Anxiolytic-like effect of *Citrus limon* (L.) Burm f. essential oil inhalation on mice

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ABSTRACT: Experimental *in vivo* study aimed to characterize the anxiolytic-like effect of the *Citrus limon* fruit peel's essential oil (CLEO) in animal models of anxiety, besides evaluating the viability J774.A1 cells *in vitro* through the MTT reduction method at the concentrations of 10 and 100 μg/mL. The anxiolytic behavior was evaluated in *Swiss* mice (n = 8) using the methodology of Elevated Plus-Maze (EPM) and Open-Field (OF). CLEO was tested by inhalation at the doses of 100, 200, and 400 μL, and as control, animals were subjected to inhalation of the vehicle (saline solution 0.9% + Tween80®) and intraperitoneal administration of diazepam (1.5 mg/kg). In the cell viability assay, it was observed that none of the concentrations showed cytotoxicity. OF test showed significant anxiolytic activity at all tested doses of OECL, compared to the control group, without changing the motor performance of the animals. Corroborating OF data, the EPM test confirmed anxiolytic activity in at least two doses of the tested oil (200 and 400 μL), justified by the number of entries and increase in the percentage of time in the open arms. The data analysis of this study evidenced that inhalation of OECL was able to induce an anxiolytic behavior in mice; however, further studies are required to ensure its safe use by the population.

Keywords: Anxiolytic. *Citrus limon*. Essential oil. Inhalation. Mice.

RESUMO: Efeito ansiolítico da inalação do óleo essencial de *Citrus limon* (L.) Burm f. em camundongos. O estudo experimental *in vivo* objetivou caracterizar o efeito ansiolítico do óleo essencial das cascas do fruto de *Citrus limon* (OECL) em modelos animais de ansiedade, além de avaliar *in vitro* a viabilidade de células J774.A1 através do método de redução do MTT nas concentrações de 10 e 100 μg/mL. O comportamento ansiolítico foi avaliado em camundongos *Swiss* (n = 8), utilizando a metodologia do Labirinto em Cruz Elevado (LCE) e do Campo Aberto (CA). O OECL foi testado por inalação nas doses de 100, 200 e 400 μL, e como controle, os grupos foram submetidos à inalação do veículo (Solução fisiológica 0,9% + Tween80®) e administração intraperitoneal de diazepam (1,5 mg/kg). No ensaio de viabilidade celular, observou-se que nenhuma das concentrações apresentou citotoxicidade. O teste de CA demonstrou atividade ansiolítica significativa em todas as doses testadas do OECL, comparada ao grupo controle, sem alterar a performance motora dos animais. Corroborando os dados do CA, o teste do LCE confirmou a atividade ansiolítica em pelo menos duas das doses do óleo testadas (200 e 400 μL), justificada pelo número de entradas e aumento na percentagem de tempo nos braços abertos. A análise dos dados deste estudo evidenciou que a inalação do OECL foi capaz de induzir um comportamento ansiolítico em camundongos; entretanto, outros estudos são necessários para garantir o seu uso seguro pela população.

Palavras-chave: Ansiolítico. *Citrus limon*. Óleo essencial. Inalação. Camundongos.

INTRODUCTION

Anxiety affects one-eighth of the total population of the world and is the most prevalent psychiatric disorder in the general population; therefore, it has become an important area of psychopharmacological research because of its prevalence in recent years (White & Clare,

2006; Lourdu-Jafrin et al., 2013). As existing pharmacotherapy is not effective for all patients, the search for better anxiolytic drugs with fewer side effects continues (Bridges et al., 2011). To date, the efficacy of the drugs for mental disorder conditions are very limited; consequently, the need for newer, better-tolerated, and more efficacious treatments remains high (Wattanathorn et al., 2007).

Aromatherapy, the therapeutic use of essential oils (EOs) from plants towards improving physical, emotional, and spiritual well-being is an inexpensive, non-invasive complementary method (Ueki et al., 2014; NCI, 2014). This technique has increased substantially over the years compared with other medical approaches, since it represents an important therapeutic instrument for health professionals who can pluralize this practice in order to provide a more comfortable therapy, with fewer adverse effects and higher treatment adherence (Kutlu et al., 2008; Gorn et al., 2009; Fayazi et al., 2011; Ischkanian & Pelicioni, 2012).

In this sense, complementary therapies using a variety of EOs from medicinal herbs have been currently tested in animals and patients as aromatherapy agents to treat mild to moderate anxiety symptoms, since they are safer and have less adverse effects than pharmacological approaches (Saeed et al. 2007; Setzer, 2009; Fayazi et al., 2011; Tsitsi et al., 2014).

EOs are volatile compounds present in high concentrations in aromatic plants, complexes, highly concentrated, constituted by secondary metabolites, and characterized by a strong fragrance (Bakkali et al., 2008; Silva et al., 2009).

Many EOs obtained from some parts of plants (or from their isolated compounds) of the *Citrus* genus (Rutaceae) have exhibited an anxiolytic activity profile by inhalation in preclinical assays with rodents – for instance orange (*Citrus aurantium*) and yuzu (*Citrus junos*) – as well as in clinical studies with bergamot (*Citrus bergamia*) and yuzu (Leite et al., 2008; Satou et al., 2012; Ueki et al., 2014; Watanabe et al., 2015).

Citrus species' EO contain derivatives of terpenes, aliphatic sesquiterpenes, oxygenated derivatives, and aromatic hydrocarbons so that the composition of the various mixtures of terpenes depends on the type of the examined species. However, the mixture of each type contains proportions of: limonene, α-pinene, β-pinene, myrcene, linalool, and terpinene (Monagemi et al., 2010).

Phytochemical analysis performed by the supplier company of the oil used in this study demonstrated the presence of the following metabolites: α-pinene, β-pinene, myrcene, *trans*-geraniol, γ-terpinene and (-)-D-limonene, the major

constituent. These compounds are also cited in the literature as chemical constituents of other species of the genus (Gonzalez-Molina et al., 2010).

The use of *Citrus limon* is very popular in daily life for a variety of problems including its use as nerve tonic and other central disorders (Khan & Riaz, 2015). Many studies also support and demonstrate the use of *C. limon* and some species of the *Citrus* genus as alternative and/or complementary therapy, but no work has been carried out to investigate the anxiolytic effects of the *C. limon* fruit peel's essential oil by inhalation. In this regard, the present study aimed to evaluate the anxiolytic-like effect of the *C. limon* essential oil (CLEO) by inhalation using behavioral animal models of generalized anxiety disorder and exploratory activity test.

MATERIAL AND METHOD
Animals

Male albino *Swiss* mice weighing between 25 and 35 g, provided by the Animal House of the Federal University of Alagoas were used in pharmacological tests. The animals were kept in polypropylene boxes in a room with controlled temperature (22 ± 1°C), with a 12-hour light/dark cycle (dark phase: 19:00-7:00), for at least 72 h before testing for acclimatization to the environment. They were fed with mineral water and a commercial solid diet *ad libitum* (Labina®, Purina, Brazil). All proceedings and experimental models were executed accordance with the experimental protocols designed in conformation with the Ethical Principles in Animal Research as adopted by the Brazilian Society of Laboratory Animal Science and by UFAL Research Ethics Committee (nº 024094/2011-14).

Drugs and treatments

Experimental procedures were performed with *Citrus limon* essential oil purchased from Ferquima (Ind. and Com. Ltda, Brazil), obtained through cold pressing method of the peels. For *in vivo* assays, CLEO doses used were: 100, 200, and 400 µL. Tween80® (2%, Sigma – EUA, in 0.9% sodium chloride isotonic solution) was used as vehicle (control group). As standard drug, Diazepam (DZP) 1.5 mg/kg (Compaz®, Cristália – Brazil) was administrated in a volume of 10 mL/kg body weight of animal. The animals (n = 8, for each group) were subjected to behavioral tests after sixty minutes of the DZP intraperitoneally (i.p.) administration, or thirty minutes of the vehicle inhalation, or seven minutes after inhalation of CLEO.

Inhalation apparatus

The inhalation apparatus, validated by Almeida et al. (2004), consists in an acrylic chamber

(36×30×29 cm, total volume = 31.32 L), whose floor was made of stainless steel grid, and the front and back walls were made of acrylic fiber containing four holes in each size (2 cm in diameter each) in which were inserted cotton balls (sized to fit the holes, in order to assure the aroma circulation inside the cage) soaked with vehicle (2 mL) or CLEO at the doses: 100, 200 and 400 µL – equivalent to concentrations: 2.15, 4.3 and 8.6 mg/L air, respectively, calculated by the expression:

$$Concentration\ of\ CLEO = \frac{number\ of\ cottons\ \times\ tested\ dose\ (mL) \times 84}{Total\ volume\ of\ the\ chamber}$$

, where 84 represents the mass of CLEO by mL (d_{CLEO} = 0,084g/mL).

The top wall contained 30 small holes for ventilation. During the inhalation exposition, animals were placed individually into the chamber for 7 min and after, submitted to behavioral tests. Chamber was always cleaned up (10% ethanol solution) between expositions.

Evaluation of cellular viability
Cytotoxicity assay

The deleterious effect of CLEO was determined by assessing the cytotoxicity on murine macrophages (J774.A1 cell line) obtained from the Cell Bank of Federal University of Rio de Janeiro (Brazil). These adherent-phenotype macrophage line was cultured in DMEM supplemented with 10% PBS at 37ºC with 95% humidity and 5% CO_2. Briefly, cell suspensions containing 2.0×10^5 cells/mL were placed in a 96-well plate in triplicate an incubated at 37ºC for 1 h. Once this time had elapsed, CLEO was added at two concentrations starting at 10 and 100 µg/mL. The cells were also cultured with milieu free from compounds or vehicle (basal growth control) or in media with DMSO 0.1% (vehicle control). Positive control (dead cells) was obtained by cellular lysis with 1% of Triton 100× in DMEM complete. After 48 h, the cytotoxicity was evaluated by mitochondrial activity of the cells via MTT reduction, through the cleavage of the tetrazolium salt, which the resulting optical density was measure by spectrophotometer (Hussain et al., 1993).

Behavioral tests
Elevated plus-maze (EPM) test

The apparatus consisted of a acrylic maze with two closed arms (30×5×15 cm) and two open arms (30×5×0.25 cm) connected by an open central area (5×5 cm). The arms were arranged such that those of the same type were opposite to each other. The maze was positioned 37.5 cm above the floor. Mice were individually placed into the centre of the maze, facing an open arm, and allowed to explore the whole apparatus for 5 min. The percentage of time spent in both arms (open or closed arm time/ total arm time x 100) and the number of entries into both arms were evaluated and used as traditional indices of the anxiety. The animals were observed in EPM between 9:00 a.m. and 13:00 p.m. (Pellow et al., 1985).

Open-field (OF) test

The Open-field consists in a rectangular acrylic box (31x31x15 cm) whose floor is dark with white lines which divides it into nine parts of similar area to assess the exploratory and behavioral activity of animals. Mice were individually placed in the center of the OF and recorded the number of behavioral parameters for 5 min (with one minute of previous adaptation): crossing (evaluated for the times which the animal passed for the rectangles with four paws - measuring locomotion activity), frequency of rearing (number of times the animals stood on their hind limbs), grooming (self-cleaning behavior), freezing (total motionless time) and the number of fecal boluses. In order to minimize the possible influence of circadian alterations, the animals were observed between 9:00 a.m. and 13:00 p.m. (Hall, 1934).

Statistical analysis

The data were expressed as the means ± SEM and analyzed by One-way ANOVA followed by Dunnett's or Bonferroni's tests compared with control and DZP groups, respectively. Data were analyzed using the GraphPad Prism software (version 5.0) and data were considered statistically significant when $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS
CLEO does not exhibit cytotoxicity

The results presented in the Figure 1 show the effects of CLEO (at 10 and 100 µg/mL) and DMSO (0.1%) against J774 murine macrophage using the MTT method. After 48 h of incubation, none of the tested doses affected the viability of J774 murine macrophages.

CLEO exhibits anxiolytic-like activity in mice
EPM test

Figure 2A shows that CLEO inhalation at the doses of 200 µL and 400 µL resulted in an increase in the number of open arms entries (CLEO 200 µL: 11.63 ± 0.9; CLEO 400 µL: 13.88 ± 1.1; $p < 0.01$ and $p < 0.001$, respectively) compared to the respective control group (CTRL: 8.0 ± 0.3;); in addition, the higher dose was also able to reduce the visits to the closed arms (CLEO 400 µL: 11.25 ± 0.5; $p <$
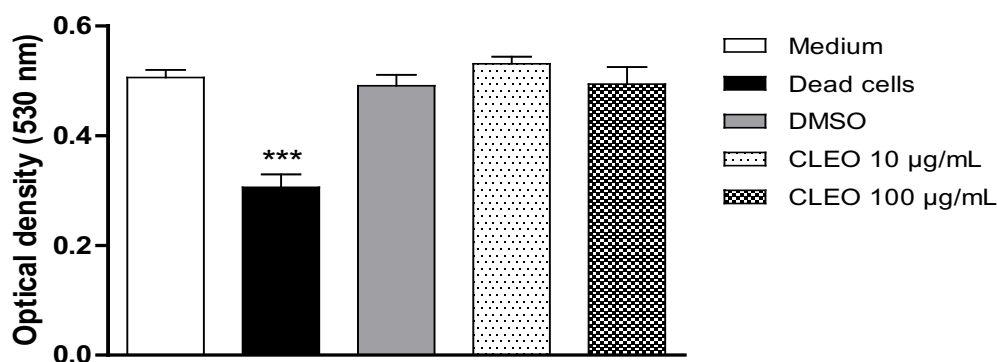
FIGURE 1. Effects of *Citrus limon* essential oil against J774 line cells in MTT assay. Dimethyl sulfoxide 0.1% (DMSO); *Citrus limon* essential oil (CLEO 10 and 100 µg/mL). The columns and vertical bars represent the means ± SEM in triplicate. ***$p < 0.001$ compared with dead cells using ANOVA and Dunnett's test as the *post hoc* test.

0.05) compared to the same group (CTRL: 14.5 ± 0.5) – Figure 2B. DZP treatment (i.p.), as expected, increased significantly the number of entries into open arms (DZP: 17.13 ± 0.6; $p < 0.001$), as well as decreasing the visits to the closed arms (DZP: 5.37 ± 0.6; $p < 0.001$) compared to control.

The ANOVA for the %TOA (Figure 2C) revealed significant differences among the groups treated with essential oil or DZP compared to CTRL, in which the animals which were submitted at inhalation of the doses of 200 and 400 µL spent more time in the open arms (CLEO 200 µL: 45.23 ± 3.5%; CLEO 400 µL: 49.46 ± 3.5%; both, $p < 0.001$) than the CTRL group (CTRL: 30.46 ± 0.8%), also observed with DZP treatment (DZP: 73.35% ± 1.2%; $p < 0.001$). The same analysis for the parameter %TCA (Figure 2D) also showed difference among the groups treated with CLEO in the same doses (CLEO 200 µL: 36.34 ± 2.6%; CLEO 400 µL: 32.16 ± 3.0%; both, $p < 0.001$), and DZP (DZP: 15.44 ± 1.6%; $p < 0.001$), standard drug, compared to CTRL group (CTRL: 59.55 ± 1.2%).

OF test

According to Table 01, it was observed that treatment with DZP resulted in significantly decreasing ($p < 0.001$) of emotional parameters – rearing and grooming –, as well as autonomic stimulation represented by the number of fecal boluses ($p < 0.05$), beyond what this drug was also able to significantly reduce the number of crossings ($p < 0.001$) and increase the time in which the animals remained motionless, compromising the animals locomotor system, characteristic effect of anxiolytic drugs (benzodiazepines) used in clinical practice.

All three tested doses of CLEO were also able to significantly reduce the number of rearings and groomings (both, $p < 0.001$), however without changing the locomotion parameters (crossing and immobility time), which suggests that inhalation of this essential oil exhibits anxiolytic activity without to modify motor performance, unlike it is observed in the conventional pharmacotherapy.

CLEO doses did not statistically differ among themselves in the evaluated parameters. On the other hand, these same doses were statistically different for the rearing ($p < 0.05$, for CLEO 100 and 200 µL; $p < 0.001$, for CLEO 400 µL) and immobility time ($p < 0.001$, for all three doses) compared to the DZP treatment, so that in this test the behavioral records of the animals were similar at the three doses.

DISCUSSION

In the modern world, anxiety disorders have become common ailments and are usually associated with other psychiatric disorders, like depression (Linck et al., 2010). Despite the availability of treatment with several anxiolytic drugs currently on the market, many of these pharmaceutical options, such as benzodiazepines, are fairly nonselective and may cause significant adverse effects such as dependence, tolerance, withdrawal syndrome or muscle relaxation (Souto-Maior et al., 2011).

It is widely accepted that more than 80% of drug substances are either directly derived or developed from a natural compound (Maridass & Britto, 2008). Numerous herbal medicines, or their isolated actives, 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 sources of treatments for anxiety disorders, aromatherapy has grown in importance as an area
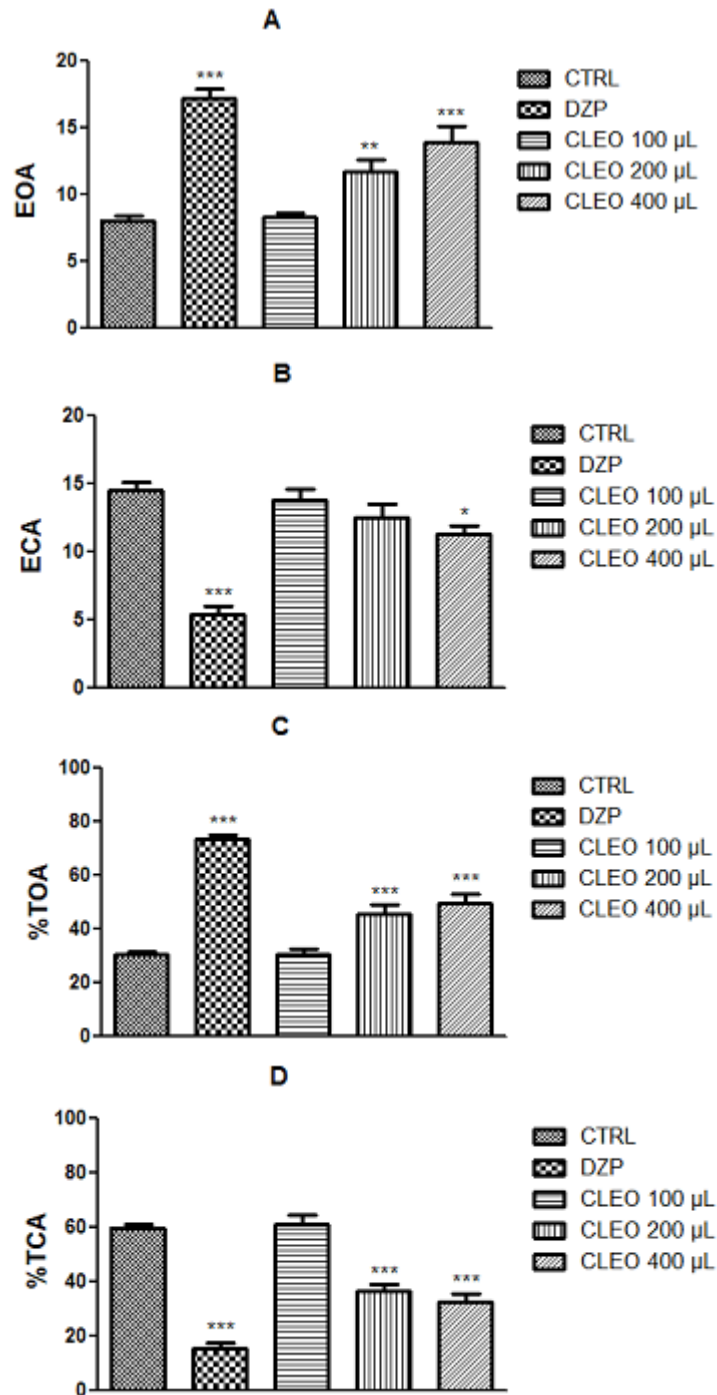
FIGURE 2. Effects of *Citrus limon* essential oil (CLEO) inhalation in mice in Elevated Plus Maze. **A.** Numbers of entries into open arms (EOA). **B** Numbers of entries into closed arms (ECA). **C**. Spent time percentage into open arms (%TOA). **D.** Spent time percentage into closed arms (%TCA). Control (CTRL: 2.0 mL, inhalation); Diazepam (DZP: 1.5 mg/kg, i.p.); *Citrus limon* essential oil (CLEO: 100, 200, and 400 µL, inhalation). The columns and vertical bars represent the means ± SEM of eight mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with vehicle group using ANOVA and Dunnett's test as the *post hoc* test.

TABLE 1. Effects of *Citrus limon* essential oil inhalation in mice in the open-field test. Control (CTRL: 2.0 mL, inhalation); Diazepam (DZP: 1.5 mg/kg, i.p.); *Citrus limon* essential oil (CLEO: 100, 200, and 400 µL, inhalation). The values represent the means ± SEM of eight mice. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with vehicle group; +$p < 0.05$ and +++$p < 0.001$ compared with DZP group; using ANOVA and Bonferroni's test as the *post hoc* test.

Treatment	Crossing	Rearing	Grooming	Fecal boluses	Immobility time
CTRL	44,4 ± 3,4	27,7 ± 2,9	10,9 ± 2,3	1,8 ± 0,2	15,9 ± 2,1
DZP	27,1 ± 3,2···	0,5 ± 0,4···	0,0 ± 0,0···	1,0 ± 0,7·	188,8 ± 16,7···
CLEO 100 µL	38,8 ± 2,5	11,0 ± 1,8···+	0,6 ± 0,6···	1,2 ± 0,4	28,3 ± 4,2+++
CLEO 200 µL	37,2 ± 4,8	10,0 ± 1,7···+	0,0 ± 0,0···	1,0 ± 0,4·	27,1 ± 6,6+++
CLEO 400 µL	32,8 ± 3,2	14,7 ± 2,7··+++	0,0 ± 0,0···	1,2 ± 0,4	44,6 ± 8,5+++

in alternative medicine with proven high efficacy in reducing stress and improving mood disorders and vital signs (Bradley et al., 2007; Steflitsch & Steflitsch, 2008; Boehm et al., 2012).

According to Umezu et al. (2002), although the odor of EOs appears to exert an active effect, this odor alone is not effective due to an adaptation that occurs in olfactory receptor cells. The same author has suggested that EOs may possess psychoactive activity, modifying brain function pharmacologically (Umezu, 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 often processed (Bradley et al., 2007). 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, mainly regarding to the first-pass effect (Bradley et al., 2007).

Among the EOs, those of citrus fragrances have been popularly used as therapies for their effects on mood states and depression (Agra et al., 2008). *Citrus limon* L. species is widely used to treat or lighten insomnia and anxiety disorders, and so this work aimed to evaluate the potential anxiolytic effect of *C. limon* (sicilian lemon) essential oil. Then, the investigation was primarily undertaken to detect a possible cytotoxic activity of the CLEO against macrophages from J774.A1 lineage.

The development of new bioactive drugs from essential oils requires *in vitro* studies that can identify possible toxicity because provide important results about its mode of action and thus lead to next steps for studying and future use in humans. Toxicity studies with *in vitro* assays have demonstrated that tests with cell cultures can be used successfully, since they are reproducible, fast, sensitive, affordable for the execution, and mainly biocompatibilities with *in vivo* results (Authier et al., 2013).

Our results demonstrated that tested doses did not affect the viability of this cell lineage, unlike from the observed in other studies. Cytotoxic effects of *Citrus sinensis* essential oil were demonstrated by MTT assay in a study with SW480 and HT-29 cells in different times of incubation (Chidambara-Murthy et al., 2012). Similarly, the incubation of LAN-1 and SK-N-SH cells with growing concentrations of *Citrus bergamia* juice ranging from 0.5 to 10% for 24, 48 and 72h reduced cell proliferation in a concentration-dependent manner (Navarra et al., 2014).

Thus, based on our data, our results demonstrated to be safe and gave us support to continue with behavioral tests. These behavior animal models have contributed to the screening of new psychopharmacological tools as well to understanding the information about molecular mechanisms involved in anxiety and for screening and developing new medications for their treatment that would be unfeasible in humans (Kumar et al., 2013).

The test battery employed here showed to be adequate, since the classical anxiolytic, diazepam, exhibited its typical effects in the Open-field and Elevated plus-maze tests which are best experimental methods for the measurement of anxiety, because they are typically assessed by measuring the repeated decrease in a fear-related behavior in a context environmental – for example, freezing and elevation to the floor (Griebel & Holmes, 2013; Mansouri et al., 2014).

The results obtained have clearly shown anxiolytic-like effects of *C. limon* essential oil, through the reduction of emotional parameters and/ or increasing of anxiolytic behaviors presented by the animals exposed to the aroma in the animal models used.

Despite the fact that two doses (200 and 400 µL) demonstrated anxiolytic activity in both tests, the highest dose seemed to be the best one, as it gave good results. It is not possible to talk about a dose-dependent response since, in the EPM, the oil-treated groups only differed from the control group

and in the OF, and all the doses seem to exert similar effects. This lack of dose/effect relation is common when dealing with a mixture of compounds instead of a pure substance.

In the EPM, inhalation of CLEO was able to induce an increasing in EOA and %TOA at the doses 200 and 400 µL similar to DZP treatment. The frequency and the time spent into open arms are the largest parameters of reduction of anxiety on the EPM, since the open area represents extremely aversion by the rodents. This trend is enhanced by anxiolytic drugs and suppressed by anxiogenic agents (Bourin et al., 2007).

Our results corroborate other studies of anxiolytic activity with species from *Citrus* genus. The experiments carried out by Faturi et al. (2010) in EPM with OE of *C. sinensis*, at the same doses and route of our study, demonstrated that the intermediate and maximum tested doses show significant statistically differences compared to the vehicle group, regarding the number EOA and %TOA.

Saiyudthong & Marsden (2011) have also demonstrated that the inhalation of *Citrus bergamia* essential oil at the concentrations 2.5 and 5.0% significantly increased the percentage of EOA and %TOA on the EPM, which shows that the species of the *Citrus* genus has high potential to trigger anxiolytic effect.

Some authors suggest that substances which modify locomotor activity can act in the EPM test providing a false-positive result (Silva et al, 2007; Gomes et al., 2008). To confirm this result of the anxiolytic effect by itself and not of changing locomotor activity by OECL, it was carried out the open-field test, which is used to analyze behaviors based on natural conflict situations reflected by exploration and aversion to open and light environments (Sestakova et al., 2013).

The total number of crossings during the test is considered exploratory activity index (Lotufo et al., 2004). Thus, the model can discriminate whether the tested substance exhibits an anxiolytic effect or nonspecific depressant, acting in brain regions also involved with the animal's motor system (Silva et al., 2006).

In our findings, we observed that exposure at *C. limon* aroma reduced the number of rearing and grooming - behaviors indicative of anxiolytic activity. In addition, it was observed in this test that there were no changes in the motor parameters (crossing and immobility time), which discards the possibility of false-positive from CLEO inhalation on the EPM.

Furthermore, the results demonstrated that treatment with the DZP also produced a decrease of emotionality parameters (rearing and grooming), however it reduced the number of crossings and increased immobility time, which is expected, since benzodiazepines may cause decrease of locomotor activity and sedation (Deng et al., 2010).

Thereby, development of new anxiolytics that do not induce sedative effects and/or inhibit locomotion would be highly useful (Park et al., 2005). Hence, CLEO might serve as an appealing alternative therapeutic target, because the behavior analyzed in the animals is suggestive of anxiolytic-like effect with no motor impairment.

CONCLUSION

The combined results of this study allow us to conclude that *Citrus limon* essential oil shows no toxicity *in vitro* according to the test performed and its use for inhalation route demonstrated anxiolytic-like effect in animals with no motor impairments although more pre-clinical and clinical investigations are necessary in order to find out the significance of this treatment and mechanisms involved. Furthermore, our findings appear very relevant, because the results shown were obtained from a natural product, which indicate potential clinical applications in the future, particularly for the high occurrence of patients which suffer with anxiety disorders or current drug therapy.

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