

# Essential Oils from *Citrus latifolia* and *Citrus reticulata* Reduce Anxiety and Prolong Ether Sleeping Time in Mice

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## ABSTRACT

Essential oils (EO) from *Citrus reticulata* and *Citrus latifolia* were submitted to classical experimental procedures, such as light-dark box and marble-burying tests with male Swiss mice, to evaluate anxiolytic activity. Sedative activity was also investigated with EO from *C. aurantium* using the sleeping time induced by ether inhalation. EOs were administered 30 min before the experiments in doses ranging from 0.5 to 2.0 g/kg (w/v). EO from *C. latifolia* showed a positive effect on light-dark box parameters, and those from both *C. reticulata* and *C. latifolia* were able to reduce the number of buried marbles. Positive results were obtained using experimental procedures related to generalized anxiety (light-dark box) and obsessive-compulsive disorders (marble-burying), suggesting some differences in the spectrum of anxiolytic activity. EO from all citrus species significantly increased the duration of sleep induced by ether inhalation, disregarding the potential interaction with hepatic enzymes, which is a limitation of barbiturate sleeping time. On the other hand, this effect was detected only with higher doses, emphasizing that specificity of this action should be investigated. Results obtained with EOs from *C. reticulata* and *C. latifolia* confirm the central nervous system activity previously described for *C. aurantium*. Further investigation is warranted to guarantee the safe use of EOs by the population.

**Keywords:** light-dark box test, marble-burying test, sleeping time induced by ether inhalation

**Abbreviations:** i.p., intraperitoneally (administration in the peritoneal cavity); p.o., per os (administration by oral route)

## INTRODUCTION

Traditional populations in several countries usually refer to *Citrus* species as useful in reducing symptoms of anxiety or insomnia. Habitually used as decoction (Giron *et al.* 1991; Longuefosse and Nossin 1996) or infusion (Tortoriello and Romero 1992; Vásquez *et al.* 1997) of flowers and/or leaves, *Citrus* preparations have been most frequently mentioned for treatment of problems related to the nervous system in inquiries made in Mexico (Tortoriello and Romero 1992) as well as in Puerto Rico (Hernandez *et al.* 1984).

Sedative and anxiolytic effects were previously described, in mice, with the essential oil obtained from the peel of *Citrus aurantium* L. (Carvalho-Freitas and Costa 2002; Pultrini *et al.* 2006). These activities occurred in the absence of toxic or deleterious effects.

The related species *Citrus latifolia* Tanaka and *Citrus reticulata* Blanco are also commonly cited as minimizing central nervous system disorders (Lawless 1995) and are more frequently encountered in backyards and orchards in Brazil than *C. aurantium*. Thus, the present study aimed to investigate the anxiolytic and sedative properties of essential oil obtained from ripe fruit peels of *C. latifolia* and *C. reticulata*.

## MATERIALS AND METHODS

### Plant material extraction and identification of main compounds

Fruits from *Citrus latifolia* Tanaka, *Citrus reticulata* Blanco and *Citrus aurantium* L. were collected from adult plants, producing fruits for 6-8 years. Immediately after collection, fruits were washed in potable water, peeled and the essential oil (EO) from peels was extracted by hydrodistillation in a Clevenger apparatus.

The EO from each plant was kept in amber flasks and stored in a freezer until used in the pharmacological assays. Samples of the EO were suspended in 1 ml of ethyl acetate (P.A., Merck, Germany) and 1 µl of this solution was analyzed by gas chromatography coupled with mass spectrometry (GC/MS, Shimadzu, model QP-5000) equipped with a fused silica capillary column DB-5 (30 m × 0.25 mm 0.25 µm). The electron impact technique (70 eV) was used; injector temperature was 240°C and that of the detector was 230°C. The carrier gas was helium at the working rate of 1.7 ml/min. The column temperature was initially 50°C and was then gradually increased at a rate of 5°C/min up to 180°C and after that up to 240°C at a rate of 8°C. For detection of the oil components, we used a flame ionization detector, set up at 230°C. The main compounds were identified through comparison of substance mass spectrum with the GC/MS database (NIST 62.lib), literature and retention index (McLafferty and Stauffer 1989; Adams 1995).

### Animals

Experimental procedures were carried out using male Swiss mice (45-55 days old), from the colony at the Central Animal House of UNESP, maintained for at least 1 week prior to the experiments at the Animal House of the Department of Pharmacology under controlled room temperature (21 ± 2°C) and a regular light/dark cycle (12/12h). Mice had free access to food (balanced feed Nuvilab CR-1 produced by Nuvital Nutrientes S/A, Brazil) and potable water until 2 hours before treatments. During the experimental procedure, mice were maintained in a sound-attenuated room under controlled temperature, with no other simultaneous activity being carried out. All experimental protocols were designed according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Bioscience Institute/UNESP-Ethics Committee for Animal Research.

## Treatments and behavioural evaluation

In all procedures, mice were randomly assigned to experimental groups and EO treatments were administered orally at doses varying from 0.5 to 2.0 g/kg (w/v) 30 min before the experimental trial. EO was suspended in polyoxyethylene sorbitan monooleate (Tween 80® – 12% (v/v) in saline, Synth, Brazil) at a constant volume of 10 ml/kg. Negative control groups were treated (*p.o.*) with the vehicle (TW) in the same proportion, whereas positive control groups were treated (*i.p.*) with imipramine (IM, 30 mg/kg; Hydrochloride, Sigma, Brazil) or diazepam (DZP, 1.0 or 5.0 mg/kg; Valium, Roche, Brazil) both dissolved in saline at 0.9% (w/v).

### Marble-burying test

The experimental procedure was based on classic descriptions (Poling *et al.* 1981; Broekkamp *et al.* 1986), with slight modifications (Pultrini *et al.* 2006). Only pre-selected mice that had presented a stable burying score were used in the experimental protocol. Each mouse was individually placed in a covered cage with 25 glass marbles uniformly distributed on a 5-cm layer of sawdust. Animals were left in the cage with marbles for 30 minutes after which they were removed and the number of marbles that were totally hidden by sawdust was counted.

### Light-dark box test

According to the protocol described by Costall *et al.* (1989), each animal was individually placed in the centre of the light compartment, facing the dark compartment, and observed for 5 min after the first entry into the dark compartment. Details of the apparatus were previously described (Pultrini *et al.* 2006). During this period, the number of shuttle crossings and the time spent in the light compartment was recorded.

### Rota-rod test

Since a positive effect in the procedures to evaluate anxiolytic activity could be due to motor impairment (Rodgers *et al.* 1997), the integrity of the motor system was evaluated in treated mice by means of the Rota-rod rotating bar (Dunham and Miya 1957). Mice were classified as “able” or “unable”, according to their ability in walk under the apparatus at 5 rpm for 1 min, with tolerance of up to three falls.

### Sleeping time induced by ether inhalation

EO from *Citrus aurantium* was active in augmenting the pentobarbital sleeping time (Carvalho-Freitas and Costa 2002). The sedative activity could be evaluated without interfering in the hepatic metabolism by inducing sleep through ether inhalation, since a massive amount is eliminated by the respiratory cycle, without hepatic metabolism, as suggested by Tsuji *et al.* (1996). In this manner, the EOs of *C. aurantium*, *C. latifolia* and *C. reticulata* were submitted to this evaluation. Following the protocol described by Helli6n-Ibarrola *et al.* (2006) with some procedural modifications, mice were individually placed in a cylindrical glass chamber (a flask of 3L capacity) saturated for 20 min with vapour 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 sec, after loss of postural reflex (sleep latency), was then withdrawn and placed in an individual cage to record the time to recover the postural reflex (sleep duration).

### Statistical analysis

Quantitative data were compared by non-parametric analysis of variance (Kruskal-Wallis) followed by Mann-Whitney Test when necessary; proportions were compared by Fisher's Exact Test. In all cases, results from experimental groups were compared with the vehicle group (TW), and differences were considered significant when associated with  $p \leq 0.05$ .

## RESULTS

### EO composition

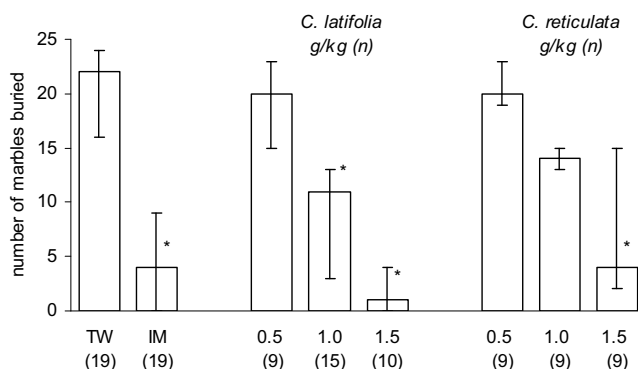
The monoterpenoid limonene was the main compound in all EOs from *Citrus* species tested, present in the respective proportions of 58% in *Citrus latifolia* and 90% in *Citrus reticulata*. EO from *Citrus aurantium* also presented a high proportion of limonene and was detected at 87% in this study, which is similar to the amounts of greater than 90% that have been reported in previous works (Carvalho-Freitas and Costa 2002; Pultrini *et al.* 2006).  $\beta$ -pinene and  $\gamma$ -terpinene were present in important concentrations only in *C. latifolia* (13 and 17%, respectively) and, except for  $\gamma$ -terpinene in *C. aurantium* (4%), all other compounds were present in amounts lower than 2%.

### Marble-burying test

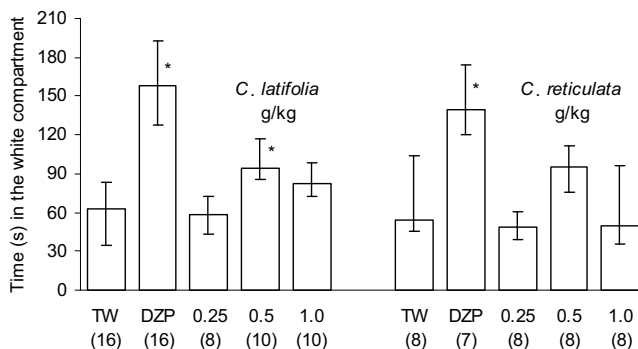
Treatment with *C. latifolia* EO at the doses of 1.0 and 1.5 g/kg significantly decreased the number of marbles buried, as did the treatment with *C. reticulata* at 1.5 g/kg. The results are presented in Fig. 1.

### Light/dark box test

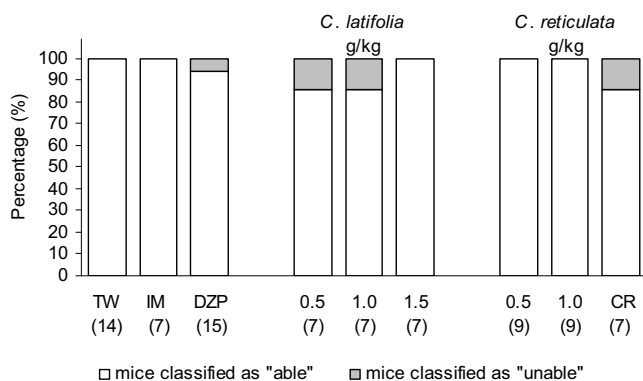
The results with the light/dark box are summarized in Fig. 2. After treatment with 0.5 g/kg of *C. latifolia* EO or DZP at 1.0 mg/kg, the time spent in light compartment was verified to be longer, while the group treated with 1.0 g/kg showed a non-significant tendency ( $p = 0.0563$ ). The number of transitions was not modified by any of the EO doses from



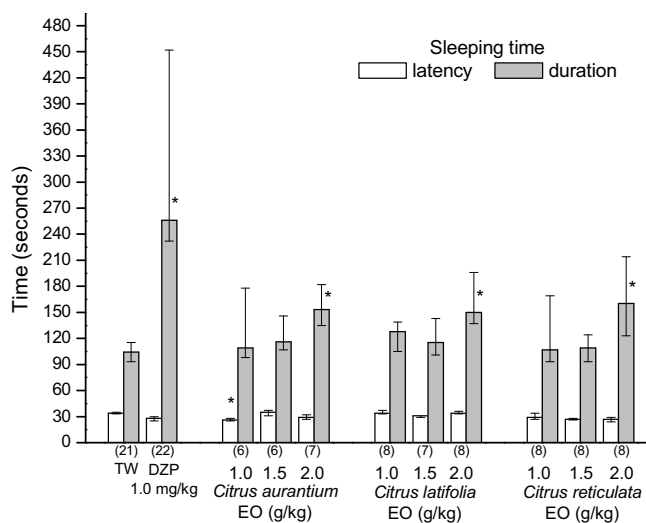
**Fig. 1** Median and interquartile range (Q1-Q3) of number of entirely hidden glass marbles after treatment with essential oil (EO) from *Citrus latifolia* or *Citrus reticulata* at 0.5, 1.0 or 1.5 g/kg (*p.o.*). IM: imipramine 30 mg/kg (*i.p.*); TW: vehicle, 10 ml/kg (*p.o.*). Numbers in parentheses are the number of animals in each group. \*  $p \leq 0.05$  relation to TW group (Kruskal-Wallis followed by Mann-Whitney Test).



**Fig. 2** Median and interquartile range (Q1-Q3) of time (in seconds) spent in the illuminated side of light/dark box treatment with essential oil (EO) from *Citrus latifolia* or *Citrus reticulata* at 0.25, 0.5 or 1.0 g/kg (*p.o.*). DZP: diazepam 1 mg/kg (*i.p.*); TW: vehicle, 10 ml/kg (*p.o.*). Numbers in parentheses are the number of animals in each group. \*  $p \leq 0.05$  in relation to TW group (Kruskal-Wallis followed by Mann-Whitney Test).



**Fig. 3** Effects of rota-rod test after treatment with essential oil (EO) from *Citrus latifolia* or *Citrus reticulata* at 0.5, 1.0 or 1.5 g/kg (*p.o.*). IM: imipramine 30 mg/kg (*i.p.*); TW: vehicle, 10 ml/kg (*p.o.*). Numbers in parentheses are the number of animals in each group.



**Fig. 4** Median and interquartile range (Q1-Q3) of time (in seconds) relative to latency and sleep duration induced by diethyl ether inhalation after treatment with essential oil (EO) from *Citrus aurantium*, *Citrus latifolia* or *Citrus reticulata* at 1.0, 1.5 or 2.0 g/kg (*p.o.*). DZP: diazepam 5 mg/kg (*i.p.*); TW: vehicle, 10 ml/kg (*p.o.*). Numbers in parentheses are the number of animals in each group. \*  $p \leq 0.05$  in relation to TW group (Kruskal-Wallis followed by Mann-Whitney Test).

either *C. latifolia* or *C. reticulata*. DZP and 0.5 g/kg of *C. latifolia* EO were able to augment the rearing number (data not shown).

### Rota-rod test

The percentage of animals classified as able or unable to stay on the rota-rod is presented in **Fig. 3**. None of the EOs showed a significantly impaired performance, denoting no effect upon motor activity. In the same manner, the reference drugs, diazepam and imipramine, displayed no deleterious effect upon motor coordination at effective doses in procedures to evaluate anxiety.

### Sleeping time induced by ether inhalation

Sleep latency was affected only after treatment with *C. aurantium* EO at 1.0 g/kg. EOs of *C. reticulata* and *C. latifolia* significantly increased sleep duration after treatment at the highest dose evaluated. Data are presented in **Fig. 4**.

## DISCUSSION

Traditional preparations using *Citrus* species are popularly used as an alternative to treat anxiety and insomnia, among other central nervous system disorders. Results obtained

with EO from *C. latifolia* and *C. reticulata* in experimental models were suggestive of anxiolytic and sedative activities, as previously observed with *C. aurantium* (Carvalho-Freitas and Costa 2002; Pultrini *et al.* 2006).

EOs from *Citrus* species showed positive activity in experimental models, which reflects both generalized anxiety and obsessive-compulsive disorders. In the light/dark box test, the experimental procedure that reflected generalized anxiety disorder (Graeff and Zangrossi 2002), EOs were active at 0.5 g/kg, while in the marble-burying test related to obsessive-compulsive disorder (Broekkamp *et al.* 1986; Ichimaru *et al.* 1995), EOs were active at higher doses (1.0 and 1.5 g/kg). The clinical therapeutic activity for these disorders is obtained from distinct classes of compounds such as benzodiazepine derivatives and tricyclic antidepressants which are reference drugs effective against generalized anxiety and obsessive compulsive disorders, respectively. Thus, the profile of activity observed with EOs denotes a wide spectrum, since they were active in experimental models related to both anxiety disorders.

The marble-burying test displayed a remarkable dose-dependent effect, with the high dose drastically reducing the number of buried marbles. On the other hand, in the light-dark box test, an inverted U-shaped curve was observed. This is a well-known phenomenon in toxicology (Calabrese and Baldwin 2003) which is related to corticosteroid action on the brain cells (Joëls 2006) and was described for tricyclic antidepressants almost 30 years ago (Ericksen 1979). This type of dose-response association has already been suggested for *C. aurantium* EO due to its interference in chemical and electrically-induced seizures (Carvalho-Freitas and Costa 2002).

The ether-induced effect on sleep discards a potential interaction with hepatic enzymes, a limitation of barbiturate sleeping time, but this effect was detected only at higher doses (2.0 g/kg), emphasizing that specificity of this action should be investigated.

Results obtained with EO from *C. reticulata* and *C. latifolia* confirm the central nervous system activity previously described for *C. aurantium*. This is a specific action, since it was observed in the absence of motor impairment as evaluated on the rotating bar.

Limonene, the main compound, presents biological activity related to depression of the central nervous system, since treatment with this compound provokes reductions in spontaneous activity, rearing and grooming in the open field test and is able to increase the barbiturate sleeping time (Vale *et al.* 2002). However, behavioural effects in the light-dark box, marble-burying test and sleep induced by ethylic ether inhalation were similar among the EOs obtained from all evaluated species, despite the different limonene proportions in their composition (58% in *C. latifolia* and around 90% in *C. reticulata* and *C. aurantium*). Other compounds are present in different proportions in EOs, such as  $\beta$ -pinene, whose EO proportions are 13% in *C. latifolia*, 0.35% in *C. reticulata* and 1% in *C. aurantium*. Similarly,  $\gamma$ -terpinene represents 17% of *C. latifolia* EO, is undetectable in *C. aurantium* and presents 4% proportion in *C. reticulata*. Linalol presents 3% in *C. aurantium*, 0.4% in *C. latifolia* and 1% in *C. reticulata*. The similar behavioural profile observed in mice, despite the relative differences in main compound amounts present in *Citrus* species, suggesting that the observed activity cannot be attributed exclusively to any one of these compounds. It is more likely that the biological profile of an EO results from the synergism among its constituent compounds.

In general, we can conclude that EOs from *C. latifolia*, *C. reticulata* and *C. aurantium* peels presents anxiolytic and sedative activity in rodents, without motor impairment. Positive results in anxiety experimental procedures are related to both generalized anxiety and obsessive-compulsive disorders. Observed results are probably due to a synergistic action of the common compounds present in the three species. These results call for the continuity of the toxicological evaluation after chronic administration, in order to guar-

rantee the safer use of Citrus by the population, as well as to ascertain the pharmacological action mechanism related to the biological activity.

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