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Anti-tumor Effects of Essential Oils of Red Clover and RagWeed on MCF-7 Breast Cancer Cell Line

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Abstract

Introduction: Breast cancer is one of the most common malignancies among Iranian women. Nowadays, the use of traditional medicinal plants has emerged as a tempting complementary to the treatment of breast cancer due to minimal side effects and less documented drug resistance. The aim of this study was to examine the anticancer effects of the essential oils of red clover and mature fruit of *Bassia scoparia* on MCF-7 cells.
Methods: Essential oils were extracted from *Bassia scoparia* and red clover plants, by a Clevenger condenser and mass spectrometry was performed for qualitative analysis, then MCF-7 and HU02 cells were treated with different concentrations of these essential oils at different time intervals. The viability of cells was measured by the MTT assay. The results were analyzed using the one-way ANOVA and Tukey's test.
Results: Essential oils of Kochia scoparia and red clover have cytotoxic effects on MCF7 cells. The MTT analysis showed decreased cell viability percentage in treated cells ($P < 0.05$). In addition, the microscopic examination of cells treated with essential oils revealed morphological changes of apoptotic cells in MCF-7 cells.
Conclusions: The findings of this study show that essential oils of *Bassia scoparia* and Red clover have toxic and anticancer effects on MCF-7 cells.

INTRODUCTION

Breast cancer is the most common invasive cancer in women. It is the leading cause of death worldwide and accounts for about 18% of cancer cases in women. The incidence of this cancer is much higher in developed countries compared to developing countries, which is associated with multiple reasons. The life expectancy is probably one of these causes, and lifestyle, dietary habits, fertility, environment, and physical activity play important roles in the incidence of breast cancer as well. Several systemic chemotherapeutic agents such as 5-fluorouracil, trastuzumab, cyclophosphamide, doxorubicin, bevacizumab, and tamoxifen are used to treat this disorder. However, despite their anticancer effects, these drugs have adverse side effects such as cardiac toxicity, bone marrow failure, thrombocytopenia, and mucositis. Therefore, the use of novel and more effective drugs with minimum side effects, including traditional herbal drugs and natural substances, may be useful to prevent the development and progression of breast cancer [1, 2]. Red clover (*Trifolium pratense L.*) is one of the forage plants belonging to the *Trifolium* species of the leguminous family. It grows in temperate and humid regions and plays an important role as livestock feed. Its flowers have a sweet and delicious flavor and have been traditionally used as a seasoning in salads, soups, desserts, and beverages all over the world [3]. This plant contains flavonoids, coumarins, coumestans, and isoflavones, which can be used orally or topically. Red clover is believed to be effective in the treatment of cancers related to hormonal disorders. It is also used to reduce the rate of hot flushes in women with premature menopause as part of their cancer treatment. Early studies have also shown that isoflavones in red clover may be useful in treating prostate and colon cancers, but there

is still insufficient evidence in this regard [4].
Bassia scoparia fruit is an annual plant of Chenopodiaceae family, which is usually harvested in autumn and dried in the sun throughout China. It is widely used as a medicinal plant for treating dysuria and skin disease in China and Japan. *Bassia scoparia* has particularly been used for the treatment of breast masses and chest pain. Various types of compounds, including triterpenoid glycosides, saponins, and alkaloids have been isolated from this herb. Furthermore, the anti-inflammatory and anti-allergic effects of *K. scoparia* have been demonstrated. Recently, it was reported that *Bassia scoparia* fruit also has potential antitumor effects, but its anticancer mechanism has not been determined [5]. Hey Yeon Han et al. showed that the methanol extracted from the dried fruit of *K. scoparia* (MEKS) inhibits cell proliferation and induces apoptosis in MDA-MB-231 breast cancer cell line [6]. They also showed the apoptotic effect of essential oil of *K. scoparia* fruit on oral squamous cell carcinoma (OSCC) cell line using the MTT method [7]. In this study, the possible cytotoxic and anticancer effects of *K. scoparia* and red clover on the MCF-7 breast cancer cell line were investigated.

METHODS

The MCF-7 and HU02 Cell Lines

The MCF-7 cell line (a breast cancer cell line) and HU02 (a human fibroblast cell line) were purchased from National Center of Genetic and Biological Reserves of Iran. Cell culture materials were purchased from Gibco Company (USA), but the flasks and microplates were acquired from Griner Company (Germany).

Collection of Plant Samples

The samples of *Trifolium pratense L.* and *Bassia scoparia*, which were harvested from Boroujerd city of Lorestan Province, were confirmed by Faculty of Natural Science, and deposited at the Herbarium of the Department of Boroujerd Islamic Azad University. Next, the collected plant materials were dried in a dark place and after drying were packed in paper bags in which they were preserved until the experiments were conducted.

Essential Oil Extraction

The selected method to isolate essential oils is dependent upon the original composition of the plant (alkaloids, flavonoids, terpenes, and sugars) as well as the purity degree of the final product. In this study, a Clevenger condenser was used for the extraction of essential oils using distillation. First, 20g of a chopped and dried sample from both plants' leaves were placed in the flask (1000 mL) and 400-500 mL distilled water was added to it. Before extraction, the trays were washed with acetone, water, alcohol, and eventually water, respectively. Next, the water temperature was increased to boiling temperature. Heating continued until the first drop of liquid appeared in G-part at the end of the cooling part. Consequently, the essential oil was collected and kept in sealed bottles in the refrigerator (+4˚C). This process lasted 3-5 hours.

Mass Spectrometry

Qualitative analysis was performed using an Agilent 7980 gas chromatograph (GC) equipped with an Agilent 5975C mass selective detector (MS). The MS detector was operated in the electron impact system with the interface temperature of 280°C. Also, the IE source temperature was set to 150°C, analyzer temperature was set to 230°C, and interface temperature between GC and MS was set on 420˚C.

Cell Culture

In this experimental study, MCF-7 and HU02 cells were grown in a RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 2 mM/L glutamine, 100 unit/mL penicillin, and 100 µg/mL streptomycin in a 5% CO2 incubator (Memmert) at a temperature of 37°C under standard cell culture conditions.

Cell Viability Measured by the MTT Assay

The effects of different concentrations of *Bassia scoparia* and *Trifolium pratense L.* on cell viability were examined by the MTT [3-(4,5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] colorimetric method. Briefly, the cells were put into a 96-well culture plate at a density of 10 000 cells/well and incubated with various concentrations of *K. scoparia* and *Trifolium pratense L.* for 24h. After removing the culture medium, the cells were incubated with MTT solution (5 mg/mL in PBS) for 3 hours, and the resulting formazan was solubilized using 150-200 µL of DMSO (Sigma). The absorbance was measured at 570-590 nm in a plate reader (BioTeK ELx800).

Microscopic Examination

The effects of different concentrations of essential oils of *Bassia scoparia* and red clover on morphology of MCF-7 and HU02 cells were examined by microscopic observation. For this purpose, first the cells were treated with various concentration of essential oils of *Bassia scoparia* and red clover for 24, 48 and 72 hours. Then, the morphological changes were examined by an invert microscope (Olympus).

Statistical Analysis

The SPSS 20 software was used to perform statistical analyses. The significance of differences between the ex-

perimental variables was determined using the one-way ANOVA analysis and Tukey's test. A probability level of $P<0.05$ was considered as statistically significant.

RESULTS

2mL of essential oils was extracted from 400g dried leaves of *K. scoparia* and red clover by the distillation method. The results of extraction were analyzed using the GC/MS mass spectrometry and are presented in Table 1 and 2. According to these results, the main compounds in the essential oil of *K. scoparia* included α-thujaplicin, phytoene, butylated hydroxytoluene, dictamnol, phytol, n-docosane and the important compounds of red clover were hexanal 2-ethylfuran, 2-methyl-2, 4-hexadiene, and cyclopentanol.

Table 1: Essential Oil Composition of Aerial Parts of Red Clover Samples Collected in Boroujerd

Number	Contents (Shoot)	Area	RT (min)
1	Isobutane	1847771	1.89
2	Methoxyformamide	1523033	1.97
3	Pentanal	552258	2.701
4	2-propen-1-ol	706088	2.814
5	Cyclopentanol	116639	3.078
6	2-ethylfuran	216453	3.357
Number	**Contents (Flower)**	**Area**	**RT (min)**
1	Isobutane	2146156	1.826
2	Methoxyformamide	3610489	1.974
3	Pentanal	1254088	2.699
4	2-propen-1-ol	3608496	2.812
5	2-methyl-2,4-hexadiene	221914	3.344
6	Hexanal	247138	6.165

Table 2: Essential Oil Composition of *K. scoparia* Samples Collected in Boroujerd

Number	Contents	Area	RT(min)
1	*n*-eicosane	2000	2.61
2	*n*-henicosane	2100	3.1
3	*n*-docosane	2200	3.17
4	*n*-tricosane	2300	1.23
5	*n*-tetracosane	2400	1.87
6	Isopropyl tiglate	976	0.91
7	*n*-decane	999	0.66
8	P-methyl anisol	1019	0.43
9	Camphenilone	1082	2.11
10	Maltol	1108	2.09
11	*Z*-damascone	1358	2.33
12	Orcinol	1371	0.92
13	*E*-damascenone	1385	1.12
14	O-methyl eugenol	1401	1.33
15	α-thujaplicin	1412	16.89
16	Dictamnol	1430	6.51
17	Butylated hydroxytoluene	1512	7.49
18	*n*-hexadecane	1600	0.76
19	2,6,10,-tetramethylpentadecane	1688	0.64
20	*n*-heptadecane	1700	1.49
21	Phytone	1790	9.11
22	*n*-octadecane	1800	0.56
23	Phytoene	1815	1.19
24	*n*-nonadecane	1900	2.92
25	Phytol	1943	5.51
26	Isophytol	1948	0.98

Essential Oils of Red Clover and *K. scoparia* Cause Morphologic Changes in MCF-7 Cells

The morphological changes related to essential oils of red clover and *K. scoparia* fruit on the MCF-7 cancer cell line and natural HU02 fibroblast cell line were examined by microscopic observation after treatment of these cells with different concentrations of essential oils for 24, 48, and 72 hours. As shown in figure 1, the HU02 cells treated with different concentrations of essential oils of red clover and *K. scoparia* showed no significant morphological changes as compared to control cells. These results showed that the essential oils of red clover and *K. scoparia* had no effect on control cells while morphological changes in the treated MCF-7 cancer cells were significant as compared with MCF-7 control cells. As shown in figure 1, morphological changes are visible as star-like shapes, vacuolation, cytoplasmic and cellular shrinkage as well as pyknotic nuclei, which are attributes of apoptotic cells. These observations indicated the cytotoxic effects of essential oils of red clover and *K. scoparia* on MCF-7 cancer cells. Interestingly, as shown in figure 1, the observed morphological changes were more frequent and obvious with increasing dose and duration of cell treatment by essential oils of red clover and *K. scoparia*, indicating the toxic effects of these essential oils in a dose- and time-dependent manner on MCF-7 cancer cell lines.

Red Clover Essential Oil Induced Cell Death in MCF-7 Cells

The viability of MCF-7 cells was assessed by the MTT assay after treatment of cells with various concentrations of red clover's essential oil for 24, 48, and 72 hours, as shown figure 2 Next, the cells were stained using MTT solution (containing tetrazolium salt), and the absorbance was finally measured by an ELISA reader. As presented in figure 2, the cytotoxic effect of the essential oil of red clover on MCF-7 cells was dose- and time-dependent. In addition, it was indicated that the percentage of viable cells was significantly decreased ($P<0.05$) at 15 and 30 µg/mL concentrations of essential oils of red clover. Table 3 shows the results related to one of the triplicate mean cell viability assays as mean ± standard deviation (Mean ± SD). Moreover, the minimum inhibitory concentration (IC50) value of red clover's essential oil on growth of MCF-7 cells was observed in 125 µg/mL concentration. The percentage of viable cells treated with red clover's essential oil was calculated using the following formula as compared with control cells:

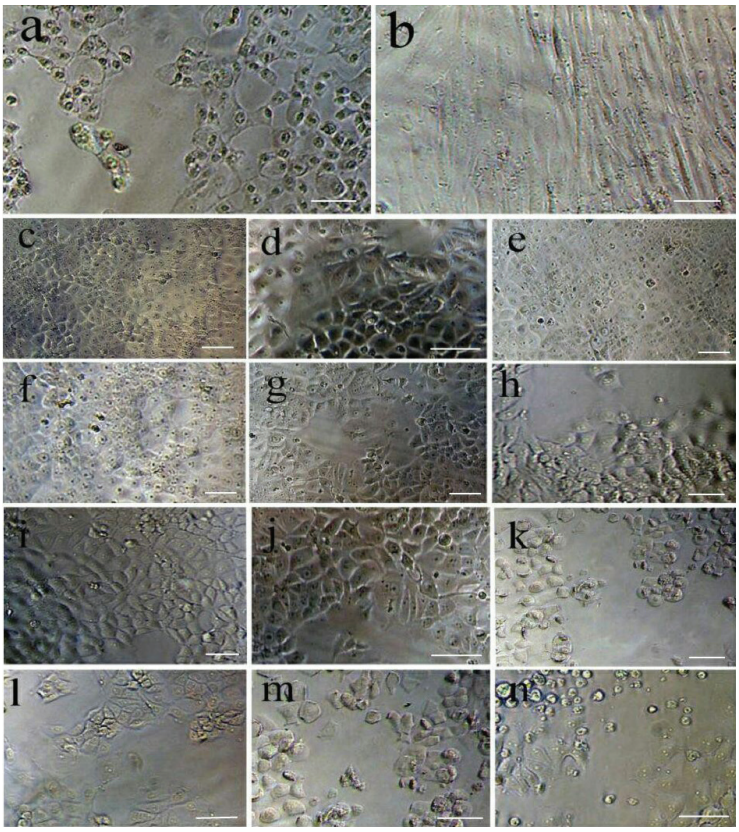

Figure 1: Morphologic Changes in MCF-7 and HU02 Cells Treated with Essential Oils with a Dose of 100µg/mL of Red Clover and *K. scoparia*.
a: MCF-7 control cells; b: HU02 cells; c-e: MCF-7 cells treated with red clover essential oils; f-h: MCF-7 cells treated with *K. scoparia* essential oils; i-k: MCF-7 cells treated with red clover essential oils, l-n: MCF-7 cells treated with broom grass essential oils within 24, 48, and 72 hours.
All the pictures have a 50-micrometer scale bar.

Table 3: Test Results of One of the Triplicate Mean Cell Viability Tests for MCF-7 Cells After Incubation with Red Clover's Essential Oil as Mean ± SD

Time (hour)	Control	15µg/ml	30µg/ml	60µg/ml	125µg/ml	250µg/ml
24	100	97.83 ± 1.44	88.42 ± 1.95	62.51 ± 1.73	57.63 ± 0.91	47.33 ± 0.17
48	100	93.18 ± 0.55	81.33 ± 0.63	61.05 ± 0.11	54.19 ± 1.78	47.19 ± 0.54
72	100	90.34 ± 0.21	69.73 ± 1.49	60.48 ± 0.77	51.88 ± 1.41	47.05 ± 1.83

Table 4: Test Results of one of the Triplicate Mean Cell Viability Tests for MCF-7 Cells After Incubation with Essential Oil of *K. scoparia* as Mean ± SD

Time (hour)	Control	15µg/ml	30µg/ml	60µg/ml	125µg/ml	250µg/ml
24	100	95.35 ± 0.82	73.69 ± 0.34	59.83 ± 0.25	46.2 ± 0.98	41.9 ± 1.53
48	100	89.22 ± 0.76	68.83 ± 0.80	53.7 ± 0.12	45.66 ± 0.36	38.16 ± 0.77
72	100	85.49 ± 0.37	60.94 ± 0.33	51.4 ± 0.54	44.97 ± 0.75	38.09 ± 1.4

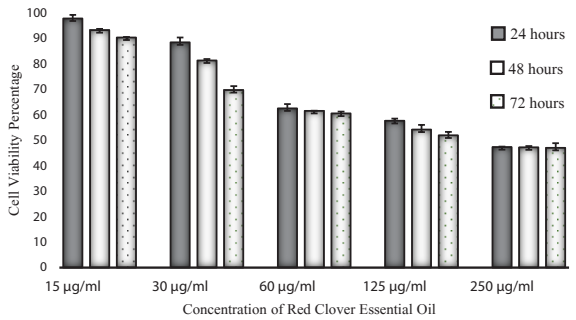

Figure 2: The Results of One of the Triplicate MTT Tests.
Results show the toxicity effects and cell death of red clover's essential oil in 30µg/mL concentration in a dose- and time-dependent manner, which is significant (P<0.05), but in higher concentrations of 60, 125 and 250µg/mL, the decrease in cell viability is only dose-dependent.

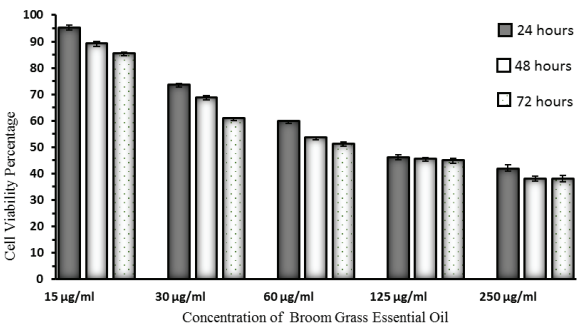

Figure 3: The Results of One of the Triplicate MTT Tests.
Results show the toxicity effects of essential oils of k. scoparia in 30µg/mL concentration in a dose- and time-dependent manner, which is significant (P<0.05) but in higher concentrations of 60, 125, and 250µg/mL, cell viability reduction is dose-dependent only.

(light absorbance of cells treated with red clover' s essential oil)/(the average of light absorbance in control cells)×100

Essential Oil of *K. scoparia* Induced Cell Death in MCF-7 Cells

The viability of MCF-7 cells was examined by the MTT assay after the treatment of MCF-7 cells with different concentrations of the essential oil of *Bassia scoparia* for 24, 48, and 72 hours, as presented in figure 3. As shown in this figure, the cytotoxic effects of essential oil of *K. scoparia* on MCF-7 cells were dose- and time-dependent, which was similar to the results of the cell viability assay of MCF-7 cells treated with red clover essential oil. Moreover, it was revealed that the percentage of dead cells was significantly increased ($P < 0.05$) at 15 and 30 µg/mL concentrations of essential oil of *K. scoparia*. The results of one of the triplicate tests of the MTT assay have shown in Table 3 as mean ± SD. As shown in figure 3 and Table 4, the mean cell viability was significantly decreased by increasing concentrations of essential oils up to 60, 125, and 250µg/mL, while increased incubation time in these concentrations did not significantly change the mean cell viability. Furthermore, the 125 µg/mL concentration of *K. scoparia* essential oil is the minimum inhibitory concentration (IC50) value of MCF-7 cells proliferation. The percentage of viable cells treated with the essential oil of *K. scoparia* was calculated using the above formula.

DISCUSSION

Recently, many studies have been conducted to evaluate the anticancer effects of various essential oils and extracts, all of which demonstrate the ability of such compounds to inhibit the growth of a wide range of pathogenic microorganisms and food spoilage agents, as well as other factors affecting tumor growth. Because these compounds are completely natural, they have much less harmful effects on human health and environment as compared with chemical agents [8]. Iran has the history of several thousand years of civilization and a rich Islamic culture as well as a unique geograph-

ic location where a large number of plant species can be grown. The number of plant species in Iran is five times that of the whole European continent [9]. In general, 11,000 plant species have been identified and collected from Iran, which are classified in 180 families and 1200 genera and could be considered as one of the richest sources available for health and economic aspects [10]. In this study, essential oils of red clover and *Bassia scoparia* fruit were used to assess their antitumor and antitoxic effects on MCF-7 breast cancer cells. Different studies have been conducted to examine the effect of essential oil of *Bassia scoparia* on apoptosis induction as well as its effects on preventing the proliferation of different cancer cells, including breast cells, oral squamous cell carcinoma (OSCC) cell line and hematologic malignancies such as Hodgkin's lymphoma. These studies demonstrated the antitumor and inhibitory effects of the essential oil of *K. scoparia* fruit on the proliferation of cancer cells. The present study showed the antitumor and antitoxic effects of essential oils of red clover and *Bassia scoparia* on MCF-7 cells. The analysis of essential oils of the plants indicated that the most frequent components in the essential oil of *K. scoparia* included α-thujaplicine, phytoene, and butylated hydroxytoluene, dictamnol, phytol, and n-docosane. In addition, the most frequent compounds in red clover included hexanal, 2-ethyl furan, 2-methyl-2,4-hexadiene, and cyclopentanol. Biljana Kaurinovic et al. identified β-myrcene (4.55%), p-cymene (3.59%), limonene (0.86%) and tetrahydroionone (1.56%) as the most frequent compounds in red clover [11]. Examining the results of mean cell viability using the MTT method reveals the sensitivity of MCF-7 cancer cells to essential oils of red clover and *K. scoparia* fruit. In similar studies, the cytotoxic effect of essential oil of *K. scoparia* on MDA-MB-231 cancer cells (breast cancer cell line) and oral squamous carcinoma cell line (OSCC) has been shown by Hey-yeon et al. [6]. Mazzio and Soleiman found the antitumor effect of the essential oil of *K. scoparia* in their study [12]. Furthermore, a study by Hey Yeon et al. on the effect of essential oil of *K. scoparia* on the morphology of MDA-MB-231 cells showed morphological changes similar to apoptotic cells, including vacuolation, cytoplasmic and cellular shrinkage as well as pyknotic nuclei. These changes were also observed in the present study on MCF-7 cells treated with essential oils of red clover and *K. scoparia* fruit. Morphological changes and apoptotic characteristics in MCF-7 calls were reinforced by increasing concentrations of essential oils of red clover and *K. scoparia* as well as increasing incubation time. Han H-Y et al. showed that the essential oil of *K. scoparia* fruit prevents cell cycle by arresting the G1 phase of cell cycle, which in turn decreases the percentage of viable cells [6]. As we know, reactive oxygen species (ROS) are the intrinsic activators of apoptosis capable of activating apoptosis from the extrinsic path. Han H-Y et al. indicated that the essential oil of *K. scoparia* fruit significantly increased the ROS levels. They believed that an increase in intrinsic ROS triggers both intrinsic and extrinsic apoptosis pathways and prevents cell cycle. Furthermore, Wang et al. reported that Momordin IC, a triterpenoid-rich saponin in different medicinal plants, including *K. scoparia*, has antitumor effects in HepG2 cells [13]. Han H-Y et al. also stated in their study that further research on the effective factors present in the fruit of this plant, such as Mormordin (), which also exist in other plants like *M. balsamina* and Ampelopsis radix [14], can raise *K. scoparia* as a useful herbal medicine in treating or preventing breast cancer. Chen et al. showed that treating cancer cells with demethylated cantharidin from (NCTD) P21 resulted in 3-caspase inhibitor activation, which consequently led to apoptosis in HepG2 cells [15].

Based on our findings, the essential oils of red clover and *K. scoparia* inhibit the proliferation of the MCF-7 breast cancer cell line. In this regard, further studies will be helpful to understand possible molecular apoptotic mechanisms of essential oils of red clover and *K. scoparia* and to identify other major compounds in these plants as well as their potential antitumor characteristics.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

ETHICS APPROVAL

The use of cell lines in this study does not involve ethical considerations.

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