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

André Costa Gargano# • Celso A. R. Almeida Costa# • Mirtes Costa*

Department of Pharmacology, Institute of Bioscience, UNESP - São Paulo State University, 18618-000, Botucatu, São Paulo, Brazil
#both authors contributed equally to the work.
*Corresponding author: mcosta@ibb.unesp.br

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-ence *Citrus* species as useful in reducing symptoms of anxiety or insomnia. Habitually used as decoction (Giron et al. 1991; Longuefossé and Nossin 1996) or infusion (Tortorello 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 (Tortorello and Romero 1992) as well as in Puerto Rico (Hernández 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 monoooleate (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 (Polutrini 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 Hellín-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 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 flask of the cap. 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

Statistical analysis

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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 ethyl 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 /g533-pinene, /g534-terpinene and /g535-camphene, all evaluated species, despite the different limonene proportions in their composition. These results call for the continuity of the toxicological evaluation after chronic administration, in order to gua-
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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