Biological activities and chemical composition of the stems and roots of *Helichrysum oligocephalum* DC grown in Iran

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Abstract: *Helichrysum* has long been used medicinally, proving to be beneficial in treatment of acne, asthma, bronchitis and circulatory problems, and lymphatic system diseases. The objective of this research was to study the antioxidant and antibacterial activities and chemical composition of the compounds derived from the stems and roots of cultivated *H. oligocephalum* using gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS). The primary components found in the stem oil were ortho-vanillin (51.0%) and carvacrol (16.0%), and those found in the root oil were 1,8-cineole (30.6%) and isobornyl acetate (13.9%). Stem and root oils of *H. oligocephalum* demonstrated antibacterial activity, particularly in relation to Gram-positive bacteria. In a β-carotene/linoleic acid bleaching assay, the root oil of *H. oligocephalum* demonstrated an antioxidant effect. Antioxidant capacity measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was 1205.0 for the stem oil and 722.8 µg/ml for the root oil.

Keywords: *Helichrysum oligocephalum*; essential oil; ortho-vanillin; 1,8-cineole; antibacterial; antioxidant.

INTRODUCTION

Helichrysum has long been used in traditional medicine to combat a variety of ills, including headache; inflammation (Apendino *et al*., 2007; Sala, del Carmen Recio, Schinella, *et al*., 2003; Sala, del Carmen Recio, Giner, *et al*., 2002); respiratory ailments such as allergies and asthma, bronchitis and coughs; and harmful fungi and microorganisms (Chinou, Roussis, Perdetzoglou & Loukis, 1996; Mastelic, Politeo, Jerkovic & Radosevic, 2005; Nostro, Cannatelli, Musolino, Procopio & Alonzo, 2002; Rossi *et al*., 2007). It has also been used medicinally as an astringent, cicatrisant, diuretic and expectorant and in treatment of the liver, skin, and lymphatic and nervous systems. Studies have demonstrated that it has antioxidant properties (Maffei Facino, Carini, Franzoi, Pirola & Bosisio, 1990; Schinella *et al*., 2007). The oil from the flower heads of various species of *Helichrysum* has nonmedical applications in aromatherapy and as a fixative in cosmetics, perfumes and soaps. Its flower heads contain essential oil that is sought by the perfume industry and aromatherapy. It is nontoxic, nonirritating and nonsensitizing.

Helichrysum is a highly aromatic herb which grows to a height of 60 cm. Its brightly coloured flowers resemble those of its close relative the daisy. The oil has a pungent, honeylike aroma reminiscent of some teas. A member of the Compositae family, *Helichrysum* belongs to the subfamily Asteroideae and the Gnaphalieae tribe and is commonly referred to as everlasting or immortelle. It is indigenous to the Mediterranean although also widely cultivated in the Balkans, France, Italy and Spain. Nineteen species are found in Iran, of which eight are endemic. Its endemism rate in Iran (number of endemic species of *Helichrysum* in Iran/number of *Helichrysum* species in Iran) is ca 42% (Mozaffarian, 1996). Research over the last 20 years on oil composition of plants in the genus *Helichrysum* has concerned the Mediterranean species *H. amorginum* (Chinou, Bougatsos & Perdetzoglou, 2004), *H. italicum* (Chinou, Roussis, Perdetzoglou, Tzakou & Loukis, 1996; Chinou, Roussis, Perdetzoglou, Tzakou & Loukis, 1997; Roussis, Tspukatou, Petrakis *et al*., 2000; Weyerstahl *et al*., 1986), *H. picardii* (Puerta, Garcia, Saenz & Gil, 1993), *H. italicum* ssp. *serotinum*, *H. stoechas* ssp. *stoechas* (Roussis, Tsoukatou, Chinou, Petrakis & Ortiz, 2000), *H. rupestre* and *H. ainbiguu* (Roussis, Tsoukatou & Chinou, 1998), as well as the Greek *H. italicum italicum, H. italicum microphyllum*, *H. stoechas harrelieri, H. orientale*, *H. amorginum*, *H. taenari*, *H. heldreichii* and *H. doerfleri* (Chinou, Roussis, Perdetzoglou, Tzakou & Loukis, 1997; Roussis, Tsoukatou, Petrakis, *et al*., 2000). Comparison of the essential oils derived from everlasting grown in Croatia and Greece has shown them to vary significantly in chemical composition. Oil from Croatia is similar to that of plants of Italian origin (Bianchini, Tomi, Costa & Bernardini, 2001), although its chemical composition varies in relation to locality, season and vegetation cycle (Bianchini, Tomi, Costa & Bernardini, 2001; Blažević, Petričić, Stanić & Maleš, 1995; Paolini *et al*., 2006). The primary components of the essential oil of Croatian *Helichrysum* have been found to be α-cedrene, α-curcumene, α-pinene, γ-curcumene, geranyl acetate, nerol and neryl acetate.

Analysis of essential oils of numerous plants have shown them to contain primarily esters of low fatty acids, often acetate, and only infrequently such esters as propionate and butanoate. Mastelic, Politeo, Jerkovic & Radosevic (2005) found numerous monoterpene, sesquiterpene and

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nonterpene compounds (hydrocarbons, alcohols, carbonyls, esters, ethers and phenols) in the essential oil of *Helichrysum italicum*. However, due to numerous peak overlaps in the chromatograms, leading to insufficient separation and identification, their analysis must be considered unreliable, particularly in regard to esters. According to our early findings, everlasting oil contains numerous ester bonded acids.

Only three reports analysing the essential oils of the species of *Helichrysum* grown in Iran have been published. Investigators found similar compounds in *H. oocephalum* (Firouznia, Akbari, Rustaiyan, Masoudi, Bigdeli & Tabatabaei-Anaraki, 2007), *H. oligocephalum* (Ebrahim Sajjadi, Jafari, & Naderian, 2009), *H. leucocephalum* and *H. artemisioides* (Javidnia, Miri, Soltani, & Khosravi, 2009) to those observed in our research. These studies identified the primary components of the essential oil of *H. oocephalum* as β-caryophyllene, menthone, dodecane and menthol. Those of *H. oligocephalum* were thymol carvacrol, 1,8-cineole, camphor and β-caryophyllene. Rosifoliol, β-caryophyllene and α-humulene were the main constituents found in *H. leucocephalum,* and hexadecanoic acid, β-caryophyllene and α-humulene were the primary components identified in the oil of *H. artemisioides*. Our own study revealed similar compounds.

The purpose of our research was to investigate in detail the antioxidant and antibacterial effectiveness and chemical composition of the essential oil obtained from the stems and roots of the cultivated *H. oligocephalum*. For this purpose, fresh stems and roots of *H. oligocephalum* were subjected to hydrodistillation and the volatile acids thus obtained isolated and then analysed using by GC and GC-MS.

MATERIALS AND METHODS

Plant material

The samples of stems and roots of *H. oligocephalum* were collected in July 2004 during the flowering stage, from 15 km north of Khorramabad in Lorestan Province, Iran, at an altitude of 1700 m. Voucher specimens (No.3797) were deposited with the of Research Institute of Forests and Rangelands Herbarium (TARI), Tehran, Iran.

Isolation of oil

Stems (200 g) and roots (250 g) of *H. oligocephalum* were hydrodistilled for 3 h with a Cleavenger-type apparatus following the parameters described in the British pharmacopoeia (European Pharmacopoeia Maisoneuve, 1983). The oil was decanted and dried over anhydrous sodium sulphate.

Qualitative and quantitative analyses

Most constituents were identified using GC and comparing the retention indices with either those of the literature or authentic compound samples kept in our laboratories. The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8-C_{28}) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with one of three sources: NIST 02 and Wiley 275 libraries, the literature, or our homemade library. Relative concentrations of components were calculated based on GC peak areas without using correction factors (Adams, 2007; Joulain & König, 1998; Unwin Brothers, 1991).

Antimicrobial assay

The broth dilution method was employed to determine the minimum bactericidal concentration and thus evaluate antibacterial activity (Foti, Daquino & Geraci, 2004; Salehi, Sonboli, Eftekhari, Nejad-Ebrahimi, & Yousefzadi, 2005). Five species of bacteria identified by the Research Centre of Science and Industry, Tehran, Iran, were tested: Gram-positive *Staphylococcus aureus* (PTCC 1113), *S. epidermidis* (PTCC 1349) and *S. saprophyticus* (PTCC 1379) and Gram-negative *Escherichia coli* (PTCC 1330) and *Pseudomonas aeruginosa* (PTCC 1310).

After obtaining microorganisms with enrichment culture in 1 mL of Mueller-Hinton broth incubated at 37°C for 12 h, the microorganisms were cultured on Mueller-Hinton agar medium. The following procedure was employed to measure antibacterial activity: 40 µL of diluted essential oil (40 µL oil in 2 mL DMSO 10%) was added to a 200 µL microbial suspension (1 loop from medium in physiological serum was compared with a 0.5 McFarland standard) in well 1 in a microplate, and 100 µL from this well was add to a 100 µL microbial suspension in well 2, and this continued until 8 wells in the microplate were filled. Following this, the microplates were incubated at 37°C for 24 h.

Radical scavenging test (DPPH assay)

The DPPH assay is generally considered to involve a hydrogen atom transfer reaction, but some researchers have asserted an electron transfer mechanism as well, based on kinetic data. We used an established DPPH radical-scavenging activity (RSA) assay method with minor modifications to evaluate the RSA of the stem and root oils of *H. oligocephalum.* A 10 mg/ml stock solution was prepared for each oil and for the synthetic standard antioxidant butylated hydroxytoluene (BHT). Each solution was diluted in methanol. Dilutions of different concentrations ranging from 1 to 5×10^{-10} mg/ml were prepared. Two ml of each diluted solution was mixed with the same amount of freshly prepared 80 µg/ml DPPH solution and left standing for 30 min in the dark at room temperature to allow any reaction to take place. Ultraviolet absorbance of these solutions was recorded on a spectrometer at 517 nm using a blank containing the

same concentration of oil or BHT without DPPH. Percent of inhibition (I%) of free radical DPPH was the computed using the following equation:

$$(1)\ I\% = \left[\frac{\left(A_{blank} - A_{sample}\right)}{A_{blank}}\right] \times 100$$

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 performed three times, and IC_{50} values were reported as means ± SD of triplicates.

β-Carotene/linoleic acid bleaching assay

In the β-carotene/linoleic acid bleaching assay, the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation was measured to compute antioxidant activity. The Miraliakbari-Shahidi method (2008) was used, slightly modified. A stock solution was prepared containing 0.5 mg *β*-carotene, 1 ml chloroform, 25 µl linoleic acid and 200 mg Tween 40. The chloroform was evaporated under vacuum, and 100 ml of aerated distilled water then added. The samples (2 g/l) were dissolved in DMSO, and 350 µl of each sample solution added to test tubes containing 2.5 ml of the above mixture. The test tubes were incubated for 2 h in a hot water bath at 50°C, together with two blanks, one containing the antioxidant BHT as a positive control and the other containing the same volume of DMSO in place of the extracts. The test tube with BHT maintained its yellow colour throughout the incubation period. The absorbance was measured at 470 nm on an ultraviolet spectrometer. Antioxidant activity (I%) of each sample was computed using as follows:

$$(1)\ I\% = \left[\frac{\left(A_{\beta\text{-carotene after 2 h assay}} - A_{\text{initial }\beta\text{-carotene}}\right)}{A_{blank}}\right] \times 100$$

where $A_{\beta\text{-carotene after 2 h assay}}$ is the absorbance of *β*-carotene remaining in the samples after 2 h assay, and $A_{\text{initial β-carotene}}$ is the absorbance of *β*-carotene at the beginning of the experiments (Park, Koo, Ikegaki & Contado, 1997). All tests were performed three times, and I% values were reported as means ± SD of triplicates.

Gas chromatography and GC-MS

GC analysis was performed on a Shimadzu 15A gas chromatograph equipped with a capillary column used was DB-5 (50 m × 0.2 mm, film thickness 0.32 µm). Split/splitless injector and flame ionization detector were heated at (250°C). N_2 was used as carrier gas (1 mL/min). The oven temperature was kept at 60°C for 3 min and then heated to 220°C with a 5°C/min rate and kept constant at 220°C for 5 min. Relative percentage amounts were calculated from peak area using a Shimadzu C-R4A chromatopac integrator without the use of correction.

GC-MS analysis was performed using a Hewlett-Packard 5973 with a HP-5MS column (30m × 0.25mm, film thickness 0.25µm). The oven temperature was kept at 60°C for 3 min and programmed to 220°C at a rate of 5°C/ min and kept constant at 220°C for 5 min. The flow rate of Helium as carrier gas was (1 mL/min). MS were taken at 70 eV.

Identification of the constituents of the oil was made by comparison of their mass spectrums and retention indices (RI) with those given in the literature and those authentic samples (Adams, 2007).

RESULTS

Essential oil analysis

The percentage chemical compositions of the essential oil in the two part of *H. oligocephalum* oils are listed in Table 1. 23 (from stem oil) and 50 (for root oil) components representing 94.8% and 92.4% respectively, of the oil were identified. The main components of the oil were: ortho-vanillin (51.0%) and carvacrol (16.0%) for stem oil and trans-caryophyllene (27.0%), caryophyllene oxide (7.6%) for root oil.

Antimicrobial assay

The results of the antibacterial screening showed that *T. pinnatum* oil was active against the Gram-positive bacteria *Staphylococcus aureus* (PTCC 1113), *Staphylococcus epidermidis* (PTCC 1349) *Staphylococcus saprophyticus* (PTCC 1379) [14.3, 12.6, 16.2 (for stem) oil and 12.0 13.3, 10.0 (for root oil) mm diameter respectively]. The same oil showed inhibitory activity against the Gram-negative bacteria *Escherichia coli* (PTCC 1330) while *Pseudomonas aeruginosa* (PTCC 1310) [11.0, 11.3 (for stem oils) and 10.0, 13.6 (for root oil) mm diameter respectively) (table 2).

Radical scavenging test and β-carotene/linoleic acid model

In the present study, the weakest radical scavenging activity was exhibited by the root oil (722.8 ± 4.3µg ml^{-1}). The positive control BHT demonstrated stronger activity than any of the samples. The I% capacity of the stem oil (25.3 ± 1.2) was found to be superior to the sample.

DISCUSSION

Essential oil analysis

Table 1 lists the proportions of constituents isolated in the two *H. oligocephalum* oils. The stem oil contained were found (8.3%) in the stem oil and two sesquiterpenes

Table 1: Percentage composition of the stem and root oils of *Helichrysum oligocephalum*

Compound	[a]RI	[b]Stem oil	[b]Root oil	Compound	[a]RI	[b]Stem oil	[b]Root oil
Camphene	953	2.9	-	α-Ylangene	1298	0.4	-
α- Pinene	939	1.2	5.9	Carvacrol	1300	16.0	-
1,8- Cineole	1033	4.3	30.6	β-Bourbonene	1372	1.4	-
Acetophenone	1065	0.7	1.0	β-Caryophyllene	1442	1.4	-
Linalool	1098	0.6	-	Geranyl acetone	1453	0.5	-
Nonanal	1102	0.4	3.6	α-Humulene	1454	0.8	-
Camphor	1143	1.5	10.1	β-Selinene	1494	0.5	-
Eugenol	1280	1.1	2.0	Pentadecane	1500	-	3.2
Isopulegol acetate	1281	0.8	-	δ-Cadinene	1524	-	2.9
Ispbornyl acetate	1285	2.9	13.9	Elemol	1549	-	1.4
(Z)-Ethyl-isomenthone	1289	1.2	-	Caryophyllene oxide	1581	1.6	-
Thymol	1290	1.5	3.6	α-Cadinol	1653	1.6	-
ortho-vanillin	1292	51.0	2.0	β-Bisabolene	1673	0.5	-

[a]Retention indices using a DB-5 column. [b]Relative area (peak area relative to total peak area).

Table 2: Antibacterial activity of the stem and root oils of *Helichrysum oligocephalum* based on dilution method and using four reagents*

Bacterial Species	Gram-+/–	Stem oil	Root oil	Gentamicin	Penicillin	Cefazolin	Norfloxacin
Staphylococcus aureus PTCC 1113	+	14.3	12.0	0.0	0.0	15.7	0.0
Staphylococcus epidermidis PTCC 1349	+	12.6	13.3	30.3	21.0	30.3	31.0
Staphylococcus saprophyticus PTCC 1379	+	16.2	10.0	0.0	25.0	20.0	11.0
Escherichia coli PTCC 1330	–	11.0	10.0	19.0	0.0	17.7	28.3
Pseudomonas aeruginosa PTCC 1310	–	11.3	13.6	15.6	0.0	15.3	30.3

*Values are the mean diameter of inhibitory zones (mm)

(4.3%) in the root oil. In a related study, analysis of the essential oil of *H. cymosum* isolated 50 components, composing 92.4% of the overall composition. The primary constituents proved to be trans-caryophyllene (27.0%), caryophyllene oxide (7.6%), *p*-cymene (7.5%), δ-3-carene (6.8%) and *α*-fenchene (6.2%). The essential oil of *H. fulgidum* was found to contain 28 components, comprising 88.2% of the total oil. The primary constituents were found to be caryophyllene oxide (12.4%), β-pinene (8.7%), spathulenol (7.8%), α-muurolol (7.3%), *β*-bourbonene (7.1%) and camphor (5.3%) (Bougatsos, Ngassapa, Runyoro & Chinou, 2004).

Comparison of the chemical composition of the oils investigated in this study with *H. oligocephalum* shows little similarity, although the plants are members of the same genus. Caryophyllene oxide was the only component isolated in significant quantities in all three species (Bianchini, Tomi, Bernardini *et al*., 2003).

In a previous investigation the oil obtained from *H. oocephalum* was identified as containing β-caryophyllene (13.5%), menthone (10.8%), dodecane (9.1%) and menthol (8.9%) among the 18 components detected (Firouznia, Akbari, Rustaiyan, Masoudi, Bigdeli, & Tabatabaei-Anaraki, 2007). In the essential oil of *H. oligocephalum* grown in Isfahan, Iran, 82 components were detected; thymol (14.4%), carvacrol (1.7%), 1,8-cineole (2.2%), camphor (1.9%) and β-caryophyllene (4.9%) were found to be the major constituents (Ebrahim Sajjadi, Jafari, & Naderian, 2009). In the oils of *H. leucocephalum* and *H. artemisioides* 92 and 48 components representing 93.0% and 92.4% respectively were identified. Rosifoliol (22.3%), β-caryophyllene (10.1%) and α-humulene (9.0%) comprise the primary constituents of the oil of *H. leucocephalum,* and hexadecanoic acid (14.7%), β-caryophyllene (10.6%) and α-humulene (7.7%) were the major constituents of the oil of *H. artemisioides* (Javidnia, Miri, Soltani & Khosravi, 2009).

Comparison of the chemical composition of the *H. oligocephalum, H. leucocephalum* and *H. artemisioides* oils with that found in our research does show much similarity. In previous investigations the oil obtained from *H. oligocephalum, H. leucocephalum* and *H. artemisioides* grown in the province of Isfahan, Iran, was rich in monoterpenes, and the oil of *H. oligocephalum* grown in a province of north of Khorramabad (Lorestan Province), Iran, had a composition of more than 50%

monoterpenes. Monoterpenes in the oil of *H. oocephalum* represented 29.8% of the total components detected. Some of these differences could be due to environmental factors and ecological conditions.

Antimicrobial assay

The *in vitro* antibacterial activity of *H. oligocephalum* stem and root oils against five species of bacteria, including both Gram-positive and Gram-negative bacteria and those associated with gastrointestinal, respiratory, skin and urinary diseases, was investigated by determining the minimum bactericidal concentration with the broth dilution method. Our sample showed activity particularly against Gram-positive bacteria, as evidenced by the lower minimum inhibitory concentration values found in Gram-positive bacteria (table 2). Gram-negative bacteria did not appear to be sensitive to the oil.

Radical scavenging test and β-carotene/linoleic acid model

Antioxidant effectiveness of the stem and root oils of *H. oligocephalum* was evaluated by two different test systems, DPPH and β-carotene/linoleic acid model system. In the DPPH method, the antioxidants react with the stable free radical, i.e. 1,1-diphenyl-2-picrylhydrazyl (deep violet colour), converting it to 1,1-diphenyl-2-picrylhydrazine, with the degree of discolouration indicating the free radical scavenging potentials of the sample/antioxidant. Known antioxidants such as ascorbic acid, cysteine, glutathione, tocopherol and polyhydroxy aromatic compounds (hydroquinone, pyrogallol, etc.) have been found to reduce and decolourize 1,1-diphenyl-2-picrylhydrazyl by their hydrogen-donating ability. In this investigation, the weakest RSA occurred with root oil (722.8±4.3 μg ml^{-1}).

In the β-carotene/linoleic acid model system, β-carotene undergoes rapid discolouration in the absence of an antioxidant. This is because of the free radicals generated by the coupled oxidation of β-carotene and linoleic acid. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from its diallylic methylene group attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and broken down in part, and the system loses its chromophore and characteristic orange colour, which is monitored spectrophotometrically. The auto-oxidation of linoleic acid without volatiles and extracts accompanies the rapid increase of peroxides. According to Farag, Badei and El-Baroty (1989), there is a relationship between inhibition of the hydroperoxide formation and the presence of some phenolic nuclei in essential oils and extracts. The antioxidative effectiveness in natural sources has been reported to be mostly due to phenolic compounds. The I% capacity of the stem oil (25.3 ± 1.2) was found to be superior to the sample (table 3).

Table 3: Antioxidative capacities of the stem and root oils of *Helichrysum oligocephalum*

Plant oils	Test system	
	DPPH IC_{50} (μg/ml)	β-Carotene/linoleic acid (% inhibition rate)
Stem *H. oligocephalum*	1205.0±0.6	25.3±1.1
Root *H. oligocephalum*	722.8±1.8	36.1±2.1

[a]Results are means of three different experiments.

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