EUROPEAN ACADEMIC RESEARCH
Vol. VII, Issue 12/ March 2020

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Biodegradable nanoparticles loaded with *Lippia alba* essential oil: a sustainable alternative for *Aedes aegypti* larvae control

JÉSSICA M. S. DA SILVA
MAXWALDO DA S. RABELO
SUZAN X. LIMA
ANA LUISA F. ROCHA
Laboratório de Polímeros Nanoestruturados (NANOPOL)
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil
HENRIQUE D. DA FONSECA FILHO
Departamento de Física (DF)
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil
ANDRÉ C. DE OLIVEIRA
WANDERLI P. TADEI
Instituto Nacional de Pesquisas da Amazônia (INPA)
Laboratório de Malária e Dengue, Manaus/AM, Brasil
FRANCISCO C. M. CHAVES
Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)
Unidade Amazônia Ocidental, Manaus/AM, Brasil
JAQUELINE DE A. BEZERRA
Instituto Federal de Educação
Ciência e Tecnologia do Amazonas (IFAM), Manaus/AM, Brasil
MATHEUS M. BIONDO
Laboratório de Polímeros Nanoestruturados (NANOPOL)
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil
PEDRO H. CAMPELO
Laboratório de Polímeros Nanoestruturados (NANOPOL)
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil
Faculdade de Ciências Agrárias
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil
EDGAR A. SANCHES
Laboratório de Polímeros Nanoestruturados (NANOPOL)
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil
Departamento de Física (DF)
Universidade Federal do Amazonas (UFAM), Manaus/AM, Brasil

Abstract

Poly-ε-caprolactone (PCL) nanoparticles were loaded with essential oil (EO) from Lippia alba aiming the development of a larvicidal controlled-release formulation. Chromatographic analysis (GC-MS) revealed that the EO is mainly constituted of carvone (54,54%) and limonene (21,49%). Larvicidal assays were performed

using the EO in natura, and LD_{50} and LD_{90} were found about 106 µg/mL and 130 µg/mL, respectively. AFM and DLS techniques allowed to estimate the average diameter of the unloaded and loaded nanoparticles around (136 ± 2) and (266 ± 6) nm, respectively. Nanoparticles loaded with an absolute concentration of 500 µg/mL of EO containing preservatives were stable for 50 days. The controlled-release assays presented faster release at neutral and basic pH due to the destabilization of the formulation that approaches its isoelectric point. Larvicidal assays using the encapsulated EO were carried out against A. aegypti larvae. The mortality time was considerably reduced from 72 h for the EO in natura to 24 h for the formulation containing encapsulated EO. The loaded nanoparticles may present different mechanisms of interaction with larvae when compared to the EO in natura. These data suggest that the proposed formulation can represent an alternative for larvicidal control of A. aegypti.

Key words: Poly-ε-caprolactone nanoparticles, *Aedes aegypti*, *Lippia alba*, Controlled Release.

1 INTRODUCTION

Aedes aegypti Linn. vector have been transmitted serious human viral diseases (Leta et al., 2018; Shanmugaraj et al., 2019). The encapsulation of essential oils (EO) within biodegradable nanoparticles represents an alternative tool to control larvae, since mosquitoes have been presenting resistance to conventional insecticides (Karunaratne et al., 2018; Zhu et al., 2016).

Carriers based on biopolymeric nanoparticles are effective in encapsulating bioactive compounds, preserving its functionality, as well as increasing its time of action (Banik et al., 2016; Kamaly et al., 2016). For this reason, EO have been considered an alternative, natural bioactive to control a range of pests (Mar et al., 2018) due to their complex chemical composition. Among the EO presenting pronounced biological activities, *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson (Verbenaceae) (Glamočlija et al., 2011), known in Brazil as "erva-cidreira" (Islam et al., 2018; Santos et al., 2016), has been

applied aiming at different purposes: in addition to being widely distributed throughout Brazil, it has been used in traditional medicine as a tranquilizer, as well as for gastrointestinal diseases.

The combination of biopolymeric nanoparticles and EO to control pests represents an interesting area of research (Ghaderi-Ghahfarokhi et al., 2017) due to the prolonged action of the bioactive compound, as well as to the carrier biodegradation. These materials can reduce the risks of toxicity and environmental contamination. However, the knowledge of both core and carrier is fundamental for the development of controlled-release formulations (Souza et al., 2014). Poly-ε-caprolactone (PCL) is known for its biodegradability, as well as good encapsulating property as carrier (Karuppuswamy and Reddy, 2015; Othman et al., 2016). For this reason, PCL-based nanoparticles have been successfully used to encapsulate bioactive molecules, and represent a good alternative for encapsulation of EO.

PCL-based nanoparticles containing the encapsulated EO from *L. alba* were synthesized by the nanoprecipitation method (Christofoli et al., 2015). The developed formulation was fully characterized by Atomic Force Microscopy (AFM), Dynamic Light Scattering (DLS), zeta potential, and Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR). In addition, some stability parameters such as Encapsulation Efficiency (EE), pH and electrical conductivity were evaluated as a function of time. Since Brazil and most tropical and subtropical countries have drastically suffered with the diseases transmitted by the *A. aegypti* vector, the larvicidal efficiency of the developed formulation was tested in this work aiming an alternative domestic larvae control.

2. MATERIALS AND METHODS

2.1. Essential oil characterization

L. alba leaves were collected in Manaus/AM-Brazil at the Brazilian Agricultural Research Corporation (EMBRAPA, voucher specimen n° 191732, SISGEN n° A26CD5E). A portion of 150 g of powdered leaves were subjected to a Clevenger-type apparatus for 2.5 h at 100 °C. Yield was obtained by the ratio of the extracted EO volume to the mass of dried leaves. The refraction index (RI) was measured at 20 °C

using an Atago Master Refractometer. Density was calculated at 20 °C based on previous work (Mar et al., 2018).

GC-FID and GC-MS analyses were carried out on a Shimadzu™ GC2010-FID and GCMS-QP2010 instruments, respectively, using a DB-5 (0.25 mm × 30 m, 0.25 µm coating thickness) fused silica capillary column. The operating conditions were based on the work reported by (Mar et al., 2018). The chemical constituents were identified from a C_7–C_{30} *n*-alkane homologous series, as well as through published data (Adams, 2007), Wiley 7.0 Registry of Mass Spectral Data (McLafferty et al., 1991) and Pherobase (El-Sayed, 2014).

2.2. DPPH• radical scavenging ability and acetylcholinesterase (AChE) inhibitory assay

The DPPH• radical scavenging ability of the EO was evaluated according to previous report (Molyneux, 2004). Trolox was used as positive control from 3.9 µg/mL to 1,000.0 µg/mL. The AChE inhibitory assay was performed according to (Senol et al., 2015). Neostigmine was used as positive control from 31.2 µg/mL to 1,000.0 µg/mL. Assays were carried out in triplicate.

2.3. PCL-based nanoparticles

PCL-based nanoparticles were prepared according to previous report (Christofoli et al., 2015), with modifications. Solutions I and II were prepared. Solution I was constituted of PCL (0.15 g) and acetone (15 mL). Solution II was prepared using Tween 80 (0.05 g) and distilled water (30 mL). Solution I was heated to 40 °C until complete solubilization of PCL. The EO was mixed to solution I, which was added drop-by-drop to solution II, allowing the nanoparticles precipitation. Acetone was totally removed. The pH of the formulation was adjusted to 4 using HCl 1M. Finally, two preservatives were added under constant stirring: phenoxyethanol/2-methyl-2H-isothiazolin-3-one (NE, 15 µL) or sodium benzoate (SB, 0.015 g). The formulations were maintained at 25 °C.

2.4. Atomic Force Microscopy (AFM) analysis

The 2D topographic AFM images of the unloaded and loaded PCL-based nanoparticles were obtained on an Atomic Force Microscope (Innova, Bruker). The operating conditions were as follow: area of (10 × 10) μm^2; contact mode of operation; (296 ± 1) K; (40 ± 1)% RH; 512 × 512 pixels; scan rate of 1 Hz. Particle size distribution was estimated using the program ImageJ (Schneider et al., 2012).

2.5. Dynamic Light Scattering (DLS) and zeta potential

DLS equipment coupled in a Zetasizer Nano ZS90 device (Malvern Instruments, Malvern, UK) was used for particle size distribution. The zeta potential values (in mV) were measured from pH = 3 to 10 at 25 °C. Measurements were performed in triplicate.

2.6. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Spectroscopic analysis (ATR-FTIR) was performed on a Shimadzu IR Prestige-21 spectrophotometer, IRsolution software version 1.6, in the range of 1.000 to 4.000 cm^{-1}.

2.7. Encapsulation efficiency (EE) and stability evaluation

Nanoparticles were separated by centrifugation (20.000 rpm) and the supernatant absorbance was used to determine the amount of free EO. The EE was estimated by the ratio between the amount of encapsulated EO and the absolute concentration used in formulation. The pH and electrical conductivity parameters were evaluated for 50 days using a pHmeter Kasvi and a Bel conductivity meter model W12D, respectively. Measurements were performed in triplicate.

2.8. *In-vitro* controlled-release of essential oil

Controlled-release assays were carried out using dialysis tubing cellulose membrane suspended in water (pH = 4, 7 and 10) at 25 °C. Absorbances were measured at regular time on an UV–vis spectrophotometer. The concentration of the released EO was determined using a standard curve (Ghasemishahrestani et al., 2015). The experiments were carried out in triplicate.

2.9. Larvicidal bioassay and formulation efficiency

Groups of 10 *A. aegypti* larvae in the 3rd larval stage were placed in vials containing 1 mL of distilled water, 100 µL of larval feed and 100 µL of EO/DMSO solutions (70 to 150 mg/mL). DMSO (1 mL) and Bti (1 mg/mL) were used as negative and positive control, respectively. The experiment was carried out in triplicate containing five replicates. Data were analyzed using the POLO PC® program (Russell et al., 1977), with fiducial limit of 95% (Barci et al., 2009).

Larvicidal bioassay was also conducted to evaluate the formulation efficacy. Groups of 10 larvae in the 3rd larval stage were placed in vials containing 10 mL of formulation. The formulation was diluted in water (pH = 7) in the proportions of 1:1, 1:0.6; 1:0.4; 1:0.25; 1:0.1. The formulation containing unloaded nanoparticles (10 mL) and thymol (3 µg/mL) were used as negative and positive control, respectively.

3. RESULTS AND DISCUSSION

3.1. Essential oil characterization

The optimum time of EO extraction was found to be 120 min (Camêlo et al., 2011; Da Silva et al., 2017). The relative density of the EO was measured around 0.936 g/cm^3 and the estimated refraction index (RI) was 1.492. The EO yield was 0.62% (w/w). However, it should be optimized according to the season and site collecting. A yield of 0.21% (Santos et al., 2016) was reported previously considering the same amount of dried leaves.

The chemical constituents of the EO are shown in **Table 1**. Ten constituents were identified, representing 97.24% of the EO *in natura*. Monoterpenes (82.9%) and sesquiterpenes (11.1%) represent the main constituent groups. Carvone (54.5%) and limonene (21.5%) were quantified as major constituents, totalizing 76.0% of the whole chemical composition. For this reason, this EO was classified as a chemotype III. The EO from leaves of *L. alba* collected in the Northern Brazil presented three chemotypes (Da Silva et al., 2017). Some studies reported similar chemical composition of *L. alba* essential; however, quantitative changes were observed in *L. alba* grown in different regions (Castro et al., 2002; Silva et al., 2006).

	Constituent	AI_{calc}	$AI_{lit.}$	(%)
1	butyl acetate	801	807	3.24
2	mircene	990	988	3.61
3	limonene	1026	1024	21.49
4	*γ*-terpinene	1049	1054	1.06
5	linalool	1100	1095	0.68
6	carvone	1244	1239	54.54
7	piperitetone	1340	1340	1.51
8	α-copaene	1377	1374	1.86
9	*E*-cariophyllene	1420	1417	3.25
10	germacrene D	1483	1480	4.80
11	*δ*-cadinene	1525	1522	1.20
Total				**97.24**
Total monoterpenes				82.89
Hydrocarbon monoterpenes				26.16
Oxygenated monoterpenes				56.76
Total sesquiterpenes				1.11
Other constituents				3.24

AI: Arithmetic index relative to C_7–C_{30} *n*-alkanes on HP-5 column.

Table 1. Essential oil composition from leaves of *Lippia alba*.

3.2. DPPH• radical scavenging activity and acetylcholinesterase (AChE) inhibitory assay

The evaluation of the DPPH• radical scavenging of the EO *in natura* (1 mg/mL) presented no significant activity. (Puertas-Mejía et al., 2002) reported low activity of the EO from *L. alba* (chemotype III) using 2.8 x 10^5 mg/mL. This chemotype do not present phenolic groups nor flavonoids, which are closely associated to the DPPH• radical scavenging.

AChE has an important role in the nervous system (Gutierres et al., 2014), being the target site of inhibition by organophosphorus and carbamate pesticides (Lionetto et al., 2012). The tested EO presented no significant enzymatic activity in concentration up to 1,000.0 µg/mL. The AChE inhibitory activity of limonene was reported previously; however, carvone presents weak inhibition (Abdelgaleil et al., 2009). Although limonene is one of the chemical constituents of the tested EO, the high concentration of carvone may have influenced its enzymatic activity. In order to improve the AChE activity, higher concentrations of EO would be required (López and Pascual-Villalobos, 2010).

3.3. Larvicidal assay using the essential oil *in-natura*

EO have been considered as potential sources of bioactive compounds (Mar et al., 2018). The EO *in natura* from *L. alba* can be considered an alternative controlling agent of *A. aegypti* larvae. After 72 h of exposure, a moderate larvicidal potential was observed. LD_{50} and LD_{90} were found around (105.9 ± 0.1) µg/mL and (129.7 ± 0.1) µg/mL, respectively.

Although the EO *in natura* did not present significant antioxidant nor enzymatic activity, some studies reported its larvicidal and insecticidal effects (Vera et al., 2014). Furthermore, isolated carvone or limonene also showed significant effect against *C. quinquefasciatus*, *A. aegypti* and *A. stephensi* larvae. The LD_{50} values of 8.83 mg/mL, 12.01 mg/mL and 14.07 mg/mL were obtained for the isolated limonene, respectively. (Da Botas et al., 2017) also reported the larvicidal activity against *A. aegypti* of limonene obtained from the EO of *B. reticularia*. Some other reports also confirmed the larvicidal and insecticidal activity of the isolated limonene against *A. aegypti*, *A. albopictus* and *Sitophilus oryzae* (Cheng et al., 2009; Liu et al., 2013). The synergistic interaction between the EO constituents may results in larvicidal effect by contact, fumigation or ingestion (Mendes et al., 2017).

3.4. 2D topographic AFM images

The synthesized unloaded and loaded nanoparticles presented almost spherical morphology, as shown in **Figure 1**. The unloaded nanoparticles presented average size around (136 ± 2) nm and polydispersity index of 0.14. On the other hand, the nanoparticles loaded with EO presented average size around (266 ± 6) nm and polydispersity index of 0.22. The increase of the average diameter of the nanoparticles is due to the encapsulation of EO (Yilmaz et al., 2019). This result indicates that the EO was successfully encapsulated within the PCL-based nanoparticles.

3.5. Average diameter size and surface charge

DLS analysis was carried out to evaluate the average diameter of the produced PCL-based nanoparticles. The unloaded nanoparticles presented average size diameter between 236 nm and 275 nm and

polydispersity index of 0.2. (Schaffazick et al., 2003) reported that average diameters less than 300 nm and polydispersity index equal to or below 0.2 are attributed to uniform and stable colloidal system.

Table 2 shows the zeta potential values as a function of pH, allowing to correlate these values with the diameter size of the unloaded nanoparticles. The average diameter of the unloaded nanoparticles estimated by DLS was larger than those found by AFM. Prior to the AFM analysis, the colloidal system was subjected to a drying process to form a film over a glass slide, which may have influenced the average size of the nanoparticles. The zeta potential values presented, in module, higher surface charge at pH = 4, allowing the formulation stability. The isoelectric point was verified close to pH = 10. Then, instability may occur between pH from 5 to 10. The negative values of zeta potential may be due to the carbonyl radicals of the polymeric molecular structure. Nanoparticles were synthesized using a non-ionic surfactant and, according to the scientific literature, the surfactant interacts with the hydrophobic sites of PCL, resulting in the exposure of the surfactant chain radicals. This may result in negative charges on the nanoparticles surface, decreasing the zeta potential value and presenting the steric hindrance stabilization mechanism.

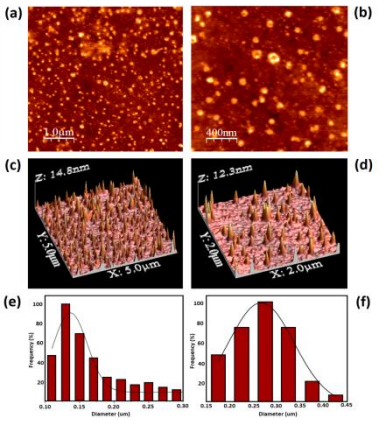

Figure1. (a-b) 2D topographic AFM images of the unloaded and loaded PCL-based nanoparticles, respectively; (c-d) 3D topographic AFM images of the unloaded and loaded nanoparticles, respectively and (e-f) diameter size frequency (%) of the unloaded and loaded nanoparticles, respectively.

Table 2. Zeta potential (mV) and nanoparticle size in different pH.

pH	Zeta potential (mV)	Nanoparticle size (nm)	PDI
3	-5.37 ± 0.06	244.0 ± 0,4	0.24
4	-8.02 ± 0.07	243 ± 5	0.18
5	-3.6 ±0.2	236 ± 1	0.24
6	-2.8 ± 0.9	275 ± 4	0.34
7	-1.6 ± 0.1	252 ± 3	0.27
8	-1.5 ± 0.4	269 ± 2	0.34
9	-1.2 ± 0.2	227 ± 1	0.27
10	-0.9 ± 0.1	238 ± 3	0.24

According to the steric stability, the emulsifier joins the nanoparticle surface, improving stability. On the other hand, the electrostatic stability occurs due to the repulsion between the particles resulting from their high surface charge (Campelo et al., 2017). Thus, values equal to or greater than 30 mV (in modulus) are important to the formulation stability (Roland et al., 2003). For this reason, the electrostatic stabilization is not the main mechanism of the developed formulation proposed here (da Rosa et al., 2015).

3.6. ATR-FTIR analysis

Figure 2 shows the ATR-FTIR spectra of the EO *in natura*, the unloaded and loaded nanoparticles. The bands found between 1,500 and 1,000 cm^{-1} are of complex descriptions. Due to the mixture of chemical constituents, some bands may be overlapped. For this reason, only the main vibrational modes of the major constituents (carvone and limonene) were identified.

The band at 3,333 cm^{-1} was assigned to the H_2C=R stretching of limonene, and a band at 1,676 cm^{-1} is due to the stretching of unsaturated carbonyl present in carvone. Angular deformation of CH_2–CH_3 related to primary or secondary carbons was observed in the regions of 1,440 and 1,369 cm^{-1}. Bands at 1,246 cm^{-1} and 1,110 cm^{-1} were assigned to the C=O bond (Gualdrón et al., 2013; Ramamoorthy and Rajiv, 2014; Zhang et al., 2017).

The spectra of the unloaded nanoparticles (in blue) and loaded with EO (in green) are quite similar. The band located at 1,639 cm^{-1} was attributed to the ester groups of PCL. The narrow and intense characteristic bands observed in the EO spectrum were not observed in the spectra of the unloaded or loaded nanoparticles. This fact

confirms the encapsulation of the EO within the developed PCL-based nanoparticles. For this reason, the ATR-FTIR technique can represent an efficient qualitative tool to verify the encapsulation of EO.

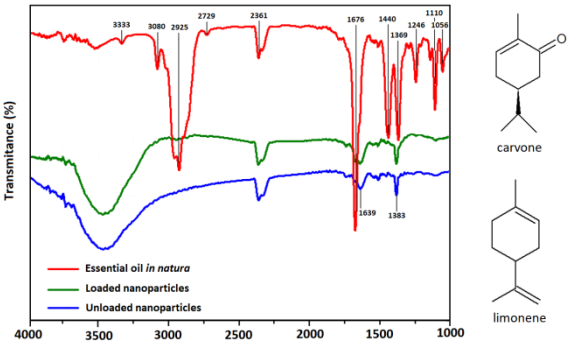

Figure 2. ATR-FTIR spectra of the EO *in natura*, the unloaded and loaded nanoparticles.

3.7. Formulation stability

EE was measured for all formulations until reach 70% (Xing et al., 2005). The encapsulation of EO promoted by the nanoprecipitation method was highly efficient, as shown in **Figure 3**. The initial EE was found around 95 – 100%. Some studies also reported high EE for formulations containing PCL-based nanoparticles (Grillo et al., 2012). The formulation containing encapsulated EO and no preservative presented considerable decreasing of EE after 30 days, reaching 66%. This fact occurred due to nanoparticle degradation, altering organoleptic properties such as color and smell. On the other hand, the addition of the preservatives NE and SB increased the formulation stability. The EE for both formulations presented similar behavior over time, reaching 70% simultaneously after 50 days.

The centrifugation test confirmed the preliminary stability of the formulation, resulting in no phase separation. Then, physical parameters such as pH and electrical conductivity were evaluated over time at 25 °C for the followed formulations: *(i)* unloaded nanoparticles with no preservative, *(ii)* loaded nanoparticles with no preservative, *(iii)* loaded nanoparticles containing the preservative NE and *(iv)* loaded nanoparticles containing the preservative SB. All

formulations were initially maintained at pH = 4, according to the zeta potential data.

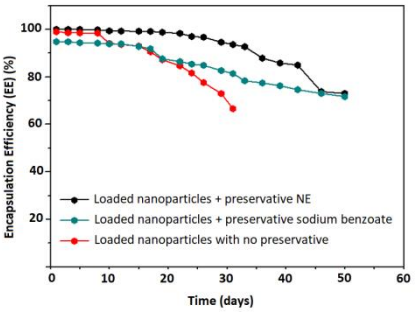

Figure 3. Encapsulation efficiency (EE) as a function of time.

The pH values increased over time, as shown in **Figure 4**. The *(i)* unloaded nanoparticles with no preservative presented increase of pH reaching 4.9 after 18 days, and then destabilized completely. The *(ii)* loaded nanoparticles with no preservative reached pH = 6, being stable for 30 days, and then destabilized. The formulations containing preservatives also presented increase of pH over time but reached 70% of EE after 50 days. The *(iii)* loaded nanoparticles containing the preservative NE presented initial pH = 4 and reached 6.9 after 50 days. The *(iv)* loaded nanoparticles containing the preservative SB presented initial pH = 4, increasing to 6.2. This formulation showed a sudden decrease in pH after 25 days, which may be related to interactions between its constituents.

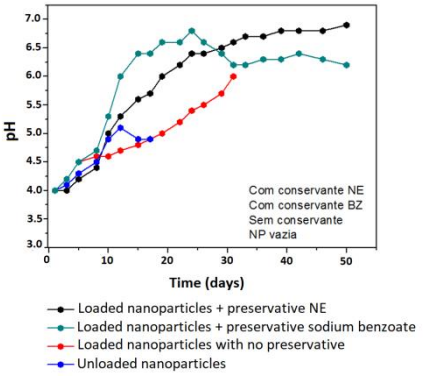

Figure 4. pH values of formulations as a function of time.

For all formulations the increasing of pH was correlated with decreasing of EE. This fact may be explained by the PCL degradation (Nobes and Marchessault, 1999). In addition, such degradation resulted in EO release from nanoparticles, decreasing the EE and increasing the pH values. This result corroborates with the zeta potential data, showing that the destabilization of the formulations tends to reach the isoelectric point.

The electrical conductivity of the formulations is shown in **Figure 5**. After addition of preservatives the electrical conductivity changed; however, without destabilizing the formulation. It is known that the preservative SB dissociates in water. Thus, cations from this preservative may have interacted with the negatively charged carriers available in the formulation, resulting in decreasing of electrical conductivity to about 460 µS/cm. On the other hand, the formulation containing the preservative NE presented initial electrical conductivity around 1890 µS/cm, which is slightly higher than those observed for the unloaded and loaded nanoparticles containing no preservative.

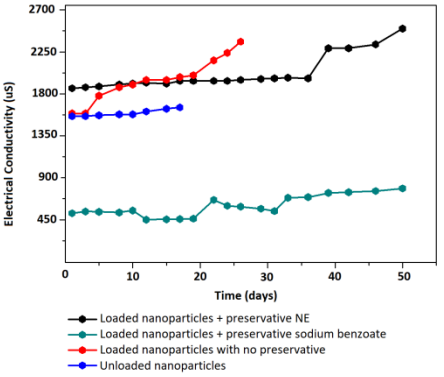

Figure 5. Electrical conductivity of formulations as a function of time.

All formulations presented a gradual increase of electrical conductivity over time, except for the unloaded nanoparticles containing no preservative. This increase may be related to release of EO due to the destabilization of the formulation. According to GC/MS data, the EO from *Lipia alba* is rich in chemical constituents

containing electronegative groups which, in solution, can increase the electrical conductivity values.

These data show that both preservatives NE and SB can be used in the proposed formulations. However, more regular behavior over time (as a function of EE, pH and electrical conductivity) was observed in the formulation containing the preservative NE. For this reason, this developed formulation was chosen to be applied in the larvicidal bioassay.

3.8. *In-vitro* controlled-release of essential oil

Figure 6 shows the controlled-release curves fitted by the Korsmeyer-Peppas model. The dashed horizontal line represents the reference value of LD_{90} obtained using the EO *in-natura* for the larvicidal bioassay. The LD_{90} was reached in all controlled-release assays. However, a significant difference in the releasing time was observed. At neutral and basic pH, the LD_{90} was reached after 50 min. However, at acid pH this concentration was reached after 180 min. The controlled-release curves at pH = 7 and 10 are similar, being rapid in the first minutes and gradual after approximately 25 min. The maximum EO concentration was released after 270 min, reaching 400 mg/mL. In contrast, the maximum EO concentration released at pH = 4 was about 160 mg/mL after 270 min. According to the zeta potential data, the optimum pH that guarantee the stability of the formulation is acid (pH = 4). Thus, at this pH a rapid release of EO, as well as higher released concentration, was not observed. However, when the formulation was allowed to release the EO at neutral or basic pH, rapid release and higher released concentration of EO was observed. This fact may be explained by the destabilization of the formulation when the pH value approaches the isoelectric point.

The controlled-release curves were better adjusted by the Korsmeyer-Peppas model (Korsmeyer and Peppas, 1983). Values of n were observed between 0.63 and 0.67 (**Table 3**), which reveal that the developed PCL-based nanoparticles released the EO according to the anomalous transport mechanism.

Table 3. Controlled-release kinect parameters according to the Korsmeyer-Peppas model.

parameters	pH 4	pH 7	pH 10
K	3.77	11.83	10.07
n	0.67	0.63	0.66
R^2	0.9604	0.9604	0.9801

3.9. Larvicidal bioassays

The loaded nanoparticles containing the preservative NE was submitted to the larvicidal bioassay with different proportions of water/formulation, as shown in **Table 4**. The LD_{90} was found around 129.7 µg/mL. The formulation showed high larvicidal activity: the undiluted and 1:1 (250 µg/mL) diluted formulation promoted 100% larvicidal effect after 24 h. These data corroborate with those found in the larvicidal bioassay considering only the EO *in-natura*. The 1:0.6 diluted formulation promoted 100% larvae mortality after 48 h. Since in this case the EO concentration is similar to the LD_{90} value, higher percentage of larvae mortality was observed in the first 24 h (87 ± 1) %, reaching 100% after 48 h. Dilutions below 1:0.6 did not result in 100% larvae mortality. However, the 1:0.25 dilution presented an expressive larvicidal potential after 24 h, reaching almost 80% larval mortality.

These results allowed important hypotheses about the observed larvicidal effect. The LD_{50} and LD_{90} were obtained only after 72 h when the larvicidal assay was performed using the EO *in-natura*. However, larvae mortality was observed before 24 h for some concentrations. This fact suggest that the formulation may present different mechanisms of action when compared to the EO *in-natura*. The nanoparticles may have penetrated the larvae cell membrane. This can be suggested because the mortality time was considerably reduced (from 72 h for the EO *in-natura* to 24 h for the loaded nanoparticles). Some authors report that most larvae ingest larger proportions of organic particulate matter up to around 50 µm (Eisenberg et al., 2000), allowing the incorporation of bioactive compounds in nanoparticles and facilitating the ingestion.

Table 4. Larvicidal bioassay using the colloidal system containing the preservative NE in different proportions of water/formulation.

	24 h			48 h		
Concentration (mg/mL)	**Concentration (v/v)**	**M**	**T (%)**	**Concentration (v/v)**	**M**	**T (%)**
500	Undiluted formulation	10.0	100.0 ± 0.0	Undiluted formulation	0.0	0.0 ± 0.0
250	1:1	10.0	100.0 ± 0.0	1:1	0.0	0.0 ± 0.0
125	1:0.6	8.7	87 ± 1	1:0.6	1.3	13 ± 1
62.5	1:0.25	7.7	77 ± 1	1:0.25	1.7	17 ± 1
31.2	1:0,4	1.7	17 ± 1	1:0.4	4.7	47 ± 2
15.6	1:0.1	0.3	3.0 ± 0.5	1:0.1	0.0	0.0 ± 0.0
--	Control	0.0	0.0 ± 0.0	Control	0.0	0.0 ± 0.0

M: Average number of dead larvae obtained in triplicate; T(%): Percentage of dead larvae.

Studies on the application of biodegradable nanoparticles as larvicidal formulations are still little explored. However, in this work the toxicity of the EO from *L. alba* was maintained after its encapsulation. The larvicidal results obtained from the loaded nanoparticles allowed the proposition of an application. As the results show that this formulation can be diluted to a ratio of 1: 0.25 to obtain larvicidal efficacy up to 24 h, it would be an alternative for domestic larvicidal control.

CONCLUSIONS

A larvicidal formulation based on the EO from *L. alba* encapsulated in PCL-based nanoparticles was successfully developed. Although no antioxidant nor enzymatic action was observed at the tested concentrations of EO, its larvicidal effect was observed. This larvicidal property is most probably due to the major chemical constituents of the EO (carvone and limonene), or also due to the synergism between the various compounds. The EE of the formulation containing the preservatives NE and SB exhibited similar behaviors over time. Both formulations reached EE = 70% simultaneously after 50 days. The larvicidal bioassay based on the loaded nanoparticles showed high *in vitro* activity against the tested larvae. We can suggest that nanoparticles penetrated through the larval cell membrane because the LD_{90} was reached in reduced time. The encapsulation of natural

and bioactive compounds becomes an interesting alternative for larvicidal application, besides the proposed formulation does not present harmful chemical components to the environment. This formulation can be diluted to a ratio up to 1:0.25 to obtain larvicidal efficacy for 24 h, representing a good alternative for domestic larvicidal control.

ACKNOWLEDGMENTS

The authors thank FAPEAM (Amazonas Estratégico Processo 062.01305 / 2018) and CNPq (MCTI / CT-AGRONEGÓCIO / CT-AMAZÔNIA, Grant Number 403496/2013 and Universal Grant Number 401508 / 2016-1) for the financial support and fellowships.

REFERENCES

1. Abdelgaleil, S.A.M., Mohamed, M.I.E., Badawy, M.E.I., El-arami, S.A.A., 2009. Fumigant and Contact Toxicities of Monoterpenes to *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) and their inhibitory effects on acetylcholinesterase activity. J. Chem. Ecol. 35, 518–525. https://doi.org/10.1007/s10886-009-9635-3.
2. Adams, R.P., 2007. Identification of essential oil components by gas chromatography/mass spectrometry, 4th ed, Journal of the American Society for Mass Spectrometry. Allured Books. https://doi.org/10.1163/_q3_SIM_00374.
3. Banik, B.L., Fattahi, P., Brown, J.L., 2016. Polymeric nanoparticles: The future of nanomedicine. Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 8, 271-299. https://doi.org/10.1002/wnan.1364
4. Barci, L.A.G., de Almeida, J.E.M., de Campos Nogueira, A.H., do Prado, A.P., 2009. Determinação da CL90 e TL90 do isolado IBCB66 de *Beauveria bassiana* (Ascomycetes: Clavicipitaceae) para o controle de *Rhipicephalus* (Boophilus) *microplus* (Acari: Ixodidae). Rev. Bras. Parasitol. Vet. 18, 34–39. https://doi.org/10.4322/rbpv.018e1006.
5. Camêlo, L.C.A., Blank, A.F., Ehlert, P.A.D., Carvalho, C.R.D., Arrigoni-Blank, M. de F., Mattos, J., 2011. Caracterização morfológica e agronômica de acessos de erva-cidreira brasileira [*Lippia alba* (Mill.) N. E. Br.]. Sci. Plena 7, 1–8.
6. Campelo, P.H., Junqueira, L.A., Resende, J.V. de, Zacarias, R.D., Fernandes, R.V. de B., Botrel, D.A., Borges, S.V., 2017. Stability of lime essential oil emulsion prepared using biopolymers and

ultrasound treatment. Int. J. Food Prop. 20, S564–S579. https://doi.org/10.1080/10942912.2017.1303707.

7. Castro, D.M., Ming, L.C., Marques, M.O.M., 2002. Composição fitoquímica dos óleos essenciais de folhas de *Lippia alba* (Mill.)N.E.Br. em diferentes épocas de colheita e partes do ramo. Rev. Bras. Plantas Med. 4, 75-99.
8. Cheng, S.S., Chang, H.T., Lin, C.Y., Chen, P.S., Huang, C.G., Chen, W.J., Chang, S.T., 2009. Insecticidal activities of leaf and twig essential oils from *Clausena excavata* against *Aedes aegypti* and *Aedes albopictus* larvae. Pest Manag. Sci. 65, 339–343. https://doi.org/10.1002/ps.1693.
9. Christofoli, M., Costa, E.C.C., Bicalho, K.U., de Cássia Domingues, V., Peixoto, M.F., Alves, C.C.F., Araújo, W.L., de Melo Cazal, C., 2015. Insecticidal effect of nanoencapsulated essential oils from *Zanthoxylum rhoifolium* (Rutaceae) in *Bemisia tabaci* populations. Ind. Crops Prod. 70, 301–308. https://doi.org/10.1016/j.indcrop.2015.03.025.
10. Da Botas, G.S., Cruz, R.A.S., De Almeida, F.B., Duarte, J.L., Araújo, R.S., Souto, R.N.P., Ferreira, R., Carvalho, J.C.T., Santos, M.G., Rocha, L., Pereira, V.L.P., Fernandes, C.P., 2017. *Baccharis reticularia* DC. and Limonene Nanoemulsions: Promising Larvicidal Agents for *Aedes aegypti* (Diptera: Culicidae). Molecules 22, 1–14. https://doi.org/10.3390/molecules22111990.
11. da Rosa, C.G., de Oliveira Brisola Maciel, M.V., de Carvalho, S.M., de Melo, A.P.Z., Jummes, B., da Silva, T., Martelli, S.M., Villetti, M.A., Bertoldi, F.C., Barreto, P.L.M., 2015. Characterization and evaluation of physicochemical and antimicrobial properties of zein nanoparticles loaded with phenolics monoterpenes. Colloids Surfaces A Physicochem. Eng. Asp. 481, 337–344. https://doi.org/10.1016/j.colsurfa.2015.05.019.
12. Da Silva, A.C.C., Barbosa, F.G., Mafezoli, J., Da, M., De Oliveira A E Terezinha, C.F., De Oliveira, F., 2017. HS-SPME as an efficient tool for discriminating chemotypes of *Lippia alba* (Mill.) N. E. Brown. Quim. Nov. 40, 42–46. https://doi.org/10.21577/0100-4042.20160156.
13. Eisenberg, J., Washburn, J., Schreiber, S., 2000. Generalist feeding behaviors of *Aedes sierrensis* larvae and their effects on protozoan populations. Ecol. Soc. Am. 81, 921–935. https://doi.org/10.2307/177167 https://www.jstor.org/stable/177167.
14. El-Sayed, A.M., 2014. The Pherobase: Database of pheromones and semiochemicals. http://www.pherobase.net/database/control/control-approach-Mass%20trapping-all.php.

15. Ghaderi-Ghahfarokhi, M., Barzegar, M., Sahari, M.A., Ahmadi Gavlighi, H., Gardini, F., 2017. Chitosan-cinnamon essential oil nano-formulation: Application as a novel additive for controlled release and shelf life extension of beef patties. Int. J. Biol. Macromol. 102, 19–28. https://doi.org/10.1016/j.ijbiomac.2017.04.002.
16. Ghasemishahrestani, Z., Mehta, M., Darne, P., Yadav, A., Ranade, S., 2015. Tunable synthesis of gelatin nanoparticles employing sophorolipid and plant extract, a promising drug carrier. World J. Pharm. Pharm. Sci. 4, 1365–1381.
17. Glamočlija, J., Soković, M., Tešević, V., Linde, G.A., Colauto, N.B., 2011. Chemical characterization of Lippia alba essential oil: An alternative to control green molds. Brazilian J. Microbiol. 42, 1537–1546. https://doi.org/10.1590/S1517-83822011000400041.
18. Grillo, R., dos Santos, N.Z.P., Maruyama, C.R., Rosa, A.H., de Lima, R., Fraceto, L.F., 2012. Poly(e-caprolactone) nanocapsules as carrier systems for herbicides: Physico-chemical characterization and genotoxicity evaluation. J. Hazard. Mater. 231–232, 1–9. https://doi.org/10.1016/j.jhazmat.2012.06.019.
19. Gualdrón, A.F., Becerra, E.N., Peña, D.Y., Gutiérrez, J.C., Becerra, H.Q., 2013. Inhibitory effect of Eucalyptus and *Lippia Alba* essential oils on the corrosion of mild steel in hydrochloric acid. J. Mater. Environ. Sci. 4, 143–158.
20. Gutierres, J.M., Carvalho, F.B., Schetinger, M.R.C., Agostinho, P., Marisco, P.C., Vieira, J.M., Rosa, M.M., Bohnert, C., Rubin, M.A., Morsch, V.M., Spanevello, R., Mazzanti, C.M., 2014. Neuroprotective effect of anthocyanins on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia in rats. Int. J. Dev. Neurosci. 33, 88–97. https://doi.org/10.1016/J.IJDEVNEU.2013.12.006.
21. Islam, M., Amin, M., Ahmed, M., Khatun, S., Rahman, ML, Siddiqui, S., Rahman, MA, Kudrat, E., Mannan, M., 2018. *In-vitro* antimicrobial activity of essential oils and different organic extracts of *Lippia alba*. J. Phytochem. Biochem. 2, 1–5.
22. Kamaly, N., Yameen, B., Wu, J., Farokhzad, O.C., 2016. Degradable controlled-release polymers and polymeric nanoparticles: mechanisms of controlling drug release. Chem. Rev. 116, 2602–2663. https://doi.org/10.1021/acs.chemrev.5b00346.
23. Karunaratne, P., De Silva, P., Weeraratne, T., Surendran, N., 2018. Insecticide resistance in mosquitoes: Development, mechanisms and monitoring. Ceylon J. Sci. 47, 299. https://doi.org/10.4038/cjs.v47i4.7547.

24. Karuppuswamy, P., Reddy, J., 2015. Polycaprolactone nano fibers for the controlled release of tetracycline hydrochloride. Mater. Lett. 141, 180–186. https://doi.org/10.1016/j.matlet.2014.11.044
25. Korsmeyer, R., Peppas, N., 1983. Macromolecular and modeling aspects of swelling controlled systems. In Controlled Release Delivery Systems, eds. 77–90.
26. Leta, S., Beyene, T.J., De Clercq, E.M., Amenu, K., Kraemer, M.U.G., Revie, C.W., 2018. Global risk mapping for major diseases transmitted by *Aedes aegypti* and *Aedes albopictus*. Int. J. Infect. Dis. 67, 25–35. https://doi.org/10.1016/j.ijid.2017.11.026.
27. Lionetto, M.G., Caricato, R., Calisi, A., Schettino, T., 2012. Acetylcholinesterase inhibition as a relevant biomarker in environmental biomonitoring: new insights and perspectives, in: Visser, J.E. (Ed.), Ecotoxicology around the Globe. Nova Science Publishers, Inc., pp. 87–115.
28. Liu, X.C., Dong, H.W., Zhou, L., Du, S.S., Liu, Z.L., 2013. Essential oil composition and larvicidal activity of *Toddalia asiatica* roots against the mosquito *Aedes albopictus* (Diptera: Culicidae). Parasitol. Res. 112, 1197–1203. https://doi.org/10.1007/s00436-012-3251-9.
29. López, M.D., Pascual-Villalobos, M.J., 2010. Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. Ind. Crops Prod. 31, 284–288. https://doi.org/10.1016/J.INDCROP.2009.11.005.
30. Mar, J.M., Silva, L.S., Azevedo, S.G., França, L.P., Goes, A.F.F., dos Santos, A.L., de A. Bezerra, J., de Cássia S. Nunomura, R., Machado, M.B., Sanches, E.A., 2018. *Lippia origanoides* essential oil: An efficient alternative to control *Aedes aegypti, Tetranychus urticae* and *Cerataphis lataniae*. Ind. Crops Prod. 111. https://doi.org/10.1016/j.indcrop.2017.10.033.
31. McLafferty, F.W., Stauffer, D.B., Loh, S.Y., 1991. Comparative evaluations of mass spectral data bases. J Am Soc Mass Spectrom 2, 438–440. https://doi.org/10.1016/1044-0305(91)85011-T.
32. Mendes, L.A., Martins, G.F., Valbon, W.R., da Silva de Souza, T., Menini, L., Ferreira, A., da Silva Ferreira, M.F., 2017. Larvicidal effect of essential oils from Brazilian cultivars of guava on *Aedes aegypti* L. Ind. Crops Prod. https://doi.org/10.1016/j.indcrop.2017.07.034.
33. Molyneux, P., 2004. The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26, 211–219. https://doi.org/10.1287/isre.6.2.144.

34. Nobes, G.A.R., Marchessault, R.H., 1999. Enzymology of the synthesis and degradation of polyhydroxyalkanoates, in: Biopolymers. ACS Symposium Series, Canada, pp. 110–145. https://doi.org/10.1021/bk-1999-0723.ch009.
35. Othman, R., Vladisavljević, G.T., Nagy, Z.K., Holdich, R.G., 2016. Encapsulation and controlled release of rapamycin from polycaprolactone nanoparticles prepared by membrane micromixing combined with antisolvent precipitation. Langmuir 32, 10685–10693. https://doi.org/10.1021/acs.langmuir.6b03178.
36. Puertas-Mejía, M., Hillebrand, S., Stashenko, E., Winterhalter, P., 2002. *In vitro* radical scavenging activity of essential oils from Columbian plants and fractions from oregano (*Origanum vulgare* L.) essential oil. Flavour Fragr. J. 17, 380–384. https://doi.org/10.1002/ffj.1110.
37. Ramamoorthy, M., Rajiv, S., 2014. L-carvone-loaded nanofibrous membrane as a fragrance delivery system: Fabrication, characterization and *in vitro* study. Flavour Fragr. J. 29, 334–339. https://doi.org/10.1002/ffj.3209.
38. Roland, I., Piel, G., Delattre, L., Evrard, B., 2003. Systematic characterization of oil-in-water emulsions for formulation design. Int. J. Pharm. 263, 85–94. https://doi.org/10.1016/S0378-5173(03)00364-8.
39. Russell, R.M., Robertson, J.L., Savin, N.E., 1977. POLO: A New Computer Program for Probit Analysis. Bull. Entomol. Soc. Am. 23, 209–213. https://doi.org/10.1093/besa/23.3.209
40. Santos, N., Pascon, R., Vallim, M., Figueiredo, C., Soares, M., Lago, J., Sartorelli, P., 2016. Cytotoxic and antimicrobial constituents from the essential oil of *Lippia alba* (Verbenaceae). Medicines 3, 22. https://doi.org/10.3390/medicines3030022.
41. Schaffazick, S.R., Guterres, S.S., De Lucca Freitas, L., Pohlmann, A.R., 2003. Caracterização e estabilidade físico-química de sistemas poliméricos nanoparticulados para administração de fármacos. Quim. Nova 26, 726–737. https://doi.org/10.1590/S0100-40422003000500017
42. Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.
43. Senol, F.S., Orhan, I.E., Ustun, O., 2015. In vitro cholinesterase inhibitory and antioxidant effect of selected coniferous tree species. Asian Pac. J. Trop. Med. 8, 269–275. https://doi.org/10.1016/S1995-7645(14)60329-1.
44. Shanmugaraj, B., Malla, A., Ramalingam, S., 2019. Epidemiology, clinical features and transmission of re-emerging arboviral infection

chikungunya. Asian Pac. J. Trop. Biomed. 9, 135. https://doi.org/10.4103/2221-1691.256726.

45. Silva, N.A., Oliveira, F.F., Costa, L.C.B., Bizzo, H.R., Oliveira, R.A., 2006. Caracterização química do óleo essencial da erva cidreira (*Lippia alba* (Mill.) N. E. Br.) cultivada em Ilhéus na Bahia. Rev. Bras. Plantas Med. 8, 52–55.
46. Souza, J.M., Caldas, A.L., Tohidi, S.D., Molina, J., Souto, A.P., Fangueiro, R., Zille, A., 2014. Properties and controlled release of chitosan microencapsulated limonene oil. Brazilian J. Pharmacogn. 24, 691–698. https://doi.org/10.1016/j.bjp.2014.11.007.
47. Vera, S.S., Zambrano, D.F., Méndez-Sanchez, S.C., Rodríguez-Sanabria, F., Stashenko, E.E., Duque Luna, J.E., 2014. Essential oils with insecticidal activity against larvae of *Aedes aegypti* (Diptera: Culicidae). Parasitol. Res. 113, 2647–2654. https://doi.org/10.1007/s00436-014-3917-6.
48. Xing, F., Cheng, G., Yi, K., Ma, L., 2005. Nanoencapsulation of capsaicin by complex coacervation of gelatin, acacia, and tannins. J. Appl. Polym. Sci. 96, 2225–2229. https://doi.org/10.1002/app.21698
49. Yilmaz, M.T., Yilmaz, A., Akman, P.K., Bozkurt, F., Dertli, E., Basahel, A., Al-Sasi, B., Taylan, O., Sagdic, O., 2019. Electrospraying method for fabrication of essential oil loaded-chitosan nanoparticle delivery systems characterized by molecular, thermal, morphological and antifungal properties. Innov. Food Sci. Emerg. Technol. 52, 166–178. https://doi.org/10.1016/J.IFSET.2018.12.005.
50. Zhang, S., Zhou, Y., Jin, S., Meng, X., Yang, L., Wang, H., 2017. Preparation and structural characterization of corn starch–aroma compound inclusion complexes. J. Sci. Food Agric. 97, 182–190. https://doi.org/10.1002/jsfa.7707.
51. Zhu, F., Lavine, L., O'Neal, S., Lavine, M., Foss, C., Walsh, D., 2016. Insecticide resistance and management strategies in urban ecosystems. Insects 7, 1–26. https://doi.org/10.3390/insects7010002.