Phytomedicine 18 (2011) 393–401

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

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

Cristina Wasowski, Mariel Marder*

Instituto de Química y Fisicoquímica Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 (C1113AAD), Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Aloysia virgata
Phyllocladane diterpenoids
Anxiety
$GABA_A$ receptor
Benzodiazepine binding site ligand

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).

* Corresponding author. Tel.: +54 11 4964 8289; fax: +54 11 4962 5457.
E-mail address: mmarder@qb.ffyb.uba.ar (M. Marder).

0944-7113/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.phymed.2010.08.017

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.

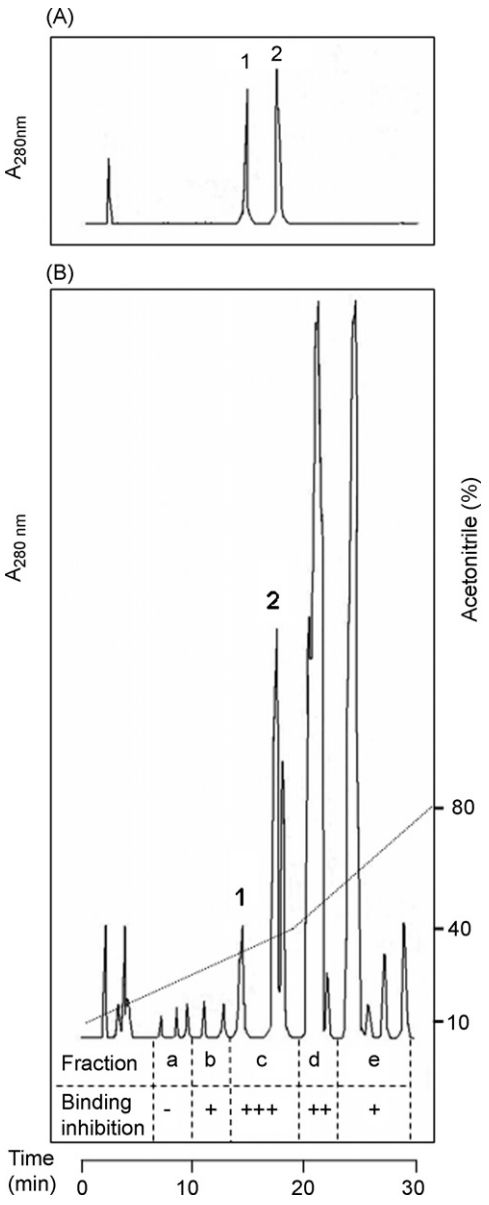

Fig. 1. Analytical HPLC chromatograms of: (A) the pure isolated compounds **1** and **2**; (B) the ethyl ether extract of *Aloysia virgata*. HPLC fractionations were performed as indicated in Materials and methods. For biochemical testing fractions **a**–**e** were recovered from a HPLC fractionation 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-