

Effects of the essential oil from *Citrus aurantium* L. in experimental anxiety models in mice

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Abstract

Citrus aurantium L. is popularly used to treat anxiety, among other indications suggesting central nervous system action. Previous studies showed anxiolytic effect in the essential oil from peel in mice evaluated on the elevated plus maze [Carvalho-Freitas, M.I.R., Costa, M., 2002. Anxiolytic and sedative effects of extracts and essential oil from *Citrus aurantium* L. Biological and Pharmaceutical Bulletin 25, 1629–1633.]. In order to better characterize the activity of the essential oil, it was evaluated in two other experimental models: the light–dark box and the marble-burying test, respectively related to generalized anxiety disorder and to obsessive compulsive disorder. Mice were treated acutely by oral route 30 min (single dose) or once a day for 15 days (repeated doses) before experimental procedures. In light–dark box test, single treatment with essential oil augmented the time spent by mice in the light chamber and the number of transitions between the two compartments. There were no observed alterations in the parameters evaluated in light–dark box after repeated treatment. Otherwise, single and repeated treatments with essential oil were able to suppress marble-burying behavior. At effective doses in the behavioral tests, mice showed no impairment on rotarod procedure after both single and repeated treatments with essential oil, denoting absence of motor deficit. Results observed in marble-burying test, related to obsessive compulsive disorder, appear more consistent than those observed in light–dark box.

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Keywords: *Citrus aurantium*; Anxiety; Black and white box; Marble-burying test; Rotarod test; Mice

Introduction

Preparations from peel, flowers and leaves of *Citrus aurantium* L. (Rutaceae) are popularly used in order to minimize central nervous system disorders. *Citrus aurantium* is the most frequently plant used as sedative by patients at the outpatient clinics of five health-care centers in Puerto Rico (Hernandez et al., 1984) and is popularly cited by the same activity in Guatemala (Girón et al., 1991), México (Tortoriello and Romero, 1992), Italy (De Feo and Senatore, 1993), Martinique (Longuefosse and Nossin, 1996) and Spain (Vásquez et al., 1997). Ambient odor of orange (*C. sinensis*) diffused in the waiting room in a dental office results in a lower level of state anxiety in patients (Lehrner et al., 2000).

Previous studies in our laboratory (Carvalho-Freitas and Costa, 2002) showed central nervous system effects in the essential oil from peel in mice, after single treatment. Anxiolytic activity was determined by increase in time spent in open arms of the elevated plus maze, and sedative activity by enlargement of barbiturate-induced sleeping time. There was not observed any impairment upon attention state or motor activity in mice treated with essential oil.

In the present study, essential oil from peel was evaluated in two other experimental models after single or repeated treatment, in order to better characterize their anxiolytic effect. The light–dark box is a sensitive model to detect activity in disorders related to generalized anxiety (Costall et al., 1989) and the marble-burying test is more related to obsessive compulsive disorder (Ichimaru et al., 1995). To avoid unspecific effects due to motor impairment, the integrity of motor system was evaluated using the rotarod test.

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Material and methods

Plant material and extraction

Fruits were harvested from adult plants in an orchard at the UNESP (São Paulo State University) campus and the essential oil was immediately extracted by hydro distillation. Peels were immersed in double their volume of distilled water and boiled in the Clevenger apparatus until total extraction. Essential oil from several extractions were mixed in a pool and stored in a freezer in amber flasks in 2 ml aliquots until the pharmacological assays.

Essential oil composition

Samples of the essential oil was suspended in 1 ml of ethyl acetate (P.A., Merck, Germany) and 1 μ l of this solution was analyzed by gas chromatography coupled with mass spectrometer (GC/MS, Shimadzu, model QP-5000) equipped with a fused silica capillary column DB-5 (30 m \times 0.25 mm \times 0.25 μ m). The electron impact technique (70 eV) was used and injector temperature was 240 °C and that of the detector was 230 °C. The carrier gas was helium at the working rate of 1.7ml/min. The column temperature was initially 50 °C and then was gradually increased at the rate of 5 °C/min up to 180 °C and after up to 240 °C at the rate of 8 °C. For detection of the oil components we used a flame ionization detector, set up at 230 °C. The identification of the components of the essential oils was effected through of comparison of substance mass spectrum with the database of the GC/MS (NIST 62.lib), literature and retention index (McLafferty and Stauffer, 1989; Adams, 1995).

Animals

All experimental procedures were carried out using adult male Swiss mice (30–45 g) from the Central Animal House of UNESP colony and maintained under standard environmental conditions: temperature 21 °C \pm 2, 12 h light–12 h dark cycle at the Animal House of the Department of Pharmacology during at least 1 week before experiments. Food and water were given ad libitum until 2 h before the experimental procedures. Experimental protocols were designed according to Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA), and were approved by the Bioscience Institute/UNESP–Ethical Committee for Animal Research (CEEAA).

Treatments

Treatment with essential oil (0.5 or 1.0 g/kg) was delivered orally by gavage after suspension in polyoxyethylenesorbitan monooleate (Tween 80–12% v/v in saline, Synth, Brazil) in a constant volume of 10 ml/kg. Positive control group was intraperitoneally treated with diazepam (DZP: Valium, Roche,

Brazil) dissolved in 0.9% w/v NaCl solution. Pilot experiments showed no differences between Tween (p.o.) and Saline (i.p.) for parameters evaluated in the experimental procedures. In this way, Tween (12% v/v in saline, 10 ml/kg) was chosen to treat negative control group due to their use in essential oil solubility. For single-treatment schedule, mice were treated for 30 min before experimental procedure whereas for repeated-treatment schedule animals were treated once a day for 15 consecutive days and exposed to experimental procedure 30 min after the last treatment. Tests were run between 8:00 a.m. and 4:00 p.m. in an illuminated room with controlled temperature and attenuated sound.

Light–dark box test

The experimental procedure was described by Crawley and Goodwin (1980) and slightly modified (Costall et al., 1989; Bourin and Hascoët, 2003). The test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior in response to novel environment and light. The acrylic test box (46 \times 27 \times 30 cm high) was divided at one third of the greatest dimension to form two compartments: the smaller was black with a dark cover and the larger was white and directly illuminated with a 20 W fluorescent lamp. The compartments were connected by an opening 7.5 \times 7.5 cm located at floor level in the center of the black division. During the experimental session, each mouse was individually placed at the center of the white compartment facing the opening and recorded by remote video for 5 min after the first entrance in the dark compartment to register: (a) the time spent in the white compartment, (b) the number of transitions between the two compartments and (c) the number of rearings in the white compartment.

Marble-burying test

The procedure is based on defensive burying that could be elicited in rodents in response to aversive stimuli; and glass marbles provide an effective unconditioned stimulus that provokes burying (Poling et al., 1981; Broekkamp et al., 1986). Mice were previously selected to choose those that presented a stable burying score (over 13 out of 25 marbles) for 3 consecutive days before the test. Thirty minutes after treatments, animals were individually placed in 27 \times 16 \times 13 cm high cages with 25 glass marbles, 1.5 cm in diameter, uniformly distributed on a 5-cm layer of sawdust. A smooth ceiling with a few holes covered the cages so that the mice could not cling to avoid the aversive stimulus provided by marbles. The mice were left in the cage with marbles for 30 min after which they were removed to count the number of marbles that were totally hidden by sawdust.

Rotarod test

The procedure was described by Dunham and Miya (1957) and is suitable for detecting motor impairment due to

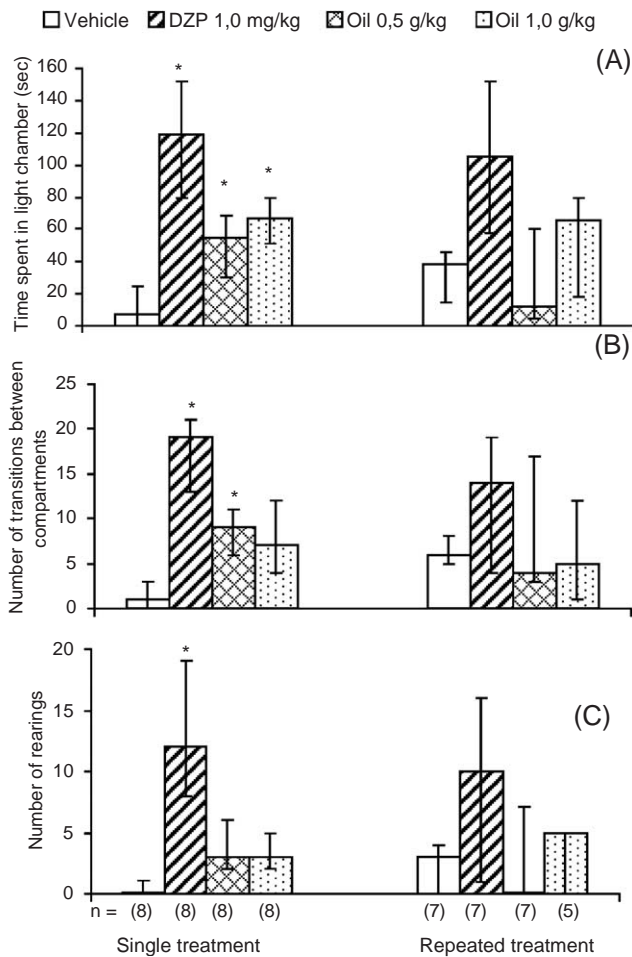


Fig. 1. Effects on light–dark box test after single or repeated treatment with oil: essential oil from *Citrus aurantium* peel, p.o.; DZP: diazepam 1.0 mg/kg, i.p.; vehicle: Tween 80 12%, 10 ml/kg, p.o. (A) Total time spent in the white compartment; (B) number of transitions between white and black compartments; (C) number of rearings in white compartment. Results are expressed as median and interquartile range (Q_1 – Q_3). Numbers in parentheses are the number of animals in each group. * $p \leq 0.05$ as compared with the vehicle group (Kruskal–Wallis followed by Mann–Whitney U -test).

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, rotating at 5 rpm. After 30 min of treatments, each mouse was placed on the apparatus to register the total time performed on the rotating bar in 3 successive trials up to 1 min. To avoid a bias on account of inability not related to drug treatment, on the previous day animals were evaluated to select those that had the ability of walking on the bar, under the same conditions used in the test.

Statistical analysis

Quantitative data were compared by non-parametric analysis of variance (Kruskal–Wallis) followed by Mann–Whitney's U -test and proportions were compared by Fisher's Exact Test. Data from experimental groups were compared with the vehicle group, and differences were considered significant when $p \leq 0.05$.

Results

Essential oil composition

Essential oil used in biological evaluation was composed by two monoterpenoids: limonene (97.83%) and myrcene (1.43%) and one aldehyde: octanal (0.45%). The remaining 0.29% in composition was not identified.

Light–dark box test

Results from light–dark box test are presented in Fig. 1. After single treatment, essential oil at 0.5 and 1.0 g/kg and diazepam at 1.0 mg/kg augmented the time spent in light compartment (A). Significant increase in the number of transitions between compartments was observed in groups treated with diazepam and with essential oil at 0.5 g/kg (B). The group treated with 1.0 g/kg of essential oil showed an increase in transitions number but, due to the bigger dispersion in data, there was no statistical significance. Number of rearings was significantly augmented only in the positive control group treated with diazepam, in spite of the tendency observed with essential oil in both doses administered (C). After repeated administration for 15 days, none of treatments was able to modify the parameters on the light–dark box test.

Marble-burying test

Previous selection of animals reduces the dispersion of the individual number of buried marbles; and repeated burying tests had no effect of habituation, i.e., mice scoring 13 or more on day one maintained high scoring on successive days (data not shown). Treatment with essential oil (0.5 and 1.0 g/kg) and diazepam (2.0 mg/kg) significantly decreased the number of marbles buried after single and after repeated treatments (Fig. 2).

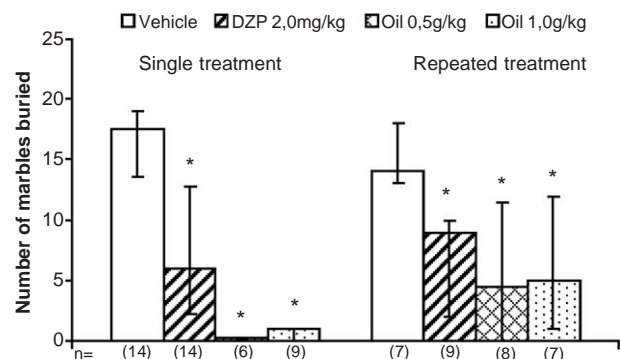


Fig. 2. Effects on marble-burying test after single or repeated treatment with oil: essential oil from *Citrus aurantium* peel, p.o.; DZP: diazepam 2.0 mg/kg, i.p.; vehicle: Tween 80 12%, 10 ml/kg, p.o. Results are expressed as median and interquartile range (Q_1 – Q_3). Numbers in parentheses are the number of animals in each group. * $p \leq 0.05$ as compared with the vehicle group (Kruskal–Wallis followed by Mann–Whitney U -test).

Rotarod test

Results are presented in Fig. 3, as percentage of animals that were able or unable to stay on the bar rotating at 5 rpm for 1 min. Mice submitted to a single treatment with 1.0 g/kg of essential oil showed ability similar to the vehicle treated group; and the decrease in percentage of animals in the group treated with 2.0 mg/kg of diazepam was not statistically significant. After repeated treatment with diazepam at 2.0 mg/kg, mice showed a significantly impaired performance.

Discussion

Citrus aurantium L. is popularly used as an alternative to treat anxiety, among other central nervous system disorders, and the essential oil obtained from peel showed anxiolytic activity after a single administration in mice, denoted by the increase in time spent in open arms of the elevated plus maze (Carvalho-Freitas and Costa, 2002). Biological effect could be due to one specific compound in the preparation or, more common, it results of synergic effect among several compounds. The main compound present in the essential oil from *Citrus aurantium* is limonene (97.83%), followed by myrcene (1.43%), which is present in around one tenth of that amount. Both compounds have biological activity related with depression of central nervous system. Mice treated with limonene or myrcene shown decrease in spontaneous activity, rearing and grooming in the open field test and were able in increase the barbiturate sleeping time (Vale et al., 2002). In the same study limonene and myrcene were not able to modify parameters of anxiolytic activity evaluated on elevated plus maze.

The aim of this study was to evaluate the essential oil in two other experimental procedures in order to confirm the anxiolytic effect suggested by elevated plus maze. Essential oil was evaluated after single (30 min before experimental procedure) and repeated (once a day for 15 consecutive days) treatments in the light–dark box and marble-burying tests.

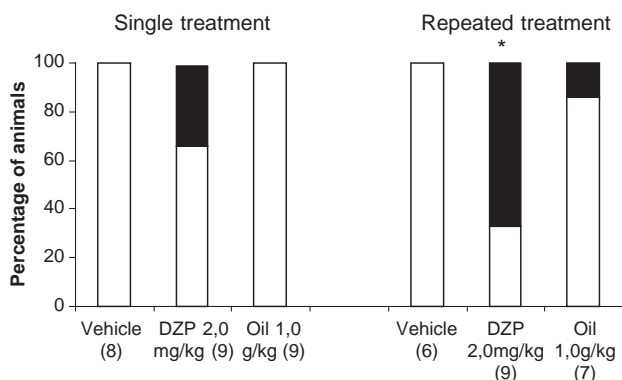


Fig. 3. Effects of rotarod test after single or repeated treatment with oil: essential oil from *Citrus aurantium* peel, p.o.; DZP: diazepam, i.p.; vehicle: Tween 80 12%, 10 ml/kg, p.o. Results are expressed as percentage of animals that are able (open columns) and unable (closed columns) to stay on the rotating bar at 5 rpm for 1 min. * $p \leq 0.05$ as compared with the vehicle group (Fisher's Exact Test).

In the light–dark box test described by Crawley and Goodwin (1980), anxiety is generated by the conflict between desires to explore and to retreat from an unknown and well illuminated space. This experimental procedure had an ethological approach, based on innate response of fear in the face of aversive situations and/or stimuli reflecting the generalized anxiety (Crawley and Goodwin, 1980; Graeff and Zangrossi, 2002).

The evaluated parameters were the number of transitions between the white and black compartments, the time spent and the number of rearings in the white compartment. Despite the lack of reservations that the rearing number reflects the exploratory activity, there remains some discussion about the appropriate parameter to evaluate anxiolytic activity.

According to Young and Johnson (1991) the time spent in the illuminated compartment, rather than the number of transitions, is the most consistent and usual parameter for evaluating anxiolytic activity, while Lepicard et al. (2000) affirm that anxiety activity could be evaluated by the number of transitions and by the time in the white chamber, emphasizing that the latter is the most robust indicator in the anxiety study and that the first is also a sign of exploratory activity. Graeff and Zangrossi (2002) assert that both the time spent in light compartment and the number of transitions are important measures to evaluate anxiolytic activity.

In our experimental procedure after a single treatment with essential oil, mice showed a significant increase in both the time spent in the light compartment (Fig. 1A) and in the number of transitions between chambers (Fig. 1B). There was no significant modification in the number of rearings (Fig. 1C), in spite of the tendency observed. After treatment for 15 days none of the treatments were effective in significantly altering the parameters evaluated in the light–dark box test. This behavioral profile was similar to that presented by animals treated with 1.0 mg/kg of diazepam, recognized as an effective drug in treating generalized anxiety. The reduction in efficacy after repeated treatment with diazepam – and perhaps with essential oil – could be due to the development of tolerance, already described in mice after 2 weeks of continuous use of diazepam (Flaishon et al., 2003). In humans, results concerning tolerance after long-term treatment of anxiety with benzodiazepines are not well established. Results show decrease in effect after 8 weeks of lorazepam, but tolerance was not detected after 6 months of clorazepate or 16 weeks of lorazepam and alprazolam (Carlson and Roy-Byrne, 2002).

A different interpretation for the lack of effect after repeated treatment is due to increase in parameters on vehicle group, when compared with single treatment. The observed tendency (not statistically significant) to increase in parameters after repeated treatment may perhaps involve the mice habituation to diary manipulation for treatment. In these conditions animals could be less afraid to experimental procedure, feeling safe for light compartment exploration. Considering that mice from treated groups also were submitted to diary manipulation, the absence of difference between vehicle and treated group is, in fact, due to the lack of effect of treatment with diazepam or essential oil.

The marble-burying test in an experimental model based on the defensive burying behavior elicited in rodents in response to aversive stimuli. Anxiolytic drugs suppress the marble-burying behavior, although other classes of drugs also reduce or abolish this defensive display, indicating absence of pharmacological specificity. The experimental procedure appears related to obsessive–compulsive disorder, characterized by obsession – recurrent thoughts which lead to accentuated anxiety and discomfort – and/or compulsion – stereotyped behavior or rituals to alleviate the anxiety (Gyertyan, 1995; Ichimaru et al., 1995). Two points support this view: effective drugs to treat obsessive–compulsive disorder, via the selective inhibitors of serotonin reuptake, reduce the number of marble buried; and, mice previously exposed to glass marbles continue in intensive burying activity, even in the absence of marbles, a supposed compulsive behavior (Gyertyan, 1995).

Essential oil was effective in suppressing the marble-burying behavior after single and repeated treatment, in both administered doses. The reference drug, diazepam, also was able to reduce burying behavior but, in contrast to the light–dark box, essential oil had a more intense activity.

Positive results in these experimental procedures could be due to motor impairment that hinders the execution of behaviors such as avoiding the illuminated compartment or burying the marbles (Rodgers et al., 1997). In order to discard the possibility of false-positive results, the integrity of the motor system was evaluated by rotarod test, devised to detect neurological impairment including ataxia, sedation or muscle relaxing (Dunham and Miya, 1957). Performance of animals treated with the larger dose of essential oil was similar to that of the vehicle group, either after single or repeated treatments, denoting the integrity of the motor system in mice treated with it. On the other hand, 67% of mice in the group treated for 15 days with 2.0 mg/kg of diazepam were not able to stay under the revolving bar for 1 min (Fig. 3). Benzodiazepines are not the prime drug to treat obsessive compulsive disorder, and it was not effective to reduce the number of marble burying after treatment with 1.0 mg/kg (data not shown), the effective dose in the light–dark box test. Due to tolerance to sedative effect and motor impairment, enhancement in rotarod performance was predictable after repeated treatment with diazepam. The observed decrease in performance could be due to the higher diary dose (2.0 mg/kg), resulting in a toxic impairment, suggesting a false-positive effect of diazepam on marble-burying test.

In our previous study (Carvalho-Freitas and Costa, 2002) mice treated with the same doses of essential oil used in the present work showed no impairment in spontaneous ambulation or rearing (exploratory measures) and in freezing and grooming behaviors (emotionality measures). By combining these results we can conclude that essential oil from *Citrus aurantium* peel presents anxiolytic activity in rodents without any motor impairment, even after 15 consecutive days of treatment. Positive results were obtained in experimental models clearly related to generalized anxiety and in a putative model for obsessive–compulsive disorder. Studies designed for

identify the possible neural or hormonal mechanisms involved in the activity of essential oil and for toxicological investigation, including hepatic interaction and chronic treatment could be planned in order to contribute information to guarantee the safer use of this preparation.

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