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ORIGINAL ARTICLE

INFLUENCE OF SINAPIC ACID ON INDUCTION OF APOPTOSIS IN HUMAN LARYNGEAL CARCINOMA CELL LINE

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ABSTRACT

We investigated the influence of sinapic acid on the induction of apoptosis in Human laryngeal carcinoma cell line (HEp-2). **Methods:** In vitro cytotoxicity were determined by MTT assay, morphological changes, levels of reactive oxygen species, mitochondrial membrane potential and cell cycle analysis by flowcytometry. **Results:** Based on the results, we determined the effective dose of sinapic acid as 125.23µM/ml for 24 hr and 117.81 µM/ml for 48hr. Cytotoxicity effects of sinapic acid was confirmed by treatment of HEp-2 cell with IC_{50} concentration of sinapic acid resulted in sequences of events marked by the enhance the apoptosis accompanied by loss of cell viability, modulation of reactive oxygen species and cell cycle arrest through the induction of G_0/G_1 phase arrest on HEp-2 cells. **Conclusion:** Our study suggests that the sinapic acid possesses the cytotoxic effect of cancer cells and a novel candidate for cancer chemoprevention.

Keywords: Apoptosis; HEp-2 cell line; Mitochondria; Cell cycle analysis; Sinapic acid.

1.INTRODUCTION

Oral cancer is the third most common cancer and most common cancer death among male in the world population (Thun *et al*, 2010). Chemoprevention is emerged as a good novel therapy in the recent years and involves the use of natural, synthetic and semi-synthetic compounds to suppress and inhibit the malignant transformation (Hong and Sporn, 1997). Most of the anticancer compounds are in the nature of phenolic acids and these compounds plays a major role in antioxidants as chemoprevention (Arpita *et al*, 2010). Natural products are important sources of new bioactive molecules, due to the structural diversity of their constituents. Between 2005 and 2007, thirteen new drugs are derived from phytochemicals and have been approved by FDA, with five of them being the first members of new classes. The discovery of effective anti-cancer drugs from natural products plays an important role in cancer chemotherapy (Li and Vederas, 2011).

Recently, much attention has been focused on the protective function, especially antioxidative effect of naturally occurring compounds and on the mechanisms of their action.

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Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of

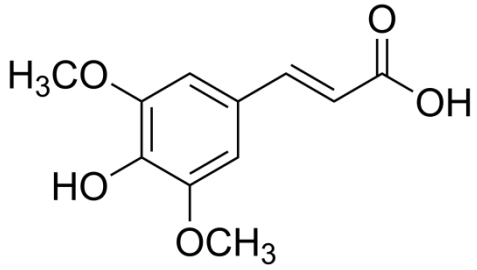


Figure 1. Structure of Sinapic acid

oxidative damage in living system (Pandey and Rizvi, 2009). Sinapic acid (4-hydroxy-3, 5-dimethoxy cinnamic acid) (Figure.1) is a small naturally occurring carboxylic acid, a member of phenylpropanoid family, widely distributed in edible plants such as cereals, nuts, oil seeds and berries (Zou *et al*, 2002). It possesses a wide variety of biological activities, especially as antioxidants and antibacterial activity *in vitro* (Nowak *et al*, 1992). Previous report were documented that; sinapic acid is a potent antioxidant capacity higher than ferulic acid and sometimes

comparable to that of caffeic acid (Nenadis *et al*, 2007). To our knowledge there are no scientific reports available on the literature for in vitro inhibition of growth and induction of apoptosis in human laryngeal epithelial cancer cell line HEp-2 by sinapic acid. However, in the present study, we evaluated the effect of sinapic acid on inhibition of growth induction and apoptosis in HEp-2 cancer cells.

2.MATERIALS AND METHODS

Cell culture and drug treatment

Human laryngeal epithelial cancer cell line (HEp-2) used in our study was obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were placed into $25cm^2$ tissue culture flasks and grown in Minimum essential medium (Eagle) supplemented with 10% Fetal bovine serum (FBS), 1% glutamine, 100 IU/ml penicillin and 100µg/mL streptomycin at 37ºC under a humidified 5% CO_2 atmosphere. Cells were treated with different concentrations of sinapic acid (25-250µM/ml) and the cytotoxicity was observed by MTT (3-(4,5-dimethyl-2-thiaozolyl)-2,5-diphenyl-2H tetrazolium bromide) assay. IC_{50} values were calculated and the effective dose was used for further study.

Determination of effective dose of sinapic acid (MTT assay)

In the present study, MTT assay was used to determine the effective dose of sinapic acid. MTT is reduced by living cells to insoluble purple MTT formazan crystals using succinate, and the pyridine nucleotide cofactors, NADH and NADPH as substrates. These results in a yellow to blue color changes were quantified. The viability of cultured cells was determined by assaying for the reduction of MTT to formazan as described by Mosmann, (1983) with some modifications. In brief, HEp-2 oral cancer cells were seeded in 96 well microtiter plate (5×10^3 cells/well), and then the cells were incubated with sinapic acid at different concentrations ranging from 25-250 µM for 24 and 48 hr. The untreated cells served as control. After the incubation the cells were washed twice with phosphate buffered saline (PBS). MTT (100 µM/0.1 mL of PBS) was added to each well. Cells were incubated at 37ºC for 4 hr, and DMSO (100 µL) was added to dissolve the formazan crystals. An equal volume of DMSO was added to stop the reaction and to solubilize the blue crystals. Samples were transferred into culture plates and the absorbance was measured calorimetrically at 590 nm.

Determination of Reactive Oxygen Species

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is a probe used for the highly sensitive and quantifiable detection of ROS. The non-fluorescent DCFH-DA diffuses into the cells and is cleaved by cytoplasmic esterases into 2',7'-dichlorodihydrofluorescein (H_2DCF) which is unable to diffuse back out of the cells. In the presence of hydrogen peroxide, H_2DCF is oxidized to the fluorescent molecule dichlorofluorescein (DCF) by peroxidases. The fluorescent signal emanating from DCF can be measured and providing an indication of intracellular ROS concentration was determined by the method of Rastogi *et al*, (2010).The levels of oxidative stress were determined quantitatively and qualitatively by the production of ROS by DCFH-DA. Briefly, an aliquot of the HEp-2 cells (8×10^6 cells/mL) were treated with IC_{50} concentration of sinapic acid for 24 and 48 hr, then made up to a final volume of 2 mL in normal phosphate buffered saline (pH 7.4). An 1 mL aliquot of cells were taken, to which 100 µL DCFH-DA (10 µM) was added and incubated at 37 °C for 30 min. we have observed the cells microscopically and the images were captured using a Nikon fluorescence microscope.

Mitochondrial membrane potential (ΔΨm)

Rhodamine 123 (Rh-123) is a lipophilic cationic dye, highly specific for mitochondria. Rh 123 uptake into mitochondria is driven by mitochondrial membrane potential that allows the determination of cell population with active integrated mitochondrial functions. Loss of ΔΨm leads to depolarization of mitochondrial membranes leading to collapse of mitochondrial functions ensurimg cell death. ΔΨm measurement was carried out essentially as described by Scaduto and Grotyohann, (1999). The cells $1x10^6$ cells/mL were cultured in 6-well plate and treated with IC_{50} concentration of sinapic acid for 24 and 48 hr and the untreated cells served as control. The cells were then stained with Rh-123 dye (10µM/mL) and the cells were incubated in CO_2 incubator for 30 minutes. The cells were washed by the addition of warm PBS and the mitochondrial depolarization patterns of the cells were observed in the fluorescence microscope using blue filter.

Apoptosis studies with AO/EB staining method

The ethidium bromide/acridine orange stain (EBr/AO stain) is a viability stain that detects apoptotic cells. Ethidium bromide is a dye that is only able to pass through the membrane of a dead or dying cell. Acridine orange is a membrane permeable dye that stains all the cells. Each dye that is taken up by a cell fluoresces AO makes a cell green, and EB makes a cell red (Baskic *et al*, 2002). Apoptotic studies were performed with a staining method utilizing AO and EBr according to the method of Lakshmi *et al*, (2008). The HEp-2 cells were grown in 6 well plates (5×10^3) for 24 and 48hr, and then treated with IC_{50} concentration of sinapic acid were incubated in CO_2 incubator for 24h, then the cells were trypsinized and stained with 1:1 ratio of AO/EBr. Stained cells were immediately washed again with PBS and viewed under a fluorescence microscope using blue filter with a magnification of 40x.

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by staining the DNA with Propidium iodide (PI) as described previously Chakraborty *et al*, (2012) with some modifications. The PI fluorescent nucleic acid dye is capable of binding and labeling double-stranded nucleic acids, making possible to obtain a rapid and precise evaluation of cellular DNA content by flow cytometric analysis, and subsequent identification of hypodiploid cells. In briefly, cells are seeded T^{25} flask at a density of 1×10^6 cells/flask. After, IC_{50} concentrations of sinapic acid were added to each flask incubated for 24 and 48hr, and then the cells were trypsinized, harvested and fixed in 70% ice cold ethanol in cell culture tubes and stored at 20°C until use. The cells

were centrifuged, the cell pellets were resuspended with PI (40 µM/mL in PBS) solution containing RNase (100 µM/mL). The stained cells were analyzed using fluorescence activated cell sorter (FACScan, Becton-Dickinson) with 488 nm argon ion laser using MAC Cell-Quest™ Software. The cell cycle distribution was analyzed using PI signals were collected using the 585/42 band pass filter. The data acquired were analyzed using quest software.

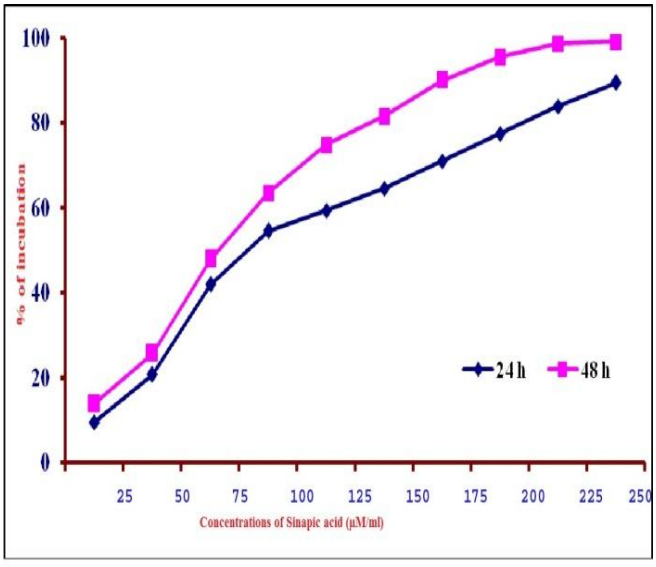
Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using statistical package of social science (SPSS) version 12.0 for windows. The values are mean ± S.D. for six samples in each group. *p* values <0.05 were considered as level of significance.

3.RESULTS

The cytotoxic effect of sinapic acid was examined on cultured HEp-2 cancer cells by exposing the cells to 25-250 µM concentrations for 24 and 48 hr. The graph was plotted as percentage of inhibition on (X-axis) against the concentration of sinapic acid (Y-axis). The IC_{50} value was determined by the concentration of the drug which reduces the absorbance to half that of the control and the cell viability was depicted in Figure. 2. Sinapic acid can effectively inhibit the viability of HEp-2 cell lines in a dose-dependent manner. The IC_{50} values of sinapic acid were 125.23 µM/ml for 24 hr and 117.81 µM/ml for 48 hr, obviously; sinapic acid at a concentration of 125.23 µM/ml for 24hr shows higher cytotoxic activity. These results suggest that sinapic acid can effectively inhibit the proliferation of the HEp-2 cells.

Figure 2. MTT Assay on sinapic acid treated HEp-2 cells HEp-2 cells were exposed to various concentrations of sinapic acid (25-250 µM). The cytotoxicity activity was measured by MTT assay and IC_{50} values were calculated (125.23 µM/ml for 24 hr and 117.81 µM/ml for 48 hr).



Furthermore, we study the effect of sinapic acid on ROS production in HEp-2 by using DCFH-DA in flurometrically. Figure 3 shows the fluorescence intensity of DCFH-DA in HEp-2 cells after exposure to sinapic acid at IC_{50} concentrations for 24 and 48 hr, and the bar diagram of the fluorescence intensity of ROS production in control and sinapic acid treated HEp-2 cells shown in figure 3. The intracellular ROS levels were increased in sinapic acid treated cells as compared to the untreated cells. The ROS level were observed after treatment with sinapic acid 108.32 ± 7.32 for 24 hr and 76.41 ± 7.09 for 48 hr. There were decreased ROS levels significantly in untreated cells as dose dependent manner.

Figure 3. The effect of sinapic acid on HEp-2 cells stained with DCFH-DA for 24 and 48 hr. Generation of ROS levels during sinapic acid treatment was measured spectrofluorimetrically by DCFH-DA staining for 24 and 48 hr. Values are given as mean ± SD differ significantly at P < 0.05 vs control (DMRT).

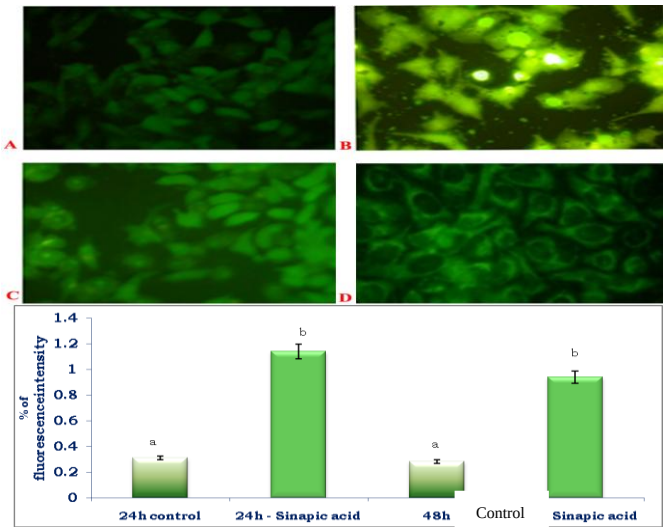
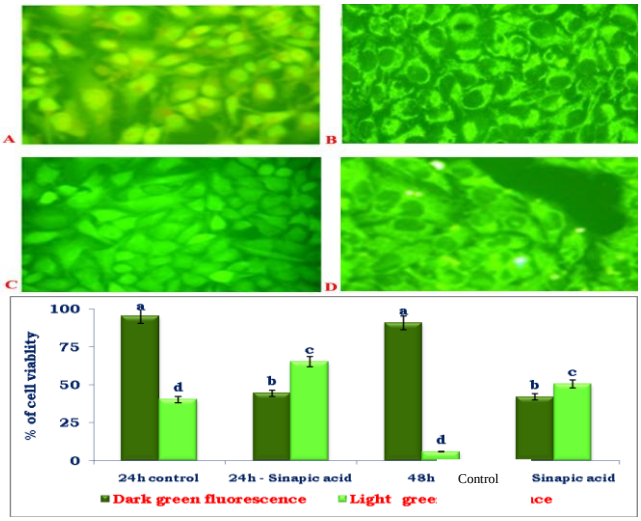


Figure 4 Shows the accumulation of rhodamine 123 within the active mitochondria of HEp-2 cells after exposure to sinapic acid at IC_{50} concentration for 24 and 48hr incubation. The results shows significantly, increase in the progressive loss of red Rh 123 aggregates fluorescence by increasing the incubation time and the concentration of sinapic acid determines the significant increase in the cytoplasm diffusion of green monomer fluorescence. In the IC_{50} concentration; partial or 50% loss of red fluorescence were observed, due to loss of mitochondrial trans-membrane potential in the cancer cells. It has been also observed that there was significantly increased fluorescence intensity (123.76 ± 7.81 for 24 hr; 134.78± 9.76) were seen in untreated HEp-2 cells.

Figure 4. The effect of sinapic acid on HEp-2 cells stained with rho 123 for 24 and 48 hr. The reduction of mitochondrial membrane potential in sinapic acid treated HEp-2 cells were measured spectrofluorimetrically by rhodamine 123 staining for 24 and 48 hr. Values are given as mean ± SD differ significantly at P <0.05 vs. control (DMRT).



AO/EB staining was done on HEp-2 cells treated with IC_{50} concentration of sinapic acid for 24 and 48 hr were shown in figure 5. The changes determined by the colour variation of cells. Figure. 5-A & C shows that the living cells of HEp-2 cells were stained bright green in spots. The stained HEp-2 cells with IC_{50} concentration of sinapic acid for 24 and 48 hr showed gradual increase in the apoptotic death (orange), whereas necrotic (red) cells were shown in Figure 5-B & D and percentage of apoptosis feature (apoptotic cells and necrotoic cells) also was shown in figures 5. Sinapic acid treated tumor cells characterized by the membrane blebbling, chromatin condensation innumerable micronuclei in cells, thus showing the apoptotic features.

Figure 5. The effect of sinapic acid on HEp-2 cells stained with AO/EBr for 24 and 48 hr. The apoptotic feature of sinapic acid treated HEp-2 cells was measured by dual staining with AO/Ebr staining. The values are expressed as mean ± SD from the six independent experiments p<0.05.

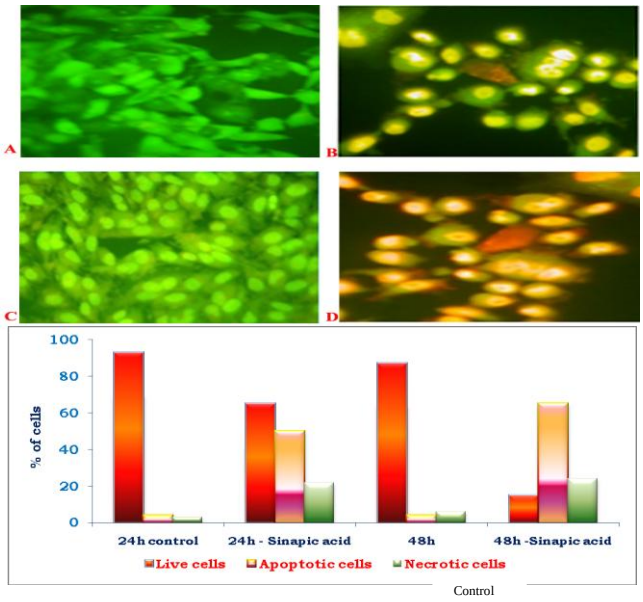


Figure 6. The effect of sinapic acid on cell cycle checkpoint potential on HEp-2 cells in 24 and 48 hr as revealed in the FACS analysis. The experiments were performed three times, sinapic acid treated cells showed a cell cycle arrest in G_0/G_1 phase at 24 and 48 hr for HEp-2 cells. Values are given as mean ± SD differ significantly at P < 0.05 (DMRT).

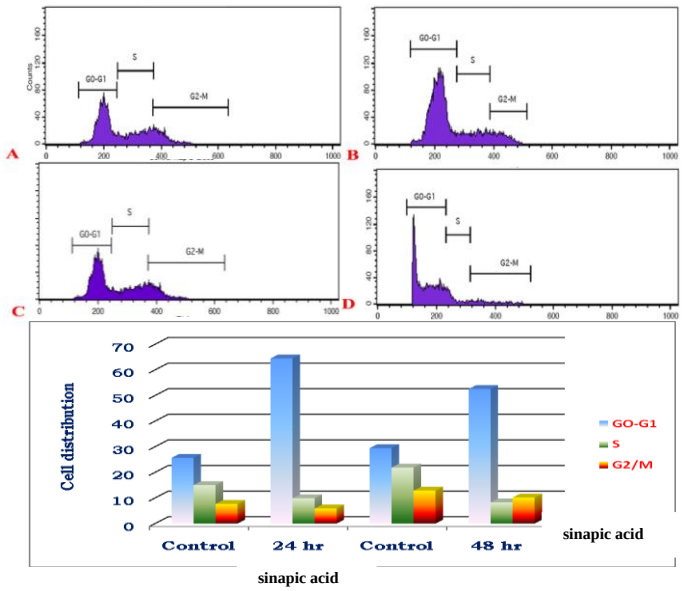


Figure 6 Shows the effect of sinapic acid on cell cycle processes by flow cytometry with propidium iodide staining.

In the treatment of HEp-2 cells with IC_{50} concentration of sinapic acid for 24 and 48 hr, the G_o/ G_1 phase were significantly decreased when compared to control or untreated cells. This result indicates that in the treatment of sinapic acid the cells undergo DNA fragmentation, so that their DNA content below that of the S and G_2/M phase. Sinapic acid treated with HEp-2 cells for 24 and 48 hr shows cell cycle arrest at the G_0/G_1 phase. The quantitative analysis of the histograms to determine the percentage of cells in the G_0/G_1, S, and G_2/M phases for the control and sinapic acid treated cells for 24 and 48hr are shown in figure 6

4.DISCUSSION

The results of the study suggest that sinapic acid induces apoptosis in cancer cells by analysis of apoptotic factors. Sinapic acid and other phenylpropanoids are present in vegetables and grains, e.g., *Brassica juncea* L., hazelnut, pea, cabbage, wheat, rye or brown rice (Kern *et al*, 2005). Profound studies reported that sinapic acids were effectively inhibited the growth of human oral, breast and colon carcinoma cell lines (Hudson *et al*, 2000). The apoptosis inducing effect of sinapic acid was confirmed by ROS, MMP and double AO/EtBr staining assays and cell cycle analysis. Our results revealed that sinapic acid is more potent to inhibit the cell proliferation and induce apoptotic cell death in HEp-2 cell line.

In the present study, we examined the cytotoxic potential of sinapic acid on HEp-2 cell lines using the MTT assay. The results of the MTT assay conclude that, the sinapic acid caused cytotoxicity in a dose and time dependent manner, the IC_{50} values obtained after 24 hr treatment of different concentrations of sinapic acid (125.23µM/ml for 24 hr).

ROS plays a very important role in apoptosis induction under both physiological and pathological conditions. Accumulation of ROS coupled with an increase in oxidative stress has been implicated in the pathogenesis of several diseases including cancers (Thannickal and Fanburg, 2000). Increasing evidence suggests that phytochemicals induces apoptosis associated with oxidative stress derived from ROS and mitochondrial dysfunction. ROS is critical for the metabolic and signal transduction pathways associated with cell growth and apoptosis (Chen *et al*, 2007). However, excessive production of ROS leads to oxidative stress and cellular damage. Several anticancer agents, including anthracyclines, cisplatin, bleomycin, and irradiation currently used for cancer treatment, it have been shown to cause increased intracellular ROS generation. Moreover, some studies have reported that anticancer agents from natural sources such as emodin and quercetin induce apoptosis in human cancer cells through ROS dependent mechanism (Hockenbery *et al*, 2002). The results of this study showed that the intracellular ROS levels were significantly increased in HEp-2 cells treated with IC_{50} concentration of sinapic acid at time and dose dependent manner.

Mitochondria have been shown to play a central role in the apoptotic process, because both the intrinsic pathway and the extrinsic pathway can converge at the mitochondrial level and trigger mitochondrial membrane permeabilization

(Kuo *et al*, 2010). The changes induced in the ΔΨm have been previously reported to represent a determinant in the execution of cell death. Most of the conventional anticancer treatments are thought to induce cell death through indirect activation of the mitochondria dependent pathway of apoptosis, a pathway often found altered in drug-resistant cancer cells (Ma *et al*, 2012). Chen *et al*, (2009) demonstrated that galic acid shows ROS-mediated anti-cancer activity through the reduction of ΔΨm human prostate cancer cells. However, there is also accumulating evidence that ΔΨm loss is a late event in the apoptotic process. In this study, we demonstrated that sinapic acid on the intracellular ROS by increasing its level in the cell; it is possible that considerable increase in intracellular ROS level enhances the cell death by the treatment of sinapic acid. This result is in agreement with a recent study of Ma *et al.*, (2012) reported that, an active natural anthraquinone derivative of emodin induces apoptotic cell death through the associated with loss of ΔΨm.

More than 50% of neoplasms undergo aberrations in the apoptotic machinery which leads to abnormal cell proliferation (Reed, 2002). Accumulated evidences indicated that the most of chemotherapeutic agents halt tumor cells proliferation via induction of apoptosis (Pommier *et al*, 2004). Most of current anti-cancer drugs such as camptothecin, vincristine, taxol, etoposide and paclitaxel are plant-derived compounds. These bioactive phytochemicals are known to exert their anti-cancer activity through different mechanisms, including altered carcinogen metabolism, induction of DNA repair systems, immune activation, suppression of cell cycle progression and induction of apoptosis (Cragg and Newman, 2005). In the present study, we examined sinapic acid can induces apoptosis in the HEp-2 cell lines in a dose dependent manner confirmed by typical morphological changes as the apoptotic cells e.g. membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, apoptotic bodies and loss of adhesion were observed in the sinapic acid treated HEp-2 cell lines. This observation is in agreement with recently published studies on Dumitria *et al*, (2010) documented that mistletoe extract, rich in phenolic acids presents anti-carcinogenic potential on A2780 tumor ovarian cells. Previously, Kampa *et al*, (2004) suggest that phenolic acids exert a apoptotic activity. Furthermore, the direct interaction with the AhR, the interaction with the NOS system and the pro-apoptotic effect of phenolic acids provide insights about their mode of action in T47D human breast cancer cells. According to above cited results; the finding of the results sorted that sinapic acid used in the treatment of cancer have strong apoptotic activities in HEp-2 cell lines.

The induction of apoptosis and inhibition of cell proliferation are revealed to the activity of signal transduction molecules involved in the cell cycle. The premitosis G_2/M phase and pre-replication G_1/S phase which are specifically regulated by specific genes are the two check points for DNA damage (Bartek and Lukas, 2001). It is also reported that phenolic acids found to arrest (specific genes) cell cycle progression either at G_1/S phase or G_2/M phase boundaries (Chen and Huang, 1998). In our study the cell cycle arrest was analyzed by the flow cytometry techniques. The results of flow cytometry showed that the sinapic acid induce an early G_0/G_1 phase arrest in IC_{50} concentration of sinapic acid at 24 and 48 hrs. A reduced number of S-phase and an increase in the G_1 peak were the characteristic of the early stage arrest of G_1/S phase cell cycle arrest.

These results showed that the sinapic acid may be a potent cytotoxicity effects on HEp-2 cancer cells, by inhibiting the growth of the cancer cells, through the apoptotic cell death and cell cycle arrest. Thus, the present study suggests that sinapic acid may be a promising anticancer therapeutic agent for oral cancer cell lines.

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