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Central nervous system activities of two diterpenes isolated from *Aloysia virgata*

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ABSTRACT

Using the guide of a competitive assay for the benzodiazepine binding site in the γ-aminobutyric acid type A receptor ($GABA_A$), two active diterpenes were isolated from the aerial parts of *Aloysia virgata* (Ruíz & Pavón) A.L. Jussieu var. *platyphylla* (Briquet) Moldenke. These compounds, identified as (16*R*)-16,17,18-trihydroxyphyllocladan-3-one (**1**) and (16*R*)-16,17-dihydroxyphyllocladan-3-one (**2**) on the basis of spectral data, competitively inhibited the binding of [^{3}H]-FNZ to the benzodiazepine binding site with $K_i \pm$ S.E.M. values of 56 ± 19 μM and 111 ± 13 μM, respectively. The behavioral actions of these diterpenes, intraperitoneally (i.p.) administered in mice, were examined in the plus-maze, holeboard, locomotor activity and light/dark tests. Compound **1** exhibited anxiolytic-like effects in mice evidenced by a significant increase of the parameters measured in the holeboard test (the number of head dips at 0.3 mg/kg and 3 mg/kg, the rears at 1 mg/kg and the time spent head-dipping at 3 mg/kg), in the plus-maze assay (the percentage of open arm entries at 1 mg/kg) and in the light/dark test (the time in light and the number of transitions at 1 mg/kg). Compound **2** augmented the number of rearings in the holeboard apparatus (at 0.3 mg/kg and 1 mg/kg) and the locomotor activity (at 1 mg/kg). These results reveal the presence of neuroactive compounds in *Aloysia virgata*.

Introduction

Anxiety is an important adaptive mechanism vital to an organism's survival, but excessive anxiety can be very disabling, and symptoms of anxiety create immense socioeconomic burdens in modern society. Clinically, excessive anxiety often presents in the form of discrete disorders, which are very common and often comorbid with other psychiatric and medical illnesses (Liberzon et al. 2003).

Currently, two types of treatment are available for anxiety disorders, cognitive-behavioral therapy and pharmacologic agents. Beta blockers or benzodiazepines are used for time-limited and predictable anxiety disorders, whereas selective serotonin reuptake inhibitors, selective serotonin-norepinephrine reuptake inhibitors, tricyclic antidepressants, buspirone, or monoamine oxidase inhibitors are preferred for chronic or recurrent anxiety disorders (Pillay and Stein 2007). In recent years, studies using herbal remedies and supplements to treat mild to moderate anxiety disorders have emerged. Data support the effectiveness of some popular herbal remedies and dietary supplements; in some of these products the potential for benefit seems greater than that for harm with short-term use in patients with anxiety but there is a lack of rigorous studies in this area (Ernst 2006).

Several converging lines of evidence, from molecular, animal, and clinical studies have demonstrated that the γ-aminobutyric type A ($GABA_A$) receptor complex plays a central role in the modulation of anxiety. While currently available therapeutic agents that act on this receptor (e.g., benzodiazepines) are effective anxiolytics, they are limited by side effects, tolerance, and abuse potential (Lader 1999).

There is now an impressive array of natural products of plant origin that are known to influence the function of ionotropic receptors for GABA. The major chemical classes of such natural products are flavonoids, terpenoids, phenols and polyacetylenic alcohols (Johnston et al. 2006). The interaction of flavonoids with benzodiazepine modulatory sites on $GABA_A$ receptors led to the great interest in flavonoids as positive modulators of such receptors (Marder and Paladini 2002).

Aloysia virgata (Ruíz & Pavón) A.L. Jussieu var. *platyphylla* (Briquet) Moldenke, popularly named "niño rupá guasú" or "salvia guasú" in South America, is a medicinal plant used in folk medicine for a variety of indications, but there is no information concerning the effect of this plant on animal behavior. In Bolivia, Paraguay and Argentina it is popularly used as carminative, diaphoretic, stimulant, stomachic, tonic, anticatarrhal and antirheumatic (Bassols and Gurni 1996). Therapeutic action of other species of Aloysia (i.e., *Aloysia triphilla* and *Aloysia polystachya*) includes febrifuge, sedative, anxiolytic, stomachic, diuretic and antispasmodic activities (Hellión-Ibarrola et al. 2006).

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The present work was conducted to investigate the presence of neuroactive compounds in *Aloysia virgata*, using the guide of a competitive assay for the benzodiazepine binding site in the $GABA_A$ receptor. This study led to the isolation and identification of two diterpenes and their behavioral profiles are also described.

Materials and methods

Plant material

Aerial parts of *Aloysia virgata* (Ruíz & Pavón) A.L. Jussieu var. *platyphylla* (Briquet) Moldenke were collected in Caacupé, Departamento Cordillera, Paraguay. Its identification was carried out by Ing. G. Giberti at the Botany Museum of the School of Pharmacy and Biochemistry of Buenos Aires, where a voucher specimen has been deposited (number 9161).

High performance liquid chromatographies (HPLC) specifications

HPLC fractionations were performed using a LKB Pharmacia apparatus for analytical HPLCs and an ISCO apparatus, adapted for high liquid fluxes, to perform semi-preparative HPLCs. C18 reversed phase Vydac columns (The Separation Group, Hesperia, CAL, USA) were used for analytical (5 μm, 0.46 cm × 25 cm) and semi-preparative (5 μm, 1 cm × 25 cm) purposes. Each extract was properly injected into the column and eluted using an aqueous acetonitrile gradient. The flow rates for analytical and semi-preparative fractionations were 1 ml/min and 5 ml/min, respectively; and monitoring of the effluent was at 280 nm.

Biochemical assay ([^{3}H]-flunitrazepam binding assay)

A radioligand binding assay was used to evaluate the putative action of the extracts or the isolated compounds on the benzodiazepine binding site of the $GABA_A$ receptor complex. The binding of [^{3}H]-flunitrazepam ([^{3}H]-FNZ) (81.8 Ci/mmol; obtained from PerkinElmer Life and Analytical Sciences, Boston, MA, USA) to the benzodiazepine binding site in washed crude synaptosomal membranes from rat cerebral cortex was determined as previously described (Marder et al. 2003). In the competition assays, the incubations were done with [^{3}H]-FNZ 0.4 nM in the presence of 0.6–300 μM concentrations of (16*R*)-16,17,18-trihydroxyphyllocladan-3-one (**1**) and of 3–900 μM concentrations of (16*R*)-16,17-dihydroxyphyllocladan-3-one (**2**). Diazepam was used as positive control in concentrations between 1 nM and 100 nM. In saturation assays, increasing concentrations of [^{3}H]-FNZ (0.2–7.2 nM) were incubated in the presence of vehicle, compound **1** 100 μM or compound **2** 200 μM. Non-specific binding was measured in the presence of FNZ 10 μM and represented 5–15% of the total binding. After incubation, the assays were terminated by filtration under vacuum through Whatman GF/B glass-fiber filters followed by washing three times with 3 ml each of incubation medium. Individual filters were incubated overnight with scintillation cocktail (OptiPhase 'HiSafe' 3) before measuring radioactivity in a Wallac Rackbeta 1214 liquid scintillation counter.

Extraction and isolation of the active constituents

Dry aerial parts of *Aloysia virgata* (50 g) were powdered and suspended in 0.75 l of 70% ethanol and the mixture was kept 24 h at 37 °C, with stirring. The filtrate was concentrated in vacuo to remove the ethanol and then it was extracted with an equal volume of petroleum ether, which was discarded. The aqueous remaining phase was extracted three times with an equal volume of ethyl ether and this ether phase was evaporated to dryness in vacuo (yield 1.32 g). The aqueous remaining phase from this step yield 5.75 g. Samples of the ethyl ether phase and the aqueous remaining phase were submitted to the biochemical assay and only the ether extract showed the presence of ligands for the benzodiazepine binding site.

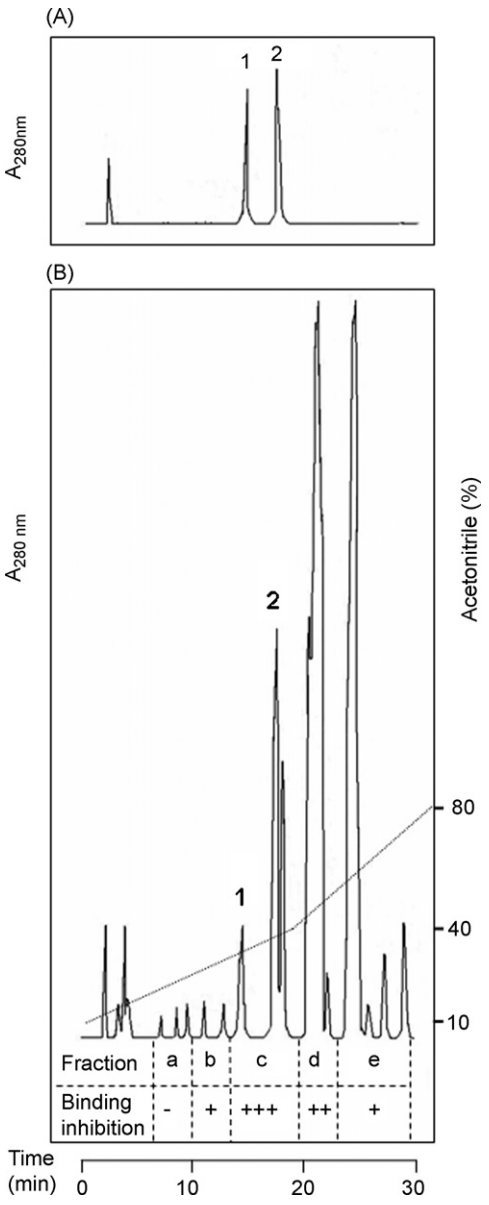
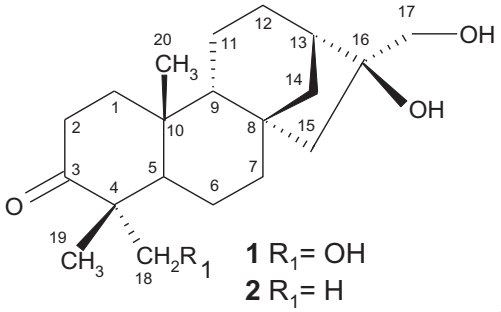


Fig. 1. Analytical HPLC chromatograms of: (A) the pure isolated compounds **1** and **2**; (B) the ethyl ether extract of *Aloysia virgata*. HPLC fractionations were performed as indicated in Materials and methods. For biochemical testing fractions **a**–**e** were recovered from a HPLC fractionation of 1 mg of the dry *A. virgata* ethyl ether extract. The capacity of the different fractions obtained to bind to the benzodiazepine binding site of the $GABA_A$ receptor is indicated as: inhibition > 80% (+++); inhibition 40–80% (++); inhibition 20–40% (+); and inhibition < 20% (−). The retention times of the active isolated compounds, **1** and **2**, were 15.3 min and 18.1 min, respectively.

Fig. 1 shows the results of an analytical HPLC of the ethyl ether extract phase. Fractions **a**–**e** were recovered for use in the *in vitro* benzodiazepine binding site binding assays. Medium to high affinity ligands were present in fraction **c**.

The isolation of the benzodiazepine binding site ligands from the ethyl ether extract of *Aloysia virgata* was performed by successive semi-preparative HPLC. After the evaporation of the solvent in vacuo, the active fractions were crystallized from water to give the isolated active compounds **1** (44 mg) and **2** (80 mg). These compounds showed a degree of purity of approx-

Table 1
^{1}H and ^{13}C NMR spectral data of compounds **1** and **2** in $CDCl_3$ (500 MHz).



Position	**1**		**2**	
	δ_C	δ_H (mult., *J*, Hz)[a]	δ_C	δ_H (mult., *J*, Hz)[a]
1	37.3	1.86 (*m*)	38.2	1.85 (*ddd*, 3.9, 7.1, 13.2)
		1.35 (*dd*, 3.2, 9.6)		1.42 (*m*)
2	35.1	2.57 (*m*)	34.0	2.49 (*ddd*, 7.3, 10.8, 15.8)
		2.25 (*ddd*, 2.8, 5.7, 16.5)		2.36 (*ddd*, 4.1, 10.8, 15.8)
3	219.7		217.8	
4	52.3		47.4	
5	48.6	1.85 (*m*)	55.3	1.36 (*m*)
6	21.6	1.31 (*m*)	21.3	1.38 (*m*)
		1.58 (*m*)		1.47 (*m*)
7	40.3	1.66 (*m*)	40.6	1.69 (*m*)
		1.55 (*m*)		1.53 (*m*)
8	43.3		43.5	
9	55.4	1.16 (*dd*, 4.6, 12.3)	55.8	1.13 (*dd*, 4.6, 12.3)
10	36.8		37.1	
11	20.8	1.25 (*m*)	19.7	1.3 (*m*)
		1.58 (*m*)		1.55 (*m*)
12	26.5	1.70 (*m*)	26.7	1.68 (*m*)
		1.52 (*m*)		1.46 (*m*)
13	43.7	2.03 (*m*)	44.0	1.92 (*d* br, 3.4)
14	47.9	2.06 (*m*)	48.1	2.08 (*ddd*, 2.3, 4.3, 11)
		1.06 (*d*, 11.2)		1.09 (*d*, 11.2)
15	44.4	2.03 (*m*)	44.6	2.05 (*dd*, 1.6, 14.6)
		1.21 (*d*, 14.7)		1.24 (*d*, 14,6)
16	84.3		84.3	
17	65.2	3.70 (*d*, 11)	65.6	3.76 (*d*, 11)
		3.54 (*d*, 11)		3.62 (*d*, 11)
18	66.8	3.55 (*d*, 11.2)	26.8	1.06 (*s*)
		3.31 (*d*, 11.2)		
19	17.1	1.02 (*s*)	21.6	1.00 (*s*)
20	14.7	0.92 (*s*)	14.5	0.97 (*s*)

[a] Assignments were made using HMQC and HMBC data.

imately 98%, based on analytical HPLC experiments. They were identify by UV (Shimadzu 160A spectrophotometer with the compounds dissolved in methanol) and ^{1}H and ^{13}C NMR spectrometry (500 MHz multinuclear Brukker Avance II 500 apparatus with the sample dissolved in chloroform-d_3); including ^{1}H,^{1}H-COSY; ^{13}C,^{1}H-HMQC; and ^{13}C,^{1}H-long-range (HMBC) experiments; as (16*R*)-16,17,18-trihydroxyphyllocladan-3-one (**1**) and (16*R*)-16,17-dihydroxyphyllocladan-3-one (**2**) (Table 1). Compound **1**: UV λ_{max} 206.5 nm and 270.5 nm. Compound **2**: UV λ_{max} 206.5 nm and 275 nm. Their structures, as well as their relative configuration were determined trough extensive spectrometric data analysis and by comparison with those reported in literature (Jaensch et al. 1990; Liu et al. 2003).

Animals

Adult male Swiss mice weighing 25–30 g were used in the pharmacological assays and adult male rats (200–300 g) Wistar strain for biochemical studies, both were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. For behavioral assays mice were housed in groups of five in a controlled environment (20–23 °C), with free access to food and water and maintained on a 12 h/12 h day/night cycle, light on at 06:00 AM, except for the light/dark assay where the light was on at 6:00 PM. Housing, handling, and experimental procedures complied with the recommendations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and CICUAL (Institutional Committee for the Care and Use of Laboratory Animals, University of Buenos Aires, Argentina). All efforts were taken in order to minimize animal suffering. The number of animals used was the minimum number consistent with obtaining significant data. The animals were randomly assigned to any treatment groups and were used only once. The behavioral experiments were performed between 10:00 AM and 2:00 PM.

Drugs solutions and experimental procedures

The compounds isolated from *Aloysia virgata*, flumazenil (Richet S.A., Argentina) and diazepam (Roche Diagnostics, Argentina) were dissolved by using the sequential addition of dimethylsulfoxide, a solution of 0.25% Tween 80 and saline; up to final concentrations of 5%, 20% and 75%, respectively. The mice were intraperitoneally (i.p.) injected 20 min before performing the pharmacological tests. In the

blockade experiments flumazenil was i.p. injected 35 min prior to testing. The volume of i.p. injections was 0.15 ml/30 g of the body weight. In each session, a control group receiving only vehicle was tested in parallel.

Behavioral studies

Holeboard assay

This assay was conducted in a walled black Plexiglass arena with a floor of 60 cm × 60 cm and 30 cm high walls, with four centered and equally spaced holes in the floor, 2 cm in diameter each as previously described (Fernández et al. 2006) and illuminated by an indirect and dimly light. Each animal was placed in the center of the holeboard and allowed to freely explore the apparatus for 5 min and the number of holes explored, the time spent head-dipping and the number of rearings were measured.

Elevated plus-maze test

The elevated plus-maze set-up consisted of a maze of two open arms, 25 × 5 cm, crossed by two closed arms of the same dimensions, with free access to all arms from the crossing point. The closed arms had walls 15 cm high all around. The maze was suspended 50 cm from the room floor. Mice were placed on the central part of the cross facing an open arm. The number of entries and the time spent going into open arms were counted during 5 min under red dim light. The total exploratory activity (number of entries in both arms) was also determined (Lister 1987).

Locomotor activity test

The spontaneous locomotion activity was measured in a box made of Plexiglass, with a floor of 30 cm by 15 cm and 15 cm high walls as previously described (Fernández et al. 2006). The locomotor activity was expressed as total light beam counts per 5 min.

Light/dark transition test

The light/dark box consisted of a fully automated Plexiglass box monitored by computer with two compartments, distinguished by wall color, illumination and size; one light area (30 cm long × 21 cm wide × 21 cm height) illuminated by a 60-W light (400 lx) in the ceiling of the compartment and with white walls, and a smaller dark compartment (14 cm long × 21 cm wide × 21 cm height) with black walls and not illuminated. An opening door (6 cm × 3 cm) located in the center of the partition at floor level connected the two compartments.

The model is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior in response to novel environment and light. The experiments were performed between 10:00 AM and 2:00 PM, in the middle of the dark phase. Animals were placed in the center of the dark area facing the wall opposite to the door. To reduce any neophobic response to the test situation, the light–dark compartments are previously dirty with mice other than those used during the test. Mice are always tested in a soiled apparatus, and there is no cleaning between trials. The following parameters were recorded during 5 min: (1) latency time of the first crossing to the light compartment, (2) the number of crossings between both compartments and (3) the total time spent in the illuminated zone of the cage (Bourin et al. 1992).

Statistical analyses

For the competition binding, data were analyzed by nonlinear regression of specific bound vs radioligand concentration. Regressions for two (full model) and one (reduced model) binding site(s) were further compared by the extra sum-of-squares *F* test. According to the best fit model, the affinity constant (K_d) and number of binding sites (B_{max}) were estimated and compared (GraphPad Prism version 5.0® software). K_i values were calculated using the Cheng-Prusoff/Chou equation: $K_i = IC_{50}/[1 + (L/K_d)]$, where K_i refers to the inhibition constant of the unlabeled ligand, IC_{50} is the concentration of unlabeled ligand required to reach half-maximal binding, K_d refers to the equilibrium dissociation constant of the radioactive ligand and L refers to the concentration of radioactive ligand. For saturation binding, data were fitted by nonlinear regression using the equation $y = (B_{max})\cdot(x)/(K_d + x)$, where y is specifically bound [^{3}H]-ligand in dpm, B_{max} is maximal binding, and x is the concentration of [^{3}H]-ligand.

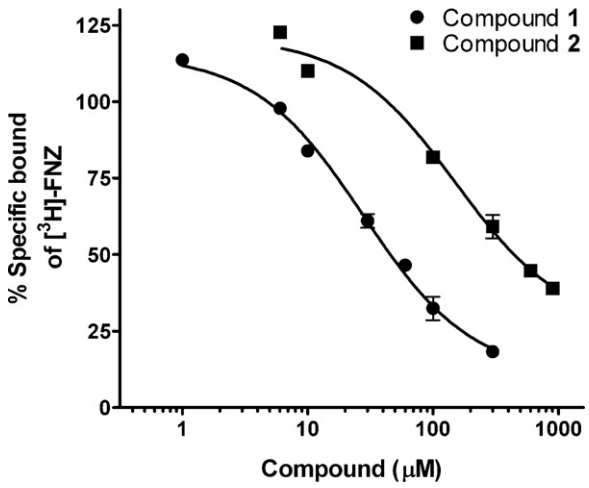


Fig. 2. Competition curves of compounds **1** and **2** for [^{3}H]-FNZ binding to washed crude synaptosomal membranes of the rat cerebral cortex. Mean ± S.E.M. values from a representative experiment performed in duplicate and replicated four times are shown.

The effects of the compounds in mice were analyzed by one-way analysis of variance (ANOVA) and post hoc comparisons between treatments and vehicle were made using Dunnett multiple comparison test. The blockade experiments were analyzed by one-way analysis of variance (ANOVA) and post hoc comparisons between individual treatments were made using Newman–Keuls multiple comparison test. Significance levels were set at $P < 0.05$.

Results

*Effects of compounds **1** and **2** on [^{3}H]-FNZ binding*

Compounds **1** and **2** inhibited the binding of [^{3}H]-FNZ to the benzodiazepine binding site with K_i ± S.E.M. of 56 ± 19 μM and 111 ± 13 μM, respectively ($n = 4$) (Fig. 2). Concentrations higher than 0.9 mM were not evaluated due to the limited solubility of the compounds. Diazepam, used as a positive control, gave a $K_i = 0.0070 \pm 0.0005$ μM ($n = 5$), as expected (Marder and Paladini 2002). In all cases, data obtained were best fitted to one site binding hyperbola. Scatchard plot analysis of saturation curves (Fig. 3) showed that both the compound **1** 100 μM and **2** 200 μM competitively inhibited the binding of [^{3}H]-FNZ with a significant decrease in [^{3}H]-FNZ binding apparent affinity (K_d) [$F(2,22) = 12.44$, $P = 0.0002$] and no change in maximal binding (B_{max}) [$F(2,22) = 0.89$, $P = 0.4270$]. K_d ± S.E.M. and B_{max} ± S.E.M. values for vehicle and compound **1** were 0.80 ± 0.18 nM and 2.29 ± 0.16 pmol/mg protein, and 2.43 ± 0.50 nM and 2.61 ± 0.21 pmol/mg protein, respectively; while the values for compound **2** were 5.39 ± 0.49 nM and 2.53 ± 0.12 pmol/mg protein.

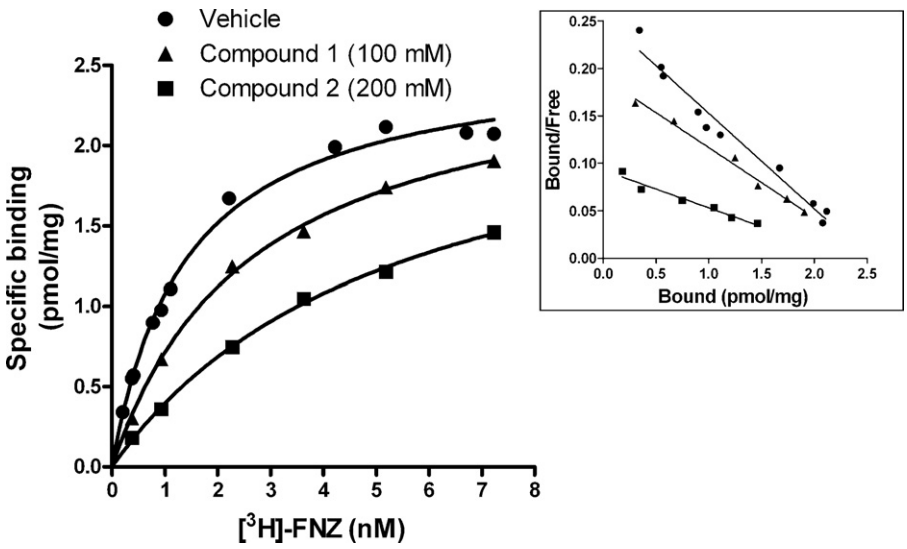


Fig. 3. Effects of compound **1** (100 μM) and **2** (200 μM) on [^{3}H]-FNZ binding to washed crude synaptosomal membranes of the rat cerebral cortex. Saturation isotherms and Scatchard plot (inset) of [^{3}H]-FNZ (0.1–7.2 nM) are shown. Data were fitted to one site binding hyperbolas and the apparent affinity constant (K_d) and the number of binding sites (B_{max}) were estimated. Data are from a representative experiment done in duplicate and replicated three times.

*Behavioral effects of diterpenes **1** and **2** in the holeboard and the locomotor activity tests*

The effect of compound **1** in the hole board and locomotor activity tests is shown in Fig. 4. ANOVA indicated a significant effect on the number of holes explored [$F(5,64) = 8.03$, $P < 0.0001$], the time spent head-dipping [$F(5,64) = 5.86$, $P = 0.0002$], the number of mouse rearings [$F(5,64) = 4.45$, $P = 0.0015$] and the locomotor activity counts [$F(5,68) = 3.19$, $P = 0.012$]. Comparisons between the vehicle control group and experimental groups (Dunnett's procedure) indicated that compound **1** increased the number of head dips and the time spent head-dipping at 0.3 mg/kg ($P < 0.05$) and 3 mg/kg ($P < 0.001$). Meanwhile, at 1 mg/kg, the number of rearings was augmented ($P < 0.01$). A similar behavior could be observed with mice treated with diazepam at 0.3 mg/kg (number of head dips and time spent head-dipping, $P < 0.05$) and 1 mg/kg (number of rearings $P < 0.05$). Locomotor activity of mice was not modified by compound **1** at the doses tested (Fig. 4B). Diazepam, instead, increased locomotor activity at 1 mg/kg ($P < 0.05$).

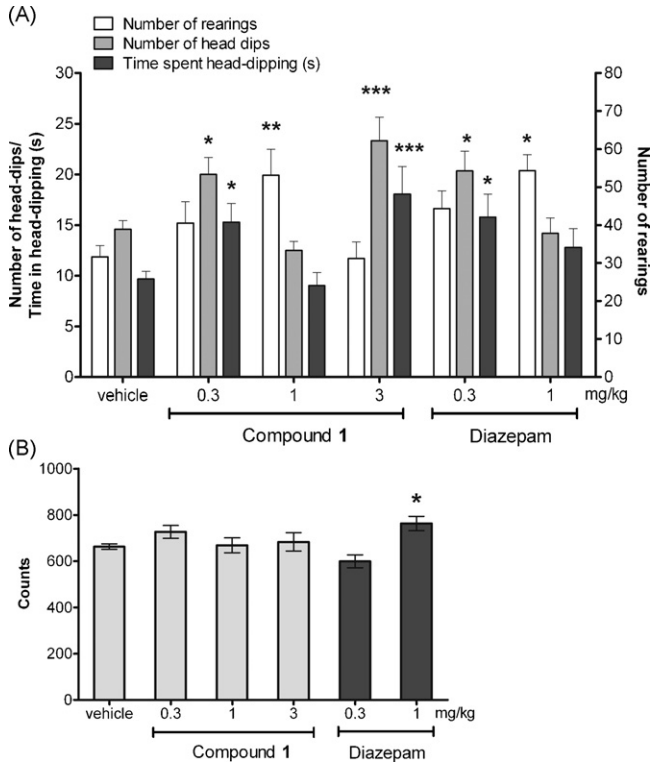


Fig. 4. Effect of the i.p. injection of compound **1** and diazepam in the holeboard and locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of (A) the holeboard parameters and (B) spontaneous locomotor activity counts; registered in 5 min sessions. The symbols denote significance levels: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different from vehicle; Dunnett's multiple comparison test after ANOVA ($n = 6$–30 mice/group).

The results of the holeboard and locomotor activity assays of compound **2** are presented in Fig. 5. In this case ANOVA indicated a significant effect on the number of mouse rearings [$F(4,41) = 4.73$, $P = 0.0031$] and the locomotor activity counts [$F(4,47) = 4.44$, $P = 0.004$], but the number of holes explored [$F(4,41) < 1$, $P = 1.00$] and the time spent head-dipping [$F(4,41) = 1.80$, $P = 0.1464$] were unaffected. Control and experimental groups compared with Dunnett's procedure revealed that diterpene **2** significantly increased the number of rearings at 1 mg/kg and 3 mg/kg ($P < 0.05$, Fig. 5A) and the locomotor activity of mice at 1 mg/kg ($P < 0.01$, Fig. 5B).

*Effects of compounds **1** and **2** in the plus-maze test*

The effect of compound **1** in the plus-maze test is shown in Fig. 6. ANOVA of the results obtained yielded statistically significant differences in the percentage of open arms entries [$F(4,60) = 5.82$, $P = 0.0005$], percentage of time spent in open arms [$F(4,60) = 4.53$, $P = 0.0029$], total entries [$F(4,60) = 8.85$, $P < 0.0001$] and closed entries [$F(4,60) = 3.75$, $P = 0.0087$]. Compound **1**, at 1 mg/kg, significantly increased the percentage of open arm entries compared to control mice (Dunnett's procedure, $P < 0.01$, Fig. 6A). The number of closed arm entries and the total arm entries were not modified by compound **1** at the doses tested (Fig. 6B). Diazepam (1 mg/kg) augmented the percentage of open arm entries ($P < 0.001$), the percentage of time spent in open arms ($P < 0.001$), the number of closed arm entries ($P < 0.01$) and the total arm entries ($P < 0.001$).

Compound **2** failed to significantly modify the parameters evaluated in this assay (Fig. 7A and B).

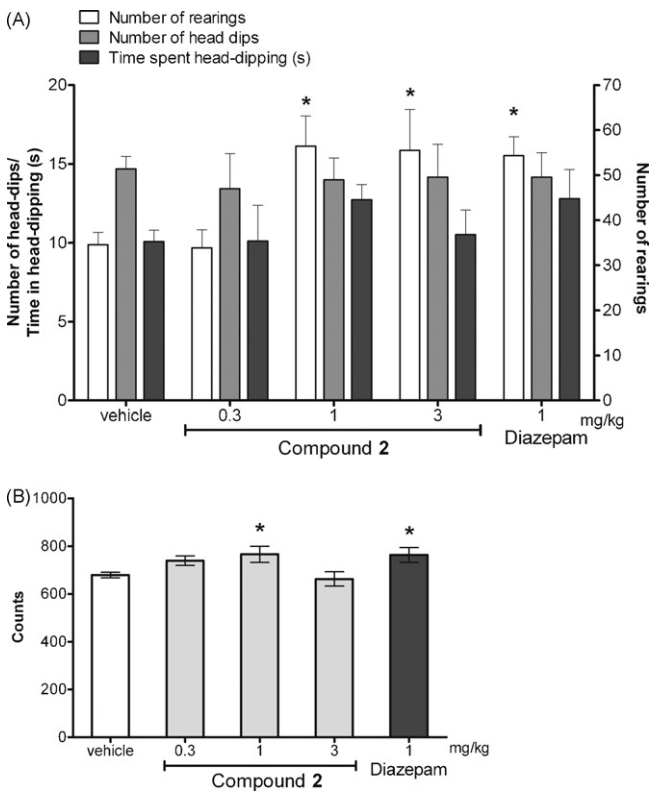


Fig. 5. Effect of the i.p. injection of compound **2** and diazepam in the holeboard and locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of (A) the holeboard parameters and (B) spontaneous locomotor activity counts; registered in 5 min sessions. The symbols denote significance levels: $^{*}P < 0.05$ significantly different from vehicle; Dunnett's multiple comparison test after ANOVA (n = 6–20 mice/group).

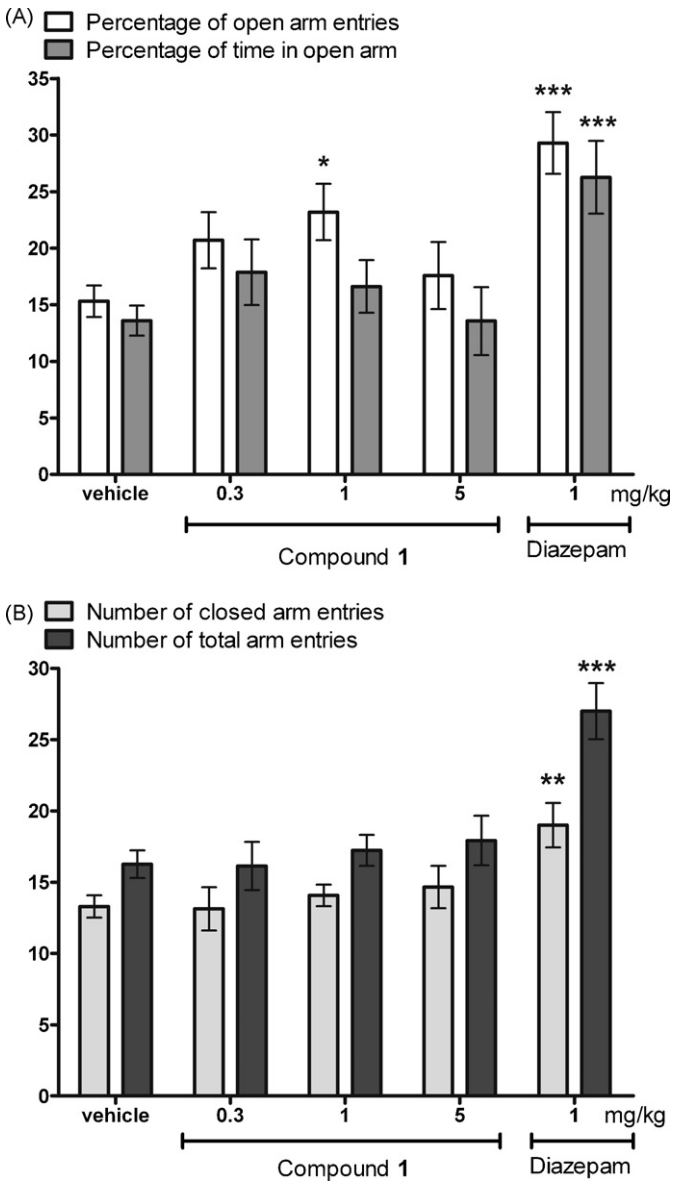


Fig. 6. Effect of the i.p. injection of compound **1** and diazepam in the plus-maze test in mice. Results are expressed as (A) percentages (mean ± S.E.M.) of open arm entries and time spent in open arms; (B) number of closed arm entries and total arm entries; registered in 5 min sessions, 20 min after an i.p. injection of vehicle or the drugs. The symbols denote significance levels: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ significantly different from vehicle; Dunnett's multiple comparison test after ANOVA (n = 7–23 mice/group).

*Anxiolytic effect of compound **1** in the light/dark transition assay*

In further to corroborate the anxiolytic activity of diterpene **1** observed in the holeboard and plus-maze tests we decided to use the light/dark assay (Table 2). In this assay ANOVA indicated a significant effect on the time spent in the light area [$F(2,25) = 8.87$, $P = 0.0012$], the number of inter-compartment transitions [$F(2,25) = 23.54$, $P < 0.0001$] and on the latency time to enter into the light area [$F(2,25) = 4.35$, $P = 0.0258$]. Comparisons between the vehicle control group and experimental groups (Dunnett's test) indicated that compound **1**, at 1 mg/kg, significantly increased the time spent in the light area and the number of transitions (both $P < 0.05$). Diazepam, at 0.3 mg/kg, significantly decreased the latency to cross to the light area ($P < 0.05$) and augmented the time spent in the light area and the number of transitions (both $P < 0.001$).

*Effects of the administration of flumazenil on the anxiolytic activity of diterpene **1** in the holeboard, plus-maze and the locomotor activity tests*

Flumazenil (5 mg/kg, i.p.), a specific antagonist for the benzodiazepine binding site of the $GABA_A$ receptor, showed no significant effect by itself in the holeboard, locomotor activity and plus-maze tests. A pre-treatment of mice with flumazenil (5 mg/kg) totally reverted the anxiolytic-like effect of compound **1**, at the dose of 3 mg/kg for the number of head dips [$F(3,37) = 5.95$, $P = 0.0020$] and the time spent head-dipping [$F(3,37) = 5.84$, $P = 0.0023$] in the holeboard test ($P < 0.01$, $P < 0.05$, respectively, significantly different from diterpene **1**, Newman–Keuls multiple comparison test). The percentage of open arm entries of mice injected with 1 mg/kg of diterpene **1** in the plus-maze assay was also significantly affected by the pre-treatment of mice with flumazenil (5 mg/kg) [$F(3,37) = 4.96$, $P = 0.0054$] ($P < 0.05$, significantly different from diterpene **1**, Newman–Keuls multiple comparison test) (Table 3).

Discussion

The presence of diterpenoids in *Aloysia virgata* was previously reported. These compounds were named as "hoffmanniaketone" and its "monoacetate", identified by comparison of their NMR spectra with those reported in the literature. Unfortunately, no spectral data was shown in the article (de Oliveira et al. 2005). "Hoffmanniaketone" was isolated for the first time from *Hoffmania strigillosa*, together with its 17-(acetyloxy) derivative, and

Table 2
Effects of the administration of diterpene **1** and diazepam on the light/dark transition test in mice.

Groups	Doses (mg/kg)	Latency time (s)	Transition number	Time spent in light area (s)
Vehicle	–	167.90 ± 30.61	6.33 ± 2.04	46.44 ± 17.44
Diazepam	0.3	60.88 ± 19.67[a]	29.38 ± 2.40[b]	146.60 ± 12.25[b]
Diterpene **1**	1	99.27 ± 23.39	14.00 ± 2.35[a]	110.90 ± 17.16[a]

Diterpene **1** and diazepam were given i.p. 20 min prior the test. Values represent mean ± S.E.M.
[a] $P < 0.05$ drug vs. control groups (Dunnett's test after ANOVA), $n = 8–11$.
[b] $P < 0.001$ drug vs. control groups (Dunnett's test after ANOVA), $n = 8–11$.

originally assigned the structure of (16*R*)-16,17-dihydroxy-*ent*-phyllocladan-3-one using chiroptical data (Jaensch et al. 1990). Later, Liu and co-workers (2003) made a thorough spectral comparison between this compound isolated from *Hoffmania strigillosa* with calliterpenone and found these compounds to be identical.

In the present work, through a bioassay-guided fractionation procedure, two neuroactive diterpenes were isolated from the aerial parts of *Aloysia virgata*. These compounds were identified by ^{1}H and ^{13}C NMR spectrometry, as (16*R*)-16,17,18-trihydroxyphyllocladan-3-one (**1**) and (16*R*)-16,17-dihydroxyphyllocladan-3-one (**2**) (calliterpenone), according to Liu and co-workers (2003). Phyllocladane diterpenoids isolated from the plant *Callicarpa macrophylla* Vahl (Verbenaceae) have been found to promote plant growth and alleviate the effects of growth retardant allelochemicals. These novel activities pave the utility of these compounds as natural plant growth promoters and for alleviation of the growth retarding effects produced by allelochemicals. In particular, the effect of calliterpenone was observed in *Mentha arvensis*, which is a source of commercial menthol (Haider et al. 2009). No neuroactive effects were reported for these kinds of diterpenes.

In this work, compounds **1** and **2** exhibited a moderate affinity for the benzodiazepine binding site of the $GABA_A$ receptor, with $K_i \pm$ S.E.M. values of 56 ± 19 μM and 111 ± 13 μM, respectively. In the saturation experiments, the ability of these diterpenes to modify the K_d without any significant change in the B_{max} suggests that these compounds interact competitively at this site. The $GABA_A$ receptor is a member of the ligand gated ion channel superfamily and GABA is the major inhibitor transmitter in the central nervous system. Binding of GABA to the $GABA_A$ receptor activates a chloride ion flux through the channel and ligands for the benzodiazepine binding site modulate the inhibitory effects of GABA (Wang et al. 1999). Such ligands for the benzodiazepine binding sites are classified as positive allosteric modulators, antagonists or negative allosteric modulators according to their spectrum of intrinsic efficacy toward the $GABA_A$ receptor (Gardner et al. 1993). Positive allosteric modulators increase the frequency of chloride channel openings without altering channel conductance or duration of openings. Therapeutically, they are used as anxiolytics, anticonvulsants, sedatives, hypnotics and muscle relaxants. In order to investigate the *in vivo* effects of compounds **1** and **2**, their possible central activities were studied in several behavioral animal models, such as the elevated plus-maze, holeboard and light/dark tests. Separately from the other experiments spontaneous locomotor activity was measured automatically.

Many animal models of anxiety examine the natural behavioral patterns of mice and rats to develop ethologically based behavioral tasks. These include 'approach–avoidance' tasks in which animals are exposed to an aversive/threatening environment e.g. open, elevated arms of the elevated plus-maze, light arena (light/dark exploration test), and open field tests (holeboard assay), with anxiety-like behavior in each case, inferred from increased avoidance. Other models include social interaction tests, punishment-based conflict procedures, defensive burying tests, etc. (Cryan and Holmes 2005).

In preliminary studies of the light/dark transition test in which mice were placed in the center of the lit area, we obtained disperse results with control and diazepam (the reference drug) treated mice. So, in the present study, animals were placed in the center of the dark area, where we found that mice demonstrated a stable response.

In our study, compound **1** at 0.3 and 3 mg/kg significantly increased the number of head dips and the time spent head-dipping in the holeboard test. Head-dipping behavior is sensitive to changes in the emotional state of the animal, and it has been suggested that the expression of an anxiolytic state may be reflected by an increase in head-dipping behavior (Takeda et al. 1998). On the other hand, compound **1** showed its anxiolytic-like effect in mice in the plus-maze test, at 1 mg/kg, by an increase in the percentage of open arm entries. Meanwhile, in the light/dark transition test, the same dose of this compound significantly increased the time spent in the light area and the number of transitions, providing further evidence for its anxiolytic-like effect. Locomotor activity of mice was not modified by compound **1** at the doses tested.

Diazepam has been used as a typical anxiolytic and has been frequently employed in behavioral pharmacology as a reference compound for potentially anxiolytic-acting substances. In the present work it was found that diterpene **1** was as effective as diazepam in the holeboard test, at 0.3 mg/kg and 1 mg/kg, and displayed a striking anxiolytic effect at 3 mg/kg; whereas, in the

Table 3
Effects of the administration of flumazenil on the anxiolytic activity of diterpene **1** in mice.

Groups	Locomotor activity (counts, $n = 8–15$)	Hole board test ($n = 8–14$)		Plus-maze test ($n = 6–17$)			
		Number of rearings	Number of head dips	Time spent head-dipping (s)	Number of closed arms entries	Percentage of open arms entries	Percentage of time in open arms
Vehicle	688 ± 27	30.5 ± 5.7	14.1 ± 1.2	7.7 ± 0.8	15.5 ± 1.0	13.8 ± 1.8	14.5 ± 2.0
Flumazenil	687 ± 27	41.1 ± 5.3	16.6 ± 2.0	10.8 ± 1.2	16,5 ± 1.2	18.5 ± 3.3	19.1 ± 4.5
Diterpene **1**	688 ± 29	29.0 ± 3.4	24.2 ± 2.7[a]	14.5 ± 1.8[a]	13,5 ± 1.4	25.4 ± 2.4[a]	17.8 ± 2.6
Flumazenil/diterpene **1**	601 ± 30	27.3 ± 4.3	13.7 ± 2.2[b]	8.6 ± 1.5[c]	15,7 ± 1.6	15.9 ± 3.5[c]	17.8 ± 4.0

Flumazenil (5 mg/kg) was i.p. administered 15 min before the i.p. injection of vehicle or diterpene **1**. Diterpene **1** (3 mg/kg for the hole board and locomotor activity tests, and 1 mg/kg for the plus-maze assay) and vehicle were i.p. injected 20 min prior the tests. Values represent mean ± SEM. Newman–Keuls multiple comparison test after ANOVA.
[a] $P < 0.01$, significantly different from vehicle.
[b] $P < 0.01$, significantly different from diterpene **1**.
[c] $P < 0.05$, significantly different from diterpene **1**.

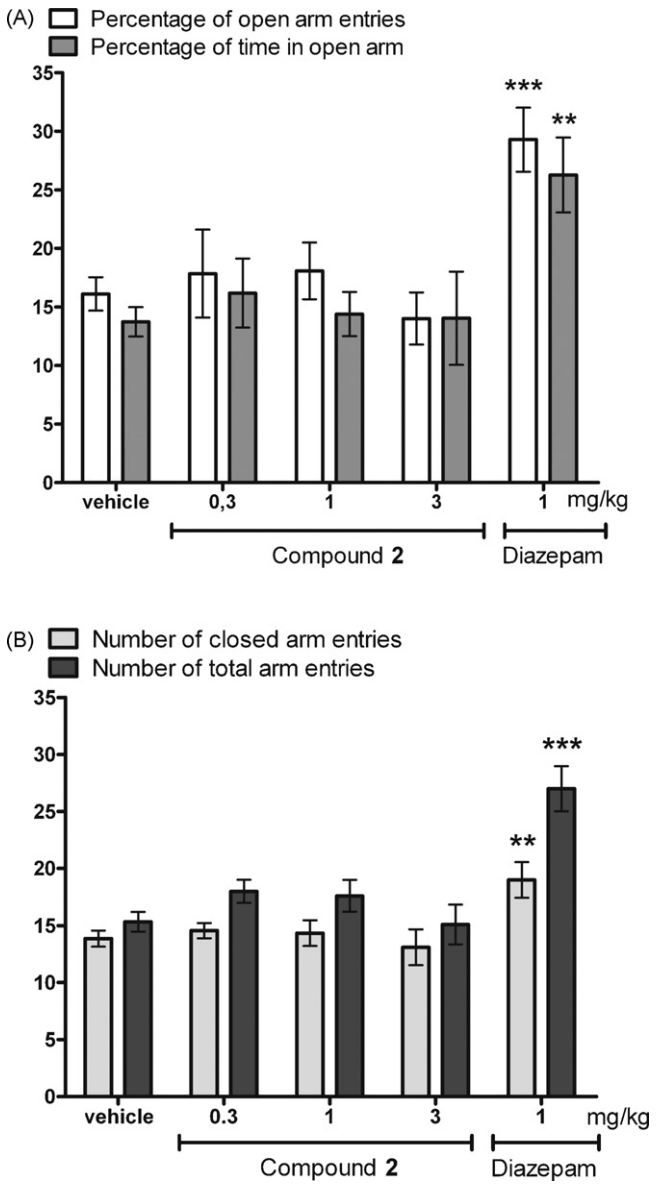


Fig. 7. Effect of the i.p. injection of compound **2** and diazepam in the plus-maze test in mice. Results are expressed as (A) percentages (mean ± SEM) of open arm entries and time spent in open arms; (B) number of closed arm entries and total arm entries; registered in 5 min sessions, 20 min after an i.p. injection of vehicle or the drugs. The symbols denote significance levels: $^{***}P < 0.001$, $^{**}P < 0.01$ significantly different from vehicle; Dunnett's multiple comparison test after ANOVA ($n = 10–27$ mice/group).

plus-maze and light dark assays, compound **1** showed a moderate anxiolytic effect. Although the affinity for the central benzodiazepine binding site of this diterpene is low, its anxiolytic-like effect would be mediated via this site, as flumazenil, a specific central benzodiazepine binding site blocker, totally reverted its action *in vivo*. While the responses in these tests at specific doses were robust and statistically significant, they did not observe a graded dose–response relationship. Complex plant derivatives can contain multiple pharmacophores and can have multiple modes of action, so it is possible that other mechanisms are engaged at different doses of diterpene **1**.

Although the structures of diterpenes **2** and **1** are very similar, the lack of one hydroxyl group in position 18 in compound **2** seems to confer a different pharmacological profile on this compound. Interestingly, diterpene **2** displayed higher horizontal and vertical activities of mice. This compound, at 0.3 mg/kg and 1 mg/kg significantly increased the number of rearings and augmented the spontaneous locomotor activity of mice at 1 mg/kg. While compound **2** exerted an increase locomotor behavior, it failed to modify the parameters measured in the plus-maze test at the doses tested. As no bioavailability studies were made, it should be considered that these diterpenes may be metabolized to active derivatives that could be the responsible of the biological activity observed *in vivo*.

There are many psychotropic drugs and psychotherapies available for the treatment of patients with anxiety disorders, but overall clinical outcomes and the standard of care for most patients are far from optimal. An increasing number of herbal products have been introduced into psychiatric practice in the past decade. There are also a large number of herbal medicines whose therapeutic potential has been assessed in a variety of animal models, and whose mechanisms of actions have been investigated through neurochemical approaches. These studies have provided useful information for the development of new pharmacotherapies from medicinal plants for use in clinical psychiatry (Zhang 2004).

To summarize, the data presented here indicate, for the first time, the presence of neuroactive phyllocladane diterpenoids in *Aloysia virgata*. One of these diterpenes (**1**) exerted an anxiolytic effect in mice evidenced in the holeboard, plus-maze and light/dark tests, which may be mediated by an interaction with the benzodiazepine binding site of the $GABA_A$ receptor. Meanwhile, diterpene **2** displayed higher horizontal (locomotion) and vertical (rearings) activities i.e. increased novelty-induced behavioral activation of mice.

Once again, natural products seem to be interesting sources of biologically active constituents that exhibit central nervous system effects.

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