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ORIGINAL ARTICLE

Natural Antioxidants as a Tool to Protect the Organs from Tumors Induced Ehrlich Ascites in Mice

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ABSTRACT

The objective of this study was to estimate if pre-treatment with pomegranate or beetroot juice and their combination for EAC which induces tumor in mice has the antitumor effect. Therefore, antioxidant activity DPPH scavenging was determined. The organs liver, spleen, and kidney were weighted to body weight in mice injected with EAC and treated by cisplatin, pomegranate, beetroot, and both of each pomegranate and beetroot and compared with the mice normal group. Single-cell suspension from the spleen was determined and RNA was extracted from the liver using a total RNA isolation kit and mRNA gene expression in EAC Cells was quantitative Real-Time PCR (RT-qPCR) analysis. The results showed that the pomegranate and beetroot contained high amounts of phenolic acid and flavonoid compounds and the highest antioxidant activity, which gave the highest DPPH scavenging activity**.** Moreover, the changes in the organ weight of vital organs were determined and compared with the control group. Because homologous recombination appears a new objective for cancer treatment by pomegranate and beetroot juice as an alternative to anticancer drugs. Therefore, pomegranate and beetroot juice have antitumor and antiproliferative influences on the Ehrlich tumor. Therefore, the pomegranate or beetroot and their combination were homologous recombinations that sensitize cells to growth inhibition and apoptosis.

Keywords: Pomegranate, Beetroot juice, Ehrlich Ascites, Cisplatin, Cancer

INTRODUCTION

Pomegranate contained the antioxidants polyphenolic. Therefore, antioxidant activity has been to play an absolutely necessary role in various human diseases. Therefore, inhibition of free radicals by adding antioxidants is a therapeutic strategy to reduce disease risk[1,2].

Beetroot is a root vegetable and had contained rich amounts of polyphenols, vitamins, minerals, and water-soluble pigments[3]. It has obtained publicity due to its biological activity and usefulness as a functional food for health promotion and disease prevention[4].

The DPPH stable radical model is closely utilized to estimate free radical scavenging activity. Antioxidants into interacting with DPPH, which happens to transfer hydrogen atoms to DPPH and consequently the color tone indicates the scavenging activity of the sample and can be easily seen as a color change from purple to yellow[5].

Moreover, Kim et al. [6] explained that the plant kingdom which had contained more compounds that inhibits tumors and cell poisoning due to its fiber content and natural health-promoting antioxidants.

Cancer is the absence of the normal cell cycle, resulting in a disturbance of growth and differentiation characterized by malignant growth. Cancer can occur at any time and in any organ. Furthermore, the Ehrlich tumor is adenocarcinoma that can happen in almost all strains of mice[7].

Pomegranate would be demonstrated to apply anticancer activity, which it has contented high amounts of natural antioxidants[8]. Thus, pomegranate induces apoptosis and prevents the activation of inflammatory pathways and prostate cancer. Therefore, it was suggested that the use of pomegranate or its polyphenols has been for therapeutic purposes in cancer[9].

Recent research has given evidence that eating beetroot has physiological influences which can promote clinical results for many diseases, such as high blood pressure, diabetes, and cardiovascular. It is a rich source of phytochemical compounds containing vitamins, antioxidants, and betalains[10]. Clifford et al.[4] found that antioxidants and betalains have anti-inflammatory and anti-cancer properties.

Therapy with medicinal plants is a modern strategy in the killing of cancer cells. Moreover, the medicinal plants and its ingredient inhibit the carcinogenesis process[11].

Therefore, pomegranate and beetroot juice have various activities that could be involved in their beneficial clinical effects and are of great interest to biomedical research .Thus, the target of this research was to estimate if pre-treatment with pomegranate and beetroot juice in an EAC tumor model has an antitumor effect and if pomegranate and beetroot juice affects the expression of antioxidant genes.

MATERIALS AND METHODS

Materials:

Pomegranate and beetroot were obtained from the local market. Fresh juices were obtained by the electric mixer.

Ehrlich Ascites Carcinoma (EAC) was purchased from National Research Cancer Cairo, Egypt. Chemicals and plants Cisplatin (cis-diamminedichloroplatinum) and other kits for the determination of various parameters of biological experimental, in addition, all chemical uses in the study were purchased from Sigma-Aldrich Corp., USA.

Fifty-four female mice (8 weeks of age) were obtained from the National Organization for Drug and Control Research, Giza, Egypt, as well as, the mice were fed on the basal diet according to Pell et al.[12].

Methods

Preparation of crude aqueous extract from pomegranate and beetroot: Pomegranate pulp was separated and the beetroot was cut into small pieces. The pomegranate and beetroot were extracted by ethanol 70% and this step was repeated twice. The filtrate was then concentrated and stored in a refrigerator until further analysis according to Muyenga et al.[13].

Evaluation of DPPH free radical activity: The DPPH free radical activity was evaluated and calculated in pomegranate and beetroot juice using the method described by Shekhar and Anju[14].

Biological experimental: Experimental mice were adapted for 7 days on a basal diet and randomly divided into six groups, nine mice for each group. The 1st group namely the control negative group (nine mice) injected with phosphate buffer saline (PBS) (10^6 mg/mice i.p) and fed a basal diet for four weeks according to Lala et al.[15].

The five groups (45 mice) were injected by EAC at a dose of 0.25×10^6 cells/mice to induce cancer. The five groups were re-divided to positive EAC control group 2nd was also fed the basal diet only. The third group of EAC was treated with chemotherapy by injecting cisplatin (10 mg/mice) twice from 5-7 days after EAC injection and fed on a basal diet only for four weeks. The fourth EAC group was taken orally from pomegranate juice (1 ml/mice) on an empty stomach daily. The fifth EAC group was taken orally from beetroot juice (1ml/mice) on an empty stomach daily. The sixth EAC group was taken orally from a mixture of pomegranate

and beetroot juice (1ml/mice) on an empty stomach daily. Mice were taken orally/ day from the different juices as nutritional therapy before two weeks of injected EAC.

The biochemical in the serum of the different groups of mice were determined after the end of the experimental period. The organs of the liver, spleen, and kidney were weighted to body weight in mice injected with EAC and treated by cisplatin, pomegranate, beetroot, and both of each pomegranate and beetroot and compared with the mice normal group.

Preparation of single-cell suspension from spleen: Sacrifice mice spleens were grouped in 10 ml phosphate buffer saline (PBS) and it was centrifuged undercooling for 5 min and the supernatant was discarded. The pellet was re-suspended in lysis buffer 5 ml/spleen and diluted lysis buffer with 20–30 ml PBS. Cell count and viability analyses were performed with Rubinstein et al. [16].

Cell-cycle analysis: Spleen cell suspensions were counted using a hemocytometer with trypan blue dye exclusion assay [17,18]. Cell suspensions were treated with PBS and fixed with 70% ethanol on cold ice for 24 hrs. Fixed cells were treated with 25 mg/ml RNAase-A at 37°C for 30 min and then with propidium iodide (5 mg/ml, Sigma, USA) solution for 30 min in the dark. Cell-cycle analysis was performed using a flow cytometer (Becton Dickinson BD FACSCalibur, USA)

Gene expression analysis using quantitative Real-Time PCR (RT-qPCR): RNA is extracted from the liver using a total RNA isolation kit (analytic Jena-Germany). DNA (cDNA) was then obtained from 1 µg of purified RNA using a reverse transcription syntheses kit (QIAGEN, Germany). Real-time PCR reactions were performed using Hot Start Taq DNA Polymerase.

Oligonucleotide primers for IGF-1 were 5'-TGCTCTTCAGTTCGTGTG-3' (sense) and: 5'-ACATCTCCAGTCTCCTCAG -3' (antisense) and GAPDH used were 5'- ACCACAGTCCATGCCATCAC-3' (sense) and: 5'-TCCACCACCCTGTTGCTGTA-3' (antisense). Cycle Threshold (CT) was utilized to define the expression level in control cells and cells therapy with EAC with or without pomegranate and beetroot extract. The gene level was calculated by Yuan et al.[19]. The results were expressed as the ratio of reference gene to target gene according to Ding et al.[20].

Statistically analysis: All results are expressed as ± SD and the P values were estimated by ANOVA using the LSD test, in addition, the SPSS program was statistically analyzed according to Armitage and Berry[21].

RESULTS AND DISCUSSION

DPPH scavenging for pomegranate and beetroot extract: Table (1) and Figure (1) showed the effect of antioxidants on DPPH radical scavenging from beetroot and pomegranate extract at different concentrations. The results indicated that the scavenging of DPPH radicals increased with increasing extract concentration from beetroot and pomegranate.

Jalal et al. [5] revealed that the percent DPPH and ABTS-radical scavenging activity of pomegranate peel powder were significantly higher than the pomegranate seed powder. Therefore, it was noticed that pomegranate could find many applications as functional food components due to its antioxidant characteristics.

The same table showed that the IC_{50} DPPH values of beetroot and pomegranate extract, were determined on the basis of radical scavenging activities of the extract. It was observed that the beetroot extract was at a higher level than pomegranate extract which was less effective in the DPPH radical scavenging activity. The IC_{50} value is the concentration of the extract required to scavenge 50% of the radicals and it is used to measure the free radical scavenging activity[22].

The reducing power of the beetroot extract increased with increasing concentrations and the IC_{50} expressed of DPPH-free radical activity ranged from 0.133mg/ml to 0.275 mg/ml. A significant correlation was observed between all phytochemical components and scavenging activity. Therefore, 0.5 mg/ml of ethanol extract completely removed hydroxyl radicals, and 75% of superoxide radicals[22].

Organs' weight in mice in different groups: Table (1) shows the relative weight of the liver, spleen, and kidney to body weight in mice injected with EAC and treated by cisplatin, pomegranate, beetroot, and both of each pomegranate and beetroot and compared with the mice normal group. The results noticed that no significant change in the weight of organs was observed in any of the treatment groups. These results were confirmed by Chanda et al. [23] who did not find significant changes in the relative weight of necessary organs in treated groups compared to the control group.

Table (1): Percent of DPPH scavenging for pomegranate and beetroot extract

Concentrate µg/ml	DPPH scavenging%	
	Pomegranate	Beetroot
1000	88.2	68.3
500	81.1	54.4
250	72.6	46.0
125	62.9	35.5
62.5	57.2	27.4
31.25	48.5	22.4
15.625	39.5	18.7
7.8125	31.0	14.2
3.9	18.7	6.8
1.95	13.5	1.6
IC50	39.66 ug/ml	340.5 ug/ml

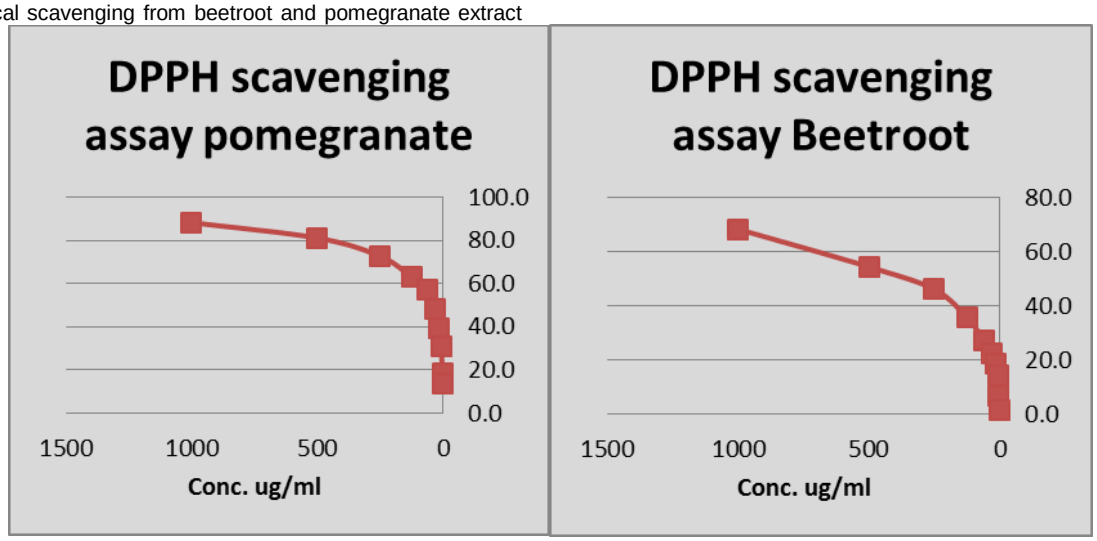

Figure (1): DPPH scavenging for pomegranate and beetroot extract

Table (2): Organ's weight of mice different groups

Groups	Liver	Spleen	Kidney
Normal	5.61±0.60	0.50±0.06	1.21±0.14
EAC	4.60±0.29	0.43±0.24d	1.09±0.21
EAC+CIS	4.60±0.88	0.39±0.09	1.02±0.12
P+ EAC	5.32±0.63	0.68±0.21	1.22±0.29
BR+ EAC	6.53±1.02	0.64±0.23	1.41±0.17
P+BR+ EAC	6.07±0.85	0.85±0.25*	1.38±0.40

Data are mean ± SD of six mice.

Spleen Cell-cycle: Figure (2) and Table (3) observed that the cell cycle analysis was performed for spleen cell suspensions using a hemocytometer and trypan blue dye exclusion assay. The percent distribution of Sub G1, G0/1, S phase %, and G2/M of the EAC cells before and after therapy by CIS and pre-treatment pomegranate or beetroot juice, and their combination were measured. Group (2) used to be injected with EAC cells only. The results demonstrated (sub G1%), (G0/1%), (S phase%) and (G2/M%) as 5.6%, 60.1%, 11.9%, and 21.3% respectively, which recorded excessive values compared to the healthy group .While the therapy by way of cisplatin group and pre-treated groups recorded the highest percentage of sub G1% as 15.1, 56.9, 40.2, and 37.0% than the EAC control group. In addition, the curve of the G0/1 phase was reduced as follows: 58.2%, 23.0%, 24.9%, and 26.2%. The S phase % had a gradually west value in pomegranate, cisplatin, beetroot, and their combination pomegranate and beetroot groups respectively (3.8%, 5.9%, 9.8%, and 10.2%) than the EAC control group. Also, the mitotic phase (G2/M) obtained the same gradual peak values by recording low values in CIS and all pre-treated groups respectively (10.3%, 10.8%, 19.2%, and 20.0% respectively) than to the EAC control group. Flow cytometry revealed pomegranate, beetroot, and their combination may be inhibiting the cellular proliferation of EAC cells with the aid ofG0/G1 phase arrest, also involved sub-G1 cell cycle stopping cause to a become greater in cells in the sub-G1 phase (Figure 2) and Table (3).

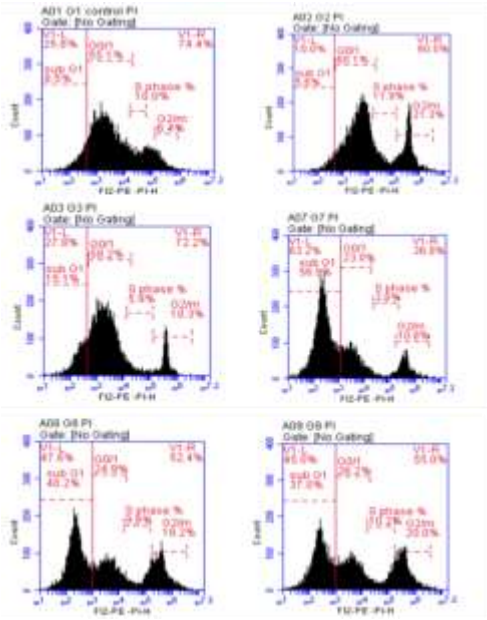

Figure (2): Spleen cell cycle phase

Pomegranate and beetroot juice inhibited ascites fluid throughout the treatment period via the anti-inflammatory effect of pomegranate and beetroot juice that was confirmed by inhibiting tumor cell count and decreasing the splenocyte count and these results in agreement with Wu et al.[24], who clarifies that the effect of nicotinic acid was increased by increasing the dose. Usually, in cancer and cancer chemotherapy drugs the major problems that are being encountered are myelosuppression and anemia.

Winkler et al.[25] found that beetroot extract is able to reduce inflammation in mononuclear cells. The beneficial influence of beetroot extract may be connected with this anti-inflammatory ability[26].

Lin et al.[27] showed that compared to untreated cells as only 1.3% of cells were in the sub-G1 phase, cells treated with doses of 10, 50, and 100 μM bakuchiol indicated that the percentage of cells in the sub-G1 phase was significantly increased.

Table (3): The percentages of spleen cells in different phases of the cell cycle of normal, EAC and pre-treated mice groups

	Sub G1	G0/1	S phase	G2/m
Normal	9.5±1.5	55.1±1.9	10.0±1.0	6.4±0.85
EAC	5.6±0.6	60.1±0.95	11.9±1.2	21.3±0.9
EAC+CIS	15.1±0.6**	58.2±1.65*	5.9±0.95**	10.3±035**
P+ EAC	56.9±0.9**	23.0±0.85**	3.8±0.25**	10.8±0.3**
BR+ EAC	40.2±1.2**	24.9±0.8**	9.8±0.8**	19.2±1.2*
P+BR+ EAC	37.0±1.0**	26.2±0.7**	10.2±1.0*	20.0±1.0*

Data are mean ± SD of nine mice. *Significant P-value < 0.05 001compared to positive control (EAC) with LSD post-test. EAC, Ehrlich ascitic carcinoma; CIS, Cisplatin; P: Pomegranate, BR: Beet Root, PB: Pomegranate+ Beet Root

Effect of pomegranate and beetroot on mRNAgene expression in EAC Cells using quantitative Real-Time PCR (RT-qPCR) analysis: The effect of pomegranate and beetroot on mRNA gene expression in EAC cells (RNA was extracted from liver tissue) was analysed by microarray and reverse-transcription-quantitative PCR (RT-qPCR) and the results are reported in Table (3) and Figure (3). According to the results, it could be noticed that the pomegranate juice group observed a great elevate in the expression level of mRNA- IGF1 (CT) by 3.43 % than in the control group negative and 11.38% than in the control group positive, followed by beetroot juice and their combination.

Nonetheless, GAPDH and RQ were significantly become well by pomegranate or beetroot juice, and their combination was compared to the control group negative and positive. Moreover, mRNA- IGF1 and IGF1 were significantly increased when mice have taken orally pomegranate or beetroot juice, and their combination compared to the control group was negative and positive in addition to mice group cisplatin. The improvement of these results from mice EAC Cells that induce cancer and pre-treatment with pomegranate or beetroot and their combination may be due to pomegranate or beetroot having contained high amounts of phenolic acid and flavonoids compounds and the highest antioxidant activity which homologous recombination sensitizes cells to growth inhibition and apoptosis.

Table (3): Effect of pomegranate and beetroot on mRNAgene expression in EAC Cells

Groups	mRNA-IGF1 (CT)	GAPDH (CT)	mRNA-IGF1 (ΔCT)	(IGF1) (ΔΔCT)	RQ (IGF1)
Normal	29.13	31.96	-2.83	-0.02	1.01
EAC	26.98	31.91	-4.93	-2.12	4.34
EAC+CIS	23.16	28.83	-5.67	-2.86	7.26
P+ EAC	30.13	29.64	0.49	3.3	0.10
BR+ EAC	28.91	28.71	0.20	3.01	0.12
P+BR+ EAC	28.16	27.18	0.98	3.79	0.07

CT: Cycle threshold, Δ CT: Delta cycle threshold, RQ: Relative quantitative of gene expression in relation to a housekeeping gene (GAPDH); IGF: insulin growth factor

In agreement with the behavior of the pomegranate mRNA considerable elevated DJ-1 protein by 1.5-fold than the control. DJ-1 can protect cells and tissues from oxidative stress through several antioxidants like superoxide dismutase. In addition, this protein stabilizes nuclear factor-binding erythroid-2 (NRF2), a key regulator of antioxidant proteins and detoxification enzymes[28].

Pomegranate fruits, illustrate cancer preventive role due to being had contained high amounts of antioxidants and other

valuable ingredients. Moreover, the antitumor activities of pomegranate were evident by modulating cell signaling pathways including transcription factors, apoptosis, and angiogenesis[11].

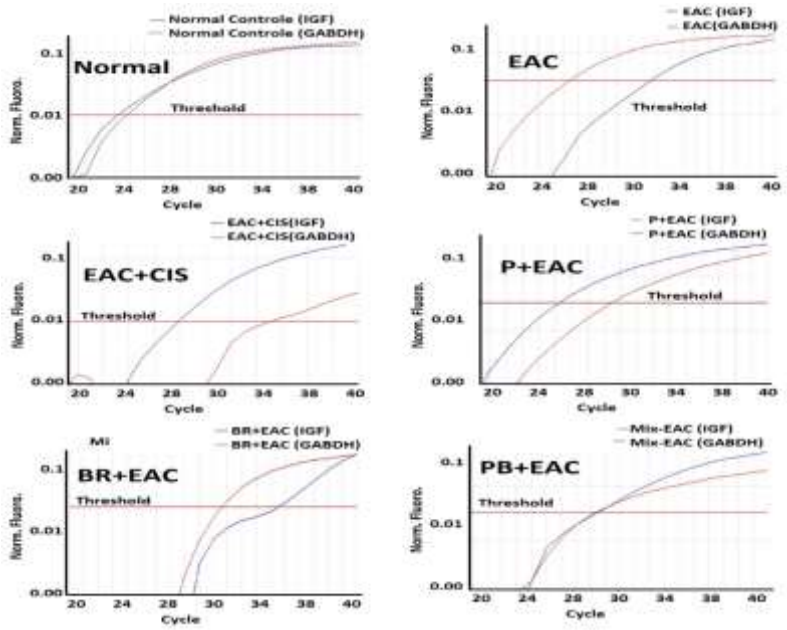

Figure (3): Effect of pomegranate and beetroot on mRNA gene expression in EAC Cells

CONCLUSION

Pomegranate and beetroot contained high amounts of phenolic acid and flavonoid compounds and the highest antioxidant activity as DPPH. Moreover, the tumors induced by Ehrlich Ascites in mice were pre-treatment using pomegranate and beetroot and their combination. Thus could be observed that the pomegranate and beetroot which that homologous recombination sensitizes cells to growth inhibition and apoptosis.

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