



**UNIVERSIDADE FEDERAL DA PARAÍBA
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PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA
CELULAR E MOLECULAR**



JONATHAS DIEGO LIMA SANTOS

**ESTUDOS ESTRUTURAIS DE FOSFOLIPASES A₂ ISOLADAS DA
PEÇONHA DE *Bothrops jararacussu* E DESCRIÇÃO DE INIBIDORES
HÁBEIS EM TRATAMENTO DE OFIDISMO.**

**JOÃO PESSOA – PARAÍBA
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Orientadora: Daniela Priscila Marchi Salvador

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Dissertação de mestrado avaliada em: _____/_____/_____

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Toda boa dádiva e todo dom perfeito são lá do alto, descendo do Pai das luzes, em quem não pode existir variação ou sombra de mudança (Tiago 1.17).

RESUMO

O Brasil é o país com maior índice de acidentes ofídicos registrados. As serpentes peçonhentas que ocorrem no país pertencem às famílias Viperidae e Elapidae. Acidentes ofídicos causam a liberação de substâncias farmacologicamente ativas com efeitos locais como hemorragia, edema, necrose tecidual, miotoxicidade, cardiotoxicidade, neurotoxicidade e citotoxicidade, além de efeitos sistêmicos como coagulação sanguínea, sangramentos espontâneos, insuficiência renal e respiratória. Peçonha de serpentes são fontes ricas de proteínas, peptídeos, compostos orgânicos e inorgânicos. As fosfolipases A₂ (PLA₂s) são as proteínas mais abundantes nas peçonhas dos diferentes gêneros de serpentes e apresentam efeitos tóxicos e farmacológicos de grande interesse médico-científico. O presente estudo teve por objetivos caracterizar físico-quimicamente epitopos B-lineares de três PLA₂s isoladas da peçonha de *Bothrops jararacussu* reconhecidos pelos soros antiofídicos; analisar comparativamente, *in silico*, os epitopos B-lineares reconhecidos pelos soros que estão presentes e/ou ausentes em demais PLA₂s isoladas de peçonhas de serpentes; relatar moléculas isoladas de extratos vegetais que possibilitem inibir os efeitos tóxicos e farmacológicos das PLA₂s. Experimentos de síntese paralela de peptídeos realizadas em membranas de celulose permitiram identificar, por quimiluminescência, doze regiões das PLA₂s (BthTX-I, BthTX-II e BthA-I) presentes na peçonha de *Bothrops jararacussu* reconhecidas por soros antiofídicos (antibotrópico e/ou anticrotálico). Análises comparativas entre PLA₂s possibilitaram identificar que algumas regiões específicas que foram reconhecidas pelos soros antiofídicos são responsáveis pelas atividades neurotóxica, miotóxica e anticoagulante desencadeadas pelo ofidismo. Portanto, o presente trabalho possibilita elucidar regiões de interação molecular soro-proteína bem como auxiliar na proposição de novos inibidores.

Palavras-chave: Fosfolipase A₂, Peçonha de serpentes, *Bothrops jararacussu*, inibidores de fosfolipase A₂.

ABSTRACT

Brazil is the country with the highest rate of reported snakebites. The venomous snakes that occur in the country belong to the Viperidae and Elapidae families. Snakebites cause the release of pharmacologically active substances with local effects such as hemorrhage, oedema, necrosis, myotoxicity, cardiotoxicity, neurotoxicity and cytotoxicity, as well as systemic effects such as blood coagulation, spontaneous bleeding, renal and respiratory failure. Snake venoms are rich sources of proteins, peptides, organic and inorganic compounds. The phospholipase A₂ (PLA₂s) are the most abundant proteins in the snake venom of different genera and exhibit toxic and pharmacology effects of strong medical and scientific interest. The aim of this study was to characterize physico-chemically B-linear epitopes of three phospholipases A₂ (BhtTX-I, BhtTX-II and BthA-I) isolated from *Bothrops jararacussu* snake venom recognized by the therapeutic horse anti-ophidic sera; *in silico* comparative analysis of the B-linear epitopes recognized by the sera that are present and / or absent in other PLA₂s isolated from snake venoms; and describe molecules isolated from plant extracts that are able to inhibit the toxic and pharmacological effects of PLA₂s. Parallel spot synthesis peptides experiments on cellulose membranes allowed identifying, by chemiluminescence, twelve PLA₂s regions recognized by anti-ophidic sera (anti-bothropic and/or anti-crotalic). Comparative analyzes between PLA₂s enabled to identify that some regions recognized by anti-ophidic sera are responsible for neurotoxic, anticoagulant and myotoxic activities performed by snakes. Therefore, this work enables interaction regions elucidate molecular serum-protein as well as assist in proposing new inhibitors.

Keywords: Phospholipases A₂, Snakes venom, *Bothrops jararacussu*, phospholipases A₂ Inhibitors.

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PLA₂	Fosfolipases A ₂ (E. C. 3. 1. 1. 4.)
BthTX-I	Bothropstoxina I, Lys49-PLA ₂ básica purificada da peçonha de <i>Bothrops jararacussu</i>
BthX-II	Bothropstoxina II, Asp49-PLA ₂ básica purificada da peçonha de <i>Bothrops jararacussu</i>
BthA-I	Bothropstoxina, Asp49-PLA ₂ ácida purificada da peçonha de <i>Bothrops jararacussu</i>
CEATOX	<i>Centro de Assistência Toxicológica de Campina Grande - PB</i>

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1. INTRODUÇÃO

1.1. Acidentes ofídicos

As serpentes são bem adaptadas aos mais diversos ambientes exercendo papel importante na relação entre os seres vivos e no equilíbrio da teia alimentar de diversos animais (CARDOSO et al., 2003). A maior biodiversidade de serpentes é encontrada nas regiões tropicais. O Brasil possui aproximadamente 382 espécies e, destas, 55 são consideradas peçonhentas (THE REPTILE DATABASE, 2012).

Serpentes peçonhentas são encontradas em todo território brasileiro, pertencentes as famílias Viperidae e Elapidae. A família Viperidae tem por representantes os gêneros *Bothrops*, *Lachesis*, *Bothriopsis*, *Bothropoides*, *Rhinocerothis*, *Bothrocophias* e *Caudisona*, responsáveis pela maioria dos acidentes ofídicos em humanos e, por isso, de grande preocupação na saúde pública brasileira (MELGAREJO, 2003). Os representantes destas famílias possuem dentição do tipo solenóglifa e fosseta loreal, características típica de serpentes peçonhentas (BERNARDE, 2011). A família Elapidae é representada por 27 espécies (BERNARDE, 2011) classificadas em dois gêneros, *Micrurus* e *Leptomicrurus*. O gênero *Micrurus* esta presente em todo território nacional enquanto que o *Leptomicrurus* é encontrado apenas na região oeste amazônica (BERNARDE, 2011). Serpentes da família Elapidae apresentam dentição proteróglifa e ausência de fosseta loreal e estão envolvidas em apenas 1% dos acidentes ofídicos registrados no Brasil.

A World Health Organization (2012), estima que mundialmente ocorram aproximadamente 421 mil envenenamentos e 20.000 mortes, mas adverte que esses números podem ser, ainda maiores, devido aos muitos casos de acidentes ofídicos não notificados, e ainda, a não procura dos acometidos pelo serviço público ou privado de saúde para tratamento, dados estes, que não entram na estatística. O Brasil é o país da América do Sul com maior índice de envenenamento registrado com, aproximadamente, 25 mil casos anuais (MINISTÉRIO DA SAÚDE, 2011). Os acidentes ofídicos ocorrem com maior frequência nos períodos de intensa atividade humana no campo e nas estações quentes e úmidas, as quais coincidem, na maioria dos estados, com o período mais chuvoso, de janeiro a abril (LEMOS et al., 2009).

As serpentes do gênero *Bothrops* (hoje subdividida em cinco gêneros, *Bothrops*, *Bothriopsis*, *Bothropoides*, *Rhinocerothis* e *Bothrocophias*), são responsáveis por 90% dos

casos de acidentes ofídicos notificados no Brasil com letalidade de 0,31%, seguido pelo gênero *Caudisona* (cascavel) com 7,7% e letalidade de 1,87%, *Lachesis* (surucucu) com 1,4% e letalidade de 0,95% e *Micrurus* (corais verdadeiras) com 0,5% e letalidade de 0,36% (BERNARDE, 2011). No Estado da Paraíba, segundo dados apresentados pelo Centro de Assistência Toxicológica de Campina Grande, PB – CEATOX, a ocorrência destes acidentes é de 75,7% para as serpentes do gênero *Bothrops*, *Bothriopsis*, *Bothropoides*, *Rhinocerophis* e *Bothrocophias*, 14% para o gênero *Caudisona*, 0,7% para o gênero *Micrurus* e 2,1% para o gênero *Lachesis*. Ainda, de acordo com o CEATOX, os acidentes acometem principalmente trabalhadores rurais do sexo masculino, com faixa etária entre 12 e 20 anos e maiores de 50 anos (BOCHNER; STRUCHINER, 2003).

Devido à significância de envenenamento por serpentes, justifica-se a necessidade da realização de estudos que visem à produção de soros antiofídicos mais eficazes no tratamento dos efeitos locais e sistêmicos causados pelo ofidismo que proporcione um melhor êxito no terapêutico, bem como o desenvolver tratamentos complementares a soroterapia. A elucidação dos mecanismos moleculares da interação entre proteínas presentes nas peçonhas de serpentes com inibidores de efeitos tóxicos e farmacolé ainda pouco explorada, e, portanto, um caminho a seguir para auxiliar no desenvolvimento de métodos mais hábeis para o combate aos problemas de saúde causados pelos efeitos tóxicos da peçonha (ROTHSCHILD, 1979).

1.2. Peçonha de serpente

A peçonha de serpentes é rica em substâncias farmacológica e bioquimicamente ativas. A composição química varia de espécie para espécie e sua ação depende da natureza dos elementos presentes na peçonha e da interação biológica entre estes elementos (VARANDA; GIANNINI, 1994). Peçonha é uma substância tóxica que pode ser inoculada através de dispositivos que o próprio animal possui como dentes ou ferrões. A peçonha de serpentes é constituída por uma complexa mistura de proteínas, enzimas, peptídeos e compostos inorgânicos. Dentre as proteínas que compõem estas peçonhas encontram-se as fosfolipases A₂ (PLA₂), serinoproteínas, metaloproteínas, L-aminoácido oxidase, fragmentos DC, lectinas do tipo C e proteínas secretadas ricas em cisteínas (ALAPE-GIRÓN et al., 2008).

Segundo Daltry et al., (1996), serpentes da mesma espécie podem diferir na composição da peçonha devido a idade (indivíduos jovens possuem composição da peçonha

diferente de indivíduos adultos), posição geográfica em que se encontram e a diversidade de animais disponíveis para sua alimentação, pois a função primordial da peçonha é a imobilização dos animais capturados e auxílio na alimentação.

Análise de transcritos da glândula de veneno da serpente *Bothrops jararacussu* realizada por Kashima et al., (2004) demonstrou que o peçonha desta serpente compreende PLA₂s, metaloproteases, lectinas do tipo C, proteínas indutoras de apoptose, serina proteases, L-aminoácido oxidases, albuminas, entre outros componentes. Os resultados obtidos por Kashima et al., (2004) revelaram que a maior parte dos genes expressos nas glândulas são de fosfolipases A₂ (35% dos transcritos), sendo 83% destes de BthTX-I (Bothropstoxina-I, uma Lys49-PLA₂ básica), 8% de BthTX-II (Botropstoxina-II, uma Asp49-PLA₂ básica) e 9% é BthA-I (Asp49-PLA₂ ácida). Estes dados apresentados sugerem uma significativa importância do papel das proteínas PLA₂s (destacando as Lys49-PLA₂ básicas) para os efeitos causados pelo empoçoamento. Uma vez que a maior parte de transcritos veneno serem de fosfolipases A₂, torna-se relevante o estudo de inibidores que atuem produzindo decréscimo nas atividades enzimática e toxicológica causada por esta classe proteica.

Estudos de compostos presentes em peçonhas ofídicas têm tido êxito e seus resultados estão sendo aplicado na produção de diversos fármacos, um exemplo clássico de um fármaco desenvolvido com base na observação do desencadeamento do efeito sistêmico causado pelo empoçoamento é o captopril (captoten), um dos medicamentos mais vendidos no mundo e eficaz no controle da hipertensão. Este medicamento possui como princípio ativo um oligopeptídeo rico em prolina retirado da peçonha da serpente *Bothrops jararaca*, o piroglutamil. (HAYASHI e CAMARGO, 2005). Outros medicamentos produzidos a partir de compostos da peçonha de serpentes são:

- **Eptifibatide (integrelín)** um heptapeptídeo cíclico extraído da peçonha da serpente *Sistrurus miliarius barbouri* que age como inibidor de integrina sendo utilizado em tratamentos anti-trombóticos (FERNANDES, 2010).
- **Batroxobina** uma enzima trombina encontrada no DNA da peçonha da espécie *Bothrops atrox*, utilizada no tratamento de doenças trombóticas pois, converte fibrinogênio em fibrina produzindo uma rede não flexível de ligações não cruzadas que é rapidamente removida do sistema circulatório pelo sistema

fibrinolítico resultando em um efeito final desfibrinante (GEMPELER-MESSINA et al., 2001).

- **Ancrod (ViprinexTM)** uma molécula derivada da peçonha da serpente *Agkistrodon rhodostoma* que tem ação anticoagulante, por reduzir a viscosidade sanguínea nas artérias, ajudando a combater a dor e melhorando a mobilidade.

1.3 Fosfolipases A₂: caracterização e classificação

As primeiras publicações referentes às fosfolipases A₂ (PLA₂s) realizadas por Hanahan et al., (1960) e por Doery e Pearson (1961) caracterizam estas enzimas como promotoras da hidrólise de substratos lipídicos fosfatados. Devido às descobertas posteriores de várias proteínas com atividade igualmente as acima citadas, este grupo de proteínas passou a ser mais bem estudada e, conseqüentemente, suas ações hoje, são melhores compreendidas.

As fosfolipases são classificadas em fosfolipases C, fosfolipases D, fosfolipases A₁ e fosfolipases A₂, de acordo com a posição da clivagem na cadeia do substrato lipídico (Figura 1). As PLA₂s catalisam a reação da hidrólise de fosfolipídios na posição sn-2 resultando na produção de ácidos graxos e lisofosfolipídios (VALENTIN; LAMBEAU, 2000). Um dos produtos de sua atividade catalítica, o ácido araquidônico, participa de rotas de síntese de compostos bioativos como os tromboxanos, leucotrienos e prostanóides (prostaglandinas e prostaciclinas), importantes intermediários em processos celulares diversos como resposta inflamatória, proteção de mucosas e do endotélio, neurotransmissão, controle de temperatura (Murakami; Kudo, 2004) e mediador da dor (Booting, 2004). Enquanto que o lisofosfolipídio resultante da reação de hidrólise dos fosfolipídios vai atuar na desestabilização e remodelamento de membranas celulares.

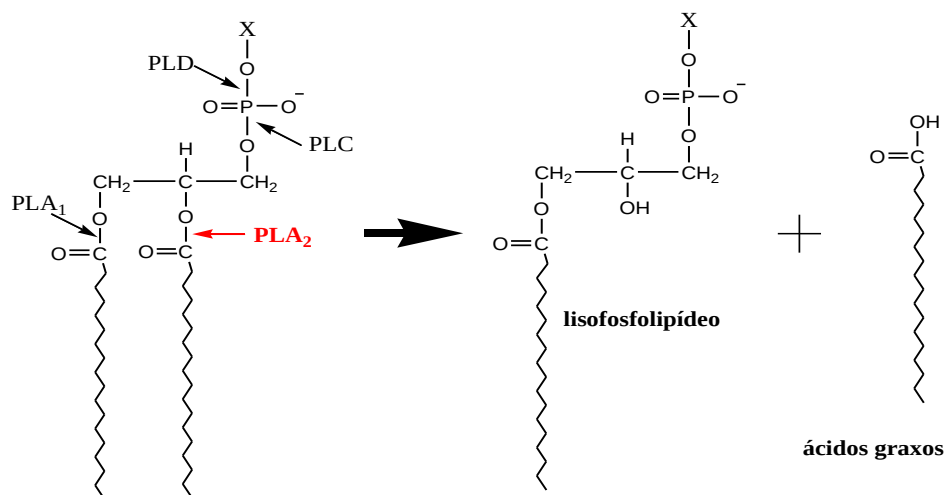


Figura 01. Classificação das fosfolipases de acordo com a posição da clivagem na região polar da cadeia do substrato lipídico.

As PLA₂s são encontradas em diversos organismos na natureza, tanto em vegetais como, animais (moluscos, artrópodes, répteis e mamífero), fungos, bactérias e vírus (KINI, 2003; KUCHLER et al., 1989; LEE et al., 2005; MCINTOSH et al., 1995; RYU et al., 2003; VALENTIN E LAMBEAU, 2000). Estas enzimas são identificadas pela Joint Commission on Biochemical Nomenclature (JCBN IUPAC –IUBMB) com código **E.C.3.1.1.4**. (A numeração 3 refere-se a hidrolases, 3.1 hidrolases que agem em ligação éster, 3.1.1 hidrolases de éster carboxílico E 3.1.1.4 para fosfolipases A₂) .

As PLA₂s podem ser encontradas tanto em meio extra como intracelular. De acordo com Six e Dennis (2000) as PLA₂s intracelulares têm massa molecular variando entre 26 kDa e 114 kDa com aproximadamente 231 e 1014 aminoácidos, respectivamente. Participam de processos envolvidos na transdução de sinais, remodelamento de membrana (BROWN et al., 2003) e importantes processos celulares como, resposta inflamatória, neurotransmissor, hemostasia, proteção do endotélio e da mucosa gástrica. O produto dos ácidos graxos resultante da hidrólise de glicerofosfolipídios é o ácido araquidônico, que participa da via de síntese dos leucotrienos, tramboxanos, prostaglandinas e prostaciclina, mediadores dos processos acima citados (MURAKAMI e KUDO, 2002).

As PLA₂s extracelulares possuem massa molecular baixa com relação às intracelulares variando entre 13 kDa e 18 kDa com aproximadamente 110 e 70 aminoácidos, respectivamente (SIX e DENNIS, 2000), porém são ricas em pontes, possuindo em média sete pontes de dissulfeto, sendo seis delas bem conservada (resíduos 27-131, 29-45, 44-109, 51-

102, 61-95 e 85-100) com algumas variações em algumas PLA₂s. De acordo com (MURAKAMI *et al.*, 2010), estas proteínas estão estruturalmente inativas quando em meio intracelular, porém possuem um peptídeo sinal na extremidade N-terminal para ativação e exportação para o meio extracelular e, assim, podendo realizar suas funções (DENNIS, 1994). Ações como neurotoxicidade, miotoxidade, cardiotoxidade, indução de edema, indutora de necrose tecidual, pró- e anticoagulante, pró- e antiinflamatória, hemolítica, citotóxica e inibidora de agregação plaquetária e atividade letal são alguns dos diversos efeitos biológicos causados pelas PLA₂s extracelulares (KINI, 2003).

As PLA₂ foram primeiramente classificadas em grupos I e II considerando-se a localização e quantidade de pontes de dissulfeto que compunha a enzima (HEINRIKSON *et al.*, 1977; DUFTON e HIDER, 1983). DENNIS (1994) classificou as fosfolipases A₂ em quatro classes: I, II, III e IV. As PLA₂s da classe IV é caracterizada por sua origem intracelular, por possuir um alto peso molecular e por apresentar afinidade a um substrato específico gerando como produto da hidrólise o ácido araquidônico, já as classes I, II, e III são enzimas extracelulares, caracterizadas por apresentarem um número alto de pontes dissulfeto (5 a 7) e, por possuírem baixo peso molecular (12 a 15 kDa).

O avanço das técnicas e ferramentas de biologia molecular permite a descobertas de um número cada vez maior de PLA₂s, Six e Dennis, (2000) propuseram um sistema de classificação para as fosfolipases A₂ em onze classes (I a XI) com subdivisões, por exemplo: IA e IB (Tabela 1), utilizando quatro critérios de classificação: a capacidade de catalisar fosfolipídios na posição *sn*-2, a sequência de aminoácidos, aspectos de homologia entre as sequências e a variação de *splice* nos genes produzindo proteínas distintas.

TABELA 1: Classificação das Fosfolipases A₂ proposta por SIX e DENNIS (2000).

Classe e subdivisões	Fonte	KDa	Pontes dissulfeto	NCBI
I A	Serpente	13-15	7	P47712
I B	Mamíferos	13-15	7	AAD32135
II A	Humano/Serpentes	13-15	7	AAC32823
II B	Serpente de víbora	13-15	6	AAD41722
II C	Rato	15	8	NP_003551
II D	Humano e Rato	14-15	7	NP_036532
II E	Humano	14-15	7	AAF36541
II F	Rato	16-17	6	AAF04500
III	Escorpião e Humano	15-18	8	P00630
IV A	Humano	85		P47712

IV B	Humano	114		AAD32135
IV C	Humano	61		AAC32823
V	Mamífero	14	6	NP_000920
VI A1	Macrofagos	84-85		AAD41722
VI A2	Linfócitos B humanos	88-90		NP_003551
VI B	Musculo cardíaco humano	88		BAA94997
VII A	Humano/ Rato	45		Q15102
VII B	Humano/ bovino	40		Q99487
VIII A	Cérebro humano	26		Q15102
VIII B	Cérebro humano	26		Q29459
IX	Serpente do caracol	14	6	AABB33555
X	Humano	14	8	NP_003552
XI A	Arroz	12.4	6	CAB40841
XI B	Arroz	12.9	6	CAB40842

Fonte: SIX e DENNIS (2000)

Em 2006, Schaloske e Dennis reclassificaram a superfamília das PLA₂s considerando a similaridade na sequência de aminoácidos e na estrutura como a variação das posições e quantidade das pontes de dissulfeto e variações no tamanho dos *loops* (alças presentes na estrutura tridimensional da proteína). Assim, atualmente as PLA₂s podem ser divididas em 5 tipos distintos: PLA₂s secretoras (sPLA₂), PLA₂s citosólicas (cPLA₂), PLA₂s independente de Ca²⁺ (iPLA₂), PLA₂s lisossomais e PLA₂s PAF acetil-hidrolases (PAF-AH). Os cinco tipos de PLA₂s ainda são subdivididos de acordo com as distinções no mecanismo catalítico (His/Asp hidrolases, Ser/Asp hidrolases ou Ser/His/Asp hidrolases), bem como, os aspectos funcionais e estruturais, totalizando 15 classes (I a XV) com subdivisões (Tabela 02) para classificar fosfolipases A₂:

- sPLA₂: (grupos IA e IB; IIA, IIB, IIC, IID, IIE e IIF; III, V, IX, X, XIA e XIB; XII e XIV),
- cPLA₂: (grupo IVA, IVB, IVC, IVD e IVE),
- iPLA₂: (grupo VIA, VIB, VIC, VIE e VIF),
- PAF-AH: (grupos VIIA e VIIB; VIIIA e VIIIB)
- Lisossomal PLA₂s: (grupo XV).

TABELA 2: Reclassificação das Fosfolipases A₂ por Schaloske e Dennis (2006).

Classe e subdivisões	Fonte	kDa	Pontes dissulfeto
I A	Serpente	13-15	7
IB	Humano/serpentes	13-15	7

IIA	Humano/Serpentes	13-15	7
II B	Serpente de víbora	13-15	6
II C	Serpente	15	8
IID	Humano/camundongo	14-15	7
II E	Humano/camundongo	14-15	7
II F	Humano/camundongo	16-17	6
III	Humano/camundongo/escorpião	15-18	8
IVA	Humano/camundongo	85	
IVB	Humano	114	
IVC	Humano	61	
IVD	Humano/camundongo	92-93	
IVE	Camundongo	100	
V	Humano	14	6
VIA1	Humano	84-85	
VIA2	Humano/camundongo	88-90	
VI B	Humano/ camundongo	88-91	
VI C	Humano/ camundongo	146	
VID	Humano	53	
VIE	Humano	57	
VIF	Humano	26	
VIIA	Humano/Camundongo	45	
VII B	Humano/Bovino	40	
VIIIA	Humano	26	
VIIIB	Humano	26	
IX	Rato do caracol	14	6
X	Humano	14	8
XI A	Arroz	12.4	6
XI B	Arroz	12.9	6
XII	Humano/Camundongo	19	7
XIII	Parvovirus	>10	0
XIV	Fungos/Bactérias	13-19	2
XV	Humano/Bovino	45	

Fonte: Schaloske e Dennis (2006)

1.4 Fosfolipases A₂: estrutura e função

PLA₂s extracelulares da classe IIA presentes nas famílias Elapidae e Viperidae (SIX; DENNIS, 2000) apresentam em sua estrutura primária de 120 a 123 aminoácidos que seguem o modelo de numeração da PLA₂ de pâncreas bovino proposta por Renetseder et al., (1985).

A estrutura terciária básica das PLA₂s isoladas de peçonhas de serpentes é composta por uma região de N-terminal (resíduos 1-12) representada por uma hélice- α I (“h1”) – região envolvida na ligação e orientação de substratos à proteína (canal hidrofóbico das PLA₂s); em

seguida tem-se a hélice curta (*short helix*) formada pelos resíduos 18-23. Entre os resíduos 25-34 está localizado o *loop* de ligação do íon Ca^{2+} seguido pela hélice- α II (“h2”) constituída pelos resíduos 40-55, a qual se liga a uma curta folha β antiparalela (β -wing) (resíduos 75 ao 77 e 82 ao 84). Após a região de β -wing, os resíduos 90-107 formam a hélice- α III (“h3”) que é seguida do *loop* de C-terminal (resíduos 108-133) (Figura 2). Estes três últimos seguimentos, “h2”, β -wing e “h3” alocam resíduos constituintes do sítio catalítico (His48, Asp49, Tyr52 e Asp99) das PLA₂s (ARNI e WARD, 1995; DE AZEVEDO et al., 1999).

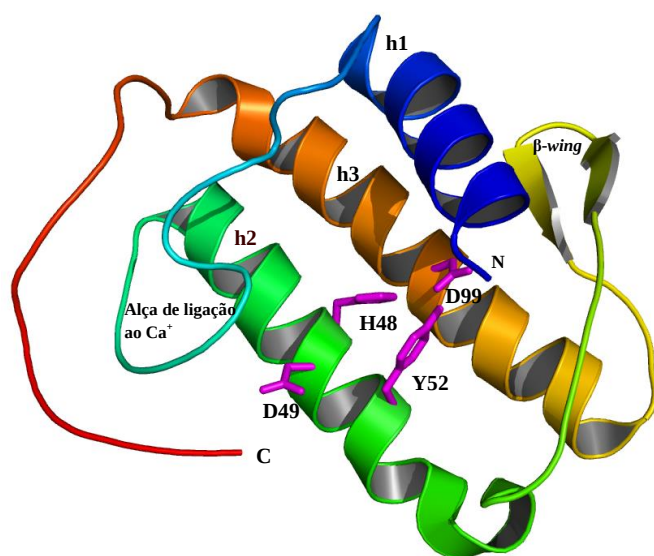


Figura 2: Enovelamento de uma PLA₂ (isoforma CB1 da crotoxina B isolada da peçonha de *Crotalus durissus terrificus* – PDB: 2qog) – programa “Py Mol” (De Lano, 2002).

A estrutura quaternária das PLA₂s é variável, tanto em solução como no estado cristalino, apresentando conformações monoméricas (FERNANDES et al., 2010), diméricas (MARCHI-SALVADOR et al., 2009), triméricas (FREMONT et al., 1993) e tetraméricas (MARCHI-SALVADOR et al., 2008).

As PLA₂s de peçonha de serpentes apresentam um variedade de atividades como neurotoxicidade, miotoxicidade, citotoxicidade, anticoagulante, hipotensiva, cardiotoxica, indutora de edema e bactericida (KINI e EVANS, 1989; Gutiérrez e Ownby, 2003; KINI, 2003). Tradicionalmente, as Lys49-PLA₂s são consideradas miotoxinas não-neurotóxicas, uma vez que não induzem paralisia *in vivo*. Enquanto que as PLA₂s neurotóxicas apresentam o ácido aspártico na posição 49 (Asp49-PLA₂s) que catalisa a hidrólise de glicerolfosfolipídios na posição sn-2, e Lys49 caracterizada pela ausência de atividade enzimática.

O resíduo de ácido aspártico na posição 49 das PLA₂s é um dos principais responsáveis pela alta atividade catalítica destas enzimas. Este resíduo coordena a ligação do íon cálcio no

sítio de ligação de Ca^{2+} , cofator necessário à liberação de ácidos graxos e lisofosfolípidos produtos resultantes da hidrólise de fosfolípidos por fosfolipases A_2 (LEE et al., 2001).

Mutações são mudanças na composição química de um gene, incluindo mudanças na sequência de nucleotídeos e, conseqüentemente, nos aminoácidos, podendo ser por substituição, deleção ou inserção (POLLARD e ERNSHAW, 2006). Uma das mutações mais encontradas em PLA_2s é a observada na posição 49 da proteína, em que ocorre a troca do ácido aspártico (D – carregado negativamente) por Lisina (K – carregado positivamente). Esta alteração impede que a enzima oriente o cálcio para ligar ao *loop* de ligação de Ca^{2+} e, dessa forma, a função catalítica da enzima é perdida, pois a capacidade de hidrolisar é dependente de cálcio, conseqüentemente, não há liberação de ácido graxo fazendo com que os produtos de sua via metabólica não sejam sintetizados (LEE et al., 2001) (Figura 3).

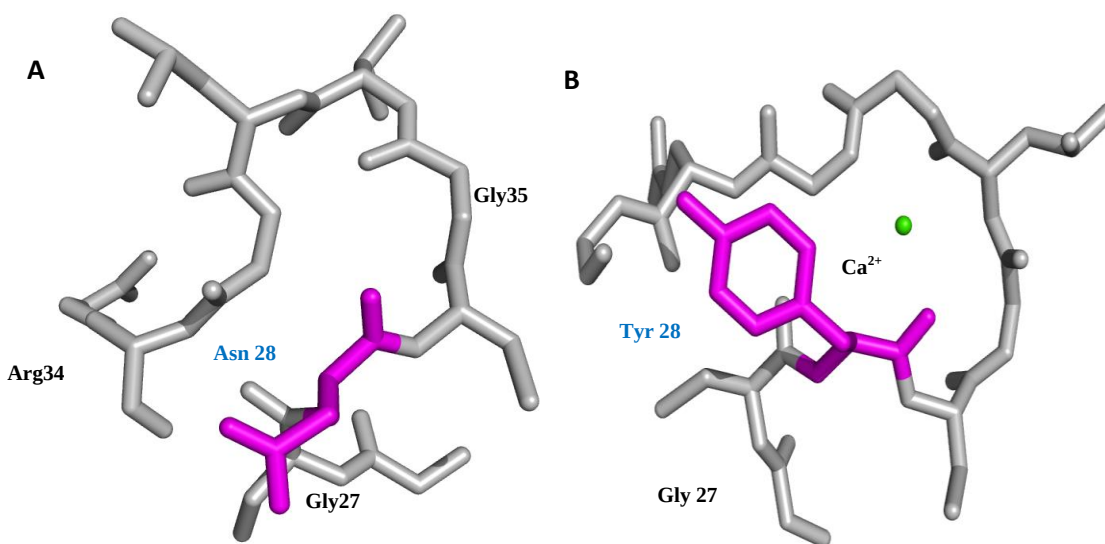


Figura 3. Região de ligação do íon cálcio (Resíduos 27-35). A. BthTX-I (K49- PLA_2). B. BthA-I (Asp49- PLA_2). Em A, destacado em magenta o resíduo asparagina (Asn28) que impede a ligação do co-fator cálcio a região de *loop*. Em B destacado o aminoácido tirosina (Tyr28) que permite a ligação do íon Ca^{2+} ao loop de ligação de cálcio. “Py Mol” (De Lano, 2002).

As $\text{Lys49-PLA}_2\text{s}$, quando comparada com as demais PLA_2s , apresentam alta similaridade tanto na sequência primária de aminoácidos como na estrutura secundária e terciária, porém não apresentam atividade catalítica, mas são altamente tóxicas (LOMONTE et al., 2003). Outras substituições menos frequentes também ocorrem na posição 49 como a substituição Asp-Ser ($\text{Ser49-PLA}_2\text{s}$), cuja alteração causa distorção estrutural na região

catalítica da proteína e consequente perda de atividade catalítica, preservando, no entanto, a ação miotóxica e citotóxica (GEORGIEVA et al., 2012). Outra substituição presente nas PLA₂s é a Asp→Arg (Arg49-PLA₂s), (CHIJIWA, 2006) miotóxica e indutora de edema, mas cataliticamente inativa (MURAKAMI, 2007).

1.5 Inibidores de fosfolipase A₂

Atualmente, o tratamento mais utilizado no combate aos efeitos causados por acidentes ofídicos é a aplicação de soro antiofídico. A produção de antivenenos no Brasil é restrita a três laboratórios oficiais: Instituto Butantan – IB, São Paulo (SP), Instituto Vital Brazil - IVB Niterói, Rio de Janeiro (RJ) e Fundação Ezequiel Dias – FUNED, Minas Gerais (MG), os quais têm trabalhado na elaboração de soros cada vez mais eficazes e específicos para combater os efeitos da peçonha de diversos gêneros de serpentes. Os soros antiofídicos comumente produzidos são:

- Antibotrópico: usado em envenenamentos por serpentes dos gêneros *Bothrops*, *Bothriopsis*, *Bothropoides*, *Rhinocerophis* e *Bothrocophias*
- Anticrotálico: aplicado em envenenamentos por serpentes do gênero *Caudisona*;
- Antilaquético: utilizado para envenenamentos por serpentes do gênero *Lachesis*;
- Antielapídico: aplicado em envenenamentos por serpentes dos gêneros *Micrurus* e *Leptomicrurus*;
- Antibotrópico/Crotálico: utilizado em casos de acidentes envolvendo serpentes tanto dos gêneros *Bothrops*, *Bothriopsis*, *Bothropoides*, *Rhinocerophis* e *Bothrocophias* quanto como do gênero *Caudisona*;
- Antibotrópico/Laquético: soro combinado aplicado em envenenamentos duvidosos entre os gêneros para acidentes com jararacas e surucucus.

Devido à grande quantidade e intensidade de acidentes ocorrentes em todo o planeta, elucidar os mecanismos tóxicos e farmacológicos desencadeados pelos envenenamentos e inferir possíveis substâncias para inibir os efeitos ofídicos tem um caráter emergencial.

As plantas têm sido utilizadas como um dos grandes recursos nas áreas médica e farmacêutica, principalmente, no uso de suas propriedades para tratamentos de doenças.

Propriedades bactericida (DEY e DE, 2012a), antifúngica (LOUIS et al., 2011), antioxidativa (DEY e De, 2012b), larvicida (RAGHAVENDRA et al., 2011), antifertilizante (SHAH et al., 2010), imunomoduladora (LATORRE et al., 2009), antidiabética (PALSAMY e MALATHI, 2007), anti-inflamatória (KARACA et al., 2009) e diurético (BALA et al., 2011) são alguns exemplos, que mostram o potencial terapêutico vegetal.

Relatos do uso das propriedades das plantas medicinais contra peçonha de serpentes são conhecidos no mundo (SARWAR et al., 2011). Pesquisadores têm se empenhado em descobrir compostos vegetais com capacidade para inibir efeitos do ofidismo, a partir de biomoléculas e sintéticas. (HAGE-MELIM et al., 2009). Experimentos têm demonstrado que diversas moléculas sintéticas e/ou naturais são hábeis inibidores dos efeitos tóxicos desencadeados pelo emponhamento. Dentre os **compostos naturais** temos: ácido aristolóquico (GIRISH e KEMPARAJU, 2005), ácido rosmarínico (TICLI et al., 2005), 4-nerolidilcatecol (NÚÑEZ, 2005) e ácido elágico (SILVA et al., 2008); os **extratos vegetais**: *Casearia sylvestris* (Cavalcante et al., 2007), *Mandevilla velutina* (BIONDO et al., 2003) e *Hypericum brasiliense* (ASSAFIM et al., 2011); Exemplos de **compostos sintéticos**: sesquiterpenos lactonas (ALVARENGA, 2008) e nitrostireno (VILLAR, 2008).

O uso de técnicas da biologia molecular como a purificação e sequenciamento proteico, cristalização e elucidação da estrutura tridimensional, síntese e simulações de peptídeos e uso de ferramentas computacionais para entendimento da biologia molecular são apenas algumas vias importantes para estudos desta natureza. Um aspecto importante no estudo de moléculas inibidoras de fosfolipases A_2 é a elucidação estrutural destas proteínas na forma nativa ou na presença de inibidores, uma vez que a relação estrutura-função é intrínseca.

Estudos de neutralização com toxinas isoladas da peçonha de serpentes do gênero *Bothrops* demonstram que o uso do soro anticrotálico ou antibotrópico específicos e isolados não inibe totalmente efeitos causados pelas PLA_2 s. Quando a mistura do soro antibotrópico e anticrotálico é utilizada observa-se uma maior eficiência na redução dos efeitos causados pelo ofidismo (Queiroz et al., 2008). No entanto, os mecanismos moleculares da neutralização pela mistura dos soros ainda não são bem entendidos, o que torna um campo a ser explorado no meio científico.

2. OBJETIVOS

Objetivos Gerais

Caracterizar bioquimicamente B-epitopos lineares de três fosfolipases A₂ isoladas da peçonha de *Bothrops jararacussu* reconhecidos por soros antiofídicos através da síntese paralela de peptídeos realizadas em membranas de celulose e realizar análises comparativas *in silico* das regiões identificadas; Relatar, por meio de revisão bibliográfica, moléculas sintéticas e isoladas de extratos vegetais utilizadas para inibir PLA₂s.

Objetivos Específicos

- Identificar regiões PLA₂s: BthTX-I, BthTX-II e BthA-I isoladas da peçonha de *Bothrops jararacussu* reconhecidas por soros antiofídicos (anticrotático, antibotrópico e/ou pela mistura de ambos)
- Caracterizar bioquimicamente regiões das PLA₂s: BthTX-I, BthTX-II e BthA-I reconhecidas por soros antiofídicos (anticrotático, antibotrópico e/ou pela mistura de ambos).
- Destacar a importância dos B-epitopos lineares de BthTX-I, BthTX-II e BthA-I para as atividades biológicas destas proteínas por análise comparativa entre PLA₂s isoladas de venenos de serpentes.
- Relatar inibidores moleculares naturais e sintéticos isolados de extratos vegetais utilizados como método terapêutico alternativo no combate aos efeitos causados pelo ofidismo ou para o desenho de fármacos específicos.

3. METODOLOGIA, RESULTADO, DISCUSSÃO.

APÊNDICE I

“Snake venom PLA₂s inhibitors from Brazilian plants: Synthetic and natural molecules”

Snake venom PLA₂s inhibitors isolated from Brazilian plants: Synthetic and natural molecules

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Abstract

Ophidian envenomation is an important health problem in Brazil and other South American countries. In folk medicine, especially in developing countries, several vegetal species are employed for the treatment of snakebites in communities that lack prompt access to serum therapy. However, the identification and characterization of the effects of several new plants, or their isolated compounds, which are able to inhibit the activities of snake venoms (or their toxic components) is extremely important and such studies are imperative. Snake venom contains several organic and inorganic compounds; phospholipases A₂ (PLA₂s) are one of the principal toxic components of venom. PLA₂s display a wide variety of pharmacological activities, such as neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, antiplatelet, hypotensive, hemolytic, hemorrhagic and edema-inducing effects. PLA₂ inhibition is of pharmacological and therapeutic interest as these enzymes are involved in several inflammatory diseases. This review describes the results of several studies of plant extracts, and their isolated active principles, when used against crude snake venoms or their toxic fractions. Isolated inhibitors (natural or synthetic), such as steroids, terpenoids and phenolic compounds (flavonoids, coumestans, nitro compounds and other hydroxylated phenolic molecules), are able to inhibit PLA₂s from different snake venoms. The design of specific inhibitors of PLA₂s might help in the development of new pharmaceutical drugs, more specific antivenoms, or even as alternative approaches for treating snakebites.

Keywords: Snake venom PLA₂ inhibitors, PLA₂ inhibitors isolated from plants, synthetic inhibitors, phenolic compounds, molecules from secondary plant metabolism.

Introduction

Venomous snakebites represent an important risk for public health worldwide, especially in tropical regions where these accidents are more common. Snake venom contains several organic and inorganic compounds, among which phospholipase A₂ enzymes (PLA₂s, E.C. 3.1.1.4, phosphatide sn-2 acylhydrolases) are one of the most important for its effect. The PLA₂ class includes several polypeptides with similar enzymatic functions; however, these proteins exert a variety of relevant toxic actions, such as neurotoxicity and myotoxicity. PLA₂s show considerable homology in their amino acid sequence (Higuchi et al., 2007; Ponce-Soto et al., 2007), as well similarity in their tertiary structures (Arni and Ward 1996; Corrêa et al., 2008; Marchi-Salvador et al., 2008; Marchi-Salvador et al., 2009; Fernandes et al., 2010). Venoms of different snake species are used as sources of PLA₂, due to the abundance of these enzymes and the fact that the purification of these molecules is relatively simple (Villar et al., 2008).

The apparent contradiction between structural uniformity and functional diversity, exhibited by PLA₂s, has attracted much interest from the scientific community. According to Ohno et al. (1998), this diversity of pharmacological and toxic effects may have been evolutionarily acquired by positive Darwinian selection of the coding exons of these activities.

Small variations among PLA₂ isoforms may be used for the study of structural and functional relationships of these proteins. Moreover, research regarding natural and synthetic inhibitors that are able to neutralize the toxic effects promoted by these enzymes is being carried out in an attempt to explain the physiopathological mechanisms of these molecules (Cavalcante et al., 2007; Marcussi et al., 2007; Oliveira et al., 2008). Furthermore, knowledge about the mechanism of toxicity exhibited by these proteins may assist the discovery and development of new anti-inflammatory drugs, cellular lesions and

therapies for several diseases, including Parkinson's, Alzheimer's, and even cancer (Cummings et al., 2000; Araya and Lomonte, 2007; Lomonte et al., 2010; Samy and Gopalakrishnakone, 2010).

Several PLA₂ inhibitors have been obtained from natural resources, such as invertebrate and vertebrate animals and plants (Ticli et al., 2005). Plant extracts have become a promising alternative to substitute traditional snake antivenom, which often are unavailable in emergency situations (Soares et al., 2004; da Silva et al., 2012). After studying plants commonly used to treat snakebites in South America, Soares and collaborators (2004) reported 56 vegetal species that exhibited anti-inflammatory activity caused by crude snake venoms or by their isolated components.

Treatment of snake bites is still carried out using traditional antivenom therapy (Gutiérrez et al., 2007). However, although antivenom therapy is effective for the majority of cases, some side effects exist for these treatments, including adverse reactions on the skin, gastrointestinal tract, respiratory and circulatory system (García et al., 2002; Schmidt, 2005). Moreover, snake antivenom therapy is usually unable to prevent the progress of local effects (Cardoso et al., 2003). Given the limitations of traditional therapy, research focusing on the interactions between PLA₂s and their natural or synthetic inhibitors could allow the development of alternative treatments for the toxic and pharmacological effects of snake bites.

1. PLA₂ inhibitors isolated from plants

Plants are used in traditional medicine to treat the effects of venomous snake bites. Pharmacological studies have shown that fractions of these plant extracts have anti-inflammatory, antiviral, and antivenom properties (Mors et al., 2000; Ticli et al., 2005). The effect of specific molecules from these plant extracts may be attributed to the presence of multiple factors, such as low molecular weight chemical compounds, and the abundance of chemical and pharmacological properties (Soares et al., 2004).

Borges et al. (2000) reported that the aqueous extract of *Casearia sylvestris* (Flacourtiaceae), a native vegetal specie found in Brazilian open pastures, had the ability to inhibit myotoxic, anti-coagulant and edema-inducing activities from *Bothrops moojeni*, *B. pirajai*, *B. neuwiedi* and *B. jararacussu* venoms and its Asp49 and Lys49-PLA₂ isolated toxins. In addition, Borges et al. (2001) emphasized that *C. sylvestris* was able to neutralize hemorrhagic activity caused by the *B. pirajai*, *B. jararacussu*, *B. asper*, *B. moojeni* and *B. neuwiedi* venoms. Cavalcante and colaboradores (2007) showed that the *C. sylvestris* aqueous extract demonstrated protective effects against muscle damage induced by two Lys49-PLA₂ toxins (PrTX-I from *B. pirajai* and BthTX-I from *B. jararacussu* snake venoms) and prevented the neuromuscular blockage induced by all PLA₂ toxins.

Mandevilla velutina (Apocynaceae) is a perennial plant from the Brazilian *cerrado* that has been studied for its anti-inflammatory activity, as well as its antagonist effect on bradykinin, a vasodilator (Biondo et al., 2003). These authors reported that the aqueous extract of this plant was an effective inhibitor of phospholipase A₂ activity and some toxic effects, such as hemorrhage, caused by venom from snakes of the *Bothrops* and *Crotalus* genus. In a posterior study, the same research group reported that extracts from *Mandevilla illustris* were able to completely inhibit the activity of the Crotoxin B, the basic Asp49-PLA₂, isolated from *Crotalus durissus terrificus* venom (Biondo et al., 2004).

The anti-hemorrhagic properties of the aqueous extract of *Pentaclethra macroloba* (Fabaceae), an ethnomedicinal plant found in the Amazon region, was evaluated against snake venoms and displayed a full inhibition of hemorrhagic and nucleolytic activities induced by several snake venoms. Additionally, a partial inhibition of myotoxic, lethal, enzymatic and edema activities of snake venoms, and their isolated PLA₂s, was observed (da Silva et al., 2005).

Almeida et al. (2004) showed that the aqueous extract of *Tabernaemontana catharinensis* (Apocynaceae), which is encountered in some countries of South America, was able to inhibit the crotoxin complex, isolated from *C. d. terrificus* venom, and was

also able to partially neutralize the myotoxicity of *B. jararacussu* snake venom and its basic PLA₂s (Veronese et al., 2005).

The aqueous extract of the aerial parts of *Bauhinia forficata* (Fabaceae), a native species native to Asia and well adapted and developed in several regions of Brazil, was assayed against the fibrinogenolytic and coagulant activities of *C. d. terrificus* and *B. jararacussu* crude venoms and was found to neutralize these effects. Moreover, the extract efficiently inhibited the edema induced by *C. d. terrificus* venom and its isolated PLA₂ (Oliveira et al., 2005).

Mendes et al. (2008) reported that the aqueous extract of *Schizolobium parahyba* (Fabaceae), a plant found in the Mata Atlântica of southeastern Brazil, contains compounds that can inhibit some enzymatic and biological activities induced by *Bothrops pauloensis* (current *Bothropoides pauloensis*) and *C. d. terrificus* snake venoms as well as by their isolated neuwiedase toxins (metalloproteinase), BnSP-7 (basic Lys49-PLA₂ from *B. pauloensis* venom) and Crotoxin B.

The ethanolic extract of the aerial parts of *Blutaparon portulacoides* (Amaranthaceae), an herbaceous plant that occurs mainly in the Atlantic bush, caused a reduction in edema formation and in the leukocyte influx induced by Lys49-PLA₂ isolated from *B. jararacussu* venom (Pereira et al., 2009).

In 2005, Maiorano et al., evaluated aqueous extracts prepared from dried or fresh roots, stems or leaves of *Mikania glomerata* (Asteraceae), a plant found in the Mata Atlântica in Southeastern Brazil and popularly known as "Guaco". The *M. glomerata* extract efficiently neutralized different toxic, pharmacological, and enzymatic effects induced by *Bothrops* and *Crotalus* snake venoms. The phospholipase A₂ activity and the edema induced by *C. d. terrificus* venom were inhibited by approximately 100 and 40%, respectively, and this inhibition was also partially observed with the *Bothrops* venoms. The hemorrhagic activities of *B. alternatus*, *B. moojeni*, *B. neuwiedi* and *B. jararacussu* venoms were significantly inhibited by *M. glomerata* extract, while the clotting activities

of *C. d. terrificus*, *B. jararacussu* and *B. neuwiedi* venoms were totally inhibited. In addition, Floriano et al. (2009) reported clinical and laboratory alterations in mice caused by the combination of *M. glomerata* leaf extract and antiophidian serum against *C.d. terrificus* venom.

Nazato and collaborators (2010) found that the methanolic extract of the bark from *Dipteryx alata* (Fabaceae) (a native species of the Brazilian Savanna, found principally in Minas Gerais, Goiás, Federal District and Mato Grosso) decreased the neurotoxicity and myotoxicity of *B. jararacussu* crude venom. In another study by Puebla et al. (2010), the extract from *D. alata* was fractionated and its compounds were evaluated against the neuromuscular blockade caused by *B. jararacussu* venom.

The ability of the ethanolic extract of *Hypericum brasiliense* (Hypericaceae), a plant found mainly in the southeastern and southern regions of Brazil, to neutralize some effects induced by *B. jararaca* venom was investigated using biological assays. *H. brasiliense* extracts were able to inhibit some pharmacological effects such as lethality, edema, hemorrhage, hemolysis and proteolysis, as well as fibrinogen or plasma clotting (Assafim et al., 2011).

In 2012, Dey and De published a review that evaluated several pharmacological studies on plant efficacies against snakebites. The authors compiled studies from a number of plants, or their fractions, that were active against snake venoms and concluded that folk knowledge is relevant. However, clinical tests should be performed with these plant extracts or fractions to assess the effect of the compounds used for the treatment of snakebites.

Recently, Samy, Gopalakrishnakone and Chow (2012) published an extensive revision on the therapeutic application of natural inhibitors of snake venom PLA₂s, covering molecules from the primary metabolism of different organisms, such as glycoproteins (PLIs), peptides and lipids, as well as from secondary metabolism, exemplified by terpenoids, alkaloids, flavonoids and other molecules. These authors

concluded that the biotechnological potential of PLA₂ inhibitors may provide therapeutic molecular models with antiophidian activity to supplement conventional serum therapy or for the development of novel antivenom therapeutics. Additionally, inhibitors isolated from medicinal plants may also be an essential tool in isolated communities (Soares et al., 2005; Marcussi et al., 2007).

2. Structural characterization of PLA₂ inhibitors

The main classes of PLA₂ inhibitors are the phenolic compounds, which include flavonoids, coumestans and nitro compounds, steroids and terpenoids (mono, di and triterpenes) and polyphenols (vegetable tannins). There is also mention in the literature of other molecules such as carbohydrates, lipids and proteins, although this article emphasizes molecules originating from plant secondary metabolism.

2.1. Phenolic Compounds

2.1.1. Flavonoids

Polyphenolic secondary metabolites are commonly able to bind to biological polymers and some of these have been shown to inhibit PLA₂s. Examples include quercetin, a strong lipoxigenase inhibitor, naringenin, artemetin, kaempferol and galangin, among several other flavonoids. Primetin (Figure 1), 5,8-Dihydroxyflavone, isolated from *Primula sp* (Primulaceae), is known for its ability to inhibit toxins from snake venoms, its structural form may be seen in Figure 1. Flavonoids usually exert their inhibitory effect via hydrophobic interactions with the A and B rings and aromatic or hydrophobic amino acid residues in the protein (Soares et al., 2005; Ticli et al., 2005; Lattig et al., 2007).

2.1.2. Coumestans

Eclipita alba (Asteraceae) is a native plant from Brazil and other tropical and subtropical areas of the world whose medicinal properties are widely known. *E. alba* was genetically engineered using *Agrobacterium rhizogenes* LB9402 to enhance the production of secondary wedelolactone metabolites, which are coumestan compounds with activity against basic PLA₂s. This mutant strain was found to reduce the phospholipase A₂ activities, and myotoxic and neurotoxic effects of the *C. d. terrificus* and *B. jararacussu* snake venoms (Mors et al., 2000, Diogo et al., 2009). Analogs of wedelolactone molecule (Figure 1) were able to antagonize the release of creatine kinase induced by *B. jararacussu* venom even at concentrations as low as 30µM (Da Silva et al., 2001).

2.1.3. Nitro compounds

Batina et al. (2000) isolated an alkaloid from *Tabernaemontana catharinensis* (Apocynaceae) named 12-methoxy-4-methylvoachalotine (Figure 1), and reported a strong inhibitory effect against lethality and myotoxic activities induced by *C. d. terrificus* venom.

2.1.4. Other phenolic compounds

Ar-Turmerone (Figure 1) is a phenolic compound isolated from the *Curcuma longa* (Zingiberaceae) plant that has a strong effect against the hemorrhage and lethality caused by *B. jararaca* and *C. d. terrificus* snake venom (Ferreira et al., 1992).

Extracts from *Piper umbellatum* and *P. peltatum* (Piperaceae) were shown to inhibit the myotoxic activities of PLA₂s isolated from *Bothrops* snake venoms (Núñez et al., 2005). Fractionation of these plant extracts revealed that 4-nerolidylcatechol, a hydroxylated phenolic compound (Figure 1), was responsible for at least part of the inhibitory effect against the group I, II and III PLA₂s.

In 2008, Da Silva et al. studied the half maximal inhibitory concentration (IC₅₀) of ellagic acid (Figure 1), extracted from *C. sylvestris*, against BthTX-II, a basic Asp49-PLA₂

from *B. jararacussu* snake venom, and concluded that this compound was effective at competitively inhibiting the induction of edema, myotoxicity and enzymatic activity, incurred by this PLA₂.

The first structural analysis of aristolochic acid (Figure 1), isolated from *Aristolochia* sp (Aristolochiaceae), was performed by Vishwanath and Gowda (1987). In this study, the interaction of aristolochic acid, an alkaloid, with PLA₂ from *Vipera russelli* was characterized as non-competitive inhibitive. This compound has also been shown to reduce the induction of edema by this enzyme. Additionally, Vishwanath et al. (1987) emphasized that the interaction between aristolochic acid, from *Aristolochia radix*, and three PLA₂s from *Trimeresurus flavoviridis* resulted in the inhibition of hemolytic and edema induction by competitive inhibition. Chandra et al. (2002) reported the crystal structure of the complex formed between the PLA₂ isolated from *V. russelli* venom and aristolochic acid. In this study, the interaction between aristolochic acid and PLA₂ was competitive and the final model consists of a dimer of PLA₂ and one molecule of aristolochic acid located in the binding site of molecule A; this interaction was stabilized by three hydrogen bonds and hydrophobic contacts.

Chlorogenic and caffeic acids (Figure 1) can interact with proteins via hydrophobic contacts and hydrogen bonds, inhibiting enzyme function and acting as antidotes. Strong interactions may induce conformational changes in the protein structure (Mors et al., 2000). In 2011, Shimabuku and collaborators crystallized PrTX-I (basic Lys49-PLA₂ from *B. pirajai* snake venom) in the presence of the inhibitor, caffeic acid, and the electron-density map unambiguously indicated the presence of three caffeic acid molecules interacting with the C-terminus of the protein.

Rosmarinic acid (Figure 1) is a hydroxylated phenolic compound isolated from *Cordia verbenacea* (Boraginaceae). This compound demonstrates anti-myotoxic properties and inhibits edema induced by crude *B. jararacussu* snake venom and its basic PLA₂s (Soares et al., 2005, Ticli et al., 2005). The three dimensional structure of the

PrTX-I – rosmarinic acid complex was elucidated by Santos et al. (2011), where rosmarinic acid was observed located at the entrance to the hydrophobic channel monomer A of the PrTX-I dimer via an interaction between hydrogen bonds and hydrophobic contacts in the same monomer. Interactions were also observed between rosmarinic acid and a residue of the C-terminal region of the monomer B. The interaction between the rosmarinic acid molecule with the hydrophobic channel (monomer A) and the C-terminal region (myotoxic site, monomer B) suggests two mechanisms of myotoxicity inhibition (Santos et al., 2011).

2.2. Steroid Compounds

Sterol and cholesterol molecules present well known antidote activities against snake venom. Steroids can form complexes that are stabilized via Van der Waals interactions, as well as by hydrophobic interactions (Mors et al., 2000). Anti-myotoxic and anti-hemorrhagic effects of the *Eclipta prostrata* (Asteraceae) extract and its components, sitosterol and stigmasterol (Figure 1), were observed against *B. jararaca*, *B. jararacussu* and *Lachesis muta* snake venoms (Mors et al., 2000). Previously, Mors et al. (1989) reported that sitosterol and stigmasterol, isolated from *E. prostrata*, prevented the lethality of the *C. d. terrificus* venom in a dose-dependent manner.

2.3. Terpenoids

The neo-clerodane, diterpenoid, isolated from the aerial parts of *Baccharis trimera* (Asteraceae) demonstrate anti-hemorrhagic and anti-protolytic properties against *Bothrops* snake venoms (Januário et al., 2004).

Several pentacyclic triterpenes, such as oleanolic acid, lupeol, ursolic acid, taraxerol, taraxasterol, α,β -amyrin and friedeline, exhibit activity against snake venoms (Mors et al., 2000). Triterpenoids, isolated from *Betula alba* (Betulaceae), including pentacyclic triterpenes betulin and betulonic acid (Figure 1), exhibited anti-

phospholipasic A₂ activity. Docking (*in silico* experiments) indicated betulinic acid as the best PLA₂ inhibitor, due to its direct insertion in the catalytic site on the enzyme, with a very low energy value (Soares et al., 2005).

2.4. Synthetic inhibitors

Edunol (Figure 1) is a pterocarpan with a chemical structure similar to those of the inhibitors extracted from the roots of *Harpalyce brasiliiana* (Fabaceae). Edunol was obtained via chemical synthesis and the compound showed anti-myotoxic, anti-proteolytic and anti-PLA₂ activities against *B. jararacussu* crude venom (da Silva et al., 2004, Soares et al., 2005).

Elaidoylamide, the amide of trans-9-octadecenoic acid (Figure 1), is a powerful synthetic inhibitor of a neurotoxic Asp49-PLA₂ from *Vipera ammodytes meridionalis* venom. In 2003, Georgieva and collaborators isolated the neurotoxic complex from *V. a. meridionalis* venom, dissociated the basic PLA₂ from the complex and crystallized it with elaidoylamide. This final structure contained two identical homodimers and one molecule of elaidoylamide bound simultaneously to the substrate-binding sites of each homodimer (Georgieva et al., 2004).

Villar et al. (2008) demonstrated that synthetic inhibitor derivatives from nitrostyrene that contain typical nitro groups at the *ortho*, *meta* and *para* positions on the aromatic ring were more efficient against the enzymatic, edematogenic, and myotoxic activities of PLA₂s from *B. jararacussu* venom. da Silva et al. (2009), performing molecular modeling studies between Asp49-PLA₂ from *C. adamanteus* venom and synthetic derivatives polyhydroxy phenolic compounds, concluded that some conformations of these groups might positively influence enzymatic activity inhibition.

Isolated inhibitors (natural or synthetic) can be important tools for understanding the mechanisms of action of PLA₂s from snake venoms and, consequently, these results might be helpful for the design of a drug that specifically inhibits PLA₂s. However, the

synthesis of compounds analogous to their natural equivalents, based on chemical characteristics or with minor structural modifications, is often necessary. The synthesis of compounds could be justified by the low amount of these compounds available in vegetal extracts or to adjust some specific chemical characteristics. For this reason, some researchers have isolated and characterized new compounds or produced synthetic analogues for use in the commercial production of pharmaceutical drugs.

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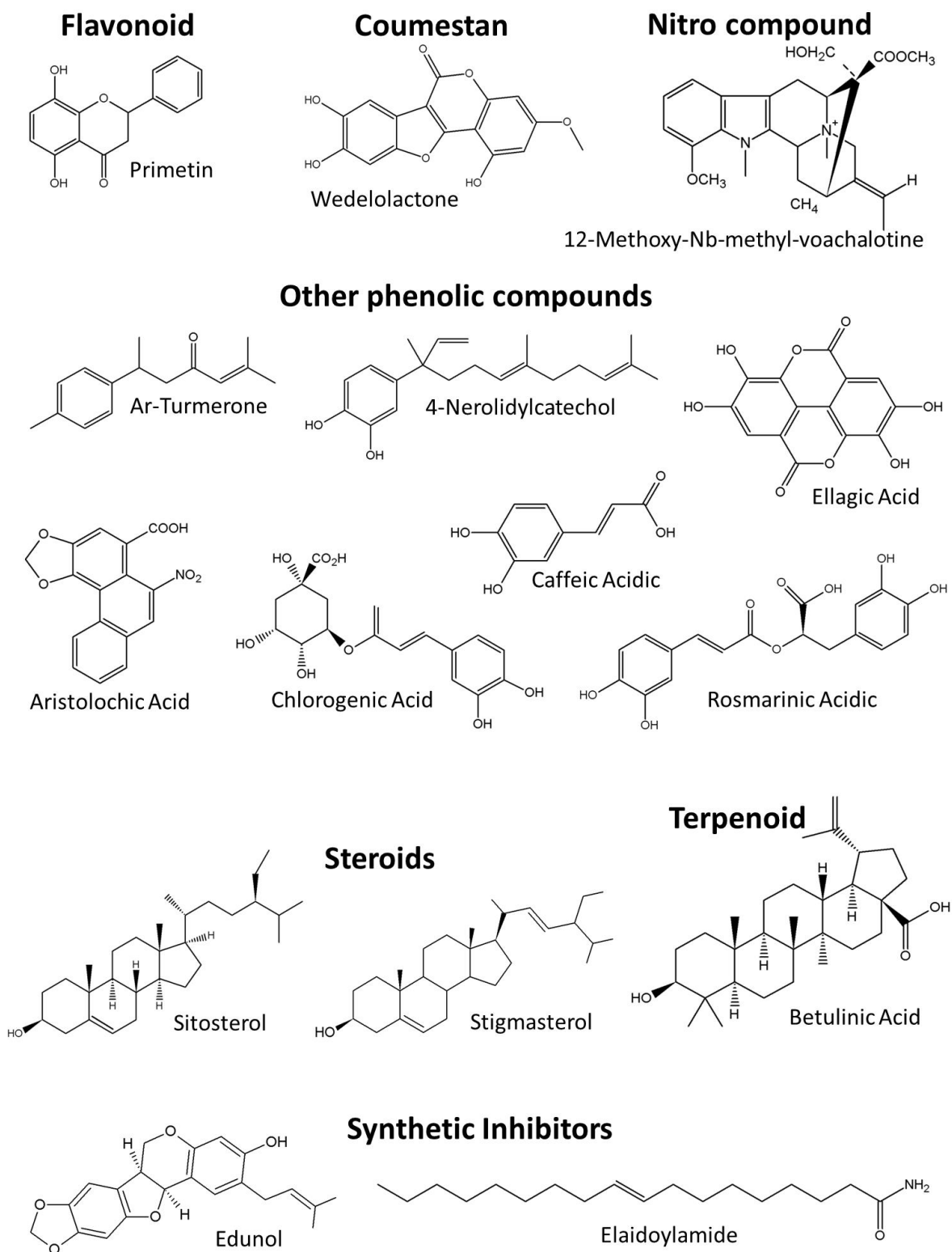
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Figure List:

Figure 1



Legend List

Figure 1: Structures of bioactive compounds with inhibitory potential against the snake venom or its phospholipase A₂ fraction. Draw using ACD/ChemSketch program (www.acdlabs.com).

APÊNDICE II

“Neutralization of B-epitopes of the BthTX-I, BthTX-II and BthA-I, phospholipases A₂ from *Bothrops jararacussu* snake venom, recognized by anti-bothropic and anti-crotalic horse sera”

Neutralization of B-epitopes of BthTX-I, BthTX-II and BthA-I, phospholipases A₂ from *Bothrops jararacussu* snake venom, recognized by anti-bothropic and anti-crotalic horse sera

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Highlights

A mixture of therapeutic antivenom sera recognized six distinct epitopes within snake venom PLA₂s through IgG binding.

Anti-bothropic horse serum exclusively recognized two epitopes in BthTX-1.

Anti-crotalic horse serum recognized two epitopes in BthA-I, one in BthTX-I and one in BthTX-II.

ABSTRACT

The benefits from treatment with antivenom sera are indubitable. However, the mechanism for toxin neutralization has not been completely elucidated. A mixture of anti-bothropic and anti-crotalic horse sera has been reported to be more effective in neutralizing the effects of *Bothrops jararacussu* snake venom than anti-bothropic serum alone. This study determined which regions in the three PLA₂s from *B. jararacussu* snake venom are bound by antibodies in tetravalent anti-bothropic and monovalent anti-crotalic commercial horse sera. Mapping experiments of BthTX-I, BthTX-II and BthA-I using two small libraries of 69 peptides each revealed six major IgG-binding epitopes that were recognized by both anti-bothropic and anti-

crotalic horse sera. Two epitopes in BthTX-I were only recognized by the anti-bothropic horse serum, while anti-crotalic horse serum recognized four unique epitopes across the three PLA₂s. Our studies suggest that the harmful activities of the PLA₂s present in the venom of *B. jararacussu* are neutralized by the combinatorial treatment with both antivenom sera through their complementary binding sites, which provides a wide coverage on the PLA₂s. This is the first peptide microarray of PLA₂s from *B. jararacussu* snake venom to survey the performance of commercial horse antiophidic sera. Regions recognized by the protective antivenom sera are prime candidates for improved venom cocktails or a chimeric protein encoding the multiple epitopes to immunize animals as well as for designing future synthetic vaccines.

Key words: Phospholipase A₂; Peptide array; SPOT-synthesis; Synthetic peptides; B-epitopes; Antigenic determinants.

1. Introduction

Every year, 2.5 million people are bitten by snakes in South America with approximately 100,000 deaths as a result. Administration of specific antivenoms has been the most efficient treatment for snake envenoming. The effectiveness of anti-bothropic horse serum for the neutralization of the toxic and pharmacological effects of *Bothrops jararacussu* venom has been investigated by many groups (dos Santos et al., 1992; de Roodt et al., 1998, 1999; Oshima-Franco et al., 2001; Zamunér et al., 2004; Beghini et al., 2007), yet an understanding of the mechanism has not been elucidated.

Among the bioactive proteins from *Bothrops sp* snake venoms, the phospholipases A₂ (PLA₂s, E.C. 3.1.1.4) are regarded as one of the most important protein classes. PLA₂s are enzymes that catalyze the hydrolysis of 2-acyl ester bonds of 3-sn-phospholipids producing fatty acids and lysophospholipids (Gutiérrez and Lomonte, 1997). In addition to their catalytic role, they show a wide variety of pharmacological activities, such as neurotoxicity, myotoxicity, anti-coagulant and cardiotoxicity (de Paula et al., 2009). An analysis of the *B. jararacussu* venom gland transcriptome showed that 35% of transcripts are PLA₂s with 83% encoding BthTX-I (Bothropstoxin-I, a basic Lys49-PLA₂), 8% BthTX-II (Botropstoxin-II, a basic Asp49-PLA₂) and 9% BthA-I (Acidic Asp49-PLA₂) (Kashima et al., 2004).

A large number of PLA₂s have been purified, characterized and several three dimensional structures have been solved for PLA₂s from the *Bothrops* genus (revised by Stábéli et al., 2012). Correlative studies have been performed with the predicted structures to understand the sites of pharmacological activity (Soares et al., 2001; Lomonte et al., 2003; Soares and Giglio, 2003; Murakami et al., 2007; Montecucco et al., 2008; Teixeira et al., 2011). The PLA₂s as a family are intriguing because despite the significant sequence and structural similarities between members, they present a diverse spectrum of activities, which may or may not be related to their primary catalytic activity (Higuchi et al., 2007; Tsai et al., 2007). This diverse pharmacological profile is suggested to have been acquired through evolution by a positive darwinian selection in the protein-coding exons by an accelerated evolutionary process that has resulted in many variants with diverse pharmacological effects (Ohno et al., 1998).

The superfamily of PLA₂ can be divided into five principal groups of enzymes: the secreted PLA₂s (sPLA₂s), the cytosolic PLA₂s (cPLA₂s), the Ca²⁺-independent PLA₂s (iPLA₂s), the platelet-activating factor acetylhydrolases (PAF-AH) and the lysosomal PLA₂s (Schaloske and Dennis 2006). The sPLA₂s are further subdivided into seventeen classifications according to their molecular mass and the number of disulfide bonds (Schaloske and Dennis 2006). The snake (Viperidae) venom PLA₂s (svPLA₂s) are classified as GIIA, which contain seven disulfide bonds and have a molecular mass around 13-15 kDa (Schaloske and Dennis 2006). The GIIA svPLA₂s can be further subdivided into two main types according to the amino acid residue in the 49th position: Asp49-PLA₂ and Lys49-PLA₂ (Arni et al., 1995). The Asp49-PLA₂s are the enzymes responsible for cellular membrane disruption through Ca²⁺-dependent hydrolysis of phospholipids with myotoxicity activity or not. The Lys49-PLA₂s do not display catalytic activity, but can exert a pronounced and localized myotoxic effect that is not neutralized by serum therapy (Howard et al., 1980; Chang, 1985).

In 1911, Brazil demonstrated that the action of the *B. jararacussu* venom could not be efficiently neutralized with a specific antivenom or anti-bothropic horse serum alone. For the best treatment of the bite of this snake, it was suggested that therapeutic should be associated with anti-crotalic horse serum. Later, experiments were conducted to confirm that the

administration of both the anti-bothropic and anti-crotalic horse sera provided a more effective neutralization for the myotoxic, coagulant and/or lethal activities than one sera used alone (de Roodt et al, 1999; Queiroz et al., 2008). This was not restricted only with bothropic-crotalic antivenom since it was recently observed for venom from Australian snake species (O'Leary and Isbister 2009).

Other immunochemical studies using rabbit antibodies against a synthetic peptide (residues 1-15) of BthTX-I (Angulo et al., 2001) and an anti-NN-XI_a-PLA₂ from *Naja naja* venom (Basavarajappa et al, 1993) showed that the enzymatic activity of these PLA_{2s} was inhibited in a dose-dependent manner by antibodies. However, the lethal and neurotoxic symptoms were not neutralized in experimental animals (Basavarajappa et al., 1993). Further studies have demonstrated cross-reactivity between BthTX-I and the crotoxin of *Crotalus durissus cascavella*, but the common and specific antigenic determinants were not identified (Oshima-Franco et al, 2001; Beghini et al., 2007).

Overall, the mechanisms associated with the capacity to neutralize myotoxic and anti-coagulant activities of snake venoms remain unknown along with the observed protective synergic effects of combining therapeutic sera. In this study, we report the identification and structural characterization of the linear B-cell epitopes of the three PLA_{2s} from *B. jararacussu* snake venom recognized by neutralizing anti-bothropic and anti-crotalic commercial horse sera. The results suggest that the best performance of the monovalent anti-crotalic serum to neutralize *B. jararacussu* PLA_{2s} may be due to the recognition of different epitopes rather than cross-reactivity or other factors such as the affinity of the antibodies. Our observations reinforce the importance of defining the mechanisms leading to the neutralization of the highly toxic proteins in venom by commercial antivenom to drive production of more protective treatments.

2. Material and methods

2.1. Material

Amino acids for peptide synthesis were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Super SignalR West Pico chemiluminescent substrate was from Pierce

Biotechnology (Rockford, IL, USA). Amino-PEG₅₀₀-UC540 cellulose membranes were obtained from Intavis Bioanalytical Instruments (Koeln, Germany). Pyperidine, acetonitrile and trifluoroacetic acid were from Fluke. A peroxidase-labeled rabbit anti-horse immunoglobulin serum was from KPL (Gaithersburg, MD, USA). Bovine serum albumin, 3,3',5,5'-tetramethylbenzidine (TMB) and Tween 20 were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Amicon centricon 10 filters were from Millipore (Billerica, MD, USA). CDP-Star® Substrate was from Applied Biosystems (Foster City, CA, USA). Sequence reagents and all other reagents and chemicals were from Calbiochem-Merck (Darmstadt, Germany).

2.2. Anti-bothropic and anti-crotalic horse sera

Tetravalent anti-bothropic (*B. jararacussu*, *B. jararaca*, *B. neuwiedi* and *B. alternatus*) and monovalent anti-crotalic (*C. d. terrificus*) horse sera were produced and kindly provided by the Vital Brazil Institute, Niteroi, RJ, Brazil.

2.3. Synthesis of the cellulose-membrane-bound peptide array

Two libraries of sixty-nine, 14-mer peptides were designed to represent a consecutive overlapping coverage that was offset by nine amino acids across the entire coding region (121-122 amino acids) of the three PLA_{2s} present in the venom of *B. jararacussu*. Sequences were obtained from the UniProtKB – Protein knowledgebase (<http://www.uniprot.org/>): BthTX-I (Swiss-Prot ID.: Q90249), BthTX-II (Swiss-Prot ID.: P45881) and BthA-I (Swiss-Prot ID.: Q8AXY1). The peptides were automatically prepared onto Amino-PEG₅₀₀-UC540 cellulose membranes according to standard SPOT synthesis protocols (Frank, 2002) using an Auto-Spot Robot ASP-222 (Intavis Bioanalytical Instruments AG, Köln, Germany). In brief, coupling reactions were followed by acetylation with acetic anhydride (4%, v/v) in *N,N*-dimethylformamide to render peptides unreactive during the subsequent steps. After acetylation, Fmoc protective groups were removed by the addition of piperidine to render nascent peptides reactive. The remaining amino acids were added by this same process of coupling, blocking and deprotection until the expected desired peptide was generated. After the addition of the last amino acid in the peptide, the amino acid side chains were deprotected using a solution of dichloromethane–trifluoroacetic acid–triisobutylsilane (1:1:0.05, v/v/v) and

washed with methanol. Membranes containing the synthetic peptides were either probed immediately or stored at -20 °C until needed. Negative controls [without peptide; IHLVNNESSSEVIVHK (*Clostridium tetani*) precursor peptide] and positive controls were included in each assay.

2.4. Screening of SPOT membranes

SPOT membranes were washed with TBS (50 mM Tris-buffer saline, pH 7.0) and blocked with TBS-CT (50 mM Tris-buffer saline, 3% casein, 0.1% Tween 20, pH 7.0) at room temperature under agitation or overnight at 4° C. After extensive washing with TBS-T (50 mM Tris-buffer saline, 0.1% Tween 20, pH 7.0), two membranes presenting the same peptide library were incubated separately for two hours with either horse anti-crotalic or anti-bothropic sera (1:250) in TBS-CT and then washed again with TBS-T. Afterwards, the membranes were incubated with alkaline phosphatase-labeled sheep anti-horse IgG (1:5000 in TBS-CT) for one hour, and then washed with TBS-T and CBS (50 mM citrate-buffer saline, pH 7.0). Chemiluminescent CDP-Star® Substrate (0.25 mM) with Nitro-Block-II™ Enhancer (Applied Biosystems, USA) was added to complete the reaction.

2.5. Scanning and measurement of spot signal intensities

Chemiluminescent signals were detected on MF-ChemiBis 3.2 (DNR Bio-Imaging Systems, Israel) at a resolution of 5 MP. The digital image file was analyzed with TotalLab Software (Nonlinear Dynamics, USA) to quantify signal intensities and to define the empirical probability that the intensity from a spot was distinct from that of the background signal using algorithms that compared the intensities between background, spot area and negative controls.

2.6. Sequence analysis

Fifty PLA₂s isolated from the venom of snakes from *Bothrops*, *Crotalus* and *Lachesis* genera were selected and their amino acid sequences were aligned, compared and analyzed. The sequences were obtained from the UniProt Knowledgebase (<http://www.uniprot.org/>). The

sequences were clustered using two criteria: physical-chemical property (acidic or basic) and the amino acid residues at position 49 (lysine or aspartic acid). The theoretical isoelectric point (pI) of all the selected sequences was calculated according to the amino acids sequence with the ProtParam tool (Gasteiger et al., 2005) from the ExPASy Proteomics Server (<http://www.expasy.ch/tools/protparam.html>). The multiple sequence alignment (MSA) of the selected sequences was generated within the web server T-Coffee (Notredame et al., 2000), using the program default parameters. Manual improvements were made to adjust the alignment performed by T-Coffee with the numbering system proposed by Renetseder et al. (1985).

2.7. Hydropathy

A hydropathy plot with a window size of nine was used to span the epitopes throughout the hydrophobicity of the PLA₂s over the length of the peptide sequence (Kyte and Doolittle 1982).

3. Results

3.1. Identification and mapping of linear epitopes using synthetic peptides

The epitopes recognized by therapeutic horse antivenom sera in the three major PLA₂s present in the venom of *B. jararacussu*, BthTX-I, BthTX-II and BthA-I, were mapped using the parallel Spot-synthesis strategy. Two peptide libraries were designed to more precisely define the epitopes recognized by anti-bothropic and/or anti-crotalic horse serum. Each consisted of 69 peptide sequences of fourteen amino acids each that overlapped by nine amino acids and covered the entire protein sequences of the three PLA₂s. A representative experiment, which shows results identical to three independent assays, is presented in Fig. 1.

The analysis of spot signal intensity for the synthesized peptides from the three PLA₂s sequences in cross-reactivity with the anti-crotalic and anti-bothropic horse sera showed a total of 12 epitopes. Two of the epitopes were specifically recognized by the anti-bothropic horse serum, while four epitopes were restricted to the activity of the anti-crotalic horse serum. The

other six epitopes interacted with antibodies in both antivenom sera, however there were differences in the signal intensities.

The two immunodominant antigenic determinants present in the BthTX-I (Cys84–Asn89 and Lys116–Asp130) were recognized exclusively by the anti-bothropic horse serum, while one (Gln11–Lys20) was bound by the anti-crotalic horse serum and two others (Cys27–Gly30 and Gly59–Tyr73) by both horse antivenom sera. On the other hand, four major IgG-binding epitopes were recognized in BthTX-II: three (Leu17–Tyr25, Pro37–Cys45 and Gly80–Thr89) by both antivenom sera and one (Thr70–Glu78) solely by the anti-crotalic horse serum. In the BthA-I, two epitopes (Tyr52–Tyr73 and Phe106–Phe119) were recognized specifically by the anti-crotalic horse serum and one (Ser17–Tyr25) by both of sera (Table 1).

Overall, each of the epitopes displayed a relatively strong reactivity (containing 4-14 amino acids extension). However, the strongest intensity was observed with the antigenic determinant Thr70–Glu78, from the basic Asp49-PLA₂ (BthTX-II) either with the anti-bothropic and anti-crotalic horse sera (Fig. 1A and Fig. 1B, spots B12 and B11, respectively). Other important epitopes were the Cys84–Asn89 and Lys116–Asp130 from BThTX-I and Leu17–Tyr 25 and Pro37–Cys45 from BThTX-II – Fig. 1C shows the list of synthesized peptides. Fig. 1A and 1B present the immunological assay and the signal intensity of reactivity for each peptide with anti-bothropic and anti-crotalic horse sera, respectively.

3.2. Spatial location of the most reactive heavy chain hIg-epitopes

The oligomeric structure of BthTX-I, BthTX-II and BthA-I proteins were solved by X-ray crystallography and are available in the protein data bank (<http://www.pdb.org>) under the PDB accession numbers: 3I3I (Fernandes et al., 2010), 2OQD (Correa et al., 2008) and 1U73 (Magro et al., 2004), respectively. Fig. 2 displays the spatial localization of the epitopes identified by the SPOT-synthesis array experiments. Two of the BthTX-II epitopes (Thy70–Glu78 and Gly80–Thr89) were localized in a β -wing region, while all of other linear epitopes were located in coil/loop structures in the PLA₂s protein structures. The hydropathy plots of the three proteins, shown in the Fig. 3, also suggested that all of the epitopes were present on the surface of the proteins.

3.3. Sequence analysis

The sequences of fifty PLA₂s were selected and grouped into three sub-groups: *a.* Lys49-PLA₂ (fourteen from the *Bothrops* genus and one from the *Crotalus* genus); *b.* basic Asp49-PLA₂ (seven from the *Bothrops* genus and ten from the *Crotalus* genus); *c.* acidic Asp49-PLA₂ (eight from the *Bothrops* genus, eight from the *Crotalus* genus and two from the *Lachesis* genus) (Fig. 4). Individual identifiers, accession numbers and theoretical isoelectric points (*pI*) of the PLA₂s sequences are presented in Table 2.

Shared amino acids sequence from the 12 epitopes recognized by the reaction between the *B. jararacussu* PLA₂s and anti-crotalic/anti-bothropic horse sera were analyzed by a multiple sequence alignment between the fifty PLA₂s selected sequences.

Two antigenic determinants present in the Lys49-PLA₂s, which reacted positive only for the anti-bothropic horse serum, were identified as Cys84–Asn89 and Lys116–Asp130. The ⁸⁴CGENN⁸⁹ epitope of BthTX-I was identified in the three-dimensional structure within a β -wing region (Fernandes et al., 2010), which was considered to have an acidic characteristic (theoretical *pI* = 4.0). The Glu86, Asn88 and Asn89 residues can be important for the specific interaction with the protein and the anti-bothropic horse serum, since the replacement of Asn88→Gly and Asn89→Thr observed in BthTX-II allows the recognition of this region by both antivenom sera. The ¹¹⁶KYRYHLKPFCKKAD¹³⁰ epitope was situated in the C-terminal region (Fernandes et al., 2010) which, in *Bothrops* genus proteins, is considered responsible for the myotoxic activity observed in Lys49-PLA₂s (Chioato et al., 2002). The Lys116–Asp130 epitope has a basic characteristic (theoretical *pI* = 9.75) that was rich in positively charged amino acids and differed from most of the acidic Asp49-PLA₂s, which presented theoretical *pI*'s of approximately 4.0. This positively charged region can be exerted a strong influence in the interaction with the anti-bothropic horse serum and the BthTX-I.

Four epitopes were specifically recognized by the anti-crotalic horse serum: Gln11–Lys20 (BthTX-I), Thy70–Glu78 (BthTX-II), Tyr52–Tyr73, and Phe106–Phe119 (BthA-I) (Fig. 4). In BthTX-I the ¹¹QETGKNPAK²⁰ region in the three dimensional model, was present in a transition region corresponding to the end of alpha helix I followed by the Ca²⁺-binding loop (Fernandes et al., 2010). This epitope showed a basic characteristic (theoretical *pI* equal

to 8.59). The comparative analysis of snake venom PLA₂s amino acid sequences showed that the glutamine in position 11 was conserved in all of the Lys49-PLA₂s from the *Bothrops* genus and this residue may be responsible for the interaction between this epitope and the anti-crotalic horse serum, since this is the only change observed when compared with the same region of BthTX-II which is not recognized by this serum. The proline in position 18 was present in almost all Lys49-PLA₂s or was replaced by alanine, serine or glycine in basic PLA₂s. In BthTX-II, the acidic ⁷⁰TDRYSYSRE⁷⁸ (theoretical pI = 5.73) epitope was three-dimensionally located in the β-wing region (Correa et al., 2008) and the comparative analysis showed that the ⁷⁷RE⁷⁸→⁷⁷WK⁷⁸ replacement observed in Lys49-PLA₂s does not allow the interaction between this same region and the anti-crotalic horse serum. In BthA-I, the ⁵²YGKVTGCDPKIDSY⁷³ epitope (theoretical pI = 8.14) was located, in three dimensional model, between the final alpha helix II and the beginning of the β-wing (Magro et al., 2004). The Val55 is conserved in acidic PLA₂s from *Bothrops* genus and, when it is replaced to leucine or methionine (basic PLA₂s), the interaction of this region with the anti-crotalic horse serum is not observed. The other BthA-I epitope Phe106–Phe119 had an acidic characteristic (theoretical pI = 6.04) and, in the three dimensional model, it was located in the C-terminal loop of this protein (Magro et al., 2004). The comparative sequence analysis for the residues 106-119 from BthA-I and others PLA₂s highlighted the following amino acids: Phe106 (absent in basic PLA₂s from *Crotalus* genus), Lys110 (present only in acidic Asp49-PLA₂s from *Bothrops* genus and Bmoj2), Asp114 (present only in acidic Asp49-PLA₂s from *Bothrops* genus) and Trp118 (exclusive to the acidic Asp49-PLA₂s from *Bothrops* and *Lachesis* genera and present in most of acidic PLA₂s from *Crotalus* genus). All of these amino acids may be essential for the recognition of this region exclusively by anti-crotalic horse serum.

Six other epitopes were recognized by both antivenom sera: Cys27–Gly30 and Gly59–Tyr73 from BthTX-I; Leu17–Tyr25, Pro37–Cys45 and Gly80–Thr89 from BthTX-II; and Ser17–Tyr25 from BthA-I. The ²⁷CNCG³⁰ region represented the Ca²⁺-binding loop in the three dimensional structure of BthTX-I (Fernandes et al., 2010). The acidic Cys27–Gly30 epitope (theoretical pI = 5.51) was a conserved region in Lys49-PLA₂s that was recognized by both antivenom sera and presented a single change that differentiated it from Asp49-PLA₂s. The Asn28 was conserved in Lys49-PLA₂s, but this position in the Asp49-PLA₂s was occupied exclusively by tyrosine and this amino acid residue can be responsible by the

interaction with both of antivenom sera, once that the Asn28→Tyr replacement in Asp49-PLA₂s not allows the interaction with any antivenom sera. The other epitope from BthTX-I recognized by both of antivenom sera is the ⁵⁹GCDPKKDRY⁷³ (theoretical pI = 8.18) region located nearly from the β-wing (Fernandes et al., 2010). The preceding region of the β-wing (⁷⁰KDRY⁷³) in BthTX-I interacted with both of antivenom sera. This same region in BthTX-II (⁷⁰TDRY⁷³) and BthA-I (⁷⁰IDSY⁷³) interacted only with the anti-crotalic horse serum. In BthTX-I, the lysine at position 70 could be crucial due to its positive charge for the interaction of this sequence with both of the antivenom sera. Furthermore, this amino acid was present in the Lys49-PLA₂s from *Bothrops* genus with the exception of the sequences Bnuf1, Bgod1 and Bgod2. Moreover, the comparative analysis with the selected PLA₂s shows that the Gly59 and Asp67 can be important amino acids residues to interacted with the antivenom sera because when the replacements Gly59→Asn and Asp67→Lys occurred in BthTX-I, this interaction is not observed. The epitopes Leu17–Tyr25 (BthTX-II – theoretical pI = 5.52) and Ser17–Tyr25 (BthA-I – theoretical pI = 5.24) represented the same position in both of the Asp49-PLA₂s and it was located nearly from the Ca²⁺-binding loop, important catalytic region from PLA₂s. Although, other two epitopes from BthTX-II located in the final of Ca²⁺-binding loop (³⁷PKDATDRCC⁴⁵) and in the β-wing (⁸⁰GVIICGEGT⁸⁹) displayed acidic characteristic, theoretical pI equal to 5.95 and 4.0, respectively.

4. Discussion

The therapeutic action of antivenom serum is based on neutralizing the normal, detrimental activity of enzymes present in venom. Neutralization most likely occurs by the formation of complexes between antibodies in the antivenom and their corresponding target antigens in the venom. This complex formation can lead to structural changes that prevent the normal function of the native antigen. It also is possible that antibody binding could cause steric hinderance that interferes with the interaction of the antigen with cellular targets. In either case, indentification of the epitopes bound by antivenom serum antibodies will improve treatment protocols.

In the case of *B. jararacussu* snake venom, the most effective treatment involves the administration of a combination of anti-bothropic and anti-crotalic sera to neutralize the

myotoxic, coagulant and lethal activities of the venom than when one of these antivenom sera is used alone (dos Santos et al., 1992; de Roodt et al., 1998, 1999). It is evident that each of the individual antivenoms delivers antibodies that are necessary for neutralizing the effect of the venom. Considering the proteins present in venom, the PLA₂s are the principle enzymes responsible for the harmful effects. Since the performances of the individual antivenom sera are not well understood, we focused on determining the antigenic determinants present in the PLA₂s proteins from *B. jararacussu* venom that are bound by antibodies present in the individual anti-bothropic and anti-crotalic horse sera.

The mapping experiments presented in Fig. 1 showed the immunogenicity of the array of peptides that was synthesized to represent the three PLA₂s from *B. jararacussu* snake venom. Two antigenic determinants were recognized by the anti-bothropic horse serum, four antigenic determinants by the anti-crotalic horse serum and six peptides were recognized by both antivenom sera (Table 1). While cross reactivity has been described for distinct proteins from snake venoms (de Roodt et al., 1998; Oshima-Franco et al., 2001; Beghini et al., 2007), which may reflect genetic relationship within proteins of the same family in various species and/or repetitive segments in distinct toxins, the use of spot synthesis peptide array employed here provided more detail of the common and unique epitopes bound by the two commercial horse antivenom sera. The advantages of this micro-immunoassay employing cellulose immobilized peptides over other different assays as classical ELISA for screening of antigenic peptide-arrays has been extensively discussed (Copeland et al., 2004; Henderson and Bradley 2007). In our assays it was employed a cellulose membrane derivatized with amino-PEG₅₀₀ to attach the amino acids. The advantage of this link over that using beta-alanine is the neglected background generated.

The Lys49-PLA₂s are proteins that exhibit various toxic effects including oedema, membrane depolarization (Kihara et al., 1992) and myonecrotic activity (Montecuso et al., 2008). The myotoxic activity is an important and severe behavior displayed by Lys49-PLA₂s, which was associated with the significant number of positively charged residues located in the N-terminal region (Arni et al., 1995). The results from the peptide array demonstrate that the amino acid sequence for one of the epitopes recognized by anti-crotalic horse serum was from the sequence ¹¹QETGKNPAK²⁰, which encompasses this N-terminal region (Table 1). A comparative analysis of this epitope with the selected snake venoms sequences indicated that

these residues are conserved in Lys49-PLA₂s and may exert strong influence on the myotoxic action exhibited by this family of proteins (Selistre-de-Araujo et al., 1996). Angulo and collaborators (2001) showed that rabbit antibodies obtained against the N-terminal peptide ¹SLFELGKMILQETGK¹⁵ from BthTX-I was able to block the myotoxic activity of the toxin. This suggests that the neutralization of the myonecrotic action caused by Lys49-PLA₂s could occur by the interaction with the anti-crotalic horse serum with this specific region, which is present only in BthTX-I. Furthermore, the three dimensional molecular model (Fig. 2) placed this epitope between the alpha-helix I and the beginning of the Ca²⁺-binding loop suggesting a possible molecular mechanism for the action of binding of an antibody.

Experiments that included site-directed mutagenesis (Ward et al., 2002, Chioato et al., 2007) and synthetic peptide immunogenicity (Lomonte et al., 2010) suggested that the C-terminal region of Lys49-PLA₂s acts as a heparin-binding site (Lomonte et al., 1994) and as a domain for myotoxic activity (Calderón and Lomonte., 1998). Our results showed that the C-terminal of BthTX-I contains the epitope ¹¹⁶KYRYHLKPFCKKAD¹³⁰, which was specifically recognized by anti-bothropic horse serum. The myotoxic activity have been attributed to this segment however it contributes several positively charged residues, a critical fact that may determine the specific neutralization of this important region by the anti-bothropic horse serum.

Kini and Iwanaga (1986) suggested that residues between the positions 83-95 were involved in the myotoxic pre-synaptic action and neurotoxicity of PLA₂s and in our studies, the epitope ⁸⁴CGENN⁸⁹ were neutralized specifically by the anti-bothropic horse serum. This specificity may be related with the physical chemical characteristics of the amino acid residues that constitute this sequence, especially the conserved Glu86. The Glu86 is conserved in basic PLA₂s from *Bothrops* genus along with the asparagine dyad (Asn88/89) can be observed only in Lys49-PLA₂s. However, in acidic Asp49-PLA₂s, the Glu86 was substituted by the amino acid residues glycine or aspartic acid. In basic Asp49-PLA₂s from *Crotalus* genus, this residue was replaced by an oppositely charged amino acid (Lys86) (Fig. 4). The replacement of charged residues by a glycine at position 86 in the acidic Asp49-PLA₂s from *Bothrops* genus is probably responsible for the absence of interaction between these regions in BthA-I with either antivenom sera studied.

Moreover, the ⁸⁰GVIICGEGT⁸⁹ region from BthTX-II interacted with both antivenom sera suggesting that the hydrophilic dyad composed by Asn88 and Asn89, present in BthTX-I, mediated the interactions only with antibodies present within anti-bothropic horse serum. However, the amino acid sequence analysis suggested that the residues Glu86, Asn88 and Asn89 are critical for the neutralizing of the myotoxic activity carried on Lys49-PLA₂s by interaction with the anti-bothropic horse serum.

The ²⁷CYCG³⁰ region is conserved within the Asp49-PLA₂s and in the three dimensional model corresponded to a Ca²⁺-binding loop that coordinates the Ca²⁺ ion, an essential cofactor to the catalytic action of PLA₂s (Selistre-de-Araujo et al., 1996). The Ca²⁺-binding domain was not present in Lys49-PLA₂s due to a substitution of the tyrosine residue at position 28 by asparagine. This specific adjustment caused a conformational change in the Ca²⁺-binding loop and, consequently, a loss of the catalytic activity of PLA₂s (Kaiser et al., 1990). As indicated by the results of the spot synthesis experiments, both of the antivenom sera interacted with the epitope ²⁷CNCG³⁰ from BthTX-I. It can be suggested that the presence of an aromatic amino acid at position 28 prevented the interaction of the Asp49-PLA₂s with the antivenom sera analyzed.

The BthA-I presents a highly catalytic, platelet aggregation inhibition, oedema induction, hemolytic and hypotensive activities (Fully et al., 2004). However, it is not myotoxic, cytotoxic or lethal (Magro et al., 2004). It was proposed that the lysine at position 69 and the glycine or glutamic acidic at position 53 are essential for the anticoagulant effect displayed by this acidic Asp49-PLA₂ (Carredano et al., 1998). In addition, it appears that the key regions related to the pharmacological effects of this acidic Asp49-PLA₂ is in the C-terminal loop, the region ¹⁷SGVLQYL²³ (between alpha helix I and Ca²⁺-binding loop) and the lysine at position 69 (Magro et al., 2005). Our results showed that two regions of BthA-I was specifically bound by anti-crotalic horse serum (⁵²YGKVTGCDPKIDSY⁷³ and ¹⁰⁶FRNDKDTYDIKYWF¹¹⁹) and only one region (¹⁷SGVLQYALSY²⁵) reacted with both antivenom sera. Thus our results indicated that the major pharmacological activities of BthA-I are most likely neutralized by the anti-crotalic horse serum more than by the anti-bothropic horse serum, but that the association of both sera could better inhibit the pharmacological activity of this toxin.

The comparative analysis of PLA₂s sequences allowed a survey of the glycine residue at position 53. It was highly conserved in Asp49-PLA₂s except for Cdte3 and Bjmo3, which have a glutamic acid at this position. The lysine residues at positions 54 and 69 were conserved in PLA₂s from snake venoms. In addition, we observed that the amino acid residues Phe106, Lys110, Asp114 and Trp118 were conserved in the acidic Asp49-PLA₂s from the *Bothrops* genus. However, the epitopes Tyr52–Tyr73 and Phe106–Phe119 were specifically recognized by anti-crotalic horse serum and not by anti-bothropic horse serum, which suggests that the anticoagulant activity of BthA-I was best neutralized by the anti-crotalic horse serum.

Toxins with similar biological actions usually present structural similarities, which are reflected in their antigenic cross-reactivity and consequent neutralization by heterologous antivenom sera. Only a few reports have shown antigenic cross-reactivity between *B. jararacussu* and *C. durissus ssp* venoms that specifically focused on the PLA₂s from both venoms (de Roodt et al., 1998, 1999; Oshima-Franco et al., 2001; Beghini et al., 2007; Correa-Netto et al., 2010). Lomonte (2012) identified the linear B-epitopes of miotoxin II, a Lys49-PLA₂ from *B. asper* snake venom by PepSetsTM-ELISA assay using rabbit antivenom serum and a therapeutic polyvalent Crotalinae (mixture of *B. asper*, *Crotalus simus* and *Lachesis stenophrys* snakes venoms) horse serum. However, these results are not compatible with those obtained by our studies. This incompatibility can be justified due to different techniques used in these experiments, and should be considered that the preparation of antivenom serum was performed in different animal and by venoms from different snake species, making it impossible to compare with our results. Conversely the linear B-epitopes of Lys49-PLA₂ of *B. asper* snake venom were determined by PepSetsTM-ELISA assay using rabbit sera anti-purified Lys49 and a therapeutic polyvalent Crotalinae (mixture of *B. asper*, *Crotalus simus* and *Lachesis stenophrys* venoms) horse sera (Lomonte, 2012). Therefore, as these experiments were realized using distinct antigenic preparations and sera of a different animal it was difficult compare these results with those obtained in our study.

The results of our antigenic map reinforce the need for the application of multiple antivenom sera whereas only two epitopes were detected specifically by the anti-bothropic horse serum in relation to four epitopes to the anti-crotalic horse serum. Together, it is proposed that; (1) the improved performance of the application of both antivenom sera is a

result of synergism due to specificity rather than shared antigenic determinants, (2) the therapeutic contributions of the anti-crotalic horse serum can be linked to the interaction of its antibodies to important regions of BthTX-II and BthA-I and (3) the anti-bothropic horse serum appears to neutralize the sites of BthTX-I that are proposed to be myotoxic.

The commercial anti-bothropic horse serum produced in Brazil by the Vital Brazil Institute and other institutes are prepared by hyperimmunization of horses with a pool of venoms from *B. jararacussu*, *B. jararaca*, *B. moojeni*, *B. alternatus* and *B. neuwiedi* while the anti-crotalic serum is produced using only *Crotalus durissus terrificus* venom. A major question that remains unresolved is why the immunization of horses with distinct antigenic proteins (*Crotalus sp* proteins x *Bothrops sp* proteins) results in a product that, individually, is deficient to overcome the detrimental effects of a snake bite, but when applied jointly gives a neutralizing response. Is possible that the intraspecific snake venom variations have great implications in the preparation of venom pools for the generation of antivenoms as suggested recently (Gutiérrez et al., 2010). Independent of this fact, additional experiments are needed to determine if some epitopes have a predominant recognition when mixing different *Bothrops sp* snake venoms, i.e. a dilution effect. The identification of the individual epitopes involved in the neutralization of the PLA₂s observed with the commercial antivenom sera provides a new direction for the design of immunization protocols to generate more effective treatments.

5. Conclusions

In conclusion, the peptide arrays formed directly onto cellulose membranes allowed the identification of the major antigenic determinants in the three most important PLA₂s (BthTX-I, BthTX-II and BthA-I) isolated from *B. jararacussu* snake venom recognized by commercial anti-bothropic and anti-crotalic horse sera. The cross-reactive epitopes located in the Lys49-PLA₂, the major protein of this venom, recognized two specific epitopes located in a region of the enzyme responsible for the myotoxic action, which contributes to the deleterious effects of snake venom. In addition, the ability of the anti-crotalic horse serum to neutralize the anticoagulant activity was most likely associated with the acidic Asp49-PLA₂. This study provides proof that the mixture of anti-crotalic and anti-bothropic horse sera is qualitatively more effective in neutralizing the effects unleashed of *B. jararacussu* snakebite.

Conflict of interest

The authors declare no conflict of interest.

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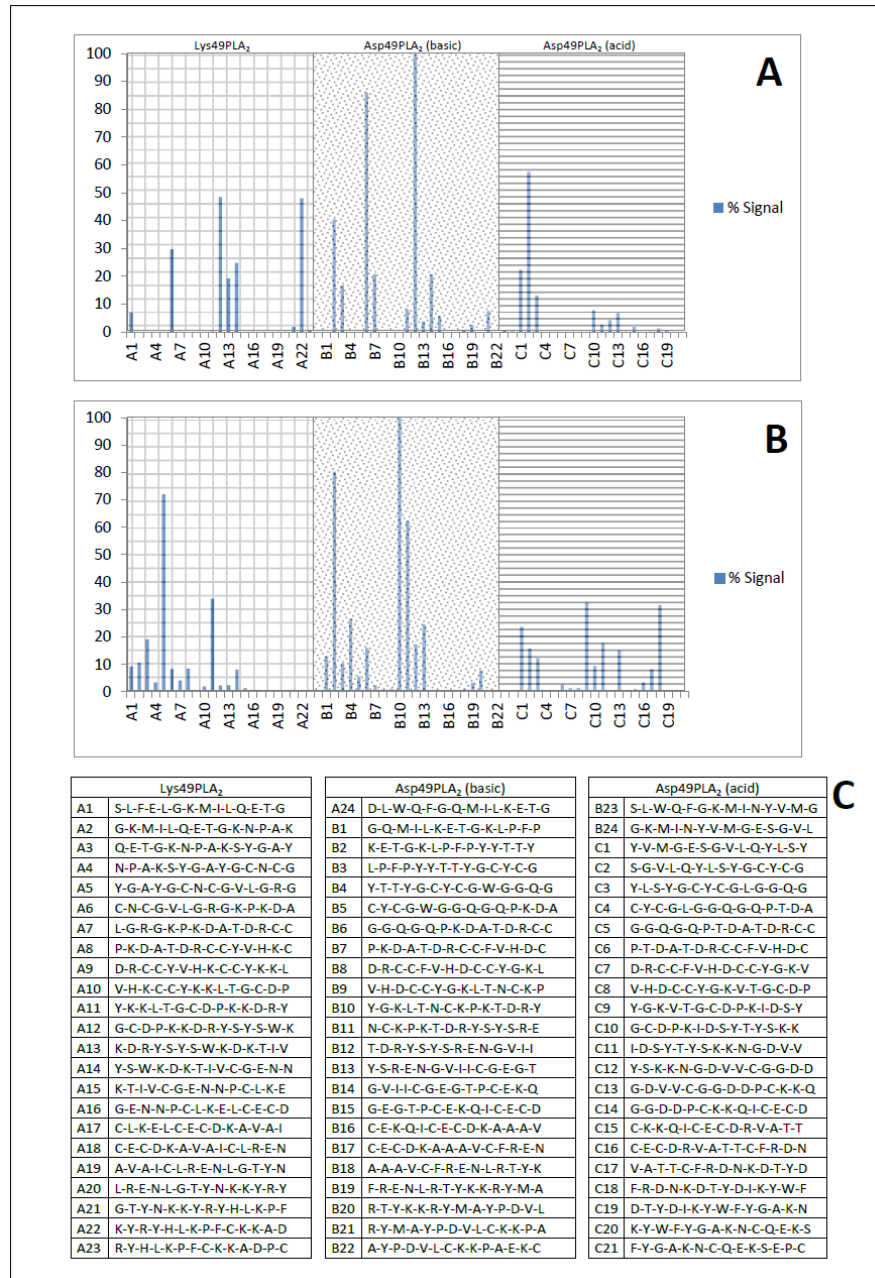
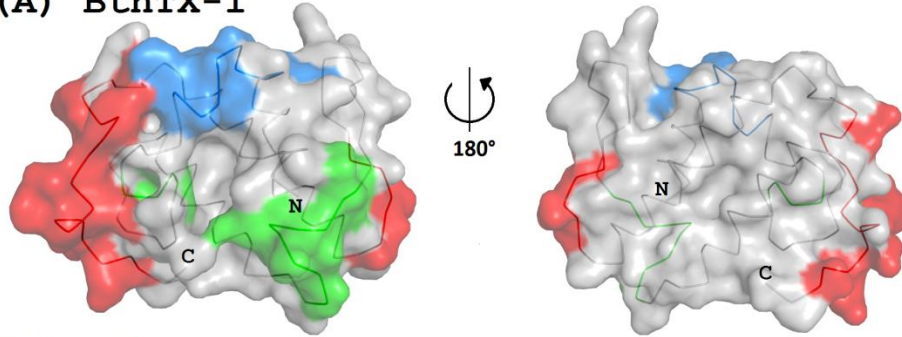


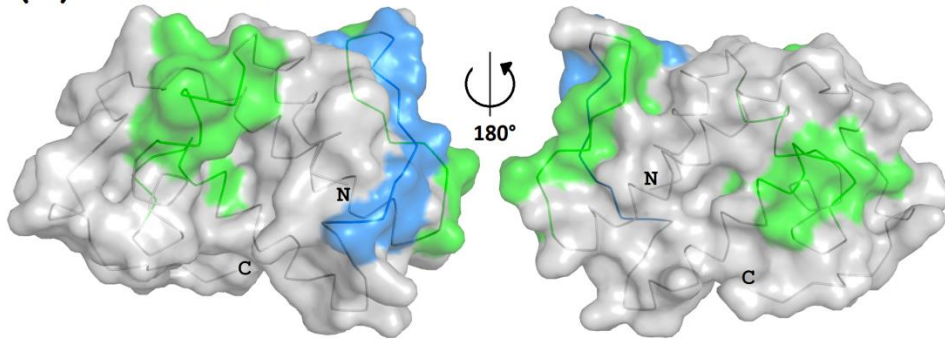
Figure 1: Array interaction of PLA₂s peptide library from *Bothrops jararacussu* snake venom with antibodies in antivenom sera. Anti-bothropic (A) and anti-crotalic (B) horse sera were presented to peptides synthesized on membranes by Spot-synthesis. The peptides, listed in (C) represented the coding regions of BthTX-I (A1-A23), BthTX-II (A24-B22) and BthA-I (B23-C21) PLA₂s. Each peptide was identified by the Spot-synthesis membrane position numbering. Peptide C22 (positive control, not shown) had the highest signal intensity, which was arbitrarily assigned as 100%. C20 and C21 were negative controls. Spot intensities below 20% were considered as background. The 69 overlapping peptides, synthesized here,

encompassed the entire amino acid sequence of the PLA_{2s}, and consisted of 14 residues each with an overlap of 9 residues between peptides.

(A) BthTX-I



(B) BthTX-II



(C) BthA-I

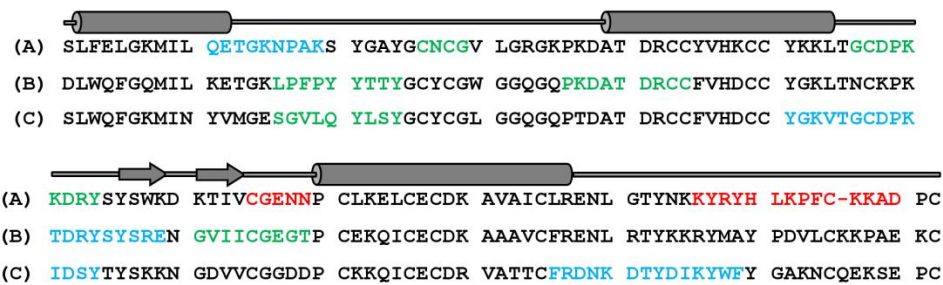
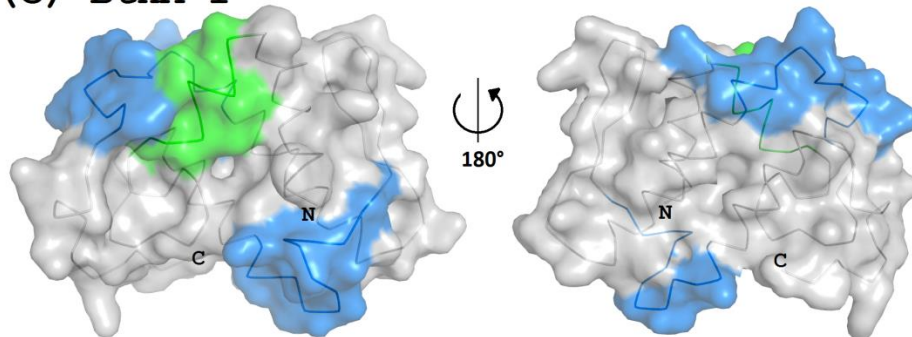


Figure 2: Spatial localization of the specific reactive epitopes in three dimensional structures of BthTX-I, BthTX-II and BthA-I from *Bothrops jararacussu* snake venom. The residues in PLA₂s bound by antibodies are highlighted in red (anti-bothropic horse serum), blue (anti-crotalic horse serum) and green (both sera). The structures of the BthTX-I (PDB ID: 3I3I), BthTX-II (PDB ID: 2OQD) and BthA-I (PDB ID: 1U73) were obtained from protein data bank (<http://www.pdb.org>) and drawn using PyMol (DeLano, 2002).

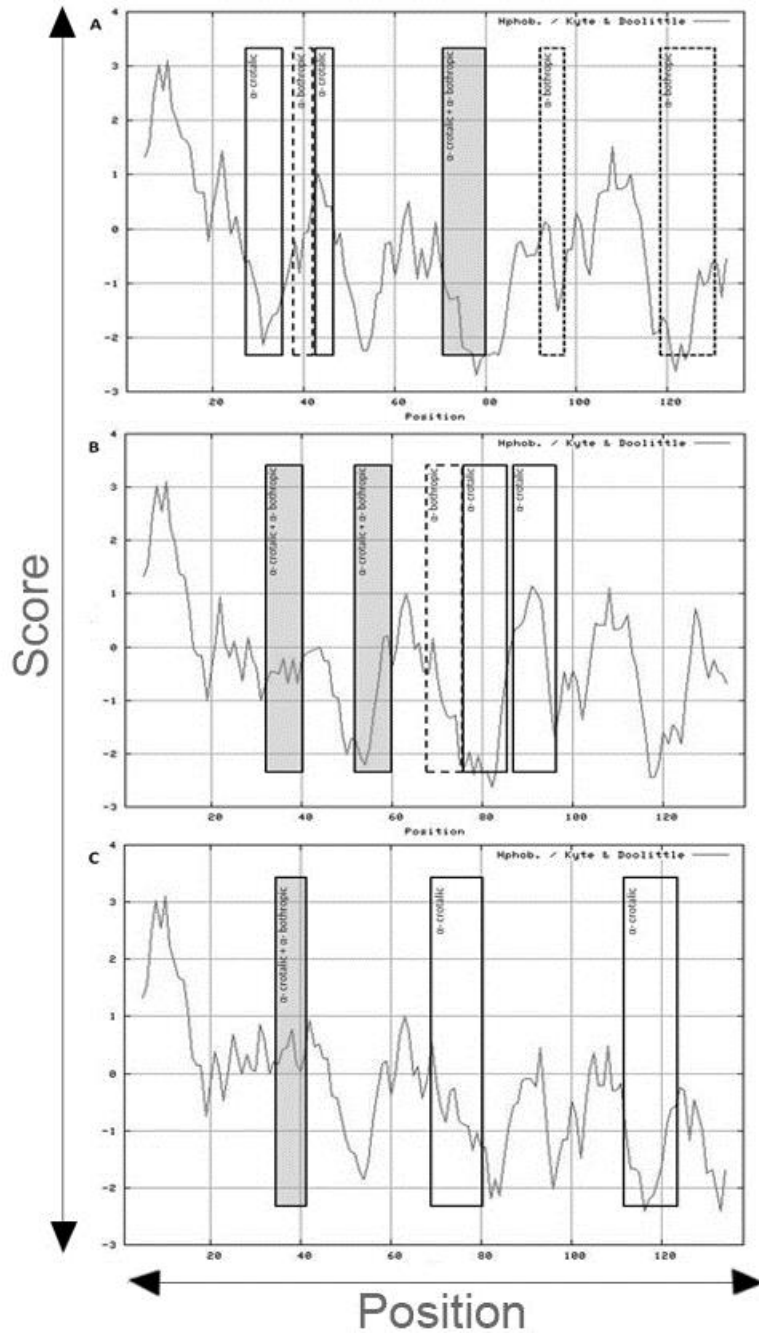


Figure 3: Hydrophobicity plots of the three PLA₂ proteins. Panels A (BthTX-I), B (BthTX-II) and C (BthA-I) were determined with window sizes between 7-11 residues according to Kyte and Doolittle (1982) to maximize the information content for the plots. Gray rectangles mark the epitopes recognized by both antivenom sera. Solid rectangles encompass epitopes recognized exclusively by the anti-crotalic horse serum and the dashed rectangles enclose the epitopes recognized solely by the anti-bothropic horse serum.

	1	11	21	31	41	51	61	71	81	91	101	111	121	131	
Bjsu1	SLFELGKMIL	QETG-KNPAK	SYGAYG-CNCG	VLGRG-KPKDA	TDRCCVHHC	CYKKLT-G	C----	DPKK	DRYS--YSWKDK	TIVCGE-NNP	CLKELCECDK	AVAI CLRNL	GTYNK KYRY-H	LK-PFCKKAD	--PC
Bpir1	SLFELGKMIL	QETG-KNPAK	SYGAYG-CNCG	VLGRG-KPKDA	TDRCCVHHC	CYKKLT-G	C----	NPKK	DRYS--YSWKDK	TIVCGE-NNP	CLKELCECDK	AVAI CLRNL	GTYNK KYRY-H	LK-PFCKKAD	--DC
Bpir2	SLFELGKMIL	QETG-KNPAK	SYGAYG-CNCG	VLGRG-KPKDA	TDRCCVHHC	CYKKLT-G	C----	NPKK	DRYS--YSWKDK	TIVCGE-NNP	CLKELCECDK	AVAI CLRNL	GTYNK KYRY-H	LK-PFCKKAD	--DC
Basp1	SLFELGKMIL	QETG-KNPAK	SYGAYG-CNCG	VLGRG-KPKDA	TDRCCVHHC	CYKKLT-G	C----	NPKK	DRYS--YSWKDK	TIVCGE-NNS	CLKELCECDK	AVAI CLRNL	NTYNK KYRY-Y	LK-PLCHKAD	--AC
Basp2	SLVLEGMIL	QETG-KNPLT	SYGVYG-CNCG	VGGRH-KPKDG	TDRCCVHHC	CYKGMT-D	C----	DPKK	DRYS--YSWKDK	TIVCDE-NNP	CLKELCECDK	AVAI CLRNL	DTYNK KYKI-Y	PK-PFCKKAE	--PC
Basp3	SLVLEGMIL	QETG-KNPVT	SYGAYG-CNCG	VLGRG-KPKDA	TDRCCVHHC	CYKKLT-G	C----	NPKK	DRYS--YSWKDK	TIVCGE-NNS	CLKELCECDK	AVAI CLRNL	DTYNK KYKNNY	LK-PFCKKAD	--PC
Basp4	SLVLEGMIL	QETG-KNPLT	SYGVYG-CNCG	VGGRH-KPKDG	TDRCCVHHC	CYKGMT-D	C----	DPKK	DRYS--YSWKDK	TIVCDE-NNP	CLKELCECDK	AVAI CLRNL	DTYNK KYKI-Y	PK-PFCKKAE	--PC
Bmoj1	SLFELGKMIL	QETG-KNPAK	SYGVYG-CNCG	VGGRG-KPKDA	TDRCCVHHC	CYKKLT-G	C----	DPKK	DRYS--YSWKDK	TIVCGE-NNS	CLKELCECDK	AVAI CLRNL	DTYNK KYRYNY	LK-PFCKKAD	--PC
Bmoj2	SLVLEGMIL	QETG-KNPAK	SYGAYG-CNCG	VLGRG-KPKDA	TDRCCVHHC	CYKKLT-N	C----	DPKK	DRYS--YDWKDK	TIVCGE-ENP	CLKELCECDK	AVAI CLRNL	GTYNK K-RDVK	LK-PFCDKGR	--DC
Bpal1	S-FELGKMIL	QETG-KNPAK	SYGAYG-CNCG	VLGRG-QPKDA	TDRCCVHHC	CYKKLT-G	C----	DPKK	DRYS--YSWKDK	TIVCGE-NNP	CLKELCECDK	AVAI CLRNL	GTYNK KYRY-H	LK-PFCKKAD	--PC
Batx1	SLVLEGMIL	QETG-KNPLT	SYGAYG-CNCG	VGGRG-KPKDA	TDRCCVHHC	CYKGMT-D	C----	DPKK	DRYS--YSWKDK	TIVCGE-KNS	CLKELCECDK	AVAI CLRNL	DTYNK KYKNNY	LK-PFCKKAD	--AC
Bnuf1	NLYQLWKIL	QETG-KNAAP	SYGFYG-CNCG	VGSRG-KPKDA	TDRCCVHHC	CYKALT-D	C----	SPKT	DSYS--YSWKDK	TIVCGK-NNP	CLKQCECDK	AVAI CLRNL	DTYNK KYKI-Y	PK-PLCHKAD	--DC
Bgod1	SMYQLWKIL	QETG-KNAVP	SYGLYG-CNCG	VGSRG-KPKDA	TDRCCVHHC	CYKKLT-D	C----	SPKT	DSYS--YSWKDK	TIVCGD-NNP	CLKQCECDK	AVAI CLRNL	DTYNK KYKI-Y	PK-PLCHKAD	--AC
Bgod2	SVYELGKMIL	QETG-KNAAT	SYGFYG-CNCG	VGSRG-KPKDA	TDRCCVHHC	CYKKLT-D	C----	NSKT	DRYS--YSWKDK	TIVCGD-NNP	CLKQCECDK	AVAI CLRNL	NTYNK KYKI-H	MK-PFCKKPD	--AC
Catx1	SLVLEGMIL	QETG-KNPIT	SYGIYG-CNCG	VGSRH-KPKDG	TDRCCVHHC	CYKKLT-D	C----	DPKM	DGYT--YSFKDK	TIICDV-NNP	CLKQCECDK	AVAI CLRNL	DTYNK KYKI-Y	PK-FLCHKPD	--TC
Bjsu2	DLWQFGQML	KETG-KLPFP	YYTTYG-CYCG	WGGQG-QPKDA	TDRCCVHDC	CYKGLT-N	C----	KPKT	DRYS--YSRENG	VIIICGE-GTP	CEKQICECDK	AAAVCFREN	RTYK KYMA-Y	PD-VLCHKPA	E-KC
Bpir3	DLWQFGQML	KETG-KLPFP	YY-TYGGCYCG	VGRRG-GLGTH	TDRCCVHDC	CYKGLT-G	C----	PKTD	DRYS--YSRLDL	TIVCGE-DDP	CK-ELCECDK	AAAVCFREN	GTYNK KYRY-H	LK-PCHKAD	K-PC
Bmar1	DLWQFGQML	KETG-KIPFS	YYGAYG-CYCG	WGGRGKPKAG	TDRCCVHDC	CYKGLT-S	C----	PKTD	DRYS--YSRLDL	TIVCGE-DDP	CK-ELCECDK	AAAVCFREN	GTYNK KYRY-H	LK-SCHKAD	K-PC
Bmar2	DLWQFGQML	KETG-KIPFP	YYGAYG-CYCG	WGGRGKPKAG	TDRCCVHDC	CYKGLT-S	C----	PKTD	DRYS--YSRLDG	TIVCGE-DDP	CK-ELCECDK	AAAVCFREN	GTYNK KYRY-H	LK-SCHKAD	K-PC
Bmoj3	DLWQFNKMIK	KEVG-KLPFP	FYGAYG-CYCG	WGGRGKPKDG	TDRCCVHDC	CYKGLT-G	C----	PKWD	DRYS--YSWKDI	TIVCGE-DLP	CE-EICECDR	AAAVCFYEN	GTYNK KYMK-H	LK-PCHKAD	Y-PC
Bgod3	NLLQFNKMIK	IMTK-KNAVP	FYTSYG-CYCG	WGGRG-KPKDA	TDRCCFEHDC	CYEKLT-D	C----	SPKT	DIYS--YSWKSG	VIIICGE-GTP	CEKQICECDR	AAAVCFGNL	RTYK KYMF-Y	PD-FLCTDPS	E-KC
Basp5	SLIEFAKMIL	EETK-RLPFP	YYTTYG-CYCG	WGGQG-QPKDA	TDRCCVHDC	CYKGLS-N	C----	KPKT	DRYS--YSRKSG	VIIICGE-GTP	CEKQICECDR	AAAVCFREN	RTYK KYMA-Y	PD-LLCHKPA	E-KC
Cdte1	HLLQFNKMIK	PETR-KNAIP	FYAFYG-CYCG	WGGRG-RPKDA	TDRCCVHDC	CYKGLA-K	C----	NTKW	DIYP--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdte2	HLLQFNKMIK	PETR-KNAVP	FYAFYG-CYCG	WGGQG-RPKDA	TDRCCVHDC	CYKGLA-K	C----	NTKW	DIYR--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdte3	HLLQFNKMIK	PETR-KNAVP	FYAF-G-CYCG	WGGQR-RPKDA	TDRCCVHDC	CYEKVT-K	C----	NTKW	DFYR--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdte4	HLLQFNKMIK	PETR-KNAVP	FYAFYG-CYCG	WGGQR-RPKDA	TDRCCVHDC	CYKGLT-K	C----	NTKW	DIYR--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdcu1	SLQFNKMIK	PETR-KSGVP	FYAAYG-CYCG	WGGQ--RPKDP	TDRCCVHDC	CYKGLT-K	C----	NTKW	DIYS--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	NTYK YGMY-Y	PD-SRCRGP	EXTC
Cdcu2	SLVQFNKMIK	PETR-KSGLP	FYAAYG-CYCG	WGGQ--RPKDA	TDRCCVHDC	CYKGLA-K	C----	NTKW	DIYS--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGP	EXTC
Cdru1	SLQFNKMIK	PETR-KNAIP	FYAFYG-CYCG	WGGQG-RPKDA	TDRCCVHDC	CYKGLA-K	C----	NTKW	DFYR--YSLRSG	YFQCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdru2	SLVQFEKMIK	EETG-KNAVP	FYAFYG-CYCG	WGGRG-RPKDA	TDRCCVHDC	CYEKLV-K	C----	NTKW	DFYR--YSLRSG	YFQCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdru3	HLLQFNKMIK	PETR-KNAIP	FYAFYG-CYCG	WGGRG-RPKDA	TDRCCVHDC	CYKGLA-K	C----	NTKW	DIYP--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Cdco1	HLLQFNKMIK	PETR-RNAIP	FYAFYG-CYCG	WGGRG-RPKDA	TDRCCVHDC	CYKGLA-K	C----	NTKW	DFYR--YSLKSG	YITCGK-GTW	CEEQICECDR	VAAECRLRSL	STYK YGMY-Y	PD-SRCRGS	E-TC
Bjsu3	SLWQFGKMIN	YVMG-ESGVL	QYLSYG-CYCG	LGGQG-QPTDA	TDRCCVHDC	CYKGLT-G	C----	DPKI	DSYT--YSKNG	DVVCQG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-Y	GA-KNCQES	E-PC
Bpir4	NLWQFGKMIN	KIAG-ESGVF	KYLSYG-CYCG	LGGQG-QPTDA	TDRCCVHDC	CYKGLT-G	C----	DPKI	DSYT--YSKNG	DVVCQG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-Y	GA-KNCQES	E-PC
Bmoj4	NLWQFEMIL	KIAG-TSGFL	FYSSYG-CYCG	WGGHG-RPQDA	TDRCCVHDC	CYKGLT-G	C----	NPKT	DSYT--YSEENG	DVVCQG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-Y	PA-KNCQES	E-PC
Bins1	NLWQFGKMIN	YVMG-QSVVY	KYFYSG-CYCG	WGGIG-QPRDA	TDRCCVHDC	CYKGLT-G	C----	DPKT	DSYT--YSKNG	DVVCQG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-Y	GA-KNCQES	E-PC
Basp6	NLWQFGQMS	DVMR-KNVVF	KYLSYG-CYCG	WGGIG-QPKDA	TDRCCVHDC	CYKGLT-G	C----	DPKM	DIYTYTSKNG	DVVCQG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-Y	GA-KNCQES	E-PC
Balt1	NLVQFKTIL	KIAG-RSVVY	KY-FYG-CYCG	WGGIG-QPRDA	TDRCCVHDC	CYKGLT-N	C----	NPKT	ATYS--YSEENG	ALVCGG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-L	PP-KNCQES	E-PC
Bery1	SLVQFETIL	KIAG-RSGVW	YYSYG-CYCG	SGGQG-RPQDA	SDRCCVHDC	CYKGLT-D	C----	DPKA	DVYT--YSEENG	VVVCQG-DDP	CKKQICECDR	VAAECFRDNK	DTYD KYWF-F	PA-KNCQES	E-PC
Bjar1	DLWQFGQMN	DVMR-EYVVF	NYLYSG-CYCG	WGGIG-KPRDA	TDRCCVHDC	CYKGLT-G	C----	NPKT	DSYTYTSEENG	DVVCQG-DDL	CKKQICECDR	VAAECFRDNK	DTYD KYWF-Y	GA-KNCQES	E-PC
Cdca1	SLLDFEMIL	KVAK-KSGLL	WYSAYG-CYCG	WGGQG-RPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKM	VSYT--YSVKNG	EIICED-DDP	CKKQICECDR	VAAECFRDNK	PSYD KYRQ-F	PA-ENCREEP	E-PC
Cvir1	SLVQFETIL	KIAG-RSGLL	WYSAYG-CYCG	WGGHG-LPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKT	VSYT--YSEENG	EIVCGG-DDP	CGTQICECDK	AAACFRDNI	PSYD KYWF-F	LP-KNCRGDP	E-PC
Cvir2	SLVQFEMIL	KVAK-RSGLF	WYGAYG-CYCG	WGGQG-RPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKT	VSYT--YSVKNG	EIICED-DDP	CKKQICECDR	VAAECFRDNI	PSYD KYRQ-F	PA-ENCREEP	E-PC
Cvir3	SLVQFETIL	KIAG-RSGLL	WYSAYG-CYCG	WGGHG-LPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKT	VSYT--YSVKNG	EIICED-DDP	CKKQICECDR	VAAECFRDNI	PSYD KYRQ-F	PA-ENCREEP	E-PC
Cvir4	SLVQFEMIL	KVAK-RSGLF	WYGAYG-CYCG	WGGQG-RPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKT	VSYT--YSVKNG	EIICED-DDP	CKKQICECDR	VAAECFRDNI	PSYD KYRQ-F	PA-ENCRGDP	E-PC
Cada1	SLVQFETIL	KVAK-RSGLL	WYSAYG-CYCG	WGGHG-RPQDA	TDRCCVHDC	CYKGLT-N	C----	NPKT	VSYT--YSEENG	EIVCGG-DDP	CGTQICECDK	AAACFRDNI	PSYD KYWF-F	PP-KNCRGDP	E-PC
Cada2	SLVQFETIL	KVAK-RSGLL	WYSAYG-CYCG	WGGHG-RPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKT	VSYT--YSEENG	EIVCGG-DDP	CGTQICECDK	AAACFRDNI	PSYD KYWF-F	PP-KNCRGDP	E-PC
Catx2	SLVQFETIL	KIAG-RSGLL	WYSAYG-CYCG	WGGHG-LPQDA	TDRCCVHDC	CYKGLT-D	C----	NPKT	VSYT--YSEENG	EIICG-DDP	CGTQICECDK	AAACFRDNI	PSYD KYWF-F	PP-KNCRGDP	E-PC
Lste1	HLLQFGDLID	KIAG-RSGFW	YGFYG-CYCG	LGGRG-RPQDA	TDRCCVHDC	CYKGLT-G	C----	DPKK	DIYT--YSEENG	AIIVCGG-DNP	CKKEICECDR	DAACFRDNL	DTYD KYWF-F	PN-KYCKES	E-PC
Lmut1	M--QFGDLIN	KIAR-RNGIL	YYSFYG-CYCG	LGGRG-RPQDA	TDRCCVHDC	CYKGLT-G	C----	NPKN	DIYT--YSEENG	AIIVCGG-DNP	CKKEICECDR	DAACFRDNL	DTYD KYWF-L	PR-KYCKES	E-PC

Figure 4: Multiple sequence alignment of PLA₂s amino acid sequences of the snake venoms from the *Bothrops*, *Crotalus* and *Lachesis* genera. The sequences were clustered into three groups according to their physical-chemical property (acidic or basic) and the type of amino acid (lysine or aspartic acid) at position 49. Basic Lys49-PLA₂s are gray, basic Asp49-PLA₂s basic are yellow and acidic Asp49-PLA₂s are shown in pink. The gray gaps were included to adjust the sequences for the numbering system proposed by Renetseder *et al.* (1985) and the black gaps were used to adjust the alignment for all sequences. The amino acid residues from BhtTX-I, BhtTX-II and BthA-I that bound antibodies are highlighted in red (anti-bothropic horse serum), blue (anti-crotalic horse serum) and green (both antivenom sera).

Table 1

Summary of the epitopes identified using commercial anti-bothropic and anti-crotalic horse sera. The PLA₂s sequences from *Bothrops jararacussu* snake venom were obtained from the Swiss-Prot database (<http://www.uniprot.org/>).

Sequences Accession Number	Protein Identification	Anti-Crotalic Horse Serum	Anti-Bothropic Horse Serum
Q90249	BhtTX-I Lys49-PLA ₂	Gln11–Lys20	–
		Cys27–Gly30	Cys27–Gly30
		Gly59–Tyr73	Gly59–Tyr73
		–	Cys84–Asn89
		–	Lys116–Asp130
P45881	BhtTX-II Asp49-PLA ₂ basic	Leu17–Tyr25	Leu17–Tyr25
		Pro37–Cys45	Pro37–Cys45
		Thr70–Glu78	–
		Gly80–Thr89	Gly80–Thr89
Q8AXY1	BthA-I Asp49-PLA ₂ acidic	Ser17–Tyr25	Ser17–Tyr25
		Tyr52–Tyr73	–
		Phe106–Phe119	–

Table 2

List of the individual snake venom PLA₂s from *Bothrops sp*, *Crotalus sp* and *Lachesis sp* used for comparative analysis with the species name, individual identifiers, their accession numbers for Swiss-Prot, TrEMBL or PDB and theoretical isoelectric points. Enzymes are divided into three groups; Lys49-PLA₂s, basic Asp49-PLA₂s and acidic Asp49-PLA₂s.

Species	Individual Identifier	Accession number	Isoelectric point
Lys49-PLA ₂ s			
<i>Bothrops jararacussu</i>	Bjsu1	sp Q90249	9.03
<i>Bothrops pirajai</i>	Bpir1	sp P58399	8.97
<i>Bothrops pirajai</i>	Bpir2	sp P82287	9.03
<i>Bothrops asper</i>	Basp1	sp P24605	9.10
<i>Bothrops asper</i>	Basp2	sp P0C616	8.70
<i>Bothrops asper</i>	Basp3	sp Q9PVE3	9.10
<i>Bothrops asper</i>	Basp4	tr B0FM89	8.70
<i>Bothrops moojeni</i>	Bmoj1	sp Q9I834	8.95
<i>Bothrops moojeni</i>	Bmoj2	sp P82114	8.97
<i>Bothropoides pauloensis</i>	Bpal1	sp Q9IAT9	8.97
<i>Bothrops atrox</i>	Batx1	sp Q6JK69	8.87
<i>Atropoides nummifer</i>	Bnuf1	sp P82950	8.71
<i>Cerrophidion godmani</i>	Bgod1	sp P81165	8.61
<i>Cerrophidion godmani</i>	Bgod2	sp Q8UVU7	8.90
<i>Crotalus atrox</i>	Catx1	sp Q8UVZ7	8.60
Basic Asp49-PLA ₂ s			
<i>Bothrops jararacussu</i>	Bjsu2	sp P45881	8.50
<i>Bothrops pirajai</i>	Bpir3	pdb 1GMZ	8.35
<i>Bothrops marajoensis</i>	Bmar1	sp P86804	8.61

<i>Bothrops marajoensis</i>	Bmar2	sp P86803	8.49
<i>Bothrops moojeni</i>	Bmoj3	sp P0C8M1	8.17
<i>Cerrophidion godmani</i>	Bgod3	tr Q6EER5	8.36
<i>Bothrops asper</i>	Basp5	sp P20474	8.72
<i>Crotalus durissus terrificus</i>	Cdte1	sp P62022	8.74
<i>Crotalus durissus terrificus</i>	Cdte2	sp P24027	8.74
<i>Crotalus durissus terrificus</i>	Cdte3	sp P0CAS7	8.74
<i>Crotalus durissus terrificus</i>	Cdte4	sp P0CAS5	9.16
<i>Crotalus durissus cumanensis</i>	Cdcu1	sp P86806	8.83
<i>Crotalus durissus cumanensis</i>	Cdcu2	sp P86805	8.62
<i>Crotalus durissus ruruima</i>	Cdru1	sp P0CAS3	8.75
<i>Crotalus durissus ruruima</i>	Cdru2	sp P0CAS4	8.51
<i>Crotalus durissus ruruima</i>	Cdru3	sp P86169	8.74
<i>Crotalus durissus collilineatus</i>	Cdco1	sp P0CAS2	8.85

Acidic Asp49-PLA₂s

<i>Bothrops jararacussu</i>	Bjsu3	sp Q8AXY1	5.20
<i>Bothrops pirajai</i>	Bpir4	sp C9DPL5	4.90
<i>Bothrops moojeni</i>	Bmoj4	tr G3DT18	4.91
<i>Bothrops insularis</i>	Bins1	sp Q8QG87	5.05
<i>Bothrops asper</i>	Basp6	sp P86389	5.05
<i>Bothrops alternatus</i>	Balt1	sp P86456	6.69
<i>Bothrops erythromelas</i>	Bery1	tr Q2HZ28	4.67
<i>Bothrops jararaca</i>	Bjar1	sp P81243	4.58
<i>Crotalus durissus cascavella</i>	Cdca1	tr C9E7C4	5.14
<i>Crotalus viridis viridis</i>	Cvir1	tr Q800C4	4.99
<i>Crotalus viridis viridis</i>	Cvir2	tr Q800C3	5.39

<i>Crotalus viridis viridis</i>	Cvir3	tr Q800C1	5.10
<i>Crotalus viridis viridis</i>	Cvir4	tr Q800C2	5.78
<i>Crotalus adamanteus</i>	Cada1	sp P00623	5.06
<i>Crotalus adamanteus</i>	Cada2	tr F8S100	4.91
<i>Crotalus atrox</i>	Catx2	sp P00624	4.64
<i>Lachesis stenophrys</i>	Lste1	sp P84651	4.95
<i>Lachesis muta</i>	Lmut1	tr D3IVZ4	5.38

Reference Sequences: sp: Swiss-Prot and tr: TrEMBL databases (<http://www.uniprot.org/>), and pdb: Protein Data Bank (<http://www.pdb.org> /pdb/home/home.do).

4. CONCLUSÕES

➤ Foram identificadas doze regiões reconhecidas pelo soro. Em BthTX-I duas regiões foram reconhecidas pelo soro antibotrópico (C⁸⁴-N⁸⁹ e K¹¹⁶-D¹³⁰), uma região pelo soro anticrotático (Q¹¹-K²⁰) e duas regiões (C²⁷-G³⁰ e G⁵⁹-Y⁷³) por ambos os soros. Em BthTX-II, três epitopos B-lineares (Leu¹⁷-Tyr²⁵, Pro³⁷-Cys⁴⁵ e G⁷⁹-T⁸⁹) foram identificados por ambos os soros e um pelo soro anticrotático (T⁶⁹-D⁷⁷). Em BthA-I, três regiões foram reconhecidas, sendo Y⁵²-Y⁷³ e F¹⁰⁶-F¹¹⁹ pelo soro anticrotático e S¹⁷-Y²⁵ pelos dois tipos de soro.

➤ Os B-epitopos lineares das fosfolipases A₂ isoladas da peçonha de *Bothrops jararacussu* reconhecidos pelos soros antiofídicos localizam-se em regiões responsáveis por atividade catalítica, neurotóxica, miotóxica e anticoagulante, cujas ações poderão ser inibidas ou atenuadas pela interação soro-proteína.

➤ O cálculo teórico do ponto isoelérico (pI) de cada B-epitopo determinado e posterior análise comparativa entre PLA₂s isoladas de peçonhas de serpentes nos possibilitou concluir que a maioria das regiões reconhecidas pelos soros anticrotático, antibotrópico ou por ambos, tem característica básica, evidenciando uma possível essencialidade dos aminoácidos lisina e arginina presentes na maioria dos B-epitopos para a interação entre o soro antiofídico comercial de cavalo e as PLA₂s de *Bothrops jararacussu*.

➤ O uso de inibidores vegetais como tratamento alternativo ou complementar para o ofidismo pode ser uma opção viável pela indisponibilidade de soros antiofídicos em locais adversos ou por cultura popular.

➤ Extratos vegetais brutos e moléculas ativas isoladas presentes em diversas espécies de plantas encontradas em território brasileiro possuem propriedades inibidoras de PLA₂s presente na peçonha de serpentes.

➤ Moléculas sintéticas desenhadas a partir de similares naturais têm sido utilizados com sucesso para inibir os efeitos tóxicos causados pelo ofidismo.

➤ Inibidores de origem vegetal ou sintética podem ser importantes ferramentas para auxiliar o entendimento dos mecanismos de ação de proteínas isoladas (como por exemplo, as fosfolipases A₂) de peçonhas de serpentes.

➤ A identificação de regiões específicas das PLA₂s isoladas da peçonha de *B. jararacussu* contribui para o desenvolvimento de produtos farmacêuticos para tratamento e diagnóstico de ofidismos, podendo gerar fármacos seletivos e quites para diagnosticar os diversos gêneros de serpentes envolvidas em acidentes ofídicos.

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