Screening chemical composition of essential oils and antioxidant activities of two *Artemisia* species from Iran

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Abstract

Essential oils of two wild-growing *Artemisia* species collected from west of Iran during the flowering stage were obtained by hydro-distillation and analyzed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC–MS). Under the optimum extraction and analysis conditions, 23 and 19 constituents were identified in *A. fragrans* and *A. hausskenechtii* which represented 92.96% and 90.20% of the oils, respectively. The main constituents of *A. hausskenechtii* were camphor (42.50), 1,8-cineol (20.91 %) and isoborneol (7.27%). Chrysanthenone (41.05%), 1,8-cineol (11.09%), and n-pentane (9.07%) were the major components of essential oils in *A. fragrans.* Antioxidant activity was assayed by two complementary test systems, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and β-carotene/linoleic acid systems. Antioxidant activity of polar sub-fraction of *A. hausskenechtii* was found to be higher than those of the others in DPPH assay while essential oil in *A. hausskenechtii* had the highest level of antioxidant activity in β-carotene/linoleic acid test (54.2± 0.5 µg/ml and 80.2±0.8% inhibition rate, respectively).

Key words: *Artemisia*; antioxidant activity; essential oil

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Introduction

Antioxidant molecules and enzymes protect cellular membranes and organelles from the damaging effects of reactive oxygen species (ROS), which are formed both during normal cellular metabolism and unwanted environmental conditions (Chaparzadeh and Yavari, 2013). To minimize the harmful effects of ROS, plants have evolved an effective scavenging system composed of antioxidant molecules and antioxidant enzymes (Karimi and Souri, 2013).

Free radicals and other reactive oxygen species cause oxidation of biomolecules including proteins, amino acids, unsaturated lipids and DNA, and ultimately produce molecular alterations related to aging, arteriosclerosis and cancer (Gardner, 1997), Alzheimer's disease (Butterfield and Lauderback, 2002), Parkinson's disease, diabetes and asthma (Zarkovic, 2003). Human body is equipped with an inherent defense system which can quench free radicals present in almost all cells (Halliwell and

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Gutteridge, 1990). An imbalance between free radical production and their removal by the body's antioxidant system leads to a phenomena known as 'oxidative stress' (Abdollahi et al., 2004; McCord, 2000). In this situation, an external supply of antioxidants is necessary to regain a balance between free radicals and antioxidants.

Nowadays, academic researchers and food industries are searching for alternative sources of chemical preservatives as protection agents against lipid oxidation. Eugenol, limonene, carvacrol, and geraniol have been already identified as natural food preservatives with no mutagenic effects (Guimarães et al., 2010). However, utilization of chemical antioxidants with high activity such as tertiary butyl hydroquinone (TBHQ), can threaten the human health. Essential oils of medicinal plants and spices containing phenolic compounds contain antioxidants that have been widely used in pharmaceutical, sanitary, cosmetic, agricultural and food industries around the world (Sharafati et al., 2013).

Asteraceae contributes to 10% of flowering plants. This is why it is regarded as the largest group of flowering plants (Zareh, 2005). Asteraceae involves 1,600 genera and 25,000 accepted species which are of great importance (Panero and Funk, 2008). The genus *Artemisia* (Asteraceae) is one of the largest and most widely distributed of the approximately 60 genera in the tribe anthemideae. This genus comprises a variable number of species, ranging from 200 to over 400, predominantly distributed in the northern temperate region of the world in the 0-50 cm precipitation area (Tan et al., 1998). 29 of them are reported in Iran some being endemic (Mozafarian, 1988). As reported, some substances from the genus have shown antimalarial, antiviral, antitumor, antipyretic, antihemorrhagic, anticoagulant, antianginal, antioxidant, antihepatitis, antiulcerogenic, antispasmodic, anticomplementary, and interferon-inducing activity (Tan et al., 1998).

Several secondary metabolites characterize the chemical composition of the genus *Artemisia*. A survey of the literature indicates that almost all classes of compounds are present in the genus, with particular reference to terpenoids and flavonoids (Mucciarelli and Maffei, 2002).

Artemisia fragrans Willd, commonly known as Chao, is a species growing in Armenia, Iran, Russia, and neighboring regions. This perennial herb grows wild in the Azerbaijan, Mazandaran, Qazvin, and Tehran provinces of Iran (Rechinger, 1986) and is famed for its strong fragrance. The species possesses antibacterial properties (Shafaghat et al., 2009). Previous phytochemical studies on this species revealed the presence of several sesquiterpen lactones with germacrane, eudesmane, guaian and elemane frameworks (Safaei-Ghomi et al., 2012).

This study was conducted to investigate the essential oil compositions and antioxidant activities of the essential oils and methanol extracts of *Artemisia fragrans* and *Artemisia hausskenechtii.*

Materials and Methods

Plant material

The aerial parts of *Artemisia fragrans* and *Artemisia haussknechtii* were collected during full flowering stage between 22th May and 10th Sep. 2012 from Lorestan Province, Southwestern Iran. The voucher specimen was prepared and authenticated. The herbarium sample was deposited at the Herbarium of Agriculture and Natural Resources Center of Lorestan Province, Khoramabad, Iran.

Isolation of the essential oils

The aerial parts (140.0 g) of *A. fragrans* and *A. haussknechtii* were air-dried at room temperature in the shade and the oils were obtained by hydro-distillation using Clevenger-type apparatus for 3, 2.5, and 3 h, respectively. After decanting and drying over anhydrous sodium sulfate, the sample were kept in bottles covered in aluminum foil at 4 °C to prevent the negative effects of light, especially direct sunlight.

Preparation of methanol extracts

The air-dried and finely ground samples were extracted using the method described by Sokmen et al. (1999). Briefly, the samples weighing about 100 g were extracted in a Soxhlet

with methanol (MeOH) at 60 °C for 6 h. The extracts were then filtered and concentrated *in vacuo* at 45 °C yielding a waxy material. The resulting extracts were suspended in water and partitioned with chloroform ($CHCl_3$) to obtain water-soluble (polar) and water-insoluble (non-polar, chloroformic) sub-fractions. Extracts were concentrated, dried, and kept in the dark at +4 °C until tested.

Analysis of the oils

FID-GC was carried out using a Hewlett-Packard 6890 with DB-5 capillary column (phenyl methyl diloxane, 25 m × 0.25 mm i.d., 0.25 µm film thickness); carrier gas, He; split ratio, 1:25, and flam ionization detector. Temperature progamme: 60° C (2 min) rising to 240 °C at 4 °C/min, injector temperature, 250 °C, detector temperature, 260 °C. GC-MS was performed using Hewlett-Packard 6859 with quadrupole detector, on a HP-5 column (see GC), operating at 70 eV ionization energy, using the same temperature program and carrier gas as above. Retention indices were calculated by using retention times of n-alkanes that were injected after the oils at the same chromatographic conditions according to Van Den Dool method (Van Den Dool and Krats, 1963).

Identification of compounds

The constituents of the essential oils were identified by calculation of their retention indices under temperature-programmed conditions for *n-alkanes* (C_6–C_{24}) and the oil on a DB-5 column under the same chromatographic conditions. Identification of individual compounds was made by comparison of their mass spectra with those of the internal reference mass spectra library or with authentic compounds and confirmed by comparison of their retention indices with authentic compounds or with those reported in the literature (Adams, 2001). For quantification purpose, relative area percentages obtained by FID were used without the use of correction factors.

Antioxidant activity

DPPH assay

Radical-scavenging activities (RSA) of *A. fragrans* and *A. haussknechtii* essential oils and extracts were determined using a published DPPH radical-scavenging activity assay method (Sarker et al, 2006) with minor modifications. Briefly, stock solutions (10 mg/ml each) of the essential oils, extracts, and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions were made to obtain concentrations ranging from 1 to 5×10^{-10} mg/ml. Diluted solutions (2 ml each) were mixed with 2 ml of freshly prepared 80 µg/ml DPPH methanol solution and allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Ultraviolet (UV) absorbencies of these solutions were recorded on a spectrometer at 517 nm using a blank containing the same concentration of oils or extracts or BHT without DPPH. Inhibition of free radical DPPH in percent (I%) was calculated as follow:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

β-Carotene/linoleic acid bleaching assay

The method described by Miraliakbari and Shahidi (2008) was used with slight modifications. A stock solution of *β*-carotene and linoleic acid was prepared with 0.5 mg of *β*-carotene in 1 ml chloroform, 25 µl of linoleic, acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of aerated distilled water was then added to the residue. The samples (2 g/l) were dissolved in DMSO and 350 µl of each sample solution was added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h, together with two blanks, one contained the antioxidant BHT as a positive control and the other contained the same volume

of DMSO instead of the extracts. The test tube with BHT maintained its yellow color during the incubation period. The absorbencies were measured at 470 nm on an ultraviolet spectrometer. Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

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

where $A_{\beta\text{carotene after 2 h assay}}$ is the absorbance of *β*-carotene after 2 h assay remaining in the samples and $A_{\text{initial } \beta\text{-carotene}}$ is the absorbance of *β*-carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

Assay for total phenolics

Total phenolic constituents of the polar and nonpolar sub-fractions of methanol extracts of *A. fragrans* and *A. haussknechtii* were determined by literature methods involving Folin–Ciocalteu reagent and gallic acid standard (Slinkard and Singleton, 1977). Solutions of the extracts (0.1 ml each) containing 1000 µg of the extracts were taken individually in volumetric flasks, 46 ml of distilled water and 1 ml Folin–Ciocalteu reagent were added, and the flasks were thoroughly shaken. After 3 min, 3 ml of 2% Na_2CO_3 solution were added and the mixtures were allowed to stand for 2 h with intermittent shaking. Absorbencies were measured at 760 nm. The same procedure was repeated for the all standard Gallic acid solutions (0–1000 mg/0.1 ml) and a standard curve was obtained with the following equation:

$$\text{Absorbance} = 0.0012 \times \text{gallic acid } (\mu g) + 0.0033$$

Total phenols of the extract, as Gallic acid equivalents, was determined using the absorbance of the extract measured at 760 nm as input to the standard curve and the equation. All tests were carried out in triplicate and phenolic contents as Gallic acid equivalents were reported as means ± SD of triplicate determinations.

Total flavonoid content (TFC*)*

Total flavonoid content was determined using the Dowd method as adapted by Arvouet-Grand et al. (1994). Briefly, 1 ml of 2% aluminum trichloride ($AlCl_3$) in methanol was mixed with the same volume of the extracts (2000 µg). Absorption readings at 415 nm were taken after 10 min against a blank sample consisting of a 1 ml extract solution with 1 ml methanol without $AlCl_3$. The concentrations of flavonoid compounds were calculated according to the following equation obtained from the standard quercetin graph:

$$\text{Absorbance- } 0.026 \text{ quercetine } (\mu g) - 0.0060 \ (R^2: 0.9977)$$

Results

Chemical composition of the essential oils

The chemical composition results found for the essential oils from two Artemisia species in the order of their elution from a low-polar HP-5MS column are presented in Table 1. The essential oil of *A. fragrans*, characterized by high monoterpenes content (77.67%), the main components are Chrysanthenon (41.05%), 1,8-cineol (11.09%) and n-pentane (9.07%) followed by 5,5-Dimethyl-1-ethyl-1,3-cyclopentadiene (5.78%), isoborneol (3.54%) and filfolone (3.21%). Also the A. hausskenechtii oil was characterizedto have higher monoterpenes compound content (83.81%). camphor (42.50), 1,8-cineol (20.91 %) and isoborneol (7.27%) were themain components, followed by camphene (5.42 %) 2,5-octadiene (3.51%).

Antioxidant activity

The reduction ability of DPPH radicals' formation was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.

The scavenging ability of the essential oils and methanolic extract are shown in Table 2. Free radical scavenging activity of the extracts is concentration dependent and lower IC50 value reflects better protective action.

Table 1
Chemical composition of essential oils of Artemisia species

NO	Compound	RI	*A. fragans*	*A. haussknechtii*
1	n-pentane	500	9.07	-
2	Acetic acid	737	0.23	-
3	Hexanal	802	0.18	-
4	5,5-Dimethyl-1-ethyl-1,3-cyclopentadiene	833	5.78	-
5	Cumene	931	2.11	-
6	α-pinene	935	0.59	2.59
7	Camphene	947	0.39	5.42
8	1,3,5-heptatriene	952	-	0.25
9	3,5-octadiene	959	-	0.25
10	2-allyl-4-pentanal	966	-	0.63
11	Hemimellitene	967	1.53	-
12	α-terpinene	1013	-	0.63
13	p-cymene	1024	2.04	1.06
14	1,8-cineol	1031	11.09	20.91
15	trans-ocimene	1048	-	0.12
16	γ-terpinene	1057	-	0.80
17	4-(2-methylpropyl)-phenol	1102	0.92	-
18	Filfolone	1113	3.21	-
19	Isophorone	1119	3.54	-
20	2,5-octadiene	1123	-	3.51
21	Chrysanthenone	1128	41.05	-
22	Trans-Pinocarvone	1139	1.91	-
23	Camphor	1143	-	42.50
24	Cis-Chrysanthenol	1161	1.61	-
25	Borneol	1166	0.7	-
26	terpinene-4-ol	1179	0.57	-
27	Eucarvone	1181	0.38	-
28	α-terpineol	1182	-	0.64
29	β-fenchyl alcohol	1183	-	-
30	Dihydrocarvone	1186	0.2	-
31	Isoborneol	1196	-	7.27
32	Chrysanthenyl acetate	1225	0.41	-
33	Thymol	1278	1.74	-
34	Cis-Jasmone	1280	3.71	-
35	Endobornyl acetate	1289	-	0.56
36	Carvacrol	1291	-	0.25
37	geranyl acetate	1362	-	1.42
38	β-caryophyllene	1414	-	0.3
39	α-farnesene	1459	-	1.09
40	Valeranone	1630	-	-
41	1-octadecene	1790	-	-
42	Tricosane	2300	-	-
43	Pentacosane	2500	-	-
44	Heptacosane	2700	-	-
Total			92.96	90.20

The strongest free radical scavenging activity was exhibited by polar sub-fraction *A. hausskenkechtii* (IC50=54.2±0.5 µg/ml). Free radical scavenging capacity of polar sub-fraction *A. fragrans* was determined (IC50=122.4± 0.9 µg/ml). Polar sub fraction of methanolic extracts of *A. hausskenechtii* and *A. fragrans* exhibited stronger activity than non-polar extracts. When compared to BHT, the Polar sub fraction of methanolic extracts is the less effective radical scavenger.

Table 2
Antioxidative capacities of the essential oils and methanol extracts of *Artemisia* species[a]

Plant oils, methanol extracts and controls	Test system	
	DPPH IC_{50} (µg/ml)	β-Carotene/linoleic acid (% inhibition rate)
A. fragrans oil	165.7±1.4	55.2± 0.4
*A. hausskenkechtii*oil	288.6±3.1	80.2±0.8
A. fragrans polar sub-fraction	122.4±0.9	32.8±0.4
A. fragrans non-polar sub-fraction	412.7±4.1	63.5± 0.5
A. hausskenkechtii polar sub-fraction	54.2± 0.5	44.7±0.4
A. hausskenkechtii non-polar sub-fraction	544.5±6.6	75.9±0.9
BHT	17.8 ± 0.3	97.1 ± 0.9

a Results are means of three different experiments.

In β-carotene/linoleic acid model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of b-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene group attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and broken down in part; subsequently the system loses its chromophore and characteristic orange color, which is monitored spectrophotometrically.

In contrast to DPPH test system, essential oil and non-polar sub fraction of methano extracts showed remarkable activity profile in β-carotene/linoleic acid system (80.2±0.8% , 75.9%±0.9, 63.5%±0.5, 55.2%±0.4 for oil of *A. hausskenechtii*, non-polar sub fraction extract of *A. hauskenechtii*, non-polar sub fraction extract of *A. fragrans* and essential oil of *A. fragrans* respectively).

Assays for total phenolics and flavonoids

Total phenolic assay was carried out based on the absorbance values of the various extract solutions, reacted with Folin–Ciocalteu reagent and compared with the standard solutions of pyrocatechol equivalents as described above. Data obtained from the total phenolic assay supports the key role of phenolic compounds in free radical scavenging and/or reducing systems. As expected, amount of the total phenolics were very high in polar sub fraction of methanol extracts of *A. hausskenechtii* and *A. fragrans* (120.3 ± 1.3 and 91.8±1.1 µg GAEs/mg extract respectively). It was followed by non-polar sub fraction of methanol extract with a value of 31.6± 0.3 and 29.7± 0.3 µg GAEs/mg (Table 3). It is extremely important to point out that; there is a positive correlation between antioxidant activity potential and amount of phenolic compounds of the extracts. On the other hand, polar sub fraction of methanol extracts has been found to be rich in flavonoids with a value of 40.5±0.7µg QEs/mg. and 23.4±0.5µg QEs/mg. for *A. fragrans* and *A. hausskenechtii.*

Table 3
Amount of total phenolic and flavonoids in the methanol extracts of Artemisia species[a]

Sample	Phenolic content (µg GAEs/mg extract)[b]	Flavonoid content (µg QEs/mg extract)[c]
A. fragrans polar sub-fraction	91.8 ± 1.1	40.5 ± 0.7
A. fragrans non-polar sub-fraction	29.7± 0.3	12.8 ± 0.6
A. hausskenkechtii polar sub-fraction	120.3 ± 1.3	23.4 ± 0.5
A. hausskenkechtii non-polar sub-fraction	31.6± 0.3	9.8 ± 0.5

[a] Results are means of three different experiments.

[b] GAEs, Gallic acid equivalents.

[c] QEs: quercetin equivalents.

Discussion

Previous studies showed that bornane derivatives and 1,8-cineole are major characteristic components of many species of Artemisia genus. Camphor (a bornane derivative) and 1,8-cineole as the main component of *A. haussknechtii* oil were the major constituents of the essential oils of *A. annua* (Juteau et al., 2002) *A. diffusa*, (Khazraei-Alizadeh and Rustaiyan, 2001) four Artemisia species growing in western Canada (Lopes-Lutz et al., 2008) and three Artemisia species from Turkey (Kordali et al., 2005).

The result of one research using hydrodistillation and head space liquid phase microextraction techniques showed 56 components in the essential oil of *A. haussknechtii* which were collected from Yazd province in Iran and camphor (40.83%), 1,8-Cineole (26.84%), cis-davanone (4.77%), and linalool (4.44%) were the main components (Miraliakbari and Shahidi, 2008).

1,8-cineole and camphor, were also found to be the major components of the essential oil obtained from leaves and flowers of *A. fragrans* collected from the northwestern part of Iran (Movafeghi et al., 2010) and from its aerial parts at the flowering stage collected from Mazandaran (Morteza-Semnani et al., 2005). Furthermore,1,8-cineole and α-thujone were also detected in considerable mounts in the oil of *A. fragrans* collected from Tabriz region of Iran (Barazandeh, 2003). Shafaghat et al. (2009) reported that the essential oil from *A. fragrans* leaves and roots contained chrysanthenone (23.8%), 1,8-cineole (23.7%), β-caryophyllene (9.6%), pcymene (7.7%), filifolide-A (5.7%) and filifolone (5.7%), and camphor (67.0%) and camphene (16.9%) as the main constituents,respectively. 1,8-cineole (22.8%), chrysanthenone (18.16%), α-pinene (8.33%) and mesithylene (7.4%) were the major components of *A. aucheri* essential oil obtained by microextraction method (Safaei-Ghomi et al., 2005).

According to a previously published report (Delazar et al., 2007) the two major constituents of the essential oil obtained from the aerial parts of *A. fragrans* and *A. austriaca* cultivated in Tabriz were 1,8-cineole (11.48% and 27.97%, respectively) and camphor (54.92% and 40.59%, respectively. We attributed the great variability and the diversity observed in the chemical composition of this essential oil to the geographical location, ecological conditions (Santos-Gomes and Fernandes-Ferreira, 2001), genetic factors geology, part of the plant and the method used to obtain the essential oil. In fact, these factors influence the plants biosynthetic pathways and, consequently, the relative proportion of the main characteristic compounds (Chryssavgi et al., 2008).

It seems that this activity is mostly related to the presence of the phenolic compounds such as flavonoids and phenolic acids in the polar fraction. The key role of phenolic compounds as scavengers of free radicals is emphasized in several reports (Dueneset al., 2006; Thĕriault et al., 2006).

Some reports on the antioxidant properties of these species are documented. Essential oils of two species (*A. absyssinca* and *A. afra*) are tested for antioxidant activity using TIC screening method (Burits et al., 2001)

The high bleaching activity of the plant extract obtained in this test may be a consequence of the presence of allyl and / or benzyl containing compounds. Occurrence of compounds with allylic and/orbenzylic hydrogens, such as terpenoids, was also reported in plants of the Artemisia genus (Bang et al., 2008).

In conclusion, our study can be considered as the first report on the in vitro antioxidant properties of the essential oil and methanol extracts prepared from *A. hausskenechtii.* Owing to its strong excellent protective features exhibited in antioxidant activity tests, the essential oil and extracts from the herbal parts of *A. hausskenechtii* could be concluded as a natural source that can be freely used in the food industry as a culinary herb.

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