

Full Length Research Paper

Volatile constituents and behavioral change induced by *Cymbopogon winterianus* leaf essential oil in rodents

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Cymbopogon winterianus Jowitt ('Java citronella') is an important essential oil yielding aromatic grass cultivated in India and Brazil and its volatile essential oils extracted from its leaves are used in perfumery, cosmetics, pharmaceuticals and flavoring industries. However, there is no report on any psychopharmacological study of *C. winterianus* leaf essential oil (LEO) available to date. In this study, the pharmacological effects of the LEO were investigated in animal models and its phytochemical analyses. GC-MS analysis showed a mixture of monoterpenes, as citronellal (36.19%), geraniol (32.82%) and citronellol (11.37%). LEO exhibited an inhibitory effect on the locomotor activity of mice, an antinociceptive effect by increasing the reaction time in the writhing and capsaicin tests. All doses induced a significant increase in the sleeping time of animals not having modified however, the latency. The LEO did not alter the remaining time of the animals on the rota-rod apparatus. These results suggest a possible central effect.

Key words: *Cymbopogon winterianus*, essential oil, CNS, behavioral effects, analgesic.

INTRODUCTION

Traditional health care is utilized by the majority of the low income population in Brazilian northeast. This is especially true of treatment for mental health problems.

Besides, it have been described as a hypothetical potential to affect chronic conditions such as anxiety, depression, headaches, pain treatment or epilepsy, which does not respond well to conventional treatments (Carlini, 2003). A great number of scientists and organizations turn their attention to traditional therapies in order to find and conserve important resources (Akerele, 1990). However, medicinal plants have been an important source of new drugs with biological activity (Quintans-Júnior et al., 2008a, 2011. The genus *Cymbopogon*

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Abbreviation: LEO, Leaf essential oil; EO, essential oil.

Spreng (Poaceae) is characterized by its species possessing great variability in morphology and chemotypes. Most species of the genus are aromatic and yield volatile oils of important commercial values (Blank et al., 2007). *Cymbopogon winterianus* Jowitt (Java citronella) is an important essential oil yielding aromatic grass cultivated in India mainly in the lower hills of Assam, Karnataka and Southern Gujarat. The steam volatile essential oils extracted from its leaves are used in perfumery, cosmetics, pharmaceuticals and flavoring industries (Tanu et al., 2004). The main traditional use is as a repellent (Tawatsin et al., 2001). Folk medicine practitioners in northeastern Brazil use the infusion of the fleshy leaves and unguent for the treatment of anxiety, sedative and pain disorders (Quintans-Júnior et al., 2008b). However, there is little published information about biological effects of this plant. Menezes et al. (2010) demonstrate that *C. winterianus* leaf essential oil (LEO) induces hypotension due to a decrease in peripheral vascular resistance secondary to vasodilatation and these effects appear to be mainly mediated by Ca^{+2} channel blocking. Additionally, preliminary study realized in our laboratory with the LEO showed anticonvulsant and antinociceptive properties in rodents (Quintans-Júnior et al., 2008b; Leite et al., 2010).

The aim of this study was to perform phytochemical screening of the LEO and to investigate its central nervous system (CNS) activity.

MATERIALS AND METHODS

Plant material and essential oil extraction

Leaves were collected in February 2007 from the cultivation of the *C. winterianus* genotypes established at the Research Station "Campus Rural" of the Federal University of Sergipe (10°C $55'$ S, 37°C $11'$ W), Brazil and a voucher sample was deposited in the Herbarium of the Department of Biology of the same University. Plants were cut 20 cm above soil level in Spring at 09:00 h and dried at 40°C in a forced air oven (Marconi®, Brazil) for 5 days. The essential oil (EO) of those leaves were extracted by hydrodistillation for 3 h (Carvalho-Filho et al., 2006), using a Clevenger-type apparatus (British Pharmacopoeia, 1988). The oils were separated from the aqueous phase and kept in the freezer (-20°C) until further use. The oil content was estimated based on dry herbage weight using three samples of 75 g of dry leaves (American Spice Trade Association, 1985). 3.4% essential oil content was obtained.

Gas chromatography – mass spectrometry

Oil sample analysis was performed on a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system comprising a AOC 20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column J and W Scientific DB-5MS (Folsom, CA, USA) fused silica capillary column (30 cm x 0.25 mm i.d., 0.25 μm coating thickness, composed of 5% phenylmethylpolysiloxane), helium (99.999% purity) was used as carrier gas at a constant flow

of 1.2 ml/min and an injection volume of 0.5 μl was employed (split ratio of 1:83) injector temperature 250°C and ion-source temperature 280°C . The oven temperature was programmed from 50°C (isothermal for 2 min), with an increase of $4^{\circ}\text{C}/\text{min.}$, to 200°C , then $10^{\circ}\text{C}/\text{min}$ to 300°C , ending with a 10 min isothermal at 300°C . The mass spectra were taken at 70 eV with scanning speed of 0.85 scan/s from 40 to 550 Da.

Gas-chromatography (GC-FID)

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) equipment, under the following operational conditions: capillary ZB-5MS column (5% dimethylpolysiloxane) fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm coating thickness) from Phenomenex (Torrance, CA, USA), under the same conditions GC-MS. The essential oil composition was reported as a relative percentage of the total peak area.

Identification of essential oil constituents

Identification of individual components of the essential oil was performed by computerized matching of the acquired mass spectra with those stored in NIST21 and NIST107 mass spectral library of the GC-MS data system. Retention indices (RI) for all compounds were determined according to the method of Van den Dool and Kratz (1963) for each constituent as previously described (Adams, 2007).

Drugs

Polyoxyethylene-sorbitan monolated (Tween 80) and cremophor was purchased from Sigma (USA) and Diazepam (DZP) was obtained from Cristália (Brazil). All drugs and the essential oil were administered orally (*per os*, p.o.) in volumes of 0.1 ml/10 g (mice).

Animals

Male Swiss mice (28 to 32 g), with 2 to 3 months of age, were used throughout in this study. The animals were randomly housed in appropriate cages at $22 \pm 1^{\circ}\text{C}$ on a 12 h light/dark cycle (lights on 06:00 to 18:00) with free access to food (Purina) and water. They were used in groups of 8 animals each. Experimental protocols and procedures were approved by the Universidade Federal de Sergipe Animal Care and Use Committee (CEPA/UFS N°010/07).

Acute toxicity (LD₅₀)

This test was performed according to a method described by Lorde (1983), with modifications, where the acute toxicity of LEO was assessed by orally route (*per os*, p.o.). Groups of 10 animals each were separated and received doses of 500, 750, 1000, 2000 or 3000 mg/kg of LEO. The animals were observed daily for 48 h and a number of deaths of animals were registered and lethal dose 50% (LD₅₀) calculated (Litchfield and Wilcoxon, 1949).

Behavioral effects

Behavioral screening of the mice was performed following

parameters described by Almeida et al. (1999) and animals ($n = 8$, each group) were observed at 0.5, 1.0 and 2.0 h after *per os* (p.o.) administration of LEO (25, 50 and 100 mg/kg). Control group received saline/tween-80 0.2% (vehicle).

Locomotor activity

Mice were divided into four groups of 10 animals each. Vehicle (saline/tween-80 0.2%) and LEO (25, 50 and 100 mg/kg, p.o.) were injected. The spontaneous locomotor activity of the animals was assessed in a cage activity ($50 \times 50 \times 50$ cm) in 0.5, 1 and 2 h after administration (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. Mice were placed on a horizontal rotation rod set at a rate of 9 rpm (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. Sixty minutes after the administration of either vehicle (saline/tween-80 0.2%), LEO (25, 50 and 100 mg/kg, p.o.) or diazepam (1.5 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 1 h.

Pentobarbital-induced hypnosis

Sodium pentobarbital, at a hypnotic dose of 50 mg/kg (i.p.), was injected into four groups ($n = 10$) of the mice 60 min after pretreatment with saline/tween-80 0.2% (vehicle) and LEO (25, 50 and 100 mg/kg, p.o.), respectively. The latency (the interval between the injection of sodium pentobarbital and the loss of the righting reflex) and duration of sleeping time (the interval between the loss and recovery of the righting reflex) were recorded (Elisabetsky et al., 1995).

Acetic acid-induced writhing

This study was performed according to Koster et al. (1959). Mice ($n = 8$, per group) were injected intraperitoneally (i.p.) with 0.85% acetic acid at a dose of 10 ml/kg. LEO (25, 50, and 100 mg/kg, p.o.). The reference drug, morphine (MOR, 3 mg/kg), was solubilized in saline + 1 drop of Tween-80 0.2% (vehicle) and was administered i.p. to different groups of the mice 1 h before the acetic acid injection. Subsequently, the writhing was counted for 20 min after a latency period of 5 min.

Capsaicin-induced nociception

The method used was similar to that described previously (Sakurada et al., 1992). Mice were individually placed in a transparent Plexiglas cage ($25 \times 15 \times 15$ cm) observation chamber. Following the adaptation period, 20 μ l of capsaicin (1.6 μ g/paw prepared in a phosphate-buffered solution) was injected under the skin of the dorsal surface on the right hind paw. The mice were pretreated with LEO (25, 50 and 100 mg/kg, p.o.) 60 min before injection of the algogen. The control animals received a similar volume of vehicle. After this process, pairs of mice were placed individually in different Plexiglas cage for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered indicative of

nociception.

Statistical analysis

The data obtained were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS

GC-MS analysis showed a mixture of monoterpenes, being citronellal (36.19%), geraniol (32.82%) and citronellol (11.37%) as the main compounds in the EO (Table 1).

The LD₅₀ calculated to *per os* (p.o.) administration of the LEO in mice was 1,953.8 mg/kg with confidence interval of 1,580.9 to 2,326.7 mg/kg. LEO at doses of 25, 50, 100, 200 and 400 mg/kg (p.o.) showed depressant activity on CNS based on the following behavioral alterations in animals after 0.5, 1 and 2 h treatment: decrease of the spontaneous activity, palpebral ptosis, ataxia, analgesia and sedation. These effects were dose-dependent.

The doses of 25, 50 and 100 mg/kg (p.o.) LEO caused a significant decrease of ambulation (number of squares crossed) at 0.5, 1 and 2 h after administration (Figure 1). As shown in Figure 2a, LEO at all doses did not affect the latency of pentobarbital-induced hypnosis. However, LEO at 25, 50 and 100 mg/kg (p.o.) significantly increased the sleeping time compared with control group animals (Figure 2b).

In the rota-rod test, LEO-treated mice did not show any significant motor performance alterations with doses of 25, 50 and 100 mg/kg. As might be expected, the CNS depressant diazepam (1.5 mg/kg) reduced the time of treated animals on the rota-rod apparatus (Figure 3).

Figure 4 shows that LEO was significantly ($p < 0.001$) reduced, in a dose-dependent manner, the number of writhing movements induced by the p.o. administration of the acetic acid solution. In the capsaicin test, LEO significantly reduced the licking time compared with the control group (Figure 5) only in higher doses.

DISCUSSION

In this study, the pharmacological effects of the *C. winterianus* leaf essential oil (LEO) were investigated in animal models and it characterized a psychopharmacological effect of this essential oil on the CNS. The results obtained and the LD₅₀ values represent a low toxicity of LEO and they were similar to the ones observed for other essential oils (Fandohan et al., 2008). The LEO increases the sleeping time induced by sodium pentobarbital in a dose-dependent manner, decrease ambulation without alter motor coordination performance

Table 1. Chemical composition and retention indices of the constituents of the EO.

RT(min) ^a	Compound ^b	(%) ^c	RI ^d
8.452	6-Metil-5-hepten-2-one	0.23	984
8.600	Myrcene	0.16	988
9.975	Limonene	2.37	1028
10.225	β -(Z)-Ocimene	0.35	1035
10.600	β - (E)-Ocimene	0.23	1045
11.533	Not identified	0.28	1071
12.558	Linalool	1.39	1099
14.350	Isopulegol	1.11	1148
14.525	Citronellal	36.19	1152
14.725	Iso-isopulegol	0.33	1158
16.508	N-decanal	0.46	1206
17.242	Citronellol	11.37	1226
17.642	Neral (Z-citral)	4.53	1237
18.125	Geraniol	32.82	1251
18.717	Geranial (E-citral)	5.84	1267
21.592	Citronellyl acetate	0.75	1348
22.572	Geranyl acetate	1.17	1377
23.958	β -Caryophyllene	0.42	1417
Total		99.62	

^aRetention time; ^bcompounds listed in order of elution from an DB-5MS column; ^cpercentage based on FID peak area normalization; ^dcalculated using the equation of Van den Dool and Kratz (1963).

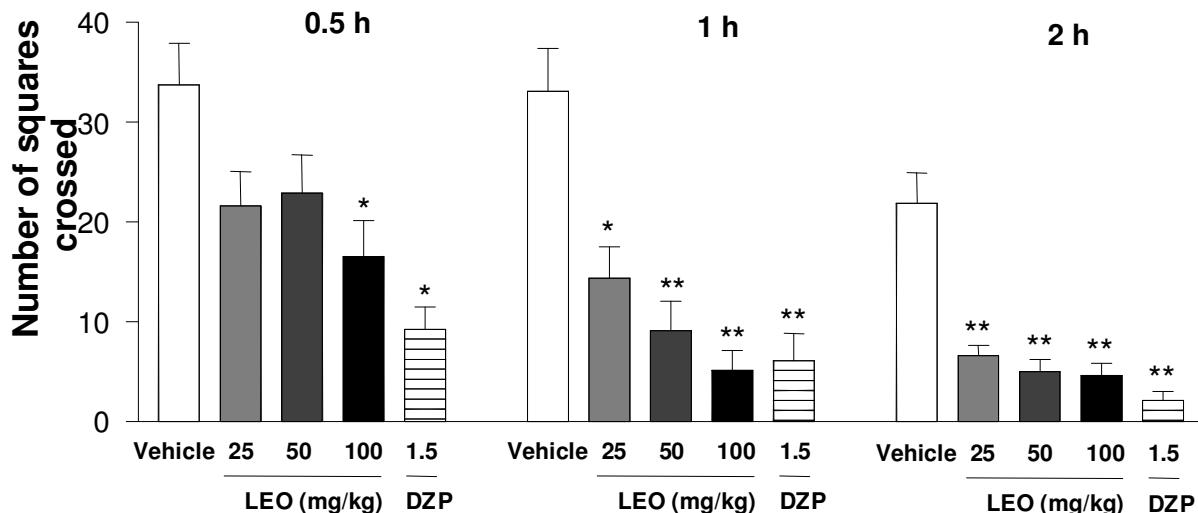


Figure 1. Effect of LEO on locomotor activity of mice. The parameters evaluated were the number of squares crossed in activity cage. Values are the mean \pm SEM for 10 mice; statistical differences versus control group were calculated using ANOVA, followed by Dunnett's test ($n = 10$). * $p < 0.05$ or ** $p < 0.01$.

of animals. Additionally, LEO produced significantly analgesic effect at all doses in the writhing and capsaicin tests.

A general pharmacological screening with the LEO demonstrated some behavioral change in mice, as

decrease of the spontaneous activity, palpebral ptosis ataxia, analgesia and sedation. These signals showed possible evidence that the effects on CNS are similar to drugs that reduce the CNS activity (Fernández-Guasti et al., 2001; Morais et al., 2004).

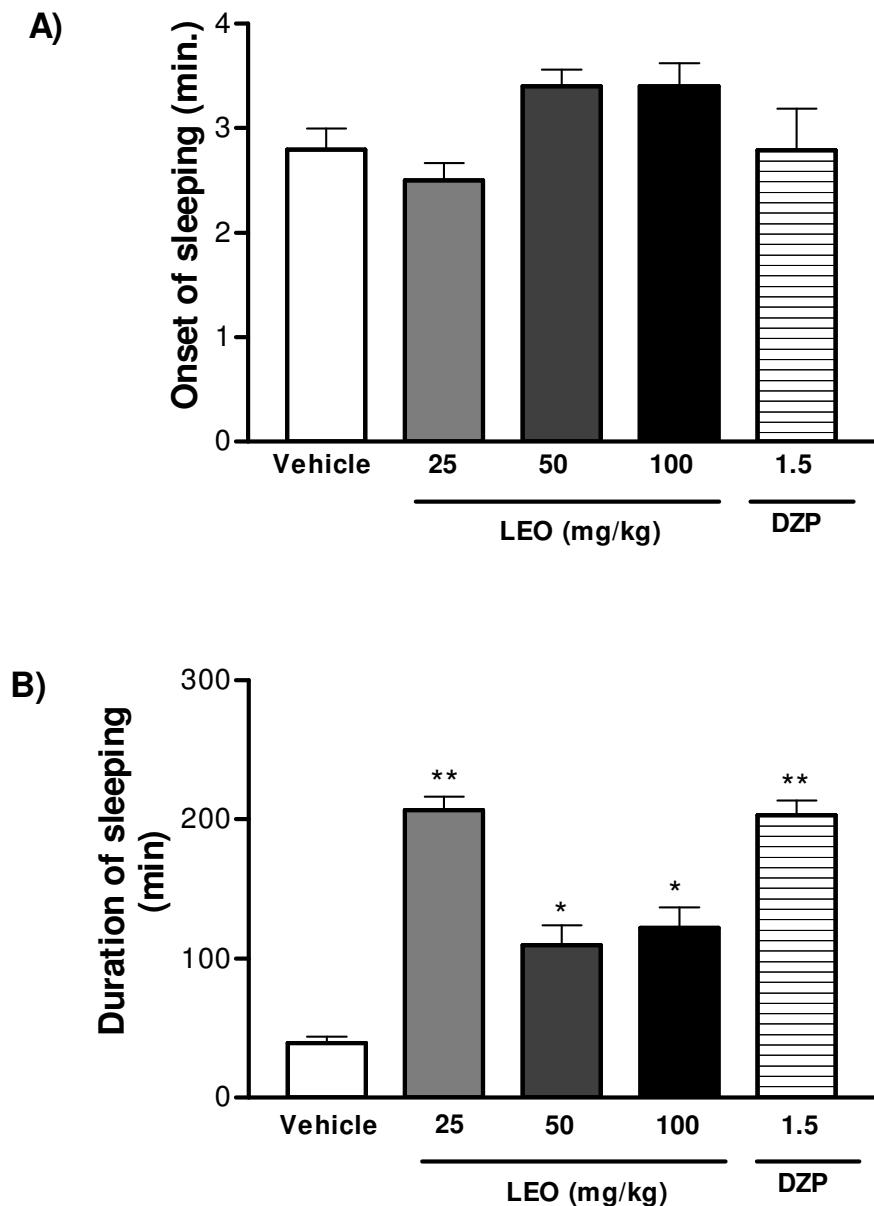


Figure 2. Effect of LEO on pentobarbital-induced hypnosis in mice. The parameters evaluated were the onset of sleeping (A) and duration of sleeping (B). Values are mean \pm SEM for 10 mice, * $p < 0.01$; ** $p < 0.001$, as compared to vehicle

LEO caused a significant reduction of ambulation of animals in the test of spontaneous movement after 0.5, 1 and 2 h of its administration in the doses of 25, 50 and 100 mg/kg, that corroborates with the hypothesis of the LEO reduces the CNS activity, it was reported that reduction of the ambulation of the animals is characteristic of psychopharmacological drugs (Fernández-Guasti et al., 2001).

The LEO 25, 50 and 100 mg/kg (p.o.) had an increase in the total time of sleep of the animals, but did not have an increase in the latency for the induction of sleep

compared with the control group. It is established that the potentiation of the time of sleep induced by pentobarbital must be a sedative or hypnotic action that is attributed to the involvement of central mechanisms in the regulation of sleep (N'Gouemo et al., 1994) and involves the enhancement of the GABAergic system (Steinbach and Akk, 2001; Sivam et al., 2004).

Previous studies suggested that the CNS depression and the nonspecific muscle relaxation effect can reduce the response of motor coordination (Gonçalves et al., 2008). We did not see any interference with the motor

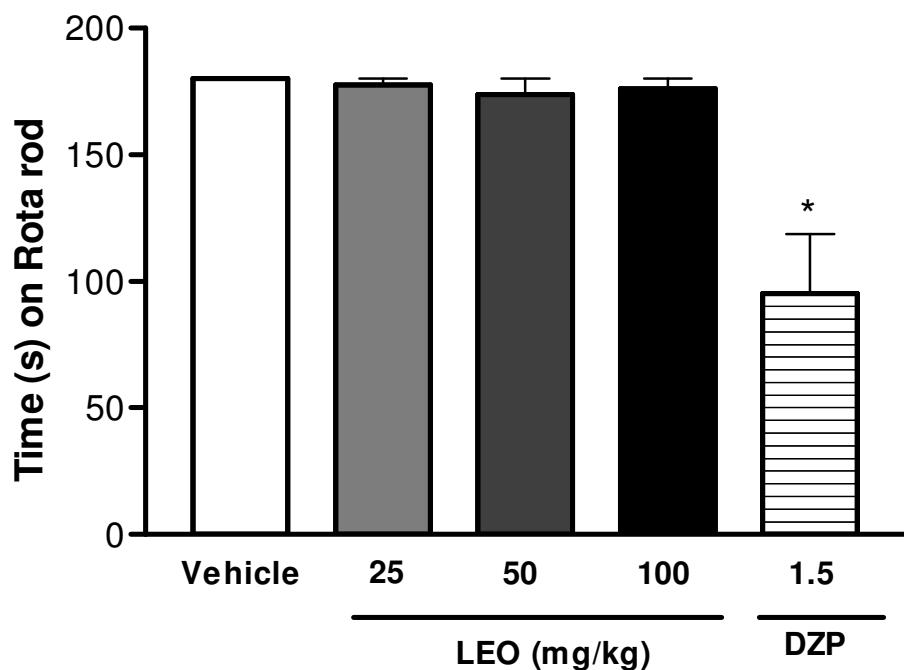


Figure 3. Time (s) on the Rota-rod observed in mice after p.o. treatment with vehicle (control), LEO (25, 50 and 100 mg/kg) or Diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s after drug treatment. Statistical differences versus control group were calculated using ANOVA, followed by Dunnett's test ($n = 10$) * $p < 0.05$.

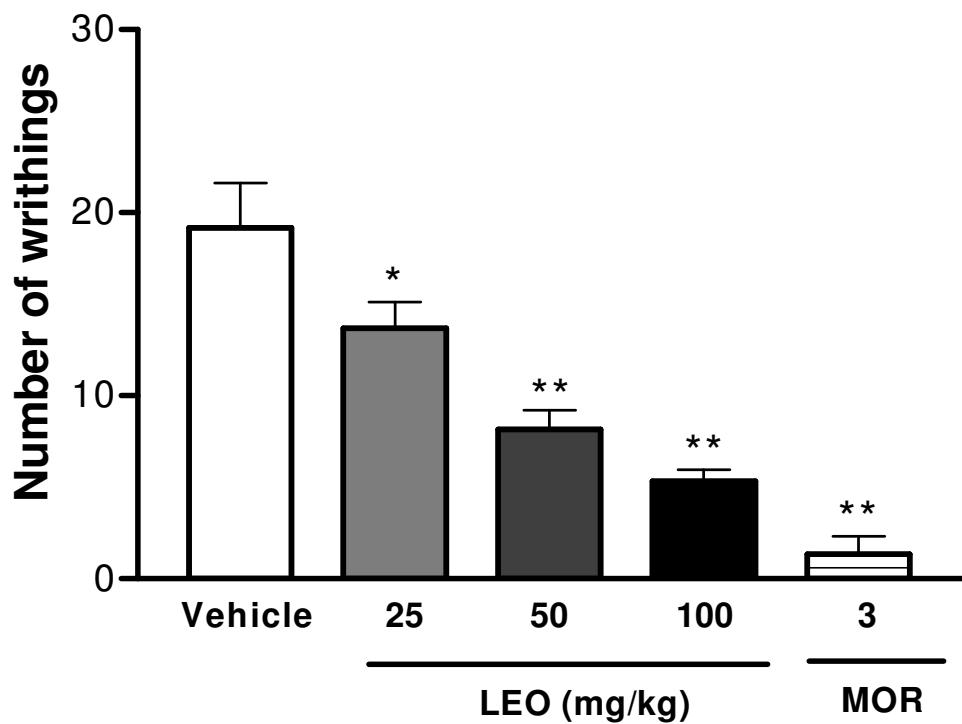


Figure 4. Antinociceptive effect of LEO in the acetic acid-induced writhing test in mice. Vehicle (control), LEO (25, 50 and 100 mg/kg, p.o.) or morphine (MOR) were administered 60 min before acetic acid injection. Values are mean \pm SEM for 10 mice, * $p < 0.05$ or ** $p < 0.001$, when compared with control, one-way ANOVA.

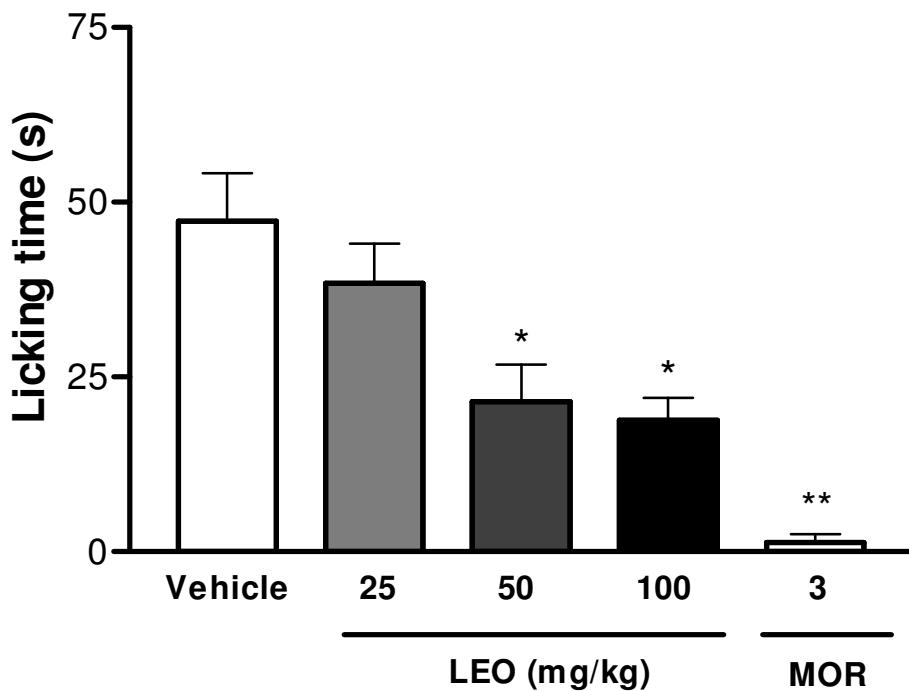


Figure 5. Antinociceptive effect of LEO in the capsaicin test in mice. Vehicle (control), LEO (25, 50 and 100 mg/kg, p.o.) or morphine (MOR) were administered 60 min before acetic acid injection. Values are mean \pm SEM for 10 mice, * p < 0.01 or ** p < 0.001, when compared with control, one-way ANOVA.

coordination of the animals in the rota-rod test, therefore, eliminating a nonspecific muscle relaxation effect of LEO at the doses used.

Acetic acid-induced is a standard, simple and sensitive test for measuring analgesia induced by both opioids and peripherally acting analgesics (Hunskaar and Hole, 1987). In this test, pain is elicited by the injection of an irritant such as acetic acid into the peritoneal cavity which produces episodes of characteristic stretching (writhing) movements and inhibition of the number of episodes by analgesics is easily quantifiable. Oral administration of LEO produced marked inhibition of the abdominal constriction. However, although, the writhing response test is very sensitive, it has a poor specificity as an analgesic screening test

Sakurada et al. (1992) proposed the capsaicin-induced pain model for the study of compounds that act on pain of a neurogenic origin. Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate) nitric oxide and proinflammatory mediators in the periphery and transmits nociceptive information to the spinal cord (Sakurada et al., 2003). The results indicate a significant reduction in neurogenic nociception caused by the intraplantar injection of capsaicin, showing that LEO caused significant effects in this model. LEO may be good candidates for the treatment of neuropathic conditions, in

which effective treatment is difficult (Akada et al., 2006).

CG-MS analyses showed a mixture of monoterpenes (main compounds): citronellal (36.19%), geraniol (32.82%) and citronellol (11.37%). Biological activities described for eugenol and citronellal include myorelaxant, anticonvulsant, antinociceptive and sedative (Quintans-Júnior et al., 2008b; Melo et al., 2010). Quintans-Júnior et al. (2010a) demonstrate analgesic effect of the citronellal on orofacial nociception in rodents. Another article published by our group demonstrate anti-convulsant activity of many monoterpenes, such as carvacrol, (–)-borneol and citral (Quintans-Júnior et al., 2010b). Additionally, De Sousa et al. (2006) demonstrated that citronellol possesses anticonvulsant activity due to the reduction of neuronal excitability mainly through the voltage-dependent Na⁺ channels and by facilitation of the inhibitory synaptic input by simply activating GABA_A.

Based on the results obtained, it is possible to suggest that LEO has CNS activities, as hypnotic, sedative and antinociceptive, which might involve a central GABAergic system. Pharmacological, toxicological and chemical studies are continuing, in order to characterize the precise mechanism(s) responsible for the CNS action and also to identify other monoterpenes present in *C. winterianus* leaf essential oil. Finally, the CNS action demonstrated in this study supported at least in part, the ethnomedical uses of this plant.

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