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Antioxidant, anticholinesterase, and antimicrobial activities and fatty acid constituents of *Achillea cappadocica* Hausskn. et Bornm.

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Abstract: This study was the first fatty acid analysis and biological activity report on an endemic *Achillea* species, *Achillea cappadocica* Hausskn. et Bornm. The major compounds in the fatty acid were identified as oleic (34.7%), palmitic (23.1%), and linoleic acids (20.6%). The methanol and water extracts indicated higher ABTS cation radical scavenging activity than that of α-tocopherol and BHT at 100 μg/mL. The acetone extract showed 70.62% inhibitory activity against butyrylcholinesterase at 200 μg/mL. The acetone and methanol extracts exhibited moderate antimicrobial activity.

Key words: Asteraceae, *Achillea cappadocica*, fatty acid, antioxidant, anticholinesterase, antimicrobial

1. Introduction

In Turkey, the genus *Achillea* (Asteraceae) is represented by 50 species and 58 taxa; 31 taxa are endemic.[1–3] *Achillea* species, which are known as civan perçemi, pireotu, yilan çiçeği, ormaderen, buyucan, kiliçotu, and çoban kirpiği in Turkish, are widespread all over the world, and many of them have been used in traditional medicine as an antiinflammatory, diuretic, and emmenagogue and for wound healing.[4–7] They also possess antiemetic, antiviral, antiallergic, and antiirritant effects.[8–10] They are rich in sesquiterpene lactones, lignans, and flavonoids.[11,12] Many studies such as on antioxidant, anticholinesterase, antimicrobial activity, and fatty acid composition have been conducted on *Achillea* species.[13–16]

A literature survey showed that there were no previous phytochemical and biological reports on an endemic *Achillea* species, *A. cappadocica* Hausskn. et Bornm.[1] We aimed to evaluate the antioxidant, anticholinesterase, and antimicrobial activities of the petroleum ether, acetone, methanol, and water extracts of *A. cappadocica* with their total flavonoid and phenolic contents in the current study. The petroleum ether extract was also analyzed to determine its fatty acid composition by GC/MS. β-Carotene-linoleic acid test system, DPPH free and ABTS cation radical scavenging activity, and cupric reducing antioxidant capacity assays were carried out to indicate the antioxidant activity. The anticholinesterase and antimicrobial potentials of the extracts were determined by Ellman and disk diffusion methods, respectively.

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2. Results and discussion

Synthetic antioxidants (BHA, BHT, PG, etc.) have been commonly used to inhibit lipid oxidation in foods containing lipids. Numerous studies have shown that synthetic products could cause several health problems (cancer, cardiovascular diseases, etc.). Alzheimer's disease, which is a neurodegenerative disease, is a health problem in elderly people all around the world.[17] Since ancient times, plants and their extracts have been used in traditional medicine to treat various diseases. Scientific research has focused on medicinal plants, their extracts, and secondary metabolites to indicate their biological potentials since products of natural origin have fewer or no side effects compared to synthetics. For this reason, consumers have preferred to use medicinal plants and products derived from plants to protect their health.

As part of our research on the biological activity of medicinal plants and their secondary metabolites, the antioxidant, anticholinesterase, and antimicrobial potentials of an endemic species, *A. cappadocica*, were investigated along with its fatty acid profile.[18] The present study is the first report on the fatty acid composition of *A. cappadocica* . Marcincakova et al. reported that the main fatty acid constituents of *A. millefolium* were oleic (39.43%), palmitic (22.59%), and linoleic acids (19.49%).[19] As shown in Table 1, the fatty acid profile of *A. cappadocica* was found to be similar to that of *A. millefolium*. Seventeen components were identified, constituting 99.4% of the petroleum ether extract in which the major components were also oleic (34.7%), palmitic (23.1%), and linoleic acids (20.7%).

Table 1. GC-MS analysis of *A. cappadocica* petroleum ether extract.

Rt (min)[a]	Constituents[b]	Composition (%)
12.00	Lauric acid	0.3
12.75	Nonanedioic acid	0.1
14.39	10-Undecenoic acid	0.3
18.60	Myristic acid	2.5
24.63	9-Hexadecenoic acid	2.1
25.27	Palmitic acid	23.1
29.75	Phytol	1.8
30.64	Linoleic acid	20.6
30.77	Oleic acid	34.7
30.86	Linolenic acid	4.0
31.00	Vaccenic acid	2.0
31.54	Stearic acid	4.4
36.55	Eicosane	0.6
37.38	Arachidic acid	1.2
39.36	Docosane	0.6
42.65	Pentacosane	0.5
43.82	Behenic acid	0.5
	Total	99.4

[a] Retention time (in minutes) [b] Compounds listed in order of elution from a HP-5 MS column. A nonpolar Phenomenex DB-5 fused silica column

The antioxidant activity of the petroleum ether (ACP), acetone (ACA), methanol (ACM), and water (ACW) extracts prepared from the whole plant material was evaluated using different in vitro methods. The inhibition of lipid peroxidation was determined by β-carotene-linoleic acid test system, their scavenging capacity by DPPH free radical and ABTS cation radical decolorization methods, and their reducing potential by

CUPRAC assay with their total flavonoid and phenolic contents. BHT and α-tocopherol were used as reference compounds in the antioxidant assays. The acetone and water extracts, having almost the same inhibition of lipid peroxidation, exhibited moderate activity in the β-carotene bleaching method at 100 μg/mL (Figure 1). The methanol extract, which possessed the richest phenolic and flavonoid contents among the tested extracts, showed moderate DPPH free radical scavenging activity at 100 μg/mL comparable to those of the standard compounds (Table 2; Figure 2). The acetone, methanol, and water extracts indicated over 80% inhibition in ABTS cation radical scavenging assay at 100 μg/mL (Figure 3). None of the extracts had a cupric reducing effect.

Table 2. Total phenolic and flavonoid contents of the extracts.[a]

Samples	Phenolic content (μg PEs/mg extract)[b]	Flavonoid content (μg QEs/mg extract)[c]
ACP	90.23 ± 2.76	46.86 ± 0.03
ACA	98.05 ± 2.76	47.96 ± 0.36
ACM	101.95 ± 2.68	50.14 ± 0.42
ACW	92.19 ± 0.00	49.63 ± 1.59

[a] Values expressed are means ± SD of 3 parallel measurements [b] PEs, pyrocatechol equivalents (y = 0.0128 x + 0.0324 R^2 = 0.9924) [c] QEs, quercetin equivalents (y = 0.1701 x – 0.0778 R^2 = 0.9939)

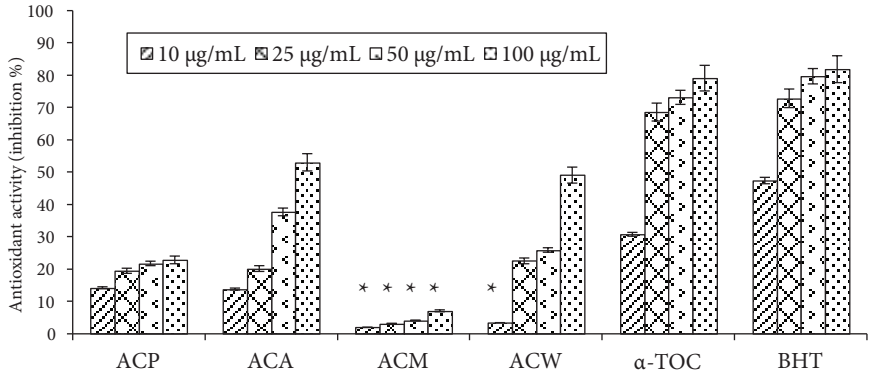

Figure 1. Inhibition (%) of lipid peroxidation of the extracts, α-tocopherol, and BHT by β-carotene bleaching method. Values are means ± SD, n = 3, P < 0.05, significantly different with Student's t-test. *The results were not significantly different.

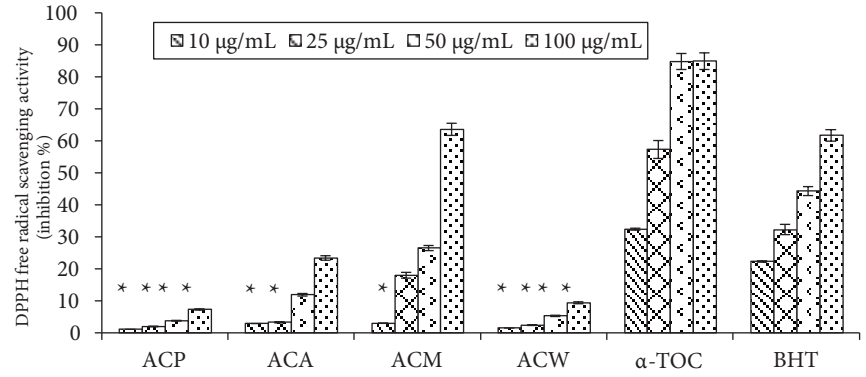

Figure 2. DPPH free radical scavenging activity of the extracts, α-tocopherol, and BHT. Values are means ± SD, n = 3, P < 0.05, significantly different with Student's t-test. *The results were not significantly different.

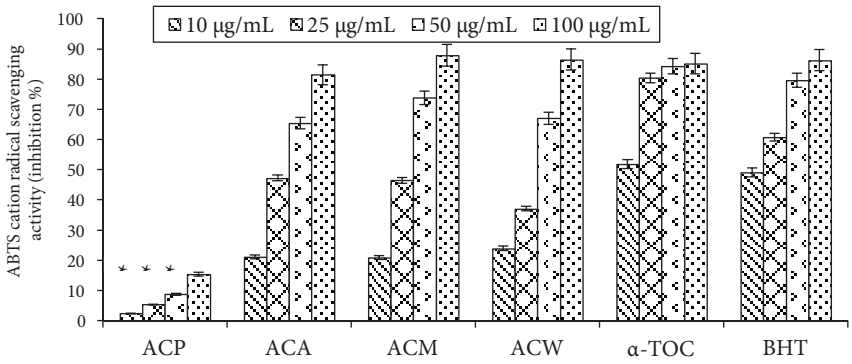

Figure 3. ABTS cation radical scavenging activity of the extracts, α-tocopherol, and BHT. Values are means ± SD, n = 3, P < 0.05, significantly different with Student's t-test. *The results were not significantly different.

Mekinic et al. reported that 80% ethanol extract of *A. millefolium* showed the highest antioxidant and acetylcholinesterase capacities among the tested plants.[16] In the present work, all of the extracts were inactive against acetylcholinesterase. As shown in Table 3, the acetone extract possessed the same inhibitory activity against butyrylcholinesterase (70.62%) as compared with a reference compound, galanthamine, at 200 μg/mL. Other treatments did not show any anticholinesterase activity. The acetone and methanol extracts exhibited moderate antimicrobial activity (inhibition zone < 20–12 mm) at 30 mg/mL concentration against *E. coli* and *C. albicans* (Table 4). Results obtained from the disk diffusion method did not make it necessary to indicate the minimum inhibition concentration (MIC).

Table 3. Anticholinesterase activity of the extracts and galanthamine at 200 μg/mL.[a]

Samples	Inhibition % against AChE	Inhibition % against AChE
ACP	3.94 ± 0.93	17.22 ± 1.41
ACA	22.70 ± 1.21	70.62 ± 1.57
ACM	NA	21.09 ± 3.80
ACW	NA	NA
Galanthamine[b]	85.09 ± 0.40	70.22 ± 1.46

[a] Values expressed are means ± SD of 3 parallel measurements and they were calculated according to negative control.
[b] Standard drug, NA: Not active

To the best of our knowledge, the current study is the first investigation on *A. cappadocica.* This work showed that the petroleum ether extract that possessed nonpolar compounds did not have antioxidant, anticholinesterase, or antimicrobial effects. In addition, the acetone, methanol, and water extracts having polar compounds showed strong ABTS cation radical scavenging activity. Further phytochemical and biological studies on the polar extracts of *A. cappadocica* could be carried out to identify their active compounds.

3. Experimental

3.1. Plant material

A. cappadocica Hausskn. et Bornm.(whole plant) was collected and identified by Dr Y Yeşil (Department of Pharmaceutical Botany, Faculty of Pharmacy, İstanbul University), from southeastern Turkey (Kürecik,

Malatya) in August (2012). This specimen has been stored at the Herbarium of İstanbul University (ISTE 98070).

Table 4. Antimicrobial activity of the extracts.

Microorganisms						
		E. coli	*S. pyogenes*	*S. aureus*	*P. aeruginosa*	*C. albicans*
	10 mg/mL	NZ*	NZ	NZ	NZ	NZ
ACP	20 mg/mL	NZ	NZ	NZ	NZ	NZ
	30 mg/mL	NZ	NZ	NZ	NZ	NZ
	10 mg/mL	10 ± 0.3	9 ± 0.7	NZ	NZ	10 ± 0.4
ACA	20 mg/mL	12 ± 0.5	9 ± 0.5	9 ± 0.6	NZ	13 ± 0.2
	30 mg/mL	13 ± 0.5	10 ± 0.2	11 ± 0.2	NZ	13 ± 0.3
	10 mg/mL	10 ± 0.6	NZ	NZ	NZ	NZ
ACM	20 mg/mL	10 ± 0.4	NZ	10 ± 0.3	NZ	NZ
	30 mg/mL	13 ± 0.2	12 ± 0.3	12 ± 0.5	NZ	NZ
	10 mg/mL	NZ	NZ	NZ	NZ	NZ
ACW	20 mg/mL	NZ	NZ	NZ	NZ	NZ
	30 mg/mL	NZ	NZ	NZ	NZ	NZ
IPM (10 μg per disk)		19 ± 1.4	39.5 ± 0.7	27.5 ± 0.7	12 ± 0	-
Nystatin (30 μg per disk)		-	-	-	-	25 ± 0.5

* Inhibition zone diameter (in mm), NZ: No zone, IPM (Imipenem): Positive control, Nystatin: Positive control

3.2. Esterification of total fatty acid and GC/MS conditions

A hundred milligrams of the petroleum ether extract was refluxed in 0.1 M KOH solution in 2 mL of methanol for 1 h, the solution was cooled, and 5 mL of water was added. The aqueous mixture was neutralized with 0.5 mL of HCl solution and it was extracted with diethyl ether:hexane (1:1; 3.5 mL). The separating organic phase was washed with 10 mL of water and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and then fatty acid methyl esters were obtained.[17] The analyses were performed using a Thermo Scientific Polaris Q GC-MS/MS. The GC/MS procedure described by Sabudak et al. was applied.[20]

3.3. Preparation of the extracts

The plant material (100 g) was dried and powdered, and it was sequentially macerated with petroleum ether (250 mL × 3), acetone, methanol, and water for 24 h at 25 °C. After filtration, the solvents were evaporated to obtain crude extracts.

3.4. Determination of total flavonoid and phenolic contents

The concentrations of flavonoid and phenolic contents of the crude extracts were expressed as quercetin and pyrocatechol equivalents, respectively, and they were calculated according to the following equations:[21,22]

Absorbance = 0.1701 quercetin (μg) – 0.0778 (R^2 = 0.9939)

Absorbance = 0.0128 pyrocatechol (μg) + 0.0324 (R^2 = 0.9924)

3.5. Antioxidant activity of the extracts

3.5.1. β-Carotene bleaching method

First, 0.5 mg of β-carotene in 1 mL of chloroform was added to a linoleic acid (25 μL) and Tween 40 emulsifier (200 mg) mixture. After evaporating the chloroform, 100 mL of distilled water saturated with oxygen was added, followed by shaking; 160 μL of this mixture was transferred into different test tubes containing 40 μL of the sample solutions at different concentrations. The emulsion was added to each tube and the zero time absorbances of the values were read at 470 nm. The mixture was incubated for 2 h at 50 °C.[23]

3.5.2. Free radical scavenging activity method

First, 160 μL of 0.1 mM DPPH solution in methanol was added to 40 μL of sample solutions in methanol at different concentrations. After 30 min the absorbance values were read at 517 nm. The DPPH free radical scavenging potential was calculated using the following equation:[24]

DPPH scavenging effect (Inhibition %) = $\frac{A_{control}-A_{sample}}{A_{control}} \times 100$

$A_{Control}$ is the initial concentration of the $DPPH^{\bullet}$

A_{Sample} is the absorbance of the remaining concentration of $DPPH^{\bullet}$ in the presence of the extracts or positive controls.

3.5.3. ABTS cation radical decolorization assay

First, 7 mM ABTS in H_2O was added to 2.45 mM potassium persulfate to produce $ABTS^{\bullet+}$ and solution was stored in the dark at 25 °C for 12 h. The prepared solution was diluted with ethanol to get an absorbance of 0.700 ± 0.025 at 734 nm. $ABTS^{\bullet+}$ solution (160 μL) was added to each sample solution at different concentrations. After 30 min, the percentage inhibition at 734 nm was read for each concentration relative to a blank absorbance (methanol). The following equation was used to calculate the scavenging capability of $ABTS^{\bullet+}$:[25]

$ABTS^{\bullet+}$ scavenging effect (Inhibition %) = $\frac{A_{control}-A_{sample}}{A_{control}} \times 100$

3.5.4. Cupric reducing antioxidant capacity (CUPRAC) method

The petroleum ether and acetone extracts were dissolved in methanol, and methanol and water extracts in distilled water to prepare their stock solution at 1000 μg/mL concentration. Aliquots of 61 mL of 1.0 $\times$ 10^{-2} M copper(II) chloride, 61 μL of NH_4OAc buffer (1 M, pH 7.0), and 61 μL of 7.5 $\times$ 10^{-3} M neocuproine solution were mixed; $x\mu$L of sample solution (2.5, 6.25, 12.5, and 25 μL) and (67 $-x)\mu$L of distilled water were added to make the final volume 250 μL. The tubes were stopped, and after 1 h the absorbance at 450 nm was measured against a reagent blank.[26]

3.6. Anticholinesterase activity of the extracts

All samples were dissolved in ethanol to prepare their stock solution at 4000 μg/mL concentration. Aliquots of 150 μL of 100 mM sodium phosphate buffer (pH 8.0), 10 μL of sample solution, and 20 μL BChE (or AChE) solution were mixed and incubated for 15 min at 25 °C, and DTNB (10 μL) was added. The reaction was then initiated by the addition of butyrylthiocholine iodide (or acetylthiocholine iodide) (10 μL). The final

concentration of the tested solutions was 200 μg/mL.[27] The hydrolysis of these substrates was monitored using a BioTek Power Wave XS at 412 nm.

3.7. Antimicrobial activity of the extracts

The extracts were tested using the disk diffusion method against gram-positive bacteria (*Streptococcus pyogenes* ATCC19615, *Staphylococcus aureus* ATCC 25923), gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922), and a yeast (*Candida albicans* ATCC10231), which were purchased from Refik Saydam Sanitation Center (Turkey).[28] Bacterial strains were cultured overnight in nutrient broth (NB) at 37 °C and *C. albicans* at 30 °C. The nutrient agar plates were inoculated with 100 μL of microorganism culture (10^8 CFU/mL). Fifteen microliters of the extracts (10, 20, 30 mg/mL) was loaded onto a sterile filter paper disk (6 mm) and placed on the inoculated plates. The seeded plates were incubated at 37 °C for bacteria (24 h) and 30 °C for yeast (48 h). The same procedure was applied for the positive controls, imipenem (IPM) and nystatin for bacteria and yeast, respectively.

3.8. Statistical analysis

The results of the antioxidant and anticholinesterase activity assays were mean ± SD of 3 parallel measurements. The statistical significance was estimated using Student's t-test. P values <0.05 were regarded as significant.

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