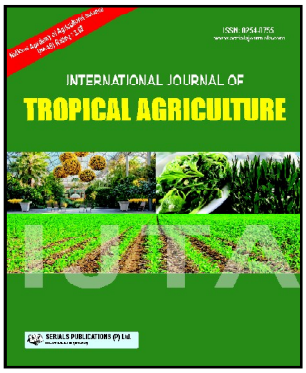

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Punica Granatum Possesses *in Vitro* Cytotoxicity Against Lung Cancer Cells

Navneet Kour[1], Vikas Sharma[1], Arti Heer[1] and Shashank K Singh[1#]

[1] Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu-180 009, J&K, India

[#] Cancer Pharmacology Division, Indian Institute of Integrative Medicine, Canal Road Jammu-180001, J&K, India.

Abstract: In the search of potential anticancer agents from minor fruits, the present research work was carried out to examine the *in vitro* cytotoxic potential of minor fruit *i.e., Punica granatum* (anaar) against nine human cancer cell lines from eight different origins such as MCF-7 and T-47D (breast), SF-295 (CNS), HCT-116 (colon), A-549 (lung), MDA-MB-435 (melanoma), OVCAR-5 (ovary), PC-3 (prostate), A-498 (renal). Methanolic extract of the fruit was used as test material and *in vitro* anticancer activity was determined *via* SRB assay at 100 µg/ml. Results revealed that anaar showed *in vitro* cytotoxicity against four human cancer cells of four different origins (colon, lung melanoma, renal) with growth inhibition range of 72-93%. At lower concentration of 50 µg/ml, the fruit exhibited significant *in vitro* cytotoxic effect against lung cancer cells (A-549). Further, IC_{50} values were calculated and it was observed that anaar showed IC_{50} = 21 in case of lung cancer cells. To conclude, pomegranate possess certain constituents with cytotoxic properties that can be used for developing anticancer agents especially for lung cancer therapy and to provide a great service to cancer patients, studies are required for the isolation of active ingredients from this minor fruit.

Key words: Cancer cells, *In vitro* cytotoxicity, *Punica granatum,* SRB assay.

INTRODUCTION

Cancer is one of the most life threatening diseases, which represents a substantial burden in the community and appears to be a prime cause of concern. Multidisciplinary scientific investigations are making best efforts to combat the disease, but the sure-shot, perfect cure is yet to be brought into the world of medicine. Fruits consumed in our daily diet

could be a solution to this deadly disease by providing chemo protective and chemotherapeutic remedy. *Punica granatum* fruit extract rich in ellagitannins proved their efficacy as anticancer agents especially against breast and colon cancer[1,2,3,4]. Anticancer activities of standardized whole fruit, pulp and peel extract of Egyptian pomegranate has also been reported[5]. *In vitro* studies stated that several pomegranate products inhibit prostate cancer cell growth, induce apoptosis of several prostate cancer cell lines, suppress invasive potential of PC-3 cells and decrease proliferation of DU-145 prostate cancer cells[6,7]. Moreover, there is strong, consistent evidence that high intake of fruits protect against various cancers and these protective effects of high fruit consumption are attributed to the active micronutrients (vitamins and minerals) and non-nutritive components (phytochemicals) that exhibit a potential for modulating human metabolism in a manner favorable for the prevention of cancer. In view of the above, *in vitro* anticancer potential of *P. granatum* has been investigated against nine human cancer cell lines (A-498, A-549, HCT-116, MCF-7, T-47D, MDA-MB-435,OVCAR-5, PC-3, SF-295) from eight different tissues (renal, lung, colon, breast, melanoma, ovarian, prostate, CNS) respectively.

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium, dimethyl sulfoxide (DMSO), EDTA, fetal bovine serum (FBS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin and 5-flurouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally with the brand Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd. from Ramesh Traders, Panjthirthi-Jammu, J&K.

Fruit Material and Preparation of Extracts

P. granatum was authenticated at site by Dr. Vijay Bahadhur Singh, I/c Rainfed Research Station for Subtropical Fruits (RRSSF), Raya, SKUAST-Jammu and enough quantity of fresh fruit was collected.The freshly collected fruit was chopped, shade-dried and ground into powdered form. The methanolic extract of the fruit was prepared by percolating the dried ground plant material (100 g) with 95% methanol and then concentrating it to dryness under reduced pressure. Stock solutions of 20 mg/ml were prepared by dissolving 95% methanolic extract in DMSO. Stock solutions were prepared at least one day in advance and were not filtered. The microbial contamination was controlled by addition of 1% gentamycin in complete growth medium *i.e.* used for dilution of stock solutions to make working test solutions of 200 µg/ml.

Cell Lines/cultures and Positive Controls

The human cancer cells–A-498 (renal), A-549 (lung), HCT-116 (colon), MCF-7 (breast), MDA-MB-435 (melanoma), OVCAR-5 (ovarian), PC-3 (prostate), SF-295 (CNS) and T-47D (breast) were obtained from National Centre for Cell Science, Pune, India and National Cancer Institute, Frederick, USA. These human cancer cells were further grown and maintained in RPMI-1640 medium. Doxorubicin, 5-Fluorouracil, Mitomycin-C, Paclitaxel and Tamoxifen were used as positive controls.

In Vitro Assay for Cytotoxic Activity

Extract was subjected to *in vitro* anticancer activity against various human cancer cell lines[8]. In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator (Hera Cell, Heraeus; Asheville, NCI, USA). The cells at sub-confluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 µl of cells (10^5 cells/ml) was transferred to a well of 96-well tissue culture

plate. The cells were allowed to grow for 24 h. Extracts (100 µl/well) were then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay which estimates cell number indirectly by staining total cellular protein with the dye SRB was performed to assess growth inhibition. The SRB staining method is simpler, faster and provides better linearity with cell number. It is less sensitive to environmental fluctuations and does not require a time sensitive measurement of initial reaction velocity[9]. The cell growth was stopped by gently layering 50 µl of 50% (ice cold) trichloroacetic acid on the top of growth medium in all the wells. The plates were incubated at 4°C for 1 h to fix the cells attached to the bottom of the wells. Liquid of all the wells was then gently pipetted out and discarded. The plates were washed five-times with distilled water and air-dried. SRB 100 µl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min.

The unbound SRB was quickly removed by washing the cells five-times with 1% acetic acid. Plates were air-dried, tris buffer (100 µl, 0.01 M, pH 10.5) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. Suitable blanks (growth medium and DMSO) and positive controls (prepared in DMSO and distilled water) were also included. Each test was done in triplicate and the values reported were mean values of three experiments.

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

- OD Change in presence of control = Mean OD of control – Mean OD of blank
- OD Change in presence of test sample = Mean OD of test sample – Mean OD of blank
- % Growth in presence of control = 100/OD change in presence of control
- % Growth in presence of test sample = % Growth in presence of control × OD change in presence of test sample
- % Inhibition by test sample = 100 – % Growth in presence of test sample

The growth inhibition of 70% or above was considered active while testing extracts, but in testing of active ingredients at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

RESULTS AND DISCUSSION

P. granatum fruit part was observed to be cytotoxic to four human cancer cell lines derived from four different tissues as it showed 93% growth inhibition of HCT-116, 84% of A-549, 80% of MDA-MB-435 and 72% of A-498 cancer cells (Table 1). When evaluated at lower concentrations of 50, 30, 10 and 1 µg/ml, anaar exhibited 81% growth inhibition at 50 µg/ml against A-549 cancer cells (Table 2). Further, IC_{50} values were calculated and it was observed that pomegranate showed IC_{50} = 20 in case of lung cancer cell line *i.e.,* A-549 (Figure 1). In the present investigation, results revealed that methanolic extract from *P. granatum,* showed (cell line specific) *in vitro* cytotoxic activity against various human cancer cell lines. What is quite remarkable in these observations is that the cytotoxic effect shown by the extract was much stronger than that shown by standard drugs for cancer (serving as positive controls in present investigation). According to IC_{50} values, it was observed that anaar can be explored further and active ingredients from the fruit can act as lead molecules for the development of drugs for lung carcinoma. The data was compared with literature values and it was found that the data was in good agreement with the published data. Pomegranate fruit extracts/constituents possess

Table 1
Growth inhibitory effect of Anaar along with positive controls against human cancer cells

			Human cancer cell lines from eight different tissues								
			Breast	*Breast*	*CNS*	*Colon*	*Lung*	*Melanoma*	*Ovarian*	*Prostate*	*Renal*
			MCF-7	*T-47D*	*SF-295*	*HCT-116*	*A-549*	*MDA-MB–435*	*OVCAR-5*	*PC-3*	*A-498*
Generic name of the fruit	*Extract*	*Conc. (µg/ml)*	*Growth Inhibition (%)*								
Punica granatum	*Methanolic*	*100*	*55*	*59*	*00*	*93*	*84*	*80*	*61*	*29*	*72*
	Positive controls (standard drugs)	*Conc. (µM)*									
	Doxorubicin	1	–	–	71	–	–	–	–	–	–
	5-Fluorouracil	20	–	–	–	65	–	–	70	–	–
	Mitomycin-C	1	–	–	–	–	–	–	–	63	–
	Paclitaxel	1	77	72	–	–	71	–	–	–	70
	Tamoxifen	1	–	–	–	–	–	75	–	–	–

Growth inhibition of 70% or more in case of extracts has been indicated in bold numbers.
Mark (–) indicates that particular human cancer cell line was not treated with that particular positive control.

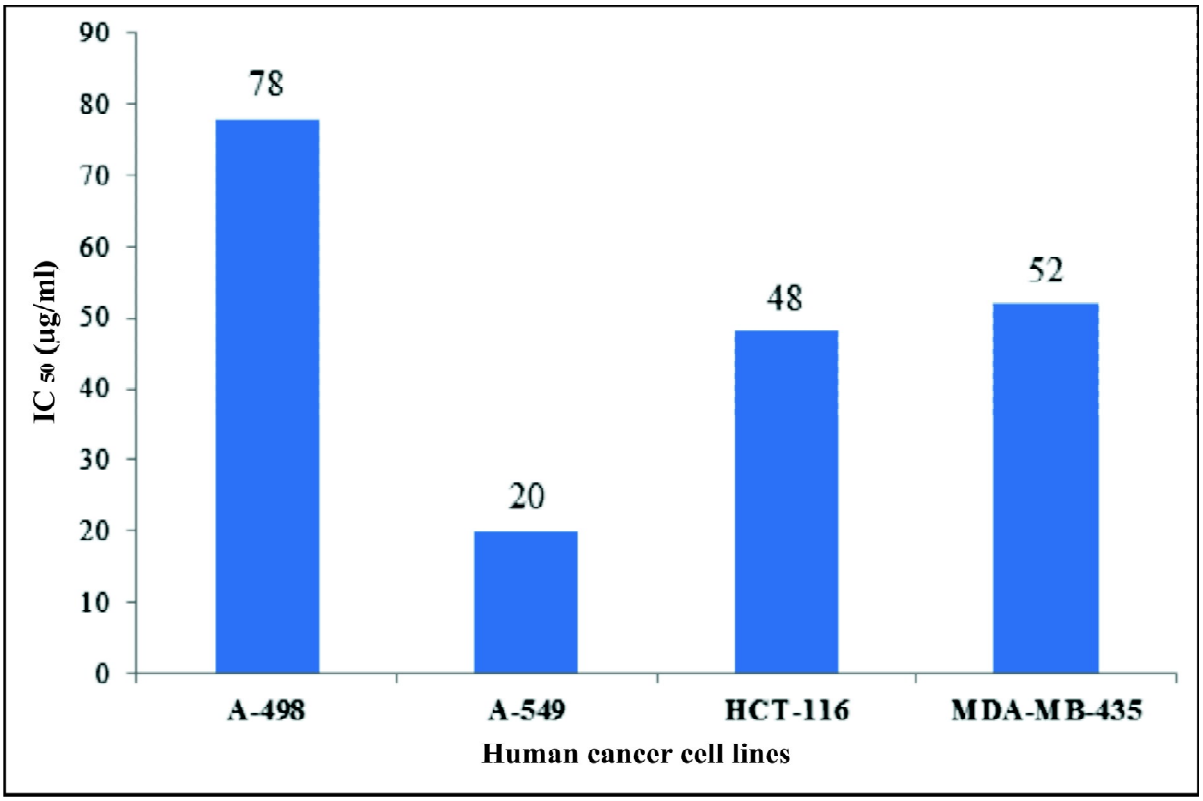

Figure 1: IC_{50} values of Anaar (*Punica granatum*) e xtract

Table 2
Growth inhibitory effect of Anaar at lower concentrations against human cancer cells

			Human cancer cell lines from four different tissue			
			Colon	*Lung*	*Melanoma*	*Renal*
			HCT-116	*A-549*	*MDA-MB-435*	*A-498*
Genericname of the fruit	*Extract*	*Conc.(μg/ml)*	*Growth inhibition (%)*			
Punica granatum	*Methanolic*	*50*	*52*	*81*	*48*	*23*
		30	31	57	18	03
		10	08	41	11	25
		1	06	21	26	42
Positive controls (standard drugs)		*Conc.(μM)*				
5-Fluorouracil		20	65	–	–	–
Paclitaxel		1	–	71	–	70
Tamoxifen		1	–	–	75	–

Growth inhibition of 70% or more in case of extracts has been indicated in bold numbers

Mark (–) indicates that particular human cancer cell line was not treated with that particular positive control

immense anticarcinogenic properties[10,11]. Pomegranate, consumed as whole fruit or juice possess anti-proliferative, pro-apoptotic and anti-angiogenic effects and there are several publications on the anticarcinogenic effects of pomegranate[12,13,14,15,16]. Our *in vitro* cytotoxicity assays conducted with appropriate positive controls displayed very interesting and significant results from medical point of view.

Cancer is becoming a big load on families and economies. Cancer cases related deaths on rise in J&K during past four years with the total of 11,815 cancer cases and 5,198 mortality cases have been reported in the state during the current year. Cancer research has, therefore, become a major area of scientific research supporting the foundations of modern biology to a great extent. Chemotherapy is a major treatment modality for cancer, but most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non tumor cells. Therefore, the research for alternative drugs of natural origin, which are less toxic, endowed with fewer side effects and more potent in their mechanism of action, is an important research line. Fruits have long history for the treatment of various diseases including cancer and active principles from these fruits are used to control the advance stages of malignancies in clinical settings. These natural products now have been contemplated of exceptional value in the development of effective anticancer drugs with minimum host cell toxicity. A number of exciting researches suggest that vegetables, fruits, whole grains, herbs, nuts and seeds contain an abundance of polyphenolic compounds, terpenoids, sulphur compounds, pigments and other natural antioxidants, that have been associated with protection from or treatment of conditions such as cancer. Therefore, we can say that natural products have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer.

This research work has two fold importance: First, the *in vitro* anticancer efficiency of anaar from Jammu region with IC_{50} values against the lung cancer cells have not been reported in the literature. Secondly, the results from the investigation forms a good basis for the selection of these this minor fruit of Jammu for further phytochemical and pharmacological analysis and offer us new drugs

from natural sources which would be less toxic and more potent for the efficient management of cancer.

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