Antimicrobial, antioxidant, cytotoxic and apoptotic activities of *Satureja khuzestanica*

Satureja Khuzestanica'nın Antimikrobiyal, Antioksidant, Sitotoksik ve Apoptotik Aktivitesi

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ABSTRACT

Objective: Natural products with antimicrobial effect are currently investigated in order to eliminate the use of synthetic antibiotics that cause the resistance of microorganisms. This study determines the antibacterial, antioxidant activity, and also the cytotoxic and apoptotic effect of *Satureja khuzestanica* on various cancer cell lines.
Materials and methods: The individual constituents were identified by their identical retention indices referring to known compounds from the literature. Methanol and ethanol extracts of *S. khuzestanica* (25 and 50 mg/$_{mL}$) were screened against four Gram-positive and four Gram-negative bacteria. The plant extracts were tested for their antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The interaction between the extracts and plasmid DNA were analyzed by agarose gel electrophoresis. In addition, the cytotoxic activity of the extracts was evaluated on MCF-7, DLD-1, osteosarcoma, and fibroblast cell line. Finally, gene expression of *caspase*-3, *Bax* and *Bcl*-2 were investigated by real-time PCR.
Results: Our result indicated that both extracts showed good inhibitory activity against both Gram-negative (*Escherichia coli* ATCC 35218) and positive (*Bacillus subtilis* ATCC 6633) bacteria. In addition, the methanol extract of *S. khuzestanica* had strong antioxidant activity (IC50=37.0±0.3 µg/mL). The extracts showed a strong effect on plasmid DNA. The methanol extract of *S. khuzestanica* showed a good concentration-dependent cytotoxicity. Finally, IC50=47.00a±1.21µg/mL, ethanol, and water extract had apoptotic effect in MCF-7 cell line.
Discussion: MCF-7 was detected as the most sensitive cell line therefore further studies should be done on this plant extract as a potential anticancer agent against breast cancer.

Key Words: *Satureja khuzestanica*, plasmid DNA, MCF-7, cytotoxicity

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ÖZET

Amaç: Antimikrobiyal etki gösteren doğal ürünler, mikroorganizmaların direncine neden olan sentetik antibiyotiklerin kullanımını ortadan kaldırmak için araştırılmaktadır. Bu çalışmada, *Satureja khuzestanica* bitkisinin antibakteriyel, antioksidan aktivitesi ve çeşitli kanser hücre hatlarında sitotoksik ve apoptotik etkisi araştırılmıştır.
Yöntem: Tek tek bileşenler, literatürden bilinen bileşiklere atıfta bulundukları özdeş alıkonma endeksleri ile tanımlanmıştır. *S. khuzestanica*'nın (25 ve 50 mg / mL) metanol ve etanol özütünün dört Gram-pozitif ve dört Gram-negatif bakteri üzerindeki etkisi belirlendi. Bitki özütlerinin 2,2-difenil-l-pikrilhidrazil (DPPH) radikaline karşı antioksidan aktiviteleri hesaplandı. Özütlerin plazmid DNA üzerine etkisi agaroz jel elektroforezi ile analiz edildi. Ayrıca, özütlerin MCF-7, DLD-1, osteosarkom ve fibroblast hücre hatları üzerinde üzerinde sitotoksik aktiviteleri araştırıldı. Son olarak, gerçek zamanlı PZR ile özütlerin *kaspaz*-3, *Bax* ve *Bcl*-2'nin gen ifadesi üzerine etkisi araştırıldı.
Bulgular: Bizim sonuçlarımız, her iki özütünün de hem Gram-negatif (*Escherichia coli* ATCC 35218) hem de pozitif (*Bacillus subtilis* ATCC 6633) bakterilere karşı iyi bir inhibitör aktivite gösterdiğini ortaya çıkarmıştır. Ayrıca, *S. khuzestanica*'nın metanol özütünü kuvvetli bir antioksidan aktivitesine sahip olduğu bulunmuştur (IC50 = 37.0 ± 0.3 µg / mL). Özütler plazmid DNA üzerinde güçlü bir etki göstermiştir. *S. khuzestanica*'nın metanol özütü konsantrasyon bağımlı güçlü sitotoksik aktivite göstermiştir. Son olarak, IC50, 47.00a ± 1.21µg / mL olarak hesaplanmış, etanol ve su özütü MCF-7 hücre hattında apoptotik etki göstermiştir.
Sonuç: MCF-7 en hassas hücre hattı olarak tespit edilmiştir. Meme kanserine karşı potansiyel antikanser ajan olarak ileri çalışmalar yapılması önerilmektedir.

Anahtar Sözcükler: *Satureja khuzestanica*, plasmid DNA, MCF-7, sitotoksite

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INTRODUCTION

Using medicinal plant to treat diseases is becoming popular in human societies (1). Aromatic plants, known for both aromatic and antiseptic properties, are used in spices and natural food preservatives, perfume industry for aromatherapy, etc. (2, 3). During the last decades, natural products with antimicrobial effect have been investigated for eliminating the use of synthetic antibiotics that cause the resistance of microorganisms and have side effects to human health (4).

Most aromatic plants belonging to the family of Lamiaceae, such as *Satureja, Sideritis, Salvia, Origanum*, and *Thymus* are annual or perennial semi-bushy aromatic plants that inhabit arid, sunny, stony, and rocky habitats (5). Several Satureja species locally known as 'Kekik' exist in Turkey (6). There are about 30 species of *Satureja* in the world. *Satureja khuzestanica* is an endemic species in Iran (7). Distribution of the genus *Satureja* overlaps the region of southern and southeastern Europe, Asia Minor, northern Africa, and Mediterranean area. *Satureja* species are commonly used for making herbal tea, flavoring agents (condiment and spice), and medicinal purposes. Infusion and decoction of aerial parts of *Satureja* species are used to produce a tonic, carminative, digestive, and expectorant and for the treatment of colds in Iranian traditional medicine. Due to the presence of secondary metabolites such as flavonoids, steroids, terpenoids, and tannins, they are known for their healing properties for a long time and have been used as traditional remedies to treat various ailments such as cramps, muscle pains, nausea indigestion, diarrhea, and infectious diseases (8).

Recently, antimicrobial (9,10), antiviral (11), antioxidant (12,13), antiproliferative (14), antiprotozoal (15), antifungal (16), anti-inflammatory, antinociceptive (17,18), antidiarrheal, and vasodilatory (19,20) activities have been reported from different species of *Satureja*. This plant is also used in ethnomedicine as an analgesic and antiseptic agent. Medicinal properties of the genus *Satureja* have made it one of the most popular plants throughout the world (10).

The polyphenols have the antioxidant, antiproliferative, and apoptosis-inducing properties related to the prevention of diseases such as cancer (20). Also, flavonoids, as polyphenolic compounds existing in plants, have potential anti-allergic, anti-inflammatory, antimicrobial, and anticancer activities (21).

S. khuzestanica is a subshrub, branched stem, 30 cm high, densely leafy, broadly ovaiate-orbicular plant covered with white hairs. In a previous study on essential oil analysis and its antimicrobial activity, carvacrol was found as a dominant compound of *S. khuzestanica*. The essential oils of *S. khuzistanica* mainly consist of carvacrol (80.6%), p-cymene (4.8%), myrcene (1.5%), γ-terpinene (2.1%), and terpinene-4-ol (2.1%). Extract and essential oil of this plant and carvacrol (as the main constituent of its essential oil) have shown propitious antimicrobial and antioxidant activities (22,23).

Bcl-2 and *Bax* are two important regulator genes in the mitochondrial apoptotic pathway. Bax promotes cell death through permeabilization of the mitochondrial outer membrane in response to different cellular stresses. In contrast, *Bcl*-2 prevents apoptosis by inhibiting the activity of *Bax*. Therefore, the balance between pro- and anti-apoptotic members of this family can determine the cellular fate. *Bax* and *Bcl*-2 are the major members of a *Bcl*-2 family whose potential roles in tumor progression and prognosis of different human malignancies have been of interest in various studies during the last decade (24, 23).

Breast cancer is one of the most common female cancers in the world (24). Improvement in the survival and quality of life of breast cancer patients requires the design and discover new therapies and drugs that are effective and less side effect. As far as we know, there is no report on the anticancer activity of an extract of *S. khuzestanica*. Thus, many studies have been recently undertaken to investigate the effects of this herb extract to discover its therapeutic potentials. The aim of this research is to study the antibacterial, antioxidant effects of the methanolic, ethanolic, and water extracts of *S. khuzestanica* against some important human pathogenic bacteria. In addition, cytotoxicity and apoptotic effect of *S. khuzestanica* were tested against MCF-7, osteosarcoma, DLD-1, and fibroblast cell lines by MTT assay and real-time PCR. To the best of our knowledge, this is the first study that focuses on the determination of antioxidant, cytotoxic, and apoptotic activities of the extract on *S. khuzestanica*.

MATERIAL and METHODS

Plant material

S. khuzistanica were collected from Lorestan Province of Iran during May and June 2013 at an altitude of 1170 m. The plant used in this study was identified by Dr. Shahsavari in the Herbarium of Agricultural Science Faculty, Khoramabad University of Iran.

Preparation of the extracts

The leaves of *S. khuzestanica* were dried at room temperature. *S. khuzestanica* powder (30 g) was added to 450 mL ethanol, methanol, and water at ambient temperature for 24 h. The suspension was filtered through a Whatman filter paper. The solvent was evaporated with a rotary evaporator at 40℃. The dried extracts were stored in the dark at +4℃ until further use.

Gas chromatography/ mass spectrometry

Fid- GC was carried out using a Hewlett-Packard 6890 with HP-5 capillary column (phenyl methyl siloxane) 25 m, 0.25 mm (i.e., the ratio of 1:25), and a flame ionization detector. Temperature programmer: 60℃ (2 min) rising to 240℃ at 4℃/min: injector temperature 250℃, detector temperature, 260℃. GC-MS was performed using a Hewlett-Packard 6859 apparatus with a quadruple detector on an HP-5 column operating at 70 eV ionization energy, using the same temperature programmer and carrier gas. Retention indices were calculated using retention times of n-alkanes that were injected after the oil chromatography (5).

Antimicrobial activity

Microorganisms and growth conditions

For the bioassays, we used eight bacteria *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Escherichia coli* (ATCC 35218) *Bacillus subtilis* ATCC 6633, *Bacillus cereus* NRRL-B-3711, P. vulgaris (NRRL B-123), and *Enterococcus faecalis* ATCC 292112, and antifungal activity against *Candida albicans* ATCC 10231 and *Candida tropicalis* ATCC 13803. All the organisms tested were collected from Gazi University, Ankara, Turkey. The bacteria species were resuspended in Mueller Hinton broth at 35-37℃. The turbidimetry of the suspension was adjusted to the McFarland 0.5 turbidity standard and, then, spread over Mueller Hinton Agar culture medium plates(3).

Disc diffusion assay

In this method, concentrations of methanolic, ethanolic, and water extracts of *S. khuzestanica* (25 and 50 mg/mL) were prepared and sterilized using membrane filters of 0.4 µm. Then, 50 µl of each concentration of the extracts was added to each sterile blank disc (Padtan Teb, Iran). In the following, from the fresh culture of *H. pylori*, 1 McFarland solution in brucella broth (Himedia) was prepared and 100 µl of the solution was taken by sterile swab and cultured on brucella agar medium containing 5% defibrinated sheep blood 7% inactivated fetal bovine serum (FBS). The dried discs of the extracts were placed on the culture on appropriate distances. After placing the discs, the media were kept in a "CO2" incubator (with 10% "CO2") at 37℃ for 72 h. Antibacterial activity was determined by measuring the zone of inhibition around the test discs. The growth inhibition diameter was an average of three different measurements. The result was expressed as the diameter of inhibition zone and compared with standard antibiotic Ampicillin (Amp, 10 µg) and Chloramphenicol (C, 30 µg).

Antioxidant activity

Determination of DPPH radical scavenging activity

Antioxidant activity of the extracts was determined based on its ability to react with the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical (Yamasaki et al. 1994). Briefly, 0,1 mmol/ L solution of DPPH was added to *S. khuzestanica* extracts solutions at different concentrations and after 30 min incubation, the absorbance was measured at 517 nm. Inhibition percentage of free radical DPPH (I%) was calculated using the following formula:

$I\% = (A_{blank} - A_{sample}/A_{blank})X100$,

Where A_{blank} is the absorbance of the control reaction and A_{sample} is absorbance of the test extract. Extract concentration providing 50% inhibition IC_{50} was calculated from the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate. BHT and ascorbic acid were used as a positive control.

Total phenolic content

Total phenolics constituents were determined using Folin-Ciocalteau reagent (Singleton & Rossi, 1965). 100µL of each plant extract (1 mg/mL) or gallic acid standard solutions was mixed with 200 µL of Folin-Ciocalteau reagent (1,1 diluted with distilled water) and 2 mL of aqueous "Na2CO3" 1M. After standing for 1 h at room temperature, absorbance was measured at 765 nm. Results were expressed as mg Gallic acid equivalents (GAE) /g DW.

Cytotoxicity assay

Cell lines

The human cancer cell line MCF-7 (breast cancer), DLD-1 (colorectal adenocarcinoma), osteosarcoma, and normal gingival fibroblast cells were obtained from Kirikkale University. All cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% fetal bovine serum, l- glutamine (3 mM), streptomycin (100 mg/mL), and penicillin (100 IU/mL). Cells were grown in a humidified atmosphere of 95% air and 5% "CO2" at 37℃.

Treatment of cell lines

Stock solutions (100 mg/mL) of methanol extract, prepared in dimethyl sulfoxide (DMSO), were dissolved in the corresponding medium to the required working concentrations. All cancer cell lines and fibroblast cells (5000 cells per well) were seeded into 96-well microtiter plates and 24 h later after the cell adherence. The cells were treated with the extracts (ranging from 3.125 to 100 µg/mL) except for the control cells to which a nutrient medium only was added. Nutrient medium (RPMI 1640) was incubated for 48 h.

Determination of cell survival

The effects of extracts on cancer cell survival were determined by MTT test. Briefly, 20 µl of MTT solution was added to each well. Samples were incubated for further 4 h at 37℃ in 5% "CO_2" and humidified air atmosphere. The supernatant was removed and replaced with 100 µL of DMSO. The optical density of wells was measured with a microplate spectrophotometer reader. Optical density was measured at 570nm. Each test was repeated three times (29).

Statistical analysis

SPSS 11 were used for statistical analyses**.** Experimental results were expressed as mean ± S.D of three parallel measurements. *P-values < 0.05* were regarded as significant.

The Double Stain Apoptosis Detection

Hoechst 33342/PI provides a rapid and convenient assay for apoptosis based upon fluorescent detection for the compacted state of the chromatin in apoptotic cells. The Double Stain Apoptosis Detection contains two ready-to-use dyes bound to DNA. Hoechst 33342, a kind of blue-fluorescence dye stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells. Propidium iodide (PI), a red-fluorescence dye, is only permeant to dead cells (25).

RNA Extraction and cDNA Synthesis

RNA was extracted using the High Pure RNA Isolation Kit (Roche, Germany). Total cDNA was synthesized using the First Strand cDNA Synthesis Kit (Fermentas, USA), according to the manufacturer's protocol. The samples were eluted with 50 µl of RNase free water and stored at -80℃. RNA concentration was determined using the NanoDrop method.

Quantitative RT-PCR

Quantification of gene expression was done by LightCycler®480. PCR solution (20 µl) was composed of 5 µl cDNA, 10µl LightCycler®480ProbesMaster, and 1µl Real Time ready Assay (Primer). The thermal treatment parameters consist of 1 cycle at 95℃ for 10 minutes, 1 cycle at 95℃ for 10 min, and 45 cycles at 95℃ for 30 sec, at 60℃ for 30 sec, and at 72℃ for 1 sec.

Polymerase Chain Reactions (PCR) amplifications were also done without cDNA template, as a negative control, to monitor the reagents utilized in the assay for possible contamination. In order to confirm the reliability of the results achieved with GAPDH as a housekeeping gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference genes for gene expression analysis.

The LightCycler®480 software was used to determine the amplification cycle in which product accumulation was above the threshold (Ct). Real time-PCR Ct values were analyzed using the Advanced Relative Quantification method.

Interactions of DNA with the Extracts

The interactions of plasmid DNA with extracts were studied by agarose gel electrophoresis. The extracts were incubated with plasmid DNA at 37℃ for 24 and 48 h. The extracts /DNA mixtures were loaded onto the 1% agarose gel with loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol). Electrophoresis was carried out in 0.05 M Tris base, 0.05 M glacial acetic acid and 1 mM EDTA (TAE buffer) for 3 h at 60V [6,51]. Afterward, the gel was subsequently stained with ethidium bromide (0.5 µg/mL), visualized under UV light using a transilluminator (BioDoc Analyzer, Biometra), and photographed with a video-camera and saved as a TIFF file(25).

RESULTS

In the present study, the extract yield percentages obtained from *S. khuzestanica* were 17.5, 16.8, and 22.54% methanol, ethanol, and water, respectively. Overall, water extraction gave better recovery percentages.

Identification of components

The linear retention indices for all the compounds were determined by connecting the sample with a solution containing homologous series of C8-C22 n-alkanes. The individual constituents were identified by their identical retention indices, referring to known compounds from the literature and also by comparing their mass spectra either with the known compounds or with the Wiley mass spectral database (Table 1).

Table 1. Gas chromatography/ mass spectrometry. Fid- GC was carried out using a Hewlett-Packard 6890 with HP-5 capillary column (phenyl methyl siloxane. 25 m, 0.25 mm i.e., ratio, 1:25, and flame ionization detector. Temperature programmer: 60ºC (2 min) rising to 240 ºC at 4 ºC/min: injector temperature 250 ºC, detector temperature, 260 ºC. GC-MS was performed using a Hewlett-Packard 6859 with a quadruple detector, on a HP-5 column, operating at 70 eV ionization energy, using the same temperature programmer and carrier gas as above. Retention indices were calculated by using retention times of n-alkanes that were injected after the oil chromatographic.

NO	Compound Name	Ret.Time	Similarity	KI_{STD}	KI_{CAL}	Area (%)
1	α-Thujene	9.5	96	930	931	0.61
2	α-Pinene	9.8	98	939	940	0.42
3	Camphene	10.4	97	954	959	0.05
4	β-Pinene	11.3	97	979	988	0.12
5	Myrcene	11.5	94	991	995	1.16
6	6-Methyl-3,5-heptadien-2-one	11.7	86	-	1001	0.38
7	3-Octanol	12.0	96	991	1011	0.04
8	3-Carene	12.2	93	1031	1017	0.1
9	α-Terpinene	12.5	95	1017	1026	0.25
10	para Cymene	12.9	93	1025	1038	3.35
11	β-Ocimene	13.4	95	1050	1053	0.02
12	ɣ-Terpinene	13.9	97	1060	1068	0.67
13	cis Sabinene hydrate	14.5	92	1070	1087	0.44
14	Terpinolene	14.8	95	1089	1096	0.07
15	Linalool	15.3	96	1097	1112	1.02
16	4-Terpineol	18.1	97	1177	1201	1.94
17	α-Terpineol	18.7	95	1189	1218	0.17
18	Thymol	21.6	93	1290	1315	0.28
19	Carvacrol	22.7	89	1299	1353	86.29
20	β-Caryophyllene	25.2	93	1419	1443	0.13
21	Geranyl acetone	25.7	97	1455	1463	0.18
22	α-Franesene	27.1	95	1506	1512	0.33
23	β-Bisabolene	27.3	96	1506	1522	0.97
24	α-Bisabolene	28.1	89	1507	1553	0.15
25	Caryophyllene oxide	29.7	94	1583	1616	0.53
26	β-Udesmol	30.0	89	1651	1630	0.25
27	α-Bisabolol	32.0	89	1686	1711	0.08

Antimicrobial activity

The antimicrobial and antifungal activity of the extracts was measured by disc diffusion method. The antimicrobial and antifungal activity of the extracts was found within the 25-50 mg/mL concentration range. The inhibition zones of the bacteria and fungi were in the range of (14 ±0.4) mm to (22.3 ±0.4) mm, respectively. According to the disc diffusion method, all concentrations used were inhibitory for all reference bacterial strains. The most susceptible organisms were the Gram-positive *B. subtilis and S. aureus* with large inhibition zones even at the lowest extracts.

Among the Gram-negative bacteria, *E. coli* were highly sensitive to the extracts at different concentrations in the disc diffusion method. The methanol extract was found as the most effective extract in each concentration (25, 50 mg/mL) against all the bacteria strains (Table 2). It was also seen that water extract had no antimicrobial and antifungal effect. Antimicrobial effect of the extracts increased according to concentrations. Ampicillin (10 mg) and chloramphenicol (30 mg) were used as standard antibacterial agents and ketoconazole (50 mg) was used as an antifungal control.

Table 2. Antimicrobial activity of methanol and ethanol extract of *S. khuzestanica*

Microorganism	Ethanol extract (µM)	Methanol extract (µM)
B. cereus NRRL-B-3711	25 ± 0.3	25± 0.5
B. subtilis ATCC 6633	12,5± 0.7	12,5± 0.2
S.aureus ATCC 25923	12,5± 0.5	12,5± 0.3
E. coli ATCC 25922	6,25± 0.2	6,25± 0.8
E. coli ATCC 35218	6,25± 0.0	12,5± 0.5
E.fecalis ATCC 292112	12,5± 0.4	6,25± 0.5
P. vulgaris (NRRL B-123)	25± 0.7	12,5± 0.8
P.aureginosa ATCC 27853	6,25± 0.5	12,5± 0.0
C. albicans ATCC 10231	6,25± 0.3	6,25± 0.2
C. tropicalis ATCC 13803	12,5± 0.4	12,5± 0.0

About 50% DMSO (solvent of the extracts) was used as a negative control by disc diffusion method. The highest antifungal activities were observed against *Candida albicans*.

Antioxidant activity

Free radical-scavenging activity potentials of test plant extracts were evaluated for estimating their antioxidant capacities. The DPPH results of *S. khuzestanica* extracts in different concentrations are presented in (Fig. 1) the positive standard (BHT), methanol, ethanol, and water extracts showed significant antioxidant activities.

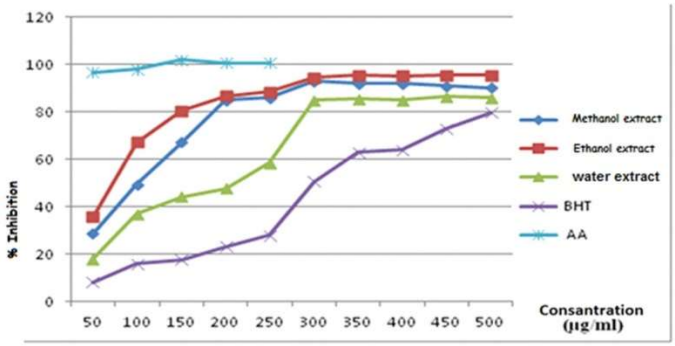

Figure 1. DPPH radical scavenging activities methanol, ethanol and water extracts of *S. khuzestanica*.

To determine antioxidant capacity, 10 concentrations (mg/mL) were tested for each extract. The lower IC_{50} value indicates a higher antioxidant activity. The ethanol extract IC_{50}=37,7±0,8 µg/mL exhibited a higher DPPH scavenging activity than those of methanol IC_{50}=51,3±0,8 µg/mL and water extracts IC_{50}=65,5±0,5 µg/mL. Also, DPPH scavenging activities of the methanol and ethanol extracts were higher than the standard BHT. DPPH radical scavenging activity of test samples decreased in the order of ethanol>methanol>BHT >water.

All extracts of the studied plants exhibited antioxidant activities. Phenolic compounds of these plants could be contributed to their antioxidant activities. Some studies reported a positive correlation between total phenolic content and antioxidant activity. These results indicated that *S. khuzestanica* extracts have different antioxidant capacities. Also, the broad range of activity suggests that multiple mechanisms are responsible for the antioxidant activity. The multiple antioxidant activities of *S. khuzestanica* detected in this study demonstrate the potential application value of these extract.

Amount of total phenolic constituents

Phenolic content of gallic acid was used as a standard; the contents of phenolic in plant extracts were expressed as gallic acid equivalents to help the comparison. The standard curve equation is,

y (absorbance)=0.0085x (µg gallic acid)-0.0209, R^2=0.9858.

The absorbance value was inserted into the equation above and the total amount of phenolic compound was calculated by the same equation. The total phenolic contents of methanol, ethanol, and water extracts were 218,45±1,004 µg/mg, 222,55±,946 µg/mg, and 210,67±0,926 µg/mg, respectively. In general, higher phenolics contents were observed in the ethanol extract. The ethanol extract of the plant showed slightly higher phenolic content compared to methanol and water extracts. The results of total phenolic content showed that after ethanol, the methanol, BHT, and water extract contained a high amount of phenolic compounds, in the order of their appearance.

Cytotoxicity assay

The cytotoxicity of extracts was tested against MCF-7, osteosarcoma, DLD-1, and fibroblast cell lines, using MTT. The concentrations of the compounds were made as 1 mg stock solution and serially diluted to give six concentrations ranging from 100 µg to 3.125 µg. The results obtained from three independent experiments were averaged to give the relative cell viability at each concentration. The relative cell viability values were shown in (Fig. 2).

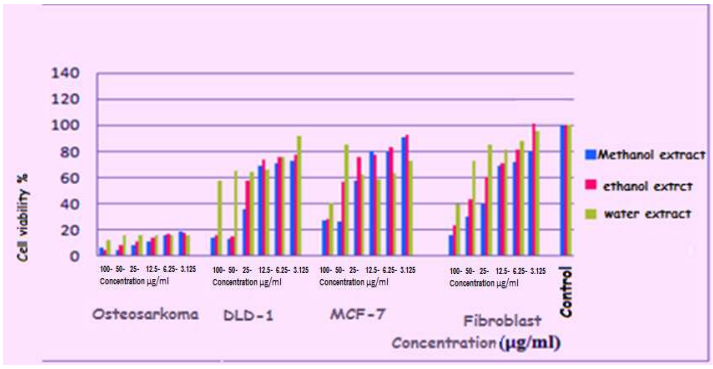

Figure 2. Relative cell viability (%) of *S. khuzestanica* extracts(methanol, ethanol, water) in different cancer cell lines (Osteosarcoma DLD-1,MCF-7,Fibroblast) following exposure of cells with various concentrations between 100 µg/mL -3.25 µg/mL for 24 h.

The methanol extract exhibited strong activity against MCF-7 cells. The cytotoxicity of the methanol extracts on MCF-7 and DLD-1 cells for 24 h is presented in Fig. 2. Also, IC50 values of the methanol extract for MCF-7 was 30.125 µg/mL while for DLD-1 it was 26.92. The IC50 values of the methanol, ethanol, and water extracts are shown in (Table 3).

Table 3. The IC_{50} values of the methanol,ethanol and water extracts on various cancer cell lines

Cell lines	Extracts	IC_{50} values (µg/ml)
Fibroblast	Methanol	29,86
	Ethanol	33,92
	water	62,41
MCF-7	Methanol	30,125
	Ethanol	40,125
	water	40,125
Osteosarcoma	Methanol	47,94
	Ethanol	63,56
	water	65,24
DLD-1	Methanol	26,92
	Ethanol	36,03
	water	61,75

The cytotoxic activity of methanol and ethanol extracts on MCF-7 cells was higher than those of other cancer cell lines. The cytotoxic activity of methanol, ethanol, and water extracts on all cancer cell lines decreased in the order of methanol>ethanol>water; also the results indicated that the cytotoxicity order of extracts was Fibroblast > MCF-7> DLD-1> osteosarcoma. The results indicated that the cytotoxicity of methanol and ethanol extracts was influenced by the concentration (100-3.125 $\mu g/_{mL}$). Methanol extract exhibited a remarkable cytotoxic effect on MCF-7 and DLD-1cells. Among all cancer cells, only MCF-7 cells were highly susceptible to methanol and ethanol extract.

Hoechst 33342 and PI double-staining assay

Since apoptosis is the major mechanism of most anticancer agents, we investigated the apoptosis-inducing activity of *S. khuzestanica* extracts using Hoechst 33342 and PI double-staining assay.

The results showed that the nuclei of cancer cells not treated with *S. khuzestanica* extracts exhibited diffuse staining of chromatin. Also, cells not stained by PI did not present a brighter blue fluorescence (control). With the treatment of *S. khuzestanica* extracts, more nuclei exhibited brighter blue fluorescence, indicating the nuclear condensation in apoptotic cells (Fig 3, 4). Furthermore, the apoptosis-inducing activity of methanol, ethanol, and water extracts on all cancer cell lines decreased in the order of methanol>ethanol>water. These results suggested that *S. khuzestanica* extracts could induce apoptosis in cancer cell lines.

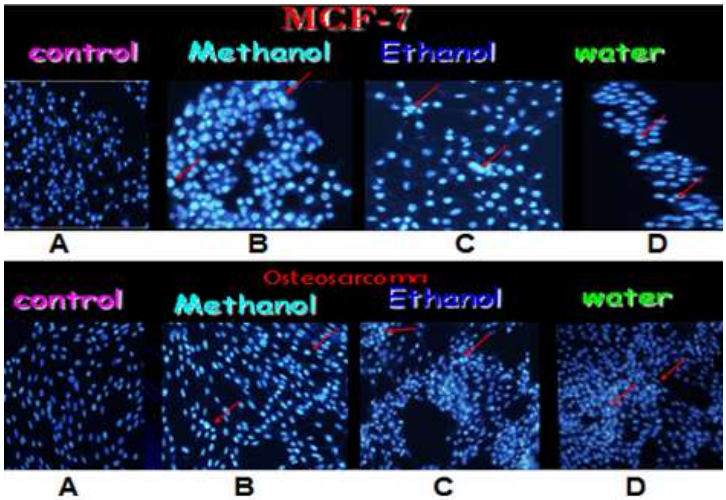

Figure 3. Analysis of apoptosis by double-staining with Hoechst 33342/PI in MCF-7 and osteosarcoma cancer cell line that treated with methanol, ethanol and water extract of *S. khuzestanica*, observed under the fluorescence microscopy. The control cells were untreated with extracts.

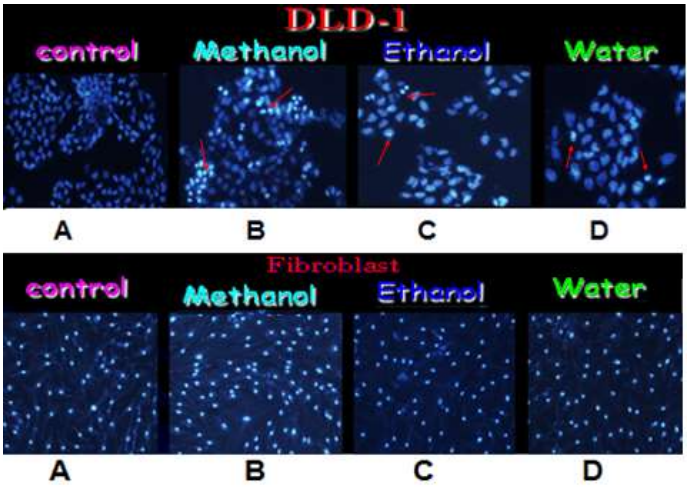

Figure 4. Analysis of apoptosis by double-staining with Hoechst 33342/PI in DLD-1and fibroblast cancer cell linet that treated with methanol, ethanol and water extract of *S. khuzestanica,* observed under the fluorescence microscopy. The control cells were untreated with extracts.

Evaluation of dependence between Bcl-2 and Bax expression

The relative level of mRNA expression of *Bax* and *Bcl*-2 genes and *Bax/Bcl*-2 ratio was evaluated in MCF-7, DLD-1, osteosarcoma, and fibroblast cell lines. The statistical analysis showed no significant correlation between relative level expression of *Bax* and *Bcl*-2 with DLD-1, osteosarcoma, and fibroblast cell lines. According to our results, *Bax*/*Bcl*-2 ratio was significantly *($P < 0.05$)* correlated with MCF-7 cell line (Fig. 5). Compared to other results, the lower levels of this ratio may lead to resistance of human cancer cells to apoptosis. Thus, *Bax/Bcl*-2 ratio can affect tumor progression and aggressiveness. However, there is a stronger possibility that ethanol and water extract of *S. khuzestanica* may affect the expression level and clinical roles of molecules involved in apoptosis, especially *Bax* and *Bcl*-2.

Gene expression analysis was performed using the relative quantification method and compared to controls according to the Advanced Relative Quantification method.

A Relative Quantification analysis was used to compare two ratios: the ratio of a target DNA sequence to a reference DNA sequence in an unknown sample, and the ratio of the same two sequences in a standard sample called a "calibrator". The results of MCF-7 value are shown in (Fig. 5).

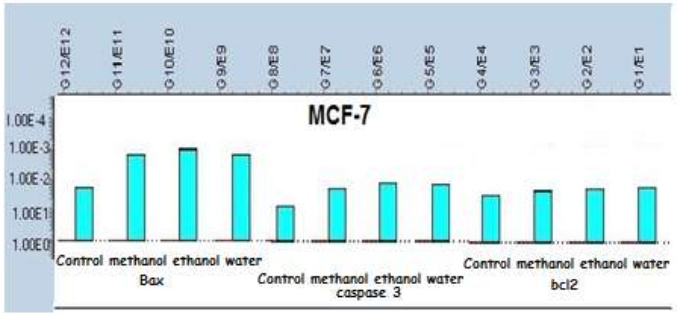

Figure 5. The Results displays height of the left bar indicates the target/reference ratio of the Bax and Caspase 3 value in MCF-7 cell lines and the right bar shows the bcl2 value in MCF-7 cell lines.

Interaction of methanol and ethanol extracts of S. khuzestanica with plasmid DNA

Electrophoretograms were used to investigate the interaction of plasmid DNA for decreasing concentrations of methanol and ethanol extracts of *S. khuzestanica* Lane P with untreated plasmid DNA. These concentrations (in µM) are as follows: lanes 1 to 7 apply to plasmid DNA interacting with decreasing concentrations of the extracts: lane 1: 10000; lane 2: 5000; lane 3 2500; lane 4: 1250; lane 5: 625; lane 6: 312; lane 7: 156. The top, middle, and bottom bands correspond to form II (single nicked open circular), form I (covalently closed circular or supercoiled), and form III (linear) plasmids, respectively.

When the extracts interact with plasmid DNA, the DNA damage due to the conformational change can be visualized via gel electrophoresis (Fig. 6). In the electrophoretograms, the lane (p) applies to untreated plasmid DNA as a control, while the lanes 1 to 7 apply to plasmid DNA interacted with decreasing concentrations of the extracts (from 10000 µM to 156 µM). The extracts have a strong effect on plasmid DNA, especially at lanes 3 and 4, where in the intensity and mobility of form I was decreased. In the case of lanes 5, 6, and 7, the extracts (except methanol at 48 h) indicate a very little effect on the mobility and intensity of plasmid DNA (Fig. 6).

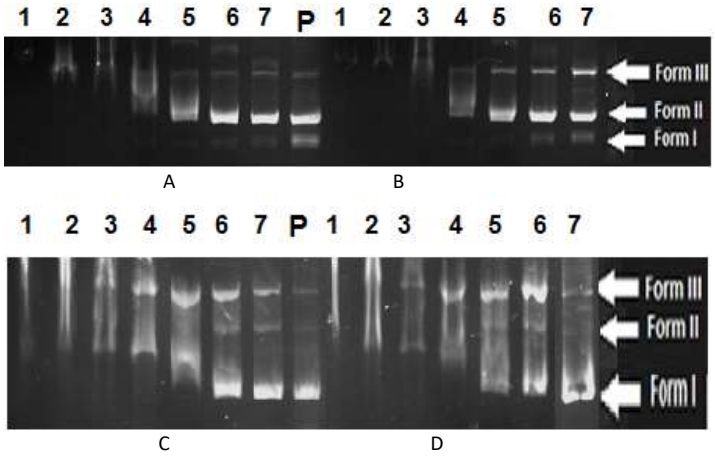

Figure.6. Electrophoretograms applying to the interaction of plasmid DNA with decreasing concentrations of methanol and ethanol extracts. for 24 (A), (B), 48 (C) a (D) hours incubation respectively. Lane P applies untreated pBR322 plasmid DNA. Concentrations (in µM) are as follows: lanes 1 to 7 apply to plasmid DNA interacted with decreasing concentrations of the extracts: : lane 1: 10000; lane 2: 5000; lane 3 2500; lane 4: 1250; lane 5: 625; lane 6: 312; lane 7: 156 (µM). The top and the bottom bands correspond to form II (single nicked open circular), form I (covalenty closed circular or supercoiled) and form III (linear) plasmids, respectively.

DISCUSSION

Satureja is an analgesic and antiseptic plant found in the southern parts of Iran. The composition of the essential oils of wild and cultivated *S. khuzistanica* and the antioxidant, antidiabetic, antihyperlipidemic, and reproduction stimulatory properties of this plant have been recently reported from Iran. Carvacrol is one of the most important components of many species including those belonging to *Satureja* genus. This phenolic compound shows antiseptic, antibacterial, antifungal as well as antinociceptive and anti-inflammatory properties in *Satureja* spp (23,26).

The results of antimicrobial activity showed that among the extracts studied in this work, methanol extract had a more powerful antimicrobial effect than ethanol and water extracts against important eight human pathogenic bacteria.

According to antimicrobial results, ethanol, methanol, and water extracts of *S. khuzestanica* had different antimicrobial effects against these pathogens, and these effects may result from their phytochemical possessions including phenolic compounds (6).

Antimicrobial effect of the extracts was different, depending on the type of microorganisms; thus, the Gram-positive bacterium *S. aureus* has a higher sensitivity compared to Gram-negative bacteria *E. coli*. Previous works showed that the Gram-positive bacteria are more sensitive to plant extracts than the Gram-negative ones, due to differences in the cell structure of Gram-negative and Gram-positive bacteria. An explanation for this behavior is that Gram-positive bacteria have a higher mucopeptide content in their cell wall composition while Gram-negative bacteria have only a thin layer of mucopeptide and most of their cell structure is lipoprotein and lipo polysaccharides. Thus, Gram-negative bacteria are more resistant. These observations are consistent with the results obtained by (27) who showed the high antimicrobial effect of these extracts. It seems that the presence of thymol, carvacrol, P-cymene, and gamma-terpinene cause the strong antimicrobial effect of these extracts. Several mechanisms are discussed to explain the antimicrobial effect. Many of the previous studies demonstrated that the members of the genus *Satureja* show a high antimicrobial activity due to the presence of thymol, carvacrol, and their precursors (18).

The biological precursor of carvacrol, p-cymene, is hydrophobic and causes expansion of the cytoplasmic membrane. When combined with carvacrol in vivo, p-cymene incorporates into cytoplasmic membranes, facilitating transport of carvacrol across them (Baren, et al. 2006). Thus, the antimicrobial activity of carvacrol is increased by the presence of its precursor p-cymene, owing to described synergistic effect.

The antimicrobial effect of thymol and carvacrol is due to the damage in membrane integrity induced by a change in pH hemostasis. Also, by the equilibrium of inorganic ions, although p-cymene does not have antimicrobial activity, the antimicrobial activity of thymol or carvacrol is increased (28).

The flavonoids are almost ubiquitous in plants and are powerful chain-breaking antioxidants acting as metal chelators and free radical scavengers (29). Nitrogen compounds (alkaloids), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites in plants also play important roles in antioxidant activity (1).

Several antioxidant compounds have different polarities; therefore, different solvents are frequently used for the isolation of antioxidants. Antioxidant activity of the extracts is drastically dependent on the type of solvents (30).

According to our antioxidant results, the extract dissolved in ethanol had the most powerful antioxidant activity in DPPH free radical scavenging methods and contained the highest phenolic compounds. According to Miliauskas and colleagues, there is a correlation between flavonoids and antioxidant activity. Also, a significant correlation was found between the content of phenolic compounds and the DPPH free radical scavenging (31).

Our antimicrobial results were also in agreement with the literature that exhibited a good correlation between antimicrobial and antioxidant activity. The methanol and ethanol extracts of *S. khuzestanica* were detected with strong antioxidant and antimicrobial activities. Although several studies have reported antioxidant and antimicrobial activities of some *Satureja* species, our results proved that *S. khuzestanica* has strong antioxidant activities. Among species studied, *S. khuzestanica* was found to be more active against studied microorganisms than the other species.

In the cytotoxic assay, ethanol, methanol, and water extracts exhibited cytotoxic effects on MCF-7, DLD-1, and osteosarcoma cells. Methanol extract had a more powerful cytotoxic effect than ethanol extract on MCF-7, DLD-1, and osteosarcoma cells.

There are some reports that contribute to the relationship of cytotoxicity with antioxidant activity. Therefore, antioxidant potential of the methanol extract may contribute to its cytotoxic activity.

These phenolic compounds could be responsible for the observed cytotoxic activity of the methanol and ethanol extract. Since the cytotoxic activity of *S. khuzestanica* extracts has not been reported up to now, additional research is needed to investigate its cytotoxicity.

Dysregulation of the mitochondrial pathway of apoptosis is one of the most important events during carcinogenesis. Bcl-2 protein family, including antiapoptotic (Bcl-2 and Bcl-xl) and proapoptotic (Bax and Bak) members play an essential role in the regulation of this pathway. According to our results, Bax/Bcl-2 ratio was significantly *($P < 0.05$)* correlated with MCF-7 cell line.

CONCLUSION

Our analysis on the antibacterial, antioxidant cytotoxicity and apoptotic effect of *S. khuzestanica* in different cancer cell lines provided a better insight into understanding the therapeutic strategies and molecular mechanisms of breast cancer in order to predict genetic risk. Thus, it would be possible to obtain a novel drug that could potentially be less toxic and more cost-effective against cancer. Our results suggest that these methanolic and ethanolic extracts might have the potential to be used in, for example, anticancer agents.

Conflict of interest

No conflict of interest was declared by the authors.

Acknowledgments

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