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Composition and the *in vitro* Antimicrobial Activities of the Essential Oils of *Achillea wilhelmsii* C. Koch. and *Achillea lycaonica* Boiss & Heldr.

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The genus *Achillea* (Asteraceae) is represented by 42 species (48 taxa) in Turkey and 22 of which are endemic to Turkey. *Achillea wilhelmsii* and *Achillea lycaonica* collected from different localities in Turkey, were subjected to hydrodistillation to yield essential oils which were subsequently analyzed by GC and GC/MS. The main constituents of the oils were identified and antimicrobial bioassays were applied. Camphor (39.62 %) was the main component in the oil of *Achillea wilhelmsii*. The oil of *Achillea lycaonica* contained L-camphor (43.19 %) as main constituent. All tested microorganisms were inhibited by the essential oils. On the other hand both essential oils showed strong antifungal activity against *Alternaria brassicola* than other tested microfungi.

Key Words: *Achillea lycaonica*, *Achillea wilhelmsii*, Essential oil, Chemical composition, Antimicrobial activity.

INTRODUCTION

The antiseptic qualities of aromatic and medicinal plant and their extracts have been recognized since antiquity. Plant essential oils are generally isolated from nonwoody plant material by distillation methods and are variable mixtures of terpenoids and variety of low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes and acyclic esters. Terpenes are among the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants[1].

The essentially Eurasia genus *Achillea* L. (Asteraceae: *Anthemideae*) contains about 120 species throughout the world. According to recent studies on the Turkish *Achillea*, this genus is represented by 42 species (48 taxa)

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of which 22 are endemic to Turkey[2-4], including *Achillea lycaonica* Boiss. & Heldr. which is one of the materials of present research.

Several *Achillea* species are used as an appetizer, wound healer, diuretic, carminative or menstrual regulator, cosmetic and fragrance properties[5,6]. Previously chemical compositions, antimicrobial and antioxidant properties of some *Achillea* species were reported[7-11].

EXPERIMENTAL

Information on the plant material used in this study is given in Table-1. Air-dried aerial parts were hydrodistilled for 3 h using a Clevenger-type apparatus. The voucher specimens were deposited at the Herbarium of the Department of Biology, Inönü University, Malatya, Turkey (Table-1).

The GC analyses were carried out using Hewlett-Packard 6890 GC with FID. A HP-5 MS capillary column (30 m × 0.25 mm i.d. 0.25 μm film thickness) was used. Helium was used as a carrier gas (1.4 mL/min). The column was temperature programmed as follows: 5 min at 45 °C; then at 3 °C/min to 220 °C and held for 10 min. The injector and detector temperatures were to 220 and 250 °C, respectively. Injection was carried out automatic mode. Samples [0.5 μL of the oil solution in hexane (1:100)] was injected by the splitless technique into helium carrier gas. The percentages were obtained from electronic integrator (EI) using flame ionization detection (FID, 220 °C).

GC/MS analyses of the essential oils were carried out on Hewlett Packard 5970A mass selective detector (MSD), directly coupled to a HP 6890 GC. The column, temperature programme and injection were performed as described above. Injection was carried out automatic mode. Library search was carried out using Wiley Library. EI mass spectra were measured at 70 eV ionisation voltage over the mass range 10-400 μ.

The constituents of the oils were identified by matching their mass spectra and retention indices and the components identified in the oils are listed in Table-1.

Antimicrobial screening: The agar disc diffusion method was employed for the determination of antimicrobial screening[12]. Suspension of the tested microorganisms (10^8 CFU/μL) was spread on the solid media plates. Each test solutions are prepared in DMSO. Than filter paper discs (6 mm in diameter) were soaked with 20 μL of the stock solutions and placed on the inoculated plates. After keeping at 2 °C for 2 h, they were incubated at 37 °C for 24 h for bacteria and *Candida albicans*. The diameter of the inhibition zones were measured in mm (Table-2).

Determination of minimum inhibitory concentration (MIC): The antimicrobial action of the oils was investigated by microdilution broth susceptibility assay[12] with the following microorganisms: *Escherichia coli*

TABLE-1
INFORMATION ON *Achillea* sp. AND ESSENTIAL OIL COMPOSITION

Achillea sp.	Collection site and date	Collector number	Components	(%)
Achillea wilhelmsii C.Koch.	Hakkari: At the fork of Hakkari and Yuksekova, 1500 m	TA 1405	Artemisia triene	0.39
			γ-Terpinene	0.19
			Camphene	2.06
			β-Pinene	0.22
			2,5,5-Trimethyl-3,6-heptadien-2-ol	16.10
			1,8-Cineole (Eucalyptol)	7.21
			Artemisia alcohol	17.92
			Camphor	39.62
			L-Camphor	9.44
			α-Terpineol	0.88
			Trans-(+)-carveol	0.26
			Myrcene	0.46
			(+) Spathulenol	1.57
			Caryophyllene oxide	0.66
			Δ-Cadinene	0.68
			β-Eudesmol	2.34
			Monoterpene hydrocarbons	42.94
			Oxygenated monoterpenes hydrocarbons	51.81
			Oxygenated sesquiterpene hydrocarbons	3.91
			Sesquiterpene hydrocarbons	1.34
			Total	**100**
Achillea lycaonica Boiss&Heldr.	Sivas: Between Sivas and Ulas, 9km, 1300 m 29 vi 2002	TA 1479	α-Pinene	0.64
			Camphene	3.08
			1,8-Cineole (Eucalyptol)	5.96
			Artemisia alcohol	21.18
			Camphor	16.48
			L-Camphor	43.19
			(-) - Borneol	1.12
			Borneol-L	0.87
			Terpinene-4-ol	1.46
			Myrcene	0.50
			(+) Spathulenol	2.62
			Δ-Cadinene	0.85
			β-Eudesmol	1.96
			Monoterpene hydrocarbons	4.22
			Oxygenated monoterpenes hydrocarbons	90.26
			Oxygenated Sesquiterpene hydrocarbons	4.58
			Sesquiterpene hydrocarbons	0.85
			Total	**99.91**

TABLE-2
INHIBITION ZONES ACCORDING TO AGAR DISC DIFFUSION METHOD (mm)

Micro-organisms	Serial dilution (100 µL Stock + µL H_2O)				
	Essential Oil and control materials	Stock Solution	100	200	300
E. coli ATCC 25292	*A. wilhelmsii* C. Koch.	8	7	6	6
	A. lycaonica Boiss & Heldr.	8	6	6	6
	Chloramphenicol				23
	Streptomycin				17
S. aureus ATCC 6538	*A. wilhelmsii* C. Koch.	12	8	7	6
	A. lycaonica Boiss & Heldr.	10	7	6	6
	Chloramphenicol				16
	Streptomycin				12
P. aeruginosa ATCC 27853	*A. wilhelmsii* C. Koch.	12	8	7	6
	A. lycaonica Boiss & Heldr.	10	7	6	6
	Chloramphenicol				24
	Streptomycin				15
E. aerogenes NRRL 3567	*A. wilhelmsii* C. Koch.	12	8	7	6
	A. lycaonica Boiss & Heldr.	10	7	7	6
	Chloramphenicol				21
	Streptomycin				15
P. vulgaris NRRL 123	*A. wilhelmsii* C. Koch.	12	10	8	6
	A. lycaonica Boiss & Heldr.	11	9	8	6
	Chloramphenicol				21
	Streptomycin				20
C. albicans	*A. wilhelmsii* C. Koch.	10	8	7	6
	A. lycaonica Boiss & Heldr.	12	11	8	8
	Ketoconazol				27

ATCC 25292, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* NRRL 3567, *Proteus vulgaris* NRLLB 123, *Candida albicans* OGU. Stock solutions of essential oils were prepared in DMSO. Serial dilutions of essential oils were prepared using sterile distilled water placed in 96-well microtiter plates. Freshly grown bacterial suspensions in double strength Mueller Hinton Broth (Merck) and yeast suspension of *Candida albicans* in yeast medium were standardized 10^8 CFU/µL (McFarland No: 0.5). 100 µL of each microbial suspension was then added to each well. The wells including sterile distilled water only served as growth control. The last row containing only the serial dilutions of antibacterial agent without microorganism was used as negative control. After incubation at 37 °C for 24 h, the first well without turbidity was determined as the minimal inhibitory concentration (MIC) (Table-3).

TABLE-3
MINIMUM INHIBITORY CONCENTRATION (μg/mL) OF *Achillea* ESSENTIAL OILS

Microorganisms	Sources	A	B	
Escherichia coli	ATCC 25292	125.00	125.00	-c
Staphylococcus aureus	ATCC 6538	125.00	62.50	-c
Pseudomonas aeruginosa	ATCC 27853	125.00	62.50	-c
Enterobacter aerogenes	NRRL 3567	31.25	62.50	-c
Proteus vulgaris	NRLLB 123	31.25	125.00	-c
Candida albicans	OGU	62.50	31.25	*

A = *Achillea wilhelmsii* C. Koch.; B = *Achillea lycaonica* Boiss & Heldr.; c = Chloramphenicol; * = Ketoconazole

Antifungal studies: In order to obtain conidia, the fungi were cultured on Czapex Dox Agar and malt extract Agar medium in 9 cm petri dishes at 25 °C, for 10 d. Harvesting was carried out by suspending the conidia in a 1 % (w/v) sodium chloride solution contain 5 % (w/v) DMSO. The spore suspension was then filtered and transferred into tubes and stored[13] at -20 °C.

Screening for antifungal activities of the stock solution of the essential oils were performed qualitatively using the disc diffusion method (Table-4) against saprophytic fungi namely *Aspergillus flavus*, *A. niger*, *Penicillium expansum* and *Alternaria brassicola* cultured on malt extract and Czapex Dox Agar medium. For this reason one loop drop the spore suspension was applied onto the centre of the petri dishes. 20 μL stock solution of the essential oil was applied onto sterile paper discs (6 mm in diameter) and placed in the petri dishes and incubated at 25 °C for 3 d. The test solution is prepared in DMSO. The inhibition of fungal growths expressed in percentage terms was determined on the growth in test plates compared to the respective control plates as given % inhibition[14].

$$\text{Inhibition \%} = 100\ (C-T)/C$$

(C = Diameter of fungal growth on the control, T = Diameter of fungal growth on the test plate). The activities of the essential oils have been compared with the activity of standard fungicide ketoconazol.

TABLE-4
ANTIFUNGAL ACTIVITY DATA FOR THE ESSENTIAL OILS

Micro-organisms	*Achillea wilhelmsii* C. Koch.			*Achillea lycaonica* Boiss & Heldr.			Ketoconazol		
	T	C	I	T	C	I	T	C	I
A. flavus	25	55	54.545	21	55	61.818	9	55	83.63
A. niger	10	20	50.000	9	20	55.000	18	20	10.00
P. expansum	20	50	60.000	18	50	64.000	8	50	84.00
A. brassicola	12	45	73.333	12	45	73.333	22	45	54.00

C = Diameter of fungal growth on the control; T = Diameter of fungal growth on the test plate; I = Inhibition (%)

RESULTS AND DISCUSSION

Water distillation of dried aerial parts of *A. wilhelmsii* yielded 0.3 %, while that of *A. lycaonica* yielded of 0.9 % (v/w) (calculated per weight of dried material). 16 Compounds were identified from the essential oil of *Achillea wilhelmsii* and 13 compounds were from *Achillea lycaonica*. Both of the oils were characterized by high number of monoterpenes and acyclic monoterpenoids. Camphor (39.62 %), 2,5,5-trimethyl-3,6-heptadien-2-ol (16.10 %), *Artemisia alcohol* (17.92 %) in the oil of *Achillea wilhelmsii*; L-camphor (43.19 %), camphor (16.48 %) and *Artemisia alcohol* (21.18 %) were the main components of *Achillea lycaonica* (Table-1). In earlier studies eucalyptol, camphor and/or α-terpineol have been determined as major components in many *Achillea* species[15,16] and antimicrobial potentials of 1,8-cineole and camphor were determined[17].

It was interesting to note that the oil composition of *A. lycaonica* and *A. wilhelmsii* were different from the previous studies. An earlier work reported that the essential oil of *A. lycaonica* contain *trans*-sabinene hydrate (9.3 %) and caryophyllene oxide (7.2 %) as major components[9]. In that report, they determined the essential oil of *A. wilhelmsii* that was described from naplaret and it contains fragranyl acetate (25.5 %), camphor (36.1 %) and caryophyllene oxide (12.5 %) as major components. Apart from camphor, these differences can probably be attributed to the genetic differences or different ecological conditions of the plant materials.

All tested microorganisms were inhibited by the essential oils of *A. wilhelmsii* and *A. lycaonica*. The inhibition of *Enterobacter aerogenes* and *Proteus vulgaris* were more strongly inhibited by the oil of *Achillea wilhelmsii* (31.25 μg/mL) comparing to other microorganisms. On the other hand, the inactivation of *C. albicans* was affected strongly by *A. lycaonica* (31.25 μg/mL) (Table-2).

These essential oils were screened *in vitro* in order to evaluate their antifungal activity against *Aspergillus flavus, A. niger, Penicillium expansum* and *Alternaria brassicola*. The results show that the both essential oils are more toxic against *Alternaria brassicola* than other tested microfungi under the identical experimental conditions.

It is already stated that the samples containing high amount of the monoterpenes results in the influence of antibacterial and antifungal activities[18,19]. Table-1 shows oxygenated monoterpene hydrocarbons and monoterpene hydrocarbons dominate within both samples with high percentages.

The tested plant essential oils appear to be effective against a wide spectrum of microorganisms, both pathogenic and saprophytic. These compounds may be able to control a wide range of microbes but there is also the possibility that they cause an imbalance in the gut microflora. Further

studies on therapeutic applications of essential oils should be undertaken to investigate these issue, especially when considering the substantial number of analytical studies carried out on these natural products. Essential oils, which often contain the principal aromatic and flavouring components of herbs and spices, if added to foodstuffs, would cause no loss of organoleptic properties, would retard microbial contamination and therefore reduce the onset of spoilage[1].

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