Cytotoxic, mutagenic and genotoxic evaluation of crude extracts and fractions from *Piper jericoense* with trypanocidal action

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ABSTRACT

The current Chagas disease treatment is based on two drugs, nifurtimox and benznidazole, which is considered unsatisfactory, not only because of the narrow therapeutic range but also because of the associated toxicity. Natural products are considered an important source of biologically active compounds against various infectious organisms. Numerous *Piper* species are used in traditional medicine to treat parasitic diseases. In this paper, we study the activity of extracts and fractions obtained from *Piper jericoense* plant against epimastigote, trypomastigote and amastigote forms of *Trypanosoma cruzi*. In addition, we evaluated the cytotoxic, mutagenic and genotoxic activities of the F4 fraction obtained from one of the more promising extracts. We obtained four extracts, one of which presented low toxicity and high trypanocidal activity. This extract was separated into eight fractions, and the F4 fraction presented better results than the other extracts and had a higher selectivity index than the reference drug, benznidazole. This fraction was not cytotoxic, mutagenic or genotoxic.

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1. Introduction

Chagas disease caused by *Trypanosoma cruzi* affects 17 million people worldwide (Moncayo and Silveira, 2009). The current Chagas disease treatment is based on two drugs, nifurtimox (nfx) and benznidazole (bz). However, their use is characterized by toxicity, and their efficacy against chronic stage disease is unreliable (Castro et al., 2006). Therefore, there is an urgent need to search for novel, effective and non-toxic drugs for Chagas disease treatment.

Natural products, especially those derived from plants, are considered an important source of biologically active compounds against various infectious organisms, especially parasites. Many studies related to the activity of plant extracts *in vitro* against *T. cruzi* have been published. Numerous *Piper* species are used in traditional medicine to treat parasitic diseases and more specifically to treat *T. cruzi* (Da Silva Mota et al., 2009; Luize et al., 2006; Martins et al., 2003). Although a few reports on the toxicity of this class of compounds have been published (Flores et al., 2000; Lopes et al., 2008), the effect of these extracts and compounds on cell viability and DNA damage has not been studied extensively.

To contribute to the development of new chemotherapeutic agents, the aim of this study was to evaluate the *in vitro* biological activity of extracts and fractions from *Piper jericoense* plant against epimastigote, trypomastigote and amastigote forms of *T. cruzi* parasites and to compare the extracts' activity with the reference drug, benznidazole. We also evaluated the mutagenic, cytotoxic and genotoxic activity of one of the main components obtained from this plant.

2. Materials and methods

2.1. Plant materials

Leaves of *P. jericoense* were collected in locations where the plants grow naturally in Puerto Triunfo (Antioquia), Colombia. Taxonomic characterization was performed in the Herbarium of the University of Antioquia, Colombia, where the voucher specimen is located.

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2.2. Obtaining and preparation of extracts

Approximately 100 g of leaves from the plant were dried at room temperature for 10 d, pulverized and subjected to exhaustive, successive extraction with petroleum ether, dichloromethane, ethyl acetate and methanol in a percolator at room temperature and concentrated in a vacuum to give the corresponding extract. All extracts were monitored by thin layer chromatography periodically on silica gel chromatoplates (Merck®).

The dried extracts were solubilized in dimethyl sulfoxide (DMSO 1%), sonicated for 30 min and diluted to the required concentration for the specific test to be performed.

2.3. Fractionation of the crude extract obtained from ethyl acetate Piper jericoense

The ethyl acetate extract obtained from *P. jericoense* was subjected to silica gel column chromatography, eluting with a step-gradient of petroleum ether-ethyl acetate (0–100%), to obtain eight fractions (F1–F8) collected according to their thin layer chromatography (TLC) profiles. This extract was selected because of its promising activity against *Trypanosoma* and low cytotoxicity (see Section 3).

2.4. In vitro trypanocidal activity

2.4.1. Epimastigotes

Epimastigote cells from four Colombian strains of *T. cruzi*, genotyped as TC I, were cultivated by successive subcultures every 7 d at 28 °C in liver infusion tryptose (LIT) medium supplemented with 10% FBS (fetal bovine serum) (Camargo, 1964). Parasites in logarithmic growth phase were distributed in 96-well, flat-bottom microtiter plates at a concentration of 5×10^6 cells/mL. Each well was incubated for 72 h with increasing concentrations of the extract and fractions in the range 3.1–100 µg/mL dissolved in DMSO. The evaluation of the extract and fractions was performed according to the methodology described by Zapata (2004) using the enzymatic MTT micromethod (Weniger et al., 2001). Untreated parasites and parasites treated with 1% DMSO but maintained under the same conditions were used as controls. Benznidazole was used as the reference drug. Two independent assays were carried out in triplicate. The inhibitory concentration (IC_{50}) was calculated using GraphPad PRISM v5.01.

2.4.2. Trypomastigotes

DA strain trypomastigote forms were obtained by infection of the Vero cells with parasite obtained from 30-day-old cultures, according to the protocol established by Contreras et al. (1999). After 24 h, the parasites that did not infect cells were removed by successive washing. Cells were maintained until they obtained trypomastigote forms or until they reached their maximum confluence. One hundred µL of trypomastigotes were inoculated intraperitoneally in young male mice (20 days old, Swiss strain). Infected blood was collected at the maximum peak of parasitemia, which was determined by counting parasites following the method described by Brener (1962). Infected blood was diluted with uninfected blood to a concentration of 5×10^5 parasites/well. The F4 fraction was evaluated in triplicate in 96-well plates at a final volume of 200 µL/well. DMSO and PBS were used as negative controls, while benznidazole was used as the reference drug. The plate was incubated at 4 °C; after 24 h, the number of trypomastigotes was counted using a Neubauer chamber. The activity of the extract and fraction was expressed as the percentage of trypomastigote deaths.

2.4.3. Amastigotes

Trypomastigotes were obtained as described previously. Vero cells were seeded at a density of 5×10^4 cells/well in 24-well plates with a round coverslip at the bottom of each well. Subsequently, 5×10^5 trypomastigotes were added to each well with fresh medium to a final volume of 2 mL. After cell infection, the culture medium was removed and the crude extracts were added to a non-toxic concentration. After 48 h, the coverslips were fixed and stained with Giemsa stain (Sigma®). The number of amastigotes/100 cells was estimated. All the assays were performed in triplicate.

2.5. Cytotoxicity assays

Cytotoxicity of the crude extracts and fractions was determined in three cell lines and in human lymphocytes. U937 cells were cultivated in RPMI (Roswell Park Memorial Institute) 1640 Sigma®, and HepG2 and Vero cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) Sigma® (Mendonça et al., 1999). For the assays, 10^4 cells/well were seeded onto test plates containing the dilutions of the crude extracts and fractions and incubated at 37 °C and 5% CO_2 for 72 h. For each assay, the conventional drug benznidazole was included. Each evaluation was assessed in triplicate. After the incubation period, cell viability was assessed by the MTT reduction method as described by Weniger et al. (2001). Data were analyzed using GraphPad PRISM version 5.01. The results were expressed as the percent reduction in cell viability compared to the control. Two trials were conducted separately under the same conditions in the laboratory. We calculated the Pearson coefficient of variation (%CV) for each extract (Reed et al., 2002).

For cytotoxicity in lymphocytes, lymphocytes were extracted from 5 mL of whole blood from healthy, adult volunteers. Subsequently, a 1:1 mixture was made of PBS free of Ca^{2+} and Mg^{2+}, and cell viability was assessed by the trypan blue exclusion method as described by Sarma et al. (2000). For the trial, we used the samples that maintained a cell viability greater than 85% during pretreatment (Sarma et al., 2000). A total of 5×10^5 cells were consecutively incubated with each concentration of treatment for 3 and 12 h, and the post-treatment viability was measured for those doses that maintained viability above 85% (Henderson et al., 1998).

2.6. Alkaline single-cell microgel-electrophoresis (comet assay)

For this procedure, we followed the methodology proposed by Singh et al. (1988) with some modifications. A total of 5×10^4 lymphocytes were incubated at 37 °C for 1 h in the presence of the extract to be tested in two concentrations, one which retained 85% viability of lymphocytes (C1) and half of this (C2) in a final volume of 250 µL PBS. As a negative control, DMSO was used at a final concentration not exceeding 1% and 100 mM H_2O_2 was used as positive control. Cells were observed using a Nikon® fluorescence microscope using a green filter at a wavelength of 540 nm and 40× magnification. Three independent assays were each performed in duplicate. For each treatment, 150 cells were analyzed. DNA damage was measured based on the length (µm) of the DNA fragments' migration and quantified into four categories based on standard deviations calculated from the migration length in µm as follows: category 1, 0 to 25 µm (more than 1 SD negative control), without damage; category 2, 26 to 50 µm, low damage; category 3, 51 to 75 µm, moderate damage; and category 4, greater than 76 µm, high damage (Brugés and Reguero-Reza, 2008). For comparative purposes, we determined the weighted damage index (WDI) calculated as WDI = 1n1 + 2n2 + 3n3 + 4n4, where n1 is the number of cells classified as "no damage", n2 is the number cells with low damage, n3 is the number of cells with moderate damage, and n4 is the number of cells with high damage (Brugés and Reguero-Reza, 2008)).