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Eplingiella fruticosa leaf essential oil complexed with β-cyclodextrin produces a superior neuroprotective and behavioral profile in a mice model of Parkinson's disease

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ABSTRACT

Evidence indicates that oxidative stress has an important role in the onset and progression of Parkinson's disease (PD). Antioxidant agents from natural products have shown neuroprotective effects in animal models of PD. *Eplingiella fruticosa* is an aromatic and medicinal plant of the Lamiaceae family that include culinary herbs. The essential oil (EPL) has anti-inflammatory and antioxidant activities. Cyclodextrins are used to enhances pharmacological profile of essential oil. We obtained the EPL from leaves and complexed with β-cyclodextrin (EPL-βCD). Phytochemical analysis showed as main constituents: β-caryophyllene, bicyclogermacrene and 1,8-cineole. We evaluated the effects of EPL and EPL-βCD (5 mg/kg, p.o. for 40 days) on male mice submitted to the progressive reserpine PD model. Behavioral evaluations, lipid peroxidation quantification and immunohistochemistry for tyrosine hydroxylase were conducted. EPL delayed the onset of catalepsy and decreased membrane lipid peroxides levels in the striatum. EPL-βCD also delayed the onset of catalepsy, reduced the frequency of oral diskynesia, restored memory deficit, produced anxiolytic activity and protected against dopaminergic depletion in the striatum and SNpc. These findings showed that EPL has a potential neuroprotective effect in a progressive PD animal model. Further, EPL-βCD enhanced this protective effects, suggesting a novel therapeutic approach to ameliorate the symptoms of PD.

1. Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder (Barbosa et al., 2006; Mayeux, 2003). The prevalence increases with age, reaching 1–2% of the population over 60 years (Mayeux, 2003; Van Den Eeden et al., 2003). PD pathology is characterized by the progressive loss of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) and accumulation of α-synuclein aggregates, known as Lewy's bodies (Rocha et al., 2018). Although PD's etiology remains insufficiently understood, there is evidence of the involvement of apoptotic events (Ye et al., 2016), mitochondrial dysfunctions (Pozo Devoto and Falzone, 2017), structural changes in proteins (Rocha et al., 2018), excitotoxicity (Dexter and Jenner, 2013) and oxidative stress (Loeffler et al., 2017).

Although classic motor symptoms are determinant for PD diagnosis (Holdorf et al., 2013), non-motor symptoms (autonomic and sensory dysfunctions, anxiety, sleep disturbances, fatigue, and cognitive deficits) may appear previously or concomitantly to motor symptoms (Pfeiffer, 2012; Zhu et al., 2016; Pontone et al., 2009; Chahine et al., 2017; Garcia-Ruiz et al., 2014; Sartor et al., 2017; Leão et al., 2015). PD therapy focus on the symptomatic treatment of motor impairments, rather than precluding dopaminergic neuronal injury (Politi et al., 2018). Indeed, current therapy for PD combines the gold-standard dopaminergic reposition with L-DOPA in combination or not with other agents (monoamine oxidase B (MAOB) and catechol O-methyl transferase (COMT) inhibitors, dopaminergic agonists, and cholinergic

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Abbreviations

ANOVA	analysis of variance
βCD	β-cyclodextrin $CaC_2O_4xH_2O$ calcium oxalate monohydrate
CDs	cyclodextrins
CEDEME	Center of the Development of Experimental Models for Medicine and Biology
CNS	central nervous system
COMT	*catechol O-methyl transferase*
CTR	vehicle
DS	dorsal striatum
DAB	3,3-diaminobenzidine
DSC	differential scanning calorimetry
EPL	essential oil of *E. fruticosa*
EPL-βCD	essential oil of *E. fruticosa* complexed with β-cyclodextrin
EPM	elevated plus maze
GC-MS/FID	mass spectrometry and flame ionization detector
LC	locus ceruleus
MAOB	monoamine oxidase B
Na_2SO_4	sodium sulphate
NOR	novel object recognition test
NPs	natural products
OF	open field
RI	retention index
ROD	relative optical densitometry
PBS	phosphate-buffered saline
PC	paste complexation
PD	Parkinson's disease
PM	physical mixture
RES	reserpine
SNpc	substantia nigra pars compacta
SC	slurry complexation
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TH	tyrosine hydroxylase
TG-DTG	thermogravimetry/derivative thermogravimetry
TOA	time spent in open arms
UFS	Federal University of Sergipe
VTA	ventral tegmental area

blockers) (Jankovic and Aguilar, 2008). Importantly, treatment is frequently associated with motor and non-motor side effects, such as dyskinesia and hallucinations (Lees et al., 2009; Stayte and Vissel, 2014; Leão et al., 2015; Politi et al., 2018), leading to a poor efficacy/side effect relationship in advanced stages of PD. This scenario emphasizes the need for novel antiparkinsonian drugs designed to delay or mitigate progressive courses in neurodegeneration.

Natural herbs and food supplements are possible sources of substances able to treat or prevent neurodegenerative mechanisms. In this regard, the genus *Eplingiella* (Lamiaceae) (Brazilian Biodiversity Information System; previously classified by The Plant List database as *Hyptis*) comprises important species with medicinal and culinary uses, such as *Eplingiella fruticosa* (Salzm. Ex Benth) Harley & J.F.B. Pastore, *E. cuniloides* (Epling) Harley & J.F.B. Pastore and *E. brightoniae* Harley (2014). They are characterized by their shrub size, with small xeromorphic leaves and flowers with short pedicels (Harley and Pastore, 2012; Harley, 2014). *E. fruticosa* (as basionym: *Hyptis fruticosa* Salzm. ex Benth.,), popularly known as 'alecrim de tabuleiro' ('board rosemary'), is widely distributed in the northeastern region of Brazil (Silva et al., 2017). Its infusion is used by the local population as an analgesic treatment (Silva et al., 2006). Previous studies showed that its extract have antinociceptive (Silva et al., 2006), vasorelaxant (Moreira et al., 2010), anti-inflammatory (Andrade et al., 2010) and antioxidant properties (de Lima et al., 2013).

The components of this species, such as other essential oils, are non-polar compounds with hydrophobic properties, which may interfere with the therapeutic use in systemic pharmaceutical formulations (Siqueira-Lima et al., 2017). Some technological tools have optimized the pharmacological effects of these non-polar compounds, for example the use of cyclodextrins (CDs) (cyclic oligosaccharides able to form host-guest complexes with hydrophobic molecules) (Oliveira et al., 2017; Araújo-Filho et al., 2017). CDs can protect the essential oils against heat, light degradation, oxidation, evaporation and moisture, as well as make them easily soluble in water and easy-to-handle powders (Siqueira-Lima et al., 2017). Recently, studies involving essential oils and drugs commonly used in the clinic (for example, steroidal anti-inflammatory and anticancer drugs) have shown that, when complexed or encapsulated on CDs (host), present an improvement in the bioavailability and pharmacological effect (Quintans et al., 2013; Gidwani and Vyas, 2015; Brito et al., 2015; Santos et al., 2017). Therefore, the complexation of CDs appears to be promising for improving the biological effects of essential oils, especially in the treatment of chronic diseases (de Oliveira et al., 2015; Diniz et al., 2018).

In view of the biological activities described to *E. fruticosa*, and due to the variety of secondary metabolites commonly found in its essential oil with action on central nervous system (CNS), we aimed to investigate the possible neuroprotective effect of the leaf essential oil (non-complexed, EPL or complexed with β-cyclodextrin, EPL-βCD) on the reserpine-induced progressive model for PD in mice.

2. Materials and methods

2.1. Collection of the botanical material and extraction of EPL

The leaves of *E. fruticosa* were collected in São Cristóvão city, Sergipe State, Brazil (11°01'47″ S, 37°20'64″ W, err: ± 23587 WGS84) during March 2014. Voucher specimens (registry number ASE 39138) were prepared and deposited at the Department of Biology of the Federal University of Sergipe (UFS), Brazil. The specie was identified by PhD Marla I. U. Oliveira at UFS. The dried leaves were used to extract essential oil from EPL (970 g) by hydrodistillation in a Clevenger-type apparatus for 3 h. After extraction, the leaf essential oil was dried with anhydrous sodium sulphate (Na_2SO_4), filtered and stored in freezer. The yield (w/w) was calculated based on the fresh weight of plant material.

2.2. GC-MS/FID analysis

We performed phytochemical analysis using a mass spectrometry and flame ionization detector (GC-MS/FID) system (QP2010 Ultra, Shimadzu, Japan) equipped with an AOC-20I auto injector (Shimadzu, Japan). We performed the assay under the following experimental conditions - silica capillary column: DB-5MS (30 m × 0.25 mm x 0.25 mm), injector temperature: 230 °C, detector temperature: 250 °C, temperature program: the column was initially 50 °C, then raised to 200 °C at a rate of 4 °C/min, and finally held at 200 °C for 10 min, electron impact: 70 eV, carrier gas: helium at 1.2 ml/min, scanning speed 0.84 scan/sec from m/z 40–550 Da. The identification of the compounds was performed by computerized matching of the acquired mass spectra with those stored in the mass spectral libraries (NIST107 and NIST21; WILEY8), followed by the calculation of the retention index (RI) obtained with an equation proposed by Van Den Dool and Kratz (1963) for each constituent, as previously described (Adams, 2007).

2.3. Preparation of inclusion complex containing EPL and β-cyclodextrin

The inclusion complexes containing EPL and β-cyclodextrin were prepared according to Siqueira-Lima et al. (2014). These samples were prepared based on the majority compound's molecular weight of EPL (β-caryophyllene - 204 g) in a 1:1molar ratio. The physical mixture was prepared by adding EPL (204 mg) in an agate mortar containing β-cyclodextrin (1135 mg) under manual stirring followed by storage in sealed glass vials. Paste complexation was prepared through homogenization of EPL (204 mg) and β-cyclodextrin (1135 mg) in water (2 ml) directly in an agate mortar. Then, the material was dried at room temperature (in a desiccator) until a glass film was formed, which was removed by manual trituration and stored in airtight glass containers. Finally, the slurry complexation was performed by adding EPL (204 mg) to a solution of β-cyclodextrin (1135 mg, in 20 ml), maintained under magnetic stirring at 400 rpm for 36 h constantly (Quimis Q 261A21, Brazil). The EPL-βCD mass ratio was maintained as described for inclusion complex preparation and the mechanical mixture was stored in airtight glass containers.

2.4. Physicochemical and morphological characterization of the complexes

2.4.1. Thermal analysis

Differential scanning calorimetry (DSC) and Thermogravimetry/derivative thermogravimetry (TG/DTG) curves were obtained as described by Serafini et al. (2012). DSC curves were performed in a DSC-60 cell (Shimadzu, Japan) using aluminum crucibles containing about 1 mg of samples, under a dynamic nitrogen atmosphere (50 ml/min) and at a heating rate of 10 °C/min in the temperature range from 25 °C to 500 °C. The DSC cell was verified with indium (m.p. 156.6 °C; $\Delta H_{melting}$ = 28.54 J/g) and zinc (m.p. 419.6 °C). The TG/DTG curves were obtained with a TGA 60 (Shimadzu, Japan) thermobalance in the temperature range of 25–900 °C, in platinum crucibles containing approximately 3 mg of samples, under a dynamic nitrogen atmosphere (50 ml/min) and at a heating rate of 10 °C/min. The TG/DTG was verified with calcium oxalate monohydrate ($CaC_2O_4xH_2O$), according to the ASTM standard (ASTM, 1993).

2.4.2. Moisture determination

The moisture contents of the EPL, β-cyclodextrin, physical mixture (PM), paste complexation (PC) and slurry complexation (SC) were determined by the Karl Fischer method using a KF 1000 Analyzer (Brazil) and Hydranal (Merck, USA) as the titrating solution. The analyses were performed in triplicate.

2.4.3. Scanning electron microscopy (SEM)

The samples were mounted on carbon tape and then covered with gold ions. Subsequently, the samples were visualized in a JEOL Model JSM-7410-F scanning electron microscope, at an accelerated voltage of 1 kV.

2.5. Animals

Six-month-old male Swiss mice (40–60 g) were obtained from the Center of the Development of Experimental Models for Medicine and Biology – CEDEME (Federal University of São Paulo). All animals were housed in groups of five per cage (30 cm × 37 cm × 16 cm), under controlled conditions of ventilation, temperature (23 ± 1 °C) and a 12/12 h light/dark cycle (lights on 7:00 a.m.), with free access to water and food. All experimental procedures were approved by the local ethics committee for animal use (Protocol CEUA/UNIFESP n° 1073230415/2015) and are in accordance to the Brazilian law for the use of animals in research (Law number 11.794). All efforts were made to minimize animal pain, suffering or discomfort.

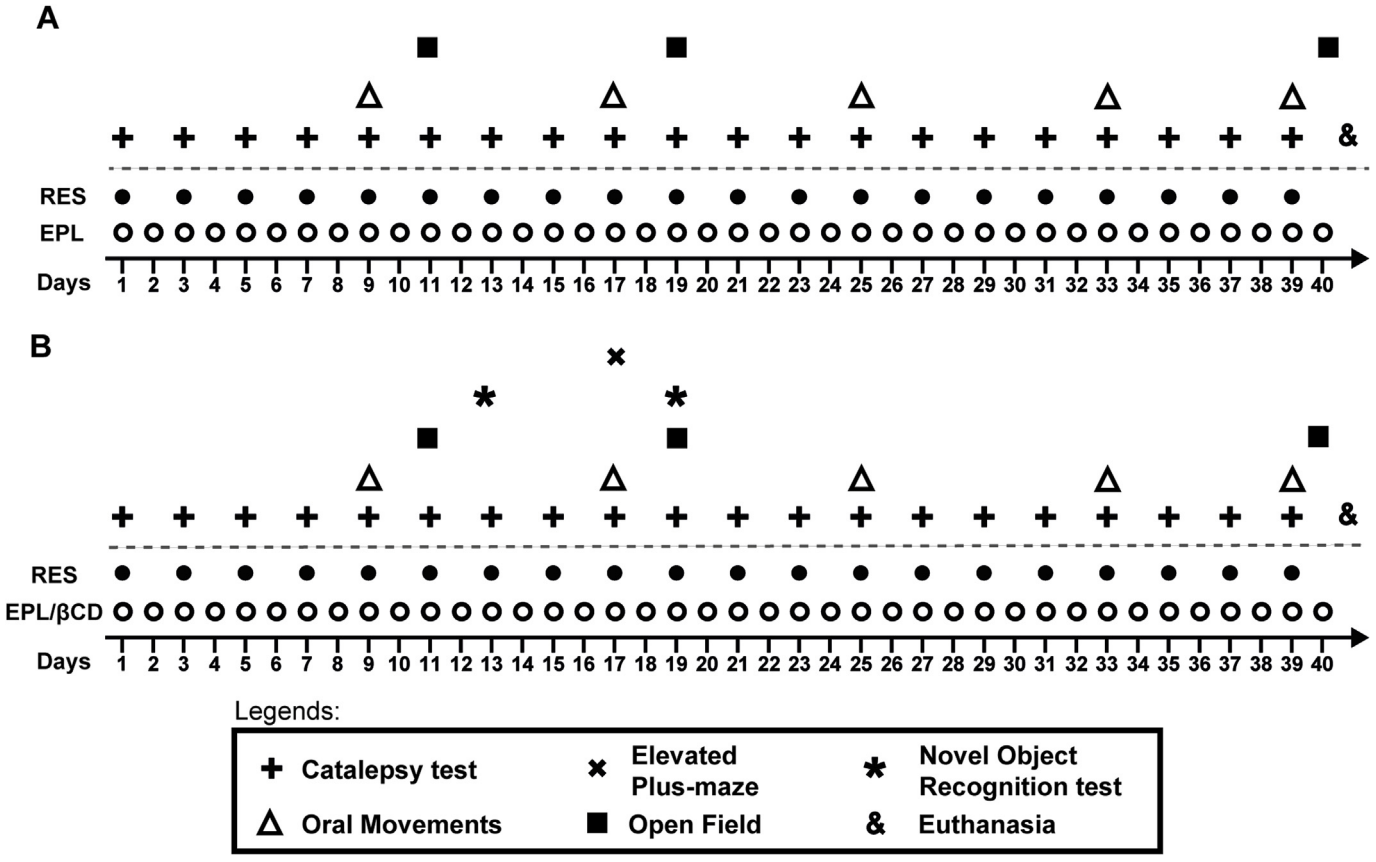

Fig. 1. Schematic representation of the experimental designs of experiments I (A) and II (B).

2.6. Drugs

Reserpine (RES, #R0875) and β-cyclodextrin (≥97% purity, #C4767) were purchased from Sigma Chemical Co. (USA). RES was dissolved in glacial acetic acid (1%) and then diluted to the correct concentration with distilled water. Vehicle consisted of the same amount of acetic acid and water as in the RES solution. Both RES and vehicle were injected subcutaneously (s.c.). EPL was suspended in 0.9% saline solution with Tween 80 and its vehicle (CTR) consisted of 0.9% saline/Tween 80. EPL-βCD was suspended in distilled water, which was used as vehicle control. EPL, EPL-βCD or CTR were administered orally (p.o.) by gavage.

2.7. General procedures and experimental design

Before the beginning of experimental procedures, animals were gently handled during 10 min for five consecutive days. The apparatuses were cleaned with 5% alcohol solution after each behavioral session, and all behavioral data were registered and analyzed through the video-tracking software Any-maze (Stoelting, USA), except for the catalepsy, the oral movements and sensibility tests evaluation, that were manually registered by experimenters blind to treatment.

We conducted two experiments (Fig. 1), in which the animals were randomly assigned to groups (Table 1) that received subcutaneous (s.c.) injections of vehicle (CTR) or 0.1 mg/kg of reserpine (RES) at the volume of 10 ml/kg, every 48 h during 40 days of treatment. Moreover, mice received oral (p.o.) administrations of EPL vehicle (CTR), or EPL (5 mg/kg) or EPL-βCD (5 mg/kg) at a volume of 10 ml/kg body weight every day during 40 days. During the treatment period, mice were submitted to behavioral tests (Table 1) that were conducted before the following injection. Further, 48 h after the last injection of reserpine, the animals were anesthetized and euthanized, and the brains were removed in order to evaluate oxidative stress by quantification of membrane lipid peroxides (thiobarbituric acid reactive substances, TBARS) or immunohistochemical analysis of tyrosine hydroxylase.

2.8. Behavioral testing

2.8.1. Catalepsy test

The catalepsy behavior was assessed by placing the animal's forepaws on a horizontal bar positioned 5 cm above the bench surface. Catalepsy was defined as an immobile posture (keeping both forepaws on the bar) and was measured up to a maximum of 180 s. Three trials for each animal in each observation day were carried out and the results were analyzed considering the mean value of these trials (Santos et al., 2013).

2.8.2. Oral movements

Mice were individually placed in a transparent glass box (20 cm × 20 cm x 15 cm) with mirrors positioned under and behind it to allow behavioral quantification when animal faced away from the observer. The frequency of vacuous chewing movements (mouth openings in the vertical plane not directed toward physical material) were measured continuously for 5 min (Santos et al., 2013).

2.8.3. Open field (OF)

Locomotor activity was observed in a circular arena (50 cm in diameter) with 40 cm high walls, made of wood and painted black. Animals were placed in the center of the apparatus to freely explore it during 5 min. Distance travelled in the whole arena (in meters) and maximum speed (in centimeters/second) were evaluated (Santos et al., 2013).

2.8.4. Novel object recognition test (NOR)

The NOR test was performed in the same circular arena used in the OF test. The animals were presented to two identical objects in the sample phase and after a retention interval (1 h) they were tested in the presence of a familiar object in the same place of the sample phase and a new object. The objects used in the training and testing phase were different among the animals. Throughout the repetitions of the sessions, different objects were used, each with two copies, and all the objects were made of the same material differing in color, size and shape. Objects had no ethological significance and a pilot test was initially conducted to evaluate whether the animals had any preference for the selected objects (data not shown). The objects were heavy enough not to be moved by the animals or, otherwise, they were fixed in the OF. After each series the objects used as well as the OF were cleaned with 5% alcohol to avoid the presence of olfactory tips. The duration of the sessions was 5 min each (Santos et al., 2013).

2.8.5. Elevated plus-maze (EPM)

The EPM is a cross-shaped wooden apparatus containing two opposite enclosed arms (27.5 cm × 6.5 cm x 18 cm) and two open arms (27.5 cm × 6.5 cm). The background of the device was painted black in order to increase the contrast and allow software tracking of animal movements. The animals were placed in the center of the maze and videotaped for 5 min. The behavioral evaluation included distance travelled, maximum speed and time in open arms (%TOA - percentage of time spent in open arms) (Doukkali et al., 2016).

2.9. Lipid peroxidation

After euthanasia by decapitation, the brains were removed and the dorsal striatum (DS) were dissected and weighed. After this procedure, the samples were pooled and homogenized in 0.1 M phosphate buffer (1:5 ratio), after centrifugation for 15 min at 3500 rpm and 4–5 °C to obtain at least 150 µl of the homogenate supernatant. Quantification was performed by the method described by Tanizawa et al. (1981). The reaction was started by adding 250 µl 3% SDS, 1.5 ml of 2 M acetic acid buffer, and 1.5 ml of 0.8% thiobarbituric acid (TBA) to 50 µl of tissue homogenate. Finally, the volume was quenched with 4 ml of Milli Q water and then heated at 95 °C for 60 min and chilled with ice water.

Table 1
Details of the experimental groups and behavioral tests.

Experiment	Groups (N of animals)	Treatment	Behavioral tests/Days of analysis
Essential oil ***(EPL)***	CTR-CTR (12)	RES vehicle + EPL vehicle	*Catalepsy every two days throughout the treatment*
	CTR-EPL (12)	RES vehicle + EPL	*Oral movements after the 4*th, *8*th, *12*th, *16*th *and 19*th *injection*
	RES-CTR (12)	RES + EPL vehicle	*Open field after the 5*th, *9*th *and 20*th *injection*
	RES-EPL (12)	RES + EPL	*Novel object recognition test after the 6*th *and 9*th *injection*
Essential oil complexed with β-cyclodextrin ***(EPL-βCD)***	CTR-CTR (7)	RES vehicle + βCD-EPL vehicle	*Catalepsy every two days throughout the treatment*
	CTR-βCD-EPL (7)	RES vehicle + βCD-EPL	*Sensitivity test after the 3*rd, *6*th, *7*th *e 13*th *injection*
	RES-CTR (7)	RES + βCD-EPL vehicle	*Oral movements after the 4*th, *8*th, *12*th, *16*th *and 19*th
	RES-βCD-EPL (7)	RES + βCD-EPL	*Open field after the 5*th, *9*th *and 20*th *injection*
			*Novel object recognition test after the 6*th *and 9*th *injection*
			*Elevated plus maze after the 8*th *injection*

Then, 2.5 ml of an n-butanol: pyridine (15:1) mixture was added and samples were stirred vigorously. The amount of thiobarbituric acid reactive substances (TBARS) formed was measured by the reaction with the thiobarbituric acid at 532 nm from the top phase formed with the n-butanol: pyridine mixture. The amount of TBARS formed was then measured and the results expressed in nanomoles of TBARS per gram (g) of wet tissue, by plotting the obtained values against a known TBARS concentration curve.

2.10. Tyrosine hydroxylase (TH) immunohistochemistry

All animals were anesthetized with intraperitoneal injection of xylazine hydrochloride (10 mg/kg), ketamine hydrochloride (100 mg/kg), acepromazine (1 mg/kg) and fentanyl (0.5 mg/kg). Then, they were perfused transcardially with 200 ml phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4. The brains were removed from the skull, postfixed in the same fixative solution (paraformaldehyde in PBS) for 24 h and transferred to a solution containing sucrose 30% in 0.1 M PBS, pH 7.4. Each brain was serially cut in the coronal plane into 50 μm tick sections with a cryostat microtome (Leica, Germany) at a temperature of −20 °C. The sections were placed sequentially in five compartments (one section per compartment, 250 μm apart) and stored in antifreeze solution. Following tissue processing, we performed immunohistochemistry for TH (DS; Substantia Nigra Pars Compacta, SNpc; and Ventral Tegmentar Area, VTA), using a free-floating protocol. Sections were washed 4 times with PBS (pH 7.4) for 5 min each, and consecutively washed with 0.0003% H_2O_2 solution for 20 min to reduce endogenous peroxidase activity. For detection of TH, sections were incubated with rabbit anti-tyrosine hydroxylase polyclonal antibody (cat #AB152 Chemicon, USA, 1:10,000) diluted in triton x-100 0.4% and PBS (pH 7.4) with 2% albumin serum, for 18–24 h at room temperature. Afterwards, sections were incubated with goat biotinylated anti-rabbit IgG (Vector Labs, USA, 1:5000) diluted with triton x-100 0.4% NaCl and PBS (pH 7.4) for 2 h at room temperature, followed by washing steps, and incubated with avidin-biotin-peroxidase solution (ABC Elite kit, Vector Labs, USA) for another 2 h. The reaction was developed by adding of 3,3-diaminobenzidine (DAB, Sigma-Aldrich, USA) and 0.01% H_2O_2 0.1 M phosphate buffer solution for 1–2 min. Then, we left sections to dry, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Entellan (Merck, Germany). All sections were immunostained concomitantly, to minimize possible background differences between samples. Sections were examined under brightfield illumination with an optical microscope (Nikon Eclipse Ni-E; 10x), attached with a digital camera (Motic 5.0) to record images.

In order to estimate the number of TH + cells (SNpc, VTA) and TH-immunoreactivity in striatum fibers, we analyzed four sections of each animal: one at rostral level, two at median level and one at caudal level. All TH + cells of SNpc and VTA on each section were counted by a researcher blind to treatment and the mean of the four measures was registered. Additionally, TH-immunoreactivity in DS fibers was assessed by analysis of relative optical densitometry (ROD) using ImageJ software (version 1.48, NIH, USA). Four random fields were chosen in the DS and the mean pixels were calculated (n = 5–7 per group). For this analysis, the mean pixels in the target area (DS) were subtracted from the mean values of a control region (random field of the same size in the same image that was not stained for TH, *i.e.* cortex or *corpus calosum*). All final values were normalized by the mean value of control group.

2.11. Data analysis

Kolmogorov-Smirnov's and Levene's tests were used to analyze normality of data and homogeneity of variance, respectively. Parametric tests were used accordingly to data distribution and homogeneity of variance. Two-way analysis of variance (ANOVA) with repeated measures was applied to catalepsy, sensitivity test and oral movement parameters to assess effects throughout treatment and withdraw phases. Two-way ANOVA was applied to OF, EPM, TBARS and immunohistochemistry. Both tests were followed by Tukey's *post hoc* test to highlight differences between strain and treatment groups, with the exception of lipid peroxidation that was followed by Sidak's *post hoc* test. New object recognition test was analyzed by paired-samples t tests. Results were expressed as mean + SEM and exact p-values were expressed for each factor and factor interactions for the two-away ANOVA, besides differences highlighted by the Tukey's or Sidak's post hoc. Significant differences was considered by $p \leq 0.05$.

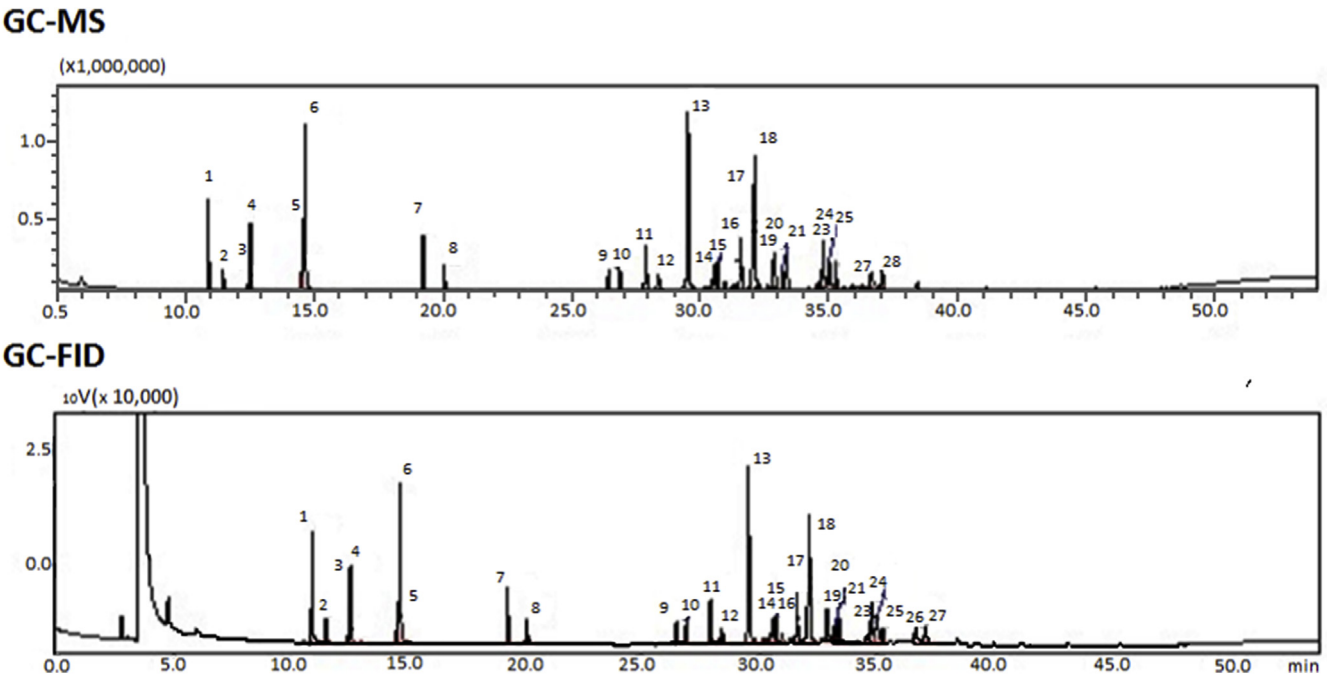

Fig. 2. Chromatographic profile of *E. fruticosa* essential oil.

3. Results

3.1. Characterization of the compounded essential oil

The phytochemical screening of the EPL was obtained by gas spectrometer analysis coupled to mass spectrometry (Fig. 2) and the CG/MS-FID analysis of the EPL follows the composition on Table 2.

3.2. Characterizations of the complexes obtained by the PM, PC and SC methods

DSC curves are shown in Fig. 3A. The EPL curve showed a thermal event in the range of 37–202 °C relative to its volatilization. The βCD showed three endothermic events, the first in the range of 30–116 °C, which is related to dehydration, the second between 210 and 230 °C, related to the transition phase and the third in the range of 278–345 °C, characteristic of the melting point, followed by the degradation of the βCD. Similar results were obtained in other studies (Serafini et al., 2012; Yang et al., 2016). The physical mixture showed a similar thermal profile to the βCD, indicating that no oil had been complexed. However, the PC and SC methods showed a reduction in the temperature of the dehydration and melting events compared to the βCD. Additionally, the phase transition events could not be observed in the PC and SC curves when the guest molecule was complexed inside the βCD cavity, as observed in the study by Kayaci and Uyar (2011) about complexation of vanillin and βCDs. More recently, Menezes et al. (2015) studied the complexation of *Hyptis pectinata* in βCD and also observed that during the complexation process, the temperatures of the characteristic thermal events found in βCD such as phase transition and melting point are reduced. As we also found this, it suggests the procedure was also successful in our study.

Fig. 3B and Table 3 show the TG/DTG results for the samples. The EPL showed weight loss (Δm = 100%) in the range of 34–171 °C, and this event was related with the decomposition process, which corresponds to the volatilization event shown by DSC. The βCD showed four steps of weight loss. The first related to a dehydration (Δm = 13%, 34-171 °C confirmed by Karl Fischer titration: 13.7%) event previously shown by DSC. The second event did not show a significant mass loss because it relates to the transition phase event shown by DSC, which corresponds to the structural reorganization of the molecule. The other mass loss events were related to decomposition (Δm = 78.8%, 307-500 °C) followed by degradation (Δm = 5.9%, 500-900 °C). For the PM mass losses, we can observe the highest percentage in the first step (12.2%) corresponding to the surface oil (once 11.6% corresponded to water loss as determined by the Karl Fischer method). In the second step (171-300 °C) the weight loss of 4.2% is related to a low quantity of complexed oil (probably any monoterpene with a high affinity with the βCD cavity). The PC and SC methods showed a reduction in the percentage of water and surface oil, suggesting the complexation replaces water in the βCD cavity with EPL. In the second step, we observed an increase of mass loss related to complexed oil (PC: 6.2% and SC: 4.0% in the range of 171–300 °C). After release of complexed oil, the βCD in the samples started the degradation process (300 °C) as can be observed in Fig. 3B.

Fig. 4 shows the SEM analysis of the samples at magnifications of 500 and 800x. By examining the images, we could see the morphology of the surface of the βCD and the other samples. The SEM studies of βCD revealed that crystals were polyhedral in form and of a bigger size. In respect of PM, it was possible to observe clearly the oil covering the crystalline surface of the βCD, and this observation agrees with the highest value of superficial oil reported in the TG curve. This result was also reported by Menezes et al. (2015) in a study of *Hyptis pectinata* essential oil using the same complexation methods. The microphotographs of the samples prepared by PC and SC showed a strong reduction in the particle size and morphological changes due to the processing methods employed, which provoked a high degree of particle agglomeration, although the wettability of the EPL increased as previously described by Azevedo et al. (2011).

Therefore, these results suggested that PC is the best method for the EPL-βCD complex formation and is the most promising formulation for use in the pharmacological studies.

3.3. Neuroprotective effect the EPL and EPL- βCD

3.3.1. Catalepsy behavior

In previous studies we have robustly shown that reserpine progressively increase the catalepsy behavior across repeated treatment in rodents (Santos et al., 2013; Leão et al., 2015; Brandão et al., 2017; Campêlo et al., 2017; Lins et al., 2018; Souza et al., 2018). In this study, our results confirmed the progressive increase of catalepsy time from the 19th or 23rd day onwards ($p < 0.05$, Fig. 5A and C respectively). The administration of the EPL delayed the onset of the motor impairment ($p < 0.05$, Fig. 5A). Moreover, the treatment with EPL-βCD delayed the initiation of increased catalepsy time until the 29th day, and promoted a shorter time of catalepsy throughout the treatment compared to reserpine only ($p < 0.05$, Fig. 5C).

3.3.2. Oral movements

The analysis of oral movements revealed a significant effect of reserpine and absence of effect of EPL. Indeed, reserpine only and reserpine plus EPL groups showed increased vacuous chewing movements at the 25th day when compared to control groups ($p < 0.05$, Fig. 5B and D). On the other hand, coadministration with EPL- βCD promoted a decrease in vacuous chewing at the 25th and 39th days compared to RES-CTR ($p < 0.05$, Fig. 5D).

Table 2
Analysis of constituents of the essential oil extracted from *Eplingiella fruticosa*.

Peak	RT (min)	Compound	%GC-MS	%GC-FID	ERRI exp.*	RIL lit.**
1	10.895	α-pinene	5.74	6.79	934	932
2	11.471	Canfene	1.30	1.63	950	946
3	12.373	Sabinene	0.33	0.37	974	969
4	12.533	β-pinene	4.47	5.10	979	974
5	14.521	Limonene	1.65	1.68	1031	1024
6	14.643	1,8-cineole	12.09	11.06	1035	1031
7	19.232	Camphor	4.32	4.32	1151	1141
8	20.051	Borneol	1.94	2.28	1171	1165
9	26.437	δ-elemene	1.59	1.74	1343	1335
10	26.868	α-cubebene	1.96	1.95	1355	1345
11	27.889	α-ylangene	3.72	3.45	1384	1373
12	28.370	β-elemene	1.17	1.68	1398	1389
13	29.515	β-Caryophyllene	14.78	14.16	1432	1417
14	30.172	*trans*-muurrola-3,5-diene	2.06	1.81	1462	1451
15	30.511	α-humulene	2.41	2.36	1452	1462
16	30.669	*cis*-murrola-4(14),5-diene	0.60	0.93	1475	1465
17	30.943	*trans*-murrola-4(14),5-diene	4.48	4.13	1493	1493
18	31.571	Bicyclogermacrene	14.15	12.68	1510	1500
19	32.102	*cis*-calamenene	4.30	3.65	1535	1528
20	32.863	*trans*-cadina-1,4-diene	1.65	1.33	1545	1533
21	33.179	α- cadinene	3.52	3.16	1550	1537
22	33.351	NI	0.43	1.04	1590	–
23	34.561	Espatulenol	4.01	3.64	1590	1577
24	34.764	Caryophyllene oxide	2.25	2.98	1595	1582
25	34.990	Globulol	1.60	1.66	1611	1590
26	35.259	Epi-α-cadinol	1.60	2.18	1638	1657
27	36.626	α-cadinol	1.88	2.21	1669	1652

NI, not identified. RT (recovery time), %GC-MS (Gas chromatography – mass spectrometer), %GC-FID (Gas chromatography – flame ionization detector), ERRI (Experimental relative retention index). RIL (Retention index literature).

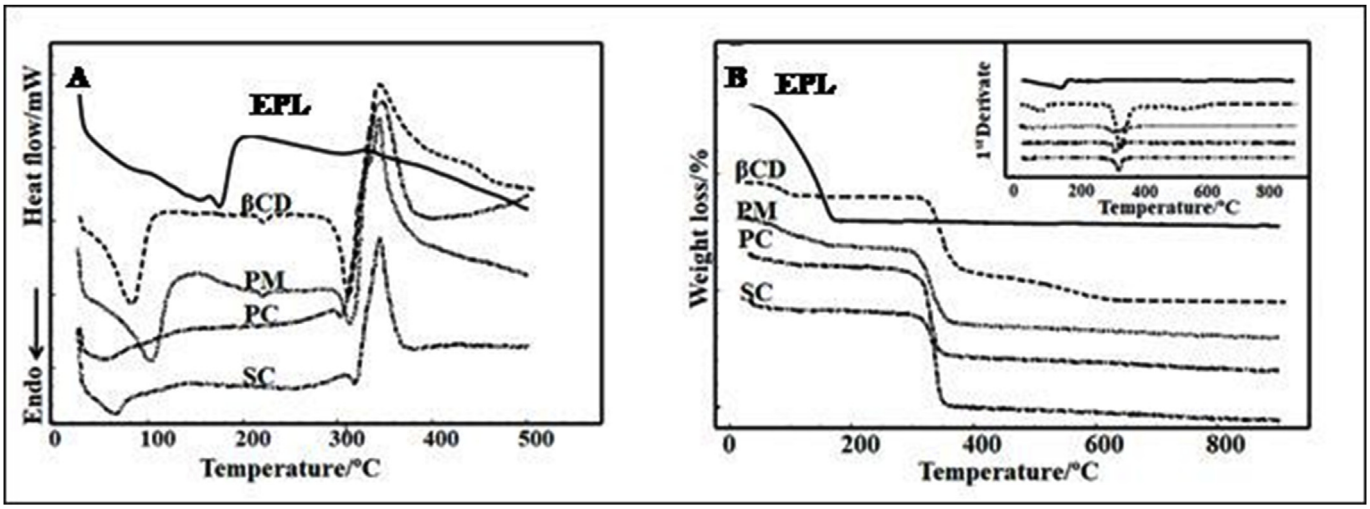

Fig. 3. Thermal analysis of essential oil from *Eplingiella fruticosa* (EPL), β-cyclodextrin (βCD), physical mixture (PM), paste complexation (PC) and slurry complexation (SC). A) DSC curves: heat flow (mW) versus temperature (°C). B) TG/DTG curves: mass loss (%) versus temperature (°C).

Table 3
Percentage of weight loss shown by the thermogravimetry/derivate thermogravimetry (TG/DTG) curves and water content of the β-cyclodextrin (βCD), physical mixture (PM), paste complexation (PC) and slurry complexation (SC) samples obtained by the Karl Fischer method.

Sample	1st step/% 34-171 °C	2st step/% 171-300 °C	3st step/% 307-500 °C	4st step/% 500-900 °C	% H_2O
EPL	100.0	–	–	–	0.5 ± 0.1
βCD	13.0	1.5	78.8	6.7	13.7 ± 0.4
PM	23.8	4.2	66.1	5.9	11.6 ± 0.3
PC	10.8	6.2	74.2	8.8	9.5 ± 0.1
SC	8.6	4.0	81.4	6.0	10.9 ± 0.2

3.3.3. Motor activity in OF

As expected, the analysis of showed that RES-treated animals had lower distance travelled from the 11th day onwards ($p < 0.05$, Table 4). Moreover, the RES-EPL group showed lower total distance travelled on the 11th day ($p < 0.05$) when compared to the CTR-CTR. However, EPL-βCD-treated animals had decreased distance travelled only from the 40th day (Table 4), suggesting neuroprotective effect.

3.3.4. Quantification of TBARS (lipid peroxidation)

Reserpine increased the amount of thiobarbituric acid reactive substances (TBARS) and EPL treatment reduced the level of TBARS in striatum ($p = 0.05$, Fig. 6).

3.3.5. Novel object recognition test

On the 19th day, no differences were observed in training sessions (Fig. 7A, C). In the test session, control animals spent more time exploring the novel object compared to the familiar object ($p < 0.05$), but not RES-CTR and RES-EPL groups (Fig. 7B), i.e. these groups had an impairment on memory. On the other hand, RES-EPL-βCD-treated animals also spent more time exploring the new object in comparison to the familiar object ($p < 0.05$, Fig. 7D), suggesting a protective effect on memory. No differences were observed on the 13th day (data not shown).

3.3.6. Anxiety-like behavior (EPM)

During the evaluation anxiety-like behaviors, animals treated with EPL-βCD spent more time in the open arms when compared to CTR-CTR and the RES-CTR ($p < 0.05$). Moreover, EPL-βCD-treated mice demonstrated higher distance travelled when compared to RES-CTR group ($p < 0.05$, Table 5). No differences were found for maximum speed.

3.3.7. Immunohistochemistry

As expected, there was a decrease in the number of TH + cells in SNpc in the reserpine group ($p < 0.05$, Fig. 8A and B). However, treatment with EPL-βCD increased the reactive cell number ($p < 0.05$, Fig. 8B). Regarding the DS, there was a decrease in the density of TH-immunoreactivity in RES-CTR relative to CTR-CTR ($p < 0.05$, Fig. 8C), as well as an increase in RES-EPL-βCD when compared to RES-CTR group ($p < 0.05$, Fig. 8C). No differences were found for VTA (Fig. 8D).

4. Discussion

Our study investigated the effects of leaf essential oil from *E. fruticosa* (non-complexed, EPL, and complexed with β-cyclodextrin, EPL-βCD) in mice submitted to the animal model of progressive parkinsonism induced by repeated treatment with a low dose of reserpine. Chronic treatment with the EPL (5 mg/kg, p.o.) delayed catalepsy onset and decreased TBARS levels in the SD, while treatment with EPL-βCD delayed the onset of catalepsy, reduced the frequency of oral diskynesia (vacuous chewing), restored the short-term memory deficit, promoted an anxiolytic effect and protected against dopaminergic imbalances (TH + cells) in the DS and SNpc.

During recent decades, studies have used neurotoxic approaches to model pathophysiological characteristics in animal models of PD. Reserpine irreversibly blocks the vesicular monoamine transporter-1 and 2 (VMAT1 and 2), which prevents the reuptake of monoamines into the vesicles and results in cytoplasmic accumulation followed by monoamine depletion in these cells (Henry et al., 1998). Further, monoamines are metabolized in the cytoplasm generating reactive oxygen species that eventually result in increased oxidative stress (Bilska et al., 2007; Fuentes et al., 2007; Leão et al., 2015) and autophagy-related neuronal death (Lee et al., 2015). Reserpine-treated rodents develop cognitive impairments such as memory loss (Aguiar et al., 2009; Carvalho et al., 2006; Santos et al., 2013) previously to motor disorders (tremor, stiffness and hypokinesia) (Fernandes et al., 2012; Santos et al., 2013; Leão et al., 2015). These alterations are similar to those found in Parkinson's disease (Colpaert, 1987; Kaur and Starr, 1997). For this reason, it is a suitable pharmacological model for the discovery of new drugs for PD treatment (Leão et al., 2015).

Our research group previously reported that the repeated administration of RES at a low dose is able to induce progressive motor and cognitive deficits accompanied by increased striatal lipid peroxidation (Fernandes et al., 2012; Leão et al., 2017) and tyrosine hydroxylase depletion in the SD and SNpc (Santos et al., 2013; Campêlo et al.,

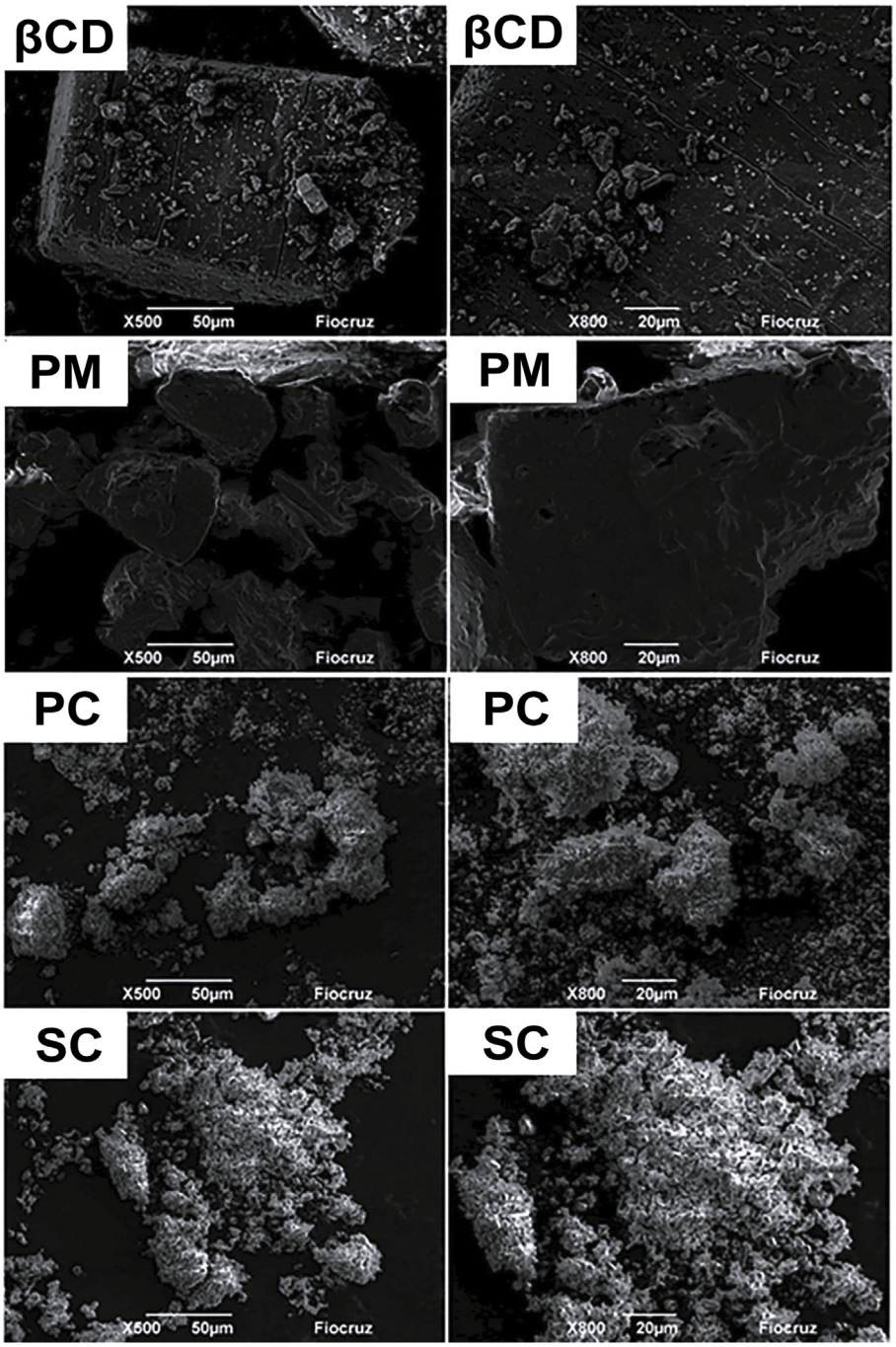

Fig. 4. Scanning electron microscopy (SEM) photomicrographs of β-cyclodextrin (βCD), physical mixture (PM), paste complexation (PC) and slurry complexation (SC) at 500 and 800x magnification.

2017). It is also important to highlight that besides altered motor function and non-motor effects, and consequences to dopaminergic functioning, this protocol of reserpine treatment also induces other features compatible with PD such as increased alpha-synuclein levels (Leão et al., 2017) and neuroinflammation parameters (unpublished data).

Our results corroborate those previous findings, as RES-treated mice showed a motor progressive impairment evaluated by catalepsy behavior and motor parameters in open field. On the other hand, EPL and EPL-βCD-treated animals delayed the effects of RES on catalepsy time, and the delay in EPL-βCD-treated mice was more pronounced compared to the EPL group. Thus, the EPL-βCD produces a longer lasting effect enhancing the protective effect against the difficulty to start a movement in the catalepsy test. Furthermore, the animals of RES-CTR group showed a progressive decrease in the total distance travelled and average speed (data not shown) at the 10th, 19th and 40th day of treatment. Conversely, EPL-βCD group (but not EPL-treated mice) presented delayed motor deficits, which only occurred on the 40th day. Once again, EPL-βCD reduced the magnitude of motor impairments compared to the RES-treated group.

Still regarding the effects of RES on motor activity, previous studies demonstrated that the repeated treatment results in facial muscle tremor, mouth opening in the vacuum and tongue protrusions (Neisewander et al., 1994). These facial automatisms are compatible with the parkinsonian tremor because they are produced or exacerbated by dopaminergic antagonists, cholinergic drugs and dopamine

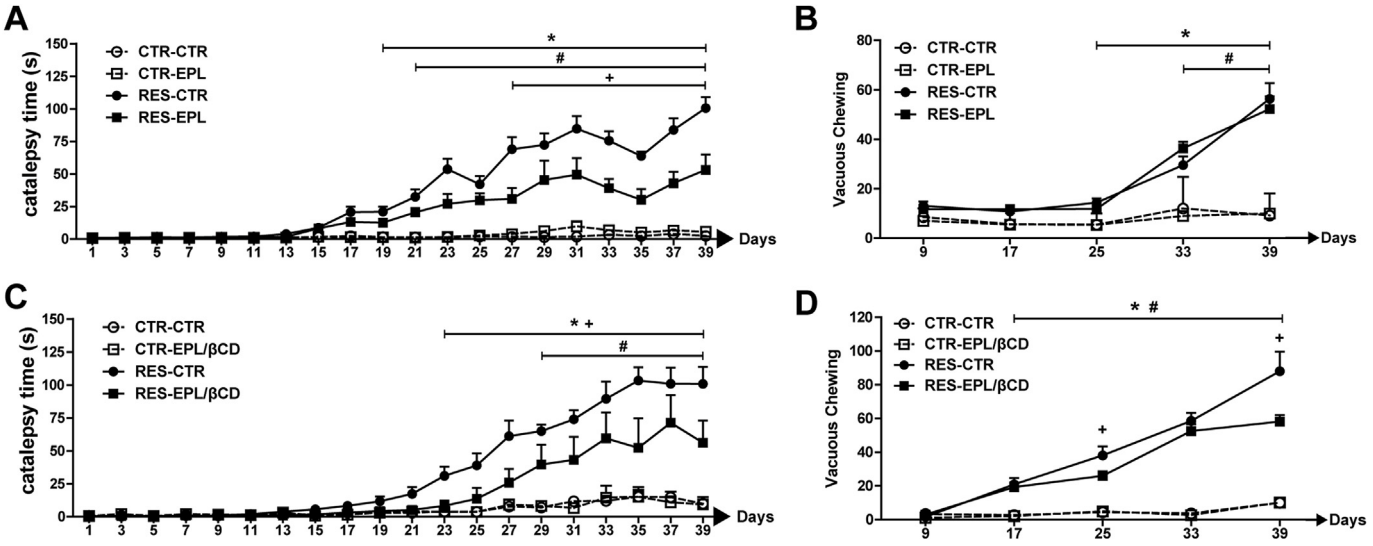

Fig. 5. Effects of repeated administration of EPL (5 mg/kg), EPL-βCD (5 mg/kg) and RES (0.1 mg/kg) on catalepsy behavior of mice (A,C) and on oral movements of mice (B, D). Data are expressed as mean ± SEM. *p < 0.05 RES-CTR compared to CTR-CTR; #p < 0.05 RES-EPL compared to CTR-CTR; +p < 0.05 RES-EPL compared to RES-CTR (Two-way ANOVA with repeated measures followed by Tukey's post hoc test).

Table 4
Effects of repeated administration of EPL and EPL-βCD (5 mg/kg) and RES (0.1 mg/kg) to mice in spontaneous motor activity on OF.

Day	Treatment	Distance (m)	Maximun speed (cm/s)
11th	CTR-CTR	10.1 ± 0.4	48 ± 9.2
	CTR-EPL	9.7 ± 0.7	45 ± 5.4
	RES-CTR	6.1 ± 0.5*	55 ± 9.4
	RES- EPL	7.4 ± 0.6*	53 ± 10.3
	CTR-CTR	16.7 ± 2.3	26.3 ± 1.3
	CTR-EPL-βCD	19.9 ± 1.0	28.9 ± 1.6
	RES-CTR	16.0 ± 1.3	28.4 ± 1.6
	RES-EPL-βCD	14.8 ± 1.9	26.0 ± 1.7
19th	CTR-CTR	5.8 ± 0.7	59 ± 25.3
	CTR- EPL	6.9 ± 1.2	46 ± 14.4
	RES-CTR	2.2 ± 0.3*	25 ± 4.3
	RES- EPL	3.5 ± 0.6	29 ± 3.5
	CTR-CTR	11.0 ± 2.0	25.2 ± 2.0
	CTR-EPL-βCD	13.5 ± 2.0	28.5 ± 1.3
	RES-CTR	7.5 ± 1.0	25.5 ± 2.9
	RES-EPL-βCD	8.7 ± 1.2	26.5 ± 1.5
40th	CTR-CTR	6.6 ± 0.9	98 ± 26.1
	CTR- EPL	7.6 ± 0.6	72 ± 16.4
	RES-CTR	1.8 ± 1.5*	103 ± 23.7
	RES- EPL	2.7 ± 0.9	101 ± 27.5
	CTR-CTR	9.0 ± 2.5	25.4 ± 1.7
	CTR-EPL-βCD	10.1 ± 1.4	26.0 ± 1.5
	RES-CTR	2.0 ± 0.2*#	16.3 ± 0.6*#
	RES-EPL-βCD	3.2 ± 0.5#	17.7 ± 0.5#

Data are expressed as mean ± SEM. *p < 0.05 compared to CTR-CTR and *p < 0.05 compared to CTR-EPL-βCD (Two-way ANOVA followed by Tukey's post hoc test).

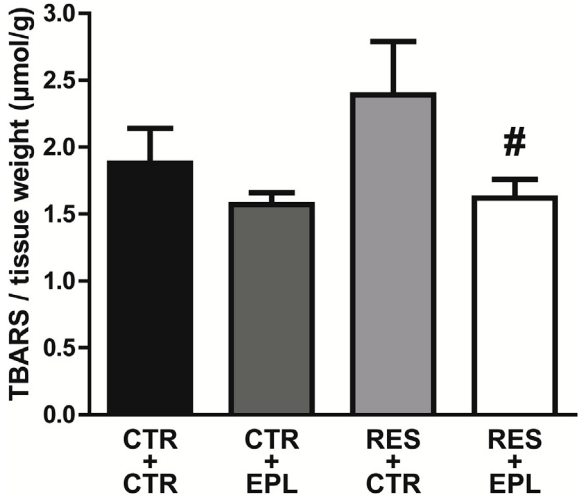

Fig. 6. Effects of repeated administration of EPL (5 mg/kg) and RES (0.1 mg/kg) on TBARS level in the dorsal striatum of mice. Data are expressed as mean ± SEM. #p < 0.05 RES-EPL compared to RES-CTR (Two-way ANOVA followed by Sidak's post hoc test).

depletion (Salamone et al., 1998). In our study, the treatment with RES promoted an increase in the number of vacuous chewing from the 25th day until the end of the treatment. The EPL did not alter the number of vacuous chewing movements. However, EPL-βCD reduced the frequency of this behavior, suggesting again the prevention of the RES-induced motor impairments.

Despite motor symptoms are the signs in which diagnosis is based (Shulman, 2007), studies have shown that non-motor symptoms usually precede those cardinal signs, such as autonomic dysfunctions, sensory alterations, fatigue, neuropsychiatric, sleep and emotional disorders, among others (Garcia-Ruiz et al., 2014). Besides motor assessment, the experimental protocol used in our study included the cognitive and emotional evaluation. Indeed, repeated low-dose treatment with RES allows the detection of the onset of non-motor deficits prior to the motor impairments. Notwithstanding, EPL-βCD (but not EPL) restored short-term memory deficits, because EPL-βCD-treated animals spent more time exploring the new object relative to the familiar object after the 19th day of treatment. Furthermore, repeated administration of EPL-βCD resulted in anxiolytic effect in animals submitted to EPM evidenced by the longer permanence of these animals in the open arms of the apparatus when compared with CTR-CTR group.

Previous studies demonstrated that the β-cyclodextrin is used as a host-guest agent that can improve the solubility and stabilizes the non-polar compounds (such as essential oils and terpenes). Moreover, this procedure can improve absorption by the gastrointestinal tract and enhance the bioavailability, thus successfully favoring the biological effects of guest molecules (Quintans-Júnior et al., 2013; Pragadheesh et al., 2013; Serafini et al., 2012; Menezes et al., 2012; Lima et al., 2016). These aspects explain the different effects observed in the treatments with EPL and EPL-βCD in PD model.

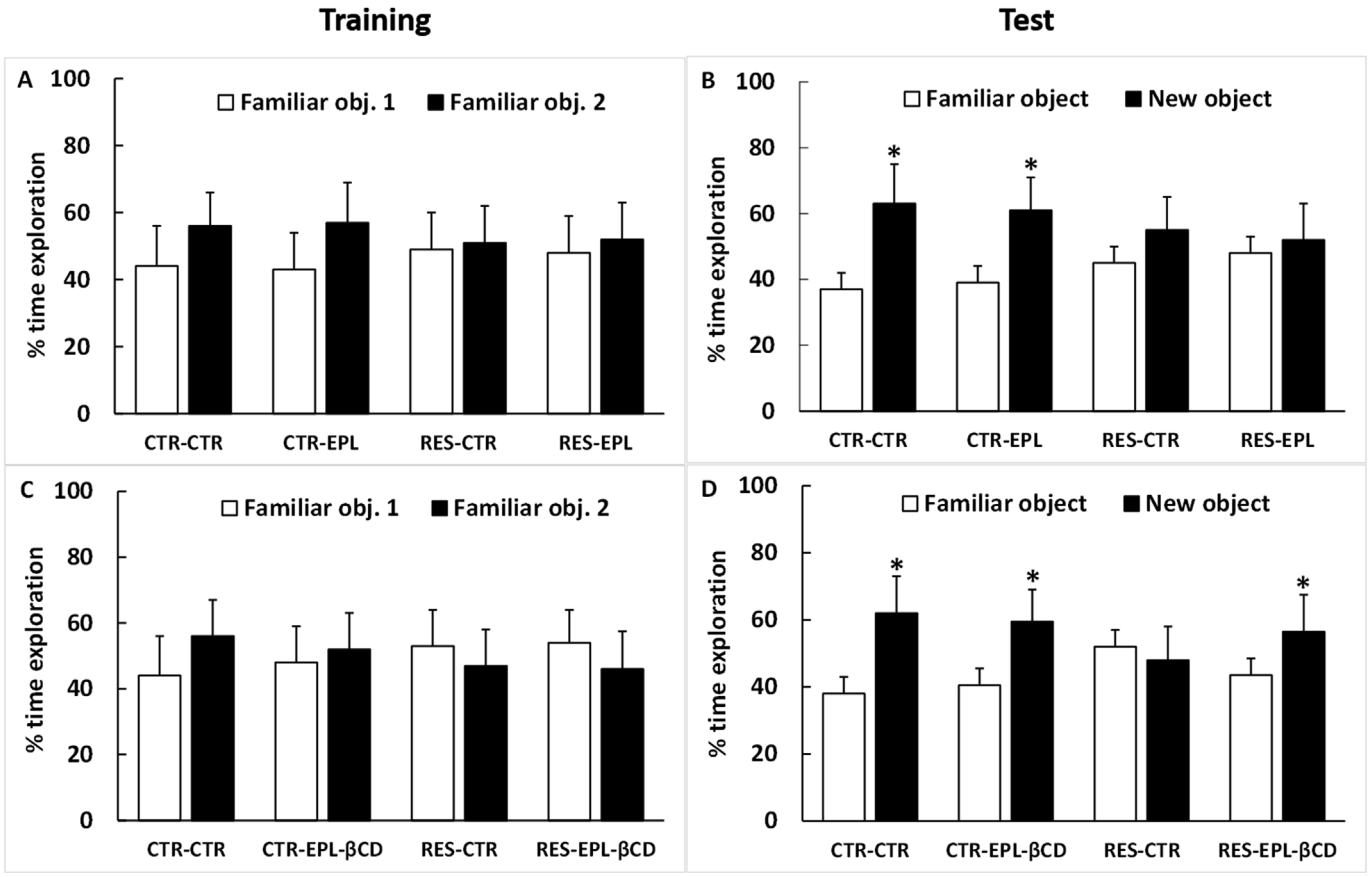

Fig. 7. Effects of administration of EPL (5 mg/kg), EPL-βCD (5 mg/kg) and RES (0.1 mg/kg) on object recognition task of mice on the 19th day. (A,C) training session; (B,D) test session. *p < 0.05 compared to familiar object. (Student's t-test for paired samples).

Table 5
Effects of repeated administration of EPL-βCD (5 mg/kg) and RES (0.1 mg/kg) of mice in EPM.

Treatment	% TOA	Distance (m)	Maximum speed (cm/s)
CTR-CTR	26.1 ± 4.4	11.9 ± 1.4	24.6 ± 2.2
CTR-EPL-βCD	41.9 ± 4.3*#	13.3 ± 1.3#	25.6 ± 1.7
RES-CTR	27.2 ± 3.1	8.8 ± 0.3	23.3 ± 2.5
RES-EPL-βCD	37.3 ± 2.5	9.3 ± 0.8	22.3 ± 1.4

Data are expressed as mean ± SEM. *p < 0.05 compared to CTR-CTR; #p < 0.05 compared to RES-CTR (Two-way ANOVA followed by Tukey's post hoc test). %TOA: percentage of time spent in open arms.

The neuroprotective effect of the EPL and the EPL-βCD may be associated with the antioxidant capacity of this compound (Andrade et al., 2010) and its constituents (de Almeida et al., 2014). Dopaminergic depletion is closely linked to catalepsy and diskynesia (Abílio et al., 2003, 2004), and antioxidant compounds may play an important role in the prevention of neurochemical impairments to dopaminergic neurons induced by reserpine (Sarmento-Silva et al., 2014).

The EPL reduced the production of TBARS, one of the byproducts of the lipid peroxidation process, in the SD of mice treated with RES, suggesting that the protective effect of the oil essential may be related to its antioxidant action. In the study by Abílio et al. (2003) there was an increase in the frequency of tongue protrusion in the oral movements test and the GSSG/GSH ratio after acute RES administration in rats, which was restored after the antioxidant vitamin E treatment. Likewise, in another study, Abílio et al. (2004) described the role of catalase on the resistance to RES induced oral movements, which was impaired by the catalase inhibitor aminotriazole. Additionally, Sarmento-Silva et al. (2014) showed that the administration of alpha-tocopherol (vitamin E) was able to prevent motor and cognitive deficits in rats when subjected to the repeated low-dose reserpine PD model. Thus, these findings corroborate the hypothesis that the protective effects found in our study are related to a possible antioxidant action.

Moreover, the chemotype of *E. fruticosa* assessed here demonstrating that EPL was rich in terpenes: β-caryophyllene (14.78), bicyclogermacrene (14.15), 1,8-cineole (12.09), α-pinene (5.74) and β-pinene (4.47). Several terpenes have action on CNS and can modulate different neurotransmission systems or improve the natural antioxidant action of the nervous tissue that may contribute to PD effects (Gertsch et al., 2008; González-Burgos and Gómez-Serranillos et al., 2012). Additionally, the EPL main compounds, such as α-terpene, β-terpene and linalol, are substances that have yielded great interest due to their broad spectrum of biological activity, including neuroprotection (González-Coloma et al., 2011). Among the most studied terpenes are carotenoids, which inhibit ROS activity by reducing the amount of free radicals (Edge et al., 1997). In addition, studies *in vitro* showed that β-caryophyllene and bicyclogermacrene, also compounds of EPL, seem to act in important neurotransmissions (such as adrenergic, opioid, endocannabinoid and serotonergic systems) (Gertsch et al., 2008; Siqueira-Lima et al., 2017; Quintans-Júnior et al., 2017). In this respect, these terpenes may contribute, at least in part, to the neuroprotective profile, mainly by synergistic actions. In this context, a new research on the effects of isolated compounds from EPL on the RES model is needed to elucidate whether the effects observed in our study are due to a synergistic effect of the constituents of the EPL or whether it is due to specific compounds.

5. Conclusion

Taken together, these findings suggested that EPL have a potential

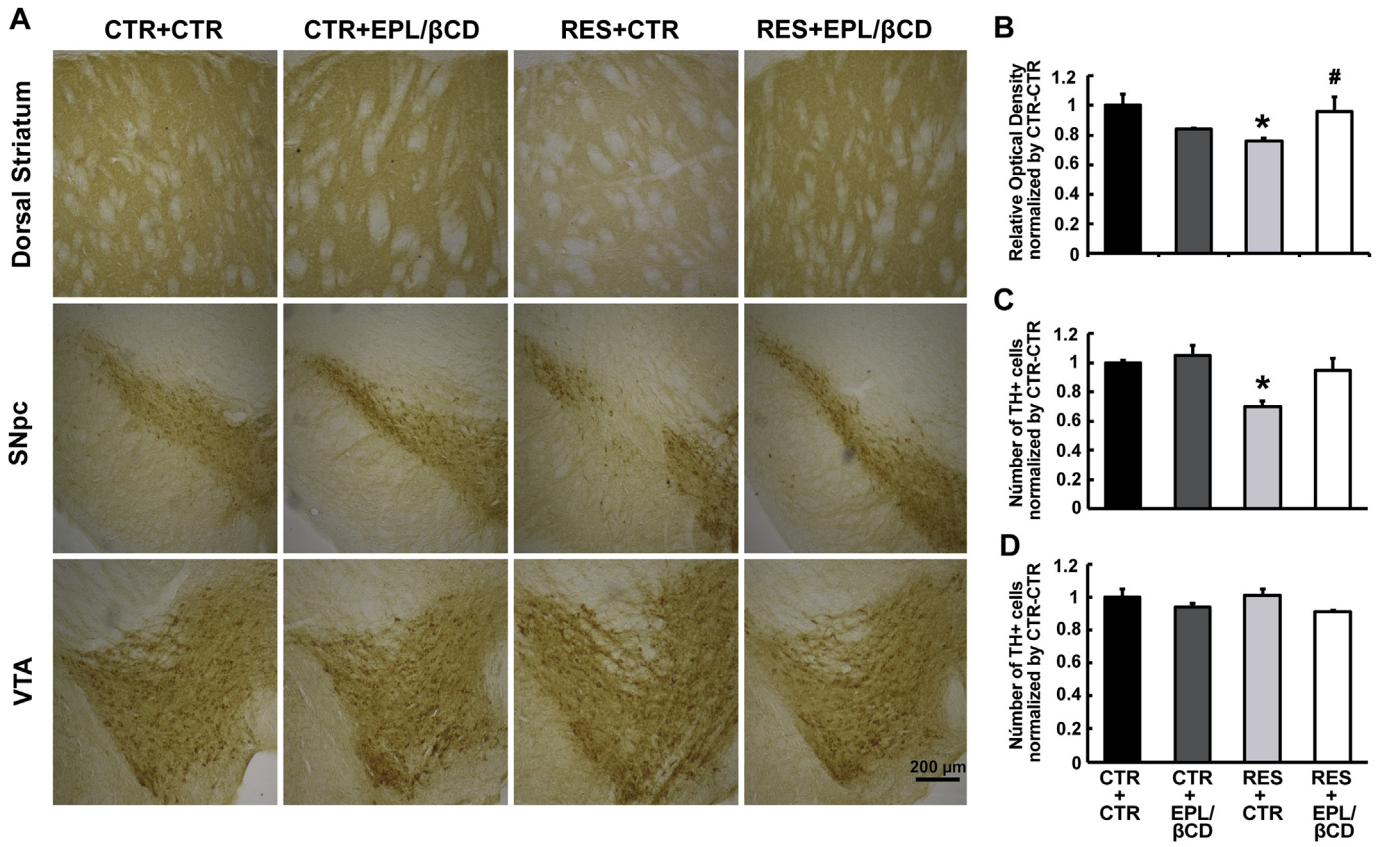

Fig. 8. Effects of repeated administration of EPL-βCD (5 mg/kg) and RES (0.1 mg/kg). (A) Representative photomicrographs of brain coronal sections; (B) TH-immunoreactivity fibers of DS; (C) TH + cells of SNpc; (D) TH + cells of VTA, both normalized by CTR values. Data are expressed as mean ± SEM. *$p < 0.05$ compared to CTR-CTR. $^{\#}p < 0.05$ compared to RES-CTR. (One-way ANOVA followed Tukey's post hoc test).

neuroprotective profile for PD, probably mediated by an antioxidant-related action. Further, our results suggest that EPL-βCD might present an important draft of drug to the study of new compounds for the treatment of PD.

Author contributions

JIABF, AMM and, AMR designed the study. JIABF, AMM, JMMB, JRS, AJOM, PPM, MDC, AASA performed the experiments and collected the data. JIABF, AMM, AHFFL, LJQJ and AMR analyzed data and wrote the manuscript. JRS, LJQJ, RHS and AMR contributed with theoretical discussions and technical insights. JIABF, AHFFL, LJQJ and AMR contributed in analysis and writing, and revised the final manuscript.

Conflicts of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.11.056.

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