

# Biological Activity of *Matricaria Chamomilla* Essential Oils of Various Chemotypes



## Authors

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## Key words

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## ABSTRACT

The essential oil of *Matricaria chamomilla* L., which is commonly used for medicinal and cosmetic purposes, can be differentiated between several chemotypes. In the current study, six essential chamomile oil samples of various origins (four of commercial sources, one of cultivation, one of wild collection) were examined regarding their composition and biological activities, i. e., antibacterial, antifungal, mosquito repellent, and larvicidal effects. GC-MS analyses revealed that the samples largely varied in composition and could be attributed to various chemotypes. In contrast to the other two samples, the four commercial samples were unusually high in *trans*- $\beta$ -farnesene. The overall antimicrobial effects were only moderate, but it could be shown that a higher content in  $\alpha$ -bisabolol and a smaller in  $\alpha$ -bisabolol oxides A and B had a positive effect on overall activity. All samples had a biting deterrent effect comparable to DEET. Higher concentrations of (*Z*)- and (*E*)-spiroethers improved larvicidal activity, whereas *trans*- $\beta$ -farnesene had the opposite effect. In conclusion, the importance of  $\alpha$ -bisabolol for the biological activity of chamomile essential oil could be demonstrated.

## Introduction

German chamomile [*Matricaria chamomilla* L., syn. *Matricaria recutita* L., *Chamomilla recutita* (L.) Rauschert, Asteraceae] is among the most valued medicinal plants worldwide. The dried flower heads

as well as the essential oil are used as a medicinal drug (*Matricariae flos* and *Matricariae aetheroleum* [1] against a broad spectrum of symptoms in the form of tisanes, baths, inhalations, and mouthwashes [2]. Therapeutic indications include inflammatory condi-

tions, bacterial infections, and lesions of the skin and mucosa (e. g., oral cavity, gastrointestinal tract, and respiratory tract), spasms, and ulcers of the gastrointestinal tract as well as insomnia and nervous disposition [2].

Phytochemical analyses have revealed that besides flavonoids (e. g., apigenin, luteolin) and their glycosides as well as coumarins (e. g., herniarin, umbelliferone), the essential oil can be considered crucial for the biological activities of chamomile flowers [3]. The essential oil is notably used in pharmaceutical and cosmetic preparations for its antibacterial [4], antifungal [5], antioxidant [6], and anti-inflammatory [7, 8] properties. The essential oil is mainly comprised of oxygenated sesquiterpenes of the bisabolane type. The blue colour typical for the majority of chamomile oil samples originates from chamazulene, an artefact of the distillation process derived from the nonvolatile sesquiterpene lactone matricin [7].

Within the species *M. chamomilla*, a chemical polymorphism regarding essential oil composition was first shown by Schilcher [9] and has been substantiated by several studies [10–14].

In the present study, the compositions of four commercial chamomile essential oil samples as well as a cultivated one and one

sample of wild-growing populations were analysed regarding their composition, and antibacterial and anticandidal effects. Moreover, samples from Serbia, Hungary, South Africa, and India were evaluated against biting deterrent and larvicidal activities against *Aedes aegypti* L. Up to now, the influence of chemotype on biological activities of chamomile essential oils has not been evaluated thoroughly, which was another aim of our study.

## Results

In the present study, the chemical compositions of four commercial essential oil samples, a sample of cultivated plants as well as a sample of collected wild plants were elucidated by means of simultaneous GC-FID and GC-MS. ► **Table 1** presents the results of the GC-FID and GC-MS analyses. In total, 114 compounds could be identified, amounting to 84.5–95.5 %. All samples had a low content in monoterpenes (> 3 %) except for sample D from India (11.9 %, with > 2 %  $\beta$ -pinene and linalool) and sample F (6.6 %) but were dominated by sesquiterpenes (50–84 %). While samples A, C, and D were mainly composed of sesquiterpene hydrocarbons, no-

► **Table 1** Chemical composition determined by simultaneous GC-FID and GC-MS of six essential oil samples of *M. chamomilla* L. of various origins.

Compound	RI#	Sample/provenance					
		A/Hungary	B/South Africa	C/Serbia	D/India	E/Hungary	F/India
hexanal	800	–	–	–	–	tr	–
ethyl 2-methyl butanoate	848	0.1	0.1	–	–	0.1	0.3
ethyl isovalerate	851	–	–	–	–	tr	–
(Z)-3-hexenol	852	tr	–	–	–	–	0.1
hexanol	862	–	–	–	–	–	tr
tricyclene	930	–	–	–	0.3	–	–
$\alpha$ -pinene	941	tr	–	tr	1.2	–	0.1
isopropyl 2-methylbutanoate	943	tr	tr	tr	–	0.1	0.2
3-buten-2-one	948	–	–	–	–	0.5	–
camphene	957	–	–	–	1.5	–	tr
isoamyl propanoate	964	–	–	–	–	–	0.1
sabinene	980	–	–	0.1	–	tr	0.5
6-methyl-5-hepten-2-one	984	tr	0.1	tr	tr	0.1	0.5
$\beta$ -pinene	985	tr	–	–	2.3	–	–
6-methyl-5-hepten-2-ol	989	–	–	–	–	–	tr
myrcene	991	–	–	tr	0.1	–	0.1
2-pentylfuran	994	0.1	0.1	–	–	tr	–
yomogi alcohol	997	tr	0.1	0.1	–	0.4	0.4
octanal	1001	0.1	0.1	–	tr	0.1	0.1
(Z)-3-hexenyl acetate	1003	–	–	–	–	–	tr
$\delta$ -3-carene	1017	tr	–	–	–	–	–
$\alpha$ -terpinene	1023	–	–	–	–	tr	tr
p-cymene	1031	0.1	0.1	0.2	1.6	0.1	0.2
limonene	1035	0.1	0.1	tr	0.1	tr	0.1
(Z)- $\beta$ -ocimene	1036	0.1	tr	0.1	–	0.1	0.1
1,8-cineole	1038	–	0.1	–	1.0	0.3	0.3
(E)- $\beta$ -ocimene	1050	0.6	0.2	0.6	0.2	0.3	0.9
(E)-2-octenal	1055	–	–	–	–	tr	–
3-methyl-2-cyclohexen-1-one	1055	–	–	–	–	tr	–

► Table 1 Continued

Compound	RI#	Sample/provenance					
		A/Hungary	B/South Africa	C/Serbia	D/India	E/Hungary	F/India
artemisia ketone	1062	0.3	0.4	0.6	0.1	0.5	2.0
$\gamma$ -terpinene	1065	0.1	tr	0.2	tr	0.2	0.9
1-nonen-3-ol	1067	–	–	–	–	tr	–
octanol	1068	–	0.1	–	–	tr	0.1
(Z)-sabinene hydrate	1072	–	tr	–	–	–	tr
(Z)-linalool oxide	1076	–	–	–	tr	–	–
artemisia alcohol	1086	0.1	0.1	0.1	–	0.5	0.6
(E)-linalool oxide	1092	–	–	–	tr	–	–
(Z)-3-hexenyl propanoate	1098	–	–	–	–	tr	0.1
isoamyl 2-methylbutyrate	1101	–	–	–	–	–	0.1
linalool	1102	–	–	–	2.2	0.1	0.1
nonanal	1102	0.1	0.1	–	–	tr	–
acetate of yomogi alcohol	1111	–	–	–	–	tr	–
hexyl isobutanoate	1146	–	–	–	–	tr	–
(E)-pinocarveol	1150	–	–	–	0.1	–	–
4-acetyl-1-methyl-cyclohexene	1155	–	–	–	–	tr	–
camphor	1154	–	–	–	–	–	0.3
(E)-chrysanthemol	1160	–	–	–	–	0.1	0.1
(E)-2-nonenal	1165	–	–	–	–	–	0.2
lavandulol	1167	–	–	–	–	–	0.2
isoborneol	1167	–	–	–	–	0.1	–
artemisyl acetate	1169	tr	0.2	–	–	0.2	–
borneol	1177	–	–	–	–	0.1	0.2
terpinen-4-ol	1186	–	–	–	–	0.1	0.3
hexyl butanoate	1189	–	–	–	–	tr	–
$\alpha$ -terpineol	1198	–	–	tr	1.8	0.2	0.2
decanal	1204	–	–	–	–	tr	–
$\gamma$ -terpineol	1204	–	–	–	0.3	–	–
4,8-dimethyl-7-nonen-2-one	1227	–	–	–	–	0.1	–
(Z)-3-hexenyl-2-methylbutanoate	1231	–	–	–	–	tr	tr
(Z)-3-hexenyl-3-methylbutanoate	1234	–	–	–	–	0.1	0.1
4,8-dimethyl-3,7-nonadien-2-one isomer 1	1238	–	–	–	–	0.3	–
benzyl propanoate	1258	–	–	–	–	–	0.1
geranial	1274	–	–	–	0.1	–	–
4,8-dimethyl-3,7-nonadien-2-one isomer 2	1275	–	0.4	–	–	1.2	–
methyl geranate	1275	0.1	–	0.3	–	–	0.3
lavandulyl acetate	1288	–	–	–	–	tr	–
(E,E)-2,4-decadienal	1319	–	–	–	–	tr	–
$\delta$ -elemene	1352	–	–	0.2	–	–	–
$\alpha$ -copaene	1359	–	–	0.1	–	–	–
capric acid	1360	0.3	0.4	0.1	0.6	0.5	–
benzyl isovalerate	1398	–	–	–	–	–	0.1
tetradecane	1399	–	0.1	–	–	–	–
$\beta$ -elemene	1406	0.2	0.1	0.2	0.2	0.1	0.2
$\alpha$ -isocomene	1411	0.2	0.3	0.4	0.3	–	–
2,5-dimethoxy- <i>p</i> -cymene	1423	–	0.3	–	–	–	–
(Z)- $\beta$ -farnesene	1434	–	–	–	0.7	–	–
(E)- $\beta$ -caryophyllene	1442	0.2	0.2	0.3	0.4	0.1	0.1

► Table 1 Continued

Compound	RI#	Sample/provenance					
		A/Hungary	B/South Africa	C/Serbia	D/India	E/Hungary	F/India
(E)- $\alpha$ -bergamotene	1448	–	0.3	–	–	–	–
geranyl acetone	1453	–	0.2	–	–	–	–
(E)- $\beta$ -farnesene	1460	38.5	18.7	38.4	24.1	3.3	7.2
(Z,Z)- $\alpha$ -farnesene	1475	–	–	–	1.0	–	–
dihydrosesquicineole	1479	0.2	0.2	0.3	0.6	0.4	1.4
allo-aromadendrene	1486	0.1	0.2	0.1	–	–	–
$\gamma$ -curcumene	1490	0.1	0.8	–	–	0.1	–
(Z,E)- $\alpha$ -farnesene	1497	–	–	0.6	0.6	–	–
(E,Z)- $\alpha$ -farnesene	1496	0.6	0.4	–	0.9	–	–
pentadecane	1499	0.1	0.2	–	–	–	–
germacrene D	1503	2.3	1.4	3.1	1.0	0.5	1.6
(E,E)- $\alpha$ -farnesene	1510	5.8	1.3	5.6	4.5	0.2	0.8
bicyclogermacrene	1518	1.9	1.2	2.0	–	0.4	1.1
$\beta$ -bisabolene	1519	–	–	–	1.5	–	–
(Z)- $\gamma$ -bisabolene	1529	–	–	–	0.5	–	–
$\beta$ -sesquiphellandrene	1535	–	0.5	–	–	–	–
$\gamma$ -cadinene	1537	0.2	–	0.1	–	–	0.1
$\delta$ -cadinene	1539	0.2	0.3	0.4	0.2	tr	0.1
trans- $\gamma$ -bisabolene	1544	–	–	–	0.3	–	–
isohumbertiol B	1546	–	–	–	–	0.1	–
(Z)-nerolidol	1551	–	–	–	–	tr	–
(E)- $\alpha$ -bisabolene	1552	–	–	–	2.7	–	–
(E)-nerolidol	1568	0.2	1.4	0.4	0.2	0.8	0.2
(E)-dendrolasin	1580	0.2	0.5	0.1	0.2	0.1	–
spathulenol	1602	0.7	5.7	0.6	0.7	0.8	–
caryophyllene oxide	1610	–	–	–	–	0.1	–
$\tau$ -cadinol	1661	0.4	0.8	0.3	0.2	0.2	0.5
$\alpha$ -bisabolol oxide B	1676	3.9	12.1	5.8	5.9	3.1	17.0
$\alpha$ -bisabolol	1703	1.2	1.9	11.5	5.7	38.3	6.2
(E,E)-farnesol	1704	–	–	–	5.6	–	–
epi- $\alpha$ -bisabolol	1705	–	–	–	–	0.5	–
$\alpha$ -bisabolone oxide A	1707	3.0	8.5	2.8	5.2	0.1	7.3
(E,Z)-farnesol	1728	–	–	–	4.0	–	–
chamazulene	1763	1.3	4.4	8.0	0.6	21.6	6.8
$\alpha$ -bisabolol oxide A	1770	24.1	6.9	6.3	6.0	1.0	25.0
guajazulene	1808	–	–	–	–	0.2	–
phytol	1848	0.3	1.1	0.3	0.5	–	–
(Z)-spiroether	1887	5.5	10.4	1.3	2.6	0.1	6.0
(E)-spiroether	1900	0.6	1.6	0.2	0.3	16.8	0.6
palmitic acid	1954	–	–	–	–	0.3	–
<b>Sum</b>		<b>94.2</b>	<b>84.5</b>	<b>91.7</b>	<b>90.9</b>	<b>95.5</b>	<b>92.2</b>

"tr" = trace, "–" = not detectable.

tably (E)- $\beta$ -farnesene, oxygenated sesquiterpenes prevailed in samples B and F ( $\alpha$ -bisabolol oxides A and B,  $\alpha$ -bisabolone oxide A) as well as sample E (main compound  $\alpha$ -bisabolol). The latter had also notably high concentrations of azulenes (21.8%) and spiroethers (16.9%), not only compared to the other samples (< 10%) but also other samples from Hungary [15]. As both  $\alpha$ -bisabolol [8, 16] and chamazulene [17–19] proved to possess anti-inflammatory and

highly antioxidant properties [14, 17, 20], this combination is highly preferable for pharmaceutical and cosmetic use.

All of the commercially available samples were found to have a remarkably high content of *trans*- $\beta$ -farnesene (18.7–38.5%) compared to sample E from the wild collection in Hungary (3.3%) and sample F from North India (7.2%). Among the reasons for these unusually high levels, the developmental stage of the harvested

plants as well as the use of vegetative plant parts [21] can be discussed. These results are supported by a study by Su et al. who showed that the activity of (*E*)- $\beta$ -farnesene synthase, and thus (*E*)- $\beta$ -farnesene production, is influenced by the plant part investigated (highest in leaves, lowest in disc flowers) and the developmental stage of the plant (highest in semi-opened flowers, lowest in fully opened flowers) amongst other factors like distillation time [22].

In ► **Table 2**, the results of the antimicrobial activity screening are given. As demonstrated before, chamomile essential oil exerts antibacterial effects [4, 20], particularly against gram-positive bacteria like *Staphylococcus aureus* as well as anticandidal activity [14] against *Candida albicans*. However, gram-negative bacteria, especially *Pseudomonas aeruginosa*, were less susceptible. In this respect, this result was predictable as *P. aeruginosa* is protected against terpenoids and other chemicals by its outer membrane and specific efflux mechanisms [23]. Compared to other essential oils, the overall antimicrobial activity was mediocre, but our findings go along with those of Satyal et al. [14]. While our findings corroborate previous findings concerning the antibacterial effects of pure  $\alpha$ -bisabolol [24], there is also evidence for a negative effect of  $\alpha$ -bisabolol oxides A and B on antimicrobial activity as shown by samples A and F.

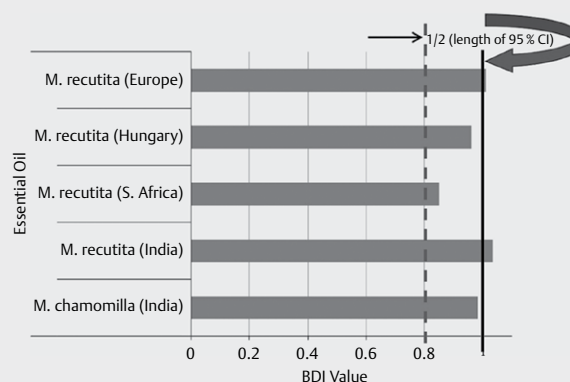
The *in vitro* K&D system used in this study specifically quantified the mosquito biting deterrent properties of five essential oils of *M. chamomilla*. Biting deterrent indices (BDI) of the essential oils against *Aedes aegypti* are given in ► **Fig. 1**. All the essential oil samples showed biting deterrent activity above the negative control ethanol, and the activity of all samples was similar to DEET at 25 nmol/cm<sup>2</sup>. A high content in  $\alpha$ -bisabolol as well as monoterpenes might have a positive influence on biting deterrence, but the differences were minimal.

Data on the toxicity against the larvae of *A. aegypti* is presented in ► **Table 3**. The toxicity of sample B (South Africa) with an LD<sub>50</sub> of 2.9 ppm was significantly higher than all the other samples, followed by sample F (India) with an LD<sub>50</sub> of 8.2 ppm. Samples A (Hungary), C (Serbia), and D (India) exhibited similar mortalities with LD<sub>50</sub> values of 60.5, 49.3, and 45.4 ppm, respectively. Sample E was not included in these assays. The high toxicity of samples B and F might be explained by its relatively high content in spiroethers (12.0 and 6.6 %, respectively). Polyines (or polyacetylenes), especially those found in the genus *Artemisia*, have been known to possess high larvicidal activity for a long time [25]. In contrast, higher

yields of sesquiterpene hydrocarbons, especially (*E*)- $\beta$ -farnesene, had a negative effect on toxicity in *Aedes* larvae in this study.

## Discussion

Since 1973 [9], the chemical polymorphism of German chamomile regarding its essential oil has been established. Originally, Schilcher [10] proposed four chemotypes based on varying contents in sesquiterpene compounds: Type A, mainly found in Central, South, and East Europe, is dominated by bisabolol oxide B, Type B (South America) by bisabolol oxide A, and Type C by  $\alpha$ -bisabolol, while Type D is composed of comparable quantities of these three sesquiterpenes. Additionally, a variant low in matricin (prochamazulene) was mentioned. This classification has been used by many authors [13, 15, 26, 27], however, Type A and B have been interchanged. Later, Horn et al. [28] could identify four chemotypes as follows: Type I dominated by  $\alpha$ -bisabolol and matricin, Type IIa by  $\alpha$ -bisabolol oxide A and matricin, Type IIb by  $\alpha$ -bisabolol oxide B and matricin, and Type III high in  $\alpha$ -bisabolone oxide but devoid of  $\alpha$ -bisabolol and matricin. Horn et al. [28] as well as Massoud and Franz [29, 30] also elucidated the genetically controlled mechanisms for the formation of  $\alpha$ -bisabolol and its derivatives as well as



► **Fig. 1** Biting deterrent indices (BDI) of five essential oils (10  $\mu$ g/cm<sup>2</sup>) of *Matricaria chamomilla* L. from various parts of the world against female *A. aegypti*. Ethanol was the solvent control and DEET at 25 nmol/cm<sup>2</sup> was used as positive control. Mean BDI falling between 1/2 length of 95% CI and 1 are statistically similar to DEET.

► **Table 2** Minimal bactericidal/fungicidal concentrations ( $\mu$ g/mL) of six essential oils of *M. chamomilla* L. of various origins.

Test strain	Essential oils						Controls				
	A	B	C	D	E	F	Ac	Ci	Ce	Na	Va
<i>S. aureus</i> ATCC 6538	4000	2000	2000	4000	2000	8000	0.5	0.125	0.125	0.063	0.5
<i>E. coli</i> ATCC 25922	4000	2000	4000	2000	2000	8000	0.5	0.25	0.25	0.125	0.25
<i>S. abony</i> ATCC 6017	4000	2000	4000	2000	2000	8000	0.5	0.5	0.5	0.5	0.5
<i>P. aeruginosa</i> ATCC 9027	4000	4000	8000	4000	4000	8000	32	1.0	2.0	4.0	8.0
							Am	Fl	It	Ke	Vo
<i>C. albicans</i> ATCC 10231	2000	2000	2000	2000	2000	4000	0.25	0.25	0.25	0.023	0.016

Ac = amoxicillin, Ci = ciprofloxacin, Ce = cefazolin, Na = nalidixic acid, Va = vancomycin; Am = amphotericin B, Fl = fluconazole, It = itraconazole, Ke = ketoconazole, Vo = voriconazole.

► **Table 3** Toxicity of essential oils of *M. chamomilla* from various parts of the world against 1-day-old larvae of *A. aegypti* 24 h post-treatment.

Essential oil	<i>Aedes aegypti</i>			
	LC <sub>50</sub> (95 % CI) *	LC <sub>90</sub> (95 % CI)	χ <sup>2</sup>	DF
Sample A (Hungary)	60.5 (54.2–67.8)	99.9 (86.3–124.4)	59.8	48
Sample B (S. Africa)	2.9 (2.6–3.2)	4.4 (3.9–5.2)	54.6	48
Sample C (Serbia)	49.3 (43.2–56.6)	103.4 (85.7–135.3)	79.3	48
Sample D (India)	45.4 (40.3–51.1)	81.7 (69.9–102.5)	67.6	48
Sample F (India)	8.2 (7.4–9.0)	12.6 (11.3–15.6)	52.2	48
Permethrin * *	0.0034 (0.003–0.0038)			

\* LC<sub>50</sub> and LC<sub>90</sub> values are in ppm, 95 % CI = confidence interval.

chamazulene and identified the responsible genes, thus assigning genotypes to the chemotypes. As some of these chemotypes form locally distinct populations, they could also be termed “chemodem” [31]; however, this term is rarely used in literature. Moreover, some authors suggested spiroether [14, 32] and trans- $\beta$ -farnesene [14] chemotypes; however, Satyal et al. [14] analysed the essential oil of the aerial parts. Interestingly, the European Pharmacopoeia differentiates between only two chemotypes, one rich in  $\alpha$ -bisabolol (10–65 %) and one dominated by bisabolol oxides (29–81 %) [1].

Considering that a high trans- $\beta$ -farnesene level might also be ascribed to a premature harvesting or use of stems and leaves, the samples in the current study could be characterised as follows: Sample A (Hungary) is a representative of the  $\alpha$ -bisabolol oxide A/matricin type, and sample E (Hungary) is typical for the  $\alpha$ -bisabolol/matricin type, both of which are commonly found in Hungary [15]. Sample F (North India) can also be attributed to the  $\alpha$ -bisabolol oxide A/matricin type (though also rich in  $\alpha$ -bisabolol oxide B), which is apparently common for this region [33] as well as the Ganges plains [34]. Sample B (South Africa) might represent the  $\alpha$ -bisabolol oxide B/matricin type, whereas sample C (Serbia) could be an example of Schilcher’s Type D, which is prevalent in this geographical region [35, 36]. Sample D (India) might also be attributed to Type D, even though it is also rich in  $\alpha$ -bisabolone oxide and poor in chamazulene. However, the limited data on used cultivars, harvesting, and essential oil production of the commercial samples should be pointed out.

In conclusion, our study could demonstrate that the quantitative differences regarding the chemical composition of *M. chamomilla* essential oils crucially influence biological activities such as antimicrobial, insect repellent, and larvicidal effects. To our knowledge, this is the first published study that directly compared chamomile essential oils of different provenance and chemotype in this respect. Regarding essential oil composition, essential oils of chemotypes rich in  $\alpha$ -bisabolol, chamazulene, and spiroethers should be preferred over chemotypes dominated by trans- $\beta$ -farnesene and  $\alpha$ -bisabolol oxides for use in pharmaceutical and cosmetic products as these compounds have been shown once more to have positive effects on various activities of chamomile essential oil.

## Materials and Methods

### Plant material

Three essential oil samples were purchased from Paul Kaders (Hamburg, Germany): sample A (Lot. nr. 122300/260810, Hungary), sample B (Lot. nr. 12302/260810, South Africa), and sample D (Lot. nr. 122306/260810, India). Sample C (Lot. nr. 38595, Serbia) was purchased from Unterweger (Thal-Assling, Austria). Sample E was prepared in 2009 by Beata Gosztola and authenticated by Prof. Eva Nemeth (Corvinus University, Budapest, Hungary) from plants of a wild-growing population of *M. chamomilla*. For sample F, the plants were cultivated and harvested by Dr. Virendra Singh at the CSIR-Institute of Himalayan Bioresource Technology (Palampur, India) from April to May 2014, and authenticated by V. K. Kaul. The aerial parts containing 95 % flowers were distilled using a Clevenger-type apparatus; the oil yield was 0.1 %.

### Essential oil analysis

GC-FID and GC-MS analyses as well as identification and quantification of the compounds were carried out as described earlier [37]. Retention indices [38] were compared to retention indices of reference compounds and from literature data [39–42] to confirm the peak data. Quantification of compounds was performed using normalised peak area calculations of the FID chromatogram.

### Microbiological assays

The antimicrobial effects of the essential oils were tested against the following strains of microorganisms: *S. aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Salmonella abony* ATCC 6017, *P. aeruginosa* ATCC 9027, and *C. albicans* ATCC 10231, all obtained from culture collections of The National Bank of Industrial Microorganisms and Cell Cultures (NBIMCC, Bulgaria). Nutritional agar, Mueller-Hinton broth and agar as well as Sabouraud dextrose broth and agar were obtained from the National Center of Infectious and Parasitic Diseases (NCIPD, Bulgaria). For positive controls, HiComb MIC test strips were obtained from HiMedia Laboratories Ltd. Antibacterial and anticandidal susceptibility testing was carried out as described in detail previously [43].

### Mosquito bioassays

Both the larvae and adult mosquitoes used in these studies were from the laboratory colonies maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary

Entomology, USDA-ARS, Gainesville, Florida. Maintenance conditions and bioassays are described in detail elsewhere [44].

For biting bioassays, two sets of replications, consisting of six treatments (four test compounds, DEET at 25 nmol/cm<sup>2</sup>, and ethanol-treated organdy as a solvent control) each with five females per treatment, were conducted on two different days using a newly treated organdy and a new batch of females in each replication. Treatments were replicated nine times.

Regarding the larval bioassay, larvae that showed no movement in the well after manual disturbance of water were recorded as dead. A series of 5 concentrations ranging between 125 and 0.98 ppm were used in different treatments to obtain a range of mortality. Permethrin (46.1 % *cis*/53.2 % *trans*; Chemical Service) was used as a positive control. Treatments were replicated ten times for each of the treatments.

### Statistical analyses

Proportion not biting (PNB) and biting deterrence index (BDI) were calculated as described before [44]. A BDI value of 0 indicates an effect similar to ethanol, while a value significantly greater than 0 indicates a biting deterrent effect relative to ethanol. BDI values that were not significantly different from 1 are statistically similar to DEET. BDI values were analysed using SAS Proc ANOVA [single factor: test compound (fixed)], (SAS Institute 2007), and means were separated using the Ryan-Einot-Gabriel-Welsch Multiple Range Test. To determine whether confidence intervals (CIs) include the values of 0 or 1 for treatments, Scheffe's multiple comparison procedure with the option of CLM was used in SAS. LC<sub>50</sub> values for larvicidal data were calculated by using SAS, Proc Probit. Control mortality was corrected by using Abbott's formula [45]. Toxicity was compared among treatments based on non-overlapping 95 % CIs [46].

### Conflict of Interest

The authors declare that they have no conflict of interest.

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