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Neuropharmacological studies of *Piper auritum* Kunth (Piperaceae): antinociceptive and anxiolytic-like effects

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P. auritum Kunth leaves commonly known as “hierba santa” or “acuyo” or false Kava is used in Mexican Traditional Medicine as a tranquilizing and appetite stimulant agent, as well as a remedy for the relief of headache. The objective of the present work was to evaluate the effects of organic and aqueous extracts of *Piper auritum* Kunth (Piperaceae) leaves on the Central Nervous System (CNS) in mice. For acute toxicity evaluation, the LD₅₀ of all extracts were determined according to Locke's method. Effects of both the organic and aqueous extracts of *P. auritum* leaves were tested in sodium pentobarbital-induced sleep, hot plate and open field tests. The anxiolytic-like effects were determined in burying behavior and hole-board models. In this study we demonstrated for the first time that both organic and aqueous extracts of the leaves of *P. auritum* possess antinociceptive effect while at higher doses the organic extracts exert a depressant effect on the CNS in mice. The aqueous extract showed antinociceptive and anxiolytic-like effects in the model tested, without affecting the locomotor activity of experimental animals. Acute toxicity tests show that the intake of extracts of different polarities of *P. auritum* involves no health risks. The present study supports, in part, the uses of *P. auritum* leaves as a tranquilizer, sedative agent, and as a remedy for the relief of pain in Mexican traditional medicine.

Key words: Depressant, sedative, *acuyo*, *hierba santa*, analgesic, antianxiety.

INTRODUCTION

Piper auritum Kunth belongs to the Piperaceae Family which has members widely distributed throughout the tropical and subtropical regions of the world (Berger, 1983; Jaramillo et al., 2001). The members of the *Piper* genus have various commercial and medicinal applications. Certain *Piper* species produce peppers used as spices worldwide; these peppers are valued for their organoleptic properties such as seasoning (Lopes et al., 2012). Traditional medicine has used various *Piper* species for many applications including antipyretic, analgesic and toothache treatment (Guerrini et al., 2009). Plants from the *Piper* genus have also been used to treat neurological and mental disorders (Bourbonnais-Spear et al., 2005). For example, it was reported that *Piper tuberculatum* can exert both anxiolytic and antidepressant

effects (Bezerra et al., 2007). In one study, the ethanol extract of *P. capense* exhibited moderate binding activity to the GABA_A-benzodiazepine receptor (Stafford et al., 2005). In other study *Piper laetispicum* showed antidepressant and antinociceptive effects in mice (Yao et al., 2009), and in a separate study methanol extract of *Piper chaba* can exert depressant and antianalgesic effects on the CNS in rodents (Ur-Rahman et al., 2005). *Piper methysticum* also known as kava-kava, is probably the most studied *Piper* species and can exert anti-anxiety, antidepressant, analgesic, muscle relaxing and anticonvulsant effects (Sarris et al., 2009; LaPorte et al., 2011; Garret et al., 2003; Anke and Ramzan, 2004; Spinella, 2002; Sharma et al., 2012). While *Piper nigrum* (black pepper) is the most common spice in the

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world, it can also be used as an analgesic, antioxidant, CNS stimulant, and anti-inflammatory drug and for the treatment of epilepsy (Srinivas et al., 1999; Bafdyopadhyay et al., 1990; Fu et al., 2010; Boucbonnais-Spear, 2005; Jirovetz et al., 2002; Gayasuddin et al., 2013; Moghadamnia et al., 2010; Al-Baghdadi et al., 2012).

In Mexico *P. auritum* is commonly known as “hierba santa” or “acuyo” or false Kava (Aguilar et al., 1994). As with many other species of the *Piper* genus *P. auritum* is used in seasoning. As a folk remedy, this plant is employed in a variety of uses such as a sudorific agent, an appetite stimulant, a hypoglycemic agent, an antioxidant, an antibacterial and an anti-inflammatory and analgesic agent (Montemayor, 2007; Martínez, 1969). This plant may be used to treat fever, headache, stomachache, and susto” (fear), and to promote lactation (Andrade-Cetto and Heinrich, 2005; García et al., 2007; Monzote et al., 2010). Additionally, this plant has been used as an aphrodisiac, a stimulant, and as a marihuana (*Cannabis sativa* L.) substitute (Schultes and Hofmann, 1982). Regarding its chemical composition, a variety of secondary metabolites are known to be present in *P. auritum*, for example piperidine amides such as piperine, an antidepressant and neuroprotector agent (Fu et al., 2010; Prashantha et al., 2012). Aporphine alkaloids, such as 1,2,3-trimethoxy-4,5-dioxo-6a,7-dehydroaporphine (Hansel and Leuschse, 1975; Perez-Gutierrez et al., 2013), flavonoid compounds such as 5,3'-dihydroxy-7,4'-dimethoxyflavone, and phenylpropenoids such as eugenol have also been isolated (Nair et al., 1989; Ampofo et al., 1987; Gupta and Arias, 1985). While the chemical analysis of the essential oil revealed the presence of terpenoids such as α -thujene, limonene, β -pinene, γ -terpinene, β -caryophyllene, and linalool among others, the major component of the essential oil is safrol (Gupta and Arias, 1985; Monzote, et al., 2010).

In spite of its uses in traditional medicine, a systematic investigation of the effect of *P. auritum* on the CNS has never been undertaken. Thus, the objective of this study was to evaluate the behavioral effects of hexane, ethyl acetate, methanol and aqueous extracts of the leaves of *P. auritum* on antinociceptive and sedative experimentals in mice models. Acute toxicity of the extracts was also determined by measuring LD₅₀. Furthermore, the aqueous extract was evaluated for anxiolytic-like effects in burying behavior and hole-board paradigms in the mice model.

MATERIALS AND METHODS

Vegetal material

Leaves of *P. auritum* Kunth (Piperaceae) were collected in Guadalajara, Jalisco State, Mexico. The species was authenticated by Botanist M en C. Abigail Aguilar from the “Herbario de Plantas Medicinales del Instituto Mexicano del Seguro Social”, and a voucher specimen (voucher num. IMSSM15722) was deposited in

the Herbario de Plantas Medicinales del IMSS.

Preparation of extracts

Aqueous extract

Air-dried and finely ground leaves (10 g) of *P. auritum* were extracted by boiling in distilled water (90 mL) for 10 min. The resulting solution was lyophilized in a Telstar freeze dryer at -50°C and 0.01 mBar resulting in a corresponding extract yield of 21%. The extract was stored at 4°C until the pharmacological assays.

Organic extracts

Dried ground leaves of *P. auritum* (100 g) were successively extracted with hexane (18.5 %), ethyl acetate (EtOAc; 23 %), and methanol (MeOH; 35.8 %). Evaporation of the solvents under a vacuum yielded the respective extracts.

Animals

Adult male Swiss Webster mice (weighing 20 to 30 g) were used. All animals were housed eight per cage in a temperature (20 to 21°C)-controlled room under inverted light: dark conditions (12:12 h, lights on at 22:00 h). All behavioral evaluations were performed between 10:00 and 14:00 h. Animals had *ad libitum* access to Purina rodent chow and water. Animals were handled in agreement with the general principles of laboratory animal care (NIH publication # 85-23, revised in 1985) and the 'Norma Oficial Mexicana' (NOM-062-ZOO-1999). All the experimental sessions were videotaped and analyzed by an observer unaware of the treatment conditions.

Drug and dosage

All drugs in this study were intraperitoneally (i.p.) injected in a total volume of 10.0 mL/kg body weight. Sodium pentobarbital (SP) (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in an isotonic solution (0.9 % NaCl). Diazepam (Dz) (Hoffmann-La Roche, Mexico City, Mexico) and ibuprofen (IB) (Aldrich-Sigma, Mexico City, Mexico) were dissolved in 1.0 % propylene glycol. Control animals received the same volume of the vehicle (isotonic solution, 0.9 % NaCl).

The pharmacological assays were performed with aqueous dilutions (1-2 % Tween 80) of the extracts. Doses are expressed as milligrams of dry extract per kilogram of body weight per mouse.

Pharmacological evaluations

For habituation, animals received a daily i.p. injection of saline solution (0.1 mL/10 g) for five days before treatments were initiated. Diazepam (Dz) was used as a reference drug in both the sedative and anti-anxiety tests, and ibuprofen was used as a reference in the antithermonociceptive test. Doses and latencies for the extracts were obtained from previous pilot studies.

Sodium pentobarbital-induced sleeping time (SPT)

The sedative and hypnotic effects of organic and aqueous extracts of *P. auritum* in combination with sodium pentobarbital (SP) were evaluated. For this purpose, twenty independent groups (eight mice per group) received hexane, EtOAc, methanol or aqueous extract (1, 10, 100, 200, and 500 mg/kg i.p., respectively) 60 min before the

administration of SP (42 mg/kg, i.p.). Two other groups received Dz (1.0 or 2.0 mg/kg, i.p., respectively) 30 min before the administration of SP (42 mg/kg); these two groups served as positive control. An independent group was injected with the vehicle 60 min before the i.p. administration of the 42 mg/kg dose of SP and this group served as a negative control.

Each mouse was placed on a warm table and was carefully observed for the onset of uncoordinated movements corresponding to the sedative phase of the test. Loss of the righting reflex related to the sleep phase and the duration of sleep were also observed. The time the elapsed between the loss and recovery of the righting reflex was considered as the sleeping time (Tingli et al., 2007; Estrada-Reyes et al., 2010).

Exploratory Behavior Test (OFT)

Spontaneous locomotor activity was measured immediately after completing the hole-board test; it was tested using an open field apparatus made of an opaque-Plexiglas box (40 cm × 30 cm × 20 cm) with the floor divided into 12 equal squares. Each animal was gently placed in a corner of the apparatus and its behavior was videotaped during a 5 min session. An observer, blind to the pharmacological treatment, registered the number of times the animal entered each square (counts per 5 min) and the rearing number (number of times the animal stood on its hind legs) was also recorded for 5 min (López-Rubalcava et al., 2006).

Hot Plate Test (HPT)

Extracts were administered at doses of 1.0, 10, 100 or 200 mg/kg (i.p.) to groups of eight mice 60 min before the beginning of the test. Three groups of animals received IB at doses of 30, 60, and 90 mg/kg 30 min before beginning the test. These groups served as positive controls. One group received the vehicle and served as a negative control. Each mouse was introduced into a glass cylinder (20 cm in diameter and 25 cm in height) placed at the center of a metal plate (Uge baseline, model DS 37) adjusted to 53±0.5°C. Within several seconds, the animals displayed specific responses evoked by the thermal stimulation; the flexor anti-analgesic reflex behavior was recorder as flexion latency (in seconds; s). If the mouse did not respond within 50 s, the test was terminated and the mouse was immediately removed from the hot plate to avoid tissue damage and returned to its home cage. Animals were tested one at a time and were not habituated to the apparatus prior to testing. Each animal was tested only once (Wesolowska et al., 2006; Estrada-Reyes et al., 2010).

Anxiolytic-like effect: Burying Behavior Test (BBT)

Mice were individually tested in a cage that had exactly the same dimensions as home cages (15 cm × 24 cm × 11 cm) but with an electrified prod (7 cm long) emerging from one of its walls, 2 cm above the bedding material consisting of fine sawdust. Every time the animal touched the prod it received an electric shock of 0.3 mA. The source of the shock was a constant current shocker (La Fayette Instruments Co., model 5806). The prod remained electrified throughout the test. Immediately after the placement of the animal in the cage, its behavior was registered for 10 min. Once the animal received the first shock, it typically moved towards the prod recognizing it as the aversive stimulus. The animal then sprayed and pushed a pile of bedding material ahead with rapid alternating movements of its forepaws. The parameters registered in this anxiety test were the burying behavior latency (time, in seconds, from the first shock to the display of the burying behavior) and the cumulative burying behavior (cumulative time in seconds,

that the animals spent burying the prod). In this test, a decrease in the cumulative burying behavior is interpreted as a reduction in anxiety (Pinel and Treit, 1978). On the other hand, an increase in burying behavior latency is considered to reflect a decreased reactivity (the readiness of the animal to respond to a certain condition) (Estrada-Reyes et al., 2009; López-Rubalcava et al., 2006).

Anxiolytic-like effect: Hole Board Test (HBT)

The hole-board set-up apparatus is a wooden box of 60 cm × 30 cm × 30 cm with four equidistant holes (2 cm diameter) on the floor, with light intensity during testing of 270-300 lux. Extracts were administered in independent form at doses of 1.0, 10, and 100, mg/kg, i.p. to groups of eight mice each. After 60 min, each mouse was placed in the center of the hole-board and the number of head-dips into the hole and the number of rears was recorded over a 5 min period. A head-dip was registered if a mouse put its head in a hole at least up to the eye level; repeated dips into the same hole were not counted unless these were separated by locomotion and rearing is scored when mice raise themselves on the hind legs and the fore-paws rest on a partition wall.

Two independent groups of eight animals received Dz (1.0 or 2.0 mg/kg) 30 min before the test was conducted and these groups served as a reference standard. One group receiving only the vehicle served as control. After each trial, the floor of the apparatus was carefully cleaned to remove traces of previous paths. An increase in the number of head dips and the number of rears compared to the controls were considered to indicate an anxiolytic-like effect, while the decrease in these variables was considered a sedative effect (Viola et al., 1995; Estrada-Reyes et al., 2010).

Acute toxicity (LD₅₀)

Acute toxicity was determined by administration through the i.p. route according to Lorke's method as LD₅₀ (Lorke, 1983). Briefly, in the first stage, the aqueous extract was intraperitoneally administered at doses of 10, 100, and 1000 mg/kg to three groups of three mice each. The animals were observed for 1 h for signs and symptoms of toxicity. Later observations were made every 24 h for 7 days. In the second phase, doses of 1600, 2900, and 5000 mg/kg were administered to three groups of four, five, and six animals, respectively. These mice were carefully observed until they either completely recovered or died. The surviving animals were observed for 14 days, and their time of mortality recorded.

Statistical analysis

The treated and control groups in all tests were analyzed using a Kruskal-Wallis analysis of variance on ranks (*p < 0.05, **p < 0.01, and ***p < 0.001) followed by the Mann-Whitney rank sum test. All statistical analyses were carried out using the Sigma Stat Program (version 3.5, Jandel Scientific), and graphics were generated using the Sigma Plot Program (version 10.0, Jandel Scientific).

RESULTS

Sodium pentobarbital-induced sleeping time (SPT)

For the preliminary central activity assessment of *P. auritum* leaves, polar and non-polar organic extracts (hexane, ethyl acetate, MeOH and aqueous) were

Table 1. Effect of hexane, EtOAc, methanol and aqueous extracts of *P. auritum* and diazepam (Dz) on sodium pentobarbital-induced sleeping time (SPT).

| Treatment | | Dose (mg/kg) | | |
|-------------|-----|------------------------------------|------------------------------------|------------------------------------|
| | | Se L minutes | S L minutes | S T minutes |
| Extract+ SP | | | | |
| Vehicle | --- | 1.19 ± 0.05 | 3.27 ± 0.13 | 15.04 ± 0.79 |
| Dz | 1.0 | 1.13 ± 0.10 | 2.86 ± 0.15 | 24.84 ± 2.05** |
| | 2.0 | 1.20 ± 0.27 | 2.97 ± 0.09 | 38.01 ± 2.60*** |
| Hexane | 1 | 1.30 ± 0.09 | 3.61 ± 0.32 | 21.33 ± 2.32 |
| | 10 | 1.37 ± 0.07 | 3.43 ± 0.10 | 20.33 ± 3.44 |
| | 100 | 1.32 ± 0.10 | 3.81 ± 0.52 | 79.24 ± 5.20*** |
| | 200 | 1.55 ± 0.11 | 3.43 ± 0.20 | 96.24 ± 6.10*** |
| | 500 | 1.43 ± 0.10 | 3.22 ± 0.54* | 122.82 ± 4.50*** |
| | | H= 13.43, df= 7, (p= 0.06) | H= 13.71, df= 7, (p= 0.056) | H= 56.38, df= 7, (p≤ 0.001) |
| EtOAc | 1 | 1.20 ± 0.10 | 3.50 ± 0.17 | 19.74 ± 1.67 |
| | 10 | 1.36 ± 0.17 | 3.53 ± 0.37 | 15.94 ± 1.54 |
| | 100 | 1.53 ± 0.52 | 4.52 ± 1.02 | 36.11 ± 2.64*** |
| | 200 | 1.28 ± 0.09 | 2.83 ± 0.11** | 46.96 ± 2.68*** |
| | 500 | 1.51 ± 0.15 | 3.21 ± 0.18** | 34.22 ± 2.75*** |
| | | H= 3.28, df= 7, (p= 0.085) | H= 18.55, df= 7, (p= 0.010) | H= 51.7, df= 7, (p≤ 0.001) |
| MeOH | 1 | 1.43 ± 0.10 | 3.16 ± 0.13 | 19.60 ± 3.01 |
| | 10 | 0.71 ± 0.12** | 1.78 ± 0.015*** | 49.17 ± 4.83*** |
| | 100 | 1.41 ± 0.18 | 3.10 ± 0.10 | 37.51 ± 7.46*** |
| | 200 | 0.73 ± 0.15 | 2.13 ± 0.17 | 44.25 ± 4.19*** |
| | 500 | 1.17 ± 0.07 | 2.77 ± 0.16*** | 57.14 ± 2.56*** |
| | | H= 21.48, df= 7, (p= 0.003) | H= 33.90, df= 7, (p≤ 0.001) | H= 43.93, df= 7, (p≤ 0.001) |
| Aqueous | 1 | 1.37 ± 0.12 | 3.11 ± 0.11 | 19.26 ± 3.26 |
| | 10 | 1.06 ± 0.04 | 3.17 ± 0.12 | 21.61 ± 3.98 |
| | 100 | 0.83 ± 0.07** | 2.44 ± 0.23 | 22.97 ± 4.3 |
| | 200 | 1.15 ± 0.10 | 3.23 ± 0.20 | 39.80 ± 2.09*** |
| | 500 | 0.97 ± 0.08* | 2.80 ± 0.16 | 42.02 ± 2.46*** |
| | | H= 17.77, df= 7, (p= 0.013) | H= 15.67, df= 7, (p= 0.013) | H= 46.93, df= 7, (p≤ 0.001) |

SeL = sedative latency; SL = sleeping latency; ST = sleeping time. All results are expressed as means ± SEM (n = 8-10 animals in each group). Comparisons were made by using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Mann-Whitney U test: *p < 0.05, **p < 0.01 and ***p = 0.001 compared with the control group.

prepared and investigated with the sodium pentobarbital-induced sleeping time test, the most common sedative and depressant test model in mice. As shown in Table 1 neither the intraperitoneal injection of any extracts tested, nor Dz (1.0 and 2.0 mg/kg) resulted in a decrease in the sedative latency time (hexane: p= 0.06, EtOAc: p= 0.085, MeOH: p= 0.003, and aqueous: p= 0.013).

The duration of the sleeping time as a result of SP treatment was significantly prolonged in a clearly dose-dependent manner by the hexane extract (H= 56.38, df= 7, P≤ 0.001), while the EtOAc and MeOH extracts also caused a significant increase in the sleeping time (H=

51.7, df= 7, p≤ 0.001) and (H=43.93, df= 7, p≤ 0.001), respectively. This effect was not dose-dependent and it did not reach the same levels as the hexane extract. The aqueous extract only caused significant effect at higher doses tested (200 and 500 mg/kg) (H= 46.93, df= 7, p≤ 0.001). These results demonstrate that the intraperitoneal administration of hexane extract produced a more remarkable depressant effect (as determined by the longer duration of sleep), whereas the more polar extracts (MeOH and aqueous) were less effective in prolonging the sleeping time. These results show that *P. auritum* exerts a depressant effect on the CNS.

Exploratory behavior test (OFT)

As shown in Figures 2A and B, the hexane extract in different doses (10 to 500 mg/kg) caused a significant reduction in spontaneous activity in mice undergoing the open field test as measured both by exploratory count number and rearing number ($H= 61.87$, $df= 8$, $p\leq 0.001$ and $H= 43.35$, $df= 8$, $p\leq 0.001$, respectively). The EtOAc extract when administered at a dose of 1 mg/kg produced an increase in the ambulatory activity in comparison to control groups ($p\leq 0.001$); this activity gradually decreased in a dose-dependent manner until a significant reduction was observed at doses of 200 and 500 mg/kg (counts number: $H= 64.92$, $df= 8$, $p\leq 0.001$ and rearing number: $H= 51.68$ $df= 8$, $p\leq 0.001$). At these doses, the response was similar to Dz (given at a 1- to 4- mg/kg dose).

In general, the highest doses of all treatments (hexane: 200 and 500 mg/kg, EtOAc: 200 and 500 mg/kg, MeOH: 500 mg/kg, aqueous: 500 mg/kg and Dz: 2 and 4mg/kg) induced a significant decrease in both the number of exploratory counts and rearing number observed during the open field test. Any treatment at a dosage below 100 mg/kg did not affect significantly the ambulatory activity of the experimental mice.

Hot plate test (HPT)

Figure 1 shows the results of the hot plate test. All compounds under investigation exhibited analgesic effects in mice in comparison to the control group. A single administration of hexane, EtOAc, MeOH, or aqueous extracts (with doses ranging from 1 to 200 mg/kg), produced a statistically significant elevation of nociceptive threshold which was measured by an increased latency of flexion in hot plate test. However, several differences were observed among the treatments. While the hexane, EtOAc, and MeOH extracts increased the flexion latency ($H= 34.18$, $df= 4$, $p\leq 0.001$, $H= 29.69$, $df= 4$, $p\leq 0.001$, and $H= 25.02$, $df= 4$, $p\leq 0.001$, respectively), at the highest doses (100 and 200 mg/kg), the organic extracts also caused a sedative effect in the SPT (sodium pentobarbital-induced sleeping time). These results indicate that the hexane, EtOAc and MeOH extracts exerted an antinociceptive effect against thermal stimulus at the lowest dosage used (1 to 100 mg/kg). Unlike those compounds, the intraperitoneal administration of aqueous extract of *P. auritum* prolonged the response towards the thermonociceptive stimulus as measured by an increase showing a prolongation of flexion latency ($H= 29.10$, $df= 4$, $p\leq 0.001$) without affecting the ambulatory activity of mice. Moreover, this extract showed the clearest dose-dependent analgesic effect when compared to the other extracts and control groups. Remarkably, the potency of the aqueous extract was found to be higher than that of ibuprofen which was used as a positive control ($H= 19.14$, $df= 3$, $p\leq 0.001$).

Table 2. Burying behavior latency (seconds).

| Treatment (mg/kg) | Mean | Std. Error |
|--------------------------|--------|------------|
| <i>P. auritum</i> | | |
| 0.00 | 74.71 | 6.35 |
| 0.5 | 74.57 | 5.99 |
| 1.0 | 70.71 | 8.04 |
| 10 | 100.28 | 12.23 |
| 100 | 85.14 | 9.15 |
| Diazepam | | |
| 0.0 | 74.71 | 6.35 |
| 0.5 | 83.14 | 10.64 |
| 1.0 | 59.14* | 5.40 |

$H = 11.09$, $df = 6$, $p = 0.086$. All of the results were expressed as the averages \pm SEM of groups of 8 animals each. Comparisons were made by using a Kruskal-Wallis one way analysis of variance on ranks, followed by Mann-Whitney *U*-test: *** $P < 0.001$.

Anxiolytic-like effect

Although administration of the organic extracts at low doses produced an increase in both rearing and exploratory count numbers in the OFT (Figure 2A and B) which is considered an exploratory activity index, these extracts failed in the BBT at all doses tested (data not shown). In contrast, the aqueous extract induced a dose-dependent anxiolytic-like effect in both the BBT and HBT, as described below.

The effect of Dz and aqueous extract of *P. auritum* on the BBT is shown in Figure 3. Both compounds elicited a significant decrease in burying behavior. Dz was given at doses of 0.5 and 1.0 mg/kg and the aqueous extract at 1, 10, and 100 mg/kg doses ($H= 55.43$, $df= 6$, $p\leq 0.001$). Table 2 shows the action of diazepam and aqueous extract on the latency of burying behavior. None of the doses tested for either treatment affected this parameter ($H= 11.09$, $df= 6$, $p= 0.086$).

With regard to general activity, only the highest dose of diazepam (4.0 mg/kg) diminished the exploratory behavior of the mice thus producing a decrease in rearing number. In contrast, the aqueous extract of *P. auritum* did not affect the mice's general activity at any dose when assessed by the BBT (Figure 2A and B). It is interesting to note that the LD_{50} of aqueous extract of *P. auritum* was 10-fold higher than its highest anxiolytic (BBT) dose. These results indicate that the anxiolytic-like actions of the aqueous extract of *P. auritum* are specific and are not due to collateral motor effects.

Anxiolytic-like effect Hole-Board Test (HBT)

Figure 4 shows the effect of both Dz and aqueous extract

Table 3. Determination of LD₅₀ of organic and aqueous extracts of *P. auritum* by i.p. route in mice.

| Dose (mg/kg) | First experimental phase | | | |
|------------------|---------------------------|-------|-------|---------|
| | hexane | EtOAc | MeOH | Aqueous |
| 10 | 0/3 | 0/3 | 0/3 | 0/3 |
| 100 | 0/3 | 0/3 | 0/3 | 0/3 |
| 1000 | 0/3 | 0/3 | 0/3 | 0/4 |
| | Second experimental phase | | | |
| 1000 | 0/1 | 0/1 | 0/1 | 0/1 |
| 1600 | 2/2 | 2/2 | 0/2 | 0/2 |
| 2900 | - | - | 0/3 | 0/3 |
| 5000 | - | - | 1/5 | 5/5 |
| LD ₅₀ | 1264 | 1264 | >2900 | 3800 |

of *P. auritum* on the performance of mice in the HBT. Diazepam significantly increased both the rearing number (Figure 4A) and head dipping number at doses of 0.5 and 1.0 mg/kg, while the aqueous extract produced a similar effect at doses of 1 and 10 mg/kg (Figure 4B). Both parameters were significantly decreased relative to the control group (for head dipping number $H= 52.39$, $df= 8$, $p\leq 0.001$ and for the rearing number $H= 55.56$, $df= 8$, $p\leq 0.001$) when the compounds were administered at the highest doses tested (2.0 and 4.0 mg/kg for Dz and 100 mg/kg for the aqueous extract of *P. auritum*). These results demonstrated that the aqueous extract produced an anxiolytic-like effect similar to Dz. With regard to general activity, only the highest dose of Dz (4.0 mg/kg) decreased this parameter, while none of the doses of the aqueous extract affected the general activity or produced any signs of toxicity.

Acute toxicity LD₅₀

The acute toxicity of the compounds was determined according to Lorke's method with the results shown in Table 3. Animals treated with hexane extract of *P. auritum* exhibited alterations in some of their behavioral responses. These animals became remarkably quiet and a considerable decrease in locomotor activity was observed, but the extract did not cause animal death until 24 h after administration at a dose of 1600 mg/kg. The LD₅₀ was measured at 1264 mg/kg. Treatment of animals with 2900 mg/kg of the EtOAc extract produced toxic manifestations that persisted until the animals' deaths within the first hour after administration. This extract also showed an acute toxicity with a LD₅₀ of 1264 mg/kg.

A similar evaluation of acute toxicity of the MeOH and aqueous extracts revealed that both were slightly toxic (LD₅₀> 2900 and LD₅₀= 3800 mg/kg, respectively) according to Lorke's classification.

DISCUSSION

As previously discussed, the leaves of *P. auritum* have traditionally been used by patients as a sedative and as a stimulant agent and also as a form of relief for headache and stomachache. On the other hand, *P. methysticum* is used to brew a mildly narcotic drink used in traditional ceremonies and it has been widely commercialized as an anxiolytic and relaxant drug in North America and Mexico. *P. auritum* belongs to the same genus as *P. nigrum* (black pepper) and *P. methysticum* (kava kava). False Kava is the term applied to plants that in appearance resemble true Kava (*Piper methysticum*); due to this *P. auritum* is used in the same way as *P. methysticum*. Furthermore, during the last years *P. auritum* has been commercialized as a more productive alternative to *P. methysticum* (Kava Kava). In contrast to this, there are no pharmacological studies until now that validate or refute properties attributed to *P. auritum*. Taking into account the above, the aim of this work was to evaluate the effects on CNS of *Piper auritum* Kunth (Piperaceae) leaves in behavioral model in mice.

It has been previously demonstrated that the sedative effects of drugs can be evaluated in laboratory animals by measurement of the sleep time induced by pentobarbital administration (Carpando et al., 1994). Therefore, in order to test whether *P. auritum* exhibits sedative or depressant effects on the CNS, hexane, ethyl acetate, methanol and aqueous extracts of *P. auritum* leaves were each separately dissolved in a suitable vehicle and were administered i.p. to mice. The effect of each treatment was subsequently determined by testing the pentobarbital-induced sleeping time.

It is important to note that although the extracts *per se* did not have sedative effects, animals treated with the non-polar extracts (hexane and EtOAc) were calm and relaxed, while treatment with the polar extracts (MeOH and aqueous) did not produce any behavioral change in the experimental animals. Additionally, neither diazepam nor any of the extracts modified the latency time of sedation. However, all organic (100, 200 and 500 mg/kg doses) and aqueous extracts (up 200 mg/kg dose) were able to increase pentobarbital-induced sleeping time significantly.

Interestingly, when hexane extract was administered in combination with SP, it was able to prolong the actions of pentobarbital almost ten times beyond that of Dz in this test. Although it has been reported that prolongation of sleeping time induced by sodium pentobarbital may be due to an inhibition in hepatic metabolism, it has also been demonstrated that effects of pentobarbital on the CNS are mediated through the GABA/benzodiazepine receptor complex. Based on this finding the prolongation of pentobarbital sleep produced by *P. auritum* is a good index of CNS depressant activity (Kaul and Kulkarni, 1978; Petty, 1995).

The depressant effect of *P. auritum* was confirmed by results obtained in the OFT. A comparison between the

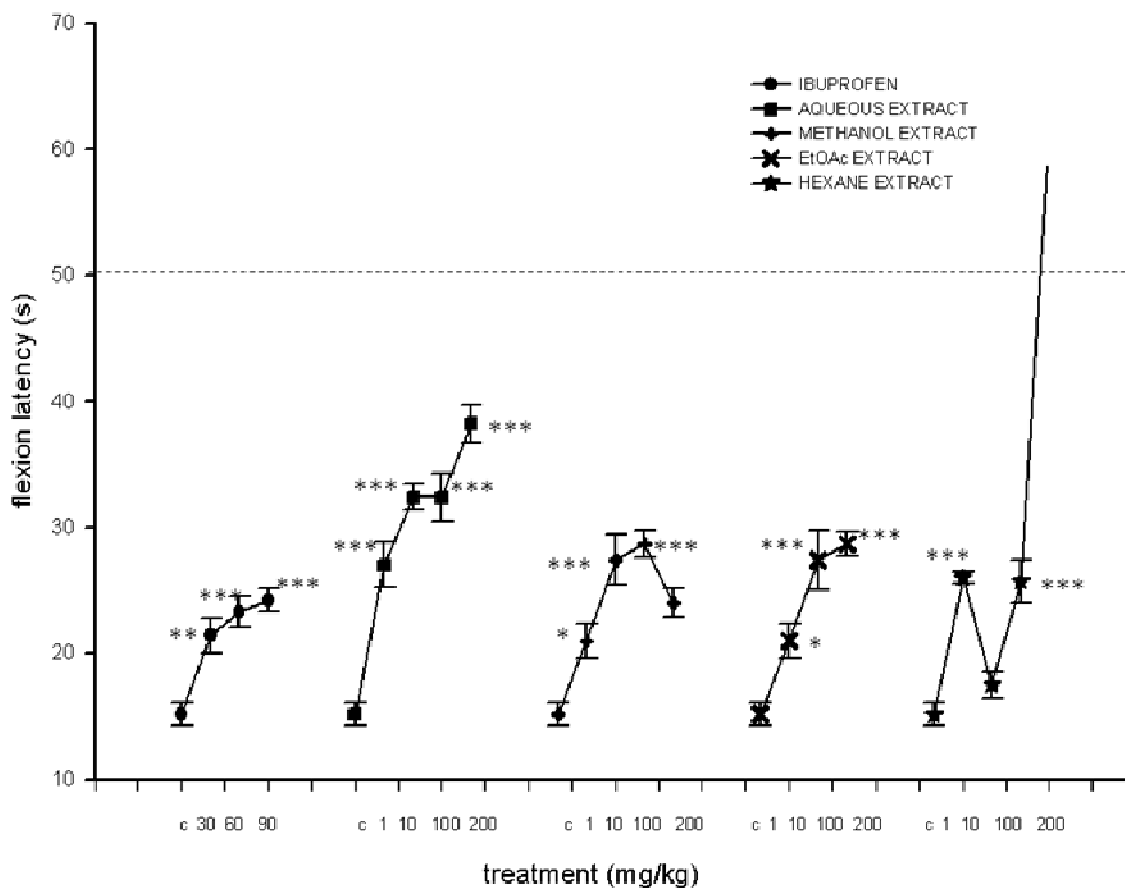


Figure 1. Effects of organic and aqueous extracts of *P. auritum* on Hot Plate Test (HPT). Ibuprofen: $H= 19.14$, $df= 3$, $P\leq 0.001$; Hexane extract: $H= 34.182$, $df= 4$, $P\leq 0.001$; Methanol extract: $H= 25.020$, $P\leq 0.001$; Ethyl acetate extract: $H= 29.695$, $df= 4$, $P\leq 0.001$; Aqueous extract: $H= 29.10$, $df= 4$, $P\leq 0.001$. All results are expressed as means \pm SEM ($n = 8-10$ animals in each group). Comparisons were made by using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Mann-Whitney U test: * $p < 0.05$, ** $p < 0.01$ and *** $p = 0.001$ compared with the control group.

non-polar extracts (hexane and EtOAc) and polar extracts (MeOH and aqueous) shows that while the non-polar (hexane and EtOAc) extracts induced a significant decrease in the ambulatory activity of experimental animals when administered at the same dose that was used effectively in the SPT, the polar extracts caused an increase in both rearing and count number when independently administered at both 1 and 10 mg/kg dose. When the polar extracts were administered at 200 and 500 mg/kg they produced a decrease in these same parameters. The profile shown by the aqueous extract was similar to that of Dz, a benzodiazepine anxiolytic-hypnotic commonly used in clinical practice and was used as a positive control for this test.

The hot plate method is one of the most common tests for evaluating the antinociceptive efficacy of drug/compounds in rodents (Carter, 1991). Our findings show that in comparison to vehicle-treated control animals, all extracts (organic and aqueous) suppressed the thermociceptive response in mice that underwent the

hot plate test. In addition, all extracts elicited a stronger antinociceptive effect than ibuprofen, which is a potent non-steroid anti-inflammatory agent and can cause analgesia by a central action that has been reported to be effective in the hot plate test (Jurna and Brune, 1990).

The intraperitoneal administration of hexane extract resulted in an increase in flexion latency in comparison with the control group. Additionally, at a dose of 200 mg/kg, this extract was able to abolish completely the response to thermal stimulus, although a dose-dependent response was not observed. As we have previously mentioned, the hexane extract produced a sedative effect three times greater than that of Dz in the SPT and significantly reduced the spontaneous locomotor activity of animals tested in the OFT when it was given at the same dose (200 mg/kg).

The EtOAc and MeOH extracts showed a significant antinociceptive effect against thermal stimulus when given at a low dosage (1 to 100 mg/kg). However, at a dose of 200 mg/kg, both extracts also caused a sedative

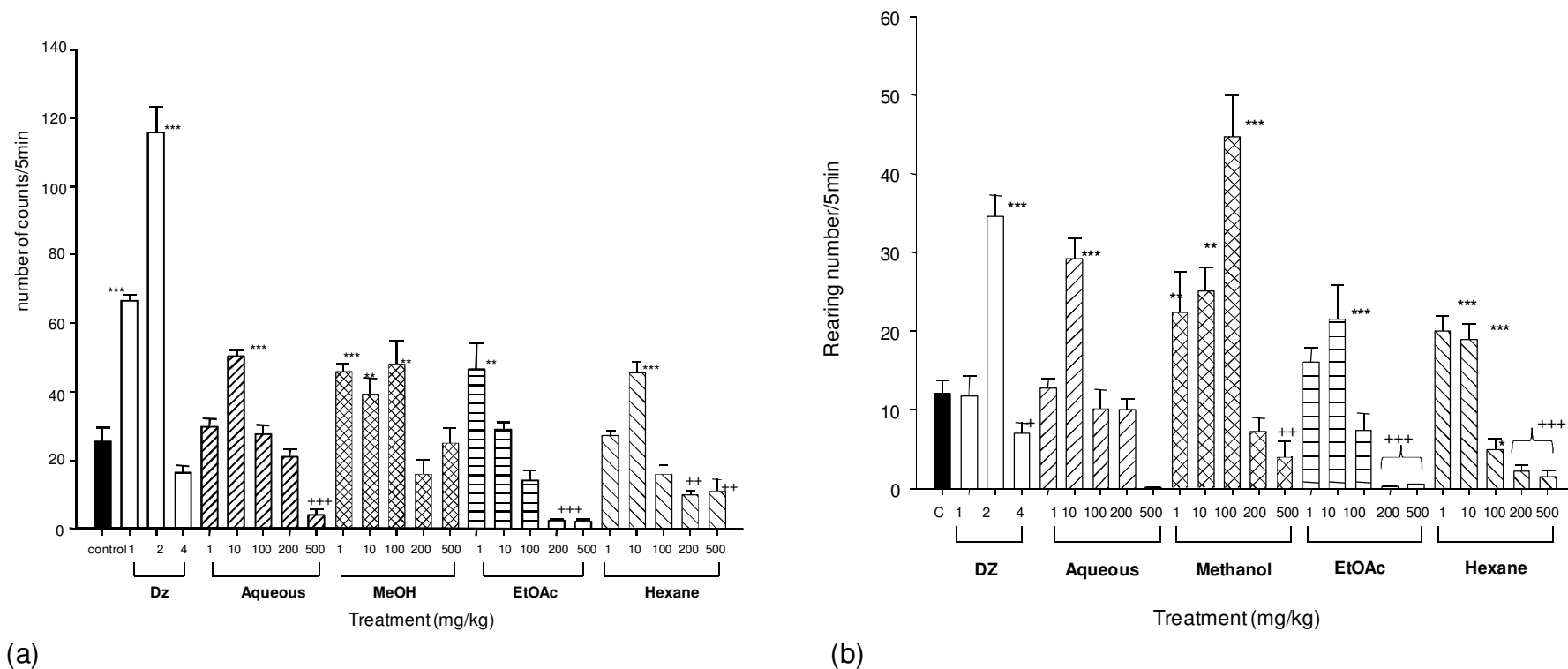


Figure 2. Effect of extracts of *P. auritum* on locomotor activity in the open field test (OFT). (ai) Aqueous extract: $H = 64.30$, $df = 8$, ($P \leq 0.001$); ii) methanol extract: $H = 54.14$, $df = 8$, ($P \leq 0.001$); iii) EtOAc extract: $H = 64.92$, $df = 8$, ($P \leq 0.001$); iv) Hexane extract: $H = 61.87$, $df = 8$, ($P \leq 0.001$). (bi) Aqueous extract: $H = 41.08$, $df = 8$, ($P \leq 0.001$); ii) Methanol extract: $H = 37.42$, $df = 8$, ($P \leq 0.001$); iii) EtOAc extract: $H = 51.68$, $df = 8$, ($P \leq 0.001$); iv) Hexane extract: $H = 43.35$, $df = 8$, ($P \leq 0.001$).

effect in the mice. It is therefore possible that the depressant effect of organic extracts could modify its antinociceptive action at doses higher than 100 mg/kg. In addition, terpenoids such as linalool and limonene have been reported as sedative agents, (Peana et al., 2003) and eugenol, an aromatic molecule that elicits an antinociceptive effect via the capsaicin receptors (Soo-Hyun et al., 2011) are abundant in *P. auritum* thus, the presence of these could explain some effects of hexane and ethyl acetate extracts. Further studies are being

carried out in our laboratory in order to find the active principles responsible for these effects as well as to examine their possible mechanisms.

The aqueous extract of *P. auritum* when administered at a dosage between 0.5 to 100 mg/kg prolonged the mice's response towards the thermociceptive stimulus without affecting its ambulatory activity in the OFT or producing sedative effect in the SPT. Moreover, this extract showed the best dose-dependent antinociceptive effect when compared to the organic extracts,

vehicle-treated control and ibuprofen-treated positive control. These results provide support for the traditional use of *P. auritum* as a remedy for relieving pain and a tranquilizer drug.

Other applications of *P. auritum* leaves include their use in the preparation of teas or infusions employed as traditional remedies to treat nervous conditions such as "susto" (fear), and as stimulant. Thus, we carried out the evaluation of the anxiolytic like effects of aqueous extract of *P. auritum*.

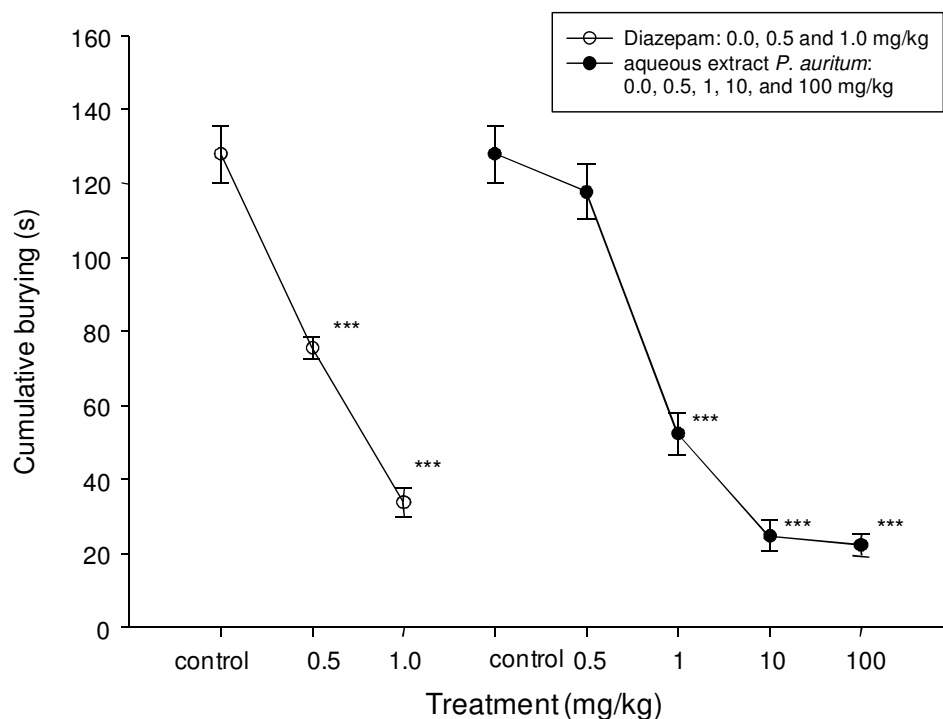


Figure 3. Effect of diazepam and aqueous extract of *P. auritum* on the Burying Behavior test (BBT). $H = 55.43$, $d f = 6$, ($p \leq 0.001$).

The organic extracts failed to influence the behavior of the mice in the hole-board test, indicating a lack of anxiolytic-like effects. The reasons underlying this lack of anxiolytic-like action are presently unknown; however, it could be due to a sedative action. At a high dose, the hexane, EtOAc, and MeOH extracts could be acting differently thus masking or blocking their anxiolytic-like actions.

In contrast, the aqueous extract did elicit an anxiolytic-like effect. Our findings demonstrate that the intraperitoneal administration of the aqueous extract of *P. auritum* produced anxiolytic-like effects in two different animal models of anxiety (the BBT and HBT). The burying behavior paradigm that was used in this study has been largely validated for the study of both anxiolytic and anxiogenic drugs (Lopez-Rubalcava et al., 2006). In BBT, treatment with the aqueous extract of *P. auritum* resulted in a dose-dependent decrease in burying behavior, a response considered to reflect anxiolytic-like actions (De Boer et al., 1991). These results are similar to those observed when using the same paradigm with benzodiazepines such as diazepam (López-Rubalcava, 1996). In the BBT, aqueous extract of *P. auritum* induced clearly anxiolytic-like effects at 1.0 and 100 mg/kg doses.

Burying behavior latency was also measured in this test. This parameter is considered to inversely reflect the animals' reactivity. Interestingly, the aqueous extract of *P. auritum* did not modify the reactivity of the mice when administered at the same dose that elicits the previously

described anxiolytic-like effect.

The HBT has been shown to be notably sensitive to potential anxiolytic activity of many drug, active principles and extracts of plants (File and Pellow, 1985). The hole-board test offers a simple method of measuring the natural tendency of mice to dip their heads into holes and the response or reactivity of an animal to an unfamiliar environment (Boissier and Simon, 1962; Mendonça et al., 2008). In our study, both diazepam (0.5 and 1.0 mg/kg, i.p.) and aqueous extract of *P. auritum* (1 and 10 mg/kg, i.p.) increased the head-dipping and rearing number compared with control group without modifying the locomotion of the mice. It is important to mention that the profile of behavioral effects produced by the aqueous extract was similar to the produced by diazepam, which is a classical drug that was also effective in this test.

It has been reported that some drugs, extracts or active principles with anxiolytic-like effects can affect mice behavior in the OFT due to undesirable side effects (López-Rubalcava et al., 2006). However, *P. auritum* did not produce any modification in locomotor activity of experimental animals. Furthermore, the aqueous extract had an antinociceptive effect during the HPT in a dose-dependent manner, and only at higher doses (200 and 500 mg/kg) did this treatment produce a CNS depressant effect. The aqueous extract of *P. auritum* clearly produced a dose-dependent, anxiolytic-like effect. This conclusion is based on the results of two specific experiments. The first result is the selective decrease in

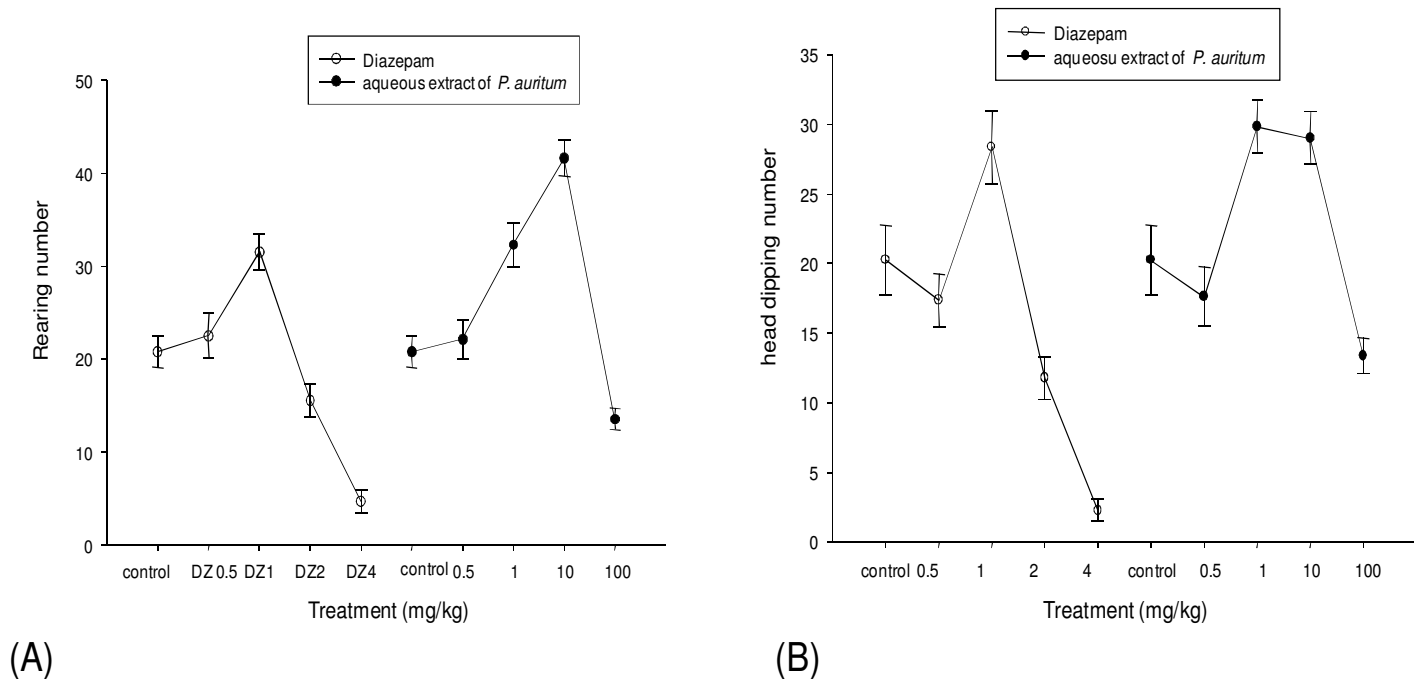


Figure 4. Effect of Diazepam (Dz) and aqueous extract of *P. auritum* on Hole Board Test (HBT). (A) $H = 55.56$, $df = 8$, $P \leq 0.001$; (B) $H = 52.39$, $df = 8$, $p \leq 0.001$.

burying time observed in the BBT and the second is the increase in the rearing and head dipping in the HBT.

Another aim of the present study was to evaluate the possible toxic risks of selected *P. auritum* extracts. To accomplish this goal, the acute toxicity resulting from the intraperitoneal administration of both organic and aqueous extracts was measured according Lorke's method. As mentioned in the result section, at low doses (100 and 200 mg/kg), some adverse effects (hypo-activity and general flaccidity) were seen with the administration of the hexane extract while at higher doses (1600 mg/kg) this toxic manifestations persisted until death within a period of 36 h with an LD_{50} of 1264 mg/kg. Similarly, the intraperitoneally EtOAc treatment produced mortality at LD_{50} of 1264 mg/kg of body weight but the death occurred within the first hour after the EtOAc administration. Finally, the administration of MeOH and aqueous extracts produced toxic manifestations with LD_{50} at 2900 and 3800 mg/kg, respectively. According to Lorke's method, they are considered lightly toxic.

In summary, with this study we have demonstrated for the first time that both organic and aqueous extracts from leaves of *P. auritum* exert an antinociceptive effect on mice at lower doses while at higher doses, the organic extracts act as a depressant effect on the CNS at higher doses. Interestingly, the aqueous extract possesses an antinociceptive effect that is even more potent than ibuprofen and an anxiolytic-like effect similar to those produced by diazepam without affecting the locomotor activity of the experimental animals. In addition, the lack

of toxicity implies that the intake of *P. auritum* does not involve significant health risks. Taken together, these results support the use of *P. auritum* in traditional medicine as an anxiolytic, tranquilizing and sedative agent as well as a remedy for the relief of pain. Moreover, this work encourages the therapeutic use of *P. auritum* as an alternative to *P. methysticum* in anxiety disorder treatments.

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Declaration of interest

The authors reported no conflicts of interest.

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