Iran J Pharm Res. January-December 2026; 25(1): e169138 https://doi.org/10.5812/ijpr-169138

Published Online: 2026 November 22 **Research Article**

Comparative Cytotoxicity and Pro-Apoptotic Effects of Sweet and Sour Iranian Pomegranate Seed Extracts on MCF-7 Breast Cancer Cells and Human Fibroblasts

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Received: 20 December, 2025; **Revised:** 10 November, 2026; **Accepted:** 17 November, 2026

Abstract

Background: Pomegranate (*Punica granatum* L.) contains bioactive compounds with potential anticancer properties. However, comparative evidence on the cytotoxic and pro-apoptotic effects of sweet and sour pomegranate seed extracts remains limited.

Objectives: The present study compared the cytotoxicity, apoptosis-related molecular responses, and cell-cycle effects of ethanolic extracts of sweet and sour Iranian pomegranate seeds in MCF-7 breast cancer cells and HFF-1 fibroblasts.

Methods: Cytotoxicity was assessed using the MTT assay, and IC_{50} values were determined by nonlinear regression. The effects of the IC_{50} concentrations on BAX, BCL-2, and CASP8 expression were evaluated by quantitative real-time PCR. Apoptosis and cell-cycle distribution were further analyzed by flow cytometry after 72 h of treatment.

Results: Both extracts showed selective cytotoxicity toward MCF-7 cells, whereas HFF-1 viability remained above 85% at concentrations up to 200 µg/mL. The IC_{50} values were 82.50 µg/mL for the sweet extract and 78.51 µg/mL for the sour extract, indicating greater cytotoxic potency of the sour extract. The sour extract markedly increased BAX expression and suppressed BCL-2 expression, producing a pro-apoptotic shift. Both extracts increased CASP8 expression. Flow cytometric analysis showed greater apoptosis and G1-phase accumulation after treatment with the sour extract. The sub-G1 population reached 81.25% with the sour extract versus 66.84% with the sweet extract, whereas G1-phase cells accounted for 80.22% and 43.35%, respectively.

Conclusions: The findings suggest that differences between the two cultivars may influence their biological effects, and they support further investigation of the cultivars' bioactive constituents and underlying mechanisms. Overall, both extracts exhibited selective cytotoxic and pro-apoptotic effects against MCF-7 cells, with greater activity observed for the sour extract.

Keywords: Pomegranate, MCF-7 Cells, Breast Neoplasms, Fibroblasts, Apoptosis

1. Background

Breast cancer remains the most prevalent malignant disease among women worldwide (1, 2), with over 2.3 million new cases reported in 2022 alone (3). It continues to be the leading cause of cancer-related morbidity and mortality, accounting for nearly one in four cancer diagnoses and one in six cancer deaths among women (1). Despite higher incidence rates in developed countries, mortality is disproportionately greater in developing regions owing to limited access to early detection and effective treatment (4, 5). These global disparities underscore the urgent need for equitable healthcare strategies to improve outcomes and reduce the burden of breast cancer across diverse populations (2). Subtypes such as triple-negative breast cancer exhibit aggressive behavior and lack targeted therapies, and their treatment therefore relies on toxic chemotherapies that induce resistance and damage normal tissue. Current breast cancer therapies, including conventional and targeted approaches, often

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How to Cite: Tavangar S, Hadi F, Hejazi SH, Mozdoori N. Comparative Cytotoxicity and Pro-Apoptotic Effects of Sweet and Sour Iranian Pomegranate Seed Extracts on MCF-7 Breast Cancer Cells and Human Fibroblasts. Iran J Pharm Res. 2026;25(1):e169138. doi: https://doi.org/10.5812/ijpr-169138

involve multidrug combinations of cytotoxic chemotherapeutics and pathway-selective small-molecule inhibitors. However, these treatments are frequently associated with systemic toxicity, intrinsic or acquired therapy resistance, and the emergence of drug-resistant cancer stem cell populations. These limitations highlight the need for alternative strategies against therapy-resistant metastatic breast cancer (6). Natural products, such as dietary phytochemicals and nutritional herbs, are being investigated as promising alternatives because of their documented human consumption, lack of detectable systemic toxicity, and minimal off-target side effects (6-8). Pomegranate (*Punica granatum* L.), native to Iran and long associated with traditional diet and medicine, is rich in polyphenols, anthocyanins, and hydrolyzable tannins, and its antioxidant activity has been found to be greater than that of red wine and green tea (7, 9, 10). Its derivatives, such as juice, peel extracts, and seed oil, exhibit anti-inflammatory, antiproliferative, and antiestrogenic activities in breast cancer models (7, 9, 11). Pomegranate polyphenols possess strong anticancer activity through various mechanisms, including antiestrogenic, antiproliferative, antiangiogenic, anti-inflammatory, and antimetastatic effects (9). Indeed, studies have demonstrated that pomegranate extracts induce cell cycle arrest and dose- and time-dependent cytotoxicity, as well as inhibit the invasion, migration, and viability of breast cancer cells (9, 12, 13). Although numerous mechanistic studies have been conducted in cell lines and animal models, larger and better-controlled clinical studies are required to fully establish the effectiveness and safety of pomegranate extracts in the management of breast cancer (9, 14).

2. Objectives

The present study adds a fresh dimension, as prior studies have focused on pomegranate juice, peel, and oil, whereas comparative studies of sweet versus sour seed cultivars, which are integral to Iranian diets, represent a new line of inquiry. Therefore, this study aimed to compare the effects of ethanolic extracts from sweet and sour Iranian pomegranate seeds on cytotoxicity, apoptosis induction, and cell-cycle distribution in MCF-7 human breast cancer cells with those in normal human fibroblasts.

3. Methods

3.1. Extraction Procedure

Seeds from sweet and sour pomegranate (*Punica granatum* L.) cultivars were air-dried and ground into a fine powder (particle size 0.25 – 1.0 mm). The powdered seeds (50.0 g of each type) were extracted with a mixture of distilled water and 80% ethanol (Merck, Germany) at a 1:10 (w/v) ratio. The mixtures were shaken regularly and kept in sealed containers for 72 h at ambient temperature. After extraction, the solutions were filtered through Whatman No. 40 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator (KIKA WERKE) at a controlled temperature (40 °C) and rotation speed. The dried extracts were collected, weighed, and stored at –20 °C in airtight, sterile glass vials until use. The extraction yield was approximately 12% (w/w), corresponding to about 6.0 g of dried extract per 50.0 g of starting material. All extracts were used within two months of preparation (15). Stock solutions of the extracts were prepared in absolute ethanol and sterile-filtered through a 0.22 µm syringe filter. The final ethanol concentration in all cell culture treatments was adjusted to 0.1% (v/v).

3.2. Cell Culture

The MCF-7 human breast cancer cell line was obtained from the Pasteur Institute of Tehran, Iran. Human foreskin fibroblasts (HFF-1 cell line; code RSCB0564) were provided by the Stem Cell Bank of Royan (Isfahan, Iran). MCF-7 cells were maintained in DMEM/F12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Bio-Iran Idea) and 1% penicillin/streptomycin (Pen/Strep; Idezist Co., Iran). Fibroblasts were cultured in DMEM/F12 medium containing 10% FBS, 1% Pen/Strep, 1× non-essential amino acids, and 1% L-glutamine. Both cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

To expand the cell population, three consecutive passages were performed. When the desired cell density was reached, the cells were detached using 0.25% trypsin-EDTA. Cell line authentication was confirmed by short tandem repeat profiling (for MCF-7) and by verification of the supplier's certificate of analysis (for HFF-1). All cultures were routinely screened for mycoplasma contamination using a PCR-based detection kit (SinaClone, Iran) and were confirmed to be negative before experimental use.

3.3. Cytotoxicity Assay

The cytotoxic effects of sweet and sour pomegranate seed ethanolic extracts were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MCF-7 cells and human fibroblasts (HFF-1) were seeded in 96-well plates at a density of 5×10^3 cells/well and allowed to adhere overnight. The cells were then treated with a series of concentrations (25, 50, 100, and 200 µg/mL) of each ethanolic extract for 24, 48, and 72 h. Vehicle controls (culture medium containing 0.1% v/v

ethanol, the highest solvent concentration used) and a positive control (1 µM doxorubicin) were included in every experiment. The seeding density was selected to provide a consistent starting cell number while minimizing excessive confluence during the treatment period. Cell culture and maintenance were performed according to the protocols recommended by the respective cell sources. The population doubling time was not independently determined in the present study.

After each incubation period, the medium was replaced with fresh medium containing 0.5 mg/mL MTT (prepared from a 5 mg/mL stock solution in PBS), and the cells were incubated for 2 h at 37 °C. The formazan crystals were dissolved in dimethyl sulfoxide, and absorbance was measured at 570 nm using a microplate reader (BioTek, USA). To rule out direct chemical reduction of MTT by the extracts, control wells containing each extract concentration plus MTT (without cells) were run in parallel; no significant reduction was observed.

Cell viability and growth inhibition percentages were calculated using the following formulas:

$$(\%)\text{Viability} = \frac{\text{Treated cell OD}}{\text{Control OD}} \times 100$$

$$(\%)\,\text{Inhibition of growth} = 100 - \text{Viability}$$

The half-maximal inhibitory concentration (IC_{50}) was determined by non-linear regression of sigmoidal dose-response curves using GraphPad Prism 9.0. Each experiment was performed in three independent biological replicates.

3.4. Molecular Biology

Total RNA was extracted using RNX-plus (SinaClon, Iran) according to the manufacturer's instructions. RNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo), and RNA samples deemed suitable for downstream analysis based on the quality assessment performed at the time were selected for cDNA synthesis. The individual RNA concentration values were not retained in the laboratory records. From 1 µg of total RNA, cDNA was synthesized using the Easy™ cDNA Synthesis Kit (ParsTous, Iran).

The qPCR was performed on a Rotor-Gene Q_real-time PCR system (Qiagen, Germany) using SinaClon SYBR Green PCR Master Mix (SinaClon, Iran). Each 25 µL reaction contained 12.5 µL of Master Mix, 1 µL each of forward and reverse primer (10 pmol/µL), 3 µL of diluted cDNA (1:10), and 7.5 µL of nuclease-free water. The primer sequences used in this study are presented in Table 1. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Melting-curve analysis was performed at the end of each run (65 – 95 °C, in increments of 0.5 °C) to assess amplification specificity.

3.5. Flow Cytometry

MCF-7 cells were seeded in 6-well plates (15,000 cells/well) and treated with the IC_{50} of each extract for 72 h. Cells were harvested, fixed in 70% ice-cold ethanol overnight at 4 °C, and then treated with RNase A (100 µg/mL) for 30 min at 37 °C. DNA was stained with DAPI (300 nM) for 30 min at room temperature in the dark. Flow cytometric analysis was performed using a Partec CyFlow instrument (Partec, Germany). A minimum of 50,000 singlet events per sample was acquired. Single cells were gated based on forward scatter area versus height (FSC-A vs FSC-H) to exclude doublets and aggregates, and debris was excluded based on low forward- and side-scatter signals (FSC-A vs SSC-A). The cell cycle distribution (G1, S, and G2 phases) and the apoptotic (sub-G1) population were analyzed. The complete numerical data for each biological replicate are provided in Supplementary Table S1. The sub-G1 population was used as an estimate of DNA fragmentation-associated cell death and was interpreted as an indicator of apoptosis rather than as definitive proof of apoptosis.

3.6. Statistical Analysis

Statistical comparisons were performed using two-tailed t tests in Microsoft Excel 2013. The IC_{50} values were determined by nonlinear regression using GraphPad Prism 9.0. Results are presented as mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant.

4. Results

4.1. Evaluation of Viability and Cytotoxicity of Pomegranate Seed Extracts

The MTT assays demonstrated selective cytotoxicity of sweet and sour pomegranate seed extracts toward MCF-7 cells, with limited effects on human fibroblasts. Treatment with 25, 50, 100, and 200 µg/mL of the extracts for 24, 48, and 72 h maintained fibroblast viability above 90% under most conditions, with only minor decreases after prolonged exposure (Figure 1A,B).

In contrast, MCF-7 cells showed a substantial loss of viability following treatment with both extracts (Figure 1C,D). The response was not consistently time- or concentration-dependent. For the sweet extract, IC_{50} values

Table 1. Primer Sequences Used in This Study

Gene	Accession No.	Primer Sequence (5′ → 3′)	Product Size, bp	Annealing Temp., °C	Efficiency, %	Reference
GAPDH	NM_002046.7	F: 5′-GTCGGAGTCAACGGATT-3′; R: 5′-AAGCTTCCCGTTCTCAG-3′	186	60	100	(16)
Casp-8	NM_001080125	F: 5′-AGAAGAGGGTCATCCTGGGAGA-3′; R: 5′-TCAGGACTTCCTTCAAGGCTGC-3′	142	60	98	(17)
Bax	NM_001291429.2	F: 5′-CAGGATGCGTCCACCAAGAAG-3′; R: 5′-GCAAAGTAGAAAAGGGCGACAAC-3′	150	60	102	(18)
BCL-2	NM_000657.3	F: 5′-CATGTGTGTGGAGAGCGTCAA-3′; R: 5′-GCCGGTTCAGGTACTCAGTCA-3′	132	60	99	(19)

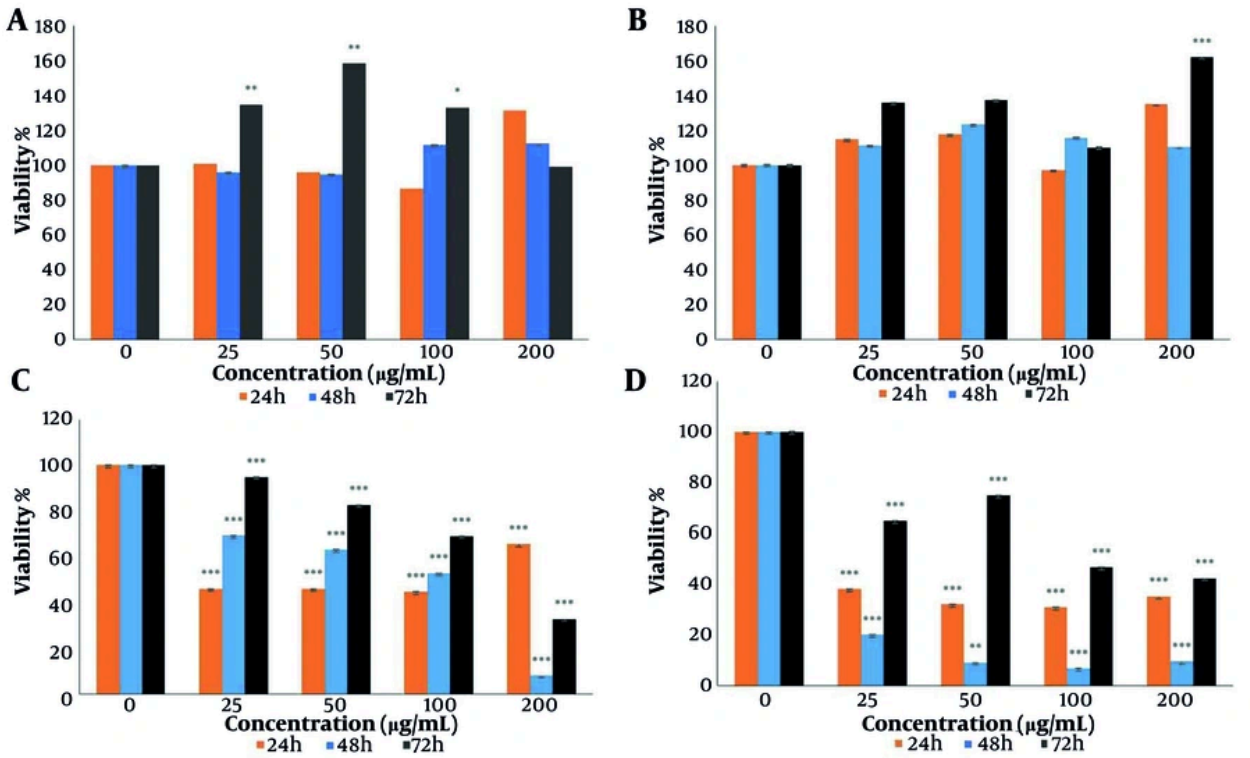


Figure 1. Viability of (A, B) fibroblast and (C, D) MCF-7 cells following treatment with sweet and sour pomegranate seed ethanolic extracts. Sweet (A) and sour (B) extracts did not significantly affect fibroblast viability. In contrast, both sweet (C) and sour (D) extracts reduced MCF-7 cell viability at 24, 48, and 72 hours across different concentrations. The reduction was statistically significant, although no consistent dose- or time-dependent pattern was observed. Statistical significance: * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ compared to untreated control cells.

were 287.3, 41.82, and 82.50 µg/mL at 24, 48, and 72 h, respectively, indicating a nonlinear temporal response. The sour extract showed greater cytotoxicity, with IC_{50} values of 7.42, 14.69, and 78.51 µg/mL at the corresponding time points (Figure 2). The strongest effect was observed at 48 h. At 200 µg/mL, both extracts markedly reduced MCF-7 viability, with the sour extract showing greater potency.

Previous work reported limited cytotoxicity of aqueous sweet and sour pomegranate seed extracts, particularly at lower concentrations (20). The greater activity observed here with ethanolic extracts may reflect more efficient extraction of lipophilic or moderately polar bioactive compounds. Therefore, subsequent mechanistic experiments focused on the ethanolic extracts. Because 50% growth inhibition was not reached in HFF-1 cells at the highest tested concentration (200 µg/mL), the selectivity index (SI) was estimated to be > 2.43 for the sweet pomegranate seed extract and > 3.57 for the sour

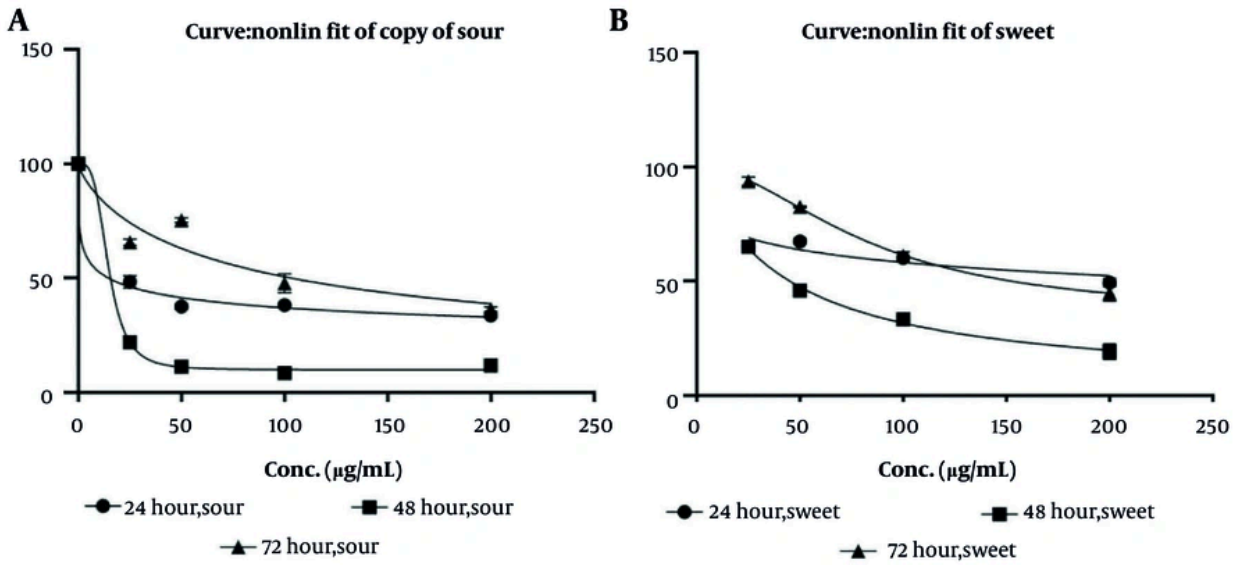


Figure 2. Time-dependent IC_{50} values of sweet and sour pomegranate seed ethanolic extracts on MCF-7 breast cancer cells. The sour extract exhibited significantly greater cytotoxicity at all time points, with the lowest IC_{50} value.

pomegranate seed extract. Direct comparison of the IC_{50} estimates showed that the sour extract had significantly lower IC_{50} values than the sweet extract at all time points ($P < 0.001$ for both comparisons).

4.2. Real-Time PCR Analysis of the Impact of Pomegranate Seed Extract on Apoptotic Genes

Real-time PCR analysis showed marked modulation of apoptosis-related genes in MCF-7 cells treated with the 72-h IC_{50} concentrations of the two extracts (Figure 3). The sour extract induced an approximately 444-fold upregulation of the pro-apoptotic gene Bax, whereas Bax expression after treatment with the sweet extract decreased to approximately 0.39-fold of the control level.

Despite this decrease, sweet extract treatment caused a much stronger suppression of the anti-apoptotic Bcl-2 gene, reducing its expression to approximately 0.005-fold of control. Consequently, the relative Bax/Bcl-2 ratio increased approximately 78-fold, indicating a strong shift toward a pro-apoptotic state. In sour extract-treated cells, Bax induction was accompanied by even greater suppression of Bcl-2 (approximately 0.002-fold of control), resulting in a pronounced pro-apoptotic shift. These reciprocal changes support the involvement of the intrinsic mitochondrial apoptotic pathway in the response to both extracts. Direct comparisons between the sweet and sour extract groups revealed significant differences in Bax ($P < 0.001$) and Bcl-2 ($P < 0.005$) expression.

Caspase-8 expression also increased, by approximately 3.33-fold and 1.80-fold following sour and sweet extract treatment, respectively, suggesting that extrinsic apoptotic signaling may also contribute to the observed cell death. Overall, the sour extract produced the strongest pro-apoptotic molecular response, characterized by marked Bax induction, Bcl-2 suppression, and increased Caspase-8 expression.

4.3. Cell Cycle Assessment via Flow Cytometry of MCF-7 Cells Treated with Pomegranate Seed Extracts

Cell cycle distribution and apoptosis were assessed by flow cytometry after 72 h of treatment with the IC_{50} of each extract. Quantitative analysis of the DNA content histograms revealed that both extracts significantly induced apoptosis and cell cycle arrest (Figure 4 and Figure 5); however, the sour pomegranate seed extract exerted a markedly stronger effect.

The sour extract resulted in 81.25% of cells in the sub-G1 population, compared with 66.84% following sweet extract treatment. It also produced pronounced G1 arrest, with 80.22% of cells in G1 and only 0.83% in G2. In comparison, sweet extract treatment resulted in 43.35% of cells in G1 and 11.51% in G2. These findings indicate that the sour extract

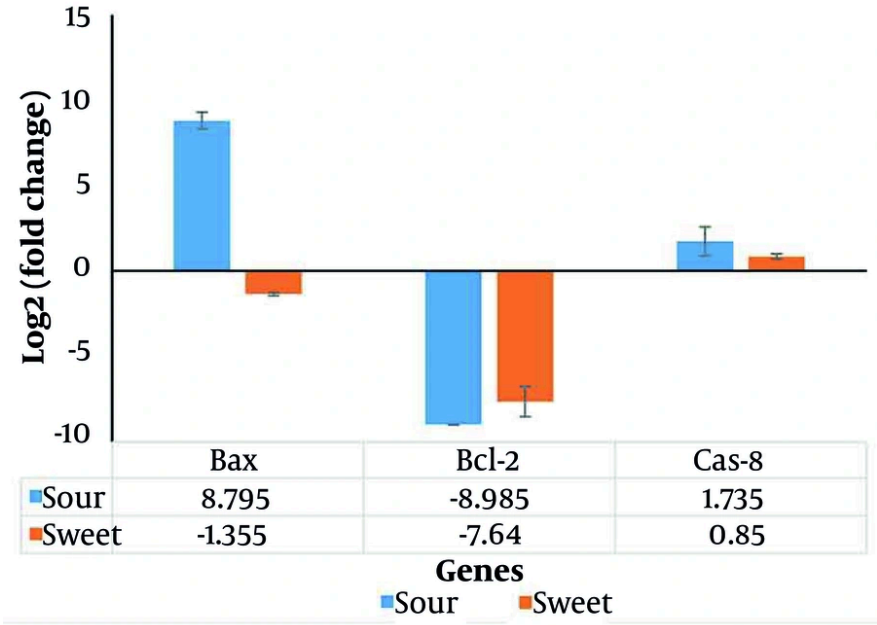


Figure 3. Expression of Apoptotic Genes in MCF-7 Cells Treated with Pomegranate Seed Extracts. Note: Gene expression was normalized to GAPDH and calculated using the $2^{(-\Delta\Delta Ct)}$ method. Data represent mean ± SD from biological replicates. Fold change is relative to the control group (set to 1).

more effectively induced both apoptotic cell death and G1-phase cell cycle arrest. Compared with the sweet extract, the sour extract induced a significantly higher G1 fraction and a lower G2 fraction ($P < 0.001$ for both comparisons), as well as a significantly lower S-phase fraction ($P < 0.005$).

Pomegranate and its bioactive compounds have demonstrated considerable anticancer potential, with mechanisms ranging from the induction of apoptosis to the modulation of critical signaling pathways (7). In addition to apoptosis, both extracts induced significant cell cycle arrest, predominantly in the Go/G1 phase, with the sour extract again causing a more pronounced effect (80.22% of cells in G1). This finding aligns with previous reports that pomegranate-derived compounds, such as ellagic acid, can inhibit proliferation by arresting breast cancer cells in the Go/G1 phase (21, 22). Although some studies have reported arrest in the G2/M phase (12, 23), the G1 arrest observed here effectively halts DNA synthesis and prevents cell division, synergizing with the apoptotic signals to inhibit cancer growth.

5. Discussion

This study directly compared the efficacy of seed extracts from two distinct pomegranate cultivars, sour and sweet, against MCF-7 breast cancer cells. Our findings indicate the superior cytotoxicity of the sour extract, as evidenced by its lower IC_{50} value (78.51 µg/mL) compared with that of the sweet extract (82.50 µg/mL) after 72 hours of treatment.

The selectivity of these extracts is a crucial attribute for the development of safe therapeutic agents. The difference in cytotoxicity between the extracts likely arises from the distinct phytochemical compositions of the cultivars. The anticancer properties of pomegranate are closely linked to its rich content of polyphenols, such as ellagitannins and anthocyanins (7). The phytochemical composition and biological activity of pomegranate extracts may vary depending on the plant part and extraction conditions (24). The concentration and specific composition of these compounds vary significantly among cultivars, with some varieties exhibiting stronger cytotoxic effects (25). It is plausible that the sour extract possesses a more potent or bioavailable profile of these key phenolic compounds, which would account for its enhanced cytotoxic activity. The relatively high IC_{50} values observed can be attributed to the use of crude ethanolic extracts, which contain a complex mixture of active and inert compounds without prior purification. Because the extracts were not subjected to phytochemical profiling, the specific constituents

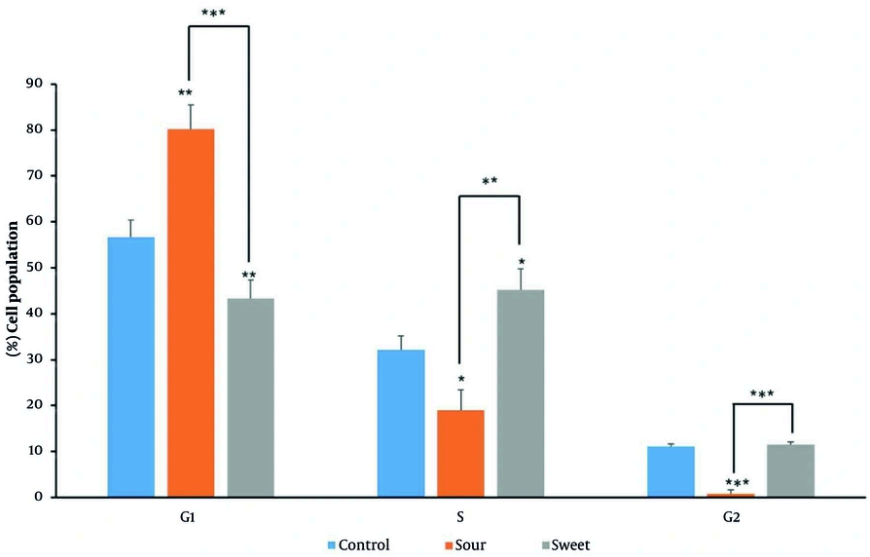


Figure 4. Percentage of Apoptosis and Cell Cycle Arrest in MCF-7 Cells Treated for 72 Hours with IC_{50} doses of ethanolic Extracts of Pomegranate Seeds. Statistical significance: * = P < 0.05, ** = P < 0.01, and *** = P < 0.001 compared to untreated control cells.

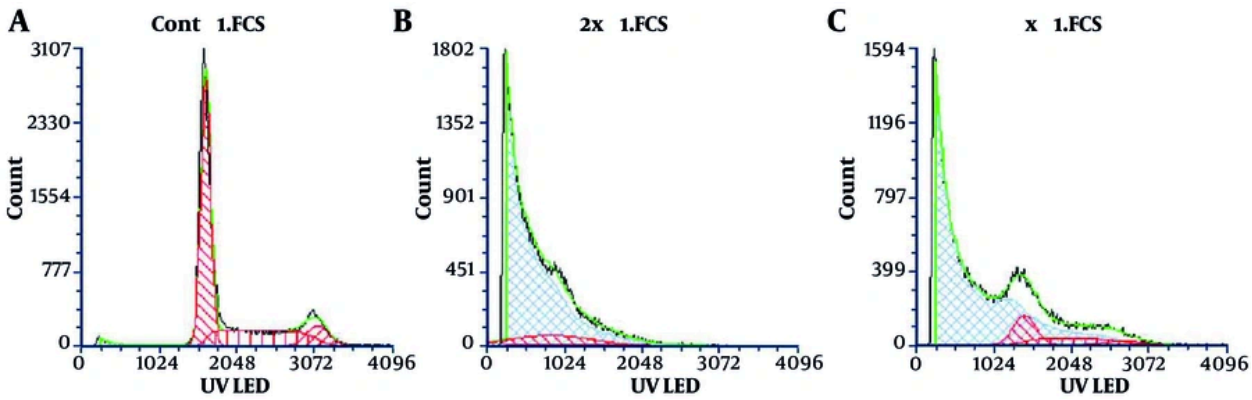


Figure 5. Flow Cytometry result depicting MCF-7 cell groups treated with control (A), ethanolic extract of sour (B) and sweet (C) pomegranate seed.

responsible for the observed biological effects could not be identified, and any role attributed to polyphenols or other compounds remains speculative. Nevertheless, both extracts exhibited marked selective cytotoxicity, significantly inhibiting MCF-7 cell viability while showing minimal toxicity toward normal human fibroblasts (20).

Collectively, the molecular and flow-cytometric findings suggest that apoptosis-related processes may contribute to the cytotoxic effects of both extracts. The marked Bax/Bcl-2 imbalance, increased Caspase-8 expression, high sub-G1 population, and G1 arrest were particularly pronounced following treatment with the sour extract. Apoptosis-associated morphological alterations have also been reported following treatment with plant-derived compounds. Eizadifard and Tafrihi (26) observed chromatin condensation, nuclear fragmentation,

and DNA degradation in A-375 melanoma cells treated with *Ferula gummosa* gum, suggesting the involvement of apoptotic cell death (26). These effects suggest that the sour extract exerts stronger anticancer activity through the combined modulation of apoptotic signaling and cell-cycle progression. However, sub-G1 accumulation alone cannot definitively distinguish apoptosis from other forms of cell death associated with DNA fragmentation.

Beyond direct cytotoxicity, pomegranate extracts may interfere with cancer-promoting pathways such as estrogen signaling. Compounds derived from pomegranate ellagitannins, such as urolithin B, have shown anti-aromatase activity, which could suppress the proliferation of estrogen-responsive breast cancer cells (27). The potential multitarget effects of pomegranate, together with its accessibility and previously reported safety profile, have led to suggestions that it may have a role as a dietary adjunct in breast cancer management (9).

One limitation of this study is the use of GAPDH as the sole reference gene without independent validation of its expression stability under the experimental conditions. The observed variation in GAPDH Ct values between the control and treated groups may have influenced the normalized gene expression estimates. Therefore, the results, particularly the approximately 444-fold increase in BAX expression in the group treated with the sour extract, should be interpreted with caution. Future studies should validate multiple reference genes to ensure reliable qPCR normalization.

Taken together, the present findings indicate that sweet and sour pomegranate seed extracts have distinct biological effects on MCF-7 cells. The sour extract demonstrated greater overall cytotoxicity and a stronger pro-apoptotic phenotype, whereas the sweet extract also induced a marked pro-apoptotic shift, primarily through suppression of Bcl-2. These differences may reflect cultivar-dependent variation in phytochemical composition rather than differences in potency alone. Because crude extracts were used, the specific compounds responsible for the observed effects could not be identified. Although the extracts showed differential cytotoxic effects in vitro, their therapeutic relevance cannot be established from the present study. Their in vivo bioavailability, pharmacokinetic behavior, systemic toxicity, and achievable tissue concentrations remain unknown and require further investigation before their potential therapeutic application can be considered.

Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

AI Use Disclosure: The authors declare that no generative AI tools were used in the creation of this article.

Authors' Contribution: S. T. performed the experiments, analyzed the data, conducted the statistical analysis, and contributed to writing the manuscript. F. H. and S. H. H. supervised the study and conceived and designed the experiments. S. H. H. analyzed and interpreted the data and results. F. H. wrote and revised the manuscript. N. M. performed the data analysis and contributed to the critical revision and editing of the manuscript. All authors critically reviewed the manuscript, approved the final version for publication, and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interests Statement: The authors declare no conflict of interests.

Data Availability: The dataset presented in this study is available from the corresponding author upon request, either during submission or after publication.

Funding/Support: The authors declare no funding/support.

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