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SDE-prepared Oil Analysis and Evaluation of Antioxidant and Antibacterial Potentials of *Varthemia persica* DC.

Maryam Akhbari[1], Mohammad-Reza Delnavazi[2], Maryam Karimi[1], Rafi Almasi[3], Saeed Tavakoli[1,2]*

[1]*Essential Oil Research Institute, University of Kashan, Kashan, Iran.*

[2]*Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.*

[3]*School of Chemistry, College of Science, University of Tehran, Tehran, Iran.*

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ABSTRACT

Background: *Varthemia persica* DC. (Asteraceae) is an aromatic plant which is used as anti-spasmodic, anti-emetic and for some kidney conditions. In the present study the plant aerial parts from Ghamsar (GS) and Ghohrud (GR) regions (Kashan, Iran) were investigated for SDE (Simultaneous distillation and solvent extraction) prepared oil composition, antioxidant and antibacterial activity. ***Methods:*** The oils were analyzed using GC and GC-MS. The plant oils and total methanol extracts were subjected to antioxidant evaluation in DPPH and β-carotene/linoleic acid assays and antibacterial screening using disk diffusion and micro-well dilution methods. ***Results:*** A total of 46 compounds were identified, of which α-pinene was characterized as the main compound in both GS (37.6%) and GR (53.8%) oil samples. Total extracts demonstrated moderate antioxidant activity in DPPH (RC_{50}; 79.2 ± 2.9 µg/ml for GS and 73.9 ± 3.4 µg/ml for GR samples) and β-carotene/linoleic acid assays (Inhibition percentage; 69.6 ± 2.4 % for GS and 70.2 ± 3.6 % for GR samples). Extracts and volatile oils exhibited considerable antibacterial activity especially against *Staphylococcus epidermidis*. ***Conclusion:*** The results of study report SDE-prepared oil of *V. persica* as a source of α-pinene. Favorable antioxidant and antibcterial activity of this plant also highlight it as an apropriate candidate to determination of its bioactive compounds.

Introduction

Nowadays, more recognizing of the important role of antioxidants in disease prevention and growing bacterial resistance to current antibiotics, have led researchers to be more interested in medicinal plants as potential sources of new antioxidant and antibacterial compounds.[1,2]

Varthemia persica DC. ("Atre-sang" in Persian) belonging to the Asteraceae family is an aromatic perennial plant widely distributed throughout the eastern and central parts of Iran.[3] In Iranian folk medicine, the aerial parts of *V. persica* are used as anti-spasmodic and anti-emetic as well as for the treatment of some kidney conditions.[4] The aromatic aerial parts of this species are also used by indigenous people as a flavor in preparation of some pickles. Previous phytochemical investigations have showed occurrences of flavonoids (luteolin, quercetin and kaempferol) and chlorogenic acid in aerial parts extracts, together with some sesquiterpens as a major group of constituents in volatile oils of *V. persica*.[4-8]

In order to more evaluation of medicinal potentials of this aromatic species, the aim of present study was to analysis the SDE-prepared volatile oils and determination of *in vitro* antioxidant and antibacterial activity of the oils and total methanol extracts of the *V. persica* aerial parts from "Ghamsar" and "Ghohrud", two different regions in center of Iran. To the best of our knowledge, this is the first report on antioxidant and antibacterial properties of *V. persica*.

Material and Methods

Plant materials

The aerial parts of *V. persica* were collected during its flowering stage in May 2012 from Ghamsar (GS) (altitude: 1850 m, average annual rainfall: 180 mm) and Ghohrud (GR) (altitude: 2600 m, average annual rainfall: 220 mm) regions located in Kashan county, Isfahan province, center of Iran. The voucher specimen of the plant was deposited at the herbarium of Kashan Botanical Garden, Research Institute of Forests and Rangelands, Kashan, Iran. (Voucher no. HKBG 0091).

Volatile oil extraction

The air-dried and ground aerial parts of plant samples (100 g each) were individually subjected to volatile oil extraction by simultaneous distillation and solvent extraction method (SDE) for 2 h using n-pentane as a solvent.[9] The obtained solutions were dehydrated using

***Corresponding Author:** Saeed Tavakoli (MSc), Essential Oil Research Institute, University of Kashan, Iran. Tel/fax: +98 3623624392, E-mail: tavakoli.sae@gmail.com

sufficient amount of anhydrous sodium sulfate and followed by evaporation of their solvent at the room temperature (25 °C) to get pure pale yellowish oils.
The volatile oils (0.5 and 0.6% (v/w) in yields for Ghamsar and Ghohrud plant samples, respectively) stored in amber glass vials under nitrogen atmosphere at 4°C until analysis.

GC and GC-MS analysis

GC-MS analysis of the volatile oils were carried out on a Hewlett-Packard 6890 gas chromatograph with silica HP-5 column (60 m × 0.25 mm i.d., 0.25 µm film thickness) coupled with a mass detector (Hewlett-Packard model 6973 HP). The carrier gas (Helium) flow rate was 1 ml/min. The oven temperature was maintained first at 60 °C for 30 min and was then raised to 250 °C at a rate of 5 °C per minute. The injection temperature was 250 °C and the sample (1 µl) was injected with a split ratio of 1:90. The mass spectra were obtained by electron ionization at 70 eV. The retention indices (RI) were calculated for all compounds using a homologous series of n-alkanes injected in conditions equal to the samples. Identification of the constituents was based on computer matching with the Wiley275.L library and direct comparison of the retention indices and fragmentation pattern of the mass spectra with those for standard compounds data published in the literature.[10,11]
In order to achieve relative percentages of the identified compounds, GC analysis of the volatile oils were also performed using an Agilent HP-6890 gas chromatograph equipped with a FID detector. The FID detector temperature was 290 °C and the operation was performed under the same conditions as described above for GC-MS analysis.

Preparation of total methanol extracts

The air-dried and powdered plant samples (50 g each) were individually subjected to extraction using Soxhlet apparatus for 8 h with methanol as a solvent. The obtained total extracts were concentrated using a rotary evaporator under the maximum temperature of 45 °C and the residuals were completely dried using a vacuum oven at 45 °C and 7.5 torr.

Antioxidant activity evaluation

DPPH bleaching and β-carotene/linoleic acid assays were used to evaluation of *in vitro* antioxidant activity of the volatile oils and total extracts.

DPPH free radical scavenging activity assay

Free radical scavenging activity of the samples were measured using 2,2-diphenyl-1-picryl hydrazyl (DPPH) method described by Delazar *et al.*[12] Briefly, the stock sample solutions (10 mg/ml each in methanol) were diluted serially with methanol to obtain concentrations ranging from 10 to 1.9×10^{-2} mg/ml. Diluted solutions (2 ml each) were mixed with 2 ml of DPPH solution (80 µg/ml in methanol) and were kept 30 min at 25 °C in dark for any reaction to take place. Ultraviolet (UV) absorptions of the solutions were measured with spectrophotometer (Cintra 6, GBC, Australia) at 517 nm. A synthetic antioxidant, butylated hydroxytoluene (BHT), was also used as a positive control. All tests were performed three times and RC_{50} values (the concentration causing 50% reduction in absorbance of DPPH) were reported as Mean ± SD.

β-Carotene/linoleic acid assay

Protection of β-Carotene from oxidation via neutralization of hydroperoxides arising from linoleic acid is the basis of this method for determination of antioxidant activity.[13] For this purpose, the method described by Miraliakbari & Shahidi was used with slight modifications.[14] Briefly, 0.5 mg of β-carotene solution (diluted in 1 ml chloroform) was mixed with 25 µl of linoleic acid and 200 mg of tween 40. The chloroform was evaporated under vacuum and 100 ml of oxygenated distillated water was then added to the residue. 350 µl from the each oil and extract sample solutions (2 mg/ml in DMSO) was added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a water bath at 50 °C for 2 h. In each assay, butylated hydroxytoluene (BHT) was used as a positive control. The absorbencies were measured using a spectrophotometer (Cintra 6, GBC) at 470 nm. Inhibition percentage (I%) of the samples were calculated using the following equation (A; Absorbance):

$$I\% = (A_{\beta\text{-carotene after 2 h assay}} / A_{\text{initial }\beta\text{-carotene}}) \times 100 \quad \text{Eq.(1)}$$

All tests were performed three times and inhibition percentages were reported as Mean ± SD.

Antibacterial activity determination

Bacterial strains

Potential antibacterial activity of the volatile oils and extracts were individually tested against a set of eight gram positive and gram negative bacterial strains; *Escherichia coli* (ATCC 10536), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29737), *Klebsiella pneumonia* (ATCC 10031), *Staphylococcus epidermidis* (ATCC 12228), *Shigella dysenteriae* (PTCC 1188), *Proteus vulgaris* (PTCC 1182), *Salmonella paratyphi*-serotype A (ATCC 5702). Bacterial strains were provided from the Iranian Research Organization for Science and Technology (IROST) and were cultured overnight at 37 °C in nutrient agar (NA).
30 mg/ml using DMSO as a solvent and filtered through 0.45 µm Millipore filters for sterilization. 100 µl of suspension containing 10^8 CFU/ml of bacteria was spread on to the nutrient agar (NA). The impregnated discs (6 mm in diameter) with 10 µl of the

Disk diffusion assay

Evaluation of antibacterial activity of the samples were carried out using agar disc diffusion method.[15,16] Plant sample solutions were prepared at the concentration of

volatile oils or the extracts solutions (300 µg/disc) and DMSO (as negative control) were placed on the inoculated agar. All plates were incubated at 37 °C for 24 h and the diameters of inhibition zones (mm) were measured. Gentamicin (10 µg/disc) and rifampin (5 µg/disc) were also used as positive controls.

Micro-well dilution assay

Minimal inhibition concentration (MIC) values of the plant extracts and volatile oils were determined using micro-well dilution assay for bacterial strains sensitive to examined samples in disc diffusion assay.[17] The inocula of the bacterial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The extract and volatile oil samples were dissolved in 10% DMSO (500 µg/ml) and serial two-fold dilutions were made in a concentration range of 7.8–500 µg/ml in 10 ml sterile test tubes containing brain heart infusion (BHI) broth. The 96-well plates were prepared by dispensing 95 µl of the cultures media and 5 µl of the inoculums into each well. A 100 µl aliquot from the stock solutions of the samples initially prepared at the concentration of 500 µg/ml was added into the first well. Then, 100 µl from their serial dilutions were transferred into six consecutive wells. The last well containing 195 µl of the cultures media without the test materials and 5 µl of the inoculums on each strip was used as negative control. The final volume in each well was 200 µl. Gentamicin and rifampin were used as positive controls. Contents of each well were mixed on the plate shaker at 300 rpm for 20 s and then incubated at appropriate temperature for 24 h. Bacterial growth was determined by the presence of a white pellet on the well bottom and confirmed by plating 5 µl samples from clear wells on NA medium. The MIC value was defined as the lowest concentration of the plant extract required for inhibiting the growth of each microorganism. All tests were repeated two times.

Results and Discussion

Chemical composition of the volatile oils

A total of forty-seven compounds were identified as a result of GC and GC-MS analyses of the SDE-prepared volatile oils samples of *V. persica* from Ghamsar and Ghohrud regions (Table 1). The results showed that hydrocarbon monoterpenes (44.7% for GS and 66.1% for GR plant samples) were the main group of constituents and α-pinene (37.6 for GS and 53.8% for GR samples) was the main compound of both volatile oil samples (Table 1).

Our study introduces hydrocarbone monoterpenes as main constituents of the *V. persica* oil, whereas all previous studies on this species have reported sesquiterpenes as major constituents of its oil (Table 2).[4,7,8] Furthermore, α-pinene, a major compound of our analyzed oil samples, has also been reported in the amounts less than 1.5% from previous studies on the volatile oil of *V. persica*.[4,7,8]

Table 1. Chemical composition of the *V. persica* volatile oil samples from Ghamsar and Ghohrud regions, center of Iran.

No.	Compounds[a]	Composition (%)		Rt[b]	RI[c]	RI[d]
		Ghamsar	Ghohrud			
1	β-γ-Hexenol	1.6	2.7	5.4	849	851
2	1-Hexanol	0.4	1.0	5.7	862	861
3	α-Pinene	37.6	53.8	8.0	950	949
4	Camphene	1.3	2.0	8.2	956	953
5	β-Pinene	0.9	1.1	8.9	981	981
6	β-Myrcene	0.6	0.8	9.3	993	993
7	Methyl 4-methylhexanoate	0.4	1.2	9.5	999	996
8	Octanal	-	0.8	9.7	1005	1004
9	α-Terpinene	0.3	0.3	10.2	1018	1018
10	p-Cymene	0.6	0.8	10.5	1026	1025
11	Limonene	-	3.0	10.7	1032	1031
12	Cineole	2.5	-	10.8	1035	1034
13	β-(E)-Ocimene	2.0	1.9	11.3	1049	1050
14	γ-Terpinene	0.7	0.8	11. 8	1061	1061
15	1-Octanol	0.4	0.4	12.1	1070	1070
16	Terpinolene	0.6	1.0	12.9	1090	1088
17	Nonanal	0.9	0.5	13.5	1105	1106
18	Fenchol	-	0.5	14.0	1117	1117
19	α-Campholenal	1.0	0.4	14.5	1128	1126
20	Camphene hydrate	2.6	0.3	15.4	1150	1145
21	p- Menth-3-en-8-ol	1.5	-	15.6	1157	114
22	Borneol	-	1.4	16.2	1170	1166
23	p-Menthan	3.1	-	16.3	1173	1172
24	Borneol	1.5	-	16.4	1175	1173
25	Terpinen-4-ol	0.5	-	16.7	1183	1182
26	α-Terpineol	-	1.7	17.3	1195	1187

Table 1. (Continued).

27	Levomenthol	2.8	-	17.4	1200	-
28	Decanal	0.8	0.4	17.8	1209	1207
29	trans-Carveol	0.6	-	18.8	1231	1217
30	Pulegone	5.2	-	19.6	1251	1237
31	Piperitone	1.5	-	20.1	1263	1253
32	bornyl acetate	1.0	1.0	21.2	1289	1289
33	2-Undecanone	-	0.5	21.5	1295	1296
34	Isomenthyl acetate	1.0	-	21.6	1300	1298
35	Pulespenone	2.7	-	23.8	1352	1348
36	Eugenol	0. 7	0.5	24.3	1364	1363
37	α-Ylangene	0.7	0.5	24.8	1375	1375
38	Italicene	0.6	1.8	26.1	1408	1402
39	β-Caryophyllene	7.1	5.0	26.9	1429	1425
40	Humulene	0.5	-	28.1	1459	1454
41	γ-Curcumene	2.2	2.0	29.2	1485	1485
42	β-Selinene	1.4	1. 7	29.5	1494	1490
43	α-Selinene	1.6	2.0	29.9	1502	1497
44	δ-Cadinene	1.3	0. 7	30.8	1527	1524
45	α-Calacorene	1.6	1.0	31.6	1548	1542
46	Caryophyllene oxide	1.0	-	33.2	1590	1587
	Hydrocarbon monoterpenes	44.7	66.1			
	Oxygenated monoterpenes	27.5	5.3			
	Hydrocarbon sesquiterpenes	17.2	14.7			
	Oxygenated sesquiterpenes	1.0	-			
	Hydrocarbon non-terpenes	-	-			
	Oxigenated non-terpenes	5.1	7.5			
	Total identified	95.5	93.6			

A dash (-) indicate the absence of compound in the volatile oil. [a] Compounds listed in order of elution from HP-5MS column; [b] Retention times; [c] Retention indices to C8–C24 n-alkanes on HP-5MS column; [d] Literature Retention indices.

Table 2. The results of volatile oil analysis of the *V. persica* in previous and present studies.

Location of collection	Date of collection	Method	Yield (%)	Main group (%)	Main compounds (%)
Elburz mountains (Tehran, North of Iran)	July 2002	SD[a]	0.1%	Hc-St[d] (58.2%)	β-Bourbonene (17.8%) β-Caryophyllene (10.8%)
Haraz Mountain (Kerman, South of Iran)	Sept. 1998	HD[b]	0.08%	Ox-St[e] (53.0%)	β-eudesmol (31.7%) Spathulenol (23.5%)
Karkas mountains (Natanz, Center of Iran)	-	SD	0.1%	Hc-St (44.9%)	δ-Cadinene (9.7%) Germacrene D (4.9%)
Ghamsar region (Kashan, Center of Iran)	May 2012	SDE[c]	0.54%	Hc-Mt[f] (44.7%)	α-Pinene (37.6%) β-Caryophyllene (7.1%)
Ghohrud region (Kashan, Center of Iran)	May 2012	SDE	0.60%	Hc-Mt (66.1%)	α-Pinene (53.8%) β-Caryophyllene (5.0%)

A dash (-) indicate not reported. [a] Steam-distillation; [b] Hydro-distillation; [c] Simultaneous distillation and solvent extraction; [d] Hydrocarbon sesquiterpens; [e] Oxygenated sesquiterpens; [f] Hydrocarbon monoterpens.

Although the influence of factors such as time of plant collection, altitude and regional climate is undeniable,[11] considering the overall similarity between the results of our analyzed samples, it seems the method of volatile oil extraction is the most important factor involved in appearance of mentioned differences. The results of some comparative studies have also shown that simultaneous distillation and solvent extraction (SDE) method provides more yield of oil containing higher amounts of highly volatile compounds (e.g. monoterpenes), in comparison with some earlier methods such as hydro-distillation (HD) and steam-distillation (SD).[18-20]

In the mentioned earlier methods (HD and SD) which have been used in previous studies on *V. persica* volatile oils, possible imbalance between evaporation and condensation of volatile compounds could be led to the loss of oil, particularly its highly volatile portion.

The use of n-pentane as a solvent in SDE method, however, improves the trapping of the volatile compounds and enhances the yield of volatile oil extraction.

Antioxidant activity

The results of *in vitro* antioxidant activity of the total methanol extracts and volatile oils are given in Table 3. In the DPPH free radical scavenging assay, both extract samples showed a moderate antioxidant activity with the RC_{50} values of 79.2 ± 2.9 µg/ml for GS and 73.9 ± 3.4 µg/ml for GR samples, in comparison with BHT, a synthetic commercial antioxidant (RC_{50}; 19.7 µg/ml) (Table 3). Oxidation of β-carotene was also effectively inhibited by the extracts (69.6 ± 2.4 % for GS and 70.2 ± 3.6 % for GR samples) in β-carotene/linoleic acid assay (Table 3).

According to occurrence of well-known antioxidant phenolic compounds such as flavonoids (luteolin, quercetin and kaempferol) and chlorogenic acid in the aerial parts of *V. persica*, a part of the observed antioxidant activity may be related to the presence of above mentioned flavonoids.[5,6,21,22]

Despite of the favorable antioxidant effects of extract samples, both of the oil samples showed a weak antioxidant activity, RC_{50} values of 825.4 ± 12.7 µg/ml and 786.6 ± 8.2l µg/ml in DPPH and 19.6 ± 1.7 % and 18.3 ± 2.4 % (I%) in β-carotene/linoleic acid assays (Table 3).

Table 3. Antioxidant activity of the oils and extracts of *V. persica* from Ghamsar and Ghohrud regions, center of Iran.

	Total extract		Volatile oil		
	Ghamsar	Ghohrud	Ghamsar	Ghohrud	BHT
DPPH assay (RC_{50}, µg/ml)	79.2 ± 2.9	73.9 ± 3.4	825.4 ± 12.7	786.6 ± 8.2	19.7 ± 0.8
β-Carotene assay (I%)	69.6 ± 2.4	70.2 ± 3.6	19.6 ± 1.7	18.3 ± 2.4	87.6 ± 4.9

Table 4. Antibacterial activity of the volatile oils and extracts of *V. persica* plant samples from Ghamsar and Ghohrud regions, center of Iran.

	Ghamsar sample				Ghohrud sample				Antibiotics			
	Extract		volatile oil		Extract		volatile oil		Rif[a]		Gen[b]	
Bacteria	IZ[c]	MIC[d]	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
S. paratyphi	-	-	-	-	-	-	-	-	-	-	21	500
S. aureus	11	>500	-	-	20	250	-	-	10	250	21	500
S. dysenteriae	-	-	-	-	-	-	-	-	8	250	18	500
E. coli	-	-	12	500	-	-	18	250	11	500	20	500
K. pneumoniae	12	500	-	-	20	500	-	-	7	250	22	250
B. subtilis	14	250	-	-	18	500	-	-	13	15	21	500
P. vulgaris	-	-	-	-	-	-	-	-	10	125	23	500
S. epidermidis	30	250	15	>500	31	250	18	500	40	250	35	500

A dash (-) indicates no antibacterial activity. [a] Rifampin; [b] Gentamicin; [c] Inhibition zone in diameter (mm) around the impregnated discs; [d] Minimal inhibition concentrations (as µg/ml).

Antibacterial activity

As a result of antibacterial activity evaluation of the extract and volatile oil samples, total extracts showed considerable antibacterial activity against *B. subtilis*, *K. pneumonia*, *S. aureus* and *S. epidermidis* that among these, the most sensitive bacterial strain was *S. epidermidis* (Table 4). The volatile oil samples also had considerable antibacterial activity against *E. coli* and *S. epidermidis* (Table 4).

Previous antibacterial investigation of *V. iphionoides*, has also reported its antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli* and some other tested microorganisms.[23,24] Flavonoids and chlorogenic acid which are previously reported from *V. persica* and their antibacterial activity have been reported during former studies might be involved in this observed antibacterial activity of this species.[5,6,25,26] Sesquiterpene lactones, one of the bioactive principles present in of Asteraceae family plants with potentially antimicrobial activity, may also have a role in appearance of antibacterial effects of *V. persica*.[27] Alkylation reaction between α, β-unsaturated cyclopentanone and bionucleophile -SH

groups (Michael addition) is one of the main mechanisms proposed for antimicrobial activity of sesquiterpene lactones.[27]

Conclusion

The results of our study report simultaneous distillation and solvent extraction (SDE) as an appropriate method for voltile oil extrction of *V. persica*. Considerable antioxidant and antibcterial activity of *V. persica* also highlight this aromatic plant species as a good candidate for further and more in-depth studies, in order to determination of its bioactive principles and introduction it as a safe and effective natural flavour and preservative for use in food industries.

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