Chemical Composition and Biological Effects of *Artemisia maritima* and *Artemisia nilagirica* Essential Oils from Wild Plants of Western Himalaya

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Key words
- *Artemisia nilagirica*
- *Artemisia maritima*
- *Asteraceae*
- antifungal activity
- antibacterial activity
- biting deterrence
- larvicidal activity

Abstract

Artemisia species possess pharmacological properties that are used for medical purposes worldwide. In this paper, the essential oils from the aerial parts of *Artemisia nilagirica* and *Artemisia maritima* from the western Indian Himalaya region are described. The main compounds analyzed by simultaneous GC/MS and GC/FID were camphor and 1,8-cineole from *A. maritima*, and camphor and artemisia ketone from *A. nilagirica*. Additionally, the oils were evaluated for their antibacterial, antifungal, mosquito biting deterrent, and larvicidal activities. *A. nilagirica* essential oil demonstrated nonselective antifungal activity against plant pathogens *Colletotrichum acutatum, Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*, whereas *A. maritima* did not show antifungal activity. Both *Artemisia* spp. exhibited considerable mosquito biting deterrence, whereas only *A. nilagirica* showed larvicidal activity against *Aedes aegypti*. Antibacterial effects assessed by an agar dilution assay demonstrated greater activity of *A. maritima* essential oil against *Staphylococcus aureus* and *Pseudomonas aeruginosa* compared to *A. nilagirica*.

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Bibliography
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Introduction

The genus *Artemisia* is a member of the *Asteraceae* family and consists of more than 800 species, most of them used worldwide for medicinal purposes mainly due to their precious essential oils. *Artemisia nilagirica* (C.B. Clarke) Pamp., commonly known as "mugwort" or "Indian wormwood", grows in Europe, North Africa, Asia as well in the USA [1]. The shrub is generally 60–160 cm high, the papery and elliptic leaves are arranged in an opposite pattern, and the clustered flowers are pale yellow and arranged in panicles. There is great variation in the chemical constituents of the essential oil, not only due to the region but also to the altitude at which the plants are harvested, since in India they can be found at altitudes of over 2000 m [2, 3]. The plant's physiological age also plays an important role in the oil composition [4], as essential oil compositions change during different growth and development phases to ensure the plant's protection against external stress, pests, and insects. The effects of *A. nilagirica* essential oil from South India were investigated by Leeja et al. [5], who demonstrated mosquito larvicidal activity against *Aedes albopictus*. Essential oil obtained from plants from the same region showed antifungal activity against *Phytophthora capsici* [6]. Significant anti-dermatophytic activity of Indian *A. nilagirica* essential oil against *Epidermophyton floccosum* and *Trichophyton violaceum* was shown in *in vivo* guinea pig experiments [7]; an agar diffusion test revealed activity against *Trichophyton rubrum, Microsporum canis,* and *Microsporum gypseum* [8]. Sati et al. [9] recently showed that *A. nilagirica* essential oil from northern India demonstrated antifungal activity against *Rhizoctonia solani, Sclerotium rolfsii*, and *Macrophomina phaseolina*.

Artemisia maritima L. (syn. for *A. brevifolia* Wall. ex DC [10, 11]) is a local plant commonly growing wild in the cold desert Himalayan region of India, Afghanistan, and Pakistan at altitudes of 4000 m [12] and higher [13]. The leaves are ground into a powder and mainly used as a folk medicine treatment for digestive diseases [14], whereas the powder from the stem is used as a remedy to treat erythremia [15]. The strong aromatic shrub prefers dry and stony regions with sandy soil and can also be found in the cold desert area of Himachal Pradesh [10]. Inouye et al. [16] examined the volatile ingredients by GC/MS of six *A. maritima* samples collected in different locations in the Himalaya region in Pakistan and found 1,8-cineole

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and camphor as main constituents in all of the samples. The ethyl acetate extract of *A. maritima* showed potent antifungal activity [17]. The antispasmodic and bronchodilatory effect of *A. maritima* essential oil due to calcium channel blocker activity was confirmed by Shah et al. [11].

Although *A. nilagirica* and *A. maritima* are both used in traditional medicine throughout the Himalayan region and have been described in multiple publications, to our knowledge, none of these investigations give a complete and detailed description of their essential oil contents and activity. This paper reports the GC/MS analysis of *A. nilagirica* and *A. maritima* essential oils obtained from wild plants from the western Himalayan area in India. Additionally, their antibacterial, antifungal, and mosquito biting deterrent as well as larvicidal activities are described.

Results and Discussion

In the present investigation, *A. nilagirica* and *A. maritima* leaf essential oils were analyzed using simultaneous GC/FID and GC/MS in two columns differing in polarities (SE 54 and Carbowax 20 M), length, and diameter. **Tables 1** and **2** show the results of the qualitative and quantitative analyses of the essential oils, respectively. A total of 91 compounds, mainly monoterpenes, accounting for 87.1–85.4% depending on the column, were obtained and identified from *A. nilagirica* oil and 93 compounds (96.5%) from *A. maritima* essential oil.

The essential oil of *A. maritima* possessed the highest amount of camphor (44.3–44.4%) followed by 1,8-cineole with 27.0–27.3% (**Table 1**), which is in accordance with the reports by Inouye et al. [16] and Shah et al. [11]. Camphene was found at 5.9% and other mono- and sesquiterpenes contributed < 1.5% to the essential oil. Generally, the composition of *A. maritima* essential oil varied between investigations published in the literature. Some older publications reported α-thujone (63.3%) [13] or limonene (60–85%) as major constituents [18]. Sah et al. [19] identified chrysanthenone (25.7%) as the main compound followed by 23.6% 1,8-cineole in *A. maritima* essential oil from the Himalayan region. These data illustrate that published reports are inconsistent, and this may be due to differences in location, soil type, altitude, and physiological stage of development. The altitudinal influence was demonstrated by an investigation of Jaitak et al. [20] in western Himalaya in India. Essential oil from plants collected in the Pooh area (2500 m) contained 1,8-cineole (23.8%) and chrysanthenone (17.5%) as the major constituents, whereas oil from the Rhongtong pass at 3500 m showed significantly higher concentrations with 37.3% and 38.1%, respectively. The Lahaul-Spiti oil (4500 m) contained 1,8-cineole (44.2%), borneol (10.9%), and camphor (9.2%) as the main components.

The constituents of *A. nilagirica* essential oil compared to *A. maritima* possessed camphor (12.4–12.6%), artemisia ketone (10.2–10.3%), caryophyllene oxide (6.8–7.4%), 1,8-cineole (5.3%), and borneol (5.3%) as the major compounds (**Table 2**). Haider et al. [4] found similar results for Northern Indian *A. nilagirica* oil concerning the main constituents, but higher concentrations for caryophyllene oxide (28.6%), borneol (35.8%), and camphor (46.9%). In other reports, the differences in essential oil composition of *A. nilagirica* are qualitative rather than quantitative: (i) The analysis of the aerial parts of *A. nilagirica* from northern and southern India showed α-thujone and β-thujone as major components [6, 21]. (ii) When plants were harvested during the flowering stage, β-caryophyllene (22.8%) was identified as the main constituent of the oil [2, 5]. (iii) The altitudinal influence was investigated by Badoni and coworkers [3]. Plants collected in lower regions around 500 m demonstrated α-thujone (37%) as the major constituent [3, 6]. Whereas plants from 1200 m altitude showed high concentrations of 4-nitrobenzenoic acid-4-methoxyphenyl ester (22.1%) [3].

Olfactory evaluation of *A. maritima* oil, which was light brown in color, revealed an herbal, somewhat woody and terpenic odor with a tender green note. The odor of green-colored *A. nilagirica* essential oil was described as fresh herbal, thujone-like with a tender spicy and camphoraceus note.

Although the overall antimicrobial activity of both of the essential oils proved to be rather moderate against bacteria strains *Staphylococcus aureus, Salmonella abony, Escherichia coli*, and *Pseudomonas aeruginosa* [minimal inhibitory concentration (MIC) between 2000 and 8000 µg/mL], *A. maritima* essential oil was more active against *S. aureus* and *P. aeruginosa* compared to *A. nilagirica* (**Table 3**). *Candida albicans* was the most sensitive microorganism to both essential oils (MIC 1000 µg/mL). Ahameethunisa and Hopper [22] screened six organic solvent extracts of *A. nilagirica* for potential antimicrobial activity against fifteen bacterial strains, including *S. aureus, P. aeruginosa*, and *E. coli*. None of the extracts were active against *S. aureus*. Due to the fact that the organic extracts showed different activity against the same strains, it was concluded that the nonvolatile constituents, i.e., alkaloids, flavonoids, tannins, and amino acids, appeared to be jointly responsible for the antimicrobial effects [22]. To our knowledge, the antimicrobial data of neither *A. maritima* nor *A. nilagirica* essential oil were described in the recent scientific literature. However, antimicrobial activity was reported for *Artemisia scoparia* and *Artemisia capillaris* collected in Korea (MIC 1600–12 800 µg/mL against *S. aureus* and *E. coli*) [23] as well as *Artemisia lehmanniana* [24] and *Artemisia kermanensis* from Iran [25]. *Artemisia lavandulaefolia* showed significant antimicrobial activity against *P. aeruginosa* and *S. aureus* (MIC 250 µg/mL and 125 µg/mL, respectively) [26].

Although high percentages of 1,8-cineol are known to be associated with the antibacterial properties of essential oils e.g. *Artemisia scoparia* and *Salvia ringens* [23, 27], antimicrobial studies from the literature on single compounds revealed interesting results. Investigations published by Gupta [28] and Cha [23] reported moderate to no activity of 1,8-cineol against *S. aureus, P. aeruginosa, E. coli*, and *C. albicans* (MICs between 3200 and > 18 100 µg/mL) amongst other different genera of bacteria. These findings are in accordance with the results of Klein and coworkers [29]. An investigation of the pairwise combination of 1,8-cineol, linalool, carvacrol, α-pinene, and α-terpineol in different concentrations though, showed a significant synergistic effect for most of the combined monoterpenes leading to higher antibacterial activity against various bacteria [29], whereas some combinations led to antagonistic effects. These results impressively demonstrate the complex interaction of essential oil compounds – even trace components – and their unpredictable influence on its activity, which makes investigations on single main compounds in this context rather questionable.

Mosquito biting deterrent and larvicidal activity of *A. nilagirica* and *A. maritima* essential oils were evaluated against *Aedes aegypti. In vitro* testing of the oils showed a high level of mosquito biting deterrent activity at 10 µg/cm^2 for both *Artemisia* species (**Fig. 1**). Biting deterrent activity was similar between two *Artemisia* spp. and was lower than that of *N,N*-diethyl-meta-toluamide (DEET), which was used as a positive control, at 4.8 µg/

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Table 1 Chemical composition of the essential oil of *A. nilagirica* (in % peak area) determined by GC-FID and GC-MS.

Compound	RI[#]	% Area$_{FID}$	RI[##]	% Area$_{FID}$
hexanal			1066	tr
(E)-2-hexenal			1223	0.1
(Z)-3-hexenol			1356	tr
hexanol			1336	0.1
(Z)-4-heptenal	891	0.1		
santolinatriene	908	0.1		
tricyclene	930	0.2	1008	0.1
α-pinene	941	1.1	1019	1.2
α-fenchene			1048	tr
camphene	957	2.9	1052	2.9
benzaldehyde	961	0.2		
1-octen-3-ol	975	0.9	1433	0.9
sabinene	978	0.4	1114	0.5
3-octanone			1239	tr
6-methyl-5-hepten-2-one	982	0.1	1315	0.1
β-pinene	984	1.0	1105	1.0
myrcene	990	1.5	1149	1.5
2-pentylfurane			1209	tr
dehydro-1,8-cineole			1185	0.1
3-octanol			1376	tr
yomogi alcohol	995	0.4	1382	0.4
α-terpinene	1020	0.1	1169	0.1
p-cymene	1027	2.6	1254	2.6
(Z)-β-ocimene			1216	0.1
limonene	1032	1.8	1187	1.8
β-phellandrene			1198	tr
1,8-cineole	1036	5.3	1211	5.3
artemisia ketone	1058	10.2	1327	10.3
(E)-β-ocimene			1235	tr
γ-terpinene	1061	0.5	1233	0.5
cis-sabinene hydrate	1070	1.6	1450	1.7
artemisia alcohol	1081	0.7	1484	0.9
terpinolene	1091	0.1	1269	0.1
linalool	1102	0.7	1534	0.2
trans-sabinene hydrate	1103	0.7	1498	0.7
nonanal	1104	0.1	1381	tr
cis-thujone	1114	0.5	1403	0.5
trans-thujone	1124	0.2	1421	0.2
trans-chrysanthenol	1125	0.5	1447	0.5
cis-*p*-menth-2-en-1-ol	1125	0.3	1544	0.3
trans-*p*-mentha-2,8-dien-1-ol	1129	0.3	1606	0.3
trans-*p*-menth-2-en-1-ol	1133	0.2	1609	0.2
4,8-dimethyl-1,3,7-nonatriene	1135	0.3		
chrysanthenone	1136	0.6	1489	0.4
cis-*p*-mentha-2,8-dien-1-ol	1136	0.2	1649	0.2
trans-pinocarveol	1155	0.3	1635	0.3
trans-verbenol	1157	0.4	1652	0.6
camphor	1161	12.6	1503	12.4
lavandulol			1652	0.3
isoborneol	1165	0.6	1639	0.3
isothujol	1168	0.3	1641	0.1
borneol	1181	5.3	1686	5.3
pinocarvone			1549	0.2
terpinen-ol-4	1183	3.2	1591	3.3
trans-p-mentha-1(7),8-dien-2-ol	1190	0.2	1772	0.3
p-cymen-8-ol	1193	0.2	1833	0.1
α-terpineol	1202	1.1	1689	1.3
trans-piperitol	1204	0.1	1731	0.1
myrtenal	1212	0.4	1602	0.2
myrtenol			1769	0.3
cis-piperitol	1219	0.1	1728	0.1
trans-carveol	1221	0.7	1817	0.6
verbenone	1222	0.1		
cis-*p*-mentha-1(7),8-dien-2-ol	1230	0.2	1864	0.2
cumin aldehyde	1233	0.1	1763	0.2
cis-carveol	1234	0.1		

continued

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Table 1 *Continued*

Compound	RI[#]	% Area$_{FID}$	RI[##]	% Area$_{FID}$
carvone	1257	0.2	1723	0.3
chrysanthenyl acetate			1505	0.1
perilla aldehyde	1281	1.0	1766	1.1
lavandulyl acetate	1284	0.8		0.7
bornyl acetate	1299	1.6	1563	1.5
perilla alcohol	1303	0.9	1979	0.8
eugenol	1364	0.2	2139	0.3
bornyl propanoate	1384	0.1	1620	0.2
α-copaene	1398	0.2	1480	0.3
3-exo-hydroxybornyl acetate	1416	2.8	1941	2.9
α-gurjunene	1423	0.2	1507	0.2
bornyl isobutanoate	1425	0.1		
(E)-caryophyllene	1444	1.7	1583	1.8
α-humulene	1482	0.3	1647	0.3
germacrene D	1509	0.3	1698	0.4
γ-cadinene	1532	0.3	1737	0.1
δ-cadinene	1544	0.5	1735	0.5
α-calacorene	1562	0.2	1888	0.2
trans-nerolidol	1568	0.2		
β-calacorene	1582	0.1		
spathulenol	1609	0.6	2106	0.5
caryophyllene oxide	1611	7.4	1966	6.8
humulene epoxide II	1637	0.7		
β-eudesmol	1678	4.3	2208	4.0
α-eudesmol			2201	0.4
Total		87.1		85.4

[#] 50 m × 0.25 mm × 1.0 µm SE-54; [##] 60 m × 0.25 mm × 0.25 µm CW20M; tr = trace compound (< 0.1 %)

Table 2 Chemical composition of the essential oil of *A. maritima* (in % peak area) determined by GC-FID and GC-MS.

Compound	RI[#]	% Area$_{FID}$	RI[##]	% Area$_{FID}$
isovaleric aldehyde	646	tr	912	0.1
butanol	651	tr	1129	0.0
isoprenyl methyl ketone	666	tr	985	0.0
amyl alcohol	729	tr		
ethyl isobutanoate	751	0.1	953	0.1
hexanal	797	tr		
ethyl 2-methylbutanoate	846	0.3	1041	0.3
ethyl isovalerate	848	0.3	1058	0.3
isopropyl 2-methylbutanoate			1132	0.0
hexanol			1356	0.0
santolinatriene	908	0.1		
ethyl tiglate	921	tr		
tricyclene	930	0.3	1008	0.3
α-pinene	941	0.5	1019	0.6
propyl 2-methylbutanoate			1227	0.0
camphene	957	5.9	1062	5.9
thuja-2,4(10)-diene	961	0.1		
benzaldehyde	962	tr		
1-octen-3-ol	975	tr	1433	0.0
sabinene	978	0.6	1114	0.7
β-pinene	984	0.4	1105	0.4
myrcene	990	0.3	1149	0.3
dehydro 1,8-cineole	996	0.3	1185	0.3
octanal	1000	tr	1283	0.0
α-phellandrene	1011	tr	1160	0.0
amyl isobutyanoate	1013	tr		
α-terpinene	1020	0.1	1169	0.1
santolina alcohol			1395	0.0
p-cymene	1027	0.7	1254	0.6
limonene	1032	0.1		
1,8-cineole	1036	27.3	1211	27.0
γ-terpinene	1061	0.3	1233	0.3
octanol	1064	0.1	1554	0.1
cis-sabinene hydrate	1070	0.1		
ethyl heptanoate			1381	0.1

continued

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Table 2 *Continued*

Compound	RI[#]	% Area$_{FID}$	RI[##]	% Area$_{FID}$
camphenilone	1089	0.1	1448	0.1
terpinolene	1091	0.1	1269	0.1
p-cymenene			1423	0.0
linalool	1102	0.2	1534	0.1
nonanal			1381	0.0
isoamyl 2-methylbutanoate	1103	0.1	1279	0.1
trans-sabinene hydrate	1103	0.1	1498	0.1
cis-thujone	1114	0.3	1415	0.3
trans-thujone	1124	1.0	1433	1.0
isochrysanthenone			1427	0.1
dehydro sabina ketone	1131	0.3	1617	0.2
chrysanthenone	1136	0.2	1489	0.3
trans-pinene hydrate	1138	0.3		
cis-verbenol			1674	0.3
trans-pinocarveol	1155	0.7	1635	0.8
cis-pinene hydrate	1157	0.2	1627	0.3
trans-verbenol			1652	0.1
camphor	1161	44.4	1503	44.3
cis-chrysanthenol	1172	1.0	1738	1.0
sabina ketone	1173	0.2		
mentha-1,5-dien-8-ol			1656	0.0
3-thujanol			1549	0.1
cis-pinocamphone			1537	0.1
δ-terpineol	1179	0.3	1669	0.2
pinocarvone	1181	0.5	1549	0.4
borneol	1181	0.9	1686	1.0
cis-3-hexenyl butanoate			1602	0.1
terpinen-4-ol	1191	1.5	1591	1.5
p-cymen-8-ol	1193	0.2	1833	0.1
α-terpineol	1202	0.5	1689	0.4
myrtenol	1210	0.5	1785	0.5
myrtenal	1212	0.6	1619	0.6
cis-piperitol	1219	0.1	1744	0.1
verbenone	1222	0.1		
cis-carveol			1859	0.1
cumin aldehyde	1233	0.1	1763	0.2
isopropylphenol	1238	0.1	2184	0.1
carvone	1257	0.9	1723	1.0
cis-piperitone epoxide	1268	0.1		
geranial	1269	0.1		
cis-chrysanthenyl acetate	1271	0.6	1561	0.9
p-cymen-7-ol			2077	0.0
bornyl acetate	1299	1.2	1563	1.0
trans-sabinyl acetate			1641	0.2
phenylpropionic acid ethyl ester	1358	0.1	1868	0.2
α-terpinyl acetate	1358	tr		
eugenol	1364	0.3	2129	0.1
cis-jasmon			1938	0.1
methyl cinnamate			2055	0.0
α-copaene	1398	0.1		
(E)-caryophyllene	1444	0.1	1583	0.2
(E)-*β*-farnesene	1473	tr		
ethyl *trans*-cinnamate	1479	0.1		
germacrene D	1509	0.3	1698	0.4
artedouglasia oxide C	1550	tr	2003	0.0
artedouglasia oxide A	1562	tr		
spathulenol	1609	0.1	2114	0.1
furomyrcenol			2016	0.1
Total		96.5		96.5

[#] 50 m × 0.25 mm×1.0 µm SE-54; [##] 60 m × 0.25 mm × 0.25 µm CW20M; tr = trace compound (< 0.1 %)

cm^2. The essential oil of *A. nilagirica* showed 100 % larvicidal mortality at 120 ppm. Permethrin (positive standard) at 0.025 ppm gave 100 % mortality in the screening bioassays. These data corroborate the findings of Panneerselvam et al. [30], who reported 41 % mortality in four different larval instars and pupae of *Anopheles stephensi* at a concentration of 200 ppm of the extract increasing to 94 % at 600 ppm in *Ae. aegypti*. Additionally, their results showed significant repellency of the leaf extract against

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Table 3 Antimicrobial activity of the essential oils of *A. nilagirica* and *A. maritima* (agar dilution assay, MIC in µg/mL).

Aroma sample	Test microorganism				
	S. aureus ATCC 6538	*E. coli* ATCC 25922	*S. abony* ATCC 6017	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231
A. maritima	2000	2000	2000	4000	1000
A. nilagirica	4000	2000	2000	8000	1000
Ciprofloxacin	0.25	0.15	0.25	1	–
Cefazolin	0.50	2	2	4	–
Amphotericin B	–	–	–	–	0.25
Fluconazole	–	–	–	–	0.25

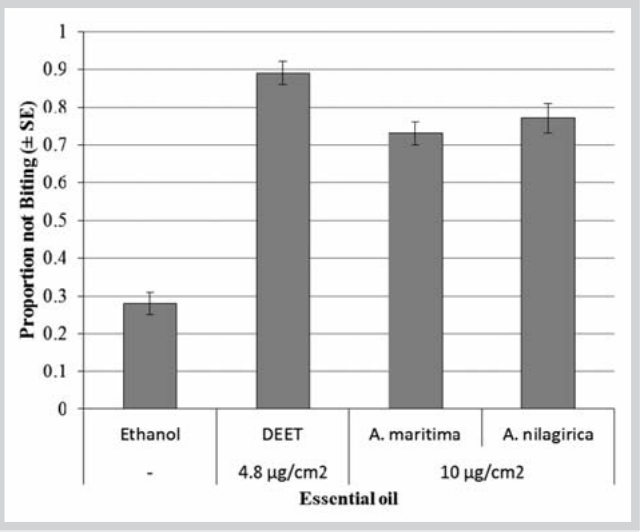

Fig. 1 The proportion of not biting values using *A. nilagirica* and *A. maritima* essential oils against female *Ae. aegypti*. DEET was tested at 5.8 µg/cm^2 and the essential oils were tested at 10 µg/cm^2. Ethanol was the solvent control and DEET was used as a positive control.

these insects in an *in vivo* experiment. Leeja et al. [5] studied larvicidal activity of *A. nilagirica* essential oil against *Ae. albopictus* and found the LC_{50} value of 5 µg/mL. Although the experimental methods of the investigations were different, the essential oil of *A. nilagirica* appears to be more active than the extract, leading to the conclusion that the volatile constituents might be responsible for the larvicidal and mosquito repellent activity. Nevertheless, our investigation showed no larvicidal activity from *A. maritima* at the highest dose of 120 ppm.

A. nilagirica and *A. maritima* essential oils were evaluated in this study for antifungal activity using direct bioautography assays against strawberry anthracnose causing three *Colletotrichum* species, *Colletotrichum acutatum, Colletotrichum fragariae*, and *Colletotrichum gloeosporioides. A. nilagirica* essential oil demonstrated clear zones (5.5 ± 0.7 mm and 11.5 ± 0.7 mm) of fungal growth inhibition, 80 and 160 µg/spot, respectively, against all three *Colletotrichum* species. At the high concentration, *A. nilagirica* essential oil showed better activity using direct bioautography compared with the growth inhibition provided by the fungicide captan (clear zones of 18.5 ± 1.3 mm). Captan is a well-known, multi-site inhibitor fungicide with no systemic activity and is often used as a protectant fungicide in commercial strawberry production to prevent anthracnose of fruits and plants caused by *Colletotrichum* species [31]. *A. nilagirica* essential oil also exhibited potent antifungal activity against phytopatogenic fungi *R. solani, S. Rolfsii*, and *M. phaseolina* [21]. ED_{50} values of the oil were higher than bavistin, a commercial antifungal drug, but lower than azadirachtin. These findings showed a higher activity of *A. nilagirica* oil against the tested fungi than the botanical reference azadirachtin [21]. Two other investigations demonstrated moderate antifungal activity at 0.50 mg/mL against two toxigenic strains of *Aspergillus flavus* (Navjot 4 NSt and Saktiman 3 NSt), an aflatoxin producing storage fungus [32, 33]. However, *A. maritima* essential oil did not show any antifungal activity against these plant pathogens even at the highest concentration. This finding was supported by Sharma et al. [34] who reported moderate to little antifungal activity of *A. maritima* essential oil against *Rhizoctonia bataticola* and *S. rolfsii*. At a concentration of 0.50 mg/mL, the oil showed 86.8% inhibition of mycelial growth of *S. rolfsii* but only 28.1% of *R. bataticola*. These results indicate little antifungal activity of *A. maritima* essential oil against *S. rolfsii* (ED_{50} 290.8) and *R. bataticola* (ED_{50} 868.1).

The lack of antifungal activity of *A. maritima* essential oil could be attributed to high contents of cyclic monoterpenoids (71.7%). The antifungal activity of *A. nilagirica* could be due to other compounds rather than camphor and 1,8-cineole, since their concentration is low in the investigated oil with 12.8% and 5.3%, respectively. Synergistic effect of more than one individual compound could be responsible for the activity [29]. Further studies should be carried out to investigate one-dimensional thin-layer chromatography coupled with direct bioautography assays against *Colletotrichum* and other species.

In conclusion, the investigated *Artemisia* essential oils showed different larvicidal, antimicrobial, and antifungal activities. *A. nilagirica* oil was more active against fungal strains and had better larvicidal activity, whereas the essential oil of *A. maritima* showed slightly better antimicrobial activity. Both oils exhibited good mosquito deterrent activity and should be further explored to find their use as alternative phytochemicals to replace synthetic chemicals.

The goal of this study was to investigate the activity of two *Artemisia* essential oils from wild growing plants, which are used in local traditional medicine, collected from areas that are known for their special climate [35]. These annually changing conditions play an important role in the plant's production of essential oils [36, 37]. An investigation of the oils' compositions under well-defined ecological conditions (e.g., glass house) to access possible chemotypes [35, 37] and to standardize the essential oil composition for the market could be an interesting subject for further research.

Materials and Methods

Plant material and isolation procedure

A. maritima was collected wild from the Lahaul-Spiti cold desert area of western Himalaya, at an altitude of 4000 m in August 2009. *A. nilagirica* was collected around Palampur at an altitude

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of 1400 m in western Himalaya in August 2010. The samples were identified by a taxonomist (Brij Lal) of the Biodiversity Division (IHBT Palampur). Voucher specimens (*A. maritima* 6553 and *A. nilagirica* 0899) were deposited at the Herbarium-PLP of the Institute of Himalayan Bioresource Technology, Palampur, India. The leaves and aerial parts of both species were air-dried at room temperature (about 25 °C) separately in the shade. In a glass Clevenger-type apparatus of 5 kg capacity, the leaves and aerial parts, including inflorescence of *A. maritima*, were hydrodistilled until total recovery of the essential oil. For *A. nilagirica* essential oil, the aerial parts without inflorescence were used. The oil samples were dried by anhydrous sodium sulphate, filtered, and stored at 4 °C until analysis. *A. nilagirica* yielded a green-colored oil (0.2% w/v), while the oil of *A. maritima* (0.25% w/v) was light brown in color.

Essential oil analysis

Using a Finnigan ThermoQuest TraceGC with two split/splitless injectors, an FID detector, and a Finnigan Automass quadrupole mass spectrometer, simultaneous GC/FID and GC/MS analyses were carried out in one instrument on two capillary columns of different polarity. One inlet was coupled to a 50 m × 0.25 mm × 1.0 µm SE-54 fused silica column (CS Chromatographie Service), the other injector was connected to a 60 m × 0.25 mm × 0.25 µm Carbowax 20 M column (J & W Scientific, Inc.). An X connector (cross union) was attached to the two columns at the outlet. Its two other joints were coupled to the FID and MS detectors with a short (ca. 20 cm) 0.1 mm ID fused silica restrictor column as a GC/MS interface and a 1 m × 0.25 mm deactivated fused silica column as a transfer line for the FID.

Using this configuration resulted in FID and MS chromatograms with almost identical retention times, thus facilitating the peak assignment of the FID chromatogram. The carrier gas was helium, with a constant flow rate of 1.5 mL/min for the operated column, while the unused column was held at a low flow rate of 0.1 mL/min. The injector temperature was 230 °C, FID detector temperature 250 °C, GC-MS interface heating 250 °C, ion source 150 °C, EI mode at 70 eV, and scan range 40–300 amu. The following temperature program was used: 46 °C for 1 min to 100 °C at a rate of 5 °C/min.; 100 °C to 230 °C at 2 °C/min; and 230 °C for 13.2 min.

Finnigan XCalibur 1.2 software was used for identifying the compounds by correlating mass spectra to databases of NIST 2008 (Scientific Instrument Services, Inc.), Wiley 8th ed., Adams [38], MassFinder (Hochmuth Scientific Consulting), and our own library. Retention indices were calculated and compared to data of reference compounds and the literature [39–42] to confirm the peak data. Quantification was performed using normalized peak area calculations from the FID chromatograms.

Olfactory evaluation

Olfactory evaluation was conducted by one droplet of each essential oil sample being applied to commercially available paper blotters. Each sample was examined by a panel consisting of a professional perfumer and two aroma chemists over 90 min to control odor progression.

Antimicrobial testing

The antimicrobial effects of both essential oils were tested against the gram-positive bacteria *S. aureus* (ATCC 6538), as well as the gram-negative bacteria *E. coli* (ATCC 25922), *S. abony* (ATCC 6017), and *P. aeruginosa* (ATCC 27853). Additionally, an antifungal testing against *C. albicans* ATCC 10231 was performed. All strains were deposited in the Microbial Culture Collection of the Department of Biochemistry and Microbiology (University of Plovdiv, Bulgaria) and bacterial strains were stored on nutritional agar (NA, HiMedia Laboratories Ltd.). The fungal strain was stored on Sabouraud dextrose agar (SDA, HiMedia Laboratories Ltd.).

Bacterial cultures were prepared by inoculating about 2 mL of Mueller-Hinton broth (MHB; HiMedia Laboratories Ltd.) with two to three colonies selected from NA. Cultures were incubated at 37 °C for 24 h on a rotary shaker at 220 rpm. For preparation of the inocula, overnight cultures were diluted in sterile MHB to achieve an absorbance corresponding to 0.5 McFarland turbidity standards (1.0–1.5 × 10^8 CFU/mL) using a bench top densitometer for measuring turbidity of the cell suspensions (DEN-1, Grant Instruments, Ltd.). The yeast culture was prepared following the same procedure, except Sabouraud dextrose broth (SDB, HiMedia Laboratories Ltd.) was used instead of MHB. Antimicrobial activity of the aroma samples was evaluated by the Broth Microdillution Test in accordance with the CLSI broth microdilution method in Nunc™ 96-well microtitration plates [43,44]. Stock solutions prepared by dispersing the respective aroma sample in 2% DMSO (Sigma-Aldrich Co.) were added to the culture broth to obtain dilutions with final sample concentrations, after inoculation with microbial test suspension, between 8000 µg/mL and 125 µg/mL. Controls consisting of inoculated broth without an aroma sample and without DMSO as well as with DMSO were also prepared. The DMSO concentration in the broth dilution assay was low in order to keep the effect on bacterial growth to a minimum. Subsequently, the 96-well microtitration plates were incubated at 37 °C for 24 h for bacterial cultures and 48 h for the yeast culture. Following incubation, the absorbance of samples was measured by a microplate reader (ELx800 absorbance microplate reader, Bio-Tek Instruments, Ltd.). MIC was defined as the lowest concentration of essential oil which resulted in an absorbance reduction of > 90% compared to the observed absorbance of control samples without essential oil. For positive controls, standard antibacterial antibiotic HiComb™ MIC test strips of Ciprofloxacin and Cefazolin and antifungal HiComb™ MIC test strips of Amphotericin B and Fluconazole (HiMedia Laboratories Ltd., 100% purity) were evaluated. All tests were performed in duplicate.

Antifungal testing

Direct bioautography assay against plant pathogens: Isolates of *C. acutatum, C. fragariae*, and *C. gloeosporioides* were obtained from B.J. Smith, Small Fruit Research Station, ARS-USDA, Poplarville, MS. *C. fragariae* (isolate CF63), *C. acutatum* (isolate CAGoff), and *C. gloeosporioides* (isolate CG162) were used for all pathogen and bioautography studies. Isolate CF63 is one of the most virulent isolates at infecting strawberry plants and inducing both crown and fruit rot [45]. Conidia were harvested from 7- to 10-day-old cultures by flooding the plates with 5 mL of sterile distilled water and dislodging the conidia by softly brushing the colonies with an L-shaped glass rod. Conidial suspensions were filtered through a sterile miracloth (Calbiochem-Novabiochem Corp.) to remove mycelia. Conidia concentrations were determined photometrically [31] from a standard curve based on the percent of transmittance (%T) at 625 nm, and suspensions were then adjusted with sterile distilled water to a concentration of 1.0 × 10^5 conidia/mL. Extracts and fractions containing antifungal compounds were indicated by clear zones of fungal growth inhibition directly on silica gel chromatography plates using modifications of thin-layer chromatography (TLC) bioautographic assays [31]. TLC plates for bioautography assays were spotted with 80 and

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160 µg/spot of crude plant extracts and chromatographed in one dimension. Fungicide standards benomyl, cyprodinil, captan (all > 98%; Chem Service, Inc.), and azoxystrobin (> 98%; Syngentia) were used as controls at 2 mM in 2 µL of EtOH. To detect biological activity directly on the TLC plate, silica gel plates were sprayed with one of the three spore suspensions adjusted to a final concentration of 3.0×10^5 conidia/mL with liquid potato dextrose broth (PDB, Difco) and 0.1% Tween-80. Using a 50-mL chromatographic sprayer, each TLC plate with a fluorescent indicator (250 µm, silica gel GF Uniplate, Analtech, Inc.) was sprayed lightly (to a damp appearance) three times with the conidial suspension. Inoculated plates were then placed in a 30 × 13 × 7.5 cm moisture chamber (398-C, 100% relative humidity, Pioneer Plastics, Inc.) and incubated in a growth chamber at 24 ± 1 °C and a 12-h photoperiod under 60 ± 5 µmols · m^{-2} · sec^{-1} light. The inhibition of fungal growth was measured four days after treatment. The sensitivity of each fungal species to each test compound was determined by comparing the size of the inhibitory zones.

Mosquito biting tests

Insects: Ae. aegypti used in these studies were obtained from a laboratory colony maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida [46]. For biting deterrence bioassays, pupae were maintained in the laboratory at 27 ± 2 °C and 60 ± 10% RH, and 5- to 13-day-old adult females were used. For larval bioassays, the eggs were hatched and the larvae were maintained at a temperature of 27 ± 2 °C and 60 ± 10% RH in a photoperiod regimen of 12 : 12 h (L: D).

Mosquito biting bioassays: Experiments were conducted by using a six-celled *in vitro* Klun and Debboun (K&D) module bioassay system developed by Klun et al. [47] for quantitative evaluation of bite deterrent properties of candidate compounds. Briefly, the assay system consists of a six-well reservoir with each of the 3 × 4 cm wells containing 6 mL of blood. As described by Ali et al. [48], a feeding solution consisting of CPDA-1 and ATP was used instead of blood. *A. maritima* and *A. nilagirica* essential oils were tested in this study. Treatments were applied at 10 µg/cm^2 and DEET (97%; Sigma Aldrich) at 4.8 µg/cm^2 was used as a positive control. All the treatments were freshly prepared in molecular biology grade 100% ethanol (Fisher Scientific Chemical Co.) at the time of bioassay.

The temperature of the solution in the reservoirs was maintained at 37.5 °C by continuously passing warm water through the reservoir using a circulatory bath. The reservoirs were covered with a layer of collagen membrane (Devro). The test compounds were randomly applied to six 4 × 5 cm areas of organdy cloth and positioned over the membrane-covered CPDA-1 + ATP solution with a Teflon separator placed between the treated cloth and the six-celled module to prevent contamination of the module. A six-celled K&D module containing five female mosquitoes per cell was positioned over the cloth treatments covering the six CPDA-1 + ATP solution membrane wells, and trap doors were opened to expose the treatments to these females. The number of mosquitoes biting through the cloth treatments in each cell was recorded after a 3-min exposure, and mosquitoes were prodded back into the cells to check the actual feeding. These mosquitoes were then squashed to determine the numbers that had actually imbibed the solution. Three sets of five replications each with five females per treatment were conducted on three different days using a newly treated organdy and a new batch of females in each replication. Treatments were replicated 14 times.

Larval bioassays

Bioassays were conducted using the system described by Pridgeon et al. [49] to determine the larvicidal activity of the essential oils against *Ae. aegypti*. Five 1-day-old larvae were transferred to individual wells of a 24-well tissue culture plate in a 30–40 µL droplet of water. Fifty µL of larval diet of 2% slurry of 3 : 2 beef liver powder (Now Foods) and Brewer's yeast (Lewis Laboratories Ltd.) and 1 mL of deionized water were added to each well by using a Finnpipette stepper (Thermo Fisher). *A. maritima* and *A. nilagirica* essential oils were diluted in DMSO. Eleven microliters of the test chemical was added to the labeled wells, while 11 µL of DMSO was added to the control treatments. After treatment application, the plates were swirled in clockwise and counter clockwise motions and front and back and side to side five times to ensure even mixing of the tested compounds. Permethrin (positive standard, 46.1% *cis* – 53.2% *trans*, Chemical Service) at 0.025 ppm gave 100% mortality in the screening bioassays. Larval mortality was recorded 24 h post-treatment.

Statistical analyses

The proportion of not biting insects (PNB) was calculated using the following formula:

$$\text{PNB} = 1 - \frac{\text{Total number of females biting}}{\text{Total number of females}}$$

The proportion of not biting data was analyzed using SAS Proc ANOVA (SAS Institute 2007), and the means were separated using the Ryan-Einot-Gabriel-Welsch multiple range test.

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Conflict of Interest

The authors declare no conflict of interest.

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References

1 *Rani PN, Moorthi C, Senthamarai R, Kathiresan K.* A study to explore the pharmacognostic and phytochemical screening of *Artemisia nilagirica* leaves found in Nilgiris district of Tamil Nadu. Int J Pharm Sci 2012; 4: 441–447
2 *Haider F, Kumar N, Naqvi A, Das Bagchi G.* Oil constituents of *Artemisia nilagirica* var. *septentrionalis* during different growth phases at subtropical conditions of North Indian plains. J Essent Oil Res 2007; 19: 5–7
3 *Badoni R, Semwal DK, Rawat U.* Altitudinal variation in the volatile constituents of *Artemisia nilagirica*. Int J Ess Oil Therapeutics 2009; 3: 66–68
4 *Haider F, Kumar N, Naqvi A, Das Bagchi G.* Oil constituents of *Artemisia nilagirica* var. *septentrionalis* growing at different altitudes. Nat Prod Commun 2010; 5: 1959–1960
5 *Leeja L, Thoppil JE.* Essential oil composition and mosquito larvicidal activity of *Artemisia nilagirica* (C.B. Clarke) Pampan. from South India. J Phytol Res 2004; 17: 155–158
6 *Shafi PM, Nambiar MKG, Clery RA, Sarma YR, Veena SS.* Composition and antifungal activity of the oil of *Artemisia nilagirica* (Clarke) Pamp. J Ess Oil Res 2004; 16: 377–379
7 *Kishore A, Dubey NK, Chansouria JPN.* Antimycotic activity of the essential oil of *Artemisia nilagirica*. Flavour Fragr J 2001; 16: 61–63
8 *Vijayalakshmi A, Rakesh T, Ravichandiran V.* Characterization and evaluation of antidermatophytic activity of the essential oil from *Artemisia nilagirica* leaves growing wild in Nilgiris. Int J Pharm Sci 2010; 2: 93–97
9 *Sati SC, Sati N, Ahluwalia V, Walia S, Sati OP.* Chemical composition and antifungal activity of *Artemisia nilagirica* essential oil growing in northern hilly areas of India. Nat Prod Res 2013; 27: 445–448
10 *Kumar E, Bath ZA, Kumar V, Zargar MI.* A short review on *Artemisia maritima* Linn. Int J Res Phytochem Pharmacol 2011; 1: 201–206
11 *Shah AJ, Gilani AH, Abbas K, Rasheed M, Ahmed A, Ahmed VU.* Studies on the chemical composition and possible mechanisms underlying the antispasmodic and bronchodilatory activities of the essential oil of *Artemisia maritima* L. Arch Pharm Res 2011; 34: 1227–1238
12 *Ashraf M, Hayat MQ, Jabeen S, Shaheen N, Khan MA, Yasmin G. Artemisia* L. species recognized by the local community of northern areas of Pakistan as folk therapeutic plants. J Med Plants Res 2010; 4: 112–119
13 *Mathela CS, Kharkwal H, Shah GC.* Essential oil composition of some Himalayan *Artemisia* species. J Ess Oil Res 1994; 6: 345–348
14 *Khan SM, Harper DM, Page S, Ahmad H.* Residual value analyses of the medicinal flora of the Western Himalayas: the Naran valley, Pakistan. Pak J Bot 2011; 43: 97–103
15 *Gilani SS, Abbas SQ, Shinwari ZK, Hussain F, Nargis K.* Ethnobotanical studies of Kurram Agency, Pakistan through rural community participation. Pak J Biol Sci 2003; 6: 1368–1375
16 *Inouye, S, Amano M, Abe S, Amin M, Karim F.* Volatile aroma constituents to twenty herbs growing wild in Karakoram/Himalaya in Pakistan (1). Aroma Res 2006; 7: 354–361
17 *Inouye S, Uchida K, Abe S.* Antimicrobial activity, especially anti-*Trichophyton* vapor activity of twenty herbs growing wild in the Karakoram (Pakistan Himalayas). Aroma Res 2007; 8: 282–288
18 *Hethelyi E, Eseko I, Grosz M, Gergel M, Palinkas J.* Capillary GC analysis of *Artemisia* essential oils. Kozmetika 1995; 44: 117–120
19 *Sah S, Lohani H, Narayan O, Bartwal S, Chauhan NK.* Volatile constituents of *Artemisia maritima* Linn. grown in Garhwal Himalaya. J Ess Oil Bear Plants 2010; 13: 603–606
20 *Jaitak V, Singh B, Kaul VK.* Variability of volatile constituents in *Artemisia maritima* in western Himalaya. Nat Prod Res 2008; 22: 565–568
21 *Sati SC, Sati N, Ahluwalia V, Walia S, Sati OP.* Chemical composition and antifungal activity of *Artemisia nilagirica* essential oil growing in northern hilly areas of India. Nat Prod Res 2013; 27: 45–48
22 *Ahameethunisa AR, Hopper W.* Antibacterial activity of *Artemisia nilagirica* leaf extracts against clinical and phytopathogenic bacteria. BMC Complement Alternat Med 2010; 10: 6
23 *Cha JD, Jeong MR, Jeong SI, Moon SI, Kim JY, Kil BS, Song YH.* Antimicrobial activity of the essential oils of *Artemisia scoparia* and *A. capillaris*. Planta Med 2005; 71: 186–190
24 *Kazemi M, Dakhili M, Davari M.* Constituents and antimicrobial activity of essential oil of *Artemisia lehmanniana* Bunge from Iran. J Ess Oil Bear Plants 2011; 15: 392–398
25 *Kazemi M, Dakhili M, Dadkhah A, Yasrebifar Z, Lafijani K.* Composition, antimicrobial and antioxidant activities of the essential oil of *Artemisia kermanensis* Podl., an endemic species from Iran. J Med Plant Res 2011; 5: 4993–4998
26 *Zhang J, Shi B, Gao P, Wang J, Wu W.* Composition, *in vitro* antioxidant and antimicrobial activity of the essential oils from *Artemisia lavandulaefolia* DC. J Med Plant Res 2012; 6: 1284–1288
27 *Tzakou O, Pitarokili D, Chinou IB, Harvala C.* Composition and antimicrobial activity of the essential oil of *Salvia ringens*. Planta Med 2001; 67: 81–83
28 *Gupta N, Saxena G, Kalra SS.* Antimicrobial activity pattern of certain terpenoids. IJPBS 2011; 2: 87–91
29 *Klein G, Rüben C, Upmann M.* Antimicrobial activity of essential oil components against potential food spoilage microorganisms. Curr Microbiol 2013; 67: 200–208
30 *Panneerselvam C, Murugan K, Kovendan K, Mahesh KP.* Mosquito larvicidal, pupicidal, adulticidal and repellent activity of *Artemisia nilagirica* (Famils: Dompositae) against *Anopheles stephensi* and *Aedes aegypti*. Parasitol Res 2012; 111: 2241–2251
31 *Wedge DE, Nagle DG.* A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. J Nat Prod 2000; 63: 1050–1054
32 *Kumar R, Dubey NK, Tiwari OP, Tripathi YB, Sinha KK.* Evaluation of some essential oils as botanical fungitoxicants for the protection of stored food commodities from fungal infestation. J Sci Food Agric 2007; 87: 1737–1742
33 *Kumar R, Mishra SK, Dubey NK, Tripathi YB.* Evaluation of *Chenopodium ambrosioides* oil as a potential source of antifungal, antiaflatoxigenic and antioxidant activity. Int J Food Microbiol 2007; 115: 159–164
34 *Sharma PK, Raina AP, Dureja P.* Evaluation of the antifungal and phytotoxic effects of various essential oils against *Sclerotin rolfsii* (Sacc) and *Rhizoctonia bataticola* (Taub). Arch Phytopath Plant Protect 2009; 42: 65–72
35 *Singh N, Kaul VK, Megeji NW, Singh V, Shuja PS.* Essential oil composition of three accessions of *Dracocephalum heterophyllum* Benth. cultivated at Palampur, India. NPC 2008; 22: 927–936
36 *Schmidt E.* Production of essential oils. In: Baser KH, Buchbauer G, editors. Handbook of essential oils. Boca Raton: CRC Press; 2010: 89–90
37 *Nemeth E, Bernáth J, Tarján G.* Quantitative and qualitative studies of essential oils of Hungarian *Achillea* populations. JHSMP 2007; 13: 57–69
38 *Adams RP.* Identification of essential oil components by gas chromatography/quadrupole mass spectrometry. Carol Stream: Allured Publishing; 2007
39 *Joulain D, Koenig WA.* The atlas of spectral data of sesquiterpene hydrocarbons. Hamburg: EB Verlag; 2008
40 *Heller SR, Milne GWA.* EPA/NIH mass spectral data base. Washington DC: US Government Printing Office; 1978, 1980, 1983
41 *Stenhagen E, Abrahamsson S, McLafferty FW.* Registry of mass spectral data. New York: Wiley; 1974
42 *Swigar AA, Siverstein RM.* Monoterpenes. Milwaukee: Aldrich Chemical Co.; 1981
43 *Clinical and Laboratory Standards Institute.* Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; CLSI approved standard document M7-A7, 7th edition. Wayne: CLSI; 2006
44 *Clinical and Laboratory Standards Institute.* Reference method for broth dilution antifungal susceptibility testing of yeasts: CLSI approved standard M27-A3, 3rd edition. Wayne: CLSI; 2008
45 *Smith BJ, Black LL.* Morphological, cultural, and pathogenic variation among *Colletotrichum* species isolated from strawberry. Plant Dis 1990; 74: 69–76
46 *Pridgeon JW, Meepagala KM, Becnel JJ, Clark GG, Pereira RM, Linthicum KJ.* Structure-activity relationships of 33 piperidines as toxicants against female adults of *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 2007; 44: 263–269
47 *Klun JA, Kramer M, Debboun M.* A new *in vitro* bioassay system for discovery of novel human-use mosquito repellents. J Am Mosq Control Assoc 2005; 21: 64–70
48 *Ali A, Cantrell CL, Bernier UR, Duke SO, Schneider JC, Khan I. Aedes aegypti* (Diptera: Culicidae) biting deterrence: structure-activity relationship of saturated and unsaturated fatty acids. J Med Entomol 2012; 49: 1370–1378
49 *Pridgeon JW, Becnel JJ, Clark GG, Linthicum KJ.* A high throughput screening method to identify potential pesticides for mosquito control. J Med Entomol 2009; 46: 335–341

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