IJPSR (2021), Volume 12, Issue 7 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 18 July 2020; received in revised form, 12 December 2020; accepted, 10 June 2021; published 01 July 2021

EVALUATION OF METHANOL EXTRACT OF *ALOYSIA GRATISSIMA* VAR. GRATISSIMA LEAVES ON BEHAVIOR AND ANXIETY IN MICE

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Keywords:

Sleeping time, Elevated plus maze, Open Field, Hole Board

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ABSTRACT: Anxiety disorders are among the most prevalent and high-cost diseases. Medicinal plants are a very good option to treat, and several have shown the ability to reduce anxiety. We report here the effect of *A. gratissima* var. *gratissima* (Agg) on behavior and anxiety in mice. The methanol extract of leaves was tested in order to verify the safety, effect on sleeping time and behavior. Anxiety was tested through the Open field, Hole board, and elevated plus maze tests, with 50, 100, 200, and 400 mg/kg of Agg, and diazepam as positive control drugs. The results showed that *A. gratissima* var. gratissima did not cause signs of toxicity or sedative effect, and the general behavior of mice was not altered with any dose. All doses of Agg and diazepam, after the mice were submitted to the Open field and Hole board, evidenced a significant increase in ambulation, and a significant decrease in immobility time, related to the control group. On Hole board, a significant increase in number and time spent in exploration indicated a possible anxiolytic-like effect, that was confirmed by the results obtained in the elevated plus-maze, where mice increased the number of entries and time spent in open arms, and decreased the number of entries and time spent in closed arms of the labyrinth. Al the results evidenced the safety of Agg, and denoted an anxiolytic-like effect in mice.

INTRODUCTION: Anxiety disorders, affecting up to 33.7% of the population, are the most prevalent mental disorders and are associated with a high cost in health. A substantial under-recognition and under-treatment of these disorders have been demonstrated [1]. Persistent fretfulness, distractedness, and a sort of whole-body clenching to a full-blown panic attack constitute the variety of clinical manifestations of anxiety, which are indicators of a possible threat to homeostasis.

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DOI: 10.13040/IJPSR.0975-8232.12(7).3858-65

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(7).3858-65

Anxiety requires treatment when it appears in the absence of any threat or in disproportionate relation to a threat, and the individual is not capable of leading a normal life [2].

According to a report by the World Health Organization (WHO), of all the patients admitted to mental health services in Paraguay, anxiety disorders are the fourth most attended cause in any of its units (representing 10 - 15% of all patients), being the highest prevalence in women [3]. Psycho-therapy and/or medication are the treatment option for anxiety disorders. Anti-anxiety medications can help reduce the symptoms of anxiety, panic attacks, or extreme fear and worry. Monoaminergic neuro-transmitters such as serotonin (5-hydroxytrypt-amine or 5-HT) and dopamine (DA), and gamma-

aminobutyric acid (GABA) and glutamate are involved in the neurobiological basis of anxiety. Pharmacological treatment is based on regulating the functions of some of the compounds mentioned above [4], and usually, benzodiazepines are the first-choice drug, which has benefits and drawbacks. Some benefits of benzodiazepines are that they are effective in relieving anxiety, and some drawbacks are that people can build up a tolerance, become dependent on them, may have withdrawal symptoms if they suddenly stop taking them, or their anxiety may return [5].

To overcome these disadvantages that arise from the use of benzodiazepines, we look for medical alternatives with a better therapeutic profile. A treatment option is medicinal plants, several of them have been studied, and their anxiolytic effects have been demonstrated. Such is the case of *Valeriana officinalis*, whose roots, rhizomes, and stolons have sesquiterpenes and iridoids such as valerenone, valerianol, valerenic acid among others, which have shown some GABAergic activity, reducing anxiety levels [6]; *Passiflora incarnata*, whose aerial parts contain flavonoids and alkaloids to which the sedative and anxiolytic effects are attributed [7], among others. In our research group, we have previously investigated the anxiolytic-like effect of *Kyllinga brevifolia* rhizomes [8]; and *Aloysia polystachya* leaves extract [9], and both demonstrated the ability to reduce anxiety and also antidepressant like effect [10-12].

Previous studies on *A. gratissima* showed the antidepressant- and neuroprotective activities of the aqueous extract [13], in addition to an *in-vitro* antioxidant effect [14]. A variety of *A. gratissima* [15], *Aloysia gratissima* var. *gratissima (*Verbenaceae), locally known as poleo´i, is an aromatic shrub that blooms in December and grows from Mexico to central Argentina. Its aerial parts are used as an infusion in throat and stomach conditions, as a diaphoretic, digestive, aphrodisiac, and tonic [16]. Considering that no scientific report related to safety, sedative, or anxiolytic-like effects of *A. gratissima* var. *gratissima* was found in the scientific literature, the health problems arising from anxiety in Paraguay and around the world, and in the search for new alternatives with pharmacological effectiveness to treat anxiety disorder, we report here the acute toxicity, behavioral profile, sleeping time, and the *in-vivo* anxiolytic effect in mice of the methanol extract of *A. gratissima* var. *gratissima*.

MATERIALS AND METHODS:

Plant Material and Extraction: *Aloysia gratissima* (Gillies & Hook. Ex. Hook.) Tronc. Var. *gratissima* (Verbenaceae) leaves were collected in Paraguari, Paraguay. A voucher specimen was filed with the herbarium of Facultad de Ciencias Químicas, Universidad Nacional de Asunción (No 4.579). Dried and powdered samples were extracted with methanol, the powder along with solvent was kept in a beaker and sonicated 30 min. at room temperature (three times), then filtered. The sample residue was subsequently extracted with methanol by a conventional reflux method for 1 h (repeated twice), then filtered and evaporated under reduced pressure. The material obtained was stored at room temperature in a desiccator and suspended in distilled water to be used for biological assays. All solutions were prepared freshly on the test days.

Drugs: Diazepam (Valium Roche Laboratory, Argentina); sodium pentobarbital (Nembutal) from Abbott (Japan) were used; methanol was purchased from JT Baker.

Experimental Animals and Ethical Issues: Adult Swiss albino male and female mice, of average weight 25-35 g, acclimatized at room temperature 23-25 °C, with 12:12 h light-dark cycle, in a humidity-controlled environment (50-60%) were used. They received daily standard animal pellets and water *ad libitum* and kept fasted prior to the experiment. All studies and research were done according to scientific principles in compliance with international standards of animal welfare; the Bioethical Committee of the Facultad de Ciencias Químicas approved the study design (CEI 402/18). The minimum number of animals and duration of observation required to obtain consistent data were used, all individuals involved in this study had the necessary expertise and training [17].

Acute Toxicity Study and General Behaviour Effect: Following the fixed doses methods described by OECD guidelines [18], the acute toxicity study was done in female Swiss albino mice weighing 20-30 g. The methanol extract of *A. gratissima var gratissima* (Agg) was administered

orally in different doses (5; 50; 300; 500 and 2000 mg/kg), and 24 h observation was performed to identify toxic effects. In the search for signs of toxicity, the appearance of altered locomotion, sedation, stimulation, piloerection, increased respiratory rate, and death was sought. Additionally, all animals were observed during 14 days searching for delay adverse events. After that time, the mice were dissected, and the organs and fundamental tissues (heart, kidney, spleen, lung, liver, stomach, and intestine) were observed. In order to test the general effect, at the same time, behavioral, physiological, and neurological alterations and neurotoxicity symptoms such as changes in the skin and coat, eyes and mucous membranes, as well as respiratory, circulatory, autonomic, and central nervous system changes, tremors, seizures, salivation, diarrhea, lethargy, sedation, and coma, were recorded according to a standardized observation grid. All groups were observed in simultaneous comparison with a control group given vehicle (0.1mL/10g body weight) at 0, 5, 15, 30, 60, and 120 min after drug administration and also 24 and 48 hours later [19].

Pentobarbital-Induced Hypnosis: Six groups of male mice (n=8) were orally treated with vehicle (water, 0.1 mL/10 g body weight); 50, 100, 200 y 400 mg/kg of Agg, or with diazepam (0.5 mg/kg i.p.). Each animal was injected with sodium pentobarbital (35 mg/kg, i.p.) 1 h after the vehicle or extract treatment and twenty minutes after diazepam. The latency to the loss of righting reflex (induction time in seconds) and the time required to recover righting reflex or awakening (sleeping time in minutes) were registered for each animal as previously described [19].

Open Field Test: Exploratory behavior and general activity in male mice were observed in the open-field test. Open field is an enclosure square with surrounding walls that prevent escape (height: 17 cm, length: 30 cm; width: 30 cm), and a black floor marked with white lines in 10 cm^2 areas. Animals were randomly distributed into groups of eight animals. One hour after treatment with vehicle (water, 0.1 mL/10 g body weight), Agg (50, 100, 200 mg//kg, p.o.) or diazepam (0.5 mg/kg, i.p.), each mice was placed in the center of the arena and the distance moved, (peripheral and central area), time spent immobile, rearing, grooming, and defecation were recorded the first 5 min [20]. The open field apparatus was cleaned with ethanol solution (10%) before placing the next mouse, and the test was carried out in a light-controlled room (red light, 15 W).

Hole-Board Test: The hole-board apparatus consisted of a Plexiglas cage of dimensions 40 cm x 40 × 15 cm; the black floor was divided into squares (10 cm × 10 cm), and contained 16 evenly spaced holes (diameter: 2 cm). Each mouse distributed in one of six groups (n=8), one hour after Agg extract administration (50, 100 and 200 mg//kg, p.o.), water (0.1 mL/10 g body weight) or 20 minutes after diazepam (0,5 mg/kg, i.p.) was placed in the center of the hole bored and allowed to explore the apparatus for 5 min. The number and time spent in head-dipping in the holes were recorded, as well as ambulation (peripheral and central area), rearing, grooming, and defecation[20]. The apparatus was cleaned with a 10% ethanol solution between the trials and the test was carried out in a light-controlled room (red light, 15 W).

Elevated Plus-Maze Test (EPM): The plus-maze apparatus consisting of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm, walls 15cm) with an open roof which was 40 cm elevated from ground level in a room illuminated with red light (15 W), was used to observe anxiolytic behavior in animals [21-22]. Each mouse was placed on the elevated plus-maze apparatus, in the center with its head facing the open arms, 60 min after the administration of vehicle and the extract of Agg (50, 100, 200 and 400 mg//kg, p.o.), or 20 min after diazepam (0,5 mg/kg, i.p.). Their behavior was observed for 5 min, and parameters such as frequency and duration in open arms and in closed arms were recorded.

The EPM apparatus was thoroughly cleaned after each trial with ethanol (10%). Subsequently, the percentage of frequency and time spent in open arms and in closed arms were calculated.

Statistical Analysis: The data were presented as mean ± standard deviation (SD), the statistical analysis was performed using analysis of variance (ANOVA) of one factor followed by Dunnett's post *hoc* test as appropriate using GraphPad Prism 7.0 software (GraphPad Software, Inc., CA).

Differences were considered to be statistically significant when $p < 0.05$.

RESULTS:

Acute Toxicity, Effects on General Behavior and on Pentobarbital Induced Sleep of *A. gratissima* var *gratissima* Methanol Extract: The acute oral toxicity evaluation of the methanol extract of *A. gratissima* var *gratissima* was performed in female mice. During the observation period, none of the four tested doses (5, 50, 300, and 2000 mg/kg) caused signs of toxicity or death of animals. The general behavior of the animals was not altered with any of the doses tested.

The results obtained in the sodium pentobarbital-induced sleep test showed that diazepam could prolong the duration of sleep (190.4±61.19, $p<0.5$, regarding control group (Veh.), 135.6±44.6), and no differences were found in either sleep latency or sleep time between the control group and the groups receiving treatment (50, 100, 200 and 400 mg/kg of *A. gratissima* var. *gratissima*: 124.4±15.6; 99.29±31.2; 105.9±11.3; 126.8±28.5; respectively).

Effect of *A. gratissima var gratissima* on the Open-Field and Hole-Board Tests: We tested the ambulatory (total, center, and peripheral) and emotional (rearing, grooming, and defecation) behavior in the open field and in the hole board tests in mice administered with 50, 100, 200 mg/kg of Agg extract. As anxiolytic positive control drug was used diazepam [23], and with the dose used, no impairment in locomotion was observed (0.5 mg/kg, i.p.). A significant difference in peripheral (Agg 50, 89.00±14.08; Agg 100, 95.00±25.49; and Agg 200, 79.13±19.46; control, 46.9±14.13) and central (Agg 50, 19.0±7.5; Agg 100, 13.3±4.9; and Agg 200, 13.3±9.3; control, 2.7±1.4) displacement was observed between the control group and the groups treated with diazepam and all tested doses of the extract **Fig. 1A, B**. Likewise, the statistical difference is significant in the immobility time (sec) between the vehicle group (12.1±3), compared with the diazepam group and also with the three tested doses of the extract (Agg 50, 3.9±2.5; Agg 100, 6.8±4.4; Agg 200, 4.4±3.9, **Fig. 1C**.

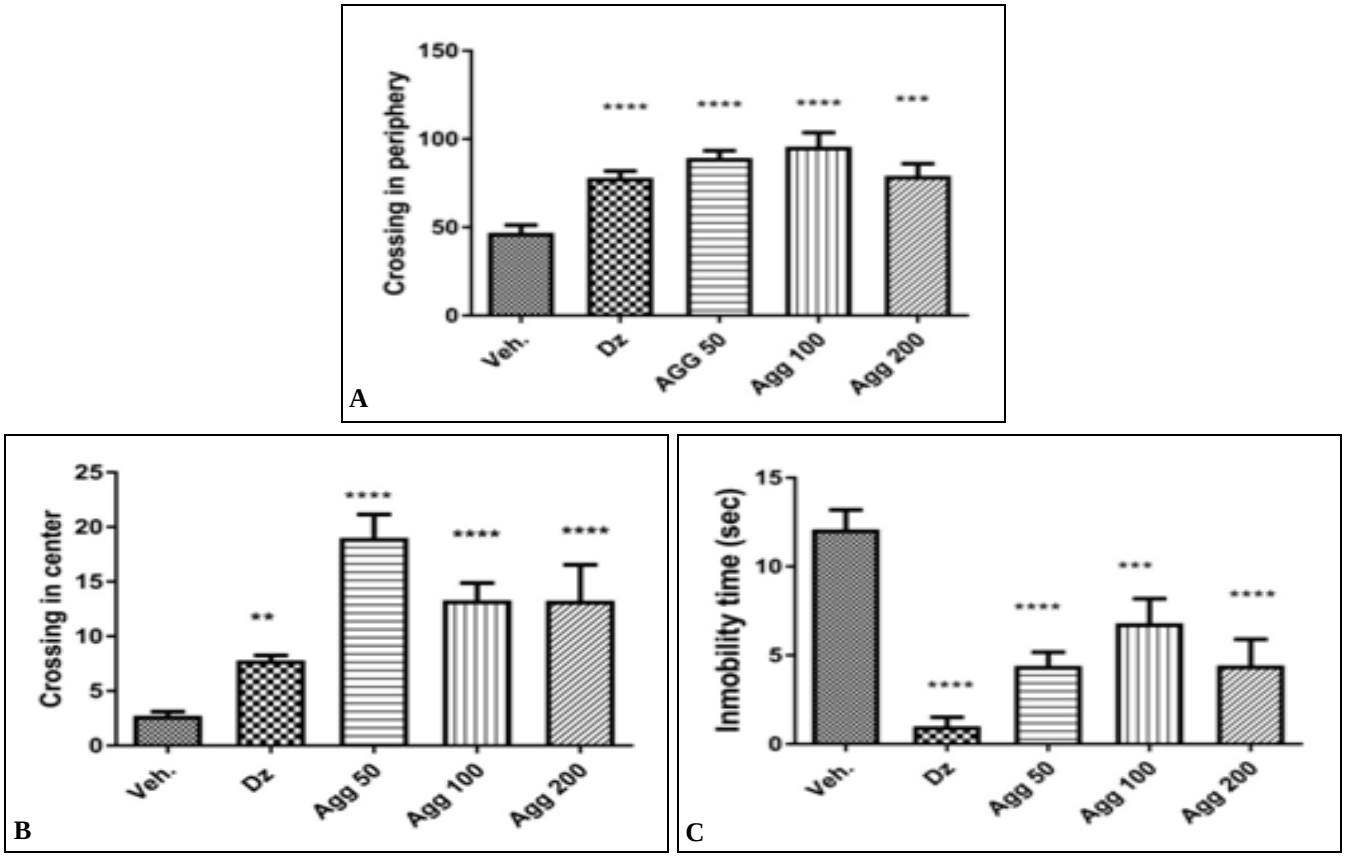

FIG. 1: EFFECT OF *A. GRATISSIMA VAR. GRATISSIMA* ON OPEN FIELD PARAMETERS IN MICE (n=8). A: NUMBER OF CROSSING IN PERIPHERY; B: NUMBER OF CROSSING IN CENTER; C: IMMOBILITY TIME. Data are expressed as media ±SD after one way ANOVA, followed by Dunnet posttest. ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$

In the hole board trial, a statistically significant difference was also observed between the number of head dipping (Agg50, 45.88±15.75; Agg 100, 39.75±20.45; Agg 200, 46.71±10.09; control, 8.67± 3.16) and also in the exploration time between the vehicle group, and the groups (Agg50, 26.25±9.6; Agg100, 21.4±8.4; Agg200, 32.29±7.04, control 5.1±3.3) treated with the anxiolytic diazepam (number of head dipping, 35.3±11; exploration time, 35.88±6.6) and the three doses of extract tested **Fig. 2A** and **B**. Additionally, the evaluation of emotional parameters (rearing, grooming, and number of feces) was performed in the hole board and open field trials; the results showed no significant difference between the different groups.

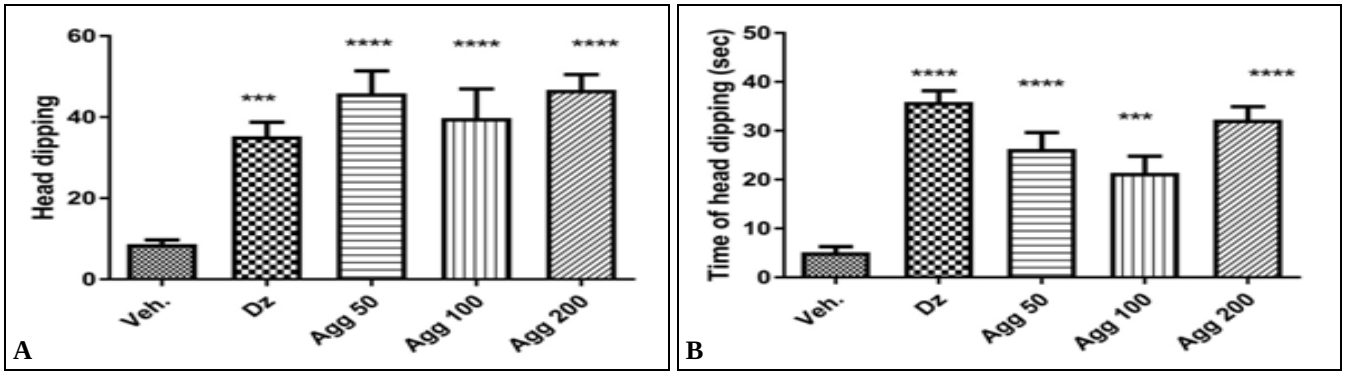

FIG. 2: NUMBER (A) AND TIME OF HEAD DIPPING (B) ON HOLE-BOARD OF *A. GRATISSIMA* VAR. *GRATISSIMA* IN MICE (n=8). Data are expressed as media ±SD after one way ANOVA, followed by Dunnet posttest. *** p<0.001; **** p<0.0001

Effect of *A. gratissima* var *gratissima* on the Elevated Plus-Maze Test: The extract of *A. gratissima* var. *gratissima* exhibited anxiolytic-like effects after oral treatment with 50 and 100 mg/kg as determined by the increase in the number of open arm entries (Agg50, 6.83±0.98; Agg100, 7.67±3.88 Agg200 6±1.55; 5.5±1.52; Veh., 2.83± 0,75; Dz 8±2.53 **Fig. 3A**); 50, 200 and 400 mg/kg of Agg also increased the time spent in the open arms of the plus maze (Agg50, 224±22.4; Agg100, 141±79.1; Agg200, 173±54; Agg400, 200±39.2; Veh., 102±42; Dz 235±20 **Fig. 3B**).

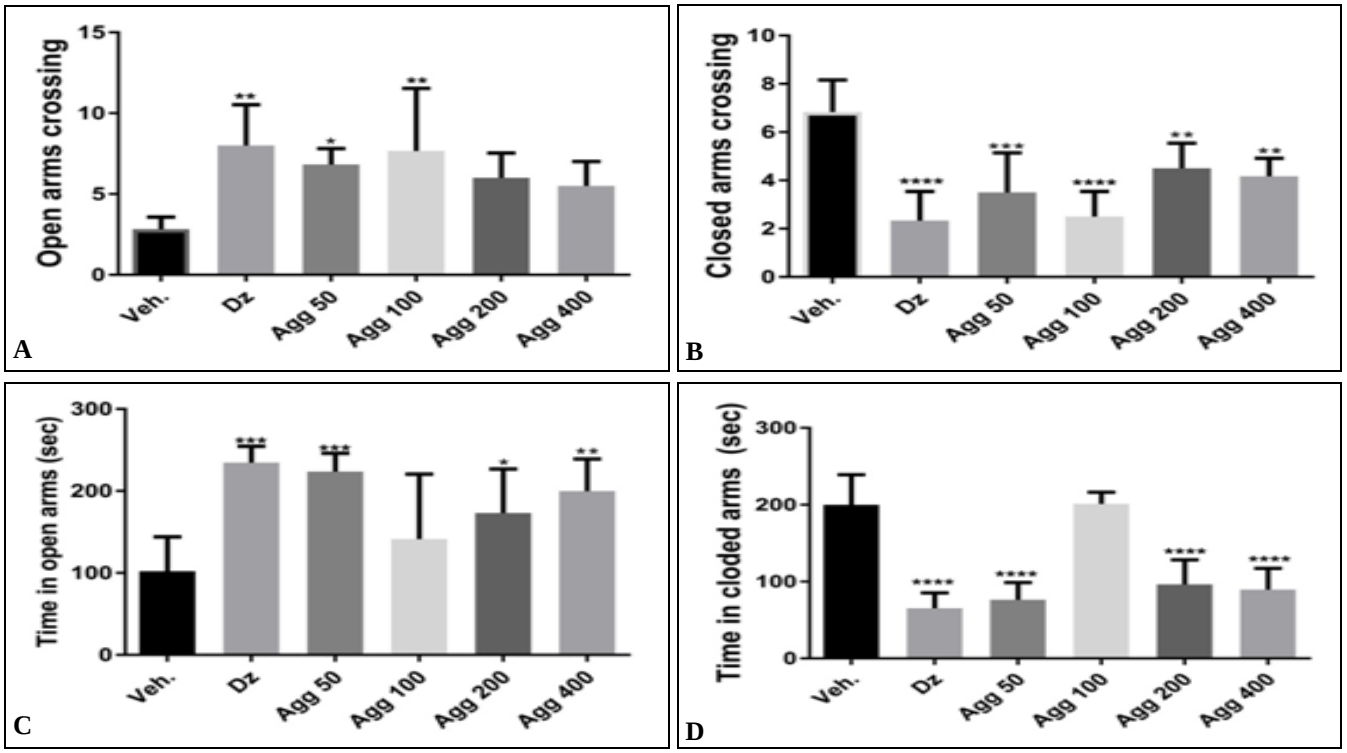

FIG. 3: NUMBER OF CROSSING ON OPEN ARMS (A) AND CLOSED ARMS (C), AND TIME SPENT IN OPEN (B) AND CLOSED ARMS (D) ON ELEVATED PLUS MAZE OF *A. GRATISSIMA* VAR. *GRATISSIMA* IN MICE (n=8). Data are expressed as media ±SD after one way ANOVA, followed by Dunnet posttest. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001

On the other hand, the number of entries and the time spent in the enclosed arms were reduced by the treatment with Agg. Thus, 50, 100, 200 and 400 mg/kg were statistically different from vehicle group in numbers of entries (Agg50 3.5±1.64; Agg 100 2.5±1.05; Agg200 4.5±1.05; Agg400 4.17 ±0.75; Veh. 6.83±1.33; Dz 6.83±1.33, **Fig. 3C**); and 50, 200 and 400 mg/kg were statistically different from vehicle group in time spent in the closed arms (Agg50 76.3±22.4; Agg100 201±15.2; Agg200 96.3±32.1; Agg400 89.5±28; Veh. 187± 47.6; Dz 65.3±20; **Fig. 3D**). Additionally, in all parameters the data showed that diazepam affected in a statistically significant manner when compared to the control group.

DISCUSSION: The acute toxicity test showed that this plant is safe since no signs and symptoms of toxicity or death were observed in the animals used in the test, like the finding in *A. gratissima* [14]. The results obtained in the barbiturate-induced sleep time test seem to indicate that this plant lacks depressant or stimulant effects on the Central Nervous System [24].

In the open field trial, the increase in exploratory activity and the decrease in immobility time were verified with all the tested doses of the extract of *A. gratissima* var. *gratissima*, when compared with the vehicle-treated group, this result is compatible with a possible anxiolytic effect. When compared the number and time of exploration of mice that received diazepam or the extract with vehicle-treated mice, it was evidenced that all of them differed significantly from the control group. This indicated the potential anxiolytic effect of the sample tested and agreed with the results obtained in the open field trial.

By verifying a statistically significant difference between the number and the time spent in exploring, between vehicle and diazepam groups in the hole-board assay, the anxiolytic effect of diazepam was confirmed, thus validating the method for the evaluation of an anxiolytic activity. Mice exposed to an unfamiliar surrounding tend to reduce their mobility and exploration due to their fear of being preyed upon by predators. If the mice are previously treated with an anxiolytic substance such as diazepam, their mobility within the apparatus will be considerably increased, as well as the number and time of explorations carried out in each hole. Therefore, a potential anxiolytic effect can be evaluated in the hole-board test [25].

In general terms, the methanol extract of *A. gratissima* var. *gratissima* increased the entry and residence time of mice treated in the open arms of the elevated plus-maze, and therefore decreased the entry and residence time in the closed arms. These results confirmed the suspected anxiolytic effect of this plant. The elevated plus-maze test is a validated method to verify the anxiolytic effects of chemical substances in rodents [21], by counting the number of entries and the measure of the residence time in open and closed arms. Rodents naturally show an aversion to open and high places and prefer to spend more time in closed arms. Anxiolytic like-effect substances such as diazepam cause rodents to increase their entry and residence time in open arms [26-27].

Further studies are required to determine the chemical composition of the tested extract, as well as to elucidate the mechanism of action by which the extracts exert their effect on biological systems, an interaction with the GABAA receptor can be presumed since in all tests, there are similarities in the responses with diazepam [4]. However, it is worth mentioning that other plants of this family have been studied, and the main components have been reported to be phenolic acids and carotenoid [14], which are associated with an important variety of biological activity.

In other Verbenaceae species, such as *Aloysia polystachya*, the anxiolytic and antidepressant like activity was determined [9, 11, 28, 29, 13], and phenyl-ethanoids glycoside and flavonoids from the hydroethanolic extract of leaves was described [28]. Additionally, phenylethanoid glycoside, particularly the main component acteoside or verbascoside was determined to have inhibitory activity of mono-amine oxidase-A, and both anxiolytic and anti-depressant activity have been associated to the presence of this component [28-29].

There is evidence that suggests that the cannabinoid receptor subtype 2 (CB2) has a role in anxiety and depression disorders [30-31]. Furthermore, it has been proven that β-caryophyllene was able to decrease the symptoms of anxiety and depression

due to its agonist effect on the CB2 receptor [32]. The essential oil of *A. gratissima* var. *gratissima* has been studied and the presence of both mono-terpenes and sesquiterpenes was determined in its composition [16]; among the sesquiterpenes β-caryophyllene is the main component.

CONCLUSION: The methanol extract of *A. gratissima* var. *gratissima* has no central nervous system depressant or stimulant effect, and anxiolytic - like effects were evidenced in mice according to the Hole Board tests (indicative test) and elevated plus maze test (confirmatory test). The results obtained support ethnobotanical knowledge, showing that it continues to be a good source of information to obtain plants with a therapeutic effect.

ACKNOWLEDGEMENT: The authors would like to thank Professor R. Degen who identified the plant material, Professor N. Alvarenga for providing the extract, and Mr George López for the English wording review.

CONFLICTS OF INTEREST: The authors declare that they have no conflicts of interest.

FINANCIAL SUPPORT: This research was partially supported by research grant from Rectorado Universidad Nacional de Asunción (Project FCQ 04/16) and Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay.

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How to cite this article:
Kennedy ML, Taboada T, Snead E, Diarte EMG, Coronel CM, Arrúa W, Heinichen OY, Montabetti Y, Hellión-Ibarrola MC, Ibarrola DA and Campuzano-Bublitz MA: Evaluation of methanol extract of *Aloysia gratissima* var. *gratissima* leaves on behavior and anxiety in mice. Int J Pharm Sci & Res 2021; 12(7): 3858-65. doi: 10.13040/IJPSR.0975-8232.12(7).3858-65.