Composition and Antimicrobial Activity of the Essential Oils of *Micromeria cristata* subsp. *phrygia* and the Enantiomeric Distribution of Borneol

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Water-distilled essential oils from herbal parts of *Micromeria cristata* (Hampe) Griseb. subsp. *phrygia* P. H. Davis (Endemic) (Lamiaceae) collected from three different localities were analyzed by GC-MS. The major component characterized in the three oils was borneol (27−39%). Other main components were determined as camphor (9−15%), caryophyllene oxide (4−6%), and *trans*-verbenol (4−6%) in the oils. Enantiomeric distributions of borneol and camphor in the oils were determined on a fused silica Lipodex-E capillary column using a multidimensional GC-MS system. The three essential oils and both enantiomers of borneol have been evaluated for their antimicrobial activity. They showed inhibitory effects on Gr (−) and Gr (+) pathogenic microorganisms.

Keywords: *Micromeria cristata subsp. phrygia; essential oils; enantiomeric distribution; borneol; GC-MS; MD-GC-MS; antimicrobial activity*

INTRODUCTION

The genus *Micromeria* (Lamiaceae) is represented in Turkey by 14 species and 22 taxa, 12 of them being endemic (*1*). In Turkey, *Micromeria* species are used as herbal teas due to their pleasant aroma and medicinal properties and as a substitute for mint in folk medicine. In Gaziantep, *M. congesta* is locally known as "Kaya yarpuzu" and is used against kidney stones and stomachache. In Adana, *M. fruticosa* subsp. *brachycalyx* is locally known as "Viks otu" and is used against stomach problems. In contrast, in İzmir, *M. juliana*, which is locally known as "Yosma otu" or "Nane", is used as an appetizer, carminative, and stimulant. In Kahramanmaraş and its villages, *M. myrtifolia*, locally known as "Kayakekiği", "Altınbaşcayı", is used commonly as an aromatic herbal tea (*2*). Several *Micromeria* species have been reported as antiseptic, abortifacient, antirheumatic, CNS stimulant, and tonic. They are also used against heart disorders, indigestion, and headache and as a topical anesthetic in toothache and wounds, inflamed eyes, skin infections, chest pains, and colds (*2*, *3*).

Here, we report for the first time on the essential oil composition and the enantiomeric distribution of the main constituents borneol and camphor in *Micromeria cristata* (Hampe) Griseb. subsp. *phrygia* P. H. Davis. Preliminary antimicrobial investigation of the oils and the enantiomers of borneol was also performed.

MATERIALS AND METHODS

General. Optical rotations were measured on an Oriel Pol S-2 polarimeter. Chromatographic materials and solvents were obtained from Aldrich/Merck. Compounds were compared with authentic standards (Aldrich), and a library search was carried out using the Wiley GC-MS Library and the TBAM Library of Essential Oil Constituents.

Plant Material. The air-dried plant materials, collection places and dates, and yields of essential oils are given in Table 1. Voucher specimens have been deposited at the Herbarium of the Faculty of Pharmacy, Anadolu University in Eskisehir, Turkey (ESSE).

Isolation of the Essential Oils. Plants materials were water distilled for 3 h using a Clevenger-type apparatus. The percentage yields of the oils calculated on a moisture free basis are shown in Table 1.

Gas Chromatograpy—Mass Spectrometry (GC-MS). The oils were analyzed by GC-MS using a Hewlett-Packard GCD system. An HP-Innowax FSC column (60 m × 0.25 mm i.d., with 0.25 μm film thickness) was used with helium as carrier gas (1 mL/min). The GC oven temperature was kept at 60 °C for 10 min, programmed to 220 °C at a rate of 4 °C/min, then kept constant at 220 °C for 10 min, and then programmed to 240 °C at a rate of 1 °C/min. *n*-Alkanes were used as reference points in the calculation of the relative retention indices (RRI). The split ratio was adjusted at 50:1. The injector temperature was 250 °C. MS were recorded at 70 eV. The mass range was from *m*/*z* 35 to 425. Relative percentage amounts were calculated from total ion chromatograms (TIC) by the computer (Table 2).

Multidimensional GC-MS. Chiral separations were performed on a multidimensional gas chromatography−mass spectrometry (MD-GC-MS) system. Two Hewlett-Packard GC 6890 systems with MSD and a Gerstel multicolumn switching (MCS) system were used. The cooled injection system (CIS) was kept at 40 °C for injection. Helium was used as carrier gas (1 mL/min).

Table 1. Plant Materials Used in This Study

code	collection site	collection date	yield[a] (%)	ESSE[b]
A	Afyon: Sultan Mountains, Çay	July 1994	0.05	10642
B	Isparta: Ulubolu	July 1997	0.03	12460
C	Kütahya: Murat Mountain, Hamam-Sobalan province	July 1994	0.08	10460

[a] Calculated on a moisture free basis. [b] Herbarium of the Faculty of Pharmacy, Anadolu University in Eskisehir, Turkey.

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Table 2. Composition of the Essential Oils of *M. cristata* Subsp. *phrygia*

	component	A	B	C	RRI[a]		component	A	B	C	RRI[a]
1	α-pinene	0.1	0.1	tr[b]	1032	65	geranyl acetate	tr	0.4	0.5	1765
2	camphene	0.2	0.1	0.1	1076	66	*trans*-linalool oxide (pyranoid)		0.1		1770
3	limonene	0.1	0.1	0.3	1203	67	δ-cadinene	0.4	0.2		1773
4	isoamyl alcohol (= 3-methyl-1-butanol)	0.1			1212	68	γ-cadinene	0.2	0.2		1776
5	1,8-cineole	0.1	0.1	0.1	1213	69	campholene alcohol	0.1			1782
6	γ-terpinene	0.1	tr	0.1	1255	70	*ar*-curcumene		0.1		1786
7	5-methyl-3-heptanone	0.1	0.1	tr	1267	71	*p*-methyl acetophenone	0.2	0.1	0.1	1797
8	*p*-cymene	0.8	0.3	0.4	1280	72	methyl salicylate	0.1			1798
9	terpinolene	0.1	tr	0.1	1290	73	cumin aldehyde	0.3	0.1	0.1	1802
10	octanal	0.1			1296	74	myrtenol	0.7	1.1	0.8	1804
11	6-methyl-5-hepten-2-one	0.1			1348	75	3,7-guaiadiene		0.1		1810
12	hexanol	0.1			1360	76	2-tridecanone	0.1			1815
13	3-octanol	1.8	2.3	0.9	1393	77	β-damascenone	0.1	0.1	0.1	1838
14	nonanal	0.3	0.1	0.1	1400	78	*trans*-carveol	0.2	0.6	0.8	1845
15	γ-campholene aldehyde	0.1	0.2	tr	1435	79	*cis*-calamenene	0.2	0.2		1853
16	*trans*-linalool oxide (furanoid)	0.2	0.3	0.3	1450	80	carvon-1,2-oxide		0.1		1856
17	β-thujone	0.1	0.1	0.1	1451	81	*p*-cymen-8-ol	0.5	0.5	0.6	1864
18	α,*p*-dimethylstyrene	0.1			1452	82	(*E*)-geranylacetone	0.3	0.3		1868
19	1-octen-3-ol	0.1	tr		1452	83	1-methylnaphthalene	0.1			1884
20	heptanol	0.1			1463	84	α-calacorene	0.1	0.1		1941
21	eucarvone	0.1	0.1	0.1	1465	85	β-ionone	0.1	0.1		1958
22	*trans*-sabinene hydrate	0.3	0.9	0.2	1474	86	1-dodecanol	0.1	0.1		1973
23	camphenilone	0.2	0.3	0.2	1474	87	isocaryophyllene oxide		0.3	0.3	2001
24	menthone	0.1			1475	88	caryophyllene oxide	3.7	5.5	3.8	2008
25	*cis*-linalool oxide (furanoid)	0.1	0.2	0.2	1478	89	methyleugenol	0.1			2030
26	3-nonanol	0.1	0.1	tr	1496	90	humulene epoxide I		0.3		2045
27	α-copaene		0.3		1497	91	norbourbonone	0.3	0.6	0.2	2046
28	α-campholene aldehyde	0.5	0.7	0.6	1499	92	(*E*)-nerolidol	0.1	1.1	2.0	2050
29	decanal	0.2			1506	93	humulene epoxide II	0.2		0.3	2071
30	chrysanthenone	0.2	0.3	0.2	1522	94	cubenol	0.1			2080
31	camphor	14.5	9.1	10.7	1532	95	octanoic acid	0.2			2084
32	dihydroachillene	0.3		0.2	1547	96	heneicosane	0.2	0.1		2100
33	linalool	0.6	1.9	2.1	1553	97	cumin alcohol	0.1	0.1		2113
34	*cis*-sabinene hydrate	0.1	0.8	0.2	1556	98	hexahydrofarnesyl acetone	1.0	0.7	0.4	2131
35	octanol	0.6			1562	99	α-cedrol	0.3	0.4		2149
36	1-methyl-4-acetylcyclohex-1-ene	tr	0.1	0.1	1568	100	*nor*-copaanone		0.5		2179
37	*trans*-*p*-menth-2-en-1-ol	0.1	0.1	0.1	1571	101	nonanoic acid	1.1			2192
38	pinocarvone	1.0	0.8	0.7	1586	102	thymol	0.5	0.2		2198
39	bornyl formate	0.4	2.2	2.6	1588	103	*ar*-turmerol		0.1		2214
40	bornyl acetate	3.0	1.3	1.6	1597	104	carvacrol	4.0	0.6	0.1	2239
41	β-elemene	tr	0.1		1600	105	α-cadinol		0.7	0.2	2255
42	6-methyl-3,5-heptadien-2-one			0.1	1602	106	cadalene	0.6			2256
43	terpinen-4-ol	1.6	0.1	1.5	1611	107	decanoic acid	0.5			2300
44	β-caryophyllene	tr	1.8	1.5	1612	108	tricosane	0.2			2300
45	hotrienol	0.4	0.1	0.2	1616	109	caryophylla-2(12),6(13)-dien-5β-ol (= caryophylladienol I)	0.1			2316
46	*trans*-*p*-mentha-2,8-dien-1-ol			0.2	1639						
47	myrtenal	1.3	1.1	1.1	1648	110	caryophylla-2(12),6(13)-dien-5α-ol (= caryophylladienol II)	0.3	0.1		2324
48	bornyl isobutyrate	0.1			1651						
49	pulegone	0.2			1662	111	caryophylla-2(12),6-dien-5α-ol (= caryophyllenol I)	0.7	0.2		2389
50	*cis*-verbenol	1.5	0.5	1.1	1663						
51	*trans*-pinocarveol	1.5	1.8	1.8	1664	112	caryophylla-2(12),6-dien-5β-ol (=caryophyllenol II)	0.4	0.6	0.1	2392
52	*p*-mentha-1,5-dien-8-ol	0.2	0.1	0.2	1674						
53	isoborneol	0.3	0.2	0.2	1682	113	undecanoic acid	0.1			2400
54	*trans*-verbenol	3.8	5.6	5.0	1683	114	tetracosane	0.1			2445
55	γ-selinene	0.1	0.2		1690	115	pentacosane	0.2			2500
56	heptadecane	0.1	0.1		1700	116	dodecanoic acid	0.6			2503
57	α-terpineol	0.4	0.2	0.5	1706	117	phytol	0.1	0.1		2622
58	borneol	26.9	31.4	39.3	1719	118	heptacosane	0.2			2700
59	verbenone	0.9	0.7	0.7	1725	119	pentadecanoic acid	0.1			2822
60	germacrene D	0.5	2.3	2.2	1726	120	nonacosane	0.3	0.1		2900
61	α-muurolene	0.1			1740	121	hexadecanoic acid	1.5	0.1		2931
62	β-bisabolene	0.1	0.1		1741						
63	geranial			0.1	1742		total	89.6	86.7	89.1	
64	carvone	0.6	0.4	0.7	1751						

[a] RRI, relative retention indices calculated against *n*-alkanes (percentage calculated from TIC data). [b] tr, trace (<0.1%).

Precolumn. An HP-Innowax fused silica capillary column (60 m × 0.25 mm i.d., with 0.25 μm film thickness) was used. The GC oven temperature was kept at 60 °C for 10 min, programmed to 220 °C at a rate of 4 °C/min, kept constant at 220 °C for 10 min, programmed to 240 °C at a rate of 1 °C/min, and kept constant at 240 °C for 40 min. The FID detector temperature was 250 °C.

Main Column. A Lipodex E [octakis(3-*O*-butyryl-2,6-di-*O*-pentyl)-γ-cyclodextrin] (70% in OV 1701) (25 m × 0.25 mm i.d.) was used. The temperature program for borneol was 40 °C for 39 min, programmed to 120 °C at a rate of 1 °C/min, and then kept constant at 120 °C for 1 min.

Isolation of Borneol. The compound was isolated by column chromatography. Silica gel 60G (10 g, Merck 7734) was used as packing material and was filled with wet hexane (column size: 10 × 500 mm). *n*-Hexane/diethyl ether was used as eluant in a gradient system. Essential oil (50 mg) was applied to the column and *n*-hexane/diethyl ether (70:30)

Table 3. Relative Amounts (Percent, GC-MS) and Enantiomeric Distributions (Percent) of Borneol and Camphor in the *M. cristata* subsp. *phyrgia* Essential Oils

	borneol			camphor		
code	% GC-MS	(1*R*)-(+)	(1*S*)-(−)	% GC-MS	(1*R*)-(+)	(1*S*)-(−)
A	29.9		100.0	14.5	0.1	99.0
B	31.4		100.0	9.1	0.1	99.0
C	39.3		100.0	10.7	0.1	99.0

eluted borneol (12 mg). This compound was separated using the γ-cyclodextrin chiral column and identified as (1*S*)-(−)-borneol by MD-GC-MS in comparison with authentic samples (*4*) (Table 3).

Antimicrobial Assay. A microdilution broth susceptibility assay was used for the evaluation (*5*). Stock solutions of essential oils and compounds were prepared in DMSO. Serial dilutions were prepared in sterile distilled water in a 96-well microtiter plate from 2000 to 1.94 μg/mL for the essential oils and from 1000 to 0.97 μg/mL for the pure compounds. Freshly grown bacterial suspensions in double-strength Mueller−Hinton broth (Merck) and yeast suspension of *Candida albicans* in yeast medium were standardized to 10^8 CFU/mL. Sterile distilled water served as growth control. One hundred microliters of each microbial suspension was then added to each well. The last row containing only the serial dilutions of antimicrobial 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 inhibition concentration (MIC). Human pathogens *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Proteus vulgaris*, and *Salmonella typhimurium* were obtained from the culture collection of the Microbiology Department in Anadolu University, and *Candida albicans* was obtained from the culture collection of Osmangazi University, Medical Faculty, Microbiology Department.

RESULTS AND DISCUSSION

Chemical Composition of the Essential Oils. Water-distilled essential oils from aerial parts of *Micromeria cristata* subsp. *phrygia*, collected from three different localities, were analyzed using a GC-MS system. The components identified are given in Table 2 with their relative percentages. One hundred and eight compounds were identified in the Afyon sample (A), representing 89.6% of the total components detected. Eighty-six compounds were characterized in the Isparta sample (B), representing 86.7% of the total. Finally, in the Kütahya sample (C), 61 compounds representing 89.1% of the oil were identified.

Borneol (A, 26.9%; B, 31.4%; C, 39.3%) was found as the main constituent in all essential oils. The second major component was identified as camphor (A, 14.5%; B, 9.1%; C, 10.7%). The enantiomeric purity of borneol and camphor in the oils of *M. cristata* subsp. *phrygia* was investigated, resulting in the detection of enantiomerically pure (1*S*)-(−)-borneol (100%) and (1*S*)-(−)-camphor (99%) in all samples. Their optical purity was determined by chiral separation and supported by subsequent specific rotation (Table 3). The isolation of (1*S*)-(−)-borneol was described in detail under Materials and Methods and was confirmed with a commercial sample, whereas (1*R*)-(+)-borneol was isolated using similar conditions from *Salvia tomentosa* (*4*) for reconfirmation of the absolute configuration and subsequent antimicrobial evaluation against various human pathogens.

Borneol is a colorless, crystalline monoterpene, which occurs in essential oils. Borneol has been reported to have antibacterial, antifungal, antispasmodic, choleretic, and tranquilizing effects (*6*−*11*). The effects of individual enantiomers are not known. Borneol occurs abundantly in nature as a single enantiomer or, less frequently, as the racemate. (+)-Borneol has been found in rosemary, lavender, olibanum, and *Dryobalanops* species. (−)-Borneol occurs particulary in *Pinus*, *Abies*, and citronella oils. Commercial borneol is often (−)-borneol, and it contains up to 40% isoborneol (*6*, *7*). The (+) form has a camphoraceous odor and a slightly sharp, earthy−peppery note different from that of the (−) form. (−)-Borneol has a camphoraceous and evident woody odor (*12*). In a previous study, both enantiomers of borneol were reported in different essential oils (*13*).

Camphor is a colorless and crystalline volatile compound. Well-known physicochemical properties of camphor were previously reported (*4*, *14*). Medicinal uses and clinical pharmacology as well as the toxicity of camphor have recently been reported (*15*−*17*).

Antimicrobial Activity. The antimicrobial evaluation (Table 4) of the essential oils and the main components resulted in an activity range such as in the case of *P. vulgaris* with MIC values of 62.5−125 μg/mL. However, *S. typhimurium* was inhibited profoundly by the essential oil of the Isparta (B) sample. *C. albicans* was also strongly inhibited by A and C essential oils compared with the reference substance Ketoconazole. Although percentage amounts of the main compound in the oils did not differ much, the (−) enantiomer of camphor was previously shown by us to have relatively more activity against various microorganisms (*18*).

This study reveals that the isolated essential oils have biological activities against human pathogenic microorganisms as seen in Table 4, supporting various ethnomedical uses of this plant and thus calling for more detailed investigations of biological activities.

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Table 4. Antimicrobial Activity (MIC Values, Micrograms per Milliliter) of Borneol-Containing Samples

pathogen	source	A	B	C	D[a]	E[b]	standard
E. coli	ATCC 25922	62.5	250	250	125	250	62.5[c]
S. aureus	ATCC 6538	250	250	125	250	125	7.81[c]
P. aeruginosa	ATCC 27853	250	250	125	250	125	250[c]
E. aerogenes	NRLL 3567	125	125	125	125	125	125[c]
P. vulgaris	NRLLB 123	125	62.5	62.5	125	125	31.25[c]
S. typhimurium	NRRLB 4420	62.5	31.25	62.5	125	125	62.5[c]
C. albicans	O.G.Ü.	62.5	125	31.25	125	250	125[d]

[a] D = (−)-borneol isolated from *M. cristata* subsp. *phyrgia*. [b] E = (+)-borneol isolated from *S. tomentosa*. [c] Chloramphenicol. [d] Ketoconazole.

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