The Effect of *Dracocephalum kotschyi* Alcoholic Extract on the *BCL2* and *BAX* Expression in SKBR3 Cell Line

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**ABSTRACT**

**Background:** Breast cancer is the most prevalent malignancy among females worldwide, and its incidence is growing among Iranian women. Also, Iranian patients with breast cancer are younger than their Western counterparts. In this study, we aimed to investigate the effect of *Dracocephalum kotschyi* alcoholic extract on cell proliferation and viability, as well as the expression level of *BCL2* and *BAX* genes in breast cancer cell lines. *Dracocephalum kotschyi* belongs to the Labiatae family, a wildflower that has long been used in Iranian traditional medicine.

**Materials and Methods:** In this experimental research, the SKBR3 cell line was selected for treatment with the *Dracocephalum kotschyi* alcoholic extract. Cell proliferation and cell viability were evaluated in the presence of different concentrations of the *Dracocephalum kotschyi* alcoholic extract (10, 50, 100, and 500 µg/mL) using MTT-method. Additionally, the impact of the *Dracocephalum kotschyi* alcoholic extract on the expression of *BCL2* and *BAX* genes was assessed using a quantitative real time-polymerase chain reaction.

**Results:** The obtained results indicated the anticancer characteristics of *Dracocephalum kotschyi* alcoholic extract (10, 50, 100, and 500 µg/mL) on cell proliferation and cell viability. In the presence of *Dracocephalum kotschyi* alcoholic extract, the half-maximal inhibitory concentration value of SKBR3 cells was 102.1 at 24 h. Moreover, *Dracocephalum kotschyi* alcoholic extract decreased the expression of *BCL2* and increased the expression of *BAX*.

**Conclusion:** *Dracocephalum kotschyi* holds promises for designing anticancer medicines and investigations on breast cancer.

**Keywords:** *BAX*, *BCL2*, Breast cancer, *Dracocephalum kotschyi*
this regard [1]. Breast cancer is highly prevalent among Iranian women. However, little is known about the epidemiological features of breast cancer among Iranian patients [2]. Breast cancer comprises almost one-third of all malignancies among females. Breast cancer is the second cause of cancer mortality after lung cancer; this cancer is the leading cause of death in American women aged 40-55 years [3].

The primary objective of breast cancer follow up is the diagnosis of potentially treatable relapses [4]. In Iran, breast cancer is among the main category of diagnosed cancers among women and is the fifth most common cause for death [5]. Despite advances in cancer treatment over the past decades, there are many deficiencies in this regard. Plant-derived compounds have been a significant source of clinically helpful anti-cancer agents [6]. Dracocephalum kotschyi, from Labiatae family, is an endemic plant in Iran; this is one of the pharmaceutical and aromatic herbs that can be found in the mountains [7, 8]. One hundred compounds, such as terpenoids, flavonoids, and alkaloids were extracted and identified from Dracocephalum kotschyi. Phenolic compounds, including caffeic acid, chlorogenic acid, phenylpropanoids, and flavonoids in Dracocephalum genus are probably accountable for its antioxidant activity.

Moreover, methoxylated flavones, such as apigenin, luteolin, isokaempferid, crisimaritin, pendulatin, and xanthomicrol have anticancer effects. Luteolin has numerous biological properties, including anti-inflammatory, antioxidant, and anticancer activities [9]. Anticancer therapeutics act by activating the BCL2-regulated cell death pathway. BCL2 requires BAX, or its close relative (BAK1) to block the death of mammalian cells [10]. The BAX gene has a vital role in regulating apoptosis, which is a member of the Bcl-2 gene family; it encodes a 21-kDa protein related to Bcl-2 [11]. Various Bcl-2 family members are generally over- or under-expressed in human breast cancer. The Bcl-2 expression has been related to accurate prognosis, while the reduced expression of BAX has been associated with poor clinical results in human breast cancer. The comprehensive role of Bcl-2 family members is in regulating mammary epithelial cell survival is salient to affect the extension of the novel therapeutic outcome of this cancer [12].

The high expression of Bcl-2 proto-oncogene is observed in 50%-70% of breast cancers. Such overexpression of Bcl-2 leads to resistance to chemotherapy and radiation therapy [13]. Few studies have been conducted on Dracocephalum kotschyi, especially on the effect of this plant on BCL2 and BAX expression in breast cancer. Therefore, the current study investigated the prepared extractions of Dracocephalum kotschyi (0, 10, 50, 100, and 500 µg/mL) on BCL2 and BAX expression in breast cancer cell line (SKBR3) and its cell proliferation and cell viability activity.

Materials and Methods

Preparation of Dracocephalum kotschyi extract

In this study, the leaves and aerial parts of Dracocephalum kotschyi were collected from Semirom City, Isfahan Province, in the central region of Iran. One hundred grams of dried and powdered desired parts of Dracocephalum kotschyi was chopped and soaked in 250 mL ethanol. Next, the Dracocephalum kotschyi extracts (10, 50, 100, and 500 µg/mL) were prepared, similar to our previous study [14].

Cell culture

Briefly, the human breast cancer cell line (SKBR3) was obtained from the Pasteur Institute of Celluar Bank of Iran (Tehran, Iran). The cells were cultured at 37°C, under 5% CO2, in DMEM (Dulbecco’s modified Eagle’s medium (Gibco, USA) fully fitted based on the National Cell Bank of Iran (NCBI) suggestion. Cell line culture was treated with the different concentrations of Dracocephalum kotschyi extract (0, 10, 50, 100, and 500 µg/mL) for 24 h to investigate the effect of Dracocephalum kotschyi alcoholic extracts on the SKBR3 cell proliferation and viability as well as BCL2 and BAX expression.

MTT assay

To investigate the impact of Dracocephalum kotschyi alcoholic extract on cell proliferation and cell viability of SKBR3, we used MTT assay. SKBR3 cell line was treated with the various concentrations of Dracocephalum kotschyi extract (0, 10, 50, 100, and 500 µg/mL) for 24 h. Next, MTT dye (Sigma-Aldrich, USA) (dissolved in DMSO) was added to the last concentration of 100µg/mL. The combination was incubated for 4 h at 37°C in a CO2 incubator. The violet crystal was dissolved in DMSO for 30 min. The absorbance was measured by a spectrophotometer at 540 nm (BioTek, Winooski, VT, USA). All experiments were repeated in three separate trials for 4 times. To determine the half maximal Inhibitory Concentration (IC50), the following formula was applied: Cell proliferation inhibition percentage = mean absorption in extract wells/mean absorption in control wells [15].

RNA extraction and cDNA synthesis
The RNeasy Mini, RNA isolation kit (Qiagen) was used to extract the total RNA from the cells according to the above-mentioned protocol. RNA purity was calculated on the 260/280 nm absorbance ratio after measuring by a NanoDrop spectrometer (Thermo Scientific, Waltham, MA, USA). The synthesis of cDNA was performed by a QuantiTect Reverse Transcription Kit (Qiagen), based on the producer’s instruction.

**Real time polymerase chain reaction assay**

The BCL2 and BAX expression were evaluated using a real-time PCR assay with SYBR Premix Ex Taq II (Takara, Kusatsu, Shiga Prefecture, Japan). Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was selected to normalize mRNA expression levels. In a total volume of 10 μL, cDNA products were added to a master mix comprising 5 μL SYBR Premix Ex Taq II (Takara, Kusatsu, Shiga Prefecture, Japan), 0.5 μL forward primer, 0.5 μL reverse primer, and 3 μL DEPC-treated water. The PCR was run at 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 20 s. The mRNA levels were calculated using the $2^{-\Delta\Delta C_{T}}$ method. In each PCR reaction, a negative control was run with no cDNA template. The procedure was repeated three times with cDNA template synthesized from the same RNA. Primers were designed using the National Center for Biotechnology Information (NCBI) Website and Gene Runner software. Table 1 lists the primer sequences applied for real-time PCR.

**Statistical analysis**

The obtained data were analyzed by Graph Pad Prism (Graph Pad, San Diego, USA). The Kolmogorov–Smirnov test was applied to measure the normality of data. One-way Analysis of Variance (ANOVA) was used to compare the achieved data. Statistical significance was set at the P<0.05. The obtained data were presented as Mean±SD.

**Results**

**Cell proliferation and cell viability analysis**

Table 1. Primer sequences (5'-3')

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>CGGTGGGTCATTGTGTGTG</td>
<td>CGGTTAGGTACTCAGTCCC</td>
<td>90</td>
</tr>
<tr>
<td>BAX</td>
<td>CGAGAGGTCTTTTTCCGAGT</td>
<td>GTGGGCTCCCCAAGTGG</td>
<td>242</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCACCTCCTCACCTTGACG</td>
<td>CCACCACCTGTGTGTAG</td>
<td>107</td>
</tr>
</tbody>
</table>

The MTT assay was applied to investigate the impact of *Dracocephalum kotschyi* alcoholic extract on cell proliferation and cell viability in the SKBR3 cell line after 24 h. In the presence of *Dracocephalum kotschyi* alcoholic extract, the IC50 value of SKBR3 cells was 102.1 at 24 h. The findings indicated a statistically significant difference between increasing the concentration of *Dracocephalum kotschyi* alcoholic extract and cell proliferation and cell viability after 24 h.

Our data indicated that the *Dracocephalum kotschyi* alcoholic extract significantly reduced cell proliferation and cell viability in a dose-dependent manner. By increasing the concentration of the *Dracocephalum kotschyi* alcoholic extract (10, 50, 100, and 500 μg/mL), the cell proliferation rate reduced, compared to the controls (Figure 1). Moreover, by increasing the concentration of the *Dracocephalum kotschyi* alcoholic extract (50, 100, and 500 μg/mL), the cell viability rate decreased. However, there was no significant difference in cell viability rate at the concentration of 10 μg/mL. Eventually, the most reduction in cell viability was observed at 500 μg/mL (Figure 2).

**BCL2 expression analysis**

To explore the effect of *Dracocephalum kotschyi* alcoholic extract on BCL2 gene expression, we investigated the expression level of the BCL2 gene under the influence of the different concentrations of *Dracocephalum kotschyi* alcoholic extract using real-time PCR. The obtained data demonstrated after treatment with the *Dracocephalum kotschyi* alcoholic extract the expression of the BCL2 gene reduced. However, the expression level of BCL2 at the concentrations of 10 μg/mL and 50 μg/mL did not significantly decrease. In contrast, we observed that after treatment with the concentrations of 100 μg/mL and 500 μg/mL, the BCL2 gene expression significantly reduced in a dose-dependent manner. The highest reduction in BCL2 expression was observed after treatment with 500 μg/mL of the *Dracocephalum kotschyi* alcoholic extract (Figure 3).
BAX expression analysis

The effects of the various concentration of *Dracocephalum kotschyi* alcoholic extract on BAX expression was examined by real-time PCR method. We observed that *Dracocephalum kotschyi* alcoholic extract increased the BAX expression in a dose-dependent manner; however, our findings indicated a higher expression in the concentration of 50 µg/mL, compared to the concentration of 100 µg/mL. Additionally, the highest increase in the expression level of BAX was observed at 500 µg/mL of the *Dracocephalum kotschyi* alcoholic extract (Figure 4).

Discussion

In the current study, we examined the effect of *Dracocephalum kotschyi* alcoholic extract at different concentrations (10, 50, 100, and 500 µg/mL) on cell proliferation, cell viability, as well as *BCL2* and *BAX* expression in breast cancer cell line. In this regard, the SKBR3 cell line was selected. This cell line was treated with different concentrations of *Dracocephalum kotschyi* alcoholic extract (0, 10, 50, 100, and 500 µg/mL). Moreover, cell proliferation and cell viability were examined by MTT assay. The findings demonstrated a significant decrease in cell proliferation in a dose-dependent manner.
We also observed a significant reduction in cell viability; however, there was no significant difference at the concentration of 10 μg/mL compared to the controls. Next, the impact of various concentrations of *Dracocephalum kotschyi* alcoholic extract on *BCL2* and *BAX* expression in the SKBR3 cell line was evaluated. Our data demonstrated that the *BCL2* expression was reduced under the influence of *Dracocephalum kotschyi* alcoholic extract; however, there was no statistically significant difference in the expression of the *BCL2* at the concentrations of 10 and 50 μg/mL.

In contrast, we observed an overexpression of *BAX* expression at the various concentrations of *Dracocephalum kotschyi* alcoholic extract. However, the difference was not statistically significant at the concentration of 10 μg/mL. Additionally, the increased expression of *BAX* at the concentration of 50 μg/mL was higher than that of 100 μg/mL. The obtained results suggested that *Dracocephalum kotschyi* is a promising candidate for future anticancer research; however, higher concentrations should also be considered, too.

The controlled liberation of medicines to the exact site of the disease using a nanocarrier vehicle increases the remedial efficiency of the drugs. Most cancer-induced deaths are because of drug resistance [16]. *Dracocephalum kotschyi* inhibits the multiplication of tumor cells; therefore, it can be used as a new medicine against cancer. Iranian researchers have introduced an anticancer drug (Spinal-Z) extracted from *Dracocephalum kotschyi* leaves and Peganum harmala seeds, which strongly affects leukemia [17]. Besides, *Dracocephalum kotschyi* Boiss has a flavonoid, named xanthomicrol which is helpful in antiproliferative activity against cancer cells. Faghihinia et al. isolated 8 flavonoid aglycones from the aerial parts of *Dracocephalum kotschyi*, acknowledged as luteolin, naringenin, apigenin, isokaempferide, cirsimaritin, penduletin, xanthomicrol, and calycopterin. The methoxylated hydroxyflavones (cirsirarin, penduletin, xanthomicrol, and calycopterin) indicated anti-tumor properties [18].

Sonboli et al. have investigated the cytotoxicity and antioxidant activity of the essential oil of *Dracocephalum surmandinum* in several cell lines. K562, MCF-7, and PC12 cell lines were treated with the various concentrations of *Dracocephalum surmandinum* essential oil and its major constituents (perilla aldehyde and limonene). The cell viability decreased after 48 h by 50% at 16 μg/mL in K562, 14 μg/mL in MCF-7, and >100 μg/mL in PC12 cells [17]. It can separate the Bcl2 family into two subgroups, including pro-death and anti-death members. Bak and Bax have been categorized between the pro-death members, as the last gateway of cytochrome c release. In contrast, the anti-apoptotic Bcl-2 members prevent mitochondrial protein liberation by interacting with Bak and Bax [19].

The existence of nuclear factor-κB, Bax, and Bcl-2 factors in *Dracocephalum kotschyi* is required in research as the associate agents in the infusion of cell cycle arrest and apoptosis using apigenin in human prostate carcinoma cells [20]. Investigating anti-cancerous plant compounds affecting the mechanisms and molecular interactions suggested that several phytochemicals with the anticancer potential from various plants led to Bax up-regulation and Bcl-2 down-regulation [21]. Esmaeili et al. have examined the effect of a flavonoid compound isolated from *Dracocephalum kotschyi* (calycopterin) in hepatoblastoma cancer cells. The western bolt analysis has suggested that this flavonoid leads to overexpression of the Bax but decreases the level of Bcl-2 [19].

In conclusion *Dracocephalum kotschyi* can be beneficial for preparing new anticancer medicines. The obtained results indicated the promising results of the alcoholic extract of *Dracocephalum kotschyi* aerial parts on cell proliferation and cell viability in breast cancer cell line. Moreover, the alcoholic extracts of *Dracocephalum kotschyi* down-regulates *BCL2* expression and up-regulates *BAX* expression. More investigations in this area with different types and concentrations of extraction could reveal the anticancer effects of *Dracocephalum kotschyi* more.

Ethical Considerations

Compliance with ethical guidelines

There was no ethical considerations to be considered in this research.

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Authors contribution's

Contributing to study design: Mansoor Salehi and Nasrin Hadi; Contributing to all experimental work and molecular experiments, statistical analysis, and interpretation of data: Fatemeh Ketabchi, Faezeh Namazi, Nasrin Hadi, Farinaz Khosravian; Contribution to evaluate the findings: Mansoor Salehi, Mansoor Salehi, Nasrin Hadi, and Farinaz Khosravian; Approving the final manuscript: All authors.
Conflict of interest
The authors declared no conflicts of interest.

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References