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Volatile Composition, Antimicrobial and free radical scavenging activities of essential oil and total extract of *Helichrysum leucocephalum* Boiss.

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Abstract

The present study investigated the composition of essential oil and evaluation antimicrobial and antioxidant of the ethanolic extract of the aerial parts of *Helichrysum leucocephalum*. Gas chromatography/mass spectrometry (GC/MS) revealed the presence of 43 compounds which represents 96.8% of the total oil. Carvacrol (20.36%), acetophenone (11.17%) and azulene (7.09%) were identified as the main principal components of the essential oil. *H. leucocephalum* ethanolic extract exhibited a high phenol content (114.13±0.89 mg gallic acid equivalent (GAE)/g of dry plant) while total flavonoid content was found to be 43.26±0.70 mg quercetin equivalent (QE)/g of dry plant). In order to evaluate antioxidant efficacy of the essential oil and ethanolic extract, DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and ferric reducing antioxidant power. (FRAP) assays were conducted. The ethanolic extract demonstrated significant DPPH free radical Inhibitory activity (IC_{50}=61.35±0.07 µg/mL) and ferric reducing ability (IC_{50}= 113.43±0.58 µg/mL) while the essential oil declared a weak antioxidant properties. Antimicrobial screening revealed higher degree of inhibition for essential oil of the aerial parts of *H. leucocephalum* against *Staphylococcus aureus* and *Escherichia coli* both with MIC value, 16 µg/mL. Additionally the essential oil of H. leucocephalum exhibited antifungal properties when tested against *Candida albicans* (MIC_{50}, 32 µg/mL).

Keywords: Helichrysum leucocephalum, antimicrobial, antioxidant, Phenol, flavonoid.

1. Introduction

The rising demand for herbal drugs have so far directed the attention of pharmaceutical scientists towards medicinal plants as traditional sources of drugs for the treatment of various diseases (1). In this context, Iran stands out as one of the places with large plant biodiversity and many unexplored species with respect to their pharmacological potential. Among various families of plants so far investigated, the Asteraceae family deserves some remarkable interests. The genus *Helichrysum*, one of the largest genera in the family Asteraceae includes around 600 species, widespread throughout the different regions of the globe in the form of annual plants, shrubs and trees (2, 3). They are commonly known as "*Gole Bi Marg*" in persian, showing distribution of 19 species in Iran that occur on the mountainous area with clay soil, calcareous rocks, dry slopes and steppe areas (4). *Helichrysum leucocephalum* Boiss. one of the six endemic species of the country is the subject of this investigation. *Helichrysum* species have so far demonstrated diverse therapeutic properties such as hepatoprotective, antiallergic, anticholeretic, anti diarrheal and antidiabetic effects. These re-

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markable properties among *Helichrysum* species is attributed to the presence of flavonoids as their principal constituents (5).

This plant is used in folk medicine for the treatment of kidney stone and gall bladder disorders(6). A thorough literature search declared antidibatic, antioxidant, anti lipid peroxidation and and antimicrobial effect of other *Helichrysum* species but no published report on antimicrobial potential of this particular species (7, 8). Therefore this study was undertaken to characterise the volatile oil composition of *H. leucocephalum* and further assess antimicrobial characteristics and free radical scavenging properties of both essential oil and the plant ethanolic extract.

2. Material and methods

2.1. Chemicals and reagents

Culture media, chemicals, solvents and reagents were purchased from Merck Chemical Company, Damstadt, Germany. DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), gallic acid, quercetin and the standard antibiotics were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Plant material

The aerial parts of *H. leucocephalum* were collected from Saman area in Chahar Mahalo Bakhtiari province in July 2016. A specimen was deposited in the Herbarium of Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran under a voucher number MRCH-95-107.

2.3. Essential oil extraction

The essential oil was extracted from the powdered aerial parts of the plant by hydrodistillation in a Clevenger type apparatus over a period of 3 hours. The yield of the oil obtained was 0.15% v/w. The resulted oil was dried over anhydrous sodium sulfate and stored at 2 ºC for further chemical and pharmacological analysis.

2.4. Preparation of ethanolic extract

The powdered plant material (30 g) was extracted with ethanol 96% for 3hrs using a soxhlet apparatus. The extract was concentrated under reduced pressure at 40 ºC on a rotary evaporator and further concentrated in a speed vaccuum concentrator and freeze dried in a Christ Alpha 1-4 LD Freeze dryer, Martin Christ, Germany and stored at 2 ºC pending analysis.

2.5. GC/FID and GC/MS analysis

To achieve the best separation conditions the oil was initially injected into an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID). The inlet and FID temperatures were 250 and 300 ºC respectively. Column type was HP-5MS (30 m×0.25 mm×0.25 µm film thickness). Oven temperature was linearly programmed from 60 to 220 ºC with an increasing rate of 5 ºC/min and hold at this temperature for 10 min. Nitrogen was used as carrier gas with a flow rate of 1ml/min. The above method was further applied to a GC/MS instrument (Agilent 7890A, MSD 5975C). Helium was used as carrier gas. Column type and flow rate were the same as those used for the GC/FID run. Interface temperature was adjusted at 280 ºC. Ionization energy for the recorded mass spectra was 70 ev and the mass range were m/z 30-600. Identification of compounds using GC/MS was based on comparison of their mass spectra with Wiley 7 nl and Adams libraries (9).

2.6. Determination of total phenol

Total phenolic content of the ethanolic extract was determined using a Folin-Ciocalteu spectrophotometric method (10). To develop a standard calibration curve, 0.5 mL of gallic acid solutions of various concentrations ranging from 6.25-200 mg/L were mixed with 5 mL of 10% Foiln-Ciocalteu reagent and 4 ml of 1.0 M sodium carbonate solution. Proper dilutions of the extract was oxidized with Folin-Ciocalteu reagent and neutralized by sodium carbonate solution as given for standard. The absorbance of solution was recorded at 765 nm after 15 min against Folin-Ciocalteu reagent as blank using a PG instrument T90 UV spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalent (GAE)/g of dry plant material using the expression from the calibration curve ($Y=0.00571x-0.0843$, $R^2=0.9985$). Where Y is the absorbance and x is

the gallic acid equivalent.

2.7. Determination of total flavonoid content

Total flavonoid content of the extract was determined by an aluminum chloride colorimetric assay as described previously (11). This colorimetric procedure is based on an aluminum (III) complex formation reaction. For preparation of calibration curve 5 ml of 2% aluminum chloride solution in methanol was mixed with the same volume of quercetin (0-80 mg/L) as a standard. Absorption was measured at 415 nm on a PG-T90 UV spectrophotometer after 10 min. For quantitative determination of total flavonoid content, a solution of 300 mg/L of extract in methanol was prepared and the analysis was performed using the above procedure. For the control, methanol was used instead of the extract while the blank contained 2 ml of extract and 2 mL of methanol. Total flavonoid content was expressed as mg of quercetin equivalent (QE)/g of dry plant material using the equation obtained from the calibration curve ($Y=0.02985\ x+0.04811$, $R^2=0.9978$). Where Y is the absorbance and x is the quercetin equivalent.

2.8. DPPH Free radical scavenging assay

DPPH radical scavenging activity of the extract and quercetin were determined using a previously reported procedure (12). Twenty microliters of various concentration of the methanolic solution of extract and essential oil (6.25-200 µg/mL) were mixed with 200 µL solution of 100 mM DPPH in methanol. The mixture was left in the dark for 30 min and the absorbance was measured at 490 nm using a Biotek, ELX800 microplate reader. A sample containing 20 µL of methanol and 200 µL of DPPH solution served as control while a solution of equal amount of extract in methanol was used as the blank. All tests were conducted in triplicate. Percentage inhibition of extract against DPPH was calculated using the following equation:

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

2.9. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was performed according to a procedure described by Benzie and Strain (13). Solutions of acetate buffer 300 mM pH 3.6, Ferric chloride (III) 20 mM and TPTZ 10 mM (in 40 mM HCl) were separately prepared. The FRAP reagent was freshly prepared by mixing the above solutions including acetate buffer, TPTZ and $FeCl_3$ at the ratio of 10:1:1 (v/v/v) respectively. Briefly 20 µL of extract was mixed with 180 µL of FRAP reagent and the absorbance was measured at 593 nm after 10 min incubation at 37 °C using an Epoch microplate spectrophotometer. Control contained 20 µL of methanol and 180 µL of FRAP reagent without extract. A sample containing extract and the solvent served as blank. Quercetin was used as antioxidant standard and positive control. The absorbance of tested samples were compared to a $FeSO_4$ standard curve and the values were expressed as Ferrous Equivalent (FE), i.e. the concentration of extract which shows the same absorbance as 1 mmol ferrous ion(Fe^{+2}). An antioxidant compound or extract causes reduction of Fe^{3+} to Fe^{2+} ion leading to an increase in the absorption at 593 nm due to the formation of a blue Fe^{2+}/TPTZ complex.

2.10. Antimicrobial screening

The essential oil and the ethanolic extract were evaluated for antibacterial and antifungal activity against 4 human pathogenic strains including 2 gram-negative and gram-positive bacteria and 2 fungal strains. All microorganisms were obtained from Persian type culture collection (PTCC) Tehran, Iran. The microorganisms used in the present study were *Escherichia coli* (PTCC 1396), *Staphylococcus aureus* (PTCC 1337), *Aspergillus niger* (PTCC 5154) and *Candida albicans* (PTCC 5027). Ampicillin (Amp), Gentamycin (Gen) and Fluconazole (Flu) were used as standard antibiotics.Antimicrobial screening was performed by determination of minimum inhibitory concentration (MIC) using broth and agar dilution methods (14, 15).

3. Results and discussion

Table 1 represents the chemical composition of leaf essential oil of *H. leucocephalum*. Carvacrol (20.36%), acetophenone (11.17%), azulene (7.09%) α-pinene (6.36%) and hexadecanoic acid (5.52%) were identified as the major constituents of the oil (Table 1, Figure 1). Among the various

Table 1. Chemical composition of essential oil of aerial parts of *H. leucocephalum*.

Compound	KI^{cal}	%	Compound	KIcal	%
2-Hexenal	849	1.0	*t*-Cadinol	1646	0.88
2-Heptanone	889	0.70	Tetradecanoic acid	1761	2.68
α-Pinene	935	0.60	Phytone	1846	0.93
Camphene	950	1.60	Hexadecanoic acid	1962	5.52
Benzaldehyde	960	0.80	Manoyl oxide	2000	2,05
Hexanoic acid	971	0.40	1- Phytol	2112	0.36
6-Methyl-5-Hepten-2-one	985	1.16	Linoleic acid	2130	0.55
dl-Limonene	1029	0.65	Tricosane	2298	0.31
1,8-Cineole	1206	0.4			
Acetophenone	1208	1.9	Total		96.8
cis-Linalool oxide	1245	0.5	Monoterpene		40.24
trans-Linalool oxide	1256	0.7	Fatty acid		21.75
Camphor	1262	0.4	Aromatic ketone		11.17
Borneol	1267	1.8	Aromatic hydrocarbon		7.09
cis-1,4-Menthol	1174	1.16	Diterpene		2.41
Azulene	1174	7.09	Sesquiterpene		2.40
α-terpineol	1192	0.68	Aliphatic ketone		1.58
Pulegone	1242	0.42	Aliphatic aldehyde		0.4
Carvotanacetone	1246	0.54	Unknown compounds		3.2
Nonanoic acid	1274	5.50			
Anethole	1287	0.67			
Thymol	1291	4.94			
Carvacrol	1302	20.36			
Decanoic acid	1370	0.40			
Carvacryl acetate	1374	0.33			
trans-Caryophyllene	1425	0.67			
trans-Geranylacetone	1454	0.70			
α-Humulene	1459	0.32			
β-Ionone	1489	0.35			
Eudesma-4(14),11-diene	1492	0.41			
α-Selinene	1500	0.41			
Myristicin	1525	0.39			
δ-Cadinene	1528	0.49			
Dodecanoic acid	1562	1.09			
β-Selinene	1590	1.89			

KI^{cal}, Kovats retention indices relative to C_7 -C_{30} *n*-alkanes on HP-5MS column

classes of volatile components, monoterpene hydrocarbons (40.24%) showed the highest contribution (Table 1). Fatty acids with a total amount of 21.75%, was found to be the second major class of the oil chemical constituents. In addition aromatic ketones (11.17%), aromatic hydrocarbons (7.09%), diterpenes (2.41%), sesquiterpenes (2.40%) and aliphatic aldehydes (1.58%) were detected in the oil.

3.1.Total Phenol and Flavonoid

Table 2 shows total phenol and flavo-

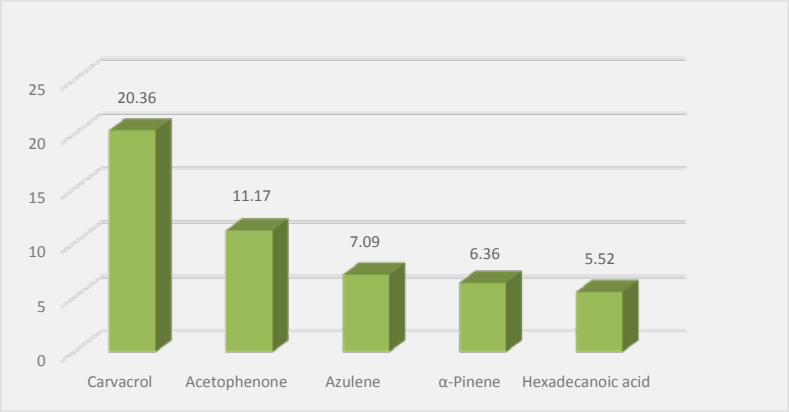

Figure 1. Major constituents of essential oil of *H. leucocephalum* Boiss.

noid contents of the ethanolic extract of H. leucocephalum. Total phenol content of the extract was found to be 114.13±0.89 mg GAE/g while total flavonoid content was determined and found to be 43.26±0.70 mg quercetin equivalent (QE)/g of dry plant (Table 2). *H. leucocephalum* extract was therefore found to be a rich source of phenolic compounds including flavonoids. These two groups of compounds have been reported earlier as the major constituents of another *Helichrysum* species (16).

3.2.Antioxidant screening

The results of antioxidant screening and the relevant IC50 values of the extract and essential oil obtained from DPPH and FRAP assays are given in Table 2. The extract and essential oil showed IC50 values of 61.35±0.07 and 285.07±2.35 µg/ml against DPPH free radical respectively. The FRAP value of ethanolic extract was found to be 113.43±0.58 µg/ml while it was 336.53±1.35 µg/ml for the essential oil (Table 2). These findings declared thst the extract bears DPPH free radical inhibitory properties and a moderate ferric reducing antioxidant capacity but the essential oil is almost devoid of antioxidant activity in the performed assays.

3.3. Antimicrobial screening

Table 3 presents the results of antimicrobial screening of the oil obtained from *H. leucocephalum*. Based on the results the oil showed significant inhibition against the growth of all tested microorganisms at concentrations ranging from 8-256 µg/ml. Antibacterial and antifungal potential of the were assessed in terms of minimum inhibitory concentration (MIC) and the results were recorded (Table 3). The essential oil demonstrated antibacterial activity against both Gram-positive and Gram-negative bacteria. The MIC vaules against both *S. aureus* and *E. coli* were found to be 16 µg/mL while the extract showed MIC values of 256 and 128 µg/mL against *S.aureus* and *E. coli* respectively (Table 3). Among the tested fungal strains, *H. leucocephalum* essential oil exhibited MIC value of 256 µg/mL against C. albicans while its MIC value was found to be 128 µg/mL when tested against A. niger (Table 3). Also the MIC values for the representative antibiotics against different test organisms are shown in Table 3.

Table 2. Total phenol and flavonoid contents and antioxidant activity of *H. leucocephalum* ethanolic extract.

Sample	Total Phenol Content[1]	Total Flavonoid Content[2]	DPPH Assay IC_{50} (µg/ml)	FRAP Assay IC_{50} (µg/ml)
Ethanol extract	114.13±0.89	43.26±0.70	61.35±0.07	113.43±0.58
Essential oil	-	-	285.07±2.35	336.53±1.35
Quercetin	-	-	26.51 ± 0.06	8.69 ± 0.03

[1]mg GAE /g of dry plant material.

[2]mg QE /g of dry plant material.

Table 3. Antimicrobial activity of essential oil of aerial parts of *H. leucocephalum* Boiss.

Gram-positive bacteria		Essential Oil/ Ext./ Antibiotics				
		Ext.	EO	Amp	Gen	Flu
Gram-positive bacteria		MIC (µg/ml)				
Staphylococcus aureus	PTCC 1337	256	16	16	16	8
Gram-negative bacteria						
Escherichia coli	PTCC 1396	128	16	16	8	16
Fungi						
Aspergillus niger	PTCC 5154	128	64	16	16	8
Candida albicans	PTCC 5027	256	32	8	16	16

MIC: Minimum Inhibitory Concentration

EO: Essential Oil, Ext.: Extract, Amp: Ampicillin, Gen: Gentamicin, Flu: Fluconazole

As evidenced, the essential oil of the aerial parts of *H. leucocephalum* exhibits significant antimicrobial activity against all tested microorganisms and therefore could be considered as a source of natural antimicrobial compounds while the extract was found to be devoid of antibacterial and antifungal activity.

On the basis of the results obtained in the present study, it is concluded that the ethanolic extract of *H. leucocephalum* which contain large amounts of phenolic compounds, exhibit remarkable antioxidant and free radical scavenging activities in DPPH assay and also declared a moderate ferric reducing antioxidant power. Thymol and Carvacrol, are well documented antimicrobial phytochemicals while acetophenone and azulene are among the volatile constituents of *H. leucocephalum* essential oil that have been suggested as lead compounds for synthesis of active antifungal products (18,19). Phytol and hexadecenoic acid are other components of the oil with known antimicrobial activity which deserve mentioning (20, 21). Therefore the observed antimicrobial activity of the essential oil is mainly justified by the presence of these active antimicrobial compounds. Furthermore the *in vitro* free radical scavenging assays clearly indicated the free radical scavenging and ferric reducing ability of *H. leucocephalum* ethanolic extract. Antioxidant activity have been previously detected in another *H. pseudoplicatum* Nab. (22). The results of present research uncovered the antioxidant properties of *H. leucocephalum* ethanolic extract. From the inspection of literature, the antioxidant activity of ethanolic extract could partly due to the presence compounds like galangin, a 3,5,7-trihydroxyflavonoid, chlorogenic type compounds such as 3,4- and 3,5-dicaffeoyl quinic acids and other classes of compounds like steroids and terpenoids detected in the genus *Helichrysum* (23).

4. Conclusion

The results of antioxidant assays in the current study might be helpful in developing products useful for preventing the progress of oxidative stresses and thus deserves further investigation. The essential oil of *H. leucocephalum* is also worthy of further inspection in order to isolate and characterise the active volatile chemical components responsible for manifestation of antimicribial characteristics.

5. Statistical analysis

Experiments were performed in triplicate. The IC_{50} values (µg/mL) were calculated for all tested samples using probit test in SPSS software. Data were expressed as Mean±SD. Statistical differences between treatments were identified using one way ANOVA followed by Tukey post test and significant differences between controls and samples mean values determined using comparison test at a level of $P<0.05$.

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Conflict of Interest

None declared.

6. References

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