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The anxiolytic-like effect of an essential oil derived from *Spiranthera odoratissima* A. St. Hil. leaves and its major component, β-caryophyllene, in male mice

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ABSTRACT

Spiranthera odoratissima A. St. Hil. (manacá) is used in folk medicine to treat renal and hepatic diseases, stomachache, headaches and rheumatism. A central nervous system (CNS) depressant effect of the hexane fraction from the ethanolic extract of this plant has been described. β-caryophyllene, the main component of this essential oil, is a sesquiterpene compound with anti-inflammatory properties that has been found in essential oils derived from several medicinal plants.

This work is aimed to evaluate the pharmacological activity of the essential oil obtained from *S. odoratissima* leaves (EO) and its major component on the murine CNS; we aimed to evaluate a possible anxiolytic-like effect and the underlying mechanisms involved. In an open field test, EO (500 mg/kg) and β-caryophyllene (50, 100 and 200 mg/kg) increased the crossing frequency ($P<0.05$) and, EO (250 and 500 mg/kg) and β-caryophyllene (200 mg/kg) increased the time spent in the center ($P<0.05$) without altering total crossings of the open field. EO and β-caryophyllene did not alter the number of falls in the rota-rod test ($P>0.05$). In the pentobarbital-induced sleep test, EO (500 mg/kg) and β-caryophyllene (200 and 400 mg/kg) decreased the latency to sleep ($P<0.05$), and EO (125, 250 and 500 mg/kg) ($P<0.001$) and β-caryophyllene (200 and 400 mg/kg) ($P<0.05$ and $P<0.001$) increased the sleep time. In anxiety tests, EO (500 mg/kg) and β-caryophyllene (100 and 200 mg/kg) increased head-dipping behavior ($P<0.05$) in the hole-board test, entries ($P<0.05$) into and time spent ($P<0.05$) on the open arms of the elevated plus maze (EPM), and number of transitions ($P<0.05$) and time spent in the light compartment ($P<0.05$) of a light–dark box (LDB). We further investigated the mechanism of action underlying the anxiolytic-like effect of EO and β-caryophyllene by pre-treating animals with antagonists of benzodiazepine (flumazenil) and 5-HT_{1A} (NAN-190) receptors prior to evaluation using EPM and LDB. The anxiolytic-like effects of EO were significantly reduced by pre-treatment with NAN-190 ($P<0.05$) but not flumazenil ($P>0.05$). The anxiolytic-like effects of β-caryophyllene were not blocked by either NAN-190 or flumazenil ($P>0.05$). In conclusion, these results suggest that the essential oil derived from *S. odoratissima* produces an anxiolytic-like effect without altering motor performance and that this effect is mediated by 5-HT_{1A} but not via benzodiazepine receptors. In addition, the major component, β-caryophyllene, also has an anxiolytic-like effect that may contribute to the effects of EO, but this effect does not seem to be mediated via 5-HT_{1A} or benzodiazepine receptors.

1. Introduction

Anxiety and stress disorders are among the most common of all chronic diseases. The prevalences of these disorders are increasing in many countries, and these disorders have a much earlier age of onset than other chronic conditions (Kessler and Greenberg, 2002). As an example, in Brasilia city (the capital of Brazil), anxiety disorders affect 12.1% of the population and are the most commonly diagnosed psychiatric disorders (Almeida Filho et al., 1992).

Since the introduction of benzodiazepines in the 1960s, they have been the most commonly prescribed treatment for anxiety, remaining

Abbreviations: CNS, central nervous system; EO, essential oil obtained from *S. odoratissima* leaves; EPM, elevated plus maze test; LDB, light–dark box test; UFG, Universidade Federal de Goiás; NAN-190, 1-(2-Methoxyphenyl)-4-[4-(2-phthalimido) butyl] piperazine hydrobromide; p.o., orally; i.p., intraperitoneally; s.c., subcutaneously; SEM, standard error of the mean; GAD, generalized anxiety disorder; CB1, cannabinoid 1 receptor; CB2, cannabinoid 2 receptor; 5-HT_{1A} receptor, 5-hydroxytryptamine 1A receptor.

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the mainstay of pharmacological treatment in anxiety disorders. However, they have prominent side effects, such as sedation, myorelaxation, ataxia, and amnesia, and they can cause pharmacological dependence (Lader and Morton, 1991). Thus, new therapies for the treatment of anxiety disorders are necessary, and the study of medicinal plants could provide new therapeutic options (Faustino et al., 2010).

The anxiolytic-like effect of plants from Rutaceae family has already been demonstrated; such plants include *Citrus aurantium* L. (Carvalho-Freitas and Costa, 2002), *Casimiroa edulis* La Llave ex Lex. (Molina-Hernández et al., 2004; Mora et al., 2005) and *Ruta chalepensis* L. (Gonzalez-Trujano et al., 2006).

Spiranthera odoratissima A. St. Hillaire (Rutaceae) is a shrub that is found in the Brazilian Cerrado region and popularly known in Brazil as *manacá*. Its flowers are whitish and very aromatic, its fruits possess a unique seed and its roots are woody, with yellow-gold coloration (Almeida et al., 1998). In folk medicine, its leaves are used as a blood purgative and in the treatment of renal and hepatic diseases (Salles et al., 1997), while the roots are used to as an appetite stimulant and to treat stomachache, headaches, sore muscles, and hepatic dysfunction (Silva, 1998). In the state of Goiás, Brazil, these roots are also used to treat rheumatism (Tresvenzol et al., 2006).

According to Matos et al. (2003), the aqueous fraction from the ethanolic extract of manacá leaves shows analgesic and anti-inflammatory activities in the acetic acid-induced writhing, croton oil-induced ear edema and carrageenan-induced peritonitis models. Similar results were obtained with the ethanolic extract of manacá roots, which are also characterized by the central nervous system depressant activity (Matos et al., 2004). The evaluation of the effects of the hexane fraction from the ethanolic extract of *S. odoratissima* leaves on the murine CNS indicates that this fraction has active substances that increase the sleep time in a pentobarbital-induced sleep test (Matos et al., 2006).

β-Caryophyllene, the major component of *S. odoratissima* essential oil (Chaibub and Paula, 2009), is a sesquiterpene compound found in the essential oils of many different species (Gertsch et al., 2008; Molina-Jasso et al., 2009). Among the pharmacological activities of this compound are anti-inflammatory (Fernandes et al., 2007; Medeiros et al., 2007; Passos et al., 2006) and antispasmodic activities (Leonhardt et al., 2010); it has also been used as a local anesthetic (Ghelardini et al., 2001) and gastric cytoprotector (Tambe et al., 1996), to stimulate natural killer cells (Standen et al., 2006). It has also been shown to be neuroprotective in human neuroblastoma (Chang et al., 2007).

Then, this work is aimed to evaluate the neuropharmacological activity of the essential oil from *S. odoratissima* leaves in the CNS, with special attention to possible anxiolytic-like effects, as well as the roles of $5HT_{1A}$ and $GABA_A$/benzodiazepine receptors in these effects. The identification of the active component(s) is one step in the proposed development of an herbal drug; therefore, this paper includes examination of the anxiolytic-like effect and possible mechanism of action of the essential oil's major compound, β-caryophyllene.

2. Material and methods

2.1. Animals

Male adult Swiss mice weighing approximately 30 g (n = 765) were used in all experiments. All animals were used only once. The animals were provided by the Central Animal House of Federal University of Goiás (Universidade Federal de Goiás — UFG); they were housed in groups of 20 mice/cage and were kept in a room with controlled temperature (25 ± 1 °C) and lighting (light/dark cycle of 12 h, lights on at 7 am), with food and water ad libitum. The animals were kept in the laboratory for an adaptation period of at least 1 h before the experiments. All experimental protocols were developed in accordance with the principles of ethics and animal welfare recommended by Brazilian Science Society of Laboratory Animal (Sociedade Brasileira de Ciência em Animais de Laboratório) and were approved by the UFG Institutional Ethical Committee (Protocol 104/2008).

2.2. Plant material and essential oil extraction

S. odoratissima A. St.-Hil. leaves were collected in December 2007 near the town of Senador Canedo, Goiás, Brazil (762 m, 16°45′45.2″ S, 49°07′06.8″ W) and were authenticated by Prof. Dr. José Realino de Paula (Pharmacy Faculty — UFG). A voucher specimen was deposited at the Herbarium of the UFG (UFG — 30,275). The leaves were dried at room temperature and crushed, and the essential oil (EO) was extracted by hydrodistillation for approximately 3 h using a Clavenger-type apparatus, with a yield of 2.3%. The EO was subjected to gas chromatographic mass spectrometry in SHIMADZU QP5050A equipment, and the chemical components were identified by comparing the mass spectra and the retention indexes with the literature. The major components identified were β-caryophyllene (20.64%), gamma-muurolene (17.7%), bicyclogermacrene (14.73%), delta-cadinene (13.40%), gamma-cadinene (4.59%) and cubenol (3.12%) (Chaibub and Paula, 2009).

2.3. Drugs

EO and β-caryophyllene (Sigma — Brazil) were emulsified with 2% Tween 80 (Sigma — USA) and dissolved in distilled water. Sodium pentobarbital (Abbott — Brazil) was dissolved in saline. Diazepam (Cristália — Brazil) was dissolved in distilled water. It is well known that benzodiazepines act as anxiolytics at low doses and that they induce sedation and myorelaxant effects at higher doses (Novas et al., 1988). Therefore, we used diazepam (1 mg/kg) as a positive control for anxiolytic-like effects, and diazepam (5 mg/kg) as a positive control for sedative and myorelaxant effects. Flumazenil (União Química — Brazil) and NAN-190 (1-(2-methoxyphenyl)-4-[4-(2-phthalimido) butyl] piperazine hydrobromide — Sigma — USA) were used as pretreatments to verify the role of $GABA_A$/benzodiazepine and $5\text{-}HT_{1A}$ receptors, respectively.

2.4. Behavioral procedures

2.4.1. General behavior test

Experimental groups of mice (n = 5 per group) were treated orally (p.o.), intraperitoneally (i.p.) or subcutaneously (s.c.) with OE at doses of 10, 30, 100, 300 or 1000 mg/kg or with β-caryophyllene at doses of 50, 100 or 200 mg/kg, whereas control groups received vehicle (2% Tween 80 in distilled water, 10 mL/kg) by the same routes. The animals were observed in free ambulation on a flat surface for 3 min, again at 5, 10, 20, 30 and 60 min, and 4, 8, 24 and 48 h after the treatments and after 4 and 7 days of treatment. The observed effects were noted using a standard pharmacological screening approach, adapted from Malone (1977).

2.4.2. Rota-rod test

In this test, the animals were pre-selected in a training session 24 h before the test based on their ability to remain on the bar (at 12 rpm) for 2 min. Groups of pre-selected animals (n = 9) were treated (p.o.) with vehicle (2% Tween 80, 10 mL/kg), EO (125, 250 or 500 mg/kg), β-caryophyllene (50, 100, 200 or 400 mg/kg) or diazepam (1 or 5 mg/kg). Sixty minutes after the treatment, the animals were placed with all four paws onto the bar and the number of falls was evaluated. The maximum time allowed was 1 min, and the maximum number of falls allowed was three (Dunham and Miya, 1957).

2.4.3. Open-field test

Experimental groups of 9 mice were treated (p.o.) with vehicle (2% Tween 80, 10 mL/kg), EO (125, 250 or 500 mg/kg), β-caryophyllene (50, 100 or 200 mg/kg) or diazepam (1 or 5 mg/kg). Sixty minutes

after the treatment, the animals were placed in the center of an open-field arena made of clear Plexiglas in which the bottom was divided into eight areas; the observed parameters were as follows: total number of crossings, central area crossings, time spent in central area, rearing and grooming behaviors and fecal bolus (Archer, 1973; Siegel, 1946).

2.4.4. Pentobarbital-induced sleep test

Experimental groups of 9 mice were treated (p.o.) with vehicle (2% Tween 80, 10 mL/kg), EO (125, 250 or 500 mg/kg), β-caryophyllene (50, 100, 200 or 400 mg/kg) or diazepam (1 or 5 mg/kg). Sixty minutes after the treatment, the animals received sodium pentobarbital (50 mg/kg i.p.), and the time elapsed between the administration of pentobarbital until the loss of the righting reflex was recorded as the sleep latency, and the time elapsed between the loss and voluntary recovery of the righting reflex was recorded as the sleep time (Carlini and Burgos, 1979).

2.4.5. Hole-board test

Experimental groups of 9 mice were treated (p.o.) with vehicle (2% Tween 80, 10 mL/kg), EO (125, 250 or 500 mg/kg), β-caryophyllene (50, 100 or 200 mg/kg) or diazepam (1 or 5 mg/kg). Sixty minutes after treatment, the animals were placed into the center of a perforated board with the bottom divided into nine squares of equal areas; the number of head-dips into the holes and the number of squares crossed (with all four paws) were recorded over a 5 min period (Clark et al., 1971).

2.4.6. Elevated plus maze test

Experimental groups of 9 mice were treated (p.o.) with vehicle (2% Tween 80, 10 mL/kg), EO (125, 250 or 500 mg/kg), β-caryophyllene (50, 100 or 200 mg/kg) or diazepam (1 or 5 mg/kg). Sixty minutes after treatment, the animals were individually placed at the center of a plus maze (purchased from Insight Scientific Equipment — Brazil) and observed for 5 min (Lister, 1987; Pellow et al., 1985). The test occurred under a red light and was fully recorded for later analysis, which consisted of counting the number of entries into the open and enclosed arms and the time spent by the animal in the open and enclosed arms. The number of entries into and time spent in the open arms was later converted into percentage of total entries and time, respectively, and these percentages were used as a measure of anxiety. (Anxiolytic compounds reduce the animal's aversion to the open arms and promote the exploration thereof.)

2.4.7. Light–dark box test

Experimental groups of 9 mice were treated (p.o.) with vehicle (2% Tween 80, 10 mL/kg), EO (125, 250 or 500 mg/kg), β-caryophyllene (50, 100 or 200 mg/kg) or diazepam 1 or 5 mg/kg. Sixty minutes after treatment, the animals were placed in the center of the light area facing the dark area opening; the number of transitions between the two compartments and the time spent in the light area were recorded over a 5 min period (Crawley and Goodwin, 1980).

2.4.8. Mechanisms involved in the anxiolytic-like activities of EO and β-caryophyllene

To investigate the possible mechanisms underlying the anxiolytic activities of EO and β-caryophyllene, the animals were intraperitoneally pre-treated with flumazenil (6 mg/kg; 15 min pre-treatment), an antagonist of $GABA_A$/benzodiazepine receptors (Savić et al., 2004) or NAN-190 (0.5 mg/kg; 30 min pre-treatment), an antagonist of 5-HT_{1A} receptors (Huo et al., 2010).

2.5. Statistical analysis

The results were expressed as the means ± standard errors of the mean (SEM). Significant differences between experimental groups were detected by one-way ANOVA (analysis of variance) followed by Student–Newman–Keuls test (more than two groups), or unpaired Student's *t* test (two groups). The results without normal distribution were analyzed using Kruskal–Wallis's test followed by Dunn's test. Effects were considered significant at $P \leq 0.05$.

3. Results

3.1. General behavioral testing

Upon subcutaneous administration of EO (300 and 1000 mg/kg), decreases in spontaneous ambulations (10–20 min and 10–60 min, respectively) and environmental alienations (10–20 min and 10–30 min, respectively) were observed; with EO (1000 mg/kg) palpebral ptosis (20–60 min) and analgesia (10–30 min) were observed. Upon intraperitoneal administration of EO (100, 300 and 1000 mg/kg), a decrease in spontaneous ambulations (60 min, 10–60 min, and 5 min–4 h, respectively) were observed; with EO (300 and 1000 mg/kg) analgesia (30–60 min and 10–60 min, respectively) and environmental alienations (both at 30–60 min) were also observed. Only at the 1000 mg/kg dose abdominal writhing (at 10 min) and one death (within 8 h of the treatment) were observed. Among the oral treatments, only the 1000 mg/kg dose decreased the animals' spontaneous ambulation (60 min) and induced analgesia (20–60 min). Upon intraperitoneal administration of β-caryophyllene (200 mg/kg), abdominal writhing was observed at 30–60 min and 24 h. No other alterations were observed upon subcutaneous, intraperitoneal or oral treatment with β-caryophyllene at the doses we evaluated.

3.2. Rota-rod test

Neither EO nor β-caryophyllene (at any dose) altered the number of falls in the rota-rod test, whereas diazepam (at 5 mg/kg only) significantly increased the number of falls, as expected [KW = 30.83, $P<0.001$; Dunn, $P<0.001$ vs. vehicle group, Table 1].

3.3. Open-field test

Treatment (p.o.) with EO, β-caryophyllene and diazepam did not alter grooming behavior [ANOVA: $F(8,72) = 0.8482$, $P>0.05$]; the total number of crossings [ANOVA: $F(8,72) = 9.874$, $P<0.001$] was reduced only by diazepam (5 mg/kg) ($P<0.001$ vs. vehicle). The fecal bolus [ANOVA: $F(8,72) = 15.533$, $P<0.001$] was reduced by p.o. treatment with EO (125, 250 and 500 mg/kg) ($P<0.05$, $P<0.05$ and $P<0.001$ vs. vehicle group, respectively) and diazepam (1 and 5 mg/kg) ($P<0.05$ and $P<0.001$ vs. vehicle group, respectively), while β-caryophyllene treatment did not alter this parameter ($P>0.05$ vs. vehicle group). The number of central area crossings [ANOVA: $F(8,72) = 15.473$, $P<0.001$] was increased by EO (500 mg/kg) ($P<0.001$ vs. vehicle), β-caryophyllene (50, 100 and 200 mg/kg) ($P<0.05$, $P<0.05$ and $P<0.01$ vs. vehicle group, respectively) and diazepam (1 mg/kg) ($P<0.01$ vs. vehicle), while diazepam (5 mg/kg) reduced this parameter ($P<0.001$ vs. vehicle). Rearing behavior [ANOVA: $F(8,72) = 32.450$, $P<0.001$] was decreased by EO (500 mg/kg) ($P<0.05$ vs. vehicle) and diazepam (5 mg/kg) ($P<0.001$ vs. vehicle), while β-caryophyllene had no effect ($P>0.05$ vs. vehicle). The time spent in the central area [ANOVA: $F(8,72) = 21.855$, $P<0.001$] was increased by EO (500 and 250 mg/kg) ($P<0.05$ and $P<0.001$ vs. vehicle, respectively), β-caryophyllene (200 mg/kg) ($P<0.05$ vs. vehicle) and diazepam (1 mg/kg) ($P<0.001$ vs. vehicle), while diazepam (5 mg/kg) reduced this parameter ($P<0.05$ vs. vehicle) (Table 2).

3.4. Pentobarbital-induced sleep test

The sleep latency [ANOVA: $F(9,80) = 8.628$, $P<0.001$; Fig. 1A] was decreased by p.o. treatment with EO (500 mg/kg) ($P<0.05$ vs. vehicle), β-caryophyllene (200 and 400 mg/kg) ($P<0.05$ and $P<0.05$ vs.

Table 1
Effects of essential oil from *Spiranthera odoratissima* leaves (EO) and β-caryophyllene on the rota rod test.

Groups	Number of falls
Vehicle	0.2 ± 0.15
EO 125	0.4 ± 0.20
EO 250	0.6 ± 0.27
EO 500	0.6 ± 0.27
β-car 50	0.1 ± 0.26
β-car 100	0.1 ± 0.31
β-car 200	0.4 ± 0.52
β-car 400	0.2 ± 0.42
Dzp 1	0.6 ± 0.70
Dzp 5	2.3 ± 0.31♦♦♦

Data expressed as mean ± SEM of nine mice. Vehicle (2% Tween, 10 mL/kg, p.o.), EO: essential oil from *Spiranthera odoratissima* leaves (125, 250 and 500 mg/kg, p.o.), β-caryophyllene (100, 200 and 400 mg/kg, p.o.) and DZP: diazepam (1 and 5 mg/kg, p.o.).
♦♦♦ $P \leq 0.001$ compared with control group using Kruskal–Wallis's test and Dunn's test as the *post hoc* test.

vehicle, respectively) and diazepam (1 or 5 mg/kg) ($P<0.05$ and $P<0.001$ vs. vehicle, respectively). The sleeping time [ANOVA: $F(9,80) = 128.53$, $P<0.001$; Fig. 1B] was increased by EO (125, 250 and 500 mg/kg) ($P<0.001$, $P<0.001$ and $P<0.001$ vs. vehicle, respectively), β-caryophyllene (200 and 400 mg/kg) ($P<0.05$ and $P<0.001$ vs. vehicle, respectively), and diazepam (1 and 5 mg/kg) ($P<0.001$ and $P<0.001$ vs. vehicle, respectively).

3.5. Hole-board test

Head-dipping behavior [$F(8,72) = 14.058$, $P<0.001$; Fig. 2A] was increased by p.o. treatment with EO (250 and 500 mg/kg) ($P<0.01$ and $P<0.05$ vs. vehicle, respectively), β-caryophyllene (100 and 200 mg/kg) ($P<0.05$ and $P<0.05$ vs. vehicle, respectively) and diazepam (1 mg/kg) ($P<0.05$ vs. vehicle), while diazepam (5 mg/kg) reduced head-dipping behavior ($P<0.05$ vs. vehicle). With regard to the number of squares crossed [ANOVA: $F(8,72) = 5.522$, $P<0.001$; Fig. 2B], only diazepam (5 mg/kg) reduced this parameter ($P<0.01$ vs. vehicle).

3.6. Elevated plus maze test

The number of entries into the open arms [ANOVA: $F(8,72) = 8.777$, $P<0.001$; Fig. 3A] was increased by p.o. treatment with EO (500 mg/kg) ($P<0.01$ vs. vehicle), β-caryophyllene (100 and 200 mg/kg) ($P<0.05$ and $P<0.05$ vs. vehicle, respectively) and diazepam (1 mg/kg) ($P<0.01$ vs. vehicle group), while diazepam (5 mg/kg) reduced this parameter ($P<0.05$ vs. vehicle group). The time spent in the open arms

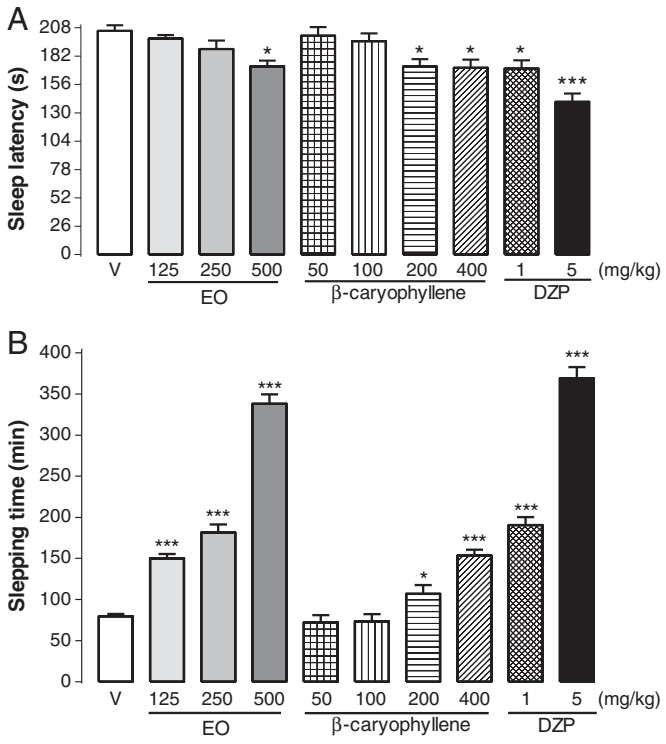

Fig. 1. Effect of essential oil (EO) from *Spiranthera odoratissima* leaves and β-caryophyllene on the sleep latency, in seconds (A) and sleeping time, in minutes (B), of the pentobarbital-induced sleep test in mice. Vehicle (V, 2% Tween 80, 10 mL/kg p.o.), essential oil (EO, 125, 250 or 500 mg/kg p.o.), β-caryophyllene (50, 100, 200 or 400 mg/kg p.o.) and diazepam (DZP, 1 or 5 mg/kg p.o.). The columns and vertical bars represent the means ± SEM of nine mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.

[ANOVA: $F(8,72) = 9.776$, $P<0.001$; Fig. 3B] was increased by EO (500 mg/kg) ($P<0.05$ vs. vehicle), β-caryophyllene (100 and 200 mg/kg) ($P<0.05$ and $P<0.01$ vs. vehicle, respectively) and diazepam (1 mg/kg) ($P<0.001$ vs. vehicle group), while diazepam (5 mg/kg) reduced this parameter ($P<0.05$ vs. vehicle group). With regard to the total number of entries into the closed arms [ANOVA: $F(8,72) = 4.153$, $P<0.001$; Fig. 3C], only diazepam (5 mg/kg) reduced this parameter ($P<0.05$ vs. vehicle).

3.7. Light–dark box test

In the light–dark test, the number of transitions between the light and dark compartments [ANOVA: $F(8,72) = 8.760$, $P<0.001$; Fig. 4A]

Table 2
Effects of essential oil from *Spiranthera odoratissima* leaves (EO) on the open-field test.

Groups	Open field test parameters					
	Total crossing	Central area crossing (%)	Time spent in the central area (s)	Rearing	Grooming	Fecal bolus
Vehicle	97.9 ± 5.15	50.1 ± 3.36	118.1 ± 8.77	72.3 ± 3.05	4.0 ± 0.57	3.0 ± 0.25
EO 125	106.0 ± 7.62	52.1 ± 3.01	114.3 ± 8.31	67.5 ± 3.35	4.0 ± 0.71	1.9 ± 0.23*
EO 250	97.9 ± 6.99	53.0 ± 1.45	155.2 ± 10.62*	63.1 ± 2.64	4.1 ± 0.89	1.8 ± 0.33*
EO 500	85.3 ± 9.59	68.6 ± 2.90***	214.1 ± 10.50***	58.4 ± 3.46*	3.4 ± 0.58	0.5 ± 0.27***
β-car 50	103.5 ± 5.57	61.9 ± 1.89*	135.7 ± 7.25	68.5 ± 3.16	3.8 ± 0.56	3.5 ± 0.36
β-car 100	94.6 ± 5.71	59.7 ± 1.95*	121.2 ± 7.03	66.2 ± 3.09	3.6 ± 0.54	3.2 ± 0.42
β-car 200	112.9 ± 7.15	65.2 ± 2.17**	158.6 ± 8.68*	64.3 ± 2.97	3.6 ± 0.54	3.8 ± 0.48
Dzp 1	130.89 ± 7.56*	62.2 ± 2.30**	167.5 ± 12.98**	82.8 ± 3.75*	4.2 ± 0.60	1.6 ± 0.20*
Dzp 5	51.6 ± 5.18***	36.4 ± 2.19***	52.4 ± 10.31***	13.5 ± 3.45***	2.3 ± 0.56	0.3 ± 0.26***

Data expressed as mean ± SEM of nine mice. Vehicle (2% Tween, 10 mL/kg, p.o.), EO: essential oil from *Spiranthera odoratissima* leaves (125, 250 and 500 mg/kg, p.o.), β-car: β-caryophyllene (50, 100 and 200 mg/kg, p.o.), DZP: diazepam (1 and 5 mg/kg, p.o.).
*** $P \leq 0.001$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.
* $P \leq 0.05$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.
** $P \leq 0.01$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.

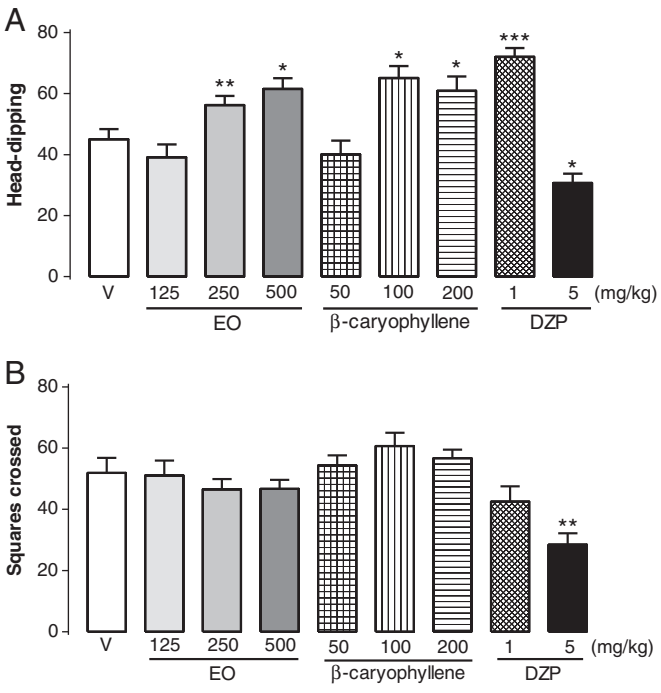

Fig. 2. Effect of essential oil (EO) from *Spiranthera odoratissima* leaves on the number of head-dipping behavior (A) and the number of squares crossed (B), as evaluated in hole-board test. Vehicle (V, 2% Tween 80, 10 mL/kg p.o.), essential oil (EO, 125, 250 or 500 mg/kg p.o.), β-caryophyllene (50, 100 or 200 mg/kg p.o.) and diazepam (DZP, 1 or 5 mg/kg p.o.). The columns and vertical bars represent the means ± SEM of nine mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.

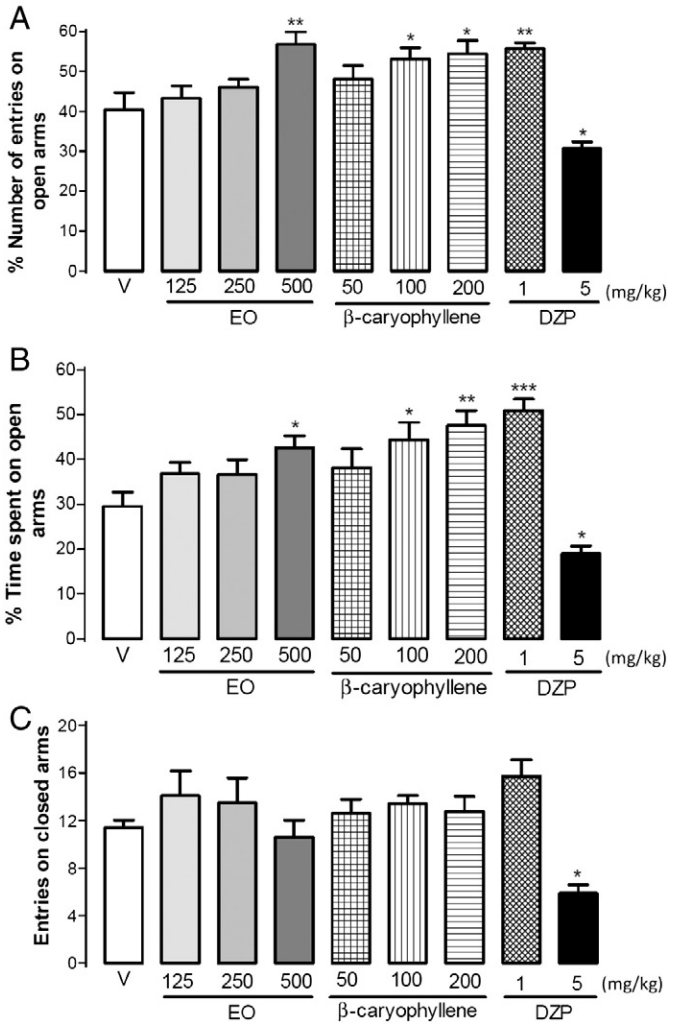

Fig. 3. Effect of essential oil (EO) from *Spiranthera odoratissima* leaves and β-caryophyllene on the entries (A) and time spent (B) on open arms and total entries in closed arms (C), as evaluated in the elevated plus maze test. Vehicle (V, 2% Tween 80, 10 mL/kg p.o.), essential oil (EO, 125, 250 or 500 mg/kg p.o.), β-caryophyllene (50, 100 or 200 mg/kg p.o.) and diazepam (DZP, 1 or 5 mg/kg p.o.). The columns and vertical bars represent the means ± SEM of nine mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.

was increased by p.o. treatment with EO (500 mg/kg) ($P<0.05$ vs. vehicle), β-caryophyllene (100 and 200 mg/kg) ($P<0.05$ and $P<0.05$ vs. vehicle, respectively) and diazepam 1 mg/kg ($P<0.01$ vs. vehicle), while this parameter was reduced ($P<0.05$ vs. vehicle) upon diazepam (5 mg/kg) treatment. The time spent in the light compartment [ANOVA: $F(8,72) = 22.698$, $P<0.001$; Fig. 4B] was increased by treatment with EO (250 and 500 mg/kg) ($P<0.05$ and $P<0.001$ vs. vehicle, respectively), β-caryophyllene (100 and 200 mg/kg) ($P<0.05$ and $P<0.05$ vs. vehicle, respectively), and diazepam (1 mg/kg) ($P<0.05$ vs. vehicle), while diazepam (5 mg/kg) reduced this parameter ($P<0.05$ vs. vehicle).

3.8. Mechanisms involved in the anxiolytic-like effects of EO

In both the elevated plus maze and light–dark box tests, the anxiolytic-like effect of EO was not significantly decreased when flumazenil was injected before oral administration of EO (500 mg/kg). However, this effect was significantly decreased when mice were pretreated with NAN-190 (Figs. 5 and 6). Meanwhile, the anxiolytic-like effect of β-caryophyllene (200 mg/kg) was not significantly altered by either flumazenil or NAN-190 pretreatment (Figs. 5 and 6).

4. Discussion

In the current work, we examined the behavioral effects of oral treatment with the essential oil derived from *S. odoratissima* leaves and its major component, β-caryophyllene, using well-validated animal models of CNS activity, namely the rota-rod, open-field, pentobarbital-induced sleep, hole-board, elevated plus maze and light–dark box tests. We also assayed the role of $GABA_A$/benzodiazepine and $5HT_{1A}$ receptors in the effects of EO and β-caryophyllene effects.

The effects on general behavioral test observed upon EO treatment, such as decreased spontaneous ambulation, presence of analgesia, environmental alienation and palpebral ptosis are suggestive of central depressant drugs. This methodology indicates the optimal route and doses for other in vivo biological tests. The group of mice that was treated with these compounds orally exhibited an absence of deaths and behaviors suggestive of neurotoxicity throughout the observational period, making this route the most promising for further evaluations using doses up to 1000 mg/kg. Therefore, we chose doses of 125, 250 and 500 mg/kg for further tests aimed at assaying possible anxiolytic-like effects and verifying a dose–response relationship.

A deficit in motor coordination would very likely affect performance in the behavioral tests. Therefore, we investigated the motor effects of EO in the rota-rod test, a classical animal model used to evaluate peripheral neuromuscular blockade and the motor coordination (Dunham and Miya, 1957). Our findings showed that EO (125–500 mg/kg), unlike diazepam (5 mg/kg), had no significant effect on motor coordination (Table 1).

The open-field test is designed to study exploratory activity (Crawley, 1985); in this test, EO administration did not alter the

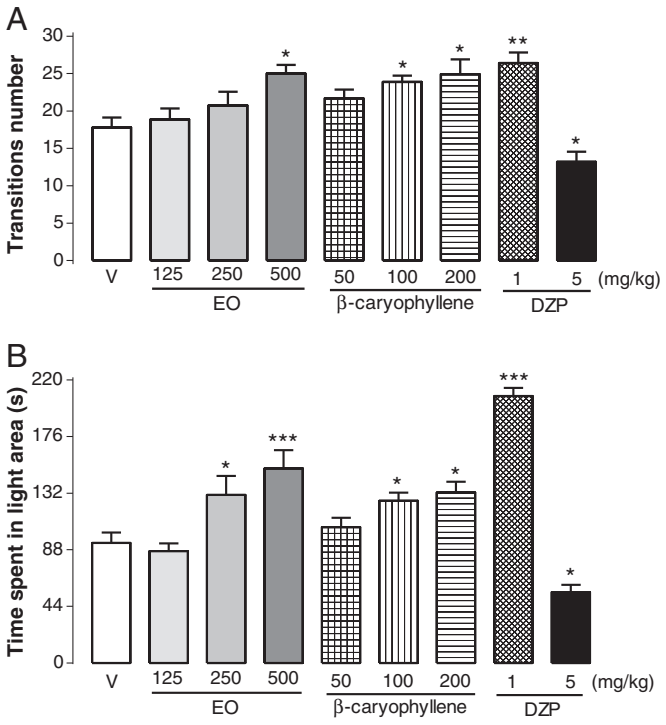

Fig. 4. Effect of essential oil (EO) from *Spiranthera odoratissima* leaves and β-caryophyllene on transitions number (A) and time spent in light compartment (B), as evaluated in the light–dark box test. Vehicle (V, 2% Tween 80, 10 mL/kg p.o.), essential oil (EO, 125, 250 or 500 mg/kg p.o.), β-caryophyllene (50, 100 or 200 mg/kg p.o.) and diazepam (DZP, 1 or 5 mg/kg p.o.). The columns and vertical bars represent the means ± SEM of nine mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with control group using ANOVA and Student–Newman–Keuls test as the *post hoc* test.

crossing number (Table 2), which supports the lack of alteration in motor coordination previously observed in the rota-rod test. The decrease in rearing behavior seems to be unrelated to a loss of motor coordination or to neuromuscular blockade; instead, it may be caused by a central depressant activity. EO treatment also increased the preference for the central area, enhancing the crossing number and the time spent in the central area of the apparatus (Table 2); an increase in these parameters could be indicative of anxiolytic-like effects (Prut and Belzung, 2003). EO, at all doses tested, reduced the fecal bolus (Table 2). Because defecation is a good indicator of emotionality in animals, its reduction can be associated with an anxiolytic-like effect; however, it can also be attributed to other physiological factors not associated with anxiety states (Angrini et al., 1998; Archer, 1973).

The pentobarbital-induced sleep test was assessed to confirm the putative depressant-like effects observed in the previous tests. In this test, CNS depressant drugs classically decrease the sleep latency and increase the sleeping time (Carlini and Burgos, 1979); EO showed both effects, confirming its depressant-like effect (Fig. 1). This EO CNS depressant-like effect could be due to anxiolytic-like properties; to verify this hypothesis, we used the hole-board, elevated plus maze and light–dark box tests.

The hole-board test is useful for modeling anxiety in animals; in this test the expression of an anxiolytic-like state may be reflected by an increase in head-dipping behaviors (Crawley, 1985; Takeda et al., 1998). Our results showed that EO increased the number of head dips without altering the number of squares crossed (Fig. 2) indicating an anxiolytic-like effect without any change in locomotion.

The elevated plus maze test is one of the most widely employed tests for evaluation of anxiolytic/anxiogenic properties. This test is based on the observation that the natural behavior of rats or mice is to display an aversion to open spaces; therefore, avoidance of the open arms is interpreted as anxiogenic behavior (Belzung and Griebel, 2001; Bertoglio and Carobrez, 2005). The anxiolytic-like effectiveness of a drug can be demonstrated by an increase in exploration of the open arms (time and entries into open arms), while the opposite holds true for drugs with anxiogenic-like effects (Bertoglio and Carobrez, 2005; Lister, 1987; Rodgers and Cole, 1994). The number of closed arm entries provides a control measure of motor activity (Bourin et al., 2007). In the present study, EO increased the entries into and time spent in the open arms without altering the number of entries into closed arms (Fig. 3), indicating an anxiolytic-like effect without motor impairment.

In the light–dark box test, anxiety is generated by the conflict between the desire to explore and to retreat from an unknown and well-illuminated space (Crawley and Goodwin, 1980) and can be evaluated according to the number of transitions into and the time spent in the light chamber (Graeff and Zangrossi, 2002; Lepicard

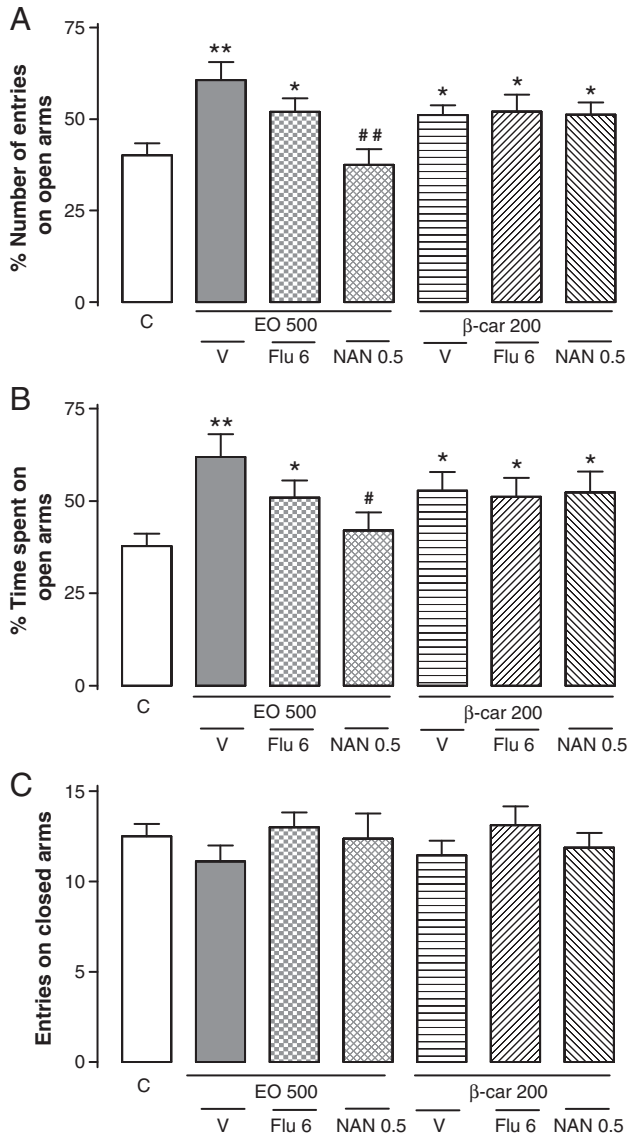

Fig. 5. Influence of the pretreatment with flumazenil or NAN-190 on the anxiolytic-like effect of essential oil (EO) from *Spiranthera odoratissima* leaves and β-caryophyllene on the entries (A) and time spent (B) on open arms and total entries in closed arms (C), in the elevated plus maze test. Control (C, 2% Tween 80 10 mL/kg i.p. and p.o.), essential oil (EO, 500 mg/kg p.o.), β-caryophyllene (β-car, 200 mg/kg p.o.), vehicle (V, 2% Tween 10 mL/kg i.p.), NAN-190 (NAN, 0.5 mg/kg i.p.) and flumazenil (Flu, 6 mg/kg i.p.). The columns and vertical bars represent the means ± SEM of nine mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with control group using unpaired "*t*" test. $^{\#}P \leq 0.05$, $^{\#\ \#}P \leq 0.01$ compared with OE or β-car group using unpaired "*t*" test.

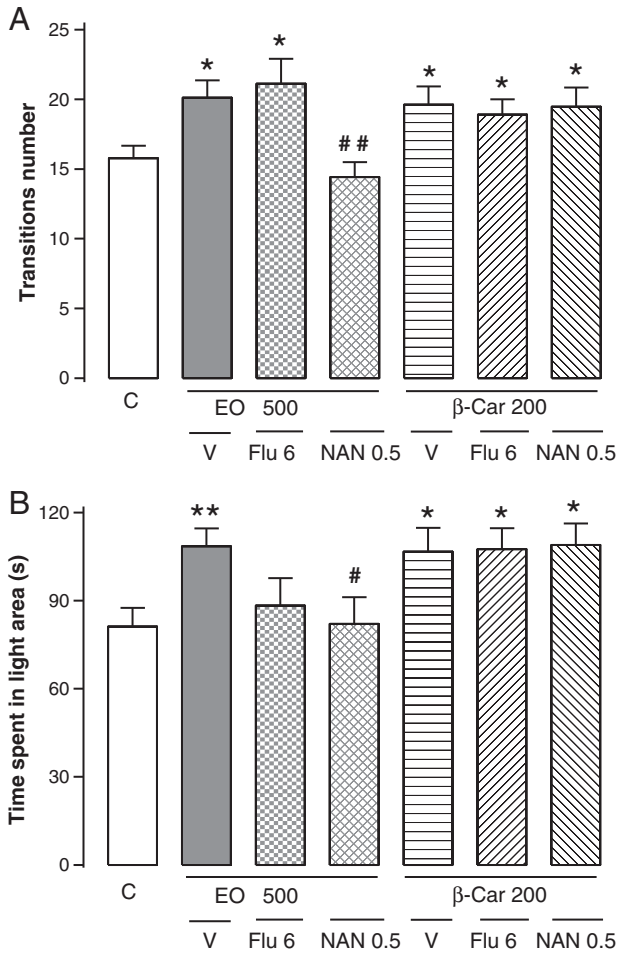

Fig. 6. Influence of the pretreatment with flumazenil or NAN-190 on the anxiolytic-like effect of essential oil (EO) from *Spiranthera odoratissima* leaves and β-caryophyllene on transitions number (A) and time spent in the light compartment (B), in light–dark box test. Control (C, 2% Tween 80 10 mL/kg i.p. and p.o.), essential oil (EO, 500 mg/kg p.o.), β-caryophyllene (β-car, 200 mg/kg p.o.), vehicle (V, 2% Tween 80 10 mL/kg i.p.), NAN-190 (NAN, 0.5 mg/kg i.p.) and flumazenil (Flu, 6 mg/kg i.p.). The columns and vertical bars represent the means ± SEM of nine mice. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ compared with control group using unpaired "*t*" test. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ compared with OE or β-car group using unpaired "*t*" test.

et al., 2000) where an increase in these parameters is considered to reflect anxiolytic-like properties. Our results showed that EO treatment increased these two parameters (Fig. 4), corroborating the anxiolytic-like effect previously shown in the hole-board and elevated plus maze tests. Therefore, the results obtained in these three methods show that EO has an anxiolytic-like activity without the motor impairment characteristic of sedative effects.

Benzodiazepines are the most widely prescribed CNS depressants, with selective activity at the inhibitory $GABA_A$ receptor complex; they act to potentiate the inhibitory effect of GABA by enhancing the frequency of chloride channel opening and thus enhance the chloride flux through the $GABA_A$ receptor/chloride channel (Lilly and Tietz, 2000). As expected, diazepam (1 mg/kg) produced an anxiolytic-like effect while diazepam (5 mg/kg) produced motor impairment.

To determine if the mechanism underlying the anxiolytic-like effects of EO involves the benzodiazepine system, we pretreated the animals with flumazenil, a well-known competitive antagonist that binds the benzodiazepine site of the $GABA_A$ receptor, at a dose that antagonizes the anxiolytic effects of diazepam in the elevated plus maze and light–dark box tests (data not shown). Our results show that the effects of EO were not significantly altered by flumazenil treatment (Figs. 5 and 6), indicating that EO's anxiolytic-like effects do not involve the benzodiazepine site of the $GABA_A$ receptor.

The 5-hydroxytryptamine 1A ($5\text{-}HT_{1A}$) receptor is a potential target for the treatment of psychiatric disorders, notably anxiety and depression (File, 1996). This receptor is a G_i-coupled-receptor that, when activated, inhibits adenylyl cyclase activity and enhances K^+ currents (Raymond et al., 1999). $5\text{-}HT_{1A}$ receptors are located at presynaptic and postsynaptic sites (Blier et al., 1993). Buspirone, an anxiolytic drug with partial agonist properties at this receptor, has efficacy in the treatment of patients with Generalized Anxiety Disorder (GAD) (Goa and Ward, 1986; Graeff et al., 1996), and a meta-analysis has indicated that buspirone has comparable efficacy to benzodiazepines in the management of GAD (Gammans et al., 1992). Therefore, the $5\text{-}HT_{1A}$ receptor is a pharmacological target that could be relevant to the mechanism of action of EO. Additionally, the anxiolytic drugs that act on the $5\text{-}HT_{1A}$ receptor are known as "anxioselective" because they do not possess the side effects commonly caused by benzodiazepine treatment, such as motor impairment and a decrease in spontaneous locomotion in animals (Harada et al., 2006; Siemiatkowski et al., 2000; Stefanski et al., 1992).

The possible involvement of the $5\text{-}HT1_A$ receptor complex in EO's mechanism of action was also tested in the elevated plus maze and light–dark box tests. EO's anxiolytic-like effects were antagonized by NAN-190 (Figs. 5 and 6), a $5\text{-}HT_{1A}$ receptor antagonist. Therefore, the EO anxiolytic-like activity seems to be mediated via $5\text{-}HT_{1A}$ receptor activation.

Recently, Foong and Bornstein (2009) found that NAN-190 may also block α2-adrenoceptors, raising a concern about the selectivity of NAN-190 for the $5\text{-}HT_{1A}$ receptors and suggesting that EO's effects may be partially mediated by α2-adrenoceptors.

Celedônio (2008) showed that β-caryophyllene, the major component of EO, has anti-nociceptive effects in the hot plate and formalin- and glutamate-induced pain tests, tests, which are sensitive to CNS drugs. Thus, it is probable that β-caryophyllene is active in CNS when administered orally. Therefore, this compound was tested to verify if it has anxiolytic-like effects similar to those of EO and if it shares the same mechanism of action.

Using the rota-rod test (Dunham and Miya, 1957), we assessed the motor coordination of animals treated with β-caryophyllene (100, 200 or 400 mg/kg). Galindo et al. (2010) observed that β-caryophyllene, at doses of up to 50 mg/kg p.o., does not produce motor impairment; in accordance with this result, we showed that this compound, at doses of up to 400 mg/kg p.o., does not produce motor impairment (Table 1).

β-Caryophyllene was then tested in the pentobarbital-induced sleep test (Carlini and Burgos, 1979). Galindo et al. (2010) showed that β-caryophyllene (at doses of up to 50 mg/kg) has no effect in this test; our results are in accordance with this, given that in our hands the 100 mg/kg dose has no effect. However, the 200 and 400 mg/kg doses decreased the latency and increased the sleep time; these effects were similar to, albeit less pronounced than, those of EO (Fig. 1). Galindo et al. (2010) have also suggested that even at subactive doses this compound may increase the depressive effect of other substances.

In the anxiety tests, β-caryophyllene (100 and 200 mg/kg) had anxiolytic-like effects, increasing the head-dipping behavior in the hole-board test (Fig. 2) and the exploration in the open arms of the EPM (Fig. 3A and B) and in the light area of the LDB (Fig. 4), without demonstrating evidence of motor impairment in the EPM (Fig. 3C). It is noteworthy that the anxiolytic-like dose of β-caryophyllene (100 mg/kg) is proportional to the anxiolytic-like dose of EO (500 mg/kg), suggesting that β-caryophyllene may contribute to the effects of EO; however, the effectiveness of β-caryophyllene was similar to or less than the effectiveness of EO.

After determining that β-caryophyllene has an anxiolytic-like activity, the possible mechanisms of action were studied, specifically by evaluating the role of $GABA_A$/benzodiazepine and $5\text{-}HT_{1A}$

receptors via pre-treatment with flumazenil and NAN-190. Neither of the antagonists was able to antagonize the anxiolytic-like effects observed (Figs. 5 and 6), suggesting that non-$GABA_A$/benzodiazepine, non-5-HT_{1A} receptors are involved.

The identification of the effects of the major component contributes to the standardization of the herbal preparation and safeguards the quality and the reproducibility of EO's effects; however, in this case this compound does not have the same mechanism of action as EO. This implies that this component likely is not the most important determinant of the anxiolytic-like effect that involves the serotonergic system; however, it may act to increase the effects of other compounds, as suggested by Galindo et al. (2010).

In the literature, there are no studies regarding other EO components. Only β-caryophyllene has been evaluated in various pharmacological tests, which commonly demonstrate its analgesic and anti-inflammatory properties (Celedonio, 2008; Fernandes et al., 2007; Medeiros et al., 2007; Passos et al., 2006). To our knowledge, this is the first study of the anxiolytic-like effects of β-caryophyllene.

Recently, Gertsch et al. (2008) demonstrated that β-caryophyllene binds selectively to cannabinoid receptor 2 and acts as a full agonist. Cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptors are GTP-binding protein (G-protein) coupled receptors that were first cloned in the early 1990s (Matsuda et al., 1990; Munro et al., 1993). Some reports have suggested that the serotonergic system can be modulated by the cannabinoid system via the CB1 receptor (Bambico et al., 2007; Haj-Dahmane and Shen, 2011; Lau and Schloss, 2008; McLaughlin et al., 2009).

For a long time it was believed that the CNS expresses only CB1, but studies have demonstrated the presence of CB2 in various CNS structures, including the spinal cord, microglial cultures, brainstem and cortex (Morgan et al., 2009; Onaivi et al., 2006). CB2 has become an attractive therapeutic target because this receptor has not been associated with CNS side effects caused by CB1 activation (Gertsch et al., 2008). Recent results from mice with genetically modified CB2 suggest that CB2 signaling is involved in the regulation of emotional behaviors including anxiety (Marco et al., 2011); thus, the cannabinoid system may be involved in the anxiolytic-like effects of β-caryophyllene. The possibility of using a preparation (the essential oil) that acts on two systems (serotonergic and cannabinoid) may widen the spectrum of cellular targets, thus resulting in a more favorable clinical effect.

Overall, beneficial results can be obtained with drugs or plant species with proven efficacy in the treatment of GAD (Faustino et al., 2010; Sousa et al., 2008); therefore, the use of OE may represent a new therapeutic option in the treatment of GAD, a hypothesis needs to be better evaluated and proved in clinical studies.

5. Conclusions

EO and β-caryophyllene show anxiolytic-like effects without altering motor coordination, and β-caryophyllene may contribute to the effects of EO. EO's anxiolytic-like effects seem to be mediated by 5-HT_{1A} receptors, while the anxiolytic-like effects of β-caryophyllene seem to be independent of 5-HT_{1A} and benzodiazepine receptors.

Contributors

BAC and JRP extract the essential oil.

PMG, MVMN, IFF, RCL and JOF executed the experiments and analyses.

PMG, TCMDL and EAC managed the literature searches and analyses.

PMG, JRP, TCMDL and EAC designed the study and wrote the protocols.

PMG, TCMDL and EAC wrote the manuscript.

All authors contributed and approved the final manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

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