Introduction:
Breast cancer is the second most common cancer after lung cancer in women. Given that herbal preparations have long been used to treat cancer, this study aimed to investigate the cytotoxic effect of hydroalcoholic extract of Kelussia odaratissma Mozaff and Thymus daenesis Celak on MCF-7 cancer cell line.

Materials and methods:
MCF-7 cancer cells and normal fibroblasts were cultured in DMEM medium containing Fetal Bovine Serum (FBS) and antibiotic. The cells were exposed to different doses of hydroalcoholic extract of Kelussia odaratissma Mozaff and Thymus daenesis Celak (0.156, 0.312, 0.625, 1.25, 2.5 mg/ml) and were incubated for 24, 48 and 72 hours. After the incubation period, modified colorimetric MTT method was used to determine the cellular toxicity of the extract.

Results:
The results of the MTT test showed that hydroalcoholic extract of Kelussia odaratissma Mozaff and Thymus daenesis Celak had dose-dependent and time-dependent anti-cancer effect on MCF-7 cancer cells. The highest percentage of cell death was observed with the highest concentration of the extract and after 72 h of incubation (p< 0.05). The extract did not show significant cytotoxicity on normal fibroblasts.

Conclusion:
Hydroalcoholic extract of Kelussia odaratissma Mozaff and Thymus daenesis Celak has cytotoxic effects on MCF-7 cancer cells; however, this extract does not have any cytotoxic effects on normal fibroblasts. It appears that this extract can be used for cancer treatment with further research.

Keywords: Kelussia Odarattissma Mozaff, Thymus Daenesis Celak, MCF-7, MTT Assay
common type of cancer and the second cause of mortality after lung cancer among them (2).

This type of cancer is a complex disease with many risk factors. Breast cancer has remained largely resistant to current therapeutic strategies and many patients develop metastases and ultimately lose their lives (3).

Since many chemical drugs cause digestive disorders and kidney failure, scientists are trying to find drugs with fewer side effects. As a result, medicinal plants have received a lot of attention (4). Many herbs and spices contain elements that can be used to prevent cancer. They can affect different stages of initiation and growth of cancer cells (5).

Wild celery, locally known as Bakhtiari celery and scientifically known as Kelussia odoratissima, is an aromatic, medicinal, herbaceous, and perennial plant of the Apiaceae family. It has branched, hollow, grooved stems with a height of 20 to 60 cm. Its flower sometimes reaches 200 cm in height. The most important habitat of the plant in Iran is in the southwest and Bakhtiari Zagros mountains (6). Phthalide, flavonoid, and terpenoid compounds have been reported in the plant so far (7). The plant has anti-inflammatory, anti-viral, anti-diabetic, anticancer and anti-poisoning effects due to its flavonoids compounds mainly accumulated in seeds, stems, and inflorescences of the plant (6). Phthalides are another group of effective compounds in the plant that pose it a food additive, a chemical anticancer, and liver protection agent and stomach ulcer protection agent (8). Various test results showed that these compounds inhibit gastric tumors by 67-83 percent (6). Studies also found that some compounds available in the seeds and flowers of this plant are potential inhibitors of liver cancer and human melanoma growth (9).

Thymus daenensis Celak is a shrub in the mint family Lamiaceae (10). It is an herbaceous and perennial plant with multiple thick stems and a height of 25-30 cm. T. daenensis is distributed in Chahar Mahal-Bakhtiari, Fars, Hamedan, Ilam, Markazi, Kohgiluyeh-Boyer-Ahmad, and Kurdistan provinces (11). T. daenensis contains tannin, flavonoid, glycoside, caffeic acid and rosmarinic acid compounds (12). According to Barazandeh and Bagherzadeh, thymol, Paracemenu, gamma-terpinene, carvacrol and betacaryophyllene are the main compounds of T. daenensis (13). T. daenensis has tonic, digestive, antispasmodic, carminative, anti-fungal, antibacterial, anti-convulsive, anti-worm, anti-rheumatic, expectorant, and antioxidant properties (14).

The anticancer and anti-carcinogenic properties of T. daenensis have recently received much attention (15). Hamta et al. suggested that the compounds in the hydroalcoholic extract of Thymus vulgaris were responsible for the induction of apoptosis in 4T1 cancer cells. The cytotoxic and anticancer properties of the extract were attributed to its phenolic compounds (15). Keramati et al. also showed that the hydroalcoholic extract of Thymus vulgaris inhibited and cured abnormal growth of precancerous lesions and carcinomas of DMBA (7, 12-Dimethylbenz[a]anthracene) in rats (16).

Cell culture is a modern study method that can be found in almost all branches of science. One goal of cell culture is the study of cells in terms of growth, nutritional needs and growth inhibitors. Therefore, the study of the cell cycle, development of cancer cell growth
methods, and gene expression modulation need in-vitro cell culture (4).

MCF-7 is the human breast cancer cell line, first isolated in 1970 from a 69-year-old Caucasian woman with a metastatic breast cancer. This cell line was used as a useful model for studying cancer ever since (17).

The present study was conducted to investigate the cytotoxic effect of mixed Kelus and Thymus daenensis hydroalcoholic extract on the MCF-7 cell line.

**Materials and Methods**

**Hydroalcoholic extraction of Kelus and Thymus daenensis**

The leaves and stems of Kelus and Thymus daenensis were collected from Chaharmahal-Bakhtiari province and their hydroalcoholic extracts were prepared by the rotary method. The leaves and stems of plants were first dried in shade. Then they were powdered by mechanical grinding and then poured into separate cylinders and the solvent was added. The solvent was 90% ethanol mixed with water. The amount of ethanol was such that completely covered the powders. The resulting solution was placed in an oven at 50 °C for 72 hour, then it was removed from the oven and passed through filter papers. The filtered solution was then gradually and on several occasions placed in a rotary stearic device to be condensed (4). Finally, 50 percent hydroalcoholic extract of Kelus and 50 percent hydroalcoholic extract of Thymus daenensis were mixed, solved in DMEM and filtered by a 0.2-micron filter. The resulting extract was used to prepare different doses.

**Cell culture**

The trial was conducted in the cellular-developemental laboratory of Shahrekord Islamic Azad University. The MCF-7 cell line of breast cancer and normal fibroblast cell line were obtained from the Iranian Biological Research Center. The DMEM culture medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin were used for cell culture of MCF-7 and fibroblast cells. They were cultured and incubated under standard conditions (at 37 °C, 5% CO₂, and 95% humidity). After three passages, the cells were used for subsequent phases. Cell count and the number of living cells with hemocytometer slides were performed using Trypan blue (Sigma). For this, 0.4 g of Trypan blue powder was dissolved in 10 ml of distilled water and then filtered and kept in the refrigerator. After cell trypsinization, 10 µl of cells available in 1 ml of the culture medium were removed and 90 µl of the culture medium was poured in a microtube plus 20 µl of trypan blue and well homogenized. In the next step, 25 µl of the solution was poured on a Neubauer slide and its cells were counted under an inverted microscope (Invert, Nikon, Japan). This had to be done in a short time as cells could be lost because of trypan blue.

**Investigating the toxicity of the mixed Kelus and Thymus daenensis hydroalcoholic extract by MTT test**

The MTT test was used to measure the cytotoxic effect of the mixed Kelus and Thymus daenensis hydroalcoholic extract. In this method, the yellow salt of methylthiazolyl-tetrazolium bromide (MTT) is converted to an insoluble purple formazan compound by mitochondrial dehydrogenase enzymes of active cells.
The optical density of the compound can be measured after dissolving in Dimethyl Sulfoxide (DMSO), with the Eliza reader device at 492-630 nm wavelengths (18). After the flask bed was covered by cells, the cell layer adhered to the bottom of the flask was removed enzymatically using Trypsin/EDTA 0.25% (Bioidea Co.) and transferred to sterile test tubes and centrifuged for 5 minutes at 1200 rpm. The cells were then suspended in the new culture medium with a Pasteur pipette and a cell suspension was prepared. The cells were counted using a Neubauer counting chamber used for white cell counting. The resulting number was divided by 4 and multiplied by the reverse dilution factor and by $10^4$. The resulting number indicated the number of cells in a milliliter of culture medium. After counting, the cells were transferred into flat-bottom, 96-well cell culture plates, $10^4$ cells per well. The plates were then incubated at 37 °C for 24 hours. After that, the supernatant was removed slowly and carefully and new medium and mixed Kelus and Thymus daenensis hydroalcoholic extract was added to all wells at concentrations of 0.156, 0.312, 0.625, 1.25, 2.5 mg/ml (19). Serum-containing media without extract were added to the control wells. The cells in the first, second, third, fourth and fifth wells were exposed to 2.5, 1.25, 0.625, 0.312, and 0.156 mg/ml concentrations of mixed Kelus and Thymus daenensis hydroalcoholic extract. The MCF-7 cancer cells and the normal fibroblast cells in the control group were exposed to DMEM medium. After incubation for 24, 48 and 72 hours, plates were removed from the incubator, the supernatant of each well was completely removed by a sampler and the cells were washed with 100 µl Phosphate-buffered saline (PBS). Then, 80 µl of culture medium and 20 µl of yellow MTT solution (Sigma Co., 50 mg of yellow MTT powder in 10 ml of PBS and filtered through a 0.2-micron filter) were added and the plates were incubated for 3 hours. Later, the supernatant was removed completely and each well was washed with 100 µl of PBS. Then 100 µl of DMSO was added to each well to dissolve the formazan crystals. The color changes were then read by Elisa reader at 492-630 nm wavelengths. The following formula was used to convert the optical density (OD) to the percentage of viable cells, and the viability percentage was calculated after 24, 48 and 72 hours.

$$100 \times \frac{OD_{control}}{OD_{Test}} = \text{viability percentage}$$

The concentration of the tested compound that reduced cell viability to half was considered as IC50 (The half maximal inhibitory concentration).

**Statistical Methods**

The study data was entered into Excel 2010 software and analyzed in SPSS version 23. The difference between groups with a minimum P<0.05 was considered significant. Repeated measures analysis of variance (rANOVA) was used for data analysis.

**Results**

The effect of different concentrations of mixed Kelus and Thymus daenensis hydroalcoholic extract on the viability of MCF-7 cancer cells at various time periods using the MTT method

The statistical analysis of the results showed that the during the 24-hour incubation, the viability reduced with increasing the dose of mixed Kelus and Thymus daenensis hydroalcoholic extract...
in the MCF-7 cell line such that the viability reduced from 92.22% at 0.156 mg/ml concentration to 14.18% at 2.5 mg/ml concentration. The difference was statistically significant (P<0.05). In the 48-hour incubation, a dose-dependent reduction of viability was observed as the viability reduced from 87.14% at 0.156 mg/ml concentration to 12.77% at 2.5 mg/ml concentration. The difference was statistically significant (P<0.05). A reduction of viability was also observed in the 72-hour incubation, where the viability reduced from 73.53% at 0.156 mg/ml concentration to 10.69% at 2.5 mg/ml concentration. The difference was statistically significant (P<0.05).

The results of analysis using rANOVA showed a significant difference in all concentrations and in all three time periods in this cell line (P<0.05). The highest toxicity effect was observed at the concentration of 2.5 mg/ml with 72-hour incubation (Figure 1). The IC50 of mixed Kelus and Thymus daenensis hydroalcoholic extract for MCF-7 cancer cells was obtained at the 0.625 mg/ml concentration.

The effect of different concentrations of mixed Kelus and Thymus daenensis hydroalcoholic extract on the viability of fibroblast normal cells at various time periods using the MTT method

Fibroblast cells were treated with different concentrations of mixed Kelus and Thymus daenensis hydroalcoholic extract (0.156, 0.312, 0.625, 1.25, 2.5 mg/ml) for 24, 48 and 72 hours. The results of the MTT method showed that the mixed Kelus and Thymus daenensis hydroalcoholic extract had little effect on normal fibroblast cells (Figure 2).

The MCF-7 cancer cell growth after 72 hours of treatment with mixed Kelus and Thymus daenensis hydroalcoholic extract at a concentration of 2.5 mg/ml (Figure 1B) was inhibited compared to the control group (not treated with mixed Kelus and Thymus daenensis hydroalcoholic extract) (Shape 1A). The MCF-7 cancer cells after exposure to a concentration of 5.2 mg/ml of mixed Kelus and Thymus daenensis hydroalcoholic extract were distorted and their morphology changed, indicating the cytotoxicity effect of the mixed Kelus and Thymus daenensis hydroalcoholic extract.

Table 1: The effect of different concentrations of mixed Kelus and Thymus daenensis hydroalcoholic extract on the viability of MCF-7 cancer cells at various time periods using the MTT method

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration</th>
<th>0.156</th>
<th>0.312</th>
<th>0.625</th>
<th>1.25</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>92.22±2.17</td>
<td>86.11±3.53</td>
<td>74.74±2.26</td>
<td>41.96±3.26</td>
<td>14.18±0.898</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td>87.14±2.32</td>
<td>80.09±2.16</td>
<td>68.79±4.55</td>
<td>30.91±3.08</td>
<td>12.77±0.740</td>
<td></td>
</tr>
<tr>
<td>72 hours</td>
<td>73.53±3.58</td>
<td>61.15±2.37</td>
<td>54.57±1.70</td>
<td>24.63±1.70</td>
<td>10.69±1.22</td>
<td></td>
</tr>
</tbody>
</table>

* The mean viability had a significant difference in different concentrations (P<0.05).
** The mean viability had a significant difference in different time periods (P<0.05).
*** The numbers represent the mean ± SD.
Table 2: The effect of different concentrations of mixed Kelus and Thymus daenensis hydroalcoholic extract on the viability of normal fibroblast cells at various time periods using the MTT method.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration</th>
<th>0.156 ±0.06</th>
<th>0.312 ±0.74</th>
<th>0.625 ±0.86</th>
<th>1.25 ±1.29</th>
<th>2.5 ±0.24</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours</td>
<td>107.07±1.06</td>
<td>104.65±0.74</td>
<td>100.49±0.86</td>
<td>95.67±1.29</td>
<td>91.35±0.24</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td>101.66±2.22</td>
<td>99.13±1.33</td>
<td>94.53±1.07</td>
<td>90.89±1.10</td>
<td>85.74±1.03</td>
<td></td>
</tr>
<tr>
<td>72 hours</td>
<td>99.12±0.40</td>
<td>95.42±0.82</td>
<td>89.74±1.34</td>
<td>86.99±0.29</td>
<td>82.07±0.63</td>
<td></td>
</tr>
</tbody>
</table>

* The mean viability has a significant difference in different concentrations (P<0.05); the 0.156 and 0.312 concentrations were not significantly different (p =0.079).

** The mean viability has a significant difference in different time periods (P<0.05).

*** The numbers represent the mean ± SD.

Figure 1: A

Figure 1: B

Figure 1: (A) MCF-7 cancer cells in the control group without treatment with mixed Kelus and Thymus daenensis hydroalcoholic extract; (B) MCF-7 cancer cells after 72 hours of treatment with the mixed Kelus and Thymus daenensis hydroalcoholic extract at a concentration of 2.5 mg/ml (×10).
Discussion

The results showed that the mixed Kelus and Thymus daenensis hydroalcoholic extract had a cytotoxic effect on MCF-7 cancer cells causing apoptosis, while having little toxicity on normal fibroblast cells.

Many herbs and spices have pharmacological and biochemical properties like antioxidant and anti-inflammatory properties that appear to play a role in the anticancer and antimutagenic activities in the cell. Since tumor development is closely related to inflammation and oxidative stress, an antioxidant or anti-inflammatory compound can have anticancer properties (20).

According to Saeedi et al. and Sajjadi et al., Kelussia odoratissima Mozaff has some compounds such as flavonoids (Limonene, myrcene, camphene, camphor, 3-O-methyl ether, etc.), Ferulic acid, Phthalide ((Z)-ligustilide, (E)-3-butyldiene phthalide, (E)-ligustilide, etc.), caffeic acid, terpenoids (α-terpinen-al, α-Pennine, β-Pennine, etc.) (21, 22). Kelus also contains phenolic and flavonoids compounds and thus has free radical-scavenging and antioxidant properties (7).

Thymus daenensis has many applications in traditional medicine including its antimicrobial activity against fungal and bacterial isolates, and its antioxidant properties are somewhat approved because of having phenolic compounds (23). Its therapeutic properties can be due to its flavonoids and phenolic compounds (24).

Phenolic compounds and flavonoids have several biological properties such as antioxidant, anti-inflammatory, trapping free radicals, etc. These compounds also prevent or delay oxidative damages in lipids and other important molecules and prevent the development of cancer and coronary heart disease (25).

Studies have shown that plant extracts rich in phenolic compounds and flavonoids protect the cells by reducing oxidative stress. Phenolic compounds are aromatic plant secondary metabolites, widely distributed throughout the plant with numerous biological effects such antioxidant and antibacterial activities (26). Phenolic compounds including vitamins, pigments, and flavonoids, have antimutagenic and anticancer properties. The antioxidant activity of phenolic compounds in plants is mainly due to their regenerating power and chemical structure, which enable them to neutralize free radicals, form complexes with metal ions and turn off the singlet and triple oxygen molecules. Phenolic compounds inhibit oxidation reactions by donating electrons to free radicals (27). Hence, it is likely that phenolic compounds and flavonoids in the mixed Kelus and Thymus daenensis hydroalcoholic extract reduce oxidative stress by free radical-scavenging.

Rana et al. (2008) showed that thyme extract improved antioxidant potential and thus helped to prevent oxidative stress (28). Angel et al. (2009) showed that antioxidant compounds such as phenolic compounds were contributing factors in cytotoxicity of A.Campesteris. Phenolic compounds protect cells against active oxygen species (29).

Studies showed a significant difference between the three treatment time periods (24, 48 and 72 hours), such that by increasing the time and concentration of hydroalcoholic extract of Thymus daenensis, cytotoxicity effects on MCF-7 cancer increased, while having little cytotoxicity on normal fibroblast cells. The results of this study are consistent
with the results from Evaluation of the Effects of the hydroalcoholic extract red cabbage on Growth inhibition of breast cancer cell line MCF-7 and Human foreskin fibroblast (HFF) cells by Mahdian et al. suggesting that the red cabbage hydroalcoholic extract inhibited cancer cell growth dose and time-dependently, while having no toxicity on normal fibroblast cells in any time period (30).

Cytotoxic effects of hydroalcoholic extract of green and ripe blueberries on three cell lines MCF-7 (Human breast adenocarcinoma), HepG2 (Liver cancer) and CHO (Normal hamster ovary) with MTT method by Rezaei et al. showed that the extract had a significant dose- and time-dependent cytotoxic effect on cancer cells, while having no toxicity on normal fibroblast cells. Their results are consistent with the results of the present study (31). These results could be the first step in examining and identifying anticancer compounds. However, studies have shown that plant compounds and their derivatives can be considered part of the standard protocols for cancer treatment and an effective tool for prevention and treatment of cancer. Due to the extensive diversity of plants, researchers have a long way ahead for research in this area.

Conclusion
The results of this study suggested that the mixed Kelus and Thymus daenensis hydroalcoholic extract had a dose- and time-dependent anticancer effect on MCF-7 cancer cells and can inhibit the growth of these cells, such that by increasing the time and dose, cancer cell growth is inhibited more. The mixed Kelus and Thymus daenensis hydroalcoholic extract had no significant effect on normal fibroblast cells.

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Conflict of interest
The Authors declare that there is no conflict of interest in this paper.

References:


