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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 $GABA_A$ receptor. These results suggest a possible CNS depressant and anticonvulsant activities in mice that needs further investigation.

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-Garcıa 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), polyoxyethylene-sorbitan monolated (Tween 80) were purchased from Sigma (USA) and Diazepam (DZP) from Cristália (Brazil). Agents were orally (*p.o.*) or intraperitoneally (*i.p.*) administrated 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 Piaui, Brazil, and their voucher was deposited at the Graziella Barroso Herbarium of the Federal University of Piaui 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 C_8-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

experimental groups, after thirty days of treatment with EO, the mice were pre-treated with flumazenil (FLU) (10 mg/kg, *i.p.*) and thirty min before received EO (150 mg/kg, *p.o.*) or standard drug (DZP, 2.0 mg/kg, *i.p.*). After thirty min of treatment with EO or DZP, the mice were treated with PTZ (*i.p.*) a single dose of 60 mg/kg (*i.p.*) (File & Pellow, 1986). The anticonvulsant activity of EO and DZP in mice pretreated with FLU was assessed.

Picrotoxin (PIC)-induced convulsion

Mice were divided into five groups (n=7 per group), control group received vehicle (saline/Tween 80 0.5%) and standard group was treated with diazepam (DZP, 2 mg/kg, *i.p.*). The remaining groups were treated with 50, 100 and 150 mg/kg of EO (*p.o.*). After thirty days of treatment with EO, the mice were treated with PIC at a dose of 8 mg/kg (*i.p.*). Immediately after the administration of the convulsant agent, mice were individually placed in plastic boxes and observed during 24 h for the time onset of clonic convulsion (latency), percent clonic convulsion and deaths. The incidence of deaths was noted until 24 h after the injection of PIC (Lehmann et al., 1988; Ngo Bum et al., 2001).

Statistical analysis

Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. The incidence (%) of clonic or tonic-clonic convulsions as well as the mortality were evaluated by Fisher`s Exact Test. Differences were considered to be statistically significant when $p<0.05$.

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.

Table 1. Chemical composition and retention indices of the constituents of the *Citrus limon* (L.) Osbeck, Rutaceae, essential oil.

RT (min)[a]	Compounds[b]	(%)	IK[c]
4.785	Limonene	52.77	1025.5
6.365	Linalool	1.73	1100
7. 137	*cis*-Limonene-oxide	2.68	1129.3
7.253	*trans*-limonene-oxide	7.13	1133.7
7.686	Citronellal	2.77	1150
10.141	Neral	6.85	1238.5
11.030	Geranial	5.49	1268.9
13.062	NI	6.62	1337.8
13.857	Nerol	4.04	1363.3
14.441	Geranyl acetate	9.92	1384.2
	Total identified	93.38	

NI: Not identified; [a]Retention time; [b]Compounds listed in order of elution from an DB-5MS column; [c]Kovats indices were calculated against *n*-alkanes (C_9-C_{18}) on a DB-5MS column.

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 ($p<0.001$).

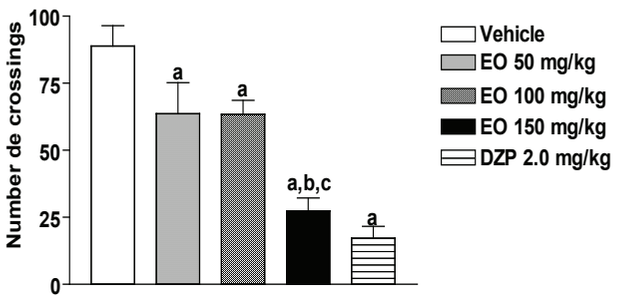

Figure 1. Effects of *Citrus limon* (L.) Osbeck, Rutaceae, essential oil (EO, 50, 100 and 150 mg/kg, *p.o.*) or diazepam (DZP, 2.0 mg/kg, *i.p.*) of mice locomotor activity. The parameters evaluated were the total number of pulses of crossings in activity cage. Values are the mean±S.E.M. (n=7 per group). [a]$p<0.001$ (Fisher`s test) compared to control; [b]$p<0.001$ (Fisher`s test) compared to EO 50 group; [c]$p<0.001$ (Fisher`s test) compared to EO 100 group.

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).

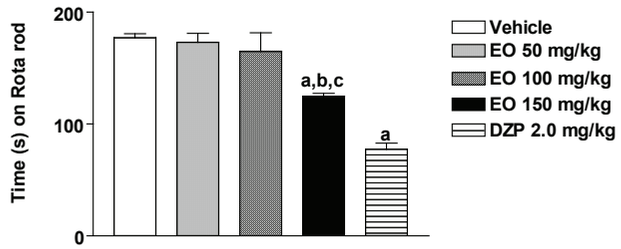

Figure 2. Time (s) on the Rota-rod observed in mice after oral route treatment with Vehicle (Control), *Citrus limon* (L.) Osbeck, Rutaceae, essential oil (EO, 50, 100 and 150 mg/kg, *p.o.*) or DZP (2.0 mg/kg, *i.p.*). The motor response was recorded for the following 180 s after drug treatment. Values are the mean±S.E.M. (n=7 per group). [a]p<0.001 (Fisher`s test) compared to control; [b]$p$<0.001 (Fisher`s test) compared to EO 50 group; [c]p<0.001 (Fisher`s test) compared to EO 100 group.

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,

Table 2. Effects of *Citrus limon* (L.) Osbeck, Rutaceae, essential oil (EO) on PTZ-induced convulsion in mice.

Treatments	Dose (mg/kg)	Latency (s)	% Inhibition of convulsion	% Inhibition of death
Vehicle	-	126.3±11.9	0	0
EO 50	50	229.0±22.6[a]	0	0
EO 100	100	345.8±38.0[b]	10	20[e]
EO 150	150	552.5±33.5[c]	85[d]	40[d]
EO+FLU	150+10	241.8+38.9	0	15[e]
DZP	2	852.9±10.0[c]	100[d]	100[d]
DZP+FLU	2+10	161.2+15.2	0	15

Values are the mean±S.E.M. mice (n=7 per group). [a]p<0.05 (ANOVA followed by t-Student-Neuman-Keuls test) compared to control; [b]p<0.01 (ANOVA followed by t-Student-Neuman-Keuls test) compared to control; [c]p<0.001 (ANOVA followed by t-Student-Neuman-Keuls test) compared to control; [d]p<0.001 (Fisher`s test) compared to control; [e]$p$<0.05 (Fisher`s test) compared to control.

Table 3. Effects of *Citrus limon* (L.) Osbeck, Rutaceae, essential oil (EO) on PIC-induced convulsion in mice

Treatments	Dose (mg/kg)	Latency (s)	% Inhibition of convulsion	% Inhibition of death
Vehicle	-	596.2±14.5	0	0
DZP	2	1436.0±10.0[a]	100[a]	100[a]
EO 50	50	598.5±21.01	0	0
EO 100	100	597.5±45.9	10	10
EO 150	150	1205.9±29.3[a]	60[a]	60[a]

Values are the mean±S.E.M. (n=7 per group). [a]p<0.001 (Fisher`s test) compared to control.

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 this context, to assess 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 its convulsant effect by inhibiting the activity of gamma aminobutyric acid (GABA) at $GABA_A$ receptors. GABA is the the epilepsy key inhibitory neurotransmitter. The enhancement and inhibition of the neurotransmission of GABA frequently attenuate and enhance convulsion, respectively (Westmoreland et al., 1994). Since the *C. limon* essential oil postponed the occurrence of convulsions, it is probable that it had been caused by the neurotransmission GABAergic activation (Nicoll, 2001; Rang et al., 2003).

In order to determine the role of GABA-BZD receptors participation in the *C. limon* essential oil-induced anticonvulsant effects, flumazenil (FLU), a specific antagonist of the benzodiazepine site in the GABA-BZD receptor complex, was used (File & Pellow, 1986). Mice pretreated with FLU in the PTZ-induced convulsion model suggest that the EO may facilitate the inhibitory activity of the GABAergic system probably through a competitive agonist action in the BZD site of the GABA receptors. The significantly effect on the motor coordination, in higher doses, can support this theory, since GABAergic drugs usually are sedative (Pedersen et al., 2009).

According to Nicoll (2001), picrotoxin, a $GABA_A$-receptor antagonist, produces seizures by blocking the chloride-ion channels linked to $GABA_A$-receptors, thus preventing the entry of chloride ions into the brain inhibiting, consequently, the brain transmission (Löscher & 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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