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Neurobehavioral and toxicological activities of two potentially CNS-acting medicinal plants of *Piper* genus

Janaina Jardim Lopes[a], Camila Marx[a], Rafaela Ingrassia[a], Jaqueline Nascimento Picada[b], Patrícia Pereira[a], Alexandre de Barros Falcão Ferraz[c,*]

[a] Laboratório de Farmacologia e Toxicologia, Programa de Pós-Graduação em Genética e Toxicologia Aplicada, ULBRA, Canoas, RS, Brazil
[b] Laboratório de Genética Toxicológica, Programa de Pós-Graduação em Genética e Toxicologia Aplicada, ULBRA, Canoas, RS, Brazil
[c] Laboratório de Fitoquímica, Programa de Pós-Graduação em Genética e Toxicologia Aplicada, ULBRA, Canoas, RS, Brazil

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ABSTRACT

Plants from the genus *Piper* are economically useful and some species have been indicated because of their medicinal properties in the central nervous system. However, few studies about toxicity and neurobehavioral effects have been conducted. In this study, two *Piper* species, *P. amalago* and *P. mikanianum* were investigated in rats to determine acute toxicity and to evaluate the ansiogenic/ansiolytic properties in the elevated plus-maze and the effects on locomotion and exploration in an open field. Additionally, genotoxic activities were evaluated, using the comet assay in several tissues and the micronucleus assay in bone marrow. The phytochemical analysis of both *Piper* species leaves suggests the presence of amide, essential oils, flavonoids and phenolic compounds. The LD_{50} of *P. amalago* and *P. mikanianum* were estimated as 2,545 and 1,661 mg/kg, respectively. The behavioral and genotoxic parameters were determined after an intraperitoneal administration of *P. amalago* (250 or 420 mg/kg) or *P. mikanianum* (160 or 270 mg/kg). Both plants decreased the number of entries and time spent in the open arms in the plus-maze test, indicating an anxiogenic effect. Only *P. mikanianum* affected locomotion and exploration in the open field behavior test. No genotoxic or mutagenic effect was observed. Our results suggest that these *Piper* species act on the central nervous system, without induce genetic toxicity.

1. Introduction

The genus *Piper* belongs to the Piperaceae family and encompasses over 700 species widely distributed throughout the tropical and subtropical regions of the world. Members of the *Piper* genus have commercial, economical, and medicinal importance. Economically, Piperaceae are employed worldwide in the production of pepper in spice markets. Plants from the genus *Piper* have been used for a number of practical applications, like remedies in many traditional medicinal systems, such as traditional Chinese medicine, the Indian Ayurvedic system, and folklore medicines of Latin America and West Indies (Parmar et al., 1997).

The traditional medicine has indicated the use of *Piper* species for many applications, such as antidiarrheic, antipyretic, expectorant (Rahman et al., 2005), antileishmanial (Sarkar et al., 2008), analgesic, toothache and wound treatment (Guerrini et al., 2009), diuretic and to treat headache (Benitez and Valois, 2004). In an ethnopsychiatry study with Maia healers, the authors reported the wide use of plants from *Piper* to treat neurological/mental disorders (Bourbonnais-Spear et al., 2005). Similarly, *Piper* species were reported to exert an anticonvulsant effect (Nsour et al., 2000), and the ethanolic extract of *P. capense* (both leaf and tuber extracts) exhibited moderate activity to the $GABA_A$-benzodiazepine receptor (Stafford et al., 2005).

Among the *Piper* species, *P. methysticum* (kava-kava) is the most well-known species from this family. Kava-kava extracts are widely used for the treatment of anxiety (Shinomiya et al., 2005). Apart from this, more recently this plant has been utilized to induce relaxation, restful sleep, and to soothe headaches and fatigue (Weiss et al., 2005).

P. mikanianum is a native species to Rio Grande do Sul, Brazil. It is used to treat stomach diseases, abortion and in the treatment of amenorrhea and leucorrhea (Alice et al., 1995). *P. amalago*, distributed from Mexico to Brazil, is used to alleviate chest pains and as anti-inflammatory agent (Parmar et al., 1997). However, there is no report about toxicity of these plants.

Numerous studies have been conducted on the biological properties of kava-kava extracts, but these investigations are mainly related to effects on the nervous central system. Thus, the general purpose of the present study was to search, in the *Piper* genus, for other CNS bioactive species, by analyzing the central effects of the methanolic extracts from the leaves of *P. mikanianum* and *P.*

* Corresponding author. Tel.: +55 51 3477 9214; fax: +55 51 3477 9239.
E-mail address: alexandre.ferraz@ulbra.br (A.d.B.F. Ferraz).

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amalago. In addition, we investigated possible genotoxic effects, in order to contribute to the evaluation of health risks involved in the intake of infusions of these plants.

2. Materials and methods

2.1. Animals

Male Wistar rats (2–3 months of age; 200–250 g) were used in this study. All animals were maintained in a controlled temperature environment. Five animals were kept in cages under 12 h light/dark cycles. The animals were allowed free access to food and water. A minimum of nine rats was used for each treatment group. All procedures involving animals were conducted in accordance with the Ethics Committee of Lutheran University of Brazil (CEP/ULBRA 2006-002A) and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

2.2. Plant material

The aerial parts of *P. amalago* and *P. mikanianum* were collected in April 2008, in Morro Reuter and Picada Café, respectively. These cities are located in Rio Grande do Sul state, Southern Brazil, and the plants were identified by Prof. Dr. Sérgio Bordignon. The specimens were deposited in the Herbarium of the Lutheran University of Brazil (HERULBRA) as Bordignon & Apel 3123 and Bordignon & Apel 3125, respectively. The leaves of both plants were dried under the shade for several days and then powdered.

2.3. Preparation of extracts

Thirty grams of *P. amalago* and *P. mikanianum* dried and powdered leaves were treated with 300 ml of methanol (Merck®) for 24 h. The samples were then filtered through Whatman number 1 filter paper and the marc was extracted with another 300 ml of methanol. This procedure was repeated for 5 days, after which the methanolic solutions were combined and evaporated in a rotary evaporator at 45 °C until dry.

2.4. Drugs and pharmacological procedures

P. amalago and *P. mikanianum* were dissolved in 5% polyssorbate 80 (tween) and saline. Thirty minutes prior to the behavior experiment, 9–10 animals per group received an intraperitoneal (i.p.) injection of saline, tween (Merck®) 5%, *P. amalago* 250 mg/kg (PA1), *P. amalago* 420 mg/kg (PA2), *P. mikanianum* 160 mg/kg (PM1) or *P. mikanianum* 270 mg/kg (PM2), as a 2 ml/kg body weight dose. Doses were chosen based on LD_{50} results.

2.5. Acute toxicity studies (LD_{50})

The acute toxicity studies (LD_{50}) were carried out as described by Navarro et al. (2005) with minor modifications. Animals received 0 (saline and tween 5%), 500, 1,000, 1,500 or 2,000 mg/kg of the extract (i.p. injections). The mortality was noted after dosing for a 14-day period.

2.6. Phytochemical analysis

Plants were subjected to qualitative chemical screening for the identification of the major classes of active chemical constituents. The phytochemical profile of *P. amalago* and *P. mikanianum* leaves were determined according to methodology described by Harborne (1984).

2.7. Open field behavior and habituation

Animals were exposed to a 40 cm × 50 cm × 60 cm open field divided into 12 identical white squares described by black lines. Animals were placed in the rear left square and allowed freedom to explore the environment for 5 min. Crossings of black lines and rearings performed were counted and used as measures of locomotion and exploration (Viana et al., 2007).

The habituation test was conducted after 24 h, when the same animals were again tested for open field behavior, for 5 min. Long-term retention of habituation to a novel environmental can be considered a type of learning. The decrease in the number of rearings performed between the first and the second exploration sessions was considered as a measure of habituation (Viana et al., 2007).

2.8. Elevated plus-maze test

The apparatus consists of a platform (10 cm × 10 cm), two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm), arranged in such a way that the two arms of each type are opposite to each other. The maze wall was 50 cm high, and the tests were conducted under dim red light. The animals received the injections 30 min before the test. They were then placed individually on the central platform of the plus-maze. During a 5-min test period, the numbers of entries and the time spent in open and enclosed arms were recorded (Viana et al., 2007). Benzodiazepine diazepam (Valium®, Roche) was utilized as positive control; it is a standard anxiolytic and is also employed in behavior pharmacology as a reference compound (Rex et al., 2002).

2.9. Comet assay

The alkaline comet assay in peripheral blood, liver and brain tissues was carried out as previously described (Tice et al., 2000), with minor modifications (Rodrigues et al., 2009). Blood samples were collected from a tail veil, 3 and 24 h after the injections, while liver and brain samples were dissected 24 h after the injections. Each piece of forebrain and live was placed in 0.5 ml of cold phosphate-buffered saline (PBS) and finely minced in order to obtain a cell suspension. The cell suspensions (5 μl) were embedded in 95 μl of 0.75% low melting point agarose (Gibco BRL) and spread on agarose-precoated microscope slides. After solidification, slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0), with freshly added 1% Triton X-100 (Sigma) and 10% DMSO for 48 h at 4 °C. The slides were subsequently incubated in freshly prepared alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min, at 4 °C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to perform DNA electrophoresis. The slides were then neutralized (0.4 M Tris, pH 7.5), stained with silver and analyzed using a microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each animal. Cells were also visually scored according to tail size into five classes ranging from undamaged (0) to maximally damaged (4), resulting in a single DNA damage score to each animal, and consequently to each studied group. Therefore, the damage index (DI) can range from 0 (completely undamaged, 100 cells × 0) to 400 (with maximum damage, 100 cells × 4) (Pereira et al., 2009).

2.10. Micronucleus assay

The micronucleus assay was performed according to the US Environmental Protection Agency Gene-Tox Program (Mavournin et al., 1990). Bone marrow from both femurs was collected from each animal 24 h after the administrations. The tissue was suspended in fetal calf serum and smears on clean glass slides were

prepared as in a previous report (Picada et al., 1997). Slides were air-dried, fixed in methanol, stained in 10% Giemsa and coded for a blind analysis. To avoid false negative results and as to obtain a measure of toxicity on bone marrow, the polychromatic erythrocytes: normochromatic erythrocytes (PCE:NCE) ratio was scored in 1,000 cells. The incidence of micronuclei (MN) was observed in 2,000 PCE for each animal (Rodrigues et al., 2009). Cyclophosphamide (Genuxal®, Asta Medica) was utilized in the positive control group.

2.11. Statistical analysis

Data from LD_{50} were examined using the Probit's analysis. Data from elevated plus-maze and open field test are expressed as mean ± S.E.M. These data were examined using the one-way ANOVA followed by the Duncan's test. Habituation results were analyzed using the Paired *t*-test. The statistical evaluation of data from comet assay and micronucleus assay was carried out using the Tukey's test. In all comparisons, $p \leq 0.05$ was considered as indicating statistical significance.

3. Results

3.1. Phytochemical analysis

The phytochemical analysis of *P. mikanianum* and *P. amalago* leaves allowed observing a similar behavior between the two species. In both plants the presence of phenolic compounds, essential oil, flavonoids and amides was indicated.

3.2. Acute toxicity studies (LD_{50})

The LD_{50} of *P. amalago* in rats was estimated to be 2,545 mg/kg i.p. with an observation period of 14 days, and the LD_{50} of *P. mikanianum* was estimated to be 1,661 mg/kg under the same conditions.

3.3. Open field behavior and habituation

We verified the effect of pretest administration of crude extract of *P. amalago* (250 or 420 mg/kg) and *P. mikanianum* (160 or 270 mg/kg) in open field behavior of rats. There were no significant differences among control groups and groups that received *P. amalago* in either parameter observed. The locomotor activity (represented by crossings number) was not affected in the animals that received the 160 mg/kg dose of *P. mikanianum* (mean ± S.E.M. = 77.4 ± 16.3), but the group that received *P. mikanianum* 270 mg/kg was able to decrease the number of rearings (mean ± S.E.M. = 11.8 ± 3.0; $p < 0.05$) and crossings (mean ± S.E.M. = 46.2 ± 11.2; $p < 0.05$) performed, suggesting that in this concentration the plant affects exploration and locomotion capabilities of these animals in this task (Fig. 1).

After 24 h the test was repeated. The groups that received *P. mikanianum* 160 mg/kg increased the exploratory activity significantly (mean ± S.E.M. = 35.6 ± 3.6; $p < 0.05$) in comparison to the first day, which reflects a decrease in habituation of the animals to the apparatus (Fig. 2).

3.4. Elevated plus-maze test

P. mikanianum (160 or 270 mg/kg; mean ± S.E.M. = 203.8 ± 31.5 and mean ± S.E.M. = 231.5 ± 26.9, $p < 0.05$) and *P. amalago* (250 or 420 mg/kg; mean ± S.E.M. = 238.6 ± 15.3 and mean ± S.E.M. = 196.9 ± 25.9, $p < 0.05$) significantly increased the time spent in closed arms when compared with saline, which may suggest an anxiogenic effect of these species. The diazepam group (used as positive control) increased the number of entries (mean ± S.E.M. = 6.7 ± 0.9; $p < 0.05$) and time spent (mean ± S.E.M. = 154.1 ± 13.6; $p < 0.05$) in the open arms when compared with the control group (Fig. 3). The animals that received tween were not affected in this task.

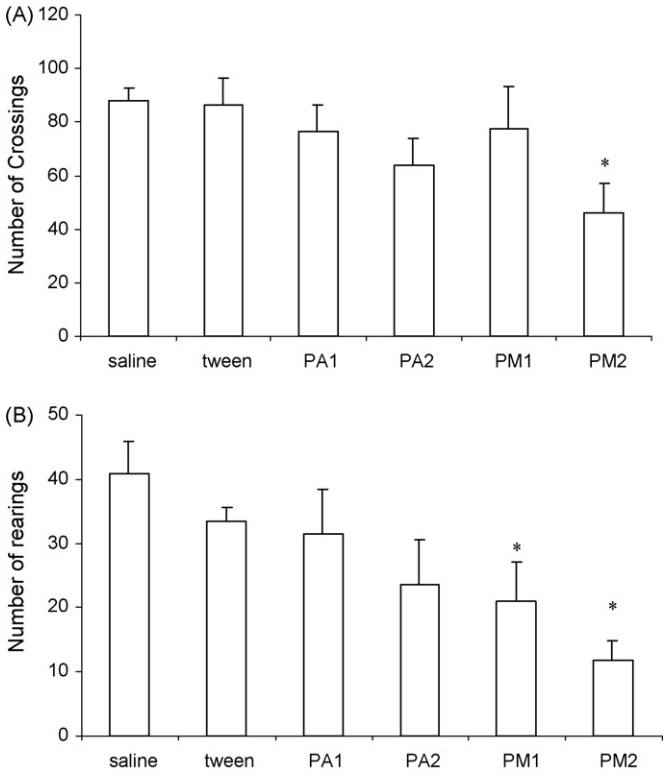

Fig. 1. Effect of pretest administration of *P. amalago* (250 or 420 mg/kg) and *P. mikaninum* (160 or 270 mg/kg) on crossings (A) and rearings number (B) performed during a 5-min exploration of an open field. Animals received an intraperitoneal injection of saline, vehicle, *P. amalago* (PA) or *P. mikaninum* (PM) 30 min prior to being exposed to the locomotor behavior task in the open field. Data are expressed as mean ± S.E.M., $N = 9–10$ animals per group; $^*p < 0.05$ compared to the control group.

3.5. Comet assay

P. amalago and *P. mikanianum* did not show any genotoxic effect on blood, liver and brain tissues from the treated groups when compared to the tween control group (Table 1).

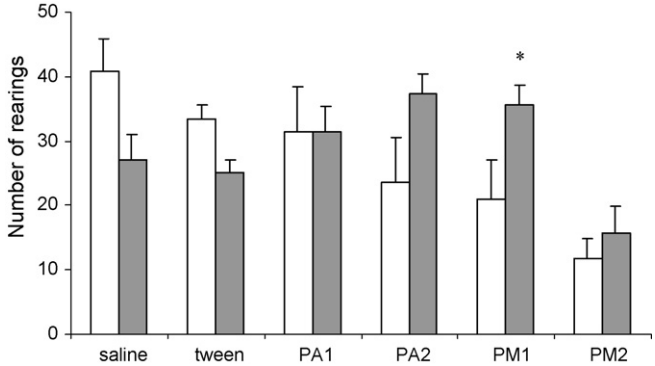

Fig. 2. Effect of pretest administration of *P. amalago* (250 or 420 mg/kg) and *P. mikaninum* (160 or 270 mg/kg) on habituation to open field. Animals received an intraperitoneal injection of saline, vehicle, *P. amalago* (PA) or *P. mikaninum* (PM) 30 min prior to training. White columns: training; gray columns: test (24 h after training). Data are expressed as mean ± S.E.M., $N = 9–10$ animals per group; $^*p < 0.05$ compared to the saline group; ANOVA/Duncan's test.

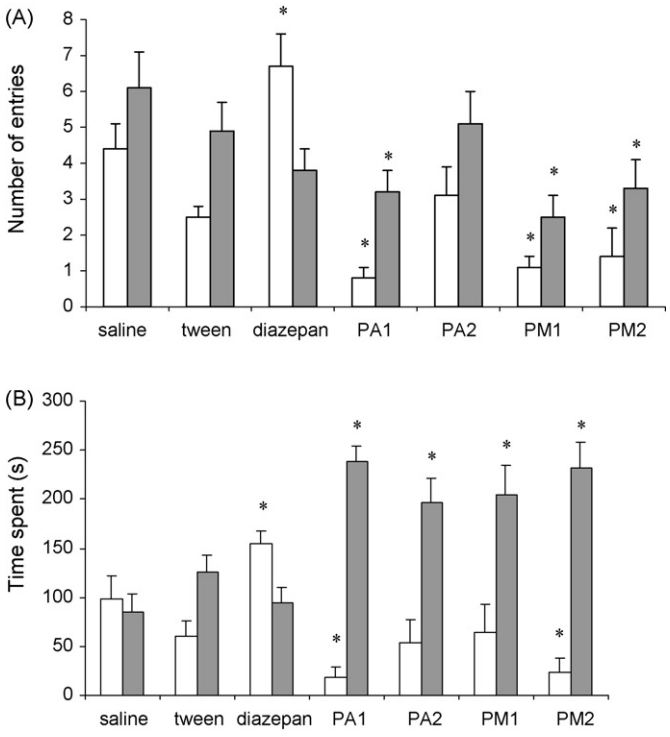

Fig. 3. Effect of pretest administration of *P. amalago* (250 or 420 mg/kg) and *P. mikanianum* (160 or 270 mg/kg) on the number of entries (A) and time spent (B) in open and closed arms. Animals received an intraperitoneal injection of saline, vehicle, diazepam (1 mg/kg), *P. amalago* (PA) or *P. mikanianum* (PM) 30 min prior to being exposed to the plus-maze. White columns: open arms; gray columns: closed arms. Data are expressed as means ± S.E.M., $N = 10$ animals per group; $^{*}p < 0.05$ compared to the control group; ANOVA/Duncan's test.

Table 1
Comet assay in blood, brain and liver of rats treated with an intraperitoneal injection of tween, *P. amalago* (PA) or *P. mikanianum* (PM).

	Damage index (mean ± SD)			
	Blood 3 h	Blood 24 h	Liver 24 h	Brain 24 h
tween	5.6 ± 4.2	7.4 ± 4.2	13.7 ± 1.2	13.5 ± 4.6
PA 250 mg/kg	4.4 ± 2.6	9.8 ± 4.2	14.0 ± 3.7	9.6 ± 5.2
PA 420 mg/kg	4.6 ± 2.4	9.8 ± 4.6	11.2 ± 4.1	16.7 ± 7.3
PM 160 mg/kg	3.6 ± 3.6	10.8 ± 8.1	14.2 ± 3.1	11.8 ± 3.9
PM 270 mg/kg	2.0 ± 1.4	7.4 ± 3.3	15.0 ± 2.4	11.3 ± 2.7
PC[a]	197.2 ± 34.8[b]	173 ± 42.4[b]	233 ± 40.3[b]	301.0 ± 48.6[b]

$N = 5$ animals per group. Damage index: can range from 0 (completely undamaged. 100 cells × 0) to 400 (with maximum damage 100 cells × 4).

[a] PC: positive control: hydrogen peroxide. Cells from the tween group were treated *ex vivo* with hydrogen peroxide 0.20 mM.

[b] $p < 0.001$: statistically significant difference from the tween groups (ANOVA. Tukey' test).

3.6. Micronucleus test

There was no significant difference in frequency of micronuclei in any of the groups, suggesting that both extracts did not induce mutagenic activity (Table 2). As expected, the micronuclei frequency was increased in the positive control group (cyclophosphamide; $^{***}p \leq 0.001$). A similar PCE/NCE ratio was detected in all groups, indicating no toxicity in bone marrow of the rats in the tested experimental conditions (data not shown).

4. Discussion

The LD_{50} was determined for the two species studied (*P. amalago* 2,545 mg/kg and *P. mikanianum* 1,661 mg/kg). These results and

Table 2
Micronucleus frequency in bone marrow of rats treated with an intraperitoneal injection of tween, *P. amalago* (PA 250 or PA 420 mg/kg) or *P. mikanianum* (PM 160 or PM 270 mg/kg). The samples were collected 24 h after the injections.

MNPCE in 2,000 PCE per animal (mean ± SD)	
tween	7.3 ± 5.1
PA 250	13.3 ± 3.1
PA 420	10.8 ± 1.5
PM 160	11.4 ± 2.6
PM 270	7.5 ± 1.3
PC[a]	25.2 ± 6.4[***]

$N = 5$ animals per group. MNPCE: micronucleated polychromatic erythrocytes (PCE).

[a] PC: positive control: cyclophosphamide 20 mg/kg.

[***] $p < 0.001$: statistically significant difference from the tween group (ANOVA/Tukey' test).

those described in similar studies in the literature (Mukinda and Syce, 2007; Veerappan et al., 2007) enable to establish that doses between 10 and 20% of the LD_{50} should be used to perform the behavior experiments. According to Veerappan et al. (2007), the LD_{50} of 1,000 mg/kg, calculated from intraperitoneal administration, may indicate a relatively safe use of the compound or extract in study. The values of LD_{50} found in this study are above this value, which may indicate that *P. amalago* and *P. mikanianum* leave extracts do not present high toxicity.

The results obtained in the open field test showed that the crude extract of *P. mikanianum* in both doses tested was able to reduce the number of rearings, but only a 270 mg/kg dose decreased the number of crossings. The acute administration of 250 or 420 mg/kg of *P. amalago* extract did not alter the locomotor and exploratory activity in the same task.

When the animals were exposed again to the apparatus (24 h after training), *P. mikanianum* 160 mg/kg was able to significantly increase the number of rearings performed. Other groups treated with *P. amalago* (PA1 and PA2) and *P. mikanianum* (PM2) also presented an increase in exploratory activity 24 h after training, but not significantly, compared to the control group. These results suggest that both species impair the animals habituation, which can be related to a decrease in memory acquisition.

We believe that the effect on the locomotor and exploratory activities of the *P. mikanianum* may be related to the presence of amides, since previous studies have shown that some amides, found in species of the genus *Piper,* exert effects on the central nervous system. For example, piplartine, present in *P. tuberculatum*, led to antidepressant and anxiolytic effects when administered to mice (Felipe et al., 2007). The study by Pan et al. (2005), which aimed to investigate the constituents responsible for the antidepressant action of *P. laetispicum* observed by forced swing test in mice, isolated and identified three amides. Compounds isolated from *Piper* such as kavapyrones and piperine have been shown to depress the central nervous system activity, exhibiting sedative, anticonvulsant, and relaxing effects (Bourbonnais-Spear et al., 2005). In another study the authors observed that the anti-depression like activity and cognitive enhancing effect of piperine were comparable to the positive control (Wattanathorn et al., 2008). In addition, this same compound displayed central nervous system depressant by antagonism of electroshock induced seizures and muscle relaxation in mice (Lee et al., 1984).

The effects of kava-kava on locomotor activity (open field test with computerized tracking) were demonstrated in the work of Garret et al. (2003), where this species was administered to mice as intraperitoneal doses of between 32 and 326 mg/kg. The locomotor activity was reduced on a dose-dependent manner by kava-kava, suggesting a sedative effect of this species.

In this study, according to the results of the elevated plus-maze, the animals treated with both species of *Piper* remained in the closed arms for longer, as compared to the presence within the open arms of the apparatus, in relation to the control group. In this task, compounds with anxiolytic activity decreased the animal's aversion to open arms, thus promoting exploration (Melo et al., 2006). Here, the anxiogenic activity was verified by the decrease in the exploration of open arms. The results suggest that *P. amalago* and *P. mikanianum* were able to cause an anxiogenic effect at the doses tested.

According to Garret et al. (2003), diazepam, a drug widely used in therapy as a sedative and anxiolytic, increases the time spent in open arms, causing a decrease in anxiety. In this research, a group of animals received diazepam 1 mg/kg in order to compare the results and validate this behavior task. The anxiolytic activity of diazepam was observed.

In the work of Felipe et al. (2007), the effects on anxiety of the amide piplartine were evaluated using the elevated plus-maze in mice. The administration of this compound as 50–100 mg/kg doses was able to significantly increase the number of entries and time spent in the open arms of the apparatus, suggesting that piplartine has anxiolytic activity. The anxiolytic effects of the administration of standardized extract of kava-kava (30% of kavalactonas) as doses of 120, 180 and 240 mg/kg were evaluated through the elevated plus-maze in rats.

This study indicated that kava-kava presented a dose-dependent anxiolytic effect in the elevated plus-maze, but only the lowest doses showed significant difference as compared to the control group (Rex et al., 2002). When the species *P. amalago* and *P. mikanianum* were evaluated, an anxiogenic effect was observed. In previous studies on other species of *Piper* on the same behavior task, the anxiolytic effect was dominant in a dose-dependent manner. The doses investigated of *P. amalago* and *P. mikanianum* were chosen based on the values of LD_{50} (between 10 and 20%), which may be higher than those with pharmacological activity of interest, meaning also a dose-related effect. The absence of anxiolytic effect observed in this study may be due the higher doses evaluated producing a saturation effect on central receptors.

Hasenöhrl et al. (2007) showed that standardized extracts of rhizomes of *Zingiber officinale* did not influence the rats' behavior on the elevated plus-maze when doses of 1 and 10 mg/kg were administered, though the 100 mg/kg led to fewer excursions to and less scanning of the open arms, indicating the anxiogenic properties of the plant when used at higher dosages. The authors concluded that is possible that the extract produces a biphasic dose–response effect on the behavior of rats in the plus-maze with an anxiolytic-like action under the low dosage, and an anxiogenic-like effect under the high dosage.

In the study conducted by Rex et al. (2002) it is possible to observe that at the highest dose (240 mg/kg) the parameters did not present statistical significance. It is interesting to note that the 240 mg/kg dose is one of the lowest doses evaluated in the present paper. In accordance with this data, Wattanathorn et al. (2008) conclude that piperine possesses anti-depression like activity and cognitive enhancing effect, especially when used as low doses.

The comet assay revealed no genotoxic effect (Table 1), since there was no induction of DNA damage after intraperitoneal administration of both doses of *P. amalago* and *P. mikaninaum* in blood, liver and brain tissues. The blood tissue collected 3 h after administrations could indicate recent DNA damages. This period of collecting is important to evaluate drugs inducing DNA damage directly, without metabolizing (Hartmann et al., 2003). Furthermore, 24 h after withdrawal, no genotoxic effect was observed, suggesting that neither extract presents either direct or indirect genotoxic compounds. The liver receives the major of the exogenous compounds, independently of their polarity, acting as a metabolizing organ. Several authors reported cases of hepatotoxicity induced by the use of *P. methysticum* (kava-kava), one of the most studied species of *Piper* genus (Wooltorton, 2002; Stickel et al., 2003). Here, *P. amalago* and *P. mikanianum* did not induced DNA damage in liver tissue, suggesting no toxic effect on the liver of the animals. These data are in accordance with the Sorrentino et al. (2006) study that evaluated the safety of ethanolic kava extract in rats submitted a chronic treatment.

Although behavioral deficits have been elicited for both extracts, in the brain tissue it was not observed an increased DNA damage in comparison to tween control group (Table 1). In fact, kava-kava extracts and piperine have demonstrated neuroprotective activities (Dajas et al., 2003; Chonpathompikunlert et al., 2010), despite hepatotoxicity (Wooltorton, 2002; Stickel et al., 2003), suggesting that compounds present in the genus *Piper* are capable to cross the blood–brain barrier and produce beneficial effects.

In other studies, drugs inducing behavioral impairs have shown genotoxic effects in brain tissue (Pereira et al., 2007, 2009). However, the studied extracts here are complex mixture, containing flavonoids, amides and other phytochemicals. Thus, various available compounds on CNS could interact with DNA, independently of their ability to induce neurobehavioral effects.

There was not difference in the proportion PCE/NCE for treatment of animals with extracts comparing with tween control group, indicating *P. amalago* and *P. mikanianum* were not cytotoxic to bone marrow. The frequency of micronucleus did not increase significantly in extract-treated groups (Table 2), suggesting no mutagenic effect in chromosomal levels. These findings suggest that both extracts do not show genetic toxicity after a single dose.

Furthermore, the pharmacological mechanisms that might account for the observed anxiogenic effects have yet to be determined. While blockade of serotonin, including blockade of 5-HT receptors, has in general been shown to lead to anxiolytic effects using the elevated plus-maze, such effects are not always consistent (Hasenöhrl et al., 2007). In fact, we noted that the extracts from leaves of *P. amalago* and *P. mikanianum* act on the central nervous system. These data point to the need to further investigate extracts when used as lower doses, in an attempt to verify if the anxiogenic effect would be maintained.

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