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Full Length Research Paper

Evaluation of the acute toxicity, genotoxicity and mutagenicity of ethanol extract of *Piper aduncum*

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The genus *Piper* is widely distributed in tropical and subtropical regions. It has many ethnopharmacological uses, such as an antifungal and antiparasitic, but its toxicity has not yet been fully elucidated. In a fixed dose assay 2 g/kg (p.o.), the ethanol extract of aerial parts (stems and leaves) of *Piper aduncum* was administered and no signs of toxicity were detected, either during the behavioral observations or in the autopsies, which indicates a reasonable lack of toxicity for the extract. In the comet assay, no significant difference was found between the extract of *P. aduncum* and negative control. In assessing mutagenicity by the micronucleus and *Saccharomyces cerevisiae* test, there was no statistically significant difference between the extract and the negative control. From the results obtained, it appears that crude ethanol extract of *P. aduncum* does not damage DNA and was considered to have low acute toxicity.

Key words: Genotoxicity, mutagenicity, acute toxicity, medicinal plant, *Piper aduncum.*

INTRODUCTION

Ancient uses of medicinal plants have shown, over the years, the potential harmful effects of certain plants. From a scientific point of view, research has shown that many substances, including natural products, are potentially toxic and therefore should be used with care, respecting their toxicological risks (Veiga-Junior et al., 2005). In most cases, the adverse effects of commonly used plants are not well documented in the literature and their long-term use by humans is usually correlated with low toxicity. However, studies have shown that many foods and traditional plants used for their medicinal properties have mutagenic effects (Elgorashi et al., 2003; Higashimoto et al., 1993; Kassie et al, 1996; Schimmer et al., 1988). In Brazilian folk medicine, Piper (Piperaceae) is an example of plants that are widely used. The Piperaceae family has more than 2000 species distributed worldwide in tropical and subtropical regions (Quijano-Abril et al., 2006). This plant is of commercial, economic and medicinal importance; for instance, the plants of this family are employed in production of pepper (Ma et al., 2004).

The phytochemistry of this genus is characterized by the presence of amides, benzoic acids, terpenes, phenylpropanoids, lignans and various alkaloids (Ma et al., 2004; Tsai et al., 2005). Species of *Piper* sp are commonly used as a natural insecticide. It is pharmacologically used to treat anxiety, and also as an antispasmodic in the treatment of bacterial and fungal infection (Ghoshal et al., 1996; Silva et al., 2007). *Piper methysticum* (Kava) has been compared pharmacologically to benzodiazepines such as diazepam or alprozolam and is recognized as a “natural” alternative to these anti-anxiety medications (Singh and Singh, 2002).

Antifungal activity has been described for different species of *Piper*, including: *Piper dilatatum* (Svetaz et al., 2010; Terreaux et al., 1998); antiparasitic activity of prenylated benzoic acid derivatives from *Piper heterophyllum* and *Piper aduncum* (Flores et al., 2009)

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and antioxidant activity related to prenylated hydroquinone and benzoic acid derivatives from *Piper crassinervium* (Yamaguchi et al., 2006) and cytotoxic activity against tumor cell lines, antifungal, anti-platelet aggregation, anxiolytic and antidepressant effects of piplartine, an alkaloid isolated from *Piper tuberculatum* (Bezerra et al., 2008).

There have been concerns over the use of Piperaceas, mainly relating to Kava hepatotoxicity. Kava is composed of approximately 18 active compounds collectively referred to as kavalactones or kavapyrones. It is well-known that the use of kava has been involved in several cases of liver toxicity in humans, including several who required liver transplants after using kava supplements (Whittaker et al., 2008).

Other Pipers have been also toxicologically evaluated. Sousa et al. (2008) investigated the acute toxicity of essential oil from *P. aduncum*, in which a dose of 3 g/kg caused no alterations in the behavioral, macroscopic and histological analysis. Déciga-Campos et al. (2007) also observed low acute toxicity of *Piper sanctum* (>5 g/kg) in male mice ICR and for *Piper cubeba*, the ethanol extract at concentrations of 1.0 and 1.5 g/kg demonstrated moderate mutagenic action (Junqueira et al., 2007).

However, no toxicological information related to ethanol extract from aerial parts of *P. aduncum* was found in the literature during the preparation of this manuscript. This study assesses the acute toxicity, genotoxicity and mutagenicity of the ethanol extract of the leaves of *P. aduncum* using specific techniques, such as acute toxicology through fixed dose in female rats; analysis of genotoxicity by the comet assay and analysis of mutagenicity using the micronucleus test on bone marrow cells and the *Saccharomyces cerevisiae* Strain.

MATERIALS AND METHODS

Plant material

Ethanol extract obtained from aerial parts of *P. aduncum* L. was prepared in the phytochemistry Laboratory of the Universidade do Vale do Itajaí (UNIVALI). The plant material was identified by Prof. Oscar Benigno Iza (Universidade do Vale do Itajaí) and a sample of the specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí), under number HJOI 9768.

The dried vegetal material (200 g) of aerial parts (stems and leaves) *P. aduncum* was extracted by maceration with 95% ethanol at room temperature, for a period of seven days. Solvent removal was carried out under reduced pressure at temperatures below 45°C, until the desired concentrations were achieved, in order to obtain the ethanol extracts (4.1 g).

Animals

The study was carried out using female *Wistar* rats (220 to 250 g, with 2 to 3 months old), obtained from the Central Animal house of UNIVALI. The animals were kept in cages with free access to water and food (Special Nuvilab CR1), in a room with controlled temperature (22°C ± 2°C) and light/dark cycle natural (12 h each). The animals used in the present study were housed and cared for in accordance with the Federal Government legislation on animal care. The experiments were also authorized by Ethical Committee for Animal Care of the Universidade do Vale do Itajaí, Itajaí, Santa Catarina, Brazil (124/09, CEP-UNIVALI).

Acute toxicity fixed test dose

The ethanol extract from the leaves of *P. aduncum* at a dose of 2 g/kg (0.2 g/mL Tween 80:0.9% NaCl [0.021:1]) was administered orally to a group of 5 animals (female) after 12 h of fasting. The control group received saline (3 mL/kg). After administration of the extract, the animals were observed individually for the first 12 h, at 15, 30 and 60 min and every 4 h. Toxic signs of a general nature were observed, with the following parameters: general activity, trembling voice, irritability, response to touch, grasping the tail, twisting, (writhing, body tone, strength of grip, ataxia, tremors, convulsions, stimulation, Straub tail, hypnosis, anesthesia, lacrimation, ptosis, piloerection, respiratory frequency and cyanosis. The animals were observed for 14 days and were weighed at 0, 7 and 14 days after administration of the extract. At the end of the observation period, the number of deaths was recorded and all the survivors were euthanized and autopsied. The macroscopic characteristics of the lungs, kidneys, intestines, liver, uterus, spleen and heart were observed (OECD, 2001).

Comet assay *in vitro*

For the *in vitro* comet assay, bone marrow cells of untreated rats were used. First, the slides were covered with 1% agarose. The animals were sacrificed and the right femur was removed. The pool of cells was extracted by washing with cold fetal calf serum. The cell suspension was centrifuged (1000xg/10 min/5°C) and cell viability was checked with 0.4% trypan blue dye. The suspension was standardized at 300.000 cells/mL and the slides mounted with 20 µL of cell suspension + 90 µL agarose low melting point 1.5% (Low Melting Point) and incubated for 15 min at 4°C. The *P. aduncum* extract was then applied, at 10 and 100 µg/mL, methyl metanesulfonate used as positive control at 40 and 60 µM (Mckelvey-Martin et al., 2008; Tice et al., 2000) and DMSO 1% as negative control (solvent). The slides were incubated for 1 h (5% of CO_2/37°C) and kept in a lysis solution (2.5 M NaCl,100 mM Na_2-EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 10 mL of DMSO,1 mL of Triton-X) for at least 30 minutes. After lysis, the slides were transferred to the electrophoresis cube cell containing buffer (200 mg/mL NaOH, 74.45 mg/mL EDTA pH ~10) at 4ºC in a current of 25 V, 300 mA for 30 min. After electrophoresis, the slides were neutralized with Tris-buffer (pH 7.4) and dried at room temperature. Staining was performed with ethidium bromide (20 µg/mL) and observed by fluorescence microscopy using an Olympus CKX41SF2/CKX41-RFA (Olympus, Japan). One hundred nuclei were analyzed per slide and 3 slides were prepared for each sample. The comets were classified into categories of migration according to the degree of DNA damage, as class 0 (undamaged nuclei and without tails), class 1 (with nuclei and with tails less than the diameter of the nucleus), class 2 (core tail length between 1 and 2 times the diameter of the nucleus), class 3 (nuclei with a tail that was more than 2 times the size of the nucleus) and class 4 (total DNA fragmentation). The injury score was calculated as the sum of

the number of cells in each class, by multiplying the value of the score (0 to 4), with the index up to 400 points of damage (Mckelvey-Martin et al., 2008; Tice et al., 2000).

Micronucleus test

The micronucleus test was performed 24 h after administration of ethanol extract of *P. aduncum* at 2 g/kg (p.o.). For the negative control, saline was used (3 mL/kg, p.o.) and for the positive control, methyl methanesulfonate (MMS) (50 mg/kg, i.p.). After 24 h, the right femur of the rats was removed and the epiphyses cut. The spinal canal was washed, using a syringe containing fetal bovine serum. The cells were obtained by centrifugation at 1000 xg/10 min/5°C and the viability obtained by 0.4% trypan blue dye. The slides were prepared using 50 µL of cell suspension, fixed with cold methanol and stained with Giemsa for 10 min. The slides were then washed and dried for examination under a light microscope. One thousand polychromatic cells were assessed per slide. The relationship between monochromatic and polychromatic erythrocytes was also established and evaluated (Mavournin et al., 1990; OECD, 1997).

Mutagenicity on the *S. cerevisiae* strain

The haploid wild-type *S. cerevisiae* strain XV185-14c (MATa, ade2-2, arg4017, his1-7, lys1-1, trp5048, hom3-10) was provided by Dr. R. C. Von Borstel (Genetics Department, Alberta University, Canada). Complete broth medium (YPD), containing 0.5% yeast extract, 2% bactopeptone, and 2% glucose were used for routine growth. The synthetic complete medium (SC) was the minimal medium [0.67% yeast nitrogen base without amino acids, 2% glucose, 2% bacto-agar and 0.25% $(NH_4)_2SO_4$] supplemented with 2% adenine, 5% lysine, 1% histidine, 2% leucine, 2% methionine, 2% uracil, 2% tryptophan and 24% threonine w/v. For the mutagenesis test, the deficient media, lacking lysine (*SC-lys*), histine (*SC-his*) or homoserine (*SC-homo*) were used to confirm these activities. Saline solution (0.9% NaCl) was used to dilute the cell suspension.

Exponential phase cultures ($2x10^7$ cells/mL) were obtained by inoculating the cells that had been removed from the stationary phase and placed in 5 mL of fresh YPD medium. Cells were harvested and washed twice with saline. Both the cell concentration and the percentage of budding cells in each culture were determined using a Neubauer chamber.

A suspension of $2x10^8$ cells/mL of the yeast strain XV185-14c grew until the exponential phase was incubated for 3 h in several different *P. aduncum* extract concentrations (50, 100, 250 and 500 µg/mL). Survival was determined based on SC (3 to 5 days, 28°C) and mutation induction (LYS+, HIS+ or HOMO+ revertants) was performed in appropriate deficient media. While *his 1-7* is a non-suppressible missense allele and reversions result from mutations at the locus itself (Hawthorne and Leopold, 1974), lys 1-1 is a suppressible ochre nonsense mutant allele, which can be reverted either by locus-specific or by forward mutation in a suppressor gene and it is believed that homo 3-10 contains a frameshift mutation due to its response to a range of diagnostic mutagens (Zimmermann, 1975).

Assays were repeated at least three times, with plating done in triplicate for each dose. 4-nitroquinolein-1-oxide (4NQO, 5 µM) was used as mutagenic positive control.

Statistical analysis

The data were expressed as mean ± standard deviation. The values were analyzed using one way analysis of variance (ANOVA), followed by the Dunnett's multiple comparison test where appropriate. p values < 0.05 were considered significant.

RESULTS AND DISCUSSION

The use of plants and herbal preparations in the treatment of diseases is very common in the rural communities of Brazil. Their safety has been demonstrated in long-term usage, with knowledge accumulated over centuries. To further improve the safety of medicinal plants, the investigation of acute toxicity must be the first step in toxicological analysis (Organisation for Economic Co-operation and Developement [OECD], 1997) as well as the use of further sub-chronic, chronic and *in vivo* mutagenicity assays like the micronucleus test.

After oral treatment, by gavage, of female Wistar rats with a high dose of *P. aduncum* extract (2 g/kg), in the acute toxicity fixed test dose, none of the following signs were observed: irritability, alteration in touch response, abnormal hindquarter tone or body tremors, seizures, straub movement, hypnosis, signs of anesthesia, increased or decreased urination or defecation, piloerection or cyanosis, comparable to negative control.

The macroscopic examination of animal viscera was completely normal; after carefully examination, any apparent and significant features or differences from the norm (control) were recorded. The microscopic examination of organs was only performed if the necropsy showed evidence of gross pathology in animals surviving 24 or more hours after the initial dosing. As no sign of pathology was observed during macroscopic analysis, compared to the negative control, histopathological examinations were not performed (OECD, 2001). No significant changes in body weight were observed, compared to the control group, during the 14 days of the experiment.

The rational of OECD 420 (2001) permits an acute toxicological classification, using the globally harmonized system (GSH), without pre-determination of LD50. The GSH classification system suggests that if any mortality was observed when the substance or mixture of substances were tested up to category 4 (2 g/kg), it is classified as category 5 or low acute toxicity with a LD50 superior to 2 g/kg. Also, recognizing the need to protect animal welfare, testing at 5 g/kg is discouraged and should only be considered related to use of the substance to directly protect humans.

The genotoxic potential of *P. aduncum* extract was determined using the Comet assay. This is a sensitive,

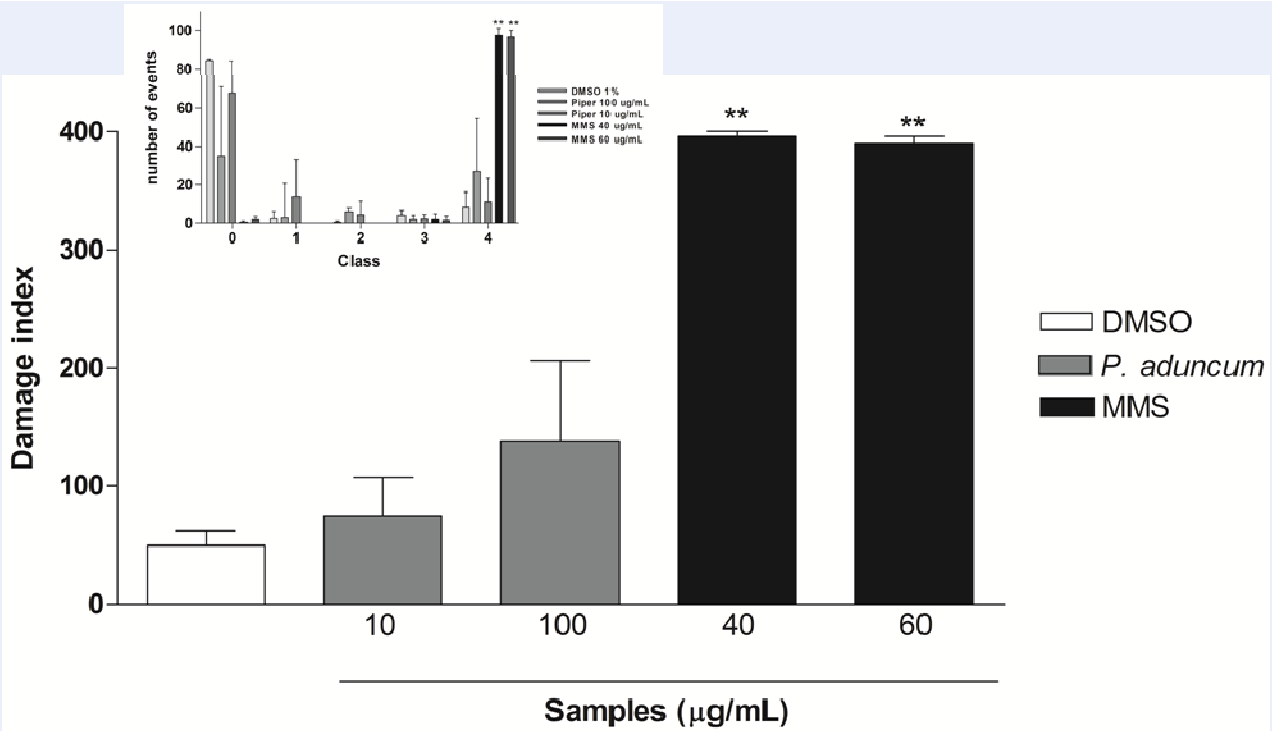

Figure 1. Score of lesions caused by the ethanol extract of *Piper aduncum* (10 and 100 µg/mL) observed in the comet assay, when compared to negative controls and positive. Insert: Number of events caused by the extract of *Piper aduncum* group. ** Significant after analysis of variance (ANOVA) followed by Dunnett test. Asterisks denote statistical differences compared with the negative control (** $p < 0.01$).

reproducible, simple and quick electrophoretic technique for detecting the presence of single strand breaks and DNA lesions in alkali-sensitive sites. It is also an excellent method of evaluating DNA damage in eukaryotic cells and is widely used *in vitro* tests to evaluate the genotoxicity of compounds. Because DNA damage is associated with cell death, it is critical that the highest dose tested not induce excessive cytotoxicity. The cytotoxicity must be evaluated concurrently with each Comet experiment. When measuring viability at the end of exposure period, a general approach has been used to avoid the testing of doses that decrease viability, compared to the concurrent control culture, by more than 30% (Tice et al., 2000), justifying the use of the limit concentration of 100 µg/mL, responsible for a reduction of 20.2 ± 6.4% in cell viability, using tripan blue exclusion test.

The classification of the comet class, from 0 to 1 was predominant in the extract of *P. aduncum* (10 and 100 µg/mL) and the negative control 1% DMSO (Figure 1), indicating a low genotoxicity potential, compared to the negative control. Similar results were also observed by Junqueira et al. (2007), with predominant classes of 0 and 1 for the ethanol extract of *Piper cubeba*. The positive control (MMS), at both concentrations (40 and 60 µg/mL), showed a higher amount of class 4 comets (Figure 1), which indicates an important genotoxic effect.

The damage index (Figure 1) of the extract was not statistically different between the doses of 10 (50 ± 55) and 100 µg/mL (152 ± 119), or when compared to the negative control DMSO (49 ± 17). The positive control MMS 40 µg/mL (396 ± 5) and MMS 60 µM (396 ± 8) presented a statistically significant difference ($p < 0.01$) when compared with the control group.

The low genotoxic and mutagenic potential of the *P. aduncum* extract was confirmed by micronucleus and *S. cerevisiae* assays. The mutagenicity of the extract of *P. aduncum* was evaluated in bone marrow polychromatic erythrocytes of Wistar rats, after 24 h of oral administration and related to the number of micronucleous in these cells. There were no statistically significant differences for the ethanol extract obtained from *P. aduncum* and the negative control (saline). As a percentage of micronuclei (0.9 ± 0.1%) compared to the negative control group (1.3 ± 0.5%) only the positive control group MMS 50 mg/kg (10.0 ± 2.6%), as expected,

Table 1. Percentage of polychromatic erythrocytes (PCE) micronucleous (PCEMN) in the micronucleus test performed with ethanol extract of *Piper aduncum*.

Samples	Dose (mg/kg)	PCEMN/1000 PCE	
		Number	%
Saline		12.7 ± 4.5	1.27 ± 4.5
Piper sp.	2000	9.5± 1.4	0.95 ± 1.4
MMS	50	100.2 ± 25.9	10.02 ± 2.6**

The data are presented as (mean ± standard deviation). ** Significant after analysis of variance (ANOVA) followed by Dunnett test. Asterisks denote statistical differences compared with control (** p <0.01).

Table 2. Reversion of point mutation for (his 1-7), ochre allele (lys 1-1) and frameshift mutations (hom 3-10) in haploid XV185-14c strain of *Saccharomyces cerevisiae* performed with ethanol extract of *Piper aduncum*.

Extract (µg/mL)	Survivor (%)	Revertants *His* 1/10^7 survivors	Revertants *Lys* 1/10^7 survivors	Revertants *Hom* 3/10^7 survivors
0	100	2.50 ± 0.87	3.42 ± 0.65	1.91 ± 0.33
50	98.7	2.73 ± 1.32	3.51 ± 0.65	1.91 ± 0.33
100	99.1	2.82 ± 1.84	3.85 ± 0.83	2.03 ± 0.70
250	96.2	2.95 ± 1.51	4.56 ± 0.54	1.98 ± 0.67
500	90.3	3.21 ± 2.73	5.02 ± 0.71	2.42 ± 0.83
NQO	87.4	215 ± 1.69	45.84 ± 2.92	5.12 ± 1.50

4NQO at 5 µM.

different from the negative (Table 1). There were no significant differences in the ratio polychromatic and monochromatic erythrocytes, indicating that the *P. aduncum* extract presented no cytotoxicity in rat bone marrow cells at the high dose tested. This result, correlated with Comet assay, confirms the low genotoxicity and mutagenicity, in the presence or absence of biological barriers.

If in the micronucleous test, at one dose level of at least 2 g/kg body weight using a single treatment, produces no observable toxic effects, and if genotoxicity would not be expected based upon data from structurally related substances, then a full study using three dose levels may not be considered necessary (Tice et al., 2000).

The extract of *P. aduncum* was tested for its mutagenic effect in the haploid strain of *S. cerevisiae* (XV185-14c) and it was verified that the extract did not induce mutations to lysine and histidine (a locus-specific mutation) and homoserine (frameshift events), and also did not reduce cell viability compared to the negative control, as shown in Table 2. The negative control showed 100% cell viability during the test and the number of revertants His (1/10^7 survivors); Lys (1/10^7 survivors) and Hom (3/10^7 survivors) were 2.5 ± 0.87; 3.42 ± 0.65 and 1.91 ± 0.33, respectively. The positive control (NQO), showed cell viability of 87.4% and increased mutation of revertants of 215.53 ± 1.69; 45.84 ± 2.92 and 5.12 ± 1.50, respectively.

In conclusion, the *P. aduncum* ethanol extract was classified as having a low acute toxic effect on female rats, with no evidence of behavioral or anatomic modification. *In vitro* genotoxic potential by the single cell electrophoresis (comet assay) on bone marrow cells was negative and the *in vivo* mutagenicity by micronucleus on bone marrow cells and *in vitro S. cerevisiae* strain mutation was not observed, confirming low mutagenicity.

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