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Review

The standardized extract of *Loeselia mexicana* possesses anxiolytic activity through the γ-amino butyric acid mechanism

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ABSTRACT

Ethnopharmacological relevance: Loeselia mexicana (Lam.) Brand has been used in Mexican Traditional Medicine to treat "espanto" or "susto" (fear), which is a culturally affiliated syndrome whose symptomatology comprises loss of appetite, difficulty in sleeping, and also nausea and fatigue, with a sensation of fear or risk – real or imagined – to external stimuli.

Aim of the study: The anxiolytic effect of the standardized methanol extract of *Loeselia mexicana*, with regard to its content of coumarin daphnoretin, was researched utilizing the elevated plus maze (EPM) in order to demonstrate whether the biological effect produced by the plant is antagonized by drugs that block γ-amino butyric acid (GABA)ergic transmission.

Materials and methods: The methanolic extract of *Loeselia mexicana* (LmMeOH) was tested at different doses on the EPM and then the interaction of this extract was evaluated in the same model with different GABAergic drugs, such as flumazenil (FLU) 10 mg/kg, bicuculline (BIC) 5 mg/kg, pentylenetetrazole (PTZ) 10 mg/kg, and picrotoxin (PTX) 2 mg/kg. The effect of all of these treatments was evaluated by means of the open field test (OFT). Coumarin content was measured by the high performance liquid chromatography (HPLC) technique.

Results: The 200- and 400-mg/kg doses of methanolic extract containing 3.14 and 6.28 mg of daphnoretin, respectively, induced an anxiolytic effect in the EPM without modification of the spontaneous motor activity. The anxiolytic activity of 200 mg/kg of methanolic extract in EPM-exposed mice was antagonized by PTX, BIC, and FLU, but not by PTZ.

Conclusion: The data presented here indicate that the *Loeselia mexicana* Brand methanolic extract possesses a significant anxiolytic effect that appears to be mediated in part by activation of the GABAergic system.

Contents

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1. Introduction

In Mexican Traditional Medicine, "espanto" or "susto" (fear) is a culturally affiliated syndrome that is most commonly found among Mexican Indigenous communities and is a cause of the use of major medical care due or its frequency in incidence and damage to the health of individuals. The definition of "susto" in Mexican Traditional Medicine comprises a person experiencing a sensation of fear or risk – real or imagined – when confronted by external stimuli. The person concerned presents symptoms of headache, deterioration, loss of appetite, insomnia, and also nausea and fatigue (Zolla et al., 1988), with symptoms of anxiety (Argueta, 1994). The anthropological definition of "susto" is similar to that of medical disorder termed anxiety, which can be defined as "maladaptive, either because it is too intense or because it is inappropriately caused by events that present no real danger" (Kandel et al., 2000).

Anxiety is the most common psychiatric disorder with a prevalence lasting throughout the lifetime of 16.6% of persons that significantly affects their Quality of life (Qol) (Somers et al., 2006; Olatunji et al., 2007) and their familial, social, and economic environments. Studies of the pathophysiology of anxiety are vast, and there is strong evidence that the neurotransmitter system of γ-amino butyric acid (GABA) is important in the pathogenesis of psychiatric disorders such as anxiety disorders. This neurotransmitter acts through two different types of receptors: $GABA_A$ and $GABA_B$ (Whiting, 2003). The benzodiazepines are anxiolytic and sedative drugs that continue to be prescribed; these substances modulate $GABA_A$ receptors that, despite their high potency and efficacy, induce side effects such as muscle relaxation and sedation, and that after use in the medium- to long-term use produce physical dependence, tolerance, ataxia, and memory impairment (O'Brien, 2005), in addition to ethanol potentiation, muscle relaxation, and amnesia, among others (Huen et al., 2003). Despite this, the great structural diversity of $GABA_A$ receptors allows them to present as important target sites of action for new anxiolytics that are likely without the undesirable effects of benzodiazepines.

An interesting source of new anxiolytic substances comprises medicinal plants, whose metabolic diversity leads to study in search of important therapeutic agents. Chemicals isolated from medicinal species possess the ability to influence the action of $GABA_A$ receptors. Examples of compounds that inhibit GABA actions include bicuculline (BIC), which is isolated from *Dicentra cucullaria* (Curtis et al., 1970), picrotoxin (PTX), isolated from *Anamirta cocculus* (Jablonski and Jackson, 2008), and the agonist Muscimol, isolated from *Amanita muscaria* (Johnston et al., 1968), in addition to the group consisting of flavonoids and terpenes, which also exert an effect on the nervous system by modulating this central neurotransmitter system (Zhang, 2004).

In Mexico, species of the Loeselia (Polemoniaceae) genus are utilized to treat "susto"-related symptoms, such as *Loeselia coerulea* (Cav.) G. Don (Argueta, 1994). The latter plant is employed in mainly in Oaxaca State (in southeastern Mexico) and *Loeselia mexicana* (Polemoniaceae), commonly known as "espinosilla" and "futto". The later is used in Mexican Traditional Medicine to treat fever, digestive disorders such as dysentery, stomach pain, and swelling, diarrhea, vomiting, and headache, and against dandruff, hair fall, and shock or "susto" (Argueta, 1994; Sánchez-González and Granados-Sánchez, 2008).

Loeselia mexicana is native to the American Continent and is found growing from southern U.S. to Central America in warm climates at altitudes of between 800 and 3900 m (Argueta, 1994; Sánchez-González and Granados-Sánchez, 2008). Pharmacological investigations have shown that the plant extracts possess antidiarrheal activity in rats and mice (Vargas et al., 1998; Pérez et al., 2005), and it was also observed that a chloroform–methanol (1:1) extract produced inhibition of spontaneous contractions in isolated ileum of rat (Rojas et al., 1999).

Chemically, the presence of flavonols such as quercetin, kaempferol, and glycoflavones has been detected (Smith, 1977). In addition, the isolation has been described of a pentacyclic triterpene (Jiménez et al., 1989), and also the presence of the following three coumarins: daphnoretin, and its precursors scopoletin and umbelliferone. Of these, the bicoumarin daphnoretin possessed significant anxiolytic activity in Institute for Cancer Research (ICR) male mice in the elevated plus maze (EPM) model (Navarro-García et al., 2007).

Several medicinal plants with activity against central nervous system (CNS) disorders in pharmacological studies with animal models and in clinical pharmacology are now successful examples of phytomedicines that are, in the majority of cases, standardized extracts of the contents of some compound. For example, *Hypericum perforatum* as a standardized extract is a pharmaceutical preparation with clinical usefulness in mild to moderate depression (Kasper et al., 2010).

Additionally, bearing in mind the ongoing study of *Loeselia mexicana* as a standardized extract, the objective of this study was to evaluate its anxiolytic effect in the EPM test, to measure possible sedative activity in the open field test (OFT), and to standardize the methanol extract based on its daphnoretin concentration employing the high performance liquid chromatography (HPLC) analytical technique. Moreover, we examined whether the plant's anxiolytic effect is due to its interaction *in vivo* with the γ-amino butyric acid (GABA)ergic system in the EPM after administration of the methanolic extract with the following selected $GABA_A$ antagonists: pentylenetetrazole (PTZ), and picrotoxin (PTX) due to their capacity to induce anxiety in the EPM by reducing the number of entries into open arms and time spent by the mice passing through these (Vivian et al., 1994; Holmes and Rodgers, 1998).

Additionally, we used flumazenil (FLU, an antagonist of the benzodiazepine binding site on the $GABA_A$ receptor) and bicuculline (BIC, a competitive antagonist of GABA on the $GABA_A$ receptor). All of these substances constitute a useful pharmacological tool in the

search for possible interaction between the GABAergic system and the anxiolytic effect of medicinal plant extracts (López-Rubalcava et al., 2006) and other anxiolytic substances (Kayir and Uzbay, 2006).

2. Materials and methods

2.1. Plant material

Loeselia mexicana was collected in March 2008 in its natural habitat in the municipality of Hueyapan, Morelos State, Mexico. The plant was identified by Biologist Margarita Avilés, and a sample of the specimen was deposited in the Botanical Garden Herbarium of the National Institute of Anthropology and History of State of Morelos (INAHM) in Cuernavaca, Morelos, Mexico, with voucher number INAHM-2002.

2.2. Preparation of the methanolic extract

The plant material was dried at 45 °C for 5 days and the aerial parts (400 g) were mixed with methanol and allowed to stand at room temperature for 3 days. The liquid was drained and filtered. The operation was repeated until the material was exhausted. The methanolic extract (LmMeOH) was concentrated under reduced pressure, and evaporation of the solvent yielded a residue of 60 g (15%).

2.3. High performance liquid chromatography (HPLC) analysis (coumarin content)

HPLC analysis of the *Loeselia mexicana* methanolic extract was performed on a Waters 2695 separation module system equipped with a Waters 2996 photodiode array detector and Empower Chromatographic Manager version 1 software (Waters USA). Analysis was performed with a Merck supersphere RP-18 column (5 μm, 4 mm × 125 mm, and 100 Å). The mobile phase consisted of a gradient mixture of 0.5% TFA solution (solvent A) and MeCN (solvent B). The gradient system was started with solvent A (100%) during 1 min. The concentration of solvent B was gradually increased (1 min) to 10%. This mixture was maintained for 2 min; during the following minute, solvent B was increased to 20%. This concentration was maintained for 2 min and changed to 30% of solvent B in the next 2 min and maintained for 6 min; during the next minute, solvent B was increased to 40% for 3 min; during the following minute, solvent B concentration was changed to 80%. These conditions were maintained for 3 min. Finally, the solvent B concentration was increased to 100% (lineal change during 3 min). The subsequent 2 min were employed to restore the initial conditions. The chromatographic method had a run time of 28 min (flow rate = 1 mL/min). Previously isolated coumarin (daphnoretin, Navarro-García et al., 2007) from *Loeselia mexicana* was injected by triplicate, and Diode ray detection (DAD) spectra were recorded at 190–600 nm. This coumarin displayed a retention time of 16.90 min.

2.4. Calibration curve of daphnoretin

Four ascending concentrations (15.625, 31.25, 62.5, and 125 mg/mL; 20 μL) of daphnoretin were injected by triplicate in the described HPLC method. The area under each chromatographic peak recorded at 340 nm was utilized to generate the standard calibration curve (R^2 = 0.993). Injection of the *Loeselia mexicana* methanolic extract (20 μL, 3 mg/mL) displayed data corresponding to a concentration of 15.73 mg daphnoretin/g extract.

2.5. Animals

Albino mice (ICR strain) weighing 30–36 g were utilized (Harlan, Mexico City), and all animals were housed eight per cage and maintained under laboratory conditions at 25 °C under a normal 12 h:12 h light/dark schedule with lights turned on at 07:00 a.m. and free access to water and food (pellets, Harlan Rodent Lab Diet). The mice were allowed at least 3 weeks to adapt to the laboratory environment prior to experiments. Experiments were carried out between 8:00 a.m. and 13:00 p.m. in a special adjacent, noise-free room with controlled illumination. All studies were conducted in accordance with the Official Mexican Norm NOM-062-ZOO-1999 (technical specifications for the production, care, and use of laboratory animals). The experimental protocol was approved by the Institutional Research Committee of the Mexican Institute of Social Security (IMSS) (IMSS-2005/1/I/066). Minimum number of animals and duration of observation time required to obtain consistent data were employed.

2.6. Drugs

Diazepam (DZP, 1.5 mg/kg, Laboratorios Cryopharma, México) was utilized as the anxiolytic, while pentylenetetrazole (PTZ, 75.0 mg/kg, Sigma), picrotoxin (PTX, 2 mg/kg, Sigma), flumazenil (FLU, 10 mg/kg, Sigma), and bicuculline (BIC, 5 mg/kg, Sigma) were employed as the anxiogenic (GABAergic antagonists). Carboxymethyl cellulose solution (CMC 1%, Sinergia, S.A. de C.V., AKUCELL® 0305, México) was utilized as the non-drug vehicle.

2.7. Pharmacological evaluations

2.7.1. Experimental protocol

The methanolic extract of *Loeselia mexicana* (LmMeOH) was administered orally (OP) for 24, 18, and 1 h prior to the biological tests. Doses were 100, 200, and 400 mg/kg. The anxiolytic drug used as positive control was DZP at 1.5 mg/kg by Intraperitoneal pathway (IP), while CMC 1% solution (100 μL/10 g) was employed as negative group (administered OP).

Then, on evaluating the anxiolytic effect of LmMeOH, the interaction of the latter with different $GABA_A$ receptor antagonists was tested in the elevated plus maze (EPM, for anxiolytic activity) and in the open field test (OFT, for sedation activity). A 200-mg/kg dose of the extract was selected; it was administered OP for by 24, 18, and 1 h before test. Subsequently, the antagonist drugs (PTZ, 10 mg/kg; PTX, 2.0 mg/kg; BIC 5.0 mg/kg, or FLU 10.0 mg/kg) were administered IP 30 min prior to the biological assays.

2.7.2. Elevated plus maze test

The EPM test is the most frequently used model for assessment of the anxiolytic activity of a new substance, including herbal remedies (Zhang, 2004; Carobrez and Bertoglio, 2005), in rodents (Pellow et al., 1985; Lister, 1987). The apparatus was made of Plexiglas and consisted of two open arms (30 cm × 5 cm) and two closed arms (30 cm × 5 cm) with 25-cm walls. The arms extended from a central platform (5 cm × 5 cm) and the maze was elevated 38.5 cm from the floor of the room.

Each animal was placed in the center of the maze, facing one of the closed arms. Number of entries and time spent in closed and open arms were recorded during a 5-min test. Entry into an arm was defined as the animal placing all four paws in the arm. All tests were taped with a video camera. After each test, the maze was carefully cleaned with a wet tissue paper (10% ethanol solution). Conventional spatial-temporal measurements comprised number of entries (all four paws in open or enclosed arms and expressed as percentage of total entries into open arms [% EOA]) and time spent

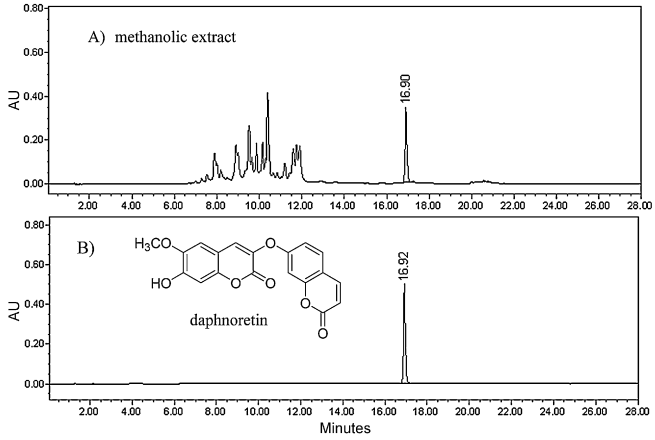

Fig. 1. Chromatograms of (A) methanolic extract (3 mg/mL, 20 μL) and (B) anxiolytic coumarin from *Loeselia mexicana* (62 μg/mL, 20 μL). Both samples were analyzed at λ = 330 nm.

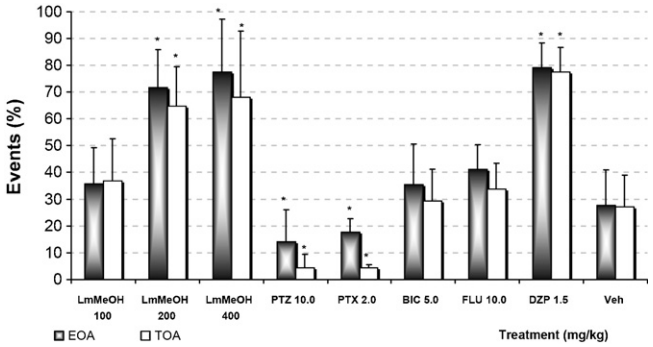

Fig. 2. Effect of different doses of the methanolic extract of *Loeselia mexicana* (LmMeOH) and the effect of different GABAergic drugs administered to ICR mice. Percentage of entries (EOA) and percentage of time (TOA) on open arms in the elevated plus maze (EPM) were measured. DZP, diazepam; PTZ, pentylenetetrazole; PTX, picrotoxin; BIC, bicuculline; FLU, flumazenil; Veh, vehicle (1% CMC). ANOVA followed by post hoc Bonferroni test. $^{*}p < 0.05$ ($n = 7$, mean ± S.D.; data were compared with the Veh group).

in open or enclosed arms (expressed as percentage of time spent in open arms [% TOA]).

2.7.3. Open field test (OFT)

The OFT was employed to evaluate the exploratory activity of the animal (Archer, 1973). Ambulatory behavior was assessed in an OFT. The apparatus consisted of transparent acrylic walls and a box with a black floor measuring 30 cm × 30 cm × 15 cm in height. The floor of the arena was divided into nine squares that were equal in area. At the beginning of each trial, a mouse was placed in the central square and allowed to explore the arena freely. Number of squares crossed (with the four paws) and number of rearings were counted in a 5-min session. The equipment was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.8. Statistical analysis

Statistical analysis was performed with an SPSS 11.0 software program and based on Analysis of variance (ANOVA) followed by the Bonferroni test (multiple comparisons). A significant difference was established with respect to the control group when the p value was <0.05.

3. Results

3.1. High performance liquid chromatography (HPLC) analysis (coumarin content)

Coumarin compounds displayed a retention time of 16.90 min for daphnoretin (λ_{max} = 210, 343 nm). Fig. 1 comprises a representative chromatogram of the *Loeselia mexicana* methanolic extract, which contains daphnoretin (R_T = 16.92 min; λ_{max} = 209, 343 nm). Total concentration of this coumarin, daphnoretin, content was 15.73 of methanolic extract (1.57%).

3.2. Elevated plus maze (EPM) test

The anxiolytic DZP at 1.5 mg/kg induced an increase in the percentage of EOA and TOA; both parameters were significantly different when compared with the animals with vehicle ($p < 0.05$). The methanolic extract of *Loeselia mexicana* at 200 and 400 mg/kg caused an increase of EAO and TOA in EPM-exposed mice; such changes were significantly different when the treatments were compared with those of the control group ($^{*}p < 0.05$) (Fig. 2). The 100 mg/kg dose did not change the behavior of animals with respect to the control ($p > 0.05$) (Fig. 2).

$GABA_A$ receptor antagonists were also tested in the EPM. PTZ (10.0 mg/kg) induced a significantly decrease of EOA and TOA when data were compared with those of the control group ($^{*}p < 0.05$); PTX also significantly diminished these parameters when it was administrated at 2.0 mg/kg to EPM-exposed mice ($^{*}p < 0.05$). On the other hand, BIC and FLU did not induce any statistical changes in EOA and TOA in comparison with animals with the vehicle ($p > 0.05$) (Fig. 2).

3.3. Interaction of LmMeOH with $GABA_A$ antagonists in EPM

TOA and EOA parameters were not modified when the LmMeOH extract was co-administered with PTZ (10.0 mg/kg). Therefore, the data were not significantly different between the presence and absence of this GABA antagonist drug ($p > 0.05$), but there is a statistical difference with respect to the control group ($^{*}p < 0.05$) (Fig. 3).

The LmMeOH-induced increase of TOA and EOA was completely blocked when the mice received 2.0 mg/kg of PTX, or 5.0 mg/kg of BIC, or 10 mg/kg of FLU. These substances induced a significant decrease in TOA and EOA in mice in the EPM when the data were compared with those of the LmMeOH-administered group

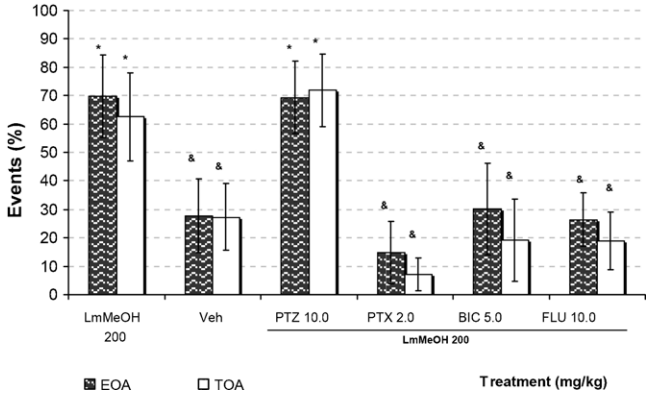

Fig. 3. Behavior of mice that received the methanolic extract of *Loeselia mexicana* (LmMeOH) and GABAergic drugs in the elevated plus maze (EPM). Percentage of entries (EOA) and percentage of time (TOA) on open arms in the EPM were measured. PTZ, pentylenetetrazole; PTX, picrotoxin; BIC, bicuculline; FLU, flumazenil; Veh, vehicle (1% CMC). ANOVA followed by post hoc Bonferroni test. $^{*}p < 0.05$ ($n = 7$, mean ± S.D., when data were compared with Veh). $^{\&}p < 0.05$ ($n = 7$, mean ± S.D., when data were compared with LmMeOH).

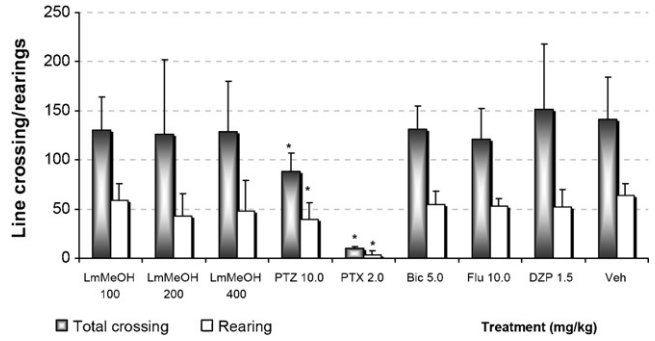

Fig. 4. Effect of different doses of methanolic extract from *Loeselia mexicana* (LmMeOH) and GABAergic drugs administered to ICR mice. Total crossings and rearings were measured in the open field test (OFT). DZP, diazepam; PTZ, pentylenetetrazole; PTX, picrotoxin; BIC, bicuculline; FLU, flumazenil; Veh, vehicle (1% CMC). ANOVA followed by post hoc Bonferroni test. $^{*}p < 0.05$ ($n = 7$, mean ± S.D., data were compared with the Veh group).

($^{\&}p < 0.05$), but these were not significantly different from those of the vehicle-administered group ($p > 0.05$) (Fig. 3).

3.4. Open field test (OFT)

Any LmMeOH treatment of 100, 200, and 400 mg/kg and any treatment with DZP caused changes in total crossings and rearings, that is, the parameters measured in the OFT, with respect to the control (vehicle) group ($p > 0.05$) (Fig. 4).

Administration of PTZ at 10.0 mg/kg induced a significant decrease in total crossings and rearings of OFT-exposed animals in comparison with mice that received only the vehicle ($^{*}p < 0.05$) (Fig. 4). Antagonists BIC and FLU at 5.0 and 10.0 mg/kg, respectively, did not produce statistical changes in total crossings and rearings in the OFT ($p > 0.05$) (Fig. 4).

3.5. Interaction of LmMeOH with $GABA_A$ antagonists in OFT

Animals treated with LmMeOH, to a greater degree than with any of the GABAergic antagonists, showed a decrease in total crossing and rearing parameters in the OFT (Fig. 5); however only PTZ- and PTX-treated mice presented behavior that was statistically different with respect to that of the vehicle group ($^{*}p < 0.05$).

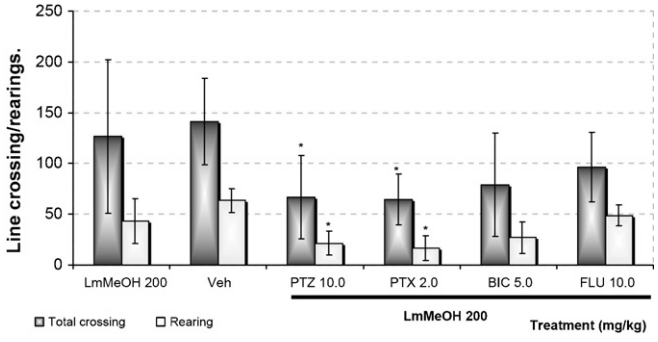

Fig. 5. Behavior of mice that received the methanolic extract of *Loeselia mexicana* (LmMeOH) and GABAergic drugs administered to ICR mice in open field test (OFT). Total crossings and rearings were measured in the OFT. Veh, vehicle (1% CMC); PTZ, pentylenetetrazole; PTX, picrotoxin; BIC, bicuculline; FLU, flumazenil. ANOVA followed by post hoc Bonferroni test. $^{*}p < 0.05$ ($n = 7$, mean ± S.D., data were compared with Veh control group).

4. Discussion

Anxiety disorders comprise a major public health problem and, according to the World Health Organization (WHO), are the most prevalent psychiatric disorders worldwide. For related problems caused by synthetic drugs, the search for therapeutic alternatives has been conducted largely by means of the study of medicinal plants (Zhang, 2004). However, while the number of these researches is growing, many of these medicinal plants have not yet been studied. Thus, for example, to our knowledge there is no data in the literature on experimentation with a plant of the family Polemoniaceae, to which *Loeselia mexicana* belongs. In fact, there is only one report showing that the compound isolated from this plant, daphnoretin, possesses an anxiolytic effect in the EPM (Navarro-García et al., 2007). The present work shows that administration of methanol extract of aerial parts of the plant has a marked anxiolytic effect at doses of 200 and 400 mg/kg, comparable with that of diazepam, in the EPM test. The *Loeselia mexicana* extract did not change the behavior of animals by subjection of these to OFT compare with the controls. In this test, an increase in locomotion is considered a stimulant effect, while a decrease is related with a sedative effect (Prut and Belzung, 2003).

The EPM paradigm is widely used in the assessment of novel substances or extracts for the evaluation of their anxiolytic activity employing mice (Belzung and Griebel, 2001) or rats (Rodgers et al., 1997). There are many data in the literature supporting its pharmacological validation with the use of anxiolytics such as benzodiazepines, which are allosteric activators of the $GABA_A$ receptor in EPM, the effect of which is to cause an increase in the number of crossings onto the open arms of EPM and consequently induce an increase in the time that the animals spend on these arms. It is assumed, then, that substances or plant extracts that produce a similar effect are defined as anxiolytics.

This work showed that the *Loeselia mexicana* methanolic extract possesses significant anxiolytic activity in the EPM model, and although this model is not sufficient to establish the mechanistic profile by which this effect is exerted, it may be suggested that such activity would likely result from an interaction between the active compounds of the extract with the GABAergic system through the activation of the $GABA_A$ receptor, which can induce neuronal membrane hyperpolarization, thus reducing nerve excitability, as do the benzodiazepines (Rodgers et al., 1997). The methanolic extract of *Loeselia mexicana* also appears to exert its anxiolytic effect by binding to this protein, because three of the four GABAergic antagonists tested (FLU, PTX, and BIC) blocked the biological activity of this plant. There are other plant species with an anxiolytic effect in the EPM at a dose similar to that employed of *Loeselia mexicana*; for example, *Piper methysticum* (Kava-kava) exerts its effect at doses range of 30–240 mg/kg, and it has been shown that its pharmacological properties include GABA enhancement in $GABA_A$ receptors (Singh and Singh, 2002), and the polar hydroalcoholic extract of *Euphorbia hirta* possesses marked anxiolytic activity at 200 mg/kg in rats placed in the EPM. The effect of this extract is also mediated by activation of the GABAergic system (Anuradha et al., 2008). Moreover, the standardized commercial extract of *Passiflora incarnata* 375 mg/kg exerts an anxiolytic effect, which is blocked by FLU (Grundmann et al., 2008).

In the present work, when PTZ was administered, we noted that this drug induced an anxiogenic effect at a 10-mg/kg dose. The mechanism of action by which PTZ induces a $GABA_A$ receptor blockade is not widely known, but early findings in the early 1980s indicate that one of its probable sites of action is the binding site for benzodiazepines. However, some of the effects exerted by PTZ were not antagonized by FLU, as might be expected of a substance acting on this site (Rehavi et al., 1982); therefore, some authors suggest that PTZ does not act on the

benzodiazepine binding site (Ramamjneyulu and Ticku, 1984). Co-administration of *Loeselia mexicana* with PTZ 10 mg/kg did not block the anxiolytic effect of the plant, which makes it clear that this species possesses anxiolytic effects, but not by acting on the PTZ binding site on the $GABA_A$ receptor.

It was noted that PTX causes an anxiogenic effect on EPM, which also is accompanied by a strong sedative effect, as indicated by the decrease in the number of total vertical stretching crossings during the OFT. PTX induces an anxiogenic or pro-anxiety effect on a large number of biological anxiety models through blocking Cl^- channel-activated GABA (Dalvi and Rodgers, 2001). It is postulated that this antagonist interacts with other substances at the same site, which is collectively referred to as the ligand to the PTX binding site, which appear to be effective blockers in several preparations (Bell-Horner et al., 2000). The anxiogenic drug completely blocked the anxiolytic effect of the *Loeselia mexicana* methanolic extract in the EPM.

This work showed that the anxiolytic activity of the methanol extract of *Loeselia mexicana* is blocked by PTX, but not by PTZ; regarding the previous information, the form of antagonism of both substances is carried to overlapping sites on the receptor, but not to the same sites in both (Dibas and Dillon, 2000). This might suggest that the plant exerts a selective effect on $GABA_A$ at the PTX, but not at the PTZ, site, and that there are even preferences in terms of the receptor isoforms to which the PTX binds.

Administration of BIC at doses of 1.0 and 5.0 mg/kg does not induce a change from the vehicle, indicating a lack of anxiogenic effects. It has been found that BIC provoked no specific effects in animal anxiety models (Dalvi and Rodgers, 1996); for example, bilateral injection in the hippocampal CA1 area produced no significant changes in the behavior of animals in the EPM (Rezayat et al., 2005), which agrees with our results.

However, when BIC is co-administered at a 5.0 mg/kg dose with the *Loeselia mexicana* methanolic extract at 200 mg/kg, the combination significantly reduced the plant's anxiolytic effect, with this activity indicating the action of some component of the plant on the $GABA_A$ receptors. It is noteworthy that although BIC is a competitive antagonist for the $GABA_A$ receptor, it is likely that part of the blockade imposed by this substance on the anxiolytic activity of *Loeselia mexicana* is in effect for any of the chemicals in it that occupy the site that corresponding to GABA, which leads to an inhibition of membrane hyperpolarization and a GABAergic transmission blockade.

FLU is widely reported in studies in which the purpose was to demonstrate the specific involvement of $GABA_A$ and benzodiazepine receptors in the mechanism of action of substances or of a plant extract with anxiolytic activity (Zhang, 2004). FLU is a $GABA_A$ receptor antagonist in the benzodiazepine binding site that possesses the ability to induce anxiety (File et al., 1982). It has been shown that the effect of the administration of 1.0 mg/kg of DZP is reversed by this antagonist at the previously mentioned dose (Poleszak, 2008). In this work, co-administration of *Loeselia mexicana* with FLU caused a significant decrease in anxiety parameters, indicating an interaction of the plant with the benzodiazepine receptor.

All data show that the extract exerts an anxiolytic effect by acting on the GABAergic transmission system. This may be mediated by the presence of groups of compounds such as coumarins that are found in the plant, such as daphnoretin, which was first isolated in this species and which demonstrated such activity (Navarro-García et al., 2007). In fact, effective doses of 200 and 400 mg/kg of LmMeOH contain 3.14 and 6.28 mg of the daphnoretin, respectively, according to calibration curve analysis by HPLC. Thus, these amounts of coumarin fall within the range of doses (3.75, 7.5 mg/kg) with anxiolytic activity reported by Navarro-García et al. (2007); according to these data, 100 mg/kg doses (that possess 1.57 mg of daphnoretin) did not produce any effect on mice in the EPM, thus coinciding with the Navarro report that coumarin doses of up to 1.87 mg did not induce an anxiolytic effect. Thus, the probability of this major component in the methanolic extract being responsible for the biological activity reported herein is high.

Coumarins are compounds found in abundance in nature, although few data have been shown them to possess some activity on the CNS; for example, it was reported that coumarin, 1,2-benzopyrene, isolated from the species *Hygrophila tyttha* L., is able to induce anxiolytic effects in ICR mice exposed to the EPM and the burying test, in addition to its having an anticonvulsant effect (Ariza et al., 2007).

Coumarin, 1,2-benzopyrene has been isolated from other plant species; other substances derived from it include dihydropyranocoumarins, which exerted neuroprotective effects against glutamate exitotoxicity (Kang et al., 2005). Others have the ability to inhibit the activity of enzymes such as Monoamine oxidase (MAO) (Matos et al., 2009), which are important targets of action for the treatment of anxiety and depression (Stahl and Felker, 2008). For example, scopoletin, a precursor of daphnoretin, which was isolated from *Hibiscus syriacus*, is capable of inhibiting MAO in a concentration-dependent fashion with values of 19.7 μg/mL (Yun et al., 2001); however, the remaining precursor, umbelliferone, isolated from *Dictamnus albus*, did not (Jeong et al., 2006). Other coumarins isolated from *Psoralea corylifolia* roots with antidepressant activity demonstrated inhibition of MAO-A and MAO-B (Chen et al., 2005).

Yet other compounds, such as phellopterin, imperatorin, and by kangelicol, isolated from the methanol extract of *Angelica dahuricae*, possess the ability to inhibit the binding of diazepam in the $GABA_A$ receptor *in vitro* (Bergendorff et al., 1997). Coumarins isolated from *Angelica gigas* roots have been reported to have activity on the CNS, such as inhibition of acetylcholinesterase enzyme and anti-amnesic actions. A recent study indicates that 1,2-benzopyrene at doses of 20 mg/kg IP, is capable to increase levels of the amino acids glutamate, taurine, and GABA in the prefrontal cortex; whereas DZP increased GABA and taurine levels and decreased glutamate and glycine levels in the same brain area. In hippocampus, only glutamate is reduced after administration of this coumarin; therefore, this compound stimulates the release of endogenous amino acids (Pereira et al., 2009); thus, it is probably modulating the organism's response to the presence of psychiatric and neurodegenerative diseases, among others.

The anxiolytic effect of *Loeselia mexicana* appears to be mediated by its action on the benzodiazepine binding site and the Cl^- channel of the $GABA_A$ receptor complex; this modulation suggests that this plant induces a rapid biological effect, which should be highlighted because it is a candidate for producing an anxiolytic phytomedicine that, in addition to being effective, could be fast-acting, such as benzodiazepines, but without the muscle-relaxation and sedation effects of these. It is, therefore, necessary to continue the detailed study of the toxicology and pharmacology of dependence and tolerance in order to demonstrate the safety of the extract for clinical use.

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