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ORIGINAL PAPER

Larvicidal and repellent activity of *Hyptis suaveolens* (Lamiaceae) essential oil against the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae)

Barbara Conti · Giovanni Benelli · Guido Flamini · Pier Luigi Cioni · Raffaele Profeti · Lucia Ceccarini · Mario Macchia · Angelo Canale

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Abstract Lamiaceae have traditionally been used in developing countries for their insecticidal and repellent properties against several insect species. In our research, the essential oil (EO) extracted from fresh leaves of *Hyptis suaveolens* (Lamiaceae), and its main constituents were evaluated for larvicidal and repellent activity against the Asian tiger mosquito, *Aedes albopictus* Skuse (Diptera: Culicidae), currently the most invasive mosquito worldwide. *H. suaveolens* EO had insecticidal activity against *A. albopictus* larvae and mortality was dosage dependent. At the highest dosages of 450 and 400 ppm, there were no significant differences on larval mortality, as mortality ranged between 98.33% and 93.33%, respectively. At dosages ranging from 250 to 350 ppm, mortality rates were lower and not significantly different from each other. Terpinolene was found to be the most effective pure compound. Efficacy protection from *H. suaveolens* EO, at dosages ranging from 0.03748 to 0.7496 μg cm^{-2} of skin, was evaluated during 150 min of observation. Results indicated that this EO had a significant repellent activity (RD_{50}=0.00035 μg cm^{-2}; RD_{90}= 0.00048 μg cm^{-2}), with differences in repellency rates, as a function of both concentration and observation time. Protection time ranged from 16 to 135 min. These results clearly evidenced that the larvicidal and repellent activity of *H. suaveolens* EO could be used for the development of new and safer products against *A. albopictus*.

B. Conti (✉) · G. Benelli · R. Profeti · A. Canale
Department of Tree Science, Entomology
and Plant Pathology "G. Scaramuzzi", University of Pisa,
Via San Michele degli Scalzi 2,
56124 Pisa, Italy
e-mail: bconti@agr.unipi.it

G. Flamini · P. L. Cioni
Department of Pharmaceutical Sciences, University of Pisa,
Via Bonanno Pisano 33,
56126 Pisa, Italy

L. Ceccarini · M. Macchia
Department of Agronomy and Agro-ecosystem Management,
University of Pisa,
Via del Borghetto 80,
56124 Pisa, Italy

Introduction

Mosquitoes are the most important insects worldwide in terms of public health importance. Mosquito-borne diseases (such as malaria, filariasis, yellow fever, dengue fever and viral encephalitis) amount to a large proportion of health problems in developing countries (James 1992). Mosquitoes are also important as human pests in Europe, since their bite causes a local skin reaction and, in some cases, serious allergic and systemic reactions such as angioderma and urticaria (Peng et al. 1999). Moreover, after the introduction in Europe of new dangerous species, such as *Aedes albopictus* Skuse, many cases of chikungunya (a very severe disease transmitted by *Aedes* spp.) were recently reported in France and Italy (Angelini et al. 2007; Ledrans et al. 2007).

It is well known that one way to reduce the mosquito populations is targeting larvae with organophosphate applications and with insect growth regulators, such as diflubenzuron and methoprene (Yang et al. 2002). Repeated use of these synthetic insecticides can lead to the development of resistance or to undesirable effects on non-target organisms or to human health (Brown 1986; Severini et al. 1993). Treatments with *Bacillus thuringiensis* (var *israeliensis*)

can be a solution, but in several cases they are not suitable against *A. albopictus* (Kamgang et al. 2011). For these reasons, there is a worldwide need to find alternatives to synthetic insecticides. Botanical pesticides are effective, environmentally friendly, easily biodegradable and often less expensive than the synthetic ones (Govindarajan et al. 2011 and references therein). Among these products, essential oils (EOs) are well known for their antibacterial, antifungal and insecticidal activities (Cheng et al. 2003). Many active ingredients isolated and identified from plant extracts can exert toxic activity against mosquito larvae (Cheng et al. 2004; Rahuman et al. 2008; Mathew et al. 2009; Conti et al. 2010; Hafeez et al. 2011). Moreover, they can be used as ovicidal, oviposition deterrents, growth and reproduction inhibitors (Rajkumar and Jebanesan 2005; Pushpanathan et al. 2006) or adult repellents (Gleiser et al. 2011 and references therein). It is known that plants contain various chemicals endowed with unique biological activities (Farnsworth and Bingel 1977), caused by secondary metabolites, who act as attractants or deterrents (Fisher 1991). The number of EOs showing repellent properties against mosquitoes continues to grow (Amer and Mehlhorn 2006; Gillij et al. 2008; Gleiser and Zygadlo 2009; Maheswaran and Ignacimuthu 2011). In some cases, the repellent activity of these compounds is higher or it has longer duration than synthetic chemicals (Moore et al. 2002; Omolo et al. 2004).

Lamiaceae have traditionally been used in developing countries for their insecticidal and repellent properties against several insect species (Ngamo et al. 2007). *Rosmarinus officinalis* L. and *Lavandula angustifolia* Milller (Lamiaceae) EOs showed moderate larvicidal activity (Conti et al. 2010) but a noticeable repellent and ovicidal effect against several mosquito species (Prajapati et al. 2005), possibly caused by α-terpinene, carvacrol and thymol (Choi et al. 2002). Lamiaceae species of the *Hyptis* genus—which included more than 400 species—are highly aromatic and grow in tropical regions, mainly in Africa and America. Several studies have shown that *Hyptis suaveolens* (L.) Poiteau EO has useful insecticidal properties against mosquitoes (Amusan et al. 2005; Jaenson et al. 2006) and many stored products pests (Peerzada 1997; Othira et al. 2009; Conti et al. 2011). Moreover, its chemical composition and biological activity change as a function of plants origin and their collecting period (Tchoumbougang et al. 2005; Noudjou et al. 2007).

As further studies are important to improve the knowledge of new plant extracts and their pure constituents for their use against mosquito species, this study investigates the chemical composition of *H. suaveolens* EO, extracted from plants cultivated in Tuscany (Italy), and its larvicidal and repellent activity against the Culicidae mosquito *A. albopictus*. Furthermore, the effectiveness of its main EO constituents as larvicides was measured.

Materials and methods

H. suaveolens cultivation

Plants were grown at the Department of Agronomy and Agroecosystem Management (University of Pisa) (Fig. 1). Seeds of *H. suaveolens* (from Nepal) were placed on filter paper moistened in Petri dishes, placed in a climatic chamber [alternating temperature of 20–30°C, photoperiod 8:16 (*L*:*D*)] and left to germinate, between February and April 2010. Seedlings (germination 83%) were transferred to nurseries and then placed in a cold greenhouse for ca. 40 days. The young plants were transplanted in June 2010, at a density of 4.5 plant m^{-2} in a silt–loam soil (sand, 15.5%; silt, 65.5%; clay, 18.0%; organic matter, 1.15%; pH 8.1), with a rather shallow water table, above a depth of 120 cm. Urea (50 kg ha^{-1} of N), 100 kg ha^{-1} of triple superphosphate (P_2O_5) and 100 kg ha^{-1} of potassium sulphate (K_2O) were used as fertilisers. Irrigation and mechanical weed control were used for the entire cultivation period. The biomass was collected at the beginning of October 2010.

Essential oil extraction and analysis

Leaves were dried in the shade, at room temperature until constant weight, and then coarsely ground and hydrodistilled in a Clevenger-type apparatus for 2 h. Gas chromatography (GC) analyses were carried out with an HP-5890 series II instruments equipped with HP-WAX and HP-5 capillary columns (30 m×0.25 mm, 0.25 μm film thickness), working with this temperature program, 60°C for 10 min, ramp of 5°C/min up to 220°C; injector and detector temperatures 250°C; carrier gas was nitrogen (2 ml/min); detector dual FID; split ratio 1:30; and injection of 0.5 μl. Component identification was carried out, for both columns,

Fig. 1 Plants of *Hyptis suaveolens* (L.) Poiteau (Lamiaceae)

Table 1 Composition of the essential oil of *Hyptis suaveolens* used in the biological assays

Constituents	l.r.i.	Leaves
(*E*)-2-Hexenal	855	0.2
α-Thujene	931	1.1
α-Pinene	939	2.6
Sabinene	977	21.9
α-Pinene	980	7.2
Myrcene	992	0.4
α-Phellandrene	1,005	0.2
δ-3-Carene	1,011	0.5
α-Terpinene	1,018	2.9
p-Cymene	1,027	0.3
Limonene	1,030	5.5
(*Z*)-β-Ocimene	1,941	Tr
Phenylacetaldehyde	1,044	Tr
(*E*)-β-Ocimene	1,051	Tr
γ−Terpinene	1,062	4.0
cis-Sabinene hydrate	1,070	0.6
Artemisia alcohol	1,084	tr
Terpinolene	1,089	9.6
trans-Sabinene hydrate	1,099	0.5
Nonanal	1,103	Tr
exo-Fenchol	1,117	0.7
cis-p-menth-2-en-1-ol	1,122	0.5
cis-p-mentha-2,8-dien-1-ol	1,139	Tr
trans-p-menth-2-en-1-ol	1,142	0.3
Sabina ketone	1,156	Tr
4-Tepineol	1,179	7.3
p-Cymen-8-ol	1,185	0.2
α-Terpineol	1,190	0.4
Eugenol	1,358	Tr
α-Copaene	1,376	Tr
(*E*)-β-Damascenone	1,382	Tr
β-Elemene	1,391	0.2
(*Z*)-Caryophyllene	1,405	Tr
β-Caryophyllene	1,418	16.1
trans-α-bergamotene	1,439	3.1
α-humulene	1,455	0.9
(E)-β-Farnesene	1,459	0.3
β-Chamigrene	1,475	Tr
β-Selinene	1,485	1.0
Bicyclogermacrene	1,494	2.3
α-Bulnesene	1,505	0.2
(*Z*)-γ-Bisabolene	1,515	Tr
δ-Cadinene	1,524	Tr
Spathulenol	1,576	1.7
Caryophyllene oxide	1,581	2.2
T-cadinol	1,641	0.2
β-eudesmol	1,649	0.2
Selin-11-en-4-α-ol	1,653	0.4
trans-α-Bergamotol	1,691	2.5
Hexahydrofarnesylacetone	1,845	Tr
Abietatriene	2,054	1.3
Abietadiene	2,080	0.6

by comparing their retention times with those of pure authentic samples and by means of their linear retention index (LRI), relative to the series of *n*-hydrocarbons.

Gas chromatography/electron impact mass spectroscopy (GC/EIMS) analyses were performed with a Varian CP-3800 gas chromatograph, equipped with an HP-5 capillary column (30 m×0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions are as follows: injector and transfer line temperatures 220°C and 240°C, respectively; oven temperature programmed from 60°C to 240°C at 3°C/min; carrier gas helium at 1 ml/min; injection of 0.2 μl (10% hexane solution); and split ratio 1:30. Constituent identification was based on the comparison of retention times with those of authentic samples; this implied comparing their LRIs with the series of *n*-hydrocarbons and using computer matching against commercial (NIST 98 and ADAMS) and home-made library mass spectra (built up from pure substances and components of known oils and MS literature data, refer to Stenhagen et al. 1974; Massada 1976; Jennings and Shibamoto 1980; Swigar and Silverstein 1981; Davies 1990; Adams 1995). Moreover, molecular weights of all identified substances were confirmed by GC/CIMS, using methanol as the chemical ionization gas.

Mosquitoes rearing conditions

Mosquito larvae and adults of *A. albopictus* originated from field-collected eggs, deposited by wild females on a bar of masonite placed outdoors in a dark vase containing water. Egg batches were collected daily and kept moist for 24 h; then they were placed in laboratory conditions [25±1°C, 65±5% relative humidity (R.H.), natural summer photoperiod] in 100-cc glass tubes and submerged in mineral water for hatching. Newly emerged larvae were isolated in groups of five specimens in 100-cc glass tubes, with mineral water and a small amount of cat food. The larvae were examined daily, until they reached the fourth instar; they were then used for bioassays (within 12 h) or to obtain adults. Adults were stocked in cages (300 specimens/cage, sex ratio 1:1), held at 25±1°C, 65±5% R.H., natural summer photoperiod and supplied with 10% sucrose solution on a cotton wick.

Larvicidal activity

Three groups of 20 fourth instar larvae were isolated in 250-ml beakers and exposed to dosages of 50, 100, 150, 200, 250, 300, 350, 400 and 450 ppm of EO in mineral water with 0.1% of Tween® 80 for 24 h (WHO 1981); 250-ml beakers with the same number of larvae (for three replicates) and mineral water with 0.1% of Tween® 80 were used as control. Mortality was recorded after 24 h, at the end of the test, during which no food was given to the larvae. Larval mortality was reported as an average of three replicates; mortality percentage rates were corrected using Abbott's formula (Abbott 1925) and they were used to calculate the LC_{50} values.

Main constituents (Table 1) identified in *H. suaveolens* EO (sabinene, α-pinene and β-pinene, limonene, terpinolene, β-caryophyllene and 4-terpineol) were purchased from Sigma-Aldrich® and tested—singularly or in blend—for larvicidal activity at the same dosage contained in 450 ppm of EO, at which 100% of larval mortality was previously obtained. Compound solutions were prepared in 250-ml beakers with mineral water containing 0.1% Tween® 80. Three groups of 20 fourth instar larvae were used for the test and 250-ml beakers with 20 larvae (three replicates) and mineral water with 0.1% of Tween® 80 were used as control.

Repellent activity

H. suaveolens EO repellency was evaluated using the human bait technique to simulate the condition of human skin on which repellents will be applied, as reported by Schreck and Mc Govern (1989), Gleiser et al. (2011) and Kamsuk et al. (2006). Tests were conducted during the summer of 2011. Groups of 150 nulliparous, nonblood-fed, starved female of *A. albopictus* (7–10 days old) were placed, in order to facilitate viewing, into Plexiglass cylindrical laboratory cages (diameter, 35 cm; length, 60 cm). Each cage had a cotton stockinet access sleeve on the front. *A. albopictus* is a day-biting mosquito; therefore, testing period was between 08:00 and 16:00 hours. Ten volunteers were chosen amongst susceptible to mosquito bites and non-allergic subjects. They had no contact with lotions, perfumes, oils or perfumed soaps on the day of the bioassay. After cleaning their hands in distilled water, they protected their forearms with a thick fabric sleeve and wore a latex surgical glove, in which a dorsal square area 5×5 cm was cut open.

Table 2 Mean percentages (%) of main chemical classes of the *H. suaveolens* essential oil volatiles

Constituents	Mean percentages (%)
Monoterpene hydrocarbons	55.2
Oxygenated monoterpenes	10.5
Sesquiterpene hydrocarbons	24.1
Oxygenated sesquiterpenes	7.2
Diterpenes	1.9
Non-terpene derivatives	0.2
Total identified	99.1

Table 3 Mortality obtained in larvicidal test conducted with *Hyptis suaveolens* essential oil against fourth instar larvae of *Aedes albopictus*

Concentration of EO (μg/cm^2 of skin)	Mortality (%±SE)	LC_{50} (ppm)	95% CL LCL UCL	Regression equation
450	98.33±0.29a			
400	93.33±0.76ab			
350	78.33±1.61bc			
300	70±0.50cd	240.3	212.8–271.3	y=3.073x+1.548
250	65±0.10cd			
200	40±0.50de			
150	20±0.50ef			
100	5±0fg			
50	3.33±0.29fg			
Control	1±0g			

Each datum represents the mean of three replicates, each setup with twenty larvae. Data followed by the same letters are not statistically different ($P<$ 0.05, one-way ANOVA, Tukey–Kramer HSD test). LC_{50} = lethal concentration (in parts per million) that kills 50% of the exposed larvae

LC lethal concentration, *95% CL* confidence limit at 95%, x=concentration in parts per million, y percentage of mortality

Table 4 Mortality obtained in larvicidal test conducted with the seven main constituents of the *Hyptis suaveolens* essential oil tested at the concentration detected in 450 ppm of the oil

Compound	Dose (ppm)	Mortality (% ± SE)
(1) Sabinene	153.0	3.33±0.29b
(2) α-Pinene	14.4	6.67±0.28 b
(3) β-Pinene	36.8	3.33±0.29b
(4) Limonene	26.0	3.33±0.29b
(5) Terpinolene	48.0	43.33±0.76a
(6) β-Caryophyllene	50.4	3.33±0.29b
(7) 4-Terpineol	11.2	0b
Blend (1+2+3+4+5+6+7)	–	55±0.13a
Control	0	0b

The blend dose was the sum of the one to seven compound relative concentrations. Compounds were tested singularly or in blend against fourth instar larvae of *Aedes albopictus*. Each datum represents the mean of three replicates, each setup with 20 larvae. Means followed by different letters are significantly different ($P<0.05$, one-way ANOVA, Tukey–Kramer HSD test)

Table 5 Efficacy protection of *Hyptis suaveolens* essential oil at different dosages against *Aedes albopictus*, during 150 min of observations

Concentration of EO (μg/cm^2 of skin)	Efficacy protection (% ± SE) after different times of observation					
	15 min	30 min	60 min	90 min	120 min	150 min
0.03748	94.20±0.08bcd	80.60±1.22def	77.00±0.71efg	72.60±1.29fg	66.80±0.55fgh	42.00±0.90i
0.1874	100±0a	100±0a	91.20±0.73cde	76.80±1.26efg	63.40±1.07ghi	49.20±0.95hi
0.3748	100±0a	100±0a	100±0a	94.20±0.30bcd	76.40±0.73efg	64.40±0.48gh
0.5622	100±0a	100±0a	100±0a	100±0a	81.60±1.01defg	70.40±0.48fg
0.7496	100±0a	100±0a	100±0a	99.60±0.09ab	98.00±0.02abc	91.40±0.50cde

Each datum represents the mean of five replicates. Means followed by different letters are significantly different ($P<0.05$, two-way ANOVA, by Tukey–Kramer HSD test)

Mosquito-exposed skin was firstly treated with 100 μl of ethanol, as negative control, and then with 100 μl of EO in ethanol solution (dosages ranging from 0.0375 to 0.750 μg/cm^2, refer to Table 5). All concentrations were replicated five times. Firstly, the control hand was exposed in the cage for 3 min, during which the number of probing mosquitoes was recorded. Immediately after, the hand was withdrawn and treated with repellent formulation; then it was re-exposed to mosquitoes in the same test cage. The number of probing mosquitoes in a 3-min exposure period was recorded. The percentage of repellency obtained from five replicates—expressed as percentage protective efficacy (PE%)—was calculated at each dosage using this formula: PE% = [(number probing untreated hand − number probing treated hand)/number probing untreated hand] × 100 (Fradin and Day 2002).

To calculate the full protection time (PT), the test was repeated every 5 min for the lowest concentration and every 10 min for the other concentrations, until either two bites occurred in a single exposure period or one bite occurred in each of two consecutive exposure periods. The period of time, between repellent application and the first two bites in a single exposure or two bites in successive observations, was recorded as the complete protection time. Each PT is an average of five replicates. Each concentration has been tested for a total of 150 min. During the tests, the control and the treated hands were regularly interchanged, to verify the mosquitoes' readiness to bite. On rare occasions, when no mosquito attempted to bite the untreated hand, trial was discarded and test was repeated with a new mosquito cage, to ensure that the lack of bites was due to repellency and not to mosquitoes being unwilling to have a blood meal at the time. To calculate the RD_{50} values, the EO was tested at dosages of 9.370, 5.622, 3.748, 1.874, 0.3748, 0.1874 and 0.093710^{-3} μg cm^{-2} (WHO 2009).

Table 6 Repellent activity of the *Hyptis suaveolens* essential oil

Value (μg/cm^2 of skin)		95% CL		Regression equation
		LCL	UCL	
RD_{50}	0.00035	0.00023	0.00052	y=8.256×+30.38
RD_{90}	0.00048	0.00014	0.0016	

RD_{50} = repellency dose (in micrograms per square centimeter of skin) that repel 50% of *Aedes albopictus*, RD_{90} = repellency dose (in micrograms per square centimeter of skin) that repel 90% of *Aedes albopictus*

RD repellency dose, *95% CL* confidence limit at 95%, *LCL* lower confidence limit, *UCL* upper confidence limit, *x* concentration in parts per million, *y* percentage of mortality

Statistical analysis

EO larvicidal activity data were transformed into arcsine square root percentage values, before statistical analysis. Data were processed by JMP®, using a general linear model (GLM) with one factor, the concentration: $y_j=\mu+C_j+e_j$, in which y_j is the observation, μ is the overall mean, C_j is the dosage (j=1–9) and e_j the residual error. Averages were separated by Tukey–Kramer HSD test (Sokal and Rohlf 1981). Only probability level $P<0.05$ was used, for the significance of differences between means, to simplify statistical analysis. Data on larvicidal activity of pure compounds were transformed into arcsine square root percentage values and analysed using a GLM as described above, in which C_j is the compound (j=1–7). Repellency data were analysed using a GLM (JMP® SAS, 1999) with two factors with interactions, dosage and time: $y_j=\mu+C_j+T_j+C_j\times T_j+e_j$ in which y_j is the observation, μ is the overall mean, C_j the dosage (j=1–5), T_j the time (j=1–5), $C_j\times T_j$ the interaction between dosage and time and e_j the residual error in the interaction between oil and dosage.

Median lethal concentration and median repellent dosage (LC_{50} and RD_{50}) were calculated by using SigmaPlot© software (Systat Software Inc., CA, USA). Bottom and top parameters were fixed to 0 (0% mortality and no repellence, respectively) and 100 (100% mortality and full repellence, respectively).

Table 7 Protection time (in minutes) for the tested five concentrations of the *Hyptis suaveolens* essential oil

Concentration of EO (μg/cm^2 of skin)	Protection time (min±SE)
0.03748	16.0±2.95c
0.1874	70.2±7.56b
0.3748	97.0±4.25b
0.5622	128.0±6.79a
0.7496	134.8±9.97a

Each datum represents the mean of five replicates. Means followed by different letters are significantly different ($P<0.05$, two-way ANOVA by Tukey–Kramer HSD test)

Results and discussion

H. suaveolens cultivation

H. suaveolens plants have reached an average height of 85 cm in mid-October. At the end of August, they have reached a height of about 80 cm. Biomass obtained after about 4 months from transplanting was 2,184 gpt^{-1}. Dry matter produced was 431.3 gpt^{-1}. In our environment, *H. suaveolens* did not reach the flowering stage.

Chemical composition of *H. suaveolens* EO

H. suaveolens is polymorphic in its essential oil composition because many different compositions have been reported (Grassi et al. 2008). Several chemotypes have been described, such as β-caryophyllene type from Nigeria (Iwu et al. 1990), 1,8-cineole/sabinene from India (Mallavarapu et al. 1993), three 1,8-cineole, α-terpinolene and fenchone/fenchol types from El Salvador (Grassi et al. 2008).

The essential oil obtained from the leaves of plants grown in Pisa (Table 1) cannot be ascribed to a precise chemotype, as it contains high percentages of sabinene (21.9%), β-caryophyllene (16.1%), terpinolene (9.6%) and 4-terpineol (7.3%). Globally, 52 constituents were identified, accounting for 99.1% of the whole essential oil. From a chemical classification point of view (Table 2), monoterpene hydrocarbons were the most represented volatiles (55.2%), followed by sesquiterpene hydrocarbons (24.1%) and oxygenated monoterpenes (10.5%). In addition, smaller amounts of oxygenated sesquiterpenes (7.2%) and diterpenes (1.9%) were detected. Non-terpene derivatives were present in very small amounts (0.2%).

Larvicidal activity

It has been acknowledged that ethanolic extracts of *H. suaveolens* caused high mortality rate (80%) on yellow fever mosquito *Aedes aegypti* (L.) larvae, at concentrations of 0.9 and 0.3 ppm (Amusan et al. 2005). By contrast, *H. suaveolens* petroleum leaf extracts did not show any effective larvicidal activity against *Culex* spp. mosquito larvae (Okigbo et al. 2010). Data reported by Tamprasit and Indrapichate (2004) showed that *H. suaveolens* crude extract manifested a larvicidal activity higher than the one from *Lantana camara* crude extract, with a synergic effect if these extracts were mixed. To our knowledge, the larvicidal activity of *H. suaveolens* EO against mosquito species was not evaluated. Our results clearly demonstrated that *H. suaveolens* EO had insecticidal activity against *A. albopictus* larvae, thus widening the extraction methods of its larvicidal botanical components. Larval mortality was dosage dependent. It was found that there are significant differences in mortality rates, as a function of EO concentration ($F=43.68$, $df=8$, $P<0.0001$) (Table 3). At the highest dosages of 450 and 400 ppm, there were no significant differences on larval mortality, with mortality percentage rates ranging from 98.33% and 93.33%, respectively. At dosages ranging from 250 to 350 ppm, mortality rates were lower and not significantly different from each other.

LC_{50} value of *H. suaveolens* EO was 240.3 ppm (Table 3). Tests with *A. aegypti* and EOs from different species of *Hyptis* showed that LC_{50} of *Hyptis fruticosa* and *Hyptis pectinata* were 502 and 366 ppm, respectively (Silva et al. 2008). It must be noted that these values were

Table 8 Repellent activity against mosquitoes, through different application methods of *Hyptis suaveolens* (L.) Poiteau (Lamiaceae)

Mosquito species	*H. suaveolens* cultivation site	Application method	Repellent activity	Reference
Aedes albopictus	Pisa, Italy	Essential oil	Effective	This study
Aedes aegypti	Guinea-Bissau	Ethyl acetate extract	Not effective	Jaenson et al. 2006
Anopheles gambiae	Kenya	Potted plants in semi-field trials	Not effective	Seyoum et al. 2002a
Anopheles gambiae	Kenya	Whole plants in house	Effective	Seyoum et al. 2002b
Anopheles gambiae	Kenya	Fresh or smouldering whole plants	Effective	Seyoum et al. 2002b
Anopheles gambiae	Kenya	Thermal expulsion	Not effective	Seyoum et al. 2002b
Culicidae spp.	Guinea-Bissau	Smoke by burning whole plants	Effective	Pålsson and Jaenson 1999a, b
Culicidae spp.	Guinea-Bissau	Fresh or smouldering whole plants	Effective	Pålsson and Jaenson 1999b

relatively higher, with respect to those obtained in our study. Moreover, *H. suaveolens* ethanolic extracts showed a high toxicity against *A. aegypti* larvae, with LD_{50}=0.60 ppm and LD_{90}=1.45 ppm (Amusan et al. 2005).

Table 4 illustrates the larvicidal activity of α-pinene and β-pinene, β-caryophillene, sabinene, terpinolene, limonene and 4-terpineol—tested on its own or blended—in the same concentration at which they were detected in 450 ppm of the EO. There was a significant larvicidal effect of the whole blend, with respect to the control and to α-pinene and β-pinene, β-caryophillene, sabinene, limonene and 4-terpineol. On the contrary, it was evident that terpinolene toxicity was not statistically different from the one of the whole blend (43.33% and 55%, respectively). Terpinolene toxicity has been well acknowledged with other insect species, such as *Sitophilus zeamais* Motschulsky, *Tribolium castaneum* (Herbst) (Wang et al. 2009), *Callosobruchus chinensis* (L.) and *Sitophilus oryzae* (L.) (Park et al. 2003). We suppose that the blend larvicidal activity is probably due to the sum of individual toxicities of the main constituents.

Repellent activity

To our knowledge, there is no reported data in literature on *H. suaveolens* EO repellent activity against *A. albopictus*. Table 5 summarises the results of repellency tests—expressed as PE%—at different dosages of *H. suaveolens* EO, during 150 min of observation. The results indicated that the EO had a significant repellent activity (RD_{50}= 0.00035 μg cm^{-2}; RD_{90}=0.00048 μg cm^{-2}, Table 6), with relevant differences in repellency rates, as a function of both concentration (F=119.55, df=4, P<0.0001) and time of observation (F=151.62, df=5, P<0.0001). At lower dosage, the EO gives a PE of 94.2% in the first 15 min of observation. At dosage of 0.1874 μg/cm^2, *H. suaveolens* EO offered a PE of 100% for at least 30 min, and after 60 min, it reached 91.2%. At a medium dosage of 0.3748 μg/cm^2, *H. suaveolens* EO reached a similar value (94.2% of PE) after 90 min. Two higher dosages (0.5622 and 0.7496 μg/cm^2 of skin) gave almost complete protection (99.6% and 100% PE, respectively) for 90 min. Table 7 shows protection times for the five concentrations of the *H. suaveolens* EO tested. The results indicated a significant effect of the EO concentration (F=51.01, df=4, P<0.0001); protection time ranged between 16 and 135 min. Our observations of *H. suaveolens* EO efficacy as repellent improve previous evidence from several studies, in which the repellent activity of *H. suaveolens* was proven through different application methods (Table 8). In fact, it is known that placing *H. suaveolens* branches or whole plants in houses was one of the most effective methods, in western Kenya, to repel malaria vector *Anopheles gambiae* s.s. Giles (Seyoum et al. 2002b). By contrast—in semi-field conditions—it was observed that *H. suaveolens* potted plants did not significantly repel *A. gambiae* mosquitoes (Seyoum et al. 2002a). Moreover, studies performed in Guinea Bissau, West Africa showed that smoke produced by burning whole plants of *H. suaveolens*, indoors at night, significantly repelled mosquitoes (Pålsson and Jaenson 1999a, b). Pålsson and Jaenson (1999b) reported that fresh or smouldering whole plants of *H. suaveolens* were used in Guinea Bissau, to reduce the number of mosquitoes indoors at night, with a repellent activity ranging from 85.4% to 66.5% (for smouldering and fresh plants, respectively). Similar results were obtained with the same method in western Kenya, against *A. gambiae* (Seyoum et al. 2002b). However, any significant repellent effect was recorded against this latter mosquito species, when *H. suaveolens* flowers and leaves were tested through thermal expulsion method (Seyoum et al. 2002b). Finally, ethyl acetate extracts of *H. suaveolens* from Guinea Bissau strongly reduced the probing activity of *A. aegypti* (Jaenson et al. 2006).

Conclusions

The present article improves the knowledge about the composition and the main constituents of the EO of an important tropical Lamiacea, such as *H. suaveolens*. Our data, compared with those reported in literature, confirm that its EO can have different chemical content as a function of the habitat where plants are grown. Investigation on larvicidal activity against *A. albopictus* demonstrated that *H. suaveolens* EO had insecticidal properties, thus widening its spectrum of action. Moreover, the study on EO repellency improved previous evidence from several studies, in which the repellent activity of *H. suaveolens* was proven through different application methods. Its insecticidal and/or repellent activity could be used for the development of new and safer products against *A. albopictus* larvae and adults.

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References

Abbott WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18:265–267

Adams RP (1995) Identification of essential oil components by gas chromatography-mass spectroscopy. Allured, Carol Stream

Amer A, Mehlhorn H (2006) Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae). Parasitol Res 99:466–472

Amusan AAS, Idowu AB, Arowolo FS (2005) Comparative toxicity effect of bush tea leaves (*Hyptis suaveolens*) and orange peel (*Citrus sinensis*) oil extract on larvae of the yellow fever mosquito *Aedes aegypti*. Tanz Health Res Bull 7:174–178

Angelini R, Finarelli AC, Angelini P, Po C, Petropulacos K, Macini P, Fiorentini C, Fortuna C, Venturi G, Romi R, Majori G, Nicoletti L, Rezza G, Cassone A (2007) An outbreak of chikungunya fever in the province of Ravenna, Italy. Euro Surveill 12:3260

Brown AWA (1986) Insecticide resistance in mosquitoes: a pragmatic review. J Am Mos Contr Assoc 2:123–139

Cheng SS, Chang HT, Chang ST, Tsai KH, Chen WJ (2003) Bioactivity of selected plant essential oils against the yellow fever mosquito *Aedes aegypti* larvae. Bioresour Technol 89:99–102

Cheng S, Liu J, Tsai K, Chen W, Chang S (2004) Chemical composition and mosquito larvicidal activity of essential oils from leaves of different *Cinnamomum osmophloeum* provenances. J Agric Food Chem 52:4395–4400

Choi W, Park B, Ku S, Lee S (2002) Repellent activity of essential oils and monoterpenes against *Culex pipiens pallens*. J Am Mosq Control Assoc 18:348–351

Conti B, Canale A, Bertoli A, Gozzini F, Pistelli L (2010) Essential oil composition and larvicidal activity of six Mediterranean aromatic plants against the mosquito *Aedes albopictus* (Diptera: Culicidae). Parasitol Res 107:1455–1462

Conti B, Canale A, Cioni PL, Flamini G, Rifici A (2011) *Hyptis suaveolens* and *Hyptis spicigera* (Lamiaceae) essential oils: qualitative analysis, contact toxicity and repellent activity against *Sitophilus granarius* (L.) (Coleoptera: Dryophthoridae). J Pest Sci 84:219–228

Davies NW (1990) Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicon and carbowax 20M phases. J Chromatogr 503:1–24

Farnsworth NR, Bingel AS (1977) Natural products and plant drugs with pharmacological, biological or therapeutic activity. Springer, Berlin

Fisher PR (1991) The role of gaseous metabolites in phototaxis by *Dictyostelium discoideum* slugs. FEMS Microbiol Lett 77:117–120

Fradin MS, Day JF (2002) Comparative efficacy of insect repellents against mosquito bites. New Engl J Med 347:13–18

Gillij YG, Gleiser RM, Zygadlo JA (2008) Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. Bioresour Technol 99:2507–2515

Gleiser RM, Zygadlo JA (2009) Essential oils as potential bioactive compounds against mosquitoes. In: Imperato F (ed) Recent advances in phytochemistry. Research Signpost, Kerala, pp 53–76

Gleiser RM, Bonino MA, Zygadlo JA (2011) Repellence of essential oils of aromatic plants growing in Argentina against *Aedes aegypti*. Parasitol Res 108:69–78

Govindarajan M, Mathivanan T, Elumalai K, Krishnappa K, Anandan A (2011) Mosquito larvicidal, ovicidal, and repellent properties of botanical extracts against *Anopheles stephensi*, *Aedes aegypti*, *and Culex quinquefasciatus* (Diptera: Culicidae). Parasitol Res 109:353–367

Grassi P, Nuñez MJ, Reyes TSU, Franz C (2008) Chemical variation in the essential oil composition of *Hyptis suaveolens* (L.) Poit. (Lamiaceae). Nat Prod Comm 3:1137–1140

Hafeez F, Akram W, Shaalan EA (2011) Mosquito larvicidal activity of citrus limonoids against *Aedes albopictus*. Parasitol Res 109:221–229

Iwu MM, Ezueufwu CO, Okunji CO, Sanson DR, Tempesta MS (1990) Antimicrobial activity and terpenoids of the essential oil of *Hyptis suaveolens*. Int J Crude Drug Res 28:73–76

Jaenson TGT, Pålsson K, Borg-Karlson AK (2006) Evaluation of extracts and oils of mosquito (Diptera: Culicidae) repellent plants from Sweden and Guinea-Bissau. J Med Entomol 43:113–119

James AA (1992) Mosquito molecular genetics: the hands that feed bite back. Science 257:37–38

Jennings W, Shibamoto T (1980) Qualitative analysis of flavour and fragrance volatiles by glass capillary chromatography. Academic, New York

Kamgang B, Marcombe S, Chandre F, Nchoutpouen E, Nwane P, Etang J, Corbelle V, Paupy C (2011) Insecticide susceptibility of *Aedes aegypti* and *Aedes albopictus* in Central Africa. Par Vect 4:79

Kamsuk K, Choochote W, Chaithong U, Jitpakdi A, Tippawangkosol P, Riyong D, Pitasawat B (2006) Effectivenes of *Zanthoxylum piperitum*-derived essential oil as an alternative repellent under laboratory and field application. Parasitol Res 100:339–345

Ledrans M, Quatresous I, Renault P, Pierre V (2007) Outbreak of chikungunya in the French territories, 2006: lessons learned. Euro Surveill 12:3262

Maheswaran R, Ignacimuthu S (2011) A novel herbal formulation against dengue vector mosquitoes *Aedes aegypti* and *Aedes albopictus*. Parasitol Res. doi:10.1007/s00436-011-2702-z, online first

Mallavarapu GR, Ramesh S, Kaul PN, Bhattacharya AK, Rao BRR (1993) The essential oil of *Hyptis suaveolens* (L.) Poit. J Essent Oil Res 5:321–323

Massada Y (1976) Analysis of essential oils by gas chromatography and mass spectrometry. Wiley, New York

Mathew N, Anitha MG, Bala TSL, Sivakumar SM, Narmadha R, Kalyanasundaram M (2009) Larvicidal activity of *Saraca indica*, *Nyctanthes arbor-tristis* and *Clitoria ternatea* extracts against three mosquito vector species. Parasitol Res 104:1017–1025

Moore SJ, Lenglet A, Hill N (2002) Field evaluation of three plant-based insect repellents against malaria vectors in Vaca Diez province, the Bolivian Amazon. J Am Mosq Control Assoc 18:107–110

Ngamo TSL, Goudoum A, Ngassoum MB, Mapongmestsem PM, Lognay G, Malaisse F, Hance T (2007) Chronic toxicity of essential oil of 3 local aromatic plants towards *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae). Afr J Agric Res 2:164–167

Noudjou F, Kouninki H, Ngamo LST, Maponmestsem PM, Ngassoum M, Hance T, Haubruge E, Malaisse F, Marlier M, Lognay GC (2007) Effect of site location and collecting period on the chemical composition of *Hyptis spicigera* Lam. an insecticidal essential oil from North-Cameroon. J Essent Oil Res 19:597–601

Okigbo RN, Okeke JJ, Madu NC (2010) Larvicidal effects of *Azadiracta indica*, *Ocimum gratissimum* and *Hyptis suaveolens* against mosquito larvae. J Agric Technol 6:703–719

Omolo MO, Okinyo D, Ndiege IO, Lwande W, Hassanali A (2004) Repellency of essential oils of some Kenyan plants against *Anopheles gambiae*. Phytochem 65:2797–2802

Othira JO, Onek LA, Deng LA, Omolo EO (2009) Insecticidal potency of *H. spicigera* preparations against *Sitophilus zeamais* (L.) and *Tribolium castaneum* (Herbst) on stored maize grains. Afr J Agric Res 4:187–192

Pålsson K, Jaenson TGT (1999a) Plant products used as a mosquito repellents in Guinea Bissau, West Africa. Act Trop 72:39–52

Pålsson K, Jaenson TGT (1999b) Comparison of plant products and pyretroid-treated bednets for protection against mosquitoes (Diptera: Culicidae) in Guinea-Bissau, West Africa. Medical Entomology 36:144–148

Park IK, Lee SG, Choi DH, Park JD, Ahn YJ (2003) Insecticidal activities of constituents identified in the essential oil from leaves of *Chamaecyparis obtusa* against *Callosobruchus chinensis* (L.) and *Sitophilus oryzae* (L.). J Stor Prod Res 39:375–384

Peerzada N (1997) Chemical composition of the essential oil of *Hyptis suaveolens*. Molecules 2:165–168

Peng Z, Yang J, Wang H, Simons FER (1999) Production and characterisation of monoclonal antibodies to two new mosquito *Aedes aegypti* salivary protein. Insect Biochem Mol Biol 29:909–914

Prajapati V, Tripathi AK, Aggrawal KK, Khanuja SPS (2005) Insecticidal, repellent and oviposition-deterrent activity of selected essential oils against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. Bioresour Technol 96:1749–1757

Pushpanathan T, Jebanesan A, Govindarajan M (2006) Larvicidal, ovicidal and repellent activities of *Cymbopogan citrates* Stapf

(Graminae) essential oil against the filarial mosquito *Culex quinquefasciatus* (Say) (Diptera: Culicidae). Tropical Biomed 23:208–212

Rahuman AA, Venkatesan P, Gopalakrishnan G (2008) Mosquito larvicidal activity of oleic and linoleic acids isolated from *Citrullus colocynthis* (Linn.) Schrad. Parasitol Res 103:1383–1390

Rajkumar S, Jebanesan A (2005) Repellency of volatile oils from *Moschosma polystachyum* and *Solanum xanthocarpum* against filarial vector *Culex quinquefasciatus* Say. Tropical Biomed 22:139–142

Schreck CE, Mc Govern TP (1989) Repellents and other personal protection strategies against *Aedes albopictus*. J Am Mosq Control Assoc 5:247–252

Severini C, Romi R, Marinucci M, Rajmond M (1993) Mechanism of insecticide resistance in field populations of *Culex pipiens* from Italy. J Am Mosq Control Assoc 9:164–168

Seyoum A, Kabiru EW, Lwande W, Killeen GF, Hassanali A, Knols BGJ (2002a) Repellency of live potted plants against *Anopheles gambiae* from human baits in semi-field experimental huts. Am J Tro Med Hyg 67:191–195

Seyoum A, Pålsson K, Kung'a S, Kabiru EW, Lwande W, Killeen GF, Hassanali A, Knots BGJ (2002b) Traditional use of mosquito-repellent plants in western Kenya and their evaluation in semi-field experimental huts against *Anopheles gambiae*: ethnobotanical studies and application by thermal expulsion and direct burning. Trans R Soc Trop Med Hyg 96:225–231

Silva WJ, Dória GAA, Maia RT, Nunes RS, Carvalho GA, Blank AF, Alves PB, Marçal RM, Cavalcanti SCH (2008) Effects of essential oils on *Aedes aegypti* larvae: alternatives to environmentally safe insecticides. Biores Technol 99:3251–3255

Sokal RR, Rohlf FJ (1981) Biometry. Freeman and Company, New York

Stenhagen E, Abrahamson S, McLafferty FW (1974) Registry of mass spectral data. Wiley, New York

Swigar AA, Silverstein RM (1981) Monoterpenes. Aldrich Chemical Company, Milwaukee

Tamprasit P, Indrapichate K (2004) Biological control of the yellow fever mosquito (*Aedes aegypti* Linn.) using leaf extract of chan (*Hyptis suaveolens* (L.) Poit) and hedge flower (*Lantana camara* Linn.). The 10th Word Congress on Clinical Nutrition, 30 November–3 December, Punket, Thailand. Abstract book p. 177

Tchoumbougang F, Amvam Zollo PH, Fecam Boyom F, Nyegue MA, Bessière JM (2005) Aromatic plants of Tropical Central Africa. XLVIII. Comparative study of the essential oils of four *Hyptis* species from Cameroon: *H. lanceolata* Poit., *H. pectinata* (L.) Poit., *H. spicigera* Lam. and *H. suaveolens* Poit. Flavour Fragr J 20:340–343

Wang JL, Li Y, Lei CL (2009) Evaluation of monoterpenes for the control of *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motschulsky. Nat Prod Res 23:1080–1088

WHO (1981) Instruction for determining the susceptibility or resistance of mosquito larvae to insecticide. WHO/VBC/81.807. Control of tropical diseases, World Health Organization, Geneva

WHO (2009) Guidelines for efficacy testing of mosquito repellents for human skin. WHO/HTM/NTD/WHOPES/2009.4. Control of neglected tropical diseases, World Health Organization, Geneva

Yang YC, Lee SG, Lee HK, Kim MK, Lee SH, Lee HS (2002) A piperidine amide extracted from *Piper longum* L. fruit shows activity against *Aedes aegypti* mosquito larvae. J Agric Food Chem 50:3765–3767