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In vitro evaluation of anticancer activity of methnaolic extract of *annona reticulata* linn. (ramphal) leaves on different human cancer cell lines

Abstract

Cancer is one of leading cause of death worldwide and sustained focus on development of novel anticancer agents from medicinal plants. Traditional medicinal systems are based on plant as a source of medicine for the treatment of chronic debilitating and life threatening disease and disorders. Annona reticulate Linn. is widely used in the traditional medicine for the treatment of various disease conditions. Methanolic leaves extract of *Annona reticulata* Linn. Was investigated for anticancer potential using sulforhodamine B (SRB) cytotoxicity assay against colon cancer (HCT15), Human lung cancer (Hop65) and Human hepatoma (HEPG2) cell lines. Adriamycin was used as a standard to compare the results. The extract exhibited anticancer effect against all the cell lines. The median growth inhibition (GI50) concentration for extract was <10µg/ml against Human lung cancer and hepatoma cell line, showing moderate anticancer efficacy of methanolic extract against these cell lines. In conclusion, methanolic leaves extract of *Annona reticulata* Linn. Possess moderate anticancer activities in vitro which could be attributed to the presence of anticancerous phytochemicals like acetogenins.

Keywords: medicinal plant, *annona reticulata* linn, anticancer activity, sulforhodamine b (srb) assay

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Abbreviations: LC, lethal concentration; TGI, total growth inhibition; HCT, human colon cancer; GI, growth inhibition; SRB, sulphorhodamine B

Introduction

Cancer is deadly disease and a major cause of mortality. The incidences of cancer are continuously increasing worldwide.[1] According to WHO, the incidences of cancer will reach to 15million until 2020. Abnormal uncontrolled growth of cells leads to the development of cancer.[2] From the ancient time, plants and plant based products are the first choice as medicine. WHO specified that till date near about 20,000 plant species studied for medicinal purposes.[3] The phytochemicals obtained from plant such as alkaloids, terpenoids, flavonoids, glycosides and phenolics exhibits meticulous physiological effects. These effects mainly include desirable beneficial effects.[4] The plant based medicines were found to be safer than synthetic chemotherapeutic agents. Toxicity, manifestation of deleterious side-effects, and a narrow margin of error are the major limitations in the use of chemotherapeutic agents.[1] Development of new anticancer agent having minimum or no side effects is the need of time.

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.[5] 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.[6] It also contains minerals such as Ca, P, K, Mg, Na, Cl, S, Mn, Zn, Fe, Cu, Se, Co, Ni and Cr.[5] In account with the medicinal properties of Annona reticulate Linn. was targeted for the investigation of anticancer property.

Materials and methods

Chemicals

All the solvents and reagents were of analytical grade purity and obtained from Rankem (India).

Collection and authentication of plant material: The leaves of *Annona reticulata* Linn. was identified and authenticated by Dr. Gachande B.D., Botanist, Associate Professor of Botany department, N. E. S. Science College, Nanded, India.

Extract preparation: Collected *Annona reticulata* Linn. leaves were converted to small pieces and dried under the shade at room temperature. Powder of dried leaves (200g) was prepared using grinder. Methanolic extract was obtained by Soxhlet extractor using 1 L methanol for 8 h at 640C and sample was concentrated with the help of rotary evaporator. The 21 % yield of methanol extract was obtained.

Preliminary phytochemical investigation: Methanolic extract leaves of *Annona reticulata* Linn. was investigated for phytochemicals using standard procedures.[7]

Anticancer activity using SRB assay: Sulphorhodamine B (SRB) assay was employed for screening of anticancer activity of methanolic leaves extract of *Annona reticulata* Linn using Human colon cancer cell line (HCT15), Human lung cancer cell line (Hop65) and Human hepatoma cell line (HEPG2). Cell lines were cultured in medium RPMI 1640 containing 10% fetal bovine serum, 2mM L-glutamine and inoculated into 96 well microtiter plates in 100µL at plating densities. Cell were inoculated and microtiter plates were incubated at 37°C, 5 % CO2, 95% air and 100% relative humidity for 24h prior to addition of extract. Initially extract was solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored

frozen prior to use. During extract addition, an aliquote of frozen concentrate (1mg/ml) was thawed and diluted to 100µg/ml, 200µg/ml, 400µg/ml and 800µg/ml with complete medium containing test article. Aliquots of extract (10µl) was mixed to appropriate microtiter wells containing 90µl of medium and final extract concentrations of 10µg/ml, 20µg/ml, 40µg/ml, 80µg/ml were obtained. The plates with extract concentrations were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. 50µl of cold 30 % (w/v) TCA (final concentration, 10% TCA) was added to fix the cells in situ and incubated for 60 minutes at 4°C. Supernatant fluid was discarded; plates were washed five times with tap water and air dried. In each well sulforhodamine B (SRB) solution (50µl) at 0.4 % (w/v) in 1 % acetic acid was added and it was incubated for 20 minutes at room temperature. One staining is completed, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid. The plates were air dried and bound stain was subsequently eluted with 10mM trizma base and absorbance was observed at a reference wavelength of 540nm with 690nm. The percentage growth was calculated on a plate-by-plate basis for extract wells relative to control wells. Adriamycin (Doxorubicin), standard anticancer drug was used as positive control. The percentage growth was calculated at each of the extract concentration levels by using the six absorbance measurements time zero [(Tz), control growth (C), and test growth in the presence of extract at the four concentration levels (Ti)]. The percent growth was calculated using following formula.

Table 1 % Control growth of cell lines in presence of methanolic extract of Annona reticulata Linn. leaves and standard adriamycin

	% Control Growth			
Concentrations (µg/ml)	10	20	40	80
Human colon cancer cell line (HCT15) Methanolic extract				
Experiment 1	76.5	69.8	54.1	21.5
Experiment 2	62.2	60.7	52.9	25.8
Experiment 3	65.5	62.8	46.4	14.8
Average values	68.1	64.5	51.1	20.7
Adriamycin (ADR)				
Experiment 1	-11.9	-21.6	-25.3	-39.8
Experiment 2	-28.1	-28.5	-36.1	-47.4
Experiment 3	-46.6	-60	-62.8	-63.7
Average values	-28.9	-36.7	-41.4	-50.3
Human lung cancer cell line (Hop65) Methanolic extract				
Experiment 1	46.4	45.2	44.9	44.2
Experiment 2	40	39.4	37.9	37.5
Experiment 3	38.8	36.6	35.8	32.2
Average values	41.7	40.4	39.5	38
Adriamycin (ADR)				
Experiment 1	13.6	4.9	3.5	0.6
Experiment 2	7.6	6.5	1.4	-7
Experiment 3	6.3	4.3	-4.1	-17.7
Average values	9.1	5.2	0.2	-8
Human hepatoma cell line (HEPG2) Methanolic extract				
Experiment 1	49.1	41.3	35.6	32
Experiment 2	35.4	30.4	30.4	24.7
Experiment 3	41	26.5	15.1	12.4
Average values	41.8	32.7	27	23
Adriamycin (ADR)				
Experiment 1	-10.8	-18.8	-43.3	-51.9
Experiment 2	-19.9	-22.1	-43.1	-53.1
Experiment 3	-20.4	-25.4	-51.8	-56.4
Average values	-17	-22.1	-46.1	-53.8

Percent growth = (average absorbance of the extract well/ average absorbance of the control wells) X 100

Percentage growth inhibition, growth inhibition, total growth inhibition (TGI) and LC50 was calculated. Percentage growth inhibition was calculated as [(Ti-Tz) / C-Tz)] x 100 for concentration for which Ti≥Tz (Ti-Tz) positive or zero and [(Ti-Tz)/Tz)] x 100 for concentration for which Ti<Tz (Ti-Tz) negative. Dose response parameters for each test article were calculated. GI50; Growth inhibition of 50% was calculated from [(Ti-Tz)/(C-Tz)] x 100= 50. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti=Tz. The LC50, concentration which inhibits 50% of protein measured from beginning was calculated from [(Ti-Tz)/Tz)] x 100= -50. GI50 value of ≤20µg/ml is considered to demonstrate activity.

Above three parameters were calculated only when the level of activity was observed. The values were expressed as greater or less than maximum or minimum concentration tested when the effect was not reached or exceeded.[8,9]

Table 2 Lethal concentration value LC50 (µg/ml), total growth inhibition (TGI) and median growth inhibition (GI50) for tested methanolic extract of *Annona reticulata* Linn. and adriamycin

Cell line	Name of Drug	Lc50	Tgi	Gi50
HCT15	Extract	>80	>80	39.1
	Adriamycin	61.9	21.8	<10
HOP62	Extract	>80	>80	<10
	Adriamycin	>80	54.5	<10
HEPG2	Extract	>80	>80	<10
	Adriamycin	61.2	25.4	<10

Results and Discussion

Phytochemical screening

Preliminary phytochemical screening of methanolic extract of *Annona reticulata* Linn. Leaves showed the presence of major phytochemicals such as alkaloid, amino acids, flavonoid, glycosides, phenolic compound, proteins, steroids and triterpenoid.

Anticancer activity

Anticancer efficacy of methanol extract of *Annona reticulata* Linn. Leaves was screened using Human colon cancer cell line (HCT15), human lung cancer cell line (Hop65) and human hepatoma cell line (HEPG2) at 10, 20, 40 and 80 (µg/ml) concentrations (Table 1 & 2).

Extract inhibited percent control growth of all the cell lines in dose dependent manner. The effect is more in Human colon cancer cell line (HCT15) and Human hepatoma cell line (HEPG2) at 80 µg/ml. While, the effect is least in Human lung cancer cell line (Hop65). The lethal concentration value (LC50) and total growth inhibition (TGI) for all the cell lines were >80µg/ml. The median growth inhibition (GI50) concentration for extract was <10µg/ml against Human lung cancer and hepatoma cell line, showing anticancer efficacy of methanolic extract. However, median growth inhibition (GI50) concentration against Human colon cancer cell line is 39.1µg/ml which indicated no anticancer effect of extract.

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.[10] Plants of Annonaceous family are rich in acetogenins, an anticancer property phytochemical.[6] The presence of acetogenin may be attributed to anticancer effect of methanolic extract of *Annona reticulata* Linn.

Conclusion

The present study was carried out to explore anticancer potential of methanolic extract of *Annona reticulata* Linn. Maximum anticancer activity was observed against Human lung cancer (Hop65) and hepatoma (HEPG2) cell line which might be correlated to its acetogenins and other anticancerous phytochemicals. In future, work will be needed to isolate bioactive constituents of fresh *Annona reticulata* Linn. Leaves extract to locate potential anticancer phytochemical.

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Conflicts of interest

Author declares there are no conflicts of interest.

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