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MICROBIOLOGY

Anti-*Leishmania amazonensis* activity of the marine sponge *Dercitus (Stoeba) latex* (Porifera) from São Pedro and São Paulo Archipelago, Pernambuco, Brazil

ANNA L.S. BARRETO, ARIADNE N. ALONSO, DANIEL C. DE MORAES, JOSÉ A.R. CURVELO, KILDARE MIRANDA, MARISTELA B. PORTELA, ANTÔNIO FERREIRA-PEREIRA, THAIS SOUTO-PADRÓN & ROSANGELA MARIA DE A. SOARES

Abstract: The search for new therapeutic strategies for leishmaniasis treatment is essential due to the side effects of available drugs and the increasing incidence of resistance to them. Marine sponges use chemical compounds as a defense mechanism, and several of them present interesting pharmacological properties. The aim of this study was to evaluate the *in vitro* activity of the aqueous extract of the marine sponge *Dercitus (Stoeba) latex* against *Leishmania amazonensis*. MIC and toxicity against mammal cells were evaluated through broth microdilution assays. Transmission electron microscopy analysis was performed to assess possible effects on *L. amazonensis* ultrastructure. Arginase and proteolytic activities were measured by spectrometric methodologies. The extract of *Dercitus (Stoeba) latex* displayed antileishmanial activity and moderate toxicity against peritonial macrophages. Ultrastructural changes were observed after the growth of *L. amazonensis* promastigotes in the presence of the extract at 150 $\mu g.ml^{-1}$ (IC_{50}), mainly on acidocalcysomes. The extract was able to inhibit the activity of arginase and serine proteases. This study shows that *Dercitus (Stoeba) latex* aqueous extract may be a novel potential source of protozoa protease inhibitors and drugs that are less toxic to be used in the treatment of *L. amazonensis* infections.

Key words: Arginase, *Dercitus (Stoeba) latex*, *Leishmania amazonensis*, leishmaniasis, proteolytic activity.

INTRODUCTION

Leishmaniasis refers to a group of diseases caused by protozoa *Leishmania* and is transmitted by the bite of an infected sandfly. Clinically, the infectioncan progress in several ways, such as a chronic skin ulcer (cutaneous leishmaniasis), an erosive mucosal disease with severe facial disfigurement (mucocutaneous leishmaniasis), or a life-threatening systemic infection with hepato-splenomegaly and bone marrow involvement (visceral leishmaniasis) (Psicopo & Mallia 2009, David & Craft 2009).

Leishmania species express molecules, such as proteases, that play different roles in host tissue invasion and immune evasion mechanisms (Soares et al. 2003, McKerrow 2018). Among them, arginase, a metalloenzyme involved in the hydrolysis of L-arginine and the regulation of nitric oxide synthesis, plays an important role in the subversion of macrophage function during the disease (Balaña-Fouce et al. 2012).

Pentavalent antimonials are the first-choice therapeutic regimen for the treatment

of leishmaniasis. Whenever it does not work or cannot be prescribed, amphotericin B, pentamidine, or paromomycin serve as a second-choice line of treatment. Miltefosine, the only anti-*Leishmania* drug available for oral treatment, is normally prescribed for visceral leishmaniasis, and azoles, such as fluconazole, have been used for the treatment of the cutaneous form. However, the development of resistant strains has often led to therapeutic failure (Mans et al. 2016, Ponte-Sucre 2017, Rodrigues et al. 2006).

The relevance of non-conventional drugs in the treatment of infectious diseases has increased remarkably in recent years. Marine organismsoffer an unprecedented opportunity for pharmacological exploitation, mainly because their metabolites have already been reported as rich sources of novel chemical compounds which may lead to more effective drugs (Haefner 2003, Mollica et al. 2012).

Sponges are marine, sessile, filter-feeding multicellular animals (without true organs) that use chemical compounds as a defense mechanism. Their extracts and compounds have been reported to present antibacterial (Mangalindan et al. 2000), antiviral (Da Silva et al. 2006), antifungal (Wattanadilok et al. 2007), and even antileishmanial activities, such as seen on those obtained from *Cliona varians* (Moura et al. 2006), *Haliclona exigua* (Dube et al. 2007), *Plakortis angulospiculatus* (Kossuga et al. 2008), and *Dragmaxia undata* (Carballeira et al. 2011).

In the present study, the antileishmanial activity of the endemic brazilian marine sponge *Dercitus* (*Stoeba*) *latex* (Moraes & Muricy 2007) collected from São Pedro and São Paulo Archipelago was evaluated.

MATERIALS AND METHODS

Sponge sampling and identification

São Pedro and São Paulo Archipelago is one of the smallest and most isolated set of islands in the world, located 1000 km from the city of Natal, Rio Grande do Norte State, Northeastern Brazil. The sponge was manually collected by scuba diving (0˚55'N 29˚21'W) and preserved in 70% ethanol. The specimen was photographed *in situ* using a Nikonos V camera with 35 mm Nikkor lens and close-up kit. The positive film was digitalized using a Nikon Coolscan IV ED scanner (Moraes & Muricy 2007). Samples of the sponge were stored at -20˚ C for later evaluation.

Preparation of extracts

The sponge was washed with distilled water and transported to the laboratory under refrigeration. Then, it was kept in milli-Q water for 72 hours at 4° C for the extraction. Thus, the extract was lyophilized in Speed Vac® and resuspended in sterile distilled water to a stock solution of 100 $mg.ml^{-1}$.

Parasite

Leishmania (Leishmania) amazonensis Josefa strain (MHOM/BR/75/Josefa), originally isolated from a human case of cutaneous leishmaniasis, was kindly gifted by Dra Elvira Saraiva (Instituto de Microbiologia Paulo de Góes, UFRJ, Brazil). Promastigote forms were maintained by weekly transfers in 25-cm^2 culture flasks with Schneider´s insect medium (Sigma Aldrich®, St. Louis, USA), pH 7.2, supplemented with 10% fetal calf serum (FCS) (Cultlab®, São Paulo, Brazil) and gentamicine sulphate (Schering-Plough®, São Paulo, Brazil) (80 $\mu g.ml^{-1}$) at 26° C.

Cytotoxicity assay

Mice were subjected to intraperitoneal stimulation with thioglycolate for 96 hours.

Then, peritoneal macrophages were obtained and incubated ($5x10^5$/well) on 96-well plates with various concentrations (promastigotes IC_{10}, IC_{50} and MIC) of *D.* (*S.*) *latex* extract at 37° C in 5% CO_2 for 24 h. *In vitro* cytotoxicity of the extract on peritoneal murine macrophages performed after intraperitoneal stimulation for 96 hours by thioglycolate was assessed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann 1983). Firstly, macrophages were treated with 5 $mg.ml^{-1}$ MTT and incubated for 3h at 37° C. Afterwards, dimethyl sulfoxide (DMSO) was added for 1 h to solubilize formazan crystals, and absorbance was measured at 540 nm. Cell viability was expressed as a percentage of average viable control cells. Three independent experiments were carried out in triplicates.Results were expressed as the concentration able to reduce cell viability by 50% (CC_{50}), in comparison to positive control. This protocol was approved by ethical committee of the UFRJ Health Sciences Center under the number IMPG020.

Antileishmanial activity

Activity against L. amazonensis promastigotes

Cells ($1x10^6$ $parasites.ml^{-1}$) were incubated at 26° C for 120 h in fresh medium (Schneider´s insect medium) supplemented with 10% FCS in the absence (control parasites) or presence (treated parasites) of several concentrations (2 $\mu g.ml^{-1}$ to 1000 $\mu g.ml^{-1}$) of *D.* (*S.*) *latex* aqueous extract. Glucantime (Sanofi-Aventis®) at 30 $\mu g.ml^{-1}$ to 470 $\mu g.ml^{-1}$ and Amphotericin B at 3 $\mu g.ml^{-1}$ to 50 $\mu g.ml^{-1}$ were used as control drugs. Parasite viability was assessed before and after incubation by evaluating cell motility and trypan blue staining. The 100% inhibitory concentration (MIC) and 50% inhibitory concentration (IC_{50}) were determined by linear regression analysis using Microsoft Excel® (Microsoft Corporation, WA, USA).

Activity against L. amazonensis amastigotes

Peritoneal macrophages adhered to glass coverslips were washed with PBS pH 7.2 and incubated with promastigotes ($5x10^6$ $parasites.ml^{-1}$) at stationary growth phase in DMEM medium (Thermo Fisher Scientific, MA, USA) supplemented with 2% FCS at 37° C in 5% CO_2 for one hour. After interaction, cells were washed in PBS and incubated with DMEM supplemented with 10% FCS in the presence or absence of *D.* (*S.*) *latex* extract at promastigotes IC_{50}. After 24h and 48h, the material was washed, fixed with methanol for 5 minutes, dehydrated with acetone-xylol, and stained with 36% Giemsa. Infected macrophages were visualized by light microscopy and counted. Three independent experiments were carried out in triplicate.

Transmission electron microscopy

Parasites incubated for 120 h in the presence or absence of *D.* (*S.*) *latex* aqueous extract at promastigotes IC_{50} were fixed in 4% formaldehyde, 2.5 % glutaraldehyde, and 0.1 M sodium cacodylate buffer (pH 7.2) for 1h at room temperature. Cells were post-fixed in 1% OsO_4, dehydrated and embedded as previously described (Bisaggio et al. 2006). Ultrathin sections obtained with a Reichert UltraCut S ultra microtome were stained with uranyl acetate and then analyzed in a FEI Morgagni F 268 transmission electron microscope, operating at 80 kV, equipped with a Megaview G2 Camera. Alternatively, cells were grown in the presence or absence of IC_{50} extract for 72h, washed in PBS and placed on Formvar®-coated grids (Agar Scientific, United Kingdom) and subsequently observed in the transmission electron microscope. A descriptive analysis of morphometric parameters was performed using ImageJ® (National Institutes of Health, MD, USA).

Morphometric parameters such as number, diameter, circularity and absolute volume were measured.

Determination of arginase activity

Arginase enzyme activity was measured as previously described (Kropf et al. 2005), with slight modifications. Promastigotes were incubated for 72 h in the absence or presence of *D.* (*S.*) *latex* aqueous extract at promastigotes IC_{50} concentration. The parasites were lysed with 0.1% Triton X-100, and then 25 mM Tris-HCl pH 8.3 was added. After this, 10 mM $MnCl_2$ was added, and the enzyme was activated by heating for 10 min at 56° C. Arginine hydrolysis was conducted by incubating the lysate with 0.5 M L-arginine (pH 9.7) at 37° C for 15-20 min. The reaction was stopped with the addition of an acidic solution (H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). Urea concentration was measured at 540 nm in spectrophotometer (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) after addition of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95° C for 30 min.

Anti-Proteolytic activity

The effect of *D.* (*S.*) *latex* on the proteolytic activity of *L. amazonensis* was assessed as previously described (Kamboj et al. 1993), using the fluorogenic substrate Z-Phe-Arg-4-methoxy-β-naphthylamide (Sigma Aldrich®, St. Louis, USA). Briefly, 40 µg of a proteic extract obtained from *L. amazonensis* and 20 µg of trypsin were diluted in 50mM phosphate buffer pH 5.5. Then, 2.5 µl of the fluorogenic substrate Z-Phe-Arg-4-methoxy-naphthylamide was added and incubated at 37° C for 1 hour with [1-[N-[(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane] (E64), phenylmethylsulfonyl fluoride (PMSF), or *D.* (*S.*) *latex* aqueous extract. The reaction was stopped by the addition of 150 µl of 5% trichloroacetic acid. Substrate cleavage was measured at 380 and 450 nm for fluorescence excitation and emission, respectively.

Statistical analysis

All experiments were repeated at least three times, all systems were performed in triplicate sets, and the results were expressed as means ± standard deviation. Data were analysed by Student´s *t*-test, and $P<0.05$ was considered statistically significant.

RESULTS

Antileishmanial activity and cytotoxicity assay

D. (*S.*) *latex* aqueous extract inhibited the growth of *L. amazonensis* promastigotes in a dose dependent manner (data not shown), reaching absolute inhibition at 372 µg.ml^{-1} and the 50% inhibitory concentration (IC_{50}) was 150 µg.ml^{-1}. The control drugs Amphotericin B and Glucantime exhibited IC_{50} of 4 µg.ml^{-1} and 60 µg.ml^{-1}, respectively. The IC_{50} of sponge extract against *L. amazonensis* promastigotes, its CC_{50} against mouse peritoneal macrophages, and the selective indexes are presented in Table I.

Treatment with the IC_{50} of the sponge extract reduced the macrophage infection by 32.6%

Table I. Inhibition of promastigote growth and peritoneal murine macrophages citotoxicity by *D. (S.) latex* aqueous extract.

	Leishmania amazonensis	**Peritoneal macrophages**	**SI**
MIC (µg/ml)	372 ± 0.65	540 ± 0.06	1.45
IC_{50} / CC_{50} (µg/ml)	150 ± 0.65	174 ± 0.06	1.16

within 24 hours, and no significant difference was observed after a 48h-incubation (data not shown). Furthermore, it was observed a 21% reduction in the number of amastigotes per macrophage.

Transmission electron microscopy

Electron microscopy analysis (Figure 1) showed different degrees of damage in treated parasites, including the discontinuity of the nuclear membrane (Figure 1d, f, black arrows), formation of concentric structures with membranes (Figure 1e, black arrow) and the presence of several axonemes (at least three) in the cytoplasm (Figure 1g, black arrow). These findings indicate the presence of multiple flagella or the internalization of a single one, probably coiled-appearing several times in the cut, suggesting effects on the cell cycle. Changes in the electron density of acidocalcisomes were also observed (Figure 1h, i, black arrows). Moreover, descriptive analysis of morphometric parameters revealed an increase in the number of acidocalcisomes and the circularity of these organelles after a 72h-incubation with the IC_{50} extract (Figure 2 and Table II).

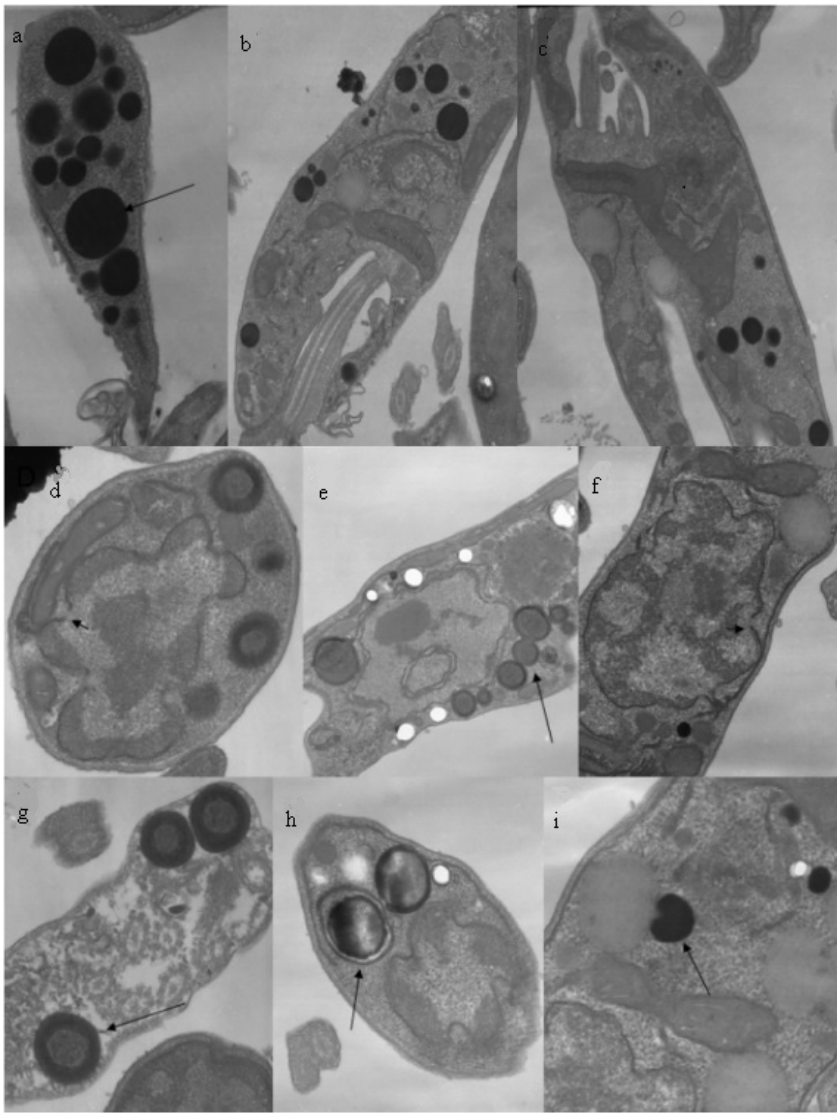

Figure 1. Analysis by TEM of *L. amazonensis* promastigotes in absence (a-c) or presence (d-i) of IC_{50} *D. (S.) latex* aqueous extract. Different degrees of damage were observed in treated cells, including the discontinuity of the nuclear membrane (d and f arrowhead), formation of concentric structures with membranes (e, black arrow), and the presence of several axonemes (at least three) in the cytoplasm (g, black arrow). Changes in the electron density of acidocalcisomes were also observed (h, i, black arrows).

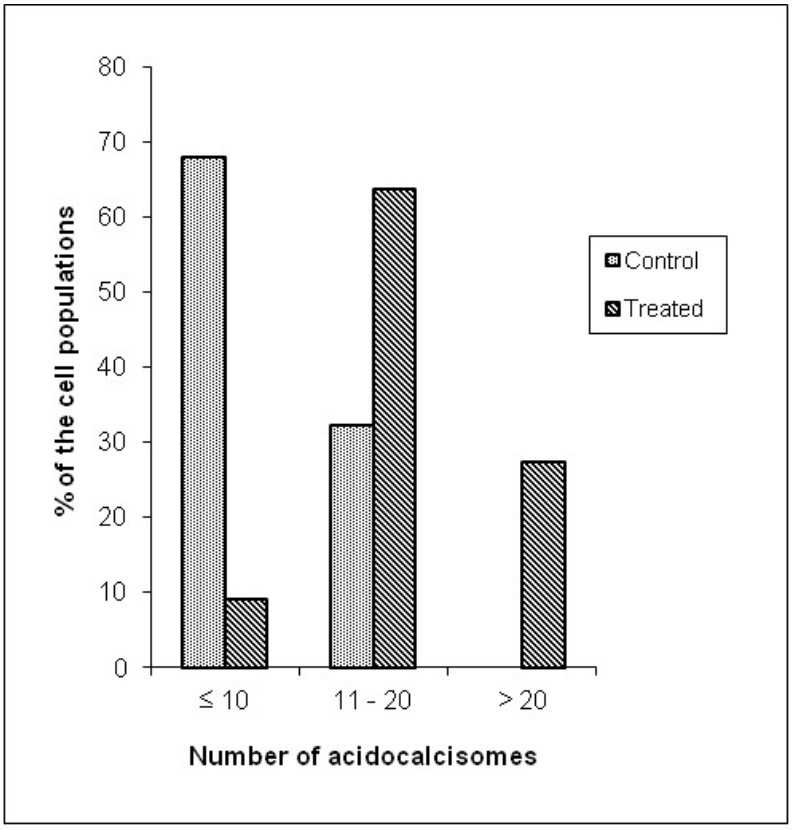

Figure 2. Numeric analysis of the acidocalcisomes in *L. amazonensis*. The number of the organelles was analysed and compared between control and treated cells. The cells treated with the sponge aqueous extract were subdivided into two groups representing cells between 11 and 20, and more than 20 acidocalcisomes per cell. Results are expressed in percentage of cell populations.

Table II. Numerical and circular changes in acidocalcisomes of *L. amazonensis* promastigotes grown in the presence of the extract IC_{50} for 72 hours. The (*) represent statistically significant data.

	Number of acidocalcisomes	Mean circularity (nm)	Mean diameter (nm)	Absolute volume (x 10^6 nm^3)
Control	9 ± 3	0,98 ± 0,03	0,26 ± 0,1	0,016 ± 0,02
Treated	17 ± 9*	0,94 ± 0,06*	0,26 ± 0,1	0,019 ± 0,02

Determination of arginase activity

Arginase activity was reduced in 28.66% in the presence of the sponge aqueous extract as demonstrated by the results shown in Figure 3.

Anti-Proteolytic activity

Data obtained revealed that the incubation of the protein cell extract with BSA in phosphate buffer pH 5.5 led to an inhibition of 35% of the proteolytic activity. This result was better than that observed with PMSF, a classic serine protease inhibitor (data not shown). Then, an assay was performed in order to analyze the hydrolysis of a specific fluorogenic substrate for serine protease. When the cysteine proteases were inhibited by E64, it was observed the inhibition of fluorescence emission when PMSF or aqueous extract of *D. (S.) latex* (IC_{50}) were added to the system (Figure 4). Treatment with E64 led to a significant hydrolysis of the fluorogenic substrate. Moreover, treatment with PMSF and aqueous extract *D. (S.) latex* significantly decreased substrate cleavage.

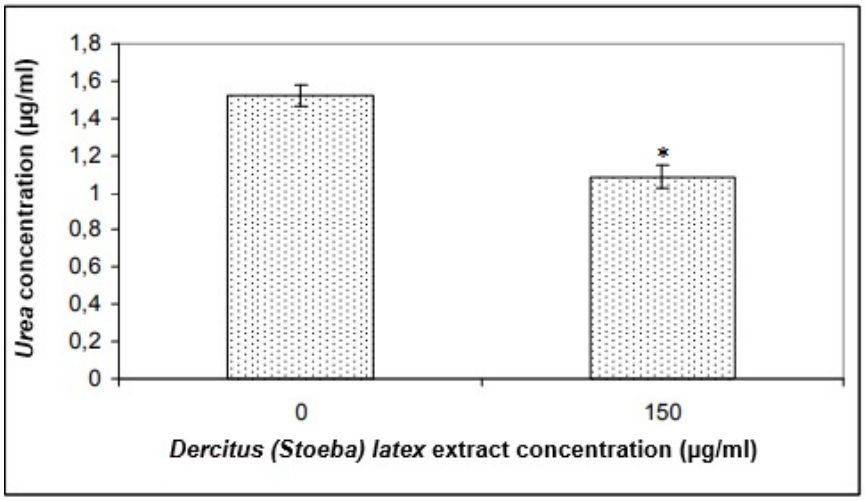

Figure 3. Effect of the aqueous extract of *Dercitus* (*Stoeba*) *latex* on arginase activity of *L. amazonensis* promastigotes. Parasites were grown on Schneider medium at 26 °C for 72 h in the presence of 150 µg.ml^{-1} *Dercitus (Stoeba) latex* aqueous extract. Then, parasites were washed, lysed, and arginase activity was measured as described at Materials and Methods section. Values represent means +/- standard deviation of three different experiments. (*): *P* < 0.05.

DISCUSSION

Natural products have been successfully used in the search for compounds with anti-*Leishmania* activity (da Silva et al. 2018). Although most of natural compounds with antimicrobial activity are derived from plant, the use of marine substances seems to be very promising (Donia & Hamman 2003, Laport et al. 2009). In this study, *D.* (*S.*) *latex* aqueous extract presented antileishmanial activity against promastigote forms, and microscopy analysis revealed damage on several structures, including acidocalcisomes. These structures are dense acidic organelles with a high concentration of phosphorus, presented mostly in the form of polyphosphates complexed with calcium and other elements. Several functions have been attributed to the acidocalcisomes, such as the storage of high energy compounds, calcium and other cations, and the regulation of the intracellular pH and osmolarity (DoCampo & Huang 2015). Despite the changes in the number and shape of acidocalcisomes revealed in this study, protozoan susceptibility to antimicrobial drugs seems to be unrelated, as demonstrated by the use of sitamaquine (López-Martín et al. 2008). However, a recent study using nelfinavir, an HIV protease inhibitor, reported the accumulation of this drug in acidocalcisomes of resistant strains of *L. amazonensis*, suggesting that an increase in the number of this vesicle might be considered as a resistant profile feature (Kumar et al. 2013). Furthermore, an inositol triphosphate receptor located in acidocalcisomes of *Trypanosoma brucei* was recently identified and considered essential for the growth and the establishment of an efficient animal infection (Huang et al. 2013). All these characteristics point to acidocalcisome as an important multifunctional organelle associated to crucial biological processes among protozoa.

The ability to survive and multiply within macrophages is a feature of several infectious agents, including *Trypanosoma cruzi* and *Leishmania* spp. In order to sustain a chronic infection, parasites must subvert macrophage-accessory cell activities and ablate the development of protective immunity (Alexander et al. 2002). Nevertheless, the most important mechanism for the killing of *Leishmania* and leishmaniasis control is the production of nitric oxide by macrophages (Holzmuller et al. 2006). The inhibition of arginase activity may influence parasite viability in *L. major* and *L. infantum*–infected macrophages (Iniesta et al. 2001). It is postulated that among *Leishmania*

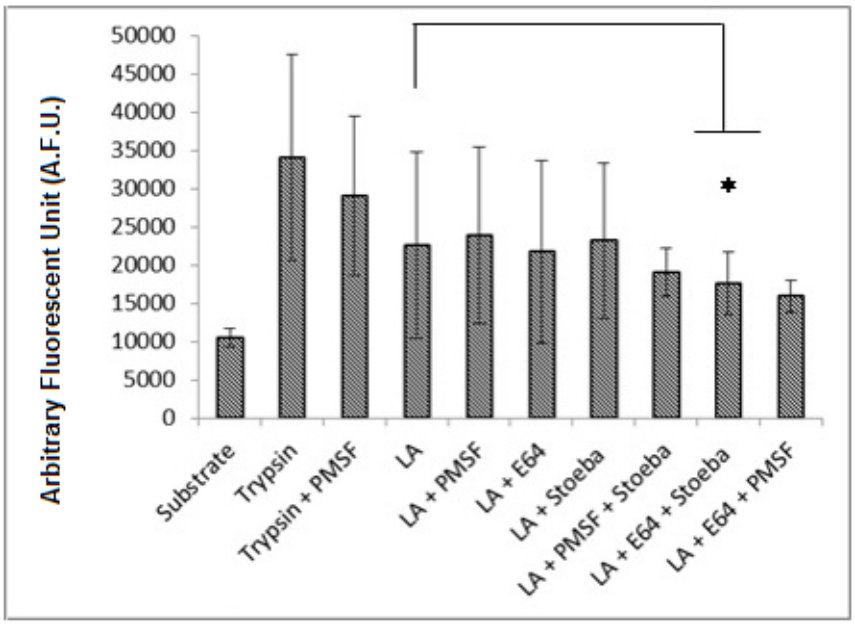

Figure 4. Effect of *Dercitus (Stoeba) latex* aqueous extract on the proteolytic activity of *L. amazonensis*. Hydrolysis of the fluorogenic substrate Z-Phe-Arg 4-methoxy-β-naphthylamide by *L. amazonensis* (LA) promastigotes cell extract in the presence or absence of proteolytic inhibitors PMSF and/or E64 and/or *D. (S.) latex* aqueous extract. Trypsin was used as control. The results represent the average of three experiments in triplicate ± standard deviation. (*): *P* < 0.05.

species, arginase activity must modulate nitric oxide synthase activity by using L-arginine, which is a common substrate for enzymatic activities (Boucher et al. 1999, Wanasen et al. 2008). In *L. amazonensis*, the arginase protein is concentrated in glycosomes both in promastigotes and amastigotes, suggesting that its location is important for enzyme activity, which modulates the L-arginine intracellular levels. Knockout parasites were able to infect macrophages but were not able to sustain the infection. It could be explained by a failure in modulating the availability of L-arginine to host cell and leading to an increased production of NO by infected macrophages (Da Silva et al. 2008). Promisingly, besides the effect on acidocalcisomes, the sponge aqueous extract was able to reduce arginase activity of *L. amazonensis* promastigotes, reinforcing the ability of this extract of modulating distinct mechanisms of *L. amazonensis* virulence.

Analysis of proteolytic enzymes of pathogenic organisms might lead to the design of powerful chemotherapeutic agents against these microorganisms (Grandgenett et al. 2007, Casgrain et al. 2016). The most studied classes of proteases in *Leishmania* spp. are cysteine-, metallo-, and serine proteases, since they are directly related to virulence. These enzymes cleave host proteins, neutralize immune response, disrupt fibronectin and extracellular matrix, overall enhancing promastigotes infectivity (Mottram et al. 2004). Then, proteases promote the survival of the parasite and the interaction with host tissues (Silva-Lopez et al. 2005). Results obtained suggest that *D.* (*S.*) *latex* aqueous extract may inhibit *L. amazonensis* serine proteases activity. Silva-Lopez and colleagues (2007) showed that the serine proteases inhibitors TPCK, benzamidine, and (ShPI-1), isolated from the sea anemone *Stichodactyla heliantus* affected cell viability and caused ultrastructural changes in the flagellar pocket, changes in membrane parasites, and the formation of intracellular vesicular bodies in *L.* amazonensis (Silva-Lopez et al. 2007). Nogueira and coworkers (2013) also showed a potent serine protease inhibitor purified from sea anemone which showed significant anti–*T. cruzi*

and moderate anti–*L. amazonensis* activities (Nogueira et al. 2013).

From the results obtained in our study, it was observed that the aqueous extract of *D. (S.) latex* has antileishmanial activity, since it was able to disrupt meaningful cellular components and inhibit enzymes associated with virulence in the parasite. The study of the extract of *D. (S.) latex* is promising and contributes to the advancement in the search for new, more effective, and less toxic drugs that can be used in the treatment of cutaneous leishmaniasis. Furthermore, this study shows that marine animals may be a source of protozoa protease inhibitors. The limitation of a study carried out with extracts in which there is no isolation and identification of compounds is understood; however the contribution to the confirmation of the potential of marine sponges as a source of new drugs is considered.

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ANNA L.S. BARRETO[1,2]
https://orcid.org/0000-0002-4987-9074

ARIADNE N. ALONSO[1,3]
https://orcid.org/0000-0003-2969-9524

DANIEL C. DE MORAES[1]
https://orcid.org/0000-0001-9782-3617

JOSÉ A.R. CURVELO[1]
https://orcid.org/0000-0003-3240-941X

KILDARE MIRANDA[4]
https://orcid.org/0000-0001-9799-773X

MARISTELA B. PORTELA[5]
https://orcid.org/0000-0001-5290-3744

ANTÔNIO FERREIRA-PEREIRA[1]
https://orcid.org/0000-0002-0474-8388

THAIS SOUTO-PADRÓN[1,*]

ROSANGELA MARIA DE A. SOARES[1]
https://orcid.org/0000-0001-7469-6950

[1]Universidade Federal do Rio de Janeiro, Instituto de Microbiologia Paulo de Góes, Laboratório de Bioquímica Microbiana, Av. Carlos Chagas Filho, 373, Ilha do Fundão, 21949-902 Rio de Janeiro, RJ, Brazil

[2]Instituto Brasileiro de Medicina de Reabilitação (IBMR), Centro Universitário, Av. das Américas, 2603, 22631-602 Rio de Janeiro, RJ, Brazil

[3]Laboratório Richet Medicina Diagnóstica, Avenida das Américas, 13331, 22790-701 Rio de Janeiro, RJ, Brazil

[4]Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, 21949-902 Rio de Janeiro, RJ, Brazil

[5]Universidade Federal Fluminense, Faculdade de Odontologia, Rua Mario Santos Braga, 28, 24020-140 Niterói, RJ, Brazil

Correspondence to: **Anna Léa Silva Barreto**
E-mail: annalsbarreto13@gmail.com

**In memorian*

Author contributions

Anna Lea Silva Barreto: Study concept, Study design, Literature research, Experimental procedures (all), Data acquisition, Data analysis, Statistical analysis, Manuscript elaboration, Manuscript editing, Manuscript review. Ariadne Nunes Alonso: Literature research, Experimental procedures (proteolytic activity, morphometric analysis of T.E.M.), Data acquisition, Data analysis. Daniel Clemente de Moraes: Literature research, Statistical analysis, Manuscript elaboration, Manuscript review. José Alexandre R. Curvelo: Study design, Literature research, Statistical analysis, Manuscript elaboration, Manuscript review. Kildare Miranda: Study design, Experimental procedures (T.E.M.), Manuscript review, Data acquisition. Maristela Barbosa Portela: Study concept, Study design, Data analysis, Statistical analysis, Manuscript editing, Manuscript review. Antônio Ferreira-Pereira: Guarantor of the integrity of the study, Definition of intellectual content, Manuscript editing, Manuscript review. Thais Souto Padron: Study design, Experimental procedures (T.E.M.), Data acquisition. Rosangela Maria A. Soares: Guarantor of the integrity of the study, Study concept, Definition of intellectual content, Manuscript editing, Manuscript review.