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Research Article

ASSESSMENT OF ANTINEOPLASTIC POTENTIAL OF *ANNONA RETICULATA* LINN. ON HUMAN CANCER CELL LINES

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ABSTRACT

Objective: The present study was undertaken to establish the antineoplastic potential of *Annona reticulata* on human cancer cell lines, *viz.,* squamous cell carcinoma 9 (SCC9), MCF-7 (human breast adenocarcinoma), A549 (lung adenocarcinoma), and HCT116 (colorectal carcinoma) cells.

Methods: The study was performed through MTT, cell cycle (G2M), and DNA fragmentation assays. MTT assay was taken as the platform assay for cytotoxicity and cells inhibited in MTT assay were subjected to cell cycle analysis and DNA fragmentation, respectively.

Results: The extracts showed dose dependant growth inhibition of SCC9 , MCF-7, A549 and HCT116 cells. In contrast to HCT116 and SCC9 cells, which exhibited cytotoxicity at a higher concentration with IC50 value of 149.2 and 168.4 µg/ml, the extract treated A549 and MCF-7 cells exhibited significant cytotoxicity at a lower concentration with IC50 value of 86.4 and 92.1 µg/ml respectively. The treated A549 and MCF-7 cells showed cell cycle arrest up to 7.07% and 14.42% respectively, at G2/M phase of cell cycle. DNA fragmentation was not observed in both A549 and MCF-7 cells treated with *A.reticulata* extract at a concentration of up to 320 µg/ml.

Conclusion: Encouraging preliminary results emphasize the necessity for further research on characterization of individual compounds from this extract and advocate it as a good source of anticancer agent for certain types of cancer.

Keywords: *Annona reticulata*, Anticancer activity, MTT, G2M and DNA fragmentation.

INTRODUCTION

Cancer is one of the most dreaded diseases of the 20th century and increasing incidentally in 21st century. It is inevitably one of the most studying, but yet unsolved non-communicable human disease and a group of disease characterized by uncontrolled cell division leading to the abnormal growth of the tissue [1]. It is an idiopathic disease for which doctors and scientist are constantly trying to evolve new effective drug for its treatment. There is no other disease which parallels cancer in diversity, its origin, nature, and treatment. International Agency for Research on Cancer estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and aging of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025. Rise in incidence of lung, breast, oral, and colorectal cancer are a cause of concern. The most commonly diagnosed cancers worldwide were those of the lung cancer (1.8 million, 13.0% of the total), breast cancer (1.7 million, 11.9%), and colorectal cancer (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) [2]. Oral squamous cell carcinoma (SCC) is one of the most common malignant tumors in human; it is the fifth most common malignancy worldwide and a major cause of cancer morbidity and mortality in India representing approximately 40-50% of all cancers. The highest incidence rates have been observed in the Indian subcontinent [3].

Chemotherapy, being a major treatment modality used for the control of advanced stages of malignancies and as a prophylactic against possible metastasis, exhibits severe toxicity on normal tissues. The side effects of such drugs make it a necessity for a new improved drug. Herbal medicines have been used since the dawn of civilization to maintain health and to treat various diseases. They maintain the health and vitality of individuals and also cure diseases including cancer without causing toxicity [4].

Annona reticulata, commonly known as bullock's heart or ramphal plant, is widely distributed all over India. They are rich in annonaceous acetogenins. Acetogenins from the leaves were found to be selectively cytotoxic to certain human tumors [5].

Leaf contains anonaine, roemerine, norcorydiene, corydine, norisocorydine, dienone isocorydine, norlaureline, glaucine hyperoside, rutin and quercetin, n-hexacosanol, n-octacosanol, n-triacontanol, 16 hentriacontanone, campsterol, stigmasterol, and sitosterol. Leaf also contains essential oils, carvone, linalool, and (+) O-methyl armepavine [6]. However, species of the Annonaceae family have also been targeted for investigation due to appurtenant substances in the acetogenins, a class that has been isolated from different parts of the plant [7]. The present study is aimed at establishing the antineoplastic potential of *A. reticulata.*

METHODS

Fresh leaves of *A. reticulata* were collected from Vadamalapeta, Chittoor district, Andhra Pradesh, and it was authenticated at National Herbarium of Medicinal Plants and Repository of Raw Drug, Foundation for Revitalization of Local Health Traditions University, Yelahanka, Bengaluru.

Preparation of crude leaf extract

Leaves were shade dried and powdered, 5 g leaf powder of *A. reticulata* was extracted with methanol using Soxhlet apparatus for 4 hrs, and it was concentrated using Rota evaporator (Rotavap PBV-7D).

MTT assay

70-80% confluent cells were collected, viability was checked and cells centrifuged. Wells were seeded with 50,000 cells/well of SCC9, MCF-7, A549, and HCT116 in a 96 well plate, respectively, and incubated for 24 hrs at 37°C in an incubator with 5% CO_2. Sample to be tested was from 0 to 320 µg/ml (2 fold variation) concentration in DMEM

or RPMI-1640 media without FBS and incubated for 24 hrs. Add 100 µl/well (50 µg/well) of the MTT (5 mg/10 ml of MTT in 1X PBS) working solution to the respective wells and incubate for 3-4 hrs. After incubation with MTT reagent, MTT reagent was discarded by pipetting without disturbing cells, and 100 µl of DMSO was added rapidly to solubilize the formazan. Absorbance was measured at 590 nm using T. CAN plate reader [8-14].

Cell cycle (G2M) analysis

Culture 5 × 10^5 cells in a 6-well plate containing 2 ml of complete media. After overnight or confluent or 24 hrs of incubation, spent media was removed. Wash once with 1X PBS. Cells are starved with serum free media for 24 hrs. After 24 hrs starvation, reticulata extract was added in 1 ml/well media containing serum for 24 hrs in SCC9 and MCF-7 cells, respectively. After 24 hrs of treatment, media was removed, washed once with 1X PBS, and finally cells were collected by Trypsin-ethylene diamine tetraacetic acid (EDTA) (Collect both floating and adherent cells). At room temperature, Pellet 1-5 × 10^5 cells/ml at 1500 rpm for 5 minutes and discard the supernatant. Resuspend the cell pellet gently with 2 washes in 1X PBS. Cell pellet was fixed overnight at 4°C in 1000 µl of fixing solution (containing 15% FBS and 15% PBS in 70% ethanol). Centrifuge at 1500 rpm for 5 minutes at room temperature and discard the supernatant. Cell pellet was washed two times with cold 1X PBS. Cells were incubated for 1 hr at room temperature in 500 µl of propidium iodide (PI) solution containing 0.05 mg/ml PI, 0.1 mm EDTA, and 0.05 mg/ml RNaseA in 1X PBS. The percentage of cells in various stages of cell cycle in compounds/samples treated and untreated populations were determined using FACS Caliber (BD Biosciences, San Jose, CA) and analyzed by Flow Jo 7.5.5 (Tree Star Ashland OR) [15-17].

DNA fragmentation or ladder assay

1.5×10^6 cells in a p-35 dish containing 2 ml of complete media were cultured, after 24 hrs of incubation, it was treated with 320 µg/ml of extract in 1 ml/well media without serum for 24 hrs in SCC9 and MCF-7 cells, respectively. After 24 hrs treatment, adherent and detached cells were harvested and washed with phosphate buffered saline (1X PBS). Cells were lysed with a lysis buffer composed of 50 mM Tris-HCl, 10 mM EDTA-4Na, and 0.5% sodium-*N*-lauroylsarcosinate (pH 7.8). The lysates were incubated in the lysis buffer containing 0.33 mg/ml RNase A at 50°C for 30 minutes and then further incubated in the lysis buffer containing 0.33 mg/ml proteinase K at 50°C for 30 minutes. Equal amount of DNA was electrophoresed on 2% agarose gel. Gels were stained with 0.5 mg/ml ethidium bromide for 15 minutes and visualized under UV light [18].

RESULTS

MTT assay

MTT assay was performed with SCC9 cells against ascending concentration of A.*reticulata* extract and measured at 590 nm, yielding IC50 value of 168.4 µg/ml (Fig. 1).

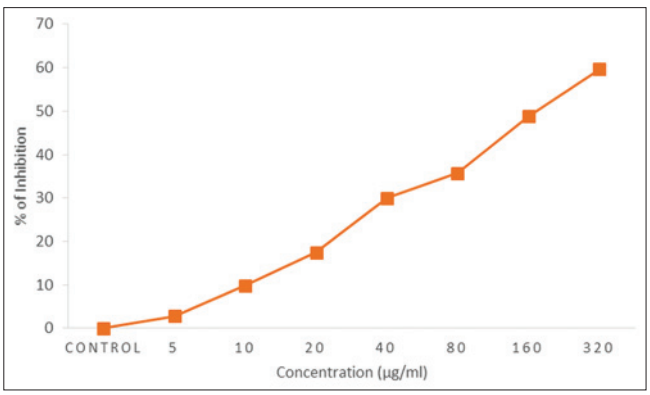

Fig. 1: Percentage of inhibition by *Annona reticulata* extract on SCC9 cells

MTT assay was performed with HCT116 cells against ascending concentration of A. *reticulata* extract and measured at 590 nm, yielding IC50 value of 149.2 µg/ml (Fig. 2).

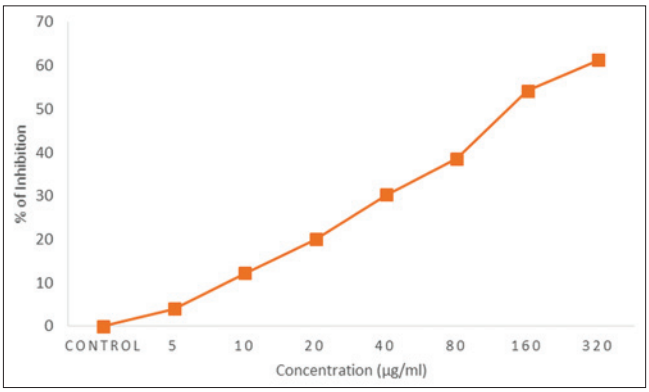

Fig. 2: Percentage of inhibition by *Annona reticulata* extract on HCT116 cells

MTT assay was performed with MCF-7 cells against ascending concentration of *A. reticulata* extract and measured at 590 nm, yielding IC50 value of 92.1 µg/ml (Fig. 3).

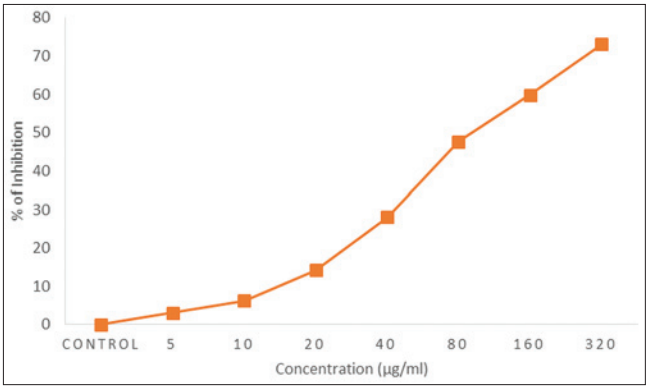

Fig. 3: Percentage of inhibition by *Annona reticulata* extract on MCF-7 cells

MTT assay was performed with A549 cells against ascending concentration of *A. reticulata* extract and measured at 590 nm, yielding IC50 value of 86.4 µg/ml (Fig. 4).

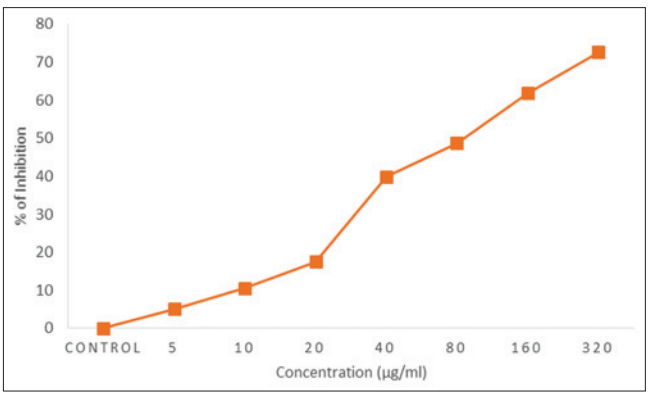

Fig. 4: Percentage of inhibition by *Annona reticulata* extract on A549 cells

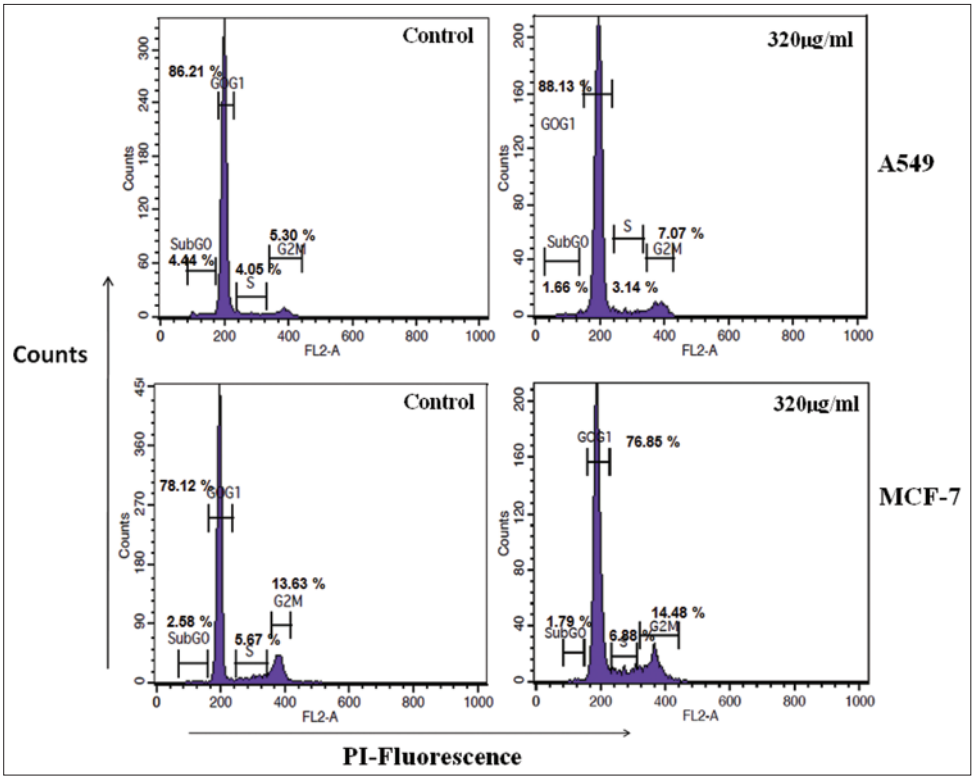

Fig. 5: Cell cycle (G2M) analysis showing percentage of cell cycle arrest

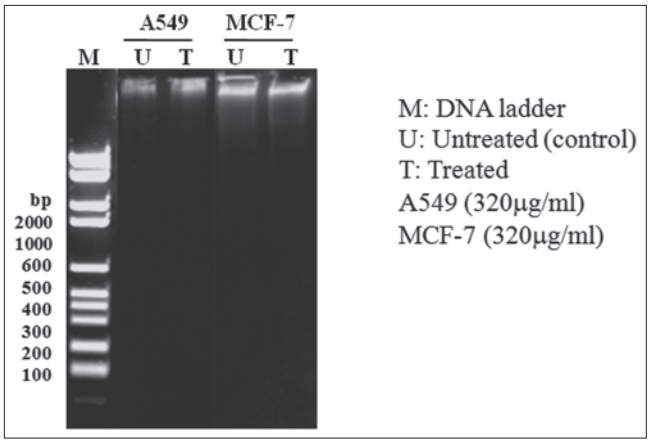

Fig. 6: DNA fragmentation in extract treated as well as untreated cells

Cell cycle analysis

A. reticulata extract treated A549 and MCF-7 cells showed cell cycle arrest up to 7.07% and 14.48%, respectively, at G2/M phase of cell cycle compared to control (untreated cells) (Fig. 5).

DNA fragmentation or ladder assay

DNA degradation was not observed in *A. reticulata* extract treated as well as untreated control A549 and MCF-7 cells showing a single band when electrophoresed in 2% agarose gel (Fig. 6).

DISCUSSION

Plant-derived compounds have played an important role in the development of several clinically useful anticancer agents, which might be effective in prevention, cure, and management of various cancers. The methanolic leaf extract of *A. reticulata* has shown significant cytotoxic potentials in some of the experiments employed in this study. Lesser side effects may make naturally occurring compounds a better choice than synthetic compounds. In many cases, the actual compounds isolated from the plants might not serve as the drug, but they serve as leads to the development of potential anticancer agents.

In spite of much progress in the treatment of cancer by the modern system of medicines and therapy using synthetic drugs, search for newer natural drugs continues because of several complications such as cell injury, bone marrow depression, impaired growth, sterility, and hair loss that are associated with the prevalent modern cancer chemotherapy. The phytochemical and pharmacological activities of *A. reticulata* components suggest a wide range of clinical application in lieu of cancer chemotherapy. Recent reports revealed that the plant exerted selective cytotoxicity and that the acetogenins present in the leaves are responsible for this specific cytotoxic effect.

Anticancer activity of *A. reticulata* leaf extract was studied on a various cancer cell lines. The extracts showed dose dependant growth inhibition of SCC9 (oral cancer), MCF-7(Breast cancer), A549 (Lung Cancer) and HCT116 (Colorectal Cancer) cells. In contrast to HCT116 and SCC9 cells, which exhibited cytotoxicity at a higher concentration with IC50 value of 149.2 and 168.4 µg/ml, the extract treated A549 and MCF-7 cells exhibited significant cytotoxicity at a lower concentration with IC50 value of 86.4 and 92.1 µg/ml respectively. The treated A549 and MCF-7 cells showed cell cycle arrest up to 7.07% and 14.42% respectively, at G2/M phase of cell cycle. Reports indicate that microtubule depolymerization agents, which arrest the cell cycle in G2/M phase act through several types of kinases, leading to phosphorylation cascades and that induce activation of cyclin *B1/cdc2* complex and *bcl-2* phosphorylation [19].

Apoptosis represents a major protective mechanism against cancer, maintaining normal cell numbers in tissue, and deleting cells with severe DNA damage. It is also an energy-requiring process, characterized by morphological changes, nuclear condensation, plasma membrane blebbing, and the action of an endonuclease that digests DNA into small fragments. It is confirmed that cell factors have a close

relationship with apoptosis, such as *bcl-2* gene acts to inhibit apoptosis, while *bax* gene induces apoptosis. The effect of an anticancer drug was determined in part by how readily the tumor cells undergo apoptosis. Annonaceous acetogenins exhibited 300 times higher anticancer effect than taxol [20]. Annonacin, a monotetrahydrofuran isolated from *A. reticulata* is reported to arrest cancer cells at G1 phase and cause cytotoxicity in a bax and caspase -3 related pathway. Extract-treated cells were analyzed for DNA fragmentation, which is a typical hallmark of apoptotic cell death [21]. Qualitative analysis of DNA fragmentation was performed using conventional agarose gel electrophoresis to resolve oligonucleosomal DNA fragments. Unfragmented single band instead of unambiguous laddered electrophoretic patterns of oligonucleosomal DNA fragments were observed following a 24 hrs exposure of both A549 and MCF-7 cells with *A. reticulata* extract at a concentration of upto 320 µg/ml.

CONCLUSION

Efficacy against some of the cancer cell lines is evident in MTT and cell cycle assay and might have potential for the development of therapeutically active compounds, which could serve as precursors or chemical templates for the design of an effective, more potent and safe antineoplastic drug which may be more potent than existing drugs of its class. These encouraging preliminary results emphasize the necessity for further research on characterization of individual compounds from this extract and advocate it as a good source of anticancer agent.

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