



Methanolic extract of *Indigofera tinctoria* induces apoptosis in HCT116 cells

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ABSTRACT

Objective: To determine the anticancer activity of the methanol extract of *Indigofera tinctoria*, which induces apoptosis in HCT 116 colon cancer cell line.

Methodology and results: the effect of the methanolic extract of *Indigofera tinctoria* on HCT 116 cells was determined by cell viability, DNA fragmentation and comet assay. Treatment of HCT 116 cell lines with various concentrations of the extracts inhibited growth and induced apoptosis in a dose-dependent manner.

Conclusion and application of results These results indicate that methanol extract of *I. tinctoria* exhibits antiproliferative effect on HCT 116 cells via apoptosis, and it may be a potential candidate in the field of anticancer drug discovery.

Key words: *Indigofera tinctoria*, cytotoxicity, HCT 116 cell line, anticancer, apoptosis

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INTRODUCTION

Cell death can occur through several different mechanisms, of which the most widely described are apoptosis and necrosis. A significant physiological consequence of cell death by apoptosis is that the apoptotic cells are immediately phagocytosed by macrophages. Therefore, the release of intracellular molecules that cause secondary disturbance to the surrounding tissue is limited to a low level compared with necrosis, which causes further tissue destruction and inflammation (Cohen, 1993; Earnshaw, 1995). Recently, there has been a global trend towards the use of natural bioactive substances found in fruits, vegetables, and herbs, as antioxidants and functional foods (Farr, 1997; Wang *et al.*, 1997; Kitts *et al.*, 2000; Lee & Lim,

2001). Some of these substances are believed to have potential as cancer chemopreventive or therapeutic agents (Pezutto, 1997; Christou *et al.*, 2001; Mukherjee *et al.*, 2001). Most of these substances exert their chemotherapeutic activity by blocking the cell cycle progression and triggering apoptotic cell death. Therefore, the induction of apoptosis in tumor cells has become an indicator of the tumor-treating ability of naturally derived bioactive substances (Smets, 1994; Paschka *et al.*, 1998).

Recent attention has also focused on the development of target organ-specific apoptosis inducers as novel cancer-preventive and therapeutic approaches (Mukherjee *et al.*, 2001). Flavonoids are commonly found in most plants and

are integral parts of the human diet (Gamet-payrastre *et al.*, 1999). These compounds exert a remarkable spectrum of biological activities affecting the basic cell functions, such as growth (Formica & Regelson, 1995), differentiation (Plaumann *et al.*, 1996), and apoptosis (Caltagirone *et al.*, 2000). Flavonoids are also known to have anticarcinogenic, anti-inflammatory, antibacterial, immune stimulating, and antiviral activities (Duarte *et al.*, 1993; Gao *et al.*, 1999; Wong & Mclean, 1999), and their beneficial effects have been attributed to the inhibition of the enzymes involved in signal transduction and to their antioxidant properties. Many studies have shown that flavonoids inhibit PI3- kinase, protein kinase C, protein tyrosine kinase, and some transcriptional factors, and that such inhibition leads to cell growth arrest and tumor cell death (Gamet-payrastre *et al.*, 1999; Yang *et al.*, 1998; Miranda *et al.*, 1999).

The use of plant extracts and plant derived compounds for treatment of cancer is well documented in Ayurveda. *I. tinctoria* is an annual

herb of 4 - 6 feet height that is cultivated in India, china and other countries as a source of indigo. The herb is widely used in the Indian system of medicine for epilepsy, nervous disorders, bronchitis and liver ailments (Singh *et al.*, 2001). Extensive research of the last few decades has revealed that the herbal extract is useful as an anti-cardiovascular (Tadigoppula *et al.*, 2006). It has been used to protect against hepatoxicity induced by ccl4 and liver anti oxidant (Sreepriya *et al.*, 2001).

The family of bis-indoles known generically as indirubins (Hoessel *et al.*, 1999) are the main constituents of *Indigofera tinctoria* a product from Chinese medicine used to treat myelogenous leukemia and which posses cytotoxic activity (Cragg *et al.*, 2005). No studies describing the anticancer potential of *Indigofera tinctoria* have been reported. In the present study an attempt is made to explore the anticancer potential and mechanism of action of the components of *I. tinctoria*.

MATERIALS AND METHODS

Preparation of extracts: *I.tinctoria* (Fabaceae) was purchased from nursery of Government Siddha Medical College, Arumbakkam, Chennai, India. Its botanical identification was done by Dr. Jayaraman, Director, Plant Anatomy Research Centre, Tambaram, Chennai. The voucher specimen (001/TN/2007) was deposited at the herbarium, Department of Botany, Presidency College, Chennai, India. The fresh leaves were shade dried, powdered and 200g samples extracted successively with 600 ml of methanol (60–80 °C) in a soxhlet extractor for 18–20 h. The extract was concentrated to dryness under reduced pressure and controlled temperature (40–50 °C) to form a dark brown solid, weighing 500 mg (25 % w/w). The methanol extract of *I. tinctoria* was screened for phytochemicals (Kabir *et al.*, 2005).

Cell line and culture medium: Human colon cancer cell line HCT 116 was purchased from American Type Culture Collection (ATCC). HCT 116 cells were cultured in Dulbecco's modified Eagle's (DEME) medium, supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.1% streptomycin in a 37 °C incubator with 5% CO₂.

Cytotoxicity and antiproliferative activity: Cytotoxicity was determined by the MTT assay as indicated by Mosmann (1983). Briefly, cells were plated in microtiter (ELISA) plates at an initial density of 1x10⁴ cells /well. After incubation for 24 h at 37 °C, cells were treated with different concentrations of Methanol extract of *Indigofera tinctoria* and incubated for 24 h. MTT solution was added to each well and further incubated for 4 h at 37°C, optical density was read with an ELISA reader at 550 nm.

Antiproliferative activity was determined by a Tryphan blue exclusion assay (Jones & Sneft, 1985). For the Tryphan blue exclusion assay, cells were cultured in a 35 mm dish and exposed to various concentrations of methanol extract of *indigofera tinctoria* for 7 days. The cells were trysinized, washed with phosphate buffered saline (PBS) and Tryphan blue dye solution was added to the cell suspension. Viable cells were counted with a hemocytometer.

Nucelar Staining with DAPI: Cells were washed with PBS and fixed with 3.7% Para formaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 4,6-diamidino-2-phenylindole

(DAPI, Sigma) solution for 10 min at room temperature. The cells were washed twice with PBS and analyzed through fluorescence microscope.

Detection of apoptosis by TUNEL assay: Apoptosis was evaluated by DNA fragmentation according to TUNEL assay using Apoptosis Detection Kits, Catalog Number: TA 300, R&D systems, Oxon, United Kingdom. Briefly, cells were plated on the poly-L-lysine-coated slides. Then, the cells were air-dried in a tissue culture hood for 1h. Subsequently, the cells were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde in PBS in a Copline jar for 25 min at room temperature and rinsed twice with PBS. The cells were then immersed in 0.2% (v/v) Triton X-100 solution for 5 min and rinsed with PBS. Control and positive control cells were treated with DMSO and DNase 1, respectively. The cells were equilibrated with equilibration buffer at room temperature for 5 min. Subsequently, TdT enzyme reaction mixture and biotinylated nucleotide mixture were added to the cells; then, the cells were covered with coverslips and incubated for 1h at 37 °C. The reaction was terminated by immersing the slides in alcohol and then incubating with streptavidin HRP solution in PBS for 30 min, rinsed with PBS and finally incubated with TACS Blue label (TBL) solution until a light brown background developed. The stained cells were immediately observed under the light microscope.

Detection of apoptosis by DNA fragmentation assay: Detection of small DNA fragments was done according to the method of Yvonne *et al.* (2001) with some modifications. Briefly, 2×10^6 cells were harvested and the pellets were lysed with 20 μ L of a solution containing 50 mM tris (hydroxymethyl) aminomethane, 10mM EDTA and 0.5% (w/v) sodium lauryl sarcosinate. RNase A (0.25mg/ml) was added and the samples incubated at 50 °C for 1 h. The condensate was spun down and proteinase K (5mg/mL) was added. The samples were incubated at 50 °C for a further hour before being loaded into the wells of a 1.5% agarose gel. Electrophoresis was carried out in 1.5% agarose gels prepared in TBE buffer (0.45 M boric acid and 2 mM EDTA, pH 8), at

V/cm DNA was visualized under UV light on a transilluminator (312nm) after ethidium bromide staining, and photographed using a digital camera.

Detection of apoptosis (Comet assay): The ability of comet assay to quantify DNA strand breaks and alkali labile sites has been widely demonstrated, but it is still a relatively new technique for detecting biological phenomena like apoptosis, also characterized by DNA fragmentation. The comet assay was performed as previously described by (Thierry *et al.*, 1999), 10^5 cells were suspended in 140 μ l pre warmed low melting point (LMP) agarose (0.5% PBS) without calcium or magnesium; 65 μ l of the suspension was rapidly spread on frosted microscope slides pre coated with 80 μ l of normal agarose (0.8% in PBS Ca⁺⁺, Mg⁺⁺-free) and covered with a cover slip (24x32mm). After gelling for 10 min at 0°C, the cover slip was gently removed and the third layer of 80 μ l LMP agarose was added. Slides were then put on a tank filled with lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, PH 10, 10% dimethyl sulfoxide and 1% Triton X-100 both freshly added) for 1 hour at room temperature. The slides were then removed from lysis solution and incubated in a fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) for 40 minutes at room temperature to allow unwinding of DNA. Electrophoresis was then carried out at room temperature in fresh electrophoresis buffer for 24 minutes at 0.7 Volts/cm and 300 mM. After electrophoresis, slides were gently washed twice for 5 minutes in fresh neutralization buffer (0.4 M Tris-HCl, pH 7.5). After drying overnight at 4 °C, slides were stained with 50 μ l of ethidium bromide solution (20 μ g/ml) and covered with a cover slip. A total of 200 randomly selected individual cells were visually analysed and comets were systematically classified into 3 categories for qualitative evaluation, i.e. undamaged cells (UC), damaged cells (DC) with intermediate level of damage and highly damaged cells (HDC). HDC were characterized by an extensive DNA fragmentation which allowed 90% of the DNA to migrate during electrophoresis, forming the comet tail. Damaged cells were never observed in the present study.

RESULTS

Cytotoxicity of methanol extract of *Indigofera tinctoria* on HCT 116 was observed to exhibit a dose dependent trend. The IC 50 value of the methanol extract as calculated from the graph was 1 μ g/ml (Fig. 1). Compared to the control cells, cells treated with the

methanol extract were significantly inhibited ($40 \pm 3\%$) after incubation for 3 days with 5 μ g/ml of the extract of *Indigofera tinctoria*. When the concentration of methanol extract was increased to 25 and 50 μ g/ml the



inhibition rate increased to 70 ± 2.3 and $97.8 \pm 1.0\%$, respectively, after 7 days incubation (Fig. 1B).

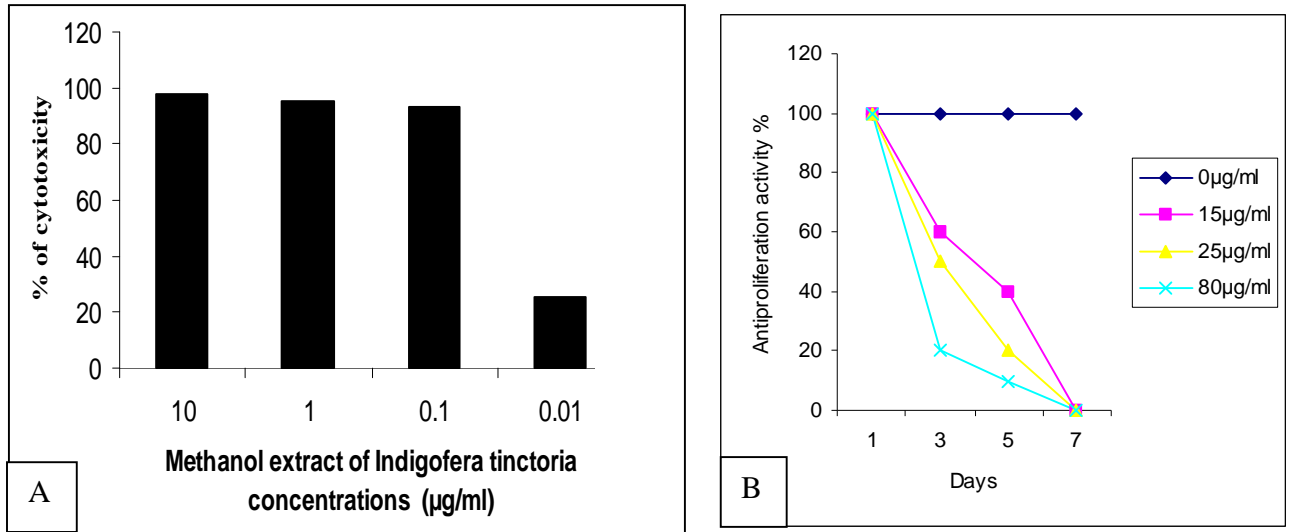


Figure 1: Cytotoxic and antiproliferative effects of Methanol extract of *Indigofera tinctoria* on HCT 116 cells. (A) Cytotoxic activity detected by MTT assay after exposing cells to various concentrations of Methanol extract of *Indigofera tinctoria* for 24 h. (B) Antiproliferative effects detected by Trypan blue exclusion assay. Cells were treated with Methanol extract of *Indigofera tinctoria* ranging in doses from 15 to 50 µg/ml for 7 days. Control cells were treated with 0.1% DMSO.

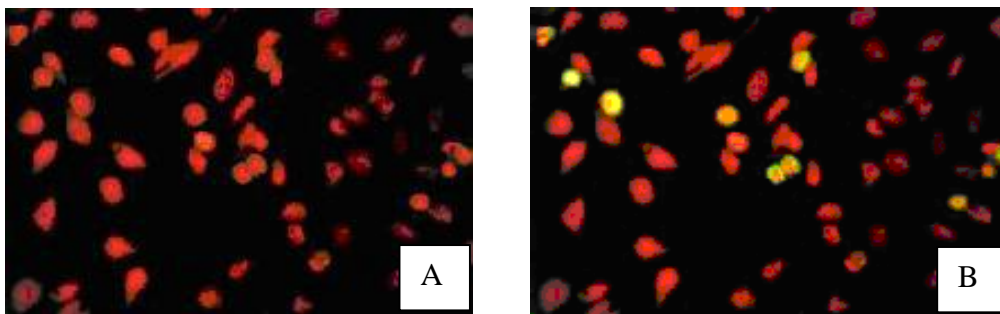


Figure 2: Morphological changes of HCT 116 cells after treatment with Methanol extract of *Indigofera tinctoria* for 48h followed by DAPI staining. (A) Control cells treated with 0.1% DMSO and (B) cells treated with 25 µg/ml Methanol extract of *Indigofera tinctoria*. Light yellow colour cells indicate apoptotic bodies of nuclear fragmentation. Magnification x 100.

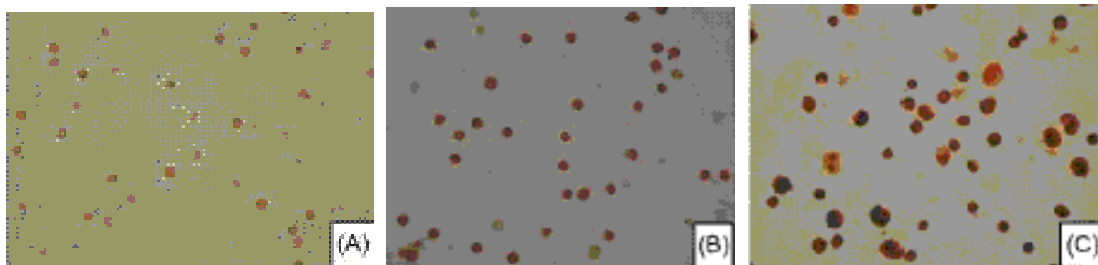


Figure 3: Cytotoxic activity of Methanol extract of *Indigofera tinctoria* on cancer cells. HCT 116 cells were treated with 0.1% DMSO (A, negative control), methanol extract of *Indigofera tinctoria* (B, 25 µg/ml) or DNase I (C, positive control) for 24 h and subjected to TUNEL assay. Nuclei of the HCT 116 cells stained dark brown were observed after cell treatment with Methanol extract of *Indigofera tinctoria* and Dnase I, whereas no stained nucleus was detected in control cells treated with DMSO. Magnification x 100.

Nuclei with condensed chromatin and apoptotic bodies, which are typical characteristics of apoptosis, were observed in HCT 116 cells incubated with methanol extract of *Indigofera tinctoria*, and the number of apoptotic cells increased as the concentration of methanol extract of *Indigofera tinctoria* increased (Fig. 2). The TUNEL assay showed the nuclei of cells treated with MEIF were stained dark brown, but very few were stained in the control cells (Fig. 3).

By the Comet assay, single-cell visual analysis revealed only the presence of undamaged (UC) and highly damaged cells (HDC). No intermediate damage

(DC) was detected regardless of the dose and the time of exposure. HDC were detectable after 1 hr treatment by 50 methanol extract of *Indigofera tinctoria*.

Gel electrophoresis results revealed fragmentation in cells treated with 50 µg/ml concentration of methanol extract of *Indigofera tinctoria*, while DNA fragments were absent in the control cells (Fig. 5). Phytochemical screening of the methanol extract of *Indigofera tinctoria* showed presence of alkaloids, glycosides, flavonoides and saponins while carbohydrates and tannins were not detected.

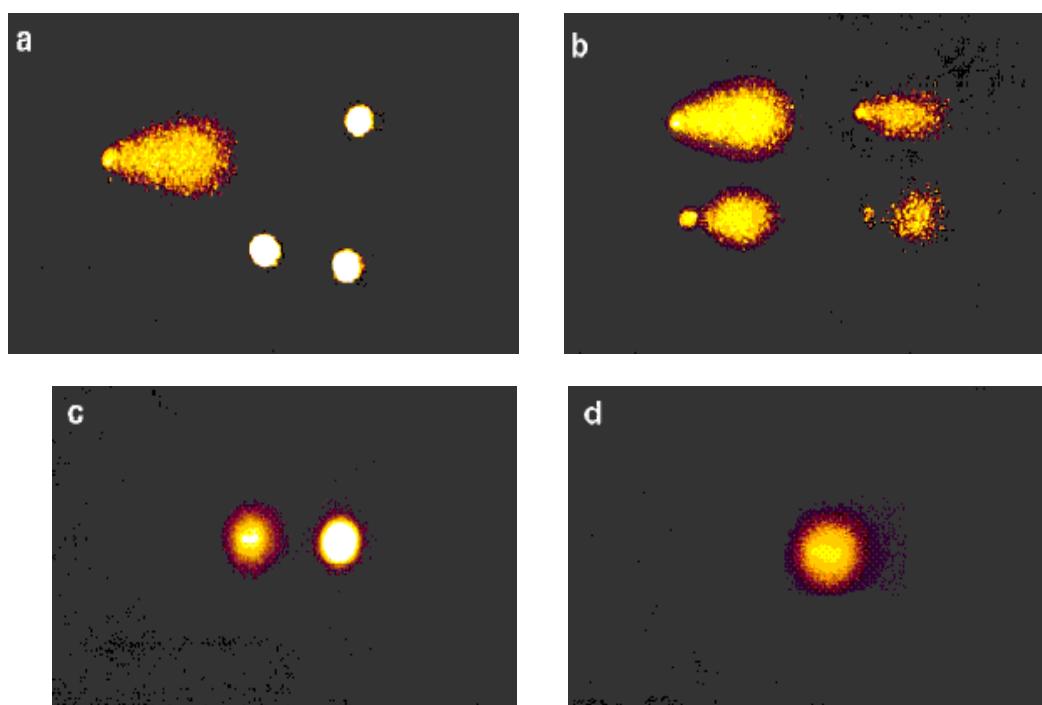


Figure 4: Induction of apoptosis by methanol extract of *Indigofera tinctoria* on HCT 116 cells (Comet assay). Photomicrographs of undamaged and highly damaged cells. (a, b): comet assay with electrophoresis where undamaged cells and highly damaged cells are visible. c, d: comet assay without electrophoresis where (c) an intact nucleus, a very small intact nucleus with a wide halo and (d) a faint halo without visible nucleus are visible.

DISCUSSION

Current studies on development of effective cancer preventive approaches have focused mainly on the utilization of natural bioactive agents that can induce selective apoptosis in cancer cells (Mukherjee *et al.*, 2001). Flavonoids exert various pharmacological properties and are believed to be beneficial compounds for cancer chemoprevention (Wei *et al.*, 1994;

Plaumann *et al.*, 1996; Caltagirone *et al.*, 2000). Methanolic extracts have been screened for anticancer properties because traditional practitioners believed that mostly the polar compounds were responsible for the claimed anticancer potential.

In this study, the methanol extracts of *Indigofera tinctoria* showed significant cytotoxic activity

of HCT116 cell line. The activities of this plant may be due to the presence of highly complex glycosides, flavonoids, alkaloids and saponins (Tan *et al.*, 2005). Our findings are similar to the data previously reported on the anticancer activity in extracts of *Silvia letasiova*. In the present study, we found that the methanol extract of *I. tinctoria* was cytotoxic and induced apoptosis in HCT 116 cells. The cells exposed to methanol extract of *Indigofera tinctoria* exhibited morphological and biochemical changes that characterize apoptosis as shown by loss of cell viability, chromatin condensation and DNA fragmentation. Since apoptosis is regarded as a new target in discovery of anticancer drugs, these results confirm the potential of *Indigofera tinctoria* as an agent of chemotherapeutic and cytostatic activity against human colon cancer cells. Further investigation is under progress to elaborate this possibility.



Figure 5: Effects of various concentration of methanol extract of *Indigofera tinctoria* on the DNA fragmentation of HCT 116 cells. Cells treated with DMSO are negative control. Lane 1 (50 µg/ml), Lane 2 (25 µg/ml), Lane 3 (10 µg/ml), Lane 4 (DMSO).

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