© 2023 Journal of Pharmacy & Pharmacognosy Research, 11 (3), 367-380, 2023
ISSN 0719-4250
https://jppres.com

DOI: https://doi.org/10.56499/jppres22.1570_11.3.367

Original Article

Iridoid esters from *Valeriana pavonii* Poepp. & Endl. as $GABA_A$ modulators: Structural insights in their binding mode and structure-activity relationship

[Ésteres iridoides de *Valeriana pavonii* Poepp. & Endl. como moduladores $GABA_A$: Perspectivas estructurales de su modo de unión y relación estructura-actividad]

Sara E. Giraldo[1,†], Mauricio Bedoya[2,3,†], Carlos Peña-Varas[4,5], Paula A. Santana[6], Isabel L. Bazzocchi[7], Ignacio A. Jiménez[7], Mariel Marder[8], Nadezdha E. Vergel[9], Mario F. Guerrero[9], David Ramírez[4*]

[1]Escuela de Ciencias Básicas y Aplicadas, Universidad de La Salle, 111711, Bogotá, Colombia.
[2]Centro de Investigación de Estudios Avanzados del Maule (CIEAM), Vicerrectoría de Investigación y Postgrado, Universidad Católica del Maule, Talca 3466706, Chile.
[3]Laboratorio de Bioinformática y Química Computacional (LBQC), Departamento de Medicina Traslacional, Facultad de Medicina, Universidad Católica del Maule, Talca 3466706, Chile.
[4]Departamento de Farmacología, Facultad de Ciencias Biológicas, Universidad de Concepción. Concepción, Chile.
[5]Departamento de Ciencias de la Computación, Facultad de Ingeniería, Universidad de Concepción, Concepción, Chile.
[6]Facultad de Ingeniería, Instituto de Ciencias Químicas Aplicadas, Universidad Autónoma de Chile, Santiago, Chile.
[7]Instituto Universitario de Bio-Orgánica Antonio González, Departamento de Química Orgánica, Universidad de La Laguna, Avenida Astrofísico Francisco Sánchez 2, 38206 La Laguna, Tenerife, Canary Islands, Spain.
[8]Universidad de Buenos Aires. Consejo Nacional de Investigaciones Científicas y Técnicas. Instituto de Química y Fisicoquímica Biológicas Prof. Dr. Alejandro C. Paladini, Facultad de Farmacia y Bioquímica, Junín 956 (C1113AAD), Buenos Aires, Argentina.
[9]Departamento de Farmacia, Facultad de Ciencias, Universidad Nacional de Colombia, 111321, Bogotá, Colombia.
†These authors equally contributed to this work.
*E-mail: dramirezs@udec.cl

Abstract

Context: *Valeriana pavonii* Poepp. & Endl. (*Caprifoliaceae*), is a plant used in traditional medicine as a tranquilizer in Colombia. Valerian extracts have been widely used since ancient times for their sedative and anxiolytic properties; however, the way its active metabolites, including iridoids, interact on their respective targets is not fully understood.

Aims: To isolate and identificate active iridoid esters from *V. pavonii*. Perform *in vitro* inhibition assays and computational analyses to study their possible interaction on the benzodiazepine site of the $GABA_A$ receptor.

Methods: Two compounds were obtained from dichloromethane and petroleum ether fractions of *V. pavonii*, respectively, by chromatographic techniques. The structural elucidation was performed by NMR and spectroscopic analyses. *In vitro* inhibition assays of the binding of ^{3}H-flunitrazepam (^{3}H-FNZ) for the benzodiazepine binding site of the $GABA_A$ receptor (BDZ-bs of the $GABA_A$ receptor) were carried out.

Results: Two iridoid esters, hydrine-type valepotriates (compounds **1** and **2**), were reported for the first time in *V. pavonii*. Both iridoids, **1** and **2**, inhibited the binding of ^{3}H-FNZ on the BDZ-bs of the $GABA_A$ receptor (40% at 300 µM). Docking studies and MMGBSA calculations revealed that these compounds exhibited molecular interactions with crucial residues of the benzodiazepine site, similar to those observed for drugs like flunitrazepam, diazepam, and flumazenil.

Conclusions: These findings contribute to understanding the *in vivo* activity of extracts of *Valeriana pavonni* on the central nervous system, which showed promising effects, especially as anticonvulsants, sedative-hypnotics, and antidepressants, through the modulation of the GABAergic system by hydrine-type valepotriates and its derivatives.

Keywords: ensemble molecular docking; iridoids; $GABA_A$ modulators; $GABA_A$ receptor; valerian.

Resumen

Contexto: *Valeriana pavonii* Poepp. & Endl. (*Caprifoliaceae*), es una planta utilizada en la medicina tradicional como tranquilizante en Colombia. Los extractos de valeriana han sido ampliamente utilizados desde la antigüedad por sus propiedades sedantes y ansiolíticas; sin embargo, la forma como sus metabolitos activos, entre ellos los iridoides, interactúan sobre sus respectivas dianas no es del todo conocida.

Objetivos: Aislar e identificar ésteres iridoides activos de *V. pavonii*. Realizar ensayos de inhibición *in vitro* y análisis computacionales para estudiar su posible interacción en el sitio benzodiazepínico del receptor $GABA_A$.

Métodos: Se obtuvieron dos compuestos a partir de fracciones de diclorometano y éter de petróleo de *V. pavonii*, respectivamente, mediante técnicas cromatográficas. La elucidación estructural se realizó mediante RMN y análisis espectroscópicos. Se llevaron a cabo ensayos de inhibición *in vitro* de la unión del ^{3}H-flunitrazepam (^{3}H-FNZ) al sitio de unión de las benzodiacepinas del receptor $GABA_A$ (BDZ-bs del receptor $GABA_A$).

Resultados: Dos ésteres iridoides, valepotriatos de tipo hidrina (compuestos **1** y **2**), fueron reportados por primera vez en *V. pavonii*. Ambos iridoides, 1 y 2, inhibieron la unión de ^{3}H-FNZ en el BDZ-bs del receptor $GABA_A$ (40% a 300 µM). Los estudios de docking y los cálculos MMGBSA revelaron que estos compuestos presentaban interacciones moleculares con residuos cruciales del sitio de la benzodiazepina, similares a las observadas para fármacos como el flunitrazepam, el diazepam y el flumazenil.

Conclusiones: Estos hallazgos contribuyen a comprender la actividad *in vivo* de extractos de *Valeriana pavonni* sobre el sistema nervioso central, los cuales mostraron efectos prometedores, especialmente como anticonvulsivantes, sedantes-hipnóticos y antidepresivos, a través de la modulación del sistema GABAérgico por valepotriatos de tipo hidrina y sus derivados.

Palabras Clave: acoplamiento molecular en conjunto; iridoides; moduladores $GABA_A$; receptor $GABA_A$; valeriana.

ARTICLE INFO
Received: December 23, 2022.
Accepted: April 21, 2023.
Available Online: May 11, 2023.

AUTHOR INFO
ORCID: 0000-0001-6652-8869 (SEG) 0000-0002-0641-607X (IAJ)
0000-0002-3542-7528 (MB) 0000-0003-0190-598X (MM)
0000-0003-2741-8813 (CPV) 0000-0002-6092-5094 (NEV)
0000-0002-5742-4926 (PAS) 0000-0003-1789-1818 (MFG)
0000-0002-7698-2704 (ILB) 0000-0003-0002-1189 (DR)

INTRODUCTION

The GABA receptor family is one of the main families implicated in the mechanism of action of drugs that behave as central nervous system depressants. The GABA receptor family, also known as GABA (gamma aminobutyric acid) activated heteropentameric chloride channels, belongs to the superfamily of ligand-activated ion channels. GABA, the main inhibitory neurotransmitter of the central nervous system, exerts its physiological effects by binding to three different types of receptors on the neuronal membrane: $GABA_A$, $GABA_B$, $GABA_C$ ($GABA_A$-rho). When activated, either by an endogenous ligand or by an agonist, the $GABA_A$ receptor exerts an inhibitory effect, hyperpolarizing the neuron (the permeability of the membrane to chloride ions is increased) and reducing the likelihood of an action potential occurring (Savage et al., 2018). GABA type A receptor subunits are encoded by 19 different genes that have been grouped into eight subclasses according to sequence homology (α_1–6, β_1–3, γ_1–3, δ, ε, θ, π, ρ1–3). The combination of subunits in the most abundant receptor subtypes in the brain represent combinations of 2α and 2β subunits, together with a single γ 2 or δ subunit. GABA type A receptors can be allosterically modulated by benzodiazepines, barbiturates, steroids, anaesthetics, anticonvulsants, and many other drugs, by binding to allosteric sites on the receptors, modulating the flux of chloride ions induced by GABA (Wasowski and Marder, 2012).

Different studies based on preclinical *in vitro* and animal models and supported by clinical evidence have also demonstrated the effects that active metabolites isolated from plants, including alkaloids, terpenoids and flavonoids, have exerted on the GABAergic pathway, acting as allosteric modulators (on $GABA_A$ or $GABA_B$ receptors) or as enzymatic modulators (inhibition of GABA transaminase, GABA-T or glutamic acid decarboxylase, GAD) (Savage et al., 2018; Wasowski and Marder, 2012).

Recently, studies about the isolation of metabolites from different species of the *Valeriana* genus (formerly *Valerianaceae*, now *Caprifoliaceae*) are carried out to evaluate their mechanism of action and their potential pharmacological use in the treatment of different diseases, especially those related to the central nervous system. Valeriana genus comprises about 200 species, however, *Valeriana officinalis* L. ("European valeriana") is the best known and studied (Patočka and Jakl, 2010).

About *Valerian* species, valerenic acid and valerenol, sesquiterpenes found in *V. officinalis* roots, have been shown to act as positive allosteric modulators for the benzodiazepine binding site of the $GABA_A$ receptor (BDZ-bs of the $GABA_A$ receptor) through the modulation of $GABA_A$ receptors containing the β_3 subunit (Benke et al., 2009). Among the natural monoterpenoids are iridoids called valepotriates (valeriana-epoxy-triesters) in species of the *Valerian* genus (Bos et al., 2002).

A wide variety of iridoids isolated from the species of the genus *Valeriana* have shown, among others, neuroprotective (Tan et al., 2016), cytotoxic, antibacterial (Liu et al., 2017), antioxidant (Wang et al., 2017), and more recently anti-inflammatory (Tang et al., 2022; Wang et al., 2020) and antiviral effects (Quan et al., 2022). However, there are few studies related to their anticonvulsant effects.

In Colombia, the stems of *Valeriana pavonii* Poepp. & Endl. (*V. pavonii*) are used in traditional medicine as a tranquilizer, especially for insomnia and anxiety problems. *V. pavonii* is a plant with long stems, liana type, that grows wildly in different regions of Colombia (Garcia, 1992). Currently, alcoholic extracts of *V. pavonii* are used for the manufacture of herbal products in Colombia (called phytotherapeutics), formulated as sedatives, and also for anxiety and sleep disorders. According to previous phytochemical studies by Thin Layer Chromatography (TLC), the stems were characterized by containing terpenoid, alkaloid, and flavonoid-type compounds (Parra, 2003). However, through pharmacological screening studies on the central nervous system, the alkaloid, dichloromethane, and petroleum ether fractions were the most active, from which isovaleramide and iridoid esters (hydrine-type valepotriates) were isolated, as described below.

In previous studies it was shown that the alkaloidal fraction from *V. pavonii* presented anticonvulsant effects in a maximum electroshock-induced seizure model (MES) (100 to 400 mg/kg, p.o., in mice), and antidepressant effects in a behavioural despair test (100 mg/kg, p.o., in mice); while hypnotic effects at 500 mg/kg (p.o., in mice) in the model of sleep induction by sodium pentobarbital were observed with the ethanolic extract (Arévalo et al., 2006; Celis et al.,

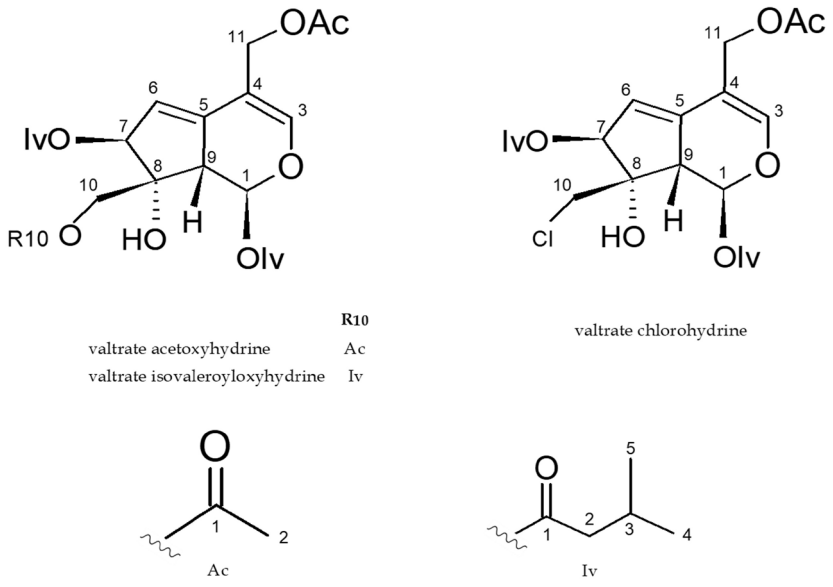

Figure 1. Hydrine-type valepotriates isolated from anticonvulsant dichloromethane fraction of *V. pavonii*.

Ac: acetyl, Iv: isovaleryl. (Giraldo et al., 2013).

2007). It was reported in subsequent tests that the dichloromethane fraction obtained from the ethanolic extract and the petroleum ether fraction obtained from the methanolic extract of *V. pavonii*, exhibited anticonvulsant effects, achieving a protection index around 90% in MES, at doses of 35 and 65 mg/kg, p.o., respectively. Biological activity was attributed to the presence of alkaloid and iridoid-type metabolites detected by TLC in these fractions (Giraldo et al., 2008).

Subsequently, isolation and identification studies of active metabolites were performed: Isovaleramide, a metabolite isolated from active alkaloid fraction, showed a significant effect achieving a protection index around 90% in MES (100 mg/kg, p.o. in mice) (Giraldo et al., 2010). Moreover, three already known iridoid esters (hydrine-type valepotriates) reported for the first time in this species, valtrate acetoxyhydrine, valtrate isovaleroyloxyhydrine, and valtrate chlorohydrin, were isolated from dichloromethane fraction, which had exhibited anticonvulsant activity (Fig. 1). Binding assays showed that the molecular mechanism of these compounds was not fully related to the BDZ-bs of the $GABA_A$ receptor (Giraldo et al., 2013).

Although a wide variety of iridoid esters-type metabolites have been isolated from different species of the genus *Valeriana*, to date there are no computational studies of these metabolites to explain their possible binding mode on the $GABA_A$ receptor. Here, the isolation of two other iridoid esters from the stems of *V. pavonii* is reported, along with *in vitro* assay of binding to the BDZ site of the $GABA_A$ as well as computational analysis to describe their binding mode. For this purpose, molecular modeling studies on the $GABA_A$ receptor were performed and the interactions at the benzodiazepine binding site are described. Different in-house scripts were developed to automatically run ensemble docking simulations in different $GABA_A$ receptor conformations (extracted from molecular dynamics simulations), and a KNIME workflow to analyze how the sampled conformations of the studied compounds interact at the binding site. These results contribute to gain structural insights about how active metabolites such as iridoids, isolated from species of the *Valerian* genus modulate the GABAergic system, which is involved in central nervous system disorders such as epilepsy, insomnia, and anxiety.

MATERIAL AND METHODS

Plant material

V. pavonii stems were collected in the rural area of the San Antonio del Tequendama municipality from department of Cundinamarca (1549 m, Colombia), 4°37'1''N (latitude) and 74°21'0''W (longitude). Botanical identification was carried out at the Herbario Nacional Colombiano of the Universidad Nacional de Colombia and two voucher specimens (Col 495179 and Col 495756) were deposited as indicated in previous studies (Celis et al., 2007).

Extraction and isolation

The ethanolic extract of *V. pavonii* (EE), was obtained from the stems by solid-liquid extraction at room temperature and drying by evaporation under reduced pressure, while the dichloromethane fraction (DF) was obtained from the EE according to previous studies (Giraldo, et al., 2013). The petroleum ether fraction of *V. pavonii* (PEF) was obtained from the methanolic extract (ME) as described below. Dry and crushed stems of *V. pavonii* (about 750 g) were subjected to liquid-solid extraction at room temperature with methanol (1:4). Three extraction processes of 24 h each were carried out. The solvent was removed by evaporation under reduced pressure, obtaining the crude methanolic extract (122.2 g). To obtain the iridoids the following protocols were followed (Nishiya et al., 1994). A quantity of methanolic extract (80 g) were dissolved in water and subjected to successive extractions with petroleum ether, ethyl acetate, and butanol, then by evaporation at reduced pressure the respective dry extracts were obtained. The fractions in ethyl acetate and butanol were not included in this study. Under this procedure, 9.9 g of PEF was obtained.

Compound **1** was obtained from dichloromethane fraction of *V. pavonii* (DF) according to the previous report (Giraldo, et al., 2013), as follows: column chromatography with silica gel 60 (0.063-0.200 mesh, Merck®), column chromatography with sephadex LH-20 (20-100 mm, Sigma Aldrich®) and flash column chromatography (silica gel, 254 nm / TLC, Machery Nagel®). Solvents of increasing polarity were used. Initially partially purified fractions were obtained, which according to their follow-up in TLC (eluent: $CHCl_3$:MeOH, 9.8:0.2, iridoids specific staining reagent: HCl:AcOH 8:2), were combined in smaller groups of fractions. One of these fractions (207.0 mg) was purified by preparative thin layer chromatography (SIL-G-100/UV 254 nm, 1 mm, Macherey Nagel®), eluting with a solution of n-hexane:Et_2O (1:1), subsequently preparative thin layer chromatography (TLC nano, silica gel 60/UV 254 nm, 0.25 mm, Macherey Nagel®) was used eluting with a solution of Toluene-EtOAc (8:2), obtaining compound **1**: Light yellow viscous liquids; (2.4 mg, 0.02% with respect to DF); $[\alpha]^{20}_D$ +93.53 (*c* 0.17, $CHCl_3$); IR cm^{-1} (KBr) 3498 cm^{-1} (hydroxyl group), 1737 cm^{-1} (carbonyl ester group), 1611 and 1643 cm^{-1} (diene double bond characteristics) (Fig. S2). The structural elucidation was performed by one-dimensional (^1H, ^{13}C, DEPT135, DEPT90) and two-dimensional NMR experiments (COSY, HSQC, HMBC and ROESY) (Figs. S1, S4-S9) and comparison of their spectral data with those reported in the literature (Table S1).

Compound **2** was obtained from PEF, as follows: 5.9 g of the fraction was purified by column chromatography (silica gel 60, 0.063-0.200 mesh, Merck®) eluting with a solution of n-hexane:$CHCl_3$:MeOH in gradient. Initially, 82 major fractions were obtained, which subsequently, according to their follow-up by TLC (eluent: $CHCl_3$:MeOH, 9.8:0.2, iridoids specific staining reagent: HCl:AcOH 8:2), were joined in 14 groups of fractions (A-N). The pooled fraction (E-G), corresponding to 941.0 mg, was purified by column chromatography (silica gel 60, 0.063-0.200 mm Merck®) eluting with a gradient solution of $CHCl_3$:MeOH, obtaining 17 groups of fractions as monitored by TLC. (EG1-EG17). The EG8 fraction (124 mg) was purified by preparative thin layer chromatography (silica gel 60, HF 254 + 366/TLC Merck®) using as eluent a $CHCl_3$:MeOH (9.85:0.15) solution, obtaining compound **2**: Dark yellow viscous liquid (17.0 mg, 0.3% with respect to PEF); $[\alpha]^{20}_D$ +49.94 (*c* 0.39, MeOH); IR cm^{-1} (KBr) 3477 cm^{-1} (hydroxyl group), 1737 cm^{-1} (carbonyl ester group), 1612 cm^{-1} and 1644 cm^{-1} (diene double bond) (Fig. S12). The structural elucidation was performed by one-dimensional (^1H, ^{13}C, DEPT135, DEPT90) and two-dimensional NMR experiments (COSY, HSQC, HMBC and ROESY) (Figs. S11, S14-S19) and comparison of their spectral data with those reported in the literature (Table S2).

Experimental techniques

Spectroscopic studies were carried out at the Instituto Universitario de Bio-Orgánica Antonio González (La Laguna University) and at the Universidad Nacional de Colombia by IR (HP/FT-IR 1600 Perkin Elmer® and FTIR Bruker® IF 55) and UV (UV/VIS Spectrophotometer V-560 Jasco Corporation®) analysis. The optical rotation results were determined by Polarimeter 343 Perkin Elmer®. Structural elucidation was performed by one-dimensional (^1H, ^{13}C, DEPT135, DEPT90) and two-dimensional NMR experiments (COSY, HSQC, HMBC and ROESY), recorded in $CDCl_3$ (chloroform-d, 99.8%, Sigma-Aldrich®) or MeOD (Methanol-d4, 99.8%, Scharlau®) in Bruker DRX® 400 MHz and Bruker Avance® 400 and 600 MHz equipment. The chemical shifts (δ) were reported in ppm relative to the internal standard of tetramethylsilane (TMS) and the coupling constants (*J*) in Hz. The mass and molecular formula were confirmed by electronic impact using low and high mass spectrometry resolution (HREIMS) (VG Micromass ZAB-2F and Micromass Autospec).

In vitro assay of binding to the BDZ site of the $GABA_A$ receptor

Adult male Wistar rats weighing 200–300 g, used for binding assays, were obtained from the Central Animal House of the School of Pharmacy and Biochemistry, University of Buenos Aires. Housing, handling, and experimental procedures complied with the recommendations and regulations set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Guide for the Care and Use of Laboratory Animals, 2011) and the Institutional Committees for the Care and Use of Laboratory Animals of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina (CICUAL, protocol's approved code numbers: CUDAP: EXP-FYB N_: 0058084/2015, N_ CICUAL FFyB: 02052016-63).

This test was carried out evaluating the inhibition caused by the compounds isolated from *V. pavonii* (300 µM) of the binding of tritiated flunitrazepam (^{3}H-FNZ) to $GABA_A$/BDZ-bs (81.8 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) in washed crude synaptosomal membranes from rat cerebral cortex, which contained 0.2 - 0.4 mg of protein. The samples were incubated at 4°C for 60 min with 0.4 nM ^{3}H-FNZ according to the previous report (Giraldo et al., 2013). For each compound, the test was performed in triplicate. The displacement of the ^{3}H-FNZ binding to BDZ-bs was determined through a liquid scintillation counter according to previously described procedures. Values are expressed as mean ± S.E.M (Marder et al., 2003; Wasowski et al., 2002).

Computational studies

Human $\alpha_1\beta_2\gamma_2$ $GABA_A$ cryo-electron microscopy structure (PDB code: 6D6U) was used for molecular dynamics simulation and molecular docking. Conformation A was chosen due there is no evidence about the difference between both conformations (A and B) reported for this channel. In addition, the transmembrane domain of the γ_2-subunit in conformation B, was comparatively more disordered than the rest of the receptor (Zhu et al., 2018).

To study how both compounds **1** and **2** interacts with $GABA_A$ receptor, a systematic pipeline including molecular dynamics simulations, massive docking and binding free energy calculations was employed. This pipeline has been successfully used to study how different ligands interacts with membrane proteins (Ramírez et al., 2017**;** 2019). Briefly, one hundred different $GABA_A$ conformations were obtained from 100-ns molecular dynamics simulation (MDs) applying restrictions on the protein backbone (force constant 0.5 kcal × mol^{-1} × Å^{-2}), then both compounds **1** and **2** were docked into the extracted $GABA_A$ conformations. The relative binding free energy of the obtained poses was calculated using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) method. Finally, clustering analysis of all the poses was performed to find the most likely interacting conformations. The most populated clusters and the most energetically favorable pose regarding MM-GBSA energy of each cluster were selected to describe the binding mode. Initially a different molecular dynamics protocol was explored, 500 ns of molecular dynamics simulation without applying any restriction. However, it was observed that the binding site was closed and a validation by re-docking the flumazenil ligand was not achieved with this approach.

Molecular Dynamics simulation (MDs)

$GABA_A$ crystal was prepared using the protein preparation wizard module of Maestro suite (Jacobson et al., 2002; 2004; Wizard, 2019). The missing residues in chain E (233-236, and 287-291) were completed using "crosslink protein" tool in Maestro. The co-crystallized ligand flumazenil at the binding site was removed, and its binding site was used as a reference in this study (holo-like). Protonation states of amino acids were assigned at pH 7.0 with PROPKA (Olsson et al., 2011). The protein was embedded into a pre-equilibrated POPC bilayer and solvated using the TIP3P water model. A total of 27 chloride ions were added to neutralize the system and then, the ion concentration was set to 0.15 M NaCl.

The Desmond membrane relaxation protocol (consisting of 6 stages) was used. The first stage consists of a 100 ps Brownian dynamics in an NVT ensemble at 10 K, applying restrictions on the heavy atoms of the protein (force constant 50 kcal × mol^{-1} × Å^{-2}). The second stage corresponds to a Brownian dynamics of 20 ps in NPT ensemble at 100 K, applying restrictions to the heavy atoms of the protein (force constant 20 kcal × mol^{-1} × Å^{-2}) and to the membrane (except for the hydrogen atoms) in the z-direction (force constant 5 kcal × mol^{-1} × Å^{-2}). The third stage consisted of a 10 ps simulation in an NPγT ensemble at 100 K, applying restrictions to the heavy atoms of the protein (force constant 10 kcal × mol^{-1} × Å^{-2}) and phosphorus and nitrogen atoms of the membrane in the z direction (2 kcal × mol^{-1} × Å^{-2}). The fourth stage consisted of heating from 100 K to 300 K during 150 ps using the same restrictions as the previous step, but gradually releasing the restrictions. The fifth stage consisted of a 100 ps simulation in NVT ensemble applying a restriction to the protein backbone (force constant 5 kcal × mol^{-1} × Å^{-2}). Finally, the sixth stage consisted of a simula-

tion of 100 ps in an NPγT ensemble at 300 K applying a restriction to the protein backbone (force constant 5 kcal × mol^{-1} × $Å^{-2}$).

After a proper system equilibration, a 100 ns - MDs in NPγT semi-isotropic ensemble was performed applying restrictions to the protein backbone (force constant 0.5 kcal × mol^{-1} × $Å^{-2}$) with constant surface tension of 0.0 bar × Å. Temperature and pressure were kept constant at 300 K and 1.01325 bar respectively by coupling to a Nose-Hoover Chain thermostat (Cheng and Merz, 1996) and Martyna-Tobias-Klein barostat (Martyna et al., 1994) with an integration time step of 2 fs. The simulations were performed with Desmond (Bowers et al., 2006) and the OPLS2005 force field (Banks et al., 2005).

Ensemble docking

To find the interacting mode of both compounds **1** and **2** with the $GABA_A$ receptor and considering the flexibility of the amino acids side chains, ensemble docking simulations were performed in the $GABA_A$ structures collected every 1 ns (100 structures) from the total 100 ns – MDs. Molecular docking calculations were done with Glide v.7.4 with the standard precision (SP) mode (Friesner et al., 2004). The grid boxes center was defined at the flumazenil binding site described in the cryo-electron microscopy structure of $GABA_A$ (PDB code: 6D6U) at the α_1-γ_2 interface (α_1: F100, H102, Y160, S205, S206, T207, Y210 and γ_2: Y58, F77, A79, T142), which is also located at the BDZ-bs of the $GABA_A$ receptor (Sigel and Buhr, 1997).

A cubic box with the axial length of 20 Å was used. Compounds **1** and **2** were prepared using LigPrep module in Maestro (Schrödinger, 2018b). Energy minimization in the gas phase using Macromodel (Schrödinger, 2018a) with the OPLS-2005 force field was performed. For the pose generation in every docking calculation, it was enabled the strain correction term for the GlideScore. A maximum of 10 poses were generated by docking simulation. An in-house script with the automated ensemble docking protocol is openly available in GitHub (https://doi.org/10.5281/zenodo.4739319).

Docking post-processing

To re-score docking solutions the binding free energy of all complexes were calculated using the MM-GBSA method (Gohlke and Case, 2004; Hou et al., 2011). This method combines energy from molecular mechanics according to the atoms parametrization in a given force field and uses implicit solvation models to estimate the binding energy in a molecular complex. The MM-GBSA method has been used as an alternative to reassign the score of the poses because it has shown more accurate results than the classical docking score (Guimarães and Cardozo, 2008). The binding energy was calculated as the difference between the energy of the complex and the energy of the protein and the ligand separately according to the equation [1].

$$E_{binding} = E_{complex} - E_{protein} - E_{ligand} \quad [1]$$

And the change in the binding free energy was calculated using the equation [2].

$$\Delta G_{Bind} = \Delta H - T\Delta S \approx \Delta E_{MM} + \Delta G_{Sol} - T\Delta S \quad [2]$$

Where ΔE_{MM}: $\Delta E_{internal} + \Delta E_{electrostatic} + \Delta E_{vdw}$; and ΔG_{Sol}: $\Delta G_{PB/GB} + \Delta G_{SA}$. The $\Delta E_{internal}$ corresponds to the bond, angle, and dihedral energies. $\Delta E_{electrostatic}$ and ΔE_{vdw} refer to electrostatic and van der Waals energies, respectively. $\Delta G_{PB/GB}$: is the electrostatic solvation energy, which corresponds to the polar contribution, while ΔG_{SA}: corresponds to the non-electrostatic solvation component that corresponds to the non-polar contribution. In this study, the Generalized Born (GB) model to calculate the polar contribution was used.

To process the different conformers obtained for both compounds **1** and **2**, all the re-scored poses were extracted from the protein structures, merged, and clustered. The conformers of compounds **1** and **2** were clustered using the Conformer-cluster based on cartesian RMSD script available in the Schrödinger suite software. To perform the clustering, only the heavy atoms of the ligands and a RMSD cutoff of 2 Å was considered using the average linkage method (Bottegoni et al., 2006). Then, the most populated clusters for each compound were selected to analyze how **1** and **2** interacts with $GABA_A$ at the BDZ-bs. To analyze the frequency of receptor-ligand interactions in the selected clusters, scripts included on Schrödinger Suite (v.2020-1) and *in-house* functional workflow (Peña-Varas and Ramírez, 2021) built on KNIME (Berthold et al., 2009) were used. All the scripts as well as the workflow designed in this study are openly available in Zenodo (https://doi.org/10.5281/zenodo.4739319).

Prior to the docking of compounds **1** and **2** and for the validation of the docking protocol, a re-docking of the crystallized flumazenil ligand was done. Then a clustering was performed with the same parameters mentioned before and the experimental flumazenil pose was obtained in the most populated cluster, which also had the most favorable average binding free energy among the first two clusters (where about 50% of the generated poses are located). This cluster represents 31.7% of the obtained poses and the average binding free energy value of the poses within the cluster was -67.47 kcal/mol.

The same validation protocol was performed for the 500 ns of molecular dynamics simulation approach without any constraint. A clustering of the binding site was performed, and the inertia was cal-

culated to select the clusters that best describe all the conformational variability obtained (Fig. S22). Thirty clusters of $GABA_A$ receptor were selected using the "elbow method" (Yuan and Yang, 2019), and flumazenil was re-docked into the centroid structure of the selected clusters. With this approach, the crystallographic pose within the most populated clusters (first two clusters, where about 50% of the generated poses are located) was not obtained. However, the experimental ligand pose was obtained in another cluster, the most energetically favorable, with an average free energy of -50.16 kcal/mol. However, this cluster represents only 9.43% of the generated poses. According to these results, the size of the binding site was analyzed, and it was found that in the 500 ns MDs, the binding site closes and maintains a volume with a median value of 96.89 A^3 in the thirty frames chosen. For the simulation where protein backbone was constrained, the binding site volume has a median value of 422.68 A^3 (Fig. S23). This allows us to conclude that without restrictions, and due to the characteristics of the binding site studied in this work, the pocket is closed and does not give rise to known interactions such as those described in the cryo-electron microscopy structure of flumazenil with $GABA_A$.

Statistical analysis

For the *in vitro* inhibition assays of binding to the BDZ-bs of the $GABA_A$ receptor, tests were performed in triplicate, for each compound. Values were expressed as mean ± S.E.M (Marder et al., 2003) using GraphPad Prism8 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Identification and structural elucidation of iridoids

The chemical structures of compounds **1** and **2** are presented in Fig. 2. Their structural elucidation allowed them to be identified as a diene-type valepotriate hydrines, which present at C-1, C-7, and C-11, the own substitutions of the valtrate (Thies, 1968). By means of the one-dimensional (^1H, ^{13}C) and two-dimensional NMR experiments (COSY, ROESY, HMBC, and HSQC) it was possible to fully elucidate the chemical structure of the compounds. The analysis of the ROESY spectra of the compound confirmed their relative configuration. By HREIMS, the mass and molecular formula of the compounds were determined, corresponding to m/z: 468.2343, $C_{24}H_{36}O_9$ (calcd. 468.2359) for compound **1** (Fig. S10) and m/z: 454.2221, $C_{23}H_{34}O_9$ (calcd. 454.2203) for compound **2** (Fig. S20).

According to the spectroscopic study and by comparing the (^1H and ^{13}C) NMR data recorded for these compounds with those reported, compound **1** was identified as valerjatadoid A, isolated from *Valeriana jatamansi* Jones (*V. jatamansi)* (Yang et al., 2015) (Table S1, Fig. 2) and compound **2** corresponds to valeriandoid F, isolated from *V. jatamansi* (Xu et al., 2012), (Table S2, Fig. 2). The compound isolated from *V. jatamansi* recorded an optical rotation value of $[\alpha]^{25}_D$: -51.7 (*c* 0.37, CH_2Cl_2), while the compound **2** isolated from *V. pavonii* obtained an optical rotation value of $[\alpha]^{20}_D$ +49.94 (*c* 0.39, MeOH), suggesting compound **2** is an enantiomer of valeriandoid F, and thus, a new reported compound.

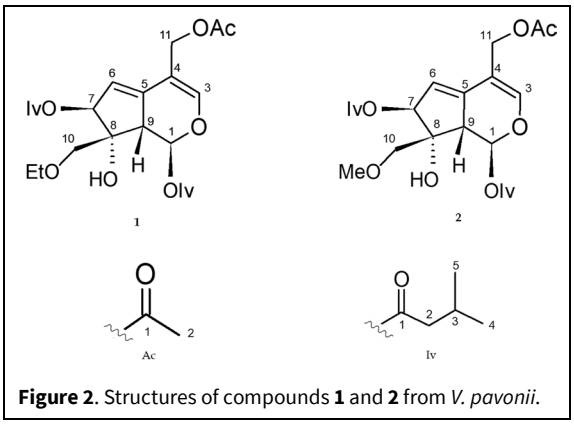

Figure 2. Structures of compounds **1** and **2** from *V. pavonii*.

In vitro assay of binding to the BDZ site of the $GABA_A$ receptor

In this study at concentrations of 300 µM, the compounds did not fully displace the ^3H-FNZ bound to BDZ-bs present in the synaptosomal membranes of rat cerebral cortices and exhibited values below 50%. Both compounds **1** and **2** reached about 40% inhibition of the binding ^3H-FNZ to the BDZ-bs of the $GABA_A$ receptor (mean ± S.E.M. of compound **1** = 40.3 ± 0.9 % and compound **2** = 43.8 ± 1.3 %) (Fig. S21).

Computational studies

Previously, it has been described that subtype $\alpha_1\beta_2\gamma_2$ is the most abundant $GABA_A$ receptor localized in the brain (Sur et al., 2001). For this work, it was hypothesized that the binding of the isolated and tested compounds in the synaptosomal membranes described in the benzodiazepine binding site assay with the displacement of the ^3H-FNZ, is mainly in the $GABA_A$ receptor. Accordingly, the structure of the $GABA_A$ receptor $\alpha_1\beta_2\gamma_2$ subtype was selected to describe the binding mode.

A massive docking protocol of both compounds **1** and **2**, was performed in 100 different conformations of the $GABA_A$ receptor extracted from the MDs to include the flexibility of the residues that integrate the benzodiazepine binding site. The ensemble docking

approach has shown to deliver more accurate results than a single structure-based docking (Ellingson et al., 2015; Huang and Zou, 2007). Therefore, multiple docking studies were performed on 100 $GABA_A$ structures in their holo-like forms. To determine the pose with the highest probability of occurrence, a re-scoring of the docking poses was done by calculating the binding free energy for all the obtained poses, followed by cluster analysis of all the re-scored conformers. For the compound **1**, a total of 867 conformers clustered into 54 clusters were obtained. For the compound **2**, 891 conformers were clustered into 49 clusters (Table S3). Fig. 3A shows that for the most populated clusters for compounds **1** and **2** were integrated by 337 and 342 conformers, respectively.

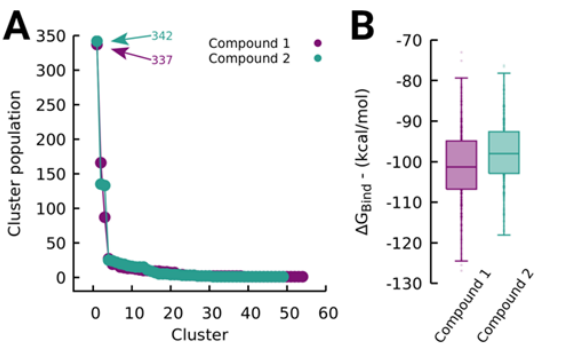

Figure 3. Cluster analyses and MM-GBSA binding energy.

(**A**) Cluster analysis of the poses obtained with the docking calculation of compounds **1** and **2**. The most populated cluster of each compound is pointed with an arrow. (**B**) Binding free energy (ΔG_{bind}) calculated with the MM-GBSA method for the most populated clusters of part A is shown.

The most populated cluster for compound **1** and **2** corresponds to 38.8% and 38.3% of the total poses generated for each compound by molecular docking, respectively. Fig. 3B shows the binding free energy calculated with the MM-GBSA method for the most populated clusters of each compound. A variable and similar energy distribution is observed for the two compounds, which agrees with the experimental assays where a similar displacement was obtained with ^{3}H-FNZ of 40.3 ± 0.9 % and 43.8 ± 1.3 % for compounds **1** and **2**, respectively.

For further analysis, the different moieties of both compounds were classified into Core (central part of the molecules), R1, R2, R3, and R4 moieties (Fig. 4). The moiety R4 is the only one that differs between both compounds **1** (ethoxymethyl) and **2** (methoxymethyl). The interactions between the most populated clusters and $GABA_A$ are described in the Fig. **5**. The residues F100, H102, K156, Y160, V203, S205, S206, T207, Y210, and V212 from subunit α_1, as well as residues Y58, F77, A79, M130, L140, and T142 from subunit γ_2 are involved in the binding site of both clusters. Both clusters present similar conformations into the described binding site.

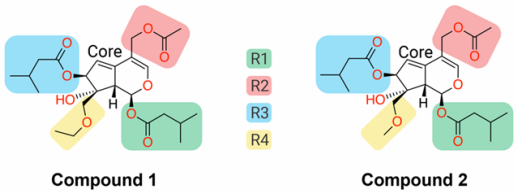

Figure 4. Compounds **1** and **2** with the Core and R1-R4 moieties highlighted.

The conformers with the lower ΔG_{bind} energy of each cluster were also analyzed. For the compound **1**, the conformer with the lowest binding energy presents an ΔG_{bind} = -126.88 kcal/mol (Fig. 6A), and for the compound **2**, the conformer with the lowest binding energy presents an ΔG_{bind} = -118.07 kcal/mol (Fig. 6B). Both conformers present similar interaction binding modes, where all interacting groups (Core, R1, R2, R3 and R4) are oriented towards the same direction, and at the same position. The RMSD between the common atoms for both conformers is 1.16 Å, which allows us to hypothesize that both compounds interact with $GABA_A$ at the same binding site, and in a remarkably similar mode. Compounds **1** and **2** are observed to overlap with the crystallized flumazenil antagonist on the $GABA_A$ receptor (Fig. 6C). The flumazenil rings are also located in the center of the binding site, the same happens for compounds **1** and **2**.

In the flumazenil crystal at the $GABA_A$ receptor, the diazepine ring is against the phenyl ring of residue γ_2-F77, however, the rings of 1 and 2 are parallel to the phenyl ring of residue γ_2-F77 (Fig. 6C). This shows that although compounds **1** and **2** overlaps in a similar way to flumazenil, there are differences in the orientation of the ligands rings as well as the interacting residues at the BDZ-bs.

To further study how both compounds **1** and **2** interacts with $GABA_A$, all the conformers of the most populated clusters by categorizing one by one conformer interaction (Fig. 7) were also analyzed. The results show that the most frequent interactions for both compounds are hydrophobic, and some hydrogen bonds were also identified. As expected, the main difference between both compounds when interacts with $GABA_A$ lies on the R4 groups, which varies between **1** and **2**. It is observed that compound **1** interacts with residues γ_2-F77, γ_2-M 130, γ_2-T142, α_1-Y160, α_1-T207, and α_1-Y210, whereas compound **2** does not present such interactions.

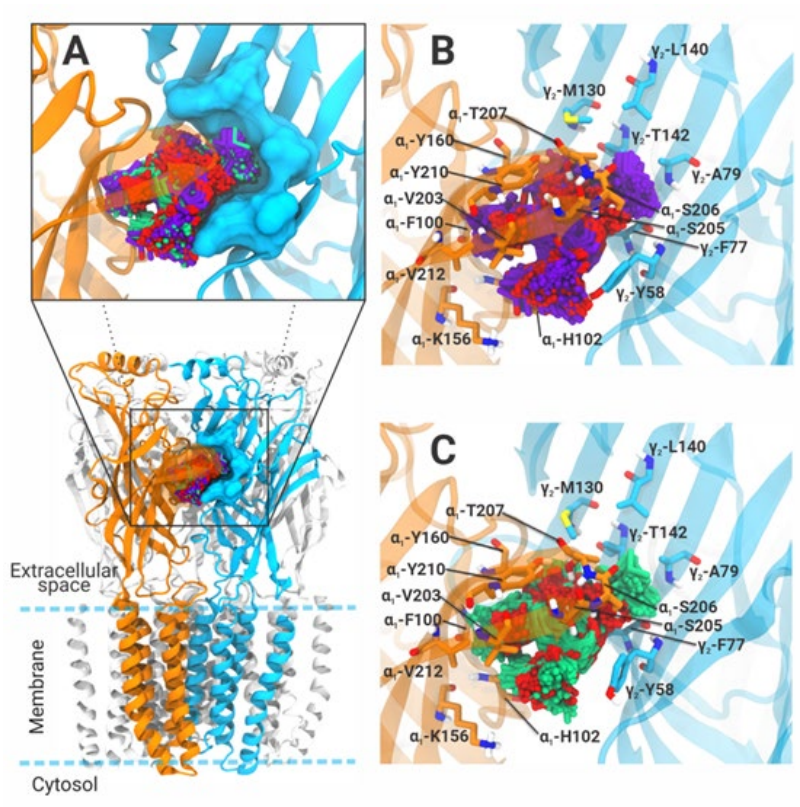

Figure 5. Binding mode of the compounds **1** and **2** most populated clusters. $GABA_A$ subunits α_1 and γ_2 are shown in orange and blue, respectively. The other chains are represented in gray carton. (**A**) Conformers of both clusters at the $GABA_A$ receptor benzodiazepine binding site. Compounds **1** and **2** are shown in purple and green, respectively. The interacting residues at the $GABA_A$ binding site are displayed for both clusters of compounds **1** (**B**) and **2** (**C**).

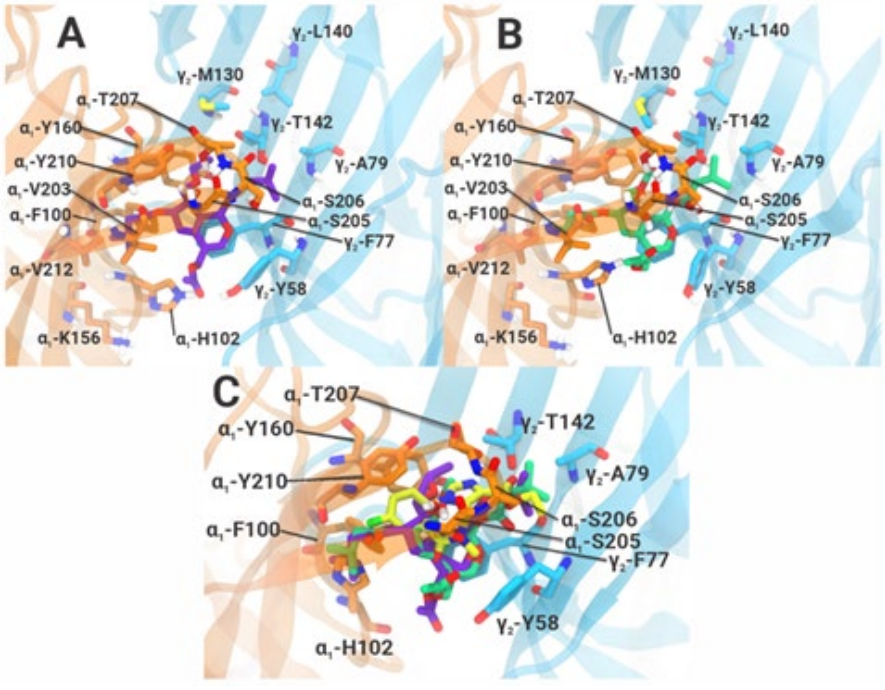

Figure 6. Binding mode of the most energetically favorable poses of compounds **1** (**A**) and **2** (**B**). $GABA_A$ subunits α_1 and γ_2 are shown in orange and blue, respectively. (**C**) Compounds **1**, **2** and crystallographic structure of the competitive benzodiazepine antagonist flumazenil are shown in violet, green and yellow, respectively. Only flumazenil binding site residues are shown.

DISCUSSION

In this study, iridoid esters-type compounds (hydrine-type valepotriates) were isolated from DF (compound **1**) and from PEF (compound **2**) of *V. pavonii*. It was previously reported that these fractions showed anticonvulsant *in vivo* activity in MES, with significant protection values of 90% in doses of 35 and 65 mg/kg, p.o. respectively (Giraldo, et al., 2008; 2013). In other *in vivo* test, it was found that PEF achieved a protection percentage of 60% in pentylentetrazole (PTZ) induce seizure model (42.5 mg/kg, s.c.) at a dose of 100 mg/kg, p.o. in mice. Both animal models are widely used for the study of drugs with

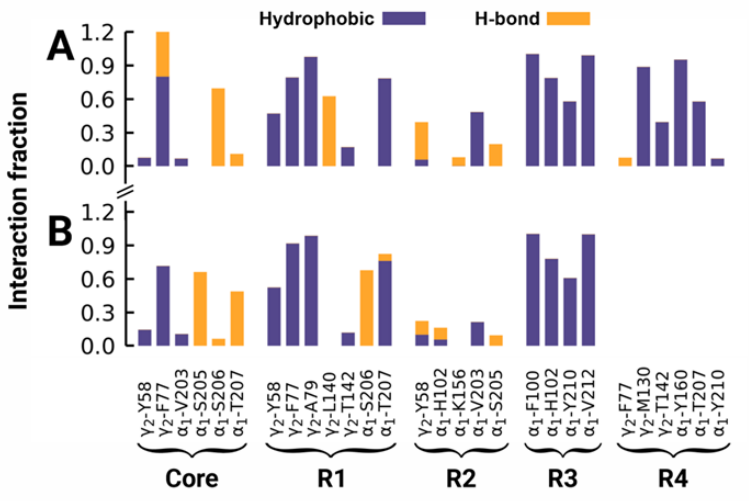

Figure 7. Interaction fraction of the most populated clusters of compounds **1** (**A**) and **2** (**B**). Core, R1, R2, R3, and R4 moieties are shown in Fig. 4.

potential antiepileptic activity, whether they inhibit generalized tonic-clonic seizures (MES model) or generalized myoclonic seizures and also absence seizures (PTZ model), being the $GABA_A$ receptor one of the most widely studied drug targets.

Although the results obtained in the *in vitro* binding assay showed partial inhibition activity of ^{3}H-FNZ binding to BDZ-bs of the $GABA_A$ receptor, it should be noted that they show differences with respect to those obtained in previous reports, where iridoid esters-type compounds (valepotriate hydrines) isolated from *V. pavonii* only achieved inhibition percentages below 30%: valtrate acetoxyhydrin (11%), valtrate isovaleroyloxyhydrin (14%) and valtrate chlorohydrin (34%) at concentrations of 300 µM (Giraldo, et al., 2013). It is highlighted that the percentage inhibition of compounds **1** and **2** are close to those obtained for isovaleramide, an anticonvulsant molecule isolated from *V. pavonii*, which achieved an inhibition of 42% in the same test (Giraldo et al., 2010). Isovaleramide is an amide derived from valerenic acid, whose chemical structure is similar to valproic acid, derived from natural valeric acid, whose mechanism of action is related to the inhibition of GABA transaminase but also blocking voltage-gated ion channels (Rahman and Nguyen, 2020).

A few previous studies have demonstrated the modulation of the GABAergic system by iridoid-type metabolites isolated from species of the genus *Valerian*, focusing mainly on valtrate derivatives. In this regard, valtrate isolated from *V. jatamansi* showed antiepileptic effects *in vivo* in MES and PTZ at doses of 5, 10 and 20 mg/kg. Its mechanism of action was explained by a significant up-regulation dose-dependent of the expression of the $GABA_A$ receptor, GAD65 (glutamic acid decarboxylase65, GAD isoform) and the Bcl2 protein (inhibits neuronal apoptosis during seizures) in the brain of chronic epileptic rats by western blot assays (Wu et al., 2017). More recently, a fraction of valepotriates from *V. edulis* (subsp. procera, Kunth, Mexico) evaluated at a dose of 100 mg/kg (i.p.) in PTZ in rats, showed a decrease in seizures, a significant decrease in seizure severity, and a significant increase in latency. In a molecular docking interaction study for the $GABA_A$ receptor, valtrate was the compound with the best docking score, followed by isodihydrovaltrate and homovaltrate, all of them exhibit hydrogen bond interactions with Thr256 from chain D, as well as hydrophobic contacts with Ile255 and Ala252 from chain B (González-Trujano et al., 2021).

Different factors could be contributing to the *in vivo* anticonvulsant activity of both DF and PEF of *V. pavonii*, enriched fractions of iridoid esters (hydrine-type valepotriates). Within these factors, there could be possible synergistic effects, in which valepotriates not yet unidentified act as allosteric modulators for the benzodiazepine binding site of the $GABA_A$ receptor, or act via GABAergic by regulating enzymes involved either with the biosynthesis of GABA (GAD: glutamic acid decarboxylase) or with its catabolism (GABA-T: GABA transaminase). Additionally, there could be other complementary mechanisms of action such as voltage-gated sodium channel blocking, which also explains the results obtained *in vivo* in the MES assay.

To describe how both compounds **1** and **2** interacts with the $GABA_A$ receptor, a systematic computational pipeline was implemented, involving molecular dynamics of the $GABA_A$ receptor in the holo-like form, followed by exhaustive docking of both compounds in different (100) conformations of the receptor, then

the docking solutions were re-scored by MM-GBSA, merged and clustered to study the interaction mode of both iridoids at the BDZ-bs. To perform this protocol in the best way, MD simulations of the receptor in the holo-like form with and without constraints were carried out, and it was found that when no constraints are applied, the binding pocket closes (Fig. S23), which drastically changes the site and does not allow to reproduce binding modes for known ligands (flumazenil – PDB code: 6D6U) by using docking simulations. For this reason, the massive docking protocol on structures derived from constrained MDs was used, in the same way as other authors have done for similar protocols applied on membrane proteins (Peña-Varas et al., 2022; Ramírez et al., 2017). In addition, it has been shown that molecular docking methods are often more successful when starting from protein structures in the bound state than in the unbound state, or when the set of structures has a higher similarity to the bound state (Ritchie, 2008; Zacharias, 2010; Zhang et al., 2017).

With the systematic pipeline implemented here, one significant cluster for each compound (Fig. 3A) was found. Both clusters interact similarly with $GABA_A$ at the BDZ-bs, with the key residues F100, H102, K156, Y160, V203, S205, S206, T207, Y210, and V212 from subunit α_1, as well as residues Y58, F77, A79, M130, L140, and T142 from subunit γ_2 (Fig. 6A-B). However, further interaction analysis (Fig. 7) reveals differences in the R4 moiety interaction, this is because the methoxymethyl moiety (R4) of compound **2** is not long enough to establish proper hydrophobic contacts with $GABA_A$. However, those interactions do not seem to explain how these compounds interact at the BDZ-bs because the experimental data obtained from the *in vitro* assay showed percentages of inhibition of the binding of ^{3}H-FNZ to BDZ-bs between 40% and 44%. On the other hand, the contacts of the R2 and R3 groups were similar for **1** and **2**, and some differences between both compounds were observed in the interactions of the core and the R1 moieties (Fig. 7). Both compounds showed a high interaction fraction with the F77 residue of the γ_2 subunit (core). Compound **1** had the highest interaction fraction with residue F77, with a hydrophobic fraction of a 0.8 and hydrogen bond of 0.39. Compound **2** presented the highest interaction fraction with groups R1 and R3, and residue F77, 0.9 being hydrophobic interactions, and 0.7 hydrogen bonds.

Furthermore, the conformers of the most populated clusters presented several interactions that have been previously described for other active molecules that bind at the binding site of benzodiazepines between the α_1-γ_2 interface of the $GABA_A$ receptor. Mutation studies have shown that γ_2-Y58 residue is relevant for the binding of flunitrazepam (Kucken et al., 2000). The residue γ_2-F77 has shown to be relevant for binding diazepam (Buhr et al., 1997a), flumazenil, CL218,872 and methyl-b-carboline-3-carboxylate (Wingrove et al., 1997). Residue γ_2-T142 is implicated in the selectivity of zolpidem and eszopiclone, and residue α_1-T206 is implicated in the affinity of eszopiclone, diazepam, and zolpidem (Buhr et al., 1997b; Hanson et al., 2008). Other authors have described through theoretical studies that residues γ_2-Y58, γ_2-F77, α_1-F100, γ_2-T142, α_1-Y160, α_1-S206, α_1-T207 and α_1-Y210 are involved in the interaction of clonazepam, and flunitrazepam with $GABA_A$, while α1-F100, α_1-Y160, α_1-T207, α_1-Y210, γ_2-Y58, γ_2-F77, and γ_2-T142 are involved in the flurazepam, Ro144513, zolpidem, and eszopiclone binding site (Amundarain et al., 2019). Those studies show that interactions found between different benzodiazepines and the $GABA_A$ receptor are common with the contact interactions for the compounds **1** and **2** reported here. It is the first time that a complete interaction profile of two metabolites (isolated from *V. pavonii* stems) against the $GABA_A$ receptor is reported, the systematic conformational sampling of both compounds using MD simulations and an ensemble docking protocol, together with the rescoring by MM-GBSA of docking solutions in order to better rank the different poses, followed by the conformational clustering allow us to gain structural insights in the ligand-$GABA_A$ interactions at the BDZ-bs. The protocol implemented here, and the findings obtained can be used to study how different phytochemicals interact with the GABAergic system, as well as to enhance the drug design process.

CONCLUSION

These results contribute to the study of the neuropharmacological profile of *Valeriana pavonii*, a native species from Colombia used in traditional medicine as a tranquilizer, especially for insomnia and anxiety problems. In this study, two iridoid esters from *V. pavonii* stems were identified; compound **1** and **2**, isolated from dichloromethane and petroleum ether fraction, respectively. Both compounds presented binding affinity for the BDZ-bs of the $GABA_A$ receptor. The computational results allow us to describe how both compounds interact at their binding site, presenting a similar interaction profile, which reflects the registered biological activity against $GABA_A$ receptors. These findings help us to explain why the ethanolic extract and the dichloromethane, petroleum ether and alkaloid fractions of *Valeriana pavonii* have shown promising effects on the central nervous system, especially as anticonvulsants and sedative-hypnotics, which could be related to the modulation of the GABAergic system by hydrine-type valepotriates and their derivatives. Further *in vitro*/*in silico*

studies are needed to better understand the mechanisms of action of these compounds, evaluating their action on other key molecular targets, such as voltage-gated sodium channels.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This research was funded by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT), grant number 1220656; the Agencia Nacional de Investigación y Desarrollo (ANID) grant number ATE220043 and FOVI210027; Spanish MINECO co-funded by the European Regional Development Fund (FEDER), grant number RTI2018-094356-B-C21; Universidad de Buenos Aires (UBA, UBA-CyT), grant number 20020150100012BA; and Departamento Administrativo de Ciencia, Tecnología e Innovación–Colciencias, grant number 110140820449. S.G. thanks the Departamento de Farmacia of the Universidad Nacional de Colombia for the technical and scientific support for the development of this research and the Universidad de La Salle, Bogotá, for academic support for the publication of this study. M.B. acknowledges FONDECYT-ANID for his postdoctoral grant Nº 3210774.

REFERENCES

Amundarain MJ, Viso, JF, Zamarreño, F, Giorgetti, A, Costabel M (2019) Orthosteric and benzodiazepine cavities of the α1β2γ2 $GABA_A$ receptor: insights from experimentally validated *in silico* methods. J Biomol Struct Dyn 37: 1597–1615. https://doi.org/10.1080/07391102.2018.1462733

Arévalo D, Martínez CA, Rincón J, Guerrero MF (2006) Fracción alcaloidal obtenida de *Valeriana pavonii* Poepp con actividad anticonvulsivante. Rev Col Cienc Quím Farm 35: 168–176.

Banks JL, Beard HS, Cao Y, Cho AE, Damm W, Farid R, Felts AK, Halgren, TA, Mainz, DT, Maple JR, Murphy R, Philipp DM, Repasky MP, Zhang LY, Berne BJ, Friesner RA, Gallicchio E, Levy RM (2005) Integrated Modeling Program, Applied Chemical Theory (IMPACT). J Comput Chem 26: 1752–1780. https://doi.org/10.1002/jcc.20292

Benke D, Barberis A, Kopp S, Altmann KH, Schubiger M, Vogt KE, Rudolph U, Möhler H (2009) $GABA_A$ receptors as *in vivo* substrate for the anxiolytic action of valerenic acid, a major constituent of valerian root extracts. Neuropharmacology 56: 174–181. https://doi.org/10.1016/j.neuropharm.2008.06.013

Berthold MR, Cebron N, Dill F, Gabriel TR, Kötter T, Meinl T, Ohl P, Thiel K, Wiswedel B (2009) KNIME-the Konstanz information miner: version 2.0 and beyond. ACM SIGKDD Explor News 11: 26–31. https://doi.org/10.1145/1656274.1656280

Bos R, Woerdenbag HJ, Pras, N (2002) Determination of valepotriates. J Chromatogr A 967: 131–146. https://doi.org/10.1016/S0021-9673(02)00036-5

Bottegoni G, Cavalli A, Recanatini M (2006) A comparative study on the application of hierarchical-agglomerative clustering approaches to organize outputs of reiterated docking runs. J Chem Inf Model 46: 852–862. https://doi.org/10.1021/ci050141q

Bowers KJ, Chow E, Xu H, Dror RO, Eastwood MP, Gregersen BA, Klepeis JL, Kolossvary, I, Moraes MA, Sacerdoti FD (2006) Scalable algorithms for molecular dynamics simulations on commodity clusters. In: SC 2006 Conference, Proceedings of the ACM/IEEE. p. 43. https://doi.org/10.1109/SC.2006.54

Buhr A, Baur R, Sigel E (1997a) Subtle changes in residue 77 of the γ subunit of α1β2γ2 $GABA_A$ receptors drastically alter the affinity for ligands of the benzodiazepine binding site. J Biol Chem 272: 11799–11804. https://doi.org/10.1074/jbc.272.18.11799

Buhr A, Schaerer MT, Baur R, Sigel E (1997b) Residues at positions 206 and 209 of the α1 subunit of γ-aminobutyric acidA receptors influence affinities for benzodiazepine binding site ligands. Mol Pharmacol 52: 676–682. https://doi.org/10.1124/mol.52.4.676

Celis CT, Rincón J, Guerrero MF (2007) Actividad farmacológica sobre el sistema nervioso central del extracto etanólico y de la fracción alcaloidal de *Valeriana pavonii*. Rev Col Cienc Quím Farm 36: 11–22.

Cheng A, Merz KM (1996) Application of the Nosé-Hoover chain algorithm to the study of protein dynamics. J Phys Chem 100: 1927–1937. https://doi.org/10.1021/jp951968y

Ellingson SR, Miao Y, Baudry J, Smith JC (2015) Multi-conformer ensemble docking to difficult protein targets. J Phys Chem B 119: 1026–1034. https://doi.org/10.1021/jp506511p

Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy J Med Chem 47: 1739–1749. https://doi.org/10.1021/jm0306430

García B (1992) Flora medicinal de Colombia, Botánica médica, Tomo 3. Bogotá, DC: Tercer Mundo.

Giraldo SE, Rincón J, Guerrero MF (2008) Metabolitos de tipo iridoide y alcaloide aislados de *Valeriana pavonii* con potencial actividad anticonvulsivante. Rev Salud UIS 40: 169–171.

Giraldo SE, Rincón J, Puebla P, Marder M, Wasowski C, Vergel NE, Guerrero MF (2010) Isovaleramida, principio anticonvulsivo aislado de *Valeriana pavonii*. Biomedica 30: 245–250. https://doi.org/10.7705/biomedica.v30i2.187

Giraldo SE, Rincón J, Guerrero MF, López I, Jiménez IA, Marder M, Wasowski C, Vergel NE (2013) Valepotriate hydrines isolated from an anticonvulsant fraction of *Valeriana pavonii* Poepp. & Endl. Lat Am J Pharm 32: 1224–1230.

Gohlke H, Case D A (2004) Converging free energy estimates: MM-PB (GB) SA studies on the protein–protein complex Ras–Raf. J Comput Chem 25: 238–250. https://doi.org/10.1002/jcc.10379

González-Trujano ME, Contreras G, López CA, Hidalgo FJ, Navarrete A, Sánchez CG, Magdaleno VM (2021) Anticonvulsant activity of *Valeriana edulis* roots and valepotriates on the pentylenetetrazole-induced seizures in rats. J Ethnopharmacol 265: 113299. https://doi.org/10.1016/j.jep.2020.113299

Guide for the Care and Use of Laboratory Animals (2011) Committee for the Update of the Guide for the Care Animals and Use of Laboratory. National Academies Press: Washington, DC, USA.

Guimarães CRW, Cardozo M (2008) MM-GB/SA rescoring of docking poses in structure-based lead optimization. J Chem Inf Model 48: 958–970. https://doi.org/10.1021/ci800004w

Hanson SM, Morlock EV, Satyshur KA, Czajkowski C (2008) Structural requirements for eszopiclone and zolpidem binding to the γ-aminobutyric acid type-A ($GABA_A$) receptor are different. J Med Chem 51: 7243–7252. https://doi.org/10.1021/jm800889m

Hou T, Wang J, Li Y, Wang W (2011) Assessing the performance of the MM/PBSA and MM/GBSA methods: I. The accuracy of binding free energy calculations based on molecular dynamics

simulations. J Chem Inf Model 51: 69–82. https://doi.org/10.1021/ci100275a

Huang SY, Zou X (2007) Ensemble docking of multiple protein structures: considering protein structural variations in molecular docking. Proteins Struct Funct Bioinforma 66: 399–421. https://doi.org/10.1002/prot.21214

Jacobson MP, Friesner RA, Xiang Z, Honig B (2002) On the role of the crystal environment in determining protein side-chain conformations. J Mol Biol 320: 597–608. https://doi.org/10.1016/S0022-2836(02)00470-9

Jacobson MP, Pincus DL, Rapp CS, Day TJF, Honig B, Shaw DE, Friesner RA (2004) A hierarchical approach to all-atom protein loop prediction. Proteins Struct Funct Bioinforma 55: 351–367. https://doi.org/10.1002/prot.10613

Kucken AM, Wagner DA, Ward PR, Teissére JA, Boileau AJ, Czajkowski C (2000) Identification of benzodiazepine binding site residues in the γ2 subunit of the γ-aminobutyric acidA receptor. Mol Pharmacol 57: 932–939.

Liu YH, Wu PQ, Hu QL, Pei YJ, Qi FM, Zhang ZX, Fei DQ (2017) Cytotoxic and antibacterial activities of iridoids and sesquiterpenoids from *Valeriana jatamansi*. Fitoterapia 123: 73–78. https://doi.org/10.1016/j.fitote.2017.09.011

Marder M, Viola H, Wasowski C, Fernández S, Medina JH, Paladini AC (2003) 6-Methylapigenin and hesperidin: New valeriana flavonoids with activity on the CNS. Pharmacol Biochem Behav 75: 537–545. https://doi.org/10.1016/S0091-3057(03)00121-7

Martyna GJ, Tobias DJ, Klein ML (1994) Constant pressure molecular dynamics algorithms. J Chem Phys 101: 4177–4189. https://doi.org/10.1063/1.467468

Nishiya K, Kimura T, Takeya K, Itokawa H (1994) Sesquiterpenoids and iridoid glycosides from *Valeriana fauriei*. Phytochemistry 36: 1547–1548. https://doi.org/10.1016/S0031-9422(00)89759-9

Olsson MHM, Søndergaard CR, Rostkowski M, Jensen JH (2011) PROPKA3: consistent treatment of internal and surface residues in empirical pKa predictions. J Chem Theory Comput 7: 525–537. https://doi.org/10.1021/ct100578z

Parra PA (2003) Pasantía en un Laboratorio Nacional de Elaboración de Productos Farmacéuticos con base en especies vegetales. Trabajo de Grado, Universidad Nacional de Colombia. Bogotá, D.C.

Patočka J, Jakl J (2010) Biomedically relevant chemical constituents of *Valeriana officinalis*. J Appl Biomed 8: 11–18. https://doi.org/10.2478/v10136-009-0002-z

Peña-Varas C, Kanstrup C, Vergara-Jaque A, González-Avendaño M, Crocoll C, Mirza O, Dreyer I, Nour-Eldin H, Ramirez, D (2022) Structural insights into the substrate transport mechanisms in GTR transporters through ensemble docking. Int J Mol Sci 23: 1595. https://doi.org/10.3390/ijms23031595

Peña-Varas C, Ramírez D (2021) Docking post-processing: Frequency interactions from cluster of conformers (Version 1.0). Zenodo, Article 1. https://doi.org/10.5281/zenodo.4739319

Quan LQ, Zhou Y, Liu D, Chen CH, Li HM, Li RT (2022) Iridoids and sesquiterpenoids from *Valeriana jatamansi* and their anti-influenza virus activities. Bioorg Chem 121: 1–16. https://doi.org/10.1016/j.bioorg.2022.105692

Rahman M, Nguyen H (2020) Valproic acid. In: StatPearls. StatPearls Publishing: Treasure Island (FL), PMID: 32644538.

Ramírez D, Arévalo B, Martínez G, Rinné S, Sepúlveda FV, Decher N, González W (2017) Side fenestrations provide "anchor" for a stable binding of A1899 to the pore of TASK-1 potassium channels. Mol Pharm 14: 2197–2208. https://doi.org/10.1021/acs.molpharmaceut.7b00005

Ramírez D, Bedoya M, Kiper AK, Rinné S, Morales-Navarro S, Hernández-Rodríguez EW, Sepúlveda FV, Decher N, González W (2019) Structure/activity analysis of TASK-3 channel antagonists based on a 5, 6, 7, 8 tetrahydropyrido [4, 3-d] pyrimidine. Int J Mol Sci 20: 2252. https://doi.org/10.3390/ijms20092252

Ritchie D (2008) Recent progress and future directions in protein-protein docking. Curr Protein Pept Sci 9: 1–15. https://doi.org/10.2174/138920308783565741

Savage K, Firth J, Stough C, Sarris J (2018) GABA-modulating phytomedicines for anxiety: A systematic review of preclinical and clinical evidence. Phyther Res 32: 3–18. https://doi.org/10.1002/ptr.5940

Schrödinger LLC (2018a) Schrödinger Release 2018-4, MacroModel. Schrödinger LLC New York, New York.

Schrödinger LLC (2018b) Schrödinger Release 2018-4, LigPrep. Schrödinger LLC New York, New York.

Sigel E, Buhr A (1997) The benzodiazepine binding site of $GABA_A$ receptors. Trends Pharmacol Sci 18: 425–429. https://doi.org/10.1016/S0165-6147(97)90675-1

Sur C, Wafford KA, Reynolds DS, Hadingham KL, Bromidge F, Macaulay A, Collinson N, O'Meara G, Howell O, Newman R (2001) Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. J Neurosci 21: 3409–3418. https://doi.org/10.1523/JNEUROSCI.21-10-03409.2001

Tan YZ, Yong Y, Dong YH, Wang RJ, Li HX, Zhang H, Guo DLe, Zhang SJ, Dong XP, Xie XF (2016) A new secoiridoid glycoside and a new sesquiterpenoid glycoside from *Valeriana jatamansi* with neuroprotective activity. Phytochem Lett 17: 177–180. https://doi.org/10.1016/j.phytol.2016.07.020

Tang JX, Quan LQ, Xie K, Zhou Y, Ye RR, Liu D, Li HM (2022) Jatavaleridoids AH, eight new iridoids from the roots and rhizomes of *Valeriana jatamansi* Jones. Fitoterapia 162: 1–9. https://doi.org/10.1016/j.fitote.2022.105286

Thies PW (1968) Die konstitution der valepotriate: Mitteilung über die wirkstoffe des baldrians. Tetrahedron 24: 313–347. https://doi.org/10.1016/0040-4020(68)89032-5

Wang F, Zhang Y, Wu S, He Y, Dai Z, Ma S, Liu B (2017) Studies of the structure-antioxidant activity relationships and antioxidant activity mechanism of iridoid valepotriates and their degradation products. PLoS One 12: 1–16. https://doi.org/10.1371/journal.pone.0189198

Wang H, Song Z, Xing H, Shi Z, Wu P, Zhang J, Tuerhong M, Xu J, Guo Y (2020) Nitric oxide inhibitory iridoids as potential anti-inflammatory agents from *Valeriana jatamansi*. Bioorg Chem 101: 1–9. https://doi.org/10.1016/j.bioorg.2020.103974

Wasowski C, Marder M (2012) Flavonoids as $GABA_A$ receptor ligands: the whole story? J Exp Pharmacol 4: 9–24. http://dx.doi.org/10.2147/JEP.S23105

Wasowski C, Marder M, Viola H, Medina JH, Paladini AC (2002) Isolation and identification of 6-methylapigenin, a competitive ligand for the brain $GABA_A$ receptors, from *Valeriana wallichii*. Planta Med 68: 934–936. https://doi.org/10.1055/s-2002-34936

Wingrove PB, Thompson SA, Wafford KA, Whiting PJ (1997) Key amino acids in the γ subunit of the γ-aminobutyric $acid_A$ receptor that determine ligand binding and modulation at the benzodiazepine site. Mol Pharmacol 52: 874–881. https://doi.org/10.1124/mol.52.5.874

Wizard PP (2019) Protein Preparation Wizard. Epik: Schrödinger, LLC, New York, NY.

Wu A, Ye X, Huang Q, Dai WM, Zhang JM (2017) Anti-epileptic effects of valepotriate isolated from *Valeriana jatamansi* Jones and its possible mechanisms. Pharmacogn Mag 13: 512–516. http://dx.doi.org/10.4103/0973-1296.211027

Xu J, Guo Y, Jin DQ, Zhao P, Guo P, Yamakuni T, Ohizumi Y (2012) Three new iridoids from the roots of *Valeriana jatamansi*. J Nat Med 66: 653–657. https://doi.org/10.1007/s11418-012-0631-5

Yang B, Zhang JF, Song HZ, Gu MC, Zhao HJ, Xiong YK (2015) Two new iridoid esters from the root and rhizome of *Valeriana jatamansi* Jones. Helv Chim Acta 98: 1225–1230. https://doi.org/10.1002/hlca.201400389

Yuan C, Yang H (2019) Research on K-value selection method of K-means clustering algorithm. J 2: 226–235. https://doi.org/10.3390/j2020016

Zacharias M (2010) Accounting for conformational changes during protein-protein docking. Curr Opin Struct Biol 20: 180–186. https://doi.org/10.1016/j.sbi.2010.02.001

Zhang Z, Ehmann U, Zacharias M (2017) Monte Carlo replica-exchange based ensemble docking of protein conformations. Proteins Struct Funct Bioinforma 85: 924–937. https://doi.org/10.1002/prot.25262

Zhu S, Noviello CM, Teng J, Walsh RM, Kim JJ, Hibbs RE (2018) Structure of a human synaptic $GABA_A$ receptor. Nature 559: 67–72. https://doi.org/10.1038/s41586-018-0255-3

AUTHOR CONTRIBUTION:

Contribution	Giraldo SE	Bedoya M	Peña-Varas C	Santana PA	Bazzocchi IL	Jiménez IA	Marder M	Vergel NE	Guerrero MF	Ramírez D
Concepts or ideas	x	x			x	x	x		x	x
Design	x	x							x	x
Definition of intellectual content	x	x							x	x
Literature search	x	x								x
Experimental studies	x	x								
Data acquisition	x	x	x	x	x	x	x	x		x
Data analysis	x	x	x	x	x	x	x	x		x
Statistical analysis			x	x	x	x	x	x		
Manuscript preparation	x	x					x			x
Manuscript editing	x	x	x	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x	x	x	x

Citation Format: Giraldo SE, Bedoya M, Peña-Varas C, Santana PA, Bazzocchi IL, Jiménez IA, Marder M, Vergel NE, Guerrero MF, Ramírez D (2023) Iridoid esters from *Valeriana pavonii* Poepp. & Endl. as $GABA_A$ modulators: Structural insights in their binding mode and structure-activity relationship. J Pharm Pharmacogn Res 11(3): 367–380. https://doi.org/10.56499/jppres22.1570_11.3.367