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DIFFERENTIAL EFFECTS OF *ANNONA SQUAMOSA* SEED EXTRACTS: ANTIOXIDANT, ANTIBACTERIAL, CYTOTOXIC AND APOPTOTIC STUDY.

[1]BIBA, V. S; [2]JEBA MALAR, P.W AND [*1]REMANI, P

[1]Division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram, Kerala, India. 695011
[2]Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, Kanyakumari, Tamil Nadu, India.629502.

ABSTRACT

The aim of this study was to investigate antioxidant, antibacterial, cytotoxic and apoptotic activities of *Annona squamosa* L seed petroleum ether extract (ASPE). Antioxidant activity was assessed by DPPH scavenging activity, antibacterial activity was tested using broth dilution technique and antiproliferative activity was evaluated by MTT assay. The apoptosis inducing capacity was detected by Acridine Orange – Ethidium Bromide Staining, Hoechst Staining and Annexin V expression was assessed by flowcytometry. The extract showed antimicrobial activity and free radical scavenging activity, RSA_{50} of ASPE was 70.5 µg/ml. The active fraction showed IC_{50} value 33.8 µg/ml of KB cells. Acridine Orange Ethidium Bromide and Hoechst staining exhibited apoptotic features and Flowcytometry revealed increased levels of Annexin-V expression after treatment with the extract. These results suggest that the active fraction isolated from *Annona squamosa* seed extracts have strong antibacterial, antioxidant and antitumor activities.

KEY WORDS: ***Annona squamosa*, antioxidant, antibacterial, cytotoxic, apoptotic**

REMANI P
Division of Cancer Research, Regional Cancer Centre, Thiruvananthapuram, Kerala, India. 695011

INTRODUCTION

Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times[7] . Natural products from plants are proven templates for new drug development [17] and have many interesting biological activities. *Annona squamosa*, family Annonaceae, is gaining a lot of importance for its therapeutic potentials[12]. Literatures of many research works prove that every partof *A. squamosa* possess medicinal property[12]. Different parts of *A. squamosa* are used in folkloric medicine for the treatment of various diseases[25]. In Ayurveda, fruits are considered as a good tonic, enrich blood, increases muscular strength, cooling, lessens burning sensation and tendency to biliousness, sedative to heart and relieves vomiting. The crushed leaves are sniffed to overcome hysteria and fainting spells they are also applied on ulcer and wounds[21]**.** Ethanolic extract of *Annona squamosa* showed significant antioxidant activity and anticancerous activity[16,3.] *A. squamosa* contains flavanoids which express strong antibacterial activity[5,18 .] It is reported to contain alkaloids, carbohydrates, fixed oils, tannins & phenolics[16]. Earlier reports from our laboratory showed cytotoxic effects of *A. squamosa* on Dalton's lymphoma cells and HeLa cells[2]. The present study describes the antioxidant, antibacterial, antiproliferative and apoptotic activity of the seed extracts from the seeds of *Annona squamosa*.

MATERIALS AND METHODS

Cell culture

The Nasopharyngeal carcinoma (KB) cell line was obtained from the National Centre for Cell sciences (NCCS) Pune and cells were cultured in DMEM media supplemented with 10% Foetal Bovine Serum (FBS) at 37^0C,50% $C0_2$ environment.

Test microorganisms

The microbial strains used were identified strains and obtained from the National Chemical Laboratory (NCL), Pune, India. The bacterial strains studied include gram negative bacteriae *Escherichia coli* (ATCC 8739), and gram positive bacteriae *Staphyllococcus aureus* (ATCC 23564).

Microbial culture conditions

The bacterial cultures were maintained in Nutrient agar plates. Overnight cultures were used in all experiments by inoculating a single colony of each type of culture in respective 5 ml Nutrient broth and incubating at 37^0C for 18-24 hours in a rotory incubator.

Preparation of seed extract

The seeds of *Annona squamosa* were collected from Thiruvananthapuram district, Kerala state, authenticated by the taxonomist and a voucher specimen TBGT 57051 has been kept in the herbarium of Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, Thiruvananthapuram. The shade dried and pulverized seeds were used for soxhlet extraction in a soxhlet apparatus using petroleum ether as the solvent and concentrated by using rotatory evaporator . These samples were further purified by column chromatography and TLC. Active fraction was Identified by MTT assay and most active petroleum ether- chloroform fraction (ASAF) was used for further study. The ASAF was dissolved in dimethyl sulfoxide (DMSO) and used for the experiments.

Determination of antioxidant activity by DPPH method

The radical scavenging activity of the extract was determined by the method described by Krings and Berger[14]. To 1ml of 0.1mM solution of DPPH in ethanol, added 3 ml of ASPE extract (25-800 µg/ml) in distilled water and kept for 30 minutes. The color change of the reaction mixture was then read at 517 nm[1]. The percent DPPH decolourization of the sample was calculated as scavenged = (Absorbance of control-Absorbance of test)/absorbance of control x100.

Assay of antibacterial activity by Broth dilution technique

Minimum Inhibitory Concentration of ASAF towards gram negative bacteria *E.coli* and gram positive bacteria *Staphylocoocus aureus* were determined using the broth dilution technique. A loopful of bacterial culture was taken from an overnight culture and was inoculated into 1 ml of nutrient broth. The inoculated culture was kept in a rotary shaker at 37^0C for 2 hours at 120 rpm and the organisms were seeded for the experiment once the optical density (O.D) reached 0.3 at 650 nm. 100 µl of the culture was added to all the wells of a 96 well micro titre plate. Different concentrations (0.019mg/ml to 0.625mg/ml) of the ASAF dissolved in 10% DMSO were serially diluted and added in triplicates into the cell culture. The negative control was maintained by treating the bacterial cells with media containing 10% DMSO. Penicillin (50 µg/ml) was used as positive control against gram negative strains and Streptomycin (50 µg/ml) against gram positive strains. The plated cells were incubated at 37^0C for 48 hours. After the incubation period, optical density was read at 620 nm in a multiwell plate reader. Minimum inhibitory concentration i.e. 50 % death of *Staphylococcus aureus* and *E.coli* was calculated. The experiments were done in quadruplicates.

Cell viability (MTT) assay

MTT assay was performed according to the method of Scudiero et al 1988. Normal human lymphocytes[4] and KB cells were cultured in 96 well plates with various concentrations of the ASAF (25 µg/ml to 800 µg/ml)s incubated for 12, 24, 48 hours in a 5% Co_2 incubator. Untreated cells served as control. At the end of incubation MTT [3-(4,5- dimethylthiazol 2-yl)-2,5 – diphenyltetrazolium bromide] and lysis buffer [20% Sodium Dodecyl Sulfate (SDS) in 50% Dimethylformamide] were added[23]. The optical densities were measured at 570 nm and cytotoxicity was calculated.

% of growth inhibition = (100 – absorbance of treated cells/ absorbance of control) x 100

Acridine orange (AO) and ethidium bromide (EB) double staining

KB cells were treated with ASAF 15 and 30 µg/ml and incubated for 24 h. To detect the morphological features of apoptotis Acridine orange (100 µg/ml) mixed with ethidium bromide (100 µg/ml) in 1x PBS solution was added to each well, and the apoptotic cells were observed under the fluorescent microscope[11].

Hoechst staining 33342

The ASAF treated cells (15 µg/ml and 30 µg/ml) washed twice with PBS and stained with Hoechst 33342 for 1 hour at room temperature. Then the cells were washed with PBS and the Hoechst stained nuclei were visualised by fluorescence microscopy at 350- 460 nm.

Annexin V status by flowcytometry

Annexin V- FITC/PI double staining of the cells were performed with the Annexin V FITC kit (BD science U.S.A). KB cells were treated with ASAF at a concentration of 15 µg/ml and 30 µg/ml for 24 hours. The cells were trypsinized, rinsed twice with PBS and resuspend in 1X binding buffer. Then the cells were labelled with 5 µl FITC conjugated AnnexinV and 5 µl PI according to manufactures instructions. After incubation in the dark for 15 minutes at room temperature 400 µl binding buffer was added and the samples were immediately analysed by Flow cytometry.

Statistical analysis

The results are represented as the mean $\pm$ SD. The data was analysed by using Excel and Graph pad.

RESULTS

Free Radical Scavenging Activity

The antioxidant activity of ASPE at 800 µg/ml was found to be 67.5±1.5 with reference to standard Gallic acid 97.25±0.9 (Figure 1a). The 50 % Radical Scavenging activity (RSA_{50}) of ASPE was found to be 70.5 µg/ml (Figure1b).

Inhibition of the growth of microorganisms

The active fraction (ASAF) showed significant inhibition in the growth of microbial strains. The results revealed that at the lowest concentration of ASAF (0.019 µg/ml) the inhibitory effect on *Staphylococcus aureus* was 44±0.8 % in 24 hours . At the highest concentration (0.625 µg/ml) the inhibitory

effect was 76.25±0.95 % with reference to positive control streptomycin (94 ±1.1 %) (Figure 2a). Likewise, the lowest concentration of ASAF (0.019 µg/ml) showed inhibitory effect of 40±1.41% towards E.coli and at highest concentration (0.625 µg/ml) inhibitory effect was 70.75±0.95% in 24 hours with reference to positive contol Pencillin (97.25± 0.5) (Figure 3a)). It was also noticed that as the concentration level of ASAF increased from 0.019µg/ml – 0.625µg/ml, the inhibitory effect was also found to be increased. Minimum inhibitory concentration of ASAF was found to be 0.04 mg/ml and 0.05mg/ml for *Staphylococcus aureus* and *E.coli* respectively. (Figure 2b & 3b).

Inhibition of tumour cell proliferation

The results of the cell viability assay showed that ASAF could inhibit the growth in KB cells in a concentration and time dependent manner. When the cells were treated with ASAF for 12, 24 and 48 hours at 800 µg/ml the cytotoxic effect observed was 67.5±1.91,69.5±1.291,72.75 ±1.707 respectively (Figure 4a). When the concentration level increased from 25 µg/ml to 800 µg/ml the cytotoxic effect was also found to be increased. The IC_{50} of ASAF on KB cells was found to be 33.81 µg/ml. (Figure 4b).

Lymphocyte viability.

To ascertain whether ASAF specifically inhibit the proliferation of normal cells, their effect was assessed on human lymphocytes in culture. There was no significant toxicity found even after the treatment with 800 µg/ml at 24, 48 and 72 hours (Figure 5).

Induction of apoptosis by Active Fraction

Dual staining by acridine orange and ethidium bromide revealed morphological features of apoptosis such as nuclear fragmentation and chromatin condensation after 24 hours of treatment with ASAF (Figure 6). Hoechst staining showed a greater intensity of blue colour in all the treated cells compared to control cells, which is due to the condensed chromatin, the characteristic feature of apoptosis (Figure 7). There was a concentration dependent increase in the percentage of apoptotic cells after 24 hours of treatment.

Annexin v status

Number of Annexin positive cells could be observed after 24 hours treatment with the active fraction indicating apoptosis induction at a concentration of 15µg/ml and 30µg/ml (Figure 8).

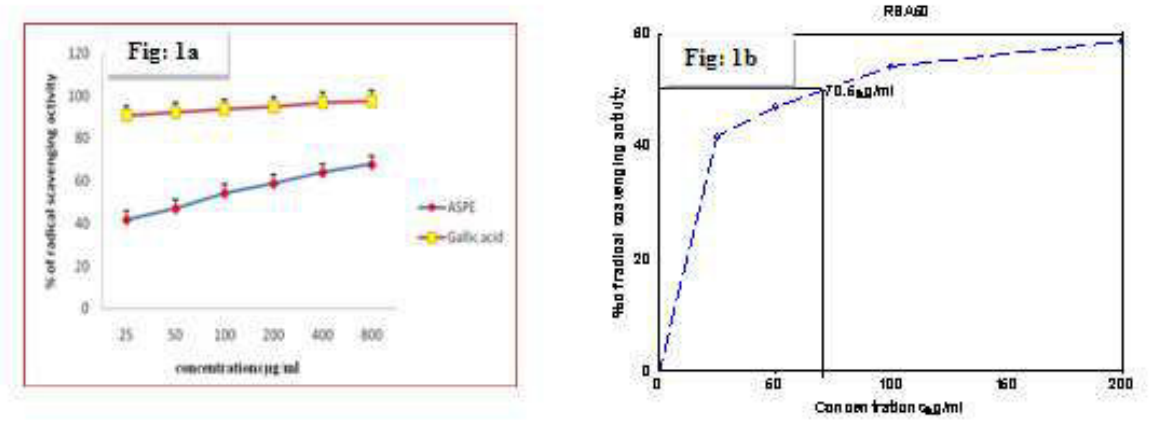

Figure 1

(a) % of radical scavenging activity by ASPE N=4, mean ±S.

(b) RSA_{50} of Annona squmosa seed petroleum ether extract.

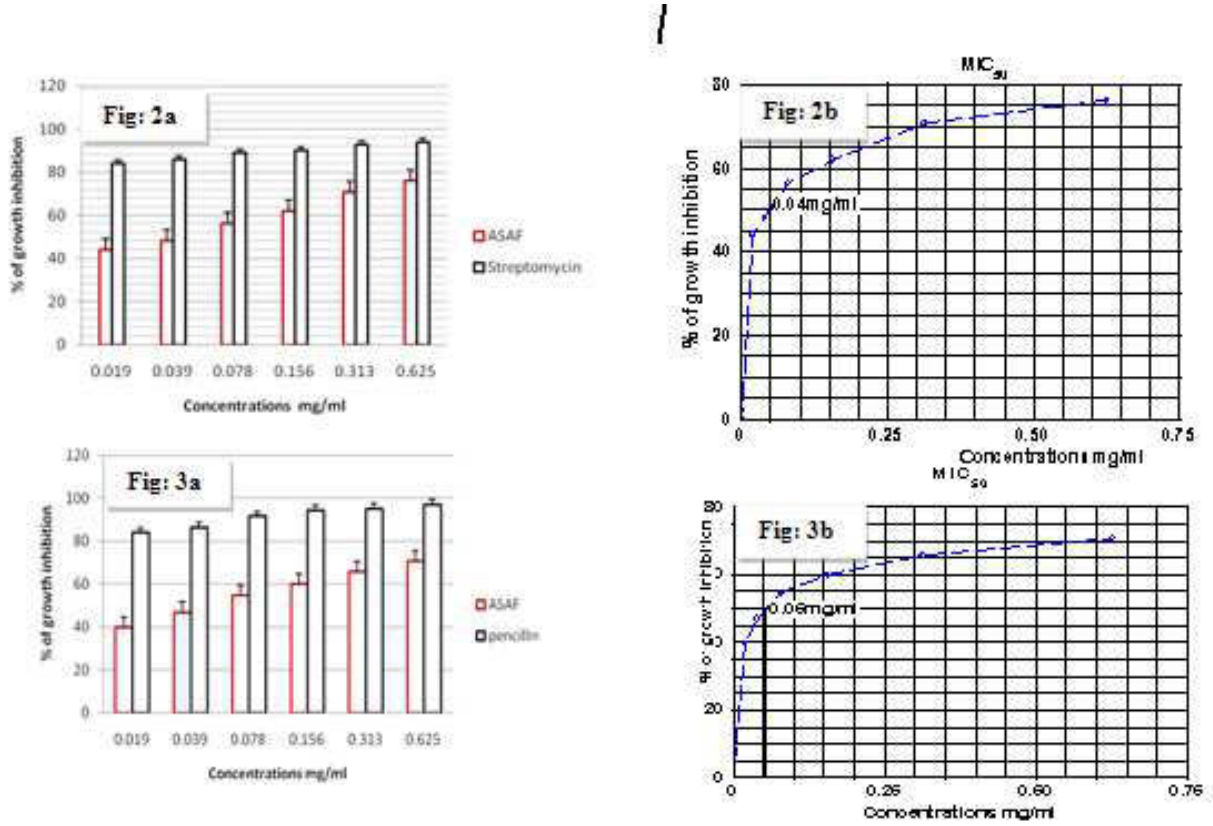

Figure 2

(a) % of growth inhibition by ASAF on Staphylococcus aureus at 24 hours. N=4,mean ±SD. (b) Minimum inhibitory concentration 50 % of growth inhibition by ASAF on Staphylococcus aureus at 24 hours . 3(a) % of growth inhibition by ASAF on E.coli at 24 hours. N=4,mean ±SD. Figure (b) Minimum inhibitory concentration 50 % of growth inhibition by ASAF on E.coli at 24 hours.

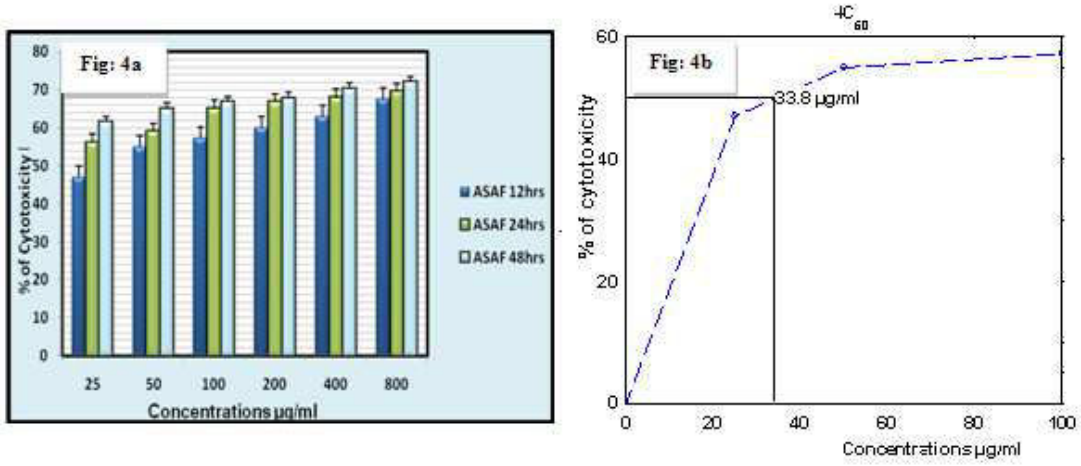

Figure 4

(a): % of ASAF on human cancer cell line KB at 12, 24 & 48hours. N=4,mean ±SD. (b) IC 50 value of ASAF on human cancer cell line KB at 12hours.

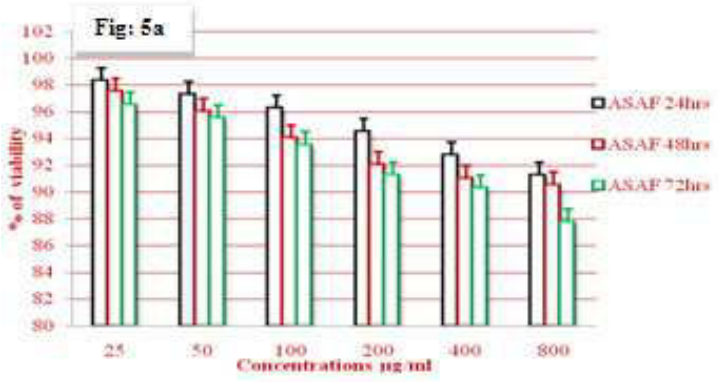

Figure 5
Lymphocyte viability assay of ASAF on normal human lymphocyte .

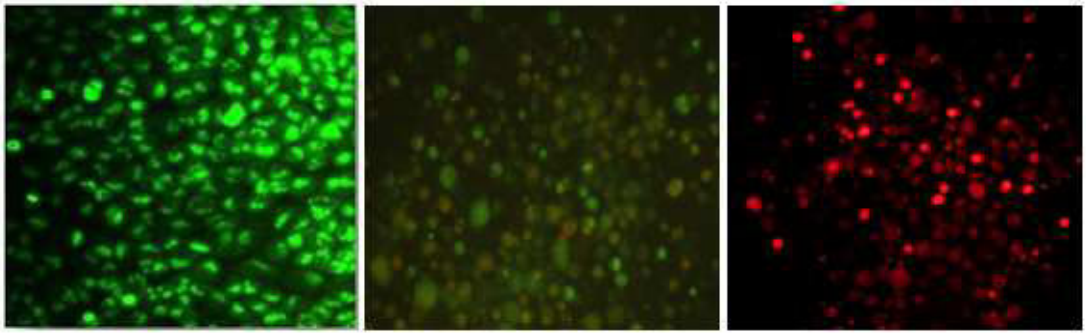

Figure 6
Acridine orange/ ethidium bromide double staining to analyze the apoptotic morphology of ASAF treated tumor cells for 24 hours. (a) Untreated KB control cells (b) KB cells treated with ASAF 15µg/ml. (c) KB cells treated with ASAF 30µg/ml. Viable cells green in colour. Late apoptotic cells red in colour under fluorescent microscopy.

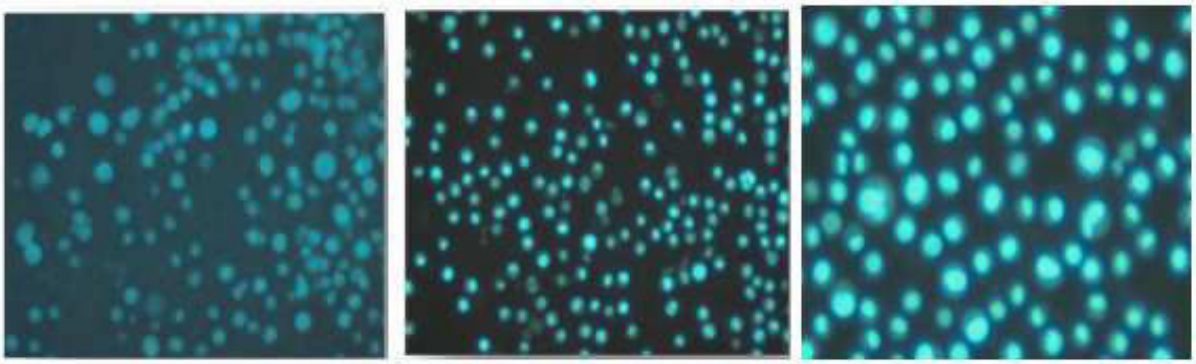

Figure 7
Hoechst staining: ASAF induced apoptosis in KB cells for 24 hours stained with Hoechst 33342 (10µM). (a) Untreated KB control cells (b) KB cells treated with ASAF 15µg/ml. (c) KB cells treated with ASAF 30µg/ml and examined by fluorescence microcopy.

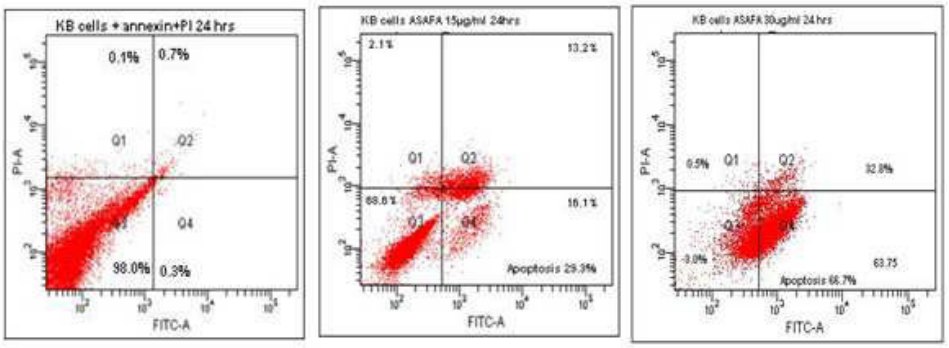

Figure 8

Flow cytometric analysis of Annexin V staining in KB cells treated by ASAF for 24 hours. (a) Untreated KB control cells (b) KB cells treated with ASAF 15µg/ml (c) KB cells treated with ASAF 30 µg/ml . Flow cytometry profile represents Annexin-V-FITC staining in x axis and PI in y axis. The number represents the percentage of early apoptotic cells (lower right quadrant) and late stage (upper right quadrant).

DISCUSSION

Annonaceae plants have received much attention, since they produce many complex compounds that are useful as antioxidants, antimicrobial and antiproliferative agents. In the present study, we have assessed the antioxidant, antibacterial and antiproliferative activity of the active fraction from the seeds of *Annona squamosa*. DPPH assay was carried out to measure the ability to remove or scavenge free radicals and the results showed that RSA_{50} of ASPE was 70.5 µg/ml. DPPH method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound[13]. This method utilizes the stable 1,1–diphenyl–2– picrylhydrazyl (DPPH) radical. It is based on the reduction of ethanolic solution of coloured free radical DPPH by free radical scavengers. Earlier reports on the ethanolic extract of the bark of *Annona squamosa* showed significant antioxidant activity using DPPH radical scavenging activity[16]. In our study the results of Broth dilution assay indicated that the ASAF was effective in inhibiting gram positive S. *aureus*, and gram negative E.coli. Earlier studies by Jayshree et al[8] also showed antibacterial activity to the leaves of *Annona squamosa*. Antimicrobial agents are very important in the treatment of cancer to prevent the secondary infections. Many phytochemicals acting as antimicrobial agents in the plant's defence are likewise active against human pathogenic organisms, and various studies report on the antimicrobial activities of crude plant extracts[22].Cell viability assay revealed antiproliferative activity to ASAF. In the present study, we have been using MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay to detect the anti-proliferative activity of Active fraction of *Annona squamosa* in Nasopharengeal carcinoma cells. We observed that ASAF (25 – 800µg/ml) at 12, 24 and 48 hours caused marked cell growth inhibition in a dose- and time-dependent manner, meanwhile there is no toxic effect in human normal lymphocytes. These studies indicated the viability of MTT assay as a tool for biomonitoring the phytochemical studies, conveniently providing drug sensitivity information[6,15]. In this study we found that ASAF of the plant belonging to the Annonaceae family has compounds in it which has the ability to kill tumor cells. Analysis of cell death using fluorescence microscopy indicates that ASAF causes several morphological changes to the tumor cell as a prelude to cell destruction. Acridine orange and ethidium bromide dual staining revealed the characteristic features of apoptosis like chromatin condensation, nuclear fragmentation and membrane blebbing.The Hoechst dye stain the DNA and

allow visualization of condensed chromatin of apoptotic cells. The number of cells manifesting morphologic features of apoptosis such as chromatin condensation and the loss of nuclear envelope were observed. It is mainly used for the identification of late apoptotic cells. The outstanding feature of apoptosis is its remarkable stereotyped morphology, showing condensation of nuclear hetero chromatin, cell shrinkage, loss of positional organization of organelles in the cytoplasm and membrane blebbing [9,10,25].Flow cytometry data of Annexin V staining confirmed that ASAF could induce apoptosis on KB cells. During apoptosis, phosphatidyl serine becomes expressed on the cell surface where it is recognized by receptors expressed by phagocytic cells. Earlier studies showed that organic and aqueous extracts from the seeds of *A. squamosa induced apoptosis in* MCF-7, breast carcinoma and K-562, erythro leukemia and COLO-205 colon carcinoma cells. Treatment of these cells with the extract resulted in the induction of reactive oxygen species (ROS) generation and reduce intracellular glutathione levels. In addition down regulation of BCl-2 and phosphytidyl externalization by Annexin V staining suggested induction of apoptosis . The extracts from *Annona squamosa* also caused significant apoptotic induction in a rat histiocytic tumor cells AK-5 cells [19 20]. These studies reveal that the extract from *Annona squamosa* seeds induces cell death by apoptosis. The apoptosis induction property of the compound is a hallmark to its cytotoxic potential. Induction of apoptosis in cancer cells is recognized as an efficient strategy for cancer chemotherapy.

CONCLUSION

Annona squamosa seed extract showed significant antioxidant activity, antimicrobial activity and antiproliferative activity. This study suggests that the active fraction isolated from the seed of *Annona squamosa* is a promising candidate to be exploited further to develop as pharmacologically active agents.

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