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The Antiproliferative Activity of Flavanoidal Fraction of *Indigofera tinctoria* is Through Cell Cycle Arrest and Apoptotic Pathway in A-549 Cells

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Abstract: The aerial parts of the plant was investigated for its antiproliferative activity in human Non Small Cell Lung Cancer cells A-549. The results showed that the flavanoidal fraction of methanolic extract of the aerial parts of the plant inhibited the proliferation of A-549 cells as measured by MTT assay. Flow cytometric analysis showed that flavanoidal fraction of methanolic extract of *I. tinctoria* blocked cell cycle progression in G0/G1 phase. In addition flavanoidal fraction of methanolic extract of *I. tinctoria* induced A-549 cell apoptosis as determined by propidium iodide staining. We suggests that flavanoidal fraction of methanolic extract of *I. tinctoria* activity might be potentially contribute to its overall chemo preventive effect against lung cancer and can possibly be considered for future therapeutic application.

Key words: *Indigofera tinctoria*, A-549, MTT, G0/ G1, apoptosis, chemoprevention

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 (Cheng *et al.*, 2003; Kim *et al.*, 2003).

Indigofera tinctoria (*I. tinctoria*), a widely distributed shrub found throughout India belong to the family Fabaceae. The aerial parts of the plant have shown to possess antihepatotoxic activities. The alcoholic extract of the leaves possess antihepatotoxic effect against D-galactosamine and carbontetrachloride induced damage in liver (Sreepriya *et al.*, 2001). Indigtone an active compound isolated from the leaves of this plant possess hepatoprotective activity (Singh *et al.*, 2004). Further, the plant has proved to be more effective against chronic myelogenous and other leukemia's (Steriti, 2002). Indiubin, the active constituent from the leaves of this plant is a promising anticancer drug (Han, 1994). Juice of the leaves has a great repute as a cure for hydrophobia being administered both internally and externally (Chopra, 1956). Ayurveda has already described this plant as a stimulant, alternative and deobstruent (Chunekar and Nighatu, 1993). In the traditional systems of Indian

medicine extract of the plant is used for the management of several hepatic and nervous disorders (Chatterjee and Pakrashi, 1992). Indirubin, the component responsible for the anticancer activity of *I. tinctoria* yielded marked inhibition of Lewis lung carcinoma and walker carcinoma thereby suggesting that the plant poses significant antineoplastic activity (Xiujuan, 1981; Rui, 1995).

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. 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 (Hengartner, 2000; Brown and Wooters, 1999).

In the present study, we evaluated whether the flavanoidal fraction of *I. tinctoria* exerted apoptotic mode of cell death on A-549 cells accompanied by cell cycle arrest if any.

MATERIALS AND METHODS

Chemicals: Fetal Bovine Serum (FBS), Penicillin G, Streptomycin and amphotericin B were obtained from Hi Media. Dimethylsulfoxide (DMSO) and Ham's F 12K medium was purchased form GIBCO. MTT and propidium

iodide was purchased from SRL, Laboratories India. All the experiments were conducted in King Institute of Preventive Medicine, Chennai, India.

Preparation of extract: *Indigofera tinctoria* belongs to the family Fabaceae. The aerial parts of the plant were taken for the study. Eight kilogram of the aerial parts were shade dried and continuously extracted with methanol. The methanolic extract is referred to as the crude extract. It was run in TLC with benzene and ethyl acetate in the ratio of 9:1 as mobile phase. The flavanoidal fractions of the plant were taken for the study.

Test for flavanoids: Fifty milligram of sample was dissolved in 5 mL of distilled water. To this few drops of 5% ferric chloride was added. A dark green colour indicates the presence of phenolic compounds. Most phenolic compounds belong to flavanoids and lignin that was tested by following test reagents.

Alkaline reagent test: An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavanoids.

Magnesium+Hydrochloric acid reductions: Fifty milligram of the extract dissolved in 5 mL alcohol and few fragments of magnesium and concentrated HCl were added; the appearance of pink to crimson colour indicates the presence of flavanol glycosides.

A-549 cell and maintenance: A-549 cell lines were obtained from the NCCS, Pune, India. A-549 cells were cultured in Ham's F-12K medium supplemented with 10% FBS with 100 U mL⁻¹ of penicillin and streptomycin 100 U mL⁻¹. 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 the maintenance is strictly in accordance with the standard methods. The cells were dissociated with trypsin phosphate versenal glucose in phosphate buffered saline. The stock cultures were grown in 25 cm² tissue culture flasks and the cytotoxic experiments were carried out in 24 well plates (Tarson India Private Limited, Kolkata, India).

Morphological analysis by light microscopy: After 48 h incubation with IT the A-549 cells were washed with PBS. The cells were observed for morphological changes under light microscope at 100X and photographed.

MTT assay: Cell viability was assessed using MTT as described by Mossman, 1983 and the cytotoxic index or the IC₅₀ was calculated.

Propidium iodide staining: The propidium staining and its subsequent apoptotic evaluation was carried according to Belloc *et al.* (1994).

Hoechst 333258 staining: Apoptotic nuclear morphology was assessed using Hoechst 333258 staining as described by Chen *et al.* (2002) with slight modification.

Cell cycle arrest by flow cytometric analysis: This analysis was carried according to Nicotelli (1991).

RESULTS AND DISCUSSION

In the present study, the morphological changes were observed in cells by light Microscopy. As shown from Fig. 1, the flavanoidal portion of *I. tinctoria* caused cytolytic and nuclear changes in cell morphology. Cell mitosis was scarce compared to control. Progressive structural alterations and reduction in the number of cells were observed on treatment with various concentrations viz. (10-50 µg) of flavanoidal fraction of *I. tinctoria* extract (Fig. 1a-d). The drug treated cells showed the clumping of cells, disintegration of cytoplasm and loss of normal architecture of cells.

The MTT assay that measures the formazan product at 570 nm clearly proves the cytotoxicity of the flavanoidal portion of *I. tinctoria*. As evident from the Fig. 2, the cytotoxicity was observed at increasing concentrations i.e., 10, 20, 30, 40 and 50 µg (p<0.05). Even at 30 µg, less than 50% of cell viability is observed while at 50 µg, only 10% of cell viability was seen. MTT is cleaved by tetrazolium ring by succinate dehydrogenase 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). Even at 30 µg, less than 50% of cell viability is observed while at 50 µg, only 10% of cell viability was seen.

Figure 3 shows the fluorescent microscopic pictures of the flavanoidal portion of *I. tinctoria* treated and untreated A-549 cells assessed for apoptosis at 400x. The propidium iodide stained cells at a concentration of 40 and 50 µg mL⁻¹ 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 µg mL⁻¹ (Fig. 3b, c). The untreated A-549 cells were shown in Fig. 3a. By morphological analysis with propidium iodide staining, cell clumping and formation of apoptotic bodies characteristic of apoptosis (Zimmerman *et al.*, 2001; Lieberthal *et al.*, 1998) were seen in cells cultured with flavonoidal fraction of *Indigofera tinctoria*, but very few of these were seen in control cells.

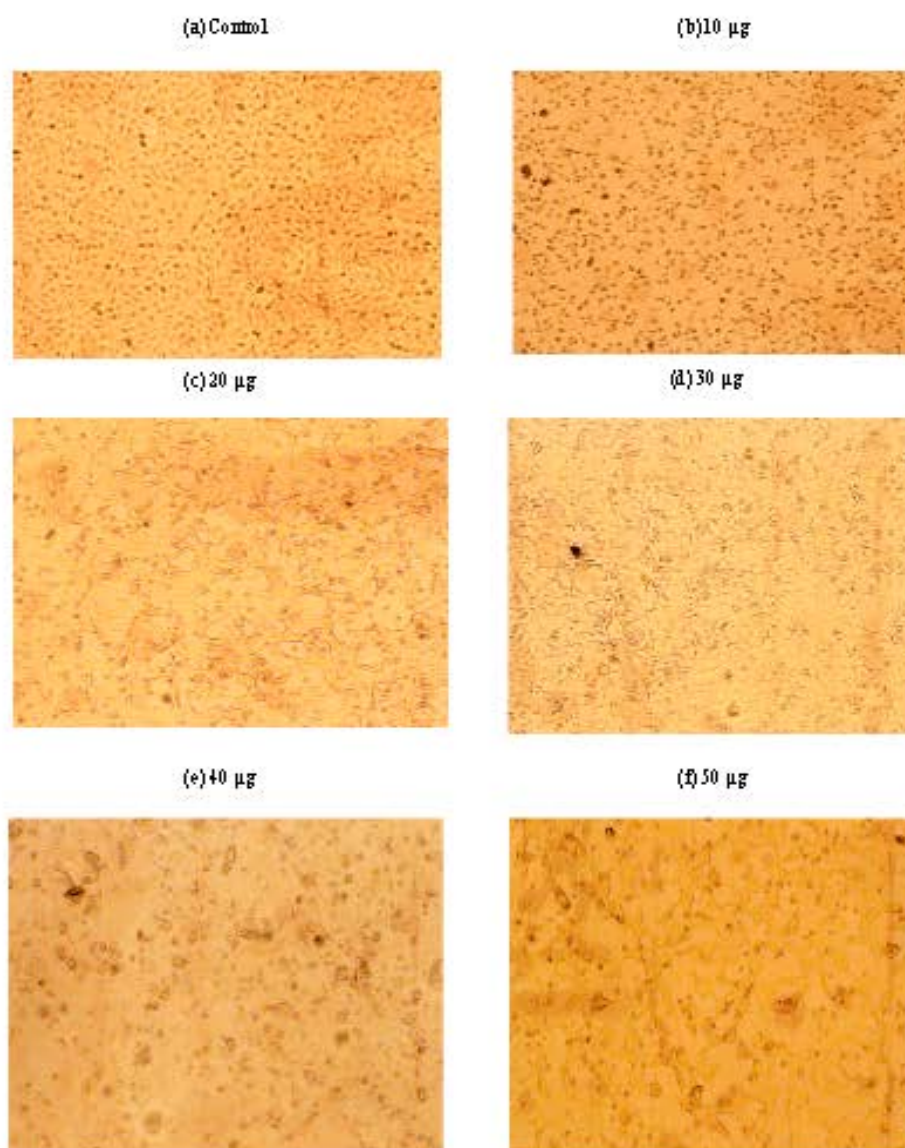


Fig. 1: Morphological changes induced by flavanoidal fraction of *Indigofera tinctoria* as observed under a light microscope (100X)

Figure 4 shows the evaluation of apoptosis in A-549 cells at 48 h after adding flavanoidal fraction of *I. tinctoria* and analyses by fluorescence microscopy using chromatin stain Hoechst 333258. Figure 4a showing the untreated A-549 cells, whereas Fig. 4b (40 µg) and Fig. 4c (50 µg) shows the condensed nuclei or apoptotic bodies in apoptotic cells after staining with Hoechst 333258. Flavanoids like quercetin, Kaempferol, Hesperetin, Hesperidin, Naringenin and Naringenin were found to induce apoptosis in neoplastic cells (Yanagihara *et al.*,

1993; Spinozzi *et al.*, 1994). The plant is a rich source of the above mentioned flavanoids. In this study, when cells were treated with flavanoids, morphological changes were consistently observed that eventually led to the detachment of cells from the monolayer. Using Hoechst 333258 to stain DNA, the nuclei of control cells and cells treated with flavanoids were compared. Present study showed DNA (chromatin) condensation was observed in the treated cells, suggesting the presence of apoptosis in these cells (Searle *et al.*, 1974).

Fluorescence microscopy was used to determine whether flavanoids were enriched in the cell membrane. Due to lipid solubility, the flavanoids are expected and found to incorporate in to lipid bilayer (Saija *et al.*, 1995;

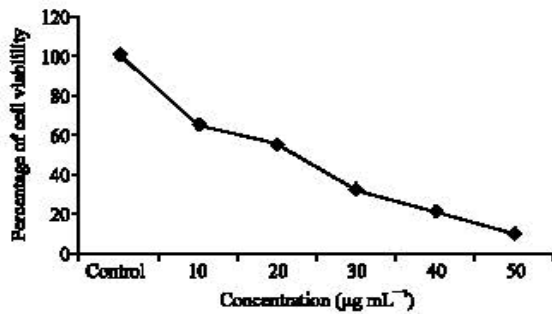
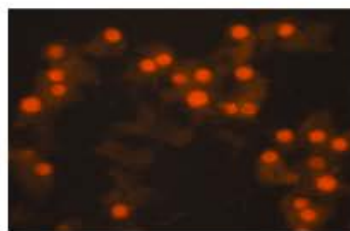


Fig. 2: The (%) cell viability of flavanoidal fraction of *Indigofera tinctoria* on A 549 cells by MTT assay

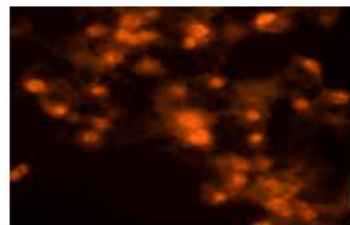
Price and Middleton, 1986). The biological effects of the flavanoids were due to its nuclear deposition (DNA interaction) or membrane incorporation. However it should be noted that chemical reactivity of structurally different flavanoids of *Indigofera tinctoria* are essential for their antiproliferative activity.

Figure 5a shows the flow cytometric analysis of untreated A 549 cells. Figure 5b shows the drug treated A 549 cells. It was found that 1.40% of the cells were in the G1 phase, 52.27% in the S phase and 9.61% of cells in the G2 phase in the control cells of A 549 cells. The flavanoidal fraction of *I. tinctoria* (50 µg) treated cells had 65.28% of the cells in the G1 phase, 27.67% in the S phase and the remaining 7.26% cells in the G2 phase, indicating the accumulation of cells in the G1 phase of cell growth by the drug.

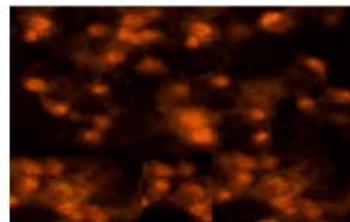
To confirm the apoptosis induced by the drug on A 549 cells, flow cytometry was employed. In the cells



(a) Control A-549 cells

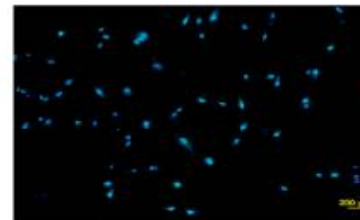


(b) A-549 cells treated with 40 µg of *I. tinctoria*

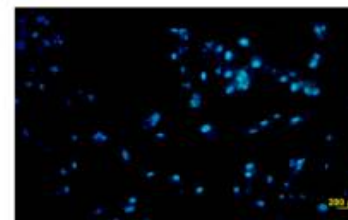


(c) A-549 cells treated with 50 µg of *I. tinctoria*

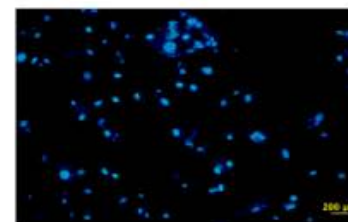
Fig. 3: Morphological changes induced by flavanoidal fraction of *Indigofera tinctoria* as observed under a fluorescent microscope after staining with propidium iodide (400X)



(a) Control A-549 cells



(b) A-549 cells treated with 40 µg of *I. tinctoria*



(c) A-549 cells treated with 50 µg of *I. tinctoria*

Fig. 4: Evaluation of apoptotic effect of flavanoidal fraction of *Indigofera tinctoria* determined by Hoechst 333258 staining

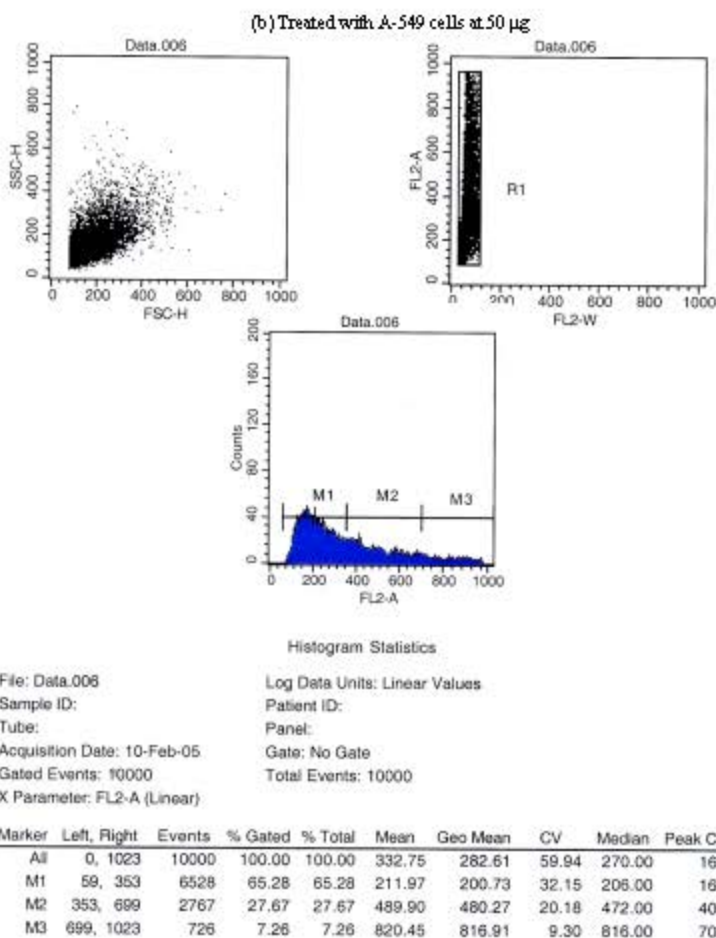
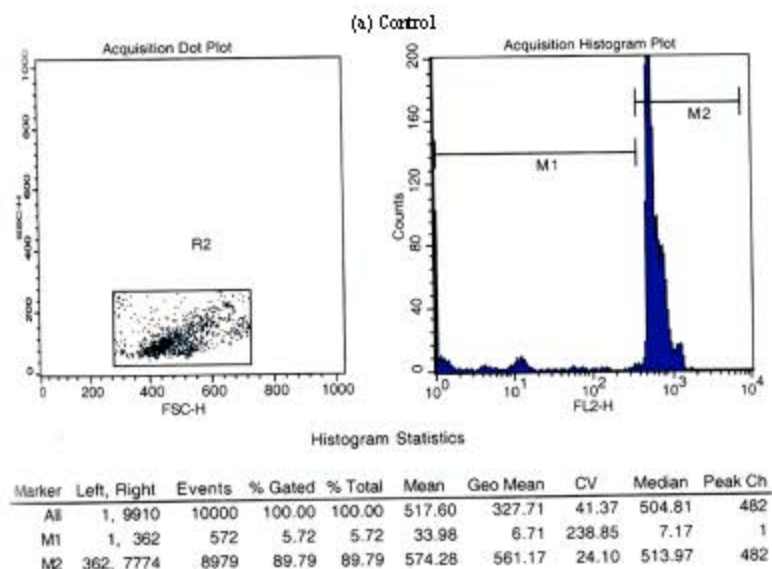


Fig. 5: Apoptotic evaluation of flavanoid fraction of *Indigofera tinctoria* evaluated by flow cytometric analysis

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 flouoro-chrome, Propidium Iodide (PI), a special DNA peak (usually called sub-G1 peak) appeared. The G0/G1 population in the flavanoidal fraction of *Indigofera tinctoria*-treated A-549 cells was increased after 24 h at 50 µg mL⁻¹. Our cell cycle analysis by flow cytometry showed that there was a prominent increase in the G0/G1 DNA upon flavanoidal fraction of *I. tinctoria* 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 (May and May, 1999). In other *in vitro* models, flavonoids have also been shown to affect cell-signaling and cell cycle progression (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. Present data suggests that flavanoidal fraction of *I. tinctoria* could cause a significant accumulation of cells in G0/G1 phase in human lung adenocarcinoma cells (A-549) after 24 h. Present data clearly showed that increase of G0/G1 phase cells was accompanied by decrease of S phase cells. Thus the blockage effect of flavanoidal fraction of *I. tinctoria* occurred at G1/S transitions and thus increase of cell numbers in G1 phase was clearly due to decrease of cells in S phase. The flavanoidal compounds indirubin and quercetin may be responsible for bringing out this effect. 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 (Surh, 1999).

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.

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