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Antioxidant and anticholinergic properties of *Citrus sinensis* (L.) Osbeck (Rutaceae) essential oil in mice hippocampus

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This study evaluated anti-acetylcholinesterase and antioxidant potentials in mice hippocampus treated with essential oils (EO) of *Citrus sinensis* (L.) Osbeck (orange) *in vitro* and *in vivo*. The acetylcholinesterase (AChE) activity was evaluated by using an adapted spectrophotometric method by Ellman after administration (30 consecutive days) in albino mice at doses of 50.00 mg/kg (EO 50), 100.00 mg/kg (EO 100) and 200.00 mg/kg (EO 200). The results showed that there was a significant decrease on enzymatic activity of AChE in mice hippocampus treated with essential oil of *C. sinensis* at doses of 50.00 mg/kg (vehicle= 9.89±0.19, OE 50= 2.63±0.21) [P <0.05], 100.00 mg/kg (vehicle= 9.89±0.19, EO 100= 1.65±0.15) [P <0.05] and 200.00 mg/kg (vehicle= 9.89±0.19, EO 200= 2.38±0.12) [P <0.05] when compared with vehicle group (0.05% Tween 80 dissolved in saline 0.9%). Concerning antioxidant activity, there was a significant reduction (P <0.05) of 20% on lipid peroxidation level in mice hippocampus treated with a dose of 200.00 mg/kg. The results obtained from the current studies showed that the essential oil of *C. sinensis* has considerable antioxidant and activity and inhibitory effect on AChE.

Keywords: Antiacetylcholinesterase activity, Antioxidant activity, *Citrus sinensis*, Essential oil.

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Introduction

Plants that have favourable effects to cognitive disorders, including antiacetylcholinesterase, anti-inflammatory and antioxidant activities or other relevant pharmacological activities, are of potential interest for clinical use in Alzheimer's treatment[1]. Some species of *Citrus* have several biological activities, including antibacterial, antifungal, antiviral, antioxidant, analgesic, and anti-inflammatory effects[2]. Species of genus *Citrus* are rich in flavonoids, essential oil, coumarins and pectins[3].

Citrus sinensis (L.) Osbeck (Rutaceae) is a medicinal plant known as orange in countries such as Brazil, Venezuela, Mexico, and Ecuador. Previous studies about the volatile oil of this fruit peels showed anxiolytic and hypnotic activities[4]. *C. sinensis* also showed *in vitro* potent effect against rotavirus[5] and antimicrobial activity[6]. C. sinensis is used in popular medicine in Brazil and other countries to treat anxiety, insomnia and as an anticonvulsant, suggests a depressive action on the central nervous system (CNS), and other properties[7].

Very important studies on essential oils (OE) have been developed in recent years. Clinically important aromatic species such as *Nigella sativa*, *Acorus gramineus*, *Lavandula angustifolia*, *Eucalyptus globulus*, *Mentha piperita*, *Rosmarinus officinalis*, *Jasminum sambac*, *Piper nigrum* and other plants have been reported with neuroprotective effects[8]. In another study, the **n**europharmacological effects of essential oil from the leaves of *Croton conduplicatus* Kunth and possible mechanisms of action involved were suggested[9].

In a previous study, an animal model of Alzheimer was used for pharmacological assessment[10]. The results suggest that the fennel essential oil inhalation ameliorates beta-amyloid (1-42)-induced anxiety and depression in laboratory rats. This study aims to evaluate antiacetylcholinesterase and antioxidant potentials in mice hippocampus treated with essential oils of *Citrus sinensis* leaves *in vitro* and *in vivo*.

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Materials and Methods

Chemical, materials, plant material and essential oils

Fresh leaves of *C. sinensis* were identified and collected by Dr Chistiane Mendes Feitosa in February 2010, at the city of Picos, State of Piaui, Brazil. A voucher specimen was deposited at the Graziela Barroso. Herbarium of the Federal University of Piaui with number 27.163. Fresh leaves (2.0 kg) were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus. The essential oils obtained were dried over anhydrous sodium sulphate and stored in a dark glass bottle at 4 °C until use.

Gas chromatography experiment conditions

The oils obtained from the leaves were analyzed by GC/MS using a GC-17 A/MS-QP505A (Shimadzu, Japan) instrument under the following conditions: dimethylpolysiloxane DB-1 fused-silica capillary column (30.00 m × 0.25 mm); carrier gas, helium (1.00 mL/min); injector temperature, 35–180°C at 4 °C/min, then 180–250 °C at 10 °C/min; mass spectra electron impact 70 eV. Individual components were identified by spectrometric analyses using two computer library MS searches and Kovat's indices as a pre-selection aid. Visual mass spectra comparison data from the literature were used for confirmation[11].

Evaluation of acetylcholinesterase (AChE) inhibitory activity by thin-layer chromatography (TLC) assay positive and false-positive method

TLC method in this study was carried out according to the procedure described previously[7]. All samples were dissolved in methanol to prepare solutions of 10 and 5 mg/mL. Then, 1.5 µL of each sample was spotted on the silica gel TLC plate and developed with chloroform:methanol (9:1) after which the enzyme inhibitory activities were detected using Ellman's method "in situ" on the plate[12]. The developed plates were sprayed with 1 mM DTNB and 1 mM ATCI in buffer A. It dried for 3-5 minutes, then an enzyme solution of AChE from an electric eel (type VI-s lyophilized, 261 U/mg solid, 386 U/mg protein) dissolved in buffer A (500 U/mL stock solution) was diluted with buffer A to obtain 5 U/mL enzyme and was then sprayed on the plate[13]. Yellow backgrounds with white spots for inhibiting compounds were visible after about 5 min. These observations must be recorded within 15 min because they fade after 20-30 min.

To rule out false-positive results from samples in the TLC or in the microplate assay that may occur due to a spontaneous reaction between DTNB and thiocoline, 5 units/mL of AChE were premixed with 1 mM ATCI in buffer A and incubated for 15 min at 37 ^{o}C. This enzyme-substrate mixture was used as a thiocoline spray[13]. As described above, the plates were sprayed with thiocoline. White spots on a yellow background were observed for false-positive compounds.

Spectrophotometric method

The inhibitory effect on acetylcholinesterase activity was evaluated using an adaptation of spectrophotometric method[12,14]. All conditions were identical to those described in earlier publication[7]. Neostigmine was used as standard and the experiment was carried out in quintuplicate. IC_{50} (50% inhibitory concentration) values were obtained through Log-Probit. Used a spectrophotometer was Biosistem SP220 for the inhibitory activity quantitatively. Initially, 100 µL of the sample (concentrations of de 0.1, 0.05, 0.025 µg/mL e 0.0125 µg/solution) in 50 mM Tris-HCl pH 8, and 10% methanol) were mixed with 100 µL AChE l of 0.22 U/mL (22 U of enzyme diluted in 100 µL of 50 mM Tris-HCl pH 8, 0.1% bovine serum albumin, BSA) and 200 µL buffer (50 mM Tris HCl pH 8, 0.1% (BSA). the mixture was incubated for 5 minutes at 30 ° C, then 500 µL was added acid 5,5-dithiobis (2-nitrobenzoic acid) - DTNB (in concentration of 3 mM Tris-HCl pH 8, 0.1 M NaCl, 0.02 M MgCl2) and 100 µL of Acetylthiocholine iodide (ATCI, 4 mM in water). A blank was also prepared by replacing AChE with 100 µL of buffer (50 mM Tris-HCl buffer pH 8, 0.1% BSA). All samples were analyzed in triplicate. The reaction was monitored for 5 min at 412 nm. The drug neostigmine was used as a standard and was used as a negative control Buffer (0.1% methanol in 50 mmol/L Tris-HCl pH 8, 10%). The percentage of inhibition of the isolated substance and neostigmine were calculated according to equation 1. Anti–acetylcholinesterase activity (I %) was calculated as the following:

$$I\ (\%) = (1 - V0\ Sample/V0\ Blank) \times 100$$

where, V0 Sample and V0 Blank represent the initial velocities of samples and blank. IC_{50} values were obtained through Log-Probit plotting.

Drugs

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All doses were expressed in mg/kg and were administered orally (o.r.) in a

volume of 10 mL/kg. The essential oil of *C. sinensis* was emulsified with 0.05% Tween 80 dissolved in 0.9% saline (vehicle). Animals (n= 10 per group) were treated with essential oils of *C. sinensis* (o.r.; 50.00; 100.00, and 200.00 mg/kg) for 30 consecutive days and 30 min before of experiments. Negative control received vehicle (10.00 mL/kg). Drug dosages of essential oil of *C. sinensis* were determined from dose-response studies and observation of dose currently used in animal's studies (data not shown).

Animals

Adult male *Swiss* albino mice (25.00-30.00 g) from the Federal University of Piaui were used. They were housed in polypropylene cages (11×17×28 cm^3) with wood shavings as bedding under controlled conditions of light (12 h light-dark cycle, light on at 7 am) and temperature (25±1 °C) and poorly illuminated with a 15-V red light. Animals were evaluated during the light period (8-10 a.m.). They had free access to water and food except 30 min before and during the experiments. Animal care followed the official governmental guidelines in compliance with the Society Policy and was approved by the Ethics Committee of the Federal University of Piauí, Brazil (# 003/2011).

Determination of AChE activity

The samples were also tested on TLC plates. In this method, OE showed activity, through the yellow field and white halos on a plate. Was not detected false-positive results.

Effects of acute administration of essential oil of *C. sinensis* at doses 50.00, 100.00, and 200.00 mg/kg on AChE activity was determined in mice hippocampus. The determination of the enzymatic activity of AchE in mice hippocampus treated orally for 30 consecutive days at doses of 50.00, 100.00 and 200.00 mg/kg (EO 50, EO 100, and EO 200 groups, respectively), a single serving per day and sacrificed 1 h after the last day of treatment with essential oil of *C. sinensis*. Hippocampus were homogenized in phosphate buffer (pH 8.0, 0.10 mol/L). 10% homogenate (5.00 µL) was added to a bucket containing 500.00 µL of the buffer, 895.00 µL of distilled water and 50.00 µL 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) 0.01 M. Then, the bucket was removed and it was added acetylthiocholine iodide (ATCI) 0.075 mol/L. The absorbance was recorded during for 3 first min at 412 nm. Enzyme activity was expressed as nanomoles of acetylthiocholine hydrolyzed/mg protein/min[15].

Determination of antioxidant and oxidative markers

Hippocampus homogenates (10%) of EO 50 (n= 10), EO 100 (n= 10), EO 200 (n= 10) and negative control (n= 10) groups were centrifuged (800 *x* g, 10 min), the supernatant was collected and used to analyze the tissue total proteins, lipid peroxidation levels (nmol de MDA/g of tissue), nitrite content (nM), reduced glutathione (µg/g weight of tissue) as per previously reported method[16].

Statistical analysis

The results were expressed as mean±standard error of mean (S.E.M.). Statistical analysis was performed using one-way ANOVA for multiple comparisons and followed by Student–Newman–Keuls as post hoc test by Graph Pad Prism (version 6.0; Graph Pad San Diego, California, USA.). Differences were considered significant at $P < 0.05$.

Results

The analysis of essential oil of *C. sinensis* leaves

The analysis revealed that the essential oil of *C. Sinensis* leaves is a mixture of these compounds: myrcene (0.64%), limonene (20.14%), trans-*β*-ocimene (0.73%), linalool (2.58%), citronellal (1.23%), citronellol (30.42%), neral (1.71%), geranial (31.42%) and *β*-caryophyllene (2.04%) (Fig. 1). The oil yield was 0.17%, calculated based on the volume of oil obtained and the weight of fresh plant material (Table 1, Fig. 2).

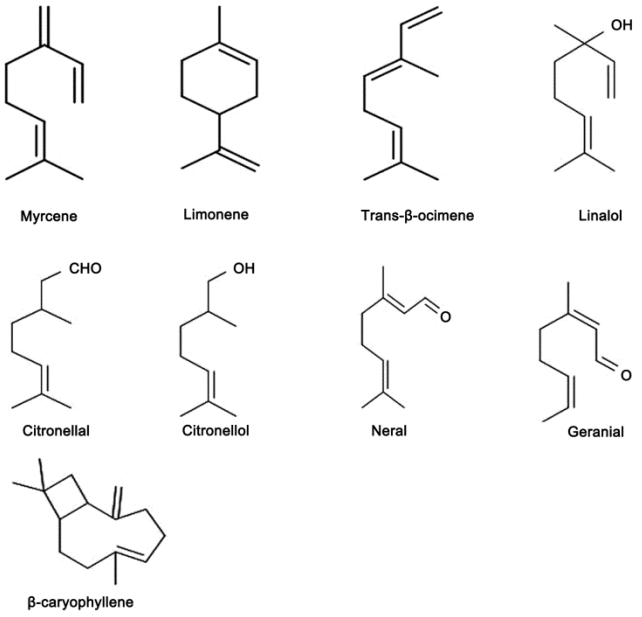

Fig. 1 — Structure of chemical constituents of essential oil from *Citrus sinensis*

Table 1 — Yield the chemical composition of essential oil extracted from leaves of *Citrus sinensis*

Peak	Constituents	MW	MF	(%)	*KI Calculated	*KI Literature
1	Myrcene	136.24	$C_{10}H_{16}$	0.64	989.30	991.00
2	Limonene	136.24	$C_{10}H_{16}$	20.14	1030.30	1031.00
3	Trans-*β*-ocimene	136.24	$C_{10}H_{16}$	0.73	1046.00	1050.00
4	Linalol	154.25	$C_{10}H_{18}O$	2.58	1113.80	1104.00
5	Citronellal	154.25	$C_{10}H_{18}O$	1.23	1151.30	1153.00
6	Citronellol	142.24	$C_9H_{18}O$	30.42	1242.90	1233.00
7	Neral	152.24	$C_{10}H_{16}O$	1.71	1245.90	1240.00
8	Geranial	138.21	$C_9H_{14}O$	31.42	1277.80	1270.00
9	*β*-caryophyllene	204.36	$C_{15}H_{24}$	2.04	1413.30	1418.00
Total yield (%)				90.91	-	-

MW= Molecular weight, MF= Molecular formule, *KI= Kovat's índices

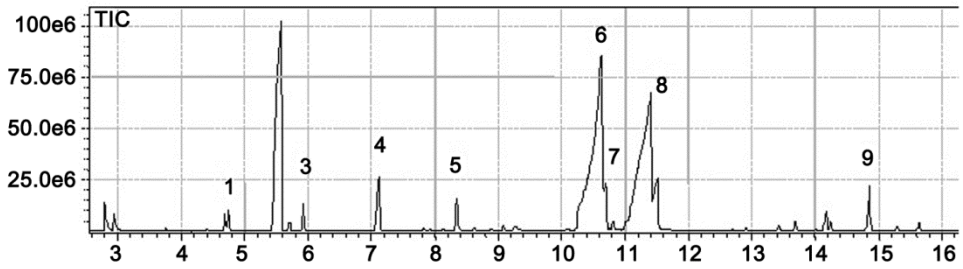

Fig. 2 — Chromatogram of essential oil from *Citrus sinensis* in GC/MS Shimadzu (Model GC 17A). Myrcene (1), limonene (2), trans-β-ocimene (3), linalol (4), citronellal (5), citronellol (6), neral (7), geranial (8) and *β*-caryophyllene (9)

Acetylcholinesterase (AChE) inhibitory activity

The qualitative results for inhibition of AChE in TLC showed that the essential oil of *C. sinensis* (1.00 mg/mL) inhibited the enzyme by the appearance of yellow backgrounds with white spots for inhibiting compounds were visible after about 5 min. In the quantitative study, the IC_{50} values were 1.87 and 63.00 µg/mL for neostigmine and essential oil of *C. sinensis*, respectively.

Acetylcholinesterase activity in mice hippocampus

There was a significant decrease on enzymatic activity of AChE in mice hippocampus treated with essential oil of *C. sinensis* for 30 consecutive days at doses of 50.00 mg/kg (vehicle= 9.89±0.19, OE 50= 2.63±0.21) [P <0.05], 100.00 mg/kg (vehicle= 9.89±0.19, EO 100= 1.65±0.15) [p <0.05] and 200.00 mg/kg (vehicle= 9.89±0.19, EO 200= 2.38±0.12) [P <0.05] when compared with vehicle group (0.05% Tween 80 dissolved in saline 0.9%). In turn, the animals treated with a dose of 100 mg/kg showed a 38% reduction on AChE activity when compared with group EO 50 (EO 50= 2.63±0.21; EO 100= 1.65±0.15) [P <0.05]. There was a decrease by 73, 83, and 76% of AChE activity observed in hippocampus of animals treated during 30 days at doses of 50.00 mg/kg [P <0.05], 100.00 mg/kg [P <0.05] and 200.00 mg/kg [P <0.05], respectively, when compared to the control group (Fig. 3).

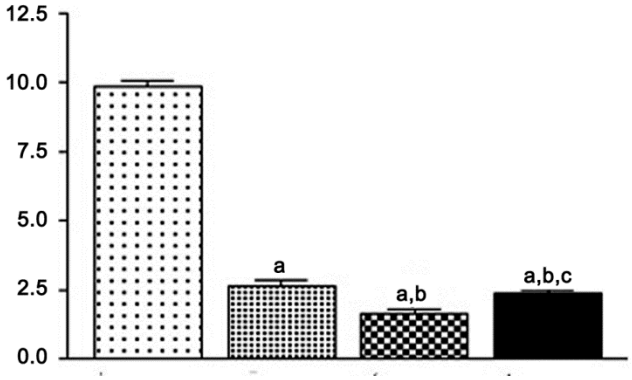

Fig. 3 — Acetylcholinesterase (AchE) activity in mice hippocampus treated with essential oil (EO) of C. sinensis. Values represent as mean ±S.E.M.

The statistical analysis used ANOVA and *t*-Student-Neuman-Keuls as *post hoc*. [a]p <0.05, when compared with vehicle (0.05% Tween 80 dissolved in 0.9% saline);[b]p <0.05, when compared with essential oil of C. sinensis at dose of 50.00 mg/kg (EO 50).

Antioxidant and oxidative markers

Antioxidant activity results are shown in Table 2. There were significant 19.6, 15.8 and 11.5% reductions after treatment at dose of 200.00 mg/kg (0.876±0.13), EO 50 (1.040±0.04) and EO 100 (0.99±0.04), respectively (P <0.05) in lipid peroxidation level when compared with vehicle (1.09±0.07). Thereby reducing oxidative stress and nitrite formation that had a significant reduction (P <0.05) in all groups provides protection against brain injuries to permanent changes neurochemical.

Table 2 — Effects of essential oil (EO) of *C. sinensis* on lipid peroxidation level, nitrite formation and glutathione (GSH) level in mice hippocampus

Treatments (n=10)	Lipid peroxidation level (nmol de MDA/g of tissue)	Nitrite formation (nM)	GSH level (µg/g weight of tissue)
Vehicle	1.09±0.07	80.29±2.56	887.10±7.58
EO 50	1.04±0.04	65.00±3.42[a]	869.00±3.36
EO 100	0.99±0.04	62.86±2.27[a]	831.00±2.63[a,b]
EO 200	0.88±0.13[a,b,c]	56.43±3.60[a,b,c]	771.90±1.83[a,b,c]

Values represent as mean±S.E.M. The statistical analysis used ANOVA and *t*-Student-Neuman-Keuls as *post-hoc*. [a]P <0.05, when compared with vehicle (0.05% Tween 80 dissolved in 0.9% saline); [b]P <0.05, when compared with essential oil of C. sinensis at dose of 50.00 mg/kg (EO 50); [c]P <0.05, when compared with essential oil of C. sinensis at dose of 100.00 mg/kg (EO 100).

Nitrite formation also had significant reductions of 19, 21.7, and 29.7% in mice hippocampus treated with the dose of 50.00 mg/kg (65.00±3.42), 100.00 mg/kg (62.86±2.27) and 200.00 mg/kg (56.43±3.60) mg/kg, when compared with vehicle (80.29±2.56), respectively (P <0.05). GSH level also reductions of 11.2, 7.11, and 13% in mice hippocampus treated with the dose of 50.00 mg/kg (869.00±3.36), 100.00 mg/kg (831.00±2.63), and 200.00 mg/kg (771.90±1.83) when compared with vehicle (887.1±7.58), respectively(P <0.05) (Table 2).

Discussion

Alzheimer's disease (AD) is histopathologically characterized by massive synaptic loss and neuronal death observed in brain regions responsible for cognitive functions, including the cerebral cortex, hippocampus, entorhinal cortex and striatum ventral[17]. The hippocampus structure is essential for memory. It shrinks dramatically in individuals affected by Alzheimer's disease, degenerative disease that affects memory. A promising approach for treating AD is to boost the acetylcholine level in the brain using AChE inhibitors[1].

There was a significant decrease on the enzymatic activity of AChE in mice hippocampus treated with essential oils of *C. sinensis* leaves for 30 consecutive days at doses of 50.00 mg/kg. There were decreases of 73, 83, and 76% AChE activity observed in hippocampus of animals treated during 30 days at doses of 50 mg/kg [P <0.05], 100.00 mg/kg [P <0.05], and 200.00 mg/kg [P <0.05], respectively when compared to the control group.

The inhibitory effect of OE was assessed using the TLC method and considered positive. In the previous studies for the detection of AChE, yellow background with white spots for inhibiting compounds was visible after about 5 min. The AChE activity was determined by a method that is based on the measure of the initial speed of production of thiocholine proportion of acetylthiocholine. This is accompanied by a continuous reaction of thiol with the 5,5'-ion-ditiobis-2-nitrobenzoate to produce the yellow anion of the 5-thio-2-nitro-benzoic acid, whose colour is measured at 412 nm[16]. Then, confirmation of AChE inhibition by *C. sinensis* oils was confirmed by the spectro–photometric method.

The value of AchE inhibition concentration of essential oils of *C. sinensis* was IC_{50}= 63.00 µg/mL and inhibition of standard (neostigmine) with IC_{50} = 1.87 µg/mL. The aromatic species *Salvia lavandulaefolia* and *R. officinali*s demonstrated some valuable therapeutic effects with less strong *in vitro* bioactivity[18]. Indeed, the essential oils of *S. lavandulaefolia* suggested to be relevant in the treatment of dementia of the Alzheimer's type[19] exhibited an IC_{50} of 50.00 µg/mL[20] while that of *R. Officinalis* with an IC_{50} value of 70.00 µg/mL[21] enhanced the performance and overall quality of memory in healthy adults. The essential oil of *S. lavandulaefolia* demonstrated also significant effects on cognition[22]. The analyzed results show that the treatment with essential oils of *C. sinensis* in mice significantly decreased AChE activity in the hippocampus, which justifies the search for inhibitory compounds in the specie.

Other articles deal with the anti-acetylcholinesterase and antioxidant activities of essential oils[18,23]. The anti-acetylcholinesterase activity was registered for oils essential elements of *Eucalyptus camaldulensis* (IC_{50}= 18.98 µg/mL) and *Ocimum canum* (IC_{50}= 36.16 µg/mL), whose results were lower than those found for *C. sinensis* and may be related to the majority presence of 1,8-cineol in these chemotypes[18]. Species of *Citrus* showed high inhibitory concentrations of AChE, *Citrus aurantifolia* (235.5±3.5 µg/mL), *Citrus aurantium* (66.6±3.8 µg/mL) and *Citrus bergamia* (243.6±3.7 µg/mL)[24], when compared to *C. sinensis*, in these species limonene was the main compound. The effects of EO on pathological targets of AD and dementia including amyloid deposition, neurofibrillary tangles, cholinergic hypofunction, oxidative stress and glutamatergic abnormalities were focused. EOs were effective on several pathological targets and have improved cognitive performance in

animal models and human subjects[8] In another study[25] showed effects of lavender (*L. austifoila*) essential oil on central nervous system well-established targets, such as MAO-A, SERT, GABAA and NMDA receptors as well as *in vitro* models of neurotoxicity, this study suggests that lavender essential oil may exert pharmacological properties via modulating the NMDA receptor, the SERT as well as protective effects in the neurotoxicity induced by hydrogen peroxide.

The essential oil of *Citrus limon* Osbeck and other species of the genus Citrus presented significantly reduced lipid peroxidation level and nitrite content, but they increased the GSH levels and SOD, catalase, and GPx activities in mice[26,27]. These results highlight that oxidative stress in the hippocampus can occur during neurodegenerative diseases, proving that hippocampal damage induced by oxidative process plays a crucial role in brain disorders and also imply that a strong protective effect could be achieved by essential oil of *C. limon* as an antioxidant. GC/MS analysis showed a mixture of monoterpenes among which limonene (52.77%), geranyl acetate (9.92%), and *trans*-limonene oxide (7.13%) were the main compounds of essential oil of *C. limon*[26].

Other studies evaluated the effects of acute treatment with essential oil in the acquisition of spatial memory in rats using the paradigm of the Morris water maze[23]. The essential oil is mainly composed by limonene (24.14%), citronellol (30.42%), and geranial (31.42%). The results of the open field demonstrated that animals did not exhibit locomotor changes when treated with the essential oils of *C. sinensis*, the results in the water maze were significantly lower than the negative control group, which indicates an increased memory capacity in the treated animals[27].

Important study[28] showed that components of lemon essential oil attenuate dementia induced by scopolamine. The anti-dementia effects of s-limonene and s-perillyl alcohol compounds in the essential oil of lemon were observed using the passive avoidance teste and the open field habituation test, this lemon EO showed strong ability to improve memory impaired by scopolamine.

Conclusion

This study suggests that *C. sinensis* essential oil have antioxidant activity, with a 19.6% reduction in lipid peroxidation, a 29.7% reduction in the formation of nitrite radicals and a 13% reduction in GSH levels at the highest tested dose. (200.00 mg/kg). In addition to revealing AChE inhibitory effects *in vitro* (IC_{50} of 63.00 µg/mL) and *in vivo*, with 73, 83, and 76% inhibition in the applied doses of 50.00, 100.00 and 200.00 mg/kg in the hippocampus of mice, which justifies the search for inhibitors in this species.

Conflict of interest

The authors declare that there is no conflict of interest.

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