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Research article

Chemical composition, insecticidal and biochemical effects of two plant oils and their major fractions against *Aedes aegypti*, the common vector of dengue fever

Abdulrhman A. Almadiy *

Department of Biology, Faculty of Arts and Sciences, Najran University, 1988 Najran, Saudi Arabia

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ABSTRACT

In an attempt to reduce the massive application of the toxic chemical pesticides, essential oils (EOs) of *Achillea biebersteinii* and *Juniperus procera* were obtained through hydrodistillation and analyzed using (GC–FID) and (GC–MS). α-terpinene and *p*-cymene were detected as the major components in the EO of *A. biebersteinii*, while eugenol and *β*-caryophyllene were the major constituents in the EO of *J. procera*. The plant EOs and major fractions act as considerable mosquitocides against *Aedes aegypti* L, the common transmitter of Dengue fever. The EOs and major fractions were tested at 6.25, 12.5, 25, 50 and 100 μl/l. Insect mortality was time and dose-dependent, and the adult stage was more sensitive than larvae. At a concentration of 50 μl/l, 24 post treatment larval and adult mortality ranged between (40.3 and 89.3%) and (51.4 and 95.6%), respectively. The LC_{50} values ranged between 12.2 and 70.1 μl/l against larvae and between 10.1 and 63.12 μl/l against adults. All of the crude EOs were more potent than their major fractions. Eugenol and *β*-caryophyllene showed strong mosquitocidal activity than *p*-cymene and α-terpinene. The corrected percentage mortality was increased over time with all of the test materials. In terms of lethal time required to kill 50% of the population (LT_{50}), a concentration of 100 μl/l of *J. procera* EO showed LT_{50} values of 2.3 and 1.7 h against larvae and adult, respectively. The EOs induced considerable inhibition of acetylcholinesterase activity, where *J. procera* crude oil (IC_{50} = 13.12mM) and eugenol (IC_{50} = 19.65mM) were the most potent. Results proved that the test plant oils and their major fractions could be developed as natural pest control agents to control *A. aegypti*.

1. Introduction

Mosquitoes are serious arthropod pests acting as transmitters of several diseases, like malaria, dengue fever, yellow fever, filariasis, encephalitis, and Zika virus, causing serious health problems to humans. Dengue is considered nowadays as one of the most serious viral diseases transmitted by mosquitoes in the globe, being endemic in over 100 countries, especially in the tropical areas, and nearly 40 % of population in the globe are at risk of infection (WHO, 2012a). *Aedes aegypti* L. (Diptera: Culicidae) is the primary arthropod acting as vector of dengue worldwide. This mosquito has adapted to the urban environment and can use human containers for egg laying and development (WHO, 2012b). Bites of the infected female *Aedes* mosquito, which is a daytime feeder is the main causative of arbovirus transmission to human (Eldridge, 2005). The infected female mosquitoes take longer time to have a blood meal than uninfected ones, thus increase the efficacy of *A aegypti* as a dengue viral transmitter (Platt et al., 1997). To date, and except for yellow fever, no vaccines or specific treatments are available, therefore, mosquito suppression mainly using insecticides is the main control strategy for such diseases. The indiscriminate application of these chemicals caused toxic hazards on the environment, non-target organisms, and human (Benelli, 2015; Pavela, 2015a, b). Of a great concern, resistance of *A. aegypti* versus conventional insecticides has been confirmed by many authors in different regions worldwide (Polson et al., 2011; Dias and Moraes, 2014). To overcome these problems, it is critical to identify novel mosquitocides with different modes of action to increase the available choices of pesticides for use in public health control (Benelli, 2015). The ideal insecticide should be effective, specific, sustainable, ecologically sound, low toxic to mammalian and cost effective (Nenaah, 2014a, b). Recently increased attention had been given to botanicals as natural pesticides against arthropod pests. Among plant metabolites, EOs from different plant species have been extensively reported as natural

* Corresponding author.
E-mail address: aalmadiyaseer@yahoo.com.

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Table 1. Chemical profile of the plant oils under investigation.

[a,b]Components	Concentration (%)			
	[c]RI exp.	[d]RI lit.	*A. biebersteinii*	*J. pocera*
n-hexanol	862	863	0.1	0.2
Heptanal	902	901	Tr	0.1
Tricyclene	918	920	0.2	–
α-Thujene	922	924	0.1	tr
α-Pinene	928	932	1.3	5.7
Camphene	942	946	1.3	tr
Sabinene	967	969	0.4	–
1-Octen-3-ol	978	973	–	0.2
β-Myrcene	988	988	–	0.3
α-Terpinene	1016	1014	23.7	0.3
p-Cymene	1021	1020	21.4	tr
β-Phellandrene	1026	1025	–	0.2
1,8-Cineole	1030	1027	4.1	–
Z-*β*-Ocimene	1036	1038	tr	0.1
E-*β*-Ocimene	1046	1044	–	tr
γ-Terpinene	1054	1055	0.7	tr
Terpinolene	1084	1086	–	0.2
Linalool	1100	1095	0.5	tr
cis-p-Ment-2-en-1-ol	1120	1118	1.1	–
trans- p-Menth-2-en-1-ol	1121	1119	1.4	–
Camphor	1140	1141	7.3	0.2
Pinocarvone	1157	1160	1.8	tr
Borneol	1163	1165	0.6	–
Terpinen-4-ol	1175	1174	3.1	2.1
α-Terpineol	1190	1188	0.9	–
Myrtenol	1192	1194	0.5	tr
Carvacrol methyl ether	1239	1241	0.1	tr
cis-Ascaridole	1241	1240	2.2	–
Piperitone	1252	1249	2.9	tr
Carvenone oxide	1260	1261	1.7	–
Bornyl acetate	1285	1187	1.4	tr
Thymol	1288	1289	0.7	tr
Carvacrol	1296	1297	2.1	–
Eugenol	1355	1356	0.9	78.4
α-Copaene	1370	1374	0.4	–
β-Elemene	1391	1389	–	0.3
β-Caryophyllene	1412	1417	0.5	9.3
α-Humulene	1451	1452	0.6	0.6
Germacrene D	1480	1484	0.7	–
β-Bisabolene	1508	1505	–	0.1
d-Cadinene	1517	1522	0.1	tr
Eugenol acetate	1546	1545	-	3.1
Spathulenol	1574	1576	0.5	tr
Caryophyllene oxide	1580	1582	0.7	0.1
Total	-	-	96.8	98.1
%Yield (ml/100 g dry wt.)	-	-	0.77	0.92

tr = trace (<0.05%).

[a] Compounds are listed in the order of their elution from a HP-5MS column.

[b] Identification methods: a, based on comparison of RT, RI and MS with those of authentic compounds; b, based on comparison of mass spectrum with those reported in Wiley, Adams and NIST 08 MS libraries.

[c] Linear retention index on HP-5MS column, experimentally determined using homologous (C_5–C_{30}) *n*-alkane series (Adams, 2007).

[d] Linear retention index based on Adams (2007) or NIST 08 (2008) and literature.

control agents against different arthropod pests including mosquitoes. In this context, EOs affect mosquitos as larvicides, adulticides, pupicides, ovicides, repellents and growth inhibitors (Amer and Mehlhorn, 2006; Pavela, 2015a, b; Benelli, 2015; Sarma et al., 2019; Benelli et al., 2020). Plant EOs are environmentally acceptable, since they are biodegradable, have low mammalian toxicity, show varying action mechanisms with different sites of action, hence development of insecticide resistance becomes limited (Pavela, 2015a). The genus *Achillea* (Asteraceae) is one of the most widely distributed genera of the Asteraceae family, widespread over the Northern hemisphere mostly in Europe, Asia, North

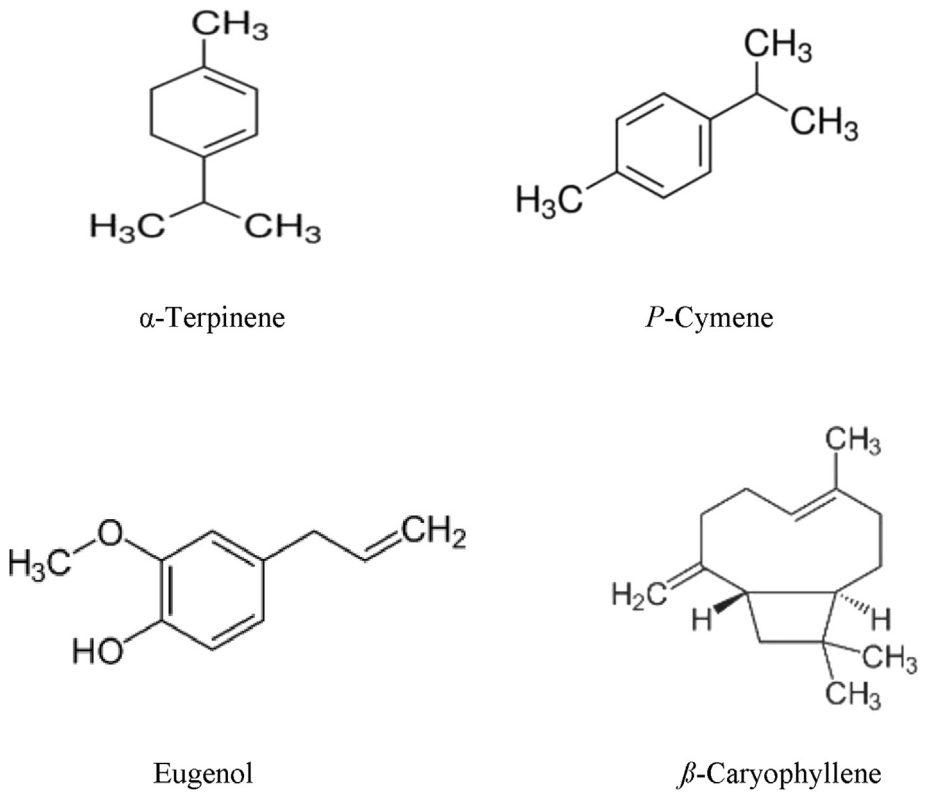

Figure 1. Chemical structure of the major fractions of *A. biebersteinii* and *J. procera* EOs.

America and the Middle East (Si et al., 2006). Traditionally, the aerial parts of different *Achillea* species are widely used in folk medicine as natural remedies for anti-inflammatory, antispasmodic, stomachic and antiseptic purposes (Sartoratto et al., 2004). There are many reported biological activities of *Achillea* plants, especially *A. biebersteinii*, which including antimicrobial, antioxidant and insecticidal activities (Calmasur et al., 2006, Nenaah, 2014a, b). In the literature, different parts, especially leaves and fruits of Juniper, *Juniperus procera* (Cupressaceae), is widely investigated as a source of natural ingredients with potential antimicrobial, anticancer, antioxidant and insecticidal activities (Burits et al., 2001; Tumen et al., 2013; Abd El-Ghany and Hakamy, 2014). The current study demonstrates an investigation of the chemical composition, mosquitocidal and biochemical properties of EOs isolated from two local plant species, namely *A. biebersteinii* and *J. procera* and some of their major fractions against *A. aegypti*, the common mosquito transmitter of Dengue fever.

2. Materials and methods

2.1. Chemicals

Monoterpene hydrocarbons, oxygenated monoterpenes, and sesquitepenes, all of analytical grad were purchased from Sigma-Aldrich Chemical Ltd. (St Louis, MO, USA) and used for retention index correlations. Dimethyl sulfoxide (DMSO) analytical grade was bought from Carlo Erba (Milan, Italy).

2.2. Insect culture

A culture of *A. aegypti* was established depending on an original culture reared for several generations (8 generations) away from any insecticidal contamination at the Department of Biology, Faculty of Science King Abdulaziz University, Saudi Arabia. Insects were reared in our laboratory for adaptation until obtaining the first generation (F_1) for use in bioassays. Larvae were reared in plastic trays containing tap water and artificial foods (brewer's yeast, dog biscuits, and ponds algae (ratio 3:1:1). Adults were maintained in plastic cages provided with 10 % sucrose solution. Conditions of the experiments were 25 ± 2 °C and 75–85% RH. under 14:10 light and dark photoperiod. The 3rd instar larvae and adults were included in bioassays.

2.3. Extraction of EOs

The aerial parts of *A. biebersteinii* and fruits of *J. procera* were collected from Aseer Province, Saudi Arabia. Powdered samples from the plants under investigation, 500 g each were hydrodistiled using a modified Clevenger-type apparatus for 6 h to produce EOs. Anhydrous Na_2So_4 was added to remove moisture after extraction. Based on the dry weight (w/w) of the extracted aerial parts of each plant, percentage yield of each oil was determined and the oils were stored at 4 °C for further experiments.

2.4. Gas chromatography–mass spectrometry

Chemical profile of the plant EOs was made by gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS) using an Agilent 6890N gas chromatograph equipped with a mass selective detector (Agilent 5973N). The (GC–FID) and (GC–MS) were equipped with an HP-5MS (30 m × 0.25 mm × 0.25 m) capillary column. Initial temperature of (GC-MS) oven was held at 50 °C for 2 min, rising to 150 °C at 2 °C/min and increased to 250 °C at 10 °C/min, then kept for 5 min. One microliter of 1% solution of each oil (diluted in *n*-hexane) was injected at an injection temperature of 250 °C. Helium at a flow rate of 1.0 mL/min was used as a carrier gas. Spectra of the oil fractions of EOs were measured from 50 to 550 m/z. The retention index of each of the isolated fractions was calculated relative to its authentic standard using a homologous *n*-alkanes series (C_5–C_{30}) as

Table 2. Larvicidal activity of the plant oils and major fractions against *A. aegypti* 24 h post treatment.

Plant oil	Concentration (µl/l)	% mortality (±SD)	LC_{50} *(95 % fl)	LC_{95} *(95 % fl)	Slope (±S. E.)	Chi^2 ($df = 5$)
J. procera	6.25	30.4 ± 1.3^{hi}	12.2 (9.4–16.8)	33.3 (29.7–41.2)	2.4 ± 0.18	1.88
	12.5	54.6 ± 2.0^{f}				
	25	70.4 ± 0.8^{d}				
	50	89.3 ± 1.2^{bc}				
	100	100.0 ± 0.0^{a}				
Eugenol	6.25	23.4 ± 0.8^{i}	23.6 (19.5–28.4)	58.4 (52.8–66.7)	3.2 ± 0.28	3.24
	12.5	34.6 ± 1.5^{h}				
	25	55.2 ± 1.2^{f}				
	50	70.8 ± 0.7^{d}				
	100	96.3 ± 1.4^{b}				
β-Caryophyllene	6.25	13.4 ± 0.9^{jk}	52.3 (46.1–61.1)	100.2 (91.3–118.7)	2.9 ± 0.30	3.07
	12.5	20.3 ± 1.2^{ij}				
	25	32.6 ± 1.6^{hi}				
	50	47.1 ± 0.9^{g}				
	100	74.7 ± 1.8^{cd}				
A. biebersteinii	6.25	14.5 ± 1.2^{kl}	38.3 (33.1–47.5)	84.6 (76.4–96.6)	3.6 ± 0.30	2.88
	12.5	22.3 ± 1.4^{ij}				
	25	40.8 ± 0.8^{h}				
	50	61.5 ± 2.0^{e}				
	100	84.0 ± 2.2^{c}				
α-Terpinene	6.25	8.7 ± 1.4^{l}	74.6 (65.1–87.4)	161.2 (152.5–184.1)	3.4 ± 0.44	3.12
	12.5	12.5 ± 1.5^{k}				
	25	20.9 ± 1.3^{ij}				
	50	37.4 ± 1.9^{h}				
	100	61.8 ± 5.3^{e}				
p-Cymene	6.25	10.2 ± 1.6^{l}	58.1 (53.3–72.1)	153.5 (142.7–177.0)	3.4 ± 0.44	3.12
	12.5	16.6 ± 1.8^{j}				
	25	28.4 ± 3.3^{hi}				
	50	46.3 ± 2.6^{g}				
	100	68.2 ± 7.7^{d}				
Control	-	0.0 ± 0.0	-	-	-	-
F value	-	398.3	-	-	-	-

Each datum represents the mean of six replicates, each set up with 20 individuals (n = 120).
Means within the same column followed by the same letter(s) are not significantly different ($P \leq 0.05$) (Tukey's b HSD test).
* fl = fiducial limits.

described earlier (Adams, 2007) and by comparing their mass spectra with those found in NIST 8.1 library and Wiley Mass Finder 3.1 commercial libraries. Quantification of EO constituents was done by normalization of GC–FID peak area without correction factors.

2.5. Purification and characterization of major constituents of EOs

Each of the test EOs (20 ml) was chromatographed using a silica gel column (kieslgel 60 Merck; 230–400 mesh) using a mobile phase system composed of *n*-hexane / acetone, starting with fractions of 100 ml *n*-hexane (10×), 2% acetone / *n*-hexane (20×), 5% acetone/*n*-hexane (10×), 10% acetone/*n*-hexane (5×), and finally acetone solvent system (5×). Fractions that showed insecticidal activity, were gathered and purified on a Sephadex LH-20 column using 70% methanol. For further purification, the developed fractions were collected and redeveloped on pre-coated TLC plates (silica gel G 60, F_{254}, Merck, Suwanee, GA), using a selective eluent system for each fraction (Pothier et al., 2001; Matysik et al., 2016), which afforded two main fractions for each EO. Same fractions were pooled and successively washed using $CHCl_3$ and MeOH for crystallization. Crystallized samples were compared by their retention indices in relation to their reference standards. Structures of the isolated fractions were established by spectroscopic equipments including ^{1}H, ^{13}C-NMR spectra on Bruker AMX500 [500 MHz (1H)] instruments using $CDCl_3$ or DMSO-d6 as solvent with TMS as internal standard.

2.6. Mosquitocidal activity

Insecticidal activity of the plant EOs and their major fractions was investigated against *A. aegypti* according to the WHO protocol (WHO, 2005). Test materials were dissolved in DMSO and tested at 6.25, 12.50, 25, 50, and 100 µl/l depending on a preliminary susceptibility test using the test EOs and their major fractions. The late 3rd instar larvae and adult female (5–6 days old) were included in this bioassay. For larval stage bioassay, one ml of the test concentrations of each material was added to a 500-mL glass beaker containing 249 mL of dechlorinated water. To this mixture, 20 late third instar larvae of *A. aegypti* were introduced. Application of the EOs and major fractions on the adult stage was done in a wind tunnel (Annex 3 in WHO, 2009 for equipment specifications and procedural details), where adults were exposed to the same concentration used for larvae. In each case, 20 sugar-fed, blood-starved adult female (5–6 days old) were placed in a cage covered with a mesh with openings sized 1.2 × 1.6 mm. The cage was placed in the tunnel, and 1 ml of each of the test concentrations of the test

Table 3. Adulticidal activity of the plant oils and major fractions against *A. aegypti* 24 h post treatment.

Plant oil	Concentration (µl/l)	% mortality (±SD)	LC_{50} *(95 % fl)	LC_{95} * (95 % fl)	Slope (±S. E.)	Chi^2 ($df = 5$)
J. procera	6.25	37.4 ± 2.1^{gh}	10.1 (8.6–12.5)	27.3 (22.7–34.2)	2.1 ± 0.24	0.48
	12.5	58.8 ± 3.4^{e}				
	25	77.2 ± 2.7^{c}				
	50	95.6 ± 1.8^{ab}				
	100	100.0 ± 0.0^{a}				
Eugenol	6.25	30.4 ± 0.8^{hi}	18.3 (13.5–24.2)	46.1 (40.8–60.7)	4.1 ± 0.88	2.26
	12.5	39.6 ± 1.5^{gh}				
	25	64.2 ± 1.2^{d}				
	50	80.4 ± 0.7^{c}				
	100	100.0 ± 0.0^{a}				
β-Caryophyllene	6.25	17.0 ± 0.8^{jk}	46.4 (40.6–57.8)	94.7 (84.0–107.8)	3.1 ± 0.40	2.33
	12.5	28.4 ± 1.6i				
	25	32.8 ± 1.4^{h}				
	50	53.3 ± 2.1^{ef}				
	100	80.5 ± 2.4^{c}				
A. biebersteinii	6.25	20.5 ± 1.2^{j}	30.2 (23.1–39.5)	72.6 (66.4–86.3)	3.6 ± 0.44	3.44
	12.5	30.3 ± 1.4^{h}i				
	25	46.8 ± 0.8^{f}				
	50	68.5 ± 2.0^{d}				
	100	92.0 ± 0.0^{b}				
α-Terpinene	6.25	12.7 ± 1.4^{k}	66.8 (57.2–781.5)	135.2 (123.4–151.1)	3.2 ± 0.38	3.44
	12.5	15.5 ± 1.5^{jk}				
	25	24.9 ± 1.3^{ij}				
	50	41.4 ± 1.9^{g}				
	100	63.8 ± 5.3^{d}				
p-Cymene	6.25	16.6 ± 2.63^{jk}	54.1 (47.3–68.6)	123.5 (112.7–138.0)	2.7 ± 0.33	3.86
	12.5	20.6 ± 1.81^{j}				
	25	31.4 ± 3.23^{h}				
	50	49.4 ± 2.67^{f}				
	100	75.2 ± 7.07^{c}				
Control	-	0.0 ± 0.0	-	-	-	-
F value		364.8				

Each datum represents the mean of six replicates, each set up with 20 individuals (n = 120).
Means within the same column followed by the same letter(s) are not significantly different (P ≤ 0.05) (Tukey's b HSD test).
* fl = fiducial limits.

Table 4. Time-dependent mortality of *Aedes aegypti* treated with the plant oils and their major fractions at 100 µl/l.

Plant oil	% mortality (Mean h ± S.E.)								
	Stage	1	2	4	8	12	16	20	24
J. procera	Larva	24.56 ± 2.14^{b}	50.76 ± 4.12^{a}	63.11 ± 3.00^{b}	76.09 ± 2.20^{ab}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
	Adult	33.44 ± 2.22^{a}	56.07 ± 3.05^{a}	68.00 ± 3.03^{a}	81.62 ± 2.40^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
Eugenol	Larva	20.22 ± 2.45^{b}	37.55 ± 3.11^{c}	50.22 ± 3.06^{c}	61.08 ± 2.88^{c}	84.62 ± 2.22^{b}	91.64 ± 2.30^{b}	93.18 ± 0.00^{b}	95.22 ± 2.40^{b}
	Adult	23.43 ± 2.10^{b}	42.82 ± 2.18^{b}	58.33 ± 3.26^{b}	69.66 ± 2.00^{b}	78.20 ± 3.14^{bc}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
β-Caryophyllene	Larva	6.00 ± 0.00^{c}	12.22 ± 1.80^{e}	21.12 ± 1.72^{f}	40.09 ± 2.83^{f}	58.23 ± 2.21^{e}	65.28 ± 2.25^{de}	68.18 ± 2.10^{e}	75.48 ± 2.33^{de}
	Adult	8.00 ± 0.00^{c}	15.08 ± 1.14^{de}	29.00 ± 1.02^{e}	47.26 ± 2.20^{e}	65.22 ± 2.08^{d}	72.55 ± 2.06^{d}	77.62 ± 4.22^{d}	79.22 ± 4.22^{d}
A. biebersteinii	Larva	5.00 ± 0.00^{cd}	16.00 ± 1.55^{de}	32.08 ± 3.03^{e}	53.09 ± 2.65^{d}	70.23 ± 1.41^{c}	82.23 ± 2.26^{c}	86.84 ± 2.12^{c}	88.22 ± 3.12^{c}
	Adult	8.11 ± 1.02^{c}	22.00 ± 2.15^{d}	39.22 ± 3.03^{d}	58.09 ± 2.07^{c}	71.44 ± 2.32^{c}	88.34 ± 2.25^{b}	91.56 ± 3.00^{b}	96.56 ± 3.00^{ab}
α-Terpinene	Larva	2.00 ± 0.00^{e}	8.22 ± 1.02^{f}	10.12 ± 1.03^{g}	26.09 ± 2.44^{h}	44.23 ± 2.27^{g}	52.28 ± 2.11^{f}	56.18 ± 2.1^{f}	60.48 ± 2.11^{g}
	Adult	5.00 ± 0.00^{d}	10.08 ± 1.14^{e}	19.00 ± 1.02^{f}	33.26 ± 2.76^{g}	50.22 ± 2.11^{f}	57.55 ± 2.91^{ef}	61.62 ± 4.55^{ef}	66.22 ± 4.22^{f}
p-Cymene	Larva	4.00 ± 0.00^{d}	7.22 ± 1.35^{f}	14.12 ± 1.03^{fg}	35.09 ± 2.22^{fg}	53.23 ± 2.23^{f}	61.28 ± 2.25^{e}	66.18 ± 2.1^{e}	71.48 ± 2.08^{e}
	Adult	6.00 ± 0.00^{c}	11.02 ± 1.14^{e}	19.00 ± 1.02^{f}	40.26 ± 2.09^{f}	58.22 ± 2.44^{e}	66.55 ± 2.49^{de}	72.62 ± 4.22^{d}	77.22 ± 4.22^{d}
Control	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
*F-value	-	160.5	145.2	321.8	117.4	105.2	94.0	110.3	102.3

Each datum represents the mean of six replicates, each set up with 20 insects (n = 120).
In the same column, means followed by the same letters are not significantly different (p ≤ 0.05) as determined by Tukey's HSD test.
* All F- values are significant at (P ≤ 0.001).

Table 5. *LT_{50} values against *Aedes aegypti* treated with the plant oils at 100 µl/l.

Plant oil	Stage	*LT_{50} (Mean h ± SE)	95% (fiducial limits)	Slope (±S.E.)	Chi^2 ($df = 5$)
J. procera	Larva	2.3	1.7–2.9	2.4 ± 0.43	2.32
	Adult	1.7	1.1–2.4	3.7 ± 0.32	2.43
Eugenol	Larva	4.1	3.3–5.3	2.8 ± 0.42	2.24
	Adult	3.4	2.8–4.7	2.6 ± 0.62	3.55
β-Caryophyllene	Larva	10.7	8.9–13.1	2.1 ± 0.41	2.18
	Adult	8.8	7.4–11.2	2.3 ± 0.37	2.65
A. biebersteinii	Larva	7.7	6.3–9.8	2.8 ± 0.32	3.21
	Adult	7.3	6.2–9.1	3.5 ± 0.48	2.80
α-Terpinene	Larva	15.8	12.3–18.2	3.2 ± 0.54	2.12
	Adult	15.3	11.9–16.9	2.7 ± 0.32	2.82
p-Cymene	Larva	11.5	9.3–14.1	3.1 ± 0.43	3.17
	Adult	10.1	8.2–12.6	2.9 ± 0.48	2.43

* LT_{50}: Time elapsed that required to achieve 50% mortality by the applied concentration.

oils or major fractions dissolved in DMSO was applied using a syringe. Treated females were then transferred to plastic cages (20 × 20 × 20 cm). For each concentration, a control group (DMSO and distilled water) was included. Six replicates were considered for each concentration tested against larvae as well as adults (a total number of 120 individuals). Treatments and control sets were kept at the same laboratory conditions described for rearing and mortality was recorded after 24 h of treatment, during which no food was offered. Dead insects were identified by probing with a brush and identified by a failure to move.

2.7. Time dependent mortality

In this bioassay, the plant EOs and their major fractions were tested against the 3rd instar larvae and adults of *A. aegypti* at the high concentration tested at the current study (100 µl/l) in order to determine their bioactivity rate on the insect mortality. The methodology of this experiment was the same as described in the previous section with the only difference being that insect mortality was recorded at regular intervals of 1, 2, 4, 8, 12, 16, 20, and 24 h post-exposure. Larvae or adults that showed no signs of movement after mechanical stimulation were considered dead. Experiments were performed in six replicates with a simultaneous control. The time elapsed, which required to achieve 50% mortality of *A. aegypti* by the tested concentration (LT_{50}) was estimated.

2.8. In vitro inhibition of acetylcholinesterase (AChE) activity

In this experiment, we used the larval protein to investigate the AChE inhibitory effect of the test botanicals. Five grams of *A. aegypti* larvae were homogenized in 15 ml of ice-cold phosphate buffer (50 mM), pH 7.4 using a glass tissue grinder (Wheaton Industries Inc., Millville, NJ, USA). The resulting homogenates were filtered through two layers of cheesecloth. The filtrates were centrifuged (5000 rpm for 30 min at 4 °C). AChE inhibition was determined in the developed supernatants, which used as enzyme source using acetylthiocholine iodide (ATChI) as a substrate (Ellman et al., 1961). Twenty microliter from the enzyme together with one hundred microliter dithiodinitrobenzoic (DTNB) were added to 0.1 M phosphate buffer (pH 8.0; 2.8 ml). Twenty microliters of each botanical solution prepared in acetone and Triton-X 100 (at concentration of 0.01%) was added to this mixture. The EOs and their fractions were tested at concentrations of 2.5, 5, 10, 20, 40, 60, 80, and 100 mM. Acetone (20 µl) was added in control treatments. The reaction was allowed to start by adding ATChI (30 µl of 15 mM) followed by incubation at 37 °C for 10 min. The change in absorption at 412 nm was monitored on Sequoia-Turner Model 340 spectrophotometer. Triplicates were considered for each experiment. Activity of AChE (ΔOD/mg protein/min) was calculated for each treatment and control. The Inhibitory effect of AChE was determined as percentage according to Lowry et al. (1951) as follows:

AChE inhibition % = [1 − SAT/SAC] × 100, where SAT is the specific enzyme activity in treatment and SAC is specific enzyme activity in control. The concentration of each botanical that caused 50% inhibition of substrate hydrolysis (IC_{50}) were determined. All applicable international and national ethical guidelines for the care and use of animals were followed.

2.9. Data analysis

Abbott's formula (Abbott, 1925) was employed to adjust the recorded mortality data for mortality in control when it exceeded 5%, and expressed as percentages. Probit analysis was done to

Table 6. Inhibition of acetylcholinesterase from *Aedes aegypti* larvae by the test plant oils.

Plant oil	IC_{50}[a] (mM)	95% confidence limits		Slope[b] ± SE	(χ^2) ($df = 4$)	*P* value
		Lower	Upper			
J. procera	13.12	11.22	14.07	1.88 ± 0.24	1.86	0.814
Eugenol	19.65	16.06	24.44	2.02 ± 0.18	1.02	0.733
ß-Caryophyllene	33.22	28.22	39.43	2.88 ± 0.74	1.66	0.644
A. biebersteinii	27.13	22.15	35.02	2.66 ± 0.26	1.42	0.831
α-Terpinene	53.18	47.88	62.55	1.78 ± 0.21	1.42	0.565
p-Cymene	44.72	38.04	53.19	1.49 ± 0.32	2.03	0.468
Methomyl	2.19×10^{-3}	1.62×10^{-3}	3.36×10^{-3}	1.39 ± 0.11	3.18	0.308

[a] The concentration causing 50% enzyme inhibition.
[b] Slope of the concentration-inhibition regression line.

estimate LC_{50} and LC_{90} (lethal concentrations) and their 95% fiducial limits by fitting a probit regression model to the observed relationship between percentage mortality and logarithmic concentrations (Finney, 1971). Means (±S.E.) mortality data were recorded and significant difference between means in treatments and control were compared using ANOVA at 5% probability level, Individual pairwise comparisons were made using Tukey's HSD. The time elapsed that required to achieve 50% mortality of *A. aegypti* by the test concentration (LT_{50}) as well as the concentration of each of the test materials causing 50% inhibition in AChE activity (IC_{50}) was estimated using probit analysis (Finney, 1971). Data analysis was achieved using the SPSS (Statistical Package Social Science) software version 23.0.

3. Results

3.1. Chemical composition of EOs

Chemical profile and the amount yielded from each plant oil are found in Table 1. A total number of 31 compounds constituting 98.7 % were identified in *A. biebersteinii* EO. The major constituents detected were *α*-Terpinene (23.7%), *P*-Cymene (21.4%), and Camphor (7.3%). While, data in Table 1 revealed that eugenol (78.4%), *β*-caryophyllene (9.3%), and terpinen-4-ol (4.1%) were the major oil fractions of *J. procera*.

3.2. Identification of the major constituents of EOs

Based on the chromatographic data, two main fractions with insecticidal activity were detected in each of the test EOs. In case of *A. biebersteinii* EO, the first fraction (fractions 7–13, 2.1 g) was purified using a mobile phase composed of 2% *n*-hexane/acetone/chloroform (1:0.1:0.1) to give 1.1 g of 1-isopropyl-4-methyl-1,3-cyclohexadiene, *p*-mentha-1,3-diene (α-terpinene). The second fraction (fractions 19–23, 1.86 g) was eluted using a solution of 10% acetone⁄*n*-hexane to produce 0.83 g of 1-methyl-4-(1-methylethyl) benzene (*p*-Cymene). For *J. procera*, the first fraction (fractions 4–9, 2.45 g) was purified using a solution of 2% acetone⁄*n*-hexane as an eluent to give 1.4 g of 2-methoxy-4-prop-2-enylphenol (eugenol). The second fraction (fractions 12–15, 1.28 g) was purified using petroleum ether-ethyl acetate (10:1) to give 0.32 g of (1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene (*β*-caryophyllene). Spectroscopic equipments including ^{1}H, ^{13}C-NMR spectra on Bruker AMX500 [500 MHz (1H)] instruments using $CDCl_3$ or DMSO-d6 as solvent with TMS as internal standard, and ^{1}H–^{1}H COSY, and HR-MS were used to elucidate the structure of the major oil fractions (Figure 1). Spectroscopic data of the oil fractions were compared with their references and literature.

3.3. Mosquitocidal activity

Mortality data of the third instar larvae and adults of *A. aegypti* are shown in Tables 2 and 3. It is clear that significant ($P < 0.001$) mortality was evident in all the tested concentrations after 24 h of treatment. The larval and adult mortality was time and dose-dependent, where the highest mosquitocidal activity was observed in case of *J. procera* EO. After 24 h of treatment using a concentration of 6.25 μl/l, the larval mortality ranged between 10.2 and 30.4%, whereas increased to 68.2–100 % at 100 μl/l. At the same assaying conditions, adult mosquito was more susceptible to EOs, where mortality ranged between 16.6 and 37.4 % at lower concentration, and between 75.2–100% at the higher concentration. After 24 h of treatment, a dose of 100 μl/l of the oils of *J. procera* and *A. biebersteinii* completely controlled adults of *A. aegypti*, whereas caused 100 and 93.3% mortality against larvae, respectively. The LC_{50} values were 12.2, 23.6, 38.3, and 70.1 μl/l for *J. procera* oil, *A. biebersteinii* oil, eugenol, and α-Terpinene against larvae, respectively. In case of adults, the LC_{50} values ranged between 10.1 and 63.12 μl/l.

3.4. Time–response mosquitocidal bioassay (LT_{50})

The corrected percentage mortality of *A. aegypti* was increased significantly over time with all of the tested plant oils and major fractions (Tables 4 and 5). The lethal time (LT_{50}) decreased remarkably in case of *J. procera* crude oil and eugenol more than the other treatments (Table 6). In terms of lethal time to kill 50 % of the population of *A. aegypti*, 100 μl/l of *J. procera* EO showed LT_{50} values of 2.3 and 1.7 h against larvae and adult, respectively. Whereas, eugenol had 4.1 and 3.4 h against larvae and adult, respectively. In case of the oil of *A. biebersteinii*, these values were 7.7 and 7.3 h against larvae and adults, respectively. Results revealed a significant chi-square value ($P < 0.0001$ level), higher slopes, the lower confidence limits at 95 % of LT_{50} and positive correlation between mortality and the time of exposure having *R* values close to 1 in each case were observed.

3.5. Inhibitory effect of (AChE) activity

In vitro inhibition of AChE by the test oils and major fractions in terms of IC_{50} are presented in Table 6. Results showed that, all tested plant oils had considerable inhibitory effects on AChE activity isolated from the larvae of *A. aegypti*. Data showed that *J. procera* EO ($IC_{50} = 13.12$ mM) caused the highest inhibitory effect, followed by eugenol ($IC_{50} = 19.65$ mM). The IC_{50} value of *A. biebersteinii* was 27.13 mM. The bicyclic sesquiterpene, *β*-caryophyllene had a pronounced activity, while the monoterpene hydrocarbons, α-terpinene and *p*-cymene showed weak to moderate activities. All of the test plant oils and major fractions showed AChE inhibitory activity less than that of the tested standard insecticide, methomyl ($IC_{50} = 2.19 \times 10^{-3}$ mM).

4. Discussion

Chemical profile of the EOs studied herein was similar to previous reports concerning the test species belonging to the Saudi flora or similar biota. For example, *α*-terpinene (29.2%) and *P*-Cymene (22.9%) were abundant in *A. biebersteinii* EO growing wild in the southern region of Saudi Arabia (Al-Said et al., 2016). Whereas, ascaridol, camphor and *p*-Cymene are reported as the major fractions in the Egyptian and Jordanian *A. biebersteinii* oil (Bader et al., 2003; Nenaah, 2014a, b). In different studies, eugenol was reported as the main component of *J. procera* EO (Ramadan et al., 2015). Differences were observed both in the composition and the abundant components in the test EO with similar species belonging to other flora (Adams, 1990; Rahimmalek et al., 2009; Nenaah, 2014b), which have been attributed to several environmental differences (climatic, seasonal and geographical), chemotype and genetic variations, nutritional status of the test plant, time of harvesting and reasons related to the extraction procedures (Figueiredo et al., 2008). All of these factors could affect the chemical composition and the abundant components of the plant oil under investigation.

As the vectors of serious pathogens, mosquitoes represent a great threat to humankind. These arthropod pests are combated mainly by the repeated use of chemical insecticides. Because of the well confirmed health and environmental impacts of chemical pesticides, the high cost of control strategies and the development of insecticide resistance reported with the major vectors of diseases, there is a renewed interest to develop low-cost, environmentally friendlier, and efficient alternatives that have the potential to replace or even minimize the excessive application of conventional insecticides. Herein, EOs oils of *J. procera* and *A. biebersteinii* and their major fractions exhibited significant mortality against larvae and adults of *A. aegypti*. The activity of the EOs was time and dose-dependent. In terms of LT_{50}, EO of *J procera* showed a fast insecticidal potential with LT_{50} of 2.3 and 1.7 h against larvae and adults, respectively. In case of the monoterpenoid, eugenol, LT_{50} values were 4.1 and 3.4 h, respectively. The two plant oils contain mainly monoterpenes as abundant components. Results reported herein agreed with previous

reports, where EOs rich in phenylpropanoids and monoterpenes were found to be the most active chemical groups that demonstrated pesticidal activity against pest insects including mosquito vectors (Arriaga et al., 2007; Feitosa et al., 2009; Pavela, 2015a; b; Sarma et al., 2019). As recently discussed by Pavela (2015b), plant EOs with mosquitocidal LC_{50} values lower than 50 μg/ml can be considered as extremely promising sources of botanical pesticides. Therefore, EOs of *J. procera* and *A. biebersteinii* and many of their major components are considered as promising mosquito control tools against *A. aegypti*.

In the literature, EOs from different plant species exhibited remarkable insecticidal activity against a wide array of mosquito vectors, including *A. aegypti* (Dias and Moraes, 2014). *Thymus serpyllum*, *Juniperus virginiana* and *Amyris balsamifera* were among the most active plant oils tested (LC_{50} = 1 mg/L) (Amer and Mehlhorn, 2006). In related studies, LC_{50} values of *Stemodia maritima* stem EO is 22.9 mg/L against larvae of *A. aegypti*, whereas that from the leaves was 55.4 mg/L (Arriaga et al., 2007). Feitosa et al. (2009) stated that *Rollinia leptopetala* leaf EO exhibited LC_{50} of 34.7 mg/L against the same mosquito. The EO of *Chenopodium ambrosioides* had LC_{50} of 9.1 and 17.5 ppm against *Aedes aegypti* and *A. arabiensis*, respectively (Massebo et al., 2009). EOs are mixtures of major and minor constituents and their insecticidal activity is correlated mainly with their major constituents such as α-terpinene, *P*-cymene, camphor, carvacrol, thymol, and eugenol (Pavela, 2015a, b; Sarma et al., 2019). Herein, eugenol, the major monoterpene in the EO of *J. procera* showed a considerable mosquitocidal activity against *A. aegypti*. This monoterpene reported as effective insecticide against a wide array of mosquito vectors including *A. aegypti* (Barbosa et al., 2012). As described in our study, β-caryophyllene, the bicyclic sesquiterpene of *J. procera* EO was a promising mosquitocide against *A. aegypti*. These results are in a good accordance with the findings of many authors, where β-caryophyllene is proved as insecticide and repellant against pest insects (de Elguea-Culebras et al., 2017; Francomano et al., 2019).

Many reports proved that the activity of plant EOs could be correlated to the presence of several components with demonstrated insecticidal activities, such as α-terpinene, α-pinene, p-cymene, β-caryophyllene, thymol and carvacrol (Shaalan et al., 2005; Dai et al., 2020), although synergism with other minor constituents has to be taken into consideration, as each oil fraction can play a role in penetration, affinity and distribution within living cells. Accordingly, for biological efficacy, it is more appropriate to investigate the entire oil and some of its major components, as synergistic interaction between the essential oil fractions becomes a common phenomenon (Nenaah, 2014a, b). The use of such oil mixtures as pest control strategy is also appropriate to overcome the problem of pest resistance, where developing of resistance within the insect's body against such mixtures becomes limited and need enough time and a large enough population for selection to occur (Nenaah, 2014c).

According to our results, the test oils and their major fractions caused a pronounced inhibition of acetylcholinesterase activity from larvae. Most of the test EOs induced death symptoms, which attributed mainly to a neurotoxic mode of action, hence trials should be carried out to explain the side effects of such oils and their components on mammals and/or beneficial non-target organisms. It is well known that the insecticidal activity of plant EOs and/or their major components, especially monoterpenes are mainly correlated with their ability to inhibit of acetylcholinesterase activity, blocking of octopamine receptors of insects or GABA-gated chloride channels (Pavela, 2015).

5. Conclusions

Based on their considerable mosquitocidal and biochemical effects on *A. aegypti*, EOs under investigation and their major components appear to be promising candidates to control the major vector of Dengue fever. In all treatments, the whole EO was more active than its major fractions and *J procera* and its monoterpenoid, eugenol were the most potent bio-insecticides against *A. aegypti*. Most of these EOs are classified as medicinal plants and may be incorporated in several pharmaceutical preparations, therefore considered less harmful to mammals than most of the conventional insecticides. After the required toxicological assessments, the test oils could be incorporated as eco-friendly mosquitocides against *A. aegypti*.

Declarations

Author contribution statement

Abdulrhman Almadiy: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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