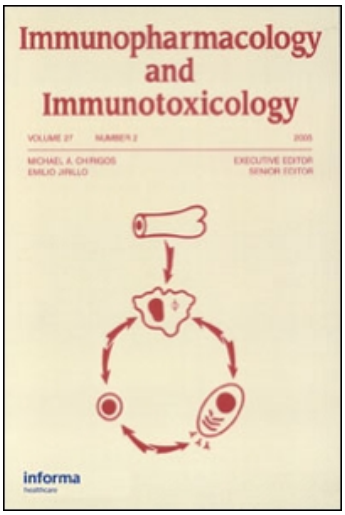

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INVITED REVIEW

Inhibitors of Snake Venoms and Development of New Therapeutics

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Natural inhibitors of snake venoms play a significant role in the ability to neutralize the degradation effects induced by venom toxins. It has been known for many years that animal sera and some plant extracts are competent in neutralizing snake venoms. The purpose of this review is to highlight the recent work that has been accomplished with natural inhibitors of snake venoms as well as revisiting the past research including those found in plants. The biomedical value of these natural inhibitors can lead to the development of new therapeutics for an assortment of diseases as well as contributing to efficient antivenoms for the treatment of ophidic accidents.

Keywords Endothermic Animals, Hemorrhagic Activity, Metalloprotease Inhibitors, Plant Extracts, Proteolytic Activity, Snake Venom.

INTRODUCTION

Snake venoms are a heterogeneous mixture of organic and inorganic substances that act as functional molecular groups that allow these animals the confinement, immobilization, and digestion of their prey, along with being a defense mechanism against enemies and natural predators. Consequently, in their accidental contacts with human beings, venomous snakes constitute a problem of Public Health, becoming a complex biomedical challenge that begins with the study and characterization of their venom components. Inside the venom's organic components, several toxins have been found with elaborate mechanisms of actions, many with enzymatic activities that alter tissue

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integrity. The manifestations of local tissue damage, such as hemorrhage and myonecrosis are among some of the most dramatic as a result of Viperidae snake envenoming. The severity can extend from a light local hemorrhage, in mild envenoming, to muscular necrosis and lethal bleeding in the most serious cases.[1]

Hemorrhage and myonecrosis are the results of the combination of several factors present in venom. Hemorrhage is the primary consequence of the enzymatic factors degradative action on the subcellular muscle proteins and endothelium produced by hemorrhagic toxins, which are in general snake venom metalloproteases (SVMPs).[2] These hemorrhagins, together with the action of secondary factors, produce blood incoagulability allowing not only an altered permeability of the blood vessels, especially of the small ones, but also inducing the exit of plasma and red blood cells to extra-vascular spaces.[1,3,4]

Myonecrosis is a result of toxins (generally basic), classified as phospholipases A_2 (PLA_2s). The PLA_2s secreted in snake venoms (with myotoxic and neurotoxic activities), contain highly preserved sequences that belong to a super-molecular family with very diverse non-toxic variants. The PLA_2s have a high distribution, in glandular or digestive tissues of mammals and reptiles, acting as elements of modulations in different physiological processes.[5–8] As enzymes, the PLA_2s are able to catalyze the glycerophospholipids hydrolysis, liberating fatty acids, and lysophospholipids responsible for cellular membrane damages.[8]

In nature, by phylogenetic evolution throughout millions of years, mammals and some reptiles have developed a natural resistance to snake toxins. They have naturally occurring inhibitors which neutralize the toxins found in venom. Both snakes and mammals have been shown to possess protease inhibitors neutralizing the hemorrhagic or proteolytic effects of venom proteins. The sera of certain snakes, both venomous and nonvenomous, have been reported to contain these metalloprotease inhibitors known as antihemorrhagic factors.[9–16] Early publications reported on the resistance of snakes against their own or other snake venoms, in which snakes were allowed to bite each other to determine resistance.[9] Later investigations became more quantitative and used serum protection tests and LD_{50} to determine the degree of neutralization.

However, as early as 1895, resistance of mammals (hedgehog, *Erinoaceous europaeus*) against snake venom was first reported.[17] It was not until half a century later that Vellard reported the resistance of the common opossum (*Didelphis marsupialis*) from South America to the toxic effects of snake venom.[18,19] Later it was discovered that several other mammals have the ability to resist the effects of snake venom toxins.[12,20–24]

As reviewed by Domont et al.[25] and Pérez and Sánchez,[26] metalloprotease inhibitors are the main factors isolated from resistant animals that are responsible for the inhibition of the toxic effects of snake venoms. These

inhibitors are known to be mostly non-enzymatic, heat stable and acidic glycoproteins with molecular masses ranging from 52 to 90 kDa. A variety of plant extracts also contain rich sources of compounds that have many pharmacological activities including the inhibition of snake venom proteolytic activities; however, they have been scientifically validated in only a few cases.[27]

Natural protease inhibitors are important because of their potential use in medicine. In order to understand their significance, their relationship with snake metalloproteases and other toxins must be reviewed. Metalloproteases are enzymes which split peptide bonds in proteins and are involved in many biological and pathological processes. These metalloproteases are in a delicate balance with protease inhibitors to control biological functions. Matrix metalloproteases (MMPs) or matrixins are a family of zinc endopeptidases that function in the degradation of extracellular matrix proteins and play a key role in tissue degradation during embryogenesis, tissue growth, and wound healing in mammals.[28] They also belong to the metalloproteinase class which is one of the four major classes of proteases (serine, cysteine, aspartyl. and metalloproteases). These classes of proteases have been implicated in cancer and AIDS. Such proteases involved in these diseases could be targeted and neutralized more efficiently if specific inhibitors could be designed.

Both native and synthetic inhibitors have been considered for therapeutic approaches to cancer but the large size of the molecules was a problem in tissue penetration.[28] Synthetic matrix metalloprotease inhibitors (MMPIs) have been developed since the early 1980s. Most of these inhibitors were peptide derivatives of shorter sequences which would neutralize the effects of metalloproteases.[29–31] The British Biotech inhibitor batimastat (BB-94) was shown to reduce metastasis of melanoma, mammary carcinoma, and colorectal tumor cells in experimental metastasis assays.[29] Marimastat is another type of synthetic MMPIs that is being used in comparative phase III trials.[32] However most of these protease inhibitors used to treat pathological processes are problematic due to poor specificity and low bioavailability.[28]

The fact that mammalian and venom metalloproteases are similar and both possess natural protease inhibitors that regulate them makes them quite intriguing. In pathological processes such as cancer, one critical step in the initiation of tumor metastasis is known to be the unregulated degradation of basement membranes by MMPs.[33] Studies with native tissue inhibitors of metalloproteases (TIMPs) have shown that they can prevent the growth and spread of experimental tumors.[29–31] Similarly, protease inhibitors from resistant animals neutralize the hemorrhagic effects of snake venom metalloproteases.

The biological and biochemical effects of snake venom toxins suggest that their action highly depends of their proteolytic activity. This activity has been described with detail for hemorragins[1,34] and myotoxins.[35] The basal membrane proteolytic destruction has been prevented by antivenoms;

however, they have not always demonstrated to be completely effective or impede the damage of the lesion.[36–44]

The possibility of inhibiting toxin activity has been explored using peptidic inhibitors designed to interact specifically through non-covalent associations with their lateral chains. Among these inhibitors, coupled to the peptidic sequence, there are diverse ligands (carboxylic, sulfhydryl, chloromethyl, pyro-glutamates. or hydroxamates), which block the proteolytic activity of the enzyme located in the catalytic center where the Zn^{++} ion is present. The inhibitory potential of these synthetic peptids depends on the sequence of chosen peptides.[1,45] Based on the same principle, the presence of endogenous inhibitors has also been explored in venoms. Pyroglutamic tripeptides in *Crotalus atrox* and *Bothrops asper* have been described.[46,47] They have the ability to inhibit, *in situ*, type HT hemorrhagins, and it is believed that after venom inoculation, they lose their inhibitory action when venom components are disseminated in the tissues, which diminishes the hemorrhagin K_i constant. Under these conditions, in the affected tissues, the venom components favor their target molecules in muscle plasmatic membrane or basal membrane of the capillaries. These small, inhibitory peptides are thus limited to the venom glands, not allowing the hydrolysis of venom toxins before being injected in their prey.[46–47]

Non-specific protease inhibitors, together with TIMPs, contribute in containing the catalytic potential of MMPs in a living system.[48] Both TIMPs and MMPs are necessary in maintaining an equilibrium balance of normal physiological processes. When this equilibrium is disrupted, the catalytic potential of MMPs results in abnormal physiological complications such as inflammation, osteoporosis, cancer, atherosclerosis, and other diseases. Protease endogenous inhibitors have been valued in inhibiting venom proteolytic activities which include metalloproteases. Anai et al.[49] investigated the *in vitro* endogenous inhibitory ability of human and rat sera on the proteolytic activity of jararafibrasa I, a hemorrhagic/fibrinolytic enzyme purified from *Bothrops jararaca* venom. Both sera, especially that of rat were able to inhibit the enzymatic activity of the jararafibrasa I, and this inhibitory activity was depended on the α-1-macroglobulin and murineglobulin in the rat serum, and on the α-2-macroglobulin in human serum. They also demonstrated by SDS-PAGE that these macroglobulins formed a complex with jararafibrasa I. It has been thought that the formation of these complexes is an important part of the mechanism of action of the well-known inhibitors. Venom enzymes, upon finding a vulnerable area in the peptide sequence of the inhibitor that connects the globulins, hydrolizes it and produce an irreversible conformational change inactiving the inhibitor.[50] Therefore, the ability of these macroglobulins to inhibit hemorrhagic activity caused by venom toxins is questionable for humans. For example, the inactivation of antithrombin III occurs when it is incubated with Crotalidae, Viperidae and Colubridae venoms.[51]

Understanding the properties of natural protease inhibitors is important in the study of protease inhibition in pathological processes. The focus of this article is to provide a review of studies that have been undertaken with natural inhibitors found in reptiles, mammals, and plant extracts capable of neutralizing the toxic effects of snake venoms.

INHIBITORS IN SERA OF REPTILES

It has been known for many years that different species of snakes are resistant against their own venom and venom of other species in the same family. This resistance is almost certainly caused by specific sera inhibitors that are far more efficient than rat and human sera inhibitors. Noguchi, in 1909,[52] reported this observation and hypothesized that resistance was caused by the lack of tissue receptors in the resistant snakes. Early information demonstrated that the natural resistance was a plasma protector effect, as a result of a previous non lethal envenoming.[53] Nichol et al.,[9] attempting to elucidate the "immunity" of the *Crotalus molossus* snakes to their own venom, confronted different size snakes. It was reported that the snakes were resistant and supported a higher lethal dose of their own venom, being able to inhibit larger volumes of venom than humans or mammals. Later reports, gave as a resistance indicator the period between the administration of a well-known dose of venom and the death, also establishing the effects of different snakes and their venoms.[54] They proved that the resistance was better among closely related species, and that the venom varied in its composition and susceptibility to the inhibition of their lethal action. This study was confirmed by a hemoglobin digestion assay, where the proteolytic activity of *C. adamanteus* venom was inhibited more effectively than *Agkistrodon piscivorus* venom by *Lampropeltis getulus floridana* serum.[55] Omori-Satoh et al.[11] established these findings by purifying an antihemorrhagic factor from serum of the *Trimeresurus flavoviridis* snake. This factor, with a molecular weight of 70 kDa and an isoelectric point (pI) of 4.0, migrated near the albumin and the alpha-globulin area, inhibiting the lethal and hemorrhagic toxicity of the hemorrhagic HR-1 fraction and reduced the basal membrane rupture induced by the HR-2 hemorrhagin.

Different research papers have accounted for and described the existence of serum factors that neutralize venoms of diverse species of snakes.[16,26] These works reflected that the specificity of the resistance diverges among species, and that the inhibitory activity it is not restricted to a single pathological injury, but can have an effect on a variety of degradative functions, such as myonecrosis, coagulopathies, and hemorrhages.

Ovadía and Kochva[12] evidenced a 56 kDa thermoresistant serum protein of *Vipera palestinae* that could neutralize the neurotoxic and hemorrhagic

activities of their own venom. Nahas et al.[56] found that *Bothrops jararaca* and *Waglerophis merremii* snakes presented factors that inactivated the coagulation ability of several venoms. These factors migrated together with the plasma fibrinogen bands, which have a higher inhibitory ability than the sera of these snakes. The proteinase inhibitor in the *B. jararaca* serum was purified by Tanizaki et al.,[57] it presented a similar molecular weight to other described inhibitors (between 50 and 90 kDa), and it also neutralized the proteolysis induced by Bothropasin and the J protease, both *B. jararaca* non-specific metalloproteases that hydrolized casein. Borkow et al.[58] purified an antihemorrhagic factor from *B. asper* (BaSAH1) serum having a molecular weight of 66 kDa and a pI of 5.2. It proved to be more effective in neutralizing the hemorrhagic activity of *B. asper* BaH1 toxin than the horse polyvalent antivenom.

Yamakawa and Omori-Satoh[59] determined the complete amino acid sequence of the antihemorrhagic factor isolated from the *Trimeresurus flavoviridis* serum. This factor was a glycoprotein composed by 323 residual amino acids and three asparagines at positions 123, 185 and 263, linked to oligosaccharides chains. Huang et al.[60] purified the first small inhibitor, between 47 and 36 kDa, in the presence and absence of mercaptoethanol. This was also a glycoprotein, but different to the previously purified inhibitor in reptile sera, with a marked specificity for *Trimeresurus* snakes. Later, three endogenous inhibitors in *T. mucrosquamatus*, pyroGlu-Asn-Trp (pENW), pyroGlu-Gln-Trp (pEQW), or pyroGlu-Lys-Trp (pEKW) were confronted against the SVMP, TM-3, of the same species, to study the disposition adopted by both crystalized molecules. The interaction of the inhibitors with TM-3 caused a slight displacement of some residuals near the active area of the enzyme, providing a space for the carboxylic C-terminal groups of the inhibitors that are exchanged with the metallic molecules in this center.[61]

Three different kinds of phospholipase A_2 (PLA_2) inhibitory proteins (PLIalpha, PLIbeta, and PLIgamma) were separated from the Chinese mamushi, *Agkistrodon blomhoffii siniticus* sera. PLIalpha was an inhibitor that inhibited selectively the group-II acidic PLA_2s from Crotalidae venom. PLIbeta was a 160-kDa glycoprotein having a trimeric structure composed of 50-kDa subunits. PLIgamma was a 100-kDa glycoprotein containing 25-kDa and 20-kDa subunits and inhibited PLA_2s from Elapidae venom PLA_2s (group I), Crotalidae and Viperidae venom PLA_2s (group II), and honey-bee PLA_2 (group III).[62]

A snake's natural resistance is not only limited to venomous species.[10,15,63,64] A serum protein inhibiting the enzymatic activity of the basic phospholipase A_2 from the venom of the same snake (*Agkistrodon blomhoffii siniticus*) was also purified from a nonvenomous Colubridae snake, *Elaphe quadrivirgata*. The purified inhibitor was a 150 kDa glycoprotein having a trimeric structure, composed of two homologous 50 kDa subunits. The inhibitor

inhibited solely group II basic PLA_2s and did not inhibit other types of PLA_2s.[65] In general, the successive reports of purified proteases inhibitors in venom, isolated from snake sera, coincide in that they are glycoproteins with a variable molecular weight between 36 and 80 kDa, and a mechanism of action that in spite of not being immunogenic, is very specific and stable for the toxin type which form inactive complexes.

INHIBITORS IN SERA OF THE GENUS DIDELPHIS

The *Didelphis* genus contains one of the broadly distributed species of mammals in the American continent. The species distributed in Venezuela are the *D. marsupialis* and *D. albiventris*.[66] Because of their natural resiliency, *D. marsupialis* together with their homologous *D. virginiana*, distributed in Center and North America,[67] are animals that have been utilized in biomedical investigations.

The first well-known report about the natural resistance of the Didelphidae family was carried out by Vellard,[18] and in this and a later report,[19] he considered that: "the only possible hypothesis to explain the *Didelphis* resistance to the venoms, is a progressive immunity developed in carnivorous animals of night life that should have frequently occasion of finding venomous snakes, killing them to feed." This report included three species of *Didelphis*: *D. marsupialis*, *D. aura,* and *D. azarae*. Individual results were not discriminate for each species. The studied opossums resisted the lethality and pathogenic actions of intramuscular injections of venom, and their sera neutralized *Crotalus terrificus, Bothrops neuwiedii* and *B. jararaca* venoms.

The Virginia opossum (*D. virginiana*) was first reported by Kilmon[20] to have a natural resistance to different venoms of various snake species. The study consisted of injecting venom into opossums and determining their effects. A dose of 15 mg/kg of *Agkistrodon p. piscivorus* venom was injected into *D. virginiana*, which corresponded to more than 5 lethal doses for 15 kg dogs. This dose did not provoke lethality in these animals but recovered their normal hemodynamia 10 minutes after administration of the venom. These animals did not display edema, ecchymosis, or remarkable necrosis. All opossums survived with no obvious effects.

Werner and Vick,[21] using the well-known LD_{50}, determined that the *D. virginiana* is resistant to *Crotalus adamanteus*, *C. atrox*, *A. c. contortrix*, *A. piscivorus*, *A. h. brevicaudata* and *A. bilineatus* venoms. However, it was susceptible to the venoms of Viperidae *(Bitis arietans)*, Elapidae (*Naja naja*, *N. naja atra*, *N. snowy* and *Micrurus fulvius)* and Hydrophidae (*Laticaudata semifasciata)*. These results proposed the use of the *D. virginiana* as an animal model for the study of venom resistance. Werner and Faith[68]

conducted a study in which Crotalidae snake venoms were incubated with opossum, horse and dog sera, and antivenin. The incubated solutions were injected into mice, and lethal effects were compared. It was concluded that the dog and horse sera did not neutralize the snake venom while the opossum and antivenin had approximately equivalent protective effects. It was determined that the venoms of *A. piscivorus*, *A. contortrix*, *C. atrox,* and *C. adamanteus* were reduced in their lethal effects as the amount of *D. virginiana* sera increased in concentration.

Pérez et al.[22] conducted a study in which 40 warm-blooded animals were surveyed for antihemorrhagic activity and for precipitating factors. The results indicated that 17 animal sera neutralized *C. atrox* venom. However, the opossum was the one that had the highest antihemorrhagic activity with a dilution factor of 256. It was assumed that the opossum antihemorrhagins were naturally occurring antibodies, but Menchaca and Pérez[69] provided evidence, by purifying a 68 kDa *D. virginiana* antihemorrhagic factor, that the antihemorrhagins involved in neutralization were not immunoglobulins but most likely other types of proteins. This hypothesis was further confirmed by Mckeller and Pérez[70] when they reported the inability of the *D. virginiana* to produce significant antibody levels against various inoculations with *C. atrox* venom. It is possible that this absence in the immune response was due to a rapid "blocking" of venom molecules by the natural inhibitor molecules, regardless of the used immunization protocol. With regard to the complete serum, a 204.8 increment in the specific activity was achieved after purification. Tarng et al.[71] isolated antihemorrhagic factors in opossum serum by using a monoclonal antibody immunoadsorbent assay. Soto et al.[72] studied the antihemorrhagic and antiproteolytic activities in *D. virginana* and *N. micropus* sera against 25 species of Crotalidae snake venoms. Both species neutralized the hemorrhagic activity of all venoms. All the venoms showed hemolytic and varied proteolytic activities in presence of the sera. These variations in the proteolysis of the different venoms challenged by the sera of both animals indicate the presence of multiple inhibitory factors.

Melo et al.[73] reported that *B. jararacussu* venom incubated with heparin or the purified acidic component of *D. marsupialis* serum was not able to produce an increase in the levels of sarcoplasmic enzymes, especially in the creatinine kinase (CK) produced. They suggested that this inhibitor effect was due to the ability of the complex formation between the inhibitors or the heparin with the basic myotoxins and not the catalytic ones. Perales et al.,[74] using the electrophoresis in cellulose acetate and conventional PAGE as approaches of purity, obtained two heterogeneous inhibiting fractions between 42 and 58 kDa from *D. marsupialis* against *B. jararaca* venom. Similar reports have demonstrated that in *D. marsupialis* species, an antibothropic complex also exists with antiedema and antihemorrhagic properties, which is

an inhibitor of venom metalloproteases [75,76] and at least six times more effective than conventional antivenoms.[77]

Catanese and Kress[78] purified a 52 kDa *D. virginiana* metalloproteinase inhibitor, named oprin (opossum proteinase inhibitor), which inhibited proteases from *C. atrox* venom. Furthermore, Catanese and Kress,[79] purified a new inhibitor, which was partially sequenced and immunologically screened with an opossum cDNA liver, and clones for the α_1-PI were isolated. The result demonstrated a 389-residue protein named α_1-PI that, contrary to $\alpha_1\beta$-G previously described, had homologies from 51 to 58% with proteases inhibitors of the α_1-PI type in mammals and humans. In spite of their similarity, *D. virginiana* α_1-PI conserved their anti-metalloprotease activity under inoperative conditions for the human α_1-PI. These reports strengthened the hypothesis that the mechanism of natural resistance in certain animals is due to a similar protein inhibitor as the macroglobulins that can bind to the metalloproteases neutralizing its hemorrhagic and degenerative capacities. Many early reports pointed to the existence of a large, single inhibitor protein.

Pifano et al.[80] also reported the neutralizing property of *D. marsupialis* sera against the hemorrhagic and proteolytic activities of *B. lanceolatus* venom using the immunoglobulin-free supernatant from ammonium sulfate precipitated sera. They demonstrated that *B. lanceolatus* venom was inactivated by the F-0.1 fraction isolated from *D. marsupialis* sera. This fraction conserved its antihemorrhagic and antiproteolytic characteristics. The fraction F-0.1 was further purified by running it under non-reducing SDS-PAGE conditions and obtaining eight bands that were extracted by electroelution.[81] A unique 97 kDa band neutralized the hemorrhagic activity of *B. lanceolatus* venom. This protein had a considerably larger molecular weight than the fraction previously purified from *D. marsupialis* sera, but similar to those described in *B. jararaca* sera.[57] It is quite probable that the inhibiting factor could be a dimeric structure affected by mercaptoethanol.

Perales et al.[82] suggested the dimeric nature of the serum inhibitors in marsupials after the chromatography of an antibothropic fraction (ABF) of 84 kDa from whole sera of *D. marsupialis*, *Philander opossum*, *Lutreolina crassicaudata* and *Metachirus nudicaudatum*. The ABF was rechromatographed resulting in two bands corresponding to two subunits of 48 and 43 kDa. Neither presented homologies with any other well-known protein. Lovo-Farah et al.[83] also isolated a 43 kDa protein with antibothropic activity. This protein (DA2-II), separated from an initial fraction (DA2), showed homology in the N-terminal sequence with the 48 kDa protein previously described by Perales et al.[82] for *D. marsupialis, P. opossum*. and *L. crassicaudata.*

Neves-Ferreira et al.[84] purified two antihemorrhagic proteins from *D. marsupialis* serum. Their masses by mass spectrometry were 40318 AMU for DM40 and 42373 and 43010 AMU for DM43, demonstrating the presence

of isoforms for the latter. Molecular masses of 44.8 and 47.3 kDa were achieved by SDS-PAGE for DM40 and DM43, respectively. N-terminal sequences of the first 17 residues of DM40 and DM43 were indistinguishable with the exception of the substitution of R9 for P9. Both inhibitors demonstrated isoelectric points lower than 3.5, and glycosylation percentages varied from 20.5 to 29.0%. Both were homologous to oprin, an analogous inhibitor from *D. virginiana* serum, and both formed stable complexes with jararhagin and inhibited its hemorrhagic and enzymatic effects. Neves-Ferreira et al.[85] analyzed structurally and functionally the DM43 inhibitor which showed homology to the human α_1B-glycoprotein, a plasma protein of unknown functions, and also showed partial homology to the transcripts of the immunoglobulin genes superfamily.

Rodríguez-Acosta et al.[86] reported that the neurotoxic activities of rattlesnake (*C. vegrandis*) venom caused by phospholipases activities (mainly crotoxin) were also neutralized by opossum (*D. marsupialis*) sera. This study not only demonstrated that the protease activity was inhibited by these sera, but the phospholipase activities were also inhibited. In general, many of the venom phospholipases A_2 (vPLA$_2$s) have demonstrated their ability to bind to soluble and membrane proteins belonging to the type-C lectins superfamily (probably responsible for the myotoxic effect of the vPLA2s), the type-M (responsible for the neurotoxic effects of the vPLA$_2$s receptor of PLA$_2$), and the surfactant lung protein. vPLA$_2$s also link to proteins like pentraxin, reticulecalbin, and Xa factor. The vPLA$_2$s are also associated with inhibitor sera proteins belonging to the type-C lectins superfamily ("three fingers" and proteins rich in leucines repetitions).[87,88] The structural and functional similarity of these different inhibitor proteins and other acceptor proteins of PLAs suggests that the physiological functions of the interaction among these molecules is not limited only to the protection against the toxic activity, but may also have a regulator role in the vPLA$_2$s activities.[89] Recently, Pineda et al.[90] established the protective action of the factor (s) isolated from *D. marsupialis* sera on hemorrhagic and proteolytic effects induced by *Porthidium langsbergii hutmanni* venom and its proteolytic fraction P2Plh. The inhibiting fraction, 0.15Dm, had an ED_{50} for P2Plh of 198 mg/kg, which was 43.13% higher compared to that of crude *D. marsupialis* sera (470 mg/kg).

The possibility that the natural inhibition of the toxicity of different venom components lay in one molecule has been initially misled for serum of *D. virginiana*,[78,79] and later for *D. marsupialis*. This came to light with the reported existence of the DM40 and DM64 proteins.[84,91] The fact that the DM43 protein did not have antimyotoxic activity and it did not form a complex with jararhagina C containing no metalloproteinase domain[85] reinforced the idea that the *Didelphis* natural inhibitors are very specific, and that this specificity has been a functional coevolution with the varied toxins present in the venoms.[8,92]

Reports suggest the existence of more than one protein in the sera of these opossums involved in the inhibition of venom proteolytic activities. Based on previous studies relating to the growing divergence of the number of inhibitors characterized, the question arises on how these different inhibitors function. It is possible to consider certain aspects:

1. varied inhibitors can exercise their neutralizing ability in an isolated manner, as units with a high specificity for the "target enzyme",
2. inhibitors can act as subunits with other inhibitors to favor inhibition,
3. inhibitors can exist as a mixture of specific and nonspecific inhibitors that act separately, and
4. they can exist in a combination of some or all of the above aspects.

Contrary to their structure and mechanism of action, the most coincidental aspect attributed to the inhibitors is their acidic nature (a pI near 4). It is feasible that the acidic portion of the inhibitors is complemented with the basic portion of the toxins, and in this manner the probability of approaching their active forms is favored.[35]

INHIBITORS IN THE SERA OF OTHER RESISTANT MAMMALS

Although the opossum has been the animal most studied for its ability to neutralize snake venom, other neutralization studies have been carried out with other mammalian sera. As stated earlier, the first studies on the resistance of warm-blooded animals to snake venoms were carried out in 1895 by Phisalix and Bertrand.[17] They were able to determine that the *Erinaceus europeus* was 40 times more resistant than the guinea pig, and the resistance was due to a serum factor, since the *E. europeus* serum was able to protect the guinea pig against the same venom. Phisalix and Bertrand[17] reported that the hedgehog (*E. europaeus*) had resistance against the venom of the European viper (*Vipera berus*), and later de Wit and Westrom[93,94,95] found at least ten protease inhibitors in its sera. The natural resistance to venoms has also been reported for other endothermic animals for over a century.

The ichneumon mongoose (*H. ichneumon*) and the Indian mongoose (*H. edwardsii*) were also shown to have a natural tolerance to snake venom.[15,96,97] Ovadía and Kochva[12] reported the lethal neutralizing effect of sera of several species of mammals (*H. ichneumon*, *Felis tames*, *Oryctologus cuniculus*, *Homo sapiens*, *E.europeus* and *M. auratus*) in mice via intravenous administration of six venoms (*Vipera palaestinae*, *Echis colorata*, *Pseudocerastes fieldi*, *Aspis cerastes*, *Walterinnesia aegyptia,* and *Naja nigricollis*). The major resistance was observed in the hedgehog (*E. europeus*), the hamster (*M. auratus*), and the mongoose (*H. ichneumon*). The mongooses, in particular,

were resistant to 20 LD_{50} of *W. aegyptia* venom and to 10 LD_{50} of *N. nigricollis*; however, their sera did not protect mice from these venoms; and thus, the natural resistance in these species was due to nonhumoral factors. In this study, the *H. ichneumon* showed resistance to some neurotoxins of Viperidae and Elapidae snakes. The hamster (*M. auratus*) was also shown to be resistant but to a lesser degree than the mongoose.

Three antihemorrhagic factors were isolated from *H. edwardsii* by Tomihara et al.[97] The factors were named AHF-1, AHF-2 and AHF-3 and were purified by a combination of gel filtration on a Sephadex G-200 column and high performance liquid chromatography. The three antihemorrhagic factors neutralized the hemorrhagic activity of HR-1 and HR-2, the hemorrhagic proteins from the venom of the habu snake. These factors had molecular weights of approximately 65 kDa, and pI values between 2 and 11.0 and were stable at temperatures ranging from 0 to 60°C. This was the first evidence for the presence of different antihemorrhagic factors against snake venom in mammalian sera. Precipitant tests revealed that the antihemorrhagic factors were not immunoglobulins, but were similar to those reported by Menchaca and Pérez.[69] In addition, the three antihemorrhagic factors also neutralized hemorrhagic factors of various snakes such as *A. b. blomhoffi*, *B. jararaca* and *Bitis a. arietas.*[15]

Pérez et al.[23] through an observation of how a grey woodrat (*N. micropus*) could survive the bite of a rattlesnake, used serum protection and antihemorrhagic tests to determine the neutralizing ability of *N. micropus*, rattlesnake and venom immunized goat sera against *C. atrox* venom. The sera of *N. micropus* were able to neutralize the hemorrhagic activity of *C. atrox* venom with a titer of 64, while snake and goat sera neutralized with a titer of 256. On the other hand, immunized goat sera were able to withstand 77 mg/kg body weight of venom, followed by 47 and 29 mg/kg for snake and woodrat sera, respectively. The LD_{50} for *C. atrox* venom in *N. micropus* was 1121 mg/kg body weight which was 140 times more resistant than control laboratory mice. In light of the woodrat study, these same investigators[22] explored the resistance of 40 species of mammals to *C. atrox* venom, detecting resistance in 16 of these animals.

Not all the animals that presented resistance to *C. atrox* venom at a high titer of antihemorrhagic activity were able to form rings in the precipitation tests. Seven warm-blooded animals had significant antihemorrhagic titers ranging between 16 and 256. Aside from the *D. virginiana* sera having the highest neutralizing ability, it was reported that the gray woodrat (*N. micropus*), Mexican ground squirrel (*Spermophilus mexicanus*), hispid cottonrat (*Sigmodon hispidus*) also had high resistance against *C. atrox* venom. The capacity of neutralization for *N. micropus* serum was further tested on six different snake venoms from the Crotalidae, Elapidae and Viperidae families, resulting in a much higher sera protection test against the venoms in the Crotalidae family

than that of the Viperidae or Elapidae families. Furthermore, Perez et al.[98] reported the resistance of the *S. hispidus*, *N. micropus*, and *D. virginiana* sera and muscle tissue extract against *C. atrox* venom. Very little antihemorrhagic activity was associated with the tissue extract. The sera of all three animals were able to neutralize the hemorrhagic activity, with *D. virginiana* being the highest followed by equal neutralization by *N. micropus* and *S. hispidus* sera. In the LD_{50} assay, both *D. virginiana* and *N. micropus* did equally well requiring about 1121 mg/kg, where as only 172 mg/kg was required in the presence of *S. hispidus* sera.

Pichyangkul and Pérez[99] isolated an antihemorrhagic factor from the sera of *S. hispidus*. It was purified by DEAE Sephadex and flat-bed isoelectrofocusing. It had no gelatinase or caseinase activity, and had a molecular weight of 90 kDa and pI of 5.4. The purified factor overcame 20 times the neutralizing capacity of the complete serum without forming a precipitate with *C. atrox* venom. It was suggested in this study that the factor was not antibody but probably an alpha-globulin. García and Pérez[100] reported the isolation of an antihemorrhagic factor of *N. micropus* serum. The factor had antihemorrhagic activity against *C. atrox* venom which was 8.9 times higher than the original crude woodrat serum. The factor had a pI of 4.1 by column isoelectric focusing and a molecular weight of 54 kDa by gel permeation column chromatography. The protein maintained its antihemorrhagic activity between 0 and 56°C and in a pH range of 3–10. To test if the antihemorrhagic proteins were immunoglobulins, a precipitant test was done between antihemorrhagin and crude *C. atrox* venom. However, no precipitation occurred indicating that the antihemorrhagins must not be immunoglobulins. This factor was also found to be similar in its characteristics to the factors of the Virginiana opossum (*D. virginianana*) and to the hispid cottonrat (*S. hispidus*). In another experiment, the antiproteolytic activity of these sera was evaluated for nine venoms of different species. *Didelphis virginiana* serum were the only that inhibited the proteolytic activity of *C. atrox* venom.

De Wit[101] studied the ability of Prairie vole (*Microtus ochrogaster*) and Woodrat (*N. floridana*) sera to neutralize the hemorrhagic activity against Osage copperhead (*Agkistrodon contortrix phaeogaster*) venom. Furthermore, de Wit and Weström[95] purified and characterized the *E. europeus* inhibitors, which was an antihemorrhagic macroglobulin fraction, composed of α_1-macroglobulin, α_1-β-macroglobulin and β-macroglobulin, and were indistinguishable according to their antihemorrhagic, proteolytic and immunoelectrophoreic activities.

Using hemorrhagic activity inhibition tests, the inhibitory capacity of the *E. europeus* muscular extracts was determined against 17 species of snake venoms. In the muscles of these animals reasonable titers of antihemorrhagic activity were obtained for the venoms of *Bitis* (3.5 to 18 mg) and *Vipera* (5.9 to 15 mg) snakes. The animals studied were obtained from areas free of snakes. The muscular macroglobulins responsible for the hemorrhage inhibition

presented a 700 kDa molecular weight, similar to the weights of the plasma macroglobulins. This evidence does not reinforce the idea of a resistance mediated by previous contacts or immunizations.[102]

Omori-Satoh et al.[103] compared the antihemorrhagic activity of the skeletal muscle extracts from several mammals. The extracts of the *E. europeus* (0.50 mg), *Crocidura russula* (2.05 mg) and *Talpa europaea* (2.95 mg) required smaller quantity than the rabbit (negative), mouse (18.5 mg), or hamster (18.0 mg) to neutralize the minimum hemorrhagic dose (2.5 mg) of *Bothrops jararaca* venom.

Poran et al.[104] proposed that the defenses developed by *Spermophilus beecheyi* species was the product of a long association between the *S. beecheyi* and its predator the *Crotalus viridis oreganus.* They demonstrated wide intraspecific and interspecies variations in the levels of natural resistance, reflected by LD_{50} differences, and approaches like mortality, necrosis, and time of scaring, when were compared with different populations of the *S. beecheyi* and the susceptible species *S. parryii*. Martínez et al.[24] were able to purify antihemorrhagic and antiproteolytic factors in another squirrel species, *S. mexicanus*. After having purified the factor using a five-step protocol, they obtained an increment in the specific activity of 27 times with regard to the complete serum. The factor had a molecular weight of 52 kDa and a pI of 4.9.

Biardi et al.[105] demonstrated that the California ground squirrels' (*Spermophilus beecheyi*) sera inhibited venom proteases from the venom of the Northern Pacific Rattlesnake (*C. o. oreganus*). These authors suggest that evolutionary specialization due to sera from rattlesnake-abundant habitats inhibited *C. o. oreganus* venom more effectively than venom from two allopatric rattlesnake species, *C. v. viridis* and *C. atrox*. Furthermore, Biardi et al.[106] analyzed four California ground squirrel populations for their ability to neutralize digestive and hemostatic effects of venom from three rattlesnake species. In Douglas ground squirrels (*S. b. douglasii*), they found that animals from a location where snakes are common showed greater inhibition of venom metalloprotease and hemolytic activity than animals from a location where snakes are rare. Effects on general proteolysis were not different. Douglas ground squirrels also reduced the metalloprotease activity of venom from sympatric Northern Pacific Rattlesnakes (*C. o. oreganus*) more than the activity of venom from allopatric Western Diamondback Rattlesnakes (*C. atrox*), but enhanced the fibrinolysis of sympatric venom almost 1.8 times above baseline levels. Two Beechey ground squirrel (*S. b. beecheyi*) populations had similar inhibition of venoms from Northern and Southern Pacific Rattlesnakes (*C. o. helleri*), despite differences between the populations in the locally prevalent predator. However, the venom toxins inhibited by Beechey squirrels varied among venom from Pacific Rattlesnake subspecies, and between these venoms and venom from allopatric Western Diamondback

Rattlesnakes. Blood plasma from Beechey squirrels showed highest inhibition of metalloprotease activity of Northern Pacific Rattlesnake venom, general proteolytic activity and hemolysis of Southern Pacific Rattlesnake venom, and hemolysis by allopatric Western Diamondback venom. These results reveal previously cryptic variation in venom activity against resistant prey that suggests reciprocal adaptation at the molecular level.

Galan et al.[107] characterized the venoms from two Southern Pacific Rattlesnakes for their ability to be neutralized by sera of *D. virginana*, *S. mexicanus*, *N. micropus* and *S. hispidus*. Opossum and Mexican ground squirrel sera did not neutralize the hemorrhagic activity of the venom of one of the two snakes used in this study. The sera of gray woodrats and hispid cotton rats neutralized all hemorrhagins in both *C. helleri* venoms. This is the first reported case in which opossum serum has not neutralized hemorrhagic activity of pit viper venom.

The sera of any animal are well known as one of the most heterogeneous fluids. However, based on these inhibitors' biochemical behaviors and low isoelectric points, it is possible to produce rational schemes of purification that may eventually lead us to the three dimensional structures that would allow the understanding of there interactions with venom toxins. The existence of multiple factors motivates to design strategies that facilitate to obtain qualitative and quantitative antivenom fractions, simplifying purification and defining the characterization of their components.

INHIBITORS IN PLANT EXTRACTS

Pharmacological properties of plant compounds had been presumed as snakebite antidotes.[108–112] These compounds are reported to have antihemorrhagic, antimyotoxic, anti-inflammatory, antiedematogenic, analgesic activities and protection from venom lethal activity. The fact that plant compounds are still very much in their infancy for treatment of snake envenoming and not to mention the stigma of being viewed as folk medicine, only a few of these studies have been recently scientifically validated and have been proposed to have important antiophidian properties in conjunction with conventional antivenoms.[27,113–122] Medicinal plants continue to be basically unnoticed and neglected; however, protective activity of plant extracts such as resverotrol (3,4′5-trihydroxy trans-stilbene) from *Cissus assamica* have been established in biological tests for snakebite envenoming.[123] Decrease of venom-induced effects of *N. nigricollis* in rodent by *Parkia biglobosa* extracts[124] and activation of coagulation activity by *Mucuna pruriens* seed compounds has also been reported.[125]

Several mixtures from plants used as all-purpose inflammatory agents also inhibit enzymes (e.g., phospholipase A_2) from snake venom.[126–127] Many of these plant compounds are hypolaetin-8-glucoside and associated

flavonoids. Stimulation of the immune system might also help to lower snakebite effects and improve the envenomation recovery by contributing to a faster elimination of the venom.[128] For example, chlorogenic acid operates as a treatment by linking to proteins through hydrophobic interactions and hydrogen bonds presenting anticomplementary action of the classical pathway.[129] A further direct antivenom action of these plant compounds would be the formation of complexes with venom components thus making them incapable to be active on receptors, or to act by competitive blocking of the receptors.[128] For instance, phenolic compounds, particularly complex polyphenols such as tannins, can link with proteins.[127]

Antivenom plant extracts include flavonoids (rutin, isoscutellarein, kaempferol, quercetin, hesperidin) protocatechic acid, caffeic acid derivatives (chlorogenic acid, cynarin), a catechin-gallo-catechin tannin, coumarins (bergapten), ar-turmerone, alkaloids (aristolochic acid), triterpenoids, triterpenes, coumestans (wedelolactone), sterols (sitosterol, stigmasterol, beta-amyrin), triterpenoid glycosides, lignoflavonoids and alkaloids (allantoin).[127,130,131] The structural similarities of certain plant chemicals used for snakebites are of an isoflavone skeleton, acidic in nature, and have a deoxygenated functionality. [131] Compounds from plants known to protect mice from ophitoxemia are usually nitrogen-free, low molecular weight which includes phenolics, phytosterols (β-amyrin and sitosterol) and triterpenoids[132,133] although exceptions were described with an alkaloid (12-methoxy-4-methylvoachalotine).[134] It is thought that these plant micromolecules interact with venom toxins and/or cell receptors displaying analgesic and anti-inflammatory properties.[135]

In the West Indies, Venezuela, South and Central America *Aristolochia rugosa* and *A. trilobata* are plants used against snakebites.[126,130,136–138] Aristolochic acid inhibits the activity of snake venom phospholipase (PLA_2) by forming a 1:1 complex with the enzyme.[112,126,139] Given that phospholipases participate in the inflammatory and pain route, their inhibition could improve the treatment of snake envenomations.[140] Moreno[139] proposed that aristolochic acid inhibits inflammation stimulated by immune complexes, and nonimmunological agents (croton oil or carrageenan). In Trinidad *Bauhinia excisa* vine decoction has been used for snakebites.[141]

Castro et al.[142] found total inhibition of *Bothrops asper* hemorrhagic activity with the ethanolic, ethyl acetate and aqueous extracts of plants having catequines, flavones, anthocyanines and tannins. These substances inhibitory effects are possibly due to the chelation of the zinc responsible for the catalytic activity of metalloproteases present in hemorrhagic venoms. Diminution in the strength of the envenomation effects may also be reached by a neutralization of the venom enzymes, peptides, polypeptides and active proteins.[112] Some plants that are used for *Crotalus* snake envenoming act by inhibiting venom proteolytic activities such as crotoxin-induced hemolysis, myotoxicity and hemorrhagic.[143]

Shamans in the northwest region of Colombia use *Costus lasius* and *Dendropanax arboreus* for snakebites and *D. arboreus* is also used by the Tacana in the Bolivian Amazon.[144] The active component is an acetylenic compound.[145] Other active substances in the leaf extract are dehydrofalcarinol, a diynene, falcarindiol, dehydrofalcarindiol, and two new polyacetylenes (dendroarboreols).[146] An ethanolic extract of *C. lasius* (leaves, branches and stem) to some measure neutralized *B. atrox* venom when it was experimentally injected into mice.[147] *Costus speciosus* have diosgenin, and beta-glucosidase which converts a furostanol glycoside (protogracillin) to a spirostanol glycoside (gracillin).[148,149] Mice isolated skeletal muscles *in vitro* exposure to *B. jararaca*, *B. jararacussu* and *Lachesis muta* venom and myotoxins (bothropstoxin, bothropasin and crotoxin) were inactivated by an *Eclipta prostrata* aqueous suspension containing wedelolactone, stigmaterol and sitosterol. Stigmaterol and sitosterol were less effective than wedelolactone, but interacted synergistically with it.[109] Most likely, these effects were the anti-proteolytic and antiphospholipase A_2 activities of *E. prostrata* and its constituents, which have anti-inflammatory actions and are known as anti-venom compounds.[126,127,130,131] Aqueous extracts of the plant *E. prostrata* leaves inhibited the liberation of creatine kinase from isolated rat muscle exposed to the crude venom.[108] Wedelolactone diminished the *Crotalus viridis viridis* (Prairie Rattlesnake) and *Agkistrodon contortrix laticinctus* (Broadbanded Copperhead) venoms' myotoxic effects and the effects of two myotoxins (PLA_2) isolated from both snakes.[143]

In Africa, snakebites in rural areas are usually treated with plant extracts.[150] Reports are established for *Steganotaenia araliacea*, *Combretum collinum*, *Solanum incanum* and 3 species of *Grewia* (*G. bicolor*, *G. fallax* and *G. truncata*). Additionally, species inside *Vernonia*, *Erythrina (E. abyssinica)* and *Sansevieria* (*S. kirkii*) genera were also document to inhibit snake venoms.[151] Another African plant used for snakebite treatments is the *Guiera senegalensis* (Combretaceae). The leaf extract of this plant, when preincubated with the venoms of *Echis carinatus* and *Naja nigricollis,* protects the mortality of mice after intra-peritoneal injection.[127] *Mucuna pruriens* extracts have been shown to have antagonizing effects against elapidae[152] and viperidae[153] venoms. In 2001, Guerranti et al.[125] examined the effect of *M. pruriens* extract (MP101UJ) on prothrombin cleavage by *E. carinatus* venom. Their studies showed that the protective effect of MP101UJ against *E. carinatus* venom was primarily prophylactic in which no direct inhibition of the toxic components occurs. The protection of mice when injected with MP101UJ days prior to venom introduction could be due to the formation of antibodies, as previously demonstrated by Aguiyi et al.,[152] and later confirmed by Guerranti et al.[154] Stem bark extract from the Nigerian *Parkia biglobosa* (Mimosaceae) significantly inhibited neurally evoked twitches caused by the venom *N. nigricollis* on chick biventer cervicis muscle preparation. The extract also protected the

cytotoxic actions of *Echis ocellatus* and *N. nigricollis* venoms on C_2C_{12} muscle cells. Furthermore, egg embryos exposed to lethal concentrations of *E. ocellatus* venom were protected and completely inhibited the hemorrhagic activity. Lethal activity was not protected when mice were injected i.p. with extract preincubated with *N. nigricollis* venom, whereas, 40% of the mice treated with extract and *E. ocellatus* venom survived.[124] Aqueous extracts from four West African plants (*Schumanniphyton magnificum*) bark (Rubiaceae), *Mucuna pruriens* var. *utilis* leaves (Leguminosae), *Strophanthus gratus* and *Strophanthus hispidus* leaves (Apocynaceae)) all generated a dose-related augment in the coagulation time of blood provoked by *E. carinatus* venom,[126] and perhaps would be valuable against bites from *Bothrops* species that produces hemorrhage at the bite site due to the inhibition of the coagulation activity.[155]

In Colombia, an extract of branches and leaves from *Passiflora quadrangularis* had reasonable neutralizing capability against the hemorrhagic effect of *Bothrops atrox* venom.[156] The plant contains passiflorene, nor-epinephrine, 5-hydroxytryptamine and flavonoids.[157] Núñez et al.[122] determined the inhibiting capability of 12 ethanolic extracts of plants against edema, defibrinating and coagulant effects of *B. asper* venom from Antioquia and Chocó, Colombia. The plants used were from leaves and branches of *Bixa orellana* (Bixaceae), *Ficus nympaeifolia* (Moraceae), *Struthanthus orbicularis* (Loranthaceae) and *Gonzalagunia panamensis* (Rubiaceae); the stem barks of *Brownea rosademonte* (Caesalpiniaceae) and *Tabebuia rosea* (Bignoniaceae); the whole plant of *Pleaopeltis percussa* (Polypodiaceae) and *Trichomanes elegans* (Hymenophyllaceae); rhizomes of *Renealmia alpinia* (Zingiberaceae), *Heliconia curtispatha* (Heliconiaceae) and *Dracontium croatii* (Araceae), and the ripe fruit of *Citrus limon* (Rutaceae). All extracts inhibited edema to a certain extend in a dose-dependent manner (59–76%); ten extracts neutralized the defibrinating effect by 100% and nine prolonged the venom induced coagulation time.

In Brazil, the aqueous extract of *Tabernaemontana catharinensis* inhibits the lethal activity (2 LD_{50}) of *Crotalus durissus terrificus* venom and 2 LD_{50} of crotoxin. Extracts and fractions of *T. catharinensis* also had potent antitumoral activity against human breast carcinoma (SK-BR3) cells.[158] The extract from *Cordia verbenacea* inhibited the edema induced by *B. jararacussu* venom. The extract of *C. verbenacea* was identified as rosmarinic acid, which contains anti-inflammatory and antimyotoxic properties against snake venoms and isolated toxins.[119] The aqueous extract from the plant *Pentaclethra macroloba* (EPema) fully inhibits the hemorrhagic and nucleolytic activities induced by several snake venoms.[113] EPema was able to completely inhibit the hemorrhagic activity of *B. atrox* and *B. jararacussu* venoms. This hemorrhagic inhibition is suggested to be an interaction of EPema with divalent metal ions and/or metalloproteases found in snake venom.

In the Republic of China, melanin extract from *Thea sinensis* Linn. (Black tea) was reported to have neutralizing effects against venoms of *A. contortrix*

laticinctus, *A. halys blomhoffii*, and *C. atrox*, with the greatest effect against venom of the *A. h. blomhoffii*. As far as inhibiting PLA_2 activity, 33.5, 33.9 and 37 % inhibition was achieved against the venoms of *C. atrox*, *A. c. laticinctus* and *A. h. blomhoffi*, respectively.[159]

In Thailand, *Barleria lupulina* is known as an anti-inflammatory and is used against snakebites.[160] Substances from *B. lupulina* leaves were acetylshanzhiside methyl ester, acetylbarlerin, shanzhiside methyl ester, ipolamiidoside, iridoid glucosides and barlerin.[161] The butanolic extracts of *Eclipta prostrata* inhibited the lethal activity of 2 LD_{50} of *Calloselasma rhodostoma* venom; however, an increase of extract diminished the survival rate of mice to 39%. The extracts had a partial inhibitory effect on the hemorrhagic activity, showed a very low anti-phospholipase A_2 activity, and no anticaseinolytic activity was present.[162] Plant polyphenols from the aqueous extracts of *Pentace burmanica*, *Pithecellobium dulce*, *Areca catechu* and *Quercus infectoria* were tested to inhibit lethal activity against 4 LD_{50} of *Naja kaouthia* venom but at varying tannin concentrations. The extracts were also able to inhibit 1 minimum necrotizing dose (MND), whereas *P. burmanica* extract required higher tannin content. Furthermore, inhibition of acetylcholinesterase activity was almost completly achieved by *P. dulce*, *P. burmanica* and *A. catechu* extracts. *Quercus infectoria* required a higher tannin concentration to achieve inhibition to the same degree.[121] Tannin is found throughout the plant kingdom and is used as astringent, for wound healing, as an anti-inflammatory agent, among others. Tannin produces a complex with proteins, including snake enzymes, allowing a natural healing process, reducing loss of fluids and preventing external toxins from being re-absorbed.[163]

In India, the methanolic root extracts of *Vitex negundo* Linn. and *Emblica officinalis* Geartn. significantly inhibited the lethal activities of venoms from *V. russelii* and *N. kaouthia*. The hemorrhagic, coagulant, defibrinogenating and inflammatory activity of *V. russelii* was also effectively inhibited by both of these plant extracts.[164] Root extract of *Mimosa pudica* inhibited the hyaluronidase activity of three Indian snakes, *N. naja*, *V. russelii* and *E. carinatus*. The hyaluronidase activity was decreased to 0, 20 and 25% in the venoms of *E. cariantus*, *N. naja* and *V. russelii*, respectively using root extract concentration of 400 μg/mL. The protease activity was decreased to 0% at 200, 300 and 400 μg/mL of extract in the venoms of *N. naja*, *V. russelii* and *E. carinatus*, respectively[165] The seed extract of *Tamarindus indica* (Leguminosae) inhibits, in a dose-dependent manner, the PLA_2, protease, hyaluronidase, L-amino acid oxidase and 5'-nucleotidase enzyme activities from venom of *V. russelli.*[116] In addition, the extract inhibits the degradation of the Bβ chain of human fibrinogen and indirect hemolysis caused by this venom.

Hyaluronidases are found in venoms and are known as the "spreading factors" because they degrade the hyaluronic acid present in the endothelial lining of tissues, which in turn facilitate the diffusion of toxins into the tissue

of victims.[166] A glycoprotein isolated from the plant *Withania somnifer*, inhibits the hyaluronidase activity of both viper (*Daboia russellii*) and cobra (*Naja naja*) venoms.[117]

Extract of *Casearia sylvestris* (Flacourtiaceae) inhibits the toxic effects of some *Bothrops* and *Crotalus* venom including their purified PLA_2.[114] Neuromuscular blockade was significantly prevented (93–97% protection) when preincubating isolated PLA_2 with *C. sylvestris* extract. Root extracts of B-sitosterol and stigmasterol isolated from the root extract of *Pluchea indica* Less. (Asteraceae) significantly neutralized the lethal (up to 6.2 LD_{50}), hemorrhagic (up to 5 MHD), defibrinogenation (up to 1 MDD), edema (up to 3 MED) and PLA_2 (up to 6 units) activities of venom of the *Daboia russellii*. Furthermore, the lethality (up to 4 LD50), cardiotoxicity (up to 3 MCTD), neurotoxicity (up to 2 MNTD), respiratory changes (up to 2 MAD) and PLA_2 (up to 8 units) activities from venom of the cobra *Naja Kaouthia* were also affected by this plant extracts.

ALTERNATIVE METHODOLOGIES FOR OBTAINING SNAKE VENOM INHIBITORS

In reptiles and mammals, sera metalloproteases inhibitors have been conventionally obtained by slow processes, with low quantitative yield involving the death, or eventual sacrifice of the animal.[167,168] However, other production methodologies have been proposed.[169] They have proven the capacity of cellular synthesis that possesses the marsupial's hepatocytes, which hold an elaborated apparatus of molecular expression developed to respond to the antihemorrhagins necessities and diverse organic molecules.[78,79]

The primary culture techniques of long term hepatic spheroids of mammals have been characterized as a well differentiated model of *in vitro* study, where cells coexist with a great metabolic capacity and of cytoarchitectonic rearrangement.[170–173] Based on the hypothesis that it is possible to obtain different antihemorrhagins (metalloproteasas inhibitors) from the supernatant of *D. marsupialis* hepatic spheroids primary cultures, characterizing and purifying these molecules from a less heterogeneous source of proteins is an attractive option.

Sánchez et al.[174] informed that the metalloprotease inhibitors bound to a specific antihemorrhagin can be detected with monoclonal antibodies produced against the antihemorrhagin in a five-step Western Blot analysis. A monoclonal antibody against a hemorrhagic inhibitor was developed (DV-2LD#2) that binds specifically to the antihemorrhagin of *D. virginiana* sera previously incubated with venom. The specificity of the antibody, DV-2LD#2, was determined for its inhibitory ability of the antihemorrhagins in the sera. The DV-2LD#2 does not react nor inhibits the antihemorrhagins of the sera of *N. micropus*. It was proposed that partially purified hemorrhagic

fractions could be detected without having to depend on the life and costs of experimental animals.

The purification of molecules responsible for the natural inhibition initially advanced with antihemorrhagic activity studies.[20,69,75,79,100] However, the last reports[84,85] and the generation of assays with more specific and innovative techniques have demonstrated that it is possible to discern among the different antitoxic properties, and that we can venture in the idea that the inhibition responsible for the natural resistance could be attributed, in the case of *Didelphis*, to different molecules with distinct homology grades.

CONCLUSIONS

Antivenoms produced in horses and sheep still remain the widely used treatment for snake envenoming to date, despite the continuing efforts to develop new and improved treatments. Antivenoms at times do not provide sufficient protection against the varying toxic and lethal effects of venoms due in part to the well-documented interspecies venom variation.[175–179] It is perhaps feasible to propose a combination of natural animal inhibitors, plant extracts and traditional manufactured antivenoms as an effective treatment in ophidic accidents.

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