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In vitro Study of Antibacterial Activity on Multi-Resistant Bacteria and Chemical Composition of the Chloroform Extract of Endemic *Centaurea drabifolia* subsp. *cappadocica*

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The antimicrobial activity of the *n*-hexane, chloroform, ethyl acetate and ethanol extracts of the aerial parts of *C. drabifolia* S.M. subsp. *cappadocica* (DC.) Wagenitz (Asteraceae) was evaluated against microorganisms including multi-antibiotic resistant bacteria using the paper disc diffusion method. The chemical composition of the chloroform extract of this plant was determined by gas chromatography and gas chromatography-mass spectrometry. The chloroform extract exhibited significant antibacterial activity against all the bacteria tested, except *Stenotrophomonas maltophilia* MU63. The major compounds of the chloroform extract were spathulenol (14.1%), caryophyllene oxide (12.5%), octadecanol (10.2%), ethyl palmitate (7.7%), [*Z,Z*]-10,12-hexadecadienal (6.0%), 3-hydroxy *p*-anisaldehyde (5.9%) and pentacosane (5.8%).

Keywords: *Centaurea drabifolia* ssp. *cappadocica*, chemical composition, antimicrobial activity.

There is an increasing interest in medicinal plants as an alternative to synthetic drugs, particularly against microbial agents because of the growth of antibiotic resistance [1]. About 20,000 plant species used for medicinal purposes are reported by WHO [2].

Many members of the genus *Centaurea* have long been used in Anatolian folk medicine [3a-3c]. The aerial parts of the plants are known in Turkey as 'peygamber cicegi, zerdali dikeni, coban kaldiran, and timur dikeni' [3a,4]. *C. drabifolia* subsp. *cappadocica* is an endemic species of Turkey. It is native to south Anatolia and the eastern part of central Anatolia, and grows on rocky slopes at altitudes of 1300-1600 m [4]. This species has no popular use described in the academic literature. The aim of this study was to identify the chemical composition and evaluate the antimicrobial activity of extracts of this species against different microorganisms including multi-resistant bacteria.

Ethanol, *n*-hexane, chloroform and ethyl acetate extracts of *C. drabifolia* ssp. *cappadocica* were investigated for their antimicrobial activities. Nine standard test microorganisms (*Micrococcus luteus* NRRL B-4375, *Bacillus subtilis* ATCC 6633, *Streptococcus mutans* CNCTC 8/77, *Staphylococcus aureus* ATCC 25923, *Enterobacter aerogenes* RSKK 720, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10239, *Candida tropicalis* RSKK 665), and multi-resistant strains of *S. maltophilia* and various species of *Staphylococcus* were used. The antibiotic resistance patterns of the multi-antibiotic resistant bacteria are shown in Table 1.

The ethyl acetate and ethanol extracts inhibited all of the Gram-negative bacteria, including the multi-antibiotic resistant strains of *S. maltophilia*. The chloroform extract inhibited all *S. maltophilia* strains, except for *S. maltophilia* MU 63.

Table 1: Antibiotic resistance patterns of *S. maltophilia* and *Staphylococcus* species.

Strains	Resistance Patterns
S. maltophilia MU 23	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 25	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 52	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, TVA, AM PRL, ATM, SAM, AMC
S. maltophilia MU 53	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 63	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 64	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, SXT, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 69	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, SXT, TVA, AM, PRL, ATM, SAM, AMC
S.maltophilia MU 94	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 99	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, SXT, TVA, AM, PRL, ATM,SAM, AMC
S. maltophilia MU 136	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
S. maltophilia MU 137	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
Staphylococcus xylosus MU 34	P, AK, DA, E, CN, OX, TEC
S. xylosus MU 35	P, DA, E, C, OX, TE
S. xylosus MU 37	P, AK, DA, E, CN, TEC, TE
S. xylosus MU 42	P, AK, DA, CN, OX, TE
S. aureus MU 38	P, AK, DA, CN, ME, TEC, TE, OX
S. aureus MU 40	P, AK, CN, C, ME, OX, TE
S. aureus MU 46	P, AK, DA, E, CN, TE, OX
Staphylococcus sp. MU 28	P, AK, DA, E, CN, TE
Staphylococcus capitis MU 27	P, AK, DA, E, CN, TE
Staphylococcus epidermidis MU 30	P, AK, DA, CN, OX, TEC, TE
Staphylococcus lentus MU 43	P, AK, DA, CN, OX, TE

MEZ: Mezlocillin (75 µg), TIM: Ticarcillin+clavulanic acid (75+ 10 µg), CAZ: Ceftazidime (30 µg), FEP: Cephepim (30 µg), CRO: Ceftriaxone (30 µg), CTX: Cefotaxime (30 µg), KF: Cephalothin (30 µg), IPM: Imipenem (10 µg), P: Penicillin (10 U), AK: Amikacin (30 µg), TOB: Tobramycin (10 µg), NET: Netilmicin (30 µg), CN: Gentamicin (10 µg), TE: Tetracycline (30 µg), NOR: Norfloxacin (10 µg), C: Chloramphenicol (30 µg), TVA: Trovafloksasin (10 µg), AM: Ampicillin (10 µg), PRL: Piperacillin (100 µg), ATM: Aztreonam (30 µg), SAM: Sulbactam + Ampicillin (10 µg + 10 µg), AMC: Amoxicillin + Clavulanic acid (20 µg + 10 µg), CIP: Ciprofloxacin (5 µg), SXT: Trimetoprim+sulfamethoxazole (1.25 µg + 23.75 µg), DA: Clindamycin (2 µg); E: Erythromycin (15 µg); ME: Methicillin (5µg); OX: Oxacillin (1µg); TEC: Teicoplanin (30 µg).

Multidrug resistance is common and increasing among Gram-negative nonfermenters, and a number of strains have now been identified that exhibit resistance to essentially all commonly used antibiotics, including antipseudomonal penicillins and cephalosporins, aminoglycosides, tetracyclines, fluoroquinolones, trimethoprim-sulfamethoxazole, and carbapenems [5]. *S. maltophilia* is one of the important members of this group.

S. maltophilia has received much attention in the last decade because of its role as a pathogenic microorganism in an increasing number of clinical syndromes [6], such as bacteremia, infections of the respiratory and urinary tracts, skin and soft tissue infections, biliary tract infection, meningitis, serious wound infections, conjunctivitis and endocarditis [7]. The treatment of infections caused by this microorganism is difficult because *S. maltophilia* is frequently resistant to most of the widely used antibiotics [8]. This *in vitro* study provided evidence that the extracts of *C. drabifolia* subsp. *cappadocica* are potentially rich source of antibacterial agents against multi-antibiotic resistant *S. maltophilia*.

The chloroform extract was active against all the tested Gram-positive bacteria, including the multi-antibiotic resistant strains of *Staphylococcus*. The antibacterial activities of the chloroform extract on the Gram-positive bacteria were higher than on Gram-negative bacteria.

Staphylococci are among the most commonly encountered pathogens in clinical practice. *S. aureus* is a major cause of nosocomial infections, food poisoning, osteomyelitis, pyoarthritis, endocarditis, toxic shock syndrome, and a broad spectrum of other disorders [9]. In recent years, several species of coagulase negative staphylococci (CNS) have been recognized as opportunistic pathogens and have been implicated in human infections and disease, especially in immune-compromised and seriously ill patients. In recent years, there has been an alarming increase in nosocomial staphylococcal infections by strains with multiple drug resistance [10].

The ethyl acetate and ethanol extracts had antibacterial activity on all of the tested Gram-negative bacteria, including the multi-antibiotic resistant strains of *S. maltophilia*. The ethanol extract also inhibited the Gram-positive bacteria, except *S. capitis* MU 27. The chloroform extract inhibited the all of the Gram-positive and Gram-negative bacteria, except *S. maltophila* MU 63.

The results indicate that the chloroform extract of *C. drabifolia* subsp, *cappadocica* has a capacity to inhibit the growth of multi-antibiotic resistant strains. For this reason, the chemical composition of its chloroform extract was determined. Twenty compounds were detected using GC and GC-MS (Table 4). The percentage composition of the chloroform extract was determined with a Class-GC

Table 2: Antimicrobial activities of the various extracts of *C. drabifolia* subsp. *cappadocica* on Gram-negative bacteria.

Strains	Inhibition zone (mm)			
	n-Hexane extract	Chloroform extract	Ethanol extract	Ethyl acetate extract
E. aerogenes RSKK 720	-	11	15	11
P. aeruginosa ATCC 27853	-	16	15	13
E. coli ATCC 25922	11	13	14	12
S. maltophila MU 23	15	11	10	14
S. maltophila MU 25	13	11	15	19
S. maltophila MU 52	14	10	15	16
S. maltophila MU 53	13	11	19	18
S. maltophila MU 63	11	-	14	12
S. maltophila MU 64	-	12	16	13
S. maltophila MU 69	-	9	10	15
S. maltophila MU 94	-	12	11	11
S. maltophila MU 99	12	12	15	19
S. maltophila MU 136	-	11	13	14
S. maltophila MU 137	14	9	12	16

(-) : No activity

Table 3: Antimicrobial activities of the various extracts of *C. drabifolia* subsp. *cappadocica* on Gram-positive bacteria and yeasts.

Strains	Inhibition zone (mm) of the extracts			
	n-Hexane extract	Chloroform extract	Ethanol extract	Ethyl acetate extract
M. luteus NRRL B- 4375	9	13	12	12
B. subtilis ATCC 6633	10	14	14	10
S. aureus ATCC 25923	11	17	14	-
S. mutans CNCTC 8/77	-	16	10	10
S. capitis MU 27	-	22	-	16
Staphylococcus sp. MU 28	-	15	15	17
S. epidermidis MU 30	-	19	17	-
S. xylosus MU 34	-	21	14	11
S. xylosus MU 35	-	16	13	19
S. xylosus MU 37	-	16	14	10
S. aureus MU 38	-	14	16	-
S. aureus MU 40	14	16	12	13
S. xylosus MU 42	9	16	16	18
S. lentus MU 43	14	20	12	10
S. aureus MU 46	11	17	15	14
C. albicans ATCC 10239	-	-	-	-
C. tropicalis RSKK 665	-	-	-	-

(-) : No activity

computer program. The major compounds were spathulenol (14.1%), caryophyllene oxide (12.5%), octadecanol (10.2%), ethyl palmitate (7.7%), [*Z,Z*]-10,12-hexadecadienal (6.0%), 3-hydroxy-*p*-anisaldehyde (5.9%) and pentacosane (5.8%).

The components of the extract were separated into five classes: oxygenated monoterpene hydrocarbons (11.3%), sesquiterpene hydrocarbons (5.5%), oxygenated sesquiterpenes (30.0%), aromatic alcohols (3.8%) and others (49.4%). Of the oxygenated sesquiterpenes, spathulenol (14.1%) and caryophyllene oxide (12.5%) were the main components.

Sesquiterpenes have been reported to have potent antimicrobial activity and play a critical role in plant defense mechanisms [11]. Spathulenol and caryophyllene oxide are also known to exhibit antibacterial activity [12a,12b,13]. In this study, these are the most abundant constituents of the chloroform extract.

Table 4: Chemical composition of chloroform extract of *C. drabifolia* subsp. *cappadocica*

No	Compounds[a]	RI[a]	Percentage (%)	Methods
1	2-Amino, *p*-cymene	842	4.9	b
2	5-Amino, 2-methoxyphenol	916	3.8	b
3	*cis*-7-Decen-1-al	973	4.9	b
4	[*E-E*]-2,4-Decadienal	1024	1.7	a, b
5	3-Hydroxy, *p*-anisaldehyde	1082	5.9	a, b
6	β-cyclocitral	1135	2.9	a, b
7	Vanillin	1197	2.5	a, b
8	[*E-E*]-2,4-Dodecadienal	1256	1.5	b
9	β-Caryophyllene	1298	2.6	a, b
10	*cis*-α-Bisabolene	1307	2.8	a, b
11	Caryophyllene oxide	1324	12.5	a, b
12	Spathulenol	1355	14.1	a, b
13	[*Z,Z*]-10,12-Hexadecadienal	1478	6.0	b
14	2-Methyl hexadecanol	1527	1.6	b
15	Ethyl palmitate	1619	7.7	a, b
16	[*Z*]-9-Octadecenal	1674	3.0	b
17	Hexahydro farnesyl acetone	1746	3.4	a, b
18	Heneicosane	1867	1.9	a, b
19	Pentacosane	2103	5.8	b
20	Octadecan-1-ol	2149	10.2	a, b
	TOTAL		100.0	

a: co-injection with authentic compounds, b: MS,
[a] : In DB-5 fused silica capillary column

To our knowledge, this is the first study of the antimicrobial activity and chemical composition of extracts of *C. drabifolia* subsp. *cappadocica*. Our results indicate that the chloroform extract of this species has a capacity to inhibit the growth of pathogenic bacteria, especially multi-antibiotic resistant strains of *S. aureus*. These extract may be useful as alternative antimicrobial agents for multi-antibiotic resistant Staphylococci.

Experimental

Plant material and extraction: *C. drabifolia* ssp. *cappadocica* (Asteraceae) was collected at the flowering stage from Mugla, Turkey. A voucher specimen (Herbarium No: O.V. 4448) has been deposited in the Herbarium of University of Mugla, Turkey. The air dried and powdered aerial parts of *C. drabifolia* ssp. *cappadocica* were extracted successively with *n*-hexane, chloroform, ethyl acetate and ethanol in a Soxhlet apparatus. Solvents of all the extracts were removed under low vacuum using rotary evaporation. Crude extracts were maintained at +4ºC until investigated for antimicrobial activity.

Microorganisms: Three Gram-negative, 4 Gram-positive and multi-antibiotic resistant bacteria, and 2 yeasts were used. The strains of MU coded were obtained from Mugla University Culture Collection.

Disc diffusion assay: The antibacterial activity was based on a disc diffusion method [14-16] using bacterial cell suspensions whose concentration was equilibrated to 0.5 McFarland standard after the bacteria cultured. Each bacterial suspension (100 µL)

was spread on a Mueller–Hinton agar plate. Sterile paper discs (6 mm diameter) were impregnated with 20 µL of each extract dissolved in the solvent used for extraction at 25 mg/mL. The discs were allowed to dry and then placed on the inoculated agar. The plates were incubated at appropriate temperature and time for microorganisms. Discs of *n*-hexane, chloroform, ethanol, and ethyl acetate were used as controls. After the incubation time, the zones of inhibition were measured. The experiment was performed in triplicate.

Gas chromatography (GC) and GC-MS analysis: GC and GC/MS analyses were performed under the experimental conditions as reported earlier [13]. Identification of the components was based on GC retention indices and computer matching of MS with those of standards (NIST & Wiley, and a personal library of 320 spectra), as well as by comparison with the fragmentation patterns of MS reported in the literature [17] and, whenever possible, by co-injection with authentic compounds.

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