Research Article

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

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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*. Because 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 way of enabling the use of essential oils in water as larvicides. This study comprised the development of *Lippia gracilis* essential oil (LGEO) and β-CD inclusion complexes for control of *Ae. aegypti*.

RESULTS: Thermal analysis clearly showed the formation of complexes using kneading and co-evaporation methods. Gas chromatography analysis showed that kneading without co-solvent (KW) gave the highest content (∼ 15%) of the LGEO major component. Moreover, KW showed that the complex had a 50% lethal concentration (LC_{50}; 33 ppm) lower than that of pure LGEO (39 ppm); in other words, complexing LGEO with β-CD improved the larvicidal activity. In addition, LGEO complexed with β-CD (KW) was not harmful to non-target organisms at the concentrations needed to control *Ae. aegypti* larvae.

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

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

1 INTRODUCTION

Aedes aegypti is the main vector of yellow fever, dengue and emerging arboviruses such as chikungunya and Zika. These diseases are responsible for a great deal of morbidity and mortality around the world. Moreover, microcephaly in newborns and Guillain-Barré syndrome have been diagnosed and associated with Zika virus.[1–3]

Because vector control is one of the most effective ways to prevent the aforementioned diseases, several million dollars are spent each year in attempts to eradicate *Ae. aegypti*.[2] Many synthetic larvicides, such as organophosphates (e.g. temephos), have been used in several countries. However, intensive use of these pesticides presents several problems including resistance in mosquito larvae and toxic effects on the environment via contamination of the soil, water and air.[4] To circumvent these problems, natural products (e.g. essential oils) with larvicidal activity have been studied.[5]

Essential oils are excellent candidates for larvicides due to their high activity, availability in tropical countries and affordability.[6] *Lippia gracilis* essential oil (LGEO) is composed of a mixture of terpenes and sesquiterpenes, with carvacrol as its major constituent, and has demonstrated strong larvicidal activity against *Ae. aegypti* larvae.[2] Because of its low aqueous solubility, easy oxidation and volatility, LGEO formulations are required that avoid degradation and have increased solubility, and incorporate the essential oil within a viable larvicide.[7,8]

Several formulations have been developed to optimize the use of larvicides such as nanoemulsions,[9] *in situ* gelling nanostructured surfactant systems[10] and cyclodextrins.[11]

Cyclodextrins are cyclic oligomers composed of six, seven or eight α-D-glucopyranose units on a ring-like structure. They are

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commonly available in three types: alpha (α), beta (β) and gamma (γ), with enclosed cavities that are ~ 4.7–5.3 (α), 6.0–6.5 (β) and 7.5–8.3 Å (γ) in diameter.[12,13] The external part of a cyclodextrin is hydrophilic and the interior cavity is hydrophobic, enabling the solubilization of nonpolar solutes in water.[13]

Among the cyclodextrins, β-cyclodextrin (β-CD) is the most commonly used due to its availability, price and cavity size, which is suitable for a wide range of guest molecules. Formation of an inclusion complex formation is determined by the properties of the guest (molecular size, geometry and polarity), which should be suitable for the size of the β-CD cavity.[14]

Here, we study the complexation of LGEO with β-CD, using kneading and co-evaporation methods, and evaluate the influence of co-solvent in the preparation. Phase solubility studies were performed and the inclusion complexes were evaluated. In addition, the LGEO content (%) was measured and the inclusion complex with the highest LGEO content was evaluated for its biological activity against *Ae. aegypti* larvae.

2 MATERIALS AND METHODS

2.1 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 no. 14734), located at a research farm 'Campus Rural da UFS'. Defoliation was performed manually, and leaves were dried in an oven with forced air circulation, at 40 °C, for 5 days. β-CD and carvacrol (~ 98%) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other reagents were from Synth (Diadema, SP, Brazil).

2.2 Methods

2.2.1 Essential oil extraction

Extraction of the essential oil was performed in the Laboratory of Plant Genetic Resources and Essential Oils of the Federal University of Sergipe. Some 75 g of dried leaves were subjected to hydro-distillation in a Clevenger-type apparatus for 140 min to yield a yellowish oil. The essential oil was separated from the aqueous phase, Na_2SO_4 was added to remove the remaining water and the resulting oil was refrigerated until further analysis or preparation of the inclusion complexes.

2.2.2 Identification of essential oil constituents

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

Percentage composition was calculated using the peak normalization method. Peaks were identified by comparison with their Kovats Retention Indices,[15] relative to an *n*-alkane homologous series (C_8–C_{18}), and obtained under the same conditions as 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 the NIST107, NIST21 and Willey8 (80% of similarity index) mass spectral library, in the GC–MS data system.

2.2.3 Phase solubility studies

Phase solubility diagrams were acquired in accordance with the Higuchi and Connors method.[16] 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, 2×10^{-3}, 4×10^{-3}, 6×10^{-3}, 8×10^{-3} and 10×10^{-3} mol L^{-1} under magnetic stirring for 24 h. Samples were kept in a thermostatic bath at 25, 35, and 45 °C, then centrifuged at 3000 rpm for 10 min and filtered with an ultrafiltration membrane (0.45 µm). Quantification was performed in triplicate using UV–Vis equipment (FEMTO 800XI) at 266 nm. The results were plotted with LGEO concentration as a function of β-CD concentration. The stability constant ($K_{1:1}$) was obtained according the equation proposed by Higuchi and Connors[16]:

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

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

Thermodynamic parameters were calculated as a function of temperature and the stability constant. Changes in enthalpy (ΔH) were determined using the Van't Hoff equation:

$$\frac{d\ln}{dT} = \frac{\Delta H}{R} \times \frac{1}{T^2} \tag{2}$$

Furthermore, changes in the Gibbs' free energy (ΔG) and entropy (ΔS) were calculated using Eqns 3 and 4, respectively

$$\Delta G = -RT \ln K \tag{3}$$

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

2.2.4 Samples and preparation of inclusion complexes

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

2.2.4.2 Method 2. In the co-evaporation (CE) system, β-CD and LGEO (1:1 M ratio based on the molecular mass of carvacrol) were mixed in 20 mL of distilled water (CW) or a distilled water/ethanol (75:25) (CWE) mixture for 36 h with magnetic stirring (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.[19,20]

2.2.4.3 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.[8]

2.2.5 Physicochemical properties of inclusion complexes

2.2.5.1 Thermal analysis. Thermoanalytical measurements were obtained in a DSC-50 cell (Shimadzu®, Kyoto, Japan) using ~ 2 mg of the sample in aluminum crucibles under a dynamic nitrogen atmosphere (100 mL min^{-1}), and a heating rate of 10 °C min^{-1}, over a temperature range of 30–600 °C. Indium (m.p. 156.6 °C; $\Delta H_{melt.} = 28.54$ J g^{-1}) and zinc (Sigma-Aldrich; m.p. 419.6 °C) were used to calibrate the Differential Scanning Calorimetry (DSC) cells. Thermogravimetry (TG) curves were obtained using a thermobalance, model TGA-50 (Shimadzu), over a temperature range of 30–600 °C, using alumina crucibles with ~ 3 mg of samples under a dynamic nitrogen atmosphere (100 mL min^{-1}) and a heating rate of 10 °C min^{-1}. Thermogravimetry/Differential Thermogravimetry (TG/DTG) was calibrated using a $CaC_2O_4 \cdot H_2O$ standard (Sigma-Aldrich) in accordance with ASTM.

2.2.5.2 Moisture determination. β-CD, LGEO, PM and inclusion complex moisture contents were determined by Karl Fischer titration using a Metrohm® potentiometric titrator (Model Titrando 836). The analyses were performed in triplicate at 25 °C.

2.2.5.3 X-Ray diffraction analysis. β-CD, PM and inclusion complex (KW, KWE, CW and CWE) crystallinity was evaluated in a Rigaku D/MAX 2000 diffractometer with CuKα (1.5406 Å) over a range of 10–30° (2θ), using the powder X-ray diffraction (XRD) method.

2.2.5.4 Fourier transform infrared spectroscopy. Fourier transform infrared (FTIR) spectra were acquired using a PerkinElmer spectrometer, over a range of 4000–400 cm^{-1}, resolution of 4 cm^{-1} and 16 scans. The solid samples (β-CD, PM and inclusion complexes) were ground and mixed thoroughly with KBr. The liquid sample (LGEO) was prepared using the KBr window technique.

2.2.5.5 Scanning electron microscopy. β-CD and the KW inclusion complex were analyzed by scanning electron microscopy (SEM) (JEOL, model JSM-6510) with an low vacuum acceleration voltage of 5 kV and a magnitude of 5000 and 10 000×. Samples were placed on copper strips, attached to a blade and covered with gold film.

2.2.6 Quantification of inclusion of LGEO in β-CD by gas chromatography

2.2.6.1 Gas chromatography conditions. The analyses were performed in a Shimadzu QP 5050A equipped with an 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 50 °C for 1.5 min, increased at a rate of 4 °C min^{-1} to 200 °C, then 10 °C min^{-1} to 250 °C, ending with a 5 min isothermal at 300 °C. Mass spectra were taken at 70 eV with a scanning speed of 0.50 scan s^{-1} from 40 to 450 Da.

2.2.6.2 Inclusion content of LGEO in β-CD. To determine the inclusion content, the adsorbed oil and total oil were extracted from the inclusion complexes (KW, KWE, CW and CWE). To obtain the adsorbed oil, 3 g of powder and 20 mL of hexane were stirred for 20 min. The suspension was then filtered and the residue washed three times with 10 mL of hexane and concentrated in a rotary evaporator. Thereafter, the internal standard menthol (2 mg) solubilized with 1 mL of hexane was added and further analyzed by gas chromatography (GC) under the previously mentioned conditions. To obtain the total oil, 0.2 g of inclusion complexes, 4 mL of hexane and 8 mL of distillated water were stirred for 20 min and kept at a constant temperature of 85 °C. The suspension was filtered using a filter paper and the residue washed three times with 10 mL of hexane and concentrated in a rotary evaporator. 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 corresponds to the amount of LGEO complexed in the β-CD cavity plus the adsorbed oil. The difference between the adsorbed and total oil was used to determine the LGEO content.[8]

2.2.7 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 used to prepare 100 mL of aqueous solutions in the range of 5–120 ppm in disposable cups. To each cup was added 20 third-instar Rockefeller *Ae. aegypti* larvae. A dispersion of Tween-80 (0.1 mL) and water (19.9 mL) was used as the control. After 24 h of treatment, the mortality count was conducted.[2]

The inclusion complex LGEO/β-CD chosen to perform the larvicidal activity was KW, which showed the highest complexation, is prepared without using organic solvent and therefore is environmentally safe. The test was performed at room temperature (25 ± 2 °C) and in triplicate. The concentrations of the complex varied from 5 to 120 ppm, similar to the LGEO content in the formulation. Probit analysis was used to determine the lethal concentration 50% (LC_{50}).[21] A Student's *t*-test was also performed.

2.2.8 Artemia sp. lethality test

To evaluate the toxicity of LGEO and the inclusion complex LGEO/β-CD (KW) towards a non-target species, the acute (24 h) mortality of brine shrimps (*Artemia* sp. *nauplii*) was tested by adapting the methodology of Meyer *et al.*[22] *Artemia* sp. cysts (100 mg) were incubated in 1000 mL of standard artificial saline (35%, w/v) under illumination and aeration. After 18–24 h of incubation, the nauplii (stages II to III) were transferred to a vessel containing 200 mL of saline, under illumination for 24 h. A series of solutions at concentrations of 5 to 500 ppm in 10 mL of saline containing 10 *Artemia* sp. (triplicate) were used. Nauplii were exposed to these solutions for 24 h, after which mortality was counted. Probit analysis was performed to calculate LC_{50} value. Furthermore, a Student's *t*-test was performed.

3 RESULTS AND DISCUSSIONS

The use of inclusion complexes containing β-CD is a viable way of increasing the solubility of essential oils in water.[11] Because a variety of physicochemical properties are involved in the formation of inclusion complexes between the 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 media (water and water/ethanol, 75:25) were evaluated in terms of LGEO complexation with β-CD. In addition, when using essential oils as the guest, it is important to determine the main componentsof the inclusion, especially those previously shown to have activity against *Ae. aegypti* larvae.

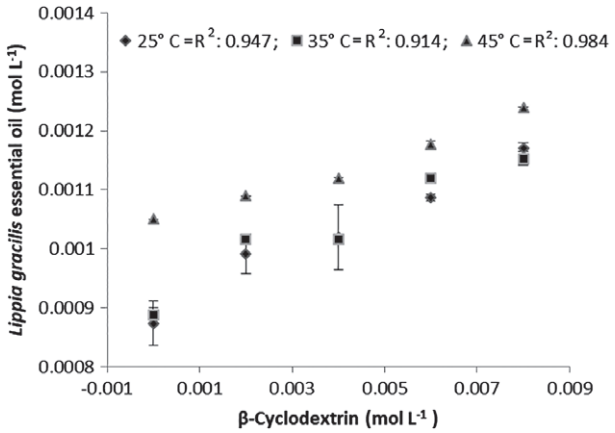

Figure 1. Phase solubility diagram of *Lippia gracilis* essential oil (LGEO) in the function of β-cyclodextrin concentrations at 25, 35 and 45 °C.

Table 1. Calculated stability constant ($K_{1:1}$) and thermodynamic parameters regarding inclusion at different temperatures

Temperature (°C)	$K_{1:1}$ (M^{-1})	ΔH (KJ mol^{-1})	ΔG (KJ mol^{-1})	ΔS (J $mol\ K^{-1}$)
25	40	−23.45	−9.14	−48.02
35	33	−23.45	−8.93	−47.14
45	22	−23.45	−8.17	−48.05

Because larvicides are released into the environment, there is a risk of non-target species toxicity. In view of this, the toxicity of LGEO and its β-CD inclusion complexes on *Artemia* sp. was evaluated.

First, a phase solubility diagram was constructed. According to Higuchi and Connors,[16] a phase solubility diagram is constructed using the total molar concentration of the 'guest' (LGEO) as the ordinate and the total molar concentration of the 'host' (β-CD) as the abscissa. These phase diagrams are classified into two types, A and B. In the 'A' diagram, the solubility of the 'guest' increases with 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 and guest is 1:1. The 'B' diagram is observed when insoluble complexes are formed.

The phase solubility diagram was determined in water at three different temperatures (25, 35 and 45 °C) (Fig. 1). The obtained curves were of the A_L type, where LGEO solubility increases linearly with β-CD concentration; in other words, the ideal molar ratio between β-CD and LGEO is 1:1. All tested temperatures exhibited A_L type curves and similar results were found by Wang *et al.*[19]

The stability constant ($K_{1:1}$) and thermodynamic parameters ΔH, ΔG and ΔS are presented in Table 1. It was observed that $K_{1:1}$ decreased with increasing temperature, indicating that the inclusion process is exothermic,[23] as confirmed by the thermodynamic data ($\Delta H < 0$). Because it is exothermic, the inclusion process is expected to be spontaneous (considering that ΔG must be negative in the expression $\Delta G = \Delta H - T\Delta S$). This was confirmed by the thermodynamic data, in which $\Delta G < 0$ and $\Delta S < 0$. These results are also in agreement with Jun *et al.*[24]

DSC and TG analysis are interesting tools with which to determine the formation of inclusion complexes with cyclodextrins.[25] In Fig. 2, LGEO shows three endothermic peaks at T_{peak} 107, 170 and

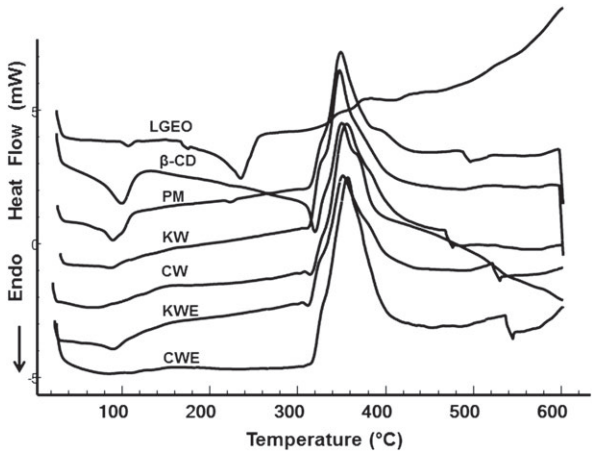

Figure 2. Differential Scanning Calorimetry curves for *Lippia gracilis* essential oil (LGEO), β-cyclodextrin (β-CD), physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE) in a dynamic nitrogen atmosphere (50 mL min^{-1}) at a heating rate of 10 °C min^{-1}.

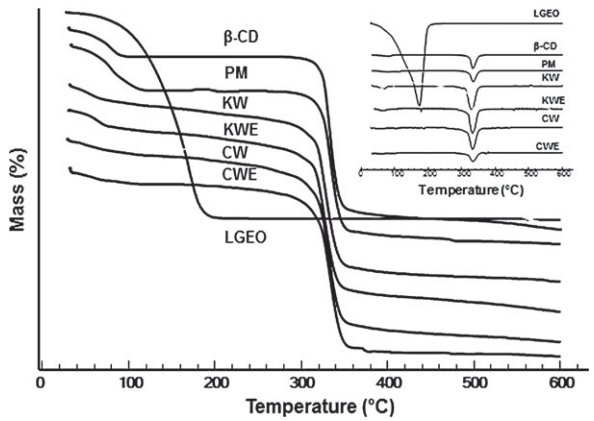

Figure 3. Thermogravimetry curves of *Lippia gracilis* essential oil (LGEO), β-cyclodextrin (β-CD), physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE). (Inset) DTG curves of LGEO, β-CD, PM, KW and KWE, CW and CWE in a dynamic nitrogen atmosphere (100 mL min^{-1}) and at a heating rate of 10 °C.

230 °C, possibly corresponding to release of the remaining water molecules or a volatilization process. The DSC curve of β-CD, which shows a wide and strong endothermic effect over the interval 34–119 °C (DSC T_{peak} = 99 °C) is also seen in the TG curve (Fig. 3) with a 13.4% weight loss, which corresponds to the release of water molecules. Above 300 °C, decomposition and elimination of carbonaceous material occur.

PM showed an endothermic event at 30 to 120 °C (DSC T_{peak} at 89 °C), which is probably related to the evaporation of LGEO and the release of water from β-CD. Using TG analysis, the previous observation is confirmed due to the appearance of a pronounced weight loss (21.7%) during the first event in the temperature range 30–120 °C (Table 2). In both DSC and TG curves, superposition of the thermal events of 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 PM by Menezes *et al.*[26]

LGEO/β-CD complexes demonstrated no endothermic events within the oil volatilization range (T_{peak} 170 and 230 °C), which

Table 2. Loss of mass for *Lippia gracilis* essential oil (LEGO), *β*-cyclodextrin (*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

Sample	Mass loss (%)			Karl Fisher % water
	First step	Second step	Third step	
LGEO	20.0[a]	78.2[b]	–	1.77
β-CD	13.4[a]	–	74	12.12
PM	21.7[a]	0.3[c]	72.2	9.45
KW	10.0[a]	7.6[c]	76	9.84
KWE	10.2[a]	7.8[c]	77.6	10.43
CW	7.2[a]	6.4[c]	81.9	7.89
CWE	7.5[a]	7.7[c]	77.6	9.46

[a] Percentage loss in mass up to 120 °C.
[b] Percentage loss in mass regarding LGEO at 120–200 °C.
[c] Mass loss attributed to LGEO release at 120–280 °C.
[d] Thermal decomposition at 280–400 °C.

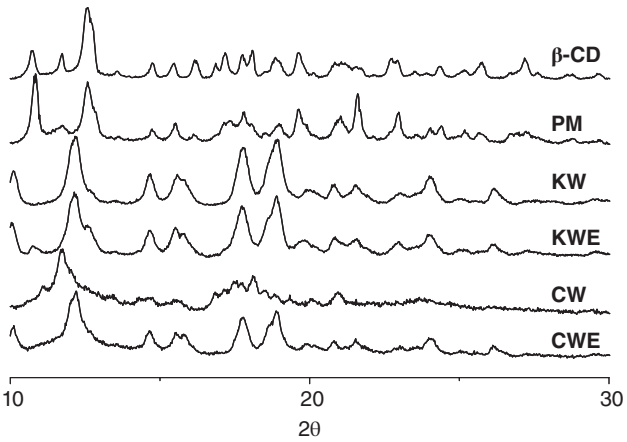

Figure 4. X-Ray diffraction pattern of the inclusion complexes obtained by kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE), *β*-cyclodextrin (*β*-CD) and physical mixture (PM).

suggests that the LGEO is in 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%) over the temperature range 120–280 °C, which may be related to the release of LGEO, confirming that the guest is within the host using any of the four methods.[27] The KW/KWE inclusion complexes show a loss of mass greater than that for CW/CWE, suggesting better complexation using the kneading method of preparation.

Nevertheless, TG analysis is not able to distinguish between losses in mass due to essential oil and water from inclusion complexes. Thus, the TG results were complemented using Karl Fischer titration to determinate the amount of water in the samples. As shown in Table 2, the complexes showed a decrease in the percentage of water compared with pure *β*-CD. This may be attributed to complex formation, as water molecules originally found in the *β*-CD cavity were replaced by LGEO molecules.[11]

XRD is commonly used to detect the formation of *β*-CD inclusion complexes.[25] In Fig. 4, *β*-CD shows sharp peaks at 10.7°, 12.6°, 14.7 19.6° and 22.7°, which may be related to its crystalline nature. Crystallinity peaks for *β*-CD were also detected in the PM. Nevertheless, the XRD profile of the LGEO/*β*-CD inclusion complexes diverges significantly from that of *β*-CD alone, resulting in a new diffraction pattern, and confirming the complexation of LGEO in *β*-CD. Similar results were reported by Wang *et al*.[19] who found a different diffraction pattern after complexation. Similar patterns for the inclusion complexes were obtained using the KW and KWE methods. Shrestha and co-workers[28] observed that using ethanol in the kneading method to complex tea tree oil and *β*-CD led to crystallization of the complex, similar to complexes prepared using water.

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

The LGEO FTIR spectrum (Fig. 5) exhibits stretching vibrations (υ) in the band of the group O—H in 3431 cm^{-1}; υ C—H in 2966 and 2865 cm^{-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 (Fig. 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 1300–1000 cm^{-1} (for C—O—C stretching vibration).[29]

The spectra of inclusion complexes LGEO/*β*-CD prepared by different methods (KW, KWE, CW and CWE) were dominated by *β*-CD bands, presenting a shift of 3383 cm^{-1} (for OH stretching vibrations) to lower wavenumbers and disappearance of the LGEO characteristic peaks at 2966, 2865 and 1460 cm^{-1}, thus suggesting interaction between the host and guest.

The composition of LGEO was similar to that observed previously by Cruz and co-workers.[30] The major components are carvacrol (46.76%), *p*-cimene (10.70%), *γ*-terpinene (13.85%), and thymol (4.99%) (Table 3). In addition, the amount of the major component of LGEO (carvacrol) in the LGEO/*β*-CD inclusion complexes was 15.25%, 4.53%, 5.39%, and 13.07% for KW, KWE, CW and CWE, respectively (Table 3).

In this study, the KW method resulted in higher inclusion content for the main components of LGEO (carvacrol, thymol, *p*-cimene and *γ*-terpinene) compared with the co-evaporation method, which is attributed to molecular disorder in the co-evaporation method due to the use of higher amounts of solvent, which minimizes the chances of complexation. In addition, longer sample preparation might result in greater loss of LGEO during the process. However, the opposite occurred when ethanol was used as the co-solvent. It is probable that the ratio of water to ethanol (75:25) did not provide ideal conditions for complexation in the kneading method. Galvão and co-workers[11] showed that a water/ethanol ratio of 50:50 using the kneading method resulted in a higher inclusion content than when pure water was used. They attributed this to changes in the system's dielectric constant because ethanol improved the solubilization conditions for *Citrus sinensis* essential oil in the preparation system, therefore increasing complexation.

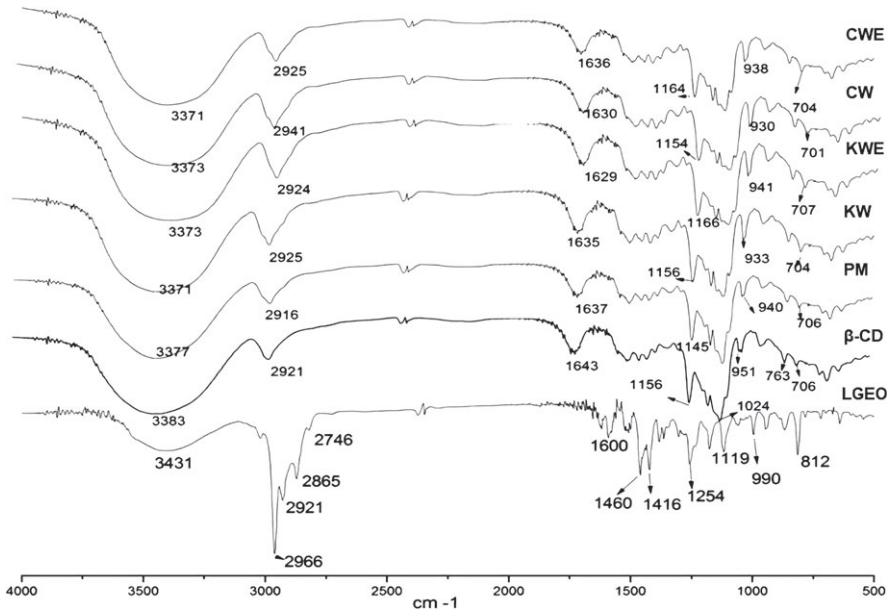

Figure 5. Fourier transform infrared spectra of *Lippia gracilis* essential oil (LGEO), β-cyclodextrin (β-CD), physical mixture (PM), kneading prepared with water (KW) and with water/ethanol (KWE), co-evaporation prepared with water (CW) and with water/ethanol (CWE).

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)

			Samples											
LGEO			KW			KWE			CW			CWE		
RT[a]			S	T	C	S	T	C	S	T	C	S	T	C
(min)	Components	%	(%)			(%)			(%)			(%)		
7.267	α-Tujhene	0.83	0.24	0.54	0.30	0.29	0.41	0.12	0.45	0.66	0.21	0.36	0.38	0.02
7.483	α-Pinene	0.16	0.09	0.23	0.14	0.11	0.36	0.25	0.18	0.29	0.11	0.10	0.29	0.19
9.375	Micene	2.39	0.74	1.63	0.89	1.04	1.75	0.71	1.62	2.43	0.81	1.02	1.13	0.11
10.367	α-Terpinene	2.27	0.64	1.55	0.91	1.11	1.26	0.15	1.23	1.93	0.70	0.67	0.82	0.15
10.642	*p*-Cimene	10.70	4.33	10.16	5.83	7.96	0.09	1.13	7.98	11.97	3.99	5.44	6.37	0.93
10.783	Limonene	0.19	0.49	0.40	−0.09	2.76	0.75	−2.01	4.59	2.05	−2.54	2.78	1.22	−1.56
10.933	1,8 cineol	0.22	0.73	1.44	0.71	1.37	2.63	1.26	1.94	2.56	0.62	0.74	0.26	−0.48
11.883	γ-Terpinene	13.85	3.80	8.08	4.28	7.86	11.52	3.66	7.00	10.61	3.61	4.08	4.75	0.67
13.442	Linalool	0.60	0.08	0.30	0.22	0.24	0.34	0.1	0.27	0.33	0.06	0.19	0.76	0.57
16.392	Menthol (IS)	–	64.34	37.43	–	35.30	20.17	–	37.30	17.91	–	57.92	37.97	–
16.500	Terpinene-4-ol	0.56	0.33	0.62	0.29	0.46	0.69	0.23	0.69	0.76	0.07	0.32	0.58	0.26
20.492	Thymol	4.99	1.08	2.58	1.50	2.64	3.02	0.38	2.05	3.21	1.16	1.53	2.60	1.07
20.808	Carvacrol	46.76	14.15	29.40	15.25	35.83	40.36	4.53	30.29	35.68	5.39	20.58	33.65	13.07
25.058	E-caryophyllene	5.78	2.78	1.12	−1.66	1.23	2.83	1.6	1.31	2.74	1.43	1.15	1.83	0.68
25.683	Aromadendren	0.48	0	0.09	0.09	0	0.19	0.19	0.04	0.23	0.19	0	0.37	0.37
26.258	α-Humulene	0.75	0.29	0.08	−0.21	0.08	0.21	0.13	0.08	0.20	0.12	0.08	0.17	0.09
27.417	Viridiflorene	0.84	0.12	0.16	0.04	0	0.38	0.38	0	0.41	0.41	0	0.38	0.38
30.142	Spathulenol	0.67	1.60	0.20	−1.4	0.10	0.28	0.18	0.11	0.29	0.18	0.32	0.93	0.61
30.325	Caryophylene oxide	0.51	0.98	0.15	−0.83	0.07	0.36	0.29	0.11	0.35	0.24	0.40	0.40	0.40

[a] Retention index calculated using Kovats Retention Indices relative to an *n*-alkane homologous series (C_8–C_{18}).
[b] S, superficial oil (%); T, total oil (%); C, complexed oil (%); IS, internal standard.

The use of ethanol as a co-solvent in the co-evaporation method had a positive impact on the inclusion content. According to Del Valle and co-workers,[31] ethanol increases van der Waal's interactions between the host and the guest by modifying the hydrophobicity of the aqueous phase. This depends on variables such as the alcohol concentration and the preparation method (temperature, solvent amount, stirring time).

Even though the inclusion complexes prepared using KW and CWE showed similar inclusion contents for the main components of LGEO (carvacrol, thymol, *p*-cimene and γ-terpinene), the

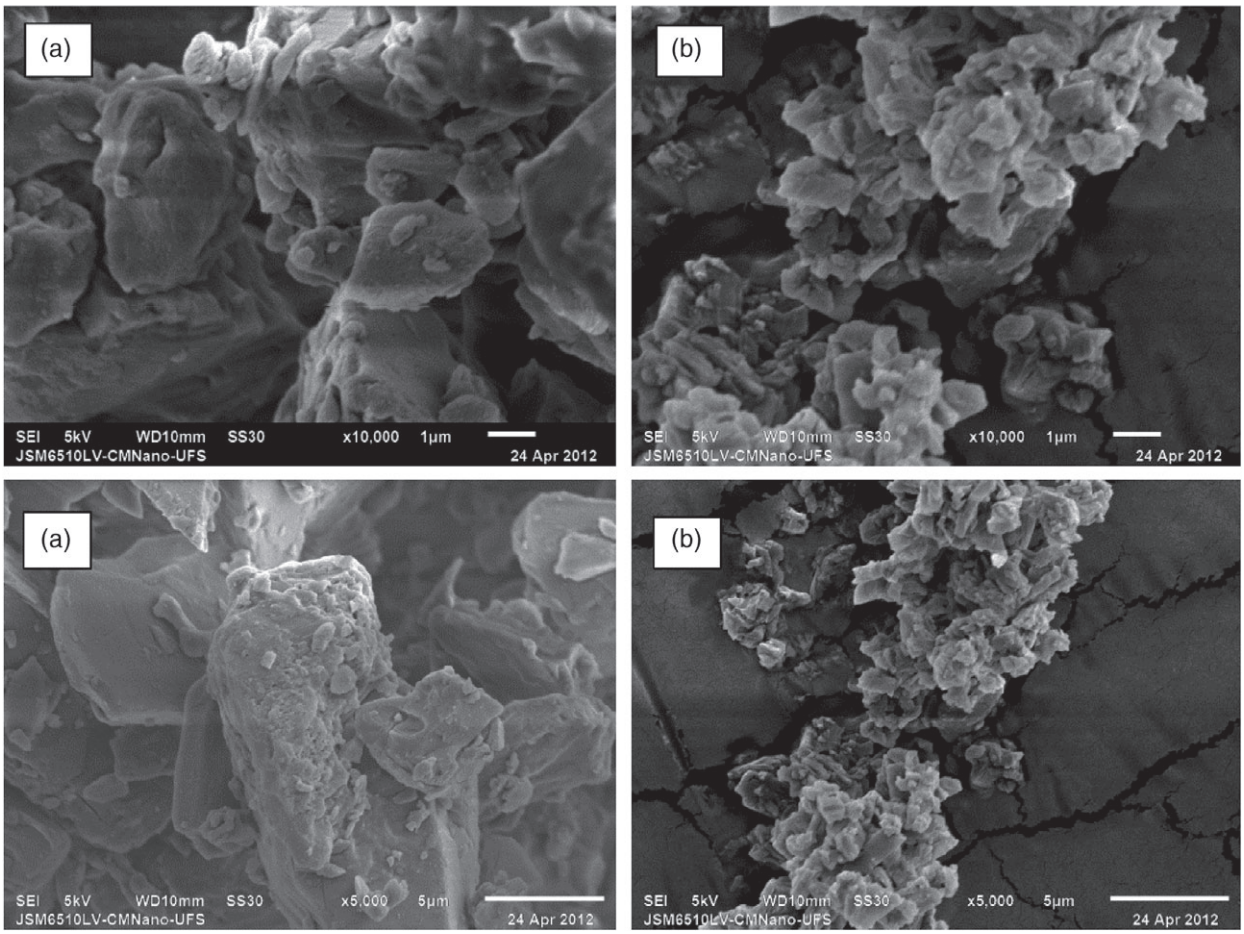

Figure 6. SEM photographs of β-cyclodextrin (a); and inclusion complex LGEO/β-cyclodextrin obtained by kneading prepared with water (KW) (b).

KW method was chosen because it did not use organic solvent (ethanol), which is desirable for formulations with biological application. Thus, the KW inclusion complex was further analyzed by SEM, larvicidal activity and lethality against non-target organisms.

The morphology of β-CD was studied using SEM. As shown in Fig. 6(a), the particles are of irregular size and shape. However, LGEO/β-CD inclusion complexes prepared by the KW method (Fig. 6b) showed drastic changes in particle shape, resulting in clusters, which suggests the formation of a complex and supports previous characterizations. Songkro and co-workers,[29] also found that an inclusion complex with citronella oil, citronellol and citronellal demonstrated drastic differences in the shape and size of particles compared with pure β-CD.

Marreto and co-workers[8] investigated the essential oil used in this work. They used the slurry and paste complexation methods, but a different type of cyclodextrin (hydroxypropyl-β-cyclodextrin) and observed that the paste complexation method gave almost complete oil retention (99.8%) in comparison with the slurry method. Nevertheless, the slurry method showed a better inclusion profile for the active and major terpenes found in LGEO. Similar results were found in this study using β-CD as a host for LGEO, the kneading method (also known as the paste method) was more effective in complexing LGEO than the co-evaporation method. In addition, this work evaluated the influence of ethanol in the complexation media, larvicidal activity and toxicity to non-target animals.

LGEO and its major constituent (carvacrol) showed larvicidal activity similar to that found by Silva and co-workers.[2] LGEO induced mortality in *Ae. aegypti* larvae after 24 h, with an LC_{50} of 39 ppm (38–42 95% CI). The inclusion complex prepared using the KW method had a LC_{50} of 33 ppm (31–35 95% CI). Thus, the inclusion complex β-CD/LGEO had greater larvicidal activity ($P < 0.01$, Student's *t*-test) against *Ae. aegypti* larvae compared with pure LGEO, suggesting that complexation with β-CD improved the efficacy of LGEO, probably by increasing its solubility.

Lethality assay against non-target organisms demonstrated that LGEO induced mortality in *Artemia* sp. with an LC_{50} of 30.5 ppm[30,31]; this is 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[32] reported a lethality of LGEO against *Artemia* sp. of 23.6 ppm, which is close to the value found here. However, the complexation of LGEO with β-CD showed lower lethality against non-target organisms with a LC_{50} of 150.6 ppm (148–156), that is fivefold higher ($P < 0.0001$, Students' *t*-test) than the LC_{50} needed to control *Ae. aegypti* larvae, and so less harmful to non-target organisms.

4 CONCLUSIONS

This work indicated that an inclusion complex between LGEO and β-CD formed effectively and improved the efficacy of LGEO against *Ae. aegypti* larvae. Phase solubility studies demonstrated

that the ideal molar ratio between LGEO and β-CD is 1:1. Moreover, thermodynamic data revealed that the complexation tends to be spontaneous ($\Delta S < 0$) and exothermic ($\Delta H < 0$). Characterization using DSC, TG, Karl Fisher, XRD, FTIR, and SEM proved formation of the inclusion complex. DSC and TG showed formation of inclusion complexes by kneading and co-evaporation methods. The inclusion content results showed that the KW method was best for preparing a LGEO/β-CD complex, giving the highest inclusion content (15.25%). Moreover, LGEO/β-CD complex (KW) improved larvicidal activity (39 to 33 ppm), and decreased toxicity towards non-target animals (30.5 to 150.1 ppm) in comparison with pure LGEO, with no harm to aquatic ecosystems at the concentrations needed to control *Ae. aegypti*. In conclusion, the product studied may be an attractive alternative for the control of *Ae. aegypti*, being biodegradable and economically viable.

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