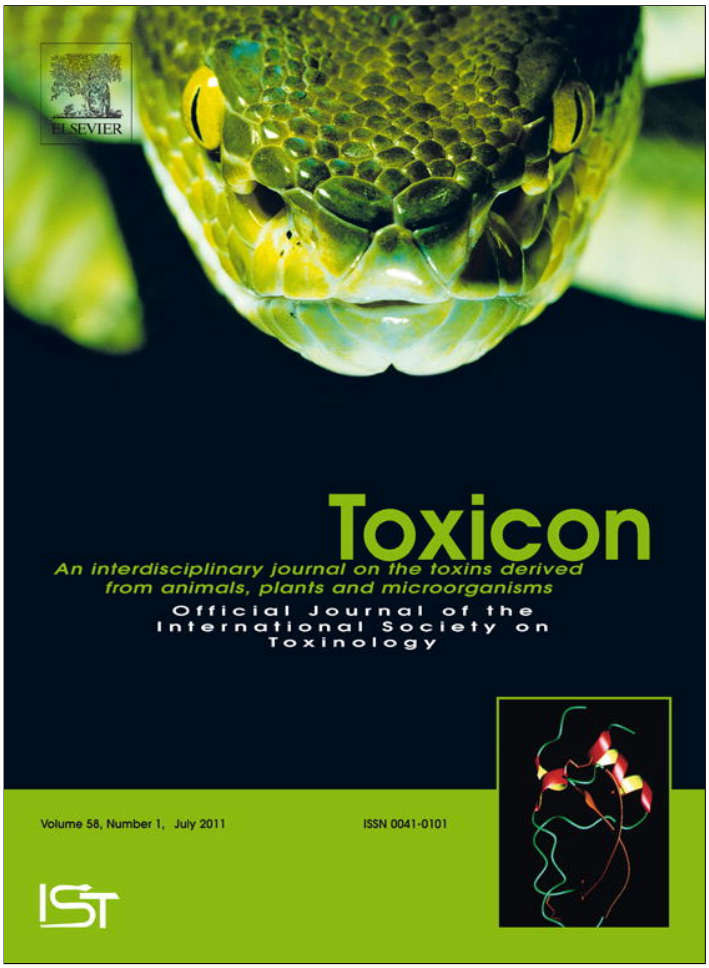

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A comparative study of the effects of venoms from five rear-fanged snake species on the growth of *Leishmania major:* Identification of a protein with inhibitory activity against the parasite

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ABSTRACT

Leishmania parasites of several species cause cutaneous and visceral disease to millions of people worldwide, and treatment for this vector-borne protozoan parasite typically involves administration of highly toxic antimonial drugs. Snake venoms are one of the most concentrated enzyme sources in nature, displaying a broad range of biological effects, and several drugs now used in humans were derived from venoms. In this study, we compared the effects of the venoms of the South American rear-fanged snakes *Philodryas baroni* (PbV), *Philodryas olfersii olfersii* (PooV) and *Philodryas patagoniensis* (PpV), and the North American rear-fanged snakes *Hypsiglena torquata texana* (HttV) and *Trimorphodon biscutatus lambda* (TblV), on the growth of *Leishmania major*, a causative agent of cutaneous leishmaniasis. Different concentrations of each venom were incubated with the log-phase promastigote stage of *L. major*. TblV showed significant anti-leishmanial activity (IC_{50} of 108.6 μg/mL) at its highest concentrations; however, it induced parasite proliferation at intermediate concentrations. PpV was not very active in decreasing the parasitic growth, and a high final concentration (1.7 mg/mL) was necessary to inhibit proliferation by only 51.5% ± 3.6%. PbV, PooV and HttV, at final concentrations of 562, 524 and 438 μg/mL respectively, had no significant effect on *L. major* growth. The phospholipase A_2 of TblV (trimorphin) was isolated and assayed as for crude venom, and it also exhibited dose-dependent biphasic effects on the parasite culture, with potent cytotoxicity at higher concentrations (IC_{50} of 0.25 μM; 3.6 μg/mL) and stimulation of proliferation at very low concentrations. Anti-leishmanial activity of TblV appears to be solely due to the action of trimorphin. This is the first report of anti-leishmanial activity of rear-fanged snake venoms, and these results suggest novel possibilities for discovering new protein-based drugs that might be used as possible agents against leishmaniasis as well as tools to study the biology of *Leishmania* parasites.

1. Introduction

Leishmaniasis is an anthropozoonosis caused by cutaneous infection with protozoan parasites of the genus *Leishmania* following the bite of an infected sandfly (commonly *Phlebotomus* sp.). It is a severe disease affecting millions of people in Africa, America, Europe and Asia (Desjeux, 1992). Because of its prevalence and morbidity, this disease has been considered a serious health problem by the World Health Organization (WHO) and is statistically the second most important parasitic disease worldwide (Rath et al., 2003).

Currently, the main drugs used for the treatment of patients are pentavalent antimonials, whereas the antifungal

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drug amphotericin B (a polyene macrolide antibiotic) is considered an efficient treatment option for many antimonial-resistant strains of *Leishmania* (Rosenthal et al., 2009). Other drugs, such as allopurinol, miltefosine and pentamidine also represent important options for leishmaniasis treatment (Loiseau and Bories, 2006). However, effective new anti-leishmanial compounds are needed due to the high costs, development of parasite resistance and side effects related to those drugs currently in use. Importantly, no licensed human vaccine for leishmaniasis is available.

A productive, more recent approach to the search for more efficient and less toxic chemotherapeutic agents has been the screening of natural compounds able to inhibit protistan growth. Snake venoms, one of the most concentrated enzyme sources in nature, are complex mixtures of proteins, peptides and small organic molecules whose composition varies phylogenetically and as a function of other factors, overall displaying a broad range of biological effects (Mackessy, 2010). Previous studies have shown that the venom of the front-fanged snakes *Bothrops marajoensis* (Costa Torres et al., 2010), *Bothrops moojeni* (Tempone et al., 2001), *Bothrops jararaca* (Gonçalves et al., 2002), *Crotalus durissus terrificus*, *Crotalus durissus cascavella*, *Crotalus durissus collilineatus* (Passero et al., 2007), *Cerastes cerastes* and *Naja haje* (Fernandez-Gomez et al., 1994) can inhibit the growth of different *Leishmania* species. However, at present no venoms from rear-fanged snakes, most species of which do not represent a risk to humans, have been evaluated for anti-protist activity.

There is tremendous potential for discovering novel bioactive compounds in rear-fanged snake venoms, because this is the most speciose group of advanced snakes and relatively few studies have investigated their composition and biological activities (Mackessy, 2002). The current study evaluates new sources of potential anti-leishmanial compounds from rear-fanged snake venoms. In this study, we compared the effects of the venoms of the South American rear-fanged snakes *Philodryas baroni* (PbV), *Philodryas olfersii olfersii* (PooV) and *Philodryas patagoniensis* (PpV), and the North American rear-fanged snakes *Hypsiglena torquata texana* (HttV) and *Trimorphodon biscutatus lambda* (TblV), as well as a purified PLA_2 from *T. b. lambda* venom, on the growth of the promastigote stage of *Leishmania major*, a causative agent of cutaneous leishmaniasis, as an initial screen of their potential anti-leishmanial activity.

2. Materials and methods

2.1. Rear-fanged snake venoms

Pools of *P. patagoniensis* and *P. o. olfersii* venoms were obtained from wild specimens captured in northeastern Argentina and then maintained at the serpentarium of the local Zoo, Corrientes, Argentina. Specimens were extracted by introducing a 100-μL micropipette under each fang, according to a procedure described previously (Ferlan et al., 1983).

Hypsiglena torquata and *T. biscutatus* venoms were obtained from wild specimens captured in Colorado and Arizona, USA (scientific collecting permits MCKSY000221 and 0456), and maintained in the UNC Animal Facility. Four captive-born specimens of *P. baroni* were obtained from the Dallas Zoological Park and were also maintained in the UNC Animal Facility. All housing and handling procedures were approved by the UNC-IACUC (protocols #9204.1 & 9401). Extraction of snakes utilized a previously published method (Hill and Mackessy, 1997; Rosenberg, 1992).

After extraction, all venoms were centrifuged, lyophilized and kept frozen at −20 °C. When required, venoms were dissolved in 0.01 M phosphate buffer saline (PBS), pH 7.4, and filtered through a 0.22 μm Millipore filter to remove insoluble material and to sterilize.

2.2. Protein concentration determination

Protein concentration was assayed in triplicate according to Bradford (1976) as modified by BioRad Inc. (Hercules, CA, USA), using bovine gamma globulin as a standard.

2.3. Purification of phospholipase A_2 (PLA_2) from TblV

TblV was dissolved in 25 mM HEPES buffer containing 100 mM NaCl (pH 6.8). Two hundred microliters (4 mg) of the venom was injected onto a TSKgel G2000 SWXL size exclusion column (7.8 mm i.d., 30 cm, 5 μm) (TOSOH Bioscience LLC, Tokyo, Japan) at a flow rate of 0.15 mL/min using the same buffer (Waters HPLC), and chromatograms were recorded using Empower software. Fractions were collected and assayed for PLA_2 activity (see below). Selected fractions were electrophoresed on NuPAGE® Novex® 12% Bis-Tris gels (Invitrogen, Inc., Carlsbad, CA, USA). Active fractions were pooled and applied to a Protein and Peptide C_{18} column (5 μm, 4.6 mm i.d. × 250 mm) (VYDAC, Hesperia, CA, USA) equilibrated with solvent A [0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water], using a Waters HPLC system. Bound proteins were eluted with 0–100% increasing linear gradient of solvent B [0.1% TFA/80% acetonitrile (ACN)] at a flow rate of 1 mL/min for 90 min. The elution profile was monitored at 220 and 280 nm.

2.4. PLA_2 activity assay

PLA_2 enzyme activity was determined by the method of Holzer and Mackessy (1996), using 4-nitro-3-(octanoyloxy) benzoic acid as substrate. The assay buffer was 10 mM Tris–HCl (pH 8.0) containing 10 mM $CaCl_2$ and 100 mM NaCl.

2.5. SDS-PAGE

Crude venoms and purified PLA_2 fractions were subjected to SDS-PAGE using NuPage Bis-Tris gels (Invitrogen, Inc., Carlsbad, CA, USA), MES/SDS running buffer and 24 μg venom/lane as described previously (Weldon and Mackessy, 2010). Following staining with Coomassie brilliant blue R-250 and destaining, the gel was imaged using an AlphaImager (Cell Biosciences, Inc., Santa Clara, CA, USA).

2.6. Mass spectrometry

In order to determine the molecular mass of the purified PLA_2, approximately 1 μg protein in 50% ACN containing

0.1% TFA was spotted onto a MALDI sample holder, mixed with an equal volume of 10 mg/mL sinapinic acid in 50% ACN containing 0.1% TFA, and allowed to dry. Mass spectrum was obtained using a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer.

For unequivocal identification of the purified PLA_2, gel bands of interest were excised, destained and subjected to reduction with DTT, alkylation with iodoacetamide, and then in-gel digestion with mass spectrometry grade Trypsin Gold (Promega, Madison, WI, USA), following the manufacturer's instructions. The tryptic peptide mixtures were purified and concentrated using ZipTip® C18 pipette tips (Millipore Corporation, Billerica, MA, USA). The peptides eluted from the ZipTip® tips were dried in a Speed-Vac and redissolved in 5 μL of 50% ACN containing 0.1% TFA. Digests (1 μL) were spotted onto a MALDI sample holder, mixed with an equal volume of 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50% ACN containing 0.1% TFA, dried, and analyzed with a Bruker Ultraflex II MALDI-TOF/TOF mass spectrometer. Singly-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were sequenced by collision-induced dissociation tandem mass spectrometry. Spectra were interpreted using the online form of the MASCOT program at http://www.matrixscience.com.

2.7. Parasite viability assays

L. major parasites (LV39, RHO/SU/59/P, Neal, or P strain) were obtained as a generous gift from R. Titus (Colorado State University, Ft. Collins, CO, USA). The parasites were isolated from infected foot pads of C57BL/6 mice 7–10 days after infection and maintained *in vitro* as previously described (Sacks and Perkins, 1984) in Schneider's *Drosophila* medium (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS), hemin (0.005%), penicillin (100 U/mL), streptomycin (100 μg/mL), gentamicin (50 μg/mL), $CaCl_2$ (0.6 mg/mL), and $NaHCO_3$ (0.4 mg/mL). Promastigotes in logarithmic phase of growth (1×10^6 parasites/mL, 100 μL/well) were used for parasite viability assays which were performed in 96-well microplates, according to a previous method (Mukherjee et al., 2009) with some modifications. Briefly, venom samples were added to cultured cells at various final concentrations; amphotericin B was utilized as positive control at a final concentration of 100 μg/mL. Controls containing only parasites were also included. Plates were incubated at 26 °C for 72 h, and parasite viability was determined colorimetrically by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (e.g., Passero et al., 2007; Costa Torres et al., 2010) according to the manufacturer's instructions (ATCC, Manassas, VA, USA). The IC_{50} values were calculated from the dose-response curves of each experiment using Microsoft-Excel software. Experimental samples were evaluated in triplicate and experiments were repeated at least twice.

2.8. Statistical analysis

The data are presented as mean ± standard deviation (SD). Results were analyzed using one-way analysis of variance (ANOVA) with the Holm-Sidak method for multiple comparisons versus control group. Statistical analyses were performed using the software SigmaStat for Windows, version 3.5. A value of $p < 0.05$ indicated statistical significance.

3. Results and discussion

The effect of the whole venom from front-fanged snakes on *Leishmania* promastigotes has been well documented (Costa Torres et al., 2010; Fernandez-Gomez et al., 1994; Gonçalves et al., 2002; Passero et al., 2007; Tempone et al., 2001). Nonetheless, no reports in the literature describe the effects of the venom of rear-fanged snakes on *Leishmania* parasites, or even on any other kind of microorganism. Venoms from *P. baroni*, *P. o. olfersii*, *P. patagoniensis*, *H. t. texana* and *T. b. lambda* contain a diversity of protein/peptide components (Fig. 1) and the activity of these "colubrid" venoms toward *L. major* promastigote proliferation was assessed.

Trimorphodon venom showed significant anti-leishmanial activity (Fig. 2), exhibiting an IC_{50} value of 108.6 μg/mL against the promastigote stage. An intriguing stimulant effect on parasite growth was observed at intermediate concentrations of the venom (48 μg/mL). *P. patagoniensis* venom showed low activity toward parasitic

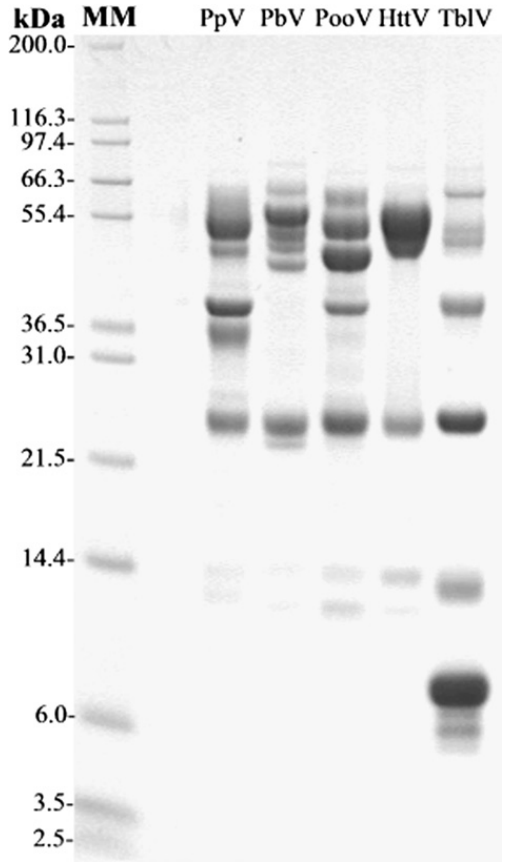

Fig. 1. SDS-PAGE of venoms from five rear-fanged snake species (24 μg) under reducing conditions on NuPAGE Novex 12% Bis-Tris gel; stained with Coomassie Brilliant Blue. Novex Mark 12 molecular mass standards (MM) are on the left lane of the gel.

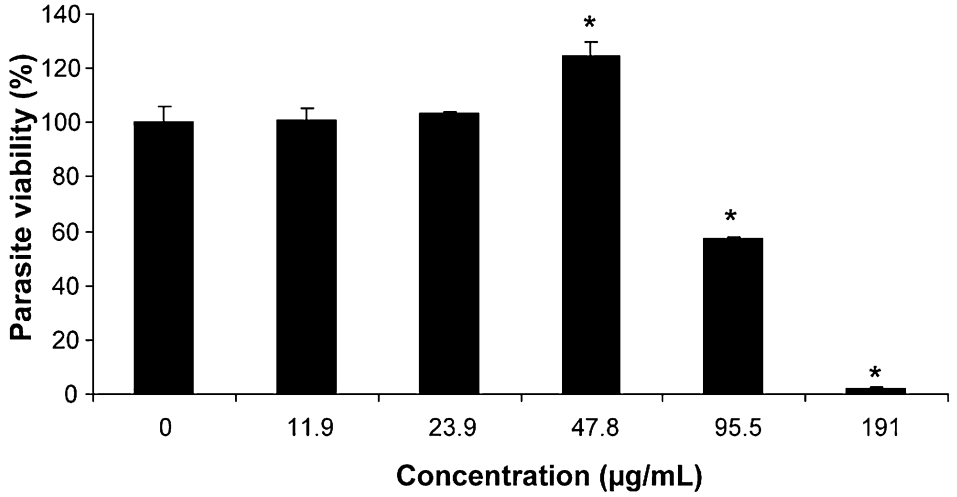

Fig. 2. Effects of different concentrations of TlbV on the log-phase promastigote stage of *L. major*. The initial parasite density was 1×10^6 cells/mL. Parasite viability was determined by MTT assay after 72 h of incubation. The graph presents means ± SD ($n = 3$). *$p < 0.05$ compared to the control group.

growth (Fig. 3), and a very high final concentration of 1.7 mg/mL was necessary to inhibit parasite proliferation by only 51.5 ± 3.6%. This venom represents an example of non-specific effects against parasite growth and results provide rationale for *not* considering it further for possible drug screening. Venoms from the other three species (PbV, PooV and HttV), at final concentrations of 562, 524 and 438 µg/mL respectively, had no significant effect on *L. major* growth (data not shown).

We recently observed that one of the most significant differences among the five rear-fanged snake venoms tested is the presence of PLA_2 activity in TblV only (Peichoto et al., 2010). In addition, a comparison of size exclusion HPLC chromatograms revealed the presence of three differential protein peaks in TblV (Peichoto, unpublished data). Only one of these contained anti-leishmanial activity (Fig. 4a), and it corresponded to the PLA_2 active fraction of the venom. This enzyme was further purified by C_{18} reversed-phase HPLC (Fig. 4b), and SDS-PAGE demonstrated a high degree of purity (Fig. 4c). Mass spectroscopic analysis revealed a single peak with a molecular mass of 13,962 Da (Fig. 4d). This enzyme was previously named trimorphin, the PLA_2 isolated in native form from the venom of the Sonoran Lyre Snake *T. b. lambda* by Huang and Mackessy (2004). SDS-PAGE-separated protein bands were excised, in-gel digested with trypsin and the resulting peptides were analyzed by MALDI-TOF peptide mass fingerprinting (Fig. 4e) followed by MALDI-TOF/TOF. The MS/MS spectrum of the fragmented singly-charged peptide ion ($m/z = 2419.692$) was matched by MASCOT to an internal sequence, NVNCEGDNDECGAFVCECDR, from a PLA_2 (trimorphin) also sequenced from the venom gland transcriptome of *T. biscutatus* (Fry et al., 2008). When assayed for anti-leishmanial activity under similar conditions used for assessing crude venom effects, trimorphin was very active against *L. major* (Fig. 5), exhibiting an IC_{50} value of 0.25 µM (3.6 µg/mL). This value is comparable to that exhibited by gyroxin, the fraction from the venom of *C. d. cascavella* most active against

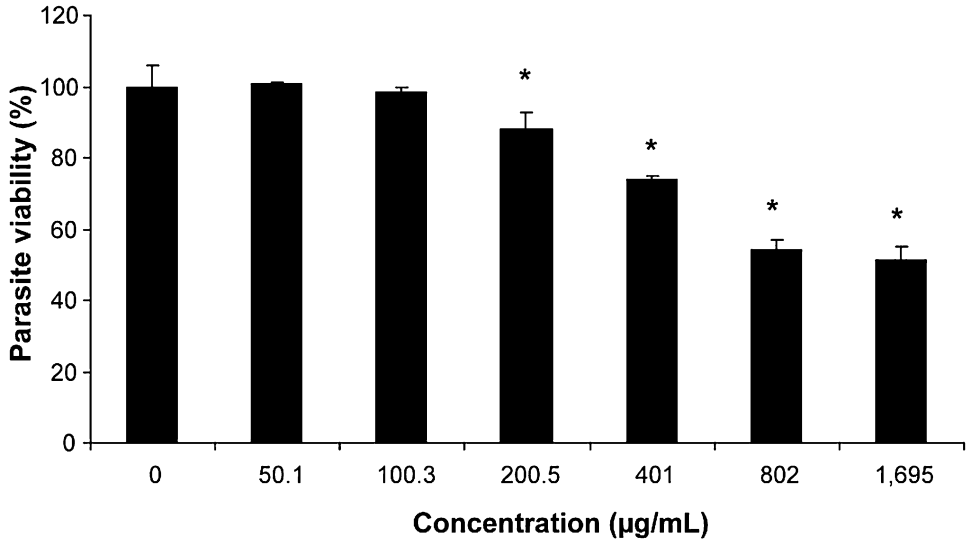

Fig. 3. Effects of different concentrations of PpV on the log-phase promastigote stage of *L. major*. The initial parasite density was 1×10^6 cells/mL. Parasite viability was determined by MTT assay after 72 h of incubation. The graph presents means ± SD ($n = 3$); note that exceptionally high concentrations of this venom were required to obtain statistically significant effects on parasites. *$p < 0.05$ compared to the control group.

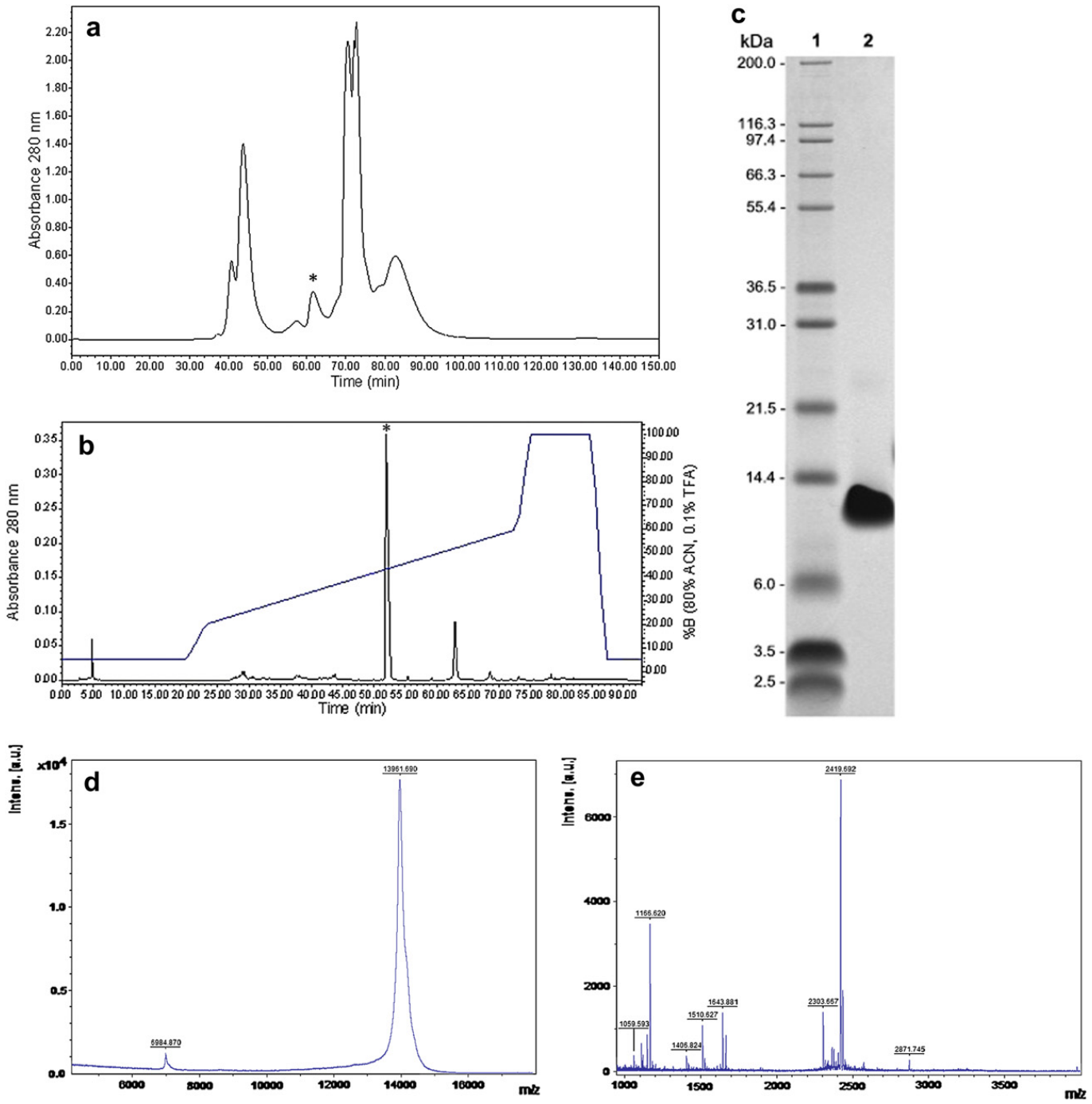

Fig. 4. (a) Size exclusion HPLC separation of crude TblV (4 mg). Proteins eluting between 60 and 65 min (*) showed anti-leishmanial and PLA_2 activities. (b) Second step for purification of PLA_2 on analytical reversed-phase HPLC. The PLA_2 peak from SE-HPLC was applied to a C_{18} column, and the resulting highly purified PLA_2 (*) was eluted with a 20–60% linear gradient of solvent B. (c) SDS-PAGE of PLA_2 from TblV under non-reducing conditions. Lane 1: Novex Mark 12 protein standards. Lane 2: RP-HPLC purified PLA_2 (trimorphin), approximately 10 μg protein; note lack of contaminant bands. (d) MALDI-TOF mass spectrum of the intact enzyme. A single peak with a molecular mass of 13,962 Da is seen, and the 6.98 kDa peak is the doubly charged ion of the enzyme. This data also shows that the isolation method produces a highly purified product. (e) MALDI-TOF mass spectrum of trimorphin in-gel digested with trypsin. MS/MS CID sequence of peptide 2419.692 matched internal sequence of PLA_2 of *Trimorphodon biscutatus*.

Leishmania amazonensis promastigotes (Passero et al., 2007). However, it is less potent than amphotericin B, a standard anti-leishmanial drug, which showed an IC_{50} of 0.2 μg/mL against *L. major* (Sabina et al., 2005), though on a molar basis it shows comparable potency. The amount of purified trimorphin required to eliminate parasite viability is approximately 3.3% of the amount of crude venom required to accomplish the same decrease in viability; significantly, PLA_2 represents about 3.2% of the total protein of the venom. Thus, it is likely that the anti-leishmanial activity of TlbV can be accounted for solely by the mass of trimorphin present in this venom.

Snake venom PLA_2s are multifunctional proteins that induce important biological effects, such as inflammation, myotoxicity and other deleterious effects following snake envenomation (Doley et al., 2010). However, these enzymes

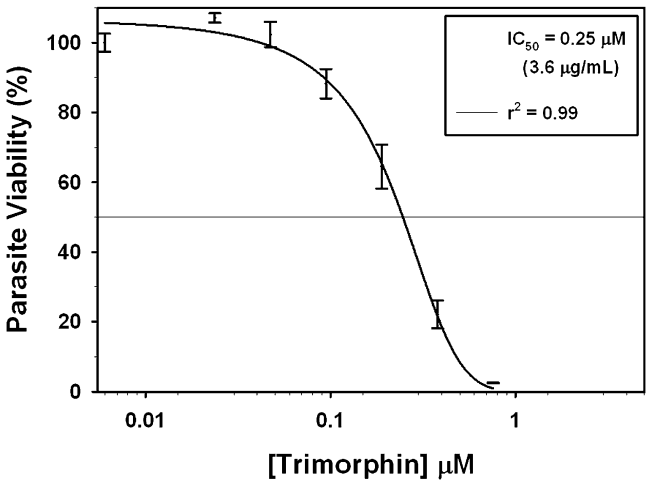

Fig. 5. Effects of different concentrations of trimorphin (PLA_2 isolated from TlbV) on the log-phase promastigote stage of *L. major*. The initial parasite density was 1×10^6 cells/mL. Parasite viability was determined by MTT assay after 72 h of incubation. The graph presents means ± SD ($n = 3$). Trimorphin molar concentrations are based on a mass of 13.962 kDa.

have been extensively studied and used in some applied areas of biomedicine: detection of severe pre-eclampsia, general anesthetic action, treatment of rheumatoid arthritis, as bactericidal agents in lachrymal glands and other tissues, as a new class of HIV inhibitors by blocking host cell invasion, and as potential antimalarial agents (Costa et al., 2008; Soares et al., 2004; Stabeli et al., 2006). In addition, some snake venom PLA_2s show antitumoral activities (Chen et al., 2008; Kessentini-Zouari et al., 2010).

Purified snake venom PLA_2s also show a diversity of effects on parasites, such as *Leishmania* protozoans. MjTX-II, a myotoxic PLA_2 from *B. moojeni* venom that is catalytically inactive, proved to be an effective parasiticidal agent against several *Leishmania* species (*L. amazonensis*, *Leishmania braziliensis*, *Leishmania donovani* and *L. major*) (Stabeli et al., 2006). Moreover, Costa et al. (2008) reported that two myotoxic PLA_2s from *Bothrops brazili* venom (MTX-I, catalytically active, and MTX-II, catalytically inactive) displayed effective parasiticidal activity against *Leishmania* species (*L. amazonensis* and *L. braziliensis*). On the other hand, a PLA_2 from *B. marajoensis* venom exhibited neither significant enzymatic activity nor inhibitory activity against the growth of the parasites *L. amazonensis* and *Leishmania chagasi* (Costa Torres et al., 2010). Furthermore, a PLA_2 isolated from *C. d. collilineatus* was not deleterious toward *L. amazonensis* until after 24 h incubation, but it increased the infectiveness of this parasite in experimental mice (Passero et al., 2008). Thus, not all snake venom PLA_2s are able to inhibit the growth of *Leishmania* parasites.

PLA_2s present in eukaryotic cells mediate important phenomena such as cell migration, growth, differentiation and apoptosis (Farooqui and Horrocks, 2005; Schaeffer et al., 2009). This class of enzymes has been obtained from snake venoms and then used as models to explain a great number of pathophysiological phenomena occurring in human beings, since its structure is similar to human PLA_2s (Rufini et al., 1999). Some snake venom PLA_2s, such as the PLA_2 isolated from *Agkistrodon blomhoffii siniticus*, induce the proliferation of human hepatoma HEP3B cells (Ma et al., 2006). Mora et al. (2005) also observed a similar effect of PLA_2 isolated from *Bothrops asper* venom on lymphoblastoid CRL-8062 cells, and Rufini et al. (1999) reported an increase in multiplication of smooth muscle cells in culture by PLA_2 isolated from *Vipera ammodytes*. Furthermore, Passero et al. (2007) suggested that the presence of PLA_2 (the basic sub-unit of crotoxin) in the venom of *C. d. collilineatus* is involved in the increase of the number of promastigotes when treated with this venom. Probably for this reason, the crude venom from *C. d. collilineatus* increased parasite numbers by approximately 50% at 44.3 μg/mL, while at higher concentrations the venom showed anti-leishmanial activity (IC_{50} = 281 μg/mL). A similar effect was observed in the present study using TblV. However, the IC_{50} for this "colubrid" venom is less than one half the IC_{50} value for *C. d. collilineatus* venom (Passero et al., 2007).

In the present work, we demonstrate that PLA_2 from TblV also induces opposing dose-related effects, similar to investigations that have shown these biphasic dose-response relationships in other pharmacological and toxicological phenomena (Conolly and Lutz, 2004). A possible explanation for this kind of biphasic response is the hormesis hypothesis, which is related to the adaptive responses of cells and organisms to a moderate stress (Calabrese and Baldwin, 2001; Mattson, 2008). As PLA_2s are important mediators of complex intracellular signaling pathways, lower concentrations of these enzymes could trigger favorable stimuli for cell growth and proliferation. However, under higher concentrations, the toxicity of their enzymatic activity could result in direct damage of the cell membrane, or even in the triggering or exacerbation of unfavorable intracellular changes. Further investigations should clarify not only the actions of PLA_2s on *Leishmania* but also their mechanisms of interferences with various biological phenomena of prokaryotic or eukaryotic cells.

In conclusion, this is the first report of rear-fanged snake venoms with activity against a pathogenic parasite which is able to infect not only humans but also domestic and wild mammals worldwide. Thus, these results open new possibilities to finding protein-based drugs that might be used as possible agents against leishmaniasis. Furthermore, because of the dose-dependent biphasic action of this toxin, PLA_2 from TblV may be a useful molecular tool to investigate the biology of *Leishmania* sp. Snake venom PLA_2s are multifunctional proteins with promising biotechnological applications, and we are continuing to investigate the action of this protein in leishmaniasis as well as identifying specific protein regions with anti-leishmanial activity.

Conflict of interest

The authors declare that there are no conflicts of interest.

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