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# Biological characterization of the Amazon coral *Micrurus spixii* snake venom: Isolation of a new neurotoxic phospholipase $A_2$

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ABSTRACT

The *Micrurus* genus is the American representative of Elapidae family. *Micrurus spixii* is endemic of South America and northern states of Brazil. Elapidic venoms contain neurotoxins that promote curare-mimetic neuromuscular blockage. In this study, biochemical and functional characterizations of *M. spixii* crude venom were performed and a new neurotoxic phospholipase $A_2$ called MsPLA$_2$-I was isolated. *M. spixii* crude venom caused severe swelling in the legs of tested mice and significant release of creatine kinase (CK) showing its myotoxic activity. Leishmanicidal activity against *Leishmania amazonensis* ($IC_{50}$ 1.24 μg/mL) was also observed, along with antiplasmodial activity against *Plasmodium falciparum*, which are unprecedented for *Micrurus* venoms. MsPLA$_2$-I with a *Mr* 12,809.4 Da was isolated from the crude venom of *M. spixii*. The N-terminal sequencing of a fragment of 60 amino acids showed 80% similarity with another $PLA_2$ from *Micrurus altirostris*. This toxin and the crude venom showed phospholipase activity. In a mouse phrenic nerve-diaphragm preparation, *M. spixii* venom and MsPLA$_2$-I induced the blockage of both direct and indirect twitches. While the venom presented a pronounced myotoxic activity, MsPLA$_2$-I expressed a summation of neurotoxic activity. The results of this study make *M. spixii* crude venom promising compounds in the exploration of molecules with microbicidal potential.



## 1. Introduction

In the Americas, the snakes known as coral snakes or corals are those belonging to the genus *Leptomicrurus*, *Micrurus* and *Micrurroides*, which are representatives of the Elapidae family in this part of the world (Urdaneta et al., 2004; Dal Belo et al., 2005). This genus presents a greater variability of species in the areas surrounding the equator (Salazar et al., 2011). *Micrurus spixii* (Fig. 1A) is endemic in five countries of South America: Brazil, Colombia, Venezuela, Bolivia and Peru. In Brazil *M. spixii* is endemic in the states of Acre, Amazonas, Mato Grosso, Pará, Rondônia and the *Micrurus* genus has about 27 other species dispersed throughout the country (Bernarde and Gomes, 2012; Ciscotto et al., 2011) (Fig. 1B).

The genus *Micrurus* is responsible for 0.4% of the accidents and the mortality coefficient is 0.63% having relevant importance in Brazilian public health (Moraes et al., 2003). In this country, *Micrurus* bites of all the genera follow the general pattern for treatment in snakebite cases, consisting in the protocol of applying a hyperimmune heterologous anti-elapid serum (made from

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**Fig. 1.** (A) Juvenile specimen of *Micrurus spixii*, collected in the municipality Rolim de Moura, Rondônia, Brazil. (Photo courtesy of Cleópatra Caldeira). (B) Map of the hit area of *M. spixii*, in South America. The marked area corresponds to hits in Brazilian territory and in the countries surrounding Brazil (adapted from Kelly et al., 2009).

equine immunization). The pool used for this process is obtained exclusively from *Micrurus corallinus* and *Micrurus frontalis* venoms (Tanaka et al., 2010; Corrêa-Netto et al., 2011) neglecting other species of equal importance and national occurrence.

The venom of the *Micrurus* genus follows the elapidic pattern in its biological actions, and its curare-mimetic actions are the most important cause of muscle paralysis and damage to respiratory function (Da Silva Jr. et al., 1991). Preliminary studies have shown that there is occurrence of intense cardiotoxic and myotoxic activity (MacIsaac and Weis, 1971); these activities were also described later, showing that there is variability in the proteins which constitute these venoms (Cecchini et al., 2015). The range of actions and chemical compositions of venoms from the genus *Micrurus* is an adaptation to different habitats, which is an useful evolutionary tool for this species (Tanaka et al., 2010). This variation in proteins and actions of the venom would enable snakes to have a predator-prey type selectivity making their diet specialized (Bernarde, 2011; Moreira et al., 2010).

In *Micrurus* venom there is a considerable quantity of different toxins such as C-type lectins, natriuretic peptides precursors (NPP), L-amino acid oxidase (LAAO), metalloproteinases, Kunitz-type inhibitors, serine proteases, lysosomal acid lipase, hyaluronidase and alkaline phosphomonesterases (Corrêa-Netto et al., 2011; Leão et al., 2009). The main toxins presents in *Micrurus* venoms are α-neurotoxins 3FTx (three-finger toxins) and β-neurotoxins or phospholipase $A_2$ ($PLA_2$) with variable proportions in the proteome of poisons of various species being primarily responsible for toxicity (Castro et al., 2015). The $PLA_2$s are considered to be important toxins in snake venoms, especially in the Elapidae family.

In cases of accidents, $PLA_2$ acts directly causing changes in the permeability of cell membranes by cleaving the structural lipids at the 2-sn-phospholipid bond causing the release of lysophospholipids (Costa et al., 2008; Rossetto et al., 2006). Those toxins are responsible for neurotoxic, myotoxic, and edematogenic activities (Castro et al., 2015) and in some species of *Micrurus*, act on blood coagulation, play an important role in homeostatic changes triggered by the venoms (Cecchini et al., 2015). Therefore, the aim of this study is to promote the biochemical and functional characterization of crude venom from *M. spixii*, to purify and characterize one neurotoxic $PLA_2$, in addition to demonstrating the microbicidal potential of this venom species.

## 2. Material and methods

### 2.1. Venoms and animals

The lyophilized crude venom of *M. spixii* was provided by Serpentarium Bioagents Batatais (SP), and acquired with the company Perubiotech EIRL™ of Huanáco (Huanáco State, Peru). These samples were kept frozen in a domestic freezer at −20 °C. The use of mice is regulated and authorized by protocol nº 058/06-EAEC (UNESP-Botucatu). This study was licensed by CGEN (010627/2011-1) and IBAMA (27131-2).

### 2.2. Functional characterization

#### 2.2.1. Phospholipase activity

A sample of 10 μL of *M. spixii* crude venom, a sample of 10 μL of *Bothrops jararacussu* crude venom and another containing the same volume of $MsPLA_2$-I were used. Both fractions were analyzed for phospholipase activity with a colorimetric reaction using as a reagent 5 mg of 4N3OBA (4-nitro-3-octanoyloxy benzoic acid) that was solubilized in 5 mL of acetonitrile as previously described by Petrovic et al. (2001). It was necessary to make buffer 1 (for phospholipase activity): 10 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$ pH 8.0. Then, the dry aliquot of 4N3OBA was solubilized in 2 mL of buffer 1, with vigorous stirring and kept protected from light until use. For activity, in a flat-bottom plate 190 μL of 4N3OBA solution and 10 μL of sample were added to $MsPLA_2$-I, with an initial concentration of 200 μg/mL. Immediately, the plates were introduced in the thermal spectrophotometer stabilized at 37 °C, and the optical density (OD) was determined to be 425 nm at 1 min kinetic

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