Revista Brasileira de Farmacognosia 29 (2019) 778–784

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

A herbal oil in water nano-emulsion prepared through an ecofriendly approach affects two tropical disease vectors

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ARTICLE INFO

Article history:
Received 9 January 2019
Accepted 1 May 2019
Available online 4 July 2019

Keywords:
Colloidal system
Droplet size distribution
Miniemulsion
Larvicide

ABSTRACT

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson, Verbenaceae, is considered a great source of a bioactive volatile oil. Due to the wide range of known chemotypes, its chemical analysis is very important. Among the several activities of this volatile oil, a potential larvicidal action against Culicidae species is highlighted. However, the low water miscibility of volatile oils limits their application in aqueous media. Oil in water nano-emulsions are in the spotlight of novelty to solve this main problem. Thus, the aim of the present study was to obtain this nanostructured system with *L. alba* volatile oil (citral chemotype) and evaluate its larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus* larvae. The major compounds were geranial (30.02%) and neral (25.26%). Low mean droplet size (117.0 ± 1.0 nm) and low polydispersity index (0.231 ± 0.004) were observed and no major changes were observed after seven days of storage. LC_{50} values against *C. quinquefasciatus* and *A. aegypti* third-instar larvae were respectively 38.22 and 31.02 ppm, while LC_{90} values were, respectively, 59.42 and 47.19 ppm. The present study makes use of a low energy, solvent-free and ecofriendly method with reduced costs. Thus, this paper contributes significantly to phyto-nanobiotechnology of larvicidal agents, opening perspectives for the utilization of *L. alba* volatile oil in integrated practices of vector control.

Introduction

Tropical neglected and emergent diseases transmitted by mosquito vectors are a main problem in developing countries. *Aedes aegypti* develop a main role in some arboviruses, including dengue, chikungunya fever, zika and urban yellow fever (Consoli and Oliveira, 1994; Kucharz and Cebula-Byrska, 2012; Lima-Camara, 2016). In case of dengue, a remarkable public health problem is observed in the last years in several tropical and subtropical countries, being around 3.9 billion people worldwide in risk, distributed in more than 128 countries (WHO, 2018). Estimates indicate around 390 million cases per year and 96 million with clinical signs (Bhatt et al., 2013). Another vector is the mosquito *Culex quinquefasciatus*, which is related to the transmission of lymphatic filariasis. Approximately 1 billion of people are in risk in 72 countries including at least 36 million people disfigured and incapacited by the disease (WHO, 2016; Ramaiah and Ottesen, 2014). It is estimated that, in India, the loss due to lymphatic filariasis is around $1 billion per year due to treatment costs and lost productivity (Norris et al., 2012). Moreover, emerging tropical diseases which can be spread by mosquitoes may turn public health problem in some regions. This problem is highlighted by the recent cases of zika virus in Brazil (Zanluca et al., 2015). However, the risk of occurrence of these diseases in developed countries, including those from Europe should also be considered (Tappe et al., 2014). Zika virus infection and related clusters of cases of microcephaly and other neurological disorders, especially in newborns, are an emerging public health issue of international concern (ECDC, 2016).

Despite the synthetic insecticides have been currently used for pest management, they are associated to several disadvantages, such environmental toxicity and insect resistance. Moreover, the toxicity of these products to non-target organism turns the research

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https://doi.org/10.1016/j.bjp.2019.05.003

aiming to generate green-ecofriendly pesticides very promising (Tavares et al., 2009). The plant based insecticides, including volatile oils, are considered alternatives to conventional synthetic compounds (Czaja et al., 2014; Isman, 2015). These complex volatile mixtures or their isolated compounds are especially interesting in vector-borne disease control programs (Dias and Moraes, 2014), being considered potential more environmentally safe insecticides (Silva et al., 2008). Despite some toxicity to aquatic plankton or small vertebrates may occur, it depends on the concentration and time of exposure. Therefore, considering that flow water is expected on water reservoirs, consequent dilution and biodegradation should minimize this possibility (Pavela, 2015).

Some plant species are remarkable volatile oil producers, such *Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson, Verbenaceae. Ethnopharmacological indications of this species include its utilization as calmative, for treatment of indigestion, stomachache, diarrhea, intestinal problem and inappetence (Cartaxo et al., 2010). It belongs to the Verbenaceae family and it is recognized by a large number of chemotypes, evidenced by various volatile oil phytochemical profiles (Hennebelle et al., 2008). A significant correlation between P450-inhibition and repellent activity of some volatile oils against *A. aegypti* was observed, including the one obtained from *L. alba* volatile oil (Ramirez et al., 2012). Adulticidal (Muñoz et al., 2014) and larvicidal (Muñoz et al., 2014; Vera et al., 2014) evaluation of the *L. alba* volatile oil were also carried out. This last approach has been considered a main strategy for novel larvicidal products against disease vectors. However, despite the great advantages of volatile oils as source of bioactive compounds, the intrinsic immiscibility in water and loss due to evaporation of volatiles are still main concerns. On this context, nanostructured systems, such nanoparticles and nano-emulsions, are a great alternative to solve these main problems (Flores et al., 2011).

Nanotechnology is an emerging area for novel products in different industrial segments due to the ability that nanostructured systems have to modify properties of the bulk material. Nanoformulations containing botanical insecticides have great potential due to several advantages that can be achieved by the nano-scale, including enhancement of physical stability, protection against chemical degradation, controlled release, better water-solubility and even reduction of evaporation loss, which is a special advantage if an volatile oil is used (Oliveira et al., 2014; Kah and Hofmann, 2014). Oil in water nano-emulsions are thermodynamically unstable dispersed systems of fine spherical droplets of immiscible oil in water. These colloids are often stabilized by surfactants, which develop a main role in the kinetic stability of the nano-emulsions during storage (McClements, 2012). The aqueous external phase allows the dilution of the oil in water nano-emulsions, allowing a great advantage regarding the availability of natural oils in water for larvicidal purposes against mosquito larvae (Rodrigues et al., 2014; Duarte et al., 2015; Oliveira et al., 2016, 2017). The nano-size range of nano-emulsion droplets is still subject of discussion. Often, the accepted definitions indicate that it is related to droplets with mean diameter around 20 to 100–500 nm, where the upper limit varies according to different authors (Solans and Solè, 2012). Nano-emulsions, also called miniemulsions, can be obtained by high-energy methods and low energy methods. The first group makes use of devices that provide a high input of energy in order to reduce the droplet size, including high-pressure homogenizers, sonication or high-shear stirrers. However, this approach often elevates costs of the process and turn more difficult the practical application. On another hand, low energy methods include the change in the surfactant spontaneous curvature (phase inversion). The phase inversion methods are phase inversion temperature (PIT), which is performed under constant composition and changing temperature, and phase inversion composition (PIC), which is performed under constant temperature and changing composition. A self-emulsification (SE), also called spontaneous emulsification, without any change in the spontaneous curvature of the surfactant can also induce the formation of nano-droplets, being sometimes erroneously included as part of low energy methods (Solans and Solè, 2012).

One would expect a step of evaporation, often under reduced pressure, if a volatile solvent, that is able to rapidly diffuse to the external phase, is used during the SE. On this context, some loss of volatile oil components may occur. Regarding the PIT, the intrinsic increment of temperature is a critical point that may also induce loss of the volatiles from the volatile oil. Thus, the PIC can be considered the optimal method for nano-emulsification of volatile oils, since the change in the spontaneous curvature is triggered at constant temperature, being reached even at room temperature and avoiding the loss of volatile oil during the process. These low energy methods make possible to achieve fine droplets using simple stirring equipment and therefore, make possible to obtain these novel delivery systems using a low cost approach which can be easily spread for practical applications.

To our knowledge, most of the studies aiming to generate larvicidal aqueous nano-emulsions with bioactive volatile oils still focus on high energy methods. On this context, the present paper aims to prepare for the first time a nano-emulsion with *L. alba* by a simple titration method and evaluate its larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus* larvae.

Materials and methods

Plant material

Leaves of *Lippia alba* (Mill.) N.E.Br. ex Britton & P.Wilson, Verbenaceae, were obtained from twenty individuals, randomly chosen, at the Medicinal Garden of São Raimundo Community, in the municipality of Santarém (Pará, Brazil) (S 02^0 33′ 28.0″ e W 05^0 54′ 19.8″), in the year 2015. The plant identification was performed by Dra. Fátima Regina Gonçalves Salimena and a voucher sample was deposited under the register number CESJ 65276 at the Juiz de Fora Federal University, Brazil.

Extraction of volatile oil

The leaves were dried at 40 °C using a forced air oven (Solab Mod 102/336, Piracicaba, SP, Brazil) and kept protected from the light under room temperature until the extraction of the volatile oil, which was obtained by hydrodistillation using a Clevenger type apparatus (Ming et al., 1996). After water boiling, the time of extraction was 120 min. The extraction was performed in triplicate and each replicate was carried out using 100 g of dried leaves. After the extraction, samples containing volatile oil were centrifuged with anhydrous sodium sulphate in order to remove residual water and stored in amber glass vials at 5 °C. The volatile oil yield was calculated according to Santos et al. (2004) and it is related to the dry plant material used in the extraction. The relative density was determined according to the Brazilian Pharmacopeia (Anvisa, 2010) using a pycnometer.

Gas-chromatograph analysis of the volatile oil

The qualitative and quantitative analyses of the volatile oil were carried out using a GCMS-QP2010 Ultra (Shimadzu Corporation, Tokyo, Japan). The qualitative analysis was performed using a gas-chromatograph coupled to mass spectrometer mode with auto injector AOC-20i and CGMS Solution software that includes the compounds library from Willey, NIST, ADAMS and FFNSC 2. Rxi-5 ms fused-silica capillary column (Restek Corporation, Bellefonte, PA) with 30 m (length) × 0.25 mm (inner diameter) × 0.25 μm (film

Table 1
Chemical composition of volatile oil from leaves of *Lippia alba* extracted by hydro distillation. Relative abundance of each volatile compound is expressed as percentage in the volatile oil.

Compound	Rt (min)	RI (Calc)	RI (Lit)	%
2*E*-Hexenal	4.045	846	846	0.06
α-Thujene	5.700	926	924	0.10
Benzaldehyde	6.575	958	952	0.04
Sabinene	6.985	973	969	0.35
3,5-Dimethyl-4-heptanone	7.065	975	973	0.04
6-Methyl-5-hepten-2-one	7.300	984	981	1.40
Myrcene	7.465	990	988	0.31
α-Phellandrene	7.935	1005	1002	0.10
Isoamylisobutyrate	8.265	1014	1007	0.03
α-Terpinene	8.340	1016	1014	0.18
p-Cymene	8.605	1024	1020	1.35
Limonene	8.785	1029	1024	9.11
1,8-Cineole	8.865	1031	1026	0.04
(*E*)-β Ocimene	9.410	1046	1044	0.45
Bergamal	9.835	1058	1051	3.13
cis-Sabinene hydrate (IPP vs OH)	10.130	1066	1065	0.08
Terpinolene	10.955	1088	1086	0.04
NI	11.260	1097		0.08
Linalool	11.355	1099	1095	0.95
α-Pinene oxide	11.560	1104	1099	0.12
NI	12.965	1138		0.15
Geijerene	13.115	1142	1138	0.12
exo-Isocitral	13.180	1143	1140	0.26
trans-α-Necrodol	13.400	1149	1144	0.25
Citronellal	13.525	1152	1148	0.34
Z-Isocitral	14.005	1163	1160	1.10
Borneol	14.085	1165	1165	0.10
Borneol	14.460	1174	1165	0.06
Terpinen-4-ol	14.570	1177	1174	0.27
NI	14.655	1179		0.07
E-Isocitral	14.775	1182	1177	1.51
NI	15.280	1194		0.23
cis-9 Piperitol	15.535	1200	1195	0.05
trans-Carveol	16.325	1218	1215	0.05
Citronellol	16.760	1229	1223	1.42
cis-*p*-Mentha-1(7),8-dien-2-ol	17.010	1234	1227	0.14
Neral	17.410	1244	1235	25.26
Carvone	17.470	1245	1239	0.07
Geraniol	17.870	1254	1249	0.17
Geranial	18.720	1274	1264	30.02
Citronellyl acetate	22.070	1353	1350	0.04
Eugenol	22.215	1357	1356	0.13
Neryl acetate	22.540	1364	1359	0.11
α-Copaene	23.065	1377	1374	0.13
Geranyl acetate	23.350	1383	1379	0.35
β-Bourbonene	23.445	1386	1382	0.11
β-Cubebene	23.665	1391	1387	0.31
β-Elemene	23.740	1393	1389	0.30
Sesquithujene	24.310	1406	1405	0.16
α-Cedrene	24.580	1413	1410	0.17
α-Funebrene	24.650	1415	1413	0.06
E-Caryophyllene	24.875	1420	1417	0.43
β-Copaene	25.275	1430	1430	0.14
α-Humulene	26.265	1454	1452	0.10
E-β-Farnesene	26.390	1457	1454	0.15
allo-Aromadendrene	26.565	1462	1458	0.20
γ-Muurolene	27.405	1482	1478	4.33
γ-Amorphene	27.945	1496	1495	1.14
α-Muurolene	28.165	1501	1500	0.19
Z-α-Bisabolene	28.480	1509	1506	0.13
Geranyl isobutanoate	28.650	1514	1514	0.19
NI	28.745	1516		0.28
7-*epi*-α-Selinene	28.960	1521	1520	0.11
δ-Cadinene	29.075	1524	1522	0.45
cis-Calamenene	29.535	1536	1528	0.09
α-Copaen-11-ol	29.735	1541	1539	0.06
Elemol	30.090	1551	1548	5.24
E-Nerolidol	30.605	1564	1561	0.71
Globulol	31.950	1598	1590	0.66
1,10-di-*epi*-Cubenol	32.795	1621	1618	0.05
Eremoligenol	33.130	1630	1629	0.11
γ-Eudesmol	33.220	1632	1632	0.39
Hinesol	33.755	1647	1640	0.05
β-Eudesmol	33.910	1651	1649	0.54
α-Eudesmol	34.015	1654	1652	0.52
Bulnesol	34.815	1675	1670	0.05
Germacra-4(15),5,10(14)-trien-1-alpha-ol	35.220	1686	1685	0.13
Z-α-*trans*-Bergamotols	35.615	1697	1690	0.26
Curcumenol	37.055	1738	1733	0.14
Total Identified				98.31

Rt, retention time; Rt (calc), retention index experimentally calculated; Rt (lit), retention index from literature; NI, not identified.

thickness). The analysis conditions were as follows: injector temperature, 250 °C; carrier gas, helium (99.995%); oven temperature, 60–240 °C at a rate of 3 °C/min; helium (99.995%); flow rate, 1 ml min^{-1}); split injection with split ratio of 1:20; volume of injection, 1 μl of volatile oil hexane solution (1%, v/v); ionization by electron impact, 70 eV; temperature of ion source, 200 °C; transference line, 250 °C. The mass spectra were acquired using an automatic scan of 0.3 s and mass fragments at *m/z* 35–400 were recorded. The retention indices were calculated by the interpolation of the retention times of volatile compounds to the retention times of a homologous series of C8-C20 *n*-alkanes (Sigma–Aldrich), according to the Van den Dool and Kratz linear equation (Van den Dool and Kratz, 1963). The identification of the compounds was performed by comparison of their retention indices and mass spectra (molecular mass and fragmentation pattern) to the aforementioned libraries (Adams, 2007; NIST, 2011; Mondello, 2011). The quantitative analysis was carried out using a gas chromatograph coupled to flame ionization detector (FID) mode at the same conditions of GCMS, except by the carrier gas, which was hydrogen on the GCFID.

Preparation and characterization of nano-emulsions

The nano-emulsions containing volatile oil of *L. alba* were prepared by a low energy titration method (Ostertag et al., 2012). Formulations were constituted by 90% (w/w) of water, 5% (w/w) of *L. alba* volatile oil and 5% (w/w) of polysorbate 80 at a final mass of 25 g. The volatile oil and surfactant (s) were pooled together and stirred for 30 min at 800 rpm using a magnetic stirrer (Fisatom, Brazil). After this period, water was added at a controlled flow rate (3.5 ± 5 ml/min) and the system was stirred for 60 min. The nano-emulsion was used for larvicidal assay immediately after preparation. It was also characterized by dynamic light scattering (Zetasizer ZS, Malvern, UK) after storage under room temperature (25 ± 2 °C) and protected from light. Prior to the droplet size and polydispersity index (PDI) measurements, the nano-emulsion was diluted in deionized water (1:25). Results represent measurements performed in triplicate and are expressed as mean ± SD.

Larvicidal assay

Aedes aegypti (Rockefeller strain) and *Culex quinquefasciatus* third instar larvae were obtained from the Arthropoda Laboratory (Universidade Federal do Amapá, Brazil). The biological assay was performed under controlled conditions, being larvae kept at 25 ± 2 °C, relative humidity of 75 ± 5% and a 12 h light:dark cycle. The experimental evaluation was performed according to the World Health Organization protocol (WHO, 2005) with some modifications. All the experiments were performed in triplicate with ten third-instar larvae in each sample. The nano-emulsion was diluted in distilled water and the experimental concentrations were expressed as the *L. alba* volatile oil content in aqueous media. The control group was constituted by deionized water. Mortality levels were recorded after 24 and 48 h of exposure. The correction of mortality levels were performed if mortality in control group was

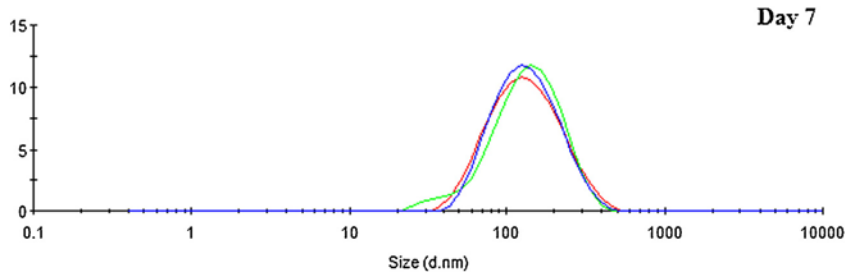

Fig. 1. Droplet size distribution of nanoemulsion prepared with *Lippia alba* volatile oil (HLB 15). Day 1: Size – 117.0 ± 1.0 nm; Pdi: 0.231 ± 0.004. Day 7: Size: 116.2 ± 0.3 nm; Pdi: 0.205 ± 0.001.

Table 2
Physicochemical properties of major compounds (>3%) from *Lippia alba* volatile oil. Data obtained from www.chemspider.com.

Compound	LogP*	Water solubility (25 °C, mg/l)**	Surface tension (dyne/cm)*	%
Limonene	4.45	4.581	25.9 ± 3.0	9.11
Bergamal	3.00	212.0	26.0 ± 3.0	3.13
Neral (citral b)	3.17	84.71	27.1 ± 3.0	25.26
Geranial	3.17	84.71	27.1 ± 3.0	30.2
Muurolene<gamma>	6.55	0.05378	29.2 ± 5.0	4.33
Elemol	4.75	1.99	35.3 ± 3.0	5.24

* ChemSpider - (Predicted - ACD/Labs - Percepta Platform - PhysChem Module).
** ChemSpider (Predicted - EPIsuite™ - Estimated from Log Kow).

between 5 and 20%, according to the Abbott's formula as follows: Mortality (%) = 100 (X – Y)/X, where X is the percentage of survival in the untreated control group and Y is the percentage of survival in the treated groups.

Statistical analysis

The statistical analysis was carried out using the software GraphPad Prism 6.0 (San Diego, California, USA). The paired t-test was used for comparison of droplet size after storage. Probit analysis was performed with 95% confidence interval for LC_{50} and LC_{90} determination using the software Statgraphics Centurion XV version 15.2.11.

Results and discussion

The extraction of *L. alba* yielded 2.2 ± 0.23% (v/w) of volatile oil, which presented a yellow color with citric scent. The relative density of the volatile oil was 0.82 ± 0.03 g/ml at 25 °C. The phytochemical profile obtained by gas-chromatographic analysis revealed the presence of eighty compounds that corresponded to 98.31% of the volatile oil, being 74 of them properly identified (Table 1). The major compounds were geranial (30.02%) and neral (25.26%), followed by limonene (9.11%). Citral is constituted by the two isomers geranial and neral (Dewick, 2009) and these compounds are used to distinguish a well established chemotype of *L. alba* (Pascual et al., 2001). The monoterpenes are bioactive compounds constituted by two isoprene units with a wide range of functionalization and some of them were previous reported as larvicidal agents. Limonene presented a LC_{50} of 37.0 ± 2.08 ppm against third to fourth *A. aegypti* larvae (Silva et al., 2008).

Fig. 1 shows the droplet size distribution, obtained through dynamic light scattering, of the nano-emulsion prepared with *L. alba* volatile oil. It revealed a low mean droplet size (117.0 ± 1.0 nm) and low polydispersity index (0.231 ± 0.004). No significant statistical difference was observed for droplet size (p = 0.3783), suggesting kinetic stability during 7 days of storage (size: 116.2 ± 0.3 nm; PDI: 0.205 ± 0.001). The main mechanism involved in the destabilization of nano-emulsions is the Ostwald ripening, which occurs when some component of the oily dispersed phase has some degree of solubility in the external phase and led to increase in droplet size (Tadros et al., 2004). However, the absence of differences in the results obtained in the present study suggest that a desired particle size distribution was maintained, suggesting the physical stability of the *L. alba* volatile oil nano-emulsion during this period and protection against the Ostwald ripening. This is a great advantage, since it would be stable in field during the expected larvicidal activity period.

Rao and McClements (2012) investigated the effects of *in silico* physicochemical parameters on the stabilization of nano-emulsions prepared with lemon oil. According to these authors, the presence of some compounds with high logP and low water solubility would lead to better stabilization, in contrast to Ostwald ripening, through a mechanism called "compositional ripening". Table 2 summarizes some of the physicochemical characteristics of the major compounds (>3%) of *L. alba* volatile oil used in the present study. In fact, the utilization of lipophilic components added to the internal phase of nano-emulsions is a well-known strategy to enhance the stability of the colloid. Therefore, it is fascinating that the intrinsic characteristic of an volatile oil, with compounds differing regarding the physicochemical parameters, may enhance itself the stability of the formulation, avoiding any extra adjuvant and contributing to a more "green nano-emulsion".

The titration method that was used in the present study is probably associated to a phase inversion composition process to produce the observed fine droplets. A previous study that was carried out with orange oil, which has the botanical insecticide limonene as main constituent, or limonene itself, indicated that these nano-emulsions presented mean droplet size around 260 nm or 340 nm, respectively. The concentration of the non-ionic surfactant polysorbate 80 was 2.5 times greater than the oil concentration (10% of oil and 25% of surfactant) (Ostertag et al., 2012). Considering that we achieved even lower mean droplet size using an equal amount of surfactant (5% of polysorbate 80), when compared to the oil concentration (5% of *L. alba* volatile oil), this should be considered an advantage, since it reduces the costs associated to energy with no impairment to nanosize and nano-emulsion stability. Considering not only the cost, but the ecofriendly approach of the nano-emulsion prepared with a biodegradable volatile oil and non-ionic surfactants, the absence of any utilization of organic solvent should also be considered an advantage.

Table 3
Mortality levels (% ±SD) after treatment of *Culex quinquefasciatus* third-instar larvae with oil in water nanoemulsion prepared with *Lippia alba* volatile oil.

	Control	2.5 ppm	10 ppm	25 ppm	50 ppm	100 ppm	200 ppm
24 h	3.33 ± 5.7	0 ± 0	0 ± 0	13.33 ± 11.5	46.67 ± 15.3	83.3 ± 15.3	100 ± 0
48 h	3.33 ± 5.7	0 ± 0	3.33 ± 5.7	20 ± 20	76.67 ± 5.8	100 ± 0	100 ± 0

Table 4
Mortality levels (% ±SD) after treatment of *Aedes aegypti* third-instar larvae with oil in water nanoemulsion prepared with *Lippia alba* volatile oil.

	Control	2.5 ppm	25 ppm	50 ppm	100 ppm	200 ppm
24 h	3.33 ± 5.7	3.33 ± 5.7	13.33 ± 15.3	93.33 ± 5.8	100 ± 0	100 ± 0
48 h	6.66 ± 5.8	13.33 ± 5.8	20 ± 17.3	100 ± 0	100 ± 0	100 ± 0

Table 3 shows the influence of the nano-emulsion prepared with *L. alba* volatile oil on *C. quinquefasciatus* third-instar larvae. Low mortality was observed on the control group (3.33 ± 5.7%), being attributable to a spontaneous mortality that may occur during the experiment. No mortality was observed on the group treated with the nano-emulsion at 2.5 ppm (24 and 48 h) neither in the group treated with nano-emulsion at 10 ppm after 24 h of treatment. The mortality on this group was observed only after 48 h, but it was similar to that observed in the control group. Percentage of mortality higher than 50% was observed only after 48 h of experiment on the group treated with the nano-emulsion at 50 ppm (76.67 ± 5.8%). The group treated with the nano-emulsion at 200 ppm reached 100% of mortality in the first 24 h of experiment, while this percentage of mortality was reached after 48 h of experiment on the group treated with the nano-emulsion at 100 ppm.

Regarding the recorded mortalities during all experiment (24 and 48 h), no statistically significant difference was observed among the control group and the groups treated at 2.5, 10 and 25 ppm ($p > 0.05$). Significant differences were observed on this period, when compared the control group and treated groups at 2.5 and 10 ppm, to the groups treated at 50, 100 and 200 ppm ($p < 0.0001$). The mortality on the group treated with the nano-emulsion at 25 ppm statistically differed from the mortality of the group treated with the nano-emulsion at 50 ppm (24 h: $p < 0.01$; 48 h: $p < 0.0001$) and groups treated with the nano-emulsion at 100 and 200 ppm (24 h,48h: $p < 0.0001$). After 24 h of exposure, it was observed a significant difference among the mortality observed after treatment with the nano-emulsion at 50 ppm and mortalities of groups treated with the nano-emulsion at 100 ppm ($p < 0.001$) and 200 ppm ($p < 0.0001$). However, no significant difference among mortality of the group treated with the nano-emulsion at 50 ppm and the groups treated with the nano-emulsion at 100 and 200 ppm ($p > 0.05$), after 48 h. No statistical significant difference was observed between mortality of groups treated with the nano-emulsion at 100 and 200 ppm ($p > 0.05$), during all the treatment. Significant difference between the mortalities as function of exposure time was observed only on the groups treated with the nano-emulsion at 50 ppm ($p < 0.001$) and 100 ppm ($p < 0.05$).

After 24 h of treatment, the analysis of the data indicated that the percentage of deviance explained by the model was 95.1466% and the adjusted percentage was 87.1984%. The equation of regression estimated by the model was $y = -2.05443 + 0.0326513^{*}C$, being y the mortality and C the concentration of the tested nano-emulsion. The p-value for the model and p-value for the residuals were, respectively, 0.0000 and 0.6550. The test Chi-Squared ($x^2 = 47.8835$ and $p < 0.001$) indicated that the mortality is associated to the tested concentration. The estimated LC_{50} and LC_{90} values, with the lower limit and upper limit, are respectively, 62.92 (48.13–86.02) ppm and 102.17 (80.83–152.21) ppm. After 48 h of treatment, analysis of the data indicated that the percentage of deviance explained by the model was 99.6897% and the adjusted percentage was 92.6707%. The equation of regression estimated by the model was $y = -2.31126 + 0.0604685^{*}C$, while the p-value for the model and p-value for the residuals were, respectively, 0.0000 and 0.9963. The association between the mortality and the concentration was still maintained ($x^2 = 56.8116$ and $p < 0.001$).The estimated LC_{50} and LC_{90} values, with the lower limit and upper limit, are respectively, 38.22 (29.30–52.57) ppm and 59.42 (47.16–93.00) ppm.

Table 4 shows the influence of the nano-emulsion prepared with *L. alba* volatile oil on *A. aegypti* third-instar larvae. Low mortality was also observed on the control group (24 h = 3.33 ± 5.7% and 48 h = 6.66 ± 5.8%). Higher percentage of mortality was observed against *A. aegypti*, highlighted by 93.33 ± 5.8% after 24 h on the group treated with the nano-emulsion at 50 ppm. The absence of any living larvae (100% of mortality) was observed after 48 h of treatment on this group and also after 24 h of treatment with the nano-emulsion at 100 and 200 ppm.

After 24 and 48 h of exposure, no statistical significant difference was observed among the mortalities of control group and groups treated with the nano-emulsion at 2.5 and 25 ppm ($p > 0.05$). The mortalities recorded during all the experiment (24 and 48 h) revealed that significant differences were observed among control group and groups treated with the nano-emulsion at 2.5 and 25 ppm, when compared to the groups treated with the nano-emulsion at 50, 100 and 200 ppm ($p < 0.0001$). No statistical significant difference was observed among groups treated with the nano-emulsion at 50, 100 and 200 ppm ($p > 0.05$), during all the experiment. No time-dependent alteration on mortality was observed ($p > 0.05$).

After 24 h of treatment, analysis of the data indicated that the percentage of deviance explained by the model was 97.481% and the adjusted percentage was 89.6391%. The equation of regression estimated by the model was $y = -2.83916 + 0.081801^{*}C$, while the p-value for the model and the p-value for the residuals were, respectively, 0.0000 and 0.7327. The model indicated an association of mortality with the tested concentration ($x^2 = 49.7232$ and $p < 0.001$). The estimated LC_{50} and LC_{90} values, with the lower limit and upper limit, are respectively, 34.71 (25.17–45.05) ppm and 50.37 (41.27–76.72) ppm. After 48 h of treatment, analysis of the data indicated that the percentage of deviance explained by the model was 91.5587% and the adjusted percentage was 83.8744%. The equation of regression estimated was $y = -2.45803 + 0.0792463^{*}C$, while the p-value for the model and the p-value for the residuals were, respectively, 0.0000 and 0.2219. The association between the mortality and the concentration was still maintained ($x^2 = 47.6598$ and $p < 0.001$). The estimated LC_{50} and LC_{90} values, with the lower limit and upper limit, are respectively, 31.02 (21.47–41.40) ppm and 47.19 (37.93–73.57) ppm.

The carvone-type volatile oil of *L. alba* was assayed against *A. aegypti* larvae, presenting LC_{50} and LC_{90} values of 110.1 ppm and 211.5 ppm, respectively (Muñoz et al., 2014). Another study carried out with *L. alba* volatile oil from carvone-type indicated a LC_{50} value of 42.2 ppm against *A. aegypti* third to fourth instar larvae (Vera et al., 2014). The lowest tested concentration of *L. polystachya*

or L. turbinate volatile oils that induced more than 50% of mortality on *C. quinquefasciatus* after 24 h of treatment were 160 ppm or 60 ppm, respectively (Gleiser and Zygadlo, 2007). Thus, the results obtained in the present study with the nano-emulsion prepared with *L. alba* volatile oil from citral chemotype suggest a stronger activity then those of previous literature data of volatile oils from another chemotypes of this species or another *Lippia* species against Culicidae larvae.

The LC_{50} value of citral against *A. aegypti* third-instar larvae was 49.19 (45.19–54.06), after 24 h of exposure (Lee and Ahn, 2013). *R*-limonene and *S*-limonene slighly differed, regarding the LC_{50} values, against *A. aegypti* third-instar larvae. The values were, respectively, 27 and 30 ppm (Santos et al., 2011). Limonene presented a LC_{50} value of 40 (34–47) ppm against *Culex quinquefasciatus* third-instar larvae (Pavela, 2015). D-limonene found on the volatile oil from roots of *Toddalia asiatica* (L.) Lam. exhibited larvicidal activity against *Aedes albopictus* with LC_{50} value of 19.84 (17.96–21.67) ppm (Liu et al., 2013) after 24 h of exposure. Botas et al. (2017) tested a nano-emulsion of this compound against *Aedes aegypti* fourth-instar larvae, obtaining LC_{50} value of 91.25 (74.17–111.62) ppm after 24 h of exposure. Geranial and neral isolated from the *Magnolia salicifolia* bark induced 100% mortality on *Aedes aegypti* 4th instar larvae, at a concentration 100 ppm and after in 24 h of exposure (Kelm et al., 1997).

An oil in water nano-emulsion prepared with neem oil presented a LC_{50} value around 25.99 ppm against *C. quinquefasciatus*, when mean droplet size was 93.0 ± 0.33 nm (Anjali et al., 2012). The nano-emulsified eucalyptus oil was also evaluated against this vector larvae and showed a stronger activity, when compared to the bulk oil. However, the LC_{50} was not estimated on this investigation (Sugumar et al., 2014). Larvicidal assays of nano-emulsions containing copaiba oleoresin (Rodrigues et al., 2014) and rosemary volatile oil (Duarte et al., 2015) suggested their potential against *A. aegypti*, however, LC_{50} values were also not determined on these studies.

The nano-emulsion prepared with *Pterodon emarginatus* oleoresin was more active against *A. aegypti* (LC_{50} = 34.75 ppm) (Oliveira et al., 2016), when compared to *C. quinquefasciatus* (LC_{50} = 56.70 ppm) (Oliveira et al., 2017). Thus, the results obtained in the present study can be considered in accordance with literature data for nanostructured systems evaluated against this two larvae species. Silver nanoparticles containing herbal extract were also considered slightly more active against *A. aegypti*, when compared to *C. quinquefasciatus* larvae. The LC_{50}/LC_{90} values were around 28.67/53.24 ppm and 31.27/58.11 ppm, respectively (Muthukumaran et al., 2015). To our knowledge, most of the studies still aim to obtain larvicidal metal-based nanostructured system instead of nano-emulsions. Considering the biodegradable nature of the nano-emulsions and less impairment to the environment (e.g. less bioaccumulation), this can be considered an advantage when compared to metallic nanoparticles, which also make use of more expensive raw materials.

Conclusions

The *L. alba* volatile oil presented the compounds geranial and neral as major constituents, being in accordance with the expected citral chemotype. The bioactive nano-emulsion with low mean droplet size and low polydispersity index presented low LC_{50} values against *C. quinquefasciatus* and *A. aegypti* third-instar larvae, respectively, 38.22 and 31.02 ppm, suggesting its potential as larvicide. We believe that our results contribute significantly to the nanobiotechnology of botanical larvicidal against disease vectors, since it provides an easily produced, solvent-free, low energy and ecofriendly nanoproduct. The fact that *L. alba* can be widely cultivated and therefore attend the demand for a further nano-emulsion production is also a great advantage. Moreover, the utilization of an inexpensive technique is special interesting, since future production would not make use of high cost equipment, opening perspectives for utilization both in developed or developing countries.

Author's contributions

RMAF runned the laboratorial work as part of his academic thesis and drafted the manuscript. JLD and AEMFMO contributed to the nano-emulsion preparation and characterization. RASC contributed to the statistical analysis. RSA and JCTC contributed to analysis of data. RHVM contributed to volatile oil extraction and characterization. RNPS and CPF are advisors of the doctorate student, conceived and supervised the study, analyzed the data, drafted and revised the manuscript.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgements

Authors would like to thank PROPESPG/UNIFAP (PAPESQ) for the financial support and INCT RENOFITO by the network collaboration.

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