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β-cyclodextrin inclusion complexes containing *Citrus sinensis* (L.) Osbeck essential oil: An alternative to control *Aedes aegypti* larvae

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ABSTRACT

The development of β-cyclodextrin (β-CD) complexes is an interesting way for increasing the aqueous solubility of essential oils. The aim of this study was to prepare inclusion complexes of *Citrus sinensis* essential oil (CSEO) with β-CD using paste complexation (with and without co-solvent) and co-precipitation methods. Additionally, the physicochemical properties of the inclusion complexes using thermal analysis, X-ray diffraction, Fourier transform infrared spectroscopy, and scanning electron microscopy were evaluated. Furthermore, CSEO content (%) and solubility of complexes were measured. The biological activity against the *Aedes aegypti* Linn. larvae was further evaluated. For comparison purposes, a physical mixture between β-CD and CSEO was prepared and evaluated. Thermal analysis clearly indicated the formation of complexes by paste and co-precipitation methods. The headspace/gas chromatography quantitative analysis showed inclusions contents higher than 50%. On the other hand, the product revealed LC_{50} of 23.01 ppm, close to CSEO LC_{50} 21.5 ppm.

1. Introduction

Aedes aegypti is the vector known to carry yellow and dengue fever, diseases responsible for a number of morbidity and mortality around the world [1,2]. Although several million dollars are spent attempting to eradicate the vector. Many synthetic insecticides have been used along the past years. Organophosphates, such as, temephos, have been used as larvicide in several countries [3,4].

However, due to the resistance of pesticides and toxic effects on the environment by contamination of soil, water and air, new methods using biotechnology alternatives to chemistry-based solutions have been developed in order to control *A. aegypti* [5–7]. Essential oils are outstanding candidates, since they are, in some cases, highly active, readily available in tropical countries, and economically viable.

Essential oils are complex mixtures of volatile organic compounds produced as secondary metabolites in plants. They are constituted by hydrocarbons (frequently mono- and sesquiterpenes) and oxygenated compounds (alcohols, esters, ethers, aldehydes, ketones, lactones, phenols, and phenol ethers) [8], which have been largely employed for their properties observed in nature, i.e., for their antibacterial, antifungal, and insecticidal activities [9]. In addition, essential oils have been suggested as potential tools for *A. aegypti* chemical control, given their larvicidal activity. Furthermore, essential oils are economically viable, biodegradable, and do not show toxic effects on non-target population [10].

Citrus sinensis (L.) Osbeck essential oil (CSEO) is essentially a mixture of terpenes, hydrocarbons and oxygenated compounds which are chemically unstable [11]. Due to its unsaturated nature, terpene and sesquiterpene compounds oxidize easily under the influence of light, air, and moisture. About 98% of *R*-limonene is found in the CSEO, the remaining two percent consists of a mixture of other terpenes (including myrcene, α-pinene, and some aldehydes monoterpenes) and aliphatic aldehydes (decanal, octanal, and others) [11].

The main constituent of CSEO, *R*-limonene, has an intense larvicidal activity against *A. aegypti* larvae [12–14], which makes this oil a potential candidate to control *A. aegypti* larvae population. However, due to its low aqueous solubility, easy oxidation, and volatility, the development of formulations is required to avoid CSEO degradation and to increase solubility as well as to incorporate the essential oil in a viable larvicidal product.

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Cyclodextrins are cyclic oligosaccharides constructed from α-(1,4)-linked-D-glucopyranose units, in a ring formation. The most common of these originally occurring, ring-shaped molecules, are the α (alpha), β (beta), and γ (gamma) cyclodextrins formed by six, seven, and eight glucose units, respectively, in which the enclosed cavities are approximately 4.7–5.3, 6.0–6.5, and 7.5–8.3 Å in diameter [15,16]. The nonpolar characteristic of the interior cavity of cyclodextrins makes them ideal for solubilizing nonpolar solutes, whereas the polarity of their exterior enables them and their guest to become soluble in water [17].

β-cyclodextrin (β-CD) has been widely applied due to its availability and cavity size suitable for a wide range of guest molecules. However, β-CD has a relatively low aqueous solubility (1.85 g/100 mL) limiting the applicability of its inclusion complexes [18]. Various methods have been introduced to enhance its solubility, including the addition of urea, metal salts, ethanol, and 2-propanol. Co-solvents are widely used in the formation of β-CD inclusion complexes [18].

Thus, the aim of the present work was to study the complexation of CSEO with β-cyclodextrin, using co-precipitation and kneading methods. The complexes were characterized by thermal analysis, X-ray diffractometry (XRD), Fourier transform-infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). In addition, the CSEO content (%) and the complexes solubility were measured. Finally, the inclusion complexes were evaluated for their biological activity against *A. Aegypti* larvae.

2. Material and methods

2.1. Material

The *C. sinensis* fruits used in this study were acquired in a market located in Aracaju City, Sergipe State-Brazil, in a single lot and sodium sulfate (Na_2SO_4) by Synth®. β-CD was obtained by Sigma–Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Methods

2.2.1. Essential oil extraction

The *C. sinensis* peels were dried in a circulating-air oven at 40 °C for 3 days, grinded and submitted to hydrodistillation for 3 h in a Clevenger-type apparatus to yield a yellowish oil. The essential oil obtained was separated from the aqueous phase by addition of sodium sulfate, and kept in a freezer until further analysis and preparation of inclusion complexes.

2.2.2. Identification of essential oil constituents

The CSEO was analyzed by GC/MS using QP5050A equipped with 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 carrier gas at 1.0 mL/min; injector split at 250 °C (split ratio 1/83); detector at 280 °C, 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 10 min isothermal at 300 °C. The mass spectra were taken at 70 eV with scanning speed of 0.50 scan/s from 40 to 500 Da.

Percentage composition was calculated using the peak normalization method. The peak identification was determined by comparing them with their Kovats retention indices, relative to a *n*-alkane homologous series (C_8–C_{18}), 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 NIST05 and Willey8 mass spectral library, in the GC/MS data system.

2.2.3. Samples and preparation of inclusion complexes

2.2.3.1. Method 1. The inclusion complexes were obtained by the kneading method with molar ratio of 1:1 β-CD:CSEO (based on *R*-limonene molecular weight) either in distilled water (paste complexation with water-PW) or distilled water:ethanol [1:1] (paste complexation with water and ethanol-PWE). β-CD and CSEO were weighed and homogenized in a glass mortar. Either distilled water or distilled water:etanol [1:1] mixture was added gradually, under constant manual agitation, until the formation of a paste. 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 airtight glass containers [19–20].

2.2.3.2. Method 2. In the co-precipitation (CP) system, β-CD was solubilized in 10 mL of distilled water for 30 min in a magnetic stirrer at 60 °C. After this stage, the mixture was cooled down to 30 °C, then CSEO with molar ratio 1:1 β-CD:CSEO (based on *R*-limonene molecular weight), dissolved in 10 mL of ethanol, was slowly added to the solution, and kept under stirring for 30 min. Then the sample was submitted to vacuum filtration and stored in a desiccator for improved conservation [21–24].

2.2.3.3. Method 3. A physical mixture (PM) was prepared by addition of CSEO to a glass mortar containing powdered β-CD under manual agitation. The CSEO/β-CD mass ratio was maintained, as described for the inclusion complex preparation, and the PM was stored in airtight glass containers [21].

2.2.4. Physicochemical properties of inclusion complexes

2.2.4.1. Thermal analysis. Thermoanalytical measurements were performed in a DSC-50 cell (Shimadzu®) using approximately 2 mg of the sample in aluminum crucibles under dynamic nitrogen atmosphere (50 mL/min), and heating rate of 10 °C/min· in the temperature range of 30–600 °C. The DSC cell was calibrated with indium (m.p. 156.6 °C; $\Delta H_{fus.} = 28.54\ J\ g^{-1}$) and zinc (m.p. 419.6 °C). The TG curves were carried out using a thermobalance, model TGA-50 (Shimadzu®), in the temperature range of 30–600 °C, using alumina crucibles with approximately 2 mg of samples under dynamic nitrogen atmosphere (50 mL/min) and heating rate of 10 °C/min. TG/DTG was calibrated using a $CaC_2O_4{\cdot}H_2O$ standard in conformity to ASTM.

2.2.4.2. Moisture determination. Physical mixture and inclusion complexes moisture contents were determined by Karl Fischer titration using a Potentiometric Titrator Metrohm® Titrando 836. The analyses were performed in triplicate.

X-ray diffraction analysis (XRD)

The crystallinity of β-CD, PM, and complexes (PW, PWE, and CP) were investigated in a Rigaku DMAX 2000 diffractometer with CuKα (1.5406 Å) in the range of 10–30° (2θ), using the powder XRD method.

2.2.4.3. Fourier transform infrared spectroscopy (FTIR). FTIR spectra were obtained on a PerkinElmer®, 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 ground and mixed thoroughly with KBr. Then, KBr discs were produced by compressing the mixed samples in a hydraulic press. The liquid sample (CSEO) was prepared using the KBr window technique.

2.2.4.4. Scanning electron microscopy (SEM). The morphology of β-CD and inclusion complex PWE (the best method of complexation demonstrated in others analysis), was analyzed by scanning electron microscope (model JSM-6510) with LV

acceleration voltage of 5 kV and a magnitude of 5000 and 4000×. The samples were placed on copper strips and attached to a blade and then covered with a gold film.

2.2.5. Quantification of inclusion of CSEO in β-CD by headspace gas chromatography

2.2.5.1. Headspace/GC–FID conditions. The analyses were performed in a headspace extractor, CTC Analytics (model: Combi Pal), coupled to a gas chromatograph (model GC 2010 Shimadzu®), equipped with a flame ionization detector (FID), with capacity of 250 °C. *R*-limonene was used as a chemical marker [11].

The GC–FID was equipped with a 5% diphenyl/95% diphenylsiloxano RTX-5 (Restek, USA, Bellefonte), 30 m long, 0.32 mm i.d. and 0.1 mm film thickness (crossbond®) column. The oven was programmed to start with a temperature of 50 °C for 15 min and heating ramp of 2 °C/min to 80 °C, followed by 14 °C/min to 150 °C. The temperature of both the injector and the detector was set to 230 °C, and finally, the temperature of 40 °C for the syringe with injection volume of 250 μL of the headspace. For extraction, the sample was incubated at 30 °C for 20 min with stirring of 500 rpm every 20 s in Combi-pal injector.

2.2.5.2. Inclusion content of CSEO in β-CD. To determine the inclusion content, the adsorbed oil was extracted from the inclusion complexes (PW, PWE, and CP) and PM surfaces by washing 3 g of powder with 20 mL of hexanes, followed by stirring for 20 min. The suspension was then filtered and residue washed with 10 mL of hexanes three times [21]. After the extraction process, 10 mg of the residue was placed in a 10 mL volumetric flask, and its volume was completed with ethanol. Samples were homogenized for 3 min in a vortex. Then, 500 μL of this solution was transferred to the chromatography analysis vial and added to 5 mL of water. Inclusion content was calculated as:

$$\text{Inclusion content}(\%) = \frac{\text{mass of recovered limonene in inclusion complex}}{\text{mass added limonene in inclusion complex}} \times 100$$

For discussion purposes, the dielectric constant of the preparation system was calculated by:

$$\varepsilon_{\text{system}} = \frac{\varepsilon_A \times \%A + \varepsilon_B \times \%B + \ldots \varepsilon_n \times \%n}{100} \quad (2)$$

where $\varepsilon_{\text{system}}$ – dielectric constant of the system; ε_A – dielectric constant of the solvent A; ε_B – dielectric constant of the solvent B; ε_n – dielectric constant of the "*n*" solvents; % A – Solvent A percentage; % B – solvent B percentage; % *n* – "*n*" solvent percentages.

2.2.6. Solubility measurement

Solubility of the complexes in water was determined by the above headspace–gas chromatography (HS/GC–FID) method in duplicate. A total of 150 mg inclusion complex (PW, PWE, and CP) was placed in 5 mL of water, under magnetic stirring for 24 h at 25 ± 1 °C. The content was centrifuged at 2500 rpm for 20 min and the clear supernatant solution was accurately diluted in water for analysis [25].

2.2.7. Larvicidal activity

For the determination of CSEO and its major component (*R*-limonene) larvicidal activity 100 mg of CSEO or *R*-limonene were dispersed in Tween-80 (0.25 mL). Mineral water (4.75 mL) was further added to the previous dispersion and stirred in a vortex, resulting in a 20,000 ppm solution. This solution was used to prepare 100 mL aqueous solutions in the range of 5–70 ppm in disposable cups. Twenty third-instar Rockefeller *A. aegypti* larvae were added to each cup. An aqueous solution of Tween-80 (0.1 mL) and water (19.9 mL) was used as control. A mortality count was conducted 24 h after treatment.

The larvicidal activity of inclusion complexes (PW, PWE, and CP) was performed using only the complex CSEO/β-CD which presented the highest level of complexation. The test was performed in triplicate. The concentrations of the complex varied in the range 5–70 ppm of equivalent CSEO in the formulation. No Tween-80 was added and the remaining conditions were similar to the previously established. Statistical analysis was performed by Probit analysis with the goal to determining the lethal concentration able to exterminate 50% of the larvae (LC_{50}) [26].

3. Results and discussions

The CSEO was obtained in 9.65% yield. Seven components in the CSEO were identified. The major component was *R*-limonene [96.30%], followed by mircene [2.11%], α-pinene [0.66%], including some other minor components. Moreover, the specific rotation of CSEO (+110.5) was measured to confirm the *R*-(+)-limonene [27].

Thermal analysis is an important tool to identify the formation of inclusion complexes with cyclodextrins [28]. In Fig. 1, the CSEO shows two endothermic peaks, one at 61.8 °C and another at 178.9 °C, both possibly corresponding to the process of volatilization or evaporation [29]. The DSC curve of β-CD, displayed a wide and strong endothermic effect in the interval 34–119 °C (DSC T_{peak} = 97.4 °C), also shown in curve TG/DTG (Fig. 2) with weight loss of 13.42%, which correspond to the release of water molecules. Furthermore, above 300 °C decomposition and removal of carbonaceous material occurs.

The DSC curve of PM exhibited a broad endothermic event between 30 and 110 °C (DSC T_{peak} at 89.6 °C) related to the evaporation of the essential oil and the water release from the β-CD. This observation can be confirmed through the TG/DTG analysis (Table 1), in which the PM exhibited a pronounced weight loss [14.13%], in the first event in the temperature range of 30–113 °C.

The complexes CSEO/β-CD showed endothermic events in a range of 80–170 °C, DSC T_{peak} = 100.7 °C, 69.9 °C, and 80.2 °C, represented by PW, PWE, and CP, respectively. In the TG/DTG

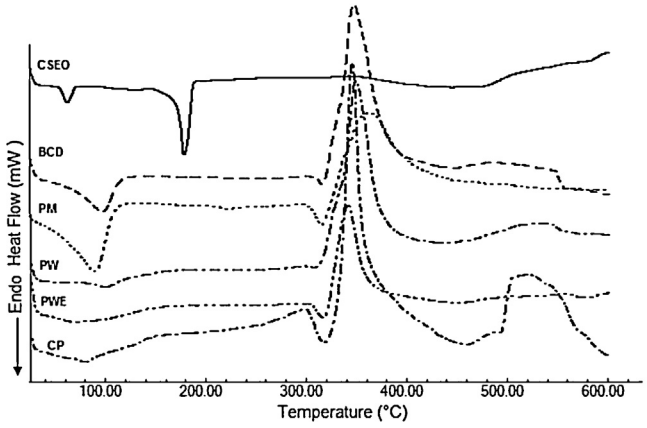

Fig. 1. DSC curves of *Citrus sinensis* essential oil (CSEO), β-cyclodextrin (β-CD), physical mixture (PM), paste complex water (PW), paste complex water/ethanol (PWE), and co-precipitation (CP) in dynamic nitrogen atmosphere (50 mL/min) and rate heat 10 °C/min.

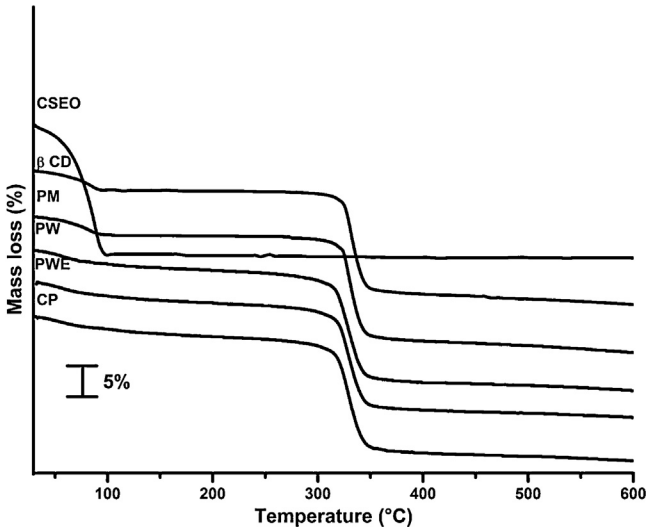

Fig. 2. TG curves of *Citrus sinensis* essential oil (CSEO), β-cyclodextrin (β-CD), physical mixture (PM), paste complex water (PW), paste complex water/ethanol (PWE), and co-precipitation (CP) in dynamic nitrogen atmosphere (50 mL/min) and rate heat 10 °C/min.

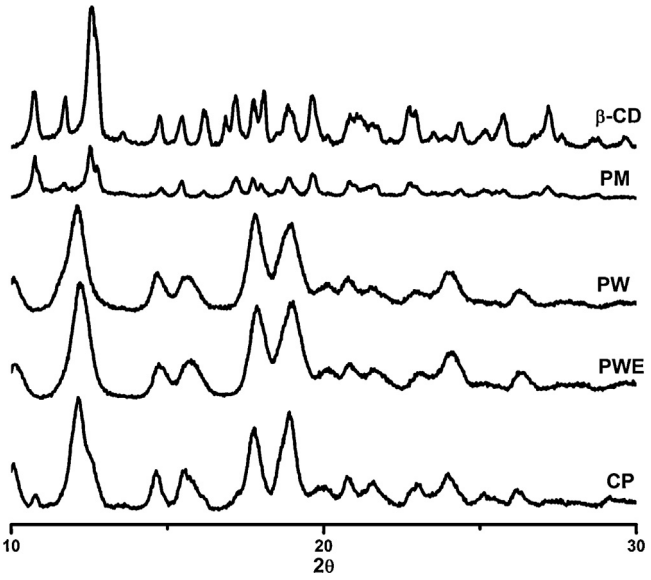

Fig. 3. X-ray diffraction pattern of β-cyclodextrin (β-CD), physical mixture (PM), paste complex water (PW), paste complex water/ethanol (PWE), and co-precipitation (CP).

curves, the complexes showed loss of mass (PW – 7.37%, PWE – 7.74%, and CP – 7.26%) in the range of 120–280 °C, which may be related to the release of CSEO, suggesting that the guest is included in the host by using any of the three methods.

It is important to note, however, that the results obtained by thermogravimetric analysis could not be solely used to distinguish the mass losses of oil and water from inclusion complexes. Thus, these results were complemented by water determination analysis using Karl Fischer titration. As shown in Table 1, the complexes are displaying a decrease in the percentage of water as compared to pure β-CD. According to Serafini and co-workers [30], this decrease is due to complex formation, since water molecules originally found in the cavity of β-CD were replaced by guest molecules.

The X-ray diffraction method is usually employed to detect the formation of β-CD complexes [28]. As shown in Fig. 3, β-CD has sharp peaks at 10.71°, 12.57°, 19.64°, 22.75°, and 27.17°, which are related to the crystalline nature of the material. Similarly, peaks of crystallinity of β-CD were detected in the physical mixture. On the other hand, XRD patterns of the CSEO:β-CD inclusion complexes differed considerably of β-CD alone, resulting in a new profile of diffraction, suggesting the complexation of CSEO in β-CD [31]. There were few differences between the profiles of the crystalline inclusion complexes obtained from kneading and co-precipitation methods, as well as there were no significant differences between the X-ray diffraction of the complex obtained without co-solvent (PW), and the complex obtained with co-solvent (PWE).

Table 1
Mass losses for *Citrus sinensis* essential oil (CSEO), β-cyclodextrin (β-CD), physical mixture (PM), paste complex water (PW), paste complex water/ethanol (PWE), and co-precipitation (CP), and moisture contents obtained by Karl Fisher titration.

Sample	Mass loss (%)			Karl Fisher % water
	1st step	2nd step	3rd step	
CSEO	94.7[a]	1.4	0.82	0.12
β-CD	13.4[b]	0.1	74.03	12.12
PM	14.1[c]	1.91[d]	74.63[e]	11.34
PW	11.5[c]	7.37[d]	77.15[e]	10.54
PWE	10.5[c]	7.74[d]	77.24[e]	10.45
CP	10.3[c]	7.26[d]	75.63[e]	10.28

[a] Percentage of the CSEO evaporates up to 120 °C.
[b] Percentage of water releasing up to 120 °C.
[c] Mass loss related to evaporation of the CSEO and water release up to 120 °C.
[d] Mass loss probably attributed to CSEO release in the interval from 120 to 280 °C.
[e] Thermal decomposition in the interval from 280 to 600 °C.

The FTIR spectrum of CSEO (Fig. 4) showed stretching vibrations (υ) in the bands of the group C=C in the range 1650–1500 cm^{-1}; υ CH in the range of 3100–2800 cm^{-1}, and angular deformation bands in the region of CH 1000–650 cm^{-1}. The FTIR spectrum of β-CD pure (Fig. 4) 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).

The spectra of inclusion complexes CSEO/β-CD obtained by different methods (PW, PWE, and CP) were practically dominated by the bands of β-CD, showing only a shift of β-CD from 2927 cm^{-1} (for CH stretching vibrations) to lower wavenumbers and the disappearance of characteristic peaks from CSEO in 2966 cm^{-1}, 2838 cm^{-1}, and 1437 cm^{-1}, thus providing an indication of interaction of the CSEO in the cavity of β-CD.

In the PWE and CP inclusion complexes, the β-CD characteristic band in the range (3600–3200 cm^{-1}) shifted to lower wavenumbers, which may be related to use of the co-solvent, ethanol, in the complexes preparation.

The surface morphology of pure β-CD observed in the scanning electron microscopy (SEM), as shown in Fig. 5a, presents particles of irregular size and shape.

On the other hand, the inclusion complexes CSEO/β-CD paste complexation obtained by water–ethanol (Fig. 5b) showed dramatic changes in the particle shape, resulting in clusters, suggesting a change of the structures, indicating the formation of a complex. This observation is in agreement with Songkro and co-workers [32], who observed that an inclusion complex with citronella oil, citronellol, and citronellal, had drastic changes in the form and size of particles.

The inclusion content for the CSEO/β-CD complexes was 57.3%, 78.5%, and 50.4% to PW, PWE, and CP, respectively as well as 1.7% for the physical mixture. PWE was the method that best complexes CSEO, possibly due to the use of the co-solvent ethanol ($\varepsilon = 30$), which modified the system dielectric constant of $\varepsilon_{systemPW} = 70$ to

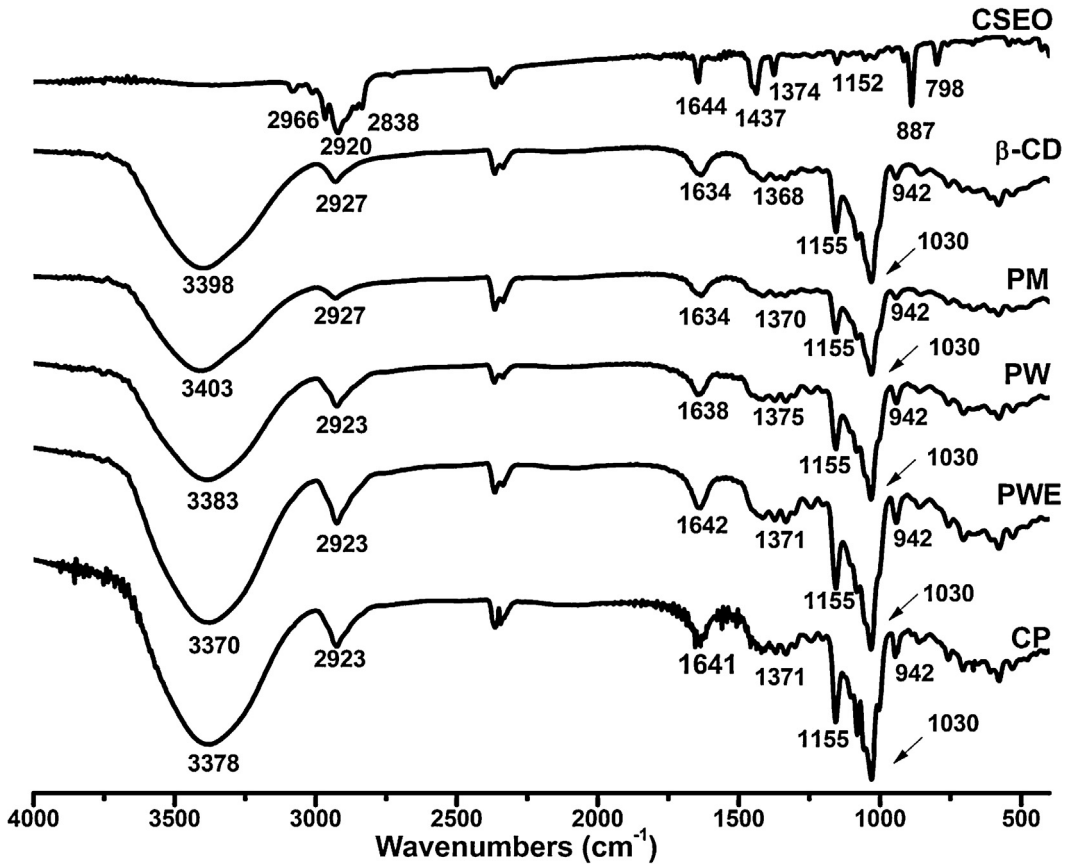

Fig. 4. FTIR spectra of *Citrus sinensis* essential oil (CSEO), β-cyclodextrin (β-CD), physical mixture (PM), paste complex water (PW), paste complex water/ethanol (PWE), and co-precipitation (CP).

$\varepsilon_{systemPWE} = 50$, enabling an improvement in the solubilization conditions of the oil in the preparation system, and therefore increasing complexation. According to Huang and co-workers [33], the use of alcohol as a co-solvent modifies the hydrophobicity of the aqueous phase, providing greater stability by increasing van der Waals interactions between the guest and the host, depending only on factors, such as the concentration of alcohol, cavity size, and structure of the guest.

The kneading method resulted in higher levels of inclusion content compared to the co-precipitation method, which is attributed to the occurrence of molecule disorder in the co-precipitation method, minimizing the chances of complexation. Additionally, longer sample preparation might result in higher loss of CSEO in the process.

The low level of inclusion obtained for the physical mixture, compared to the complexes, may be explained by the lack of water in the system. According to Loftsson and Brewster [34], the replacement of high enthalpy water molecules in the cavity β-CD by other substrate would be the driving force for complexation.

CSEO measured aqueous solubility was 0.00416 mg mL^{-1}. However, after the inclusion complex formation, the oil solubility raised to values around 0.057, 0.073, and 0.069 mg mL^{-1} to the obtained complex by PW, PWE, and CP, respectively. It was possible, then, to raise CSEO solubility to around 13, 17, and 16 times from the inclusion complexes with β-CD optimizing its use in aqueous media.

The larvicidal activity of CSEO and *R*-limonene were similar to those of Silva et al. [2]. OECS induced 100% mortality of *A. aegypti*

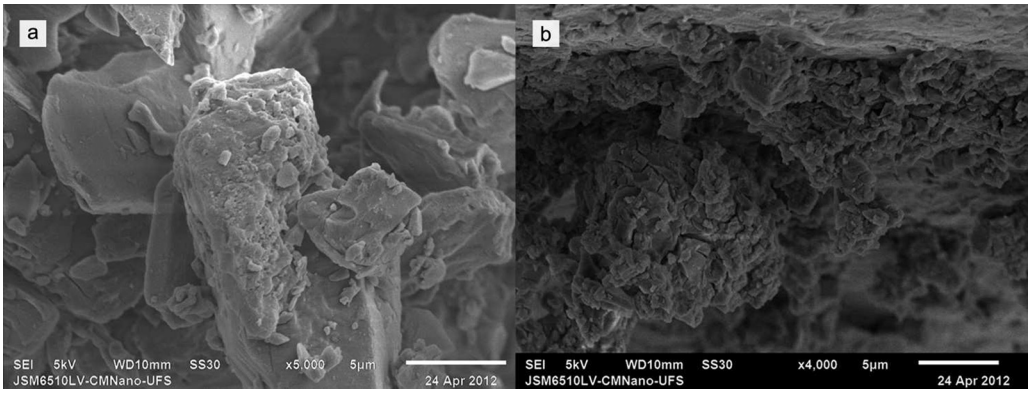

Fig. 5. Photographs SEM of β-cyclodextrin (a); and inclusion complex CSEO/β-cyclodextrin (b).

larvae after 24 h at 30 ppm (LC_{50} = 21.5 ppm, 21.3–21.7), and *R*-limonene induced 100% mortality at 50 ppm (LC_{50} = 26.8 ppm, 26.5–27.3). The complex obtained by the method PWE induced 93% mortality at 50 ppm (LC_{50} = 23.0 ppm, 22.6–23.3). CSEO and the complex PWE exhibit higher larvicidal activity against the larvae of *A. aegypti* than *R*-limonene (CSEO major component). Consequently, minor components are probably acting synergistically to achieve the experimental larvicidal action.

4. Conclusion

This study demonstrated that CSEO was effectively complexed in β-CD, and the formed product was, in fact, effective against the larvae of *A. aegypti*. The characterization of the complexes (DSC, TG/DTG, Karl Fisher, XRD, FTIR, and SEM) suggested their inclusion complexes formation. Thermal analysis clearly indicated the formation of complexes by paste and co-precipitation methods used to obtain the inclusion complexes. The results of HS/GC–FID quantitative analysis revealed that the best method of obtaining a CSEO/β-CD complex was the PWE, with the largest inclusion content [78.5%], and once the larvicidal activity of this complex is close to CSEO pure, this product becomes an interesting alternative, as well as, environmentally safe (biodegradable, non-toxic, and economically viable) for controlling *A. aegypti* larvae.

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