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ResearchArticle

PRELIMINARY STUDY ON SALUBRIOUS EFFECT OF SYRINGIC ACID ON APOPTOSIS IN HUMAN LUNG CARCINOMA A549 CELLS AND *INSILICO* ANALYSIS THROUGH DOCKING STUDIES

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ABSTRACT

Objective
To analyse the anticancer activity of Syringic acid (SA) in lung carcinoma A549 cell line and investigate the mechanism of *Bcl-2*inhibition by molecular docking analysis.
Methods
The antiproliferative activity of SA was analysed by MTT assay. Apoptosisratio of SAtreated cells was detected by Acridine orange/Ethidium bromide staining.Nuclear morphology of SA treated cells was assessed by propidiumiodode staining method. Molecular docking was done withBcl-2 by Autodock4v4 of human origin and the interaction was studies using pymol.
Results
Preliminary study with MTT assay revealed that SA had cytotoxicity toward A549 lung cancer cells with an IC50 of 30µM. AO/EB staining and PI staining confirms that there is a significant increase in the percentage of apoptotic cells and nuclei respectively of SA treated cells. Further, molecular docking analysis revealed SA inhibited *Bcl-2* with a binding score of –6.54 Kcal forming three Hydrogen bonds.
Conclusion
These findings suggest that Syringic acid has potential therapeutic benefit and promises to be a weapon against lung cancer.

Keywords: Lung Cancer, Apoptosis, Syringic acid, Molecular docking

INTRODUCTION

Cancer is a serious health threat to humans and is a leading cause of death worldwide. Cancer results from a cascade of biological events that fundamentally alters the normal functions of cells. It is a hyper proliferative disorder that involves transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis [1].International Agency for Research on Cancer reported that more than 18% of patients are generally diagnosed with lung cancer and has been the major cause of death [2]. In India, as of July 2002, a total of 41,000 lung cancer cases would have been diagnosed. Over 70 % of patients with lung cancer are in Stages III and IV when diagnosed which makes curative surgery difficult [3]. Lung cancer can be further classified into small and non-small cell lung cancer (NSCLC), where NSCLC constitutes 75-80% of total lung cancer victims.Apoptosis, also known as programmed cell death, is important not only during development and tissue homeostasis, but also plays a major role in the pathogenesis of the varieties of human disorders. Studies in diverse model organisms have explored the molecular mechanisms underlying the apoptotic signaling pathways in initiation, mediation, execution, and regulation of apoptosis [4]. The cardinal morphological features of apoptosis are cell shrinkage accompanied by transient but violent bubbling and blebbing. Several studies have documented the involvement of Bcl-2 family [5].B-cell lymphoma2 (Bcl-2) may inhibit a central step in an apoptotic cell death pathway or protect an essential cellular constituent that is a target of apoptotic programs [6]. Thus inhibition of the anti-apoptotic Bcl-2 protein can force the cell towards apoptosis.

In the past few decades, a large number of plant-derived bioactive compounds such as paclitaxel, vinblastine, and camptothecin have been isolated and are widely used to treat various types of cancers. It is the mandate to develop better therapeutic agents with enhanced activity against lung cancer. Currently, Functional foods and Nutraceuticals are receiving widespread interest in terms of prevention of several chronic diseases, such as cardiovascular disease and cancer [7]. Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants [8]. Syringic acid (SA) (4-hydroxy-3, 5-dimethoxybenzoic acid) is a naturally occurring major phenolic compound found in many plants and food compounds. The main sources of SA are swiss chard, olives, walnuts, dates, spices and pumpkin [9,10] It has been reported that SA is present abundant in cereals such as barley, maize, millet, oat, rice, rye, sorghum, and wheat [11] and in plants like *Raphanussativus* L [12], *Hemidesmusindicus* [13]*Tageteserecta* Linn flower [14]. It shows a vast array of biological activities contributing towards the protection of human health. Various biological activities like antioxidant, antiproliferative [15] anti-endotoxic [16] and anti-cancer [17] activity of SA were reported earlier. The administration of SA could suppress hepatic fibrosis in chronic liver injury [18]. SA decreased proliferation in leukaemia cells and induced apoptosis by raising the level caspase 3, 8, and 9 activities [19].

Molecular mechanism underlying triggering anti-anticancer activity by SA is not explored till now. Hence the present study was aimed to investigate the anti-canceractivity of SA on A549 cell line and identify the mechanism behind the activity *Bcl-2*-SA interaction was studied by molecular docking using *in silico* methods.

MATERIALS AND METHODS

CHEMICALS

Syringic acid, Dimethyl sulfoxide and (DMSO) and MTT were purchased from Sigma Aldrich Chemicals. Pvt. Ltd. (USA). All the other chemicals used were extra pure and of molecular biology grade and cell culture tested.

CELL PREPARATION AND CULTURING

The A549 lung adenocarcinoma cell line was procured from National Centre for Cell Science (NCCS), Pune. Cells were maintained in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), with 100units/mL penicillin and 100µg/mL streptomycin. Cells were cultured in a humidified

atmosphere with 5% CO_2 at 37 °C. Cells were grown in 75cm^2 culture flask and after a few passages, cells were seeded for experiments. The experiments were done at 70 to 80% confluence. Upon reaching confluence, cells were detached using 0.25% Trypsin-EDTA solution.

CELL PROLIFERATION ASSAY OR MTT ASSAY

Proliferation of A549 cells was assessed by MTT assay. The proliferation test is based on the colour reaction of mitochondrial dehydrogenase in living cells by MTT. Cells were plated in 96-well plate at a concentration of 2 × 10^4 cells/well 24 h after plating. After 24h of cells incubation, the medium was replaced with 100µl medium containing Syringic acid at different concentrations (1 – 1000µM/ well) and incubated for 24h. Untreated cells served as control and received only 0.1% DMSO in which the drug was prepared. At the end of treatment period, media from control and drug-treated cells was discarded and 20µl of MTT (5mg/ml PBS) was added to each well. Cells were then incubated for 4h at 37°C in CO_2 incubator. MTT was then discarded and the coloured crystals of produced formazan were dissolved in 200µl of DMSO and mixed effectively by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570nm. Optical density of each sample was compared with control optical density and graphs were plotted.

CELL MORPHOLOGY

Cell morphology of SA treated cells for was assessed using an inverted optical microscopeand recorded.Concentrations of 10, 20, 30, 50, 75 and 100 µM ofSA for 24 hrs were used for the morphological studies. The 0.1% DMSO treated cells were served as negative control.

ETHIDIUM BROMIDE/ACRIDINE ORANGE STAINING

Ethidium bromide/acridine orange staining was carried out by the method of [20]. A549 cells were plated at a density of 1×10^4 in 48-well plates. They were allowed to grow at 37°C in a humidified CO_2 incubator until they were 70–80% confluent. Then cells were treated with 15µM/ml and 30µM/ml of drug for 24h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and drug treated were mixed with 100µl of dye mixture (1:1) of ethidium bromide and acridine orange) and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at 10x magnification. A minimum of 300 cells was counted in each sample at two different fields. The percentage of apoptotic cells was determined by [% of apoptotic cells = (total number of apoptotic cells/total number of cells counted) ×100].

PROPIDIUM IODIDE STAINING

Propidium iodide staining was carried out by the method of [22]. A549 cells were plated at a density of 1 × 10^4 in 48-well plates. They were allowed to grow at 37°C in a humidified CO_2 incubator until they are 70–80% confluent. Then cells were treated with 15µM/ml and 30µM/ml of drug for 24 h. The culture medium was aspirated from each well and the cells were gently rinsed twice with PBS at room temperature, before fixing in methanol: acetic acid (3:1 v/v) for 10 min, and stained with 50µg/ml Propidium iodide for 20min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscopy and at least 1 ×10^3cells were counted for assessing apoptotic cell death.

MOLECULAR DOCKING

Molecular Docking study was carried out for the SA with VEGF by Autodock 4.2. In-silico generation of ligand (SA) were done by ACD ChemSketch and files were saved in MDL Molfiles. 2D of SA (.mol format) was converted into 3D structures (.pdb format) by Open Babel GUI version 2.0.2. Structure of BCL-2 (1VPP) of *Homo sapiens*was taken from protein data bank (PDB) (http://www.rcsb.org/pdb/) as target for docking. Molecular docking using Lamarckian Genetic Algorithm [22] was carried out to find out the binding mode of SA with VEGF on the basis of calculated ligand-protein pairwise interaction energies. Autodock uses empirical force field for the energy minimization of molecules.Docking was carried out with standard docking protocol on the basis of a population size of 150 randomly placed individuals; a maximum number of 2.5 *10^7 energy evaluations. Ten independent docking runs were carried out for each ligand and results were clustered according to the 1.0 Å rmsd criteria. The interaction between target and ligand were analysed using pymol and hydrogen bond distances were calculated.

RESULTS

MTT ASSAY

The cytotoxic effects of SA in A549 lung carcinoma cells were analyzed using MTT assay. As shown in Figure 1, the percentage of viable cells was significantly decreased with respect to treatment of SA with increasingconcentrations after 24h and 48h.50% of viable cells was observed at 30µM on A549cells line at 24 hrs. From this result the IC_{50} value of SA in A549 cells was calculated 30µM.Hence, for further studies SA of 15 and 30µM concentrations for 24 hrs were considered as low and high dose respectively.

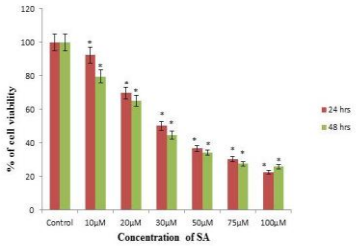

Figure 1. The Effects of SA on Cell Viability in A549 cells.
Each Bar represents the Mean±SEM of six independent observations. '*' represents statistical significance between control vs SA treated groups at p<0.05 level using Newman-Keul's test.

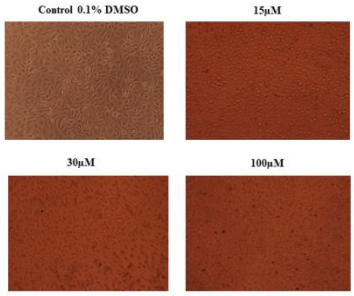

Figure 2 : Morphological changes induced by different concentrations of SA in A549 cells

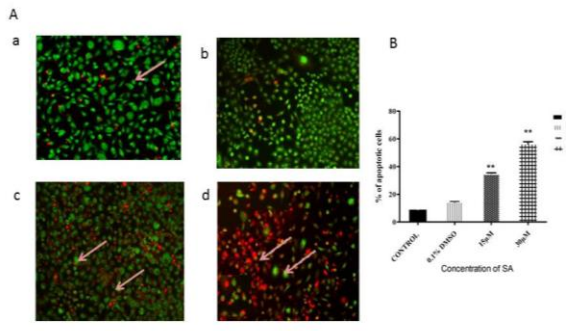

Figure 3: Effect of SA on apoptosis in A549 cells A) AO/EB staining of SA treated A549 cell a) Control b) 0.1% DMSO c) 15 µM d) 30 µM. Viable cells DNA was stained by Acridine orange and their nuclei were bright green , while apoptotic cells DNA were stained by Ethidium bromide and appears orange to red color and both indicated by arrows.B) Grap representing % of apoptotic cells (**) p<0.01 statistically significant compared to that of control.

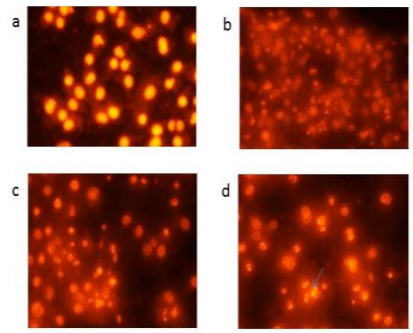

Figure 4: Effect of apoptosis in A549 cells by propidium iodide staining a) Control b)0.1% DMSO c)15 µM d) 30 µM. The nucleus of the treated cells were more condensed and fragmented compare to that of control shown by arrow.

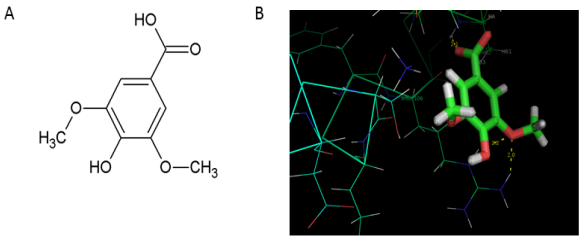

Figure 5: A) Structure of SA in chemsketch B) Docked complex in Pymol showing hydrogen bond interaction to the active site of BCL2 protein of human origin

Table1: Hydrogen bond distance between SA and *Bcl-2*

Residues (aminoacid)	Receptor Atom	Ligand Atom	Distance(Å)
Ala 110	H	O	2.2
Arg 106	H	O	2.1
Arg 106	O	O	2

CELL MORPHOLOGY

Figure 2 shows morphological changes to A549 cells after SA treatment for 24 h. The change in the morphology of treated cells can be observed in dose dependent manner. Cytotoxicity was increases with increase in SA concentration. SA induced cell shrinkage, rounding of the cell and reduction in number of viable cells. These changes indicate that SA induced apoptosis in A549 cells.

ACRIDINE ORANGE/ ETHIDIUM BROMIDE STAINING(AO/EB)

AO/EB staining of was performed to analyse the percentage of apoptotic cells of SA treated and control cells. Viable cell's DNA was stained by Acridine orange and their nuclei were bright green, while apoptotic cell's DNA were stained by Ethidium bromide and appears orange to red color was shown by arrows in Figure 3.SA treated cells significantly decreased the percentage of living cells in dose dependent manner. At 30 µM there is a 5 fold in apoptotic ratio compared to that of control.

PI STAINING

SA treated and untreated A549 cells was analysed for its cell morphology under fluorescence microscope after staining ofthe nuclei with propidium iodide. It was observed that there is a significant difference between control and SA treated cells. After SA treatment there were more apoptotic cells. The nuclei was condensed and fragmented to form apoptotic bodies upon treatment (Figure 4)whereas, the control cells do not show any nuclear fragmentation.

MOLECULAR DOCKING

To investigate the molecular interaction behind the mechanism of inhibition between SA and BCL-2 molecular docking analysis was done using Autodock 4v2 and the interaction was viewed using pymol. Structure of SA was drawn using ADL Chemsketch(Fig. 5A). The results of the binding energy, length and number of hydrogen bonds formed with ligand and the active site were briefed in table 1. SA shows binding energy of --6.55 kcal/mol and formed three hydrogen bond with the active site of BCL-2 (Fig 5B) thereby effectively inhibits the anti-apoptotic protein.

DISCUSSION

Non small lung cancer is the most commonly diagnosed type of lung cancer, accounting for approximately 85% of all cases [23]. More than 70 % of them are in Stages III and IV when diagnosed making curative surgery difficult. Hence there is an urgent necessitynew strategies aimed at better understanding the molecular mechanism by which potential anticancer chemicals act and to further provide the basis for more effective treatments [24]. Treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell apoptosis in tumor cells [25]. Thus an alternative therapy by indentifying effective anticancer agents using natural compound has substantial hope in anticancer therapy. Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants [8]. Emerging studiesshow an increasing demand for phenolic compounds to counter the activity of cancerous transformation of cells. Syringic acid (SA) (4-hydroxy-3, 5-dimethoxybenzoic acid) is a naturally occurring phenolic compound found in many plants and food compounds. Recently Syringic acid from *Tamarixaucheriana* possesses antimitogenic and chemo-sensitizing activities in human colorectal cancer cells [26].Thus an attempt has been made to study the anticancer activity of SA in A549 cells.

In the present study MTT assay revealed that SA caused cytotoxicity in A549 cells in dose dependent manner. IC50 value was observed at 30µM concentration of SA which significantly reduced the viability of cells compared to that of control. The morphology of the cells was assessed under light microscope after treating with SA. Cytotoxicity was increased with increase in SA concentration. SA induced cell shrinkage, rounding of the cell and reduction in the number of viable cells indicating the sign of apoptosis upon treatment of higher concentration. SA has shownantiproliferative activity against human colon carcinoma cell lines HT-29 and HCT 116 also increased the apoptotic marker BAX [27]. Cell morphology of SA induced apoptosis was done by staining the cells with a combination of the fluorescent DNA-binding dyes AO and EB (dual staining). AO stains DNA bright green, hence the living cell nuclear chromatin appears green. EB stains DNA orange, but is excluded by viable cells, thus death cells chromatin appears orange-red [28]. The result reveals that SA induced apoptosis in A549 cells in a dose dependent manner. There were significant apoptotic cells observed at 30µM compared to that of control. There is a 5 fold increase of apoptotic ratio observed in 30µM of SA treated cells. It is worth to state that SA exhibited no toxicity to normal HUVEC cell line [19].Propidium iodide staining was done to stain the nuclei of treated and control cells for additional confirmation SA induced apoptosis. SA treated A549 cells shows condensed nuclei and fragmented to form apoptotic bodies. As these results suggest the preliminary confirmation of anticancer activity of SA in A549 cells, a molecular docking analysis was done to analyse the mechanism of apoptosis at protein level. Docking of small molecule compounds into the binding site of a receptor and estimating the binding affinity of the complex is an important part of the structure based drug design process [29]. Targeting the anti-apoptotic *Bcl-2* family of proteins can improve apoptosis and thus overcome drug resistance to cancer chemotherapy[30]. SA reported to also increase the ratio of the apoptotic-related protein Bax/Bcl-2, and reduced Akt activation [19]. Thus *Bcl-2* of human origin was selected and SA was docked against it. The results obtained (Table 1) revealed that the interaction of *Bcl-2* with SA has three hydrogen bond interactions with a docking score of -6.55 kcal/mol. Hydrogen bond formation of ligand and receptor has a distance ranging from 2.0-2.2Å. In summary, the present research work clearly establishes that Syringic acid exerts cytotoxicity in A549 cells. However mRNA and Protein expression analysis of pro and anti-apoptotic proteins will further confirm the activity.

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