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Armeniacae Semen Regulates Apoptosis and Cell Cycle Progression in MCF-7, MDA-MB-231, and T47D Breast Cancer Cell Lines

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Abstract

Background: Amygdalin is a glycoside ingredient of rosacea plants that exerts an antitumor effect by blocking the growth of the tumor cells. Therefore, we aimed to use the Armeniacae semen, a member of the Rosacea family, which contains a large amount of the amygdalin, to evaluate its antiproliferative effect on MCF-7, MDA-MB-231, and T47D breast cancer cell lines.

Method: In this experimental study, we prepared the aqueous, ethyl acetate, and hydro-alcoholic extracts of the Armeniacae semen. The MCF-7, MDA-MB-231, and T47D cell lines were treated with different doses of the extracts for 12, 24, 36, and 48 hours; cell viability was investigated with MTT test and cell apoptosis was detected by use of double staining fluorescent. Cell cycle progression was analyzed using a BD Cycle TEST PLUS DNA Kit. We also assessed Bcl2, Bax, and caspase-3 mRNA expression.

Results: The best IC_{50}s belonged to hydro-alcoholic extract of the Armeniacae semen in all three cell lines for the 48-hour treatment. We observed a significant increase in Bax and caspase-3 mRNA expression and a noticeable reduction in Bcl2 mRNA compared with the controls. Application of amygdalin to MCF-7, MDA-MB-231, and T47D cell lines increased the number of G0/G1 cells and reduced the number of cells at G2/M phasecompared to the controls.

Conclusion: This study showed that the hydro-alcoholic extract of Prunus armeniaca had antitumor effects on breast cancer cell lines as it inhibited the cell cycle at G0/G1 phase and apoptosis induction in the MCF-7, MDA-MB-231, and T47D cell lines.

Keywords: Armeniacae semen, Breast neoplasms, Cell cycle, Caspase 3, Proto-oncogene proteins c-bcl-2, Bax

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Introduction

Today, breast cancer is the most common cancer among women. It is among the most deadly diseases in this stratum.[1] According to the most recent data from the National Cancer Institute, 61% of breast cancers are diagnosed prior to metastasis, 31% are detected after they have spread to extra-breast lymph nodes, and 68% are diagnosed following lymph node metastasis.[2] This heterogeneous disease mainly spreads to the bone and is almost always associated with bone breakdown.[3] Histologically, ductal carcinoma and lobular carcinoma are the two most prevalent types of breast cancer. According to the recent data, the latter is growing more than the former.[4] Cancer is usually associated with death and its incidence is increasing worldwide; thus, one of the major challenges for researchers is to find an efficient and low-cost approach to treatment.[5] Medicinal plants have always been important sources for discovering new therapeutic agents for human diseases.[6] New compounds do not entail the problems of anticancer drugs on the market. The most common issues associated with these drugs are drug resistance and side-effects such as hair loss, anorexia, and nausea (characteristic of cytotoxic drugs).[7] Rosaceous family is one member of these medicinal plants with an important ingredient called amygdalin.[8] Many studies have revealed the antitumor features of this compound. Amygdalin regulated apoptosis and adhesion in Hs578T triple-negative breast cancer cells;[9] it also induced apoptosis in bladder cancer cells.[10] Armeniacae semen down-regulated special genes involved in the cell cycle in the colon cancer cell line;[11] Persicae semen extract induced apoptosis in human promyelocytic leukemia (HL-60) cells.[12] Furthermore, amygdalin from apricot kernels affected bladder cancer cell adhesion and invasion in vitro.[13] In this connection, our objective was to evaluate the antiproliferative and apoptotic effects of Armeniacae semen which includes large amounts of amygdalin on MCF-7, MDA-MB-231, and T47D cell lines; we also aimed to investigate the cell cycle progression and caspase-3, Bcl2, and Bax gene expression under the influence of amygdalin in order to compensate for the lack of coherent studies in this field.

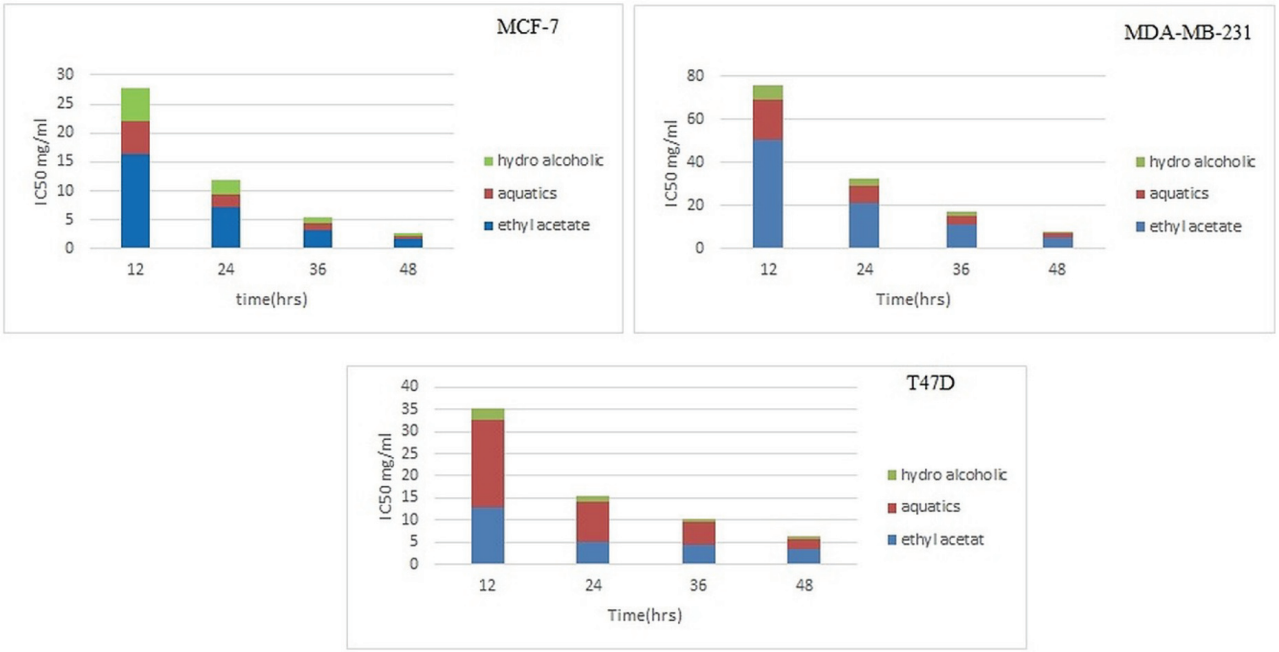

Figure 1. Aqueous, hydro-alcoholic, and ethyl acetate extract IC50s for MCF-7, MDA-MB-231, and T47D cell lines at 12, 24, 36, and 48 hours, showed that the best IC50s belonged to the hydroalcoholic extract of the Armeniacae semen in all three cell lines with 48-h treatment.

Materials and Methods

Cell culture

This experimental study was a test-tube lab research on breast cancer cell lines. MCF-7, MDA-MB-231, and T47D cell lines were purchased from the Pastor Institute of Iran. The cells were cultured in RPMI1640 containing 20mM HEPES-buffer and glutamax1% (Biosera, France) supplemented with 10% heat-inactivated FBS (fetal bovine serum) (Gibco, USA) and 100µg/mL penicillin/streptomycin (Biosera). The cultures were incubated at 37°C with 5% CO_2 and 95% humidity. The medium was changed every 2-3 days. Following appropriate growth, trypsin 25% was used to remove the cells from the flask and transfer them to plates.

Preparation of extracts

We hatched 200 grams of the Armeniacae semen from the shell and dried it in the shade for a week. The seeds were then crushed, and dry powder was macerated in a petroleum ether-solvent to remove oils. After oil extraction, the seed powder was allowed to completely dry and the solvent to evaporate. To prepare an aqueous extract, 50 g of the powder was weighed and macerated in 90 °C water for 30-45 minutes. The rest of the powder was utilized to provide ethyl acetate and hydro-alcoholic extract; 500 mL of ethyl acetate was added to the powder; after 48 hours, the solvent was removed and a new ethyl acetate solvent was added again. The procedure was repeated three times. The above processes were also repeated for 60% methanol. The extracts from each step were filtered and transferred to a rotary balloon (Heidolph, Germany) and concentrated at 100 rpm at 40 °C.

MTT assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay was used to check cell viability. The cells were seeded at a concentration of 1×10^4 cells/well in a 96-well plate. All three cell lines were treated with aqueous, methanol, and ethyl acetate extracts of the Armeniacae semen at 0.125, 0.25, 0.5, 1 mg/mL concentrations for 12, 24, 36, and 48 h. Following 4h incubation with 50µl MTT (Sigma, USA), the

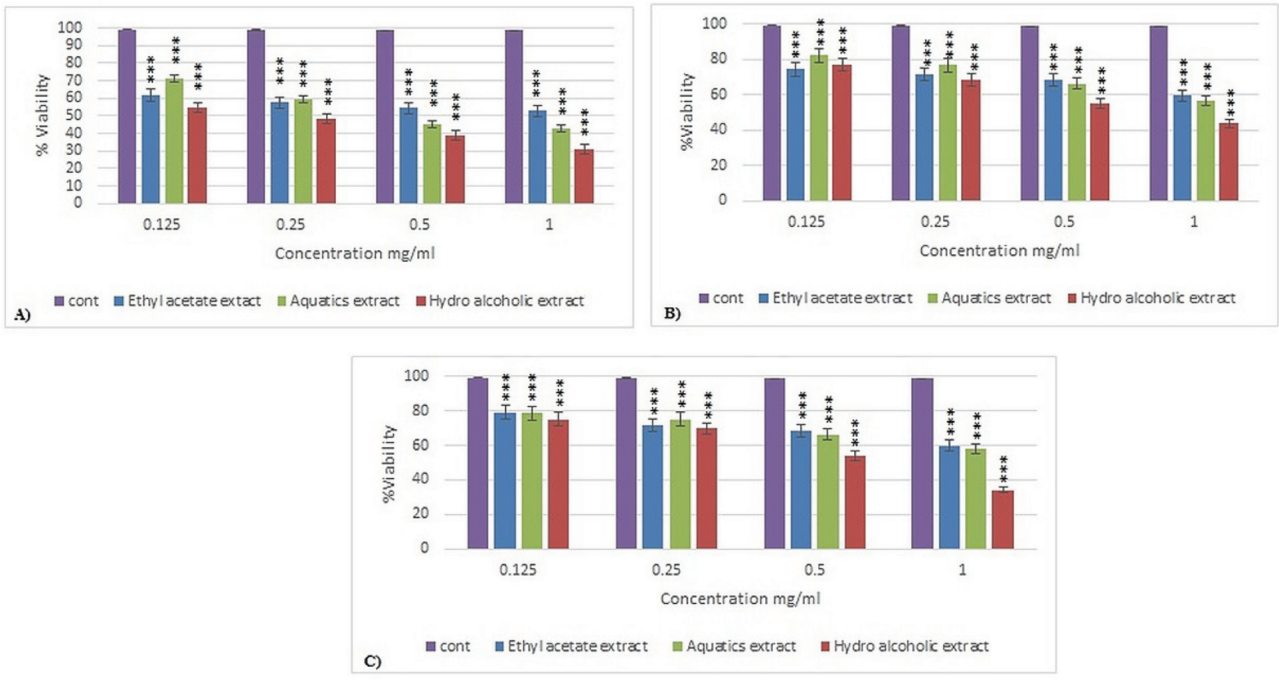

Figure 2. Effect of aqueous, methanol, and ethyl acetate extracts of the Armeniacae semen on the viability of Mcf-7, MDA-MB-231, and T47D cells treated with different concentrations of extracts for 48 h (the optimum time). After adding 50µl of MTT-labeling reagent, the cells were incubated for 4 h, before 100 µl DMSO solution was added. The experiments were repeated at least three times. The data represent the mean (± standard deviation, SD) of the three independent experiments, each performed in triplicate and presented relative to the controls. (A). Mcf-7 (B). MDA-MB-231. (C). T47D.

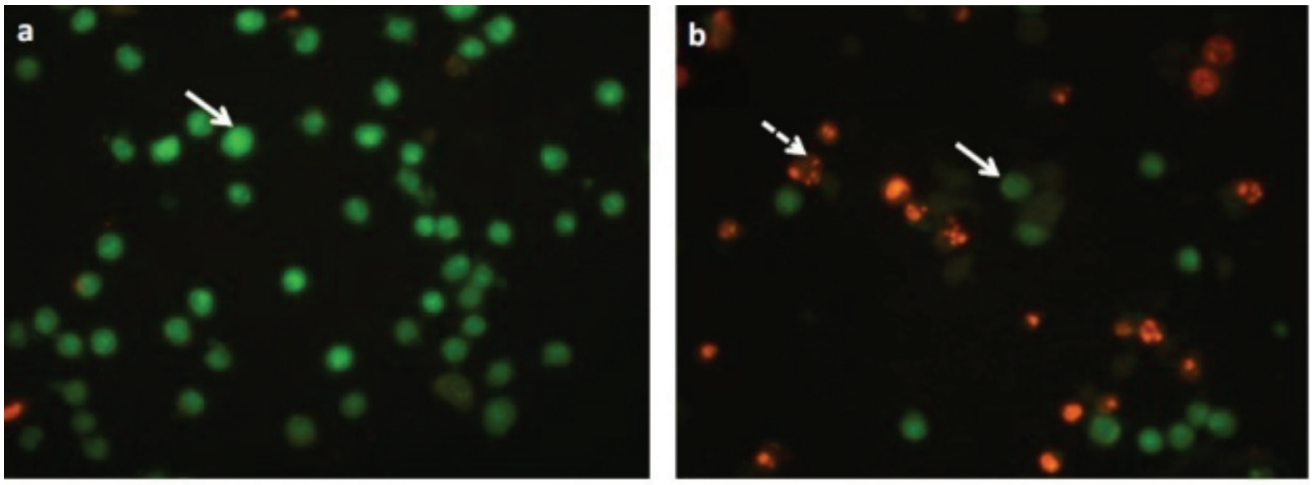

Figure 3. Photo taken from MCF-7 cells by fluorescent microscope showed that all the cells were green (alive and healthy) in the control group. In other groups treated with hydro-alcoholic extract, the cells were orange-red, hence apoptotic.

supernatant was removed and 100µl DMSO (Sigma-Aldrich, USA, Biologic Grade) was added to dissolve MTT. After 15 minutes of incubation, the OD was read with an ELISA plate reader (BioTek ELx808, USA) at 492 nm. The assay was performed at least three times. Viability percentages of each cell line and IC_{50}s of all extracts were calculated using the Excel 2013 software. The optimum time and the best IC_{50}s were considered for further analysis.

Double staining fluorescent and analysis with fluorescent microscope

Fluorescent microscopy and ethidium bromide / acridine orange staining is among the methods for detecting cell apoptosis based on cell morphology.

To perform this test, the cells were grown at a concentration of 3×10^5 cells/ well on a six-well plate. After 24 h, the cells were treated with hydro-alcoholic extract containing the best IC_{50} (each cell line was treated with IC_{50} doses of their own hydroalcoholic extract). A well was left empty as control. The cells were incubated for 48h (the optimum time specified with MTT assay). After incubation, the cells were trypsinized, and the suspension was transferred into a falcon. 4 µL of two ethidium bromide / acridine orange dyes was added to cell precipitate at a concentration of 1 mg / mL and analyzed via fluorescent microscopy.

Cell cycle analysis

For cell cycle analysis, tumor cell populations, after being exposed to the extract, were stained

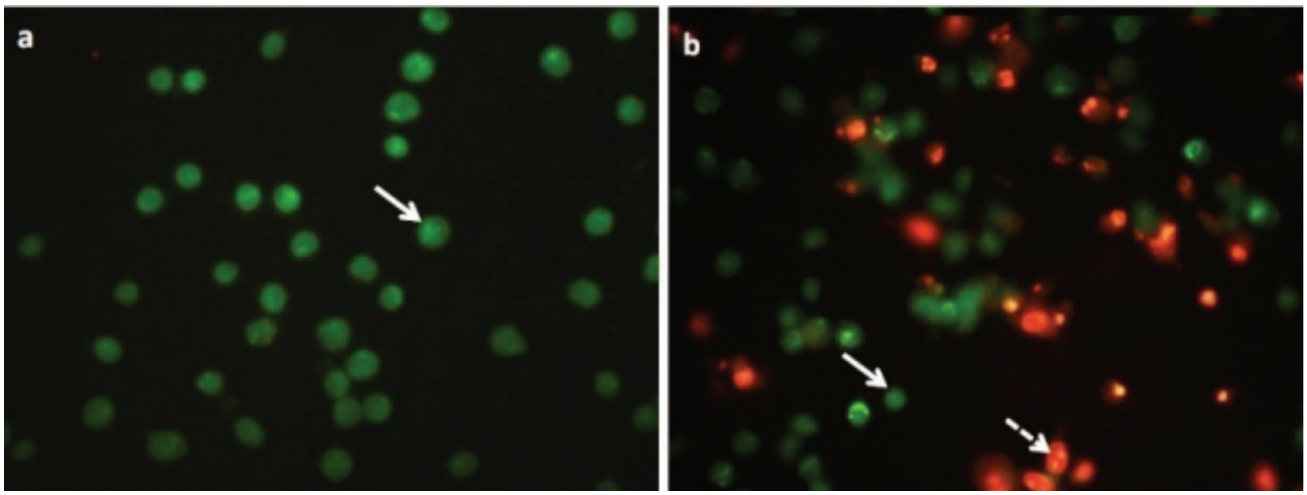

Figure 4. Photo taken from MDA-MB231 cells by fluorescent microscope showed that all the cells were green (alive and healthy) in the control group. In other groups treated with hydro-alcoholic extract, the cells were orange-red, hence apoptotic.

with propidium iodide by use of BD Cycle TEST PLUS DNA Kit (BD Biosciences); flow cytometry was then performed with a BD FACS Calibur Flow Cytometry Machine (BD Biosciences_ USA). Data were collected using BD Cell FIT software, and cell cycle progression was analyzed using the ModFit software (BD Biosciences). The number of cells gated in each phase was reported as %.

RT-PCR

RT-PCR was conducted to identify Bcl2, Bax, and caspase-3 mRNA expression. The cells were collected after 48 hours of treatment with the extract that had the best IC_{50} on the MTT test; moreover, total RNA was extracted through the use of Trizol (Qiagen, Germany) method. Afterwards, according to kit manufacturer's instructions, 1µg total RNA was converted to cDNA using the PrimeScript 1st strand cDNA Synthesis kit (Takara, Japan). Using the primers in table 1, RQ-PCR assay was carried out to investigate the level of Bcl2, Bax, and caspase-3 mRNA expressions in drug-treated and control groups. B2-microglobulin (B2M) was employed as a house-keeping gene for the normalization of RT-qPCR data. All tests were performed in triplicate. The fold change of Bcl2, Bax, and caspase-3 mRNA in treated cells relative to untreated cells was computed by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All experiments were performed in duplicate and repeated three times. The data were expressed as mean±SD for all experiments. IC_{50} was calculated using the Excel 2013 software and cell cycle analysis was done using the BD Cell FIT software. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used to detect the significant differences between the control and treated groups. Statistical significance were defined at $*P<0.05$, $**P<0.01$, and $***P<0.001$ compared to the corresponding controls.

Results

Hydro-alcoholic extract had the best IC_{50} on the MTT test. Based on the cell treatment with the aqueous, methanol (Hydro-alcoholic), and ethyl acetate extracts of the Armeniacae semen at of 0.125, 0.25, 0.5, 1 mg/mL concentrations for 12, 24, 36, and 48h, the best IC_{50}s belonged to the hydro-alcoholic extract of the Armeniacae semen in all three cell lines with 48-h treatment. $IC5_0$s of the aqueous extract were 0.491±2.65, 1.794±3.32, and 2.402±3.41 mg/mL, IC_{50}s of hydro-alcoholic extract were 0.198±2.04, 0.693±1.17, and 0.532±1.27 mg/mL, and IC_{50}s of ethyl acetate extract were 1.709±1.96, 5.179±2.31, and 3.410±2.74 mg/mL for MCF-7, MDA-MB-231, and T47D cell lines in 48 hours,

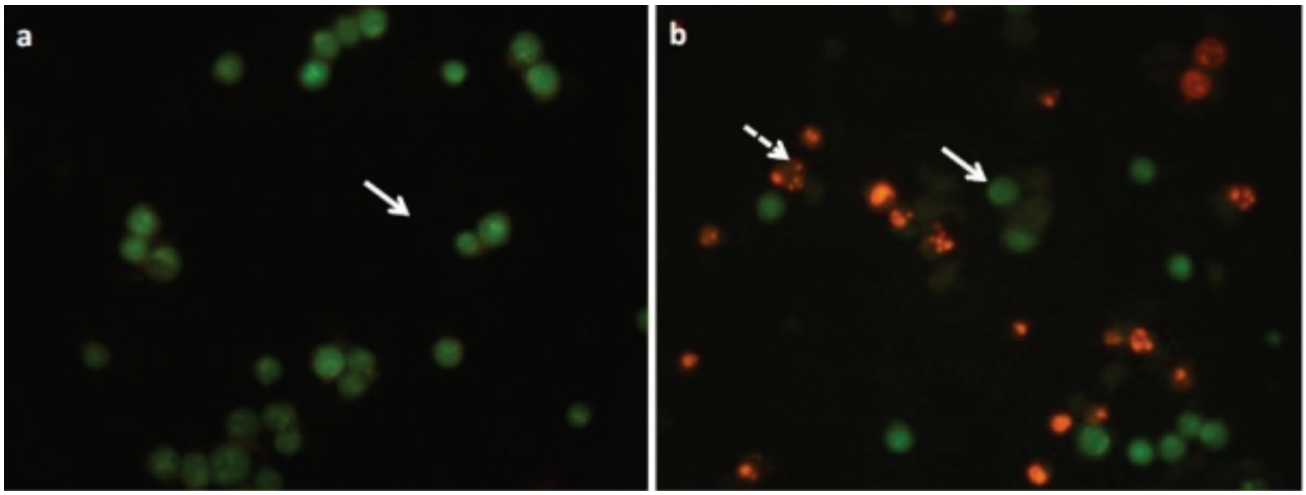

Figure 5. Photo taken from T47-D cells by fluorescent microscope showed that all the cells were green (alive and healthy) in the control group. In other groups treated with hydro-alcoholic extract, the cells were orange-red, hence apoptotic.

Note: Investigation of morphological changes induced by apoptosis in breast cancer cell lines by IC_{50} dose of extract. (a) Control cells (b) cells treated with hydro-alcoholic extract. Apoptosis was assessed by acridine orange-ethidium bromide staining by fluorescent microscopy after 48 h of incubation with 40× magnification. White arrows indicate living cells, and dash arrows indicate late apoptosis.

respectively (Figure1). It was shown that cell proliferation was inhibited in a dose-dependent manner with all extracts (Figure 2).

Analysis of apoptosis by double staining fluorescent in MCF-7, MDA-MB-231, and T47D cell lines with ethidium bromide / acridine orange staining and analysis with fluorescent microscope

Fluorescent microscopy studies in the presence of control and hydro-alcoholic extract at IC_{50} concentration (each cell line was treated with the IC_{50} concentration of its own hydro-alcoholic extract) showed that all the cells were green (alive and healthy) in the control group. Acridine orange is absorbed by living cells and binds to the DNA of the cells, so chromatins are seen in green under a fluorescent microscope. In other groups treated with hydro-alcoholic extract, the cells were orange-red, hence apoptotic. Ethidium bromide is only absorbed by the dead cells, binds to the DNA of the cells, and indicates them by red-orange. This test was performed to confirm the presence of apoptosis on all three breast cancer cell lines (Figures 3-5).

Cell cycle progression

Amygdalin modulated cell cycle progression is dependent on the tumor cell line. Application of hydro-alcoholic extract to MCF-7 increased the number of G0/G1 (P=0.0148) cells and reduced the number of G2/M cells (P=0.0116). In MDA-MB-231 cells, the number of G0/G1-phase cells increased (P=0.0127), but the number of cells in the G2/M-phase decreased (P=0.0364). In T47D cells, amygdalin exposure caused G2/M-phase reduction (P=0.0014) and an S-phase increase (P=0.0132) compared with the controls (Figure 6).

Effect of the hydro-alcoholic extract of Armeniacae semen on Bcl2, Bax, and caspase3 gene expression

Molecular examination, 48 hours after the treatment of MCF-7, MDA-MB-231, and T47D cell lines, showed a significant increase in Bax and caspase-3 mRNA expression and a noticeable reduction in Bcl2 mRNA in test samples compared with the controls (Figures 7-9).

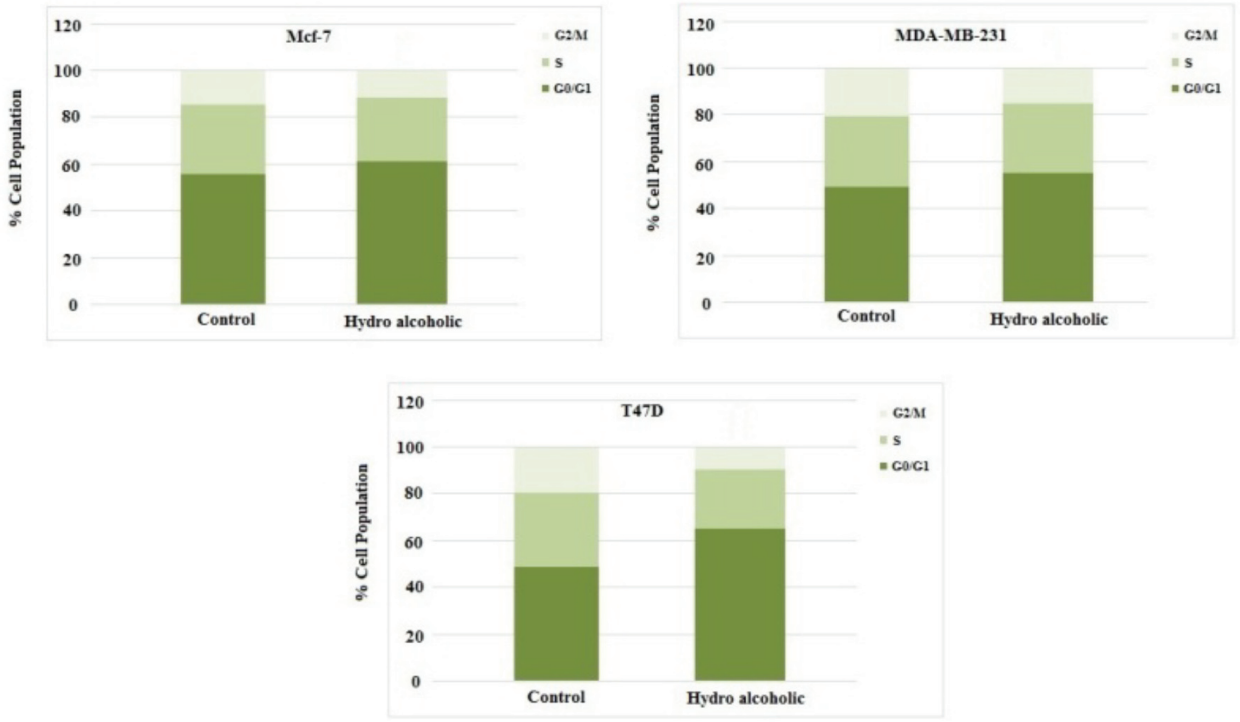

Figure 6. Cell cycle analysis of Mcf-7, T47D, and MDA-MB-231 cultures pretreated with amygdalin for 48 h (controls remained untreated). The cell population is expressed as percentage of the total cells analyzed. One representative experiment of three is shown.

Table 1. Bcl2, Bax, caspase-3, and B-2 microglobulin primers

Gene	Primer(5′-3′)	PCR product size (bp)
Casp3 (Forward)	TCTGGTTTTCGGTGGGTGTG	137
Casp3 (Reverse)	CGCTTCCATGTATGATCTTTGGTTC	
B2M (Forward)	CTCCGTGGCCTTAGCTGTG	69
B2M (Reverse)	TTTGGAGTACGCTGGATAGCCT	
Bcl2 (Forward)	CTGCACCTGACGCCCTTCACC	119
Bcl2 (Reverse)	CACATGACCCCACCGAACTCAAAGA	
Bax (Forward)	GTGCACCAAGGTGCCGGAAC	205
Bax (Reverse)	TCAGCCCATCTTCTTCCAGA	

Discussion

In the present study, it was found that, the cell viability in all three cell lines of MCF-7, MDA-MB-231, and T47D was dependent on dose and cell type; tumor cell survival decreased with the increase in the extract dose, particularly in the hydro-alcoholic extract. In the cell lines treated with the hydro-alcoholic extract of Armeniacae semen, analysis of apoptosis by double staining fluorescent confirmed the presence of apoptosis on all three breast cancer cell lines. Amygdalin modulated cell cycle progression, depending on the tumor cell line, augmented the number of G0/G1 cells and reduced the number of G2/M cells. Molecular examination showed a significant increase in caspase-3 and Bax and a decrease in Bcl2 genes expression compared with the controls 48 hours after the treatment of MCF-7, MDA-MB-231, and T47D cell lines.

Today the pharmacological activities of herbal

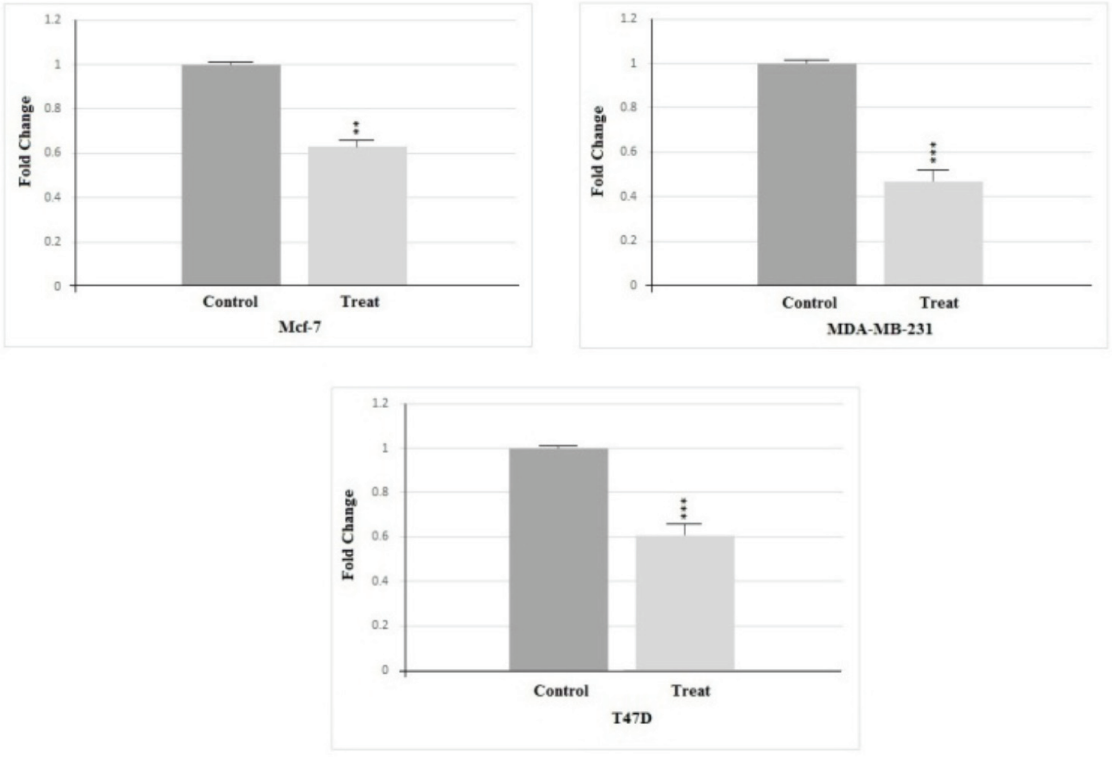

Figure 7. RT-PCR analysis results of the mRNA Levels of Bcl-2. A noticeable decrease was detected in Bcl2 mRNA in test samples compared with the control group. $P<0.01$ in MCF-7, $P<0.001$ in MDA-MB-231, and $P<0.001$ in T47D cell lines, respectively.

compounds (as cancer inhibitors) have raised the interest of the scientific community.[14] Many anticancer plants are known to have therapeutic effects via inducing apoptosis and enhancing the immune system.[15] Many studies have shown the antiproliferative effects of rosaceous family which contains a large amount of amygdalin.[8]

In 2016, Hye Min Lee and Aree Moon revealed that amygdalin regulated apoptosis and adhesion in Hs578T triple-negative breast cancer cells.[9] Makarevic J et al. (2014) showed that amygdalin induced apoptosis in bladder cancer cells.[10] In addition, in 2005, Hae-Jeong Park et al. reported that Armeniacae semen down-regulated special genes involved in the cell cycle in the colon cancer cell lines.[11] In 2003, it was observed that Persicae semen extract inducedd apoptosis in human promyelocytic leukemia (HL-60) cells.[12]

In the current study, according to MTT assay results, tumor cell survival decreased with the increase in the extract dose, especially in the hydro-alcoholic extract. Park HJ et al. (2005) reported that the extract from the amygdalin had a dose-dependent cytotoxic effect on human colon cancer (SNU-C4).[11] They also showed that the treatment of human chronic myeloid leukemia (K562 cell line) with amygdalin at different concentrations resulted in a reduction in cell viability in a dose-dependent manner.[16] These findings confirm that amygdalin has antiproliferative properties.

In our study, fluorescent analysis of apoptosis in MCF-7, MDA-MB-231, and T47D cell lines indicated the presence of apoptosis. Amygdalin increased the number of G0/G1 cells and reduced the number of G2/M cells in all three breast cancer cell lines. Hyun-Kyung Chang et al. (2005) revealed that amygdalin induced apoptosis in prostate cancer cells.[8] Makarevic´ J, et al. (2014), using the Annexin-V-FITC/PI kit, assessed the

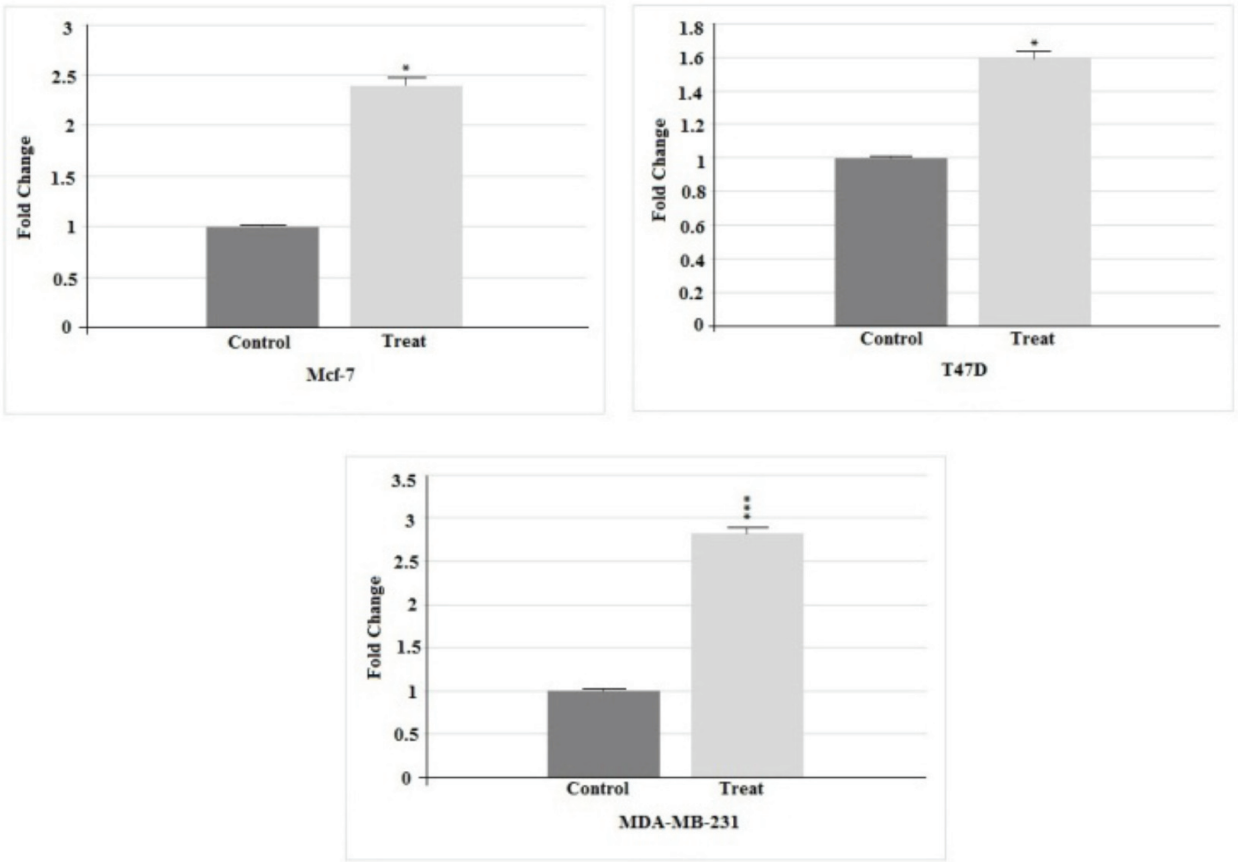

Figure 8. RT-PCR analysis results of the mRNA levels of Bax. Results showed a significant increase in Bax mRNA expression. $P<0.05$ in MCF-7, $P<0.05$ in T47D, and $P<0.001$ in MDA-MB-231 cell lines, respectively.

treatment of UMUC-3, TCCSUP, and RT-112 bladder cancer cell lines with amygdalin concentrations of 1, 10, and 25 mg/mL. They showed a dose-dependent relationship between amygdalin concentration and Annexin V positive cells. Furthermore, amygdalin modulated cell cycle progression depending on the tumor cell line.[10] Hee-Young Kwon et al. (2003) showed that Persicae semen extract induced apoptosis in human promyelocytic leukemia (HL-60) cells.[12] Therefore, it can be concluded that amygdalin has the ability to induce apoptosis and regulated the cell cycle progression in cancer cell lines.

In the present study, the mRNA expression level of caspase-3, Bax, and Bcl2 genes was assessed to specify the effect of amygdalin extract on the induction of cell death. Results showed a significant increase in caspase-3 and Bax and a decrease in Bcl2 genes expression compared with the control group. Similarly, Hyun-Kyung Chang et al. determined that amygdalin induced apoptosis through regulating Bax and Bcl2 expressions in human DU145 and LNCaP prostate cancer cells; they also showed that amygdalin significantly increased caspase-3 activity in both cell lines.[8] Lee et al. reported that amygdalin induced apoptosis in Hs578T breast cancer cells through the caspase-3 pathway.[9] In 2013, Yu Chen et al. showed that in amygdalin-treated HeLa cells, the expression of Bax protein increased and that of Bcl2 protein decreased in a dose-dependent manner. As a result, amygdalin regulates apoptosis by increasing the levels of proapoptotic proteins and reducing the levels of antiapoptotic ones.

In previous studies on breast cancer, researchers used commercial amygdalin; meanwhile, in the present study, we used Armeniacae semen extract which contains amygdalin. Others only treated the cells for 24 hours with amygdalin, whereas 24, 36, and 48 hours were considered for the

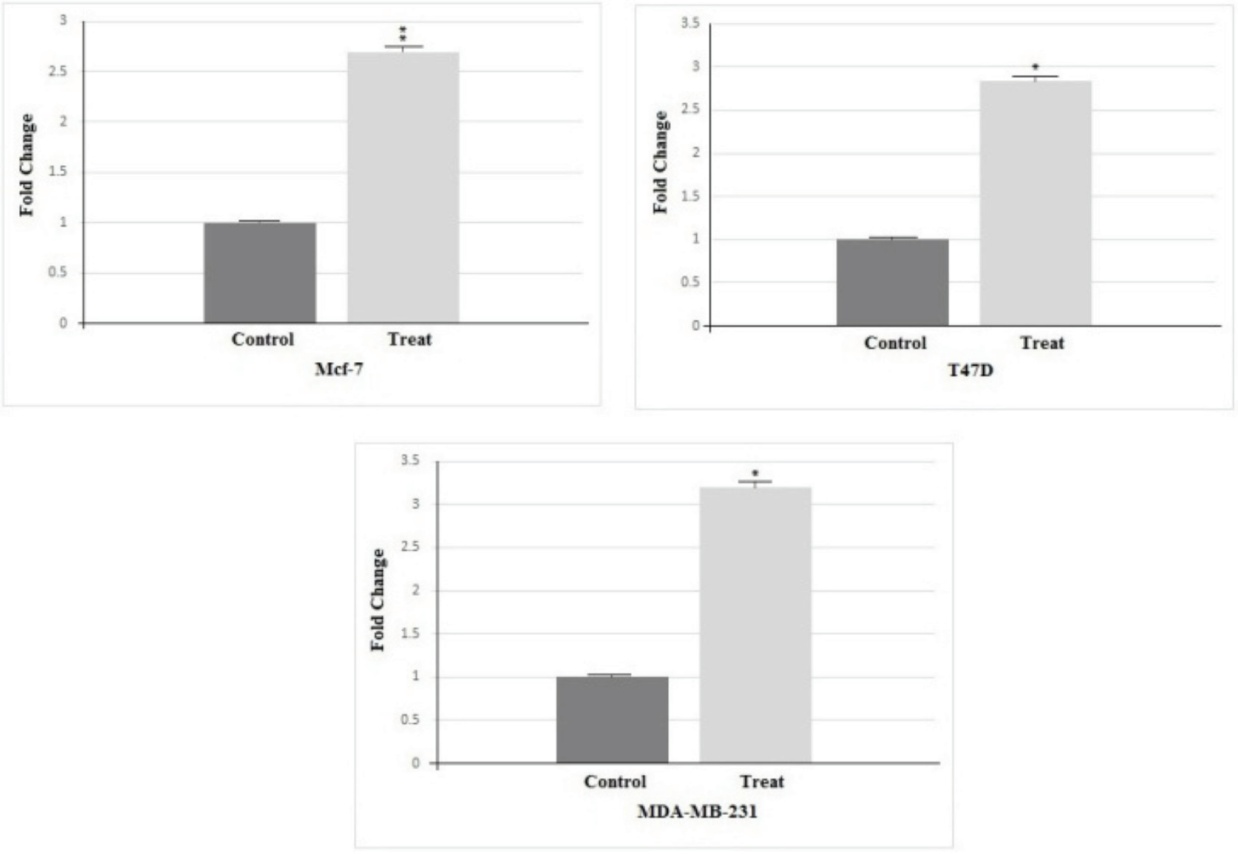

Figure 9. RT-PCR analysis results of the mRNA Levels of caspase3. A significant increase was observed in caspase3 mRNA expression. $P<0.01$ in MCF-7, $P<0.05$ in MDA-MB-231, and $P<0.05$ in T47D cell lines, respectively.

treatment in the present research. In those studies, the levels of Bcl2, Bax, and procaspase-3 were measured using immunoblot; while, we employed PCR for this purpose. Adhesion assay was performed (in those studies) to investigate the effect of amygdalin on adhesive phenotype of Hs578T cells, which we refused to do because that phenotype was not the focus of the work.

Conclusion

The present in vitro study determined that the hydro-alcoholic extract of Armeniacae semen had antiproliferative effects on MCF-7, MDA-MB-231, and T47D cell lines via inducing apoptosis; this was done through increasing the levels of proapoptotic proteins, decreasing antiapoptotic proteins, and regulating cell cycle progression in cancer cell lines. However, future studies should focus on the isolation of the effective ingredients of the hydro-alcoholic extract and evaluate their apoptotic molecular mechanisms in animal and human cancer cell lines.

Acknowledgments

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Conflicts of Interest

None declared.

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