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Anxiolytic-like effects of inhaled linalool oxide in experimental mouse anxiety models

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ABSTRACT

Linalool oxide is a monoterpene that is found in some species of aromatic plants. The effects of the inhalation of linalool oxide (0.65%, 1.25%, 2.5% and 5.0% w/w) in the elevated plus-maze and light/dark box tests as animal models of anxiety were investigated in adult male mice and compared with the effects of the reference anxiolytic diazepam (0.5 and 2.0 mg/kg), administered intraperitoneally. Additionally, the effects of inhaled linalool oxide were investigated in the rotarod test. Linalool oxide significantly increased the number of visits to the open arms of the elevated plus-maze and the amount of time spent there as well as the total number of entries. In the light/dark box test, inhalation of linalool oxide led to an increase in the time spent by the mice in the brightly-lit chamber and in the number of times the animal crossed from one compartment to another. Performance on the rotarod was unaffected. Thus, inhaled linalool oxide was found to have anxiolytic properties in both animal models, without causing any motor deficit. These results suggest that inhalation of linalool oxide may be a useful means of counteracting anxiety.

1. Introduction

Anxiety disorders are common in the general population and basic pharmacological treatment of these disorders incurs significant costs, thus constituting an important public health problem in developed countries (Almeida et al., 2004; Bradley et al., 2007b). Furthermore, the treatment of anxiety with the anxiolytic drugs currently on the market today involves problems such as adverse and undesirable effects. In an attempt to resolve these issues, interest has increased in alternative therapies such as aromatherapy, which represents a possible alternative to standard pharmacological treatment and has been used for several conditions such as chronic pain, depression and anxiety (Bradley et al., 2007b; Abuhamdah and Chazot, 2008).

Essential oils (EOs) are concentrated volatile compounds formed as secondary metabolites in aromatic plants and characterized by a strong odor (Bakkali et al., 2008; Silva et al., 2009). They have been widely used in folk medicine and aromatherapy (Umezu et al., 2002; Almeida et al., 2004). Studies have shown that certain EOs decrease anxiety-related behavior in humans and animals (Bowers, 2006; Bradley et al., 2007b). Their biological effects may be the result of the synergistic effect of all the molecules together or only of the principal molecules present in larger concentrations in these oils (Bakkali et al., 2008).

Linalool is a monoterpene frequently found as a major component of EOs obtained from various species of well known aromatic plants such as *Lavandula angustifolia* Mill., *Melissa officinalis* L., *Rosmarinus officinalis L.* and *Cymbopogon citratus* DC (Elisabetsky et al., 1995; Batista et al., 2008; Linck et al., 2009). It is widely used in the manufacture of fragrances for shampoos, soaps and detergents (Mitić-Ćulafić et al., 2009).

Recent studies have shown that, when inhaled, linalool is clearly sedative, inducing hypothermia, reducing ambulation and increasing pentobarbital-induced sleep time in mice (Linck et al., 2009). In addition, its anxiolytic profile has been demonstrated in the light/dark box test and in social interaction and aggressive behavior tests (Linck et al., 2010).

Linalool oxide is another substance that can also be found in some herbal EOs, albeit as a minority component. It is a monocyclic alcohol that can be formed from linalool by natural oxidation or may be produced by synthetic processes such as biotransformation of linalool using the fungus *Aspergillus niger*. (Demyttenaere and Willemen, 1998; Hilmer and Gatfield, 2004; Santos et al., 2008).

The objective of this study was to evaluate the possible anxiolytic effects of inhaled linalool oxide in mice by using the elevated plus-

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maze test and the light/dark box test as animal models of anxiety, and to investigate the presence of a nonspecific muscle relaxant effect of this substance by using the rotarod test.

2. Materials and methods

2.1. Animals

Male Swiss (*Mus musculus*) albino mice (25–35 g) from the *Professor Dr. Thomas George* animal laboratory at the Laboratory of Pharmaceutical Technology, Federal University of Paraíba were used in these experiments. Animals were kept in polypropylene boxes covered with sawdust and maintained at controlled temperature (21 ± 1 °C), with a 12-hour light/dark cycle (lights on at 6 a.m.). Food (Purina® pellets) and water were given ad libitum until 1 h prior to the experimental procedures. All experiments were performed between 12:00 p.m. and 5:00 p.m. Experimental protocols and procedures were approved by the Animal Experimentation Ethics Committee (CEPA/LTF) and are in accordance with EC Directive 86/609/EEC.

2.2. Drugs and treatments

Linalool oxide (Sigma-Aldrich, Brazil) was diluted with polyoxyethylene sorbitan monooleate (Tween 80 0.2% v/v in distilled water, VETEC, Brazil) to prepare linalool oxide emulsions (0.65%, 1.25%, 2.5% and 5.0%) minutes prior to the experiments. Diazepam (Merck, Brazil) was dissolved in saline solution and used as a positive control (0.5 mg/kg or 2 mg/kg). It was administered intraperitoneally (i.p.) at a volume of 0.1 ml/10 g of body weight. Negative control groups consisted of inhaled 0.2% Tween or intraperitoneal saline solution.

2.3. Inhalation apparatus

The inhalation apparatus consisted of an acrylic box measuring 36 × 30 × 29 cm. The floor consisted of a stainless steel grid onto which the animals were placed individually. There were four holes (each measuring 2 cm in diameter) on the front and back walls into which drug-embedded cotton wool was placed, alternating between 2.5 ml of saline solution and 2.5 ml of linalool oxide emulsion. Each animal was individually evaluated in the test shortly after 7 min of exposure to the inhalation chamber. Each animal was exposed only once, and after four animals had been exposed, the cotton wool containing the linalool oxide was replaced to ensure that the concentration of the drug was maintained.

2.4. Elevated plus-maze test

The elevated plus-maze (EPM) test was first evaluated for rats (Pellow et al., 1985) and later adapted for mice (Lister, 1987). The openness of the apparatus combined with its elevation serves to generate behavioral changes predominantly associated with anxiety (Schmitt and Hiemke, 1998). It relies on the natural anxiety-related behavior of rodents to remain in shadows, close to walls and to avoid heights (Bradley et al., 2007a). The apparatus was made of gray acrylic material (Insight®) and consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm) radiating from a central platform (5 × 5 cm) to form a plus sign. The apparatus was elevated to a height of 38.5 cm and placed inside a sound-attenuated room. At the beginning of the trial, an animal was placed on the central platform of the maze, facing an open arm. The number of entries and the time spent in the open arms, as well as the number of crossings between compartments, were counted during a 5-minute test period. Entry into an arm was considered valid only when all four paws of the animal were inside that arm (Biala and Kruk, 2008).

2.5. Light/dark box test

This test is based on the innate aversion of rodents to brightly lit areas and on their spontaneous exploratory behavior in response to a novel environment and to light (Pultrini et al., 2006). The apparatus consisted of an acrylic box 28 cm wide, 14 cm high and 13.4 cm long, divided into two separate compartments, occupying two-thirds and one-third of the total volume, respectively. The larger chamber was white and directly lit by a 60-watt light bulb, while the smaller chamber was entirely black and enclosed under a dark cover. The compartments were connected by an open door located at floor level in the center of the box. At the beginning of the test, the mice were placed individually at the center of the brightly lit chamber, facing the opening, and were allowed to explore the entire apparatus for 5 min. During this experimental session, the time spent in the well-lit compartment and the number of transitions between compartments was registered. A compartment entry was considered valid when the animal's four paws were inside that chamber.

2.6. Rotarod test

This procedure was first described by Dunham and Miya, 1957 and is suitable for detecting motor impairment due to pharmacological agents such as skeletal muscle relaxants or central nervous system depressants (Pultrini et al., 2006). The rotarod treadmill (Ugo Basile, Model 7750, Italy) consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks of 25 cm in diameter rotating at 7 revolutions per minute (rpm). Mice were submitted to three training sessions 24 h prior to testing and those lasting <90 s were rejected. Motor performance was evaluated immediately prior to treatment (basal) and at 30, 60 and 120 min after treatment.

2.7. Statistical analysis

The data obtained were evaluated using ANOVA followed by Bonferroni multiple comparisons test. Data were analyzed using the GraphPad Prism software (version 5.0) and experimental groups were compared with the vehicle group. *P*-values<0.05 were considered statistically significant.

3. Results

3.1. Elevated plus-maze test

Fig. 1 shows that both linalool oxide inhalation (0.65%, 2.5% and 5.0% w/w) and intraperitoneal diazepam resulted in an increase in the number of open entries compared to the respective control groups ($p<0.05$ and $p<0.01$, respectively). As illustrated in Fig. 2,

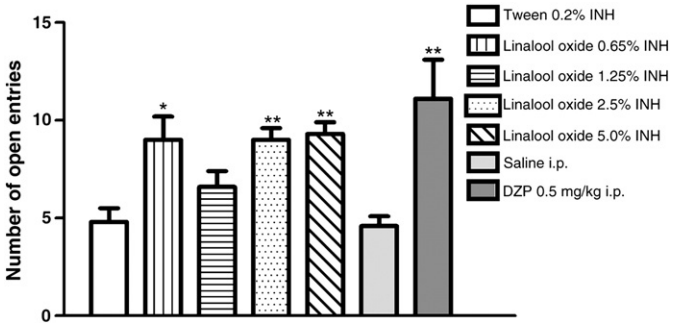

Fig. 1. Effect of linalool oxide on the number of open entries in EPM test. Evaluation of different concentrations of linalool oxide by inhalation route (INH) and diazepam administered intraperitoneally (i.p.) in male mice. Data are expressed as mean ± SEM. N = 8. * $p<0.05$, ** $p<0.01$ compared with controls; ANOVA/Bonferroni multiple comparisons test.

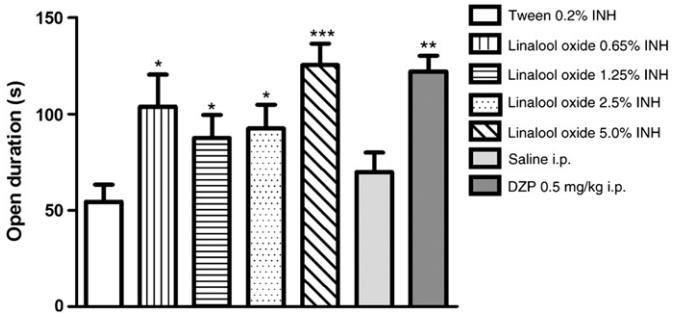

Fig. 2. Effect of linalool oxide on the open duration (s) in EPM test. Evaluation of different concentrations of inhaled linalool oxide and diazepam i.p. in male mice. Data are expressed as mean ± SEM. N = 8. * p<0.05, ** p<0.01, *** p<0.001 compared with controls; ANOVA/Bonferroni multiple comparisons test.

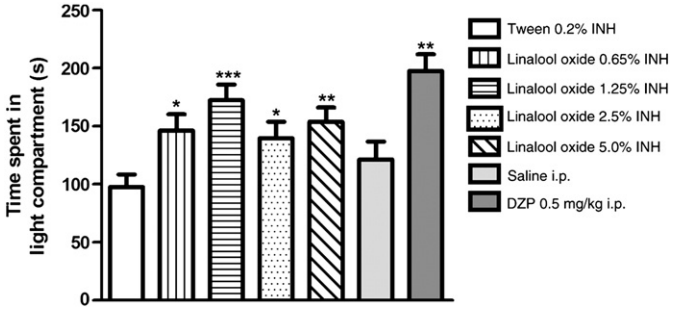

Fig. 4. Effect of linalool oxide on the time (s) spent in light compartment in light–dark box test. Evaluation of different concentrations of inhaled linalool oxide and diazepam i.p. in male mice. Data are expressed as mean ± SEM. N = 8. * p<0.05, ** p<0.01, *** p<0.001 compared with controls; ANOVA/Bonferroni multiple comparisons test.

the time spent in the open arms was also significantly affected by all the concentrations of linalool oxide and diazepam ($p<0.05$, $p<0.01$ and $p<0.001$, respectively). The effects of linalool oxide inhalation (0.65%, 2.5% and 5.0% w/w) and intraperitoneal diazepam resulted in significant increases in the total number of entries into the arms ($p<0.05$, $p<0.01$ and $p<0.001$, respectively) (Fig. 3).

3.2. Light/dark box test

The results of the light/dark box test are shown in Figs. 4 and 5. As shown in Fig. 4, all the linalool oxide and diazepam groups spent significantly more time in the brightly lit chamber compared to the respective control groups ($p<0.05$, $p<0.01$ and $p<0.001$). In addition, there was a significant increase in the number of crossings between the compartments as a consequence of the effect of 0.65%, 2.5% and 5.0% linalool oxide and diazepam compared to controls ($p<0.05$, $p<0.01$ and $p<0.001$) (Fig. 5).

3.3. Rotarod test

Previous treatment with linalool oxide failed to modify motor performance on the rotarod, unlike the significant effect observed only 30 min following treatment with diazepam used as positive control (Fig. 6).

4. Discussion

In the modern world, anxiety disorders have become common ailments and are usually associated with other psychiatric disorders (Linck et al., 2010). Despite the availability of treatment with the arsenal of anxiolytic drugs currently available on the market, many of these pharmaceutical options, such as benzodiazepines, are fairly nonselective and may cause significant adverse effects such as dependence, withdrawal syndrome or muscle relaxation.

Many plants exert recognized medicinal effects on the central nervous system and are able to act on chronic conditions such as anxiety and depression that do not respond well to conventional therapeutic treatments (Blanco et al., 2009). Due to the constant need to identify new treatments for anxiety disorders, aromatherapy has grown in importance as an area in alternative medicine with proven high efficacy in reducing stress and improving mood disorders (Bradley et al., 2007a; Steflitsch and Steflitsch, 2008).

According to Umezu et al. (2002), although the odor of OEs appears to exert an active effect, this odor alone is ineffective because of an õadaptation that occurs in olfactory receptor cells. The same author has suggested that EOs may possess psychoactive activity, affecting brain function pharmacologically (Umezu, 1999, 2000; Umezu et al., 2001).

When inhaled through the nose and olfactory cortex, the odors of EOs seem to exert a direct effect on the limbic system where anxiety and emotions are usually generated (Bradley et al., 2007a). This gives inhalation an advantage over other routes of administration such as, for example, the oral route, and eliminates the possibility of any chemical changes to the oil as it passes through the digestive system (Bradley et al., 2007a).

Linalool, a monoterpene found in high concentrations in the EOs of various species of aromatic plants, has been used in traditional medical systems to relieve symptoms and cure a variety of diseases (Peana et al., 2002). It is a mono-oxygenated monoterpene containing a chiral center. The anxiolytic activity of this metabolite has been reported in the literature. Linalool oxide is a di-oxygenated monoterpene containing two chiral centers. The more complex stereochemistry of

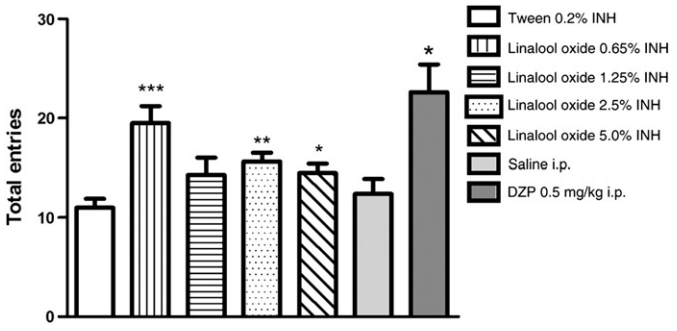

Fig. 3. Effect of linalool oxide on the total arms entries in EPM test. Evaluation of different concentrations of inhaled linalool oxide and diazepam i.p. in male mice. Data are expressed as mean ± SEM. N = 8. * p<0.05, ** p<0.01, *** p<0.001 compared with controls; ANOVA/Bonferroni multiple comparisons test.

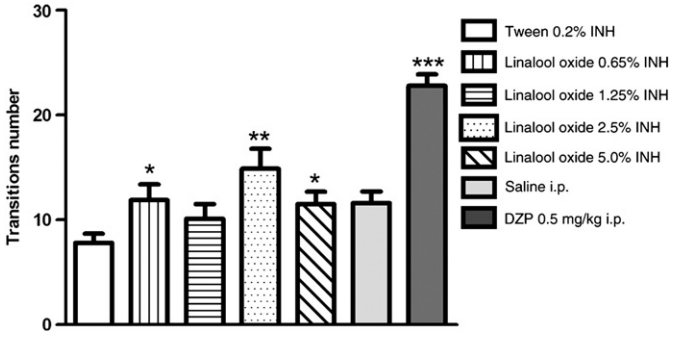

Fig. 5. Effect of linalool oxide on the transitions number between compartments in light–dark box test. Evaluation of different concentrations of inhaled linalool and diazepam i.p. in male mice. Data are expressed as mean ± SEM. N = 8. * p<0.05, ** p<0.01, *** p<0.001 compared with controls; ANOVA/Bonferroni multiple comparisons test.

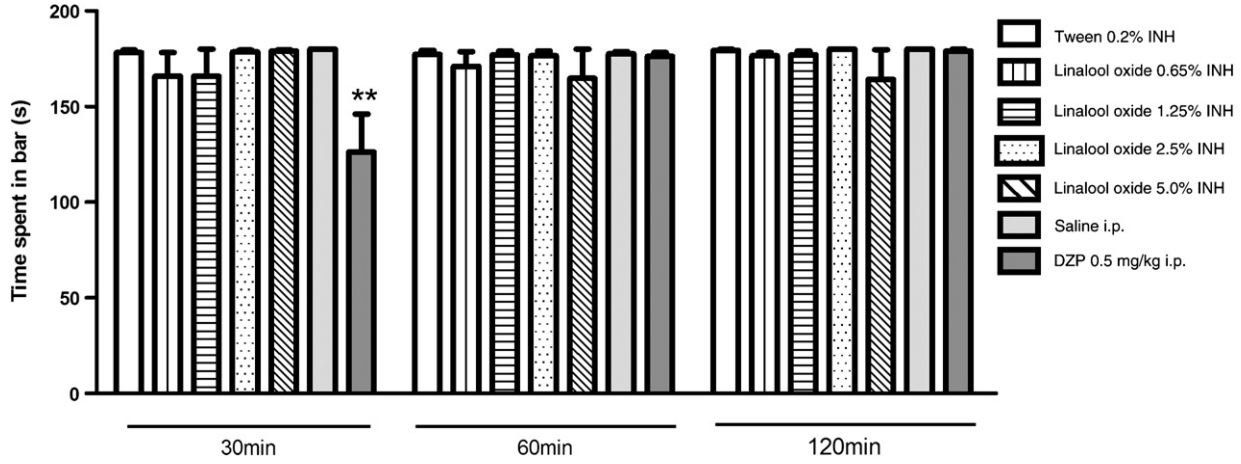

Fig. 6. Effect of linalool oxide upon the ability of mice to remain on the bar in rotarod test. Evaluation of different concentrations of inhaled linalool oxide and diazepam i.p. in male mice. Data are expressed as mean ± SEM. N = 8, ** $p<0.01$ compared with control; ANOVA/Bonferroni multiple comparisons test.

this metabolite may confer greater stereoselectivity in receptors that are associated with anxiolytic activity. We believe that linalool oxide has a better pharmacological profile compared to that of linalool. In fact, it is known that 2,5-substituted tetrahydrofuran subunits are found in many biologically active natural products and contribute to the pharmacological action of various natural substances (McLaughlin, 2008; Bermejo et al., 2005). There is a growing interest in this chemical group. Therefore, these experimental results with linalool oxide represent the first report of the anxiolytic activity of a representative natural molecule of the 2,5-substituted tetrahydrofuran group. Investigation of its mechanism of action should be an objective of future studies.

\In the present study, the effect of linalool oxide was investigated in mice, being administered by inhalation at concentrations of 0.65%, 1.25%, 2.5% and 5.0%. Results show that the anxiolytic drug profile of this monoterpene is similar to that of linalool in mice when a 3% concentration is inhaled (Linck et al., 2010).

The EPM test is a useful and valid animal model for measuring anxiety by investigating aspects of physiological and pharmacological behavior (Martinez et al., 2005; Bessa et al., 2005). This method is able to reproduce anxiolytic or anxiogenic effects in rodents in which anxiolytic substances tend to increase the number of entries into the open arms of the device and the time spent there, while anxiogenic substances have the opposite effect (Lister, 1987, 1990; Pellow and File, 1986; Santos et al., 2006; Daza-Losada et al., 2008). In this study, linalool oxide increased the number of entries into the open arms of the device (Fig. 1) and the time spent in the open arms of the maze (Fig. 2), behaviors indicative of anxiolytic activity and similar to the positive pattern effects expected following the use of diazepam (Deng et al., 2010). The total number of entries in the maze is calculated by adding the number of times the animal goes into the open arms of the device plus the number of times it goes into the closed arms. The total distance traveled by the animals is a measure of locomotor activity (Faturi et al., 2010), and the results showed that linalool oxide increased ambulation in these animals (Fig. 3). The anxiolytic effects of certain drugs such as benzodiazepines are accompanied by decreased locomotor activity and sedation (Park et al., 2005). Development of new anxiolytics that do not induce sedative effects and/or inhibit locomotion would be highly useful (Park et al., 2005). Since OXL stimulated locomotor activity in this experiment, it is reasonable to conclude that OXL has a better anxiolytic profile compared with other drugs.

In order to obtain further knowledge on the possible anxiolytic profile of linalool oxide, the light/dark box test was also performed. This test is widely used with rodents as a model for screening anxiolytic and anxiogenic drugs, based on the innate aversion of rodents to well-lit areas and the spontaneous exploratory behavior of these animals (Wei et al., 2007). An analogy with anxiety is generated by the conflict between the rodent's desire to explore and its aversion to unknown spaces and brightly lit areas (Pultrini et al., 2006). The anxiolytic-like effect of a drug may be reflected in an increase in the time spent in the well-lit compartment and by an increase in the number of times the animal crosses from one compartment to the other (Bradley et al., 2007b). The results were consistent with data in the literature, showing an increase in the time spent in the brightly lit compartment (Fig. 4) and an increase in the number of movements from one compartment to the other (Fig. 5). In the light/dark test, diazepam also caused an increase in the exploratory behavior of animals by increasing the time they spent exploring the well-lit area of the box (Fig. 4) as well as the number of movements between compartments (Fig. 5), confirming its role as a positive control of anxiety.

The rotarod test was performed to complement the other experiments and to investigate whether linalool oxide would cause muscle relaxation in the animals. This test is useful for evaluating the integrity of motor coordination in animals based on their ability to continue walking on a rotating bar for a certain period of time (Capasso et al., 1996). It can be used to detect physical disabilities due to pharmacological agents such as muscle relaxants and central nervous system depressants (Sen and Chaudhuri, 1992; Pultrini et al., 2006). When the performance of animals treated with a certain drug is investigated using the rotating bar and is found to be similar to that of the control group, this suggests that motor coordination has not been impaired in those animals (Blanco et al., 2009). Linalool oxide inhalation did not appear to cause muscle relaxation or motor coordination deficit, since there was no decrease in the time spent on the bar compared to the control group.

The combined results of this study allow us to conclude that linalool oxide exerts an anxiolytic effect on mice without causing motor impairment. Together with other studies conducted in this area, this study may lead to further investigation aimed at discovering new drugs for the treatment of anxiety disorders.

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