Original Article

In vitro Antimicrobial and Cytotoxicity Assays of *Satureja bakhtiarica* and *Zataria multiflora* Essential Oils

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ABSTRACT

In the present study, the antimicrobial and the antitumor activities of the essential oils obtained from *Satureja bakhtiarica* and *Zataria multiflora* were evaluated *in vitro*. The essential oils were extracted using a clevenger apparatus and then the chemical composition was analyzed by GC-MS. An MTT cytotoxicity assay was employed to test effects of the essential oil on human normal embryonic kidney cells (HEK) and cancerous cell lines (human breast cell MDA-MB-231 and human ovary cancer cell SKOV3). The essential oil of *S. bakhtiarica* showed high antitumor activity on SKOV3 and MDA-MB-231 cell lines (IC_{50} of 74.6µg/ml and IC_{50} 83.76µg/ml, respectively). Its activity on normal HEK cell lines was interesting (IC_{50} of 102.03µg/ml) compared with tumor cell lines. Besides that, essential oil of *Zataria* showed least antitumor activity on both SKOV3 and MDA-MB-231cell lines (IC_{50} of 112.2µg/ml and IC_{50} 141.76µg/ml, respectively) compared with *S. bakhtiarica* essential oil. Both oils also showed good antibacterial and antifungal activities. The MBC values of the strains sensitive to the essential oil were in the ranges of 0.3% to 2%. The essential oils from *S. bakhtiarica* and *Z. multiflora*, which exhibited antimicrobial and antitumor activities, deserve further research into the chemoprevention and treatment of human ovary and breast cancers as well as infectious diseases. Therefore, further studies are necessary to evaluate this oil in animal models (*in vivo*) for future drug applications.

Keywords: Cytotoxicity, Antimicrobial, Essential oil, *Satureja bakhtiarica*, *Zataria multiflora*.

INTRODUCTION

Recently, there is a worldwide popularity and scientific interest to screen plant essential oils[1]. The use of medicinal plants as anti-bacterial and anti-inflammatory drugs in folk medicine is a practice common in Iran, although in most cases the active principles of the plants are unknown[2]. Multiple drug resistance in human pathogenic microorganisms has been developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases[3]. This situation forced scientists to search for new antimicrobial substances from various sources, including medicinal plants. Nowadays there are a huge number of papers available which contain conclusive information about the anti-bacterial and anti-inflammatory activities of essential oils from various aromatic plants[3]. Besides that, the diverse therapeutic potentials of essential oils have attracted the attention of many researchers to investigate their anti-cancer activity. Early reports had indicated that essential oil components, especially the monoterpenes, have multiple pharma-cological effects on the mevalonate pathway of metabolism which may account for the tumor suppressive activity exhibited by such terpenes[3]. *Zataria multiflora* is a thyme-like plant (belonging to the Lamiaceae family) that grows wild only in Iran, Pakistan and Afghanistan. This plant is a valuable medicinal and condimental plant in Iran. It has several traditional uses as an antiseptic, carminative, stimulant, diaphoretic, diuretic, anesthetic, anti-spasmodic and analgesic[4]. *S. bakhtiarica* (belonging to the Lamiaceae family), an endemic plant and one of the most important of the twelve species of Iranian *Satureja*, is also widely distributed in the southern region of Iran[5]. It has antispasmodic, anti-diarrheal, antioxidant, sedative and antimicrobial properties[6-10]. To our knowledge, no reports are known which have included the antitumor activity of the essential oil of *S. bakhtiarica* in Iran. Besides that, a few studies reported the antimicrobial and antitumor of *Z. multiflora* in Iran.

The aim of this work was to evaluate the antitumor and antimicrobial activities of *S. bakhtiarica* and *Z. multiflora* under laboratory conditions (*in vitro*).

MATERIALS AND METHODS

Plant material

Apparently healthy leaves of *Satureja bakhtiarica* and *Zataria* were collected during the flowering period, from the mountain region of Fars Province in Iran and identified by the Department of Botany of the Sari Agricultural University. A voucher specimen was deposited in the Herbarium of Faculty of Agriculture.

Isolation of essential oil

Air-dried plant material (100 g) was hydro-distilled for 3 h using a Clevenger type apparatus. The essential oils were collected over water, separated and dried over anhydrous sodium sulfate. They were stored in sealed vials at 4-6^{0}C prior to chemical analysis and antitumor activity evaluation.

Gas chromatography mass spectrometry Analysis

GC-MS analysis of the oil was conducted using a Hewlett Packard 6890 instrument operating on EI mode and equipped with a 5MS-HP fused silica column (30 m × 0.25 mm × 0.25 µm film thickness capillary columns). Helium (99.99 %) was used as the carrier gas at a constant flow of 1 mL/min. The oven temperature was held at 60°C for 1 min, then programmed to 210°C at a rate of 6°C/min, and then held for 10 min. The injector and detector (FID) temperatures

were kept at 250°C and 280°C, respectively. The components of the oil were identified by comparison of their MS with those obtained from authentic samples and/or the NIST/NBS and Wiley mass spectral database. They were also confirmed by comparison of their retention indices (RI)[11] and retention times (RT), either with those of authentic compounds or with published data[12].

Strains of pathogens

The microorganisms used in this study were *Staphylococcus aureus* (51153), *E. Coli* (53218), *Pseudomonasa aeruginosa* (27853), *Streptococcus pyogenes*, *Candida Albicans* (10231 BBL) and *Trichophyton mentagrophyton* were provided from the Institute pasture, Tehran- Iran.

Test for Antimicrobial Activity

The antimicrobial activity of essential oils was tested by the agar well diffusion method on Muller Hinton Agar (MHA). Using a cork borer, five wells (6 mm in diameter) were made in the agar medium (one in center and four wells were at corner) and inoculums containing 1.5×10^6 CFU/ml of the test bacteria were spread onto the surface of the medium with a sterile swab. In the case of essential oil, 4 %, 5 %, 6.8 %, 10 % and 20 % of the essence was pipette into the wells, whilst 50µl of DMSO served as a control. Gentamycine (10µg/ml) disk was used as positive control. For antifungal activity nistatine disk (30µg/ml) was used. The agar plates were incubated for 24h at 37^0C and the diameter of the zone of inhibition surrounding the wells was measured. Assays were performed in triplicate and the data are shown as the mean ± standard deviation (SD).

Cell lines and cell culture medium

A normal cell line (human embryonic kidney cells, HEK) and cancerous cell lines (human breast cell MDA-MB-231 and human ovary cancer cell SKOV3) were used in this study. All cell lines, which obtained from Pasteur Institute, Tehran, Iran, were cultured in RPMI 1640 medium (Sigma), supplemented with 10 % inactivated fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 mg/ml). The medium was then sterilized by filtering through 0.22 mm microbiological filters and kept at 4°C before use. Cells were grown at 37^0 C, under a 5 % CO_2 atmosphere and at 90 % humidity for 24h. Cell counts were determined.

In vitro cytotoxicity assay

The cytotoxic effects of the essential oils against tumor and normal cell lines were determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-iphynyltetrazolium bromide] assay. The cell suspension (1×10^4 cells) was placed in a 96-well flat-bottomed tissue culture plate. After that the cells were treated with different volumes of essential oils. After determining the dry weight of essential oil (12 mg/ml), different concentrations were prepared in 1 % DMSO-PBS solution. One hundred micro liters of each concentration of essential oil (10, 25, 50, 100 and 200 µg/ml diluted in RPMI 1640 medium), were added into the plate. Cells treated with a cytotoxic drug, methotrexate, were used as a positive control, whereas untreated cells (DMSO) were used as a negative control. After 24h incubation at 37°C, cell viability was evaluated using MTT assay. The assay was performed in triplicate for each of the concentrations. Briefly, the supernatants were removed from the wells and 200 ml MTT (Sigma) solution (2 mg/ml in PBS) was replaced. The plates were incubated for 2 h at 37°C, and 125 ml of DMSO was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker for 15 min and the absorbance at 595 nm was read by a multi-well spectro-photometer. The mean of the cell viability values was compared to the control to

determine the effect of the essential oil on cells and percentage of cytotoxicity was plotted against concentrations of the extract. The percentage of cytotoxicity was calculated as following:

% Cytotoxicity = 1- [(OD extract treated-OD blank) / (OD control-OD blank)] ×100.

RESULTS AND DISCUSSION

The qualitative and quantitative analyses of the *S. bakhtiarica* essential oil are depicted in Table 1. We found thirteen main constituents in which phenols (37.4 %), thymol (22.6 %) and *p*-cymene (19.3 %) were prominent. The results obtained in the qualitative and quantitative analyses of the *Z. multiflora* essential oil are also shown in Table 2. Fifteen main constituents were identified in which phenol (56.35 %), thymol (13.82.6 %), *p*-cymene (8.79 %), γ-terpinene (3.36 %) and α-pinene (2.07 %) were predominant.

Figure 1 illustrates the cytotoxicity effects of *S. bakhtiarica* essential oil at different concentrations ranging from 10µg/ml to 200µg/ml. Two cancerous cell lines, (human breast cell MDA-MB-231 and human ovary cancer cell SKOV3), were used to determine anticancer properties of *S. bakhtiarica* leaf essential oil. To determine the antitumor activity of essential oil against cancer cells, cytotoxicity MTT assay was carried out. At maximum concentration (200 µg/ml), essential oil showed the highest activity on cancer cells. No significant difference was shown in the cytotoxicity activity of essential oil between both cancerous cells at the lowest concentration; however, SKOV3 cell lines (IC_{50} of 74.6µg/ml) were more sensitive than MDA-MB-231 cells (IC_{50} 83.76µg/ml) at high concentration of essential oil. Its activity on normal HEK cell lines was also interesting compared with tumor cell lines. Normal HEK cell lines were least sensitive to cytotoxicity of essential oil particularly at about 100µg/ml concentrations (IC_{50} of 102.03µg/ml).

Figure 2 also depicts the cytotoxicity effects of *Z. multiflora* essential oil at different concentrations ranging from 10µg/ml to 200µg/ml on all tested cells. Similarly, at maximum concentration (200 µg/ml), essential oil showed the highest activity on cancer cells. Additionally, no significant difference was shown in the cytotoxicity activity of essential oil between both cancerous cells at the lowest concentration, but at higher concentration SKOV3 cell lines (IC_{50} of 112.2µg/ml) were more sensitive than MDA-MB-231 cells (IC_{50} 141.76µg/ml). Interestingly, essential oil at low concentrations has the least cytotoxicity effects on normal cells (157.6µg/ml). Normal HEK cell lines were least sensitive to cytotoxicity of essential oil particularly at low concentrations (IC_{50} of 163.2µg/ml).

The results regarding the preliminary tests on antibacterial and antifungal activities of the essential oil from S. *bakhtiarica* and *Z. multiflora* against four human pathogenic bacteria and two fungi are indicated in Table 3. The results show that bacterial growth was suppressed by both tested essential oils. Antimicrobial activity of essential oils in all concentrations (from 4 % to 20 %) was found for tested bacteria, and the inhibitory effect of the oils increased in proportion to their concentrations. Both essential oils showed good antibacterial and antifungal activities against to all test bacteria and fungi compared to tested antibiotics. However, S. *bakhtiarica* essential oil showed greater antimicrobial activity (with lower MIC) for tested bacteria and fungi than *Z. multiflora.* The MIC value of the sensitive bacteria was in the range of 0.3 % to 1 %, while the MIC value of the sensitive fungi was in the range of 1-2 %. *E. coli* and *S. Aeruginosa* were found to be the most sensitive bacteria to essential oils showing the MIC 0.5 % and 0.3 % for S.

bakhtiarica and 0.8% and 0.4% for *Z. multiflora* oils. Control treatment (DMSO) did not show an inhibitory effect on any of the bacteria.

Plant extracts are extensively used in the traditional medicine of Iran. Essential oils have been used as flavoring agents in food and beverages and, due to the presence of antimicrobial compounds, they have a potential for food preservation[13]. Moreover, researchers have been interested in biologically active compounds isolated from plant species for the elimination of pathogenic microorganisms because of the resistance that has developed to antibiotics[14]. Recently, many studies have focused on the antimicrobial activity of the essential oil and extracts of *Satureja* species and *Z. multiflora*. These studies have revealed that the genus has antimicrobial activity against human, food and plant pathogens[15-17] due to the presence of phenolic components, such as thymol and carvacrol. A large number of studies have reported that the essential oils of *Satureja* species are among the most potent essential oils regarding their antimicrobial properties[18-20]. In our pervious study, we reported the antibacterial activity of *S. bakhtiarica* against 10 human pathogenic bacteria, in which it showed good activity against all tested bacteria[21]. Besides that, our previous study demonstrated the biological and anti-parasite activities of *S. bakhtiarica* essential oil against *Leishmania major*[3]. Several studies have also reported the cytotoxicity properties of some *Satureja* species, such as *S. kitaibelii*[22], *S. Montana*[23] and *S. khuzistanica*[24]. Here, the *in vitro* anti-tumor activity of *S. bakhtiarica* essential oil against human cancer cell lines was evaluated. In study by Yousefzade *et al*., (2013), they reported that *S. khuzistanica* essential oil significantly reduce cell viability of Vero, SW 480, MC F7, and JET 3 cancer cells in a dose-dependent manner, with the IC_{50} values calculated for each cell type being, respectively, 31.2µg/ml, 62.5µg/ml, 125µg/ml, and 125µg/ml[24]. Our results also demonstrated that the essential oil from S. *bakhtiarica* has a strong antitumor activity on human tested tumor cell lines. Approximately 50 % of tumor cells growth was inhibited at 70-80µg/ml which was comparable with other studies. This evidence suggested for the first time *S. bakhtiarica* essential oil has a good antitumor activity against tested cancer cell lines. Furthermore, we also reported that *S. bakhtiarica* has remarkable antibacterial and antifungal activities. *Z. multiflora* essential oil also showed good antibacterial and antifungal activities; however, it showed least activity compared with *S. bakhtiarica*. Similarly it showed a good antitumor activity against to tested cell lines. Previous studies also reported remarkable antibacterial activity of *Z. multiflora* against some species. For instance, in study by Malekinejad *et al*., (2012), they reported a significant antibacterial effect of *Z. multiflora* against *Aeromonas hydrophila*[4].

The reliable antimicrobial and antitumor activities of *S. bakhtiarica* and *Z. multiflora* are essential oils are most likely attributable to their phenolic compounds, particularly thymol, carvacrol and *p*-cymene. Both studied plants have played an important role in Iranian traditional medicine. In light of the modern pharmacological and clinical investigations, they are valuable medicinal and condimental plants that have anti-microbial, antioxidative, anti-inflammatory, and antitumor activities. Further study and clinical trials based on traditional administration of this plant is needed in order to obtain information regarding the practical effectiveness of *S. bakhtiarica* essential oil to prevent the growth of tumor cells, and its use as an antitumor agent for cancer diseases in humans. Furthermore, further activity directed fractionation of its compositions is needed in order to establish a causal relationship

between given molecular species and the antitumor activity detected.

CONCLUSION

This study revealed that phenol, thymol, and *p*-cymene were the main chemical compounds of *Satureja bakhtiarica* and *Zataria multiflora* essential oils. Both essential oils, particularly the essential oil of *Satureja bakhtiarica*, showed good antibacterial activity. Furthermore, they had also the ability to inhibit tumor cell growth. Therefore, the essential oils could be used as potential natural antibacterial and antitumor agents; however, further studies *in vivo* are needed to clarify the mechanism of action and their potential use.

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Table 1. The composition of the essential oil from *S. bakhtiarica*

S. N.	Compounds	*S. bakhtiarica* (%)	RI
1	β-Myrcene	0.8	988
2	α-Terpinene	0.9	1017
3	Borneol	1.9	1131
4	Thymol	22.6	1268
5	Phenol	37.4	1298
6	Caryophyllene oxide	2.0	1567
7	Carvacrole	0.2	1280
8	Limonene	0.9	1030
9	P-Cymene	19.3	1010
10	γ-Terpinene	5.0	1060
11	L-Linalool	4.9	1076
12	Terpineol	0.7	1189
13	β-Caryophyllene	2.2	1418

Table 2. The composition of the essential oil from *Zataria*

S. N.	Compounds	*Zataria* (%)	RI
1	α-pinene	2.07	938
2	β-myrcene	1.91	988
3	α-terpinene	1.21	1017
4	Thymol	13.82	1268
5	Phenol	56.35	1298
6	Acetatecarvacrol	1.61	1284
7	Caryophyllene oxide	0.47	1567
8	Carvacrole	2.88	1280
9	P-cymene	8.79	1010
10	γ-terpinene	3.36	1060
11	β-Caryophyllene	1.28	1418
12	Cineole	0.75	1033
13	Cyclohexene	0.73	1202
14	α-terpineol	0.88	1189
15	Coraxeniolide	0.9	1247

Table 3. Antimicrobial activity of two tested essential oils

		Microbial Species					
		E. coli	*S. Aeruginosa*	*Staph. Aureus*	*Strep Ppyogen*	*C. Albicans*	*T. Metaphyton*
Bakhtiarica	4%	14.83±0.25	19.3±0.17	16.03±0.3	15.8±0.37	10.8±0.4	11.9±0.15
	5%	18±0.26	21.36±0.23	18±0.24	16.76±0.66	12±0.75	17.4±0.51
	6.6%	19.2±0.1	25.43±0.15	20.63±0.37	16.9±0.2	12.4±0.3	19.5±0.47
	10%	22.66±0.2	29.43±0.35	23.73±0.35	18.9±0.2	19.8±0.43	22.7±0.32
	20%	21.2±0.41	32.23±0.2	27.6±0.43	24.4±0.4	28.7±0.6	27.7±0.52
	MIC	0.5	0.3	0.7	0.7	1	1
Zataria	4%	12.86±0.92	18.33±0.32	14.1±0.2	15.2±0.55	10.03±0.32	10.6±0.45
	5%	14.96±0.2	20.43±0.41	14.9±0.3	15.33±0.47	10.16±0.4	15.86±0.15
	6.6%	15.23±0.2	22.6±0.26	15.1±0.2	18.4±0.45	11.96±0.73	16.78±0.35
	10%	16.56±0.2	25.83±0.3	17.23±0.47	18.7±0.45	19.3±0.37	18.43±0.15
	20%	21.2±0.41	19.13±0.2	25.36±0.45	30.43±0.15	28.06±0.8	24.43±0.56
	MIC	0.8	0.4	1	0.9	1	2
N.T	MIC	-	-	-	-	22.1±0.61	19.36±1.1
G.M	MIC	11.73±0.45	16.26±0.8	18.9±0.5	17.6±0.1	-	-

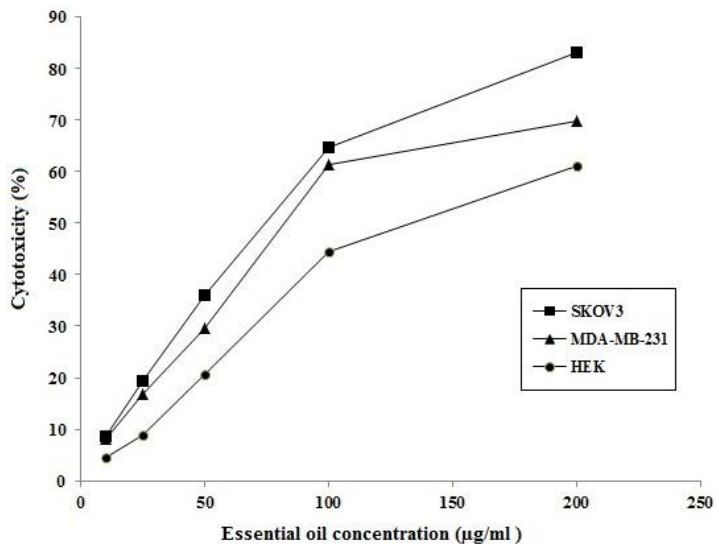

Figure 1. The dose-dependent cytotoxic effect on SKOV3, MDA-MB-231 and HEK cell lines of the essential oils (EO) of S. *bakhtiarica*. Cytotoxicity was measured as the reduced change in absorbance in cultures containing EO at 595 nm as compared with control untreated cultures. Each point represents the average from three separate measurements, each done in triplicate, and the standard deviation of the mean

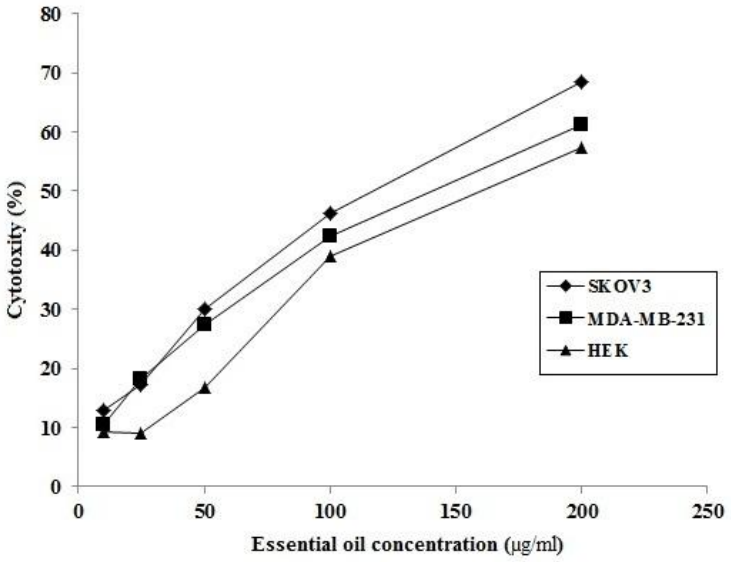

Figure 2. The dose-dependent cytotoxic effect on SKOV3, MDA-MB-231 and HEK cell lines of the EO of *Z. multiflora.* Cytotoxicity was measured as the reduced change in absorbance in cultures containing EO at 595 nm as compared with control untreated cultures. Each point represents the average from three separate measurements, each done in triplicate, and the standard deviation of the mean