Influence of Sinapic Acid on Induction of Apoptosis in Human Laryngeal Carcinoma Cell Line

Kalaimathi Janakiraman, *Suresh Kathiresan and Arokia Vijayaanand Mariadoss

1Department of Biochemistry, Bharathiar University, Coimbatore-641 046,
2Department of Biochemistry & Biotechnology, Faculty of Science, Annamalai University, Annamalai Nagar, Tamilnadu, India- 608 002.

Article History: Received 5th Feb, 2014, Accepted 21st April, 2014, Published 22nd May, 2014

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 IC50 concentration of sinapic acid resulted in sequences of events marked by the enhancement the apoptosis accompanied by loss of cell viability, modulation of reactive oxygen species and cell cycle arrest through the induction of G0/G1 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).

Phenolic compounds, which are widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of 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 25cm² 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 37°C under a humidified 5% CO₂ 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-thiazoyl)-2,5-diphenyl-2H tetrazolium bromide) assay. IC₅₀ 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 into 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 (5×10⁴ 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 37°C 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 (H₂DCF) which is unable to diffuse back out of the cells. In the presence of hydrogen peroxide, H₂DCF 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 x 10⁶ cells/mL) were treated with IC₅₀ 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 ensuring cell death. ΔΨm measurement was carried out essentially as described by Scaduto and Grotzohn, (1999). The cells 1x10⁶ cells/mL were cultured in 6-well plate and treated with IC₅₀ 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 CO₂ 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 (5x10⁴) for 24 and 48hr, and then treated with IC₅₀ concentration of sinapic acid were incubated in CO₂ incubator for 24hr, 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 T²5 flask at a density of 1x10⁶ cells/flask. After, IC₅₀ 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 observed microscopically and the images were captured using a Nikon fluorescence microscope.
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 CellQuest™ 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 IC₅₀ 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 IC₅₀ 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 24 hr 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 necrotic 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/EB for 24 and 48 hr. The apoptotic feature of sinapic acid treated HEp-2 cells was measured by dual staining with AO/EtBr staining. The values are expressed as mean ± SD from the six independent experiments p<0.05.

In the treatment of HEp-2 cells with IC_{50} concentration of sinapic acid for 24 and 48 hr, the G_{0}/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 anthracylines, 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 gallic 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 Dumitriia 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 G2/M phase and pre-replication G1/S phase which are specifically regulated by specific genes are the two checkpoint for DNA damage (Bartek and Lukas, 2001). It is also reported that phenolic acids found to arrest (specific genes) cell cycle progression either at G1/S phase or G2/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 G0/G1 phase arrest in IC50 concentration of sinapic acid at 24 and 48 hrs. A reduced number of S-phase and an increase in the G1 peak were the characteristic of the early stage arrest of G1/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.

5. REFERENCES


****
Introduction

Oral cancer is a serious and developing issue in various parts of the world, is the sixth most common cancer on the planet, and 3 – 4% of all diseases in western nations (Ferlay et al., 2004). Various etiologic factors have been implicated in the improvement of oral SCCs, for example, tobacco smoking, biting and alcohol abuse (Johnson et al., 2001).

7,12-dimethylbenz(a)anthracene (DMBA), a class of aromatic polycyclic hydrocarbons, which are repercussions of the ignition of tobacco and other natural substances (Lee et al., 2002). This agent may prompt generation of free radicals and oxidative stress through the creation of superoxide, hydrogen peroxide and nitric oxide in cells.
Reactive oxygen species (ROS) participate in a variety of chemical reactions with biomolecules leading to a pathological condition known as oxidative stress (Cooke et al., 2003). Oxidative stress is initiated by free radicals like hydroxyl, peroxyl and superoxide radicals, which become stable through electron pairing with biological macromolecules such as proteins, lipids and DNA (Burton and Jauniaux, 2011). Oxidative stresses have implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases. The roles of antioxidants are to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention (Ganie et al., 2011). Vitamins C and E satisfy various natural exercises, including safe incitement, immune stimulation, scavenging free radicals and modifying the metabolic initiation of cancer-causing agents (Van Poppel and Vanden Berg, 1997). Vitamin E is a powerful oxygen radical scavenger that shields cells from cancer-causing chemicals by inhibiting LPO and free radical mediated consequences.

Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense, different human diseases and aging process. Keeping in view of the demand for developing natural antioxidants drugs, the present study was aimed to investigate its antioxidant, free radical scavenging activity using various antioxidants assays system. However, to the best of our knowledge no scientific studies has been undertaken to evaluate chemopreventive effect of sinapic acid against oral cancer. Therefore, we determine the free radical scavenging activity, antioxidant and anticarcinogenic potential of sinapic acid during 7,12 dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis.

Materials and Methods

Chemicals

Sinapic acid, DMBA, reduced glutathione (GSH), 2, 2'-dipyridyl, 2, 4-dinitro phenylhydrazine (DNPH), 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB), 2, 2'-diphenyl-1-picrylhydrazyl radical (DPPH), 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from S.D. Fine chemicals Mumbai, India and were of analytical grade.

Animals

Male golden Syrian hamsters, eight to ten weeks old, weighing 80-120g were purchased from National Institute of Nutrition, Hyderabad. The animals were housed in polypropylene cages at room temperature (27±2°C) with relative humidity (55±5%) in an experimental room, the LD (light: dark) cycle is almost 12:12h. The animals were provided with standard pellet diet (M/s Kamdhenu Ltd., Bangaluru,) and water ad libitum.. The recommendations of Institutional Animal Ethical Committee (Committee for the purpose of control and supervision of experiment on animals (Regn. no 1416/a/11/CPCSEA, Muthayammal College of Arts and Science, Rasipuram, India) for the care and use of laboratory animals were strictly followed throughout the study.

Experimental design

A total number of 32 hamsters were divided into 4 groups of 8 animals each. Group I animals were served as untreated control.
Animals in groups II were induced oral carcinogenesis by painting with DMBA in liquid paraffin three times a week for 14 weeks. Group II received no other treatment. Groups III animals were painted with DMBA and treated with sinapic acid (50mg/kg). Group VI animals were orally administered with Sinapic acid alone throughout the experimental period. The experiment was terminated at the end of 16th week all animals were sacrificed and samples were collected.

Biochemical assays

The reduced glutathione level was determined by the method of Beutler and Kelly (1963). The level of vitamin C was determined by the method of Omaye et al. (1979). Vitamin E in plasma and erythrocyte was estimated by the method of Palan et al. (1991) as well as in buccal mucosa was estimated by the method of Desai, (1984). Thiobarbituric Acid Reactive Substances (TBARS) in erythrocytes were estimated for method of Donnan (1950) and TBARS in plasma were assayed by the method of Yagi (1987) and in tissue was estimated by Ohkawa et al., (1979). Superoxide dismutase activity in plasma, erythrocyte and buccal mucosa was assayed by the method of Kakkar et al. (1984). The activity of catalase was assayed by the method of Sinha (1972). Cytochrome P450 and b 5 in liver and buccal mucosa tissues microsomes were estimated by the method of Omura and Sato, (1964).

Statistical Analysis

The data were expressed as mean ± S.D with 8 animals in each group. Values were analysed using SPSS/15.0 software. Hypothesis testing methods were included with analysis of variance (ANOVA) followed by least significance difference (LSD). P values of > 0.05 were considered statistically significant.

Results and Discussion

Figure 1 Shows enzymatic antioxidants such as SOD, CAT and GPx levels were decreased in plasma and erythrocytes whereas buccal tissue GPx levels were increased in DMBA treated animals. Oral administration of Sinapic acid (50 mg/kg bw) to DMBA painted animals were significantly restored the levels of SOD, CAT and GPx when compared to control animals. Animals were treated with Sinapic acid showed no significant differences were observed compared to control animals.

Figure 2 Shows plasma and erythrocyte GSH, Vit-C and Vit-E levels were decreased and simultaneously increased in buccal tissue in DMBA painted animals. Oral administration of Sinapic acid (50 mg/kg bw) to DMBA painted animals were more effective and significantly brought back the levels of GSH, Vit-C and Vit-E when compared to control animals. Animals treated with Sinapic acid alone showed no significant differences were observed compared to control animals.

Figure 3 Shows the status of phase I (cytochrome p450) and phase II (GST and GR) detoxification enzymes in the buccal mucosa and liver of control and experimental hamsters in each group. The phase I enzymes activity were significantly increased in buccal and liver tissue whereas phase II enzymes were increased in buccal mucosa and simultaneously decreased in liver tissue in tumor bearing hamsters as compared to control hamsters. Oral administration of sinapic acid (50mg/kg) to hamsters treated with DMBA significantly recover the status of phase I and phase II detoxification enzymes. Animals treated
with sinapic acid alone showed no significant differences were observed compared to control animals.

In the present study, chemopreventive potential of Sinapic acid was assessed by checking status of lipid peroxidation, antioxidants and phase I detoxification proteins. Reactive oxygen species (ROS) generated by mitochondria or from other intracellular or extracellular destinations can cause cell damage and initiate diverse degradation forms (Davies, and Hochstein, 1982). ROS, for example, superoxide anion (O$_2^{-}$), singlet oxygen (1O$_2$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (•OH), were primarily formed when blended capacity oxidases framework by action of polycyclic aromatic hydrocarbons like 7,12-dimethyl benz(a)anthracene (Giri et al., 1995). These oxyradicals can attach covalently nucleophilic locales on cell macromolecules accordingly bringing out carcinogenic reactions. Subsequently, DMBA-affected hamster buccal pouch carcinogenesis has been broadly utilized as an animal model for advancement of chemopreventive medications for oral cancer. Present study, topical application of DMBA to the hamster buccal pouch for 16 weeks brought about decently created OSCC with exceptionally mean tumor trouble associated with extreme hyperplasia, hyperkeratosis, and dysplasia.

The relationship between lipid peroxidation and the rate of cell extension, seen in our study exhibits that the tumor cells multiple broadly when lipid peroxidation is irrelevant. The thiobarbituric acid responsive substances in plasma were essentially expanded while its level in buccal tissue was fundamentally lessened in DMBA treated hamsters. The tumor cells in buccal tissue demonstrated a particularly low level of peroxidation making an ideal air for the multiplication of disease cells. Data of the present study showed that lipid peroxidation incited by oxidative stress brought about DNA harm. Oral administration of Sinapic acid altogether adjusted the lipid peroxidation and improves the status of antioxidants. The more huge impact of Sinapic acid supplementation was seen at the dosage of 50 mg/kg body weight which was comparable to control group.

In DMBA treated animals, the level of plasma and erythrocytes SOD, CAT, GPx and GSH were diminished. At the same time expanded GPx and GSH levels in buccal mucosa show that oxidative anxiety is brought about by DMBA. SOD is the first cancer prevention protein to manage oxyradicals by quickening the dismutation of superoxide (O$_2^{-}$) to hydrogen peroxide (H$_2$O$_2$). Catalase is a catalyst that changes over H$_2$O$_2$ to nonpartisan items, O$_2$ and H$_2$O (Vennila et al., 2010). GPx is a initiated protein against oxidative damage and this, requires glutathione as a cofactor. It catalyzes the oxidation of GSH to GSSG to the detriment of H$_2$O (Soujanya et al., 2011). Subsequently, the ROS rummaging movement of CAT, GPx and GSH are viable when it is trailed by the neighboring of SOD, which requires an extra source to repress oxidative anxiety (Weydert et al., 2006). Oral administration of Sinapic acid (50mg/kg) was modestly initiated SOD, CAT, GPx and emphatically enacted GSH to DMBA treated hamsters. Sinapic acid alone group showed no huge contrasts when compared to the control group. Lessened activities of Vit-C and Vit-E in the blood and an increase in buccal tissue of DMBA treated hamsters that the exercises of these catalysts were impeded because of rehashed exacerbation by the carcinogen. A reclamation of Vit-C and Vit-E taking after the oral administration of Sinapic acid (50mg/kg) shows that Sinapic acid triggers the antioxidant impacts at endogenous level.
Figure 1 Changes the enzymatic antioxidants (SOD, CAT and GPx) activities in plasma, erythrocyte and buccal mucosa of control and experimental animals

Values are expressed as the mean ± SD for 6 hamsters in each group. Values that are not sharing a common superscript letters in the same column differ significantly at p<0.05 (DMRT). X - The amount of enzyme required to inhibit 50% NBT reduction. Y - Micromoles of H₂O₂. Z - Micromoles of glutathione utilized/min utilized/sec.
Figure 2 Changes the non-enzymatic antioxidants (GSH, Vit-C and Vit-E) activities in plasma, erythrocyte and buccal mucosa of control and experimental animals.

Values are expressed as the mean ± SD for 6 hamsters in each group. Values that are not sharing a common superscript letters in the same column differ significantly at p<0.05 (DMRT). X - The amount of enzyme required to inhibit 50% NBT reduction. Y - Micromoles of H₂O₂. Z - Micromoles of glutathione utilized/min utilized/sec.
**Figure 3** Changes in the activities of Phase I (Cytochrome p450) and Phase II enzymes (GST and GR) in buccal mucosa and liver of control and experimental animals.

Values are expressed as the mean ± SD for 6 hamsters in each group. Values that are not sharing a common superscript letters in the same column differ significantly at p<0.05 (DMRT). X - Micromoles of cytochrome P450, P - Micromoles of CDNB conjugated with GSH/ minute; Q - Micromoles of p-nitroaniline formed/ hr; R - Micromoles of NADPH oxidized/ hr.
The cytochrome P450 (oxidizing phase I metabolizing enzymes) are multigene group of constitutively expressed and inducible hemoproteins with a central part in the oxidative metabolic system (Kirton et al., 2005). DMBA requires metabolic actuation by to structure diol epoxide and different ROS that are known to increase intracellular oxidation can cause severe DNA damage, lipids and proteins (Gao et al., 2005). Oral administration of Sinapic acid on DMBA treated hamsters restored the status of phase I enzymes in the liver and buccal mucosa suggests that Sinapic acid may have assumed critical part in the detoxification of carcinogens, assisted either in the hindrance of the metabolic activation of DMBA.

The oxidized metabolites of cancer causing xenobiotics are detoxified by phase II metabolizing enzymes. Glutathione S-transferases (GSTs), glutathione reductase (GR) a family of phase II detoxification enzymes, play an important part in securing cellular macromolecules from genotoxic chemicals and cytotoxic agents (Reszka and Wasowicz, 2001; Coles et al., 2003). In this present study, the GST and GR levels of 7,12-dimethylbenz[a]anthracene (DMBA)-treated hamster were discovered to be up-regulated in buccal mucosa and down-regulated in the liver when compared to the control. These irregular GST and GR levels are in charge of xenobiotic biotransformation. Oral administration of Sinapic acid (50 mg/kg) to DMBA-treated hamsters resumed these enzymes level. Accordingly, Sinapic acid implantation was found to induce GST and GR activities that can efficaciously eliminate xenobiotics. In conclusion of present study, treatment at the dose of 50 mg/kg significantly recovers the antioxidant and phase I and Phase II detoxification enzymes activities to near normal levels. This indicates that Sinapic acid at a dose 50 mg/kg/bw fixed effective dose for further studies.

References


