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Article

# Antioxidant Properties of *Croton zehntneri* Pax et Hoffm. Essential Oil and Its Inclusion Complex with β-Cyclodextrin Prepared by Spray Drying

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*Croton zehntneri* Pax et Hoffm. is a plant from Northeastern Brazil, whose main component of its essential oil is estragole. Its thermal instability and low aqueous solubility prevent its technological application. This study aimed to prepare and characterize the *Croton zehntneri* essential oil (CZEO) complexed with β-cyclodextrin (β-CD), and to evaluate antioxidant activity of free and complexed-CZEO *in vitro* systems by the elimination of 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH$^{\bullet}$), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS$^{\bullet+}$), hydroxyl and nitric oxide, as well as their ability to transfer electrons by the reducing potential. The infrared spectroscopy and thermal techniques demonstrated the formation of the inclusion complex (Est/β-CD) obtained by spray drying method. The free CZEO and its Est/β-CD at 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8 μg mL$^{-1}$ showed the ability to remove DPPH$^{\bullet}$ ($EC_{50}$ (half maximal effective concentration) 26.06 and 9.46 μg mL$^{-1}$, respectively) and ABTS$^{\bullet+}$ ($EC_{50}$ 22.73 and 4.47 μg mL$^{-1}$, respectively), nitric oxide inhibition ($EC_{50}$ 17.65 and 2.68 μg mL$^{-1}$, respectively), hydroxyl radical sequestration ($EC_{50}$ 23.42 and 2.34 μg mL$^{-1}$, respectively) and reducing potential ($EC_{50}$ 46.48 and 12.47 μg mL$^{-1}$, respectively). The formation of the inclusion complex significantly increases its antioxidant potential.

**Keywords:** *Croton zehntneri*, estragole, β-cyclodextrin, antioxidant potential

## Introduction

The *Croton zehntneri* Pax et Hoffm. species belongs to the family Euphorbiaceae, popularly known as "*canela de cunhã*", "*canelinha*" or "*canela-brava*". It is commonly used in traditional medicine as a sedative, appetite stimulant and to relieve intestinal ailments. The antinociceptive, antimalarial, antimicrobial, and other bioactivities of its essential oil have already been demonstrated.[1-5] Estragole (4-methoxy-2-propenylbenzene) is a phenylpropanoid found in essential oil of *C. zehntneri* and has pharmacological activities such as antimicrobial,[1] anti-inflammatory,[6] antispasmodic[7] and antioxidant,[8] as well as local anesthetic[9] and central nervous system depressant,[10] thus, allowing it as a constituent for a possible drug.

Although estragole has various pharmacological activities, its physicochemical properties limit its use in *in vivo* systems. Compounds with low aqueous solubility often exhibit lower performance *in vivo*, such as low bioavailability, more influence of dietary status, interpatient variability, and incomplete release of pharmaceutical forms when compared to those with higher aqueous solubility. In addition, low solubility results in numerous obstacles to developing new pharmaceutical forms, given the low number of technological options and significant challenges

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in designing and validating properly discriminatory dissolution assays potentially applicable to *in vitro*/*in vivo* correlation.[11,12] According to Kfoury *et al*.,[13] the use of carriers such as cyclodextrins (CD) could be appropriate to improve estragole stability and increase its aqueous solubility, which amplifies its bioavailability.

Studies[14,15] have shown that the complexation of essential oils with cyclodextrins increases pharmacological activity as antioxidant potential, influencing or preventing oxidative damage. Thus, this study aims to prepare and characterize the Est/β-CD and to evaluate the antioxidant activity of free and complexed CZEO (*Croton zehntneri* essential oil) by the elimination of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radicals, nitric oxide (NO) inhibition, hydroxyl radical ($OH^{\bullet}$) sequestration, as well as their ability to transfer electrons for their reducing potential.

## Experimental

### Plant material

The leaves and branches were collected in February 2014 in the municipality of Simões, state of Piauí, Brazil, by Professor Dr Sidney Gonçalo de Lima of the Federal University of Piauí (UFPI). The plant material was identified and deposited in the Graziela Barroso Herbarium of the UFPI under accession number 27273.

### Essential oil extraction

The CZEO from dried leaves was continuously extracted for 3 h using a Clevenger-type distillation apparatus, dried with anhydrous sodium sulfate (Dinâmica, São Paulo, Brazil), weighed, stored in a capped vial protected with aluminum foil and kept in a refrigerator (4 °C). The oil was solubilized in ethyl acetate (Neon, São Paulo, Brazil) for gas chromatography (GC) and mass spectrometry analysis (MS).

### GC-MS conditions

For the chemical characterization of CZEO, it was used a gas chromatograph coupled to mass spectrometer (GC-MS), a Shimadzu® chromatograph, CGMSQP2010 SE model equipped with AOC-5000 (Shimadzu, Kyoto, Japan) automatic injector, and SLB-5ms column (30 m × 0.25 mm × 0.25 μm). The analysis conditions were as follows: helium as carrier gas at a flow rate of 1 mL $min^{-1}$, a temperature of 250 °C in the injector; heating ramp: starting at 60 °C (remaining for 3 min), followed by a heating rate of 3 °C $min^{-1}$ to 240 °C, maintaining this temperature for 10 min; the detector temperature was 250 °C; injection volume of 1 μL. The MS conditions were a single quadrupole ion detector operating electron impact (70 eV, 45 to 450 Da). The identification and relative quantification of the constituents were obtained based on the areas of the corresponding chromatographic peaks, determination of the Kovats index, and comparison with the database records and the literature (Wiley, NIST, Pherobase) with a similarity of 95% or greater. The *n*-alkanes pattern solution ($C_8$-$C_{20}$) was used for the calculation of the Kovats index, and this value was compared to the one described in the literature.[16-18]

### Inclusion complex preparation by spray drying method

The Est/β-CD (estragole/β-CD inclusion complex) was prepared according to the method proposed by Jantarat *et al*.[19] with some modifications. The inclusion complex was prepared in a 1:1 molar ratio, defined according to the chemical composition of the essential oil (95.24% estragole). Equimolar quantities of CZEO and β-CD were solubilized in ethanol (Synth, São Paulo, Brazil) and distilled water (1:9) heated to 45 °C, respectively. The two solutions were mixed and stirred with the aid of a magnetic stirrer for 90 min; then the complex was dried using a spray dryer (BUCHI B-290, BUCHI, Flawil, Switzerland) under the following conditions: inlet temperature 105 °C, pressure 0.9 bar and sample flow of 3 mL $min^{-1}$. The yield was calculated, and the Est/β-CD was stored in a desiccator until the analyses were performed.

### Physical mixture preparation (PM)

The PM (CZEO and β-CD in a 1:1 ratio) was homogenized with the aid of mortar and pestle for 10 min. The PM was stored in a hermetically sealed vial.

### Inclusion complex characterization

About 100 mg of the Est/β-CD was washed with 5 mL of *n*-hexane (Bio-Grade, San Francisco, USA), and the volume was reduced to about 1 mL, followed by GC-MS analysis to investigate the presence of uncomplexed CZEO.

### Quantification of CZEO components

For concentration determination of the CZEO components present in the Est/β-CD, an analytical curve was prepared from several dilutions of estragole standard (Sigma-Aldrich, Steinheim, Germany) in *n*-hexane, obtaining seven dilutions ranging from 5 to 500 mg $L^{-1}$.

These solutions were analyzed by GC-MS, as previously described. All the dilutions are prepared in triplicate.

The total amount of CZEO in the Est/β-CD was determined by the method of extraction with solvent widely used in our research group (data previously published by Fonseca *et al.*).[20] Briefly, 100 mg of Est/β-CD were solubilized with 4.0 mL of distilled water in a test tube sealed with a Bakelite screw cap and extracted with 4.0 mL of *n*-hexane. For this, the mixture was heated in a water bath at a temperature of 85 ± 2 °C for 15 min with intermittent shaking. Then, it was cooled to room temperature. The *n*-hexane phase containing the essential oil was collected with a pipette, and the aqueous phase was subjected to two successive extractions with *n*-hexane (2 × 4.0 mL). The combined extracts were appropriately diluted and analyzed by GC-MS for quantification of estragole in the Est/β-CD.

To evaluate the thermal stability, we decided to analyze the CZEO, PM, and Est/β-CD by head-space (HS)-GC-MS. This experiment was done similarly to that proposed by Silva *et al.*,[21] with modifications. About 10 μL of CZEO and 100 mg of PM, and Est/β-CD were transferred to a sealed headspace vial and subjected to incubation at 45 °C for 0 and 60 min. After the incubation period, 0.5 mL of the sample vapor was analyzed by HS-GC.

### Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum of CZEO, β-CD, PM, and Est/β-CD were obtained in the middle IR region (4000-600 $cm^{-1}$) on a Vertex 70 spectrometer (Bruker, Massachusetts, USA). The spectra were obtained from 64 scans with 4 $cm^{-1}$ resolution. CZEO was pressed into KBr plates. The β-CD, PM, and Est/β-CD were triturated with potassium bromide (KBr) and then pressed into 1 mm tablets under 9-ton pressure. A KBr disk was used as blank. IR spectra were smoothed, and the baseline was corrected.

### Raman spectroscopy

The Raman spectra analysis of CZEO, β-CD, PM, and Est/β-CD were obtained with a Bruker Senterra dispersive Raman microscope (Bruker, Massachusetts, USA) with a charge-coupled device (CCD) detection system, with a ×20 objective. The excitation wavelength was 785 nm, obtained using a solid-state laser. The measurements were calibrated with crystalline silicon and the resolution was about 3 $cm^{-1}$ and for each spectrum, ten accumulations of 10 s.

### Differential scanning calorimetry (DSC)

DSC thermal analysis (DSC-60, Shimadzu, Kyoto, Japan) was performed in an atmosphere of nitrogen with a flow of 10 mL $min^{-1}$ employing a sample mass of approximately 5.5 mg, packed in a hermetically sealed alumina sample holder. CZEO, β-CD, PM, and Est/β-CD were analyzed at 28-600 °C with a heating rate of 10 °C $min^{-1}$.

### Thermogravimetry-derivative thermogravimetry (TG/DTG)

Thermogravimetry measurements (TG) were performed in duplicate by thermo scale (TGA 60, Shimadzu, Kyoto, Japan) in a nitrogen atmosphere at a flow rate of 10 mL $min^{-1}$, with a sample mass equivalent to about 2.0 ± 0.2 mg, packed in alumina crucible and analyzed in the temperature range 28-600 °C with a heating rate of 10 °C $min^{-1}$. Before the tests, the instrument was calibrated by using an aluminum and zinc sample.

### Antioxidant activity against DPPH$^{\bullet}$ radical

In all antioxidant assays were used CZEO and Est/β-CD solutions at various concentrations (0.9 to 28.8 μg $mL^{-1}$), prepared in 0.9% saline solution containing 0.05% Tween 80.

The experimental procedure was performed as previously described by Oliveira.[22] Briefly, solutions of CZEO and Est/β-CD at various concentrations (0.9 to 28.8 μg $mL^{-1}$) was mixed with DPPH$^{\bullet}$ (100 μM) for a 30 min reaction. Absorbance values (517 nm) were expressed as inhibition of DPPH$^{\bullet}$ radical concentration concerning the system (100% DPPH$^{\bullet}$ radical). For comparison, the same experimental procedure was used for Trolox (140 μg $mL^{-1}$) and β-CD (28.8 μg $mL^{-1}$).

### Antioxidant activity against ABTS$^{\bullet+}$ radical cation

The experimental procedure was performed as previously described by Oliveira *et al.*[23] Initially, the ABTS$^{\bullet+}$ radical cation was formed to prepare an ABTS$^{\bullet+}$ solution with an absorbance of 1.00 ± 0.05 at 734 nm. Solutions of CZEO and Est/β-CD were prepared at room temperature in various concentrations (0.9 to 28.8 μg $mL^{-1}$) and mixed with ABTS$^{\bullet+}$ for a 6 min reaction. Absorbance values (734 nm) were expressed as inhibition of ABTS$^{\bullet+}$ radical cation concentration concerning the system (100% ABTS$^{\bullet+}$ radical). For comparison, the same experimental procedure was used for Trolox (140 μg $mL^{-1}$) and β-CD (28.8 μg $mL^{-1}$).

### Antioxidant activity against nitric oxide (NO)

The experimental procedure was performed as previously described by Oliveira *et al.*[23] Briefly, sodium

nitroprusside ($Na_2[Fe(CN)_5NO]$) (20 mM in phosphate buffer, pH 7.4) reacted with CZEO solutions and Est/β-CD at various concentrations (0.9 to 28.8 μg $mL^{-1}$) for 60 min at 37 °C. After the reaction time, Griess reagent was added to determine the absorbance values (540 nm) that were expressed as inhibition of nitrite ions formation relative to sodium nitroprusside (100% nitrite ions). For comparison, the same experimental procedure was used for Trolox (140 μg $mL^{-1}$) and β-CD (28.8 μg $mL^{-1}$).

### Antioxidant activity against deoxyribose degradation

The experimental procedure was performed as previously described by Oliveira *et al.*[23] Briefly, CZEO solutions and Est/β-CD at various concentrations (0.9 to 28.8 μg $mL^{-1}$) reacted for 40 min at 50 °C with the Fenton reaction system generated by a reaction medium containing 50 mM 2-deoxyribose, 3.2 mM ferric chloride ($FeCl_3$), 100 mM hydrogen peroxide ($H_2O_2$) and 20 mM buffer phosphate (pH 7.4). After the reaction time, trichloroacetic acid (10%) was added, followed by 1% thiobarbituric acid (TBA). Finally, the reaction mixture was heated for 15 min (95 °C) to determine the absorbance values (532 nm) that were expressed as inhibition of 2-deoxyribose degradation relative to the system (100% hydroxyl radical). For comparison, the same experimental procedure was used for Trolox (140 μg $mL^{-1}$) and β-CD (28.8 μg $mL^{-1}$).

### Reducing potential

The experimental procedure was achieved as previously described by Oliveira *et al.*[23] In brief, CZEO solutions and Est/β-CD at various concentrations (0.9 to 28.8 μg $mL^{-1}$) reacted with 200 μL potassium ferricyanide (1%) and 200 μL sodium phosphate buffer (200 mM, pH 6.6) for 20 min (50 °C). After the reaction time, 200 μL trichloroacetic acid (10%), 200 μL distilled water, and 125 μL ferric chloride (0.1%) were added to determine absorbance values (700 nm). For comparison, the same experimental procedure was used for Trolox (140 μg $mL^{-1}$), and β-CD (28.8 μg $mL^{-1}$).

### Statistical analysis

All results were presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Newman-Keuls test as a post hoc test. Results were considered statistically significant when $p < 0.05$. All statistical analyzes were performed using GraphPad Prism.[24]

## Results and Discussion

### Chemical composition of *C. zehntneri* essential oil (CZEO)

The CZEO was obtained from the hydrodistillation of 98.55 g of dried leaves in a 3.2% yield (oil mass/dried leaves mass). Subsequently, the essential oil extracted was analyzed by GC-MS, allowing the identification of 99% of the integrated constituents. The main constituents identified were myrcene, eucalyptol, estragole (as the main constituent), and spathulenol. Their respective structures are shown in Figure 1.

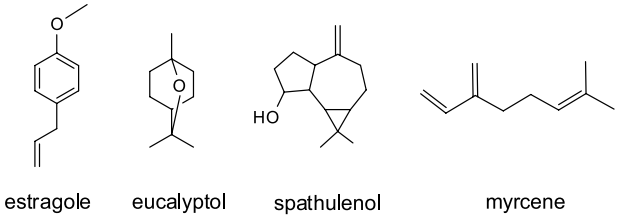


**Figure 1.** Chemical structures of the constituents identified in the CZEO.

Table 1 demonstrates the components identified in CZEO, with estragole (95.24%) being the major component. Myrcene, eucalyptol, and spathulenol appear in low relative abundance.

**Table 1.** Chemical composition of the *C. zehntneri* essential oil of dried leaves

| Retention time / min | Compound | RI[a] | RI[b] | Area / % |
|---|---|---|---|---|
| 16.205 | myrcene | 991 | 995 | 0.39 |
| 18.109 | eucalyptol | 1033 | 1031 | 2.61 |
| 23.655 | estragole | 1195 | 1189 | 95.24 |
| 35.193 | spathulenol | 1575 | 1577 | 0.75 |

[a]Experimental relative retention index: *n*-alkanes ($C_9$-$C_{24}$) were used as reference points in the calculation of relative retention indices; [b]relative retention indices.[17]

The literature shows a variation in the concentration of the major chemical constituents of the CZEO leaves depending on the place of collection. Thus, the literature reports the composition of different chemotypes from *C. zehntneri*: anethole: for the specimens collected in Tianguá (CE, Brazil, 74.5%) and Viçosa do Ceará (CE, Brazil, 47.34%);[25,26] estragole: Tianguá (CE, Brazil, 57.0%), Araripe (CE, Brazil, 61.0%), Valença (PI/Brazil, 90%), and Simões (PI, Brazil, 85-96%);[25,27,28] for those collected in Ubajara and Croatá da Serra (CE, Brazil): *E*-anethole (89.1%) to chemotype 1, eugenol (84.2%) to chemotype 2, and estragole (90,2%) to chemotype 3.

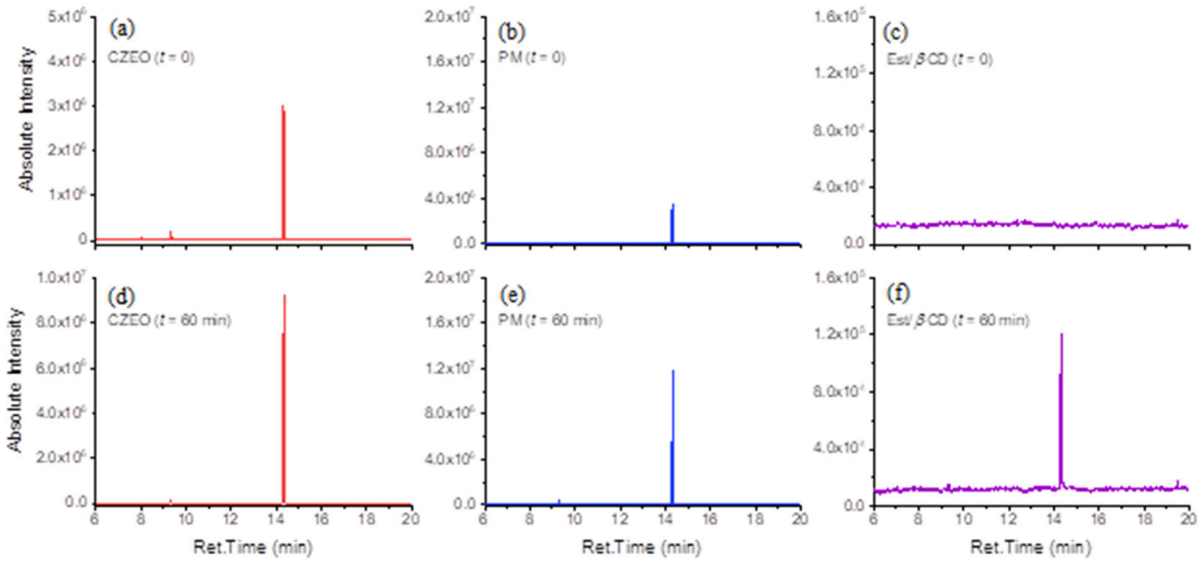


**Figure 2.** Chromatographic profile (HS/GC-MS, SCAN mode) of the *Croton zehntneri* essential oil (CZEO), inclusion complex with β-cyclodextrin (Est/β-CD), and physical mixture (PM) subjected to heat stress at 45 °C in headspace apparatus for (a-c) 0 and (d-f) 60 min.

When compared to literature data, our study evidenced some differences in the chromatographic profile as well as in quantitative composition and eessential oil yield (about 3.2%).

## Characterization of the inclusion complex (Est/β-CD)

Our research group has already quantified estragole in the Est/β-CD prepared by the spray drying method in previous work.[20] An analytical curve was constructed with different concentrations of standard estragole, and from the equation of the straight line, it was possible to quantify the estragole mass in the Est/β-CD, thus in 100 mg of Est/β-CD complex has 1.2 mg of estragole.

The Est/β-CD was washed with *n*-hexane and analyzed by GC-MS, and no adsorbed essential oil component was detected. On the other hand, the spray drying technique should remove any excess weakly adsorbed or uncomplexed CZEO. This phenomenon may be explained by the relatively high temperatures used during the spray drying process that can volatilize estragole. This fact may justify the absence of estragole, the main component of the essential oil, in the hexane washing experiments.

By analyzing Figures 2a-2f, it was possible to confirm the Est/β-CD formation and/or evaluate its thermal stability. In Figures 2d, 2e and 2f, it is possible to observe the volatilization of the essential oil components with the following order of intensity: CZEO > PM >>> Est/β-CD. This effect is probably due to the protective character that β-CD promotes next to the volatile components,[21] reducing the volatilization loss of CZEO components present in PM and more pronounced in Est/β-CD.

## Infrared spectroscopy with Fourier transform (FTIR)

The IR spectrum of CZEO, β-CD, PM, and Est/β-CD are shown in Figure 3. The infrared spectrum of β-CD showed a broad absorption band of 3700 to 3075 $cm^{-1}$ typical of O–H stretch and a band at 2928 $cm^{-1}$ for C–H stretches. In approximately 1026 and 1154 $cm^{-1}$ bands are identified for the C–O–C, and C–O stretches, respectively, present in the α-1,4 glycosidic bonds and in the hydroxyl of the alcohol function of CD. The bands observed at 1638 and 1611 $cm^{-1}$ are assigned to C=C stretching mode, while the 1511 $cm^{-1}$ mode was attributed for aromatic ring stretching. An intense band at 1247 $cm^{-1}$ is related to bond stretching C–O of the aryl compound, which is

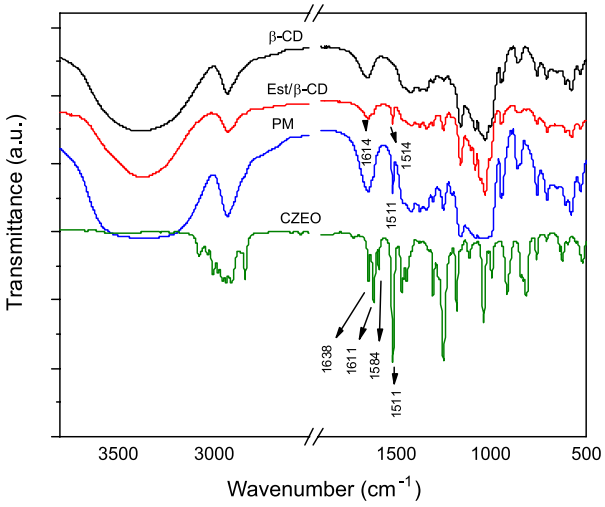


**Figure 3.** FTIR (KBr) spectrum of *Croton zehntneri* essential oil (CZEO), inclusion complex with β-cyclodextrin (Est/β-CD), β-cyclodextrin (β-CD), and physical mixture (PM).

characteristic of estragole (main compound of CZEO). The infrared spectrum of the PM has characteristic bands of both β-CD and CZEO, which suggests a discrete interaction between them.

Because of the low analyte concentration in the inclusion complex, the absorption spectrum in the infrared region shows only slight changes, those referring to bands around 1500 and 1600 $cm^{-1}$ (C–C stretching). In the CZEO and PM the stretching associated with the C–C bond appears at 1511 $cm^{-1}$; however, it decreases in intensity and shifts to a higher frequency 1514 $cm^{-1}$ in the Est/β-CD. In the range 1585-1640 $cm^{-1}$, changes were observed only around 1611 $cm^{-1}$, which is shifted to a higher frequency in the inclusion complex (1614 $cm^{-1}$). In general, this region is also masked by the absorption bands of β-CD. However, when we compare both Est/β-CD and the PM spectra, there is a reduction in the intensity of the band assigned to water (1647 $cm^{-1}$) in β-CD, probably due to substitution by the analytes (mainly estragole). These data show that our observations were consistent with those found in the literature.[20,29,30] The interaction of CZEO with β-CD can change the force constant in these vibrational modes, justifying the possible reduction in signal intensity that can be further analyzed by Raman spectroscopy.[20] This same effect has already been observed when inclusion complexes were prepared using β-CD.[20,31]

### Raman spectroscopy

Raman spectroscopy has been suggested to be an excellent tool to estimate inclusion complex formation. The Raman spectrum of CZEO, β-CD, PM, and Est/β-CD are shown in Figure 4. The presence of the guest molecules within the host cavity is mainly detected by Raman scattering in 1640, 1608, 845, 817, and 639 $cm^{-1}$. The Raman spectra of CZEO and β-CD are similar to those obtained by Fonseca *et al.*[20] and, as previously noted, the characteristic bands for estragole around 1608 $cm^{-1}$ (aromatic ring C=C) and 1640 $cm^{-1}$ (stretch C=C) have higher intensity in the complex (Est/β-CD) than in the PM. This fact shows that even with the low estragole content determined by GC-MS, the Raman spectroscopy technique can be used in elucidating the formation of the inclusion complex.

The band at 1640 $cm^{-1}$ (stretch C=C) appears in the PM at low intensity and disappears in the Est/β-CD. The band at 1608 $cm^{-1}$ (attributed to C=C vibration) is also shifted to lower frequency (1601 $cm^{-1}$) in the Est/β-CD. The bands at 845 and 818 $cm^{-1}$ (C–H aromatic ring) and 638 $cm^{-1}$ (*p*-aromatic ring) remain in the PM and disappear in the Est/β-CD.

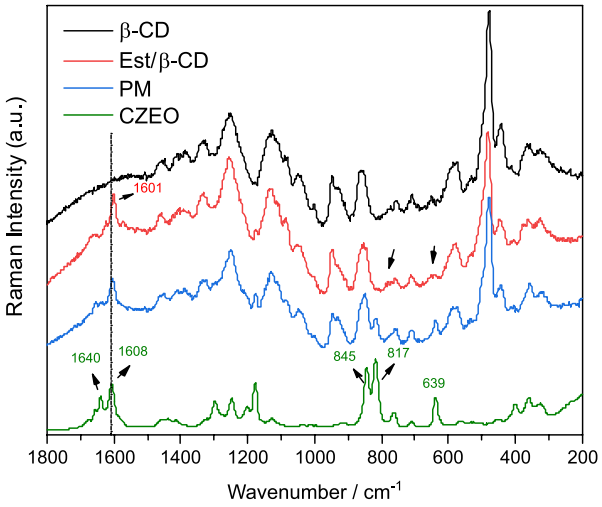


**Figure 4.** Raman spectra of *Croton zehntneri* essential oil (CZEO), inclusion complex with β-cyclodextrin (Est/β-CD), β-cyclodextrin (β-CD) and physical mixture (PM).

### Differential scanning calorimetry (DSC)

The endothermic peak at 127 °C of β-CD and 118 °C of PM indicate the exit of water molecules from the hydrophobic cavity;[29,30] in the PM, it is also possible to identify another endothermic event at 95 °C representative of the volatilization of the CZEO components. Aguiar *et al.*,[29] analyzing the inclusion complex of CZEO with β-CD prepared by the "co-precipitation" method, observed that this thermal event was significantly reduced probably due to the displacement of the water present in the cavity of the β-CD by the CZEO components. In the present work, the reduction of the intensity of the thermal event characteristic of water loss also occurred, but to a lesser extent, being compatible with the quantification determined by GC-MS. The endothermic peak at 189 °C of CZEO disappears in the thermogram of the Est/β-CD, which may represent greater stability of CZEO in the complex as compared to the free form (Figure 5).

### Thermograms TG/DTG

In the DTG curves of the free CZEO and Est/β-CD, it was verified that the thermal decomposition of the complex occurs at 326 °C, a temperature higher than the decomposition of CZEO that has a boiling point of 228.1 °C and close to temperature of decomposition of β-CD which is 327.4 °C. It shows an increase in thermal stability for the complexed CZEO, probably due to CZEO interactions with the β-CD cavity (Figure 6).

The TG curve of the PM showed the superposition of the thermal behavior of the β-CD and CZEO, with mass loss of 16.97%, against 14.49% (β-CD) and 12.12% (Est/β-CD).

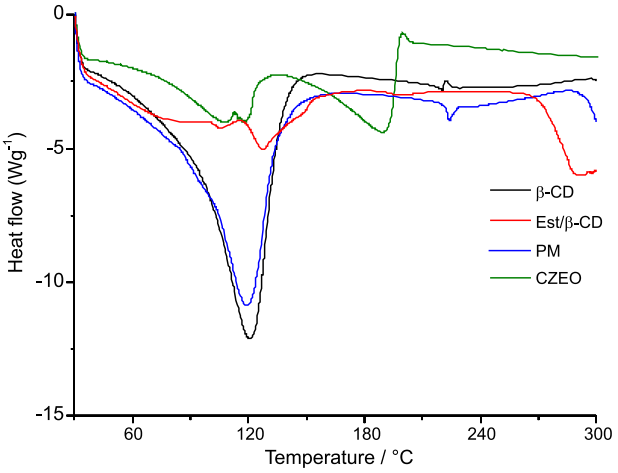


**Figure 5.** DSC thermograms of *Croton zehntneri* essential oil (CZEO), inclusion complex with β-cyclodextrin (Est/β-CD), β-cyclodextrin (β-CD) and physical mixture (PM).

It can be attributed mainly to water loss and the release of part of the CZEO. The mass loss above 280 °C in β-CD, PM, and Est/β-CD corresponds to thermal degradation, representing 72.67, 70.72, and 74.46%. The highest mass loss (74.46%) in Est/β-CD can be attributed to the increased thermal stability of CZEO components.

## Antioxidant activity

All the antioxidant results of CZEO and Est/β-CD are shown in Figure 7. It was observed that the antioxidant capacity of Est/β-CD *in vitro* systems was significantly different when compared to CZEO ($p < 0.05$). No significant antioxidant capacity was demonstrated when evaluating β-CD alone in all *in vitro* methodologies (Figure 7).

In this study, the method involving the sequestration of stable free radical DPPH• was analyzed by decreasing the absorbance of the DPPH solution at 517 nm, in which the purple DPPH• radical is reduced to form yellow DPPH.[29] Based on this principle, the result showed that Est/β-CD complex increases the antioxidant capacity compared to free CZEO. When both results are compared, it is possible to observe an increase in the antioxidant capacity of Est/β-CD against the DPPH• radical of 25.83, 25.85, 30.24, 30.13, 34.61 and 32.58% in the concentrations of 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8 μg mL$^{-1}$, respectively. According to these results, Est/β-CD $EC_{50}$ (half maximal effective concentration) was 9.46 μg mL$^{-1}$, which was 2.75 times lower than CZEO $EC_{50}$ 26.06 μg mL$^{-1}$. Similar results regarding the increased antioxidant capacity of complexed compounds with β-CD were obtained for other compounds (mangiferin, rutin, and coumarin), demonstrating that the results obtained in the present study against the DPPH• radical agree with previous studies.[32,33]

The $ABTS^{•+}$ radical cation produced by oxidation of the ABTS solution by potassium persulfate has blue/green coloration, and the reaction with an antioxidant compound is monitored by decreasing the absorbance of the reaction at 734 nm. Thus, the results demonstrated that low

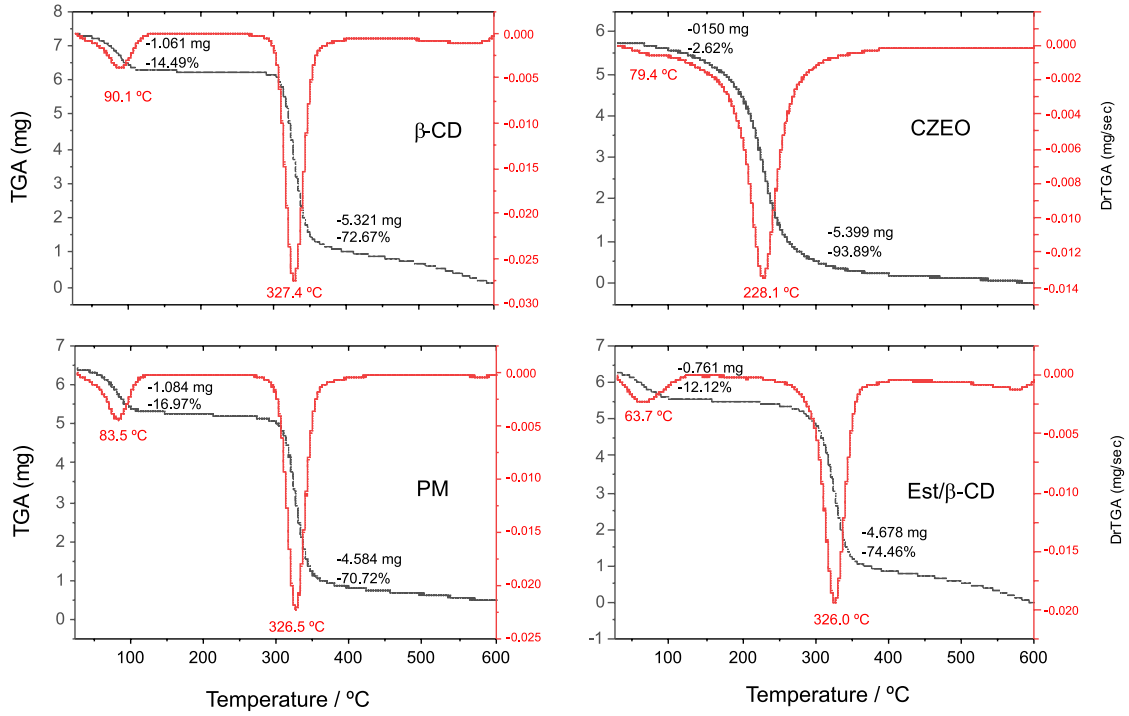


**Figure 6.** TG curves (black line) and DTG curves (red line) of *Croton zehntneri* essential oil (CZEO), inclusion complex with β-cyclodextrin (Est/β-CD), β-cyclodextrin (β-CD) and physical mixture (PM).

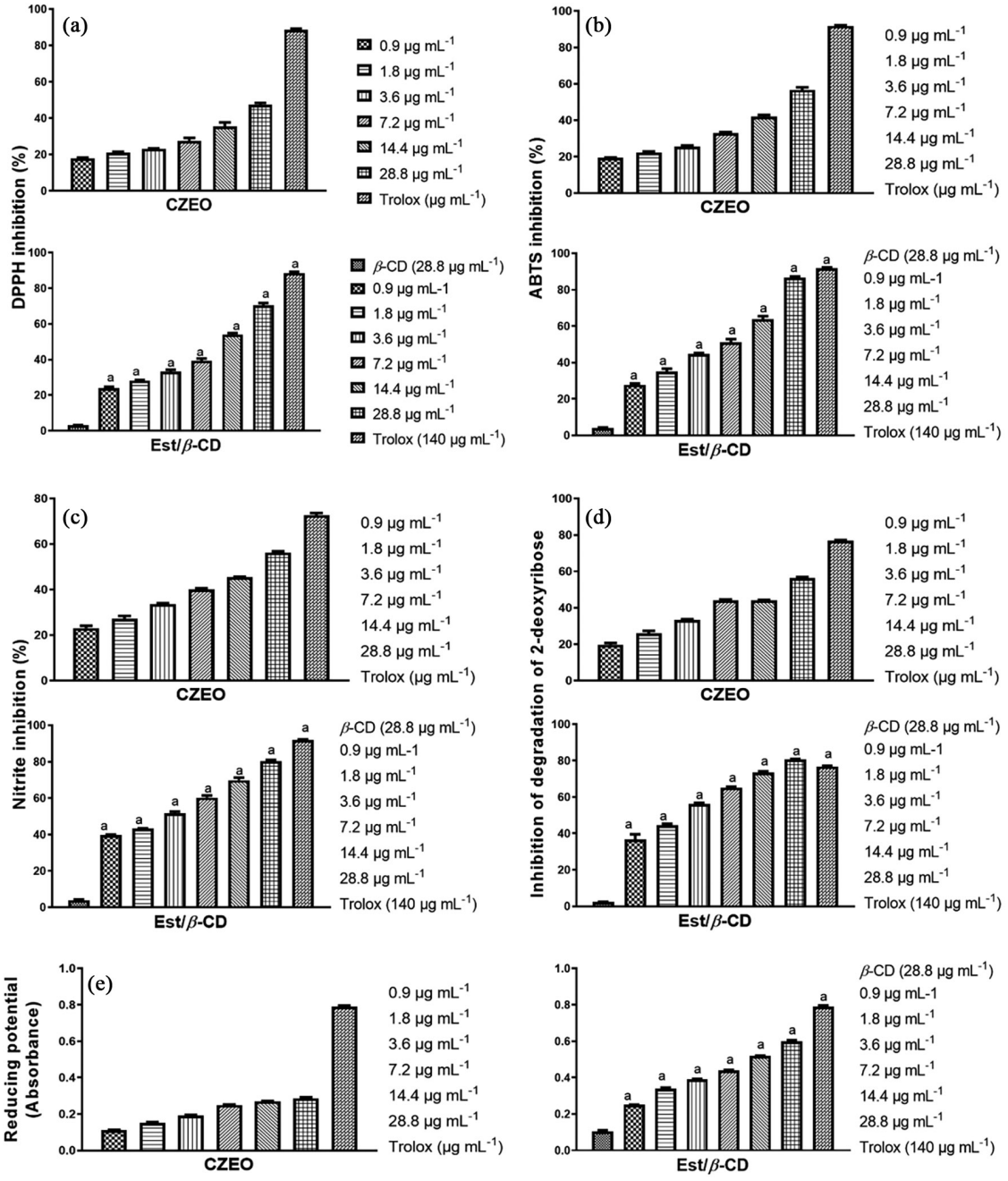


**Figure 7.** Antioxidant activity of *Croton zehntneri* essential oil (CZEO) and its inclusion complex (Est/β-CD) at different concentrations (0.9 to 28.8 μg $mL^{-1}$) in non-biological systems. (a) DPPH inhibition assay; (b) $ABTS^{+}$ inhibition assay; (c) nitrite production assay; (d) 2-deoxyribose degradation assay, and (e) reducing potential assay. The values represent the mean ± SEM of the *in vitro* values, n = 3, of the duplicate experiments. Trolox 140 μg $mL^{-1}$ was used as an antioxidant standard. [a]$p < 0.05$ in relation to β-CD (ANOVA and Neuman-Keuls as post hoc test).

concentrations of Est/β-CD complex showed antioxidant capacity by inhibition of $ABTS^{\bullet+}$ radical cation. As shown in Figure 7, the antioxidant capacity was proportional to the increase of concentration. When the results are compared to CZEO, it is possible to observe an increase of the Est/β-CD antioxidant capacity against $ABTS^{\bullet+}$ radical of 30, 36, 44, 43, 50, 34.3 and 34.6% at concentrations of 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8 μg $mL^{-1}$, respectively. Consequently, this antioxidant result presented by Est/β-CD resulted in the $EC_{50}$ 4.47 μg $mL^{-1}$, which is five times lower than the CZEO $EC_{50}$ 22.73 μg $mL^{-1}$. Similar results regarding the increased pharmacological potential of compounds complexed with β-CD were obtained for other compounds (carvacrol and thymol), demonstrating that the results obtained in the present study agree with previous studies.[13,34,35]

Determining antioxidant capacity by nitric oxide elimination was based on the principle that sodium nitroprusside in an aqueous solution at physiological pH

spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions ($NO_2^{\bullet-}$), which can be calculated employing a Griess reagent.[36] The principle of this reaction was used in this antioxidant assessment and according to the result obtained, CZEO and Est/β-CD at concentrations of 0.9, 1.8, 3.6, 7, 2, 14.4 and 28.8 μg $mL^{-1}$ reacted with nitric oxide and were able to inhibit nitrite ion production.

When the antioxidant capacity of Est/β-CD is compared to CZEO, an increase in the antioxidant capacity of Est/β-CD of 37.12, 34.92, 33.52, 34.91 and 30% was observed at concentrations of 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8 μg $mL^{-1}$, respectively. Consequently, this antioxidant result presented by Est/β-CD resulted in the $EC_{50}$ 2.68 μg $mL^{-1}$, which was 6.5 times lower than the CZEO $EC_{50}$ 17.65 μg $mL^{-1}$.

The antioxidant method against the hydroxyl radical is based on the degradation of 2-deoxyribose by the hydroxyl radicals generated by the Fenton reaction, and the degradation product produces the malonaldehyde compound, which when heated with thiobarbituric acid at an acid pH forms a complex that can be measured at 532 nm.[37] Thus, when an antioxidant compound is added to the mixture and reacts with hydroxyl radicals, there is a decrease in deoxyribose degradation rate and absorbance values at 532 nm. The principle of this reaction was used in this antioxidant evaluation and according to the obtained result, Est/β-CD presented potential to sequester hydroxyl radicals. When the antioxidant capacity of Est/β-CD compared to free CZEO, a significant increase in antioxidant capacity of 46.36, 41.52, 40.77, 32.4, 39.92, 30.63% was observed at concentrations of 0.9, 1.8, 3.6, 7.2, 14.4 and 28.8 μg $mL^{-1}$, respectively. The antioxidant result presented by Est/β-CD resulted in the $EC_{50}$ 2.34 μg $mL^{-1}$, which is ten times lower than the CZEO $EC_{50}$ 23.42 μg $mL^{-1}$.

The reducing capacity of CZEO and Est/β-CD were evaluated by transforming the yellow potassium ferricyanide to the green potassium ferrocyanide. This color change occurs through the electron transfer capability, which is a critical indicator of the antioxidant capacity of CZEO and Est/β-CD. The redox reaction mediated processes such as free radical scavenging and/or inhibition of lipid peroxidation. In this sense, the antioxidant result presented by Est/β-CD resulted in $EC_{50}$ 12.47 μg $mL^{-1}$, which was 3.7 times lower than CZEO $EC_{50}$ 46.48 μg $mL^{-1}$.

## Conclusions

In this study, the inclusion complex CZEO with β-cyclodextrin was successfully prepared by the spray drying method and characterized by IR, Raman, DSC, TG/DTG and GC-MS. However, due to the low concentration of CZEO in the Est/β-CD, slight differences were observed in the DSC, TG/DTG and IR techniques. The Raman and HS-GC-MS techniques were more efficient in characterizing the inclusion complex. Finally, the results of the antioxidant assay clearly showed that Est/β-CD increases the antioxidant potential of CZEO *in vitro*.

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### Author Contributions

Antônio J. P. Sousa was responsible for formal analysis, methodology, project administration, and writing original draft; George L. S. Oliveira for investigation and methodology; Lorenna Fonseca for formal analysis and methodology; Marcio S. Rocha was responsible for formal analysis, methodology, and visualization; Mahendra Rai was responsible for resources, supervision, and visualization; Francisco E. P. Santos was responsible for data curation; Sidney G. de Lima for investigation, project administration, supervision, writing review, and visualization.

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