Saber, Universidad de Oriente, Venezuela. Vol. 28 Nº 1: 40-61. (2016)
ISSN: 2343-6468 Digital / Depósito Legal ppi 198702SU4231 ISSN: 1315-0162 Impreso / Depósito Legal pp 198702SU187

COMPARATIVE ANALYSIS OF BIOLOGICAL AND BIOCHEMICAL VENOM CHARACTERISTICS OF NORTH AND SOUTH AMERICAN *Crotalus* (SERPENTES: VIPERIDAE) SNAKE SPECIES: AN INTRODUCTORY APPROXIMATION TO UNDERSTAND THEIR BIOLOGICAL FUNCTIONS

UN ANÁLISIS COMPARATIVO DE LAS CARACTERÍSTICAS BIOLÓGICAS Y BIOQUÍMICAS DE LOS VENENOS DE SERPIENTES *Crotalus* (SERPENTES: VIPERIDAE) DE NORTE Y SURAMÉRICA: UNA APROXIMACIÓN INTRODUCTORIA PARA ENTENDER SUS FUNCIONES BIOLÓGICAS

ELDA E. SÁNCHEZ[1], ALEXIS RODRÍGUEZ-ACOSTA[2]

[1]Texas A&M University-Kingsville, National Natural Toxins Research Center, Kingsville, USA, [2]Universidad Central de Venezuela, Instituto Anatómico, Laboratorio de Inmunoquímica y Ultraestructura, Caracas, Venezuela
E-mail: rodriguezacosta1946@yahoo.es

ABSTRACT

In this study, venom samples of rattlesnakes from the United States of America and Venezuela Bolivarian Republic were tested for their gelatinase, fibrinolytic, hemorrhagic and coagulant activities, as well as inhibition of platelet function. Electrophoretic titration curves on polyacrylamide gel were used to compare protein composition. There were different protease activities in the venoms. Hemorrhagic activity showed high geographic variation, but northern specimens had a higher trend in this parameter. Differences in venom protein conformation were palpable between snakes from the North and South America. There was a distinct protein composition among all venoms, several venoms presented hemorrhagic and fibrinolytic activities, and different performance on platelet assays. These results demonstrate the interesting differences between North and South America *Crotalus* venoms.

KEY WORDS: Coagulation, fibrinolysis, hemorrhagic, platelet function, snake venom.

RESUMEN

En este estudio se examinaron muestras de veneno de serpientes de cascabel de los Estados Unidos de América y de la República Bolivariana de Venezuela para evaluar sus actividades de gelatinasa, fibrinolítica, coagulantes y hemorrágicas, así como la inhibición de la función plaquetaria. Se utilizaron curvas de valoración electroforética en gel de poliacrilamida para comparar la composición de las proteínas. Se evidenciaron diversas actividades de proteasas en los venenos. La actividad hemorrágica mostró gran variación geográfica, pero las muestras del Norte mostraron la tendencia a una mayor intensidad en este parámetro. Las diferencias en conformación de proteínas de los venenos fueron palpables entre serpientes del norte y América del sur. Hubo una composición distinta entre todos los venenos; varios presentaron actividad hemorrágica y fibrinolítica y diferente comportamiento en los ensayos con plaquetas. Estos resultados demostraron las diferencias interesantes entre los venenos de *Crotalus* de Norte y Suramérica.

PALABRAS CLAVE: Coagulación, fibrinólisis, hemorragia, agregación plaquetaria, veneno de serpiente.

INTRODUCTION

Snake venoms are secretions of enzymatic and non-enzymatic proteins extremely complicated in their composition. Venoms from snakes of the Viperidae family induce a complex representation of local and systemic physiopathological alterations including pain, paralysis, bleeding, swelling, myonecrosis, coagulation, cardiovascular shock, and renal failure. In addition, they contain toxins such as disintegrins, which inhibit platelet aggregation and function and interact with extracellular matrices, which have biomedical applications (Rengifo and Rodríguez-Acosta 2004).

The United States of America (USA) has approximately 16 species of rattlesnakes (Campbell and Lamar 2004). It has been considered that 7,000-8,000 people per year are envenomated in this country and about 5 of those people die. Venomous snakebites reported to U.S. poison centers in all states except Hawaii are as follow: 98% were by Viperidae snakes, mainly *Crotalus* (CDC 2015), while the remaining 2% were by elapids. In general, 77% of victims were male, 70% were adults > 20 years, and 12% were less than 10 years. The frequent hospital

Recibido: junio 2015. Aprobado: septiembre 2015
Versión final: febrero 2016

admission rate was 53%, and the consequences were usually more severe with rattlesnake envenomations. Indigenous US venomous snakebites result in considerable morbidity and mortality (Seifert *et al*. 2009). Deadly snakebites are attributed to the eastern and western diamondback rattlesnakes, and the great majority of snakebites happen in warm weather states like Texas, Florida, California and New Mexico. Bites from rattlesnakes are exceptionally rare in the states near the Canadian borders. The state of Maine, for instance, has only one species (*Crotalus horridus*), and it is seldom noticed only in the southern areas of the state (Campbell and Lamar 2004).

Eighty percent of snakebite accidents in South America are due to *Bothrops.* Alternatively, *Crotalus* produce roughly 12% of accidents (Rodríguez-Acosta *et al*. 2000). Venezuela has approximately 5 species of rattlesnakes (Lancini 1979, Navarrete *et al*. 2009). The data of accidents caused by snakebites in Venezuela, registered at the morbidity statistics of the Direction of Epidemiology and Strategic Analysis of the Ministry of Health, during the years of 1996-2004, considered that an average of 5,976 cases per year were venomous snakebites in Venezuela, with a higher incidence during the year 2004 (7,486 incidents) and nearly 109 of those people die (De Sousa *et al*. 2005, Benítez *et al*. 2007, MPPS 2009, 2011, De Sousa *et al*. 2013). The current work compares biochemical and hemostatic venom activities from seven United States and two Venezuelan rattlesnakes venoms.

MATERIALS AND METHODS

Venoms and snakes

Seven venoms were obtained from an existing collection in the Serpentarium of Texas A&M University-Kingsville, Kingsville, Texas, USA: *Crotalus atrox*, *Crotalus horridus horridus*, *Crotalus molossus molossus*, *Crotalus mitchelli stephensi*, *Crotalus scutulatus scutulatus*, *Crotalus scutulatus scutulatus* Type B and *Crotalus viridis viridis*.

Crotalus durissus cumanensis and *Crotalus vegrandis* venoms from adult snakes were obtained from Lagunetica and Carrizales towns (Miranda state) and Uracoan savannahs (Monagas state) Venezuelan geographical locations, and maintained at the Serpentarium of the Tropical Medicine Institute of the Universidad Central de Venezuela, Caracas, Venezuela.

Venoms from USA were pools from the same specimens. Venezuelan venoms were collected and pooled from at least 10 individual species and all of them were centrifuged using a centrifuge Beckman Avanti 30 at 10,000 *g* for 5 minutes, and immediately filtered using a filtrate unit Millipore MillexHV (0.45µm). Then lyophilized and kept at -90ºC until use.

Experimental animals

Rabbits

To test subcutaneous hemorrhagic activity, New Zealand rabbits (*Oryctolagus cuniculus*) were used. Rabbits with 2 kg weight were obtained from the National Natural Toxins Research Center of the Texas A & M University-Kingsville, Kingsville, Texas and from the Tropical Medicine Institute Animal Facility, Universidad Central de Venezuela, Caracas, Venezuela.

Ethical statement

Expert personnel arranged all the experimental events relating to the use of live animals. Pertinent regulations as well as institutional guidelines agreeing to protocols approved by the National Natural Toxins Research Center, Texas A & M University-Kingsville, Texas, USA and the Institute of Anatomy of the Universidad Central de Venezuela following the norms obtained from the guidelines for the care and use of laboratory animals, published by the US National Institute of Health (NIH 1985).

Electrophoretic titration curves (ETC)

To determine the protein concentration and the isoelectric points (pIs) from all the studied venoms, electrophoretic titration curves were carried out. Samples were lyophilized and reconstituted in deionized water at 3 mg/mL. A pH gradient of 3-9 was established using IEF 3-9 (PhastGels, Amersham Biosciences ®, Piscataway, NJ, USA) gels. The gels were then rotated 90º and 3 µL samples were applied in the center of the gel. The proteins were then separated and silver stained as recommended by Amersham Pharmacia PhastSystem ™ manuals.

Purification of proteins

Molecular exclusion chromatography

A total of 200 µL (35 mg/mL) of each venom was fractionated by molecular exclusion chromatography using a Waters ™ ProteinPak 60 exclusion (1-20 kDa) column. The elution was performed with 0.02 M sodium phosphate pH 6.5, and an isocratic flow rate of 0.5 mL/min on a Waters™ 510 HPLC System. Proteins were detected using a Waters ™ 484 Tunable Detector

Dialysis and protein concentration

Venom fractions obtained by chromatography were desalted using a Pharmacia G25 HiTrap column (5,000 molecular weight cutting), and concentrated by freeze-drying (6 Freezone Labconco, Kansas, MO, USA) at -40˚C.

Blood sample collection

The blood samples were obtained using a gravity flowing system, which cause minimal trauma to platelets. Eighteen milliliters of human blood were collected on a tube containing 2 mL 1% sodium citrate. After blood collection, the tube was softly inverted twice to ensure total blood citration. Blood was aliquoted into 2 mL samples prior to its use.

Analyses of activated clot time, clot rate and platelet function

Glass bead activated cuvettes (Kit gbAC, Sienco ®, USA) were used for the detection of the activated clot time, clot rate and platelet function using on a Sonoclot ® and a Platelet Function Analyzer (Sienco ®, Inc. Wheat Ridge, CO, U.S.A). Citrated human blood was incubated at 37ºC for at least 5 min prior to use. A total of 13 µL of 0.25 M $CaCl_2$ was added to the wall of the cuvette, and 20 µL containing 13 µg of venom or venom fraction were added to the other side of the cuvette. A total of 300 µL of citrated human blood was then added, and the analyzer was initiated. The data was analyzed using a "Signature Viewer ™" provided by Sienco® on an IMac computer.

Hemorrhagic analysis

To determine the hemorrhagic activity of crude venom and fractions, the modified Omori-Satoh *et al*. (1972) method was utilized. One hundred microliters of crude venom (0.1 mg/mL) or fraction was intracutaneously (i.c.) injected on the back of a New Zealand rabbit. The rabbit was sacrificed after 18 h and skin was removed. Hemorrhagic activity was established by the presence of a hemorrhagic spot on the rabbit's skin. Specific hemorrhagic activity was determined by dividing the size of the hemorrhagic spot (mm) by the amount of injected protein (µg). This activity was compared with minimum hemorrhagic dose (MHD: 2.5 µg) of *Crotalus atrox* crude venom.

Fibrinolytic analysis

To measure the fibrinolytic activity of fractions, the modified Bajwa *et al*. (1980) method was used. Three hundred microliters of fibrinogen (9.4 mg/mL) and 12 µL thrombin solution (38.5 U/mL) solution were added to each well of a 24 wells plate and gently agitated. The plate was kept at room temperature until content solidified; then the plate was incubated at 37ºC for 3h. Twenty microliters from each fraction or venom sample were added to each well and incubated at 37ºC for another 15 h. Then, 700 µL of 10% trichloroacetic acid were placed on each well to stop the reaction and decanted after 10 min. Specific fibrinolytic activity was calculated by dividing the cleared fibrin area (millimeters) by the amount of protein (µg) in each well.

Hide powder azure analysis

A modified method of Rinderknecht *et al*. (1968) was used to test the proteolytic activity. Eight milligrams of powder azure was diluted in 2 mL of 0.02 M Tris-HCl, pH 8.0, and 100 µL of venom (0.1 mg/mL) fraction was added. Each sample was incubated at 37ºC for 1 h and agitated at intervals of 5 min. After incubation, each sample was centrifuged at 420 x *g* for 5 min. The supernatant was transferred to a vial and absorbance was measured at 595 nm. The specific activity was calculated by dividing the absorbance by the amount of used protein (mg).

Gelatinase activity

A method by Huang and Perez (1980) was used to test the gelatinase activity of crude venom and fractions. An X-ray film (Kodak X-OMAT) was rinsed with distilled water and incubated at 37°C for 45 min. After incubation, the film was entirely dried and 20 µL of ½ serial diluted crude venom or fractions (starting at 50 µg protein) were placed on the film containing a gelatin coating.

The X-ray film was incubated for 2 h at 37°C in a humid incubator. Washing the film with distilled water and observing a clear area determined hydrolysis of gelatin. Serial dilutions were performed to determine the minimum amount of venom required to cause a clear spot on the film. The titer was defined as the reciprocal of the highest dilution that caused a clear spot on the film. The specific gelatinase activity was calculated by dividing the titer by the amount of protein (µg) applied on the film. The assay was repeated 3 times.

Hydrolysis of b-chain of insulin

To detect the proteolytic activity for each venom fraction, a P/ACE 5500 (Beckman, USA) capillary electrophoresis was used. Ten µL of the venom fractions (0.06 mg/mL) were incubated at room temperature for 1 h with 10 µL B-chain of insulin (0.5 mg/mL) and 10 µL of 0.1 M sodium borate, pH 8.3. The mixture was then separated during 10 min at 20 kV, using 0.1 M sodium borate, pH 8.3, in a 75 µm I.D x 50 cm (800 x 100 aperture) capillary tube. The proteins were determined at 214 nm.

RESULTS

Electrophoretic titration curves (ETC)

Electrophoretic titration curves (ETC) used in this preliminary study showed that venoms were a mixture of complex molecules. Although the ETCs are used primarily to determine the optimal conditions for the separation of molecules by ion exchange chromatography, it can also be used to indicate purity and complexity. Figure 1 shows the ETCs for 9 venoms used in this preliminary study. The ETCs represent two venoms from Venezuela, *C. d. cumanensis* and *C. vegrandis*, and the remaining from the USA. *Crotalus vegrandis* has the majority of venom proteins with isoelectric points (pIs) less than 6, while *C. d. cumanensis* have several proteins with pIs greater than 6. With the exception of *C. m. molossus and C. m. stephensi*, the USA snake venoms have the majority of their proteins with pIs less than 6.

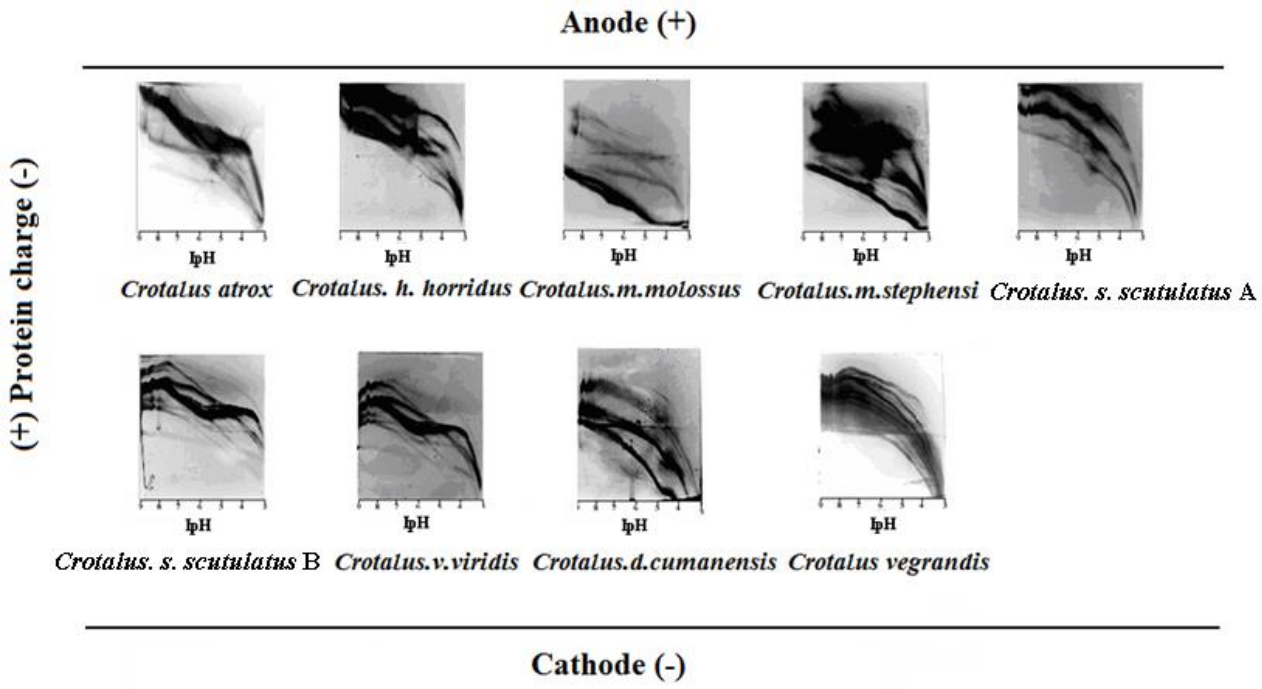


Figure 1. Electrophoretic titration curves of crude venom. The ETCs represent two venoms from Venezuela, *C. d. cumanensis* and *C. vegrandis*, and the remaining from the USA. *Crotalus vegrandis* has the majority of venom proteins with isoelectric points (pIs) less than 6, while *C. d. cumanensis* have several proteins with pIs greater than 6. With the exception of *C. m. molossus and C. m. stephensi*, the USA snake venoms have the majority of their proteins with pIs less than 6.

Analyses of activated clot time, clot rate and platelet function

Venoms were tested with human blood in a Sonoclot analyzer (Control: Fig. 2). Nine different snake venoms were used to determine their impact on activated clot time (coagulation), clot rate and platelet function in human blood. Five venoms (*C. atrox*, *C. v. viridis*, *C. s. scutulatus*, *C. s. scutulatus* B, *C. m. stephensi* and *C. h. horridus*) from the United States of America significantly affected blood coagulation, while *Crotalus m. molossus* venom had minimal effects on the hemostasis pathway. Two Venezuelan venoms (*C. d. cumanensis* and *C. vegrandis*) showed procoagulant activity (Figs. 3A and 3B).

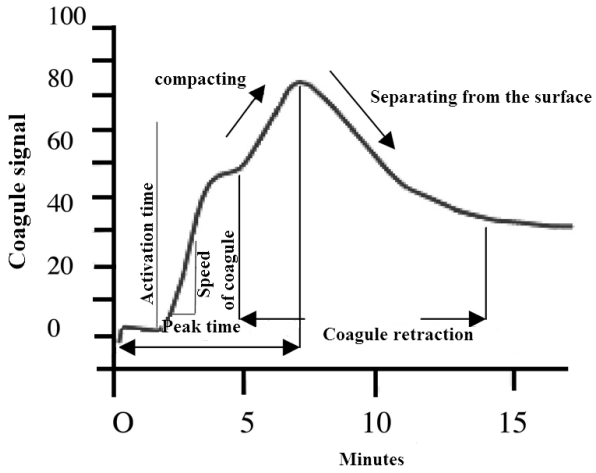


Figure 2. Normal human blood signature evaluated by Sonoclot analyzer. The instrument measures the activated clot time, clot rate, and platelet function (clot retraction) as well as fibrinolysis.

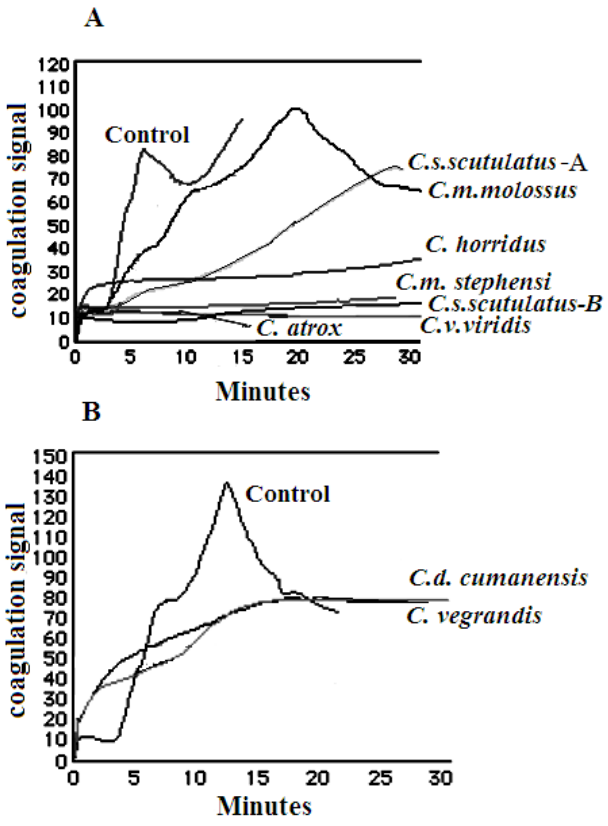


Figure 3. The effect of snake venom on human blood. Nine different snake venoms were used to determine their impact on human blood. Control graphs were normal blood signatures without venom. (A) Five venoms (*C. atrox*, *C. v. viridis*, *C. s. scutulatus* A, *C. d. stephensi* and *C. h. horridus*) from United States affected blood significantly. Two venoms (*C. d. molossus* and *C. s. scutulatus* B) did not have much effect on coagulation. (B) Two Venezuelan venoms (*C. d. cumanensis* and *C. vegrandis*) showed significant procoagulant activity.

Molecular exclusion chromatography and activities of *Crotalus* venoms and fractions

A total of 200 µL (35 mg/mL) of each venom was fractionated using a Waters ™ ProteinPak 60 (1-20 kDa) column. The elution was performed with 0.02 M sodium phosphate pH 6.5, and an isocratic flow rate of 0.5 mL/min on a Waters™ 510 HPLC System. Proteins were detected using a Waters ™ 484 Tunable Detector. The gray shaded areas on the maps indicate the species' geographical locations. Information on the proteolytic activities of fractions and their effects on human blood can be found in the tables. Activation clot time, clot rate and platelet function were analyzed by a Sonoclot Analyzer. The numbers in parentheses and the numbers that follow are averages and ranges, respectively, for normal human blood.

Proteolytic activities

All venoms separated by molecular exclusion liquid chromatography were tested for proteolytic activities (i.e. fibrinolytic, gelatinase, hide powder azure, and hydrolysis of β chain of insulin) from the collected fractions.

***Crotalus atrox* venom**

The results from eight fractions of *C. atrox* venom collected are shown in Figure 4 and Table 1. Eight chromatographic fractions were collected. Fraction 1 was the only one that hydrolyzed β chain of insulin (capillary electrophoresis). Fractions 1, 2, 4 and 7 had hemorrhagic activity. Fraction 1 also had fibrinolytic and gelatinase activities. Fractions 1 and 4 tested with hide powder azure method had proteolytic activity. Fractions 1, 6, 7 and 8 showed procoagulant activity. Fractions 1, 5-8 inhibited platelet function.

***Crotalus horridus* venom**

Crotalus horridus venom produced nine fractions by chromatography (Fig.5 and Table 2). Beta chain of insulin was hydrolyzed by fractions 1 and 2. Fractions 4 had hemorrhagic activity. Fraction 1 had fibrinolytic and gelatinase activities. Fractions 3 tested with hide powder azure method had proteolytic activity. Fractions 1 showed procoagulant activity when tested with the Sonoclot analyzer. Fractions 1, 8 and 9 inhibited platelet function.

***Crotalus molossus molossus* venom**

Nine fractions were collected from *C. d. molossus* venom (Fig.6 and Table 3). Fractions 3 and 5-9 hydrolyzed β chain of insulin. All fractions had hemorrhagic activity. The fibrinolytic activity was observed in 1, 2, and 7-9 fractions, the gelatinase activity was found in fractions 1-3 and 5-8. Fractions 1, 7 and 8 tested with hide powder azure method had proteolytic activity. Fractions 5 and 7 showed procoagulant activity when tested with the Sonoclot analyzer. Fractions 1-3 and 5-9 inhibited platelet function.

***Crotalus mitchelli stephensi* venom**

A total of 10 fractions were collected from *C. mitchelli stephensi* venom (Fig.7 and Table 4). Beta chain of insulin was hydrolyzed by all fractions. All fractions, except fraction 10 had fibrinolytic and hemorrhagic activities. Fractions 1-7 had gelatinase activity. Fractions 1-9 tested with hide powder azure method had proteolytic activity. Fraction 1 was the only one that contained procoagulant activity when tested with the Sonoclot analyzer. Fractions 1, 4, 6, 8 and 9 inhibited platelet function.

***Crotalus scutulatus scutulatus* Type A venom**

Eight fractions were collected from *C. scutulatus scutulatus* venom (Fig.8 and Table 6). Beta chain of insulin was hydrolyzed by fraction 6. Fractions 2-8 had hemorrhagic activity. Fractions 1 and 7 had fibrinolytic activity. None of the fractions had gelatinase activity. Fraction 1 was the only one that had proteolytic activity when tested by the hide powder azure method, and was the only one with procoagulant activity. Fractions 1, 2 and 5, 6 inhibited platelet function.

***Crotalus scutulatus scutulatus*- Type B venom**

Crotalus scutulatus scutulatus Type B venom yielded eight fractions (Fig. 9 and Table 5). Fractions 1 and 2 hydrolyzed β chain of insulin. Fractions 1, 2, 4 and 7 had hemorrhagic activity. Fraction 1 was the only one with fibrinolytic and gelatinase activities. Fractions 1 tested with hide powder azure method had proteolytic activity. Fractions 1 and 6-8 had procoagulant activity when tested with the Sonoclot analyzer. Fractions 1, 5-8 inhibited platelet function.

***Crotalus viridis viridis* venom**

From *C. v. viridis* venom, nine fractions were collected (Fig. 10 and Table 7). None of the fractions hydrolyzed β chain of insulin. Fractions 1 and 7 had hemorrhagic activity. Fraction 1 and 3 had fibrinolytic activity. Fractions 1 and 2 had gelatinase activity. Fractions 1 and 4 tested with hide powder azure method had proteolytic activity. None of the fractions had procoagulant activity when tested with the Sonoclot analyzer. Fraction 1 was the only one that inhibited platelet function.

***Crotalus durissus cumanensis* venom**

From *C. d. cumanensis* venom, seven fractions were collected (Fig. 11 and Table 8). Beta chain of insulin was hydrolyzed by fractions 1 and 2. Fractions 1 and 4 had hemorrhagic activity. Fraction 1 had fibrinolytic, gelatinase activities, and proteolytic activity when tested with hide powder azure method. Fractions 1-4 had procoagulant activity when tested with the Sonoclot analyzer. Fractions 3-5 and 7 inhibited platelet function.

***Crotalus vegrandis* venom**

Nine fractions were collected from *C. vegrandis* venom (Fig. 12 and Table 9). Beta chain of insulin was hydrolyzed by fraction 1. Fractions 1 and 4 had hemorrhagic activity. All fractions showed fibrinolytic activity. Fraction 1 also had gelatinase and proteolytic activity when tested with hide powder azure method. Fraction 1 had procoagulant activity; this fraction had effects on the coagulant rate and platelet function when tested with the Sonoclot analyzer. Fractions 2, 3, 5 and 6 had effects on the coagulant rate and platelet function.

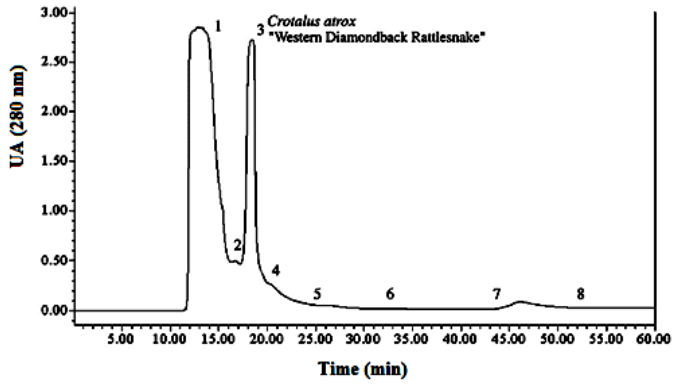
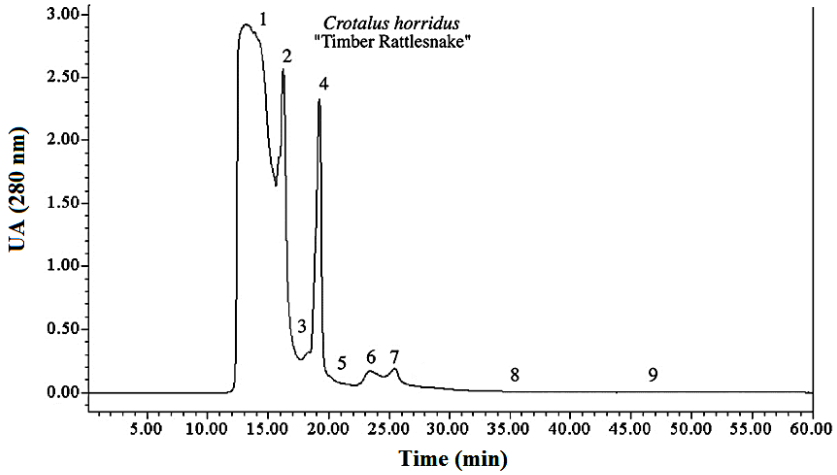


Figure 4. *Crotalus atrox* molecular exclusion liquid chromatography and geographical distribution.

Table 1. *Crotalus atrox* venom.

Fractions	1	2	3	4	5	6	7	8
Capillary electrophoresis	+	-	-	-	-	-	-	-
Hemorrhagic	+	+	-	+	-	-	+	-
Fibrinolytic	+	-	-	-	-	-	-	-
Gelatinase	+	-	-	-	-	-	-	-
Azure Powder	+	-	-	+	-	-	-	-
Clot activation (172) 128-213	<60*	147	146	154	111	81	73	70
Clot rate (20) 15-26	8	21	1.5	11	4.25	8.5	8.5	10
Platelets function (4) 3-5	0	5	4	3	2	2	2	2

* Coagulation rate too fast to measure.



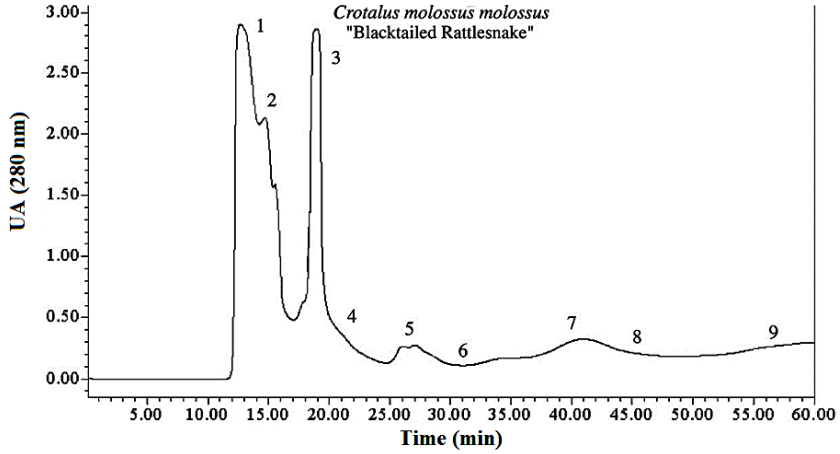
Crotalus horridus

Figure 5. *Crotalus horridus horridus*.

Table 2. *Crotalus horridus horridus* venom.

Fractions	1	2	3	4	5	6	7	8	9
Capillary electrophoresis	+	+	-	-	-	-	-	-	-
Hemorrhagic	-	-	-	+	-	-	-	-	-
Fibrinolytic	+	-	-	-	-	-	-	-	-
Gelatinase	+	-	-	-	-	-	-	-	-
Azure Powder	-	+	-	-	-	-	-	-	-
Clot activation (172) 128-213	1	303	N.D	175	204	163	190	195	149
Clot rate (20) 15-26	*	9.9	N.D	18	13.5	19	11.5	9.2	18
Platelets function (4) 3-5	0	3	N.D	4	4	5	4	2	1

*Coagulation rate too fast to measure.
N.D: None determined.

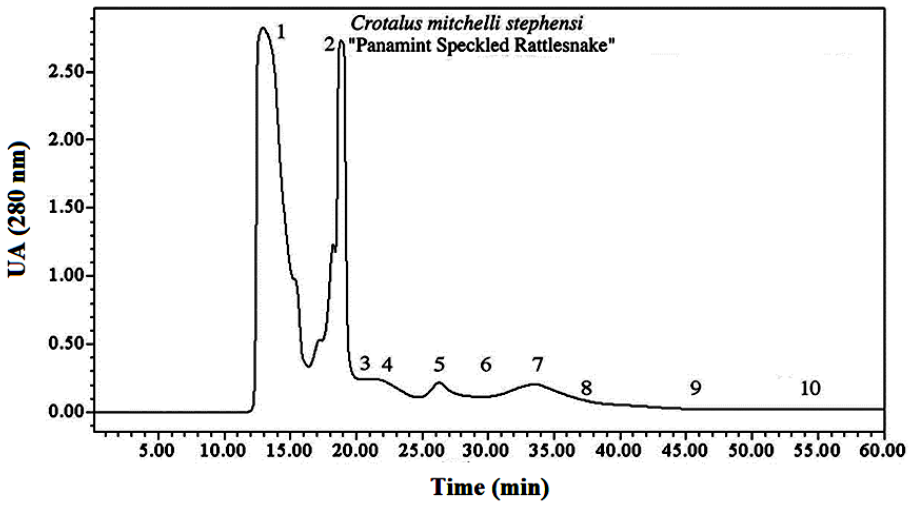


Crotalus molossus molossus

Figure.6. *Crotalus molossus molossus* venom.

Table 3. *Crotalus molossus molossus* venom.

Fractions	1	2	3	4	5	6	7	8	9
Capillary electrophoresis	-	-	+	-	+	+	+	+	-
Hemorrhagic	+	+	+	+	+	+	+	+	+
Fibrinolytic	+	+	-	-	-	-	+	+	+
Gelatinase	+	+	+	-	+	+	+	+	-
Azure Powder	+	-	-	-	-	-	+	+	-
Clot activation (172) 128-213	275	353.5	224	22.5	96	188	57	138.5	116.5
Clot rate (20) 15-26	3.05	0.9	3.85	13.5	3.55	13.5	4.45	8.75	2.6
Platelets function (4) 3-5	1	0	1	4	2	4	3	1	0



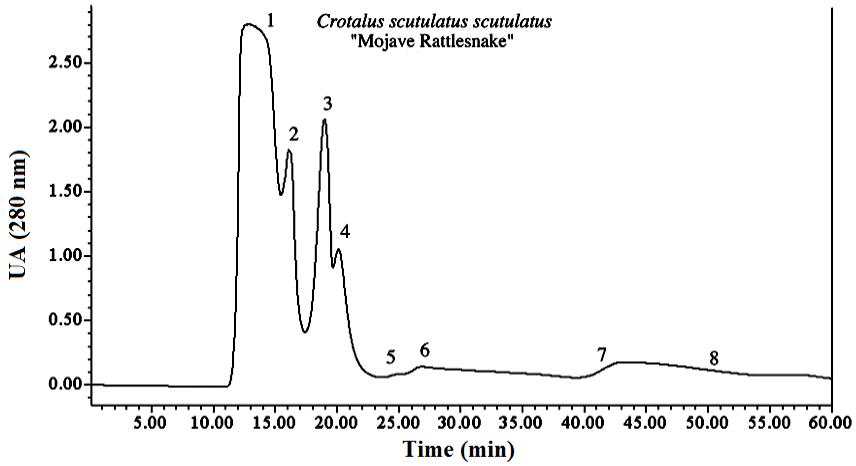
Crotalus mitchelli stephensi

Figure 7. *Crotalus mitchelli stephensi* venom.

Table 4. *Crotalus mitchelli stephensi* venom.

Fractions	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Capillary electrophoresis	-	-	+	-	+	+	+	+	-	
Hemorrhagic	+	+	+	+	+	+	+	+	+	
Fibrinolytic	+	+	-	-	-	-	+	+	+	
Gelatinase	+	+	+	-	+	+	+	+	-	
Azure Powder	+	-	-	-	-	-	+	+	-	
Clot activation (172) 128-213	>60	179.5	167.5	157	137	136	136.5	132	184	183
Clot rate (20) 15-26	*	11.1	9.55	7.9	4.55	9.6	13	12.2	7.4	13
Platelets function (4) 3-5	2	3	3	1	4	2	4	1	2	3

*Coagulation rate too fast to measure.



Crotalus scutulatus scutulatus

Figure 8. *Crotalus scutulatus scutulatus*, Type A venom.

Table 5. *Crotalus scutulatus scutulatus* Type A venom.

Fractions	1	2	3	4	5	6	7	8
Capillary electrophoresis	-	-	-	-	-	+	-	-
Hemorrhagic	-	+	+	+	+	+	+	+
Fibrinolytic	+	-	-	-	-	-	+	-
Gelatinase	-	-	-	-	-	-	-	-
Azure Powder	+	-	-	-	-	-	-	-
Clot activation (172) 128-213	<60	167	204	259	288	250	225	178
Clot rate (20) 15-26	*	3.8	11	7.85	7.2	6.1	8.6	14
Platelets function (4) 3-5	0	1	4	3	2	0	3	3

*Coagulation rate too fast to measure.

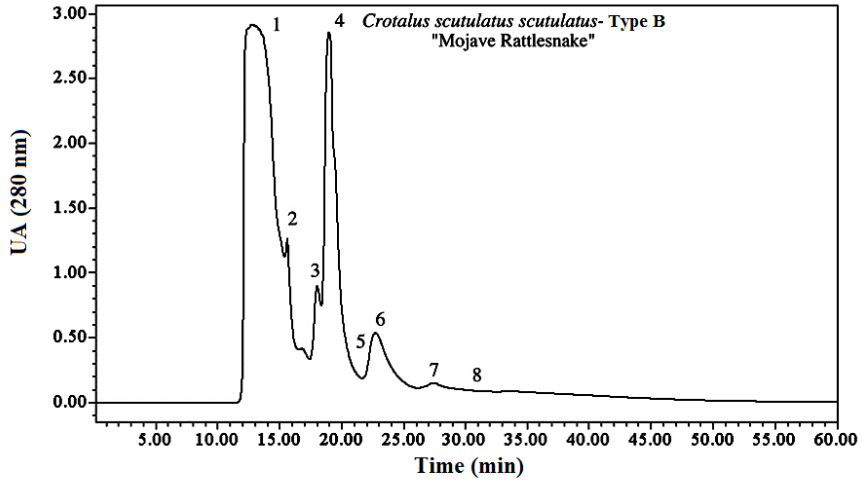
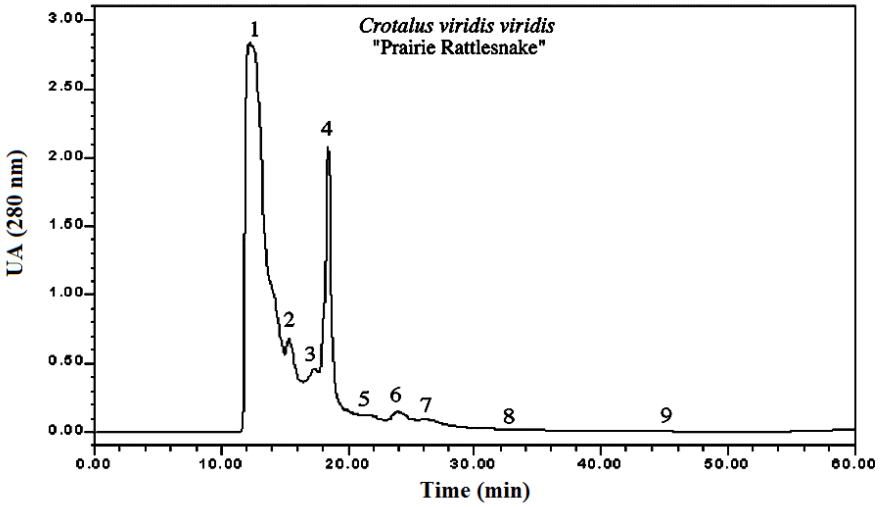


Figure 9. *Crotalus scutulatus scutulatus* Type B venom.

Table 6. *Crotalus scutulatus scutulatus* Type B venom.

Fractions	1	2	3	4	5	6	7	8
Capillary electrophoresis	+	+	-	-	-	-	-	-
Hemorrhagic	+	+	-	+	-	-	+	-
Fibrinolytic	+	-	-	-	-	-	-	-
Gelatinase	+	-	-	-	-	-	-	-
Azure Powder	+	-	-	-	-	-	-	-
Clot activation (172) 128-213	>60	147	146	154	111	81	73	70
Clot rate (20) 15-26	*	21	1.5	11	4.25	8.5	8.5	10
Platelets function (4) 3-5	0	5	4	3	2	2	2	2

*Coagulation rate too fast to measure.



Crotalus viridis viridis

Figure 10. *Crotalus viridis viridis* venom.

Table. 7. *Crotalus viridis viridis* venom.

Fractions	1	2	3	4	5	6	7	8	9
Capillary electrophoresis	-	-	-	-	-	-	-	-	-
Hemorrhagic	+	+	+	+	+	+	+	-	-
Fibrinolytic	+	+	+	-	-	-	-	-	-
Gelatinase	+	+	-	-	-	-	-	-	-
Azure Powder	+	+	+	+	-	-	-	-	-
Clot activation (172) 128-213	103	184	155	170	183	192	175	183	192
Clot rate (20) 15-26	1	10	15	18	19	17	20	19	20
Platelets function (4) 3-5	0	3	4	5	5	5	5	5	5

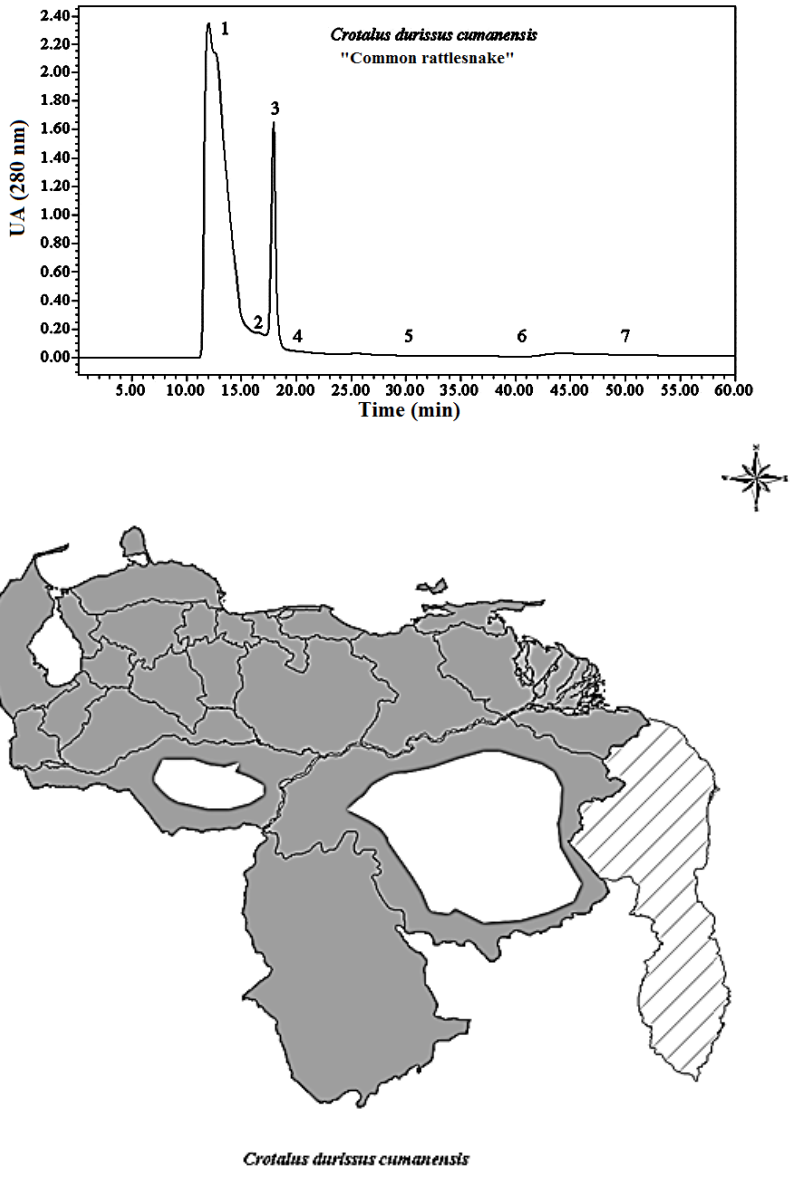
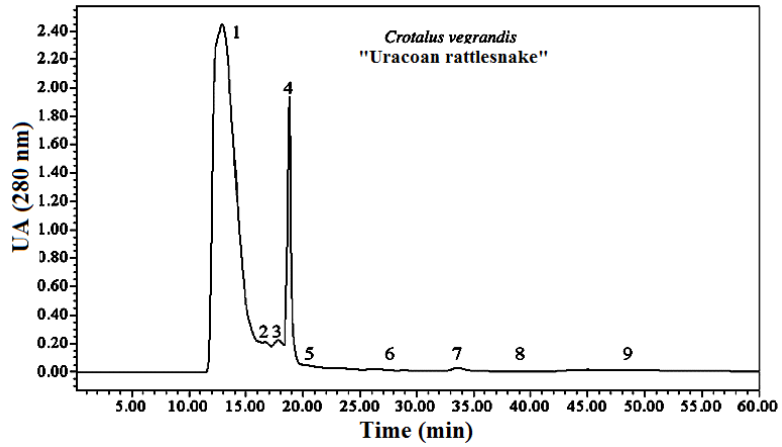


Figure 11. *Crotalus durissus cumanensis* venom.

Table 8. *Crotalus durissus cumanensis* venom.

Fractions	1	2	3	4	5	6	7
Capillary electrophoresis	+	+	-	-	-	-	-
Hemorrhagic	+	+	+	+	-	-	-
Fibrinolytic	+	-	-	-	-	-	-
Gelatinase	+	-	-	-	-	-	-
Azure Powder	+	-	-	-	-	-	-
Clot activation (172) 128-213	<60	<60	<60	<60	126	121	136
Clot rate (20) 15-26	*	16	16	16	5.6	24	5.2
Platelets function (4) 3-5	0	3	2	1	2	3	1

*Coagulation rate too fast to measure.



Crotalus vegrandis

Figure 12. *Crotalus vegrandis* venom

Table 9. *Crotalus vegrandis* venom.

Fractions	1	2	3	4	5	6	7	8	9
Capillary electrophoresis	+	-	-	-	-	-	-	-	-
Hemorrhagic	+	+	+	+	-	-	-	-	-
Fibrinolytic	+	+	+	+	+	+	+	+	+
Gelatinase	+	-	-	-	-	-	-	-	-
Azure Powder	+	-	-	-	-	-	-	-	-
Clot activation (172) 128-213	<60	158	84	125	163	166	179	178	188
Clot rate (20) 15-26	*	5	6	12	7	9	14	13	19
Platelets function (4) 3-5	0	1	1	1	1	1	2	2	4

*Coagulation rate too fast to measure.

DISCUSSION

In this work variation in electrophoretic titration curves, chromatographic profiles and proteolytic activities induced by nine venoms of different *Crotalus* species from different regions of the United States of America and Venezuela were studied. Venoms collected from different snakes were identified by herpetologists to circumvent species misidentification. Results showed that all venoms from the USA and Venezuela generally differed, as expected, in chromatographic elution profiles by molecular exclusion chromatography, however, they share some similarities. Hemorrhagic and pro-coagulant activities, proteolytic activity on fibrinogen, B-chain of insulin, hide powder azure, and gelatin were detected in most samples, although potencies varied.

Disparity in snake venom constitution is an omnipresent event at all taxonomic stages. Numerous aspects including geographic distribution, phylogeny, season, age and prey predilection may have the tendency to change venom composition. Aguilar *et al*. (2007) found that there existed significant differences in *C. d. cumanensis* venom when tested for their hemostatic functions from snakes that were collected in different geographical locations. On the other hand, Girón *et al*. (2008) found differences in hemostatic functions in the venom of two narrowly related *Bothrops colombiensis* found in very close proximities. To date, intraspecies variation of components in snake venoms is very well recognized (Aguilar *et al*. 2007, Jurado *et al*. 2007, Alape-Girón *et al*. 2008, Salazar *et al*. 2008). In contrast, some research concludes that venom of different snake species contain similar components (Tsai *et al*. 1994, Wang *et al*. 1996).

Rattlesnake venoms are complex mixtures of different toxins with enzymatic and non-enzymatic activities on the factors involved in the hemostatic pathway. Some of the lethal effects of rattlesnake venoms seem to be due in high degree to low molecular weight, non-enzymatic peptides (Schaeffer *et al*. 1978, Da Silva *et al*. 2009). However, venom enzymes are highly responsible for the deleterious effects of envenomation. Many of these effects are linked to the actions of metalloproteinases (Suntravat *et al*. 2013), serine proteinases, neurotoxins such as crotamine and crotoxin (Hernández *et al*. 2007), phosphodiesterases, hyaluronidases, L-amino acid oxidase as well as other proteolytic enzymes with hemostatic properties. In general, these venom constituents have been associated to local tissue damage and necrosis that is mainly observed after envenomation from North American *Crotalus*; however, these effects are observed only in a few South American venomous snakes. Rattlesnake metalloproteinases and procoagulant enzymes are responsible for fibrinogen disintegration after envenomation (Rodríguez-Acosta *et al*. 1998a,b, Aguilar *et al*. 2007, Suntravat *et al*. 2013, Dagda *et al*. 2014); these enzymes transform fibrinogen to a soluble fibrin gel (Weiss *et al*. 1969). In early times, several authors described various elements with esterase-like or amidolase-like activity in some Crotalidae and Viperidae venoms. These fibrinogen-stimulating enzymes, broadly circulated in *Crotalus* venoms, are mainly represented by single-chain glycosylated serine proteases that exhibit extraordinary macromolecular selectivity and are habitually denoted as thrombin-like enzymes, which have aided as structural models to expand our perception of the structure–function associations

of blood clotting elements (Reid *et al*. 1963, Morse *et al*. 1967, Damus *et al*. 1972, Mitrakul 1973, Aguilar *et al*. 2001). Furthermore, fibrinogenolytic activities in both North and South American *Crotalus* venoms have been found when experimental concentrations were assayed (Aguilar *et al*. 2007, Da Silva *et al*. 2009, Salazar *et al*. 2009). It is remarkable that the hemorrhagic activity in venom of the genus *Crotalus* (widely described in the American continent) increases from south to north, and is highly present in North American *Crotalus*, and so far, almost non-present in South American *Crotalus* species and subspecies (Rodríguez-Acosta *et al*. 1998a).

Viperidae venom components such as serine and metalloproteinases have been described as enzymes interfering in the regulation and maintenance and control of the multifaceted hemostatic system; and therefore, some of these enzymes, such as protein C activator (PROTAC) (Pentapharm Ltd) and DEFIBRASE (American Diagnostica Inc.), have been used in the treatment of hemostatic syndromes (Marsh and Williams 2005). The spectrum of activities displayed by these molecules comprises hemorrhagic, procoagulant, anticoagulant, platelet activation, and bradykinin-releasing effects (Braud *et al*. 2000). Established by their functions, the procoagulant snake venom proteinases were classified as C and D prothrombin activators, factor I (thrombin-like enzymes), factor V, factor VII and factor X (Kini 2005).

Making a preliminary identification of different toxins that all of these venoms possess, allows the assembly of a map of interesting molecules, from the biomedical point of view, which could stimulate their characterization by different venom researchers.

Some venoms from the USA (*C. mitchelli stephensi*, *C. scutulatus scutulatus*-A) and all those from Venezuela (*C. vegrandis* and *C. d. cumanensis*) contained neurotoxins (not tested in the present work) (Glenn and Straight 1985, Kaiser and Aird 1987, Rael *et al*. 1992, 1993, Bush and Cardwell 1999, Aguilar *et al*. 2007, Hernández *et al*. 2007). Glenn and Straight (1985) analyzed *C. s. scutulatus* venoms from north of Tucson to the extreme southeastern region of Arizona, and found that specimens from north of Tucson produced venom with lethal toxicity (LD_{50}) in mice to be 2.0 to 6.0 mg/kg. These corresponded to the LD_{50}s reported for *C. s. scutulatus* in the area of Phoenix, Arizona labeled as type B venom. Contrarily, the venom LD_{50} of specimens from extreme southeastern Arizona, including animals near Tucson, fluctuated from 0.22 to 0.46 mg/kg, corresponding to LD_{50}s for *C. s. scutulatus* type A venom (Glenn *et al*. 1983). Samples with type A venom have been captured in California, Nevada, Utah and some parts of Arizona. As it is observed in the current work, the type A venom compared with Type B venom showed differences in their chromatographic profiles. Weak proteolytic activity and no hemorrhagic activity is indication of the presence of Mojave toxin as described by Glenn and Straight (1985), and Rael *et al*. (1993). These results come to ratify the geographical differences of *C. s. scutulatus* venom in the state of Arizona. Northern Mojave rattlesnakes live from southern Nevada, the Mojave Desert of California, southwestern Utah, Arizona, western Texas (Trans-Pecos area) and the southern region of New Mexico, irradiating to Mexico as distant south as Queretaro state.

Crotalus d. cumanensis venom is commonly recognized as neurotoxic, displaying crotamine, gyroxin, convulxin and crotoxin activities, among others (Aguilar *et al*. 2007, Cavalcante *et al*. 2015). *Crotalus vegrandis* venom is also neurotoxic, containing crotoxin isomers (Kaiser and Aird 1987, Aird *et al*. 1989, Pifano and Rodríguez-Acosta 1995, 1996). Most of these rattlesnakes present high phospholipase A2 activity. However, Wang *et al*. (2010) showed the absence of phospholipase A2s in most *Crotalus horridus* venom due to a translation blockage.

Mice when injected with crude venom evidenced neurological symptoms typical of depolarizing neuromuscular blocking neurotoxins (Hawgood and Smith 1977), consisting of ataxia, flaccid or spastic paralysis of respiratory muscles, edginess, convulsions and death. It seemed that interspecies variation of neurotoxins venoms was larger than intraspecies variations.

In the current work, all venoms tested from both countries had hemostatic activities. The higher variability of venoms definitely diverge in their constituents and therefore in their toxicity (Salazar *et al*. 2007). Hemorrhagic and fibrinolytic activities play an important role in *Crotalus* snake envenomation, stimulating coagulopathies and local and systemic bleeding (Saravia *et al*. 2002, Aguilar *et al*. 2007). *Crotalus horridus*, *C. m. stephensi*, *C. s. scutulatus* B, *C. v. viridis* and *C. atrox* were those who had the

lowest coagulation activation time, while *C. s scutulatus* A, *C. m. molossus*, *C. d. cumanensis* and *C. vegrandis* presented coagulation signals over 70 at 20 min.

The inhibition of platelet function by venom and venom fractions are probably mediated by disintegrins, which characterize a family of low molecular weight, cysteine-rich polypeptides that bind specifically to integrins $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ present on platelets and leading to inhibition of platelet aggregation (Da Silva *et al*. 2009).

Several authors (Daltry *et al*. 1996, Aguilar *et al*. 2007) established an important association between geographical variation and venom composition and theorized that geographical disparity in venom constitution may be a sign of natural selection for digesting and/or killing diverse prey (Pifano and Rodríguez-Acosta 1995) in different territories. Other support reveals that the constitution of snake venom is effectively altered by bio-ecological reasons including climate, soils, habitat, and prey (Chijiwa *et al*. 2000, Jorge Da Silva and Aird 2001). The geographical regions, habitats and climates of a snake's locations in the USA (i.e. states like Arizona, New Mexico and Texas) and Venezuela are profoundly different. In the USA, there exist regions that are hot and have extensive rainfall, with winters generally short, and the spring and fall seasons are relatively long with mild temperatures.

In Venezuela, the summers are very hot with temperatures nearly at 38°C for many days, and with tropical or torrid zones where the entire Country receives the rays of the sun directly than those zones in upper latitudes; the usual annual temperature is higher and there are no seasons, but only dry and wet periods with minor changes of temperature.

One of the factors that can impact venom toxicity and produce variable results is environmental condition. Both countries have different habitats, climates and preys; so that it is utterly certain that they vary from each other, which could explain the variation of toxins in these different venoms. Nonetheless, a comparative analysis of enzymatic and other toxic activities of *Crotalus* venoms found in diverse geographical areas of the American continent is still absent. The statistics concerning victims' age, sex, activity, time of day, and anatomic site of bites do not fluctuate significantly from those reported for rattlesnake bites in both countries.

Diverse form and structure of American snake venoms as well as difference in sequences of venom proteins are a result of the expression varying genomes. Variance in the phylogenetic trees established on dissimilar morphological structures and protein sequences may signify that diverse parts of the genome in a single snake have their particular history of evolution since the genome is an assortment of genes of unlike evolutional accounts. It was suggest that the main dynamic force for evolution is interspecies gene exchanges, instead of divergence of species from a single ancestor by the addition of mutations, and the genus *Crotalus* probably has been initiated from numerous ancestors instead of a single one. However, monophyly of rattlesnakes has never been disproved, as confirmed by the presence of a rattle (Murphy *et al*. 2002). *Crotalus* represent a clade spreading in all the American Continent and probably is descending of multiple ancestors from Colubroidea superfamily (Lawson *et al*. 2005).

The preliminary study that has been done with these venoms provides the foundation for further analysis of many of these interesting proteins that will most certainly provide valuable data that may lead in the design of drugs that could be used for the treatment of thrombo-embolic diseases as well as other disorders.

ACKNOWLEDGEMENTS

Funding for the research was provided by grants from the CDCH de la UCV PG: 09-8760-2013, Caracas, Venezuela, and NCRR/BMRG, Viper Resource Grant #S 8P40OD01960-10 3P40OD01096-10S1 (NNTRC, Texas A & M University-Kingsville), and the Robert A. Welch Foundation Department Grant # AC-0006 (TAMUK-Department of Chemistry).

The authors declare that there is no conflict of interests regarding the publication of this paper.

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