Atividade antimicrobiana da peçonha de *Lachesis muta rhombeata*

Antibacterial activity of *Lachesis muta rhombeata* venom

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ABSTRACT

Increase of death cases caused by bacterial infections has encouraged researchers to investigate different biological sources that contain bioactive molecules as new therapeutic agents for microbial infections such as animals' Venoms. Thus, the aim of this study was to evaluate antibacterial activity of

Palavras chaves: Infecção, bactéria, peçonha de cobra, Viperidae, atividade antimicrobiana.
Lachesis muta rhombeata snake venom. Venom antibacterial activity was analyzed by disc diffusion and microdilution. The results demonstrated that the L. m. rhombeata venom did not present significant antibacterial activity against gram-negative bacteria Klebsiella pneumoniae ATCC 700603 and Escherichia coli ATCC 25922, however, the venom presented moderated activity against Pseudomonas aeruginosa ATCC 27853 (inhibition halo = 11.4 ± 0.5 mm). Regarding the gram positive bacterial, the L. m. rhombeata venom presented antibacterial activity against the Staphylococcus. aureus ATCC 29213 (inhibition halo = 15.4 ± 1.1 mm), however, the venom did not exhibit activity against methicillin-resistant S. aureus (MRSA) ATCC 33591. In the microdilution method, the L. m. rhombeata venom showed higher activity against S. aureus ATCC 29213 (MIC = 64 µg/mL), moderate activity against P. aeruginosa ATCC 27853 (Minimum Inhibitory Concentration (MIC) = 256 µg/mL), weakly active against E. coli ATCC 25922 (MIC = 512 µg/mL) and inactive for K. pneumoniae ATCC 700603 and MRSA ATCC 33591 (MIC ≥ 512 µg/mL). Considering the results obtained in this study, L. m. rhombeata venom is a promising alternative as an antibacterial agent, especially against S. aureus.

Keywords: Antimicrobial activity; bacteria; Infection; Lachesis muta rhombeata; snake venoms; Viperidae.

1 INTRODUCTION

Infections caused by antimicrobial resistant bacteria have become a worldwide public health problem. Researches estimates that until 2050s, deaths attributed to infections caused by bacteria with resistance profile can reach 10 million people per year (O’ NEILL, 2014). These data represent a global concern for humans, animals and the environment due to rapid bacterial multiplication, dissemination, persistence, and occurrence of mutations in antimicrobial resistance genes (DAVIES; DAVIES, 2010). In addition, the development of new antibiotics is no longer considered an economically viable investment for the pharmaceutical industry due to lack of success and low financial gains by bringing new antibiotics to market (VENTOLA, 2015; JACKSON; CZAPLEWSKI; PIDDOCK, 2018) in the reduction of exploration on new molecules with antimicrobial potential by the pharmaceutical industry over the years (LUSHNIAK, 2014; JACKSON; CZAPLEWSKI; PIDDOCK, 2018).

This problem has led to a wider search for antimicrobial agents from animal and plant sources. Therefore, studying different biological sources increases the possibilities to discover new molecules with therapeutic potential for the treatment of these infections. As an example of a drug made from ophthalmic venoms, we can mention Captopril®, a widely known antihypertensive drug developed from a peptide isolated from the venom of the Brazilian snake Bothrops jararaca. This example demonstrates the importance of ophidian venom as an important source to be explored for the discovery of new therapeutic agents (FERREIRA, 1998; DE LIMA et al., 2010; WAHEED; MOIN; CHOUHDHARY, 2017).

Snake venoms have a wide range of molecules with potential antimicrobial activity. Examples include L-amino oxidase (LAAO), phospholipase A2 (PLA2) and metalloprotease enzymes, as well
as multi-class peptides such as cardiotoxins, crotamine and cysteine-rich secretory proteins (CRISPs) (DE OLIVEIRA JUNIOR; E SILVA CARDOSO; FRANCO, 2013). Brazil has a wide diversity of species of venomous snakes, including the largest viper (Viperidae family) in the world. Belonging to the genus *Lachesis* (collectively called “bushmasters”), these animals are distributed in remote forest areas in Central and South America. The *Lachesis muta* species is the only representative of the genus in Brazil and can be found in the Amazon Rainforest and Atlantic forest, from the state of Ceará to Rio de Janeiro. Frequently, snakes distributed in the Atlantic Forest regions are referred to as a subspecies, the *Lachesis muta rhombeata* (CAMPBELL; LAMAR, 2004). Similar to the venom of other *Lachesis* species, the proteomic analysis of *L. muta rhombeta* venom demonstrated that substance was rich in bradykinin-enhancing peptides (BPP-like peptides), serine proteases, metalloproteases and PLA2, as well as containing lectins, CRISPs and L-amino oxidase to a lesser extent (PLA et al., 2013).

Given the diversity of molecules with antimicrobial potential present in snake venoms and in the absence of studies with *L. m. rhombeata* in this area, this study aimed to investigate the antimicrobial activity of this venom against human pathogenic bacteria.

2 MATERIAL AND METHODS

2.1 MATERIAL

The lyophilized venom of the *Lachesis muta rhombeata* subspecies was supplied by the Laboratory of Venomous Animals and Toxins of the Federal University of Pernambuco (LAPTx - UFPE). Müeller-Hinton Broth (MHC), Müeller-Hinton Agar (MHA) and Brain Heart infusion (BHI) were obtained from Himedia® (Vadhani, Mumbai, IND) and Sigma-Aldrich (St. Louis, Missouri, USA). Standard bacterial strains were obtained from the American Type Culture Collection (ATCC).

2.2 BACTERIA AND GROWING CONDITIONS

Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853) and gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591) cultivated on nutrient agar and incubated at 35 ± 2 °C were used in this work.

2.3 PREPARATION OF VENOM SOLUTION

Initially, 10.24 mg of the *L. m rhombeata* venom was weighed and diluted in 10 mL of 0.9% saline to obtain a stock solution at a concentration of 1024 µg/mL. The sample was sterilized by filtration with a 0.22 µm filter (Vertical® Chromatography).
2.4 EVALUATION OF ANTIBACTERIAL ACTIVITY

For the evaluation of the antibacterial activity of *L. m. rhombeata* venom were used disc diffusion and broth microdilution methods according to the *Clinical and Laboratory Standards Institute* (CLSI, 2018).

2.5 DISC DIFFUSION METHOD (KIRBY-BAUER)

Bacteria were diluted in sterile 0.9% saline and adjusted at 0.5 McFarland scale concentration by spectrophotometry at 630 nm and then seeded using the plate-counting technique in petri dishes containing Müeller-Hinton Agar. Posteriorly, 20 µL of the venom at a concentration of 1024 µg/mL was applied to dry and sterile filter paper discs measuring 6 mm in diameter. Finally, the discs were uniformly distributed on the plates that were incubated at 35 ± 2 ºC for 24 h. The result was achieved by measuring the inhibition halos. The tests were performed on different days in triplicate.

The classification of antibacterial activity by disc method was performed according to the Qiao and Sun (QIAO; SUN, 2014): inhibition halos ≤ 10 mm corresponds to inactive molecule; inhibition zone between 10 < inhibition halos ≤ 13 mm represents moderate activity; 13 < inhibition halo ≤19 mm is active and inhibition halos > 19 mm is very active.

2.6 MICRODILUTION METHODS

Initially, Müeller-Hinton broth was added to each well of the microdilution plates. Then, the *Lachesis muta rhombeata* venom in the concentration range of 1 to 512 µg/mL was added by serial dilution. Afterwards the bacteria were adjusted at 0.5 on the MacFarland scale concentration, diluted (1:10) and seeded into the wells to obtain a final concentration of 10^5 CFU/well. Finally, the plates were incubated at 35 ± 2 ºC for 24 h. The minimum inhibitory concentration (MIC) was determined as the lowest concentration capable of inhibiting microbial growth by colorimetric analysis using resazurin. Minimum bactericidal concentration (MBC) was determined after MIC results. An aliquot of wells with no visible growth was inoculated on Müeller-Hinton agar and the plates were incubated at 35 ± 2 ºC for 24 h. After this period, MBC was determined as the lowest concentration where there was no microbial growth.

The classification of antibacterial activity by the microdilution method was performed according to Holetz *et al.*, (2002) and Ayres *et al.*, (2008): MIC > 1000 µg/mL corresponds to inactive molecule; 500 < MIC ≤ 1000 µg/mL corresponds to poor activity; 100 < MIC ≤ 500 µg/mL corresponds to moderate activity and MIC ≤ 100 µg/mL corresponds to active molecule.
2.7 STATISTICAL ANALYSIS

All halo sizes represent the mean triplicate concentration ± standard deviation.

3 RESULTS

The results obtained in this study demonstrated that in the disc diffusion method, the *L. m. rhombeata* venom showed no antibacterial activity against gram-negative bacteria *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922, but the venom showed moderate activity against *Pseudomonas aeruginosa* ATCC 27853. Regarding gram-positive bacteria, the venom presented antibacterial activity against *S. aureus* ATCC 29213. However, this venom was not active against MRSA ATCC 33591 (Table 1).

**Table 1**: Evaluation of the antibacterial activity of *Lachesis muta rhombeata* venom by the disk diffusion method. Halo sizes represent the mean triplicate concentration ± standard deviation.

| Bacteria                          | Inhibition halo | Classification |
|-----------------------------------|-----------------|----------------|
| *Klebsiella pneumoniae* ATCC 700603 | 5.7 ± 0.3 mm    | INT            |
| *Escherichia coli* ATCC 25922     | 6.2 ± 1.2 mm    | INT            |
| *Pseudomonas aeruginosa* ATCC 27853 | 11.4 ± 0.5 mm   | MOD            |
| *Staphylococcus aureus* ATCC 29213 | 15.4 ± 1.1 mm   | ATV            |
| Methicillin-resistant *Staphylococcus aureus* (MRSA) | 0 mm            | INT            |
| ATCC 33591                        |                 |                |

ATCC: American Type Culture Collection; INT: inactive; MOD: moderate activity; ATV: active.

In microdilution of venom of *L. m. rhombeata* showed antibacterial activity against some strains used in this study (Table 2). The venom showed moderate activity against *P. aeruginosa* ATCC 27853 and activity against *S. aureus* ATCC 29213, weakly active against *E. coli* ATCC 25922 and inactive for *K. pneumoniae* ATCC 700603 and MRSA ATCC 33591. Venom of *L. m. rhombeata* did not present MBC at the tested concentrations, indicating its bacteriostatic effect against *S. aureus*, *E. coli* and *P. aeruginosa*.

Moreover, from these results, it can be concluded that both methods used in this study presented similar results (Table 1 and Table 2).
Table 2: Evaluation of the antibacterial activity of *Lachesis muta rhombeata* venom by the microdilution method

| Bacteria                                           | MIC      | Classification |
|----------------------------------------------------|----------|----------------|
| *Klebsiella pneumoniae* ATCC 700603                 | > 512 µg/mL | INT            |
| *Escherichia coli* ATCC 25922                      | 512 µg/mL | WEA            |
| *Pseudomonas aeruginosa* ATCC 27853                | 256 µg/mL | MOD            |
| *Staphylococcus aureus* ATCC 29213                 | 64 µg/mL  | ATV            |
| Methicillin-resistant *Staphylococcus aureus*      | > 512 µg/mL | INT            |
| (MRSA) ATCC 33591                                  |          |                |

MIC: minimum inhibitory concentration; ATCC: American Type Culture Collection; INT: inactive; WEA: weakly active; MOD: moderate activity; ATV: active.

**4 DISCUSSION**

Natural products and their derivatives represent more than 30% of current pharmaceutical products available on the market and are one of the main sources of innovative therapeutic agents (PIMENTEL et al., 2015). Snake venoms are a rich source of compounds that exhibit a variety of pharmacological activities. However, the antimicrobial activity of the venom of *L. m. rhombeata* subspecies is not well explored. In this work, tests with the *L. m. rhombeata* venom revealed its ability to inhibit the growth of certain bacteria, especially *Staphylococcus aureus*.

Snake venoms have been studied for their antimicrobial properties. Studies performed by San et al., (SAN et al., 2010) using the *Calloselasma rhodostoma* venom belonging to the *Viperidae* family, demonstrated inhibitory effects with MIC of 125 µg/mL when tested against *S. aureus*.

Ferreira et al., (2011) evaluated the antibacterial activity of four venoms of different snakes of the *Viperidae* family, the species *Agkistrodon rhodostoma, Bothrops atrox, Bothrops jararaca* and *Lachesis muta* using the venom at a concentration of 20,000 µg/mL against strains of gram-positive and gram-negative bacteria. The results obtained in their studies showed that the *L. muta* subspecies had no antibacterial activity against the bacterial strains tested. In the disc diffusion tests using this venom the following results were obtained: 2 ± 1 mm inhibition zone against *S. aureus*, 1 ± 1 mm against *P. aeruginosa* and 0 mm against *K. pneumoniae*. On the other hand, in our study, the venom of the subspecies *L. m. rhombeata* at a concentration of 1024 µg/mL showed activity ranging from inactive, moderately active and active against the same bacteria studied by Ferreira et al., (2011).

According to Alape-Girón et al., (2008), the chemical composition and biological activities of ophidian venoms may vary among different families, genera, species, specimens and age. Variations that occur between species of the same genus are called interspecific variations, while those that occur between individuals (specimens) of the same species are called intraspecific
variations. Thus, ophidian venom may change due to several factors such as seasonal variations, eating habits, geographic location in which the animal lives, ontogenic, interpopulational, intrapopulational and interindividual variations (DALTRY; WÜSTER; THORPE, 1996; CASEWELL et al., 2013; DOWELL et al., 2018; ZANCOLLI et al., 2019).

In Brazil, the *Lachesis muta* is found in two isolated regions, namely the Amazon Rainforest and Atlantic Forest remnant areas in the Northeast and Southeast states. Distinct geographical regions such as these, subject to different climates and prey source, may influence the eating habits of these animals which, together with other factors such as seasonality, may contribute to a variation of venom composition within the same species (SANTOS et al., 1995; CAMPBELL; LAMAR, 2004; UETZ; FREED; HOŠEK, 2019).

In previous studies using the disc diffusion methodology, other venomous snakes from the Viperidae family had already shown promising antimicrobial potential against gram-positive and gram-negative bacteria. The venom of the subspecies *Daboia russelli siamensis* has been evaluated and found to be very active against *S. aureus* (25.2 ± 0.84 mm) and moderately active against *E. coli* (14.8 ± 0.83 mm) (SAMY et al., 2008). In this same study, they also evaluated the action of *Daboia russelli russelli* and *Agkistrodon halys* venoms. *Daboia russelli russelli* venom was very active against *S. aureus* (29.4 ± 0.89 mm) and *Proteus vulgaris* (26.4 ± 0.98 mm), and moderately active for *P. mirabilis* (16.8 ± 0.84 mm). Similarly, *Agkistrodon halys* venom was very active against *S. aureus* (24.1 ± 1.23 mm) and moderately active against *P. vulgaris* (15.4 ± 0.74 mm) and *P. mirabilis* (17.2 ± 0.83 mm) (SAMY et al., 2008).

Regarding the evaluation of the antibacterial activity of snake venoms of the Viperidae family using the broth microdilution method, San *et al.*, and Oguiura *et al.*, (SAN et al., 2010; OGUIURA et al., 2011) evaluated the antimicrobial property of *Calloselasma rhodostoma* and *Crotalus durissus rattlesnake* venoms against *S. aureus* and obtained MICs of 125 µg/mL and ≥ 200 µg/mL, respectively. Concerning the study of isolated toxins, Salama *et al.*, evaluated the antimicrobial activity of the purified L-amino acid oxidase enzyme from *Cerastes vipera* venom against *E. coli* and *S. aureus* and obtained MIC of 20 µg/mL while against *P. aeruginosa* and *K. pneumoniae* the MIC found was 40 µg/mL. By comparison, these reports show that *L. m. rhombeata* (MIC = 64 µg/mL) may be a promising source of antimicrobial agents against *S. aureus* (SALAMA et al., 2018).

The venom of *L. m. rhombeata* was not very promising when tested against gram-negative bacteria. Possibly, the molecules present in the venom must enter the microorganisms to develop their antibacterial activity, thus differences in cell wall composition may influence this process. In gram-positive bacteria, the cell wall is relatively thick but porous and comprises multiple layers of peptidoglycans intercalated with teichoic and lipoteichoic acids. On the other hand, gram-negative
bacteria have an outer membrane containing lipopolysaccharide (LPS) situated above a thin layer of peptidoglycans forming a barrier to the passage of outer molecules (FULY et al., 2000; WIEGAND et al., 2013; DUEÑAS-CUELLAR et al., 2015).

Studies related to molecules completely isolated from Viperidae venoms revealed different enzymatic classes with antibacterial properties, such as phospholipases A2, metalloproteases and LAAOs (DE LIMA et al., 2005; WEI et al., 2006; XU et al., 2007; OKUBO et al., 2012; SAMY et al., 2012; SUDHARSHAN; DHANANJAYA, 2015).

Some phospholipases A2 exhibit an enzymatic degradation effect on the phospholipids from plasma membrane of gram-positive and gram-negative bacteria, resulting in bacterial membrane lysis (PÁRAMO et al., 1998; DE LIMA et al., 2005; SAMY et al., 2008; SUDHARSHAN; DHANANJAYA, 2015). In addition, it has been reported that these molecules can depolarize, create pores, affect the spread and distribution of phospholipids and increase bacterial membrane permeability, as well as cause damage to intracellular targets after peptide internalization and inhibition of important metabolic pathways for the bacterial proliferation [34,39,40]. Antimicrobial studies performed with the enzyme PLA2 R49 isolated from Protobothrops mucrosquamatus venom show that this molecule exhibits bacteriostatic activity against Bacillus subtilis, P. aeruginosa and Salmonella typhimurium (WEI et al., 2006). Another PLA2 isolated from Bungarus faciatus venom was strongly active against S. aureus and E. coli (XU et al., 2007), demonstrating that phospholipases may exhibit activity against bacteria of clinical interest.

Samy et al., (2012) isolated a metalloprotease from Agkistrodon halys venom that showed inhibitory activity against Bacillus pseudomallei, P. vulgaris and S. aureus. According to these authors, one of the possible mechanisms of action of these enzymes would be by altering the arrangement of phospholipids in the bacterial membrane.

Okubo et al., (OKUBO et al., 2012) demonstrated that LAAOs isolated from Bothrops mattrugrosensis venom had antibacterial activity with MIC between 2 and 8 µg/mL when tested against gram-negative bacteria such as K. pneumoniae, E. coli and P. aeruginosa and MIC between 8 and 32 µg/mL when tested against gram-positive bacteria such as S. aureus and Streptococcus pyogenes. A possible mechanism of action for LAAOs had already been suggested by Okubo et al., (2012), where the interaction of these enzymes with the bacterial membrane would induce increased permeability and H2O2 production which is important to induce cell damage. Besides this, another mechanism was proposed by Wang et al., (2011), where the induction of cell damage caused by lipid peroxidation would cause oxidative degradation of lipids present in the bacterial cell membrane, as well as DNA chain breakage leading to a reduction in bacterial development.
Regarding the constituents of *L. muta* and *L. muta rhombeata* venoms, proteomic studies have shown that the major proteins in the venom are serine proteases (21-35%), metalloproteinases (18-38%) and PLA2s (2-13%). On the other hand, the LAAO content comprises only 0.5-10% (SANZ et al., 2008; MADRIGAL et al., 2012; PLA et al., 2013). Considering these results, it is possible to suggest that the metalloproteases and/or PLA2s of *L. muta rhombeata* venom play an important role in their antibacterial action.

Given the above, it is concluded that the venom of the *Lachesis muta rhombeata* subspecies has antimicrobial potential, being very active against *S. aureus*. As perspectives of this study, we aim to purify the possible antimicrobial molecules from *L. muta rhombeata* venom and evaluate their potential against bacteria of human clinical interest.

### 5 CONCLUSION

Natural products play an important role in the development of new drugs and are a major source of therapeutic agents for infectious diseases. Therefore, the search for new antimicrobial agents using snake venoms is promising. The results of this work suggest that *Lachesis muta rhombeata* venom is a promising alternative as a source of future antibacterial agents, presenting moderate activity against gram-negative bacteria, such as, *E. coli* and *P. aeruginosa*, and high activity against gram-positive bacteria, specially *S. aureus*. The purification of *L. muta rhombeata* venom active molecules is an important step to confirm the biotechnological potential of this venom and to develop promising new therapeutic agents for bacterial infections.

### INTEREST CONFLICT

The authors declare that there is no conflict of interest.

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