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Original Article

Effects of pomegranate seed extract and ellagic acid on miR-16-5p and miR-34a-5p expression, cell cycle, and apoptosis in MCF-7 cells

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Abstract

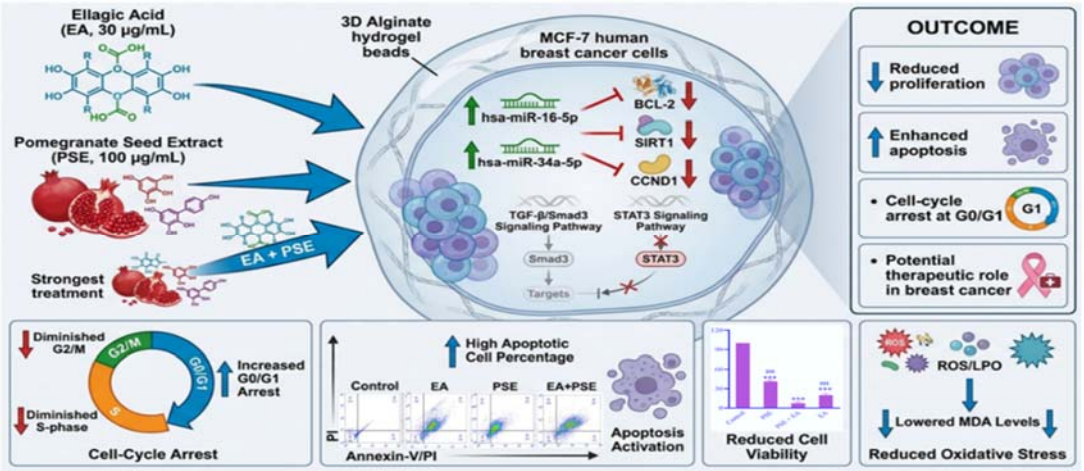
Background and purpose: The expression of *Homo sapiens (has)-miR-16-5p* and *hsa-miR-34a-5p* with anti-tumor effects is downregulated in cancer cells. To maintain these gene expressions in cancer cells, we evaluated the effects of pomegranate seed extract (PSE) and ellagic acid (EA) on *hsa-miR-16-5p* and *hsa-miR-34a-5p* expression, cell cycle regulation, and apoptotic induction in the MCF-7 cells encapsulated in the alginate hydrogel, a 3D culture system.

Experimental approach: MCF-7 cells were encapsulated in 3-D alginate scaffolds and cultured (experimental groups: EA, PSE, EA and PSE, and the control group). Cell viability (using MTT assay), cell apoptosis (*via* flow cytometry), the level of malondialdehyde (MDA), and the expression levels of *hsa-miR-16-5p, hsa-miR-34a-5p*, B-cell lymphoma 2 *(BCL-2),* cyclin D1 *(CCND1),* and sirtuin 1 *(SIRT1)* were measured (via real-time PCR).

Findings/Results: The combination of PSE and EA exhibited the greatest effects on MCF-7 cells. EA and PSE increased the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p* and decreased the expression of the *SIRT1* and *CCND1* genes. In addition, the antiapoptotic *BCL-2* gene was downregulated in the experimental group. Both antioxidants significantly increased the population of MCF-7 cells in the G1 phase. Additionally, antioxidants reduce the level of MDA in cancer cells.

Conclusion and implications: EA and PSE antioxidants increased *hsa-miR-16-5p* and *hsa-miR-34a-5p* expression, induced apoptosis, decreased cell proliferation, and stopped cancer cells in the G1 phase. Therefore, they can be considered promising compounds for helping the treatment of breast cancer.

Keywords: Breast cancer; Ellagic acid; *hsa-miR-16-5p*; *hsa-miR-34a-5p*; Pomegranate seed extract.



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INTRODUCTION

Breast cancer (BC) is a high-risk malignancy that has serious effects on the health of women (1). According to the World Health Organization (WHO), approximately 1.3 million women are confirmed to receive timely treatment every year (2). Although various methods are used to treat BC, including surgical procedures, hormone therapy, chemotherapy, and radiotherapy, some of these methods, such as chemotherapy, also have destructive effects on healthy cells (3). Therefore, it is necessary to find and develop methods for the effective treatment of cancer that destroy as many cancer cells as possible and cause the least damage to healthy cells. These methods affect the expression levels of disordered proteins and genes, such as microRNAs (miRNAs), and activate or deactivate a cascade of cellular signaling pathways, resulting in damage to and death of cancer cells.

Studies have shown that miRNAs are secreted by tumor cells and play a role in cancer control as oncogenes or tumor suppressors (3). An important aspect of cancer research is the interaction between cancer cells and the surrounding microenvironment, which is mediated by miRNAs. miRNAs, a single type of short noncoding RNA of 19-25 nucleotides, are key regulators that control the progression and metastasis of BC (4). miRNA gene expression controls oxidative stress and DNA damage in cancer. By inhibiting tumor-suppressive miRNAs, oncogenic miRNAs, which act as tumor-promoting onco-miRNAs and lead to cancer progression, can be upregulated. Tumor-suppressive mRNAs bind to target genes, holding diverse functions through their target genes. miRNAs are involved in various biological cell functions, such as cell growth and proliferation, survival and death, differentiation, migration, and invasion. The ability of miRNA molecules in the development of biopharmaceuticals against BC has been extensively reported (5). The categories of miRNAs with antitumor activity include *Homo sapiens (has)-miR-16-5p* and *hsa-miR-34a-5p* (6,7). Despite the tumor suppressive effects of *hsa-miR-16-5p* and *hsa-miR-34a-5p*, their expression decreases in cancer cells (8,9). Targeting miRNAs is an important treatment option. Therefore, it is important to maintain their expression in cancer cells to aid in cancer treatment. On the other hand, an important obstacle in cancer treatment is the alteration of miRNA expression, leading to decreased apoptosis of cancer cells and increased resistance to treatment (10). Recent studies have shown that foodstuffs can modulate carcinogen-induced adaptations in terms of miRNA expression. Therefore, it is necessary to use dietary compounds such as herbal compounds to induce apoptosis in cancer cells and for cancer treatment (8,11). Vegetables and fruits containing flavonoids and antioxidants have been found to have an inhibitory effect on tumor cells (12). Pomegranate seed extract (PSE) and its polyphenolic compounds, such as ellagic acid (EA), have antioxidant and anticancer effects (13). EA has antiallergic, anti-inflammatory, antidiabetic, anticancer, and antimicrobial effects. EA, which inhibits estrogen carcinogenesis through modulated miRNAs (miR-122, -127, and -375) and their target genes (*cyclin D1* (*CCND1*), *forkhead box O3 (FoxO3a), FoxO1,* cyclin G1 (*CCNG1*), and B-cell lymphoma 2 (*Bcl-2*)), can prevent breast cancer (14). In addition, various other antioxidant substances, such as arachidonic acid, punicic acid, and gallic acid, are found in pomegranate seeds. The potential anticancer and anti-inflammatory effects of pomegranate seeds are attributed to interactions among compounds (15).

Previous studies have shown that the transforming growth factor/Smad3 (TGF-β/Smad3) and signal transducer and activator of transcription 3 (STAT3) signaling pathways stop the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p.* Furthermore, EA and PSE inhibit these signaling pathways; thus, PSE and EA may increase the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p* (Fig. 1).

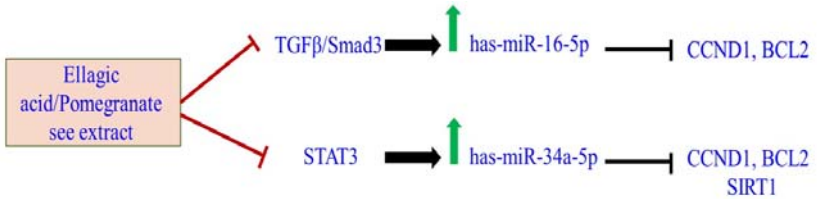

Fig 1. Ellagic acid and pomegranate seed extract inhibit the TGF-β/Smad3 and signal transducer and STAT3 signaling pathways, which can stop the expression of the anticancer miRNAs *hsa-miR-16-5p* and *hsa-miR-34a-5p*. TGF-β/Smad3, transforming growth factor/Smad3; STAT3, signal transducer and activator of transcription 3; CCND1, cyclin D1; BCL2, B-cell lymphoma 2.

The most important strategies used today for basic cancer research and the development of effective treatments include the analysis of clinical specimens (biopsies), animal models, and *in vitro* models. To evaluate cancer treatments, including the measurement of the effect of new anticancer drugs, research is first conducted *in vitro* on cells or tissues (16). *In vitro* research does not have the difficulties of using biopsies, such as heterogeneity and small sample size. Also, although animal research is of great importance, it is expensive and cumbersome, and often cannot properly reflect the vital aspects of human tissues due to interspecies biological differences. On the other hand, animal studies related to cancer are mainly conducted in immunodeficient mice, which may affect tumor growth; therefore, most studies are conducted in the laboratory. *In vitro* cell culture methods mainly include two-dimensional (2D) and 3D culture. In the past, 2D culture methods were the focus of researchers. However, the limitations of these methods, such as the lack of assessment of the roles of other cells, cell-cell and cell-extracellular matrix interactions, the roles of factors in the tumor microenvironment, such as exosomes, miRNAs, and the absence of a dynamic microenvironment, led to the introduction of 3D culture methods. Cells are cultured in 3D models under laboratory conditions. One of the important 3D culture methods is scaffold-based culture, which has become increasingly important in medical research, especially in oncology (17).

Therefore, in this study, we investigated the effects of PSE and EA on the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p*, the regulation of the cell cycle, and the induction of apoptosis in MCF-7 cancer cells encapsulated in alginate hydrogels in a 3D culture system. If the tested compounds demonstrate an ability to increase *hsa-miR-16-5p* and *hsa-miR-34a-5p* expression while reducing the survival of cancer cells, further research could support their potential as therapeutic agents for breast cancer (BC).

MATERIALS AND METHODS

Preparation of the PSE

The gardens of Najaf Abad in Isfahan Province were used to harvest the fruit in order to produce the PSEs. Pomegranate seeds were dried and made into powder. They were then placed on a shaker in 70% ethanol (CAS No. 64--17--5, Merck, Germany) for 4 h. A Buchner funnel was used to filter the extract. One gram of the desired extract was dissolved in 10 mL of annular water. Then, 1 mL of the stock solution was diluted with 9 mL of Dulbecco's modified Eagle medium-F12 (DMEM-F12, Gibco, USA) and stored at -20 °C. A total of 100 µg/mL of the prepared extract was used in the experiment (18,19).

Preparation of EA

EA (E2250, Sigma, USA) was purchased from Kian Perto Tajhiz Company. A stock solution of EA (30 µg/mL) was prepared in dimethyl sulfoxide (DMSO, CAS No. 67-68-5, Sigma, USA) and filtered before use (20).

Preparation of the scaffold (1.5% alginate solution)

A total of 1.5 g of sodium alginate powder (CAS No. 9005-38-3, Sigma, USA) was dissolved in 100 mL of 0.9% sodium chloride (CAS No. 7647-14-5, Sigma, USA) and then filtered with a 0.2-µm filter (CAS No. Z358215, Sigma, USA) (21).

Encapsulating MCF7 cells in the alginate hydrogel

The MCF-7 human breast cancer cell line was purchased from the Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran. MCF7 cells were cultured at 37 °C with 95% humidity and 5% CO_2 in DMEM-F12 supplemented with 10% fetal bovine serum (FBS, CAS No. F4135, Sigma, USA), 1% penicillin, and streptomycin antibiotic solution (CAS No. P4333, Sigma, USA). When the MCF-7 cell density reached 80% (by visual inspection), the cells were trypsinized (Cat No. N0100-780, Cegrogen, Germany) and mixed with 10,000 cells with 100 µL of alginate solution after counting. The alginate-cell mixture was subsequently added to 102 mM calcium chloride solution (CAS No. C34006 MSDS, Sigma, USA) in a 12-well plate (Biologix, Germany; 2.5 mL per well). After 15 min, the alginate beads were created, the calcium chloride was subsequently drained, and 2.5 mL of sodium chloride (0.9%) solution was added to each well. EA and PSE were then added to the wells according to the group instructions (1 mL to each well) (22,13).

MTT assay

In the different culture groups, including those treated for 5 days. EA group (1000 µg/mL EA, 1 mL EA + 1 mL DMEM-F12), PSE group (1000 µg/mL, 1 mL PSE and 1 mL DMEM-F12), EA and PSE group (respectively 500 µL of EA + 500 µL of PSE + 1 mL of DMEM-F12), and the control group (untreated MCF7 cells, 2 mL of DMEM-F12), the supernatant medium was removed, and 400 µL of DMEM F12 and 40 µL of MTT solution (CAS No. M5655, Sigma, USA; 5 mg/mL) were added to each well of the 12-well plate and incubated for 4 h. Next, the contents of each well were removed, 400 µL of DMSO was added, and the mixture was incubated in the dark at room temperature for 2 h. Finally, the optical density (OD) was read at 570 nm with a microplate reader (ZEISS, Germany) (23).

Apoptosis assays

An Annexin-V-fluorescein isothiocyanate (FITC) kit (BMS500FI/300CE, eBioiscience, Germany) was used to examine cell apoptosis. A suspension of MCF-7 cells (5×10^4 cells) was prepared in 2 mL of phosphate-buffered saline (PBS) and transferred to a flow cytometry tube. After the cells were centrifuged (at 1400 rpm for 8 min), the supernatant was removed, and the mixture was deposited into the sediment of each tube. Five hundred microliters of binding buffer mixture were added, followed by adding 5 µL of Annexin-FITC and 5 µL of propidium iodide (PI; BMS500FI/300CE, eBioiscience, Germany) to each tube. The mixture was incubated for 5 min in the dark, after which the number of apoptotic cells was evaluated at 488 and 530 nm *via* a flow cytometry device (BD FACSCaliburTM flow cytometer, Becton Dickinson, San Jose, USA) (24).

Cell cycle analysis

To analyze the cell cycle distribution *via* flow cytometry, cultured MCF-7 cells (5×10^5 cells) were trypsinized (CAS No. D8537, Sigma, USA), fixed after washing with 70% ethanol, and fixed for 24 h at -20 °C. The cells were subsequently centrifuged (at 1800 rpm at 4 °C for 7 min) and incubated with 0.1% Triton™ X100 (CAS No. 9036-19-5, Sigma, USA), which contains RNase A (CAS No. R6148.70856, Sigma, USA), which was stored on ice for 30 min and stained with PI (CAS No. P4170, Sigma, USA), and the cells were analyzed in the growth (G)0-G1, G1, synthesis (S) and G2- mitosis (M) phases via flow cytometry (BD FACSCalibur™ flow cytometer, Becton Dickinson, San Jose, USA) (25).

MDA assay for ROS analysis

To assess lipid peroxidation (LPO), the level of malondialdehyde (MDA) was measured using a kit (NS-15022, NS-15023; Navandsalamat, Iran). The level of MDA is considered an important biomarker for measuring reactive oxygen species (ROS) levels. Fifty milliliters of tetrachloric acid was mixed with 50 mL of thiobarbituric acid and an equal volume of hydrochloric acid. Then, 50 mL of the sample was mixed with 100 mL of the abovementioned mixture. The samples were subsequently centrifuged at 3000 rpm for 10 min. The absorbance of the samples was measured at 535 nm (26). Finally, the MDA concentration of each sample was obtained *via* the following equation:

$$\text{MDA concentration}\left(\frac{\text{nmol}}{\text{mL}}\right) = \frac{\text{Optimal density} \times \text{total volume}}{1.56 \times 105 \times \text{sample volume}} \times 10\text{^}6$$

RNA isolation and qRT-PCR

The expression of genes and miRNAs was measured ($2^{-\Delta\Delta CT}$) *via* quantitative real-time polymerase chain reaction (qRT-PCR). This was performed according to the kit protocol (Cat. No. FABRK001, Favorgene, Taiwan). The SYBR™ Green and Master Mix method (Cat. No. DQ385-40 h, BIOFACT High ROX, Korea) was used for RT-PCR, and the details of the procedure are included in Table 1. Finally, the extracted RNA was stored in a fridge at -70 °C (27). A cDNA synthesis kit (BR441-Biofact, Korea) was used to synthesize cDNA. *GAPDH* (CAS No. 39705, Sigma, USA) was used as a reference gene, and for miRNAs, *U6* (CAS No. 15-288-22314F, Sigma, USA) was used as the reference gene (28). Tables 2 and 3 list the primers (Metabion, Germany) used for qRT-PCR.

Table 1. The procedure of RT-PCR.

Reaction phase	Temperature and time	Number of cycles
Initial activation	95 °C for 15 min	1 cycle
Double-strand disruption	95 °C for 15 s	
Annealing	60 °C for 1 min	40 cycles
Extension	72 °C for 30 s	
Melting curve from	60 °C to 95 °C in 3/ intervals for 1 min	1 cycle

Table 2. Primers for real-time polymerase chain reaction.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
BCL-2	GGATCCAGGATAACGGAGGC	GGCAGGCATGTTGACTTCAC
CCND1	GAGGAGCTGCTGCAAATGG	TCTGTTTGTTCTCCTCCGCC
SIRT1	TGGGTACCGAGATAACCTTCTG	GTGCCAATCATAAGATGTTGCTG
GAPDH	GCTCATTTCCTGGTATGACAACG	CTCTCTTCCTCTTGTGCTC TTG
U6	CGCTTCGGCAGCACATATAC	AAATATGGAACGCTTCACGA

Table 3. Primers for real-time polymerase chain reaction.

miRNAs	Stem-loop (5'-3')	Forward primer (5'-3')	universal reverse primer (5'-3')
hsa-miR-16-5p	GTTGGCTCTGGTGCAGGGTC CGCCAA	GTTTGGTAGCAGCACGTA AATA	GTGCAGGGTCCGAGGT
hsa-miR-34a-5p	GTTGGCTCTGGTGCAGGGTCCACAA CC	GTGTGGCAGTGTCTTAGCT	GTGCAGGGTCCGAGGT

Data analysis

IBM SPSS 28.0.1 software was used. First, the data distribution was checked *via* the Kolmogorov-Smirnov method. The data are presented as mean ± SD. The significant differences among all groups were evaluated *via* one-way ANOVA followed by Tukey's Post Hoc test. FlowJo software (v10.10) was used to analyze flow cytometry data. *P*-values < 0.05 were considered statistically significant.

RESULTS

Cell culture

Inverted microscope observations revealed the proliferation of the MCF-7 cell line in monolayer culture with an epithelial-like morphology (Fig. 2A). In the 3D culture system, the encapsulated cells in the alginate beads were round (Fig. 2B).

Viability of the MCF7 cell line

As shown in Fig. 3, the viability of the cells in the groups treated with PSE, EA, or PSE + EA for 5 days was compared with that of the control group. The analysis of the results indicated that the percentage of viable cells in all treatment groups was significantly lower than that in the control group. Additionally, the viability of the cells in the group treated with PSE + EA (7.567 ± 2.20%) was significantly lower than that in the EA (19.80 ± 2.38%) and PSE (41.23 ± 2.14%) groups.

Apoptosis assays by flow cytometry

As shown in Fig. 4A and B, the percentage of apoptotic cells in the groups treated with PSE, EA, or PSE + EA was compared with that of the control group (5.115 ± 0.1626). The analysis of the results indicated that the percentage of apoptotic cells in all treated groups significantly increased compared to the control group. Additionally, among the treated groups, PSE + EA induced the highest percentage of apoptotic cells (97.90 ± 0.1485), while PSE treatment led to a lower percentage of apoptosis (71.50 ± 2.121).

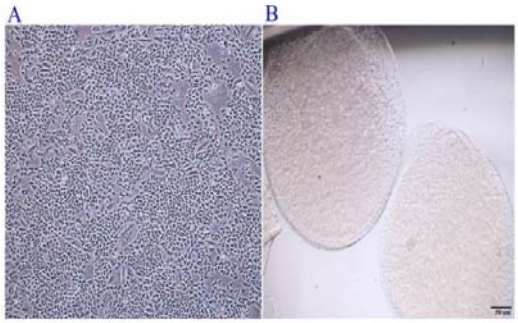

Fig. 2. Morphology of MCF-7 cells in (A) 2D with star-shaped morphology and (B) 3D with a round and small morphology cultures imaged by inverted microscopy at actual magnifications of 40× and 10×.

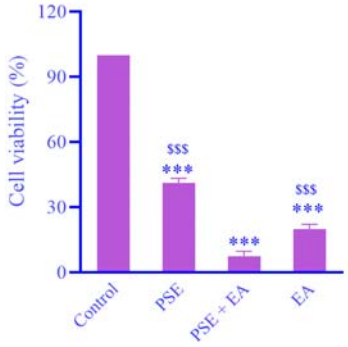

Fig. 3. MTT assay results showing that the viability of the MCF7 cells in the groups treated with PSE, EA, and PSE + EA was lower than that in the control group. Data are presented as mean ± SD. ***$P < 0.001$ indicates significant differences compared to the control group; $^{\$\$\$}P < 0.001$ vs PSE + EA group. EA, Ellagic acid; PSE, pomegranate seed extract.

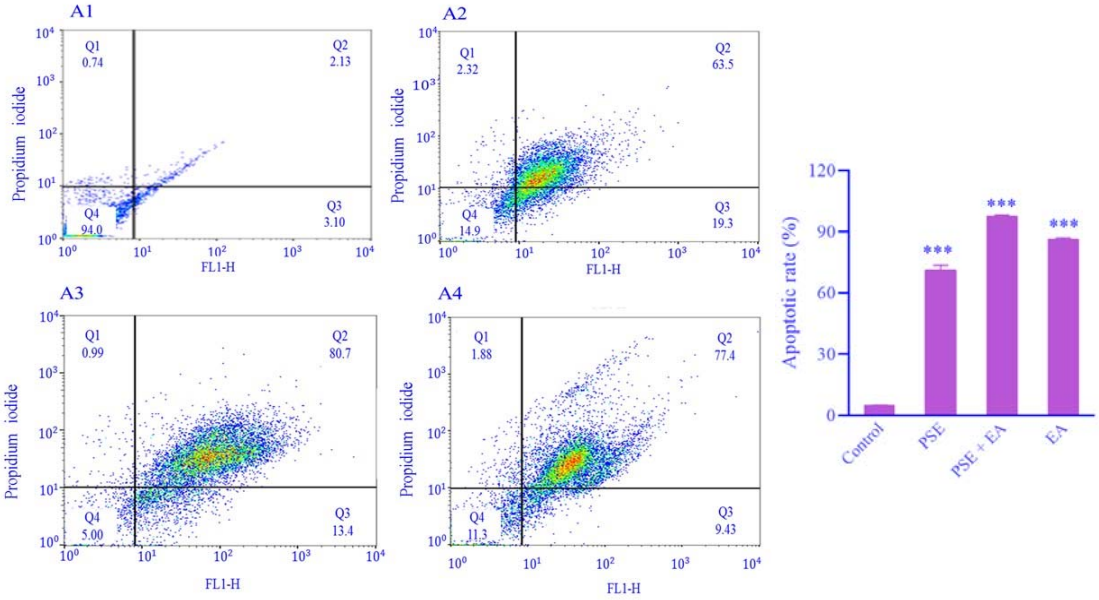

Fig. 4. Results of the histogram flow cytometric analysis by propidium iodide and annexin V staining for cell apoptosis. (A1) Apoptosis rate of untreated MCF-7 cells, (A2) cells treated with PSE, (A3) cells treated with PSE + EA, and (A4) cells treated with EA. (B) The percentage of apoptotic cells in the groups that received different treatments. Data are expressed as mean ± SD, n = 3. ***$P < 0.001$ indicates significant differences compared with the control group (untreated MCF-7 cells). EA, Ellagic acid; PSE, pomegranate seed extract; Q1, necrotic; Q2, late apoptotic; Q3, early apoptotic; Q4, alive.

Cell cycle

According to the results shown in Figs. 5 and 6, the percentages of cells distributed in different stages of the cell cycle after 5 days in the groups treated with PSE, EA, and PSE + EA and the percentage of cells distributed in the control group (un-treated MCF7 cells) were compared, and the analysis of the results revealed that the distribution percentage of cells in the G0/G1 phase in all the mentioned treatment groups was significantly greater than that in the control group (53.25 ± 0.35). Among the treatment groups, the percentage of cells distributed in the PSE + EA group (93.95 ± 0.07) was greater than that in the PSE group (84.80 ± 0.28).

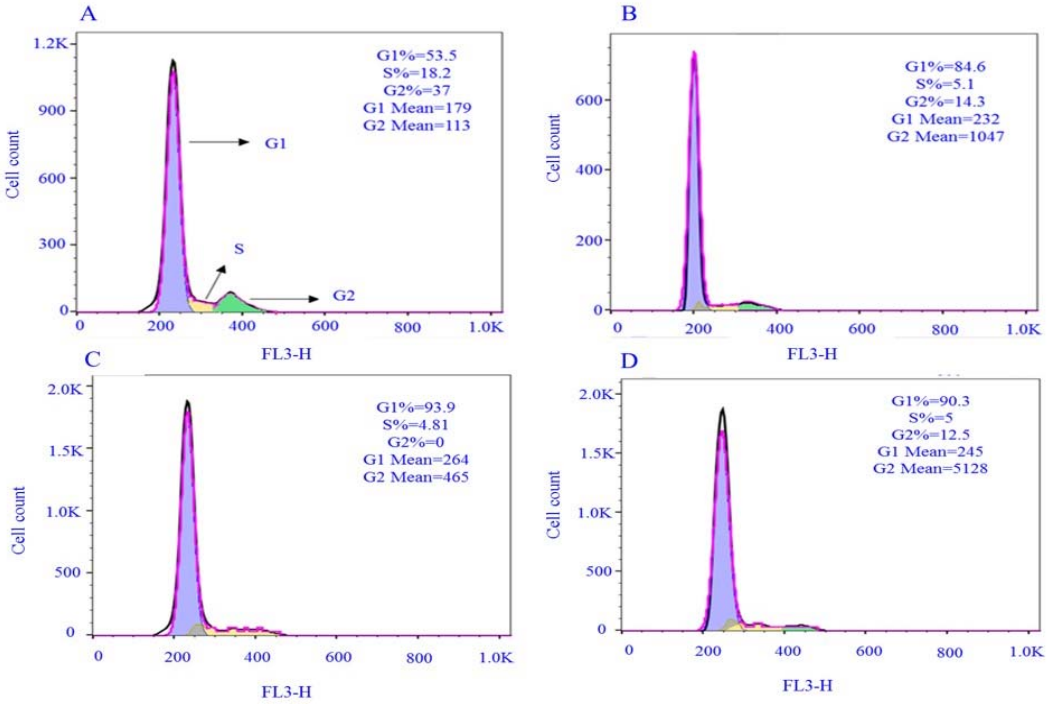

Fig. 5. Flow cytometry analysis (using propidium iodide) of the MCF7 cells' cycle. (A) Control group (untreated MCF7 cells); (B) PSE-treated cells; (C) cells received PSE + EA combination; (D) EA-treated cells. EA, Ellagic acid; PSE, pomegranate seed extract.

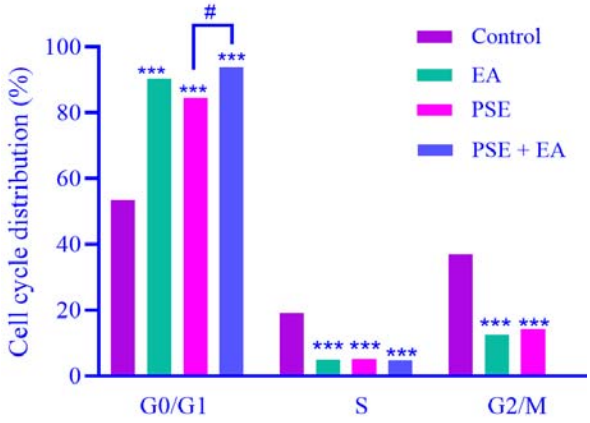

Fig. 6. Flow cytometric analysis of the MCF7 cells. Data are expressed as mean ± SD, n = 3. ***$P < 0.001$ indicates significant differences compared with the control group (untreated MCF-7 cells); $^{\#}P < 0.05$ between designated groups. EA, Ellagic acid; PSE, pomegranate seed extract.

Additionally, the percentage of cells in the S phase of the cell cycle was significantly lower in all the mentioned treatment groups in comparison with the control group (19.10 ± 0.14). In addition, among the treatment groups, the percentage of cells distributed in the PSE + EA group (4.90 ± 0.13) was lower than that in the other treatment groups.

The percentage of cells in the G2/M phase of the cell cycle was the lowest in the PSE + EA group (00.00 ± 0.00) than in the control group (37.05 ± 0.07). Besides, the other treatments led to a significant decrease (EA: 12.25 ± 0.35 and PSE: 14.15 ± 0.21) compared to the control group.

Concentration of ROS by the MDA assay

According to the results shown in Fig. 7, the average MDA concentration (nmol/mL) after 5 days was significantly lower in the groups treated with PSE, EA, or PSE + EA than in the control group. No significant difference was observed among the treatment groups.

Gene expression

RT-PCR was used to measure gene expression in the treatment groups. As shown in Fig. 8A and B, the expression of *hsa-miR16-5p* and *hsa-miR-34a-5* significantly increased in all treatment groups compared to the control group. Additionally, the expression of *hsa-miR16-5p* and *hsa-miR-34a-5p was greater* in the PSE + EA group than in the PSE group. Overall, the results of RT-PCR revealed the highest expression of *miR16-5p* and *hsa-miR-34a-5p* in the group treated with PSE + EA.

As shown in Fig. 8C-E, the gene expression levels of *cyclin D1 (CCND1)*, *BCL2*, and *sirtuin 1 (SIRT1*) were significantly lower in all treatment groups than those in the control group. Additionally, the lowest levels of *CCND1*, *BCL2*, and *SIRT1* gene expression were detected in the group treated with PSE + EA ($P < 0.001$).

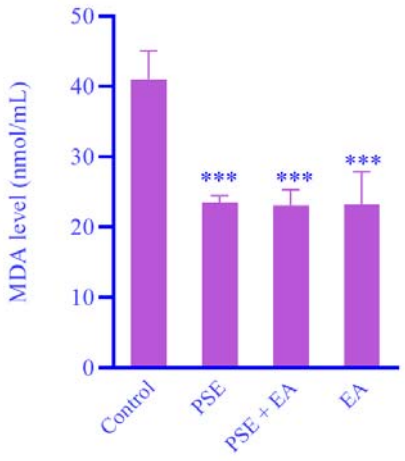

Fig. 7. The average concentration of MDA in the groups treated with PSE, EA, and PSE + EA. Data are expressed as mean ± SD, n = 3. ***$P < 0.001$ indicates significant differences compared with the control group (untreated MCF-7 cells). EA, Ellagic acid; PSE, pomegranate seed extract; MDA, malondialdehyde.

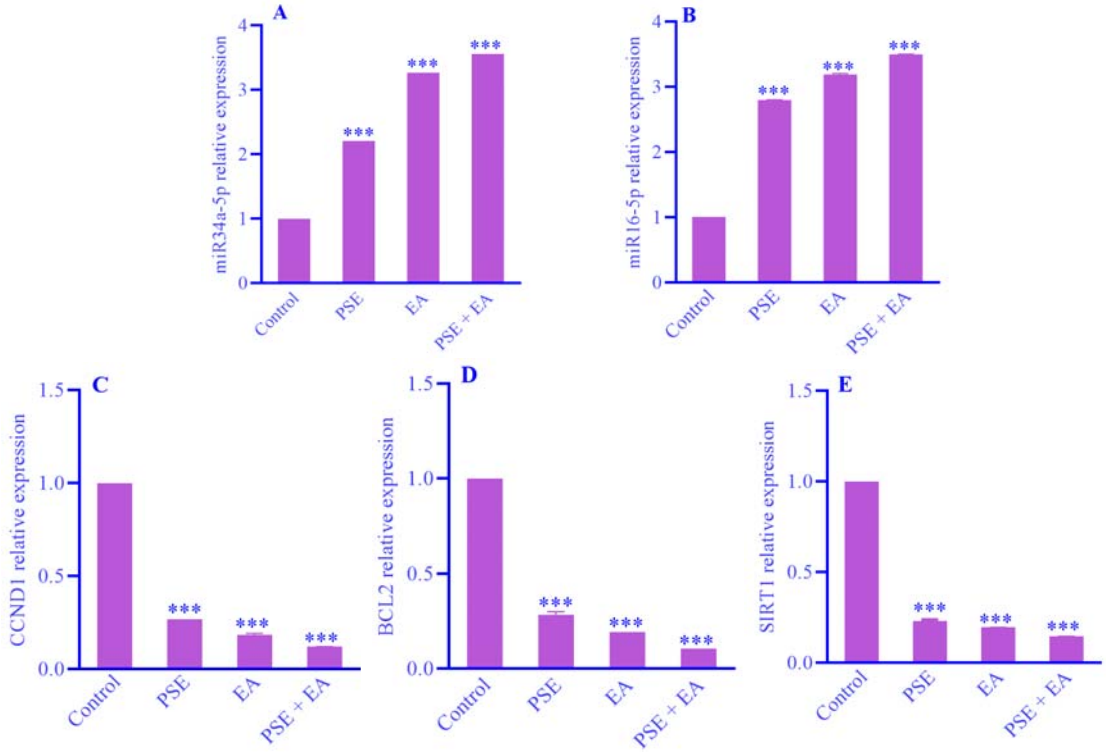

Fig. 8. Real-time polymerase chain reaction was used to measure the gene expression in the MCF7 treatment groups. Data are expressed as mean ± SD, n = 3. ***$P < 0.001$ indicates significant differences compared with the control group (untreated MCF-7 cells). EA, Ellagic acid; PSE, pomegranate seed extract;

DISCUSSION

Many studies have investigated the inhibitory and antitumor effects of herbal compounds such as PSE and EA on the metastasis and progression of breast tumors (13,29). These preclinical trials provide valuable insight into the potential benefits of PSE and EA in cancer (30). Further investigations are needed to clarify their mechanisms of function and their effects on safety in the human body. The pomegranate (*Punica granatum* L.) belongs to the Punicaceae family (31). EA is the key material and abundant phenolic acid of PSE and is responsible for most biological activities, including the modulation of miRNA expression. MiRNAs play a key role in regulating essential processes in cancer cells, including apoptosis, metastasis, and cell division. In this study, we investigated the effects of EA at 30 µg/mL (31) and PSE at 100 µg/mL (13,31), both alone and in combination, on the expression of hsa-miR-16-5p, hsa-miR-34a-5p, and their target genes (BCL2, SIRT1, and CCND1), as well as on the viability, apoptosis, and cell cycle of the MCF-7 human breast cancer cell line. To mimic the natural environment of the body, the cells were encapsulated in the alginate hydrogel as a 3D culture (32).

Bishayee *et al*. reported that EA has inhibitory effects on BC (33). It also plays a role in inhibiting the progression of colon cancer, prostate cancer, and liver cancer (34,35). EA and PSE inhibit the expression of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and the TGF-β/Smad3 signaling pathway (36,37). Additionally, Chen *et al.* (38) reported that EA has anti-proliferative effects on MCF-7 breast cancer cells *via* the modulation of TGF-B/Smad3 and STAT3 signaling, modulating various genes, which are involved in the regulation of cell differentiation, proliferation, apoptosis, angiogenesis, and metastasis; thus, EA inhibits the proliferation of MCF-7 cells. The PSE also has important effects on cell division. The cell cycle consists of 4 phases, including growth (G1), which includes cell growth, RNA production, and protein synthesis for DNA replication during S phase, DNA synthesis (S), continued growth and preparation for cell division (G2), and the final stage includes mitosis (M) (39). The regulation of the cancer cell cycle is the most important mechanism for cancer suppression and treatment (40,41). Based on the results of our study, EA and PSE increased the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p* and decreased the expression of *CCND1*, which is a cell cycle promoter gene, leading to cell cycle arrest in MCF7 cells in the G0/G1 phase and preventing their reproduction and growth. *CCND1* augmentation is the most commonly observed genetic change in cancer (29). Cizmarikova *et al.* (42) reported that EA, by decreasing the expression of *CCND1*, *CCND2*, and *CCND3* and suppressing the expression of two proteins governing the cell cycle, namely, CDK2 and WEE1 kinase, leads to the induction of apoptosis and cell cycle arrest in the G0/G1 phase in cancer cells (42). Additionally, EA suppresses the JAK/STAT3 pathway and inhibits cell division, cell proliferation, differentiation, invasion, and tumor angiogenesis (42,43).

In addition to reducing cell proliferation, our study revealed that EA and PSE can lead to an approximately 90% reduction in MCF7 cell viability by increasing the expression of *hsa-miR-16-5p*, *hsa-miR-34a-5p*, and their target genes, such as reducing the expression of the antiapoptotic gene of *BCL-2*. Cheshomi *et al*. reported that EA and other pomegranate compounds associated with changes in the expression of genes related to apoptosis and inflammation, including *P53*, Bcl-2-associated X-protein (*BAX*), apoptotic peptidase activating factor 1 (*APAF1), BCL2, NF-kB, IL-8*, and *TNF-α*, led to increased death of stomach cancer cells in mice (31). Previous studies have shown that PSE reduces the viability of MCF-7 cells (13). Ozkan *et al*. (18) reported that punicalagin, one of the most important extracts of PSE, has effective cytotoxicity on the MCF-7 BC cell line and decreases the survival of cancer cells.

We also noted that these two herbal compounds, which modulate the expression of miRNAs, such as *hsa-miR-16-5p* and *hsa-miR-*

34a-5p, play important regulatory roles in controlling the progression and viability of BC. *hsa-miR-16-5p* can modulate apoptosis by targeting *BCL2* (43,44). *Sadeghi et al.* (7) revealed that *hsa-miR-16-5p* is expressed at low levels in most human cancers, such as BC. Ruan *et al*. (45) reported that downregulation of *hsa-miR-16-5p* in patients with BC effectively stimulated tumor cell migration, proliferation, and DNA synthesis in the cell cycle but inhibited apoptosis. We demonstrated that PSE and EA can enhance the expression of *hsa-miR-16-5p* and decrease the expression of its target genes, including *BCL2*.

hsa-miR-34a-5p is another tumor suppressor miRNA that exerts antitumor effects by targeting genes such as *SIRT1*, which is closely associated with the TGF-β1/Smad3 pathway (46,47). In a study by Anjaly *et al.* (48) EA decreased the expression of *SIRT1* and led to prostate cancer cell death. The present study, consistent with previous studies, revealed that EA and PSE treatments resulted in elevated expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p* and diminished expression of *SIRT1, BCL-2*, and *CCND1*. This modulation consequently promotes cell death and reduced survival rates in cancer cells.

These two compounds also arrested the cell cycle of MCF7 cells in the G0/G1 phase, reduced the number of cells in the S and G2 phases, and generally prevented the proliferation and growth of cancer cells. The results of the present study indicated that the effectiveness of EA was greater in preventing cancer cell proliferation and reducing cell viability compared with that of PSE. Furthermore, the combined treatment with EA and PSE exerted a synergistic effect on cytotoxicity relative to their individual applications. A controversial issue that is important in current research related to the development of cancer is the role of ROS in cancer progression. Compared with normal cells, malignant cells have increased levels of ROS, which are involved in both the beginning and progression of cancer. As a result, antioxidant compounds limit the excessive production of ROS, genes that respond to oxidative stress can be suppressed, and the proliferation of cancer cells can be prevented (49,50). ROS can lead to lipid peroxidation, which is monitored by measuring the level of MDA. In this context, this study also revealed that the antioxidant properties of EA and PSE led to a decrease in MDA production and likely play a role in the upregulation of *hsa-miR-16-5p* and *hsa-miR-34a-5p* expression. EA has also been previously shown to reduce MDA production and mediate the development of TGF-β1 through the activation of Notch signaling (50). Additionally, *SIRT1* related to *hsa-miR-34a-5p* leads to the inhibition of lipid peroxidation (45).

The results of this study revealed stronger effects of the combination of the antioxidants EA and PSE on reducing cell viability, increasing apoptosis, and arresting the cell cycle in BC. EA had stronger anticancer effects than PSE. We predict that EA and PSE inhibit the TGF-β/Smad3 and STAT3 signaling pathways and, owing to the inhibitory effects of TGF-β/Smad3 and STAT3 on the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p*, lead to increases in *hsa-miR-16-5p* and *hsa-miR-34a-5p* expression in cancer cells. Therefore, EA and PSE can be used alone or in combination to improve BC treatment, which can be a new way to increase treatment efficiency. However, more studies are needed to determine the precise procedures of their functions. Additionally, despite the importance of laboratory studies, animal models and clinical trials are needed in the future.

CONCLUSION

This is the first study on the medicinal properties of EA and PSE, revealing that they can effectively increase the expression of *hsa-miR-16-5p* and *hsa-miR-34a-5p* and their target genes, such as *BCL-2*, *SIRT1,* and *CCND1*, which are involved in cell death and the cell cycle in 3D cultures of MCF-7 cells in alginate hydrogels. Thus, EA and PSE, through the aforementioned pathways, led to decreased cell viability and cell cycle arrest. Although the current findings are promising, more *in vivo* studies are needed to confirm their prohibitive effects on BC.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

B. Hashemibeni designed the project. Z. Sadeghi wrote the manuscript. Z. Sadeghi experimented. Z. Sadeghi and M. Malekzadeh analyzed the data and designed the illustrations. Sh. Ghaedamini helped with the culturing of cells. M. Sharifi conducted the molecular experiments and qRT-PCR analysis. B. Hashemibeni revised and edited the manuscript. All authors have read and approved the final version of the article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

Ethics approval and consent to participate

The Research Ethical Committee of Isfahan University of Medical Sciences approved the study protocols. IR.MUI.REC.1401.060.

AI declaration

The authors did not use any AI-assisted technologies in the preparation of this manuscript.

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