BIODIVERSITY DIVERSITY OF ACTIVE TOXINS AND PROTEOMIC ANALYSIS OF LANSBERG'S MAPANARE (*Porthidium lansbergii hutmanni*) VENOM SNAKE AND ITS IMPACT ON ENVENOMING

Diversidad Boquímica De Las Toxinas Activas Y El Análisis Proteómico Del Veneno De La Serpiente Mapanare De Lansberg (Porthidium Lansbergii Hutmanni) Y Su Impacto En El Envenenamiento

María Eugenia Pineda[1], Alba Marlene Vargas[1] and Alexis Rodríguez-Acosta[2]*

[1]Unidad de Biotecnología de la Facultad de Farmacia, Universidad Central de Venezuela, Caracas, Venezuela. mariupinex@gmail.com; avapino1104@gmail.com[2]Laboratorio de Inmunoquímica y Ultraestructura del Instituto Anatómico, Universidad Central de Venezuela, Caracas, Venezuela.rodriguezacosta1946@yahoo.es Immunochemistry and Ultrastructural Laboratory of the Anatomical Institute, Universidad Central de Venezuela, Caracas, Venezuela. Email: rodriguezacosta1946@yahoo.es

ABSTRACT

The venom characterization of Lansberg's hognose viper (*Porthidium lansbergii hutmanni*) was described using a combination of SDS-PAGE are 1 and 2d electrophoresis, MALDI TOF/TOF and tandem mass spectrometry (LC-MS / MS), as well as some proteolytic activities. This study shown the existence of metallo and serine proteases, cysteine-rich proteins (CRISPs), and L-amino acid oxidases (LAAOs), which appeared in variable amounts. It was also described, for the first time in this venom, the presence of the glutaminil-cyclase (vQC) that had been only before observed in species of *Crotalus atrox, C. godmani, C. d. terrificus* and *M. fulvius* of the American continent, but not in *Porthidium* genus. The presence of toxins with a gelatinolytic and caseinolytic activity was also defined. The crude venoms are excellent sources of enzymatic and non-enzymatic actions, which should be purified and characterized with the aim of their possible use as therapeutic agents, in antitumor and haemostatic treatments.

Key words: Lethality; MALDI-TOF/TOF; *Porthidium lansbergii hutmanni;* proteolytic; proteomic; LC-MS/MS; venom

RESUMEN

La caracterización del veneno del *Porthidium lansbergii hutmanni* se realizó utilizando una combinación de electroforesis (SDS-PAGE) de una y dos dimensiones, por MALDI TOF / TOF y espectrometría de masas en tándem (LC-MS/MS), así como algunas actividades proteolíticas usando caseína y gelatina. Este estudio reveló la existencia de metalo y serino proteasas, proteínas ricas en cisteína (CRISP) y L-aminoacido oxidasas (LAAOs), que aparecieron en cantidades variables. También se describió, por primera vez en este veneno, la presencia de la glutaminilciclasa (vQC) que solo se había observado anteriormente en especies de *Crotalus atrox*, *C. godmani*, *C. durissus terrificus* y *M. fulvius* del continente americano, pero no en el género *Porthidium*. También se definió la presencia de toxinas con actividades gelatinolítica y caseinolítica. Los venenos crudos son una excelente fuente de acciones enzimáticas y no enzimáticas, que deben ser purificadas y caracterizadas con el objetivo de su posible uso como agentes terapéuticos, en tratamientos antitumorales y hemostáticos.

Palabras clave: Letalidad; MALDI-TOF/TOF; *Porthidium lansbergii hutmanni;* proteólisis; proteómica; LC-MS/MS; veneno

Recibido: 08/01/2019 Aceptado: 01/07/2019

INTRODUCTION

Lansberg's hognose viper (*Porthidium lansbergii hutmanni*) (*P.l.h*) snakebite is an important medical condition in Margarita and Coche Islands (Nueva Esparta State, Venezuela) [17]. This venom experimentally produces oedema, necrosis, bleeding (skin bruising, gastrointestinal haemorrhage and haematuria) and lymphatic vessel damage, with degradation of extracellular matrix effects [17, 43]. The development of *P.l.h* snakebite has been roughly studied, and the basic pathogenic mechanism of this envenomation remains an open question. It has been generally assumed that haemostatic complications were linked to serine and metalloproteases activities, but this venom is a cocktail of toxins with various enzymatic and non-enzymatic molecules, which are responsible for the numerous symptoms and signs that express the patients, which are caused by the pathophysiological venom action. Therefore, it is helpful to explore by proteomics methods the *P.l.h* venom to find the *P.l.h* snakebite-associated biomarkers for addressing the mechanism of this envenomation. A comprehensive view offered by general proteomics can provide novel insights into the etiology of *P.l.h* snakebite and identify new opportunities for the prediction, prevention, and treatment of this accident. New findings in mass spectrometry technologies have transformed the global vision of the measurement of isolated molecules, peptides, or total proteins up to a broad analysis of all the protein components that make up cellular cells and how they relate and function orchestrated. Mass spectrometry has become a methodology that answers many analytical questions, in order to try to dissect the molecular complexity of biological entities [4,7].

In the present work, it was initiated a proteome and mass spectrometry technologies studies from samples of *P.l.h,* venom using SDS-PAGE and 2 dimensions (2D) electrophoresis, MALDI/TOF/TOF and tandem mass spectrometry (LC-MS/MS), as well as particular proteolytic activities, in order to isolate and characterize toxins that have relevant activities, which could be useful in medical therapy, such as coagulation and cancer disorders.

MATERIALS AND METHODS

Reagents

Electrophoresis: Reagents (BIO-RAD, USA), IPG Strips pH 3-10, 11 cm (BIO-RAD, USA); casein (Merck and Riedel de Haen, Germany); Immunoblotting: Equine peroxidase-coupled-equine IgG antibody (Santa Cruz Biotechnology, CA, USA), Nitrocellulose membrane (BIO-RAD, USA), SuperSignal West Pico® chemiluminescence development kit (ThermoScientific, USA); MALDI-TOF/TOF: α-Cyano-4-hydroxycinnamic acid matrix (α-CHCA) (SIGMA, Mo, USA), Acetonitrile, Trifluoroacetic Acid and diethyl ether (SIGMA, Mo, USA); LC-MS / MS: OFFGEL RoomTemp HighRes® Kit (Agilent Technologies, USA), IEF pH 3-10 24 centimetres (cm) strips (GE Healthcare, USA), Swine Trypsin (PROMEGA), Electro spray calibrate solution 63606 and Calibration Tune Mix ESI (SIGMA-Fluka, USA); working solutions: reagents of high purity ≥98% (Merck and Riedel de Haen, Germany).

Software

For the statistical analyses, the Prism® program (GraphPad, Software) [60] was used; for the one dimension gels analysis, QuantityOne® (BIO-RAD, USA) software was utilised; while for the two-dimensional gels electrophoresis analysis, the PDQuest® program (BIO-RAD) was employed [48].

In the MALDI/TOF mass spectrometry experiments, Compass 1.2 SR1 for Flex Analysis (BrukerDaltonics) software was used. The LC-MS/MS analyses the Compass 1.2 SR1 program for Microtof / Maxis® (Bruker-Daltonics) was utilised [9].

Experimental animals

Male mice (*Mus musculus*) (NIH strain) weighing 20 to 22 grams (g) were purchased from the National Institute of Hygiene "Rafael Rangel" (Caracas, Venezuela) animal facility. The mice were kept in cages under room temperature conditions, twelve hours (h) natural light with water and food *ad libitum* until used.

Ethical statement

Trained staff prepared all the experimental methods relating to the use of live animals. Applicable regulations as well as institutional guidelines, according to protocols approved by the Institute of Anatomy of the Universidad Central de Venezuela Ethical Committee, following the norms obtained from the guidelines for the care and use of laboratory animals, published by the US National Institute of Health [38].

Venom

Porthidium l. hutmanni (*P.l.h*) venom was obtained by manual milking of 11 adult specimens of both sexes, from Margarita Island, Nueva Esparta State (Venezuela), geographically located at 10º 51 '50 "- 11º 11' 06" NL 63º 46 '40 "- 64º 24' 32" WL. The island has ~ 1150 square kilometres (km^2) (115.000 hectares (Ht) of territory (FIG.1), which represents 0.13% of the national land. The *P.l.h* snakes have been trapped throughout the insular territory and the environment where it lives has particular physical and natural characteristics. The climate has produced a mixture of ecological areas from the tropical desert brushwood, in the low and dry zones, with a wide range of altitudinal floors of mountains, and tropical dry forest, roofed by the trade winds ("vientos aliseos" [2]. The area of the island with greater precipitation corresponds to the piedmont zone ("Cerro Copey"), which is a premontane humid forest, with average annual temperatures of 28^0C and an atmospheric humidity of around 50%. The annual rainfall is less than 500 millimetres (mm). The few rains cause superficial and underground water resources to be scarce, because in spite of the existence of geological conditions conducive to their storage,

the recharge is not significant [41]. All snakes were taken to the Research Laboratory Serpentarium, Faculty of Pharmacy of the Universidad Central de Venezuela. The snakes were milked upon reaching the Serpentarium, after their adaptation for a week (w). The venom, once obtained, was crystallized under vacuum in a desiccator containing $CaCl_2$ as a desiccant, and maintained at 4 ° C (Frigidaire FGVU21F8QF Vertical Freezer, USA), until its subsequent use.

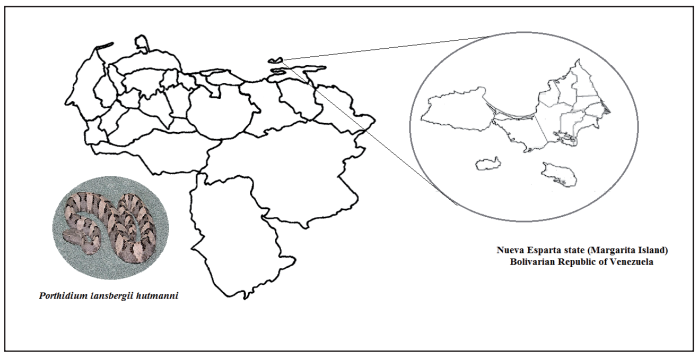

FIGURE 1. *Porthidium lansbergii hutmanni* GEOGRAPHICAL DISTRIBUTION IN MARGARITA ISLAND (VENEZUELA)

Protein determination

The protein venom content was determined by Lowry *et al.* method [30].

Lethality determination

Five groups containing 5 mice weighing 18–22 g were used to determine the LD_{50} for *P.l.h* venom. The mice were intraperitoneally injected with 200 microlitres (µL) of serially diluted sample ranging from 17.33 to 132 micrograms (µg). Deaths during a 48-h period were recorded. The LD_{50} was calculated by the Spearman-Kärber method [49].

Proteomic characterization of the *P.l.h* venom

SDS-PAGE analysis of venom

Twelve percent (12%) of polyacrylamide gel electrophoresis (SDS-PAGE) were carried out following Laemmli. [26] method. Briefly, *P.l.h* venom sample was dissolved at a concentration of 5 µg/µL in a protease inhibitors cocktail, composed of 4-(2-aminoethyl)-benzene-sulphonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin and disodium EDTA and then diluted to the optimum concentration for visualization (venom: 2µg/µL), in 0.5 M Tris-HCl buffer, pH 6.8, 10% SDS, 1% glycerol and 0.02% bromophenol blue. A 5 µL aliquot of the sample and 5 µL of pre-stained markers of wide molecular weight range were distributed into the gel. The samples were subjected to the electric constant current of 100 volts (V) until complete the run (about 120 min). Afterward, the relevant gels selected for staining were placed in a solution containing 12% volume/volume (v/v) phosphoric acid, 10% weight/volume (w/v) ammonium sulphate, 20% v/v methanol and 0.12% w/v Coomassie G-250 blue, according to Blue Silver staining protocol [8], which has a sensitivity of 1 nanogram (ng) per band. Then, the gels were washed with deionized water to remove excess dye, and digitised by densitometry (Densitometer GS-900™ Bio-Rad, USA). Each experiment was performed in duplicate.

Two-dimensional (2D) electrophoresis of *P.l.h* venom

In order to have the proteomic map of the *P.l.h* venom, two-dimensional (2D) electrophoresis was performed. The analysis of the venom was carried out in duplicate to obtain two gels per sample. The methodology consisted in the dissolution of 100 µg of *P.l.h* venom, in 50 µL of protease inhibitors cocktail. In turn, 150 µL of 8M Urea, 2M Tiourea, 4% CHAPS, 20 millimolar (mM) DTT and 0.5% v/v of a commercial ampholyte mixture were added. The resulting solution was placed on a commercial strip that contained immobilized on its surface, a linear pH gradient comprised between pH 3 and pH 10, which were used for the determinations. After electrophoresis, one lane was removed from the unstained gel, and incubated in SDS sample buffer containing 3% 2-ME for 30 min. This treated gel strip was then relocated to the top of a second 12.5% SDS gel, before electrophoresis in the second dimension. The obtained gels were stained with Blue Silver [8]. They were digitised by densitometry, and the images obtained were analysed, in order to estimate the isoelectric point and the molecular weights of the visualised spots.

Those spots visualized in the gels, but not in all of the gel that formed the experiment, were taken into account for the results of this work. The differences between the intensity of the spots were not compared, i.e. the analysis was only performed from a qualitative perspective.

Proteomic analysis by Matrix-Assisted Laser (MALDI TOF TOF) of *P.l.h* venom

This assay was carried out in the Proteomics Unit, Structural Biology Centre, Instituto Venezolano de Investigaciones Científicas (IVIC). For the *P.l.h* venom analysis by mass spectrometry (MS) [32], the proteins venom was initially separated by 10% SDS-PAGE electrophoresis under reducing conditions. Then, the gel was stained with Coomassie blue staining. After the bands visualization, small portions of them were cut and each of these fragments was distained with 30% acetonitrile (ACN) and 250mM ammonium bicarbonate. At that point, they were dehydrated with aqueous solution at 90 % of ACN and subsequent digestion of proteins, by incubation of the gel fragments at 37°C for 18 h, covering them with a solution of 12.5 ng/µL trypsin in 50mM ammonium bicarbonate. The triptych peptides obtained were concentrated and purified by using ZIP-TIP C18® tips, using an aqueous solution containing 60% ACN and 1% formic acid as eluent. The ionization of the samples for mass spectrometry analysis was carried out by MALDI (Matrix-

Assisted Laser Desorption / Ionization). Each sample was mixed in a 2: 1 ratio with a suspension of α-Cyano-4-hydroxycinnamic acid (α-CHCA), prepared in 50% ACN and 0.05% trifluoroacetic acid (TFA) and allowed to dry over a MALDI plate, at room temperature until their complete crystallisation.

The analysis by mass spectrometry was performed on a spectrometer equipped with a TOF/TOF (Time-Of-Flight/ Time-Of-Flight) type analyser, operated in a reflectron mode. The peptides MS/MS analysis that generated the most intense signal was carried out using the MASCOT program (MS/MS Ion Search®, MatrixScience) [32] with the following parameters: Taxonomy: AllEntries, Variable modifications: oxidation (M), deamidated (NQ), Fixed Modifications: propionamited (C); against the database of the NCBI (National Center for Biotechnology Information, USA). In addition, the peptide alignment sequences obtained through the manual interpretation of the spectra was performed, with non-redundant sequences through the BLAST® program (NCBI), considered as valid, those results with an E-Value <1E-04.

Proteomic analysis by liquid chromatography coupled to Tandem Mass Spectrometry (LC-MS / MS) of *P.I.h* venom

In order to obtain more proteomic information *P.I.h* venom was subjected to liquid chromatography, coupled to tandem mass spectrometry (MS/MS). Briefly, 100 microgram (ug)/100 microliters (uL) of venom was incubated for 15 minutes (min) with 5 miliMol (mM) DTT and subsequently for another 15 min with 10mM iodoacetamide. Afterward, the sample was incubated at 37°C with trypsin in a 1:40 ratio enzyme: substrate. The obtained sample composed of a mixture of triptych peptides, was placed in a fractionator (3100 OFFGEL Fractionator, Agilent, USA), for the fractionation of these peptides according to their isoelectric point. The products obtained were recovered in 24 liquid phase fractions of 150µL each. A linear fixed gradient strip of pH 3-10 of 24 centimeters (cm) was used. During the process, a voltage/ hour ratio of 50 kilovolts (kV) was reached at a 4500 V. Current: 50 microAmpere (uA) and Power: 200 microWatts (mW). Then, the fractions obtained were injected into a high performance chromatographic system, equipped with a C18 reverse phase precolumn (5 µm and 2 cm in length) and a C18 chromatographic column (5 µm and 10 cm in length). A linear gradient was established between a solution Phase A: composed of 0.1% formic acid in deionized water and a solution Phase B: composed of 0.1% formic acid in ACN. This gradient was increasingly established from 0% from Phase B to 80% from Phase B in 60 min. Each of the eluates continued through an interface coupled to a tandem mass analyser (MS/MS) time of flight-quadrupole. The ionization of the peptides was performed by ESI (Electrospray ionization) with voltage in the capillary of -4500V, Gas flow desolvation: 2L/ min, Gas nebulizer: 5.8 psi and temperature: 160ºC. The mass range analysed was 50-3000 Da/z. Calibration was carried out using a commercial calibrator solution. The analysis and searches of the obtained monoisotopic masses for the peptide fragments were carried out according to MALDI TOF/TOF.

Evaluation of the proteolytic activity of *P. l. h* venom

Determination of proteolytic activity on gelatine

The methodology proposed by Terra *et al*. [53], with minor modifications was followed. Discontinuous 12.5% polyacrylamide gels copolymerized with 1% gelatine were run. The *P.l.h* venom and samples were prepared at a concentration of 2 µg/µL in 0.5 M Tris buffer, pH 6.8, 10% SDS, 1% glycerol and 0.02% bromophenol blue, placing 3µL of each sample in the gel. In addition, as positive control, 1 µL (2 µg/µL) of *Bothrops colombiensis* venom was added. Then, after electrophoresis, the gel was equilibrated in a 2.5% Triton X-100 solution, under stirring for 1 h at room temperature, washed with double distilled water (two washes of 10 min each) and incubated at 37°C for 18 h, in a buffer solution Tris-HCl 20 mM pH 7.4, 150 mM NaCl, 5 mM CaCl2. The proteolytic activity on gelatine was evidenced by zones of degradation in the gel, generated by the gelatine staining with Coomasie R-250 Blue, which were presented as translucent areas on a blue background.

Determination of proteolytic activity on casein

Proteolytic activity was carried out using casein (40µg) as substrate. Briefly, 1mL of 1% casein in Tris HCl 0.2M buffer, pH 8.5 and (5 µg/0.05 mL) of *P.I.h* venom. The reaction mixture was incubated for 15 min at 37^{0}C, stopping the reaction with 0.44 M trichloroacetic acid (TCA) at 5^{0}C for 30 min. Then, the sample was centrifuged (Beckman Avanti 30, USA) at 5000xg for 10 min. The specific activity was measured by the formation of acidic products soluble at 280 nm, converting the values of optical density (OD) (280 nm) to µg of L-tyrosine measured at 660 nm 5 and expressed in units of activity/mg of protein.

Statistical analysis

In the case of differences between experimental groups, the comparison was made using one-way Analysis of Variance (ANOVA), using as a statistical test, the Dunett test considered as significant those results with an error probability P <0.01. Analysis was completed using SPSS version 2.0 [35]

RESULTS AND DISCUSSION

Proteomic approach has permitted far-reaching studies of protein expression in different venoms in isolated conditions and/ or time situations. New improvements of procedures in this area have initiated new opportunities to achieve applicable information on toxinology processes. In the current work, the focal proteomics techniques and their application to *P.I.h* venom analysis were studied.

One of the limitations of the MALDI-TOF system is the difficulty in detecting low molecular weight proteins, which because of this characteristic, generate few peptides (FIG.2). The system is also not able to detect more than one component of a mixture [9] that

is why it was combined several protein exploration methods to obtain better information.

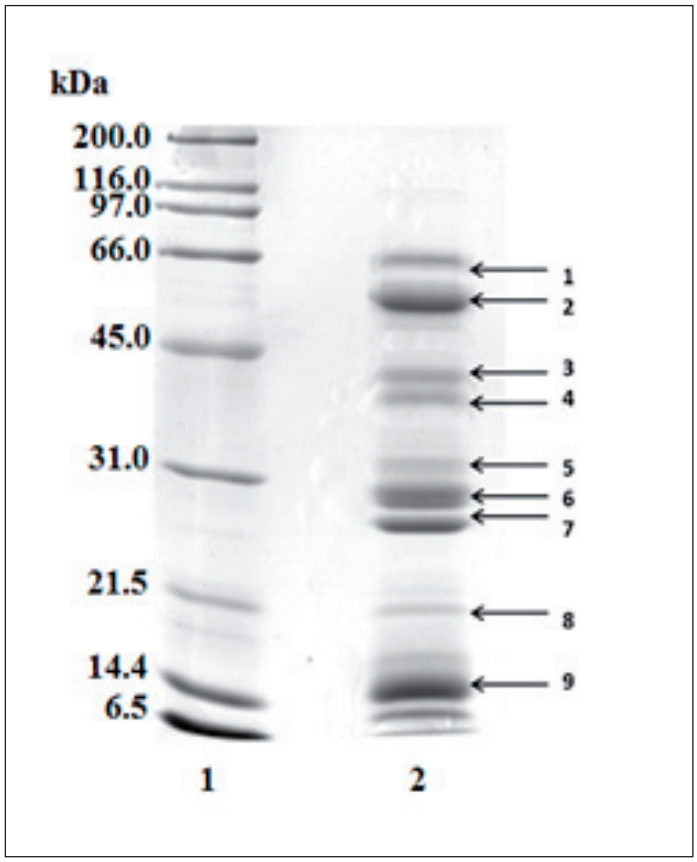

FIGURE 2. SDS-PAGE OF *P.l.h* UNDER REDUCING CONDITIONS FOR MALDI/TOF-TOF. The arrows indicate the cut bands for analysis by MALDI/TOF-TOF

Porthidium lansbergii hutmanni found in Margarita Island (Venezuela) is the most common venomous snake in this region [17]. Its venom is quite moderate as compared to the rattlesnake *Crotalus durissus cumanensis* described in the same areas [17]. The venoms of *Porthidium lansbergii hutmanni* and *Crotalus durissus cumanensis* lethality showed LD_{50} value of 2.51±0.16 mg/kg, respectively, on experimental animals inoculated intraperitoneally. In mammals, *Pl.h venom* can produces numerous pathological effects, such as local pain, haemorrhage, oedema, blistering, necrosis, lymphatic vessel damage and degradation of extracellular matrix [17]. Commonly, its envenomation is not considered a threat to an adult person because of its small size and the little amount of injected venom [17, 43]. However, *P.l.h* venom toxins cause muscles necrosis, the cascade coagulation activation or blockade of some of their factors. Tissue damage happens by endothelial cells injury, habitually produced by phospholipases A_2 and metalloproteases, interfering with homeostasis on diverse levels. Metalloproteases enzymes produce haemorrhage damaging of many constituents of vessel walls extracellular matrix proteins, such as laminin, nidogen, fibronectin, collagen type IV and proteoglycans from the endothelial basal membrane, besides cleavage of large proteins such as fibrinogen [22], favouring the dissemination of venom fractions through the cell membranes [14]. In this venom, phospholipases A_2 display myotoxic and haemotoxic activities, causing local and systemic degeneration of the skeletal muscles by hampering the integrity of the muscle cell membranes [17]. Haemostatic activity of phospholipases involves the inhibition of blood coagulation factor cascade [11] synergistically acting in conjunction with serine proteases, which effectively disturb the coagulation victim system, affecting components of blood coagulation system, fibrinolysis, and platelets function [41].

The venoms from Viperidae family description have showed contain up more than 100 proteins fit in restricted protein families [5]. When *P.l.h* venom was run on 2DE gels 101 spots were found. As soon as the identification using MALDI TOF/TOF was carried out, less than 5 protein families were established. It could be argued that these proteins are highly modified post-translationally, an occurrence frequently described for Asiatic and South American snakes of Viperidae family. This was evidenced by the clearly visible spot sequences in the gels [6,48,55].

For separation of *P.l.h* proteins by one dimension (1-DE) SDS-PAGE and two-dimensional (2-DE) methods, it was necessary to isolate the molecules from fresh crude venom that allowed having good electrophoretic results. The *P.l.h* proteins needed to be denatured, disaggregated, solubilized, and treated to reduce disulphide bridges. The SDS-PAGE of *P.l.h* venom showed several venom bands at locations in which the high molecular weight metalloproteases and L-amino acid oxidase (LAAO) are frequently described [31]. Here was also observed a larger presence of bands on metalloproteases and LAAO locations, more than phospholipases. Previously, the presence of these two groups in almost similar amounts had been reported [18,20,48]. FIG. 3 shows the *P.l.h* venom electrophoretic profiles. The distribution of protein bands occurred within gel regions corresponding to wide molecular weights range. In this venom 11 protein bands were evident. The high intensity corresponded to the ~ 60, 54, 29, 26 and 12 kDa molecular weights. The lower intensity bands corresponded to 45, 39, 36 and 20 kDa. In the high molecular weight region only two low intensity bands of 170 and 140 kDa were evident.

The two-dimensional electrophoresis was carried out with *P.l.h* crude venom, which was separated in two successive steps. The isoelectric focusing (IEF), where the molecules migrate on pH gradient polyacrylamide gel immobilized by amphoteric buffers to achieve a point (pH) where its charge was equivalent to zero (isoelectric point or pI). Then, the venom proteins were yield to an electrophoresis with perpendicular direction to IEF on polyacrylamide gel sodium sulphate electrophoresis (SDS-PAGE), being separated agreeing with its molecular weight. In the example of 2-DE gels, 101 spots (FIG. 4 and TABLE I), each enclosing one or more proteins, and various post-translational

TABLE I
MOLECULAR WEIGHTS AND ISOELECTRIC POINTS (IP) ANALYSIS OF THE TWO-DIMENSIONAL (2D) PROTEIN SPOTS IN A PROTEOMIC MAP OF *P.l.h* VENOM

Molecular weight	Isoelectric Point	Molecular weight	Isoelectric Point	Molecular weight	Isoelectric Point
10.67	5.33	34.27	6.62	64.89	7.82
10.69	5.97	34.31	5.65	65.30	7.24
10.75	5.60	34.33	5.43	65.40	7.37
10.76	5.13	34.49	6.38	65.67	7.10
11.12	9.99	34.81	7.70	65.99	7.54
11.82	8.70	34.83	7.34	66.35	6.99
12.90	9.96	35.29	6.61	66.48	7.70
16.17	6.89	35.33	6.38	66.60	7.22
16.72	6.51	35.46	8.33	66.75	7.99
21.93	4.88	35.49	6.15	66.76	8.09
29.67	6.10	35.58	6.78	66.95	6.89
29.69	6.36	35.70	8.75	67.43	7.09
29.69	5.87	35.94	5.82	67.94	7.37
29.72	6.58	35.95	5.48	68.12	7.23
29.78	5.53	35.95	5.64	68.35	7.00
29.79	4.93	35.97	5.30	68.57	7.55
29.84	5.23	36.08	6.14	72.57	5.60
29.87	6.78	39.51	6.40	72.71	5.80
31.60	6.10	43.25	8.49	72.90	5.51
31.83	6.34	45.12	6.81	73.01	5.70
32.32	6.09	45.26	7.16	73.24	8.39
32.46	5.85	45.27	9.98	73.31	8.18
32.54	6.34	45.34	6.98	73.33	8.63
32.63	5.65	46.11	10.00	73.69	9.90
32.66	4.99	47.02	8.98	74.39	8.00
32.75	6.58	47.22	8.46	74.70	8.63
33.60	5.89	49.05	10.00	74.82	8.39
33.64	5.47	51.90	10.00	74.90	8.02
33.65	6.90	56.39	7.58	74.93	8.19
33.66	5.68	63.74	7.37	75.06	7.37
34.19	6.90	63.82	8.12	75.30	9.90
34.21	5.25	64.30	3.00		

modifications were simply distinguished in the form of sequences of spots, aligned on the vertical or horizontal axis.

Then, in the gels using computer programs [35], the background material was taken away. The spots were compared and the data analysed for quantification of protein volumes or intensities. Regarding two-dimensional electrophoresis (2D), this was used to preliminary study the of *P.l.h* proteome. The *P.l.h* venom proteins isoelectric focusing and its subsequent run on polyacrylamide gel were achieved. Then, the two-dimensional proteomic venom map was obtained (FIG. 4). One hundred and one protein spots were showed in the experimental gels, distributed in a range between 10.67 and 75.30 kDa, which had predominance between ~ 30-40 kDa (42.10%) and 60-80 kDa (35.80%), and in smaller proportion in ~ 41-59 kDa (11.60%), 10-20 kDa (8.40%) and 21-29 kDa (1%) molecular weights. All proteins showed isoelectric points with values between 4.9 and 10, predominantly in the pI <7.5 range (66.31%). The location of these spots on the protein map of *P.l.h*

venom is illustrated in FIG. 4, while their molecular weights and isoelectric points are summarized in TABLE I. The combination of 2D and proteomic analysis by MALDI TOF/TOF examination of post-translational protein modification discovery was carried out. Additionally, the 2D assay for high-molecular mass protein examination was more appropriate [56].

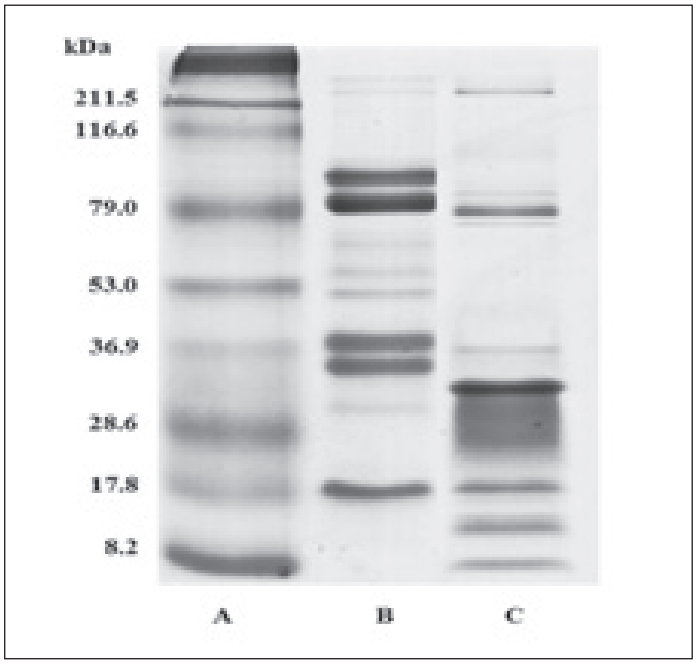

FIGURE 3. SDS-PAGE OF VENOMS. A) MOLECULAR WEIGHT MARKER; B) *P.L.H* VENOM; C) *B. colombiensis* Venom.

In the present work, proteomic analysis of *P.l.h* venom by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and MALDI TOF/ TOF showed that the metallo and serine proteases, cysteine-rich proteins (CRISPs)[44], LAAOs, and glutaminil-cyclase (vQC) occurred in variable amounts [TABLE II].

The 2D electrophoresis identified components fluctuating between 10.67 and 75.30 kDa, and molecular mass ranging from 8 to 116 kDa (FIG. 3). Several horizontal series of spots were observed. A total of ~ 101 spots were saved for analysis from diverse regions of the gel. TABLE III summarizes the results obtained from *P.l.h* venom by analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). During the analysis it was possible to identify several proteins, most of them corresponding to L-amino acid oxidases (LAAOs) forms, metallo and serine proteases, cysteine-rich proteins (CRISPs), and glutaminil-cyclase (vQC). The presence of a protein serine protease-like, with ability to induce platelet aggregation, and a protein rich in cysteine residues, which revealed similarity with a domain rich cysteine toxin called Piscivorin was also determined.

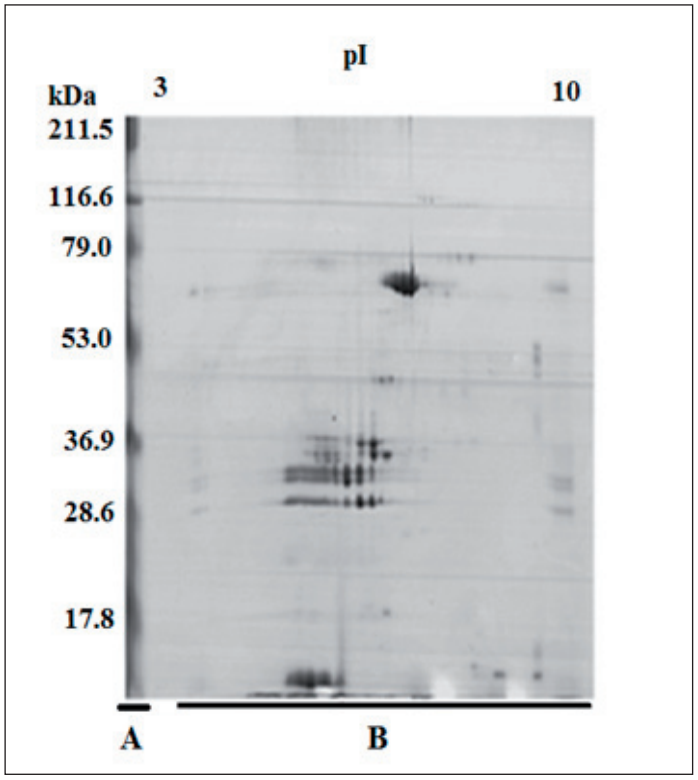

FIGURE 4. TWO-DIMENSIONAL GEL ELECTROPHORESIS IMAGE OF THE VENOM OF PORTHIDIUM. L. HUTMANNI VENOM (A) MOLECULAR WEIGHT MARKERS. (B) P.L.H VENOM. TWO HUNDRED MICROGRAMS WAS RUN ON AN ISOELECTRIC FOCUSING (IEF) (PH 3 TO 10) AND 12.5% SDS-PAGE (M/V), DEVELOPED BY COMASSIE BLUE STAINING.

The CRISPs are responsible for disturbing homeostasis through several mechanisms, counting inhibition or activation of some factors in blood coagulation and blocking ion channels [34, 58, 59]. These proteins also can produce a deadbolt of the calcium and potassium channels and thus, the inhibition of smooth and skeletal muscles contraction and alteration of sanguineous vessels [57].

Here, cysteine-rich venom proteins (CRISPs) exhibited low abundance in analysed samples. This protein had been previously identified in this genus [21]. CRISPs also have broadly distribution in the venoms of the most important snake's families (Viperidae, Elapidae and Colubridae) [13,57]. It is generally described in Viperidae as a single protein [43]. These snake venom secretory proteins are a group of proteins, which are capable of disrupting homeostasis through various mechanisms, including inhibiting or activating factors in blood coagulation, blocking ion channels and displaying neurotoxic activity [13,34,58].

L-amino acid oxidases (LAAOs) in *P.l.h* venom were evident in the SDS-PAGE (> 100 kDa), mass spectrometry LC-MS/MS and mass spectrometry MALDI/TOF-TOF. Crotalid venoms are the richest sources of L-amino acid oxidases with the

TABLE II
IDENTIFICATION OF THE *P.I.h* VENOM PROTEINS BY MASS SPECTROMETRY MALDI/TOF-TOF

Identification				**BLAST analysis**		
Bands	**Sequence identified peptide**	**Identified Protein**	**Taxonomy**	**Score BLAST**	**E value**	**% sequence**
2	R.ETDYEEFLEIAR.N	GI:347595686 GI:347602321 Y 34 more L-aminoacid oxidase	*Protobothrops flavoviridis* *Bothrops jararaca*	50.3	$3e^{-07}$	100
2	R.NDEEGWYANLGPMR.L	GI:82090465 GI:33355627 Y 51 more L-aminoacid oxidase	*Trimeresurus stejnegeri*	58.3	$9e^{-09}$	100
3	IYEIVNTLNVIYR	GI:82228618 GI:297593940 VLAIP-B Zinc-Metalloprotease	*Macrovipera lebetina* *Echis coloratus*	47.3	$3e^{-05}$	100
4	R.YIELVIVADHR.M	GI:308212508 GI:332321851 Y 94 more Jerdonitin Zinc-Metalloprotease	*Bothrops neuwiedi* *Crotalus viridis viridis*	47.7	$2e^{-05}$	100
6	R.NPEIQNEIIDLHNYLR.R	GI:46395675 and 58 more Tripurin Protein rich in cysteine	*Trimeresurus purpureo maculatus*	64.7	$4e^{-12}$	100

exception of some *C. scutulatus scutulatus* [52]. Concerning, *Porthidium.l.hutmanni* venom composition, it varies in its own family and individual snake venom, which contains more than one type of LAAOs [12,51]. They also have distinct molecular masses [12,32] and functions [37,54]. LAAOs can lead to apoptosis of vascular endothelial cells in some tumours by its ability of producing hydrogen peroxide during amino acid oxidation in catalysed reactions [54]. This group of proteins also influence platelet aggregation inducing haemorrhages [45].

In the current work, *P.I.h* venom also have demonstrated by mass spectrometry and SDS-PAGE that the prevalent cluster of proteins of *P.I.h* venom were the metalloproteases, which as is already recognised, it exists in high amounts in the Viperidae venom, but also they have been described in Elapidae and Colubridae families [27,36]. The description of Snake Venom Metalloproteases (SVMPs) proposed three basic groups (from P-I to P-III) established on their protein domain [14]. The spectrum of activity of these enzymes is very wide, but all are directly or indirectly involved in the haemostasis disorders. In this venom were seen metalloproteases belonging to all three groups, some of them may be implicated in haemorrhagic effects, fibrinogenolysis, inhibition of platelet aggregation, activation of prothrombin and factor X, and acting on inhibitors of serine proteases [14,36,45],

TABLE III
IDENTIFICATION OF *P.L.H VENOM* PROTEINS BY MASS SPECTROMETRY LC-MS / MS

Identification				BLAST analysis		
Fraction	**Peptide Sequence**	**Identified Protein**	**Taxonomy**	**Score BLAST**	**E Value**	**% sequence**
1	K.DCADIVINDLSLIHQLPK.E	GI:426205815 GI:5565692 GI:347602329 L-aminoacid oxidase	*Crotalus durissus cumanensis* *Crotalus atrox* *Sistrurus catenatus edwardsi*	68.1	$7e^{-12}$	100
1	R.NDEEGWYANLGPMR.L	GI:60729671 GI:33355627 L-aminoacid oxidase	*Trimeresurus stejnegeri*	51.0	$9e^{-09}$	100
1	R.NDKEGWYANLGPMR.L	GI:538259839 GI:347602324 GI:75570145 GI:401021343 L-aminoacid oxidase	*Protobothrops flavoviridis* *Bothropoides pauloensis* *Gloydius blomhoffii* *Lachesis muta*	57.9	$1e^{-08}$	100
2	K.DCGDIVINDLSLIHQLPK.E	GI:347602324 Precursor L-amino acid oxidase	*Bothropoides pauloensis*	68.1	$7e^{-12}$	100
2	K.KNDVLDKDIMLIR.L	GI:34148047 Serine protease platelet aggregant	*Bothrops jararaca*	52.4	$6e^{-07}$	100

Continue…… Identification				BLAST analysis		
Fraction	**Peptide Sequence**	**Identified Protein**	**Taxonomy**	**Score BLAST**	**E Value**	**% sequence**
4	K.SAGQLYEESLGK.V	GI:10120751 GI:48425312 GI:75570145 GI:82088273 GI:538260091 A chain, L-aminoacid oxidase	*Calloselasma rhodostoma* *Agkistrodon halys pallas* *Gloydius blomhoffii* *Gloydius halys* Ovophis okinavensis	45.2	$1e^{-04}$	100

4	K.SAGQLYQESLGK.A	GI:70797645 GI:284018108 GI:395406796 L-aminoacid oxidase	*Daboia russellii siamensis* *Daboia russellii russellii*	44.8	$2e^{-04}$	100
8	K.CGENIYMSSIPIK.W	GI:48428840 Piscivorin, cysteine rich protein	*Agkistrodon piscivorus piscivorus*	55.8	$3e^{-08}$	100
9	R.MWQNDLHPILIER.Y	GI:538259821 GI:380846513 GI:380846523 and 10 more glutaminilcyclase	*Protobothrops flavoviridis* *Cerrophidion godmani* *Sistrurus catenatus tergeminus*	57.9	$4e^{-09}$	100

without ruling out the occurrence of other anticoagulant proteins, identified in this venom. Early studies proposed that even though the *Porthidium* venom has an important assortment of serine proteases, these enzymes were unable to coagulate human plasma or fibrinogen [17], therefore these proteases lack of thrombin-like activities.

One of the most interesting finding was the detection of venom glutaminil-cyclase (vQC) in the *P.l.h* venom, whose presence had been only before observed in American continent species of *Crotalus atrox, C. godmani, C. d. terrificus* and *M. fulvius* [60], but not in *Porthidium* genus. Among numerous snake venom constituents, glutaminyl cyclase (vQC) is one of the least understood protein family and none of its members has been purified or characterized until 2014 [50, 60]. Although these proteins were present in the venom in small quantities was observed that had more than 96% sequence similarities among vQCs and approximately 75% sequence identities between vQCs and human secretory QC (hQC) [60]. The vQC glycoprotein of 43 kDa was described from *C. atrox* venom, and its N-terminal sequence was established. Glutaminil-cyclase catalyzes N-terminal pyroglutamate (pGlu) development on proteins or peptides and the discharge of ammonia or water molecules [47]. This change seems to be important for structural stability, resistance to aminopeptidase degradation, and interaction of the proteins or peptides with their associates [48]. The presence of vQC activities in *P.l.h* and several snake venoms can be contemplated as one of the protein families present in the huge spectrum of snake venom proteins that have been already described, but that have been neglected [15]. In human medicine, hQC has been involved on infectious diseases and inflammatory process [19], but its minimal presence and a single form of vQC existing in sample venom put forward its internal management guidance for posttranslational venom proteins adaptation. This finding is in divergence to the characteristics of other toxins in the venom families, which have experienced enhanced evolution to produce variations with different activities [15]. The present work offers for the first time new evidence about the presence of vQC-like in the venom of *Porthidium genus*.

The *P.l.h* venom showed proteolytic (gelatinolytic and caseinolytic) activities very interesting and with comparable results to several *Bothrops* venoms. This has been widely reported and attributed mainly to the presence of serine and metalloproteases [40,46]. The electrophoretic profile of *P.l.h* venom (6 µg) on polyacrylamide gels copolymerized with gelatine (FIG.5) showed the presence of a degradation band around 37 kDa; while 2 µg of *B. colombiensis* venom (positive control) induced six bands of lysis with the predominance of intensity of ~ 48 kDa band and five weaker bands around 194, 91, 36, 29 and 26 kDa regions. Regarding casein activity, *P. l. hutmanni* venom showed proteolytic activity on casein (107.9 U^{cas}). This activity was proportional to the used concentrations (data not shown).

The proteolytic activity value, on a non-specific substrate such as casein (in units of casein = U^{cas})(107.9 U^{cas}) obtained for *P.l.h* venom was similar to *B. venezuelensis* (116 U^{cas}) [29], *Atropoides picadoi* (103 U^{cas}) [1,28], being lower than reported for other Central American lineage species such as *Atropoides nummifer* (217 U^{cas}) and *Cerrophidium godmanni* (172 U^{cas}) [1,28], while it was higher than reported for other Viperidae such as *Bohtrops asper* (76 U^{cas}) and *Lachesis muta* (85 U^{cas}); even higher than the found in the *Porthidium ophyromegas* (34 U^{cas}) and *Porthidium nasutum* (42 U^{cas}) venoms [1,28]. The proteolytic activity value, on another non-specific substrate such as gelatine showed that metalloproteases from Viperidae snakes are regularly linked with extensive haemorrhaging, nonetheless can produce myonecrosis, fibrinogenolysis, and also degrade the extracellular matrix [39]. As it is known, gelatine is a denatured form of collagen and this assay tested proteolytic activity. Proteases

such metalloproteases are the most important enzymes liable for the degradation of the extracellular matrix proteins [3]. The *P.l.h* venom demonstrated gelatinase/proteolytic activity, but much less than the positive control of *B. colombiensis,* the *P.l.h* venom possessed electrophoretic bands with a molecular weight consistent with snake venom type II metalloproteases (FIG.2), suggesting that this toxins was responsible for the heightened gelatinase activity of the venom.

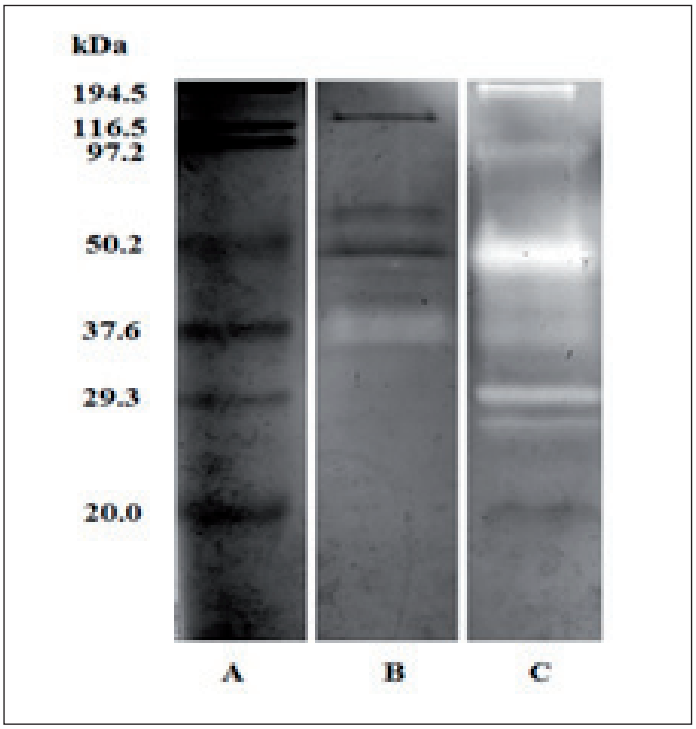

FIGURE 5. GELATINOLYTIC ACTIVITY OF *P.l.h* VENOM. A) molecular weight marker; B) 6 µg ***P.l.h*** **venom; C) 2** µg ***B. colombiensis*** **venom.**

The phospholipases A2 (PLA_2) were identified varying in their isoelectric point located in the acidic and basic spot locations, displaying similar molecular weight. These secreted PLA_2 proteins are typical of Viperidae family [7]. Most phospholipases A_2 present in snake venoms have comparable amino acid sequence and three-dimensional structure, but wide range of neurotoxic, cardiotoxic and myotoxic activities, as well as anti-coagulant, anti-platelet, haemolytic and cell membrane damage actions, which have been defined [23]. *Bothrops colombiensis* possesses high proportion of phospholipases A_2. But, *Porthidium* species contain lower amounts of these enzymes, which only inducing slight muscle damage [17,28]. Nevertheless, the presence of PLA_2, even in lower proportions is able to develop anticoagulant properties, acting together with haemostatic proteases, resulting in the inhibition of coagulation complex formation, primarily over hydrolysis of phospholipids-induced inhibition of the intrinsic tenase complex [2]. Likewise, PLA_2, can directly affect platelet membranes: at low concentrations are able to produce aggregation, but, acting at high concentrations they operate as inhibitors [24].

Among *P.h.l* venom serine proteases, it was identified a platelet aggregant with a 100% identification of a protease from *Bothrops jararaca* [33] possibly involved in thrombosis. Blood coagulation is an exceptionally structured physiological route involving cells i.e. platelets, coagulation cascade proteins, finishing with the factor Xa (FXa)-mediated transformation of the prothrombin zymogen to active thrombin. Until now, although having profuse metalloproteases and serine proteases, *Porthidium* spp. lack of procoagulant activity on human plasma, in contrast to bothropic venoms [17]. The absence of coagulating capacity in *Porthidium* group venom had been early reported, by *Atropoides picadoi* [16], *P. nasutum* and *P. ophryomegas* [16,28], *P. lansbergii lansbergii* [21] and *P. lansbergii hutmanni* [17]. However, the presence of this platelet aggregant fraction could be the first fraction described in this venom that can generate thrombosis. This platelet aggregant could aggregate human platelets, either isolated or in whole blood. In the prothrombinase complex on the platelet surface, FXa cleaves prothrombin at Arg-271, generating the inactive precursor prethrombin-2, which is further attacked at Arg-320-Ile-321 to yield mature thrombin [10, 25]. The present work findings suggest that alternative platelet aggregation by proteases can modify the delicate procoagulant-anticoagulant equilibrium toward thrombosis.

CONCLUSIONS

P. lansbergii hutmanni venom has demonstrated with the previous assays being a venom with high lethal, haemorrhagic, proteolytic and procoagulant activity, whose description will have an enormous utility among the veterinarians and human clinicians, who have to deal with these accidents in its geographical distribution areas. The conformation of previously undefined venoms, and their characteristic components, also will allow the releases of new potentials interesting molecules with therapeutic activity for many diseases treatments. In addition, this will provide new toxins that will allow the improving of the production of better antivenoms for the envenomed victims.

ACKNOWLEDGMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. We would like to thank .We express our sincere gratitude to the Pharmacy Faculty Serpentarium of the Universidad Central de Venezuela.

Author contributions

Conceived and designed the experiments: MEP and ARA. Performed the experiments: MEP and AMV. Contributed materials/analysis tools: AMV and ARA. Wrote the paper: MEP and ARA. All authors analysed the results and approved the final version of the manuscript.

Conflict of interest and disclosure

The authors declare that they have not conflict of interest. The authors confirm that there are no financial disclosures for this study

BIBLIOGRAPHIC REFERENCES

[1] ANGULO, Y.; ESCOLANO, J.; LOMONTE, B.; GUTIÉRREZ, J.M.; SANZ, L.; CALVETE, J.J. Snake venomics of Central American pitvipers: clues for rationalizing the distinct envenomation profiles of *Atropoides nummife*r and *Atropoides picadoi*. **J. Proteome. Res**. 7:708-719. 2008.

[2] ATANASOV, V.N.; DANCHEV, D.; MITEWA, M.; PETROVA, S. Hemolytic and anticoagulant study of the neurotoxin vipoxin and its components-basic phospholipase A2 and an acidic inhibitor. **Biochem.** 74: 276–280. 2009.

[3] BALDO, C.; JAMORA, C.; YAMANOUYE, N.; ZORN, T.M.; MOURA-DA-SILVA, A.M. Mechanisms of vascular damage by hemorrhagic snake venom metalloproteinases: Tissue distribution and *in situ* hydrolysis. **PLoS. Negl. Trop. Dis.** 4: e727. 2010.

[4] BREKER, M.; GYMREK, M.; MOLDAVSKI, O.; SCHULDINER, M. Loqate-localization and quantitation atlas of the yeast proteome. A new tool for multiparametric dissection of single-protein behavior in response to biological perturbations in yeast. **Nucleic. Acids. Res**. 42: 726-730. 2014.

[5] CALVETE, J.J.; JUAREZ, P.; SANZ, L. Snake venomics. Strategy and applications. **J. Mass. Spectrom**. 42: 1405–1414. 2007.

[6] CALVETE, J.J. Antivenomics and venom phenotyping: A marriage of convenience to address the performance and range of clinical use of antivenoms. **Toxicon**. 56: 1284–1291. 2010.

[7] CALVETE, J.J. Venomics: integrative venom proteomics and beyond. **Biochem. J.** 474: 611-634. 2017.

[8] CANDIANO, G.; SANTUCCI, L.; PETRETTO, A.; LAVARELLO, C.; INGLESE, E.; BRUSCHI, M.; GHIGGERI.; G.M.; BOSCHETTI, E.; RIGHETTI, P.G. Widening and diversifying the proteome capture by combinatorial peptide ligand libraries via Alcian Blue dye binding. **Anal. Chem**. 87:4814-4820. 2015.

[9] CHERNUSHEVICH, I.V.; LOBODA, A.V.; THOMSON, B.A. An introduction to quadrupoletime-of-flight mass spectrometry. **J. Mass. Spectrom**. 36(8):849-865. 2001.

[10] DEGUCHI, H.; TAKEYA, H.; GABAZZA, E.C.; NISHIOKA, J.; SUZUKI, K. Prothrombin kringle 1 domain interacts with factor Va during the assembly of prothrombinase complex**. Biochem. J.** 321: 729-735. 1997.

[11] DOLEY, R.; ZHOU, X.; KINI, R.M. Snake venom phospholipase A2 enzymes. In: Mackessy, S.P. (Ed), **Handbook of Venoms and Toxins of Reptiles**. CRC Press, Boca Raton, FL, USA. Pp 173–206. 2010.

[12] DU, X.Y.; CLEMETSON, K.J. Snake venom L-amino acid oxidases. **Toxicon**. 40: 659-665. 2002.

[13] ESTRELLA, A.; SÁNCHEZ, E.E.; GALÁN, J.; TAO, W.A.; GUERRERO, B.; NAVARRETE, L.; RODRÍGUEZ-ACOSTA A. Characterization of toxins from the broad-banded water snake *Helicops angulatus* (Linnaeus, 1758): isolation of a cysteine-rich secretory protein, Helicopsin. **Arch. Toxicol**. 85: 305–313. 2011.

[14] FOX, J.; SERRANO, S. Timeline of key events in snake venom metalloproteinase research. **J. Proteomics**. 72: 200-209. 2009.

[15] FRY, B.G.;, SCHEIB, H.; VAN DER WEERD, L.; YOUNG, B.; MCNAUGHTAN, J.; RAMJAN, S.F.; VIDAL, N.; POELMANN, R.E.; NORMAN, J.A. Evolution of an arsenal: structural and functional diversification of the venom system in the advanced snakes (Caenophidia). **Mol. Cell. Proteomics**. 7: 215-246. 2008.

[16] GENE, J.A.; ROY, A.; ROJAS, G.; GUTIÉRREZ, J.M.; CERDAS, L. Comparative study on the coagulant, desfibrinating, fibrinolytic, and fibrinogenolytic activities of Costa Rican Crotalinae snake venoms and their neutralization by a polyvalent antivenom. **Toxicon**. 27: 841-848. 1989.

[17] GIRÓN, M.E.; ESTRELLA, A.; SÁNCHEZ, E.E.; GALÁN, J.; TAO, A.; GUERRERO, B.; SALAZAR, A.M.; RODRÍGUEZ-ACOSTA, A. Purification and characterization of a metalloproteinase, Porthidin-1, from the venom of Lansberg's hognosed pitvipers (*Porthidium lansbergii hutmanni*). **Toxicon**. 57: 608-618. 2011.

[18] GUO, C.; LIU, S.; YAO, Y.; ZHANG, Q.; SUN, M.Z. Past decade study of snake venom L-amino acid oxidase . **Toxicon** 60: 302-311. 2012.

[19] HELLVARD, A.; MARESZ, K.; SCHILLING, S.; GRAUBNER, S.; HEISER, U.; JONSSON, R.; CYNIS, H.; DEMUTH, H.U.; POTEMPA, J.; MYDEL, P. Glutaminyl Cyclases as novel targets for the treatment of septic arthritis. ***J. Infect. Dis***. 207: 768–777. 2013.

[20] IZIDORO, L.F.; SOBRINHO, J.C.; MENDES, M.M.; COSTA, T.R.; GRABNER, A.N.; RODRIGUES, V.M.; DA SILVA, S.L.; ZANCHI, B.; ZULIANI, J.P.; FERNANDES, C.F.; CALDERON, L.A.; STÁBELI, R.G.; SOARES, A.M. Snake venom L-amino acid oxidases: trends in pharmacology and biochemistry. Biomed. Res. Int. 2014: 196754. 2014.

[21] JIMÉNEZ-CHARRIS, E.; MONTEALEGRE-SANCHEZ, L.; SOLANO-REDONDO, L.; **CAMACHO,** E.; CASTRO-HERRERA, F.; FIERRO-PÉREZ, L.; LOMONTE, B. Proteomic and functional analyses of the venom of Porthidium lansbergii lansbergii *(Lansberg's hognose viper)* from the Atlantic Department of Colombia**. J. Proteom.** 114: 287-299. 2015**.**

[22] KAMIGUTI, A.S.; ZUZEL, M.; THEAKSTON, R.D.G. Snake venom metalloproteinases and disintegrins: Interactions with cells. **Braz. J. Med. Biol. Res.** 31:853–862. 1998.

[23] KINI, R.M. Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes. **Toxicon.** 42: 827-840. 2003.

[24] KINI, R.M. Anticoagulant proteins from snake venoms: Structure, function and mechanism**. Biochem. J.** 397: 377–387. 2006.

[25] KOMAROV, A.; PANCHENKO, E.; DOBROVOLSKY, A.; KARPOV, Y.; DEEV, A.; TITAEVA, E.; DAVLETOV, K.; ESHKEEVA, A.; MARKOVA, L. D-dimer and platelet aggregability are related to thrombotic events in patients with peripheral arterial occlusive disease. **Eur. Heart. J.** 23:1309-1316. 2002.

[26] LAEMMLI, U.K. Cleavage of structural proteins during the assemble of the head bacteriophage T4. **Nature.** 227: 680-685. 1970.

[27] LEMOINE, K.; GIRÓN, M.E.; AGUILAR, I.; NAVARRETE, L.F.; RODRIGUEZ-ACOSTA, A. Proteolytic, hemorrhagic, and neurotoxic activities caused by *Leptodeira annulata ashmeadii* (Serpentes:Colubridae) Duvernoy's gland secretion. **Wildern. Environm. Med**. 15: 82-89. 2004;

[28] LOMONTE, B.; REY-SUÁREZ, P.; TSAIA, W.; ANGULO, Y.; SASA, A.M.; GUTIÉRREZ, J.M.; CALVETE, J.J. Snake venomics of the pit vipers *Porthidium nasutum, Porthidium ophryomegas*, and *Cerrophidion godmani* from Costa Rica: Toxicological and taxonomical insights. **J. Proteomics**. 75: 1675-1689. 2012.

[29] LÓPEZ, J.C.; VARGAS, A.M.; SCANNONE, H.; FERNANDEZ, Y. Chromatographic, electrophoretic and enzymatic study of the whole venom and fraction I of the Venezuelan snake *Bothrops venezuelensis* **(Tigra mariposa). Rev.** Cientif. FCV-LUZ. IX (4): 314-320. 1999.

[30] LOWRY, O.; ROSENBROUGH, N.; FARR, A.; RANDALL, R. Protein measurement with the Folin phenol reagent. **J. Biol. Chem**. 93:265-275. 1951.

[31] MACKESSY, S.P. Fractionation of red diamond rattlesnake (*Crotalus ruber ruber*) venom: protease, phosphodiesterase, L-amino acid oxidase activities and effects of metal ions and inhibitors on protease activity. **Toxicon**. 23: 337–340. 1985.

[32] MARGRES, M.J.; MCGIVERN, J.J.; WRAY, K.P.; SEAVY, M.; CALVIN, K.; ROKYTA, D.R. Linking the transcriptome and proteome to characterize the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*). **J. Proteomics**. 96: 145-148. 2014.

[33] MATSUI, T.; HAMAKO, J.; TITANI, K. Structure and function of snake venom proteins affecting platelet plug formation. **Toxins (Basel)**. 2: 10-23. 2010.

[34] MORRISSETTE, J.; KRÄTZSCHMAR, J.; HAENDLER, B.; EL-HAYEK, R.; MOCHCA-MORALES, J.; MARTIN, B.M.; PATEL, J.R.; MOSS, R.L.; SCHLEUNING, W.D.; CORONADO, R. Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors. **Biophys .J.** 68: 2280-2288. 1995.

[35] MOTULSKY, H. J. GraphPad Statistics Guide. In linea:http://www.graphpad.com/guides/prism/7/statistics/index.htm. 5/07/2017.

[36] MOURA-DA-SILVA, A.M.; BUTERA, D.; TANJONI, I. Importance of snake venom metalloproteinases in cell biology: Effects on platelets, inflammatory and endothelial cells. **Curr. Pharm. Des**. 13: 2893–2905. 2007.

[37] NATHAN, I.; DVILANSKY, A.; YIRMIYAHU, T.; AHARON, M.; LIVNE, A. Impairment of platelet aggregation by *Echis colorata* venom mediated by L-amino acid oxidase or H_2O_2. **Thromb. Haemost.** 48: 277-282. 1982.

[38] NATIONAL INSTITUTE OF HEALTH (NIH). Principles of laboratory animal care. Maryland, National Institute of Health of United States, Publication Series 85-23. Pp1-126. 1985.

[39] OLIVEIRA, A.K.; PAES-LEME, A.F.; ASEGA, A.F.; CAMARGO, A.C.; FOX, J.W.; SERRANO, S.M. New insights into the structural elements involved in the skin haemorrhage induced by snake venom metalloproteinases. **Thromb. Haemost.** 104: 485-497. 2010.

[40] ORTIZ, C.; LAZO, F.; BELLIDO, C.; GONZALES, E.; YARLEQUÉ, A. Variaciones en las actividades enzimáticas del veneno de la serpiente *Bothrops atrox* "jergón", de tres zonas geográficas del Perú. **Rev. Peru. Med. Exp. Sal .Pub**. 29: 198-205. 2012.

[41] PHILLIPS, D.J.; SWENSON, S.D.; MARKLAND, F.S. JR.

Thrombin-like snake venom serine proteinases. In: Mackessy, S.P. (Ed). **Handbook of Venoms and Toxins of Reptiles**. CRC Press, Boca Raton, FL, USA. Pp. 139–154. 2010.

[42] PIFANO, F. Investigacion y docencia en Medicina Tropical. **Arch. Ven. Med. Trop. Parasitol**. 4: 1-203. 1961.

[43] PINEDA, M.E.; GIRÓN, M.E.; ESTRELLA, A.; SÁNCHEZ, E.; AGUILAR, I.; FERNÁNDEZ, I.; VARGAS, A.M.; SCANNONE, H.; RODRÍGUEZ-ACOSTA, A. Inhibition of the hemorrhagic and proteolytic activities of Lansberg's Hognose Pit Viper (*Porthidium lansbergii hutmanni*) venom by opossum (*Didelphis marsupialis*) Serum: Isolation of *Didelphis marsupialis* 0.15Dm Fraction on DEAE-Cellulose Chromatography. **Immunopharmacol. Immunotoxicol** . 30: 883–896. 2008.

[44] RAMAZANOVA, A.S.; STARKOV, V.G.; OSIPOV, A.V.; ZIGANSHIN, R.H.; FILKIN, S.Y.; TSETLIN, V.I.; UTKIN, Y.N. Cysteine-rich venom proteins from the snakes of Viperinae subfamily-Molecular cloning and phylogenetic relationship. **Toxicon.** 53: 162–168. 2009.

[45] SAJEVIC, T.; LEONARDI, A.; KRIŽAJ, I. Haemostatically active proteins in snake venoms. **Toxicon.** 57: 627–645. 2011.

[46] SALAZAR, A.M.; RODRÍGUEZ-ACOSTA, A.; GIRÓN, M.E.; AGUILAR, I.; GUERRERO, B. A comparative analysis of the clotting and fibrinolytic activities of the snake venom (*Bothrops atrox*) from different geographical areas in Venezuela. **Thromb. Res**. 120: 95-104. 2007.

[47] SEIFERT, F.; SCHULZ, K.; KOCH, B.; MANHART, S.; DEMUTH, H.U.; SCHILLING, S. Glutaminyl cyclases display significant catalytic proficiency for glutamyl substrates. **Biochem**. 48: 11831–11833. 2009.

[48] SERRANO, S.M.T.; SHANNON, J.D.; WANG, D.; CAMARGO, A.C.M.; FOX, J.W. A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: An approach to understanding venom proteomics. **Proteomics.** 5: 501–510. 2005.

[49] SPEARMAN- KÄRBER, R. Alternative methods of analysis for quantal responses. In: Finney, D (Ed). **Statistical method in biological assay**. London: Charles Griffin. Pp 1– 78. 1978.

[50] STEPHAN, A.; WERMANN, M.; VON BOHLEN, A.; KOCH, B.; CYNIS, H;, DEMUTH, H.U.; SCHILLING, S. Mammalian glutaminyl cyclases and their isoenzymes have identical enzymatic characteristics. **FEBS. J.** 276: 6522–6536. 2009.

[51] STILES, B.G.; SEXTON, F.W.; WEINSTEIN, S.A. Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian king brown or mulga snake) venom. **Toxicon**. 29:1129-1141. 1991.

[52] TAN, C.H.; TAN, K.Y.; FUNG, SY.; TAN, N.H. Venom-gland transcriptome and venomproteome of the Malaysian king cobra (*Ophiophagus hannah*) **BMC. Genom.** 16: 1–21. 2015.

[53] TERRA, R.M.; PINTO, A.F.; GUIMARÃES, J.A.; FOX, J.W. Proteomic profiling of snake venom metalloproteinases (SVMPs): insights into venom induced pathology. **Toxicon**. 54: 836-844. 2009.

[54] TORII, S.; NAITO, M.; TSURUO, T. Apoxin I, a novel apoptosis-inducing factor with L-amino acid oxidase activity purified from Western diamondback rattlesnake venom. **J. Biol. Chem**. 272: 9539–9542. 1997.

[55] VEJAYAN, J.; SHIN-YEE, L.; PONNUDURAI, G.; AMBU, S.; IBRAHIM, I. Protein profile analysis of Malaysian snake venoms by two-dimensional gel electrophoresis. **J. Venom. Anim. Toxins. Incl.Trop. Dis.** 16: 623–630. 2010.

[56] VEJAYAN, J.; TANG, M.S.; HALIJAH, I. The role of conventional two-dimensional electrophoresis (2DE) and its newer applications in the study of snake venoms. In: Heazlewood, J.L.; Petzold, C.J. (Eds.) **Proteomic Applications in Biology**. In Tech, Rijeka, Croatia. Pp 226–252. 2012.

[57] YAMAZAKI, Y.; MORITA, T. Structure and function of snake venom cysteine-rich secretory proteins. **Toxicon.** 44: 227–231. 2004

[58] YAMAZAKI, Y.; KOIKE, H.; SUGIYAMA, Y.; MOTOYOSHI, K.; WADA, T.; HISHINUMA, S.; MITA, M.; MORITA, T. Cloning and characterization of novel snake venom proteins that block smooth muscle contraction. **Eur. J. Biochem**. 269: 2708-2715. 2002.

[59] YAMAZAKI, Y.; MORITA, T. Snake venom components affecting blood coagulation and the vascular system: structural similarities and marked diversity. **Curr. Pharm. Des**. 13: 2872-2886. 2007.

[60] YING-MING, W.; KAI-FA, H.; INN-HO, T. Snake venom glutaminyl cyclases: Purification, cloning, kinetic study, recombinant expression, and comparison with the human enzyme. **Toxicon.** 86: 40-50. 2014.