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Toxicovenomics and antivenom profiling of the Eastern green mamba snake (*Dendroaspis angusticeps*)

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Running title: Proteomics of green mamba venom

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Abstract

A toxicovenomic study was performed on the venom of the green mamba, *Dendroaspis angusticeps*. Forty-two different proteins were identified in the venom of *D. angusticeps*, in addition to the nucleoside adenosine. The most abundant proteins belong to the three-finger toxin (3FTx) (69.2%) and the Kunitz-type proteinase inhibitor (16.3%) families. Several sub-subfamilies of the 3FTxs were identified, such as Orphan Group XI (Toxin F-VIII), acetylcholinesterase inhibitors (fasciculins), and aminergic toxins (muscarinic toxins, synergistic-like toxins, and adrenergic toxins). Remarkably, no α-neurotoxins were identified. Proteins of the Kunitz-type proteinase inhibitor family include dendrotoxins. Toxicological screening revealed a lack of lethal activity in all RP-HPLC fractions, except one, at the doses tested. Thus, the overall toxicity depends on the synergistic action of various types of proteins, such as dendrotoxins, fasciculins, and probably other synergistically-acting toxins. Polyspecific antivenoms manufactured in South Africa and India were effective in the neutralization of venom-induced lethality. These antivenoms also showed a pattern of broad immunorecognition of the different HPLC fractions by ELISA and immunoprecipitated the crude venom by gel immunodiffusion. The synergistic mechanism of toxicity constitutes a challenge for the development of effective recombinant antibodies, as it requires the identification of the most relevant synergistic toxins.

(197 words)

Keywords: *Dendroaspis angusticeps*; Green mamba; Snake venom; Proteomics; Toxicovenomics: Antivenoms.

Biological significance

Envenomings by elapid snakes of the genus *Dendroaspis*, collectively known as mambas, represent a serious medical problem in sub-Saharan Africa. The development of novel antivenoms and of recombinant neutralizing antibodies demands the identification of the most relevant toxins in these venoms. In this study, a bottom-up approach was followed for the study of the proteome of the venom of the Eastern green mamba, *D. angusticeps*. Forty-two different proteins were identified, among which the three-finger toxin (3FTx) family, characteristic of elapid venoms, was the most abundant, followed by the Kunitz-type proteinase inhibitor family. In addition, several other protein families were present in the venom, together with the nucleoside adenosine. No α-neurotoxins were identified within the family of 3FTxs in the venom of *D. angusticeps,* in contrast to the venom of *D. polylepis*, in which α-neurotoxins are largely responsible for the toxicity. With one exception, HPLC fractions from *D. angusticeps* venom did not kill mice at the doses tested. This underscores that the toxicity of the whole venom is due to the synergistic action of various components, such as fasciculins and dendrotoxins, and probably other synergistically-acting toxins. Thus, the venoms of these closely related species (*D. angusticeps* and *D. polylepis*) seem to have different mechanisms to subdue their prey, which may be related to different prey preferences, as *D. angusticeps* is predominantly arboreal, whereas *D. polylepis* lives mostly in open bush country and feeds mainly on mammals. It is therefore likely that the predominant clinical manifestations of human envenomings by these species also differ, although in both cases neurotoxic manifestations predominate. Polyspecific antivenoms manufactured in South Africa and India were effective in the neutralization of venom-induced lethality in mice and showed a pattern of broad immunorecognition of the various venom fractions. It is necessary to identify the toxins responsible for the

synergistic mode of toxicity in this venom, since they are the targets for the development of recombinant antibodies for the treatment of envenomings.

1. Introduction

The Eastern green mamba (*Dendroaspis angusticeps*) is a highly venomous elapid found primarily in southeastern Africa (Figure 1). First described by Smith in 1848 [1], *D. angusticeps* is a relatively small mamba species, averaging 1.4 m in length. Due to its arboreal, shy, and elusive nature, human envenomings are less frequent than those inflicted by the more territorial *Dendroaspis polylepis* (black mamba) [2,3]. Adult specimens of *D. angusticeps* have a brilliant emerald to lime green coloration, providing them with an excellent camouflage in their natural habitat of the tropical rainforests in the coastal lowlands of Southeast Africa [4]. *D. angusticeps* is, however, also found in areas with coastal bush, dune, and montane forest [5], as well as in closer proximity to humans, when residing in farm trees, such as citrus, mango, coconut, and cashew [6]. Due to its color and habitat, *D. angusticeps* is often mistaken for a harmless tree snake, why people often do not take proper precaution [2,3]. *D. angusticeps* preferably preys on warm-blooded animals, such as rodents, bats, birds, and nestlings, but also on eggs [4].

Despite a low number of human envenomings reported, but due to its potent neurotoxic venom, *D. angusticeps* is classified as a category 1 snake, which is the highest level of medically important snakes, according to the WHO [7]. Furthermore, its high abundance, particularly in Kenya, Tanzania, Mozambique, Malawi, eastern Zimbabwe, and the Republic of South Africa, makes this a snake of high epidemiological relevance [7]. Severe envenomings by *D. angusticeps* can lead to rapid mortality within only 30 minutes of a bite [6]. The typical clinical manifestations include swelling of the bitten area, dizziness, nausea, difficult breathing, irregular heartbeat, and respiratory paralysis [6]. These life-threatening symptoms may escalate rapidly, but deaths are rare when effective antivenom is administered timely [6].

Given the medical importance of *D*. *angusticeps,* it is necessary to have a thorough understanding of the composition of its venom, as well as of the underlying mechanisms for venom pathophysiology in human victims. Furthermore, preclinical assessment of antivenoms is critical for predicting efficacy of snakebite envenoming therapy, which may be used to guide clinicians in the treatment of snakebites by *D*. *angusticeps*. Currently, only the SAIMR Polyvalent Snake Antivenom from the South African Vaccine Producers is claimed to be effective against *D*. *angusticeps*, although it is possible that other polyvalent antivenoms raised against the venoms of other mamba species may be effective in neutralization of *D*. *angusticeps* venom.

The venom of *D*. *angusticeps* has not undergone a full proteomics evaluation, and its quantitative protein composition is not known. Nevertheless, several biochemical and pharmacological studies have been performed on different toxins from *D*. *angusticeps* venom [8–11]. These studies report that this venom contains several neurotoxins, such as the fasciculins [10] and dendrotoxins [8,9], which are unique to the *Dendroaspis* genus [12,13]. This venom also contains a number of other toxins of the three-finger toxin family (3FTx), such as muscarinic toxins, adrenergic toxins, and synergistic-type toxins [14–16].

The dendrotoxins, of structural similarity to the Kunitz-type serine protease inhibitors, target the presynaptic voltage-gated potassium channels with high specificity, facilitating the release of acetylcholine from the presynaptic nerve terminals, causing excitatory activity [17,18]. Other important neurotoxins of the 3FTx family present in *D*. *angusticeps* venom are the fasciculins, which prolong the presence of acetylcholine in the neuromuscular junction by inhibiting acetylcholinesterase, leading to muscle fasciculations [10]. Although the venom composition of *D*. *angusticeps* has not been elucidated, a study of the venom of the closely related and more feared relative, *D*.

polylepis (black mamba), has recently been reported [19]. According to this study, *D*. *polylepis* venom is dominated by α-neurotoxins from the 3FTx family and dendrotoxins (BPTI-type/Kunitz type protease inhibitors). It is therefore of relevance to study the venom proteome of *D*. *angusticeps* in order to identify similarities and differences with that of *D*. *polylepis*.

Toxicovenomics defines the recent convergence between toxicological evaluation of toxins and venomics [20,21]. Together with antivenomics, this tool may help provide a better understanding of *D*. *angusticeps* venom, the relative importance of different proteins for toxicity, and how venom toxicity may best be abrogated. While previous investigations of *D*. *angusticeps* have focused on the biochemical and pharmacological features of the toxins, recent advances in the field of venomics and antivenomics facilitate development of novel antivenoms through rational and knowledge-based interpretation of pharmacological relevant toxins [22].

Here, we report the first full toxicovenomics analysis of *D*. *angusticeps*, a quantitative estimation of its venome, and a preclinical and immunochemical assessment of three antivenoms against *D*. *angusticeps* venom.

2. Materials and Methods

2.1 Snake venom

Venom from *D*. *angusticeps* was obtained from Latoxan SAS, Valence, France, from a pool of 50 specimens collected in Tanzania.

2.2 Venom separation by reverse-phase HPLC and SDS-PAGE

The 'snake venomics' analytical strategy [23] involving fractionation of crude venom by a combination of RP-HPLC and SDS-PAGE separation steps, was followed.

158 Venom (2 mg) was dissolved in 200 µL of water containing 0.1% trifluoroacetic acid
159 (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C_{18} column (250 x
160 4.6 mm, 5 µm particle; Teknokroma). Elution was carried out at 1 mL/min by applying
161 a gradient towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–
162 15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9
163 min, as previously described [24]. Fractions, collected manually, were dried in a
164 vacuum centrifuge, redissolved in water, reduced with 5% β-mercaptoethanol at 100 °C
165 for 5 min, and further separated by SDS-PAGE in 15% gels. Proteins were stained with
166 colloidal Coomassie blue G-250, and gel images were acquired on a ChemiDoc®
167 recorder using ImageLab® software (Bio-Rad).
168
169 *2.3 Protein identification by tandem mass spectrometry of tryptic peptides*
170 Protein bands were excised from the polyacrylamide gels and subjected to
171 reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-
172 gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate
173 at 37 °C. Tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and
174 analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied
175 Biosystems). Peptides were mixed with an equal volume of saturated α-cyano-
176 hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 µL) onto an
177 Opti-TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra
178 were acquired using 500 shots at a laser intensity of 3000. Selection of the ten most
179 intense precursor ions was done automatically and their TOF/TOF fragmentation
180 spectra were acquired using 500 shots at a laser intensity of 3900. External calibration
181 in each run was performed with CalMix® standards (ABSciex) spotted onto the same
182 plate. Resulting spectra were searched against the UniProt/SwissProt database for

Serpentes (20150217) using ProteinPilot® v.4 and the Paragon® algorithm (ABSciex) at ≥ 95% confidence, or, in few cases, manually interpreted and the deduced sequences searched using BLAST (http://blast.ncbi.nlm.nih.gov) for protein family assignment by similarity.

2.4 Relative protein abundance estimations

Relative abundance of the venom proteins was estimated by integrating the areas of their chromatographic peaks at 215 nm, roughly corresponding to peptide bond abundance, using the ChemStation® software (Agilent) [23]. In the case where HPLC peaks contained several electrophoretic bands, their percentage distributions were assigned by densitometry, using ImageLab® (Bio-Rad). Finally, for electrophoretic bands in which more than one protein was identified by MALDI-TOF-TOF, their percentage distributions were estimated on the basis of the corresponding intensities of the intact protein ions, as observed in the nESI-MS analysis. Intensities lower than 5% (relative to the major protein ions in such mixtures) were considered as traces.

2.5 Adenosine analysis

The presence of the nucleoside adenosine was determined by spiking a sample of 2 mg of venom with 10 µg of adenosine and separating it by RP-HPLC as described in section 2.2. If the adenosine coincided with a peak already present in a crude venom sample (as judged by the increment in the height of the peak), and if this venom peak showed an ESI-MS spectrum essentially identical to adenosine, the identity of venom component was judged to be adenosine. Further confirmation of the molecular identity of adenosine was obtained by acquiring its collision-induced dissociation MS/MS spectrum in positive mode, using the Enhanced Product Ion tool of Analyst v1.5 in the

QTrap3200 mass spectrometer, to show the expected reporter ion transition 268 → 136. Nucleoside abundance was estimated by deriving un-spiked nucleoside concentration from integrating the areas of both spiked and un-spiked chromatographic peaks.

2.6 In vitro enzymatic activities

2.6.1. Phospholipase A_2 activity

Assay of PLA_2 activity was carried out using the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) [25]. Twenty-five µL of solution containing various amounts of venom were mixed with 200 µL of 10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0, and 25 µL of NOBA to achieve a final substrate concentration of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at 405 nm in a microplate reader. For comparative purposes, the activities of the venoms of *D. polylepis* and the viperid snake *Bothrops asper* were also assessed.

2.6.2 Proteinase activity

Proteinase activity was assayed by adding 20 µg of venom to 100 µL of azocasein (10 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$ buffer, pH 8.0), and incubated for 90 min at 37 °C. The reaction was terminated by addition of 200 µL of 5% trichloroacetic acid, and after centrifugation (5 min, 6000 *g*), 150 µL of supernatants were mixed with 100 µL of 0.5 M NaOH, and absorbances were recorded at 450 nm. The absorbance of azocasein incubated with distilled water alone was used as a blank, being subtracted from all readings [26]. For comparative purposes, the activities of the venoms of *D. polylepis* and the viperid snake *Bothrops asper* were also assessed.

233

234 *2.7 Toxicological profiling*

235 *2.7.1 Animals*

236 *In vivo* assays were performed in CD-1 mice of both sexes, provided by Instituto
237 Clodomiro Picado, following protocols approved by the Institutional Committee for the
238 Use and Care of Animals (CICUA), University of Costa Rica. Mice were provided food
239 and water *ad libitum*.

240

241 *2.7.2 Toxicity of crude venom and isolated venom fractions*

242 The lethality of the whole venom and venom fractions was tested by intravenous
243 (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of
244 venom or venom fractions were dissolved in phosphate-buffered saline (PBS; 0.12 M
245 NaCl, 0.04 M sodium phosphate buffer, pH 7.2) and injected in the caudal vein, in a
246 volume of 100 µL. Deaths occurring within 24 h were recorded, and LD_{50}s were
247 calculated by probits [27], using the BioStat® software (AnalySoft).

248 The acute toxicity of venom fractions was initially screened by selecting a dose
249 based on fraction abundance in the venom and assuming a venom yield of 75 mg for *D.*
250 *angusticeps* (http://snakedatabase.org/pages/LD50.php#legendAndDefinitions),
251 Laustsen et al.'s Toxicity Score [20], and 50 kg as the weight of a human being. On this
252 basis, a cutoff dose (mg/kg) was selected and tested for each fraction. Fractions that
253 were not lethal at this dose (corresponding to a Toxicity Score below 7) were considered
254 as having insignificant acute toxicity, whereas fractions which did kill mice at this level
255 were further evaluated, and precise LD_{50}s were determined for them.

256

257 *2.8 Antivenoms*

Polyspecific antivenoms from the following manufacturers were used: (a) SAIMR (South African Institute for Medical Research) Polyvalent Snake Antivenom from South African Vaccine Producers (Pty) Ltd (batch number BC02645, expiry date 07/2016); (b) Snake Venom Antivenom (Central Africa) from VINS Bioproducts Ltd (batch 12AS13002, expiry date 04/2017); (c) Snake Venom Antivenom (African) from VINS Bioproducts Ltd (batch 13022, expiry date 01/2018). In addition, the monospecific *Micrurus nigrocinctus* Anticoral Antivenom from Instituto Clodomiro Picado (batch 5310713ACLQ, expiry date 07/2016) was used for comparison in certain experiments.

2.9 Immunoreactivity of antivenoms against crude venom and venom fractions by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 1.0 μg of each HPLC venom fraction, or crude venom, dissolved in 100 μL PBS. After a washing step, wells were blocked by adding 100 μL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma), and incubated at room temperature for 1 h. Plates were then washed five times with PBS. A dilution of each antivenom in PBS + 2% BSA was prepared. 100 μL of antivenom solution was added to each well in triplicates and incubated for 2 h. Plates were then washed five times with PBS. 100 μL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline phosphatase in PBS + 1% BSA) was then added to each well. The plates were incubated for 2 h, and then washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, pH 7.4). Development of color was attained by addition of 100 μL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8) and absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific).

2.10 Double immunodiffusion of antivenoms against venoms from D. polylepis and D. angusticeps

Agarose was dissolved in 30 mL of PBS to attain 1% concentration, and poured into a Petri dish. Six holes were punched in the gel, and 50 µl of antivenom was placed in the center well, while 30 µl of solutions of *D. angusticeps* and *D. polylepis* venoms were added to the surrounding wells at variable concentrations (0.5, 1 and 2 µg/µL). After overnight incubation at room temperature, agarose gels were photographed using a ChemiDoc® recorder and ImageLab® software.

2.10 Neutralization studies with antivenoms

Mixtures containing a fixed amount of venom and variable dilutions of antivenoms were prepared using PBS as diluent and incubated at 37 ºC for 30 min. Controls contained PBS instead of antivenom. Aliquots of 100 µL of the solutions, containing 4 LD_{50}s of venom (64 µg/mouse), were then injected i.v. into groups of four mice (18-20 g). Deaths occurring within 24 h were recorded for determining the neutralizing capacity of antivenoms. Neutralization was expressed as the Median Effective Dose (ED_{50}) of antivenom, defined as the ratio mg venom/mL antivenom at which 50% of the injected mice were protected. The ED_{50}s as estimated by probits, as described in Section 2.6.2.

3.0 Results and Discussion

3.1 Venomics

SDS-PAGE separation of venom proteins revealed similarities and differences between the venoms of *D. angusticeps* and *D. polylepis* (Figure 2). Both venoms

showed predominantly low molecular mass bands, in addition to a number of bands of a wide range of molecular masses, including some large proteins with molecular masses above 100 kDa. When SDS-PAGE was run under non-reducing conditions, the venom of *D. angusticeps* showed more bands than that of *D. polylepis*. In particular, *D. angusticeps* venom presented three bands of molecular masses between 18 and 22 kDa, which were absent in the venom of *D. polylepis*. In turn, *D. polylepis* venom had a band of 37 kDa, absent in *D. angusticeps* venom. In contrast, with the exception of a 25 kDa band in the venom of *D. angusticeps*, the majority of these intermediate molecular mass bands were not observed in reduced gels, indicating that these bands were comprised of higher order protein complexes.

A bottom-up proteomic characterization of *D. angusticeps* venom was carried out. Using RP-HPLC, the venom was resolved into 29 fractions, where the first three eluting from the column did not contain proteins as evidenced by electrophoresis. After SDS-PAGE separation, the remaining 26 fractions were resolved into 63 bands (Figure 3), of which 59 resulted in positive identifications upon in-gel digestion and MALDI-TOF-TOF analysis, whereas 4 remained unknown. In total, 42 different proteins were identified within these bands (Table 1). In certain cases, exemplified by fraction number 5, the bands separated by SDS-PAGE contained the same protein in both monomer and dimer forms.

Fractions 1-3 did not contain proteins according to SDS-PAGE. Due to its high abundance, fraction 1 was analyzed by direct infusion using nESI-MS/MS, which revealed a component with a molecular mass of 268 Da. Upon collision-induced dissociation, this ion produced a fragment of 136 Da, corresponding to the characteristic transition of adenosine. Furthermore, spiking with adenosine as described in section 2.5, provided an estimation that 0.75% of the chromatographic signal of the venom

corresponded to this nucleoside. Presence of a substantial amount of adenosine was also observed in the venom of *D. polylepis* [19]. Adenosine might play an auxiliary role in the toxicity of mamba venoms owing to its vasodilatory effect, as previously suggested [19].

The overall protein composition of *D. angusticeps* venom was determined by assigning the identified proteins to families and expressing these as percentages of total protein content (Figure 4). The most abundant components belong to the three-finger toxin family (3FTx; 69.2%) and the family of Kunitz-type proteinase inhibitors, which includes the dendrotoxins (KUN; 16.3%). The 3FTxs in elapid venoms all share a common structural architecture with a distinct protein fold, comprising between 60 and 80 amino acids in length, containing a small, globular, hydrophobic core with four or five conserved disulfide bridges, from which three β-stranded loops extend [28–30]. This makes this group of toxins resemble three outstretched fingers [31]. Despite the common structural motif, a diverse array of functions has been associated with 3FTxs [32].

All 3FTxs found in *D. angusticeps* venom belong to the short chain subfamily, but attained to different sub-subfamilies (Figure 4). The majority of 3FTxs in this venom belong to the Orphan Group XI (from Toxin FV-III), whose function has not yet been established [29], followed by aminergic toxins (Muscarinic toxin 2, Muscarinic toxin 4, Synergistic-like protein, and Adrenergic toxins) [16]. A further 8.4% of 3FTxs were attained to fasciculins (all from the acetylcholinesterase inhibitory sub-subfamily), which are unique to *D. angusticeps* [33].

Interestingly, the proteomic analysis of the 3FTxs of *D. angusticeps* venom did not reveal the presence of α-neurotoxins, perhaps the most studied 3FTxs from elapid venoms. α-neurotoxins bind with high affinity to the nicotinic cholinergic receptor at

358 the motor end-plate of the neuromuscular junction, causing a blockage in neuromuscular
359 transmission and flaccid paralysis, generally inducing death by respiratory failure [34].
360 α-neurotoxins show the highest Toxicity Score values among the fractions of *D*.
361 *polylepis* venom [19]. Their absence in the venom of *D*. *angusticeps* marks a significant
362 difference between these two mamba venoms and suggests that the predominant
363 mechanisms for prey immobilization in these venoms might be different.

364 Another type of neurotoxins unique to the *Dendroaspis* genus, and found in our
365 proteomic analysis of *D*. *angusticeps* venom, is comprised by the dendrotoxins, which
366 are homologous to Kunitz-type serine proteinase inhibitors [35]. Dendrotoxins interact
367 and inhibit the presynaptic voltage-gated potassium channels, thus exerting a facilitatory
368 effect associated with excitability [18,35]. The venom of *D*. *angusticeps* has a lower
369 relative content of Kunitz-type proteinase inhibitors, but a higher content of 3FTxs,
370 when compared to the venom of *D*. *polylepis* [19]. The combined action of the
371 fasciculins and dendrotoxins results in enhanced skeletal muscle excitability and
372 contraction, probably leading to respiratory arrest.

373 Other protein families found in lower proportions in the venom of *D*.
374 *angusticeps* include metalloproteinases (SVMP; 6.7%), cysteine-rich secretory proteins
375 (CRISP; 2.1%), and traces of Galactose-binding lectins (GAL; < 0.5%), peptidases
376 (PEP; < 0.1%), hyaluronidases (HYA; < 0.3%), and nerve growth factors (NGF < 0.1%)
377 (Figure 4). An extremely low PLA_2 activity was observed *in vitro* for *D*.
378 *angusticeps* venom (Figure 5A), in agreement with previous findings [36]. The
379 proteomic analysis, however, did not identify any PLA_2 in this venom, implying that
380 such enzyme would be present only in trace amounts. Alternatively, the very low PLA_2
381 activity recorded for this venom may correspond to low levels of non-specific
382 hydrolysis of the NOBA synthetic substrate caused by other enzymes. The negligible

383 content of PLA_2s in *Dendroaspis* venoms contrasts with the characteristic high amounts
384 and activity of this enzyme in many other elapid venoms [37,38]. Also, despite the
385 presence of 6.7% of SVMPs in the venom proteome, very low proteinase activity was
386 observed for *D. angusticeps* venom when using azocasein as substrate (Figure 5B). This
387 observation mirrors the negligible activity described for *D. polylepis* venom [19]. It is
388 likely that *Dendroaspis* SVMPs have a restricted substrate specificity, as occurs in
389 SVMPs from other elapid venoms [39,40].
390
391 *3.2. Toxicity of venom fractions*
392 Toxicity testing was performed for most venom fractions (Table 2). Using the
393 Toxicity Score defined by Laustsen et al. [20], a cut-off Toxicity Score value of 7,
394 below which a fraction would be deemed to not be of medical relevance for lethality,
395 was chosen for screening the fractions. From Table 2 it is evident that the vast majority
396 of the fractions did not induce lethality in mice when tested individually. Only fraction
397 8 (containing Rho-elapitoxin-Da1b and Fasciculin-2) was shown to be lethal at the
398 doses tested, with an LD_{50} of 0.58 mg/kg (95% confidence limits: 0.17-1.23 mg/kg) and
399 a toxicity score of 10.9. A previous study showed that an 'angusticeps-type' toxin,
400 which corresponds to a fasciculin, induced respiratory arrest in mice within minutes
401 after an i.v. injection of a dose of 1 mg/kg, and also caused cardiovascular alterations
402 [41]. Nevertheless the Toxicity Score of fraction 8 contrasts with the overall Toxicity
403 Score of 117.6 for the whole venom, suggesting that different toxins in *D. angusticeps*
404 venom may act in a synergistic manner, thereby potentiating each other's toxic effects,
405 leading to higher toxicity for whole venom. To further investigate the possible
406 synergism between toxins in the venom, fractions 4-12 were combined in equivalent
407 amounts (according to mass), and the LD_{50} was determined to be 1.36 mg/kg (95%

confidence limits: 0.96-1.66 mg/kg), corresponding to a Toxicity Score of 51.7, providing further evidence for the presence of synergism. The identity of the toxins acting synergistically is presently unknown; however, it is suggested that fasciculins and dendrotoxins, and probably other synergistically acting proteins, might be involved in this phenomenon. It should be kept in mind that the solvents used in RP-HPLC separation, particularly acetonitrile, denature some venom components, especially SVMPs; thus, the toxicity of SVMP fractions cannot be assessed with our approach. Nevertheless, elapid SVMPs are unlikely to play a key role in lethality. In support of this, it was previously shown that the LD_{50} of *D. polylepis* venom was not significantly altered after incubating venom with RP-HPLC solvents [19].

Despite its lack of α-neurotoxins, the venom of *D. angusticeps* is quite effective in killing mice rapidly after injection, as observed in our toxicity experiments with crude venom, where the controls receiving 4 LD_{50}s of venom on average died within 10 minutes. Previous studies highlighted two main toxic activities when *D. angusticeps* whole venom is tested in experimental systems. On various nerve-muscle preparations, this venom augmented the responses to indirect stimulation [35], possibly due to the combined action of dendrotoxins and fasciculins. Then, prolonged exposure to higher venom concentrations resulted in failure of muscle contraction. Additionally, the venom induced hypotension in various animal models, an effect that was blocked by the muscarinic cholinergic antagonist atropine [42]. This effect could be caused by the 3FTxs, previously characterized from this venom, that act on muscarinic cholinergic and adrenergic receptors [14–16]. Thus, the combined action of the various neurotoxin types present in *D. angusticeps* venom may result in a complex series of neuromuscular and cardiovascular effects, which result in effective prey immobilization in the absence of the action of α-neurotoxins. This toxicological scenario, and the existence of

433 synergistic effects, complicates the selection of the most relevant toxins towards which
434 antibodies should be raised in order to abrogate venom toxicity. This challenging task
435 demands the identification of the most relevant synergistic toxins.
436
437 *3.3 Immunoprofiling and neutralizing ability of antivenoms*
438 Three polyspecific antivenoms, which are distributed in sub-Saharan Africa,
439 were investigated for their ability to neutralize *D. angusticeps* venom and their ability to
440 recognize both whole venoms and venom fractions. The SAVP antivenom showed the
441 highest neutralizing ability against *D. angusticeps* venom, with an ED_{50} (mg venom
442 neutralized per mL antivenom) of 4.0 mg/mL (95% confidence limits: 1.7-10.0 mg/mL).
443 VINS African antivenom also neutralized the lethal activity of the venom, with an ED_{50}
444 of 2.4 mg/mL (95% confidence limits: 1.4-4.0 mg/mL). On the other hand, VINS
445 Central African antivenom failed to neutralize *D. angusticeps* venom at the lowest
446 venom/antivenom ratio tested (1.0 mg venom/mL antivenom). These results bear a
447 relationship with the fact that the venom of *D. angusticeps* is included in the
448 immunization mixture for the manufacture of SAVP antivenom, whereas the two VINS
449 antivenoms do not include this venom during immunization. The two VINS products
450 do, however, include the venoms of other *Dendroaspis* species, according to their leaflet
451 information. Gel immunodiffusion tests of the three antivenoms indeed revealed that
452 cross-reactive antigens between *D. angusticeps* and *D. polylepis* venoms exist,
453 evidenced by the SAVP antivenom, which produced the strongest precipitin bands with
454 identity or partial identity patterns (Figure 6). Cross-reactivity between at least some
455 components of these two venoms would explain the neutralization obtained with the
456 VINS antivenom, despite these being produced without using *D. angusticeps* venom. It
457 would be relevant to perform detailed studies on the antigenic relationships of the main

toxicologically-relevant components of *Dendroaspis* venoms, such as the various types of 3FTxs and dendrotoxins, in order to have a knowledge base for selecting the venoms or toxins to be used for preparing antivenoms. Interestingly, gel immunodiffusion results, regarding the intensity of precipitates, showed a better correlation with the neutralization potencies observed for the three antivenoms compared to their ELISA titration curves against immobilized crude venoms, which showed only minor differences in binding among them (Figure 7). Although the SAVP antivenom displays a slightly stronger binding when comparing the three antivenoms on the basis of volume, differences are less evident when the antivenoms are evaluated based on their protein concentrations (Figure 7). In general, solid-phase immunoassays of antivenoms against crude venoms do not always predict their neutralizing efficacy, as antibodies may bind to highly immunogenic venom components that may not have a key role in toxicity.

To further investigate the immunorecognition patterns of the antivenoms, binding of their antibodies to the different venom fractions was measured by ELISA. From Figure 8, it is evident that a somewhat similar recognition pattern exists for the different antivenoms. However, not only does the SAVP antivenom in general display stronger binding to the venom fractions compared to the VINS antivenoms, but SAVP antivenom also shows a much stronger binding to the fractions in the first part of the chromatogram (4-10), containing the 3FTxs and the dendrotoxins. These findings, based on the use of immobilized venom fractions rather than crude venoms, better agree with the *in vivo* neutralization studies described above.

Observations performed on mice injected with mixtures of venom and antivenom in the neutralization experiments revealed that, at some venom/antivenom ratios, mice were protected from death, but nevertheless showed evident manifestations

of toxicity, such as reduced mobility (without paralysis) and congestion of the eyes. This suggests that toxins responsible for these effects are not fully neutralized, at some of the tested venom/antivenom ratios. Since these toxins may play an important role in envenomings, it would be relevant to assess whether these non-lethal manifestations of toxicity are neutralized or not in the evaluation of an antivenom. For instance, in the case of SAVP antivenom, complete neutralization of lethality and of these additional manifestations was observed at a venom/antivenom ratio of 1.0 mg/mL. In contrast, at ratios of 2 and 3 mg venom/mL antivenom, lethality was abrogated, but reduced mobility and eye congestion were present to some extent. At ratios of 4 mg venom/mL antivenom and higher, lethality was not completely neutralized. Similar observations were performed with VINS African antivenom, whereby complete neutralization of lethality and the other effects was achieved at 0.5 mg venom/mL antivenom, whereas at 1 mg/mL the additional effects were observed, and lethality was abrogated. These findings underscore the relevance of identifying the most relevant toxins in the venom of *D. angusticeps* in order to ensure that neutralizing antibodies against them are included in heterologous or recombinant antivenoms in the future.

4.0 Concluding remarks and outlook

The venom proteome of *D. angusticeps* was characterized by a bottom-up approach. It shows a predominance of 3FTxs and Kunitz-type proteinase inhibitors, with additional less abundant components of various protein families. A remarkable feature of this venom is the absence of α-neurotoxins, in sharp contrast with the venom of the closely related species *D. polylepis*. The toxicity analysis of RP-HPLC fractions revealed that only one fraction was lethal to mice at the doses tested, and that the lethality of whole venom was much higher than what would be expected based on the

lethality of individual fractions. This highlights the presence of synergism between various venom components, such as dendrotoxins, fasciculins, and probably aminergic 3FTxs of various types. South African polyvalent antivenom and one Indian antivenom were effective in the neutralization of venom lethality, in agreement with a pattern of immunorecognition of the various RP-HPLC fractions. On the basis of the synergism observed in the overall toxicity of this venom, the development of an effective combination of recombinant neutralizing antibodies demands the identification of the most relevant synergistic toxins that need to be neutralized – a task that awaits future research efforts.

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648

Figure legends

Figure 1: (A) *Dendroaspis angusticeps* **(B)** Distribution of *D*. *angusticeps* in Africa.

Figure 2: SDS-PAGE comparing crude venom of *Dendroaspis angusticeps* and *D*. *polylepis* under non-reduced (A) and reduced (B) conditions. Various amounts of each venom were separated in 15% gels and stained with Coomassie Blue G-250. Molecular mass markers (M) are labeled to the right, in kDa.

Figure 3: Separation of *Dendroaspis angusticeps* venom proteins using RP-HPLC **(A)**, followed by SDS-PAGE **(B)**. Two mg of venom were fractionated on a C_{18} column and eluted with an acetonitrile gradient (dashed line), as described in Methods. Further separation of protein fractions was performed by SDS-PAGE under reducing conditions. Molecular weight markers (M) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in Table 1.

Figure 4: Composition of the *Dendroaspis angusticeps* venom proteome according to protein families (A) and three-finger toxin sub-subfamilies (B), expressed as percentages of total protein content. **KUN**: Bovine pancreatic trypsin inhibitors/Kunitz inhibitors (dendrotoxins); **3FTx**: Three-finger toxins; **SVMP**: Metalloproteinases; **GAL**: Galactose-binding lectins; **PEP**: Peptidases; **HYA**: Hyaluronidases; **KTC**: Prokineticins; **NGF**: Nerve growth factors. **CRISP**: Cysteine-rich secretory proteins. *Proteins in this fraction (Mambalgins) are not classified to a sub-subfamily; however they are known to inhibit acid sensing ion channels. **MIX**: Fractions of different

673 members of the 3FTx family for which percentages were not determined; sub-
674 subfamilies in this group include: Aminergic toxin, Antiplatelet toxin, Orphan group XI,
675 and Acid sensing ion channel inhibitor.
676
677 **Figure 5: (A)** Comparison of the phospholipase A_2 activity of 20 μg of the venoms of
678 *Dendroaspis angusticep*s, *Dendroaspis polylepis,* and *Bothrops asper*, on 4-nitro-3-
679 octanoyloxybenzoic acid synthetic substrate. **(B)** Comparison of the proteolytic activity
680 of 40 μg of venoms of *D. angusticep*s, *D. polylepis*, and *B. asper*, on azocasein
681 substrate. Venoms from both species of *Dendroaspis* show extremely low
682 phospholipase A_2 and proteinase activities. Each bar represents mean ± SD of
683 triplicates.
684
685 **Figure 6:** Gel immunodiffusion assay of antivenoms against the venoms of
686 *Dendroaspis angusticeps* (Da) and *Dendroaspis polylepis* (Dp). Antivenoms (50 μL)
687 were added to the central wells, and solutions of various concentrations of venoms (30
688 μL) were added to peripheral wells. **(A):** VINS African antivenom. **(B):** VINS Central
689 Africa Antivenom. **(C):** SAVP antivenom. **(D):** *Micrurus nigrocinctus* antivenom.
690
691 **Figure 7:** ELISA titrations of antivenoms against immobilized crude venoms of
692 *Dendroaspis angusticeps* (A and C) and *Dendroaspis polylepis* (B and D) **SAVP**:
693 SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers, **VINS**
694 **African**: Snake Venom Antiserum (African) from VINS Bioproducts Ltd., **VINS**
695 **Central Africa** Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd.
696 Normal horse serum was used as a negative control. Each point represents mean ± SD

of triplicate wells. Antivenom titrations are represented as volumetric dilutions in A and B, or as protein concentrations in C and D.

Figure 8: ELISA-based immunoprofiling of antivenoms against HPLC fractions of *Dendroaspis angusticeps* venom. Binding of the equine antibodies to the immobilized venom fractions was detected as described in Methods. Normal horse serum was used as a negative control. For identification of venom fractions see Table 2. **(A) SAVP**: SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. **(B): VINS African**: Snake Venom Antiserum (African) from VINS Bioproducts Ltd., **VINS Central Africa** Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. Each bar represents mean ± SD of triplicate wells.

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Table 1: Assignment of the RP-HPLC isolated fractions of *Dendroaspis angusticeps* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak*	%	Mass (kDa)▼	Peptide ion		MS/MS-derived sequence **	Conf (%)	Sc	Protein family ***	Related protein, code
			m/z	z					
4.i	2.7	11.8	2014.9	1	AKQCLPFDYSGCGGNANR	99	13	BPTI/KUN	Delta-dendrotoxin
			1815.8	1	QCLPFDYSGCGGNANR	99	15		*D. angusticeps*; P00982
			1045.6	1	KIPSFYYK	98.5	6		
4.ii			1501.7	1	ASIPAFYYNWAAK	99	14	BPTI/KUN	Long epsilon-dendrotox.R55 *D. angusticeps*; Q7LZS8
5a	0.5	16.1	1030.5	1	PAFYYNQK	99	7	BPTI/KUN	Alpha-dendrotoxin
			1143.6	1	IPAFYYNQK	99	14		*D .angusticeps*; P00980
			1709.8	1	CYDKIPAFYYNQK	99	16		
			1356.5	1	FDWSGCGGNSNR	99	12		
5b	4.3	11.7	1030.5	1	PAFYYNQK	99	11	BPTI/KUN	Alpha-dendrotoxin
			1236.6	1	LCILHR(N^{da})PGR	99	10		*D. angusticeps*; P00980
			1143.6	1	IPAFYYNQK	99	14		
			1709.8	1	CYDKIPAFYYNQK	99	22		
			1356.5	1	FDWSGCGGNSNR	99	19		
6a.i	1.3	12.9	2039.9	1	SIGGVTTEDCPAGQNVCFK	99	17	3FTx	Muscarinic toxin 2
			2196.0	1	SIGGVTTEDCPAGQNVCFKR	99	20		*D. angusticeps*; P18328
6a.ii			1253.6	1	MIWTYDGVIR	99	9	3FTx	Thrombostatin
									D. angusticeps; P81946
6b.i	0.9	12.1	1143.6	1	IPAFYYNQK	99	11	BPTI/KUN	Alpha-dendrotoxin
			1709.8	1	CYDKIPAFYYNQK	99	10		*D. angusticeps*; P00980
			1356.5	1	FDWSGCGGNSNR	99	14		
6b.ii	trace		1253.6	1	MIWTYDGVIR	99	12	3FTx	Thrombostatin
									D. angusticeps; P81946
6b.iii	0.9		2196.0	1	SIGGVTTEDCPAGQNVCFKR	99	11	3FTx	Muscarinic toxin 2
									D. angusticeps; P18328
7a	0.5	16.4	1253.6	1	MIWTYDGVIR	99	7	3FTx	Thrombostatin

									D. angusticeps; P81946
7b.i	1.1	14.0	1557.7	1	AILTNCGENSCYR	99	10	3FTx	Dendrotoxin A (fragm) *D. angusticeps*; Q9PS08
7b.ii			1253.6	1	MIWTYDGVIR	99	10	3FTx	Thrombostatin *D. angusticeps*; P18328
7c.i	4.6	12.0	2821.2	1	LICYNQLGTKPPTTETCGDDSCYK	99	12	3FTx	Thrombostatin
			1253.6	1	MIWTYDGVIR	99	16		*D. angusticeps*; P81946
			1409.7	1	MIWTYDGVIRR	98	10		
7c.ii	trace		1356.6	1	FDWSGCGGNSNR	99	11	BPTI/KUN	Alpha-dendrotoxin *D. angusticeps*; P00980
8a	3.0	13.7	1517.6	1	GCGCPPGDDNLEVK	99	7	3FTx	Fasciculin-2
			1304.5	1	CCTSPDKCNY	99	10		*D. angusticeps*; P0C1Z0
			1344.6	1	TMCYSHTTTSR	99	10		
			1685.8	1	AILTNCGENSCYRK	99	15		
			1557.7	1	AILTNCGENSCYR	99	17		
8b.i	3.4	11.5	2426.2	1	DTIFGITTQNCPAGQNLCFIR	99	13	3FTx	Rho-elapitoxin-Da1b *D. angusticeps*; P86419
8b.ii			1253.6	1	MIWTYDGVIR	97.7	6	3FTx	Thrombostatin *D. angusticeps*; P81946
9a	1.8	15.8	1507.7	1	SIGGISTEECAAGQK	99	8	3FTx	Synergistic-like protein *D. angusticeps*; P17696
9b	3.2	12.9	1557.7	1	AILTNCGENSCYR	99	12	3FTx	Fasciculin-1
			1685.8	1	AILTNCGENSCYRK	93.6	6		*D. angusticeps*; P0C1Y9
			1344.6	1	TMCYSHTTTSR	50.2	4		
9c.i	4.4	11.0	1507.7	1	SIGGISTEECAAGQK	99	6	3FTx	Synergistic-like protein *D. angusticeps*; P17696
9c.ii	6.6		1216.4	1	CL(E^{dh})FTYGGCK	99	11	BPTI/KUN	Protease inhibitor 1 *W. Aegyptia*; C1IC50
10a	0.6	15.7	1557.8	1	AILTNCGENSCYR	68.8	5	3FTx	Dendrotoxin A (fragm) *D. angusticeps*; Q9PS08
10b	1.6	12.9	1304.5	1	CCTSPDKCNY	99	9	3FTx	Fasciculin-1
			1344.6	1	TMCYSHTTTSR	99	12		*D. angusticeps*; P0C1Y9
			1566.7	1	GCGCPPGDDYLEVK	99	18		

			1685.8	1	AILTNCGENSCYRK	99	14		
			1557.7	1	AILTNCGENSCYR	99	16		
10c.i	4.6	10.8	1165.7	1	MGPKLYDVSR	99	7	3FTx	Synergistic-like protein
			1507.8	1	SIGGISTEECAAGQK	99	13		*D. angusticeps*; P17696
10c.ii			1253.7	1	MIWTYDGVIR	99	7	3FTx	Thrombostatin
									D. angusticeps; P81946
11a.i	1.3	15.1	1288.7	1	EMLVAIHCCR	99	10	3FTx	Toxin F-VIII
			2035.0	1	GCGCPSKEMLVAIHCCR	99	11		*D. angusticeps*; P01404
11a.ii	trace		1356.6	1	FDWSGCGGNSNR	96.7	6	BPTI/KUN	Alpha-dendrotoxin
									D. angusticeps; P00980
11b.i	6.5	11.5	1288.7	1	EMLVAIHCCR	99	11	3FTx	Toxin F-VIII
									D. angusticeps; P01404
11b.ii	trace		2724.4	1	VCTPVGTSGEDCHPASHKIPFSGQR	99	10	KTC	Toxin MIT1
									D.polylepis; P25687
11b.iii	trace		1557.8	1	AILTNCGENSCYR	99	6	3FTx	Dendrotoxin A (fragm)
									D. angusticeps; Q9PS08
11c.i	9.0	10.4	1288.7	1	EMLVAIHCCR	99	14	3FTx	Toxin F-VIII
			1281.6	1	EMLVAIHCCR	99	12		*D. angusticeps*; P01404
			2035.0	1	GCGCPSKEMLVAIHCCR	99	15		
11c.ii	trace		2434.1	1	GCGCPTAMWPYQTECCKGDR	99	17	3FTx	Toxin S4C8
									D. jamesoni; P25683
12a	1.2	15.3	1645.9	1	WQPPWYCKEPVR	80.2	5	BPTI/KUN	Kunitz-type calcicludine
									D. angusticeps; P81658
12b.i	trace	11.1	1294.7	1	GTCCAVSLWIK	99	12	KTC	Toxin MIT1
			2724.4	1	VCTPVGTSGEDCHPASHKIPFSGQR	99	13		*D.polylepis*; P25687
			2209.1	1	MHHTCPCAPNLACVQTSPK	99	26		
			2296.1	1	VCTPVGTSGEDCHPASHKIPF	91.8	6		
12b.ii	3.0		1441.7	1	FCYHNIGMPFR	99	11	3FTx	Mambalgin-3
									D. angusticeps; C0HJB0
12c.i	trace	10.1	1645.9	1	WQPPWYCKEPVR	99	11	BPTI/KUN	Kunitz-type calcicludine
			1669.8	1	CLPFLFSGCGGNANR	99	15		*D. angusticeps*; P81658

			1797.9	1	KCLPFLFSGCGGNANR	98.7	7		
12c.ii	3.5		2034.9	1	GCGCPSKEMLVAIHCCR	99	13	3FTx	Toxin F-VIII
			1288.7	1	EMLVAIHCCR	99	14		*D. angusticeps*; P01404
13a.i	trace	24.4	1669.8	1	CLPFLFSGCGGNANR	99	7	BPTI/KUN	Kunitz-type calcicludine
									D. angusticeps; P81658
13a.ii	0.4****		1288.6	1	EMLVAIHCCR	99	11	3FTx	Toxin F-VIII
									D. angusticeps; P01404
13a.iii			1441.7	1	FCYHNIGMPFR	99	13	3FTx	Mambalgin-3
									D. angusticeps; C0HJB0
13b.i	trace	19.8	1669.8	1	CLPFLFSGCGGNANR	99	10	BPTI/KUN	Kunitz-type calcicludine
			1645.8	1	WQPPWYCKEPVR	99	9		*D. angusticeps*; P81658
13b.ii	1.1****		1288.6	1	EMLVAIHCCR	99	9	3FTx	Toxin F-VIII
			2034.9	1	GCGCPSKEMLVAIHCCR	99	19		*D. angusticeps*; P01404
13b.iii			1441.7	1	FCYHNIGMPFR	99	15	3FTx	Mambalgin-3
									D. angusticeps; C0HJB0
13c.i	trace	15.4	1669.8	1	CLPFLFSGCGGNANR	99	13	BPTI/KUN	Kunitz-type calcicludine
			1645.8	1	WQPPWYCKEPVR	99	12		*D. angusticeps*; P81658
13c.ii	2.9****		2034.9	1	GCGCPSKEMLVAIHCCR	99	16	3FTx	Toxin F-VIII
			1288.6	1	EMLVAIHCCR	99	9		*D. angusticeps*; P01404
13c.iii			1441.7	1	FCYHNIGMPFR	99	16	3FTx	Mambalgin-3
									D. angusticeps; C0HJB0
14a	0.4	19.1			Negative				
14b.i	0.1	15.4	1797.9	1	KCLPFLFSGCGGNANR	99	16	BPTI/KUN	Kunitz-type calcicludine
			1669.8	1	CLPFLFSGCGGNANR	99	17		*D. angusticeps*; P81658
			1645.8	1	WQPPWYCKEPVR	99	13		
			1164.5	1	WQPPWYCK	95.1	5		
14b.ii	1.2		2034.9	1	GCGCPSKEMLVAIHCCR	99	13	3FTx	Toxin F-VIII
									D. angusticeps; P01404
14b.iii			1288.6	1	EMLVAIHCCR	99	11	3FTx	Mambalgin-3
			1441.7	1	FCYHNIGMPFR	99	12		*D. angusticeps*; C0HJB0

15a	0.1	23.5	1669.8	1	CLPFLFSGCGGNANR	99	7	3FTx	Kunitz-type calcicludine *D. angusticeps*; P81658
15b	1.1	18.3			Negative				
15c.i	0.1	15.0	1797.9	1	KCLPFLFSGCGGNANR	99	15	BPTI/KUN	Kunitz-type calcicludine
			1669.8	1	CLPFLFSGCGGNANR	99	18		*D. angusticeps*; P81658
			1645.8	1	WQPPWYCKEPVR	99	14		
			1010.5	1	FQTIGECR	95.4	9		
			1164.5	1	WQPPWYCK	86.5	5		
15c.ii	1.1		2034.9	1	GCGCPSKEMLVAIHCCR	99	7	3FTx	Toxin F-VIII
			1288.6	1	EMLVAIHCCR	99	15		*D. angusticeps*; P01404
15c.iii			1441.7	1	FCYHNIGMPFR	99	13	3FTx	Mambalgin-3 *D. angusticeps*; C0HJB0
16a	trace	21.2	1097.6	1	NPNPVPSGCR	99	6	NGF	Uncharact.prot (frag)
			1781.9	1	HWNSYCTTTHTFVK	99	20		*O.hannah*; V8NP13
			1413.7	1	CRNPNPVPSGCR	80.7	9		
	0.1		996.6	1	WYYIVPR	98.6	9	3 FTx	Muscarinic toxin 4
			2197.1	1	YSDITWGCAATCPKPTNVR	65.7	6		*D. angusticeps*; Q9PSN1
16b.i	0.5****	18.5	996.6	1	WYYIVPR	99	10	3FTx	Muscarinic toxin 4
			2197.1	1	YSDITWGCAATCPKPTNVR	97.7	8		*D. angusticeps*; Q9PSN1
16b.ii			1288.7	1	EMLVAIHCCR	99	9	3FTx	Toxin F-VIII *D. angusticeps*; P01404
16b.iii	trace		1669.8	1	CLPFLFSGCGGNANR	99	6	BPTI/KUN	Kunitz-type calcicludine *D. angusticeps*; P81658
16c.i	1.0	16.2	2358.2	1	SIFGITTEDCPDGQNLCFKR	99	13	3FTx	Toxin AdTx1
			2197.1	1	SIFGITTEDCPDGQNLCFK	99	17		*D. angusticeps*; P85092
			2253.0	1	GCAATCPIPENYDSIHCCK	99	21		
16c.ii			2329.2	1	SIFGITTENCPDGQNLCFKK	99	14	3FTx	Muscarinic toxin 4
			1124.7	1	KWYYIVPR	99	9		*D. angusticeps*; Q9PSN1
16c.iii			1291.7	1	NIWTFDNIIR	97	8	3FTx	Dendroaspin

			996.6	1	WYYIVPR	93	10		*D.jamesoni*; P28375
16d	1.4	15.0	2329.2	1	SIFGITTENCPDGQNLCFKK	99	18	3FTx	Muscarinic toxin 4
			1124.7	1	KWYYIVPR	99	11		*D. angusticeps*; Q9PSN1
			2197.1	1	YSDITWGCAATCPKPTNVR	99	24		
			996.6	1	WYYIVPR	98.6	10		
	trace		1669.8	1	CLPFLFSGCGGNANR	99	11	BPTI/KUN	Kunitz-type calcicludine
			1645.9	1	WQPPWYCKEPVR	98.5	9		*D. angusticeps*; P81658
17a.i	trace	21.5	1097.6	1	NPNPVPSGCR	99	8	NGF	Uncharact.prot (frag)
			1413.7	1	CRNPNPVPSGCR	99	11		*O.hannah*; V8NP13
			1709.9	1	FIRIDTACVCVISR	99	8		
			1781.9	1	HWNSYCTTTHTFVK	99	15		
17a.ii	0.2		1288.7	1	EMLVAIHCCR	99	9	3FTx	Toxin F-VIII
									D. angusticeps; P01404
17b	1.4	15.4	2197.1	1	YSDITWGCAATCPKPTNVR	99	16	3FTx	Muscarinic toxin 4
			996.6	1	WYYIVPR	98.9	10		*D. angusticeps*; Q9PSN1
18a	2.0	18.0			Negative				
18b	1.4	16.1	2197.1	1	SIFGITTEDCPDGQNLCFK	99	11	3FTx	Toxin AdTx1
									D. angusticeps; P85092
19	1.0	15.4	1390.7	1	GCGCPLTLPFLR	99	10	3FTx	Toxin C13S1C1
									D. angusticeps; P18329
20a.i	0.1	57.4	2053.0	1	TKPAYQFSSCSVQEHQR	99	10	MP	SVMP 1
			1256.7	1	VTL(D^{na})LFGKWR	99	14		*M. fulvius*; U3EPC7
								MP	SVMP-Hop-13 (Frag)
20a.ii			1297.6	1	SAECPTDSFQR	99	9		*H.bungaroides*; R4G7J1
20b	0.1	19.9	1346.6	1	TCEENSCYKR	99	11	3FTx	Toxin C13S1C1
			1319.9	1	SLPKIPLIIIGR	99	15		*D. angusticeps*; P18329
			1390.7	1	GCGCPLTLPFLR	99	12		
			1333.7	1	CGCPLTLPFLR	96.4	8		
20c	0.5	16.1	1333.7	1	CGCPLTLPFLR	99	11	3FTx	Toxin C13S1C1
			1390.7	1	GCGCPLTLPFLR	99	13		*D. angusticeps*; P18329
			1346.6	1	TCEENSCYKR	99	13		

			1319.9	1	SLPKIPLIIIGR	99	16		
20d	0.7	15.2	1346.6	1	TCEENSCYKR	99	10	3FTx	Toxin C13S1C1
			1319.9	1	SLPKIPLIIIGR	99	15		*D. angusticeps*; P18329
			1390.7	1	GCGCPLTLPFLR	99	12		
21	2.1	29.9	2551.3	1	YLYVCQYCPAGNIIGSIATPYK	99	14	CRISP	CRiSP
			1193.7	1	QIVDKHNALR	99	10		*Micropechis ikaheka*;
			1349.8	1	QIVDKHNALRR	62.1	7		A0A024AX20
			1537.7	1	NMLQMEWNSDAAQ	99	21		
22.i	1.1	70.7	1234.7	1	VTLNLFGEWR	99	12	MP	SVMP
									Macrovipera lebetina;Q98995
22.ii			2053.9	1	TKPAYQFSSCSVQEHQR	99	11	MP	SVMP 1
									M. fulvius U3EPC7
22.iii			1508.9	1	RNPQCILNKPLR	99	11	MP	SVMP
23a.i	0.6	69.5	1234.7	1	VTLNLFGEWR	99	10	MP	SVMP
									Echis coloratus; E9JG68
23a.ii			1508.8	1	NRPQCLLNKPLR	99	13	MP	SVMP
									Bothrops erythromelas;
									Q8UVG0
23b	0.3	51.3	1256.7	1	VTL(D^{na})LFGKWR	99	13	MP	SVMP 1
			2053.0	1	TKPAYQFSSCSVQEHQR	99	12		*M. fulvius* U3EPC7
24a.i	0.3	74.4	1967.0	1	KYIEFYVVVDNKMYR	99	9	MP	Scutatease-1
			1838.9	1	YIEFYVVVDNKMYR	96	6		*Notechis scutatus*; B5KFV7
24a.ii			1508.9	1	RNPQCILNKPLR	99	13	MP	SVMP ussurin
									Gloydius ussuriensis;Q7SZD9
24a.iii			2053.1	1	TKPAYQFSSCSVQEHQR	97.7	7	MP	SVMP 1
									M. fulvius; U3EPC7
24b	0.9	50.0	1256.7	1	VTL(D^{na})LFGKWR	99	14	MP	SVMP 1
			2053.0	1	TKPAYQFSSCSVQEHQR	99	14		*M. fulvius*; U3EPC7
25a.i	0.6	71.9	1881.8	1	NGHPCQNNQGYCYNR	99	14	MP	SVMP-Hop-50 (Fragm)
									H.bungaroides R4G7I9
25a.ii			1852.9	1	TDIVSPPVCGNYFVEVG	99	15	MP	SVMP
25a.iii									*Ovophis okinavensis*;U3TBS9
			1087.6	1	EHQEYLLR	99	10	MP	SVMP atrase-A.

									Naja atra; D5LMJ3
25b.i	0.2	52.2	1118.6	1	LVLNTFQAGR	99	10	GAL	Galectin (Frag) *O.hannah*; V8NHB1
25b.ii			2053.0	1	TKPAYQFSSCSVQEHQR	99	9	MP	SVMP 1
			1256.7	1	VTL(D^{na})LFGKWR	98.5	8		*M. fulvius*; U3EPC7
25c	0.5	22.5			Negative				
26a.i	0.2	70.1	2009.9	1	NGHPCQNNQGYCYNRK	99	9	MP	SVMP-Hop-50 (Fragm)
			1881.8	1	NGHPCQNNQGYCYNR	99	14		*H.bungaroides*; R4G7I9
26a.ii									SVMP
			1852.9	1	TDIVSPPVCGNYFVEVG	99	16	MP	*Ovophis okinavensis*;U3TBS9
									SVMP-Hem-2 (Frag)
26a.iii			1607.9	1	GATVGLAYVGSLC(N^{da})PK	99	15	MP	*Hemiaspis signata*; R4G2W9
26b	0.2	54.4	2412.2	1	TFHGLGVIDWENWRPQWDR	99	12	HYA	Hyaluronidase
			1904.0	1	HSDSNAFLHLFPESFR	99	12		*M. fulvius*; U3FYQ4
			2032.0	1	KHSDSNAFLHLFPESFR	99	11		
			1243.7	1	NDQLIWLWR	99	11		
								HYA	
			2780.4	1	APMYPNEPFLVFWNAPTTQCQLR	99	17		Hyaluronidase *O.hannah*; V8P1Z9
26c.i	0.6	39.3	1811.9	1	YIEFYVVVDNEMYK	99	12	MP	SVMP-Sut-51 (Frag) *Suta fasciata*; R4FIX4
26c.ii			1234.7	1	VTLNLFGEWR	99	11	MP	SVMP *Echis coloratus*; E9JG68
26d.i	0.3	35.9	1759.9	1	VYEMVNALNTMYRR	99	9	MP	SVMP mocarhagin *Naja mossambica*; Q10749
26d.ii			1811.9	1	YIEFYVVVDNEMYK	99	12	MP	SVMP-Sut-51 (Frag) *Suta fasciata*; R4FIX4
26d.iii			1234.7	1	VTLNLFGEWR	99	9	MP	SVMP *Echis coloratus*; E9JG68
26e	0.1	22.8	1118.7	1	LVLNTFQAGR	99	12	GAL	Galectin (Frag)
			1026.5	1	WGDEQVH(K^{fo})	98.3	12		*O.hannah*; V8NHB1
26f	0.1	21.1	1118.6	1	LVLNTFQAGR	99	12	GAL	Galectin (Frag) *O.hannah*; V8NHB1

27a.i	1.0	78.0	1838.9 1967.0	1 1	YIEFYVVVDNKMYR KYIEFYVVVDNKMYR	99 99	8 7	MP	Scutatease-1 *Notechis scutatus*; B5KFV7
27a.ii			3059.3	1	(A^{ca})AKDDCDLPEICTG(Q^{da})SAECPMDSFQR	99	13	MP	SVMP-Ver17 (Frag) *Vermicella annulata*; R4FIY6
27b	0.1	57.2	1462.8	1	TQEL(P^{ox})SILFSVGR	99	9	MP	Endoplasmic ret. aminopeptidase 1-like protein *Crotalus horridus*. T1E6L9
27c	0.1	45.9	1605.8 1234.7	1 1	SFG(D^{dh})(W^{di})RETDLLPR VTLNLFGEWR	99 68.9	12 6	MP	SVMP man SVMP *Echis coloratus*; E9JG68
27d	0.1	42.4	1605.8	1	SFG(D^{dh})(W^{di})RETDLLPR	99	10	MP	SVMP man
28i	0.5	48.8	1297.6	1	SAECPTDSFQR	99	10	MP	SVMP Australease-1 *P. australis*; B5KFV3
28ii			2191.0	1	(RT^{dh})KPAYQFSSCSVQEHQR	99	18	MP	SVMP 1 *M. fulvius* U3EPC7
28iii			2432.1	1	(N^{da})LVVAVI(M^{dt})A(H^{ox})EMGHNLGIHHDR	99	10	MP	SVMP man
29	0.9	-	-	-	-	-	-	Unknown	-

*Numbers correspond to peaks obtained by RP-HPLC separation; letters (a, b, c, d, e, f) correspond to bands in SDS-PAGE gels; and i, ii, iii correspond to different matching proteins identified in the same electrophoretic band.

** Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot®, ▼: reduced SDS-PAGE mass estimations, in kDa. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: ^{da}: deamidated; ^{dh}: dehydration; ^{fo}: formylated; ^{ox}: oxidized; ^{na}: Na cation; ^{ca}: carboxamidomethyl; ^{di}: deoxidized; Man: manual interpretation of spectrum

*** Protein family abbreviations: 3FTx: three-finger toxin; A_2; CRISP: cysteine-rich secretory protein; HYA: hyaluronidase BPTI/KUN: bovine pancreatic trypsin inhibitor/Kunitz inhibitor; MP: Metalloproteinase; KTC: prokineticin; GAL: galactose binding galectin

****It was not possible to determine the specific percentages of the two proteins of the three finger toxin family using ESI in these bands, as they were of similar mass, however the percentage was attained to the 3FTx in calculating total venom composition.

Table 2: Lethality and Toxicity Score of RP-HPLC fractions of the venom of *D. angusticeps*

Peak	%	Protein family	LD_{50} (mg/kg) (95% conf.)	Reported LD_{50} (mg/kg)	Toxicity score[1] % / LD_{50} (kg/mg)
Whole venom	100		0.85 (0.61-1.23)	1.13*	117.6
	70.3	Fractions 4-12 (equal amounts according to mass)	1.36 (0.96-1.66)		51.7
4	2.7	**BPTI/Kunitz inhibitor** Delta-dendrotoxin *D. angusticeps*; P00982 Long epsilon-dendrotox.R55 *D. angusticeps*; Q7LZS8	>0.40	Delta-dendrotoxin: 15 [43]	<7
5	4.8	**BPTI/Kunitz inhibitor** Alpha-dendrotoxin *D. angusticeps*; P00980	>0.71	23 [44]	<7
6	3.0 (1:1 mix)	**BPTI/Kunitz inhibitor** Alpha-dendrotoxin *D. angusticeps*; P00980 **3FTx** Muscarinic toxin 2 *D. angusticeps*; P18328	>0.45		<7
7	6.2	**3FTx** Thrombostatin *D.angusticeps*; P81946	>0.92		<7
8	6.3	**3FTx** Fasciculin-2 *D.angusticeps*; P0C1Z0 Thrombostatin *D.angusticeps*; P81946 Rho-elapitoxin-Da1b *D.angusticeps*; P86419	0.58 (0.17-1.23)	Fasciculin 2 >20 [33]	10.9
9	15.9 (2:1:2 mix)	**BPTI/Kunitz inhibitor** Protease inhibitor 1 *W. aegyptia*; C1IC50	>2.38		<7

		3FTx Fasciculin-1 *D.angusticeps*; P0C1Y9 Synergistic-like protein *D.angusticeps*; P17696		
10	6.8	**3FTx** Fasciculin-1 *D.angusticeps;* P0C1Y9 Synergistic-like protein *D.angusticeps;* P17696 Thrombostatin *D.angusticeps*; P81946 Dendrotoxin A (fragm) *D.angusticeps*; Q9PS08	>2.11	<3.3
11	16.8	**3FTx** Toxin F-VIII *D.angusticeps*; P01404	>2.52	<7
12	7.7 (2:2:1 mix)	**3FTx** Mambalgin-3 *D.angusticeps*; C0HJB0 Toxin F-VIII *D.angusticeps*; P01404 **BPTI/Kunitz inhibitor** Kunitz-type calcicludine *D.angusticeps*; P81658	>1.1	<7
13	2.1	**3FTx** Mambalgin-3 *D.angusticeps*; C0HJB0 Toxin F-VIII *D.angusticeps*; P01404	>0.64	<7
14	1.7	**3FTx** Mambalgin-3 *D.angusticeps*; C0HJB0 Toxin F-VIII *D.angusticeps*; P01404 **BPTI/Kunitz inhibitor** Kunitz-type calcicludine *D.angusticeps*; P81658	>0.26	<7
15	2.4	**3FTx** Mambalgin-3 *D.angusticeps*; C0HJB0 Toxin F-VIII *D.angusticeps*; P01404	>0.35	<7

16	3.0	**3FTx** Toxin F-VIII *D.angusticeps*; P01404 Toxin AdTx1 *D.angusticeps*; P85092 Muscarinic toxin 4 *D.angusticeps*; Q9PSN1	>0.44	<7
17	1.6	**3FTx** Toxin F-VIII *D.angusticeps*; P01404 Muscarinic toxin 4 *D.angusticeps*; Q9PSN1	>0.24	<7
18	3.4	**3FTx** Toxin AdTx1 *D.angusticeps*; P85092	>0.50	<7
19	1.0	**3FTx** Toxin C13S1C1 *D.angusticeps*; P18329	>0.15	<7
20	1.4	**3FTx** Toxin C13S1C1 *D.angusticeps*; P18329	>0.21	<7
21	2.1	**CRiSP** CRiSP *Micropechis ikaheka*; A0A024AX20	>0.31	<7

*: http://snakedatabase.org/pages/LD50.php#legendAndDefinitions

[1]Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD_{50}) for CD-1 mice by i.v. injection. In the case of crude venom, the % abundance was 100%.

[2]Mix indicates that the fraction did not contain a pure, isolated toxin, but instead a mixture of 2–4 different toxins in variable ratios indicated in the table.

Figure 1
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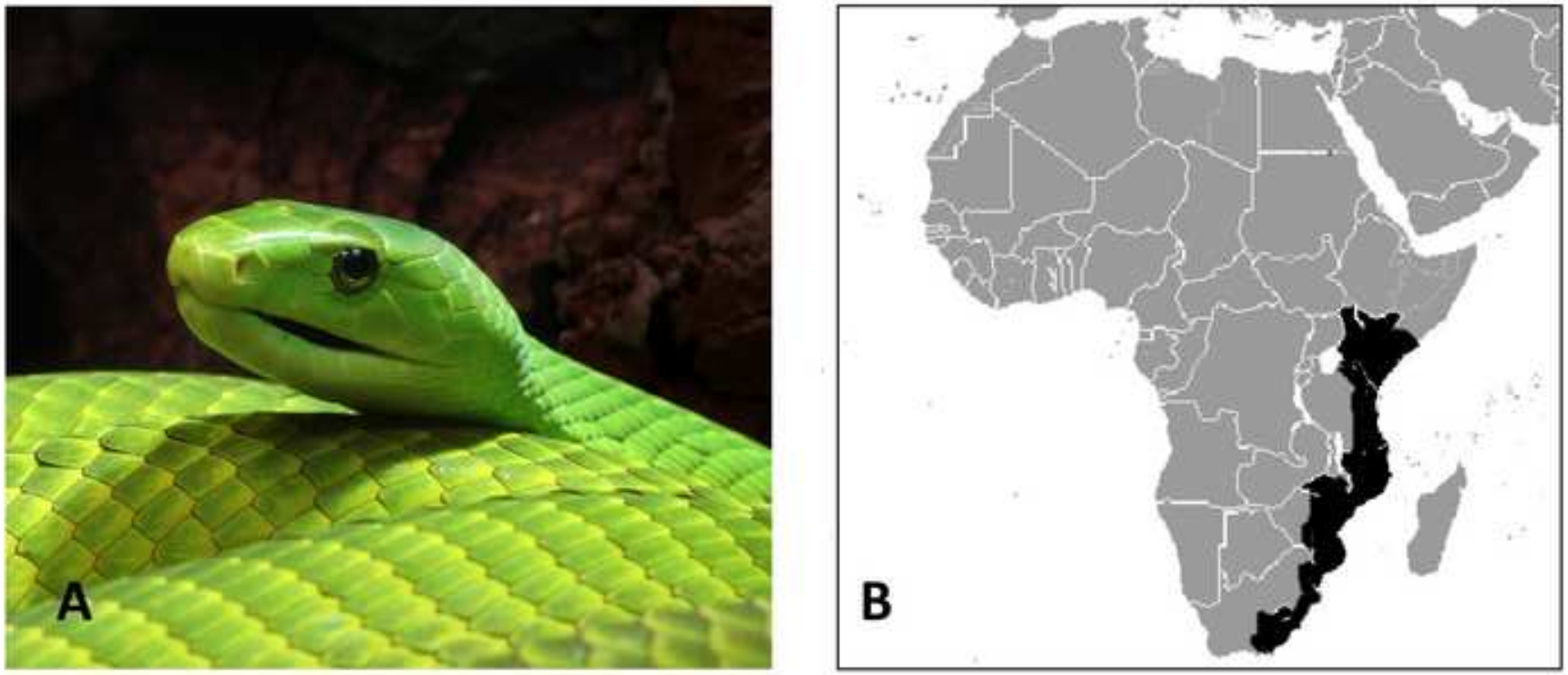

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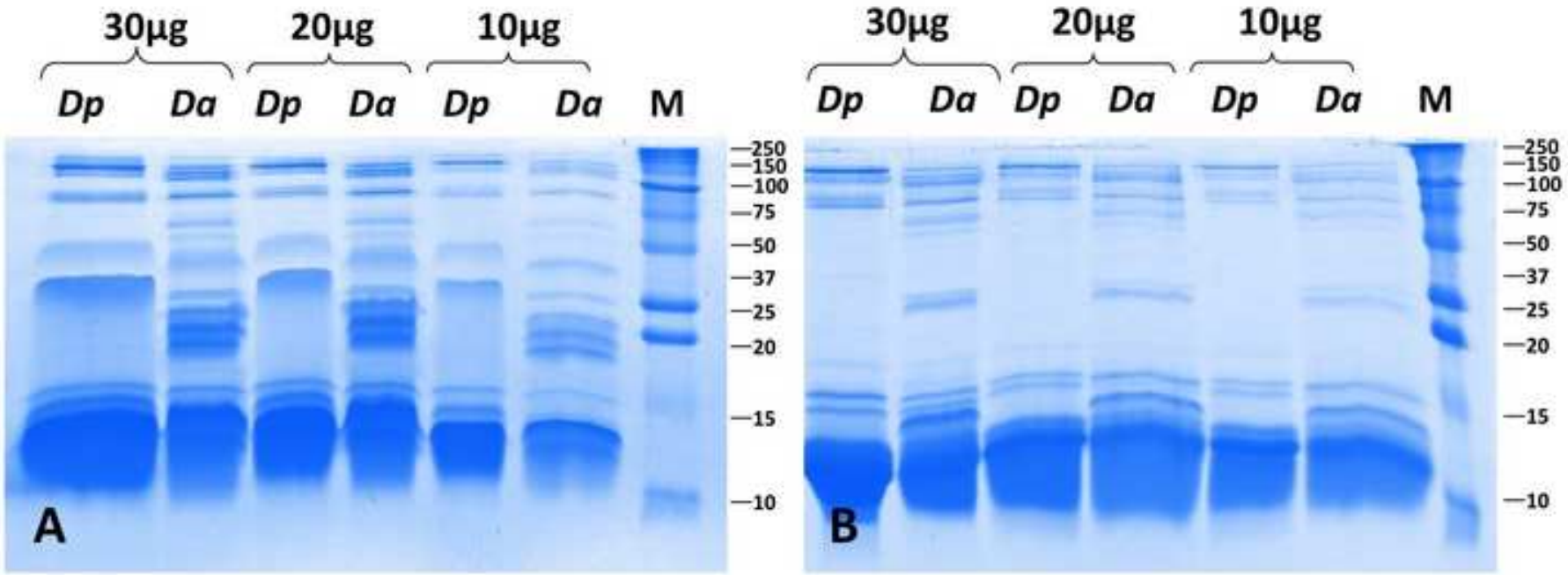

Figure 3

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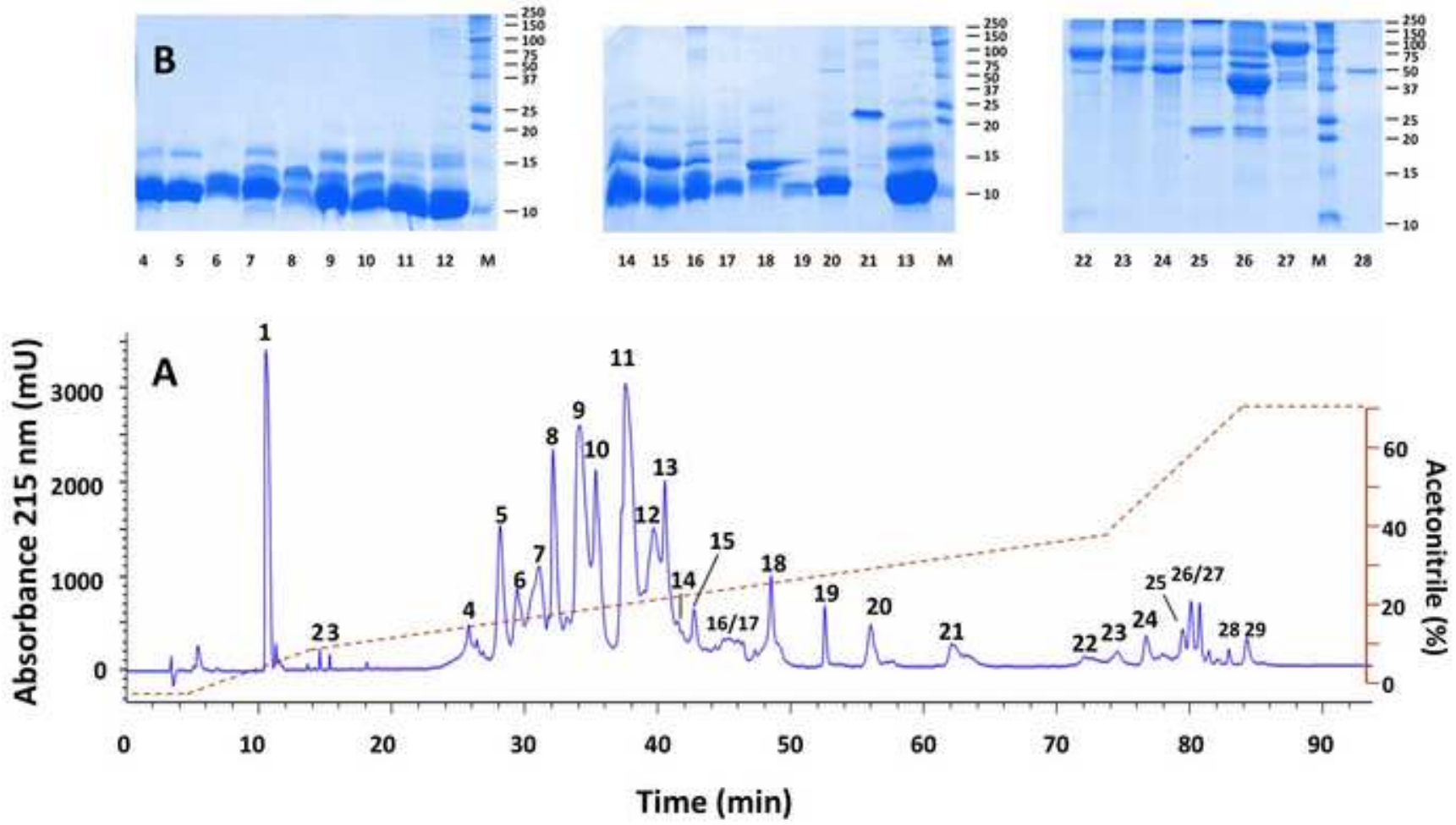

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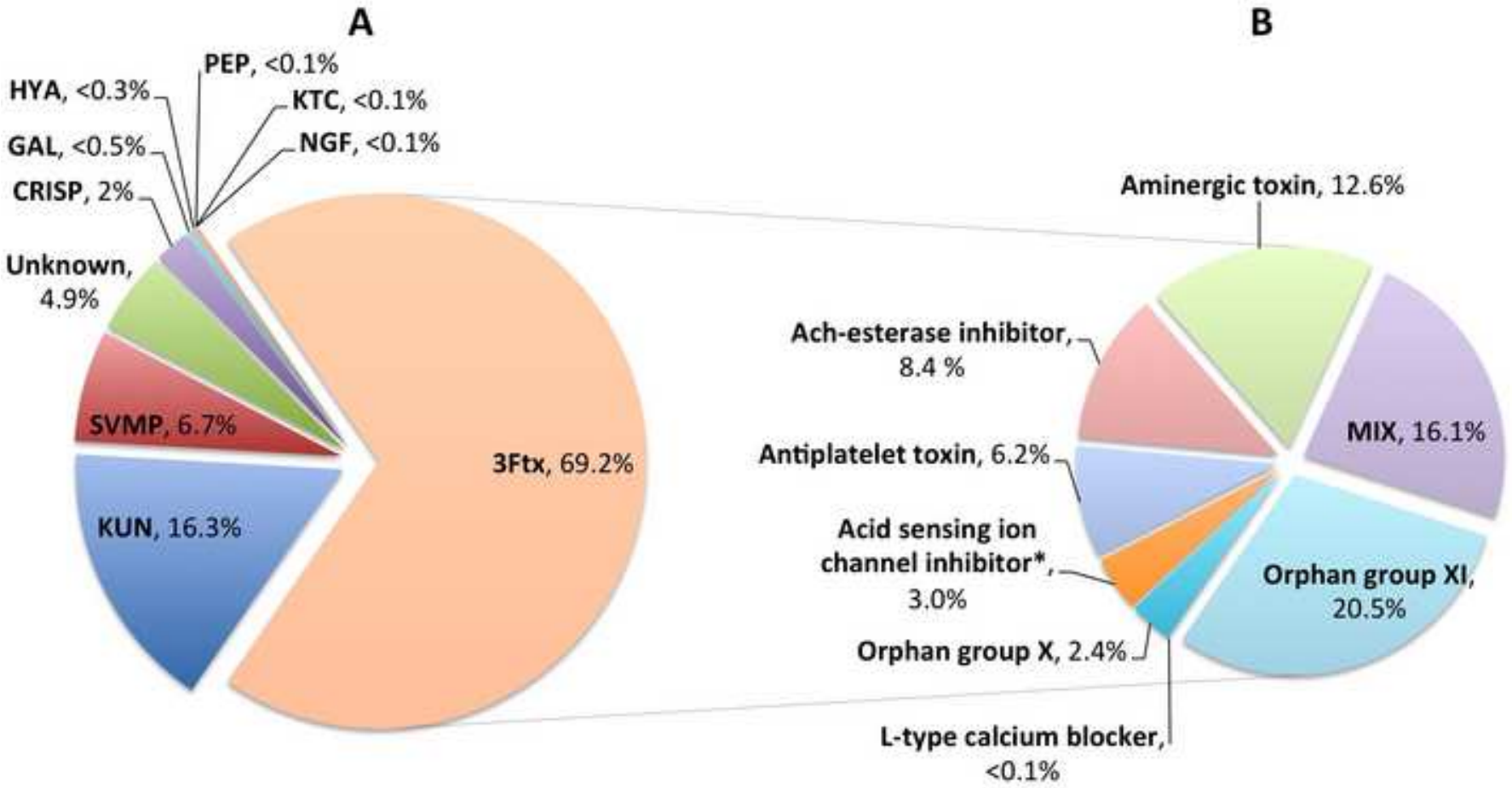

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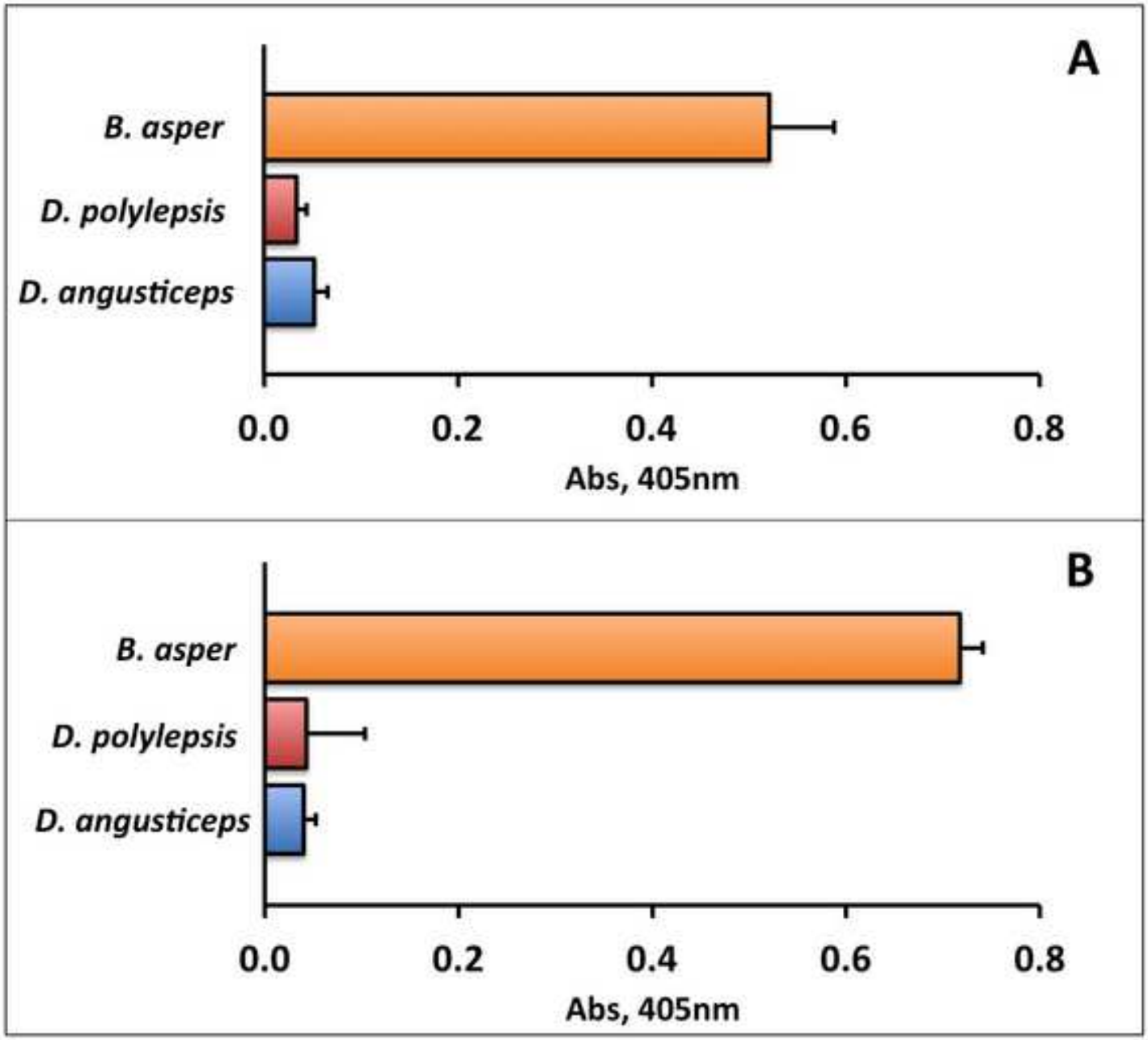

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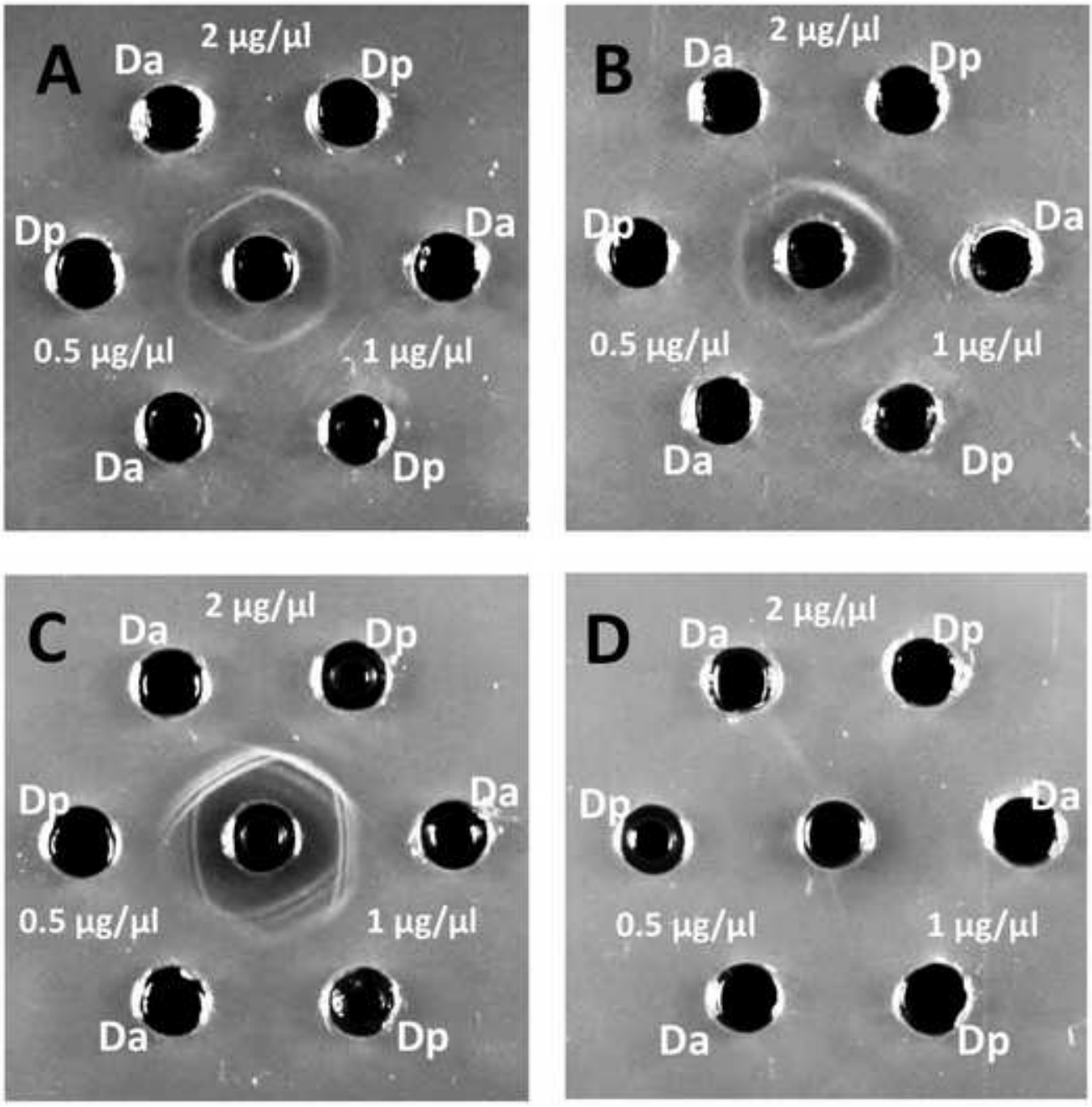

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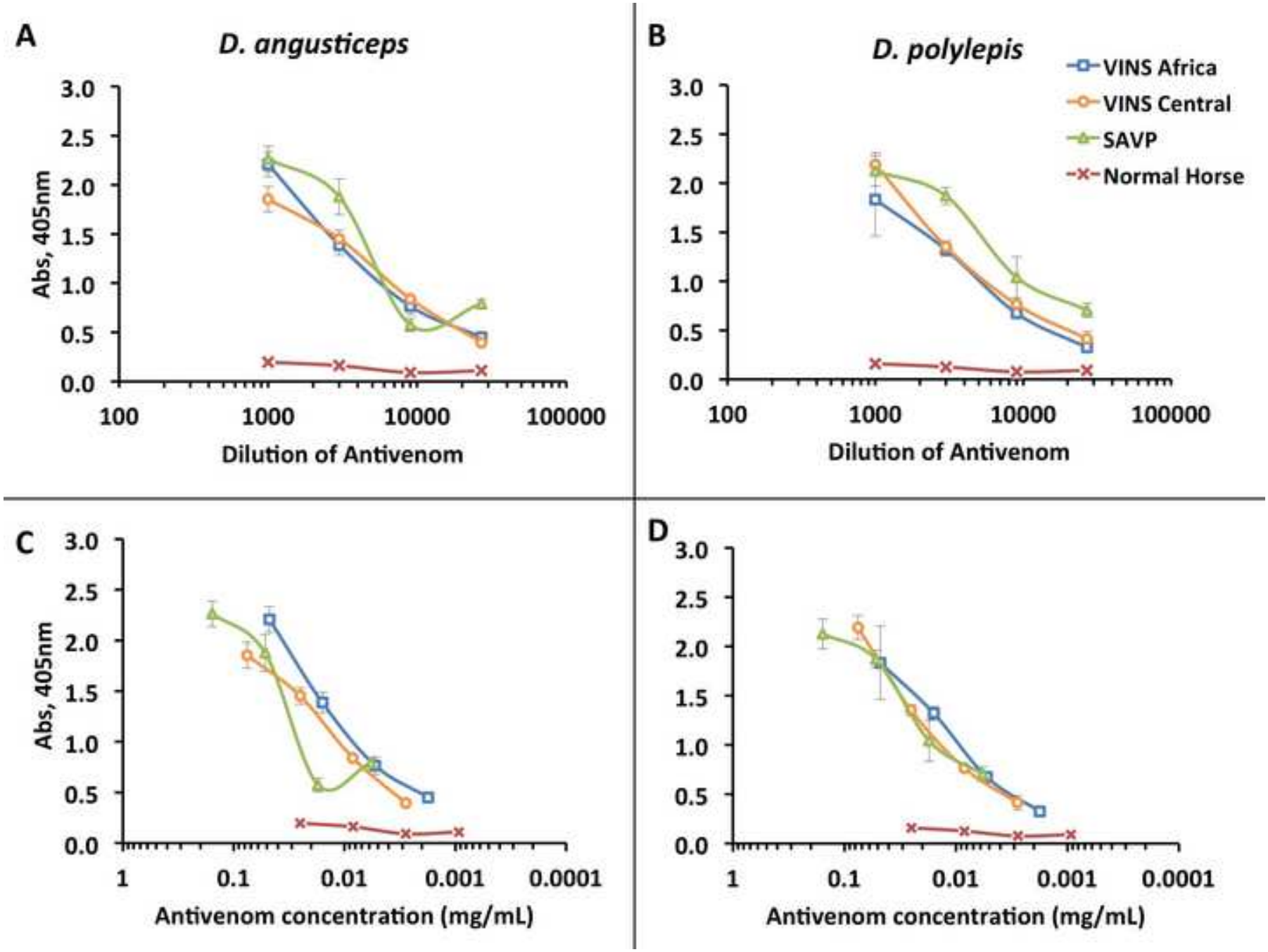

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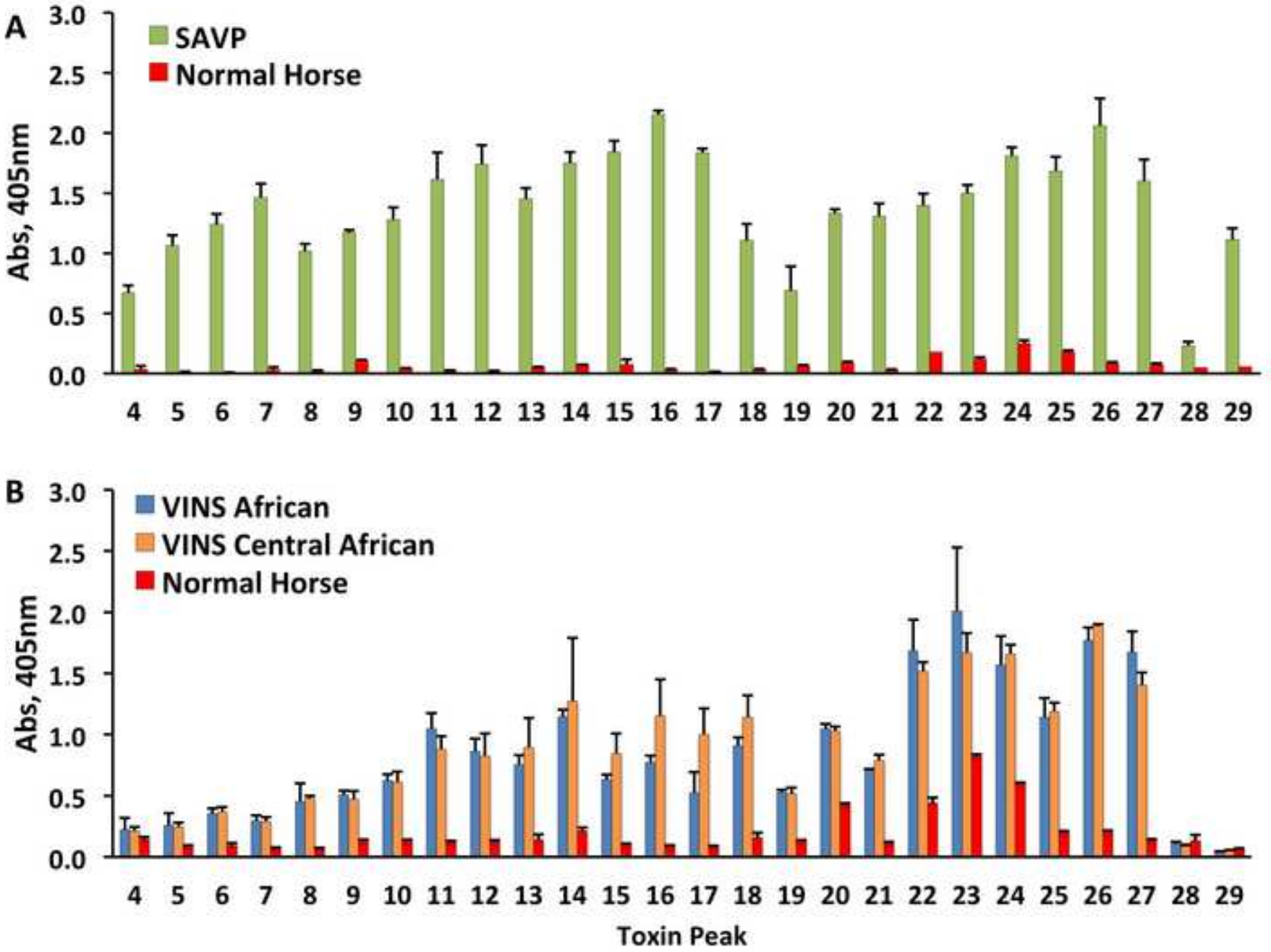

***Conflict of Interest**

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