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RESEARCH ARTICLE

IN VITRO EVALUATION OF ANTI-LEISHMANIA AND ANTI-TRYPANOSOMA CRUZI ACTIVITY OF VIPERIDAE VENOMS

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ABSTRACT

The chemotherapy available for the treatment of Leishmaniosis and Chagas is restricted to a few medicines. Moreover, snake venoms have been studied because they have active substances with therapeutic potential. Thus, the aim of this study was to evaluate the *in vitro* effects of total venom of *Crotalusdurissuscascavella*, *Crotalusdurissusterrificus* and *Bothropsjararaca* in *Leishmania chagasi* and *Trypanosoma cruzi*.RAW264.7 and LLC-MK2 cells, *L. chagasi* promastigote and trypomastigotes of *T. cruzi.* In *L.chagasi* amastigotes of *T. cruzi* was carried out *in situ* ELISA test and MTT test. The toxins concentration of 500 µg/mL showed 100% mortality on the cells studied. Since of the 250 µg/mL concentration had obtained less than 50% cytotoxicity. These results presented high toxicity in trypomastigotes and amastigotes of *T.cruzi*. In promastigotes, the venom of *C. d. terrificus* caused 71.1% mortality at a concentration of 250 µg/mL and the IC50 of 63.5 to 155.0 mg/mL. In *L. chagasi* amastigotes, 250 µg/mL the *Bothrops jararaca* venom caused 96% mortality and a lower IC50 value with the range of 55.6 to 90.9 µg/mL. Therefore, the venoms of *C. d. terrificus* and *B. jararaca* in the concentrations tested had anti-Leishmaniosis and anti-Chagas disease activity.

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INTRODUCTION

In the Viperidae family, the subfamily Crotalinae stands out, to which belongs the *Crotalus, Bothrops* and *Lachesis* genera (Melgarejo, 2003). Biochemical analysis of snake venoms are useful in the diagnosis, monitoring and clinical prognosis of several animal diseases (Silva *et al*., 2010). One of the main reasons for studying snake venoms is the isolation of substances that have pharmacological effects in low concentrations (Ticli, 2006). Recently, *Crotalus* snakes have attracted attention due to their potential as a source of new pharmacological substances or hypotensive effects (Lopes *et al*., 2014; Evangelista *et al*., 2011). Several studies describe the anti-parasitic effects of snake venoms, mainly against protozoa, with only one promising report on the leishmanicidal effect of *C. d. cascavella* venom. The Trypanosomatidae family is one of major economic and medical interest and belongs to a group of exclusively parasitic, unicellular flagellates, including many important pathogens (Aslett *et al*., 2009). These protozoa have been intensively studied since some are pathogenic for humans and domestic animals, causing diseases such as Leishmaniosis and Chagas disease, which have a high incidence in Latin America (Motta *et al*., 2013). Approximately 37 million people have different forms of Leishmaniosis and Chagas disease worldwide, caused by *Leishmania* spp. and *T. cruzi*, respectively (Lopes *et al*., 2010). The chemotherapeutic protocol available for the treatment of both diseases is restricted to a few drugs, which have limited efficacy and undesirable adverse effects (Nunes, 2008). With the emergence of *Leishmania* spp. and *T. cruzi* protozoa resistance to drugs used in human and veterinary medicine and their intense adverse effects, this study aimed to evaluate *in*

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vitro effects of *C. d. cascavella*, in comparison to *C. d. terrificus* and *B. jararaca* venoms, in promastigotes and amastigotes of *L. chagasi*, amastigotes and trypomastigotes of *T. cruzi* and to analyze the toxicity in RAW 264.7 and LLC-MK2 cells.

MATERIALS AND METHODS

Ethics Committee

This project was approved by the Committee on Animals Research and Ethics of State University of Ceará (CEUA – UECE) (Protocol12641492-0). Measures were taken to protect all personnel and animals involved on this project, as well as preserving the environment during the research.

Collection of C. d. cascavella, C. d. terrificus and B. jararaca venoms

The lyophilized venom from *C. d. cascavella* was kindly provided by Professor Dr. Miriam Camargo Guarnieri, from Department of Zoology and Center of Biological Sciences, Federal University of Pernambuco, Brazil. The lyophilized venoms from *C. d. terrificus* and *B. jararaca* were donated by Professor Dr. Patrick Jack Spencer of the Institute of Nuclear and Energy Research at the University of São Paulo, IPEN, Brazil. Venoms were diluted in phosphate buffered saline (PBS) and stored at -80°C.

Collection of L. chagasi and *T. cruzi*

Promastigotes of *L. chagasi* were grown in cell culture bottles with M199 medium (Cultilab®) supplemented with 10.0% inactivated fetal calf serum (FCS) (Cultilab®), sodium bicarbonate (Sigma-Aldrich®), HEPES buffer (Sigma-Aldrich®), bovine hemin (Inlab®), gentamicin 30.00 µg/mL (Inlab®) and 5.0% human male sterile urine. Parasites were incubated at 27 °C. After a week, cultures were examined under a light microscope to observe their viability. Trypomastigotes of *T. cruzi* were grown in culture medium RPMI 1640 (Sigma-Aldrich®) supplemented with 10.0% FCS (Cultilab®), HEPES buffer (Sigma-Aldrich®), 200 mM L-glutamine (Gibco®) and 30.00 µg/mL gentamicin (Inlab®). This culture was maintained in cell culture bottles at 27ºC. After a week, cultures was analyzed in a light microscope to determine their viability.

Polyacrylamid gel electrophoresis (SDS-PAGE)

The protein profile of *C. d. cascavella*, *C. d. terrificus* and *B. jararaca* crude venoms were evaluated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 15% in denaturing conditions. Crude venoms were diluted in a concentration of 500.00 µg/mL in PBS and submitted to a 100W voltage. Fractions were colored in 0,1% Brilliant Blue G250, 45% methanol and 10% acetic acid (SDS-PAGE-CBB).

Cytotoxicity in RAW 264.7 and LLC-MK2 cells

Cell line RAW 264.7 derived from murine monocytes were cultured in Dulbecco (Cultilab®). The cell line LLC-MK2, originated from monkey kidney fibroblasts, were cultured in RPMI 1640 (Sigma-Aldrich®). Both cell lines were counted in a Neubauer chamber and diluted at a concentration of $1x10^6$ cells/mL and plated on 96-wells plate. *C. d. cascavella*, *C. d. terrificus* and *B. jararaca* venoms were tested in cell lines at 500.00, 250.00, 125.00, 62.50, 31.25, 15.62, 7.81 and 3.90 µg/mL concentrations. As negative control, Dulbecco (Cultilab®) was used for the RAW 264.7 plate and RPMI 1640 (Sigma-Aldrich®) for the LLC-MK2 plate. Positive control was tested with 10.0% sodium dodecyl sulfate (10.0% SDS) in both plates. All compounds were tested in triplicate. Plates were incubated in a 5.0% CO_2 atmosphere at 37 °C for 24 h. After the incubation period, MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was performed as described by Tempone (2005) and plates were analyzed by an ELISA reader at 570 nm.

L. chagasi promastigote and T. cruzitrypomastigote tests

L. chagasi promastigote and *T. cruzi*trypomastigotes were diluted at a concentration of $1x10^6$ cells/mL. Each culture was plated in 96-wells plates. Venoms were tested against these parasites in 500.00; 250.00; 125.00; 62.50; 31.25; 15.62; 7.81 and 3.90 µg/mL concentrations, respectively. The positive control was performed with 50 µg/mL Pentamidine (Ithaca®) in both cultures. Negative control promastigotes was tested with M199 medium (Cultilab®) and tripomastigotes with RPMI 1640 medium (Sigma-Aldrich®). All compounds were tested in triplicate. After, plates were placed in a cell culture incubator at 27 °C for 24 h. The activity of trypomastigotes and promastigotes was assessed by MTT assay using the methodology of Tempone (2005). Plates were read in an ELISA reader at 570 nm.

L. chagasi amastigotes test

Plating of RAW 264.7 cells was performed at a concentration of $1x10^5$ cells/well in 96-wells plates, which were maintained for 24 h in a CO_2 incubator at 37 °C for adherence. After the incubation period, *L. chagasi* promastigotes were added to the plate at a concentration of $1x10^6$/well and waited infection over 24 h at 27ºC. After such period, the infection was assessed by light microscopy and wells were aspirated leaving only infected cells. Then, venoms were added in 500.00, 250.00, 125.00, 62.50, 31.25, 15.62, 7.81 and 3.90 µg/mL concentrations. For positive control 50.00 µg/mL of pentamidine (Ithaca®) was added and Dulbecco (Cultilab®) was used as a negative control. All compounds were tested in triplicate. The plate was incubated for 24 h at 27 ºC. After this period, 10.0% formaldehyde was added to all wells for cell fixation and *in situ* ELISA procedures were performed. A solution of 0.01% saponin (Merck®) with 1.0% bovine serum albumin (BSA, Sigma-Aldrich®) diluted in 1x phosphate buffered saline (PBS) was added and plates were incubated for 30min at 37 °C. Subsequently, a new solution of 5.0% skimmed milk diluted in PBS and incubated for 30min at 37 °C was added. The plate was rapidly washed three times in a PBSLT solution (PBS (1x), 3.0% skimmed milk and 0.05% Tween 20). After drying, rabbit serum anti-*L. chagasi* diluted in 10.0% FCS and PBSLT was added in each well. This solution was incubated overnight at 37 ºC. Then, the plate was slowly re-washed three times with PBSLT. The conjugated anti-rabbit IgG (Sigma-Aldrich®) was diluted in PBSLT, plated, and incubated at 37 °C for 60min. Again, three washes

with PBSLT were performed and the chromogen ortho-phenyldiamine (OPD, Sigma-Aldrich®) was added for 30min in a dark chamber. Finally, t 4N hydrochloric acid (4N HClNovaquímica®) was added and the plate read in a microplate reader using a 492nm filter.

T. cruzi amastigotes test

LLC-MK2 cells plating was performed at a $1x10^5$ cell/well concentration in 96-well plates, which were maintained for 24h in a CO2incubator at 37 °C for adherence. After the incubation period, *T. cruzi* trypomastigotes were added to the plate at a $1x10^6$ trypomastigotes/well concentration and incubated at 27 ºC for 24hfor development of infection. After, the infection was assessed by light microscopy and wells were aspirated leaving only the infected cells. Then, venoms were added in the following concentrations: 500.00, 250.00, 125.00, 62.50, 31.25, 15.62, 7.81 and 3.90 µg/mL. For positive control 50.00 µg/mL Pentamidine (Ithaca®) was added and RPMI 1640 (Sigma-Aldrich®) was used as negative control. All compounds were tested in triplicate. The plate was incubated for 24h at 27ºC. After this period, ELISA *in situ* was performed, as described for *L. chagasi*amastigotes, using rabbit serum anti-*T.cruzi*. The plate was then read on a microplate reader using a 492nm filter.

Statistical analysis

IC_{50} values (drug concentration able to inhibit 50% of parasites) in the range of 95% (95% Confidence Interval) were calculated using a nonlinear regression curve. One-way ANOVA and comparative analysis between treatments was performed by Tukey's parametric test.100% survival was based on the viability control OD containing only promastigotes or amastigotes, and/or murine monocytic cells after normalization using the statistical software GraphPad Prism 6.0.

RESULTS

Polyacrylamid gel electrophoresis (SDS-PAGE)

The SDS-PAGE showed that *C. d. cascavella* and *C. d. terrificus* venoms had similar molecular profiles, differently from *B. jararaca* venom (Fig. 1).

Cytotoxic activity of C. d. cascavella, C. d. terrificus and B. jararaca venoms in RAW 264.7 and LLC-MK2 cell lines

The cytotoxicity assay on RAW 264.7 cells revealed that venoms had cytotoxicity in 100.0% cells at the highest concentration tested (500.00 µg/mL). The other concentrations also showed cytotoxicity, but with a percentage below 36.0%. The venom of *C. d. cascavella* had a IC_{50}= 236.10-354.60µg/mL for the RAW survival rate of 264.7 cells (Fig. 2). Also, IC_{50} values of *C. d. terrificus* and *B. jararaca* venoms ranged from 248.50 to 305.00µg/mL and from 247.10 to 351.40µg/mL, respectively. All venoms tested demonstrated similar results in the cell line LLC-MK2 and all concentrations used in this experiment proved cytotoxic. Only the highest concentration of venoms (500.00 µg/mL) had100.0% cells mortality, which was below 50.0% in the other concentrations. IC_{50} values of *C. d. cascavella*, *C. d. terrificus* and *B. jararaca* venoms varied from 230.20 to 329.00, 227.70 to 341.00, and 260.90 to 366.80 µg/mL (Fig. 2), respectively. These IC_{50} values had no significant differences ($P<0.05$) when compared to venoms tested in the RAW 264.7 cell line.

Activity of the venoms in L. chagasi promastigotes and T. cruzitrypomastigotes

The 500.00µg/mL venom concentration studied had higher cytotoxic activity against *L. chagasi* promastigotes, followed by the concentration of 250.00µg/mL (Fig. 3). The 500.00 µg/mL concentration was not considered ideal since it had 100.0% cytotoxicity in both RAW 264.7 and LLC-MK2 cells, proving to be highly toxic. The optimal concentration was 250.00 µg/mL since it did not had a high cytotoxicity. *C. d. terrificus* venom had better leishmanicidal activity at this concentration, achieving 71.1% of promastigote mortality. *C. d. cascavella* and *B. jararaca* venoms also had cytotoxicity at 250.00 µg/mL concentration and caused a 67.1 and 55.0% mortality, respectively, in promastigotes. Other concentrations had no statistically satisfactory results ($P<0.05$). IC_{50} values for *C. d. terrificus* were the lowest (63.50 to 155.00µg/mL), while *C. d. cascavella* and *B. jararaca* venoms had higher inhibitory concentrations in *L. chagasi* promastigotes (from 180.50 to 237.90 and from 103.90 to 241.90 µg/mL, respectively). All venoms studied had no efficacy against *T. cruzi* trypomastigotes. The concentration that had the greatest activity against these parasites was 500.00µg/mL, of which the survival percentage was 9.3% to *C. d. cascavella venom*, 5.9% to *C. d. terrificus* venom and 13.4% to *B. Jararaca venom* (Fig. 3). In the three venoms studied, the 250.00 µg/mL concentration was not effective against the parasites, with no significant statistical difference. The trypomastigotes survival values on the concentration of 250.00µg/mL were approximated, representing 44.1% for the *C. d. cascavella* venom, 45.4% for the *C. d. terrificus* venom and 43.7% for the *B. Jararaca* venom. All other concentrations had mortalities below 30.0%. IC_{50} values had no significant differences when compared the three venoms studied. IC_{50} values obtained from *T. cruzi* trypomastigotes varied from 192.60 to 267.90 µg/mL for the *C. d. cascavella*, from 81.50 to 213.00 µg/mL for the *C. d. terrificus* and from 137.60 to 234.10 µg/mL for the *B. jararaca* venoms.

Activity of the venoms in L. chagasi and T. cruzi amastigotes

Results obtained by ELISA *in situ* showed that the highest concentration studied (500.00 µg/mL), caused the highest leishmanicidal activity against *L. chagasi* amastigotes. The *B. jararaca* venom caused the highest mortality rate (99.5%; Fig. 4). *C. d. cascavella* venom caused 85.9% mortality in *L. chagasi* amastigotes while of the *C. d. terrificus* venom caused a 31.9% of mortality. At the 250.00 µg/mL concentration, the *B. jararaca* venom expressed a 96.0% mortality rate while the *C. d. cascavella* and *C. d. terrificus* venoms had 11.8 and 15.7% mortality, respectively. At the 125.00 µg/mL concentration only *B. jararaca* venom had a valid mortality (60.7%). All other concentrations had mortalities below 40.0%. IC_{50} values of the venoms studied had significant differences, with the *B. jararaca* presenting the lowest values, ranging from 55.60 to 90.90µg/mL. The *C. d. cascavella* venom had an IC_{50} ranging from 181.60 to 495.50µg/mL, while of the *C. d. terrificus* did not have a IC_{50} statistically

favorable (from 402.90 to 1253.00µg/mL). The ELISA *in situ* performed with *T. cruzi* amastigotes revealed that the 500.00 µg/mL concentration had greater trypanocidal activity. The *B. jararaca* venom had the highest mortality rate of 90.4%, while the venom of *C. d. cascavella* had the second highest mortality rate of *T. cruzi* amastigotes (72.5%), and the venom of *C. d. terrificus* had the lowest mortality rate (57.7%; Fig. 4). All other concentrations had no statistically satisfactory trypanocidal activity, with a survival rate of 56.7% for *T. cruzi* amastigotes. The IC_{50} of *C. d. terrificus* venom had the best results among the venoms studied (77.90-293.80µg/mL). The *C. d. cascavella* venom had values varying from 126.50 to 387.30µg/mL and *B. jararaca* venom with values between 209.20 and 496.80µg/mL.

DISCUSSION

The electrophoresis demonstrated that venoms of *C. d. cascavella* and *C. d. terrificus* had similar molecular profiles with four bands on the same level. Although *C. d. cascavella* and *C. d. terrificus* had bands of different molecular weights when compared to *B. jararaca*, they had a band in common in the region close to the molecular weight of 35 kDa. Cell lines RAW 264.7 and LLC-MK2 did not survive at the highest concentration of venoms studied. Therefore this concentration can be discarded for future *in vivo* tests. From the 250.00µg/mL concentration of *C. d. terrificus*, *C. d. cascavella* and *B. jararaca* venoms, the survivability of these cells increased and the RAW 264.7 had better results than LLC-MK2 cells. In this study, IC_{50} values for *C. d. terrificus* were the lowest (63.50 to 155.00µg/mL) while *C. d. cascavella* and *B. jararaca* venoms had higher inhibitory concentrations in *L. chagasi* promastigotes expressing (from 180.50 to 237.90 and 103.90 to 241.90 µg/mL, respectively). Other studies have been conducted with *C. d. terrificus*, *C. d. cascavella* and *C. d. collilineatus* venoms on *L. amazonensis*promastigotes. Results showed a higher leishmanicidal activity of *C. d. terrificus* venom (IC_{50}=4.70-1.72µg/mL), while *C. d. cascavella* had an IC_{50} from 9.41 to 1.21 µg/mL.*C. d. collilineatus* venom had a low leishmanicidal activity at higher concentrations: IC_{50} varied from 9.50 to 281.00µg/mL and also increased the numbers of parasites in 50.0% at the IC_{50} from 44.30 to 2.18µg/mL (Passero *et al*. 2007). These results differ from that obtained in this study with *L. chagasi* promastigotes. These species of *Leishmania* are biologically distinct, thus, different clinical presentations can be displayed (Camargo; Barcinski, 2003).

Snake venoms can be sources of new bioactive molecules with potential effects on the growth of *T. cruzi* and *Leishmania* spp. Changes in the structure and growth of *T. cruzi*epimastigotes, amastigotes and trypomastigotes and *L. major* promastigotes were analyzed after treatment with crude venom of *B. jararaca*. No growth of promastigotes and epimastigotes occurred at a 100.00 µg/mL concentration (IC_{50}=0.10-0.30µg/mL). Structural observation performed in the bloodstream trypomastigotes, in intracellular amastigotes, as well as axenic cultures of promastigotes and epimastigotes had increased mitochondrial volume and disorganization of kinetoplast (Gonçalves *et al*. 2002). Deolindo *et al*. (2005) found that the growth of *T. cruzi* epimastigostes was inhibited after treatment with *B. jararaca* venom and the IC_{50} obtained was 10.00 µg/mL. Cellular molecular observations revealed increased mitochondrial volume, disorganization of kinetoplast, condensation of cytoplasm, and loss of mitochondrial membrane potential. Several studies on snake venom cytotoxicity were conducted in intracellular parasites, but few studies have been reported with *C. d. terrificus*. Reports of anti-Leishmania activity of snake venoms on the *L. chagasi* species are also scarce. Results showed that 250.00 and 125.00µg/mL concentrations of *C. d. terrificus* and *B. jararaca* venoms had a higher mortality in the protozoa studied. Thus, further study of toxic effects of the crude venom and its isolated fractions of the analyzed snakes may contribute to further research on the pathophysiology and mechanism of action of these toxins in visceral leishmaniosis and Chagas disease, as well as provide new pharmacological and therapeutic tools for *in vivo* studies.

Conclusions

We conclude that the studied venoms were 100% cytotoxicity in RAW 264.7 and LLC-MK2 cells at the concentration 500 mg / mL and 50% in lower concentrations. However, the venom of *Crotalusdurissus terrificus* showed higher mortality rate in promastigotes and *Bothrops jararaca* showed better activity against *Leishmaniachagasi* amastigotes. Moreover, we find that the trypomastigotes, the venom of the *Bothrops jararaca* showed higher toxicity while in amastigotes of *Trypanosoma cruzi*, the venom of *Crotalusdurissus terrificus* expressed greater toxicity. Therefore, we showed that the snakes venom's *Crotalusdurissus terrificus* and *Bothrops jararaca* have leishmanicide activity and trypanocidal, can be considered as a new therapeutic and pharmacological research tool for the treatment of visceral leishmaniasis and American trypanosomiasis.

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Conflict of Interests

All authors declare there is no potential conflicts of interest including employment, consultancies, stock ownership, honoraria, paid expert testimony and patent applications/registrations related to the current manuscript.

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