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Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae)

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Abstract

The in vitro antimicrobial and antioxidant activities of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae) were investigated. GC-MS analysis of the essential oil resulted in the identification of 36 compounds constituting 90.8% of the total oil. Eucalyptol, camphor, α-terpineol, β-pinene, and borneol were the principal components comprising 60.7% of the oil. The oil strongly reduced the diphenylpicrylhydrazyl radical (IC_{50} = 1.56 μg/ml) and exhibited hydroxyl radical scavenging effect in the Fe^{3+}–EDTA–H_2O_2 deoxyribose system (IC_{50} = 2.7 μg/ml). It also inhibited the nonenzymatic lipid peroxidation of rat liver homogenate (IC_{50} = 13.5 μg/ml). The polar phase of the extract showed antioxidant activity. The oil showed antimicrobial activity against *Streptococcus pneumoniae*, *Clostridium perfringens*, *Candida albicans*, *Mycobacterium smegmatis*, *Acinetobacter lwoffii* and *Candida krusei* while water-insoluble parts of the methanolic extracts exhibited slight or no activity. This study confirms that the essential oil of *Achillea millefolium* possesses antioxidant and antimicrobial properties in vitro.

Keywords: *Achillea millefolium*; Antioxidant activity; Antimicrobial activity; Methanol extracts; Essential oil; GC-MS

1. Introduction

It has long been recognized that naturally occurring substances in higher plants have antioxidant activity. Recently, there is a growing interest in oxygen-containing free radicals in biological systems and their implied roles as causative agents in the aetiology of a variety of chronic disorders. Accordingly, attention is focused on the protective biochemical functions of naturally occurring antioxidants in the cells of the organisms containing them (Larson, 1988; Halliwell, 1997). Antioxidants in the oils are important in the stabilization of free fatty acids (Six, 1994; Baldioli et al., 1996). The antioxidant activity of phenols and other compounds present in oils has been well and widely studied by several authors (Litridou et al., 1997; Visioli et al., 1998; Yoshida and Takagi, 1999). Besides, reports concerning the local uses, pharmacological features of the extracts and the essential oils of several *Achillea* species have been cited in the literature. (Rustaiyan et al., 1998; Chalchat et al., 1999; Simic et al., 2000).

The genus *Achillea* (Family: Asteraceae, Section: Santolinoidea) is represented by about 85 species mostly found in Europe and Asia and a handful in North America (Könemann, 1999). Forty species of *Achillea* are widely distributed in Turkey (Davis, 1982). As far as ethnopharmacologic background is concerned, *Achillea millefolium* is a well-known species amongst the members of *Achillea* (Asteraceae) (Chandler et al., 1982). It is known as "Civanperçemi" and used in folk remedies as an appetizer, wound healer, diuretic, carminative or menstrual regulator (Baytop, 1999). To the best of our knowledge, information concerning the in vitro antioxidant features of *Achillea millefolium* subsp. *millefolium* has not been found in the literature.

As a part of our continuing research project on the in vitro antimicrobial and antioxidant activities of several *Achillea* species, we previously reported antimicrobial activities of the essential oils isolated from *Achillea setacea* and *Achillea teretifolia* (Ünlü et al., 2002). The aim of this study was

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to evaluate the in vitro antioxidant and antimicrobial properties of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* (Asteraceae).

2. Materials and methods

2.1. Collection of plant material

The herbal parts of *Achillea millefolium* subsp. *millefolium* were collected in Kızıldağ Pass, 130 km east of Sivas, when flowering late-July 2001. The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas, Turkey (CUFH-Voucher No.: ED 6358).

2.2. Preparation of the methanolic extracts

The air-dried and finely ground sample was extracted by using the method as described elsewhere (Sökmen et al., 1999). The resulting extract (19.78%, w/w) was suspended in water and partitioned with chloroform ($CHCI_3$) to separate less polar, water-insoluble compounds. The water-soluble (16.36%, w/w) and non-soluble parts (3.42%, w/w) were then lyophilised and kept in the dark at +4 °C until tested.

2.3. Extraction of the essential oil

The air-dried and ground aerial parts of plants collected were submitted for 3 h to water-distillation using a Clevenger-type apparatus to produce an oil in 0.6% (v/w) yield. Oil was dried over anhydrous sodium sulphate and, after filtration, stored at +4 °C until tested and analyzed.

2.4. GC-MS analysis

The analysis of the essential oil was performed using a Hewlett Packard 5890 II GC, equipped with a HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm) and a HP 5972 mass selective detector. For GC-MS detection, an electron ionisation system was used with ionisation energy of 70 eV. Helium was the carrier gas, at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. Column temperature was initially at 50 °C, then gradually increased to 150 °C at a 3 °C/min rate, held for 10 min and finally increased to 250 °C at 10 °C/min. Diluted samples (1/100 in acetone) of 1.0 μl were injected manually and splitless. The components were identified based on the comparison of their relative retention time and mass spectra with those of NBS75K library data of the GC-MS system, literature data (Adams, 2001) and standards of the main components. The results were also confirmed by the comparison of the compounds elution order with their relative retention indices on non-polar phases reported in the literature (Adams, 2001).

2.5. Antioxidant activity

2.5.1. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the extract or crude oil for hydroxyl radicals, which attack to deoxyribose leading to the formation of thiobarbituric acid reaction system (TBARS), generated from the Fe^{3+}–ascorbate–EDTA–H_2O_2 system (Kunchandy and Rao, 1990). The formed TBARS were measured by using the method described elsewhere (Ohkawa et al., 1979). Experiments were carried out in triplicate. All reagents were prepared freshly.

Inhibition (I) of deoxyribose degradation in percent was calculated in following way:

$$I = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

where A_0 is the absorbance of the control reaction (containing all reagents except the test compound), and A_1 is the absorbance of the test compound. The IC_{50} value represented the concentration of the compounds, that caused 50% inhibition.

2.5.2. Inhibition of superoxide radicals

Superoxide radical generated by the xanthine–xanthine oxidase system was determined spectrophotometrically by monitoring its ability to reduce nitroblue tetrazolium (NBT) (Robak and Gryglewski, 1988). Percent scavenging of superoxide was calculated from the optical density of the treated and control samples.

2.5.3. DPPH assay

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described elsewhere (Cuendet et al., 1997). Tests were carried out in triplicate.

2.5.4. Inhibition of lipid peroxide formation

The reaction mixture contained 0.1 ml of 25% (w/v) rat liver homogenate in 40 mM, pH;7.0 Tris–HCl buffer, 30 mM KCl, 0.16 mM ferrous iron, various concentrations of the extract, and positive controls; BHT, curcumin, 0.06 mM ascorbic acid in a final volume of 0.5 ml. As positive controls, BHT and curcumin had their own control reactions containing all related reagents except the test compounds. The mixture was then incubated at 37 °C for 1 h (Bishayee and Balasubramanian, 1971). The lipid peroxide formation was measured by using the method described elsewhere (Ohkawa et al., 1979). The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compounds with those of controls not treated with the extracts. Calculations were done as mentioned in hydroxyl radical scavenging method.

2.6. Antimicrobial activity

2.6.1. Microbial strains

The methanolic extracts (both water-soluble and water-insoluble parts) and the essential oil of *Achillea millefolium* were individually tested against a panel of microorganisms including *Staphylococcus aureus* ATCC #25923 and for minimum inhibitory concentration (MIC) test, ATCC #29213, *Streptococcus pneumoniae* ATCC #49619, *Moraxella catarrhalis* ATCC #49143, *Bacillus cereus* ATCC #11778, *Acinetobacter lwoffii* ATCC #19002, *Enterobacter aerogenes* ATCC #13043, *Escherichia coli* ATCC #25922, *Klebsiella pneumoniae* ATCC #13883, *Proteus mirabilis* ATCC #7002, *Pseudomonas aeruginosa* ATCC #27853, *Clostridium perfringens* KUKENS-Turkey, *Mycobacterium smegmatis* CMM 2067, *Candida albicans* ATCC #10239 and *Candida krusei* ATCC #6258. Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar (MHA), with the exception of *Streptococcus pneumoniae* (MHA containing 50 ml citrate blood/l) and *Clostridium perfringens* (in anaerobic conditions). Yeasts were cultured overnight at 30 °C in Sabouraud dextrose agar.

2.6.2. Antimicrobial screening

Two different methods were employed for the determination of antimicrobial activities; agar well-diffusion method for the methanol extracts (water-soluble and water-insoluble parts) and agar disc diffusion method for the essential oil. MICs of the essential oil against the test organisms were determined by broth microdilution method. All the tests were performed in duplicate and repeated twice. Modal values were selected.

2.6.3. Agar well-diffusion method

The water-soluble extracts were weighed and dissolved in phosphate buffer saline (PBS; pH 7.0–7.2), 10 mg/ml, water-insoluble parts were dissolved in dimethylsulphoxide (DMSO), 10 mg/ml. Both extracts were filter-sterilised using a 0.45 μm membrane filter. Each microorganism was suspended in sterile saline and diluted at ca. 10^6 colony forming unit (cfu)/ml. They were "flood-inoculated" onto the surface of MHA. The wells (8 mm in diameter) were cut from the agar and 0.06 ml of extract solution was delivered into them. After incubation for 24 h at 37 °C, all plates were examined for any zones of growth inhibition, and the diameter of these zones were measured in millimetres.

2.6.4. Disc diffusion method

Disc diffusion method was employed for the determination of antimicrobial activities of the essential oil (NCCLS, 1997). Briefly, a suspension of the tested microorganism (0.1 ml of 10^8 cells per ml) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 15 μl of the oil or the fraction and placed on the inoculated plates. These plates, after staying at 4 °C for 2 h, were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeasts. The diameters of the inhibition zones were measured in millimetres. Amikacin, clindamycin and ciprofloxacin were individually used as positive controls for bacteria.

2.6.5. Determination of minimum inhibitory concentration (MIC)

A broth microdilution broth susceptibility assay was used, as recommended by NCCLS, for the determination of MIC (NCCLS, 1999). All tests were performed in Mueller Hinton broth (MHB; BBL) supplemented with Tween 80 detergent (final concentration of 0.5% (v/v), with the exception of the yeasts (Sabouraud dextrose broth-SDB + Tween 80). Bacterial strains were cultured overnight at 37 °C in MHA and the yeasts were cultured overnight at 30 °C in SDB. Test strains were suspended in MHB to give a final density of 5×10^5 cfu/ml and these were confirmed by viable counts. Geometric dilutions ranging from 0.036 mg/ml to 72.00 mg/ml of the essential oil were prepared in a 96-well microtiter plate, including one growth control (MHB + Tween 80) and one sterility control (MHB+Tween 80+test oil). Plates were incubated under normal atmospheric conditions at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeasts. The MIC of amikacin, clindamycine and ciprofloxacine was individually determined in parallel experiments in order to control the sensitivity of the test organisms. The bacterial growth was indicated by the presence of a white "pellet" on the well bottom.

3. Results and discussion

3.1. Chemical composition of the essential oil

Thirty-six compounds were identified and constituted 90.8% of the total oil. The essential oil of *Achillea millefolium* was characterized by a high number of monoterpenes. Eucalyptol (24.6%), camphor (16.7%), α-terpineol (10.2%), β-pinene (4.2%), and borneol (4.0%) were the principal components comprising the 59.7% of the essential oil (Table 1).

Changes in the composition of *Achillea millefolium* essential oil were reported as being related to maturation of the plant, with increasing amounts of monoterpenes in relation of the sesquiterpenes (Rohloff et al., 2000).

Eucalyptol, camphor, and/or α-terpineol have been found as major compounds in many other *Achillea* species (Rustaiyan et al., 1998; Chalchat et al., 1999; Simic et al., 2000).

The studied essential oil displayed different chemical profile from those observed from *Achillea millefolium* plants of other geographical origin. *Achillea millefolium* essential oil from Cuba (Pino et al., 1998) was found to include caryophyllene oxide at a percentage of 20%, while *Achillea millefolium* oil from five clones from Russia (Orth

Table 1
Chemical composition of the essential oil from *Achillea millefolium* subsp. *millefolium*

Compound[a]	R_t[b]	Composition (%)
Thujene	9.412	0.1
α-Pinene	9.669	2.4
Camphene	10.323	2.4
Benzaldehyde	10.948	0.2
Sabinene	11.523	2.8
β-Pinene	11.592	4.2
2,3-Dehydro-1,8-cineole	12.355	0.6
α-Terpinene	13.545	0.5
Eucalyptol	14.496	24.6
γ-Terpinene	15.666	1.0
Terpinolene	17.123	0.2
Linalool	17.807	0.6
Camphor	20.166	16.7
Borneol	21.117	4.0
Terpinen-4-ol	21.692	2.8
α-Terpineol	22.515	10.2
Myrtenol	22.663	0.3
Fragranol	23.486	0.5
7-Methyl-3-methylene-6-octen-1-ol	23.694	0.2
3,7-Dimethyl-3,6-octadien-1-ol	24.408	0.6
Chrysanthenyl acetate	25.716	0.8
Bornyl acetate	26.866	0.1
Myrtenyl acetate	28.888	0.1
Eugenol	30.196	0.2
α-Copaene	31.019	0.1
Caryophyllene	32.991	0.4
β-Farnesene	34.002	0.3
γ-Curcumene	35.588	0.2
Zingiberene	36.262	0.3
Nerolidol	39.464	0.1
Caryophyllene oxide	40.604	0.7
γ-Eudesmol	43.647	1.8
β-Eudesmol	44.945	1.6
Bisabolol oxide II	45.381	3.8
Bisabolone oxide	47.354	3.3
α-Bisabolol	47.443	2.1
Others not identified (33)		9.2
Total		100

[a] Compounds listed in order of elution from a HP-5 MS column.
[b] Retention time (as minutes).

et al., 1999) were characterized by sesquiterpenes with high chamazulene contents (46–74%) in the four clones and high β-caryophyllene content (38–45%) in the one clone.

The fraction of sesquiterpenes in the essential oil of *Achillea millefolium* (section Santolinoidea) of Turkey origin has been found in remarkable amounts, but was qualitatively different from the mentioned above (Table 1).

3.2. Antioxidant activity

The antioxidant activity of *Achillea millefolium* extract (water-soluble part) was examined by comparing it to the activity of known antioxidants such as curcumin, ascorbic acid and BHT by the following four in vitro assays; inhibition of DPPH radical and the oxygen radicals such as lipid peroxides, superoxides, and hydroxyl radicals. Since the water-insoluble part of the extract is partly soluble in aqueous test media and its colour interfered the spectroscopic measurements, only water-soluble part and essential oil could be tested for their antioxidative capacity. All results are reported in Table 2.

As can be seen from Table 2, the extract improved 50% inhibition at higher concentrations, indicating lesser antioxidant capacity than positive controls, but in the superoxide radical inhibition case, it was found to be more effective than ascorbic acid. Hydroxyl radical scavenging and lipid peroxidation were not tested with ascorbic acid since this chemical was already present in the test medium. On the other hand, results in Table 2 demonstrated the strong ability of the essential oil to act as a donor for hydrogen atoms or electrons. The reduction of the stable radical DPPH to yellow coloured diphenylpicrylhydrazine was obtained with an $IC_{50} = 1.56\,\mu g/ml$ instead of 3.90, 7.92, 19.30 μg/ml for ascorbic acid, curcumine, and BHT, respectively. Both hydroxyl radical scavenging and the lipid peroxidation inhibition of the oil were also better than curcumine and BHT. This could be assigned to the presence of some phenolic compounds (Table 1). The antioxidative effectiveness in natural sources was reported to be mostly due to phenolic compounds (Hayase and Kato, 1984). Phenolic compounds were reported to play an important role in inhibiting autoxidation of the oils (Ramarathnam et al., 1986). In order to determine the antioxidant nature of the oil, its main components, e.g. eucalyptol, camphor, β-pinene, borneol, terpinen-4-ol, α-pinene were all tested individually and none exhibited antioxidative activity in all methods employed. Antioxidant activity of eucalyptol and terpinen-4-ol were previously reported using two different methods; aldehyde/carboxylic acid assay and lipid peroxidation (Lee and Shibamoto, 2001) with prolonged incubation periods (30 days and 18 h, respectively) in contrast to our experimental procedure employing 30–60 min incubation period. These results implicate that the main components in the total oil might synergize each other or other components involve in these activities.

3.3. Antimicrobial activity

Water-insoluble parts of the methanolic extracts were found to have moderate activity against *Clostridium perfringens* and the yeasts. No activity was exhibited by the water-soluble parts. The water-insoluble parts exhibits, in many cases, greater activity than the water-soluble (aquatic) ones (Sökmen et al., 1999).

The essential oil possessed stronger antimicrobial activity than the extracts tested. The oil exhibited moderate activity against *Streptococcus pneumoniae*, *Clostridium perfringens* and *Candida albicans*, and weak activity against *Mycobacterium smegmatis*, *Acinetobacter lwoffii* and *Candida krusei* (Table 3). The growth inhibitions of test microorganisms ranged from 4.5 mg/ml (w/v) to 72.00 mg/ml (w/v) with

Table 2
Effects of methanolic extracts (water-soluble part) and essential oil of *Achillea millefolium* and positive controls on the in vitro free radical (DPPH, superoxide and hydroxyl) and lipid peroxidation generation

Sample	IC_{50} (μg/ml)			
	DPPH	Superoxide	Hydroxyl	Lipid peroxidation
Extract	45.60 ± 1.30	304.00 ± 5.10	407.30 ± 4.05	892.67 ± 13.0
Oil	1.56 ± 0.03	nt	2.70 ± 0.03	13.50 ± 0.07
Curcumin	7.92 ± 0.30	11.04 ± 0.17	14.28 ± 0.08	40.83 ± 0.15
Ascorbic acid	3.90 ± 0.15	1390.00 ± 2.90	nt	nt
BHT	19.30 ± 0.05	nt	32.00 ± 1.20	17.80 ± 0.04

nt: not tested.

Table 3
Antimicrobial activity of the essential oil and the methanolic extracts of *Achillea millefolium* subsp. *millefolium*

Microorganism	Essential oil		MeOH[c]		The MIC of Antibiotics[d]			
	DD[a]	MIC[b]	H_2O	$CHCl_3$	AK	CF	CM	FC
Staphylococcus aureus	8	72.00	na[e]	na	2.00	0.25	nt[f]	nt
Streptococcus pneumoniae	14	4.50	na	na	nt	nt	0.125	nt
Moraxella catarrhalis	na	na	na	na	nt	nt	nt	nt
Bacillus cereus	10	72.00	na	10	nt	nt	nt	nt
Acinetobacter lwoffii	15	18.00	na	na	nt	nt	nt	nt
Enterobacter aerogenes	7	72.00	na	na	nt	nt	nt	nt
Escherichia coli	na	na	na	na	2.00	0.015	nt	nt
Klebsiella pneumoniae	9	72.00	na	na	nt	nt	nt	nt
Proteus mirabilis	na	na	na	na	nt	nt	nt	nt
Pseudomonas aeruginosa	na	na	na	na	1.00	0.25	nt	nt
Clostridium perfringens	12	4.50	na	12	nt	nt	0.25	nt
Mycobacterium smegmatis	12	9.00	na	na	nt	nt	nt	nt
Candida albicans	21	4.50	na	12	nt	nt	nt	128.00
Candida krusei	16	18.00	na	12	nt	nt	nt	64.00

[a] DD, disc diffusion method as recommended by NCCLS. Diameter of zone of inhibition (mm) including disk diameter of 6 mm.
[b] MIC, minimum inhibitory concentration. Values given as mg/ml (for the essential oil) and as μg/ml (for antibiotics).
[c] MeOH, methanolic extracts. Diameter of zone of inhibition (mm) including well diameter of 8 mm.
[d] AK, amikacin; CF, ciprofloxacin; CF, clindamycin, FC, fluconazole.
[e] na, not active.
[f] nt, not tested.

the lowest MIC value against *Streptococcus pneumoniae*, *Clostridium perfringens*, *Candida albicans* at 4.5 mg/ml (w/v).

Eucalyptol (1,8-cineole) and camphor are well-known chemicals with their pronounced antimicrobial potentials (Pattnaik et al., 1997; Tzakou et al., 2001). Antimicrobial activities of borneol have also been previously reported by other investigators (Knobloch et al., 1989; Tabanca et al., 2001).

In conclusion, our observations confirm that oil of *Achillea millefolium* possess strong antioxidative activity but low antimicrobial activity in vitro.

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