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Original Article

# Anti-inflammatory activity and acute toxicity studies of hydroalcoholic extract of *Herissantia tiubae*

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ABSTRACT

Hydroalcoholic extract of aerial parts of *Herissantia tiubae* (K. Schum.) Brizicky, Malvaceae, was evaluated in experimental models of inflammation and toxicity. For toxicity assays, male and female Swiss mice were orally treated with hydroalcoholic extract of *H. tiubae* (2000 mg/kg) and analyzed by consumption of water and food, body weight, mortality and rates of major organ weights, as well as biochemical and hematological indexes. For anti-inflammatory effect, phlogistic agents such as carrageenan or acetic acid were used to evaluate paw edema, cell migration and cytokine production. It was also investigated the hydroalcoholic extract of *H. tiubae* in RAW 264.7 macrophage lineage by nitric oxide and cytokine productions. Swiss mice treated with hydroalcoholic extract of *H. tiubae* showed low toxicity and (50 or 100 mg/kg) was able to reduce significantly ($p < 0.01$, $p < 0.001$) polymorphonuclear cell migration, TNF-α and IL-1β production in the carrageenan-induced peritonitis. However the hydroalcoholic extract of *H. tiubae* (50, 100 or 200 mg/kg) did not reduce carrageenan-induced paw edema. Additionally, hydroalcoholic extract of *H. tiubae* did not present cytotoxicity at concentrations of 6.25, 12.5, 25 or 50 μg/ml but induced significantly decrease of NO, TNF-α and IL-6 production in macrophage lineage. This study suggests that hydroalcoholic extract of *H. tiubae* has anti-inflammatory activity by inhibiting cell migration mainly by decreasing the inflammatory cytokine levels at the inflamed site independently of the anti-edematogenic effect.

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## Introduction

Malvaceae family has a wide variety of natural compounds with pharmacological properties such as anti-inflammatory, analgesic, anti-rheumatic, among others (Falcão-Silva et al., 2009). *Herissantia tiubae* K. Schum.) Brizicky is one of the species of this botanic family largely found in tropical regions of South America, especially in northeastern Brazil and it is popularly known as "mela-bode" or "lava-prato". The plant is used in folk medicine to treat influenza and fever (Albuquerque et al., 2007). Phytochemical investigation of *H. tiubae* demonstrated the presence of polyoxygen flavonoids, triterpenes, steroid, phenolic compounds and two glycosylated flavonoids (kaempferol 7-*O*-α-L-rhamnopyranoside and 4′,5-dihydroxy-3,6,7,8,3′-pentamethoxyflavone) (Silva et al., 2009). Therefore, the aim of this study was to investigate the anti-inflammatory activity of the hydroalcoholic extract of the aero parts of the *H. tiubae* (HtE) and its toxicity using different experimental models.

## Material and methods

### *Animals*

Male and female Swiss mice ($n = 6$/per group, 6–8 weeks, 25–30 g) were used throughout the study. The animals were provided from Prof. Thomas George Vivarium of the Biotechnology Center (CBiotec) from Federal University of Paraíba (UFPB), PB, Brazil. All experimental protocols were approved and performed in accordance with the recommendations of Commission of Ethics for Use of Animals (CEUA) from UFPB, which was recorded under number 0508/12. Animals were kept in polypropylene cage, at room

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temperature (25 ± 2 °C), under 12 h light/dark cycle, and free access to food and water.

*Plant material and preparation of hydroalcoholic extract*

Aerial parts of *Herissantia tiubae* (K. Schum.) Brizicky, Malvaceae, were collected in January 2010 in the city of Juazeirinho, Paraiba, Brazil. It was identified by Dr. Maria de Fatima Agra from UFPB. A voucher specimen (n° 2434) is deposited in the Herbarium Lauro Pires Xavier – JPB at the same University. The aerial parts of *H. tiubae* (1 kg) were dried at 40 °C in a circulating air oven for 96 h and ground to powder. Dried and powdered plant material was submitted to extraction by maceration with ethanol–water (70:30) as a solvent at room temperature for 72 h. The ratio of plant material:solvent was 20:80 (w/v) and at the final extraction process the material was filtered and concentrated in rotaevaporator, thus obtaining the HtE.

*Chromatography of the HtE*

HtE was successively partitioned with hexane, $CHCl_3$, EtOAc and butanol. Kaempferol was isolated from the ethyl acetate extract and subjected to a Sephadex LH-20 gel column eluted with MeOH. Kaempferol was quantified by means of High Performance Liquid Chromatography (HPLC) with ultraviolet detection. Calibration curves to kaempferol were constructed by using the standard addition method. The separation of kaempferol was achieved using a Prominence Chromatographic System (Shimadzu®, Tokyo, Japan) equipped with LC-20AT multi solvent delivery system, degassing system DGU-20A5, autoinjector SIL-20A, oven CTO-20A column and detection by electron spectroscopy in the ultraviolet-visible region with diode array SPD-M20A UV-VIS. Data were collected and integrated through software Class VP V6.14 SP1. The mobile phase consisted of a mixture of methanol:water:$H_3PO_4$ (1:1:0.01, v/v) pH controlled at 3.1 and the flow rate of 1.2 ml/min in the gradient mode, where the proportion of the organic phase constitutions by 47% for 18 min, from 80% in 23 min and returning to 47% after 28 min. To perform the chromatographic runs, we used a C18 column (Phenomenex®) dimensions 25 cm × 4.6 mm × 5 µm, the UV detector with the wavelength of 351 nm, injection volume of 10 µl temperature 50 °C.

*Treatment with HtE*

For *in vivo* experiments, the HtE in 2% Tween 20 (Vetec®) and distilled water (vehicle) was orally (*p.o.*) administered at doses of 50, 100 or 200 mg/kg. The untreated control group received an equal volume of the vehicle. For *in vitro* experiments, the HtE was dissolved in dimethylsulfoxide (DMSO), the stock solution was sterilized using a disposable filter unit of 0.22 mm in porosity (Millipore Millex™) and used in the follow range of concentrations 0, 6.25, 12.5, 25, 50, 100, 200 or 400 µg/ml.

*Acute toxicological test*

Groups of male and female ($n = 6$) Swiss mice were treated orally with HtE (2000 mg/kg) or vehicle. The animals were observed for signs of general toxicity in intervals of 0, 15, 30 and 60 min, 4 and 24 h later and daily for 14 days (Hibbs et al., 1988). During these times, occurrence of central nervous system changes was analyzed: hyperactivity, irritability, aggressiveness, tremors, convulsions, catatonia, analgesia, anesthesia, ptosis, decreased touch response, ambulation, cleaning capacity, raise, and autonomic nervous system changes: diarrhea, constipation, defecation, urination, muscle tone, among others (Almeida et al., 1999). Throughout the experiment, the consumption of water and food intake and weight gain were observed. On day 14$^{th}$, the treated animals and non-treated animals were euthanized by anesthetic: sodium thiopental (Thiopentax R, Cristalia – Pharmaceutical Chemicals) and organs were removed: heart, liver, kidneys, spleen and thymus to determine its indexes. The weight gain for each animal was determined using the formula:

$$\%\text{ of weight gain} = \left(\frac{\text{animal weight on first challenge}}{\text{animal weight on last challenge}}\right) - 1 \times 100$$

The index of the weight organs was calculated following the formula below:

$$\text{Index} = \frac{\text{organ weight (mg)}}{\text{animal weight (g)}}$$

*Evaluation of biochemical and hematological parameters*

On the 14$^{th}$ day animals fasted for 6 h were anesthetized with sodium thiopental and orbital sinus blood was collected using a heparinized Pasteur pipette and transferred into tubes (Eppendorf). The blood was analyzed for hematological (erythrocyte and leukocyte counts) and biochemical parameters (urea, creatinine, uric acid, alanine transaminase-ALT, aspartate transaminase-AST, albumin, total protein, triacylglycerides, glucose and total cholesterol).

*Carrageenan-induced mice paw edema*

Groups of Swiss mice ($n = 6$) were treated (*p.o.*) with vehicle, indomethacin (10 mg/kg-Roche®) or HtE (50, 100 or 200 mg/kg) 1 h before administration of carrageenan at 2.5% (Sigma-Aldrich®) injected subcutaneously into the plantar region of the left hind paw and phosphate buffer saline (PBS) in right hind paw. Negative control group received 20 µl PBS injections in both paws. Paw diameter was measured with a digital micrometer at 1, 2, 3, 4, 6 and 24 h after stimulation. Results were expressed as difference between the diameter of left and right paws (De Vasconcelos et al., 2011).

*Carrageenan induced peritonitis*

Mice ($n = 6$) were orally treated with HtE (50 or 100 mg/kg), indomethacin 10 mg/kg or vehicle 1 h before carrageenan (1%) intraperitoneal injection. The basal group received saline. After 4 h the animals were euthanized by xylazine and ketamine overdose and the peritoneal cavity washed with 2 ml of sterile cold PBS, followed by a one-min massage and collection of the fluid (Guerra et al., 2011; Pinheiro et al., 2013). Exudates were centrifuged (10 min, 266 g, 4 °C) and the pellet of cells resuspended in 1 ml of PBS (4 °C), diluted in Turk solution in the ratio of 1:40 and total cells were counted in at Neubauer camera under optical microscope (Nikon E200, Melville, NY – EUA). Differential cell measurement was made in cytocentrifuge – 254 × g, 15 min (Cytospin – Bio Research, Washington – USA), slide stained in Fast Panoptic (RenyLab) and counted under optical microscope (100× objective). For each slide a minimum of 100 cells were counted in optical microscope under 1000 magnification (Sousa et al., 2010).

*Cytotoxic assay*

RAW 264.7 macrophage lineage (ATCC®, Rockville, MD, USA) was cultured in RPMI-1640 medium (streptomycin 10 mg/ml; penicillin 6 mg/ml, kanamycin 2 mg/ml, fetal bovine serum 10% – Gibco®, without phenol red). The cytotoxic effect of the extract were evaluated by MTT assay as first described by Mosmann (1983) with the modifications suggested by Denizot and Lang (1986). Cells were seeded in 96-well plates ($2 \times 10^5$ cells/well) and incubated for 4 h. After this period, cells were treated with HtE (0, 6.25, 12.5,

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