Advanced Pharmaceutical Bulletin

Adv Pharm Bull, 2014, 4(Suppl 2), 583-590
doi: 10.5681/apb.2014.086
http://apb.tbzmed.ac.ir

TUOMS Publishing Group

Research Article

Growth-Inhibitory and Apoptosis-Inducing Effects of *Punica granatum L. var. spinosa* (Apple Punice) on Fibrosarcoma Cell Lines

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Article info

Article History:
Received: 28 July 2014
Revised: 27 December 2014
Accepted: 28 December 2014
ePublished: 31 December 2014

Keywords:
· Apoptosis
· Fibrosarcoma cell line
· Punica granatum L. var. spinosa

Abstract

Purpose: *Punica granatum L. var. granatum* (Pomegranate), an herbaceous plant found in Iran, The aim of this study was to investigate the cytotoxic effects, induction of apoptosis, and the mechanism of cell death of ethanol extract from *Punica granatum L. var. spinosa* on the mouse fibrosarcoma cell line, WEHI-164.

Methods: Various parts of the herbs were extracted from fruit using ethanol as the solvent, and the cytotoxicity and cell viability of the ethanolic extract were determined by the MTT assay. To determine whether necrosis or apoptosis is the predominant cause of cell death, cell death detection was performed using the ELISA method. The induction of apoptosis was confirmed using the terminal deoxynucleotidyl transferase- (TdT-) mediated dUTP nick end labeling (TUNEL) assay. Moreover, a sensitive immunoblotting technique was used to examine the production of Caspase-3 and Bcl_2 proteins.

Results: Our findings suggested that the ethalonic extract of *Punica granatum L. var. spinosa* altered cell morphology, decreased cell viability, suppressed cell proliferation and induced cell death in a time- and dose-dependent manner in WEHI-164 cells (IC_{50} = 229.024µg/ml), when compared to a chemotherapeutic anticancer drug, Toxol (Vesper Pharmaceuticals), with increased nucleosome production from apoptotic cells. Induction of apoptosis by the plant extract was proved by the decrease of pro-Caspase-3 and Bcl_2 proteins and quantitatively confirmed by Immunoblotting analysis.

Conclusion: The results obtained from the present study have demonstrated the growth-inhibitory effect of Ethanol Extracts from *Punica granatum L. var. spinosa*, and clearly showed that apoptosis was the major mechanism of in-vitro cell death induced by the extract.

Introduction

Cancer is one of the major causes of mortality and morbidity throughout the world.[1] Conventional cancer treatments including surgery, chemotherapy, and radiotherapy, used separately or in combination, have limitations and side effects. This highlights the need for the development of novel therapies to improve the survival and quality of life of patients with cancer. Hence, the development of novel therapeutic compounds without significant adverse side effects is considered as an important area for immunopharmacological studies. Indeed, it is conceivable to therapeutically induce cancer cell apoptosis.[2,3] Apoptosis remains a highly coordinated cell suicide mechanism that normally occurs during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. It is characterized by a series of morphological and biochemical alterations such as blebbing of the plasma membrane, condensation of nuclear heterochromatin, cell shrinkage, formation of apoptotic bodies, loss of positional organization of organelles in the cytoplasm and formation of apoptotic bodies.[4] Under normal circumstances, damaged cells will undergo apoptosis that is associated with the rapid engulfment and removal of cell corpses by phagocytic cells. In addition, apoptosis is considered to be a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. On the other hand, in the case of cancer cells, mutations may have occurred that prevent cells from undergoing apoptosis.[1,5,6] Dysregulation of apoptosis plays a critical role in the development of cancers, and mediates resistance to cancer therapy.[7] The ability of apoptosis to modulate the life or death of a cell is recognized for its profound therapeutic potential.[5,6] Therefore, many forms of anti-cancer therapy focus on inducing apoptosis in cancer cells. Several Studies revealed a high frequency of apoptosis in spontaneously regressing tumor and in tumor treated with cytotoxic anti-cancer agents. A considerable number of studies have been dedicated to

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the apoptosis and its potential role in intervention of the lethal properties of antineoplastic agents in cancer cells. It is now well-established that anticancer agents induce apoptosis, so that disruption of apoptotic pathways can reduce treatment sensitivity.[8,9] Therefore, Apoptosis seems to be a reliable marker for the evaluation of potential anti-cancer agents. Plants have a long history of use in traditional medicine to treat and prevent human diseases including cancer. It is significant that over 60% of currently used anticancer agents are derived from natural sources, including plants, marine organisms and microorganisms.[10] A wide variety of natural compounds possesses significant cytotoxic as well as chemopreventive activity through induction of apoptosis in cancer cells.[11] Plant extracts used in traditional medicine exhibit a similar property.[12-14] Historically, plant-derived extracts have been used in traditional medicine for maintaining health, enhancing overall immune status, as well as prevention and treatment of different diseases.[14,15] Some of these substances are supposed to be of the potential value as cancer chemopreventive and therapeutic agents within the human body. The species *Punica granatum L. var. granatum***,** (pomegranate), a ancient fruit native to Iran, has been widely used by traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases, including cancer, cardiovascular disorders, diabetes, male infertility, Alzheimer's disease, aging, and AIDS.[15-17] Several recent studies have demonstrated that *Punica granatum L. var. granatum* extract mediates a wide spectrum of biological activities including antibacterial, antiviral, antifungal, cytotoxic and immuno-potentiating activities. The *Punica granatum L. var. granatum* tree (pomegranate), specifically its fruit, possesses a vast ethnomedical history and represents a phytochemical reservoir of heuristic medicinal value.[15,16,18] The pomegranate tree/fruit can be divided into several anatomical compartments including seed, juice, peel, leaf, flower, bark, and root, each of which has an interesting pharmacologic profile; For example, Juice and peel possess potent antioxidant activities, while juice, peel and oil are all weakly estrogenic and heuristically of interest for the treatment of menopausal symptoms as well as sequellae. The use of juice, peel and oil has also been found to be of anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion and angiogenesis.[15,16] A large number of *in-vitro*, *in-vivo* and preclinical studies have been performed over the last decade to assess the antioxidant, anticarcinogenic and anti-inflammatory properties of pomegranate constituents, focusing on the treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections and antibiotic resistance as well as ultraviolet radiation-induced skin damage.[16] Collectively, the present study was conducted to examine the *in vitro* cytotoxic activities of a highly standardized ethanolic extract of *Punica granatum L. var. spi*nosa (PGS) and to determine the possible mechanism of cell death in malignant and nonmalignant cell lines, using the MTT cytotoxicity assay. Cell death detection (ELISA) and TUNEL assay were performed to detect apoptotic chromatin in glomerular membranes, and an immunoblotting assay was also carried out to survey the expression of Caspase-3 and Bcl_2 proteins.

Materials and Methods

Preparation of Plant Extract

Fresh *Punica granatum L. var. granatum* (Pomegranate) fruits were collected from the southeast of Golestan province, Iran (Ramian) in July 2013, and their identification confirmed by Dr. Mazandarani from the Medicinal Plant Research Center of Islamic Azad University of Gorgan, Iran. The voucher specimen was deposited in the herbarium as mentioned above, with the number 315HRCMP. Pomegranate seeds and peels were manually separated, shade dried at room temperature and then ground into a fine powder with mortar and pestle. Only fine powder was collected and stored in air-tight containers, completely protected from heat, humidity and light. The ethanolic extract was extracted with macerating 100 g of powdered dry plant material in 500 ml of 70% ethanol solvent for 48 h at room temperature using percolator apparatus (2-liter volume). The extract was removed from percolator, filtered through Whatman filter paper (NO.4) and dried under reduced pressure at 37°C with rotator evaporator. The ethanol extract was filtered, concentrated using a rotary evaporator and then evaporated to dryness. Briefly, the concentrated extract was dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to make a stock solution of 10 mg/ml. the stock solution was lyophilized and stored at -20°C until use. The working solutions of 0.2 mg/ml were prepared by diluting 20 µL of the stock solution into 980 µL sterile serum-free culture medium (RPMI 1640), at different concentrations. It is should be noted that the percentage of DMSO in the experiment should not exceed 0.5. All stock and working solutions were stored at 4°C.

Cell Cultures

The mouse fibrosarcoma cell line (WEHI-164) and mouse non-malignant cell line (L929) were obtained from Pasteur Institute of Iran, national cell bank of Iran (NCBI). The cells were cultured in RPMI-1640 (SIGMA) containing 10% heat inactivated fetal calf serum (FBS)**,** 100 unit/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen Gibco), and incubated at 37°C in a humidified incubator with 5% CO_2 atmosphere for 2 weeks.

Once grown up to approximately 60% confluence, the cells were passaged. Having harvested from sterile T75 culture flasks (Nunc, Denmark), the cell numbers were immediately counted using a Neubauer hemocytometer and cell viability was determined by trypan blue exclusion assay. Approximately ten thousand cells from log-phase cultures were seeded in 100 µL of RPMI-164 medium supplemented with 10% fetal bovine serum per

well of 96-well flat-bottom culture plates (Nunc, Denmark). Exponentially growing cells were treated with different concentrations of PGS extract (peel and seed), and incubated for defined times at 37°C.[3,15] Proliferative response and cell death of the PGS extract-treated cells were determined using Trypan blue, MTT, cell death ELISA and TUNEL assays.

Dye Exclusion Assay

Cellular cytotoxicity induced by the treatment with PGS extract was measured by trypan blue exclusion assay. Briefly, cells were seeded into 96-well plates at a density of 1×10^4 cells per well and treated with or without (as a control) the crude extract at different doses for 24 and 48 hours. At the end of the incubation period, the cells were harvested and washed twice with PBS. The cell pellet was then resuspended in 0.5 mL PBS. Then, 20 µL of cell suspension was mixed with equal volume of 0.4% trypan blue (Sigma, USA Merck) and counted using a Neubauer haemocytometer (Weber, England) under clear-field microscopy (Nikon, Japan). The experiment for each extract and control were conducted twice in triplicate. The cells were then stained with trypan blue dye (Merck) and live cells were enumerated. Cell counts were expressed as mean ± standard deviation (SD).

MTT Cell Cytotoxicity Assay

The MTT colorimetric assay detects the reduction of the metabolic dye [3-(4, 5-dimethylthiazolyl)-2, 5-diphenyl-tetrazolium bromide] by mitochondrial dehydrogenase to blue formazan product, reflecting the normal function of mitochondria in metabolically active cells, allowing the measurement of cell cytotoxicity and viability. In this light, the effects of the extract on the cell proliferation were first assessed using MTT (Sigma).

Briefly, viable cells were seeded onto 96-well tissue culture plates (Nunc, Denmark) at a density of 1×10^4 cells/100 µL per well, and then incubated overnight at 37°C. The next day when cells approximately reached >80% confluence, the media were replaced with 200 mL of fresh complete medium containing peel and seed extracts of PGS at the various concentrations of 0, 10, 20, 50, 100, 200, 300, 400, 500 µg/mL for 24- and 48-hour treatment periods at 37 °C in triplicate. It should be noted that no extract was added to the negative-control well and that Toxol (Paclitaxel: plant-derived chemotherapeutic anticancer drug from *Taxus brevifolia* L.) was used as a positive control.[3,19] After 24 and 48 hrs of incubation, the medium was removed, cell layers were washed with phosphate buffered saline (PBS, Invitrogen Gibco), 100 µL of the MTT solution (0.5 mg/mL in PBS) in RPMI 1640 without FCS was added to each well and the cells were incubated for 4 h at 37 °C in a humidified atmosphere according to the manufacturer's protocol. After incubation period, the cell cultures were centrifuged at 1000 g for 5 min, and the supernatants were discarded. Then, 100 µL of solubilizing reagent, dimethyl sulphoxide (DMSO, 1%, 1000 µl, Sigma Aldrich, USA) and Sorenson buffer were added to each well, and gently and thoroughly mixed at room temperature for 1 h in order to dissolve the formazan crystals formed.

The absorbance of each well was measured at 570 nm using an ELISA plate reader. The absorbance readings of untreated cells were considered to be 100% confluence. The mean optical density (OD) ± SD for each group of triplicates was measured in three independent experiments. The whole procedure was repeated for three times. The inhibitory rate of cell growth was calculated using the following formula:

$$\% \text{ Growth inhibition} = (1 - \text{OD extract treated}) / \text{OD negative control} \times 100)$$

Cell viability was expressed as the ratio (%) of optical density values for treated cells to control cells (% of control). The mean extract concentration that was cytotoxic to 50 % of the cells (IC_{50}) was calculated from multiple runs.

Cell death detection

Quantification of apoptotic cell death was determined by Cell Death Detection ELISA ^{PLUS} (Roche Applied Science, Switzerland), which measures cytoplasmic histone-complexed DNA fragments (nucleosomes) produced in cytoplasm of the apoptotic cells during apoptosis. Briefly, after incubation with the PGS extract (at concentrations determined by MTT assay) for 24 h, the WEHI-164 cells were pelleted and lysed. Mouse monoclonal antibodies directed against single-strand DNA and histones (H_1, H_2a, H_2b, H_3 and H_4) specifically detected both mononucleosomes and oligonucleosomes derived from cells undergoing apoptosis. Biotinylated anti-histone antibodies then fixed the antibody-nucleosome complexes to the streptavidin-coated microtiter plate. The anti-DNA antibodies were conjugated with horseradish peroxidase, reacting with the substrate ABTS [2,2V-azino-di(3-ethylbenzthiazolinsulfonate)] to form a colored product. All the remaining steps were performed according to the manufacturer's instructions. The resulting color development, being directly proportional to the amount of nucleosomes (single-strand DNA and histones H_1, H_2a, H_2b, H_3 and H_4) captured in the antibody sandwich, was measured at 405 nm (with reference wavelength at 490 nm) using a Benchmark microtiter plate reader (Bio-Rad). The results were expressed as the percentages of apoptosis and necrosis, calculated from the ratio of absorbance of treated (apoptotic) samples to that of untreated (control) ones.[3,15,20,21]

TUNEL Assay

To assess the cell death caused by apoptosis, DNA chromatin morphologic features were determined by an In Situ Cell Death Detection Kit, POD (Roche, Germany). The assay was carried out according to the manufacturer's guidelines. Briefly, cells were cultured on glass slides and analyzed 24 hours after treatment with PGS peel extract (230 µg/ml). Cells on coverslips were washed twice with PBS, air-dried and then fixed for 60

minutes in freshly prepared 4% paraformaldehyde/PBS (Sigma-Germany), pH 7.4, at room temperature. Afterwards, the cells were again washed twice with PBS and incubated with 3% H_2O_2/methanol (Merck, Germany) for 10 minutes. Following washing with PBS, the cells were permeabilized using 0.2% Triton X-100/PBS (Sigma, Germany) for 2 minutes at 4°C. Samples were incubated with 50 µl of TUNEL reaction mixture for 2 hours at 37°C in a dark, humidified chamber, sealed with parafilm. Omission of TdT provided the negative control for the assay, and preincubation of cells with 10 µg/mL DNase I in 50 mM Tris- HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mg/mL BSA for 10 min at room temperature to induce DNA strand breaks artificially served as positive control. All the cells were washed with PBS and incubated for 30 min in a humidified chamber, at 37°C with 50 µL converter-PODS (Anti-fluorescein antibody, Fab fragment from sheep, conjugated with horse-radish peroxidase). After briefly rinsing with PBS, the samples were incubated for 10 min with 100 µL DAB substrate (Sigma, Germany) in the dark. Finally, the samples were mounted and analyzed under light microscope where the apoptotic cells could be detected as highly condensed shrunken dark brown cells.

SDS-PAGE and Electroblotting

To determine caspase 3 and bcl_2 protein levels involved in apoptosis, SDS-PAGE and Immunoblotting analysis were carried out. To this end, the cells were treated with PGS peel extract (230 µg/ml) for 24 hours and washed with cold PBS. Cell lysates were prepared by incubating cells in RIPA lysis buffer [1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS plus protease inhibitor cocktail Tablet (Roche)] on ice for 25 min. Cellular debris was removed from lysates by centrifugation at 15000 g for 20 minutes at 4°C. The total protein concentration was quantified using NanoDrop (1000 Spectrophotometer Wilmington, DE, and USA). Equal amounts of protein (50µg) were mixed with one volume of sample buffer, boiled for 5 min and electrophoretically separated on 12% of SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore; Billerica, MA), and the membrane was incubated for 1 hour in blocking buffer (5% non-fat dry milk in PBS) at room temperature. The membrane was probed with primary monoclonal antibodies against human caspase 3, bcl_2 or β-actin (1:1000) (Cell signaling technology, Beverly, MA) for 3 hours and then washed three times with PBS. After reaction with secondary antibody conjugated with horseradish peroxidase (1:3000) for 1 hour, the protein bands were visualized by ECL detection reagents (Amersham Pharmacia Biotech, Arlington Hts, IL) according to manufacturer's protocol. The optical density of bands was measured using Image J software. The intensities of each band were compared with that of β-actin, and relative intensity ratios were calculated.

Results

Inhibitory effects of PGS on proliferation of WEHI-164 cells

The MTT assay is a standard colorimetric method for the measurement of viable cell number. MTT is reduced to purple formazan in the mitochondria of living cells and directly related to the number of viable cells. The effects of PGS were examined in time- and dose-response experiments after 24 and 48 hours, at the concentrations of 10 to 500 µg/ml. Proliferation of WEHI-164 cells was significantly inhibited by PGS in a dose-dependent manner. Growth inhibitory effect of peel extract on the WEHI-164 cells in 24 and 48 hours was 83.44±2.3% and 86.67±1.1%**,** respectively (Figure 1A), with more than 85% suppression. The concentrations producing 50% growth inhibition (IC_{50}) of the PGS peel extract potently suppressed proliferation of WEHI-164 cells with the IC_{50} value (229.024µg/ml). On the other hand, seed extract induced no significant suppression on the proliferation of WEHI-164 cells (Figure 1B). More importantly, the peel extract induced no significant suppression on the proliferation of normal L929 cells (Figure 1C). Surprisingly, more than 83% and 90% growth inhibition were observed following a 24-h incubation period with 500 and 15 µg/ml of peel extract and Toxol (positive control), respectively (Table 1).

Table 1. Comparison of cytotoxic activity of PGS peel extract (a) and Toxol (b) on the WEHI-164 cells after 24 hours. The data represent the mean ± SEM of three independent experiments. P<0.01.

	Concentration (µg/ml)	Growth inhibition %
(a)	500	83.44
	400	77.67
	300	64.33
	200	42.67
(b)	15	90.76
	10	71.69
	5	63.88
	3.5	51.79

The Effect of PGS on Cell Death of WEHI-164 Cells

As determined by MTT assay, peel extracts (at the concentrations of 50, 100, 200 and 300 µg/ml) and positive control (especially used for cell death detection ELISA kit) in 24 hours were chosen for the WEHI-164 cell line in cell death detection ELISA. The results suggest that Apoptotic cell death is most likely not the only mechanism involved in downregulation of cell growth by PGS extract. Necrosis remained at a low level (<10%) over the whole dose range (Figure 2). Taken together, the possible mechanism was via induction of apoptosis. As shown in Figure 2, the significant increase in nucleosome production at 50-300 µg/ml of PGS extracts after incubation for 24 hours but the ratio of apoptosis was constant approximately 28.4±3.5% to 57.3±1.4 %.

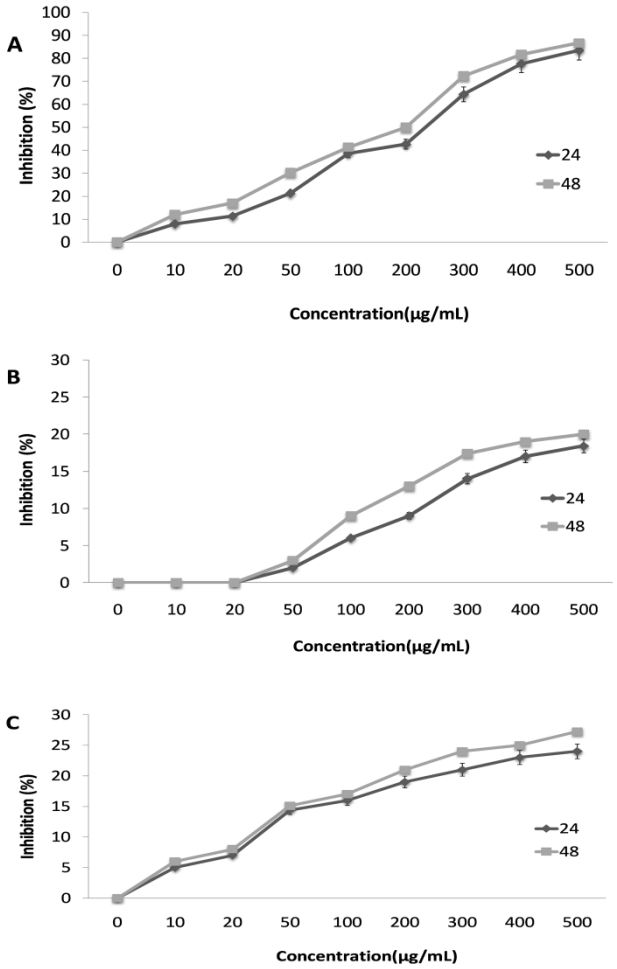

Figure 1. The effects of PGS peel extract on proliferation of WEHI-164 cells (A), seed extract on WEHI-164 cells (B) and peel extract on L929 cells (C) after 24- and 48-hour exposure periods, with increasing concentrations (10–500µg/ml). The inhibitory responses were assessed by the MTT assay. The data represent the mean ± SEM of three independent experiments. P<0.01.

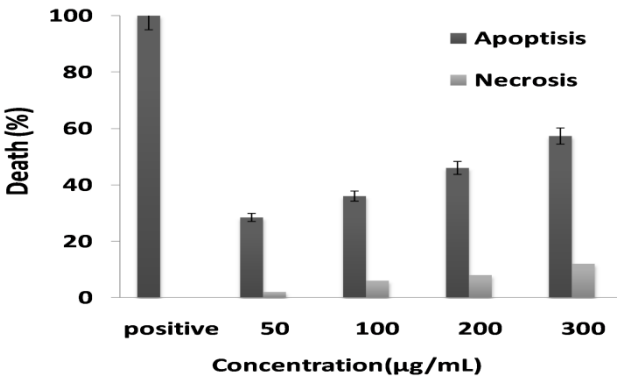

Figure 2. The effects of PGS peel extracts on cell viability of WEHI-164 cells. The cells were incubated with peel extracts at the concentrations of 50, 100, 200 and 300 µg/ml as well as positive control (especially used for cell death detection ELISA kit) for 24 h. The induced apoptosis (internucleosomal DNA fragmentations) was then measured by cell death detection ELISA. The data represented the mean ± SEM of three independent experiments.

Quantification of apoptosis by TUNEL assay

The TUNEL assay was performed to determine whether or not peel extract of PGS induced a decrease in cell viability, and whether cytotoxicity contributed to apoptotic or necrosis death in WEHI-164 cells in vitro. In addition, this assay was used to examine the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks. For *in situ* end labeling, terminal transferase was applied to add labeled UTP to the 3' end of the DNA fragments in individual cells after apoptosis. Cells were incubated with 230 µg/mL of PGS for 24 h and then determined using TUNEL assay. As shown in Figure 3, the apoptotic cells that displayed apoptotic body formation were observed more frequently in the group treated with peel extract (230 µg/ml) for 24 hours, compared to the control cells. The WEHI-164 cells treated with peel extract exhibited condensed and fragmented nuclei (Figure 3b), typical morphological features of apoptotic cells, when compared with homogenous nuclear chromatin particularly evident in the control cells (Figure 3a). It was found that the WEHI-164 cells treated with peel extract (230 µg/ml) for 24 hours exhibited apoptotic body formation.

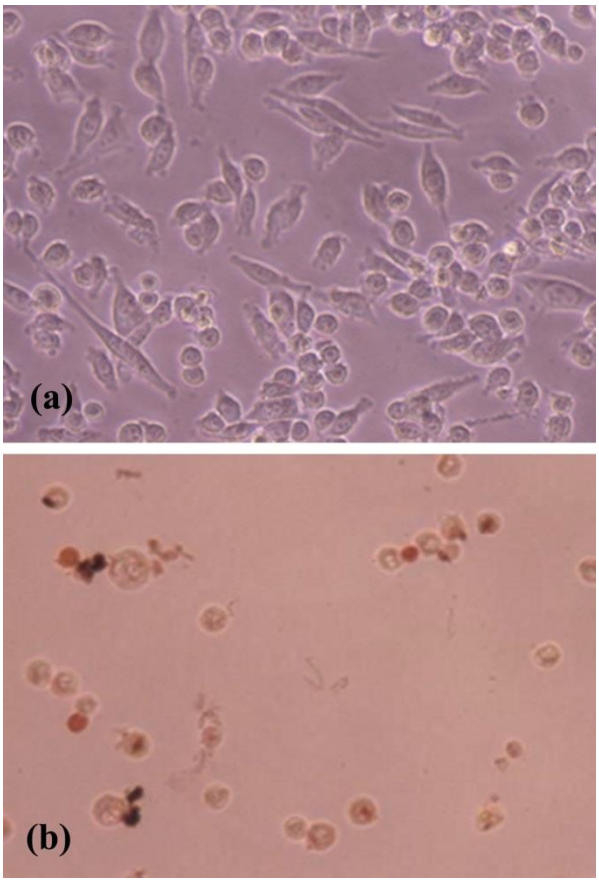

Figure 3. Morphological changes of nuclei during peel-induced apoptosis in WEHI-164 cells detected by TUNEL assay stained with DAB as chromogen. For WEHI-164 cells, (a) represents the negative control cells (without treatment) and (b) represents the cells treated with extract (230 µg) for 24 hours. In (b), apoptotic cells can be easily and reliably distinguished, as dark brown condensed figures, from non-apoptotic cells. Data represent the mean ± SEM of three independent experiments.

Electrophoresis and Immunoblotting

To obtain further support for the induction of apoptosis by PGS peel extract, immunoblotting was performed to survey the production of Caspase-3 and Bcl_2 proteins, which are the key and reliable indicators of intracellular signalling in the induction of apoptosis. To compare the effects of different concentrations of PGS peel extract on the production of Bcl_2 and Caspase-3, all the samples were unified by β-actin as an intrinsic factor. As shown in Figure 4, the expression of Bcl_2 and pro caspase-3 proteins was significantly decreased after treatment with PGS peel extract for 24h. It should be noted that because antizymogen Caspase-3 was used as a primary antibody, caspase-3 zymogene band is weakened due to its cleavage. Notably, most caspases are synthesized as inactive zymogens, and must be cleaved at two or three aspartate residues to generate the active enzyme. The figure also demonstrates the significant reduction of the Bcl_2 antiapoptotic protein in treated cells. As a consequence, both immunoblotting results confirmed that PGS peel extract can induce apoptosis in WEHI-164 cell lines.

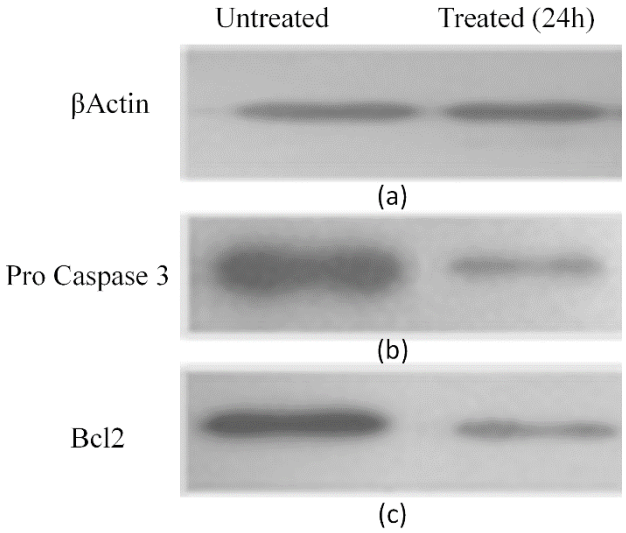

Figure 4. Effects of PGS peel extract on the expression of (a) β-actin, (b) pro-Caspase-3 and (c) Bcl_2 detected by immunoblotting in WEHI-164 cells. Cells were treated with 230 µg/ml (IC_{50}) for 24 h.

Discussion

Despite a period in which the great majority of pharmaceutical companies cut back on their use of natural products in drug discovery, there are many promising drug candidates in the current development pipeline that are of natural origin. Technical drawbacks associated with natural product research have been widely lessened, and there are plenty of better opportunities to explore the biological activity of previously inaccessible sources of natural products. With the increasing acceptance that the chemical diversity of natural products is well suited to provide the core scaffolds for future drugs, there will be further developments in the use of novel natural products in drug discovery campaigns.[22,23] On the other hand, although traditional chemotherapeutic agents kill cancer cells by indirectly inducing apoptosis, the side effects are awful and most tumor cells become resistant to drugs.[23-26] Therefore, drugs and treatment strategies able to restore the apoptotic pathways have the potential to effectively eliminate cancer cells. Herbal plants have long been the basis for nearly all medicinal therapies and remain one of the most popular alternatives for cancer prevention and treatment in many countries around the world.[22] A number of chemotherapeutic agents, with properties including apoptosis induction and anti-angiogenesis, have been isolated from natural products, such as curcumin from Curcuma longa, epicatechin gallate from tea, paclitaxel from Pacific yew, Emodin, a natural anthraquinone derivative from Rheum palmatum L. and Honokiol, a biphenyl extract from Magnolia obovata bark.[22,27-29] Pomegranate is an economically important species of the tropical and subtropical regions of the world that is widely cultivated in Iran, India, and the Mediterranean countries such as Turkey, Egypt, Tunisia, Spain, and Morocco.[30] Pomegranate components have been extensively used as a natural medicine in many cultures.[31] To evaluate the effects of PGS peel ethanolic extract on cell proliferation and to identify its therapeutic potential, we firstly found that different concentrations of the extract reduced the viability of WEHI-164 fibrosarcoma cells. By definition, a successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells. However, this ideal situation is achievable by inducing apoptosis in cancer cells. Understanding the modes of action of these compounds should provide potential useful information for their possible applications in cancer prevention and treatment. Whereas cell cycle modulation by various natural and synthetic agents is gaining widespread attention in recent years, a variety of techniques were used to assess the anti-proliferative and apoptotic effects of PGS peel ethanolic extract on cancer cells.[22,32] Usually, cells undergoing apoptosis display a very similar pattern of morphological changes, including blebbing, loss of membrane symmetry and attachment, cell shrinkage, nuclear fragmentation, and chromatin condensation.[22,33] In the present study, *in vitro* MTT assay indicated that PGS peel ethanolic extract caused growth inhibition in the WEHI-164 cells in a dose- and time-dependent manner. However, it showed less toxic effect when exposed to low concentrations towards normal cells because IC_{50} values of the PGS peel extract in 24 hours were 229.024µg/ml for WEHI-164 and had a negligible effect on proliferation of L929 cells, respectively. This claim was confirmed by statistical analysis using independent *t*-test that resulted in $P < 0.0001$, meaning that the mean differences of cytotoxicity effects of 50 µg/mL plant extract in 36 hours on WEHI-164 and L929 cells are significant. In addition, we compared the effects of PGS extract with those of Taxol, an anticancer and apoptosis inducer drug, and it should be mentioned

that the effects of PGS extract followed the same pattern as that of Taxol on the cells (Table 1). Microscopic studies also showed morphological changes of the cells. Chromatin condensation, cell shrinkage, and other alterations, characteristics of apoptotic cells, cause the morphology of treated cells with the PGS extract, change from spindle like to spherical shape and also make them lose their attachment (Figure 1). In conclusion, the plant extract induced apoptosis but not necrosis in the treated cells. Apoptosis induction was also confirmed by using TUNEL assay. As evidenced, typical apoptotic characteristic TUNEL staining was observed in treated cells (Figure 3). Apoptosis seems to be the main mechanism for cell death induced by the presence of peel extract of PGS. Apoptosis is a consequence of a highly complex and sequential cascade of cellular events. The Bcl_2 protein plays a central role in determining whether or not cells will undergo apoptosis. Due to our investigation results, the immunoblotting data, since 32KD Caspase-3 precursor was decreased in time- and concentration-dependent manner, methanolic extract of PGS peel can induce Caspase-3 activation via its proteolytic cleavage into active subunits which enact the final irreversible commitment to death. Based on our findings from the immunoblotting data, a decrease in Bcl_2 expression was observed in WEHI-164 fibrosarcoma cell line after treatment with PGS peel extract for 24 h. Caspase-3 precursor was also significantly decreased due to its cleavage after treatment with PGS for 24 h, because most caspases are synthesized as zymogens and must be cleaved to generate the active enzyme. The expression of Bcl_2 and caspase-3 proteins, markers of apoptosis, is involved in induced apoptosis in WEHI-164 cells using PGS peel extract. Taking together, the present scientific study was conducted to find out the cytotoxicity effect of PGS on the proliferation of malignant cells. It could provide further information about mechanisms involved in this toxicity. We are currently studying the intracellular mechanism of apoptosis induction by PGS extract and characterizing its active ingredients. More importantly, PGS could be considered as a promising chemotherapeutic agent in cancer treatment in future.

Conclusion

It is well established that a wide variety of natural compounds exhibit significant cytotoxic and chemopreventive properties, many of which act as triggers of apoptosis. The present study provides evidence that in-vitro cytotoxic activity of an ethanol standardized extract from wild *Punica granatum L. var. spinosa* (PGS) dose-dependently inhibits the proliferation of fibrosarcoma cancer cell line (WEHI-164) via an apoptosis-dependent pathway. However, further studies to more closely evaluate their mechanism of action may lead to new opportunities for their possible application in cancer therapy and prevention.

Acknowledgments

This work was supported by a grant from Immunology Research Center (IRC), Tabriz University of medical sciences, and Ischemic Disorders Research Center, Golestan University of Medical Sciences. The authors wish to thank the numerous people who supported or provided assistance with this report.

Conflict of Interest

The authors declare no conflict of interest.

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