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Research Article
Anticancer Potentials of Free and Nanocapsulated Sinapic Acid on Human Squamous Cell Carcinoma Cell Line: *In vitro* Study

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Abstract

Background and Objective: Sinapic acid is a cinnamic acid derivative that widely distributed in plant kingdom such as in various fruits, vegetables, cereal grains, oilseed crops, some spices and medicinal plants. Sinapic acid act as preventive and therapeutic agents in several oxidative stress related diseases, such as atherosclerosis, inflammatory injury and cancer. The current study aimed to examine cytotoxicity of free and Nano-sinapic acid on squamous cell carcinoma (HEp-2). **Material and Methods:** The present work conducted to evaluate the cytotoxicity using MTT assay, evaluation of morphological changes post cell treatment with free and Nanocapsulated Sinapic acid. Nanocapsulation was performed using Tween-80, also evaluation of cell apoptosis and related pro and anti apoptotic genes using rt-PCR concerning (Bax and BCl-2) and cell apoptosis process. Finally, geno-toxicity was traced via arranging for flow cytometry. **Results:** Data recorded revealed that the inhibitory concentration (IC_{50}) of free and nanocapsulated sinapic acid was 646.4 μM mL^{-1} and 84.74 μM mL^{-1} post treatment with free and nanocapsulated sinapic acid, respectively. Also, cytotoxicity was concentration dependent with significant difference between free and capsulated one ($p<0.05$). Cytotoxicity enhanced apoptosis process accompanied with cell cycle arrest through the induction of G_2/M and pre-G1 phases arrest compared with that of cell control. Finally, proapoptotic gene (Bax) was significantly upregulated parallel with significant down regulation of BCL-2 ($p<0.05$). **Conclusion:** Current study suggested that Nano-sinapic acid possesses more cytotoxic effect on cancer cells with lower concentration than free sinapic acid so can act better as novel candidate for cancer chemoprevention.

Key words: Sinapic acid, nanocapsulated sinapic acid, squamous cell carcinoma, apoptosis, cell cycle

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030 [1]. Oral cancer is the third most common cancer and most common cancer death among male in the world population[2]. Chemoprevention is emerged as a good novel therapy in the recent years; Most of the anticancer compounds are in the nature of phenolic acids and these compounds plays a major role in antioxidants as chemoprevention[3]. 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[4].

Nanotechnology has the power to radically change the way cancer is diagnosed, imaged and treated. Nanoparticles are capable of detecting cancer at its earliest stages, pinpointing its location within the body and delivering anticancer drugs specifically to malignant cells[5]. Sinapic acid is a cinnamic acid derivative which possesses 4 hydroxy 3, 5 dimethoxycinnamic acid is one of the phenolic acids widely distributed in edible plants such as cereals, nuts, oil seeds and berries[6]. Sinapic acid act as preventive and therapeutic agents in several oxidative stress related diseases, such as atherosclerosis, inflammatory injury and cancer[7].

AIM OF THE STUDY

The present study aimed to evaluate the cytotoxicity accompanied genotoxicity and biochemical changes regarding cell cycle profile, apoptosis and apoptotic genes (Bax/BCL-2) monitoring relatively to the reactive oxygen species as anticancer biomarkers. Also, to evaluate cytotoxicity of free and Nano-sinapic acid on squamous cell carcinoma (HEp-2).

MATERIAL AND METHODS

This research project was conducted from 2016-2018. Sinapic acid molecular formula of ($C_{11}H_{12}O_5$) (3, 5-dimethoxy-4-hydroxycinnamic acid) and a molecular weight of 224.21 g moL^{-1} was kindly supplied from (Sigma Aldrich-USA) and Nano capsulated form was prepared using bio surfactant (tween 80) (Nano-Tech Egypt for Photo-Electronics) using bio surfactant (tween 80) in methanol 50:50 (v/v). The oil solution is the water phase and the tween is the oil phase (Fig. 1).

Cell treatment: Human squamous cell carcinoma cell line (HEp-2) used in the present study was kindly supplied from Cell Culture Department-VACSERA-EGYPT. Cells were supplied in 75 cm^2 surface area tissue culture flasks and grown in Minimum essential medium (Eagle) supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine, 100 IU mL^{-1} penicillin and 100 µg mL^{-1} streptomycin at 37°C under a humidified 5% CO_2 atmosphere. Cells were treated with different concentrations of sinapic acid and Nano-sinapic acid then 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.

Cytotoxicity assay (MTT assay): In the present study, MTT assay was used to determine the effective dose of free and Nanocapsulated sinapic acid. The MTT is reduced to insoluble purple MTT formazan crystals, by mitochondrial lactate dehydrogenase enzyme (LDH) and the pyridine nucleotide cofactors, NADH and NADPH as substrates. These results in a

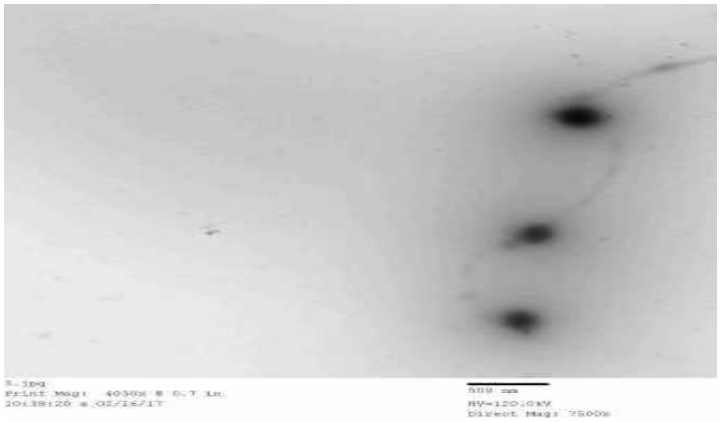

Fig. 1: TEM images illustrating spherical shape of Nano-particles of sinapic acid

yellow to blue color changes were quantified as described by Mosmann[8] with some modifications. In brief, HEp-2 oral cancer cells were seeded in 96 well microtiter plates (5×10^3 cells/well). Cells were incubated with sinapic acid at different concentrations ranging from 25-250 µM for 24 h. The untreated cells served as control. After the incubation the cells were washed twice with Phosphate Buffered Saline (PBS). The MTT (0.5 mg mL^{-1}) in PBS) added as 0.05 mL/well. Cells were incubated at 37°C for 4 h and DMSO (50 µL) was added to dissolve the formazan crystals. Samples were transferred into culture plates and the absorbance was measured calorimetrically at 570 nm.

Flow cytometry: Cell cycle analysis was performed by staining the DNA with Propidium Iodide (PI) as described previously Chakraborty *et al.*[9] 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 brief, cells are seeded in T25 flask at a density of 1×10^6 cells/flask. The IC_{50} concentration of both free and Nano capsulated sinapic acid were added to each flask incubated for 24 h. Cells were trypsinized, harvested and fixed in 70% ice cold ethanol in cell culture tubes and stored at +40°C until use. The cells were centrifuged, the cell pellets were resuspended in PI (40 µM mL^{-1} in PBS) solution containing RNase (100 µM mL^{-1}). The stained cells were analyzed using fluorescence activated cell sorter (FACScan, Becton-Dickinson) with 488 nm argon ion laser using MAC Cell-QuestTM 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.

Cell cycle analysis: Annexin V Apoptosis Detection Kit is based on the observation that soon after initiating apoptosis, cells translocate the membrane Phosphatidyl Serine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can be easily detected by staining with a fluorescent conjugate of Annexin V, a protein that has a high affinity for PS. The one-step staining procedure takes only 10 min. Detection can be analyzed by flow cytometry or by fluorescence microscopy. The kit can differentiate between apoptosis and necrosis when performing both Annexin V-FITC and Propidium Iodide (PI) staining.

Apoptosis: Control, free and Nano-capsulated Sinapic acid treated cells total RNA was extracted using GeneJET RNA Purification kit [Fermantus-UK] according to the manufacturer's protocol. The concentration and the integrity of RNA were assessed spectrophotometrically at 260/280 nm ratio. First-strand cDNA was synthesized with1 g of total RNA using a Quantitect Reverse Transcription kit [Qiagen, Germany] in accordance with the manufacturer's instructions. These samples were subsequently frozen at a temperature of -80°C until use for determination of the expression levels of P53, Bax and Bcl-2 genes using real-time PCR. Quantitative real-time PCR was performed on a Rotor-Gene Q cycler [Qiagen, Germany] using QuantiTect SYBR Green PCR kits [Qiagen, Germany] and forward and reverse primers for each gene.

Statistical analysis: All experiments were carried out in three independent tests. Data were expressed as the Mean±standard deviation (SD) and analyzed using one-way analysis of variance [ANOVA]. The results were considered statistically significant at probability <0.05.

RESULTS

Cytotoxicity assay: Regarding the cytotoxic effect of free and Nano capsulated Sinapic acid on HEp-2 cells, viability was employed using MTT assay. Data recorded revealed that the mean viability percentage of Sinapic acid treated cells was concentration dependent (Fig. 2) and the safe concentration induced 100% cell viability was at ~<20 µM mL^{-1}. And the IC_{50} values determined were 646.4 µM mL^{-1} and 84.74 µM mL^{-1} for free and Nano capsulated sinapic acid respectively indicating a significant difference ($p<0.05$) (Fig. 3).

Flow cytometric analysis: Regarding HEp-2 cells distribution post 24 h treatment with the IC_{50} value of both free and Nano capsulated sinapic acid formulae, it was noticed that the % of cells arrested at the G0/G1 phase were significantly decreased compared with that of nontreated cell control ($p<0.05$), while the % of cells at S-phase were increased compared with that of nontreated cell. Also, Data recorded revealed that there was a significant elevated arrest during the G_2-M phase in case of HEp-2 treatment with both free and Nano capsulated sinapic acid ($p<0.05$) compared with cell control . Also, there was a significant apoptotic cells in the pre-G_1 phase compared to its values in control group but there

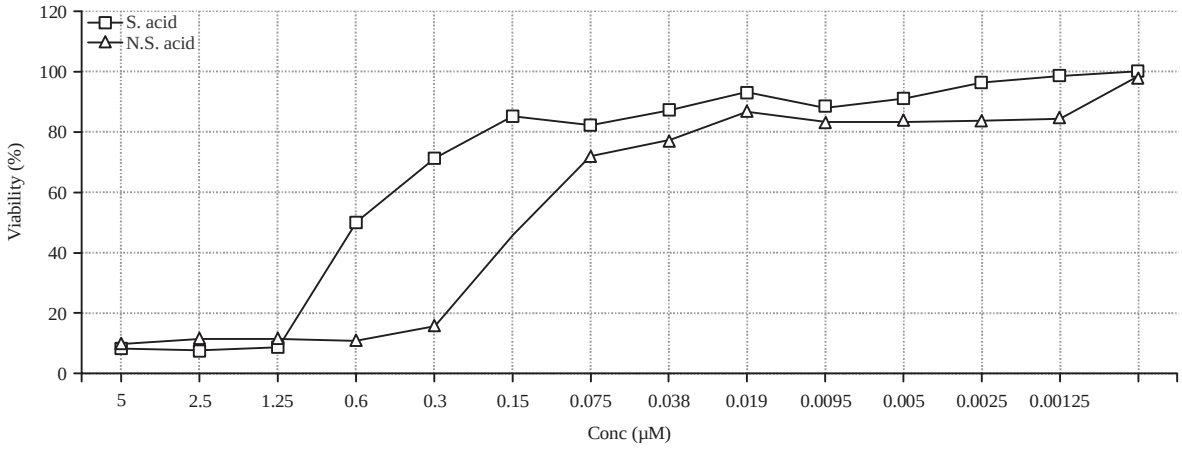

Fig. 2: Evaluation of cell viability post treatment with both free and Nano capsulated Sinapic acid using MTT assay

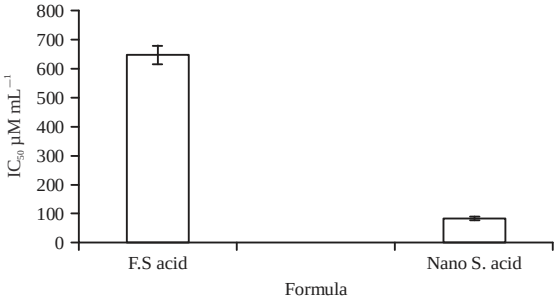

Fig. 3: Evaluation of inhibitory concentration (IC_{50}) post HEP-2 cell treatment with free and Nanocapsulated Sinapic acid

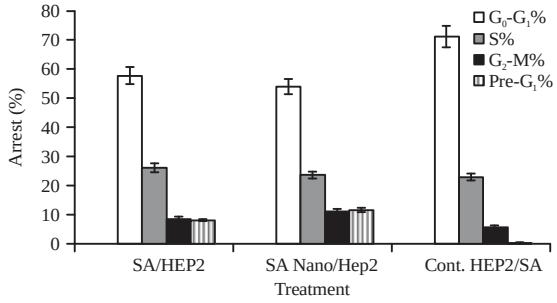

Fig. 4: The histograms to determine the percentage of cells in different phases of cell cycle for the control versus sinapic acid and Nano-sinapic acid treated cells for 24 h

are more apoptotic cells in case of Nano-sinapic acid treated cells than free sinapic acid treated cells (Fig. 4-5).

Apoptotic profile: Apoptotic cells were accumulated at G_2/M phase which proceed G1 phase, this called pre G1 apoptosis so the percentage of cells enter G1 phase decreased. Regarding the apoptotic profile of treated cells, there was a significant elevated early and late apoptotic cells and necrotic cells as well post cell treatment with free and Nanocapsulated sinapic acid respectively ($p<0.05$) (Fig. 6).

Real time PCR: Treatment of HEp-2 cells with both free and Nano-capsulated sinapic acid cause significant ($p<0.05$) upregulation of Bax gene and downregulation of Bcl-2 gene compared to control cells (Fig. 7).

DISCUSSION

Sinapic acid is a small naturally occurring hydroxycinnamic acid derivative. It is a phenolic compound and a member of the phenylpropanoid family which are assumed as therapeutically beneficial and generally not toxic[10]. Reddy and Prasad[11] have shown that phenolic compounds have health protective effects as well as are cytotoxic to cancer cells. Sinapic acid and other phenylpropanoids are present in vegetables and grains, e.g., Brassica juncea L., hazelnut, pea, cabbage, wheat or brown rice[12]. Studies reported that sinapic acids were effectively inhibited the growth of human oral, breast and colon carcinoma cell lines[13]. Therefore, this study was designed to explore the *in vitro* cytotoxicity of free and Nano-sinapic acid on squamous cell carcinoma (HEp-2). In the present study, cytotoxic potential of free and nanocapsulated was concentration dependent with significant difference. These results suggest that both formulae of sinapic acid could effectively inhibit the proliferation of the HEp-2 cells but the Nano form was more effective than the free sinapic acid. In line with present results was the findings revealed by Cheng *et al.*[14] who demonstrated that sinapic acid inhibited

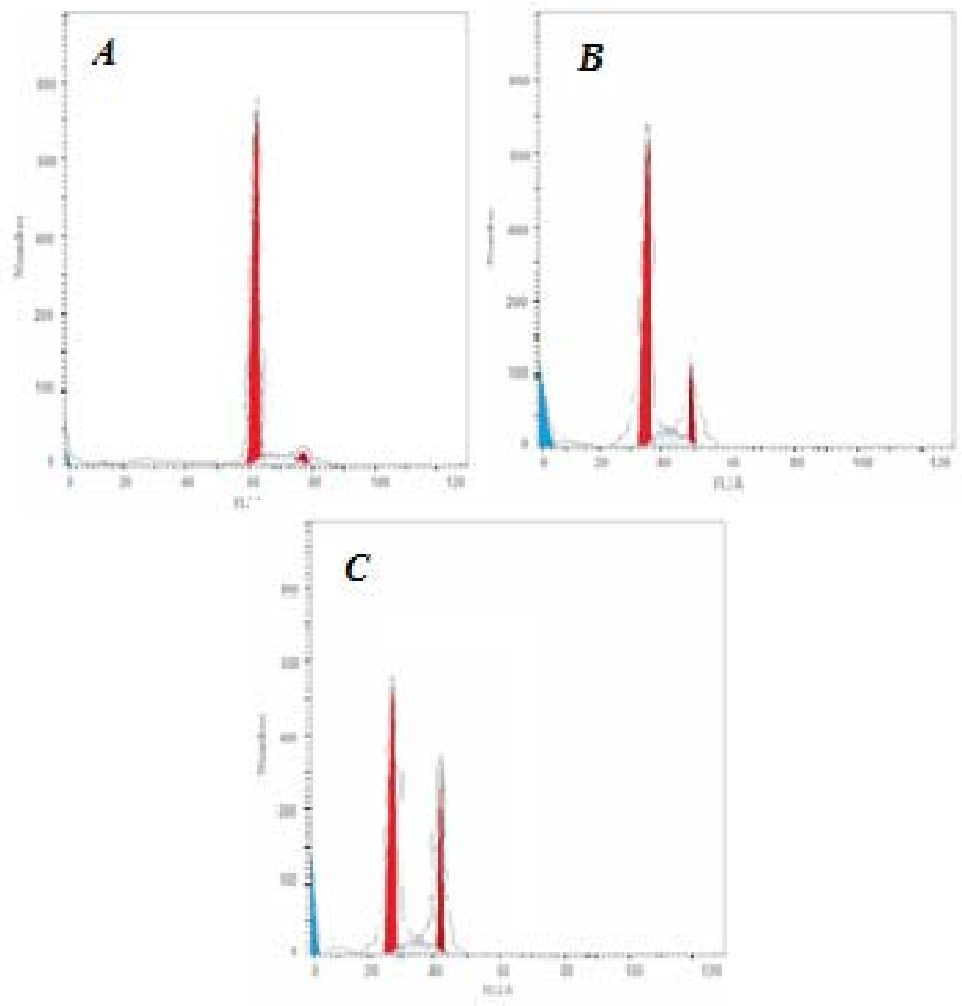

Fig. 5(a-c): Evaluation of cell cycle arrest at different phases post Hep-2 treatment with free and nano capsulated sinapic acid compared with nontreated cell control, (a) Cell control, (b) S. Acid treated cells and (c) Nano capsulated sinapic acid

the viability of colon cancer cell lines and anticancer effects was a dose and time dependent. 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[15].

Furthermore, the present results showed that the toxic effect of both formulae was accompanied with morphological changes denoting apoptosis. This observation was in accordance with Cragg and Newman[16] who reported that most of current anti-cancer drugs such as camptothecin, vincristine, taxol, etoposide and paclitaxel are plant-derived compounds and 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. Additionally, author's records were in accordance with *Pommier et al.*[17] and Janakiraman *et al.*[18] who indicated that most of chemotherapeutic agents halt tumor cells proliferation via induction of apoptosis. The suggestion that sinapic acid can induces apoptosis in HEp-2 cell line in a dose dependent manner confirmed by typical morphological changes as membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation, apoptotic bodies and loss of adhesion. In the meantime, Rugina *et al.*[19] documented that phenolic acids presents in mistletoe extract, showed anti-carcinogenic potential on A2780 tumor ovarian cells through apoptosis. Moreover, Kampa *et al.*[15] suggested that phenolic acids exert an apoptotic activity.

Apoptotic activity of sinapic acid formulae and inhibition of cell proliferation revealed that the activity of signal

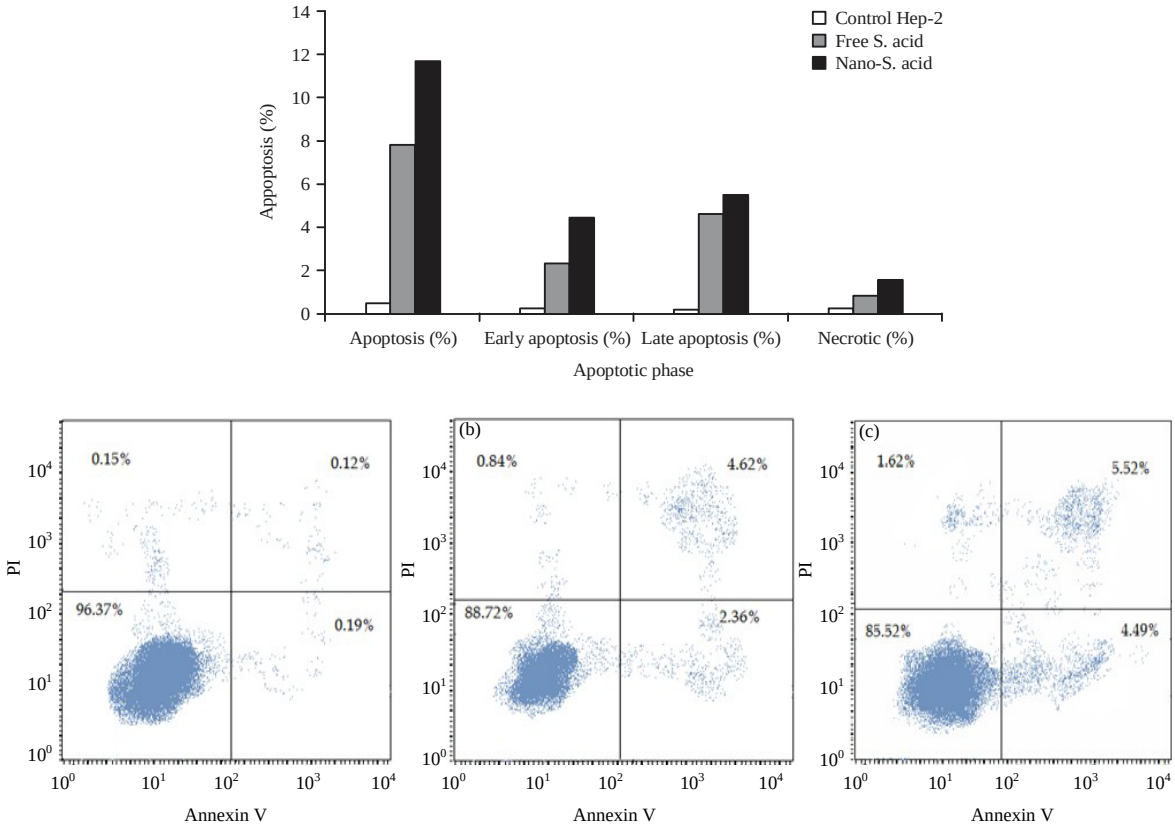

Fig. 6(a-c): Evaluation of apoptotic profile of HEp-2 post treatment with both free and Nano-capsulated sinapic acid using flow cytometry. (a) Control cell, (b) Sinapic acid treated cells and (c) Nano-capsulated sinapic acid treated cells

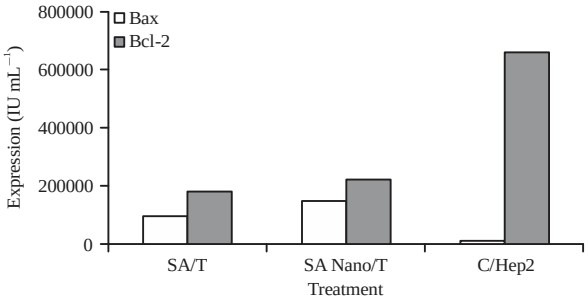

Fig. 7: Evaluation of pro and antiapoptotic gene regulation under the effect of free and Nanocapsulated sinapic acid treated Hep-2 cells compared to control cells

transduction molecules involved in the cell cycle. The pre-mitosis G_2/M phase and pre-replication G_1/S phase are specifically regulated by specific genes are the two check points for DNA damage[20]. In the present study, the cell cycle arrest profile was accompanied with apoptotic profile where the total apoptotic % was significantly elevated in case of Nanocapsulated sinapic acid than free one ($p<0.05$). These results were in agreement with Chen and Huang[21] who reported that phenolic acids found to arrest (specific genes) cell cycle progression either at G_1/S phase or G_2/M phase boundaries, while, was on the contrary of the report of Janakiraman *et al.*[18] recorded that sinapic acid induced an early G_0/G_1 phase arrest in case of cell treatment with the IC_{50} value for 24 and 48 h. Also, was in agreement with Kim *et al.*[22] reporting that differential accumulation of nanoparticles inside cells in the different phases of cell cycle, with characteristic ranking in the order G_2/M, S and G_0/G_1 arises because for times shorter than cell cycle duration, G_2/M phase cells have not yet divided and therefore have accumulated longer, whereas cells in the S phase have accumulated after cell division and G_0/G_1-phase cells have just divided and have not had time to internalize many new nanoparticles. The phenolic constituents of sinapic acid may initiate release of H_2O_2 a highly Reactive Oxygen Species (ROS) sources those can induce damage to proteins, nucleic acids and cell membranes. It is known to cause oxidative DNA damage primarily through the hydroxyl radical which results from

Fenton reaction. H_2O_2 has been reported to cause DNA damage in the form of chromosomal aberrations, single- and double- strand breaks[23].

Alteration of the p53 gene and high frequency of p53 expression were detected in a number of human solid tumors including oral cancer[24], regarding the gene expression profile our records were in accordance with Janakiraman *et al.*[18], they stated that the oral administration of sinapic acid treatment led to a significant increased expression of p53, pro-apoptotic proteins Bax and caspase 3, whereas decreased the levels of anti-apoptotic Bcl-2 that correlated well with the induction of apoptosis. Incidentally, this is the first *in vivo* report which shows that sinapic acid directly or indirectly was associated with induction of apoptosis in DMBA-induced buccal pouch carcinogenesis. Result of current study was also in agreement with Puangpraphant *et al.*[25] who reported that hydroxycinnamic acids and derivatives significantly improved the ratio of Bax: Bcl-2 protein expression and it facilitates the apoptosis in colon cancer cell lines , also on the contrary of Prasad *et al.*[26] showed that in conjunction with sinapic acid mediated apoptosis, down regulation of Bax and up regulation of Bcl-2 was noticed in DMBA-induced buccal pouch carcinogenesis as reported in human and animal tumors. And was in agreement with Hu *et al.*[27] who documented that the chemotherapeutic agent induced apoptosis in human cancer cell line and hamsters by down regulation of Bcl-2 and up regulation of Bax. Current results were concordant with Rajasekaran *et al.*[28] showed that the structural related phenolics compound of carnosic acid has significantly improve the expression of p53 and Bcl-2 during DMBA-induced oral carcinogenesis.

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[29]. Recently, Janakiraman *et al.*[18] 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[30]. 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, alternating mitochondrial membrane potential, increasing intracellular ROS, lipid peroxidation and inducing apoptosis in HT-29 and SW480 colon cancer cell lines.

Finally it can be concluded that sinapic acid as a natural derived phenolic compound can be used safely as anticancer agent that can affect the cell cycle profile inducing apoptosis that related to the expression of P53 gene upregulation and also the cell arrest was related to the ROS level in treated cells compared with nontreated cell control. Also, Nanocapsulation showed a marked change in the cell proliferation profile and there was a significant effect on cell cycle and gene expression profiles compare with free sinapic acid. Finally it is possible to recommend that the use of both free and nanocapsulated formulae are possible with the insignificant variation on the bioactivity of sinapic acid. Another capsulation agents enhance the sustained release must be tried. Finally, *In vivo* trial for Sinapic acid anticancer potential must be conducted. Also, the release and total loading efficiency of sinapic acid must be traced.

CONCLUSION

Sinapic acid possesses the cytotoxic effect on cancer cells and a novel candidate for cancer chemoprevention While Nano- sinapic acid possesses more cytotoxic effect on cancer cells with lower concentration than sinapic acid so act better as novel candidate for cancer chemoprevention.

SIGNIFICANCE STATEMENT

Sinapic acid act as preventive and therapeutic agents in several oxidative stress related diseases, such as atherosclerosis, inflammatory injury and cancer. This study was conducted based on monitoring of *in vitro* cytotoxicity using MTT assay, morphological changes determination of free and nanocapsulated Sinapic acid. Data recorded revealed that the inhibitory dose of 50 % of free and nanocapsulated sinapic acid was 646.4 and 84.74 μM mL^{-1} post treatment with free and nanocapsulated sinapic acid respectively. Cytotoxicity enhanced apoptosis process accompanied with cell cycle arrest through the induction of G_2/M and pre-G_1 phases arrest compared with that of cell control. Also, cytotoxicity was concentration dependent with significant difference between free and capsulated one ($p<0.05$). Finally, proapoptotic gene (Bax) was significantly upregulated parallel with significant down regulation of BCL-2 ($p<0.05$).

This study will help the researchers to uncover critical areas in treatment of Cancer. It can be concluded that Nano-sinapic acid possesses more cytotoxic effect on cancer

cells with lower concentration than free sinapic acid so can act better as novel candidate for cancer chemoprevention.

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