Evaluation of central nervous system effects of *Citrus limon* essential oil in mice

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Abstract: The central nervous system (CNS) depressant and anticonvulsant activities of *Citrus limon* (L.) Osbeck, Rutaceae, essential oil (EO) were investigated in animal models. The EO (50, 100 and 150 mg/kg) injected by oral route (p.o.) in mice caused a significant decrease in the motor activity of animals when compared with the control group, up to thirty days after the administration and the dose of 150 mg/kg significantly reduced the remaining time of the animals on the Rota-rod apparatus. Additionally, *C. limon* essential oil was also capable to promote an increase of latency for development of convulsions induced by pentylenetetrazole (PTZ). The administration of FLU (10 mg/kg, i.p.), GABA A-benzodiazepine (GABA-BZD) receptor antagonist, antagonized the effect of *C. limon* essential oil at higher dose. This *C. limon* essential oil was also capable to promote an increase of latency for development of convulsions induced by picrotoxin (PIC) at higher dose. In the same way, the anticonvulsant effect of the EO was affected by pretreatment with flumazenil, a selective antagonist of benzodiazepine site of GABAA receptor. These results suggest a possible CNS depressant and anticonvulsant activities in mice that needs further investigation.

Keywords: anticonvulsant

*Citrus limon* essential oil

*mice*

Introduction

The lemon [*Citrus limon* (L.) Osbeck, Rutaceae] exhibits many important natural chemical components, including citric acid, ascorbic acid, minerals and phenolic compounds, such flavonoids. Although their biological properties have always been associated with their content of vitamin C, it has recently been shown that flavonoids and other nutrients and non-nutrients (vitamins, minerals, dietary fiber, essential oils and carotenoids). Play a role in this respect (Benavente-Garcia et al., 1997; Elangovan et al., 1944). Therefore, their health-promoting effects, such as obesity, diabetes, blood lipid lowering, cardiovascular diseases, brain disorders and certain types of cancer, have been associated with their contents, especially vitamin C and flavonoids, due to their natural antioxidant characteristics (Monforte et al., 1995; Miyake et al., 1997; Miyake et al., 1998; Rice-Evans et al., 1997; Tanaka et al., 1996).

Numerous herbal medicines are recognized as active in the central nervous system (CNS), and they have at least a hypothetical potential to affect chronic conditions such as anxiety, depression, headaches or epilepsy, that do not react well to conventional treatments (Carlini, 2003). Thus, *C. limon* essential oil may possess a modulatory role in the treatment of neurodegenerative diseases, since their phenolic compound can interrupts cellular oxidative processes in the central nervous system (CNS) (Rice-Evans et al., 1997). The effects of *C. limon* essential oil leaves in CNS have not yet been determined, therefore, it would be important to conduct these studies to clarify its brain action mechanism.

Preliminary behavioral screening performed with the lemon fruit demonstrates that it promotes sleep in dementia (Wolfe & Herzberg, 1996), increasing motivational behaviour and improving disturbed behaviour (Brooker et al., 1997). Additionally, Nguyen & Paton (2008) demonstrate an antinociceptive effect of lemon fruit on unspecific and specific tests. Since the role of *C. limon* on CNS property is little understood, we decided to assess the activities of *Citrus limon* essential oil (EO) in mice.

Materials and methods

Drugs
The drugs used were: pentylenetetrazole (PTZ), picrotoxin (PIC), polyoxethylene-sorbitan monolated (Tween 80) were purchased from Sigma (USA) and Diazepam (DZP) from Cristália (Brazil). Agents were orally (p.o.) or intraperitoneally (i.p.) administered at a dose volume of 0.1 mL/10 g.

**Plant material and essential oil extraction**

The plant material was collected in February 2010, at the city of Picos, State of Piauí, Brazil, and their voucher was deposited at the Graziella Barroso Herbarium of the Federal University of Piauí under the voucher number 26.453. Samples of essential oils from the leaves of the Citrus limon (L.) Osbeck, Rutaceae, were prepared by Laboratory of Chemistry, UFPI (Matos et al., 1999).

The leaves of C. limon were dried in an oven with air renewal and circulation (model MA-037/18) at 40 °C until complete dehydration has been achieved. The essential oil was obtained by hydrodistillation in a Clevenger-type apparatus using 1,100 g of dried leaves. The oil obtained was dried over anhydrous sodium sulphate, producing yields of 0.32% (v/w). GC-MS analysis was performed in a GC-17A/MS QP5050A - GC/MS system (EI mode 70 eV, source temperature 270 °C, scanned mass ranged 43-350 amu). The operating conditions were as follows: DB-5HT (J&W Scientific, 30 m x 0.25 mm i.d. x 0.10 mm film thickness); helium as the carrier gas, flow rate of 1.0 mL min⁻¹ and with split ratio of 1:30; from 60 °C (2 min.) to 180 °C at 4 °C/min and then from 180 °C (4 min.) to 260 °C at 10 °C/min, with a final hold of 10 min at 260°C. The identity of each compound was determined by comparison of its retention index relative to C₈-C₂₀ n-alkanes (Fluka Analytical, 1.0 mL Alkane Standard Solution), as well as of its spectra with the Wiley 275. L data base (Alencar et al., 1984; 1990). The retention data (retention indices) were compared to those of the literature (Adams, 2007; Stenhagen et al., 1974).

**Animals**

Male Swiss mice (25-30 g), aging two months, were used. The animals were randomly housed in appropriate cages at 23±2 °C under 12 h light/dark cycle (lights on 6:00-18:00 pm) with access to food (Purina®) and water ad libitum. All experiments were carried out between 8 am and 18 pm in a quiet room. Experimental protocols and procedures were approved by the Ethics Committee on Animal Experiments at the Federal University of Piauí (CEEA/UFPI # 44/09).

**Behavioral effects**

Behavioral screening of the mice (n=7 per group) was performed following parameters described by Almeida et al. (1999). The mice were observed during thirty days after oral treatment of C. limon essential oil (50, 100 and 150 mg/kg). It was observed the occurrence of the following general signs of toxicity: piloerection, prostration, writhing, increased evacuation, grooming, discrete groups, dyspnea, sedation, analgesia and palpebral ptosis.

**Locomotor activity**

Mice were divided into four groups of seven animals each and were treated orally with vehicle (saline/Tween 80 0.5%; control group) or EO (50, 100 and 150 mg/kg). The spontaneous locomotor activity of the animals was assessed in a cage activity (50 cm × 50 cm × 50 cm) after thirty days of treatment (Asakura et al., 1993).

**Motor coordination test (rota-rod test)**

A Rota-rod tread mill device (AVS®, Brazil) was used for the evaluation of motor coordination (Perez et al., 1998). Initially, the mice able to remain on the Rota-rod apparatus longer than 180 s (9 rpm) were selected 24 h before the test. Thirty minutes after thirty days of administration of either C. limon essential oil (50, 100 and 150 mg/kg, p.o.), vehicle (saline/Tween 80 0.5%; control group) or diazepam (DZP, 2.0 mg/kg, i.p.), each animal was tested on the Rota-rod apparatus and the time (s) remained on the bar for up to 180 s was recorded after thirty days of treatment.

**Pentylenetetrazole (PTZ)-induced convulsions**

PTZ (60 mg/kg, i.p.) was used to induce clonic convulsions (Smith et al., 2007). Mice were divided into five groups (n=7 per group), the first group served as control and received vehicle (saline/Tween 80 0.5%) while the second group was treated with diazepam (DZP, 2.0 mg/kg, i.p.). The remaining groups were treated during thirty days with C. limon essential oil (50, 100 and 150 mg/kg, p.o.). After the treatment with EO, the mice were treated with PTZ (i.p.) a single dose of 60 mg/kg (i.p.). The latency and percent of inhibition clonic convulsions were registered during 24 h. The incidence of deaths was noted until 24 h after the injection of PTZ.

**Effects of flumazenil on PTZ-induced convulsion**

The effect of selective GABA_A-benzodiazepine (GABA-BZD) receptor antagonist, flumazenil, on the anticonvulsant activity of EO was investigated. In the
Table 1. Chemical composition and retention indices of the constituents of the Citrus limon (L.) Osbeck, Rutaceae, essential oil.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Compoundsa (%)</th>
<th>IKc</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.785</td>
<td>Limonene 52.77</td>
<td>1025.5</td>
</tr>
<tr>
<td>6.365</td>
<td>Linalool 1.73</td>
<td>1100</td>
</tr>
<tr>
<td>7.137</td>
<td>cis-Limonene-oxide 2.68</td>
<td>1129.3</td>
</tr>
<tr>
<td>7.253</td>
<td>trans-limonene-oxide 7.13</td>
<td>1133.7</td>
</tr>
<tr>
<td>7.686</td>
<td>Citronellal 2.77</td>
<td>1150</td>
</tr>
<tr>
<td>10.141</td>
<td>Neral 6.85</td>
<td>1238.5</td>
</tr>
<tr>
<td>11.030</td>
<td>Geranial 5.49</td>
<td>1268.9</td>
</tr>
<tr>
<td>13.062</td>
<td>NI 6.62</td>
<td>1337.8</td>
</tr>
<tr>
<td>13.857</td>
<td>Nerol 4.04</td>
<td>1363.3</td>
</tr>
<tr>
<td>14.441</td>
<td>Geranyl acetate 9.92</td>
<td>1384.2</td>
</tr>
</tbody>
</table>

NI: Not identified; aRetention time; bCompounds listed in order of elution from an DB-5MS column; cKovats indices were calculated against n-alkanes (C9-C18) on a DB-5MS column.

Results

Compounds of essential oil of C. limon

GC-MS analysis showed a mixture of monoterpenes, being limonene (52.77%), geranyl acetate (9.92%) and trans-limonene-oxide (7.13%) as the main compounds in C. limon essential oil (Table 1).

Behavioral effects

C. limon essential oil at doses of 50, 100 and 150 mg/kg, p.o. showed behavioral changes in animals thirty days after of treatment: decrease of spontaneous activity, palpebral ptosis, ataxia, analgesia, and sedation. Behavioral changes were more evident in the second day of treatments. These effects were apparently dose-dependent.

Locomotor activity

In doses of 50, 100 or 150 mg/kg of C. limon essential oil caused significant decreases of 28, 29 and 79% of ambulation (number of crossings) at thirty days after administration, when compared to control group (p<0.001), respectively (Figure 1). At dose of 150 mg/kg of C. limon essential oil caused significant decreases of 57 and 56% of ambulation at thirty days after administration, when compared to EO 50 (p<0.001) and EO 100 (p<0.001), respectively (Figure 1). Diazepam (2 mg/kg, i.p.) caused significant decrease of 81% of ambulation (number of crossings), when compared to control group (p<0.001).
Motor coordination (Rota-rod test)

In this test, thirty days after administration of C. limon essential oil only the dose of 150 mg/kg (p.o.) the remaining time of animals on the Rota-rod apparatus was significantly reduced in 30% (Figure 2). At dose of 150 mg/kg of EO caused significant decreases of 28 and 27% of remaining time of animals on the Rota-rod apparatus at thirty days after administration, when compared to EO 50 (p<0.001) and EO 100 (p<0.001), respectively (Figure 2). Diazepam (2 mg/kg, i.p.) caused significant decrease of 56% of remaining time of animals on the Rota-rod apparatus, when compared to control group (p<0.001).

Anticonvulsant activity

Table 1 show that PTZ, in control group, clonic convulsions induced in 100% of mice. C. limon essential oil (50, 100 and 150 mg/kg, p.o.) delayed the onset of PTZ-induced tonic convulsion significantly. C. limon essential oil (150 mg/kg, p.o.) protected 85% (p<0.001) of mice against the convulsion and reduced in 60% the mortality rate induced by PTZ (p<0.001). Diazepam (2 mg/kg, i.p.) completely protected the animals against the tonic convulsion elicited by PTZ.

As seen in Table 1, the administration of FLU (10 mg/kg, i.p.) antagonized the effect of C. limon essential oil (150 mg/kg, p.o.) and DZP (2 mg/kg, i.p.) in the prolongation of convulsion latency.

When given p.o. only the highest dose of C. limon essential oil (150 mg/kg, p.o.) increased the latency for convulsions and reduced mortality rate induced by PIC when compared to the negative control (p<0.001) (Table 3).

Discussion

In Brazilian Northeast folk medicine, C. limon (“limoeiro”) is used for treatment of dementia and oxidative damages. In the current study, the CNS depressant and anticonvulsant activities of C. limon leaf essential oil were investigated in different animal models. In pharmacological behavioral screening, the animals treated with EO showed decrease of response to the touch,
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palpebral ptosis, ataxia, analgesia, sedation and reduction of motor activity. These behavioral changes suggest a possible depressant effect on CNS and are similar to drugs that reduce the CNS activity (Morais et al., 2004; Netto et al., 2009; Almeida et al., 1999).

C. limon leaf essential oil at the highest dose (150 mg/kg) caused a significant reduction of ambulation of animals, corroborating with the hypothesis that C. limon essential oils reduce the CNS activity (Freire et al., 2006; Leite et al., 2008; Carlini, 2003; Quintans-Júnior et al., 2008).

The reduction of the locomotor activity might be due to either an inhibitory effect of the C. limon essential oil in CNS or by periphery muscular relaxant activity. So, this result indicates that EO could exhibit a sedative activity. Our GC-MS analysis revealed a mixture of monoterpenes (limonene, geranyl acetate and trans-limonene-oxide) as major compounds in EO and it can be able by inhibitory effects on CNS of mice observed during pharmacological behavioral screening after thirty days of treatment with EO (Passos et al., 2009).

In order to determine whether the C. limon essential oil produces loss of motor coordination of animals was performed to rota-rod apparatus. Once more, the results show that the highest dose produces loss of motor coordination in mice. Thus, the lack of motor coordination in the test of the Rota-rod is characteristic of a drug that reduces the CNS activity such as anxiolytics, sedatives and hypnotics (Almeida et al., 1999; Olayiwola et al., 2007; Dallmeier & Carlini, 1981).

The beginning of tonic-clonic convulsion produced by PTZ was significantly delayed by C. limon essential oil and the incidence of mortality was reduced. According to De Sarro et al. (1999), PTZ may be exerting essential oil and the incidence of mortality was reduced. Produced by PTZ was significantly delayed by Dallmeier & Carlini, 1981).

In this context, to assess whether the C. limon essential oil-induced anticonvulsant effects, flumazenil (FLU), a specific antagonist of the GABA-CBD receptors, thus preventing the entry of chloride ions into the brain inhibiting, consequently, the brain transmission (Löschner & Schmidt, 2006). Therefore, the findings of the present study suggest that C. limon essential oil (150 mg/kg, p.o.) has inhibited and/or attenuated the PIC-induced convulsions of mice by interfering with GABAergic neurotransmission (Oliveira et al., 2001).

Summarizing our data, the results propose a possible depressant CNS and anticonvulsant effects of C. limon essential oil. The precise mechanisms of possible behavioral effects of C. limon essential oil are not clear, however, GABAergic neurotransmitter system might be involved. Thus, further investigations are in progress for elucidation of this effect in CNS.

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References


According to Nicoll (2001), picrotoxin, a GABA receptor antagonist, produces seizures by blocking the chloride-ion channels linked to GABA receptors, thus preventing the chloride ions into the brain inhibiting, consequently, the brain transmission (Löschner & Schmidt, 2006). Therefore, the findings of the present study suggest that C. limon essential oil (150 mg/kg, p.o.) has inhibited and/or attenuated the PIC-induced convulsions of mice by interfering with GABAergic neurotransmission (Oliveira et al., 2001).
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