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Article *in* Journal of Medical Entomology · February 2017

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Journal of Medical Entomology, 2017, 1–7
doi: 10.1093/jme/tjw222
Research article

Repellent and Larvicidal Activity of the Essential Oil From *Eucalyptus nitens* Against *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae)

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Subject Editor: Roberto Barrera

Received 8 August 2016; Editorial decision 30 November 2017

Abstract

Dengue, chikungunya, and yellow fever are important vector-borne diseases transmitted by female mosquitoes when they feed on humans. The use of repellents based on natural products is an alternative for personal protection against these diseases. Application of chemicals with larvicidal activity is another strategy for controlling the mosquito population. The repellent and larvicidal activities of the essential oil from *Eucalyptus nitens* were tested against *Aedes aegypti* and *Aedes albopictus*, the main vectors of these arboviruses. The essential oil was extracted by hydrodistillation and then analyzed by gas chromatography–mass spectrometry. The main components of *Eucalyptus nitens* essential oil were found to be terpenes such as 1,8-cineole and *p*-cymene, followed by β-triketones and alkyl esters. The repellent activity of the essential oil against both species was significantly higher when compared with the main component, 1,8-cineole, alone. These results indicate that the repellent effect of *E. nitens* is not due only to the main component, 1,8-cineole, but also that other compounds may be responsible. *Aedes aegypti* was found to be more tolerant to the essential oil larvicidal effects than *Ae. albopictus* (*Ae. aegypti* $LC_{50} = 52.83$ ppm, *Ae. albopictus* $LC_{50} = 28.19$ ppm). The repellent and larvicidal activity could be associated to the presence of cyclic β-triketones such as flavesone, leptospermone, and isoleptospermone.

Key words: *Eucalyptus nitens*, repellent, larvicidal, *Aedes aegypti*, *Aedes albopictus*

Aedes aegypti and *Aedes albopictus* are the main vectors of several diseases that affect humans worldwide, such as dengue fever, dengue hemorrhagic fever, chikungunya, yellow fever, and Zika (Mangiafico 1971, Gubler 2002, WHO 2006a, Wong et al. 2013, Grard et al. 2014). No vaccines are available for the majority of these diseases; therefore, an essential strategy to address this problem is to control the vectors. Among the control alternatives proposed, the use of personal protection measures (like repellents) and the application of larvicides in breeding sites are the most widely accepted (WHO 2006b). The chemicals frequently used in mosquito control are temephos for larvicidal treatment in water containers (focal treatment) and *N*,*N*-diethyl-m-toluamide (DEET) as a personal repellent (Chavasse and Yap 1997). However, some vector populations have developed resistance to temephos, and DEET may exert toxic reactions under some circumstances and for some age groups; thus, alternative new products need to be explored (Snyder et al. 1986, WHO 1992, Osimitz and Murphy 1997, Abdel-Rahman et al. 2001, Briassoulis et al. 2001, Braga and Valle 2007, Melo-Santos et al. 2010, Polson et al. 2011).

Several substances of plant origin have been identified as bioactive, such as toxic, repellent, antifeedant, and/or growth and development-inhibiting potential, on arthropod pests (Coats 1994). Essential oils are particular plant products made up of volatile substances found in a variety of species (Weinzieri et al. 1994, Weinzieri 2000). The main characteristics of the essential oils are that they are easily extractable, ecofriendly and biodegradable; possess low toxicity against mammals; and are effective against wide spectra of insect pests (Tisserand and Balacs 1995; Isman 2000; Amer and Mehlhorn 2006a,b; Elango et al. 2009; Govindarajan et al. 2008; Michaelakis et al. 2009; Rahuman et al. 2009).

The amount of research focused on essential oils that could become suitable active substances for new botanical larvicides has been growing over the past few decades. Moreover, some commercial botanic products presented larvicidal effects (Xue et al. 2006). In a recent work, Pavela (2015) has shown that the essential oils with better lethal activity ($LC50 < 100$ ppm) against mosquitoes come from only five botanical families: Lamiaceae, Cupressaceae, Rutaceae, Apiaceae, and Myrtaceae. In particular, it is of interest to study *Eucalyptus spp*. (Myrtaceae) because they are cultivated over the world and others studies have shown that their oils have great larvicidal effect against *Ae. aegypti* (Zhu et al. 2006, Lucia et al.

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2007, Lucia et al. 2008, Cheng et al. 2009). However, the studies about larvicidal effects of *Eucalyptus spp*. against *Ae. albopictus* are not abundant, and they are reduced to the larvicidal effects of *Eucalyptus camaldulensis*, *Eucalyptus urophylla*, *Eucalyptus globulus*, and *Eucalyptus citriodora* (Yang and Ma 2005, Zhu et al. 2006, Zhu et al. 2008, Cheng et al. 2009).

The essential oils have been used therapeutically for centuries, and before DEET appeared, people used plant-derived materials to repel medically important arthropods, including mosquitoes (Curtis et al. 1990). Essential oils of large number of plants have been found to have repellent properties against mosquitoes (Hao et al. 2008, Mann and Kaufman 2012). Among the plant genus with promising essential oil properties to be used as repellent, *Cympogon spp*., *Ocimun spp*., and *Eucalyptus spp*. are the most studied (Nerio et al. 2010). However, only a few number of species showed repellent protection time similar to DEET (Barnard 1999, Gillij et al. 2008, Sritabutra and Soonwera 2013, Satpute et al. 2015, Tisgratog et al. 2016).

Eucalyptus nitens Maiden (shining gum) belongs to Victoria and New South Wales, Australia, and its resistance to low temperatures allows to cultivate it in the south of Argentina and Chile (FAO 1981, Prado and Barros 1989; Eldridge et al. 1994). In the past, other authors (Franich 1985, Li and Madden 1995) have reported the chemical composition of the essential oils of *E. nitens*; however, there is no recent information about their chemical components using modern techniques and nothing is known about their larvicidal or repellent effects. The aim of this study was to characterize the chemical composition of the essential oil of *E. nitens* and associate it with the larvicidal and repellent effects on *Ae. aegypti* and *Ae. albopictus*.

Materials and Methods

Plant Material and Essential Oil Extraction

One-year-old *E. nitens* plants were purchased from a forest tree nursery (Vivero Forestal Bosques Patagónicos, Bariloche, Argentina). Each plant was certified for quality and origin. Nine seedlings were planted in an experimental plot located in our research center in Buenos Aires, Argentina (34° 33′92.20″ S, 58° 30′ 95.20″ W). During summer (January, February, and March 2014), fresh leaves were harvested and dried at room temperature for 24 h. After that, samples of leaves (200 g) were hydrodistillated for 90 min using a modified Clevenger-type apparatus (Clevenger 1928). The essential oils were separated from water, dried over anhydrous sodium sulfate, and maintained under −4°C until use. The mean of the essential oil yield was estimated using the yield of each extraction calculated with the following formula: Yield (%) = mass of essential oil (g) × 100/mass of leaves (g). The essential oils were extracted in three independent distillations for each sampling procedure.

Essential Oil Composition: Analysis by Gas Chromatography–Mass Spectrometry

The essential oil samples were diluted in hexane (1 mg/ml) and analyzed in a Shimadzu GC-17A interfaced to a Shimadzu quadrupole mass spectrometer (GC-MS QP 5050A). Gas chromatography–mass spectrometry (GC-MS) analysis was performed using a DB-5MS column, where initial temperature was 60°C (3 min hold time), then increased to 100°C (2°C/min), and finally up to 240°C (7°C/min, hold time 3 min). Injector and detector temperatures were 270°C and 280°C, respectively. Helium was used as carrier gas, at a constant column pressure of 100 kPa and column flow of 1.6 ml/min. Analysis was also performed on a polar column DB-WAX. GC-MS program was: initial temperature was 40°C (3 min hold time), and then increased to a final temperature of 200°C (hold time 3 min). Column inlet pressure was set to 35 kPa (53.6 cm/seg) and column flow at 2.2 ml/min. Injector and detector temperatures were 240°C and 245°C, respectively. A split ratio of (1:30) was used for both analyses. The mass spectrums were recorded between 40 and 350 a.m.u., with ionization energy of 70 eV.

Each compound in the essential oil was identified by different methods: 1) comparing its mass spectrum obtained with those available in the database (Adams 1995; Wiley mass spectral database version 7, NIST web-book database) when the similarity index was ≥ 90%; 2) comparing linear retention index obtained in the sample by co-injection with a homologous series of n-alkanes (C_8-C_{23}, Sigma-Aldrich) with those obtained for standards compounds (Sigma-Aldrich); and 3) comparing the linear retention index with those reported in the bibliography for both columns used.

Chemicals

The following chemicals were purchased from Sigma-Aldrich (Argentina) and used as standards for essential oil analyses: (1R)-(+)-α-pinene (98%), (+)-camphene (80%), (1S)-(−)-β-pinene (98%), α-terpinene (85%), *p*-cymene (99%), (1R)-(+)-limonene (97%), 1,8-cineole (99%), γ-terpinene (97%), linalool (97%), (−)-borneol (90%), (S)-(+)-4-terpineol (96%), α-terpineol (99%), and caryophyllene oxide (99%).

Mosquito Strains and Rearing

Insecticide-susceptible strains of *Ae. albopictus* (USDA strain, Gainesville, FL, USA) and *Ae. aegypti* (Rockefeller strain, Venezuela) were used for the assays. The laboratory colony was kept in the laboratory, free of exposure to pathogens, insecticides, or repellents, at 25–27°C, 50–60% RH, and a photoperiod of 12:12 (L:D) h (Lucia et al. 2007, Gómez et al. 2011). All larval instars were fed on a mixture of rabbit pellets and yeast in a 3:1 proportion. Adult mosquitoes were fed on raisings, and a pigeon was offered three times a week for females to produce their eggs.

Repellent Activity

The repellent activity test was based on the WHO protocol (2009) with a few modifications (Gleiser et al. 2011). Non-blood-fed females (5–10-d-old) were used for the assays; these females were raised together with males, at least 5 d after pupae hatching, to ensure copulation and host-seeking behavior. The repellency assay was performed between 11:00 and 16:00 h in a polycarbonate cage (width 30 cm, height 30 cm, depth 38 cm) with a front circled window (diameter 15 cm) covered with a nylon stockinet; lateral sides made with aluminum net. A 30-cm^2 area of the forearm of a volunteer, delimited by a round-shaped paper mold, was treated with 100 µl of DEET, essential oil of *E. nitens*, or 1,8-cineole (20% in ethanol) solution. Also, ethanol was used as the control. The remaining area of the forearm was covered by a sleeve made of ethyl vinyl acetate, and the hand was protected by three latex gloves. Before each test, a control (only ethanol) was performed to ensure host-seeking behavior. If the number of mosquitoes landing (or probing) on the exposed area were more than 10 in a 1-min period, the test was continued. For the repellency tests, the forearm was introduced in the cage containing 100 female mosquitoes every 10 min for a 1-min period. The time in which two mosquitoes bit the exposed area or one bite was observed in each of two consecutive exposure periods was registered as total protection time (TPT; Schreck and McGovern 1989). Then, the duration, in minutes, of

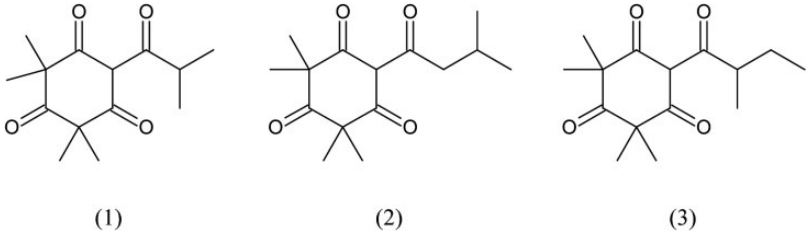

Fig. 1. Structures of cyclic β-triketones: (1) flavesone, (2) leptospermone, (3) isoleptospermone.

complete repellence was used to measure the repellent activity and expressed as TPT) (Trongtokit et al. 2005). Mosquitoes were blown up to prevent blood-feeding, in both control and treated groups. Each treatment was performed four times with different volunteers.

Larvicidal Activity

The larvicidal activity of the essential oil was analyzed by following the protocol previously used in our laboratory (Lucia et al. 2007). One milliliter of the essential oil solution was added to 199 ml of dechlorinated water in a 500-ml plastic jar and shaken for homogenization. Then, 50 ml of water containing 20 late third-instar or early four-instar larvae of *Ae. aegypti* or *Ae. albopictus* was added into the first jar. The stock solutions of the essential oil were prepared in acetone in different concentrations: 5, 7.5, 8.75, 10, 15, and 20 mg/ml (final concentrations 20, 30, 35, 40, 60, and 80 ppm, respectively). For *Ae. albopictus*, an extra solution of 8.75 mg/ml (final concentration 35 ppm) was included. Mortality of larvae was recorded after 24 h of exposure. Food was not supplied during the assay. Immobile larvae or those unable to raise to the surface were considered as dead. Five replicates were performed for each solution for both mosquito species. The assay was conducted in the same conditions used for rearing.

Statistical Analysis

To analyze differences in TPT, we performed a generalized linear model (GLM) with the function "Varident" to model the variance for each repellent, using species, the repellents, and their interaction as factors. Then, we used a posteriori Fisher's least significant difference (LSD) test to compare the differences of the means. A value of $P < 0.05$ was considered as statistically different. For larvicidal activity, a dose–response curve was obtained and then submitted to probit analysis to calculate 50 and 90% lethal concentration (LC_{50} and LC_{90}). The last parameters were expressed as parts per million in the final concentration (ppm), with a 95% confidence interval. These confidence intervals were used for comparing the susceptibility between the species and were considered statistically significant when the 95% confidence limits did not overlap. The software Infostat (Di Rienzo et al. 2014) and PoloPlus 2.0 were used for all statistical analyses.

Results and Discussion

Essential Oil Composition

The main components of the *E. nitens* essential oil were the monoterpenes 1,8-cineole (22.88%), *p*-cymene (22.46%), γ-terpinene (7.52%), and α-terpineol (2.42%). We also found three components identified as cyclic β-triketones (Fig. 1): flavesone (11.71%), leptospermone (5.94%), and isoleptospermone (1.77%). The group of cyclic β-triketones represents 19.42% of the essential oil composition. In a similar study, Li and Madden 1995 also reported the terpenes 1,8-cineole and *p*-cymene as main components of the *E. nitens* essential oil, but they did not find any cyclic β-triketone. In our case, the percentage of 1,8-cineole was low compared with other *Eucalyptus* species, and this could be related to the low value of the essential oil yield (Lucia et al. 2008; Table 1). So far, cyclic β-triketones were identified in essential oils of several species of the Myrtaceae family: *Eucalyptus, Leptospermum*, *Xanthostemon*, *Melaleuca*, and others (Hellyer 1968, Ghisalberti 1996, Van Klink et al. 1999). Flavesone was described in many *Eucalyptus* species, such as *Eucalyptus decorticans* (Bick et al. 1965, Hellyer 1968), *Eucalyptus grandis* (Batista-Pereira et al. 2006), and *Eucalyptus oblonga* (Hellyer 1968).

We also identified non-terpene esters such as isobutyl isobutyrate (6.47%) and isoamyl isobutyrate (4.99%) in the essential oil composition. There are several reports showing that these alkyl esters and other similar compounds are usually present in *Eucalyptus* essential oils: *E. grandis* (Menut et al. 1992), *Eucalyptus goniocalyx*, and *E. patens* (Menut et al. 1992, 1995).

Results from our work indicate that the essential oil of *E. nitens* has a complex chemical composition, with some monoterpenes, non-terpene esters, and cyclic β-triketones. Moreover, the presence of cyclic β-triketones such as flavesone, leptospermone, and isoleptospermone could be an important result to explain the following results about their repellent activity.

Repellent Activity

The DEET, the *E. nitens* essential oil, and its main component, 1,8-cineole, showed a repellent effect against both *Aedes* species (Fig. 2). The values of TPT of DEET ($TPT_{(min)}=196.67 \pm 41.77$ for *Ae. aegypti* and $TPT_{(min)}=196.67 \pm 86.22$ in the case of *Ae. albopictus*) were significant greater than those of the essential oil and the 1,8-cineole. The values of TPT of DEET against both mosquito species in our work were lower than in several studies (Barnard and Xue 2004, Lupi et al. 2013). Nevertheless, Frances et al. (1993, 2009) determined the TPT of DEET in *Ae. aegypti* and *Ae. albopictus* and reported similar results to those found in our work, where the probability to be stung was similar, as they used 100 females in the cage.

We found a statistical difference for the *E. nitens* essential oil against *Ae. aegypti* ($TPT_{(min)}=97.50 \pm 10.31$) in comparison with 1,8-cineole ($TPT_{(min)}=5.00 \pm 2.89$). Similar results were obtained for *Ae. albopictus* with $TPT_{(min)} = 87.50 \pm 16.52$ for the essential oil and $TPT_{(min)} = 12.50 \pm 2.50$ for 1,8-cineole. These results allows us to assume that the repellent effect of *E. nitens* is not due to the high amount of 1,8-cineole and that other compounds may be responsible for it. In other *Eucalyptus* species studied in our laboratory, it was demonstrated that essential oils with high amount of 1,8-cineole and *p*-cymene, such as in *Eucalyptus tereticornis*, showed similar TPT in comparison with 1,8-cineole alone (Naspi et al., unpublished data). The repellent effect against *Ae. aegypti* was previously demonstrated for 1,8-cineole using a 10% solution

Table 1. Chemical composition (%) of *E. nitens* essential oil analyzed by GC-MS

No.	Component	LRI		Identification	Percentage (%)[d]
		DB-5MS[a]	DB-WAX[b]	Methods[c]	
1	Isobutyl propionate	860	1,076	A,C	0.22
2	Isobutyl isobutyrate	912	1,090	A,C	6.47
3	α-pinene	929	1,012	A,B,C	0.11
4	Isobutyl methacrylate	933	1,160	A,C	0.32
5	Isoamyl propionate	965	1,174	A,C	0.24
6	α-phellandrene	1,005	1,152	A,C	<0.1
7	Isobutyl Isovalerate	1,007	1,293	A,C	0.16
8	Isoamyl isobutyrate	1012	1,193	A,C	4.99
9	Isopentyl isobutanoate	1,014	1,195	A,C	0.99
10	*p*-cymene	1,023	1,256	A,B,C	22.46
11	Limonene	1,026	1,188	A,B,C	1.12
12	1.8-cineole	1,030	1,198	A,B,C	22.88
13	*cis*-β-ocymene	1,033	1,229	A,C	0.80
14	γ-terpinene	1,054	1,235	A,B,C	7.52
15	Linalool	1,099	1,554	A,B,C	0.35
16	2-nonanol	1,103	1,528	A,C	0.20
17	4-terpineol	1,176	1,602	A,B,C	1.62
18	α-terpineol	1,192	1,703	A,B,C	2.42
19	Geraniol	1,255	1,840	A,B,C	0.28
20	*iso*-carvacrol	1,285	2,222	A,C	0.75
21	Thymol	1,293	2,173	A,B,C	0.61
22	Benzyl isobutyrate	1,295	1,789	A,C	0.31
23	Carvacrol	1,298	2,209	A,B,C	0.61
24	Phenyl Ethyl Isobutyrate	1,392	1,867	A,C	0.28
25	Flavesone	1,539	1,971	A,C	11.71
26	Spathulenol	1,578	2,106	A,C	0.53
27	*iso*-leptospermone	1,612	2,043	A,C	1.77
28	Leptospermone	1,623	2,060	A,C	5.94
29	α-eudesmol	1,660	2,240	A,C	0.28
30	β-eudesmol	1,660	2,250	A,C	0.30
		Monoterpene hydrocarbons	32.01		
		Oxygenated monoterpenes	29.52		
		Sesquiterpene hydrocarbons	–		
		Oxygenated sesquiterpenes	0.81		
		Non-terpenoid compounds (esters and alcohols)	14.18		
		Cyclic β-triketones	19.42		
		Total (%)	95.94		
		Nonidentified compounds (%)	4.06		
		Essential oil yield (%)[d]	0.14 ± 0.01		

LRI = Linear retention index in:

[a] DB-5MS

[b] DB-WAX

[c] Identification methods: (A) comparison of mass spectrum with bibliography. Comparison of LRI with authentic standard (B) and with those of bibliography (C) for both columns.

[d] Yield mean of tree extraction replicates.

and the effect lasted 16 min (Klocke et al. 1987). Thus, various essential oils with a high content of 1,8-cineole showed a repellent effect against *Ae. aegypti* (Gillij et al. 2008) and *Culex pipiens pallens* (Choi et al. 2002), but the effect did not last for more than 30 min. The low persistence of the repellent effect observed for monoterpenes could be explained by their high volatility and vapor pressure (Gleiser et al. 2011), meaning that the effect is lost as time goes by. Sesquiterpenes are also active as repellents against mosquitoes, with the advantage of having less volatility and long-lasting effect (Paluch et al. 2009; Garcia-Domenech et al. 2010). However, the *E. nitens* essential oil has low quantities of sesquiterpenes (<0.81%). Oxygenated compounds play an important role in the repellent effect of terpenes: those molecules with two substituents, such as hydroxyl group, esters, and ethers, have been found to be good repellents against mosquitoes (Wang et al. 2008). However, these oxygenated terpenes are present in low concentration in the essential oil of *E. nitens*.

Results obtained so far show that the good repellent activity of the *E. nitens* essential oil could be also attributed to the presence of non-terpenoid ketones and esters compounds, as described by Conti et al. (2013) and Jaleta et al. (2016). To date, the repellency of essentials oils with a high content of these ketones and esters has not been studied for mosquitoes. Owing to their structure, with a great number of oxygenated substituents, they may also be contributing to the repellent effect of the essential oil. All these findings may suggest that the repellent activity of the essential oil of *E. nitens* is due to esters partially (isobutyl isobutyrate and isoamyl isobutyrate) and the high content of β-triketones mainly.

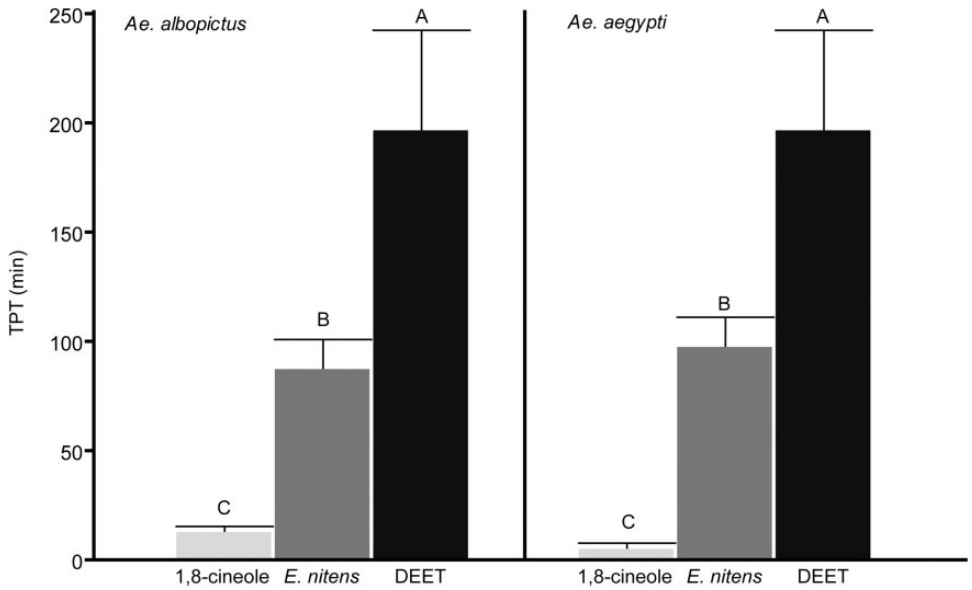

Fig. 2. Repellent activity of DEET, *E. nitens* essential oil, and 1,8-cineole against *Ae. aegypti* and *Ae. albopictus*. Each bar represents the mean TPT + standard error estimated (in minutes). Same letters are not significantly different ($P \geq 0.05$), analyzed by GLM and Fisher's LSD test.

Table 2. Larvicidal activity of *E. nitens* essential oil against *Ae. aegypti* and *Ae. albopictus*

Mosquito specie	Value (ppm)	*n*	95% CI (LCL–UCL)	Slope ± SD	Chi-square
Aedes albopictus	28.19	278	25.61–30.66	5.84 ± 0.73	18.10
Aedes aegypti	52.83	280	47.92–59.80	6.35 ± 0.63	20.16

LC_{50} = lethal concentration that kills 50% of larvae, IC = confidence interval at 95%, LCL = lower confidence limit, UCL = upper confidence limit.

Larvicidal Activity

Regarding the larvicidal activity of the *E. nitens* essential oil, the results showed greater toxicity against *Ae. albopictus* than *Ae. aegypti* larvae (Table 2), with LC_{50} values of 28.19 and 52.83 ppm, respectively. Larval mortality was dose-dependent for both mosquito species. Similar variations in the sensitivities of mosquito species to essential oils or plant extracts were demonstrated, and these differences could be attributed to physiological differences between both species (Amer and Mehlhorn 2006b). The toxicity of several monoterpenes such as γ-terpinene, *p*-cymene, limonene, and α-terpineol that are present in the *E. nitens* essential oil was previously evaluated for *Ae. aegypti* (Park et al. 2011, Lucia et al. 2013) and *Ae. albopictus* (Cheng et al. 2009, Giatropoulos et al. 2012) and may be responsible for the larvicidal activity. The larvicidal activity of many essential oils with high and low concentration of 1,8-cineole was reported in *Ae. aegypti* (Lucia et al. 2007, 2008, 2013), *Ae. albopictus* (Conti et al. 2010), and other mosquitoes species (Traboulsi et al. 2005). The hydrophobicity of the compounds is considered to be an important property, and it is strongly associated with the toxicity against mosquito larvae (Fahmy et al. 1978). Those molecules with greater hydrophobicity are more capable of penetrating the hydrophobic cuticle of mosquito larva (Welling and Paterson 1985). In addition, the essential oil of *Ruta chalepensis*, rich in long-chain ketones (C_9 and C_{11}) and an ester (C_{11}), showed similar values of LC_{50} against *Ae. albopictus* larvae (Conti et al. 2013). This suggests that the esters and triketones found in *E. nitens* also may be involved in larvae toxicity.

This is the first study showing the repellent activity of an essential oil with high content of cyclic β-triketones such as flavesone, leptospermone, and isoleptospermone against *Ae. aegypti* and *Ae. albopictus*. The repellency and larvicidal activity found in our work could be associated to the presence of these cyclic β-triketones in the *E. nitens* essential oil. This effect is supported by several works where the biological activities of β-triketones have been evaluated. Several activities such as herbicide activity (Dayan et al. 2007, Owens et al. 2013), antibacterial activity (Christoph et al. 2001), virucidal activity (Reichling et al. 2005), acaricidal activity (Jeong et al. 2009), and antitumoral activity (Riley 1994, Porter 2001) have been recognized. These triketones have also been reported for cosmetic use (The Good Scents Company 2016). In the search for new molecules of natural origin with repellent activity, these compounds could be good candidates or potential alternatives as repellents against mosquitoes. Because of this, we believe that further works are necessary to evaluate the repellent activity of the pure cyclic β-triketones on mosquitoes.

Acknowledgments

This work was supported by project PICT 2012 – 1857 from the National Agency for Science and Technology Promotion (ANPCyT-Argentina) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). A.L. and H.M. are members of the CONICET. Dr L. Harburguer ((Centro de Investigaciones de Plagas e Insecticidas); CIPEIN-UNIDEF (UNIDAD DE INVESTIGACION Y DESARROLLO ESTRATEGICO PARA LA DEFENSA)-CONICET) provided constructive suggestions and criticisms for English and grammar on an earlier version of this manuscript. We also would like to thank two anonymous reviewers for their valuable comments and suggestions, which helped improve the quality of this paper.

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