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ORIGINAL PAPER

Greek *Pinus* essential oils: larvicidal activity and repellency against *Aedes albopictus* (Diptera: Culicidae)

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Abstract The needle volatiles metabolites of seven *Pinus* spp.: *Pinus nigra* (3 samples), *Pinus stankewiczii*, *Pinus brutia*, *Pinus halepensis*, *Pinus canariensis*, *Pinus pinaster* and *Pinus strobus* from Greece were determined by gas chromatography and gas chromatography–mass spectrometry. *P. nigra* and *P. canariensis* essential oils were dominated by α-pinene (24.9–28.9 % and 15 %, respectively) and germacrene D (20.3–31.9 % and 55.8 %, respectively), whereas *P. brutia* and *P. strobus* by α-pinene (20.6 % and 31.4 %, respectively) and β-pinene (31.7 % and 33.6 %, respectively). *P. halepensis* and *P. pinaster* oils were characterized by β-caryophyllene (28.5 % and 22.5 %, respectively). Finally, β-pinene (31.4 %), germacrene D (23.3 %) and α-pinene (17.5 %) were the most abundant compounds in the needle oil of *P. stankewiczii*. Additionally the larvicidal and repellent properties of their essential oils were evaluated against *Aedes albopictus*, a mosquito of great ecological and medical importance. The results of bioassays revealed that repellent abilities of the tested essential oils were more potent than their larvicidal activities. The essential oils of *P. brutia*, *P. halepensis* and *P. stankewiczii* presented considerable larvicidal activity (LC_{50} values 67.04 mgL^{-1} and 70.21 mgL^{-1}, respectively), while the others were weak to inactive against larvae. The essential oils of *P. halepensis*, *P. brutia,* and *P. stankewiczii* presented a high repellent activity, even at the dose of 0.2 μL cm^{-2}, while in the dose of 0.4 μL cm^{-2}, almost all the tested EOs displayed protection against the mosquito.

Keywords *Pinus* taxa · Essential oil composition · *Aedes albopictus* · Larvicidal activity · Repellency

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Introduction

Mosquito-borne viruses or "moboviruses" outbreaks are strongly linked with micro- and macroclimatic and other environmental conditions (Hubálek 2008). Due to climate change and other factors, such as travelling and transportation and socioeconomic conditions, many diseases have recently appeared or reappeared as a major threat in European continent (Klasen and Habedank 2008; Becker 2008).

Aedes albopictus (Diptera: Culicidae), an invasive mosquito species, over the last three decades has spread in many countries in America, Europe, Africa, and Oceania primarily by the trade of used tyres (Enserink 2008). Rapid colonization of new habitats in the northern hemisphere from its origin has been well explained with the wide genetic variability, physiologic variability and ecological adaptation abilities of this species (Hawley 1988). The capacity to develop photoperiodic egg diapause, helped *Ae. albopictus* to survive cold winters and allowed

colonization of temperate areas (Focks et al. 1994; Mori and Oda 1981; Pumpuni et al. 1992).

Due to the fact that no vaccine is available against moboviruses, the most effective way to prevent infection is the protection from mosquito bites. The aim of current work was to study the activity of Greek *Pinus* essential oils against *Ae. albopictus* mosquitoes. For this purpose, essential oils (EOs) derived from seven different *Pinus* species were tested for their larvicidal and repellent activity under laboratory conditions.

The genus *Pinus* (Pinaceae) comprises *c*. 115 species, of monoeicious, evergreen, resiniferous trees or shrubs, widely distributed mainly in the northern hemisphere (Farjon 1984), (Gaussen et al. 1993). Essential oils play in nature an important role in the protection of the plants as antibacterial, antiviral, antifungal and insecticide agents and also against herbivores. According to ethnobotanical data, preparations of pine species have been used in the past for the treatment of different ailments (Berendes 1902). Moreover, studies have shown that essential oils from *Pinus* species exhibit a variety of pharmacological and biological effects (Macchioni et al. 2002; Tognolini et al. 2006; Kolayli et al. 2009). To the best of our knowledge, this is the first study of the larvicidal and repellent activity of pine EOs against *Ae. albopictus*. Thus, seven *Pinus* species, *Pinus nigra* Arnold, *Pinus stankewiczii* Suk., *Pinus brutia* Ten., *Pinus halepensis* Miller, *Pinus canariensis* Sweet ex Sprengel, *Pinus pinaster* Aiton and *Pinus strobus* L. were investigated as larvicidal and insecticidal agents against *Ae. albopictus*. Additionally, the chemical constituents of these essential oils obtained from hydrodistillation were identified by means of gas chromatography–flame ionization detector (GC-FID) and gas chromatography–mass spectrometry (GC-MS).

Materials and methods

Plant materials

Aerial parts of *P. nigra* (sample PNI), *P. stankewiczii* (sample PSTA), *P. brutia* (sample PBR), *P. halepensis* (sample PHA), *P. canariensis* (sample PCA), *P. pinaster* (sample PPI) and *P. strobus* (sample PST) were collected in May 2011, from J. & A. N. Diomedes Botanic Garden; all were cultivated with the exception of *P. halepensis*, which was spontaneous in the area of the botanic garden. Additionally aerial parts of *P. nigra* from natural populations were collected in May 2012 from County Korinthos (sample PNK) and from Samos island (sample PNS). Voucher specimens have been deposited in the Herbarium of the University of Athens.

Isolation of the essential oils

Fresh needles were separated from branches and were further cut in small pieces and subjected to hydrodistillation for 3 h, using a modified Clevenger-type apparatus. The oils were obtained using *n*-pentane as a collecting solvent and subsequently they were dried over anhydrous sodium sulphate and stored under N_2 atmosphere in amber vials at 4 °C until they were analysed.

Gas chromatography analysis

Gas chromatography (GC) analysis was carried out using a SRI 8610C GC-FID system, equipped with DB-5 capillary column (30 m×0.32 mm; film thickness of 0.25 μm; J & W, CA, USA) and connected to a FID detector. The injector and detector temperature was 280 °C. The carrier gas was He, at flow rate of 1.2 mL/min. The thermal programme was 60–280 °C at a rate of 3 °C/min; split ratio of 1:10. Two replicates of each oil sample were processed in the same way. The injected volume was 1 μL of diluted essential oil in *n*-pentane (10 % *v*/*v*). The integration of the peaks was calculated according to the area % as reported from the PeakSimple software.

Gas chromatography–mass spectrometry analysis

Analyses of the oils were performed using a Hewlett Packard (Hewlett Packard GmbH, Waldbronn, Germany) model 5973–6890 GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (200 °C). The transfer line temperature was 250 °C. Helium was used as carrier gas (1 mL/min) and the capillary column used was HP-5MS (30 m×0.25 mm; film thickness of 0.25 μm; Agilent, Palo Alto, CA, USA). The temperature programme was the same with that used for the GC analysis; split ratio of 1:10. The injected volume was 1 μL of diluted essential oil in *n*-pentane (10 % *v*/*v*). Total scan time of 83.33 min. Acquisition mass range of 40–400 amu.

Identification of components

The identification of the compounds was based on comparison of their retention indices (RI), their retention times (RT) and mass spectra with those obtained from authentic samples (purchased from the Sigma-Aldrich Group) and/or the NIST/NBS, Wiley libraries and the literature (Adams 2007).

Mosquito rearing

Mosquito larvae were obtained from a laboratory colony of *Ae. albopictus* which was maintained at 25±2 °C, 80 % relative humidity, and photoperiod of LD 16:8 h, in the

laboratory of Benaki Phytopathological Institute, Kifissia, Greece. Adult mosquitoes were kept in wooden framed cage (33×33×33 cm) covered by a 32×32 mesh, with easy access to 10 % sucrose solution through a cotton wick. Females were blood fed from senior author's forearm, once a fortnight. Larvae were reared in tap water-filled cylindrical enamel pans with diameter of 35 cm and 10 cm deep covered by fine muslin. Approximately 400 larvae were fed ad libitum with powdered fish food (JBL Novo Tom 10 % Artemia) in each pan until the adults emerged. Adult mosquitoes were often collected using mouth aspirator and transferred to the rearing cage. Plastic beakers with 100 mL water and strips of moistened filter paper were provided in the cage for oviposition. The eggs were kept wet for few days and then placed in the pans for hatching.

Larvicidal bioassays

The larval mortality bioassays were carried out according to the test method of larval susceptibility as suggested by the World Health Organization (WHO 2005) with modifications. Sufficient amounts of each compound were transferred to a vial, and the residual solvent was removed under high vacuum. Stock solutions of each test compound in dimethyl sulfoxide (DMSO) were prepared with a concentration of 10 % *w*/*v* (10 mg of compound in 100 μL DMSO). Twenty late third to early fourth-instar mosquito larvae were placed in 2 % *v*/*v* aqueous solution of DMSO (98 mL of tap water plus 2 mL of DMSO), followed by addition of the tested material solution. Gentle shaking to ensure a homogeneous test solution was then performed. Four replicates per dose were made, and a treatment with 98 ml of tap water and 2 mL of DMSO was included in each bioassay as control.

Repellent bioassays

The assessment of repellent activity of each compound was based on the number of mosquito landings on human skin (Giatropoulos et al. 2012, 2013). The study was conducted into a cage (33×33×33 cm) with a 32×32 mesh and with a 20-cm diameter circular opening fitted with cloth sleeve. Each cage contained 100 adult mosquitoes (sex ratio 1:1), 5–10-day-old, starved for 12 h at 25±2 °C and 70–80 % relative humidity.

Data analysis

Larvicidal effect for lethal bioassays was recorded 24 h after treatment. Data obtained from each dose–larvicidal bioassay (total mortality per milligram per litre of each concentration in water) were subjected to probit analysis in which probit-transformed mortality was regressed against $\log_{10}$-transformed dose; LC_{50}, LC_{90} values and slopes were generated (Finney 1971). Four samples were used in each experiment (n=4).

Data concerning the repellency of EOs (mosquito landings) were analysed using Kruskal–Wallis test. When significant differences were detected, Mann–Whitney U tests were carried out for pairwise comparison ($P<0.05$).

All analyses were conducted using the statistical package SPSS 14.0 (SPSS Inc., Chicago, IL, 2004).

Results and discussion

Chemical analysis

After hydrodistillation, whitish oils were obtained with a yield ranging from 0.28 to 0.79 % *v*/*w*. A total of 121 metabolites were identified, comprising 77.6–99.6 % of the total oils. Table 1 shows the composition of the *Pinus* oils in order of their elution on the HP-5 MS column.

The essential oils of *P. nigra* needles, collected from three different sites, were all characterized by the high abundance of α-pinene (24.9–28.9 %) and germacrene D (20.3–31.9 %), followed by β-caryophyllene (15.5–19.0 %). The monoterpene β-pinene was identified in a significantly lower percentage (1.7 %) in sample PNK, collected from a natural population in Korinthos, compared to the needle oil of the other two plant samples (12.8 %, 11.0 %). Our results are in accordance with the previously reported chemical analyses of *P. nigra* needle oils (Sezik et al. 2010; Politeo et al. 2011; Ustun et al. 2012; Ioannou et al. 2014).

P. stankewiczii needle essential oil was characterized by β-pinene (31.4 %) and germacrene D (23.3 %), followed by α-pinene (17.5 %) and β-caryophyllene (9.3 %). To the best of our knowledge, this is the first study on the volatiles of *P. stankewiczii*.

The needle oil of *P. brutia* was dominated by the monoterpenes α- and β-pinene (20.6 and 31.7 %, respectively) along with the sesquiterpene β-caryophyllene (14.5 %), while germacrene D was detected in lower amounts (3.9 %). Several reports on the needle oil of *P. brutia* showed mainly quantitative differentiations in comparison to our results (Roussis et al. 1995; Lahlou 2003; Bagci et al. 2011; Ustun et al. 2012; Ioannou et al. 2014), while germacrene D was not detected at all in the sample collected from Morroco (Lahlou 2003).

P. halepensis oil was characterized by the presence of β-caryophyllene (28.5 %) and an unidentified oxygenated compound (MW=290, 15.0 %), followed by α-humulene, phenyl ethyl-3-methylbutanoate (7.4 and 6.2 %, respectively) and cembrene (5.4 %), while the monoterpenes, α- and β-pinene and the sesquiterpene germacrene D, often characterizing the volatile fraction of the genus, were detected in much lower

Table 1 Chemical composition (%) of the examined oils

Constituents[a]	RI	PNI	PNK	PNS	PSTA	PBR	PHA	PCA	PPI	PST
cis-3-Hexenol	847	0.4	–	–	tr	–	tr	tr	–	–
Santene	876	–	–	–	–	–	–	–	tr	–
Tricyclene	914	–	–	–	–	–	–	tr	tr	tr
α-Thujene	929	1.2	–	tr	–	tr	tr	–	–	–
α-Pinene	928	24.9	28.9	26.4	17.5	20.6	5.0	15.0	12.1	31.4
Camphene	935	1.2	1.4	1.0	0.6	0.6	0.1	0.4	0.2	3.6
Sabinene	965	tr	0.2	tr	tr	tr	0.4	tr	tr	tr
β-Pinene	968	11.0	1.7	12.8	31.4	31.7	0.5	2.1	5.0	33.6
Myrcene	979	1.5	1.3	3.1	1.3	2.1	0.3	1.0	1.2	1.7
cis-3-Hexenyl acetate	989	–	0.1	–	–	–	–	–	–	–
α-Phellandrene	990	–	–	–	tr	tr	–	–	–	tr
δ-3-Carene	1000	–	–	–	1.5	1.4	0.7	–	–	–
α-Terpinene	1005	0.2	tr	–	tr	0.2	tr	–	–	tr
p-Cymene	1012	–	–	–	tr	tr	tr	–	tr	–
Limonene	1017	1.8	3.0	3.1	1.9	1.7	1.0	0.5	0.5	2.9
β-Phellandrene	1018	0.9	0.2	1.0	1.2	1.2	0.2	0.5	0.5	1.1
cis-Ocimene	1025	–	–	–	tr	tr	tr	–	–	–
trans-Ocimene	1038	1.7	3.0	1.6	0.4	1.6	0.4	0.2	tr	–
γ-Terpinene	1047	0.3	tr	tr	tr	0.3	0.1	tr	tr	tr
Terpinolene	1076	1.8	1.0	0.6	0.5	2.2	0.9	tr	tr	1.0
Linalool	1084	–	tr	tr	tr	0.3	0.2	–	0.6	tr
Nonanal	1089	tr	–	–	–	–	tr	–	–	–
α-Fenchol	1104	–	–	–	tr	tr	–	–	–	–
trans-Pinocarveol	1127	tr	–	–	tr	–	–	–	–	tr
Camphene hydrate	1135	–	–	–	tr	–	–	–	–	–
trans-Pinocamphone	1145	–	–	–	tr	–	–	–	–	–
Pinocarvone	1148	–	–	–	tr	–	–	–	–	–
Borneol	1154	–	–	–	tr	tr	tr	–	–	tr
cis-Pinocamphone	1158	–	–	–	tr	–	–	–	–	–
Terpinen-4-ol	1162	tr	tr	tr	tr	0.3	tr	–	–	–
α-Terpineol	1172	1.1	0.2	0.6	1.4	1.0	tr	0.2	0.5	1.1
Thymol methyl ether	1213	–	–	–	–	tr	–	–	–	–
Citronellol	1214	–	–	–	–	–	–	–	–	tr
Linalyl acetate	1235	–	0.2	0.3	–	1.2	–	–	tr	–
Bornyl acetate	1265	0.6	0.1	0.3	tr	0.5	0.2	0.4	tr	2.6
Sabinyl acetate	1266	–	–	tr	–	–	–	–	–	–
2-Undecanone	1273	–	–	–	–	–	–	–	–	tr
n-Tridecane	1280	–	–	–	–	–	–	–	0.9	–
Linalool propanoate	1318	–	–	–	tr	tr	–	–	–	–
α-Terpinyl acetate	1319	2.0	2.2	3.9	1.3	5.3	–	–	–	–
δ-Elemene	1321	–	–	–	–	–	0.3	–	–	–
α-Cubebene	1325	–	–	–	–	–	0.2	tr	0.5	–
Citronellyl acetate	1330	–	–	–	–	–	–	–	–	0.3
Neryl acetate	1338	–	–	–	–	tr	–	–	–	–
2-Dodecanone	1339	–	–	–	–	–	–	–	–	0.4
α-Ylangene	1350	tr	–	–	tr	–	tr	tr	tr	–
α-Copaene	1356	tr	–	–	tr	tr	0.9	0.4	1.9	tr
Geranyl acetate	1364	–	–	–	–	tr	–	–	–	0.4
β-Bourbonene	1368	–	–	–	0.4	tr	–	0.6	–	–

Table 1 (continued)

Constituents[a]	RI	PNI	PNK	PNS	PSTA	PBR	PHA	PCA	PPI	PST
β-Elemene	1370	–	–	–	tr	tr	–	0.3	–	–
Methyl chavicol	1380	–	–	tr	–	–	–	–	–	–
Methyl eugenol	1385	–	–	–	0.6	0.3	–	–	–	–
β-Caryophyllene	1395	19.0	17.1	15.5	9.3	14.5	28.5	5.3	22.5	8.2
β-Copaene	1412	–	–	–	0.2	–	–	–	–	–
β-Gurjunene	1413	tr	0.1	–	–	–	–	0.3	tr	–
α-Guaiene	1415	–	–	–	–	–	tr	–	–	–
6,9-Guaiadiene	1420	–	–	–	–	–	0.2	–	tr	–
cis-Muurola-3,5-diene	1430	–	–	–	–	–	0.2	–	tr	–
α-Humulene	1435	3.8	3.4	2.5	1.8	2.9	7.4	0.5	4.5	1.6
trans-Cadina-1(6),4-diene	1450	–	–	–	–	–	0.3	–	–	–
γ-Muurolene	1455	tr	tr	tr	tr	tr	0.2	tr	1.3	tr
Germacrene D	1460	20.3	31.9	22.6	23.3	3.9	0.6	55.8	2.8	2.3
Phenyl ethyl-2-methylbutanoate	1465	–	–	–	–	tr	1.0	–	0.4	–
Phenyl ethyl-3-methylbutanoate	1470	–	tr	–	0.9	0.7	6.2	–	1.4	–
epi-Cubebol	1472	–	–	–	–	–	–	–	tr	–
Bicyclogermacrene	1475	–	–	–	–	–	–	–	–	0.4
α-Muurolene	1478	0.5	0.2	0.4	0.3	tr	0.2	1.4	1.0	0.7
Cubebol	1490	–	–	–	–	–	0.1	–	–	–
δ-Amorphene	1491	tr	tr	tr	tr	–	–	0.6	tr	–
β-Selinene	1493	–	–	–	tr	–	–	–	–	–
γ-Cadinene	1495	0.7	0.2	0.9	0.4	tr	–	1.4	–	0.6
Cubebol	1498	–	–	–	–	–	–	–	0.4	–
δ-Cadinene	1502	2.0	0.7	3.0	1.3	0.3	2.7	4.2	3.3	1.6
Zonarene	1510	–	–	–	–	–	0.3	–	0.5	–
trans-Cadina-1(2),4-diene	1514	–	–	–	tr	–	tr	tr	tr	tr
α-Cadinene	1518	–	–	–	tr	–	–	0.2	tr	tr
Hedycaryol	1528	–	–	–	–	–	0.5	–	–	–
2-Tetradecanone	1538	–	–	–	–	–	–	–	–	tr
Germacrene D-4-ol	1548	–	–	–	0.2	–	–	tr	–	0.3
2-Phenyl ethyl tiglate	1554	–	–	–	0.1	–	0.8	–	–	–
Caryolphyllene oxide	1563	0.7	tr	tr	0.2	0.4	1.0	–	0.7	tr
β-Copaene-4-α-ol	1570	–	–	–	–	–	–	0.3	–	–
Salvial-4(14)-en-1-one	1574	–	–	–	tr	–	–	–	–	–
Guaiol	1580	–	–	–	–	–	2.9	–	1.0	–
Humulene epoxide II	1588	–	–	–	tr	–	0.2	–	–	–
Junenol	1600	–	–	–	tr	–	–	–	–	–
10-*epi*-γ-Eudesmol	1603	–	–	–	–	–	0.2	–	–	–
1-*epi*-Cubenol	1608	–	–	–	tr	–	0.4	–	–	–
1,10-di-*epi*-Cubenol	1609	–	–	–	–	–	–	–	tr	–
γ-Eudesmol	1615	–	–	–	–	–	0.4	–	–	–
Caryophylla-4(12),8(13)-dien-5-ol[b]	1620	–	–	–	–	–	tr	–	–	–
epi-α-Cadinol	1621	–	–	–	0.1	tr	0.2	0.4	–	tr
epi-α-Muurolol	1622	–	tr	–	0.1	tr	0.3	0.5	–	tr
Cubenol	1626	–	–	–	–	–	0.5	–	0.5	–
α-Muurolol	1627	–	–	–	tr	tr	tr	0.3	tr	tr
α-Cadinol	1634	–	–	–	0.3	tr	–	0.6	tr	0.8
β-Eudesmol	1635	–	–	–	–	–	0.4	–	–	–
α-Eudesmol	1636	–	–	–	–	–	1.6	–	–	–

Table 1 (continued)

Constituents[a]	RI	PNI	PNK	PNS	PSTA	PBR	PHA	PCA	PPI	PST
Eudesma-4(15),7-dien-1β-ol	1668	–	–	–	tr	–	–	0.4	–	–
(2*Z*,6*E*)-Farnesal	1690	–	–	–	–	–	–	tr	tr	–
Benzyl benzoate	1741	–	–	–	–	–	–	tr	–	–
(2*Z*,6*E*)-Farnesyl acetate	1802	–	–	–	–	tr	tr	0.3	–	–
Cembrene	1910	–	–	–	–	1.2	5.4	–	tr	–
Cyclohexadecanolide	1915	tr	–	–	–	–	–	–	–	–
Biformene	1925	–	–	–	–	–	1.5	–	–	–
(3*Z*)-Cembrene A	1940	–	–	–	–	–	1.0	–	–	–
Manool oxide	1965	–	0.5	–	–	–	0.2	tr	–	tr
Unidentified (81, 290)	2020	–	–	–	–	1.7	11.5	–	–	–
Dehydroabietane	2030	–	–	–	–	–	–	–	3.0	–
Manool	2036	–	–	–	–	–	tr	–	–	–
Abietadiene	2065	0.5	–	–	–	–	–	0.3	14.8	–
Abieta-8(14),13(15)-diene	2132	tr	–	–	–	–	–	tr	4.3	–
Sandaracopimarinal	2162	–	–	–	–	–	–	tr	–	–
Methyl isopimarate	2278	–	–	–	–	–	0.3	0.3	–	–
Methyl levopimarate	2284	tr	–	–	0.2	–	0.2	0.5	0.4	tr
Abietal	2290	tr	–	–	tr	–	–	0.3	–	–
Methyl daniellate	2302	–	–	–	–	–	–	–	–	0.7
Methyl dehydroabietate	2315	tr	–	–	tr	–	–	tr	–	–
Methyl abietate	2338	–	–	–	–	–	–	0.4	–	–
Methyl neoabietate	2425	tr	–	–	0.3	–	0.2	0.4	–	–
Total identified (%)		98.1	97.6	99.6	99.0	98.1	77.6	96.3	86.7	95.0
Oil yield % *v*/*w*		0.42	0.45	0.57	0.79	0.71	0.61	0.42	0.28	0.56

PNI *P. nigra*, PNK *P. nigra*, PNS *P. nigra*, PSTA *P. stankewiczii*, PBR *P. brutia*, PHA *P. halepensis*, PCA *P. canariensis*, PPI *P. pinaster*, PST *P. strobus*, "tr" trace (<0.1 %), "–" not detected

[a] Constituents listed in order of elution from a HP-5 MS column, [b] isomer not identified RI: retention indices on HP-5 MS column relative to C_9-C_{23} *n*-alkanes

[b] Isomer not identified RI: retention indices on HP-5 MS column relative to C_9–C_{23} *n*-alkanes

amounts (5.0, 0.5 and 0.6 %, respectively). There are several references to the chemical composition of *P. halepensis* needle essential oils (Ioannou et al. 2014; Ustun et al. 2012; Macchioni et al. 2003; Lahlou 2003; Dob et al. 2007). Among the studied oils, mainly quantitative differences are observed. The chemical profile of our sample seems to be analogous to that analysed by Dob et al. (2007) as the high percentage of sesquiterpenes, β-caryophyllene and α-humulene characterizes both samples. Ioannou et al. (2014) have reported an unidentified oxygenated compound (MW=290, 18.0 %) from an EO sample of *P. halepensis* with the same fragmentation pattern and analogous RI.

P. canariensis needle oil is dominated by germacrene D (55.8 %) along with α-pinene (15.0 %), whereas the percentage of β-pinene is relatively low (2.1 %). Similar qualitative pattern of the main constituents is also observed in preceding studies (Roussis et al. 1995; Pfeifhofer 2000; Hmamouchi et al. 2001; Ioannou et al. 2014).

In *P. pinaster* needle oil, β-caryophyllene (22.5 %), abietadiene (14.8 %) and α-pinene (12.1 %) were the most abundant compounds. Ottavioli et al. (2008) reported a comparable composition from a *P. pinaster* oil from Corsica. Nevertheless, several other authors have reported not only quantitative differences but also qualitative ones, as the diterpene abieta-7,13-diene, a compound abundant in our sample, was not even detected in these oils (Pauly et al. 1973; Hmamouchi et al. 2001; Lahlou 2003; Macchioni et al. 2003; Dob et al. 2005; Ustun et al. 2012; Ioannou et al. 2014). In the analysis of Ioannou et al. (2014) a high percentage of diterpenes (67.3 %) is reported; with isoabienol (19.1 %) and sclarene (18.0 %) being the major compounds, whereas in our sample no diterpenes were detected.

P. strobus essential oil was dominated by β-pinene (33.6 %) and α-pinene (31.4 %) followed by β-caryophyllene (22.5 %). In opposition, the reported analysis of the needle oil by Krauze-Baranowska et al. (2002) showed a significantly lower percentage of β-pinene (7.9 %) while α-pinene and β-

caryophyllene, the major compounds of the oil, were found in lower amounts (17.7, 12.2 %, respectively). Distinctive quantitative differences were also found between the analysis of Ioannou et al. (2014) and ours despite the qualitative similarities.

Larvicidal activity

Although the EOs of the current study were evaluated for the first time against mosquitoes, other plant species, belonging to Pinaceae family, were evaluated against several mosquito species (Sukumar et al. 1991; Dias and Moraes 2014; Amer and Mehlhorn 2006a, b).

As mentioned before, our EOs were evaluated for the first time against mosquitoes and therefore, the link with other previous studies is not an easy task. Furthermore, any relationship between the apparent activity of our EOs and their phytochemical content was not detected. Regarding their larvicidal activity, *P. brutia* (PBR) and *P. halepensis* (PHA) were found to be efficacious with LC_{50} values of 67.04 mgL^{-1} and 70.21 mgL^{-1}, respectively (Table 2). It is noteworthy that by comparing the slope of the graphical representations of concentration vs. mortality, both EOs seem to be the most active of all, resulting in an LC_{90} value of around 70 mgL^{-1}. The EO of *P. stankewiczii* (PSTA) also presented considerable toxic activity with LC_{50} value of 81.66 mgL^{-1} while the EOs of *P. strobus* (PST) and *P. nigra* from Samos (PNS) revealed a weak larvicidal activity with LC_{50} values of 127.98 mgL^{-1} and 152.65 mgL^{-1}, respectively. Previous studies showed that EO from the leaves of *P. radiata*, presented moderate toxicity on *Aedes aegypti* larvae, while *Pinus densiflora* hydrodistillate was found to have a high efficacy against larval of *Ae. albopictus*, *Ae. aegypti* and *Culex pipiens pallens* (Chantraine et al. 1998; Lee and Ahn 2013). Against mosquito larvae of *Ae. aegypti* and *Culex quinquefasciatus*, the EOs of *Pinus tropicalis* and *Pinus caribaea* had a high insecticidal activity and particularly, bioassays against *Ae. aegypti*, revealed both an ovicidal and inhibitory action of larvae development (Leyva et al. 2009, 2012). Against these two aforementioned mosquito species, the EO of *Pinus sylvestris* demonstrated also a good larvicidal toxicity (Fayemiwo et al. 2014). The rest of the EOs (PNI, PNK, PCA and PPI) were inactive at concentrations even as high as 200 mgL^{-1}. Except for EOs, the evaluation of macerating dried leaves from *P. caribaea* in different solutions, showed that the acetone extract was more active and that the larvicidal activity was correlated with the lignin concentration (Kanis et al. 2009).

Repellent activity

The results from the bioassays conducted for the evaluation of the repellent activity of tested materials (essential oils, DEET and control) are presented in Fig. 1. Significant differences in the number of landings were detected among essential oils at both doses evaluated for repellence (x square=42.412; df=10; $P<0.0001$ and x square=34.472; df=10; $P<0.0001$, respectively). In the dose of 0.4 μL cm^{-2}, almost all the tested EOs displayed protection against mosquito. Among the EOs with strong toxicity, only the oils derived from *P. halepenis* (PHA), *P. brutia* (PBR) and *P. stankewiczii* (PSTA) showed a high repellent activity even at the dose of 0.2 μL cm^{-2}. On the contrary, the EOs derived from *P. nigra* collected from Diomedes Botanic Garden (PNI) and *P. strobus* (PST) were not the same effective against mosquito. This differentiation in activity of EOs derived from plants, belonging to the same genus, is common and strongly related with their compounds. The same pattern is observed in other similar studies. Ansari et al. (2005)

Table 2 LC_{50} and LC_{90} values for the studied essential oils against third to fourth-instar larvae of *Ae. albopictus*

Essential oil	Slope (±SE)	LC_{50} (95 % CL)[a]	LC_{90} (95 % CL)[a]	x^2	df
P. nigra (PNI)		>>200			
P. nigra (PNK)		>>200			
P. nigra (PNS)	10.22±0.94	152.65 (146.06–159.78)	203.75 (191.33–221.60)	14.246	13
P. stankewiczii (PSTA)	6.97±0.62	81.66 (76.85–86.95)	124.70 (114.16–140.20)	15.553	13
P. brutia (PBR)	8.65±0.85	67.04 (60.40–72.87)	94.31 (86.06–107.70)	24.129[b]	13
P. halepensis (PHA)	7.08±0.72	70.21 (61.20–77.07)	106.51 (96.05–126.27)	37.050[b]	15
P. canariensis (PCA)		>>200			
P. pinaster (PPI)		>>200			
P. strobus (PST)	12.43±1.11	127.98 (118.47–137.03)	162.29 (150.06–184.02)	36.695[b]	13

[a] LC values are expressed in mg L^{-1} and are considered significantly different when 95 % CL fail to overlap

[b] Since goodness-of-fit test is significant ($P<0.05$), a heterogeneity factor is used in the calculation of confidence limits (*CL*)

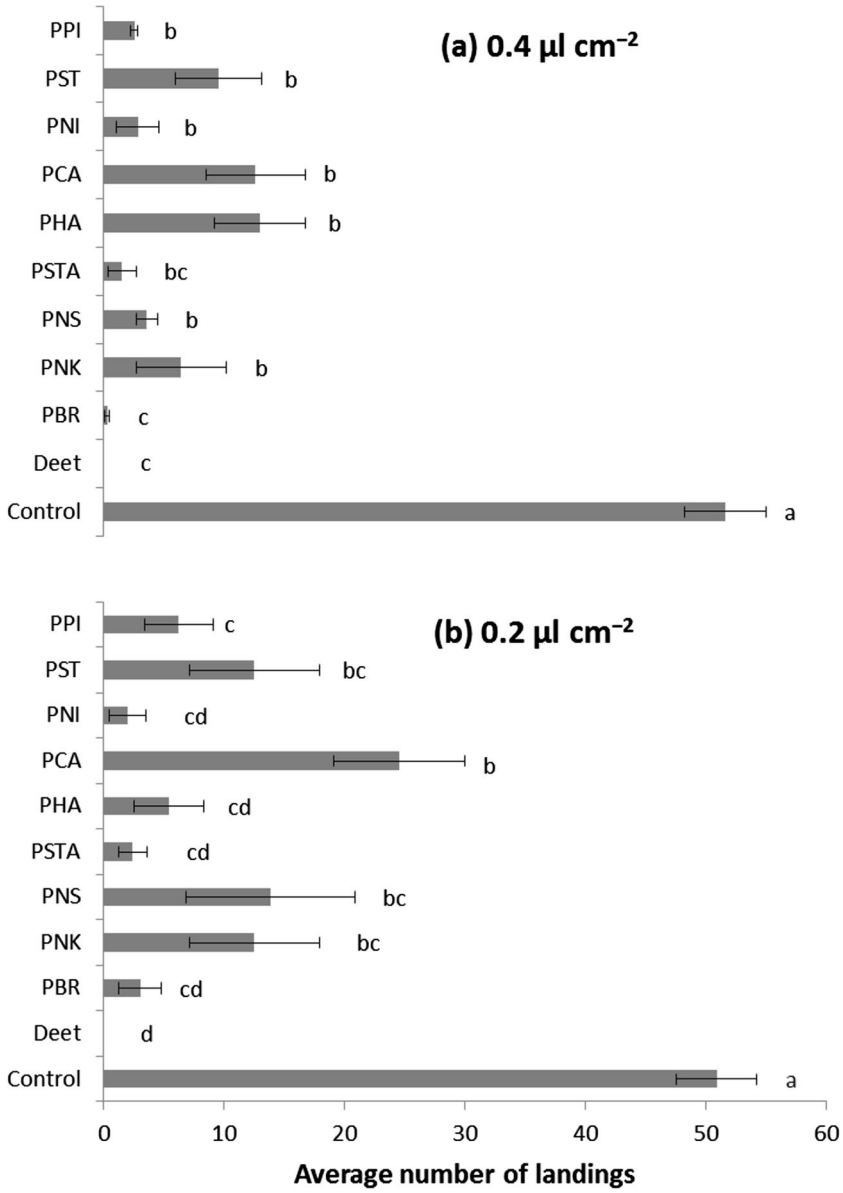

Fig. 1 Repellent activity of tested materials (essential oils plus DEET) on *Aedes albopictus* adults at **a** "high" dose of 0.4 µL cm^{-2} and **b** "low" dose of 0.2 µL cm^{-2}. Mean number of landings per 5 min exposure. Means in a column followed by the same letter are not significantly different ($P \geq 0.05$), Mann–Whitney *U* test

tested pine oil (*Pinus longifolia*) against the malaria vector *Anopheles culicifacies* and the *Cx. quinquefasciatus* for its potential use as larvicidal and/or mosquito repellent agent. Even if the pine oil showed a strong repellent action, it was not effective against mosquito larvae (as larvicidal agent). In contrast, EO from *Pinus pinea* was one of the most toxic tested materials against fourth-instar larvae of the mosquito *Culex pipiens molestus* while it was the least effective EO against mosquito bites (Traboulsi et al. 2005).

Except *Pinus* species, there also some reports from other genus belonging to Gymnospermae with repellent activity against the Asian tiger mosquito. According to Gu et al. (2009), when EOs from different parts of *Cryptomeria japonica* were evaluated against two invasive mosquito species (*Ae. aegypti* and *Ae. albopictus*), the EO from its leaf exhibited the best repellent activity.

Conclusion

In the current study, nine different EOs belonging to seven *Pinus* species were evaluated against *Ae. albopictus*. The trend in research of natural products in vector control, due to their low-risk profile for the environment and humans, has increased (Semmler et al. 2009). Accordingly, in the present study, we investigated the larvicidal and repellent action of against the invasive mosquito species and dengue vector *Ae. albopictus*. We found that repellent abilities of the tested essential oils were more potent than their larvicidal activities. Overall, our results indicate that *Pinus* EOs could serve primarily as an alternate agent of *Ae. albopictus* adult repellency for human skin protection and secondly as an alternate larval control measure.

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