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Mechanistic insights into functional characteristics of native crotamine

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ABSTRACT

The chemical composition of snake venoms is a complex mixture of proteins and peptides that can be pharmacologically active. Crotamine, a cell-penetrating peptide, has been described to have antimicrobial properties and it exerts its effects by interacting selectively with different structures, inducing changes in the ion flow pattern and cellular responses. However, its real therapeutic potential is not yet fully known. Bearing in mind that crotamine is a promising molecule in therapeutics, this study investigated the action of purified molecule in three aspects: I) antibacterial action on different species of clinical interest, II) the effect of two different concentrations of the molecule on platelet aggregation, and III) its effects on isolated mitochondria. Crotamine was purified to homogeneity in a single step procedure using Heparin Sepharose. The molecular mass of the purified enzyme was 4881.4 Da, as determined by mass spectrometry. To assess antibacterial action, changes in the parameters of bacterial oxidative stress were determined. The peptide showed antibacterial activity on *Escherichia coli* (MIC: 2.0 μg/μL), *Staphylococcus aureus* (MIC: 8–16 μg/μL) and methicillin-resistant *Staphylococcus aureus* (MIC: 4.0–8.0 μg/μL), inducing bacterial death by lipid peroxidation and oxidation of target proteins, determined by thiobarbituric acid reactive substances and sulfhydryl groups, respectively. Crotamine induced increased platelet aggregation (IPA) at the two concentrations analyzed (0.1 and 1.4 μg/μL) compared to ADP-induced aggregation of PRP. Mitochondrial respiratory parameters and organelle structure assays were used to elucidate the action of the compound in this organelle. The exposure of mitochondria to crotamine caused a decrease in oxidative phosphorylation and changes in mitochondrial permeability, without causing damage in the mitochondrial redox state. Together, these results support the hypothesis that, besides the antimicrobial potential, crotamine acts on different molecular targets, inducing platelet aggregation and mitochondrial dysfunction.

1. Introduction

Snake venoms are a promising source of biologically active substances that have specific mechanisms of action under different molecular targets (Koh et al., 2006). The biological effects caused by the *Crotalus durissus terrificus* venom are attributed to the actions of various neurotoxins such as crotoxin, convulxine, gyroxine and crotamine, as well as peptides and enzymes, including L-amino-xidase, thrombin-like phosphodiesterases, tissue-type kallikrein NAD-hydrolase, disintegrins, natriuretic peptides, growth factors, C-type lectin, and cysteine-rich secretory proteins (Bercovici et al., 1987; Bjarnason and Fox, 1994).

Crotamine, a polypeptide with an approximate molecular mass of 4.8 kDa, induces structural damage to skeletal muscle fibers with

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consequent myotoxicity and myonecrosis (Gonçalves and Arantes, 1956; Cameron and Tu, 1978; Peigneur et al., 2012). These effects are related to changes in membrane potential and the influx of sodium and potassium ions, changes in mitochondrial calcium homeostasis, and degeneration of myofibrils, without damage to the sarcolemma or the transverse tubules (Cheymol et al., 1971; Chang and Tseng, 1978; Gutiérrez and Cerdas, 1984; Ownby et al., 1988; Fletcher et al., 1996) and also is able to blocker Kv1.3 channel, such as mitoKv1.3 in mitochondrial inner membrane (Peigneur et al., 2012; Szabó et al., 2008).

The pharmacological activities of crotamine have been studied previously, including the induction of acetylcholine and dopamine release in striated tissue (Camillo et al., 2001) and of histamine into mast cells (Mancin et al., 1997), analgesia (Mancin et al., 1998), insulin secretion of β-pancreatic cells (Toyama et al., 2000), and stimulation of phagocytic activity of macrophages from the induction of nitric oxide (iNOs) and TNF-α via p38 and nFk-B (Lee et al., 2016). Crotamine was also shown to increase memory persistence in mice with no changes in mood, anxiety and locomotion (Vargas et al., 2014). In addition, crotamine has been shown to have a heterogeneous cytotoxic profile on different microorganisms, and structural and/or genetic similarities with antimicrobial peptides, such as HBD-2 and β-defensive peptides. Moreover, the toxin has shown important effects on cellular metabolism involving different signaling pathways, and due to its cellular penetration capacity, it has been studied as a peptide mediator for the release of drugs, peptides and proteins, and as an antitumor substance (Kerkis et al., 2004, 2017; Hayashi et al., 2008, 2012; Nascimento et al., 2007; Pereira et al., 2011; Rodrigues et al., 2013).

The cytotoxic effects of crotamine have been demonstrated *in vivo* and *in vitro* using tumor cell lines, which allow understanding how the molecule is capable of altering cellular homeostasis including damage to cytoplasmic organelles such as lysosomes and mitochondria (Nascimento et al., 2012). Due to its characteristics, crotamine has been recognized as a promising molecule in several biological processes. To better understand the toxicity of crotamine and its probable antibacterial action, we evaluated its effect on the oxidative stress in bacteria of clinical interest. Additionally, we studied the effects of crotamine on platelet aggregation and mitochondrial bioenergetics.

2. Materials and methods

2.1. Reagents and venom

Crotalus durissus terrificus venom, which is yellowish in color, was purchased from Koemitã Me (Mococa, SP, Brazil). Heparin-Sepharose FF and HiPrep 26/10 Desalting column were purchased from Amersham Life Science, Inc. The Müeller-Hinton agar was purchased from Himedia (India) and the platelets were from healthy donors. EGTA, sucrose, rotenone, succinate, ADP, cyclosporin, ruthenium red, N-ethylmaleimide, butylated hydroxytoluene, thiobarbituric acid and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was purchased from Sigma Chem. Co. Alfa-cyano-4-hydroxy cinnamic acid (HCCA) and Peptide calibration standard II were purchased from Bruker Daltonics. TFA, Acetonitrile from JT Baker. All of the other reagents used in this study were of analytical grade and purchased from Sigma Chem. Co, Merck and/or Sinapse Biotechnologia.

This research was approved by the Committee of Ethics in Research and Scientific Merit of the Hermínio Ometto University Center, UNIARARAS (process nº 833/2015 and 014/2017).

2.2. Purification of crotamine

C. durissus terrificus crude venom (0.1 g) was applied to Heparin-Sepharose FF (HiTrap, heparin (HP), 5 mL) column that had previously been equilibrated with 0.01 M sodium phosphate, pH 7.0. The protein was eluted in linear gradient of NaCl (0–1.5 M) at a flow rate of 2.5 mL/min, and 3 mL fractions were collected. The eluted fraction containing crotamine was concentrated in an ALPHA 2–4 LD plus Freeze-Dryer and 30 mg of the fraction were applied to HiPrep 26/10 Desalting column equilibrated with 0.05 M ammonium bicarbonate (AMBIC), pH 7.0, at a flow rate 10 mL/min, and 3 mL fractions were collected. Chromatography was performed using the ÄKTAprime and liquid chromatography system (GE Healthcare). Next, crotamine was subjected to identification and functional characterization. All purification and isolation procedures were performed at room temperature.

2.3. Identification of crotamine

The identification of crotamine was determined by mass spectrometry. A Coomassie Brilliant Blue-stained crotamine band (approximately 2 μg/spot) was cut out of the polyacrylamide gel (13%) and "in-gel" digested for peptide mass fingerprinting and for internal sequence determination. Five micrograms of isolated crotamine were reduced in 10 mM DTT, alkylated in 50 mM iodoacetamide and then trypsinized in 20 ng/μL trypsin after gel electrophoresis, according to the protocol reported by Shevchenko et al. (1996), with some modifications. Tryptic fragmentations of the peptide were scored by the cleavage of peptide bond, and charges on C-terminus (y ions) and N-terminus (b ions) were generated. Peptide identification was performed in a liquid chromatography-tandem mass spectrometer coupled to a high performance liquid chromatographer using a C18 nanocolumn (LC-MS/MS Q-TOF PREMIER™). The resulting spectra were analyzed using Mascot (Matrix Science) in the NCBI nr protein databases, with carbamidomethylation as the fixed modification. The similarity between peptide sequences was assessed with BLAST.

The molecular mass, as well as, profiling of native crotamine was determined through matrix-assisted laser desorption/ionization (MALDI-TOF/TOF) by using a Bruker Ultraflextreme equipment (Bruker Daltonics). Five micrograms of crotamine was diluted with alpha-cyano-4-hydroxy cinnamic acid (HCCA) in a saturated solution of acetonitrile containing 0.1% trifluoroacetic acid: deionized water (3:7 v/v). The parameters used to obtain the spectra were: 1000 laser shots/spectra, PIE (Pulsed ion extraction) of 100 ns, positive mode, laser frequency of 1000 Hz and negative mode of ionization. The voltage (IS1 and IS2) applied were 20 kV and 18.05 kV respectively. The voltage reflector mode (RV1 and RV2) of 21.3 kV and 10.7 kV respectively were used. For external calibration, a Bruker peptides standard II were used.

2.4. Evaluation of the antibacterial action of crotamine

2.4.1. Determination of bacterial oxidative stress

The antibacterial action was evaluated by the microdilution method according to the Clinical and Laboratory Standards Institute (Wayne, 2007) for the following bacterial species of clinical interest: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and ORSA – oxacillin-resistant *Staphylococcus aureus* (ATCC 33591). Bacterial cultures were spiked into Brain Heart Infusion Broth (BHI) enriched medium and incubated at 37 °C for 24 h. After, the microbial density was adjusted to approximately 10^8 CFU/mL. Then, 50 μL of sterile BHI culture medium, 50 μL of the diluted bacterial suspension at 10^6 CFU/mL and 100 μL of crotamine at increasing concentrations

(2.0–16 μg/μL) were added to a 96-well plate. A negative control of 100 μL of sterile ultrapure H_2O and a positive control of 100 μL of levofloxacin (30 μg/μL) were submitted to the same conditions. Plates were incubated at 37 °C for 24 h and inhibition of bacterial growth was determined spectrophotometrically on a Biotek ELX 800 microplate reader (λ_{490nm}) at 0 and 24 h. The lowest concentration capable of inhibiting bacterial growth was considered the minimum inhibitory concentration (MIC).

Bacterial oxidative stress was determined by the levels of thiobarbituric acid reactive species (TBARS) according to the methodology described by Esterbauer and Cheeseman (1990). The concentration of MDA-TBA produced was determined spectrophotometrically (λ_{535nm}) and reported in nM/mg protein, using the molar extinction coefficient ($\varepsilon = 1.56 \times 10^5\ M^{-1}cm^{-1}$). The sulfhydryl groups were determined by the spectrophotometric method (λ_{412}) as described by Faure and Lafond (1995) by reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and the levels calculated using the molar extinction coefficient ($\varepsilon = 13600\ M^{-1}cm^{-1}$).

2.5. Effect of crotamine on platelet aggregation

A pool of blood samples obtained of ten human health volunteers, previously treated with 3.2% sodium citrate, were centrifuged at 850 rpm for 15 min and at 3500 rpm to obtain platelet-rich plasma (PRP). The PRP concentration was adjusted in an aggregometer (5×10^5 platelets/mL) and the absence of platelet aggregation was confirmed. Two different concentrations of crotamine and crude venom (0.1 and 1.4 μg/μL) were incubated with 440 μL of PRP and the reaction was monitored for 5 min in a Chrono-Log Model 700 Whole Blood /Optical Lumi-Aggregometer ($\lambda_{935-955}$nm). The results transcribed by the Aggro /Link 8 software were compared with the positive control (ADP 1 nM/μL) and the negative control (PRP only) under the same conditions. Results are reported as percentage of platelet aggregation over a 5-min time period.

2.6. Mitochondrial assays

2.6.1. Animals

Male Wistar rats weighing 180–220 g were used. The Committee for Experimental Animal Care and Use of the Hermínio Ometto University Center, Brazil, approved all the experimental procedures (FHO-UNIARARAS, protocol 014–2017). Animals were kept under a 12 h light:dark cycle, at an ambient temperature of 24 ± 2 °C, with water and food provided *ad libitum*.

2.6.2. Mitochondria isolation

The animals were euthanized and their livers (10–15 g) were immediately removed, sliced into a 50 mL medium containing 250 mM sucrose, 1 mM EGTA, and 10 mM HEPES–KOH, pH 7.2, and homogenized three times in a Potter–Elvehjem homogenizer for 15 s, with 1min intervals between each. Rat liver mitochondria were isolated by standard differential centrifugation (Pedersen et al., 1978). Homogenates were centrifuged at 580 g for 5 min; the supernatant was centrifuged again at 10,300 g for 10 min. The pellets were suspended in 10 mL of medium containing 250 mM sucrose, 0.3 mM EGTA, and 10 mM HEPES–KOH, pH 7.2, and finally centrifuged at 3400 g for 15 min. The mitochondrial pellet was suspended in 1 mL of medium containing 250 mM sucrose and 10 mM HEPES–KOH, pH 7.2, and used within 3 h. During all the procedures the mitochondria were maintained at 4 °C, and the mitochondrial protein content was determined by the biuret reaction.

2.6.3. Mitochondrial respiratory rate

Mitochondrial respiration was polarographically monitored on an oxygraph (Hansatech, Norfolk, England) equipped with a Clark-type oxygen electrode (Chance and Willians, 1956). The mitochondria (1 mg of protein/mL) were incubated in 1 mL of the standard medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, 0.5 mM EGTA and 10 mM K_2HPO_4, pH 7.2, at 30 °C. Glutamate + malate and potassium succinate at 5 mM (added with 2.5 μM rotenone) were used as the oxidizable substrate for complexes I and II of the respiratory chain, respectively. The state III of the phosphorylation was initiated with 400 nmol of ADP for all assays, and crotamine was added at the time of testing (Cain and Skilleter, 1987).

2.6.4. Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in apparent turbidity at 540 nm measured with a spectrophotometer (Thermo Fisher Scientific Evolution™ 300 UV–Vis). The mitochondrial suspension (0.4 mg protein/mL) was incubated with the standard reaction medium plus 10 μM Ca^{2+}, 2.5 μM rotenone, and 5 mM succinate, at 30 °C. Kinetics evaluation was performed for 10 min (Lemaster et al., 1987) while different concentrations of crotamine were added to analyze mitochondrial swelling. To better understand the mechanisms that trigger the process, 1 μM cyclosporine A (CsA), 0.5 μM and 25 μM ruthenium red (RR), 25 μM N-ethylmaleimide (NEM) and 25 μM butylated hydroxytoluene (BHT) were used as modulators of the process. Crotamine (1000 μg/mL) was added to the standard reaction medium after the modulators at the time of testing (Lemasters et al., 1987).

2.6.5. Mitochondrial redox state

Lipid peroxidation of the mitochondrial membrane (LPO assay) was estimated from malondialdehyde (MDA) generation (Buege and Aust, 1978). The mitochondrial suspension (1 mg of protein in 1 mL) was incubated with 50 μM $FeSO_4$ plus 2 mM sodium citrate at 37 °C. After 30 min, 1 mL of 1% thiobarbituric acid (TBA, prepared in 50 mM NaOH), 0.1 mL of 10 M NaOH and 0.5 mL of 20% H_3PO_4 were added, followed by incubation for 20 min at 85 °C. The MDA-TBA complex was extracted with 2 mL of *n*-butanol and absorbance was measured at λ_{535nm}. As a positive control, hydrogen peroxide (3%) was used. MDA concentration was reported as ng/mg of protein calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Jocelyn, 1987; Devienne et al., 2007).

For quantification of sulfhydryl groups, the mitochondrial homogenate (1 mg/mL) was mixed with 5% perchloric acid, and the precipitate was resuspended with 1 mL of Tris-EDTA buffer (pH 8.0). As negative control, the same conditions were used without the addition of the peptide, and for the positive control, hydrogen peroxide (3%) was used. The produced amount of sulfhydryl groups in proteins was determined spectrophotometrically (λ_{412nm}) after addition of 0.2 mM/L DTNB and calculated using the molar extinction coefficient ($\varepsilon = 13600\ M^{-1}cm^{-1}$).

2.7. Statistical analysis

The data from the antimicrobial and platelet aggregation assays were assessed by the Kolmogorov-Smirnov normality test, which confirmed that data was parametric. Therefore, the Student's t-test was used to compare each experimental group with the control group. For the mitochondrial assays, data were subjected to analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons. The data are presented in means ± SD or means ± SEM. A $p < 0.05$ indicates significant difference between the analyzed samples. Graphs and statistical analysis were performed in GraphPad Prism 5.1 software.

3. Results

3.1. Purification and identification of crotamine

To assess the effects of crotamine on different biological systems, the molecule was isolated by bioaffinity chromatography done in one step in a Heparin Sepharose FF column (Fig. 1A). Crotamine identity and molecular mass were confirmed by LC-MS/MS Q-TOF PREMIER and MALDI-TOF/TOF mass spectrometric analysis. Three internal peptide fragments (ICIPPSSDFGK; ICIPPSSDFGKMDCR, and EKICIPPSSDFGK) determined by mass spectrometry, confirmed the molecular identity (Fig. 2A). Additionally, the purified peptide presented an experimental molecular mass of 4881.449 Da (Fig. 2B).

3.2. Bacterial oxidative stress induced by crotamine

The results demonstrate a heterogeneous response pattern depending on the sensitivity of each microorganism, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* - MRSA (ATCC 33591) (Fig. 3). After 24 h incubation the species most susceptible to crotamine showed a growth reduction with concentrations between 2.0 and 16.0 μg/μL for *E. coli*, 8.0 and 16.0 μg/μL for *S. aureus* and 4.0–16.0 μg/μL for ORSA. The MIC determined for susceptible species was 16 μg/μL after 24 h of incubation (Fig. 3A, C, D). However, *P. aeruginosa* showed resistance at all concentrations of crotamine used (Fig. 3B). Additionally, except for *P. aeruginosa*, oxidative stress was observed in all other species, evidenced by the high levels of TBARS and sulfhydryl groups (-SH) (Fig. 4A–H).

3.3. Effect of crotamine on platelet aggregation

After 5-min treatment of platelet rich plasma (5×10^5 platelets/mL) with the lowest concentration (0.1 μg/μL) of crotamine, platelet aggregation was induced at ~96%. Treatment with the highest concentration induced 100% platelet aggregation, more than with the ADP positive control (~85%). The crude venom at the two concentrations analyzed (1.4 and 0.1 μg/μL) under the same conditions demonstrated a similar effect to isolated native crotamine (Fig. 5).

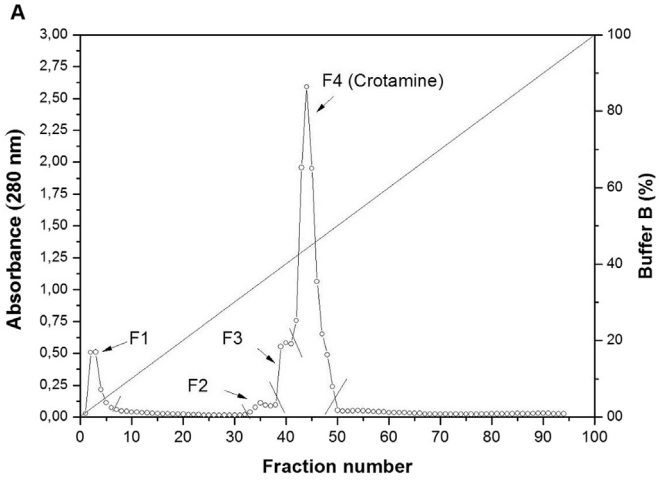

Fig. 1. Purification and isolation of crotamine. (A) Affinity chromatography of the crude venom of *Crotalus durissus terrificus* on Heparin Sepharose column (HiTrap, heparin (HP), 5 mL), which had been previously equilibrated in 0.01 M sodium phosphate, pH 7.0, and eluted in increasing 1.5 M NaCl gradient (Buffer B, 0–100%) with the same buffer at a flow rate of 2.5 mL/min. Fractions of 3 mL were collected and analyzed in a spectrophotometer (λ_{280}nm). (B) SDS-PAGE using a 13% acrylamide-bisacrylamide (w/v) gel in Tris-glycine buffer, pH 8.3. Lane 1: molecular weight markers. Lane 2: crude venom (CV) (10 μg) and Lane 3: Crotamine (10 μg).

3.4. Effects of crotamine on mitochondrial function

Crotamine, a cell-penetrating peptide, has been reported in the literature as causing mitochondrial and sarcoplasmic reticulum swelling (Fletcher et al., 1996; Nascimento et al., 2012). However, the mechanisms of this effect are unknown. Several toxins can interfere on and modify physiological mechanisms, and in many cases the preferential target is mitochondria (Bragadin, 2006). Therefore, to confirm the hypothesis that crotamine interacts with this organelle, we assessed mitochondrial oxidative phosphorylation. The results showed that crotamine was able to affect mitochondrial respiration when it is energized with succinate, a substrate for complex II of the mitochondrial respiratory chain. The respiratory control ratio (RCR) revealed decreased oxygen consumption at all crotamine concentrations (Fig. 6A). However, the ADP consumption by oxygen molecule (ADP/O) was not diminished in the groups treated with crotamine (Fig. 6B). In contrast, the velocity values in state IV (V4) had a considerable increase, demonstrating the ability of the peptide to act as an uncoupler agent of the mitochondria (Fig. 6C).

Mitochondrial swelling triggers the release of proteins located in the matrix or in the intermembrane space of mitochondria exposed to xenobiotics (Rasola and Bernardi, 2007a; b; Halestrap, 2009). The evaluation of mitochondrial swelling demonstrated the ability of crotamine to modify the permeability of the mitochondrial membrane and consequently its integrity and selectivity. This effect was expected because the tested peptide has cell-penetrating ability. Fig. 7A shows statistically significant differences ($^*p < 0.05$) between the treated and the control group (without addition of crotamine). The compound (Fig. 7B) exerted an effect at the three highest concentrations (100, 500 and 1000 μg/mL), but only at 1000 μg/mL the effect was significant. As shown in Fig. 7C, pre-incubation of mitochondria with mitochondrial swelling modulators prior to exposure to crotamine partially inhibited this effect. The swelling was prevented in the presence of 1 μM CsA, 0.5 μM and 25 μM ruthenium red, 25 μM N-ethylmaleimide and also by 25 μM butylated hydroxytoluene.

The ability of crotamine to alter the mitochondrial redox state was determined by the levels of TBARS and by monitoring the oxidation of sulfhydryl groups.

Our results demonstrate that crotamine in the concentrations used did not cause lipoperoxidation in the mitochondria compared to the positive control (H_2O_2), as shown in Fig. 8A. This data corroborates the fact that crotamine did not induce the oxidation of sulfhydryl groups present in the mitochondrial membrane (Fig. 8B).

4. Discussion

In this study, we isolated crotamine, a cell penetrating peptide (CCP) from *Crotalus durissus terrificus* venom. Crotamine was purified by a new method, in a single step chromatography, using Heparin Sepharose column. Heparin Sepharose matrix is a highly sulphated glycosaminoglycan with the ability to bind a very wide range of biomolecules, such as DNA binding proteins, serine protease inhibitors, proteases, coagulation enzymes, and growth factors (Kan et al., 1993; Farooqui et al., 1994). Due to its unique structure and surface charge distribution, heparin is able to interact strongly with many enzymes and macromolecules in two ways: a positive, cooperative binding, and a specific binding. According to Nascimento et al. (2007) the mechanism of crotamine penetration

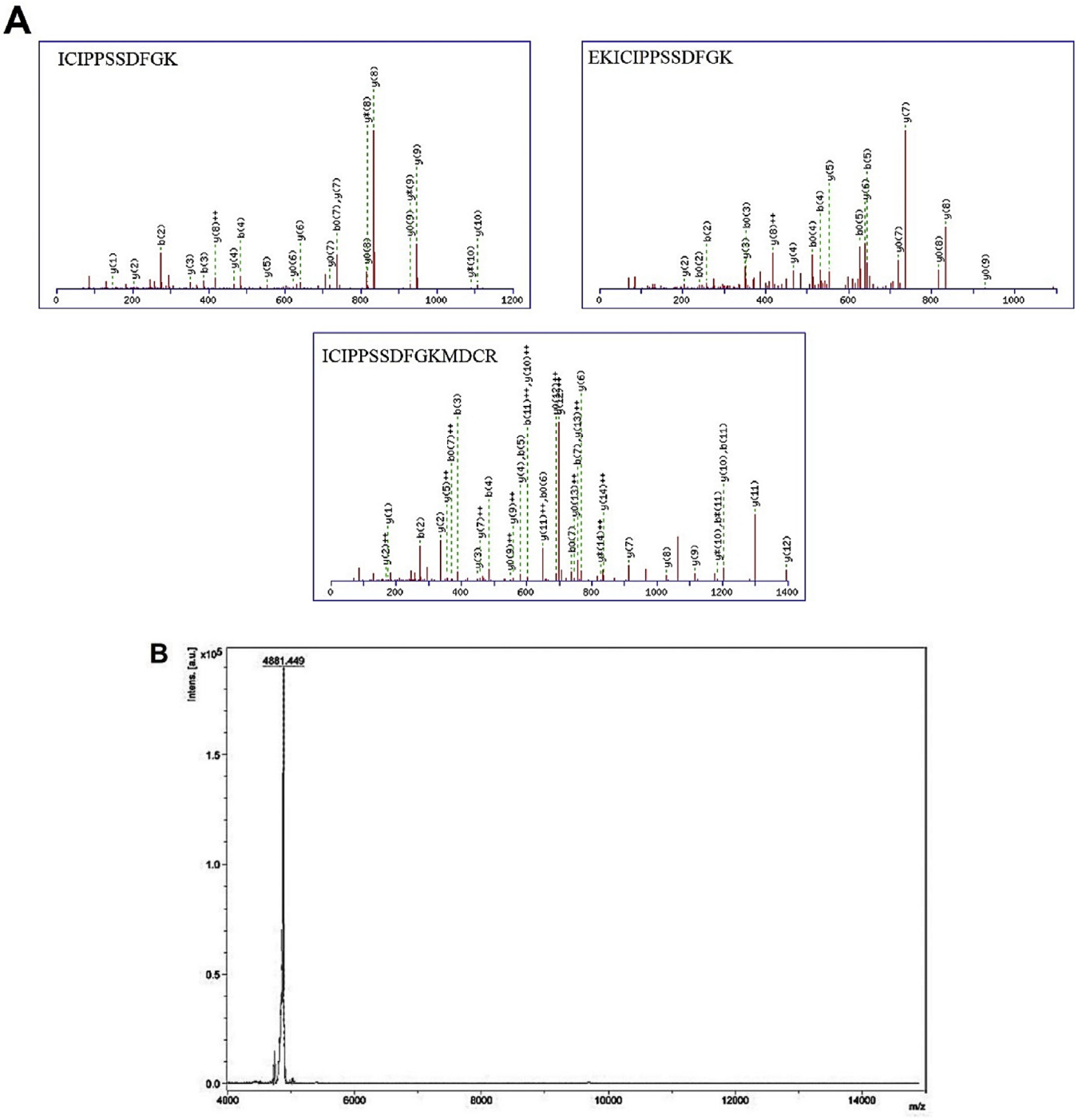

Fig. 2. Partial sequencing and identification of *C. durissus terrificus* crotamine. (A) LC-MS/MS Q-TOF PREMIER™ mass spectrometry of crotamine. Five micrograms of isolated crotamine were reduced in 10 mM DTT, alkylated in 50 mM iodoacetamide and digested with 20 ng/μL of trypsin. Protein identification was performed in a mass spectrometer coupled to an HPLC using a C18 nanocolumn. The resulting spectra were analyzed using Mascot (Matrix 5 Science) in the NCBI nr protein databases, with carbamidomethylation as the fixed modification. Similar peptide sequences were identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Peptide fragments are observed in b ions (N-terminus), and y ions (C-terminus). a*, b* and y* represent ion fragments of RKNQ that have lost ammonia (−17 Da). b° represent ion fragments of STED that have lost water (−18 Da). (B) MS spectra of crotamine. 1000 laser shots/espectro, PIE (Pulsed ion extraction) of 100 ns, positive mode, laser frequency of 1000 Hz and negative mode of ioniozation. Voltage of 20 kV (IS1) and 18.05 kV (IS2). Reflector voltage of 21.3 kV (RV1) and 10.7 kV (RV2). For calibration and equipment, Bruker peptides were used.

and cargo delivery into cells involves heparan sulfate proteoglycans interaction in the uptake phase and high cell nuclear affinity. Thus, the present method of isolation the crotamine was used considering these two mechanisms. The degree of purity of crotamine was confirmed by matrix-assisted laser desorption/ionization (MALDI-TOF/TOF) spectrometry that presented a good resolution peak. Purification process of crotamine was conducted under neutral conditions in order to confer greater stability during the isolation of the molecule, since previous studies have shown that crotamine may exhibit variable structural conformation depending on pH (Beltran et al., 1985). In addition, several studies on the isolation and biochemical characterization of crotamine have used two or

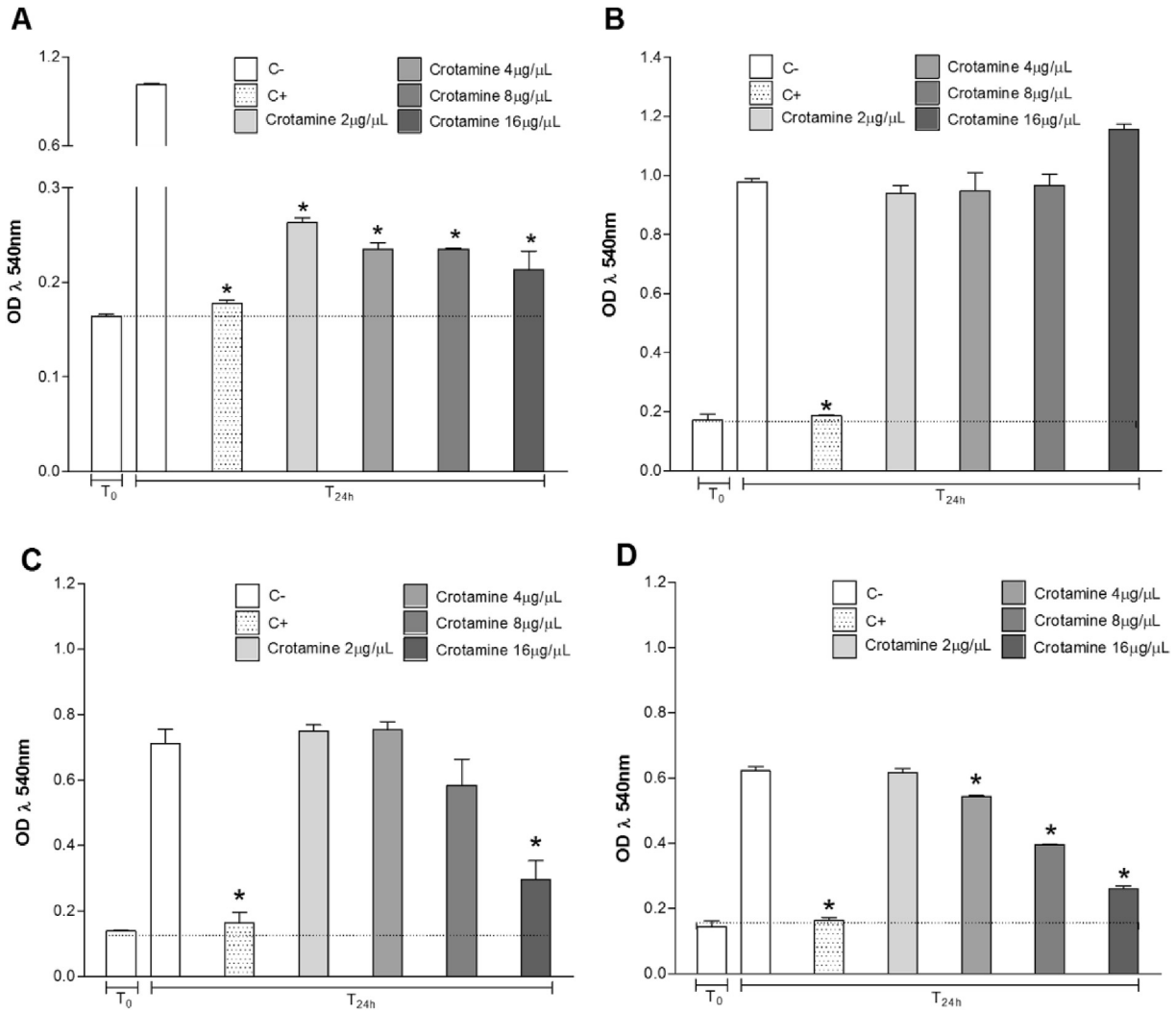

Fig. 3. Antibacterial activity of crotamine. Microdilution assay with different concentrations of the peptide (2.0–16 μg/μL) incubated for 24 h at 37 °C with 10^6 CFU/mL of (A) *E. coli*, (B) *P. aeruginosa*, (C) *Staphylococcus aureus*; (D) oxacillin-resistant *Staphylococcus aureus* - ORSA. Positive control: 30 μg/μL levofloxacin; negative control: bacterial only. Values are reported as mean ± SD (Student's t-test, *$p < 0.05$ relative to the positive control, $n = 3$).

more chromatographic steps including classical molecular exclusion associated with ion exchange (Hampe et al., 1990; Mancin et al., 1998; Boni-Mitake et al., 2001). Here, the bioaffinity-based method was selective, reproducible, and fast.

The identity of crotamine was confirmed by mass spectrometric analysis. Three internal peptide fragments (ICIPPSSDFGK; ICIPPSSDFGKMDCR and EKICIPPSSDFGK) were generated by mass fingerprinting. We identified that the first residue of the mature form of the protein was Ile. Databank analyses show that crotamine has proline and leucine/isoleucine amino acids highly conserved in the peptide. The identity of crotamine was experimentally confirmed, showing a 98% similarity. The analysis by MALDI-TOF/TOF spectrometry showed a molecular mass of 4881.449 Da, which agrees with the theoretical mass of the peptide (4885.6 Da) (Peigneur et al., 2012). Previous studies report that geographical factors, the age of the snake and intra-species genetic variations may alter the chemical composition of the venom as well as the expression of crotamine, characterizing crotamine-positive and crotamine-negative snake species (Teno et al., 1990; Francischetti et al., 2000; Saravia et al., 2002; Nicastro et al., 2003; Rádis-Baptista et al., 2003; Rádis-Bastista et al., 2004; Fadel et al., 2005). Rádis-Baptista et al. (1999) also report the presence of crotamine isoforms, revealing a single base change of the amino acid leucine replaced by isoleucine at position 19. Moreover, Toyama et al. (2000) showed by high performance liquid chromatography the existence of crotamine isoforms with very close molecular weights of 4882.03 Da and 4882.16 Da.

Currently, there is a great interest in the research and discovery of new antimicrobial drugs due to the high incidence of infectious diseases and the existence of microorganisms multiresistant to the conventional antibiotics (Roca et al., 2015). Among the highest priority microorganisms for public health are *Acinetobacter*, *Pseudomonas*, *Klebsiella*, *E. coli*, *Serratia* and *Proteus*, and other species that have acquired resistance such as *Staphylococcus aureus*, *Helicobacter pylori*, *Salmonella* spp, *Neisseria gonorrhoeae*, *Shigella* spp., and *Streptococcus pneumoniae* (WHO, 2017). Several studies have shown the antimicrobial action of crotamine on different microorganisms, including bacteria, yeasts and parasites. In these studies, the cytotoxic profile of the molecule was shown to be variable, with MICs ranging from 1 to 100 μg/mL, depending on the microorganism (Passero et al., 2007; El-Chamy-Maluf et al., 2016).

In the present study, different concentrations of crotamine (2–64 μg/μL) were used to determine and confirm the MICs on different strains of clinical interest, such as *Escherichia coli* (ATCC

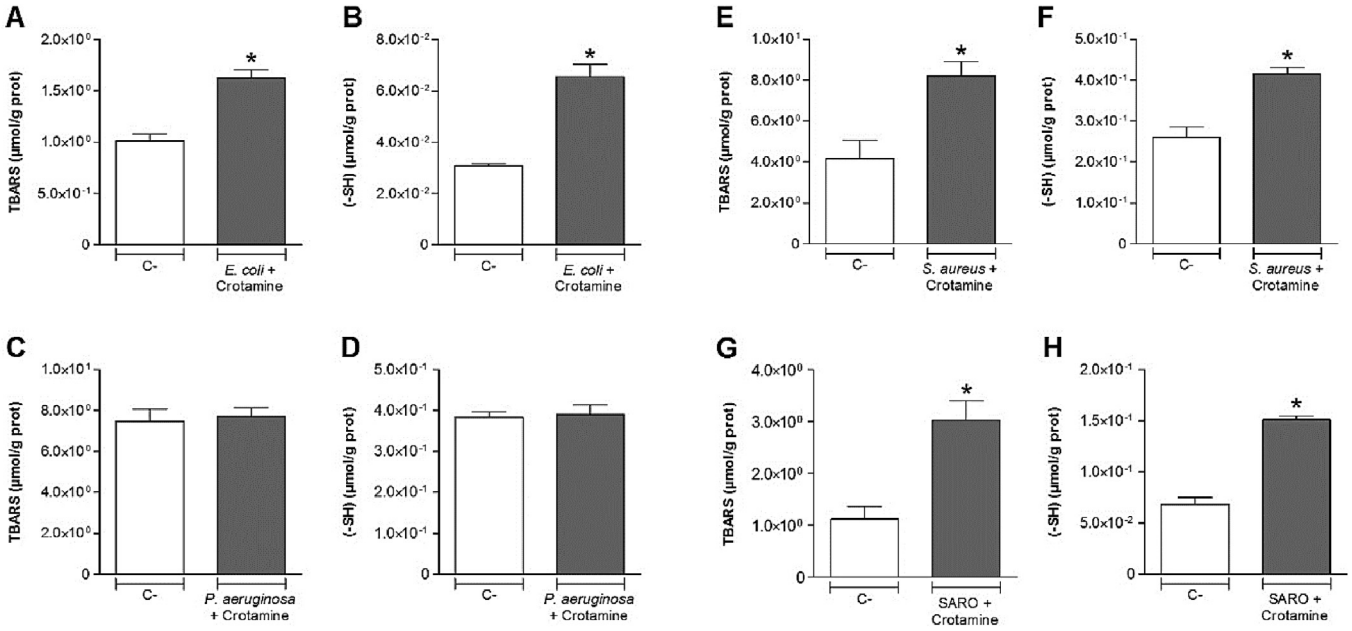

Fig. 4. Oxidative stress induced by crotamine. Determination of thiobarbituric acid reactive species levels (TBARS) and sulfhydryl groups (-SH) in bacterial homogenates (10^6 CFU/mL) after incubation with crotamine (16 µg/µL) for 24 h at 37 °C. (A, B) *E. coli*; (C, D) *P. aeruginosa;* (E, F) *Staphylococcus aureus*; (G, H) methicillin-resistant *Staphylococcus aureus* - MRSA. Negative control: bacteria only. Values are reported as mean ± SD (Student's t-test, *p < 0.05 compared to the positive control, n = 3).

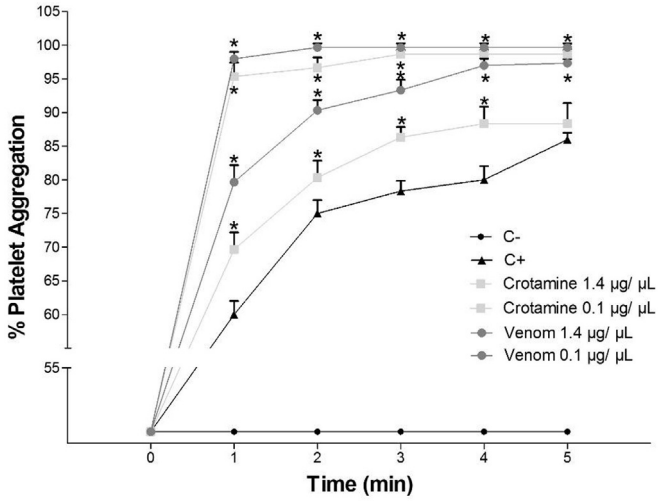

Fig. 5. Platelet aggregation induced by crotamine. Human platelet aggregation was induced by crotamine from the venom of *Crotalus durissus terrificus*. Different concentrations of the purified peptide and crude venom (0.1 and 1.4 µg/µL) were immediately added to PRP and the reaction was monitored in Chrono-Log Model 700 Whole Blood/Optical Lumi-Aggregometer ($\lambda_{935-955nm}$, 5 min, 37 °C). Reading was transcribed by Aggro/Link software 8. Data represent the average of three experiments ± SD. Results are reported as percentage of aggregation. C+: positive control (ADP 1 nM/µL); C-: negative control (PRP 5×10^5 platelets/mL) (Student *t*-test, *$p < 0.05$ compared to the positive control, $n = 3$).

25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591). A heterogeneous response pattern was verified according to the sensitivity profile for each microorganism.

The cytotoxic potential of crotamine was better evidenced on *E. coli* compared to the positive control (microorganisms alone), and growth inhibition occurred at the lowest concentration (2.0 µg/µL). However, studies performed by Oguiura et al. (2011) demonstrated that *S. aureus* species was more resistant to crotamine at up to 0.2 µg/µL concentrations than the *E. coli* species (0.025–0.1 µg/µL). Yamane et al. (2013) also found high antimicrobial activity of crotamine against *E. coli* and *S. aureus* with MICs of 0.05–0.2 µg/µL. In contrast, we found that *E. coli*, *S. aureus* and MRSA presented higher sensitivity using concentrations of 2, 4 and 8 µg/µL, respectively. However, *P. aeruginosa* was not sensitive to the peptide, even with the highest concentration.

To better understand the bactericidal activity of crotamine, our study assessed the oxidative stress caused by crotamine as a possible mechanism of action against the evaluated species. The minimum inhibitory concentration of the peptide (MIC = 16 µg/µL) produced an increase in TBARS, which suggests oxidative events on membrane lipids. Accordingly, the levels of sulfhydryl groups (-SH), resulting from an attempt to combat oxidative attack were also elevated. These results imply that crotamine induces changes in the redox state, with the generation of toxic products and byproducts that may affect membrane fluidity as well as the formation of adducts between proteins and DNA, affecting cell metabolism and growth (Hanne et al., 2000; Medeiros, 2009). In contrast, the oxidative events resulting from crotamine treatment evidenced in *E. coli*, *S. aureus* and ORSA were not observed in *P. aeruginosa*, which presented low levels of TBARS and sulfhydryl groups. These results suggest that *P. aeruginosa* was not sensitive to crotamine and thus did not experience enough oxidative damage to interfere with its metabolism. Recent studies have shown that the OhrA-peroxidase gene and its OhrR negative regulator are the main genes responsible for detection and detoxification of organic hydroperoxides (Lesniak et al., 2003; Atichartpongkul et al., 2010). In addition, *P. aeruginosa* has been shown to have multiple mechanisms of resistance to antibacterial drugs, many of which are related to target site modifications, overexpression of efflux pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM), and enzymatic hydrolysis by β-lactamases and metallo-β-lactamases. Moreover, drug resistance of *P. aeruginosa* is related with the low expression, if any, of porins such as OprC, OprD, OprE and OprF, and the expression of blocking porins, such as OprH (Young et al., 1992;

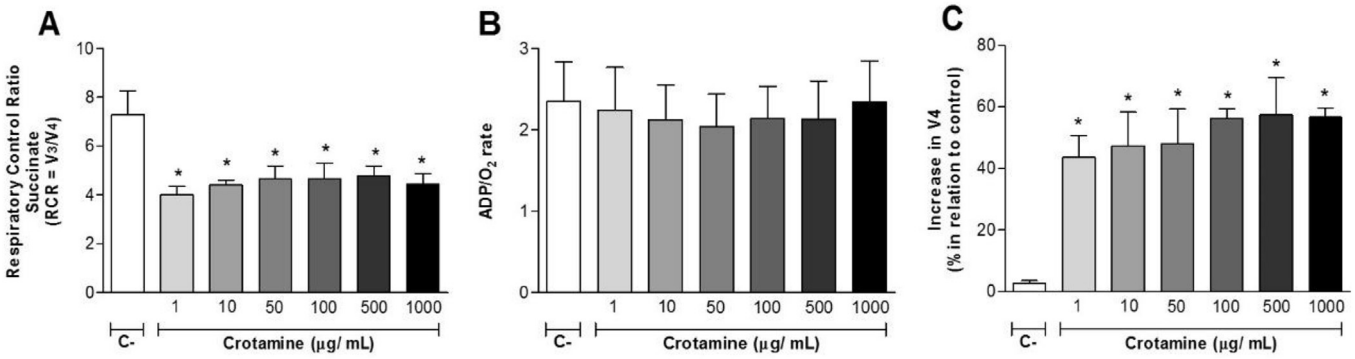

Fig. 6. Effects of crotamine (1–1000 µg/mL) on mitochondrial bioenergetics. (A) Respiratory control ratio (RCR) in liver mitochondria calculated as RCR = (V_3/V_4). (B) ADP consumption by oxygen molecule (ADP/O). (C) Increased rate of oxygen consumption in state IV (V_4). Respiratory parameters in mitochondria isolated from rat liver (1.0 mg protein/mL) evaluated using the oxygraph (Hansatech, Norfolk, England) as described in materials and methods. Data represent the mean ± SEM of five determinations with different mitochondrial preparations ($n = 5$). *Statistically significant results ($p < 0.05$) compared with the control (absence of crotamine).

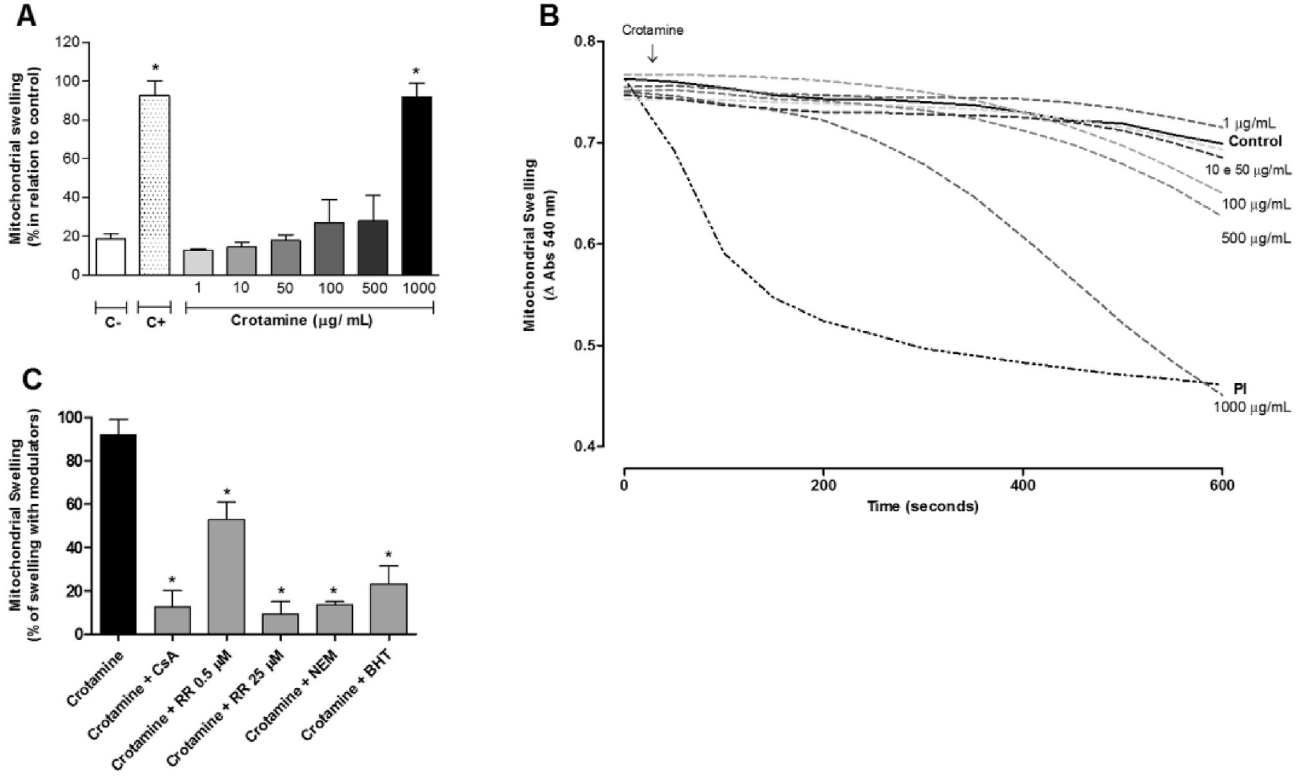

Fig. 7. Effects of crotamine (1–1000 µg/mL) on mitochondrial swelling. (A) Mitochondrial swelling monitored with the change of absorbance at 540 nm (ΔAbs_{540nm}) of the mitochondrial suspension over 10 min. C-: negative control (without crotamine), C+: positive control (1 mM inorganic phosphate). (B) The traces represent direct recordings of experiments performed for each group. Control: without crotamine; PI: 1 mM inorganic phosphate (positive control). (C) Mitochondrial swelling monitored with the presence of the modulators 1 µM Cyclosporin A (CsA), 0.5 and 25 µM ruthenium red (RR), 25 µM N-ethylmaleimide (NEM) and 25 µM butylated hydroxytoluene on the swelling induced by 1000 µg/mL crotamine. Data represent the mean ± SEM of three determinations with different mitochondrial preparations ($n = 3$). *Statistically significant results ($p < 0.05$) compared with the control.

Khan et al., 1995; Livermore, 2002; Strateva and Yordanov, 2009).

Based on different *in vitro* assays and studies on the structure-function of crotamine suggest that its bactericidal mechanism occur due to its amphipathic structure (Oguiura et al., 2011; Yamane et al., 2013; Costa et al., 2014). Crotamine presents a high positive net charge on the surface, which facilitates its binding to negatively charged surfaces driven by electrostatic interaction, as observed for other antimicrobial cationic peptides. Another hypothesis is that crotamine may induce the formation of gaps, through which ions and/or other molecules can diffuse on the outer bacterial, leading to consequent cell death (Coronado et al., 2013; Costa et al., 2014). This mechanism was observed in the studies by Oguiura et al. (2011), in which crotamine was effective against different strains of *Escherichia coli* (*E. coli* ATTC 25922; *E. coli* O157: H7; *E. coli* ML-35p), with lysis evidenced by the release of cell contents. The interaction of crotamine with cell membranes was also verified with *Candida* spp., which presented ultra-membrane alterations accompanied by loss of cellular integrity, assessed by electron microscopy (Yamane et al., 2013).

Studies using previously isolated fractions of the venom have

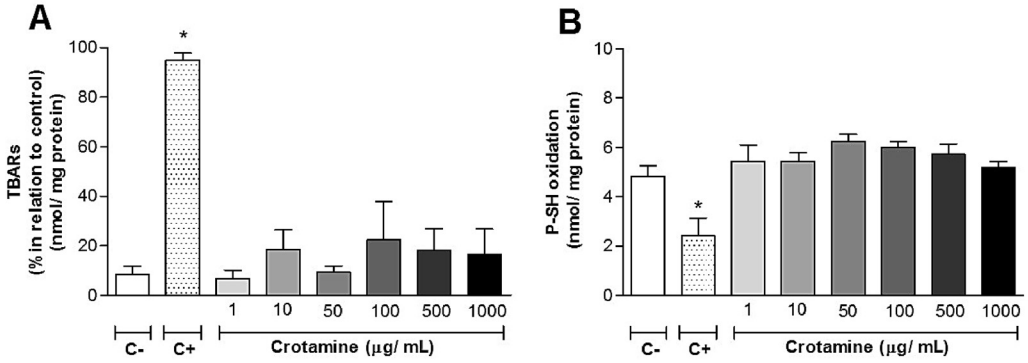

Fig. 8. Effects of crotamine (1–1000 µg/mL) in mitochondrial redox state. (A) Formation of thiobarbituric acid reactive species (TBARS) after 30 min of incubation. C-: control (without crotamine), C+: positive control (3% hydrogen peroxide – H_2O_2). (B) Evaluation of the oxidation of sulfhydryl proteins (P-SH) in the membrane using DTNB and measured at λ_{412nm}. C-: control (without crotamine), C+: positive control (3% H_2O_2). Data represent the mean ± SEM of three determinations with different mitochondrial preparations ($n = 3$). *Statistically significant results ($p < 0.05$) compared with the control.

shown a marked pro-coagulant activity for crotoxin (Fonseca et al., 2006) and gyroxine (a “thrombin-like" enzyme) (Magalhães et al., 2007; Yonamine et al., 2009), in addition to selective activation and inactivation of coagulation factors involved in platelet aggregation, coagulation and fibrinolysis (Serrano and Maroun, 2005). On the other hand, there are no reports on the effect of crotamine on platelet aggregation, as well as on mechanisms involved in this process. In our study, the effect of two different concentrations of crotamine on platelet-rich plasma was analyzed. Interestingly, platelet aggregation was induced by approximately 90% with the lowest concentration of the peptide (0.1 µg/µL) and by 99% with highest concentration (1.4 µg/µL) when compared to the ADP-control (85% aggregation). It is suggested, therefore, that crotamine acts on different platelet targets, including glycoprotein receptors, called integrins (Rivera et al., 2009; Cupit et al., 2004). These data are of great relevance in possible future applications of this molecule in the study of platelet receptors and dysfunctions.

In the literature, crotamine is described as having high tropism for kidneys, muscles and liver (Boni-Mitake et al., 2006). According to Jaeschke et al. (2002), the liver is an important target for the harmful effects of chemical substances in general, mainly due to its function in metabolism. Here, we show for the first time the interaction of crotamine on hepatic mitochondria. The tests were performed with the objective to elucidate the mechanisms of action of crotamine and its interactions with biological systems. So far, many toxins have been studied for their effects in this organelle; for example, the uncoupling of oxidative phosphorylation by juliprosopine on rat brain mitochondria (Maioli et al., 2012), the oxygen consumption and electrical membrane potential by veratrine and veratridine in isolated rat skeletal muscle and liver mitochondria (Freitas et al., 2006) and cyclosporine A-insensitive mitochondrial permeability transition induced by dehydromonocrotaline (dos Santos et al., 2009).

Mitochondrial dysfunction is a relevant mechanism for mammals, particularly in the liver, since this organelle has a central and decisive function in cellular homeostasis (Amacher, 2005). To evaluate the potential involvement of mitochondria in the interaction of crotamine with biological systems, we addressed its effects primarily in oxidative phosphorylation. Thus, we may say that the mitochondria, particularly the mitochondrial respiratory chain, are a target of crotamine, as we observed a decrease in oxygen consumption and parallel increase in the state IV rate, which indicates that the peptide has the capacity to act as an uncoupling agent. Some compounds, classified as uncouplers, increase the conductance of the protons by the internal mitochondrial membrane (Wallace and Starkov, 2000), which can trigger the mitochondrial swelling process. This phenomenon, known as mitochondrial swelling, comprises a series of steps that can induce the mitochondrial membrane permeability transition (MPT), defined as a sudden increase in the permeability of the internal mitochondrial membrane to solutes with molecular masses of up to 1500 kDa, due to the opening of a high-conductance cyclosporine-A and Ca^{+2}-dependent channels (Rasola, Bernardi, 2007a; b; Tsujimoto and Shimizu, 2007).

The interference caused by crotamine in mitochondria bioenergetic process might be responsible for the changes in mitochondrial permeability. It is also possible that a mechanisms of action of crotamine is the opening of the mitochondrial permeability pore, since the observed swelling was inhibited significantly by cyclosporine, a classic inhibitor of MPT (Broekemeier et al., 1989). In line with these findings, the observed swelling is also calcium-dependent, since it was sensitive to ruthenium red, which gives us an indication that the Ca^{+2} ion is also an important agent of MPT induced by this compound. It was also observed that NEM and BHT partially reduce mitochondrial swelling. However, no change was detected in the mitochondrial redox state. This suggests that the compound did not cause mitochondrial oxidative stress, but it is possible that crotamine is able to interact with the SH proteins of mitochondrial membranes, since stabilizing them with NEM prevented swelling. In a study using melanoma cell lines (B16F10) Nascimento et al. (2012) showed that the cytotoxic effect of crotamine involves rapid intracellular calcium release and loss of mitochondrial membrane potential and, consequently induction of apoptosis via caspases activation. Valente et al. (1998) studied the effect of three purified phospholipase A_2 isoforms isolated from *C. d. terrificus* on mitochondria and showed that PLA_2 isoforms cause a dose-dependent swelling and O_2 consumption through an enzymatic mechanism.

Mitochondria performance depended on a proton electrochemical gradient generated by respiration and maintained by the impermeability of the inner mitochondrial membrane to protons (Kehrer and Lund, 1994; Pessayre et al., 1999). It has been well documented that the decrease of ATP levels is one of the consequences of mitochondrial damage and a critical event that leads to cell death by necrosis or apoptosis (Nicotera et al., 1998; Wallace and Starkov, 2000; Szewczyk and Wojtczak, 2002). Consistent with the possibility of cell death by the effects observed in mitochondria after exposure to crotamine, it is noteworthy that in the inner mitochondrial membrane have the presence of Kv1.3, a shaker potassium channel family of the, functionally active in the

mitochondrial inner membrane - mitoKv1.3 (Szabò et al., 2008), and such as previously described this channel is target of crotamine. Therefore, the interaction of crotamine with the mitochondria can become an important ally when cell death is desired.

5. Conclusions

In conclusion, native crotamine from *Crotalus durissus terrificus* was purified with homogeneity. The purified peptide showed remarkable pro-aggregating activity on platelets. Interestingly, the molecule effect on bacteria seems to be through oxidative stress. Mechanistically, the mitochondrial respiratory chain is a target of crotamine, which indicates that the peptide has the capacity to act as an uncoupling agent in association with the opening of the mitochondrial membrane permeability pore. Thus, the activities found in this fraction of the venom can contribute to the understanding of the structure-activity relationship of these biomolecules, as well as the physiological and molecular mechanisms in different biological models. Thus this study contributes to the future use of crotamine in the development of biotechnology products with clinical application.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Ethical statement

The experiments followed the methodology recommended by the international ethical standards of the scientific committee of our university (process nº 833/2015 and 014/2017).

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.toxicon.2018.03.007.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2018.03.007.

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