Low Concentrations of Pomegranate Rind Extract Influence: Proliferation, Cytotoxicity and Apoptosis in Cancer Cell Lines

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Abstract

Background: Pomegranate (*punica granatum*) fruits are widely consumed as juice. The potent antioxidant and anti-atherosclerotic activities of pomegranate are attributed to its polyphenols including in it.

Aim: Pomegranate extract were evaluated for *in vitro* antiproliferative, cytotoxicity and apoptosis activities against Hep2 (Human larynx epidermal carcinoma) and Hela (cervical human carcinoma) cell lines.

Methods: aqueous extraction of pomegranate was prepared and *in vitro* cytotoxicity was evaluated on two cancer cell lines Hep2 (Human larynx epidermal carcinoma) and Hela (cervical human carcinoma) cell lines.

Results: the study showed that the pomegranate extract has greatest antiproliferative activity against the two cell lines used by inhibition proliferation from 13% to 73.9%. At 10 µg/ml, the extract showed inhibitory effect on Hep-2 cell line at 24 hr period of exposure gives 73.9% while the inhibitory effect on Hela cell line at 24 hr was 57.7%.

Conclusions: we concluded that pomegranate extract showed an *in vitro* antiproliferative activity, indicating its potential usefulness to treat different cancer types.

Key words: pomegranate extract, cytotoxicity, cancer cell lines.

Introduction

Punica granatum, which belongs to the family of Punicaceae, is commonly known as pomegranate, grenade, granats and punica apple [1]. *Punica granatum* has been used extensively as a traditional medicine in many countries [2] for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies [3,4].

In addition, *P. granatum* is reported to have antioxidant [5,6], anti-atherosclerotic [7,8], antibacterial [9,10] and antiviral [11] properties. The constituents of *P. granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties [12]. *Punica granatum* peel is used to treat infections found in human sexual organs as well as mastitis, acne, folliculitis, pile, allergic dermatitis, tympanitis, scalds, diarrhea, dysentery and as an antioxidant [13]. The potent antioxidant properties of pomegranate juice (PJ) have been attributed to its high content of punicalagin isomers that can reach levels >2 g/L juice [14–16]. Ellagitannins (ETs) have also been identified as active antiatherogenic compounds in PJ [17,18].

Our group and others have shown that pomegranate fruit extracts and its purified ETs inhibit the proliferation of human cancer cells and modulate inflammatory subcellular signaling pathways and apoptosis [19-22]. Pomegranate fruit extract has also been shown to significantly reduce prostate tumor growth and prostate-specific antigen (PSA) levels in athymic nude mice implanted with CWR22Rv1 prostate cancer cells [23].

Materials & Methods

*Plant material and preparation of the extract:

An aqueous extraction of pomegranate were prepared using dried rind 100 g which soaked in 250 ml boiling distilled water for about 6 hours on a hot plate and homogenized. The mixture was then filtered through a piece of soft cloth and filtered through Whatman no. 1 filter paper to remove all the residual materials. Then, it dried at 45˚C by using hot air oven, with circulatory fan, and kept at 4˚C until the use [24].

*Cytotoxicity test using ELIZA assay:

For this test, the extract were weighed (0.05 g) and dissolved in phosphate buffer saline (PBS) and dimethylsulphoxide (DMSO) to prepare extract solution at 1000 µg/ml. The following dilutions of extract were then prepared (100, 75, 50, 25 and 10) µg/ml and kept at 4˚C until the use.

*Cell line and culture:

This *in vitro* method was used to establish the effect of the water extract of pomegranate on (Hep-2 and Hela tumour cell lines). Solutions were prepared according to ICCMGR standard method. These cells were maintained in RPMI $-_{1640}$ media with 10% (v/v)

bovine serum and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and 95% air.

***Cytotoxic assay:**

The cytotoxic assay was measured using the crystal violate stain [25].

In brief, the extract re dissolved in DMSO then diluted by serum free media (SFM) and (100, 75, 50, 25 and 10) µg/ml concentrations, then tumor cells were seeded in 96- well microplate. After 24 h incubation at 37˚C, the old media was changed with new media SFM containing the serial concentrations of each extract, and then plate incubated for 24 hr of all cell lines and humidified incubator at 37˚C containing 5% CO_2. After finishing the exposure periods, the old media was replaced with 200 µl/well of crystal violate dye and incubated the plate 20 min at 37˚C, the wells were washed with warmed PBS. The plates leaved 15 min at room temperature and then the absorbency of each well was road by ELISA reader at 492 nm.

Statistical analysis:

Experiments data were analyzed using statistical Last Significant Design (LSD). Significance between control and samples was determined using students f-test. A P value ≤0.05 was considered statistically significant.

Results

The crude aqueous extract derived from the peel of *punica granatum* was evaluated as a cytotoxic agent, using the crystal violate assay, against Hep-2cell line and Hela cell line. Two different types of cancer cell lines were evaluated in order to establish if the cytotoxic effect of *punica granatum* was exclusive to any one cell line.

The inhibition values for the continuous exposure of Hep2 cells over a 72 hr period to *punica granatum* extract are shown in **Figure 1 and in table 1.** The results indicate that the extract is potently cytotoxic to the Hep2 cell line with an inhibition value 73.9% in 10µg/ml after 24 h incubation. An additional series of experiments were carried out in order to establish if the *punica granatum* extract was cytotoxic in a dose- and time-dependent manner to the Hep2. Results from these studies are presented in **Figure 1,** and clearly indicate that significant cytotoxic activity was evident after 48 h and 72 h exposure at a concentration ($P<0.05$) and this effect increased in a dose-dependent manner up to 10 µg/ml. Significant cytototoxic activity was also observed after 24 incubation and this was dose and time dependent up to 10 µg/ml, respectively ($P<0.05$). However, the cytotoxic effect of *punica granatum* in these cells abated after 24 h of incubation.

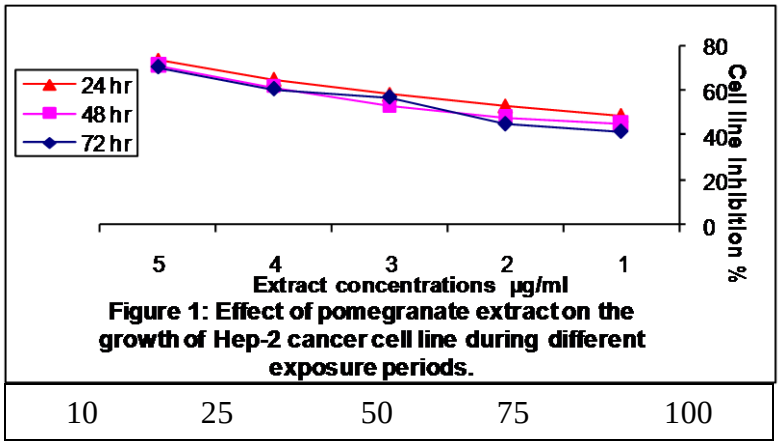

Figure 1: Effect of pomegranate extract on the growth of Hep-2 cancer cell line during different exposure periods.

Table 1: A comparison optical density of growth inhibition of Hep-2 cell line, by using different concentration of pomegranate extracts during three periods of exposure.

Conc. (µg/ml) of extract	Optical density (O.D)		
	24hrs	48hrs	72hrs
Control	1.29	1.15	1.12
100	0.662	0.634	0.65
75	0.605*	0.60	0.613
50	0.538*	0.54*	0.48
25	0.449*	0.44	0.44
10	0.337*	0.33	0.331

(*): means presence of significant differences at level ($p\leq0.05$) between the concentrations

The effects of aqueous extract of *punica granatum* against the human Hela cells are shown in **Figure 2 and in table 2.** The results show that *punica granatum* exhibited less cytotoxic activity in the Hela cells compared to the Hep cells (Fig.1) with an inhibition value 57.7 % at the same concentration 10 µg/ml after 24 hours of incubation.

Additional experiments were also performed for three days in order to determine if the extract was cytotoxic in a dose- and time-dependent manner against the Hela cells. The results in Figure 2 show that there was cytotoxic activity as early as after 24 h incubation (P>0.05) and this decreased in a dose-dependent manner up to 10 µg/ml.

The 24 h treatment of 10 µg/ml *punica granatum* extract inhibited cell proliferation of Hela cells by approximately 50% and minimum cytotoxic effect was obtained after 72 h incubation whereby only approximately 90% viable cells were present (P>0.05).

This study shows that the crude extract of *punica granatum* is more effective in inhibiting growth of Hep cells than the Hela cells.

There are no significant differences between the concentrations during periods of exposure

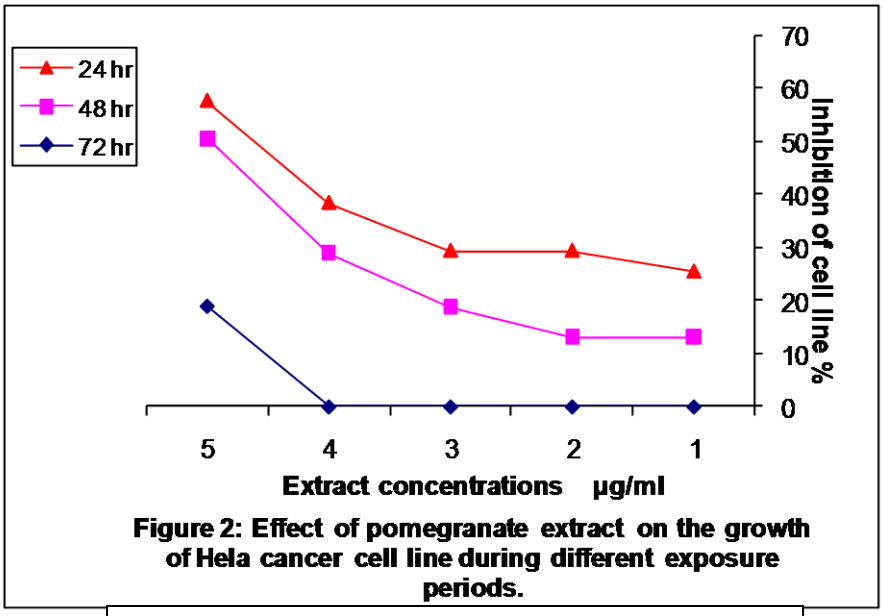

Figure 2: Effect of pomegranate extract on the growth of Hela cancer cell line during different exposure periods.

10 25 50 75 100

Table 2: A comparison optical density of growth inhibition of Hela cell line, by using different concentration of pomegranate extracts during three periods of exposure.

Conc. (µg/ml) of extract	Optical density (O.D)		
	24hrs	48hrs	72hrs
Control	0.78	0.69	0.40
100	0.58	0.60	0.38
75	0.55	0.60	0.38
50	0.55	0.56	0.38
25	0.48	0.49	0.38
10	0.33	0.34	0.314

Discussion

Recent studies have highlighted the benefits of using medicinal plants with combined antioxidants properties for the treatment from cancer types.

Pomegranate is a popular plant world wide consumed in fresh and beverage forms and has been used extensively in ancient cultures for various medicinal properties [26]. Pomegranate juice and extracts have been shown to have potent in vitro antioxidant [16, 27] and in vivo anti atherosclerotic properties [7,20, 28], attributed to its content of polyphenols including Ellagitannins and ellagic acid.

In this study we used different ranges of extract concentration until we found the best or ideal range

which give high inhibition to the two type of cells used. In present study the rind extract from pomegranate decreased the viable cell number of Hep-2 cell line but little of that appears on Hela cell lines.

In study to investigate the activity of punicalagin, ellagic acid and pomegranate tannin as anti tumor activity they found that these compound from pomegranate have the ability to decrease the viable cell number of human oral, prostate and colon tumor cells; however superior activity was obtained with pure pomegranate juice [29]. Similarly, in the apoptotic studies, pomegranate juice induced apoptosis in HT-29 (human colon cancer cells) when concentrations of punicalagin, ellagic acid and pomegranate tannin equalizes to amounts found in pomegranate juice had no effect. Only when the concentration of these compounds was raised to equivalent amount (w/w) with pomegranate juice were they able to induce apoptosis [29].

These findings support the promising results of a pair of 2003 studies in South Dakota and Japan that explored pomegranate seed oil as a safe and effective agent against skin cancer and colon cancer tumors, respectively [30,31]. In a 2002 study, pomegranate seed oil inhibited the proliferation of human breast cancer cells up to 90%, while fermented pomegranate juice polyphenols inhibited 47% of cancerous lesion formation in mammary gland cells from mice [32].

The effects of ellagic acid on cell cycle events and apoptosis were studied in cervical carcinoma (CaSki) cells. Ellagic acid at a concentration of 10 M induced G arrest within 48 h, inhibited overall cell growth and induced apoptosis in CaSki cells after 72 h of treatment. Activation of the cdk inhibitory protein p21 by ellagic acid suggests a role for ellagic acid in cell cycle regulation of cancer cells [33]

Ellagic acid, a polyphenol derived from pomegranate, has been identified to have potent antioxidant, anti-cancer, and anti-atherosclerotic properties [34].

In study examined the antileukemic activity of non edible parts of 13 common Thai tropical fruits. Their ethanolic extracts were tested for cytotoxic effects on U937, K562, HL60, Molt 4 and normal human peripheral blood mononuclear cells (PBMCs). Three of 20 crude plant extracts (kaffir lime leaves, mangosteen peels, and wampee leaves) had strong cytotoxic effects on K562, U937, and Molt4 cells. Furthermore, pomegranate peel extract had a potent cytotoxic effect on HL60 cells (8.0 µg/ml), but was non-toxic to normal PBMCs, indicating that as a potential source of antileukemic agents [35].

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