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Research paper

# Unraveling the antifungal activity of a South American rattlesnake toxin crotamine

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ABSTRACT

Crotamine is a highly basic peptide from the venom of *Crotalus durissus terrificus* rattlesnake. Its common gene ancestry and structural similarity with the β-defensins, mainly due to an identical disulfide bond pattern, stimulated us to assess the antimicrobial properties of native, recombinant, and chemically synthesized crotamine. Antimicrobial activities against standard strains and clinical isolates were analyzed by the colorimetric microdilution method showing a weak antibacterial activity against both Gram-positive and Gram-negative bacteria [MIC (Minimum Inhibitory Concentration) of 50–>200 μg/mL], with the exception of *Micrococcus luteus* [MIC ranging from 1 to 2 μg/mL]. No detectable activity was observed for the filamentous fungus *Aspergillus fumigatus* and *Trichophyton rubrum* at concentrations up to 125 μg/mL. However, a pronounced antifungal activity against *Candida* spp., *Trichosporon* spp., and *Cryptococcus neoformans* [12.5–50.0 μg/mL] was observed. Chemically produced synthetic crotamine in general displayed MIC values similar to those observed for native crotamine, whereas recombinant crotamine was overridingly more potent in most assays. On the other hand, derived short linear peptides were not very effective apart from a few exceptions. Pronounced ultrastructure alteration in *Candida albicans* elicited by crotamine was observed by electron microscopy analyses. The peculiar specificity for highly proliferating cells was confirmed here showing potential low cytotoxic effect of crotamine against nontumoral mammal cell lines (HEK293, PC12, and primary culture astrocyte cells) compared to tumoral B16F10 cells, and no hemolytic activity was observed. Taken together these results suggest that, at low concentration, crotamine is a potentially valuable anti-yeast or candicidal agent, with low harmful effects on normal mammal cells, justifying further studies on its mechanisms of action aiming medical and industrial applications.



*Abbreviations:* MIC, minimal inhibitory concentration; AMP, antimicrobial peptide; CPP, cell penetrating peptide; ATCC, American Type Culture Collection; IOC, Oswaldo Cruz Institute Collection; CD, circular dichroism; CC50, 50% cytotoxic concentration; TEM, transmission electron microscopy.

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## 1. Introduction

All animals, from arthropods to humans, express peptides able to kill microbial cells. Antimicrobial peptides (AMPs) are essential components of innate immunity for essentially all organisms, including invertebrates, vertebrate, and plants, acting as the first line of defense against invading microbes by disrupting cytoplasmic membrane functions [1]. AMPs are potential candidates for a new antibiotic generation against multiresistant bacterial strains [2,3].

Interestingly AMPs and cell penetrating peptides (CPPs) are similar in several aspects: they share physical and chemical characteristics (short length, cationic, amphipathic, and structured arranged peptides) and, consequently, the fundamental property of strong interaction with lipid membranes. However, AMPs predominantly translocate into the cell by transient pore formation in the membrane, while entry of CPPs has been shown to include both endocytic and non-endocytic routes [4–6], sometimes involving more than one single mode [7,8]. Whatever the pathway CPPs use to translocate membrane (trafficking) systems, they can serve to deliver molecules into cells and their internal compartments, including endosomes, nuclei, and mitochondria [9–11].

Crotamine is the major toxic component toward rodents found in the venom of the South American rattlesnake *Crotalus durissus terrificus*. It was initially described as being responsible for the hind limb paralysis observed in mice after venom injection [12]. The primary structure of crotamine is very similar to those of other small, non-enzymatic, myonecrotic toxins identified in different rattlesnake venoms [13,14]. The 3D structure of crotamine in solution, determined by proton NMR spectroscopy, showed that both its cysteine-pairing pattern and global fold are similar to those of the human β-defensins AMPs, regardless of their low primary structure identity [15,16]. Our group was the first to demonstrate that crotamine is also a CPP with a high specificity for actively proliferating cells [17,18]. We demonstrated that internalization of this polycationic peptide involves endocytosis and is dependent on cell membrane heparan sulfate proteoglycans [19]. Subsequently, we studied the cytotoxic effect of this toxin on highly proliferative cells and suggested the lysosomes as the primary target for crotamine toxic activity at the cellular level [20]. This led us to study and propose the potential application of native crotamine as a theranostic agent [18,21].

Crotamine is a highly basic and amphipathic toxin, particularly rich in Lys, whose 42-residues long polypeptide [YKQCH KKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG] folds into a compact structure containing an antiparallel β-sheet and an α-helix stabilized by three disulfide bridges [15,16]. This type of fold and amphipathic structure is also found in the aforementioned β-defensins [1,22], which compose the major family of AMPs [23]. Defensins possess direct antimicrobial activities against a broad range of Gram-negative and Gram-positive bacteria [15,23,24], and the interaction with glycosaminoglycans was also recently demonstrated for human β-defensin 2 (HBD2) [25]. Moreover, it has been suggested that crotamine and β-defensin share a common ancestry [16,26,27], and a potential antimicrobial activity was recently attributed to crotamine [28,29].

In the present work we aimed to characterize the antimicrobial activity of crotamine against Gram-negative and Gram-positive bacteria, and several fungi species, including clinical resistant strains. For comparison, recombinant and chemically synthesized forms of crotamine were included in these studies. In addition, denatured full-length crotamine and two synthetic linear crotamine fragments, comprising half of the positively charged amino acid residues in each, were also evaluated here. The results indicate that crotamine has strong potential as an anti-yeast or candicidal agent, with no hemolytic activity and low harmful effects on normal mammal cells. The effective antifungal activity against a number of clinical yeast strains point out the potential of crotamine as a structural model compound for the development of a new generation of antimicrobial drugs against clinical strains, potentially able to overcome the microbial resistance challenge.

## 2. Materials and methods

### 2.1. Materials

*C.d. terrificus* venom was extracted from snakes maintained at the Faculdade de Medicina de Ribeirão Preto (FMRP) serpentarium, São Paulo University, and dried under vacuum. All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). Gomesin was the same as previously described [30]. The cDNA encoding the full-length crotamine [GenBank Acc. No. AF044674] subcloned in frame into the expression plasmid vector pQE-1 (Qiagen, Hilden, Germany), named 127pQE, was kindly provided by Dr. Gandhi Rádis-Baptista (Instituto de Ciências do Mar, Universidade Federal do Ceará, Fortaleza, Brazil).

### 2.2. Preparation and biochemical characterization of the native, synthetic, and recombinant crotamine

#### 2.2.1. Purification of native crotamine from snake venom

Six hundred milligrams of crude venom were dissolved in 5 mL of 0.25 M ammonium formate buffer pH 3.5, and the bulk of crotoxin, the major venom component, was eliminated by slow speed centrifugation as a heavy precipitate that formed upon slow addition of 20 mL of cold water to the solution. Tris-base [1 M] was then added dropwise to the supernatant to raise the pH to 8.8 and the solution was applied to a CM-Sepharose FF (1.5 × 4.5 cm; former Amersham-Pharmacia, GE Healthcare, Buckinghamshire, UK) column, equilibrated with 0.04 M Tris–HC1 buffer pH 8.8, containing 0.064 M NaCl. After washing the column with 100 mL of equilibrating solution, crotamine was recovered as a narrow protein peak by raising the NaCl concentration of the diluting solution to 0.64 M. The material was thoroughly dialyzed against water (benzoylated membrane, cut off MW = 3000) and was lyophilized. Amino acid analysis after acid hydrolysis of a sample (4 N $MeSO_3H$ + 0.1% tryptamine; 24 h at 115 °C) indicated a yield of 72 mg (14.7 μmol) of crotamine and trace amounts of Thr, Ala and Val (purity > 98%).

#### 2.2.2. Expression of recombinant crotamine in bacteria

The expression and purification of the recombinant protein were performed essentially as previously described [31], except for the use of Ni–NTA agarose (Qiagen Inc., Valencia, CA) instead of glutathione-sepharose beads for crotamine. Briefly, bacteria transformed with 127pQE were grown under antibiotic selection to an absorbance reading of about 0.6 at 600 nm, when the expression of recombinant crotamine, carrying a 6xHis-tag at its C-terminus, was induced by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by incubation for 3 h at 30 °C. The bacteria were then collected by centrifugation, re-suspended in 20 mM Tris–HCl pH 8.5, 100 mM KCl, 20 mM imidazole, 10% (v/v) glycerol buffer, and then lysed by two cycles of freezing and thawing, followed by sonication. After removal of bacterial debris, the supernatant was purified by affinity chromatography using Ni–NTA magnetic agarose beads, which had been exhaustively washed with 20 mM Tris–HCl pH 8.5, 1 M KCl, 10% (v/v) glycerol buffer, in order to remove undesired bacterial proteins. The recombinant crotamine was eluted using 20 mM Tris–HCl pH 8.5, 100 mM KCl, and 100 mM

imidazole buffer. Then, the imidazole was removed after intensive washing using the centricon YM-3 filter system (Millipore Corp., Billerica, MA, USA), and aliquots of the sample were then analyzed by SDS/PAGE after quantification by Bradford.

*2.2.3. Crotamine synthesis*

Solid phase chemical synthesis of full-length crotamine [YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG] was performed on Boc-Gly-$OCH_2$-PAM resin, using an in-house chemistry tailored from the *N*,*N*-diisopropylethylamine *in situ* neutralization/2-(1*H*-benzotriazolyl)-1,1,3,3-tetramethyl uroniumhexafluorophosphate activation protocol originally developed by Schroeder et al. [32]. Specifically, Boc-amino acids (2.2 mmol) were activated in dimethylformamide (DMF) for 3 min by 2.0 mmol of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in the presence of 20% *N*,*N*-diisopropylethylamine (DIEA) (v/v), and coupled in stepwise fashion for 10 min on 0.25 mmol of appropriate PAM resin on an ABI 433A peptide synthesizer. Tri-fluoroacetic acid (TFA) (100%) was used for removal of N-Boc groups; DMF and dichloromethane (DCM) were used for batch washes throughout the entire synthesis. After chain assembly, the peptides were cleaved by anhydrous hydrogen fluoride in the presence of 5% *p*-cresol at 0 °C for 1 h followed by precipitation with cold ether. All crude peptides were purified to homogeneity by preparative C18 reversed phase high performance liquid chromatography (HPLC) on a Waters Delta Prep 600 system, and the molecular masses were verified by a Micromass ZQ-4000 single quadrupole electrospray ionization-mass spectrometer (ESI-MS). Productive oxidative folding of synthetic crotamine was achieved under thiol-disulfide exchange conditions as previously described [33,34]. Reduced crotamine was first dissolved at 2 mg/mL in pure water containing 12 mM reduced and 1.2 mM oxidized glutathione, followed by 4-fold dilution with 0.5 M sodium bicarbonate ($NaHCO_3$). After overnight stirring at room temperature, the material was purified to homogeneity by HPLC using a linear gradient of 10–30% acetonitrile (AcN) containing 0.1% TFA. The material eluted at the same percent AcN as venom-derived native crotamine. Pure fractions were verified by ESI-MS, and the 6-unit mass reduction was indicative of the formation of three disulfide bridges.

*2.2.4. Circular dichroism analysis*

Circular dichroism (CD) experiments were carried out using a Jasco 810 spectropolarimeter (JASCO International Co. Ltd., Tokyo, Japan), coupled to a Peltier Jasco PFD-425S system for temperature control. Far UV-CD spectra were collected from 190 to 260 nm and averaged over 4–8 scans, with a 1 mm path length quartz cell. A 0.5 nm step resolution, 50 nm/min speed, 8 s response time and 1 nm bandwidth were used. The experiments were performed, at 37 °C, with 20 μM crotamine in 10 mM Tris–HCl pH 7.4. All data were obtained using three different solutions of the proteins.

*2.2.5. Synthesis and purification of crotamine fragments*

Peptides were synthesized essentially as previously described by Picolo and co-workers [35]. Briefly, the syntheses of P1 ($Crot_{2-18}$ [KQSHKKGGHSFPKEKIS]) and P2 ($Crot_{27-39}$ [KMDSRWRWKSSKK]) were carried on an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan) by a stepwise solid-phase method using *N*-9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Novabiochem-EMD Chemicals Inc., San Diego, CA, USA). All the resins and Fmoc-L-amino acids were purchased from Nova Biochem (UK). Cleavage of the peptide from the resin was achieved by treatment with a mixture of TFA/1,2-ethanedithiol/ethyl methyl sulfide for 2 h at room temperature. After removal of the resin by filtration and washing twice with TFA, the crude synthetic peptide was purified by preparative reversed-phase HPLC (Shimadzu Corp.) on a YMC-Pack ODS column (20 mm × 150 mm) (YMC Co. Ltd., Shimogyo-ku, Kyoto, Japan), using a linear gradient from 3 to 20% AcN in 0.1% TFA, at a flow rate of 7 mL/min. Both, the homogeneity and the sequence of each synthetic peptide were confirmed by analytical HPLC and MALDI-TOF mass spectrometry (Amersham Biosciences, Uppsala, Sweden). The amino acid sequence of the peptides was analyzed by a gas-phase sequencer PPSQ-10 (Shimadzu Corp.) based on automated Edman degradation. All mass spectra were acquired on a Voyager Elite MALDI-TOF MS spectrometer (Applied Biosystems, Framingham, MA, USA), equipped with a delayed extraction system and 337 nm pulsed nitrogen laser. The accelerating voltage was 20 kV. Argon gas was used as the collision gas for the CID/PSD experiment. A matrix, α-cyano-4-hydroxycinnamic acid was prepared at a concentration of 10 mg/mL in 1:1 AcN/0.1%TFA.

*2.2.6. Denaturation of native crotamine*

Aiming to test the impact of the three dimensional structure on the functional properties of purified crotamine, the disulfide bonds were reduced by incubation either at 100 °C with 5 mM dithiothreitol or 10 mM β-mercaptoethanol for 10 min, or by autoclaving 1 mg/mL crotamine solution in pure water at 100 °C for 20 min [29]. Then CD experiments were then carried out, as described above, to confirm the denaturation of crotamine, after these protein-denaturing treatments and before carrying out the functional assays.

*2.3. Antibacterial and antifungal susceptibility tests*

*2.3.1. Microorganisms*

The microorganisms used in this study are from the American Type Culture Collection (ATCC), clinical isolate strains from the Microbiology Department of São Paulo Federal University (São Paulo, SP, Brazil) and from Adolfo Lutz Institute (São Paulo, SP, Brazil). Clinical strains deposited at Oswaldo Cruz Institute Collection (IOC) were coded by IOC numbers, as indicated for each strain. The microorganisms used in this study are the following: *Escherichia coli* [ATCC 25922], *Staphylococcus aureus* [ATCC 29213], *Pseudomonas aeruginosa* [ATCC 29213], *Cryptococcus neoformans* [ATCC 90112], *Candida albicans* [ATCC 36802/IOC 3704], *C. albicans* IOC 4558, *Candida krusei* [IOC 4559], *Candida glabrata* [IOC 4565], *Candida parapsilosis* [IOC 4564], *Candida tropicalis* [IOC 4560], *Candida guilliermondii* [IOC 4557], *C. neoformans* [IOC 4528], *Aspergillus fumigatus* [IOC 4526], *Trichophyton rubrum* [IOC 4527], and *Trichosporon* spp. [IOC 4569]. Other microorganisms also used here are: *C. neoformans* [IOC 4528] (clinical strain), *E. coli* [SBS363], and *Micrococcus luteus* (A270) from Pasteur Institute Collection (Paris, France); *C. albicans* (MDM8) from the Microbiology Department Collection, Biomedical Sciences Institute of São Paulo University (São Paulo, SP, Brazil), and *C. tropicalis* (Squibb 1600, L. 03/97) from Adolfo Lutz Institute Culture Collection (São Paulo, SP, Brazil).

The isolates were identified by standard methods [36] and were preserved at −80 °C. Prior to testing, each isolate was subcultured at least twice on Sabouraud dextrose agar (Himedia, Biosystems Com. Imp. Exp. Produtos Lab. Ltda, Curitiba, PR, Brazil) to ensure optimal growth characteristics.

*2.3.2. Minimal inhibitory concentration (MIC)*

The antibacterial and antifungal activities were monitored by a microbial growth inhibition assay in liquid medium as described earlier [37,38]. Briefly, 10 μL of the peptide solution were added to 90 μL of a suspension of a mid-logarithmic phase culture of bacteria at a starting $OD_{595\ nm} = 0.001$ in poor broth nutrient medium (PB: 1.0 g peptone in 100 mL of water containing 86 mM NaCl at pH 7.4; 217 mOsM). In the case of the antifungal activity the medium used

was the poor dextrose broth (1/2 PDB: 1.2 g potato dextrose in 100 mL of $H_2O$ at pH 5.0; 79 mOsM). Microbial growth was measured by monitoring the OD increase at 595 nm. The minimal inhibitory concentration (MIC) is the lowest concentration that caused 100% growth inhibition determined by optical density measurements in a microplate reader (VICTOR Multilabel Plate Reader, Perkin–Elmer), following incubation at 30 °C for 18 h in an orbital shaker for microplate (Titramax 100, Heidolph Instruments). The MIC was recorded as the *a–b* μM range, where "*a*" corresponds to the highest concentration in which the bacterial growth was observed and "*b*" corresponds to the lowest concentration of the peptide that caused 100% of inhibition microbial growth. The natural peptide gomesin is a well-studied antimicrobial peptide (AMP) from hemocytes of the spider *Acanthoscurria gomesiana* with antimicrobial activity against both Gram-positive and Gram-negative bacteria, and for this reason gomesin was used here as a positive control.

#### 2.3.3. Fungicidal assay

Fungicidal activity assays for crotamine were conducted in triplicate, using the following clinical yeast isolates: *C. albicans* [IOC 4558], *C. krusei* [IOC 4559], *C. glabrata* [IOC 4565], *C. parapsilosis* [IOC 4564], *C. tropicalis* [IOC 4560], *C. guilliermondii* [IOC 4557], and *Trichosporon* spp. [IOC 4569]. The clinical isolates (final concentration of $10^4$ cells/mL) were suspended in a growth medium containing 1.2% potato dextrose (w/v) (Difco; "half strength"). Eighty μL aliquots of this suspension were dispensed into wells of a microplate containing 20 μL of water or crotamine [4× MIC], and were incubated for 24 h at 30 °C. The same culture without crotamine was the control. After incubation, 20 μL of crotamine-treated and control (water) culture were removed and sown in Petri dishes containing nutrient agar [Luria-nutrient medium Bertani: 1% Bacto-Tryptone, 0.5% yeast extract, 1% NaCl, and 1.5% (w/v) agar]. The kinetics of killing assay induced by crotamine was monitored. This procedure, slightly modified of Klepser et al. [39], was performed only against the clinical isolate *C. albicans* [IOC 4558], which we believe to potentially reflect the expected effects on nosocomial strains. After different incubation times (10 min, 1, 2, 4, 8, 12, and 24 h), 20 μL aliquots samples were collected and sown in Petri dishes with Sabouraud dextrose agar. The number of Colony Forming Units (CFU), which represents the viable colony counts, was determined the following morning, to ensure optimal growth of all viable entities. Results were presented as the mean ± SD of triplicates.

### 2.4. Ultrastructural analysis of *C. albicans* cells treated with crotamine

#### 2.4.1. Transmission electron microscopy (TEM)

This methodology was adapted from that used by Bizerra et al. [40]. Initially, cells treated for 24 h with crotamine [10 or 25 μM, corresponding to 50 or 125 μg/mL] or gomesin [10 μM, *i.e.* 22.7 μg/mL] were washed in PBS and fixed overnight at 4 °C with 2.0% formaldehyde plus 2.5% glutaraldehyde buffered at pH 7.2 with 0.1 M sodium cacodylate. The fixed cells were post-fixed in 1% osmium tetroxide for 1 h at room temperature. After dehydration in a graded series of ethanol and treatment with propylene oxide, the cells were embedded in Epon resin. Ultrathin sections (60 nm) were contrasted with uranyl acetate and lead citrate, and they were examined using a transmission electron microscope (JEOL, JEM-1200EX II, JEOL Ltd., Tokyo, Japan).

### 2.5. Cytotoxicity assay

#### 2.5.1. Hemolytic assay

Fresh citrated human blood obtained from a healthy volunteer donor was used in this assay. The hemolytic activity assay was monitored against human erythrocytes. Serial dilutions of native crotamine [0.1–100 μM] were incubated in microcentrifuge tubes with a suspension of erythrocytes (0.4%) in phosphate-buffered saline (NaCl 0.14 M; KCl 2.7 mM; $Na_2HPO_4$ 10 mM; $KH_2PO_4$ 1.8 mM pH 7.4). As a positive control (100% lyses), erythrocytes were incubated with 0.1% Triton X-100. After 1 h at 37 °C, the samples were centrifuged (320 ×*g*, 5 min), and the absorbance of the supernatant (50 μL) was measured at 405 nm using a microtiter plate reader.

#### 2.5.2. Viability test

Cell viability after exposure to crotamine was examined using the MTT assay, whereby metabolically active mitochondrial dehydrogenases convert the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co., St. Louis, MO, USA) to insoluble purple formazan crystals at 298 nm, which was proportional to cell viability. Primary culture of mice astrocyte cells, rat pheochromocytoma PC12 cells, embryonic kidney HEK293 cells, and tumoral mice melanoma B16F10 cells were used for cytotoxicity analysis of crotamine. Cells were plated in 96-well microtitre plates at $2 \times 10^5$ cells/mL. After overnight incubation, the medium was removed and the cells were incubated with appropriate amounts of toxin in the presence of 100 μL of appropriate culture media supplemented with 10% of FBS for 24 h, at 37 °C, in an atmosphere of 5% $CO_2$ in air. At the end of incubation 20 μL of MTT solution (5 mg/mL in PBS) were added to each well. After 4 h, 100 μL of 0.04 N HCl in isopropanol were added to each well and thoroughly mixed before the plate was read on a FlexStation3™ (Molecular Devices, Sunnyvale, CA, USA, www.moleculardevices.com), using a test wavelength of 570 nm and a reference wavelength of 620 nm (MTT viability assay). Percent of cytotoxicity was calculated as 100 × (1 − [optical density at 570–620 nm with toxin]/[optical density at 570–620 nm without toxin]). Results are expressed as mean values ± SD of three independent experiments, and the 50% cytotoxic concentration ($CC_{50}$) was estimated as previously [20].

## 3. Results

### 3.1. Expression and purification of recombinant crotamine and immunochemical characterization

Fig. 1A shows the SDS/PAGE analysis of recombinant protein after single step purification using Ni–NTA magnetic agarose beads affinity chromatography. As assessed by the SDS/PAGE, the recombinant proteins yielded an apparently homogenous protein sample of about >95% purity which migrated similarly to the native crotamine (Fig. 1A). In order to further confirm the identity of the obtained recombinant crotamine, Western blotting experiments were carried out using rabbit polyclonal anti-crotamine antibody, showing the recognition of one single band of about 5 kDa for both native and recombinant crotamine (Fig. 1B).

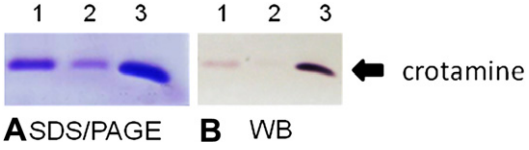


**Fig. 1.** Analysis of the native and recombinant crotamine by SDS/PAGE and Western blotting (WB). Aliquots of the recombinant (lanes 1 and 2) and native crotamine (lane 3) were analyzed by 12.5% SDS/PAGE electrophoresis stained with Coomassie Brilliant Blue (SDS/PAGE, A), and by Western blotting (WB, B) using primary antibody against crotamine. Twenty times less of each sample was used for the WB analysis compared to the SDS/PAGE.

### 3.2. Conformational analysis

The circular dichroism (CD) spectra of native, synthetic, and recombinant crotamine are shown in Fig. 2. The overall CD spectrum was similar to native and synthetic crotamine (Fig. 2), suggesting overall similar secondary structure for both. The α-helical contribution was significant for both native and synthetic crotamine, as observed in the CD spectrum (Fig. 2). On the other hand, the CD spectrum of the recombinant crotamine was more characteristic of a disordered peptide chain (Fig. 2). In the same way, the CD analysis of denatured native crotamine also showed a spectrum typical of a disordered structure independent of the denaturing protocols employed, *i.e.*, heating in the presence of 5 mM DTT or 10 mM β-mercaptoethanol, or by autoclaving 1 mg/mL crotamine solution in water (Supplementary Figure S1).

### 3.3. Antimicrobial susceptibility tests of bacterial and fungal standard strains

The MIC values of native, synthetic, and recombinant crotamine, as well as those for the derived small peptides P1 and P2, are shown in Table 1. The reliance upon such measurements was guaranteed by using gomesin, which showed an effective antibacterial activity against Gram-positive and Gram-negative bacteria strains in our hands, exactly as previously described [30]. Although gomesin clearly displayed efficient antimicrobial activity against Gram-positive and Gram-negative bacteria, in general no significant activity could be observed for either native, synthetic or recombinant crotamine [MIC values >200 μg/mL], except for *E. coli* (SBS363) that showed MIC values ranging from 12.5 to 25.0 μg/mL (Table 1). Peptide P2 also showed MIC values ranging from 17 to 34 μg/mL for Gram-negative bacteria *P. aeruginosa* [ATCC 29213] (Table 1). Another exception was for the Gram-positive bacteria *M. luteus* (*A*270), in which both native crotamine and gomesin showed MIC values ranging from 1 to 2 μg/mL (Table 1). Nevertheless, for this same strain, the recombinant crotamine showed a stronger potency compared to native crotamine, with MIC values between 0.25 and 0.50 μg/mL. Short peptide analog P1 showed no activity up to 25 μg/mL, while P2 showed MIC values ranging from 5 to 10 μg/mL for the Gram-positive bacteria *M. luteus* (*A*270) (Table 1).

In contrast, significant antifungal activity was observed against all reference yeast strains tested, including *C. albicans* [MDM8 and ATCC 36802] and *C. neoformans* [ATCC 90112] strains, with MIC values around 15–30 μg/mL for native crotamine. Interestingly, the MIC values of *C. albicans* strain MDM8 for the recombinant crotamine were significant lower [between 0.5 and 1.0 μg/mL] when compared to those obtained with the native toxin [15–30 μg/mL] (Table 1). Although linear unstructured reduced crotamine showed activity against *C. albicans* (MDM8 strain) in the range of 0.5–1.0 μg/mL, the small linear peptides analogs P1 and P2 showed no activity up to 25 μg/mL (Table 1). However, these linear peptides analogs showed similar MIC values [6–12 μg/mL] compared to those observed for the denatured native crotamine [6–12 μg/mL] (Supplementary Table 1), while structured native crotamine MIC values [15–30 μg/mL] against *C. neoformans* [ATCC 90112] was significantly higher (Table 1).

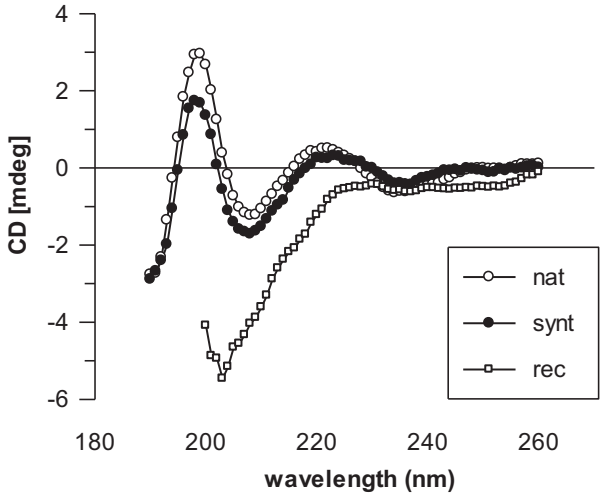


**Fig. 2.** CD spectra profile of the native, synthetic and recombinant crotamine. Circular dichroism (CD) spectra of native (○), full-length synthetic (●) and recombinant (□) crotamine is shown.

### 3.4. Antifungal susceptibility of clinical yeast isolates

Table 2 summarizes the *in vitro* susceptibilities of clinical yeast isolates to native, synthetic and recombinant crotamine, as well as to peptide analogs P1 and P2.

The MIC values obtained with native, synthetic, and recombinant crotamine were below 50 μg/mL for all tested yeasts, including *Candida* spp., *Trichosporon* spp., and *C. neoformans* strains, with an exception for synthetic crotamine that showed a little higher MIC values ranging from 50 to 100 μg/mL only for *C. guilliermondii* [IOC 4557]. The antifungal activity of synthetic crotamine was generally very similar to that observed with the native molecule. The only exceptions were for *Trichosporon* spp. and *C. guilliermondii* isolates, with lower and higher MIC values, respectively, for synthetic compared to native crotamine (Table 2). Excluding these two isolates from the analysis, the MIC values obtained with both synthetic and native crotamine were almost the same, *i.e.* ranging from 12.5 to 50.0 μg/mL (Table 2).

Interestingly, overall the MIC values obtained with recombinant crotamine [1.5–6.0 μg/mL] were about ten times lower than that for native crotamine [12.5–50.0 μg/mL] for most clinical strains evaluate here, with the single exception of *C. krusei* isolate, which showed exactly the same MIC values for both native and recombinant molecules [12.5–25.0 μg/mL]. These results show that recombinant crotamine exhibits a higher antifungal activity for almost all the yeast strains evaluated here (Table 2), demonstrating that the unstructured crotamine (Fig. 2) is a more efficient antifungal agent than the structured native crotamine, although the linear short peptide analogs were overall less efficient.

Although crotamine exhibits significant antifungal activity against the medically important yeast species, no major activity could be observed against the filamentous fungus *A. fumigatus* [IOC 4526] and *T. rubrum* [IOC 4527] for native crotamine and its linear fragment peptides P1 and P2, even at concentrations up to 125 and 100 μg/mL, respectively (Table 2).

Peptides P1 and P2 did not show any important antifungal activity, with the exception of the clinical strain *C. neoformans* [IOC 4528] and *C. krusei* [IOC 4559], which exhibited MIC values between 6 and 12 μg/mL and 35–70 μg/mL, respectively. In addition, peptide P2 showed MIC values of 35–70 μg/mL and 10–20 μg/mL for *Trichosporon* spp. [IOC 4569] and *C. guilliermondii* [IOC 4557] strains, respectively (Table 2).

### 3.5. Fungicidal effect of crotamine

The fungicidal activity of crotamine against the most important yeast species, including *C. albicans* [IOC 4558], *C. krusei* [IOC 4559], *C. glabrata* [IOC 4565], *C. parapsilosis* [IOC 4564], *C. tropicalis* [IOC 4560], *C. guilliermondii* [IOC 4557], and *Trichosporon* ssp. [IOC 4569] was also evaluated. Exponential phase culture of these yeast strains

**Table 1**
Crotamine minimum inhibitory concentrations (MICs) values for bacterial and fungal standard strains.

| Organisms | Minimum inhibitory concentration [μg/mL] | | | | | |
|---|---|---|---|---|---|---|
| | Native | P1 | P2 | Synthetic | Recombinant | Gomesin |
| Gram-negative bacteria | | | | | | |
| *Escherichia coli* [SBS363] | 12.5–25.0 | >80 | >80 | >200 | ND[a] | 1–2 |
| *Pseudomonas aeruginosa* [ATCC 29213] | >200 | >80 | 20–40 | >200 | >200 | 6–12 |
| Gram-positive bacteria | | | | | | |
| *Staphylococcus aureus* [ATCC 29213] | >200 | >80 | >40.0 | >200 | >200 | 3–6 |
| *Micrococcus luteus* (A270) | 1–2 | ND[a,b] | 6–12 | ND[a] | 0.25–0.50 | 1–2 |
| Fungus | | | | | | |
| *Candida albicans* (MDM8) | 15–30 | ND[a,b] | ND[a,b] | ND[a] | 0.5–1.0 | 0.3–0.6 |
| *Candida albicans* [ATCC 36802] | 15–30 | >100 | >100 | ND[a] | ND[a] | 12–24 |
| *Cryptococcus neoformans* [ATCC 90112] | 15–30 | 6–12 | 6–12 | ND[a] | ND[a] | ND[a] |

MDM8: USP Microbiology collection (SP, Brazil).
ATCC: The Global Bioresource Center (USA).
A270: CNRS (France).
[a] ND = non determined.
[b] ND = non observed up to 25 μg/mL.

were treated (exposed) for 24 h with 4-fold MIC concentrations [*e.g.* 200 μg/mL] of native crotamine. Under this condition no CFU were detected for all evaluated yeast strains (Table 3). In agreement with the CFU count data, the kinetics of *C. albicans* IOC 4558 killing in the presence of 4-fold MIC concentration confirmed the fungicidal activity of crotamine. A complete cell killing was observed even at short incubation times, as early as 10 min (Fig. 3).

### 3.6. Ultrastructural alterations induced by crotamine

Aiming to further investigate the effect of crotamine on *C. albicans* IOC 4558, the morphology and ultrastructure of these cells were analyzed by transmission electron microscopy (TEM) (Fig. 4). Untreated cells had both a normal cytoplasm membrane and cell wall morphology (Fig. 4A). By means of conventional TEM, we observed that control cells exhibited a cell wall with two layers: i) an electron-dense outer layer; and ii) an inner layer of low electron density, continuous with the plasma membrane. On the other hand, incubation of *C. albicans* [IOC 4558] yeast cells for 24 h in the presence of 50 or 125 μg/mL [corresponding to10 or 25 μM] of crotamine resulted in pronounced ultrastructure alterations (Fig. 4B and C, respectively). Cells tended to adopt irregular shapes and the cell wall became irregular in the layering structure with predominance of a more electron-dense layer (Fig. 4B). Plasma membranes lost their integrity and cytoplasm contents coagulated. Similar alterations were observed for treatments with gomesin (Fig. 4D).

### 3.7. Cytotoxicity effect of crotamine

Interestingly, no hemolytic activity against human erythrocytes was observed for native crotamine (triplicate) at concentrations up to 100 μM (Fig. 5). For comparison, gomesin, the antimicrobial peptide from hemocytes of the *A. gomesiana* spider, displays a certain degree of hemolytic activity, viz., 29% of cell lysis at 100 μM, as previously described [30].

In addition, to further evaluate the cytotoxic effect of crotamine, several normal cell lines including mice astrocyte primary culture cells, rat pheochromocytoma PC12 cells, embryonic kidney HEK293 cells, and tumoral mice melanoma B16F10 cells were treated with different concentrations of crotamine [0, 50, 100, and 250 μg/mL]. Then the cell viability was examined using the MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, in which metabolically active mitochondrial dehydrogenase activity converts the MTT to insoluble purple formazan crystals at a rate proportional to cell viability (Fig. 6). The 50% cytotoxic concentration ($CC_{50}$) was above 250 μg/mL for all cells lines tested, except for the tumoral B16F10 cells, which was found to be around 2 μM [~10 μg/mL], as already described by us elsewhere [20].

## 4. Discussion

Antimicrobial peptides (AMPs) are important functional molecules in innate immunity, and provide a sophisticated first line of host defense with the capacities to kill microbes directly, and to stimulate innate and adaptive defense systems [23,41,42]. In the case of the human defensins, there is direct evidence for a correlation between this polypeptide's expression and the incidence of infection [24,43–45].

The three-dimensional structure of crotamine in solution indicates that both the cysteine-pairing pattern and the global fold of this toxin are similar to those of the AMP human β-defensin [15,16]. The antimicrobial activity of the AMPs, human β-defensin, and *A.*

**Table 2**
Antifungal susceptibility of clinical isolates determined by a colorimetric broth microdilution method.

| Clinical fungus strains | Minimum inhibitory concentration [μg/mL] | | | | |
|---|---|---|---|---|---|
| | Native | P1 | P2 | Synthetic | Recombinant |
| *Candida krusei* [IOC 4559] | 12.5–25.0 | 40–80 | 40–80 | 25–50 | 12.5–25.0 |
| *Trichosporon* spp. [IOC 4569] | 12.5–25.0 | >80 | 40–80 | 3–6 | 1.5–3.0 |
| *Candida glabrata* [IOC 4565] | 25–50 | >80 | >80 | 12.5–25.0 | 1.5–3.0 |
| *Candida albicans* [IOC 4558] | 25–50 | >80 | >80 | 25–50 | 3.0–6.0 |
| *Candida parapsilosis* [IOC 4564] | 25–50 | >80 | >80 | 12.5–25.0 | 1.5–3.0 |
| *Candida tropicalis* [IOC 4560] | 12.5–25.0 | >80 | >80 | 12.5–25.0 | 3.0–6.0 |
| *Candida guilliermondii* [IOC 4557] | 12.5–25.0 | >80 | 10–20 | 50–100 | 3.0–6.0 |
| *Cryptococcus neoformans* [IOC 4528] | 15–30 | 6–12 | 6–12 | – | – |
| *Aspergillus fumigatus* [IOC 4526] | >125 | >100 | >100 | – | – |
| *Trichophyton rubrum* [IOC 4527] | >125 | >100 | >100 | – | – |

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**Table 3**
Fungicidal activity of crotamine at concentrations 4× MIC values against yeast clinical strains.

| Clinical yeast | MIC for native crotamine [μg/mL] | Crotamine [μg/mL] | CFU(N) |
|---|---|---|---|
| *Candida krusei* [IOC 4559] | 12.5–25.0 | 100 | 0 |
| *Trichosporon* spp. [IOC 4569] | 12.5–25.0 | 100 | 0 |
| *Candida glabrata* [IOC 4565] | 25.0–50.0 | 200 | 0 |
| *Candida albicans* [IOC 4558] | 25.0–50.0 | 200 | 0 |
| *Candida parapsilosis* [IOC 4564] | 25.0–50.0 | 200 | 0 |
| *Candida tropicalis* [IOC 4560] | 12.5–25.0 | 100 | 0 |
| *Candida guilliermondii* [IOC 4557] | 12.5–25.0 | 100 | 0 |

*gomesiana* spider gomesin has been characterized. Human β-defensins exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria [23,24,45,46]. Gomesin also has potent antimicrobial activity against the same bacterial species [30]. However, each animal species seems to possess numerous paralogous β-defensin genes, which have arisen by multiple duplication events followed by bursts of sequence variation [47]. The human β-defensins are a unique and diverse group of molecules displaying a characteristic triple-stranded twisted antiparallel β-sheet scaffold, whose formation seems to depend primarily on the presence of conserved disulfide bridges, but presenting few residues conserved in their sequences [47], with an extensive and distinct set of biological activities [48].

In this study, we describe for the first time an extensive analysis of the antimicrobial and antifungal activities of native, chemically-synthesized, and expressed recombinant crotamine. Although the impressive low MIC value observed for the Gram-positive bacteria *M. luteus* (*A*270), the antibacterial activity of crotamine and its analogs against most Gram-negative and Gram-positive strains studied here (Table 1) was unremarkable compared to those observed for gomesin and β-defensins. Likewise, no activity could be observed against *S. aureus* (also Gram-positive) at concentrations up to 200 μg/mL (Table 1), in contrast to that described for human defensin 5 [49]. These results confirm that crotamine exhibits a narrow spectrum of antibacterial activity, compared to the broad activity of human β-defensins [23,24,45,46]. Since *M. luteus* is the major microbial contaminant of orthodontic buccal devices and oral dispensers [50,51], further exploitation of crotamine and its analogs activities against this bacterial species is warranted.

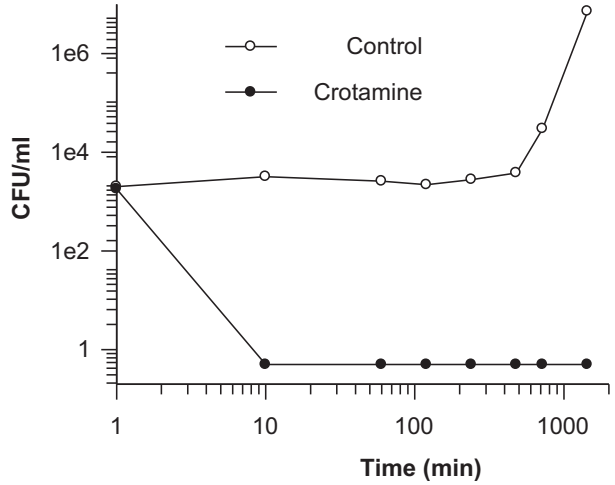


**Fig. 3.** Kinetics of *C. albicans* IOC 4558 killing by crotamine. Crotamine (200 μg/mL, ●) was added to a $10^4$ culture of *C. albicans*. As control the same culture without crotamine was utilized (○). Aliquots were removed at various times, and the number of colony forming units (*CFU*) was determined on LB agar plates after overnight incubation at 30 °C.

In contrast to the weak antibacterial effects, crotamine and its analogs were found to exhibit significant antifungal activity. In general, synthetic and native crotamine showed similar antifungal activity against all yeast clinical strains, including *Candida* species, *C. neoformans*, and *Trichosporon* spp.

Recently, two studies described the antibacterial activity of crotamine [28,29], and both studies suggested a crotamine activity against only a narrow spectrum of bacteria. Yount et al. [28] suggested an antifungal activity of crotamine against *C. albicans* isolates. However, these authors used a radial diffusion methodology in which MIC values cannot be evaluated. Oguiura et al. [29] reported that crotamine exhibited antibacterial activity only against *E. coli* isolates [ATCC 25922], with MIC values ranging from 25 to 100 μg/mL [5–20 μM], while no antimicrobial activity was observed with *P. aeruginosa*, *Salmonella typhimurium*, *S. aureus*, and *Listeria monocytogenes* isolates. These results are in good agreement with our data presented here. However, in our study the MIC values were estimated by the microdilution test, and also the antifungal activity of crotamine was evaluated using three yeast standards and 10 clinical isolates, including eight yeast species and two filamentous fungi. Interestingly, a very potent antifungal activity was observed for native crotamine against all yeast strains evaluated here. However, this toxin did not exhibit antifungal activity against the filamentous fungus *A. fumigatus* [IOC 4526] and *T. rubrum* [IOC 4527] (Table 2). Crotamine peptide fragments P1 and P2 did not show significant antifungal activity against the clinical strains studied here, with the exception for standard microbiological and clinical strains of *C. neoformans* [ATCC 90112/IOC 4528] with MIC values around 6–12 μg/mL (Tables 1 and 2). Activities with MIC values around 40–80 μg/mL were also observed for these peptides against *C. krusei* [IOC 4559] and *Trichosporon* spp. [IOC 4569], although for the latter no activity was observed for P1 up to 80 μg/mL (Table 2). Note that the MIC value for P2 [about 20 μg/mL] against *C. guilliermondii* [IOC 4557] was very close to that observed for native crotamine [12.5–25 μg/mL] (Table 2).

In summary the results presented here show that recombinant crotamine exhibits a higher antifungal activity for almost all the yeast strains evaluated here (Tables 1 and 2), demonstrating that the unstructured crotamine is a more efficient antifungal agent than the structured native crotamine, as also indicated by the use of denatured native crotamine (Supplementary material), although the linear short peptide analogs were overall less efficient.

The MICs of recombinant crotamine observed for several standard and clinical strains were generally lower than those of native crotamine (Tables 1 and 2). We believe that our bacterially-expressed recombinant crotamine represents the features of a completely reduced/linear crotamine, as confirmed by its CD spectrum (Fig. 2). Enhancement of antimicrobial activity by reduction of disulphide bonds has been described for human β-defensin 1 [32]. In addition, reduction of these bonds alters the structure of natural β-defensin, resulting in a different type of membrane interaction and a switch to different modes of action toward microbial cells underlying the antimicrobial activity [53]. Covalent dimerization via S–S bond formation could enhance such activity [53]. On the other hand, disulfide bond deletion in human defensin 5 disulfide array mutants showed an attenuation of antibacterial activity against *S. aureus* [49]. Recombinant mouse β-defensin 3 (the human homolog of β-defensin 2) was shown to present most significant antifungal activity against filamentous species compared to yeast forms, with a mild antibacterial activity [54]. Clearly, the nature and extent of S–S bond formation has

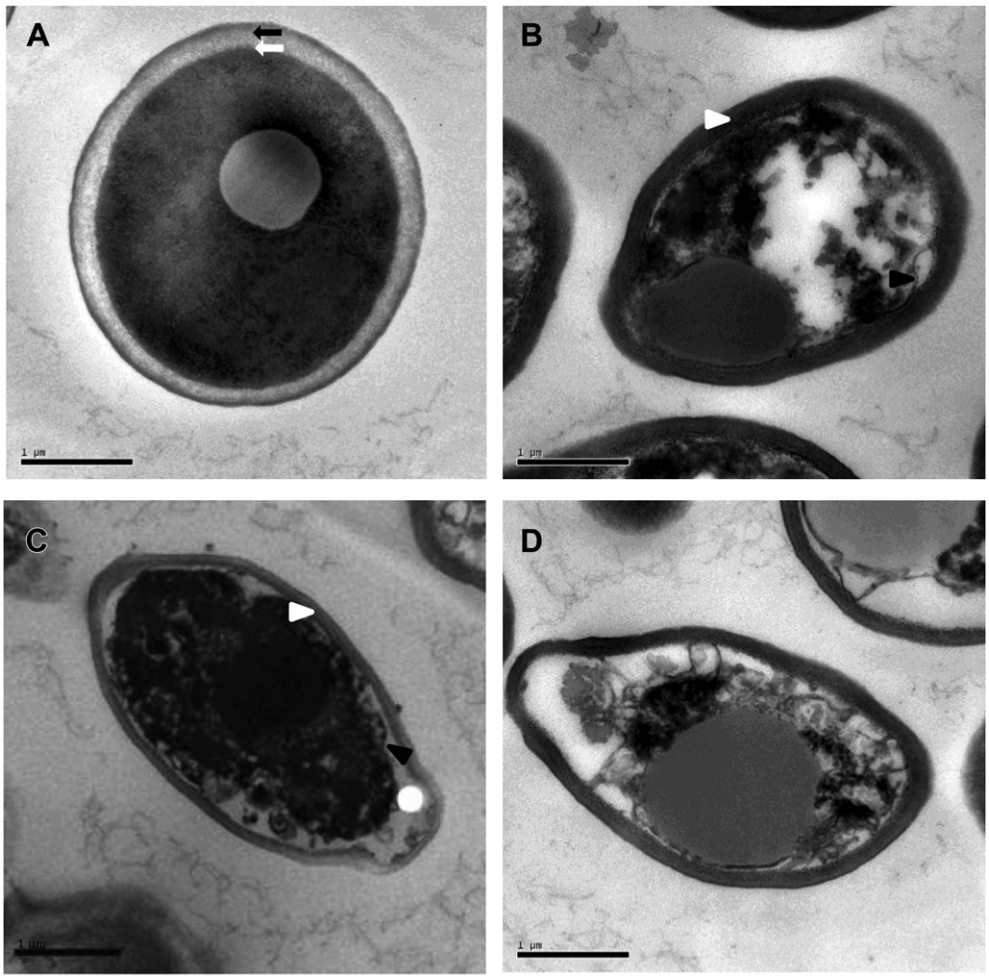


**Fig. 4.** Transmission electron micrographs of *C. albicans* cells treated with crotamine. (A) Untreated cells exhibited a normal plasma membrane and cell walls with two layers, an electron-dense outer layer and an inner layer of low electron density (arrows black and white, respectively). (B and C) The yeast cells treated with 50 and 125 μg/mL, corresponding to 10 and 25 μM of crotamine, respectively, exhibited pronounced morphological alterations, including membrane collapse (black-headed arrows), cell wall with predominance of the more electron-dense layer (white-headed arrows) and cytoplasm coagulation. Similar alterations were observed when crotamine was replaced by gomesin (D). Scale bars = 1 μm.

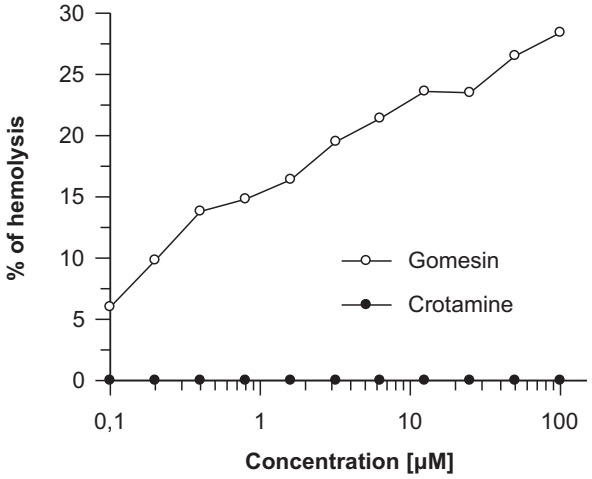


**Fig. 5.** Hemolytic assay. Crotamine (●) and gomesin (○) were incubated with human erythrocytes at concentrations ranging from 0.1 to 100 μM for 1 h at 37 °C. The hemolysis percentual was expressed in relation to a 100% lysis control (erythrocytes incubated with 0.1% Triton X-100).

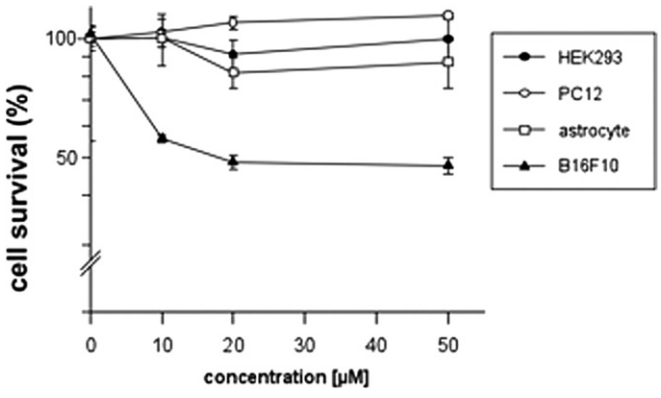


**Fig. 6.** Cytotoxic effect of crotamine. Primary culture of mice astrocyte cells, rat pheochromocytoma PC12 cells, embryonic kidney HEK293 cells and tumoral mice melanoma B16F10 cells were incubated with 0, 50, 100 and 250 μg/mL of crotamine for 24 h, and the cell survival rate was measured by the MTT assay. The percentage of cell growth in the control group was designated as 100%. Results are expressed as mean values ± SD of three independent experiments.

a major effect on antimicrobial and antifungal activity, and this is an area that requires further exploration.

In addition to the antifungal activity analysis of crotamine, we also evaluated the fungicidal activity of this toxin. The kinetics of *C. albicans* IOC 4558 killing by 4-fold MIC crotamine showed complete cell killing after only 10 min incubation (Fig. 3). Moreover, addition of 4-fold MIC of native crotamine to exponential phase cultures of several clinical yeast strains showed a major fungicidal effect after overnight incubation, with no CFU detected on LB agar plates (Table 3). Since the majority of antifungal drugs currently used for treatment of *Candida* species, *Trichosporon* spp., and *C. neoformans* fungal infections are usually fungistatic [5,52], the fungicidal activity of crotamine is an important improvement in relation to these other currently used drugs.

With respect to the ultrastructural alterations in cells treated with crotamine, we showed that *C. albicans* IOC 4558 cells subjected to 10 and 25 μM crotamine, which correspond to 50 and 125 μg/mL, respectively, exhibited pronounced morphological alterations, including membrane collapse and cytoplasm coagulation, which were very similar to the changes observed with spider gomesin (Fig. 4). Our results lead to the conclusion that crotamine antifungal activity is related to disruption of the cytoplasm membrane of fungal cells. In contrast to gomesin, native crotamine did not show hemolytic activity (Fig. 5), and showed high values for 50% cytotoxic concentration [$CC_{50} > 50$ μM] when tested against non-tumoral animal and human cells (Fig. 6).

These results strongly suggest that crotamine has potential use as a novel antifungal agent. The *Candida* species, predominantly *C. albicans*, are involved in a wide range of human infections, including invasive candidiasis, oral, and vulvovaginal infection [55]. The broad antifungal activity and the pronounced activity against the Gram-positive bacteria *M. luteus* (*A*270) observed for crotamine in this work open new perspectives for the use of this venom component in infectious disease treatments. The demonstrated successful production of crotamine by chemical synthesis or recombinant expression with biological activity further reinforces the potential feasibility of using this molecule in medical and industrial applications.

## 5. Conclusion

In conclusion, we have shown in this work that crotamine has effective antifungal activity against a number of clinical yeast strains, with great potential to contribute to the development of a new generation of antimicrobial compounds able to overcome the microbial resistance challenge. We envision new perspectives for the use of this venom component in biomedicine, as it has been described for a number of other natural toxins [56].

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## Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2012.09.019.

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