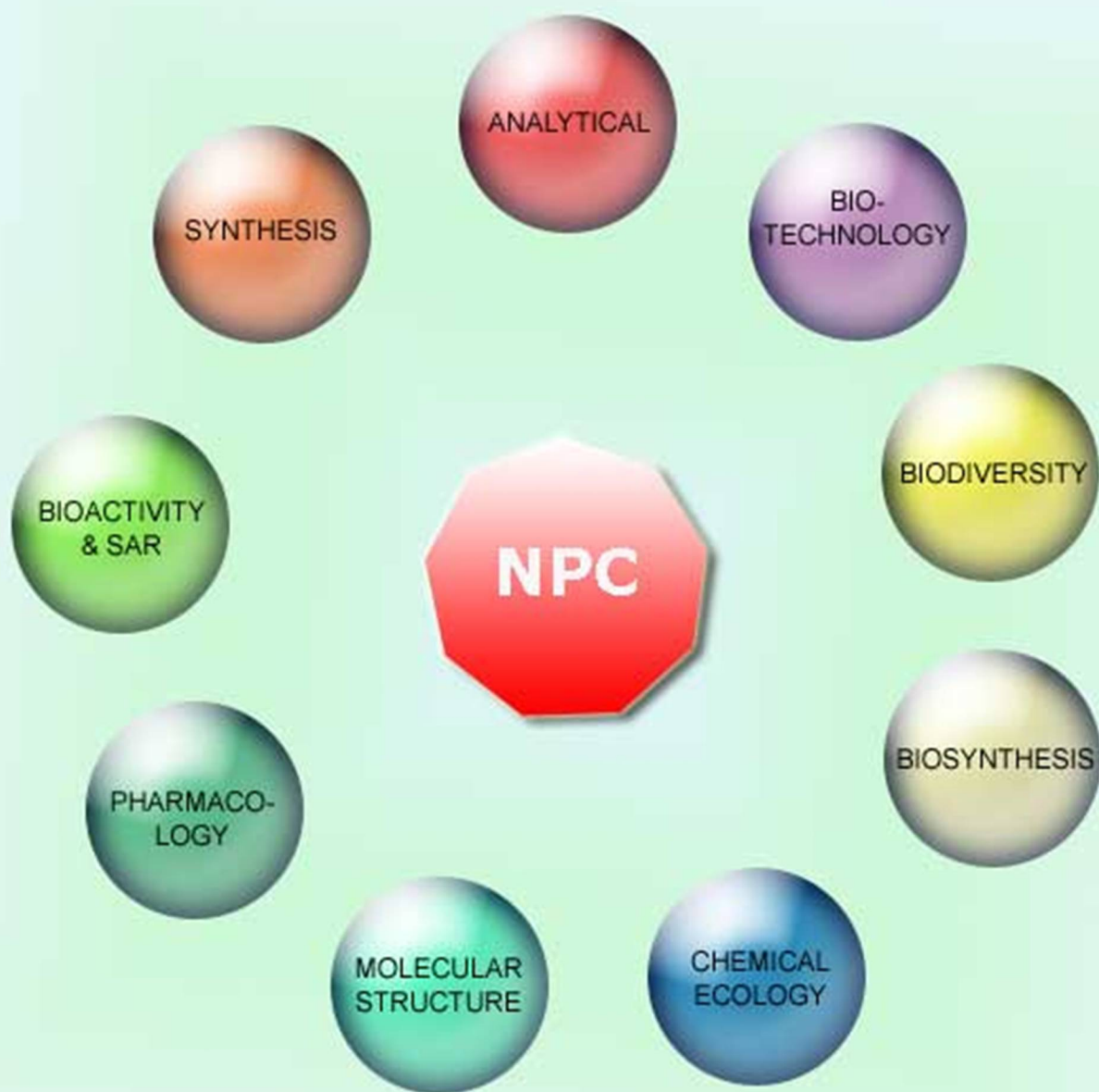


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**This Issue is Dedicated to
Professor Gerald Blunden
On the Occasion of his 72nd Birthday**

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Essential Oil Composition of Five Collections of *Achillea biebersteinii* from Central Turkey and their Antifungal and Insecticidal Activity

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The composition of the essential oils hydrodistilled from the aerial parts of five *Achillea biebersteinii* Afan samples, collected in central Turkey from Konya, Isparta and Ankara, were analyzed both by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Eighty-four components were identified, representing 87 to 99% of the total oil composition. The identified major components were 1,8-cineole (9-37%), camphor (16-30%) and *p*-cymene (1-27%). Two samples differed in piperitone (11%) and ascaridol (4%) content. The five *A. biebersteinii* essential oils were subsequently evaluated for their antifungal activity against the strawberry anthracnose-causing fungal plant pathogens *Colletotrichum acutatum*, *C. fragariae* and *C. gloeosporioides* using the direct overlay bioautography assay. The essential oils showed no antifungal activity at 80 and 160 µg/spot. In addition, *A. biebersteinii* oils and their major compounds were subsequently investigated against *Aedes aegypti* first instar larvae in a high throughput bioassay. Among the oils, only one sample from Ankara showed a notable larvicidal effect on *Ae. aegypti* larvae. The major compounds, 1,8-cineole, camphor and *p*-cymene, exhibited low mosquito larval activity, and thus the minor compounds are probably responsible for the observed activity against *Ae. aegypti* larvae. The oils showed weak activity against adult *Ae. aegypti*.

Keywords: *Achillea biebersteinii*, plant pathogens, *Colletotrichum*, bioautography, *Aedes aegypti*, adult activity, larvicidal activity.

The genus *Achillea* L. (Asteraceae) is represented by about 115 species found in the Northern Hemisphere, mostly in Europe and Asia, and commonly known as yarrows [1-3]. The genus name *Achillea* may have been derived from Achilles of Greek mythology and its historical reputation for healing wounds made it popular among the military and this association led to many of its common names: knight's milfoil, herba milifaris, staunch weed, soldiers' bloodwort and nosebleed [2,3]. Antimicrobial, antioxidant, antiinflammatory, spasmolytic, antidiabetic, antiulcer, antitumor, choleric and hepatoprotective activity, and cytotoxic effects of different *Achillea* species have been previously reported [3-9]. Phytochemical studies carried out on *Achillea* essential oils have identified sesquiterpene lactones, flavonoids, alkaloids, lignans, triterpenes alkalamides and polyacetylenes [3,4]. *Achillea* essential oils and sesquiterpene lactones have been studied by a number of investigators, but never evaluated for their potential as agrochemicals [3,4,8,10,11]. Terpenoids (1,8-cineole, camphor, borneol, pinenes, artemisia ketone, santolina alcohol, farnesane, caryophyllene and its oxides, α -bisabolol

and oxides, cubebene, germacrenes, eudesmol, farnesene, γ -gurjunene, γ -muurolene and chamazulene) are the principle components of *Achillea* essential oils [3,4]. Yarrow's healing power is mainly attributed to proazulenes, and so the chemical composition of yarrow oils has been investigated by several research groups in different countries [4,6,8,10,11] in search of novel compounds.

Scientists at the USDA, Natural Product Utilization Research Unit in Oxford (NPURU), Mississippi, in collaboration with the Deployed War-Fighter Protection (DWFP) Research Program have expanded their role in exploration and identification of new natural compounds for mosquito activity. The DWFP program emphasis is on identifying and testing new classes of chemical compound for control of insect vectors, new tools for chemical application suited to the protection of troops and human populations after natural disasters (ie. hurricanes, tsunamis), and new methods for personal protection (ie. clothing, bed netting, ointments) [12,13]. Mosquitoes can

Table 1: Collection data for samples of *Achillea biebersteinii* from Turkey.

Code	Voucher numbers	Collection site	Oil yield (v/w, %)
A	GUE-2611	Ankara: Ankara-Istanbul main road, Kızılcahamam-Pazar, Incek province	0.6
B	GUE-2612	Ankara: Yenimahalle, Yesilevler, 998 m	0.3
C	GUE-2756	Konya: Konya-Beysehir main road, 42 km to Beysehir	0.5
D	GUE-2926	Konya: Beysehir-Akşehir road, 31 km to Akşehir, 1440m.	0.3
E	GUE-2784	Isparta: Sarkikaraagac-Yalvac Road, to 15 km Yalvac, Sultan Mountain, 1300 m	0.4

Table 2: Composition of the essential oils of *Achillea biebersteinii* from five geographical locations (A-E) in Turkey

RRI	Compound	A %	B %	C %	D %	E %
1014	Tricyclene	0.3	0.2	0.1	0.1	0.1
1032	α -Pinene	3.2	0.9	2.2	1.4	1.2
1035	α -Thujene	0.2	-	0.2	0.1	0.1
1043	Santolinatriene	-	-	0.2	-	-
1076	Camphene	5.3	3.9	1.9	2.2	1.8
1118	β -Pinene	1.9	0.2	1.2	0.5	0.4
1132	Sabinene	1.0	-	0.6	-	-
1176	α -Phellandrene	-	-	0.1	0.1	-
1188	α -Terpinene	0.4	-	0.5	0.2	0.1
1195	Dehydro-1,8-cineole	0.3	-	-	0.1	0.1
1203	Limonene	0.4	0.1	0.4	0.2	0.2
1213	1,8-Cineole	36.0	8.8	36.9	35.5	34.3
1255	γ -Terpinene	0.7	-	0.7	0.2	0.2
1280	<i>p</i> -Cymene	0.6	27.0	3.4	13.3	13.4
1290	Terpinolene	0.2	-	0.2	-	0.1
1400	Nonanal	tr	-	-	-	-
1439	γ -Campholene aldehyde	tr	-	tr	-	-
1450	<i>trans</i> -Linalool oxide (Furanoid)	-	-	-	0.5	0.4
1452	α , <i>p</i> -Dimethylstyrene	-	0.1	-	0.1	0.1
1452	1-Octen-3-ol	-	-	0.1	tr	tr
1474	Camphenilone	-	0.1	-	0.2	0.2
1474	<i>trans</i> -Sabinene hydrate	tr	-	0.4	-	-
1478	<i>cis</i> -Linalool oxide (Furanoid)	-	-	-	0.4	0.4
1499	α -Campholene aldehyde	-	0.2	0.2	-	0.2
1522	Chrysanthenone	-	-	0.2	-	-
1532	Camphor	30.3	24.5	15.6	21.7	21.7
1547	Dihydrochillene	0.1	0.2	0.4	0.1	0.3
1553	Linalool	0.4	-	0.2	2.8	1.7
1556	<i>cis</i> -Sabinene hydrate	0.1	-	0.4	-	-
1571	<i>trans</i> - <i>p</i> -Menth-2-en-1-ol	0.2	1.0	0.8	0.5	0.5
1582	<i>cis</i> -Chrysanthenyl acetate	-	-	0.7	-	-
1586	Pinocarvone	0.5	0.4	-	0.1	0.2
1588	Bornyl formate	-	0.1	0.1	tr	-
1591	Bornyl acetate	1.0	0.8	2.1	0.4	1.3
1611	Terpinen-4-ol	2.4	0.1	1.7	0.8	0.6
1617	Lavandulyl acetate	-	-	0.2	0.2	0.8
1638	<i>cis</i> - <i>p</i> -Menth-2-en-1-ol	0.2	0.7	0.6	0.4	0.4
1648	Myrtenal	0.4	0.2	0.1	0.1	0.2
1651	Sabinaketone	0.3	0.1	0.3	0.2	0.3
1670	<i>trans</i> -Pinocarveol	0.6	0.5	0.3	0.2	0.3
1682	δ -Terpineol	0.7	0.1	0.6	0.3	0.4
1683	<i>trans</i> -Verbenol	-	-	0.3	0.4	0.1
1686	Lavandulol	-	-	0.2	0.8	0.4
1689	<i>trans</i> -Piperitol (=trans- <i>p</i> -Menth-1-en-3-ol)	tr	0.4	0.3	tr	-
1706	α -Terpineol	2.7	0.1	2.5	1.2	1.3
1719	Borneol	6.7	2.8	5.1	5.3	4.5
1725	Verbenone	-	-	-	-	0.1
1726	Germacrene D	0.3	-	-	-	-
1748	Piperitone	-	-	10.9	-	-
1754	<i>trans</i> -Piperitone oxide	-	0.8	-	0.3	0.4
1758	<i>cis</i> -Piperitol	tr	0.4	0.3	0.2	0.2
1764	<i>cis</i> -Chrysanthenol	-	-	0.7	-	-
1802	Cumin aldehyde	0.3	0.3	0.1	0.2	tr

1804	Myrtenol	0.2	-	0.1	0.1	-
1845	<i>trans</i> -Carveol	0.2	-	0.2	0.1	tr
1864	<i>p</i> -Cymen-8-ol	tr	0.3	0.1	0.3	0.5
1889	Ascaridol	-	4.2	0.2	0.9	1.3
1948	<i>trans</i> -Jasmone	0.2	-	0.3	0.3	0.4
2008	Caryophyllene oxide	0.1	-	0.2	0.5	0.2
2029	Perilla alcohol	tr	-	0.1	-	-
2030	Methyl eugenol	tr	-	0.1	-	-
2073	<i>p</i> -Mentha-1,4-dien-7-ol	0.4	-	0.2	0.1	tr
2084	Octanoic acid	0.3	-	tr	0.2	0.5
2113	Cumin alcohol	0.2	0.5	0.3	0.2	0.3
2131	Hexahydrofarnesyl acetone	-	0.1	0.1	-	-
2144	Spathulenol	-	0.2	0.2	0.1	tr
2181	Isothymol (=2-Isopropyl-4-methyl phenol)	-	0.2	-	0.1	0.1
2185	γ -Eudesmol	tr	-	-	-	-
2186	Eugenol	-	0.1	0.3	tr	tr
2191	Zingiberenol	-	-	0.1	-	-
2192	Nonanoic acid	-	-	tr	-	-
2198	Thymol	-	0.5	-	0.4	0.3
2221	Isocarvacrol (=4-Isopropyl-2-methyl phenol)	-	0.6	-	0.2	0.1
2239	Carvacrol	-	2.9	-	1.3	0.9
2257	β -Eudesmol	tr	1.2	0.3	0.7	0.6
2260	15-Hexadecanolide	-	0.2	0.1	0.2	0.1
2298	Decanoic acid	-	-	-	0.1	0.3
2300	Tricosane	tr	-	0.2	tr	tr
2324	Caryophylla-2(12),6(13)-dien-5 α -ol (=Caryophylladienol II)	-	-	-	0.1	tr
2392	Caryophylla-2(12),6-dien-5 β -ol (=Caryophyllenol II)	-	-	tr	-	0.1
2500	Pentacosane	tr	-	-	tr	0.1
2670	Tetradecanoic acid (=Myristic acid)	-	-	-	-	0.1
2700	Heptacosane	tr	-	-	-	-
2931	Hexadecanoic acid	tr	1.1	0.5	0.2	0.4
Total		99.3	87.1	97.6	97.4	95.3

RRI: Relative retention indices calculated against *n*-alkanes

tr : Trace (< 0.1 %);%: Calculated from FID data

lead to explosive outbreaks in human diseases which can cause high rates of morbidity and mortality. Essential oils can be an alternative source of environmentally friendly insecticides. One aspect of our research focuses on novel plant-derived fungicides for the control of important crop pathogens and pests in agriculture. Pathogens of small fruits and ornamentals, such as *Colletotrichum*, *Botrytis*, *Phomopsis* and *Fusarium*, continue to hamper the growth and profitability of many agricultural crops [14].

This study evaluated the use of *A. biebersteinii* essential oils for fungicidal activity and for toxicity against *Aedes aegypti* L. larvae and adults. The essential oils obtained by water distillation from aerial parts of *A. biebersteinii* were collected from five different geographical locations in central Turkey (Table 1). The chemical composition was determined by GC-FID and GC-MS. A total of 84 compounds were identified, representing from 87% to 99% of the total oils (Table 2). The most relevant components were 1,8-cineole (9-37%), camphor (16-30%) and *p*-cymene (1-27%). Samples A and B collected from Ankara (east central Turkey) showed different proportions of their main constituents. Sample A, collected from the northwest part of Ankara, was characterized by larger amounts of 1,8-cineole (36.0%), camphor (30.3%), and borneol (6.7%), but a low amount of *p*-cymene (0.6%). Sample B, collected from the central area of Ankara, had a low amount

Table 3: Major components of *A. biebersteinii* essential oils reported in previous studies.

Geographic regions	Major compounds	Ref
Turkey		
Sivas	piperitone (35%), 1,8-cineole (13%), camphor (9%), chrysanthenone (8%), borneol (4%)	15
Erzurum	piperitone (31%), camphor (12%), 1,8-cineole (11%)	16
Erzurum	1,8-cineole (38%), camphor (24%)	17
Ankara	piperitone (50%), 1,8-cineole (11%), camphor (9%)	18
Iran		
	1,8-cineole (8%), camphor (7%), α -fenchene (6%), santolina triene (5%)	19
	piperitone (46%), 1,8-cineole (18%), limonene (6%), <i>p</i> -cymene (5%)	20
Bouin	α -terpineol (14%), camphor (12%), spathulenol (12%)	21
Azna	spathulenol (11%), bicyclogermacrene (3%), camphor (2%), borneol (2%), germacrene D (1%)	21
Natanz	camphor (23%), 1,8-cineole (19%), germacrene D (14%), α -terpineol (9%), bicyclogermacrene (6%)	21
Razawi	1,8-cineole (46-60%), ascaridol (3-26%), <i>p</i> -cymene (6-10%), isoascaridol (2-7%)	22
Razawi	1,8-cineole (33%), carvacrol (11%), piperitone (7%)	23
Tehran	ascaridol (37%), piperitone (17%), camphor (12%), <i>p</i> -cymene (8%), piperitone oxide (6%)	24
Jordan		
Naur	<i>cis</i> -ascaridol (36%), <i>p</i> -cymene (32%), carvenone oxide (6%), camphor (5%)	25
Azerbaijan	camphor (34-38%), borneol (7-23%), 1,8-cineole (14-22%)	26

of 1,8-cineole (8.8%), but high amounts of *p*-cymene (27.0%), camphor (24.5%) and borneol (2.8%). Sample B also differed by containing 4.2% ascaridol. Sample C from southeastern Konya and sample D from northwestern Konya in central Turkey had 1,8-cineole (36.9 and 35.5%), camphor (15.6 and 21.7 %), and *p*-cymene (3.4 and 13.3 %) as their most abundant components, respectively. Piperitone was a major constituent of sample C (10.9%), but was not detected in the other four samples (Table 2). Sample E was collected from Isparta in southwestern Turkey, and its chemical profile was similar to that of sample D; 1,8-cineole (34.3%), camphor (21.7%) and *p*-cymene (13.4%) were the main components. As far as we know, this is the first report on the essential oil composition of *A. biebersteinii* from the Konya and Isparta regions.

The chemical compositions of *A. biebersteinii* essential oils reported from different geographic regions [15-26] are summarized in Table 3. Monoterpenes, with quantitative and qualitative differences, represented the largest group in these previous reports [15-26], an exception being one sample from Iran, which was found to be rich in the sesquiterpene, spathulenol. Rahimmalek *et al.* [21] reported a high level of spathulenol and a high essential oil yield, which might be due to a soil characterized by an accumulation of CaCO₃. The differences in oil composition may be attributed to different environmental factors, plant genetic type, seasonality, physiological age, and developmental stage.

The antifungal activity of *A. biebersteinii* oils was evaluated against the plant pathogens *Colletotrichum acutatum*, *C. fragariae*, and *C. gloeosporioides*. No activity was observed using direct bioautography assay with spot applications of 80 and 160 μ g/spot. Therefore, not further antifungal studies were warranted.

In searching for new forms of mosquito control, the five *A. biebersteinii* essential oils were tested against *Ae. aegypti* in a high throughput larval bioassay and adult toxicity test. Oils were evaluated in a dose-dependent manner at 500, 250, 125, 62.5 and 31.25 ppm (Table 4). Percent mortality was determined for the evaluated compounds using the first instar larvae of *Ae. aegypti*. All samples showed 100% mortality at 500 ppm, but only two (D and E) gave 100% mortality at 125 ppm. Sample B was the only active sample resulting in 20% mortality at 62.5 ppm, whereas the other samples were inactive at the same concentration. The difference in toxicity of sample B on *A. aegypti* could be due to either qualitative and/or quantitative variations in the essential oil constituents. In order to test whether the major compounds of *A. biebersteinii* oils were responsible for the larvicidal activity, 1,8-cineole, *p*-cymene and camphor were evaluated for their larvicidal effects. 1,8-Cineole produced 63.4% $\pm$ 0.58 mortality at 500 ppm and 40% $\pm$ 0 mortality at 250 ppm, camphor 50.0% $\pm$ 0.71 mortality at 250 ppm and 20% $\pm$ 0 mortality at 125 ppm, and *p*-cymene 90% $\pm$ 0.71 mortality at 125 ppm and 0% $\pm$ 0 mortality at 62.5 ppm. These pure compounds independently showed significantly weaker larvicidal activity than the unfractionated essential oil, suggesting that minor compounds are probably the active principles responsible for the observed *Ae. aegypti* larvicidal activity. The five *A. biebersteinii* essential oils were also tested on adult mosquitoes. Three samples (A, D and E) exhibited 10% mortality at 3.1 μ g/0.5 μ L concentration against *Ae. aegypti*. No adult mortality was observed for B and C samples at this same screening rate. On the basis of these results, *A. biebersteinii* essential oils did not appear to possess insecticidal compounds active at useful concentrations against mosquitoes.

Table 4: Larvicidal activity of *A. biebersteinii* essential oils against first instar larvae of *Ae. aegypti*

Sample Codes	Mortality [%]				
	500 ppm	250 ppm	125 ppm	62.5 ppm	31.25 ppm
A	100	100	40	0	0
B	100	100	40	20	0
C	100	100	20	0	0
D	100	100	100	0	0
E	100	100	100	0	0

Essential oils have not received as much attention for use as natural sources of potential biopesticides with low mammalian and environmental toxicity. Unfortunately, our data indicated that *A. biebersteinii* does not appear to have potential for agrochemical applications as either an antifungal or insecticidal agent.

Experimental

General: 1,8-Cineole (99%, Aldrich-Sigma, St., Louis, MO), $\pm$ camphor (99%, Aldrich-Sigma, St., Louis, MO), *p*-cymene (96%, Aldrich-Sigma, St., Louis, MO), and fungicide technical grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service, Inc. West Chester, PA) were purchased from commercial sources [27,28].

Samples: *Achillea biebersteinii* was collected from different localities in central Turkey (Table 1). Voucher specimens are kept at the Herbarium of the Faculty of Pharmacy, Gazi University in Ankara, Turkey.

Isolation of the essential oils: The essential oils from air-dried plant materials were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method recommended in the *European Pharmacopoeia* [29]. The obtained oils were dried over anhydrous sodium sulfate and stored at +4°C in the dark until analyzed and tested.

GC/FID and GC/MS conditions: The chemical composition of *A. biebersteinii* oils was analyzed by capillary GC and GC/MS using an Agilent GC/MSD system. The same column and analysis conditions were used for both GC and GC/MS.

The GC/MS analysis was carried out with an Agilent 5975 GC/MSD system. A Hewlett Packard-Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) was used with helium as carrier gas (0.8 mL/min). The GC oven temperature was kept at 60°C for 10 min and ramped to 220°C at a rate of 4°C/min, then held constant at 220°C for 10 min with a final programmed ramp to 240°C at a rate of 1°C/min, and held a second time at 240°C for 20 min. Split ratio was adjusted at 40:1. The injector temperature was at 250°C. The mass spectrometer was operated with an electron energy of 70 eV. Mass spectra were acquired with the instrument set to scan from *m/z* 35 to 450 at a scan rate of 3.46 scans/s. The GC analysis was carried out using an Agilent 6890N GC system. FID detector temperature was 300°C. In order to obtain the same elution order with GC/MS, simultaneous injection was done using the same column and appropriate operational conditions.

Identification of the essential oil components was carried out either by comparison of their relative retention times with those of authentic samples or by comparing their relative retention index (RRI) to a series of *n*-alkanes. Computer matching was also used for the identification of compounds using as references Wiley and MassFinder 3.1 [30,31], an in-house “Başer Library of Essential Oil Constituents” composed of genuine compounds and components of known oils, and MS literature data [32-34]. Relative concentrations of the separated compounds based on percentage were computed from FID chromatograms.

Direct bioautography assay: Detection of naturally occurring antifungal agents was used to evaluate the antifungal activity of *A. biebersteinii* essential oils against *Colletotrichum fragariae*, *C. acutatum* and *C. gloeosporioides* using direct bioautography procedures

[35,36]. One-dimensional thin-layer chromatography (1D TLC) was subsequently used to purify and identify the antifungal agents in the extracts. The sensitivity of each fungal species to each test compound was determined by comparing the sizes of the inhibitory zones. Each plate was subsequently sprayed with a spore suspension (10^5 spores/mL) of the fungus of interest and incubated in a moisture chamber for 4 days at 26°C with a 12 h photoperiod. Fungal growth inhibition was evaluated 4–5 days after treatment by measuring zone diameters. Bioautography experiments were performed multiple times using both dose- and non-dose-response formats. Fungicide technical grade standards benomyl, cyprodinil, azoxystrobin, and captan were used as controls at 2 mM in 2 µL of EtOH.

Mosquito larvae and adult mosquito assays: Larval bioassays were performed as described in [37,38]. Briefly, five *Ae. aegypti* first instar larvae were placed in individual wells of a 24-well plate containing 950 µL deionized water and 40 µL of larvae food solution, and 10 µL of either DMSO (control) or 10 µL of serially diluted test compound. After 24 h, the number of dead larvae was recorded. Serial dilutions were continued until 0% mortality was observed for each chemical. Larval mortality was recorded after 24 h of exposure. The larval assays were repeated several times on different days with 6 concentrations providing a range of 0–100% mortality. Controls included negative (untreated), carrier (DMSO), and positive (permethrin).

For assays against mosquito adults, stock chemical solutions prepared as above were diluted in acetone to a final concentration of 6.25 µg/µL. Ten adult *A. aegypti* female mosquitoes, 3-5 days post-eclosion, were cold-anaesthetized and placed on a BioQuip chill table (Rancho Dominguez, CA) set at 4°C. The test chemical (0.5 µL) was applied to the dorsal thorax of each insect using a #1702 Gas-tight Hamilton syringe mounted on a Hamilton PB600 repeating dispenser (Reno, NV), with a final dose of 3.12 µg per insect. For any chemical producing 50% or greater mortality, a second assay was performed using 1.56 µg per insect. After treatment, mosquitoes were placed in 3.5-oz plastic cups containing 10% sucrose solution and maintained at 28°C and 80% relative humidity. Controls included negative (untreated), carrier (DMSO-acetone), and positive (permethrin).

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