

COMPARATIVE STUDY ON THE TOXICITY OF *MENTHA PIPERITA* L. AND *ARTEMISIA DRACUNCULUS* L. HYDROALCOHOLIC EXTRACTS ON HUMAN BREAST CANCER CELL LINES

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ABSTRACT

As the second prevalent cancer in women, breast cancer is a global epidemiologic problem and the medications used nowadays are not very effective being associated with undesirable complications. Due to the side effects of many chemical drugs, the use of natural drugs has increased dramatically over the past decade. This study aimed to investigate the toxicity of mint [*Mentha piperita* L. (Lamiaceae)] and tarragon (*Artemisia dracunculus* L.) hydroalcoholic extracts on MCF-7, Hu -02, T-47D, MDA-MB-231, and MCF-10A cell lines *in vitro* using the MTT assay.

Extracted EOs (Essential Oils) were analyzed and identified by a GC/MS device. The effects of EOs from both plants at different concentrations (3, 7, 15, 30, 60, 125, and 500 µg/ml) were evaluated on the aforesaid cell lines for 24 h. The cytotoxicity effect was assessed by the MTT assay. The optical absorbance was measured by an ELISA system at 570 nm wavelength.

Results indicated significant cytotoxicity of EOs from mint leave on growth of the breast cancer cell lines (MCF-7, T-47D, MDA-MB-231) and normal cell lines (MCF-10A, Hu-02) in a dose-dependent manner (P<0.05).

The mint extracted EO has cytotoxic effects against MCF-7, T-47D, MDA-MB-231 breast cancer cells more than tarragon extracted EO. Both EOs causing lesser toxicity on healthy fibroblast (Hu-02) and healthy breast epithelial cells (MCF-10A).

Our findings suggest that the potential anticancer effects of mint extracted EO should be further exploited in future studies.

Keywords: Mint, tarragon, cytotoxicity, MTT assay, breast cancer, MCF

INTRODUCTION

Cancer is the second mortality cause after cardiovascular disease in many societies, including Iran (Hajian *et al.*, 2003). With an annual incidence rate of 1.2 million new patients worldwide, breast cancer is the most prevalent malignancy among women and the leading cause of cancer-related deaths among the American women (Samadi *et al.*, 2008). Chemotherapy is a conventional method used for the control of breast cancer development (Shahbazi *et al.*, 2013), but it is associated with side effects and even irreversible tissue damages in intact organs due to the selectivity of drugs used in this method (Sabzichi *et al.*, 2014); besides, drug resistance in the treatment process provides a poor prognosis (Easton *et al.*, 2006). Therefore, the use of novel herbal compounds with less toxicity and side effects is an essential research topic (Maleksabet *et al.*, 2014). Natural products, in particular flowers and plants, have been used for the treatment of various diseases during thousands of years. Due to the side effects of many chemical drugs, the use of natural drug alternatives has increased dramatically over the past decade (Hemati *et al.*, 2010). Anticancer activities have been demonstrated for a number of herbal medicines. The scant scientific evidence concerning the activation paths of herbal medicines leads to reductions in their clinical uses (Eisenberg *et al.*, 1993). Mint (*Mentha piperita*) is a herbaceous, perennial, and cross hybrid plant that propagates through germination. This plant is endemic to Europe, but is grown in most temperate regions of the world, and is spread in the majority of Iran, particularly in Alborz hillsides, the north and northeastern regions, and some other areas (Samsam *et al.*, 1997). Active ingredients of mint plant include volatile oils (1%), resins, and tannins (Amin *et al.*, 1992). The properties of this plant have been known since long time ago and its extract, leaves, and EOs are today used in traditional medicine, food industries (as a flavoring agent), and in the manufacture of cosmetics (Judy *et al.*, 2007). In the mint EO, menthol is the most abundant active ingredient, with free menthol and menthol esters comprising 30-70% of volatile oils. In traditional medicine, mint is used as stomach tonic, carminative, anti-

convulsion, and tranquilizer. Mint EO has also been shown to alleviate abdominal pain through mitigating smooth muscle spasm in the digestive tract (Nouraldini *et al.*, 2007; Samsam-Shariat *et al.*, 2007). Mint prevents cancer development as it contains flavonoids and rosmarinic acid. Additionally, EOs and extracts prepared from some mint species in other countries were reported to have antioxidant and antimicrobial effects (Golluce *et al.*, 2007).

Tarragon (*Artemisia dracunculus*), from the asteraceae family, with a vast worldwide distribution and numerous nutritional applications, and existing evidence is indicative of its beneficial effects in ailments and disease conditions (Ivorra *et al.*, 1989). Tarragon aerial organs contain EOs, the most important ingredients of which include estragole (anethole isomers), α -pinene, β -pinene, camphene, sabinene, myrcene, flandren, limonene, linalool, delta-4-carene, α -flandren, and cis- and trans-ocimene (Duke *et al.*, 2001). Different species of tarragon have been found to possess antimicrobial properties, which inhibit the growth of numerous Gram-positive and Gram-negative bacteria (Mehrotra *et al.*, 1993). The presence of antifungal effects has been confirmed in scientific reports (Mehrotra *et al.*, 1993; Kordali *et al.*, 2005). This plant is also used in the treatment of digestive problems and disorders, nausea, bloat, and hiccup (Mehrotra *et al.*, 1993).

Medicinal plants are of special value and importance in providing health in societies with regard to disease treatment and prevention, one of which include the use of antioxidant properties of these plants. The oxidative stress process caused by the free radical activity is considered as the causative agent of many diseases, such as cancers, cardiovascular disease, diabetes, Alzheimer's, and aging process, and antioxidants inhibit free radicals and reduce oxidative reaction rates (Jang *et al.*, 2010). There is a plethora of research on the discovery of plant-derived anticancer compounds. Accordingly, the compounds contained in mint and tarragon plants are expected to be utilized in the treatment of breast cancer.

MATERIAL AND METHODS

Extraction of EOs

For EO extraction, 150 g of the plant aerial organ was partially crushed, poured in a 1 L balloon of a cleverger device, and some water (4-6 times the plant weight) was added to soften the plant tissue. The contained EOs were then extracted by the device for 2 h, followed by collecting the distilled EOs.

Preparation of essential oil different dilutions

Dilutions of 3, 7, 30, 60, 125, and 500 $\mu\text{g/mL}$ were used in this study. The calculated DMSO level was $< 1\%$ in the final solution in the cellulose culture wells. DMSO is not toxic at concentrations $< 1\%$, hence its concentration is important in this respect.

Cell lines

Cell lines used here were procured from National Cell Bank of Iran (Pasteur Institute of Iran, Tehran). Cells were cultured in the RPMI1640 medium containing heat-inactivated 10% fetal bovine serum (FBS), penicillin (100 unit/mL), and streptomycin (100 $\mu\text{g/mL}$). The experimental cell lines were incubated at 37 °C with 5% CO₂ atmosphere.

MTT assay

The cytotoxicity of mint and tarragon extracts was measured by the MTT assay. In this test, the MTT (yellowish tetrazolium bromide salt) is converted into an insoluble, purple compound, formazan, by mitochondrial dehydrogenases in active cells. After being dissolved in DMSO, the optical absorbance of this compound was measured by an ELISA reader at 570 nm wavelength (Mosmann, 1983).

Cytotoxicity assessment of the obtained EOs by the MTT assay

After 24 h, 20 μL of MTT solution (5 $\mu\text{g/mL}$) was added to each well. Plates were incubated for 3-4 h, the residue was removed, and then 100 μL of DMSO was added to obtain formazan. After shaking the plates by a plate shaker for 10 min, formazan absorption was read at 570 nm using a plate reader. Cell-containing, EO-free wells were considered as the control optical density (OD) and ODs of cell-free wells with only PRMI 1640 and FBS were regarded as the blank. Cell viability was calculated by the following formula:

$$\text{Cell viability} = \text{Control OD} / \text{treatment OD} \times 100$$

Obtained results were examined by a computer and then analyzed statistically by San Raphael, (Jandel CA) SIGMASTAT™, analysis of variance (ANOVA), and Tukey's test.

RESULTS

The percentages of the compounds in Table 1 were determined by the GC/MS device. The growth inhibition of breast cancer cell lines was investigated by mint EOs. The growth rate was considered to be 100% in negative control samples and the growth rate of the cells was examined at different EO concentrations.

The highest (about 60%) and lowest (about 99%) MCF-7, T-47D, MDA-MB-231 growth inhibition rates were found at 500 and 7 μ g/ml of mint EOs, respectively. At the same EOs concentrations, the highest (about 95%) and lowest (about 99%) growth inhibition rates were observed for Hu-02 and MCF-10A normal cell lines, respectively (Fig.1).

The percentages of the compounds in Table 3 were measured by the GC/MS device. The growth inhibition of breast cancer cell lines was examined by tarragon EOs. The growth rate was considered to be 100% in negative control samples and the growth rate of the cells was examined at different EO concentrations.

The highest (about 95%) and lowest (about 99%-100%) MCF-7, T-47D, MDA-MB-231 growth inhibition rates were detected at 500 and 7 μ g/ml oftarragon EOs, respectively. At the same EO concentrations, the highest (about 95%-98%) and lowest (about 99%-100%) growth inhibition rates were recorded for Hu-02 and MCF-10A normal cell lines, respectively (Fig.2).

Figure 3 displays the growth inhibition on the MCF-7 division process affected by tarragon and mint EOs at different concentrations. Tarragon EO had the highest and similar growth inhibitory effect at concentrations of 250 and 500 mg/L, but not at 3, 7, 15, 30, 60, 125 mg/L; but mint EO exerted the inhibitory effect at all of concentrations except at 3 mg/L.

Table1. Percentages of compounds found in mint essential oil

1	9.695	1,2-propanediol
2	1.493	isopulegol 1
3	0.166]cyclohexanol,5-methyl-2-[1-methylethyl]-,cis
4	0.475	neo-menthol
5	83.037	cyclohexanol,5-methyl-2-[1-methylethyl]-,[1.alpha.,2beta.,5.alpha.]
6	0.318	1-[-]menthol
7	0.122	3-methyl-4-isopropylphenol
8	3.848	benzoic acid,
9	0.846	bis[2-ethylhexyl]

Table2. Comparison of the effect of mint EO on the MCF-7, T-47D, MDA-MB-231, Hu-02 and MCF-10A cells.

	P value	Mean MCF-7	Mean T-47D	Mean MDA-MB-231	Mean Hu-02	Mean MCF-10A	
Control	0.995218	100.0	100.0	100.0	100.0	100.0	
7	0.00155	93.201	91.101	92.402	99.999	99.999	
15	< 0.0001	90.878	89.778	88.156	99.999	99.999	
30	< 0.0001	76.351	75.251	77.333	100.0	100.0	
60	0.00197	72.297	71.195	70.121	100.0	100.0	
125	0.0008	70.777	69.117	69.354	99.873	99.873	
250	< 0.0001	60.093	59.097	58.066	97.297	98.122	
500	< 0.0001	55.701	54.555	53.344	94.679	96.444	

MCF-7 = A human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors

T47D= An ideal experimental model cell line to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer.

MDA-MB-231= An epithelial cell line, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories.

Hu-02=A human fibroblast cell line and MCF-10A= A non-tumorigenic epithelial cell line.

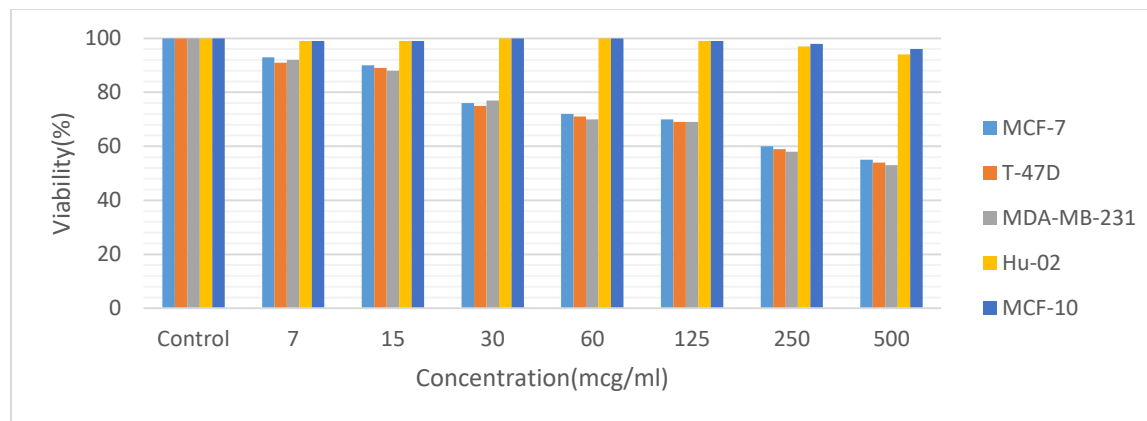


Fig-1. The effect of mint EO on the MCF-7, T-47D, MDA-MB-231, Hu-02 and MCF-10A cells.

MCF-7 = A human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors

T47D= An ideal experimental model cell line to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. MDA-MB-231= An epithelial cell line, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories. Hu-02=A human fibroblast cell line and MCF-10A= A non-tumorigenic epithelial cell line.

Table 3. Percentages of compounds found in tarragon essential oil.

1	66.879	estragole
2	0.109	camphene
3	0.240	sabinene
4	0.289	beta-pinene
5	5.276	d-limonene
6	9.099	cis-ocimene
7	9.058	1,3,6-octatriene,3,7- di methyl
8	5.276	d-limonene
9	1.162	alpha- terpinolene
10	9.058	1,3,6-octatriene,3,7- di methyl

Table 4. Comparison of the effect of Tarragon EO on the MCF-7, T-47D, MDA-MB-231 Hu-02 and MCF-10A cells.

	P value	Mean MCF-7	Mean T-47D	Mean MDA-MB-231	Mean Hu-02	Mean MCF-10A
Control	> 0.9999	100.0	100.0	100.0	100.0	100.0
7	0.8405	99.99	100.0	100.0	99.86	100.0
15	0.3146	99.997	100.0	100.0	99.638	99.838
30	0.84711	99.0	100.0	100.0	99.728	99.767
60	0.9323	99.0	100.0	100.0	99.954	99.958
125	0.6094	98.8733	99.879	100.0	98.779	99.879
250	0.55993	97.297	97.997	98.999	98.793	99.788
500	0.1088	94.6793	96.993	97.679	96.738	98.799

MCF-7 = A human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors

T47D= An ideal experimental model cell line to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer.

MDA-MB-231= An epithelial cell line, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories.

Hu-02=A human fibroblast cell line and MCF-10A= A non-tumorigenic epithelial cell line.

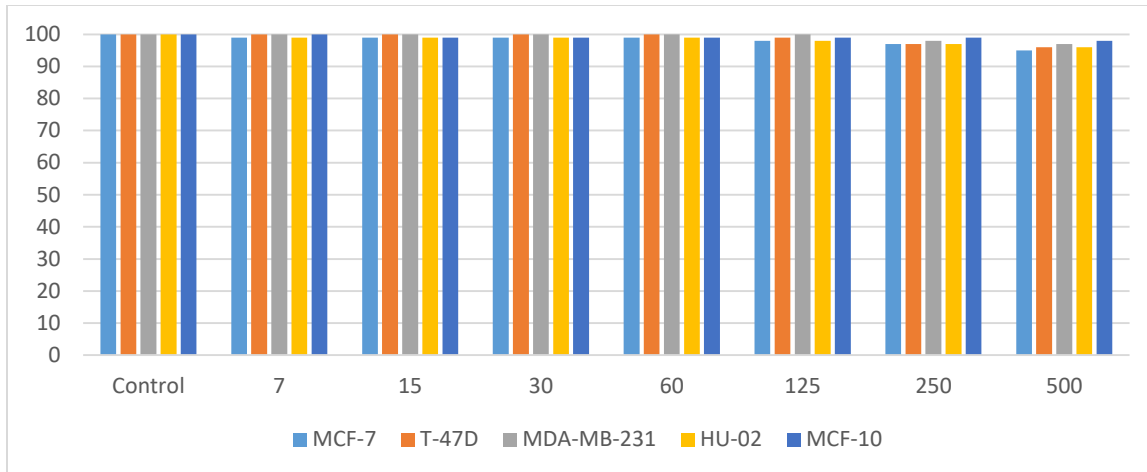


Fig-2.the effect of Tarragon EO on the MCF-7, T-47D, MDA-MB-231Hu-02andMCF-10A cells.

MCF-7 = A human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors

T47D= An ideal experimental model cell line to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. MDA-MB-231= An epithelial cell line, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories. Hu-02=A human fibroblast cell line and MCF-10A= A non-tumorigenic epithelial cell line.

Table 5. Comparison of the effect of mint and tarragon essential oil on MCF-7

	P value	Mean MCF-7	Mean Hu-02
Control	0.995218	100.0	100.0
7	0.00155	93.201	99.999
15	< 0.0001	90.878	99.999
30	< 0.0001	76.351	100.0
60	0.00197	72.297	100.0
125	0.0008	70.777	99.873
250	< 0.0001	60.093	97.297
500	< 0.0001	55.701	94.679

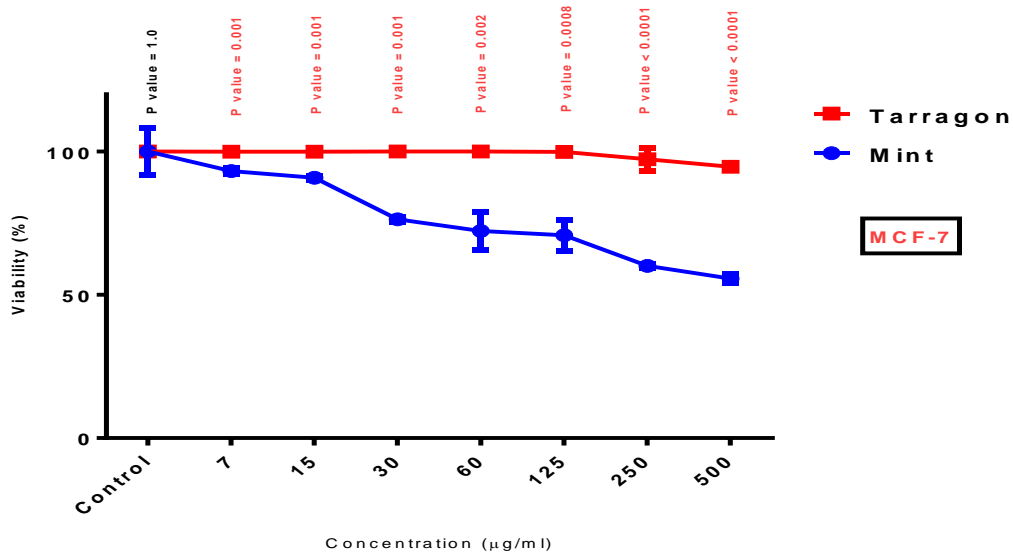
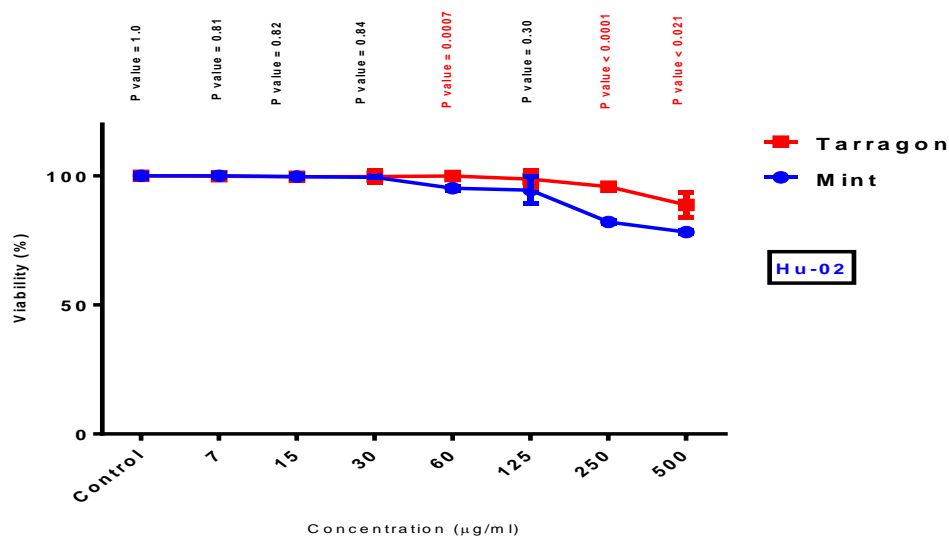


Fig. 3. Comparison of the effect of mint and tarragon EOs on MCF-7 (Two Way ANOVA).

)Two-way ANOVA (Table 6. Comparison of the effect of mint and tarragon essential oil on Hu-02

	P value	Mean MCF-7	Mean Hu-02
Control	> 0.9999	100.0	100.0
7	0.810629	100.0	99.864
15	0.816154	99.774	99.6383
30	0.839215	99.4573	99.7287
60	0.00069	95.251	99.9547
125	0.30392	94.4823	98.779
250	< 0.0001	82.135	95.7933
500	0.02138	78.2	88.7383



)Two-way ANOVA (Fig-4.Comparison of the effect of mint and tarragon essential oil on Hu-02

Figure 4 displays the growth inhibition on theHu-02division process affected by tarragon and mint EOs at different concentrations. Tarragon EO had the highest and similar growth inhibitory effect at concentrations of 250 and 500 mg/l, but not at 3, 7, 15, 30, 60,125 mg/L; but mint EO exerted the inhibitory effect at concentrations of 60,125,250,500 mg/L, but not at 3, 7, 15, 30 mg/L.

Morphological changes

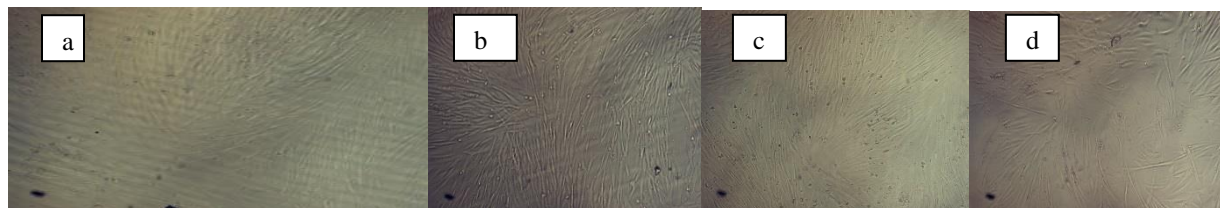


Fig-5. Hu-02 cells treated with different concentrations of mint and tarragon Eos

Hu-02 cells treated with a) 3 mg/L of mint EO, b) 500 mg/L of mint EO, c) 3 mg/L of tarragon EO, and d) 500 mg/L of tarragon EO. Cells were cultured in 96-well plate for 24 h and then treated with different concentrations of EOs. The growth inhibition was measured by the MTT assay (Fig-5).

MCF-7 cancer cells treated with a) 3 mg/L of mint EO, b) 500 mg/L of mint EO, c) 3 mg/L of tarragon EO, and d) 500 mg/L of tarragon EO. Cancer cells were cultured in 96-well plate for 24 h and then treated with different concentrations of EOs. The growth inhibition was measured by the MTT assay (Fig-6).

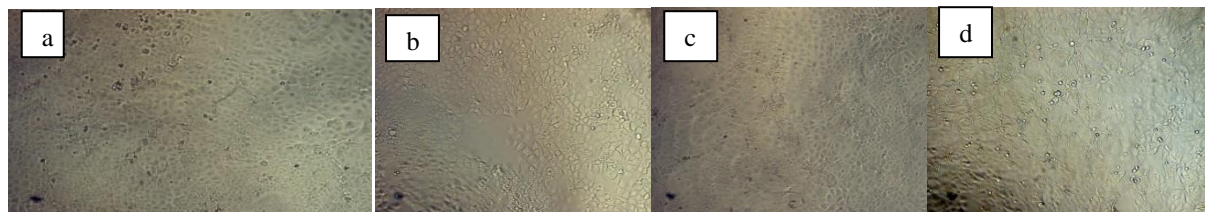


Fig-6. MCF-7 cancer cells treated with different concentrations of mint and tarragon EOs.

The MTT assay findings were also confirmed by morphological changes in cancer cells after treatments with different EO concentrations. Deformed cells separated from the surface and some cell bodies indicative of EO toxicity and cellular death were observable after the addition of EOs, in particular at high concentrations. These changes, however, were not observed in the control sample without the EO treatment.

DISCUSSION

Many studies have been carried out on the medicinal properties of plants and their extracts (Moheghi *et al.*, 2011; Meimandi *et al.*, 2015; Behdarv *et al.*, 2017; Nejad *et al.*, 2009; Seyedalipour *et al.*, 2016). Medicinal plants are very effective in nutrition and disease treatment around the world. Compounds with toxic effects, in particular cytotoxicity, which can be measured by toxicity measurement methods on cell and tissue cultures are the candidates for the synthesis of anticancer drugs for use in cancer chemotherapy (Mongelli *et al.*, 2000). Nature-derived (herbal, animal, and mineral) compounds, on the other hand, are of interest to drug manufacturers and physicians for the synthesis of novel drugs and the treatment of diseases (Deshpande *et al.*, 2008), in particular those without a definitive treatment and medication or with unknown adequate effectiveness. Little studies are available on the anticancer effects of mint and tarragon compounds. Given the evidence concerning the cytotoxicity of mint and tarragon compounds on some cancer cells, the aim of this study was to evaluate the cytotoxicity of mint and tarragon EOs on MCF-7, T-47D, MDA-MB-231 breast cancer cell lines.

The present results demonstrate that mint EO has cytotoxic and anti-proliferative effects on breast cancer cell lines (MCF-7, T-47D, MDA-MB-231) more than tarragon EO and this issue needs further investigation. The tables (2, 4, 5 and 6) indicate that there are significant differences between the toxicity of mint and tarragon Eos.

Both EOs of the two plants can be claimed to have a considerable growth inhibitory effect on the breast cancer cell lines. However, in our study, contradictory results were obtained that tarragon EO has much less cytotoxic effect than mint EO. Previous studies on tarragon plant constituents and the GC/Mass analysis indicated that its EO contains 60-70% of methyl chavicol (estragoles: 66.879%) and somepara-methoxycinnamaldehyde (the bitter substance). The other compounds include α -pinene, β -pinene, camphene, limonene, cis- and trans-ocimene, α -flandren, linalool, butyric acid, delta-4- carene, geraniol, and eugenol. In recent years, an isocoumarin, called artmedinole, has been discovered in tarragon plant. Important flavonoids of tarragon are rutin and quercetin 3-glucogalactoside, which seem to be involved in the strong anticancer and anti-atherosclerosis effects of this plant. But despite popular belief, the use of medicinal plants is not always safe. For example, it has been revealed that chronic uptake of estragole and methyl-eugenol, the two compounds found in some herbs such as tarragon, was associated with an increased risk of hepato-carcinogenicity in rodents (De Vincenzi *et al.*, 2000). Therefore, it was suggested that ingestion of products containing estragole and methyl-eugenol should be minimized (De Vincenzi *et al.*, 2000; Weinoehrl *et al.*, 2012). Due to its low cytotoxicity effect, it is better to use a little caution in using tarragon, both because of its small inhibitory effect on cancer cells and because it has two carcinogenic substances (estragole and methyl-eugenol).

Another compound that was studied here was mint EO, which had growth inhibitory effects on the cancer cell lines at different concentrations. EOs and extracts prepared from some mint species in other countries were reported to have antioxidant and antimicrobial effects (Golluce *et al.*, 2007). Mint is also added to various meat products as a flavoring agent. This family is a rich source of polyphenol compounds and hence possesses antioxidant properties. Members of this genus contain volatile EOs and are therefore cultivated in a number of countries (Sweetie *et al.*, 2007).

Most of plants have pharmacological and biochemical properties, including antioxidant and anti-inflammatory activities, which seem to be involved in cellular anti-malignancy and anti-mutagenic activities. Given that tumor progression has a very close relationship with inflammation and oxidative stress, a compound with antioxidant or

anti-inflammatory properties can be a cellular anti-malignancy agent (Shukla *et al.*, 2007). Many studies have investigated the properties of mint plant. Singh *et al.* (2011), for example, studied the antioxidant and antibacterial properties of mint plant and concluded that mint EO had a strong antibacterial effect that could be equivalent to that of gentamycin (Ramezani *et al.*, 2009). Koytchev *et al.* (1999) also examined the use of mint alcoholic extract on 66 people with a history of Herpes disease affected at least four times a year. They found that the use of mint alcoholic extract could improve resistance to this disease (Koytchev *et al.*, 1999).

A review of the literature and the present findings demonstrate that the EOs of these two plants have a high biological and pharmacological potential. It, therefore, seems that the isolation and purification of active ingredients in EOs of these plants, as well as the determination of their structures and mechanisms of action are the issues that should be considered in future studies.

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