Cutaneous secretion from the giant African snail, *Achatina fulica*, as a source of Antileishmanial compounds

Secreção cutânea do caramujo gigante africano, *Achatina fulica*, como fonte de compostos anti-*Leishmania*

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ABSTRACT
Leishmaniasis is a severe protozoan parasite disease, causing clinical symptoms from a single cutaneous ulceration to a progressive and fatal disease. The treatment is based on toxic chemotherapeutic compounds, being pentavalent antimonials the first line drugs. Natural products from Brazilian fauna, especially animal venoms and cutaneous secretions, are a rich source of novel chemical molecules which could be used as drug prototypes for the development of new therapeutics. This study describes for the first time the effective antileishmanial activity of crude cutaneous secretion from the giant African snail, *Achatina fulica*, and its mammalian cells citotoxicity and also its potential mode of action against Leishmania promastigotes. The crude secretion showed an 50% Effective Concentration (EC_{50}) of 98.37 µg/mL against *L.(L.) chagasi* promastigotes. By means of enzymatic assays, a L-amino acid oxidase (L-AAO) activity was detected in crude secretion, and the hydrogen peroxide produced by this enzyme revealed to be one of the compounds responsible for the antileishmanial effect. The use of catalase for H_2O_2 scavenging in *Leishmania* cultures incubated with crude secretion abolished 54% of parasite death. Despite a moderate toxicity of the snail cutaneous secretion on LLC-MK2 mammalian cells (EC_{50} of 83.25 µg/mL), these promising data provided the valuable information for further chromatographic isolation of novels antiparasitics, which could be a useful tool for the development of new drugs against Leishmaniasis.
Key words. venoms, secretions, *Leishmania*, *Achatina*, drugs, L-amino acid oxidase.

RESUMO
A Leishmaniose é uma doença parasitária grave que causa desde uma única ulceração cutânea até uma doença progressiva e fatal. O tratamento é baseado em agentes quimioterápicos tóxicos, sendo indicados como fármacos de primeira escolha os antimoniais pentavalentes. Os produtos naturais derivados da fauna brasileira, especialmente venenos e secreções cutâneas de anfíbios, são fontes ricas de novas moléculas químicas, as quais podem ser utilizadas como protótipos farmacêuticos no desenvolvimento de novos fármacos. No presente trabalho, é feita a descrição inédita sobre a efetiva atividade anti-*Leishmania* da secreção cutânea do caramujo gigante Africano, *Achatina fulica*, bem como a sua citotoxicidade em células de mamíferos e seu possível mecanismo de ação contra os promastigotas. A secreção bruta apresentou Concentração Efetiva 50% de 98,37 µg/mL contra promastigotas de *L.(L.) chagasi*. Por meio de ensaios enzimáticos, foi detectada a atividade L-aminoácido oxidase (L-AAO) na secreção bruta, e também foi demonstrado que o H_2O_2 gerado por esta enzima é um dos compostos responsáveis pelo efeito anti-*Leishmania*. O uso de catalase**,** para eliminar a ação do H_2O_2 nas culturas de *Leishmania*, causou a diminuição de 54% na morte dos parasitos. Apesar da ocorrência de citotoxicidade moderada da secreção bruta contra as células LLC-MK2 (CE_{50} de 83,25 µg/mL), estes são dados promissores que possibilitam isolamentos cromatográficos futuros de novos antiparasitários, os quais poderão servir como valiosas ferramentas no desenvolvimento de fármacos contra a Leishmaniose.
Palavras-chave. venenos, secreções, *Leishmania*, *Achatina*, drogas, L-aminoácido oxidase.

INTRODUCTION

The African giant snail (*Achatina fulica*, Ferussac), is one of the largest land snail species in the world, now found abundantly in tropical and subtropical regions[1]. In Brazil, the snail was supposed to be used for human consumption as "escargot". Its dispersal from the artificial breeding has widespread the risk of human contamination by *Angiostrongylus cantonensis*, a common nematode parasite found in this snail[2]. The cutaneous secretions of many animals, especially amphibians, have been representing an important source of antiparasitic and antifungal compounds[3]. *A. fulica* presents an abundant cutaneous secretion that recently has been demonstrating significant therapeutic potential, with biological activities related to antibacterial[4,5] and antiangiogenic activity in tumor cells[6]. Leishmaniasis is a complex disease where the protozoan parasite spread by a sandfly insect vector, can cause from a single cutaneous ulceration to a progressive and fatal disease. The disease is a significant health problem in many parts of the world resulting in an estimated of 12 million cases[7]. The treatment is based on toxic chemotherapeutics, which is difficulty to administer, expensive and becoming ineffective due to the emergence of drug resistance[8]. Despite the increased number of cases, no considerable effort has been made for new drug development. The lack of efficient and less toxic drugs against Leishmaniasis and the mainstay of Gaspar Vianna´s antimonial for clinical therapy for almost one century show the imperative necessity of new lead compounds[9]. In this work, we have described the potential antileishmanial activity of the crude cutaneous secretion of *A. fulica* against promastigotes, studied the *in vitro* mammalian cytotoxicity and its possible mode of action.

MATERIAL AND METHODS

Materials

M-199 and RPMI-PR$^-$ 1640 medium (without phenol red) were purchased from Sigma. Pentavalent antimony (Aventis-Pharma) and pentamidine (Eurofarma) were used as the standard drugs. Sodium dodecyl sulphate (SDS) was purchased from Merck, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Thiazol blue; MTT) was purchased from Sigma.

Animals

Animals were supplied by the Animal Breeding Facility at Instituto Adolfo Lutz de São Paulo. They were maintained in sterilized cages under a controlled environment, receiving water and food *ad libitum*. Animal procedures were performed under the approval of the Research Ethics Commission, according to the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences (http://www.nap.edu). *A. fulica* cutaneous secretion was obtained from Dr. Sylvio Rocco at Instituto Adolfo Lutz through slightly mechanical compression of 1 animal in distillated water. The secretion was immediately kept at -20°C and lyophilized. The mass of the dried secretion was weighted, diluted in Phosphate Buffered-Saline and sterilized through 0.22 μm membranes (Millipore).

Parasites

L. (L.) *chagasi* (MHOM/BR/1972/LD) was maintained in golden hamsters. Promastigotes were maintained in M-199 medium supplemented with 10% calf serum, 2% male human urine and 0.25% hemin at 24°C.

Determination of the 50% Effective Concentration (EC_{50})

The antileishmanial activity against promastigotes was determined using 96-well microplates, with pentamidine as the standard drug. Briefly, promastigotes were counted in a Neubauer haemocytometer and seeded at 1 x 10^6/well in 96-well microplates. The *A. fulica* secretion was incubated in a range concentration of 500 to 0.1 μg/mL for 48 h at 24°C. Parasite viability was determined using the MTT assay at 570 nm[10]. The antileishmanial activity against intracellular amastigotes was determined with infected macrophages, using pentavalent antimony as the standard drug. Macrophages were obtained from the peritoneal cavity of BALB/c mice and added to 24-well plate at 4x10^5/well. The parasite burden was defined as the number amastigotes/macrophage out of 500 cells. Each assay was determined in duplicate and at least in 2 independent assays[11]. The EC_{50} values were determined using a sigmoid dose-response analysis at Graph Pad Prism 3.0, considering the 95% Confidence Intervals. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

Cytotoxicity Assay

LLC-MK2 (ATCC –Rhesus Monkey Kidney Epithelial Cells) cells were kept in RPMI-PR$^-$ 1640 medium, seeded at 4x10^4/well in 96-well microplates and incubated at 37°C for 48 hours in the presence of *A. fulica* secretion in a 5% CO_2 humidified incubator. The viability of the cells was determined with the MTT[10]. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

L-amino acid oxidase (L-AAO) activity

Crude secretion of *A. fulica* (100 μg/mL) was assayed for L-AAO activity using a colorimetric assay in a 96-well microplate. The assay was derived by an adaptation of a reported peroxidase test[12]. The reaction mixture contains 100 mM Tris/HCl, pH 8.5, 3 mM L-leucine as substrate, horseradish peroxidase (10 IU/mL) and o-phenylenediamine (OPD), (0.1 mg/mL). Samples were incubated for 1 hour at 25°C, and Optical Density (O.D.) was determined at 414 nm at 10 minutes intervals on a microplate reader for kinetics. Hydrogen peroxide standards were used and the linear regression data calculated with the Graph Pad Prism 3.0 software. L-AAO activity was

expressed as nmoles H_2O_2/mL. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

Characterization of L-AAO action on promastigotes

Promastigotes of *L.* (L.) *chagasi* (1 x 10^6/well) were incubated with *A. fulica* secretion (100µg/mL) and catalase (300µg/mL) in a final volume of 150µL/well for 24 h at 24ºC in a 96-well microplate assay, in order to abolish the action of H_2O_2[13]. Control groups without *A. fulica* secretion, with or without catalase were also tested. The viability of *L.* (L.) *chagasi* was determined by the MTT assay[10]. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

Leishmania Permeability Assay

Pore formation in *L.* (L.) *chagasi* promastigote membrane was studied in the presence of *A. fulica* secretion through the ethidium bromide assay[14]. The *A. fulica* secretion was incubated at 100 µg/mL with promastigotes (1 x 10^6/well) in the presence of ethidium bromide (25 µg/mL) in 96-well microplates for different times (0 to 120 min) at 24°C. In each time a sample was added to glass coverslips and observed in a fluorescence microscopy. Each image was saved as TIFF file. Amphotericin B was incubated with parasites at the same conditions and used as internal control.

RESULTS AND DISCUSSION

Antileishmanial Activity and Cytotoxicity Studies: Determination of the 50% Effective Concentration (EC_{50})

The crude secretion of *A. fulica* showed an 50% Effective Concentration (EC_{50}) of 98.37 µg/mL (95% Confidence Interval= 81.62- 118.6 µg/mL) against *L.* (L.) *chagasi* promastigotes, showing 100% of killing activity at the highest dose (Figure 1). In order to evaluate the activity against the intracellular form of the parasite, *Leishmania*-infected macrophages (intracellular amastigotes) were incubated with the test *A. fulica* secretion at different concentrations. No detectable antileishmanial activity could be observed at the highest concentrations, as macrophage damage was clearly seen. Future isolation of the active compound in the *A. fulica* secretion by chromatographic techniques could eliminate the mammalian cytotoxic fraction and further activity against Leishmania-infected macrophages could be properly evaluated. Although antibacterial activity of *A. fulica* secretion has been described[4,5,15], neither antileishmanial nor any other antiparasitic activity was described. Kubota and co-workers[15], isolated the antibacterial factor of the *A. fulica* secretion. The glycoprotein presented a molecular weight of 160 kDa, being composed of two subunits of 70 – 80 kDa. Ehara and co-workers[5] have sequentially isolated and characterized the antibacterial compound. They named it Achacin, an antibacterial glycoprotein with L-amino acid oxidase (L-AAO) activity, which generates cytotoxic hydrogen peroxide.

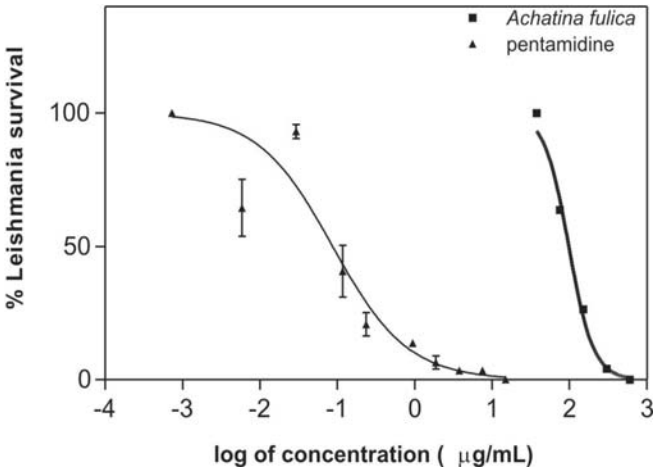


Figure 1. Determination of the 50% Effective Concentration (EC_{50}) of *A. fulica* secretion against *L.* (L.) *chagasi* promastigotes. The viability of parasites was determined through the oxidative mitochondrial function by MTT at 570 nm. Pentamidine was used as standard drug. Sigmoid dose-response curve was analyzed in Graph Pad Prism 3.0 software. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

We have also studied the cytotoxicity of the crude secretion against mammalian cells through the *in vitro* incubation with LLC-MK2 cells. The crude mucus showed a moderate citotoxicity, with an EC_{50} of 83.25 µg/mL (95% Confidence Interval= 63.70 - 108.8 µg/mL) as determined by oxidative mitochondrial function by MTT (Figure 2).

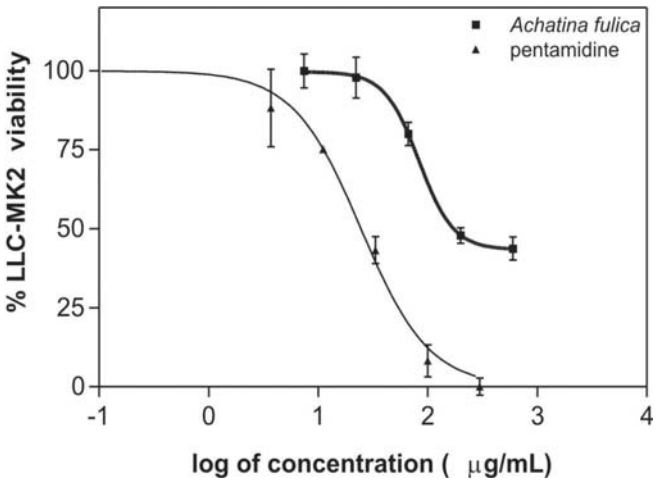


Figure 2. Cytotoxicity studies of *A. fulica* secretion against LLC-MK2 cells. Pentamidine was used as standard drug. The viability of mammalian cells was determined through the oxidative mitochondrial function by MTT at 570 nm. Sigmoid dose-response curve was analyzed in Graph Pad Prism 3.0 software. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

Biochemical Characterization of Crude Secretion: L-AAO Activity

Through enzymatic assays, we have found L-amino acid oxidase (L-AAO) activity in the crude secretion of *A. fulica*. The kinetic parameters showed that the enzyme produced 0.104 nmoles/min/mL of H_2O_2 (Figure 3). The venom of the snake *Bothrops moojeni* was used as standard and produced 0.451 nmoles/min/mL of H_2O_2. Our data about the presence of L-AAO activity in *A. fulica* secretion corroborates those observed elsewhere[5].

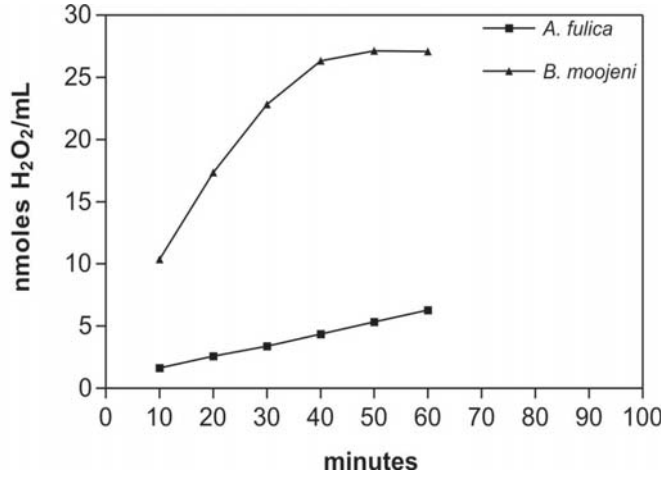
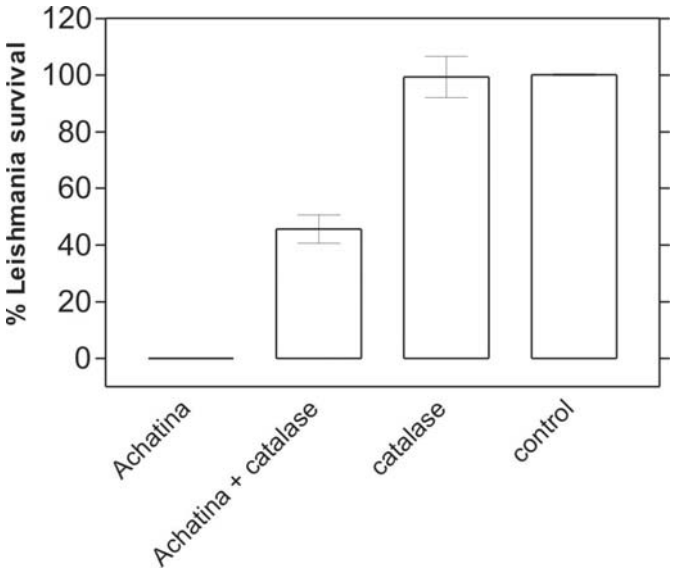


Figure 3. L-amino acid oxidase (L-AAO) activity of *A. fulica* secretion. Samples were incubated for 1 hour at 25°C with the reagents described in material and methods, and Optical Density (O.D.) was determined at 414 nm at 10 minutes intervals on a microplate reader for kinetics. Hydrogen peroxide standards were used and the linear regression data calculated with the Graph Pad Prism 3.0 software. *Bothrops moojeni* venom was used as positive control for H_2O_2 production. Data represents the mean (± S.D.) of duplicate samples from two independent assays.

***Leishmania* and *A. fulica* Secretion: Possible Mode of Action on Promastigotes**

The incubation of *L*. (L.) *chagasi* promastigotes with *A. fulica* secretion using catalase for H_2O_2 scavenging, abolished 54% of parasite death (Figure 4). L-amino acid oxidases (L-AAO) catalyzes the oxidative deamination of L-amino acids to produce the corresponding α-keto-acid, hydrogen peroxide and ammonia[16,17]. Tempone and co-workers[13] have previously demonstrated that *Leishmania* sp. is not affected by the α-keto-acid and ammonia produced by L-AAO. Consequently, our data suggest that other compounds than L-AAO presented in the crude secretion of *A. fulica* might be involved in the antileishmanial activity, corresponding to the 46% of the killing activity when incubated with catalase. The presence of other compounds like peptides and proteins might be associated to the antiparasitic effect.



We have also studied the effect of the crude secretion on the membrane of *L.* (L.) *chagasi* promastigotes through the permeability assay using ethidium bromide[14]. The fluorescent microscopy images (data not shown) suggest that the killing activity of *A. fulica* secretion on *Leishmania (L.) chagasi* might be other than pore-forming activity in the parasite membrane, as no fluorescence marker was found in the intracellular environment of promastigotes. Amphotericin B was used as a standard drug, since its pore-forming activity in *Leishmania* sp. membrane has been described[14] (data not shown). Further assays would evaluate other possible intracellular targets of *A. fulica* secretion in *Leishmania* promastigotes, by using transmission electron microscopy.

CONCLUSIONS

This is the first paper which describes the effective antileishmanial activity of the Giant African snail, *A. fulica*, and also demonstrates that other compounds than the high molecular weight enzyme L-AAO might be involved in the killing effect. This valuable information, if adequately studied, could be a useful tool for chromatographic isolation of active compounds. These results could lead in the development of new drug prototypes which could be used as novel and less toxic chemotherapeutics for neglected diseases as Leishmaniasis.

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