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Metabolite profile of *Passiflora mollisima* leaf using UHPLC-ESI-MS and its potential anxiolytic activity in mice

Perfil de metabolitos de hojas de *Passiflora mollisima* utilizando UHPLC-ESI-MS y su potencial actividad ansiolítica en ratones

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ABSTRACT

Background: Traditionally, extracts of *Passiflora mollisima* leaves are used as anxiolytics, but the existing metabolites are unknown. **Objectives**: To identify by UHPLC-ESI-MS/MS some of the metabolites and the anxiolytic activity of the leaf extract. **Materials and Methods**: The extract was subject to UHPLC-ESI-MS/MS analysis and administered intraperitoneally (VIP) to 4 groups of mice: G1: white group 0.1 mL of saline solution, G2: positive control group 1 mg kg^{-1} of diazepam and to treatment groups, namely, G3 50 mg kg^{-1} and G4 100 mg kg^{-1} and anxiety levels were evaluated with the light/dark transition test for mice. **Results**: Six flavone C-glycosides were tentatively identified, namely vicenin 2, lucenin 2, schaftoside, orientin, vitexin, and glucopyranosyl methyl luteolin. Lower levels of anxiety were observed in animals of groups G3 and G4 based on the number of transitions, with a mean of 11±2 and 21±2, respectively, compared to G1, which was 2±1 ($p<0.05$). The groups displayed a significant difference among them ($p<0.05$). There was an increase in the total time spent in darkness in G3 and G4 *Passiflora*-treated mice with a mean of 70±8 and 113±8 respectively, compared to G1, which was 8±1 with a $p<0.05$. **Conclusion**: The metabolites identified have biological activities, neuroprotective, antioxidant, anti-inflammatory, antibacterial, and anticancer effects. The results of the effects of the 50 and 100 mg Kg^{-1} doses of ethanolic extract of *Passiflora mollisima* leaves significantly decreased anxiety levels ($p<0.05$). This information contributes towards its further use in a therapeutic, clinical setting.

Keywords: Tranquilizers; anxiety; apigenin; vitexin; diazepam, UHPLC-ESI-MS/MS

RESUMEN

Tradicionalmente, extractos de hojas de *Passiflora mollisima* son utilizados como ansiolíticos, pero se desconocen los metabolitos existentes. **Objetivos**: Identificar por UHPLC-ESI-MS/MS algunos de los metabolitos y la actividad ansiolítica del extracto de las hojas. **Materiales y Métodos**: El extracto se sometió a análisis UHPLC-ESI-MS/MS y se administró por vía intraperitoneal (VIP) a 24 ratones: Grupo 1 0.1 mL de sodio cloruro 0,9%. Grupo 2 control positivo 1 mg kg^{-1} de diazepam. Y a Grupo 3 dosis de 50 mg kg^{-1} y Grupo 4 dosis de 100 mg kg^{-1} del extracto. Los niveles de ansiedad se evaluaron con la prueba de transición luz/oscuridad. **Resultados**: Se identificaron seis glucósidos C de flavona, vicenina 2, lucenina 2, schaftósido, orientina, vitexina y glucopiranosil metil luteolina. Se observaron menores niveles de ansiedad en los animales del grupo G3 y G4 en función del número de transiciones, con una media de 11±2 y 21±2, respectivamente, en comparación con G1 que fue de 2±1 ($p<0,05$). Los grupos mostraron diferencia significativa entre ellos ($p<0,05$), el tiempo total pasado en la oscuridad por los ratones G3 y G4 fué mayor con una media de 70±8 y 113±8 respectivamente, en comparación con G1 que fue de 8±1 con una $p<0,05$. **Conclusión**: Los metabolitos identificados se han relacionado con un amplio espectro de actividades biológicas, incluidas actividades neuroprotectoras, así como efectos antioxidantes, antiinflamatorios, antibacterianos y anticancerígenos. Los resultados de los efectos de las dosis de 50 y 100 mg Kg^{-1} de extracto etanólico de hojas de *Passiflora mollisima* disminuyeron significativamente los niveles de ansiedad ($p<0,05$), Esta información contribuye a su uso posterior en un entorno clínico terapéutico.

Palabras clave: Tranquilizantes; ansiedad; apigenina; vitexina; diazepam, UHPLC-ESI-MS/MS.

INTRODUCTION

Andean communities have used plants as an abundant source of food and medicines to sustain life and health throughout their history. Among the medicinal plants native to this area is *Passiflora mollisima* L.H. Bailey, a perennial vine belonging to the passion fruit family (Passifloraceae), commonly known in Peru as 'tumbo'. It is largely distributed in the neotropical region and grows in altitudes between 50 to 1,500 m. Knowledge of the use of different plant parts constitutes an important component of the lore and tradition of these communities, which use it as an herbal remedy to treat a variety of ailments and conditions, especially those requiring a sedative, anxiolytic, hypoglycemic effect (1).

There have been some studies characterizing the chemical constituents of *Passiflora* leaves (2, 3), seeds (4), and fruit (5), and they have shown to possess important biological activities such as antioxidant, antimicrobial, antihyperglycemic, and anti-cancer effects. The metabolites of the flavonoid class are responsible for much of the antioxidant activities in leaf extracts from *P. alata, P. caerulea* and *P. incarnata*. In addition, other beneficial effects related to it include antibacterial, antifungal, amoebostatic, and amebicidal activities. All this points out the possibility of using these extracts in a healthy diet and a source of health-promoting natural products (6). There is, therefore, much to be gained by discovering the actual bioactive compounds present in this plant, particularly in its leaves. Despite its importance, identifying secondary metabolites to improve its health-promoting effects has not been studied in depth. The purpose of this work was to screen plant extracts using ultra-high performance liquid chromatography and mass spectrometry to determine its bioactive compounds and to evaluate its anxiolytic activity in mice.

Materials and methods

Collection, taxonomic identification. Plant material. The pathogen-free leaves were collected from *Passiflora mollisima* trees (Figure 1) located in La Botica hill near the town of Cachicadán (La Libertad Department, Peru) between 9:00 and 15:00 hours from the La Botica hill in the town of Cachicadán located in the mountain region of the La Libertad Department (Peru). It was taxonomically identified in the Herbarium Truxillense (HUT; code 58465) of the National University of Trujillo as Passiflora tripartita var. mollisima, which is synonymous to *Passiflora mollisima*, the currently accepted name.

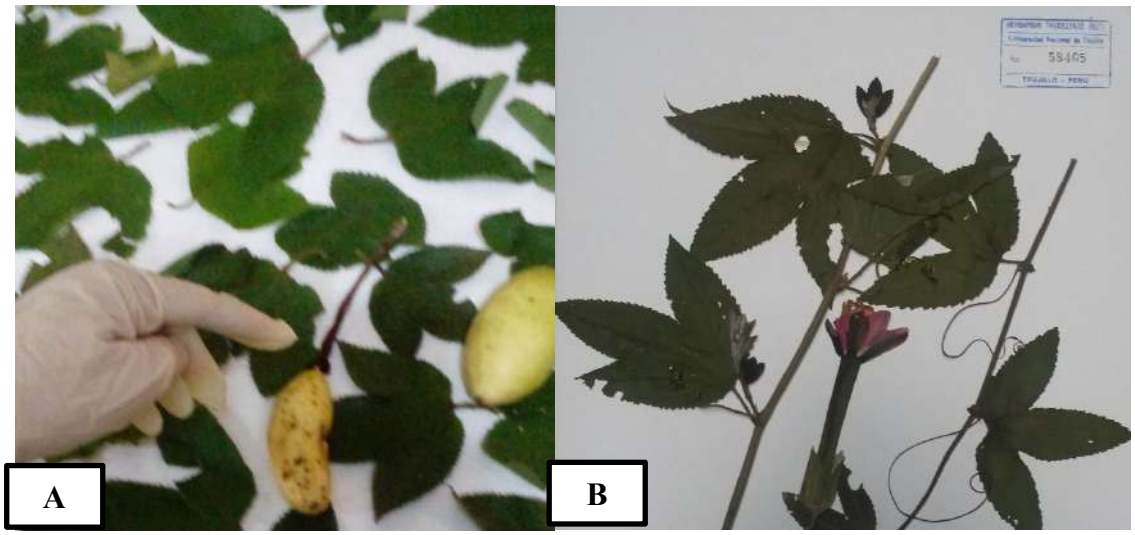

Figure 1. *Passiflora mollisima*. A: Leaves and fruit B: Leaves and flower.

Preparation of the plant samples. Leaves were dehydrated at room temperature (15-19 °C) under shade for 96 hours. The dried leaves were then mechanically ground and sorted to a 0.5 mm in diameter powder and stored in dark-colored flasks (7).

Preparation of the ethanolic extract. The stored material was macerated at the proportion of 48.5 g of pulverized dry sample per liter of 45% alcohol for 7 days at room temperature, shaking occasionally. A Halthen rotary evaporator was then used to remove the solvent (alcohol). The extract was distributed in Petri dishes, dispensing approximately 20 mL of the extract in each plate. They were subsequently placed in an oven at 37 °C for 48 hours to eliminate the remaining moisture. The extract was stored in the same plates in a sterile, dark and dry environment for its preservation. Finally, for its dosage, the dried solid extract was weighed and dissolved in physiological saline solution (0.9 % w/v) according to a pre-determined concentration (8).

Analysis by UPLC MS/MS. Ultrahigh performance liquid chromatography–electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) was the method employed for the assessment of metabolites in the biological samples. A sample of 10 mg of dry extract were weighed and dissolved in 10 mL of a mixture of LC-MS-grade acetonitrile (Supelco, Germany) and ultrapure water (50:50). It was then directly filtered into a 1.5 mL amber vial with a precut septum, using a 3 mL sterile syringe and a 0.2 µm pore x 25 mm diameter PTFE syringe filter. The prepared sample was placed on the UHPLC-ESI-MS/MS Xevo® TQ-XS® equipment (Waters, USA) having a C18 ACQUITY UPLC HSS T3 column with dimensions of 2.1 mm in diameter and 150 mm in length at 40 °C. For the chromatographic run, an aqueous solution of 0.1% of LC-MS grade formic acid (Sigma- Aldrich, USA) (phase A) and 0.1% acetonitrile formic acid (phase B) was used. The flow used was 0.3 mL/min and the phase A gradient: 0-4.17 min (97-90%), 4.17-6.25 min (90-85%), 6.25-8.34 min (85-85%)), 8.34-10.42 min (85-80%), 10.42-14.59 min (80-75%), 14.59-16.67 min (75-70%), 16.67-18.76 min (70-50%), 18.76-21.67 min (50-10%), 21.67-23.76 min (10-10%), 23.76-27.09 min (10-97%), 27.09-30 min (97-97%). A Survey Scan function was used with scanning in Scan Wave MS, scanning was from *m/z* 50-2000. The mass detector parameters were 2.5 kV capillary voltage, 40 V cone voltage with a gas flow of 150 L/h, desolvation temperature 480 °C, and 35 Ev collision energy (9).

Animals. Male, five-month-old mice, were obtained from the Bioterio of the Department of Pharmacy and Biochemistry of the National University of Trujillo. They were housed in cages in groups of 6 with access to food and water.

Dose. The animals were weighed in a laboratory balance. The treatment dose of 50 or 100 mg of the dry ethanolic leaf extract per kg of animal's mass. The control group received 1.0 mg of diazepan per kg (10). Body weight was as dose per mouse = (0.050 or 0.10 g) / (average body weight). This leaf extract amount in mg was weighed and emptied into a vial, then 2 drops of alcohol and 1 mL of a 0.9% NaCl solution were added, shaken and finally loaded into a 1 mL-syringe from which 0.1 mL of it was applied through intraperitoneal route to each individual.

The light/dark transition test. A rectangular box opens at the top (46x27x30 cm) divided into a small area (18x27 cm) and a large area (27x27 cm) was used, with an opening door (7.5 to 7.5 cm) located in the center at ground level of the box. There was a partition separating a large from a small compartment. The behavioral tests were performed between 9:00 and 18:00 hours. All cages containing the mice were brought to the behavioral testing room. The interior of the small compartment was black in color and illuminated by a dim 60 W red light, while the large compartment had bright white light illumination by a 100 W light source. Mice were placed in the dark compartment, allowing them to move freely between the two compartments with the door open for 5 min. The data were recorded using a digital camera. After each trial, all compartments were wiped with 96% alcohol to avoid bias based on olfactory cues. The parameters evaluated were: The total time spent in the illuminated compartment and the total number of transitions, which is the transition from the dark to the white compartment and vice versa (11). During a 5 minute-session, the animals were allowed to freely explore a new environment composed of two different compartments: protected (dark) and unprotected (lit). The total time was recorded. The increase in the number of transitions between the dark and bright compartment, and the increase in the time spent on the light side, were considered to be indicative of reduced anxiety. Although rodents are free to explore both compartments, they showed a clear preference to stay on the dark side.

Statistical method. Standard liquid chromatography and mass spectroscopy methods were used included the comparison of their experimental masses in high-resolution mass spectrometry with theoretically calculated masses.

Ethical considerations. This study was approved by the Institutional Review Board of the Faculty of Pharmacy and Biochemistry of the National University of Trujillo (Resolution No. 3252017/ ESCFARM),

RESULTS

This work aimed to determine whether *Passiflora mollisima* leaf extract had an potential anxiolytic effect on mice behavior. To this end, a dosage of ethanolic leaf extract was applied via intraperitoneal injection to the animals, and the chemical composition of the extract was determined using liquid chromatography and mass spectroscopy.

The adduct $[M-H]^-$ precursor ions were generated by the negative ionization mode of the Xevo® TQ-XS® mass spectrometer, which were then analyzed by the product ion mode to produce their fragments or secondary ion peaks. Six compounds were identified (Table 1). Compound (1) (9.64 min retention time) with deprotonated ion at *m/z* 593 and molecular formula $C_{27}H_{30}O_{15}$ was suggested as 6,8-di-C-β-D-glucopyranosylapigenin (vicenin 2) (12). The MS/MS fragment of the same compound was observed at *m/z* 473, indicating the possible loss of $C_4H_8O_4$ (loss of 120). It also indicated a characteristic fragment ion at *m/z* 431, most likely due to the neutral loss of $C_6H_{10}O_5$ (loss of 162 amu) of the parent ion. Another fragment ion was observed at *m/z* 311, possibly due to a loss of $C_4H_8O_4$ and $C_6H_{10}O_5$ (loss of 282 amu) from the parent ion. Compound (2) (eluted at the retention time of 9.80 min) gave a deprotonated molecular [M-H]- ion at *m/z* 609 ($C_{27}H_{30}O_{16}$) which according to the molecular composition and the fragmentation mechanism was identified as a flavonoid, possibly 6,8-di-C-β-D-glucopyranosylluteolin (luteolin 2) (12). MS/MS spectrum of this compound presented fragments at *m/z* 447, 357 and 327, indicating possible cleavage of $C_6H_{10}O_5$ (loss of 162 amu), $C_3H_6O_3$ + $C_6H_{10}O_5$ (loss of 252 amu) and $C_4H_8O_4$ + $C_6H_{10}O_5$ (loss of 282 amu) from the parent ion at *m/z* 609, respectively.

Table 1. Mass spectral information of flavonoid glycosides identified in ethanolic extracts of *Passiflora mollisima* leaves

	Compound	MF	MW	Retention time	UV	[M-H]-	Fragment
			g·mol^{-1}	min	nm	*m/z*	*m/z*
1	**Vicenin 2** 6,8-di-C-β-D-glucopyranosylapigenin	$C_{27}H_{30}O_{15}$	594.5	9.64	269, 343	593	473, 431, 311
2	**Lucenin 2** 6,8-di-C-β-D-glucopyranosylluteolin	$C_{27}H_{30}O_{16}$	610.5	9.8	269, 350	609	447, 357, 327
3	**Schaftoside** 6-C-β-D-glucopyranosyl-8-C-α-L-arabinopyranosylapigenin	$C_{26}H_{28}O_{14}$	564.5	12.15	269, 337	563	473, 443, 383, 353
4	**Orientin** 8-C-β-D-glucopyranosylluteolin	$C_{21}H_{20}O_{11}$	448.4	12.47	270, 344	447	327, 297, 285
5	**Vitexin** 8-C-β-D-glucopyranosylapigenin	$C_{21}H_{20}O_{10}$	432.4	13.48	269, 335	431	311, 283
6	**Glucopyranosyl methylluteolin** 6-C-β-D-glucopyranosyl-3'-O-methylluteolin	$C_{22}H_{22}O_{11}$	462.12	14.54	270, 347	461	371, 341, 326, 313, 298

MF: Molecular formula, MW: Molecular weight, UV: Ultraviolet radiation. [M-H]: Precursor ion

Compound (3) according to the spectrum could be 6-C-β-D-glucopyranosyl-8-C-α-L-arabinopyranosylapigenin (schaftoside) the same one that was registered in the retention time of 12.15 min in mode of negative ionization and produced a molecular ion peak at *m/z* 563 ($C_{26}H_{28}O_{14}$). A fragment also appeared at *m/z* 473 after the possible loss of the $C_3H_6O_3$ group (loss of 90 amu) of the original ion, likewise a fragmentation of ions was found at *m/z* 443 [M-H-$C_4H_8O_4$] (loss of 120 amu), *m/z* 383 [M-H-$C_3H_6O_3$-$C_3H_6O_3$] (loss of 180 amu) and *m/z* 353 [M-H-$C_4H_8O_4$-$C_3H_6O_3$] (loss of 210 amu) (12). The metabolite (4), with molecular formula $C_{21}H_{20}O_{11}$ (found at a retention time of 12.47 min) with the parent ion [M-H]- at *m/z* 447 in negative ionization mode, was identified as 8-C-β-D-glucopyranosylluteolin (orientin) according to the database (12) (Simirgiotis et al., 2013). This in turn produced daughter fragments at *m/z* 327 [M-H-$C_4H_8O_4$] (loss of 120 amu), *m/z* 297 [M-H-$C_5H_{10}O_5$] (loss of 150), 285 [M-H-$C_6H_{10}O_5$] (loss of 162 amu) (13). Compound (5) (eluting at 13.48 min) with an original ion peak at *m/z* 431 and molecular formula $C_{21}H_{20}O_{10}$ was identified as a flavonoid, 8-C-β-D-glucopyranosylapigenin (vitexin) (14).

The molecular ion of compound (6) at *m/z* 461 [M-H]- eluting at a retention time of 14.54 min, yielded the diagnostic fragment ion at *m/z* 371, which was due to cleavage of the $C_3H_6O_3$ molecule (loss of 90 amu). The presence of the daughter ion at *m/z* 341 was due to loss of $C_4H_8O_4$ (loss of 120 amu) from the parent ion, fragment *m/z* 326 was also observed by molecular cleavage of $C_4H_8O_4$ and CH_3 (loss of 135). Similarly, fragment *m/z* 313 was observed by molecular cleavage of $C_4H_8O_4$ and then CO (loss of 120 + 28 amu) and fragment *m/z* 216 [M-H-$C_6H_{11}O_5$] (loss of 163 amu). Based on spectral and literature data, compound (6) was provisionally assigned to 6-C-β-D-glucopyranosyl-3'-O-methylluteolin (13).

The identified compounds recorded Table 1 are structurally flavonoids of the C-glycosylflavone class (14), occurring as glycosides of apigenin and luteolin (15). These included the trihydroxyflavones functionally related to apigenin (vicenin 2, schaftoside, and vitexin); or the tetrahydroxyflavones related to luteolin (lucenin 2, orientin, and methyl-luteolin) (16).

Of the six structures identified; flavonoids in Figure 1: 6-C-β-D-glucopyranosyl-8-C-α-L-arabinopyranosylapigenin (schaftoside), in Figure 2E: 8-C-β-D-glucopyranosylapigenin (vitexin) and in Figure 2. TO; 6,8-di-C-β-D-glucopyranosylapigenin (vicenin 2) which are apigenin-like structures studied for their therapeutic potential to recover physiology in mammals (17).

UHPLC-ESI-MS. Mass spectra obtained from the analysis of the leaf extracts included liquid chromatography and negative-mode ESI-MS (Figure 1). Proposed compounds were first assumed by comparing their experimental masses in high-resolution mass spectrometry with theoretical calculated masses. Identification of compounds

was carried out using tandem mass spectrometry (MS/MS), which determines the structure of compounds found in heterogeneous mixtures.

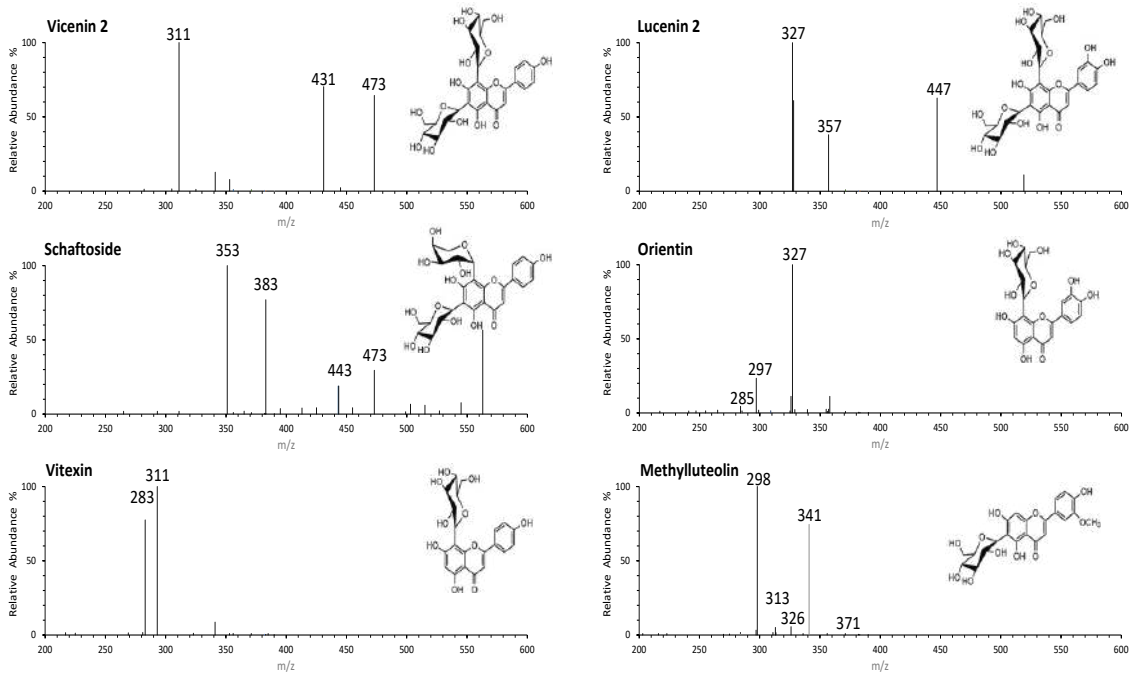

Figure 2. Mass spectrum of some the metabolites found in *Passiflora mollisima*

The compounds identified are some derivatives of lutein (Figure 2), namely, 6,8-di-C-β-D-glucopyranosylluteolin (lucenin 2), 8-C-β-D-glucopyranosyl luteolin (orientin) and 6-C-β-D-glucopyranosyl-3'-O-methylluteolin.

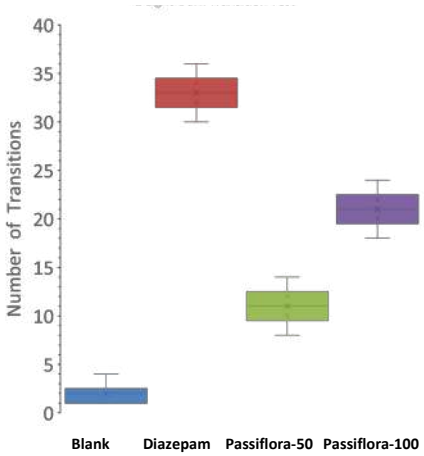

Figure 3. Behavioral evaluation in mice based on the number of transitions from illuminated to dark compartments, and visceversa, during the mouse light/dark box test (duration of test: 5 minutes). The inference is that the larger the number of transitions is associated to a less anxious behavior in mice. The doses were given by intraperitoneal administration, six mice per treatment. The Blank represents the negative control in which the experimental subjects received 0.1 mL of 0.9% saline solution. The diazepam is an anxiolytic compound used as a positive control (0.1 mL of 1 mg/kg^{-1}), Passiflora-50 and Passiflora-100 were treatment doses involving 0.1 mL of 50 or 100 mg/kg^{-1}, respectively.

Diazepam, Passiflora-50 and Passiflora-100 groups showed a notable increase in the number of transitions ($p<0.05$) in relation to the Blank group. The Passiflora treatment groups displayed a significant difference between them.

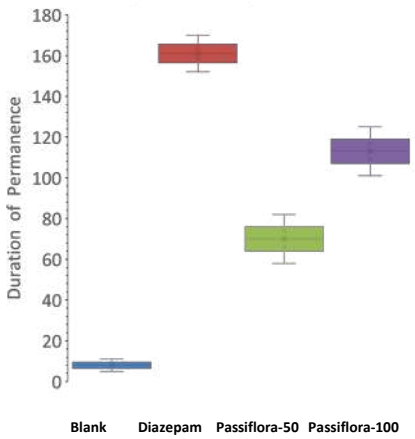

Figure 4. Behavioral evaluation in mice based on the duration of permanence in the dark compartment during the mouse light/dark box test (duration of test: 5 minutes). The inference is that the longer the permanence reflects a less anxious behavior in mice. Labels are the same as in Figure 2.

Anxiety assessment. The light and dark exploration test is based on the innate aversion of rodents to places illuminated to bright light. Anxiolytic agents increase the exploration of the illuminated area, as well as the transitions between both compartments. The light/dark transition test was originally developed

by Crawley and colleagues (18). We use the original version wherein the light chamber is larger than the dark chamber. In our protocol, a mouse is first placed in the dark chamber. Three seconds after placing the mouse in the dark chamber, the door between the chambers opened and the mouse could move freely between the two chambers. The mice tend to spend less time in the light chamber than in the dark chamber [the time spent in the dark does not include the initial time spent in the dark prior to entering the light chamber for the first time]. This is interpreted as the mice to tending avoid the light chamber, and that the time spent in the light is a good index of anxiety-like behavior.

DISCUSSION

Flavonoids are one of the largest groups of polyphenolic compounds, with more than 3,000 known structures. Within this group, flavones are the most widespread flavonoids of plant origin, which generally occur as glycosides of apigenin and luteolin. Undoubtedly, these flavones present in *Passiflora mollisima* leaves may contribute to its health-promoting effects, as it has been found in other *Passiflora* species (19). Likewise, a study on *Passiflora edulis* found that its high content of flavonoids or anthocyanins has a therapeutic potential for mammal physiology (20).

Polyphenols found in an *Passiflora caerulea* aqueous extract of fruits showed anticonvulsant activity when evaluated in Swiss albino mice induced by pilocarpine suggesting a therapeutic potential for epilepsy and neurodegeneration (19). Likewise, the purification of the anticonvulsant subfractions obtained by column chromatography led to the isolation of lucenin 2 (21).

In the renal system, vitexin was found to decrease crystal deposition and renal tissue injury in a mouse model system of calcium oxalate nephrolithiasis. It also decreased the level of the oxidant malondialdehyde (MDA) and elevated the levels of the antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione (GSH). Vitexin also reduced the levels of pyroptosis-related proteins, such as the pore-forming protein gasdermin D, and the inflammasome component NLRP3 (22).

Likewise, vitexin has been evaluated in newborn rats subjected to unilateral carotid artery ligation, generating hypoxia by measuring brain atrophy, Nissl staining, and neurobehavioral tests. This study found that a dose of 45 mg kg^{-1} of vitexin decreases cerebral edema, alteration of the blood-brain barrier and neuronal death. Vitexin attenuated the increase in hypoxia-induced factor (HIF-1α) and vascular endothelial growth factor (VEGF) leading to a long-term protective effect against ipsilateral brain loss and improved neurobehavioral outcomes (23). In studies on anxiety, vitexin has also been shown to exert sedative effects mediated by the GABA-ergic pathway (24). Other reports with the light/dark box test show that a pretreatment with vitexin significantly increased the exploration time of protected head dips and stretch attend postures (both anxiolytic behaviors) (25).

Regarding luteolin, a study showed that this metabolite has a greater inhibitory action than vitamin C in mononuclear cells, while continuing to act in polymorphonuclear cells by elevating the activity of antioxidants such as SOD and CAT enzymes (26). Studies have found that polyphenols and flavonoids can interfere with hormonal synthesis (27) and allergic responses (28).

The metabolite orientin, is a water-soluble flavonoid monomer; it inhibits fat production, promotes antioxidation and possess anti-inflammatory activity. Under this consideration, orientin has been studied as a potential effector affecting aging of the *Caenorhabditis elegans* nematode. Orientin apparently enhances the resistance to heat, oxidative, and pathogenic inputs through activation of stress responses (including heat shock response mediated by HSF-1 transcription factor, improves the response to xenobiotic and oxidation mediated by the SKN-1 gene expression, unfolded responses of mitochondria and endoplasmic protein response. Orientin could also activate key regulators of the nutrient-sensing pathway, including AMP-activated protein kinase (AMPK) and the downstream insulin transcription factor FOXO/DAF-16 to further improve cellular health status. The effective result of orientin with these mechanisms, reduces the accumulation of toxic proteins (α-synuclein, β-amyloid and poly-Q) and delays the appearance of neurodegenerative disorders in Alzheimer's, Parkinson's and Huntington disease models of the C. elegans, increasing their health and longevity (29).

Aerial parts of *Passiflora incarnata* have been studied in freeze-dried hydroalcoholic and aqueous extracts, as well as chemical constituents of the plant, finding indole alkaloids (harman, harmin, harmalin, harmol, and harmalol), maltol, and flavonoids (orientin, isoorientin, vitexin and isovitexin) and after evaluating the behavioral

effects in mice, psychotropic properties were confirmed (30), likewise our own study on the neuropharmacological and anxiolytic effect of *Passiflora mollisima* found that it had a greater anxiolytic and antidepressant effect in mice at a dose of 200 mg kg^{-1} body weight (8).

Regarding the evaluation of anxiety with the light-dark box test (Figure 3) it was observed that the diazepam group significantly increased the total number of transitions from the light to dark compartment ($p < 0.05$) in mice with respect to the white group, confirming the anxiolytic effect of the drug which agrees previous studies (18) .The light-dark box test is based on the premise that rodents, being nocturnal animals and predators, have a natural aversion to light, so they will normally spend more time in the dark compartment than in the illuminated one. On the other hand, their exploratory motivation leads them to explore new, unfamiliar environments, and makes the animal move from one compartment to another (31), thus, highlighting a lack of anxiety in these animals.

Results of *Passiflora mollisima* extract doses of 50 and 100 mg Kg^{-1} showed that the total number of transitions increased significantly ($p < 0.05$) with respect to the control group, following the same trend as the positive control (diazepam). This work is cognizant that the leaf extracts used in this experiment are a heterogeneous mixture of chemical compounds, therefore, besides the identified flavonoids, it is possible that many others also present in it may have a direct or modulatory effect on the mice behavior. Both doses of *Passiflora mollisima* demonstrate an anxiolytic effect as seen by the results in the tests.

This agrees with previous results obtained by our laboratory which found that the effect of the ethanolic extract of *Passiflora tripartita*, significantly decreased anxiety in mice (8)

The diazepam positive control, the Passiflora-50 and Passiflora-100 treatments increase overall the total number of transitions significantly ($p < 0.05$) with a 95% confidence level in relation to the Blank group, at the same time the treatment groups showed a significant difference between them. In Figure 4, as expected the diazepam group significantly increased the time spent in the illuminated compartment ($p < 0.005$), which is consistent to the anxiolytic effect of the drug. The test is based upon the natural aversion of mice to bright lighted areas and on their spontaneous exploratory behavior in novel environments (32).

This probable result is due to the pharmacological action carried out by the benzodiazepines (BZP) depends on the type of α-subunit that the GABA receptor contains. The benzodiazepine receptor site of the α1 subunit is the most abundant in the Central Nervous System, and regulates the anticonvulsant, hypnotic, and sedative actions of BZDs; this subunit is expressed primarily in the cerebral and cerebellar cortices. The BZD receptor site of the α2 subunit regulates anxiolytic actions, and its expression predominates in the temporal lobe amygdala (particularly in the central nucleus), hippocampus, and striatum. The benzodiazepine site of the α3 subunit is also known as a peripheral receptor; the pharmacological action of BZDs on this subunit is related to its muscle relaxer effect (33).

In this work, the ethanolic extract in *Passiflora mollisima* leaves showed that an anxiolytic effect on mice is apparent. Similar observations have been obtained in *Passiflora mollisima* wherein the flavonoid chrysin is thought to have affinity for the GABA receptor, thus regulating the activity of chloride channels in neurons, and thus exerting an anxiolytic effect (34). Other studies have found that systemic administration of vitexin exhibited selective protection against chemically-induced seizures, which suggests that this compound acts mainly in the modulation of GABAergic neurotransmission and/or related pathways, thus conferring to vitexin anticonvulsant and anxiolytic properties (24). There are studies that showed Vitexin and chrysin flavones are novel class of compounds with therapeutic potential against Alzheimer's disease (35).

CONCLUSIONS

The results obtained in this work show that leaf extracts from *Passiflora mollisima* imparts an anxiolytic effect on in the behavior of mice. The identified six flavonoid C-glycosides are known to possess anxiolytic activity and may be involved in the behavioral response observed. We found that combining chromatography and mass spectrometric methods maximized the coverage of m/z peak detection, and thus provided more confidence in the identification of the compounds. *Passiflora mollisima* is becoming an excellent model system to study effect of plant secondary metabolites on the central nervous system, and possibly lead to natural and clinical progress in novel therapeutics.

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AUTHORS' CONTRIBUTIONS

All authors participated equally in the development and writing of this research.

CONFLICTS OF INTEREST

The authors declare no conflict of interest in the present investigation.

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