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ORIGINAL ARTICLE

Antimicrobial peptidomes of *Bothrops atrox* and *Bothrops jararacussu* snake venoms

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Abstract

The worrisome emergence of pathogens resistant to conventional drugs has stimulated the search for new classes of antimicrobial and antiparasitic agents from natural sources. Antimicrobial peptides (AMPs), acting through mechanisms that do not rely on the interaction with a specific receptor, provide new possibilities for the development of drugs against resistant organisms. This study sought to purify and proteomically characterize the antimicrobial and antiparasitic peptidomes of *B. atrox* and *B. jararacussu* snake venoms against Gram-positive (*Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*—MRSA), Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae)* bacteria, and the protozoan parasites *Leishmania amazonensis* and *Plasmodium falciparum* (clone W2, resistant to chloroquine). To this end, *B. atrox* and *B. jararacussu* venom peptides were purified by combination of 3 kDa cut-off Amicon® ultracentrifugal filters and reverse-phase high-performance liquid chromatography, and then identified by electrospray-ionization Ion-Trap/Time-of-Flight mass spectrometry. Fourteen distinct peptides, with masses ranging from 443.17 to 1383.73 Da and primary structure between 3 and 13 amino acid residues, were sequenced. Among them, 13 contained unique sequences, including 4 novel bradykinin-potentiating-like peptides (BPPs), and a snake venom metalloproteinase tripeptide inhibitor (SVMPi). Although commonly found in *Viperidae* venoms, except for Bax-12, the BPPs and SVMPi here reported had not been described in *B. atrox* and *B. jararacussu* venoms. Among the novel peptides, some exhibited bactericidal activity towards *P. aeruginosa* and *S. aureus*, had low hemolytic effect, and were devoid of antiparasitic activity. The identified novel antimicrobial peptides may be relevant in the development of new drugs for the management of multidrug-resistant Gram-negative and Gram-positive bacteria.

Keywords Snake venom peptidome · *Bothrops atrox* · *Bothrops jararacussu* · Peptidomics · Antimicrobial peptide

Introduction

Venoms represent evolutionary innovations that have evolved for predatory and defensive purposes independently in a broad phylogenetic range of animal lineages (Fry et al. 2009; Calvete 2017; Jenner and Undheim 2017). Venoms contain protein and peptide mixtures of varying complexity acting individually or as an integrated phenotype to wreak havoc on prey internal organs. Due to their high degree of target specificity, the study of venom toxins is of growing interest for the pharmacological and biotechnological communities, as venoms are increasingly recognized as attractive subjects for chemical prospecting in the search of lead compounds for the development of novel biotechnological tools and biotherapeutics (King 2015). Venom components exhibiting a range of pharmacologic activities, e.g., antihypertensive, analgesic, antitumoral, antiparasitic, and antimicrobial, have been reported (Samy et al. 2014; Almeida et al. 2016; Dal Mas et al. 2017; Akef 2018; Zhao et al. 2018; Sala et al. 2018). Scorpine is an antimicrobial peptide that exhibits

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antibacterial activity and inhibits the sporogonic development of parasites responsible for murine malaria (Conde et al. 2000). In recombinant form, this peptide showed antibacterial activity against *Bacillus subtilis* and *Klebsiella pneumoniae*, at 5 and 10 µM, respectively, and induced 98% mortality rate towards *Plasmodium berghei* at 15 µM and 100% towards *P. falciparum* at 5 µM, in addition to inhibiting the replication of dengue virus type 2 in mosquito cells (Carballar-Lejarazú et al. 2008). Crotamine, a defensin-like peptide isolated more than 70 years ago from the venom of the South American rattlesnake *Crotalus durissus terrificus* (Gonçalves and Polson 1947; Mancin et al. 1998), has not yet revealed its full pharmacological multifunctionality. Described as a potent analgesic and a myotoxin that interacts with sodium channels on muscle cells provoking spastic paralysis via hind limb hyperextension in mice (Mancin et al. 1998; Oguiura et al. 2005), a range of other activities have been attributed to crotamine, including bactericidal activity against Gram-positive and Gram-negative strains (Cendron et al. 2014), antifungal action (Yamane et al. 2013), anti-*Leishmania* (Macedo et al. 2015), antitumor (Kerkis et al. 2010) and cell membrane penetration capability (Rádis-Baptista and Kerkis 2011; Rodrigues et al. 2012). In another study, Rosas showed that the venoms of *Crotalus durissus cascavella*, *C. d. terrificus* and *B. jararaca* were toxic for promastigotes and amastigotes forms of *Leishmania chagasi* and trypomastigotes and amastigotes of *T. cruzi* (Rosas 2013).

Antimicrobial resistance (AMR) is a complex public health challenge of broad concern in many parts of the world for the effective treatment of an ever-increasing range of infections caused by multidrug-resistant bacteria, parasites, viruses and fungi (Chen and Chopra 2009; WHO 2014, 2018). The occurrence of AMR is a natural phenomenon in microorganisms (Morse 1995). Accelerated by the selective pressure imposed by use, misuse or overuse of antimicrobial agents in humans and animals (Boni and Feldman 2005; Palmer and Feldman 2012), antibiotic-resistant pathogens have found productive reservoirs in multiple sectors of the community, including hospital settings and livestock-breeding environments, and can be also transmitted to humans through the food chain (Wielinga and Schlundt 2012). First reported in 1941, growing AMR has developed in the twenty-first century into a global phenomenon, which substantially burdens the healthcare system on a global scale (Hwang and Gums 2016). Similarly, 33,000 patients die in Europe from multidrug-resistant bacterial infections (European Centre for Disease Prevention and Control 2018; Cassini et al. 2019). Though the true annual economic costs of AMR are difficult to assess, it has been estimated to amount $300 billion to date and more than $1 trillion by 2050 worldwide (Dadgostar 2019; Chokshi et al. 2019). 2.8 million USA people become yearly infected by antibiotic-resistant bacteria strains and about 35,000 die as a result of such infections (Centers for Disease Control 2019). Similarly, 33,000 patients die in Europe from multidrug-resistant bacterial infections (European Centre for Disease Prevention and Control 2018; Cassini et al. 2019). Though the true economic burden of AMR is difficult to assess, it has been estimated to amount to $300 billion to date and more than $1 trillion by 2050 worldwide (Dadgostar 2019; Chokshi et al. 2019), and the antibiotic treatment choices for already existing or emerging hard-to-treat multidrug-resistant bacterial infections are limited.

Staphylococcus aureus can be a part of skin and nose normal flora. However, owing to its numerous virulence factors and its resistance to a multitude of antibiotics, it is among the most important human pathogens involved in post-operative wound infections, pneumonia, bone and bloodstream infections that can cause sepsis and death (David and Daum 2010). *P. aeruginosa* can be found ubiquitously in soil, plants, and hospital reservoirs of water, including showers, sinks, and toilet water. *P. aeruginosa* is a common cause of healthcare-associated infections including pneumonia, bloodstream infections, urinary tract infections, and surgical site infections. A recent report identified *P. aeruginosa* as the sixth most common nosocomial pathogen overall and second most common pathogen in ventilator-associated pneumonia in US hospitals (Weiner et al. 2016). Enterobacteria like *E. coli* and *Klebsiella* spp. are frequent colonizers of the gut in humans and other vertebrates. Untreatable and hard-to-treat infections from carbapenem-resistant Enterobacterales bacteria are on the rise in hospitals among vulnerable patients, such as pre-term infants and patients with impaired immune systems, diabetes or alcohol-use disorder. The mortality rates for *K. pneumoniae* hospital-acquired pneumonia can exceed 50% in vulnerable patients, even when treated with appropriate antibacterial drugs (Eckmann et al. 2018; Keith and Pamer 2019).

The vast majority of antimicrobial classes in use today have been derived from a limited number of ecological niches and taxonomic groups, mainly from soil *Actinomyces* (Aminov 2010). However, further explorations in the past 20+ years of this ecological niche did not produce any novel drug (Culp et al. 2019). Given the drop in the rate of discovery of novel drug classes, and the consequent shortage of new antimicrobials on the horizon to combat multidrug-resistant pathogens (Freire-Moran et al. 2011), numerous countries have championed national stewardship programs to prevent the misuse of antibiotics and promote the discovery of alternative antimicrobial agents (Doron and Davidson 2011; Betts et al. 2018; Ostrowsky et al. 2018). Mounting evidence recurrently suggests the presence in animal venoms of multifunctional peptides targeting a number of bacterial and parasitic human pathogens. Currently, there are six US FDA-approved drugs derived from venom peptides or

proteins as well as many venom peptides in clinical trials or preclinical development (Pennington et al. 2018).

Parasites and bacteria have co-evolved with humankind, and they interact all the time in a myriad of ways (Ashour and Othman 2020). The work we present here reports the isolation and functional characterization of novel *B. atrox* and *B. jararacussu* snake venom peptides that exhibit bactericidal activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*P. aeruginosa)* bacteria, with low hemolytic effect, and were devoid of antiparasitic activity. This study provides novel insights into the potential functional biodiversity of two *Bothrops* snake venoms, and portends the further development, and ultimate therapeutic utility, of snake venom peptide-derived antibiotics in the treatment of antimicrobial-resistant bacterial isolates.

Methods

Preparation of snake venoms peptidomes

Venoms pooled were from an undeclared number of adult specimens from the same populations of *Bothrops atrox* and *Bothrops jararacussu* from the Banco de Venenos do Centro de Estudos de Biomoléculas Aplicadas a Saúde-CEBio, Fiocruz Rondônia and Universidade Federal de Rondônia (UNIR).

The licenses were obtained from the Brazilian Institute of the Environment (IBAMA), the Chico Mendes Institute for Biodiversity Conservation (ICMBio) no. 12/2018, authorization for activities with Scientific Purpose no. 64385-1, and register of Genetic Heritage Management Council (CGEN) no. AFCAB61 and AEFA6FB.

Fifty mg of *B. atrox* and *B. jararacussu* venoms were dissolved in 5 mL of Milli-Q™ water and centrifuged at 4000×*g* for 30 min at 20 °C. Afterwards, the supernatant was fractionated by ultrafiltration using a 3 kDa cut-off Amicon Ultra-15 cellulose membrane filter. The filtrate fraction was lyophilized for further bioprospection and biological activities.

Reverse-phase chromatography

For individual peptide purification, the *B. atrox* and *B. jararacussu* venom peptidomes were submitted to reverse-phase high-performance liquid chromatography (RP-HPLC) on an Äkta Purifier (GE Healthcare Life Sciences) system using a C18 Aeris™ PEPTIDE XB column (3.6 µm, 150×4.6 mm), developed with a linear gradient (0–100% B in 25 min) of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 99.9% ACN (solvent B) at a constant flow rate of 1 mL/min. The eluate was monitored at 215 nm wavelength.

Mass spectrometry

The molecular masses of RP-HPLC separated peptides (isolated from the natural venoms and their synthetic forms, Fig. 1) were determined using an electrospray-ionization Ion-Trap/Time-of-Flight (ESI-IT-TOF) hybrid mass spectrometer (Shimadzu Co., Japan). To this end, the chromatographic fractions, dissolved in 50% ACN containing 0.5% formic acid, were directly delivered to the ionization source at a flow rate of 50 µL/min using a Rheodyne injector. The temperature and voltage of the interface were set at 200 °C and 4.5 kV, respectively, and the detector voltage at 1.76 kV.

For peptide ion sequencing, the full-range (50–2000 *m/z*) MS^1 spectrum of each chromatographic fraction was recorded, and a mass window including the monoisotopic isotopologue of the precursor ion of interest ± 0.5 *m/z* was selected and fragmented by collision-induced dissociation (CID) using argon at 50% energy. Fragmentation (MS^2) spectra recorded in the positive mode were analyzed using

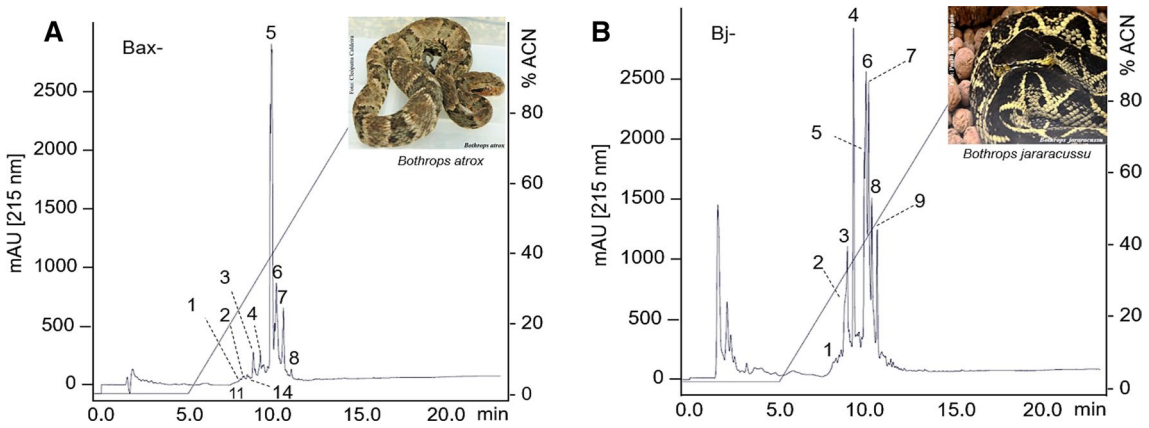

Fig. 1 Reverse-phase chromatographic profiles of the 3 kDa cut-off filtration fractions of *Bothrops atrox* (Bax) (**A**) and *Bothrops jararacussu* (Bj) (**B**) venoms

Peaks Mass Spectrometry software (Bioinformatics Solutions Inc., Canada) (Zhang et al. 2012) and the proposed assignments were manually inspected (Coutinho-Neto et al. 2013).

Synthetic peptides

The following peptides were synthesized with a degree of purity > 97% by Aminotech Research and Development, Diadema-SP, Brazil (Tables 1, 2): Pep-Bax8 (ZQPVSSPK), SVMPi-Bax/Bj (ZKW), BPP-Bax11 (GRVPDNPKAPP), BPP-Bax10 (ZKWPSPQVPP), Pep-Bj6c (ZQRFSPR) and BPP-Bj13 (ZRAPPHPPLPAPP).

Antimicrobial activity assay

Antibacterial activity towards Gram + *S. aureus* (ATCC 29213) and methicillin-resistant *S. aureus* (MRSA), and Gram-*P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 13883) and *E. coli* (ATCC 25922) of synthetic peptides Pep-Bax8 (ZQPVSSPK), SVMPi-Bax/Bj (ZKW), BPP-Bax11 (GRVPDNPKAPP), BPP-Bax10 (ZKWPSPQVPP), Pep-Bj6c (ZQRFSPR) and BPP-Bj13 (ZRAPPHPPLPAPP), and peptide fractions Bax3k and Bj3k, was assessed in accordance with the standards established by the Clinical and Laboratory Standards Institute (CLSI 2018). All bacterial strains were cultured in Luria Bertani broth (LB-BD Difco™) for 24 h (exponential phase) and the cultures adjusted to a turbidity absorbance corresponding to 0.5 on the McFarland scale, e.g., 1.5×10^6 colony-forming units/mL. The percent of bacterial growth inhibition was determined using a microdilution susceptibility testing method, whereby decreasing concentrations of peptide dry weight (250–0.48 µg/mL)

Table 2 Antimicrobial *B. atrox* and *B. jararacussu* venom peptides suggested by Boman index (BI) analysis implemented in the APD (Antimicrobial Peptides Database, https://wangapd3.com/tools.php) online platform

Peptides	Synthesized sequence	Charge	BI (Kcal/mol)
Pep-Bax8	ZQPVSSPK[a]	0	2.58
Pep-Bax7	APVDFRE	− 1	3.08
Pep-Bax4a	SLTY	0	0.29
SVMPi-Bax	ZKW[a]	0	3.34
BPP-Bax10	ZKWPSPQVPP[a]	0	1.49
Pep-Bax4b	DFFL	− 1	− 0.43
BPP-Bax11	GRVPDNPKAPP[a]	+ 1	2.64
BPP-Bax-12	ZKWPRPGPIXPP	0	2.15
Pep-Bj6a	GVVVGV	0	− 3
Pep-Bj6b	LNTLGV	0	− 0.93
Pep-Bj7	AEFELGK	− 1	1.21
SVMPi-Bj	ZKW[a]	0	3.34
BPP-Bj6c	ZQRFSPR[a]	+ 1	6.08
Pep-Bj8	PSTDLLPGV	− 1	− 0.01
BPP-Bj13	ZRAPPHPPLPAPP[a]	+ 1	1.37

Expressed in Kcal/mol, the Boman Index (BI) is a measure of the normalized free energy of protein–protein interaction computed for the amino acid sequence of a protein (Radzicka and Wolfenden 1988; Boman 2003)

[a]Peptides selected for chemical synthesis

Table 1 Peptides identified in *Bothrops atrox* (Ba) and *Bothrops jararacussu* (BJ) snake venoms by ESI-IT-TOF tandem mass spectrometry

Venom	Fraction	Sequence	*m/z*	*z*	Mass (Da)	ppm	Peptides
Bax	1–2	ZBPVSSPK[a]	427.22	2	852.43	− 14.6	Pep-Bax8
	3, 4	GRVPDNPBAPP[a]	574.31	2	1146.61	− 1.4	BPP-Bax11
	4	ZBWPRPGPEXPP	692.87	2	1383.73	5.0	BPP-Bax-12
	5	**ZBW**[a]	444.20	1	443.19	− 1.2	SVMPi -Bax
	7	SXTY	483.24	1	482.24	− 14.2	Pep-Bax4a
	5–7	ZBWPSPQVPP[a]	573.81	2	1145.59	11.3	BPP-Bax10
	8	APVDFRE	417.21	2	832.41	4.0	Pep-Bax7
	8	DFFX	271.14	2	540.26	− 1.3	Pep-Bax4b
Bj	1, 2	ZBRFSPR[a]	451.23	2	900.46	− 7.2	Pep-Bj6c
	3	ZRAPPHPPXPAPP[a]	679.36	2	1356.73	− 16.2	BPP-Bj13
	4	**ZBW**[a]	444.17	1	443.17	2.2	SVMPi-Bj
	4	PSTDXXPGV	449.74	2	897.48	− 6.6	Pep-Bj8
	5, 6	AEFEXGK	397.21	2	792.40	9.8	Pep-Bj7
	7, 8	XNTXGV	308.68	2	615.36	− 16.2	Pep-Bj6b
	9	GVVVGV	265.17	2	528.33	− 1.0	Pep-Bj6a

The only peptide shared between both *Bothrops* species is highlighted in boldface

Z represents pyroglutamic acid, *B* Gln/Lys, *Pep* peptide, *BPP* bradykinin-potentiating peptide-like, *SVMPi* snake venom metalloproteinase tripeptide inhibitor

[a]Peptides selected for chemical synthesis and functional screening

were incubated with the bacterial culture in a final volume of 200 µL in 96-well microplates, and cell growth monitored for 24 h at 37 °C. Positive control with bacterial suspension in LB broth containing 20 µg/mL of the antibiotic chloramphenicol (Sigma-Aldrich, USA) and negative control with LB broth alone were prepared. Inhibition of bacterial growth was determined spectrophotometrically at a wavelength of 630 nm using a TC 96 Elisa Microplate Reader.

Anti-*Leishmania amazonensis* activity

Leishmania amazonensis strain (IFLA/BR/ 97/PH8) was grown and maintained following the methodology established by (Ioset et al. 2009). Promastigote forms were cultured in RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid), and 40 µg/mL gentamycin (Sigma-Aldrich). The promastigote cultures were maintained in vitro using a parasitic aliquot in the stationary growth phase, which was diluted in erythrosine B dye (0.04%). Parasite concentration was adjusted to 1×10^6 cells/mL with the aid of a Neubauer chamber, and the parasites were counted by optical microscopy at 400× magnification. Living promastigotes were subcultured in RPMI 1640/FBS medium and maintained in an oven at 24 °C and 5% CO_2.

Promastigotes in the stationary phase were seeded at 1×10^6 parasites in 100 µL of RPMI 1640 complete medium per well of a 96-well plate and incubated with decreasing concentrations (200–3.12 µg/mL PBS) of synthetic or RP-HPLC purified peptides at 24 °C for 72 h. Parasites incubated with culture medium were considered as the growth control and incubation with pentamidine (5 µg/mL) at identical conditions was used as leishmanicidal reference control. After the incubation, 10 µL of a 2 mM resazurin solution in PBS was added to each well and the plate was incubated for 54 h at 24 °C. The fluorescent signal was monitored using excitation wavelength of 530 nm and emission wavelength of 590 nm. Each concentration was tested in quadruplicate and assays were repeated three times.

Anti-*Plasmodium falciparum* activity

The possible in vitro antiplasmodial activity of synthetic peptides Pep-Bax8 (ZQPVSSPK), SVMPi-Bax/Bj (ZKW), BPP-Bax11 (GRVPDNPKAPP), BPP-Bax10 (ZKWPSPQVPP), Pep-Bj6c (ZQRFSPR) and BPP-Bj13 (ZRAPPHPPLPAPP), and peptide fractions Bax3k and Bj3k, against *Plasmodium falciparum* (clone W2, resistant to chloroquine) was evaluated in human erythrocyte cultures, as described (Trager and Jensen 1976). Blood parasitic forms were maintained in RPMI 1640 (Sigma-Aldrich) medium supplemented with 50 mg/mL of AlbuMAX (Thermo-Fisher Scientific) in a set of human RBCs (type O, Rh+; hematocrit in 2%) previously washed and stored at 37 °C in 5% CO_2, 5% O_2 and balanced 5% N_2 and monitored daily by optical microscopy with immersion objective (1000×).

To obtain parasites, synchronization was performed using sorbitol. Uniformity was determined by blood smearing using a panoptic kit, fixing with methyl alcohol and staining with eosin and methylene blue, followed by visualization by optical microscopy with immersion objective (1000×). For the antiplasmodial assay, 96-well microplates were used to adjust the cultures of the synchronized parasite to a 2% hematocrit value and 0.5% parasitemia (Lambros and Vanderberg 1979). The antiplasmodial potential of the addition of serial dilutions (from 100 to 1.56 µg/mL PBS) of synthetic and RP-HPLC purified peptides, followed by incubation for 48 h at 37 °C in a 5% CO_2, 5% O_2 and balanced N_2 (Penna-Coutinho et al. 2011). Controls consisted of untreated infected red cells (negative), uninfected red blood cells (white) and the reference drug artemisinin at 50 ng/mL (positive). After the incubation, the cells were washed with PBS 1× by centrifugation at 478× for 10 min. Subsequently, a solution of 0.002 mL of an SYBR Green I (Thermo-Fischer Scientific) 10 mL in lysis buffer (20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% p/v saponin; 0.08%, v/v Triton X-100) was prepared. After centrifugation, the supernatant was discarded and 100 µL of an SYBR Green I solution in lysis buffer was added to each well, and the plate was incubated at room temperature for 30 min. Fluorescence was measured at excitation wavelength of 485 nm and emission wavelength of 535 nm. The experiment was performed in triplicate and the IC_{50} was expressed as Inhibitory Concentration (IC) index (Smilkstein et al. 2004).

Hemolytic activity

The possible hemolytic activity of synthetic peptides Pep-Bax8 (ZQPVSSPK), SVMPi-Bax/Bj (ZKW), BPP-Bax11 (GRVPDNPKAPP), and peptide fractions Bax3k and Bj3k was performed according to Stark and coworkers (Stark et al. 2002), with modifications. Briefly, human blood was collected from a healthy O^+ donor, 3.2% citrate (v/v) was added, and centrifuged at 2000×*g* for 15 min to remove plasma. The obtained erythrocytes were washed three times with phosphate-buffered saline (PBS), centrifuged for 10 min at 1000×*g*, and resuspended in PBS 4% (v/v). Peptides were serially diluted in PBS to final concentrations of 250–0.49 µg/mL. 100 µL of red blood cell suspension and 100 µL of peptide solutions were mixed and incubated for 1 h at 37 °C, followed by centrifugation for 5 min at 1000×*g*. Supernatants were transferred to a 96-well microplate and hemoglobin released was measured at 540 nm on the Biotec spectrophotometer. Incubations with 0.1% Triton

X-100, and PBS, were used as positive and negative controls, respectively.

Statistical analysis

Assays were performed in triplicate and results are presented as mean ± standard deviation. The statistical significance of the results was evaluated using the Anova test, followed by Bonferroni's post-test using GraphPad Prism 5.0 software. A value of $p < 0.05$ was considered significant.

Results and discussion

Isolation and structural characterization of *B. atrox* and *B. jararacussu* venom peptidomes

The peptide fractions of *B. atrox* (Bax) and *B. jararacussu* (Bj) venoms were obtained through membrane ultrafiltration using 3 kDa cut-off Amicon membranes and were denominated Bax3k and Bj3k, respectively. Yields were 2.6 and 3.0 mg per 100 mg of total *B. atrox* and *B. jararacussu* venom, respectively. These 3 kDa cut-off fractions were separated by RP-HPLC and 8 Bax and 9 Bj chromatographic fractions were collected (Fig. 1A, B). Eight *B. atrox* peptides (denominated Bax-1 through 8) of 3–12 amino acid primary structures and molecular masses between 443.19 and 1383.73 Da (Table 1) were characterized in 8 RP-HPLC fractions (Fig. 1A, Supplementary Fig. S1). Mass spectrometric characterization of seven peptides eluted in the 9 RP-HPLC fractions of *B. jararacussu* venom collected (Bj-1 through 9) (Fig. 1B) yielded molecular masses in the range of 443.17–1356.73 Da and amino acid sequences of 3–13 residues and (Table 1; Supplementary Fig. S1). Their molecular masses were determined by ESI-IT-TOF and their amino acid sequences deduced from the CID spectra of the corresponding monoisotopic isotopologue (Table 1, Supplementary Figure S1).

Bothrops atrox peptides Pep-Bax8, Pep-Bax4a, Pep-Bax7 and Pep-Bax4b, and *B. jararacussu* Pep-Bj6c, Pep-Bj8, Pep-Bj7, Pep-Bj6b, and Pep-Bj6a (Table 1) did not show significant BLAST hit in the non-redundant NCBI database. conversely, *B. atrox* peptides Bax-12 (ZBWPSPQVPP), Bax10 (ZBWPRPGPEXPP) and Bax11 (GRVPDNPBAPP), and *B. jararacussu* peptide Bj13 (ZRAPPHPPXPAPP) (Table 1) showed signatures, such as C-terminal PP and N-terminal Z (5-oxoproline or pyroglutamic acid), characteristically found in bradykinin-potentiating-like peptides (BPPs) (Sciani and Pimenta 2017). Proteolytically released from larger (~ 180-residue) precursors (such as *B. jararacussu* [Q7T1M3]), BPPs inhibit the angiotensin I-converting enzyme, thereby enhancing the hypotensive effect of circulating bradykinin and causing a vascular shock in the snake's prey or snakebite victim (Ferreira et al. 1970; Greene et al. 1972; Luft 2008; Sciani and Pimenta 2017). Snake venom metalloproteinase (SVMP) tripeptide inhibitors (SVMPi) ZBW, found in both *B. atrox* (Bax) and *B. jararacussu* (Bj) venom peptidomes (Table 1), are released from the N-terminal part of *B. atrox* bradykinin-potentiating-like peptides ZBWPSPQVPP, ZBWPRPGPEIPP (Table 1) and presumably from homolog *B. jararacussu* BPPs. Peptide ZBWPRPGPEIPP is identical to *B. atrox* BPP Bax-12 (Coutinho-Neto et al. 2013), and similar to ZQWPRDPAPIPP (P86721) from *B. atrox*., Tripeptide homologs to SVMPi-Bax/Bj (e.g., ZQW, ZKW, and ZNW) have been isolated from a number of *Viperinae* and *Crotalinae* venoms (Huang et al. 1998; Munekiyo and Mackessy 2005; Marques-Porto et al. 2008; Chou et al. 2013; Villar-Briones and Aird 2018) where they are present at high (mM) concentration and act as endogenous low-affinity (Ki 0.20–0.95 mM) inhibitors (Huang et al. 1998), keeping SVMPs functionally silent in the venom gland (Munekiyo and Mackessy 2005; Chou et al. 2013)

Table 3 Antimicrobial and hemolytic activities of snake venoms peptidome fractions and synthetic peptides with the chosen sequences

Names	Venom peptidomes/ synthetic peptides	MIC µg/mL (µM)					
		E. coli	*K. pneumoniae*	*P. aeruginosa*	*S. aureus*	MRSA	H%
Bax3k	*B. atrox* 3k	> 250	**250**	> 250	> 250	> 250	3.1
Bj3k	*B. jararacussu* 3k	**15.62**	**3.9**	**3.9**	**250**	> 250	1.1
Pep-Bax8	ZQPVSSPK	> 250	> 250	**125 (146.6)**	> 250	> 250	1.6
SVMPi-Bax/Bj	ZKW	> 250	> 250	> 250	**250 (564.1)**	> 250	1.7
BPP-Bax11	GRVPDNPKAPP	> 250	> 250	**125 (109.0)**	> 250	> 250	1.8
BPP-Bax10	ZKWPSPQVPP	> 250	> 250	> 250	> 250	> 250	NP
BPP-Bj6c	ZQRFSPR	> 250	> 250	> 250	> 250	> 250	NP
BPP-Bj13	ZRAPPHPPLPAPP	> 250	> 250	> 250	> 250	> 250	NP

MIC minimal inhibitory concentration, *H%* percentage of hemolysis at a maximum concentration of 250 µg/mL, *NP* not performed

Values in bold correspond to the most satisfactory bacterial inhibition results

until spontaneous disengagement of this control at the time of the snakebite (Marques-Porto et al. 2008).

Prediction and chemical synthesis of *B. atrox* and *B. jararacussu* putative antimicrobial venom peptides

Putative antimicrobial peptides were initially predicted through Boman Index (BI) analysis using the online tool implemented in the Antimicrobial Peptides Database (APD, https://wangapd3.com/tools.php) platform. This function computes the potential protein interaction index proposed by Boman based on the normalized sum of the polarity (solubility and hydrophobicity) values computed from the protein's amino acid sequence (Radzicka and Wolfenden 1988; Boman 2003). High antimicrobial potential is predicted for proteins exhibiting BI values higher than 2.48. Five out of the unique 14 Bax and Bj venom peptides characterized (Table 1) conformed to the classification of putative antimicrobial peptide and, additionally, showed features described for cell-penetrating peptides (CPPs) (Sciani et al. 2017). These peptides labeled with an asterisk in the table were synthesized for further testing. Leucine and lysine residues were included in positions with isobaric residue ambiguity, as these amino acids have been reported with higher

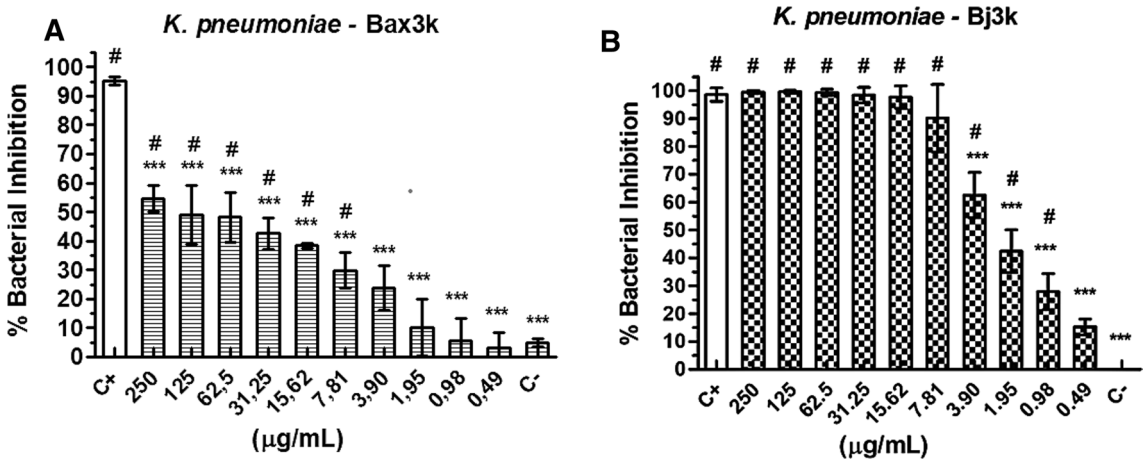

Fig. 2 Inhibition of *K. pneumoniae* growth by the whole peptidome fraction of *B. atrox* (Bax3k) (**A**), *B. jararacussu* (Bj3k) (**B**). C+, positive control, chloramphenicol (500 µg/mL); C−, negative control, bacterial suspension. The graphs show mean ± deviation ($n = 3$). Analysis of variance was carried out using Anova and Bonferroni post-test. (***) Denotes $p < 0.05$ compared to the positive control; (#) compared to the negative control

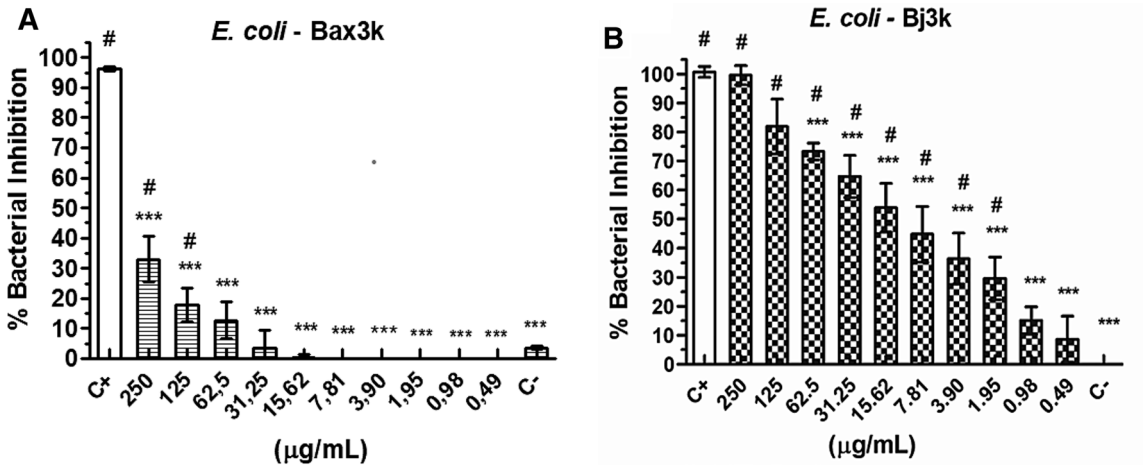

Fig. 3 Inhibition of *E. coli* growth by the whole peptidome fraction of *B. atrox* (Bax3k) (**A**) and *B. jararacussu* (Bj3k) (**B**). C+, positive control, chloramphenicol (500 µg/mL); C−, negative control, bacterial suspension. The graphs show mean ± deviation ($n = 3$). Analysis of variance was carried out using Anova and Bonferroni post-test. (***) Denotes $p < 0.05$ compared to the positive control; (#) compared to the negative control

frequency in a number of antimicrobial peptides (Wang et al. 2009; Wang and Wang 2019).

Functional analysis of *B. atrox* and *B. jararacussu* venom peptidome fractions and synthetic peptides

Antimicrobial tests were carried out with the peptide fractions Bax3k and Bj3k and their synthetic putative antimicrobial peptides. Pep-Bax8, BPP-Bax11, BPP-Bax10, Pep-Bj6c, and BPP-Bj13 showed promising results, as they inhibited, at lower concentration than the control antibiotic chloramphenicol (500 µg/mL), the growth of Gram-positive but also Gram-negative bacteria (Table 3).

At a minimal inhibitory concentration (MIC) of 250 µg/ml, Bax3k was selectively inhibited by 55% of the growth of Gram$^-$ *K. pneumoniae* (Fig. 2A). At 250 µg/mL, this *B. atrox* peptidome fraction modestly impaired the growth of *E. coli* (33%) (Fig. 3A), *P. aeruginosa* (42%) (Fig. 4A), and *S. aureus* strains (50%) (Fig. 5A). On the other hand, Bj3k exhibited inhibitory growth > 50% for both Gram$^-$ (*K. pneumoniae*, *E. coli*, *P. aeruginosa*) and Gram$^+$ *S. aureus*, except for methicillin-resistant *S. aureus* (MRSA) (Table 3). In particular, Bj3k inhibited 100% of the growth of *K. pneumoniae* and at concentrations of 250, 125 and 62.5 µg/mL (Fig. 2B), from which an IC_{50} of 3.9 µg/mL was calculated (Table 3). Bj3k also was very effective (99.8%, IC_{50} of 15.62 µg/mL) inhibiting growth of *E. coli* at minimal inhibitory concentration of 250 µg/mL (Fig. 3B), and was able to dose-dependently inhibit 100% of *P. aeruginosa* growth at concentrations of 31.25–250 µg/mL (Fig. 4B), corresponding to an IC_{50} of 3.9 µg/mL (Table 3), and reached IC_{50} growth inhibition

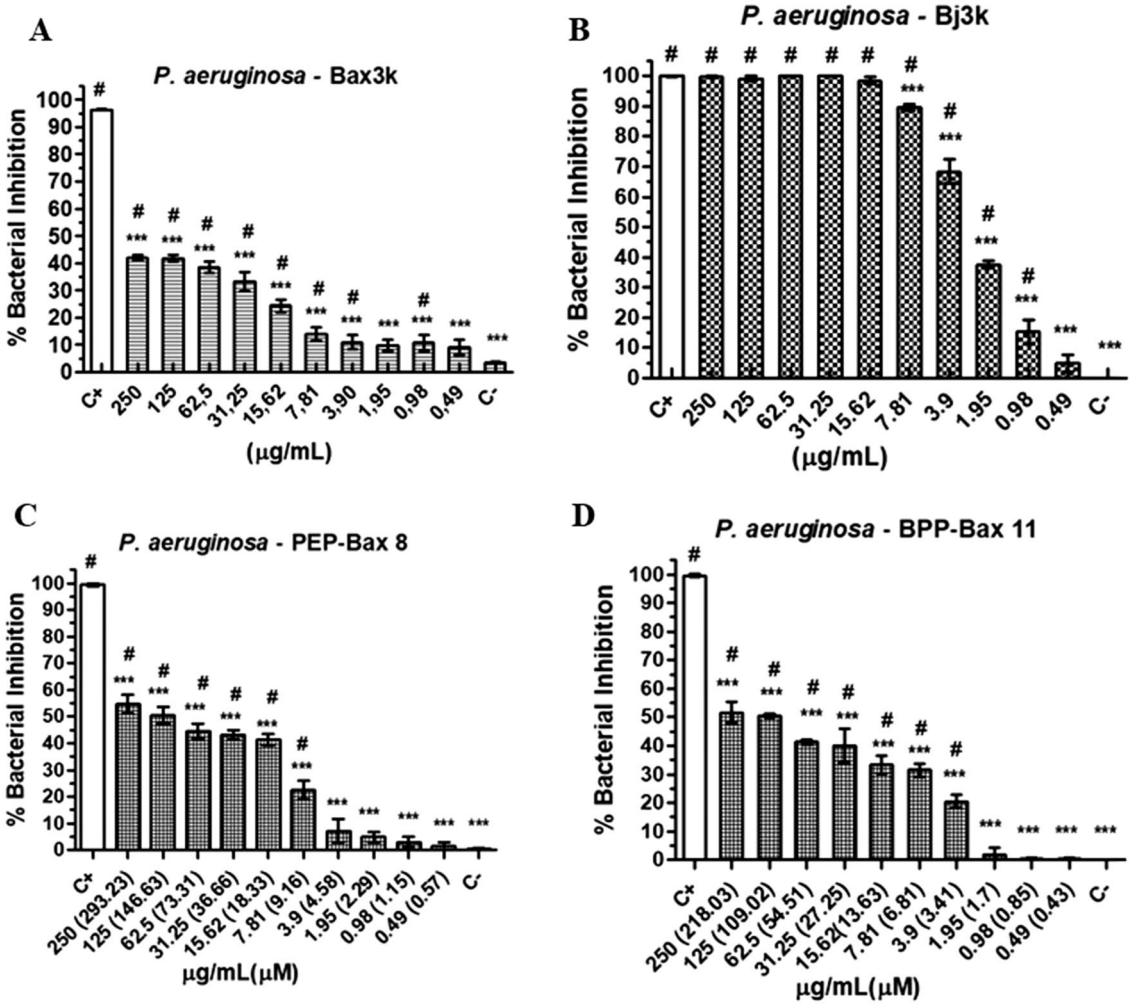

Fig. 4 Inhibition of *P. aeruginosa* growth by the whole peptidome fraction of *B. atrox* (Bax3k) (**A**), *B. jararacussu* (Bj3k) (**B**), and synthetic peptides Pep-Bax8 (**C**) and BPP-Bax11 (**D**). C+, positive control, chloramphenicol (500 µg/mL); C−, negative control, bacterial suspension. The graphs show mean ± deviation ($n = 3$). Analysis of variance was carried out using Anova and Bonferroni post-test. (***) Denotes $p < 0.05$ compared to the positive control; (#) compared to the negative control

of *S. aureus* at the maximal concentration tested (Fig. 5B). Neither the *B. atrox* and *B. jararacussu* venom peptidomes nor any of the synthetic putative antimicrobial synthetic peptide tested was capable of blocking 50% of the growth of methicillin-resistant *S. aureus* (MRSA) (Table 3). At the maximal concentration assayed (250 µg/mL), Bax3k (Fig. 6A) and Bj3k (Fig. 6B) produced 39 and 40% growth inhibition, respectively.

None of the six synthetic peptides tested showed inhibitory growth activity > 50% towards *E. coli* and *K. pneumoniae* (Table 3). However, synthetic peptides Pep-Bax8 (Fig. 4C) and BPP-Bax11 (Fig. 4D) exhibited MICs of (125 µg/mL = 146.6 µM), and (125 µg/mL = 109.0 µM), respectively, towards *P. aeruginosa* (Table 3), whereas SVMPi ZKW inhibited the growth of *S. aureus* strain ATCC 29,213 with MIC of 250 µg/mL (564.08 µM).

The positive results obtained with some bothropic venom peptides tested make us optimistic about being able to tune the peptides that showed antibacterial activity to convert them into therapeutically useful compounds. However, we are also aware that enthusiasm in peptide research has intrinsic limitation, such as immunogenicity, short half-life, proteolytic degradation, or toxicity. The hemolytic activity of peptides is the commonly considered and indicator of peptide toxicity (Ruiz et al. 2014; Kumar et al. 2020). Fractions Bax3k, Bj3k, as well as all the synthetic peptides assayed did not cause significant hemolysis in human red blood cells at the concentrations used (250–0.49 µg/mL). Maximum percentages of hemolysis triggered by peptide fractions Bax3k and Bj3k at 250 µg/mL were, respectively, 3.1 and 1.1%, and the synthetic peptides showed values between 1.4 and 1.8% (Table 3; Fig. 7).

In addition to the bactericidal activity, we tested the possible in vitro anti-*Leishmania amazonensis* and anti-*Plasmodium falciparum* activities of Bax3k and Bj3k venom peptidomes and their synthetic peptides. However, disappointingly, neither venom fractions Bax3k and Bj3k nor the synthetic Bax and Bj peptides, at the serial concentrations of 200–3.12 µg/mL and 100–1.56 µg/mL, respectively, were

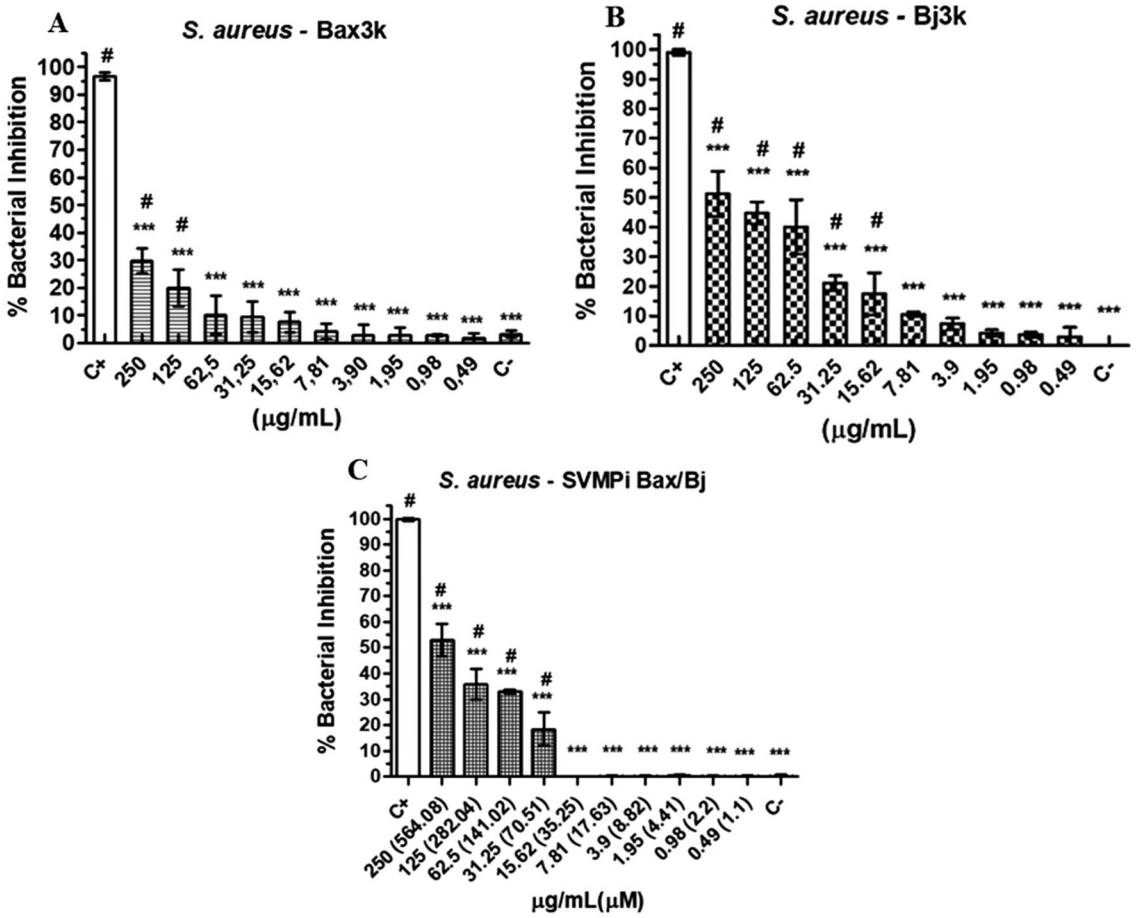

Fig. 5 Inhibition of *S. aureus* growth by the whole peptidome fraction of *B. atrox* (Bax3k) (**A**), *B. jararacussu* (Bj3k) (**B**), and synthetic peptide SVMPi-Bax/Bj (ZKW) (**C**). C+, positive control, chloramphenicol (500 µg/mL); C−, negative control, bacterial suspension. The graphs show mean ± deviation ($n = 3$). Analysis of variance was carried out using Anova and Bonferroni post-test. (***) Denotes $p < 0.05$ compared to the positive control; (#) compared to the negative control

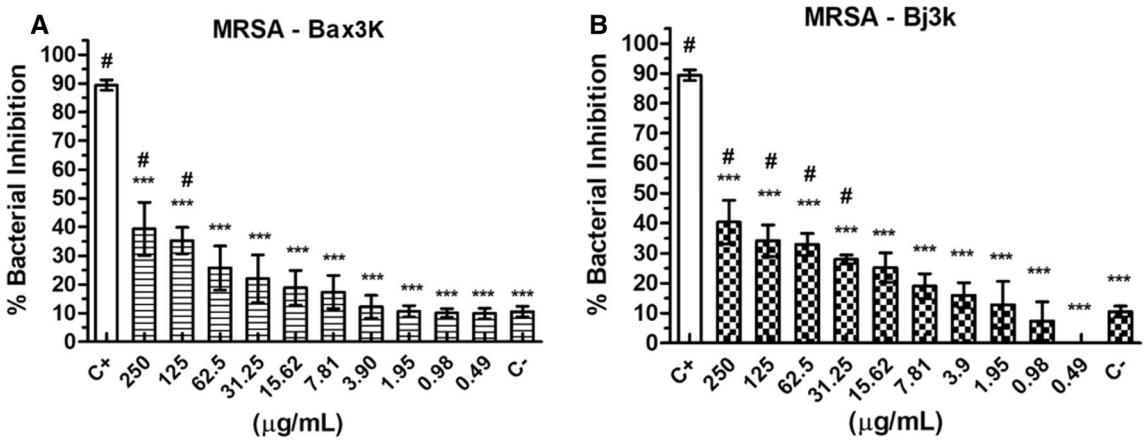

Fig. 6 Inhibition of methicillin-resistant *Staphylococcus aureus* (MRSA) growth by the whole peptidome fractions of *B. atrox* (Bax3k) (**A**), and *B. jararacussu* (Bj3k) (**B**). C+, positive control, chloramphenicol (500 µg/mL); C−, negative control, bacterial suspension. The graphs show mean ± deviation ($n = 3$). Analysis of variance was carried out using Anova and Bonferroni post-test. (***) Denotes $p < 0.05$ compared to the positive control; (#) compared to the negative control

toxic for *L. amazonensis* (IFLA/BR/97/PH8) and *P. falciparum* (clone W2 resistant to chloroquine).

Concluding remarks and perspectives

This study reports a peptidomic approach to characterize the structure and biological actions of peptides present in the venoms of *B. atrox* and *B. jararacussu* snakes. Our study identified functional differences of the venom peptidomes of *B. atrox* and *B. jararacussu* regarding their antimicrobial potential, which may aid in the design of novel antimicrobial agents. These results are in line with previous investigations reporting antimicrobial effects of whole snake venoms and peptides isolated from them. Ferreira and colleagues (Ferreira et al. 2011) evaluated the antimicrobial effect of four snakes venoms against ten clinical Gram-positive and Gram-negative bacteria strains, and found that the venom of *B. atrox* was effective against *E. faecalis* and *S. epidermidis.* Another study (Sciani et al. 2017) reported that the growth inhibitory capability of *B. jararaca* 1370 Da BPP-13a [ZGGWPRPGEIPP] against phytopathogenic fungi (*Fusarium oxysporum* and *Colletotrichum lindemuthianum)* and yeasts (*Candida albicans* and *Saccharomyces cerevisiae)* (Gomes et al. 2005) involves a cell penetration mechanism, pinpointing snake venom BPPs as multifunctional molecules.

Our finding of the inhibitory potential of the tripeptide ZKW, an endogenous inhibitor of snake venom metalloproteinases, towards the growth of *S. aureus* strain ATCC 29213 could represent a productive point of confluence between research aimed at alleviating the devasting SVMP-induced local effects associated with Viperidae snakebite envenomings and research focused on addressing the pathology associated with life-threatening infections by *S. aureus*. *S. aureus* strains are known to secrete a number of proteases that contribute to increasing their virulence. One such secretory protease involved in the pathology of staphylococcal diseases is aureolysin, a Zn^{2+}-dependent neutral metalloproteinase that cleaves plasma proteinase inhibitors α1-antichymotrypsin and α1-proteinase inhibitor and activates prothrombin in human plasma (Banbula et al. 1998; Laarman et al. 2011). In this context, our result suggests that the ZKW-mediated inhibition of the proteolytic activity of metalloproteinase virulence factors secreted by *S. aureus* strain ATCC 29,213 may underlay bacterial growth arrest. If this hypothesis holds, current efforts to find selective inhibitors of snake venom metalloproteinases (SVMPs) to block the devastating local effects of Viperidae snake venoms (Villalta-Romero et al. 2012, 2017; Gutiérrez et al. 2017) could also be relevant to identify inhibitors of virulence-aiding metalloproteases of *S. aureus*, a major bacterial human pathogen. The high IC_{50} of ZKW is consistent with the low affinity (Ki = 0.20–0.95 mM) of this class of endogenous tripeptide inhibitors of SVMP (Huang et al. 1998; Munekiyo and Mackessy 2005; Wagstaff et al. 2008; Chou et al. 2013). The crystal structure of *Trimeresurus mucrosquamatus* venom metalloproteinases TM-1 and TM-3 and their models in complex with the SVMPi ZNW (CHOU et al., 2013) provide relevant structural insights for the rational design of high-affinity peptidomimetic inhibitors for both SVMPs and the metalloproteinase virulence factors secreted by *S. aureus* strain ATCC 29213.

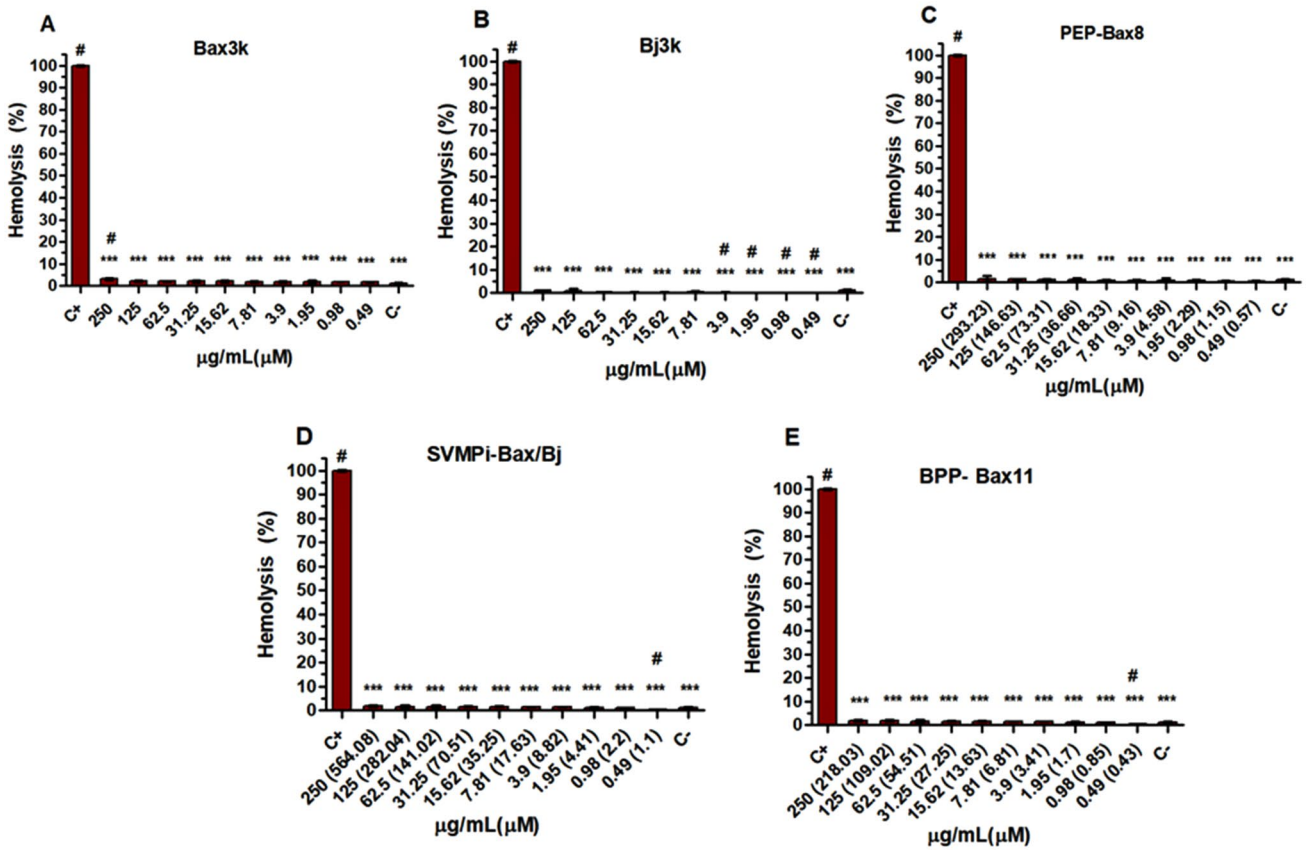

Fig. 7 Hemolytic activity of the peptide fractions of *B. atrox* (Bax3k—**A**), *B. jararacussu* (Bj3K—**B**) and peptides: Pep-Bax8 (ZQPVSSPK), **C**; SVMPi-Bax/Bj (ZKW), **D**; and BPP-Bax11 (GRVPDNPKAPP), **E**. (C+) positive control: Triton X 0.1% + erythrocytes. (C−) negative control: PBS + erythrocytes. The graphs show mean ± deviation ($n = 3$). Analysis of variance was carried out using Anova and Bonferroni post-test. (***) Denotes $p < 0.05$ compared to the positive control; (#) compared to the negative control

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Author contributions Conceptualization: CASC, LAC, AMS, and RGS. Data curation and Formal analysis: CASC, RDS, LAC, and AMS. Funding acquisition: LAC, AMS, and RGS. Investigation and Methodology: CASC, LAC, AMS, RDS, DCP, APAS, CBGT, NBM, SAC, and SLS. Writing and editing: CASC, LAC, AMS, RDS, SAC, SLS, CBGT, and DCP.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical statement The blood samples used for the tests received a favorable opinion from the Research Ethics Committee (CEP) under the number of Presentation Certificate for Ethical Appreciation (CAAE) 44899715.2.0000.0011.

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