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Study of lectin, antioxidant, cytotoxicity and anticancer properties of *Punica granatum* fruit juice against EAC cells in Swiss Albino mice

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Abstract

Natural compounds from our daily diet can suppress or reverse the progression of cancer. Fruits and vegetables consumption are inversely associated with the reduction of cancer incidence and mortality. Antioxidants from fruits have been extensively studied for their free radical scavenging activities to prevent the occurrence of chronic degenerative diseases. Pomegranate *(Punica granatum L.)* fruit is known to have interesting pharmaceuticals activities and of great interest in biomedical research now a days. This fruit has been shown to exert anticancer and antioxidant activity which is generally attributed to its high content of polyphenols due to their effects of neutralizing free radicals. Current research was aimed to find out the presence or absence of anticancer activity of pomegranate fruit juice. The hemagglutination assay was performed in 96-well microtiter U-bottomed plates to determine the presence of lectin protein at the lower concentration of 52 µg/100 µL. When pomegranate juice was injected to EAC bearing mice for 5 days, it significantly decreased cancerous cells and percentage of cell growth inhibition was 56.28%. Apoptotic changes of EAC cells from treated mice were determined by light fluorescence microscopy. Interestingly, cell growth inhibition was clearly visible with pomegranate juice significantly. Further studies can be carried out to identify the lead compound responsible for anticancer activities of *P. granatum* fruit juice.

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Introduction

Cancer is a prominent cause of global death and estimated for approximately 9.6 million deaths in 2018 (Bray *et al.*, 2018). In gross, cancer covers to any one of a large group of diseases characterized by continuous cell growth with the ability to invade other normal cells of the body.

The development of cancer is a consequence of genetic and epigenetic changes that alters the essential biological mechanisms, such as cell cycle, differentiation, angiogenesis and migration thus normal cells transform into preneoplastic lesions and then to invasive carcinoma. Before the signs and symptoms of cancer arise there is a long latent period likely for decades because the precursor lesions represent an intermediate stage with slow growth rate to progress as malignant cells. This prolonged interval provides the opportunity to block or slow down the cancer developmental events, thereby prevention is being focused to be the most practical strategy for minimizing cancer incidence (Turrini, Ferruzzi, & Fimognari, 2015).

Numerous Epidemiological studies have clearly estimated two thirds of cancer related deaths can be well managed through lifestyle variation and more specifically diatery patterns (da Silva *et al.*, 2019; Nkondjock, Robidoux, Paredes, Narod, & Ghadirian, 2006). It was also indicated that relatively high intake of fruits and vegetables significantly reduce the magnitude of risk related to cancer. Recently, considerable interest has been focused on food from plant sources for their polyphenolic content (Huang, Cai, & Zhang, 2009; Khan, Afaq, & Mukhtar, 2008). More than 2500 different non toxic phytochemicals with outstanding anticancer properties have been identified in fruits and vegetables based on their specificity of targeting multiple signaling pathways regarding to cancer (Alabri, Al Musalami, Hossain, Weli, & Al-Riyami, 2014; Khan *et al.*, 2008).

In addition, Fruits are rich in structurally and functionally diverse antioxidants that scavenge different types of free radicals and thus protect the body from various oxidative stress as well as degenerative diseases caused by over production of free radicals by cellular metabolic reactions (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012).

The pomegranate fruit is obtained from a deciduous tree *Punica granatum* L, Punicaceae is used in Ayurvedic medicine. From ancient time, it was often considered as a whole pharmacy itself for its outstanding potential to control diabetes, erectile dysfunction, cardiovascular disease, bacterial infections and antibiotic resistance, infants Brain ischemia, Alzheimer's disease, intestinal parasitesand obesity and so on (Sharma, McClees, & Afaq, 2017).

It is a rich source of polyphenolic compounds such as hydrolyzable tannins, ellagitannins, catechins, gallocatechins, anthocyanins, proanthocyanidins (flavonoids) and this mixture of different polyphenolic compounds produce unique antioxidant activity (Hajleh & Al-Dujaili, 2016). Additionally, the juicy sarcotesta of pomegranate contains chitin-binding thermostable lectin protein of 26 kDa that is gaining immense of researcher for its tremendous antibacterial, antifungal and hemolytic activity (da Silva *et al.*, 2019). However, the detailed study of antiproliferative activity is still unrevealed.

Therefore, the supreme aim of our study was to investigate anticancer activity of *P.granatum* against EAC cells in Swiss albino mice.

Materials and methods

Chemicals

DPPH (1, 1-diphenyl-2-picrylhydrazyl), BHT (butylated hydroxytoluene), DAPI (Purchased from Sigma Aldrich, USA), Trypan blue and all other reagents used in this experiment were of good reagent grade.

Collection of plant material

Pomegranate fruit was collected from Rajshahi Fruit Research Institute, Binodpur, Rajshahi. This fruit material was authenticated and identified by department of Botany University of Rajshahi.

Preparation of pomegranate juice

After collection, fresh pomegranate fruit was washed and lightly dried. Then one cup of pomegranate seeds were taken in the blender jar and half cup of water was added of it and it was blended. After blending fresh juice was obtained and stored at 4°C.

Determination of lectin activity

The lectin activity of fruit juice was determined by hemagglutination assay described by *Crreia et al*, 1995. Briefly, from the sample juice prepared by blender, 50 µl test sample was placed in the first well in a 96-well U bottom microtiter plate and then serially diluted into the successive wells with phosphate buffer saline (PBS), pH 7.4. An aliquot of 50 µl of 2 % mice blood suspension was added in each well and PBS along was added as control. The titer plate was then kept at 37°C for 30 minutes and observed the agglutination of blood. Hemagglutination activity was assessed as agglutination of RBCs at lowest concentration of extract.

DPPH radical scavenging assay

DPPH was used to evaluate the free radical scavenging activity of the juice and was tested according tothe method of *Choi et al*.,2007. A solution of 0.1mmol/L DPPH in methanol was prepared and 2.4 mL of this solution was mixed with 1.6 mL of extract in methanol at different concentrations. The reaction mixture was vortexed thoroughly and kept in dark at room temperature for 30 minutes. The absorbance of the mixture was measured spectrophotometrically at 519 nm where BHT was used as reference. The percentage of DPPH free radical scavenging activity was calculated by the following equation:

% DPPH free radical scavenging activity

$$= [(A_0 - A_1)/A_0] \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of the extract at different concentrations. Then percentage of inhibition was plotted against different concentrations and from the graph, IC_{50}value was calculated.

Experimental animals and ethical clearance

Swiss Albino mice of 5-7 weeks old, weight about 20-26g were collected from Department of Pharmacy, University of Jahangirnagar, Bangladesh and were used as experimental model for the current investigation. This research work was approved by the Institutional Animal, Medical Ethics, Bio-safety and Bio-security Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (286/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Transplantation of ascitic tumour

Ascitic fluid was drawn out from different tumour bearing Swiss albino mice at the respective log-phases of tumour cells. A three ml (3ml) syringe fitted with 20 gauge needle was used for this tumour cell aspiration. Freshly drawn fluid was diluted with normal saline (0.98% NaCl solution) and the tumour cell number was adjusted to approximately 1.7×10^6 cells/ml by counting the cell number with the help of a haemocytometer. The viability of tumour cells was observed by trypan blue dye (0.4%) exclusion assay. Cell sample showing above 90% viability were used for transplantation. Tumour suspension of 0.1 ml was injected intraperitoneally (i.p.) to each Swiss albino mouse and strict aseptic condition was maintained throughout the transplantation process.

Preparation of test samples

We prepared one dose of sample to inject mice. For this, first we dissolved 60 microliter pomegranate juice in 1 ml water and then centrifugation was performed at 14000 rpm for 5 minutes. We took the supernatant and it was considered as stock solution. Finally, 100 microliter juice was injected per mice from that stock solution.

Determination of cell growth inhibition (in vivo)

To determine the cell growth inhibition of the sample juice, two groups of Swiss albino mice (n=6) were used according to *Sur et al,* 2001. For therapeutic

evaluation 1.76×10^6 EAC cells in every mouse were inoculated on day 0. Treatments were started after 24 h of tumor inoculation and continued for 5 days. Group one received 100µl juice solutions that were injected per mice every day (i.p.). Group two was used as control. Mice in each group were sacrificed on day six and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified by using trypan blue and then counted by a haemocytometer. The total number of viable cells in every animal of the treated groups was compared with those of control (EAC treated only) group.

Brine shrimp lethality bioassay

Cytotoxicity of the fruit juice was screened against *Artemia salina* in a day *in vivo* assay according to of (Meyer *et al.* 1982). For the experiment, solution of varying concentrations such as 2.5, 5.0, 10.0, 20.0 and 40.0µl/mL were used. After 24 hours of incubation, the percentage of mortality of the nauplii was calculated for each concentration and the LD_{50} value was determined using the equation from graph.

Apoptosis assessment by DAPI staining

Collected EAC cells (1 ml) from each group of mice were centrifuged at 1200 rpm for 2 minutes. The plate was then washed with PBS for each time after centrifugation at 1200 rpm for 2 minutes for three times. The resultant cells were then incubated with 5µl DAPI staining solution in dark for 10 minutes with subsequent adding of PBS to the DAPI containing pellet and then centrifuged at 1200 rpm for 2 minutes. Finally, 200µL PBS was added to the pellet and 10µl of the supernatant was taken on a microscopic slide and observed the morphological changes of cancer cells under the fluorescence microscope (XDS-2FL, Optika, Italy).

Results

Hemagglutination assay

Hemagglutination assay is a common method to detect the presence of lectin protein in plant samples. Lectins are glycoproteins which shows different pharmacological and anticancer activities that is the most important and common one. Therefore, we studied the presence of lectin protein in pomegranate juice using hemagglutination activity. Our study shows that the juice of pomegranate was able to agglutinate up to 32 fold at the lower concentration which indicates the juice has agglutination activity and contain lectin protein. As lectin protein is carbohydrate binding protein and that's why it binds to that portion of blood. As a result, coagulation was occurred due to the presence of lectin protein specially documented for anticancer activity. So, our study indicates that juice of pomegranate has significant amount of lectin protein.

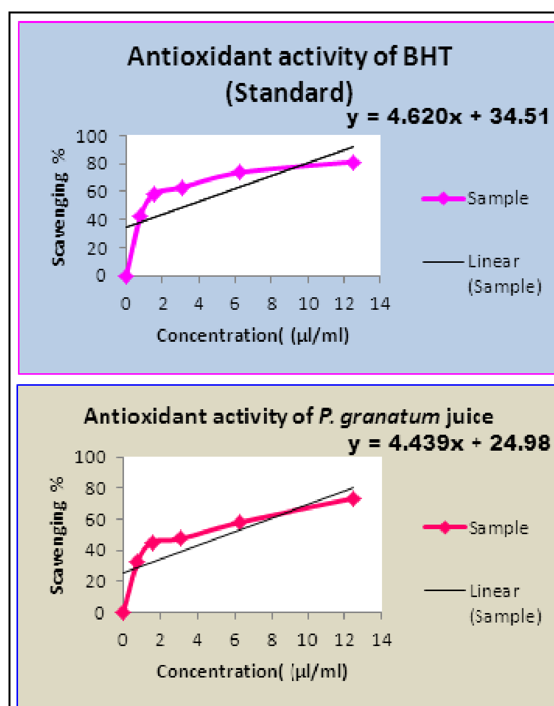

Fig. 1. Determination of DPPH free radical scavenging activity of pomegranate juice extracts compare to BHT. The calculated IC_{50}value of pomegranate is 5.63µL/ml and BHT is 3.35µL/ml.

Antioxidant activity

DPPH free radical scavenging assay was applied for determining antioxidant activity of sample at different concentrations. Free radical scavenging percentage of experimental sample and BHT standard is shown in figure 1.

Effects of Punica granatum juice on EAC cell growth inhibition

The haemocytometric observation of the EAC cells from both control and treated mice was carried out

under the inverted microscope. An average of 1.43×10^6 cancer cells were found for control mice by cell counting method using haemocytometer whereas treated mice showed only 0.12×10^6 cancer cells under the inverted light microscope. So, the number of cells was reduced at a significant level by apoptosis which was induced by pomegranate juice compared to control mice. And this proves that juice of *punica granatum* have anticancer activity against EAC cells which may be probably due to the presence of lectin protein.

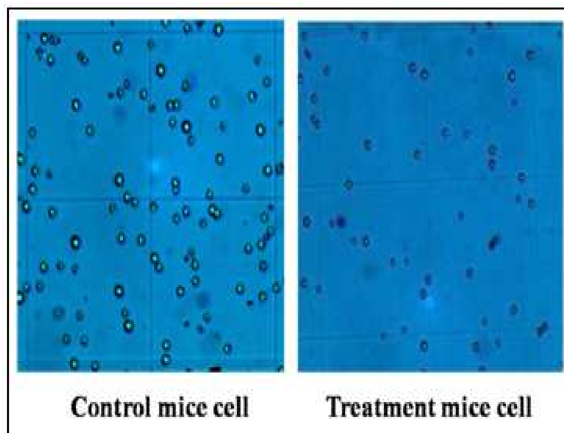

Fig. 2. Haemocytometric observation of the EAC cells of control mice were showed high density of cancer cells but the treated mice were showed the decrease of EAC cells.

Cytotoxicity Test

In brine shrimp lethality bioassay, the sample from the experimental fruit showed positive results that they are biologically active. The LD_{50} value of the test samples were evaluated in this screening and regression equation for each test sample was obtained by plotting against the logarithm of the sample concentration. After 24 hrs, the LD_{50} values of sample was calculated from the corresponding regression equation and it was found to be 67.22µL/ml which indicates mild toxicity to normal cells. In this bioassay, the mortality rate of brine shrimp nauplii by test sample was increased in different concentrations.

From these results, it is evident that fruit juice of *P. granatum* is a promising candidate for anticancer activity as it shows mild toxic effects against brine shrimp nauplii.

Fluorescence morphological examination of treated and control EAC cells

Experimental EAC nuclei were round, regular and homogeneously stained with DAPI in control group shown in Fig-4(A), Whereas, *Punica granatum* treated EAC cells were showed manifest fragmented DNA in nuclei shown in Fig-4(B).

Apoptotic morphological alterations such as membrane and nuclear condensation were also observed clearly by fluorescence microscopy. These results indicate that *Punica granatum* juice has the ability to induce apoptosis of EAC cells.

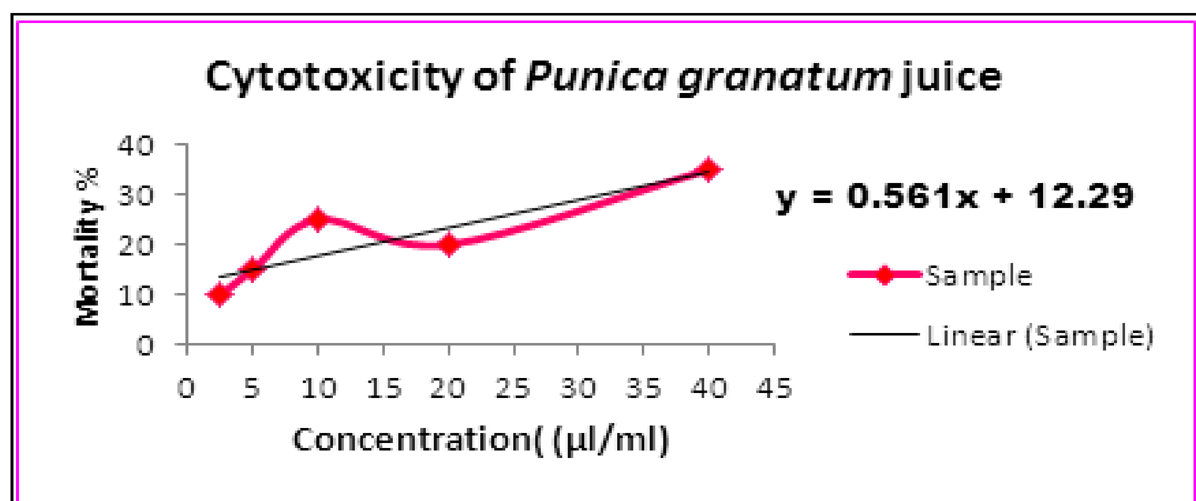

Fig. 3. Estimation of mortality percentage (% mortality) of pomegranate juice extracts at different dose levels after 24 hours and the calculated LD_{50} value of juice is 67.22µl/ml.

Discussion

Presently, Cancer prevention via dietary agents is drawing the considerable attention of both scientist and medical professionals due to the prominent anticancer potential of natural compound as well as their cost effectiveness and easy availability (Sharma *et al.*, 2017).

Hemagglutination assay was carried out in the present study to identify the presence of lectin protein in the juice of *P. granatum*. When hemagglutination experiment of mice blood was done with pomegranate juice, we found significant result. This juice agglutinated the blood up to 32 fold which proved the presence of high amount of lectin protein in pomegranate juice. Pomegranate juice had been shown to have potent *in vitro* antioxidant and anticancer activity, attributed to its content of lectin protein and gallic acid. Lectins are specific carbohydrate-binding proteins that can kill cancerous cells (Kaltner, Caballero, Ludwig, Manning, & Gabius, 2018). Several *in vitro* and *in vivo* studies had also proven that plant derived lectin protein had the ability to abolish cancer cells (Kabir *et al.*, 2013).

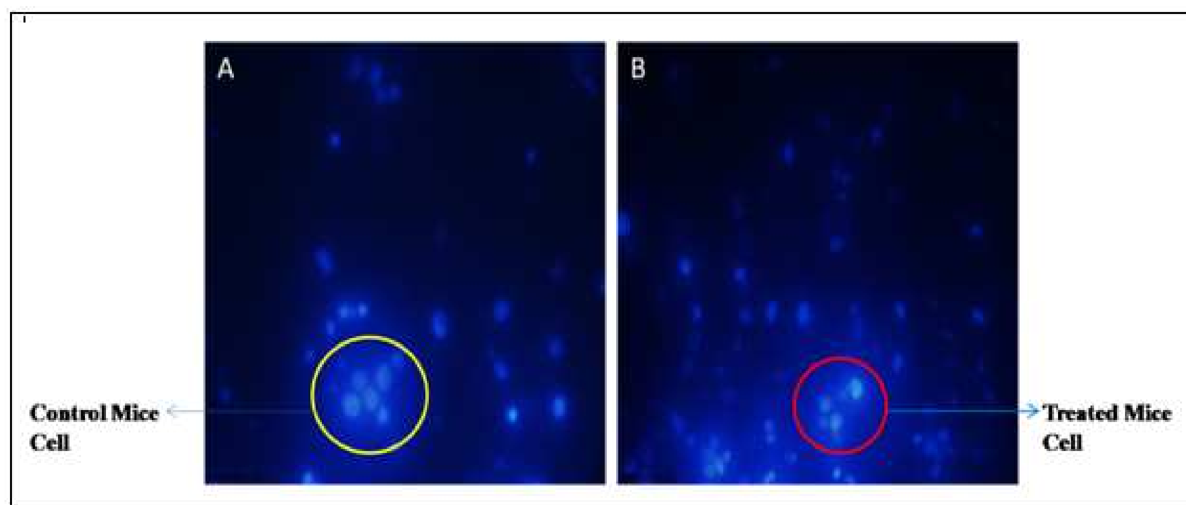

Fig. 4. The morphological changes of EAC cells under fluorescence microscope induced by *P. granatum* juice. (A) Cells of control mice showed regular and round shape but (B) Cells of sample treated mice showed morphological and structural changes.

The DPPH assay was based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives purple colour while experiment was done at 519 nm. Our sample showed purple colour at this absorption. Higher concentrations captured more free radicals formed by DPPH resulting into decrease in absorbance and increase in IC_{50} value (Chaveerach *et al.*, 2016). Pomegranate juices have shown higher level of scavenging activity at the highest concentration 100µL/ml.

Cell growth inhibition in case of pomegranate juice is 56.28% was showed against EAC cell bearing Swiss albino mice. Several studies reported that the fruit juice showed moderate cytotoxicity and anticancer activity in experimental animals (Haldar, Kar, Bala, Bhattacharya, & Mazumder, 2010). Decrease in viable cell in tumor bearing mice suggest antitumor activity against EAC cells in mice (Bala, Kar, Haldar, Mazumder, & Bera, 2010).

The brine shrimp lethality bioassay of our experimental sample was used to predict the cytotoxic activity. The experimental sample showed cytotoxicity against brine shrimp in dose dependent manner. The LD_{50} value was found to be 67.22% and experimental sample was also showed ant proliferative activity of the EAC in Swiss albino mice.

The result of EAC cells observation from both control and pomegranate treated mice under fluorescence

microscope showed different morphological changes which ultimately indicates apoptosis and also formed apoptotic body and chromatin condensation. On the other hand, control mice showed round and uniform cell size in fluorescent microscope whereas cell and nuclear shrinkage, chromatin condensation, formation of apoptotic bodies and phagocytosis by neighboring cells demonstrated the induction of apoptosis process (Kerr, Wyllie, & Currie, 1972).

Conclusion

The present study represents potent anticancer activity against EAC cell line which may be due to moderate antioxidant property and good source of lectin protein with lower toxic effects on normal cell. Therefore, the pomegranate juice can be considered as novel source for the detection and purification of bioactive compounds those will lead anticancer drug development in future through further investigations.

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