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Research Article

EVALUATION OF ANTITUMOR PROPERTIES OF *ANNONA RETICULATA* (L.) SEED ON CANCER CELL LINES

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ABSTRACT

In the present investigation a plant Annona reticulata L. Seed belonging to Annonaceae family was taken and sequential extraction was carried out using soxhlet apparatus and concentrated using rota vapour. The preliminary screening studies (MTT assay) carried out using K 562 showed that petroleum ether extract was found to be most active. Further cytotoxicity studies were done on K 562 and MCF-7. The result conferred that for K 562 maximum cytotoxicity was seen at 800µl and for MCF-7 it is 400µl. Cell death by apoptosis was confirmed by morphological changes shown during AO/EtBr dual staining and Hoechst 33342 staining. Lymphocyte viability assay was performed to check the toxicity of the extract on normal cells. Maximum viability (99.1%) was observed at concentration (100µg/ml) of extract.

KEYWORDS: *Annona reticulata, Antitumor, MTT Assay, Cytotoxicity, Apoptosis.*

INTRODUCTION

Cancer is one of the most life-threatening diseases and serious public health problems in both developed and developing countries. The loss of normal cell growth control is one of the main events in cancer development, followed by angiogenesis, metastasis, and apoptosis, considered hallmarks of cancer (Hanahan and Weinberg, 2000). Medicinal herbs are a significant source of active compounds that also could be used as lead compounds for new anticancer agents (Malhotra and Pal Singh, 2006). The development of the anti-proliferative screening by the U. S. National Cancer Institute (NCI) has contributed to the discovery of new natural anticancer agents. Nowadays over 60% of all anticancer drugs have their origin directly or indirectly from natural resources (Cragg and Newman, 2009).

Treating cancer with plant extracts has been shown to be an easy and effective approach in China. *Coix lacrymajobi* (adlay) seed oil could enhance the antitumor activity of *Ganoderma lucidum* triterpene loaded micro-emulsions (Qu *et al*., 2014). *Brucea javanica* oil (BJO) (Yan *et al*., 2015) has been widely used for treatment of lung cancer (Nie *et al*., 2012). The fatty acid methyl esters obtained from *Annona cornifolia* showed significant antifungal, antioxidant and cytotoxic potential activities (Lima *et al*., 2011, 2012).

Annona reticulata Linn. Commonly known as custard apple is a traditionally important plant used for the treatment of various ailments. Traditionally plant is used for the treatment of dysentery, fever and as insecticide (Jamkhande and Wattamwar, 2015). Different plant parts are effectively employed as folk medicine in the rural area of India. The plant is rich in several phytochemicals such as dopamine, salsolinol, coclaurine, sesquiterpenes and acetogenin (Jamkhande *et al*., 2015). It also contains minerals such as Ca, P, K, Mg, Na, Cl, S, Mn, Zn, Fe, Cu, Se, Co, Ni and Cr (Jamkhande and Wattamwar, 2015). In account with the medicinal properties, *Annona reticulata* Linn. is targeted for the investigation of anticancer property. This paper describes the screening of seed extracts of *Annona reticulata* for antitumor activity against cell lineages of different types of leukaemia cell line K562 and breast cancer cell line MCF-7 using the MTT cell viability assay.

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MATERIALS AND METHODS

The medicinal plant of *Annona reticulata* seed was shade dried and powdered. This powder was used for sequential solvent extraction. The shade dried and powdered plant materials (50g) were extracted by using three different solvents i.e., petroleum ether, (Boiling point 40^{0}C to 60^{0}C), Chloroform (Boiling point-62^{0}C) and methanol (Boiling point -65^{0}C). The extracts were concentrated by using rota vapour. 10 mg of concentrated extracts was taken and dissolved in 200µl 10% DMSO and made up to 2 ml in media and for analyzing *in vitro* cytotoxicity. DMEM medium was prepared and 10% FCS was mixed with medium before used for culture.

A-549 (Human lung adenocarcinoma cell line), K562 (leukaemia cell line) and breast cancer cell line MCF-7 were obtained from National Centre for Cell Sciences, Pune, India. All lineages were maintained in the logarithmic phase of growth in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 100 U mL-1 penicillin and 100 mg mL-1 streptomycin (GIBCO BRL, Grand Island, NY) enriched with 2 mM of L-glutamine and 10% (leukemic cells) or 5% (adherent cells) of fetal bovine serum. All cultures were maintained at 37 ºC in a humidified incubator with 5% CO_2 and 95% air. The media were changed twice weekly and they were regularly examined.

Evaluation of the cytotoxic effect against human tumor cell lines:

The *in vitro* response of crude seed extract against K562 cell line was studied using MTT assay. The K562 cell line was maintained in DMEM medium, supplemented with 10% FCS. Briefly cells in the log

phase of growth were harvested, counted and seeded (5×10^3 cells/well in 100µl) in 96 well titre plates (Axygen). PBS was added to outer wells (200µl/well). Then cultures were treated with varying concentrations of drugs 1-100 µg /ml diluted with medium. The vehicle controls were also kept that is cells and media .The plates were further incubated for 48 hours. On completion of incubation, with the drugs, media were removed without disturbing the cells, and to each cell, 100µl of 5mg/ml stock solution of MTT were added and plates were further incubated for 2 hours in dark at 37^0C in a CO_2 incubator. 100µl of lysis buffer were added to each well and the plates were further incubated for 4 hours in dark in a CO_2 incubator using multiple reader. 3 replicates were maintained for each concentration. The concentration required to reduce absorbance by 50% IC_{5o} values were derived by substituting percentage inhibition values of drugs against the closes used.

% Of Growth Inhibition =100 - Absorbance of treated cells x 100
Absorbance of control cells

MTT (3-4, 5-dimethylthiazol-2yl-2, 5 -diphenyl tetrazolium bromide) assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal which is largely impermeable to cell membrane, thus resulting in its accumulation with in healthy cells.

Isolation of lymphocytes from whole blood (Boyum, 1968):

3 ml of blood was taken in a heparinised test tube; to this 5 ml of PBS mixture was added and mixed well by inversion. This 8 ml of blood PBS mixture was then carefully layered onto 3 ml of ficoll hypaque solution taken in the conical centrifuge at 2000 rpm for 30 minutes. After centrifugation, the opaque interface containing mononuclear cells was aspirated and transferred into a clean conical centrifuge tube with a Pasteur pipette and discarded the upper layer. Further 10 ml of PBS solution was added, mixed by inversion and centrifuged at 1500 rpm for 10 minutes, the supernatant was discarded, and the pellet was re-suspended in 500 µl PBS.

Cytotoxicity studies on normal human lymphocytes - Lymphocyte viability assay:

Lymphocyte assay was used to study the *in vitro* response of AR extract against lymphocyte. From the gradient plasma interphases lymphocytes used were aspirated, washed twice with PBS and the final cell pellets were re-suspended in DMEM medium containing 10% FCS, 100µl fungicide (pH-7.4).

Cells were harvested, counted and seeded (5×10^3cells per well in 100 µl) in 96 well titre plates (Axygen) and PBS were added to the outer wells (200µl/well). After 24 hours incubation at 37^0C in 5% CO_2 to allow cell attachment, media were removed; cultures were treated with various concentrations of diluted with medium. Negative controls were also kept (cells with media). The plates were further incubated for 72 hours. On completion of incubation, with the drugs, media were removed without disturbing the cells and to each well, 100µl of 5 mg/ml stock solution of MTT were added and plates were further incubated for 2 hours in dark at 37^0C in a CO_2 incubator. 100µl of lysis buffer was added to each well, and the plates were further incubated for 4 hours in dark in a CO_2 incubator and absorbance was read using ELISA plate reader. There replicates were setup for each concentration.

Morphological determination of apoptotic cells:

For assessment of apoptosis after treatment of DLA cells for 24 hours, Acridine orange/Ethidium bromide dual staining of K562 cells was used. The mixture of fluorescent dyes consisted of acridine orange/ethidium bromide at 5µg/ml in PBS. These dyes stain the DNA and allow visualization of the condensed chromatin of apoptotic cells. Slides were observed under a fluorescent microscope. Acridine orange was observed using standard narrow band FITC excitation (excitation wavelength 450-490nm and barrier filter 520-560nm). Ethidium bromide only stains cells in late stages of apoptosis and secondary necrosis when membrane integrity has been lost. Early apoptotic cells are impermeable to the dye. The early stages of apoptosis are readily detectable using acridine orange (Hague *et al*., 1997).

Cells are cultured in test tubes (1×10^6 K562 cells) and incubated in DMEM medium with 10% FCS containing various concentrations of the petroleum ether extract in a CO_2 incubator at 37^0C for (Kirsch *et al*, 1997). For assessment of apoptosis during 3 hrs and 18 hrs after exposure to different concentration of drug treatment, Acridine orange, Ethidium bromide dual staining of unfixed DLA cells was used. These dyes stain the DNA and allow visualization of the condensed chromatin of apoptotic cells. The medium was removed the gel pelleted gently and 1µl of acridine orange (100 g) and Ethidium bromide in 1ml PBS was added to cells and immediately washed ones with buffer saline(PBS) re-suspended in 10µl of 10% glycerol in PBS and the slides analyzed by fluorescent microscopy (Nikon Diaphot, UV 410).The number of cells manifesting morphologic features of apoptosis such as chromatin condensation and the loss of nuclear envelope were counted as the function of total number of cells present in the field (Kerr *et al*.,1972).

Analysis of Cell Death (Apoptosis) Using Fluorescence Microscopy by Hoechst staining:

For assessment of apoptosis after treatment of K562 cells for 24 hours, Hoechst staining of unfixed cells was used. Hoechst stains are fluorescent DNA stains that bind preferably to A-T base pairs. This dye stains the DNA and allows visualization of condensed chromatin of apoptotic cells. Slides were observed under a fluorescent microscope. The dye is excited by UV light around 350nm and emits blue/cyan fluorescent light. It is mainly used for the identification of necrotic and late apoptotic cells.

Cells were cultured in test tubes. $1x10^6$ cells were incubated in DMEM medium with 10% FCS containing various concentrations of petroleum ether extract in a CO_2 incubator at 37ºC for 24 hours of incubation (Kirsch *et al.,* 1997). For assessment of apoptosis, 48 hours after exposure to different concentration of drug treatment, stained with 5 µM Hoechst 33342. These dyes stain the DNA and allow visualization of condensed chromatin of apoptotic cells. The number of cells manifesting features of apoptosis such as chromatin condensation and the loss of nuclear envelope (Kerr *et al*., 1972) were counted as the function of total number of cells, present in the field.

RESULTS AND DISCUSSION

The sequential extraction of the powdered plant material with a selected panel of solvents gave four extracts namely ARP (petroleum ether extract), ARC (Chloroform extract), ARE (Ethyl acetate extract) and ARM (methanol extract). These four extract were further used for screening cytotoxicity against leukaemia cell line K562 and breast cancer cell line MCF-7 by MTT assay.

***In-vitro* cytotoxicity studies:**

Among the four solvent extract, Petroleum ether extract showed the maximum cytotoxicity (Table: 1) on K562 cell lines. Since petroleum ether extract showed the highest activity further studies were conducted using this on K562 and MCF-7 cell lines.

At lowest concentration of 50µg/ml the cytotoxicity effect was 45%, 70% in MCF-7 and K562 respectively in 48 hrs. In K562 cell lines the maximum cytotoxic effect 76% was observed 800µg/ml at 48 hrs. Where as in MCF-7 cell lines, at 400µg/ml concentration (48hrs) the maximum cytotoxic effect 61% was observed. The IC_{50} of MCF-7 was found to be 88.33µg/ml (figure 1, 2, and 3).

Comparison between Suspension and Adherent cell line:

A comparative study in the cytotoxic effect of the extract against suspension cell lines (K562) and adherent cell lines (MCF-7) is given in (Fig 4). K 562 cell lines showed maximum cytotoxicity of 76% (800µg/ml) at 48 hrs, whereas MCF-7 cell lines showed maximum cytotoxicity of 61% (400µg/ml) at 48 hrs. Therefore among the two cell lines tested, K562 cells were found more sensitive to ARP and showed maximum cytotoxic effect at 400µg/ml concentration.

Lymphocyte viability assay:

To check the cytotoxicity of ARP on normal cells, normal human lymphocyte cell culture was set up and the cells were treated with different concentrations of ASP. Viability was evaluated by MTT assay. No significant cytotoxicity was observed (fig 5).

Table No. 1: Screening of K 562 cell lines by MTT Assay

Extract	% of cytotoxicity at different drug concentration				
	50µg/ml	100µg/ml	200µg/ml	400µg/ml	800µg/ml
ARP	43	45	50	55	60
ARC	20	22	25	30	35
ARE	18	19	20	23	30
ARM	15	17	20	22	25

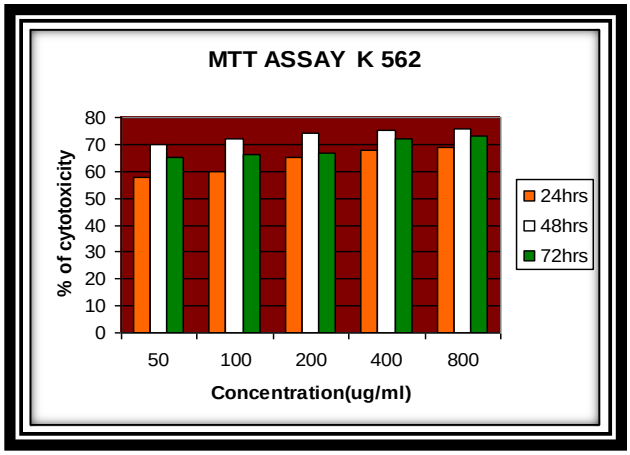

Fig. 1: ARP on K-562 – MTT assay

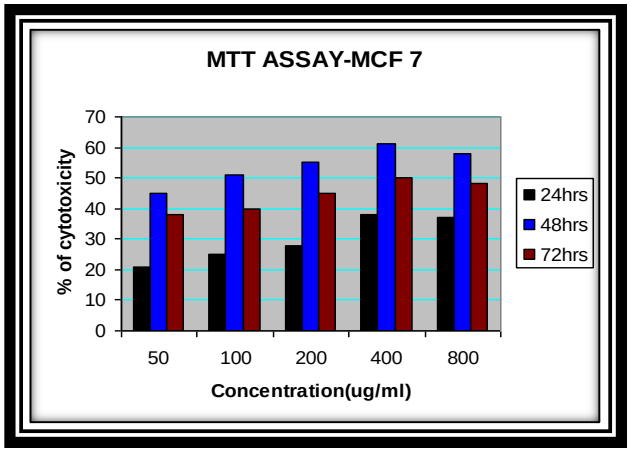

Fig. 2: MTT assay on MCF-7

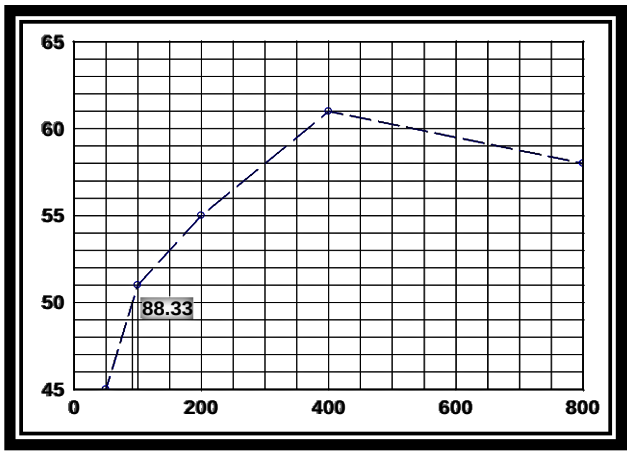

Fig. 3: IC_{50} value of MCF-7 cells

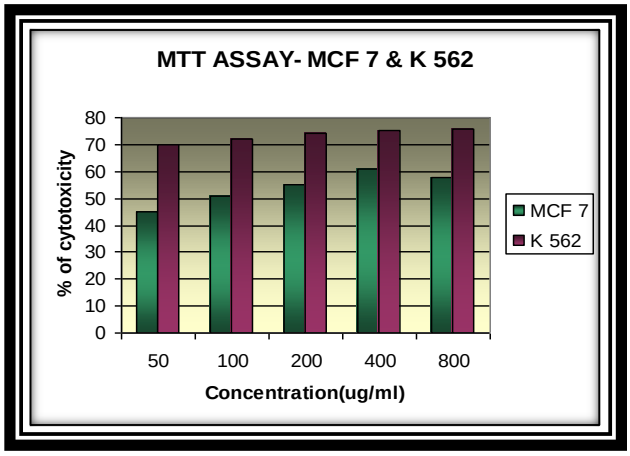

Fig. 4: Comparison between K562 & MCF-7 cell lines

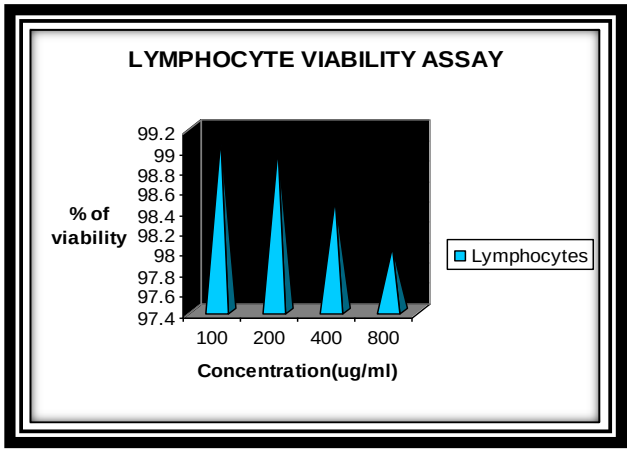

Fig. 5: Lymphocyte Viability Assay

Flouresence staining:

AO/EtBr dual staining:

AO/EtBr dual staining was used to detect morphological features of apoptosis on the ARP treated cells. On examination under fluorescent microscope viable cells were found to be green in colour and dead cells appeared orange in colour. Among the dead cells many of the cells showed condensed and membrane blebbing, which are characteristic feature of apoptosis (fig.6).

Hoechst 33342 staining:

Hoechst staining was also used for analyzing apoptosis. On the examination under fluorescent microscope cells undergoing apoptosis showed more fluorescence due to chromatin condensation and loss of nuclear envelope. While non-apoptotic cells were non fluorescent (fig.7).

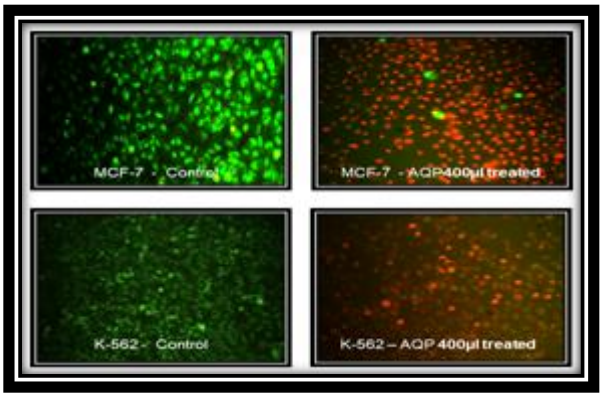

Fig. 6: AO/EtBr dual staining

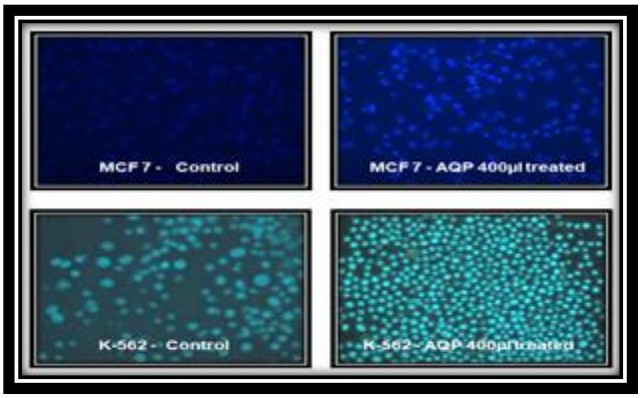

Fig. 7: Hoechst staining

CONCLUSION

Several previous studies showed that plant extract contains abundant number of phytochemicals which possess anticancer properties and might be responsible for anticancer effect of these plants extract (Gaidhani *et al*., 2013). Plants of Annonaceous family are rich in acetogenins, an anticancer property phytochemical (Jamkhande *et al*., 2015). The presence of acetogenin may be attributed to anticancer effect of petroleum ether extract of *Annona reticulate* seed.

The present study was carried out to explore anticancer potential of petroleum ether extract of *Annona reticulata* seed. Maximum anticancer activity was observed against leukemia cell line K562 and breast cancer cell line MCF-7 which might be correlated to its acetogenins and other anticancerous phytochemicals. In future, work will be needed to investigate the mechanisms of action and active principles of *Annona reticulate* may contribute to the development of novel drugs for cancer therapy in the future.

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