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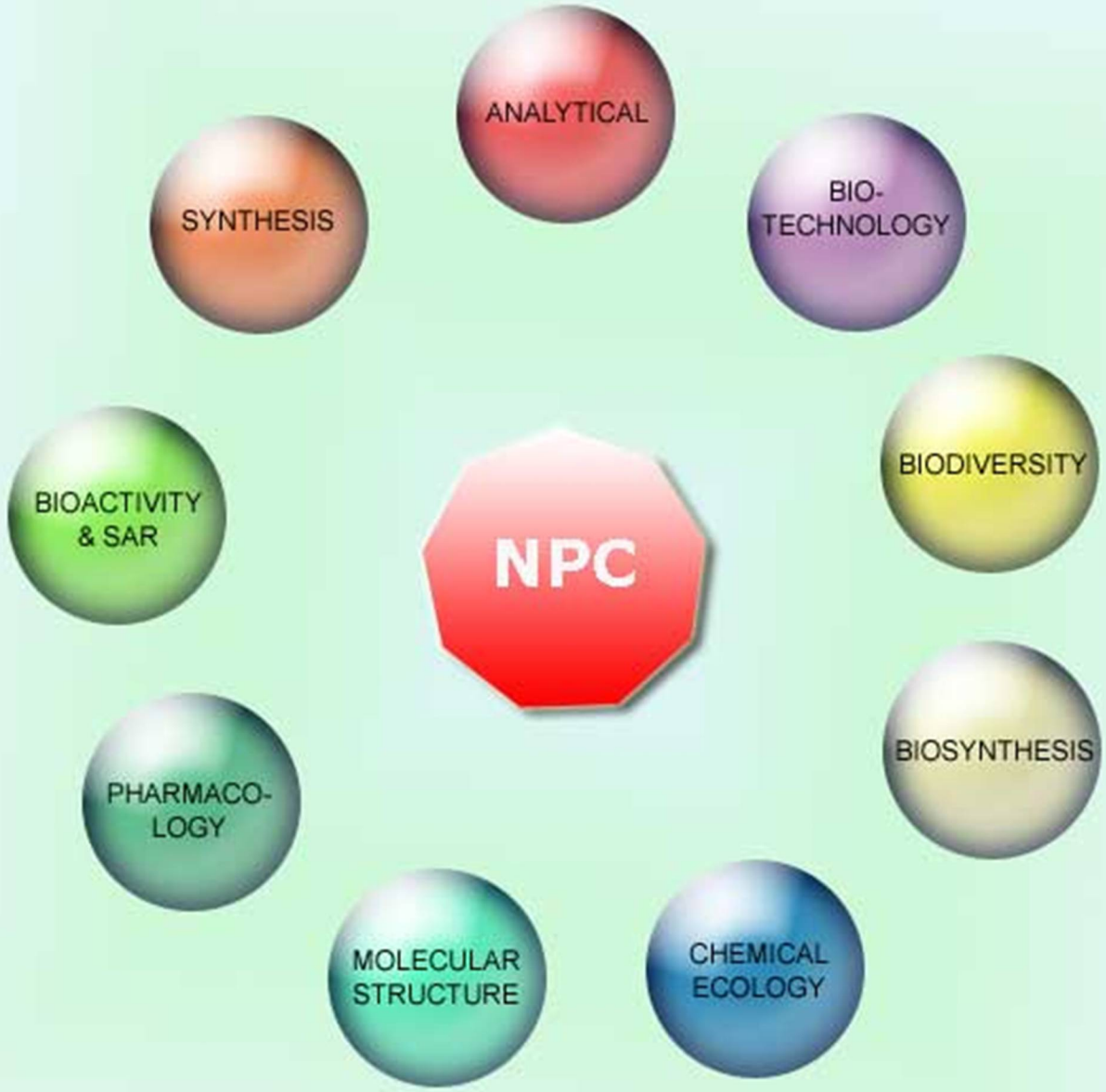

Volume 7. Issue 1. Pages 1-142. 2012
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2012
Vol. 7
No. 1
71 - 74

Antibacterial and Antiparasitic Effects of *Bothropoides lutzi* venom

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Received: May 26th, 2011; Accepted: August 9th, 2011

The therapeutic potential of toxins has aroused great interest in the scientific community. Microbial resistance is a serious current public health problem, in part because of the wide use of antimicrobial drugs. Furthermore, there are several problems in the treatment of parasitic diseases such as leishmaniosis and Chagas' disease, including the low efficacy in some clinical phases of the diseases and the loss of effectiveness of benzonidazole in the chronic phase of Chagas' disease. In this context, the aim of this work was to study the antimicrobial and antiparasitic effects of *Bothropoides lutzi* total venom (BltTV). The venom exerted an antibacterial effect on *S. aureus*, with MIC=MLC=200 µg/mL. The inhibitory effects of BltTV on promastigote forms of *Leishmania amazonensis* and *L. chagasi* were assessed by counting of viable cells after incubation with BltTV. IC_{50} values of 234.6 µg/mL and 61.2 µg/mL, were obtained, respectively. Furthermore, the venom repressed epimastigote forms of *Trypanosoma cruzi* growth. Finally, BltTV was verified to affect murine peritoneal macrophages, causing a cytotoxic effect at the highest concentrations (100 and 50 µg/mL). In conclusion, *Bothropoides lutzi* venom demonstrated antibacterial and antiparasite effects, suggesting that the venom contains some substance(s) of therapeutic value.

Keywords: *Bothropoides lutzi*, Antibacterial effect, Antiparasite effect.

The study of the therapeutic potential of toxins has aroused great interest from the scientific community as a source of molecular models for the design of new drugs [1]. The search for new substances with microbiocidal potential is of great importance because of the various pathologies caused by microorganisms, such as bacteria, fungi and parasites [2]. The development of microbial resistance has grown in recent years, in part because of the wide use of currently available antimicrobial agents. Resistance is more prevalent in nosocomial institutions, where microorganisms can develop resistance to many drugs, and some hospitalized patients are immunocompromised [3].

Furthermore, many problems exist in the treatment of parasitic diseases, such as limited antimicrobial effectiveness [4]; severe collateral effects have been related to the use of benzonidazole and pentavalent antimonial drugs for the treatment of Chagas' disease and leishmaniosis, respectively [5]. In an attempt to restrain these problems, several studies have aimed to find novel antimicrobial and antiparasitic substances from natural sources, with snake venoms representing a potential source. Many substances isolated from *Bothrops* and *Bothropoides* venoms have shown antimicrobial effects, such as miotoxin II and III from *B. asper* [6], which inhibits the growth of some Gram-positive and Gram-negative strains. Also, Izidoro *et al*. [7] demonstrated the antibacterial effect of L-amino acid oxidase (LAAO) from *B. pirajai* against *Pseudomonas aeruginosa* and *Escherichia coli.*

Previously, *B. moojeni* venom and its LAAO were demonstrated to be capable of inhibiting the growth of *Leishmania amazonensis*, *L. panamensis*, *L. chagasi* [8] and *Trypanosoma cruzi* [9]. In a similar study, *Bothrops jararaca* also showed trypanocidal and leishmanicidal effects [10].

Bothropoides lutzi is a snake found in southeastern and northeastern Brazil. Recently, some authors have made a revision genera, according to their morphological and phylogenetic of the *Bothrops, Bothriopsis* and *Bothrocophias* aspects. In this revision, *Bothrops lutzi* was removed from the *Bothrops newiedi* complex, and renamed as *Bothropoides lutzi* [11,12]. Few data are available about the biological effects of this venom, indicating the importance of studies aiming to discover new bioactive substances with therapeutic potential [13]. In this way, the aim of the present work was to evaluate the antimicrobial and antiparasitic effects of *B. lutzi* venom (BltTV).

To determine the antimicrobial activities of BltTV, four bacterial and one yeast species were diffused over the surface of Mueller-Hinton and Saubouraud-Dextrose agar, respectively. BltTV caused a visible zone of inhibition over the Gram-positive bacterium *S. aureus* at the two highest concentrations (2 and 1 mg/mL). To evaluate the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC), BltTV was seeded at different concentrations in 96-well microplates and incubated with *S. aureus.* The visible growth inhibition was verified and quantified as MIC=MLC=200 µg/mL. These MIC and MLC values suggest that the venom may have a bactericidal rather than an inhibitory effect [14].

Recently, we demonstrated the antimicrobial activity of *Bothrops leucurus* snake venom against *S. aureus* [15]; also, LAAO from

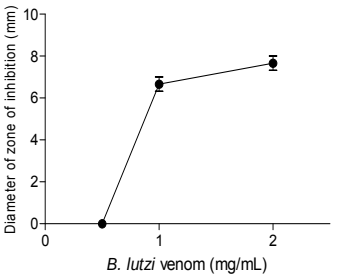

Figure 1: Antibacterial effect of *Bothropoides lutzi* (*B. lutzi*) on *S. aureus*. The data are expressed as mean±SEM of three independent experiments realized in triplicate.

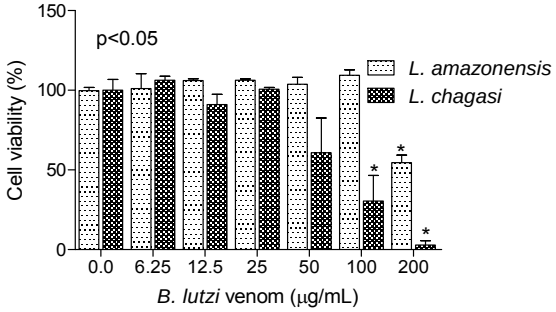

Figure 2: Leishmanicidal effect of *Bothropoides lutzi* (*B. lutzi*) venom on promastigote forms of *L. amazonensis* and *L. chagasi*. Sterile PBS, pH 7.4, was used as a negative control. The graph represents the mean ± SEM (n=3). BltTV= *Bothropoides lutzi* total venom. **p*<0.05.

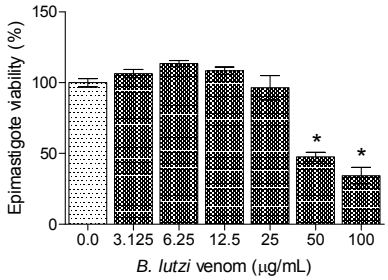

Figure 3: Trypanocidal effect of *Bothropoides lutzi* (*B. lutzi*) on epimastigotes of *Trypanosoma cruzi* strain Y. Sterile PBS, pH 7.4, was used as a negative control. The graph represents the mean ± SEM (n=3). BltTV= *Bothropoides lutzi* venom. **p*<0.05.

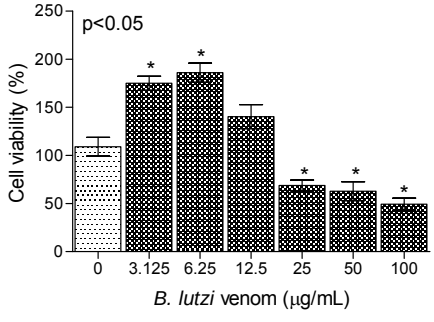

Figure 4: The effect of *Bothropoides lutzi* (*B. lutzi*) venom on murine macrophages. Sterile PBS, pH 7.4, was used as the negative control. The graph represents the mean ± SEM (n=3). BltTV= *Bothropoides lutzi* total venom. **p*<0.05.

B. marajoensis venom has been shown to inhibit the growth of *S. aureus*, *P. aeruginosa* and *C. albicans* [16]. After incubation with BltTV, promastigote forms of *L. amazonensis* and *L. chagasi* were counted in a Neubauer chamber. The venom was verified to have an inhibitory effect against both strains (Figure 2), with IC_{50}=234.6 μg/mL for *L. amazonensis* and 61.2 μg/mL for *L. chagasi*.

The trypanocidal effect of BltTV was assessed over epimastigote forms of *T. cruzi* strain Y by counting viable cells remaining after 5 days of incubation with the venom. BltTV caused a statistically significant growth inhibition at 100 and 50 μg/mL (Figure 3), resulting in an IC_{50} value of 50.1 μg/mL.

The present study demonstrates that BltTV has a similar antiparasite potential against strains of *Leishmania* and *T. cruzi*. Similar results were observed with other venoms and fractions isolated from *Bothrops* and *Bothropoides* snakes, such as *B. jararaca* and its LAAO [17]. LAAO from *B. pauloensis* and *B. jararaca* caused leishmanicidal effects against various strains [18,19], and these results were abolished by the addition of catalase, indicating the involvement of peroxide production. Our recent findings also demonstrated the leishmanicidal and trypanocidal potentials of *B. leucurus* venom, as well as a leishmanicidal effect of LAAO isolated from *B. marajoensis* venom [15,16].

Murine macrophages were extracted from the peritoneal cavity of Swiss mice and incubated with BltTV to determine its cytotoxic effect, through MTT assay. BltTV showed a dose-dependent result, where the highest concentrations (100, 50 and 25 μg/mL) induced a decrease in cell viability, whereas the lowest concentration (6.25 μg/mL) demonstrated a higher viability than the control group, treated with sterile PBS (Figure 4). Substances present in venoms are able to cause inflammatory responses by different pathways. The bothropic venoms and fractions can activate immune cells such macrophages, stimulating the release of several cytokines [20,21]. The discovery of the inhibitory potential of venoms against microorganisms must be followed up with fractionating and purification of the substances responsible for the effects observed, as well as molecular modeling methods to improve their pharmacological characteristics and reduce their toxic effects to develop synthetic analogues with adequate pharmacological and toxicological characteristics [22].

Experimental

Reagents and venom: *Bothropoides lutzi* total venom (BltTV) was obtained from the Ophiology Regional Nucleus of Ceará, Brazil (NUROF) and dissolved in sterile phosphate buffered saline at pH 7.4 (2.0 mg/mL). Chemicals and reagents used in this work were purchased from Sigma and Aldrich (Sigma Chemical Co., St. Louis, MO), Applied Biosystems, Pierce and Bio Rad (USA).

Antimicrobial activity of* Bothropoides lutzi *venom: The antimicrobial potential of BltTV was determined by a disk diffusion method [23]. Five pure microbial cultures (*Salmonella choleraesuis* subsp. *choleraesuis* sorotype *choleraesuis* ATCC 10708, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 15442 and *Candida albicans* ATCC 10231, donated by the Laboratory for Reference Materials of the Oswaldo Cruz Foundation, FIOCRUZ), were added to brain heart infusion (BHI) broth and incubated at 35ºC until they reached a visible turbidity equivalent to 0.5 on the McFarland scale (approximately 10^8 CFU/mL). The bacterial inoculums were spread on the surface of sterile Mueller-Hinton agar (Merck, Germany), and the yeast was spread on sterile Sabouraud-Dextrose agar (Merck, Germany). After 5 min, 5 mm-diameter wells were made in the agar using a sterile punch, and 25 μL of varying concentrations (2, 1, 0.5, 0.25 and 0.125 mg/mL) of BltTV were applied to the wells. Commercial antimicrobial drugs (2 mg/mL amikacin and 1.2 mg/mL ketoconazole) were used as positive controls, and the sample diluent (PBS, pH 7.4) as a negative control (absence of growth inhibition). After incubation at 35ºC for 18 h, the diameter of the zone of inhibition was recorded in mm.

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method, as described in [24], with modifications. Briefly, the microbial strains were sub-cultured, and after adjusting the density, they were diluted 1:100 to ~1.5 $\times 10^6$ CFU/mL. An inoculum of 125 µL of microbial culture was added to 25 µL of each concentration of BltTV (200, 100, 50, 25 and 12.5 µg/mL) in either BHI broth (100 µL) for bacteria or Sabouraud-Dextrose broth for *C. albicans*, in 96-well plates. Wells containing either BHI or Sabouraud-Dextrose broth with sterile PBS buffer (pH 7.4) were used as negative controls. Other wells containing culture medium and an antimicrobial agent (amikacin for bacteria and ketoconazole for yeast), and an inoculum of microorganisms was used as a positive control (growth inhibition). The plates were incubated at 35ºC for 24 h, and the MIC was assessed as the lowest sample concentration required to inhibit microbial growth (detected as a lack of visible turbidity). Three independent experiments were performed in triplicate. In addition, 25 µL aliquots were removed from the wells without visible turbidity and placed on Agar Plate-count (for bacteria) or Agar Sabouraud-Dextrose (for *C. albicans*) by the Pour-Plate Method. Colonies were counted after incubation at 35ºC for 24 h. The sample concentration that resulted in ≤0.1% of the growth of the initial inoculums (1.5 $\times 10^6$ CFU/mL) was determined as the MLC [24].

Leishmanicidal effect of **Bothropoides lutzi** ***venom:*** The leishmanicidal effect of BltTV was evaluated on promastigote forms of *Leishmania amazonensis* (MHOM/IFLA/BR/67/PH8) and *L. chagasi* (MCAN/BR/99/JP15). The parasites were cultivated in NNN/Schneider medium supplemented with 20% fetal bovine serum (FBS) and penicillin/streptomycin. The cultures were incubated at 26ºC until they reached the stationary phase of growth. The cytotoxicity was evaluated for 1×10^6 cell/mL of parasite culture in the presence of several concentrations of BltTV (200, 100, 50, 25 12.5 and 6.25 µg/mL). As a negative control, the cells were cultivated in the presence of sterile PBS. After 72 h, qualitative and quantitative analyses of the cells were carried out, and the number of promastigote forms present was quantified in a Neubauer chamber. The IC_{50} was determined by a Probit model of regression analysis.

Trypanocidal effect of **Bothropoides lutzi** ***venom:*** Epimastigote forms of strain Y of *Trypanosoma cruzi* were cultured in LIT (liver infusion tryptose) medium, as previously described [25]. Parasites were incubated at 28°C for 5-6 days until the cell density reached 5×10^7 - 10×10^8 parasites/mL. Cells were seeded at 1×10^6 cells/mL with different concentrations of BltTV (100, 50, 25, 12.5 and 6.25 µg/mL) and incubated at 26°C for 5 days; the number of viable cells was then determined by counting in a Neubauer chamber.

Cytotoxicity to murine macrophages: Macrophages, obtained from the peritoneal cavity of Swiss female mice, were placed in RPMI-1640 medium, supplemented with FBS (10%) and penicillin/streptomycin and seeded at 1×10^6 cells/mL in 96-well microplates for 2 h at 37ºC with 5% CO_2. Cells were washed twice with medium at 37ºC and further incubated with BltTV at 100, 50, 25, 12.5 and 6.25 µg/mL for 24 h at 37ºC. The viability of the macrophages was determined using MTT (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide) assay [26]. After incubation, 100 µL of the medium was aspirated, 10 µL of MTT was added to the wells, and the plate was incubated for 4 h. Following this incubation, 90 µL of a solution of 10% sodium dodecyl sulfate (SDS)/HCl was added to solubilize the MTT-formazan product. After 17 h, the plate was read with a microplate reader at 570 nm.

Statistical analysis: All the experiments were performed in triplicate, and the data are presented as mean ± SEM. The means were evaluated by either a Student's unpaired t-test or ANOVA followed by the Dunnet test, when appropriate. Values of $p<0.05$ were considered to be statistically significant. Prism version 5.0 was used for all statistical analyses.

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Natural Product Communications
2012
Volume 7, Number 1

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