

Chemical composition of endemic *Centaurea austro-anatolica* and studies of its antimicrobial activity against multi-resistant bacteria

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Hexane, chloroform, ethyl acetate and ethanolic extracts of the aerial parts of *Centaurea austro-anatolica* Hub.-Mor. (*Asteraceae*) were evaluated against microorganisms, including multiresistant bacteria, using a paper disc diffusion method. The chloroform extract exhibited significant antibacterial activity toward all bacteria tested. The chemical composition of the chloroform extract was determined by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The major compounds of the extract were caryophyllene oxide (21.32 %), spathulenol (10.86 %), *n*-tricosanol (9.58 %) and geranyl isovalerate (8.71 %).

Keywords: *Centaurea austro-anatolica* (*Asteraceae*), chemical composition, antimicrobial activity

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Many members of the genus *Centaurea* have been long used in Anatolian folk medicine (1). Various *Centaurea* species have certain biological properties, such as antimicrobial (2), antifungal (3), anti-inflammatory (4), anti-ulcerogenic (5), antioxidant (6), antiviral (7), anti-*Helicobacter pylori* (8), antiprotozoal (2), anticancer and cytotoxic properties (9).

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C. austro-anatolica is an endemic species distributed in the Aegean region of Turkey and is an East-Mediterranean element (10). The aim of this study was to identify the chemical composition and to evaluate the antimicrobial activity of the extracts of *C. austro-anatolica* against different microorganisms, including multi-resistant bacteria. The plant species was selected because many species of this genus are used in traditional medicine for various purposes and because of the absence of phytochemical and biological studies of *C. austro-anatolica*.

EXPERIMENTAL

Chemicals

Silicagel 60, used for column chromatography, and Kieselgel 60F254, used for TLC as precoated plates, *n*-hexane, chloroform, ethyl acetate and methanol were obtained from Merck (Germany). Reference standards used for co-injection in GC α -cubebene (purity 97 %), β -bourbonene (purity \geq 98 %), spathulenol (purity \geq 98 %), *n*-tricosanol (purity \geq 97 %), *n*-heptacosanol (purity \geq 98 %), 2-decenal (purity \geq 95 %), caryophyllene oxide (purity \geq 90 %), 2-buthyl-1-octanol (95 %) and β -eudesmol (purity \geq 90 %) were purchased from Fluka and Sigma-Aldrich (USA). Ascaridole (purity \geq 97 %) ascariole epoxide (purity \geq 96 %), cubenol (purity \geq 97 %), ledene oxide-(II) (purity \geq 96 %), and hexahydro farnesyl acetone (purity \geq 96 %) were obtained from the Department of Chemistry, Faculty of Sciences and Arts, University of Mugla, Turkey.

Plant material and crude extracts

C. austro-anatolica naturally growing plants belonging to *Asteraceae* were collected at the flowering stage from Mugla, Turkey. A voucher specimen has been deposited in the Herbarium of the Faculty of Arts and Sciences, University of Mugla, Turkey. The plant was identified immediately after collection and air-dried at room temperature for later analysis.

The air dried and powdered aerial parts of *C. austro-anatolica* were extracted successively with hexane, chloroform, ethyl acetate and ethanol in a Soxhlet apparatus until the last portion of the extract became colorless. Solvents of all extracts were removed under low vacuum by using rotary evaporation. Crude extracts were maintained at +4 °C until use. Crude extracts were investigated for antimicrobial activity.

Chemical composition of the chloroform extract

Column chromatography. – For CC, silica-gel 60 (63–210 μ m) was used as adsorbent in a column and mobile phases were 95:5, 90:10 and 85:15 hexane/acetone systems. The fractions were purified by TLC and subjected to GC and GC-MS analysis.

Gas chromatography (GC). – GC analysis of the extract was performed using a Shimadzu GC-17 AAF, V3, 230V LV (Japan) series gas chromatograph equipped with a FID and a DB-5 fused silica capillary column (30 m \times 0.32 mm i.d., film thickness 0.25 μ m).

The initial oven temperature was kept at 100 °C for 5 min, then programmed to 240 °C at 3 °C min⁻¹ and kept at this temperature for 30 min; injector and detector temperatures were 250 and 270 °C, respectively. Carrier gas was He at a flow rate of 1.4 mL min⁻¹, sample size was 1.0 µL, split ratio was 50:1. The percentage composition of the chloroform extract was recorded using the Class-GC 10 computer program.

Gas chromatography-mass spectrometry (GC/MS). – Solution of the dry chloroform extract in CHCl₃ (50 mg mL⁻¹) was prepared for GC and GC-MS analyses. The analysis of the extract was performed using a Varian Saturn 2100 (Varian, USA) equipped with a Agilent DB-5 MS fused silica capillary column (Agilent Technologies, USA) (30 m × 0.32 mm i.d., film thickness 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium at a flow rate of 1.7 mL min⁻¹. Injector and MS transfer line temperatures were set at 220 °C and 290 °C, respectively. The oven temperature was kept at 100 °C for 5 min, then increased to 240 °C with 3 °C min⁻¹ increment and maintained at this temperature for 25 min. Diluted samples (1/100, V/V, in methylene chloride) (1.0 µL) were injected manually in the splitless mode. The relative percentage of extract constituents was expressed as percentage by peak area normalization.

The compounds were identified by using NIST 2002, Wiley library data of GC-MS systems and a locally customized library of 320 spectra, as well as by comparison with the fragmentation patterns of the mass spectra with those reported in the literature (11) and, whenever possible, using reference substances, by co-injection with authentic compounds.

Antimicrobial assay

Microorganisms and cultivation conditions. – Gram-negative bacteria *Enterobacter aerogenes* RSKK 720, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922, Gram-positive bacteria *Micrococcus luteus* NRRL B-4375, *Bacillus subtilis* ATCC 6633, *Streptococcus mutans* CNCTC 8/77 and *Staphylococcus aureus* ATCC 25923, yeasts *Candida albicans* ATCC 10239, *Candida tropicalis* RSKK 665 and multi-resistant strains of *S. maltophilia* and various species of *Staphylococcus*, including coagulase negative *Staphylococci* (CNS), were used. The multi-resistant bacteria were obtained from the Mugla University Culture Collection.

The above mentioned bacteria, except *S. mutans*, were cultured in Nutrient Broth (NB) (Difco, USA), *S. mutans* was cultured in Brain Heart Infusion Broth (BHIB) (Difco), *C. albicans* and *C. tropicalis* were cultured in Sabouraud Dextrose Broth (SDB) (Difco). *P. aeruginosa* and *S. maltophilia* strains and the fungi were incubated at 30 ± 0.1 °C for 18–24 h and 24–48 h, respectively. Other bacteria were incubated at 37 ± 0.1 °C for 24–48 h. Inocula were prepared by adjusting the turbidity of the medium to match the 0.5 McFarland standard dilutions of this suspension in 0.1 % peptone (*m/V*) solution in sterile water inoculated on NB, BHIB, SDB to check the viability of the preparation. The cultures of microorganisms were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

Disc diffusion assay. – The antibacterial activity was based on the disc diffusion method (12, 13) using a bacterial cell suspension whose concentration was equilibrated to

the 0.5 McFarland standard dilutions. Each bacterial suspension 100 μL was spread on a Müller-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^{-1} . The discs were allowed to dry and were then placed on the inoculated agar. The plates were incubated at appropriate temperature and time for the microorganisms, as mentioned above. Discs with hexane, chloroform, ethyl alcohol and ethyl acetate were used as controls. After incubation time, the zone of inhibition was measured. The experiments were performed in triplicate.

RESULTS AND DISCUSSION

A total of 28 compounds was detected in the chloroform extract using GC and GC/MS (Table I). The typical chromatogram can be seen in Figure I.

Components of the chloroform extract were separated into five classes, including oxygenated monoterpene hydrocarbons (0.91 %), sesquiterpene hydrocarbons (3.14 %), oxygenated sesquiterpenes (60.54 %), aromatic alcohols (3.12 %) and others (32.29 %). The chloroform extract consisted mainly of oxygenated sesquiterpenes (60.54 %). Caryophyllene oxide (21.32 %) and spathulenol (10.32 %) were the prevailing oxygenated sesquiterpenes.

Three Gram-negative test bacteria, four Gram-positive test bacteria, two yeasts and multi-resistant bacteria were used in this study. If the extracts had any effect on the

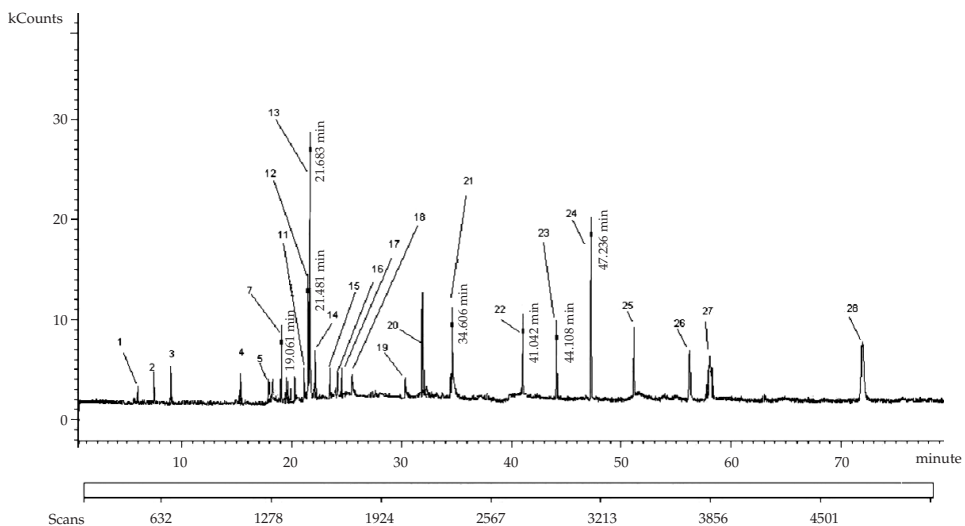


Fig. 1. Typical chromatogram of chloroform extract of *C. austro-anatolica*. For keys to the peaks see Table I.

Gram-negative and Gram-positive test bacteria, its antibacterial activity was evaluated on multi-resistant strains of *Stenotrophomonas maltophilia* and various species of *Staphylococcus*, including CNS, respectively. The antibiotic resistance patterns of the multi-resistant bacteria are shown in Table II. The results obtained by evaluation of the antimicrobial activity of the extracts are shown in Table III.

Table I. Chemical compositions of the chloroform extract of *C. austro-anatolica*

No.	Compound ^a	Percentage (%)	Method
1	3-Pentyl-2,4-pentadien-1-ol	0.91	b
2	5-Amino-2-methoxyphenol	1.27	b
3	α -Ethyl- <i>p</i> -methoxybenzylalcohol	1.85	b
4	2-Decenal	1.37	b, c
5	Ascaridol	0.96	b, c
6	2-Buthyl-1-octanol	0.87	b, c
7	7-Tetradecene	2.58	b
8	Ascaridole epoxide	0.92	b, c
9	2-Methyl-(<i>Z</i>)-4-tetradecene	0.98	b
10	α -Cubebene	1.54	b, c
11	β -Bourbonene	1.60	b, c
12	Spathulenol	10.86	b, c
13	Caryophyllene oxide	21.32	b, c
14	Cubenol	1.89	b, c
15	β -Eudesmol	1.12	b, c
16	Isoaromadendrene epoxide	0.87	b, c
17	Ledene oxide-(II)	0.94	b, c
18	<i>trans</i> -(<i>Z</i>)- α -Bisabolene epoxide	1.31	b
19	Cedrane-8,13-diol	1.17	b
20	Hexahydro farnesyl acetone	6.32	b, c
21	2,6,10-Trimethyl-9-undecenal (adoxal)	4.15	b
22	2-Methyl-1-hexadecanol	3.28	b
23	4-Methyl-docosane	2.46	b
24	<i>n</i> -Tricosanol	9.58	b, c
25	11-(1-Ethyl propyl)-heneicosane	2.81	b
26	<i>n</i> -Heptacosanol	3.97	b, c
27	(<i>Z</i>)-5-Methyl-6-heneicosen-11-one	4.39	b
28	Geranyl isovalerate	8.71	b
	Total	100	

^a In DB-1 fused silica capillary column.

b – MS

c – Co-injection with authentic compound.

Table II. Antibiotic resistance patterns of *S. maltophilia* and *Staphylococcus*

Strain	Resistance patterns
<i>S. maltophilia</i> MU 23	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
<i>S. maltophilia</i> 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
<i>S. maltophilia</i> 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
<i>S. maltophilia</i> MU 53	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
<i>S. maltophilia</i> 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
<i>S. maltophilia</i> 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
<i>S. maltophilia</i> 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
<i>S. maltophilia</i> MU 94	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
<i>S. maltophilia</i> 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
<i>S. maltophilia</i> MU 136	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, NOR, C, TVA, AM, PRL, ATM, SAM, AMC
<i>S. maltophilia</i> 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
<i>Staphylococcus xylosum</i> MU 34	P, AK, DA, E, CN, OX, TEC
<i>S. xylosum</i> MU 35	P, DA, E, C, OX, TE
<i>S. xylosum</i> MU 37	P, AK, DA, E, CN, TEC, TE
<i>S. xylosum</i> MU 42	P, AK, DA, CN, OX, TE
<i>S. aureus</i> MU 38	P, AK, DA, CN, ME, TEC, TE, OX
<i>S. aureus</i> MU 40	P, AK, CN, C, ME, OX, TE
<i>S. aureus</i> MU 46	P, AK, DA, E, CN, TE, OX
<i>Staphylococcus sp.</i> MU 28	P, AK, DA, E, CN, TE
<i>S. capitis</i> MU 27	P, AK, DA, E, CN, TE
<i>S. epidermidis</i> MU 30	P, AK, DA, CN, OX, TEC, TE
<i>S. lentus</i> MU 43	P, AK, DA, CN, OX, TE

Antibiotics dosing 6-mm discs (in $\mu\text{g cm}^{-2}$) except penicillin (in U cm^{-2}): MEZ – mezlocillin (75), TIM – ticarcillin + clavulanic acid (75 + 10), CAZ – ceftazidime (30), FEP – cephepim (30), CRO – ceftriaxone (30), CTX – cefotaxime (30), KF – cephalothin (30), IPM – imipenem (10), P – penicillin (10 U), AK – amikacin (30), TOB – tobramycin (10), NET – netilmicin (30), CN – gentamicin (10), TE – tetracycline (30), NOR – norfloxacin (10), C – chloramphenicol (30), TVA – trovafloksasin (10), AM – ampicillin (10), PRL – piperacillin (100), ATM – aztreonam (30), SAM – sulbactam + ampicillin (10 + 10), AMC – amoxicillin + clavulanic acid (20 + 10), CIP – ciprofloxacin (5), SXT – trimetoprim + sulfamethoxazole (1.25 + 23.75), DA – clindamycin (2); E – erythromycin (15); ME – methicillin (5); OX – oxacillin (1); TEC – teicoplanin (30).

Table III. Antimicrobial activity of *C. austro-anatolica* extracts

Strain	Inhibition zone (mm)			
	Hexane extract	Chloroform extract	Ethanollic extract	Ethyl acetate extract
<i>E. aerogenes</i> RSKK 720	–	13	–	–
<i>P. aeruginosa</i> ATCC 27853	–	15	13	–
<i>E. coli</i> ATCC 25922	–	20	12	–
<i>S. maltophila</i> MU 23	NT	19	13	NT
<i>S. maltophila</i> MU 25	NT	12	9	NT
<i>S. maltophila</i> MU 52	NT	12	14	NT
<i>S. maltophila</i> MU 53	NT	20	19	NT
<i>S. maltophila</i> MU 63	NT	12	15	NT
<i>S. maltophila</i> MU 64	NT	20	20	NT
<i>S. maltophila</i> MU 69	NT	11	10	NT
<i>S. maltophila</i> MU 94	NT	19	13	NT
<i>S. maltophila</i> MU 99	NT	20	11	NT
<i>S. maltophila</i> MU 136	NT	12	18	NT
<i>S. maltophila</i> MU 137	NT	11	15	NT
<i>M. luteus</i> NRRL B-4375	9	19	–	–
<i>B. subtilis</i> ATCC 6633	–	18	–	–
<i>S. mutans</i> CNCTC 8/77	–	13	–	–
<i>S. aureus</i> ATCC 25923	–	17	22	–
<i>S. capitis</i> MU 27	–	21	–	NT
<i>Staphylococcus</i> sp. MU 28	–	15	–	NT
<i>S. epidermidis</i> MU 30	–	16	18	NT
<i>S. xylosus</i> MU 34	–	21	20	NT
<i>S. xylosus</i> MU 35	–	19	22	NT
<i>S. xylosus</i> MU 37	–	20	21	NT
<i>S. xylosus</i> MU 42	–	16	21	NT
<i>S. lentus</i> MU 43	–	20	22	NT
<i>S. aureus</i> MU 38	–	14	18	NT
<i>S. aureus</i> MU 40	–	18	15	NT
<i>S. aureus</i> MU 46	–	21	18	NT
<i>C. albicans</i> ATCC 10239	–	–	–	–
<i>C. tropicalis</i> RSKK 665	–	12	12	–

NT – not tested, (–) – no activity

Since hexane and ethyl acetate extracts had no effect on Gram-negative bacteria, their antibacterial activities were not determined on antibiotic resistant *S. maltophilia* strains. Hexane extract showed no inhibition effect on Gram-positive bacteria either, except *M. luteus* or multi-resistant strains of various species of *Staphylococcus*. Chloroform extract inhibited the growth of all microorganisms, except *C. albicans* and the inhibition zones ranged between 11–21 mm. Ethanolic extract had antimicrobial effect on many of the microorganisms and the inhibition zones ranged between 9–22 mm. Ethyl acetate extract did not inhibit the growth of either Gram-positive or Gram-negative bacteria. The extracts did not exert any antifungal activity against *C. albicans*.

In this study, all strains of multi-resistant *S. maltophilia* tested were inhibited by ethanol and chloroform extracts.

The most effective extract was the chloroform extract, which exhibited a significant antimicrobial activity against all the microorganisms tested except *C. albicans*. This extract exhibited a significant effect against multi-resistant strains of *S. maltophilia* with 11–20 mm inhibition zones. Also, this extract exhibited bactericidal effect against multi-resistant *Staphylococcus aureus* and CNS.

The results indicate that the chloroform extract of *C. austro-anatolica* has a capacity to inhibit the growth of pathogenic bacteria. Sesquiterpenes, which were found in appreciable amounts, have been reported to have potent antimicrobial activity and to play a critical role in plant defense mechanisms (14). The potent activity of the chloroform extract might be attributed to its high oxygenated sesquiterpene content (60.54 %).

Caryophyllene oxide, the most abundant constituent of the extract, is known to exhibit antibacterial activity (15). It is thought that because of its high caryophyllene oxide content, the chloroform extract of *C. austro-anatolica* possesses high total antimicrobial activities.

CONCLUSIONS

Our study can be considered as the first document of the *in vitro* antimicrobial features and chemical composition of *C. austro-anatolica*. The study has shown that the chloroform extract of *C. austro-anatolica* may be useful as an alternative antimicrobial agent against multi-resistant *S. maltophilia*, *S. aureus* and coagulase negative *Staphylococci*.

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S A Ž E T A K

Kemijski sastav endemske biljke *Centaurea austro-anatolica* i ispitivanje antimikrobnog djelovanja protiv multi-rezistentnih bakterija

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Heksanski, kloroformski, etil-acetatni i etanolni ekstrakti vršnih dijelova biljke *C. austro-anatolica* Hub.-Mor. (Asteraceae) ispitivani su na antimikrobno djelovanje protiv multi-rezistentnih bakterija, koristeći difuzijsku metodu na papirnom disku. Klorofo-

mski ekstrakt pokazao je značajno antibakterijsko djelovanje protiv svih testiranih bakterija. Kemijski sastav tog ekstrakta određivan je plinskom kromatografijom (GC) i plinskom kromatografijom-spektrometrijom masa (GC-MS). Najvažniji sastojci ekstrakta bili su kariofilen oksid (21,32 %), spatulenol (10,86 %), *n*-trikožanol (9,58 %) i geranil izovalerat (8,71 %).

Ključne riječi: *Centaurea austro-anatolica* (Asteraceae), kemijski sastav, antimikrobno djelovanje

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