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Anti-proliferative effects of *Rhamnus frangula* miller leaf and bark extracts on HEK293 and MCF-7 cell lines and evaluation of Bax and Bcl-2 genes expression level in MCF-7 cells

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Abstract: *Rhamnus frangula* extracts may have anti-proliferative effects on cancer development. This study was exerted to determine the effects of *Rhamnus frangula* Miller leaf and bark extracts on the viability of MCF-7 cells compared with HEK293 cells and evaluation of Bax and Bcl-2 genes expression level in MCF-7 cells. The cell lines were randomly divided into control group (not exposed to extract) and groups exposed to 0.001, 0.01, 0.1, 1 mg/ml of *Rhamnus frangula* leaf and bark extracts. Cell viability was quantified by MTT assay. The expression of Bax and Bcl-2 genes was evaluated by quantitative Real time PCR analysis. Statistical analysis was performed using ANOVA. HEK293 cells viability significantly increased in groups exposed to 0.001 and 0.01 mg/ml and decreased in group exposed to 1 mg/ml of *Rhamnus frangula* leaf extract compared to control group ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively). Exposure of MCF-7 cells to 0.01, 0.1 and 1 mg/ml of leaf extract led to significant decrease in cell viability ($P < 0.05$). Exposure of HEK293 cells to 1 mg/ml and of MCF-7 cells to 0.1 and 1 mg/ml of *Rhamnus frangula* bark extract resulted in significant decrease in cell viability compared to control group ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively). The cytotoxic effect of leaf was higher than bark extract on MCF7 cells. 1 mg/ml of leaf and bark extracts significantly increased the expression level of pro-apoptotic Bax and decreased anti-apoptotic Bcl-2 genes in MCF-7 cells ($P < 0.01$). However, expression level of Bax gene was significantly higher in group exposed to 1 mg/ ml of leaf compared to bark extract ($P < 0.01$). Despite bark, lower leaf extract concentrations inhibit breast cancer (MCF-7) cells proliferation while have no cytotoxic effects on non-cancerous HEK293 cells. Therefore, using leaf is more appropriate than bark extract to fight against breast cancer cells. We have also shown that *Rhamnus frangula* leaf and

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bark extracts mechanism of action is occurred through Bax-dependent apoptotic pathway on breast cancer cell line.

Keywords: *Rhamnus frangula* miller, MCF-7, HEK 293, Proliferation, Apoptosis

1 Introduction

Rhamnus frangula is a shrub belonging to Rhamnaceae family in the major group Angiosperms (flowering plant). Often it is native to Europe, Africa, Siberia, The Urals and The Alborz mountains. This plant is poisonous and before using, it should be kept in a place for 12 month. This product is very suitable for the use as medical consumption materials. Some components extracted from *Frangula*, have been shown to display a wide range of biological activities including antiviral, antimicrobial, anti-tumor, anti-fibrosis, elimination of reactive oxygen species and anti-inflammatory effects (Fiedler and Landis, 2012; Howell and Blackwell, 1977; Huang et al., 2009).

Breast cancer has been recognized as a major public health problem in the world (Lacey et al., 2002), with 1,384,155 estimated new cases worldwide with nearly 459,000 related deaths (Tao et al., 2015). Breast cancers are often a type of carcinoma called adenocarcinoma and is a major cause of illness, depression, loss of sex desire, lymph nodes, bone pain, chest pain, metastatic breast cancer, brain damage (DeSantis et al., 2011; Zdenkowski et al., 2016).

Apoptosis is a complex regulating process that occurs through normal tissue homeostasis (the balance between cell proliferation and cell loss) in self-renewing tissues and plays crucial roles in tumorigenesis and antitumor therapy, thereby opening a cancer therapeutic window (Kagawa et al., 2000). The Bcl-2 gene was identified as the locus linked to the immunoglobulin heavy chain locus by the 14;18 chromosome translocation associated with lymphomas of follicular center B-cell origin that encodes a 24-kDa membrane-associated protein, apparently localized to mitochondria that have role in contributing to oncogenesis by suppressing signals that induce apoptotic cell death (Eimani et al., 2014; Strasser et al., 1991). Bax, an important homologue of Bcl-2, is an indispensable gateway to mitochondrial dysfunction and a major proapoptotic member of the B-cell lymphoma 2 (Bcl-2) family proteins that its overexpression leads to apoptosis in a wide variety of cells (Eimani et al., 2014; Liu et al., 2016). The suitable pro-apoptotic environment is prepared for the cell when the amount of Bax increases and Bcl-2 decreases (Wood et al., 2013).

Research have shown that Bcl-2 family proteins are critical checkpoints of apoptotic cell death and is one of the most promising therapeutic strategies for dysfunctional apoptosis-related diseases including cancers. In addition, investigations have proved that Bax activation induces mitochondrial membrane permeabilization, thereby leading to the release of apoptotic factor cytochrome *c* and consequently cancer cell death (Liu et al, 2016).

Studies have shown that Plant-derived compounds have been an important source of several clinically useful anti-cancer agents (Cragg and Newman, 2005; Yesil-Celiktas et al., 2010; Singh et al., 2011; Bronikowska et al., 2012). The findings also indicated that cytotoxic effect of

plant extracts was observed in the normal cell viability assays (Assadollahi et al., 2013; Shokrzadeh et al., 2009). Studies suggest that people who eat more vegetables and fruits, which are rich sources of antioxidants such as vitamin C, vitamin E, carotenoids and many other phytochemicals, may have a lower risk for some types of cancer (Hocman, 1989). The researchers have found that the different molecular frameworks of plant phenolic compounds attribute to their antioxidant properties of protection against oxidative damage, such as cancers (Dai and Mumper, 2010). The studies also suggest that flowering plants demonstrate cytotoxic activities against breast cancer cell lines (MCF-7) (Ali et al., 2014; Han et al., 2009). Recent reports revealed that some plants leaves, bark and seed extracts have anti-cancer activity against breast cancer cell lines (Al-Asmari et al, 2015). New academic research suggest that *Rhamnaceae* family has antitumor effects (Plastina et al., 2012; Kumar et al., 2011) and recent advances in cancer research have shown that *Rhamnus frangula* has anticancer effects on chronic lymphocytic leukemia cells (Kupchan and Karim, 1976). A large body of experimental data has revealed that the highest apoptosis level was found in SW872, SW982, HS 39.T, HS 5.T, HL-60, M14WM, MCF-7, and HT29 cell lines treated with *Rhamnus Frangula* (Lombardi et al., 2017). In addition, other experimental findings have demonstrated that Bax expression level was reduced in primary breast tumours and breast cancer cell lines compared with normal breast epithelium and non-malignant epithelial cell lines, and apoptosis could be induced in those cell lines with high Bax expression (De Angelis et al., 1998). Although most of studies are emphasizing on anticancer effects of Rhamnus family, some studies have shown that certain extracts of *Frangula* may have less anti-cancer effects than other herbal extracts (Tepkeeva et al., 2008).

The prevalence of breast cancer cases has dramatically increased in world in recent years (Huang et al., 2009) and unfortunately the methods of therapy still could not fulfill entirely the treatment goals. Therefore, investigations on herbal treatments have a pivotal place in anticancer studies. Although previous studies have reported the anticancer effects of *Rhamnus frangula* extracts (Fazeli et al., 2014), there are few research on the effects of *Rhamnus frangula* extracts on cancer cells at cellular and molecular level. The present study aimed to investigate the effects of *Rhamnus frangula* Miller leaf and bark extracts on viability of breast cancer (MCF-7) cells compared with non-cancerous human embryonic kidney (HEK293) cells and evaluation of Bax and Bcl-2 genes expression level in MCF-7 cells.

2 Materials and Methods

2. 1. *Rhamnus frangula* extract preparation

The selected plant was collected in different areas of Guilan province, Iran in June, 2016. *Rhamnus frangula* Miller leaf and bark were dried at room temperature in the dark and ground finely using blender. Exactly 250 g of the *Rhmnus frangula* dried leaves and dried barks were weighted by a digital scale and after grinding, 500 mL of 50% ethanol alcohol was added to the sample to cover almost all the powder surface. Then the erlen was placed in a percolator for 48 hours. The solution was filtered to remove undissolved particles to obtain clear solution. The solution was divided into glass plates and was placed at room temperature to evaporate the solvent thoroughly. Finally, it was kept in refrigerator until used (Lombardi et al., 2017; Fazeli

et al., 2014).

2. 2. Cells culture

MCF-7 and HEK293 cell line were purchased from Pasteur institute of Iran (cell bank), Tehran, Iran. The cells were cultured in RPMI 1640 (Gibco, UK) supplemented with 10% heat inactivated fetal bovine serum (Gibco, UK), 100 units/mL penicillin and 100 µg/ml streptomycin and incubated at 37 °C in 5% CO_2 incubator for 48 hrs (Abedini et al., 2016).

2. 3. Cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed for assessing cell proliferation activity and cytotoxicity in HEK293 and MCF-7 cells exposed to 0.001, 0.01, 0.1 and 1mg/ ml of *Rhamnus frangula* leaf and bark extracts. Cell viability was determined using the MTT assay 24 hours after incubation. The MTT assays were performed according to standard protocols (Kobayashi et al., 2013; Ahmadian et al., 2009).

The cells were seeded in 96-well plates with 1×10^4cells/ well and placed at 37 °C in a 5% CO2 humidified incubator until 60% confluency.

The complete growth medium was removed and the cells were serum-starved for 24 h prior to treatment. Cells incubated in culture medium alone served as a control for cell viability (untreated cells). The cells were treated with different doses of *Rhamnus frangula* leaf and bark extracts for 24 h in complete growth medium. Following the extracts treatments, the medium was removed and 100 µl of MTT solution (5 mg/mL in sterile H2O) was added to each well. The plates were incubated under 95% atmosphere air and 5% CO2 at 37 °C for 4 h. The MTT solution was removed and 200 µl aliquots of DMSO were added to each well to dissolve the formazan crystals followed by incubation for 10 min at 37 °C. Treatments were performed in triplicates, and optical densities were read at 570 nm by spectrophotometric method.

2. 4. Quantitative real time-PCR analysis

HEK239 and MCF-7 cells were seeded in dishes at 500,000 cells/10 mL/75 cm^2. One day after seeding, the medium was changed, and the cells were incubated with the test compounds for 12 h. At the end of the incubation, the cells were collected by centrifugation, washed with ice-cold PBS, and total RNA was extracted using an RNeasy midi kit (Roche, 1 828 665, Germany). Total RNA (2.5 µg) was reverse transcribed into cDNA using a Transcriptor First Strand cDNA synthesis kit (Roche, 04 379 012 001, Germany), and quantitative realtime PCR was carried out as using a LightCycler-FastStart DNA master SYBR Green I Kit (ABI, 4369016, American) and Light Cycler apparatus (Roche Diagnostics).

The Quantitative RT-PCR for Bax and Bcl2 genes was carried out using the specific primers (Table 1). GAPDH gene was used to normalize the relative expression for interested genes calculated by $2^{\Delta\Delta CT}$ method and SYBR Green kit. The presence of the expected PCR products after quantitative real-time RT-PCR reactions were confirmed by an agarose gel electrophoresis.

Table 1. Real time Primer sequence

Gene	Sequences
GAPDH	5'TGCACCACCAACTGCTTA3' (Forward) 5'GGATGCAGGGATBATGTTC3' (Reverse)
BAX	5'TGGAGCTGCAGAGGATGATTG3' (Forward) 5'GAAGTTGCCGTCAGAAAACATG3' (Reverse)
BCL-2	5'CTGCACCTGACGCCCTTCACC3' (Forward) 5'CACATGACCCCACCGAACTCAAAGA3' (Reverse)

2. 5. Statistical analysis

The data was expressed as mean± standard deviation (SD). One-way Analysis of Variance (ANOVA) and Tukey's post hoc-test were used to determine significant difference between groups ($P < 0.05$) in SPSS 20 software.

3 Results and Discussions

Figure 1 shows viability of HEK239 cells exposed to 0.001, 0.01, 0.01 and 1 mg/ml of *Rhamnus frangula* leaf and bark extracts in cell culture. According to figure 1, HEK239 cells viability significantly increased in groups exposed to 0.001 and 0.01 mg/ ml of *Rhamnus frangula* leaf extract compared to control group ($P < 0.01$ and $P < 0.05$, respectively). However, there was no significant difference between viability of HEK239 cells exposed to 0.1 mg/ml of leaf extract compared to control group. HEK239 cells viability significantly decreased in group exposed to 1 mg/ml of leaf extract compared to control group ($P < 0.01$). There was also no significant difference in cell viability between HEK239 cells exposed to 0.001, 0.01 and to 0.1 mg/ml of *Rhamnus frangula* bark extract. However, HEK239 cells viability significantly decreased in group exposed to 1 mg/ml of bark extract compared to control group ($P < 0.01$). Meanwhile, there was also significant difference in cell viability between HEK239 cells exposed to 0.001, 0.01, 0.1 and 1 mg/ml of leaf extract compared to HEK239 cells exposed to 0.001, 0.01, 0.1 and 1 mg/ml of bark extract ($P < 0.01$).

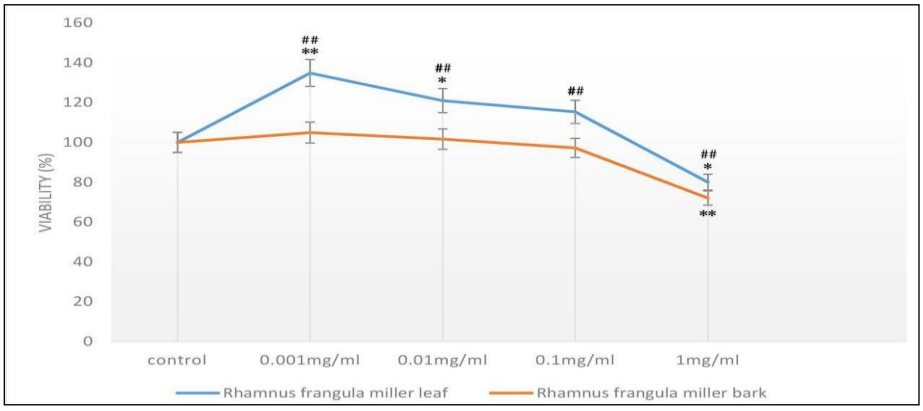

Figure 1: Effect of *Rhamnus frangula* miller leaf and bark extracts on HEK293 cells viability. The cells were treated

with different concentrations of leaf and bark extracts (0.001, 0.01, 0.1 and 1mg/ml). Data are expressed as mean ± SD (n = 3). Values are statistically significant at $^{**}P < 0.01$, $^{*}P < 0.05$ compared to control group and, $^{\#\#}P < 0.01$ compared to groups exposed to bark extract.

Figure 2 shows viability of breast cancer (MCF-7) cells exposed to 0.001, 0.01, 0.1 and 1 mg/ml of *Rhamnus frangula* leaf and bark extracts in cell culture. According to figure 2, there was no significant difference between viability of MCF-7 cells exposed to 0.001 mg/ml of *Rhamnus frangula* leaf extract compared with control group. However, MCF-7 cells viability significantly decreased in groups exposed to 0.01, 0.1 and 1 mg/ml of leaf extract compared to control group ($P < 0.05$). There was also no significant difference in cell viability between MCF-7 cells exposed to 0.001 and 0.01 mg/ml of *Rhamnus frangula* bark extract. Exposure of MCF-7 cells to 0.1 and 1 mg/ml of bark extract led to significant decrease in viability of MCF-7 cells ($P < 0.05$ and $P < 0.01$, respectively). In addition, there was also significant difference in cell viability between MCF-7 cells exposed to 0.01, 0.1 and mg/ml of leaf extract compared to MCF-7 cells exposed to 0.01, 0.1 and 1 mg/ml of bark extract. ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively). The IC50 (50% of growth inhibition) of *Rhamnus frangula* miller leaf extract and bark extract on MCF-7 breast cancer cell line were determined at 0.43 and 5 mg/ml, respectively.

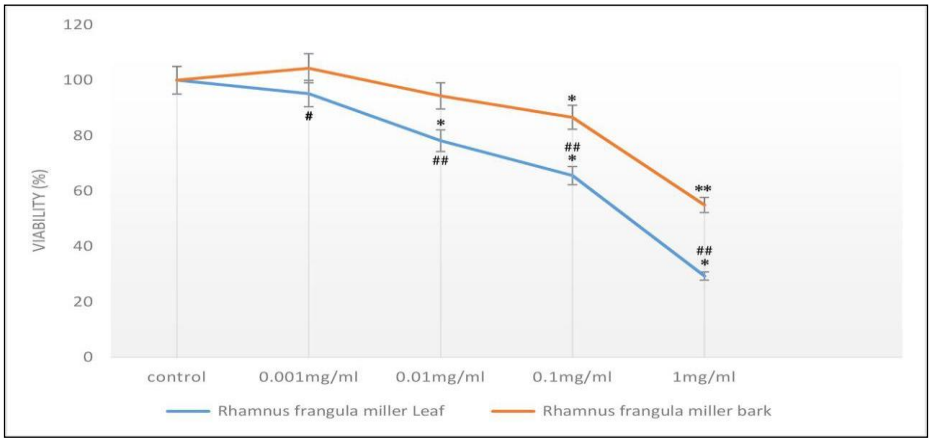

Figure 2: Effect of *Rhamnus frangula* miller leaf and bark extracts on MCF-7 cells viability. The cells were treated with different concentrations of leaf and bark extracts (0.001, 0.01, 0.1 and 1 mg/ml). Data are expressed as mean ± SD (n = 3). Values are statistically significant at $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control group and $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with groups exposed to bark extract.

Figure 3 shows expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 genes in MCF-7 cells exposed to 1 mg/ml of *Rhamnus frangula* leaf and bark extracts. According to figure 3, to examine the alteration of apoptosis regulating genes expression by *Rhamnus frangula* leaf and bark extracts in MCF7 cells, we investigated the effect of 1 mg/ml of leaf and bark extracts (as cytotoxic dose) on expression level of GAPDH, Bax and Bcl-2 genes. The results revealed that 1 mg/ ml of extracts significantly increased the expression level of pro-apoptotic Bax and decreased anti-apoptotic Bcl-2 genes in MCF7 cells ($P < 0.01$). There was no significant difference between expression of Bcl-2 gene exposed to 1 mg/ml of bark extract compared to control group but Bcl-2 gene expression significantly decreased in group exposed to 1 mg/ml of leaf extract compared to control group ($P < 0.01$). However, Bcl-2 gene expression levels

significantly decreased in group exposed to 1 mg/ml of leaf extract compared to bark extract ($P < 0.01$). Bax gene expression level significantly increased in groups exposed 1 mg/ml bark and 1 mg/ml leaf extract compared with control group ($P < 0.01$). However, expression level of Bax gene significantly increased in groups exposed to 1 mg/ml of leaf extract compared to bark extract ($P < 0.01$).

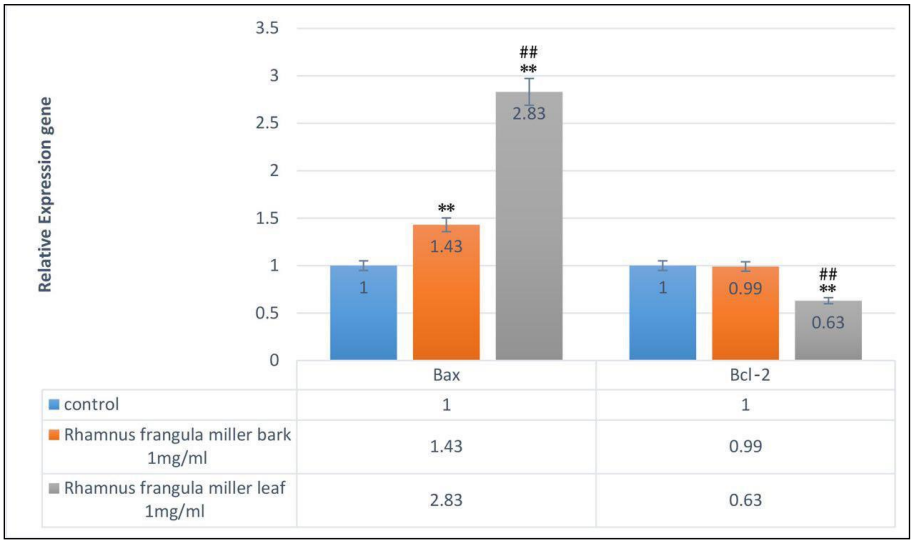

Figure 3: MCF-7 cells treated with 1 mg/ml *Rhamnus frangula* miller leaf and bark extracts. Data represents relative gene expression (Target/GADPH) mean ± SD of five experiments (n = 3). Values are statistically significant at ** $P < 0.01$ and ##$P < 0.01$ compared to control group and group exposed to bark extract, respectively.

Our findings indicated that cell viability increases in non-cancerous human embryonic kidney cells exposed to low leaf extract concentration; However, exposure of HEK293 cells to high concentrations of both leaf and bark extracts gives rise to decreased cell viability, demonstrating that low level of leaf extract may have protective effects on non-cancerous cells. In line with this finding, there are studies showing that some plants species leaf extracts exhibited a lower level of toxicity against non-cancerous cells compared with cancerous cells (Strzemski et al., 2017; Bishayee et al., 2011). However, there are plant extracts which can inhibit proliferation of the non-cancerous cells in cell culture (Medjakovic et al., 2016).

We have shown that higher concentrations of both *Rhamnus frangula* leaf and bark extracts had anti-proliferative effects on MCF-7 cells; However, only lower concentration of leaf extract could inhibit MCF-7 cells proliferation, showing greater anticancer potential of leaf than bark extract. In addition, all concentrations on MCF-7 cells had higher cytotoxic effects against MCF-7 cells when compared one to one with bark extract.

Previous studies have also reported that members of Rhamnaceae family have been used in traditional Chinese medicine for treatment of cancer (Plastina et al., 2012) and according to experimental findings, some extract components of plants belonging to Rhamnaceae family represent a large and structurally diverse class of natural products that can prevent and treat breast cancer (Bishayee et al., 2011). The leaf and bark of many medicinal plants are used in traditional medicine for the treatment of cancer, especially in breast cancer patients (Engel et al., 2011). In an experimental study, a systematic fractionation of an ethanol-water extract of the

seeds of *Rhamnus frangula* L., guided by assays for tumore-inhibitory activity, led to the isolation of aloe emodin. This compound was found to show significant anticancer activity in mice. (Kupchan and Karim, 1976) According to studies, the natural *Rhamnus frangula* compounds may be also origin from the cytotoxic effects of *Rhamnus frangula* (Hocman, 1989). However, there are reports showing that the peptide of *Frangula* extract has less anti-cancer effect than other herbal peptides (Tepkeeva et al., 2008).

Our observation demonstrated that both leaf and bark extracts induces MCF-7 cell apoptosis by Bax-dependent pathway in which Bax expression level increases and Bcl-2 expression level decrease. Our results also showed that leaf extract was more effective than bark extract in increasing of Bax gene expression level. This is why that we observed higher cytotoxic effects on MCF-7 cell by leaf extract than bark extract.

Recent advances in cancer also have demonstrated that Bax expression was reduced in primary breast tumors and breast cancer cell lines compared with normal breast epithelium and non-malignant epithelial cell lines, and apoptosis could be induced in those cell lines with high Bax expression (De Angelis et al., 1998). Insight into the possible mechanism, plants belonging to Rhamanaceae family, have cytotoxic effects on the Hela cancer, SW872, SW982, HS 39.T, HS 5.T, HL-60, M14WM cell lines and MDA-MB-468 tumor cells (Lombardi et al., 2017; Jafarian et al., 2014). In line with our findings the highest apoptosis level has been found in colon and breast cancer cells treated with Rhamnus frangula extract (Lombardi et al., 2017).

Several reports have also demonstrated that emodin existing in plants belonging to the Rhamanaceae family may have an inhibitory effect on the mammalian cell cycle, thus this compound can have anti-cancer effects in the live creatures (Srinivas et al., 2007). It has also been shown that emodin can induce apoptosis in many kinds of cancer cells. It has been demonstrated that emodin induces mitochondrial transmembrane potential loss, increase in Bax and decrease in Bcl-2 expression and mitochondrial translocation and release of cytochrome c to cytosol in certain cancer cells (Xie et al., 2014; Huang et al., 2008).

The main purpose of this study was to evaluate the induction of apoptosis in breast cancer (MCF-7) cells treated with cytotoxic concentration of hydro-alcoholic leaf and bark extracts of *Rhamnus frangula*. The concept of apoptosis could be of great importance for cancer treatment and studies on the effects of natural derived compounds on cancer cells have a place in *in vitro* and *in vivo* experiments. In summary, leaf and bark extracts of Rhamnus frangula showed significant reduction in *in vitro* breast cancer cells proliferation by inducing Bax gene dependent apoptosis. However, more studies are needed to investigate the exact molecular mechanisms underlying anticancer activity of the *Rhamnus frangula* leaf and bark extracts. Altogether, natural extracts of *Rhamnus frangula* use may hold promise as an adjuvant treatment to prevent or treat breast cancer.

4 Conclusion

Our findings indicated that *Rhamnus frangula* leaf and bark extracts have anti-proliferative effects on MCF-7 cells *in vitro* and there was greater anticancer potential for leaf than bark extract. We have demonstrated that both *Rhamnus frangula* leaf and bark extracts induce

apoptosis in MCF-7 cells by increasing of Bax and decreasing of Bcl-2 expression level. Higher Bax gene expression level in breast cancer cells exposed to leaf extract accounts for higher cytotoxic effects of leaf extract on MCF-7 cells than bark extract.

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Conflict of interests

The authors declare no conflict of interests regarding publication of this paper.

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