

## EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF *Crotalus durissus terrificus* CRUDE VENOM

### AVALIAÇÃO DA ATIVIDADE ANTIBACTERIANA DO VENENO BRUTO DE *Crotalus durissus terrificus*

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#### Abstract

Snake venoms are recognized as a promising source of pharmacologically active substances and are potentially useful for the development of new antimicrobial drugs. This study aimed to investigate the antimicrobial activity of the venom from the rattlesnake *Crotalus durissus terrificus* against several bacteria. Antibacterial activity was determined by using the plate microdilution method and the activity on the bacterial envelope structure was screened by using the crystal violet assay. The proteins in crude venom were separated by electrophoresis and characterized regarding their proteolytic activity. *C. d. terrificus* venom exhibited antimicrobial action against gram-positive and gram-negative bacteria. MIC values were defined for *Pseudomonas aeruginosa* ATCC 27853 (62.5 µg/mL), *Staphylococcus aureus* ATCC 25923 (125 µg/mL), and *Micrococcus luteus* ATCC 9341 (≤500 µg/mL). For *Salmonella enterica* serovar *typhimurium* ATCC 14028 and *Corynebacterium glutamicum* ATCC 13032, the decrease in bacterial growth was not detected visually, but was statistically significant. The crystal violet assay demonstrated that the crude venom increased bacterial cell permeability and the secreted protein profile agreed with previous reports. The results suggest that the proteins with lytic activity against bacteria in *C. d. terrificus* venom deserve further characterization as they may offer reinforcements to the weak therapeutic arsenal used to fight microbial multidrug resistance.

**Keywords:** rattlesnake venom, antimicrobial action, cellular envelope, proteolytic activity.

#### Resumo

Os venenos de serpentes são reconhecidos como uma fonte promissora de substâncias farmacologicamente ativas e potencialmente úteis para o desenvolvimento de novas drogas antimicrobianas. Esse trabalho teve como objetivo investigar a atividade antimicrobiana do veneno de *Crotalus durissus terrificus* contra várias bactérias. A determinação da atividade antibacteriana foi

realizada pelo método de microdiluição em placas e a ação na estrutura do envelope bacteriano pelo ensaio violeta de cristal. As proteínas do extrato bruto foram separadas por eletroforese e caracterizadas quanto à sua atividade proteolítica. O veneno de *C. d. terrificus* apresentou ação antimicrobiana frente bactérias gram-positivas e gram-negativas. Os valores de MIC foram definidos para *Pseudomonas aeruginosa* ATCC 27853 (62.5 µg/ mL), *Staphylococcus aureus* ATCC 25923 (125 µg/mL) e *Micrococcus luteus* ATCC 9341 ( $\leq$  500 µg/mL). Para *Salmonella enterica serovar typhimurium* ATCC 14028 e *Corynebacterium glutamicum* ATCC 13032, o decréscimo no crescimento bacteriano não foi detectado visualmente, mas foi estatisticamente significativo. O teste do cristal violeta demonstrou que o veneno bruto aumentou a permeabilidade das células bacterianas e o perfil de proteína secretada está em consonância com relatos anteriores. Os resultados sugerem que as proteínas com atividade lítica contra bactérias no veneno de *C. d. terrificus* merecem atenção para uma melhor caracterização, uma vez que podem trazer reforços para o escasso arsenal terapêutico empregado para combater a multirresistência microbiana.

**Palavras-chave:** veneno de cascavel, ação antimicrobiana, envelope celular, atividade proteolítica.

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## Introduction

Snakes of the genus *Crotalus* are commonly known as rattlesnakes. They are characterized by the presence of a terminal rattle in the tail that produces a particular noise when threatened or excited. Rattlesnakes are found in dry, sandy, and stony areas, such as open and closed fields, but are absent from coastal or dense vegetation. They measure approximately one meter in length, with yellowish-brown coloration and a robust body<sup>(1)</sup>. In Brazil, the genus is represented only by one species, *Crotalus durissus*, which has seven subspecies: *C. d. dryinas*, *C. d. marajoensis*, *C. d. ruruima*, *C. d. trigonicus*, *C. d. terrificus*, *C. d. cascavella*, and *C. d. collilineatus*<sup>(2)</sup>. *Crotalus. d. terrificus* probably originated from a stock of the nominal race (*Crotalus durissus*) in Mexico and occupied the open areas of South America. They occur in the southern Amazon rainforest, extreme southeastern Peru, Bolivia, Paraguay, Uruguay, and northern Argentina and are likely to be found in urban areas as a consequence of deforestation. The various known subspecies are parapatric forms that have been diversified by speciation<sup>(3,4)</sup>.

Snake venom is an important evolutionary adaptation essentially used for predation. They are primarily composed of a complex mixture of proteins and peptides, usually toxins, which act synergistically and exert a wide range of pharmacological activities, such as neurotoxic, hemotoxic, and cytotoxic effects on prey and humans<sup>(5)</sup>. The most common components found in rattlesnake venoms are enzymes, primarily nucleases, hyaluronidases, L-amino acid oxidases (LAAOs), different metalloproteinases, serine proteases, and phospholipases<sup>(6,7)</sup>. In *C. durissus* species, the fractionation of the venom has evidenced the presence of polypeptidic toxins, mainly crotoxin and crotamine, with smaller proportions of gyroxin and convulxin<sup>(8,9)</sup>. Together, the inoculation of these constituents in an ophidian accident can promote several physiological disorders with effects on the nervous, cardiovascular, and neuromuscular systems, and on coagulation and hemostasis<sup>(10)</sup>.

Many components of snake venoms led to drugs that act on the cardiovascular system, such as the antihypertensive Captopril®, and blood hemostasis, for example, the anti-thrombotic Integrilin®, as well as drugs with anti-inflammatory, analgesic, immunomodulatory, antineoplastic, and even antibiotic action<sup>(11–13)</sup>. The venoms of various snake species also exhibit antimicrobial activity against gram-positive, gram-negative bacteria, retroviruses, and protozoa, such as *Leishmania* and *Trypanosoma cruzi*, especially the enzymatic components such as PLAs<sub>2</sub> and metalloproteinases. The enzymes act commonly through the hydrolysis of membrane phospholipids; in addition to L-amino acid oxidases and flavoproteins, whose biological activity results from the induction of oxidative stress<sup>(14–18)</sup>.

Owing to the need for new therapeutic alternatives for the treatment of microbial infections, the investigation of *Crotalus* venom for potential antimicrobial agents is an obvious choice. Therefore, the aim of the present study was to evaluate the antimicrobial activity of *C. d. terrificus* crude venom, concomitantly screen for a possible mechanism of action on the bacterial cellular envelope, and analyze its protein profile and enzymatic activity.

## Material and Methods

The venom of *C. d. terrificus* was extracted from one adult male specimen by manual massage of the venom gland, and clarified by centrifugation at 24,000 x g for 12 min at 4 °C. The supernatant was then lyophilized as described previously by Ribeiro et al.<sup>(19)</sup>. After that, 1.0 mg of venom was diluted in 1.0 mL volume of sterile Milli-Q water (1.0 mg/mL) and stored at –20 °C. The specimen of *C. d. terrificus* was kept in captivity at the Center of Biological Studies and Research (CEPB) of the Pontifical Catholic University of Goiás (PUC-GO), Goiânia, Brazil. The experimental protocol was approved by the Ethics Committee on Animal Use at the PUC-GO (Protocol 0008/1).

Gram-positive and gram-negative bacterial strains were used as indicators in the screening tests of antibacterial activity: *Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *Corynebacterium glutamicum* ATCC 13032, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 70063, *Serratia marcescens* ATCC 14756, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Pseudomonas aeruginosa* ATCC 27853. The strains were stored in a freezer at –20 °C. For the experiments, the strains were first grown in brain heart infusion (BHI) broth for 24 h at 37 °C and subsequently on nutrient agar for 24 h at 37 °C.

A microdilution test, performed in accordance with the Clinical and Laboratory Standards Institute (CLSI)<sup>(20)</sup>, was used to evaluate bacterial susceptibility to different concentrations of the *C. d. terrificus* venom. Initially, isolated colonies from bacterial cultures were suspended in 3 mL sterile saline solution (0.85%, w/v) and the turbidity was adjusted to MacFarland standard 0.5. Subsequently, the material was diluted 1:10 (v/v) in saline solution to 10<sup>7</sup> colony forming units/mL. Double-strength Mueller-Hinton broth was prepared and 100 µL was transferred to each well of a 96-well microplate. First, 100 µL of a 1.000 µg/mL solution of *C. d. terrificus* venom was added to the first column. Thereafter, serial dilutions were performed to produce crude venom concentrations between 500 and 0.49 µg/mL. Finally, 10 µL of bacterial inoculum was added to each well, and the plates were incubated for 18 h at 35 °C. Each test was performed in triplicate. Three distinct controls were used in the experiment: (1) control of microbial growth, in which Mueller-Hinton broth and bacterial

inoculum were added to the wells; (2) control of medium sterility, in which Mueller-Hinton broth was added to the wells; (3) in which Mueller-Hinton liquid medium and 1.000 µg/mL venom solution were added to the well.

After the incubation period, 20 µL of 0.5% (w/v) triphenyl tetrazolium chloride (TTC) was added to the wells and the incubation of the plates was continued for 30 min at 35 °C. The presence of red staining was considered as evidence of bacterial growth<sup>(21)</sup>. The absorbance at 450 nm was measured in a microplate reader (TP Reader Basic, Thermo Plate) to analyze the antibacterial action of the venom. Statistical analysis of the results was performed with a t-test at a significance of 5% (p < 0.05).

Bacterial strains sensitive to the action of the venom in the microdilution tests were grown on nutrient agar. This was achieved by plating directly onto nutrient agar the content of wells with the following concentrations: twice the MIC, the MIC and half the MIC values. The plates were incubated for 24 h at 35°C. The MLC was the lowest concentration of the venom capable of causing the death of 99.9% of the microbial cells; in other words, the concentration at which no colony growth occurred the agar surface<sup>(22)</sup>.

The alterations in membrane permeability by *C. d. terrificus* venom was accessed by the crystal violet assay<sup>(23)</sup> with modifications. The standard strain of *S. aureus* ATCC 25923 was selected as the indicator microorganism for this experiment. Briefly, the strain was grown in BHI broth and suspended in 1.5 mL phosphate buffered saline (PBS) (pH 7.4) until a turbidity of 0.5 on the MacFarland scale was achieved. Then, the suspension was centrifuged at 4,500 × g for 5 min at 4 °C, and the sedimented cells were washed twice and resuspended in PBS. The bacterial suspension was further fractionated in 250 µL aliquots. These fractions were treated with venom concentrations equivalent to the half of the MIC value (MIC/2), the MIC value, and the MLC value by the addition 250 µL of the crude extract and incubated. After an initial incubation for 30 min at 37 °C, the suspensions were centrifuged for 5 min at 9,300 × g. The supernatant was discarded, and the pellet was resuspended in 500 µL PBS supplemented with 10 µg/mL crystal violet. The samples were incubated for 10 min at 37 °C and centrifuged at 13,400 × g for 15 min. The positive control was bacterial treatment with 0.25 M EDTA and the negative control consisted of untreated bacterial cells suspended in PBS.

Finally, 100 µL aliquots of the supernatant from each treatment and the controls were transferred to sterile 96-well microplates. The absorbance at 595 nm was measured in an automatic microplate reader and the results were statistically analyzed by the t-test at the 5% significance level. To calculate the percentage of crystal violet uptake by bacterial cells, the formula given below was used. The mean absorbance of the 10 µg/mL violet crystal solution in PBS was considered to be 100%:

$$\% \text{ Crystal Violet Uptake} = \frac{OD_{595nm} \text{ value of the sample}}{OD_{595nm} \text{ value of the violet crystal}} \times 100$$

To determine the protein profile, 12% polyacrylamide gel electrophoresis (SDS-PAGE) of *C. d. terrificus* venom was performed by on samples at concentrations of 50 mg/mL and 10 mg/mL<sup>(24)</sup>. The protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) to make the standard solutions<sup>(25)</sup>. Standard curves of BSA solutions between 0 and 125 mg/mL were prepared in triplicate and the absorbance at 595 nm was measured.

The proteolytic activity of the venom was verified in accordance with a previously established

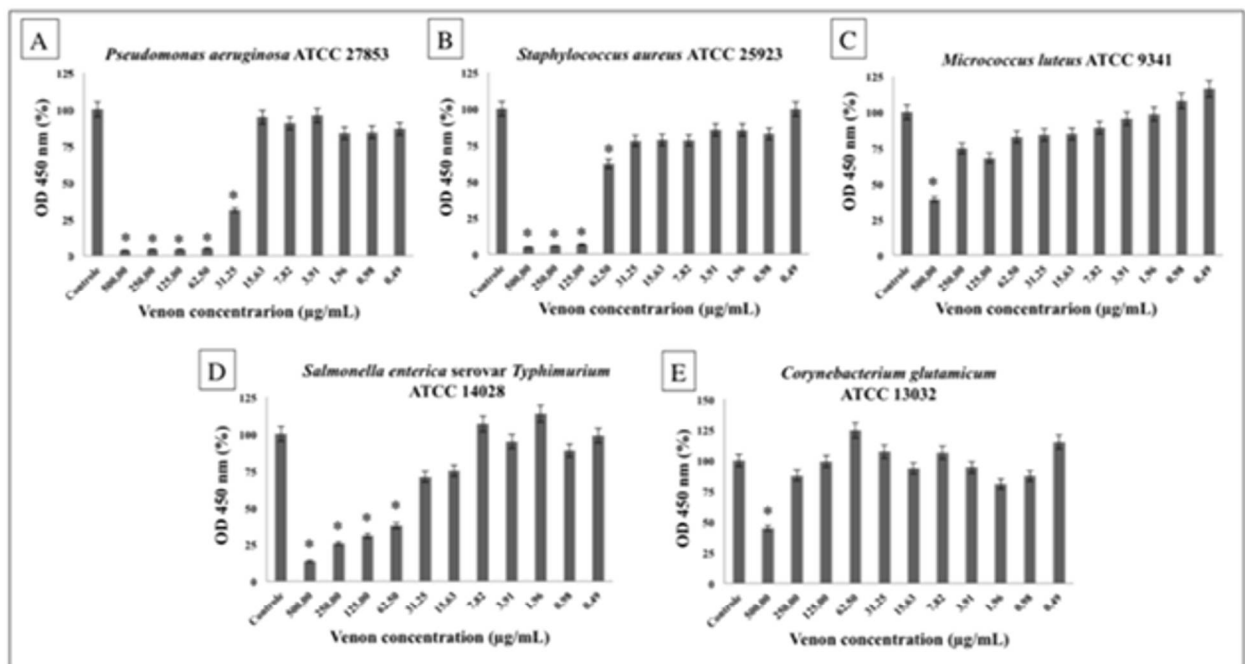
protocol<sup>(26)</sup>. In a test tube, 2.0 mL of 1.0% (w/v) casein solution in 0.1 M phosphate buffer (pH 7.0) and 1.0 mL of 0.1 mg/mL venom were added. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 4.0 mL of 5% trichloroacetic acid and incubation was continued for 30 min at room temperature. Finally, the tubes were centrifuged for 5 min at 10.000 rpm and the absorbance at 280 nm was measured.

The samples were analyzed in triplicate and the mean of the three results was used to calculate the units of caseinolytic activity through the application of the following formula:

$$U/mg = \frac{OD_{280nm} \text{ value of the sample}}{Venom(mg)} \times 100$$

## Results and Discussion

The crude venom of *C. d. terrificus* exhibited antimicrobial activity against gram-positive and gram-negative bacteria in the microdilution test. The MIC values were determined for *P. aeruginosa* ATCC 27853 (62.5 µg/mL), *S. aureus* ATCC 25923 (125 µg/mL), and *M. luteus* ATCC 9341 ( $\leq$  500 µg/mL) (Figure 1). A decrease of more than 50% was observed in the cellular proliferation of *S. enterica* serovar Typhimurium ATCC 14028 (500 to 62.5 µg/mL) and *C. glutamicum* ATCC 13032 (500 µg/mL) (Figure 1). Although inhibition was not detected visually by using the TTC, statistical analysis revealed that the results were significant.



**Figure 1** – Broth microdilution test results for *P. aeruginosa* ATCC 27853 (A), *S. aureus* ATCC 25923 (B), *M. luteus* ATCC 9341 (C), *S. enterica* serovar Typhimurium ATCC 14028 (D), and *C. glutamicum* ATCC 13032 (E). The plotted data were obtained from the average of the absorbance values ( $\lambda = 450$  nm). The “control” is equivalent to the control of bacterial growth in the absence of venom. \*p < 0.05 (statistically significant by t-test).

Some authors conceptualize the MIC as the lowest concentration of an antibiotic or chemotherapeutic agent capable of the reduction of microbial growth by at least 50%<sup>(27)</sup>. In view of this definition, the

MIC for *S. enterica* serovar Typhimurium ATCC 14028 was determined as 62.5 µg/mL and ≤ 500 µg/mL for *C. glutamicum* ATCC 13032. It is valid to stress that TTC is enzymatically reduced by living microorganisms to produce triphenyl formazan, a red stain that is held within granules in cells<sup>(21)</sup>. Thus, the remaining number of viable cells of these two bacterial species mentioned above was sufficiently effective in the reduction of TTC, so that its presence could be visually detected.

Several studies have demonstrated the antibiotic activity of *C. d. terrificus* venom against different bacteria<sup>(28,29)</sup>. Other studies have shown that the venom has antiviral activity against dengue fever, yellow fever<sup>(30)</sup>, and human immunodeficiency virus<sup>(31)</sup>, antiprotozoal activity against *Leishmania amazonensis*<sup>(32)</sup> and *Giardia duodenalis*<sup>(33)</sup>, and antifungal activity against *Candida spp.*, *Trichosporon spp.*, and *Cryptococcus neoformans*<sup>(28)</sup>.

Variations in venom composition, even among snakes from the same species, have already been reported and are probably influenced by factors such as diet and geographic distribution<sup>(34,35)</sup>, which has been demonstrated in some studies evaluating *C. d. terrificus*<sup>(36,48)</sup>. The effect of the crude venom of *C. adamanteus* from the *Crotalus* genus was evaluated by using the disc-diffusion method and was also thought inhibit the growth of *S. aureus*, *Proteus vulgaris*, and *Proteus mirabilis*, but did not exert activity against *P. aeruginosa*<sup>(27)</sup>, which was different from the results of our study of these bacteria.

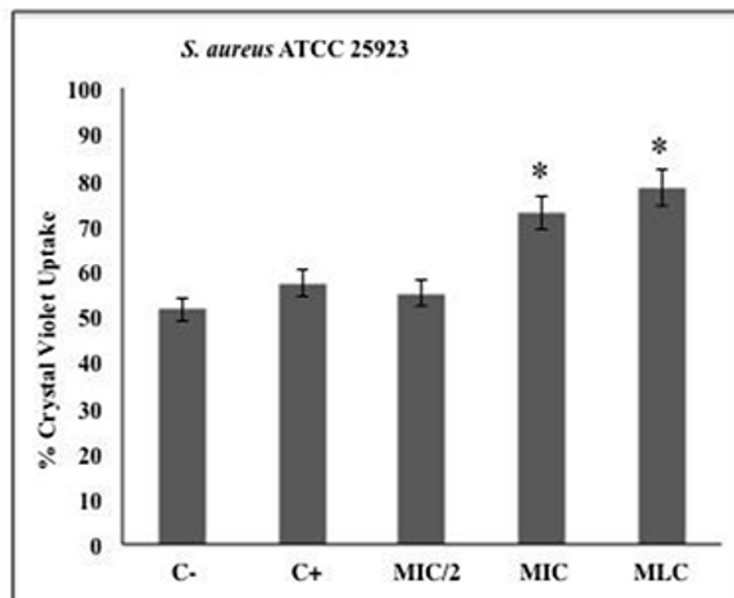
MIC was defined by a more sensitive methodology than the disk-diffusion method commonly used to evaluate the potential of antimicrobial agents<sup>(37)</sup>. Natural products are often difficult to diffuse in the culture media used in agar diffusion tests, owing to their water solubility and molecular weight characteristics. Therefore, the broth dilution method is a better option to determine their antibiotic activity<sup>(38)</sup>.

The antibacterial property of crotalic venom arises from the synergistic association of the constituent antimicrobial peptides and enzymes<sup>(39)</sup>, including phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which degrades bacterial membrane phospholipids, and L-amino acid oxidase (LAAO) (39,40). LAAO is commonly associated with the yellowish coloration of snake venoms and affects the oxidative deamination of L-amino acids, a process that generates hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Several studies have suggested that this protein plays an antimicrobial role mediated by the production of H<sub>2</sub>O<sub>2</sub>, a compound that induces the rupture of membranes in the microorganism, not by direct action on this structure, but by the promotion of oxidative stress and, consequently, induction of death in the microbial cells<sup>(40,41)</sup>. Antimicrobial peptides act on bacterial cells through multiple mechanisms and can interact directly on the membrane, through an intracellular receptor, or stimulate innate immunity<sup>(42)</sup>.

The antibacterial effects were not solely attributable to PLA<sub>2</sub>, as they may depend on the performance of other components from the non-enzymatic fraction of the venom<sup>(39)</sup>. This was corroborated by Yamane et al.<sup>(28)</sup>, who observed a weak inhibition in the growth of *E. coli*, *P. aeruginosa*, *S. aureus*, and *M. luteus* treated with crotamine, a peptide portion of the crotalic venom. The antimicrobial activity of crotamine was attributed to a mechanism involving cell permeability. This toxin is structurally similar to human β-defensins, which, among other functions, exert an antibiotic effect through their ability to form pores in the bacterial membrane. For the above-mentioned reasons, crotamine has been categorized in the family of antimicrobial peptides (AMPs)<sup>(43–46)</sup>.

The MLC of the venom defined for *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 was immediately higher than MIC, of 125 µg/mL and 250 µg/mL, respectively. The MLC determined for

*M. luteus* was the same as the MIC (MIC = MLC = 500 µg/mL). For other indicator strains, it was not possible to define MLC accurately. The uptake of crystal violet by *S. aureus* ATCC 25923 was 51.4% in the absence of venom treatment, but increased to 72.5% and 78.2% after treatment with the MIC and MLC of venom, respectively (Figure 2).



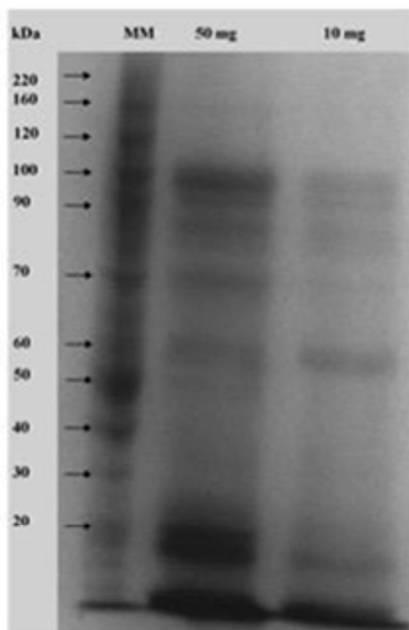
**Figure 2** – Crystal violet crystal uptake by *S. aureus* ATCC 25923 treated with 0.25 M EDTA and different concentrations of *C. d. terrificus* venom. C-: negative control (no treatment, only in PBS); C+: positive control (treatment with 0.25 M EDTA); MIC/2: venom at 62.5 µg/mL; MIC: venom at 125 µg/mL; MLC: venom at 250 µg/mL. \*p < 0.05 (statistically significant by t-test).

These results suggested that the constituents of the venom increase the permeability of the bacterial cell membrane, as crystal violet has difficulty penetrating intact membranes, but accumulates in cells with damaged membrane<sup>(23)</sup>. The alteration caused by EDTA (the positive control) is explained by the reduction in the supply of divalent cations, such as calcium (Ca<sup>2+</sup>) and magnesium (Mg<sup>2+</sup>), to the bacterial cell<sup>(47)</sup>. These ions have a fundamental role as macromolecule ligands of the plasma membrane and their restriction confers an osmotic imbalance that ultimately results in increased cellular permeability.

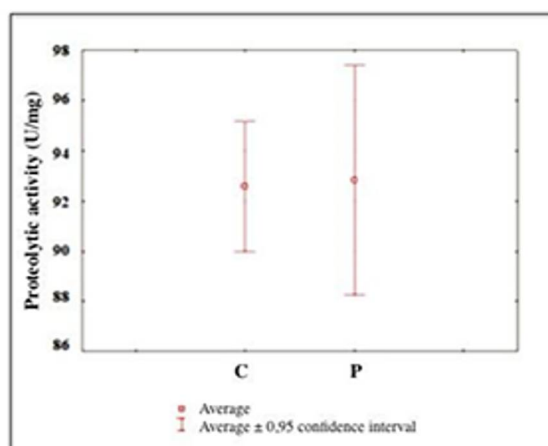
The protein profile exhibited by SDS-PAGE revealed a predominance of proteins with molecular weights below 20 kDa, and another considerable protein band in the 100 kDa range (Figure 3).

A concordant electrophoretic profile was obtained<sup>(48)</sup> when analyzing the venom of 92 *C. d. terrificus* male and female snakes of different ages kept in captivity. Different subspecies of *C. durissus* (*C. d. terrificus*, *C. d. collilineatus*, and *C. d. cascavella*) exhibited a comparable pattern of proteins that do not differ greatly, as with their molecular weights<sup>(49)</sup>.

The crude venom had a high protein content (1.903–2.674 mg/mL); our data were consistent with the reported value of 2 mg/mL protein<sup>(50)</sup>. Finally, *C. d. terrificus* venom exhibited statistically insignificant proteolytic activity (Figure 4) (p = 0.205).



**Figure 3** – SDS-PAGE of the *C. d. terrificus* crude venom. Molecular Markers (MM) (kDa) (Amersham Biosciences); 50 mg/mL and 10 mg/mL concentrations of crude venom of *C. d. terrificus*.



**Figure 4** – Proteolytic activity on casein (U/mg). C: control (casein solution without addition of the venom); P: 100 µg of *C. d. terrificus* crude venom.

The venom from *C. durissus* subspecies has negligible proteolytic activity<sup>(49)</sup>. In contrast, *Bothrops* snakes show strong proteolytic action resulting from the action of proteases, hyaluronidases, and phospholipases<sup>(51)</sup>, which cause local lesions such as edema, blisters, and necrosis<sup>(52)</sup>. The proteolytic and cytotoxic actions are closely related<sup>(53)</sup>; therefore, it was inferred that the low proteolytic action of venom also implies decreased cytotoxicity<sup>(53)</sup>.

## Conclusions

The crude venom of *C. d. terrificus* exhibited antimicrobial potential against gram-positive and gram-negative bacteria. Moreover, the crystal violet assay demonstrated that the venom increased bacterial cell permeability. The results suggested that isolated venom components may assist in the



development of new drugs with antibiotic action. However, further investigations must be performed to clarify the fraction responsible for this biological activity of the venom. Other peculiarities of the venom should also be elucidated, such as its toxicity and the characterization of its protein components.

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## Conflict of interest

The authors declare no conflicts of interest.

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