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 a control group and the groups exposed to 0.001, 0.01, 0.1, 1, and 10 mg/ml of Ziziphus spina-christi and Phlomis russeliana leaf extracts. Cell viability was quantified by the MTT assay. The expression of Bax and Bcl-2 genes was evaluated by Real-time PCR analysis. Statistical analysis was performed using ANOVA. HEK293 cell viability significantly increased in the groups exposed to 0.001, 0.01, and 0.1 mg/ml of Z.christi leaf extract and decreased in the group exposed to 10 mg/ml of P.russeliana leaf extract. MCF-7 cells viability significantly decreased in the groups exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of Z.christi leaf extract and increased in the groups exposed to 0.001 and 0.01 mg/ml of P.russeliana leaf extract. The exposure of MCF-7 cells to 1 and 10 mg/ml of P.russeliana leaf extract also led to a 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 extract also significantly increased the expression level of Bax and Bcl-2 genes in MCF7 cells. Bcl-2 gene expression significantly increased in the group exposed to 10 mg/ml of P.russeliana leaf extract. 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 has occurred through the Bax-independent apoptotic pathway on MCF-7 cells.

Keywords: Breast cancer- proliferat- apoptosis

Introduction

Phlomis russeliana, commonly known as Jerusalem or Turkish Sage, is a flowering plant of the Lamiaceae family. This plant is the herbaceous and perennial aromatic plant that the genus phlomis is widely distributed in Turkey, Iran, Turkmenistan, Afghanistan, and Iraq (Demirchi et al., 2008). Phlomis species contain monoterpenes, sesquiterpenes, aliphalic compounds, fatty acids (hexadecanoic acid), and other components such as flavonoids, iridoids, and phenylethyl alcohol, which were 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 Christ’s thorn Jujube-is a shrub belonging to the Rhamnaceae family native in northern and tropical Africa and southern and western Asia. Plant leaves contain various compounds such as phenolic, flavonoids, and alcaloids, including ziziphine, jubanine and amphibine, alpha terpinol, linalol and diverse saponins, and the roots are used to treat headaches. While, the spines or ashes of these species are applied to snake bites. Boiled leaves are applied to various surface wounds which have antihelminthic and antidiarrhetic properties to reduce eye inflammation. 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 which is the second leading cause of cancer death among women (Ghaffari et al., 2016). Many risk factors led to normal breast cells become cancerous due to the 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) that maintains the healthy survival/ death
balance in eukaryotic cells which involves the potentially determined elimination of cells (Elmore, 2007). Proteases, known as caspases cause apoptosis, and normally occurs as a homeostatic and defense mechanism in tissues, while enhanced apoptosis causes degenerative diseases and may promote carcinogenesis (Fulda, 2010). The Bcl-2 family proteins include a heterogeneous group of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) molecules which regulate programmed cell death by controlling pro-apoptotic and anti-apoptotic intracellular signals. The apoptotic signals modulate the central control points of apoptotic pathways including the expression of antiapoptotic proteins such as Bcl-2 or by down-regulation or mutation of proapoptotic proteins such as Bax (Hassan et al., 2014; McKenzie et al., 2006).

Various studies showed that herb compounds and natural materials such as Vitamins, Carotenoids, taxol, camptothecin, vincristine, and vinblastine were the important sources of several clinically useful anticancer agents (Vidhya et al., 2016; Balunas et al., 2005). Studies suggest that a rich diet in vegetables and fruits which are the rich sources of antioxidants may reduce cancer risk (Hocman, 1989). Further studies indicated that Angiosperms show the 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 cancer cells (Saracoglu et al., 1995). Moreover, several researches indicated that Phlomis samia extract could induce apoptosis so quickly in cancer cells within a few hours (Ihoulal et al., 2017).

Some studies showed that some plants belong to the Rhamnaceae family can inhibit cancer cell proliferation (Jing et al., 2015; Pawlowska et al., 2009; Shokrzadeh et al., 2009). Recent advances in cancer research have shown that high apoptosis level was found in the MCF-7 cell line treated with a member of the Rhamnaceae family (Lombardi et al., 2017). The studies also suggest that Ziziphus Jujube (Jujube) plants show numerous medicinal and pharmacological properties and have anticancer and pro-apoptotic abilities in human cervical and breast cancer cells in vitro (Abedini et al., 2016). New academic research suggests that aqueous extracts of Ziziphus spina-christi and Ziziphus spina-christi cut into small pieces, washed well in tap water, swabbed with 70% ethanol and dried in 24 to 26°C during 7-8 days. The extraction was performed using the soaking method. To this purpose, 100 grams of plant’s dry weight was mixed with 300 ml of 80% ethanol (Merck, Germany) and soaked for 24 hours. Then, Soxhlet apparatus was used for the 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 × 10⁶ cells/ml) were plated in T-25 flasks containing 5 mls of CGM and grown in a humidified incubator under 95% air and 5% CO2 at 37°C to subconfluence (90 - 95%). The culture medium was replaced every 48 hours. When the cells reached 90 - 95% confluence, 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 the complete growth medium. Cells were also stained with trypan blue (100 μl of cell suspension and 100 μl of 0.4% trypan blue), incubated for 2 minutes at room temperature, and counted using a hemacytometer.

Cytotoxicity assay
The MTT [3-(4,5-dimethy1thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to assess 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-christi leaf extracts. Cell viability was determined using the MTT assay 24 hours after incubation. The MTT assays were performed due to standard protocols. MCF-7 and HEK cells were seeded in 96-well plates with 6 × 10⁴ cells/well which placed at 37°C in a 5% CO₂ humidified incubator until 60% confluence (Kobayashi et al., 2013).
The complete growth medium was removed, and the cells were serum-starved for 24 h before 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 a 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.

**Quantitative Real Time-PCR Analysis**

HEK293 and MCF-7 cells were seeded in dishes at 500,000 cells/10 mL/75 cm². 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 RNAeasy 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 real-time PCR was carried out as using a LightCycler-FastStart DNA master SYBR Green I 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 interesting genes calculated by 2^ΔΔCT method and SYBR Green kit. After quantitative real-time RT-PCR reactions, the presence of the expected PCR products was confirmed by an agarose gel electrophoresis.

**Data analysis**

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

**Results**

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

HEK293 cell viability significantly decreased in the group exposed to 10 mg/ml of *P. russeliana* leaf extract compared to the control group (P<0.01). There was also no significant difference in cell viability among HEK293 cells exposed to 0.001, 0.01, 0.1, and 10 mg/ml of *P. russeliana* leaf extract compared to the control group. Meanwhile, there was also significant difference in cell viability between HEK293 cells exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of *P. russeliana* leaf extract compared to HEK293 cells exposed to 0.001, 0.01, 0.1, and 10 mg/ml of *Z.christi* leaf of extract (P<0.01).

The viability of MCF-7 cells exposed to 0.001, 0.01, 0.1, 1 and 10 μg/ml of *P. russeliana* and *Ziziphus spina-christi* leaf extracts in cell culture. MCF-7 cells viability significantly increased in groups exposed to 0.001 and 0.01 mg/ml of *Z.christi* leaf extract compared to the control group (P<0.01), and exposure of MCF-7 cells to 0.1 mg/ml of *Z.christi* leaf extract also led to a significant increase in viability of MCF-7 cells compared with the control group (P<0.05) (as shown in Figure 1). However, there was no significant difference between MCF-7 cells’ viability exposed to 1 and 10 mg/ml of *Z.christi* leaf extract compared to the control group. 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 of extract (P<0.01).

The viability of MCF-7 cells exposed to 0.001, 0.01, 0.1, 1 and 10 μg/ml of *Ziziphus spina-christi* and *Phlomis russeliana* leaf extracts compared to control group.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>5'TGCACCACCAACTGCTTA3' (Forward)</td>
</tr>
<tr>
<td></td>
<td>5'GGATGCAAGGGATBATGTTTC3' (Reverse)</td>
</tr>
<tr>
<td>BAX</td>
<td>5'TGAGGCTCAGAGGATGATTG3' (Forward)</td>
</tr>
<tr>
<td></td>
<td>5'GAAGTGGCGTCAAGAAAACATG3' (Reverse)</td>
</tr>
<tr>
<td>BCL-2</td>
<td>5'CTGCACCTGAGCCTCTACACC3' (Forward)</td>
</tr>
<tr>
<td></td>
<td>5'CACATGACCCACCCGAATCTAAAGA3' (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.

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0.01, 1 and 10 μg/ml of Phlomis russeliana and Ziziphus spina-christi leaf extracts in cell culture. Due to figure 2, MCF-7 cells viability significantly decreased in the groups exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of Z.christi leaf extract compared to the 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 among MCF-7 cells exposed to 0.1 mg/ml of P.russeliana leaf extract compared to control group. The 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 the control group (P<0.01). Meanwhile, there was also significant difference in cell viability among MCF-7 cells exposed to 0.001, 0.01, 0.1, 1 and 10 mg/ml of Z.christi 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 Figure2).

The 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. Due 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 the expression level of GAPDH, Bax and Bcl-2 genes. The results indicated that 1 mg/ml of Z.christi leaf extracts significantly increased the expression level of pro-apoptotic Bax and anti-apoptotic Bcl-2 genes in MCF7 cells (P<0.01). However, there was no significant difference among the expression of Bax gene exposed to 10 mg/ml of P.russeliana leaf extract compared to the control group , Bcl-2 gene expression significantly increased in the group exposed to 10 mg/ml of P.russeliana leaf extract compared to the control group.
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, the exposure of HEK293 cells to high concentrations of Phlomis russeliana extracts gives rise to decreased cell viability show that low level of Z.christi leaf extract may have protective effects on non-cancerous cells. In line with this finding, studies show some plants species leaf extracts indicated low toxicity against non-cancerous cells compared with cancerous cells (Strzemski et al., 2017; Bishayee et al., 2011). However, plant extracts can inhibit the growth of non-cancerous cells (Medjakovic et al., 2016).

We have shown that higher concentrations of Z.christi and Prusselliana leaf extracts had anti-proliferative effects on MCF-7 cells; however, only a lower concentration of Z.christi leaf extract can inhibit MCF-7 cells proliferation, and a lower concentration of Prusselliana leaf extract can promote MCF-7 cells viability show greater anticancer potential of Z.christi leaf than Prusselliana leaf extract. Besides, all concentrations of leaf extract on MCF-7 cells had higher cytotoxic effects against MCF-7 cells when compared one to one with Prusselliana leaf extract.

Previous studies have also reported that members of Rhamnaceae and lamicaeae families were used in traditional medicine for the treatment of cancer (Plastina et al., 2012; Srancikova et al., 2013); based on new research, some extract components of plants belonging to Rhamnaceae and lamicaeae 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 the MCF-7 cell line (Farmani et al., 2016). The total extract of several Phlomis species shows cytotoxicity activity against some human cancer cell lines especially including MCF-7 cell line (Sarkhail et al., 2017). However, reports show that some Rhamnaceae and lamiceae extracts have a less anti-cancer effect than other herbal extracts (Oliveira et al., 2017; Tepkeeva et al., 2008).

Our observation indicated that Z.christi and P. russeliana leaf extracts induce MCF-7 cell apoptosis via a Bax-independent pathway in which Bax expression level does not change or increases, and Bel-2 expression level also increases. Recent advances in cancer have also shown that apoptosis was induced in the MCF-7 cell line via Bax-independent pathways such as caspase-3 activation and down-regulating survivin expression (O’Donovan et al., 2003; Devarjan et al., 2002). However, apoptosis can be induced in breast cancer cell lines via a Bax-dependent pathway in which Bax expression level increases, and Bel-2 expression level decreases (De Angelis et al., 1998).

Several phytochemical reports have also indicated that flavonoids and fatty acids such as linoleic acid, oleic acid and, stearic acid existing in plants belonging to the Rhamnaceae and Lamicaeae 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 indicated that flavonoids and fatty acids can induce apoptosis in many cancer cells. It showed that flavonoids and fatty acids induce caspase-3 activation (Das et al., 2010; Wu et al., 2017). Once caspase-3 is activated, downstream death substrates are cleaved irrespective of the involvement of cytochrome c. Caspase-3 may 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). Due to our results, when Bax expression level increases, Bcl-2 expression level has also increased, and considering the results of various studies, Bcl-2 is cleaved by caspase action which is converted to pro-apoptotic protein (Ding et al., 2017; Bellows et al., 2000), Hence, we suggest that increasing Bcl-2 expression level may have associated to cleavage Bcl-2 and converting it to pro-apoptotic protein.

The main purpose of this study was to evaluate the apoptosis induction in breast cancer (MCF-7) cells treated with a 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 naturally derived compounds on cancer cells have a place in vitro and in vivo experiments. In brief, the leaf extracts of Phlomis russeliana and Ziziphus spina-christi showed a significant reduction in in vitro breast cancer cell proliferation by inducing Bax-independent apoptosis. However, more studies are needed to investigate the exact molecular mechanisms underlying the anticancer activity of Phlomis russeliana and Ziziphus spina-christi leaf extracts. Furthermore, 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 Prusselliana leaf extract concentration have anti-proliferative effects on MCF-7 cells in vitro, and there was greater anticancer potential for Z.christi than Prusselliana leaf extract. Lower Z.christi leaf extract concentration, other than inhibitory effects on cancer cell growth, has also proliferative effects on non-cancerous cells. We indicated that Z.christi and Prusselliana leaf extracts induce apoptosis in MCF-7 cells by the Bax-independent apoptosis pathway. Although the action mechanism is still not well defined, this study’s results should be useful in the future investigation of cancer studies and/ or therapy.

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Effects of Ziziphus spina-christi and Phlomis russeliana Leaf Extracts on apoptosis in Breast Cancer

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