Anti-Proliferative Effects of Ziziphus Spina-Christi and Phlomis Russeliana Leaf 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
To investigate the effects of Phlomis russeliana and Ziziphus spina-christi leaf extracts on apoptosis in breast cancer MCF-7 cells. Cell lines were divided into control group and groups exposed to 0.001, 0.01, 0.1, 1 mg/ml of Ziziphus spina-christi and Phlomis russeliana leaf extracts. Cell viability was quantified by MTT assay. The expression of Bax and Bcl-2 genes was evaluated by Real time PCR analysis. Statistical analysis was performed using ANOVA. HEK239 cells viability significantly increased in groups exposed to 0.001, 0.01 and 0.1 mg/ml of Z.christi leaf extract and decreased in group exposed to 10 mg/ml of P.russeliana leaf extract. MCF-7 cells viability significantly decreased in groups exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of Z.christi leaf extract and increased in groups exposed to 0.001 and 0.01 mg/ml of P.russeliana leaf extract. Exposure of MCF-7 cells to 1 and 10 mg/ml of P.russeliana leaf extract also led to significant decrease in cell viability. The cytotoxic effect of Z.christi was higher than P.russeliana leaf extracts on MCF7 cells. 1 mg/ml of Z.christi leaf extracts also significantly increased the expression level of both Bax and Bcl-2 genes in MCF7 cells. Despite P.russeliana leaf extract, lower Z.christi leaf extract concentrations inhibited MCF-7 cells proliferation. Ziziphus spina-christi and phlomis russeliana leaf extracts mechanism of action is occurred through Bax-independent apoptotic pathway on MCF-7 cells

Keywords: Breast cancer- proliferat- apoptosis

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Introduction
Phlomis russeliana, commonly known as Jerusalem or Turkish Sage, is a species of flowering plant of the Lamiaceae family. This plant is herbaceous and perennial aromatic plants that the genus phlomis, which is widely distributed in Turkey, Iran, Turkmenistan, Afghanistan, and Iraq (Demirchi et al., 2008). Phlomis species contain monoterpenes, sesquiterpenes, alphaliphic compounds, fatty acids (hexadecanoic acid) and other components such as flavonoids, iridoids and phenylethyl alcohol which have been used to treat various disorders such as diabetes, gastric ulcer, hemorrhoid, inflammation and wound (Yesila et al., 2005; Amor et al., 2009).

Ziziphus spina-christi - known as the Christ’s thorn Jujube- is a shrub belongs to Rhamnaceae family that is native in northern and tropical Africa and southern and Western Asia. Plant leaves contain various compounds such as phenolic, flavonoids and aecaloids, including ziziphine, jubanine and ampheline, alpha terpinol, linalol and diverse saponins and the roots are used to treat headaches, while the spines or ashes of this species are applied to snake bites. Boiled leaves are applied to various surface wounds, and also have antihelminthic and antiarrhythmic properties is used to reduce eye inflammations. The fruits are used as an emollient and astringent agent (Defni et al., 2005; Pawlowska et al., 2009; Nawwar et al., 1984; Al-Mamary et al., 2002).

Breast cancer (BC) is a major worldwide health care problem that is the second leading cause of worldwide death among women (Ghaffari et al., 2016). There are many risk factors that led to normal breast cells become cancerous because of mutation in the DNA and increase the chance of developing breast cancer (Hulka et al., 1995).

Apoptotic cell death is a genetically programmed mechanism(s) which maintains the healthy survival/ death

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balance in eukaryotic cells and involves the potentially
determined elimination of cells (Elmore, 2007). Apoptosis
is caused by proteases, known as caspases and occurs
normally as a homeostatic and defense mechanism in
tissues, while enhanced apoptosis may cause degenerative
diseases and may promote carcinogenesis (Fulda, 2010).

The Bcl-2 family proteins include a heterogeneous
group of both pro-apoptotic (BAX) and anti-apoptotic
(Bcl-2) molecules that regulating programmed cell death
through controlling pro-apoptotic and anti-apoptotic
intracellular signals. The apoptotic signals modulate the
central control points of the apoptotic pathways, including
expression of antiapoptotic proteins such as Bcl-2 or by
the downregulation or mutation of proapoptotic proteins
such as BAX (Hassan et al., 2014; McKenzie et al., 2006).

Various studies have shown that Herbal compounds
and natural materials such as Vitamins, Carotenoids, taxol,
camptothecin, vincristine and vinblastine have been an
important source of several clinically useful anti-cancer
agents (Vidhya et al., 2016; Balunas et al., 2005). Studies
suggest that a diet rich in vegetables and fruits, which
are rich sources of antioxidants, may reduce the risk of
cancer (Hocman, 1989). Further studies have indicated
that Angiosperms demonstrate cytotoxic activities
against breast cancer cell lines (MCF-7) (Ali MA et al.,
2014; Han et al., 2009). More studies have shown that
Phenyl propanoid caffeic acid, phenyl ethyl alcohol and
Phenylethyl alcohol glycosides isolated from Phlomis
Species show cytotoxic activity against several kinds of
cancer cells (Saracoglu et al., 1995). Additionally, several
research have shown that Phlomis samia extract could
induce apoptosis so quickly in cancer cells within a few
hours (Ihoual et al., 2017).

Some studies have shown that some plants belong
to Rhamnaeace family can inhibit the proliferation of
cancer cells (Jing et al., 2015; Pawlowska et al., 2009;
Shokrzadeh et al., 2009), and recent advances in cancer
research have shown that high apoptosis level was found
in MCF-7 cell line treated with a member of Rhamnaeace family
(Lombardi et al., 2017). The studies also suggest
that Ziziphus Jujube (Jujube) plant exhibit numerous
medicinal and pharmacological properties and has anti-
cancer and pro-apoptotic abilities in human cervical and
breast cancer cells in vitro (Abedini et al., 2016). New
academic research suggest that aqueous extracts of Z.
spina-christi has cytotoxic effect on cancer cell lines
(Jafarian et al., 2014). The evidences obtained from
clinical studies confirm that Ziziphus spina-christi leaf
extract has antiproliferative influence and pro-apoptotic
abilities on the MCF-7 (human breast adenocarcinoma)
cell line (Farmani et al., 2016).

In contrast, it has been reported that triterpenoids
isolated from Zizyphus jujube can inhibit foam cell
formation in macrophages and can be reason for increase
fatty in vessels (Fujiwara et al., 2011). In addition, recent
data have been shown that a member of Lamiaeae family
has low cytotoxic effects against cancer cells (Oliveira
et al., 2017).

The recent increase in the prevalence and mortality
rates of breast cancer in world (Huang et al., 2009); and
unfortunately there are so many complication and
limitation in methods of healing cancers. Therefore,
research on herbal treatments has a serious role in cancer
therapies. Although previous studies have reported the
anticancer effects of Phlomis and Ziziphus extracts
[20,21], there are few research on the effects of Ziziphus
spina-christi and Phlomis russeliana extracts on cancer
cells at cellular and molecular level. The present study
aimed to investigate the effects of Phlomis russeliana and
Ziziphus spina-christi leaf extracts on apoptosis in breast
cancer MCF-7 cells.

Materials and Methods

Cell line

The human breast cancer (MCF-7) and normal
embryonic kidney (HEK293) cell lines were obtained from
National Cell Bank of Iran (Pasteur Institute, Tehran, Iran)
(Fazeli et al., 2014).

Extracts preparation

The selected plant was collected in different areas
of Guilan province, Iran in June, 2016. The fresh leaf of
Phlomis russeliana and Ziziphus spina-christi cut into
small pieces and washed well in tap water, swabbed with
70% ethanol and allowed to dry in 24 to 26 °C during 7-8
days. Extraction was performed using soaking method. For
this purpose, 100 grams of plant dry weight was mixed
with 300 ml of 80% ethanol (Merck, Germany) and soaked
for 24 hours. The Soxhlet apparatus was then used for
extraction. The extracts were put inside the plates for 24
hours to be dried (Abedini et al., 2016).

Cell culture

The MCF-7 and HEK293 cells were maintained in
complete growth medium (CGM) supplemented with
10% FBS and 1% antibiotics (penicillin/streptomycin).
The cells (1 × 10^6 cells/ml) were plated in T-25 flasks
containing 5 ml of CGM and grown in a humidified
incubator under an atmosphere of 95% air and 5% CO2 at
37°C to subconfluence (90 - 95%). The cell culture
medium was replaced every 48 hours. Once the cells reached 90 -
95% confluency, the medium was aspirated, and the cells
monolayer was washed three times with sterile phosphate
buffered saline. The cell monolayer was treated with 1 ml
of 0.25% (w/v) trypsin-EDTA and incubated briefly at
37°C and visualized microscopically to ensure complete
cell detachment. Cells were re-suspended in complete
growth medium. Cells were also stained with trypan blue
(100 μl of cell suspension and 100 μl of 0.4% trypan blue),
icubated for 2 minutes at room temperature, and counted
using a hemacytometer. The cells were seeded at a density
of 6 × 10^3 cells/ well in 96-well microtiter tissue culture
plates prior to testosterone treatment.

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
MCF-7 and HEK293 cells exposed to 0.001, 0.01, 0.1, 1
and 10 μg/ml of Phlomis russeliana and Ziziphus spina-
chresti leaf extracts. Cell viability was determined using
the MTT assay 24 hours after incubation. The MTT assays were performed according to standard protocols. 43 MCF-7 and HEK cells were seeded in 96-well plates with \(6 \times 10^3\) cells/well and placed at 37°C in a 5% CO\(_2\) humidified incubator until 60% confluence (Kobayashi et al., 2013).

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 *Phlomis russeliana* and *Ziziphus spina-christi* leaf extracts: 0.001, 0.01, 0.1, 1 and 10 μg/ml 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 H\(_2\)O) was added to each well. The plates were incubated under 95% atmosphere air and 5% CO\(_2\) 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.

### 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 1 Kit (ABI, 4369016, American) and LightCycler apparatus (Roche Diagnostics). The Quantitative RT-PCR for *BAX* and Bcl2 genes was carried out using the specific primers (as shown in 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.

### Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA method) followed by post hoc Tukey’s multiple comparisons test in SPSS 20 software. Differences were considered significant at the P<0.05 level.

### Results

viability of HEK239 cells exposed to 0.001, 0.01, 0.01, 1 and 10 μg/ml of *Phlomis russeliana* and *Ziziphus spina-christi* leaf extracts in cell culture. HEK239 cells viability significantly increased in groups exposed to 0.001 and 0.01 mg/ml of *Z.christi* leaf extract compared to control group (P<0.01) and exposure of HEK293 cells to 0.1 mg/ml of *Z.christi* leaf extract also led to significant increase in viability of HEK293 cells compared with control group (P<0.05) (as shown in figure 1). However, there was no significant difference between viability of HEK239 cells exposed to 1 and 10 mg/ml of *Z.christi* leaf extract compared to control group. HEK239 cells viability significantly decreased in group exposed to 10 mg/ml of *P.russeliana* 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, 0.1 and 1mg/ml of *P.russeliana* leaf extract compared to control group.

### Table 1. Specific Primers for BAX, BCL-2 and GAPDH Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5’TGCACCACCAACTGCTTA3’ (Forward)</td>
</tr>
<tr>
<td></td>
<td>5’GGATGCAGGGATBATGTTC3’ (Reverse)</td>
</tr>
<tr>
<td>BAX</td>
<td>5’TGGAGCTGCAGAGGATGATTG3’ (Forward)</td>
</tr>
<tr>
<td></td>
<td>5’GAAGTTGCCGTCAGAAAACATG3’ (Reverse)</td>
</tr>
<tr>
<td>BCL-2</td>
<td>5’CTGCACCTGAGCAGGCTTACC3’ (Forward)</td>
</tr>
<tr>
<td></td>
<td>5’CACATGACCCCACCGAATCTCAAGA3’ (Reverse)</td>
</tr>
</tbody>
</table>

Figure 1. Effect of *Ziziphus Spina-Christi* and *Phlomis Russeliana* Leaf Extracts on HEK293 Cells Viability. The cells were treated with different concentrations of *Z. christi* and *P. russeliana* 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 *P. russeliana* extract.
to control group. Meanwhile, there was also significant difference in cell viability between HEK239 cells exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of P.russeliana leaf extract compared to HEK239 cells exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml Z.christi leaf extract (P<0.01).

viability of MCF-7 cells exposed to 0.001, 0.01, 0.1, 1 and 10 μg/ml of *Phlomis russeliana* and *Ziziphus spina-christi* leaf extracts in cell culture. According to figure 2, MCF-7 cells viability significantly decreased in groups exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of *Z.christi* leaf extract compared to control group (P<0.01). However, MCF-7 cells viability significantly increased in group exposed to 0.001 and 0.01 mg/ml of *P.russeliana* leaf extract compared to control group (P<0.01 and P<0.05, respectively). There was also no significant difference in cell viability between MCF-7 cells exposed to 0.1 mg/ml of *P.russeliana* leaf extract compared to control group. Exposure of MCF-7 cells to 1 and 10 mg/ml of *P.russeliana* leaf extract also led to significant decrease in viability of MCF-7 cells compared with control group (P<0.01). Meanwhile, there was also significant difference in cell viability between MCF-7 cells exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of *P.russeliana* leaf extract compared to MCF-7 cells exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of *Z.christi* leaf extract (P<0.01) (see figure 2).

Expression of pro-apoptotic *BAX* and anti-apoptotic *Bcl-2* genes in MCF-7 cells exposed to 1 mg/ml of *Ziziphus spina-christi* and 10 mg/ml of *Phlomis russeliana* leaf extracts. According to figure 3, to examine the alteration of apoptosis regulating genes expression by *Ziziphus spina-christi* and *Phlomis russeliana* leaf extracts in MCF7 cells, we investigated the effect of 1 mg/ml of *Z.christi* and 10 mg/ml of *P.russeliana* leaf extracts (as cytotoxic dose) on expression level of *GAPDH*, *BAX* and *Bcl-2* genes. The results revealed that 1 mg/ml of *Z.christi* leaf extracts significantly increased the expression level of both pro-apoptotic *BAX* and anti-apoptotic *Bcl-2* genes in
Discussion

Our findings indicated that cell viability increases in non-cancerous human embryonic kidney cells exposed to low Ziziphus spina-christi leaf extract concentration; However, exposure of HEK293 cells to high concentrations of Phlomis russeliana extracts gives rise to decreased cell viability, demonstrating that low level of Z.christi 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 demonstrated low 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 the growth of non-cancerous cells (Medjakovik et al., 2016).

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

Previous studies have also reported that members of Rhamnaceae and lamiaceae families have been used in traditional medicine for treatment of cancer (Plastina et al., 2012; Dranickova et al., 2013); and according to new research, some extract components of plants belonging to Rhamnaceae and lamiaceae families represent natural products that can prevent and treat breast cancer (Bishayee et al., 2011; Berdowska et al., 2013).

In an experimental study, Ziziphus spina-christi has antiproliferative effect on MCF-7 cell line (Farmani et al., 2016). The total extract of several Phlomis species show cytotoxicity activity against some human cancer cell lines specially including MCF-7 cell line (Sarkhail et al., 2017). However, there are reports showing that some Rhamnaceae and lamiaceae extracts has less anti-cancer effect than other herbal extracts (Oliveira et al., 2017; Tepkeeva et al., 2008).

Our observation demonstrated that both Z.christi and Prusseliana leaf extracts induces MCF-7 cell apoptosis via BAX-independent pathway which BAX expression level does not change or increases and Bcl-2 expression level also increases. Recent advances in cancer also have shown that apoptosis was induced in MCF-7 cell line via BAX-independent pathways such as caspase-3 activation and downregulating the expression of survivin (O’Donovan et al., 2003; Devarjan et al., 2002). However, apoptosis could be induced in breast cancer cell lines via BAX-dependent pathway in which BAX expression level increases and Bcl-2 expression level decreases (De Angelis et al., 1998).

Several phytochemical reports have also revealed that flavonoids and fatty acids such as linoleic acid, oleic acid and stearic acid existing in plants belonging to the Rhamnaceae and Lamiaceae family may have an inhibitory effect on the mammalian cell cycle (Amor et al., 2009; Taherghorabi et al., 2015), thus this compound can have anti-cancer effects in the live creatures (Du et al., 2013; Zafari-Shayan et al., 2016). It has also been shown that flavonoids and fatty acids can induce apoptosis in many kinds of cancer cells. It has been demonstrated that flavonoids and fatty acids induce caspase-3 activation (Das et al., 2010; Wü et al., 2017). Once caspase-3 is activated, downstream death substrates are cleaved irrespective of the involvement of cytochrome c. But caspase-3 might also amplify the upstream death cascade, including cytochrome c release from mitochondria, by cleaving Bcl-2, converting it from an anti-apoptotic to a pro-apoptotic protein (Ding et al., 2017). According to our results, when BAX expression level is increasing, Bcl-2 expression level has also increased and according to results of various studies, Bcl-2 is cleaved by caspase action and is converted to pro-apoptotic protein (Ding et al., 2017; Bellows et al., 2000), so we are suggesting that increasing Bcl-2 expression level may have associated with cleaving Bcl-2 and converting it to pro-apoptotic protein.

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 extracts of Phlomis russeliana and Ziziphus spina-christi. 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 vitro and in vivo experiments. In summary, leaf extracts of Phlomis russeliana and Ziziphus spina-christi showed significant reduction in in vitro breast cancer cells proliferation by inducing BAX-independent apoptosis. However, more studies are needed to investigate the exact molecular mechanisms underlying anticancer activity of the Phlomis russeliana and Ziziphus spina-christi leaf extracts. Altogether, natural extracts of Phlomis russeliana and Ziziphus spina-christi use may hold promise as an adjuvant treatment to prevent or treat breast cancer.

Our findings indicated that lower Z.christi and higher Prusseliana leaf extracts concentration have anti-proliferative effects on MCF-7 cells in vitro and there was greater anticancer potential for Z.christi than P.russeliana leaf extract. Lower Z.christi leaf extract concentration, other than inhibitory effects on cancer cells growth, has also proliferative effects on non-cancerous cells. We have demonstrated that both Z.christi and P.russeliana leaf extracts induce apoptosis in MCF-7 cells by BAX-independent apoptosis pathway. Although the mechanism of action is still not well defined but the results of this study should be useful in future investigation of cancer studies and/or therapy.
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