MS/MS analysis of four scorpion venoms from Colombia: a descriptive approach

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Abstract

Background: Scorpions are widely known for the neurotoxic effects of their venoms, which contain peptides affecting ionic channels. Although Colombia is recognized for its scorpion diversity, only a few studies are available describing the venom content.

Methods: In this descriptive study, we analyzed the MS/MS sequence, electrophoretic and chromatographic profile linked to a bioinformatics analysis of the scorpions Chactas reticulatus (Chactidae), Opisthacanthus elatus (Hormuridae), Centruroides edwardsii (Buthidae) and Tityus asthenes (Buthidae) from Colombia.

Results: Each scorpion showed a specific electrophoretic and chromatographic profile. The electrophoretic profiles indicate the presence of high molecular mass compounds in all venoms, with a predominance of low molecular mass compounds in the Buthidae species. Chromatographic profiles showed a similar pattern as the electrophoretic profiles. From the MS/MS analysis of the chromatographic collected fractions, we obtained internal peptide sequences corresponding to proteins reported in scorpions from the respective family of the analyzed samples. Some of these proteins correspond to neurotoxins affecting ionic channels, antimicrobial peptides and metalloproteinase-like fragments. In the venom of Tityus asthenes, the MS analysis allowed the detection of two toxins affecting sodium channels covering 50% and 84% of the sequence respectively, showing 100% sequence similarity. Two sequences from Tityus asthenes showed sequence similarity with a phospholipase from Opisthacanthus cayaporum indicating the presence of this type of toxin in this species for the first time. One sequence matching a hypothetical secreted protein from Hottentotta judaicus was found in three of the studied venoms. We found that this protein is common in the Buthidae family whereas it has been reported in other families – such as Scorpionidae – and may be part of the evolutionary puzzle of venoms in these arachnids.

Conclusion: Buthidae venoms from Colombia can be considered an important source of peptides similar to toxins affecting ionic channels. An interesting predicted antimicrobial peptide was detected in three of the analyzed venoms.

Keywords:
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Background
Scorpion venoms have evolved over 400 million years into a complex, but well elaborated library of toxins that can differ dramatically in its effects among species [1]. The diversity of protein compounds (peptides, proteins and enzymes) and non-protein compounds (salts, neurotransmitters, etc.) make these venoms a promising source of molecules with antibacterial, antifungal, antiviral, antimarial and anticancer activities [2–5], and a potential source for the design of new drugs [6,7]. The most active molecules displaying such activities are peptides that can be split into non-disulfide bridge (NDBP) and disulfide bridge (DBP) peptides, showing alpha helical linear motifs or inhibitory cysteine knots respectively. The NDBP compounds were reported recently and the main characteristic of these molecules is the lack of disulfide bridges, the cationic net charge, the sequence diversity, the hemolytic and antibacterial activity, and the relatively low molecular mass (1–4 kDa) [8]. Most of these peptides possess an amphipathic alpha-helical structure like those reported for different cationic antimicrobial molecules [3,8–15]. DBP are the major molecules described in these venoms and are characterized by containing around 30 to 70 amino acids residues and three or four disulfide bridges [3,8–15]. The major targets of these toxins are ionic channels like sodium (Nav), potassium (Kv), chlorine (Clv) or calcium (Cav) channels in the nervous system, blocking or gating the channel mechanism and thereby exhibiting a neurotoxic activity.

Despite the diversity of scorpions in Colombia, only a few studies are available describing the venom content [16–19]. No studies have been found describing the venom content of Chactas reticulatus, Opisthacanthus elatus, Centruroides edwardsii or Tityus asthenes. The only available studies report the phospholipase A2 content and activity in the venom of O. elatus, and the intraspecific biochemical differences detected in the venom of C. edwardsii from two regions in Colombia [16,17]. The genus Tityus is probably one of the most studied scorpions in South America, but from Colombia the only available studies of this genus are the proteomic analysis of Tityus pachyurus reporting specific toxins affecting Na+ and K+ channels [18,20], and the peptide content description of Tityus macracanthus [19]. There is no study available on T. asthenes. Venom from Ch. reticulatus is still completely unexplored and the venom of this species had hitherto not been described or analyzed.

Here we report the first partial amino acid sequences including the post-translational modifications (PTM) of the venom from Ch. reticulatus, O. elatus, C. edwardsii and T. asthenes, with the respective electrophoretic and chromatographic profile with analysis of their predicted antimicrobial activity and the report of different partial toxins that may affect ionic channels.

Methods
Species selection
Scorpions with epidemiologic and clinical importance in Antioquia (North-west Colombian Andean region) according to Otero et al. [21–23] Chactas reticulatus, Opisthacanthus elatus, Centruroides edwardsii and Tityus asthenes (with no or scarce previous reports), were selected for this research and kept in captivity in the serpentarium of the University of Antioquia, Medellin (COLBIOFAR-149) with water ad libitum and fed with insects (Periplaneta americana and Tenebrio molitor). One specimen of Chactas reticulatus was sourced from the municipality of El Retiro (El Salado sector) at 2100 meters above sea level (m.a.s.l), while seven specimens of Opisthacanthus elatus born in captivity from an individual from the municipality of Remedios – Antioquia were used. Four specimens of Centruroides edwardsii were from two localities in the municipality of Amaga and Medellín at 1250 m.a.s.l and 1450 m.a.s.l. respectively. Furthermore, six specimens of Tityus asthenes originating from the municipality of Carepa (Urban area) at 26 m.a.s.l. were used.

Venom extraction
Venom extraction was carried out using electro-stimulation. Metal electrodes, wetted with a saline solution, were carefully positioned on the metasoma and a block signal with an amplitude of 18V at 40-60Hz was applied twice with an interval of 5 sec using a custom-made electro-stimulator (model 01). Collected venom was transferred to dry low-protein binding vials, freeze-dried and stored at -20°C until use. These procedures were in accordance with the ethical principles in animal research adopted by the World Health Organization for the characterization of venoms. After each extraction, all animals were kept alive in captivity.

Electrophoretic profiles
All electrophoretic profiles of crude venoms were analyzed using 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) according to Laemmli [24], and stained with Coomassie blue R-250. Molecular weights were estimated using standard low range markers standards (Bio-Rad). Venoms were loaded at a concentration of 1.5 mg/ml and a final volume of 20 µL. Venom concentrations were assessed following the Biuret method using Bio-Rad Protein Assay reagent and bovine serum albumin (BSA) as standard [25–27]. A 3D scatterplot representation with the number of compounds detected in each venom based in their absence-presence in every species was performed using the software SIGMAPLOT v. 14 (Systat Software, San Jose, CA). To do so, compounds detected in each venom were grouped in four different ranges: 14 kDa to 31 kDa, 31 kDa to 45KDa, 45 kDa to 97.4 kDa, and above 97.4KDa, and plotted. Additionally, venoms showing potentially toxins affecting ion channels in the MS/MS analysis were run on 10% TRIS-TRICINE gels, and stained with Coomassie blue R-250. Molecular weights were estimated using standard broad range standards (Bio-Rad). Quantification of volumes and calculation of molecular weights were performed using the software GelAnalyzer 19.1, available at: http://www.gelanalyzer.com/ [28]. Molecular weights were calculated using the known values of the standard broad rank markers (Bio-Rad): 200 kDa, 116 kDa, 97 kDa, 66 kDa, 45 kDa, 31 kDa, 21 kDa, 14 kDa, 6 kDa. To estimate the molecular
weight, we used a simple exponential fit approximation and according to the Rf (retention factor, measured as the band distance migrated/gel length) of each analyzed band.

**Chromatographic profile**

We followed the methodology proposed by Fernandez et al. [29] and adapted by Estrada et al. [16,17] for arachnid venoms separation. One milligram of whole venom was dissolved in 200 µL of solution A (0.1% trifluoroacetic acid – 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 Prominance HPLC. Proteins were eluted by a gradient towards solution B (0.1% TFA in acetonitrile – ACN 99%) 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. The chromatographic run was monitored at 215 nm and fractions were collected, freeze-dried and stored at -20 °C until used.

**Peptide mass determination by high-resolution LC-MS**

For *Ch. reticulatus*, *C. edwardsii* and *T. asthenes* we selected the peaks with the best intensity and resolution from the RP-HPLC chromatograms. For *O. elatus*, we collected the major peak of the phospholipase region according to Estrada et al. [17], looking for a deeper characterization of this region. We wanted to see if this region exclusively contained phospholipase proteins or if there were more components co-eluting in the region. Selected dried fractions were digested and submitted to the MS/MS equipment as explained below.

**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 re-suspended in 97% purified H₂O/3% ACN/0.1% formic acid (FA). A volume of 5 µL was used for nano LC-MS/MS analysis.

**LC-MS/MS**

Fractions were 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 minutes at 300 nL/minute over the cHiPLC nanoflex system. The trap column was a Nano cHiPLC 200 µm x0.5 mm ChromXP C18-CL 3 µm x 120 Å followed by the analytical column, the Nano cHiPLC 75 µm x 15 cm ChromXP C18-CL 5 µm x 120 Å. The sample was injected into the Triple TOF 5600 and through the Nanospray III source equipped with an emission tip (New Objective, Woburn, MA, USA). 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 minute followed by a gradient of 5% B to 35% B in 90 minutes and from 35% B to 80% B in 2 minutes. Eighty percent of B was held for 5 minutes before being brought to 5% B and held for 20 minutes. PTM are reported for each containing peptide.

**Data analysis**

The data acquisition was performed monitoring 50 precursor ions at 250 ms/scan. Mascot Daemon v.2.4.0 (Matrix Science) was used for similarities searches against the different databases downloaded from the UniProt and NCBI websites. Data analysis was run in the Bindley Bioscience Center at Purdue University. Multiple sequence alignment was completed using the Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/) of MS/MS sequences from each venom with the respective similar peptide/protein.

**Bioinformatics analysis**

The search for similar peptides/proteins matching KISSV[X] NKDKI peptide was performed in Protein Information Resource (PIR) databases [30,31]. Specifically peptide matching using Apache Lucene-based search engine [32], using as query sequence the peptide without specifying the residue in the bracket [X], and each of the following residues A,I,V,S and N according to MS/MS analysis. The search was performed in the databases UniProtKB/Swiss-Prot with isoforms.

**Evaluation of the physicochemical properties**

The corresponding physicochemical properties of identified peptides following an in silico analysis, resulting in metrics for peptide length (residues), molecular weight, total hydrophobic ratio, net charge at physiological pH, and the Boman Index, were determined using the Antimicrobial Peptide Database Calculator and Predictor (APD3 http://aps.unmc.edu/AP/) [33].

**Results**

**Electrophoretic and chromatographic profiles**

The venom from each species showed a specific electrophoretic profile, and some differences were detected among the species (Figure 1A). Visibly, differences are specially observed in the high molecular mass compound (HMMC) region above 31 kDa (where HMMC like phospholipases are commonly found) (Figure 1A). Visibly, differences are specially observed in the high molecular mass compound (HMMC) region above 31 kDa (where HMMC like phospholipases are commonly found), with some compounds migrating close to 14 kDa and 21 kDa (where HMMC like phospholipases are commonly found) (Figure 1A). Venoms from *C. edwardsii* and *T. asthenes* (Buthidae) showed a very similar profile with the majority of compounds distributed above 31 kDa and with few compounds around 14 kDa (according to Figure 1B). The non-buthidae venoms from *O. elatus*, *Ch. reticulatus* shows electrophoretic profiles with visible differences; most compounds are distributed among 97 kDa and 45 kDa
A specific difference was observed below 31 kDa in the *O. elatus* venom, were at least 3 compounds were detected migrating close to 14 kDa (see Figure 1B).

As observed in the electrophoretic pattern, the chromatographic profile showed clear differences between venoms from each species (Figure 2). In all cases, we obtained complex chromatograms with good resolution and well defined peaks. As seen in the electrophoretic profile, venoms from *C. edwardsii* and *T. asthenes* (Buthidae) showed a similar profile within this sub-group, while the *Ch. reticulatus* venom showed a specific profile, displaying differences between families. Compounds from Buthidae venoms elute below 38% of ACN, while the non-Buthidae compounds elutes up to 50% of ACN or 60% of ACN for *O. elatus* and *Ch. reticulatus* respectively. In all cases, we selected well defined peaks for the MS/MS analysis (arrows in Figure 2).

**MS/MS analysis**

From all selected peaks, we obtained internal peptide sequences, matching different proteins from Buthidae scorpions. Only in the venoms from *C. edwardsii* and *T. asthenes* we found internal sequences.
Figure 2. Venom chromatographic profile. RP-HPLC chromatographic profiles of the crude venom of all scorpions using a C18 column (250 mm-4.6 mm): (A) T. asthenes, (B) C. edwardsii, (C) Ch. reticulatus and (D) O. elatus. Elution gradient used: 0-70% of acetonitrile (99% ACN in TFA 0.1%). The run was monitored at 215 nm. Arrows indicate fraction subjected to MS/MS analysis.

peptides matching neurotoxins affecting ion channels. A TRIS-TRICINE gel corroborate the presence of compounds with an estimated molecular masses similar to these neurotoxins (around to 6.5 kDa) in both venoms (see Figure 3).

The MS/MS analysis of all scorpion venoms showed toxins similar to neurotoxins affecting potassium or sodium channels, beta-neurotoxin, antimicrobial peptides and metalloproteinase-like or phospholipase-like fragments (Figure 4).

In Ch. reticulatus venom we found only one sequence that matched a hypothetical secreted protein from Hottentotta judaicus (Table 1). From the venom of O. elatus, we detected different sequences matching antimicrobial peptides, scorpine-like peptides and opistoporin, additional to the previous reported sequence matching a phospholipase A₁ from O. cayaporum (also found here) (Table 1). These compounds were all previously reported in the venom of O. cayaporum. Of the six fragments detected in C. edwardsii, three sequences showed similarity with three different peptides affecting ion channels. One fragment matched a potassium channel toxin alpha-KTx 2.2 from Centruroides margaritatus and other with a potassium channel toxin alpha-KTx 2.1 from Centruroides noxius (Table 1). Other sequences showed similarity with peptides from Hottentotta judaicus and Mesobuthus gibbosus. Venom from T. asthenes showed more than 26 hits with different proteins and peptides and the main matched organism of T. asthenes sequences belongs to a species from the same genus, Tityus discrepans (Table 1). As observed in the electrophoresis, with compounds in the range of 14-31 kDa and 31-45 kDa, some fragments from T. asthenes matched metalloproteinases (venom metalloprotease-1) and phospholipases from other Buthidae (Mesobuthus eupeus) and Hormuridae (Opisthacanthus cayaporum) scorpions.

The MS analysis of T. asthenes allowed the detection of sequences covering above 50% of two toxins affecting sodium channels. Six fragments matched one toxin affecting sodium channels (Na₇) from Centruroides noxius (Toxin Cn11), covering 84% of this toxin (Figure 5A). Additionally, two more fragments, with a similarity of 100%, covered 50% of the toxin Ts1 from T. serrulatus (Buthidae), a proven voltage-gated sodium channel (Nav) gating-modifier (see Figure 5B).

Bioinformatics analysis
In all venoms, except O. elatus, we found a sequence fragment--KISSV[IN]NKDKI – with residue number six varying between I (isoleucine) or N (asparagine), depending on the species.
Figure 3. (A) TRIS-TRICINE electrophoresis analysis of *T. asthenes* (lane #1) and *C. edwardsii* (lane #2) venoms. (B) Intensity profile of each detected lane and bands in the TRIS-TRICINE electrophoresis gel. The bottom of each image shows the respective electrophoretic run. The intensity of each band is reported in arbitrary units. (C) Estimated molecular weights of bands detected below 6.5 kDa, red box for lane #1 (*T. asthenes*) and yellow box for lane #2 (*C. edwardsii*). Molecular weight estimation according the MW calibration curve analyzed with a simple exponential fit approximation with a $R^2$ of 0.99. Rf: retention factor; MW: estimated molecular weight.

Figure 4. Matrix plot performed with presence/absence data of matched peptide family with MS/MS peptide sequence found in *Chactas reticulatus*, *Opisthacanthus elatus*, *Centruroides edwardsii* and *Tityus asthenes* venoms.
| Fragment given name | MS/MS peptide sequence | Score/Identity | Matching peptide acc. number | Matched peptide family or name | Expected peptide m/z | Z | Calculated peptide mass | Matched organism |
|---------------------|-------------------------|----------------|-----------------------------|--------------------------------|----------------------|---|-------------------------|-----------------|
| **Chactas reticulatus** |                         |                |                             |                                |                      |   |                         |                 |
| ChrP1a              | *K-ISSV[N]NKDK-I        | 26-91% [I] / 26-100% [N] | F1CJ08                     | Hypothetical secreted protein | 523.775              | +2 | 1045.54 £               | Hottentotta judaicus |
| **Opisthacanthus elatus** |                         |                |                             |                                |                      |   |                         |                 |
| Oe1                 | K-YGITNDSFFT-K-L        | 226/100%       | C5JD1                       | Phospholipase-like protein (fragment) | 646,806              | +2 | 1291,597                | Opisthacanthus cayaporum |
| Oe2                 | K-KAWNSPLANE-K-S        | 156/100%       | C7C1L2                      | Probable antimicrobial peptide | 457,584              | +3 | 1369,731                | Opisthacanthus cayaporum |
| Oe3                 | - GWINEEIQ-Q-K          | 117/100%       | P86121                      | Scorpine-like (fragment)      | 622,835              | +2 | 1243,655                | Opisthacanthus cayaporum |
| Oe4                 | R-KLGAQAMTDFIK-K        | 28/100%        | C5J889                      | Putative uncharacterized protein (fragment) | 661,859              | +2 | 1321,704                | Opisthacanthus cayaporum |
| Oe5                 | K-NFVAEKGATPS-          | 18/100%        | P83314                      | Opistoporin-2                | 617,325              | +2 | 1232,636                | Opisthacanthus cayaporum |
| **Centruroides edwardsii** |                         |                |                             |                                |                      |   |                         |                 |
| CedP1a              | K-AQFGQSAGAK-C          | 717/100%       | P40755                      | Potassium channel toxin alpha-KTx 2.2 | 482.727              | +2 | 963.477                 | Centruroides margaritatus |
| CedP2a              | *TIINVOKCTSPK-Q         | 13/100%        | P08815                      | Potassium channel toxin alpha-KTx 2.1 | 645.371              | +2 | 1288.706                | Centruroides noxius |
| CedP3a              | *K-ISSV[N]NKDK-I        | 26-91% [I] / 26-100% [N] | F1CJ08                     | Hypothetical secreted protein | 523.775              | +2 | 1045.54 £               | Hottentotta judaicus |
| CedP4a              | *R-SGTPEKER-E           | 15/100%        | P0DM19                      | Antimicrobial peptide HsAp3   | 473.236              | +2 | 944.456                 | Heterometrus spinifer |
| CedP5a              | K-TKNETGFCFLPNENK-C     | 15/100%        | F1CJ17                      | Venom peptide TKSx2           | 598.949              | +3 | 1793.825                | Hottentotta judaicus |
| CedP6a              | *K-EETWCNLIRYKK-K       | 14/100%        | A0A059UED3                  | Uncharacterized protein       | 417.720              | +4 | 1666.828                | Mesobuthus gibbosus |
| **Tityus asthenes**  |                         |                |                             |                                |                      |   |                         |                 |
| TaP1a               | *KDGYIIHGR-G            | 1259/100%      | P0CF39                      | Bactridin-1                   | 586.082              | +2 | 1171.599                | Tityus discrepans |
| TaP1b               | *K-KGSSGYCAWPACWCYGLPDNVK-I | 1259/100%     | P0CF39                      | Bactridin-1                   | 586.082              | +2 | 1171.599                | Tityus discrepans |
| TaP2a               | K-IFDYYNNK-C            | 350/100%       | C9X4K1                      | Toxin TdNa3                   | 538.775              | +2 | 1075.497                | Lychas mucronatus |
| TaP3a               | *R-KDLYLDK-N            | 53/100%        | P0CI50                      | Neurotoxin LmNaTx15.1         | 565.796              | +2 | 1129.529                | Tityus pachyurus |
| TaP4a               | ADDDLEGFSEEDKLAK-E      | 335/100%       | P0DL22                      | Toxin Tpa3                   | 632.303              | +3 | 1893.884                | Tityus pachyurus |
| Fragment given name | MS/MS peptide sequence | Score/Identity | Matching peptide acc. number | Matched peptide family or name | Expected peptide m/z | Z | Calculated peptide mass | Matched organism |
|---------------------|-------------------------|----------------|-----------------------------|--------------------------------|----------------------|---|------------------------|----------------|
| TaP5a               | R-NKINGMK-F             | 142/100%       | E4VNZ7                      | Venom metalloprotease-1       | 402.711              | +2 | 803.432                | Mesobuthus eupeus |
| TaP5b               | R-ALDQDLELR-L           | 141/100%       | P60215                      | Potassium To4                 | 602.750              | +2 | 1203.49                | Tityus obscurus  |
| TaP5c               | *R-TGSKDCPASGYIMGDR-N   | 93/100%        | Q0GY43                      | Putative beta-neurotoxin      | 565.276              | +2 | 1310.527               | Tityus pachyurus |
| TaP6a               | K- GTFCAEECTR-M         | 81/100%        | H1ZZ11                      | Hypothetical secreted protein | 523.767              | +2 | 1045.54                | Hottentotta judaicus |
| TaP7a               | K-SEYACPVIDK-F          | 66/100%        | P0DL23                      | Voltage-gated sodium channels (Nav) gating-modifier. | 876.402              | +2 | 2626.169               | Tityus serrulatus |
| TaP10a              | K-LEPADILAK-D           | 32/100%        | P15226                      | Probable antimicrobial peptide | 457.569              | +3 | 1369.735               | Opisthacanthus cayaporum |
| TaP11a              | K-YGITNDSFFTK-L         | 54/100%        | C5JB1                       | Phospholipase-like protein    | 646.810              | +2 | 1291.609               | Opisthacanthus cayaporum |
| TaP12a              | *K-ISSV[IN]NKDK-I       | 26-91% [I]/26-100% [N] | F1CJ08                      | Hypothetical secreted protein | 523.767              | +2 | 1045.54 £              | Opisthacanthus cayaporum |
| TaP13a              | K-VWDRATNK-C            | 39/100%        | P58296                      | Toxin Cn11                    | 576.246              | +2 | 1150.465               | Centruroides noxius |
| TaP13b              | *K-KGSSGYCAWPACYGLPNWVK-V | 18/100%        | P60262                      | Toxin Td1-3                   | 671.327              | +2 | 1340.628               | Tityus discrepans |

Z: indicates charge, while m/z indicates relation between mass and charge. £: indicates molecular weight calculated with asparagine residue and with lysine PTM. *Fragments with (K) acetylations, the molecular weight includes the PTM. Amino acids in square brackets indicate that we detected sequences containing both amino acids at that position.
The search for similar sequences that included the fragment, without specifying the residue in position number six [X], did match with a sequence of *Hottentotta judaicus* with accession number F1CJ08. When the search was performed with the residue “A”, it matched with seven sequences, five of *Androctonus bicolor* (A0A0K0LCB5, A0A0K0LC6, A0A0K0LC9, A0A0K0LCD6 and A0A0K0LCD7), one of *Odontobuthus dorai* (A0A0U3YCW0), and one of *Mesobuthus eupeus* (E4VP36). Finally, with one of the residues “IVSN”, it matched with a previously reported fragments in the venoms of *Androctonus amoreuxi, Pandinus imperator, Tityus fuhrmanni* and *Grosphus grandieri* [34].

**Figure 6** shows the families and species where the fragment has been reported before.

### Evaluation of the physicochemical properties

Predicted physicochemical properties of – KISSV[AIVSN] NDKDI - based in the amino acid content and the net charge of this peptide, indicates that the fragment may be part of an antimicrobial protein (Table 2). Despite all possible amino acids sequences detected in the KISSV[X]NDKDI peptide, all conformations show a net predicted charge of 2+ and a similar hydrophobic residue percentage. Only peptides with residues KISSV[AIV]NDKDI are predicted to form an alpha helix showing a higher hydrophobic residue percentage. According to our MS/MS results, only KISSV[X]NDKDI with the Isoleucine (I) amino acid residue in the sequences from *Chactas reticulatus, Centruroides edwardsii* and *Tityus asthenes* may enhance antimicrobial activity in these venoms.

### Discussion

Peptides are the dominant components of scorpion venoms and the primary source of their pharmacological diversity, becoming a natural source of bioactive compounds [6,36]. For this reason, scorpions are the focus of different studies attempting to describe the peptide content of their venoms in recent years.

Although most scorpion venom electrophoresis are carried out using TRIS-TRICINE gels to visualize low molecular mass compounds, using SDS-PAGE gels allowed us to see the rich content of HMMC in each species.

Buthidae venoms from Colombia are rich in peptides affecting ionic channels and peptides with antimicrobial activity [34]. Although *Tityus* and *Centruroides* are widely distributed and studied in Colombia, most of the studies are focused on epidemiological aspects. Only four studies are available analyzing the composition of the venom of these two epidemiologically relevant scorpion genera; two characterizing the venom from *T. pachyurus*, one characterizing the peptide content of *Tityus macrochirus*, and one reporting an intraspecific difference in the biochemical and biological activity of *C. edwardsii* venom from two populations in different regions in Colombia [16]. In the former, the authors described the presence of a potent potassium-channel blocker and putative sodium scorpion channel toxins (NaScTxs) in the *Tityus* genus [18,20]. The present work indicates that *T. asthenes* and *C. edwardsii* seems to be an important source of toxins affecting ionic channels. Matched toxins from *T. asthenes* and *C. edwardsii* correspond to neurotoxins reported in other Buthidae scorpions like *Tityus discrepans, Tityus pachyurus, Tityus obscurus, Centruroides margaritatus* or *Centruroides noxius*, from Colombia, México, Venezuela or Brazil [18,37]. In *T. asthenes* we detected 16 different fragments from this venom (TaP2a, TaP3a, TaP6a, TaP7a, TaP8a, TaP9a, TaP9b, TaP13a, TaP13b, TaP15a, TaP16a, TaP16b, TaP16c, TaP16d, TaP16e, TaP17a), matching nine different toxins affecting sodium channels and one affecting potassium channels [37–39]. All these toxins, reported in other scorpions, can inhibit sodium currents, inhibit the inactivation of the activated channels, affect sodium channel activation by shifting the voltage of activation toward more negative potentials, or block the voltage-gated potassium channels. Some of these toxins were described in other *Tityus* species from different countries. Fragments TaP16a, TaP16b, TaP16c, TaP16d, TaP16e, matching 84% of the Cn11 amino
acid sequence from *C. noxius* (Buthidae) and fragments TaP13a and TaP13b covering 50% of the toxin Ts1 from *T. serrulatus* (Buthidae) with a similarity of 100% in both cases, indicating the presence of sodium channel blockers in this venom. In the venom of *C. edwardsii*, two more fragments (CedP1a and CedP2a) were detected, matching peptides affecting potassium channels, and were described as potent inhibitors of voltage-gated potassium channels [40,41]. Although there is no previous report of the peptide or protein content of these two Buthidae scorpions from Colombia (*T. asthenes* and *C. edwardsii*), venom characterization of other Buthidae indicates that neurotoxins are the major content of these venoms, just as seen in our results [42–44]. We found four more fragments (TaP1a, TaP1b, TaP14a and CedP4a) from *T. asthenes* and *C. edwardsii* that are similar to antimicrobial peptides (AMP), active against Gram-negative and Gram-positive bacteria and fungi [13]. Many AMP have been described before in scorpion venoms [3,45], but this is the first report of the presence of these bio-active compounds in scorpion venoms from Colombia. Venom content from *O. elatus* seems to be very similar to that reported in *O. cayaporum* from Brazil [46]. All five fragments matched toxins reported in *O. cayaporum*, including a phospholipase-like protein and a probable

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**Table 2.** Physicochemical properties of the KISSV[AIVSN]NKDKI peptide calculated in APD3 (Antimicrobial Peptide Calculator and Predictor).  

| Physicochemical properties                  | A** | I** | V** | S*  | N*  |
|--------------------------------------------|-----|-----|-----|-----|-----|
| Length (residues)                          | 11  | 11  | 11  | 11  | 11  |
| Molecular weight (MW)                      | 1202.406 | 1244.487 | 1230.46 | 1218.405 | 1218.405 |
| Net charge at physiological PH (7.4)       | 2   | 2   | 2   | 2   | 2   |
| Hydrophobic residues (%)                   | 36  | 36  | 36  | 27  | 27  |
| Borman Index (kcal/mol)                    | 2.1 | 1.81| 1.89| 2.57| 2.57|
| Similar Peptide /%                         | AP01814/ 45.45 | AP02863/ 42.85 | AP02863/ 41.66 |

*According to APD3 prediction, this peptide cannot form an alpha helix that is long enough to be an AP. **Predicted short alpha-helical cationic antimicrobial peptide.
antimicrobial peptide [46]. The O. elatus phospholipase region analyzed seems to be not exclusive for the elution of this proteins but also antimicrobial peptides. We expected to find many more sequences in major fraction in the Ch. reticulatus venom (as observed in the T. asthenes venom), the lack of recovered peptide families may be due to their absence, or due to there being very few Chactas sequences in the reference database.

SDS-PAGE and TRIS-TRICINE electrophoresis together with the MS/MS analysis allowed the detection of different HMMC and low molecular mass compounds (LMMC) in both Tityus and Centruroides venoms matching molecular weighs similar to neurotoxins, phospholipases or metalloproteinases. In Colombia, this HMMC had only been described in the venom from the scorpion Opisthacanthus elatus and the spider Pandophobeteus verdolaga [17,47], but never in Buthidae scorpions. HMMC are quite commonly distributed proteins in arachnids. Their main biological activities include housekeeping functions or enzymatic activities, like phospholipases or hyaluronidase [48–52]. Despite the clinical importance of these proteins, they are among the less studied venom components. These HMMC had been previously reported in the venoms from T. bahiensis, where the 32.69% of its venom content correspond to metalloproteinases [42].

In all cases, further proteomic studies are necessary to complete the MS/MS analysis of these important sources of bioactive compounds.

In three of the four venoms analyzed by mass spectrometry, we detected a common fragment with a variant in the 6th amino acid residue where a Isoleucine (I) can be replaced by an Alanine (A), Valine (V) or cysteine (C) [34]. Is very important to consider that according to prediction results, only KISSV[X]NKDK1 with the Isoleucine (I) amino acid residue in its sequences from Chactas reticulatus, Centruroides edwardsii and Tityus asthenes may enhance an antimicrobial activity in these venoms.

Currently, 20 scorpion families are recognized [35,53], and only 45 species have a transcriptomic analysis available, including Buthidae (with 22 species) and non-buthidae families (with 23 species). Considering that the Buthidae family has the highest number of species with a transcriptome available, proteins similar with our peptide (KISSV[IN]NKDK1) have mainly been described in the family Buthidae, with some reports in the Chaetidae and Scorpionidae families. The presence of this protein mainly in the Buthidae family may suggest a recruitment of this peptide before Buthidae split from non-buthid species, as suggested by He et al. [54] for Chaerilus trisostatus and Chaerilus tryznaithe (Chaerilidae). Their evolutionary analysis showed that the NaTx, β-KTx, and bpp-like toxin types were recruited into the venom before the lineage split between Buthidae and non-Buthidae families. Similarly, Ma et al. [55] studied the evolution of the scorpion venom by comparative transcriptome analysis of venom glands and phylogenetic analysis of shared types of venom peptides and proteins between buthids and euscorpiids. This analysis revealed that at least five of the seven common types of venom peptides and proteins were likely recruited into the scorpion venom proteome before the lineage split between Buthidae and Euscorpiidae (i.e. basal in extant scorpions) with their corresponding genes undergoing individual or multiple gene duplication events.

**Conclusion**

The analyzed Buthidae venoms from Colombia may be considered a rich source of peptides similar to toxins affecting ionic channels. The Opisthacanthus elatus phospholipase region is composed not only of phospholipases but also of peptides with compounds similar to antimicrobial peptides. An interesting predicted antimicrobial peptide was detected in three of the analyzed venoms. When compared with the literature, this peptide is present in other scorpion families indicating a probable ancient peptide. The search for similar proteins that match with query peptides suggest that multiple types of venom peptides, including antimicrobial peptides, could have been recruited into the venom proteome during, before or at the basal split in the phylogeny of extant scorpions. In all cases, further proteomic studies are necessary to complete the MS/MS analysis of these important sources of bioactive compounds.

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**Availability of data and materials**

All data generated or analyzed during this study are included.

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Competing interests
The authors declare that they have no competing interests.

Authors' contributions
All authors contributed with the manuscript preparation, experiments and data analysis. All authors read and approved the final manuscript.

Ethics approval
The present study was approved by the Comité de Ética para Experimentación en Animales de la Universidad de Antioquia (CEEA, University of Antioquia – Resolución Rectoral 18084), minute no. 123-2019.

Consent for publication
Not applicable.

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