Anxiolytic-like effects of acute and chronic treatment with *Achillea millefolium* L. extract

Irinéia Paulina Baretta [a,b,*], Regiane Américo Felizardo [a], Vanessa Fávero Bimbato [a],
Maísa Gonçalves Jorge dos Santos [a], Candida Aparecida Leite Kassuya [b,c],
Arquimedes Gasparotto Junior [a,b], Cássia Regina da Silva [d], Sara Marchesan de Oliveira [d],
Juliano Ferreira [d], Roberto Andreatini [b]

[a] *Instituto de Ciências Biológicas, Médicas e da Saúde – Farmacologia, Universidade Paranaense, Umuarama 87502-210, PR, Brazil*
[b] *Departamento de Farmacologia, Setor de Ciências Biológicas, Centro Politécnico, Universidade Federal do Paraná, Curitiba 82015-690, PR, Brazil*
[c] *Faculdade de Ciências da Saúde, Universidade Federal da Grande Dourados, Dourados 79825-070, MS, Brazil*
[d] *Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria 97105-900, RS, Brazil*

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ABSTRACT

Ethnopharmacological relevance: Achillea millefolium L. (Asteraceae), known as yarrow ("mil folhas"), has been used as folk medicine for gastrointestinal disorders, inflammation, anxiety, and insomnia.
Aim: To evaluate the potential anxiolytic-like effect of hydroalcoholic extract of *Achillea millefolium* L. in animal models.
Methods: The present study evaluated the effects of the hydroalcoholic extract from the aerial parts of *Achillea millefolium* L. in mice subjected to the elevated plus-maze, marble-burying, and open-field tests. Additionally, the GABA$_A$/benzodiazepine (BDZ) mediation of the effects of *Achillea millefolium* was evaluated by pretreatment with the noncompetitive GABA$_A$ receptor antagonist picrotoxin and the BDZ antagonist flumazenil and by [³H]-flunitrazepam binding to the BDZ site on the GABA$_A$ receptor.
Results: Achillea millefolium exerted anxiolytic-like effects in the elevated plus-maze and marble-burying test after acute and chronic (25 days) administration at doses that did not alter locomotor activity. This behavioral profile was similar to diazepam. The effects of *Achillea millefolium* in the elevated plus-maze were not altered by picrotoxin pretreatment but were partially blocked by flumazenil. Furthermore, *Achillea millefolium* did not induce any changes in [³H]-flunitrazepam binding.
Conclusion: The results indicate that the orally administered hydroalcoholic extract of *Achillea millefolium* L. exerted anxiolytic-like effects that likely were not mediated by GABA$_A$/BDZ neurotransmission and did not present tolerance after short-term, repeated administration.

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1. Introduction

Numerous traditionally used plants exhibit pharmacological properties with great potential for therapeutic applications in the treatment of central nervous system disorders, such as anxiety disorders (Carlini, 2003; Faustino et al., 2010). *Achillea millefolium* L. (Asteraceae), known as yarrow ("mil folhas"), is a perennial herb that has been used for hundreds of years in folk medicine in several countries (Chandler et al., 1982; Wichtl and Bisset, 1989). In Germany and Italy, it is used for the treatment of gastrointestinal

disturbances. In Britain and North America, it is used to treat bleeding (Bruneton, 1999; Willuhn, 2002; Applequist and Moerman, 2011). In Brazil, its main indication is for the treatment of pain, wounds, inflammation, and gastrointestinal complaints (Dalsenter et al., 2004; Cavalcanti et al., 2006; Pires et al., 2009), although plant infusion or the decoction of the aerial parts of the plant is indicated for "calmness" (Manfrini et al., 2009). This latter indication is also seen in Mexico (Molina-Hernandez et al., 2004).

Previous preclinical studies have corroborated its safety and antinociceptive and antiulcer effects (Dalsenter et al., 2004; Cavalcanti et al., 2006; Pires et al., 2009). Although *Achillea millefolium* L. has been proposed as a folk remedy in the treatment of central nervous system disorders, very few data have been published supporting this claimed ethomedical action. Molina-Hernandez et al. (2004) used a conflict operant procedure and showed that the anticonflict-like actions of an aqueous extract of

the flowers of *Achillea millefolium* L. administered intraperitoneally vary according to the estrous cycle phase of female rats.

The search for new anxiolytic drugs is supported by the adverse effects associated with the current drug treatments for generalized anxiety disorder. Benzodiazepines (BDZs) can lead to disturbing effects, such as amnesia, dependence liability, and sedation. Buspirone is associated with low patient satisfaction. Moreover, antidepressants can lead to sexual dysfunction, insomnia, and gastrointestinal disturbances (Andreatini et al., 2001; Carlini, 2003; Mitte et al., 2005). Medicinal plants continue to have anxiolytic potential (Carlini, 2003; Faustino et al., 2010).

The elevated plus maze is likely the most frequently used animal model for studying anxiety and anxiolytic-like drugs (Carobrez and Bertoglio, 2005). It is based on the conflict between the fear of open areas on the open arms and neophobia (fear of new environments), which elicit avoidance behavior, and the drive to explore new environments, which elicits approach behavior (Rodgers and Cole, 1994; Rodgers and Dalvi, 1997). Thus, it is related to unconditioned behavior, although some learned behavior occurs during the experimental session (Rodgers and Cole, 1994; Carobrez and Bertoglio, 2005). Most anxiolytic-like drugs increase open arm exploration, reflected by an increase in the percentage of entries into and time spent on the open arms, at doses that do not affect locomotor activity (Lister, 1987; Borsini et al., 2002). However, debatable is whether the elevated plus maze can detect the anxiolytic-like effects of antidepressants, such as selective serotonin reuptake inhibitors (Rodgers and Cole, 1994; Borsini et al., 2002). Marble-burying behavior in mice and rats is based on the behavior of burying harmless objects (Nicolas et al., 2006; Thomas et al., 2009). This model is also related to an unconditioned behavior. Anxiolytic-like drugs decrease the number of marbles buried during a session at doses that do not alter locomotor activity (Njung'e and Handley, 1991; Borsini et al., 2002; Nicolas et al., 2006). These models have some differences. The elevated plus maze is affected by an animal's previous maze experience, whereas marble-burying behavior is stable across repeated tests (Rodgers and Cole, 1994; Carobrez and Bertoglio, 2005; Thomas et al., 2009; Gomes et al., 2011). There are some criticisms of the marble-burying model as a model of anxiety or defensive behavior (Londei et al., 1998; Thomas et al., 2009). Marble-burying behavior has been considered a perseverative behavior related to obsessive compulsive disorder (Londei et al., 1998; Costa et al., 2011; Gomes et al., 2011). However, it is sensitive to benzodiazepines and antidepressants (Njung'e and Handley, 1991; Borsini et al., 2002; Nicolas et al., 2006), which may make it suitable for screening anxiolytic-like drugs. Considering the possibility of false-positive and false-negative results with these animal models (Borsini et al., 2002; Nicolas et al., 2006), in the present study these two models were used in a complementary manner.

Thus, the objective of the present study was to evaluate the effects of acute and chronic oral treatment with the hydroalcoholic extract of the aerial parts of *Achillea millefolium* L. in mice subjected to the elevated plus maze, marble-burying, and open-field tests. Additionally, the GABA$_A$/benzodiazepine (BDZ) mediation of the effects of *Achillea millefolium* was evaluated by pretreatment with the noncompetitive GABA$_A$ receptor antagonist picrotoxin and the BDZ antagonist flumazenil and by [^{3}H]-flunitrazepam binding to the BDZ site on the GABA$_A$ receptor. Considering that apigenin is an important constituent of *Achillea millefolium* extracts (Pires et al., 2009; Tuberoso et al., 2009) and that previous studies have suggested an anxiolytic-like effect of this compound (Viola et al., 1995; Paladini et al., 1999), the effect of apigenin was also investigated, at a dose range similar to that which would be found in *Achillea millefolium* extract (0.1–1.1%; Pires et al., 2009; Tuberoso et al., 2009), in the elevated plus-maze and marble-burying test.

2. Materials and methods

2.1. Plant material

The *Achillea millefolium* used in our experiments was collected in July 2007 from the botanical garden of the Universidade Paranaense campus, Umuarama, Brazil, at an altitude of 430 m above sea level (S23°47′55–W53°18′48). The plant was identified by Dr. Mariza Barion Romagnolo (Department of Botany, Universidade Paranaense, PR, Brazil). Voucher specimens were deposited at the herbarium of the university (specimen no. 1896).

2.2. Preparation of hydroalcoholic extract

The aerial parts (leaves, stalks, and stems) of *Achillea millefolium* were air-dried in an oven at 40 °C for 4 days, and then the dry plant was cut and pulverized. The dried, powdered plant material was macerated for 7 days using 90% ethanol as a solvent. The solvent was then eliminated using a rotary vacuum evaporator under reduced pressure and lyophilized, yielding an extract of 17.4% of the dry material (Potrich et al., 2010). The hydroalcoholic extract was dissolved in a 5% Tween-80 aqueous solution and administered at doses ranging from 30 to 600 mg/kg.

2.3. Animals

Animals were adult albino Swiss male mice (30–45 g) from our breeding stock. They were housed in groups in polypropylene cages with wood shavings as bedding, under a controlled 12 h/12 h light/dark cycle (lights on at 7:00 a.m.) and controlled temperature (22 °C). The animals had free access to water and food, with the exception of 1 h before and during the experiments. The animals were not specifically handled prior to the experiments, with the exception of handling during necessary animal care (e.g., cleaning the cages) and drug administration (weighing, tail marking, and drug administration).

2.4. Drugs and treatments

The hydroalcoholic extract of *Achillea millefolium* (30–300 mg/kg), diazepam (0.75 mg/kg; Sigma, USA), and *Achillea millefolium* vehicle were administered by gavage (per os, p.o.) 1 h before the tests. Flumazenil (1.0 mg/kg, Sigma; Lolli et al., 2007; Kamei et al., 2009) and picrotoxin (1.0 mg/kg, Sigma; Stankevicius et al., 2008) were administered intraperitoneally (i.p.) 30 min before the administration of the hydroalcoholic extract of *Achillea millefolium* (300 mg/kg). All treatments were administered at a constant volume of 10 mL/kg body weight. The *Achillea millefolium* dose was based on a preliminary pharmacological screening protocol (Pires et al., 2009) with different extract doses (100, 300, and 600 mg/kg) administered to mice. At all doses used, no signs of acute toxicity (e.g., seizures, abdominal pain, and death) were observed.

The apigenin doses (0.3, 1.0, and 3.0 mg/kg; Sigma) were calculated based on apigenin content in *Achillea millefolium* extract: 0.1% (Tuberoso et al., 2009)–1.1% (Pires et al., 2009).

2.5. Behavioral tests

In the acute experiments, each animal was tested in only one behavioral test, whereas in the chronic experiment, the same animal was tested in the open-field test 5 min before testing in the elevated plus maze. The mice were habituated to the experimental room 1 h before the tests. The procedures used in the present study were in accordance with the guidelines for animal research care

from Colégio Brasileiro de Experimentação Animal (Brazilian College for Animal Experimentation) and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985). The experimental protocol was approved by the Institutional Ethics Committee of the Universidade Paranaense (approved number 13.911). The experiments were performed in the morning (8–12 h, beginning 1 h after lights on), and the illumination level was 200 lux in the experimental room.

2.5.1. Elevated plus maze

The elevated plus maze for mice consisted of two perpendicular open arms (30 cm × 5 cm) and two perpendicular closed arms (30 cm × 5 cm × 25 cm) that were open on the top. The maze was constructed of black painted wood and was elevated 45 cm above the floor (Lister, 1987). The open arms were surrounded by a wood rim (0.5 cm high) to prevent the mice from falling from the maze. One hour after oral treatment, the mouse was placed in the center of the elevated plus maze facing one closed arm and observed for 5 min. During the test period, the following variables were measured: the number of entries into and time spent on the open and closed arms and the total number of arm entries. Arm entries were defined as the entry of all four paws into the arm. Anxiolytic compounds selectively increase the percentage of time spent on the open arms (open × 100/300) and percentage of open arm entries [open × 100/(open + closed)]. These effects are observed without changes in locomotor activity, which is measured in the elevated plus maze as the total number of arm entries (number of open arm entries + number of closed arm entries; Lister, 1987; de Castro et al., 2007; Raupp et al., 2008). Before each test, the maze was cleaned with 10% (v/v) ethanol solution and dry cloths.

2.5.2. Open-field test

Open-field activity was measured in an acrylic cage (17 cm height, 30 cm length, 30 cm width) with a black floor marked with white lines in 10 cm^2 areas. One hour after drug administration, each mouse was placed in the arena, and its ambulation in the peripheral area, rearing, grooming, and defecation were recorded for 5 min (Hellión-Ibarrola et al., 2006). The number of gridlines crossed by both hind feet in a 5 min period was counted as an index of ambulation. After each trial, the open-field apparatus was wiped clean with a 10% ethanol solution.

2.5.3. Marble-burying test

One hour after drug administration, each mouse was placed individually in a propylene cage that was identical to its home cage (28 cm × 16 cm × 12 cm) and contained 5 cm deep sawdust and 24 clean glass marbles equally spaced against the wall. No food or water was present. The number of marbles that were at least two-thirds buried after 30 min was recorded (Skalisz et al., 2004; Dekeyne et al., 2008).

2.6. [3H]-flunitrazepam binding

For membrane preparation, the brains (without cerebellum) of rats were homogenized (1:10, w/v) in homogenization buffer (10 mM Tris–HCl, 300 mM sucrose, 2 mM EDTA, pH 7.4). The homogenate was centrifuged at 4 °C at 1000 × g for 10 min, and the supernatant was collected from this initial centrifugation and centrifuged again at 4 °C at 16,000 × g for 20 min. The pellet was resuspended in the same buffer and frozen at −20 °C for 48 h. After this period, membranes were defrosted, resuspended (1:20, w/v) in 50 mM Tris–HCl and 2 mM EDTA (pH 7.4), and centrifuged at 4 °C at 16,000 × g for 10 min. The pellet was resuspended in the same buffer and incubated at 37 °C for 30 min. After incubation, the pellets were centrifuged and washed in the same conditions described

above two more times. After the last centrifugation, the pellets were resuspended in 20 mM HEPES and 1 mM EDTA (pH 7.4), and the protein content was determined according to Bradford (1976).

The [3H]-flunitrazepam binding assay was performed as previously described (Vogel, 2008). The incubation was carried out in duplicate in polycarbonated tubes (total volume 500 μL) containing 50 mM Tris–HCl (pH 7.4), 0.5 mg of protein membrane in absence or presence of the crude hydroethanolic extract of plant *Achillea millefolium* (1–100 μg/ml). Diazepam (10 μM) was used as a positive control. The diazepam concentration was based on previous studies, in which the IC50 of [3H]-flunitrazepam binding was approximately 10 nM (Thomas et al., 1997; Huen et al., 2003). Thus, we used a 1000-fold higher concentration of diazepam (10 μM) to guarantee large receptor occupancy and an inhibitory effect. Regarding the concentrations of the extract (1–100 μg/ml), we tested it in a range as low as the concentration of diazepam (10 μM corresponds to 3 μg/ml) and as high as the solubility limit of the extract (100 μg/ml in aqueous solution). Incubation was started by adding 1 nM [3H]-flunitrazepam (85 Ci/mmol, Perkin-Elmer, USA), and run at ice for 60 min. The reaction was stopped by vacuum filtration, and each filter was washed with 15 mL of cold 10 mM Tris–HCl buffer. Filters were individually placed in polycarbonate tubes, and 1 mL of scintillation liquid was added. Radioactivity was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. Nonspecific binding (10–20% of total binding) was determined by adding 100 μM diazepam to the medium in parallel assays. Specific binding was considered the difference between total binding and nonspecific binding. The results are expressed as a percentage of specific binding.

2.7. Statistical analysis

The elevated plus maze, marble-burying, and open-field data were analyzed by one-way (treatment) analysis of variance (ANOVA) for independent samples followed by the Duncan test, with the exception of the total number of arm entries in the elevated plus maze and number of fecal boluses in the open-field test, which were analyzed by Kruskal–Wallis ANOVA followed by multiple comparison test. [3H]-flunitrazepam binding data were analyzed by one-way (treatment) ANOVA followed by Student Newman–Keuls' test. Statistical significance was set at $P \leq 0.05$. Data are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using Statistica software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Effects of acute and chronic administration of the hydroalcoholic extract of Achillea millefolium L. in the elevated plus maze

The results of acute and chronic administration of the hydroalcoholic extract of *Achillea millefolium* L. in the elevated plus maze are shown in Fig. 1. One-way ANOVA indicated a difference between groups in the percentage of entries in the open arms with acute and chronic treatments (acute: $F_{5,54} = 9.56$, $P < 0.001$; chronic: $F_{3,36} = 18.85$, $P < 0.001$) and in the percentage of time spent in the open arms (acute: $F_{5,54} = 22.71$, $P < 0.001$; chronic: $F_{3,36} = 48.93$, $P < 0.001$). In the acute treatment groups, diazepam and the *Achillea millefolium* extract (300–600 mg/kg) increased the percentage of entries into and time spent on the open arms compared with vehicle and the lower doses (30 and 100 mg/kg) of the *Achillea millefolium* extract (all $P < 0.05$). Similar results were seen in the chronic experiments: diazepam and the *Achillea millefolium* extract (30–300 mg/kg) increased the percentage of entries into and time spent on the open arms compared with vehicle (all $P < 0.01$). The

Table 1
Effects of acute and chronic (25 days) treatment with the hydroalcoholic extract of *Achillea millefolium* (300 mg/kg) and diazepam (0.75 mg/kg) on open-field behavior in male mice.

	Locomotor activity (number of lines crossed)	Rearing (number)	Grooming time (s)
Acute			
Vehicle	41 ± 8	8 ± 3	4 ± 1
Achillea millefolium	57 ± 7	9 ± 3	2 ± 1
Diazepam	68 ± 9	7 ± 2	3 ± 1
Chronic			
Vehicle	60 ± 5	17 ± 3	7 ± 1
Achillea millefolium	68 ± 5	12 ± 2	3 ± 1
Diazepam	69 ± 11	15 ± 3	6 ± 2

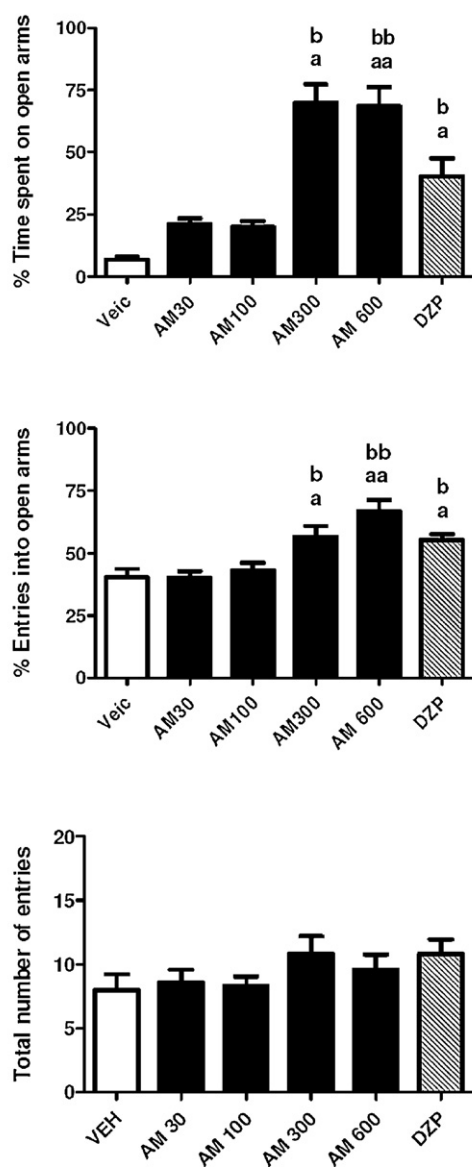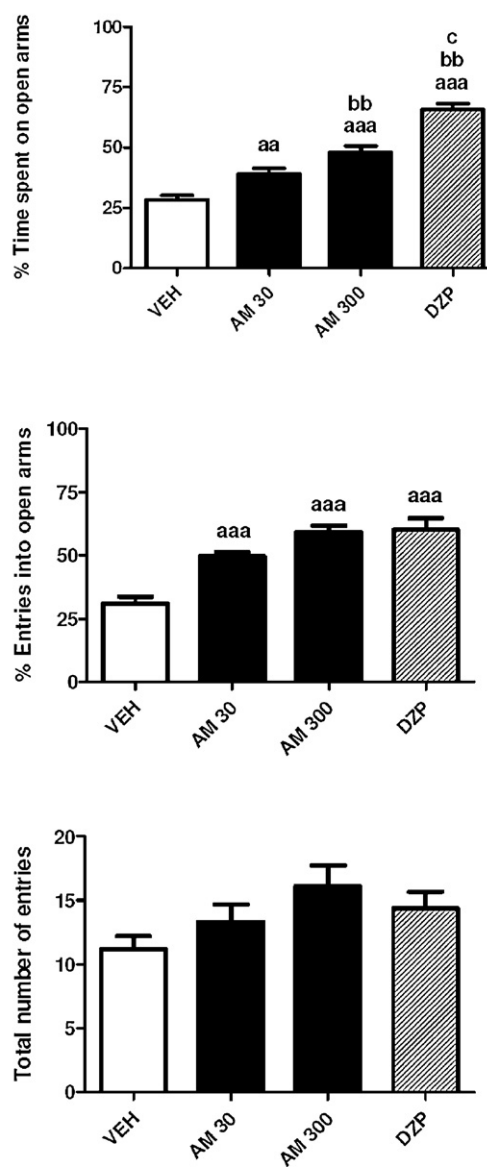Data are expressed as mean ± SEM; $n = 10$ mice/group. All treatments were administered orally.

Fig. 1. Effects of acute (left) and chronic (25 days; right) oral administration of the hydroalcoholic extract of *Achillea millefolium* (AM, 30–600 mg/kg) and diazepam (DZP, 0.75 mg/kg) on the percentage of time spent on the open arms, percentage of entries into the open arms, and total number of arm entries in 5 min in the elevated plus maze. Data are expressed as mean ± SEM ($n = 10$ mice/group). [a]$P < 0.05$, [aa]$P < 0.01$ and [aaa]$P < 0.001$, compared with vehicle; [b]$P < 0.05$ and [bb]$P < 0.01$ compared to AM 30 and AM 100 (acute) or AM 30 (chronic); [c]$P < 0.05$ compared to AM 300.
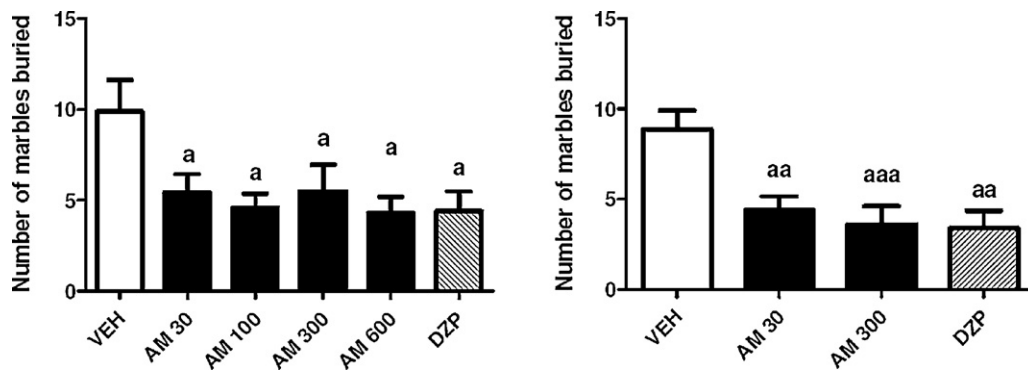
Fig. 2. Effects of acute (left) and chronic (25 days; right) oral administration of the hydroalcoholic extract of *Achillea millefolium* (AM, 30–600 mg/kg) and diazepam (DZP, 0.75 mg/kg) in the marble-burying test. A marble was considered buried when it was at least two-thirds buried with bedding material. Data are expressed as mean ± SEM (n = 10 mice/group). [a]P < 0.05, [aa]P < 0.01 and [aaa]P < 0.001 compared with vehicle.

number of total arm entries did not exhibit any differences after acute ($F_{5,54}$ = 1.20, P > 0.05) and chronic administration ($F_{3,36}$ = 2.33, P > 0.05).

3.2. Effects of acute and chronic administration of the hydroalcoholic extract of Achillea millefolium L. in the open-field test

In the open-field test, no difference was observed in locomotor activity (number of lines crossed) between groups (Table 1) after acute ($F_{3,36}$ = 2.02, P > 0.05) and chronic treatment ($F_{3,36}$ = 2.73, P = 0.057). Additionally, no difference was found in rearing and grooming after acute and chronic treatment (all Fs < 2.05, P > 0.10). No effect was found in the number of fecal boluses (acute: $H_{3,40}$ = 1.25, P > 0.05; chronic: $H_{3,40}$ = 2.54, P > 0.05).

3.3. Effects of acute and chronic administration of the hydroalcoholic extract of Achillea millefolium L. in the marble-burying test

In the marble-burying test (Fig. 2), effects were observed after acute ($F_{5,54}$ = 3.06, P < 0.05) and chronic treatment ($F_{5,54}$ = 7.52, P < 0.001). Acute *Achillea millefolium* (30, 100, and 300 mg/kg) and diazepam administration reduced the number of marbles buried compared with the vehicle group (all P < 0.05). Chronic *Achillea millefolium* (30 and 300 mg/kg) and diazepam administration also reduced marble-burying behavior (all P < 0.01).

3.4. Effects of picrotoxin and flumazenil pretreatment on the anxiolytic-like effect of acute administration of the hydroalcoholic extract of Achillea millefolium L. in the elevated plus maze and open-field test

Fig. 3 shows the effects of *Achillea millefolium* in the elevated plus maze after pretreatment with flumazenil and picrotoxin. Differences were observed between groups in the percentage of entries into the open arms ($F_{5,66}$ = 4.68, P < 0.01) and percentage of time spent in the open arms ($F_{5,66}$ = 10.10, P < 0.001). The groups treated with the *Achillea millefolium* extract exhibited a greater percentage of entries into and time spent on the open arms than the control groups (vehicle + vehicle and vehicle + flumazenil or picrotoxin; all P < 0.05), with the exception of the percentage of open arm entries between the vehicle + flumazenil and *Achillea millefolium* + flumazenil groups (P > 0.10; Fig. 4). A difference was also observed in the percentage of open arm entries between the *Achillea millefolium* + vehicle and *Achillea millefolium* + flumazenil groups (P < 0.01). The number of total arm entries was not different between groups ($F_{5,66}$ = 2.14, P > 0.05).
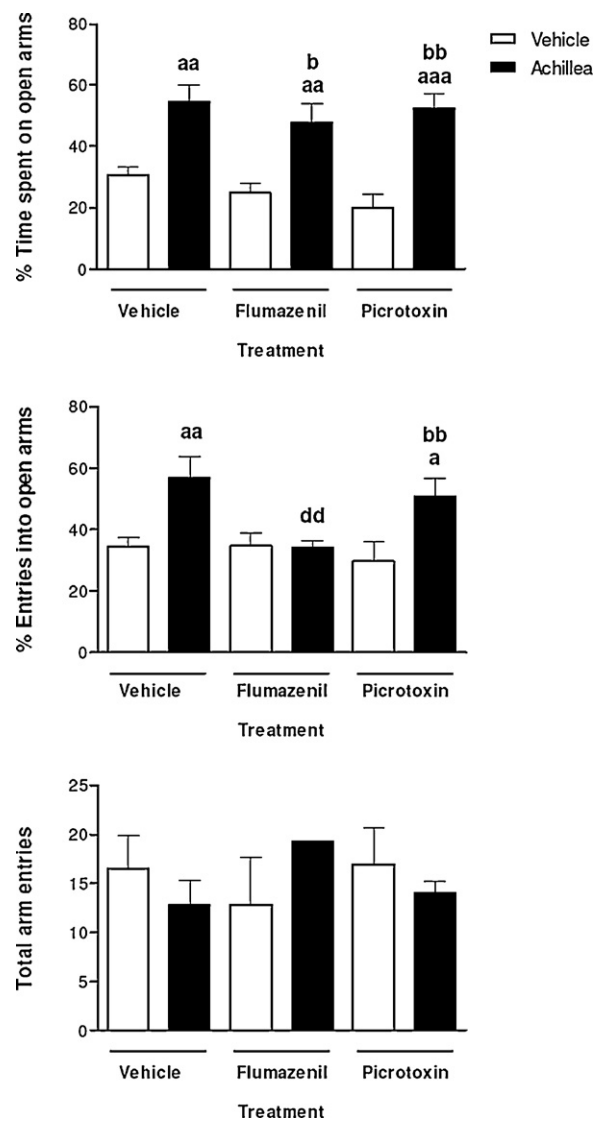

Fig. 3. Effects of acute oral administration of the hydroalcoholic extract of *Achillea millefolium* L. (300 mg/kg) following flumazenil (1.0 mg/kg, i.p.), picrotoxin (1.0 mg/kg, i.p.), or vehicle (i.p.) pretreatment on the percentage of time spent on the open arms, percentage of entries into the open arms, and total number of arm entries in 5 min in the elevated plus maze. Data are expressed as mean ± SEM (n = 12 mice/group). [a]P < 0.05, [aa]P < 0.01, [aaa]P < 0.001, compared with vehicle + vehicle; [b]P < 0.05, [bb]P < 0.01, compared with vehicle + flumazenil and vehicle + picrotoxin; [dd]P < 0.01, compared with *Achillea millefolium* + vehicle.

Table 2
Effects of acute pretreatment with flumazenil (1.0 mg/kg, i.p.) and picrotoxin (1.0 mg/kg, i.p.) on the effects of the hydroalcoholic extract of *Achillea millefolium* L. (300 mg/kg, p.o.) on open-field behavior in male mice.

	Locomotor activity (number of lines crossed)	Rearing (number)	Grooming time (s)
Vehicle + vehicle	48 ± 7	11 ± 3	9 ± 2
Achillea millefolium + vehicle	56 ± 4	14 ± 4	9 ± 3
Vehicle + flumazenil	35 ± 5	5 ± 1[*]	8 ± 1
Achillea millefolium + flumazenil	63 ± 8	13 ± 2	12 ± 2
Vehicle + picrotoxin	29 ± 8	2 ± 1[*]	6 ± 2
Achillea millefolium + picrotoxin	26 ± 7	4 ± 2[*]	4 ± 1[*]

Data are expressed as mean ± SEM; $n = 12$ mice/group.

[*] $P < 0.05$, compared with vehicle + vehicle.

In the open-field test (Table 2), changes were observed in locomotor activity ($F_{5,66} = 3.74$, $P < 0.01$) and rearing ($F_{5,66} = 6.67$, $P < 0.001$). A difference in locomotor activity was only seen between the *Achillea millefolium* + flumazenil and vehicle + picrotoxin and between the *Achillea millefolium* + flumazenil and *Achillea millefolium* + picrotoxin groups ($P < 0.03$). Rearing was reduced by vehicle + picrotoxin, vehicle + flumazenil, and *Achillea millefolium* + picrotoxin treatments compared with vehicle + vehicle, *Achillea millefolium* + vehicle, and *Achillea millefolium* + flumazenil treatments (all $P < 0.05$). No difference was seen in grooming ($F_{7,63} = 1.398$, $P > 0.05$) and defecation ($H_{7,63} = 0.68$, $P < 0.05$).

3.5. [³H]-flunitrazepam binding

The crude extract of *Achillea millefolium* (1–100 μg/ml) did not alter the specific binding of [³H]-flunitrazepam on membranes of rat brain in vitro (Fig. 4). Diazepam (10 μM) was used as a positive control and reduced [³H]-flunitrazepam-specific binding by 86 ± 1% (Fig. 4).

3.6. The effects of acute administration of Achillea millefolium, apigenin and diazepam in the elevated plus-maze and marble-burying test

The results of acute administration of apigenin in the elevated plus maze are shown in Fig. 5. One-way ANOVA indicated a difference between groups in the percentage of entries into the open arms ($F_{5,84} = 8.352$, $P < 0.0001$) and in the percentage of time spent in the open arms ($F_{5,84} = 19.11$, $P < 0.0001$). Both diazepam and the *Achillea millefolium* extract (300 mg/kg) increased the percentage of entries into open arms compared with vehicle (all $P < 0.05$). On the other hand, apigenin highest dose (3.0 mg/kg) decreased the percentage of entries into open arms when compared to other groups. *Achillea millefolium* extract (300 mg/kg) exhibited a higher percentage of time spent in open arms than other groups, including diazepam (all $P < 0.01$). Diazepam also increased time spent in
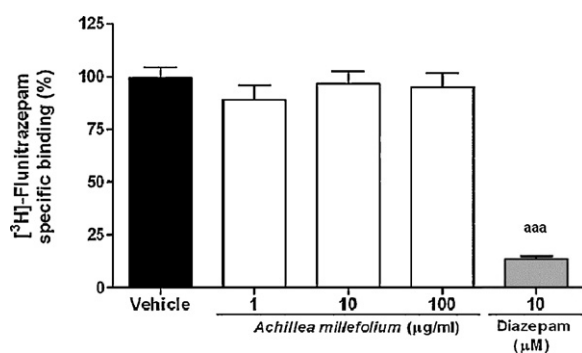

Fig. 4. Effects of the *Achillea millefolium* extract (1–100 μg/ml) and diazepam (10 μg/ml) on [³H]-flunitrazepam binding in rat brain membranes. Data are expressed as mean ± SEM ($n = 4$/triplicate). ᵃᵃᵃ$P < 0.001$, compared with vehicle.
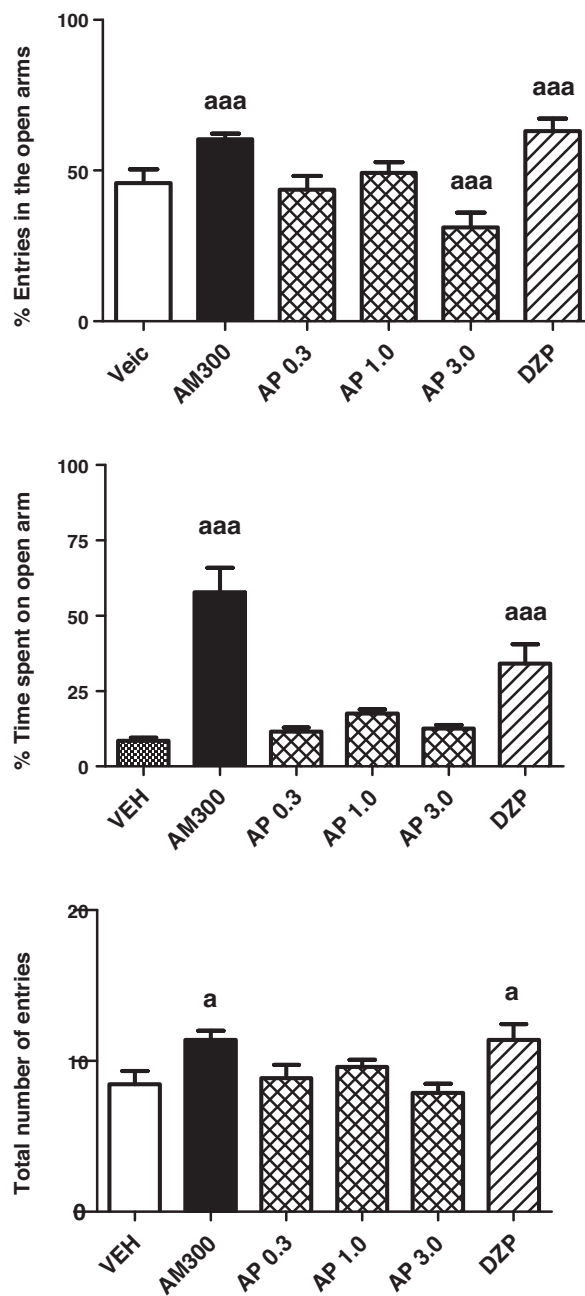

Fig. 5. Effects of acute oral administration of the hydroalcoholic extract of *Achillea millefolium* (AM, 300 mg/kg), diazepam (DZP, 0.75 mg/kg) or apigenin (AP, 0.1, 1.0, and 3.0 mg/kg) on the percentage of time spent on the open arms, percentage of entries into the open arms, and total number of arm entries in 5 min in the elevated plus maze. Data are expressed as mean ± SEM ($n = 15$ mice/group). ᵃ$P < 0.05$, and ᵃᵃᵃ$P < 0.001$, compared with vehicle.
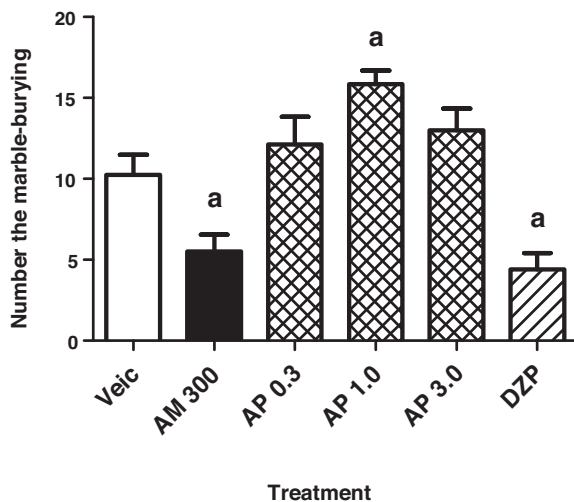
Fig. 6. Effects of acute oral administration of the hydroalcoholic extract of *Achillea millefolium* (AM, 300 mg/kg), diazepam (DZP, 0.75 mg/kg) or apigenin (AP, 0.1, 1.0, and 3.0 mg/kg) in the marble-burying test. A marble was considered buried when it was at least two-thirds buried with bedding material. Data are expressed as mean ± SEM ($n = 15$–14 mice/group). [a] $P < 0.05$ compared with vehicle.

open arms when compared to vehicle and apigenin groups. The number of total arm entries also exhibited a significant difference ($F_{5,54} = 3.84$, $P < 0.01$). *Achillea millefolium* extract (300 mg/kg) and diazepam increased the total number of arm entries when compared to vehicle group (both $P < 0.02$).

Similar results were seen in the marble-burying test (Fig. 6): diazepam and the *Achillea millefolium* extract (300 mg/kg) decreased the number of marbles buried compared to all other groups, while apigenin 1.0 mg/kg increased the number of marbles buried compared to vehicle ($F_{5,81} = 13.58$, $P < 0.0001$; all groups comparisons $P < 0.01$).

4. Discussion

The main findings of the present study were the anxiolytic-like effects of the hydroalcoholic extract of *Achillea millefolium* L. in validated animal models of anxiolytic drug screening (elevated plus maze and marble-burying test). The binding data showed that GABA/BDZ mechanisms do not mediate these effects. These results extended previous findings in female rats in an operant conflict procedure with an aqueous extract of *Achillea millefolium* L. flowers (Molina-Hernandez et al., 2004). Because the present animal models did not require water or food deprivation or shock delivery, the results also indicate that the anxiolytic-like effects of *Achillea millefolium* L. were not attributable to changes in appetitive behavior or pain sensitivity (Rodgers et al., 1997; Carobrez and Bertoglio, 2005), which can induce false-positive results in operant conflict procedures. This is an important issue because of the well-known assertion that *Achillea millefolium* exerts analgesic effects, which was partially confirmed by a reduction in writhing induced by acetic acid (Pires et al., 2009). Furthermore, the effect of the extract was not attributable to changes in locomotor activity because no changes was seen in the number of total arm entries in the elevated plus maze or in the number of lines crossed in the open-field test (Lister, 1987; de Castro et al., 2007; Raupp et al., 2008). This absence of locomotor impairment is consistent with Pires et al. (2009), whom also did not find locomotor activity or motor coordination alterations after administration of a hydroalcoholic extract of the aerial parts (with the exception of flowers) of *Achillea millefolium* L. The doses of 30 and 300 mg/kg *Achillea millefolium* extract in the present study correspond to human doses of 2.43 and 24.3 mg/kg,

respectively (Reagan-Shaw et al., 2008). Considering a person with 70 kg body weight, these doses correspond to 170 mg and 1.7 g, respectively. These doses are close to what is recommended by European Medicines Agency for oral use of *Achillea millefolium*, which is 2 g in 250 mL (infusion), once or twice daily (European Medicines Agency, 2011). Thus, the effective doses for anxiolytic-like effect found in the present study are similar to those used in traditional preparation. Interestingly, the anxiolytic-like effect of *Achillea millefolium* was seen in the elevated plus maze test after repeated treatment (25 days), suggesting the absence of tolerance to this effect after short-term administration, which is similar to diazepam.

The search for new anxiolytic drugs using ethopharmacological approaches attempts to find drugs with new mechanism of action. Benzodiazepine and GABA_A receptor antagonists (flumazenil and picrotoxin, respectively) have been used to evaluate the involvement of GABA/BDZ mechanisms in the anxiolytic-like effects of drugs (e.g., Reddy and Kulkarni, 1996; Dombrowski et al., 2006; Lolli et al., 2007; Duarte et al., 2008; Kamei et al., 2009). Picrotoxin did not alter the anxiolytic-like effects of *Achillea millefolium* in any of the anxiety measures in the elevated plus maze, whereas flumazenil blocked only the increase in the percentage of open arm entries. This effect of the combination of flumazenil plus *Achillea millefolium* extract in the elevated plus maze in the present study is puzzling because we would have expected that both open arm parameters would be the same as the pattern seen in several previous studies (e.g., Lolli et al., 2007), and both loaded heavily on the same factor in the factor analysis (e.g., Lister, 1987; Cruz et al., 1994; Rodgers and Johnson, 1995). However, one can see in the literature some studies that found a similar discrepancy (i.e., different sensitivity of percent open arm entries and percent time spent in the open arms) in response to specific drugs at certain doses, such as 20 mg/kg bretazenil and 10 mg/kg chlordiazepoxide (Cole and Rodgers, 1993), 10 mg/kg FG 7142 (Lister, 1987), and PK 8165 (Pellow and File, 1986). In this situation, the conclusion of the authors was an anxiolytic-like (Cole and Rodgers, 1993) or anxiogenic-like (Pellow and File, 1986; Lister, 1987) effect of the treatment. Furthermore, flumazenil occasionally did not reverse all of the effects of diazepam in the elevated plus maze (e.g., Duarte et al., 2008). The flumazenil plus *Achillea millefolium* extract results could be considered an anxiolytic-like effect because the percent time spent in the open arm was significantly greater than controls. The [³H]-flunitrazepam binding assay tested the putative action of the *Achillea millefolium* extract at the benzodiazepine binding site. The *Achillea millefolium* extract was not able to displace [³H]-flunitrazepam binding, whereas diazepam reduced [³H]-flunitrazepam-specific binding, suggesting that the *Achillea millefolium* extract does not have a central benzodiazepine-like action. However, although the behavioral and binding results would appear to indicate that GABA/benzodiazepine did not mediate the anxiolytic-like effect of the extract, this conclusion must be viewed carefully. Thus, the hypothesis deserves further study.

Altogether, these results suggest that the *Achillea millefolium* extract may act via a non-BDZ/γ-aminobutyric acid mechanism. This is an interesting finding since it differentiates *Achillea millefolium* from benzodiazepines, the most widely prescribed anxiolytic drug class, and even from other plants with anxiolytic effect (Houghton, 1999; Carlini, 2003; Lolli et al., 2007; Duarte et al., 2008; Benke et al., 2009). For example, flumazenil, at a dose that does not affect behavioral indices *per se*, blocked the anxiolytic-like effect of *Cymbopogon citratus* (i.e., increased the time spent in the light compartment in the light/dark test; Costa et al., 2011) and *Passiflora actinina* (i.e., increased the percent open arm time and entries in the elevated plus maze; Lolli et al., 2007).

Apigenin, in a dose range that corresponds to the content of 300 mg/kg *Achillea millefolium* extract (0.3 and 3.0 mg/kg

apigenin), did not exert any anxiolytic-like effect in the elevated plus maze or marble-burying test. These results did not corroborate some data from the literature that suggested an anxiolytic-like effect of 3.0 mg/kg apigenin in C F1 mice tested in the elevated plus maze (Viola et al., 1995). However, Zanoli et al. (2000) and Avallone et al. (2000) also did not observe any effect in the light/dark test (0.5–10 mg/kg, i.p.) and elevated plus maze (1–25 mg/kg, i.p.) in Sprague-Dawley rats. These contradictory results may be attributable to differences between animal models or species/strains. Furthermore, the intermediate dose of 1.0 mg/kg apigenin increased the number of marbles buried and the higher dose of 3.0 mg/kg apigenin decreased the percentage of entries into open arms, reflecting an anxiogenic-like effect. These results are consistent with the apigenin-induced decrease in the latency of picrotoxin-induced convulsions (Avallone et al., 2000) and apigenin-induced decrease in GABA-activated currents (Avallone et al., 2000). Under the same experimental conditions, apigenin did not reproduce the anxiolytic-like effect of *Achillea millefolium* extract, indicating that apigenin did not contribute to the anxiolytic-like effect of *Achillea millefolium* extract in the present study.

5. Conclusion

In conclusion, the present results indicate that the hydroalcoholic extract of the aerial parts of *Achillea millefolium* L. has anxiolytic-like effects. This effect did not present tolerance after short-term, repeated administration and is likely not mediated by GABA$_A$/BDZ receptors. Thus, *Achillea millefolium* L. may be a promising candidate for future development as a new anxiolytic drug.

Acknowledgments

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