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ORIGINAL RESEARCH ARTICLE

Anticancer Effects of Sinapic Acid on Human Colon Cancer Cell Lines HT-29 and SW480

Chandrasekaran Balaji, Jayachandran Muthukumaran, Rajamanickam Vinothkumar and Namasivayam Nalini*

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar-608 002, Tamil Nadu, India

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ABSTRACT

Sinapic acid is a naturally occurring phenolic acid found in fruits, vegetables, cereals, legumes and oilseeds. The present study was designed to evaluate the anti cancer effects of sinapic acid on human adenocarcinoma colon cancer cell lines HT-29and SW480. Treatment of colon cancer cells with various concentrations of sinapic acid (20 - 200 µg/ml) was evaluated to determine the IC_{50} dose at different time points 24 h, 48 h and 72 h. Lipid peroxidation marker (TBARS), antioxidant status (SOD, CAT, GPx and GSH), apoptotic morphological changes (AO/EtBr staining), mitochondrial membrane potential (JC1) and the levels of intracellular ROS (DCFH-DA) were evaluated in HT-29 and SW480 colon cancer cell lines. Our results revealed that sinapic acid suppressed the proliferation of HT-29 and SW480 colon cancer cells in a dose dependant manner. In addition, sinapic acid treatment enhanced the levels of intracellular ROS, lipid peroxidation and decreased the levels/activities of the enzymatic and non enzymatic antioxidants as compared to the sham control. Furthermore, sinapic acid treatment induced apoptosis and altered mitochondrial membrane potential in colon cancer cell lines. Overall, our results suggest that sinapic acid exhibits anticancer effects in HT-29 and SW480 colon cancer cell lines.

Key words: Apoptosis, Sinapic acid, Oxidative stress and Colon cancer.

1. INTRODUCTION

Colorectal cancer (CRC) is diagnosed in approximately 146,940 patients per year and is the second leading cause of cancer-related mortality in the developed world [1]. It is associated with changes in oxidant-antioxidant status, dysregulation of apoptosis, increased cell proliferation and angiogenesis. Age remains a fundamental risk factor but modifiable factors like obesity, physical inactivity, red meat intake, alcohol consumption and long-term smoking plays a major role in colon cancer incidence. Estimates made by the American Cancer Society for the year 2014 reveal 96,830 new cases of colon cancer, 40,000 new cases of rectal cancer and 50, 310 deaths from CRC. Incidence of CRC was low in India [2] but the incidence has increased in both the sexes due to migration of the rural population to cities and due to changes in the life style.

The colon adenocarcinoma cell lines HT-29 and SW480 are widely used as convenient *in vitro* models to investigate the potency of drugs in cancer prevention and treatment. Cancer cells are well known to aberrantly regulate the apoptotic pathways according to their need for survival and become resistant against most of the common chemotherapeutic drugs. Uncontrolled growth, invasion into surrounding tissues, and metastatic spread to distant sites are important characteristic features of tumour cells [3]. The search for anticancer agents from natural sources has been successful worldwide and recently isolated active constituents from these sources are used in pharmaceuticals for drug research to treat cancers. Numerous studies indicate that diet containing abundant vegetables, fruits and grains can reduce the risk of several cancers especially colon cancer. Some phenolic acids have been clearly shown to inhibit tumour cell growth both in vitro and *in vivo*.

***Corresponding Author:** Namasivayam Nalini, **Email:** nalininam@yahoo.com

Sinapic acid, a naturally occurring carboxylic acid, is a member of the phenylpropanoid family. Synapic acid is 4-hydroxy-3, 5-dimethoxy cinnamic acid (Figure 1). It is widely distributed in fruits, vegetables, edible plants such as cereals, nuts, oil seeds and berries [4]. Sinapic acid has several biological effects including anti-inflammatory [5], antibacterial [6] and antidiabetic [7] activities. It has been shown to possess anticancer effects in different cancer cell lines such as anti-proliferative, antiapoptotic properties and is also able to arrest the cell cycle [8]. Our present study was aimed to investigate the anticancer effects of sinapic acid on human colon cancer cell lines HT-29 and SW480.

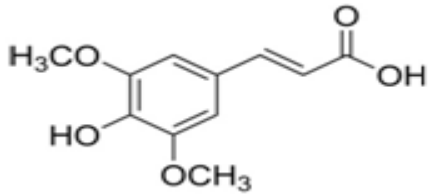

Figure 1: Structure of sinapic acid

2. MATERIALS AND METHODS

Chemicals

Sinapic acid, ethidium bromide (EtBr), JC1, fetal bovine serum (FBS) and Roswell Park Memorial Institute 1640 medium (RPMI-1640) were purchased from Sigma Chemicals Co., St.Louis, MO, USA. Trypsin EDTA, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl-2 tetrazolium bromide (MTT) were purchased from Hi-media Lab Ltd, Mumbai, India. All other chemicals and solvents utilized were of analytical grade quality and purchased from standard commercial suppliers.

Cell line and maintenance

HT-29 and SW480 cells were obtained from the National Centre for Cell Science (NCCS) Pune, India and grown as a monolayer in RPMI (Rosewell Park Memorial Institute) 1640 medium with 10% FBS (fetal bovine serum and 2% antibiotics). Stock cultures were sub-cultured every 7th day after harvesting the cells with trypsin EDTA (ethylene diamine tetra acetate) and then seeding them in tissue culture flask to maintain in an exponential phase.

Study design I (IC_{50} determination)

Cultured HT-29 and SW480 colon cancer cells were treated with different concentrations (20-200 µg) of sinapic acid for 24 h, 48 h and 72 h respectively.

Study design II (Time dependent treatment)

Cultured HT-29 and SW480 cancer cells were treated with 24 h IC_{50} dose of sinapic acid at two time intervals 24 h and 48 h respectively. Cultured HT-29 and SW480 cancer cells were divided into three groups (I-III); in each group six individual samples were processed (n = 6).

Group I : Sham control

Group II : Cells treated with sinapic acid for 24h

Group III: Cells treated with sinapic acid for 48h

Cytotoxicity assay

The cell viability was assessed by 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay Mosmann [9], which determines the metabolically active mitochondria of cells. Cells were plated in their growth medium at a density of 5000 cells/well in 96 flat bottomed well plates. After 24 h plating, sinapic acid was added at the concentrations ranging from 20 to 200 µg for 24, 48 and 72 h to study the dose as well as duration dependent cytotoxic effect. After 24, 48 and 72 h incubation, the medium was replaced with 20 µl of 5 mg/ml MTT in phosphate-buffered saline (PBS). The plates were wrapped with aluminum foil and incubated for 3 h in the dark at 37ºC. Then the purple formazan product was dissolved by using 100 µl of DMSO and the optical density was measured at 570 nm and 630 nm using a 96 well microtiter plate reader (BioRad). Data were collected for six replicates. The percentage inhibition was calculated, using the formula:

[(Mean absorbance of control cells)-(Mean absorbance of treated cells)/Mean absorbance of control cells X 100]

Unstained live morphology assay

The HT-29 and SW480 cells were grown in glass cover slip (22x22 mm) placed in six well plates at a density of $5x10^5$cells/well and allowed to settle for 24h before treatment with the IC_{50} values of sinapic acid. The medium was subsequently removed from each well of the treated and untreated HT-29 and SW480 cells, and the cover slip inverted and placed over a slide. The gross morphological changes in the treated and untreated sham control cells were observed using a differential interference phase contrast light microscope (Axio Scope A1, Carl Zesis, Germany) and photographed.

Dual staining

Apoptotic morphology was investigated by double staining with acridine orange (AO) and ethidium bromide (EtBr) as described by Spector *et al*. [10] 25 µl of cell suspension of each group (both attached, released to floating by trypsinization),

containing 5x10^{5}cells, were treated with AO/EtBr solution (1 part of 100 µg/ml AO and 1 part of 100 µg/ml EtBr in PBS) and examined using a fluorescent microscope with an UV filter (450-490 nm) and photographed.

Mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential of the sinapic acid treated and untreated colon cancer cells were measured by the fluorescent probe JC1 (5,5′,6,6″ - tetrachloro-1,1″,3,3″ tetraethyl benzimidazolyl carbocyanine iodide). The cells were grown in glass cover slip (22x22 mm), placed in six well plates and treated with IC_{50} values of sinapic acid. The cells were stained with JC1 after 24 h and 48 h exposure. The mitochondrial depolarization patterns of the cells were observed using a fluorescent microscope fitted with 485-545nm filters and photographed.

Measurement of intracellular ROS

The development of intracellular ROS was measured by 2,7-diacetyl dichlorofluorescein (DCFH-DA),which first gets hydrolysed by the cellular esterases to DCFH, where it is oxidized by ROS to yield fluorescent dichlorofluorescein (DCF). HT-29 and SW480 cells were treated with sinapic acid and incubation was continued for 24 h and 48 h. After the treatment, cells were harvested and washed with PBS, incubated with 1 ml of 10 mM DCFH-DA in PBS for 30 min in dark. The fluorescence intensity was measured at 480 nm excitation and 530 nm emission (Shimadzu RF-5301, Spectrofluorimeter) and values were calculated as follows and expressed as percent of fluorescence.

$$F = (Ft_{30} - Ft_0)/ Ft_0] \times 100$$

Where; Ft_0 is fluorescence at t=0 min and Ft_{30} fluorescence at t = 30 min

Measurement of lipid peroxidation byproducts and antioxidants

For the measurement of lipid peroxidation byproducts and antioxidants, HT-29 and SW480 cells were seeded in T_{75} flasks at a density of 1 x 10^6 cells/flask and treated with 24h IC_{50} values of sinapic acid for 24h and 48h. The cells were subsequently harvested by trypsinization, washed with PBS, suspended in 130 mM KCl, 50 mM PBS and 10 µM dithiothreitol and centrifuged at 20,000g for 15 min at 4°C. The supernatant was collected and used for biochemical estimations. The concentration of thiobarbituric acid reactive substances (TBARS), the byproducts of lipid peroxidation was measured by the method Niehaus and Samuelson [11]. The activities/levels of enzymatic and non enzymatic antioxidants such as superoxide dismutase (SOD, EC 1.15.1.1) was assayed by Kakkar *et al.* [12] catalase (CAT, EC 1.11.1.6) by the method of Sinha [13], glutathione peroxidase (GPx, EC.1.11.1.9) by the method Rotruck *et al.* [14] and reduced glutathione (GSH) by the method of Ellman [15].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and a significant difference among treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant at $p<0.05$. All statistical analyses were made using SPSS 17.0 software package (SPSS, Tokyo, Japan).

3. RESULTS

MTT assay

The MTT assay was used to make a preliminary assessment of the growth inhibitory potential of sinapic acid on HT-29 and SW480 colon cancer cells to find the respective IC_{50} values with varying concentrations of sinapic acid (20-200µg/ml) at different time points 24h, 48h and 72 h. Treatment with sinapic acid exhibited significant ($p<0.05$) growth inhibition in a dose and time dependent manner and the IC_{50} values of sinapic acid at each time point on HT-29 and SW480 colon cancer cells are represented in (**Figure 2**). The results clearly reveal that when the cells were treated with sinapic acid for a prolonged period (72 h), the IC_{50} values were bear minimum [HT-29 (85.25 µg) and SW480 (79.11 µg)]. On the other hand when the cells were treated with sinapic acid for a short time (24 h and 48h) the IC_{50} values were higher [24 h HT-29 (138.11 µg) and SW480 (112.55 µg): 48 h HT-29 (111.91 µg) and SW480 (98µg)].

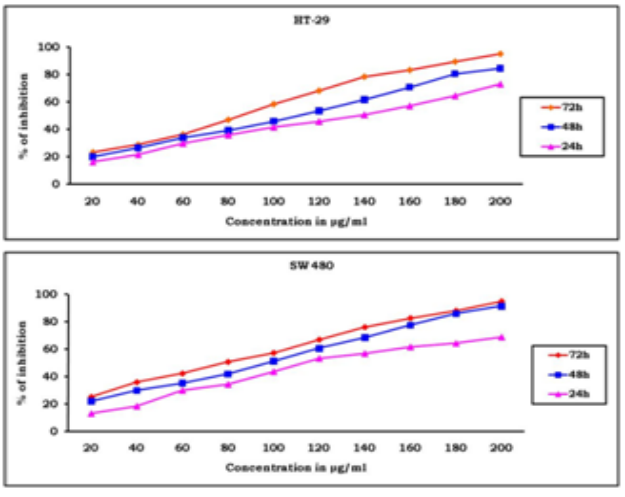

Figure 2: Effect of sinapic acid on the growth of HT-29, SW480 colon cancer cells, determined by MTT assay.

The cells were treated with different concentrations of sinapic acid (20-200 µg/ml), which affected cell viability in a time dependent manner (24 h, 48 h and 72 h respectively). Data are expressed as mean ± SD of six independent experiments.

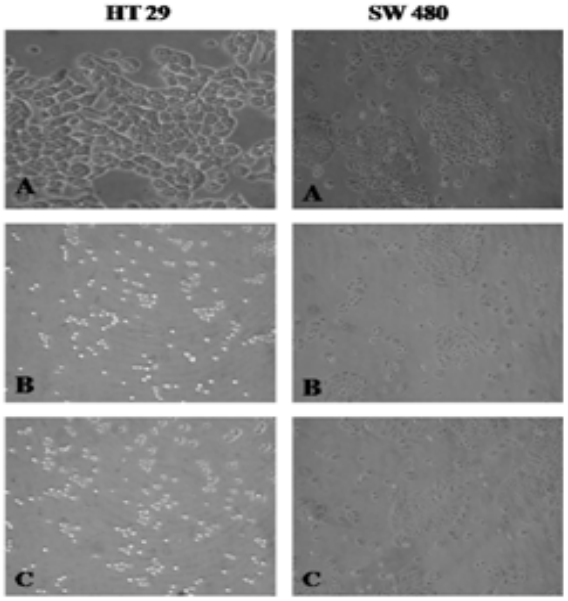

Figure 3: Effect of sinapic acid on the morphological changes in HT 29 and SW 480 colon cancer cells.

The images captured by light microscope showing intact morphology of HT-29 and SW480 control cells (A). After 24 h and 48 h sinapic acid treated cells showed swelling, irregularities in the plasma membrane and formation of blebs and vacuoles (B and C).

Apoptotic morphological changes

Morphological changes of cancer cells including viability, early and late features of apoptosis were performed using double-staining method with acridine orange/ethidium bromide (AO/EtBr). Fluorescent microscopic observations of AO/EtBr stained HT-29 and SW480 colon cancer cells, following treatment with IC_{50} concentrations of sinapic acid for 24h and 48h are shown in (**Figure 4**). Nuclei of viable cells were stained uniformly green by AO, while those of apoptotic cells exhibited yellow to orange colour, depending on the degree of loss of membrane integrity, due to co-staining with EtBr. In this study, yellowish green staining represented early apoptotic cells with condensed or fragmented chromatin, while reddish orange staining represented late apoptotic cells with condensed or fragmented chromatin and necrotic cells had a structurally normal orange nucleus.

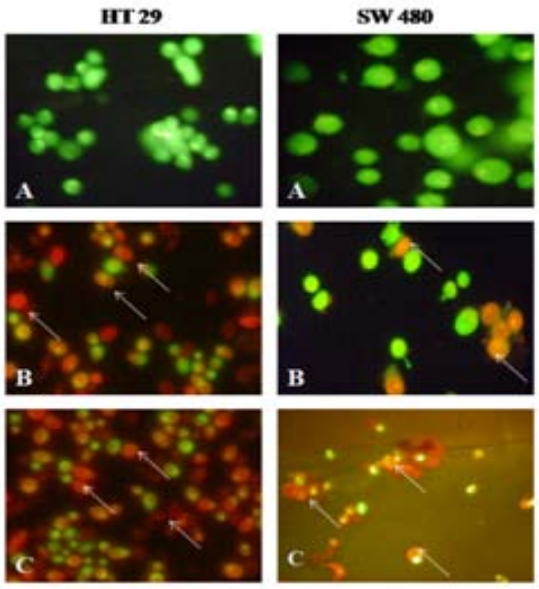

Figure 4: Effect of sinapic acid on the morphological changes in HT 29 and SW 480 colon cancer cells by AO/EtBr staining to distinguish between necrosis and apoptosis.

Uniformly stained green coloured nuclei indicate live cells (A). After 24 h and 48 h sinapic acid treatment, cells showed condensed or fragmented nucleus with green or orange-red colour indicating apoptosis (B and C) represented as white arrows heads.

Mitochondrial membrane potential (ΔΨm)

The loss of mitochondrial membrane potential is a hallmark for apoptosis. JC-1 is used mostly for detecting mitochondrial depolarization occurring in the early stages of apoptosis. It exhibits potential dependent accumulation in the mitochondria, indicated by a fluorescence emission shift from green to red. When the cells are stained with JC-1, the loss of ΔΨm is indicated by the decrease in red fluorescence and the increase in green fluorescence. In non apoptotic cells, JC-1 exists as dimer and accumulates as aggregates in the mitochondria which stains red. On the other hand, in apoptotic and necrotic cells, JC-1 exists in the monomeric form in the cytosol and stains green. In this study, we observed that cells treated with IC_{50} concentrations of sinapic acid at 24h and 48 h showed strong green fluorescence indicating the loss of mitochondrial potential, hence induction of apoptosis as compared to the sham control, whereas sinapic acid untreated cells showed red fluorescence as shown in (**Figure 5**).

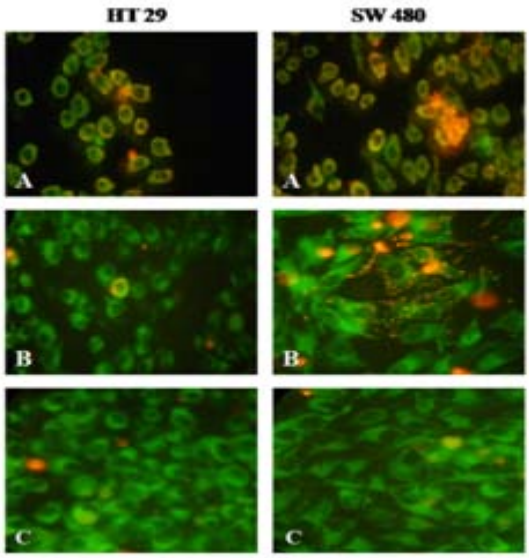

Figure 5: Effect of sinapic acid on the mitochondrial membrane potential in HT-29 and SW 480 colon cancer cells by JC1 staining. Sinapic acid untreated cells (A).

After 24 h and 48 h sinapic acid treated cells showed the green fluorescence indicating a decrease in mitochondrial membrane potential, an early event in apoptosis (B and C).

Effect of sinapic acid on intracellular ROS

HT-29 and SW480 cells on treatment with sinapic acid (figure 6) showed significant ($p< 0.05$) increase in the intracellular ROS production in a time dependent manner (24 h and 48 h) as compared to the sham control cells, indicating ROS mediated apoptosis in these cells.

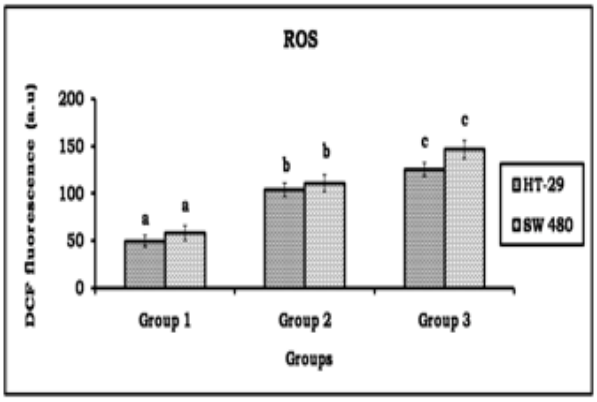

Figure 6: Effect of sinapic acid on ROS generation in HT-29 and SW480 cells.

Data are presented as the means ± SD of six independent experiments in each group. Values not sharing a common superscript letter are ($^{a-c}$) differ significantly at $p<0.05$ (DMRT).

Effect of sinapic acid on antioxidant and lipid peroxidation byproducts

(**Table 1**) shows the changes in the levels of lipidperoxidation byproducts and activities/levels of enzymatic and non enzymatic antioxidants like SOD, CAT, GPx and GSH. Treatment with sinapic acid showed a slight decrease in the activities of the enzymatic and non enzymatic antioxidants and a significant increase in the level of lipid peroxidation byproducts such as thiobarbituric acid reactive substances at 24h and 48h time points as compared to the sham control.

Table 1: Effect of sinapic acid on TBARS and antioxidant enzymes

	HT-29			SW480		
Groups	**1**	**2**	**3**	**1**	**2**	**3**
TBARS ♥	9.87±0.81^{a}	12.58±1.01^{b}	14.16±1.39^{c}	8.73±0.52^{a}	13.61±1.02^{b}	15.06±1.41^{c}
SOD ♠	15.74±1.37^{a}	11.67±0.78^{b}	10.42±0.63^{c}	9.76±0.79^{a}	8.34±0.64^{b}	7.82±0.39^{b}
CAT ♣	14.31±1.36^{a}	11.72±0.92^{b}	10.53±0.56^{b}	10.87±0.80^{a}	9.21±0.63^{b}	8.71±0.42^{b}
GPx ♦	12.46±1.17^{a}	10.58±0.76^{b}	9.32±0.65^{b}	10.32±0.89^{a}	7.62±0.58^{b}	6.70±0.46^{b}
GSH$^{£}$	21.63±1.90^{a}	17.58±1.61^{b}	15.29±1.41^{c}	18.41±1.41^{a}	15.93±1.42^{b}	14.32±1.04^{b}

♥nmoles/ml of cell lysate; ♠50% NBT reduction/min/mg protein; ♣µmoles of H_2O_2 utilized/min/mg protein; ♦µmoles of GSH utilized/min/mg protein; $^{£}$mmoles/mg protein Data are presented as the means ± SD of six independent experiments in each group. Values not sharing a common superscript letter ($^{a-c}$) differ significantly at $p<0.05$ (DMRT).

4. DISCUSSION

Cancer is a major disease burden worldwide, with colon cancer being the most common type in both men and women. Natural resources contribute to pharmaceutical and health services, since they can be used directly as pharmaceuticals or as templates for chemical synthesis of related medicinal compounds in drug development. Phenolic acids have been reported to exert chemopreventive and antitumour effects against several types of cancers including colon cancer [16]. Sinapic acid is a phenolic acid known to possess a number of biological properties. Therefore, the present study was designed to explore the in vitro anticancer activity of sinapic acid on human adenocarcinoma colon cancer cell lines HT-29 and SW480.

MTT assay is a common method of measuring viable cell number in proliferation and cytotoxicity studies. Treatment with different concentrations (20-200 µg/ml) of sinapic acid exhibited a significant effect on colon cancer cells. Our data demonstrated that sinapic acid inhibited the viability of colon cancer cell lines in

a dose and time dependent manner with IC_{50} values of 138.11µg/ml (HT-29) and 112.55µg/ml (SW480) for 24h incubation. These effects of sinapic acid on cancer cell growth could be due to its inherent antiproliferative activity. In this context, previous report indicates that sinapic acid inhibits T47D human breast cancer cells in a time and dose dependent manner [17]. Reddy and Prasad [18] have also shown that phenolic compounds have health protective effects as well as are cytotoxic to cancer cells. The cytotoxic response of sinapic acid could also be attributed to its proapoptotic effects as evident by morphological analysis.

Apoptosis is a highly regulated programmed cell death process which provides an effective non-inflammatory way to remove redundant or damaged cells from tissues thereby attaining tissue homeostasis. It is an ordered and orchestrated cellular event by which cells undergo inducible non-necrotic cellular suicide and thus it plays a crucial role in preventing carcinogenesis. So, resistance to apoptosis is an important attribute of cancer cells, which subsequently helps in invasion [19]. Various phytochemicals induce apoptosis and change the cancer cell morphology due to their proapoptotic activity and hence they are used for cancer prevention and cancer chemotherapy. In the present study, sinapic acid at IC_{50} concentrations was able to induce apoptosis in the HT-29 and SW480 colon cancer cell lines in a time and dose dependent manner which was evident by AO/EtBr doublestaining. Moreover, sinapic acid when treated with colon cancer cells at two different time points (24h and 48h) results in distinct apoptotic morphological changes like cell shrinkage, chromatin fragmentation, binucleation, cytoplasmic vacuolation, nuclear swelling, cytoplasmic blebbing and formation of apoptotic bodies. This provides morphological proof to qualitatively show that sinapic acid could induce apoptosis and inhibit colon cancer cell growth.

Mitochondria is a key organelle for the cell survival and are a source of reactive oxygen species generation (ROS) during apoptosis. Reduced mitochondrial membrane potential can lead to increased generation of ROS and apoptosis [20]. Lipophilic cationic dye (JC-1) is a fluorescent compound that exists as a monomer at low concentrations. At higher concentrations, it forms aggregates. Fluorescence of the JC-1 monomer is green, whereas that of aggregates is red. Mitochondria with intact membrane potential concentrates JC-1 into aggregates and fluoresces red thereby demonstrating increased mitochondrial membrane potential, whereas de-energized mitochondria cannot concentrate JC-1 and are thus stained green. Hence, in JC-1 staining, the apoptotic cells are identified by an increase in green fluorescence and the loss of red fluorescence [21]. In our study, the anti cancer effect of sinapic acid is revealed by its ability to decrease the mitochondrial membrane potential, and thereby induce apoptosis in HT-29 and SW480 colon cancer cells. In this context, Galati *et al*. [22] have suggested that caffeic acid and other dietary phenolic compounds alter the mitochondrial membrane potential and induce mitochondrial collapse.

ROS has been implicated as a second messenger in multiple signalling pathways and can play a significant role in the process of apoptosis by regulating the activity of certain enzymes involved in the cell death pathway [23]. In cancer cells, ROS can initiate cell transformation by causing alterations during DNA replication [24]. Enhancement of ROS production has long been associated with the apoptotic response induced by anti-cancer agents [25]. Several natural compounds like phenolic phytochemicals used for cancer treatment has shown reduced mitochondrial membrane potential leading to the increased generation of intracellular ROS and apoptosis [26]. Similar results were observed in our study when HT-29 and SW480 colon cancer cells were treated with sinapic acid. Our findings along with other reports suggest that sinapic acid acts as an antiproliferative agent through over production of ROS, apoptotic induction and the loss of $\Delta\psi_m$. Recently, Kalaimathi *et al*. [8] reported that laryngeal carcinoma cells on treatment with sinapic acid demonstrated increased intracellular ROS levels and thereby loss of mitochondrial membrane potential.

Lipid peroxidation is a free radical mediated process. Increased intracellular ROS levels are known to disrupt the biological membranes and cause cytotoxicity [27]. Previous in vitro anticancer studies reveal that the increased ROS levels and lipid peroxidation by products in primary cancer cells are associated with a decrease in antioxidants, such as SOD and CAT [28]. It is also known that many types of human cancer cells can survive in a highly oxidative state due to the decreased antioxidant protective enzymes as compared to their normal tissue counterparts [29]. Rahman [30] has also shown that increased lipid

peroxidation in cancer cells could be due to increased ROS stress as a result of depletion of antioxidant scavenger systems. But natural phytochemicals are known to deplete intracellular GSH and increase intracellular ROS to a level that can cause cell death [31]. Similarly, in our present study treatment with sinapic acid significantly increased the lipid peroxidation byproduct TBARS and decreased the enzymatic and non enzymatic antioxidants in the colon cancer cells. In this context previous reports demonstrate that plant phenolic compounds are basically antioxidants, but they also exhibit prooxidant properties in cancer cells, mainly due to the acidic environment that prevails in cancer cells and the presence of high levels of peroxidases which act on phenolics to produce phenoxyl radicals [32]. Similarly, sinapic acid a phenolic acid, could act as a prooxidant and alter the activities/levels of oxidative stress markers in colon cancer cell lines.

Thus, sinapic acid initiates cancer cell death by inhibiting cell proliferation, lowing antioxidant status, alterating mitochondrial membrane potential, increasing intracellular ROS, lipid peroxidation and inducing apoptosis in HT-29 and SW480 colon cancer cell lines. These findings suggest that sinapic acid possesses potent anticancer activity against human colon cancer. Further research is needed to determine the clinical efficacy and precise molecular mechanism by which sinapic acid inhibits cancer cell growth.

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