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Neuropharmacological and anxiolytic effects of extracts of *Passiflora tripartita* var. *mollissima* on mice

[Efecto neurofarmacológico y ansiolítico de extractos de *Passiflora tripartita* var. *mollissima* en ratones]

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Abstract: Anxiety and depression cause alterations in the physiology of an organism. Extracts from the leaves of several *Passiflora* species are traditionally used Peru and in many countries as anxiolytic and in treatment for inflammatory problems. This study aimed to determine the neuropharmacological effect of the ethanolic extract of *Passiflora tripartita* var. *mollissima* (Kunth) Holm-Niels. & P. Jørg. and its anxiolytic effect on mouse (*Mus musculus* var. *albinus*). Anxiety was evaluated with the marble burying test and the depressant effect with the Irwin test (locomotor activity, base of support, wobbly gait, immobility, escape, ease of handling, muscular strength, tight rope, inclined plane, catatonia, nociceptive reflex and death). Doses of 100 mg/Kg/body weight and 200 mg/kg/body weight by intraperitoneal route (i.p.) significantly decreased anxiety levels ($p<0.05$) in mice, and had a non-significant depressant effect in 11 of the 12 tests, showing a similar direction of correlation between diazepam and *Passiflora* extract effect. A greater anxiolytic and anti-depressant effects in mice was observed with the extract dose of 200 mg/kg/body weight with neuropharmacological manifestations found where no death was observed at any dose used.

Keywords: Diazepam; Anxiety; Depression; Fear; *Passiflora tripartita* var. *mollísima*

Resumen: La ansiedad y la depresión provocan alteraciones fisiológicas. Las especies de *Passiflora* se utilizan tradicionalmente en Perú como ansiolíticos y para tratar problemas inflamatorios. Determinar el efecto neurofarmacológico del extracto etanólico de *Passiflora tripartita* var. *mollissima* (Kunth) Holm-Niels. & P. Jørg. y su efecto ansiolítico en ratones. Se evaluó la ansiedad con el test de enterramiento de canicas y el efecto depresor con el test de Irwin. Las dosis de 100 mg/kg/peso corporal y 200 mg/kg/peso corporal por vía intraperitoneal (i.p.) disminuyeron significativamente la ansiedad ($p<0,05$) con efecto depresor no significativo en 11 de las 12 pruebas, mostrando una correlación similar entre el diazepam aplicado a dosis de 1 mg/Kg/p.c. (i.p) y el efecto de *Passiflora*. Se observó un mayor efecto ansiolítico y antidepresivo en ratones con 200 mg/kg/peso corporal encontrándose manifestaciones neurofarmacológicas pero no se observó muerte a ninguna de las dosis empleadas.

Palabras clave: Diazepam; Ansiedad; Depresión; Miedo; *Passiflora tripartite* var. *mollísima*.

INTRODUCTION

Anxiety is a state of agitation in the face of an anticipated situation of a danger potentially causing psychological manifestations at cognitive, emotional, physiological, and motor levels (Soriano *et al*., 2019). It is recognized that the psychometric distinction between anxiety and depression is controversial because both constructs share a commonality of symptoms that make the task of distinguishing them difficult (Sanz *et al*., 2012).

Anxiety decreases a person's quality of life, which is counteracted therapeutically by the administration of serotonin reuptake inhibitor (SSRI) antidepressants and norepinephrine reuptake inhibitors (SNRIs) (Reyes-Marrero & de Portugal-Fernández., 2019). Benzodiazepines, such as diazepam, bind to gamma-aminobutyric acid (GABA) receptors in various regions of the brain and spinal cord. This binding increases the inhibitory effects of GABA. Therapeutically diazepam has become an effective and safe treatment option for the acute control of anxiety, although it is not indicated for long-term management (García-Atienza *et al*., 2021) due to risk of cognitive alterations or dependency (Azevedo *et al*., 2019).

Alonso *et al*. (2018), investigated the treatment of anxiety in 21 countries and found that anxiety has possibly been adequately treated only in 9.8% of the cases. In England and Wales, the rate of hospitalization due to intoxication by psychotropic drugs and the rate of prescription affecting the central nervous system (CNS) have increased in the last 21 years (Al-Daghastani & Naser, 2022). Combination therapy or the use of complementary and alternative medicine, such as psychopharmacotherapy, cognitive behavioral therapy, and herbal medicine, is currently of particular interest. Several medicinal plants have been identified and their effective components have been isolated and characterized as having cellular and molecular targets for the central nervous system (CNS) (Allameh & Orsat, 2022).

An important plant resource used for the treatment of symptoms of anxiety and depression (as well as cultural disorders such the so-called "susto" or scare by the populations of South America) is known in Peru as "tumbo" or "puru puru", which among Andean communities this plant possesses one of the highest Cultural Importance Index (Acosta *et al*., 2017) and commercial value (Castañeda *et al*., 2019). This plant is *Passiflora tripartita var. mollissima* (Kunth) Holm-Niels. & P. Jørg, which belong to the Passifloraceae, a family largely distributed in the Neotropical region and introduced in other parts of the world (POWO, 2022). (Note: Current taxonomic work place this plant under the name *Passiflora mollissima* (Kunth.) L.H. Bailey, a name validly published in a protologue by Bailey (1916) and accepted in the latest version of the International Plant Names Index (IPNI, 2022). *Passiflora tripartita* var. *mollisima* is a synomym of *P. mollisima*).

The chemical characterization of *Passiflora* species shows a diversity of compounds such as glycosylflavonoids (orientin, isoorientin, vitexin, isovitexin) (Soulimani *et al*., 1997; Avula *et al*., 2012, Costa *et al*., 2016), the alkaloid harmane (Rehwald *et al*., 1995; Méndez *et al*., 2001), derivatives of phenolic acid, organic acids, benzophenones, flavan-3-ols, flavonols and flavones (Giambanelli *et al*., 2020), and a number of compounds with antioxidant activity (Chaparro *et al*., 2015).

Geographical origin of the plant could also exert influence the chemical proprieties and expected response to the doses. Studies have shown that environmental factors affecting provenances of *Passiflora* e.g., locality altitude, solar radiation, and temperature affect the population's ecophysiological attributes and chemical composition of the aerial parts (Fischer *et al*., 2021). This could occur on this hill of Cachicadán, considered in Peru as "La Botica" for containing abundant medicinal plants used empirically, where the plant in this study comes from, that aimed to determine the neuropharmacological effect of the ethanolic leaf extract of *Passiflora tripartita* var. *mollisima* on anxiety manifestations **in** mice. The results pointed out to an anti-depressive effect exerted by the extract and its use as an anxiolytic medication.

MATERIAL AND METHODS

The study was conducted in the Laboratory for Nutritional Metabolism and Physiology Research, Pharmacology Department, Faculty of Pharmacy and Biochemistry, National University of Trujillo (UNT) in Trujillo, Peru. All experimental animals were treated according to the recommendations of Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes

(Guillén *et al*., 2014), as well as the ethical norms of the National University of Trujillo, Peru. (UNT, 2018).

Phytochemical analysis of the alcoholic extract

The Olga Lock method (Lock de Ugaz, 2016) was used to determine the metabolites in the ethanolic and aqueous extracts (Guillén *et al*., 2014). The aqueous extraction results served as a baseline to compare with the ethanolic extraction results.

Preparation of the ethanolic extract

The leaves of *Passiflora tripartite* var. *mollisima* were collected from the slopes of La Botica hill. Cachicadán (Latitude -8.09472, Longitude: -78.14898°, altitude of 3362 m) between 9:00 am and 3:00 pm. (Taxonomic determination was carried out in the UNT Herbarium Truxillense, registration code: 58465). The procedure of Bruneton (Bruneton, 1993) was followed throughout the sample processing. The leaves were selected and dehydrated for 96 hours at 40°C before being ground in a mechanical mill, sieved and stored in glass jars (Guillén *et al*., 2014). A mass of 135.5 g of dry pulverized sample was macerated with 2.8 L of 45% alcohol for 7 days at room temperature, stirring casually. Then, it was rotary evaporated, and 20 mL of the soft extract obtained was distributed in Petri dishes. The latter were placed in an oven at 37°C. The dose of the extract was obtained based on the average weight of the studied groups.

Diazepam dose preparation

The dose of 1 mg/kg diazepam was administered to the animals trhough the intraperitoneal route (i.p.). The group of mice to be treated with diazepam had an average weight of 27 g, based on this, dose was determined using the relationship 1 mg of diazepam per kilogram of body weight of the animal, saline solution was used as a solvent, never exceeding a volume of 0.1 mL per animal.

Study animals

Male mice (*Mus musculus* var. *albinus*) of 7 to 8 weeks of age were obtained from the Vivarium of the Faculty of Pharmacy and Biochemistry of the National University of Trujillo. They were accustomed to be housed individually with free access to water and food in a ventilated environment with a cycle of 12-hour light (07:00-19:00) and 12-hour darkness (19:00-07:00). Twenty mice having an average weight of 17.8 g were subject to the marble burying test and other 24 mice (with an average weight of 27.17 g) were used for the Irwing test.

The marble burying test

Glass boxes (48-48-30 cm) with transparent walls and bottom were built especially for this test. Sawdust was employed as a substrate. The mice were randomly assigned to groups of five and the following treatments were administered by intraperitoneal route (i.p.): The negative control group (GCNM) received 0.1 mL/kg of sodium chloride (0.9% w/v LABOT Brand Lot 140542). The diazepam group (i.e., positive control, GCPM) was 1 mL of 0.9% diazepam sodium chloride (1 mg/kg MEDIFARMA Brand. Lot: 10503450) (Cedillo-Ildefonso *et al*. 2008). The Passiflora group 100 mg/Kg (GP100M) consisted of 0.1 mL of ethanolic extract with 100 mg/Kg of *Passiflora mollisima* diluted in saline solution (0.9% w/v). Finally, the Passiflora group 200 mg/Kg (GP200M) was 0.1 mL of ethanolic extract of 200 mg/Kg of *Passiflora mollisima* diluted in saline solution (0.9% w/v).

After a period of about 25 to 30 minutes after administration, each mouse individually was placed in the glass box, where it was allowed to be habituated for 10 to 12 minutes, before starting the test (Bonilla *et al*., 2014). Then, the mouse was removed from the box and 24 equidistant marbles were placed on the substrate. The individual animal was reintroduced and left for 30 minutes, after that period the animal was removed. The marbles covered at least two thirds with sawdust were counted (Rejón-Orantes *et al*., 2011).

Irwin Test

Other groups of five mice each were randomly assigned to the following Irwin treatments: the control group (GCNI), the diazepam group (GCPI), the *Passiflora* 100 mg/Kg group (GPI00I), and the *Passiflora* 200 mg/Kg Group (GP200I) (Bonilla *et al*., 2014). These treatments were administered to mice by the intraperitoneal route (i.p). The test observations consisted of qualitative recognition of neurological manifestations in the mouse, after the treatments have been administered. The items that were analyzed were locomotor activity, base of support, wobbly gait, immobility, escape, ease of handling, muscular strength, tightrope, inclined

plane, catatonia, nociceptive reflex, and death. Accordingly, it was classified as the presence or absence of a specified effect displayed (Irwin, 1968).

Statistical analysis

For the marble burying test, the ANOVA analysis of variance and the Tuckey honestly significant difference (HSD) multiple comparison tests were carried out with a 95% The confidence coefficient for the set (Riofrío, 2014). In the case of the Irwin Test that determines the neuropharmacological effects of the substance, focused on whether the extract is a depressant for the central nervous system or not. Trials 0 to 5 assessed the non-depressive effect, and trials 6 to 12 the depressive effect.

RESULTS

The phytochemical composition of the *Passiflora* samples (Table No. 1) shows the presence of important secondary metabolites, including alkaloids (detected by the Dragendorf, Hager, Mayer, and Wagner tests), phenolic derivatives (ferric chloride reaction), flavonoids (Shinoda reaction), and reducing sugars (Fehling reaction).

Table No. 1
Results of the phytochemical evaluation of *Passiflora tripartita* var. *mollisima* from the Trujillo and Cachicadán provenances

Test: metabolite			Provenance	
	Trujillo		Cachicadán	
	Aqueous extract	Ethanol extract	Aqueous extract	Ethanol extract
Dragendorff: alkaloids	-	-	-	+++
Hager: alkaloids	+	-	++	-
Mayer: alkaloids	-	-	-	++
Wagner: alkaloids	-	-	-	+
Baljet: coumarines		-		-
Shinoda: flavonoids	+	+	++	+
Rosenhein: leucoamthocyanidin	-		-	
Iron III chloride: phenolic compounds	+	+	+	+
Bortranger: quinones		-		-
Resin test: Resines		-		-
Fehling: reducing sugars	+	-	+++	+
Foam: steroidal saponins	+		-	

+++ High concentration of metabolite, ++ Median concentration of metabolite, + Low concentration of the metabolite, - Absence of the metabolite

The mice were randomly distributed by entering the glass box for 30 minutes into four groups with five individuals per group depending on the substance administered. Negative control group, Diazepam Group or positive control, *Passiflora* 100 mg/Kg Group, and *Passiflora* 200 mg/Kg Group.

In the marble burying test (Figure No. 1), the mice in the positive control group with diazepam significantly decreased the burial of spheres (p<0.05) with respect to the negative control group (GCNM). A similar sedative effect was found among mice treated with *Passiflora*-100. Mice treated with the *Passiflora*-200 did not perform any marble burying activity, they would not present anxiety.

The group treated with the lowest dose of 100 mg/kg (GP100M) of the ethanolic *Passiflora* extract showed a significant decrease in the burial of spheres (p<0.05) with respect to the negative control group and when compared with the result of the group that used Diazepam or positive control, it was observed that both have a response very similar anxiolytic.

Both administered doses of the *Passiflora* extract suggested an anxiolytic effect, due to its non-significant difference with the diazepam control group, buried 2.0 marbles (SD 2.34). The *Passiflora*-100 buried 2.8 marbles presented (SD 4.14). The *Passiflora*-200 group were in a torpor state, and buried 0 spheres, and presented a torpor condition.

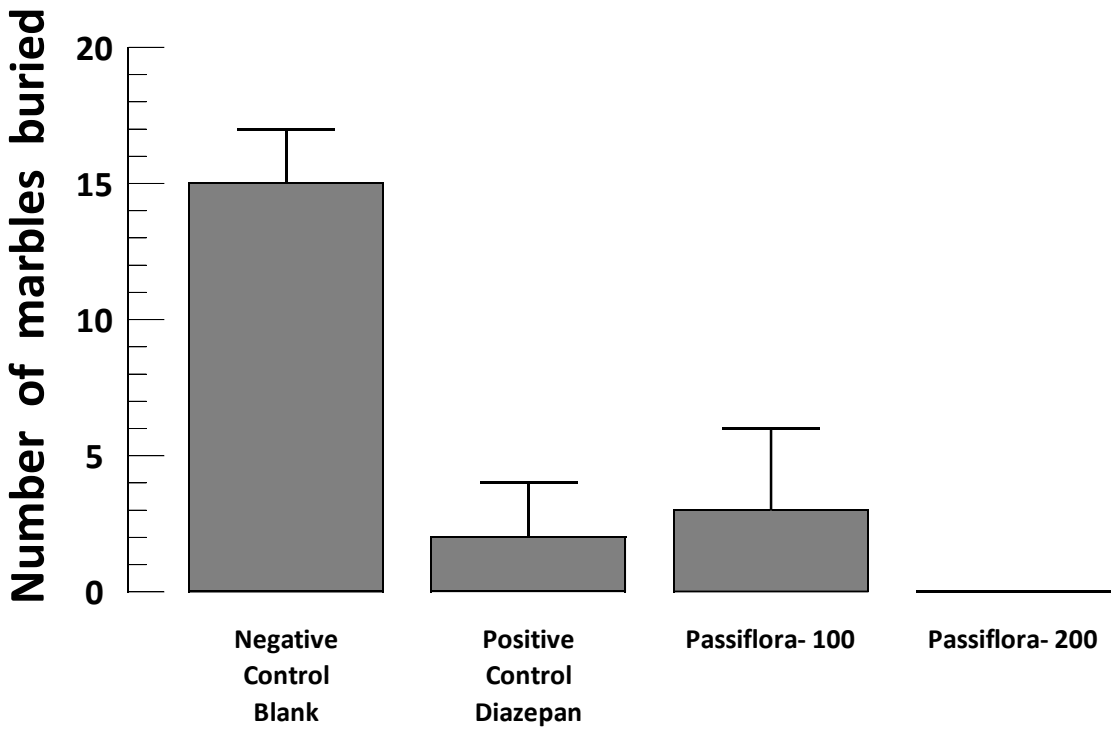

Figure No. 1
Mean and standard error of the number of buried spheres in relation to the study groups. The mice were randomly distributed by placing them in the glass box for 30 minutes. The negative control group received physiological saline solution, the positive control received diazepam dissolved in saline solution, the *Passiflora*-100 and *Passiflora*-200 treatments animals received a dose of 100 mg/Kg, and *Passiflora* 200 mg/Kg dissolved a saline solution preparation, respectively

All the results of the groups treated with diazepam, and *Passiflora* extracts compared to the negative control pointed out that there is a greater anxiolytic effect when administering the extract at a higher dose (see *Passiflora*-200 results).

To obtain a neuropharmacological profile, Irwin test was carried out in the mice, evaluating the following activities: locomotor incapacity, the base of support, wobbly gait, immobility, inability to escape, ease of handling, muscular weakness, tight rope, inclined plane, catatonia, nociceptive reflex and death (Figure No. 2).

Figure No. 2
Irwing test results. Each individual mouse was an experimental unit used only once for a specific treatment (i.e., experimental units were nested by treatment) (n=24)

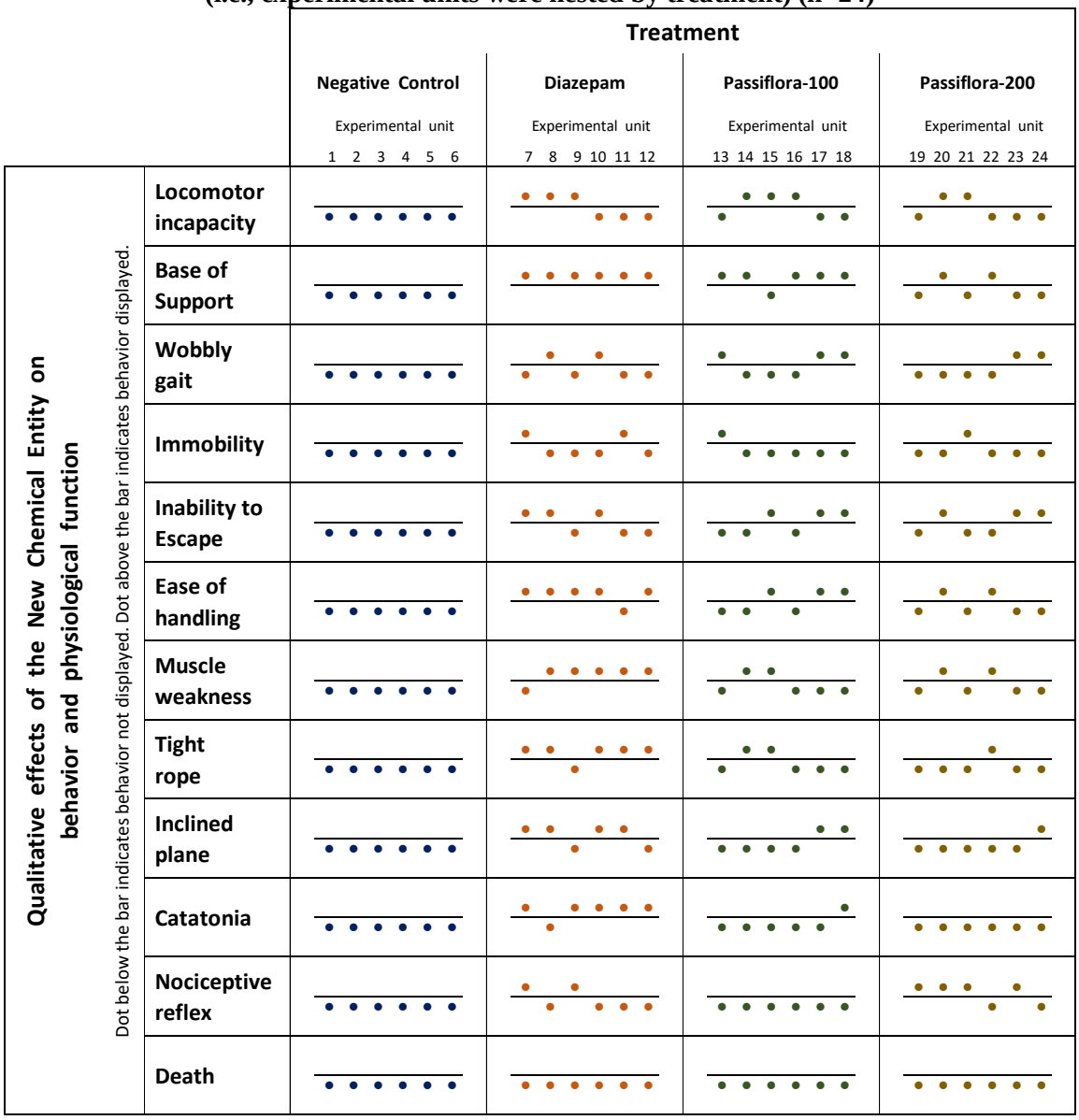

Table No. 3

Correlation between the effects obtained at a single-mouse basis mouse for each Irwing test activity (i.e pairwise comparison between the results in different test by the same mouse). In each case n=6. Individuals were nested by treatment; thus, they were different for each test and replication. Top value is the correlation of positive control (diazepam) treatment, bottom value is the correlation of *Passiflora*-100 treatment. Boxes highlighted with thick borders indicate that the correlations followed the same signed direction)

	Wobbly gait	Immobility	Inability to Escape	Muscular weakness	Tight rope	Inclined plane	Catatonia
Base of Support	- **0.45**	- **0.20**	- **0.45**	- **0.32**	- **0.32**	- **0.32**	- **0.20**
Wobbly gait		0.00 **0.45**	0.71 **0.33**	-0.45 **-0.71**	0.45 **-0.71**	0.71 **0.71**	-0.45 **0.45**
Immobility			-0.25 **-0.45**	-0.63 **-0.32**	0.41 **-0.32**	0.50 **-0.32**	0.32 **-0.20**
Inability to Escape				-0.32 **0.00**	0.63 **0.00**	0.25 **0.71**	-0.32 **0.45**
Muscular weakness					-0.20 **1.00**	-0.32 **-0.50**	-0.20 **-0.32**
Tight rope						0.63 **-0.50**	-0.20 **-0.32**
Inclined plane							-0.32 **0.63**

To evaluate the tendency of the rodent performance under different tests, a correlation analysis was carried out in which results were compared to each other on a mouse-by-mouse basis (Table No. 3).

Those animals displaying a wobbly gait did also show an inability to escape, muscular weakness, and inability to control its own movement in an inclined plane.

A high positive correlation is observed between the wobbly gait tests with an inclined plane and between escape and inclined plane, thus suggesting that a gait disorder may be associated with impairment of the central nervous system.

Negative values would indicate that there is no correlation between wobbly gait with muscular strength and tight rope, immobility and escape, immobility and muscular strength, tight rope and inclined plane, and between immobility and catatonia, there would be no correlation between muscle strength and inclined plane and muscle strength with catatonia.

DISCUSSION

The neuropharmacological profile of *Passiflora tripartita* var. *mollisima* and its effect on anxiety were evaluated with the Irwin and Marble burying tests in mice. The doses prepared from the *Passiflora* extracts were compared with the effect produced by the widely marketed anxiolytic diazepam.

In the Marble burying test (Figure No. 1), the mice in the positive control group (diazepam-treated) significantly decreased the burial of spheres ($p<0.05$) with respect to the negative control group, results that are similar to those of previous studies carried out in tests verifying the effect of asenapine acting upon anxiety behaviors, and wherein diazepam also reduced the activity of marble burying (Ene *et al.*,

2016).

Diazepam, whose mechanism of action is to increase the effects of the inhibitory neurotransmitter GABA (Abubakar *et al*., 2021) has a sedative effect, previous studies (Espitia-Camacho *et al*., 2008) found allow us to link the various effects on the central and peripheral nervous system (Salazar-Granara *et al*., 2016). In this study It was found that the total number of mice corresponding to the positive control group with diazepam passed more tests with respect to the negative control group, which is to be expected in a drug registered for this purpose and coincides with results of various tests to determine local cortical neuronal activity during sleep in mice, where they found that the slow wave amplitude of mice was significantly reduced after diazepam injection (McKillop *et al*., 2021).

A similar effect was observed in the group taking the *Passiflora* extract. Since the extract is an infusion containing a complex mixture of metabolites, its effects cannot be attributed to any specific compound in it. The dose of 200 mg/kg of the *Passiflora* ethanolic extract, the burial of spheres was observed to decrease completely (p<0.05) with respect to the negative control group, suggesting that the rodents studied were under a very strong effect of calm an even inducing sleep. the doses data of 100 mg/kg coincide with the results of the study of the ethanolic extract of *Passiflora salpoense* in "albino mice" who significantly decreased the number of buried spheres compared to the control group, when treated with doses of 50 mg/kg and 100 mg/ kg, with significance of (p<0.05) (Leiva-Salinas *et al*., 2019).

A similar effect was observed in the group taking the *Passiflora* extract. Since the extract is an infusion containing a complex mixture of metabolites, its effects cannot be attributed to any specific compound present in it. The *Passiflora*-200 group showed no burial of spheres with respect to the negative control group (p<0.05), could be explained by recognizing that the rodents were under a very strong calming effect, even soporiferous. The *Passiflora*-100 data coincide with the results of another study in *Passiflora salpoense* in "albino mice" who significantly decreased the number of buried spheres compared to the control group, when treated with doses of 50 mg/kg and 100 mg/kg, with significance of (p<0.05) (Leiva-Salinas *et al*., 2019).

There are a wide variety of effects caused by the different chemical compounds. For instance, the flavonoids such as vitexin, which has been determined in *Passiflora* (Costa *et al*., 2016), possess an antioxidant and anti-inflammatory effect *in vitro* (Marrassini *et al*., 2023). A study of patients in Vietnam showed that those undergoing breast cancer cobalt-60 radiotherapy, vitexin administration produced a significant radioprotective effect on peripheral blood cell numbers and lymphocyte transformation function (Van Hien *et al*., 2002)., which coincides with a systematic review that included nine clinical trials with treatment from one day to 30 days in people over 18 years of age, where the effects of passionflower were measured using several different tests and scales reported in most studies reduced levels of anxiety after the administration of *Passiflora incarnata* preparations, with a less evident effect in people with mild symptoms of anxiety (Janda *et al*., 2020).

On the other hand, the classes of secondary metabolites found in *Passiflora mollisima* (Table No. 1) agree with other *Passiflora* studies (García-Peña *et al*., 2009; Giambanelli *et al*., 2020). They have been reported as important components in medicinal applications, particularly alkaloids such as those derived from β-carboline (Freire *et al*., 2018). Especial reference is given to harmane alkaloids which have been found in roasted vegetables subjected to high temperatures (Wojtowicz *et al*., 2015). In a review on the harmane-carboline alkaloids, Khan *et al*. (2017) reported about the various pharmacological activities such as anxiolytic, antidepressant, antiplatelet agent, antidiabetic, inhibitor of acetylcholinesterase and myeloperoxidase. This class of alkaloids also possess antioxidant, antiparasitic, and hypotensive activities. It helps in relieving morphine withdrawal syndrome and confer antinociceptives effects but show also a tremorogenic effect and other adverse effects in learning and memory (Khan *et al*., 2017).

A literature review using SCOPUS (accessed February 2023) using "*Passiflora*" as keyword, it listed 4211 publications. However, using the keyword "*Passiflora tripartita var. mollísima*" yielded only four studies from Colombia, two of them concern with the use of the pectin found in the fruit exocarp for human consumption, which reported its hypoglycemic activity (Ortíz & Anzola, 2017) and its influence in the digestibility of lipids (Espinal-Ruíz *et al*., 2016). Another study Hernández-Rivera (2018) tested the seed oil as potential excipient for

nanoemulsion. Ramos *et al*. (2010), found the flavonoid orientin in an ethanolic leaf extract of *Passiflora tripartita*. This flavonoid was studied by Abusaliya *et al*. (2022), and found that it has an application in the treatment against cancer. The administration of orientin-containing nanoparticles in mice was reported to provide antidepressive activity in mice (Alves *et al*., 2020). The apigenin flavone glycoside vitexin was found to be the major bioactive component in ethanolic leaf extracts and fruits of *Passiflora incarnata* and found to improve the neurogenesis and protect the loss of memory in mice DBA/2 presenting sleep disorder manifestations (Kim *et al*., 2019). Polyphenols have been reported present in alkaline hydrolytic extracts from the fruit of *P. tripartite* (Domínguez-Rodríguez *et al*., 2022). Compounds of the flavonoid and polyphenol classes were also found in this study, particularly the La Botica provenance, in an Andean region, considered by the locals as a ethnobotanical resource of great value. The extensive use and proven effectiveness as an anxiolytic and antidepressive in local formulations suggests that *Passiflora tripartita* does deserve further pharmacological investigations and *in vivo* studies.

CONCLUSIONS

This study provides qualitative evidence that the ethanolic leaf extract of *Passiflora tripartita var. mollisima* produce an anxiolytic and antidepressant effects in mice, which suggest a mode of action affecting the central nervous system of the rodents. Given its broad use in traditional medicine, it would benefit to further evaluate the neuropharmacological profile of distinct provenances of *Passiflora mollisima* on a quantitative basis and promote therapeutic research at the clinical level.

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