ORIGINAL PAPER

The Journal of Venomous Animals and Toxins including Tropical Diseases
ISSN 1678-9199 | 2011 | volume 17 | issue 2 | pages 150-158

Effects of *Bothrops moojeni* venom on *Leishmania amazonensis* promastigote forms

Castilhos P (1, 2), Pereira CG (1), Silva ALN (1), Napolitano DR (2), Oliveira F (2), Souza MA (1)

(1) Laboratory of Molecular Biology, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Minas Gerais State, Brazil; (2) Laboratory of Biophysics, Federal University of Uberlândia, Uberlândia, Minas Gerais State, Brazil.

Abstract: In the present work, the effect of *Bothrops moojeni* venom fractions on *Leishmania* promastigotes was evaluated. The snake venom was fractionated into five protein fractions (E1 to E5), by ion exclusion chromatography, that were used to treat *Leishmania amazonensis* and *Leishmania braziliensis* promastigote forms whereas the viability and nitric oxide production were evaluated. It was observed that E5 venom fraction strongly inhibited *Leishmania amazonensis* nitric oxide production, while in *Leishmania braziliensis* the nitric oxide production was enhanced in all doses. *Bothrops moojeni* crude venom reduced the viability of both parasites in a dose-dependent manner and a peptide of 64 kDa was apparently degraded. *Bothrops moojeni* E5 venom fraction only reduced the viability and nitric oxide production of *Leishmania amazonensis* and no protein degradation was observed. Thus, these results suggest that *Bothrops moojeni* E5 venom fraction may offer components with a promising antileishmanial therapeutic application.

Key words: *Leishmania* spp., *Bothrops moojeni,* snake venom, nitric oxide.

INTRODUCTION

Human parasitic infections – including malaria, sleeping sickness, Chagas' disease, leishmaniasis, filariasis and schistosomiasis – have a devastating impact on global health and economic development (1). Leishmaniasis is a parasitosis caused by several species of the protozoa *Leishmania* and it is currently endemic in 88 countries (2, 3). The parasites are phagocytized by macrophages and converted into amastigotes, which multiply within and survive in parasitophorous vacuoles of these cells and infect surrounding macrophages (4). Moreover, the disease progression depends on which species of *Leishmania* is involved, as well as on the genetics and immune status of the host (5).

Parasites have different strategies to escape the host defense system and also to take advantage of host biochemical factors. In trypanosomatids, a nitric oxide (NO) pathway mediates protection against apoptotic death (6). Although macrophages trigger the host defense mechanism to neutralize the parasite, evidence shows that NO pathway, from *L* (*L.*) *amazonensis*, participates on parasite-host interaction, whereas a correlation between NO production and the amount of metacyclic forms in the culture of infective forms was found (7). The involvement of *Leishmania* spp. and NO pathway in host-macrophage interaction showed the importance of both NO pathways in the parasite-host interplay.

The antileishmanial chemotherapy relies on a limited range of drugs including amphotericin B, pentavalent antimonials, such as sodium stibogluconate (Pentostan®) and N-methyl-glucamine antimoniate (Glucantime®) (8). However, the action mechanism of pentavalent antimonials have not yet been clearly determined (8).

All these drugs are limited to some extent by their toxicity, lack of efficacy in endemic areas, and difficulty of administration because of their long term treatment and high cost (9). The effect on parasite viability was evaluated with several drugs *in vitro* and *in vivo*. For example, treatment with imidocarb reduced parasite burden in experimental *L. amazonensis* infections in mice (10). Also, it has been demonstrated that Brazilian propolis reduced *L. amazonensis* infection in macrophages (4). Currently, the chemotherapy remains the mainstay for the leishmaniasis control, since effective vaccines have yet been developed (11).

In order to develop alternative tools to subdue infectious diseases, several substances have been tested for treating the parasite, such as snake venoms, aiming at the development of future drugs that disrupt the viability of protozoan. Snake venoms comprise a complex mixture of proteins that present various physiologic effects and provoke systemic alterations such as systemic bleeding, coagulopathy, hypovolemia, hemodynamic instability and shock, and acute renal failure (12, 13). *Bothrops moojeni* is frequently found in central and south-eastern Brazil, throughout the Cerrado morphoclimatic domain, and its venom contains a wide range of proteases, which may be characterized as coagulant, anticoagulant or fibrinolytic factors that inhibit cytokines and modulate the immune system of the host (14, 15). *Bothrops jararaca* venom leads to cell death of epimastigote forms of *Trypanosoma cruzi* induced by stress, and *Bothrops moojeni* crude venom was demonstrated to kill *Leishmania* spp. *in vitro* (16). A myotoxic phospholipase A_2 homologue from *B. moojeni* venom, called MjTX-II, was characterized and displayed antiparasitic effects against *Schistosoma mansoni* and *Leishmania* spp. Thus, MjTX-II is a promising candidate for future therapeutic applications (17). In the present study, we evaluated the effect of *B. moojeni* venom and its fractions on viability, protein profile and on nitric oxide production of *Leishmania* promastigotes *in vitro*.

MATERIALS AND METHODS

Parasites

Leishmania (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8 strain) was obtained from the Zoonosis Control Center of the municipal authority of São Paulo, São Paulo state, Brazil and *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/75/M2903 strain) was obtained from the Infectious Diseases Center of the Federal University of Espírito Santo, Vitória, Brazil. Promastigote forms were grown in a brain heart infusion (BHI) medium (Oxoid, England) supplemented with 10% of fetal bovine serum (FBS) and 1 µg/mL gentamicin (Cultilab, Brazil) at 25°C. Once the culture reached the stationary phase, the parasites were harvested and centrifuged at 3,000 x *g* at 4°C for 15 minutes and then washed four times by centrifugation in a sterile phosphate buffered saline solution (PBS) pH 7.2. The concentration of parasites was adjusted to 2 x 10^5/mL in BHI medium.

Fractionation of *Bothrops moojeni* Venom by Ion Exchange Chromatography

Desiccated *Bothrops moojeni* venom was purchased from Bio-Agents Serpentarium (Batatais, Brazil) and stored at –20°C until use. *B. moojeni* crude venom (200 mg) was dissolved in 0.05 M ammonium bicarbonate buffer (AMBIC), pH 7.8, and clarified by centrifugation at 10,000 x g for ten minutes. The supernatant was chromatographed on a DEAE Sephacel column (Sigma Chemical Co., USA) (1.7 X 15 cm), previously equilibrated with 0.05 M AMBIC (pH 7.8) and eluted with a concentration gradient (0.05 M to 0.3 M) of the same buffer. Fractions of 3.0 mL/tube were collected (flow rate of 20 mL/hour) and their absorbances were read at 280 nm. The samples were pooled, lyophilized and stored at –20°C until the moment of use. Protein concentration was determined by Itzhaki and Gill (18) method using bovine serum albumin as standard.

Gel Electrophoresis

To analyze protein profile of venom fractions and *Leishmania* promastigotes treated with these fractions, a 14% polyacrylamide gel electrophoresis in presence of sodium dodecyl sulphate (SDS-PAGE) was performed as previously described (19). Electrophoresis was carried out at 20 mA/gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. The molecular weight standard proteins (Sigma Chemical Co., USA) employed were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), soybean

trypsin inhibitor (20.1 kDa) and α-lacto albumin (14.4 kDa). The slab gels were stained with Coomassie blue R-250. The relative molecular mass of the proteins was estimated by Kodak 1D image analysis software (USA).

Leishmania Promastigotes Treated with *Bothrops moojeni* Crude Venom and Fractions

Leishmania promastigotes were cultured in BHI medium, in 96-well plates (2 x 10^5 parasites/well) and incubated at 25°C for 72 hours with different doses of *Bothrops moojeni* crude venom (25, 12.5, 3.125, 1.56 µg) or venom fractions (2.5, 1.25, 0.625, 0.3125 µg) or amphotericin B (25, 12.5, 6.25, 3.125, 1.56 µg) in triplicate or were not treated (BHI medium only; negative control). Parasite viability was analyzed by 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide assay (MTT). After centrifugation of the plates the supernatant was collected and NO production was determined. These samples of treated parasites were also used to analyze the effect of *Bothrops moojeni* venom on protein profile of *Leishmania*. After centrifugation at 2,000 x *g* for ten minutes, the pellet was treated with sodium dodecyl sulphate (SDS) and 2-mercaptoethanol and heated at 100°C for three minutes and run in 14 % polyacrylamide gel.

Leishmania Promastigote Viability Tests

Parasite viability was estimated by MTT assay (20). Briefly, *L. amazonensis* and *L. braziliensis* cultures in 96-well plates (treated or not with *B. moojeni* venom, E1, E2, E3, E4, E5 fractions or amphotericin B for 72 hours) were treated with 10 µL of MTT (Sigma-Aldrich, USA) at 5 mg/mL and 90 µL BHI and incubated at 25°C for four hours. The reaction was stopped with 100 µL of isopropyl alcohol with 2.5 mM chloridric acid and incubated for ten minutes and the absorbance was read at a plate reader (Molecular Devices, USA) at 570 nm. All the assays were done in triplicate. The viability rate was calculated by the following equation:

$$\% \text{ viability} = \frac{\text{ABS test x 100}}{\text{ABS negative control}}$$

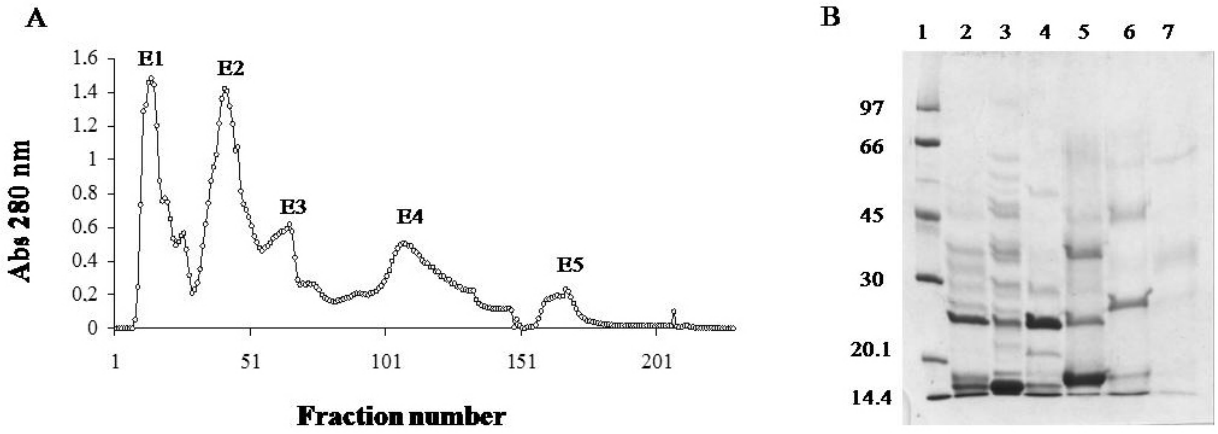

Figure 1. (**A**) Ion exchange chromatography on DEAE-Sephacel. *B. moojeni* crude venom (200 mg) was applied on the column (1.7 x 15 cm) and elution was carried out at a flow rate of 20 mL/hour with ammonium bicarbonate (Ambic) gradient buffer, pH 7.8, from 50 mM to 0.45 M. (**B**) SDS-PAGE (14%) gel in reduced condition of the fractions of the DEAE-Sephacel chromatography. Tris-glycine buffer, pH 8.3, and 20 mA. Lane 1: standard proteins; lane 2: *Bothrops moojeni* crude venom; lane 3: E1 fraction; lane 4: E2 fraction; lane 5: E3 fraction; lane 6: E4 fraction; lane 7: E5 fraction of *B. moojeni* venom. The gel was stained with Coomassie blue R-250.

Nitric Oxide Measurement

NO production in supernatants of *Leishmania* culture was estimated by accumulation of NO_2– (21). Briefly, 50 µL of Griess reagent [1 % sulfanilamide in 2.5 % H_3PO_4 and 0.1 % naphthylethylenediaminedihydrochloride (NEED) in 2.5 % H_3PO_4 (v/v)] was added to 50 µL of each sample in a 96-well plate and incubated at 25°C for 20 minutes. Blank reference and standard curve were determined. The absorbance was measured at 540 nm using a plate reader (Molecular Devices, USA). Nitrite content (µM) was quantified by extrapolation from sodium nitrite standard curve in each experiment. All the assays were performed in triplicate.

Statistical Analysis

Comparisons with control were performed by analysis of variance (one-way ANOVA). Significant differences were analyzed by the Tukey's Multiple Comparison test (Statistics Package for Social Sciences, version 10.0). Differences were considered to be significant when $p < 0.05$.

RESULTS

Profile of *B. moojeni* Crude Venom and Protein Fractions

B. moojeni venom was fractionated into five protein fractions by ion exchange chromatography in a DEAE Sephacel column and the resulting fractions were denominated E1, E2, E3, E4 and E5 (Figure 1 – A). After electrophoresis of these proteins in polyacrylamide gel at the same concentration (Figure 1 – B), E1 was shown to contain peptides markedly stained, with molecular weight of 14, 15 and 24 kDa (Figure 1 – B). E2 fraction presented strongly stained bands corresponding to 15 e 24 kDa. Three major bands were also observed for E3 fraction, with molecular weights of 36, 25, 15 kDa. E4 showed bands modestly stained with 15, 27 and 46 kDa. The protein pattern of E5 was observed as bands with lower intensity but clearly stained with molecular weights of 15 and 35 kDa (Figure 1 – B).

Effect of *B. moojeni* Venom and Protein Fraction on *Leishmania*

B. moojeni crude venom and its fractions were utilized to evaluate their effects on *Leishmania*

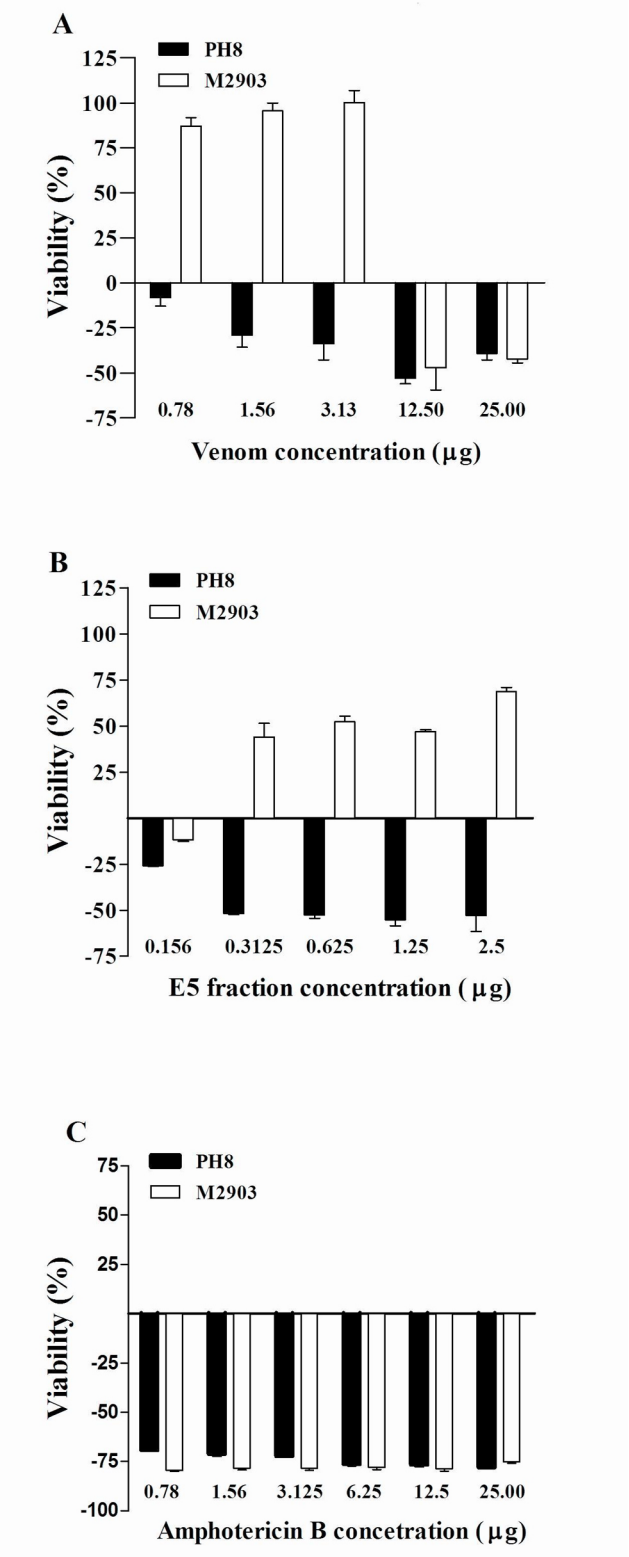

Figure 2. *Leishmania* viability by MTT method. (**A**) Parasites ($5x10^5$ cells/well) were incubated for 72 hours with *B. moojeni* crude venom; (**B**) *B. moojeni* venom E5 fraction; (**C**) amphotericin B. In the experiments, *L. amazonensis* (PH8) and *L. braziliensis* (M2903) were used. Columns shown viability (means ± standard deviations from three independent experiments) expressed in %.

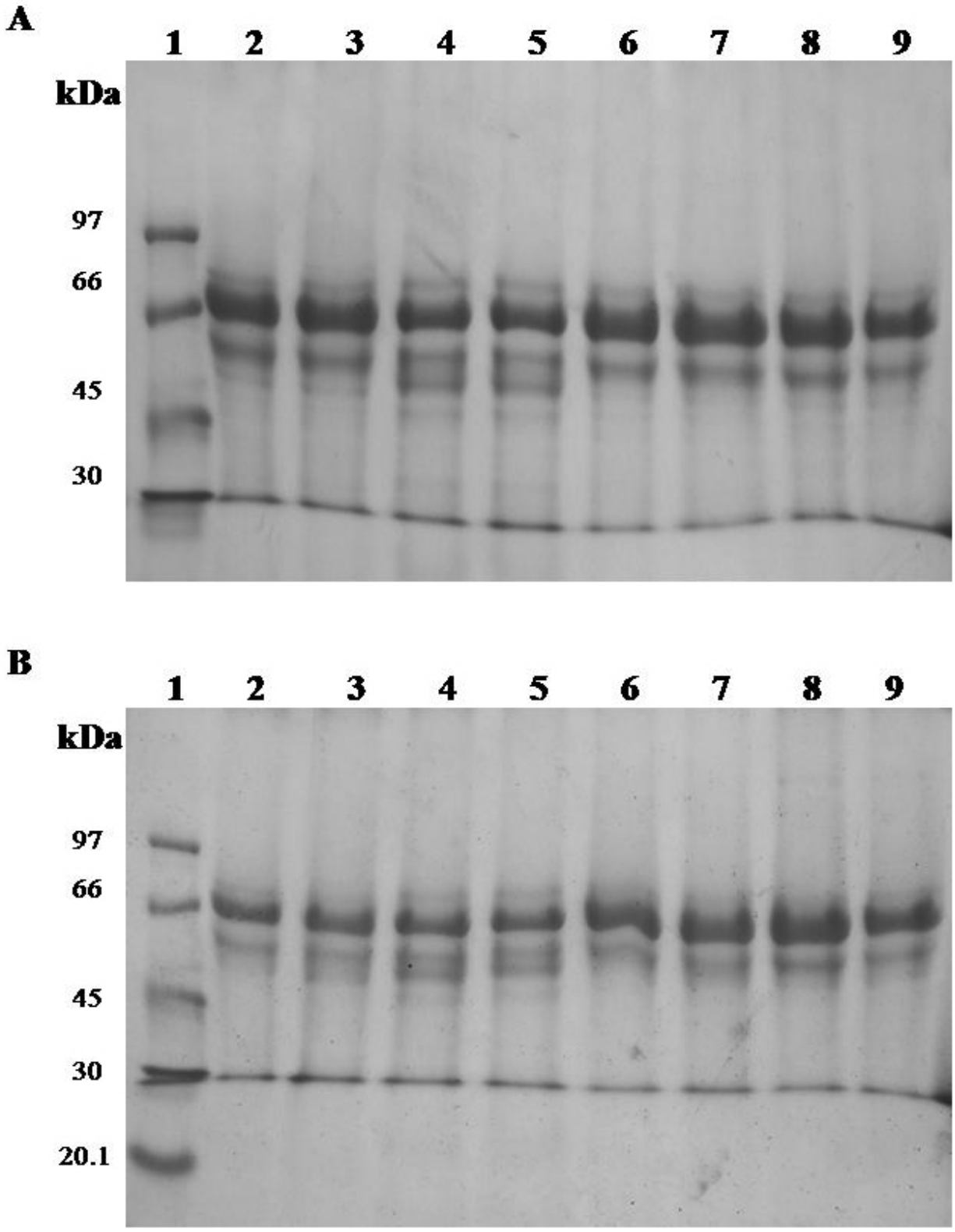

Figure 3. Protein profile of (**A**) *L. amazonensis* (PH8) and (**B**) *L. braziliensis* (M2903) by SDS-PAGE (14%) in reduced condition after treatment. Lane 1: standard proteins; lanes 2 and 3: *Leishmania* without treatment; lanes 4 and 5: treatment with *B. moojeni* crude venom (25 µg); lanes 6 and 7: treatment with *B. moojeni* venom E5 fraction (5 µg); lanes 8 and 9: treatment with amphotericin B (25 µg). The gel was stained with Coomassie blue R-250. The results presented are representative of three other experiments performed independently.

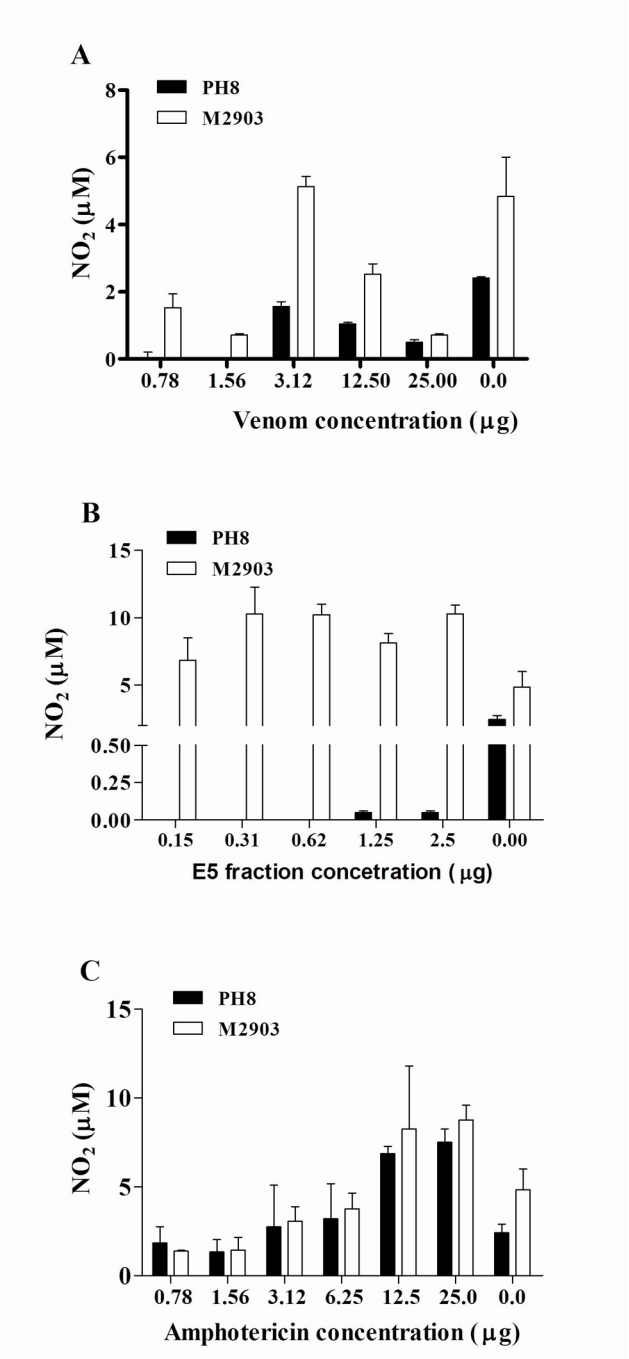

Figure 4. Effect of treatment on NO production by *L. amazonensis* (PH8) and *L. braziliensis* (M2903). NO levels were determined in supernatants from cultures of *Leishmania* treated with (**A**) *B. moojeni* crude venom, (**B**) *B. moojeni* venom E5 fraction and (**C**) amphotericin B, in different doses, or without treatment (medium). Columns shown nitrite (NO_2) levels (means ± standard deviations from three independent experiments) expressed in µM.

viability by MTT test. However, only the E5 fraction showed activity, while the others fractions (E1, E2, E3 and E4) revealed irregular viability alterations. It was observed that both *B. moojeni* crude venom and E5 fraction presented inhibitory activities on parasite viability in a dose-dependent manner (Figure 2). *L. amazonensis* was demonstrated to be sensitive to crude venom (≥ 0.78 µg) and E5 fraction (≥ 0.156 µg) (Figure 2 – A and B). *L. braziliensis* was affected only by higher doses of crude venom (25 and 12.5 µg) and lower concentration of E5 fraction (0.156 µg), and showed enhancement in viability at the highest doses of E5 fraction (Figure 2 – A and B). Besides, both *Leishmania* strains were sensitive to amphotericin B in all analyzed doses (Figure 2 – C). Statistical analysis among amphotericin B, crude venom and E5 fraction showed to be significant ($p < 0.001$). On the other hand, comparison between venom and E5 fraction showed no difference.

In addition, the protein pattern of *Leishmania* treated with crude venom, E5 fraction and amphotericin-B was analyzed on SDS-PAGE. An interesting protein profile of *L. amazonensis* treated with crude venom was observed when compared to non-treated parasites. More specifically, a peptide with 64 kDa was apparently degraded in two other peptides of 56 and 51 kDa (Figure 3 – A). However, when *L. amazonensis* and *L. braziliensis* were treated with E5 fraction or amphotericin B, no protein degradation was observed (Figure 3 – A and B).

Nitric Oxide Production by *Leishmania* after Treatment with *B. moojeni* Venom and Protein Fractions

NO levels were determined in supernatants from cultures of *L. amazonensis* and *L. braziliensis* treated with *B. moojeni* crude venom, E5 fraction or amphotericin B in different doses. By analysis of *L. amazonensis* supernatants, it was observed that crude venom (Figure 4 – A) and E5 fraction, mainly, (Figure 4 – B) strongly inhibited NO production in different doses when compared with venom and amphotericin B ($p < 0.001$). On the other hand, when supernatants from *L. braziliensis* were analyzed (Figure 4), crude venom showed irregular inhibition of NO production (Figure 4 – A), while it was observed enhancement in NO production by parasites treated with E5 fractions (Figure 4 – B) at any concentration. Treatment of *L. braziliensis* with amphotericin-B led to enhance of NO production in a dose-dependent manner (Figure 4 – C).

DISCUSSION

B. moojeni Venom Fractioning and Its Effect on Parasite Viability and Protein Pattern Expression

Biochemical studies in *Leishmania* spp. have focused on the extracellular promastigote form, instead of the intracellular amastigote form, since promastigotes are easier to culture *in vitro*. In our study, the assessment of the effects of *B. moojeni* venom on *Leishmania* promastigotes was carried out after DEAE-Sephacel chromatography. Venom fractionation by ion exclusion chromatography resulted in five fractions. A strongly stained band of 15 kDa observed in E1 fraction could probably be myotoxin (22, 23). E2 fraction presented a band corresponding to 24 kDa, and it could be metalloproteinase and the proteins present in this fraction showed substantial proteolytic activity towards azocasein and fibrinogen (data not shown). Three major bands were observed for E3 fraction, and the 36 kDa peptide appears to belong to the proteases family with an anti-thrombosis effect (24). When analyzing the protein pattern of E5 fraction, bands with lower intensity but clearly stained with molecular weight of 15 and 35 kDa were observed. However, these fractions that were detected could be corresponding to L-amino acid oxydase (LAAO), a 70 kDa protein, responsible for the yellow stain of the crude venom and also known for its anti-parasite effect (25).

In this study, the effect of *B. moojeni* venom on *Leishmania* viability was tested using MTT assay for cell viability after incubation with crude venom and its fractions obtained by chromatography. We demonstrated that crude venom reduces viability of both species, although *L* (*L.*) *amazonensis* was demonstrated to be sensitive to crude venom (≥ 0.78 µg) and *L* (*V.*) *braziliensis* was demonstrated to be sensitive only in the presence of higher doses of crude venom (25 and 12.5 µg). Among the fractions, only E5 affects *L. amazonensis* viability (≥ 0.156 µg). These results indicate that the crude venom and its fractions act differently on each species of parasite. It is possible to explain the aforementioned differences by considering the contribution of the parasite genotype to parasite virulence and pathogenesis (26).

B. moojeni venom also influenced the protein pattern of *Leishmania* spp. As a variable protein profile for *Leishmania* spp. treated or not with *B. moojeni* venom and its fractions was observed by SDS-PAGE. In fact, *Leishmania* proteins identified by various methods in various species have a wide range of characteristics (27). A band of 64 kDa presented a different profile after treatment with crude venom. These results suggest that *B. moojeni* venom changed the proteins from *Leishmania* either by inducing the production of new proteins or leading to degradation of proteins commonly produced. It was also observed that the crude venom changed the protein pattern of components from BHI medium supplemented with 10% FSB (data not shown). Based on these observations, we believe that the venom probably modified the medium components and then affected parasite growth due to environment changes. Moreover, it could lead to a parasite physiologic alteration with impact on its adaptation (28).

Crude Venom and Its Fractions Inhibited NO Production in Cultures of *Leishmania amazonensis*

L. amazonensis promastigotes produce NO, a free radical synthesized from L-arginine by nitric oxide synthase (NOS) (7). NO produced by high infective promastigotes is significantly increased when compared to low infective promastigotes. Thus, suggesting an association with parasite forms differentiation and its infectivity (7). Our findings indicated that different doses of E5 venom fraction and crude venom strongly inhibited NO production by *L. amazonensis* supernatants. On the other hand, when supernatants from *L. braziliensis* were verified, it was observed an enhancement in NO production by parasites treated with E5 venom fraction, in all doses analyzed, while the crude venom altered irregularly the NO production (Figure 4 – A). Thus, it is suggested that *L. amazonensis* infectivity could be altered due to the venom treatment, once its NO production was inhibited. The use of amphotericin B did not alter the protein profile neither NO production by *Leishmania* spp. It is worth mentioning that neither *B. moojeni* crude venom and E5 fraction nor amphotericin B decreased NO production by *L. braziliensis*. On the contrary, it was observed an increase in NO production after treatment with these drugs. Therefore, we can argue if the NO pathway would be restricted to *L. amazonensis* and not a common feature among *Leishmania* species.

Amphotericin B acts on ergosterol, a steroid present in the membrane of *Leishmania*, by increasing the permeability of the cell membrane. As consequence, it promotes an ion flux into the parasite, thus leading to parasite death (29). B. *moojeni* venom appears to act in a different way. Gonçalves *et al.* (30) demonstrated mitochondrial alterations by electronic microscopy after treatment with *Bothrops jararaca* venom and a possible explanation for the observed mitochondrial swelling may be that venom inhibited the parasite respiratory chain, thus lowering ATP levels, leading to mitochondrial swelling and finally to parasite death.

This report demonstrated *in vitro* effects of *B. moojeni* crude venom and its protein fractions on *L. amazonensis* and *L. braziliensis* promastigote forms. Our data corroborate results previously obtained by Tempone *et al.* (25), who showed that L-amino acid oxidase from crude venom of *B. moojeni* presented a *in vitro* killing effect against *Leishmania* spp. Our data showed that *in vitro* treatment of *L. amazonensis* promastigotes with *B. moojeni* E5 venom fraction and crude venom inhibited NO production and modified the protein expression profile. In addition, we observed reduced promastigote viability after treatment. Taken together, these data suggest that *B. moojeni* E5 venom fraction could have components with a promising leishmanicidal activity.

SUBMISSION STATUS
Received: November 5, 2010.
Accepted: February 10, 2011.
Abstract published online: February 10, 2011.
Full paper published online: May 31, 2011.

CONFLICTS OF INTEREST
There is no conflict.

FINANCIAL SOURCE
The National Council for Scientific and Technological Development (CNPq) and The State of Minas Gerais Research Foundation (Fapemig) provided the financial grants.

CORRESPONDENCE TO
MARIA A. SOUZA, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Av. Pará, 1720, Campus Umuarama, bloco 6T07, Uberlândia, MG, 38.400-902, Brasil. Phone: +55 34 3218 2549. Fax: +55 34 3218 2333. Email: masouza@ufu.br.

REFERENCES
1. Peters N, Sacks D. Immune privilege in sites of chronic infection: *Leishmania* and regulatory T cells. Immunol Rev. 2006;213(1):159-79.
2. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. Lancet. 2005;366(9496):1561-77.
3. World Health Organization (WHO) [homepage]. Geneva: WHO; 2011 [cited 2010 Nov 5]. Leishmaniasis. Available from: www.who.int/leishmaniasis/en/.
4. Ayres DC, Marcucci MC, Giorgio S. Effects of Brazilian propolis on *Leishmania amazonensis*. Mem Inst Oswaldo Cruz. 2007;102(2):215-20.
5. McConville MJ, Handman E. The molecular basis of *Leishmania* pathogenesis. Int J Parasitol. 2007;37(10):1047-51.
6. Piacenza L, Peluffo G, Radi R. L-Arginine-dependent suppression of apoptosis in *Trypanosoma cruzi*: contribution of the nitric oxide and polyamine pathways. Proc Nat Acad Sci. 2001;98(13):7301-6.
7. Genestra M, Guedes-Silva D, Souza WS, Cysne-Finkeltein L, Soares-Bezerra RJ, Monteiro FP, et al. Nitrite oxide synthase (NOS) characterization in *Leishmania amazonensis* axenic amastigotes. Arch Med Res. 2006;37(3):328-33.
8. Amato VS, Tuon FF, Bacha HA, Amato Neto V, Nicodemo AC. Mucosal leishmaniasis. Current scenario and prospects for treatment. Acta Trop. 2008;105(1):1-9.
9. Davies CR, Kaye P, Croft SL, Sundar S. Leishmaniasis: new approaches to disease control. Brit Med J. 2003;326(7385):377-82.
10. Rodrigues FH, Afonso-Cardoso SR, Gomes MAB, Belleti ME, Rocha A, Guimarães AHB, et al. Effects of imidocarb and levamisole on the experimental infection of BALB/c mice by *Leishmania* (*Leishmania*) *amazonensis*. Vet Par. 2006;139(1-3):37-46.
11. Palatnik-de-Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. Vaccine. 2008;26(14):1709-24.
12. Estevão-Costa MI, Diniz CR, Magalhães A, Markland FS, Sanchez E. Action of

metalloproteinases mutalysin i and ii on several components of the hemostatic and fibrinolytic systems. Thromb Res. 2000;99(4):363-73.
13. Gutiérrez JM, Escalante T, Rucavado A. Experimental pathophysiology of systemic alterations induced by *Bothrops asper* snake venom. Toxicon. 2009;54(7):976-87.
14. Marsh NA, Williams V. Practical applications of snake venom toxins in haemostasis. Toxicon. 2005;45(8):1171-81.
15. Costa LA, Fornari MC, Berardi VE, Miles HA, Diez RA. *In vivo* effect of snake phospholipase A_2 (crotoxin + cardiotoxin) on serum IL-1α, TNF-α and IL-1ra level in humans. Immunol Lett. 2001;75(2):137-41.
16. Deolindo P, Teixeira-Ferreira AS, Melo EJT, Arnholdt ACV, Souza W, Alves EW, et al. Programmed cell death in *Tripanosoma cruzi* induced by *Bothrops jararaca* venom. Mem Inst Oswaldo Cruz. 2005;100(1):33-8.
17. Stábeli RG, Amui SF, Sant'ana SD, Pires MG, Nomizo A, Monteiro MC, et al. *Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A_2 homologue: An example of function versatility of snake venom proteins. Comp Biochem Physiol Part C Toxicol Pharmacol. 2006;142(3-4):371-81.
18. Itzhaki RF, Gill DM. A micro-biuret method for estimating proteins. Anal Biochem. 1964;9(4):401-10.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol. 1983;65(1-3):55-63.
21. Green LC, Wagner DA, Glogowki J, Skipper PL, Wishnor JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem. 1982;126(1):131-8.
22. Soares AM, Andrião-Escarso SH, Ângulo Y, Lomonte B, Gutiérrez JM, Marangoni S, et al. Structural and functional characterization of a myotoxin I from *Bothrops moojeni* (caissaca) snake venom. Arch Biochem Biophys. 2000; 373(1):7-15.
23. Soares AM, Rodrigues VM, Homsi-Brandeburgo MI, Toyama MH, Lombardi FR, Arni RK, et al. A rapid procedure for the isolation of the Lys-49 myotoxin II from *Bothrops moojeni* (caissaca) venom: biochemical characterization, crystallization, myotoxic and edematogenic activity. Toxicon. 1998;36(3):503-14.
24. Bernardes CP, Santos-Filho NA, Costa TR, Gomes MS, Torres FS, Costa J, et al. Isolation and structural characterization of a new fibrin(ogen) olytic metalloproteinase from *Bothrops moojeni* snake venom. Toxicon. 2008;51(4):574-84.
25. Tempone AG, Andrade HF Jr, Spencer PJ, Lourenço CO, Rogero JR, Nascimento N. *Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase. Biochem Biophys Res Commun. 2001;280(3):620-24.
26. Smith DF, Peacock C, Cruz AK. Comparative genomics from genotype to disease phenotype in the leishmaniasis. Inter J Parasitol. 2007;37(11):1173-86.
27. Kubar J, Fragaki K. *Leishmania* proteins derived from recombinant DNA: current status and next steps. Trends Parasitol. 2006;22(3):111-6.
28. Clayton CE. Life without transcriptional control? From fly to man and back again. EMBO J. 2002;21(8):1881-8.
29. Ellis D. Amphotericin B: spectrum and resistance. J Antimicrob Chem. 2002;49(Suppl. 1):7-10.
30. Gonçalves AR, Soares MJ, Souza W, DaMatta RA, Alves EW. Ultrastructural alterations and growth inhibition of *Trypanosoma cruzi* and *Leishmania major* induced by *Bothrops jararaca* venom. Parasitol Res. 2002;88(7):598-602.