**Lippia gracilis** essential oil in β-cyclodextrin inclusion complexes: An environmentally safe formulation to control *Aedes aegypti* larvae

Juliana G. Galvão¹, Patrícia Cerpe¹, Darlisson A. Santos², Joyce K. M. C. Gonsalves¹, Adriana J. Santos¹, Rafaela K. V. Nunes¹, Ana Amélia M. Lira¹, Péricles B. Alves², Roseli La Corte³, Arie F. Blank⁴, Gabriel F. Silva⁵, Sócrates C. H. Cavalcanti¹, Rogéria S. Nunes†

¹Pharmacy Department, Federal University of Sergipe, 49100-000, São Cristóvão, SE, Brazil
²Chemistry Department, Federal University of Sergipe, 49100-000, São Cristóvão, SE, Brazil
³Morphology Department, Federal University of Sergipe, 49100-000, São Cristóvão, SE, Brazil
⁴Agronomy Department, Federal University of Sergipe, 49100-000, São Cristóvão, SE, Brazil
⁵Chemical Engineering Department, Federal University of Sergipe, 49100-000, São Cristóvão, SE, Brazil

* Corresponding author

Pharmacy Department – Federal University of Sergipe, Address: Av. Marechal Rondon, s/n, Cidade Universitária, 49000-100, São Cristóvão, Sergipe, Brasil.
E-mail: rogeria.ufs@hotmail.com Tel. +55-79-31946319/ Fax: +55-79-31946319

**Abstract**

**BACKGROUND:** One of the most efficient ways to prevent arboviruses, such as dengue fever, yellow fever, chikungunya and Zika, is by controlling their vector, the *Aedes aegypti*. Since this vector is becoming resistant to most larvicides used, the development of new larvicides should be considered. β-cyclodextrin (β-CD) complexes have been investigated as an interesting approach for enabling the use of essential oils in water as larvicides. Thus, this study consists in the development of *Lippia gracilis* essential oil (LGEO) and β-CD inclusion complexes for *Ae. aegypti* control.

**RESULTS:** Thermal analysis clearly indicated the formation of complexes by the kneading and co-evaporation methods. Gas Chromatography analysis showed that the
kneading without co-solvent (KW) presented the highest inclusion content (~15%) of the LGEO major component. Moreover, KW revealed LC$_{50}$ (33 ppm) lower than LGEO LC$_{50}$ (39 ppm), in other words, complexing LGEO in β-CD improved the larvicidal activity. In addition, the LGEO complexed in β-CD (KW) was no harmful to non-target population in the concentrations needed to control Ae. aegypti larvae.

**CONCLUSION:** The inclusion complex with LGEO is a feasible formulation being economically viable, easy-to-apply, without impact in non-target animals and, therefore a potential alternative as a larvicide for Ae. aegypti control.

**Keywords:** Aedes aegypti; inclusion complexes; larvicidal activity; arboviruses; Artemia sp.

1. **INTRODUCTION**

    *Aedes aegypti* is the main vector known to carry yellow fever, dengue, and emerging arboviruses such as chikungunya and Zika. These diseases have been responsible for a great number of morbidity and mortality around the world. Moreover, microcephaly in newborns and Guillain-Barré syndrome were diagnosed and associated with Zika virus.$^{1-3}$

    Since controlling the vector is currently one of the most effective ways to prevent the aforementioned diseases, several million dollars are spent every year in order to eradicate the vector.$^{2}$ Many synthetic larvicides such as organophosphates (e.g. temephos) have been used in several countries. Nevertheless, the intensive use of these pesticides presents several problems including resistance of mosquito larvae and toxic effects on the environment by contamination of soil, water and air.$^{4}$ In order to circumvent these problems, natural products (e.g. essential oils) with larvicidal activity have been studied.$^{5}$
Essential oils are excellent candidates as larvicides due to their high activity, availability in tropical countries, and affordability.\textsuperscript{6} \textit{Lippia gracilis} essential oil (LGEO) is composed of a mixture of terpenes and sesquiterpenes having carvacrol as its major constituent and has demonstrated a strong larvicidal activity against \textit{Ae. aegypti} larvae.\textsuperscript{2} Since LGEO presents low aqueous solubility, easy oxidation and volatility, formulations need to be developed to avoid its degradation and to increase solubility, as well as incorporating the essential oil in a viable larvicidal formulation.\textsuperscript{7,8}

Several formulations have been developed in order to optimize the use of larvicides such as nanoemulsions\textsuperscript{9}, \textit{in situ} gelling nanostructured surfactant systems\textsuperscript{10} and cyclodextrins\textsuperscript{11}.

Cyclodextrins are cyclic oligomers composed of six, seven or eight $\alpha$-D-glucopyranose units on a ring-like structure, and are commonly available in three types $\alpha$- (alpha), $\beta$- (beta) and $\gamma$- (gamma) presenting enclosed cavities approximately of 4.7-5.3, 6.0-6.5, and 7.5-8.3 Å in diameter, respectively.\textsuperscript{12,13} The external part of the cyclodextrin is hydrophilic whereas the interior cavity is hydrophobic enabling the solubilization of nonpolar solutes in water.\textsuperscript{13}

Among cyclodextrins, the $\beta$-cyclodextrin ($\beta$-CD) is the most commonly used due to its availability, price and cavity size suitable for a wide range of guest molecules. Inclusion complexes formation is determined by guest properties (molecular size, geometry and polarity) and should be suitable to $\beta$-CD size cavity.\textsuperscript{14}

Thus, this work aims the complexation of LGEO with $\beta$-cyclodextrin, using kneading and co-evaporation methods, evaluating the influence of co-solvent in the preparation methods. Phase solubility studies were performed and the inclusion complexes were evaluated. In addition, the LGEO content (%) was measured and the
inclusion complex with higher content of the major component of LGEO was evaluated for its biological activity against *Ae. aegypti* larvae.

2. MATERIALS AND METHODS

2.2 Materials

*Lippia gracilis* leaves were collected from accession LGRA-108 of the Active Germplasm Bank (AGB) of Medicinal and Aromatic Plants of the Federal University of Sergipe (Voucher n° 14734), located in the Research Farm called “Campus Rural da UFS”. Defoliation was performed manually and dried in an oven with forced air circulation, at 40°C, for five days. β-CD and carvacrol (~ 98%) was acquired by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All the other reagents were purchased from Synth (Diadema, SP, Brazil).

2.3 Methods

2.3.1 Essential oil extraction

The essential oil extraction was performed in the Laboratory of Plant Genetic Resources and Essential Oils of the Federal University of Sergipe. 75g of dried leaves were submitted to hydrodistillation in a Clevenger-type apparatus for 140 minutes to yield a yellowish oil. The essential oil was separated from the aqueous phase, Na₂SO₄ was added to remove the remaining water and the resulting oil kept refrigerated until further analysis or preparation of inclusion complexes.

2.3.2 Identification of essential oil constituents
GC/MS analysis of LGEO was performed in a GC/MS Shimadzu QP5050A equipment using a J&W Scientific (5%-phenyl-95%-dimethylpolysiloxane) fused silica column (30 m x 0.25 mm; film thickness 0.25 µm), under the following conditions: helium as carrier gas at 1.0 mL/min; injector split at 250°C (split ratio of 1/83); column temperature program 50°C for 1.5 minute, with 4°C increase per minute to 200°C, then 10°C/min to 250°C, ending with a 10 min isothermal at 300°C; and detector at 280°C. The mass spectra were performed at 70 eV and 0.50 scan/s from 40 to 500 Da.

Percentage composition was calculated using the peak normalization method. The peaks were identified by comparing them with their Kovats retention indices, relative to a n-alkane homologous series (C₈-C₁₈), and obtained by the same conditions of the sample. The identification of individual components in the essential oil was performed by computerized matching of the acquired mass spectra with those stored in NIST107, NIST21, and Willey8 (80% of similarity index) mass spectral library, in the GC/MS data system.

2.3.3 Phase solubility studies

Phase solubility diagrams were acquired according to Higuchi and Connors method. For this purpose an excess of LGEO (an amount higher than the solubility of LGEO in water) was added to 10 mL of an aqueous solution containing β-CD with crescent concentrations of 0, 2 x 10⁻³, 4 x 10⁻³, 6 x 10⁻³, 8 x 10⁻³ and 10 x10⁻³ mol.L⁻¹ under magnetic stirring for 24h. These were kept in a thermostatic bath at 25, 35, and 45°C. The samples were then centrifuged at 3000 rpm for 10 minutes and filtered with an ultra-filtration membrane (0.45 µm). Quantification was performed in triplicate on a UV-Vis equipment FEMTO 800XI at 266 nm. The results were plotted using LGEO.
concentration in function of β-CD concentration. Then, the stability constant ($K_{1:1}$) was obtained according the equation proposed by Higuchi and Connors (1965):

$$K_{1:1} = \frac{S_{\text{Slope}}}{S_0 (1 - S_{\text{Slope}})} \quad (1)$$

Where:

$S_0$ is the intercept of the line with the ordinate axis of the LGEO with no added β-CD.

Thermodynamic parameters can be calculated in function of temperature and stability constant. Enthalpy changes ($\Delta H$) were determined by Van’t Hoff equation:

$$\frac{d\ln K}{dT} = \frac{\Delta H}{R} \times \frac{1}{T^2} \quad (2)$$

Furthermore, changes in Gibbs free energy ($\Delta G$) and entropy ($\Delta S$) were calculated using equations 3 and 4, respectively:

$$\Delta G = -RT\ln K \quad (3)$$

$$\Delta S = \frac{(\Delta H - \Delta G)}{T} \quad (4)$$

2.3.4 Samples and preparation of inclusion complexes

Method 1

The inclusion complexes were obtained by the kneading method (also known as paste method) using molar ratio of 1:1 β-CD:LGEO (based on carvacrol molecular weight) either in ultrapure water (Kneading method with water - KW) or water:ethanol (75:25) (Kneading with water and ethanol - KWE). β-cyclodextrin and LGEO were weighed and homogenized in a glass mortar. Either distilled water or distilled water:ethanol (75:25) mixture was added gradually, under constant manual stirring until
the paste formation. The resulting material was then dried in a desiccator at room temperature until a glass film was formed, which was removed by manual trituration and preserved in glass containers.\textsuperscript{17,18}

\textit{Method 2}

In the co-evaporation (CE) system, β-CD and LGEO 1:1 molar ratio (based on carvacrol molecular weight) were mixed in 20 mL of distilled water (CW) or distilled water:ethanol (75:25) (CWE) mixture for 36 h in a magnetic stirrer (400 rpm). The resulting material was dried in a desiccator at room temperature until a glass film was formed, which was removed by manual trituration and stored in suitable containers.\textsuperscript{19,20}

\textit{Method 3}

A physical mixture (PM) was prepared by addition of LGEO to a glass mortar containing powdered β-CD under manual agitation. The LGEO/β-CD mass ratio was maintained, as described for the inclusion complex preparation, and the PM was stored in suitable containers.\textsuperscript{8}

\textbf{2.3.5 Physicochemical properties of inclusion complexes}

\textit{Thermal Analysis}

Thermoanalytical measurements were obtained in a DSC-50 cell (Shimadzu\textsuperscript{®} Kyoto-Japan) using approximately 2 mg of the sample in aluminum crucibles under dynamic nitrogen atmosphere (100 mL.min\textsuperscript{-1}), and heating rate of 10°C.min\textsuperscript{-1} in the temperature range of 30-600°C. Indium (m.p. 156.6°C; \(\Delta H_{\text{melt.}} = 28.54 \text{ J.g}^{-1}\)) and zinc by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) (m.p. 419.6°C) was used to

This article is protected by copyright. All rights reserved.
calibrate the DSC cells. The TG curves were performed using a thermobalance, model TGA-50 (Shimadzu® Kyoto-Japan), in the temperature range of 30-600°C, using alumina crucibles with approximately 3 mg of samples under dynamic nitrogen atmosphere (100 mL.min\(^{-1}\)) and heating rate of 10°C.min\(^{-1}\). TG/DTG was calibrated using a CaC\(_2\)O\(_4\).H\(_2\)O by Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) standard in conformity to ASTM.

**Moisture determination**

β-CD, LGEO, physical mixture and inclusion complexes moisture contents were determined by Karl Fischer titration using a Potentiometric Titrator Metrohm® (Model Titrando 836). The analyses were performed in triplicate at 25°C.

**X-ray diffraction analysis (XRD)**

β-CD, PM, and inclusion complexes (KW, KWE, CW and CWE) crystallinity was evaluated in a Rigaku DMAX 2000 diffractometer with CuK\(\alpha\) (1.5406 Å) in the range of 10-30° (2\(\theta\)), using the powder XRD method.

**Fourier transform infrared spectroscopy (FTIR)**

FTIR spectra were acquired using a Perkin Elmer®, in the range of 4000-400 cm\(^{-1}\), resolution of 4 cm\(^{-1}\) and 16 scans. The solid samples (β-CD, PM, and the inclusion complexes) were grinded and mixed thoroughly with KBr. The liquid sample (LGEO) was prepared using the KBr window technique.

**Scanning electron microscopy (SEM)**
β-CD and inclusion complex KW were analyzed by scanning electron microscope JEOL (model JSM-6510) with LV acceleration voltage of 5 kV and a magnitude of 5000 and 10000 x. The samples were placed on copper strips and attached to a blade and then covered with a gold film.

2.3.6 Quantification of inclusion of LGEO in β-CD by gas chromatography (CG)

GC conditions

The analyses were performed in a Shimadzu QP 5050A equipped with automatic injector (AOC-201). The following conditions were used: fused-silica capillary column with stationary phase DB-5MS (30 m, 0.25 mm i.d.); helium as carrier gas at 1.2 mL.min\(^{-1}\); injector split at 250°C (split ratio 1/83); column temperature program, column temperature program 50°C during 1.5 min, with 4°C increase per min. to 200°C, then 10°C/min to 250°C, ending with a 5 min isothermal at 300°C. The mass spectra were taken at 70 eV with scanning speed of 0.50 scan/s from 40 to 450 Da.

Inclusion content of LGEO in β-CD

To determine the inclusion content, the adsorbed oil and the total oil were extracted from the inclusion complexes (KW, KWE, CW and CWE). For obtaining the adsorbed oil, 3 g of powder and 20mL of hexane were stirred for 20 minutes. The suspension was then filtered and the residue washed with 10mL of hexane three times and then concentrated in a rotaevaporator. Thereafter, the internal standard menthol (2 mg) solubilized with 1 mL of hexane was added and further analyzed by GC under the previously mentioned conditions. For obtaining the total oil, 0.2 g of inclusion complexes 4 mL of hexane and 8 mL of distilled water were stirred for 20 minutes.
and kept under constant temperature at 85°C. The suspension was then filtered using a
cfilter paper and the residue washed with 10 mL of hexane three times and then
concentrated in a rotavaporator. Thereafter, the internal standard menthol (2 mg)
solubilized with 1 mL of hexane was added and further analyzed by GC under the
previously mentioned conditions. The total oil corresponding to the amount of LGEO
complexed in the β-CD cavity, plus the adsorbed oil. Then, the difference between
adsorbed and total oil was used to determine the inclusion content of the LGEO.8

2.3.7 Larvicidal Activity

LGEO (100 mg) was mixed with Tween-80 (0.25 mL) and dechlorinated water
(4.75 mL) and stirred in a vortex, resulting in a 20,000 ppm dispersion. This dispersion
was further used to prepare 100 mL of aqueous solutions in the range of 5-120 ppm in
disposable cups. To each cup was added twenty third-instar Rockefeller Ae. aegypti
larvae. A dispersion of Tween-80 (0.1 mL) and water (19.9 mL) was used as control.
After 24h of treatment, the mortality count was conducted.2

The inclusion complex LGEO/β-CD chosen to perform the larvicidal activity
was the KW which presented the highest content of complexation, and is prepared
without using organic solvent being environmentally safe. The test was performed in
room temperature (e.g. 25 ± 2°C) and in triplicate. The concentrations of the complex
varied in the range of 5-120 ppm similar to LGEO content in the formulation. Statistical
analysis using Probit analysis was performed with the goal to determining the lethal
concentration 50% (LC50).21 Furthermore, t-test of Student was performed.

2.3.8 Artemia sp. lethality test
To evaluate the toxicity of LGEO and the inclusion complex LGEO/β-CD (KW) to a non-target species, the acute (24 h) mortality of brine shrimps (*Artemia sp.* nauplii) was carried out by adapting the methodology described by Meyer et al. (1982).  

*Artemia sp.* cysts (100 mg) were incubated in 1000 mL of standard artificial saline (35%, w/v) under illumination and aeration. After 18 to 24 hours of incubation, the nauplii (II to III stage) were transferred to a vessel containing 200 mL of saline water, with illumination for 24 h. A series of concentrations ranging from 5 to 500 ppm in 10 mL of saline water containing 10 *Artemia sp.* (triplicate) were performed. The nauplii were exposed to solutions for 24 h, when the mortality was accounted. Statistical analysis using Probit analysis was performed with to calculate the lethal concentration 50% (LC₅₀). Furthermore, *t*-test of Student was performed.

### 3. RESULTS AND DISCUSSIONS

The use of inclusion complexes containing β-CD can be a viable method to increase the solubility of essential oils in water. Since a variety of physicochemical properties are involved in the formation of inclusion complexes between guest (LGEO) and host (β-CD), many characterization techniques are used to indicate the complexation. In this study, two preparation methods (kneading and co-evaporation) using two different medium (water and water:ethanol, 75:25) were evaluated regarding the complexation of LGEO with β-CD. In addition, when using essential oils as a guest, it is important to determine the inclusion contents of its main components, especially those that have previously shown activity against *Ae. aegypti* larvae.

Since larvicides are released in the environment, there is a risk for non-target species toxicity. In view of this fact, the toxicity of LGEO and its β-CD inclusion complexes on *Artemia sp.* was evaluated as well.
Firstly, the phase solubility diagram was performed. According to Higuchi and Connors (1965), phase solubility diagram is constructed using the total molar concentration of the “guest” (LGEO) as the ordinates axis and the total molar concentration of the “host” (β-CD) as the abscissas axis. These phase diagrams are classified in two types, A and B. In the “A” diagram type, the solubility of the “guest” increases with the addition of the “host”, suggesting one or more molecular interactions between the host and guest. When the solubility increases linearly (A_L), the ideal molar ratio between host-guest is 1:1 and, the “B” diagram type is observed when insoluble complexes are formed.

Phase solubility diagram was determined in water at three different temperatures (25, 35, and 45°C) (Figure 1). The obtained curves exhibited A_L type, where the LGEO solubility increases linearly with the β-CD concentration, in other words, is suggested that the ideal molar ratio between β-CD - LGEO is 1:1. All tested temperatures exhibited curves A_L type and similar results were found by Wang and co-workers (2011).

(FIGURE 1)

The stability constant (K_{1:1}) and thermodynamic parameters such as, enthalpy (ΔH), free energy of Gibbs (ΔG) and entropy (ΔS) are presented in the Table 1. It was observed that K_{1:1} decreased with rising of the temperature, indicating that the inclusion process can be exothermic. Thermodynamic data also demonstrated that inclusion process is exothermic (ΔH < 0). Since the inclusion process is exothermic, is expected that the process should be spontaneous [considering that ΔG must be negative in the expression (ΔG = ΔH - TΔS)]. This was confirmed by thermodynamic data where ΔG <
0 and ΔS <0. These results are also in agreement with those demonstrated by Jun and co-workers (2007).  

(DISTINCT TEXT)

DSC and TG analysis are interesting tools to determine the formation of inclusion complexes with cyclodextrins. In Figure 2, LGEO shows three endothermic peaks at T_{peak} 107, 170 and 230°C, both possibly corresponding to releasing of remaining water molecules or volatilization process. The DSC curve of β-CD, exhibits a wide and strong endothermic effect in the interval 34 – 119°C (DSC T_{peak}=99°C) also seen in TG curve (Figure 3) with weight loss of 13.4%, which corresponds to the release of water molecules. Above 300°C, decomposition and elimination of carbonaceous material occurs.

(Figure 2)

(Figure 3)

PM displayed an endothermic event in the interval of 30 to 120°C (DSC T_{peak} at 89°C) which is probably related to the LGEO evaporation and water release from β-CD. Through TG analysis, the previous observation can be confirmed due to the appearance of pronounced weight loss (21.7%) in the first event at the temperature range of 30 to 120°C (Table 2). In both DSC and TG curves a superposition of the thermal events of the pure LGEO (guest) and β-CD (host) is observed, which indicates that no host-guest interaction occurred in PM. Similar results were found for β-CD and geraniol physical mixture by Menezes and co-workers (2012).
LGEO/β-CD complexes demonstrated no endothermic events in the range of oil volatilization ($T_{\text{peak}}$ 170 and 230°C) which suggests that the LGEO is included into the β-CD cavity. In the TG/DTG curves, the complexes showed loss of mass (KW - 7.6%, KWE - 7.8%, CW – 6.4% and CWE – 6.7%) in the range of 120-280°C, which may be related to the release of LGEO, confirming that the guest is included in the host by using any of the four methods. The KW/KWE inclusion complexes presented a loss in mass greater than CW/CWE, suggesting a better complexation using the kneading method of preparation.

Nevertheless, TG analysis is not able to distinguish the losses in mass of essential oil and water from inclusion complexes. Thus, TG results were complemented by using Karl Fischer titration to determinate the water of the samples. As displayed in Table 2, the complexes showed a decrease in the percentage of water as compared to pure β-CD. This decrease may be attributed to complex formation, as water molecules originally found in the β-CD cavity were replaced by LGEO molecules.

X-ray diffraction method is commonly applied to detect the formation of β-CD inclusion complexes. In Figure 4, β-CD showed sharp peaks at 10.7°, 12.6°, 14.7 19.6° and 22.7°, which may be related to its crystalline nature. Peaks of crystallinity of β-CD were also detected in the physical mixture. Nevertheless, XRD profile of the LGEO:β-CD inclusion complexes diverges significantly from that of β-CD alone, resulting in a new pattern of diffraction, indicating the complexation of LGEO in β-CD. Similar results were found by Wang and co-workers (2011) in which after complexation, a different diffraction profile was found. Similar patterns of the inclusion
complexes obtained by kneading method using pure water or water:ethanol mixture were observed. Shrestha and co-workers (2017)\textsuperscript{28} observed that using ethanol in kneading method to complex tea tree oil β-CD caused crystallization of the complex, similar to those complexes prepared using water.

\underline{(FIGURE 4)}

The inclusion complexes obtained by co-evaporation presented different XRD profiles depending on the solvent used in the media. Inclusion complexes prepared by co-evaporation using water:ethanol 75:25 (CWE) presented XRD profile similar to those obtained by kneading method with few discrepancies related to the intensity of the peaks. However, the XRD profile of the samples prepared by co-evaporation using water (CW) was different from the others, suggesting that a new solid phase with lower crystallinity was formed. These differences in the XRD profile may be related to the entrapment of remained water molecules after the evaporation step. Since the co-evaporation method requires a higher amount of solvent than kneading, the presence of water may be influenced the change in crystallinity.

LGEO FTIR spectrum (Figure 5) exhibits stretching vibrations (\(\nu\)) in the band of the group O-H in 3431 cm\(^{-1}\);\(\nu\) C-H in 2966 and 2865cm\(^{-1}\), C=C in the range of 1600-1460 cm\(^{-1}\); and angular deformation bands in the region of C-H 1000-650 cm\(^{-1}\). The FTIR spectrum of pure β-CD (Figure 5) showed prominent absorption bands at 3600-3200 cm\(^{-1}\) (for O-H stretching vibrations), 3100-2800 cm\(^{-1}\) (for C-H stretching vibrations), 1634 cm\(^{-1}\) (for H-O-H bending), 1155 cm\(^{-1}\) (for C-O stretching vibration), and 1300-1000cm\(^{-1}\) (for C-O-C stretching vibration).\textsuperscript{29}
Inclusion complexes LGEO/β-CD prepared by different methods (KW, KWE, CW and CWE) spectra were dominated by β-CD bands, presenting only a shift of 3383 cm\(^{-1}\) (for OH stretching vibrations) to lower wavenumbers and the disappearance of LGEO characteristic peak at 2966 cm\(^{-1}\), 2865 cm\(^{-1}\) and 1460 cm\(^{-1}\), thus suggesting an interaction between the host and guest.

(Figure 5)

LGEO composition was similar to those observed previously by Cruz and co-workers (2013).\(^{30}\) The major components are carvacrol (46.76%), p-cimene (10.70%), γ-terpinene (13.85%), and thymol (4.99%) (Tables 3). In addition, the inclusion content of LGEO/β-CD inclusion complexes regarding the major component of LGEO (carvacrol) was 15.25%, 4.53%, 5.39%, and 13.07% for KW, KWE, CW and CWE, respectively (Tables 3).

In the present work, the kneading method using water resulted in higher levels of inclusion content of the main components of LGEO (carvacrol, thymol, p-cimene and γ-terpinene) compared to the co-evaporation method, which is attributed to the occurrence of molecule disorder in the co-evaporation method due to the use of higher amounts of solvent, minimizing the chances of complexation. Additionally, longer sample preparation might result in higher loss of LGEO in the process. However, the opposite occurred when ethanol was used as co-solvent. Probably, the ratio of water:ethanol (75:25) did not provide the ideal conditions to complexation in kneading method. Galvão and co-workers (2015)\(^{11}\) demonstrated that the ratio water:ethanol of 50:50 using the kneading method resulted in higher inclusion content than when pure water was used. They attributed this result to changes in the system dielectric constant.
promoted by ethanol enabling an improvement in the solubilization conditions of *Citrus sinensis* essential oil in the preparation system, and therefore increasing complexation.

**(TABLE 3)**

On the other hand, the use of ethanol as co-solvent in the co-evaporation method had a positive impact on the inclusion content. According to Del Valle and co-workers (2004), ethanol increases van der Waals interactions between the host and the guest by modifying the hydrophobicity of the aqueous phase. This depends on variables such as concentration of alcohol, preparation method (temperature, solvent amount, stirring time) within others.

Even though the inclusion complexes prepared by KW and CWE presented similar inclusion contents of the main components of LGEO (carvacrol, thymol, *p*-cimene and *γ*-terpinene), the kneading method using water was the preparation method chosen, since this method did not use organic solvent (ethanol), which is desirable for formulations aiming biological application. Thus, KW inclusion complex was further analyzed by SEM, larvicidal activity, and lethality test on non-target animals.

The morphology of β-CD was analyzed in the scanning electron microscopy (SEM). As shown in Figure 6A particles are distributed in an irregular size and shape. However, LGEO/β-CD inclusion complexes prepared by kneading with water (Figure 6B) presented drastic changes in the particle shape, resulting in clusters, suggesting the formation of a complex and supporting the previous characterizations. Songkro and co-workers (2012), also observed that an inclusion complex with citronella oil, citronellol, and citronellal, demonstrated drastic differences in the shape and size of particles compared to pure β-CD.
Marreto and co-workers (2008) investigated the same essential oil used in this work applying the slurry and paste complexation methods, but using a different type of cyclodextrin (hydroxypropyl-β-cyclodextrin). They observed that the paste complexation method presented almost complete oil retention (99.8%) in comparison to slurry method. Nevertheless, the slurry method showed a better inclusion profile of the active and major terpenes found in LGEO. Similar results were found in the present work using β-cyclodextrin as a host for LGEO, the kneading method (also known as paste method) was more effective in complexing LGEO in comparison to co-evaporation method. In addition, the present work evaluated the influence of the presence of ethanol in the complexation media, the larvicidal activity and the toxicity to non-target animals.

LGEO and its major constituent (carvacrol) presented larvicidal activity similar to those found by Silva and co-workers (2002). LGEO induced the mortality of *Ae. aegypti* larvae after 24 h demonstrating LC$_{50}$ of 39 ppm (38-42) (Table 5). The inclusion complex prepared by the method KW presented LC$_{50}$ of 33 ppm (31-35). Thus, the inclusion complex β-CD/LGEO exhibited higher larvicidal activity (p < 0.01, according $t$-test of Student) against the larvae of *Ae. aegypti* than LGEO pure suggesting that the complexation with β-CD improved the efficacy of the LGEO, probably, by increasing its solubility.

The lethality assay against non-target animal demonstrated that LGEO induced mortality of *Artemia* sp. LC$_{50}$ of 30.5 ppm (30-31) being lower than the lethal concentration required for *Ae. aegypti* larvae, in other words, the concentration of
LGEO needed to control *Ae. aegypti* larvae would be harmful to the aquatic ecosystem. Teles and co-workers (2010)\textsuperscript{32} reported a lethality of LGEO against *Artemia* sp. for 23.6 ppm, which is close to the one found in this work. However, the complexation of LGEO with β-CD showed a lower lethality against non-target organisms LC\textsubscript{50} of 150.6 ppm (148-156) that is 5-fold higher (p < 0.0001, according to *t*-test of Student) than the LC\textsubscript{50} needed for controlling *Ae. aegypti* larvae, therefore being less harmful to the non-target animal.

4. CONCLUSIONS

This work indicated that the inclusion complex between LGEO and β-CD was effectively formed and improved the efficacy of LGEO against the *Ae. aegypti* larvae. Phase solubility studies demonstrated that the ideal molar ratio between LGEO and β-CD was 1:1. Moreover, thermodynamic data revealed that the complexation process tends to be spontaneous (ΔS <0) and exothermic (ΔH <0). The characterizations through DSC, TG, Karl Fisher, XRD, FTIR, and SEM proved the inclusion complexes formation. DSC and TG surely exhibited the formation of inclusion complexes by kneading and co-evaporation methods. The results of inclusion content demonstrated that the best method for preparing a LGEO/β-CD complex was the KW, presenting the highest inclusion content (15.25\%). Moreover, LGEO/β-CD complex (KW) improved the larvicidal activity (39 to 33 ppm), and decreased the toxicity to non-target animals (30.5 to 150.1 ppm) in comparison to LGEO pure, being no harmful to the aquatic ecosystem in the concentrations needed to control *Ae. aegypti*. In conclusion, the product studied may be an attractive alternative to control *Ae. aegypti* being biodegradable, and economically viable.
ACKNOWLEDGMENTS

The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/Brazil) and the Fundação de Amparo à Pesquisa do Estado de Sergipe (FAPITEC/SE) for supporting funds, Herbarium of the Federal University of Sergipe (ASE) and CMNano for SEM analysis.

REFERENCES


28. Shrestha, M, Ho, TM, Bhandari B. Encapsulation of tea tree oil by amorphous


**TABLES**

**Table 1.** Calculated stability constant ($K_{1:1}$) and thermodynamic parameters regarding to the inclusion process at different temperatures (25, 35 and 45 °C).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$K_{1:1}$ (M$^{-1}$)</th>
<th>$\Delta H$ (KJ/mol)</th>
<th>$\Delta G$ (KJ/mol)</th>
<th>$\Delta S$ (J/mol.K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>40</td>
<td>-23.45</td>
<td>-9.14</td>
<td>-48.02</td>
</tr>
<tr>
<td>35</td>
<td>33</td>
<td>-23.45</td>
<td>-8.93</td>
<td>-47.14</td>
</tr>
<tr>
<td>45</td>
<td>22</td>
<td>-23.45</td>
<td>-8.17</td>
<td>-48.05</td>
</tr>
</tbody>
</table>

**Table 2.** Mass losses for *Lippia gracilis* essential oil, β-CD, physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE) and moisture contents obtained by Karl Fisher titration.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass loss (%)</th>
<th>Karl Fisher % water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1$^{\text{st}}$ step</td>
<td>2$^{\text{nd}}$ step</td>
</tr>
<tr>
<td>LGEO</td>
<td>20.0$^a$</td>
<td>78.2$^b$</td>
</tr>
<tr>
<td>β-CD</td>
<td>13.4$^a$</td>
<td>-</td>
</tr>
<tr>
<td>PM</td>
<td>21.7$^a$</td>
<td>0.3$^c$</td>
</tr>
<tr>
<td>KW</td>
<td>10.0$^a$</td>
<td>7.6$^c$</td>
</tr>
<tr>
<td>KWE</td>
<td>10.2$^a$</td>
<td>7.8$^c$</td>
</tr>
<tr>
<td>CW</td>
<td>7.2$^a$</td>
<td>6.4$^c$</td>
</tr>
<tr>
<td>CWE</td>
<td>7.5$^a$</td>
<td>7.7$^c$</td>
</tr>
</tbody>
</table>

$^a$ Percentage of the loss in mass up to 120°C;

$^b$ Percentage of the loss in mass regarding LGEO in the interval from 120 to 200°C;

$^c$ Mass loss probably attributed to LGEO release in the interval from 120 to 280°C;

$^d$ Thermal decomposition in the interval from 280 to 400°C.
Table 3. Chemical composition of *Lippia gracilis* essential oil (LGEO) and inclusion complexes kneading with water (KW), kneading with water/ethanol (KWE), co-evaporation with water (CW) and co-evaporation with water/ethanol (CWE).

<table>
<thead>
<tr>
<th>Components</th>
<th>LGEO</th>
<th>KW</th>
<th>KWE</th>
<th>CW</th>
<th>CWE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT*</td>
<td>%</td>
<td>S.</td>
<td>T.</td>
<td>C.</td>
</tr>
<tr>
<td>α – tujhene</td>
<td>7.267</td>
<td>0.83</td>
<td>0.24</td>
<td>0.54</td>
<td>0.30</td>
</tr>
<tr>
<td>α – pinene</td>
<td>7.483</td>
<td>0.16</td>
<td>0.09</td>
<td>0.23</td>
<td>0.14</td>
</tr>
<tr>
<td>Micene</td>
<td>9.375</td>
<td>2.39</td>
<td>0.74</td>
<td>1.63</td>
<td>0.89</td>
</tr>
<tr>
<td>α – terpinene</td>
<td>10.367</td>
<td>2.27</td>
<td>0.64</td>
<td>1.55</td>
<td>0.91</td>
</tr>
<tr>
<td>β-cimene</td>
<td>10.642</td>
<td>10.70</td>
<td>4.33</td>
<td>10.16</td>
<td>5.83</td>
</tr>
<tr>
<td>Limonene</td>
<td>10.783</td>
<td>0.19</td>
<td>0.49</td>
<td>0.40</td>
<td>-0.09</td>
</tr>
<tr>
<td>1,8 cineol</td>
<td>10.933</td>
<td>0.22</td>
<td>0.73</td>
<td>1.44</td>
<td>0.71</td>
</tr>
<tr>
<td>γ – terpinene</td>
<td>11.883</td>
<td>13.85</td>
<td>3.80</td>
<td>8.08</td>
<td>4.28</td>
</tr>
<tr>
<td>Linalool</td>
<td>13.442</td>
<td>0.60</td>
<td>0.08</td>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>Menthol (I.S.)</td>
<td>16.392</td>
<td>-</td>
<td>64.34</td>
<td>37.43</td>
<td>-</td>
</tr>
<tr>
<td>Terpinene-4-ol</td>
<td>16.500</td>
<td>0.56</td>
<td>0.33</td>
<td>0.62</td>
<td>0.29</td>
</tr>
<tr>
<td>Thymol</td>
<td>20.492</td>
<td>4.99</td>
<td>1.08</td>
<td>2.58</td>
<td>1.50</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>20.808</td>
<td>46.76</td>
<td>14.15</td>
<td>29.40</td>
<td>15.25</td>
</tr>
<tr>
<td>E-caryophyllene</td>
<td>25.058</td>
<td>5.78</td>
<td>2.78</td>
<td>1.12</td>
<td>-1.66</td>
</tr>
<tr>
<td>Aromadendren</td>
<td>25.683</td>
<td>0.48</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>α- humulene</td>
<td>26.258</td>
<td>0.75</td>
<td>0.29</td>
<td>0.08</td>
<td>-0.21</td>
</tr>
<tr>
<td>Viridiflorene</td>
<td>27.417</td>
<td>0.84</td>
<td>0.12</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>30.142</td>
<td>0.67</td>
<td>1.60</td>
<td>0.20</td>
<td>-1.4</td>
</tr>
<tr>
<td>Caryophyline oxide</td>
<td>30.325</td>
<td>0.51</td>
<td>0.98</td>
<td>0.15</td>
<td>-0.83</td>
</tr>
</tbody>
</table>

*a* Retention index calculated using Kovats retention indices relative to a n-alkane homologous series (C10-C18).

*b* S. oil (Superficial oil, %), T. oil (Total Oil, %) and C. oil (Complexed Oil, %).

This article is protected by copyright. All rights reserved.
Fig. 1. Phase solubility diagram of LGEO in function of β-cyclodextrin concentrations at 25, 35 and 45°C.
Fig. 2. DSC Curves of *Lippia gracilis* essential oil (LGEO), β-CD, physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE) in dynamic nitrogen atmosphere (50 mL.min⁻¹) and rate heat 10°C. min⁻¹.
Fig. 3. TG curves of *Lippia gracilis* essential oil (L GEO), β-CD, physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE); *Inset:* DTG curves of L GEO, β-CD, PM, KW and KWE, CW and CWE in dynamic nitrogen atmosphere (100 mL.min⁻¹) and rate heat 10°C.
Fig. 4. X-ray diffraction pattern of the inclusion complexes obtained by kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE), β-cyclodextrin (β-CD) and physical mixture (PM).
Fig. 5. FTIR spectra of Lippia gracilis essential oil (L GEO), β-CD, physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE).
Fig. 6. SEM photographs of β-cyclodextrin (A); and inclusion complex LGEO/β-cyclodextrin obtained by kneading prepared with water (KW) (B).