Int J Pharm Bio Sci 2013 Oct; 4(4): (B) 682 - 687

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

CYTOTOXIC AND APOPTOTIC EFFECT OF *ANNONA SQUAMOSA* SEED EXTRACT ON HUMAN AND MURINE TUMOR CELLS.

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ABSTRACT

Background/Aim: The aim of this study was to evaluate the cytotoxic and apoptotic effects of chloroform extract (ASCH) from the seeds of *Annona squamosa.* Materials and Methods: MTT assay was employed to determine the cytotoxicity of the extract on A-549 and EAC cells and IC_{50} values were calculated. The apoptotic effects induced by the ASCH was evaluated by Acridine Orange - Ethidium Bromide dual staining and Hoechst staining by Fluorescence microscopy. Results: The ASCH extract 400 µg/ml exhibited maximum cytotoxic effects of 54±1.2 and 56± 0.9 at 48 hours on human Lung adenocarcinoma cells (A-549) and murine tumor (EAC) cells respectively. IC_{50} values were 319 µg/ml and 312 µg/ml for A-549 cells and EAC cells respectively. Acridine Orange Ethidium Bromide and Hoechst staining exhibited apoptotic features such as chromatin condensation and nuclear fragmentation after treatment with the extract. Conclusions: These results suggest that the chloroform extract from *Annona squamosa* seeds have cytotoxic and apoptotic effect.

KEY WORDS: ***Annona squamosa*, murine cell, Apoptosis, Cytotoxicity**

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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[1]. The indigenous system of medicine has several plants with versatile pharmacological activities. Many of these medicinal plants have been found to be very effective in experimental as well as clinical cases of tumour therapy. The earlier studies on Annonaceae plants showed that this family is a potent source of a wide variety of secondary metabolites belonging to several categories. Different parts of *A. squamosa* are used in folkloric medicine for the treatment of various diseases[2]. Our earlier studies showed that *Annona squamosa* seed extracts contain several bioactive phytochemicals with antibacterial, antioxidant and cytotoxic properties[3,4]. In the present study we report the effect of *Annona squamosa* seed chloroform extract and its cytotoxic and apoptotic effect on Human adenocarcinoma cells (A-549) and murine tumor (EAC) cells.

MATERIALS AND METHODS

Preparation of seed extract

The seeds of *Annona squamosa* were collected from Thiruvananthapuram district, Kerala state, authenticated by 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 chloroform as the solvent and concentrated by using rotatory evaporator. The *Annona squamosa* seed chloroform extract (ASCH) was dissolved in dimethyl sulfoxide (DMSO) and used for the experiments.

Tumor cell lines

The human lung adenocarcinoma cell line (A-549) 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. Ehrlich's Ascites Carcinoma (EAC) cells were maintained as ascites tumor in Balb/c mice.

Evaluation of cytotoxicity on tumor cells by MTT assay.

The tumor cells (A-549 & EAC) were cultured in 96 well plates with various concentrations of the ASCH and incubated for 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[2]. The optical densities were measured at 570 nm and cytotoxicity was calculated. IC_{50} values were calculated using easy plot software.

Acridine orange (AO) and ethidium bromide (EB) dual staining.

A-549 cells and EAC cells were treated with ASCH 50 µg/ml and incubated for 48 hours. To detect the morphological features of apoptosis Acridine orange (100 µg/ml) mixed with ethidium bromide (100 µg/ml) in 1x PBS solution was added to each well of microtitre plate, and the apoptotic cells were observed under the fluorescent microscope[5].

Hoechst 33342 staining

The ASCH treated cells (50 µg/ml) for 48 hours were 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.

Statistical analysis

The results are represented as the mean + SD. The data was analysed by using Excel and Easyplot.

RESULTS

Effect of ASCH on tumor cell lines

The ASCH extract 400 µg/ml at 48 hours exhibited maximum cytotoxic effects 54% and 56% towards A-549 and EAC cells respectively. There was a dose dependent

increase in the percentage of growth inhibition. (Fig.1, Fig.3). The IC_{50} value of ASCH on A-549 cells & EAC cells were observed as 319µg/ml and 312 µg/ml respectively (Fig.2 & Fig.4).

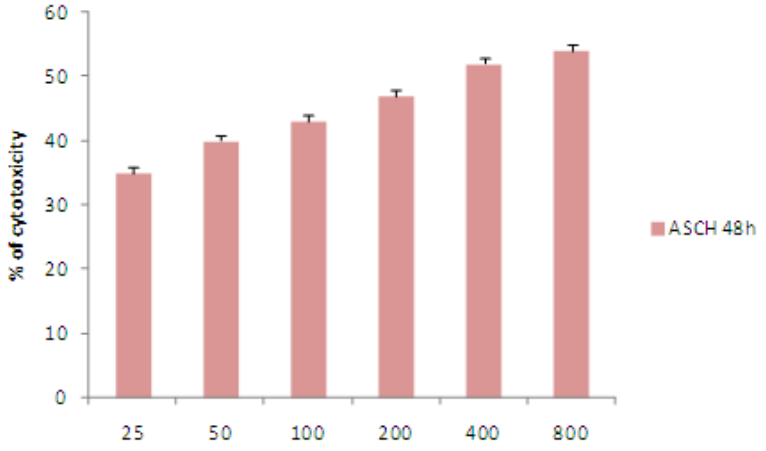

Figure 1
Percentage of cytotoxicity on A-549 cells at 48hours. N=4, mean ±SD.

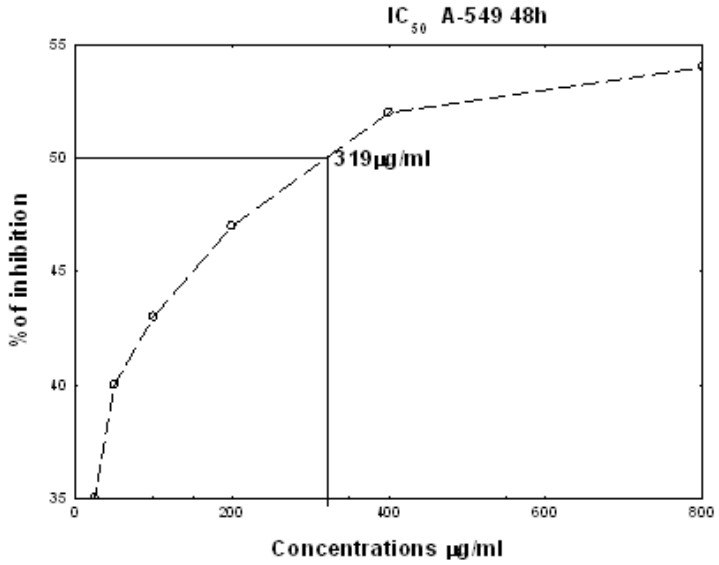

Figure 2
IC 50 value of ASCH on A-549 cells at 48hours.

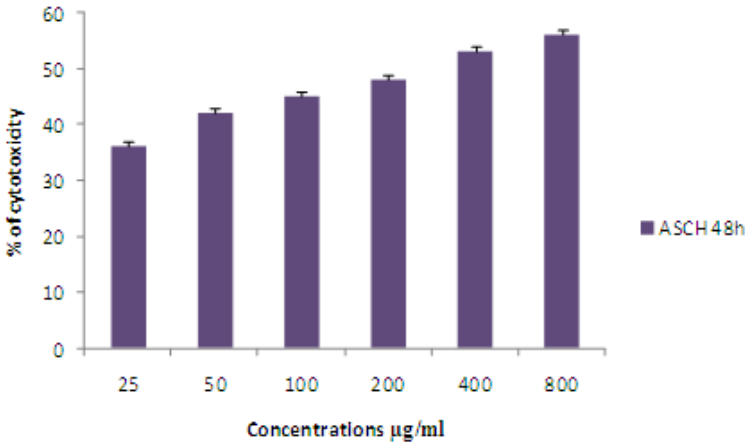

Figure 3
Percentage of cytotoxicity on EAC cells at 48hours. N=4, mean ±SD.

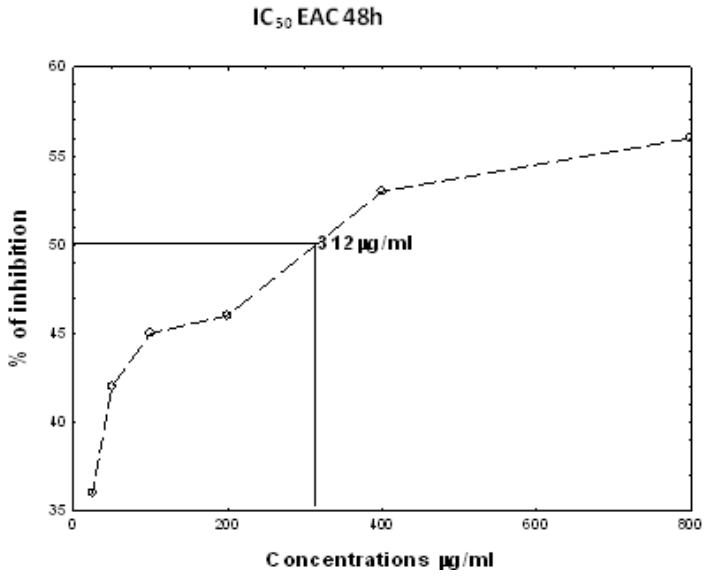

Figure 4
IC $_{50}$ value of ASCH on EAC cells at 48hours.

ASCH induced Cell Death (Apoptosis) in A-549 and EAC cells

Acridine Orange - Ethidium Bromide dual staining revealed the presence of apoptotic bodies in both A-549 cells and EAC cells after treatment with 50 µg/ml concentration. The treated cells exhibited membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation, the characteristic features of apoptosis (Fig.5).

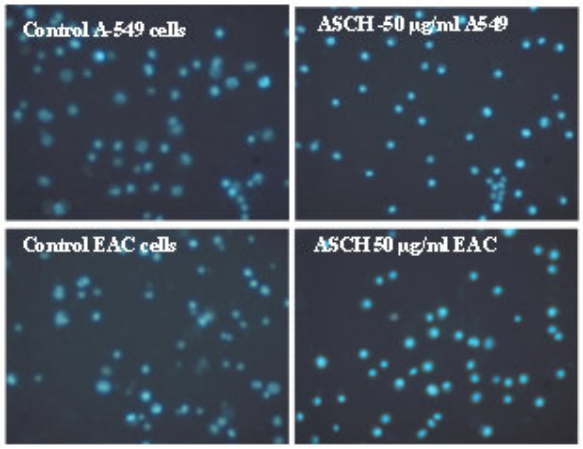

Figure 5
Effect of ASCH on A-549 cells and EAC cells (48 hours treatment) Hoechst staining.

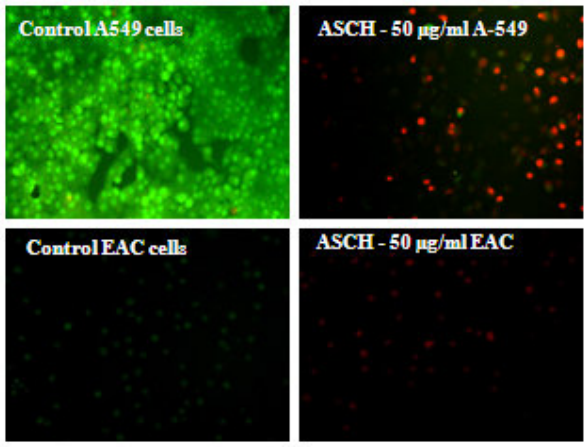

Figure 6
Effect of ASCH on A-549 and EAC cells (48 hours treatment)
Acridine orange Ethidium bromide dual staining.

Apoptosis detection by Hoechst staining
By Hoechst staining, the control cells appeared light blue in colour and the apoptotic cells appeared fluorescent blue in colour, showing chromatin condensation and DNA fragmentation after 48 hrs of incubation at a concentration of 50 µg/ml in A-549 cells and EAC cells (Fig.6).

DISCUSSION

In the present study we have evaluated the apoptotic and cytotoxic properties of *Annona squamosa* seed chloroform extract. Chloroform extract showed significant cytotoxicity towards human and murine cells. IC_{50} values were found to be 319 µg/ml and 312 µg/ml on A-549 and EAC cells respectively. Fluorescent staining by Acridine Orange Ethidium Bromide dual staining revealed the characteristic features of apoptosis such as membrane blebbing, chromatin condensation and nuclear fragmentation in the treated A-549 and EAC cells. By Hoechst staining fluorescence in the nuclear region of the treated cells, indicated the presence of DNA condensation and nuclear fragmentation. Apoptotic bodies were seen as small fluorescent masses. Earlier reports showed cytotoxic effects of *A. squamosa* fruit pericarp extract on Dalton's lymphoma cells and HeLa cells[6]. Ethanolic herbal extract residue of the seeds of *Annona squamosa* Linn. were tested against Dalton's Lymphoma Ascites (DLA) tumour cells[7]. In our previous study we have shown the apoptotic

effects of active fraction from the petroleum ether extract on Human Nasopharyngeal (KB) cells [8]. Apoptosis was assessed based on a distinct sequence of morphologic features by electron microscopy, described in 1972 by Kerr et al [9,10]. The family Annonaceae, which include *Annona squamosa* is gaining a lot of importance for its therapeutic potentials. Many compounds isolated from different members of this family have been previously reported to have anticancer properties. Literatures of many research works prove that every parts of *A. squamosa* possess medicinal property[11]. Our study shows that the cytotoxic effect of ASCH on human Lung adenocarcinoma (A-549) cells and murine tumour (EAC) cells is due to apoptosis.

CONCLUSION

The result showed that the chloroform extract from *Annona squamosa* seed*s* has the capacity to induce cytotoxicity and apoptosis on Lung adenocarcinoma cells and Ehrlic ascitic carcinoma cells. Further studies are warranted to exploit further to develop these compounds as a remarkable pharmacologically active agents.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Indian Council of Medical Research, New Delhi (Grant No. 59/47/2011/ BMS/TRM) for financial support.

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