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Compositions and the *in vitro* antimicrobial activities of the essential oils and extracts of two *Achillea* species from Iran

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Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis of the isolated essential oils obtained by steam distillation from the flowers, leaves, and stems of two plants, known to have medicinal activity, *Achillea pachycephala* Rech.f. and *Achillea santolina* L., collected from Khorasan, Northeast of Iran, as well as constituents obtained by solvent (hexane-ether and methanol) extractions of the aerial parts, resulted in the identification of 46 to 60 constituents (95.4 to 98.8% of the total oil and extracts) and 48 to 59 constituents (95.9 to 98.0% of the total oil and extracts), respectively. The hydrodistilled oil of three parts and the solvent extracts of *A. pachycephala* contain camphene, sabinene, 1, 8-cineole, camphor, borneol, terpinen-4-ol and β-caryophyllene as major constituents. In essential oils and extracts of *A. santolina,* 1, 8-cineole, camphor, terpinene-4-ol, fragranol, fragranyl acetate, α-terpinyl acetate, caryophyllene oxide, α-muurolol and some alkanes, alkanoic acids and esters were principle components. *In vitro* antimicrobial activity of essential oil of three parts and crude extracts (hexane-ether and methanol extract) of *A. pachycephala* Rech.f. and *A. santolina* L. were investigated by disc diffusion method and the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination. The studied samples were active against gram positive and gram negative bacteria. The maximum antimicrobial activities of both plants were shown by the essential oils and the hexane-ether extracts, as compared to methanolic extracts. Both oils and extracts exhibited higher activities against the gram negative tested bacterial strains.

Key words: *Achillea,* essential oil, solvent extract, antimicrobial activity.

INTRODUCTION

The genus *Achillea* L. (Asteraceae) is represented by about 115 species found in the Northern Hemisphere, mostly in Europe and Asia, and commonly known as yarrows (Radulovic et al., 2010; Benedek et al., 2008; Nemeth and Bernath, 2008). It has been represented in Iran by nineteen species including seven endemics (Mozaffarian, 2007). The *Achillea* L. species belong to the oldest medicinal plants that are used both for pharmaceutical purposes and in folk medicine .

These plants contain a complex of different

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pharmacological compounds, for example, terpenes, flavonoids, alkaloids, bitters, tannins, lignans, etc. (Aburjai and Hudaib, 2006). *Achillea* species are diuretic, emmenagogue agents, used for healing wound, curing stomachache, diarrhea and antichloristic antispasmodic, antiseptic and infection preventing properties, and have also been used to reduce sweating and to stop bleeding, amarum, stomachicum, cholagolum and carminativum (Nemeth, and Bernath, 2008; Toncer et al., 2010; DerMarderosian, 2001; Yesilada et al., 1993; Baytop, 1999; Konyalioglu and Karamenderes, 2005; Hubik, 1978; Kastner et al., 1993; Si et al., 2006).

The *Achillea* genus has a wide distributional range (Celik and Akpulat, 2008), and the differences in oil composition may be affected by different environmental factors such as plant genetic type, seasonality, and developmental stage, because it is a chemically polymorphic and perennial plant (Bezic et al., 2003). Terpenoids (1,8-cineole, camphor, borneol, pinenes, artemisia ketone, santolina alcohol, farnesane, caryophyllene and its oxides, cubebene, germacrenes, eudesmol, α-bisabolol and oxides, farnesene, γ-gurjunene, γ-muurolene and chamazulene) are the principle components of *Achillea* essential oils (Nemeth and Bernath, 2008; Si et al., 2006).

According to Nemeth's (2005) studies, within the last 15 years, an average of 54 compounds have been identified in samples of different species. Among them, the largest numbers of components (149 compounds) were found in the oils of some *Achillea* species. 1, 8-Cineole, camphor, borneol, α- and β- pinenes are among the five most abundant monoterpene components. Among the monoterpenes, 1, 8-cineole is a major component in *Achillea* species (Toker et al., 2003a; Unlu et al., 2002; Kowalczuk et al., 1998). In some *Achillea* species, essential oil components (camphor and borneol) are next ranks (Magiatis et al., 2002; Feizbakhs et al., 2003; Zen et al., 2003; Rustaiyan et al., 1998). Other most detected components are α- and β- pinenes, especially in the group *Millefolium* (Kalinkina et al., 2000). Hofmann (1993) also reported that the monoterpenes belonging to the p-menthane, thujane and pinane are the most frequent components of the oils of the *Achillea millefolium* populations.

Sesquiterpenes such as chamazulene, β-caryophyllene and oxide, eudesmol, α-bisabolol as well as its oxides and farnesene, are the most frequently constituents (Nemeth, 2005). Some researchers have reported the major constituent of several *Achillea* species as 1,8-cineole, camphor, piperitone and ascaridole in Turkey (Kusmenoglu et al., 1995; Ozen et al., 2003; Toker et al., 2003b; Baris et al. , 2006; Kordali et al., 2009).

Chemical composition of the essential oils of five *Achillea* species from Turkey has already been investigated (Toncer, 2010). The essential oils and hexane extracts of *Achillea frarantissima* and *Achillea santolina* from Egypt have been reported (El-Shazly et al., 2004).

Antimicrobial, antioxidant, antitumor, spasmolytic, antiinflammatory, antidiabetic, antiulcer, choleretic and hepatoprotective activity, and cytotoxic effects of different *Achillea* species have been previously reported (Benedek et al., 2008; Kupeli-Akkol et al., 2009; Demirci et al., 2009; Konyalioglu and Karamenderes, 2005; Iscan et al., 2006; Karamenderes and Apaydin, 2003). Essential oil composition of five *Achillea biebersteinii* from central Turkey, their antifungal, and insecticidal activity had also been investigated (Tabanca et al., 2011) .

Composition at different development stages of the essential oil of four *Achillea* species grown in Iran were established by gas chromatography/mass spectrometry (GC/MS) and gas chromatography (GC). 1, 8-Cineole (27 to 41%), α-thujone and camphor were the main compounds in the oils (Azizi et al., 2010).

There are a few reports about essential oil composition of *Achillea santolina* compared with another *Achillea* genus (Afsharypuor et al., 1996; Khafagy and El-Fatatry, 1970). Antimicrobial activity of essential oils extracted from Thai herbs and spices were reported (Wannissorn et al., 2009). Thirty-nine extracts obtained from flower heads of 13 *Achillea* species were evaluated for antimicrobial activity against nine bacteria strains and determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by NCCLS (2003) (Karaalp et al., 2009).

The chemical composition of the essential oils of *Achillea holosericea*, *Achillea taygetea*, and *Achillea fraasii* were determined by GC/MS analysis. The *in vitro* antimicrobial activity of these essential oils was evaluated against six bacteria indicating that a strain is totally inactive, while the other two strains possess moderate to strong activities mainly against the gram negative strains. The essential oil of *A. fraasii* was also active against pathogenic fungi (Magiatis et al., 2002). Potential activity of the *Achillea wilhelmsii* leaves on bacteria and effects of the leaves essential oil and methanol extract of the *A. wilhelmsii* on the growth of the bacteria were investigated (Amjad et al., 2011).

The compositions of essential oils of 19 accessions belonging to six different *Achillea* species, transferred from the natural habitats in ten provinces of Iran to the field conditions, were assessed. The relationship between the leaf areas of selected accessions with their essential oil content was also investigated. Essential oil yield of dried plants obtained by hydrodistillation ranged from 0.1 to 2.7% in leaves (Rahimmalek et al., 2009a).

The immunosuppressive activity of *Achillea talagonica* on humoral immune responses in experimental animals was studied by Rezaipoor (1999). Chloroform extract of *A. ageratum* has shown anti-inflammatory activity on chronic and acute inflammation models and has also shown a high degree of inhibition of the Hep-2 and McCoy cells compared with 6-mercaptopurine (Gomez et al., 1999, 2001). The aqueous and methanolic extracts of

A. ageratum have exhibited analgesic and anti-inflammatory activity (Garcia et al., 1997). Hydroalcoholic extract of *A. wilhelmsii* which is wildly grown in different parts of Iran has shown antihypertensive and antihyperlipidemic activity in human (Asgary et al., 2000). The composition of the volatile oil of *A. wilhelmsii* grown in Kerman (Afsharypour et al., 1996), and the composition of the essential oil of *A. wilhelmsii* collected from Kazeroon in Fars province have also been reported (Javidnia et al., 2004).

The *in vitro* antimicrobial and antioxidant activities of the essential oil and methanol extracts of *A. millefolium* ssp. millefolium Afan. were investigated (Candan et al., 2003).

The essential oil, obtained by Clevenger distillation and water-soluble and water-insoluble parts of the methanol extracts of *Achillea sintenisii* Hub. Mor. was individually assayed for their antimicrobial activities against 12 bacteria and two yeasts. No activity was exhibited by the water-soluble subfraction, whereas both the water-insoluble subfraction of the methanol extracts and the essential oil were found to be active against some test microorganisms studied (Sokmen et al., 2003). The essential oil and methanol extracts from *A. biebersteinii* Afan. were evaluated for their antimicrobial and antioxidant activities *in vitro*. The oil showed stronger antimicrobial activity than the extracts (Sokmen et al., 2004). Previous studies reported that the major constituents of *A. tenuifolia* were γ–muurolene (Jaimand and Rezaee, 2001), limonene (Shafaghat, 2009) and camphor (Rustaiyan et al., 1998, 1999; Aghjani et al., 2000).

In the present study, the flowers, leaves, and stems of the endemic *Achillea pachycephala* Rech.f. and *Achillea santolina* L., collected from Northeast of Iran, were investigated for their essential oil compositions, as well as hexane-ether and methanolic extracts and some biological activities. The essential oils were analyzed by both gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS). The essential oils were evaluated *in vitro* for their antimicrobial activities. As far as we know, this is the first report on the essential oils of *A. pachycephala* antimicrobial activities, and also, composition and antimicrobial activities of its extracts.

MATERIALS AND METHODS

Plant material

Two *Achillea* samples were collected from Khorasan-Razavi Province, Iran, in June 2011. *A. pachycephala* Rech.f. was collected from Sakhdar (latitude +36°6'49.12"N, longitude +59°5'7.66"E) in Neyshabur, and *A. santolina* L. from Jannatabad (latitude +35°35'56.30"N, longitude +61°8'25.97"E) in Torbat Jam. The plants were air dried and dried samples were crushed, then essential oils were obtained by hydrodistillation of their flowers, leaves, and stems, separately. Aerial parts of the two samples for extracts were dried and crushed. Voucher specimens of the plant were deposited in the herbarium.

Sample preparation

The flowers, leaves and stems of *A. pachycephala* Rech.f. and *A. santolina* L. were separately subjected to hydrodistillation for 3.5 to 4.0 h using an original Clevenger-type apparatus and yielded essential oils from 0.1 to 0.8% (v/w) and 0.15 to 0.7% (v/w) of dry matters, respectively. After decanting, the obtained essential oils were dried over anhydrous sodium sulfate and after filtration, stored in refrigerator at - 4°C until tested and analyzed.

The aerial parts of two plants (about 50 g) were shaken sequentially in percolation with hexane-ether (1:1, v/v) and methanol for 16 h (15 ml/g for each) at room temperature. Samples were sonicated separately for 15 min twice, and then solvents were removed subsequently under reduced pressure by rotary evaporator apparatus. The extracts were weighed and stored in refrigerator at +4°C until tested; the yield was 0.9 and 1.7% for *A. pachycephala* Rech.f., 1.2 and 1.8% for *A.santolina* L. respectively (Table 1).

Analysis of the essential oils and extracts

Gas chromatography

Samples of the oils and extracts were diluted in acetone (1:9) and 1 µl was used for analysis. GC-MS analyses of the essential oil and extracts was analyzed on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091) 413:325°C HP-5 column (30 m x 320 µm x 0.25 µm) was used with helium as carrier gas at a flow rate of 3.35 ml/min. The GC oven temperature was initially programmed at 50°C (hold for 1 min) and finally at 300°C (hold for 5 min) at a rate of 80°C/min while the trial temperature was 37.25°C.

The column heater was set at 250°C in a split less mode while the pressure was 10.2 psi with an average velocity of 66.5 cm/s and a hold-up time of 0.75 min. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID), set at 250°C.

Gas chromatography-mass spectrometry

The essential oils and extracts were analyzed by GC-MS on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091) 413:325°C HP-5 column (30 m × 320 µm × 0.25 µm) was used with helium as carrier gas at a flow rate of 3.35 ml/min. GC oven temperature and conditions were as afore described. The injector temperature was at 250°C. Mass spectra were recorded at 70 eV. Mass range was from 30 to 500 m/z.

Identification of components

Identification of components was based on their retention indices determined by reference to a homologous series of n-alkenes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Adams, 2007), and stored on the MS library (NIST 08.L database/ chemstation data system) with data previously reported in literature (McLafferty and Stauffer, 1989; Joulain and Konig, 1998).

The percentages of each component are reported as raw percentages based on total ion current without standardization. The chemical compositions of any part of the two *Achillea* are summarized in Table 2.

Table 1. Weight of plants, time and yield percent.

Parameter	*Achillea pachycephala Rech.*			*Achillea santolina L.*		
	Weight (g)	Time (h)	Yield (%)	Weight (g)	Time (h)	Yield%
Flower	105	3.5	0.8	110	4.0	0.7
Leaves	98	3.5	0.2	125	4.0	0.25
Stems	82	3.5	0.1	107	4.0	0.15
Hexane-ether extract	50	16.0	0.9	50	16.0	1.2
Methanolic extract	50	16.0	1.7	50	16.0	1.8

Antimicrobial assay of the oils and extracts

In vitro antibacterial assay of the oils were carried out according to disc agar diffusion method (Jirovets, 1999; Kumar, 2004). Antibacterial activity of the oils and extracts were tested against gram positive bacterial strains such as *Bacillus cereus* (MTCC430), *Bacillus subtilis* (MTCC441), *Staphylococcus aureus* subsp. *Aureus* (MTCC2940); and gram negative bacterial strains such as *Klebsiella pneumonia* (MTCC109), *Escherichia coli* (MTCC443), *Proteus vulgaris* (MTCC426) and *Salmonella typhi* (MTCC733), were grown in nutrient broth for 24 h (pH 7.2 to 7.4), and were used as inoculums. Mueller Hinton agar was used as the bacteriological medium. The Mueller-Hinton agar medium were poured into the plates to uniform depth (in mm) and allowed to solidify. The oils and extracts were diluted in 5% dimethylsulphoxide (DMSO). Then the microbial suspensions were streaked over the surface of media using a sterile cotton swab to ensure the confluent growth of the organism. Aliquots of 10 µl of the oil and extract at 1:2 dilutions in dimethyl sulfoxide (DMSO) were impregnated on Whatman No. 1 filter paper discs of 6 mmdiameter. These discs were aseptically applied to the surface of the agar plates at well-spaced intervals. The plates were incubated at 37°C for 24 h and observed inhibition zones including the diameter of the discs were measured. Two controls discs were also included in the test, the first was involving the presence of microorganisms without test material and the second was a standard antibiotics: streptomycin was used to control the sensitivity of the tested bacteria. Control discs impregnated with 10 µl of the solvent DMSO and streptomycin (10 µl /disc) reference for bacteria were used alongside the test discs in each experiment. The results are presented in Table 3.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for the extracts that showed total growth inhibition using the afore described method. Extract concentrations of 0.39 to 50.00 mg/ml were evaluated (Table 4). The MIC value was defined as the lowest concentration producing no visible growth (absence of turbidity and or precipitation) as observed through the naked eye and the concentration at which there was no bacterial growth after inoculation in Müeller-Hinton agar was taken as the MBC (AL.Janabi, 2011) . The experiments were performed in triplicates and repeated twice. Streptomycin was used as positive controls whilst 5% DMSO-broth mixture was used as the negative control. All the results were recorded as the mean concentration of triplicate. The standards were selected based on their availability and their presence in the extracts studied.

RESULTS AND DISCUSSION

Essential oils and extracts composition

Essential oils were obtained from the crushed flowers, leaves, and stems, by hydrodistillating separately; the hexane-ether and methanol extracts were obtained from aerial parts of *A. pachycephala* and *A. santolina,* which were immediately analyzed by both gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) system.

A. pachycephala

Fifty three compounds (98.2%) were identified in the flower's oil, whereas 47 (98.8%) and 46 (97.2%) components were characterized in the oils obtained from leaves and stems, respectively (Table 2). Camphene, sabinene, 1, 8-cineole, camphor, borneol, terpinen-4-ol and β-caryophyllene were the main constituents in the investigated parts. The principle components in the hexane-ether extract of the total aerial parts were p-cymene, 1, 8-cineole, camphor, terpinen-4-ol, fragranyl acetate, β-caryophyllene, caryophyllene oxide, C20 and C21. In methanolic extract, β-phellandrene, 1, 8-cineole, linalool, camphor, borneol, terpinen-4-ol, Z-carveol, bornyl acetate, thymol, fragranyl acetate, eugenol, methyl eogenol were the major components. 1, 8-Cineole was the most abundant constituent in five samples and all samples had this component in different amounts (16.4, 19.0 and 18.2%) in flowers, leaves and stem oils, respectively, but was higher than extracts (10.0 and 6.9%), while camphor and terpinen-4-ol were the second and third major compounds in the samples. Another distinguishing feature appeared to be the apparent absence of sabinene, p-cymene, β-caryophyllene and β-pinene in methanolic extract, which were terpinen-4-ol and thymol having more essential oils than hexane-ether extract. Also, hexadecanoic acid, eicosane and methyl linoleate were only investigated in hexane-ether extract. Monoterpenes in flowers, leaves and stem oils (22.0, 20.0 and 18.8% respectively) were more as compared to the two extracts (12.6 and 15.7%). In the oils and extracts originating from *A. pachycephala*, oxygenated monoterpenes (46.4 to 56.4%) were found to be the major class of substances, with the dominance of 1, 8-cineole (6.9 to 19.0%), camphor (7.5 to 15.0%) and borneol (2.7 to 5.2%). Sesquiterpenes (7.1 to 13.6%) and oxygenated sesquiterpenes (3.5 to 6.8%) were minor components. In the only study performed on essential oil of *A. pachycephala*, the water-distilled essential oils from aerial parts of *A. pachycephala* Rech.f., which (from

Table 2. Percentage of chemical compositions of the oil of flowers, leaves, stems and extracts of two *Achillea*.

No.	Compound	RI	*Achillea pachycephala* Rech.f.					*Achillea santolina* L.				
			Essential oil			Hexane-ether extract	Methanolic extract	Essential oil			Hexane-ether extract	Methanolic extract
			Flower	Leave	Stem			Flower	Leave	Stem		
1	Santolina triene	908	-	-	-	-	-	0.1	0.2	-	0.2	2.1
2	Tricyclene	926	Tr	0.1	-	-	0.7	-	-	-	-	-
3	α-Thujene	930	0.1	0.1	0.2	-	-	-	-	-	-	-
4	α -Pinene	939	3.1	2.1	1.9	0.7	2.1	1.2	1.4	0.9	1	2
5	Camphene	954	7.2	8	5.3	1.1	2	1.4	1.3	1.2	0.8	1.3
6	Sabinene	957	4.6	3.4	5.1	1.5	-	-	-	-	0.9	-
7	β-Pinene	979	1.3	1.7	1.6	1	-	2	1.8	1.6	1.1	2.1
8	1,8-Dehydrocineole	991	-	-	-	-	0.6	Tr	0.1	-	0.1	-
9	dehydroxy-E-Linalool oxid	993	0.1	-	-	0.2	1.7	-	-	-	-	1
10	α -Phellandrene	1002	0.2	0.3	-	0.3	1	0.1	-	0.2	0.4	0.6
11	dehydroxy-Z-Linalool oxid	1008	-	0.1	-	-	0.8	-	-	-	-	-
12	α -Terpinene	1017	0.3	-	0.1	0.2	0.7	0.7	0.8	0.2	0.1	0.4
13	p-Cymene	1024	2.1	1.7	1.9	3.5	-	1.4	1.5	0.2	0.3	1.4
14	o-Cymene	1026	0.1	-	0.1	-	-	0.8	0.6	0.4	0.4	0.1
15	β -Phellandrene	1029	0.9	1.1	1.3	1.5	5.3	0.2	0.5	0.3	0.5	0.7
16	1,8-Cineole	1031	16.4	19	18.2	10	6.9	5	4.5	3	3	6.2
17	Z- β -Ocimene	1037	0.3	0.3	0.8	0.4	-	-	-	-	-	-
18	Santolina alcohol	1040	-	-	-	0.2	1.4	0.6	-	-	0.3	-
19	E- β -Ocimene	1050	0.1	0.1	-	0.3	-	-	-	-	-	-
20	γ-Terpinene	1059	-	-	-	-	-	0.2	0.9	0.8	1	2.1
21	Artemisia ketone	1062	-	-	-	-	-	1.2	1.1	0.8	0.5	0.9
22	Z-Sabinene hydrate	1070	-	-	-	-	-	3.5	1.8	2.1	2.1	3.1
23	Artemisia alcohol	1083	-	-	-	-	-	2.1	0.6	0.5	1.1	2.2
24	Terpinolene	1088	-	-	-	-	-	0.2	0.1	0.1	-	0.8
25	p-Cymenene	1091	1.6	1.1	0.5	1.7	-	-	-	-	-	-
26	Linalool	1096	2.4	1.9	1.1	2.1	3.8	-	-	-	-	-
27	n-Nonanal	1100	0.7	0.2	0.4	-	1	-	-	-	-	-
28	E-Pinocarveol	1139	3.2	3.1	3.1	0.3	0.5	-	-	-	-	-
29	Camphor	1146	11.2	12	15	10	7.5	4.2	4.1	3.8	5.1	6.1
30	Isoborneol	1160	0.5	-	0.1	1	0.9	0.9	0.8	0.6	0.5	0.9
31	Lavandulol	1164	-	-	-	-	-	0.6	-	0.3	0.1	0.4
32	Borneol	1169	5.2	3.7	4.3	2.7	4.5	1.5	3.5	4.5	0.6	2.2
33	Octanoic acid	1171	2.1	2.3	3.1	0.9	0.2	2.3	1.7	0.9	1	0.9
34	Terpinen-4-ol	1177	4.3	3.9	3.5	5.1	7.1	6.4	7.1	6.1	4	5
35	α -Terpineol	1188	-	2.7	3.2	2.1	0.9	0.5	0.5	0.4	0.5	0.3
36	Myrtenol	1195	0.2	0.3	-	-	0.2	0.5	-	0.2	0.7	1.1

Table 2. Contd.

37	Piperitol	1199	-	-	0.9	1.1	1.8	0.7	0.7	0.9	1.2	2.3
38	Fragranol	1215	1.2	0.5	0.2	1.9	2.3	8.1	9.1	7.8	7.1	6.1
39	Z-Carveol	1229	-	0.7	0.4	0.8	3.2	-	-	-	-	-
40	Carvone	1243	0.4	Tr	-	-	-	Tr	-	-	-	-
41	Geraniol	1252	-	-	0.1	0.2	0.6	-	0.3	0.3	0.4	0.8
42	Z-Cinnamyl alcohol	1262	-	-	-	-	0.5	0.2	-	-	0.1	0.6
43	Z-Chrysanthenyl acetate	1265	0.9	-	-	0.6	-	-	-	-	-	-
44	Geranial	1267	-	0.1	1	1.2	-	-	0.1	0.3	0.2	-
45	Terpinen-7-al-α	1282	-	-	-	-	-	0.9	-	-	-	1.1
46	Bornyl acetate	1288	2.4	2.3	2.1	1.5	4.1	1.2	0.6	0.4	0.7	-
47	Thymol	1290	1.2	1.8	1.5	2.7	5.1	2.5	1.3	0.9	1.8	3.1
48	Carvacrol	1299	-	-	-	-	-	0.2	0.5	0.6	0.8	1.5
49	Fragranyl acetate	1335	2.3	2.5	1.7	3.2	4.2	28.4	34	37	20	10.9
50	α-Cubebene	1348	0.2	-	-	-	-	-	-	-	-	-
51	α-Terpinyl acetate	1352	-	-	-	0.1	-	0.6	3.4	5.1	1.1	5.2
52	Eugenol	1359	1.8	2.5	2.1	1.2	4.2	-	-	-	-	-
53	Neryl acetate	1361	-	-	-	-	-	0.2	0.2	0.1	0.1	-
54	Decanoic acid	1369	0.3	0.2	0.2	-	0.8	-	-	0.7	0.3	0.1
55	E-Myrtanol acetate	1386	-	-	-	-	0.6	0.9	1.7	1.2	1.6	2.1
56	E-α-Domascone	1393	0.2	-	-	-	-	-	-	-	-	-
57	Methyl eugenol	1403	0.9	0.4	0.1	1.2	3.4	-	-	-	-	-
58	β -Caryophyllene	1419	4.1	4.4	4.2	5.1	-	1.2	1.2	0.9	1.5	-
59	Lavandulyl isobutanoate	1424	-	-	-	-	-	0.1	0.1	0.2	0.3	1.2
60	Z-Muurola-3,5-diene	1450	0.6	-	-	0.1	-	-	-	-	-	-
61	E-β-Farnesene	1456	-	0.9	0.3	1	-	Tr	0.1	Tr	-	0.6
62	allo-Aromadendrene	1460	1.7	2.2	2	2.1	-	-	-	-	-	-
63	Germacrene D	1485	1.2	1.8	0.9	2.1	1.2	1.6	1.1	1.9	1.5	3.4
64	β -Selinene	1490	0.5	0.4	0.2	0.7	2.3	-	0.4	0.3	0.5	1.1
65	Bicyclogermacrene	1500	-	-	-	-	-	0.6	0.9	0.9	0.5	2.6
66	β -Bisabolene	1505	3	3.2	3.7	2.1	2.3	-	-	-	-	-
67	Germacrene A	1509	0.1	0.1	-	0.2	0.8	0.7	0.2	-	0.5	0.2
68	Lavandulyl -2-methyl butanoate	1511	-	-	-	-	-	2.1	1.5	0.9	Tr	-
69	γ-Cadinene	1513	0.4	0.2	0.1	0.2	0.5	0.1	-	-	-	0.5
70	Myristicin	1518	0.3	0.1	0.2	0.1	0.9	-	-	-	-	-
71	Z-Sesquisabinene hydrate	1544	-	-	-	0.2	-	-	-	-	-	-
72	Dendrolasin	1571	-	-	-	-	-	1.5	1.2	0.8	2.1	-
73	Germacrene D-4-ol	1575	0.3	0.5	0.4	0.9	1.8	-	-	-	-	-
74	Spathulenol	1578	0.2	-	-	0.7	1	-	0.1	0.6	0.3	1
75	Caryophyllene oxide	1583	3.1	3.5	2.9	3.2	0.5	1.2	1.2	1.4	3.2	4.1

Table 2. Contd.

76	10-epi-γ-eudesmol	1623	-	-	-	0.1	0.3	1.5	0.6	1.4	4.2	1.2
77	1-epi-Cubenol	1628	0.9	-	-	-	0.5	-	-	-	-	-
78	α –Muurolol	1646	-	-	-	0.3	0.9	1.1	1.9	2.1	3.6	4.1
79	β-Eudesmol	1650	-	-	0.1	0.4	1.5	1.1	-	0.1	0.2	1.3
80	Heptadecane	1700	0.7	0.2	0.4	0.3	-	0.4	0.2	0.1	0.3	-
81	Chamazulene	1731	0.9	0.8	0.6	0.8	0.3	0.4	0.3	0.2	0.3	0.3
82	2E,6E-Farnesyl acetate	1846	-	-	-	1	-	-	-	-	-	-
83	E-β-Santalol acetate	1868	0.2	0.3	0.1	-	-	1	0.2	0.1	0.5	0.1
84	Hexadecanoic acid	1960	-	-	-	1.7	Tr	-	-	-	5.2	-
85	n-Eicosane	2000	-	-	-	4	-	-	-	-	4.2	-
86	Methyl linoleate	2085	-	-	-	4.2	-	-	-	-	3.1	-
87	n-Heneicosane	2100	-	-	-	2.6	-	-	-	-	1.5	-
88	Linoleic acid	2133	-	-	-	1	-	-	-	-	-	-
89	n-Tricosane	2300	-	-	-	0.5	-	-	-	-	1.5	-
Number of identified compounds			53	47	46	60	48	53	48	50	59	49
Yield of the oil %			0.8	0.2	0.1	0.9	1.7	0.7	0.25	0.15	1.2	1.8
Monoterpene hydrocarbons			22	20	18.8	12.6	15.7	8.3	9.1	8.9	7.1	14.6
Oxygenated monoterpenes			51.8	54.6	56.4	46.4	54.8	72.4	77.2	76	55.1	57.4
Sesquiterpene hydrocarbons			11.8	13.2	11.4	13.6	7.1	4.7	4.3	3	4.5	8.4
Oxygenated sesquiterpenes			4.7	4.3	3.5	6.8	6.5	7.4	5.2	6.5	14.1	11.8
Others			7.9	6.7	7.1	18.5	11.3	3.3	2.2	1.9	16.5	1.9
Total identified			98.2	98.8	97.2	97.9	95.4	96.1	98	96.3	97.2	95.9

Tr = Trace < 0.05.

center of Iran) is endemic to Iran, was analyzed by GC and GC–MS. The oil of *A. pachycephala* was found to contain 1, 8-cineole (27.7%) and camphor (27.4%) as the major constituents (Esmaeili et al., 2006). Based on our knowledge, study on its essential oils composition has not been reported and this is the first investigation of *A. pachycephala* extracts composition.

A. santolina

107 to 125 g out of air dried flowers, leaves and stems of *A. santolina* L. were steam hydrodistilled separately, and after GC and GC-MS analysis, a total of 53, 48 and 50 chemical constituents, representing 96.1, 98.0 and 96.3% of the total content, were identified in each investigated essential oils, respectively (Table 2). 50 g of the aerial parts was extracted by a mixture (1:1) of hexane-ether, followed by GC and GC-MS analysis, after which, 59 (97.2%) components were characterized; the same amounts of plant done with methanol showed 49 (95.9%) compounds. In addition, the essential oil was also found to be rich in oxygenated monoterpenes (>72%), while solvent extracts showed minor amounts (55.1 and 57.4%). In essential oils, monoterpenes (8.3 to 9.1%), sesquiterpenes (3.0 to 4.7%) and oxygenated sesquiterpenes (5.2 to 7.4%) were less. Hexane-ether extract of *A. santolina* showed 7.1% monoterpene hydrocarbon, 4.5% sesquiterpene hydrocarbon, 14.1% oxygenated sesquiterpene and 16.5% non-terpene compounds, while methanolic extract identified 14.6, 8.4 and 11.8% monoterpene, sesquiterpene and oxygenated sesquiterpene, respectively; it also showed only 1.9% non-terpenes chemical constituents. 1, 8-Cineole (3.0

Table 3. Antibacterial activity (zoon inhibition diameter) of the oil of flowers, leaves, stems and extracts of two *Achillea* against some gram positive and gram negative bacteria as compared to streptomycin.

	Plant		Streptomycin (1 mg/m)	Gram positive bacteria			Gram negative bacteria			
				B. cereus	*B. subtillis*	*Staphylococcus*	*Escherichia*	*Klebsiella*	*Proteus*	*Salmonella*
		MTCC No.	-	430	441	2940	443	109	426	733
Zone of inhibition (mm)	*Achillea pachycephala Rech.f.*	Flower Oil in DMSO	12	11.5	11	12	19	22.5	13	25.5
		Leaf Oil in DMSO	11	10	10.5	10.5	21	18	11	27
		Stem Oil in DMSO	8.5	7	7.5	8	19.5	20.5	9.5	25
		Hexane-ether extract	12.5	15	12.5	14	24	26	12	24.5
		Methanolic extract	10	9	7.0	6	10	9	10.5	9.5
	Achillea santolina L.	Flower Oil in DMSO	13	12	14	9	25	23	26	19
		Leaf Oil in DMSO	10.5	11	12.5	7.5	21	22.5	27.5	15
		Stem Oil in DMSO	10	9	12.5	6.5	22	21.5	21	10
		Hexane-ether extract	12.5	13.5	14	7	25	24	23.5	9
		Methanolic extract	10.5	8.5	10	5	10.5	10	9	7

Table 4. Determination of MIC and MBC of tow *Achillea* extract for bacteria.

Tested bacteria	MIC (mg/ml)				MBC (mg/ml)			
	A. pachycephala		*A. santolina*		*A. pachycephala*		*A. santolina*	
	Hexane-ether	Methanolic	Hexane-ether	Methanolic	Hexane-ether	Methanolic	Hexane-ether	Methanolic
Bacillus cereus	12.5	25.0	12.5	25.0	25.0	50.0	12.5	50.0
Bacillus subtillis	6.25	12.5	12.5	25.0	25.0	50.0	25.0	50.0
Staphylococcus aureus	6.25	12.5	6.25	12.5	12.5	25.0	12.5	25.0
Escherichia coli	6.5	12.5	6.25	12.5	25.0	50.0	12.5	50.0
Klebsiella pneumonia	3.12	6.25	6.25	0.78	12.5	12.5	12.5	6.25
Proteus vulgaris	1.56	0.39	0.78	0.78	1.56	3.12	3.12	3.12
Salmunella typhi	1.56	6.25	1.56	N.T	N.T	12.5	N.T	N.T
Streptomycin	6.25	12.5	12.5	6.25	6.25	12.5	12.5	6.25

MIC: Minimum inhibitory concentration; MBC: minimum bactericidal concentration; N.T: not tested.

to 6.2%), Z-sabinene hydrate (1.8 to 3.5%), camphor (3.8 to 6.1%), terpinen-4-ol (4.0 to 7.1%), fragranol (6.1 to 9.1%), fragranyl acetate (10.9 to 37.0%) and α-terpinyl acetate (0.6 to 5.2%) were the major components in oils and solvents extract. Santolina triene (2.1%), α- pinene (2.0%), 1, 8-cineole (6.0%), camphor (6.1%), α-terpinyl acetate (5.2%), germacrene D (3.4%), caryophyllene oxide (4.1%) and α-muurolol (4.1%) in methanolic extract were higher than another extract and oils, but fragranol (6.1%), fragranyl acetate (10.9%) and 10-epi-γ-eudesmol (1.2%)

were less. Also, hexadecanoic acid (5.2%), eicosane (4.2%) and methyl linoleate (3.1%) were only found in hexane-ether extract.

Essential oils of crushed flower, leave and stem of *A. santolina* from Egyptian plant have been studied and 44 (97.62%), 43 (95.62%) and 37 (96.09%) compounds were identified respectively. Fragranyl acetate, fragranol, lavandulyl isobutyrate, terpinen-4-ol and camphor were the main constituents in the investigated parts (El-Shazly, 2004). The principle components in the hexane-ether extract of the total aerial parts were fragranyl acetate (50.7%), fragranol (8.78%) and camphor (6.6%). Mainly, oxygenated monoterpenes were identified as the major constituents. Some constituents were observed in same amounts, but some constituents were identified by different percentages.

In *A. santolina* from Jordan, terpenoids (1, 8-cineole, camphor, bomeol, pinenes, artemisia ketone, santolina alcohol, farnesane, caryophyllene and its oxides, α-bisabolol and oxides, cubebene, germacrenes, eudesmol, farnesene, γ-gurjunene, γ-muurolene and chamazulene) are the principle components (Bader et al., 2003). The variations of the essential oil content and morphological values in ten accessions of *A. santolina* collected from northwestern Iran have been investigated (Farajpour et al., 2011). Two genotypes which were gathered from Lorestan and Kurdistan provinces, gave the highest mean of essential oil content (0.2 and 0.19%, respectively). Considering the amount of essential oil in each genotype from each province, it has been mentioned that this feature varied with location, with western Iran having the highest.

Essential oil variation among and within six *Achillea* species transferred from different ecological regions in Iran to the field conditions were investigated (Rahimmalek et al., 2009b). According to the major compounds, four chemotypes were defined. Among them, four *A. santolina* collected from Natanz, Malayer, Arak and Yasooj in center and west of Iran, were studied. Their yield essential oils were between 0.1 and 0.6%, and 43 components were identified. Comphor, borneol, bornyl acetate, germacrene D and spathulenol were the major constituents.

In another experimental study, yarrow plant in late spring was collected from Sistan region, east of Iran, in 2008. The compounds of the essential oil were analyzed by GC/MS. In this study, camphor was the major compound of the essential oil (Ahmadi et al., 2011).

Antimicrobial activity

Inhibition zone diameter

In the present study, effectiveness of essential oils was also confirmed by filter paper disc diffusion assay and growth inhibitions zone diameters were measured in the presence and absence of each essential oil and extracts. Results are presented in Table 3. The inhibitory effect was compared with standard antibiotic, streptomycin.

The flowers, leaves and stems of *A. pachycephala* Rech.f. and *A. santolina* L. oils and hexane-ether extracts have shown larger growth inhibition zone diameters (6.5 to 27.5 mm) in comparison to methanolic extracts (5 to 10.5 mm). Both oils and extracts exhibited higher activities against the gram negative tested bacterial strains (7 to 27.5 mm) as compared to gram positive bacteria (5 to 14 mm), while the gram positive strain of *Staphylococcus aureus* appeared as the most resistant ones (5 to 11 mm). Gram negative strain *Proteus vulgaris* showed the most resistance against both oils and extracts of *A. pachycephala* (9.5 to 13 mm).

Salmonella typhi was very active against *A. pachycephala* oils and hexane-ether extract (24.5 to 27 mm), but it displayed moderate activities against *A. santolina* oils and extracts (7 to 19 mm). The flowers oil of *A. pachycephala* has shown 22.5 mm inhibition zone diameter against *Klebsiella pneumonia*, and 25 mm against *S. typhi*; while the leaves and stem oils and hexane-ether extract of *A. pachycephala* appeared as 27, 25 and 24.5 mm inhibition zone diameters.

The flower, leaves, stem oils, as well as hexane-ether and methanolic extracts of *A. santolina* showed 26, 27.5, 21, 23.5 and 9 mm inhibition zone diameters against *P. vulgaris* and 25, 21, 22, 25 and 10.5 mm against *Escherichia coli*, respectively (Figures 1 and 2).

In a light conclusion, *A. pachycephala* and *A. santolina* displayed moderate activities against all the tested bacterial strains, both being more active in gram negative bacteria. Acording to Ulubelen et al. (1994), caryophyllene oxide is the most efficient antibacterial property, followed by camphor and 1, 8-cineole (Aligiannis et al., 2000; Prudent et al., 1993). Thus, the antibacterial properties of the essential oils *A. pachycephala* and *A. santolina* are probably connected with their high content of 1, 8-cineol and camphor. Antimicrobial activity of *A. pachycephala* has not been reported previously. Inhibition zone diameters of *A. santolina* oils and hexane extract against two gram negative and two gram positive strains was represented (EL-Shazly, 2004). The results showed that oils of *A. santolina* exhibited moderate effect.

Determination of MIC and MBC values

The results of antimicrobial activity of the *A. pachycephala* Rech.f. and *A. santolina* L. extracts against seven bacterial strains are listed in Table 4. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determinations were obtained by disc agar diffusion method. In general, the extracts showed moderate activity against all tested microorganisms. The results for hexane-ether extract of

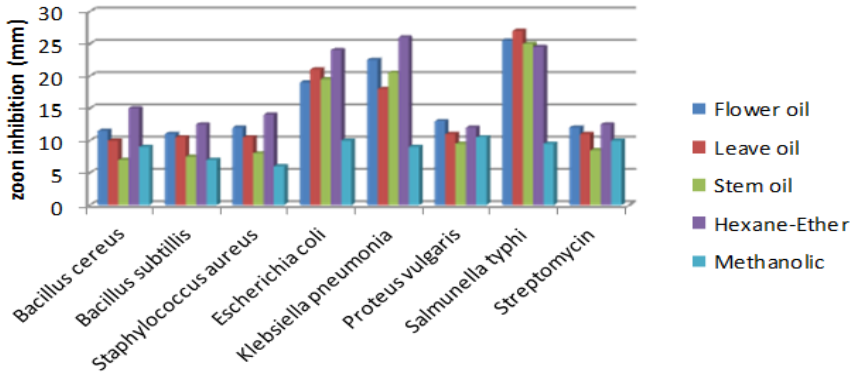

Figure 1. Zoon inhibition diameter of oils (hexane-ether and MeOH extracts of *A. pachycephala*).

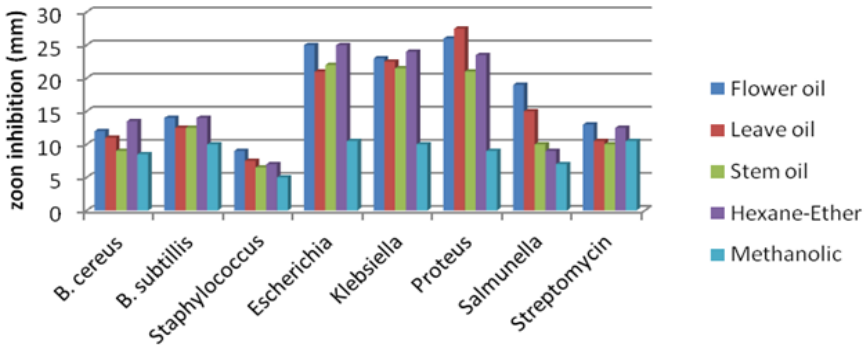

Figure 2. Zoon inhibition diameter of oils (Hexane-Ether and MeOH extracts of *A. santolina*).

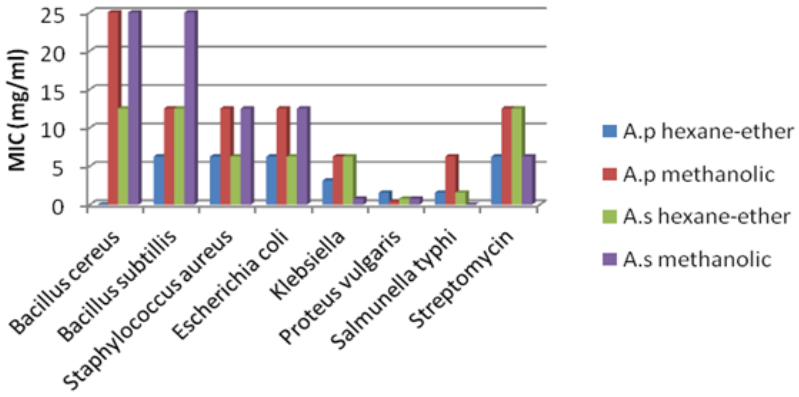

Figure 3. MIC values (mg/ml) of oils (hexane-ether and MeOH extracts of *A. pachycephala (A.p)* and *A. santolina (A.s).*

A. pachycephala indicated that MIC values against tested organisms ranged from 1.56 to 12.5 mg/ml, while MBC values ranged from 1.56 to 25.0 mg/ml; whereas, for indicated that MIC values against tested organisms ranged from 0.78 to 12.5 mg/ml while MBC values ranged from 3.12 to 25.0 mg/ml, whereas for methanolic extract, MIC values was 0.39 to 25.0 mg/ml, while MBC values ranged from 3.12 to 50.0 mg/ml.

The results for hexane-ether extract of *A. santolina* extract, MIC values was 0.78 to 25.0 mg/ml while MBC values ranged from 3.12 to 50.0 mg/ml (Figures 3 and 4). Among bacteria; *A. pachycephala* and *A. santolina*

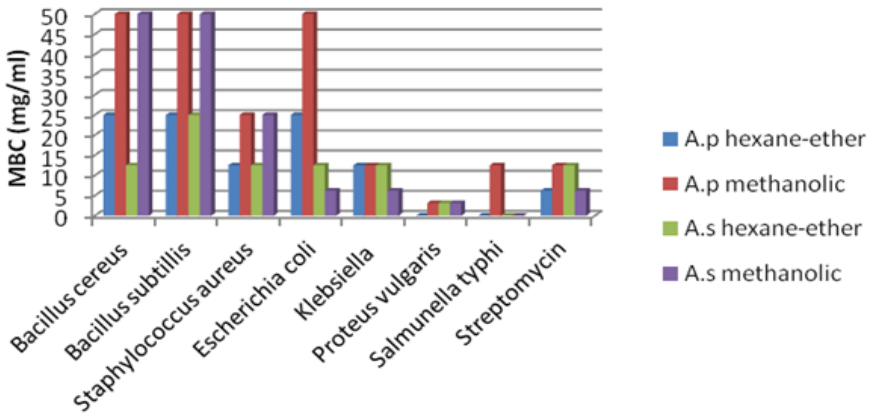

Figure 4. MBC values (mg/ml) of oils (*hexane-ether and MeOH extracts of A. pachycephala (A.p) and A. santolina (A.s)*.

extracts were the most effective against gram negative bacteria, such as *P. vulgaris* .

The effect of ethnomedicinal plants used in folklore medicine in Jordan as antibiotic resistant inhibitors on *E. coli* were investigated and MIC of methanolic extracts of some plants, such as *A. santolina* was determined (Darwish and Aburjai, 2010).

Identification of the constituents of *A. santolina* (from Sistan in Iran) essential oil and evaluation of the anti-microbial effects of its extract and essential oil were carried out (Ahmadi et al., 2011). The standard strains of *S. aureus* presented the greatest sensitivity to the stem extract and leaf extract in MIC> 0.573 and MBC> 1.146, respectively, and to the flower extract in MBC> 1.663 and MIC> 0.831, respectively. In addition, it presented an intermediate sensitivity to standard strains *E. coli* with MBC> 2.293 and MIC> 1.146, respectively, to the stem and leaf extract and MBC> 6.650 and MIC> 3.325.

This study reveals the antimicrobial susceptibility of two *Achillea* extracts (hexane-ether and methanolic extracts) against seven bacteria. It is proved by low MIC and MBC values obtained in extracts when used against each bacterial culture.

Conclusion

As a conclusion, the high concentrations of 1, 8-cineole, and borneol as well as the presence of chamazulene in *Achillea* revealed in this study and in previous reports on another *Achillea* spices is likely to account for pharmaceutical purposes and in folk medicine of plants. 1, 8-Cineole (Pattnaik et al., 1997), and borneol (Tabanca et al., 2001; Mourey and Canillac, 2002) have shown antimicrobial effects. Additionally, 1, 8-cineole (Viljoen et al. 2003) and borneol (Dai et al., 2009) have shown synergistic effects, followed by increasing antimicrobial properties of *Achillea* genus.

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