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Chemical Composition and Antimicrobial Activity of the Essential Oil of *Pimpinella puberula* (DC.) Boiss.

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ABSTRACT

In this study, the aerial parts of *Pimpinella puberula* were collected from Ramhormoz and Mashhad (Khuzestan and Khorasan Provinces) at the vegetative, flowering and seeding stages. Essential oils from the whole aerial parts as well as stem/leaf, inflorescence, unripe and ripe seeds, were isolated by hydro-distillation. The yields of essential oil obtained from the Ramhormoz samples were 0.49%, 0.31%, 3.81%, 6.01% and 1.80% w/w, and from those from Mashhad were 0.96%, 0.87%, 3.59%, 6.94% and 4.96% w/w, respectively. The oils from different parts of plant were also analyzed by GC and GC/MS. Limonene was the major constituent in all the oils (21.7%-82.4%), followed by pregeijerene (14.6%-55.4%) and geijerene (7.2%- 11.7%). Methyl eugenol and elemicine, however were only found in the Ramhormoz oil samples. The antimicrobial activity of the oils was determined using the disk diffusion method against Gram positive bacteria (*Bacillus subtilis, Bacillus cereus, Micrococcus luteus* and *Staphylococcus aureus*), Gram negative bacteria (*Yersinia entrocolitica, Klebsiella oxytoca, Serratia marcescens, Escherichia coli and Pseudomonas aeruginosa)* and yeast (*Candida albicans*). Results showed a significant difference between Gram positive and Gram negative bacteria in their susceptibility to the oil, although Gram positive bacteria were more susceptible to the antimicrobial activity of *P. puberula* oil. In addition, the antimicrobial activity of samples collected from Ramhormoz were more than of those from Mashhad.

Key words: Antimicrobial activity, Essential oils, Limonene, *Pimpinella puberula,* Pregeijerene.

INTRODUCTION

The increasing prevalence of antibiotic-resistant bacteria has led to a demand for new agents that could be used to decrease the prevalence of bacterial disease (Lima *et al.,* 2005). There is evidence that essential oils extracted from plants could be employed as antimicrobial agents in food systems. Recently, screening for new plants with antibacterial activity has been the subject of many investigations since their essential oil with its antibacterial activity could be a promising agent (Dorman and Deans, 2000)

The genus of *Pimpinella* with 23 wild species has been found in different regions of Iran, Anatolia (austro-orientalis), Jordan, Iraq, Turkey, Afghanistan and Pakistan. *P. puberula* (DC.) Boiss. grows in deserts and steppes, stony slopes and river beds. It is an annual and erect aromatic plant, about 65 cm in length, with numerous umbellae, white inflorescence and globoso-ovoideus fruits (Mozaffarian, 1996; Rechinger, 1972).

The composition of the oil of *P. anisum, P. eriocarpa, P. aurea, P. tragium, P. affinis* and *P. tragioides* has been reported, previously (Askari et al., 1998; Askari et al., 2005 a; Askari et al., 2005; Askari and Sefidkon, 2005 b; Askari and Sefidkon, 2006; Askari and Sefidkon, 2007). However, to the best of our knowledge

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no biological assays of *P. puberula* have been performed. It was decided to investigate the antibacterial activity of the essential oil of *P. puberula* against some bacterial species and fungi.

The anis root oil was characterized by a high content of β-bisabolene (52.46%) and pregeijerene (12.78%). Main constituents of the root oil of *Pimpinella peregrina* were epoxy-pseudoisoeugenyl 2-methylbutyrate (29.67%), β-sesquiphelandrene (19.83%), epoxy-pseudoisoeugenyl 2-methylpropionate (11.84%), pregeijerene (11.01%) and β-bisabolene (10.00%). The root oil of *P. major* mainly contained epoxy-pseudoisoeugenyl tiglate (56.53%) and pregeijerene (10.36%). Main constituents of the root oil of *Pimpinella saxifrage* were epoxy-pseudoisoeugenyl 2-methylbutyrate (46.24%), pregeijerene (9.18%) and germacrene B (5.44%) (Kubeczka *et al*., 1986).

The root oil of *Pimpinella major* had also reported by Bohn *et al*. from two habitats near Wurzburg (Germany) and near Riva Del Garda (Italy). The main component of both root oils was trans-epoxyseudoisoeugenyl tiglate (19.54% and 37.34% respectively). The other main components of the root oil from the German sample were δ-elemene (12.05%), Pregeijerene (9.75%), n-octanal (7.94%) and germacrene C (7.83%); while those of sample from Italy were germacrone 915.16%) and γ-elemene (9.79%) (Bohn *et al*., 1989).

The aim of this study was to determine essential oil compositions of different parts of *Pimpinella puberula* and their antimicrobial activities.

MATERIALS AND METHODS

Plant Materials

Plant materials were collected from Ramhormoz (Khuzestan Province, South of Iran) and Mashhad (Khorasan Province, North East of Iran) in the vegetative (begining of June) flowering (middle of June) and seed stages (middle of July to late September 2005). Seeds were collected in two stages: unripe seeds were collected as soon as their inflorescence formation, and ripe seeds were collected after their color changed to brown. All plant materials/sections were dried at room temperature. The herbarium specimen of Ramhormoz (No. 72144) and the Mashhad sample (No. 88419) have been deposited in the Herbarium of Research Institute of Forests and Rangelands (TARI).

Oil Isolation

Essential oils were separately extracted by hydro-distillation from the aerial parts (vegetative stage), stem/leaf and inflorescence (flowering stage) and unripe and ripe seeds (seeding stage). The dried parts of the plants were crushed to small particles. The samples were then hydrodistilled for 2 to 2.5 hours in a Clevenger type apparatus to obtain the oils. Three distillations were performed for each oil and then for all samples the replications were pooled for analysis. The oils were dehydrated over anhydrous sodium sulfate and stored in sealed vials at 4°C before analysis.

GC Analysis

The oils were analyzed using a Shimadzu GC-9A gas chromatograph equipped with a DB-5 fused silica column (30 m×0.25 mm, film thickness 0.25 μm, J and W Scientific Corporation). Oven temperature was 40°C for 5 minutes and then set to 260°C at a rate of 4°C min^{-1}. The injector and detector (FID) temperature were 270°C; helium was used as carrier gas with a linear velocity of 32 cm s^{-1}. The percentages were calculated using the area normalization method without the use of response factor correction. The retention indices were calculated for all

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compounds using a homologous series of n-alkanes.

GC-MS Analysis

GC/MS analyses were carried out on a Varian 3400 GC/MS system equipped with a DB-5 fused silica column (30 m×0.25mm, film thickness 0.25 μm, J and W Scientific Corporation); oven temperature was 50°-260 °C at a rate of 4°C min^{-1}. The transfer line temperature was 270°C, carrier gas helium with a linear velocity of 31.5 cm s^{-1}, split ratio 1/60, ionization energy 70 ev, scan time 1 sec, mass range 40-300 amu.

Identification of Compounds

The constituents were identified by comparison of their mass spectra with those in a computer library (LIBR-TR and Wiley-5 lib.) or with authentic compounds. The identifications were confirmed by comparison of their Retention indices, either with those of authentic compounds or with data in the literature (Adams, 1995).

Antibacterial Analysis

The antimicrobial activity of *Pimpinella puberula* was determined against five Gram negative bacteria, four Gram positive bacteria and yeast. Microorganisms included *Bacillus cereus* (PTCC 1247), *Bacillus subtilis* (PTCC 1023), *Micrococcus luteus* (PTCC 1169), *Staphylococcus aureus* (PTCC 1431), Yersinia *enterocolitica* (PTCC 1151), *Pseudomonas aeruginosa* (PTCC 1430), *Escherichia coli* (PTCC 1399), *Klebsiella pneumonia* (PTCC 1053), *Klebsiella oxytoca* (PTCC 1402) and *Serratia marcescens* (PTCC 1187) and *Candida albicans* (5027). These were obtained from the microbial collection of the Department of Biotechnology of the Iran Research Organization of Science and Technology (IROST) in Tehran, Iran.

The antibacterial activity was determined using disk diffusion method (European Pharmacopia) and the bacteria were cultivated on Triptic Soy Agar medium (Merck, Germany). The bacteria were suspended in a Tryptocase Soy Broth medium (Merck, Germany) with reference to the value 1 MacFarland standard 0.5 ml of standardized inoclua were placed on the surface of media and distributed uniformly. Oils were diluted with ethanol (1:5). Sterile paper disks (diameter 6 mm prepared from Whatmann number 42) were impregnated with 20 μl of diluted essential oil and placed on the surface of each inoculated plate and incubated for 24 hours at 37ºC. Tetracycline (30 μg) and gentamicin (10 μg) disks were used to compare antibacterial activity of essential oils. The zone of inhibition was measured after 24 hours' incubation.

RESULTS AND DISCUSSION

The yields of the oils from the aerial parts [AP], stem/leaf [S and L], inflorescence [IF], unripe [US] and ripe seeds [RS] of *P. puberula* from both samples have been given in Figure 1. It was proved that oil yields of generative parts (especially unripe seeds) were more than those of vegetative

Table1. Percentage of volatile oils (w/w) in different parts of *Pimpinella Puberula*.

	Arial parts (%)				
Localities	AP	SL	IF	US	RS
Ramhormoz samples	0.49	0.31	3.81	6.01	1.80
Mashhad samples	0.96	0.87	3.59	6.94	4.96

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Table2. Percentage of volatile compounds identified in the oils of *Pimpinella Puberula* (DC.) Boiss.

	Ramhormoz						Mashhad				
Compounds	RI [a]	AP [b]	SL [c]	IF [d]	US [e]	RS [f]	AP	SL	IF	US	RS
α-pinene	934	-	0.3	-	-	-	-	-	-	-	-
Sabinene	974	0.4	0.5	0.5	1.4	1.3	1.1	1.5	0.6	3.7	3.7
Myrcene	988	-	0.2	0.3	0.5	0.5	0.1	-	0.3	0.5	0.5
Limonene	1028	21.7	46.6	58.9	78.8	82.4	25.3	33.8	60.8	74.1	80.6
1,8-cineole	1031	-	0.6	-	-	1.0	-	-	-	-	-
Geijerene	1141	10.4	8.5	1.0	-	0.5	11.7	7.2	3.0	0.7	0.4
Isogeijerene C	1247	0.4	0.3	-	-	-	-	-	-	-	-
(E)-anethole	1283	0.8	-	-	-	-	-	-	-	-	-
Pregeijerene	1285	55.4	14.6	1.4	1.3	1.0	45.8	38.8	18.1	3.5	1.5
Cis-dictamnol	1379	2.1	1.8	-	-	-	1.7	1.3	0.5	-	-
Methyl eugenol	1401	-	6.4	4.6	1.0	-	5.4	12.0	10.5	16.3	13.1
β-caryophyllene	1418	0.9	0.7	0.3	-	-	1.4	0.6	0.4	-	-
Trans-dictamnol	1427	3.2	3.2	0.5	-	0.7	1.0	2.2	1.4	-	-
Bicyclogermacrene	1497	-	0.3	0.4	-	-	-	-	0.9	-	-
Elemicine	1554	1.0	14.0	27.3	13.4	7.0	-	-	-	-	-
Cubenol	1645	-	-	-	0.6	-	-	-	-	-	-
Total identified		96.3	98.0	95.2	97.0	94.4	93.5	97.4	96.5	98.8	99.8

[a] Retention indices calculated on DB-5 column; [b] Aerial parts; [c] Stems plus the leaves; [d] Inflorescence; [e] Unripe seed; [f] Ripe seed
T= Traces= Less than 0.05%.

Table 3. Antimicrobial activity of the oils of *Pimpinella puberula* obtained from Mashhad and Ramhormoz (diameter of growth zone inhibition, mm).

Microorganism	Mashhad Sample					Ramhormorz Sample						
	AP[a]	SL[b]	IF[c]	US[d]	RS[e]	AP	SL	IF	US	RS	TET[f]	GEN[g]
Pseudomonas aeroginosa	10	7	9.5	0	0	10.5	7.5	6.5	8.5	9.5	nt	14
Klebsiella oxytoca	0	0	0	0	0	0	0	0	0	0	nt	18.5
Serratia maecescens	0	0	0	0	0	0	0	0	0	0	nt	27.5
Escherichia coli	0	0	0	0	0	0	0	0	0	0	15	nt
Yercinia enterocolitica	7	8	0	10	10	0	11	0	0	0	nt	15
Bacillus cereus	19	17.5	12.5	12.5	10	32.5	27.5	25.0	27.5	18.5	36	nt
Micrococcus luteus	10	8	12.5	9	0	8.5	9.5	10	10.5	9.5	35	nt
Bacillus subtilis	0	0	0	0	0	0	9	10	0	8	22.5	nt
Staphylococcus aureus	12	8.5	11.5	8.5	3	18	13.5	11	10	11	30	nt
Candida albicans	14	13	12	15	8	15	10	0	0	0	nt	nt

[a] Aerial parts; [b] Stems plus the leaves; [c] Inflorescence; [d] Unripe seed; [e] Ripe seed [f] *Tetracycline,* [g] *Gentamicine.*
nt= Not Tested.

parts. As shown in Figure 1, for all plant parts (except IF) the yields of essential oil of the Mashhad samples were higher than those for the Ramhormoz samples.

The oils of [AP], [S and L] and [IF] were green to dark green and the oils of [US] and [RS] were pale and light yellow in color.

The oil yields of the inflorescence and seeds of *P. puberula* were considerable. Yield of *P. anisum* seed oil has been reported as 3.3% w/w by steam distillation and 3.13-10.67% by supercritical extraction (Askari *et al*., 1998; Rodrigues *et al*., 2003). Yield of seed oil of *p. eriocarpa* was 5.7%, *P. squamosa* was 4.6-7.0%, *P. serbica* was 2.02-3.25% and *P. diversifolia* was 0.3-0.85% (Askari *et al*., 2005; Mekhtieva, 1998; Ivanic *et al*., 1983; Ashraf *et al*., 1979; Melkani *et al*., 1990).

Yields of inflorescence and seed oils of many species of *Pimpinella* have been reported as followeds: *P. aurea* (1.54% and 1.97%) (Askari *et al*., 2005), *P. tragium* (0.37% and 1.33%) (Askari and Sefidkon, 2005), *P. tragioides* (0.79% and 2.49%) (Askari and Sefidkon, 2006) and *P. affinis* (1.74-1.98% and 4.05-5.33%) (Askari and Sefidkon, 2007).

The oils from different parts of *P. puberula* were analyzed by GC and GC/MS. 10, 14, 10, 7 and 8 constituents were identified in the [AP], [S and L], [IF], [US] and [RS] oils *P. puberula* from the Ramhormoz samples and 9, 8, 10, 6 and 6 constituents were identified Mashhad sample oils. Three constituents, including limonene and pregeijerene as two major compounds, were common in all the oils. Limonene was the major constituent in AP oil (21.7% and 25.3), S and L oil (46.6% and 33.8%), IF oil (58.9% and 60.8%), US oil (78.8% and 74.1%) and RS oil (82.4% and 80.6%) from the Ramhormoz and Mashhad samples, respectively, and so that limonene content increased at the growth stages. Pregeijerene and geijerene were the major constituents in the aerial parts and stem plus the leaf oils. Pregeijeren was 55.4% and 14.6% in Ramhormoz oil and 45.8% and 38.8% in Mashhad oil. Geijerene was 10.4% and 8.5% in the Ramhormoz samples and 11.7% and 7.2% in the Mashhad samples.

The other major component was methyl eugenol. Elemicine was found only in Ramhormoz samples (Table 2) while pregeijerene was found in other species of *Pimpinella*. Major constituents of the aerial parts of *P. eriocarpa* oil are: pregeijerene (59.9%), followed by Limonene (17.6%) and Elemicine (12.5%) (Askari *et al*., 2005; Askari and Sefidkon, 2007).

Pregeijerene exists in the root oil of *Pimpinella alpine* (28.1%), *P. anagodendron* (2.9%), *P. anisum* (16.4%), *P. anisoides* (48.3%), *P. cumbrae* (35.4%), *P. junionae* (46%), *P. major* (25.4%), *P. nigra* (28.3%), *P. peregrina* (14.4%), *P. saxifrage* (7.4%) and *P. tragium* (35.1%) (Kubeczka and Ullmann, 1980).

Table 3 shows the results obtained in the determining inhibitory activity of *Pimpinella puberula* essential oils on the growth of bacteria and *Candida albicans* that potentially cause infections. The control disk with ethanol showed no activity at all. Our data showed that there was no uniform response among tested bacteria. The Results showed significant difference between Gram positive and Gram negative bacteria in their susceptibility so that Gram positive bacteria were more susceptible to antimicrobial activity of this genus. The higher sensitivity of Gram positive bacteria may be explained according to their cell wall structure. In addition, differences in susceptibility among the microorganisms to the antimicrobial activity of essential oils may be explained by inherited genes on plasmids. The antimicrobial activity of AP essential oil was more than that of other parts, possibly because of the high percentage of pregeijerene. In addition, the antimicrobial activity of samples collected from Ramhormoz was more than in the Mashhad ones. The results may suggest that *Pimpinella puberula* essential oils possess compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for treatment of infectious diseases. Moreover, the results of this study

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show the need for futher research addressed at the evaluation of the antimicrobial properties of several phytochemicals, in particular pregeijerene.

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بررسی ترکیبهای شیمیایی و اثرات ضدمیکروبی اسانس *Pimpinella puberula* (DC.) Boiss.

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چکیده

اندام های هوایی گیاه *Pimpinella puberula* در مراحل رویشی، گلدهی و بذردهی از رویشگاه طبیعی آنها در رامهرمز و مشهد (استانهای خوزستان و خراسان) جمع‌آوری شدند. اسانس اندام هوایی، ساقه و برگ، گل‌آذین، بذرنارس و بذررسیده بطور جداگانه به روش تقطیر با آب تهیه شد. بازده اسانس نمونه های رامهرمز برای اندامهای فوق عبارت از: ۰/۴۹٪، ۰/۳۱٪، ۳/۸۱٪، ۶/۰۱٪ و ۱/۸۰٪ درصدوزنی بودند. به همان ترتیب بازده اسانس نمونه های مشهد عبارت از: ۰/۹۶٪، ۰/۸۷٪، ۳/۵۹٪، ۶/۹۴٪ و ۴/۹۶٪ درصدوزنی بودند. ترکیبهای تشکیل دهنده اسانس ها با استفاده از کروماتوگرافی گازی تجزیه ای (Analytical GC) و گازکروماتوگراف متصل به طیف سنج جرمی(GC/MS) و محاسبه اندیس های بازداری شناسایی شدند. در تمام نمونه‌ها مهمترین ترکیب لیمونن (۸۲/۴٪-۲۱/۷٪) بود. دیگر ترکیب های مهم در اسانس اندام هوایی و ساقه و برگ پری‌گایجرن (۵۵/۴٪-۱۴/۶٪) و گایجرن (۱۱/۷٪-۷/۲٪) بودند. متیل‌اوژنول و المیسین از دیگر ترکیب های مهمی بودند که تنها در اسانس نمونه های رامهرمز یافت شدند. اثرات ضد میکروبی اسانس‌ها با روش Disk diffusion methodبرروی باکتری های گرم مثبت (*Bacillus subtilis*، *Bacillus cereus*، *Micrococcus luteus* و *Staphylococcus aureus*) و گرم منفی (*Serratia marcescens, Escherichia coli, Kelebsiella oxytoca Yersinia entrocolitica* و *Pseudomonas aeroginosa*و مخمر (*Candida albicans*) بررسی شد. نتایج نشان داد که اختلاف قابل ملاحظه‌ای از نظر حساسیت به اسانس بین باکتری‌های گرم مثبت و باکتری‌های گرم منفی وجود ندارد. باکتری‌های گرم مثبت به اثرات ضد میکروبی اسانس *P. puberula* حساس‌تر بودند. بعلاوه اثرات ضد میکروبی اسانس نمونه های رامهرمز بیشتر از نمونه های مشهد بود.

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