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Variation in antibacterial activity of different ecotypes of *Satureja khuzestanica* Jamzad, as an Iranian endemic plant

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Satureja khuzestanica Jamzad is an endemic plant that widely distributed in South-west Iran. In the folk medicine, *S. khuzestanica* has been used as analgesic and antiseptic among the inhabitants of southern parts of Iran. The study determined variations of carvacrol content and antibacterial activity of essential oil (EO) from different ecotypes of *S. khuzestanica*. The aerial part of wild populations of *S. khuzestanica* at full flowering was collected from different natural habitats in South-west Iran. The EO of an ecotype was characterized using GC–MS. Carvacrol contents in the EOs, the main component in the EO of *S. khuzestanica* (90.8%), were determined by using HPLC. The results showed the carvacrol contents in different ecotypes ranged 42.5 - 94.8 mg/ml EO. The highest content of carvacrol in all investigated samples was obtained from Mangreh-II ecotype. The antibacterial activity of the EOs was tested against four pathogens. The inhibition zones and MIC values for bacterial strains, which were sensitive to the EOs of *S. khuzestanica*, were in the range of 12–32 mm and 0.019–0.312 mg/ml, respectively. The EO of Mangreh-II ecotype showed the strongest antibacterial activity. This ecotype had the highest amount of carvacrol in comparison with other ecotypes. The results may be used in ecotype selection program for cultivation and production of carvacrol with suppressing effect on pathogens in same growing conditions.

Keywords: Bioactivity, Essential oil, High Performance Liquid Chromatographic, *Satureja khuzestanica*

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The genus *Satureja* (the family Lamiaceae) constitutes about 200 species of herbs and shrubs, widely distributed in Mediterranean area, Asia, and boreal America[1]. *Satureja khuzestanica* Jamzad (*Marzeh–e–Khuzestani* in Persian) is an endemic plant that widely distributed in the southern parts of Iran[2]. Some members of *Satureja* are of economic importance since they have been used as culinary herbs, flavoring agents in perfumery and cosmetics[3]. The aerial parts of *Satureja* are used for herbal tea and, in traditional medicine, to treat various ailments, such as cramps, muscle pains, nausea, indigestion, diarrhoea and infectious diseases[4]. *Satureja* is famous for its medical uses as analgesic and antiseptic in folk medicine[5]. In the folk medicine, *S. khuzestanica* has been used as analgesic and antiseptic among the inhabitants of southern parts of Iran[6]. The EO and extracts isolated from *S khuzestanica* have biological properties, including antibacterial[7,8], antifungal[9], antioxidant[10,11,12], anti–diabetic[10,11], anti–hyperlipidemic[13], anti–inflammatory[14] and anti–antileishmanial[15]. These activities are mostly related to their phenolic compounds content especially carvacrol (5–isopropyl–2–methylphenol) (Fig. 1a). Carvacrol is a phenolic monoterpene presented in the EO of the wild and cultivated *S. khuzestanica*[8,12,15,16].

It is well known that yield and yield components of plants are determined by a series of factors, including plant genetic, climate, edaphic, elevation, and topography[17,18] and also an interaction of various

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Abbreviations: HPLC: High Performance Liquid Chromatographic, GC–MS: Gas Chromatography and Mass Spectrometry, EO: Essential Oil, MIC: Minimum Inhibitory Concentration, NB: Nutrient Broth, MHA: Mueller Hinton Agar, DMSO: Dimethyl Sulfoxide, PBS: Glycerol Phosphate Buffered Saline, EC: Electrical Conductivity.

factors[19]. Medicinal plants are no exception and their yield and composition of their essential oil are influenced by chemical polymorphisms or chemotypes [19,20] management practices such as harvest time as well as ecological and climatic conditions[21]. To our knowledge, no documented reports on diversity of carvacrol content and antibacterial activity of the EOs from various ecotypes of *S. khuzestanica* are available. The aims of this study were to (I) determine the variation of carvacrol content in the EOs of *S. khuzestanica* collected from various natural habitats, (II) evaluate the antibacterial activity of the EO of different ecotypes, and (III) assess the relationships between variations of carvacrol content and the environmental factors involved in different geo–ecological regions.

Methodology

Plant material

The aerial parts (up to ~ 5 cm, 0.1–0.2 kg) of wild populations of *S. khuzestanica* were collected from Khuzestan and Lorestan provinces, southwest Iran (Fig. 1b) on October 2011. The plants were identified by taxonomic references[22], which voucher number of this species is IAUSHK–35 at Research Center of Medicinal Plants, I A U, Shahrekord Branch. Soil physical and chemical characteristics including *p*H, EC, organic carbon, and nutrient level of N, P and K were determined (Table 1). Climatic data of the locations were determined using the nearest meteorology station.

Reagents and chemicals

Methanol (HPLC grade), ethanol (analytical grade), acetonitrile (analytical grade), water (HPLC grade), anhydrous sodium, NB, MHA, DMSO, and PBS were purchased from Merck Co (Darmstadt, Germany). The standard of carvacrol was purchased from ROTH (Karlsruhe, Germany). Alkan standard solution C_5–C_{24} was purchased from Sigma – Aldrich Co. (Steineheim, Germany)

Sample preparation

Harvested flowering aerial parts (leaves and flowers) were dried at room temperature. Dried plant material was powered (100 gm, and subjected to hydro–distillation (1000 ml distillated water) for 3 hrs using a Clevenger–type apparatus. Samples were dried using anhydrous sodium sulphate, and then kept in amber vials at 4°C prior to use.

Fig. 1—Structure of carvacrol, as major component in the essential oil of *Satureja khuzestanica*

Preparation of standard solution

Stock standard solutions were prepared by accurately weighing 31.35 mg thymol reference standard and 7.5 mg carvacrol into separate 50 ml volumetric flasks and dissolving in acetonitrile/water (50:50, v/v). Working standard solutions (1, 2.5 and 5 ml) were prepared by dilution from the stock standard solution. The mixture was stirred carefully and refluxed in a water bath at 90°C for one hour.

Identification of phenolic compounds using HPLC

The isolation and analysis method for carvacrol were conducted according to previously published protocols. The obtained mixture was injected to HPLC system (Kanauer, Germany). An HP 1000 series liquid chromatography system comprising vacuum degasser, quaternary pump, autosampler, thermostatted column compartment and diode array detector was used. Column Machery–NAGEL, Nucleosin–100–5 C18, Loop 20 µl was maintained at 30°C. Solvents used for separation were water (eluent A) and acetonitrile (eluent B). The gradient program was as follows: 70% A/30% B, 0–5 min; 42% A/58% B, 5–18 min; 70% A/ 30% B, 18–30 min. The calibration curves (correlation coefficient) for thymol and carvacrol were $Y = 89322\ x - 382440$ ($r^2 = 0.998$) and $Y = 74919\ x - 247838$ ($r^2 = 0.994$), respectively. Samples were filtered through a 0.45 µm membrane filter before injection. The flow rate was kept 1 ml/min. The injection volume was 20 µl, and peaks were monitored at 330 nm. The chromatographic peaks of carvacrol were confirmed by comparing their retention times and UV spectra with that of their reference standard. Working standard solutions were injected into the HPLC and peak area responses were obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried

Table 1—Geographical and climate of natural habitats of 10 populations belonging to *Satureja khuzestanica*

Region	Province	Altitude (m)	Latitude (UTM)	Longitude (UTM)	Pa	Tb	pH	E.C.d	O.Ce	Pf	Ng	Kh
Sade–e–dez	Khuzestan	578	261677	3609668	73.7	23.9	7.4	1.54	3.9	53.5	0.40	285
Lios(I)	Khuzestan	926	292201	3622110	94.5	22.2	7.6	2.45	1.3	5.0	0.22	302
Lios(II)	Khuzestan	909	290043	3623822	94.6	22.2	7.6	1.60	3.3	7.9	0.35	355
Lios(III)	Khuzestan	893	288695	3625430	94.5	22.2	7.6	1.30	3.3	7.2	0.36	355
Lios(IV)	Khuzestan	952	290040	3625205	94.5	22.2	7.8	0.96	0.3	0.7	0.05	210
Mangreh(I)	Khuzestan	656	244783	3634293	118	25	7.7	1.55	4.0	24.8	0.30	262
Mangreh(II)	Khuzestan	718	243749	3637351	118	25	7.6	0.96	3.3	7.9	0.36	259
Mangreh(III)	Khuzestan	700	243838	3635583	118	25	7.7	0.85	2.8	11.8	0.27	215
Mangreh(IV)	Khuzestan	573	246132	3633651	118	25	7.4	1.50	2.6	10.5	0.28	413
Paelm	Lorestan	523	776445	3642675	89	24	7.6	1.31	4.8	24.9	0.47	332

a P: Annual precipitation (mm), b T: Average temperature (°C), d E.C.: electrical conductivity (dS.m–1), e O.C.: organic carbon (%), f P: Available P (mg.kg–1), g N; total nitrogen (%), h K: Available K (mg.kg–1)

out from integrated peak areas of the samples using the corresponding standard graph[23,24].

Gas chromatography/mass spectrometry (GC/MS) analysis

The essential oil was analyzed using an Agilent 7890 A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP–5MS 5% phenyl methyl siloxane capillary column (30.00 m × 0.25 mm, 0.25 µm film thickness). Oven temperature was kept at 60°C for 4 min initially, and then raised at the rate of 4°C/min to 260°C. Injector and detector temperatures were set at 290°C and 300°C, respectively. Helium was used as carrier gas at a flow rate of 2 ml/min, and 0.1 µl samples were injected manually in the split mode. Peaks area percents were used for obtaining quantitative data. The gas chromatograph was coupled to an Agilent 5975 C (Agilent Technologies, Palo Alto, CA, USA) mass selective detector. The EI–MS operating parameters were as follow: ionization voltage, 70 eV; ion source temperature, 200°C. Retention indices were calculated for all components using a homologous series of *n*–alkanes (C_5–C_{24}) injected in conditions equal to samples ones. Identification of oil components was accomplished based on comparison of their retention times with those of authentic standards and by comparison of their mass spectral fragmentation patterns (WILLEY/ChemStation data system)[25].

Antibacterial test

Clinical isolates of two Gram–positive (*Staphylococcus aureus* and *Bacillus cereus*), and two Gram–negative (*Escherichia coli and Pseudomonas aeruginosa*) bacteria strains obtained from Food Microbiology Laboratory, Veterinary Medicine Faculty, (I A U) Iran. Bacteria strains were identified using polymerase chain reaction–restriction fragment length polymorphism, and conventional morphological and biochemical tests. Stock cultures of bacteria were kept in 20% glycerol PBS at –70°C. Active cultures were generated by inoculating 100 µl of the thawed microbial stock suspensions into 5 ml NB followed by overnight incubation at 37°C. The density of bacteria culture required for the test was adjusted to 1.0 McFarland standards, (1.0×10^7 CFU/ml) measured using the spectrophotometer (Eppendorf, AG, Germany). These experiments were performed by the disc diffusion method with some modification[26]. Sterile paper discs (6 mm in diameter) were impregnated with 100 µl of the essential oils and incubated at 37°C for 24 hrs in MHA. Bacteria growth inhibition was determined as the diameter of the inhibition zones around the discs (mm). The MIC values were evaluated using the broth serial dilution method according to standard methods[27]. Bacteria strains were cultured overnight at 37°C in NB. Stock solutions of the essential oil and antibiotic standard (erythromycin) were prepared in 5.0% (v/v) DMSO. Dilution series, using NB, were prepared from 0.04 to 5 mg/ml. After incubation at 37°C for 24 hrs, the microorganism growth inhibition was evaluated by measuring absorbance at 630 nm, using a spectrophotometer. Experiments were performed in triplicate but at three different times.

Statistical analyses

The data was statistically analyzed by SPSS (17.0) software, using a completely randomized design (CRD). Means of the traits were compared by Duncan's multiple range test at $p \leq 0.05$ level.

Results

The results indicated that the most of natural habitats of *S. khuzestanica* were in low altitudes (523–952 m above sea level). GC–MS analysis resulted in identification of 14 constituents of the oil composition (Table 2). Their sum constituted the bulk of the oils and ranged from 99.2% oil. The analysis of EO indicated a major compound in the EO of *S. khuzestanica* was carvacrol (90.9%), and the EO contained oxygenated monoterpenes (93%) (Table 2). The yellow EO yield of studied ecotypes of *S. khuzestanica* ranged between 1.1 to 1.4% (v/w) relative to the dried aerial parts. The highest EO yield was obtained from Paelm ecotype, and Lios ecotype produced the lowest EO yield (Table 3). In order to detect carvacrol component of *S. khuzestanica*, an HPLC method with electrochemical detection was developed. Figs. 2&3 show the chromatograms of the standard solution of the investigated carvacrol, and EO obtained under the HPLC conditions. Carvacrol had retention times *(tR)* of 14.98 min. The results of analysis of variance indicated that there was significant difference ($p \leq 0.05$) between different populations for carvacrol content (Table 3). The highest amount of carvacrol in all investigated samples was recorded in Mangreh -II population from Khuzestan province (94.8 mg/ml EO), while the lowest was observed in Lios (III) population from the same province (42.5 mg/ml EO) (Table 3). The results indicated significant variation in the antibacterial properties of essential oil (Table 3). The EOs showed strong activity (inhibition zone ≥ 20 mm), and moderate activity (inhibition zone < 20–12 mm). Attending to this, the major effectiveness was achieved by the EO from Mangreh-II population from Khuzestan province. The EOs of Mangreh and Paelm populations had the most bacteriostatic properties against three strains tested (MIC ≤ 0.019 mg/ml).

Discussion

Results of current study indicated the main compound in EO of *S. khuzestanica* was carvacrol which confirms earlier reports [8,12,15,16]. In addition, our results of the yield of the EOs extracted are in agreement with results of the yield of the oils extracted from other ecotypes that have been reported by other researchers were 1.12% (v/w) from Indimeshk (Khuzestan)[8] 0.9% (v/w) from Khoarramabad (Lorestan)[15] and 0.6 and 1.2% (v/w) for wild and cultivated plants, respectively[16]. Previous findings on other Lamiaceae plants have shown that the variation of their quantitative extract composition is attributed to the geographic direction[25,26]. EO yield was fairly strongly related to the concentrations of essential elements and percentage of organic matter in soil, and altitude, temperature and precipitation[26,28,29]. Results of our study indicated various populations of *S. khuzestanica* influenced on carvacrol amount. The highest content of carvacrol obtained from Mangreh-II population. Probably environmental factors in Mangereh region provided a better growing condition which led to a higher accumulation of carvacrol in the EO of *S. khuzestanica*. In addition, these chemical

Table 2—The essential oil components of *Satureja khuzestanica* collected from Mangreh

S. No	Compound	RT[a]	RI[b]	%[c]
1.	α–Pinene	4.081	935	0.28
2.	β–Myrcene	5.157	990	0.39
3.	α –Terpinene	5.77	1016	0.49
4.	*p*–Cymene	5.964	1023	3.11
5.	*β*–Phellandrene	6.067	1027	0.19
6.	ɣ–Terpinene	6.828	1056	1.24
7.	Linalool	7.944	1098	0.91
8.	Borneol	9.89	1162	0.35
9.	Terpinene–4–ol	10.26	1173	0.65
10.	Thymol	13.992	1291	0.19
11.	Carvacrol	14.164	1296	90.88
12.	*(Z)*–Caryophyllene	17.757	1413	0.15
13.	*β*–Bisabolene	20.464	1502	0.21
14.	Caryophyllene oxide	22.57	1574	0.18

[a] RT: Retention time (min), [b] RI: Retention indices determined on HP–5MS capillary column, [c] Calculated from TIC data.

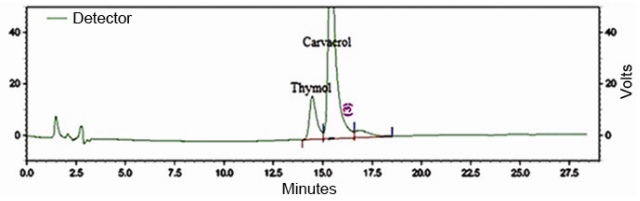


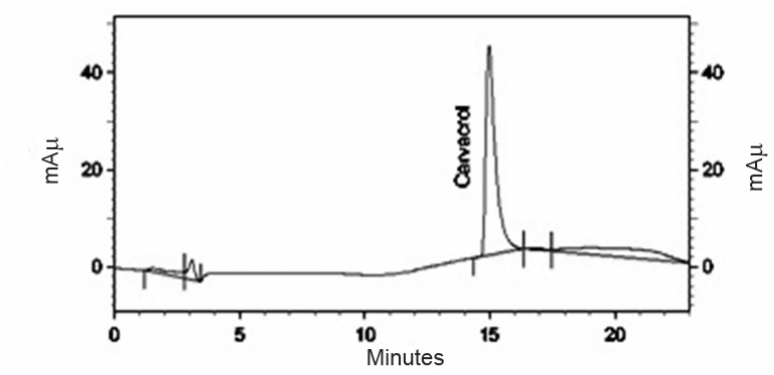
Fig. 2—HPLC chromatograms of standard solution (carvacrol t_R = 14.68 min and thymol t_R = 15.11 min)

Table 3- The carvacrol contents, zones of growth inhabitation and minimum inhibitory concentration (MIC) of 10 populations of *Satureja khuzestanica*

Population	Oil yield (%)	Carvacrol (mg/ml)	Zones of growth inhabitation (mm)				Minimum inhibitory concentration (mg/ml)			
			E.c[a]	*S.a* [b]	*P.a* [c]	*B.C* [d]	*E.c*	*S.a*	*P.a*	*B.C*
Sade–e–dez	1.29±0.21*	45.6±3.24 b†	20.0	21.3	18.0	16.3	0.078	<0.019	0.078	0.312
Lios (I)	1.23±0.13	42.7±4.22 b	17.6	22.0	19.6	15.0	0.156	0.312	0.078	0.312
Lios (II)	1.12±0.39	51.16±2.11 b	21.0	23.6	20.3	17.6	<0.019	<0.019	0.078	0.078
Lios (III)	1.27±0.31	42.5±3.99 b	18.3	19.0	14.0	12.0	0.156	0.156	0.312	0.312
Lios (IV)	1.38±0.17	65.3±5.72 ab	25.6	25.6	17.3	18.0	<0.019	<0.019	0.156	0.156
Mangreh (I)	1.17±0.11	67.1±3.91 ab	29.0	27.0	14.0	15.6	<0.019	<0.019	0.078	0.156
Mangreh (II)	1.11±0.27	94.8±4.41 a	32.3	30.0	26.0	20.6	<0.019	<0.019	<0.019	<0.019
Mangreh (III)	1.12±0.18	89.8±5.11 a	28.0	26.3	24.4	22.3	<0.019	<0.019	<0.019	0.078
Mangreh (IV)	1.24±0.07	75.4±2.57 ab	27.0	25.3	25.0	18.0	<0.019	<0.019	<0.019	0.156
Paelm	1.39±0.05	80.2±5.01 ab	30.3	28.0	24.3	14.3	<0.019	<0.019	<0.019	0.312
Erythromycin	–	–	33.1	31.1	20.5	24.1	<0.019	<0.019	<0.019	<0.019

[a] E.c*: Escherichia coli,* [b] *P.a: Pseudomonas aeruginosa,* [c] *S.a: Staphylococcus aureus,* [d] *B.c: Bacillus cereus.*

[*] Values of major compounds are given as means ± SD. [†]Means with different letter in a column are statistically significant at 5% level probability.



UV Results

Pk #	Name	Retention Time	Area	Area Percent	Height	Height Percent
1		1.533	80778	4.637	816	1.640
2		3.100	70228	4.032	4160	8.358
3	Carvacrol	14.983	1161174	66.658	43034	86.464
4		16.733	16103	0.924	299	0.601
5		19.133	413697	23.749	1462	2.937
Totals			1741980	100.000	49771	100.000

Fig. 3—UV-spectrum of carvacrol in essential oil of *Satureja khuzestanica* (population: Mangreh-(II))

differences can be most probably explained by the variability of the genetic factors as well as the existence of different chemotypes. Mangreh population might be a potential carvacrol–rich source for mass–cultivation in order to improve commercial purposes. The latitude and E.C 0.96 dS/m of Mangreh region might be introduced as the best location for the production of quantity effective materials for this plant aimed to attain the best possible results (Table 1). Yavari *et al.* [28] reported that there were positive relationship between *p*–cymene, γ–terpinene, linalool and thymol concentrations in *Thymus migricus* EO and some environmental factors. Karousou *et al.* [29] indicated that high carvacrol content in two species (*Coridothymus capitatus* and *Satureja thymbra*) is associated to the dry dwarf–shrub formations of the lowland. They also reported that the relation between oil composition and the natural habitats of the collected plants suggests the use of natural habitat unit as a tool for the assessment and prediction of variation in essential oil in a single species. In current study, most of the antibacterial activity in EO from different populations appears to be explainable by phenolic compound (carvacrol). The EO of wild populations *S. khuzestanica* have a stronger antibacterial activity as compared to the positive antibacterial standards. The phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity[30]. Shan *et al.* [31] reported that there were highly positive relationships between antibacterial activities and phenolic content of the tested extracts against each bacterium. Carvacrol, which is the main component of *S. khuzestanica* EO and extract, have been considered as biocidal, resulting in bacteria membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death[32]. The results of current study indicate that scientific studies carried out on *S. khuzestanica* having traditional claims of effectiveness might warrant fruitful results. Infusion and decoction of the aerial parts of *S. khuzestanica* used by indigenous people in Khuzestan, Ilam and Lorestan provinces exhibit some degree of antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Generally, the EO of *S. khuzestanica* aerial parts could serve as useful sources for new antimicrobial agents. Additionally, this article is the first report of the antibacterial activities of the EOs from different populations of *S. khuzestanica*.

Conclusion

It is demonstrated the potent antibacterial activity of the EO of *S. khuzestanica* against pathogens, which justifies the large use of this plant in traditional medicine. It is considered that it would be very useful to promote carvacrol chemotypes crop culture in order to guarantee the quality of products. In addition, *S. khuzestanica* might be a potential carvacrol–rich source for commercial production. However, further research is needed to evaluate the effectiveness of *S. khuzestanica* EOs and extracts in food ecosystems to establish their utility as natural antimicrobial agents in food preservation and safety. Mangreh population or ecological conditions similar to Mangreh might be a potential carvacrol–rich source for commercial production.

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Conflicts of interest

All authors have none to declare.

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