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Preclinical evaluation of repeated dose toxicity and anxiolytic activity of monoterpene R - (+) – limonene via inhalation

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R - (+) - limonene is a monoterpene found in several plant species. Studies have shown that plants that have this compound has anxiolytic activity. This study aimed to investigate the anxiolytic and toxicological activity in rats after inhalation of R - (+) - limonene for 30 min. For the anxiety tests using Elevated Plus Maze (EPM), concentrations of 1.0, 2.5 and 5.0% were determined. From the results, it was observed that the last concentration had achieved anxiolytic effect. Based on these results, it was found by biochemical and hematological tests, toxicity of 5% concentration in the period of 30 days with minor significant changes. In the hematological parameters, MCV, MCH and MCHC values were significant when compared to the control group, but were within the normal range when compared to values found in literature. In the biochemical parameters, changes occurred in AST and LDH levels. Elevated LDH levels can cause destruction of erythrocyte precursor cells in the bone marrow. In turn, AST may be associated to small changes in the liver. Therefore, the results demonstrated an anxiolytic activity with low toxicity at dose of 5% R - (+) - limonene via inhalation for 30 min.

Key words: R-(+)-limonene, inhalation, biochemical, hematological, anxiolytic, toxicity.

INTRODUCTION

The therapeutic effects of essential oils (EOs) on physical and psychological disorders are well known. Studies with aromatherapy have reported medicinal properties of aromas that have been used for thousands of years by humans in the treatment of various diseases (Kutlu et al., 2008). EOs, administered via inhalation, stimulate the olfactory nerves that have an effect on the brain, which results in behavioral changes in animals and humans (Su et al., 2009; Lawless, 2002). Their use may represent an alternative method for the treatment of disorders such as

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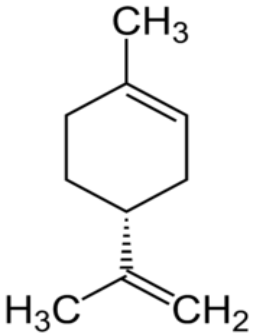

Figure 1. Chemical structure R-(+)-limonene.

anxiety (Almeida et al. 2004) and seizures (De Sousa et al., 2006).

Monoterpene R - (+) - limonene (1-Methyl-4- (1-methylethenyl) -cyclohexene) (Figure 1) is present at high proportions in citrus fruits and is marketed by many EO manufacturers (Malhotra et al., 2009). EOs are found in various plants such as *Lippia alba* (Mill.) N. E. BROWN (Verbanaceae) (Vale et al., 2002), *Artemisia dracunculus L*. (Asteraceae) (Sayyah et al., 2004), and other aromatic plant species. They present antinociceptive activity when intraperitoneally administered (Do Amaral et al., 2007). Moreover, the action of this monoterpene as a modulator of oxidative stress, also with chemopreventive activity (Manuele et al., 2010) has been reported, reducing skin tumors in animals (Chaudhary et al., 2012). Previous studies have shown that the administration of R - (+) - limonene (0.5 and 1.0%) in mice via inhalation produced anxiolytic effect in an elevated plus maze model (Lima et al., 2013).

R - (+) - limonene, found in many household products, may react with ozone to form free radical species, formaldehyde, and fine and ultra fine particles. Sunil et al. (2007) have shown that young and old mice when exposed to the product of the reaction between limonene and ozone for 3 h show an inflammatory response induction, increasing TNFa, cyclooxygenase-2 and superoxide dismutase in alveolar macrophages. This monoterpene is a potentially sensitizer compound, probably due to the presence of limonene-1,2-epoxide, a product of its oxidation (Karlberg et al., 1999). In contrast, Rolseth et al. (2002) showed that limonene-1,2-epoxide was not responsible for the limonene toxicity when investigated in culture of human pulmonary cells.

Selection of appropriate parameters, the use of a quality control concept and hematological and biochemical analyses using appropriate statistics for the clinical pathology examination of animals are very important for toxicity studies (Matsuzawa et al., 1995).

Research involving prolonged exposure to R - (+) - limonene via inhalation should be performed as limonene has many commercial applications and has been presented as environmental solvents (Rolseth et al., 2002). Therefore, this study aims to evaluate anxiety and toxicity, in accordance with the recommendations of the National Health Surveillance Agency (ANVISA, BRASIL), in rats via inhalation due to the frequent exposure of humans to this substance and its combinations.

MATERIALS AND METHODS

Animals

Wistar rats (*Rattus norvegicus*) (180 – 220 g) were divided into three groups, males (n = 10) for psychopharmacological evaluation and both genders (n = 5) for toxicological evaluation, obtained from the Thomas George Animal Facility of the Research Institute of Drugs and Medicines (IPeFarM-UFPB). All animals were kept until the day of experiments in polypropylene cages with controlled temperature (21 ± 1°C) in 12-h light-dark cycle (light starting at 06:00 am and ending at 06:00 pm). Animals had free access to food (Purina® pellets) and water, up to 60 min before experiments, which were conducted between 06:00 am and noon. All procedures and experimental protocols were approved by the Ethics Research Committee on Animal Use of the UFPB Biotechnology Center (CEUA-CeBiotec) (0606/11).

Drugs and treatments

R - (+) - limonene (97% purity) was purchased from company Dierberger Óleos Essenciais SA, Barra Bonita, Brazil and diluted with Ethoxylated Sorbitan Monooleate (Tween 80 [VETEC, Brazil], 0.2% v / v in distilled water) to prepare R - (+) - limonene (5.0%) emulsions min before the experiment. Control animals received 0.2% Tween 80 via inhalation.

Inhalation apparatus

The inhalation apparatus consisted of an acrylic box (36 × 30 × 29 cm) with a stainless steel grid at its bottom on which animals were individually placed. The front and rear panels contained four holes (each with 2 cm in diameter) with cotton balls embedded with the drug, containing 1 mL per unit of the respective substance (saline or R - (+) - limonene emulsion). The box ceiling contained 30 small holes for ventilation. Based on studies by Lima et al. (2013), the duration of drug exposure (limonene or saline) was 30 min. After each exposure session, the apparatus was cleaned and the cotton ball containing R - (+) - limonene was replaced to maintain the drug concentration in the apparatus.

Elevated plus maze test (EPM)

Three groups of 10 animals were treated with R - (+) - limonene at concentrations of 1.0%, 2.5% or 5.0% via inhalation, while the control group was treated with vehicle. After treatment with R - (+) - limonene or vehicle, animals were individually placed on the central platform facing one of the open arms of the maze, and the number of entries and time spent in each type of arm were recorded for a period of 5 min. Entry into the arm was also considered when the animal was found with all four paws within its limits (Pellow et al., 1985; Bradley et al., 2007; Grundmann et al., 2007).

Repeated dose toxicity (30 days)

Repeated dose toxicity studies were performed using animals of both genders divided into 2 groups (control and treated with 5%).

Each animal was exposed for a period of 30 min at a concentration established once a day. The total period of this protocol was 30 days. At the end of the experiment, animals were euthanized according to criteria established and blood was collected for the analysis of biochemical and hematological parameters.

Laboratory blood analysis

Sample collection was performed by bleeding of the brachial plexus and blood was collected in tubes with ethylenediamine tetraacetic acid anticoagulant (EDTA) to determine hematological parameters and in tubes with separator gel - Microtainer Becton Dickson®. Tubes were centrifuged for 5 min at 3500 rpm to obtain serum for the determination of biochemical parameters.

Hematological parameters

Hematological analysis consisted of the study of red (erythrogram) and white series (WBC) and platelet count. Erythrogram consisted of red blood cell counts, hematocrit and hemoglobin determination, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). In the WBC count, overall white cell count and counting of cell differentiation were made.

Biochemical parameters

Biochemical analyses were performed on serum samples. Dosages of glucose, urea, total cholesterol, triglycerides, uric acid, creatine kinase, albumin, amylase (enzymatic method), creatinine, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase (kinetic method) and total proteins (biuret method) were performed in automated biochemical analyzer COBAS MIRA PLUS® - ROCHE.

Statistical analysis

Data obtained for psychopharmacological tests were evaluated using the Kruskal-Wallis test followed by Dunns test. Toxicological data were treated with the "t" Student / unpaired Mann-Whitney test. These data were analyzed using GraphPad Prism software (version 5.0), with experimental group being compared with the control group. (*) P-value <0.05 was considered statistically significant.

RESULTS

Evaluation of anxiety

The animals treated with doses of 1.0, 2.5 and 5.0% showed significant differences when evaluated in the SEM. The three concentrations showed an increase in the number of entries into open arms. However, only 5% concentration showed a statistically significant increase residence time in the open arms (Figure 2).

Evaluation of repeated dose toxicity

Hematological parameters

The repeated dose toxicity test in which animals were exposed for 30 min/day for 30 days showed that some hematological parameters were changed significantly in both gender (Table 1). There was a decrease in erythrocytes, hemoglobin and hematocrit and an increase in VCM, MHCM and HCM.

Biochemical parameters

The biochemical analyses showed a statistically significant increase in glucose, AST and LDH in in both sexes treated with R - (+) - limonene, as compared to the control group (Table 2).

DISCUSSION

Evaluation of the anxiolytic effect of R - (+) - limonene in rats was examined using EPM (Figure 2). The three concentrations (1.0, 2.5 and 5.0%) tested in the study were able to increase the number of entries of rats in open arms. The concentration of 2.5% appeared high in the number of entries in the closed arms being significant only when compared this group with the control. However, to statistically analyze the four groups there is no evidence statistical difference.

In other parameter analyzed in EPM, increase in the time of permanence in the open arms at a dose of 5.0% and a decrease of this time in closed arms for the three dose levels (1.0, 2.5 and 5.0%) were observed. These data analyzed altogether show the anxiolytic effect of R - (+) - limonene, and the dose of 5.0% showed this effect when administered via inhalation in rats for 30 min.

Similar results can be found in previous studies, where the administration of R - (+) - limonene in mice at doses of 0.5 and 1.0% exhibited anxiolytic effect. It was also shown that this EO has an effect similar to diazepam when intraperitoneally administered in mice (Lima et al., 2013). This monoterpene is present at high percentages in *Citrus aurantium* EO (97.83%) having anxiolytic activity (Pultrini et al., 2006; Costa et al., 2013.). Furthermore, a product from limonene oxidation, limonene epoxide, also showed anxiolytic characteristics (De Almeida et al., 2012).

Therefore, EO concentration of 5%, considered with anxiolytic effect in a period of 30 min, was chosen for conducting toxicity tests in repeated doses. After treatment for 30 days, the levels of erythrocyte, hemoglobin and hematocrit count significantly reduced in animals exposed to 5.0% R - (+) - limonene as compared to the control group (Table 1).

The reduction on the erythrocyte and hematocrit count level can be suggestive of hemolysis caused by continuous use R - (+) - limonene (5%) via inhalation for both male and female rats, as the EO has irritant characteristics, inducing inflammatory responses (Sunil et al., 2007). However, these changes are within values

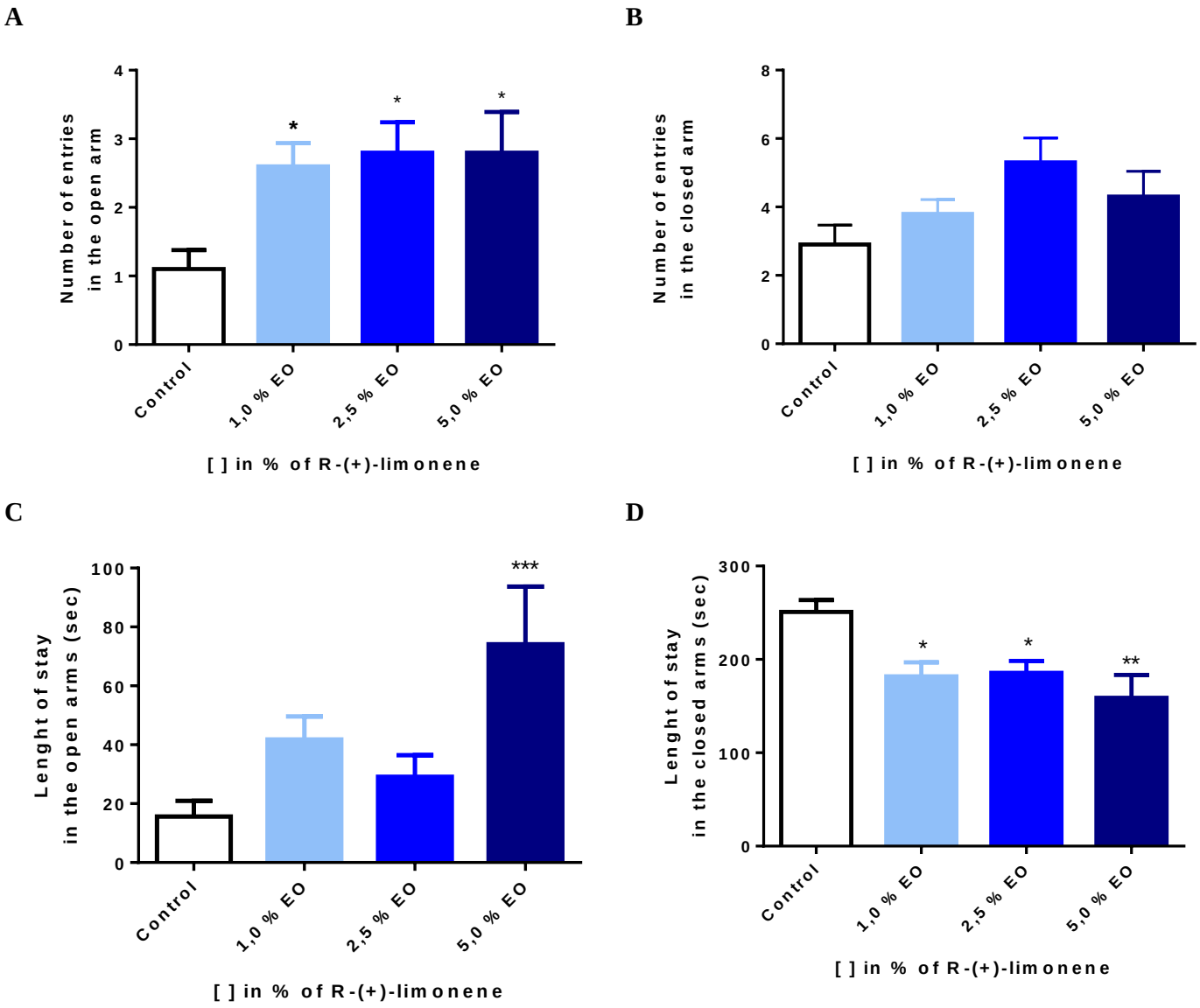

Figure 2. Effects of R-(+)-limonene v.i. (1.0, 2.5 e 5.0%) in time about 30 min the number of entries in the open arms (A) and closed (B) and time spent in the open arms (C) and arms closed (D) of EPM (n=10). Values are expressed as mean ± SEM (n=10). Kruskal-Wallis test followed by Dunns test. *p<0.05, **p<0.01, ***p<0.001.

shown by Lapchik et al. (2009) for red blood cells (5.4 – 8.5 10^6 /mm^3), hemoglobin (11.5 - 16.0 g/dL) and hematocrit (37-49%). Although high, the MCH and MCV values are very close to normal values proposed by Giknis and Clifford (2006). The MCHC values, even high when compared to the control group, are within the normal range (Giknis and Clifford, 2006).

Treatment with R - (+) - limonene in rats with repeated doses induced small changes in the biochemical profile. Some parameters such as glucose, lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) increased significantly when compared to the control group study (Table 2).

Although high when compared to the control group, glucose is within standards in according Giknis and Clifford (2006) where the range for glucose is considered 112 - 176 mg/dL. Already the LDH values showed increased compared to the control group (Table 2). High LDH can cause destruction of the erythrocyte precursor cells in the bone marrow, increasing by up to 50 times the activity of lactate dehydrogenase isoenzymes 1 and 2 (Motta, 2009).

Increases in AST levels are usually linked to liver disorders induced by drugs or hepatitis (Owen et al., 2012). However, toxicity studies with *Citrus limon* and limonene epoxide showed a decrease in AST levels

Table 1. Hematological parameters obtained from the serum of rats treated with 5% R - (+) - limonene inhalation 30 days.

Treatment	Erythrocytes (10^6/mm^3)	Hemoglobin (g/dl)	Hematocrit (%)	VCM (μ^3)	HCM ($\mu\mu$g)	MCHC (%)	Leukocytes (10/mm)	Lymphocyte (%)	Neutrophil (%)	Eosinophil (%)	Monocyte (%)	Platelets (10/mm)
						Male						
Control	8.7±0.1	14.1±0.2	41.3±0.7	47.8±0.4	16.4±0.2	33.8±0.4	4340±211.2	70.4±1.1	24.2±0.9	1.6±0.3	4.6±0.6	701.2±15.8
R-(+)-limonene (5%)	8.0±0.1*	13.9±0.2	38.9±0.6*	49.6±0.6*	17.3±0.2*	35.3±0.2*	4980±203.5	72.0±1.6	24.8±2.0	1.0±0.2	4.8±0.5	761.2±30.2
						Female						
Control	8.0±0.2	12.9±0.2	39.6±0.5	48.8±1.2	16.7±0.3	34.5±0.2	3020±609.4	77.4±1.8	17.8±1.4	0.4±0.2	4.20±0.8	678.8±57.3
R-(+)-limonene (5%)	7.6±0.1*	13.9±0.3*	37.4±0.5*	52.0±0.7*	17.8±0.1*	35.7±0.2*	4180±640.6	76.8±1.4	17.4±2.5	0.9±0.4	4.2±0.9	673.0±69.1

Values are expressed as mean ± SEM. (n=5). Test "t" de Student/Mann-Whithey. (*) $p < 0.05$. MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration).

Table 2. Biochemical parameters obtained from the serum of rats treated with 5% R - (+) - limonene inhalation 30 days.

Treatment	Glucose (mg/dL)	Urea (mg/dL)	Cholesterol (mg/dL)	Triglycerídes (mg/dL)	Uric acid (mg/dL)	Amylase (U/dL)	AST (U/l)	ALT (U/l)	Alkaline phosphatase (U/l)	LDH (U/l)	Total protein (g/dL)	Albumin (g/dL)
						Male						
Control	119.6±3.3	43.8±2.5	69.0±7.5	111.0±9.6	2.0±0.4	954.4±64.1	130.2±20.2	49.8±3.6	186.0±9.5	2788±467.5	6.9±0.2	3.3±0.1
R-(+)-limonene (5%)	143.8±5.9*	43.0±2.2	56.0±4.7	103.0±18.5	1.5±0.2	912.6±82.3	207.8±20.1*	45.4±3.8	158.6±15.7	4962±482.8*	6.5±0.2	3.0±0.1
						Female						
Control	109.6±4.9	46.0±7.2	54.8±5.2	79.6±9.8	1.6±0.1	677.6±67.1	134.8±16.3	41.6±3.9	104.4±7.7	2837±475.5	6.8±0.2	3.2±0.1
R-(+)-limonene (5%)	124.6±1.0*	42.8±1.8	57.8±3.3	90.4±7.3	2.1±0.2	710.6±16.0	251.6±39.9*	55.2±4.6	107.0±5.3	5599±226.8*	6.5±0.1	3.2±0.1

Values are expressed as mean ± SEM (n=5). Teste "t" de Student/Mann-Whithey. (*) $p < 0.05$. AST (aspartate aminotransferase), ALT (alanine aminotransferase), LDH (lactate dehydrogenase).

when orally administered (Campelo et al., 2013; De Almeida et al., 2014). In dogs and cats, increased AST and LDH levels can be caused by hemolytic diseases and decreased haptoglobin can be caused by the ruptureof erythrocytes inside blood vessels (Fighera, 2007).

Conclusion

Therefore, this study is concluded that the R - (+) - limonene 5% when administered by inhalation for 30 min produces an anxiolytic effect. Through the hematological and biochemical studies, suggest that the R - (+) - limonene in the studied concentration has a low toxicity.

Conflict of Interests

The authors have not declared any conflict of interests.

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