

Anxiolytic-like activity of *Aloysia virgata* var. *platyphylla* leaves extract in mice

Actividad ansiolítica del extracto de hojas de *Aloysia virgata* var. *platyphylla* en ratones

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Abstract

Background: Medicinal plants are part of traditional medicine and should be considered a therapeutic alternative for mental diseases. Several plants belonging to the Verbenaceae family have proved useful in treating general anxiety disorders, the most prevalent psychiatric disorders.

Objective: This research aimed to verify the extract's safety, the effect on general behavior, and the effect on sleeping time, as well as to evaluate the anxiolytic-like effect of the methanol extract of *Aloysia virgata* var. *platyphylla* (Avp), in mice.

Methodology: The toxicity test was done according to the OECD guide (mice groups n=5), and general behavior was observed during the assay. Sleeping time was assessed using the pentobarbital-induced hypnosis method (n=8). Male Swiss albino mice (n=6) were treated with 50 to 400 mg/kg of Avp extract and diazepam as a control. The anxiolytic-like effect was tested through the hole board and elevated plus-maze test.

Results: The Avp extract has no side effects in tested doses, and no central nervous system depressant activity was noted. *A. virgata* var. *platyphylla* increased exploration (number and time) in the hole board. In the elevated plus-maze, increased number and time into open arms were evidenced compared to the control group.

Conclusion: With all these results, we concluded that the Avp extract is safe and has a potential anxiolytic-like activity in the animal model used.

Keywords: *Aloysia virgata* var. *platyphylla*, sleeping time, hole board, elevated plus maze, pre-clinical, anxiolytic

Resumen

Antecedentes: Las plantas medicinales forman parte de la medicina tradicional y deben ser consideradas una alternativa terapéutica para las enfermedades mentales. Varias plantas pertenecientes a la familia Verbenaceae han demostrado su utilidad en el tratamiento de los trastornos de ansiedad, uno de los trastornos psiquiátricos más prevalentes.

Objetivo: Esta investigación tuvo como objetivo verificar la seguridad del extracto, el efecto sobre el comportamiento general y el efecto sobre el tiempo de sueño, así como evaluar el efecto tipo ansiolítico del extracto metanólico de *Aloysia virgata* var. *platyphylla* (Avp), en ratones.

Metodología: La prueba de toxicidad se realizó de acuerdo con la guía de la OCDE (grupos de ratones n=5), y se observó el comportamiento general durante el ensayo. El tiempo de sueño se evaluó mediante el método de hipnosis inducida por pentobarbital (n=8). Se trataron ratones albinos suizos macho (n=6) con 50 a 400 mg/kg de extracto de Avp y diazepam como control. El efecto ansiolítico se probó a través de la placa perforada y prueba del laberinto en cruz elevado.

Resultados: El extracto de Avp no tiene efectos secundarios en las dosis probadas y no se observó actividad depresora del sistema nervioso central. *A. virgata* var. *platyphylla* aumentó la exploración (número y tiempo) en el tablero de agujeros. En el laberinto en cruz elevado, se evidenció un mayor número y tiempo en los brazos abiertos en comparación con el grupo de control.

Conclusión: Con todos estos resultados, concluimos que el extracto de Avp es seguro y tiene una potencial actividad ansiolítica en el modelo animal utilizado.

Palabras clave: *Aloysia virgata* var. *platyphylla*, tiempo de sueño, placa perforada, laberinto en cruz elevado, preclínico, ansiolítico

Received: 06/04/2022

Accepted: 13/08/2022

Introduction

Anxiety disorders are the most prevalent psychiatric disorders. It is presumed that 3.8% of the world's population currently suffers from anxiety disorders (1). Anxiety is still an underdiagnosed disease, particularly in lower-income countries where data about the prevalence of this disease is scarce, and the treatment of mental illness has less importance (2, 3). Anxiety usually occurs without obvious symptoms but tends to manifest itself chronically, and symptoms vary in severity over time (4). The prevalence of anxiety has increased considerably in recent years, attributing this to the currently imposed lifestyle (5). Psychosocial factors include genetic vulnerability, trauma, stress, and ultimately end in neurobiological dysfunction and altered neuropsychological behavior (3). Anxiety is often related to other pathologies, such as viral diseases (6). It is also usually considered a

risk factor for cardiovascular diseases and can cause depression, which is a risk factor for cardiovascular diseases (7, 8). It is also related to rheumatological conditions (9). More recently, it has been seen that the appearance of COVID-19 increased the manifestations of anxiety in people (10).

Anxiety disorders treatment includes drugs that are quite effective. However, other considerations like adverse effects, cost, safety warnings, drug interactions, and contraindications must be considered (3, 6). Medicinal plants have been part of traditional medicine for centuries and still provide alternatives for treating several diseases (11).

The use of medicinal plants as a therapeutic alternative to treat a condition widely distributed worldwide, such as anxiety, must be considered. Many investigations have shown the anxiolytic effect of medicinal plants in preclinical trials. The roots, rhizomes, and stolons of *Valeriana officinalis* L. (Valerianaceae) have sesquiterpenes and iridoids such as valerenone, valerianol, valerenic acid, among others, which have shown certain GABAergic activity, reducing anxiety levels. The aerial parts of *Passiflora incarnata* (Passifloraceae) contain flavonoids and alkaloids to which sedative and anxiolytic effects are attributed (12, 13).

In South America, several species of medicinal plants belong to the Verbenaceae family, on which anxiolytic activity was reported. *Aloysia gratissima* var. *gratissima* (14), *A. polystachya* (15-17), *A. triphylla* (18), *Lippia alba*, *L. sidoides*, and *L. graveolens* (19-21) have shown their effectiveness as anxiolytic in preclinical studies in mice, and some have also shown anxiolytic effect in clinical trials (16). Mice treated with these plants' extract exhibited an increase in number and time spent exploring in hole board, and in the elevated plus-maze, they increased the number of entries and time spent in the open arms of the labyrinth.

Considering the large number of species belonging to the Verbenaceae family that demonstrated anxiolytic effect and the reported effect on the central nervous system of two diterpenes isolated from *Aloysia virgata* (22), in this work, the safety, the effect on sleeping time, and the anxiolytic-like activity of the methanol extract *Aloysia virgata* var. *platyphylla* (Avp) in mice were evaluated.

Materials and methods

Plant material and extraction

The leaves of the species *Aloysia virgata* (Ruiz & Pav.) Pers. var. *platyphylla* (Briq.) Moldenke belonging to the Verbenaceae family were collected in Cordillera, Paraguay. In the herbarium of Facultad de Ciencias Químicas a voucher specimen was filed (Universidad Nacional de Asunción, code Degen R 4.067). Dried and powdered leaves were extracted with methanol. First, the powder was sonicated for 30 min. at room temperature (three times) and filtered. Subsequently, the residue was extracted by a conventional reflux method (methanol, twice). The material obtained after solvent evaporation was used for biological assays.

Drugs

In our experiment diazepam (Roche) and sodium pentobarbital (Abbott, Japan) were used. Methanol (JT Baker) was used for plant extraction.

Experimental animals and ethical issues

Toxicity and general behavior tests were performed in adult Swiss albino female mice. For sleeping time and anxiolytic assays, male mice were used. All animals came from Facultad de Ciencias Químicas bioterium. They received daily standard animal pellets and water *ad libitum* in an acclimatized room (23-25°C, 12:12 h light-dark cycle, 50-60% humidity). Scientifically standardized principles in compliance with international animal welfare standards were considered, and the Research Ethics Committee of the Facultad de Ciencias Químicas approved the experiment (CEI 402/18). All animals were used once and euthanized after use by cervical dislocation.

Acute toxicity study and general behavior effect

Following the fixed doses methods described by OECD guidelines ⁽²³⁾, water (control group, n=5) or methanol extracts of *A. virgata* var. *platyphylla* (Avp) were administered orally by gavage to female mice (n=5; doses: 5; 50; 300; 500 and 2000 mg/Kg) and observed during 24 h. After that, mice were followed for 14 days looking for delayed adverse effects and finally dissected. The organs and fundamental tissues (heart, kidney, spleen, lung, liver, stomach, and intestine) were macroscopically observed, and compared with those in the control group. General effects on behavior, physiological and neurological alterations, and neurotoxicity symptoms were sought in treated animals and control group (vehicle, 0.1mL/10g body weight, n=5) 5, 15, 30, 60, 120 minutes, and 24 and 48 hours after drug administration ⁽²⁴⁾.

Pentobarbital-induced hypnosis

Six groups of male mice (n=8) were treated with vehicle (water, 0.1 mL/10 g body weight, *per os*, p.o.); 50, 100, 200, and 400 mg/kg of Avp (p.o.), or with diazepam (0.5 mg/kg, intraperitoneal, i.p.). Each animal received sodium pentobarbital (35 mg/kg, i.p.) 1 hour after the vehicle or the extract, and twenty minutes after diazepam. Induction time and sleeping time were recorded for each animal ⁽²⁵⁾.

Hole-board test

The hole-board is an experimental method used to measure anxiety and emotionality in animals. It is a box containing 16 evenly spaced holes (10×10, 2 cm diameter), the number and time spent in exploring the holes (head-dipping), as well as ambulation (peripheral and central area), rearing, grooming, and defecation were measured ⁽²⁵⁾. Six groups (male, n=8) were treated with Avp extract (50, 100, 200, and 400 mg/kg, p.o.), vehicle (water) or diazepam (0,5 mg/kg, i.p.). One hour after the treatment (20 min after for diazepam-treated mice), each animal was placed individually in the center of the hole board and allowed to explore for 5 min freely. The apparatus was cleaned after each trial with 10% ethanol. The test was carried out in a light-controlled room (red light, 15 W).

Elevated plus-maze test (EPM)

The plus maze apparatus (EPM) has been widely validated to measure animal anxiolytic behavior. The apparatus was made of transparent Plexiglas and consisted of a plus-shaped maze formed by two opposite open arms (arm length: 30.0 cm; arm width: 5.0 cm); crossed with two arms enclosed by walls (height: 15.0 cm). The open and enclosed arms converge into a central platform (5.0 cm×5.0 cm). The maze is elevated at 40.0 cm from ground level by a wood bearing, and it is placed in a room illuminated with red light (15 W) ⁽²⁶⁻²⁷⁾. After treatment (Avp extract: 50, 100, 200, and 400 mg/kg, p.o.; vehicle (water) or diazepam, 0,5 mg/kg, i.p.), each mouse was placed in the center on the elevated plus-maze apparatus, with its head towards the open arms, 60 min after the administration of vehicle and the extract of Avp, or 20 min after diazepam. Their behavior was observed for 5 min, and parameters such as frequency of entrance and duration into open and closed arms were recorded. After each test, the EPM apparatus was thoroughly cleaned with ethanol (10%). Subsequently, the number of both measured parameters was analyzed.

Data analysis

GraphPad Software, Inc., CA (GraphPad Prism 7.0) was used for the statistical analysis of data. Analysis of variance (ANOVA) of one factor followed by Dunnett's post *hoc* was performed, and data were presented as mean ± SD. When $p < 0.05$, the difference was considered statistically significant.

Results

Toxicity test and general behavior

The toxicity test revealed no adverse effects of *Aloysia virgata* var. *platyphylla*, with doses of up to 2 g/Kg of mouse body weight. Administration of the extract did not cause

the animals' death or toxicity symptoms during the 24-hour observation period. Compared to the control group, all animals presented normal behavior: postural reflex, grooming, responses to nociceptive stimuli, and water and food consumption (data not shown). After macroscopical observation, no organ alteration was evidenced with any tested doses.

Sleeping time induced by pentobarbital

In the sleep-time trial, it was observed that with the tested doses, neither the induction time (not shown) nor the total sleep time were affected compared to the control group. This suggests that *A. virgata* var. *platyphylla* has no central nervous system depressant effect. Diazepam, used as a positive control drug, prolonged the sleep time as expected (Figure 1).

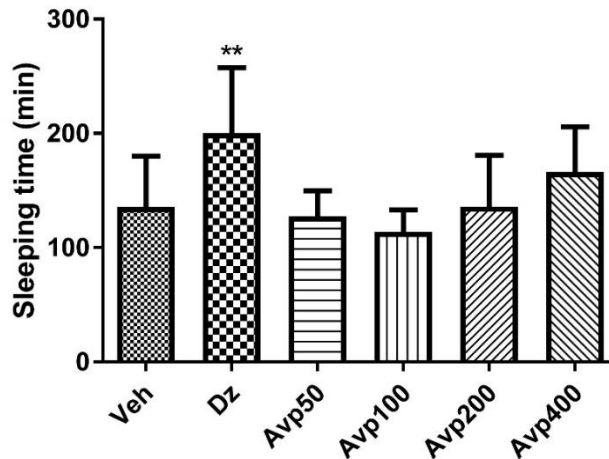


Figure 1: Sleep-time after barbiturate administration in mice orally treated with *A. virgata* var. *platyphylla* extract. Veh (vehicle), Dz (Diazepam), Avp50 (*A. virgata* var. *platyphylla* 50mg/Kg), Avp100 (100mg/Kg), Avp200 (200mg/Kg), Avp400 (400mg/Kg). Data are expressed as mean \pm SD, after one-way ANOVA, Dunnet posttest; ** $p < 0.01$, compared with Vehicle.

Hole-board assay

The number of head dipping in the hole-board test (Figure 2A) and the exploration time (Figure 2B) showed a significant difference between the vehicle group and diazepam and the groups of animals treated with some concentrations of *A. virgata* var. *platyphylla* extract.

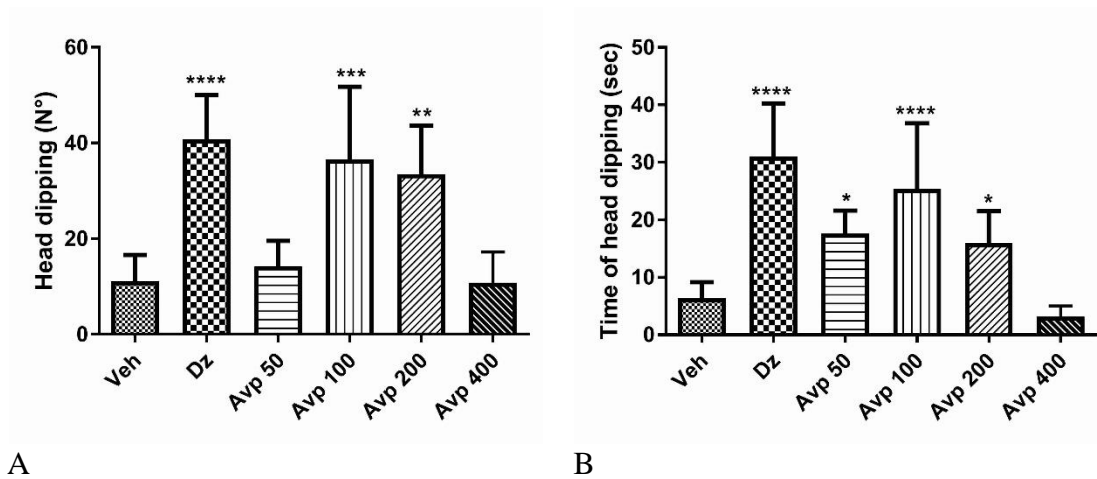


Figure 2. A) Number of head dipping and B) time of exploration in mice treated with *A. virgata* var. *platyphylla* in the Hole board apparatus. Veh (vehicle), Dz (Diazepam), Avp50 (*A. virgata* var. *platyphylla* 50mg/Kg), Avp100 (100mg/Kg), Avp200 (200mg/Kg), Avp400 (400mg/Kg). Data represent mean \pm SD (n= 8); ANOVA one way, Dunnett post-test; * p<0.05, ** p<0.01, *** p<0,001, **** p<0,0001, compared with Vehicle.

Elevated plus-maze test

The anxiolytic activity of *A. virgata* var. *platyphylla* was determined in mice using the elevated plus-maze. It was observed that the group treated with diazepam, and those that received 50 and 200 mg/kg of *A. virgata* var. *platyphylla*, increased the crossings in open arms compared to the group treated with vehicle (Figure 3A). The mean time spent in open arms was also increased in the groups treated with diazepam (p<0.0001) and when the animals received 50, 100, and 200 mg/kg (Figure 3B) compared to the control group.

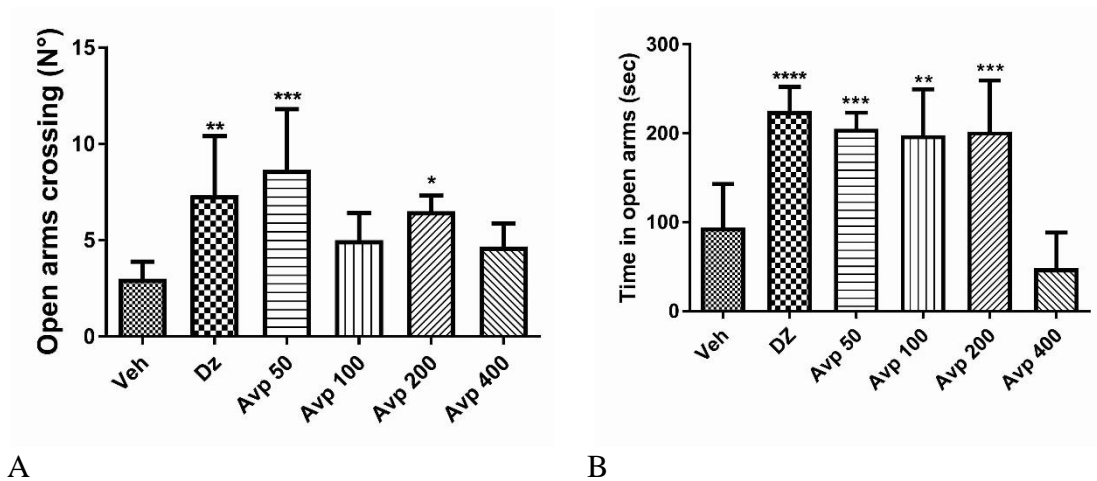
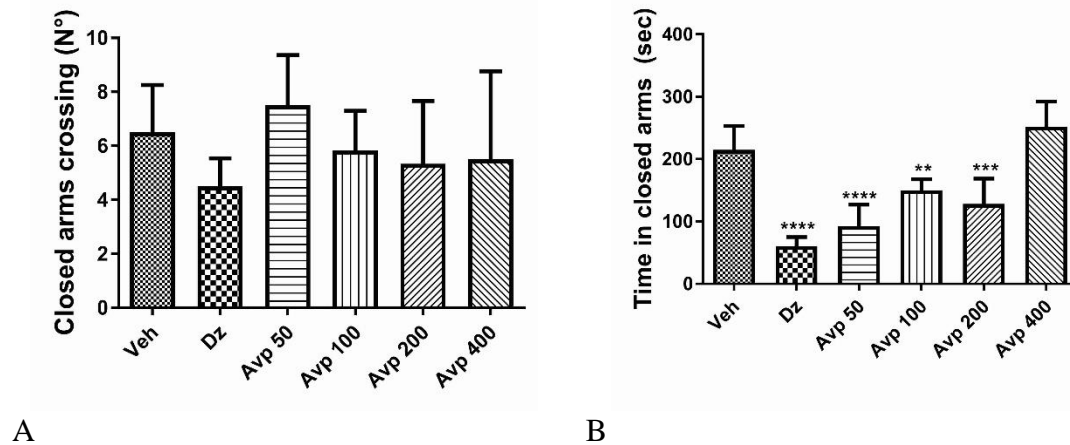


Figure 3. A) Number of crossing and B) time spent by mice in the open arms of plus-maze after treatment with *A. virgata* var. *platyphylla*. Veh (vehicle), Dz (Diazepam), Avp50 (*A. virgata* var. *platyphylla* 50mg/Kg), Avp100 (100mg/Kg), Avp200 (200mg/Kg), Avp400 (400mg/Kg). Data represent mean \pm SD (n= 8);

ANOVA one way, Dunnett post-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared with Vehicle.

In the number of crossings in closed arms, no significant differences have been observed with any of the tested doses of *A. virgata* var. *platyphylla* or diazepam compared to the control group (figure 4A). The time spent in closed arms was significantly reduced with diazepam and 50, 100, and 200 mg/kg of Avp extract (figure 4B).



A

B

Figure 4. A) Number of crossing and B) time spent by mice in the closed arms of plus maze after treatment with *A. virgata* var. *platyphylla*. Veh (vehicle), Dz (Diazepam), Avp50 (*A. virgata* var. *platyphylla* 50mg/Kg), Avp100 (100mg/Kg), Avp200 (200mg/Kg), Avp400 (400mg/Kg). Data represent mean \pm SD (n= 8); ANOVA one way, Dunnett post-test; ** $p < 0.01$, * $p < 0.001$, **** $p < 0.0001$, compared with Vehicle.**

Discussion

A. virgata var. *platyphylla* showed no adverse effect in the acute toxicity test. A similar result was observed with *A. gratissima* var. *gratissima* (14). The barbiturate-induced sleep time test indicated that this plant is devoid of depressant or stimulant effects on the central nervous system (28), while animals treated with diazepam prolonged sleep time as expected for this drug used as the positive control.

Mice treated with diazepam and 50, 100, and 200 mg/kg of the methanolic extract of *A. virgata* var. *platyphylla* significantly increased the number and exploration time in the hole board compared to the control group. This result is an indication of the anxiolytic effect. (29). Additionally, in the elevated plus maze test, both diazepam and the Avp extract (50, 100, and 200 mg/kg) increased the entries and permanency in the EPM open arms, which confirms the anxiolytic-like effect of Avp. Accordingly, the time spent in closed arms decreased in mice that received diazepam and the same doses of Avp (50, 100, and 200 mg/kg). In this case, it was verified that the trial is more sensitive for the time spent in closed arms (30) since no difference was noted in the number of entries into the closed arms. Therefore, the hole-board and plus-maze results indicated that *A. virgata* var. *platyphylla* has a potential anxiolytic effect (26). In both tests with animals, the control group was compared with diazepam (positive control to determine anxiolytic activity), and a statistically significant difference was observed for each of the parameters analyzed,

thus validating each of the determinations carried out to conclude that Avp extract has an anxiolytic-type activity.

The hole-board test offers a simple method to measure potential anxiolytic effects. In unfamiliar environments, mice show less mobility, and when treated with an anxiolytic drug such as diazepam, their mobility and the number and time of head dipping will increase considerably. These results, together with those obtained in the EPM where time spent in open arms increased and time spent in closed arms decreased in those mice treated with an anxiolytic drug, allowed us to determine that *A. virgata* var. *platyphylla* has an anxiolytic-like effect (30). Several species of Verbenaceae, particularly *Aloysia*, demonstrated an anxiolytic effect (12,14-16,18, 20-22), and our results agree with that.

Although it is still pending to determine the secondary metabolites present in this extract, phenylethanoid compounds, such as verbascoside, have been identified in this genus and associated with the anxiolytic effect. The tests showed that animals treated with verbascoside increased the percentage of entry in EPM and the time spent in open arms, as expected for substances with anxiolytic effects (31). Further experiments are required to determine the chemical composition of the extract, as well as to elucidate the mechanism of action involved in the anxiolytic effect demonstrated in biological systems. Still, it is presumed that they would act by interaction with GABA_A since, in all the tests similar response to diazepam has been observed (32).

Conclusion

Conducting this research, we determined that the extract is safe up to 2 g/Kg since there was no evidence of toxicity, nor did it affect the general behavior. The results indicated a possible anxiolytic activity according to the results observed in the hole-board and the elevated plus-maze tests. We also observed the absence of central nervous system depressant activity in the barbiturate-induced sleep test.

Authors' Contributions statement

All authors have made substantial contribution to this work, read the final manuscript, and approved the submission.

Acknowledgements

The authors really appreciate the collaboration of researchers from the Department of Botany from Facultad de Ciencias Químicas for plant collection and identification; and Mr G López and N Kennedy for revising the English language in the document.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Funding

This research was supported by Rectorado, Universidad Nacional de Asunción, Paraguay (Proyecto code FCQ/04/16), and Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay.

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