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Research article

Neurobehavioral evaluation and phytochemical characterization of a series of argentine valerian species

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ABSTRACT

Folkloric or galenic preparations of valerian roots and rhizomes have been used as sedatives/anxiolytics and sleep inducers since ancient times. "Valerianas" are plants that naturally grow in our region. Although some of them are used in folk medicine, they lack scientific information. We performed a comparative study of the phytochemical composition and the potential *in vivo* effects of ethanolic extracts of argentine valerian species: *Valeriana carnosa* Sm., *V. clarionifolia* Phil. and *V. macrorhiza* Poepp. ex DC., from "Patagonia Argentina"; *V. ferax* (Griseb.) Höck and *V. effusa* Griseb., from the central part of our country, and *V. officinalis* (as the reference plant). All these plants were rich in phenolic compounds, evidenced the presence of ligands for the benzodiazepine binding site of the $GABA_A$ receptor and were able to induce sedation as assessed by loss-of-righting reflex assays (500 mg/kg, i.p.). Mice treated with *V. macrorhiza*, *V. carnosa* and *V. ferax* extracts showed reduced exploratory behaviors while *V. clarionifolia* produced anxiolytic-like activities (500 mg/kg, i.p.) in the Hole board test. Oral administrations (300 mg/kg and 600 mg/kg, p.o.) evidenced sedative effects for *V. ferax* and anxiolytic-like properties for *V. macrorhiza*, *V. carnosa* and *V. clarionifolia* extracts. Our native valerian species are active on the CNS, validating its folkloric use as anxiolytic/sedative and sleep enhancers.

1. Introduction

The roots and rhizomes of several valerian species and other related plants (e.g. Nardus) have been the source of a peculiar fragrance mentioned in the new Testament, and of a useful natural sedative known many centuries before and recorded by Galen, Dioscorides and Pliny the Elder. The therapeutic properties of Valerian, however, were already mentioned in the Ayurvedic and Chinese Medicines (800 B. C. to 1000 A. D.) (Dweck, 1997; Houghton, 1988).

In modern times the highly respected medicinal properties of Valerian are attested by its citation in more than 20 Pharmacopoeias (Houghton, 1997) and extensive use in Europe, Asia and several other regions of the world.

Valeriana (formerly Valerianaceae) is the largest genus of the Caprifoliaceae family and contains about 200 species (The Plant List, 2013). Even though *V. officinalis* L. is the "universally" considered species, there are several other Valeriana sp. used for the same purposes, depending on the region or country in question. For example, *V. wallichii* DC (Indian Valerian), *V. fauriei* Briquet (Japanese Valerian) (Welfare, 1996) and *V. edulis* Nutt ex. Torr. & Gray (Morazzoni and Bombardelli, 1995) known as "Valeriana mexicana".

Several species of valerians naturally grow in our region. Some of these species have been mentioned in our Argentine National Pharmacopoeia as a substitute for *Valeriana officinalis* for more than 50 years, based on their known folkloric uses in this region. However, most of these species lack botanical, agronomic, phytochemical and

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pharmacological scientific information related to their quality and effectiveness as a medicine or as a dietary supplement. There have been identified 48 valerian species in Argentina (Kutschker, 2011), most of which grow along the Andes mountain range in Patagonia, while there are few described for the central region: Córdoba and San Luis (Chiapella and Demaio, 2015; Zuloaga et al., 1999).

V. carnosa Sm., *V. clarionifolia* Phil., both known as "Ñancolahuen" (or "Ñamkulawen", in Mapuzungum language, "White and hawk medicine", in English, probably in reference to the elevated areas where the species grow and where the *"ñamku"* -hawk- can be seen flying) and *V. macrorhiza* Poepp. ex DC. are species used by traditional aboriginal medicine, mainly by the Mapuche ethnicity, in the Patagonia region. Both "ñancolahuen" species are sold in health food stores and pharmacies as herbal medicines and their anatomical data have been recently described (Bach et al., 2014).

The traditional uses for *V. effusa* Griseb. and *V. ferax* (Griseb.) Höck., from other regions of the country, has been transferred through generations by oral and cultural transmission of information up to the present (Kutschker, 2011). Their subterranean organs are used to treat ailments of the lungs, stomach, liver, kidneys, waist pain and back pain, and for decay (Barboza et al., 2009). They are also used as analgesic and sedative to treat rheumatism (Ochoa et al., 2010), insomnia (Molares and Ladio, 2012), and for disorders related to heart and bladder (Estomba et al., 2006).

Several compounds have been identified in a variety of species of the valerian genus. We may mention among them: a complex essential oil (as the valerenic acid derivatives), several iridoids (named valepotriates and their decomposition products, the baldrinals; which are labile sesquiterpene esters, quite often absent in active extracts), mono- di- and sesquiterpenes, lignans (hydroxypinoresinol (Bodesheim and Hölzl, 1997)), alkaloids, flavonoids, fatty acids, etc (Houghton, 1997, 1999). Neither a particular compound nor a class of compounds proved to be fully responsible of the sedative properties (Hölzl, 1997), Furthermore, there are variable compositions of different valerians with similar activities (Hobbs, 1989; Houghton, 1999). These results have supported the present tentative consensus standing that several different compounds in valerian extracts act synergistically to produce sedative or tranquillizing effects (Hölzl, 1997). We have previously identified from the roots and rhizomes of two valerian species, *Valeriana wallichii* DC and *V. officinalis* L., 6-methylapigenin, a flavone derivative that is a ligand for the benzodiazepine binding site (BDZ-bs) of the $GABA_A$ receptor (the major neurotransmitter system of the central nervous system –CNS–) with anxiolytic properties in mice (Wasowski et al., 2002). A glycosylated flavanone, 2S-hesperidin (Marder et al., 2003) and a glycosylatedflavone, linarin (Fernández et al., 2004), with sedative-hypnotic effects were also identified by us in these species. Relevant synergistic interactions were found when these two glycosides were assayed in combination with other components of the same valerian extract (Fernández et al., 2005; Fernández et al., 2004).

In this work, a phytochemical analysis of ethanol extracts of 5 argentine valerian species, *V. carnosa* Sm., *V. clarionifolia* Phil., *Valeriana macrorhiza* Poepp. ex DC., *V. effusa* Griseb. and *V. ferax* (Griseb.) Höck. was performed. A research for the presence of ligands of the BDZ-bs of the $GABA_A$ receptor was carried out. Furthermore, the sedative, anxiolytic and enhancing of thiopental-induced properties were studied.

The studies carried out here may add information about our native plants, allowing the recognition of their popular use and encouraging the production on a larger scale. This knowledge may allow the generation of new basis to describe quality standards that could be included in new editions of Pharmacopoeias.

2. Materials and Methods

2.1. Plant material and extraction

The studies were performed on roots and rhizomes of specimens collected in Patagonia Argentina and in the province of Córdoba, Argentina, and authenticated by Dr. Hernán Bach from the "Instituto Nacional de Tecnología Agropecuaria (INTA)" and the Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina. Vouchers specimens, cited bellowed, have been deposited at the Herbarium of the Institute of Biological Resources (BAB, BAF), INTA.

Plant materials were as follows (family *Caprifoliaceae*):

- *V. carnosa* Sm.: Río Negro Province, Departamento Capital Cerro Otto: south hillside; 16-III-2012 H. G. Bach & F. O. Robbiati 578 (BAB, BAF); 41° 09′ 37′S; 71° 22′45′W.
- *V. clarionifolia* Phil.: Río Negro Province, Departamento: Pilcaniyeu: 2–3 Km North West from the National airport runway; 15-III-2012 2012 H. G. Bach & F. O. Robbiati 561 (BAB, BAF); 41° 09′ 43′ S; 71° 10′ 05′W.
- *V. macrorhiza* DC.: Neuquen Province: Departamento: Ñorquin, Copahue, Mallin: West to the camping; 13-III-2012 H. G. Bach & F. O. Robbiati 540 (BAB, BAF); 37° 49′ 0,3′S; 71° 06′ 25′W.
- *V. effusa* Griseb.: Córdoba Province. Departamento: Colón, Unquillo, Capilla de Bufo; 26-XI-2010 H. G. Bach 428 (BAB, BAF); 31° 11′ 31′S; 64° 21′47′W.
- *V. ferax* (Griseb.) Höck.: Córdoba Province. Departamento: Punilla, Camino de las altas cumbres, the Condor on the side of the provincial road; 34. 26-XI-2010 H. G. Bach 460 (BAB, BAF); 31° 37′ 9′S; 64° 43′42′W.
- *V. officinalis* L.: grown in Argentina, San Juan Province. Departamento: Calingasta, Barreal, III-2010 M. Ferres s/n (BAB 23452); 31° 33′ 48′S; 69° 27′ 56′W.

After collection, the fresh roots and rhizomes were washed, dried at room temperature and protected from light for 30 days, then a part of them were powdered. The resulting powders were submitted to the extraction and fractionation scheme shown in Figure 1. Powdered dry

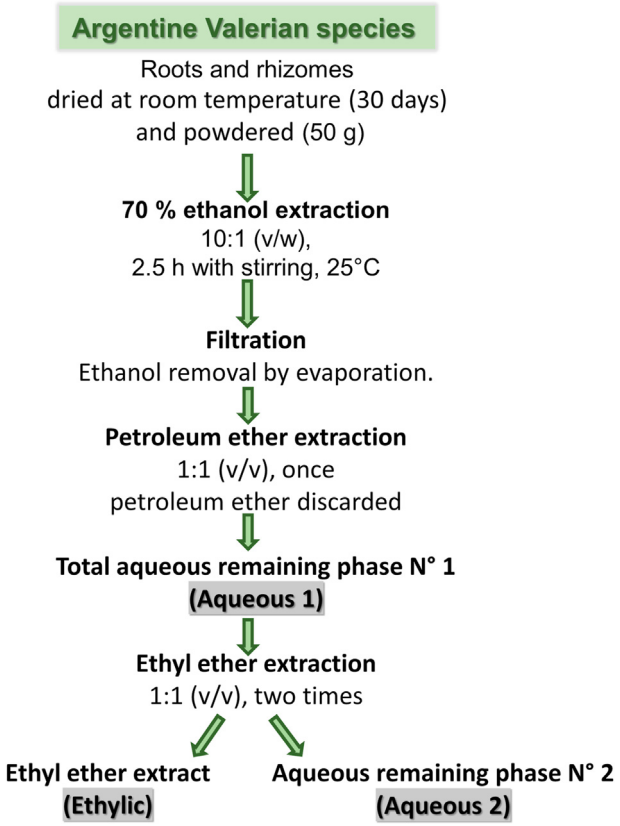


Figure 1. Flow sheet of the valerian fractionation scheme.

roots and rhizomes (50 g) of each valerian were suspended in 500 mL of 70 % ethanol and the mixture was kept 2.5 h at 25 °C, with stirring. The filtrate was concentrated to 1/3 of the original volume to eliminate most of the ethanol and extracted with an equal volume of petroleum ether, which was discarded. Two-thirds of the aqueous phase obtained was evaporated to dryness (extract Aqueous 1) and used for pharmacological evaluations. The other one third was slightly concentrated to eliminate the remained petroleum ether and was extracted twice with an equal volume of ethyl ether. Both resulting phases were evaporated to dryness (extracts Aqueous 2 and Ethylic) and the residues were stored in absence of light at -20 °C. Replicates of the same batch were evaluated. Aliquots from all the valerian extracts, before dryness, were separated for phytochemical determinations.

2.2. Chemicals and reagents

All chemicals and standard drugs like Folin- Ciocalteu reagent, aluminium chloride, gallic acid, rutin, apigenin, chlorogenic acid, hesperidin, valerenic acid and 2-amino ethyl diphenyl boronic acid ester (AEDBE) were purchased from Sigma-Aldrich®, Argentina. Sodium thiopental was obtained from Fada, Biochemie Gesellschaft m.b.H., Kundl/Tirol, Austria. Diazepam was from Hoffmann-La Roche. [^{3}H]flunitrazepam (81.8 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences, Boston, MA, USA. Diosmetin was prepared by acid hydrolysis of diosmin (from Indofine Chemical Company, USA); as previously described (Marder et al., 2003). 6-Methylapigenin was previously isolated from *Valeriana wallichii* DC (Wasowski et al., 2002). All other reagents and solvents used were of analytical or high-performance liquid chromatography (HPLC) grade.

2.3. Phytochemical analysis

2.3.1. Determination of total polyphenols content

Total polyphenols were determined by Folin–Ciocalteu method according to Makkar and cowokers (Makkar, 2003). Briefly, 50 μL of each fraction were made up to 0.5 mL with distilled water. 0.25 mL of Folin-Ciocalteu reagent and 1.25 mL of 20% aqueous sodium carbonate solution were added. Then the test tubes were vortexed. Absorbance was measured at 725 nm against blank, after 40 min. The amount of total polyphenols was expressed as mg galic acid/100 mg dry plant material. Calibration curve of galic acid was developed. All measurements were done in triplicate.

2.3.2. Determination of flavonoids

0.1 mL of each extract were added to 1.4 mL of distilled water and 0.50 mL of flavonoids reactive (400 mg of crystalline sodium acetate and 133 mg of crystalline aluminum chloride) dissolved in 100 mL of extracting solvent (140 mL methanol, 50 mL water, and 10 mL acetic acid). The absorbance was recorded at 416 nm against blank, after 5 min at room temperature. Calibration curve of rutin (a natural flavonoid) was developed. The amount of flavonoids was calculated as mg rutin/100 g dry plant material.

2.3.3. Determination of hydroxycinnamic acids

0.1 mL of each extract were added to 1.9 mL of absolute ethanol. After 5 min at room temperature, the absorbance was recorded at 328 nm against blank. Calibration curve of chlorogenic acid was developed. The amount of hydroxycinnamic acids was calculated as mg chlorogenic acid/100 g dry plant material.

2.3.4. Thin layer chromatography (TLC) characterization

Extracts were submitted to the characterization by thin layer chromatography (TLC). Analyses were conducted in silica gel F_{254} and cellulose (Merck, Darmstadt, HE, Germany) as stationary phases, employing glacial acetic acid: water (6:4 v/v) or acetone 100%, and HCl 0.1N, respectively. After chromatograms development, the plates were dried, and the compounds observed under UV light (254 and 365 nm). Then, plates were sprayed with specific chromogenic reagents according to the class of compounds investigated (5% AEDBE). The retention factors, colour and behavior of the spots were compared with chromatographic profiles of reference substances in the literature (Wagner and Bladt, 1996). Additionally, standard samples of flavonoids were employed for co-TLC analysis.

2.3.5. High-performance liquid chromatography (HPLC)

Analytical HPLC fractionations were performed using an LKB Pharmacia apparatus with C-18 reversed phase Vydac columns (5 mm, 0.46 × 25 cm) (The Separation Group, Hesperia, CA, USA). Each extract was properly injected into the column and eluted using an aqueous acetonitrile (ACN) gradient (v/v) (30 min total analysis time): 10–40% ACN, 0–20 min; 40–80% ACN, 20–30 min. Flow elution was kept constant at 1 mL/min and 200 μL of each sample was injected. The effluent was monitorized at 280 nm. The lyophilized extracts were resuspended in water (final concentration: 0.5 mg/mL) and standards were resuspended in ACN: water (1:1, v/v) (final concentration: 50 μg/mL). All analyses (Retention times (Rt)) were performed in triplicate. The identification of compounds was based on comparison of Rt, UV spectrum, and observation of the increase in the peak area after co-injection of extracts with standards.

2.4. Animals, administrations and procedures

2.4.1. Animals

Both adult male Swiss mice weighing 25–30 g, used in the pharmacological tests, and 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. For behavioral assays mice were accommodated in groups of five in controlled conditions (20–23 °C), free access to food and water, maintained on a 12h/12h day/night cycle and light on at 07:00 AM. 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 (Guid. Care Use Lab. Anim., 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). Accordingly, all possible efforts were made to minimize animal suffering and discomfort and to reduce the number of experimental subjects. Hence, the number of animals used was the minimum number consistent with obtaining significant data. Mice were randomly assigned to any treatment groups and were used only once. The pharmacological tests were evaluated by experimenters who were kept unaware of the treatment administered and were performed between 10:00 AM and 2:00 PM.

2.4.2. Administrations and procedures

The dried fractions obtained from the Aqueous 1 extracts of each valerian, were dissolved by the sequential addition of dimethylsulfoxide, a solution of 0.25% Tween 80 and saline; up to final concentrations of 5%, 20% and 75%, respectively, for the intraperitoneally (i.p.) injections. For oral gavage (p.o.) administrations the extracts were dissolved in carboxymethyl cellulose (CMC) 0.5%. Sodium thiopental was dissolved in saline.

The volume of i.p. injections and p.o. administrations were 0.10–0.20 mL/30 g and 0.30 mL/30 g of body weight, respectively. Accordingly, the assays were performed 30 min and 60 min after i.p. or p.o. administrations, respectively. Food and water were available as necessary, although mice were fasted two hours before the oral administrations.

In each session, a control group receiving only vehicle (VEH) was tested in parallel with those animals receiving drug treatment. VEH

control mice showed no significant differences in any of the tests assayed compared to mice treated with saline (data not shown). Protein concentration was determined by the method of Bradford using bovine serum albumin as standard (Bradford, 1976). Doses of the extracts used for this study were chosen based on pilot experiments and previous reports (Fernández et al., 2004; Marder et al., 2003; Wasowski et al., 2002).

2.5. Pharmacological studies

2.5.2. Behavioral studies

2.5.2.1. Sodium thiopental induced loss of righting reflex assay. The experiment was conducted using the method described by Joo et al. and Loscalzo et al. (Joo et al., 1999; Loscalzo et al., 2009). A sub-hypnotic dose of sodium thiopental (35 mg/kg) was i.p. injected to mice 30 min after a similar i.p. injection of vehicle or the aqueous 1 extracts of each valerian (500 mg/kg) (see protocol in Figure 2A). After the sodium thiopental injection, each mouse was placed in individual cages. When the mice stood still, they were gently placed on their backs, and the time they failed to right themselves was recorded. The latent period (time between thiopental sodium administration to loss of righting reflex) and duration of sleep (time between the disappearance and the reappearance of the righting reflex) was observed for each mouse. It was set a cut off of 1800 s (30 min).

2.5.2.2. Hole board assay. The behavioral profiles of mice under the influence of the extracts were assessed singly 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 apparatus and allowed to explore it for 5 min. The measured parameters were the number of head dips and the time spent head dipping (recorded automatically); the number of rearings, groomings and fecal boli depositions (counted by the observer). Rearing was taken as the number of times the mouse was standing on its hind limbs or with its forelimbs against the wall of the observation cage or in the free air. Grooming is an innate stereotyped behavior found in most animal species (Spruijt et al.,

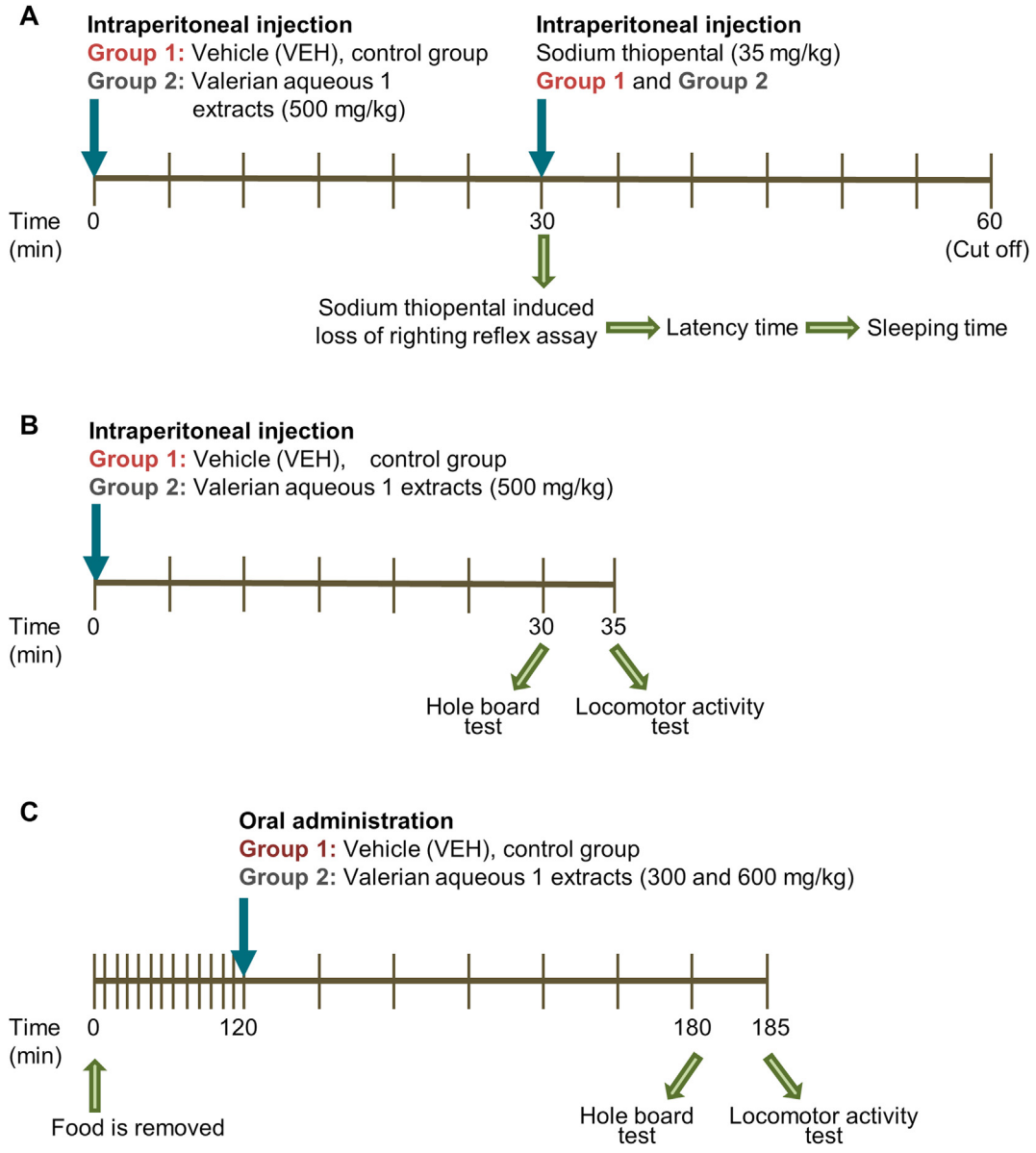


Figure 2. Protocol of administration procedures. A) Sodium thiopental induced loss of righting reflex assay after intraperitoneal (i.p.) injections; B and C) Hole board and Locomotor activity tests after intraperitoneal (i.p.) and oral administrations (p.o.) administrations, respectively.

1992). Grooming was taken as the number of body cleaning with paws, picking of the body and pubis with mouth and face washing actions.

The test was performed 30 min after the i.p. injection of 500 mg/kg of each plant or vehicle, and 1 h after the oral administration of 300 mg/kg and 600 mg/Kg of each plant or vehicle (see protocols in Figure 2B and C, respectively).

2.5.2.3. Locomotor activity. The spontaneous locomotor activity was automatically measured as previously described (Fernández et al., 2006) and was expressed as total light beam counts per 5 min. Briefly, the mice were individually placed in a Plexiglass box, with a floor of 30 cm by 15 cm and 15 cm high walls. On the walls and along the longest axis the box has 15 infrared movement sensors. Sensor interruptions measure the animal activity along a single axis (the long one). The interruption of a sensor and duration of this event were detected and recorded by a personal computer running a Visual Basic program developed specifically for this apparatus. This assay was performed, for each mouse, immediately after the Hole board (35 min after the i.p. injections, Figure 2B; and 65 min after the oral administration, Figure 2C). The Hole board apparatus and the Locomotor activity arena were cleaned with 60 % ethanol between each animal's trial. The animals were used only once.

2.6. Biochemical assays

2.6.1. Tissue preparation

For [^{3}H]-flunitrazepan binding assays, membranes were prepared according to literature (Medina et al., 1990). Briefly the brains were rapidly dissected out on ice and the different structures were homogenized in 10 volumes of 0.32 M sucrose and centrifuged at 900 x g for 10 min. The resulting supernatant was centrifuged at 100,000 x g for 30 min and the pellet washed twice in 25 mM Tris–HCl buffer pH 7.4 at 100,000 x g for 30 min, and stored at -80 °C until used.

2.6.2. [^{3}H]-flunitrazepan binding assay

A radioligand binding assay was used to evaluate the putative action of the plants on the BDZ-bs of the $GABA_A$ receptor complex. The binding of [^{3}H]-flunitrazepam (81.8 Ci/mmol) to the BDZ-bs was performed in washed crude synaptosomal membranes from rat cerebral cortex prepared as described previously (Medina et al., 1990). The extracts were added to 0.2–0.3 mg membrane protein suspended in 1 ml of 25 mM Tris–HCl buffer in the presence of [^{3}H]-flunitrazepam 0.3 nM. In the screening assays each extract was tested at 1 mg/ml in triplicate. Diazepam (10 μM) was used as positive control. Non-specific binding was measured in the presence of flunitrazepam 10 μM and represented 5–15% of the total binding. The incubations were carried out at 4 °C for 1 h. After incubation, the assays were terminated by filtration under vacuum through Whatman GF/A 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.

2.7. Statistical analyses

Data for the hole board and the locomotor activity tests were expressed as mean ± SEM (standard error of the mean) and were analysed by one-way analysis of variance (ANOVA) and *post hoc* comparisons between treatments and vehicle were made using Dunnett's multiple comparison test (Prism 8.00, GraphPad Software). For the sodium thiopental induced loss of righting reflex assay, the median (interquartile range) was calculated and Dunn's multiple comparison test was used after the Kruskal Wallis test (nonparametric analysis of variance) (Prism 8.00, GraphPad Software). Significance levels were set at $P < 0.05$.

3. Results

3.1. Phytochemical analysis

Results of total phenol, flavonoids, and hydroxycinnamic acids quantification present in all the Valerian's extracts depicted in Figure 1 are shown in Table 1. All the plant species studied exhibited high content of phenolic compounds, with values between 125.52 and 890.25 mg galic acid/100 mg dry plant material, with the following rank: *V. effusa* < *V. ferax* < *V. macrorhiza* < *V. clarionifolia* < *V. officinalis* < *V. carnosa*. Besides, all the plant tested had higher total hydroxycinnamic acids contents than flavonoids contents, with a similar rank order of *V. effusa* < *V. macrorhiza* < *V. clarionifolia* < *V. ferax* < *V. officinalis* < *V. carnosa*.

TLC and HPLC analyses of the aqueous 1 extracts of all valerian studied in this work showed the existence of hesperidin and of chlorogenic acid in their composition (Supplementary Materials Figures 1A, 1B and 1D). However, none of the aqueous 1 extracts showed the presence of valerenic acid (Supplementary Materials Figure 1C). Additionally, TLC and HPLC analyses of the ethylic extracts of *V. clarionifolia* and *V. macrorhiza* suggested the presence of diosmetin (Supplementary Materials Figures 2 and 3) and 6-methylapigenin (Supplementary Materials Figure 4), respectively. Molecular structures of the compounds are shown in Supplementary Materials Figure 5.

3.2. Pharmacological studies

3.2.1. Effect of argentine valerian species aqueous 1 extracts on sodium thiopental induced loss of righting reflex assay

The effect of valerian aqueous 1 extracts on sodium thiopental induced loss of righting reflex assay is shown in Figure 3. By the i.p. route, at the dose of 500 mg/kg, all the extracts significantly augmented the time of loss of righting reflex induced by sodium thiopental. The strength of the extracts action in decreasing order is: *V. officinalis* > *V. macrorhiza* ≅ *V. carnosa* > *V. ferax* > *V. effuse* ≅ *V. clarionifolia* (Figure 3A). Meanwhile, only *V. clarionifolia*, *V. macrorhiza*, *V. ferax* and *V. officinalis* were able to significantly reduce the latency time to the disappearance of the righting reflex of mice ($P < 0.05$, $P < 0.01$, $P < 0.05$ and $P < 0.001$, respectively) (Figure 3B).

3.2.2. Exploratory behavior of mice after argentine valerian species aqueous 1 extracts administration on the hole board and locomotor activity assays

3.2.2.1. Effects of the extracts by the i.p. route. The behavioral action of the valerian aqueous 1 extracts on the hole board and locomotor activity tests by the i.p. route, at the dose of 500 mg/kg, are shown in Figure 4. Mice administered with *V. clarionifolia*, significantly increased the number of holes explored vs. vehicle control group ($P < 0.001$) in the hole board assay. However, *V. macrorhiza* and *V. carnosa* significantly decreased the number of holes explored vs. vehicle control group ($P < 0.05$ and $P < 0.01$, respectively) in this test. Meanwhile, *V. macrorhiza*, *V. carnosa* and *V. ferax* were able to significantly reduce the time the mice spent head dipping ($P < 0.05$, $P < 0.001$ and $P < 0.05$, respectively) (Figure 4A). On the other hand, no significant differences were observed in the number of rearings, number of groomings and number of fecal boli depositions between mice injected with the extracts vs. vehicle control group (Figure 4B). Spontaneous locomotor activity of mice was significantly reduced by *V. carnosa*, at a dose of 500 mg/kg, ($P < 0.001$) (Figure 4C).

3.2.2.2. Effects of the extracts by the oral route. Those extracts of the series that showed *in vivo* activity by the i.p. route in the hole board and locomotor activity assays, were selected to be tested by the oral route. Therefore, behavioral actions of the argentine valerians aqueous 1 extracts of *V. carnosa*, *V. macrorhiza*, *V. clarionifolia* and *V. ferax* were

Table 1. Total phenolics, flavonoids and hydroxycinnamic acids content of the argentine valerian species extracts investigated.

Plant	Extract	mg galic acid/100 mg dry plant material[a]	mg rutin/100 g dry plant material[a]	mg chlorogenic acid/100 g dry plant material[a]
V. effusa	Aqueous 1	125.52 ± 6.80	8.82 ± 1.42	50.83 ± 4.70
	Aqueous 2	88.91 ± 2.04	7.55 ± 0.61	47.17 ± 1.28
	Ethylic	25.63 ± 2.31	0.98 ± 0.78	25.74 ± 1.41
V. ferax	Aqueous 1	191.56 ± 8.99	32.76 ± 4.87	273.98 ± 15.28
	Aqueous 2	133.66 ± 38.49	19.70 ± 1.18	85.21 ± 7.11
	Ethylic	123.14 ± 7.55	3.31 ± 1.10	22.81 ± 1.69
V. macrorhiza	Aqueous 1	252.85 ± 12.36	14.79 ± 0.55	74.17 ± 6.56
	Aqueous 2	145.97 ± 23.15	13.48 ± 1.90	85.44 ± 6.24
	Ethylic	87.58 ± 13.60	1.51 ± 0.31	30.83 ± 0.82
V. clarionifolia	Aqueous 1	257.36 ± 19.55	22.55 ± 1.19	166.64 ± 14.73
	Aqueous 2	178.70 ± 17.99	8.51 ± 1.59	91.96 ± 7.76
	Ethylic	20.62 ± 0.50	1.63 ± 0.14	17.20 ± 2.19
V. carnosa	Aqueous 1	890.25 ± 156.05	81.98 ± 12.46	1449.54 ± 84.04
	Aqueous 2	451.52 ± 27.38	16.95 ± 2.32	1006.81 ± 7.76
	Ethylic	75.69 ± 8.47	4.75 ± 3.37	273.93 ± 20.98
V. officinalis	Aqueous 1	736.19 ± 34.27	70.03 ± 8.89	676.38 ± 45.19
	Aqueous 2	113.82 ± 6.87	11.62 ± 1.99	140.39 ± 17.61
	Ethylic	33.74 ± 2.67	2.36 ± 0.10	30.37 ± 1.52

[a] Mean ± 2 SD of three independent determinations.

measured at the doses of 300 mg/kg and 600 mg/kg and are shown in Figure 5. *V. macrorhiza* (300 mg/kg) and *V. carnosa* (600 mg/kg) significantly increased the number of holes explored vs. vehicle control group ($P < 0.05$). Also, *V. carnosa* was able to increase the time spent head dipping ($P < 0.05$) in this test (Figure 5A).

Mice administered with *V. macrorhiza* (600 mg/kg), *V. clarionifolia* (300 mg/kg) and *V. ferax* (300 mg/kg and 600 mg/kg) significantly decreased the number of rearings in the hole board assay ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively). At the same time, number of groomings of mice were significantly reduced when mice were treated with *V. carnosa* and *V macrorhiza* at the doses of 300 mg kg ($P < 0.05$) and 600 mg/kg ($P < 0.01$), and with *V. clarionifolia* at the dose of 600 mg/kg ($P < 0.05$) (Figure 5B). On the other hand, the administration of *V. macrorhiza* at 600 mg/kg p.o. was able to significantly increase the number of fecal boli depositions of mice vs. vehicle control group ($P < 0.05$) (Figure 5B).

Spontaneous locomotor activity of mice was significantly reduced by *V. ferax*, at the doses of 300 mg/kg and 600 mg/kg, ($P < 0.001$). While *V. macrorhiza* at the dose of 600 mg/kg p.o. was able to significantly increase locomotion of mice (Figure 5C).

3.3. Biochemical studies

3.3.1. In vitro [^{3}H]-flunitrazepan binding screening

All the extracts depicted in Figure 1 were evaluated in this assay. The capacity of these extracts to inhibit the binding of [^{3}H]-FNZ to rat BDZ-bs of the $GABA_A$ receptor is shown in Figure 6. All ethylic extracts were able to displace the radioligand, at the concentration tested (1 mg/ml), meanwhile only the aqueous extract 1 of *V. effusa and V. clarionifolia* were able to inhibit the binding of this specific radioligand, at 1 mg/ml. None of the aqueous extracts 2 were active in this assay.

4. Discussion

The present study evaluated the pharmacological profile of the ethanol extracts of 5 argentine valerian species, *V. carnosa* Sm., *V. clarionifolia* Phil., *Valeriana macrorhiza* Poepp. ex DC., *V. effusa* Griseb. and *V. ferax* (Griseb.) Höck., including the commercially available *V. officinalis* L. Therefore, tests for sedative/anxiolytic-like and spontaneous locomotion were conducted in rodents. Moreover, this work presented an analysis of their phytochemical composition and a study of the possible presence of ligands for the BDZ-bs of the $GABA_A$ receptor, the major inhibitory system of the CNS.

First, all the plant species were collected in their native region and techniques for preservation and conservation were performed. According to the European Pharmacopoeia 2nd edn. (1993), the active principles of valerian are extracted, using ethanol of suitable concentration, by maceration or percolation. Similarly, the Dutch Pharmacopoeia 6th edn., 2nd printing (1966) and the German Pharmacopoeia, "Deutsches Arzneibuch", DAB 10 (1993), indicate the maceration or percolation, respectively, of one part of valerian root and 5 parts of ethanol (70%, v/v). According to this information, the extracts studied in this work were prepared by a 70 % ethanol extraction 10:1 (v/w), 2.5 h with stirring, of the powdered roots and rhizomes of these 6 valerian species.

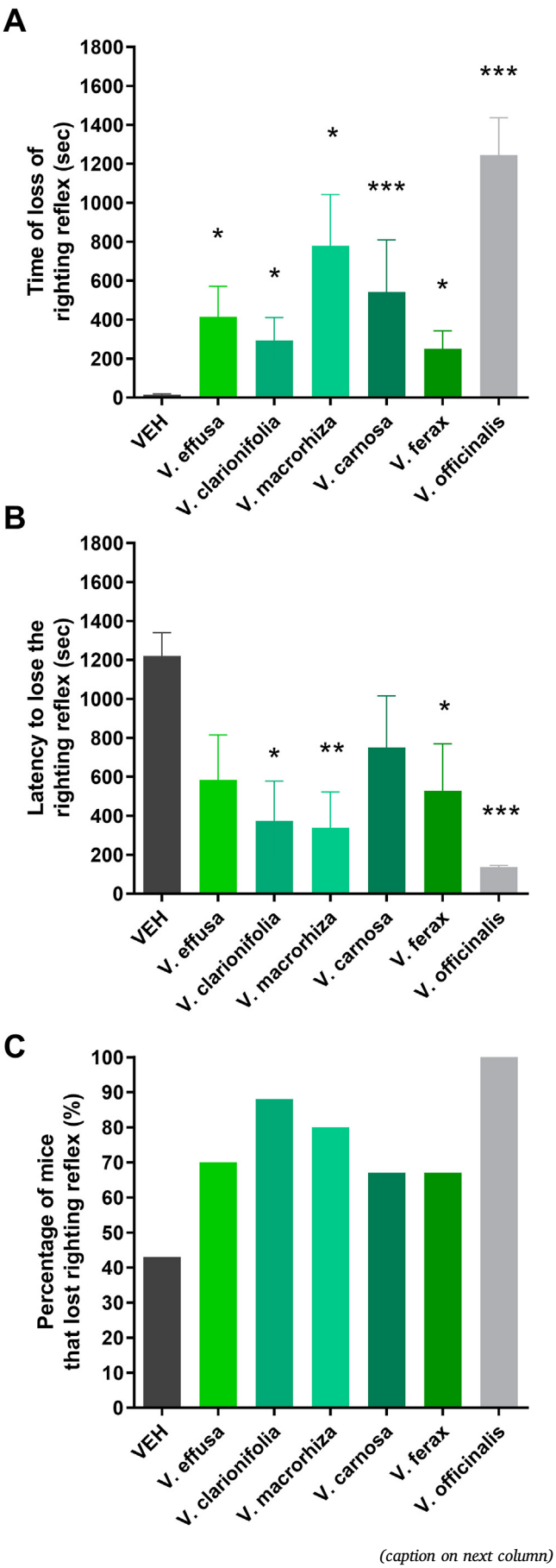
We have previously reported the presence of herperidin in the roots and rhizomes of *Valeriana officinalis* and *V. wallichii* and, for the first time, described its remarkable activity on the CNS (Marder et al., 2003). In addition, it has been described that the depressant effect of hesperidin can be partially mediated by an opioid mechanism (Loscalzo et al., 2008) and through the adenosine receptors (Guzmán-Gutiérrez and Navarrete, 2009). On the other hand, an apigenin derivative, 6-methylapigenin (4′, 5,7-dihydroxy-6-methylflavone), was also reported as a BDZ-bs ligand from the roots and rhizomes of *Valeriana wallichii* (Wasowski et al., 2002). This flavone induced anxiolytic effects in mice treated by the i.p. route and was able to potentiate hesperidin induce sedation as assessed by the loss of righting reflex assay (Marder et al., 2003). Several studies on the Valeriana genus indicate that the main active ingredients are the valepotriates, lignans, flavonoids, tannins, phenolic acids and essential oils (Kutschker et al., 2010).

Of all the Patagonian species belonging to this genus, the dry extract of the whole *V. carnosa* plant has been the most studied. It has been found that its valepotriate composition pattern, and especially its valtrates, is similar to that of *V. officinalis*. In addition, caffeoyl methyl ester and two pinoresinol-type lignans have been isolated from their extracts (Molares and Ladio, 2018) and the presence of chlorogenic and caffeic acid has been already reported in this species (Guajardo et al., 2018).

All the aqueous 1 extracts studied in this work exhibited high content of phenolic compounds, had higher total hydroxycinnamic acids contents than flavonoids contents and have no valerenic acid. In addition, we demonstrated the presence of hesperidin and of chlorogenic acid in all the plants assayed and the existence of diosmetin in *V. clarionifolia*. We have already reported the CNS action of diosmetin, a flavone derivative, which evidenced no affinity for the BDZ-bs that demonstrated sedative properties in mice (Fernández et al., 2006). Furthermore, the presence of 6-methylapigenin in *V. macrorhiza* has been acknowledged here.

These native valerian extracts possess several compounds; in consequence their pharmacological activity cannot always be attributed to only one constituent from the herbal preparation. Hence, the effect of the extracts could be due to synergistic action of the constituents on different receptors and other regulatory processes, as we have already described for *V. wallichii* and *V. officinalis* (Fernández et al., 2005; Fernández et al., 2004; Marder et al., 2003).

In the evaluation of the action of a drug on the CNS, it is critical to study its effect on animal behavior. Similarly, route of administration is one of the most important factors affecting the results of *in vivo* effects. The choice of administration route should depend upon the purpose of the assay and the nature of the test substance. It is suggested that the i.p. route is more suitable for primary screening *in vivo* assays, as it might be more sensitive than those with oral administration (Hayashi et al., 1989). Also, the i.p. route (not likely to be used in human) was commonly selected to determine the inherent toxicity of chemicals. Moreover, it must be considered that the effects of oral dose are subjected to systemic bioavailability and hepatic metabolism. Therefore, varied responses caused by the plant extracts assayed could be attributed to the different routes of administration that could lead to the production of diverse



(caption on next column)

Figure 3. Effectiveness of argentine valerian species aqueous 1 extracts on sodium thiopental induced loss of righting reflex assay in mice. Results are expressed as mean ± S.E.M. of A) time of loss of righting reflex, B) latency time to lose righting reflex and C) percentage of mice that lost the righting reflex; after 30 min of an i.p. injection of vehicle (VEH) or the Argentinian valerian species aqueous 1 extracts (500 mg/kg, i.p.) (see Figure 1). The latency to lose righting reflex was measured as the time spent between the sodium thiopental injection (45 mg/kg, i.p.) and the disappearance of the reflex (a cut off of 1800 s was set for those mice that did not lose the reflex) and the time of loss the righting reflex was quantified as the time spent between disappearance and reappearance of righting reflex (cut off of 1800 s) (see Methods). n = number of mice tested. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from vehicle; Dunn's multiple comparison test after Kruskal-Wallis test (nonparametric ANOVA).

active metabolites. Consequently, the first screening of the *in vivo* effects of the native valerian extracts were performed by i.p. administration procedures. Enhancement of barbital induced sedation is a good index of CNS depressant activity (Fujimori, 1965). Substances that have CNS depressant activity either decrease the time for onset of loss of righting reflex, prolong the duration of this loss or both. Sodium thiopental induced sedation test revealed that all valerian aqueous 1 extracts (500 mg/kg, i.p.) increased the duration of loss of righting reflex induced by this barbituric. *V. clarionifolia*, *V. macrorhiza*, *V. ferax* and *V. officinalis* extracts were also able to reduce the latency time to the disappearance of the righting reflex of mice, a parameter that is also measured in this test. Therefore, all the valerian extracts have CNS depressant activities when administered by the i.p. route.

The exploratory behavior of mice was studied on the hole board apparatus. It was reported that a decrease number of the head dips explored by the animals, as well as in the time spent exploring the holes, shows CNS depression or sedation (when accompanied by a decrease in locomotion of rodents) (File and Wardill, 1975). Furthermore, this test can be also used to study anxiolytic agents (Crawley, 1985) as it combines the assumption that head dipping of animals is inversely proportional to their anxiety-like state in a moderate environment. Therefore, increased number of head dips on the board means reduced anxiety state (Bilkei-Gorzó and Gyertyán, 1996). The head dipping behavior of mice in the hole board test after the i.p. administration of the argentine valerian aqueous 1 extract (500 mg/kg) showed that *V. macrorhiza*, *V. carnosa* and *V. ferax* were able to significantly reduce the head dipping behavior of mice, revealing their depressant properties. Meanwhile, *V. clarionifolia* significantly increased the number of holes explored evidencing its anxiolytic-like effect at this dose by an i.p. injection. On the other hand, extracts administered by oral gavage evidenced anxiolytic-like effect of *V. macrorhiza* (300 mg/kg) and *V. carnosa* (600 mg/kg), as these extracts were able to significantly increase head dipping behavior of mice vs. vehicle control group.

Another important stage in evaluating drug action on CNS is to study its effect on locomotor activity of the animal. Spontaneous locomotor activity is a parameter used to measure central excitability of animals, while its decrease may be closely related to sedation resulting from depression of the CNS. *V. macrorhiza* (600 mg/kg, p.o.) was able to induce an increase in the spontaneous locomotion of mice, whereas *V. carnosa* (after its i.p. administration) and *V. ferax* (after its p.o. administration) significantly decreased the locomotor activity of mice, as shown by the results of the locomotor activity test, reinforcing that these extracts have sedative effects.

Although *Valeriana officinalis* L. extracts have been used for treating mild sleep disorders and nervous tension, the pharmacological actions accounting for its clinical efficacy remain unclear. The effects of different commercially available preparations of this valerian extract after their oral administrations were already reported, using different behavioral paradigms in mice and rats. The data reported that none of the *Valeriana officinalis* extracts studied displayed sedative effects (500 or 1000 mg/kg). Neither spontaneous locomotor activity was reduced, nor was the

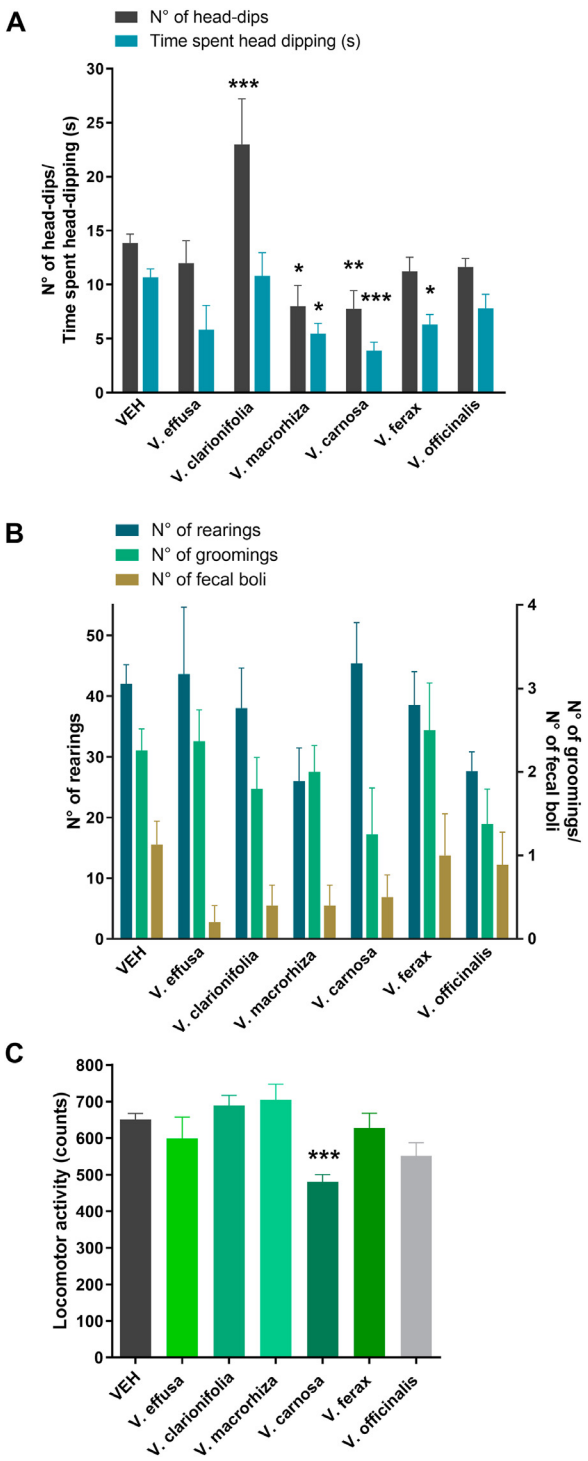


Figure 4. Effect of argentine valerian species aqueous 1 extracts in the hole-board and the locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of A) number of head dips (left scale) and time spent head dipping (s) (left scale), B) number of rearings (left scale), number of groomings (right scale) and number of fecal boli (right scale) registered in a 5 min session, 30 min after the i.p. injections and C) mean ± S.E.M. of the spontaneous locomotor activity counts registered after completing the hole board assay in 5 min sessions and 35 min after the i.p. injections of vehicle (VEH) or 500 mg/kg of the aqueous 1 extract of each valerian. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from vehicle; Dunnett's Multiple Comparison test after ANOVA. Number of animals per group ranged between 6-8, control animals (VEH) were 31.

duration of ether-induced narcosis prolonged. In contrast, pronounced anxiolytic effects were revealed in the plus maze test, a highly well-known assay to assess anxiety-like in rodents. It was proposed that not sedative but anxiolytic-like and antidepressant-like activities considerably contribute to the sleep enhancing properties of the valerian extracts studied in that work (Hattesohl et al., 2008). Accordingly, in the present study *V. officinalis* aqueous 1 extract (500 mg/kg, i.p.) could not modify the behavior of mice in the hole board and locomotor activity assays. Nevertheless, its sodium thiopental enhancing properties were evidenced, as stated above.

Another three parameters are also important to describe CNS effects of drugs in the hole board test in mice: number of rearing, grooming and fecal boli. Rearing, also known as vertical locomotion, is characterized by a vertical posture in which the animal either stands on its hind limbs while raising up its forearms in the air or placing them on the wall of the cage (Hattesohl et al., 2008). It is used as a measure of CNS activity (Aderibigbe and Agboola, 2011) as it is employed by rodents as a survival strategy; used to adapt and acclimatize to a novel environment for protection and possible escape route (Blanchard et al., 2001). The ability of an agent to increase or decrease rearing behavior in rodents has been linked to increase CNS excitation or depression; consequence of increased activities of excitatory or inhibitory neurotransmitters, respectively. Therefore, it is used to classify agents either as CNS stimulant or depressant; both locomotor and rearing activities of rodents are central excitatory behaviors and indicative of the rodent's explorative ability. A reduction in rearings in rodents points to a central inhibitory effect that can be correlated also with reduced anxiety-like behaviors, when the locomotor activity is not modified. In this study, oral gavage of *V. macrorhiza* (600 mg/kg), *V. clarionifolia* (300 mg/kg) and *V. ferax* (300 mg/kg and 600 mg/kg) significantly decreased the number of rearings in the hole board assay.

Grooming is considered a response to stress (D'Aquila et al., 2000; Moody et al., 1988; Rodríguez Echandía, Broitman and Fóscolo, 1983), novelty induced grooming has long been studied in neurobehavioral stress research in mice and rats (Enginar et al., 2008; Whyte and Johnson, 2007). Grooming is a significant factor of the rodent's behavioral repertoire, it is the initial behavioral response to stressful situations and is used by animals to lower arousal (Kalueff & Tuohimaa, 2004, 2005a, 2005b). Regulation of grooming behavior can be mediated by multiple brain regions, as well as by various endogenous agents, and psychotropic drugs. The role of grooming as a behavioral marker of anxiety has been extensively studied. Additionally, defecation, quantified by counting the number of fecal boli deposited during a session, is used as a measure of anxiety as well (Delprato et al., 2017; Ferré et al., 1995; Hall, 1934). Oral administrations of *V. carnosa* and *V macrorhiza* (300 mg/kg and 600 mg/kg), and *V. clarionifolia* (600 mg/kg) were able to significantly reduce the number of groomings of mice. Meanwhile, *V. macrorhiza* (600 mg/kg), was also able to significantly increase the number of fecal boli. So, these extracts showed anxiolytic-like activities.

Neuropharmacology is based simply on the fundamental balance between chemical excitation and inhibition. These processes are indispensable in the networks of neurons, and most neurons receive inputs from both excitatory and inhibitory synapses. The GABA system is the main inhibitory neurotransmitter system in the brain and is the target for many clinically used drugs to treat anxiety disorders and insomnia. The results obtained here suggest that the behavioral effects induced by the plant extracts studied could involve the BDZ-bs of the $GABA_A$, as all the ethylic extracts (that are a part of aqueous 1 extract) evidenced the presence of ligands for the BDZ-bs of the $GABA_A$ receptor.

In summary:

a) All the valerian extracts showed CNS depressant activities when administered by the i.p. route as they were able to induce sedation as

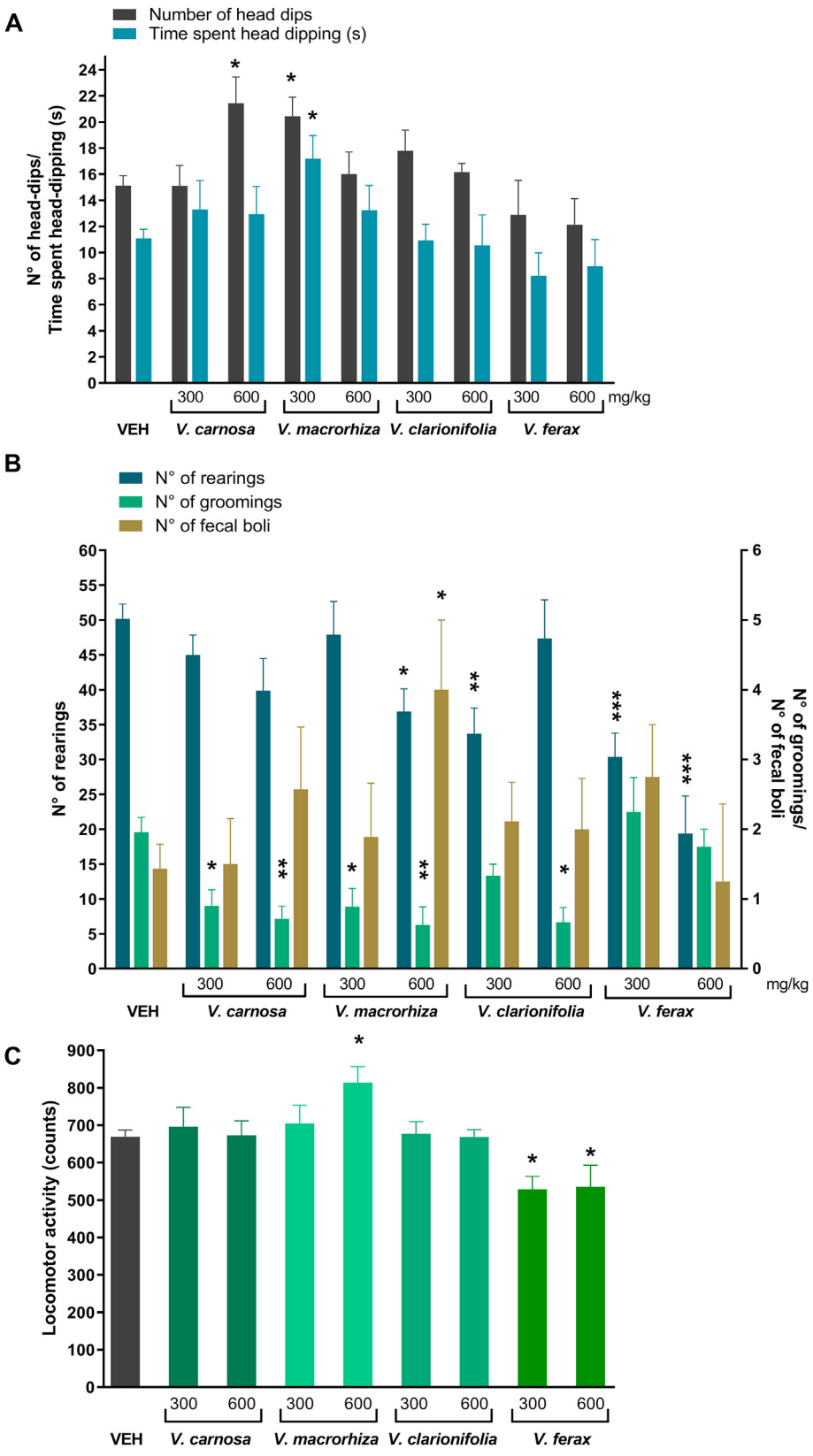


Figure 5. Effect of the oral administration of argentine valerian species aqueous 1 extracts in the hole-board and the locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of A) number of head dips (left scale) and time spent head dipping (s) (left scale); B) number of rearings (left scale), number of groomings (right scale) and number of fecal boli (right scale) registered in a 5 min session, 60 min after the oral administrations and C) mean ± S.E.M. of the spontaneous locomotor activity counts; registered after completing the hole board assay in 5 min sessions and 65 min after the oral administration of vehicle (VEH), 300 mg/kg or 600 mg/kg of the aqueous 1 extract of each valerian. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from vehicle; Dunnett's Multiple Comparison test after ANOVA. Number of animals per group ranged between 6-10, control animals (VEH) were 23.

assessed by the sodium thiopental loss-of-righting reflex assay. Furthermore, all the extracts evidenced the presence of ligands for the BDZ-bs of the $GABA_A$ receptor.

b) *V. clarionifolia* extract evidenced anxiolytic-like effects in mice as it stimulated exploratory activity, by increasing the number of holes explored in the hole board test after its i.p. administration and by reducing the number of rearings and grooming behaviors measured in this assay, with no altered locomotion, by the oral route.

c) *V. ferax* extract induced sedative effects in mice in the hole board assay, as this extract was able to decrease the exploratory behavior of

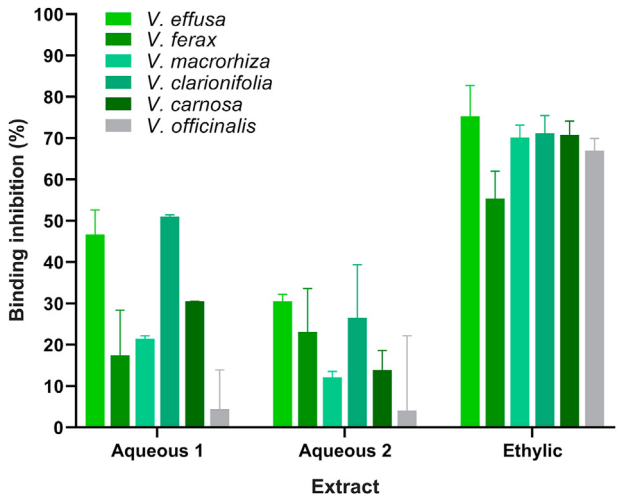


Figure 6. Capacity of the argentine valerian species extracts to inhibit the binding of [^{3}H]-flunitrazepam to the BDZ-bs of the $GABA_A$ receptor. Results are expressed as mean ± S.E.M. of percentage of binding inhibition of valerian aqueous 1, aqueous 2 and ethylic extracts (1.0 mg/kg) (see Figure 1). Inhibition <30% was considered as no inhibition. Diazepam (10 μM) showed 90 % of binding inhibition.

mice (reduced time mice spent head dipping, i.p., and decreased rearings, p.o.).

d) *V. macrorhiza* extract showed anxiolytic-like activities after oral administrations, as this plant produced an increase in the hole parameters and in the number of defecations as well as a decrease in the number of rearings and grooming behaviors measured in the hole board assay. Moreover, spontaneous locomotion of mice was augmented after its oral administrations. Otherwise, *V. macrorhiza* extract showed a decrease in exploratory behaviors after its i.p. injection, as the hole parameters of mice measured in the hole board assay were decreased, evidencing its depressant CNS activity.

e) *V. carnosa* extract showed anxiolytic-like activities after oral administrations, as this extract induced an increase in the number of holes explored and a decrease in the number of groomings measured in the hole board assay. Also, mice evidenced no motor impairment after its oral administrations. Furthermore, it showed a decrease in locomotion and exploratory behaviors after its i.p. injection, as the hole parameters of mice measured in the hole board assay were decreased, evidencing its depressant CNS activity.

5. Conclusion

Physiopathology of stress, anxiety and sleep disorders involves numerous pathways, and different neurotransmitters participate on its underlying mechanisms. This work does not provide evidence to determine the exact mechanisms responsible for the observed effects, and therefore, further studies will be needed. However, the results bring to light the potential use of these valerian extracts as an alternative natural source for the treatment of CNS disorders to offer a better quality of life to patients affected by anxiety, stress, and insomnia.

Declarations

Author contribution statement

M. Marder: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

C. Marcucci: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

J.M.A. Relats and F. Kamecki: Performed the experiments; Analyzed and interpreted the data.

H.G. Bach: Performed the experiments; Contributed reagents, materials, analysis tools or data.

B. Varela, M. Wagner, V. Pastore and N. Colettis: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

R. Ricco: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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References

Aderibigbe, A., Agboola, O., 2011. Neuropharmacological property of struchium sparganophora (Linn) O. ktze in mice. 6. Asian Journal Of Traditional Medicines 6, 104–111.

Bach, H.G., Varela, B.G., Fortunato, R.H., Wagner, M.L., 2014. Pharmacobotany of two valeriana species (Valerianaceae) of Argentinian Patagonia known as "ñancolahuen.". Latin American Journal of pharmacy 33 (6), 891–896.

Barboza, G.E., Cantero, J.J., Nuñez, C., Ariza Espinar, L., Pacciaroni, A., del, V., 2009. Medicinal plants: a general review and a phytochemical and ethnopharmacological screening of the native Argentine Flora. Kurtziana. Retrieved from. https://ri.conicet.gov.ar/handle/11336/24328.

Bilkei-Gorzó, A., Gyertyán, I., 1996. Some doubts about the basic concept of hole-board test. Neurobiology (Budapest, Hungary) 4 (4), 405–415. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/9200132.

Blanchard, D., Griebel, G., Blanchard, R., 2001. Mouse defensive behaviors: pharmacological and behavioral assays for anxiety and panic. Neurosci. Behav. Biobehav. 25 (3), 205–218. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/11378177.

Bodesheim, U., Hölzl, J., 1997. [Isolation and receptor binding properties of alkaloids and lignans from Valeriana officialis L]. Pharmazie 52 (5), 386–391. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/9229719.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/942051.

Chiapella, J.O., Demaio, P.H., 2015. Plant endemism in the Sierras of Córdoba and San Luis (Argentina): understanding links between phylogeny and regional biogeographical patterns. PhytoKeys 47 (47), 59–96.

Crawley, J., 1985. Exploratory behaviour models of anxiety in mice. Neurosci. Behav. 9, 37–44.

D'Aquila, P.S., Peana, A.T., Carboni, V., Serra, G., 2000. Exploratory behaviour and grooming after repeated restraint and chronic mild stress: effect of desipramine. Eur. J. Pharmacol. 399 (1), 43–47. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/10876021.

Delprato, A., Algéo, M.-P., Bonheur, B., Bubier, J.A., Lu, L., Williams, R.W., et al., 2017. QTL and systems genetics analysis of mouse grooming and behavioral responses to novelty in an open field. Gene Brain Behav. 16 (8), 790–799.

Dweck, A., 1997. AN INTRODUCTION TO VALERIAN Valeriana officinalis and related species. In: Houghton, P.J. (Ed.), Valerian. Harwood Academic Publishers, Amsterdam, pp. 1–19. Retrieved from. http://www.dweckdata.co.uk/Published_papers/Valerianaceae.pdf.

Enginar, N., Hatipoğlu, I., Firtina, M., 2008. Evaluation of the acute effects of amitriptyline and fluoxetine on anxiety using grooming analysis algorithm in rats. Pharmacol. Biochem. Behav. 89 (3), 450–455.

Estomba, D., Ladio, A., Lozada, M., 2006. Medicinal wild plant knowledge and gathering patterns in a Mapuche community from North-western Patagonia. J. Ethnopharmacol. 103 (1), 109–119.

Fernández, S.P., Wasowski, C., Loscalzo, L.M., Granger, R.E., Johnston, G.A.R., Paladini, A.C., Marder, M., 2006. Central nervous system depressant action of flavonoid glycosides. Eur. J. Pharmacol. 539 (3), 168–176.

Fernández, S.P., Wasowski, C., Paladini, A.C., Marder, M., 2005. Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. Eur. J. Pharmacol. 512 (2–3), 189–198.

Fernández, S., Wasowski, C., Paladini, A.C., Marder, M., 2004. Sedative and sleep-enhancing properties of linarin, a flavonoid-isolated from Valeriana officinalis. Pharmacol. Biochem. Behav. 77 (2), 399–404. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/14751470.

Ferré, P., Fernández-Teruel, A., Escorihuela, R.M., Driscoll, P., Corda, M.G., Giorgi, O., Tobeña, A., 1995. Behavior of the Roman/Verh high- and low-avoidance rat lines in anxiety tests: relationship with defecation and self-grooming. Physiol. Behav. 58 (6), 1209–1213. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/8623022.

File, S.E., Wardill, A.G., 1975. Validity of head-dipping as a measure of exploration in a modified hole-board. Psychopharmacologia 44 (1), 53–59. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/1197580.

Fujimori, H., 1965. Potentiation of barbital hypnosis as an evaluation method for central nervous system depressants. Psychopharmacologia 7 (5), 374–378. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/5837313.

Guajardo, J., Gastaldi, B., González, S., Nagahama, N., 2018. Variability of phenolic compounds at different phenological stages in two populations of Valeriana carnosa Sm. (Valerianoideae, Caprifoliaceae) in Patagonia. Bol. Latinoam. Caribe Plantas Med. Aromat. 17 (4), 381–393.

Guide for the Care and Use of Laboratory Animals, 2011. Guide for the Care and Use of Laboratory Animals. National Academies Press.

Guzmán-Gutiérrez, S.L., Navarrete, A., 2009. Pharmacological exploration of the sedative mechanism of hesperidin identified as the active principle of Citrus sinensis flowers. Planta Med. 75 (4), 295–301.

Hall, C., 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J. Comp. Psychol. 18, 385–403.

Hattesohl, M., Feistel, B., Sievers, H., Lehnfeld, R., Hegger, M., Winterhoff, H., 2008. Extracts of Valeriana officinalis L. s.l. show anxiolytic and antidepressant effects but neither sedative nor myorelaxant properties. Phytomed.– Int. J. Phytother. Phytopharmacol. 15 (1–2), 2–15.

Hayashi, M., Sutou, S., Shimada, H., Sato, S., Sasaki, Y.F., Wakata, A., 1989. Difference between intraperitoneal and oral gavage application in the micronucleus test. The 3rd collaborative study by CSGMT/JEMS.MMS. Collaborative study group for the micronucleus test/mammalian mutagenesis study group of the environmental mutagen society of Japan. Mutat. Res. 223 (4), 329–344. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/2747714.

Hobbs, C., 1989. Valerian: a literature review. HerbalGram 19–34.

Hölzl, J., 1997. The pharmacology and therapeutics of Valeriana. In: Houghton, P.J. (Ed.), Valerian. Harwood Academic Publishers, Amsterdam, pp. 55–75.

Houghton, P.J., 1988. The biological activity of Valerian and related plants. J. Ethnopharmacol. 22 (2), 121–142. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/3287008.

Houghton, P.J., 1997. The chemistry of valerian. In: Houghton, P.J. (Ed.), Valerian. Harwood Academic Publishers, Amsterdam, pp. 21–54.

Houghton, P.J., 1999. The scientific basis for the reputed activity of Valerian. J. Pharm. Pharmacol. 51 (5), 505–512. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/10411208.

Joo, D.T., Xiong, Z., MacDonald, J.F., Jia, Z., Roder, J., Sonner, J., Orser, B.A., 1999. Blockade of glutamate receptors and barbiturate anesthesia: increased sensitivity to pentobarbital-induced anesthesia despite reduced inhibition of AMPA receptors in Glur2 null mutant mice. Anesthesiology 91 (5), 1329–1341.

Kalueff, A.V., Tuohimaa, P., 2005a. Mouse grooming microstructure is a reliable anxiety marker bidirectionally sensitive to GABAergic drugs. Eur. J. Pharmacol. 508 (1–3), 147–153.

Kalueff, A.V., Tuohimaa, P., 2005b. The grooming analysis algorithm discriminates between different levels of anxiety in rats: potential utility for neurobehavioural stress research. J. Neurosci. Methods 143 (2), 169–177.

Kalueff, A.V., Tuohimaa, P., 2004. Grooming analysis algorithm for neurobehavioural stress research. Brain Res. Protoc. 13 (3), 151–158.

Kutschker, A., 2011. Gayana Botánica. Universidad de Concepción, p. 68.

Kutschker, A., Ezcurra, C., Balzaretti, V., 2010. Valeriana (Valerianaceae) de los Andes australes: biodiversidad y compuestos químicos. In: Pochettino, M., Ladio, A., Arenas, P. (Eds.), Tradiciones y Transformaciones en Etnobotánica. CYTED, La Plata, pp. 219–224.

Loscalzo, L.M., Wasowski, C., Marder, M., 2009. Neuroactive flavonoid glycosides from Tilia petiolaris DC. extracts. Phytother Res. 23 (10), 1453–1457.

Loscalzo, L.M., Wasowski, C., Paladini, A.C., Marder, M., 2008. Opioid receptors are involved in the sedative and antinociceptive effects of hesperidin as well as in its potentiation with benzodiazepines. Eur. J. Pharmacol. 580 (3), 306–313.

Makkar, H.P.S., 2003. Measurement of total phenolics and tannins using folin-ciocalteu method. In: Quantification of Tannins in Tree and Shrub Foliage. Springer, Dordrecht.

Marder, M., Viola, H., Wasowski, C., Fernández, S., Medina, J.H., Paladini, A.C., 2003. 6-methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. Pharmacol. Biochem. Behav. 75 (3), 537–545. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/12895671.

Medina, J.H., Paladini, A.C., Wolfman, C., Levi de Stein, M., Calvo, D., Diaz, L.E., Peña, C., 1990. Chrysin (5,7-di-OH-flavone), a naturally-occurring ligand for benzodiazepine receptors, with anticonvulsant properties. Biochem. Pharmacol. 40 (10), 2227–2231. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/2173925.

Molares, S., Ladio, A.H., 2012. Plantas aromáticas con órganos subterráneos de Importancia cultural en la patagonia Argentina: una aproximación a sus usos desde la etnobotánica, la percepción sensorial Y la anatomía. Darwiniana 50 (1), 7–24.

Molares, S., Ladio, A.H., 2018. Valeriana carnosa Sm. In: Medicinal and Aromatic Plants of South America. Springer Nature, Albuquerque.

Moody, T.W., Merali, Z., Crawley, J.N., 1988. The effects of anxiolytics and other agents on rat grooming behavior. Ann. N. Y. Acad. Sci. 525, 281–290. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/2455462.

Morazzoni, P., Bombardelli, E., 1995. Valeriana officinalis: traditional use and recent evaluation of activity. Fitoterapia 66, 99–112.

Ochoa, J., Ladio, A., Lozada, M., 2010. Uso de recursos herbolarios entre mapuches y criollos de la comunidad campesina de Arroyo, Las Minas (Río Negro, Patagonia Argentina). Bol. Latinoam. Caribe Plant Med y Aromat. 9 (4), 269–276.

Rodríguez Echandía, E.L., Broitman, S.T., Fóscolo, M.R., 1983. Effect of serotonergic and catecholaminergic antagonists on mild-stress-induced excessive grooming in the rat. Behav. Neurosci. 97 (6), 1022–1024. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/6651959.

Spruijt, B.M., van Hooff, J.A., Gispen, W.H., 1992. Ethology and neurobiology of grooming behavior. Physiol. Rev. 72 (3), 825–852.

The Plant List, 2013. http://www.worldfloraonline.org/.

Wagner, H., Bladt, S., 1996. Plant drug analysis.

Wasowski, C., Marder, M., Viola, H., Medina, J.H., Paladini, A.C., 2002. Isolation and identification of 6-methylapigenin, a competitive ligand for the brain GABA $_A$ receptors, from *Valeriana wallichii*. Planta Med. 68 (10), 934–936.

Welfare, M., 1996. The Japanese Pharmacopoeia XIII (XIII). Tokyo.

Whyte, D.G., Johnson, A.K., 2007. Lesions of the anteroventral third ventricle region exaggerate neuroendocrine and thermogenic but not behavioral responses to a novel environment. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292 (1), R137–R142.

Zuloaga, F.O., Morrone, O., Rodríguez, D., 1999. Análisis de la biodiversidad en plantas vasculares de la Argentina. Kurtziana 27, 17–167. Retrieved from. https://www.researchgate.net/publication/291873098_Analisis_de_la_biodiversidad_en_plantas_vasculares_de_la_Argentina.