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ORIGINAL ARTICLE

Antimicrobial activity of *Bothrops alternatus* venom from the Northeast of Argentine

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ABSTRACT. This study was undertaken to evaluate the antimicrobial activity on Gram-negative and Gram-positive bacteria of the venom from *Bothrops alternatus*, specie responsible for most of snakebites in North-eastern Argentina. This activity was investigated against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and *Enterococcus faecalis* (ATCC 29212). Inhibitory halos values and reduction of colony-forming unites (CFU) by dilution plate technique were determined. The bactericidal activity was higher for *E. coli* and *S. aureus*, in comparison to that for *P. aeruginosa*. *E. faecalis* was the most resistant organism tested to the action of venom solutions. The demonstrated bactericidal property of *B. alternatus* venom highlight it as a promising candidate for further studies according to detect which component, or components, are responsible of this activity, in order to then investigate their potential use as antimicrobial agent.

Key words: Antimicrobial, bactericidal, snake venom, *Bothrops alternatus*.

RESUMEN. La actividad antimicrobiana del veneno *Bothrops alternatus*, especie responsable de la mayoría de los accidentes ofídicos en el Nordeste de Argentina, fue evaluada sobre bacterias Gram-negativas y Gram-positivas. Esta actividad fue ensayada sobre *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), y *Enterococcus faecalis* (ATCC 29212). Se determinaron los valores de halos de inhibición y la reducción de formación de unidades formadoras de colonias (CFU) por el método de dilución en placa. La actividad bactericida fue mayor para *E. coli* y *S. aureus*; en comparación con *P. aeruginosa*, *E. faecalis* fue el microorganismo evaluado más resistente a la acción de las soluciones de veneno. La propiedad bactericida demostrada del veneno de *B. alternatus* es importante para poder luego determinar cuál componente, o componentes, son responsables de dicha actividad e investigar su potencial uso como agente antimicrobiano.

Palabras clave: Antimicrobiana, bactericida, veneno de serpiente, *Bothrops alternatus*.

INTRODUCTION

In Latin America, the majority of snakebites are caused by species of the genus *Bothrops* (Cardoso, 1985; Gutiérrez, 1995).

B. alternatus (yarará grande) and *B. diporus* (yarará chica), reptiles that belong to the *Viperidae* family, are responsible for most snake poisonings in North East of Argentina, respectively (Acosta et al, 1998).

Snake venoms are a wide mixture of proteins and peptides (90-95%), also including amino acids, nucleotides, free lipids, carbohydrates and metallic elements bound to proteins (5%) (Heise et al, 1995). Viperid venoms in particular have long been recognized for the complexity of their molecular composition. More recently, with the advent of proteomics, the extent of the venom complexity has been more clearly illustrated. For instance, Tashima et al. (2002), has demonstrated that *Bothrops cotiara* and *Bothrops fonsecai* venoms contained around 30 proteins in the range of 7-110 kDa.

Analysis of venom proteins has consistently shown high levels of intra and interspecific variation and there are a small but increasing number of studies that strongly support the idea that this variation reflects local adaptation for feeding on different prey (Tashima et al, 2008).

This active proteins and peptides are usually similar in structure but not identical to that of prey physiological systems. In the last decade, several snake venom compounds were used as important tools for the understanding of human physiological systems due to their similarity to physiological molecules (Meier et al, 1991, Vogel et al, 2004) and have yielded extensive important information on biological systems and insights into medical problems (Balsinde et al, 1999).

Envenomation is a process associated with a low incidence of bacterial infection. This feature could indicate the presence of antibacterial molecules in the snake venoms that would protect the snakes during feeding. (De Lima et al, 2005)

Several antimicrobial studies involving venom of snakes from other regions, have described the bactericidal

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activity exhibited by their enzymes, such as PLA_2, from *Bothrops asper* (Páramo et al, 1998; Lomonte et al, 1999[a,b]; Santamaría et al, 2004 and 2005); PLA_2, from *Bothrops neuwiedi pauloensis* (Soares et al, 2000; Rodríguez V. et al, 2004); PLA_2, from *Bothrops jararacussu* (Roberto et al, 2004); PLA_2, from *Bothrops pirajai* (Soares et al, 2001[a]); PLA_2, from *Bothrops moojeni* (Stábeli et al, 2005); PLA_2, from *Crotalus durissus terrificus* (Soares et al, 2001[b]; Toyama et al, 2003) metalloproteases, from *Bothrops jararacussu* (Mazzi et al, 2004); L-amino acid oxidase, from *Bothrops alternatus* (Stábeli et al, 2004); L-amino acid oxidase, from *Bothrops pirajai* (Izidoro et al, 2006). Microbicidal peptides have emerged as promising therapeutic alternatives to cope with the increasing rates of antibiotic resistance encountered worldwide (Hancock et al, 1999).

In spite of several works published on this topic, the antimicrobial activity of *Bothrops alternatus* venom from Argentina has not been yet described.

The aim of this preliminary work was to study the bactericidal activity of *Bothrops alternatus* venom, against Gram-negative and Gram-positive bacteria in order to evaluate the potential use of its components as antimicrobial agents.

MATERIALS AND METHODS

Venom. Bothrops alternatus crude venom was collected from several snakes from the North East of Argentine, vacuum dried and kept at -20 °C.

Bacterial strains and growth conditions. The bacterial strains utilized were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeuruginosa* (ATCC 27853), and *Enterococcus faecalis* (ATCC 29212). Culture conditions of bacteria followed established protocols. Bacteria were harvested and suspended in 0.01 M sodium phosphate, pH 7.4 and immediately subjected to bactericidal assays.

Bactericidal-activity assays. Two bactericidal assays were carried out:

1. Inhibitory Halo Method. 50 µl of the tested bacteria suspension were homogeneously mixed on to Petri dishes containing 15 ml of the medium. Holes were aseptically bored into the agar with a hollow punch and were filled up with 10 µl of different concentration of crude venom solutions. The plates were examined after incubation at 37 ºC for 24 h. Diameters of the growth-inhibition halos were measured. Assays were performed in triplicate. Values were expressed as mean ± SD, and statistically compared using ANOVA.

2. Dilution Plate Technique. Bactericidal activity was determined as described by Páramo et al. (1998). Briefly, bacteria were harvested from fresh agar plates and their concentrations were adjusted to 4 x 10^6 colony-forming units (CFU)/ml. 100 µl, containing 4 x 10^5 CFU, were incubated for 60 min at 37 °C with different concentrations of the crude venom solutions. The live bacteria were counted on trypticase soy agar plates. Assays were performed in triplicate. Values were expressed as mean ± SD, and statistically compared using ANOVA.

RESULTS

The bactericidal effects of *Bothrops alternatus* venom toward two Gram-positive bacteria (*E. faecalis* and *S. aureus*) and against two Gram-negative bacteria (*E. coli* and *P. aeruginosa*) were tested by measuring the inhibition halos. Our findings demonstrated that there is a dose-dependent response of this activity to venom concentrations.

Very effective bactericidal activity was observed for *S. aureus* and *E. coli* (Figure 1). Both strains were the most susceptible Gram-positive and Gram-negative bacteria, respectively. Even at the lowest concentrations tested (0.312 mg/ml), the venom displayed significant bactericidal activity against *S. aureus*, but was ineffective against *E coli*. At concentrations of 0.625 to 10 mg/ml similar inhibition halos were observed for both strains.

Intermediate potency as bactericidal agent showed *B. alternatus* venom against *P. aeruginosa*, and inhibition halos were observed from higher concentrations than 2.5 mg/ml. However, the bothropic venom not exhibited bactericidal activity against *E. faecalis* even at the highest concentration assayed (Figure 1).

We also studied the number of CFU after incubating live bacteria with various concentrations of venom solutions (Figure 2). Incubation of *B. alternatus* venom solutions with both Gram-negative bacteria and *S. aureus* at 37 °C resulted in significant bacterial killing in the 1.25-10 mg/ml range of concentration. The bactericidal activity was higher for *E. coli* and *S. aureus*, in comparison to that observed for *P. aeruginosa*. *E. faecalis* was the most resistant organism tested to the action of venom solutions (Figure 2).

DISCUSSION

This work demonstrates that *Bothrops alternatus* venom displays dose-dependent bactericidal activity against a variety of Gram-negative and Gram-positive bacteria.

Both assayed methods, Inhibitory Halo Method and Dilution plate technique, demonstrated that *E. coli* and *S. aureus* are the most susceptibly bacteria to *B. alternatus* venom action.

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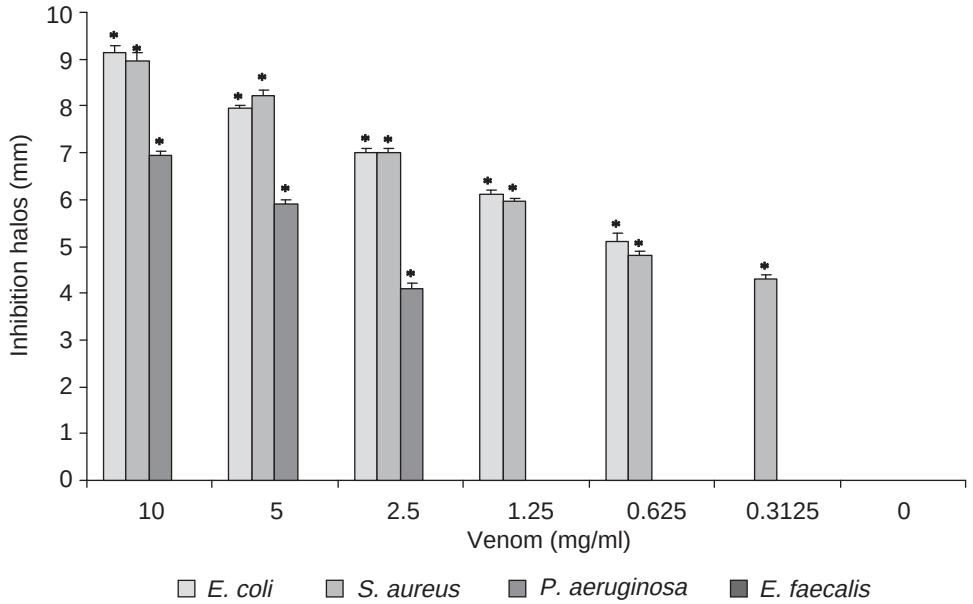
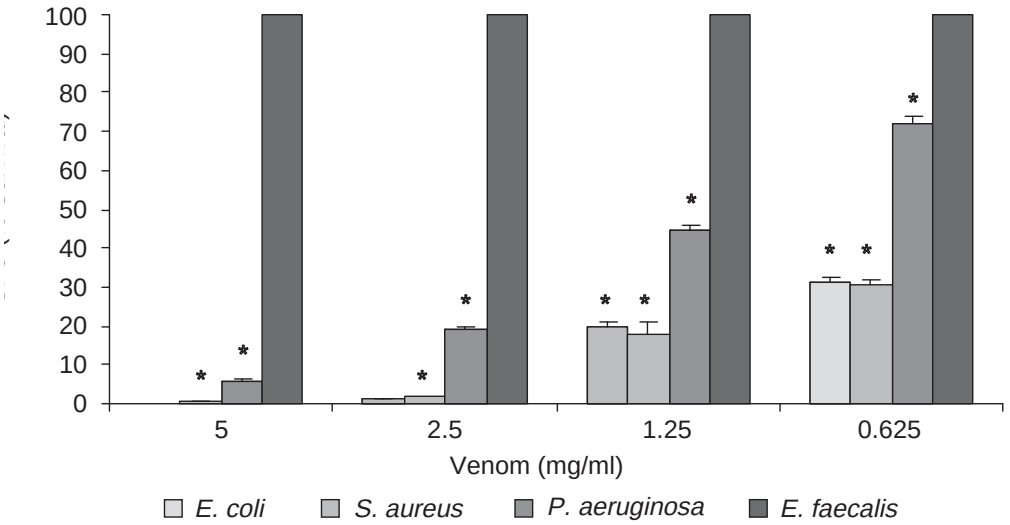


* *Significantly different to untreated control cultures, (p < 0.001).*

Figure 1. *Bactericidal activity was estimated indirectly, by comparing the diameters of inhibition halos caused by the* B. alternatus *venom. against* Staphylococcus aureus *(ATCC 25923),* Escherichia coli *(ATCC 25922),* Pseudomonas aeruginosa *(ATCC 27853) and* Enterococcus faecalis *(ATCC 29212). Values are means ± S.E. (3 replicas/treatment). Data were analyzed by one way ANOVA, Tukey test and p < 0.001 was considered significant.*



* *Significantly different to untreated control cultures, (p < 0.001).*

Figure 2. *Bactericidal activity of* B. alternatus *venom was estimated by the dilution plate technique. Surviving bacteria were counted. CFU, colony-forming units. Bars represents mean ± SD of triplicate counts.*
Data were analyzed by one way ANOVA, Tukey test and p < 0.001 was considered significant.

On the other hand, *B. alternatus* venom showed an intermediate potency as bactericidal agent against *P. aeruginosa*, and neither against *E. faecalis*, being the most resistant bacteria.

Differences in individual susceptibilities were evident, in that sense, *B alternatus* venom exhibited strongly bactericidal activity on *S. aureus*, while it was not able to kill *E. faecalis* in spite of both being Gram-positive bacteria.

It is has become increasingly clear that due to the development of antibiotic-resistant microbes, new antibacterial and antifungal peptides from natural sources have attracted the attention in recent years. The importance of bactericidal action exhibited by snake toxins is not only for a better understanding of the ophidian envenomation mechanism, but also due to their biotechnological potential as model for therapeutic agents.

In conclusion, the present study provides the first experimental evidence that *B. alternatus* venom from Argentina trigger antibacterial functions *in vitro*. Further studies will be required in order to elucidate the mechanism/s involved in this toxicity.

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