subjected to flow cytometric analysis.

RESULTS

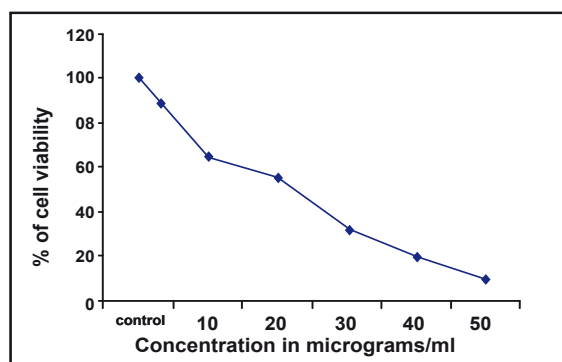
TLC analysis was carried out on Merck 1mm(20x20 cm) silica gel plate developed in solvent A (chloroform:methanol,7:1v/v) followed by solvent B (Chloroform: Acetonitrile, 7:3v/v), solvent C (Ethyl acetate: 2-propanol, 95:5,v/v) solvent D (Methylene chloride: Tetrahydrofuran,6:2v/v) and solvent E (Methylene chloride: Methanol:Dimethylformamide,90:9:1v/v/v) respectively. The area of the plate containing putative taxol was carefully removed by scrapping off the silica at the appropriate Rf and eluted with acetonitrile. Taxol was detected with 1% w/v vanillin/sulphuric acid reagent after gentle heating (Cardellina, 1991). It appeared as a bluish spot that faded to dark grey after 24 h.

FIGURE 2: SHOWING THE EFFECT OF FUNGAL TAXOL OF P.MANGIFERAEE ON CELL VIABILITY DETECTED BY TRYPAN BLUE ASSAY.



The experiments were carried out in duplicate triplicates. All the values were statistically significant at $p < 0.05$.

FIGURE 3: SHOWING THE EFFECT OF FUNGAL TAXOL OF P. MANGIFERAEE ON CELL VIABILITY DETECTED BY MTT ASSAY.



The experiments were carried out in duplicate triplicates. All the values were statistically significant at $p < 0.05$.

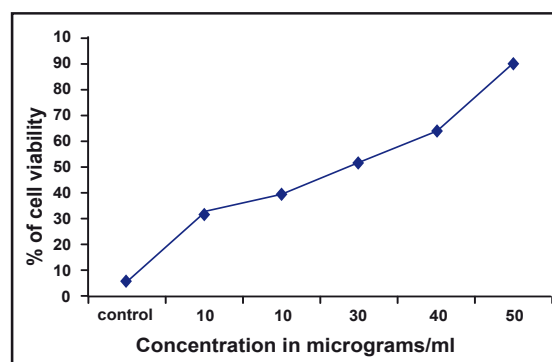
Architecture of untreated A549 cells show a typical spindle shaped cells observed under a light microscope. It exhibited typical carcinoma type morphology with a uniform monolayer (figure 2.1). In vitro incubation of A-549 cells with the methylene chloride extract of fungal taxol (1-5 μ gs) showed remarkable morphological alterations (figure 2.2-2.6) and cell death at the end of 48 hours characterized by disruption of monolayer. It caused a dose dependent cytolytic and nuclear change in cell morphology. Cell mitosis were scarce compared to untreated A549 cells.

Figure 3 showed the effect of methylene chloride extract of fungal taxol on A549 cell viability (Trypan blue uptake) after 12, 24, 48 hrs incubation. It was inferred that the incubation of methylene chloride extract of fungal taxol had led to marked decrease in viable cells. The A549 cells after 12, 24 and 48 hours incubation to serial dilutions (10-50 μ gs) showed decreased cell viability. The cell death was found to be higher after 48 hours. Incubation of cells methylene chloride extract of fungal taxol produced 48% cell viability at 50 μ gs after 24 hours and 10% cell viability at 50 μ gs after 48 hours ($p > 0.05$). The experiments were carried out in duplicate triplicates.

Cell viability was observed in a dose and time dependent manner in taxol treated cells at increasing concentrations ranging from 10,20,30,40 and 50 μ gs/ml. At 24 and 48 hours, less than 50% of cell viability was observed at a concentration of 30 micrograms/ml. Maximum cytotoxic activity was observed at a concentration of 50 μ gs/ml at 48 hours.

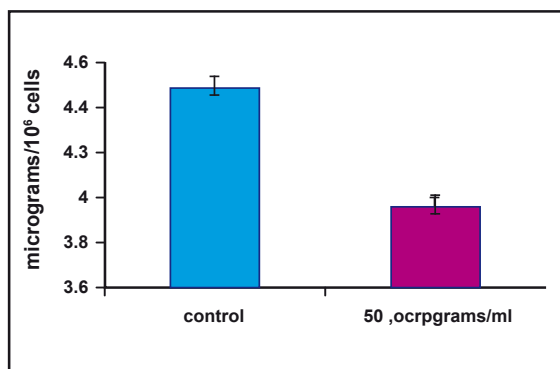
As evident from the figure 4, the cytotoxicity was observed at increasing concentrations i.e.10, 20, 30, 40 and 50 μ gs ($p < 0.05$). Even at 30 μ gs, less than

FIGURE 4: SHOWING THE EFFECT OF FUNGAL TAXOL OF P. MANGIFERAEE ON CELL VIABILITY DETECTED BY LDH ASSAY.



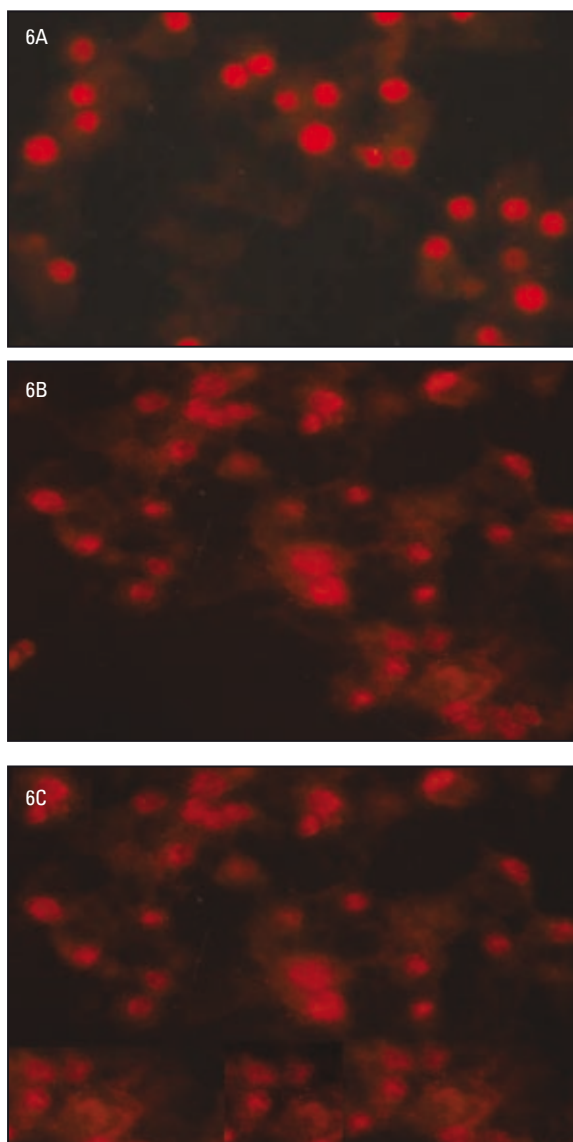
The experiments were carried out in duplicate triplicates. All the values were statistically significant at $p < 0.05$.

FIGURE 5: SHOWS THE LEVELS OF SIALIC ACID TREATED WITH FUNGAL TAXOL OF P. MANGIFERAEE.



The experiments were carried out in duplicate triplicates. All the values were statistically significant at p<0.05.

FIGURE 6: SHOWING THE EFFECT OF FUNGAL TAXOL OF P. MANGIFERAEE ON APOPTOSIS DETECTED BY PROPIDIUM IODIDE STAINING. (6A-CONTROL, 6B- 40 µGS, 6C- 80µGS).



50% of cell viability is observed while at 50 µgs, only 10% of cell viability was seen.

A dose dependent increase in the percentage of LDH leakage was observed. A maximum leakage of LDH was observed at a concentration of 50µgs/ml at 48 hours in taxol treated A549 cells. From the figure 5, the percentage of LDH release was increased at increasing concentrations of methylene chloride extract of fungal taxol which is a direct proportion to cell death. At 50 µgs, the maximum of 90% cell death was observed (p< 0.05) after 48 hours.

Fig 6 represents the levels of sialic acid in the treated and untreated A-549 cells at a concentration of 50µgs/ml. A marked reduction (p<0.05) in the levels of sialic acid was observed in the taxol treated A549 cells after 48 hours when compared to untreated cells.

Figure 7 represents the fluorescent microscopic pictures of the methylene chloride extract of fungal taxol treated and untreated A549 cells assessed for apoptosis at 400X. The propidium iodide stained cells at a concentration of 40 and 50 µgs/ml showed the clumping of cells with slight distortion. The highly condensed and fragmented nuclei that are the index of apoptosis were observed at 40 and 50 µgs/ml (fig 7b and 7c). The untreated A549 cells were shown in plate 7a.

Table 1 shows the levels of DNA, RNA and protein in taxol treated (50 µgs) A 549 and untreated A549 cells. A significant decrease (p<0.05) in the levels of these macromolecules was noted when compared to untreated cells.

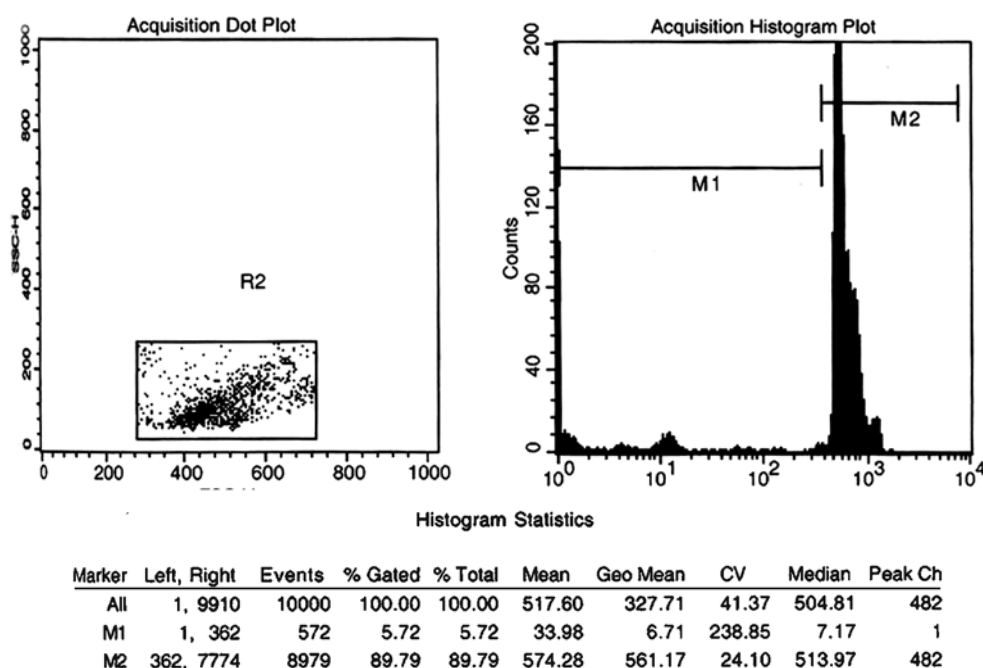
Plate 7.1 depicts the flow cytometric analysis of untreated A 549 cells. Plate 7.2 depicts the drug treated A 549 cells. Flow cytometry analysis for apoptosis study was carried out on the A549 cells after the methylene chloride extract of fungal taxol treatment. It was found that 56.06% of the cells were in the G1 phase, 8.60% in the S phase and 35.78% of cells in the G2 phase in the control cells of A 549

TABLE 1: SHOWING THE EFFECT OF FUNGAL TAXOL OF P. MANGIFERAEE ON DNA, RNA AND PROTEIN LEVELS.

Parameters	Untreated	Treated(50 µgs/ml)
DNA	10.86 ± 1.56	8.55 ± 1.71*
RNA	14.46 ± 1.75	11.88 ± 1.06*
PROTEIN	967.07 ± 32.86	917.14 ± 32.89*

The experiments were carried out in duplicate triplicates. All the values were statistically significant at *p<0.05, NS-non significant when compared to untreated group (students t-test). Values are expressed as µg/10⁶ cells.

FIGURE 7.1: SHOWS THE FLOW CYTOMETRIC ANALYSIS OF CONTROL CELLS AND FIGURE 7.2 SHOWS THE FLOW CYTOMETRIC ANALYSIS OF FUNGAL TAXOL OF P. MANGIFERAE TREATED A 549 CELLS.



cells. The methylene chloride extract of fungal taxol (5 μ g) treated cells had only 28.64% of the cells in the G1 phase, 4.4% in the S phase and the remaining 50.46% cells in the G2 phase.

DISCUSSION

A number of studies have indicated that significant cell proliferation effect against various cancer lines. In our work, we demonstrated that the methylene chloride extract of fungal taxol extract inhibited the cell proliferation in A549 by inducing apoptotic cell death. The cytotoxic effect was clearly established at increasing concentrations i.e. 1, 2, 3, 4 and 5 micrograms. The cytotoxic effect was characterized by disruption of the monolayer. (Kathiravan and Muthu- mary 2009)

Cancer chemotherapeutic as well as chemopre- ventive agents exert part of their pharmacological effect by triggering apoptotic cell death or cell cycle transition. Identification of inhibition of apoptosis by several tumor promoters and induction of apop- tosis in tumor cells, serves as a predictor of tumor treatment (Kim *et al.*, 1999). Extracts from a broad spectrum of plant species contain substances that possess antitumor activity (Dzham *et al.*, 2002).

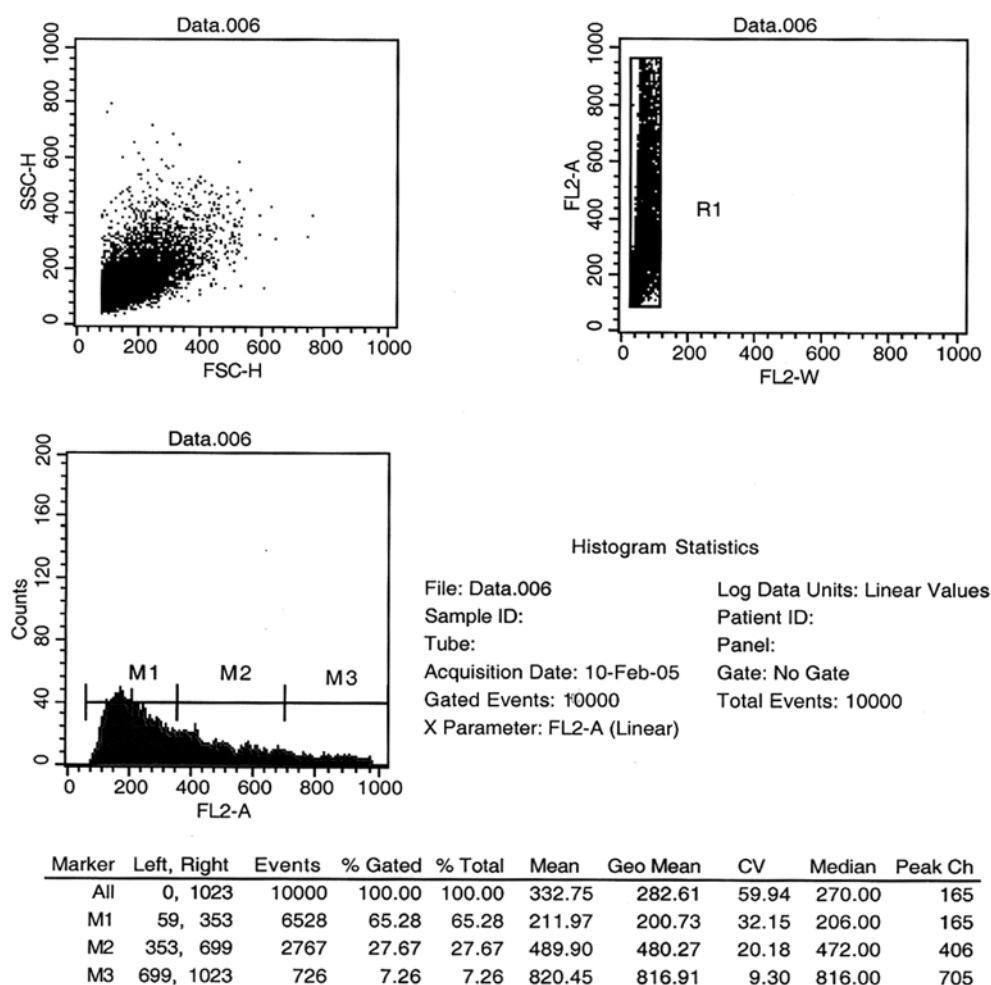
The MTT assay, which measures the formazan product at 570 nm, clearly proves the cytotoxicity of

the methylene chloride extract of fungal taxol. MTT is cleaved by tetrazolium ring by succinate tetrazo- lium reductase in active mitochondria. Metabolically active cells cleave MTT and generate a formazan product, which forms purple crystals, and colour developed is directly proportional to cell number (Mossman, 1983).

Based on the results of the cell viability and % of LDH leakage, a concentration of 5 μ g/ml for further biochemical studies (results not mentioned). LDH leakage is routinely used as an indicator of damage to plasma membrane integrity and in assessing cyto- toxic nature of the plant, as dead cells release LDH in to the culture medium (Matsuda *et al.*, 1980). In the present study at 48 hours of incubation a maxi- mum leakage of LDH leakage was observed at a concentration of 5 μ g/ml, which might be due to the cytotoxic effect of taxol leading to the loss of plasma membrane integrity. Based on the results of cell vi- ability and % LDH release, a concentration of 5 μ g/ml was taken for further studies.

An important component of the cellular response to DNA damage is the inhibition of DNA synthesis. DNA damage could be achieved through inhibition of a positive regulatory pathway of DNA syn- thesis and or costimulation of a negative regulatory pathway. The ability to inhibit DNA synthesis arises

FIGURE 7.1: SHOWS THE FLOW CYTOMETRIC ANALYSIS OF CONTROL CELLS AND FIGURE 7.2 SHOWS THE FLOW CYTOMETRIC ANALYSIS OF FUNGAL TAXOL OF P. MANGIFERAE TREATED A 549 CELLS.



from the possibility of interface with DNA replication enzymes and possibility to induce apoptosis (Kajimoto *et al.*, 2002).

Sialic acid is widely distributed in mammals and usually occurs as a terminal component at the non reducing end of carbohydrate side chains of glycoproteins and glycolipids. A increased concentration of sialic acid is observed in the cell surface and sialo groups are separated by some of these cells (Gorzycza *et al.*, 1993). Elevation of sialic acid levels is commonly found in cancer cells (Kastan *et al.*, 1992). Similar results have been observed in the present study with A 549 cells. A decrease in sialic acid levels noticed in taxol treated A549 cells of present study. This could be attributed to the presence of flavanoids in the taxol extract which could mediate the membrane permeabilisation and bring about modifications in the glycoprotein's components as secondary biochemical responses.

The PI stained cells at a concentration of 5 µg/ml showing clumping of cells with slight distortion. A growth inhibitory and apoptosis inducing activity of many flavanoids with the release of cytochrome c is already established (Bauer *et al.*, 1997; May and May, 1999). Evidence indicates that flavanoids may promote apoptotic cell death. The results of the present study reveal that taxol promotes apoptotic mode of cell death in A549 cells which may be attributed to the presence of flavanoids. Detailed investigations are required to establish the actual flavanoids involved and possible potential of taxol in cancer control and chemotherapy.

In the cells undergoing apoptosis, DNA was degraded to fragments of low molecular weight and subsequently leaked out from the cells and the DNA content was stained with a DNA-specific flouochrome, propidium iodide (PI), a special DNA peak (usually called sub-G1 peak) appeared. The G0/G1

population in the methylene chloride extract of fungal taxol treated A 549 cells was increased after 24 hours at 5 µg/ml. Our cell cycle analysis by flow cytometry showed that there was a prominent increase in the G0/G1 DNA upon methylene chloride extract of fungal taxol treatment. This increase in the G0/G1 DNA is an indication of the inhibition of DNA replication. The increase of DNA content indicated the retardation of cell cycle, which might have taken place during the G1-S transition phase. The possible mechanism of action would be down regulation of the activity of cyclin E dependent kinase, which plays an essential role for cell cycle progression at the G1/S transition stage. This inhibition of cell-cycle progression might be associated with an altered expression of cell cycle relevant regulator, including p21 and its upstream molecule p53 (Kuo, 1996).

Cell cycle control has been proven to be a major event in ensuring the accurate cell division. Abnormalities of cell cycle regulators have been associated with many carcinogenic processes. The data suggests that methylene chloride extract of fungal taxol could cause a significant accumulation of cells in G0/G1 phase in human lung adenocarcinoma cells (A549) after 24 hours. Our data clearly showed that increase of G0/G1 phase cells was accompanied by decrease

of S phase cells. Thus the blockage effect of methylene chloride extract of fungal taxol occurred at G1/S transitions and thus increase of cell numbers in G1 phase was clearly due to decrease of cells in S phase. Much research has showed that the arrest of the cells at the checkpoints of the cell cycle occurs as an event preceding the detection of apoptotic cells (DeAlbuquerque *et al.*, 2004).

There may be some factors that affect the relation of test compound effect between *in vivo* and *in vitro*, including culture conditions, tumor microenvironment, drug distribution, active metabolites and indirect inhibition effects on tumor proliferation (e.g. angiogenesis). Rather cell culture should be used to illustrate principles, concepts and mechanisms of action that may be active *in vivo*. If we see it from a drug development point, there are numerous efforts being made to find a method of delivery to achieve the concentration of crude extract in cell cultures.

The idea of study is that to show efficacy in cell cultures first, then start evaluating effects in animals.

Taxol from *Pestalotiopsis mangiferae* appears to be a promising drug for its future use as an anticancer drug. From our observation, we do agree it is only a preliminary study and it requires a further detailed study.

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