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Evaluation of acute toxicity, antibacterial activity, and mode of action of the hydroethanolic extract of *Piper umbellatum* L

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ABSTRACT

Ethnopharmacological relevance: *Piper umbellatum* L., Piperaceae, is a shrub that grows up to 3 m high. It is commonly known as "capeba" or "pariparoba" in Brazil. Tea prepared using the leaves of this plant is employed in the treatment of infections and inflammatory processes in different countries. Approximately 50 compounds, notably from the flavonoid, alkaloid, terpene, and sterol classes, have been isolated from the leaves of *Piper umbellatum*. To evaluate the acute toxicity, antibacterial activity, and mode of action of the hydroethanolic extract of *Piper umbellatum* leaves (HEPu).

Materials and methods: Acute toxicity of HEPu against CHO-K1 cells was evaluated using a cytotoxicity assay with Alamar Blue and that against mice was assessed by the Hippocratic test. Antibacterial activity of HEPu was tested using the broth microdilution method using a panel of clinically relevant bacteria, and the effects of HEPu on the bacterial membrane were analyzed in detail. A preliminary phytochemical analysis based on coloration/precipitation was performed according to procedure described in the literature. Secondary metabolites detected were analyzed and confirmed by thin layer chromatography (TLC), spectrophotometry, and high performance liquid chromatography (HPLC).

Results: *Piper umbellatum* did not appear to be toxic in the *in vitro* ($IC_{50} > 200$ μg/mL) cytotoxicity test. When administered *in vivo* at doses up to 2000 mg/kg p.o., HEPu did not cause any signs or symptoms of toxicity in mice. It demonstrated a good spectrum of antibacterial activity and its mode of action appeared to be associated with changes in the permeability of bacterial membranes; it led to increased entry of hydrophobic antibiotics, efflux of K^+, and nucleotide leakage. Preliminary phytochemical analysis revealed the presence of flavonoids, alkaloids, terpenes, and sterols in the extract. Spectrophotometric and HPLC analysis revealed the presence of the flavonoids rutin and quercetin.

Conclusion: In summary, HEPu has antibacterial activity and low acute toxicity *in vitro* and *in vivo*. Its mode of action appears to be associated with changes in the permeability of the bacterial cell wall and cytoplasmic membrane, which can at least be partly attributed to the flavonoids present in the extract.

1. Introduction

The effects of plant extracts on microorganisms have been studied and screened by many researchers in drug discovery studies in different parts of the world (Savoia, 2012; Zhang et al., 2013).

The interest in plants with antimicrobial properties has been revived because of the current problems associated with a reduction in the number of potential new antimicrobial drugs, an increase in antimicrobial resistance, and the need for treatments for recently emerging pathogens (Mahady, 2005).

From the rich biodiversity of the Amazon region of Brazil, *Piper umbellatum* L., Piperaceae, commonly known as "capeba" or "pariparoba", was analyzed in this study. This species is a perennial scrambling shrub or woody herb, 1–3 m tall, and is widely used as folk medicine in several countries. The leaves in particular have been used to create an infusion for the treatment of infectious and inflammatory diseases (Roersch, 2010).

Previous studies have shown that extracts and pure compounds derived from various parts of *Piper umbellatum* possess a wide spectrum of pharmacological activities, including antibacterial,

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antifungal, antioxidant, anti-atherogenic, cytotoxic, antimalarial, analgesic, anti-inflammatory, anti-leishmanial, and antitrypanosomal activities (Roersch, 2010; Agbor et al., 2012). However, to the best of our knowledge, there has been no published report investigating the antibacterial properties of the hydroethanolic extract of *Piper umbellatum* leaves (HEPu).

Phytochemical studies of *Piper umbellatum* have demonstrated the presence of terpenes (mainly found in the essential oil), alkaloids, flavonoids, sterols, and other classes of secondary metabolites (Roersch, 2010). The aim of this work was to evaluate the acute toxicity, antibacterial activity, and mode of action of HEPu.

2. Materials and methods

2.1. Animals

Adult male Swiss-Webster mice (25–30 g) from the Central Animal House of UFMT were used for this study. The animals were kept in propylene cages at 22 ± 2 °C with a 12 h light/dark cycle, with free access to standard Purina® Chow (Labina, Goiás, Brazil) and water *ad libitum*. They were allowed to acclimatize to the laboratory environment in this condition for 48 h. Groups of 6–10 animals were used for the experiments. The experimental protocols (number 23108.043016/10-6) were approved by the Ethic Committee for Animal Experimentation of UFMT, Brazil, in accordance with the Federal Government Legislation on Animal Care.

2.2. Microorganisms

Antibacterial activity was studied using microorganisms from the American Type Culture Collection (ATCC, Rockville, MD, USA). The ATCC bacterial strains used were *Staphylococcus aureus* 25923, *Staphylococcus epidermidis* 12228, *Streptococcus pyogenes* 19615, *Enterococcus faecalis* 29212, *Salmonella typhimurium* 14028, *Pseudomonas aeruginosa* 27853, *Shigella flexneri* 12022, *Klebsiella pneumoniae* 13883, *and Escherichia coli* 25922. The strains were maintained on slopes of Skim Milk (Oxoid), stored at −20 °C, and subcultured two days before the assays to prevent morphological and metabolic transformations.

2.3. Cell line

Epithelial cells of Chinese hamster ovary (CHO-K1) from the Rio de Janeiro Cell Bank were used to investigate the cytotoxic effects of HEPu. This cell line was grown in Dulbecco's Modified Eagle Medium (DMEM)+Ham's Nutrient Mixtures F10 (HAM F10) supplemented with 10% (v/v) fetal bovine serum (FBS) in tissue culture flasks. The cells were subcultured in fresh medium twice a week and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.4. Plant collection and extract preparation

Piper umbellatum leaves were collected in the Aripuanã Municipality of Mato Grosso State, Brazil, and the botanical identity of the plant was confirmed by Célia Regina Araújo Soares of the Herbarium of Amazonia Meridional in Alta Floresta-MT, Brazil. The *Piper umbellatum* species is not on the endangered species list and neither it was in the federal conservation area, and therefore required no permission to collect, according to Brazilian legislation [Art. 10 of 154/07 Normative Instruction of the Brazilian Institute of Environment and Natural Resources (IBAMA)]. A voucher specimen with number BI 970 was deposited at the same Herbarium.

The leaves were collected, cleaned, and dried to constant weight at 40 °C for 3 d. The dried leaves were shredded in an electric mill with a sieve having a mesh size of 40 (model TE-625 TECNAL; Piracicaba, SP). The dried powder was macerated with 75% ethanol (1:3 w/v) at 25 °C for 7 d. The macerate was filtered and concentrated under reduced pressure at 40 °C in a rotary evaporator to obtain HEPu. The residual solvent was eliminated in an incubator at 40 °C. The extract was kept protected from light and stored at 4 °C; it was solubilized in distilled water containing 2% dimethylsulfoxide (DMSO) before each test.

2.5. Phytochemical analysis

2.5.1. Phytochemical screening

Preliminary phytochemical analysis was carried out according to previously described methods (Matos, 1998), based mainly on coloration/precipitation tests. The secondary metabolites detected were confirmed by thin layer chromatography (TLC), spectrophotometric, and high performance liquid chromatography (HPLC) analyses. Q3

2.5.2. Determination of total flavonoid content

2.5.2.1. Spectrophotometric colorimetric assay. Total flavonoid content was determined using a spectrophotometric colorimetric assay as described by Peixoto Sobrinho et al. (2008) with slight modifications. Briefly, 200 μL of the diluted extract (1000 μg/mL) was transferred to test tubes. Subsequently, 400 μL of glacial acetic acid (60%), 2 mL of pyridine (20%), 1 mL of aluminum chloride (5%) and 6.4 mL of Milli-Q water were added to the tube. The samples remained in the dark for 30 min at room temperature. The absorbance of the mixture was measured in a spectrophotometer (WPA®, model Biwave II) at 420 nm against a blank prepared with Milli-Q water. The assay was performed in triplicate, and the flavonoid content was determined by interpolating the absorbance of the samples against a calibration curve constructed with rutin standard (1.0–5.0 μg/mL) and expressed as milligrams of rutin equivalent per gram of extract (mg RE/g).

2.5.2.2. HPLC analysis. The experiments were performed with an HPLC Shimadzu chromatograph model LC-10 Avp Series equipped with an LC-10AD pump, DGU-14A degasser, UV–vis (SPD–10A) detector, column oven (CTO-10A) equipped with manual injection Rheodyne (loop, 20 μL), and CLASS LC-10 integrator. The sample was analyzed using a reverse-phase Phenomenex Luna 5 μm C18 (2) (250 × 4.6 mm^2) column with direct-connect C18 Phenomenex Security Guard Cartridges (4 × 3.0 mm^2) filled with similar material as the main column. Chromatographic separation was undertaken via isocratic elution with a mobile phase consisting of 0.1% formic acid in Milli-Q water and methanol (45:55). The flow rate eluent was 1.0 mL/min at 40 °C with UV detection at 340 nm for a duration of 30 min. The standards used were rutin (Acros® 132391000) and quercetin (Sigma®, Q0125). The content of the compounds was expressed as micrograms per milligram of extract, which was calculated after correlating the area of the analyte with the calibration curves of the standards constructed at concentrations of 125, 250, and 500 μg/mL. The quercetin solution was prepared with methanol, and the solutions of the extract and rutin were prepared with the mobile phase used for elution.

2.6. Acute toxicity assays

2.6.1. Hippocratic screening

Male mice received HEPu (p.o) at doses of 500, 1000, and 2000 mg/kg body weight. Three animals were used for each dose of the extract and one control animal received the vehicle

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