Journal of Ethnopharmacology 137 (2011) 828–836

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

The GABAergic system contributes to the anxiolytic-like effect of essential oil from *Cymbopogon citratus* (lemongrass)

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ARTICLE INFO

Article history:
Received 31 March 2011
Received in revised form 13 June 2011
Accepted 1 July 2011
Available online 7 July 2011

Keywords:
Anxiolytic
Cymbopogon citratus
Essential oil
GABAergic system
Lemongrass
Sedative

ABSTRACT

Ethnopharmacological relevance: The essential oil (EO) from *Cymbopogon citratus* (DC) Stapf is reported to have a wide range of biological activities and is widely used in traditional medicine as an infusion or decoction. However, despite this widely use, there are few controlled studies confirming its biological activity in central nervous system.
Materials and methods: The anxiolytic-like activity of the EO was investigated in light/dark box (LDB) and marble-burying test (MBT) and the antidepressant activity was investigated in forced-swimming test (FST) in mice. Flumazenil, a competitive antagonist of benzodiazepine binding and the selective 5-HT_{1A} receptor antagonist WAY100635 was used in experimental procedures to determine the action mechanism of EO. To exclude any false positive results in experimental procedures, mice were submitted to the rota-rod test. We also quantified some neurotransmitters at specific brain regions after EO oral acute treatment.
Results: The present work found anxiolytic-like activity of the EO at the dose of 10 mg/kg in a LDB. Flumazenil, but not WAY100635, was able to reverse the effect of the EO in the LDB, indicating that the EO activity occurs via the $GABA_A$ receptor–benzodiazepine complex. Only at higher doses did the EO potentiate diethyl-ether-induced sleeping time in mice. In the FST and MBT, EO showed no effect. Finally, the increase in time spent in the light chamber, demonstrated by concomitant treatment with ineffective doses of diazepam (DZP) and the EO, revealed a synergistic effect of the two compounds. The lack of activity after long-term treatment in the LDB test might be related to tolerance induction, even in the DZP-treated group. Furthermore, there were no significant differences between groups after either acute or repeated treatments with the EO in the rota-rod test. Neurochemical evaluation showed no amendments in neurotransmitter levels evaluated in cortex, striatum, pons, and hypothalamus.
Conclusions: The results corroborate the use of *Cymbopogon citratus* in folk medicine and suggest that the anxiolytic-like effect of its EO is mediated by the $GABA_A$ receptor–benzodiazepine complex.

1. Introduction

Anxiety and depressive disorders are the most common mental illnesses in the world and a prominent health care problem (Herrera-Ruiz et al., 2006; Yu et al., 2007). The World Health Organization (WHO) estimates that the prevalence of these diseases ranges from 0.1 to 16.9% (Demyttenaere et al., 2004). Benzodiazepines are the major class of compounds used to treat generalized anxiety disorder and acute anxiety states (Rickels and Schweizer, 1997; Rupprecht et al., 2006). However, these compounds have a number of undesirable effects such as sedation, muscle relaxation, amnesia, dependence and tolerance (Rickels and Schweizer, 1997; Reynolds, 2008). Therefore, the search for new therapeutic agents continues, and medicinal plants have emerged as a crucial source for the development of drugs to treat neurological disorders and play an important role for patients who respond poorly to conventional treatments (Carlini, 2003; Herrera-Ruiz et al., 2006).

Cymbopogon citratus (DC) Stapf (Poaceae), commonly known as lemongrass, is widely used in traditional medicine as an infusion or decoction for treating nervous disturbances (Carlini et al., 1986). In Mexico, *Cymbopogon citratus* is used as a sedative (Tortoriello and Romero, 1992), and in Brazil, an infusion or the cold juice of the leaves has been employed as a sedative and analgesic (Hiruma-Lima et al., 2002). An antinociceptive effect of *Cymbopogon citratus* has been detected in the rodent hot plate test, an experimental procedure related to central activity (Viana et al., 2000). In the previous work (Costa et al., 2006; Blanco et al., 2009), we have demonstrated that acute treatment with the essential oil (EO) from

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doi:10.1016/j.jep.2011.07.003

Cymbopogon citratus is effective against generalized anxiety disorder and epilepsy in experimental procedures in mice.

In the current study, we investigated the effect of the EO after long-term treatment and the putative synergistic effect of the EO and diazepam (DZP), a classic anxiolytic drug. We administered repeated dosages, instead of a single dosage, to mimic human drug regimens and to allow for reductions in individual dosages. To better characterize its central nervous system activity, the EO was also evaluated in different experimental procedures related to depressive and obsessive-compulsive disorders, thus complementing the anxiolytic and sedative/hypnotic protocols. Finally, the mechanism of action of the EO was investigated through blockade of the GABAergic or serotonergic neurotransmission systems. In this manner, we sought to elucidate the pharmacological activity of the EO from *Cymbopogon citratus* in order to validate its popular use as an anxiolytic or adjuvant drug in combination with classic drugs such as benzodiazepines; such a combination could represent a novel approach for the treatment of anxiety. Regarding that EO compounds are present in moderate amounts of the tea used by population, the development of an EO-based drug would be better controlled and standardized.

2. Materials and methods

2.1. Plant material

Plant seedlings of specimens from the garden of medicinal plants (Lageado Farm, UNESP) were cultivated at the Institute of Biosciences. The plant was identified in the BOTU Herbarium, Department of Botany, UNESP, where a voucher specimen (#23031) had been deposited. Leaves of *Cymbopogon citratus* were collected between May and June of 2008 and immediately processed to obtain the EO.

2.2. Essential oil extraction and phytochemical analysis

Fresh leaves were processed with a Clevenger apparatus, and the EO was obtained by hydrodistillation, protected against light and heat, and stored until behavioral assays. Previously, the EO was analyzed by gas chromatography coupled with mass spectrometry (GC/MS) as described (Blanco et al., 2009).

Thin layer chromatography (TLC) of EO and decoction was performed on Silica gel 60 (250 μm, 10 cm × 20 cm, Sigma–Aldrich® plates) according to previously described methodology (Akisue et al., 1996). Plates were developed by using a mobile phase composed of toluene:ethyl acetate (93:7, v/v). Standards (citral, β-myrcene, and geraniol) were spotted together with plant preparations under the same conditions and Rf values were used for confirmation of their chemical identity. *Cymbopogon citratus* EO, standards, and decoction were dissolved in dichloromethane. After elution, plates were dried, sprayed with anisaldehyde–sulfuric acid or vanillin–sulfuric acid reagents and heated at 110 °C for visualization. All solvents employed for TLC were of analytical grade.

2.3. Animals

All experiments were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Biosciences Institute – Ethics Committee for Animal Research (CEEA). Adult Swiss male mice (30 days old) from the colony at the UNESP Central Animal House were used in all experiments after a one-week adaptation period at the Animal House of the Department of Pharmacology. Thus, the animals used were approximately 40–45 days old. The animals were maintained under controlled environmental conditions of temperature (21 ± 2 °C) and light (12/12 light/dark cycle) with food and water *ad libitum* until 2 h prior to the experimental procedures.

2.4. Drugs

Diazepam (DZP, Germed – EMS, Brazil) was used as the standard anxiolytic and hypnotic drug and imipramine hydrochloride (IM, Sigma–Aldrich, USA) as the standard antidepressant drug. Flumazenil (FLU, Flumazil® – Cristália, Brazil) was utilized as a competitive antagonist of benzodiazepine binding, and WAY100635 (WAY, Sigma–Aldrich) was used as a highly selective 5-HT_{1A} antagonist. For intraperitoneal injections (i.p.), DZP and IM were dissolved in isotonic saline (SAL – 0.9% NaCl). For oral administration (p.o.), the EO was dissolved in 0.01% (v/v) polyoxyethylene sorbitan monooleate (TW – Tween 80®, Sigma–Aldrich) in saline, which was used to treat control groups. All samples were freshly prepared on the test day and administered at 10 ml/kg of body weight, except FLU, which was administered at 20 ml/kg due to the concentration (0.1 mg/ml) of its commercial form.

2.5. Treatments

In a preliminary experiment, the behavioral data did not differ among the animals that received the three vehicle solutions – Tween (TW), saline and water – (data not shown), which justified our choice to include only the TW control group data for comparison. For the single treatment, mice were treated 30 min before experimental procedures, whereas for long-term treatment (WHO, 1993), animals were treated with the EO for 21 days and exposed to the experimental procedure 30 min after the last treatment. For evaluation of antidepressant activity, mice were treated three times within the 24 h before the experimental procedure. To address a possible contribution from the GABAergic system, mice were co-administered DZP (1 mg/kg, i.p.) or the EO (10 mg/kg, p.o.) 30 min before the test and then were given FLU (2 mg/kg, i.p.) 15 min before testing. To evaluate possible interference from the serotonergic system, animals were pretreated with WAY (0.1 mg/kg, i.p.) and 15 min afterwards, received the EO (10 mg/kg) or the standard drug DZP (1 mg/kg); the experimental procedure was carried out 30 min after the last drug treatment. To evaluate a putative synergism between treatments, groups of animals were simultaneously treated with non-effective doses of DZP (0.25 mg/kg, i.p.) and the EO (2.5 mg/kg, p.o.).

2.6. Neurochemical evaluation

Animals treated with TW or EO (10 mg/kg) were euthanized and their brains were rapidly dissected (not more than 3 min after extraction) on ice. Briefly, the striatum, hypothalamus, pons, and frontal cortices were dissected, weighed, and stored in liquid nitrogen until the neurochemical analyses.

Following sample collection, tissues were homogenized in 0.1 M perchloric acid by manual sonication, centrifuged at 10,000 rpm for 20 min to remove supernatant and stored at −80 °C until the determination of monoamine levels.

Neurotransmitters and their metabolites – dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA) – were measured by reverse-phase using an high performance liquid chromatography (HPLC) system (model 6A, Shimadzu, Kyoto, Japan) with an electrochemical detector as described in detail elsewhere (Felicio et al., 1996). Briefly, 20 μl samples were loaded into a sample injector and the mobile phase was delivered at a constant rate of 1.2 ml/min. The runtime for each sample was 15 min and concentrations of all neurotransmitters and

their metabolites were expressed as nanograms per gram of tissue (ng/g).

2.7. Behavioral procedures

Mouse behavior in the forced-swimming test and light/dark box test was videotaped under white light illumination using a video camera and a computer. Digital video was subsequently played, and behaviors were scored by a highly trained observer who was blind to treatment condition. In other procedures, the behaviors were registered in real time. All experimental procedures occurred between 9:00 am and 5:00 pm.

2.7.1. Marble-burying test (MBT)

The marble-burying procedure is based on a defensive burying behavior that can be elicited in rodents in response to aversive stimuli such as a shock prod, noxious food or an effective unconditioned stimulus such as glass marbles (Poling et al., 1981; Broekkamp et al., 1986). The mice used in the marble-burying procedure were previously selected to represent those that exhibit a stable burying score (at least 13 out of 25 marbles) for three consecutive days before the test (Pultrini et al., 2006). Thirty minutes after treatments with EO (5, 10 or 50 mg/kg), the animals were placed individually in cages (27 long × 16 wide × 13 cm high) with 25 glass marbles uniformly distributed on a 5-cm layer of sawdust. Each cage was covered with a smooth, acrylic, transparent plate with small punctures for ventilation. The mice were left in the cages with the marbles for 30 min, after which they were removed to count the number of marbles that were totally hidden by sawdust.

2.7.2. Forced-swimming test (FST)

This test is the most widely used and recognized pharmacological model for assessing antidepressant activity (Petit-Demouliere et al., 2005). This procedure was carried out following a modified version (Cryan et al., 2002) of the conditions proposed by Porsolt et al. (1977, 1978). The apparatus consisted of a clear Plexiglas cylinder (21 cm high × 20.5 cm diameter) filled with water (23 ± 2 °C) to a depth of 13 cm. Each animal underwent a pre-test swimming session for 15 min; the pre-tests were carried out 24 h prior to the 5-min swimming tests. At the end of the swimming sessions, the animals were removed from the water and gently dried. Mice were treated with EO at 5, 10 or 50 mg/kg according to the following schedule: the first treatment (T1) was administered 1 h after finishing the pre-test session and subsequently 5 h (T2) and 30 min (T3) before the test session. The digital video recording of each mouse in the water was evaluated using a time-sampling technique (Detke et al., 1995) to classify in 10-s bins the behavioral response into one of these three possible categories: *climbing behavior*, defined as upward-directed movements of the forepaws along the side of the swimming chamber; *swimming behavior*, defined as movements throughout the swimming chamber; and *immobility* time, defined as time during which the mouse remained floating in the water and made only the movements necessary to maintain its head above the water (Cryan et al., 2002).

2.7.3. Sleep induced by diethyl-ether (SID)

The effectiveness of the EO was measured by its capacity to reduce the latency to sleep and/or to increase the duration of sleep without interfering with hepatic metabolism (Tsuji et al., 1996). Thirty minutes after the treatments with EO (500, 1000 or 1500 mg/kg), animals were induced to sleep by inhalation of diethyl-ether in a 3-L flask. Each mouse was placed individually in a cylindrical glass chamber saturated for 20 min with vapor delivered from 5 ml of diethyl-ether absorbed on a piece of cotton bound in the coiled cap of the flask. Each mouse was left there for 60 s after loss of its postural reflex – sleep latency was defined as time until loss of the postural reflex – and then was withdrawn and placed in an individual cage to record the time to recover the postural reflex (sleep duration) (Héllión-Ibarrola et al., 2006).

2.7.4. Light/dark box test (LDB)

The light/dark box (46 long × 27 wide × 30 cm high) was divided into the typical dimensions of one-third for the dark compartment with black walls, floor and roof and two-thirds for the light compartment with white walls and floor and illuminated by a 20-W fluorescent lamp. The light and dark compartments were connected by an opening (7.5 cm × 7.5 cm) located at floor-level in the center of the partition (Crawley and Goodwin, 1980). Thirty minutes after treatment with EO (5, 10, 50 or 100 mg/kg), each animal was individually placed in the center of the light compartment, facing the dark one, and observed for 5 min after the first entry into the dark compartment. During this period, we recorded the number of shuttle crossings (an activity-exploration index), number of rearings and the time spent in the light compartment, which is the most consistent and useful measure for assessing anxiolytic-like activity. This parameter provides consistent dose–effect results for anxiolytic drugs including benzodiazepines, 5-HT_{1A} receptor agonists or 5-HT_3 receptor antagonists (Young and Johnson, 1991; Bourin and Hascoët, 2003).

2.7.5. Rota-rod test (RRT)

The rota-rod procedure was described by Dunham and Miya (1956) and is suitable for detecting motor impairment due to pharmacological agents such as skeletal muscle relaxants or central nervous system depressants. The apparatus consisted of a non-slippery plastic rod, 3.0 cm in diameter and 50 cm in length, rotating at 5 rpm at a height of 25 cm. Immediately after the LDB, each mouse was placed on the apparatus to register the total time it performed on the rotating bar in three successive trials totalling 1 min. To avoid a bias on account of inability not related to drug treatment, one day prior to testing animals were evaluated, using the same conditions as those in the test, to select those that had the ability to walk on the bar.

2.8. Statistical analysis

Quantitative data from behavioral tests were subjected to Kruskal–Wallis non-parametric variance analysis, followed by the Mann–Whitney test when appropriate. Proportions in the RRT were compared by Fisher's exact test. Neurochemical data were analyzed by unpaired *t*-test. All statistical analysis was made using GraphPad InStat® version 3.02. Contrasts were made between the treated and TW (vehicle) groups, and differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Essential oil composition

The chromatographic analysis revealed spots with similar qualitatively migration patterns. It is possible to observe the presence of the three major compounds (standards) in both *Cymbopogon citratus* EO and decoction, through TLC (Supplementary data).

The main EO compounds were identified by the retention time and retention index as monoterpene citral (71.29%), a mixture of the stereoisomers geranial and neral, or β-myrcene (16.5%). Other compounds detected in the EO include geraniol (1.28%), 6-methyl-5-hepten-2-ona (0.64%), and undecan-2-one (0.29%), as detailed in Bidinotto et al. (2010).

3.2. Neurochemical evaluation

The acute treatment with lemongrass EO did not result in neurotransmitters and it metabolites changes (DA, DOPAC, HVA, 5HT, and 5HIAA) evaluated in cortex, striatum, pons and hypothalamus (Table 1).

3.3. Effect of the EO on the forced-swimming test (FST) and marble-burying test (MBT)

The EO administered at 5, 10 or 50 mg/kg was unable to modify parameters evaluated in the FST or MBT, as shown in Table 2. Imipramine, the standard drug in both experimental procedures, significantly decreased the number of immobility events and concomitantly augmented the number of climbing and swimming events during the FST while also reducing the number of marbles buried in the MBT.

3.4. Effect of the EO on diethyl ether-induced sleep

The EO significantly decreased sleep latency after treatment at 1000 or 1500 mg/kg and increased sleep duration at the same doses, as did the standard drug DZP (5 mg/kg). Data are presented in Table 2.

3.5. Anxiolytic-like effect of the EO in the LDB

The results obtained in the LDB test after acute treatment with the EO (Fig. 1, left panel) reveal a significant increase in time spent in the LDB light chamber after acute administration of the EO at 10 mg/kg when compared to the TW group. A discrete increase in exploratory parameters (number of transitions between compartments and, especially, rearing) could be observed after treatment with either the DZP or the EO. However, neither the DZP nor the EO, administered for 21 days, was able to modify the time spent on the illuminated side of the LDB.

Additionally, the group that was repeatedly treated with saline and that received one treatment with DZP 30 min before the experimental procedure showed an anxiolytic-like effect analogous to acute DZP treatment (Fig. 1, right panel). This result validates the experimental conditions used in the LDB session and confirms the absence of an anxiolytic-like effect in mice repeatedly treated with DZP. Reductions in the number of rearings and transitions between compartments were observed after long-term treatment with 100 mg/kg of the EO, which was the same trend observed after long-term treatment with DZP (Fig. 1B and C).

3.6. The role of GABA or 5-HT neurotransmission on the anxiolytic-like effect of the EO in the LDB

Experimental sessions to evaluate the interference of flumazenil (FLU) or WAY100635 (WAY) in the acute effect of the EO (10 mg/kg) or DZP (1 mg/kg) were carried out independently; the results are presented in Fig. 2. Results from FLU or WAY alone did not differ from those obtained from the TW group, showing no *per se* effect of these blockers on evaluated parameters. Mice treated with DZP or the EO spent more time in the light chamber, when compared with the TW group – an effect not observed in mice treated concomitantly with FLU, a benzodiazepine antagonist (Fig. 2A, left panel). However, concomitant treatment with WAY not only was unable to block the effect of DZP, as expected, but also could not block the effect of the EO (Fig. 2A, right panel). Neither FLU nor WAY could reverse the effect of DZP or the EO on the exploratory or motivational parameters such as the numbers of rearings and transitions between chambers (Fig. 2B and C).

Table 1
Neurotransmitter levels and their metabolites (ng/g) after acute oral treatment with *Cymbopogon citratus* EO (10 mg/kg).

	Structures and treatments							
	Cortex		Striatum		Pons		Hypothalamus	
	TW ($N=12$)	EO ($N=8$)	TW ($N=11$)	EO ($N=7$)	TW ($N=11$)	EO ($N=8$)	TW ($N=12$)	EO ($N=8$)
DA	722.35 ± 744.32	965.83 ± 1188.11	8324.29 ± 1715.02	8712.33 ± 3507.29	26.62 ± 17.60	36.00 ± 23.71	53.08 ± 12.14	62.72 ± 14.97
DOPAC	94.63 ± 55.36	77.27 ± 57.60	437.36 ± 147.60	435.87 ± 213.41	22.44 ± 8.73	24.33 ± 13.99	58.64 ± 19.48	53.96 ± 31.37
HVA	427.32 ± 278.46	332.11 ± 218.76	1121.94 ± 320.96	892.25 ± 324.48	300.94 ± 281.20	305.98 ± 437.55	236.17 ± 127.67	390.57 ± 348.13
5HT	1246.99 ± 297.33	1246.72 ± 257.36	1686.34 ± 339.54	1739.64 ± 495.20	2601.34 ± 793.69	2549.14 ± 1005.78	1660.08 ± 437.24	1487.79 ± 290.16
5HIAA	192.72 ± 60.71	153.07 ± 33.83	317.12 ± 102.38	306.52 ± 156.38	869.54 ± 226.91	745.84 ± 271.76	341.73 ± 72.53	283.28 ± 47.89

TW (Tween). Data are presented as mean ± SD. Unpaired *t*-test.
N is different in each group as some structures could not be evaluated due to technical reasons.

Table 2
Effects of essential oil (EO) from *Cymbopogon citratus* in the forced-swimming test, marble-burying test and sleep induced by diethyl-ether inhalation.

Forced-swimming test					Marble-burying test			Sleep induced by diethyl-ether inhalation			
Treatment	*N*	Immobility	Climbing	Swimming	Treatment	*N*	Marbles buried	Treatment	*N*	Latency (s)	Sleeping time (s)
TW	7	25 (24–27)	2 (1–2)	4 (3–4)	TW	6	23 (19–24)	TW	7	47 (44–54)	102 (97–107)
IM	8	18 (16–19)***	6 (3–7)*	8 (7–11)**	IM	6	9 (6–12)*	DZP	7	34 (29–49)	177 (169–207)**
EO 5	8	26 (23–28)	2 (1–4)	2 (1–4)	EO 5	6	24 (23–24)	EO 500	8	38 (34–43)	115 (106–156)
EO 10	8	25 (24–27)	2 (1–2)	3 (1–5)	EO 10	6	20 (15–21)	EO 1000	7	35 (33–35)**	131 (116–156)*
EO 50	8	26 (25–27)	2 (1–3)	3 (2–3)	EO 50	6	21 (14–23)	EO 1500	8	29 (25–33)**	146 (123–191)**

TW (Tween); IM (imipramine hydrochloride); DZP (diazepam).
Data are expressed as median and interquartile range (Q1–Q3).
* $p \leq 0.05$ in relation to TW group (Kruskal–Wallis followed by Mann–Whitney *U*-test).
** $p \leq 0.01$ in relation to TW group (Kruskal–Wallis followed by Mann–Whitney *U*-test).
*** $p \leq 0.001$ in relation to TW group (Kruskal–Wallis followed by Mann–Whitney *U*-test).

3.7. Effect of combined administration of DZP and the EO in the LDB

The upper left corner of Fig. 3 displays the observed effect of DZP treatment at 1.0 mg/kg, the effective dose in the LDB. Thus, mice treated with 0.25 mg/kg of DZP or with 2.5 mg/kg of the EO did not spend more time than the TW group on the illuminated side of the apparatus, the main anxiety parameter in the LDB. The EO at 5.0 mg/kg showed a significant but discrete augmentation in this parameter. Furthermore, when mice were treated with a combination of DZP and the EO, at these non-effective doses, the anxiolytic-like effect was evident, show-

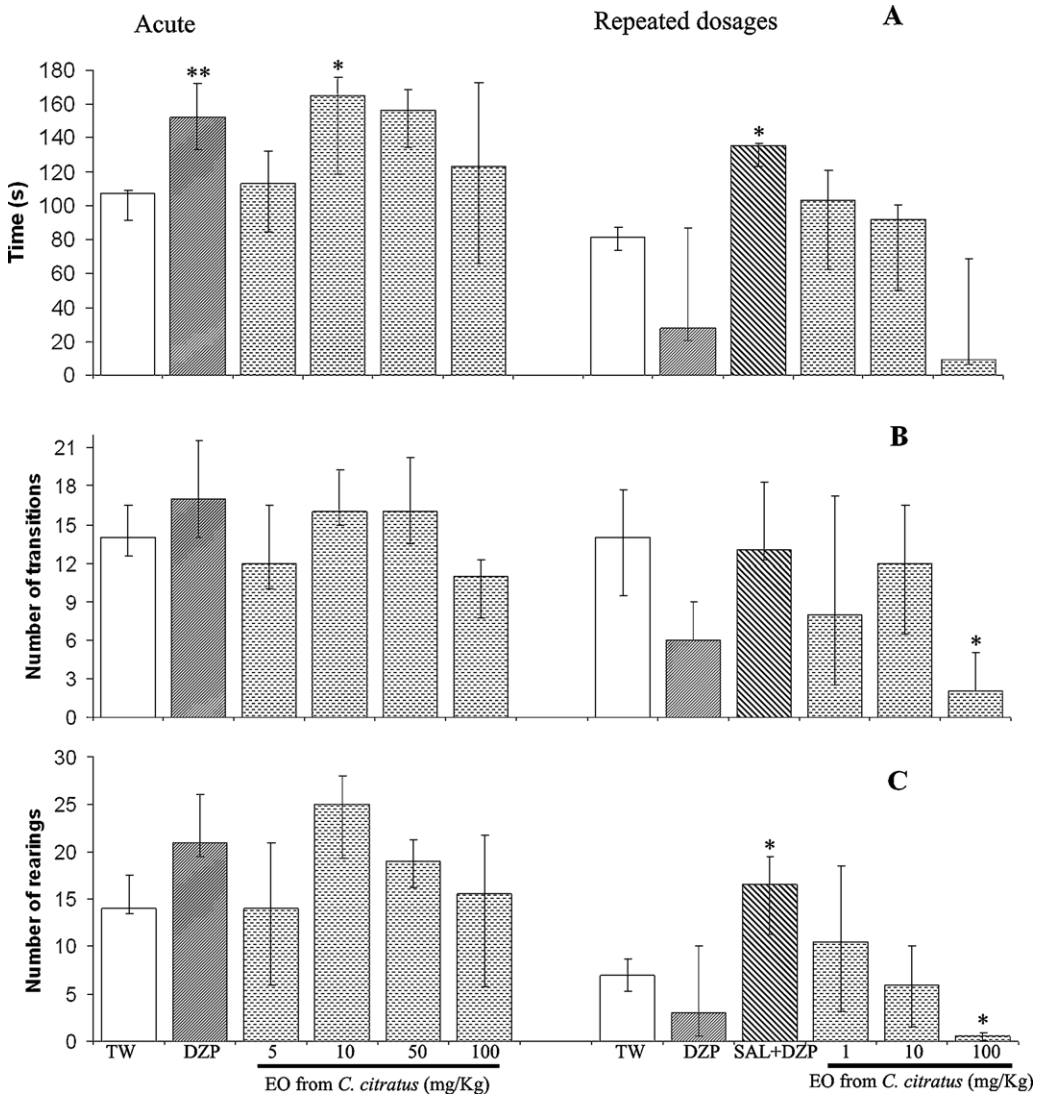

Fig. 1. Median and interquartile range (Q1–Q3) of time (in seconds) spent in the illuminated chamber (A), number of transitions (B) and number of rearings (C) in the light/dark box after acute or long-term treatment with essential oil (EO) from *Cymbopogon citratus* (p.o.); TW – vehicle, 10 ml/kg (p.o.); SAL – saline, 0.9% (p.o.); DPZ – diazepam, 1 mg/kg (i.p.). *N* = 6–8 animals per group. *$p \leq 0.05$, **$p \leq 0.01$ in relation to TW group (Kruskal–Wallis followed by Mann–Whitney *U*-test).

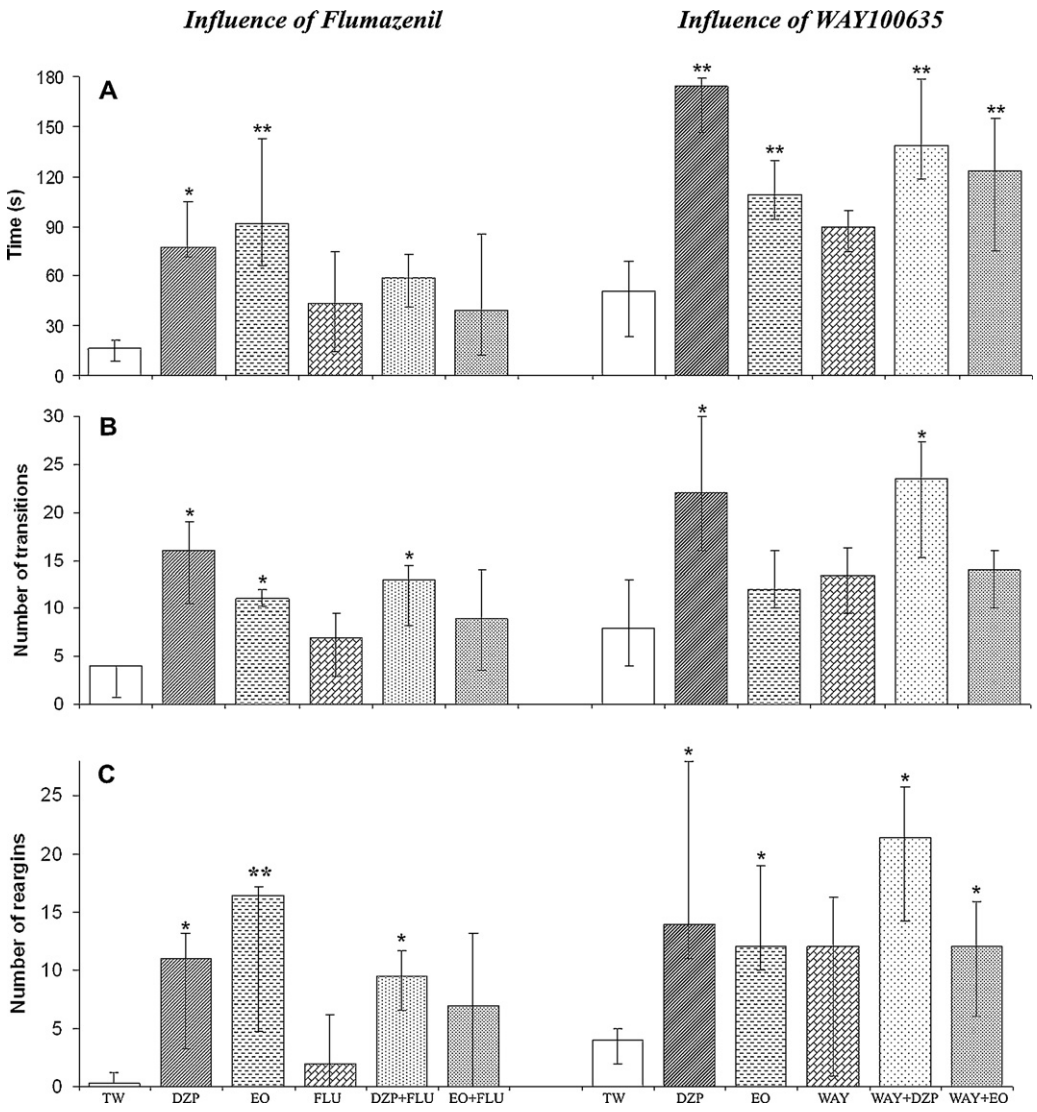

Fig. 2. Influence of flumazenil (FLU, 2.0 mg/kg, i.p.) or WAY100635 (WAY 0.1, mg/kg, i.p.) on the anxiolytic effect of diazepam (DZP, 1 mg/kg, i.p.) or essential oil (EO, 10 mg/kg, p.o.), expressed as the median and interquartile range (Q1–Q3) of time (in seconds) spent in the illuminated chamber (A), number of transitions (B) and number of rearings (C) in the light/dark box; TW – vehicle, 10 ml/kg (p.o.). $N = 6–9$ animals per group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ in relation to TW group (Kruskal–Wallis followed by Mann–Whitney *U*-test).

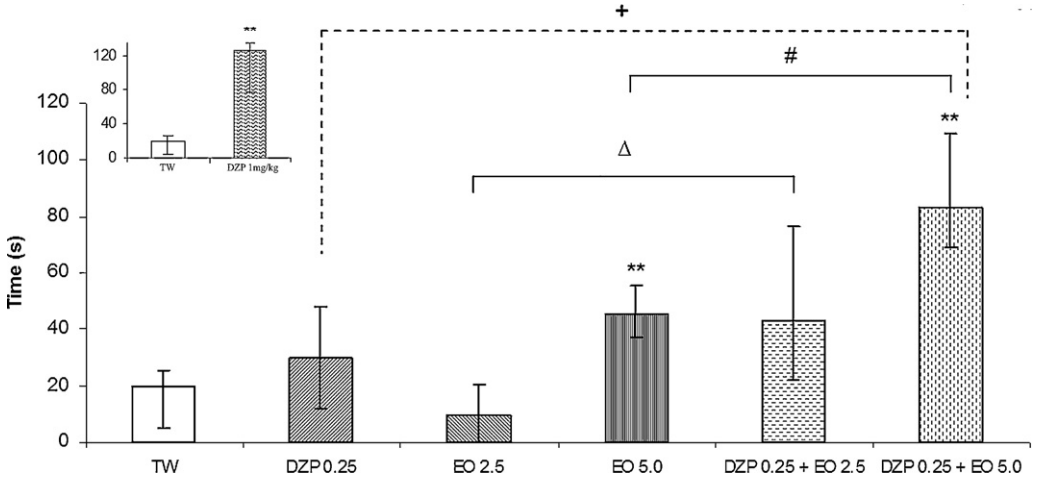

Fig. 3. Effect of co-administration of essential oil (EO – 2.5 or 5.0 mg/kg, p.o.) 30 min after an ineffective dose of diazepam (DZP – 0.25 mg/kg, i.p.) treatment, expressed as the median and interquartile range (Q1–Q3) of time (in seconds) spent in the illuminated chamber of the light/dark box. TW – vehicle, 10 ml/kg (p.o.). The small figure embedded, upper left corner, represents the observed effect after DZP treatment at 1 mg/kg under the same experimental conditions. $N = 6–9$ animals per group. $^{**}p \leq 0.01$ in relation to TW group; $^{\#}p \leq 0.05$ in relation to EO 5.0 mg/kg group; $^{\triangle}p \leq 0.05$ in relation to EO 2.5 mg/kg group; $^{+}p \leq 0.05$ in relation to DZP 0.25 mg/kg group (Kruskal–Wallis followed by Mann–Whitney *U*-test).

Table 3
Effects of treatments of essential oil (EO) on motor coordination evaluated by the rota-rod test.

Treatment	Motor impairment	
	Acute	Repeated dosages
TW	1/6	1/6
DZP 1 mg/kg	0/5	0/6
EO 1 mg/kg	Not evaluated	0/6
EO 10 mg/kg	0/5	0/7
EO 100 mg/kg	Not evaluated	1/7

TW (Tween); DZP (diazepam).
Results are expressed as the fraction of total animals in each experimental group that were unable to perform the test. Fisher's exact test.

ing the mutual potentiation of action between the EO and DZP (Fig. 3).

3.8. Effect of the EO and DZP on the rota-rod test

The rota-rod test was carried out to measure locomotor impairment in mice treated acutely or repeatedly with DZP or the EO. The results, presented in Table 3, include the number of disabled mice in relation to the total number of treated animals in each experimental group. The disabled mice, defined as those that could not stay on the rotating bar in a maximum of three attempts at 5 rpm for 1 min, did not differ statistically from the TW group, suggesting an absence of treatment-induced motor impairment.

4. Discussion

The already considerable attention directed at the use of natural essential oils from plants is continuing to increase. The widespread uses of these medicinal oils for bactericidal, antiparasitic, insecticidal and medicinal applications are examples of their pharmaceutical use (Bakkali et al., 2008; Fandohan et al., 2008).

Despite the frequent use of *Cymbopogon citratus* (lemongrass) in traditional medicine, there are few controlled studies confirming its biological activity. According to a review of herbal remedies for anxiety (Ernst, 2006), there has been only one placebo-controlled study on Brazilian lemongrass tea, conducted by Leite et al. (1986). Recently, our laboratory demonstrated the anxiolytic-like activity of the EO from *Cymbopogon citratus* in experimental models of generalized anxiety disorder and epilepsy (Costa et al., 2006; Blanco et al., 2009) at 500 and 1000 mg/kg.

The observation in mice of antinociceptive activity of the EO from *Cymbopogon citratus* at the dose of 25 mg/kg (Viana et al., 2000) and an anticonvulsant effect of the EO from *Cymbopogon winterianus* (Quintans-Júnior et al., 2008) at doses below 400 mg/kg encouraged us to evaluate the anxiolytic activity of lower doses of the EO from *Cymbopogon citratus*. This approach showed positive results in anxiety-related parameters in the LDB, in which EO was active at 10 mg/kg. The biphasic dose–response (U-shaped) curve observed in the experiment is similar to the curves extensively studied by Calabrese and Baldwin (2003). This is an example of the dose–response relation noticeable in the field of neuroscience, especially in pre-clinical screening tests for central nervous system therapeutic agents (Carvalho-Freitas and Costa, 2002; Calabrese, 2008).

The anxiolytic effect of benzodiazepines is due to their binding to $GABA_A$ receptor at a site distinct from the GABA binding site (Whiting, 2003). To determine whether the anxiolytic-like effect of the EO in the LDB occurs through the GABAergic system, mice treated with the EO or DZP were co-administered FLU, a benzodiazepine $GABA_A$ site antagonist. This procedure reversed the anxiolytic-like activity of not only the standard drug DZP but also the EO (Fig. 2). The ability of FLU, a specific $GABA_A$ receptor antagonist, to antagonize the anxiolytic-like activity of the EO strongly suggests, for the first time, a role of this receptor complex in mediating EO activity. Furthermore, according to Silva et al. (2010) the EO from *Cymbopogon citratus* administered i.p. had anticonvulsant effect, by intraperitoneal route, which was blocked by the pretreatment with flumazenil, suggesting and enhancing a possible effect on the GABAergic neurotransmission system. The authors did not exclude a possible interference of other systems (e.g., glycinergic) in such activity.

The neurochemical assessment revealed no alterations in neurotransmission mediated by dopamine and serotonin after acute oral treatment with EO. It has been suggested that serotonin could be a central neurotransmitter involved in the modulation of anxiety and anti-anxiety effects of benzodiazepines, however neurochemical studies on the effects of that class of drugs on brain serotonin metabolism/or turnover are not very consistent (Batool and Haleem, 1997). On the other hand, the diazepam seems to alter levels of dopamine in the nigrostriatum of rats only at doses greater than those with anxiolytic and anticonvulsant effects (Invernizzi et al., 1991). Further, the role of mesolimbic DA projections in the effects of benzodiazepine-site ligands is still unclear (Heikkinen et al., 2009).

Even over a large range of doses, the EO produced no effect in the experimental models of obsessive-compulsive disorder (MBT) and depression (FST). These experimental models are not sensitive to treatment with benzodiazepines but are sensitive to drugs like imipramine, which is clinically useful for treating obsessive-compulsive disorder and depression. Therefore, it was not surprising that the EO activity was not disrupted by previous exposure to the selective 5-HT_{1A} antagonist WAY100635. In our experience, WAY at 0.1 mg/kg is able to block the activity of imipramine (30 mg/kg) in the FST (data not shown). In this manner, the WAY and EO results corroborate the hypothesis that the anxiolytic-like activity of the EO is mediated by the GABAergic system.

When doses of DZP (0.25 mg/kg) and the EO (2.5 mg/kg) that were ineffective *per se* were co-administered, they had a synergistic effect on each other. In other words, mice treated with DZP and the EO at their respective ineffective doses showed a significant increase in time spent in the LDB light chamber. In addition, mice treated with effective doses of the EO (5.0 mg/kg) and DZP showed a significantly elevated light-chamber duration, when compared to the EO 5.0 mg/kg alone or with DZP (Fig. 3).

After a single treatment with the EO, mice showed a significant increase in LDB parameters, in contrast to long-term treatment, either with the EO or with the DZP, which produced no such increases (Fig. 1). In this procedure, only the animals repeatedly treated with saline that received DZP 30 min before the experimental procedure showed significant differences on the LDB anxiety parameters. Long-term treatment with the EO at 100 mg/kg produced a behavioral profile similar to that observed with DZP in the same conditions, with statistical significance in reducing the number of rearings and transitions between black and white compartments, without observable effects on motor coordination in the rota-rod test (Table 3). The reduction in efficacy after long-term treatment with DZP, and perhaps with the EO, could be due to the development of tolerance (Reynolds, 2008), as already described in mice after two weeks of continuous DZP use (Flaishon et al., 2003) and which is an EO hypothesis also assessed in other species (Pultrini et al., 2006).

The EO was effective over a wide dose range. Its sedative effects reflected in the sleep induction studies were achieved at doses that are 100-fold higher than those found effective in the LDB. In the experimental procedure involving ether-induced sleeping time, only at 1500 mg/kg did the EO decrease the latency to sleep and increase the sleeping time, which proved to be a specific central nervous system effect. According to Tsuji et al. (1996), this effect is

not related to a pharmacokinetic interaction with hepatic enzymes, a recognized limitation of barbiturate-induced sleeping time. The decrease in spontaneous motor activity (after long-term treatment) and potentiation of ether-induced sleeping time (after acute treatment at higher doses) strongly suggest central depressant activity of the EO.

To exclude any false positive results in experimental procedures related to anxiety disorders, mice underwent the rota-rod test. All animals treated with different EO doses after acute or long-term treatment showed no impairment of the locomotor system (Table 3), thus confirming that the EO from *Cymbopogon citratus* is anxiolytic in rodents without any motor impairment.

The absence of toxicity signals or symptoms in mice, whether treated acutely or repeatedly, at the doses used in the present study shows the relative safety of the EO. The absence of a deleterious effect on the morphological structure of the stomach or liver in rats treated sub-acutely with doses up to 1500 mg/kg has been described previously (Fandohan et al., 2008). However, the major compound in the EO from *Cymbopogon citratus* induced maternal toxicity in pregnant rats at doses higher than 125 mg/kg (Nogueira et al., 1995). This observation confirms that acute toxicity data limit the pre-clinical applications of the EO because a cumulative toxic effect does occur even at very low doses (Mabeku et al., 2007), reinforcing the importance of complementary toxicological studies to evaluate the safety of the EO as a complementary therapy in folk medicine.

Finally, essential oils are complex mixtures of numerous molecules. The major compounds of the EO from *Cymbopogon citratus* were identified by GC/MS (Bidinotto et al., 2010) as follows: citral (a mixture of the cis and trans isomers – neral and geranial) followed by myrcene and geraniol. According to Vale et al. (2002), citral and myrcene were not able to modify parameters of anxiolytic activity in mice, as evaluated by the elevated plus maze. These data reinforce the idea that the major compounds are not always responsible for biological activity and that such activity cannot always be attributed to only one constituent from the herbal preparation, according to the concept of a multitargeted approach as a therapeutic strategy (Wagner and Ulrich-Merzenich, 2009). Moreover, for biological purposes, it is more informative to study whole oil rather than some of its components because the concept of synergism appears to be more meaningful (Bakkali et al., 2008). This possible synergism may be present also in the decoction, once a qualitatively similar composition was observed in both EO and herbal preparation. In conclusion, the data presented hereby reinforce the traditional use of *Cymbopogon citratus* in the folk medicine due to its anxiolytic-like properties.

Conflict of interest

The authors declare that they have no conflicts of interest.

Role of the funding source

This work was supported by grants from FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo (Celso A.R.A. Costa – Proc. no. 2006/07195-8) and CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (André C. Gargano). The FAPESP and CAPES had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Acknowledgements

We are grateful to MSc. Filipe Galvão Ferreira, MSc. Flávia Bonamin and Dr. Leonardo Noboru Seito for technical assistance. We are also grateful to MSc. Lucas Tadeu Bidinotto for the improvements and advices in the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2011.07.003.

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