World Cancer Research Journal WCRJ 2020; 7: e1458

THE HYDROALCOHOLIC EXTRACT OF BANEH LEAVES (PISTACIA ATLANTICA) INDUCES APOPTOSIS IN THE BREAST CANCER CELLS

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Abstract – *Objective:* *Breast cancer is one of the most prevalent cancers among women, which has widespread in recent years in Iran. Wild Pistachio* (Pistacia Atlantica)*, known as Baneh in Iran, is a medicinal plant. The present study aimed to test the anti-tumor properties of the hydro-alcoholic extract of Baneh leaves in MCF-7 breast cancerous cells.*

Materials and Methods: *The MTT assay, morphologic analysis, and DNA fragmentation assay were conducted to evaluate the inhibitory effects of the Baneh leaves hydro-alcoholic extract on the proliferation of cancerous cells (MCF-7) and normal ones (L929). The mRNA expression levels of some apoptotic genes, including p53, caspase-8, caspase-3, bax, and bcl-2 were measured by real-time PCR.*

Results: *Data analysis demonstrated that the IC50 value for MCF-7 and L929 cells after 48-hours treatment with extract was 250 μg/mL and 400 μg/mL, respectively. The morphologic analysis and DNA fragmentation assay confirmed the occurrence of apoptosis in both L929 and MCF-7 cells after treatment with the Baneh extract at IC50 concentration. It gives the idea that up-regulation of caspase-8, caspase-3, p53, and bax genes decrease in the expression of bcl-2 gene, showing that the Baneh extract induces apoptosis through both intrinsic and extrinsic pathways of programmed cell death in MCF-7 cells.*

Conclusions: *Breast cancer cells are more sensitive to the treatment with the Baneh extract compared to normal cells, making this extract a promising candidate to be used in the preparation of anti-cancer drugs against breast cancer cells with fewer side effects on healthy cells.*

KEYWORDS: *Apoptosis, Baneh, Breast cancer, Pistacia atlantica, MCF-7 cells.*

LIST OF ABBREVIATIONS: *ANOVA: Analysis of variance, DMSO: Dimethyl sulfoxide, ELISA: Enzyme-linked immunosorbent assay, FBS: Fetal bovine serum, IC50: Inhibiting cell growth by 50%, MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, SPSS: Statistical package for the social sciences.*

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INTRODUCTION

Cancer is a malignant neoplasm that occurs as a result of the growth and proliferation of uncontrolled cells. These cells may be replicated by the circulatory and lymphatic system to other parts of the body[1]. The prevalence of cancer in the world, including Iran, is on the rise and known as the second category of non-transmissible diseases, as well as the third cause of human mortality[2]. Breast cancer has been reported as one of the most common cancers worldwide [3] and it has been also considered as one of the most prevalent cancers among Iranian women [4]. Cellular resistance to anticancer drugs is a major limitation, which can inherent or be developed during chemotherapy. Today, due to complications of chemotherapy, medicinal plants have been considered by researchers as a promising future drug/medicine for cancer management [5,6].

Wild Pistachio (*Pistacia Atlantica*), known as Baneh in Iran, from Anacardiaceae with different sub-spices, including cabulica, kurdica, and mutica, is used for medicinal purposes. Many investigations on its leaves, gum, oil, essential oil, and fruit juice have been performed showing the antimicrobial, antioxidant, anti-inflammatory, and anti-cancer properties of pistachio[7-11]. In addition, the aqueous and alcoholic extraction of pistachios decreases blood glucose levels and synthesizes liver triglycerides [12,13]. The effective roles of *Pistacia Atlantica sub-spices* kurdica in the destruction of breast cancer cells (T47D) have been reported previously. Studies on the SW742 cell line have shown that Baneh gum has a positive effect on the progression of apoptosis in these cancer cells [13]. The phytochemical evaluations suggest that Baneh has a considerable number of polyphenolic compounds, including flavonoids and anthocyanins. It has been proved that flavonoids in the diet prevent the proliferation of various cancer cells and the growth of tumors in animal studies. They can inhibit various cells in different stages of the cell cycle in several ways [14]. Therefore, researchers attempt to discover new therapeutic approaches for cancer treatment with the minimum side effects. Accordingly, different cell lines for examinations have been introduced, including Breast Cancer Cell Line (MCF-7) that can be used for therapeutic and research purposes. It has also been documented that bcl-2, bax, p53, and caspase-8 are important genes participating in the induction of apoptosis in the human cell systems. Thus, the first aim of the present study was the investigation of anti-cancer properties in MCF-7 breast carcinogenic cells and the second one was to explore the expression rates of bcl-2, bax, p53, and caspase-8 in the MCF-7 and L929 cells in IC50 concentration of the hydro-alcoholic extract of Baneh leaf.

MATERIALS AND METHODS

Plant Preparation

The fruits and leaves of Baneh were prepared in the late summer. After confirmation of the genus and species of this plant by a botanist at the Vali-e-Asr University of Rafsanjan, leaves were dried in the shade.

Preparation of Baneh leaves hydro-alcoholic extract

The fresh leaves were washed, dried in the shade, and powdered. Then, 100 g of Baneh leaves were mixed with 400 mL of 75% ethanol and incubated at room temperature for 48 hours. Later, the mixture was filtered and concentrated on the rotary evaporator under reduced pressure to be dried completely. Finally, the crude extract was kept at -20°C for further investigations.

Cell Lines

Human breast cancer cell line MCF-7, as well as L929 cell lines, were prepared from Pasteur Institute (Tehran, Iran).

Cell culture condition

The L929 and MCF-7 cell lines were cultured in the RPMI medium containing 10 mg/L streptomycin, 100 IU/mL penicillin, and 10% of fetal bovine serum (FBS) under sterile conditions in the 5% CO_2 incubator at 37°C (Memmert, Germany). The culture medium was fed every 1-2 days with a fresh medium.

Treatment of cells with hydro-alcoholic extract of Baneh leaves

In order to determine the effects of the various concentrations of Baneh leaf hydro-alcoholic extract, 5 × cells plus 1 mL of each medium were cultured in each of 12 wells, and 10, 50, 100, 150, 200, as well as 250 (µg/mL) of the extract, were added to the appropriate wells. The incubation time of the treatments was 24, 48, and 72 hours. The measurement was considered three times for each dilution, and also three wells, including cell and whole culture medium, were assigned as control.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine IC50 for the hydro-alcoholic extract of Baneh leaves. Briefly, 10 µL of MTT (5 mg/mL) was added to the well containing cells, a maximum of 100 µL of the culture medium and 5000 cells; then, the plate was incubated in a 37°C incubator for 2-4 hours. Afterward, with the naked eyes, purple deposits in the wells appeared, and when the deposition was more violet, the presence of more living cells was approved. After 4 hours, 100 µL of DMSO solution was added to each well; if necessary, the contents of each well were transferred into 1.5 mL microtube and centrifuged for 3 minutes at 1200 g to obtain a clear and colorless solution. Optical density (OD) was measured by an ELISA reader based on the intensity of the Formazan blue at 570 nm. Next, the biological power of cells was calculated using the following formula:

(Total number of cells/number of living cells) = "Bioequivalence of cells."

DNA Fragmentation

The DNA of the cells was extracted before and after treatment with different doses of the extract, and 1.5% of agarose gel was used to confirm the development of apoptosis after the effect of the extract on cells. In the cases of the apoptosis, DNA was fragmented and identified as a smear on an agarose gel.

RNA extraction and cDNA synthesize

Total cellular RNA was obtained from MCF-7 and L929 cells before and 48 hours after treatment with Baneh extract using RNA extraction kit of Cinaclone Company (Iran). First, cDNA was synthesized using a commercial kit (Parstous Company, Iran). Then, mRNA, oligo-dT, and RNase reverse transcriptase were imported to the mixture and incubated at 70°C and 4°C for 10 and 4 minutes, respectively. At that point, the premix was added and incubated at 40°C for 60 minutes.

Real-Time PCR assay

The mRNA expression level of the p53, caspase-8, caspase-3, bax, and bcl-2 genes was measured by Real-time PCR method using a Bio-Rad CFX96 real-time PCR machine (Hercules, CA, USA). All primer sequences were listed in Table 1. Each reaction consisted of 2 µL cDNA, 1 µL of primers, and 21 µL reaction buffers (SYBR Green involved); the total reaction volume was 25 µL. Real-time PCR cycles consisted of 4 minutes at 95°C for polymerase activation, following with 45 cycles of 95°C (35 s), 58°C (30 s), and 72°C (30 s). β-actin gene was used as a housekeeping gene, and the raw data was calculated via $2^{-\Delta\Delta ct}$.

TABLE 1. Primer sequences used in the study (F, forward; R, reverse).

Genes	*Primer sequences (5'→3')*
bcl-2	F: CTTCTTTGAGTTCGGTGGGG R: AAATCAAACAGAGGCCGCAT
p53	F: TGAAGCTCCCAGAATGCCAG R: GCTGCCCTGGTAGGTTTTCT
Caspase-8	F: ATTAGGGACAGGAATGGAACAC R: GGAGAGGATACAGCAGATGAAG
bax	F: TGCCTCAGGATGCGTCCACCAA R: CCCCAGTTGAAGTTGCCGTCAG
Caspase 3	F: ACTGGACTGTGGCATTGAGA R: GCACAAAGCGACTGGATGAA
b-actin	F: GGGCATGGGTCAGAAGGATT R: CGCAGCTCATTGTAGAAGGT

Statistical analysis

Statistical package for the social sciences (SPSS) version 15 (SPSS Inc., Chicago, IL, USA) was utilized for this examination. Entire investigations were completed three times in every individual example, and all of the acquired outcomes were introduced as the mean estimation of those three. Factual examination to analyze contrasts among bunches was performed by one-way ANOVA and Dunnett's technique for various correlations with a solitary control gathering. p-value < 0.05 was considered statistically significant.

RESULTS

MTT assay was utilized to assess the inhibitory effects of the Baneh leaves hydro-alcoholic extract on the growth of MCF-7 compared to mouse fibroblast L929 as normal cells under *in vitro* condition. Data analysis demonstrated that the IC50 value for MCF-7 and L929 cells after 48 hours of treatment with extract was 250 µg/mL and 400 µg/mL, respectively (Figure 1). The morphologic picture of L929 and MCF-7 cells before and after treatment is presented in Figure 2. As demonstrated in Figure 2, morpho-

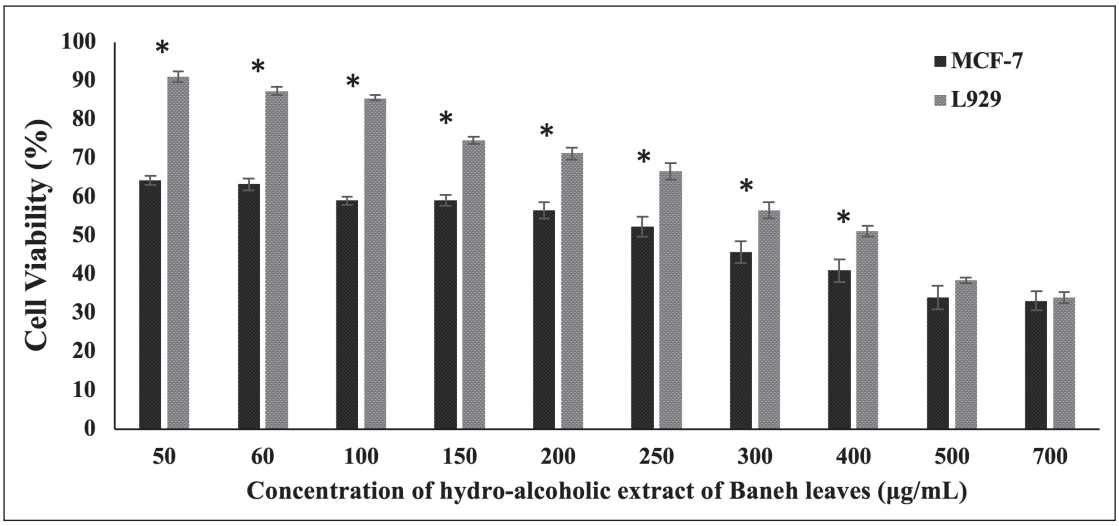

Fig. 1. Determination of IC50 in L929 and MCF7 cell lines after 48 hours treatment. MTT test showed that IC50 value for MCF-7 and L929 cells after 48 hours treatment with extract was 250 µg/mL and 400 µg/mL, respectively. *Independent sample t-test (p<0.05).

logical symptoms of apoptosis, such as membrane blebbing, cell shirking, chromatin condensation, and the formation of apoptotic bodies were observed after treatment of both cells with Baneh extract. The DNA of both cells was extracted before and after treatment with the Baneh extract at IC50 concentration and analyzed on 1.5% agarose gel to confirm the occurrence of apoptosis. The result of the electrophoresis indicated the fragmentation and smearing of extracted DNA from treated MCF-7 and L929 cells on the gel, which confirms apoptosis in these cells (Figure 3).

The results of the gene expression showed that mRNA levels of bax gene were significantly increased in MCF-7 cells (p<0.05) after 48 hours of treatment with extract, while in L929 cells, the changes on the Bax gene expression were not significant (p>0.05) (Figure 4A-4B).

Statistical analysis showed a significant down-regulation of bcl-2 (p<0.05) in MCF-7 cells, while in L929 cells the increased expression change was not significant (p>0.05) after 48 hours of treatment with extract (Figure 4C-4D).

The 48 hours treatment of MCF-7 cells with the extract led to the up-regulation of the Caspase-3 gene; however, these changes were not significant

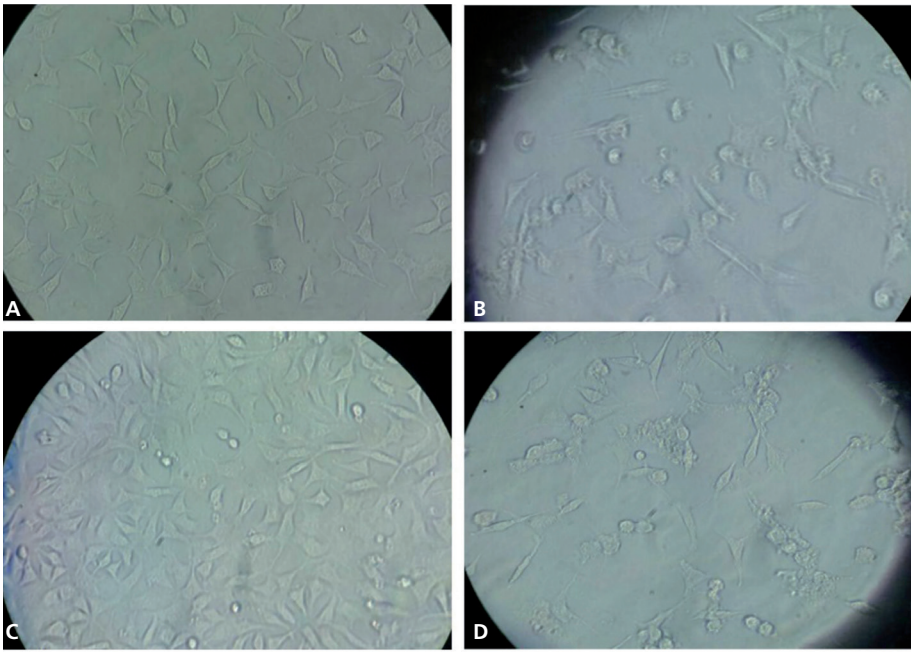

Fig. 2. Morphology of MCF-7 *(A-B)* and L929 *(C-D)* cells abefore and after treatment with Baneh extract. Baneh extract induces apoptosis in both L929 and MCF-7 cells.

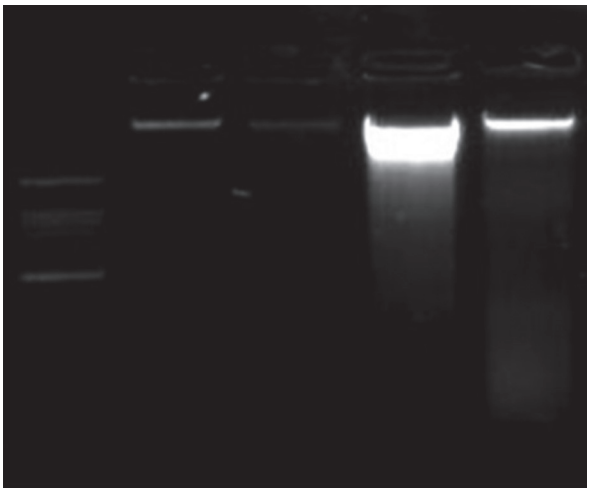

Fig. 3. DNA fragmentation assay for detection of apoptosis. Lane 1, DNA ladder, Lane 2, Gel electrophoresis of DNA isolated from un-treated MCF-7 cells. Lane 3, DNA isolated from un-treated L929 cells. Lane 4, DNA from MCF-7 cells treated with Hydro-alcoholic extract of Baneh leaves at IC50 concentration (250 µg/ mL) after 48-h. Lane 5, DNA from L929 cells treated with Hydro-alcoholic extract of Baneh leaves at IC50 concentration (400 µg/ mL) after 48-h.

(p>0.05). The results showed that the mRNA level of caspase-3 was significantly decreased in L929 cells (p<0.05) (Figure 5A-5B). It is also shown that the mRNA expression level of Caspase-8 increased in both MCF-7 and L929 cells (p<0.05) after 48 hours of treatment with Baneh extract (Figure 5C-5D).

The mRNA expression levels of p53 in both MCF-7 and L929 cells (p<0.05) were significantly increased compared to untreated cells in the same period (Figure 6).

DISCUSSION

Breast cancer is one of the most common diseases among women around the world [15]. Treatment of this disease with chemotherapy or surgery can lead to side effects [16]. Therefore, researchers are trying to achieve new approaches to support cancer treatment in order to improve the quality of life in cancer patients and reduce the chemotherapy side effects[17-25].

The outcomes proved that IC50 concentration for L929 cells at 48 hours of treatment was higher (400 µg/mL) than MCF-7 (250 µg/mL). Given the outcomes, it appears that the MCF-7 cancerous cells were more sensitive to Baneh extract than L929 as normal cells. Accordingly, the present examination was additionally intended to investigate the effec-

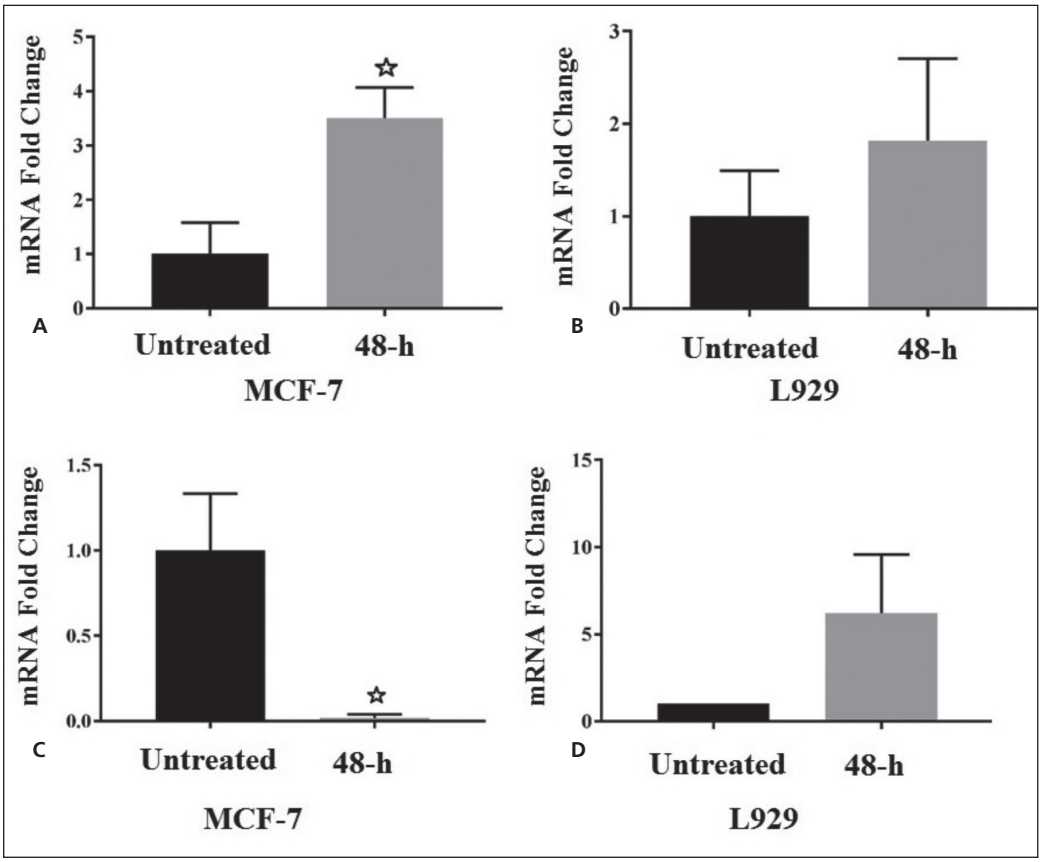

Fig. 4. The effects of the Hydro-alcoholic extract of Baneh leaves on the expression levels of bax *(A-B)* and bcl-2 *(C-D)* in MCF-7 and L929 cells, respectively. *p<0.05 indicates a significant difference with the un-treated cells.

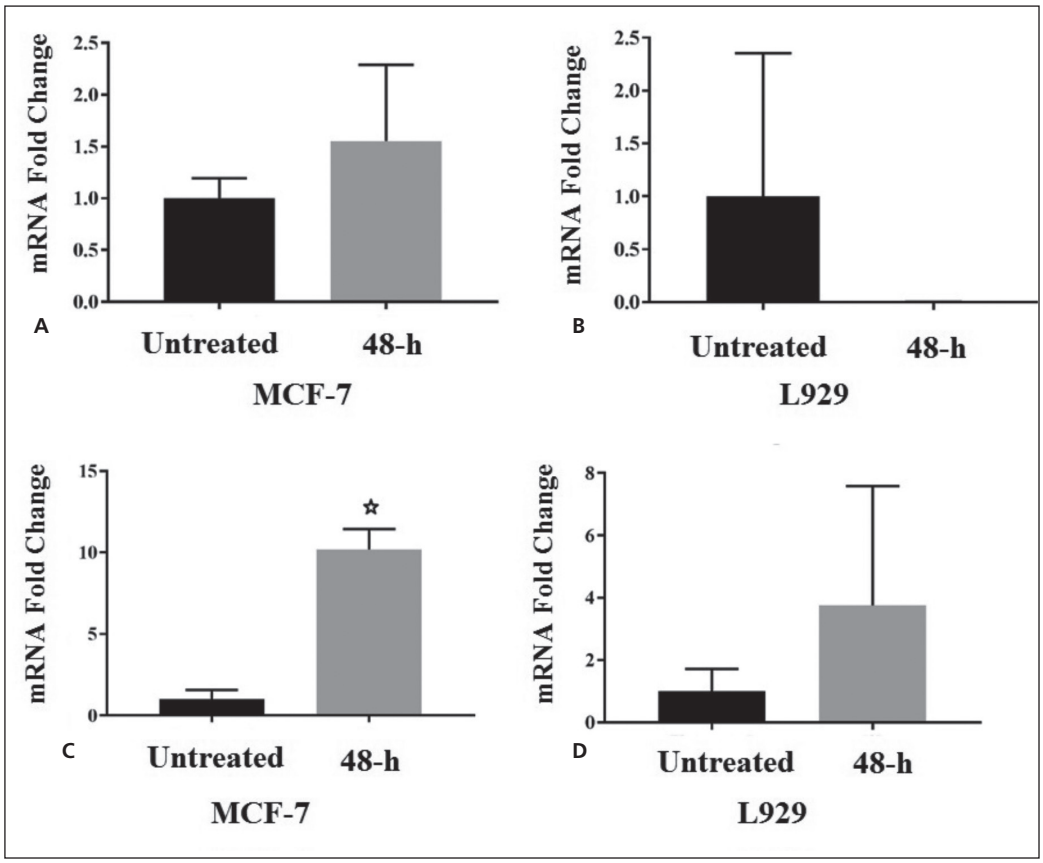

Fig. 5. The effects of the Hydro-alcoholic extract of Baneh leaves on the expression levels of caspase-3 *(A-B)* and caspase-8 *(C-D)* in MCF-7 and L929 cells, respectively. *p<0.05 indicates a significant difference with the un-treated cells.

tiveness of Baneh treatment on the expression of the anti-apoptotic genes, including p53, caspase-3, caspase-8, bax, and bcl-2. The results showed that the expressions of p53, caspase-8, and bax genes in MCF-7 cells were significantly increased after 48 hour of treatment by Baneh extract. On the contrary, the mRNA expression level of bcl-2 was significantly down-regulated, showing that both intrinsic and extrinsic apoptotic pathways were active. However, there was no change in the expression of caspase-3 gene. The data showed that bcl-2 gene expression was down-regulated in the MCF-7 cells, while it was insignificantly increased in the L929 cells; hence, it may be concluded that bcl-2 as an anti-apoptotic gene binds to pro-apoptotic gene like bax and induces the release of cytochrome c

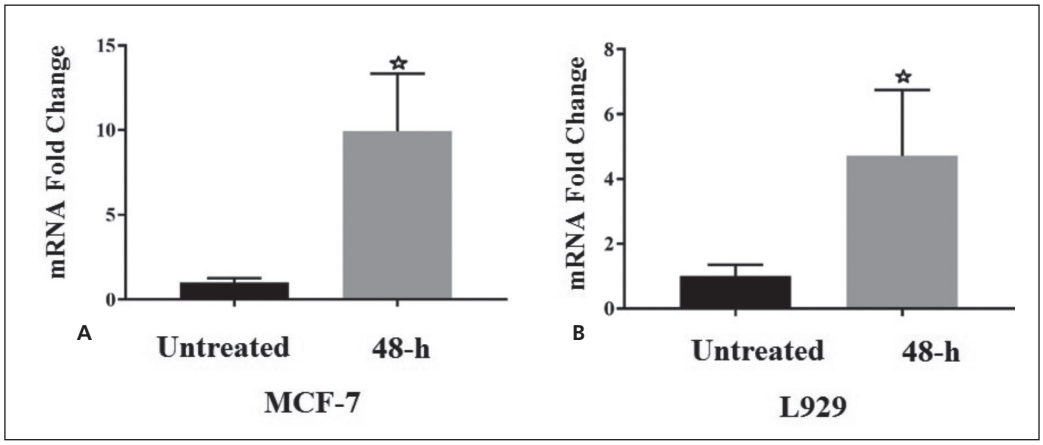

Fig. 6. The effect of the Hydro-alcoholic extract of Baneh leaves on the expression level of p53 *(A-B)* gene in MCF-7 and L929 cells, respectively. *p<0.05 indicates a significant difference with the un-treated cells.

from mitochondria, which triggers apoptosis [26]. The bcl-2 families are the central regulators of the mitochondrial cell-intrinsic apoptotic pathway [27]. Amiri et al[28] showed that the ethanol Baneh extract caused down-regulation of bcl-2 gene and up-regulation of bax and caspase-3 genes, and it was concluded that the apoptosis happened in prostate cancer cells through internal pathway. Therefore, based on both the results presented here and the related investigations, it seems that down-regulation of bcl-2 in the MCF-7 cells but not in the L929 ones, led to activation of apoptosis via intrinsic pathway following treatment of the cells with Baneh extract. In MCF-7 cells, the expression of p53, caspase-8, and bax genes were increased. It showed that over-expression of bax and down-regulation of bcl-2 triggered apoptosis via intrinsic apoptotic pathway[29]. Also, the over-expression of caspase-8 could trigger apoptosis via extrinsic apoptotic pathway in MCF-7 cells. In L929 cells, the over-expression of p53 was observed after treatment with the extract; therefore, the p53 pathway could trigger apoptosis in normal cells. The intrinsic and extrinsic apoptotic pathways were not activated in healthy cells. Caspase-3 had a major role in apoptosis as "initiator." Up-regulation of this gene in MCF-7 cells showed that the intrinsic and extrinsic apoptotic pathways were active. Due to the fact that mRNA levels of bax and bcl-2 in the cancerous cells were significantly increased and decreased, respectively, it may be concluded that Baneh extract targets the molecules in the cancerous cells and may increase the effects of the chemotherapy on the tumor cells rather than normal cells. In treated MCF-7 and L929 cells with Baneh extract, the cleavage and smearing of DNA were observed on agarose gel, indicating the occurrence of apoptosis in these cells. Also, morphological analysis revealed that the Baneh extract initiated apoptosis in both cells. Hence, the rate of apoptosis was fundamentally higher in the MCF-7 cells than L929 cells.

Baneh plant contains many antioxidants and health benefit properties; many researches in this field have been conducted on the anticancer properties of the *P. Atlanthica* sub-species mutica. For instance, Rezai et al[30] investigated the effect of mountain pistachio (*P. Atlanthica* sub-species kurdica) on the T47D breast cancer cell line. Its significant role in the degradation of cancer cells was observed by these researchers.

Balan et al[31] found that Baneh-derived compounds induce apoptosis in human intestinal cancer cells, which is in line with the result of the present study. Interestingly, Dimas et al[13] in 2009 showed that Baneh extract inhibits the growth of colorectal tumors. It was found that the products obtained from the Baneh tree as oil are more stable than that of other plant oils, such as sesame oil due to the high amount of antioxidants. Rezaei et al[32] reported about 63 compounds in Baneh oils, the most important of which are β-myrcene (8.4%), camphene (20.8%), α-pinene (8%), and limonene (8.2%). A new derivative of Baneh is isolated and identified as Hispolone, which has antioxidant properties[33].

CONCLUSIONS

According to the obtained results in the present study, the up-regulation of caspase-8, caspase-3, p53, and bax genes and the down-regulation of bcl-2 gene indicate that the Baneh extract induces the process of cell death through the intrinsic and extrinsic apoptosis pathways, causing the programmed cell death in MCF-7 cells through modulating the expression of pro-/anti-apoptotic genes and caspase signaling pathway. Therefore, the more sensitive breast cancerous cells to the treatment are compared with healthy ones; this extract can be a promising candidate to be used in the preparation of anticancer drugs against cancerous breast cells with fewer side effects on healthy cells. The described results show that Baneh leaves extract can be considered as a potential medication to induce apoptosis in cancerous breast cells without any significant effects on healthy cells. Thus, it appears that Baneh extract may be considered as a potential support treatment with conventional chemotherapy or surgery to improve the quality of life for cancer patients.

Competing interests:

The authors declare that they have no competing interests

Acknowledgments:

Authors have a chance to thanks to Molecular Medicine Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran for their warmly corporations.

Authors' contributions:

Hadis A & FR participated in the collection of data; MM participated in the study design and revised the manuscript; MHB participated in the interpretation of data and drafted the manuscript; MRH & MRM participated in the study design and revised the manuscript; Hassan A contributed to the statistical analysis of the data; MR participated in the collection of data and SKF participated in the interpretation of data, study design and revised the manuscript. All authors approved the final version of the manuscript.

Availability of data and material:

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Funding:

This study is financially supported by Rafsanjan University of Medical Sciences, Rafsanjan, Iran, under the Grant No. 95008and the Ethical Code of IR.RUMS.REC.1395.7 at Rafsanjan University of Medical Sciences.

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