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ASSESSMENT OF ANTINEOPLASTIC POTENTIAL OF *ANNONA MURICATA* LINN. IN HUMAN CANCER CELL LINES

RAJARAJAN.P[1&2*] AND SENTHILKUMAR.R[1&2]

[1]Centre for Research and PG Studies, Indian Academy Degree College, Bangalore, India.
[2]Research and Development Center, Bharathiar University, Coimbatore- 641 046, Tamil Nadu, India.

ABSTRACT

Annona produces a novel set of chemicals called annonaceous acetogenins, known to possess antineoplastic activity.The present study was undertaken to establish the antineoplastic potential of *Annona muricata* by MTT, Cell cycle (G2M) and DNA fragmentation assays. 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 cells, the treated SCC9, MCF-7 and A549 cells exhibited significant cytotoxicity at a higher concentration with IC50 value of 87.8, 151.6 and 230.6 respectively. The treated SCC9 and MCF-7cells showed cell cycle arrest up to 27.97% and 19.42% respectively, at G2/M phase of the cell cycle. DNA degradation observed in both MCF-7 and SCC9 cells treated with *A.muricata extract* at a concentration of 320µg/ml suggest that it might induce apoptosis in both MCF-7 and SCC9 cells.

KEYWORD: ***Annona muricata*, Antineoplastic activity, MTT, G2M and DNA fragmentation.**

RAJARAJAN.P
Centre for Research and PG Studies, Indian Academy Degree College, Bangalore, India.

***Corresponding author**

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 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. Rise in incidence of lung, breast, oral and colorectal cancer are a cause of concern**.** The most commonly diagnosed cancers worldwide are lung cancer (1.61 million, 12.7% of the total). Worldwide breast cancer is the second most common type of cancer after lung cancer, and the fifth most common cancer deaths. As many as 1.6 million cases are reported annually. Oral squamous cell carcinoma (OSCC) is one of the commonest malignant tumors in human, the development of which includes a number of malfunctions in gene regulation such as activation of oncogenes and inhibition of cancer suppressor genes. 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 sub-continent. It has been suggested that use of tobacco either smoked or chewed is associated with more than 70-80% of oral cancers[2]. Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide. Carcinoma of the colon and rectum is a relatively uncommon malignancy in India when compared to the western world. The age standardized rates of colorectal cancer in India has been estimated to be 4.2 and 3.2/100,000 for males and females, respectively, compared to 35.3 and 25.7, respectively, in the USA. 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. To compete with the growing pharmaceutical market, there is an importance to use and scientifically authenticate more medicinally useful plant products[3]. *Annona muricata L*. is a naturally occurring plant, traditionally used to treat various ailments including cancer and is widely distributed in India and Central America. The leaves and seeds of the tree have long been used by native peoples for an astounding variety of ailments, ranging from parasites (the seeds), to high blood pressure and cancer. Along with these properties, it is also known to have anti-bacterial, anti-fungal, and antiviral properties[4].The plant that is empirically trusted by societies to have anticancer properties are the leaves of the sour sop (Annona muricata Linn.). Based on chemotaxonomy approach, the plant family Annonaceae that have been studied has anticancer activity[5]. They are rich in annonaceous acetogenins. Sour sop (*Annona muricata*) is a traditional medicinal plant in Indonesia to treat breast cancer[6]. Among the chemical constituents found in *A. muricata*, the alkaloids (reticulin, coreximine, coclarine and anomurine) and essential oils (β-caryophyllene, δ- cadinene, epi-α-cadinol and α-cadinol) stand out. However, species of the *Annonaceae* family, have also been targeted for investigation due to appurtenant substances in the acetogenins, a class that have been isolated from different parts of the plant[7].The present study is aimed at establishing the antineoplastic potential of *Annona muricata.*

MATERIALS AND METHODS

Fresh leaves of *Annona muricata* were collected from Mavellikara, Kerala and it was authenticated at National Herbarium of Medicinal Plants and Repository of Raw Drug, Foundation for Revitalization of Local Health Traditions (FRLHT) University, Yelahanka, and Bangalore.

(i) Preparation of crude leaf extract

Leaves were shade dried and powdered, leaf powder of *annona muricata* was extracted with methanol by using soxhlet apparatus for 4 hrs. and it was concentrated by using Rota evaporator (Rotavap PBV-7D).

(ii) MTT Assay[6-12]

70-80% confluent cell lines of SCC9, MCF-7, A549 and HCT116 were collected, Checked for viability and centrifuged. 50,000 cells/well were seeded with SCC9, MCF-7, A549 and HCT116 in a 96 well plate and incubated for 24 hours at 37^{o}C with 5 % CO_2 respectively. Extract from 0-320 µg/ml (2 fold variation) concentration in DMEM or RPMI-1640 media without FBS & were incubated for 24 hrs.100 µl/well (50 µg / well) of the MTT (5 mg/10 ml of MTT in 1X PBS was added and incubated for 3 to 4 hours. After incubation with MTT reagent, MTT reagent was discarded by pipetting without disturbing cells and 100 µl of DMSO added to it so as to rapidly solubilize the formazan. Absorbance was measured at 590 nm using T.CAN plate reader**.**

(iii) Cell Cycle (G2M) Analysis[13-15]

Culture 5x10^{5}cells in a 6-well plate containing 2 ml of complete media. After overnight or confluent or 24 hours of incubation, spent media was removed and washed once with 1XPBS. Cells are allowed to starve with serum free media for 24hours. After 24 hours starvation, 320 µg/ml of *A.muricata* extract in 1ml / well media containing serum was added in SCC9 and MCF-7 cells respectively. After 24 hours of treatment, remove the media, wash once with 1X PBS and finally collected the cells by Trypsin-EDTA. (Collect both floating and adherent cells). Pellet 1-5x10^5 cells / ml at 1500 rpm for 5 minutes at room temperature and discard the supernatant. Resuspended the cell pellet gently with 2 washes in 1XPBS. 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 min at room temperature and discard the supernatant. Cell pellet was washed two times with cold 1XPBS. Cells were incubated for 1 h 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 RNase A in 1xPBS.The percentage of cells in various stages of cell cycle in compounds/samples treated and un-treated populations were determined using FACS Caliber (BD Biosciences, San Jose, CA) and analyzed by Flow Jo 7.5.5 (Tree Star Ashland OR).

(iv) DNA Fragmentation or Ladder Assay[16]

Culture 1.5x10^6 cells in a p-35 dish containing 2 ml of complete media, after 24h, treat 320µg/ml of extract in 1ml / well media without serum for 24hrs in SCC9 and MCF-7 cells respectively. After 24h 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 mMTris–HCl, 10 mM ethylene diamine tetra acetic acid (EDTA)-4Na and 0.5% sodium-*N*-lauroyl sarcosinate (pH 7.8). The lysates were incubated in the lysis buffer containing 0.33 mg/ml RNase A at 50 °C for 30 min and then further incubated in the lysis buffer containing 0.33 mg/ml proteinase K at 50 °C for 30 min. Equal amount of DNA was electrophoresed on 2% agarose gel. Gels were stained with 0.5mg/ml ethidium bromide for 15 min and visualized under UV light.

RESULTS

(i) MTT Assay

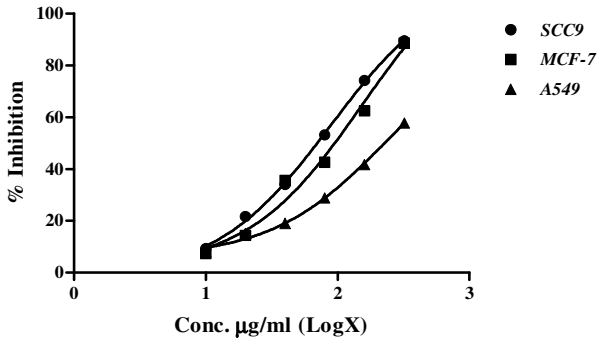
Table 1
MTT Assay of SSC9, MCF-7, A549, HCT116 with Muricata extract

Samples/Cell line	Conc. µg/ml	OD 590 nm	% Inhibition	IC_{50}
SCC9	Control	0.6949	0.00	87.8
	10	0.6302	9.31	
	20	0.5439	21.73	
	40	0.4577	34.13	
	80	0.3252	53.20	
	160	0.1795	74.17	
	320	0.0731	89.48	
MCF-7	Control	0.6037	0.00	151.6
	10	0.5593	7.35	
	20	0.5164	14.46	
	40	0.3884	35.66	
	80	0.3464	42.62	
	160	0.2265	62.48	
	320	0.0686	88.64	
A549	Control	0.9438	0.00	230.6
	10	0.868	8.03	
	20	0.8011	15.12	
	40	0.7642	19.03	
	80	0.6711	28.89	
	160	0.5501	41.71	
	320	0.3983	57.80	
HCT116	Control	0.8697	0.00	NA
	10	0.8089	6.99	
	20	0.7864	9.58	
	40	0.7455	14.28	
	80	0.7221	16.97	
	160	0.7026	19.21	
	320	0.6818	21.61	

MTT Assay was performed with SCC9, MCF-7, A549, HCT116 cells against ascending concentration of A. muricata extract and measured at 590 nm, yielding significant inhibition of cells.

Graph 1

Percentage of Inhibition by A.muricata extract on cancer cell lines

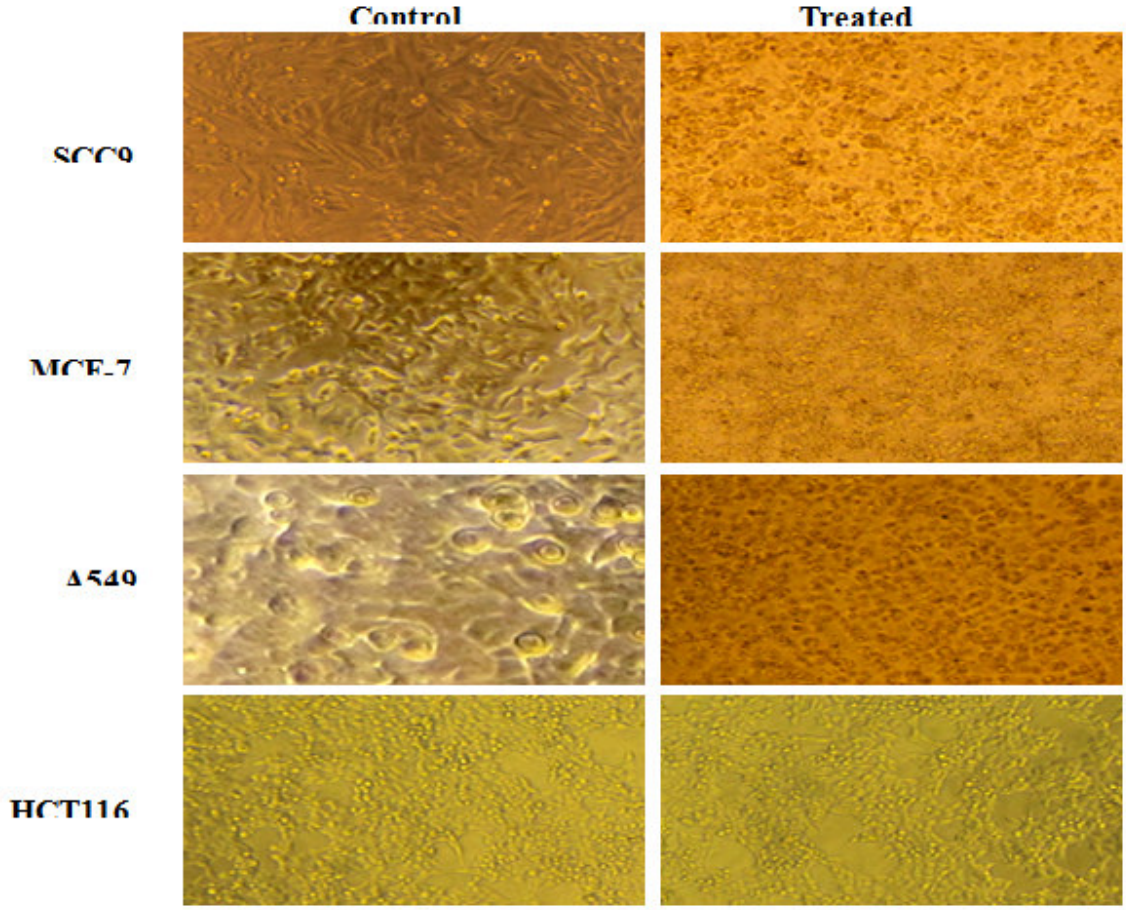


Muricata extract showed dose dependent growth inhibition of SCC9, MCF-7, A549 and HCT116 cells.

In contrast to HCT116 cells which did not show significant inhibition, extract treated SCC9, MCF-7 and A549 cells exhibited IC_{50} values of 87.8, 151.6 and 230.6µg/ml respectively.

Figure 1

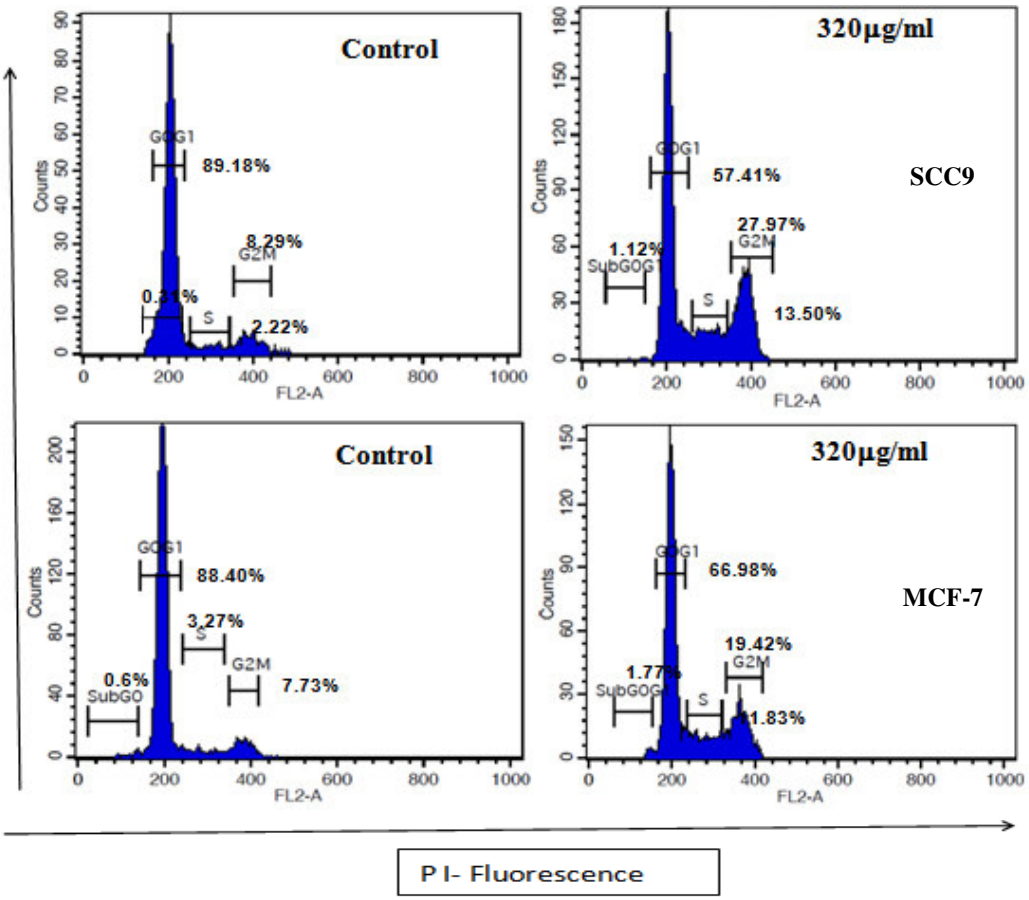
Cell morphology before and after treatment with A.muricata extract (320 µg/ml)



Change in cell morphology was observed after treatment with A.muricata extract in SCC9, MCF-7, A549 cell lines

Figure 2

Cell Cycle (G2M) analysis showing percentage of cell cycle arrest

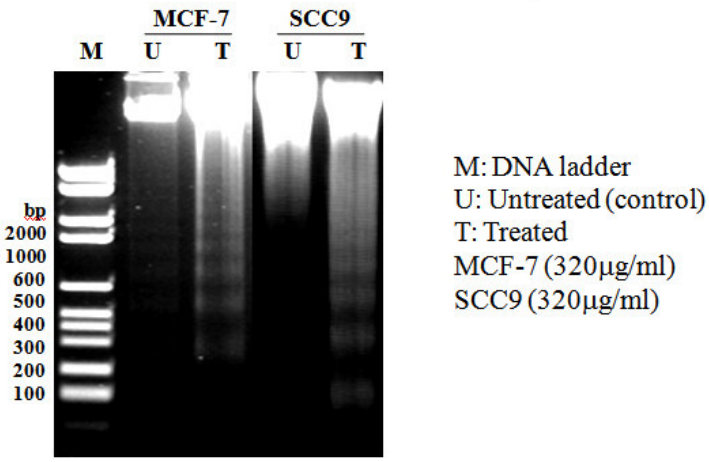


Extract treated SCC9 and MCF-7 cells showed cell cycle arrest up to 27.97% and 19.42% at G2/M phase of cell cycle at 320µg/ml respectively compared to control (untreated cells).

(iii) DNA Fragmentation or Ladder Assay

Figure 3

DNA fragmentation in extract treated and untreated cells of MCF-7 and SCC9.



DNA fragmentation was observed in A.muricata extract treated cells as compared to untreated (control) cells.

DISCUSSION

Plants have been screened for centuries to explore their various therapeutic properties which might be effective in prevention, cure and management of various diseases. The methanolic leaf extract of *Annona muricata* has shown significant cytotoxic potentials in all the experiments employed in this study. The medicinal value of a plant depends on the biological activities of the phytochemicals present in the plant. The most important of these phytochemicals are alkaloids, flavonoids, tannins and phenolic compounds**.** Plant-derived compounds have played an important role in the development of several clinically useful anticancer agents. 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 lead to the development of potential anticancer agents[4]. Numerous studies have demonstrated that the bark as well as the leaves possess hypotensive, antispasmodic, anticonvulsant, vasodilator, smooth muscle relaxant, and cardio depressant activities in animals. The phytochemical group, *Annonaceous acetogenins,* appears to play a vital role in antitumor properties. Hence, more research on graviola focus on a novel set of phytochemicals called *Annonaceous acetogenins*[7]*.* Acetogenin compounds that are produced by graviola exhibited significant antitumor effect with selective toxicity against various types of cancer cells. As a result, chemo preventive agents are preferred as an intervention approach to prevent tumor development in order to reduce the incidence of cancer. Given that the currently available treatments including chemotherapy, radiation, and surgery are associated with side effects, the need for such alternate therapies has arisen[7]. Annonaceous acetogenins, are well known for inhibition of tumor cells that are resistant to multiple drugs. Bullatacin, a bis tetrahydrofuran annonaceous acetogenin known as the most potent inhibitor of the mitochondrial respiratory chain complex I, exhibited 300 times more efficiency than taxol as tested in vivo[18]. Consequently, antineoplastic activity of *Annona muricata* leaf extract was studied on a various cancer cell lines. *Muricata* extract showed dose dependent growth inhibition of SCC9, MCF-7, A549 and HCT116 cells. In contrast to HCT116 cells which did not show significant inhibition, extract at its lower doses exhibited a significant cytotoxicity on SCC9, MCF-7 with IC_{50} values of 87.8 and 151.6µg/ml respectively, while it exhibited only a moderate cytotoxicity towards A549 cells with a comparatively higher IC_{50} value of 230.6µg/ml, clearly indicating differential cytotoxicity. An extract is said to have cytotoxic activity if the IC50 value of less than 1000 µg/mL after 24 hours contact time. The smaller the IC_{50} value of a test compound, more the toxic compound it can be. *Muricata* extract mediated SCC9 and MCF-7 cells arrest up to 27.97% and 19.42% at G2/M phase of cell cycle at 320µg/ml respectively, compared to untreated control. Studies 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 activation of cyclin *B1/cdc2* complex and *bcl-2* phosphorylation[19]. Apoptosis that represents a major protective mechanism against cancer maintain normal cell numbers in tissue and delete cells with severe DNA damage. Apoptosis is 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 which 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[20].Qualitative analysis of DNA fragmentation was performed using conventional agarose gel electrophoresis to resolve oligonucleosomal DNA fragments yielding consistent results. Unambiguous laddered electrophoretic patterns of oligonucleosomal DNA fragments were observed following a 24hrs exposure of SCC9 and MCF-7 cells.

CONCLUSION

The present study revealed that *A.muricata* exhibits extensive antineoplastic potential against oral, breast and lung cancer cell lines. Its efficacy against cancer cell lines is evident in MTT, Cell Cycle and DNA fragmentation assays. It 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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