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Cytotoxic effect of *Zataria multiflora* on gastric cancer cell line (AGS) and normal fibroblast cells

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Abstract. In this study, the cytotoxic effects of Z. multifloraessential oilwere investigated on human adenocarcinoma gastric cell line (AGS) and human fibroblast cells. The AGS cells were seeded in 96-well culture plates in the presence and absence of different concentrations of Z. multifloraessential oil(0, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml)for 24, 48 and 72 hours using MTT assay. Results showed thatessential oil of Z. multiflorashowed strong inhibitory effects on AGS cells when compared to control. The cytotoxicity effect after 24h incubation was greater than 48 and 72h and the highest cytotoxicity of Z. multifloraessential oil was related to 400 µg/ml (90.01%) . 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, AGS cells, fibroblast cells, MTT assay

1. INTRODUCTION

Gastric cancer is the fourth most common cancer. According to a global estimation, more than 930,000 new cases of gastric cancer are being diagnosed each year and a minimum of 700,000 patients die from the disease[1].Until recently, gastric cancer was the most common cancer in the world.In Iran gastric cancer is the most common cancer in male and it is reported to be the third cancer after Breast and Colorectal cancers in female.Many environmental factors including smoking, high salt intake, and a diet with an insufficient level of antioxidants are involved in the pathogenesis of gastric cancer[2, 3].

Currently, there are some inefficient treatments for cancer, including surgery, radiotherapy and chemotherapy; therefore, searching to find new effective therapies and anti-cancer drugs is one of the most important aims in medicine and pharmacology, respectively [4-6].

In recent years, due to relatively inexpensive and nontoxic properties of phytochemicals or chemopreventive agents with anti-cancer effects, it is interested in herbal medicine increased, and more than 3000 plant species have been used to treat cancer [4, 7].*Zataria multiflora* Boiss. known as Avishan Shirazi in Iran that belongs to the family Laminaceae, and it is distributed in Iran, Pakistan and Afqanistan [8, 9].Avishan Shirazi has been used traditionally as an antiseptic, anesthetic and anti-spasmodic drug [Ali]. More reports in Iran showed it also has antimicrobial activity [10, 11].Also, this plant is extensively used as a flavor ingredient in Iranian food. The essential oil of *Zataria multiflora* is extracted from the floweredbrowses of the plant, having the compoundswith important pharmaceutical, antimicrobial,and antioxidant effects [12, 13].To our knowledge, the major constituents of *Z. multiflora* Boiss. essential oil are composed of thymol and carvacrol and the other compounds are p-cymene, c-terpinene, b-caryophyllene and monoterpenes [14-16].

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This study was done with the aim of evaluating the cytotoxic effects of *Z. multiflora* essential oil on human cancer cell line and normal fibroblast cells. The human gastric cancer cell line (AGS)was studied as model of tumor. Cellular death pattern was evaluated for the determination of growth inhibitorymediatedof essence in more sensitive cells too.

2.MATERIAL AND METHODS

2.1. Plants Material

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. Essentıal Oil Extraction

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 gastric cancer cell line (AGS) were purchased from Pasteur Institute of Iran. 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 [17]. 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

Viability of cancer and normal cells treated with/without essential oil of *Z. multiflora* wasmeasured by MTT colorimetric assay. The assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells into an insoluble colored Formosan product, which can be measured spectrophotometrically after dissolving in dimethyl sulfoxide (DMSO). Briefly, cells were seeded onto 96-well microculture plates and allowed to adhere overnight. Then, each cell line was exposed to increasing concentrations of*Z. multiflora*essential oil (6.25-400µg/ml) for 24, 48 and 72 h. The first column of each microplate was assumed as negative control (containing no extract). To assay the cell viability, 20µl of MTT solution (5 mg/ml in phosphate buffered solution) was added to each well and the plate was incubated for 3 h at 37°C. Then, 200 µl of DMSO was replaced and pipetted to dissolve any

Formosan crystals. The optical density (OD) was read on an Elisa reader at a wavelength of 570 nm. The inhibitory rate of cell proliferation was calculated by the following formula: Growth inhibition (%) =[1-($OD_{control}$-$OD_{treated}$)/$OD_{control}$]×100. 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

Malignant and normal cell lines were incubated with essentialoil of *Z. multiflora*for 24, 48 and 72 h. The results of cytotoxic tests have been shown in table 1 and 2.

After 24h, its cytotoxic effect started at a concentration as little as 6.25 µg/mL and the percentage of dead cells for each concentration was found to be from 16.00 to 90.01%, in a dose-dependent manner.Proliferation of AGS cells was significantly reduced by *Z. multiflora* essential oil at 100 to 400 µg /ml after 48h.Also, After 72h the essential oil of *Z. multiflora* induced suppression on the proliferation of AGS cells with -68.55 to 41.98 percentage of cell growth inhibition (Table 1).

While, *Z. multiflora* essential oils showed significant cytotoxic effect on AGS cells at concentrations equal or above 50 µg/mL for 24 h and concentrations equal or above 50 µg/mL for 48 h, as compared to the control groups (p<0.05) but after 72h the concentrations had not significant effect on cancer cells (Figure 1 and Table 1).The 50% cytotoxic effect (IC50) of *Z. multiflora* was found to be 53.98, 46.82 and 0.312 µg/ml afetr 24, 48 and 72h respectively.

The most cytotoxic activity of the essential oils on AGS cancer cells was observed at highest concentration (400 µg/ml) after 24h incubation (90.01%) thus the *Z. multiflora* essential oils caused the most potent cytotoxic activity against AGS cells in this time. The results showed that this essence dose dependently decreased viability of cancer (Figure 1) and normal cells (Figure 2).

Table 1. Cytotoxicity activity of *Z. multiflora* essential oil against AGS cell line at different concentrations by MTT assay. Values represent the mean of three experiments (*P< 0.05, **P< 0.01, ***P< 0.001).

Concentrations of *Z. multiflora* essential oil (μg/ml)		Inhibition (%)	IC_{50} (μg/ml)	P-value
After 24h incubation				
Control	0.284±0.006	-		-
6.25	0.288±0.050	16.000		0.087
12.5	0.265±0.028	11.548		0.087
25	0.248±0.014	15.095		0.0870
50	0.172±0.006	47.801**	53.98	0.004
100	0.087±0.001	84.009**		0.001
200	0.081±0.007	86.528***		0.0006
400	0.073±0.008	90.016***		0.0001
After 48h incubation				
Control	0.134±0.012	-		-
6.25	0.134±0.005	-5.162		0.356
12.5	0.131±0.017	5.599		0.486
25	0.124±0.034	13.666		0.723
50	0.116±0.011	22.660**	46.821	0.004
100	0.113±0.012	26.195*		0.022
200	0.106±0.003	31.733*		0.045
400	0.105±0.008	35.047*		0.015
After 72h incubation				
Control	0.113±0.031	-		-
6.25	0.161±0.152	-68.555	0.321	0.374
12.5	0.048±0.003	-18.268		0.374
25	0.127±0.004	6.403		0.064
50	0.089±0.016	24.190		0.158
100	0.061±0.002	24.407		0.160
200	0.061±0.001	28.616		0.154
400	0.060±0.003	41.989		0.154

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 exhibited in 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 (*P< 0.05, **P< 0.01, ***P< 0.001)

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.

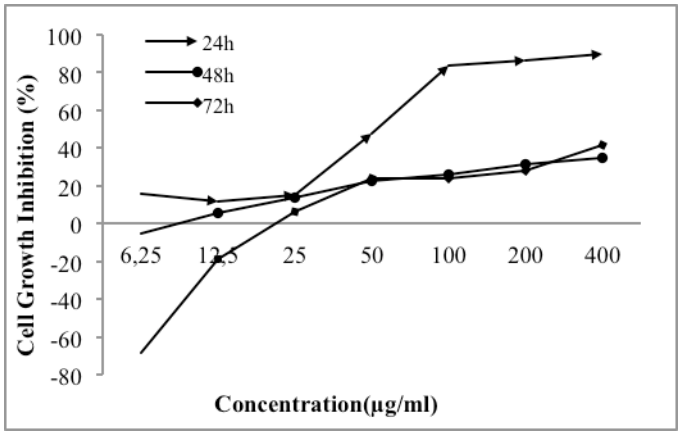

Figure 1. Concentration-cell inhibitory curves of *Z. multiflora* essential oil following 24, 48 and 72 hour exposure on AGS cells.

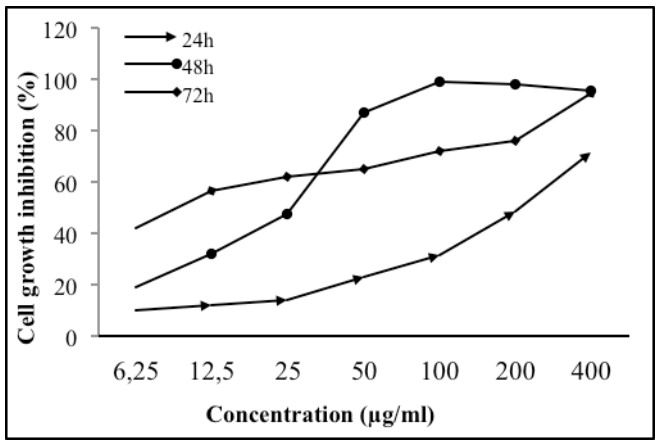

Figure 2. Concentration-cell inhibitory curves of *Z. multiflora* essential oil following 24, 48 and 72 hour exposure on human fibroblast cells.

4. DISCUSSION

Nowadays, one of the main methods of moderncancer treatment is chemotherapy (sometimes cancer chemotherapy). Most chemotherapeutic agents for cancer have different substantial short and long term side effects. Thus, in recent years major research hasbeen focused on medicinal plants and components isolated from herbs which have been considered for being nontoxic and for the prevention and treatment of certain types of cancer [18-20].

In this study, our aim was to determine whether the essential oil of *Z. multiflora*exerted an inhibitory effect on cancer cell proliferation and caused cell death. The results of our studies suggest that essence of *Z. multiflora*possess the cytotoxic effects on human cancer cell. Also, we observed that In AGS cell lines inhibition were increased (from 16.00 to 90.01%, from -5.16 to 28.61% and from-68.55% to 41.98% after 24, 48 and 72h respectively) with increasing treatment concentration.In screening result, *Z. multiflora* has shown broad cytotoxicity and it had most active cytotoxic activity on AGS cells after 24h at 400 µg/ml (90.01%).

Previous studies reported that Iranian *Z. multiflora* are strong radical scavengers and can be considered as a good source of natural antioxidant for traditional and medicinal uses [21].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 [14-16].Also some studies have shown that carvacrol can exhibit strong antitumor activity. For example, the studies revealed that carvacrol suppresses the growth of mouse B16 melanomas[22] and human larynx carcinoma Hep-2 cells [23] *in vitro* and was active against DMBA-induced lung tumors in rats [24].

Some findings suggested that the antitumor activity of carvacrol was due to prevention of prenylation of several proteins including ras[25],some results showed that its mechanism may be due to its antioxidant nature [26].Recently, the latest research showed that the anti-proliferative effects of carvacrol 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[27].

Our findings suggest that *Z. multiflora* essence may be a potential medicine for cancer and shows the efficacy of *Z. multiflora* for the cytotoxicity towards AGS cells thus suggesting protection against gastric cancer.

5. CONCLUSIONS

Based on the outcome of our study, *Z. multiflora* essential oil have an ability to induce cell growth inhibition. To the best of our knowledge, this is the first report describing significant cytotoxic effect of *Z. multiflora*essential oil against AGS cell line. Since one of the most important challenges for anti-cancer drug development is to induce cytotoxicity in malignant cells, *Z. multiflora* essential oil may be considered as a potential anti-cancer agent. This is very promising, indicating that *Z. multiflora* essential oil contains components with anti cancer activity; therefore, it can be a good candidate for further investigations to isolate, identify and test bioactive principles and elucidate the mechanisms by which *Z. multiflora*act asanticancer plant.

Overall, these findings suggest that *Z. multiflora* essential oil may be a potential therapeuticagent in cancer, and further experiments to investigate these possibilities are required.

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