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Anxiolytic-like effects of carvacryl acetate, a derivative of carvacrol, in mice

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ABSTRACT

Studies showing anxiolytic-like properties of natural products have grown. This paper evaluated if carvacryl acetate (CA) could be studied as an alternative drug to treat anxiety disorders. Elevated plus maze (EPM) tests , light-dark box (LDB) tests, and marble-burying tests (MBTs) were performed on mice. In the first protocol, the anxiolytic-like activities of CA 25, 50, 75 and 100 mg/kg at single doses were compared to those of the vehicle, buspirone 5 mg/kg (BUSP) and diazepam 1 mg/kg (DZP). In the second protocol, the anxiolytic-like actions of CA were tested for GABAergic and serotonergic systems. The time spent in the open arms (TSOA) and the number of open arms entries (NOAE) were measured in EPM; the time spent in the light box (TSLB) and the number of entries to light box (NELB) were measured in LDB; and the number of marbles buried (NMB) were measured in MBT. CA increased TSOA and NOAE in the EPM, as well as TSLB and NELB in the LDB and the NMB in the MBT. The anxiolytic-like activity of CA 25; 50; 75 and 100 mg/kg was not associated with psychomotor retardation in the open field test and in the Rota rod test, contrarily with what happened with DZP. In the second protocol, to suggest the mechanism of action of CA, flumazenil 25 mg/kg ip (FLU) and WAY 100,635 10 mg/kg ip (WAY–5-HT1A antagonist) were also used. FLU + CA100 reduced TSOA in the EPM when compared to CA100 but WAY + CA100 did not. In LDB, FLU + CA100 reduced the TSLB when compared to CA100 but WAY + CA100 did not. In the MBT, FLU + CA100 inhibited the effect of CA100 on the NMB but WAY + CA100 did not. In conclusion, CA seems to have an anxiolytic-like effect, probably due to GABAergic agonist action, without psychomotor side effects.

1. Introduction

Anxiety is a behavioral mechanism in animals to cope with difficult situations. Fear and anxiety share the same physical and mental symptoms, like avoidance, hypervigilance and an increased alert level to avoid damage (Bernick, 2010; Higgins and George, 2010).

Anxiety disorders, which are among the most prevalent psychiatric conditions in most populations, produce morbidity, frequent use of health services, impair the functional performance of the individual and comorbidities with chronic medical conditions (Campbell-Sills et al., 2013; Johansson et al., 2013).

However, most patients with anxiety disorders experience a progressive reduction of their symptoms with continuous use of selective serotonin reuptake inhibitors (SSRIs) and immediate symptom relief with benzodiazepines (BDZ), as diazepam (DZP) (Ravindran and Stein, 2010). Buspirone (BUSP) is another drug used to treat anxiety symptoms (Celada et al., 2013). These drugs which reduce anxiety are described as anxiolytic drugs and act by altering the chemical synaptic transmission in the brain.

Several studies have shown that certain substances derived from plants may have central nervous system (CNS) effects, such as the ethanolic extract from *Platonia insignis* (Costa Júnior et al., 2010), the *Citrus limon* essential oil (Campêlo et al., 2011), the *Bellis perennis* (Marques et al., 2011), the essential oil from *Eupatorium triplinerve*

Abbreviations: BDZ, benzodiazepinic; BUSP, buspirone; CA, carvacryl acetate; DZP, diazepam; EPM, elevated plus-maze test; EL, epoxy-limonene; FLU, flumazenil; GABA, gamma-aminobutyric acid; LDB, light–dark box test; MBT, marble-burying test; NF, number of falls; NELB, number of entries to the light box; NMB, number of marbles buried; NOAE, number of open arms entries; NSC, number of squares crossed; OFT, open-field test; SSRI, selective serotonin reuptake reuptake inhibitor; TSLB, time spent in light box; TSOA, time spent in the open arms; TSRB, time spent on the rotating bar; WAY, WAY 100635.

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Vahl (Melo et al., 2013), the monoterpenes isopulegol (Silva et al., 2007), 1,4 cineole (Gomes et al., 2010), linalool (Souto-Maior et al., 2011), carvone (Costa et al., 2012) and epoxy-limonene (Almeida et al., 2012).

Accordingly, this study evaluated if carvacryl acetate (CA) has potential to be studied as an alternative drug to treat anxiety disorders. CA (Fig. 1), a semisynthetic monoterpenic ester, is derived from carvacrol, a component of oregano (*Origanum vulgare* L.) oil (Manou et al., 1998). Carvacrol has also been recognized to have anxiolytic-like potential in animal models of anxiety (Melo et al., 2010).

The elevated plus-maze (EPM) test, the light-dark box (LDB) test and the marble-burying test (MBT) were applied in mice treated with a single dose of CA to identify its anxiolytic-like potential (Badgujar and Surana, 2010; Deacon, 2013).

Flumazenil (FLU), a GABAergic antagonist, and N-{2-[4-(2-metho xyphenyl)-1-piperazinyl] ethyl}-N-(2-pyridyl) cyclohexanecarboxamide – WAY 100635 (WAY), a selective antagonist of 5-HT_{1A} receptor – were applied previously to CA to suggest its mechanism of action (Dalvi and Rodgers, 1999; Castro et al., 2008).

An open-field test (OFT) and a Rota-rod test were used to evaluate if the probable action of CA on the CNS is related to sedative and muscular relaxation effects. The effect of a single dose of CA on the CNS was compared with single doses of DZP and BUSP, which are standard drugs to treat anxiety disorders (Celada et al., 2013; Ravindran and Stein, 2010).

2. Material and methods

2.1. Reagents and drugs

Polyoxyethylene sorbitan monooleate (Tween 80) (Eg Sigma Chem. Co., St. Louis, Missouri, USA) was used as the vehicle in the groups. DZP and BUSP (Union Chemical, Brazil) were used as standard anxiolytic drugs. FLU and WAY were obtained from Eg Sigma Chem. Co., St. Louis, Missouri, USA. All the administrations were in acute form – single doses – intraperitoneally (ip).

2.2. Substance preparation

DZP, BUSP, WAY and FLU were emulsified with 0.2% Tween 80 and dissolved in distilled water. DZP was administered at a dose of 1 mg/kg, which is considered an anxiolytic dose on rodents (Bert et al., 2001). BUSP was used at 5 mg/kg, which is considered an anxiolytic dose on rodents (López-Rubalcava et al., 1999). FLU was used at 25 mg/kg and WAY at 10 mg/kg. Negative controls received only 0.2% of Tween 80 dissolved in distilled water (10 ml/kg).

CA was obtained by the carvacrol acetylation procedure, which used the acylating agent acetic anhydride as catalyst. There were carvacrol (5 g, 0.033 mol), pyridine (7.5 ml) and acetic anhydride (12.5 ml) in a flask (50 ml) equipped with magnetic stirrer, coupled to a Friedrich condenser and with an inert atmosphere. Then the solution was subjected to a constant magnetic stirring and reflux for about 24 h.

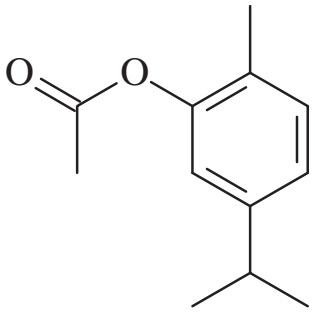

Fig. 1. Chemical structure of carvacryl acetate (5-isopropyl-2-methyl-phenyl).

The reaction mixture was poured into ice water (60 ml), and the extraction of the reaction product was done into a decantation funnel, using chloroform as a solvent extractor (3 × 60 ml). Chloroform phases were combined and washed with saturated copper sulfate (3 × 60 ml). The chloroform phases were also washed with water (3 × 60 ml) and dried with anhydrous Na_2SO_4. Subsequently, the solvent was evaporated on a rotary evaporator. The reaction product was subjected to column chromatography, using silica gel as the stationary phase and a mixture of hexane, CA (95:5), as the mobile phase.

With a 76% yield, approximately, 4779 g (0.025 mol) of CA was obtained, chemically defined as 5-isopropyl-2-methyl-phenyl (Fig. 1), having a purity of 98%, molecular weight of 192.26 g/mol, refractive index of 1497, boiling point of 94.56 °C at 760 mm Hg, enthalpy of vaporization of 48,414 kJ/mol and density 0.994 g/cm^3 (Vogel et al., 1996). Its color is yellow-green. It has an astringent and pungent taste and the characteristic odor of oregano (*Origanum vulgare* L.). CA is found in a liquid state at room temperature, with a density of 0.994 ± 0.06 g/cm^3.

The confirmation of the chemical structure of CA was performed by infrared (IR) spectroscopic data, ^{1}H NMR and ^{13}C NMR DEPT: IR (4000–400 cm^{-1}): 3050; 2950; 2850; 1750; 1500; 850. ^{1}H NMR (200 MHz, CDCl3): 7.20 (d, J = 7,80 Hz, 1H); 7,00 (d, J = 7,80 Hz, 1H); 6,90 (s, 1H); 2,95–2,75 (m, 1H); 2,30 (s, 3H); 2,15 (s, 3H); 1,26 (d, J = 6,80 Hz, 6H); ^{13}C NMR DEPT (50 MHz, $CDCl_3$): 169,1; 149,1; 147,9; 130,7; 127,0; 124,0; 119,6; 67,3; 33,4; 23,7; 20,6; 15.6.

Subsequently, CA was emulsified with 0.05% Tween 80 (Sigma Chem. Co., St. Louis, Missouri, USA) dissolved in distilled water (vehicle) and administered intraperitoneally at doses of 25, 50, 75 and 100 mg/kg for the behavioral tests in order to determine its anxiolytic-like effect.

2.3. Animals

Male *Swiss* mice (25–30 g), 2 months of age, were used throughout this study. All the animals were maintained at a controlled temperature (26 ± 1 °C) on a 12 h light/dark cycle (lights on 06:00 am–18:00 pm) with free access to water and food (Purina®). Different groups of mice were used for each test.

All the experiments were previously submitted for the approval of the Ethics Committee on Animal Experimentation of the Federal University of Piauí (UFPI) (# 013/2011).

2.4. Experimental protocols

Initially, the animals were acclimatized in a site 24 h before the behavioral experiments. In the next step, the mice were randomly divided into thirteen groups (10 mice per group), for the two protocol analysis.

In the first protocol, there were a control group, treated with the vehicle (negative control), four CA groups, with doses of 25, 50, 75 and 100 mg/kg and two groups of reference drugs (positive controls): DZP (1 mg/kg) group and BUSP (5 mg/kg) group. After 30 min from each administration, the mice were individually placed on each apparatus and observed for 5 min (EPM; LDB; MBT of OFT) or 3 min for a Rota-rod test. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried for the subsequent mouse test. For all the experiments, each mouse was tested only once. This protocol was designed to test the anxiolytic-like potential and the influence on the psychomotor activity of CA on the mice.

Once the CA anxiolytic-like potential was observed in the first protocol, the second protocol was performed. One group of mice was treated with FLU 25 mg/kg and another group with WAY 10 mg/kg. A third group was formed with the administration of FLU 25 mg/kg, 15 min before DZP 1 mg/kg administration, and a fourth group received FLU 25 mg/kg, 15 min before CA 100 mg/kg. This was the CA dose with the best anxiolytic-like potential. The prior administration of FLU and then DZP was aimed at confirming that the FLU antagonized the GABAergic effect of DZP (Almeida et al., 2012; Silva et al., 2011a). Furthermore, the

administration of FLU before CA 100 aimed to test whether CA anxiolytic-like effect is related to GABAergic action.

Still in the second protocol, there was one group pretreated with WAY 10 mg/kg 15 min before BUSP 5 mg/kg administration and one group in which WAY 10 mg/kg was administered 15 min before CA 100 mg/kg. The previous administration of WAY aimed to confirm the inhibition of the anxiolytic effect of BUSP, as well as to test whether CA anxiolytic-like activity is related to the serotonergic system.

All the groups were treated intraperitoneally 30 min before the test. In the groups in which the antagonist (FLU or WAY) was first applied, the experiments were performed 30 min after administration of the second drug (DZP, BUSP or CA 100).

2.4.1. Elevated plus-maze test

This test has been widely validated to measure anxiety in rodents (Almeida et al., 2012; Karadeli et al., 2013). It consists of two open arms (30 × 5 cm) and two enclosed arms (30 × 5 × 25 cm), with the open arms perpendicular to the enclosed arms. The open and closed arms converge to a central platform (5 × 5 cm). For both protocols, each mouse was placed individually on the central platform, facing directly a closed arm and the time spent in the open arms (TSOA) and the number of open arms entries (NOAE) was recorded during the total time of 5 min. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried. Then, the subsequent mouse was tested.

2.4.2. Light–dark box test

This model has also been widely applied for testing anxiolytic-like properties of drugs, including derivatives of natural products. The apparatus is made with acrylic and is divided into two compartments (boxes), one light and other dark, which communicate through a small entry (Almeida et al., 2012; Crawley, 1981). The dark box (black acrylic, 27 × 18 × 29 cm) is poorly lit. The light box (transparent acrylic, 27 × 18 × 29 cm) is illuminated by a light source of 60 W (400 lx). The communication between the two environments is made by an entrance of 5 × × 5 cm. The animals were initially placed individually in the dark compartment, with the face directed at the communication between the two environments and, then, observed for 5 min. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried. Then, the subsequent mouse was tested. The parameters used were the time spent in the light box (TSLB), expressed in seconds, and the number of entries to the light box (NELB).

2.4.3. Marble-burying test

It has already been shown in literature that this is an effective method for testing the anxiolytic-like properties of a particular substance (Nicolas et al., 2006). This procedure has been well described in rats by Poling et al. (1981) and in mice (Badgujar and Surana, 2010). Rodents exhibit a burying behavior in the presence of aversive stimuli as a source of shock, harmful food or inanimate objects. The administration of a substance likely to have anxiolytic effects in rodents tend to reduce the number of marbles buried (NMB) in this test. Therefore, in order to further expand the evaluation of the CA anxiolytic-like properties, this model was also added.

After treatment, the animals were placed, one at a time in the apparatus, which consisted of an acrylic chamber with the distribution of 25 glass marbles and the floor lined with sawdust. The NMB in the sawdust was reported. A marble was considered as hidden when it was at least two-thirds covered by sawdust. Then, the sawdust and marbles were washed with soap and water, cleaned with ethanol 70% and dried with paper towels. Then, the subsequent mouse was tested.

2.4.4. Open-field test

The open-field arena was made of acrylic (transparent walls and black floor, 30 × 30 × 15 cm) divided into nine squares of equal areas (Archer, 1973). The mice were divided into six groups ($n = 10$ animals). The different groups were treated with the vehicle (0.05% Tween 80 dissolved in distilled water, ip) DZP (1 mg/kg ip) and CA (25; 50; 75 and 100 mg/kg ip). After 30 min of each application, each mouse was placed individually into the apparatus and the number of squares crossed (NSC), with four legs on each square – spontaneous locomotor activity – was measured during 5 min. After each individual test session, the equipment was washed with soap and water, cleaned with ethanol 70% and dried. Then, the subsequent mouse was tested.

2.4.5. Rota-rod test

The equipment of Rota-rod was used to evaluate motor coordination produced by drugs in animals (Carlini and Burgos, 1979; Vale et al., 2002). The mice were trained before the experiment to acquire the capacity to remain for 180 s on a diameter rod, rotating at 17 rpm. Two or three trials were sufficient for the animals to learn this task. DZP (1 mg/kg, ip), CA (25; 50; 75 and 100 mg/kg, ip) and the vehicle (10 ml/kg, ip) were administered 30 min before the test in each of the experimental groups ($n = 10$). Then, the animals were placed in the four paws on the rotating bar, which is 2.5 cm in diameter is 25 cm high from the floor. The animals were observed for a period of three minutes. The ability of the mice to remain on the rota-rod for 180 s – the time spent on the rotating bar (TSRB) – and the number of falls (NF) (up to three drops) were recorded. After each test, the equipment was washed with soap and water, cleaned with ethanol 70% and dried. Then, the subsequent mouse was tested.

2.5. Statistical analyzes

All the results were presented as mean ± standard error of the mean (SEM). The data were evaluated by analysis of variance (ANOVA) followed by Student's t-test and Neuman–Keuls post hoc test. The data were analyzed using GraphPad Prism 5.0 (San Diego, CA, USA). The experimental groups were compared to the vehicle group and the two positive controls — DZP and BUSP. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Elevated plus-maze test

In the first protocol, it was observed that TSOA was increased by 44, 83, 90 and 189% in the groups treated with CA 25, 50, 75 and 100 mg/kg, respectively, when compared to the vehicle [$F(13,102.7) = 1.322$, $p < 0.0001$]. When compared to DZP, CA100 effect was 57% better. Compared to BUSP, there was a 25% better effect to CA100 [$F(13,102.7) = 1.322$, $p < 0.0001$] (Fig. 2A). NOAE in the CA100 group was increased by 208% when compared to the vehicle and 50% and 62.5% when compared to DZP and BUSP, respectively [$F(13,37.30) = 1.811$, $p < 0.0001$] (Fig. 2B). When CA100 was compared to CA75 for TSOA and NOAE, CA100 had a better effect by 52% [$F(13,102.7) = 1.322$, $p < 0.0001$] and 113% [$F(13,37.30) = 1.811$, $p < 0.0001$], respectively (Fig. 2). In addition, when compared to the vehicle, the increase in TSOA caused by CA100 was accompanied by an increase in NOAE.

In the second protocol, the inhibition of the anxiolytic effect of DZP was observed when FLU + DZP reduced the TSOA by 87% when compared to DZP (Fig. 2A) and the NOAE by 77% when compared to DZP [$F(13,102.7) = 1.322$, $p < 0.0001$] (Fig. 2B). FLU + CA100 reduced TSOA and NOAE by 150% [$F(13,102.7) = 1.322$, $p < 0.0001$] and 53% [$F(13,37.30) = 1.811$, $p < 0.0001$] when compared to CA100, respectively. FLU + CA100 reduced both TSOA and NOAE by values which were not significant when compared to the vehicle [$F(13,102.7) = 1.322$, $p > 0.05$] and [$F(13,37.30) = 1.811$, $p > 0.05$], respectively, suggesting a reversal effect on these parameters. When WAY + BUSP were compared to BUSP, there was inhibition of the anxiolytic effect of BUSP by 120% on TSOA [$F(13,102.7) = 1.322$, $p < 0.0001$] (Fig. 2A) and 76% on NOAE [$F(13,37.30) = 1.811$, $p < 0.0001$] (Fig. 2B). However,

WAY + CA100, when compared to CA100, had no statistical significance on the TSOA [F (13,102.7) = 1.322, p > 0.05] (Fig. 2A).

3.2. Light–dark box test

In the first protocol, CA 25, 50, 75 and 100 mg/kg increased TSLB by 88%, 118%, 132% and 182% when compared to the vehicle [F (13,38.66) = 1.156, p < 0.0001] (Fig. 3A). When CA100 was compared to BUSP and DZP, there was an increase respectively by 22% [F (13,38.66) = 1.156, p < 0.001] and 23% for CA100 [F (13,38.66) = 1.156, p < 0.0001] on the TSLB (Fig. 3A). Regarding NELB in comparison to the vehicle, CA25, 50, 75 and 100 increased it by 70%, 80%, 100% and 157%, respectively [F (13,14.45) = 1.873, p < 0.0001]. Additionally, CA 100 mg/kg increased the NELB by 51% when compared with DZP [F (13,14.45) = 1.873, p < 0.0001] and 63% when compared with BUSP [F (13,14.45) = 1.873, p < 0.0001] on the NELB (Fig. 3B). CA100 had a still better effect than CA75 in both TSLB and NELB respectively by 22% [F (13,38.66) = 1.156, p < 0.05] and 29% [F (13,14.45) = 1.873, p < 0.001] (Fig. 3A and B).

In the second protocol, FLU + DZP reduced TSLB by 119% [F (13,38.66) = 1.156, p < 0.0001] (Fig. 3A) and NELB by 62% [F (13,14.45) = 1.873, p < 0.0001] when compared to DZP (Fig. 3B). Furthermore, when comparing the groups WAY + BUSP and BUSP the first reduced TSLB by 37% [F (13,38.66) = 1.156, p < 0.0001, p < 0.0001] (Fig. 3A).

When FLU + CA100 was compared to CA100, there was a reduction by 110% in TSLB [F (13,38.66) = 1.156, p < 0.001] (Fig. 3A) and 157% in NELB [F (13,14.45) = 1.873, p < 0.001] (Fig. 3B). FLU + CA100 reduced both TSLB and NELB by values which were not significant when compared to the vehicle, suggesting a reversal effect on these parameters. However, when WAY + CA100 was compared to CA100 on the TSLB, there was no statistical significance [F (13,38.66) = 1.156, p > 0.05].

3.3. Marble-burying test

When compared to the vehicle, NMB of the mice treated with CA 25, 50, 75 and 100 was reduced respectively by 47%, 52%, 55% and 74% [F (13,91.45) = 4.167, p < 0.0001], which suggests the anxiolytic-like effect of CA. In addition, the CA100 reduced NMB by 35% when compared to DZP [F (13,91.45) = 4.167, p < 0.05] and by 49% when compared to BUSP [F (13,91.45) = 4.167, p < 0.0001] in the first protocol. CA100 had also a superior anxiolytic-like effect to CA75 which was 43% greater [F (13,91.45) = 4.167, p < 0.001] (Fig. 4).

In the second protocol, FLU + DZP decreased by 163% the effect of DZP on NMB [F (13,91.45) = 4.167, p < 0.0001] and WAY + BUSP reduced by 125% the effect of BUSP on NMB [F (13,91.45) = 4.167, p < 0.0001]. FLU + CA100 reduced by 174% the effect of CA100 on NMB [F (13,91.45) = 4.167, p < 0.0001], and FLU + CA100 was not significant when compared to the vehicle in this parameter [F (13,91.45) = 4.167, p > 0.05], causing a reversal of the CA100 effect. However, no significant difference was observed between WAY + CA100 and CA100 [F (13,91.45) = 4.167, p > 0.05] (Fig. 4).

3.4. Open-field test

The group treated with DZP 1 mg/kg ip had NSC reduced by 53.9% when compared with the vehicle [F (6,137.4) = 6.271, p < 0.0001] (Fig. 5). However, the mice treated with CA 25; 50; 75 or 100 mg/kg

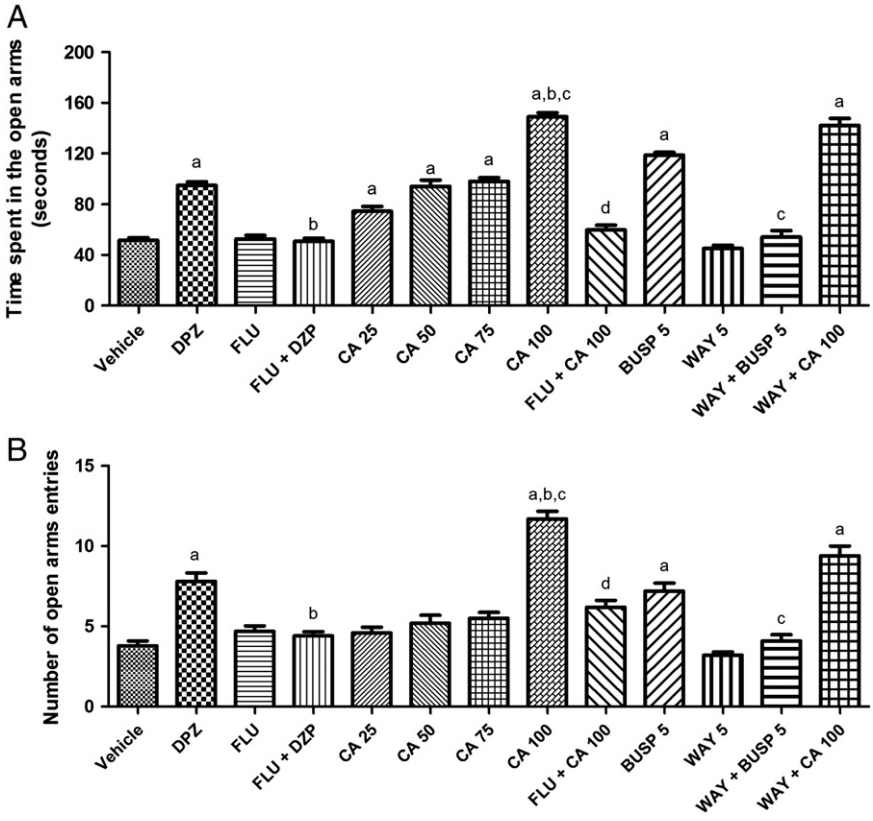

Fig. 2. Time spent in the open arms (TSOA) and number of open arms entries (NOAE) in the elevated plus-maze (EPM) test. The values were represented as mean ± SEM. [a]p < 0.0001 when compared to the control group (vehicle), [b]p < 0.0001 when compared to DZP, [c]p < 0.0001 when compared to BUSP and [d]p < 0.0001 when compared to CA100 (ANOVA followed by Student's t-test and Neuman–Keuls post hoc test). CA: carvacryl acetate; BUSP: buspirone; DZP: diazepam; FLU: flumazenil; WAY: WAY 100635.

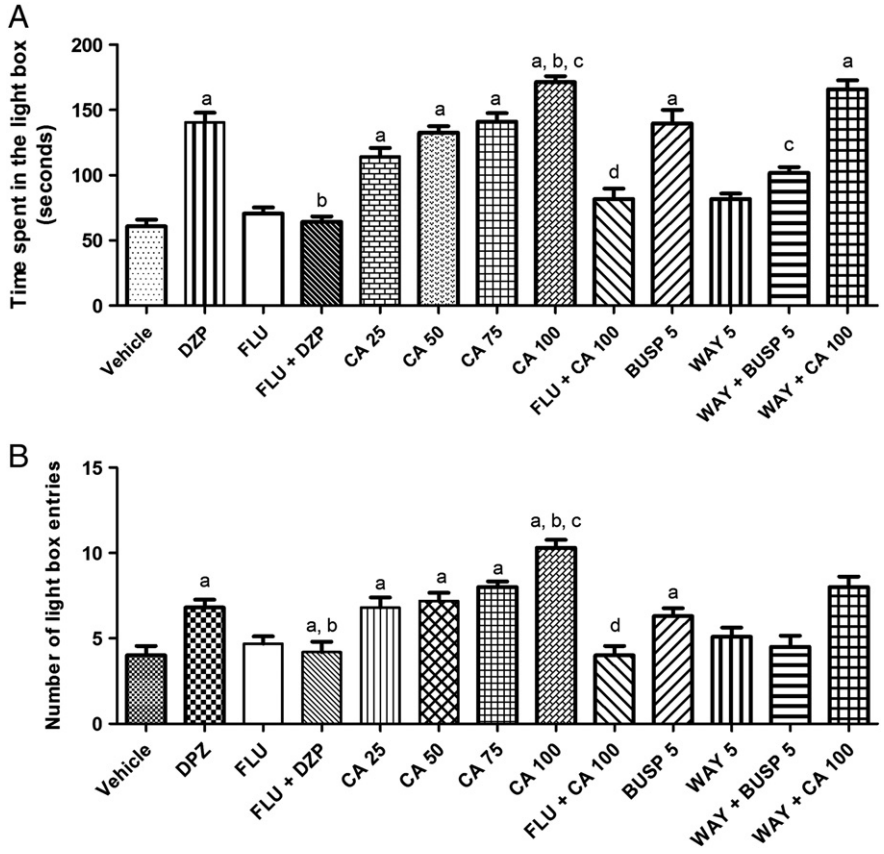

Fig. 3. Time spent in the light box (TSLB) and number of entries to the light box (NELB) in Ligh–dark box (LDB) test. The values were represented as mean ± SEM. [a]$p < 0.001$ when compared to the control group (vehicle), [b]$p < 0.001$ when compared to DZP, [c]$p < 0.0001$ when compared to BUSP and [d]$p < 0.001$ when compared to CA100 (ANOVA followed by Student's t-test and Neuman–Keuls post hoc test). CA: carvacryl acetate; BUSP: buspirone; DZP: diazepam; FLU: flumazenil; WAY: WAY 100635.

did not present any statistical difference when compared to the vehicle [$F(6,137.4) = 6.271$, $p > 0.05$].

3.5. Rota-rod test

The animals tread with DZP 1 mg/kg ip had NF increased by 121.43% when compared to the vehicle [$F(6,11.43) = 1.214$, $p < 0.05$], while the CA groups had not statistical difference when compared to the vehicle [$F(6,11.43) = 1.214$, $p > 0.05$] (Fig. 6A).

Considering the TSRB, the mice tread with DZP 1 mg/kg ip had this parameter reduced by 16.93% when compared to the vehicle [$F(6,76.37) = 1.605$, $p < 0.05$], while CA groups had no statistical difference when compared to the vehicle [$F(6,76.37) = 1.605$, $p > 0.05$] (Fig. 6B).

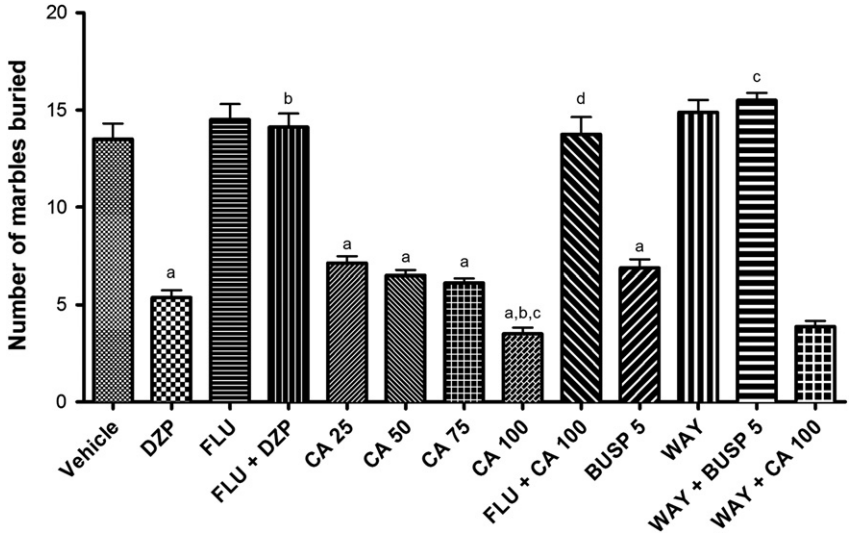

Fig. 4. Number of marbles buried (NMB) in the marble-burying test (MBT). The values were represented as mean ± SEM. [a]$p < 0.0001$ when compared to the control group (vehicle), [b]$p < 0.05$ when compared to DZP, [c]$p < 0.0001$ when compared to BUSP and [d]$p < 0.0001$ when compared to CA100 (ANOVA followed by Student's t-test and Neuman–Keuls post hoc test). CA: carvacryl acetate; BUSP: buspirone; DZP: diazepam; FLU: flumazenil; WAY: WAY 100635.

4. Discussion

In the present study we explored the anxiolytic-like effects of different doses of CA using three behavioral models (EPM, LDB and MBT) and comparing them with the effects of DZP and BUSP.

In the EPM, it is known that anxiolytic drugs such as DZP lead animals to increase the NOAE and the TSOA, whilst anxiogenic drugs induce an increase in the number of entries and in the time spent in the closed arms (Walf and Frye, 2007). Likewise, it is known that in the LDB, anxiolytic drugs tend to increase the NELB and the TSLB (Crawley, 1981). And finally, in the MBT, anxiolytic drugs tend to reduce the NMB, which also characterizes an anxiolytic-like activity (Almeida et al., 2012; Badgujar and Surana, 2010). As it is known, rodents bury a variety of conditioned and unconditioned aversive stimuli. Such burying has been considered as a species-typical defensive reaction. The rodents, when feeling threatened, tend to bury food and innocuous solids, like glass marbles (Poling et al., 1981). Therefore, a compound which reduces the NMB may have a potential to be studied as an anxiolytic drug.

In the first experimental protocol, it was found that CA had anxiolytic-like potential in EPM, LDB and MBT at all doses, and the higher the dose, the greater the effect.

In EPM, the increase on TSOA caused by CA100 was accompanied by an increase on NOAE, suggesting no psychomotor retardation at that dose of CA (Section 3.1).

In LDB, all four CA doses increased both TSLB and NELB when compared to the vehicle, corroborating that the effect of CA in this test probably did not cause psychomotor retardation (Section 3.2).

Reinforcing that CA at the doses used do not interfere in the psychomotor activity, in OFT no difference was observed on NSC, suggesting no locomotor retardation effect of CA, differently from what was observed with DZP (Section 3.4). The Rota-rod test demonstrated, additionally, no motor coordination impairment of CA 25; 50; 75 and 100 mg/kg ip, which is also the contrary to what was observed with DZP (Section 3.5). This means that the common side effects on the psychomotor activity during the treatment of anxiety disorders with benzodiazepine probably may not exist with the CA treatment (Silva et al., 2011b).

Finishing the analysis of the first protocol, it is important to consider that we previously tested the doses of CA at 1 mg/kg and 5 mg/kg and they did not demonstrate anxiolytic-like activity in any of the three models of anxiety.

In the second protocol, considering EPM results, FLU + CA100 reduced TSOA when compared to CA100 (Section 3.1), and, in LDB, FLU + CA100 reversed the anxiolytic-like effect of CA100 on both TSLB and on NELB (Section 3.2). In MBT, FLU + CA100 also reversed the effect of CA100, but not significantly when compared to the vehicle (Section 3.3). These results suggest that the anxiolytic-like potential of CA may occur, at least in part, due to a probable agonistic action on the GABAergic system.

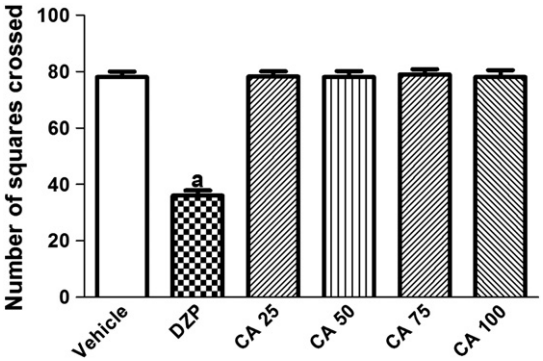

Fig. 5. Number of squares crossed (NSC) in the open-field test (OFT). The values were represented as mean ± SEM. [a]$p < 0.0001$ when compared to the control group (vehicle) (ANOVA followed by Student's t-test and Neuman–Keuls post hoc test). CA: carvacryl acetate; DZP: diazepam.

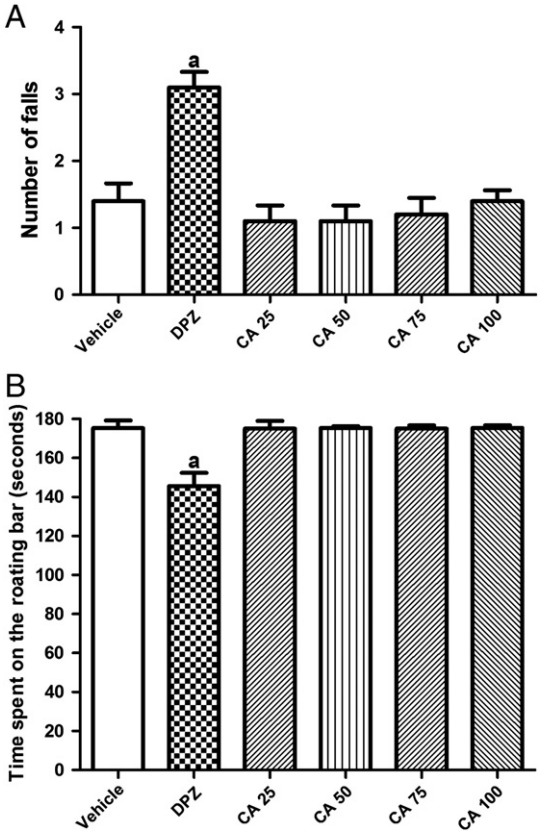

Fig. 6. Number of falls (NF) (A) and time spent on the rotating bar (TSRB) (B) in the Rota-rod test. The values were represented as mean ± SEM. [a]$p < 0.05$ when compared to the control group (vehicle) (ANOVA followed by Student's t-test and Neuman–Keuls post hoc test). CA: carvacryl acetate; DZP: diazepam.

Likewise, as it is already known that BUSP has its anxiolytic property due to an agonist action on 5-HT_{1A} receptors (Ferreira et al., 2012), pretreatment with WAY reversed the anxiolytic effect of BUSP in EPM, LDB and MBT. So, to be tested if the CA anxiolytic-like potential could be involved on the serotonergic system, a group of mice was pretreated with WAY, followed by administration of CA100.

In EPM, the WAY did not reverse the anxiolytic-like effect of CA100 on the TSOA (Section 3.1). Similarly, LDB results have suggested that the anxiolytic-like activity of CA is not related to the serotonergic system, as WAY + CA100 showed no significant difference from CA100 on TSLB (Section 3.2). This idea was corroborated in MBT, when the WAY + CA100 group caused no significant change in NMB when compared to CA100 (Section 3.3).

It is important to consider that this study was conducted on mice because low doses of CA were necessary in the experiments and the mice are considered to have good reproducibility in experimental pharmacological models. However, it should be emphasized that tests with other species of rodents will be conducted by our group to compare the results and verify the reproducibility of the same pharmacological models used in this study.

Since CA is an acetylated form of a monoterpene (carvacrol), we compared the CA anxiolytic-like effect with anxiolytic-like effects of monoterpenes and derivatives of monoterpenes obtained from other studies. A study in which epoxy-limonene (EL) was administrated at 25, 50 and 75 mg/kg in *Swiss* mice subsequently submitted to EPM demonstrated that the TSOA was increased by 47, 48.2 and 74.4%, respectively, when compared to the vehicle ($p < 0.01$) (ANOVA followed by Student's t-test and Neuman–Keuls post hoc test) (Almeida et al., 2012). However, CA showed better increases in TSOA for the doses of 50 and 75 mg/kg when compared to the vehicle (Section 3.1).

In another study, it was tested in EPM the probable anxiolytic-like activity of citral, myrcene and limonene, constituents of the essential oil of *Lippia alba* (Mill.) (Melo et al., 2010). No effects on TSOA after administration of the three monoterpenes in doses of 5, 10, 25 and 50 mg/kg ip in mice were observed. Nevertheless, there was a small yet significant effect of limonene at 5 mg/kg ip, which instead of having increased NOAE, decreased it by 30% when compared to control ($p < 0.05$). Therefore, despite being derived from a monoterpene, CA followed a different trend from citral, limonene and myrcene, but a similar tendency to carvacrol, from which CA is derived, once an anxiolytic-like property of carvacrol has already been recognized in a previous study. The CA resulted from an insertion of an acetate molecule on the chemical structure of carvacrol, which may facilitate the penetration through the blood–brain barrier since it increases the liposolubility. Thus, probably lower doses of CA may be necessary to exert the possible anxiolytic-like effect when hypothetically compared with carvacrol, increasing, therefore, a safe use of this drug in a possible clinical study in the future (Melo et al., 2010).

Thus, the results of this study demonstrate that CA presents a potential anxiolytic-like effect in three widely used experimental models of anxiety, without evidence of causing psychomotor retardation of the mice under the doses used. The evidences indicate that it probably acts on the GABAergic system, but not on the 5-HT_{1A} receptors.

5. Conclusion

By providing unpublished data about CA anxiolytic-like potential, as well as its probable mechanism of action, which seems not to interfere in the psychomotor activity in the doses used, this paper suggests that CA features a large potential to be studied as an alternative drug for the treatment of anxiety disorders.

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