Venom from *Opisthacanthus elatus* scorpion of Colombia, could be more hemolytic and less neurotoxic than thought.

Sebastián Estrada-Gómez a,b, Leidy Johana Vargas Muñoz c, Mónica Saldarriaga-Córdoba d, Juan Carlos Quintana Castillo c.

a Facultad de Química Farmacéutica, Universidad de Antioquia UdeA, Carrera 53 No. 61-30, Medellín, Postal address 050010. Colombia.

b Programa de Ofidismo/Escorpionismo, Facultad de Química Farmacéutica, Universidad de Antioquia UdeA, Carrera 53 No. 61-30, Medellín, Postal address 050010. Colombia.

c Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, Colombia, Calle 50 A No. 41-20, Medellín Postal address 050010, Colombia.

d Departamento de Ciencias, Laboratorio de Biología y Bioinformática, Universidad Iberoamericana de Ciencias y Tecnología, Padre Miguel de Olivares N°1620, Santiago de Chile, Chile.

1 Corresponding author: Tel: + 57 4 2196535; Fax: + 57 4 2631914 e-mail address: sebastian.estrada@udea.edu.co, sestradas@gmail.com postal address: Calle 70 # 52-2, Medellín COLOMBIA, zip code: 050010.
Abstract

We report the first biochemical, biological, pharmacological and partial proteomic characterization studies of the *Opisthancanthus elatus* venom (Gervais, 1844) from Colombia. The Reverse Phase High-Performance Liquid Chromatography venom profile showed 28 main well-defined peaks, most eluting between 20 and 45 minutes (18-30% of acetonitrile respectively). High-resolution mass analysis indicates the presence of 106 components ranging from 806.59742 Da to 16849.4139 Da. *O. elatus* venom showed hemolytic activity and hydrolyzed the specific substrate BapNa suggesting the presence of proteins with serine-protease activity. Collected RP-HPLC fractions eluting at 52.6, 55.5, 55.8, 56.2, and 63.9 min (PLA$_2$ region between 33-40 % of acetonitrile), showed hemolytic activity and hydrolyzed the synthetic substrate 4-nitro-3-octanoyloxy-benzoic acid, indicating the presence of compounds with phospholipases A$_2$ activity. These RP-HPLC fractions, showed molecular masses values up to 13978.19546 Da, corroborating the possible presence of the mentioned enzymes. Tryptic digestion and MS/MS analysis showed the presence of a phospholipase like fragment, similar to on described in other *Opisthacanthus* genus studies. No coagulant activity was observed. No larvicidal or antimicrobial activity was observed at concentrations evaluated. Lethal and toxic activity is expected at doses above 100 mg/kg, no neurotoxic effects were detected at lower doses. In conclusion, *O. elatus* exhibits a venom with a predominant phospholipase A$_2$ activity than thought; mammal’s neurotoxic activity is expected above the 100 mg/kg, which is very high compared to the venom from other neurotoxic scorpions.

Key words: *Opisthacanthus elatus*, scorpion, serine protease activity, phospholipase A$_2$ activity, MS/MS, mass fingerprint, proteomic.
1. Introduction

Scorpion venoms are recognized for the presence of specific peptides and proteins capable of modulate neurotransmission enhancing neurotoxic effects into various organisms such as mammals and insects among others. In over 400 million years of evolution, scorpions have developed this neurotoxic cocktail, which includes cytolytic peptides affecting different kinds of biological membranes such us those with antimicrobial activity or AMP (Harrison et al., 2014; Miyashita et al., 2010). These peptides enhancing neurotoxic effects, commonly affects ionic channels like sodium ($Na^{2+}$), potassium ($K^+$), chlorine ($Cl^-$) or calcium ($Ca^{2+}$), (Chagot et al., 2005; Possani et al., 1999; Zeng et al., 2005), with most of these neurotoxic components reported in the Buthidae family (Pimenta et al., 2001; Rodriguez de la Vega et al., 2010).

Scorpions belonging to the Hormuridae family (Lourenco, 2014) are well distributed in the African continent, Madagascar; Central America, South America and the Caribbean region, exhibiting a very stable composition with 22 species described (Lourenço and Fé, 2003; Prendini and Wheeler, 2005). Despite the wide composition of this genus, only the venom from two of the *Opisthacanthus* genera have been largely studied, *Opisthacanthus cayaporum* from Brazil and *Opisthacanthus madagascariensis* from Madagascar (Camargos et al., 2011b; Dai et al., 2002; Dai et al., 2001; Schwartz et al., 2008; Silva et al., 2009). From this venom, several compounds affecting ionic channels, or exhibiting amphipathic motifs and proteins with phospholipase activity have been described. From *O. madagascariensis*, short chain affecting ionic channels were described with molecular masses between 4 kDa and 5 kDa that act as potassium ($K^+$) channel blockers (Chagot et al., 2005; Yamaji et al., 2004). Camargos et al, described two potassium-channels toxins (KTxs) from the venom gland transcriptome of *O. cayaporum*, blocking this channels, both similar to the ones described previously in *O. madagascariensis* venom (Camargos et al., 2011a; Chagot et al., 2005). Using the ESI-MS technique, Schwartz et al. (2008) characterized the venom from of *O. cayaporum*. 
They reported 93 different components with molecular masses ranging from 229.2 atomic mass units (a.m.u.) to 61440 a.m.u., most of the compounds containing 40-45 amino acids (60% of the components with a molecular mass above 5kDa). Using Edman degradation they described, isolated and partially identified the N-terminal amino acid sequences of three possible α-helical amphipathic antimicrobial peptides and two phospholipases A2 (PLA2), the last ones with molecular masses of 14237 Da and 14518 Da respectively (Schwartz et al., 2008). With the same Opisthacanthus species, Silva et al. 2009, partially identified, a phospholipase-like protein (92 amino acid fragment) using RNA transcriptome obtained from venom glands (Silva et al., 2009). Furthermore, from these two mentioned species, various non-disulfide rich compounds were recently isolated and characterized. The main characteristic of these molecules is their lack of disulfide bridges or an inhibitory cysteine knot motif (most scorpion venoms are rich in cysteine residues), Also, their structure consists of a small double-triple stranded antiparallel β-sheet linked to a short α-helix and have relatively low molecular mass ranging from 1-4 kDa (Possani and Rodriquez de la Vega, 2006; Zeng et al., 2005). These characteristics allow the expression of an amphipathic α-helical structure like those reported for various cationic antimicrobial molecules (Cao et al., 2012a; Cao et al., 2012b; Corzo et al., 2001; Corzo et al., 2002; Diaz et al., 2009; Ramirez-Carreto et al., 2012; Zeng et al., 2005). These peptides, unlike the disulfide-rich ones, are responsible for hemolytic, antibacterial and insecticidal activities, among others (Giangaspero et al., 2001; Schwartz et al., 2008; Zeng et al., 2005).

Scorpion venoms insecticidal activities have been widely reported in scorpions belonging to the genus Buthus, Tityus, Androctonus, Mesobuthus and Centruroides from the Buthidae family and only in the genus Liocheles from the Hemiscorpiidae family (Estrada-Gomez et al., 2014; Gurevitz et al., 2007; Matsushita et al., 2007; Miyashita et al., 2007; Miyashita et al., 2010). Overall, this activity is largely mediated by the great selectivity of some peptides to ionic sodium and calcium channels (Gurevitz et al., 2007). No insecticidal activity have been reported in the Opisthacanthus genus.
In Colombia, the species *Opisthacanthus elatus* (Gervais, 1844) widely distributed in all the territory exhibiting intra-domiciliary habits, is the only species reported today for this genus, and is (Lourenço and Fé, 2003). No pharmacological or toxicological reports, from this species venom, have been published. We describe the first biological, pharmacological and biochemical characterization based on *in vitro* assays with specific enzymes substrates as well as its respective mass finger print, MS/MS partial analysis, chromatographic and electrophoretic profiles.

2. Material and Methods

2.1. Venom extraction

*O. elatus* scorpions from the Antioquia and Choco provinces in Colombia (North-west Andean region) were kept in captivity in the Universidad de Antioquia Serpentarium, with water and food *ad libitum* in a polyphagic diet of invertebrates. Venom extraction process was carried out using electro-stimulation. Copper electrodes, impregnated with water, were carefully positioned in the telson and electrical stimuli of 35 V were applied twice with an interval of 5 sec using a JRM electro-stimulator (model 06, series 007, Colombia). Collected venom was transferred to dry vials, lyophilized and stored at –20 °C until use.

2.2. Indirect Hemolytic Activity

To establish any possible venom PLA₂ activity in the *O. elatus* venom, indirect hemolysis was carried out and determined in agarose erythrocyte-egg yolk gels, according to Gutiérrez et al. (Gutierrez et al., 1988), using 0.8% agarose dissolved in PBS (0.12 M NaCl, 0.04 M sodium phosphate in distilled water), pH 7.2 and CaCl₂. An additional plate, without CaCl₂ and egg yolk, was performed to verify that hemolytic activity is due to the PLA₂ presence. RP-HPLC fractions over 250 milli Absorbance Units (mAU) were collected and used. Minimum hemolytic dose (MHeD) was
defined as the amount of venom that induced a 20 mm diameter hemolytic halo. The experiments were performed in triplicate. As positive control 2µg of Bothrops asper venom were used.

2.3. PLA₂ specific activity

PLA₂ activity was measured using the assay described by Cho and Kézdy (Cho and Kezdy, 1991) and Holzer and Mackessy (Holzer and Mackessy, 1996), modified for 96-well plates. The standard assay mixture contained 200 µL of buffer (10 mM Tris–HCl, 10 mM CaCl₂, 100 mM NaCl, pH 8.0), 20 µL of substrate 4-nitro-3-octanoyloxy-benzoic acid, 20 µL of water and 20 µL of the venom at a concentration of 5 µg/µL, or the RP-HPLC collected fractions (positive in the indirect hemolytic assay), in a final volume of 260 µL. The mixture was incubated for 60 min at 37 °C, and the absorbance was recorded at 405 nm.

2.4. Coagulant activity

Coagulant activity of venom was assessed on citrated human plasma. Samples of 100 µL of various concentrations of the venom (1.0, 0.5, 0.25, 0.125 µg/µL) were added to aliquots of 200 µL plasma, previously incubated at 37 °C. Clotting times were recorded in a HumaClot Junior coagulometer (Human; Germany), and the minimum coagulant doses (MCD) for plasma or fibrinogen were determined. The MCD corresponds to the minimal amount of venom that induces clotting in 60 seconds (Theakston and Reid, 1983).

2.5. Proteolytic activity

Azocasein (Sigma–Aldrich, St. Louis, MO) was used as substrate to measure the proteolytic activity, and to determine the presence of metalloproteases, according to Wang et al. (Wang et al., 2004) with some modifications. Briefly, 20, 10, 5, 2.5 and 1.25 µg of the venom were dissolved in 20 µl of 25 mM Tris (0.15 M NaCl, 5 mM CaCl₂), pH 7.4, (in order to obtain 1.0, 0.5, 0.25, 0.125 and 0.0625 µg/µl). These solutions were incubated with a solution of azocasein at a concentration of 10
mg/ml, previously diluted in the same buffer. After 90 min of incubation at 37 °C, the reaction was stopped adding 200 µl of trichloroacetic acid. Samples were then centrifuged at 360 g for 5 min. Supernatant (100 µl) was mixed with the equal volume of 0.5 M NaOH, and the absorbance was measured at 450 nm. Results are shown as unit of proteolytic activity, which corresponds to the amount of enzyme that induces a change in absorbance of 0.2.

A second proteolytic activity was performed to determine the presence of serine proteases enzymes following the method described by Patiño et al., (Patiño et al., 2013). The enzymatic activity was measured using the synthetic substrate N-alpha-benzoyl-DL-arginine-p-nitroanilide (BapNA). The standard assay mixture contained 50 μL of buffer (10 mM Tris–HCl, 10 mM CaCl₂, 100 mM NaCl, pH 8.0), 200 μL of substrate, 10 μL of water or enzyme in a final volume of 260 μL. After the addition of 100 µg and 50 µg of O. elatus venom, the mixture was incubated for up to 40 min at 37 °C, with the absorbance at 405 nm being recorded at 10 min intervals.

2.6. Electrophoretic profile

O. elatus crude venoms electrophoretic profile and HPLC fractions (selected and collected), were analyzed using sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) according to Laemmli (Laemmli, 1970) on 12% gels, and stained with Coomassie blue R-250. Molecular weights were estimated using standard low rank markers (Bio-Rad).

2.7. Chromatographic profile

One milligram of whole venom was dissolved in 200 µL of solution A (0.1% TFA in water) and centrifuged at 3500 g. The supernatant was then applied to a reverse-phase RESTEK C18 column (250×4.6 mm), and separated on a Shimadzu Prominence HPLC. Proteins were eluted by a gradient towards solution B (0.1% TFA in acetonitrile) as follows: 5% B for 5 min, 5–15% B over 10 min, 15–45% B over 60 min, and 45–70% B over 12 min at a flow rate of 1.0 mL/min (Fernandez et al., 2013).
The chromatographic run was monitored at 215 nm and fractions were collected, lyophilized and stored until used.

### 2.8. Peptide mass determination by High-Resolution LC-MS

Selected peaks were collected from female’s RP-HPLC venom, dried as describe above and used to complete masses analysis in a Q Exactive Q-Orbitrap (Thermo Scientific, San Juan, CA) linked to a UHPLC. For the chromatographic step, an analytical C18 Hypersil Gold Aq ™ column (2.1 mm internal diameter, 100 mm length and 1.9 µm pore size) was used. Venom components were eluted using gradient of solution B (0.1% formic acid in acetonitrile) as follows: 5% B for 2 min, 5–90% B for 13 min, 90% B for 2 more min, and 5% B for 5 more min at a flow rate of 450 µl/min.

For the spectrometric assay, ESI source was employed at 3kV and 320 °C in positive ion mode. Sheath gas flow rate was set at 40 units and auxiliary gas flow rate set at 10 units. Full scan mode MS spectra (160-2000 m/z) were acquired in the Orbitrap with a resolution R= 70,000, without fragmentation. Manual deconvolutions were used to determine the isotopic and average molecular mass composition of the components.

### 2.9. Sample Digestion

Sequence grade Lys-C/Trypsin (Promega) was used to enzymatically digest the venom samples. The samples were reduced and alkylated. All digestions were carried out in the Barocycler NEP2320 (PBI) at 50°C under 20 kpsi for 2 hours. Digested samples were cleaned over C18 spin columns (Nest Group) and dried. Resulting pellets were resuspended in 97% purified H2O/3% ACN/0.1% formic acid (FA). 5 µL of volume is used for nano LC-MS/MS analysis.

### 2.10. LC-MS/MS
Fraction eluting at 55.8 min (positive to hemolytic and NOBA activities) was run on a nano Eksigent 425 HPLC system coupled to the Triple TOF 5600 plus (Sciex, Framingham, MA). The method used for analysis was 120 min at 300 nL/minute over the cHiPLC nanoflex system. The trap column was a Nano cHiPLC 200 µm x 0.5 mm ChromXP C18-CL 3 µm 120 Å followed by the analytical column, the Nano cHiPLC 75 µm x 15 cm ChromXP C18-CL 5 µm 120 Å. The sample was injected into the Triple TOF 5600 plus through the NanoSpray III source equipped with emission tip from New Objective. Peptides from the digestion were eluted from the columns using a mobile phase A of purified H2O/0.1% formic acid (FA) and a mobile phase B of ACN/0.1 % FA. With a flow rate of 0.3 µl/min, the method started at 95% A for 1 min followed by a gradient of 5% B to 35% B in 90 min and from 35% B to 80% B in 2 min. 80% B was held for 5 minutes before being brought to 5% B and held for 20 min. The data acquisition was performed monitoring 50 precursor ions at 250 ms/scan. Mascot Daemon v.2.4.0 (Matrix Science) was used for database searches against the different databases. Some were downloaded from UniProt, NCBI or the ArachnoServer website. Samples were run in the Bindley Biosciences Center at Purdue University.

2.11. Larvicidal activity

To determine larvicidal activity, we followed the method established by the World Health Organization (WHO, 2005) with the modifications proposed by Estrada-Gómez et al (Estrada-Gomez et al., 2014). Fifty µl of O. elatus venom at different amounts (400, 300, 100, 50, 25, 12.5, 6.25 µg) were added to a tube containing 450 µl of saline solution 0.90% and 5 larvae of Aedes aegypti. A saline solution 0.90% was used as a negative control whereas a solution of Piperazine was used as a positive control. The solutions were kept at room temperature 12 hours light and dark photoperiod. The counting of death larvae (larvae with no movement) was performed on each tube 24 to 48 hours. This procedure was performed by duplicate with approval of the committee for experimentation with animals, Universidad de Antioquia (ECEA-UOFA).
2.12. Antibiotic activity

Antibiotic susceptibility tests were performed as proposed by Bauer et al. (Bauer et al., 1966), and the Clinical and Laboratory Standards Institute (CLSI) guidelines, with some modifications. *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were grown on Mueller-Hinton agar (MH) and then suspended on 5ml of MH sterile broth. Turbidity was measured at 600nm and adjusted to 0.5 absorbance which corresponds to $1.5 \times 10^{-4}$ colony forming units (CFU). Ten $\mu$l of each venom dose containing 500 and 250 $\mu$g, and RP-HPLC collected fractions were added and incubated at 37°C during 24 hours. Physiological saline solution was used as negative control and chloramphenicol as a reference control. Each test was performed in duplicate.

2.13. Toxic activity

Lethality test was carried out on female Swiss albino mice of approximately 26 g body weight as described by Valdez-Cruz *et al* (Valdez-Cruz *et al.*, 2004). Different amounts of venom from *O. elatus* were tested in parallel; 25 mg/kg, 50 mg/kg, 75 mg/kg and 100 mg/kg (group 1, group 2, group 3 and group 4 respectively). Injections were performed intraperitoneally using PBS (phosphate buffered saline, containing 0.15mM NaCl, 0.1mM sodium phosphate at pH 7.4) as vehicle and negative control. The intoxication levels were called “non-toxic”, when the animals showed no symptoms of envenoming within 20 h after testing, or showed the same symptoms as the control mice injected with 100 $\mu$l of buffer alone (PBS). “Toxic” means that the mice showed symptoms such as: piloerection, excitability, salivation, lacrimation, dyspnea, diarrhea, temporary paralysis, but recovered within 20 h. “Lethal” means that the mice showed some or all the symptoms of intoxication and died within 20 h after injection. LD$_{50}$ was calculated using the Spearman-Karber method (WHO, 1981) and TOXICALC software (Robles and Gene, 1990). Three mice were used in each doses and negative control with approval of the ECEA-UOFA.

2.14. Statistical analysis
Results were expressed as mean ± standard error media (S.E.M.) and statistical comparisons were done using an ANOVA with a Bonferroni post-test assuming a significance of p<0.05. All data analysis was done using GraphPad PRISM 5 (GraphPad Software, Inc; La Jolla, California, USA).

3. Results

3.1. Venom Enzymatic activity

*O. elatus* venom caused indirect hemolysis either in presence or in absence of calcium and egg-yolk. MHeD obtained in presence of calcium and egg-yolk was 12.5 µg, while without both, the MHeD was 100 µg ([Fig. 1A](#)). From all the analyzed RP-HPLC fractions, 5 fractions eluting at 52.6, 55.5, 55.8, 56.2 and 63.9 min (PLA₂ region between 33% and 40% of acetonitrile) enhanced indirect hemolytic activity ([Fig. 1B and 1C](#)). This activity, in the same fractions, was confirmed as being dependent on enzymatic active phospholipases A₂ (PLA₂) because the same hemolytic fractions, hydrolyzed the specific PLA₂ substrate. The venom of *O. elatus* showed proteolytic activity only against the BapNA substrate ([Fig. 1D](#)) and statistically significative differences were only found with respect blank (p<0.05) with 100 µg of venom. No coagulant activity was observed with this venom (data no shown).
Figure 1A: Hemolytic activity, using calcium, of *Opisthacanthus elatus* venom at different concentrations (100, 50, 25 and 12.5 µg) with calcium (+Ca\(^{2+}\)) and without calcium (-Ca\(^{2+}\)). C+: *Bothrops asper* crude venom (2µg).

Minimum hemolytic dose (MHeD) was defined as the amount of venom that induced a 20 mm diameter hemolytic halo. B: PLA\(_2\) region according with RP-HPLC chromatographic profile (fragment). Arrows indicates the exact peaks of phospholipase activity with retention times of 52.6, 55.5, 55.8, 56.2 and 63.9 min, the named PLA\(_2\) region. C: Hemolytic activity of *Opisthacanthus elatus* RP-HPLC fractions. Numbers in min of retention in the chromatographic profile, C+: *Bothrops asper* crude venom (2µg) and C-, phosphate buffer (PBS). D: Proteolytic activity using the substrate N-benzoyl-DL-arginine-p-nitroanilide (BapNA) with 100 µg of *O. elatus* venom. The asterisk indicates significant differences (p<0.05) with respect to blanks (BKO).

3.2. SDS-PAGE

*O. elatus* electrophoretic profile showed the presence of proteins with high and median molecular masses. Two regions were identified; a high number of proteins with a molecular mass between 31 kDa and 97.4 kDa and an considerable number between 14.4 kDa and 21 kDa (Fig. 2A). A small number of low molecular mass compounds were detected below 14.4 kDa.
Figure 2A. *Opisthacanthus elatus* crude venom SDS-PAGE profile in a 12% under reduced (R) and no reduced (NR) conditions. Venoms were loaded at a concentrations of 1.5 µg/µl. Arrows indicate low and high molecular mass components. B. *Opisthacanthus elatus* venom RP-HPLC chromatographic profile using a C18 column.
Elution gradient used: 0 – 70% of acetonitrile (99% in TFA 0.1%). Numbers indicate retention
time of fractions subjected to MS analysis. The run was monitored at 215 nm.

### 3.3. Reverse-phase chromatography

RP-HPLC venom profile showed 28 main well defined peaks (Fig. 2B), most of them (78%) eluting
between 5% and 27% of acetonitrile (before 40 min). One complex peak is observed eluting in the
region (with very similar % ACN values) where other phospholipase were reported before (around
36% of acetonitrile).

### 3.4. LC – MS analysis

Table 1 lists masses found in selected RP-HPLC fractions (see also Fig. 2B). A total of 106
molecular species were observed in the selected fractions with isotopic masses ranging from
806,59742 Da to 16849.4139 Da (Monocharged). Masses distribution showed a high amount of
compounds with molecular masses below 2000 daltons and above 10000 daltons (Fig 3). Red bold
numbers, indicates the possible phospholipases present in the HPLC fractions. LC-MS analysis shows
the co-elution of different number of peptides on each RP-HPLC fraction.

Table 1: rT: Retention time in minutes. Molecular masses in daltons, of each collected peak from female venom
RP-HPLC in figure 2B and determined by Orbitrap indicated by [M+H]+. Values shows isotopic and the
average molecular masses in daltons (Da) and the charge (Z). Bold values indicate molecular masses matching
the molecular mass rank of phospholipases A$_2$ on fractions with this activity.
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<th>Average mass (Da)</th>
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322
Figure 3: Distribution of peptide masses obtained from high resolution LC-MS analysis of crude venoms from *Opisthacanthus elatus*.

### 3.5. LC MS/MS

After the digestion step of fraction eluting at 55.8, named Oe_55_8, MS/MS analysis showed the presence of one fragment named PLA₂Hormuritoxin-Oe55_8 according to the nomenclature recommended by King *et al.* (King *et al.*, 2008). The BLAST search of the identified peptides on this work led to the determination of homology among them and some neurotoxins identified in other Brazilian species of the *Opisthacanthus* genus such as the Phospholipase-like protein (Fragment) OS=Opisthacanthus cayaporum (UniprotKB: C5J8D1|C5J8D1_OPICY) from *Opisthacanthus cayaporum*. Table 2 shows the homology percentage obtained for each toxin. Figure 4 shows the alignment of PLA₂Hormuritoxin-Oe55_8 with the PLA₆ fragment from *O. cayaporum*. 
Table 2. Protein peptide/summary of the identified peptides. MM: calculated molecular mass. Amino acid homology (AA % ID) is from clustalW. All database numbers from UniProtKB.

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Figure 4: Alignment of PLA2Hormuritoxin-Oe55_8 peptides with another sequence. Internal peptides obtained after tryptic digestion of O. cayaprum Phospholipase-like protein (Fragment) and tandem mass spectrometry analysis, as described in materials and methods, are aligned with the following peptides: Phospholipase-like protein (Fragment) OS=Opisthacanthus cayaporum (UniprotKB: CSJ8D1|CSJ8D1_95835835)

3.6. Pharmacological activities

No antimicrobial activity was detected at the concentrations evaluated (data not shown). The whole venom of O. elatus also showed neither insecticidal nor lethal activity up to a dose of 100 mg/kg because none of the proposed groups presented any of the mentioned manifestation at the doses evaluated (Data not shown).

4. Discussion

Scorpion venoms have been widely described as a source of peptides affecting ionic channels and enhancing neurotoxic effects. Most of these neurotoxic components are structurally related to short peptides. They are known to affect the function of K+ or Na+ channels and their molecular mass below 1.5 kDa, and ranging from 3.0 kDa – 4.5 kDa and 6.0 kDa – 7.5 kDa, which have been reported specially in the Buthidae family (Pimenta et al., 2001; Rodriguez de la Vega et al., 2010). Few reports
focused in the characterization of non-neurotoxicity compounds have been carried out. Only compounds with molecular masses above 9.0 kDa of *O. cayaporum* and some *Tityus* species have been described (Batista et al., 2007; Rodriguez de la Vega et al., 2010; Schwartz et al., 2008).

This is the first pharmacological, biochemical and partial proteomic report of the *Opisthacanthus elatus* venom based in *in vitro* assays with specific enzymes substrates and MS/MS analysis, allowing the detection of phospholipase A$_2$, of serine protease activity in the whole venom and some RP-HPLC fractions, and a sequence showing homology with other phospholipase-like protein from other *Opisthacanthus* species. Both enzymatic activities prevailed over the possible neurotoxic activity, which is imperceptible at doses below 100 mg/kg, a higher concentration with respect to other neurotoxic scorpions from Colombia (Estrada-Gomez et al., 2014). Arachnids venom from this group, as well as from spiders, have been proposed as an important source of neuromodulator compounds, because the presence of peptides affecting different ionic channels, like those described in the *Androctonus* genus from the Buthidae family (Estrada-Gomez et al., 2014; Estrada-Gomez et al., 2013; Gurevitz et al., 2007; King, 2007; Nicholson, 2007). From the *Opisthacanthus* genus, only the venom from the species *Opisthacanthus cayaporum* and *Opisthacanthus madagascariensis* have been studied and characterized with the description of different compounds as peptides with modulatory activity on potassium channels and proteins exhibiting phospholipase A$_2$ activity (Chagot et al., 2005; Dai et al., 2002; Dai et al., 2001; Schwartz et al., 2008; Silva et al., 2009).

The proteolytic activity enhanced by the *O. elatus* venom preliminarily indicates the presence of proteins with serine protease activity, due to the hydrolysis of the specific substrate BapNA. This kind of proteins in arachnids have been reported as responsible for fibronectinolytic and fibrinogenolytic activities, and shows molecular masses ranging from 85 kDa to 95 kDa, as reported in other arachnids (Veiga et al., 2000). The venom SDS-PAGE from *O. elatus*, shows different bands in the same range, close and above 97 kDa, a non-common protein content distribution in the scorpion
order. So far, specific assays should be performed to confirm the presence of these proteins (enzymatic parameters, structural ID and MS\textsuperscript{o} analysis, including peptides identification). \textit{O. elatus} venom and 5 RP-HPLC fractions shows a significant indirect hemolytic activity, in presence or absence of calcium and egg-yolk. This enzymatic activity is uncommon in arachnids, except in the Sicariidae family, genus Loxoceles, where phospholipases D enzymes are present, predominant and well known, enhancing dermonecrotic, inflammatory, edema, platelet aggregation, hemolysis and renal failure (Appel et al., 2008; de Souza et al., 2008; Kusma et al., 2008). The hemolytic activity detected in the fractions from \textit{O. elatus} are surprisingly similar to the halo detected with the MHD of \textit{B. asper} venom, a well-known venom for the phospholipase A\textsubscript{2} content (Andrião-Escarso et al., 2000). This hemolytic activity is suspected of depending on the presence of PLA\textsubscript{2}, although it is important to consider that cytolytic peptides may enhance this activity as well. The following assays confirm this statement. The same chromatographic fractions enhancing hemolytic activity, eluting at 52.6, 55.5, 55.8, 56.2 and 63.9 minutes (a region named here “PLA\textsubscript{2} region” eluting between 33 and 40 \% of acetonitrile), hydrolyzed the specific PLA\textsubscript{2} substrate 4-nitro-3-octanoyloxy-benzoic acid (NOBA). This substrate (NOBA) is used to determine the phospholipase A\textsubscript{2} activity, due to the high specificity for the detection of these enzymes (Holzer and Mackessy, 1996). These fractions, with this phospholipase A\textsubscript{2} activity, eluted in the same region where Schwartz \textit{et al.} partially identified the N-terminal of 2 different compounds with phospholipase A\textsubscript{2} enzymatic activity in the venom of \textit{O. cayaporum} (Schwartz et al., 2008). The hydrolyzation of the specific PLA\textsubscript{2} substrate by the mentioned fractions, undeniably confirms the presence of PLA\textsubscript{2}. The relatively stable taxonomic composition of the Hormuridae family (formerly Liochelidae) (Lourenço and Nelson, 2003) and the described results allow us to claim the presence of proteins with phospholipase activity like those described in \textit{O. cayaporum} (Schwartz et al., 2008; Silva et al., 2009). The specific partial sequence characterization analysis performed finally confirmed the presence of phospholipase A\textsubscript{2} enzymes. The fragment detected in the MS/MS analysis showed a 100\% homology with the Phospholipase-like protein isolated from \textit{O. cayaporm} (Silva et al., 2009). The less intense indirect hemolytic activity without
calcium and egg-yolk, enhanced by the venom, may indicate that not only phospholipases are responsible for this activity, and that this venom may contain peptides with lytic activity such as the amphipathic peptides, previously reported in other scorpions of the Scorpionidae family (Corzo et al., 2001), allowing the direct lysis of red blood cells. In this way, mass analysis showed a wide percentage of small peptides with molecular masses consistent with cationic peptides reported in other Opisthacanthus species and arachnids (Giangaspero et al., 2001; Pimenta et al., 2005).

High resolution mass analysis indicates the presence of 106 different protein compounds on the selected fractions with masses ranging from 806.59742 Da to 16849.4139 Da. Mass molecular values show compounds with similar values with respect to different peptides reported in other genus of the Buthidae family, such as Tityus, where compounds ranging from 3.0 kDa to 4.5 kDa and from 6.0 kDa to 7.5 kDa overlap the mass range of known toxins acting as K+ channel blockers (KTx) and long-chain Na+ channel modulators (NaScTx), respectively (Rodriguez de la Vega et al., 2010). The chromatographic profile also shows the presence of hydrophilic compounds corresponding to two peaks eluting below 20% of acetonitrile, with masses as low as 806.59742, which may correspond to small cationic peptides as reported in other scorpions (Harrison et al., 2014).

No larvicidal or antimicrobial activity was observed at the concentrations evaluated, due to the low potential activity of the venom, with respect to other Opisthacanthus venom and other arachnids, in which antimicrobial and insecticidal toxins have been characterized and isolated (Schwartz et al., 2008; Silva et al., 2009; Vargas Munoz and Estrada-Gomez, 2014). Lethal and toxic activities are expected above 100 mg/kg, because no signs of neurotoxic effects were detected at the doses evaluated. Other Buthidae scorpions from Colombia enhanced neurotoxic effects at doses of 19.2 mg/kg, showing piloerection, excitability, salivation, lacrimation, dyspnea, diarrhea, and temporary paralysis, which are classical neurotoxic symptoms of scorpion venom (Estrada-Gomez et al., 2014). This low toxicity observed is consistent with the observation that human accidents caused by Liochelidae family (formerly Ischnuridae) scorpions are not clinically important (Lourenço, 1981).
5. Conclusion.

In conclusion, the *Opisthacanthus elatus* venom from Colombia, is apparently a venom enhancing a mechanism of action dominated by the proteolytic and phospholipase A$_2$ activities, over the neurotoxic activities expected in this arachnid order. Biochemical and mass finger print indicates the presence of 5 compounds with phospholipase A$_2$ activity. Tryptic digestion and MS/MS analysis showed the presence of phospholipase-like fragment, similar to that described in other *Opisthacanthus* genus, present in one of the fraction hydrolyzing NOBA substrate. Although no neurotoxic activity was detected at the evaluated doses, mass finger print shows the presence of compounds matching molecular masses of peptides affecting K$^+$ and Na$^+$ channels.

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