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Eucalyptol prevents pilocarpine-induced seizure and neuronal damage in mice, through the cholinergic, monoaminergic and antioxidant pathways

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ABSTRACT

Current antiepileptic drugs can inhibit seizure occurrence but are not effective in preventing its onset. Moreover, they produce several side effects, which may impact the efficacy of the treatment. In addition, it has been stimulating the prospection of new molecules isolated from aromatic plants, with potential anticonvulsant and neuroprotective activities and less side effects. This study aimed to evaluate, though behavioural and neurochemical methodologies, the anticonvulsant, and neuroprotective effects of eucalyptol on mice subjected to the pilocarpine-induced seizure model. Eucalyptol (100, 200 and 400 mg/kg, p.o.) was administered in mice prior to pilocarpine (350 mg/kg, i.p.) and the following behavioural parameters were assessed: Latency to First Seizure (LFS), Seizure Intensity (SI) and Latency to Death (LD). In addition, an oxotremorine-induced tremors test was performed to evaluate the cholinergic system involvement on the eucalyptol effects. Neurochemical tests were also performed, including determination of hippocampal (HC) concentration of thiobarbituric acid reactive substances (TBARS) and nitrite/nitrate and striatal (ST) concentration of noradrenaline, dopamine, and serotonin. Lastly, histopathological, and morphometric hippocampal analysis were conducted. Eucalyptol increased the latency to first seizure and latency to death, inhibited oxotremorine-induced tremors, decreased hippocampal TBARS and nitrite/nitrate overproduction, increased striatal noradrenaline and dopamine levels and prevented hippocampal neurodegeneration. These results demonstrate the potential anticonvulsant, neuroprotective and antioxidant effects of eucalyptol, probably through a conjunction of mechanisms including muscarinic cholinergic antagonism, oxidative stress mitigation and the monoaminergic system modulation, which appears to effectively control the seizure onset.

1. Introduction

Epilepsy is a disease that affects about 70 million people worldwide. It is characterized by a transient, abnormal and synchronous hyperactivity of a population of neurons, generated in response to an imbalance between excitatory and inhibitory neurotransmitters, resulting in recurrent and rhythmic seizures (Brodie et al., 2018). Although there is a wide range of Antiepileptic drugs (AEDs) able to control most epilepsy types, none of them are able to cure neither prevent the epilepsy onset. Additionally, current AEDs often fail in controlling refractory epilepsy and exhibit severe side effects, due to their either intrinsic toxicity or different anticonvulsant drugs association, which negatively impacts the patient's life and treatment adherence (Löscher et al., 2013). New AEDs development should therefore focus on minimizing the toxicity and emerging side effects, thus improving the treatment compliance.

Natural products derived from plants and other sources have been

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increasingly studied over the years. Their numerous compounds, which exhibit unique pharmacological properties, have been widely recognised due to their traditional use in the folk medicine of many countries. These bioactive compounds usually present less toxicity and fewer side effects when compared to currently available drugs, making them desired characteristics for any drug candidate (Nóbrega de Almeida et al., 2011a). Their pharmacological activities are wide-ranging and include, but are not limited to, cancer (Fernández et al., 2021; Lenzi et al., 2018; Mitra et al., 2022), pain-related conditions (Chy et al., 2021; Ferrarini et al., 2022; Freitas et al., 2021), asthma and inflammation (Cerqua et al., 2022), and diabetes (Wang et al., 2022). Thus, it is paramount to explore the medicinal properties of natural products with the aim the developing of novel and lass harmful drugs.

In this context, essential oils and their constituents such as terpenes (the largest compound group with CNS activity) have been extensively studied demonstrating relevant anxiolytic, antidepressant, analgesic and anticonvulsant properties (Nóbrega de Almeida et al., 2011b; Passos et al., 2009). Eucalyptol is a monoterpene present as a major constituent in several essential oils and has been used as a food flavouring agent in different processed products such as soft candy, frozen dairy and non-alcoholic beverages (De Vincenzi et al., 2002) as well as an effective natural food preservative (Boukhatem et al., 2020). Moreover, regarding its medicinal properties, it has been reported to produce neuroprotection against pentylenetetrazol-induced seizure models, CNS depressant activity (de Figuêiredo et al., 2019), inhibition of autonomic neurons excitability (Ferreira-da-Silva et al., 2009) and exerts a significant antioxidant and anti-inflammatory effect (Ciftci et al., 2011; Méndez-Armenta et al., 2014; da Fonsêca et al., 2019). Therefore, it is plausible and highly relevant to investigate the effectiveness of eucalyptol in protecting the CNS from epileptogenesis.

Pilocarpine (PILO) is a muscarinic M1-receptor agonist which produces limbic motor seizures and neuronal damage, followed by status epilepticus (SE) (Turski et al., 1983). The PILO-induced seizures are followed by a latency period without seizures in whitch significant changes occur in different brain areas, but more prominently in the hippocampus (CA1, CA3 subregions), where an epileptic circuitry is generated in response to the seizure-induced neurodegeneration, resulting in the epileptogenesis establishment (Thom et al., 2009).

Several neuronal abnormalities may arise directly and/or indirectly from the PILO-induced seizure. A direct neuronal damage after seizure discharges induces neurodegeneration in the regions in which it occurs. This fist damage produces a cascade of biochemical events such as oxidative stress (induced mainly by lipid peroxidation and excessive formation of reactive oxygen/nitrogen species) (Freitas et al., 2005; Geronzi et al., 2018), monoaminergic (NA, DA and 5-HT) imbalance (de Freitas et al., 2005; Ghasemi & Mehranfard, 2018) and glutamatergic excitotoxicity (Barker-Haliski & White, 2015) which indirectly leads to neurodegeneration in different brain areas including hippocampus and striatum. Conjointly, these biochemical pathways contribute to limbic circuitry reorganization thus consolidating the epilepsy. Therefore, in the present study, we aimed to investigate whether the eucalyptol exhibits anticonvulsant, neuroprotective activities, and its underlying mechanism of action, on mice subjected to the PILO-induced seizure model.

2. Materials and methods

2.1. Animals and ethical aspects

Adult male Swiss albino mice (age: 6–8 weeks, weight: 25–30 g) from the vivarium of the Universidade Regional do Cariri – URCA, were maintained in polypropylene cages under controlled environmental conditions of temperature (23 ± 2 °C) and light (12/12 light/dark cycle) with food and water *ad libidum*. The animals were acclimatized in the experimentation room 24 h before experimental procedures. All experimental protocols were executed according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) following the 3Rs (Replace, Reduce, Refine) ethical principles of animal handling and were approved by the Ethics Committee for Animal Use – ECAU/URCA, under the registration number #n° 00113/2019.2.

2.2. Drugs and reagents

Eucalyptol (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane) 99%, oxotremorine (OXO), Atropine sulphate (ATR), pilocarpine (PILO) and Polyoxyethylene Sorbitan Monooleate (Tween 80) were purchased from Sigma Chemical, Co, (USA). Diazepam (DZP) was acquired from União Química® (Brazil). All drugs were dissolved in saline (NaCl 0.9%) and administered orally (p.o., by gavage) or intraperitoneally (i.p.) at a volume of 0.1 ml/10 g. Eucalyptol and saline solutions were emulsified in Tween 80 (0.5%) purchased from Sigma Chemical, Co, (USA).

2.3. Acute toxicity test

The acute toxicity test was performed according to the Organization for Economic Cooperation and Development (OECD) (O.f.E. Co-operation, 2002). A group of mice (n = 3) was used for behavioural evaluations, using fixed doses (2000, 300, 50, 5 mg/kg, p.o.) in each phase of the test. The test started with 2000 mg/kg orally administered and the mice were observed for 10, 30, 60 120, 180 and 240 min for 14 consecutive days. If 0–1 animal dies, the test is terminated, and the toxic dose is considered greater than or equal to the respective dose tested. If 2–3 animals die, the test proceeds to the lowest consecutive dose until the last dose (5 mg/kg). If 2–3 animals die, the compound is considered highly toxic, and the experiment should be discontinued. The eucalyptol has low non-clinical acute oral toxicity and its estimated LD50 is greater than 2000 mg/kg. Therefore, doses corresponding to 5, 10 and 20% (100, 200 and 400 mg/kg v.o.) of the estimated lethal dose were used. Another study has reported an oral toxicity equal to 3849 mg/kg (Xu et al., 2014).

2.4. Behavioural tests

2.4.1. Pilocarpine-induced seizure model

The pilocarpine-induced seizure model was established as previously described by Turski, Cavalheiro, Schwarz, Czuczwar, Kleinrok and Turski (Turski et al., 1983). Briefly, mice were divided into five groups (n = 8): saline group (SAL): pre-treated orally with saline (0.9% v/v); eucalyptol groups (E100, E200 and E400): pre-treated with eucalyptol (100, 200 and 400 mg/kg, p.o., respectively); diazepam group (DZP2): pre-treated intraperitoneally with diazepam (2 mg/kg, i.p.). Sixty minutes after saline or eucalyptol administrations and 30 min after diazepam administration, the groups received pilocarpine (350 mg/kg, i.p.; PILO350) injection. Subsequently, direct observation of each animal was conducted for 60 min to analyse the following behavioural parameters: Latency to first seizure (LFS), seizure intensity (SI) and Latency to Death (LD). The SI parameter was evaluated as described by Racine (Racine, 1972) and modified by Itzhak and Martin (Itzhak & Martin, 2000). Shortly, it consists of a 5-stage observation scale: stage 1, normal behaviour; stage 2, hyperactivity; stage 3, repeated 'vertical' movements which may represent stereotypical-like behaviour; stage 4, forelimb clonus and rearing; and stage 5, full motor seizures.

2.4.2. Oxotremorine-induced tremors test

The oxotremorine-induced seizure test was performed according to Fukuzaki, Kamenosono and Nagata (Fukuzaki et al., 2000). Briefly, mice were divided into five groups (n = 8): saline 0.9% (naïve) group (CONT); eucalyptol groups (E100, E200 and E400) and atropine group (ATR10): treated with atropine (10 mg/kg, i.p.). Eucalyptol or saline were administered to mice 60 min prior, while atropine was administered 30 min prior to oxotremorine (0.5 mg/kg, i.p.) injection. The tremors in

each animal were scored visually at 10, 20, and 30 min after the oxotremorine administration using a rating scale of 0–3. 0 = no tremor; 1 = occasional isolated twitches; 2 = moderate or intermittent tremor associated with short periods of quiescence; 3 = pronounced continuous tremor.

2.5. Neurochemical assays

For the neurochemical studies, the pilocarpine-induced seizure model was performed as described previously. It was used 3 groups (n = 8): SAL and E400 (PILO350-treated groups) and CONT (saline-treated only group). The E400 dose was utilized due to its better performance in the behavioural tests. After the seizure evaluation period, mice were euthanized by decapitation in order to rapidly remove their brains, which were placed on ice to dissect the hippocampus and striatum areas for further neurochemical analysis.

2.5.1. Determination of TBARS concentration

As an index of lipid peroxidation, we used the formation of thiobarbituric acid reactive substances (TBARS), which is widely adopted as a sensitive method for measurement of lipid peroxidation and oxidative stress, as previously demonstrated by Draper and Hadley (Draper & Hadley, 1990). In short, a potassium chloride buffer solution (1.15%) homogenate (10% w/v) was prepared from the HC areas. A 50 μL aliquot from the homogenate was removed and mixed with 200 μL of trichloroacetic acid (10%) and 200 μL of thiobarbituric acid (0.6%), then heated in a boiling water bath (95–100 °C) for 15 min. After cooling, the samples were centrifuged (4000 rpm/5 min). Then, 100 μL aliquots were removed and the TBARS were determined by spectrophotometry at 535 nm. The results were expressed as μmol of malondialdehyde (MDA)/g of tissue.

2.5.2. Determination of nitrite concentration

The nitric oxide metabolites content was determined using the methodology described by Green, Wagner, Glogowski, Skipper, Wishnok and Tannenbaum (Green et al., 1982). Briefly, A homogenate (10% w/v) was prepared from the HC area with potassium chloride (1.15%) buffer solution, which were centrifuged (4000 × rpm/10 min). 100 μL of the supernatant was incubated with 100 μL of Griess reagent (0.04 g/ml) at room temperature for 10 min. The nitrite content was measured by spectrophotometry at 570 nm. Nitrite concentration was determined from a standard nitrite curve generated using $NaNO_2$ and the results were expressed in μmol/g of tissue.

2.5.3. Determination of monoamine concentration

Striatum areas were homogenized (10% w/v) in perchloric acid (0,1 M), centrifuged (15.000 rpm/30 min at 4 °C), dialyzed and kept at 4 °C until analysed. The dialyzed samples (20 μL) were analysed for NA, DA, and 5-HT by HPLC (Shimadzu corp.) with electrochemical detection. The monoamine separation was achieved using a Shim-pack CLC-ODS (M)® C18 Column (3 μm, 4.6 mm diameter, 25 cm long) with a mobile phase composed of a citric acid (0.163 M, pH 3.0) buffer solution, octanesulfonic acid sodium (0.69 M; SOS), acetonitrile (4% v/v) and tetrahydrofuran (1.7% v/v). The monoamines were electronically detected using an amperometric detector (model L-ECD-6A, Shimadzu) by oxidation on a glassy carbon electrode at 0.85 V relative to an Ag–AgCl reference electrode. The concentration of neurotransmitters in the supernatant solutions were calculated by comparing their peak height with those of standards determined on the same day. Results were reported as μg/g of tissue.

2.6. Histopathology

Three mice groups (n = 8): naïve (no treatment received), E400 (best dose observed in the other protocols) and SAL (saline 0.9% + PILO350). E400 and SAL groups were subjected to PILO350-induced seizure model. Subsequently, hippocampal tissue samples were collected, and immersion fixed in 10% buffered paraformaldehyde (pH = 7.4), embedded in paraffin wax, and cut into 4 μm-thick sections. For histopathological examination, paraffin sections were placed on glass slides, deparaffinised, and stained with Mayer's haematoxylin and eosin. The obtained sections of bilateral hippocampal CA1, CA3 areas were subjected to morphometric quantification using optical microscopy (300 μm). The morphometric results were expressed as the number of neurons/μm2 of tissue.

2.7. Statistical analysis

The results that presented a normal distribution were represented as mean ± Standard Error of the Mean (SEM) and analysed by one-way and two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls and Bonferroni tests, respectively. The results that did not present a normal distribution were represented as median (25th percentile–75th percentile) and analysed by Kruskal–Wallis, followed by Dunns test. In both cases, the results were considered significant when $P < 0.05$.

3. Results

3.1. Pilocarpine-induced seizure model

Eucalyptol effects in the PILO350-induced seizure model are presented in Fig. 1. E100, E200 and E400 groups showed a significant increase in LFS (242, 220.28 and 290.04%, respectively; $F_{4,35} = 14.85$; $p < 0.0001$; Fig. 1A) and LD (143.49, 193.9 and 102.04%, respectively; $F_{4,35} = 21.44$; $p < 0.0001$; Fig. 1C) parameters when compared with the SAL groups. The eucalyptol groups did not show statistically significant differences in the IS (*Kruskal-Wallis statistic* = 17.52, p = 0.0015; Fig. 1B) parameter when compared with the SAL group. For all parameters assessed, the groups treated with eucalyptol did not significantly differ from each other, except for the E400 group in the LD ($p < 0.0401$) parameter. Moreover, as expected, none of the DZP2 group animals presented seizure or died during the observation period.

3.2. Oxotremorine-induced tremors test

Eucalyptol effects in the oxotremorine-induced tremors are presented in Fig. 2. E100, E200 and E400 doses reversed significantly ($F_{8, 105} = 4.47$, interaction p = 0.0001) the tremors at 10 min (41.7, 25 and 62.5%, respectively), 20 min (33.3, 33.3 and 28.6%, respectively) and 30 min (35, 2 and 25%, respectively) when compared with the CONT group, which presented the maximum tremor stage at all doses tested and evaluation time. The ATR10 (a muscarinic M1 receptor antagonist) dose, as expected, inhibited the tremors of all animals tested (tremor stage 0).

3.3. Neurochemical assays

3.3.1. Determination of TBARS and nitrite content

Eucalyptol effects on TBARS and nitrite concentration in mice hippocampus after PILO350-induced seizure are presented in Fig. 3. SAL group showed a significant increase in TBARS content (169%; $F_{2, 21} = 9.48$; $p < 0.0001$; Fig. 3A) when compared with the CONT group. The E400 dose decreased TBARS levels (65%; $F_{2, 21} = 9.48$; $p < 0.0001$; Fig. 3A) when compared with SAL group. On the other hand, Nitrite levels increased (199%; $F_{2, 21} = 61.8$; $p < 0.0001$; Fig. 3B) in the SAL group when compared with CONT group, whereas the E400 dose significantly diminished nitrate levels (49%; $F_{2, 21} = 61.8$; $p < 0.0001$; Fig. 3B) when considering the SAL group.

3.3.2. Determination of monoamine content

Eucalyptol effects on monoamine (NA, DA, and 5-HT) levels in mice

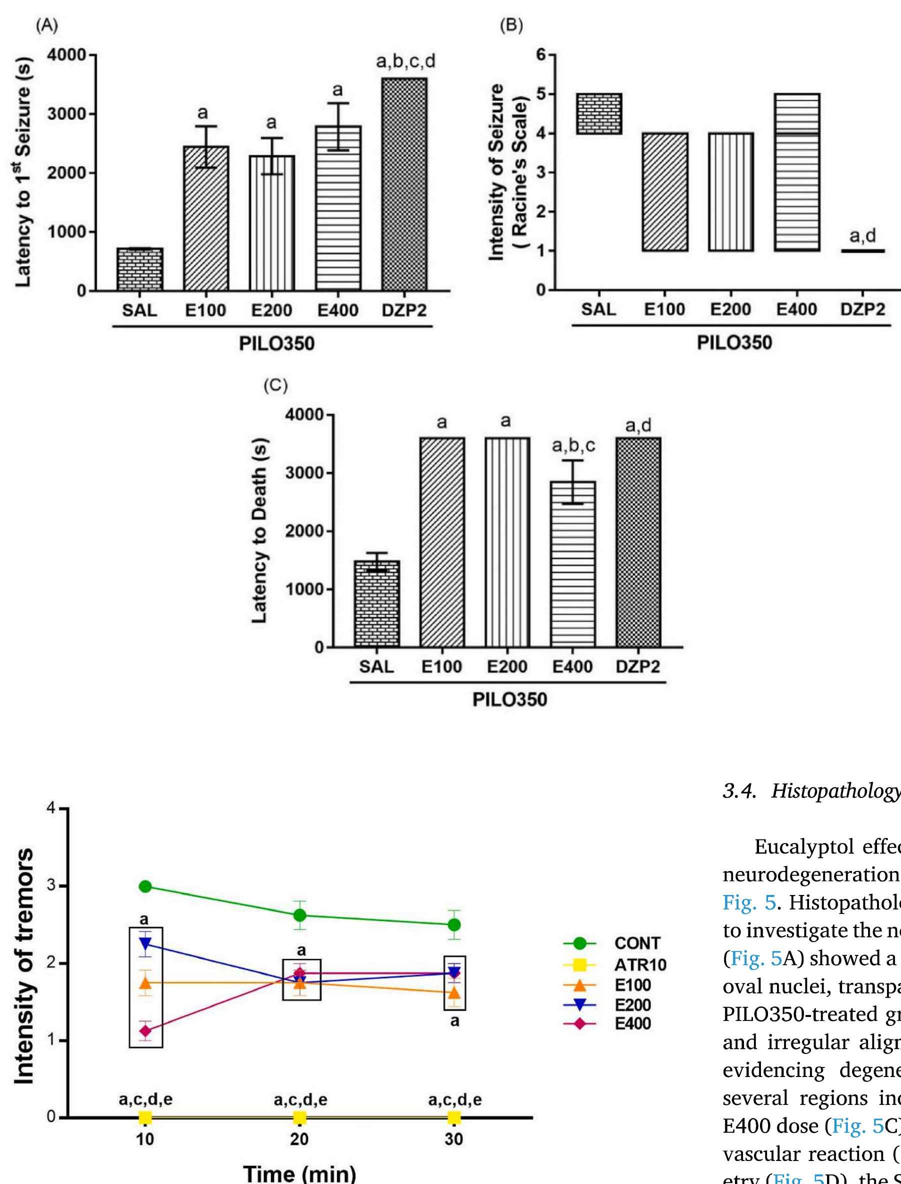

Fig. 1. Effects of eucalyptol treatment on Latency to First seizure (A), Intensity of Seizure (B) and Latency to Death (C) parameters in the pilocarpine-induced seizure test (n = 8). Values were shown as the mean ± S.E.M (Stander Error Mean) for (A) and (C), and median, minimum, and maximum for (B). They were analysed by ANOVA (A and C) followed by *Student-Newman-Keuls (post hoc)* or *Kruskal-Wallis* (B) followed by *Dunn's (post hoc)*. Significant values were expressed as "a", "b", "c" and "d" vs SAL, E100, E200, E400 and DZP2, respectively, when $p < 0.05$. The statistical date is presented for LFS ($F_{4,35} = 14.85$; $p < 0.001$), LD ($F_{4,35} = 21.44$; $p < 0.001$) and IS (*Kruskal-Wallis statistic* = 17.52; p = 0.0015). SAL = Saline 0.9%, E100 = Eucalyptol 100 mg/kg, E200 = Eucalyptol 200 mg/kg, E400 = Eucalyptol 400 mg/kg, DZP2 = Diazepam 2 mg/kg, PILO350 = Pilocarpine 350 mg/kg, LFS = Latency to First seizure, SI = Seizure Intensity and LD = Latency to Death.

Fig. 2. Effects of eucalyptol on oxotremorine-induced tremors (n = 8). Values were shown as the mean ± S.E.M (Stander Error Mean), analysed by two-way ANOVA followed by *Student-Newman-Keuls (post hoc)*. Significant values were expressed as "a", "b", "c", "d" and "e" vs CONT, E100, E200, E400 and ATR10, respectively, when $p < 0.05$. The statistical date is presented for intensity of tremors ($F_{8,\ 105} = 4.47$; p = 0.0001). CONT = Saline 0.9% (naïve), E100 = Eucalyptol 100 mg/kg, E200 = Eucalyptol 200 mg/kg, E400 = Eucalyptol 400 mg/kg, ATR10 = Atropine 10 mg/kg, OXO = Oxotremorine 0.5 mg/kg (Figure in colour). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

striatum after pilocarpine-induced seizure are presented in Fig. 4. The SAL groups presented a significant decline in the levels of NA, DA, and 5-HT (70, 90.5 and 66%; respectively; %; $F_{2,\ 15} = 22.07$, $F_{2,\ 15} = 20.5$ and $F_{11,\ 60} = 37.85$ respectively; $p < 0.0001$; Fig. 4A, B and C) when compared with their respective CONT groups. The E400 dose induced a significant rise in NA and DA levels (73.2%; $F_{2,\ 15} = 22.07$ and 482.1%; $F_{2,\ 15} = 20.5$ respectively; $p < 0.0001$ for both monoamines; Fig. 4A and B), when compared with the SAL group. Contrastingly, E400 dose induced an even greater decrease in 5-HT levels (48.70%; $F_{11,\ 60} = 37.85$; $p < 0.0001$; Fig. 4C) when compared with the SAL group.

3.4. Histopathology

Eucalyptol effects on PILO350-induced hippocampal CA1 and CA3 neurodegeneration and the cell morphometry analyses are shown in Fig. 5. Histopathological studies in hippocampal areas were performed to investigate the neuroprotective effects of eucalyptol. The CONT group (Fig. 5A) showed a regular cell alignment and intact structures, round or oval nuclei, transparent cytoplasm, and no vascular reaction. However, PILO350-treated group (Fig. 5B) presented profound neuronal damage and irregular alignment and loss of neurons and its continuities (*), evidencing degenerative process followed by vascular reaction in several regions including CA1 (arrowhead). The group treated with E400 dose (Fig. 5C), showed preserved and aligned neurons and a slight vascular reaction (arrowhead). Regarding the neuronal cell morphometry (Fig. 5D), the SAL group showed a significant decline in the number of preserved neurons (31.8%, $F_{2,\ 57} = 168.8$; $p < 0.0001$) after PILO350 administration when compared with CONT group (naïve). Contrastingly, the E400 group raised the number of preserved neurons (28.9%, $F_{2,\ 57} = 168.8$; $p < 0.0001$) when compared with the SAL group.

4. Discussion

The investigation of natural products, as a direct source of therapeutic agents, have yielded new insights into potential agents that may exhibit less toxicity and harmfulness compared with current AEDs (da Fonsêca et al., 2019; Ekstein & Schachter, 2010). New treatments for epilepsy should focus on preventing its onset as well as ameliorating the patient's response and tolerance to the treatment. Eucalyptol is a monoterpene which has been used as a flavouring agent by the food and pharmaceutical industries and has been reported to exhibit pharmacological properties on the CNS such as neuroprotection, CNS depression (de Figuêiredo et al., 2019), antioxidant and anti-inflammatory (Ciftci et al., 2011; Méndez-Armenta et al., 2014; da Fonsêca et al., 2019) activities. In the present study, we investigated the anticonvulsant, neuroprotective effects of eucalyptol against PILO-induced seizures in mice.

Initially, eucalyptol increased LFS and LD parameters at all doses tested after PILO injection, thus delaying seizure onset, and improving the animal's survival rate; All eucalyptol doses effectively prevented

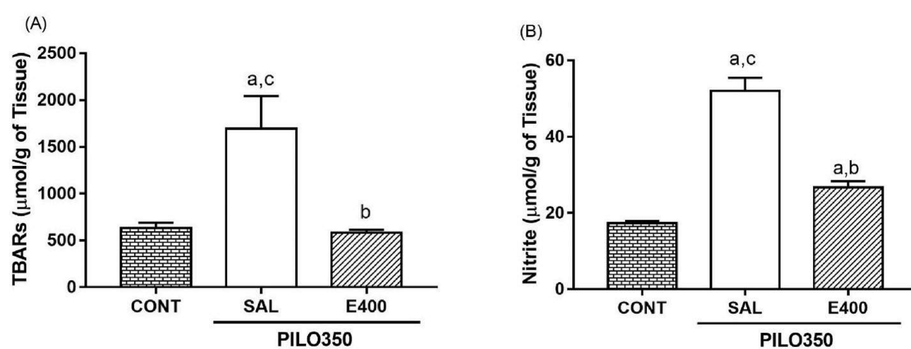

Fig. 3. Effects of eucalyptol treatment on lipide peroxidation (TBARS) (A) and nitrite content (B) in mice hippocampus after pilocarpine-induced seizure ($n = 8$). Values were shown as the mean ± S.E.M (Stander Error Mean), analysed by ANOVA followed by *Student-Newman-Keuls (post hoc)*. Significant values were expressed as "a", "b" and "c" vs CONT, SAL and E400, respectively, when $p < 0.05$. The statistical date is presented for TBARS ($F_{2, 21} = 9.48$; $p < 0.0001$) and nitrite ($F_{2, 21} = 61.8$; $p < 0.0001$). CONT = Saline 0.9% (naïve), SAL = Saline 0.9% + Pilocarpine 350 mg/kg, E400 = Eucalyptol 400 mg/kg, PILO350 = Pilocarpine 350 mg/kg.

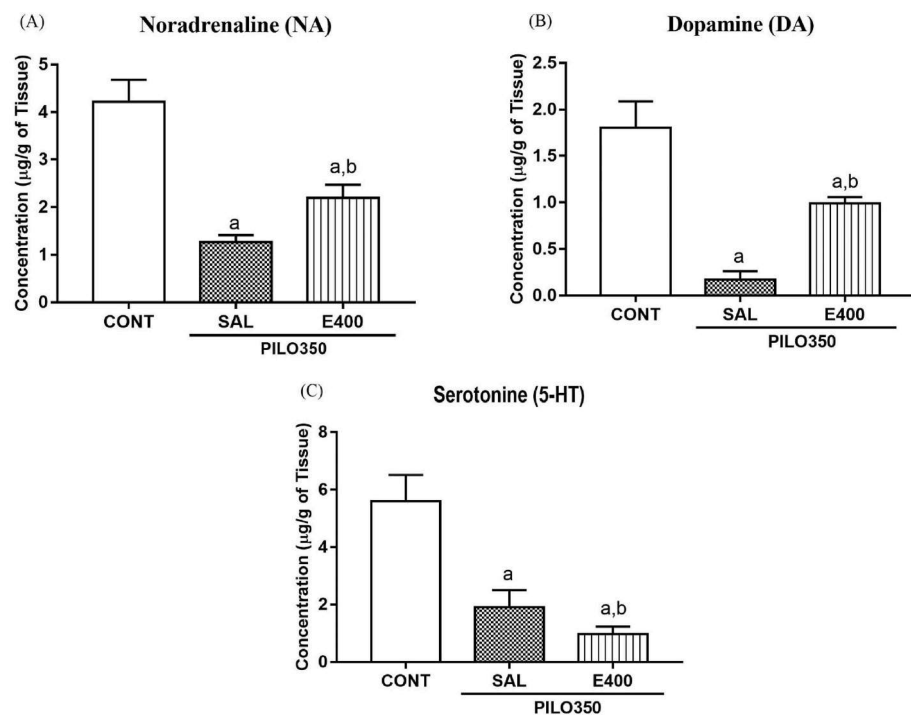

Fig. 4. Effects of eucalyptol treatment on monoamine (NA, DA, and 5-HT) levels in mice striatum after pilocarpine-induced seizure ($n = 6$). Values were shown as the mean ± S.E.M (Stander Error Mean), analysed by ANOVA followed by *Student-Newman-Keuls (post hoc)*. Significant values were expressed as "a" and "b" vs CONT and SAL, respectively, when $p < 0.05$. The statistical date is presented for NA ($F_{2, 15} = 22.07$), DA ($F_{2, 15} = 20.5$) and 5-HT ($F_{11, 60} = 37.85$) $p < 0.0001$ for all the monoamines. CONT = Saline 0.9% (naïve), SAL = Saline 0.9% + Pilocarpine 350 mg/kg, E400 = Eucalyptol 400 mg/kg, PILO350 = Pilocarpine 350 mg/kg.

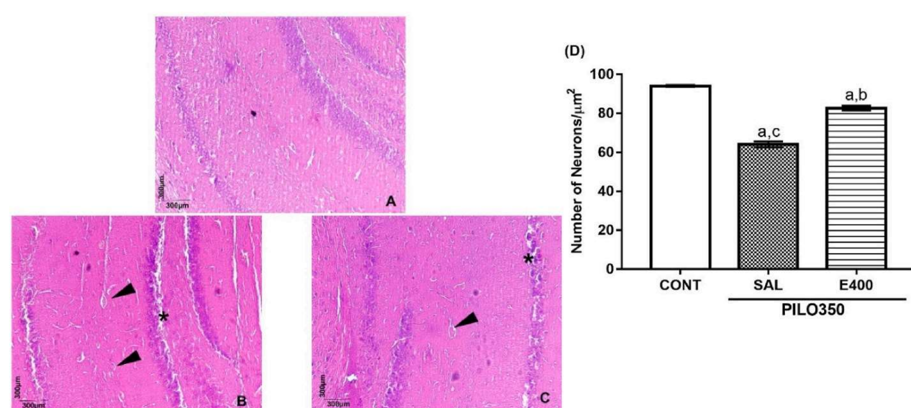

Fig. 5. Effects of eucalyptol on mice hippocampus and cell morphometry analysed in histopathological sections after PILO350-induced neurodegeneration. (A) shows preserved neurons in all regions analysed, compatible with normality. (B) presents neuronal degeneration continuities loss (*), followed by a vascular reaction in CA1 (arrowhead) in mice treated only with PILO. (C) shows preserved neurons and a slight vascular reaction (arrowhead) in mice treated with E400. (D) The morphometric results were shown as the mean ± S.E.M (Stander Error Mean), analysed by ANOVA followed by *Student-Newman-Keuls (post hoc)* and the statistical date is presented for the morphometry ($F_{2, 57} = 168,8$) and $p < 0.0001$. Significant values were expressed as "a", "b" and "c" vs CONT, SAL and E400 respectively, when $p < 0.05$. CONT = Saline 0.9% (naïve), SAL = Saline 0.9% + Pilocarpine 350 mg/kg, E400 = Eucalyptol 400 mg/kg, PILO350 = Pilocarpine 350 mg/kg.

mice's death. Although eucalyptol did not decrease SI, it showed a likely tendency to ameliorate seizure severity, as none of the animals presented generalized tonic-clonic seizures. These results suggest that eucalyptol may produce an anticonvulsant and more notably a neuroprotective effect, probably by modulating directly and/or indirectly the cholinergic system. A recent published study shows that the essential oil

of *Hyptis martiusii* Benth., and its isolated major constituent (eucalyptol) also decreased the lethality rate, the LFS and displayed a CNS depressant effect after pentylenetetrazole-induced seizure model (de Figuêiredo et al., 2019).

To investigate whether the eucalyptol activity involves the cholinergic system, the oxotremorine-induced tremors test was performed. Oxotremorine acts selectively on muscarinic cholinergic M_1-receptors in the CNS and peripheral inducing tremors (Fukuzaki et al., 2000). Eucalyptol effectively reduced the tremors probably by antagonizing the oxotremorine activity at the M_1-receptor (similarly to ATR10), thus, inhibiting the onset of tremors through the cholinergic system. Therefore, considering the protective effects of eucalyptol in the behavioural tests and the reduction of tremor intensity, eucalyptol may display a neuroprotective and anticonvulsant activity possibly by modulating the muscarinic cholinergic system likely via M_1-receptor antagonism. These eucalyptol activities have never been described before.

The neuroprotective effect of eucalyptol is evidenced by a histopathological analysis. In the PILO350-treated group, neuronal damage was observed in different hippocampal areas, especially in CA1. Contrastingly, eucalyptol attenuated hippocampal neurodegeneration, evidenced by the increased number of preserved neurons. It is known that cell loss in hippocampal CA1 and CA3 areas is a common pathological finding in TLE and an essential process in the epileptogenesis (Thom et al., 2009), due to their high neuroplasticity, susceptibility to injuries and uncontrolled growth of mossy fibres towards hippocampal cells after PILO administration (Rao et al., 2006). Therefore, these results indicate that eucalyptol acts protecting hippocampal neurons from the PILO350-induced neurodegeneration, consequently, inhibit the seizure onset arising from the hippocampus.

Based on this and considering the dose that performed better in the behavioural tests, the mice hippocampal areas were used to assess the influence of eucalyptol on TBARS and nitrite formation, which are commonly associated with oxidative stress. Our findings showed that PILO350-induced seizure increased TBARS levels and consequently lipid peroxidation. This result agrees with other studies that showed the neuronal cells' vulnerability to lipid peroxidation products which induce irreversible damage of membrane phospholipid thus contributing to epileptogenesis (Ambrogini et al., 2019; Freitas et al., 2005). Interestingly, eucalyptol pre-treatment significantly diminished the levels of TBARS in the hippocampus after PILO350 injection, demonstrating an antioxidant potential of eucalyptol. Previously, it has been revealed that eucalyptol not only promotes the reduction of TBARS and ROS, but also increases the antioxidant enzyme complex activity responsible for ROS and peroxides degradation (Ciftci et al., 2011; da Fonsêca et al., 2019).

After PILO350-induced seizure there is a consequent overproduction of NO and its metabolites which overwhelm the enzymatic and nonenzymatic antioxidant systems (Méndez-Armenta et al., 2014). Our findings demonstrated that PILO350 administration increased the levels of nitrite/nitrate in mice hippocampus, indicating that these free radicals are possibly associated with the seizure onset. These results corroborate with those found by Freitas (Freitas, 2009) who revealed that PILO administration increases the levels of nitrite after tonic-clonic seizure induced by PILO. In contrast, eucalyptol diminished the hippocampal levels of nitrite/nitrate, possibly reducing the oxidative stress and hippocampal neurodegeneration observed in the histopathological assay. A previous study suggested that eucalyptol can inhibit directly and independently superoxide (H_2O_2) and peroxide (O_2) anions, and partially modulate the activity of the superoxide dismutase (SOD) enzyme (Juergens et al., 2018). Therefore, it may be inferred that a possible mechanism by which eucalyptol prevent neurodegeneration after PILO-induced neuronal damage, is effectively controlling the oxidative stress (decreasing free radical and ROS levels) and modulating the antioxidant enzyme complex, thus preventing the hippocampal damage and seizure onset.

Monoamines such as NA, DA and 5-HT are fundamental regulators in the CNS and have been suggested to play a central role in epileptogenesis. However, neither the relative significance of monoamines individually nor conjointly has yet been fully understood, due to the great complexity of the monoaminergic systems and the diversity of epileptic syndromes which can vary in mechanisms (SvobStrac et al., 2016).

NA may expresses both anti- and proconvulsant activities, depending on its extracellular availability and receptor distribution throughout the brain regions (Ghasemi & Mehranfard, 2018). In our study, PILO350-induced seizure decreased NA levels in mice striatum, which were significantly reversed by eucalyptol pre-treatment. NA is a relevant limbic seizure regulator due to its high concentration in the limbic region; this high concentration has been used to treat medically refractory epilepsy through Vagus Nerve Stimulation (VNS) in mice hippocampus to reduce the severity and duration of seizure (Raedt et al., 2011). Thus, it is plausible to infer, since the striatum is also rich in noradrenergic neurons, that PILO350 may decrease striatal NA levels contributing to seizure onset. Furthermore, the NA increased availability, after eucalyptol administration, may stimulate postsynaptic α_2-adrenergic receptors in glutamatergic neurons, thereby suppressing glutamate (excitatory neurotransmitter) release and consequently preventing hyperexcitability and neurodegeneration (Xiao et al., 2009). Moreover, decreased expression of α_2-receptors may increase seizure susceptibility (Ghasemi & Mehranfard, 2018), suggesting that α_2-adrenergic receptor activation might prevent seizures at NA basal levels. Therefore, the increased NA levels stimulated by eucalyptol may produce striatal neuroprotection.

The dopaminergic system also appears to be involved in the limbic epilepsy onset process, however, promoting opposite effects depending on the DA receptor involved and the brain region in which they are activated. This DA opposing activity has been observed in different studies markedly suggesting that signalling from D_1 receptors is predominantly pro-epileptogenic while D_2 receptors signalling exhibit antiepileptogenic activity (Bozzi & Borrelli, 2013; Tripathi & Bozzi, 2015). Our findings demonstrate that PILO350 administration diminished DA levels in mice striatum after seizure, indicating a DA-related activity in the limbic seizure onset through the dopaminergic muscarinic receptor overstimulation. In addition to the DA levels drop observed in this study, PILO administration was reported to lower D_1 and D_2 receptors densities and elevate the D_2 dissociation constant (K_d) in mice striatum, thus lowering the DA affinity for D_2 (Nascimento et al., 2005). Both effects implicate in less inhibitory activity through D_2 activation, which could decrease the striatal hyperexcitability and prevent the seizure development in striatum. Eucalyptol pre-treatment significantly elevated the DA concentration in mice striatum after seizure, thus it is plausible to suggest that eucalyptol may display neuroprotection by elevating the DA levels and/or preventing the DA-receptors downregulation, when considering the high dopaminergic activity in the striatum and the importance of the DA levels in controlling seizure onset. However, more in-depth studies should be carried out to better understand the role of DA in the seizure process and the modulation of DA levels by eucalyptol.

Although the role of 5-HT in epileptogenesis remains unclear, there are evidences that 5-HT exerts anticonvulsant and proconvulsant effects mediated by 5-TH_1 and 5-HT_2 receptors stimulation respectively, but these effects depend on the receptor subtype (SvobStrac et al., 2016). Our results showed a significant decline in 5-HT levels in mice striatum after PILO350 injection, indicating that 5-HT is involved in the development of the limbic seizure induced by cholinergic overstimulation. This drop in 5-HT levels may induce the seizure's maintenance since 5-HT acts inhibiting the cholinergic stimulation through 5-HT_{1A} receptor activation (SvobStrac et al., 2016). In addition, decreased 5-HT levels in striatum can also produce a downregulation of the 5-HT_1 receptor, which is known to produce an anticonvulsant activity (de Freitas et al., 2005). Interestingly, eucalyptol produced an even greater decrease in 5-HT striatal levels, demonstrating that its neuroprotective effect does

not involve the elevation of 5-HT content as observed in the other monoamines evaluated in this study. Thus, this decrease in 5-HT after eucalyptol administration should be further investigated to clarify its activity on the serotoninergic system. To the extent of our knowledge, this is the first work evaluating the activity of eucalyptol on the monoaminergic system.

Eucalyptol has demonstrated to be a prospective natural compound that targets multiple pathways known to play a key role in the pathophysiology of seizure onset and epilepsy. The use of eucalyptol, either as a monotherapy or as an adjuvant therapy in combination with standard anticonvulsant drugs, can offer numerous benefits such as enhanced efficacy, amelioration of adverse effects, and potentially attenuation of resistance against typical anticonvulsant therapy, as well as improvement in the treatment response of refractory epilepsy. However, further research is necessary to better understand how eucalyptol produces its effects to maximize the benefits of combined therapy.

5. Conclusion

Eucalyptol demonstrated an anticonvulsant and neuroprotective activities, evidenced in the histopathological analysis of mice hippocampus subjected to PILO-induced seizure, probably throughout different mechanisms including: a) modulation of the muscarinic cholinergic system by antagonizing the muscarinic overstimulation; b) Mitigation of the lipid peroxidation, oxidative stress, ROS and nitrite concentration in hippocampus; c) modulation of the monoamines NA, DA and 5-HT levels in mice striatum. These activities appear to effectively control the seizure onset. However, more in-depth studies need to be performed to better understand the mechanisms underlying the eucalyptol activity on the CNS.

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Ethics approval statement

All experimental protocols were executed according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) following the 3Rs (Replace, Reduce, Refine) ethical principles of animal handling and were approved by the Ethics Committee for Animal Use – ECAU/URCA, under the registration number #n° 00113/2019.2.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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