Biological effects of extracts obtained from *Stryphnodendron adstringens* on *Herpetomonas samuelpessoai*

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We report the effect of Stryphnodendron adstringens *on the trypanosomatid* Herpetomonas samuelpessoai. *The parasites were grown at 28ºC in a chemically defined medium containing crude extract and fractions at concentrations from 100 to 5000 µg/ml obtained from* S. adstringens. *Concentrations of 500, 1000, 2500, and 5000 µg/ml both crude extract and semi-purified fraction progressively inhibited the protozoans' growth. At a concentration of 100 µg/ml, crude extract or a semi-purified (F3) fraction did not affect the growth of the protozoans. The F3-9 - F3-12 sub-fractions, at a concentration of 1000 µg/ml, also showed increased inhibitory activity on* H. samuelpessoai. *The* IC_{50} *of the crude extract and the F3 fraction were 538 and 634 µg/ml, respectively. Ultrastructural and enzymatic alterations in the trypanosomatids were also evaluated.* H. samuelpessoai *cultivated in the presence of* IC_{50} *crude extract showed considerable ultrastructural alterations, such as marked mitochondrial swelling with a large number of cristae and evident Golgi complex vesiculation, as observed by transmission electron microscopy. Cells exposed to 538 µg/ml of crude extract at 28ºC for 72 h, showed decreased activity of the enzyme succinate cytochrome* c *reductase, a typical mitochondrion marker, as compared to untreated cells*

Key words: *Herpetomonas samuelpessoai* - *Stryphnodendron adstringens* - ultrastructural alteration - antiprotozoan activity

Stryphnodendron adstringens (Mart.) Coville (Leguminosae), commonly known as "barbatimão", is a medicinal plant widely distributed in the savannah region of Brazil (Felfili et al. 1999). The stem bark of this plant contains a considerable amount of tannin (10-37%) and prominent presence of several flavan-3-ols, proanthocyanidins, and prorobinetinidins (Teixeira et al. 1990, Mello et al. 1996a, b). The crude extract, in the form of decoction or infusion, is traditionally used by the local population for the treatment of leucorrhoea and diarrhea, as well as an anti-inflammatory and healing agent (Santos et al. 1987, Mello et al. 1996a). Some studies of the crude extract from the stem bark of *S. adstringens* have revealed significant anti-inflammatory activity and gastric anti-ulcerogenic effects (Lima et al. 1998, Audi et al. 1999). It has also been observed that a decoction of the stem bark of *S. barbatiman* (syn. *S. adstringens*) accelerates the healing of wounds and decreases inflammation (Panizza et al. 1988). Crude extracts from the bark of *S. adstringens* have been tested for antiparasitic activity against the causative agent of Chagas disease, *Trypanosoma cruzi* (Herzog-Soares et al. 2002).

Previous studies have shown that the crude extract of "barbatimão" has an inhibitory activity on the growth of *Herpetomonas samuelpessoai* (Endo et al. 2000, Holetz et al. 2002), a non-pathogenic trypanosomatid isolated from the predatory insect *Zelus leucogrammus* (Hemiptera: Reduviidae) (Galvão et al. 1970). This protozoan can be cultivated in synthetic medium (Roitman et al. 1972), is sensitive to some drugs active against *T. cruzi* (Roitman & Roitman 1972), and has been used as a model to study the biology of trypanosomatids by several research groups in Brazil. Thus, *H. samuelpessoai* may be suitable as a model for screening new trypanocidal drugs.

In view of the therapeutic activity of crude extracts of *S. adstringens,* which justifies the extensive use of this plant in Brazilian folk medicine, the present study was undertaken to investigate the influence of the crude extract and fractions of "barbatimão" on morphological and biochemical alterations in *H. samuelpessoai*.

MATERIALS AND METHODS

Preparation of crude plant extract and fractionation - *S. adstringens* bark was collected during November 1999 from São Jerônimo da Serra, state of Paraná, Brazil. A voucher herbarium specimen was deposited under number HUM 3800 in the Herbarium of the State University of Maringá, Paraná, Brazil.

The bark (120 g) was dried in the dark at room temperature, powdered, and extracted by turbo-extraction in 70% acetone. Afterwards, the crude acetonic extract was evaporated under reduced pressure to yield the residue F1 (50 g). The active crude extract was re-dissolved in water and partitioned with ethyl-acetate to obtain the water-soluble fraction (F2) and the ethyl-acetate-soluble

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fraction (F3). The fraction F3 (20 g) was subjected to column chromatography on Sephadex® LH-20 with a gradient of ethanol-water 1:1, ethanol (100%), and acetone-water 7:3. Twelve sub-fractions (F3-1 - F3-12) were collected, concentrated in vacuum, and freeze-dried.

Microbial culture growth conditions and antiprotozoal activity - *H. samuelpessoai* (ATCC 30252) was grown in a chemically defined medium at 28ºC for 48 h and maintained at 4ºC. Protozoans in the logarithmic growth phase, at a concentration of 10^6 cells/ml, were incubated in defined medium in the presence of 100, 500, 1000, 2500, and 5000 µg/ml of crude extract or F3 fraction, and 1000 µg/ml of sub-fractions F3-1 - F3-12, which were added only once to the cultures. After 24, 48, 72, and 96 h at 28ºC, cell growth was estimated by counting in a Neubauer's chamber. All experiments were performed in triplicate. The IC_{50} values were determined by linear regression analysis from this inhibition percentage using statistic error limits up 10%.

Ultrastructural analysis - In order to analyse the influence of "barbatimão" on the ultrastructure of the protozoans, the cells were treated with 538 µg/ml of crude extract for 72 h at 28ºC, washed in PBS pH 7.2 0.01 M, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4ºC. The cells were washed three times with 0.1 M sodium cacodylate buffer, and postfixation was performed in 1% osmium tetroxide plus 0.8% potassium ferrocyanide and 5 mM calcium chloride for 30 min at room temperature in the dark. After postfixation, the samples were dehydrated in acetone and embedded in Epon. Sections (60-80 nm thick) were cut using a Reichert Ultracut E ultramicrotome with a diamond knife, and ribbons were collected on copper grids. After drying, the grids were stained with 5% (w/v) aqueous uranyl acetate and lead citrate, and ultrathin sections were observed in a Zeiss CEM – 900 electron microscope.

Biochemical analysis - Untreated and treated cells (with 538 µg/ml of crude extract of *S. adstringens*) were incubated at 28ºC for 72 h, and harvested by centrifugation in a Sorvall Super 21 (SL50T Rotor) refrigerated ultracentrifuge at 1000 g for 10 min. The cells were then washed three times in phosphate buffer saline (PBS) pH 7.2, resuspended in a hypotonic solution (Tris-HCl 10 mM) containing a cocktail of protease inhibitors (antipain, aprotinin, leupeptin, and pepstatin: 10 µg/ml of each), and disrupted by sonication on ice with 20 cycles of 2 s with 1 s rest between cycles in an Ultrasonic Processor (CV 33 Model). The cell disruption was monitored with a light microscope in order to avoid damage to the nuclei. The amount of cell-free extract used in individual experiments corresponded to 200 µg protein. The protein concentration was determined by a Bio-Rad protein assay kit, using bovine serum albumin as a standard and following the manufacturer's instructions.

Succinate cytochrome *c* reductase activity was measured according to Morgado-Díaz et al. (2001). The reaction mixture contained 0.2 M phosphate buffer pH 7.4; 0.003 M EDTA pH 7.4; 0.6 M succinic acid adjusted to pH 7.4 with NaOH; 0.001 M cytochrome *c*. The specific activity was measured at 30ºC, by following the reduction of cytochrome *c* at 550 nm and expressed as reduced cytochrome *c* (nanomol)/min/mg protein.

RESULTS AND DISCUSSION

S. adstringens had a marked effect on the growth of *H. samuelpessoai*. Preliminary observations indicated that this protozoan displayed a similar behavior when it was submitted to different concentrations of crude extract, F2, or F3 fractions in defined medium. Fig. 1 shows the effects of F3 (ethyl-acetate-soluble fraction) on the growth of *H. samuelpessoai* incubated at 28ºC. Growth of the protozoans was not affected at a concentration of 100 µg/ml; however, when they were cultivated for 72 h at concentrations of 500, 1000, 2500, and 5000 µg/ml, growth inhibition was 42, 70.8, 99.5, and 99.6%, respectively. The 50% inhibitory concentrations (IC_{50}) of the crude extract and the F3 fraction were 538 and 634 µg/ml, respectively.

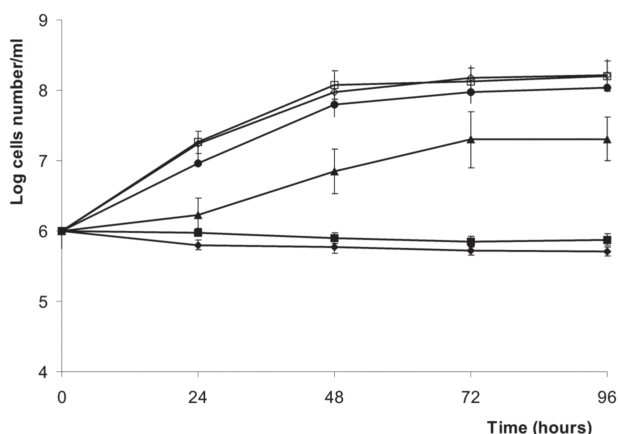

Fig. 1: effect of *Stryphnodendron adstringens* on the growth of *Herpetomonas samuelpessoai*. Parasites were incubated in defined medium in the presence of increasing concentrations of F3 fraction for 96 h at 28ºC. The cells were counted daily. ◇control; □100 µg/ml; ● 500 µg/ml; ▲ 1000 µg/ml; ■ 2500 µg/ml; ◆ 5000 µg/ml. The values presented are means ± S.D. and one representative of at least three independent experiments.

Additionally, the effect of the twelve F3 sub-fractions (F3-1 - F3-12) on the growth of the protozoans, after 72 h incubation at 28ºC was evaluated. Interestingly, the F3.9 - F3.12 sub-fractions showed the highest activity at 1000 µg/ml with 88.6% growth inhibition, when compared with the sub-fractions F3.1 - F3.4 and F3.5 - F3.8, which showed 59.7% and 70.1% growth inhibition, respectively (Fig. 2). Toledo (2002) analyzed these sub-fractions by TLC on silica gel. The chromatogram sprayed with 1% iron chloride and vanillin/sulphuric acid solutions revealed the presence of condensed tannins in the sub-fractions. It is possible that compounds of high molecular weight (oligomers, polymers) are present in the F3.9 - F3.12 sub-fractions, because condensed tannins are usually isolated in a Sephadex®LH-20 column eluting first the monomeric, and next the di-, tri-, and oligomeric flavan-3-ols compounds (Thompson et al. 1972, Toledo 2002). Thus, the inhibitory activity of these sub-fractions on the protozo-

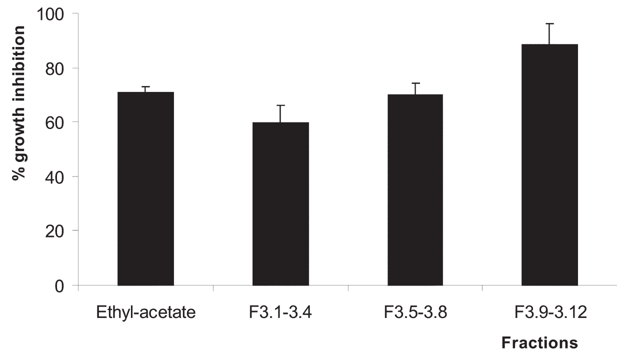

Fig. 2: effects of 1000 µg/ml of the ethyl-acetate fraction and the F3.1 - 3.4, F3.5 - 3.8, and F3.9 - 3.12 sub-fractions on growth of *Herpetomonas samuelpessoai* cultivated in defined medium at 28ºC for 72 h. The results are expressed as means ± standard deviations (n = 3).

ans may be due to the action of the compounds isolated during the purification process. Another factor that might affect the process of elevated activity of the F3-9 - F3-12 sub-fractions, may be related to the stereochemistry of the substances present in these sub-fractions. It is known that condensed tannins of high molecular weight are highly hydroxylated molecules that assume different spatial conformations, which could interact in several ways with the membrane enzymes, substrate or ions of *H. samuelpessoai*. According to the Brazilian Pharmacopoeia (Farmacopéia 2002), the bark of *S. adstringens* contains at least 20% tannins. Mello et al. (1996a, b) and Santos et al. (2002) investigated the chemical composition of this species and established that the pharmacological activity is mainly due to the content of tannins present in the bark. A study by Scalbert (1991) established the toxicity of tannin to

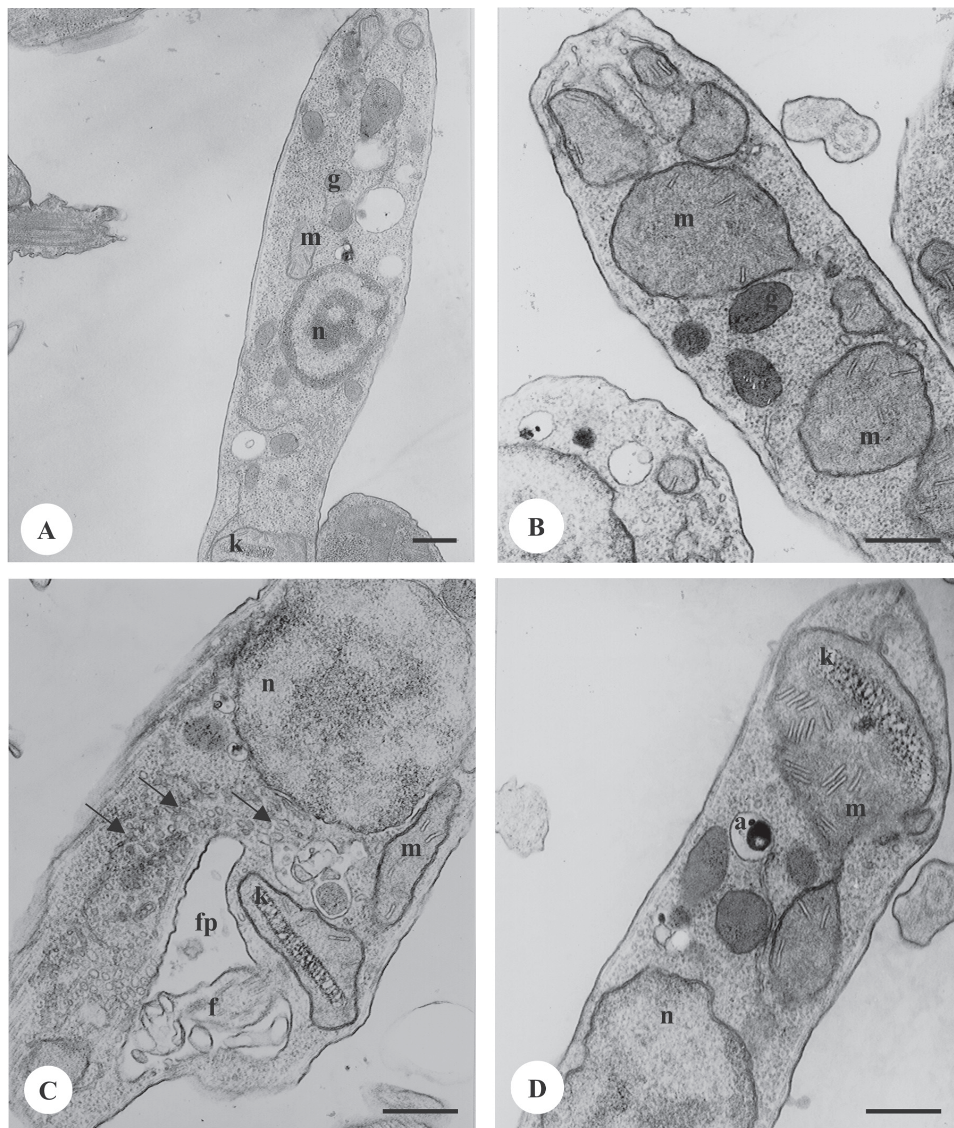

Fig. 3: transmission electron microscopy of *Herpetomonas samuelpessoai* grown in defined medium at 28ºC. A: general view of an untreated promastigote form of *H. samuelpessoai*, showing the nucleus (n), the kinetoplast (k), the glycosome (g), and the mitochondrion; B, C, and D: promastigote treated with 538 µg/ml of crude extract from *Stryphnodendron adstringens* for 72 h. Note mitochondrial swelling and cytoplasmic vesicles (arrows) - f: flagellum; fp: flagellar pocket; g: glycosome; k: kinetoplast; m: mitochondrion; n: nucleus. Bars = 1 µm

bacteria, filamentous fungus, and yeast. Three hypotheses might explain the antimicrobial mechanism of the tannins: (a) inhibition of enzyme activity by complexating with the substrate of bacteria and fungus; (b) direct action of the tannins on the microorganisms' metabolism, through the inhibition of oxidative phosphorylation; (c) a mechanism involving the complexation of tannins with metallic ions, decreasing the availability of essential ions to the metabolism of the microorganisms (Scalbert 1991, Santos & Mello 2003). Taking these considerations together, our results suggest that the tannins may be the substances responsible for inhibiting the growth of *H. samuelpessoai*.

Based on these results, we selected the crude extract to continue our biochemical and microscopic tests, because it showed the lowest IC_{50}. Therefore, the ultrastructural aspects of this protozoan were evaluated. Transmission electron microscopy of *H. samuelpessoai* exposed to "barbatimão" for 72 h revealed marked mitochondrial swelling and a large number of cristae, with a loss of cristae patterns (Fig. 3B, D) compared to untreated cells, which had mitochondria with well-defined membranes and a dense matrix (Fig. 3A). Also, treated cells exhibited an evident Golgi complex vesiculation (Fig. 3C).

The activity of the enzyme succinate cytochrome *c* reductase, a well-known mitochondrial marker related to mitochondrial metabolism involved in the respiratory sequence of this flagellate, was also observed. Cells cultivated in defined medium in the presence of the crude extract of *S. adstringens* at 28ºC for 72 h showed a decrease in the activity of this enzyme. The specific activity of the treated cells was 5.6 ± 1.03 nanomol/min/mg of protein, whereas in the untreated cells it was 9.7 ± 1.82 nanomol/min/mg of protein. Inhibition of this enzyme can influence the respiratory sequence, affecting mitochondrial function. This observation suggests an alteration in the production of adenosine-triphosphate (ATP), thus influencing the active transport of ions and molecules as well as alterations in the synthesis of macromolecules. In this regard Rebecca et al. (2003) demonstrated the action of a crude extract of "barbatimão" on hepatic energy metabolism, using isolated mitochondria and perfused rat liver. Their results indicated that the "barbatimão" extract impairs hepatic energy metabolism by uncoupling oxidative phosphorylation, inhibiting mitochondrial electron transport, and inhibiting ATP-synthase in isolated mitochondria. Inhibition of the electron flow in the respiratory sequence was confirmed by the gradual inhibition of the activity of the enzymes oxidase succinate and NADH-oxidase in disrupted mitochondria.

The results obtained in this work demonstrated that the crude extract and fractions of "barbatimão" have a dose-dependent inhibitory effect on the growth of *H. samuelpessoai*, and alter certain biochemical and ultrastructural aspects of this protozoan. Because *H. samuelpessoai* is a non-pathogenic protozoan belonging to the family Trypanosomatidae, which includes the species *T. cruzi* and *Leishmania* sp., it is an excellent model system for testing new anti-protozoal drugs. The potential of *S. adstringens* as a source of new therapeutic agents against protozoans shows promise for curing protozoan-caused diseases.

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