



Essential oil characterization of *Ocimum basilicum* and *Syzygium aromaticum* free and complexed with β -cyclodextrin. Determination of its antioxidant, antimicrobial, and antitumoral activities

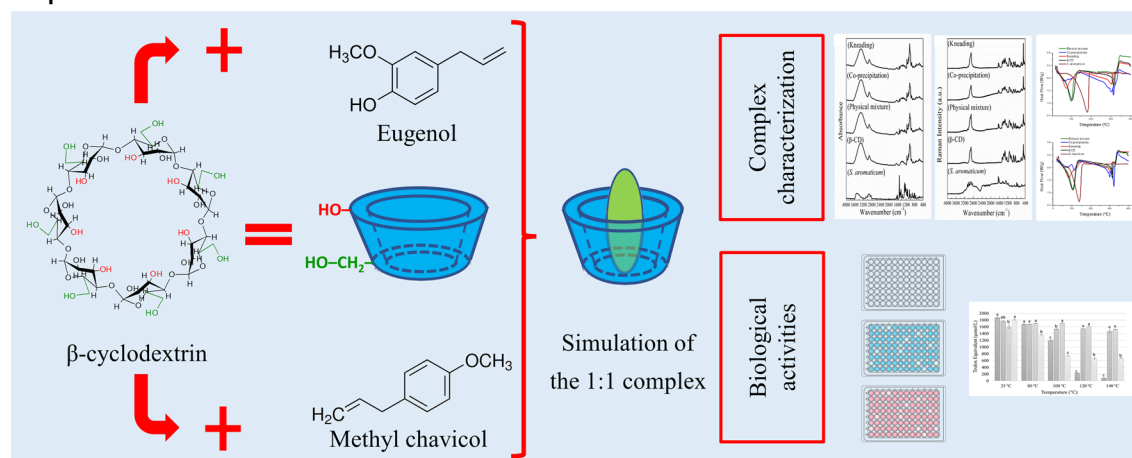
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Abstract

This research evaluated the chemical composition of *Ocimum basilicum* and *Syzygium aromaticum* and to characterize their complex with β -cyclodextrin (β -CD) using physical mixing, kneading, and co-precipitation methods. Gas chromatography coupled to a mass spectrometer showed that methyl-chavicol and eugenol were, respectively, the major compounds from *O. basilicum* and *S. aromaticum*. By the use different vibrational spectroscopic methods (ATR-FTIR and FT-Raman) and thermal analysis (DSC and TGA), it was possible to strongly suggest the formation of EO- β -CD complex. The antioxidant activity was evident in *S. aromaticum* EO due to the presence of the eugenol compound in the complexes. The antioxidant stability was evaluated at different temperatures, in which OE had its antioxidant activity reduced drastically while the complexes had a reduction of 6–13% of its activity. The antimicrobial activity was observed for EO and its complexes of *S. aromaticum* against yeasts of the genus *Candida*, in which there was a reduction of the concentration of the MIC and CFM for the complexes formed by the co-precipitation methodology. The antitumor activity against HT-29 and HeLa cells was observed in both EOs, in contrast, the cytotoxicity of EOs was also increased. The complex formed from OE *S. aromaticum* showed the best antitumor activity against HT-29 cells (IC₅₀ = 12.5 μ g/mL) with a better selectivity index (IS = 12.32). It is concluded that the techniques used were suitable for the confirmation of the complexes with β -CD, and that the complexation contributed with the stability and action of the essential oils evaluated, allowing their use in the various industrial sectors, such as pharmaceutical and food industry.

Graphic abstract



Keywords *Ocimum basilicum* · *Syzygium aromaticum* · Essential oil · β -cyclodextrin · Complexation

Introduction

Essential oils (EOs) are synthesized by plants as part of their secondary metabolism, produced from flowers to roots, and constituted in a variety of chemical compounds that may present fragrance, antimicrobial properties, antioxidant potential, antitumor action, and other characteristics [1]. Once they are natural products and generally recognized as safe (GRAS) by the Food and Drug Administration (FDA), EOs have been accepted by the population with no effort [2].

S. aromaticum is popularly known as clove. It has a set of bioactive compounds, and, therefore, a variety of biological activities [3]. Traditionally, the clove oil is used in the treatment of cuts and burns, also, as a pain reliever in toothaches. Studies have shown its activity in respiratory and digestive diseases, as well as acting as aphrodisiac, antioxidant, antimicrobial, and anti-inflammatory agent. They also have described anticancer and antimutagenic actions [4].

O. basilicum, known as basil, is abundant in OEs, and methyl chavicol (or estragole), which is the compound responsible for its pleasant taste and odor characteristics [5]. It has been used popularly as antitussive, vermifuge, headache reliever, among others [6]. In addition to popular use, studies have shown that *O. basilicum* has anticarcinogenic activity and wide pharmacological performance [7].

The use of EOs has been limited due to their low solubility in the aqueous medium [8, 9]. Also, EOs are unstable and vulnerable to degradation by light, heat, temperature, etc., when unprotected. Because of that, the use of complexing agents for microencapsulation, such as cyclodextrins (CDs), facilitates the solubility of EOs in aqueous medium, which increases their stability while preserving their antioxidant activity and bioavailability [8]. In the pharmaceutical industry, antioxidants have anticancer activity, and the improvement in the protection of antioxidant activities against external factors is necessary for the maintenance of the activity, conservation and distribution of the drug in the market [10, 11].

The solubility of compounds encapsulated with CDs is improved, due to the hydrophilic outer surface of these molecules, making it water soluble, and their interior has hydrophobic characteristics, which allow them to encapsulate numerous non-polar compounds such as EOs [12]. The most used CD is β -CD, as in addition to having an adequate cavity to house compounds with molecular masses ranging from 200 to 800 g/mol, its commercial value is reduced compared to other CDs and, consequently, has a high availability [13].

In previous works, the improvement of antioxidant stability against external factors of guava leaf oil was reported by

Rakmai et al. [14], while for the EOs in the present study are no reports of antioxidant stability at high temperatures were found. Therefore, this study aimed to identify the chemical compounds present in *S. aromaticum* and *O. basilicum* EOs and to complex them with β -CD to characterize the formation of inclusion complexes by several techniques. Also, it sought to determine the antioxidant stability of pure and encapsulated EOs on different temperatures, since no related reports were found in the literature. The antimicrobial and antitumor activities were also evaluated.

Materials and methods

Material

The main material used in this research were: *Syzygium aromaticum* EOs (Laszlo, Belo Horizonte, Minas Gerais - Origin: Indonesia) and *Ocimum basilicum* (Ferquima, Vargem Grande Paulista, São Paulo - Origin: Vietnam). The reagent used was β -cyclodextrin (Sigma-Aldrich St. Louis, MO, USA).

Methods

Essential oils characterization

The methodology used for the chemical characterization of EOs was based on Castro et al. [15] with modifications. Gas chromatography coupled to mass spectrometry (GC-MS) was used with FOCUS GC - DSQ II equipment, Thermo Electron Corporation. The column used for the analysis was Agilent DB-5 (5% phenyl/95% dimethylsiloxane stationary phase, 0.25 mm internal diameter, 30 m long and 0.1 μ m film of thickness). The initial column temperature was 60 °C, following an increase from 4 °C/min to 220 °C. After reaching this temperature, a gradient from 30 °C/min until reaching 250 °C was adopted. Helium gas was used as the carrier gas at a flow rate of 1.0 mL/min. Injector and detector temperature were 250 °C. For the reading of the oils, 1 μ L of the EO was injected in split mode (1:20). Hexane (chromatographic grade) was used to dilute the EOs.

The main compounds were characterized based on retention time (RT) compared to Kovats retention rate [16]. A standard mixture of *n*-alkanes (C8–C20; Sigma-Aldrich) was used to confirm the identified EOs compounds. The compounds of interest were confirmed [17] and presented in percentages.

Complex formation

The methodologies used for complex formation were cited by Galvão et al. [18], with modifications. The complexes were prepared by physical mixing, kneading and co-precipitation methods. The ratio of EO and β -CD in all methodologies was 1:1 according to the major component of each EO [19], that is, for the *O. basilicum* EO, the calculations were performed considering the compound methyl-chavicol, and for the *S. aromaticum* EO, eugenol. In terms of mass, per 1 g of β -CD were weighed 0.130 g of *O. basilicum* OE and 0.144 g of *S. aromaticum* OE. The ratio chosen was based on Pinto et al. [19], who complexed benzocaine in proportions of 1:1 to 1:2, and evaluated the favorable stoichiometry was 1:1 by infrared spectrometry, and the major compounds present in EOs have a molecular structure like benzocaine.

During the preparation of the physical mixture, the EO was added over the β -CD and homogenized manually in glass mortar. In the kneading process, it was made a paste with the EO and the β -CD using distilled water:ethanol (1:1) (v/v) with the aid of one glass mortar and pistil. The liquids were gradually added to the mixture until the paste was formed. It was used a desiccator at room temperature for drying the complex. By the co-precipitation method, β -CD was solubilized under agitation at 60 °C in 40 mL of distilled water. The EO was solubilized in 5 mL of ethanol. When the β -CD solution reached room temperature, the essential oil was added dropwise. This system was kept under agitation at 140 rpm for 1 h in the dark. The complex was vacuum filtered using blue strip quantitative filter paper and stored in a desiccator for drying.

All preparations were stored in a hermetically sealed jar of glass protected from light.

Complex characterization

Mangolim et al. [13] used various techniques to characterize the formation of the curcumin- β -CD complex in their research. Due to the relevance of the results found by them, their methodology was used in this study. In all assays the following samples were analyzed in duplicate: EOs, β -CD, physical mixture and the various complexes obtained. The ATR-FTIR spectra were obtained by using an infrared Fourier-transform spectrometer (FTIR) (model Vertex 70v, Bruker, Germany) accessorized with attenuated total reflectance (ATR). The spectral range was from 400 to 4000 cm^{-1} , with a resolution of 4 cm^{-1} . The results came from the averages of the 128 readings that the device performs in the ATR-FTIR analysis. For Raman spectra, the samples were analyzed using the Vertex 70v with module Ram II spectrometer, Bruker, Germany, with germanium detector, liquid cooled by nitrogen, and an Nd:YAG laser was used for excitation at 1064 nm. The analyzed wavelengths ranged from

400 to 4000 cm^{-1} . All spectra were an average of 128 scans with a resolution of 4 cm^{-1} .

Thermal analyzes of β -CD, physical mixture, and of the complexes were performed: differential scanning calorimetry (DSC) and thermogravimetry analysis (TGA). The physical and chemical properties of matter by loss of mass due to temperature were evaluated using the Netzsch equipment, model STA 409 PG LUXX®, with a heating rate of 10 °C. The temperature range used was 0 to 400 °C with a nitrogen flow of 30 mL/min. To assist in the thermogravimetric analyses, the analysis of moisture determination of β -CD, physical mixture and complexes was performed by Karl Fischer titrator (Mettler Toledo - DL31).

Retention of EOs by β -CD

The CG-MS was used to determine the percentage of retention (r) of EOs by β -CD. The methodology used was based on Kfoury et al. [20] with modifications. The EOs and their complexes were weighed and added to 10 mL of water in 22 mL headspace glass vial. The final concentrations of each EO were 1 mg/mL and their complexes/physical mixtures were weighed so that, at the end, they had the equivalent amount to 1 mg/mL of the corresponding EO. Thus, 79.4 mg and 86.9 mg of complex/physical mixture were weighed for the *S. aromaticum* and *O. basilicum* EO, respectively. The vials were left at a temperature of 25 °C for 30 min for the equilibrium of the gaseous and aqueous phases. Then, with the aid of a hermetically closed syringe, 1 mL of vapor was withdrawn from the vial and injected for GC-MS analysis. The GC settings were set as described in “Essential oils characterization” section. The r (%) was expressed as $r(\%) = (1 - \sum A_{CD} / \sum A_O) * 100$, where $\sum A_{CD}$ e $\sum A_O$ are the sum of peak areas of the compounds present in the complexes/physical mixture and in the pure EOs, respectively.

Antioxidant activity

Antioxidant activity was evaluated by determining ABTS⁺ free radical scavenging activity and DPPH free radical scavenging activity, described in the following sections. The antioxidant activity of pure and complex EOs were evaluated. For ABTS⁺ and DPPH standard Trolox curves were constructed at concentrations of 100 to 1500 $\mu\text{mol/L}$ and 50 to 2000 $\mu\text{mol/L}$, respectively. In both curves, R^2 was 0.99.

ABTS radical scavenger activity According to the methodology of Nenadis et al. [21], a solution of ABTS 7.0 mmol/L and potassium persulphate 140.0 mmol/L was prepared. The radical was prepared using 5 mL of the ABTS stock solution with 88 μL of the potassium persulphate solution, which was left to stand for 16 h in an amber vial, in the dark, at room temperature. After 16 h, the stock solution was diluted

with ethanol. The absorbance reading was taken at 734 nm, in which the solution and ABTS⁺ was adjusted by adding a stock solution or ethanol until the absorbance reached 0.700. It was used 30 µL of the sample, diluting them as needed, and 3 mL of ABTS⁺ solution, and kept in the dark for 6 min. The result of the ABTS⁺ radical scavenger activity was expressed by the equivalent concentration of Trolox in µmol/L/g sample.

DPPH radical scavenger activity The methodology used in this assay was based on Li et al. [22]. A stock solution of DPPH 6.25×10^{-5} mol/L in methanol was prepared. From the stock solution, a working solution was prepared using methanol as the diluent, in which the absorbance of this solution at 517 nm was 0.700. It was used 25 µL of the sample at a concentration of 1 mg/mL and 2 mL of the working solution. After sample preparation, they were left to stand for 30 min in the dark. Then the readings were taken at 517 nm by zeroing the apparatus with methanol. The result of DPPH radical scavenging activity was performed in triplicate and expressed as the equivalent Trolox concentration in µmol/L/g sample.

Stability of antioxidant activity The stability of the EOs was determined based on the methodology of Tomaino et al. [23] with modifications. It was weighed 1 mg of pure EOs for the physical mixture and, for the other complexes, were weighed the amount equivalent to 1 mg of EOs considering the major compound: 7.9 mg for *S. aromaticum* and 8.7 mg for *O. basilicum* EO. These EOs were placed in screw-capped glass tubes. They were kept at room temperature for 3 h or in a laboratory oven for the same period at temperatures of 80, 100, 120 and 140 °C. After that, the samples were cooled in an ice bath and immediately used for the determination of antioxidant activity. The assay was performed in triplicate and protected from light.

Antimicrobial activity

Microorganisms Antibacterial and antifungal activity was evaluated using the following strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6623, *Salmonella enteritidis*, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019 e *Candida tropicalis* ATCC 28707.

Standardization of microbial suspension The microbial suspension was standardized according to the Clinical Laboratory Standard Institute (CLSI) M07-A9 [24] and M27-A3 [25].

Minimal inhibitory concentration (MIC): microdilution method The concentration of EOs used was established based on preliminary tests. Thus, for the assays of minimum inhibitory concentration (MIC) solutions containing 8 mg/mL of *O. basilicum* and *S. aromaticum* EOs were prepared separately using 50 µL Tween 80 to solubilize the oil and MHB as solvent (test solution). For bacteria, a 100 µL volume of MHB was added to each well of a 96-well plate. Then 100 µL of the test solution was added to the wells of the first column and then, in serial dilution, until the ninth column, resulting in the following concentrations: 4000–15.63 µg/mL. For the complexes, the amount equivalent to the complexed oil was calculated, in other words, the first well contained 31.6 mg of the physical mixture with the complexes of *S. aromaticum* EO and 34.8 mg of the physical mixture with complexes of *O. basilicum* EO. For the yeasts, RPMI-1640 medium with 0.05% phenol red and 10% glucose was used. The dilution of the oils, the physical mixture, and the complexes were the same used for bacteria. Following that, 5 µL of the standard suspension of microorganisms were added to all wells and incubated at 37 ± 2 °C during 48 h for bacteria and 72 h for *Candida*. MIC (lower concentration of the drug capable of inhibiting microbial growth in vitro) was evidenced by the absence of turbidity in the culture medium. In all assays, controls were performed on a culture medium, inoculum, EO, and their complexes.

Minimum bactericidal concentration (MBC) and minimum fungicide concentration (MFC) To determine the bactericidal (MBC) and minimum fungicidal (MFC) concentration, 10 µL of culture medium from wells, in which the minimum inhibitory concentration (MIC) was positive as well as higher concentrations, were plated on Müller–Hinton agar for bacteria or Sabouraud dextrose agar for yeast. For all microorganisms, the incubation temperature was 37 ± 2 °C and the incubation time was 24 h for bacteria and 48 h for yeast. The lowest concentration of the extract in which there was no growth of microorganisms or growth of only one colony was considered as MBC/MFC.

Antitumor activity

Cell-cultivation The cells used to evaluate the antitumor activity of the EOs were from cervical carcinoma strain immortalized by the HPV 18 (HeLa), from the colon adenocarcinoma strain (HT-29) and, as normal control, the VERO epithelial cells. Cells were cultured in Eagle, modified by Dulbecco (DMEM; Gibco Invitrogen, New York, USA) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) incubated at 37 °C with moist atmosphere and tension of 5% of CO₂.

Methodology for the evaluation of cellular cytotoxicity by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) method Cytotoxicity assays followed the methodology of Mosmann [26]. From the results found using normal cells (VERO) and tumor cells (HeLa and HT-29) it was possible to verify the selectivity index represented by the equation $SI = \text{amount of } IC_{50} \text{ of VERO cells} / \text{amount of } IC_{50} \text{ of the tumor cell}$. The selectivity of tumoral cells is set on values above 1 and the higher this value the more selective the test substance is.

Statistical analysis

Results were evaluated by using the analysis of variance (ANOVA) and averages compared by using Tukey test at 5% of significance level. Microsoft Excel® application and Action Software were used.

Results and discussion

Essential oils characterization

The compounds of the EOs were identified and quantified by GC–MS. The results are presented in percentages. The main components of the *S. aromaticum* EO were α -caryophyllene (1.95%), β -caryophyllene (9.44%), and eugenol, with the last one being the majority compound with almost 87.85% of the total oil composition. In the review made by Cortés-Rojas et al. [27], it is possible to compare the compounds present in the *S. aromaticum* EO of this study. According to the authors, the α -caryophyllene compound can be up to 2.10% and the β -caryophyllene compound can range from 5 to 15%. Eugenol, being the main compound, can reach 89%.

Regarding *O. basilicum* EO, the main compounds were cis- α -bisabolene (9.41%), linalool (12.35%) and methyl-chavicol, in which the last one was the major compound with 74.40% of the total oil composition. Shirazi et al. [7] evaluated *O. basilicum* EO and reported that the major compound found in their work was methyl-chavicol, containing approximately 47% of the total EO composition; however, Lee et al. [6] obtained as the major compound in their research the linalool compound (approximately 39%).

Grayer et al. [5] considered that the different genotypes of a plant can generate variations in the composition of the EO and the concentration of its compounds. Moreover, the chemical composition of EOs is also influenced by external plant factors, which change the type and amount of compounds found [1]. In the present study, the chemical compounds present in EOs are in agreement with what the literature presents [6, 7, 27], but in different percentages.

Complex characterization

Attenuated total reflection Fourier transform infrared (ATR-FTIR)

The characterization of the complexes between EO and cyclodextrin (CD) by FTIR spectroscopic technique has been widely used. Piletti et al. [28] performed the FTIR methodology to prove the formation of the inclusion complex of eugenol, a majority compound found in *S. aromaticum* EO. Also, CD. Kfoury et al. [2] performed the same methodology for methyl-chavicol, the major compound of *O. basilicum*.

In Table 1 we have the assignments of the infrared and Raman bands of β -CD from *S. aromaticum* and *O. basilicum* EOs, while, in Fig. 1, the spectra are from regions that showed changes after encapsulation. In all analyzes, the physical mixture was read and compared with readings of the complexes formed by co-precipitation and kneading, and, also, with all regions that displacements of central positions of the bands were observed. The physical mixture showed a similar peak to the β -CD molecule, indicating that, for the complexes, there was a modification in the vibration of molecular bonds of the β -CD molecule or the compound present in the EO. In general, physical mixing was used as a standard to observe changes in the supposed encapsulation. Yet, in the co-precipitation and kneading spectra, evidence of the inclusion complex formation was observed. The displacements that occurred are associated with the characteristic vibrations of certain regions of the β -CD molecule, and, also, the supposed bonds affected in the complexation process are the bonds inside of the molecule cavity. For encapsulated compounds which have low water solubility and high volatility, the regions most likely to modify are, for example, the double-bonded regions and aromatic ring.

Inclusion complexes are formed by interactions between the guest molecule and the host; also, they are involved in hydrophobicity interactions, Van der Waals forces, hydrogen bonds, and in the release of high energy water molecules from the CD cavities [32]. In the spectrum corresponding to the EO and *S. aromaticum* complexes (Fig. 1b), a region of modification was observed. In this region, the peak at 706 cm^{-1} is related to the deformation in the plane of the β -CD glucose ring [30]; it shifted to 701 cm^{-1} while the same peak was unchanged for the physical mixture. Therefore, the peak shift only for complexes is suggestive for complexation.

For the *O. basilicum* EO spectrum, some modification regions were observed. The peak at 1610 cm^{-1} , the vibration region of the methyl-chavicol aromatic ring [30, 31], shifted to 1613 cm^{-1} in co-precipitation and 1612 cm^{-1} in kneading (Fig. 1d). The peak at 1365 cm^{-1} , corresponding to the CH scissor vibration of the β -CD molecule, shifted to 1367 cm^{-1}

Table 1 Infrared and Raman vibration modes of β -CD and OEs of *S. aromaticum* and *O. basilicum*, according to Li et al. [29], Wang et al. [30] and Yang et al. [31]

Sample	ATR-FTIR (cm ⁻¹)	Raman (cm ⁻¹)	Assignments (vibration mode)
β -CD	1365	1387	Scissors (CH)
		1338	Scissors (CH)
		1328	Scissors (CH)
		1128	Stretch (C–C)
		1083	Stretch (C–O)
		1076	Stretch (C–O)
		1021	Stretch (C–O)
		995	Symmetrical stretch (C–O–C)
		929	Glucose ring “Breath”
		852	Glucose ring “Breath”
		706	710
<i>S. aromaticum</i>	1639	478	Deformation out of the plane of glucose ring
		1639	Conjugate stretch (C=C) (Eugenol)
		1614	Aromatic ring stretch (C=C) (Eugenol)
		796	Aromatic ring deformation (Eugenol)
<i>O. basilicum</i>	1610	1611	Aromatic ring vibration (Estragole)

in both complexes (Fig. 1d). The peaks at 1076 cm⁻¹ and 1021 cm⁻¹, corresponding to the C–O stretch vibration, which mean vibrations of the functional groups within the β -CD, shifted to 1079 cm⁻¹ and 1023 cm⁻¹ in both complexes respectively. The symmetrical C–O–C stretch of β -CD was represented by the peak at 995 cm⁻¹, and it shifted to 998 cm⁻¹ in both complexes (Fig. 1e). Kfoury et al. [2] associated the changes with the reorganization of intermolecular interactions between the guest and host and/or change in the degree of hydration.

The peak at 706 cm⁻¹, corresponding to the plane deformation of the β -CD glucose ring, shifted to 701 cm⁻¹ in both complexes (Fig. 1f) [29]. In all spectral modifications observed for EO and *O. basilicum* complexes, there was no change in the spectrum of the physical mixture. These shifts indicated that there was some change in the chemical bonds of the molecules; also, it suggests that the complexation occurred and that the ATR-FTIR technique is efficient in showing the formation of complexes between the β -CD and the compounds present in the EOs.

Fourier transform Raman scattering infrared spectroscopy (FT-Raman)

The assignments of the Raman spectrum bands of the β -CD, the EOs, and the complexes are shown in Table 1, while, in Fig. 2a, the spectra are from regions that showed changes after encapsulation. As was considered for ATR-FTIR

analysis, for FT-Raman the physical mixture was used as a standard solution to observe the changes regarding the supposed encapsulation. For *S. aromaticum* EO, the peaks at 1639 cm⁻¹ and 1614 cm⁻¹ shifted, both corresponding to the C=C stretch vibration of the eugenol aromatic ring [30]. The peak at 1639 cm⁻¹ also shifted to 1638 cm⁻¹ in both complexes, but the peak at 1614 cm⁻¹ shifted to 1615 cm⁻¹ for co-precipitation and 1616 cm⁻¹ for kneading (Fig. 2b). This shifts suggest that the C=C bond of the eugenol aromatic ring changed after the β -CD complexation process.

In Fig. 2b, the 1387 cm⁻¹ and 1338 cm⁻¹ peaks related to the β -CD C–H scissoring vibration [29] were altered. The band of 1387 cm⁻¹ shifted to 1385 cm⁻¹ for co-precipitation and 1381 cm⁻¹ for kneading. However, for the band at 1338 cm⁻¹, the shift in co-precipitation was 1332 cm⁻¹ and for kneading it was 1327 cm⁻¹.

In Fig. 2c, the peak at 1127 cm⁻¹, corresponding to the β -CD C–C stretching vibration, shifted to 1134 cm⁻¹ for co-precipitation and 1136 cm⁻¹ for kneading. The peak at 1083 cm⁻¹, corresponding to the C–O stretching vibration, which are vibrations of the functional groups present within the β -CD [29], shifted to 1087 cm⁻¹ in both complexes.

In Fig. 2c, the center band at 929 cm⁻¹, corresponding to the β -CD glucose breath ring, shifted to 925 cm⁻¹ in co-precipitation and to 920 cm⁻¹ in kneading. The 852 cm⁻¹ peak, which was related to the breath ring of β -CD glucose [29], was undermined in the signal strength and shifted to 847 cm⁻¹ for co-precipitation and

Fig. 1 **a** ATR-FTIR spectrum for *S. aromaticum* EO, β -CD, physical mixture, and complexes formed from co-precipitation and kneading methodologies. **b** Magnification of $750\text{--}650\text{ cm}^{-1}$. **c** ATR-FTIR spectrum for *O. basilicum* EO, β -CD, physical mixture, and complexes formed from co-precipitation and kneading methodologies. **d** Magnification of $1200\text{--}950\text{ cm}^{-1}$. **e** Magnification of $1700\text{--}1300\text{ cm}^{-1}$. **f** Magnification of $750\text{--}650\text{ cm}^{-1}$

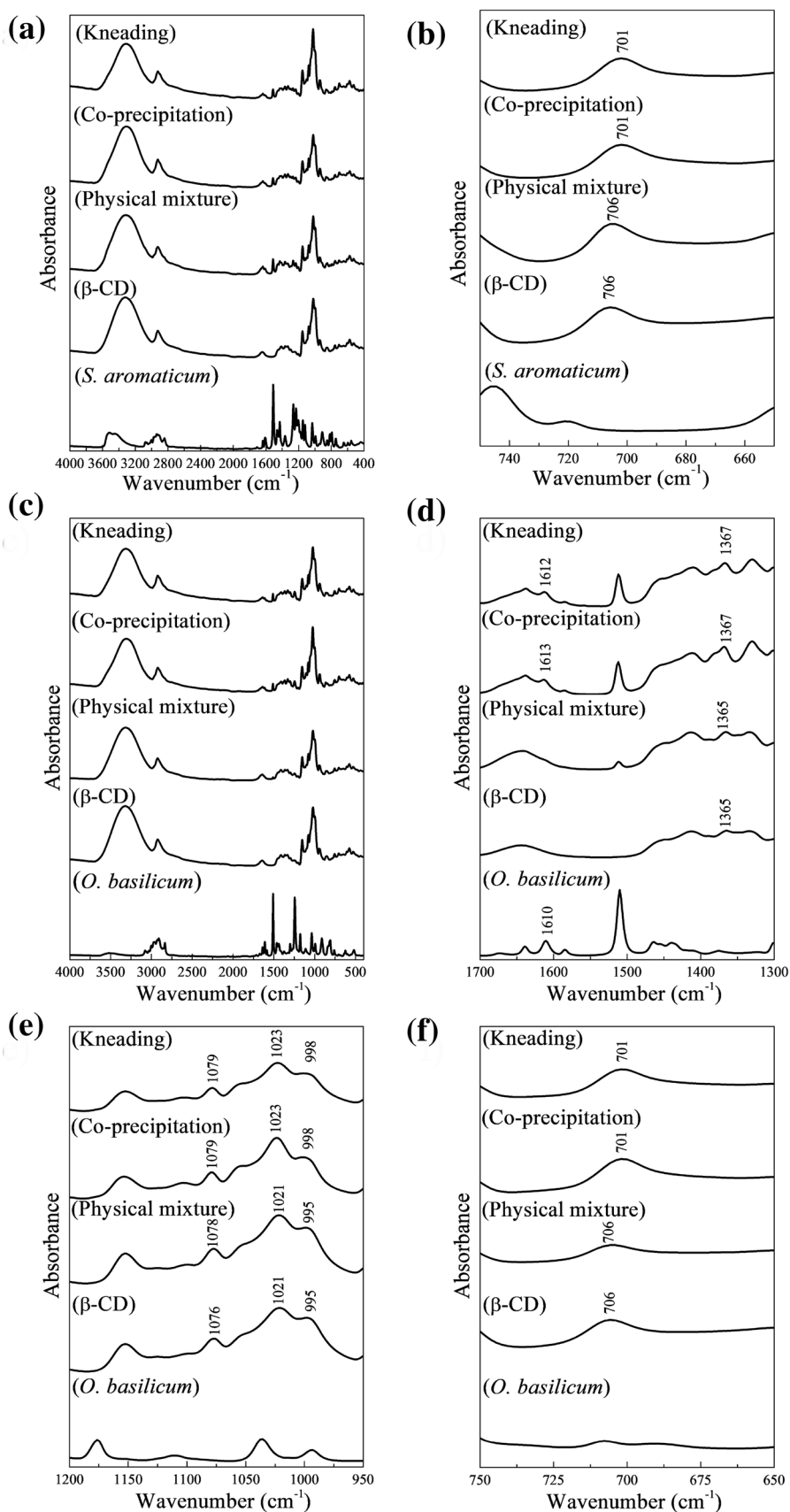
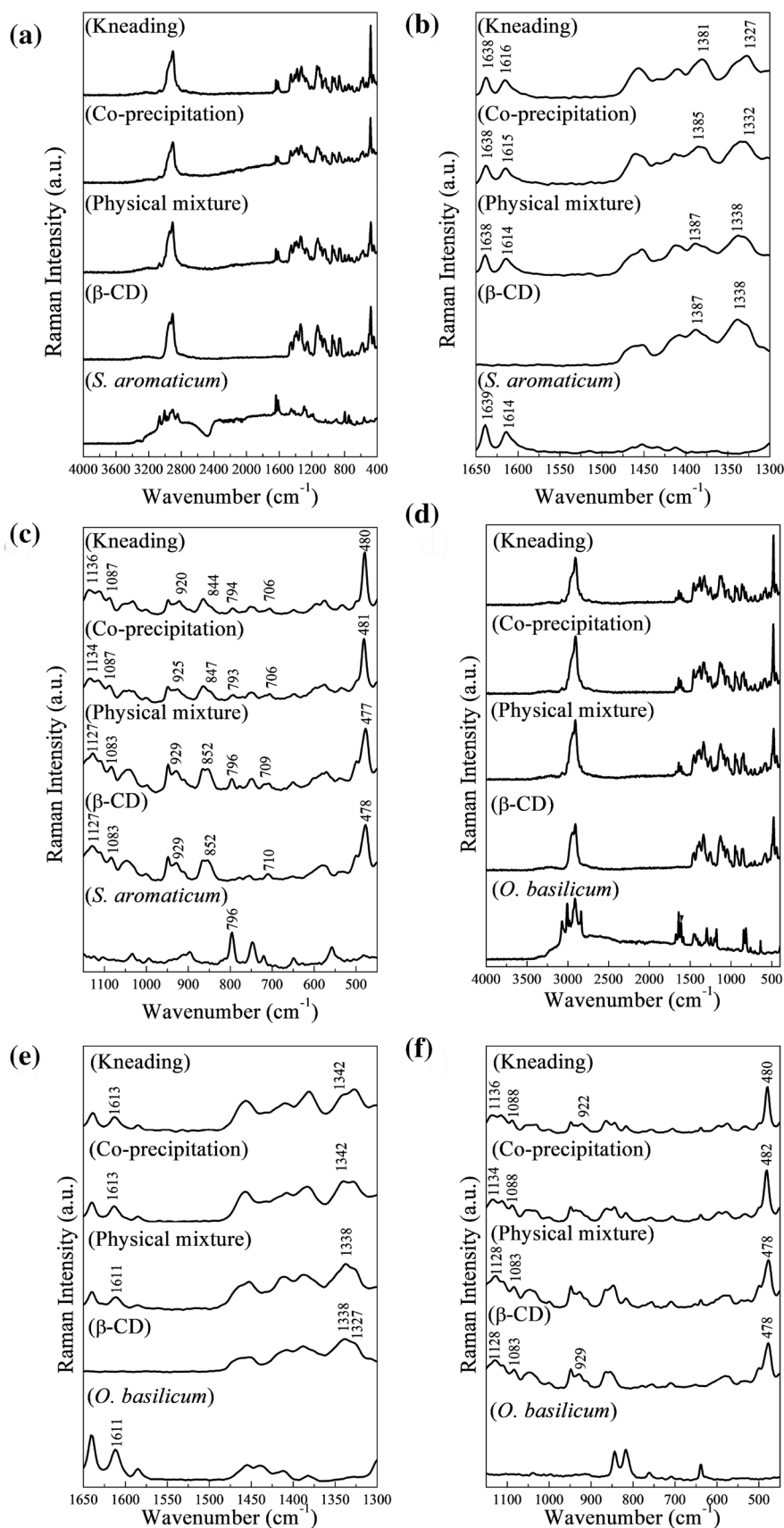


Fig. 2 **a** FT-Raman spectrum for *S. aromaticum* EO, β -CD, physical mixture, and complexes formed from co-precipitation and kneading methodologies. **b** Magnification of 1650–1300 cm^{-1} . **c** Magnification of 1200–400 cm^{-1} . **d** ATR-FTIR spectrum of *O. basilicum* EO, β -CD, physical mixture, and complexes formed from co-precipitation and kneading methodologies. **e** Magnification of 1650–1300 cm^{-1} . **f** Magnification of 1200–400 cm^{-1}



844 cm^{-1} for kneading. Eugenol showed the deformation of the aromatic ring, which is represented by the peak at 796 cm^{-1} [25], which shifted to 793 cm^{-1} in co-precipitation and 794 cm^{-1} in kneading. The peak at 710 cm^{-1} , corresponding to the deformation of the β -CD glucose ring [29], shifted in both complexes to 706 cm^{-1} . The out-of-plane deformation of the β -CD glucose ring was also observed, which is shown by the peak at 478 cm^{-1} . This peak shifted to 481 cm^{-1} for co-precipitation and 480 cm^{-1} for kneading.

For *O. basilicum* EO, the peak at 1611 cm^{-1} , related to the vibration of the aromatic ring of methyl-chavicol [30], shifted in the complexes to 1613 cm^{-1} , while for the physical mixture, it was unaffected, suggesting the detection of changes in the vibrational mode for chemical bonding. The β -CD in this region showed no peak. In both complexes, the peaks at 1338 cm^{-1} and 1327 cm^{-1} , corresponding to a β -CD CH region [29], changed. The 1338 cm^{-1} peak shifted to 1342 cm^{-1} while the 1327 cm^{-1} peak changed its conformation, which is more evident for kneading compared to co-precipitation (Fig. 2e).

The C–C stretch vibration of β -CD, represented by the band at 1128 cm^{-1} , shifted to 1134 cm^{-1} in co-precipitation and 1136 cm^{-1} in kneading (Fig. 2f). The peak at 1083 cm^{-1} , which represents the β -CD C–O stretch vibration, shifted to 1088 cm^{-1} in both complexes [29]. For the β -CD glucose ring breath vibration, the peak was at 929 cm^{-1} shifted to 922 cm^{-1} on kneading but showed no significant change in co-precipitation. In Fig. 2f, a displacement was also observed for the peak of 478 cm^{-1} peak. It corresponded to the out-of-plane deformation of the β -CD glucose ring, and it shifted to 482 cm^{-1} for co-precipitation and 480 cm^{-1} for kneading [29].

Fini et al. [33], Oliveira et al. [34] and Rocha et al. [35] used β -CD as a complexing agent for other compounds and obtained similar results as in this study. Rocha et al. [35] observed that during the formation of inclusion complexes that there was no creation of new vibrational bands in them. This indicates that the chemical bonds between guest and host molecules were not covalent, and corroborates our study. Also, it was possible to notice some shifts in the physical mixture, which may indicate the small amount of EO complexation with β -CD.

By the FT-Raman technique, it was possible to observe that the aromatic rings and double bonds of both eugenol and methyl-chavicol present in *S. aromaticum* and *O. basilicum* EOs, regions with great potential for molecular interaction, underwent changes in the vibrational mode. The same could be observed for β -CD. Therefore, the technique was able to suggest that the complexation process was efficient for both studied complexes.

Differential scanning calorimetry (DSC) and thermogravimetry (TGA)

During DSC analyzes, β -CD always has two endothermic events: the first event refers to dehydration (30–136 °C) and a second one to the process of molecular decomposition and consequent removal of carbonaceous material (296–350 °C) [9]. For the physical mixture of both EOs, the DSC curve was similar to β -CD, indicating that β -CD presented itself in a free form [31]. Yet, for the complexes, a reduction in the dehydration signal intensity was observed, suggesting that there was a displacement of water from inside the β -CD cavity and its substitution by the compounds present in the EOs. The peak position of β -CD was observed at 104 °C.

For the *S. aromaticum* EO, the endothermic peak was observed at 180 °C, corresponding to its boiling point. The endothermic peak for the inclusion complexes containing β -CD and *S. aromaticum* EO was 65 °C for co-precipitation and 74 °C for kneading. The physical mixture was observed that the endothermic peak was 99 °C, close to the peak of β -CD (Fig. 3-1a). Yet, for *O. basilicum* EO, the endothermic peak corresponding to its degradation was observed at 138 °C. For the physical mixture the peak could be observed at 108 °C, while, for the kneading procedure, the complex peak was at 83 °C and for the co-precipitation was at 111 °C (Fig. 3-1b). As the physical mixture has no significant difference in the dehydration signal when compared to inclusion complexes, according to Xi et al. [36] EO in the physical mixture has less or no affinity for β -CD, one can assume the formation of complexation by co-precipitation and kneading methodologies.

During the TGA analyzes, there was evidence of three stages in which β -CD lost mass. The first evidence occurred at a temperature below 100 °C, where β -CD lost approximately 8% of its mass, which may be associated with loss of surface water from β -CD. A second event observed near 115 °C, in which the mass loss was about 13%, suggests the evaporation of the internal water of the β -CD. Lastly, the third evidence happened around 300 °C and the β -CD mass was reduced to 23% of its initial mass, probably related to degradation [37].

For the physical mixture containing *S. aromaticum* EO, the mass loss was 20% of its initial mass higher than what we found for β -CD. For the complexes, it was observed that, at the moment when β -CD had lost 13% of its mass, the complex formed by the kneading methodology lost 10% of the initial mass and the co-precipitation 8% (Fig. 3.2a). It was possible to observe the differences between the physical mixture and the complexes formed by co-precipitation and kneading, wherein the physical mixture showed two endothermic events at approximately 100 and 140 °C, indicating an overlap of the individual thermograms of EO and β -CD, while the complexes showed only one prolonged event in the

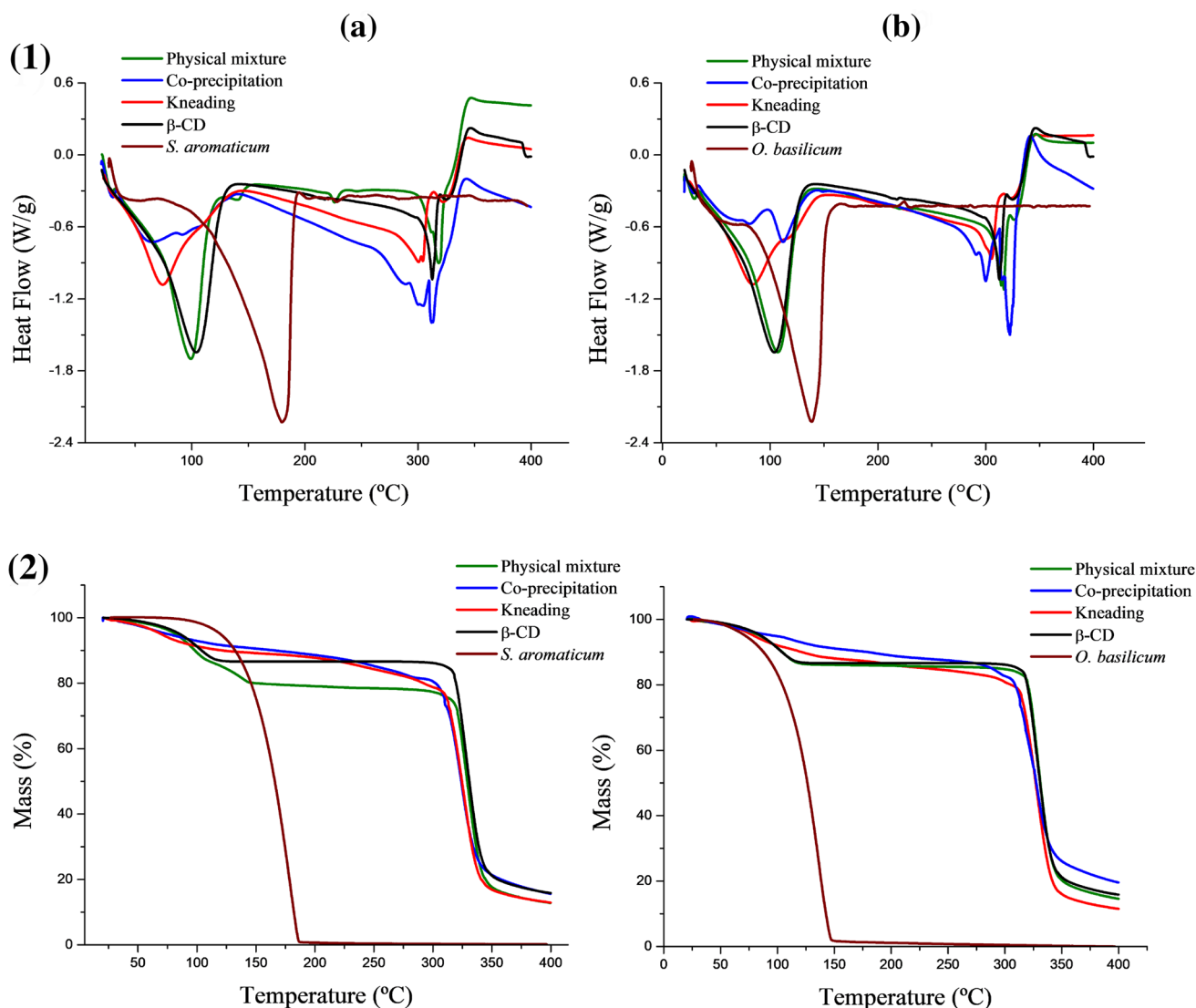


Fig. 3 (1) Differential scanning calorimetry (DSC) and (2) thermogravimetric (TGA) curves of the EO, physical mixture and of the complexes formed by the co-precipitation and kneading methodologies of **a** *S. aromaticum* and **b** *O. basilicum* EOs

temperature range of 90 to 300 °C for kneading, and from 90 °C to 285 °C for co-precipitation. The complexation was remarkable and the protection of the compounds present in the EO against the high temperature was favorable.

In the physical mixture containing *O. basilicum* EO, the mass loss was similar to β -CD, and it may suggest that the EO weakly bound the β -CD cavity and the behavior of the physical mixture was similar to that of the β -CD [31]. When analyzing the complexes, it was observed that, when the β -CD had lost 13% of its mass, the complex formed by the kneading methodology lost 10% of its initial mass and 6% for co-precipitation. The subtle fall of the complexes' masses to temperatures of 180 °C in kneading and 246 °C in co-precipitation suggests that the compounds present in the EO were protected by the β -CD and, after they passed

those temperatures, the degradation of the encapsulated compounds occurred (Fig. 3.2b) [9, 37].

To assist in the TGA analysis, Karl Fischer moisture determination tests were performed on samples of β -CD, inclusion complexes and physical mixtures. The moisture content (%) was 10.82% for β -CD. For the *S. aromaticum* EO, the moisture content was 4.78% for the physical mixture and 4.95% and 4.92% for the co-precipitation and kneading methodologies, respectively. For *O. basilicum* OE, physical mixture showed a moisture content of 5.96%, while the complexes formed by co-precipitation was 4.95% and the kneading was 4.97%. Therefore, based on the studies by Menezes et al. [38], it is possible to suggest that the loss of mass of the physical mixture and inclusion complexes are due to the evaporation of both the water present

in the β -CD cavity and the evaporation of the EO present in the inclusion complex.

Retention of EOs by β -CD

In the analysis of retention of EOs by β -CD, it was possible to observe different behaviors between the complexes and the physical mixture. For the complexes formed by the *S. aromaticum* EO the value of r (%) was 99.34% and 98.41% for the co-precipitation and kneading methodologies, respectively. For the physical mixture, it was possible to assess that the r (%) was 97.52%, reflecting the strong affinity that the compounds present in the *S. aromaticum* EO have for the hydrophobic cavity of the β -CD. Analyzing each compound separately, it was verified that for the physical mixture and the complexes the r (%) of the α -caryophyllene and β -caryophyllene compounds were approximately 99% each. Eugenol showed different r (%), for the physical mixture the r (%) was approximately 76% and for the co-precipitation and kneading methodologies it was approximately 99% and 93%, respectively. Considering the results obtained in the DSC and TGA tests and the strong retention of the compounds for the physical mixture observed in this test, it is possible to suggest that the binding of EO with β -CD is weak in the physical mixture when compared to the complexes obtained by kneading and co-precipitation.

For the *O. basilicum* OE, the R (%) was 77.38% for the co-precipitation methodology and 46.65% for the kneading methodology. In the physical mixture, the r (%) was 8.19%, indicating a lower affinity of the compounds in the “spontaneous complex” formation. In the individual analysis of the compounds for the physical mixture, the r (%) for the cis- α -bisabolene compound was 100%, while for the methyl-chavicol, the r (%) was zero; and linalool compound had an r (%) 94%. For the complex prepared by kneading, the r (%) was approximately 88% for the compounds cis- α -bisabolene and linalool and 70% for the methyl-chavicol.

In the co-precipitation methodology, the r (%) of the compounds cis- α -bisabolene, linalool and methyl-chavicol was approximately 96%, 89% and 75%, respectively. By this method, it was possible to conclude that co-precipitation was more efficient for the formation of inclusion complexes *O. basilicum* OE, corroborating the results obtained in the characterization of the complexes.

Antioxidant activity

ABTS radical scavenger activity

According to Table 2, for ABTS⁺ free radical scavenging activity values, *S. aromaticum* EO showed a satisfactory value because its major compound, eugenol, which has a reactive hydroxyl group, presented scavenging activity of 2147.0 $\mu\text{mol/g}$ of EO. For the physical mixture and the complex formed by the kneading methodology, the value of the sequestering activity was close to the value of pure EO, however, for the complex formed by the co-precipitation methodology, the value of the sequestering activity was lower. According to Kamimura et al. [39], this decrease may be due to the eugenol hydroxyl group found within the β -CD cavity and, therefore, its little availability to react with free radicals. Thus, this retention of antioxidant compounds is favorable for their protection.

The *O. basilicum* EO had a very low scavenger activity compared to the *S. aromaticum* EO, which was close to 75 $\mu\text{mol/g}$ of EO. This value may be related to the major compound (methyl-chavicol) because it does not have reactive hydroxyls, in other words, it does not have a hydrogen atom donor [2]. Therefore, the low activity presented is due to the hydroxyl present in the linalool, a compound that is present in small amounts in the oil. For the physical mixture and the complexes that were formed by co-precipitation and kneading was also observed low scavenger activity (Table 2).

Table 2 ABTS⁺ and DPPH free radical scavenging activity of pure and complex *S. aromaticum* and *O. basilicum* EOs

Sample	Method	Method (Equivalent Trolox $\mu\text{mol/g}$ sample)	
		ABTS ⁺ ($\mu\text{mol/L}$ Trolox)	DPPH ($\mu\text{mol/L}$ Trolox)
<i>S. aromaticum</i>	Essential oil	2147.0 \pm 7.1 ^a	1865.3 \pm 21.2 ^a
	Physical mix	2067.0 \pm 2.3 ^b	1810.3 \pm 14.1 ^a
	Kneading	2078.6 \pm 3.3 ^b	1762.5 \pm 23.6 ^{a,b}
	Co-precipitation	1995.3 \pm 4.7 ^c	1608.7 \pm 49.5 ^b
<i>O. basilicum</i>	Essential oil	75.3 \pm 4.7 ^a	88.7 \pm 7.1 ^a
	Physical mix	53.7 \pm 2.3 ^{b,c}	82.0 \pm 2.3 ^a
	Kneading	60.9 \pm 3.8 ^{a,b}	77.0 \pm 4.7 ^a
	Co-precipitation	37.5 \pm 3.8 ^c	25.3 \pm 2.3 ^b

^{a,b,c}Averages with equal letters in the same column do not differ significantly from each other by the Tukey test ($p \leq 0.05$)

It is also possible to observe for both EOs that the complexes formed by co-precipitation showed lower scavenger activity than kneading. This fact suggests that in the co-precipitation methodology, even having presented an r (%) of approximately 99%, during the filtration process, a small amount of the compound responsible for the scavenger activity, which was not encapsulated and therefore not retained in the filter, may have been discarded which does not happen with the other methodologies.

DPPH radical scavenger activity

Since there was great *S. aromaticum* EO scavenging activity (equivalent to 1865.3 $\mu\text{mol/g}$ OE) and for *O. basilicum* EO very low activity (equivalent to 88.7 $\mu\text{mol/g}$ EO) (Table 2), the values of DPPH free radical scavenging activity were similar to those found in ABTS⁺ free radical scavenging activity. In the *S. aromaticum* EO, the same effect was observed for the physical mixture and the complexes formed by co-precipitation and kneading. It was possible due to the possibility that the hydroxyl groups of the compounds being within the β -CD cavity and, therefore, their less availability to react with radical species [39], also this unavailability

of compounds provide their protection. Regarding *O. basilicum* EO, the sequestering activity of the complexes was low. Kfoury et al. [2] reported this low activity to the methyl-chavicol compound, which does not have hydroxyls (Table 2).

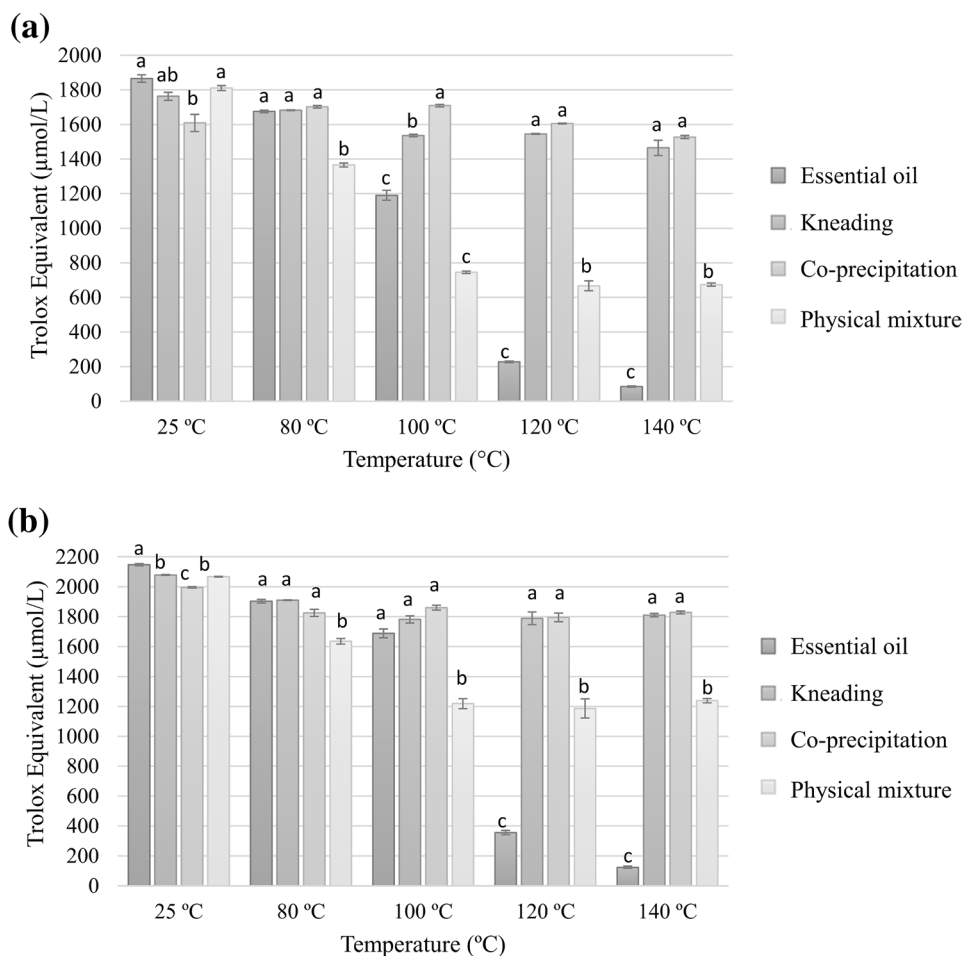
Also, for this methodology, the complexes formed by co-precipitation showed lower scavenging activity than kneading, strengthening the suggestion of loss of uncomplexed material during filtration.

Stability of antioxidant activity at different temperatures

Due to the results showed in the items 3.5.1 and 3.5.2, which demonstrated the very low free radical scavenging activity of *O. basilicum* EO, the antioxidant activity stability test at different temperatures was performed only for *S. aromaticum* EO.

In DPPH free radical scavenging activity (Fig. 4a), it was observed that, at 80 °C, pure EO had a 10% drop in free radical scavenging activity compared to 25 °C, on the other hand, the complex formed by the technique of kneading the fall was only 5%. For the complex formed by the co-precipitation methodology, a 5% increase in activity could be

Fig. 4 Stability of **a** DPPH and **b** ABTS⁺ free radical scavenging activity of *S. aromaticum* EO at temperatures of 25, 80, 100, 120 and 140 °C



observed, suggesting that eugenol within the β -CD cavity was released due to the temperature increase.

There was a sharp drop in the activity of the EO and physical mixture at 100 °C. Differently, for the complex formed by the kneading method, the drop was 13% compared to its initial value. For co-precipitation, there was a 6% increase in the activity when compared to the initial value. After passing those temperatures, pure EO sharply lost its activity, reaching almost zero. On the other hand, the physical mixture remained stable, which suggests that part of the compounds present in the EO was encapsulated by this methodology and, also, that the previously detected activity would be from compounds that were not encapsulated. For the complexes, the reduction of the final antioxidant activity compared to the initial one was 13% for the kneading methodology and 6% for the complex formed by the co-precipitation methodology, showing greater stability.

The same behavior was observed for the ABTS⁺ methodology (Fig. 4b). Therefore, the complexation promoted *S. aromaticum* EO protection, resulting in its stabilization against the temperature, especially the highest ones. Rakmai et al. [14, 40] evaluated the antioxidant stability of different EOs and their inclusion complexes against the light. In their research, they obtained clear results that the complexes were protected, thus reinforcing that CDs are excellent protective agents against different external factors.

Antimicrobial activity

For antimicrobial activities, an initial concentration of 4000 $\mu\text{g/mL}$ was used. Pure EOs showed no antimicrobial activity against the bacteria tested. Against yeast, *O. basilicum* EO showed no activity, but *S. aromaticum* EO showed a MIC of 1000 $\mu\text{g/mL}$ and an MFC of 4000 $\mu\text{g/mL}$ against *C. tropicalis* and *C. albicans*. For *C. parapsilosis* the MIC was 1000 $\mu\text{g/mL}$ and the MFC was greater than 4000 $\mu\text{g/mL}$. Because of that, only the complexes and the physical mixture obtained from *S. aromaticum* EO were tested against yeasts. It was not possible to determine the MIC for *C. tropicalis* and *C. albicans*, because until the concentration of 1000 $\mu\text{g/mL}$ medium turbidity occurred due to the low solubility of β -CD, and from 500 $\mu\text{g/mL}$ fungal growth was already observed. Even under these conditions, the MFC was evaluated starting on the concentration of 1000 $\mu\text{g/mL}$, for this was the MIC presented by pure EO. For kneading the MFC was 4000 $\mu\text{g/mL}$ and for co-precipitation, the MFC was 2000 $\mu\text{g/mL}$.

Devi et al. [41] reported that eugenol antimicrobial activity is related to increased cell membrane permeability, thus causing its disruption. From the results, it was observed that the EO obtained by the co-precipitation methodology provided better action against the yeasts, differently, for the kneading methodology, it was not possible to obtain a

favorable result. For *C. parapsilosis*, it was not possible to determine the MIC of the kneading due to the low solubility of β -CD and the consequent turbidity of the cultivation medium, but for co-precipitation, the MIC was 500 $\mu\text{g/mL}$ and the MFC was 4000 $\mu\text{g/mL}$.

Although data confirming the antimicrobial activity of the EOs studied in this research were found in the literature, the results showed only antimicrobial activity in the *S. aromaticum* EO against the tested yeasts. According to Radünz et al. [42], high antimicrobial activity is conferred to EOs with MICs up to 0.5 mg/mL, moderate to 0.6 to 1.5 mg/mL and weak above 1.6 mg/mL, in other words, *S. aromaticum* EO in our study showed moderate antimicrobial activity. According to Nascimento et al. [43], some properties, such as extraction technique, type of cultivation and harvest, and also the variation of latitudes and longitudes, may alter the results of the antimicrobial activity causing different amounts of the compound responsible for it.

Antitumor activity

The kneading and co-precipitation methodology had similar results in the spectrophotometric analyzes (ATR-FTIR and FT-Raman), but considering the ease of production of the inclusion complex by the kneading methodology, it was chosen for antitumor activity. The EO concentration was defined according to Radünz et al. [42], which were based on MIC in antimicrobial tests.

For β -CD, the antitumor activity was greater than 500 $\mu\text{g/mL}$, which shows that the CD studied has no cytotoxicity. It was observed that *O. basilicum* EO was not active against HT-29 and HeLa cells. The IC₅₀ of the inclusion complex showed that encapsulation improved the antitumor activity of *O. basilicum* EO, however, its toxicity also increased. Before the formation of the inclusion complex, the cytotoxicity to VERO cells was higher than 500 $\mu\text{g/mL}$, while for the complex the IC₅₀ was $170.0 \pm 31.8 \mu\text{g/mL}$. The selectivity index (SI) against HT-29 cells was 1.46 and for HeLa cells 0.96.

S. aromaticum EO was active against all tumor cells. The IC₅₀ for HT-29 cells was $145.0 \pm 27.8 \mu\text{g/mL}$ and for HeLa cells $190.0 \pm 14.1 \mu\text{g/mL}$, but it also showed cytotoxicity against VERO cells ($210.0 \pm 21.8 \mu\text{g/mL}$). The complexes were more active than pure EO, $132.5 \pm 17.7 \mu\text{g/mL}$ and $12.5 \pm 4.2 \mu\text{g/mL}$, respectively. In contrast of that, its cytotoxicity also increased. The SI for the HT-29 cells was 1.45 for the EO and 1.16 for complexed EO. For HeLa cells, the complex showed a significant improvement for antitumor activity, $12.5 \pm 4.2 \mu\text{g/mL}$, and the SI for the EO was 1.11 and for the complexed EO, 12.32.

Both *O. basilicum* and *S. aromaticum* EO showed antitumor activity against HT-29 and HeLa cells. Our results showed that the complex formed improves antitumor

activity, despite increasing its cytotoxicity, and this improvement may be due to increased solubility and stability of compounds responsible for antitumor activity. Trindade et al. [44] complexed carvacrol and obtained similar results, in other words, there was an improvement in the bioavailability of the active compound improving its action.

Thus, this research showed that the complexation contributed to the stability and bioavailability of the evaluated essential oils, improving their antioxidant, antibacterial and antitumor actions. The EOs are natural, safe, accepted by society and have several bioactive properties, when protected by CDs, can be used, for example, in plastic films and commercial packaging. Therefore, they can be used to protect medicines and food, ensuring the product of contamination, oxidation, among other misfortunes that may occur in the production, transportation, and marketing of the product.

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Declarations

Conflict of interest The authors declare no conflict of interest.


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