

RESEARCH ARTICLE

Anti Tumoral Properties of *Punica granatum* (Pomegranate) Seed Extract in Different Human Cancer Cells

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Abstract

Background: *Punica granatum* (PG) has been demonstrated to possess antitumor effects on various types of cancer cells. In this study, we determined antiproliferative properties of a seed extract of PG (PSE) from Iran in different human cancer cells. **Materials and Methods:** A methanolic extract of pomegranate seeds was prepared. Total phenolic content (TPC) and total flavonoid content (TFC) were assessed by colorimetric assays. Antioxidant activity was determined with reference to DPPH radical scavenging activity. The cytotoxicity of different doses of PSE (0, 5, 20, 100, 250, 500, 1000 µg/ml) was evaluated by MTT assays with A549 (lung non small cell carcinoma), MCF-7 (breast adenocarcinoma), SKOV3 (ovarian cancer cells), and PC-3 (prostate adenocarcinoma) cells. **Results:** Significant ($P < 0.01$) or very significant ($P < 0.0001$) differences were observed in comparison to negative controls at all tested doses (5-1000 µg/ml). In all studied cancer cells, PSE reduced the cell viability to values below 23%, even at the lowest doses. In all cases, IC₅₀ was determined at doses below 5 µg/ml. In this regard, SKOV3 ovarian cancer cells were the most responsive to antiproliferative effects of PSE with a maximum mean growth inhibition of 86.8% vs. 82.8%, 81.4% and 80.0% in MCF-7, PC-3 and A549 cells, respectively. **Conclusions:** Low doses of PSE exert potent antiproliferative effects on different human cancer cells SKOV3 ovarian cancer cells as most and A549 cells as least responsive regarding cytotoxic effects. However, the mechanisms of action need to be addressed.

Keywords: Pomegranate seed extract - A549 - SKOV3 - MCF-7 - PC-3 - cancer cell lines - anti proliferative effects

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Introduction

The insufficient treatment options for advanced metastatic cancers call for developing efficient methods to take down cancer. One such strategy is through chemoprevention, if possible by the use of non-toxic natural products. (Jahanban-Esfahlan et al., 2010; Valiyari S et al., 2013; Abbasi et al., 2014a; Yousefi et al., 2015). Pomegranate, used for centuries in folk medicine is now being acknowledged as a potential chemopreventive and anticancer agent. The tree/fruit consist of several components as: seed, juice, peel, leaf, flower, bark, and roots, each of which exerts intriguing pharmacologic activity (Modaeinama et al., 2015). The *Punica granatum*, especially its fruit, possesses a vast ethno medical history and represents a phytochemical reservoir of heuristic medicinal value. *P. granatum* constitute of gallic acid, ellagic acid, gallo catechins, delphinidin, cyanidin, pelargonidin and sitosterol, which possess potent antioxidant and anti cancer activity. The anticancer activity includes interference with cell proliferation, cell cycle,

angiogenesis and invasion (Lansky and Newman, 2007).

Pomegranate seed oil (PSO) comprises 12–20% of total seed weight. The oil consists of octadecatrienoic fatty acids, with a high content of cis9, trans 11, cis13 acid (i.e. punicic acid), synthesized in situ from nonconjugated octadecadienoic fatty acid, linoleic acid itself about 7% of PSO. (Singh RP et al., 2002). The fatty acid component of PSO comprises over 95% of the oil, of which 99% is triacylglycerols. Minor components of the oil include sterols, steroids, and a key component of mammalian myelin sheaths, cerebroside. Seed matrix includes lignins, fusion products of cell wall components and hydroxycinnamic acids, and potently antioxidant lignin derivatives. Poly phenolic compounds, as well as flavonoids and tannins are abundant in the peels of wild crafted compared to cultivated fruits (Syed et al., 2007).

Previous studies have demonstrated the anticarcinogenic activity of pomegranate extracts in a series of human cancer cells. In this study, we determined to study the cytotoxic properties of seed part of PG (PSE) from Iran on different human cancer cells.

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Materials and Methods

Preparation of Extracts

Pomegranate fruit were collected from Mazandaran province, Iran in 2014. The seed parts were separated, dried and grounded into fine powder using a blender. Extractions were performed in a Soxhlet apparatus with methanol. The extract were concentrated by rotary evaporator and then dried in very low pressure. The dried extracts were stored at -2°C . A stock of 10 mg of extract was prepared in 1mL dimethyl sulfoxide (DMSO) and was filtered using 0.22mm syringe filter. The percentage of DMSO in the experiment kept below 0.5).

Determination of total phenolic content (TPC)

Total phenolic content was determined with Folin-Ciocalteu Reagent (FCR) according to the a described method (Singleton and Rossi, 1965) with some modifications. Briefly, 0.5 ml of each phenolic extract was mixed with 2 ml of 7.5% sodium carbonate, and then the mixture was allowed to stand at room temperature for 2 min. After addition of 2.5 ml ten-fold Folin-Ciocalteu reagent, the mixture was incubated in the dark room for 30 min. The absorbance was measured at 720 nm by using a spectrophotometer. The results were expressed as equivalent mg of Gallic acid per 100 g of fresh mass (mg GAE/100 g FM). A standard curve for Gallic acid was plotted under the same conditions as the studied samples. All determinations were performed in triplicates.

Determination of total flavonoid content (TFC)

Total flavonoid content of the extracts were assayed by the colorimetric method described by other authors (Zhishen et al., 1999; Jahanban- Esfahlan et al., 2012), with minor modifications. CME (250 μl) was mixed with 1.25 ml of distilled water and 75 μl of a 5% NaNO_2 solution. After five minutes, 150 μl of a 10% $\text{AlCl}_3 \cdot \text{H}_2\text{O}$ solution, 500 μl of 1 M NaOH and 275 μl of distilled water were added to the mixture. The absorbance of the mixture was measured at 507 nm. The results were expressed as equivalent mg of Quercetin per 100 g of fresh mass (mg Q/100 g FM) and compared with the Quercetin standard curve, which was made under the same conditions. All determinations were performed in triplicates.

DPPH free radical scavenging activity

The DPPH radical scavenging activity was determined as described Brand-Williams et al. (Brand-Williams et al., 1995) with some modifications. Various volumes of extracts (30, 50, 70 and 100 mL) were added to 1mL of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM in methanol) and the reaction mixture shaken vigorously. After incubation at room temperature for 10 min, the absorbance of this solution was determined at 517 nm, by using a spectrophotometer. The antioxidant activity was expressed as IC₅₀ values, which were calculated by non-linear regression with a one phase exponential association equation using GraphPad Prism version 6.0.

Cell Culture

SKOV3 (human ovarian carcinoma), MCF-7(human

breast adenocarcinoma), PC-3 (Human prostate adenocarcinoma), A549 (lung non small cell cancer cells) were obtained from the Pasture Institute (Tehran- Iran). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (10 U/ml), streptomycin (10 $\mu\text{g}/\text{ml}$) and 0.2 mM sodium pyruvate. Cultures were incubated in the presence of 5% CO_2 at 37 $^{\circ}\text{C}$ and 100% relative humidified atmosphere.

MTT Assay

The cells were seeded in 96-well plates with a density of 1×10^4 cells/well/200mL and incubated for 24 h at 37C and 5% CO_2 . The cells were treated with different concentrations of solvent extracts (5, 20, 100, 250, 500, 1000 $\mu\text{g}/\text{ml}$) and 0.2% (v/v) DMSO (Merck, Darmstadt, Germany) as a negative control. Paclitaxel (plant-derived chemotherapeutic anti-cancer drug from *Taxus brevifolia*) was used as a positive control. After 72 h treatment, 10 mL of MTT reagent was added to each well. The plates were incubated at 37 $^{\circ}\text{C}$ and 5% CO_2 for 4 h. Then, 100 mL of the solubilization solution was added to each well and followed by incubation overnight at 37 $^{\circ}\text{C}$ to dissolve formazan crystals. Finally, absorbance was read using an ELISA plate reader (Bio Teck, Bad Friedrichshall, Germany) at a wavelength of 570 nm. The percentage of cytotoxicity and cell viability were calculated using following equation: % Cytotoxicity=1-(mean absorbance of treated cells/mean absorbance of negative control) and % Viability=100- % Cytotoxicity.

Statistical analysis

All the data represented in this study are mean \pm SEM of three identical experiments made in three replicate. Statistical significance was determined by analysis of variance, followed by LSD test and p-value ≤ 0.01 was considered significant. All analyses were conducted using the SPSS 16.

Results and Discussion

In recent decades, in contrast to all accomplishments in cancer treatment, it still stands out among the most common killers in the world (Jahanban-Esfahlan et al., 2015). According to the 2014 cancer statistics in the United States, Among men, cancers of the prostate, lung and bronchus, and colorectum will account for about 50% of all newly diagnosed cancers ,as prostate cancer alone will account for 27% (233,000) of incident cases in men. At the other hand, cancers of breast, lung and bronchus, and colorectum, comprises one-half of all cases in women. Breast cancer alone is expected to account for 29% (232,670) of all new cancers among women. Overall, cancers of the lung and bronchus, prostate, breast, and colorectum continue to be the most common causes of cancer death. These 4 cancers account for almost half of the total cancer deaths among men and women, with more than one-quarter of all cancer deaths due to lung cancer. An urgent need in cancer control today is to develop effective and affordable approaches to the early detection, diagnosis, and treatment of cancer(Siegel et al., 2014).

Among a plethora of options for cancer treatment

and prevention, natural products especially those rich in antioxidants, have always being regarded as safe chemopreventives to Fight cancer (Abbasi et al., 2014b). Various fractions have been extracted from the pomegranate peel and seeds using ethyl acetate, methanol and water. Extraction with methanol was reported to give a higher yield with greater antioxidant activity and peel exhibited higher activity as compared to seeds, ascribed to its phenolic composition (Singh RP et al., 2002). PSE extract from Persian cultivators showed a high content for divergent polyphenolic and flavonoid compounds ,up to 35.31 mg Q/100g FM (%TFC) and 371.5 mg GAE /100g FM (%TPC). In agreement with Singh et al findings, our results confirmed that peel extract (PPE) showed significant high amount of TFC (103.61 mg Q/100g FM) and TPC (1532.2 mg Q/100g FM) compared to the PSE (data not published). Moreover, DPPH IC50 was determined 52.6 $\mu\text{g/ml}$ for PSE and 1.5 $\mu\text{g/ml}$ for PPE. Surprisingly, cytotoxicity studies revealed that PSE exert superior anti tumoral activity than PPE.

Our results indicated that in all doses, significant or

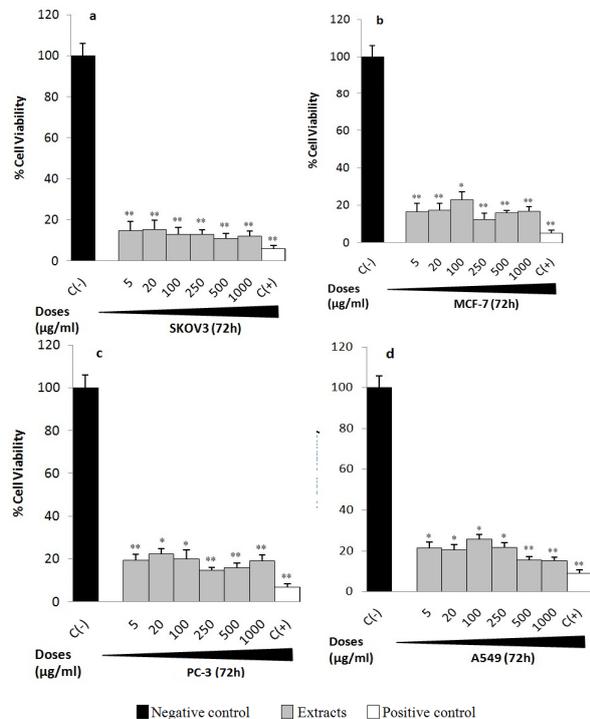


Figure 1. PSE Extract Inhibit Different Human Cancer Cell Proliferation. * $p < 0.01$; ** $p < 0.001$, Compared to the Negative Control

very significant difference was observed between treated and untreated controls in a dose independent manner (Figure1, 2). In all cell lines, 5 $\mu\text{g/ml}$ of PSE caused to $\geq 50\%$ growth inhibition in the treated cells which account for the high toxicity profile of 60% methanolic extract of PSE (Table1).

In case of ovarian cancer cells, the maximum growth inhibition of 89% was obtained at doses 500 $\mu\text{g/ml}$, value close to positive control (20 $\mu\text{g/ml}$ Toxol used as positive control and it caused to 93.76% growth inhibition of SKOV3 cancer cells) meanwhile dose 5 $\mu\text{g/ml}$ of PSE caused to the lowest growth inhibition of 85.2%. The mean growth inhibition in these cells was 86.61%.

In MCF-7 cancer cells, the maximum growth inhibition of 87.6% was obtained at doses 250 $\mu\text{g/ml}$, value

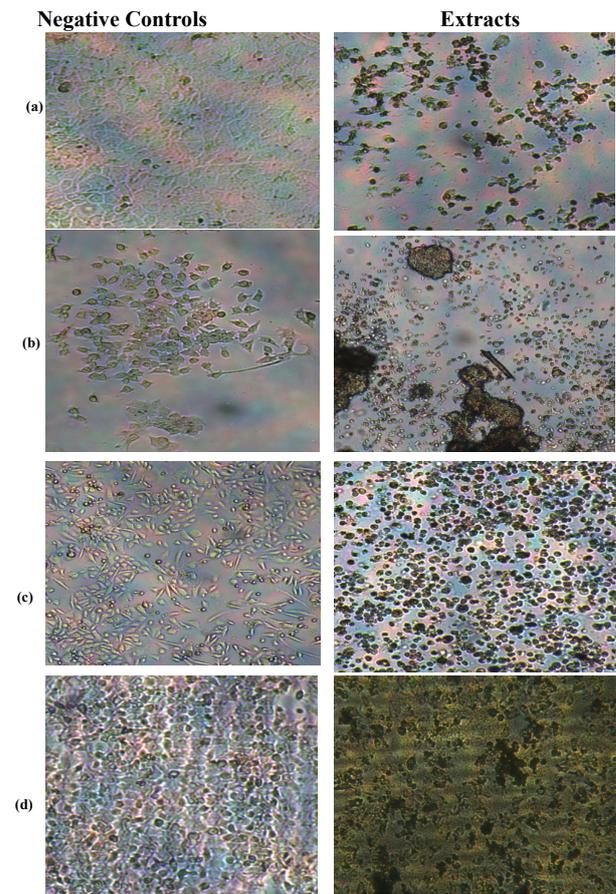


Figure 2. Cytotoxic Effects of PSE on Human SKOV3 (a), MCF-7 (b), PC-3(c) and A549 (d) Cell Lines after 72h Exposure

Table 1. Growth Inhibition % of Different Doses of PSE on Different Human Cancer Cells

Doses ($\mu\text{g/ml}$)	% Growth Inhibition of PSE (72 h)			
	SKOV3	MCF-7	PC-3	A549
1000	88.0 \pm 6.2	83.25 \pm 5.4	80.84 \pm 5.5	84.75 \pm 5.1
500	89.0 \pm 4.0	84.2 \pm 6.6	84.3 \pm 3.9	84.5 \pm 5.3
250	87.0 \pm 6.4	87.6 \pm 2.4	85.37 \pm 4.4	78.37 \pm 3.9
100	87.0 \pm 6.1	77.04 \pm 6.1	79.75 \pm 6.1	74.37 \pm 4.8
20	84.7 \pm 5.2	82.62 \pm 4.4	77.8 \pm 8.2	79.62 \pm 6.7
5	85.2 \pm 7.8	83.7 \pm 3.2	80.6 \pm 8.1	78.46 \pm 4.4
Mean \pm SEM	86.81 \pm 1.2	82.76 \pm 5.6	81.44 \pm 5.5	80.01 \pm 5.8

comparable to that of 20 μ g/ml Toxol which caused to 95.1% growth inhibition of MCF-7 breast adenocarcinoma cells. The lowest growth inhibition of 77.04% was obtained at dose 100 μ g/ml of PSE. The mean growth inhibition was 82.76 %.

In PC-3 prostate adenocarcinoma cells, PSE caused to the maximum growth inhibition of 85.37% at doses 500 μ g/ml. In this cell line, 20 μ g/ml Toxol caused to 93.1% growth inhibition of PC-3 cancer cells meanwhile dose 20 μ g/ml of PSE caused to the lowest growth inhibition 77.8%. The mean growth inhibition in these cells was 81.4% (Table1, Figure 2).

In case of A549 cancer cells, the maximum growth inhibition of 84.75% was obtained at doses 1000 μ g/ml. Dose 20 μ g/ml Toxol caused to 90.25% growth inhibition of A549 cells. In this cell line, dose 100 μ g/ml caused to the lowest growth inhibition of 74.37%. The mean growth inhibition in these cells was 80.01% (Table1, Fig 2).

In all cancer cells, PSE extract reduced the cell viability to values below 26%, even the lowest doses. In this regard, SKOV3 ovarian cancer cells were the most responsive cells to antiproliferative effects of PSE with a maximum mean growth inhibition of 86.81% vs 82.76%, 81.44% and 80.01% in MCF-7, PC-3 and A549 cells, respectively. At the other hand, it seemed that lung non small cell cancer cells were the least responsive cells to cytotoxic properties of PSE compared to the other studied cancer cells. This finding could be attributed to the high growth rate of A549 cells in the culture compared to the other studied cells, so higher doses of PSE required to results in the maximum growth inhibition of $\geq 80\%$.

In a study by Sineh Sepehr et al (2012), authors investigated cytotoxic and apoptotic effects of PPE and PSE extract of Iranian Pomegranate on proliferation of PC-3 cells. In this study, PPE caused to the maximum growth inhibition of 65% at doses 600 μ g/ml while PSE caused to no significant growth inhibition on proliferation of PC-3 cells. Pomegranate seed extract (PSE) at the highest dose (600 μ g/ml) caused to maximum growth inhibition of 35%. In our experiment, at dose 250 μ g/ml, PPE and PSE caused to the maximum growth inhibition of 84.16% and 85.37%, respectively, values comparable to 20 μ g/ml Toxol which suppressed 93.1% growth inhibition of PC-3 cells. These differences could be attributed to the lower antioxidant activity of ethanolic extracts used by these authors, meanwhile the methanolic extract used in our study showed significant content for TFC and TPC and exert a high antioxidant activity. Notably, 1000 μ g/ml PSO resulted in lower suppression than 250 μ g/ml dose suggesting that an optimal biological dose is more important and relevant than a maximally tolerated one.

In conclusion, low doses of PSE exert potent antiproliferative effects on different human cancer cells and it seemed that SKOV3 ovarian cancer cells were the most responsive cells and A549 cells were the least responsive cancer cells to the cytotoxic effects of PSE, however the mechanism of action need to be addressed.

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