ARTIGO/ARTICLE

Experimental ophitoxemia produced by the opisthoglyphous lora snake (*Philodryas olfersii*) venom

Ofitoxemia experimental produzida pelo veneno da serpente opistoglifa lora (*Philodryas olfersii*)

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ABSTRACT

Several colubrid snakes produce venomous oral secretions. In this work, the venom collected from Venezuelan opisthoglyphous (rear-fanged) Philodryas olfersii *snake was studied. Different proteins were present in its venom and they were characterized by 20% SDS-PAGE protein electrophoresis. The secretion exhibited proteolytic (gelatinase) activity, which was partially purified on a chromatography ionic exchange mono Q2 column. Additionally, the haemorrhagic activity of* Philodryas olfersii *venom on chicken embryos, mouse skin and peritoneum was demonstrated. Neurotoxic symptoms were demonstrated in mice inoculated with* Philodryas olfersii *venom. In conclusion,* Philodryas olfersii *venom showed proteolytic, haemorrhagic, and neurotoxic activities, thus increasing the interest in the high toxic action of* Philodryas *venom.*

Key-words: *Colubridae. Haemorrhage. Neurotoxic.* Philodryas olfersii. *Proteolytic activity. Venom.*

RESUMO

Várias serpentes da família Colubridae produzem secreções orais venenosas. Neste trabalho, foi estudado o veneno coletado da presa posterior da serpente opistóglifa venezuelana Philodryas olfersii. *Deferentes proteínas estavam presentes no veneno, sendo caracterizadas pela eletroforese de proteínas (SDS-PAGE) a 20%. A secreção mostrou atividade proteolítica (gelatinase) a qual foi parcialmente purificada em uma coluna de intercâmbio iônico (mono Q2). Adicionalmente, a atividade hemorrágica do veneno de* Philodryas olfersii *foi demonstrada em embriões de galinha, pele e peritônio de rato. Os sintomas neurológicos foram demonstrados em camundongos inoculados com veneno de* Philodryas olfersii. *Em conclusão, o veneno da* Philodryas olfersii *mostrou atividade proteolítica, hemorrágica, e neurotóxica, assim aumentando o interesse na elevada ação tóxica do veneno da* Philodryas olfersii.

Palavras-chaves: *Colubridae. Hemorragia. Neurotoxinas.* Philodryas olfersii. *Atividade proteolítica. Veneno.*

Philodryas genus has 16 described species[8], of which five (*baroni, chamissonis, olfersii, patagoniensis* and *viridissimus*) have been reported to cause human envenomation[7,15,21,25]. In an eight year study (1982-1990), of the 43 patients admitted to the Instituto Butantan, São Paulo, Brazil, diagnosed with *P. olfe*rsii bites, the most common clinical characteristics were local pain (37.2%), swelling (34.9%), erythema (18.6%) and ecchymosis (9.3%). The blood clotting test was performed on 11 patients and in all of them the blood was coagulable[24]. Other authors[3] have reported cases of human envenoming by *Philodryas*, showing evidence of the clinical aspects and the evolution of the symptoms of envenoming. They called attention to the similarities of these cases with those caused by the *Bothrops* genus, suggesting a more careful evaluation of the victims. In spite of these human accidents, comparatively modest experimental consideration has been given to the toxic properties of the venom produced by these opisthoglyphous snakes. This secretion contain a mixture of enzymes[6] that break down cellular organization and obstruct critical functions, such as aeration, the conduction of nervous signals and blood circulation. Analyses of some of these secretions have shown that enzymes such as phospholipase A

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and L-amino acid oxidase are not rare in the colubrid secretions studied[27]. The main aim of this study was to generate information regarding Colubridae *Philodryas olfersii* venom, thus increasing the interest in the enzymatic activities present in the toxins of this snake venom and alert physicians that this colubrid could present a serious problem for human health.

MATERIAL AND METHODS

Animals. Albino Swiss NIH strain male mice weighing 18-22g maintained under laboratory conditions and obtained from the National Institute of Hygiene "Rafael Rangel" were used. The investigation complied with the norms taken from the guide *Principles of laboratory animal care*[2].

Snake captures were carried out during evening and crepuscular tours (without transect delimitations), in different geographical Venezuelan environments, with a high emphasis on those areas of interest for the study (San Juan de los Morros, Guárico state, Venezuela), where there were museum references of opisthoglyphous snake (*Philodryas olfersii*) incidences.

Venom. Venom was collected in a 50mL plastic centrifugal tube transversely cut and covered on the top with parafilm. The snake was obliged to bite the parafilm with its opisthoglyphous fangs. The venom was milked with a capillary tube and immediately frozen in liquid nitrogen. From each milking, approximately 0.3mL of secretion was obtained.

Protein determination. The protein determination method followed that of Lowry et al[18].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis using a Dual Mini Slam Kit AE-6450 (Atto Corporation, Tokio, Japan) chamber was performed. SDS-PAGE was carried out according to the Laemmli method[16], using 20% gels under reducing conditions. Molecular weight markers (Bio-Rad) were run in parallel and the gels were stained with Coomassie Blue R-250. The *Philodryas olfersii* venom samples under analysis were dissolved at a proportion of 1:1 in the solubilizer solution: 0.5M Tris.HCl, pH 6.8, with 10% (w/v) SDS, 10% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol and 0.05% (w/v) bromo phenol blue, and heated at 100°C for 10 minutes. The molecular weight was determined by Multi-Analyst TM/ PC version 1.1 program (Bio-Rad).

Chromatographic analysis. *Philodryas olfersii* venom (20mgrs) was diluted to 1.0mL with 50mM Tris-HCl, buffer pH 7.0 and exposed to Mono Q2 column chromatography pre-equilibrated with the same buffer at 4ºC. The column was washed with three column volumes of equilibrating buffer at a flow rate of 1.0mL/min. Venom proteins were eluted with a gradient of 0-1M NaCl dissolved in 50mM Tris-HCl pH 7-9. The fraction size was 0.5mL. Protein elution was monitored at 280nm[1]. The eluting peaks were tested for proteolytic activity.

Haemorrhagic activity tested on chicken embryos. Embryonic hen eggs incubated at 37°C for five days were cleansed with 70% alcohol and the embryos extracted by breaking the eggshells. The embryos were placed on petri dishes and incubated at 37°C for three hours.

Circles of filter paper Watmann N° 2 of 3mm diameter were impregnated with 3µL (24µg) of venom and applied to the chicken embryo vitelline vein[22 26]. Circles soaked with 3µL (7.5µg) of *Bothrops venezuelensis* venom were used as a positive control and saline solution was used as a negative control.

Determination of haemorrhagic activity on mouse skin. *Philodryas olfersii* venom haemorrhagic activity was determined by a modification of Kondo's test[10 14]. One hundred microlitres of venom containing 5-50µg were injected intradermal into the abdominal skin of four male NIH Swiss albino mice. The skins were removed after six hours and the haemorrhagic spot diameters on the inside surfaces were measured[11]. Two diameters were registered for the haemorrhage spot by measuring the longest diameter of the spot and the diameter perpendicular to the first measurement. A minimal haemorrhagic dose (MHD) was taken as the end point and defined as the concentration of venom resulting in a 10mm haemorrhagic spot[5]. *Bothrops venezuelensis* venom (50µL of 5.6µg/20g of mouse weight) and saline solution were used as positive and negative controls, respectively.

Determination of haemorrhagic activity on mouse peritoneum. One hundred µL of *Philodryas olfersii* venom containing 28µg/20g of mouse weight were injected intraperitoneally into four male NIH Swiss albino mice. *Bothrops venezuelensis* venom (50µl of 5.6µg/20g of mouse weight) and saline solution were used as positive and negative controls, respectively.

Neurotoxic activity. To determine the neurological signs and symptoms which may be produced by *Philodryas olfersii* venom, six mice were subcutaneously injected with venom 0.1mL (5.6µg/20g of mouse weight) and clinically observed.

Gelatinase assay. An assay modified from Huang and Pérez[11 12] was used to test the gelatinase activity of *Philodryas olfersii* venom. The X-ray film was washed down with distilled water and incubated at 37°C for 45 min. After incubation, the film was dried completely and twenty five microlitres of crude venom, as well as ion fractions obtained by chromatography at dilutions of 1 to 128 (1.9mg/mL solution) were placed on a Kodak X-OMAT scientific imaging film with gelatine coating. Hydrolysis of the gelatine on the X-ray film was determined after four hours of incubation at 37°C in a humid incubator by washing the film with distilled water.

Serial dilutions were performed to determine the minimum amount of venom required to cause a clear spot on the X-ray film. The titre was defined as the reciprocal of the highest dilution that caused a clear spot on the X-ray film. The specific gelatinase activity was calculated by dividing the titre by the amount of protein (µg) applied to the film. The assay was repeated three times.

RESULTS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The SDS-PAGE proteins present in *Philodryas olfersii* venom are shown in Figure 1. The relative masses were determined using the Multi-Analyst TM/PC version 1.1 (Bio-Rad) programs.

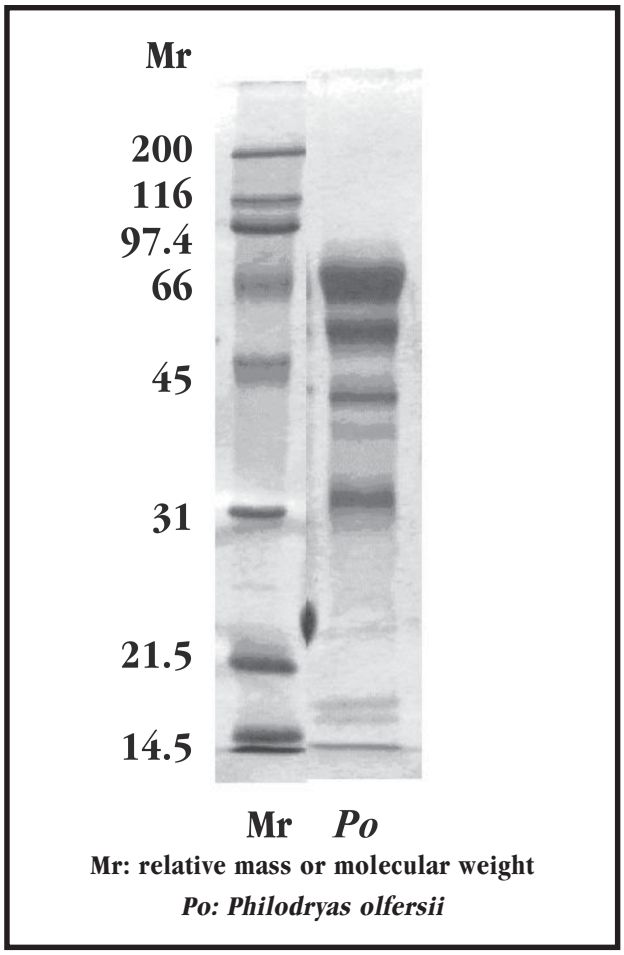

Figure 1- **Philodryas olfersii** ***venom run in a 20% polyacrylamide gel electrophoreses (SDS-PAGE)***

Ionic interchange chromatography. *Philodryas olfersii* venom run on a Mono Q2 column chromatography produced nine peaks (Figure 2). The eluting peaks were tested for proteolytic activity.

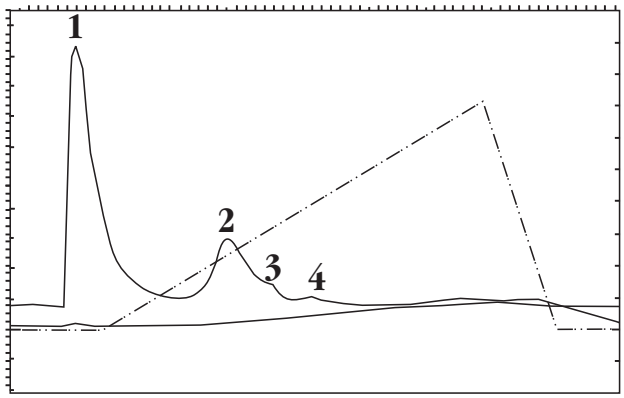

Figure 2- Separation of **Philodryas olfersii** ***venom fractions by ion-exchange chromatography on a MonoQ2 column. VENOM (20mg) was dissolved in 1.0 ml of 50mM Tris-HCl buffer, pH 7.0, and loaded on a column equilibrated with the same buffer. After elution of unbound material, a lineal NaCl gradient from 0-1M pH 7-9 was developed. Peaks 1, 2 and 4 showed proteolytic activity.***

Haemorrhagic activity tested on chicken embryos. Figure 3 shows the haemorrhagic activity of crude *Philodryas olfersii* venom on the chicken embryo vitelline vein. An obvious vascular blood extravasation was observed. Saline solution negative control and *Bothrops venezuelensis* venom positive control were also demonstrated.

Determination of haemorrhagic activity on mouse skin. *Philodryas olfersii* venom showed haemorrhagic activity when tested by intradermal injections in mice (Figure 4). The minimum haemorrhagic dose was 24μg, indicating that this venom was less active than crude *Bothrops venezuelensis* positive control venom (MHD= 5.6μg).

Determination of haemorrhagic activity on mouse peritoneum. All mice intraperitoneally injected with *Philodryas olfersii* venom showed intense haemorrhagic activity; saline solution negative control and *Bothrops venezuelensis* venom positive control were also determined (Figure 5).

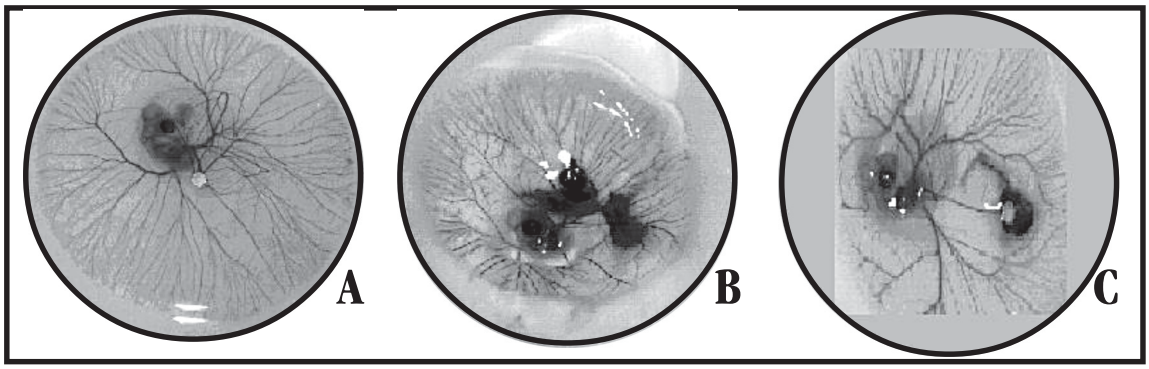

Figure 3- **Philodryas olfersii** ***venom haemorrhagic activity in chicken embryos. A: Negative control (Saline solution); B: Positive control (*** **Bothrops venezuelensis** ***venom); C:*** **Philodryas olfersii** ***venom.***

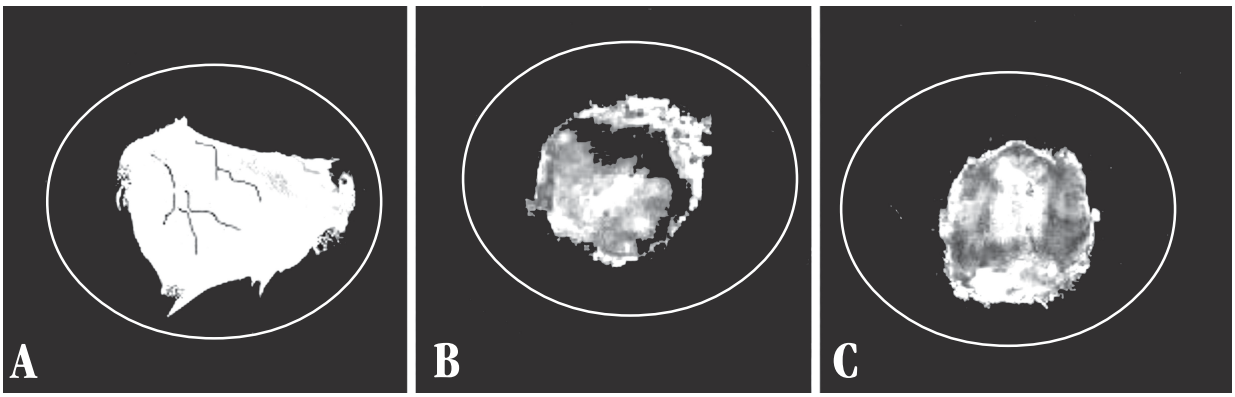

Figure 4*- Philodryas olfersii *venom haemorrhagic activity on mice skin. A: Negative control (Saline solution); B: Positive control (*Bothrops venezuelensis *venom); C:* Philodryas olfersii *venom.

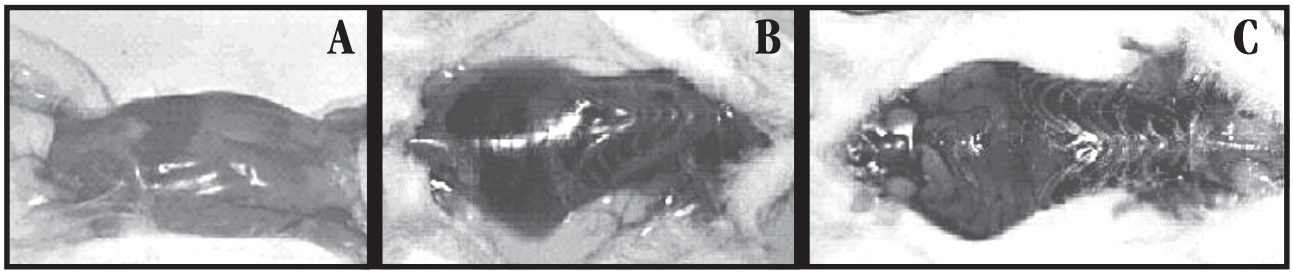

Figure 5*- Philodryas olfersii *venom haemorrhagic activity in the peritoneum. A: Negative control (Saline solution); B: Positive control (*Bothrops venezuelensis *venom); C:* Philodryas olfersii *venom.

Proteolytic (gelatinase) activity. The peaks obtained by chromatography and the secretion from crude venom were set on X-ray film showing the proteolytic activity of the crude venom up to dilutions of 1:128. Chromatography corresponding to peaks at P1, P2 and P4 demonstrated proteolytic activity: peak P1 up to dilutions of 1:128; P2 up to dilutions of 1:8 and P4 up to dilutions of 1:2.

Neurotoxic activity. *Philodryas olfersii* venom neurotoxic activity was demonstrated by observing several neurological manifestations of six mice subcutaneously injected develop during the experimental period (Table 1).

Table 1 - The results represent a pool of signs and symptoms of neurotoxic activity from 6 mice intraperitoneally inoculated with* Philodryas olfersii *venom.

Time (min)	Dyspnea	Equilibrium alterations	Posterior limbs paralysis	Flaccid paralysis	Urinary sphincter relaxation	Time of death
2		1s, 3s				
6		5s, 4s,6s	2s,4s,6s			
8			1s	2s		
15	2s,5s		5s	5s	2s,5s	2s
20						5s
23						
33	3s,4s			3s,4s		
53	1s,6s			1s	3s,4s	
63				6s	1s	3s,4s
68						1s,6s

s: represents the mouse specimen.

DISCUSSION

The biological role of neurotoxicity in *P. olfersii* is unknown, as is the case with most Colubridae. Nevertheless, neurotoxic activity in venoms is most reliable in the role of killing or debilitating a victim, rather than for digestion or some other purpose[9].

The different effects of *Philodryas olfersii* venom were studied *in vivo* in mice and chicken embryos, as well as on Kodak film with gelatine coating. Chicken embryos were used to measure haemorrhagic activity and antivenin efficiency in Viperidae and Elapidae venom[26]. This is the first time that this method has been incorporated in *Philodryas* venom studies, demonstrating its high haemorrhagic activity. In Venezuela, only a few authors[25] have described a number of electrophoretic and biological characteristics of *Philodryas viridissimus* venom. These authors found that *P. viridissimus* venom fractions shared the same molecular weight with some *Crotalus* venom fractions and they corresponded to proteolytic venom enzymes already described. In the current work, peaks 1, 2, 3 and 4 from *Philodryas olfersii* obtained by ion interchange chromatography, demonstrated a strong gelatinase activity caused by at least 3 peaks, peak 1 presented the highest activity (1:128 dilutions). Other researchers[4,5,23] make reference to the strong proteolytic activity of *Philodryas olfersii* and likewise refer to the fibrin(ogen)olytic activities and myotoxic effects, apparently caused by at least one other component of the venom.

In the present work using mice, the peritoneum and skin haemorrhagic activity produced by *Philodryas olfersii* venom was also clearly shown, indicating that this Colubridae possess a very strong haemorrhagic action on mammalian tissues. The haemorrhage seen in the vitelline vein of chicken embryos also indicated the strong proteolytic activity on the vasculature. The nature of this and other components from the venom of colubrids that cause similar effects remain unknown. Assakura et al[4] described a partially purified (HPLC) *Philodryas olfersii* venom haemorrhagic enzyme producing large haemorrhagic areas.

In the neurological assay, it was observed that *Philodryas olfersii* venom produced several neurotoxic symptoms in the inoculated mice. To the best of our knowledge, this is the first time that these different *in vivo* neurological signs and symptoms produced by *Philodryas olfersii* venom have been described. In a review of the literature, only one work describing the activity on the neuromuscular junction[23] was found. The most notable and initial activities were the equilibrium disorders, posterior limbs paralysis and flaccid paralysis, which occurred between two to six minutes after venom injection. Death, probably caused by respiratory paralysis, took place 15 min after venom injection.

For a long time opisthoglyphous snakes have been neglected by most physicians, since it was believed that their venom was of little importance and ineffective on humans, even though reports of human death envenomed by these *harmless* species have been published[17 19]. Many authors defined this venom as a Duvernoy´s gland secretion, but this term no longer applies to the colubrid families, as the glands were shown to be the same venom gland found in Atractaspididae, Elapidae and Viperidae[9].

The results obtained here regarding haemorrhagic, neurotoxic and proteolytic activities were much more convincing than expected. This should serve as a warning to medical specialists regarding the toxic potential that these species possess[13 22].

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