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Cytotoxic effect of *Zataria multiflora* on breast cancer cell line (MCF-7) and normal fibroblast cells

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Abstract. The aim of this study was to evaluate the cytotoxic effects of Zataria multifloraessential oilon the human breast cancer cell line (MCF-7) and fibroblast cells. The essential oil of Z. multiflorawas obtained by distillation. Cells were cultured with various concentrations of Z. multiflora essential oil(6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml) for 24, 48 and 72 hours by MTT testAfter 24, 48 and 72 h results revealed that the more effect of Z. multifloraessenceon the growth inhibitory of MCF-7 cell line was observed at 200 µg/ml (102.01, 105.17 and 99.96% respectively). These effects became more prominent after 48 hours. The IC50 values after 24, 48 and 72 h were 158.97, 30.44 and 36.63 µg/ml respectively. Also results showed that more effect of Z. multifloraessenceon the growth inhibitory of normal fibroblast cell was observed at 100 µg/ml after 48h (98.82%).

Keywords: Zataria multiflora, cytotoxic effect, MCF-7 cells, fibroblast cells, MTT assay

1. INTRODUCTION

Breast cancer is one of the most common malignancies in women [1, 2]. A major problem with present cancer treatment is the drastic deficiency of effective drugs for the curative therapy of tumors [3, 4]. The existence of many side effects and problems related to common cancer treatments such as chemotherapy, surgery and radiotherapy, alternative treatments were investigated frequently [5].

Natural products, especially plants have played an significant role throughout the world in treatment and prevention of human diseases for thousand of years [6]. Some medicinal plants have shown promising results against variuos types of cancers. medicinal plants used have included the use naturally occurring essential oils. Historical documents corroboratethat Iran has a long-standing history, almost 3000 years, of medical research [7]. This research has included the use of complementary medicines including herbal medicines.

Zataria multiflora Boiss is a medicinal plant that belongs to the family Laminaceae. This plant (vernacular name of Avishan Shirazi, in Iran) geographically grows in Iran, Pakistan and Afqanistan [8, 9] and has been traditionally used as an antiseptic, anesthetic and anti-spasmodic drug [8]. Also, this plant is extensively used as a flavor ingredient in Iranian food. The essential oil of *Zatariamultiflora* is extracted from the flowered browses of the plant, having the compounds with important pharmaceutical, antimicrobial, and antioxidant effects [10, 11]. The main constituents of its essential oil are phenolic compounds such as carvacrol and thymol [12].

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The aim of the present study was to examine the cytotoxic effects of *Zataria multiflora* essential oil on breast cancer cell line (MCF-7) and human normal fibroblast cells growth inhibatory.

2. MATERIAL AND METHODS

2.1. Plants Mateerial

The aerial parts of *Z. multiflora* were harvested in pre-flowering stage from wild grown plants in the Mountain regions of Fars province. Voucher specimen was deposited at the herbarium of medicinal and aromatic plants of Islamic Azad University, Qaemshahr branch, Mazandaran, Iran. The harvested plants were dried at room temperature (25°C) for 2 weeks, then, air-dried plants (in each habitat) were ground and powdered with mixer for essential oil extraction and other experiments.

2.2. Essential Oil Extractıion

The dried aerial parts of *Z. multiflora* were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus. The essential oil obtained was separated from water and dried over anhydrous sodium sulfate. The concentrated plant essential oil were dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to obtain appropriate solutions of the essence.The sub-stock solutions of 6.25, 12.5, 25, 50, 100, 200 and 400 μg/ml was prepared by diluting the stock solution into serum-free culture medium, RPMI 1640 (the percentage of DMSO in the experiment should not exceed 1). The stock and sub-stock solutions were both stored at 4 ºC.

2.3. Cell Culture

Human breast cancer cell line (MCF-7) were purchased from Pasteur Institute of Iran (NCBI C135). The cells was grown and maintained in a humidified incubator at 37 ºC and in 5% CO2 atmosphere. RPMI-1640 medium (SIGMA) was supplemented with 0.01 mg/ml heat inactivated Fetal Calf Serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin (ALL FROM INVITROGEN GIBCO) was used for cell culture. Upon reaching confluency, the cells were passaged. After being harvested from sterile T75 culture flasks, the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. Three thousand cells from log phase cultures were seeded in 100 μl of RPMI medium supplemented with 10% fetal bovine serum per well of 96-well flat-bottom culture plates [13]. Cells were incubated with the *Z. multiflora*essential oil for defined tisme (24, 48 and 72 hours). Proliferative response and cell death of the *Z. multiflora*essential oil-treated cells were determined using MTT assay and cell death ELISA Cell Viability Assay, respectively.

2.4. Cytotoxicity Analysis By Mtt Assay

Growth of tumoral cells quantitated by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. MTT assay is an accurate method to evaluate the survival of cells.This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial enzymes, and the amount of formazan produced is directly proportional to the number of living cells [14]. In brief, this color can be measured by spectroscopic methods.

There is a linear relation between the number of viable cells and absorption for each cell. This relationship allows providing accurate determination of any changes in the rate of cell proliferation [15]. In this study, after MCF-7 and fibroblast cells were incubated for 24, 48 and 72 hour with different values of essential oil, 20 µl MTT solution 0.5 mg/ml was added into the wells and incubation continued at 37°C for 3 to 5 hours. After this time the supernatant cells were removed, and instead of that 200 µl of DMSO solution was added for 15 min at room temperature. Finally, the percentage of cell viability was determined by ELISA reader in 570 nm. The percentage of cell viability was calculated using the equation: [mean optical density (OD) of treated cells/mean OD of control cells] × 100. Percent of cytotoxicity = 100 – percent of Viability [16]. The effects of essential oil were expressed by IC_{50} values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

2.5. Statistical Analysis

The data are expressed as mean ± standard deviation (SD) for at least three independent determinations in triplicate for each experimental point. SPSS Statistics 22 Software was used to perform statistical analysis. One-way ANOVA method followed by Duncan test was used to determine the differences among the groups ($P<0.05$).

3. RESULTS

The result of the present study shows that the effect of *Z. multiflora* essential oilon MCF-7 cell line and normal fibroblast cells. Results of this experiment are tabulated in Tables 1 and 2.

After 24 hours incubation, results showed significant effect on MCF-7 cells in concentration of 50, 100, 200 and 400 µg/ml compared with control, with 35.93, 86.43, 102.01 and 101.96 percent of cell growth inhibition, respectively ($p<0.05$). Also, increase in the percentage of inhibition due to increasing concentration of *Z. multiflora* essential oil was seen in MCF-7 cell line, after 48 hours and there was a clear difference in MCF-7 cell line proliferation between control and all concentrations ($p<0.05$) except 12.5 µg/ml ($p>0.05$). After 72h concentrations of 6.25, 50, 100, 200 and 400 µg/ml had shown significant effect on growth of MCF-7 cell line (Table 1).

Overall, after 24, 48 and 72 h results revealed that the more effect of *Z. multiflora*essenceon the growth inhibitory of MCF-7 cell line was observed at 200 µg/ml, equivalent102.01, 105.17 and 99.96% respectively and these effects highly significant as a comparison with the control group in 24 and 48h incubation ($p<0.01$), but the diffrence between cancer cell growth inhibition percent after 72h incubation and control group was not significant ($P>0.05$) (Table 1).

The IC_{50} values (50% inhibitory concentration) of this assay after 24, 48 and 72 h were 158.97, 30.44 and 36.63 µg/ml respectively.

Table 1. Cytotoxicity activity of *Z. multiflora* essential oil against MCF-7 cell line at different concentrations by MTT assay. Values represent the mean of three experiments.

Concentrations of *Z. multiflora* essential oil (μg/ml)	Absorbance	Inhibition (%)	IC_{50} (μg/ml)	P-value
After 24h incubation				
Control	0.226 ±0.019	-		-
6.25	0.182 ±0.002	-56.886		0.282
12.5	0.309 ±0.049	-54.553		0.280
25	0.299 ±0.026	-44.676		0.119
50	0.162 ±0.012	35.932*		0.032
100	0.074 ±0.010	86.433**	158.975	0.004
200	0.045 ±0.002	102.012**		0.007
400	0.046 ±0.001	101.961**		0.007
After 48h incubation				
Control	0.709 ±0.057	-		-
6.25	0.915±0.068	-34.193*		0.042
12.5	0.915 ±0.044	-33.679		0.781
25	0.728 ±0.035	-4.376**	30.440	0.006
50	0.488 ±0.049	35.096**		0.004
100	0.065 ±0.006	101.876**		0.004
200	0.043 ±0.0004	105.171**		0.004
400	0.046 ±0.0004	104.739**		0.004
After 72h incubation				
Control	0.698 ±0.066	-		-
6.25	0.981 ±0.048	-45.002**		0.015
12.5	0.945 ±0.041	-39.576		0.260
25	0.780 ±0.048	-13.728		0.296
50	0.612 ±0.050	12.514**	36.636	0.008
100	0.081 ±0.012	95.630**		0.006
200	0.054 ±0.0005	99.963**		0.007
400	0.055 ±0.001	99.828**		0.007

In addition, the effect of *Z. multiflora* essential oil on inhibition of normal fibroblast cells, in different concentrations of it include6.25, 12.5, 25, 50, 100, 200 and 400 μg/ml, was checked using the MTT assay after 24, 48 and 72 hours incubation and results are tabulated in Table 2. After 24h incubation, results showed lower effect on fibroblast cells compared with 48 and 72h. Nevertheless, the *Z. multiflora* essential oil was able to induce inhibitory of the fibroblast cells after 48 and 72h incubation, from 18.67 to 98.88 and from 41.65 to 94.34, respectively, as exhibitedin table 2. The Maximum cytotoxic to fibroblast cells were found at concentrations of 100 μg/ml, After 48 hours of treatment, with more cytotoxic effects on normal cells (98.88%). The IC_{50} values (50% inhibitory concentration) of this assay after 24, 48 and 72 h were 6.04, 25.11 and 66.71 μg/ml respectively.

Table 2. Cytotoxicity activity of *Z. multiflora* essential oil against normal fibroblast cells at different concentrations by MTT assay. Values represent the mean of three experiments.

Concentrations of *Z. multiflora* essential oil (µg/ml)	Absorbance	Inhibition (%)	IC_{50} (µg/ml)	P-value
After 24h incubation				
Control	0.175 ±0.009	-		-
6.25	0.161 ±0.018	9.674	6.041	0.454
12.5	0.158 ±0.003	11.803		0.069
25	0.155 ±0.007	13.734		0.060
50	0.142 ±0.009	22.836		0.081
100	0.129 ±0.008	31.360*		0.020
200	0.104 ±0.016	48.375**		0.003
400	0.071 ±0.029	71.402*		0.032
After 48h incubation				
Control	0.520 ±0.145	-		-
6.25	0.179 ±0.006	18.674		0.051
12.5	0.185 ±0.003	31.945		0.054
25	0.261±0.002	47.154	25.110	0.166
50	0.115 ±0.003	86.878		0.074
100	0.069 ±0.0008	98.823		0.062
200	0.072 ±0.001	97.736		0.065
400	0.082 ±0.002	95.507		0.065
After 72h incubation				
Control	0.386 ±0.042	-		-
6.25	0.127 ±0.040	41.654*		0.041
12.5	0.229 ±0.042	56.456*		0.030
25	0.292 ±0.005	61.838*		0.033
50	0.379 ±0.027	64.657	66.710	0.887
100	0.468 ±0.040	71.847		0.259
200	0.505 ±0.042	75.729		0.162
400	0.495±0.023	94.348		0.108

From figures 1 and 2 it can be inferred that inhibition percentage of human cancer and fibroblast cells in presence of various volumes of essential oil (6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml) after 24, 48 and 72h incubation using MTT method has been presented. Each experimental curve represents the average of a series of three different experiments.

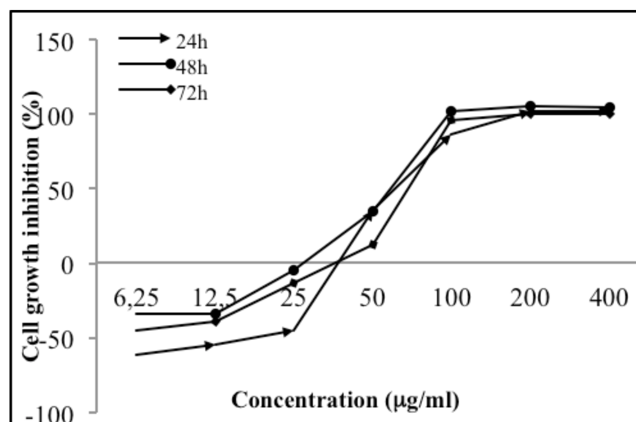

Figure 1. Concentration-cell inhibitory curves of *Z. multiflora* essential oil following 24, 48 and 72 hour exposure on MCF-7 cells.

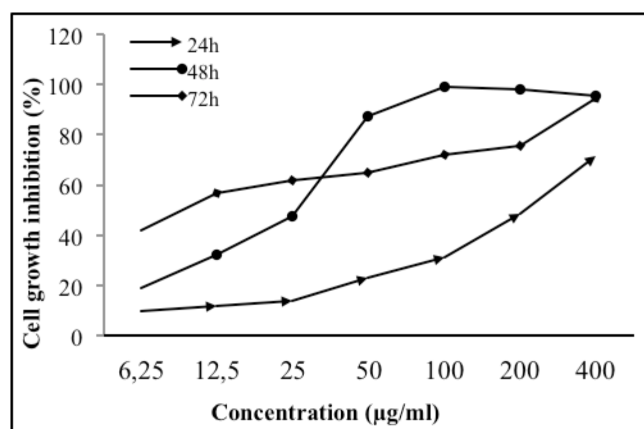

Figure 2. Concentration-cell inhibitory curves of *Z. multiflora* essential oil following 24, 48 and 72 hour exposure on human fibroblast cells.

It was found that the percentage of cancer cell growth inhibition to be increasing with increasing concentration of test compounds, thus *Z. multiflora* essential oilat 6.25 to 200 µg/ml exhibited dose-dependent inhibitory effects on the proliferation of MCF-7 cells, but slight cell inhibitory reduction was observed after 400 µg/ml than 200 µg/ml.

4. DISCUSSION

Breast cancer is one of the most common malignancies [17]. Herbal plants are potentially useful candidates for treating cancer; they can synthesize a wide diversity of chemical compounds that may influence biological functions [17-20]. Furthermore, the historically asserted chemo-preventive properties of aromatic plants are partially attributed to their volatile extracts (essential oils). These oils contain a wide variety of active phytochemicals, such as flavonoids, monoterpenes, and polyphenols, among many others [21].

The clinical trials efforts to discover medicinal plants and also, the candidate substance derived from these plants have important role in prevention and treatment of cancer. As is shown in figure 1 and 2, results of this study indicate that, the growth of MCF-7 cells in the presence of various volumes of *Z. multiflora* essential oil have been inhibited and this effect is dose dependent. Therefore, provides more evidences for its therapeutic application on cancer therapy.

The composition of the essential oil of *Z. multiflora* has been reported previously by more researchers. Alizadeh and Shaabani (2014) reported that essential oil composition in *Z. multiflora*, is rich in phenolic compounds as carvacrol and thymol and his precursors, also the extracts of *Z. multiflora* indicating good phenolic content and antioxidant activity. They showed that, Iranian *Z. multiflora* are strong radical scavengers and can be considered as a good source of natural antioxidant for traditional and medicinal uses [22]. The main constituents in *Z. multiflora* are phenolic compounds such as thymol and carvacrol and the other compounds are p-cymene, c-terpinene, b-caryophyllene and monoterpenes [23-25]. Alipour-eskandani (2011) found that the major components of *Z. multiflora* obtained from Shiraz Province of Iran was Carvacrol (71.1%) [26].

However, the efficacy of this plant against cancer may be related to terpenoid compounds. Natural terpenoids, including monoter-penoids, diterpenoids, triterpenoids and tetraterpenoids are able to inhibit tumor cell proliferation of human breast and prostate by inhibiting multiple cancer-specific targets including the proteasome, Nf-kB and Bcl-2 [27]. Another triterpenoids that found in medicinal plants are Cucurbitacins that have anticancer effects through inhibition of JAK-STAT and MAPK pathways which are important for cancer cell proliferation and survival [28]. As pRb protein and E2F1 transcription factor down-regulated by MDM2 onco-protein, the MDM2 down-regulation by essential oil of *Z. multiflora* Boiss may be a possible mechanism for the present results [29-31].

Also, previous studies have shown that carvacrol can exhibit strong antitumor activity. They revealed that carvacrol suppresses the growth of mouse B16 melanomas [32] and human larynx carcinoma Hep-2 cells [33] *in vitro* and was active against DMBA-induced lung tumors in rats [34]. Additional evidence for its antitumor activity was found: inhibition of DNA synthesis in mouse myoblasts bearing a human N-ras oncogene [35]. The further study indicated that carvacrol, dose-dependently, decreased the number of cancer cells, and total protein content and increased the degeneration of cell morphology of the human non-small cell lung cancer (NSCLC) cell line A549 [36].

Although the anti-tumor activities of carvacrol have been demonstrated earlier, the mechanisms behind this activity are still not very clear. Some findings suggested that the antitumor activity of carvacrol was not due to cytotoxicity but possibly due to prevention of prenylation of several proteins including ras [35], some results showed that its mechanism may be due to its antioxidant nature [37]. Recently, the latest research showed that the anti-proliferative effects of carvacrol in metastatic breast cancer cells (MDA-MB231) was based on the activation of the classical apoptosis response, including decrease in mitochondrial membrane potential and increase in cytochrome c release from mitochondria, decrease in Bcl-2/Bax ratio, increase in caspase activity and cleavage of PARP and fragmentation of DNA, which belong to the mitochondrial pathway of the apoptosis pathway [38].

However, the fraction of essential oil that has these potential effects and also, the involved mechanism remain as important issue to resolve. Also, it has been shown previously that the essential oil of this medicinal plant affects on p53 protein stability through regulation of MDM2 and ATM mRNA levels [31]. Thus, the results of these studies have been shown that the essential oil of *Z. multiflora* Boiss can induce p53 and RB pathways which are so important for normal cell cycle progression. Therefore, this essential oil may be efficient for the tumors that exist concomitant dysfunction of both p53 and Rb1 tumor suppressors occurs.

5. CONCLUSIONS

As it is reported in previous studies [39], in cancer therapy it is essential to apply suitable growth factors for inducing normal cell proliferation but not for malignant cells. In this study after 24h incubation with *Z. multiflora* essential oil possess this aspect; because it has a potent cytotoxic effect on MCF-7 cell growth; however, it does not have considerable inducing effect on fibroblast cells so it can be consider as potential growth factor. As long as *Z. multiflora* essential oil has less cytotoxic effects in low volumes after 24h incuabtion on human fibroblast cells compared with cancer cells, this plant oil may be an eligible candidate for use as a growth factor.

In summary, this is the evidence for the effect of *Z. multiflora* essential oil on breast cancer cell line. These findings suggest that this plant essence may be a potential medicine for cancer and further studies, as a result work, should be done for evaluation of cell death mechanisms of this plant, and also it is crucial to examine other cancer cell lines; similarly, in vivo studies are prominent to decipher the exact consequence of this plant.

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