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Constituents, Antimicrobial and Antioxidant Activities of *Pulicaria gnaphalodes* (Vent.) Bioss. Volatile Oil from Iran

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The water distilled essential oil of the aerial parts of *P. gnaphalodes* from Qom area of Iran was analyzed by GC-FID and GC/MS methods. Eighty-eight compounds, accounting for 89 % of the total oil, were indentified. The main components of the oil were myrtenol (13.2 %), citronellol (9.0 %), (E)-nuciferol (5.2%), shiromoll (5.1 %), geraniol (5.1 %) and α-pinene (5.0 %). The oil was rich in regards to oxygenated monoterpenes (48.8 %). The antimicrobial activity of the oil was measured by a disc diffusion method. The oil has shown maximum zone of inhibition against *Candida albicans* (23 ± 1.2 mm) with a value higher than that of fluconazol (18.0 ± 0.3 mm). The oil antioxidant activities were valued by DPPH assay and β-carotene-linoleic acid assays. The essential oil strongly reduced the concentration of DPPH free radical (36.0 ± 1.3 %), with an efficacy higher than that of trolox (33.0 ± 0.5 %). The oil had high inhibition against BHD standard in β-carotene bleaching test.

Key Words: *Pulicaria gnaphalodes*, Essential oil composition, Myrtenol, Citronellol, Shiromoll, Geraniol, α-Pinene.

INTRODUCTION

The relatively larg *Pulicaria* genus belongs to the plant family asteraceae (compositae)[1]. This genus is represented in the flora of Iran by five species[2]. Some *Pulicaria* species are useful in traditional medicine for the treatment of a variety of diseases and complaints. Traditionally, the roots of *P. odora* are still used for their antiinflammatory properties[3]. *P. undulata* is a medicinal plant used by people of Southern Egypt and Saudi Arabia to treat inflammation[4]. It is used also as an insect repellent[5]. Various medical activities such as antibacterial, antifungal, antioxidant, antiinflammatory and anticholinesterase, have been reported from *Pulicaria* genus[4,6-9]. *P. gnaphalodes* has been investigated chemically; a new clerodane lactone, which is closely related to hardwickiic acid, has been reported from the aerial parts of this plant from Iran[10]. Likewise, from areal parts extract of *P. gnaphalodes* from Iran have been analyzed for exudate flavonoids[11]. It was shown to accumulate mostly 6-methoxylated flavonols and flavones as external aglycones. In another report, from chloroform extract of *P. gnaphalodes*, flavonoids, sesquiterpenoid, sterol, benzoic acid derivaties and fatty acid were obtained[12]. The structures were elucidated the aid of NMR spectroscopy. In other report on extract of *P. gnaphalodes*, a new sesquiterpene-dimer of guaiane class named as gnapholide and anabsinthin of the same skeleton were identified[13]. The structures of both the compounds were elucidated with the aid of spectroscopic techniques including ^{2}D NMR. In recent report, five compounds including one phenolic acid (salicylic acid), two clerodane diterpenoids (salvifolin and salvicin) and two flavonoids (pulicarin and giperoside) were isolated from alcoholic extract of the aerial parts of endemic *P. gnaphalodes* from central Asia[14].

Also, in previous studies about the essential oils of other *Pulicaria* species, the chemical composition of the oil from aerial parts of *P. dysenterica*, growing wild in Iran[15] and Greece[16], was determined by GC and GC/MS. The main components of the Iranian sample oil were ar-curcumene (28.3 %), epi-α-cadinol (16.4 %) and (E)-coniferyl alcohol (11.0 %) while the main components in two Greek oil samples were (Z)-nerolidol (11.2 %), caryophyllene oxide (9.1 %) and (E)-nerolidol (6.6 %) (sample A) and β-caryophyllene (12.8 %), caryophyllene oxide (12.8 %) and (E)-nerolidol (6.9 %) (sample B). Although, in the literature, to our best of knowledge no report has observed about of antioxidant activity of *Pulicaria* essential oil also numerous another reports have appeared on the essential oils and its antimicrobial activity of different species of *Pulicaria*[17-23], but in the present study, the antimicrobial and antioxidant activities of the essential oil of *P. gnaphalodes* from Qom area of Iran are reported.

EXPERIMENTAL

The aerial parts of *P. gnaphalodes* were collected during the flowering stage in Qom area, province of Qom, Iran, in August 2010. Voucher specimens have been deposited at the Herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran.

Isolation of the oil: The aerial parts (152.0 g) of *P. gnaphalodes* of the plant were subjected separately to hydrodistillation using a Clevenger-type apparatus for 3 h. After decanting and drying over anhydrous sodium sulfate, the corresponding yellowish coloured oils were recovered in yields of 0.1 % (w/w), respectively. The sample were then kept in bottles covered in aluminium foil at 4 ºC to prevent the negative effect of light, especially direct sunlight.

Gas chromatography-flame ionization detector analysis was performed on a Shimadzu 15A gas chromatograph equipped with a split/splitless (ratio 1:30), injector (250 ºC) and a flame ionization detector (250 ºC). N_2 was used as carrier gas (1 mL/min) and the capillary column used was DB-5 (50 m × 0.2 mm, film thickness 0.32 µm). The column temperature was kept at 60 ºC for 3 min and then heated to 220 ºC with a 5 ºC/min rate and kept constant at 220 ºC for 5 min. Relative percentage amounts were calculated from peak area using a Shimadzu C-R4A chromatopac without the use of correction factors.

Analysis was performed using a Hewlett- Packard 5973 with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm). The column temperature was kept at 60 ºC for 3 min and programmed to 220 ºC at a rate of 5 ºC/min and kept constant at 220 ºC for 5 min. The flow rate of helium as carrier gas was (1 mL/min). MS were taken at 70 eV, mass range, 30 to 350 amu and scan time 2 scans/sec.

Identification of components: The compounds were identified by comparison of their mass spectra with the Wiley library or with published mass spectra[15]. The identifications were confirmed by comparison of KI from HP-5MS column with those reported in the NIST liberary and the Pherobase database[24,25]. The Kovats indices for all the components were determined according to the Van Den Dool method, using *n*-alkanes as standards[26].

Microorganisms: The bacteria included *Escherichia coli* (ATCC 1399) and *Staphylococcus aureus* (ATCC 1431) and fungi included *Candida albicans* (ATCC 5027). The microorganisms were obtained from the Research Center of Science and Industry, Tehran, Iran.

Antimicrobial tests: The antimicrobial activity of the essential oil was evaluated by a disc diffusion method using Mueller-Hinton and Sabouraud Dextrose agar respectively[27, 28]. A suspension of the tested microorganism (0.1 mL of a suspension of the tested microorganisms, containing 1.5×10^8 (CFU/mL) was spread on the solid media plates. Mueller-Hinton and Sabouraud Dextrose agar sterilized in a flask and cooled to 45-50 ºC were distributed to sterilized Petri dishes with a diameter of 9 cm (15 mL). A serial dilution of the oil was prepared in Mueller-Hinton and Sabouraud Dextrose Broth for bacteria and fungi respectively. The filter paper discs (6 mm in diameter) were individually impregnated with 15 µL of the *P. gnaphalodes* essential oil and then placed onto the agar plates, which had previously been inoculated with the tested microorganisms. The plates were inoculated with bacteria incubated at 37 ºC for 24 h and at 28 ºC for 72 h for the fungal strains. Ethanol (95 %) was used as a negative control in all the plates while ampicillin (10 mg/disc), gentamicin (10 mg/disc) for bacteria and fluconazol (20 mg/disc) were used as positive controls. The diameters of the inhibition zones were measured in millimetres.

Radical-scavenging capacity (DPPH assay) of the oil: The hydrogen atom or electron donation abilities of the essential oil were measured from the bleaching of the purple-coloured methanol solution of 2,2-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent[29]. In order to find out the effective dose of oil, different concentrations (0, 5, 10 and 20 % v/v in methanol) were added to DPPH reaction mixture and the concentration-dependent inhibition in DPPH radical scavenging capacity was recorded (Data not shown). Based on this, further experiments were carried out with 20 % (v/v) of the oil. Then, 50 µL of the essential oil in methanol were added to 5 mL of DPPH solution (0.004 % DPPH in methanol). Trolox (1.00 mM, 0.500 mM and 0.250 mM), a stable antioxidant, was used as reference. After incubation for 0.5 h at room temperature, the absorbance was read against the blank at 517 nm. The following formula was used to estimate the inhibitory effects of the oil extract in percent (I %): I % = (A blank - A sample/A blank) × 100. Where, a blank is the absorbance of the control reagent (containing all reagents except the test compound) and A sample is the absorbance of the test compound. All the assays were carried out in triplicate.

β-Carotene-linoleic acid assay of the oil: Antioxidant activity of the essential oil was determined using the β-carotene bleaching test as described by Taga *et al.*[30]. Approximately 10 mg of β-carotene (type I synthetic) was dissolved in 10 mL of chloroform. Two milliliters of the carotene-chloroform solution was pipetted into a boiling flask containing 20 mg linoleic acid and 200 mg Tween 40. Chloroform was removed using a rotary evaporator at 40 ºC for 5 min. Then, the residue was dissolved in 50 mL of distilled slowly with vigorous agitation, to form an emulsion. Five milliliters of the emulsion were added to a tube containing 0.2 mL of essential oil solution, prepared according to Choi *et al.*[31]. The absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50 ºC and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60 min. A sample containing 0.2 mL of ethanol instead of essential oils was used as control. Butylated hydroxyl toluene (BHT), a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as inhibition percentage with reference to the control after 60 min of incubation, using the following equation: AA = 100 (DRC-DRS)/DRC, where AA = antioxidant activity, DRC = degradation rate of the control = [ln(a/b)/60], DRS = degradation rate in presence of the sample = [ln(a/b)/60], a = absorbance at time 0, b = absorbance at 60 min.

RESULTS AND DISCUSSION

The eseential oil obtained from aerial parts of *P. gnaphalodes* are listed in Table-1 in which the percentage and retention indices of the components are given. At it is shown

from Table-1, analysis of the aerial parts oil of *P. gnaphalodes* resulted in the identification of 88 constituents, representing 89.0 % of the oil. The main components of the oil were myrtenol (13.2 %), citronellol (9.0 %), (E)-nuciferol (5.2%), shiromoll (5.1 %), geraniol (5.1 %) and α-pinene (5.0 %). The aerial parts of *P. gnaphalodes* oil were found to be rich in regards to oxygenated monoterpenes (48.8 %) while hydrocarbon monoterpenes (5.2 %) and sesquiterpenes (1.6 %) were the minor fractions of terpenoides. Aromatic compounds contained (17.0 %) phenolic derivatives was obtained as antioxidant agents. Previously, some new cadinene derivatives such as 1,8-oxidocadin-4-ene, 1(10),4-cadinadien-8a-ol, 4,10(14)-cadinadien-8-o1 and 4,10(14)-muuroladien-8-o1 were isolated from *P. gnaphalodes* essential oil[17]. Also, the oil of *P. gnaphalodes* from Tehran area was investigated[18]. The oil contained about 65 % monoterpenes, with α-pinene (34 %) and 1,8-cineole (12 %) as main constituenets and *ca.* 30 % sesquiterpenes, having a cadinane or bisabolane skeleton. Some compounds are observed in present research but major components are difference in three works. Pinane drivatives were also found in the oils of many *Pulicaria* species, especially α-pinene in the oils of *P. laciniata*[19] and *P. undulata*[20]. Cadinane derivatives the minor component of the oil of *P. gnaphalodes*, is also characteristic of the oils of *P. dysenterica*[15], *P. arabica*[22] and *P. glutinosa*[23].

TABLE-1
PERCENTAGE COMPOSITION OF THE AERIAL PARTS OF *Pulicaria gnaphalodes* OIL

	Compound	KI[a]	KI[b]	(% w/w)[c]
1	2-(*E*)-Hexenal	855	854	0.2
2	α-Pinene	939	936	5.0
3	2-Pentyl furan	992	988	0.1
4	Yomogi alcohol	999	996	0.2
5	*p*-Cymene	1025	1019	0.2
6	Limonen	1029	1024	0.1
7	1,8-Cineole	1031	1026	1.0
8	Santolina alcohol	1040	1033	0.1
9	γ-Terpinene	1060	1055	0.1
10	Artemisia alcohol	1084	1080	0.1
11	Linalool	1097	1096	0.6
12	Filifolone	1103	1097	0.1
13	*n*-Nonanal	1101	1099	0.2
14	*trans*-Pinocarveol	1139	1143	0.2
15	Comphor	1146	1149	0.4
16	Isopulegol	1150	1152	0.2
17	(*Z*)-Tagetone	1152	1157	0.2
18	Nerol oxide	1165	1158	0.3
19	*p*-Mentha-1,5-dien-8-ol	1179	1170	0.3
20	Borneol	1187	1169	0.6
21	Terpinen-4-ol	1193	1177	0.4
22	α-Terpineol	1189	1189	0.2
23	Myrtenol	1196	1180	13.2
24	γ-Terpineol	1195	1199	0.2
25	γ-Campholenyl formate	1202	1200	0.4
26	*trans*-Carveol	1215	1217	0.3
27	Citronellol	1227	1226	9.0
28	Thymyl methyl ether	1232	1235	0.2
29	(*Z*)-Citral	1238	1238	1.4
30	*cis*-Chrysanthenyl acetate	1250	1253	1.2
31	Geraniol	1254	1253	5.1
32	*cis*-Myrtanol	1251	1254	0.5
33	(*E*)-Citral	1268	1267	1.8
34	Citronellyl formate	1272	1275	0.2
35	3,7-Dimethyl-(E)-2,6-Octadien-1-ol	1278	1277	0.2
36	Thymol	1289	1290	1.1
37	Geranyl formate	1299	1298	0.1
38	Myrtenyl acetate	1325	1327	4.1
39	Citronellyl acetate	1351	1353	1.0
40	Neric acid	1354	1347	0.2
41	(*Z*)-Isogeranic acid	1360	1365	0.6
42	Neryl acetate	1362	1362	0.9
43	α-Copaene	1377	1377	0.1
44	Geranyl acetate	1381	1381	0.4
45	Methyl eugenol	1400	1404	0.7
46	α-Gurjunene	1412	1410	0.1
47	*p*-Cymene-7-ol acetate	1420	1423	0.2
48	Aromadendrene	1460	1461	0.3
49	Neryl isobutyrate	1490	1484	1.6
50	1-Pentadecene	1492	1490	0.5
51	α-Muurolene	1502	1499	0.1
52	γ-Cadinene	1518	1514	0.2
53	δ-Cadinene	1528	1524	0.6
54	Calamenene	1538	1543	0.2
55	*cis*-Sesquisabinene hydrate	1562	1560	1.2
56	α-Copaene-8-alpha-ol	1569	1555	0.3
57	Neryl-2-methyl butyrate	1577	1570	0.3
58	α-Hummulene epoxide II	1606	1606	0.2
59	Juneol	1610	1610	0.4
60	1-epi-Cabenol	1628	1629	0.2
61	10-epi- α-Muurolol	1642	1641	0.5
62	10-epi-Italicene-12-ol	1647	1645	0.4
63	Italicene-12-ol	1661	1662	0.7
64	Cadina-4,10(14)-dien-8- β-ol	1669	1675	0.1
65	(*Z*)-Nuciferal	1674	1675	1.3
66	Cadina-4,10(14)-dien-8- α-ol	1679	1680	0.4
67	Cadina-1(10),4-dien-8- α-ol	1689	1690	0.3
68	(*E*)-Nuciferal	1698	1701	3.4
69	(*Z*)-Nuciferal	1706	1705	0.6
70	(*Z*)- γ-Curcumen-12-ol	1713	1711	1.9
71	(*E*)-Nuciferol	1717	1715	5.2
72	(*E*)- γ-Curcumen-12-ol	1730	1733	0.9
73	(*Z*)- γ-Curcumen-12-yl formate	1748	1746	0.2
74	10-epi-Italicene-12-yl acetate	1766	1765	0.9
75	(*E*)-Nuciferyl formate	1781	1777	0.2
76	(*Z*)-Nuciferal acetate	1795	1793	0.2
77	(*Z*)- γ-Curcumen-12-yl acetate	1803	1808	1.4
78	Shiromool	1810	1810	5.1
79	Hexahydrofarnesyl acetone	1843	1835	0.1
80	10-epi-Italicen-12-yl isobutyrate	1894	1895	0.4
81	Italicene-12-yl isobutyrate	1914	1910	0.2
82	(*Z*)- γ-Curcumen-12-yl isobutyrate	1938	1934	0.6
83	(*E*)-Nuciferyl isobutyrate	1987	1975	0.1
84	10-epi-Italicen-12-yl isovalerate	1994	1895	0.1
85	(*Z*)-Nuciferyl-2-methyl butyrate	2022	2025	0.4
86	(*Z*)-Nuciferyl isovalerate	2034	2030	0.5
87	(*E*)-Nuciferyl isovalerate	2048	2075	0.9
88	(*E*)-Nuciferyl-2-methyl butyrate	2052	2080	1.9
	Group components			
	Hydrocarbons			
	Alkanes, alkenes, alkynes			0.5
	Alcohols			0.2
	Aldehyde			0.4
	Terpenoids			
	Monoterpene hydrocarbons			5.2

Compound	KI[a]	KI[b]	(% W/W)[c]
Oxygenated monoterpenes			48.8
Sesquiterpene hydrocarbons			1.6
Oxygenated sesquiterpenes			15.3
Aromatic compounds			17.0
Total identified			89.0

[a]Retention indices, as determined on nonpolar columns specially HP-5MS, [b]Retention indices obtained from NIST library, Pherobase database and other references, [c]Percentages obtained by FID peak-area normalization.

The antimicrobial activity of the oil are shown in Table-2. The oil has shown maximum zone of inhibition against *Candida albicans* (23 ± 1.2 mm) with a value higher than that of fluconazol (18.0 ± 0.3 mm). Previous studies showed that terpene alcohols are well-known antimicrobial compounds isolated from different plant species[32]. The moderate antimicrobial activity of *P. gnaphalodes* oil could be associated with terpene alcohols. In similar research, the composition of the essential oils obtained by hydrodistillation from the different parts of *P. arabica* from Tunisia at full flowering stage were analyzed by GC/MS[22]. The principal components of flowers essential oil were γ-cadinene (11.75 %), δ-cadinene (9.55 %), α-cadinol (8.59 %) while thymol (15.22 %), δ-cadinene (9.12 %), tau-cadinol (6.65 %) constituted the major components of stems essential oil. The roots were mainly composed of three constituents non identified, α-cadinol (2.9 %) and thymol (2.45 %). The essential oils were tested against *Fusarium solani, F. oxysporum, F. oxysporum, Phytophtora cactorum, Alternaria solani* and *Rhizoctonia solani*. The results obtained of antimicrobial activities showed that the essential oils could be considered as natural fungicide agents. Likewise, the composition of the volatile oil constituent from *P. odora* roots has been analyzed by GC/MS[23]. Twenty-seven components were identified, being thymol (47.83 %) and its derivative isobutyrate (30.05 %) the main constituents in the oil. Furthermore, the oil was tested against seven bacteria at different concentrations. A strong antibacterial activity was observed against *Streptococcus* C, *Bacillus cereus*, *Enterococcus faecalis* and *Pseudomonas vulgaris*. In each case. The activity of essential oil was higher than those of the standard antibiotic.

TABLE-2
ANTIMICROBIAL ACTIVITY OF *Pulicaria gnaphalodes* OIL AND ANTIBUTICS AGAINST STANDARD MICROORGANISMS

Microorganisms	Inhibition zone (mm)			
	Oil	AMP	GEN	FLU
Escherichia coli (ATCC 1399)	NI	NI	25.0 ± 0.3	ND
Staphylococcus aureus (ATCC 1431)	22 ± 0.7	NI	25.0 ± 0.2	ND
Candida albicans (ATCC 5027)	23 ± 1.2	ND	ND	18.0 ± 0.3

NI: no inhibition, ND: not determined, AMP: ampicillin, GEN: gentamycin, FLU: fluconazol, Values are means of three replications ± SD

The antioxidant properties of the *P. gnaphalodes* essential oil measured by DPPH and β-carotene bleaching assays are presented in Fig. 1. When compared to a standard antioxidant agent, *i.e.* Trolox, it was found that the oil strongly reduced the concentration of DPPH free radical (36.0 ± 1.3 %), with an efficacy higher than that of Trolox (33.0 ± 0.5 %). Also, the oil had high inhibition against BHD standard in β-carotene bleaching test (Fig. 1). This result together with the oil composition may suggest that antioxidant activities of the oils are likely attributable to its principle compounds and phenolic compounds[30]. Phenolic compounds such as thymol show potent antioxidant and DPPH radical scavenging activities[33]. The weak antioxidant activities of the non-phenolic components were also supported by other studies on essential[34].

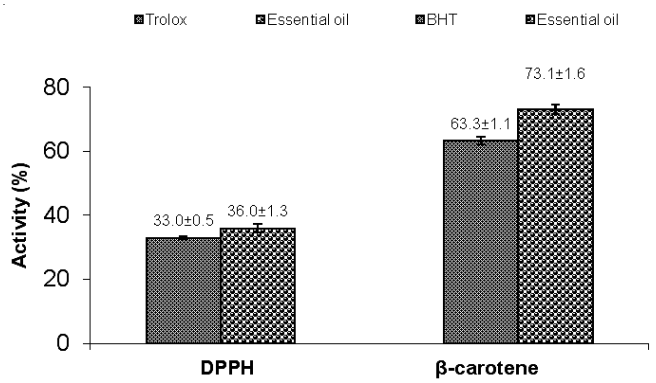

Fig. 1. Free radical-scavenging and antioxidant activities of *Pulicaria gnaphalodes* essential oil in comparison with those of the references

Conclusion

We have identified 88 constituents from *P. gnaphalodes* essential oil. The significant antimicrobial activity of *P. gnaphalodes* oil could be associated with main components especially alcohol terpenes. Also, strong antioxidant activities of the oil may be due to the presence of phenolic compounds such as thymol used for traditional applications. Therefore, *P. gnaphalodes* can be used as medicinal plant in traditional applications because it has compounds with potent antimicrobial and antioxidant activities.

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