Comparison of the biological activities in venoms from three subspecies of the South American rattlesnake (*Crotalus durissus terrificus, C. durissus cascavella* and *C. durissus collilineatus*)

Marcelo L. Santoro [a],*, Maria C.C. Sousa-e-Silva [a], Luís R.C. Gonçalves [a], Selma M. Almeida-Santos [b], Diva F. Cardoso [c], Iara L. Laporta-Ferreira [b], Mitiko Saiki [d], Clóvis A. Peres [e], Ida S. Sano-Martins [a]

[a] *Laboratory of Pathophysiology, Instituto Butantan, Av. Vital Brazil 1500, 05503-900 São Paulo-SP, Brazil*
[b] *Laboratory of Herpetology, Instituto Butantan, Av. Vital Brazil 1500, 05503-900 São Paulo-SP, Brazil*
[c] *Laboratory of Immunopathology, Instituto Butantan, Av. Vital Brazil 1500, 05503-900 São Paulo-SP, Brazil*
[d] *Research Institute of Nuclear Energy, IPEN-CNEN, São Paulo-SP, Brazil*
[e] *Department of Mathematics and Statistics, University of São Paulo, São Paulo-SP, Brazil*

Abstract

The subspecies of the South American rattlesnake, *Crotalus durissus*, are classified according to their external morphological features and geographical distribution. We have determined some biological activities of *C. durissus cascavella, C. durissus collilineatus* and *C. durissus terrificus* venoms. *C. durissus terrificus* had a significantly higher clotting activity on bovine plasma and fibrinogen, human fibrinogen and rabbit plasma. *C. durissus cascavella* presented a statistically higher phospholipase A_2 (PLA_2) activity in regard to *C. durissus collilineatus*. Their myotoxic and proteolytic activity, median lethal doses, or median platelet aggregating doses (on rabbit and human platelets) could not differentiate the three subspecies examined. However, the electrophoretic profile and the dose–response curve for edematogenic activity for *C.d. cascavella* venom were different from the others. With regard to the inorganic element content of the venoms, higher levels of Br, Cl and Mg, and a lower level of Zn, were found in *C.d. cascavella* venom. Crotamine-like activity could not be detected in *C.d. cascavella* venom. Furthermore, equine antivenom specific for *C. durissus terrificus* venom cross-reacted equally with the antigens of the three venom pools by ELISA and Western blotting. These results indicate that the venoms from the three studied subspecies of *C. durissus* were very similar, except for minor differences in paw edema-inducing activity, electrophoretic profile, phospholipase A_2 activity, crotamine-like activity and inorganic element contents of *C.d. cascavella* venom. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

South American rattlesnakes (*Crotalus durissus*) are distributed in a huge area of Brazil (Fig. 1, insert). In 1965, five subspecies had been recognized for them [24]; later, six subspecies were recognized [25]. In 1989, Campbell and Lamar reported the occurrence of seven subspecies (*C. durissus cascavella, C. durissus collilineatus, C. durissus dryinas, C. durissus marajoensis, C. durissus ruruima, C. durissus terrificus* and *C. durissus trigonicus*) [9]. Specially, *C. durissus cascavella, C. durissus collilineatus* and *C. durissus terrificus* are the subspecies most frequently received at Instituto Bu-

* Corresponding author. Fax: +55 11 8151505; e-mail: homeroda@uol.com.br

tantan and are distinguished from each other exclusively by their coloration, pattern and geographical distribution [25] (Fig. 2).

According to the Ministry of Health in Brazil, rattlesnakes account for approximately 1500 snakebite accidents annually [6]. Commercial equine antivenom specific for *Crotalus durissus terrificus* venom is utilized for treatment of victims bitten by all subspecies of rattlesnake in Brazil, but no study has demonstrated that cross-reaction of equine antivenom with venom antigens of *C. durissus cascavella* and *C. durissus collilineatus* does occur. In addition, no study has comparatively dealt with biological properties and toxicity of venoms from these three subspecies. In order to investigate this issue, firstly, our objective was to analyze comparatively the biological properties of venoms from *C. durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus* and, secondly, to study the cross-reaction of commercial crotalic antivenom with antigens of their venoms. Furthermore, due to the troublesome classification of rattlesnake subspecies, it was also our aim to evaluate if some of the biochemical and biological activities of South American rattlesnake venoms could be used as additional attributes for studying the taxonomic classification of *C. durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus*.

2. Materials and methods

2.1. Reagents and solutions

Apyrase [28] and rabbit cephalin [3] were purified as described previously. Rabbit, rat and bovine (94.3, 75.6 and 96.1% clottable, respectively) fibrinogens were prepared as described elsewhere [26]. Human fibrinogen (grade L, 82.5% clottable) was purchased from KabiVitrum (Sweden). Human factor X was purified as described by Lopes (MSc thesis, 1992). Casein ('Hammarsten'), Triton X-100 and phenol red were purchased from Merck (Germany). Soybean lecithin was purchased from Naturalis (Brazil). Human factor X-deficient plasma was obtained from Diagnostica Stago (France). Russell's viper venom containing cephalin was purchased from Diagen (Diagnostic Reagents, UK). Prostaglandin E$_1$ (PGE$_1$), *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (Hepes), ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), goat anti-horse IgG (whole molecule) antibody conjugated with peroxidase, bovine serum albumin (fraction V), bovine plasma prothrombin and creatine kinase (CK) kit were obtained from Sigma (St. Louis, MO). *Crotalus durissus terrificus* (crotamine positive) and *Bothrops* sp. specific antivenoms were obtained from Instituto Butantan. All

other reagents were of analytical grade or better. The following buffers were used: Tyrode–Hepes buffer (THB) (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO$_3$, 0.42 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 10 mM Hepes, pH 7.4); phosphate-buffered saline (PBS) (77 mM NaCl, 13.5 mM NaH$_2$PO$_4$, 61.5 mM NaH$_2$PO$_4$, pH 7.4).

2.2. Distribution of snakes and venom milking

Adult wild snakes were sent by suppliers from Northeast, Center-West and Southeast regions of Brazil (Fig. 1). Once the snakes were received at the Laboratory of Herpetology, they were separated by their region of origin (Fig. 1) and classified, according to criteria proposed by Hoge and Romano-Hoge [25], as *Crotalus durissus terrificus*, *Crotalus durissus collilineatus* and *Crotalus durissus cascavella*. Venoms were extracted manually by squeezing the glands, collected in Petri dishes, frozen and lyophilized. Venoms were then mixed and three venom pools, one for each subspecies, were obtained.

2.3. Protein, neutral hexose and inorganic element analysis

The protein content of snake venoms was determined according to Lowry et al. [39], using bovine serum albumin (Serva, Germany) as a standard. The precise concentration of albumin in the solution utilized for plotting the standard curve of protein was checked by spectrophotometry, considering the extinction coefficient of albumin at 280 nm as $\epsilon_{1\ cm}^{1\ mg/ml} = 0.66$ [56]. Neutral hexoses were determined by the phenol–sulfuric acid method [13] using D-glucose as a standard. Instrumental neutron activation was applied for elemental venom analysis [49].

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoresis of venoms was performed in 9–22.5% gradient gels [31]. Venom solutions (3.75 mg ml^{-1} in 154 mM NaCl) were mixed with equal volumes of non-reducing sample buffer (4% SDS, 124 mM Tris, 20% glycerol, pH 6.8) and maintained in boiling water for 10 min. Protein standards were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) (Sigma, St. Louis, MO). Gel was silver stained according to the procedure described elsewhere [4]. Densitometric analyses were performed using ImageMaster Software (Pharmacia Biotech, Sweden).

Fig. 1. Geographic origins of specimens of *Crotalus durissus* subspecies utilized in this study. The distribution of *Crotalus durissus* in Brazil is shown at the top right.

2.5. Median lethal dose (LD₅₀)

The lethal toxicity was determined in 18–22-g male Swiss strain mice from Animal House of the Instituto Butantan. Each venom pool was serially diluted in 154 mM NaCl, and 0.2 ml of each venom dose were injected i.p. into mice. Six groups of five animals were used for determining LD_{50} for each venom pool. Deaths occurring within 48 h were utilized for calculating LD_{50} [36].

2.6. Crotamine activity

Crotamine activity was determined by injecting 250 μg *C. durissus* venom i.p. into Swiss mice weighing 18–22 g [12]. Three mice were used in each venom assay.

2.7. Edema-forming activity

The time–course of edema was determined in Swiss strain mice injected into the subplantar surface of left hind paw with 50 μl of sterile 154 mM NaCl containing 1.5 μg of *Crotalus* venom. The contralateral paw received the same volume of sterile 154 mM NaCl. Edema was measured by plethysmography at various time intervals (30 min and 1, 2, 3 and 5 h) [63]. Results were calculated as the difference between values obtained in both paws and were expressed as percentage of increase in paw volume.

For dose–response studies, the animals were injected with 0.75, 1.5, 3.0 and 6.0 μg of each venom (in 50 μl), and the increase in paw volume was determined at the peak of edematogenic activity (at 30 min after the venom injection).

2.8. Minimum coagulant dose (MCD)

Minimum coagulant dose (MCD) [59] was determined in plasma (MCD-P) and fibrinogen (MCD-F) of different animal species (human, bovine, rabbit and rat) as described previously [53]. Citrated blood samples (1 part of 129 mM trisodium citrate and 9 parts of blood) were obtained by venipuncture of jugular veins of bovines, carotid arteries of rabbits, antecubital veins of voluntary human donors, and abdominal aorta of rats.

Blood was centrifuged at $1700 \times g$ for 15 min at 4°C for obtaining plasma. Plasma pools were prepared with at least four samples from each animal species. Fibrinogen solutions were prepared as described previously [53]. Final venom concentrations ranging from 4.7 to 600 μg ml^{-1} were tested.

2.9. Factor II and X activators

The presence of factor II and X activators in *Crotalus durissus* venoms was tested using purified bovine prothrombin and human factor X as substrates. Briefly,
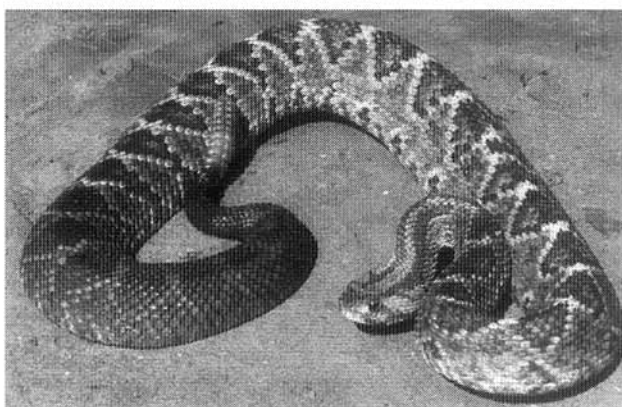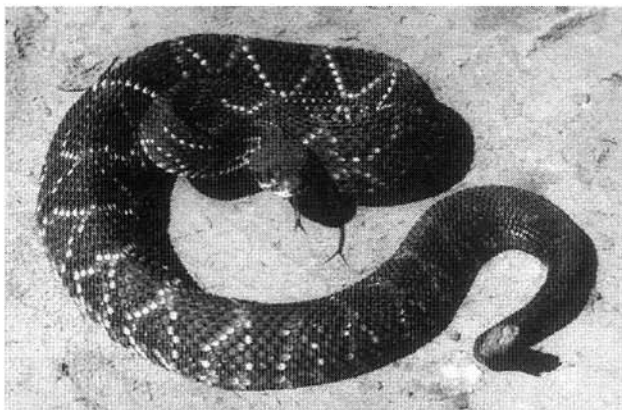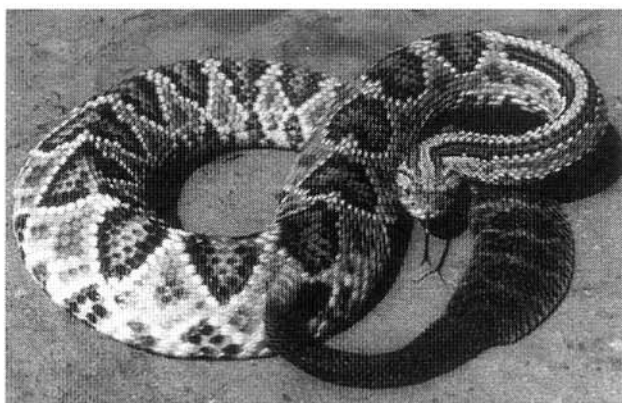

Fig. 2. Adult specimens of *Crotalus durissus collilineatus*, *Crotalus durissus terrificus* and *Crotalus durissus cascavella* are shown, respectively, from the top to the bottom of figure.

prothrombin activation was assessed by incubating 10 μl prothrombin (32 U ml^{-1} in THB) with 50 μl of THB, 50 μl of 25 mM CaCl$_2$ and 50 μl of venom solution (at 80 μg ml^{-1} in THB). After 2 min of incubation at 37°C, 20 μl of *Crotalus durissus* antivenom were added to reaction mixture in order to inhibit intrinsic venom clotting activity. After 2 min of incubation at 37°C, 50 μl of this solution was added to 200 μl of a previously warmed bovine fibrinogen solution (2 mg clottable protein ml^{-1}), and the clotting time recorded.

Factor X activation was determined in a similar manner. Ten μl of Factor X (64 U ml^{-1} in THB), 50 μl of THB, 25 μl of rabbit cephalin (1:100 dilution of the concentrated saline emulsion), 25 μl 50 mM CaCl$_2$ and 50 μl of venom solution (at 80 μg ml^{-1} in THB) were all incubated at 37°C for 2 min. After 2 min of incubation, 20 μl of *Crotalus durissus* antivenom were added to the reaction mixture. Fifty μl of this solution were taken after 2 min of incubation at 37°C, and added to 200 μl of previously warmed factor X-deficient plasma, and the clotting time recorded.

As controls, *Bothrops jararaca* venom (and *Bothrops* antivenom) was used for testing prothrombin activation; factor X activation was assessed using *Vipera russelli* venom. All clotting times were recorded on a fibrometer (BBL, Becton Dickison, USA), with constant temperature maintained at 37°C.

2.10. Platelet aggregating effective dose 50% (PA-ED$_{50}$)

Suspensions of washed human platelets were prepared as described previously [52]. For preparation of washed rabbit platelets, 6 parts of rabbit blood were collected into 1 part of ACD anticoagulant (85 mM trisodium citrate, 71.4 mM citric acid, 111 mM dextrose). For obtaining platelet-rich plasma (PRP), blood was centrifuged at $150 \times g$ for 15 min at room temperature. PGE$_1$ was added to PRP (12.5 μl of a 200 μg ml^{-1} concentration in ethanol for each 10 ml of PRP), which then remained at room temperature for 10 min. The PRP was centrifuged at $1700 \times g$ for 15 min, and rabbit platelet pellets resuspended in a modified calcium-free Tyrode solution (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO$_3$, 0.42 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 0.26 mM EGTA, 0.35% bovine albumin, 5.6 mM dextrose, 0.02% apyrase, 0.05 μg ml^{-1} PGE$_1$, pH 6.2). After remaining at 37°C for 10 min, the suspension was centrifuged at $1700 \times g$ for 15 min. This procedure was repeated once more, and finally platelet pellets were resuspended in Tyrode solution containing calcium (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO$_3$, 0.42 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 0.35% bovine albumin, 5.6 mM dextrose, 2 mM CaCl$_2$, 10 mM Hepes, pH 7.4). Platelet count was ascertained to

Table 1
Protein, neutral hexose, and inorganic element analyses of *Crotalus durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus*

Analyses	Venoms		
	C.d. cascavella	C.d. collilineatus	C.d. terrificus
Protein (mg/mg venom)	0.735	0.762	0.791
Neutral hexoses (μg/mg venom)	8.49	6.93	10.59
Inorganic element			
Na (mg/g venom)	17.20 ± 0.30^a	17.60 ± 0.30	21.10 ± 0.40
Mg (mg/g venom)	9.49 ± 0.28	4.73 ± 0.14	3.63 ± 0.35
Cl (mg/g venom)	5.79 ± 0.14	0.85 ± 0.04	1.15 ± 0.04
Ca (μg/g venom)	502 ± 39	395 ± 29	363 ± 25
Br (μg/g venom)	26.39 ± 0.09	4.34 ± 0.05	6.16 ± 0.06
Fe (μg/g venom)	2.90 ± 0.40	20.30 ± 1.10	1.80 ± 0.40
Rb (μg/g venom)	4.53 ± 0.08	8.97 ± 0.09	9.02 ± 0.08
Se (μg/g venom)	4.18 ± 0.03	4.45 ± 0.03	4.99 ± 0.02
Zn (μg/g venom)	40.60 ± 0.20	118.70 ± 0.40	102.10 ± 0.30
Sb (ng/g venom)	12.70 ± 1.10	12.50 ± 1.10	6.80 ± 0.80
Cs (ng/g venom)	71.20 ± 2.90	79.00 ± 1.20	27.00 ± 2.10
Sc (ng/g venom)	0.32 ± 0.09	0.24 ± 0.11	0.70 ± 0.13

[a] Data are expressed as mean $\pm$ SD obtained in one sample.

be 300×10^9 1^{-1} and the platelet suspension was maintained at room temperature until use.

Platelet aggregation was recorded by Born's method [5] in a Chrono-log aggregometer (model 560, Harvetown), with bar stirring speed set at 1000 rpm and constant temperature maintained at 37°C. Ten μl of two-fold serial dilutions of venoms in THB buffer were added to 400 μl of a prewarmed platelet suspension, and platelet aggregation monitored. Five minutes after the addition of venom, the percentage of platelet aggregation was recorded and used for calculating the PA-ED$_{50}$. Final venom concentrations ranging from 35.7 ng ml^{-1} to 73.2 μg ml^{-1} were tested.

2.11. Phospholipase A$_2$ activity

PLA$_2$ (EC 3.1.1.4) activity was evaluated using a modification of a previously reported method [37]. Venom was two-fold serially diluted in PBS. Fifteen μl of venom solutions were added to 1.5 ml of reaction medium (100 mM NaCl, 10 mM CaCl$_2$, 7 mM Triton X-100, 26.5% soybean lecithin, 98.8 μM phenol red, pH 7.6) in a spectrophotometer cuvette. The solution was immediately homogenized and inserted in a Micronal B382 spectrophotometer (558 nm) coupled to a Chrono-log recorder (set at 0.2 V and paper speed at 1 cm min^{-1}). Enzymatic activity was recorded and results were expressed as the maximum slope of decrease in absorbance min^{-1}. Final venom concentrations ranging from 0.16 to 9.90 μg ml^{-1} were tested. The venom concentration that produced a change in absorbance of 0.3 units min^{-1} was used for comparison of PLA$_2$ activity among subspecies of *C. durissus*.

2.12. Proteolytic activity

Proteolytic activity of venoms was determined by a slight modification of a previously described method [38]. Briefly, 500 μl of 1% casein dissolved in 50 mM Tris–HCl buffer, pH 8.0 [30,51], were incubated at 37°C with two-fold serial dilutions of venoms in THB buffer (250 μl), and after 30 min reaction was interrupted with 5% trichloroacetic acid (1 ml). Blanks were made for each venom dilution by incubating 5% trichloroacetic acid (1 ml) with 1% casein (500 μl) and venom dilutions (250 μl). After incubation for 30 min at room temperature, tubes were centrifuged at 2000 × g for 15 min, and absorbance of the supernatants was read at 280 nm. The change in absorbance induced by each venom concentration was subtracted from the respective blank, and used for evaluating proteolytic activity. Due to the low proteolytic activities of *C. durissus* venoms, the venom concentration that caused a change in absorbance of 0.03 was used for comparison among venoms. Final venom concentrations ranging from 10.4 to 1500 μg ml^{-1} were tested.

2.13. Myotoxic activity

Mice (Swiss strain, 18–22 g) were injected in the gastrocnemius muscle with 1.5 μg of *Crotalus durissus* venom in 50 μl of 154 mM NaCl. Control mice were injected with 154 mM NaCl. Blood was obtained by puncture with capillary tubes from retroorbital plexus before and at 3, 6, 9 and 24 h after injection. The myotoxic activity was evaluated by the rise of serum creatine kinase (CK) (EC 2.7.3.2) levels using a com
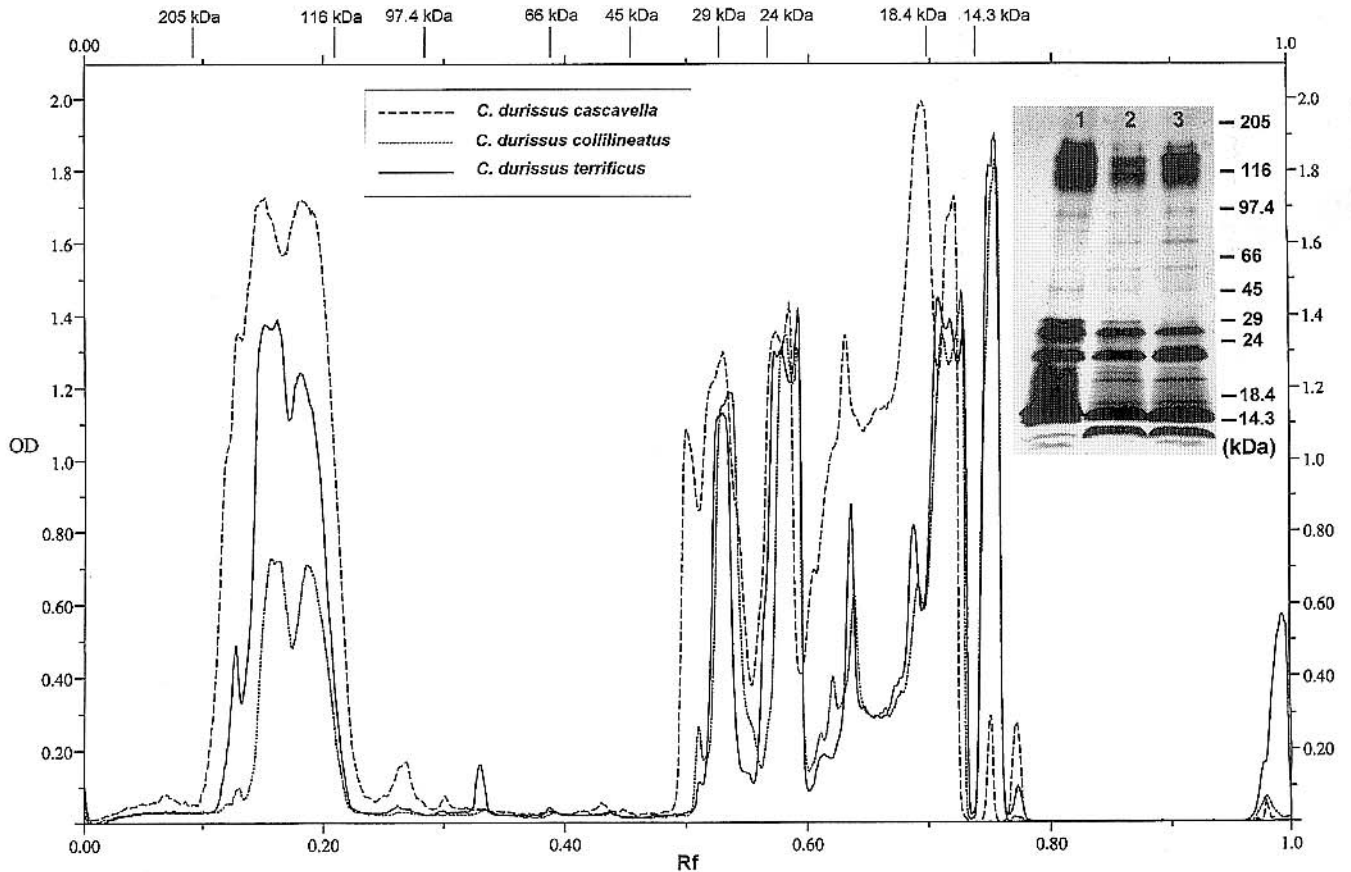
Fig. 3. Densitometric profiles of non-reduced samples of *Crotalus durissus cascavella* (1), *C. durissus collilineatus* (2) and *C. durissus terrificus* (3) venoms. Insert at the top right corner shows the SDS–PAGE, performed in 9–22.5% gradient gels as described by Laemmli [31]. Forty-seven μg of each venom sample were loaded per lane.

mercial kit. Enzyme activity was expressed in units ml^{-1}, 1 unit resulting in the phosphorylation of 1 nmol of creatine min^{-1} at 25°C.

2.14. Cross-reaction of venom antigens with commercial equine antivenom

Cross-reactivity of venom antigens from *C. durissus* subspecies with equine antivenom specific for *C. durissus terrificus* venom, produced by Instituto Butantan, was determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. *C. durissus* antivenom was titered for its antibody content in microplates coated with *C. durissus cascavella*, *C. durissus collilineatus* or *C. durissus terrificus* venoms (2 μg ml^{-1}). Antigen–antibody reaction was detected using goat anti-horse IgG (whole molecule) antibody conjugated with peroxidase, followed by orthophenylene diamine plus H_2O_2 as the enzyme substrate. Plates were read using an ELISA plate reader (Mutiskan spectrophotometer, Eflab, Helsinki, Finland); antibody titers against each venom pool were determined as the reciprocal of the highest dilution that gives an absorbance greater than 0.100 at 492 nm, since control

reaction with normal horse serum was observed below this value. For immunoblotting, the three venom pools (10 μg) were boiled for 5 min in the presence of 10 mM Na_2EDTA, under non-reducing conditions, and fractionated by SDS–PAGE in 12% polyacrylamide slab gels [31]. Antigens were transblotted onto nitrocellulose papers [60], and developed by addition of 1000-fold diluted commercial equine antivenom (Instituto Butantan) diluted in PBS plus 1% bovine serum albumin. After washing, strips were incubated for 1 h with goat anti-horse IgG (whole molecule) antibody conjugated with peroxidase and stained by addition of 4-chloro-1-α-naphthol plus H_2O_2 as the enzyme substrate. Phospholipase b (94 kDa), bovine albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa) (Pharmacia Biotech, Sweden) were used as low molecular mass standards.

2.15. Statistical analysis

For statistical comparison of the three subspecies of *Crotalus durissus*, two-way ANOVA was utilized for analysis of edematogenic and myotoxic (time–course

Table 2
Comparison of different activities among three subspecies of Brazilian rattlesnakes (*Crotalus durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus*)

Determination	Venom		
	C. d. cascavella	*C. d. collilineatus*	*C. d. terrificus*
Crotamine	Absent	Present	Present
LD$_{50}$ (μg kg^{-1})	82.3 (65.8–102.9)	86.6 (69.9–107.4)	73.4 (58.7–91.7)
MCD-P (μg ml^{-1})			
Human	22.3 (20.0–24.6)	21.9 (19.6–24.3)	19.9 (17.9–21.9)
Bovine	104.7*** (74.1–135.3)	93.4* (65.3–121.4)	45.5*,*** (39.0–52.0)
Rabbit	235.2 (75.1–395.4)	60.9* (40.4–73.3)	35.5* (31.1–39.9)
Rat	302.1 (0.0–1508.5)	346.2 (0.0–2024.0)	231.2 (0.0–889.7)
MCD-F (μg ml^{-1})			
Human	27.6 (23.5–31.7)	29.2* (25.0–33.4)	21.5* (19.5–23.6)
Bovine	51.0** (40.0–61.9)	55.3* (43.3–67.2)	31.4*,** (26.9–35.8)
Rabbit	207.3 (0.0–419.5)	51.7 (39.3–64.2)	33.3 (27.6–38.6)
Rat	245.7 (0.0–562.9)	403.3 (0.0–1203.4)	363.8 (0.0–1036.0)
Factor II activation[a] (s)	>350	>350	>350
Factor X activation[b] (s)	>500	>500	>500
Platelet-aggregating activity (ED$_{50}$) (ng ml^{-1})			
Rabbit	472.7 (0.0–2456.0)	786.1 (0.0–5364.6)	551.3 (0.0–3019.0)
Human	52.0 (0.0–105.1)	119.1 (0.0–378.6)	132.8 (0.0–464.6)
Phospholipase A$_2$ activity (μg ml^{-1})	0.99*** (0.75–1.23)	1.95*** (1.65–2.25)	1.40 (1.13–1.67)
Proteolytic activity[c] (μg ml^{-1})	1136.1 (752.8–1519.5)	844.6 (612.1–1077.1)	535.7 (401.0–670.4)

Data within parenthesis represent 95% confidence intervals.
[a] Each response was the mean of three observations. Control: *Bothrops jararaca* venom, 56.2 s.
[b] Each response was the mean of three observations. Control: *V. russelli* venom, 49.4 s.
[c] The same response was obtained with 21.1 μg ml^{-1} *B. jararaca* venom (95% confidence interval, 5.6–36.6 μg ml^{-1}).
*$P < 0.05$; **$P < 0.05$; ***$P < 0.01$.

and dose–response) activity. Values of LD$_{50}$ and their 95% fiducial limits were compared as described previously [36].

Estimates and 95% confidence intervals of MCD, PA-ED$_{50}$, PLA$_2$ and proteolytic activity were calculated by inverse linear regression [7]. Data transformations were used in order to improve fitness of the models and, for each case, a general linear model has been adjusted, and dummy variables were included in order to test the three treatments simultaneously. In order to estimate the parameters defined above, through inverse regression [7], a simple model for each treatment was generated from this general linear model. According to Brown [7], the estimator ζ and its $(1 - \gamma)100\%$ confidence interval may be calculated by:

$$(W)\left[1 + \frac{(\hat{\sigma}^2 t^2)}{(\hat{\beta}^2 S_{xx})}\right] \pm \left(\frac{\hat{\sigma} t}{\hat{\beta}}\right)\left[1 + \frac{1}{(2n)} + \frac{W^2}{2S_{xx}} + \frac{(\hat{\sigma}^2 t^2)}{2\hat{\beta}^2 S_{xx}}\right]$$

where W is the antilog of $(Z - \hat{\alpha})/\hat{\beta}$, $\hat{\sigma}^2$ is the calculated mean-square residual of the regression, n is the number of observations, t is the tabulated upper $(\gamma/2)100\%$ point of the Student t-distribution on $(n - 2)$ degrees of freedom and $S_{xx} = \Sigma(\zeta_i - \bar{\zeta})^2$. The results from different treatments were compared controlling the significance level for multiple comparisons (Bonferroni's correction). Statistical analyses were performed with the software package Stata™ 4.0.

3. Results

The protein and neutral hexose content in the three venom pools were very similar (Table 1). Most of the inorganic elements did not vary significantly. However, *C. durissus cascavella* venom showed higher concentrations of Mg, Cl, Br, and lower concentrations of Rb and Zn. In addition, the Fe content was about 10-fold higher in *C. durissus collilineatus* venom (Table 1).

Non-reduced SDS–PAGE showed very comparable protein patterns for the three venom pools (Fig. 3). The venom of *C. durissus collilineatus* presented decreased amounts of proteins with molecular masses above 97.4 kDa. Noteworthy, decreased amounts of a protein with approximately 36 kDa and of proteins with molecular masses under 14.3 kDa were also observed in *C. durissus cascavella* venom. In accordance to the later observation, *C. durissus cascavella* venom showed no crotamine-like activity (Table 2).

The three subspecies presented similar toxicity as shown by the LD$_{50}$ values in Table 2.

The three venoms showed thrombin-like activity, since they could coagulate fibrinogen as well as plasma from different species of mammals (Table 2). On the other hand, no prothrombin or factor X activator could be shown (Table 2). However, differences in substrate specificity were observed: the highest clotting activity
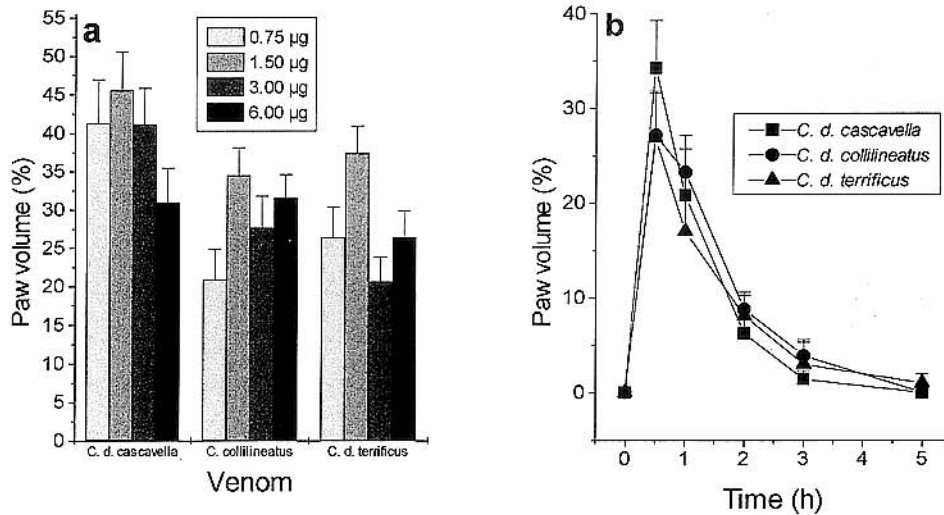
Fig. 4. Edematogenic activity of *Crotalus durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus* venoms. (a) Dose–response profiles obtained from mice injected with 0.75, 1.5, 3.0 and 6.0 μg of each venom. The increase in paw volume was determined at 30 min after the venom injection). Results are expressed as mean $\pm$ SEM ($n = 7$–9). The profile of *C. durissus cascavella* venom was statistically different from the other two profiles ($P < 0.05$). (b) Time-course of edematogenic activity measured by plethysmography. Mice were injected with 1.5 μg of each venom into the subplantar surface of hind paw. Results are expressed as mean $\pm$ SEM ($n = 5$–8).

was observed for human plasma and fibrinogen and the lowest for rat plasma and fibrinogen. (Table 2). Physiological plasma inhibitors had low affinity for thrombin-like enzymes of *C. durissus* venoms, since the MCD values were quite similar in plasma and fibrinogen of each species of mammal. *C. durissus terrificus* venom presented statistically lower MCD values in bovine plasma and fibrinogen, human fibrinogen and rabbit plasma (Table 2). *C. durissus cascavella* venom showed
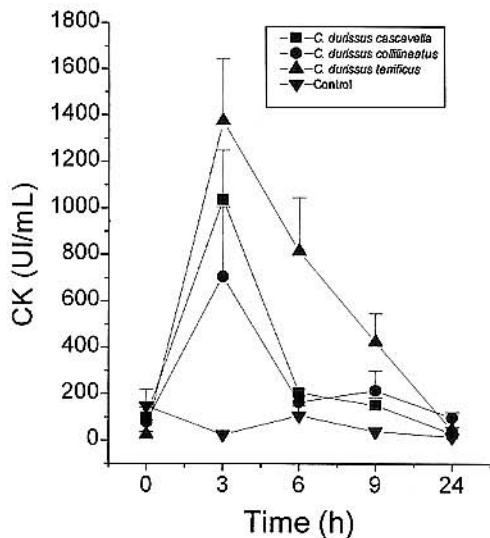

Fig. 5. Myotoxic activity of *Crotalus durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus* venoms. The three profiles of *Crotalus durissus* venoms were not statistically different. Statistical significance for the three venoms was observed only if comparisons were done separately for each time interval (*C. durissus collilineatus* vs *C. durissus terrificus* at 6 h ($P = 0.043$)). Data are expressed as mean $\pm$ SEM ($n = 3$–4 animals for each point).
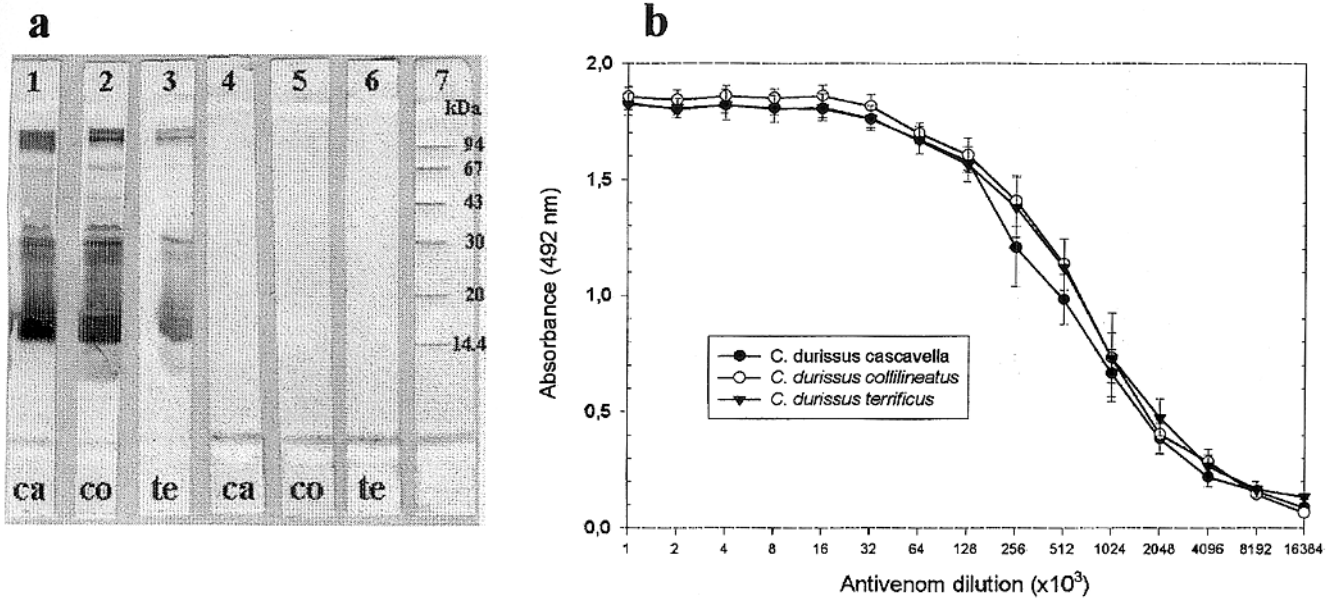
lower affinity for rabbit fibrinogen and plasma in comparison to the other two venoms, but this difference was not statistically significant (Table 2).

PA-ED$_{50}$ of *C. durissus* venoms was at least five times more potent in human than in rabbit platelets (Table 2). The highest platelet aggregating activity on rabbit and human platelets was observed for *C. durissus cascavella* venom, but this difference was not statistically significant.

C. durissus cascavella venom presented the highest PLA$_2$ activity, whereas *C. durissus collilineatus* the lowest ($P < 0.01$, *C. durissus cascavella* vs. *C. durissus collilineatus*) (Table 2). The venoms of *Crotalus durissus* subspecies showed negligible proteolytic activity, as shown in Table 2. No statistical difference among venoms was observed.

As shown in Fig. 4, the edema induced by the three venoms was not dose dependent, but the dose–response profile of *C. durissus cascavella* venom was somewhat different from those of *C. durissus collilineatus* and *C. durissus terrificus* (Fig. 4a) ($P < 0.05$). After venom injection, the edema developed rapidly, reaching its peak at 30 min, subsequently decreasing, and disappearing completely at 5 h (Fig. 4b). However, statistical analysis demonstrated that no difference was observed in the time–course of edematogenic responses among the three venoms (Fig. 4b).

The peak of myotoxic activity occurred 3 h after venom injection, and then progressively decreased (Fig. 5). *C. durissus terrificus* venom showed the highest CK values between 3 and 9 h after venom injection; the CK value at 6 h for *C. durissus terrificus* venom was statistically higher than that of *C. durissus collilineatus* ($P =$

Fig. 6. (a) Western blotting. *C. durissus cascavella* (**ca**), *C. durissus collilineatus* (**co**) and *C. durissus terrificus* (**te**) venoms were fractionated by SDS–PAGE and revealed with commercial equine antivenom specific for *C. durissus terrificus* venom (lanes 1–3) or normal horse serum (lanes 4–6), as described in Section 2. Lane 7 depicts molecular mass standards. (b) ELISA. Cross-titration curves of antibodies present in commercial equine antivenom. Data are expressed as mean ± SEM of three experiments.

0.043). However, the three time–course profiles were not statistically different from each other.

Commercial equine antivenom recognizes indistinctly venom antigens from the three *C. durissus* subspecies, both by Western blotting and ELISA (Fig. 6a,b). Noteworthily, a faint spot with molecular masses lower than 14.4 kDa was revealed by Western blotting in *C. durissus collilineatus* and *C. durissus terrificus* venom, but not in *C. durissus cascavella* (Fig. 6a). The general linear model fitted for the three ELISA titration curves shows that they are coincident ($P = 0.099$) (Fig. 6b).

4. Discussion

The rattlesnake *Crotalus durissus* is distributed in a huge continental area that reaches South and Central America [9]. Thus, biological activities of venoms may vary drastically according to the geographical origin of specimens. For example, venoms from adult specimens coming from Central America, classified as *C. durissus durissus*, present high proteolytic, hemorrhagic and edematogenic activities, and are devoid of neurotoxic activity [22]; on the other hand, venoms from specimens coming from South America (*C. durissus terrificus*) present an inversion of these activities, i.e. high neurotoxic activity and a lack of proteolytic, hemorrhagic and edematogenic activities [48,51,58]. Since no study has dealt with the properties and biological activities of venoms from the other two main subspecies of Brazil— *C. durissus cascavella* and *C. durissus collilineatus*—in

comparison with the well-studied subspecies *C. durissus terrificus*, a comparative quantification of venom activities was performed to tentatively differentiate them. For this purpose, a special statistical method—the inverse linear regression—that can calculate confidence intervals for the estimators of MCD, PA-ED$_{50}$, PLA$_2$ and proteolytic activity was utilized. The results shown herein suggested that venoms from *C. durissus terrificus* and *C. durissus collilineatus* presented identical activities for most of the tests employed, and that they also were very similar to *C. durissus cascavella* venom.

The LD$_{50}$ values reported herein did not differ significantly from each other. The value obtained for *C. durissus terrificus* venom agrees with those reported elsewhere [12,42,51]. However, Sanchez et al. [51] found out a lower i.p. LD$_{50}$ for *C. durissus collilineatus* (48.5 μg kg^{-1}). Lennon and Kaiser [35] also observed a low LD$_{50}$ for *C. durissus collilineatus* (70 μg kg^{-1}), but this value was obtained using the i.v. route. Reported values in the literature for LD$_{50}$ of *C. durissus vegrandis* (200 μg kg^{-1}, i.v.), *C. durissus durissus* (889 μg kg^{-1}), *C. durissus cumanensis* (1143 μg kg^{-1}) and *C. durissus totonacus* (2500 μg kg^{-1}) are higher than those reported herein [20,22,46,54]. However, *C. durissus ruruima* venom presents LD$_{50}$ values lower than those of *C. durissus terrificus* [12].

Organic and inorganic content of the venom pools could not properly differentiate these three subspecies. The inorganic content reported herein agrees with most of the values reported previously [33], except for the higher concentration of Cl and Br in *C. durissus cascav-*

ella, and Fe in *C. durissus collilineatus* venom. The content of neutral hexoses was the same order of magnitude reported elsewhere for *Crotalus adamanteus* and *Crotalus atrox* [44]. The analysis of electrophoretic profiles showed a great similarity among the three venoms, especially between *C. durissus collilineatus* and *C. durissus terrificus*. In comparison with the other two venoms, *C. durissus cascavella* showed minute amounts of proteins with molecular masses lower than 14.4 kDa, suggesting that crotamine would be absent. As expected, this evidence was confirmed by the absence of crotamine-like activity in *C. durissus cascavella* venom. In addition, Western blotting using commercial equine antivenom did not reveal protein bands with molecular masses lower than 14.4 kDa in *C. durissus cascavella*, which corroborates this evidence. The lack of crotamine in *C. durissus cascavella* was also observed by others [15]. Crotamine is a 4.9-kDa protein that causes paralysis associated with hyperextension of hind limbs of mice [10,34], and its differential occurrence in rattlesnake venoms was observed as early as in 1950/1951 [2]. In fact, a new subspecies has been proposed, *Crotalus terrificus crotaminicus*, based exclusively on the presence of crotamine in rattlesnake venoms [17]. However, the presence of crotamine in *C. durissus* venoms does not discriminate a new subspecies, since it may occur in venoms of different subspecies and, in addition, in different specimens of the same subspecies [2,12,17,18,21,55].

PLA$_2$ activity of the three *C. durissus* venom pools was very similar. However, *C. durissus collilineatus* showed a significantly lower PLA$_2$ activity (Table 2) and, in addition, the lowest myotoxic activity (Fig. 5). Two reports have shown that the venoms of *C. durissus ruruima* and *C. durissus durissus* possess, respectively, similar or lower myotoxic activities than *C. durissus terrificus* venom, despite their increased PLA$_2$ activities [12,22]. In fact, the myotoxicity that occurs after the administration of South American *Crotalus durissus* venom may be caused by at least two components, i.e. crotoxin and crotamine [8,19,29,41]. Despite the controversies concerning the relationship between myonecrotic and hydrolytic activities of venom PLA$_2$ [41], the myotoxicity induced by crotoxin is largely caused by its PLA$_2$ enzymatic activity [19]. Thus, the low myotoxicity of *C. durissus collilineatus* venom may be due to its low PLA$_2$ activity. Furthermore, the presence of both crotamine and a high PLA$_2$ activity provide *C. durissus terrificus* venom with the highest myotoxic activity. Interestingly, the highest PLA$_2$ activity in *C. durissus cascavella* venom is in accordance with its increased amount of a protein band of 15.4 kDa (Fig. 3), the previously reported value for the basic subunit of the crotoxin molecule [27]. Despite the lower PLA$_2$ activity in *C. durissus collilineatus*, the results reported herein do not agree with those obtained elsewhere [15], since

the crotoxin band of *C. durissus collilineatus*, shown by SDS–PAGE (Fig. 3), is nearly identical to those of the two other subspecies. However, the finding of a crotoxin isoform in *C. durissus collilineatus* venom almost identical to the *C. durissus terrificus* crotoxin agrees' with data reported herein [35].

Although the three subspecies of *C. durissus* exclusively demonstrated thrombin-like activity, and no activation of coagulation factors II and X, the clotting activities of each venom pool varied enormously in regard to the species (human, bovine, rabbit and rat) and substrate (plasma or fibrinogen) analyzed. In fact, the exclusive thrombin-like activity of *C. durissus terrificus* venom had already been reported [43]. Later, the thrombin-like enzyme of *C. durissus terrificus* was purified [1,45,47]. Despite the fact that the three venom pools coagulate plasma and fibrinogen of many mammalian species, differences in species specificity were observed. Thus, the highest clotting activity was observed for human plasma and fibrinogen, and the lowest in rat plasma and fibrinogen. In fact, our results suggest that rat plasma and fibrinogen are not a suitable model for studying clotting activity of *C. durissus* venoms. Thus, our results disagree with those reported for the purified *C. durissus terrificus* thrombin-like enzyme, which revealed the highest clotting activity in rat plasma [47]. The presence of other proteins in crude venom that interfere with the clotting activity of *C. durissus terrificus* thrombin-like enzyme may be considered. Besides, the observation of a high clotting activity of *C. durissus cascavella* venom on bovine fibrinogen disagrees with previous data showing its negligible clotting activity [62]. Interestingly, the venoms from the three subspecies of rattlesnakes analyzed herein could coagulate rabbit fibrinogen, indicating that the thrombin-like enzymes of *Bothrops jararaca* and *C. durissus* are quite different in their substrate specificity [53]. In fact, differences in substrate specificity for clotting enzymes of snake venoms may exist for adaptation to many species of prey, which may have different strategies for coagulation [53]. Other *C. durissus* subspecies—*C. durissus durissus*, *C. durissus totonacus* and *C. durissus ruruima*—also presented thrombin-like activity [12,58].

In regard to the platelet-activating activity, based on the PA-ED$_{50}$ values, all *Crotalus durissus* venoms better aggregated human platelets than rabbit platelets. In fact, convulxin, a 72-kDa glycoprotein that activates blood platelets [40], has been shown to be more active, in human than in rabbit platelets [62]. Moreover, *C. durissus cascavella* venom was the most potent among the three subspecies, probably due to the increased amounts of convulxin in this venom [23]. However, elevated amounts of protein bands of 72 kDa in *C. durissus cascavella* could not be demonstrated by SDS–PAGE (Fig. 3). Crotoxin has also been shown to

activate platelets, but it is about 50-fold less effective than convulxin in doing so [32,62]. Since crotoxin is present in great amounts in South American *C. durissus* venom, and the highest PLA$_2$ activity was observed in *C. durissus cascavella*, it remains a matter of speculation as to which one of them contributes most to platelet aggregation when the whole venom is assayed.

The three venom pools showed negligible proteolytic activities, in agreement with previous reports [12,48,51]. This finding differentiates South American from most North and Central American rattlesnakes [22,58]. The edematogenic response induced by *C. durissus terrificus* venom was not dose-dependent and has a time–course rapid and transient, which agrees with results previously obtained [57]. The results showed that the three venoms induced comparable edematogenic responses, and that the maximum edema was observed 30 min after venom injection, decreasing afterwards. The edematogenic activity of the three venoms is mild and does not reach values higher than 35%. These results are in accordance with those reported elsewhere for venoms of *C. durissus collilineatus*, *C. durissus terrificus* and *C. durissus ruruima* [12,51]. Interestingly, *C. durissus durissus* possesses a high edema-forming activity, resembling the venom properties of North American rattlesnakes [22].

In view of data obtained by ELISA and Western blotting, the results reported herein point out that commercial equine antivenom for *C. durissus terrificus* venom may be also safely utilized for treatment of victims bitten by *C. durissus cascavella* and *C. durissus collilineatus* specimens.

Despite some differences of *C. durissus cascavella* venom, in comparison with *C. durissus collilineatus* and *C. durissus terrificus*, the three venom pools showed the characteristic activities of the South American rattlesnake [48]. A similar profile of venom activities was also described for *C. durissus ruruima*, a snake that inhabits the Northern Brazilian Amazon [12]. However, other subspecies that inhabit the Northern region of South America and Central America, such as *C. durissus totonacus*, *C. durissus durissus* and *C. durissus cumanensis*, present venom characteristics that resemble North American rattlesnakes, e.g. intense proteolytic and hemorrhagic activities, and higher LD$_{50}$ values in comparison to South American rattlesnakes [20,58]. One exception to the typical characteristics of North American rattlesnake venoms is *Crotalus scutulatus scutulatus*, a species which venom may present neurotoxic activity and lower LD$_{50}$ [16], resembling South American rattlesnakes.

Our results demonstrated that activities of *C. durissus cascavella*, *C. durissus collilineatus* and *C. durissus terrificus* venoms are not sufficiently appropriate for differentiating these subspecies. Since the habitat of *C. durissus cascavella* (dry thorn scrub, the Caatinga region) is quite different from that of *C. durissus collilineatus* and *C. durissus terrificus* (semideciduous forest, the Cerrado region), and lizards are more abundant for ingestion by *C. durissus cascavella* [61] than small rodents by the other two subspecies [50], the venom of *C. durissus cascavella* may have adapted differently for the type of prey found in Caatinga region, and this condition may explain in part the few differences observed for *C. durissus cascavella* venom. In fact, it is notorious that venom toxicity may vary according to the prey species ingested by snakes [14]. Moreover, it has been proposed that a strong relationship exists between diet and venom composition [11].

In conclusion, our findings do suggest that the differences in three venom pools studied herein are very subtle, and that *C. durissus cascavella* venom seems to be the most different one. Indeed, Sanchez et al. [51] also concluded that activities of *C. durissus collilineatus* and *C. durissus terrificus* venoms are very similar. In fact, as these three subspecies of *C. durissus* present subtle differentiation of their external morphological characteristics and venom biological activities, it may be suggested that a true differentiation at subspecies level does not exist, and that the geographical origin of specimens may explain their differences.

Acknowledgements

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