

Pomegranate seed extract enhances the inhibitory effect of adipose-derived mesenchymal stem cells on breast cancer cell line in co-culture conditions

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Abstract

Background and purpose: Pomegranate seed extract (PSE) possesses anticancer activities and healing effects. Adipose-derived stem cells (ADSCs) are being considered a new candidate for cancer treatment. The purpose of this study was to investigate the effect of PSE on the cell cycle and apoptosis of the MCF-7 cell line in the co-culture condition with ADSCs.

Experimental approach: MCF-7 and ADSC cells (ratio 1/1) were cultured in a transwell plate with and without PSE (PSE-co-culture and co-culture groups). MCF-7 cells were cultured in monolayer without and with PSE (mono-culture and PSE-mono-culture groups). MCF-7 cell line was harvested on day 5 and cell viability, apoptotic activity, cell cycle, and gene expression were evaluated.

Findings / Results: The results of the MTT assay indicated that PSE at 100 µg/mL has the highest cytotoxicity on the MCF-7 in the PSE-co-culture group. The cell cycle analysis revealed that ADSCs in combination with PSE significantly increased the population of MCF-7 cells in the G1 phase, resulting in the arrest of MCF-7 cells cycle in the G0/G1 transition. In addition, the most apoptotic MCF-7 cells (41.5%) were detected in the same group. Expression of BAX and caspase3 genes were upregulated while anti-apoptotic (BCL-2) and angiogenesis inducer (VEGF) genes were downregulated in the PSE-co-culture group compared with the other groups.

Conclusion and implications: ADSCs reduced cell viability and proliferation of MCF-7 cells in co-culture conditions and adding PSE to the medium increased the apoptosis of cancer cells. This study suggests that ADSCs with PSE can suppress tumor cells.

Keywords: Adipose-derived stem cells; Apoptosis; Breast cancer; Cell cycle; Co-culture; Pomegranate seed extract.

INTRODUCTION

Breast cancer is the most common neoplasia and one of the main causes of mortality in the woman (1). Environment and lifestyle are the

cause of 90% of breast cancers and 10% of the cause is heredity (1).

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Breast cancer treatment methods are included surgery, chemotherapy, and radiotherapy (2). These therapeutic strategies have different side effects including cancer recurrence, heart failure, leukemia, pneumonitis, weight loss, skin reaction, premature ovarian dysfunction, pain, and dyspnea (3,4). A novel strategy for cancer treatment is stem cell-based therapy (5). Adipose-derived stem cells (ADSCs) are a population of stem cells with self-renewal and multipotentiality with an important role in regenerative medicine (6). ADSCs migrate to tumor sites and incorporate into the tumor stroma and interact with cancer cells (7). However, the role of support or suppression of stem cells in tumors is controversial (8). Mesenchymal stem cells (MSCs) are involved in tumorigenesis including the induction of angiogenesis (9), stimulation of epithelial-mesenchymal transition, tumor metastasis (10), and inhibition of tumor cell apoptosis (11). MSCs suppress tumor growth through inhibition of angiogenesis (12) inducing cell cycle arrest and apoptosis (13). Therefore, more appropriate approaches are needed to enhance the inhibitory effect of stem cells on cancer growth.

Antioxidants can regulate stem cell proliferation, differentiation, and migration (14). In addition, antioxidants are able to recover the morphology of cells following oxidative stress and restore the antioxidant enzymes level of endogenous impaired by oxidative stress (15,16). Studies have revealed the role of natural antioxidant-rich substances, such as vitamins, flavonoids, and polyphenols in inhibiting tumors (17,18). Pomegranate is one of the fruits rich in antioxidants with a variety of vitamins, minerals, polyphenols, isoflavones, pectin, fatty acids, proteins, crude fibers, and sugars (17,19). Pomegranate can increase the efficacy of stem cells (19). A study showed that bone marrow stem cells with pomegranate extract were more effective in experimentally-induced healing gastric ulcers than stem cells alone (20). Pomegranate extract inhibits the epithelial-to-mesenchymal transition in breast cancer stem cells (19). To date, there has been no report on the effect of ADSCs with pomegranate seed extracts (PSE) on breast cancer. Therefore, this work

examined the effect of ADSCs and PSE on breast cancer cell line (MCF-7).

MATERIALS AND METHODS

Preparation of extract

Pomegranate fruit was collected from the Shahreza region of the province of Isfahan, Iran in 2021. The seeds were separated, dried, and ground into the powder with a blender. Afterward, the powders were pulverized and soaked in 70% ethanol solvent and shook for 4 h. After 24 h and re-shaking, the extract was filtered with a Buchner funnel. This operation was repeated three times to ensure the complete extraction and it was then concentrated with a rotary apparatus (Heidolph, USA). The dried powder was obtained with a freeze dryer machine (Heto Drywinner, USA) and stored at -20 °C.

Cell culture

Human breast cancer cells (MCF-7) were purchased from Cell Bank of Iran, Pasteur Institute of Iran, Tehran, I.R. Iran. MCF-7 cells were seeded in a flask (Biologix-TC75) containing 12 mL of the medium of the completed Dulbecco's modified eagle (DMEM-F12, Gibco, USA) with 10% fetal bovine serum (FBS), 10 µg/mL penicillin, and streptomycin antibiotics (Sigma, USA) at 37 °C, 5% CO₂ conditions. The medium was changed three times per week. The cells were subcultured regularly after 80-90% confluency using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Cat No: N0100-780, Cegrogen, Germany) and counted by a hemocytometer (Boeco, Germany). ADSCs, isolated from adipose tissue in the previous study (Ethics No. IR.MUI.MED.REC.1399.1171), were thawed and cultured (21).

Preparation of appropriate concentrations of PSE

To prepare the stock solution, 0.02 g of PSE was weighed and dissolved in 10 mL DMEM/F-12 and filtered with a sterile syringe filter (PVDF 0.22 µ). The concentrations of 10, 50, 100, 200, and 500 µg/mL were then prepared. To determine the IC₅₀, 1 × 10⁴ cells/well of MCF-7 and ADSCs were seeded in 96-well plates (Biologix, Germany)

separately. PSE was added to each well at various concentrations for 24 h. MTT assay was used to determine the cell viability rate.

MTT assay

After 24 h, an MTT assay was carried out to determine cell viability. In brief, the medium of each well was discarded and the cells were washed in phosphate-buffered saline (PBS, Bio-Idea, Germany) twice. Additionally, 200 μ L of DMEM/F12 and 20 μ L of MTT solution (Sigma, USA; 5 mg/mL) were added (at 37 °C for 4 h in each well). The formazan precipitates in the cells were dissolved with dimethyl sulfoxide (DMSO, Sigma, USA). The absorption spectrum of formazan at a wavelength of 570 nm was measured.

Indirect co-culture and monolayer culture

Herein, transwell plates (12-well with a pore size of 0.4 μ m, SPL 37024) were employed. ADSCs (5×10^4 cells/well) and MCF-7 cell lines (5×10^4 cells/well) were cultured, respectively were inserted in the bottom of the well without and with PSE at 100 μ g/mL (as co-culture and PSE-co-culture groups) for 5 days. In the monolayer culture, MCF-7 cells (5×10^4 cells/well) without and with PSE (as mono-culture and PSE-mono-culture groups) were cultured, and methods were performed.

Cell cycle analysis

Cell cycle phase distributions of MCF-7 cells were analyzed *via* flow cytometry. Briefly, the cells were trypsinized and centrifuged (in all the stages: 1800 rpm, at 4 °C for 7 min). The cells were then washed with PBS and re-centrifuged. Afterward, 4 mL of 70% ethanol were added slowly to the cells for fixation. After 24 h, the cells were washed with cold PBS and suspended in propidium iodide (PI, Sigma Prod. No. P4170, Germany)) staining solution (10 μ g/mL PI, 100 μ g/mL DNase-free RNase, and 0.1% TritonTM X-100 in PBS) and

incubated for 30 min at 37 °C in the dark place. The DNA content of the cell was analyzed through BD FACSCaliburTM flow cytometer (Becton Dickinson, San Jose, USA). Results were analyzed with a FlowJo software 10.

Apoptosis assays

Annexin-V-fluorescein isothiocyanate (FITC) kit (BMS500FI/300CE, eBioscience, Germany) was performed to detect apoptotic cells. Briefly, after 5 days, the medium of each well was removed and the cells were gently washed in PBS and centrifuged (1400 rpm and 8 min). The cells were re-suspended in a binding buffer (200 μ L, 1X). Annexin (5 μ L) was added and incubated for 20 min in a dark place. The cells were then washed with binding buffer and centrifuged. Afterward, they were resuspended in binding buffer and 5 μ L of PI (20 μ g/mL) was added. The cells were analyzed *via* BD FACSCaliburTM flow cytometer using the supplied software in the instrument (BD Cell Quest software).

RNA isolation and quantitative RT-PCR

FavorPrepTM blood/cultured cell total RNA purification mini kit (Cat. No: FABRK001-Favorgene, Taiwan) was performed to extract total RNA from the cultured cells and reverse transcribed was performed by applying Thermo Scientific revert aid first-strand cDNA synthesis kit (BR441-Biofact, Korea). SYBRTM green PCR master mix (BIOFACT High ROX, Korea) was employed to detect genes expression. The reactions were performed in triplicate. Table 1 lists the DNA primers (Metabion, Germany) for the quantitative real-time polymerase chain reaction (qRT-PCR) reaction. qRT-PCR and data collection were carried out in StepOne Plus RT-PCR detection system (ABI Applied Biosystems, USA). Gene expression was normalized to the expression of GAPDH.

Table 1. Primers for the quantitative real-time polymerase chain reaction.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
BAX	TGCTTCAGGGTTTCATCCA	GGCGGCAATCATCTCTG
BCL-2	GATGGGATCGTTGCCATTATGC	CAGTCTACTTCCTCTGTGATGTTGT
Caspase3	ACTCCACAGCACCTGGTTATT	TCTGTTGCCACCTTTCGGTT
Vascular endothelial growth factor (VEGF)	CCTTGCCCTTGCTGCTCTACC	ATCCATGAACTTCACCACTTCGT
GAPDH	GCTCATTTCTGGTATGACAACG	CTCTCTTCCTCTGTGCTCTTG

Data analysis

All the tests were repeated three times and the obtained data were expressed as mean \pm SEM. Statistical analysis was conducted using SPSS software version 26 and the statistical differences between the control and treated groups, or between the two control or treated groups were evaluated utilizing one-way ANOVA followed by Tukey. In all the tests, the significance level was considered to be $P \leq 0.05$.

RESULT

Cell culture

Microscopy inverted the direct observations of MCF-7 cells after 80% confluency revealed that they were adherent and uniform in size and had good growth and edges distinct (Fig. 1A). ADSCs with 80% confluency showed that they were adherent to the spindle shape (Fig. 1B).

Preparation of appropriate concentrations of PSE

The effect of PSE on MCF-7 and ADSCs cells at various PSE concentrations was investigated *via* the MTT method. Viabilities of MCF-7 cells in 0, 10, 50, 100, 200, and 500 $\mu\text{g/mL}$ of PSE were 100 ± 1.633 , 94.5 ± 8.544 , 88.5 ± 8.386 , 56.5 ± 6.191 , 41 ± 6.831 , and $33.5 \pm 3.416\%$, respectively. Viability of ADSCs, in the aforementioned concentrations, were respectively 100 ± 4.320 , 104.5 ± 4.435 , 98 ± 651 , 56 ± 3.266 , 44.5 ± 3 , and $36.5 \pm 1.915\%$, indicating a significant

difference in cell survival between the untreated group and cells exposed to PSE at concentrations higher than 100 $\mu\text{g/mL}$ (Fig. 2, A and B).

Cell viability

Cell viability of the experimental groups was investigated *via* the MTT method. Viability in mono-culture, PSE-mono-culture, co-culture, and PSE-co-culture groups were 100 ± 1.633 , 65 ± 4.163 , 52 ± 2.828 , and $43.5 \pm 3.416\%$, respectively. In PSE-mono-culture and co-culture cell viability was reduced compared with the mono-culture group. In the co-culture group, cell viability was significantly decreased compared with the PSE-mono-culture group. The PSE-co-culture group revealed a significant decrease compared to the co-culture and PSE-mono-culture groups (Fig. 2C).

Cycle cell

The cells in the G0/G1 transition in PSE and ADSC groups increased compared to the control group. Cells accumulation in the G0/G1 transition was observed to be significant after ADSC in combination with PSE treatment, indicating cell-cycle/growth arrest in the G0/G1 transition (Fig. 3A-D). In the PSE-mono-culture group, 56.7% of the cells were in the G0/G1 phase, 20.2% in the S phase, and 18.1% in the G2/M phase. In the co-culture group, 61.5%, 19.4%, and 15.9% were in the G0/G1, S, and G2/M phase, respectively. In addition, 67.6%, 15.9%, and 11.6% of the cells respectively were in the G0/G1, S, and G2/M phase in the PSE-co-culture group (Fig. 3E).

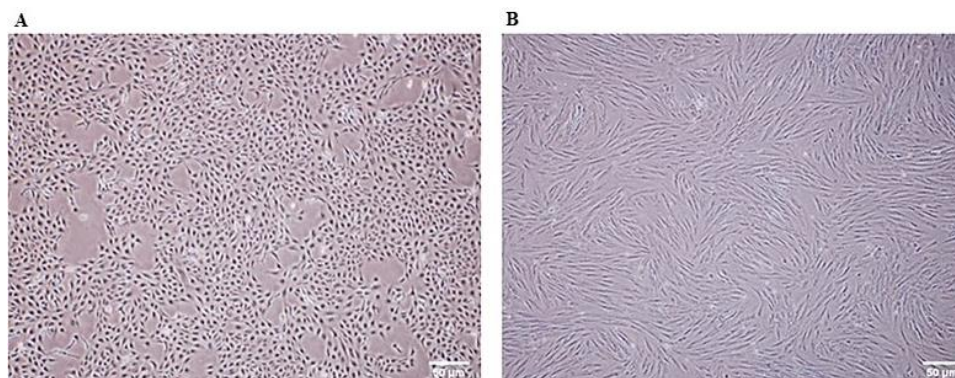


Fig. 1. Morphology of (A) MCF-7 and (B) ADSCs with 80% confluency cells imaged by inverted microscope at actual magnification 40 \times .

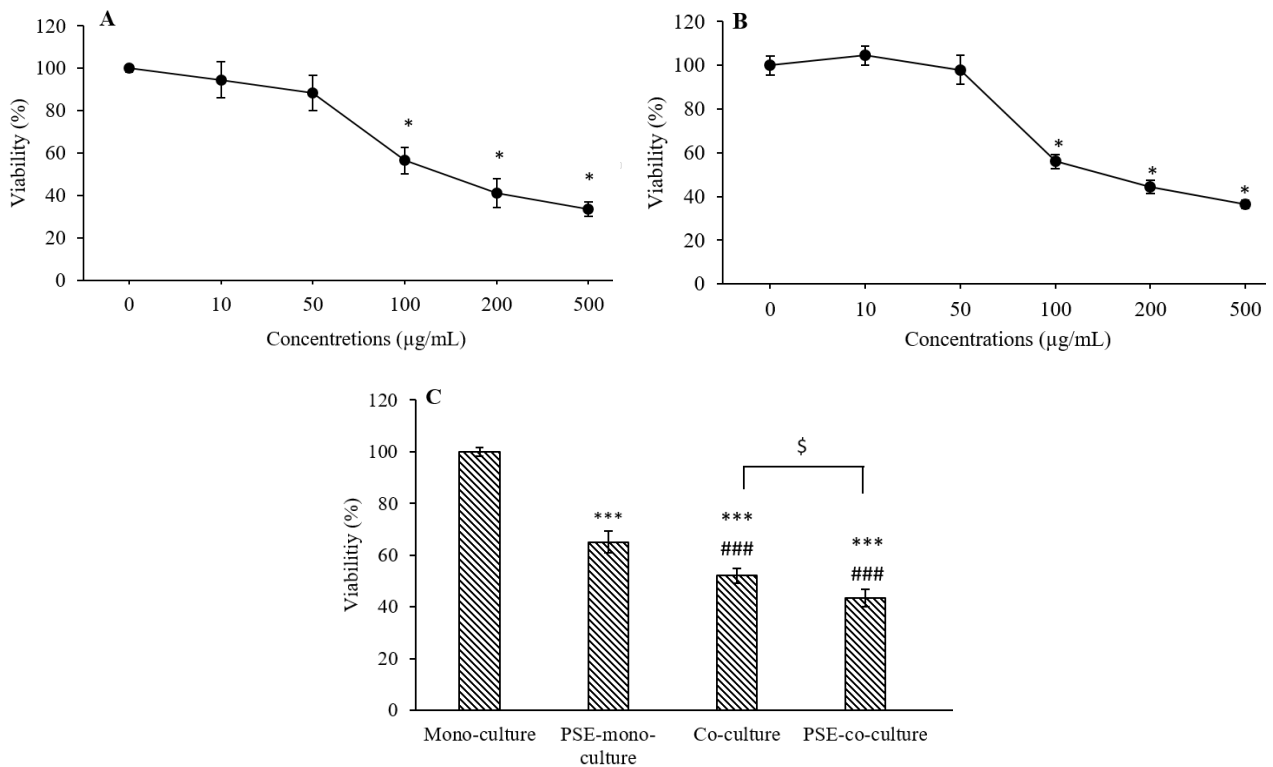


Fig. 2. Evaluation of *in vitro* cytotoxic effect of PSE against (A) MCF-7 cells and (B) ADSCs; * $P < 0.05$ indicates significant differences compared to the untreated group (0 concentration). (C) After a 5-day incubation period, PSE and ADSCs showed *in vitro* cytotoxicity against MCF-7 cells; *** $P < 0.001$ indicates significant differences compared to mono-culture, ### $P < 0.001$ versus PSE-mono-culture, and \$ $P < 0.05$ indicates significant differences between defined groups. The data from three independent experiments were reported as the mean \pm SEM. PSE, Pomegranate seed extract; ADSC, adipose-derived stem cells.

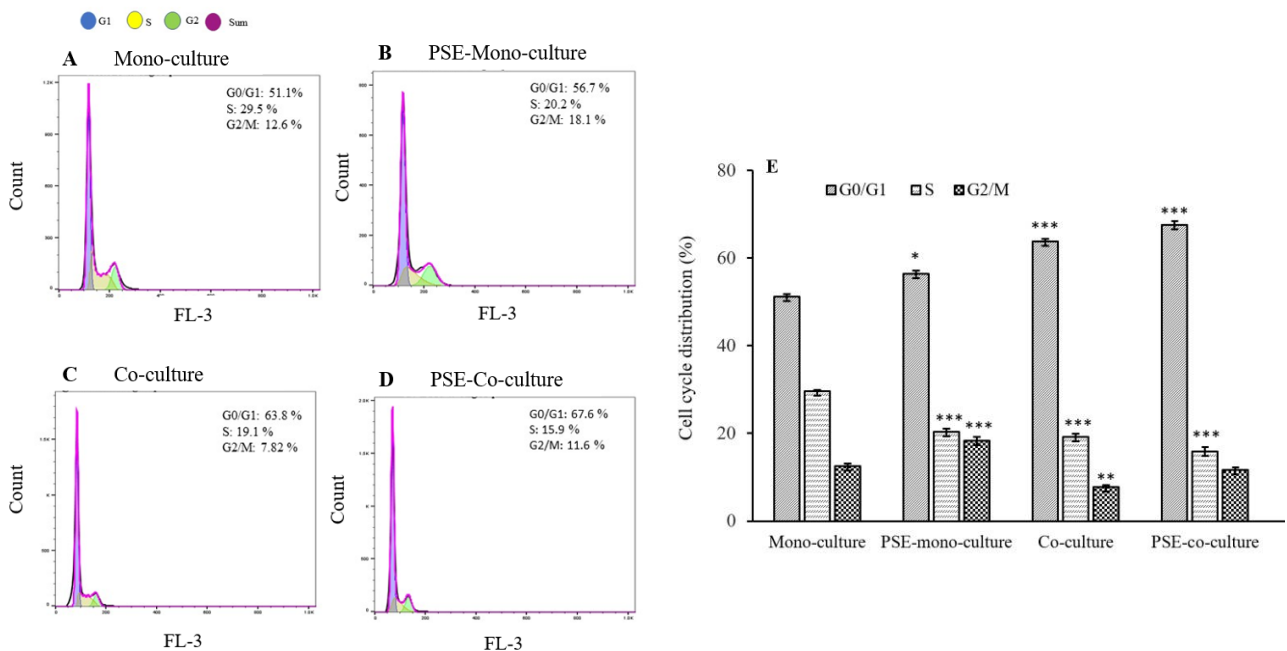


Fig. 3. Flow cytometric analysis by propidium iodide (PI, FL-3) staining. (A-D) show histograms of MCF-7 cells cycle treated with PSE and ADSCs, and (E) cells cycle distribution graph for MCF-7 cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences compared to respective mono-culture group. PSE, Pomegranate seed extract.

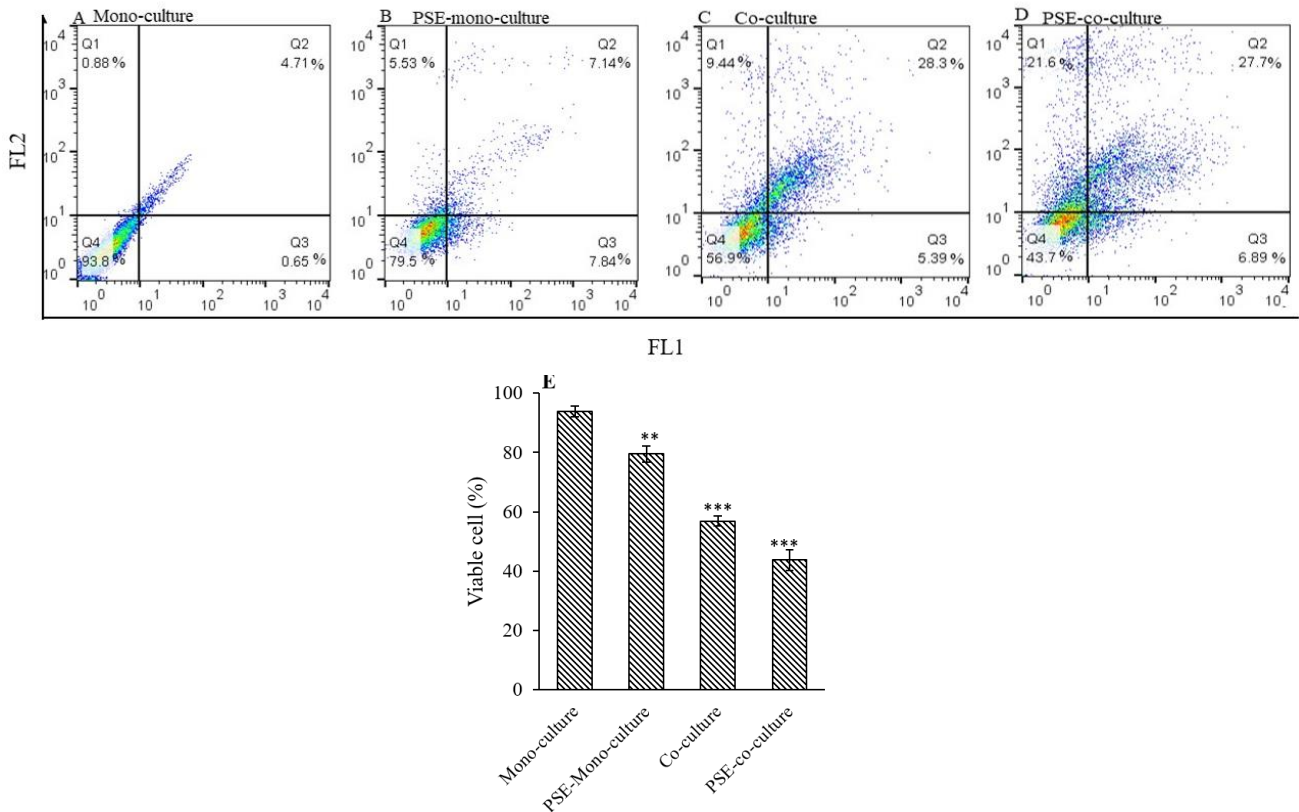


Fig. 4. Flow cytometric analysis by propidium iodide (PI) and annexin V staining. (A-D) Show histograms of MCF-7 cells treated with PSE and ADSCs, and (E) viable cells percent graph for MCF-7 cells. ** $P < 0.01$ and *** $P < 0.001$ indicate significant differences compared to mono-culture group. PSE, Pomegranate seed extract.

Apoptosis assays by flow cytometry

Figure 4. A portrays the dot plots of the staining by annexin V/PI in untreated MCF-7 cells. PSE-mono-culture group exhibited 7.84% early-stage apoptosis and 7.14% late-stage apoptosis (Fig. 4B). In the co-culture group, apoptosis in the early stages and late stages was 5.39% and 28.3%, respectively (Fig. 4C). PSE-co-culture group demonstrated apoptosis in 6.89% in early-stage, 27.7% in late stages (Fig. 4D). Figure 4E is indicating the percent viability of the cells which was significantly decreased in all cells compared with the monolayer-culture cells.

Gene expression

To elucidate the molecular mechanisms of the observed effects of PSE, we examined whether PSE alters the expression of MCF-7 cells genes, including genes involved in the apoptosis pathways (BAX and BCL-2) and angiogenesis (vascular endothelial growth factor, VEGF). Expression of BAX increased in PSE-mono-culture and co-culture groups compared with mono-culture group, but between PSE-mono-culture and co-culture groups no significant difference was observed.

The PSE-co-culture group demonstrated a significant increase in BAX gene compared to the three other groups. BCL-2 expression decreased in PSE-mono-culture and co-culture groups compared with the mono-culture group. BCL-2 also decreased in co-culture groups in comparison with the PSE-mono-culture group. The PSE-co-culture group illustrated a significant decrease in BCL-2 compared to mono-culture, PSE-mono-culture, and co-culture groups. Caspase3 was increased in PSE-mono-culture and co-culture groups compared with group mono-culture groups. The co-culture group experienced an increase of caspase3 expression compared with PSE-mono-culture. Caspase3 expression had a significant increase in the PSE-co-culture group compared with three other groups. VEGF was reduced in PSE-mono-culture and co-culture groups compared with the mono-culture group. The Co-culture group had a decline in VEGF expression compared with the PSE-mono-culture group. VEGF expression also experienced a significant decrease in the PSE-co-culture group compared with mono-culture, PSE-mono-culture, and co-culture groups (Fig. 5).

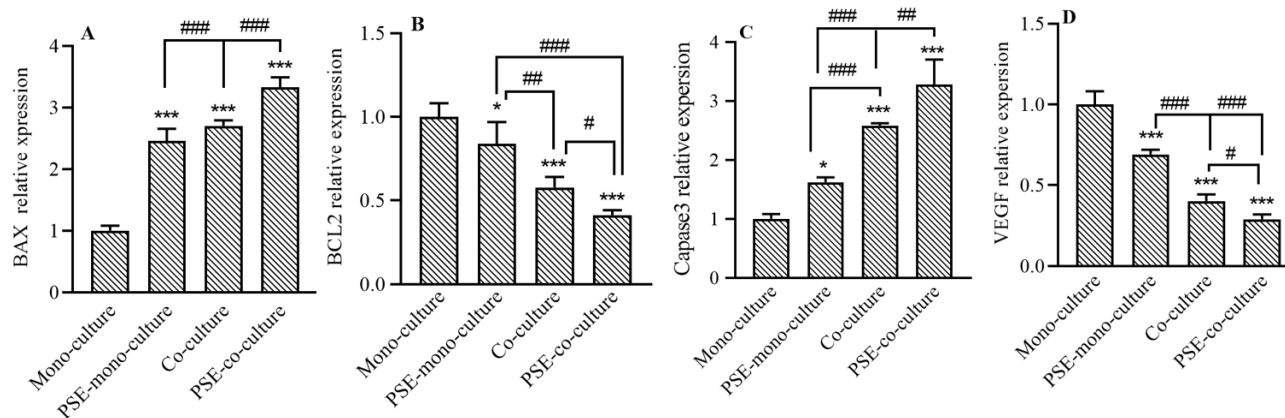


Fig. 5. Effect of PSE and ADSCs on (A) BAX, (B) BCL-2, (C) caspase 3, and (D) VEGF mRNA expression in MCF-7 cells. * $P < 0.05$ and *** $P < 0.001$ indicate significant differences compared to mono-culture group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ indicate significant differences between defined groups. PSE, Pomegranate seed extract; VEGF, vascular endothelial growth factor.

DISCUSSION

The most important finding of this study was that ADSCs in combination with PSE had higher anti-proliferative effects on breast cancer cells than ADSCs alone. The present work was conducted on human breast cancer cells (MCF-7).

MSCs are suitable targets for cell therapy in various types of cancers. However, the role of these cells in tumor tissue is controversial and the efficacy of these cells is below expectations (15). A number of pre-treatment methods appear to increase the efficacy of these cells. Vitamin E, for example, greatly increases viability, inhibits inflammation, and reduces apoptosis in stem cells (22). Nanocurcumin increases the expression level of antioxidant genes and superoxide dismutase activity in bone marrow stem cells (23,24). Consequently, antioxidants appear to be able to improve stem cell efficacy. In the current study, we showed the effect of ADSCs in combination with PSE (with 26% antioxidant activity) on MCF-7 cells. Pomegranate fruit consists of 40% arils and 10% seeds. The seeds are a rich source of total lipids; 12% to 20% of total seed weight comprises seed oil. The oil consists of linolenic, linoleic, punicic acid, oleic acid, stearic acid, and palmitic acid. The seeds also contain protein, crude fibers, vitamins, minerals, pectin, sugars, polyphenols, isoflavones (mainly genistein), the phytoestrogen coumestrol, and the sex steroid, estrone (19,25). To determine

the optimal concentration of PSE, an MTT assay was performed. PSE at concentrations higher than 100 $\mu\text{g}/\text{mL}$ had a high cytotoxic effect of 33.5% and 36.5% on MCF-7 cells and ADSCs, respectively. In addition, at concentrations lower than 100 $\mu\text{g}/\text{mL}$, cell viability was respectively found to be 94.5% and 104.5% in MCF-7 cells and ADSCs, which is close to that of the untreated group. Therefore, PSE at 100 $\mu\text{g}/\text{mL}$ as the optimal concentration was employed to treat the groups. Various studies confirm our results. Dai *et al.* revealed that pomegranate extract (100 $\mu\text{g}/\text{mL}$) inhibits the proliferation and viability of mammary cancer stem cells *in vitro* (26).

Cell survival in PSE-mono-culture and co-culture groups was decreased, indicating that ADSCs and PSE targeted the growth of MCF-7 cells. Apoptosis was noted during the co-culturing of MDA-MB-231 breast cancer cells with human umbilical mesenchymal stem cells (27). Khakoo *et al.* reported that human MSCs potently inhibit the growth of Kaposi sarcoma (28). Cell survival in PSE-mono-culture was reduced, which is in accordance with previous studies. PSE decreased MCF-7 cells viability (29,30). Viability in the PSE-co-culture group was markedly lower than that in the PSE or ADSCs alone, implying the greater toxicity of ADSCs in combination with PSE on MCF-7 cells. We suspect that PSE can increase the cytotoxic effect of ADSCs on cancer cells. Various research studies confirm that PSE suppresses breast cancer stem cell properties in

part due to the inhibition of epithelial-to-mesenchymal transition (19) and inhibits proliferation and viability (26). BMSCs can ameliorate experimentally-induced gastric injury in rats and protect the gastric tissue. The addition of pomegranate to BMSCs could lead to better results (20). No studies have been performed on the effect of stem cells in combination with pomegranate on breast cancer. Meanwhile, herein, the cytotoxic effect of stem cells or pomegranate alone on breast cancer indicated that ADSCs in combination with PSE had a greater cytotoxic effect on MCF-7 cells.

Various breast cancer exhibit types of molecular alterations and dependencies on the cell cycle (31). The cell cycle includes growth (G1), DNA synthesis (S), growth (G2) phases, and mitosis (M). During the G1 period, cells grow in size, produce RNA, and synthesize proteins for DNA formation. DNA replicates and cells continue to grow during the S phase. In the G2 phase, new proteins are produced. Nuclear and cytoplasmic divisions take place during the M stage (32). Furthermore, regulating the cancer cell cycle is one strategy in the progression of anticancer agents (32). Hence, cell cycle progression destruction is important for cancer therapy. Our results implied that there was a significant increase in DNA in the G0/G1 transition of cell cycle upon ADSCs in combination with PSE treatment in cell cycle analysis, indicating the inhibition of proliferation of MCF-7 cells. We suspect that PSE by increasing the anticancer effect of ADSCs causes arrest in the cell cycle in MCF-7 cells. The decreases in DNA in the S phase in the PSE-co-culture group can confirm the significant increase of DNA in G0/G1. Following co-culturing with rat bone marrow-derived MSCs, a recent study found an increase in the number of K562 cells in the G0/G1 transition of the cell cycle along with a decrease in the S and G2/M phases, revealing cell cycle inhibition (33). Pomegranate extract caused a significant concentration-dependent increase in the accumulation of the cells in the G0/G1 transition (26). Our work proved that ADSCs in combination with PSE caused a significant increase in MCF-7 cells number during the

G0/G1 transition compared with ADSCs or PSE alone.

The results from MTT and cell cycle assay implied that MCF7 cells might be progressing towards apoptosis. Therefore, in our work, flow cytometric analysis was carried out for apoptosis assay to investigate whether MCF7 cells are progressing towards apoptosis. Similar to the result from cell survival and cell cycle, apoptosis significantly increased as illustrated by a high number of annexin V-positive cells suggesting the reduction of MCF-7 cell viability and cell cycle arrest after the treatment with ADSCs in combination with PSE which led to the stimulation of apoptosis. Hao *et al.* observed that fullerenes improved apoptosis in ADSCs (34). Yousefi *et al.* reported that nano curcumin improved apoptosis in ADSCs (24).

Gene expression analysis confirmed the apoptosis results. BAX, BCL-2, and caspase3 are the main genes involved in the intrinsic pathway of apoptosis. Most cancer therapies target this pathway as the result of increased mitochondrial permeability and the release of cytochrome-c into the cytoplasm (35,36). Cytoplasmic release of cytochrome c activated caspase 3 and consequently, apoptosis was performed (37).

Herein, the addition of PSE along with stem cells increased the apoptosis of cancer cells through the internal pathway of apoptosis. Kalamegam *et al.* revealed that human umbilical cord Wharton's jelly stem cell increased the expression of the BAX gene and reduced the expression of BCL-2 in cancer cell lines (38). Therefore, to reduce the controversy about stem cell therapy in the treatment of cancer, we utilized these cells in combination with PSE which could be a new way to increase the efficiency of stem cell therapy in the treatment of breast cancer. Gene expression analysis confirmed these results. Increased stem cell efficacy activated the internal pathway of apoptosis in cancer cells. BCL-2 decreased and BAX and caspase3 increased (as shown in the results). These are the main genes involved in the internal pathway of apoptosis. Most cancer therapies target this pathway. Our results demonstrated that the apoptosis of cancer cells in PSE-co-culture

group cells through the internal pathway of apoptosis increased significantly.

Angiogenesis has a key role in tumor growth and VEGF is one of the main factors in cancer angiogenesis (39,40). Our results indicated that ADSCs alone can reduce VEGF expression, but the addition of PSE decreases VEGF expression significantly. Pakravan *et al.* stated that mesenchymal stem cells reduced VEGF expression in breast cancer (12). VEGF levels decreased in MCF-7 breast cancer cell lines after the treatment with pomegranate seed oil (41). We shed light on the combination of ADSCs with PSE reducing VEGF significantly. Subsequently, ADSCs and PSE can increase apoptosis in different pathways.

There are several limitations that must be taken into consideration. Primarily, we only investigated the involvement of the intrinsic apoptosis pathway in the regulation of cancer cells. Functions of other signaling apoptosis pathways, such as extrinsic apoptosis pathway, Wnt, and protein kinase B (AKT) signaling, need further investigations. Secondly, we only examined the effect of PSE on MCF-7 cells. Effect of PSE on stem cell and oxidative stress-related pathways, such as phosphatidylinositol 3-kinase (PI3K)/ AKT) PI3K/AKT, mitogen-activated protein kinase, and protein kinase C, need further studies. Thirdly, despite the importance of *in vitro* studies, animal models and clinical trials are needed for further studies.

CONCLUSION

Hence, we found that ADSCs in combination with PSE have potent anti-proliferative, growth inhibition, and antitumor activity, which was mediated through induction of cell cycle arrest. PSE reduced cancer cells in co-culture conditions and caused cancer cells cycle arrest in the G1 phase, resulting in apoptosis. Our result indicated that stem cells in combination with PSE by inducing BAX and caspase3 and reducing BCL-2 expression may stimulate the intrinsic apoptotic pathway to induce cycle cell arrest and thus cell death in MCF-7 cancer cells. The results suggest that fruit consumption should be strongly encouraged for women at risk for breast cancer.

It was also the first study to demonstrate that PSE can effectively induce genes involved in apoptosis and cell cycle in the co-culture model of MCF-7 cells and ADSCs. Although current findings are provoking, more *in vivo* studies are required to validate the preventive effects of PSE on breast cancer.

Conflicts of interest statement

All authors declared no conflict of interest in this study.

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Author's contributions

B. Hashemibeni and N. Moradi-Gharibvand conceived the original idea; N. Moradi-Gharibvand, B. Hashemibeni, and A. Safaee carried out the experiment; N. Moradi-Gharibvand and M. Setayeshmehr carried out the pomegranate seed extraction; B. Hashemibeni supervised the project. S.J. Hasheminia helped to perform the flow cytometry method; M. Kazemi conducted the molecular experiments and qRT-PCR analysis; N. Moradi-Gharibvand analyzed the data and wrote the manuscript with the support of B. Hashemibeni, L.S Khorsandi, and D. Bijan Nejad. All authors read and approved the final version of the manuscript.

REFERENCE

1. Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, et al. Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res.* 2008;25(9):2097-2116. DOI: 10.1007/s11095-008-9661-9.
2. Mao Y, Keller ET, Garfield DH, Shen K, Wang J. Stromal cells in tumor microenvironment and breast cancer. *Cancer Metastasis Rev.* 2013;32(1-2):303-315. DOI: 10.1007/s10555-012-9415-3.
3. Zhang M, Liu X, Li J, He L, Tripathy D. Chinese medicinal herbs to treat the side-effects of chemotherapy in breast cancer patients. *Cochrane Database Syst Rev.* 2007;18(2): CD004921,1-32. DOI: 10.1002/14651858.CD004921.pub2.
4. Sjövall K, Strömbeck G, Löfgren A, Bendahl P-O, Gunnars B. Adjuvant radiotherapy of women with

- breast cancer - information, support and side-effects. *Eur J Oncol Nurs.* 2010;14(2):147-153. DOI: 10.1016/j.ejon.2009.09.002.
5. Zhang Q, Xiang W, Yi D-Y, Xue B-Z, Wen W-W, Abdelmaksoud A, *et al.* Current status and potential challenges of mesenchymal stem cell-based therapy for malignant gliomas. *Stem Cell Res Ther.* 2018;9(1):228-236. DOI: 10.1186/s13287-018-0977-z.
 6. Wang J, Chen Z, Sun M, Xu H, Gao Y, Liu J, *et al.* Characterization and therapeutic applications of mesenchymal stem cells for regenerative medicine. *Tissue Cell.* 2020;64:101330,1-9. DOI: 10.1016/j.tice.2020.101330.
 7. Lazennec G, Lam PY. Recent discoveries concerning the tumor - mesenchymal stem cell interactions. *Biochim Biophys Acta.* 2016;1866(2):290-299. DOI: 10.1016/j.bbcan.2016.10.004.
 8. Rhee KJ, Lee JI, Eom YW. Mesenchymal stem cell-mediated effects of tumor support or suppression. *Int J Mol Sci.* 2015;16(12):30015-30033. DOI: 10.3390/ijms161226215.
 9. Wang Y, Liu J, Jiang Q, Deng J, Xu F, Chen X, *et al.* Human adipose-derived mesenchymal stem cell-secreted CXCL1 and CXCL8 facilitate breast tumor growth by promoting angiogenesis. *Stem Cells.* 2017;35(9):2060-2070. DOI: 10.1002/stem.2643.
 10. Luo D, Hu S, Tang C, Liu G. Mesenchymal stem cells promote cell invasion and migration and autophagy-induced epithelial-mesenchymal transition in A549 lung adenocarcinoma cells. *Cell Biochem Funct.* 2018;36(2):88-94. DOI: 10.1002/cbf.3320.
 11. Martin FT, Dwyer RM, Kelly J, Khan S, Murphy JM, Curran C, *et al.* Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Res Treat.* 2010;124(2):317-326. DOI: 10.1007/s10549-010-0734-1.
 12. Pakravan K, Babashah S, Sadeghizadeh M, Mowla SJ, Mossahebi-Mohammadi M, Ataei F, *et al.* MicroRNA-100 shuttled by mesenchymal stem cell-derived exosomes suppresses in vitro angiogenesis through modulating the mTOR/HIF-1 α /VEGF signaling axis in breast cancer cells. *Cell Oncol (Dordr).* 2017;40(5):457-470. DOI: 10.1007/s13402-017-0335-7.
 13. Allahverdi A, Arefian E, Soleimani M, Ai J, Nahanmoghaddam N, Yousefi-Ahmadipour A, *et al.* MicroRNA-4731-5p delivered by AD-mesenchymal stem cells induces cell cycle arrest and apoptosis in glioblastoma. *J Cell Physiol.* 2020;235(11):8167-8175. DOI: 10.1002/jcp.29472.
 14. Panahi M, Rahimi B, Rahimi G, Yew Low T, Saraygord-Afshari N, Alizadeh E. Cytoprotective effects of antioxidant supplementation on mesenchymal stem cell therapy. *J Cell Physiol.* 2020; 235(10): 6462-6495. DOI:10.1002/jcp.29660.
 15. Zeng W, Xiao J, Zheng G, Xing F, Tipoe GL, Wang X, *et al.* Antioxidant treatment enhances human mesenchymal stem cell anti-stress ability and therapeutic efficacy in an acute liver failure model. *Sci Rep.* 2015;5(9):1-17. DOI: 10.1038/srep11100.
 16. Fan G, Wen L, Li M, Li C, Luo B, Wang F, *et al.* Isolation of mouse mesenchymal stem cells with normal ploidy from bone marrows by reducing oxidative stress in combination with extracellular matrix. *BMC Cell Biol.* 2011;12(2):30. DOI: 10.1186/1471-2121-12-30-43.
 17. Viuda-Martos M, Fernández-López J, Pérez-Álvarez J. Pomegranate and its many functional components as related to human health: a review. *Compr Rev Food Sci Food Saf.* 2010;9(6):635-654. DOI: 10.1111/j.1541-4337.2010.00131.x.
 18. Yao H, Xu W, Shi X, Zhang Z. Dietary flavonoids as cancer prevention agents. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev.* 2011;29(1):1-31. DOI: 10.1080/10590501.2011.551317.
 19. Nallanthighal S, Elmaliki KM, Reliene R. Pomegranate extract alters breast cancer stem cell properties in association with inhibition of epithelial-to-mesenchymal transition. *Nutr Cancer.* 2017;69(7):1088-1098. DOI: 10.1080/01635581.2017.1359318.
 20. El-Azab NEE, Mansy AE, El-Mahalaway AM, Sabry D. Comparative study of the therapeutic effect of pomegranate alone or in combination with bone marrow mesenchymal stem cells on experimentally induced gastric ulcer in adult male rats: a histological and immunohistochemical study. *Egypt J Histol.* 2018;41(2):150-166. DOI: 10.21608/EJH.2018.13838.
 21. Teimourinejad A, Hashemibeni B, Salehi H, Mostafavi FS, Kazemi M, Bahramian H. Chondrogenic activity of two herbal products; pomegranate fruit extract and avocado/soybean unsaponifiable. *Res Pharm Sci.* 2020;15(4):358-366. DOI: 10.4103/1735-5362.293514.
 22. Bhatti FUR, Kim SJ, Yi AK, Hasty KA, Cho H. Cytoprotective role of vitamin E in porcine adipose-tissue-derived mesenchymal stem cells against hydrogen-peroxide-induced oxidative stress. *Cell Tissue Res.* 2018;374(1):111-120. DOI: 10.1007/s00441-018-2857-3.
 23. Nazem S, Masoumi M, Amirizadeh N, Zolghadr F, Sadeghizadeh M. Protective effect of encapsulated nanocurcumin-PEGOA against oxidative damage on human mesenchymal stem cells exposed to hydroquinone as a risk factor for leukemia. *Multidiscip Cancer Investig. J.* 2017;1(1):3-10. DOI: 10.21859/mci-01011.
 24. Yousefi F, Lavi Arab F, Jaafari MR, Rastin M, Tabasi N, Hatamipour M, *et al.* Immunoregulatory, proliferative and anti-oxidant effects of nanocurcuminoids on adipose-derived mesenchymal stem cells. *Excli j.* 2019;18(17):405-421. DOI: 10.17179/excli2019-1366.
 25. Viuda-Martos M, Fernández-López J, Pérez-Álvarez JA. Pomegranate and its many functional components

- as related to human health: a review. *Compr Rev Food Sci Food Saf.* 2010;9(6):635-654.
DOI: 10.1111/j.1541-4337.2010.00131.x.
26. Dai Z, Nair V, Khan M, Ciolino HP. Pomegranate extract inhibits the proliferation and viability of MMTV-Wnt-1 mouse mammary cancer stem cells *in vitro*. *Oncol Rep.* 2010;24(4):1087-1091.
DOI: 10.3892/or_00000959.
 27. Chao KC, Yang HT, Chen MW. Human umbilical cord mesenchymal stem cells suppress breast cancer tumorigenesis through direct cell-cell contact and internalization. *J Cell Mol Med.* 2012;16(8):1803-1815.
DOI: 10.1111/j.1582-4934.2011.01459.x.
 28. Khakoo AY, Pati S, Anderson SA, Reid W, Elshal MF, Rovira II, et al. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med.* 2006;203(5):1235-1247.
DOI: 10.1084/jem.20051921.
 29. Pinnamaneni R. Cell viability studies and anti-cancerous activity evaluation of pomegranate (*Punica granatum* L) extract. *Res J Pharm Tech.* 2020;13(1):303-307.
 30. Keta O, Deljanin M, Petković V, Zdunić G, Janković T, Živković J, et al. Pomegranate (*Punica granatum* L.) Peel Extract: potential cytotoxic agent against different cancer cell lines. *Rec Nat Prod.* 2020;14(5):326-339.
DOI: 10.25135/rnp.170.19.11.1477.
 31. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature.* 2012;490(7418):61-70.
DOI: 10.1038/nature11412.
 32. Carnero A. Targeting the cell cycle for cancer therapy. *Br J Cancer.* 2002;87(2):129-133.
DOI: 10.1038/sj.bjc.6600458.
 33. Fathi E, Farahzadi R, Valipour B, Sanaat Z. Cytokines secreted from bone marrow derived mesenchymal stem cells promote apoptosis and change cell cycle distribution of K562 cell line as clinical agent in cell transplantation. *PLoS One.* 2019;14(4):1-17.
DOI: 10.1371/journal.pone.0215678.
 34. Hao T, Zhou J, Lü S, Yang B, Wang Y, Fang W, et al. Fullerene mediates proliferation and cardiomyogenic differentiation of adipose-derived stem cells via modulation of MAPK pathway and cardiac protein expression. *Int J Nanomedicine.* 2016;11(18):269-283.
DOI: 10.2147/ijn.s95863.
 35. Pfeffer CM, Singh ATK. Apoptosis: a target for anticancer therapy. *Int J Mol Sci.* 2018;19(2):448-457.
DOI: 10.3390/ijms19020448.
 36. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell.* 2004;116(2):205-219.
DOI: 10.1016/s0092-8674(04)00046-7.
 37. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev.* 2007;87(1):99-163.
DOI: 10.1152/physrev.00013.2006.
 38. Gauthaman K, Yee FC, Cheyyatraivendran S, Biswas A, Choolani M, Bongso A. Human umbilical cord Wharton's jelly stem cell (hWJSC) extracts inhibit cancer cell growth *in vitro*. *J Cell Biochem.* 2012;113(6):2027-2039.
DOI: 10.1002/jcb.24073.
 39. Jendreyko N, Popkov M, Rader C, Barbas 3rd CF. Phenotypic knockout of VEGF-R2 and Tie-2 with an intradiabody reduces tumor growth and angiogenesis *in vivo*. *Proc Natl Acad Sci USA.* 2005;102(23):8293-8298.
DOI: 10.1073/pnas.0503168102.
 40. Mikulić D, Ilić I, Cepulić M, Orlić D, Giljević JS, Fattorini I, et al. Tumor angiogenesis and outcome in osteosarcoma. *Pediatr Hematol Oncol.* 2004;21(7):611-619.
DOI: 10.1080/08880010490501015.
 41. Costantini S, Rusolo F, De Vito V, Moccia S, Picariello G, Capone F, et al. Potential anti-inflammatory effects of the hydrophilic fraction of pomegranate (*Punica granatum* L.) seed oil on breast cancer cell lines. *Molecules.* 2014;19(6):8644-8660.
DOI: 10.3390/molecules1906864.