



***Crotalus durissus* venom: biological effects and relevant applications. A Review**

Veneno da Crotalus durissus: Efeitos biológicos e aplicações relevantes. Uma Revisão

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Abstract: Snakes have an important biological role in the ecological chain, as well as in scientific researches performed with the venoms produced by them, since the enzyme-protein fractions these substances possess have been studied as pharmacological tools for the discovery of new therapies. Snakes of the genus *Crotalus* have gained significant relevance in the scientific field, since the venom produced by these reptiles has been the target of researchers in a few decades, due to the composition and the effects that these substances can produce. In Brazil, a single species represents the genus, which is *Crotalus durissus*. This review demonstrates that even with the advancement in scientific research on the composition, role and application of the venom produced by the subspecies of *Crotalus durissus* snake, it is necessary to further study their fractions and their action potential, which also demonstrates the how rich are these active components in different fields of biomedicine.

Keywords: snake toxins, crotoxin, phospholipase A2, gyroxin.

Resumo: As serpentes possuem um papel biol gico importante na cadeia ecol gica, como nas pesquisas realizadas com os venenos por elas produzidos, uma vez que suas fra es enzim ticas-proteicas v m sendo estudadas como potenciais ferramentas farmacol gicas para descoberta de novas terapias. As serpentes do g nero *Crotalus* tem ganhado relevante espa o no campo cient fico, visto que em algumas d cadas seu veneno vem sendo alvo de pesquisadores devido sua constitui o e o efeito que essas subst ncias podem causar. No Brasil, este g nero   representado por apenas uma esp cie, a *Crotalus durissus*. Esta revis o de literatura demonstra que, mesmo com o avan o em pesquisas cient ficas sobre a composi o, atua o e aplica o do veneno da serpente *Crotalus durissus* e suas subesp cies, torna-se necess rio estudos mais aprofundados principalmente no que diz respeito

as suas frações e seus potenciais de ação, o que também demonstra a riqueza em atividade desses componentes em diversos ramos da biomedicina.

Palavras-chave: toxinas ofídicas, crotoxina, fosfolipase A2, giroxina.

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Introduction

The crotalic venom is presented as an enzyme-toxic complex of various substances. Among the enzymes are: proteases, phosphodiesterase, L-amino acid oxidase, 5'-nucleotidase and fractionation of this venom reveals toxins such as crotoxin, crotamine, gyroxin, convulxin and serine proteinases (RANGEL-SANTOS *et al.*, 2004; FONSECA *et al.*, 2006). The different components of the venom are reflected in the variations of their biological properties, toxicity and activity characteristics. The toxicity of the venoms is presented at varied times and in different clinical manifestations, which are due not only to their specific properties, but also to their toxicokinetics, composition and potency of the substance.

Snakes of the genus *Crotalus* have gained significant relevance in the scientific studies, and the venom produced by these reptiles has been the target of researchers in a few decades, due to their composition and the effects that these substances can cause. Most venoms affects

almost all types of cells and tissues of the human body where their pharmacological properties are determined by the specific amount of biologically active constituents. About 90-95% of the dry weight of snake venoms have protein properties, and these proteins are responsible for almost all of the biological effects found. Lucien Bonaparte established the proteinaceous nature of these venoms in 1843 (BON, 2006).

Therefore, it is important to study the effects of the venom of *Crotalus durissus* species, snake largely found in Brazilian territory, performing a review about the main components, aiming to better understand their biological effects and mechanisms of action.

1. General characteristics and territorial distribution

The Viperidae family presents great expertise in skull morphology, with extreme mobility of the jaw and other bones of the head. The venom of these snakes is produced by two special glands

located in the head, behind and below the eyes, which are nothing more than modified salivary glands, where saliva is the actual toxin.

They have stocks for multiple bites, and, once the venom or secretion is completely extracted, the glands will again be filled completely only after two weeks (SANTOS, 1995).

Snakes of the genus *Crotalus* are represented in Brazil by a single species, *Crotalus durissus*, which is further divided into the following subspecies: *Crotalus durissus terrificus*, found in the southeast and south regions; *Crotalus durissus collilineatus*, distributed in dry areas of the midwest region, in Minas Gerais State and in the north of São Paulo State; *Crotalus durissus cascavella*, found in the caatinga bioma of the northeast region; *Crotalus durissus ruruima* observed in the north region; and *Crotalus durissus marajoensis* observed in Marajó Island (PINHO & PEREIRA, 2001). Some authors recognize a sixth subspecies in Brazil, the *Crotalus durissus trigonicus*, found in some regions of Roraima State (CAMILLO, 1998).

2. Constituents of the venom of *crotalus durissus* species

2.1. Crotoxin and subunits

The crotoxin, which is the main polypeptide present in the venom, was isolated initially by SLOTTA & FRAENKEL-CONRAT in 1938. Silva

(1982) when isolating the crotoxin again from the subspecies *Crotalus durissus terrificus*, pointed out that this component accounted for 68% of the total weight of the venom.

According to Hendon & Fraenkel-Conrat (1971), crotoxin (24 kDa) is a β -neurotoxin that has two subunits, an acid, the crotopotin (crotoxin A) and a base, phospholipase A2 (crotoxin B).

Crotoxin has pre and postsynaptic blocking effects. Pre-synaptically, it interferes with the release of acetylcholine by blocking neuromuscular transmission and preventing the contraction of skeletal striated muscle; while postsynaptically, it acts by decreasing the action of acetylcholine and prevents membrane depolarization. It can cause paralysis of the respiratory muscles, culminating in the death of the envenomed individual. It also increases the permeability of sodium ions (BON *et al.*, 1979; GOPALAKRISHNAKONE *et al.*, 1984; RANGEL-SANTOS *et al.*, 2004). The venom of *Crotalus terrificus durissus* and crotoxin B induces protein expression of cyclooxygenase-2 by endothelial cells (MATSUBARA, 2009).

Interspecific and intraspecific variations have been described for the crotoxin complex. Intraspecific variations differ in the presence of different isoforms of subunits A and B, which possess

different potency and varied biological activity (PEREAÑEZ *et al.*, 2009). According to Rangel-Santos & colleagues (2004) even with an electrophoretic profile similar in subspecies *Crotalus durissus cascavella*, *Crotalus durissus collilineatus* and *Crotalus durissus terrificus*, there are differences in their activities, which may be related to the existence of different isoforms of crotoxin. The crotopotin acidity is justified by their amino acid composition: 12 aspartate/asparagine residues and 14 glutamate/glutamine versus 2 lysine residues and 2 arginine. The crotalic phospholipase A2 contains 11 aspartate/asparagine and 10 glutamate/glutamine residues versus 11 lysine and 12 arginine residues, characterizing the base property of the substance. The crotopotin and the phospholipase A2 are rich in cystine containing 7 and 8 disulphide bridges, respectively. The two subunits form the crotoxin, molar complex with a 1:1 ratio (BREITHAUPT *et al.*, 1974).

Crotopotin has no toxicity nor enzymatic activity of crotoxin, but enhances the toxicity. This potentiation can be explained by the fact that, when combined with component B (PLA2), the component A (crotopotin) prevents the binding of PLA 2 to low specificity and affinity sites, directing the PLA2 to sites of membrane receptors (postsynaptic) with high specificity and high affinity, which enhances the toxicity of crotoxin. These

receptors were designated as type N (neuronal) and M (muscle). The receivers of the type N were the first to be identified and are present in large quantities in rat brain membranes. These receptors bind with high affinity to neurotoxic PLA2. As for the type M receptors have been identified in skeletal striated muscle of rabbits (MATSUBARA, 2009).

Crotopotin has demonstrated the ability to potentialize the inflammatory edema (induced by carrageenan), possesses antimicrobial effect on Gram negative bacteria and elevates the renal perfusion pressure and renal vascular resistance in perfused kidneys (without, however, altering the urinary flux or glomerular filtration, in opposed to the crotoxin) (MARTINS *et al.*, 2003; OLIVEIRA, *et al.*, 2003).

Unlike the venom of *Crotalus durissus terrificus*, which contains three or four PLA2 isoforms; the venom of *Crotalus durissus cascavella* contains at least four crotoxin isoforms formed by various combinations of crotopotins with one isoform of phospholipase A2 (PLA2) (BEGHINI *et al.*, 2000). These data can serve as evidence to suggest differences in the action intensity of the venom from different subspecies.

The PLA2 induces plasma extravasation and is able to increase microvascular permeability of the skin at

the dorsal region of rats. The mechanisms of PLA2 induced edema formation involves the activation of sensory C fibers, which release neuropeptides such as substance P, which mediate neurogenic local inflammatory effects (vasoactive responses) in innervated tissues and local mast cells (by substance P), which release histamine and serotonin (CÂMARA *et al.*, 2003). FONSECA & colleagues (2006) reported that crotoxin presents two different enzymatic activities (PLA2 and serine protease) and two different types of biological actions (neurotoxic and coagulant).

2.2. *Crotamine*

The crotamine is a basic polypeptide, belonging to the family of basic polypeptidic myotoxins, which have intracellular penetration capacity through mechanisms independent of energy expenditure by interaction with proteoglycans of the extracellular matrix (MATSUBARA, 2009). The myotoxic effect is caused by this toxin, which presents synergistic activity with crotoxin. It acts mainly causing muscle contractions dependent of depolarization of the skeletal striated muscle cells by increasing the sodium ion permeability of the membrane. This effect probably occurs on these ion channels by inducing calcium influx (LOMONTE *et al.*, 2003). Additionally, it is known that crotamine actively interacts

with the lipid membranes of cells forming vacuoles and showing a myonecrotic activity.

2.3. *Convulxin*

The convulxin is a toxin with high molecular weight, belonging to the family of C-type lectins isolated from *Crotalus durissus terrificus* venom, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus* consists of a potent platelet activator. According to Lima & colleagues (2005) besides inducing platelet aggregation by binding to GPVI platelet receptor, it also causes seizures, respiratory and circulatory alterations. It is composed of two polypeptide chains (Cvx α e Cvx β) covalently associated in a trimeric structure ($\alpha\beta$)₃. The platelet induction by this toxin consists of a Ca²⁺ dependent reaction, initiated by the binding of convulxin to the GPVI platelet receptors (POLGÁR *et al.*, 1997; JANDROT-PERRUS *et al.*, 1997; KANAJI *et al.*, 2003).

Leduc & Bon (1998) cloned and sequenced both strands of convulxin of *Crotalus durissus terrificus*. In this study, a high similarity between α and β chains, with an average of 74% identity percentage was observed. The sequencing of the subunits resulted in chains comprising 158 and 148 amino acid residues in Cvx α and Cvx β , respectively, and a signal peptide of 23 amino acid residues identical for both the subunits. Toyama & colleagues (2001)

isolated and characterized a convulxin-like protein from the *Crotalus durissus collilineatus* venom with a high similarity to the convulxin of *Crotalus durissus terrificus*.

2.4. Gyroxin

The gyroxin converts fibrinogen into fibrin, thereby increasing clotting time (SANO-MARTINS *et al.*, 2001). This toxin belongs to the thrombin family and was isolated from the venom of *Crotalus durissus terrificus* (RAW *et al.*, 1986). In addition to this performance, excellent neurotoxic activity have also been described (BARROS *et al.*, 2011).

Fonseca & colleagues (2006) described gyroxin presenting two main fractions: one with thrombin-like activity and other L-amino acid oxidase (LAAO). LAAO is the only oxidase dependent FAD (flavin adenine dinucleotide) found in snake venom and toxicity possibly involves generation of hydrogen peroxide, the final product of L-amino acid oxidation. The LAAO also induces platelet aggregation, involving the activation of PLA2 (TOYAMA *et al.*, 2006). In recent studies, Barros & colleagues (2011) found that gyroxin demonstrated a high level of coagulation activity, with a minimum dose of 0.015-0.037µg/µL.

Prado-Franceschi (1990) suggests that this toxin produces a peculiar seizure syndrome in mice characterized by rapid

movements rotating the body around the longitudinal axis. The study of this enzyme could serve as a molecular model interesting for developing new drugs or therapeutic agents (MORAES *et al.*, 2004; FERRARO *et al.*, 2005), especially due to their resistance to physiological proteinase inhibitors (MATSUI *et al.*, 2000; SERRANO & MAROUN, 2005).

2.5. Natriuretic peptide

Another important component of the venom is the natriuretic peptide that was isolated from *Crotalus durissus cascavella* and was able to increase renal perfusion pressure and vascular resistance in isolated perfused rat kidneys. Increased urinary flow and glomerular filtration and reduced renal sodium tubular transport was also observed. In systemic blood pressure *in vivo* experiments, a decrease in mean arterial pressure, cardiac output and respiratory rate was observed. Therefore, this component of the venom showed renal and cardiovascular effects, with consequent hypotensive and diuretic effects (EVANGELISTA *et al.*, 2008).

3. Study of the toxic activity of the *crotalus* genus venom and fractions

Over the decades, in several studies, researchers test venoms and their components. The venom of *Crotalus durissus* species have been tested under different perspectives, and among them, the observed were: cytotoxic, antibacterial,

leishmanicide, analgesic, on hematological processes and activation of phagocytosis processes in viral infections; their influence on the nervous system; as well as activation of apoptotic pathways, or necrotic tumor cell lines phagocytosis. Some studies have also found effects of the venom on various cell types, such as macrophages, lymphocytes, platelets and tumor cells (SAMPAIO et al. 2005; YAN et al., 2006 e 2007).

Yan & colleagues (2006) tested crotoxin, the main fraction of the crotalic venom and demonstrated the occurrence of autophagy and apoptosis in chronic myeloid leukemia cell line (K562). Tamietti & colleagues (2007) reported that the venom of *Crotalus durissus* has the capacity to induce apoptosis in tumor hamster ovarian carcinoma cells (CHO-K1). Also in 2007, the same authors repeated the study using another cell line, MCF-7, derived from human breast cancer. In this experiment, the stimulation of autophagy was yet observed, however to a lesser extent and being dose-dependent as well as apoptosis by condensation and nuclear fragmentation.

Another important researched component is the crotalic PLA2. Fonseca et al (2006) tested both crotoxin and the subunits crotapotin and PLA2 in human plasma rich in platelets and verified that the concentration of 20µL of crotoxin

demonstrated potent action for platelet aggregation; while the major subunits were not able to provide the same potential when tested in separate.

The venom of *Crotalus durissus terrificus* has also been known by researchers who emphasize the analgesic activity, confirmed effect in pain experimental models (GIORGI et al., 1993; GUTIERREZ et al., 2008). More recently, Nogueira-Neto & colleagues (2008) demonstrated that the crotoxin inhibit neuropathic pain induced by transection of the sciatic nerve in rats, when applied directly onto the stumps and also when it was administered to animals.

The mechanisms involved in analgesic action of the crotoxin may also vary according to the experimental model used to assess pain. Zhang & colleagues (2006) and Zhu & colleagues (2008), by using models of acute pain, demonstrated that muscarinic and opioid receptors are not involved in the analgesic effect of the crotoxin matched against Nogueira-Neto & colleagues (2008) that reported activation of central muscarinic receptors was involved in analgesia induced by crotoxin in a model of neuropathic pain. Recently, an acid peptide identified in the venom of *Crotalus durissus terrificus*, named crotalphine, was determined as a major component in the analgesic activity, inducing a potent and long acting

antinociceptive effect in neuropathic pain (KONNO *et al.*, 2008; GUTIERREZ *et al.*, 2008).

Concerning the action of venom on microorganisms, several studies have shown that the venom of *Crotalus durissus* species has bactericidal activity for a variety of species among, such as *Burkholderia pseudomallei*, *Claribacter michiganensis* *michiganensis*, *Enterobacter aerogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Xanthomonas axonopodis* *pv. passiflorae* and can be affirmed that this activity is related to PLA2 (SOARES *et al.*, 2001; OLIVEIRA *et al.* 2003; TOYAMA *et al.* 2003; SAMY *et al.*, 2006 and 2007; DIZ FILHO *et al.*, 2009).

Oliveira & colleagues (2003) demonstrated that crotoxin isolated from the venom of *Crotalus durissus cascavella* was six times less active in Gram-negative bacteria than in Gram-positive bacteria. Also, that PLA2 present in the venom showed the same bactericidal effect on both types bacteria and that crotopotin inhibited bacterial growth rate, but the actions of the constituents varied in the way the effect was performed in the tested bacteria. The interest in some studies arose from researchers observed a very low frequency of bacterial infections that occurred in snakebite lesions, then suggesting this possible bactericidal

activity. The biological activity of the venom of these snakes on parasites was also studied. Different crotalic venoms collected from subspecies *Crotalus durissus terrificus*, *Crotalus durissus cascavella* and *Crotalus durissus collilineatus*, when tested decreased the growth rate of *Leishmania amazonensis* (PASSERO *et al.*, 2007). These results show that crotalic venoms may be protein sources with therapeutic potential against parasites causing leishmaniasis, the findings suggest that the growth of *Leishmania amazonensis* may have been affected by different routes, including the production of hydrogen peroxide and probably via recognition by cell surface receptors.

For the proteomic study, we can highlight studies involving the snakes *Crotalus durissus collilineatus* and *Crotalus durissus cascavella* when we define they had proteomes that comprehend gama proteins of 4-115 kDa, pertaining to 9 and 8 families of toxins, respectively (BOLDRINI-FRANÇA *et al.*, 2011). Also, according with those studies, venoms of *C. d. collilineatus* e *C. d. cascavella* have 20-25 toxines pertaining to following families: desintegrin, PLA2, serine proteinases, cysteine-rich secretory protein (CRISP), vascular endothelialgrowth factor (VEGF), L-amino acid oxidase, c-type lectins-like and snake

venom metalloproteinase (SMVP). Venom composition explains clinical manifestations developed in accidents caused for *Crotalus durissus*, such as, neurotoxicity, myalgia, coagulation disorders, often accompanied for myoglobinuria and acute renal failure.

4. Conclusion

In conclusion, we found several potential activities of the various components of the *Crotalus durissus* venom. Studies using bioactive natural substances are an excellent choice for the emergence of possible tools with physiopharmacological potential. Thus, snake venom and their components are widely tested. It is currently confirmed their potential antitumoral, bactericidal and leishmanicidal effects; activation of phagocytosis processes in viral infections; effect on hematological processes; biological action in the nervous system; and analgesic activity.

The venom of *Crotalus durissus* snake and subspecies of the Brazilian biodiversity, are of great value for the development of directed and advanced research lines focused on the application of the venom in several fields of biomedicine, but also for the scientific growth and the improvement of Brazilian research.

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