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ORIGINAL ARTICLE

Development of larvicide nanoemulsion from the essential oil of *Aeollanthus suaveolens* Mart. ex Spreng against *Aedes aegypti*, and its toxicity in non-target organism

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Abstract The widespread use of synthetic insecticides to control diseases such as those transmitted by the vector *Aedes aegypti* (Linnaeus, 1762) has caused widespread resistance to mosquitoes and adverse effects on non-target organisms, such as humans and animals. In this sense, nanotechnology emerges as a strategy for developing new promising insecticidal agents against this vector. Therefore, this research developed a nanoemulsion containing *A. suaveolens* essential oil, as well as to evaluate the larvicidal activity of the nanoemulsion against *A. aegypti* and toxicity in non-target mammals. The essential oil was identified by gas chromatography coupled to a mass spectrometer, and the nanoemulsions were prepared using a low-energy method and characterized by

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photon correlation spectroscopy. The larvicidal activity was evaluated against *A. aegypti* larvae at the third stage of development, and acute oral toxicity was assessed on mice. The results showed that the main compounds found were Massoialactone (64.79%), Linalool (7.83%), and (E)-β-Farnesene (6.17%). The most stable nanoemulsion was produced in HLB 15 with a particle size of 126.73 ± 0.20 nm, polydispersity index of 0.125 ± 0.01, and zeta potential of −16.25 ± 1.48 mV after 21 days. The LC_{50} and LC_{90} values in A. aegypti larvae after 24 h were 54.23 $\mu g.mL^{-1}$ and 96.96 $\mu g.mL^{-1}$, respectively. After 48 h, the values were LC_{50} of 46.06 $\mu g.mL^{-1}$ and LC_{90} of 75.31 $\mu g.mL^{-1}$. Regarding the acute oral toxicological evaluation, the results showed that despite *A. suaveolens* essential oil nanoemulsion (NEOAS) having a lethal dose (LD_{50}) greater than 2000 $mg.kg^{-1}$, histological changes were observed in the kidneys and liver of treated animals. In this sense, this study allowed the development of an ecological and low-cost larvicidal nanoformulation for the control of *A. aegypti*.

1. Introduction

The *Aedes aegypti* mosquito (Linnaeus, 1762) is considered the primary vector of several neglected and emerging tropical diseases, besides it has become a critical public health problem, since it acts on the transmission of a wide range of human pathogens of viral nature, such as dengue, chikungunya, and zika virus (Kovendan and Murugan, 2011; Peniche et al., 2019; Sumitha and Thoppil, 2016). In Brazil, according to data from the Ministry of Health (Brasil, 2020), in the period from 2008 to 2019, approximately 11.6 million diseases transmitted by *A. aegypti* were reported, with dengue concentrating 91% of cases (10.6 million cases). In addition to dengue, as of 2015, simultaneous transmission of two other diseases began to occur, chikungunya and Zika virus. Although the latter two arboviruses have milder symptoms than those of dengue, infection with the Zika virus has been of great concern to public health agencies due to its association with major congenital disorders and neurological complications such as Guillain-Barré syndrome and cognitive disabilities (Calvet et al., 2016; Oehler et al., 2014).

In many regions of the world, different strategies have been developed to reduce the prevalence of diseases transmitted by *A. aegypti*. One of the most effective tools to reduce the density of mosquitoes in their breeders, before they emerge in adults is the use of larvicides (Benelli et al., 2019, 2017; Chowdhury et al., 2009; Govindarajan et al., 2016). However, widespread resistance to synthetic insecticides and their high value has been one of the main obstacles to the economic integration of mosquito management programs (Wang et al., 2019). Also, there are cases of intoxication in non-target organisms such as humans and animals (Chaudhary et al., 2017; Mossa et al., 2018; Li et al., 2018). In this sense, there is an urgent need to develop new alternatives for mosquito control with local targets, to establish strategies and tactics for biorational management, since vaccines have limited efficacy in the control of malaria and dengue (Mahoney, 2014).

Plants can provide potential sources of mosquito control products, as they are rich in secondary metabolites such as essential oils (EOs) (Benelli et al., 2018a, 2018b; Gonzalez et al., 2014). The EOs produced by more than 17.500 species of aromatic plants have several advantages when compared to synthetic insecticides such as their rapid biodegradation in the environment, less environmental impact, and low risk for human and animal (Chermenskaya et al., 2010; Isman, 2020; Pavela, 2015; Zanuncio et al., 2016). Among the various groups of plants, the Lamiaceae family stands out for the richness of EOs in their species (Mesquita et al., 2018).

A survey carried out by Pavela (2015) on new botanical larvicides, showed that the Lamiaceae Martinov family (1820) is the group of plants that has EOs and chemical compounds with greater effectiveness in larvicide tests ($LC_{50} \leq 100$ ppm), reaching 19.7% when compared to other families that have an efficacy of 14.7% (Cupressaceae), 12.3% (Rutaceae), 11.5% (Apiaceae) and 10.6% (Myrtaceae). The main constituents of EOs belong to two phytochemical groups, terpenoids composed of low molecular weight monoterpenes and sesquiterpenes and phenylpropanoids such as volatile phenolic compounds (Echeverría and Albuquerque, 2019).

Aeollanthus suaveolens Mart. ex Spreng is an herb of the Lamiaceae family, native to the African continent, from Nigeria to Sudan and in East Africa from north to south of Tropical Africa and in a location in South Africa. In Brazil, its geographical distribution occurs mainly in the north and northeast regions (Simionatto et al., 2007; Tucker et al., 2001). Its use in folk medicine in these regions occurs mainly for the treatment of seizures, colds and flu, fever, headache, earache, to eliminate gas, rheumatic pain, asthma, "*quebranto*", insecticide, repellent, insect bite, heart problems, and menstrual cramps (Martins et al., 2020). Besides, pharmacological studies have shown anticonvulsant potential (Coelho de Souza et al., 1997), antidepressant (Santos et al., 2017), and antimicrobial (Martins et al., 2016).

A recent study by Ferraz et al. (2020) with nanoemulsion obtained from the EO of *A. suaveolens*. showed a sedative-hypnotic neuroprotective effect for zebrafish (*Danio rerio*) embryos. In these pharmacological studies, the main chemical compounds found in EO were (E)-β-farnesene, linalool, and massoia lactone. Although EOs are rich as sources of bioactive compounds, limited physical stability, low water solubility, and rapid degradation of the environment cause major concerns in the development of larvicidal products (Gnankiné and Bassolé, 2017).

In this context, nanotechnology is considered a multidisciplinary area for its diverse potential for applications, which includes promising new insecticidal agents against disease vectors (Benelli et al., 2018c; Duarte et al., 2015; Peniche et al., 2019). Among the main nanostructured systems are

nanoemulsions, which are made up of small droplets (20–200 nm) dispersed in the external phase, usually water, which are often stabilized with one or more surfactants (Mcclements, 2012). Nanoemulsions stand out for their significant properties such as the increase in physical–chemical properties, better stability, and improvement of biological activities through the increase in the area of specific surfaces (Gahruie et al., 2017; Pavela et al., 2021), also, aqueous nanoemulsions containing bioactive vegetable oils are a viable economic alternative to chemical pesticides in vector control practices.

Therefore, due to the widespread use in *A. suaveolens* folk medicine as a natural insecticide and the richness of essential oils of species of the family Lamiaceae with larvicidal potential, as well as the need to develop new control strategies for the vector *A. aegypti*, this study aimed to develop a nanoformulation of the essential oil from the species *A. suaveolens* and evaluate its larvicidal activity against *A. aegypti* and toxicity in a non-target organism mammal model.

2. Materials and methods

2.1. Plant material

A. suaveolens leaves were collected in the District of Fazendinha in the following coordinates: 00° 02′23 "S and 51° 06′29″ O, Amapá-Brazil. The identification of plant material was carried out at the Herbarium Amapaense (HAMAB) of the Institute of Scientific and Technological Research of the State of Amapá (IEPA) under registration number M.R.L.001.

2.2. EO obtaining

The crushed dry leaves were subjected to hydrodistillation for a period of 2 h in a Clevenger-type apparatus, at a temperature of approximately 100 °C, according to the Brazilian Pharmacopeia (Farmacopeia Brasileira, 2019). Then it was weighed and hermetically stored in an amber glass bottle and kept refrigerated (4 °C) until use. The yield of essential oils was calculated based on the dry weight of the leaves of each sample.

2.3. Identification of EO constituents

The identification of the constituents of the EO was performed by gas chromatography coupled to the mass spectrometer (GCMS), using Shimadzu equipment, model GCMS-QP 2010 Plus, on a DB-5HT column of the J & W Scientific brand, column 30 m, diameter 0.32 mm, film thickness 0.10 μm and nitrogen as carrier gas. The operating conditions of the gas chromatograph were: internal column pressure of 56.7 kPa, split ratio of 1:20, gas flow in the column of 1.0 mL.min^{-1} (210 °C), temperature in the injector of 220 °C, temperature in the detector, or the interface (CG-MS) of 240 °C. The initial temperature of the column was 60 °C, followed by an increase of 3 °C.min^{-1}. until it reaches 240 °C, being kept constant for 30 min. The mass spectrometer was programmed to perform readings in a range from 29 to 400 Da, in 0.5 s intervals, with ionization energy of 70 eV.

A standard mixture of n-Alkanes (Sigma-Aldrich C_8 - C_{40}) was used to verify the performance of the GC–MS system and calculate the retention index (LRI) of each compound in the sample. The standard (1 μL) of these alkanes was injected into the GC–MS system operating under the conditions described above, and their respective retention times were used as an external reference standard for calculating the LRI, together with the retention times of each compound of interest. The identification of the constituents was based on the comparison of the LRI and mass spectra of each substance with data from the literature (Adams, 2017).

2.4. Preparation of nanoemulsions

The emulsion was prepared by the low energy method according to the methodology of Ostertag et al. (2012) and Oliveira et al. (2017). The emulsions consisted of water (99.5%, w/w), EO (0.25%, w/w) and surfactant (0.25%, w/w). Initially, the oily phase (EO of *A. suaveolens* and the surfactant) was combined and stirred for 30 min on a magnetic stirrer. Then, the aqueous phase (deionized water) was added dropwise to the oil phase under continuous stirring for another 60 min at 750 rpm. The nanoemulsions were stored in glass tubes with a lid and stored at room temperature (25 ± 2 °C). The nanoemulsion had an EO concentration equal to 25.000 μg. mL^{-1}.

2.5. Determination of the required Hydrophilic-Lipophilic balance (rHLB) value

The rHLB determination of *A. suaveolens* was performed by a blend of sorbitan monooleato and Polysorbate 80 to produce the HLB 10, 11, 12, 13, 14 e 14.5; Polysorbate 80 to produce HLB of 15; and Polysorbate 80 and Polysorbate 20 to produce the HLB 15.5 e 16. The composition and preparation of the emulsions was conducted according to the emulsification method presented above. The rHLB value of the mixture was calculated according to the Eq. (1) (Botas et al., 2017).

$$rHLB = \frac{[(HLB_1 - m_1) + (HLB_2 - m_2)]}{(m_1 + m_2)} \quad (1)$$

Where HLB_1 is the HLB value of the most lipophilic surfactant, m_1 is the most lipophilic surfactant mass, HLB_2 is the HLB value of the most hydrophilic surfactant, m_2 is the mass of the most hydrophilic surfactant. The rHLB was determined as the HLB value of the surfactant that allowed us to obtain the largest possible stable system.

2.6. Characterization of nanoemulsions

The particle size (PS), polydispersity index (PdI), and zeta potential (ZP) of the nanoemulsion were determined using a Zetasizer Nano ZS (Malvern, United Kingdom), equipped with a 10 mW 'red' laser (λ = 632.8 nm), the samples were measured at a dispersion detector angle of 173° for size measurements. Each analysis was performed in triplicate and the results were expressed as mean ± standard deviation (SD). Emulsions for rHLB determination were diluted with water (1:25, v/v), for injection (Oliveira et al., 2017). The particle size, polydispersity index, and zeta potential of the optimized nanoemulsions were monitored on days 0, 7, 14, and 21.

2.7. Larvicidal bioassay

The larvicidal activity of nanoemulsion obtained from the EO of *A. suaveolens* was evaluated on the third larvae instar of the species *A. aegypti*, using a method recommended by the WHO (2005), with modifications. The larvae were obtained from the Medical Entomology Laboratory of the Institute of Scientific and Technological Research of the State of Amapá and the tests were conducted in a room of 12 m^2 (3 m × 4 m), with controlled climatic conditions: temperature of 25 ± 2 °C, relative humidity of 75 ± 5% and photoperiod of 12 h.

Five concentrations were selected after a preliminary test: 20, 40, 60, 80 and 100 $\mu g.mL^{-1}$. The nanoemulsion (25.000 $\mu g.mL^{-1}$) was diluted with distilled water in a beaker to the desired concentration and the volume of 100 mL. The entire experiment was conducted in quintuplicate with 25 larvae in each replicate (n = 150). For the negative control, the mixture of the HBL15 surfactant with distilled water was used and for the positive control, sbiotrin was used. After 24 and 48 h, the numbers of deaths were counted, being considered as such all those unable to reach the surface. The lethal concentration that causes 50% mortality in the population (LC_{50}) was determined using Probit analysis using SPSS® software [version 20.0; SPSS Inc., Chicago, IL, USA]. Tests in which the negative control mortality exceeded 20% were discarded and repeated, those with mortality between 5 and 20% were corrected using the Abbott Eq. (2) (Consoli and Oliveira, 1994).

$$\%Mortality = \frac{((\%M_T - \%M_C)X100)}{(100 - \%M_C)} \quad (2)$$

$\%M_T$ = Test Mortality Percentage;
$\%M_{NC}$ = Negative Control Mortality Percentage

2.8. Acute toxicity of A. Suaveolens essential oil nanoemulsion (NEOAS) in non-target organisms

2.8.1. Animals

Every experiment was performed by the International Animal Care Committee, following the national regulations established for animal experimentation, and was submitted to and approved by the Animal Ethics Committee of the Federal University of Amapá (CEUA - UNIFAP – 003/2019). The experiment was conducted using male and female Swiss albino mice (*Mus musculus*), from the Multidisciplinary Center for Biological Research for the Area of Science in Laboratory Animals (CEMIB) at the University of Campinas. Before the beginning of the experiments, the animals were acclimated for ten days to ascertain the behavior, eating and physiological habits. They were maintained under standard environmental conditions (12 h dark/light cycle; temperature 23 ± 2 °C). Industrialized dry food and water (Labina, Purina, Brazil) were made available *ad libitum*.

2.8.2. Experimental protocol

The investigation of acute toxicity followed, in general, the guidelines of the OECD (OECD, 2002) for testing the acute toxic dose class (Guideline 423). Initially, the animals were randomly divided into two groups (n = 6/groups; three males and three females). The first group (treated group) received a dose of 2000 $mg.kg^{-1}$ orally of a nanoemulsion obtained from the EO of *A. suaveolens* and the second group (negative control) received Tween 80. Then, the animals were observed at 30, 60, 120, 240, and 360 min and every 24 h for 14 days. During this period, changes in the sensory system, psychomotor system, and central nervous system were evaluated, as well as the daily water intake (mL) and feed (g) and body weight. On the 14th day, the animals were anesthetized with xylazine/ketamine (Flecknell, 1996; Kohn et al., 1997) and sacrificed in a CO_2 chamber, obeying the ethical principles of animal experimentation. After sacrifice, the organs (liver, spleen, heart, and kidneys) were removed for weighing and macroscopic and microscopic analysis.

2.8.3. Histopathological analysis

Histopathological analysis of the organs of animals treated with NEOAS was performed according to the methodology described by Souza et al. (2016). The organs were fixed in a 10% formalin buffer solution for 48 h. Then, they were dehydrated in different concentrations of alcohols (70%, 80%, 90%, and 100%) and then diaphanized in xylol and embedded in paraffin (Inlab). The material was stained in HE (Harris hematoxylin-LABORCLIN and yellowish eosin-INLAB). The slides were analyzed in optical microscopes (Olympus-micronal brands BX41) and photographed with an MDCE-5C USB 2.0 (digital) camera.

2.9. Statistical analysis

Data were tabulated in mean and standard deviation and analyzed using one-way Analysis of Variance with Tukey's post-test in GraphPad Prism Software v.7 (San Diego, California, USA). The results were considered significant when the p-value < 0.05 Probit analysis was performed using a 95% confidence interval to determine the LC_{50} and LC_{90}.

3. Results

3.1. Chemical composition of A. Suaveolens EO

The EO of *A. suaveolens* leaves had a 1.6% yield. The analysis by Gas Chromatography coupled to the Mass Spectrometer revealed the presence of 21 compounds, and the major compounds identified were Massoialactone (64.79%), Linalool (7.83%), and (E) -β-Farnesene (6.17%), as shown in Table 1 and Fig. S1 and Fig. S2.

3.2. Development and analysis of nanoemulsion

In this study, the characterization of the nanoemulsions considered the droplet size, PdI, and zeta potential. The results revealed that the nanoemulsions presented a bluish reflection, without sedimentation or phase separation.

The best results were obtained with the nanoemulsion prepared exclusively with polysorbate 80 as a surfactant (HBL 15). It had an average particle size of 104.83 ± 0.47 nm, a polydispersity index of 0.156 ± 0.01 (Fig. 1), and a zeta

Table 1 Chemical composition of the *A. suaveolens* EO.

N°	$LRI_{Calc.}$	$LRI_{Lit.}$	Compounds	Relative percentage (%)
1	1098	1095	Linalool	7.83
2	1194	1186	α-Terpineol	1.91
3	1222	1227	Nerol	0.59
4	1248	1249	Geraniol	4.3
5	1296	1298	Carvacrol	0.11
6	1349	1356	Eugenol	1.14
7	1357	1359	Neryl Acetate	0.3
8	1411	1424	2,5-Dimethoxy-*p*-Cymene	3.4
9	1416	1416	α-Santalene	0.74
10	1431	1411	(E)-α-Bergamotene	0.18
11	1452	1454	(E)-β-Farnesene	6.17
12	1481	1471	Massoialactone	64.79
13	1492	1493	δ-Decalactone	2.61
14	1560	1561	Nerolidol < (E)- >	0.37
15	1574	1574	Germacrene D-4-ol	0.25
16	1641	1640	Muurolol < epi-α- >	0.15
17	1653	1652	Cadinol < α - >	0.61
18	1659	1667	Pentadecen-2-one < 6Z- >	0.22
19	1668	1670	Bisabolol < epi-β >	0.05
20	1685	1685	Massoia Dodecalactone	0.1
			Total	97.55

$LRI_{Calc.}$ = Calculeted Linear Retention Index; $LRI_{Lit.}$ = Literature Retention Linear Index.

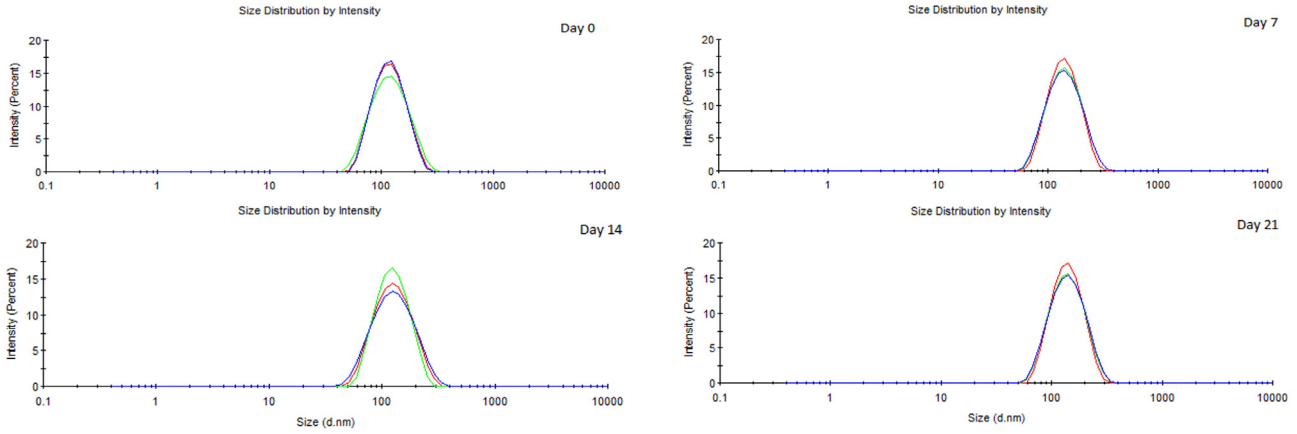

Fig. 1 Average droplet size of the nanoemulsion prepared with EO of *A. suaveolens* in rHLB 15 - Day 0: 104.8 ± 0.47 nm; Day 7: 129.53 ± 0.40 nm; Day 14: 113.26 ± 0.23 nm; Day 21: 126.73 ± 0.20 nm. The different colors represent replicates of the same sample.

potential of −13.63 ± 0.83 mV on day 0. The stability of the nanoemulsion (HBL 15) was observed over time, being found average particle size of 126.73 ± 0.20 nm, polydispersity index of 0.125 ± 0.00, and zeta potential of −16.25 ± 1.48 mV after 21 days (Table 2). The other HBL values can be seen in table S1.

3.3. Larvicidal activity of NEOAS

The larvicidal activity of nanoemulsion of NEOAS was tested in five different concentrations: 20, 40, 60, 80, and 100 μg.mL^{-1}. The results indicated that the concentrations 20 and 40 μg.mL^{-1} had low mortality after 24 and 48 h of exposure. The concentration of 60 μg.mL^{-1} showed mortality below 50% after 24 h, however, after 48 h the mortality was 76%. The concentrations of 80 and 100 μg.mL^{-1} had a mortality of 85 and 98% after 24 h, and 96% and 100% after 48 h, respectively, as can see in Table 3. Regarding the exposure time, a statistically significant difference was observed only in the group treated with NEOAS at 60 μg.mL^{-1}.

Table 2 Average particle size, polydispersity index, and zeta potential during the preparation of the nanoemulsion (HLB = 15) of the essential oil of *A. suaveolens*.

Days	Size (nm)	Pdi	Zeta potencial (mV)
0	104.83 ± 0.47	0.156 ± 0.01	−13.63 ± 0.83
7	129.53 ± 0.40	0.120 ± 0.01	−10.32 ± 0.53
14	113.26 ± 0.23	0.143 ± 0.02	−10.23 ± 0.66
21	126.73 ± 0.20	0.125 ± 0.01	−16.25 ± 1.48

PdI: Polydispersity Index

Table 3 Percentage of mortality (% ± SD) of the larvae of *A. aegypti* after treatment with different concentrations of NEOAS.

	Larvicidal activity (%)	
Concentrations (μg.mL^{-1})	24 h	48 h
Negative control	0[a]	0 [a]
20	7.2 ± 1.3[a]	7.2 ± 1.3[a]
40	15 ± 2.06[a]	18.4 ± 1.67[b]
60	46 ± 2.6[b]	76 ± 1.4[c]
80	85.6 ± 3.1[d]	96 ± 1.4[d]
100	98 ± 0.57[d]	100 ± 0[d]
LC_{50} (Positive control)	0.0034 μg.mL^{-1}	

Different letters indicate that there was a significant difference in the Tukey test ($p < 0.05$).

Table 4 shows the larvicidal effect of the nanoemulsion in terms of lethal concentration (LC_{50} and LC_{90}). Probit analysis showed a statistically significant relationship between mortality at 24 and 48 h of exposure, with the nanoemulsion concentrations assessed at a 95% confidence level.

3.4. The toxicological activity of NEOAS

The results of acute oral toxicity showed that NEOAS (2000 mg.kg^{-1}) did not cause death during the 14 days of observation. Regarding behavioral changes, it was observed that NEOAS caused changes only in the corneal reflex of an animal. The control group (Tween 80) showed no behavioral changes. No significant differences were observed in water intake, food consumption, and bodyweight of animals treated with nanoemulsion, as can be seen in Table 5.

The NEOAS (2000 mg.kg^{-1}) did not induce significant changes in the relative weight of the organs (heart, liver, lungs, and kidneys) of mice of both sexes when compared to the control (Table 6).

Histopathological analysis did not reveal changes in the organs of the control animals (Fig. 2a, 2b, 2c, and 2d), as well as in the heart of the animals treated with NEOAS (Fig. 2e). However, in the liver of animals treated with the nanoemulsion, the presence of inflammatory cells was observed (Fig. 2f). In the lungs, the presence of transudate with leukocyte infiltration was observed (Fig. 2g). In the kidneys, dilation of the capillaries of the glomerulus, a decrease in the space of the Bowman's capsule, and tubular hyperplasia was observed (Fig. 2h).

4. Discussion

The EO of *A. suaveolens* leaves had a 1.6% yield. Studies by Simionatto et al. (2007) showed that the species had a yield of 0.8%. These different productivity results can be related to several factors, such as climate, soil composition, geographic location, seasonal variation, plant organ, age, stage of the vegetative cycle, and harvest period (El-Hawary et al., 2013). Also, studies show changes in EO yield, probably influenced by the type of drying when comparing fresh plant material with dry plant material (Raduz et al., 2002; Rocha et al., 2000). However, to assess the economic viability of the EO of *A. suaveolens* it is necessary to evaluate the number of harvests per year, yield obtained and harvest duration (García-Caparrós et al., 2019).

Regarding the identification of the main compounds, Martins et al. (2016) identified the presence of (E)-β-farnesene (37.615%) and linalool (33.375%) in the EO of *A. suaveolens*, and the extraction occurred with fresh plant material, in May (rainy season). In this research, the extraction occurred with dry plant material, in April (rainy season) with most of the compounds Massoialactone (64.79%), Linalool (7.83%), and (E)-β-Farnesene (6.17%), indicating that probably there was an influence of drying on the chemical composition of the *A. suaveolens* EO, because in both studies the botanic material were collected from the same place.

Among the instability phenomena that can occur in nanoemulsions, Ostwald's maturation, which is the transport of oil from small particles to large particles through the continuous phase, is considered the main mechanism for destabilizing nanoemulsions (Artiga-Artigas et al., 2018). The evaluation of the instability of nanoemulsions can be performed through the analysis of the particle size, the polydispersity index (PdI), and the zeta potential (Oliveira et al., 2017). In this sense, the results obtained in this study suggest that NEOAS at HBL 15 showed kinetic stability and protection against Ostwald ripening during the 21-day period since its particles had a size distribution smaller than 200 nm and a PdI below 0.2 (Al - Assiuty et al., 2019).

This rHLB (15) value was the same as that found by Ferreira et al. (2019) with the EO of *Siparuna guianensis* and by Rodríguez-Rojo et al. (2012) with the EO of *Rosmarinus officinalis* L. Studies by Duarte et al. (2015) and Oliveira

Table 4 Probit linear regression parameters of NEOAS against *A. aegypti* larvae.

Time (h)	*A. suaveolens* essential oil Nanoemulsion			
	CL_{50}(LCL-UCL)	CL_{90}(LCL-UCL)	x^2 (df)	p-value
24	54.23 (42.530–66.838)	96.96 (76.460–164.759)	4.433 (3)	0.0001
48	46.06 (36.077–55.697)	75.31 (61.443–112.445)	3.937 (3)	0.0001

LC_{50}: lethal concentration causing 50% mortality and its 95% confidence interval.
LC_{90}: lethal concentration causing 90% mortality and 95% confidence interval.
χ2: heterogeneity over the regression line.
df: degree of freedom.
p: represents heterogeneity in the tested population.

Table 5 Effect of NEOAS (2000 mg.kg^{-1}) on body weight gain (g), water, and feed intake in female and male mice treated orally for 14 days. Values expressed as mean ± standard deviation. (n = 3/group).

Parameters	Control		NEOAS	
	Males	Females	Males	Females
Water (mL)	11.25 ± 3.88	10.88 ± 3.32	9.38 ± 2.77	9.81 ± 2.65
Feed (g)	5.62 ± 1.23	5.45 ± 1.59	5.42 ± 1.20	6.42 ± 1.75
Weight (g)	22.56 ± 2.68	22. 89 ± 2.96	17. 15 ± 1.73	19.93 ± 1.46

Table 6 Effect of the NEOAS (2000 mg.kg^{-1}) on the relative weight of the organs of male and female mice treated orally during 14 days of evaluation.

Animal	Organs	Relative organ weight (%)	
		Control	NEOAS
Female	Liver	5.12 ± 0.04	6.22 ± 0.16
	Kidney	0.84 ± 0.02	0.78 ± 0.01
	Heart	0.77 ± 0.05	0.75 ± 0.04
	Lungs	0.84 ± 0.02	0.74 ± 0.03
Male	Liver	6.19 ± 0.51	6.40 ± 0.33
	Kidney	0.85 ± 0.05	0.85 ± 0.02
	Heart	0.58 ± 0.03	0.68 ± 0.04
	Lungs	0.66 ± 0.05	0.78 ± 0.02

et al. (2017) found high rHLB values for EO. Rodríguez-Rojo et al. (2012) attribute the rHLB value to the hydrophilicity of the major compound of EO, in this study the main compounds found in the EO of *A. suaveolens* were Massoialactone (64.79%) and Linalool (7.83%). The most stable nanoemulsion (rHLB 15) was used to assess larvicidal activity and acute oral toxicity in non-target mammals.

EOs obtained from plants are studied as a natural compound potentially used as an alternative to common synthetic insecticides (Sanei-Dehkordi et al., 2018). However, there is no report of nanoemulsion contact toxicity obtained from the *A. suaveolens* EO in the *A. aegypti* larvae.

The NEOAS larvicidal activity shows a better result (LC_{50} = 54.23 µg.mL^{-1}) compared with other studies described in the literature. The nanoemulsion obtained from the resin oil of the fruits of *Pterodon emarginatus* showed a CL_{50} of 371.6 µg.mL^{-1} (Oliveira et al., 2017), the *Siparuna guianensis* EO presented a CL_{50} of 86.52 µg.mL^{-1} (Ferreira et al., 2019), and the *Baccharis reticularia* DC EO with LC_{50} of 221.3 µg.mL^{-1} (Botas et al., 2017).

Regarding the mechanism of action involved in the insecticidal action of the nanoemulsions prepared with EO, Casida and Durkin (2013), and Echeverría and Albuquerque, (2019) suggest that insecticidal effect can occur through enzymatic inhibition, which causes death and paralysis of insects through the neural block and signal transduction impairment. Several compounds found in EOs, such as monoterpenes and sesquiterpenes, have insecticidal activity by inhibiting the enzyme acetylcholinesterase (Botas et al., 2017; Giatropoulos et al., 2018; Hostettmann et al., 2006; Houghton et al., 2006; Regnault-Roger et al., 2012; Seo et al., 2015).

For Gnankiné and Bassolé (2017), the effect of an active compound, such as monoterpenes or sesquiterpenes, can be modulated and increased by other main compounds and/or compounds present in smaller concentrations that produce

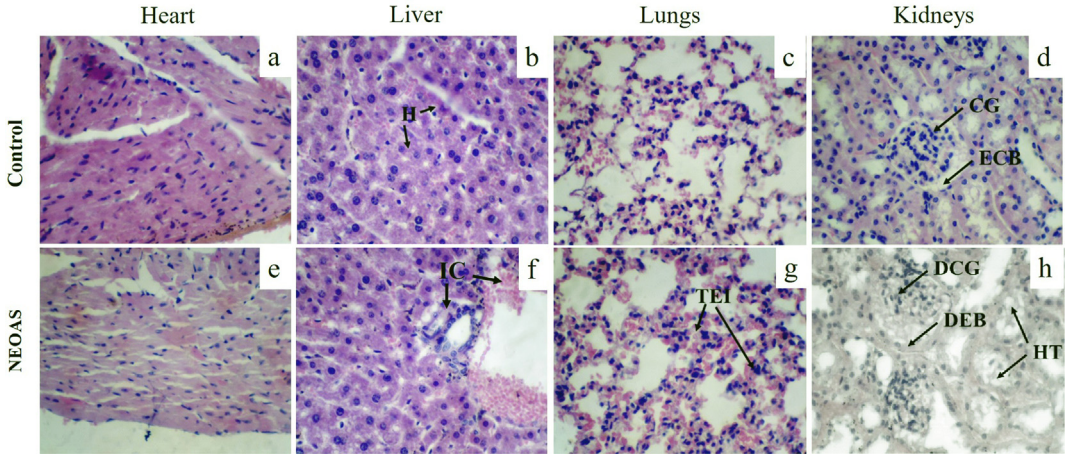

Fig. 2 Histological section of the heart, liver, lungs, and kidneys of animals treated orally with Tween 80 (control) or NEOAS (2000 mg.kg^{-1}). Normal organs of the control group are observed, with kidneys showing capillaries of the glomerulus (GC) and space of the Bowman's capsule (ECB). In the liver of animals treated with NEOAS (2000 mg.kg^{-1}), inflammatory cells (IC) were observed; In the lungs of an animal treated with nanoemulsion, an edematous transudate with leukocyte infiltration (TEI) was observed; In the kidneys of an animal treated with NEOAS, dilation of the capillaries of the glomerulus (DCG), a decrease in the space of the Bowman's capsule (DEB) and tubular hyperplasia (TH) were observed.

an additive or synergistic effect resulting in the bioactivity. In this study, the main metabolic compounds found in the *A. suaveolens* EO were Massoialactone (64.79%) (lactone), Linalool (7.83%) (monoterpene), and (E)-β-Farnesene (6.17%) (sesquiterpene). In this sense, further studies are needed to evaluate the mechanism of action of EO and the major compounds of *A. suaveolens* in the insecticidal action, as well as to correlate the release of the compounds as a function of time.

Changes in water, food, and body weight consumption are considered general health indicators for experimental animals (Hilaly et al., 2004). In this study, there were no significant differences in water consumption, feed, and the bodyweight of animals treated with NEOAS (2000 $mg.kg^{-1}$). For Raza et al. (2002) and Teo et al. (2002), the reduction in body weight and weight of the internal organ can be a simple and sensitive procedure that indicates toxicity after exposure to a toxic substance. In this study, there was no significant reduction in the relative weight of the organs when compared to the control.

Regarding the histopathological evaluation of tissues of animals treated with NEOAS (2000 $mg.kg^{-1}$), inflammatory cells in the liver of animals were observed, their lungs presented transudate with leukocyte infiltration, and the kidneys showed dilation of the capillaries of the glomerulus, decreased space in the Bowman capsule and tubular hyperplasia. Studies conducted with species of the Lamiaceae family have also demonstrated histopathological changes in the organs of animals. Caldas et al. (2013) evaluated the repeated-dose toxicity (100 $mg.kg^{-1}$ and 500 $mg.kg^{-1}$) of the EO of *Hyptis martiusii* Benth in Swiss albino mice, the results showed histopathological changes in the liver, kidneys, lungs, and spleen. The EO of *Mesosphaerum sidifolium* (2000 $mg.kg^{-1}$) showed moderate acute toxicity, with histopathological changes in the liver and kidneys of mice (Rolim et al., 2017).

In a study by Daneshbakhsh et al. (2018), the EO of *Mentha mozaffarianii* revealed microscopic lesions in the liver, kidney, stomach tissues, and small intestine. The development of formulations for controlled release has great potential for reducing the side effects of various drugs and great potency and promotes a prolonged pharmacological effect (Yilmaz and Borchert, 2005). However, due to the small size that favors rapid absorption and metabolism, nanoemulsions have the potential to have some toxic effect (Borges et al., 2018), as shown in this research.

The liver is the mammalian main detoxification organ, and the kidneys are the most important excretory organ, both of which are susceptible to chemotherapy drugs. In this study, only one type of histopathological alteration was observed in the liver of animals treated orally, showing that nanoemulsions are not toxic to this organ, without compromising its normal functioning. However, histopathological changes were observed in the kidneys of the treated animals that may compromise the normal functioning of the organ, such as, for example, the dilation of the capillaries of the glomerulus that causes the space in the Bowman capsule to decrease and prevent the passage of blood for filtration, remaining in the tubules and consequently obstructing them (Aires, 2008; Guyton and Hall, 2006).

According to Ibrahim et al. (2016), the main objective of evaluating the medicinal safety of a plant is to identify its adverse effect and to establish levels of exposure in which this effect is observed. According to the OECD (2002), NEOAS can be included in class 5, which comprises products of low toxicity with LD_{50} greater than 2000 $mg.kg^{-1}$. However, the results suggested that NEOAS is not exempt from toxic effects,since histopathological changes were observed, mainly in the kidneys and liver, therefore, its oral use in concentrations above 2000 $mg.kg^{-1}$ should be administered carefully. These results are important from a toxicological viewpoint since they allow inferring a safety limit for the use of nanoemulsion as a larvicide because, despite the histopathological changes in the organs, its LD_{50} (2000 $mg.kg^{-1}$) is higher than LC_{50} (54.23 $\mu g.mL^{-1}$) for *A. aegypti* larvae. However, future studies are needed for simultaneous evaluation of histopathological and biochemical analysis to determine the degree of toxicity of NEOAS.

5. Conclusion

The EO of *A. suaveolens* showed Massoialactone, Linalool, and (E)-β-Farnesene as major compounds. The study allowed to developing and optimize a nanoemulsion with low average particle size and low polydispersity index, which showed a potent larvicidal effect against *A. aegypti* larvae in the third instar. The NEOAS showed low acute oral toxicity for non-target organisms. In this sense, this study allowed the development of an ecological and efficient larvicidal nanoformulation, of low cost, which can be safely applied in the control of disease vectors in humans and animals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2021.103148.

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