Chapter 1

General Introduction and Review of literature

1. INTRODUCTION

Snakes are one of the most enigmatic and enthralling creations of "Nature" which have made mankind inquisitive since time immemorial. They have been always looked up with fear, fascination and are either considered sacred or evil since the age old civilizations and cultures beyond recall. Snake envenomation is a very alarming health concern in the tropical and sub-tropical countries of the world due to severe pathological conditions and lack of proper clinical management. Nevertheless, snake venom is "Blessings in disguise" i.e., in spite of its pathological aspects, some of the venom proteins are also bestowed with potential therapeutic benefits which have always attracted researchers into the venom field (1-5). Some of the multifaceted benefits of venom research include –

1) Help understand the pathophysiological action of the venom components on the physiological system of the victim.
2) Unfold the structure-function relation of the venom toxins.
3) Propose new strategies to design antidote for the better clinical management of snakebite victims.
4) Develop prototypes for the pharmaceutical industries.
5) Last but not the least; explore the possibilities to identify unique pharmacologically active molecules with potential therapeutic applications.

1.1 Snake venom

1.1.1 Snakes

Snakes are carnivorous, ectothermic reptiles which were proposed to have evolved from the burrowing or aquatic lizards during the Cretaceous period around 135 million years ago (6-8). Based on the Linnean taxonomy, snakes belong to the kingdom of Animalia, phylum Chordata, class Reptilia, order Squamata, clade Ophidian and suborder of Serpentes. They rely on the sense of hearing and smelling

to communicate with the surrounding. The sense of smell is accomplished by a pair of nostrils, a sensitive fork-like tongue and the Jacobson's organ (9). Snakes can hear low-frequency sound (200-500 Hz) by the side of the skull as they lack ears or eardrums and can feel vibrations through the ground (9). Some snakes like the pit vipers can also sense the infrared radiations with a pair of pits (even a temperature change of 0.003^{o}C) which helps in locating the warm-blooded preys (9). Ecdysis or moulting is observed in snakes intermittently throughout their life cycle which helps in the renewal of the older worn out skin and removes any kind of parasitic or fungal infections dwelling over it (10). Musking is highly species specific in snakes which act as anti-predatory response and helps attract mating partners during the breeding period (9). Snakes display a lucid and vibrant scaling pattern with different colours and markings which help them camouflage with the surrounding (11-13). They reproduce by internal fertilization and are mostly oviparous (~70%). However, a few of them like Russell's viper and Rattle snake are ovoviviparous while boa constrictor and green anaconda are reported to be viviparous in nature (14-16). Snakes are reported to be prevalent almost throughout the globe including the land and the large water bodies from the Arctic circle in the northern hemisphere and Australia in the southern hemisphere (17). However, they are absent in Antarctica, Ireland, Iceland, and New Zealand along with a few small islands of central Pacific and Atlantic ocean owing to the freezing climatic conditions not suitable for cold-blooded animals like snakes (17). Snakes mostly prefer a warmer climate which would help them maintain their body temperature. As such deserts and tropical rain forests are some of the most profusely inhabited places on earth by snakes (18,19). However, there are also reports when snakes are reported near the hot springs and at higher altitudes (20). In addition to these, snakes are also profusely distributed in the aquatic system like sea snakes (*Laticauda colubrine*, *Laticauda laticaudata*). Surprisingly, snakes are also arboreal, dwelling on the trees like the flying tree snake (Chrysopelea paradise) prevalent in South and Southeast Asia (21).

1.1.2 Venomous and non-venomous snakes

Presently 3,567 species of snakes have been discovered which are classified into 27 snake families that are categorized into 6 superfamilies (based on the recently updated

data of the reptile database http://www.reptile-database.org/db-info/taxa.html#Note2) (22). The superfamily of Acrochordoidea (1 family), Uropeltoidea (3 families), Pythonoidea (3 families), Booidea (1 family) and Typhlopoidea (5 families) include the non-venomous snakes. The finest examples of non-venomous snakes include pythons, anaconda, boas, blind snake, thread snake etc. On the other hand, the superfamily of Colubroidea is divided into 10 venomous snake families. Out of these, the elapidae and the viperidae family include some of the most venomous snakes in the world like cobras, vipers, kraits, sea and coral snakes, etc. The venomous snakes are called so because they have a venom apparatus consisting of an exocrine gland and a venom delivery system for inflicting a bite onto the prey or the victim. These venomous snakes primarily use the venom as an offensive tool in prey immobilization and digestion while secondarily as a defensive weapon to defend themselves against the predators (23,24). On the other hand, the non-venomous snakes like python, anaconda which lack the venom apparatus, use biting, grasping and constriction mechanism to subdue and strangulate their prey (6,25). A detailed list of snakes categorized into various superfamilies, families and subfamilies have been shown in Table 1.1.

Table 1.1 List of venomous and non-venomous snakes (http://www.reptile-database.org/db-info/taxa.html#Note2) (22)).

Sl No	Superfamily	Family	Subfamily	Example
1.	Acrochordoidea	Acrochordidae	-	File snake
2.	Uropeltoidea	Anomochilidae	-	Dwarf Pipe Snake
		Cylindrophiidae	-	Asian Pipe Snake
		Uropeltidae	-	Shield-tail Snake
3.	Pythonoidea	Loxocemidae	-	Mexican burrowing python
		Pythonidae	-	Python
		Xenopeltidae	-	Sunbeam Snake
4.	Booidea	Boidae	Boinae	Boa
			Ungaliophiinae	Dwarf Boa
			Erycinae	
			Calabariinae	
			Candoiinae	
			Sanziniinae	
			Charininae	
5.	Typhlopoidea	Anomalepididae	-	Dawn Blind Snake
		Gerrhopilidae	-	Blind Snake

		Typhlopidae	-	Blind Snake
		Leptotyphlopidae	Leptotyphlopinae	Slender Blind Snake
			Epictinae	
		Xenotyphlopidae	-	
6.	Colubroidea	Colubridae	Calamariinae	
			Colubrinae	
			Grayiinae	
			Sibynophiinae	
		Dipsadidae	-	
		Lamprophiidae	Aparallactinae	
			Atractaspidinae	Mole Viper
			Lamprophiinae	
			Psammophiinae	
			Prosymninae	
			Pseudaspidinae	
			Pseudoxyrhophiinae	
		Natricidae	-	
		Pseudoxenodontidae	-	
		Elapidae	Elapinae	Cobras, Coral Snake, mambas, kraits
			Hydrophiinae	Sea Snake
		Homalopsidae	-	
		Pareatidae	-	
		Viperidae	Azemiopinae	Fea's viper
			Crotalinae	Rattle Snake, New world vipers
			Viperinae	Old world vipers
		Xenodermatidae	-	
7.	Currently not assigned to any superfamily	Aniliidae	-	Pipe Snakes
		Bolyeriidae	-	Round Island Boas
		Tropidophiidae	-	Dwarf Boas
		Xenophidiidae	-	

1.1.3 Venom gland

The primitive venom producing apparatus, known as the Duvernoy's gland (located behind the eyes) was developed in the colubrids. These are anatomically and functionally different from the venom gland found in the elapids and the viperids (26). Duvernoy's gland plays a key role in prey swallowing and digestion, contrary to the advanced venom glands which also play a crucial role in prey capture and immobilization apart from digestion (26). In a generalized form, a snake venom apparatus consists of a pair of modified bilateral exocrine gland situated in the upper jaw and modified maxillary teeth called the fangs (6).

The venom apparatus is divided into 4 categories based on the structure, position and morphology of the fangs (Figure 1.1), namely;

i. *Aglyphous:* This apparatus consists of a fangless Duvernoy's gland (6). It is observed in colubrid snakes. Here the snake first firmly holds the prey; chews it and then releases toxins from the gland for digestion.
ii. *Opisthoglyphous:* In this case, the Duvernoy's gland is attached to a pair of rear fangs behind the eye at the back of the maxillary bone (6). They are efficient in releasing the venom compared to the snakes with aglyphous dentition. It is also observed in colubrid snakes where the snake holds its prey and inflicts it with the fangs to cause multiple injuries.
iii. *Proteroglyphous:* These are the front-fanged snakes where the fangs are situated anterior to the maxillary bone (6). Snakes belonging to the elapidae family harbour this dentition system.
iv. *Solenoglyphous:* These are also front-fanged snakes. However, they have long syringe like hollow fangs with a closed venom canal which is connected to a wide lumen to store a large amount of venom (6). This dentition system is observed in the viperidae family is considered to be most efficient in terms of speed of infliction and amount of venom injection (6).

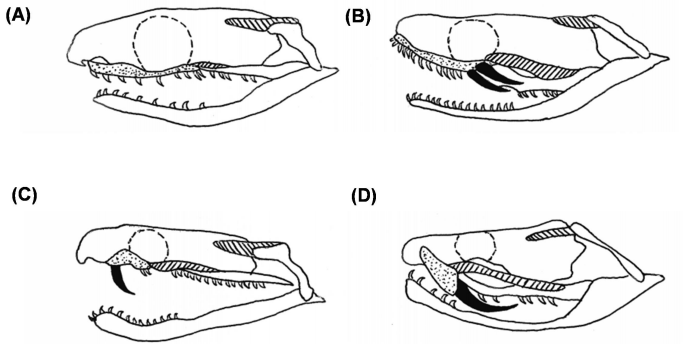

Figure 1.1: Pictorial representation of the different types of dentition present in the venomous snakes. (Adapted from Shine 1995 (27)). **(A):** Aglyphous **(B):** Opisthoglyphous **(C):** Proteroglyphous **(D):** Solenoglyphous

1.1.4 Composition of snake venom

Venom production is an expensive metabolic affair for venomous snakes along with digestion and moulting (28-30). The snake venom is a rich repertoire of both proteinaceous and non-proteinaceous components like amine, carbohydrates, lipids, nucleic acid, citrate, small organic molecules and metal ions (31-33). Out of these, ~90% of the total dry weight of the crude venom is considered to be composed of proteins while the rest comprise the non-protein components. Metal ions like Mg^{2+}, Ca^{2+}, Zn^{2+} and Cu^{2+} are reported from snake venom which function as co-factors to many proteins present in the venom (34). Snake venom proteins can be broadly classified into enzymatic and non-enzymatic protein superfamilies based on the primary sequence and three-dimensional (3D) structures (35,36). Table 1.2 and 1.3 briefly describes the various enzymatic and non-enzymatic protein families of snake venom respectively. It is reported that the venom of a single venomous snake may consist of different protein families each with various isoforms (35-37). For example, several isoforms of crotoxin, a phospholipase A_2 (PLA_2) enzyme have been reported from the venom of *Crotalus durissus terrificus* (38), Similarly, four isoforms of fibrinogenolytic serine protease activating coagulation factor V were purified from the venom of *Daboia russelii russelii* (39).

The evolution of snake venom proteins is an adaptive trait where peptide engineering occurs by natural selection based on the arm race of predator-prey relationship (40-43). It is an excellent example of convergent evolution to adapt to a particular ecological niche (42). Most of the snake venom proteins are proposed to have evolved from the normal physiological genes by the process of gene duplication and selective pressure which then gets over expressed in the venom gland (43,44). For example, the blood coagulation factor X and V are expressed in the liver of vertebrates, however, in viper venom gland, it is expressed in both liver and venom gland (44,45). Similarly, different groups of phospholipase A_2 enzymes (IB, IIA and III) are expressed in the various organs like pancreas, liver, kidney, etc. but in snake venom it is expressed as a toxin in its venom gland (44).

Table 1.2 List of enzymatic protein families of snake venom

Protein family	Characteristics	Physiological action
Phospholipase A_2 (PLA_2) enzymes	These are water soluble esterolytic enzymes which catalyse the hydrolysis of glycerophospholipid at the *sn*-2 position in the water–membrane interface liberating lysophospholipids and fatty acids. The molecular mass ranges from ~13-15 kDa with 120-130 amino acid residues and 7 disulphide bridges. They belong to group IA and IIA & B $sPLA_2$ enzymes (46).	They damage cell membrane, are myotoxic, myonecrotic, cardiotoxic, haemolytic, anticoagulant, antiplatelet, neurotoxic, induce convulsion, and inflammation (46,47).
Snake venom serine protease (SvSP)	They catalyse reactions involving kallikrein kinin, fibrinolysis, platelets and cleavage step involves acylation and deacylation. The molecular mass ranges from ~31-36 kDa.	These enzymes disrupt the various aspects of the haemostatic system (48,49).
Snake venom metalloprotease (SvMP)	They Zn-dependent enzymes which belong to M12 subfamily of metalloproteases that can be chelated by EDTA. These are classified into 3 classes based on the domain organization into PI a, PII (a, b, c, d, e) and PIII (a, b, c, d) Molecular mass ranges from ~20-85 kDa (50).	Cause local and systemic injuries which include myonecrosis, haemorrhage, edema formation, blistering and pre-digestion of prey (50).
L-amino acid oxidase (LAAO)	They catalyse the oxidation of hydrophobic L-amino acids releasing α-keto acid, ammonia and H_2O_2. The molecular mass ranges from 57-68 kDa for monomeric forms while the homodimeric forms are high molecular weight proteins (51,52).	H_2O_2 causes edema formation, ADP or collagen-induced platelet aggregation inhibition or activation, apoptosis, antibacterial effect, antiparasitic, anticoagulant, haemolytic and haemorrhagic effects (51,52).
Phosphodiesterase (PDE)	They act as endonucleases on both double and single stranded RNA and DNA releasing 5'-mononucleotides and are high molecular mass (> 90 kDa) proteins (53).	They reduce cyclic, di & trinucleotides, cause hypotension and hinder locomotion (54).
Nucleotidase (5'-NUC)	They belong to the metallophosphatase superfamily and molecular mass ranges from ~53-82 kDa (53).	They cause hypotension & paralysis (54).
Hyaluronidase	These are endo-β-glycosidases which degrade hyaluronan in the extra cellular matrix of soft connective tissues. The molecular mass ranges from 33-110 kDa and are active at neutral pH (55).	These enzymes lower interstitial viscosity, aid in rapid diffusion of venom components & cause local tissue damage (56).

Acetylcholinesterase (AChE)	These enzymes are serine hydrolases belonging to the esterase family. Catalyse the hydrolysis of acetylcholine into acetate and choline. They exist as monomers in snake venom with a molecular mass range 65-69 kDa (57).	They hinder in the transmission of acetylcholine at the synaptic and post-synaptic junctions inducing tetanic or paralysis (58).

Table 1.3 List of non-enzymatic protein families of snake venom

Protein family	Characteristics	Physiological action
Three-finger toxin (3FTx)	These are low molecular mass (~6-9 kDa) proteins with 60-74 amino acid residues. Have 3 β-stranded loops cross-linked by 4 disulphide bonds (59,60).	They are neurotoxic, cardiotoxic, anticoagulant and cause paralysis (61).
Kunitz-type serine Proteinase inhibitor (KSPI)	They are low molecular mass (~6-7 kDa) proteins having 50-60 amino acid residues with a conserved Kunitz motif typical to bovine pancreatic trypsin inhibitor (BPTI) (62,63).	They inhibit trypsin, chymotrypsin, potassium and calcium ion channels. They cause fibrinolysis, anticoagulation, hinder locomotion and haemorrhage (64,65).
Cysteine-rich secretory proteins (Crisps)/Helveprins	They are glycoproteins rich in large number of cysteine residues in the C-terminal region and have 16 conserved cysteine residues and molecular mass ranges from ~21-29 kDa (66).	They cause apoptosis, paralysis, hypothermia and inhibit cyclic nucleotide-gated ion channels in photoreceptor and olfactory cells, potassium activated smooth muscle contraction, vascular smooth muscle contraction (67-70).
Snake C-type lectin -related proteins (Snaclecs)	Based on structural and functional properties they are classified into two types- C-type lectin-related proteins and sugar binding snake lectins. Molecular mass ranges from ~26-28 kDa and are present as homodimers (71).	Cause haemorrhage and lethargy (72).
Disintegrins	They are rich in cysteine residues liberated by the proteolytic cleavage of multidomains of metalloproteases in the venom which are mostly observed in	cause inhibition of integrin receptors, inhibit cell adhesion and migration via collagen I and II, anti-angiogenic and interact with α1 and β1 of integrin (75)

	class PI and PII of metalloproteases. Have 60-70 amino acid residues and range 4-14 kDa (73,74).	
Vascular Nerve Growth factors (VNGF)	They are members of neutrotrophin family which are crucial for maintaining neuronal cells. The molecular mass ranges from ~14-32.5 kDa (76).	Cause apoptosis & vascular permeability (77).
Vascular Endothelial growth factor (VEGF)	They stimulate vascularization and angiogenesis.	Cause hypotension, increase capillary permeability, cell proliferation & migration (78).

Small organic and inorganic molecules: Purines and pyrimidines are the cleavage products of DNA degradation by PDE and 5'NUC in the snake venom. These byproducts are reported to induce hypotension, paralysis, apoptosis & necrosis (54,79). Citrate is a small non-organic molecule which is known to have a buffering and stabilizing effect on the venom (32). It inhibits metal-dependent snake venom enzymes by chelating metal ions (80). However, citrate is not reported to cause any pathological effect during snake envenomation.

Thus, during envenomation, the complex mixture of venom components work in a synergistic manner to target various vital organs and physiological system of the prey or the victim to exhibit a myriad of pharmacological effects which eventually might lead to death (43). These pathological states can be broadly classified into local signs which manifest at the site of bite followed by the systemic signs and symptoms with the progression of the venom into the blood stream of the victim. Some of the most common pathophysiological signs of snake envenomation are shown in Figure 1.2. The most profound pathophysiological signs and symptoms include necrosis, pain, swelling, blistering, respiratory distress, cardiac arrest, haemorrhage, paralysis, etc. (81). From the clinical perspective, viperidae venom is considered haemotoxic (80-90% of enzymatic protein) while the elapidae venom is classified as neurotoxic (25-70% of enzymatic protein) (31,82). Nevertheless, researchers have also isolated and

characterized some venom proteins with promising therapeutic benefits (4,5). Recently, a number of snake venom proteins have been isolated and characterized which are reported with promising therapeutic potential for the treatment of cancer, AIDs and many more incurable diseases (1-3,83).

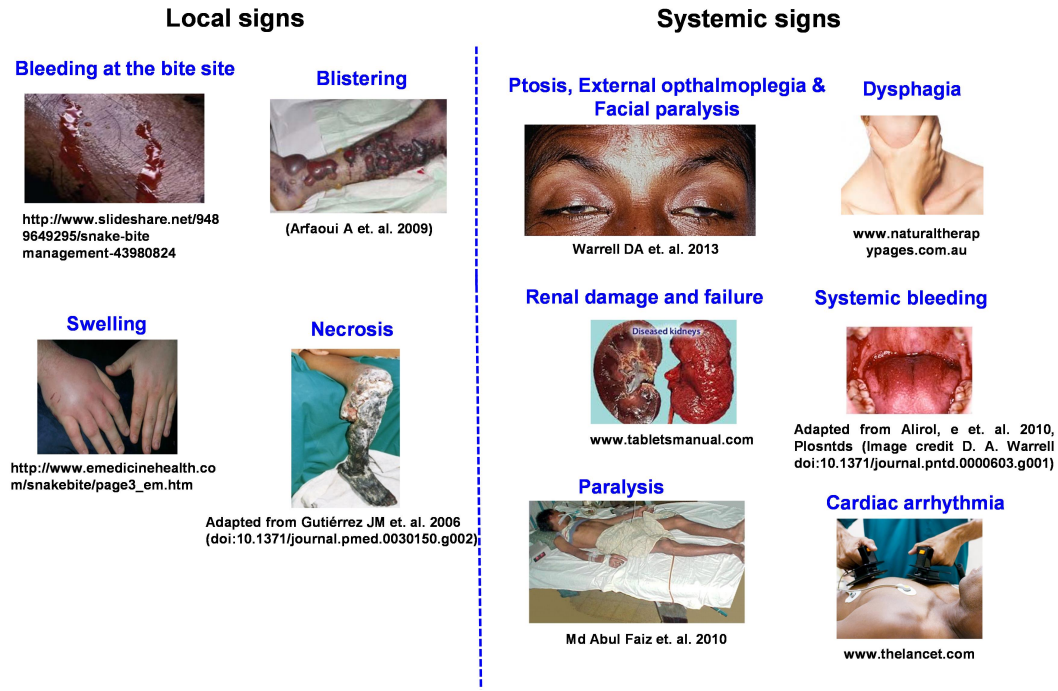

Figure 1.2: Schematic representation of the local and systemic signs and symptoms observed in snakebite victims.

1.1.5 Snakebite problem

Snake envenomation is one of the most serious medical conditions of the tropical and sub-tropical countries of the world especially Latin America, Sub-Saharan Africa, South and Southeast Asia, and Oceania (84,85). Every year thousands of people are inflicted with venomous snakebites causing a large number of morbidity and mortality. In the year 2010, Gutiérrez J.M. and co-workers reported that annually 2.5 million cases of snakebite occur across the globe out of which 85,000 cases lead to fatalities (86). Ironically, the scenario is far worst than reality in the developing countries of Asia and Africa where the medical facilities are not at par especially in the rural areas. The developing countries of Asia alone bear a burden of 1.2 million

envenomation cases which leads to more than 60,000 deaths every year (86). Snake envenomation is considered as an occupational health hazard and "disease of poverty" as it affects the lives of the rural communities like farmers, tea planters and labourers customarily (82,87).

Considering its severity on the socio-economic life of the poor people and its high rate of occurrence, the World Health Organization (WHO) had categorized snake envenomation issue as "Neglected Tropical Diseases" (NTD) in the year 2009 (87). However, recently it has been removed from the list of NTD by WHO which has lowered its importance at the social front further.

Owing to the rich vegetation and warm climatic conditions, India is an abode to ~285 species of snakes, out of which 50 species are identified to be venomous, most of which belong to the family of elapidae (cobras and kraits, water snakes) and viperidae (true vipers and pit vipers) (9). Some of the most prevalent venomous snakes of India are listed in table 1.4.

Table 1.4: List of venomous snakes in India (9,82).

Family	Species
Elapidae	*Naja naja*, *N. kaouthia*, *N. oxiana*, *N. sagittifera*, *Ophiophagus hannah, Bungarus caeruleus, B. fasciatus, B. niger, B. sindanus*
Viperidae	*Daboia russelii, Echis carinatus, Echis carinatus sochureki, Hypnale hypnale, Cryptelytrops albolabris, Cryptelytrops purpureomaculatus, Trimeresurus malabaricus, Trimeresurus gramineus, Trimeresurus macrolepis, Trimeresurus strigatus, Trimeresurus medoensis, Trimeresurus popeiorum, Trimeresurus cantori, Trimeresurus andersoni, Trimeresurus erythrurus, Trimeresurus albolabris, Trimeresurus labialis, Macrovipera lebetina, Gloydius himalayanus*

India, being an agricultural based tropical country encounters snake envenomation cases every year, especially during the monsoons and summer seasons (88). It is reported to be the worst affected area globally with respect to snake envenomation causing 35,000 to 50,000 deaths every year (88-90). Ironically, the epidemiological data available so far are based on the hospital records only, which do not actually

reflect the real envenomation scenario in India (82). This is because most of the rural victims prefer traditional healing practices due to which the envenomation cases are not even registered on hospital records (82,91). The use of traditional medicines and the wrong first aid management like the practice of tourniquets, suction, cutting and cryotherapy make the clinical symptoms of the victim very critical and deprives him or her of receiving proper medical attention at the right hour (82,92).

Considering the severity of the snakebite accidents and the dearth of proper clinical management, the Ministry of Health and family Welfare, Government of India has provided a blueprint of guidelines "National Snake Bite Management Protocol" to deal with envenomated patients more precisely (82). The only therapy available for the treatment of snakebite victims in India is the administration of the polyvalent antivenom produced against the venom of "Big Four" snakes. These includes *Naja naja* (Indian spectacled cobra), *Bungarus caeruleus* (Common krait), *Daboia russelii* (Russell's viper) and *Echis carinatus* (Saw-scaled viper) which are considered to be responsible for most of the fatal snakebites cases (93). However, the inadequate supply of antivenom, unaffordability by the rural mass, poor storage conditions and inadequate medical infrastructures in the rural dispensaries make the accessibility of this therapy to the victims quite impossible (82,92). Nonetheless, the lack of proper training, knowledge and awareness of the medical practitioners in understanding and dealing with the subtle pathological conditions of the snakebite victims make the scenario far more critical in the affected areas (86,92,94).

In addition to this, recently 4 species of cobras, 8 species of kraits, 1 species of Russell's viper and 2 sub-species of Saw-scaled viper were also found to be responsible for snakebite cases besides the "Big Four" snakes (9,92,95). Several envenomation cases were reported amongst the plantation workers in southern India due three species of viperidae family, namely Malayan pit viper (*Trimeresurus malabaricus*) and Hump-nosed pit vipers (*H. hypnale* and *H. nepa*) while *N. kaouthia* and *N. oxiana,* members of the elapidae family were reported to cause causalities in the northern, eastern and north-western parts of India (9,96-98). A specific antidote against these snakes is not available. In the case of envenomation by these snakes, the clinicians have to rely on polyvalent antivenom against the "Big Four" snake venoms.

As a result, there are incidences when the clinicians have reported inefficacy of the existing polyvalent antivenom to neutralize the toxic effects of these venomous snakes (95). Variation in the venom composition has been reported to play a critical role in the effectiveness of the antivenom. Variation at every taxonomic level of venomous snakes is observed which is advantageous for immobilization and digestion of specific prey (99), however, it manifests diversified clinical symptoms in victims. Therefore, venom variation at the interspecies and intraspecies level of venomous snakes has been extensively perused to understand the cross-reactivity and efficacy of the available polyvalent antivenom (100,101).

1.2 Venom variation

1.2.1 Venom variation at the interspecies level

Venom variation is widely observed at the genus level of venomous snakes which has complicated the pathological conditions in victims and made the cross-reactivity towards the existing polyvalent antivenom very subtle. Queiroz G.P. and co-workers reported considerable variation in the venom composition of 19 species of Brazilian snakes belonging to the genus Bothrops (*B. jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni*, *B. neuwiedi, B. atrox, B. brazili, B. bilineatus, B. castelnaudi, B. cotiara, B. erythromelas, B. insularis, B. fonsecai, B. hyoprorus, B. intapetiningae, B. leucurus, B. marajoensis, B. pirajai, B. pradoi*) based on electrophoretic profile, biological and biochemical activities (102). The commercially available anti-bothropic antivenom produced against the 5 species of Bothrops (*Bothrops jararaca*, *B. jararacussu*, *B. alternatus*, *B. moojeni*, *B. neuwiedi*) was found to be ineffective in neutralizing the tested biochemical activities of some of the venoms (102). This suggested the need to incorporate the pooled venoms of the other 14 Bothrops species for immunization during antivenom production. In another study, the proteome profile of *B. ayerbei* and *B. asper* from Cauca, Columbia displayed considerable variation in venom composition leading to exhibition of different functional activities on tested animals (103). The venom of *B. ayerbei* with 53.7% of SvMPs exhibited prominent haemorrhagic activity compared to *B. asper* which exhibited prominent myotoxic activity due to the abundance of PLA_2 enzymes (25.3%) (103).

1.2.2 Venom variation at the intraspecies level

Venom variation within the species is one of the most complex criteria which have made snakebite management in victims very challenging. The major concern arising due to venom variation at the species level is that victim envenomated with the same species of snake might manifest diverse pharmacological symptoms which make the identification of the snake very difficult. Nonetheless, it also reduces the efficacy of the antivenom produced against the same species drastically and might also demand different clinical management. This variation at the species level is mostly attributed to ontogenetic changes during the growing phase of a snake's life, the difference in gender and the most influentially geographical locations which in turn, affects the habitat, prey availability and seasonal conditions for the snake (99,104-107). This makes the production of safe and effective antivenom far more challenging.

1.2.3 Venom variation due to ontogenetic changes

Apart from the morphological changes, snakes are reported to exhibit considerable variation in the venom composition during their transition from neonate to adult. Zelanis and co-workers reported variation in venom proteome of *B. jararaca* with ontogenetic changes (108). They observed that the neonate snakes with the relative abundance of type PIII SvMP and N-glycosylated proteins preferred ectothermic small-sized prey whereas the adult snakes with abundant PI SvMP and serine proteases preferred endothermic prey (108). Moreover, the commercially available anti-bothropic antivenom which is usually produced against the pooled adult *B. jararaca* venom was reported to be ineffective in neutralizing the toxic effects induced by neonate envenomation. This suggests the need to use pooled neonate venoms along with the adult venoms for hyperimmunization of the animals during antivenom production (108). In another study, considerable variation in venom composition was reported in short-tailed pit viper (*Gloydius brevicaudus*) during ontogenetic changes by Gao J.F. and co-workers (109). The neonates venom were rich in SvMPs, C-type lectins and acidic 49PLA_2 enzymes while the adult pit viper venom were found to be rich in SvSPs and basic 49PLA_2 enzymes (109). Apart from compositional changes, allometric changes like increase in the head size, volume of venom produced and its storage in the venom gland along with the total dry weight of venom were also observed in the growing stages of snakes (110,111).

1.2.4 Venom variation due to sexual dimorphism

Interestingly, researchers have also observed a notable difference in the venom composition within a species of snake based on sexual dimorphism. Furtado and colleagues reported a significant variation in body size and venom proteome in *B. jararaca* where females were found with larger body size and 5 times more venom production than the males (112). Apart from allometric difference, considerable variation in biochemical and biological activities were also observed in the two, with females exhibiting potent haemorrhagic, hyaluronic and lethal activities while males revealed more of hydrolytic, coagulant and myotoxic activities (112). In another study, the litter of this same species has displayed variation in the biochemical activities in the two genders with males abundant in high molecular weight proteases while the females were rich in low molecular weight proteases (106).

1.2.5 Venom variation due to geographical locations

Variation in venom composition due to different geographical locations was first reported by Daltry and co-workers (105). With the change in the geographical terrain, the ecological niche of the snake changes that leads to change in diversity and availability of prey. Venom variation due to geographical location significantly hampers snakebite management as it leads to manifestation of different clinical symptoms in victims post-envenomation by snakes belonging to the same species. The elapid snake, *Naja naja* is known to be one of the most medically important snakes of India and Sri Lanka. Biogeographic variation showed the considerable difference in the proteome composition of the two snakes both qualitatively and quantitatively. This variation drastically affected the efficacy of the polyvalent antivenom produced against the Indian *Naja naja* in treating the snakebite patients of *N. naja* from Sri Lanka (113). Similarly, Tan and co-workers reported considerable variation in the venom composition of the monocled cobra, *Naja kaouthia* from three regions of Southeast Asia (Malaysia, Thailand and Vietnam) based on chromatographic, electrophoretic and tandem mass spectrometric results (114). Venom from Thailand displayed an abundance of neurotoxins while the venoms from Vietnam and Malaysia were rich in cytotoxins (114). All the three venoms also varied in lethal potencies which were also evident from their proteomic data. Nonetheless, commercially available monovalent and polyvalent antivenoms produced against the

Thailand *N. kaouthia* venom could differentially neutralize the other two venoms at different doses (114).

1.2.6 Venom variation due to diet

As the geographical location of a snake changes, various biotic and abiotic factors in its habitat changes. This affects the diversity and availability of prey which in turn affects the susceptibility of the snake venom proteins in immobilization and killing of prey. This environmental pressure eventually causes natural selection of the venom proteins for a better survivability of the snakes in the new ecological niche, leading to adaptive evolution (115). Gibbs and colleagues, evaluated the effect of ontogenetic changes and diet on a group of juvenile and adult captive Dusky Pigmy rattlesnakes (*Sistrurus miliarius barbouri*) with controlled feeding experiment with different prey (116). They reported that during the growing period until 26 months, diet did show considerable variation in the venom composition of the juvenile snakes but beyond 26 months, the overall venom composition was similar in snakes (116). However, female snakes over 26 months old did exhibit venom variation associated to prey where females fed on mouse were relatively abundant with PLA_2 enzymes (95%) and serine proteinases (>100%) compared to lizard or frog-fed female snakes (116). Moreover, the venoms of Saw-scaled vipers feeding mostly on arthropods like scorpion were found to be more toxic to the desert locust (model prey) than the snakes (African Puff Adder) feeding on vertebrate prey (117).

1.2.7 Venom variation due to seasonal changes

Snakes are cold blooded animals which are more active during the monsoon and summer seasons when the environmental temperature is warm and go for hibernation during the winters. The variation in venom composition with respect to seasonal changes was first reported by Detrait and Duguy in the venom of *Vipera aspis* (118). They observed that the snakebite cases were more severe symptomatologically during the springs while less lethal during the autumns in Europe (118). According to a study conducted by Williams and White, variation in venom composition was observed in *Pseudonaja textilis* during a 12-month study period. They observed an increase in phosphodiesterase and 5'-nucleotidase activity during the summer seasons with a

decrease in amidolytic, esterolytic and coagulant activities. The chromatographic and SDS-PAGE profiles displayed both quantitative and qualitative differences with variable cross-reactivity of the venom samples towards the brown snake antivenom during the study period (107). However, the direct effect of seasonal changes on venom variation has been a subject of debate among the researchers which needs further analysis (119,120).

1.2.8 Venom variation and limitations of antivenom

The efficacy of antivenom towards a snake venom is assessed by its ability to mitigate the toxic effects of envenomation in victims (121). Although the action of monovalent antivenom is very specific towards the venom proteins of a particular species but its use is very restricted as most of the times it is difficult to identify the snake species responsible for envenomation. In such cases, polyvalent antivenom is mostly administered to the victims. However, due to the presence of heterologous mixture of immunoglobulin molecules (against the venom proteins from different species of snake) and the large repertoire of non-specific antibodies, polyvalent antivenom is often reported with many adverse side effects across the globe (121,122).

Indian polyvalent antivenom produced against the "Big Four" snake venoms is not only widely distributed throughout the country but also supplied to its nearby developing countries like Sri Lanka, Pakistan and Bangladesh (100,123-125). However, owing to venom variation due to geographical location and species diversity, inefficacy have been often reported for Indian polyvalent antivenom (92,100,123-129). In addition to this, administration of polyvalent antivenom have been reported to manifest early adverse reactions including anaphylactic reactions like cough, nausea, vomiting, abdominal pain, respiratory distress etc., and pyrogenic reactions like fever, chills and low blood pressure (121,127). Apart from these, many patients are also reported with late serum sickness which includes fever, nausea, itching, diarrhoea, encephalopathy, lymphadenopathy, proteinuria, etc. after administration of polyvalent antivenoms (127). In a study on venom variation in Indian *Naja naja*, Shashidharamurthy and Kemparaju reported that the antivenom produced against southern *N. naja* venom was not effective in neutralizing the venom toxicity of northern or western venom (129). Hump nosed pit viper (*Hypnale hypnale*)

is identified as a potentially venomous snake of Kerala and Sri Lanka with many fatal snakebite cases, against which the Indian polyvalent antivenom is found to be ineffective (95,130,131).

These observations suggest that production of polyvalent antivenom against a few venomous snake species can neither confer complete protection against the same species from different geographical locations nor to other species of a genus. Thus, emphasizing the need to design region specific antivenom considering the epidemiological prevalence of snake envenomation. With the advent of second generation antivenomics, the pre-clinical assessment of the efficacy and specificity of the antivenom towards a pool of heterologous snake venoms have become precisely possible. Antivenomics study is based on the immunoreactivity of the antibodies of the antivenom towards the epitope bearing venom proteins (132). This helps in determining the para-specificity of the antivenom at the species-specific level (85). Moreover, the use of such techniques would also minimize the sacrifice of animals at the preliminary assessment level of the antivenom.

1.3 Overview of the haemostatic system

Haemostasis is an important physiological process of the circulatory system in vertebrates which is maintained by a delicate balance between the blood clot formation upon vascular injury and its subsequent dissolution to restore normal blood flow (133,134). It is precisely regulated by an array of components of the circulatory system including activated serine proteases, serine protease inhibitors (serpins), platelets, endothelial and sub-endothelial cells along with few components of the extra cellular matrix (133,134). Under the normal physiological condition, the optimal viscosity of the blood fluid within the blood vessels is maintained by the anti-thrombotic agents (nitric oxide, ADPase, prostacyclin, thrombomodulin, heparin sulphate, tissue factor pathway inhibitor and tissue plasminogen activator) (135-139). These agents together help maintain a non-thrombogenic environment within the blood vessel by preventing the activation and interaction of the coagulation factors which would otherwise lead to the formation of unwanted blood clots (135-139).

1.3.1 Primary Haemostasis

On the onset of a vascular injury, von willebrand factor (vWF), an adhesive multimeric protein present between the endothelial and sub-endothelial membrane gets exposed to the blood (140). The free platelets present in the blood then bind to the exposed collagen of the sub-endothelium via its major glycoprotein receptors GPIb/V/IX and the ligand vWF (140-142). This paves the onset of the primary haemostasis involving adhesion, activation (by thrombin and arachidonic acid) and aggregation of the platelets at the site of injury (141,143). This leads to the formation of a loose platelet plug at the wounded site to prevent the blood loss (Figure 1.3) (144). The activated platelet release thromboxane A_2, a vasoconstrictor, which minimizes the diameter of the blood vessel to reduce the blood flow from the wounded vessel. The activated platelets also generate a minimal amount of thrombin to facilitate the activation of platelets further and also help initiate the secondary haemostatic system (144,145).

1.3.2 Secondary Haemostasis

On the basis of the cell-based model of haemostasis proposed by Hoffman & Monroe, secondary haemostasis consists of several blood coagulation factors that participate in a series of overlapping reactions leading to initiation, amplification and propagation of fibrin mesh over the loose platelet plug formed by the primary haemostasis (146). Secondary haemostasis can be broadly divided into the extrinsic, intrinsic and the common pathway.

1.3.2.1 Extrinsic pathway

This pathway is so called because it involves the participation of an external factor, the tissue factor in its initiation. Tissue factor (TF), a cell surface receptor expressed on endothelial cells, monocytes, fibroblasts etc. comes in contact with blood only upon vascular injury. Subsequently, it binds to factor VII (FVII), an inactivated glycoprotein circulating in the blood and converts it to activated FVIIa (147,148). The activated FVIIa then forms the extrinsic tenase (Xase) complex in the presence of phospholipids (from the TF bearing cells), Ca^{2+} ions and converts factor X (FX) to FXa (149). The extrinsic tenase complex also activates factor IX (FIX) to FIXa, a key enzyme of the intrinsic pathway (150). FXa generates minor quantities of thrombin

via the prothrombinase complex (discussed later). Thus, the extrinsic pathway marks the initiation of the secondary haemostasis (Figure 1.3).

1.3.2.2 Intrinsic pathway

This pathway is so called because all the components participating here are present within the blood vessel. This pathway helps in the amplification of the thrombin burst generated in the initiation phase. Thrombin, the multi-tasking enzyme of the coagulation system activates factor VIII (FVIII), factor XI (FXI) along with the platelets for facilitating the amplification phase (151-154). In this pathway, factor XII (FXII) is first converted to XIIa by autocatalysis in the presence of kallikrein and negatively charged phospholipids. It then further facilitates the activation of FXI to FXIa (151). Eventually, FIX is converted to FIXa which then binds with FVIIIa on the platelet plug in the presence of Ca^{2+} ions to form the intrinsic tenase (Xase) complex triggering FXa generation (Figure 1.3). FXa then eventually enhances the thrombin surge in the common pathway (146,155).

1.3.2.3 Common pathway

In this pathway, the FXa generated by the Xase complexes of the extrinsic and intrinsic pathway, bind with FVa (activated by thrombin) on the platelet plug along with Ca^{2+} ions and prothrombin to form the prothrombinase complex (146) (Figure 1.3). The complex cleaves prothrombin at Arg271-Thr272 and Arg320-Ser321 to generate pro fragment and thrombin (156). Thrombin then cleaves fibrinogen at the Aα and Bβ chains forming fibrin monomers comprising of two chains each of α, β and γ which together polymerize to form the fibrin protofibrils. Thrombin also converts factor XIII (FXIII) to FXIIIa which is a transglutaminase. The activated FXIII aids in the cross-linking of fibrin molecules over the loose platelet plug, thus completing the blood coagulation process.

1.3.2.4 Fibrinolysis

Once the injured blood vessel is healed, the fibrin mesh on the platelet plug has to be dissolved to avoid unwanted thrombus formation. This is accomplished by the fibrinolytic system which converts plasminogen to plasmin by tissue plasminogen activator (157) (Figure 1.3). Plasmin then cleaves fibrin into fibrin degradation

products, thus dissolving the blood clot. The effect of plasmin is tightly regulated by its inhibitor α2-antiplasmin and α2-macroglobulin (158,159).

Once the fibrin clot is dissolved, haemostasis is restored back to its normal functioning. Hence, any malfunction to this vital physiological process leads to two major pathophysiological conditions, haemorrhage or thrombosis at large. Thrombotic disorder leads to unwanted clot formation in the blood vessel leading to hypoxia, anoxia and even death.

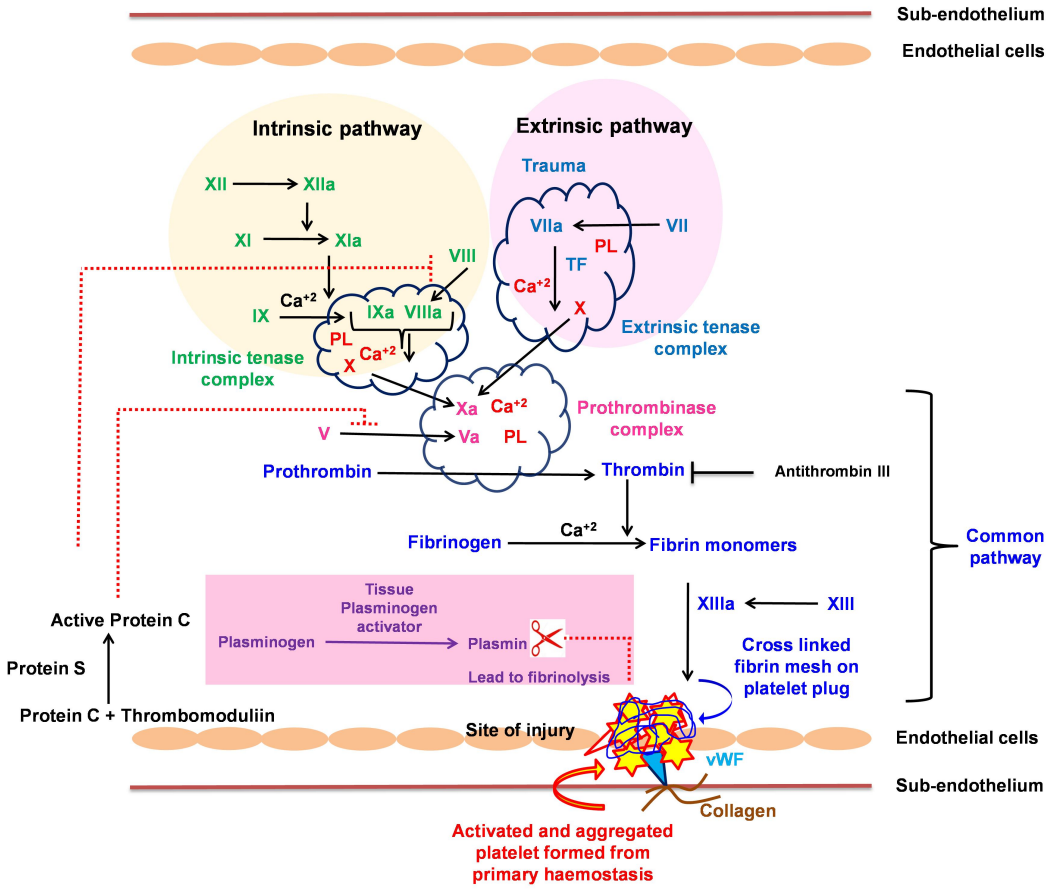

Figure 1.3: Schematic representation of the various steps involved in the haemostatic system during a vascular injury.

1.3.3 Blood coagulation factor X

Blood coagulation factor FX (Stuart-Prower factor), (58.9 kDa) is a vitamin K-dependent plasma glycoprotein which is synthesized in the hepatic cells of the liver

(160). It circulates in the blood plasma as a zymogen. It consists of a heavy and light chain both connected to each other by a disulfide bond (161,162). The light chain, which is the amino-terminal of FX is composed of Gla domain (11 γ-carboxyglutamic acid residues) with a β-hydroxyaspartic acid residue and two epidermal growth factor domain. On the other hand, the carboxyl terminal of FX which is the heavy chain comprises the activation peptide (carbohydrate moieties) and the inactive serine protease domain (163).

It is converted to its activated form, FXa by its cleavage by the Xase complex (extrinsic and intrinsic tenase complex) at Arg52-Ile53 of the carboxyl-terminal of the heavy chain. The cleavage results in the release of a 52 amino acid long activation peptide leading to the formation of the activated FXa with a molecular mass of 46 kDa.

FXa is the major convergent activated product of the extrinsic and intrinsic pathway which plays a key role in the amplification and maintenance of the thrombin burst via the common pathway, thus playing a central role in the coagulation cascade. Moreover, FXa also plays a crucial role in the proteinase-activated receptor (PAR1 and PAR2) signalling pathways which are responsible for various pro-inflammatory, anti-inflammatory responses, mitogenesis and tissue repair events in the body (164). Thus, overproduction of FXa not only leads to unregulated coagulopathy causing thrombotic disorders (pulmonary embolism, venous thromboembolism and deep vein thrombosis) but also causes various pathological conditions regulated via the PARs signalling pathway like cancer, arthritis, lung and kidney disease (164). Hence, it is very much essential to control the production of FXa for a proper functioning of the physiological system. This raises the need to look for the natural or artificial inhibitors of FXa. Although thrombin is the most crucial component of the coagulation cascade with multiple roles in protease activation along with PAR signalling pathway but it is advantageous to target FXa inhibition over thrombin (165). This is because lesser amount of inhibitor is required to inhibit the effect of FXa compared to impede the thrombin surge during pathological events, thus making the drug administration safe and effective (165). Some of the direct and

indirect inhibitors of FXa are already in the market while some are undergoing clinical trials.

1.3.3.1 Direct and Indirect inhibitors of FXa

The direct inhibitors of FXa can exert inhibitory effect directly on free FXa or prothrombinase complex bound FXa (165). The first FXa inhibitor was DX-9065a, an isoxazole derivative containing basic amidine groups (166). Although it exhibited potent antithrombotic effect in venous and arterial thrombosis, however, it was stopped due to its poor oral bioavailability of only 2% to 3% in humans owing to the higher amount of amidine (167-170). Rivaroxaban is a reversible direct inhibitor of FXa. It is recommended as an oral anticoagulant in the treatment of Venous Thromboembolism (VTE) due to its 60-80% bioavailability, faster action, half-life of 5-9 h in plasma, safe and faster elimination from the body and does not require monitoring post administration (171,172). Apixaban is also a reversible direct inhibitor of FXa. It is recommended for oral administration for VTE treatment due to better bioavailability, lower drug interaction, multiple clearance routes and a half-life of 10-15 h (171,172). Edoxaban (DU-176b), an oral direct inhibitor of FXa is also reported to inhibit tissue factor-induced platelet aggregation in human under *in-vitro* conditions (173). It has a half-life of 6-10 h and is undergoing phase III trials (171,172). It was found to be more potent inhibitor of FXa compared to fondaparinux, an indirect FXa inhibitor (173). These three FXa inhibitors have completed the phase III trials and are considered safe for the treatment of venous thrombosis based on their pharmacodynamics and pharmacokinetics data (174).

The indirect inhibitors of FXa, on the other hand activate antithrombin to exhibit their inhibitory effect on FXa (171). Fondaprinux is a low molecular weight heparin which is recommended for treatment of patients diagnosed with pulmonary embolism (PE), deep vein thrombosis (DVT) and heparin induced thrombocytopenia (HIT) (175,176). It is synthesized as five sugars moieties (pentasaccharide) on heparin and is administered into patients subcutaneously. It is widely accepted anticoagulant drug owing to its optimal bioavailability, half-life of 17-21 h in plasma, safe elimination from the body and minimal food and drug interaction (171,175). Idraparinux, on the other hand, is a polymethylated derivative of fondaprinux (177). It exhibits higher

binding affinity for antithrombin and has a prolonged half-life of 80 h in plasma (171). Although a number of promising FXa inhibitors are undergoing successful clinical trials, however, they pose some inevitable limitations like (178)–

1. Use of anticoagulant therapy always carries the risk of bleeding which demands the need of proper prophylactic treatment to reverse the effect of the inhibitor.
2. The choice of anticoagulant therapy varies from patient to patient.
3. Careful screening to analyse the performance of the anticoagulant drug on the human population.
4. No specific antidotes are available so far for the new oral anticoagulants.
5. Oral FXa inhibitors are reported with variable after effects on routine tests of coagulation and as such demand precise monitoring assays to quantity drug levels.
6. Lack of simple monitoring assays to assess the effect of the oral FXa inhibitors prior to surgery in patients complicates their administration.

Therefore, there is a need for safer inhibitors of FXa with lesser bleeding risk and more monitoring accessibility. As such a number of inhibitors of FXa have been isolated and characterized from various venomous animals with potential therapeutic benefits (179-183).

1.4 Snake venom toxin families affecting haemostatic system

The components of the haemostatic system are one of the most susceptible physiological targets of the snake venom proteins. Owing to this, there has been an expedition amid the researchers to harness these proteins from snake venom for understanding their mechanism of action as well as for therapeutic applications. The snake venom toxins affecting the haemostatic system can be largely classified into procoagulants and the anticoagulants (184). Procoagulants toxins are those venom components that shorten the process of blood coagulation either by activating the zymogenic blood clotting factors or converting the soluble fibrinogen to insoluble fibrin (184). The anticoagulants, on the other hand, delay the process of blood coagulation enzymatically by phospholipid hydrolysis or non-enzymatically by

interacting with the clotting factors (184). The various snake venom procoagulant and anticoagulant proteins targeting the coagulation factors are shown in Figure 1.4 and 1.5 respectively.

1.4.1 Procoagulant toxin families

Phospholipase A_2 enzymes: These enzymes are reported to induce procoagulant effect by the lytic products released during the phospholipid hydrolysis that participate in a number of signalling pathways. The hydrolysis of platelet phospholipids by the snake venom PLA_2 enzymes release arachidonic acid (AA) and or platelet aggregation factor (PAF) that induce platelet aggregation at the site of injury facilitating blood coagulation (143). Bothropstoxin-II (Bthtx-II), a Asp-49 PLA_2 enzyme isolated from the venom of *Bothrops jararacussu* induce platelet aggregation and secretion via multiple signal transduction pathways (185).

Snake venom metalloprotease (SvMP): RVV-X is a factor X activator isolated from the venom of *Daboia russelii* (Russell's viper) (186). It belongs to PIIId- class of metalloprotease containing metalloprotease (MP), disintegrin, cysteine-rich secretory and a C-type lectin-like domain. It is reported that during the activation of FX to FXa, the snaclec subunit of RVV-X binds to the Gla domain of FX which aids in the cleavage of the heavy chain of FX at Arg52-Ile53 by its MP subunit (187). The FXa generated then participates in the subsequent blood clotting process. Other FX activators reported so far include VLFXA from *Vipera lebetina*, VAFXA-1 and VAFXA-II isolated from the venom of *Vipera ammodytes ammodytes* which also activate FX in a similar way (188,189). The prothrombin activators are categorized into 4 groups based on the mechanism of action and the co-factor requirement, namely A, B, C and D (190,191). Group A and B belong to metalloproteases where the former does not require any co-factor for its activity while the later requires Ca^{2+} ions for the proteolytic action (191). Both the groups cleave prothrombin at Arg(323)-Ile(324) releasing meizothrombin which is then autocatalysed to form α-thrombin (187,191). Bothrojaractivase (22.829 kDa), a PI-class metalloprotease is a group A prothrombin activator isolated from the venom of *Bothrops jararaca* (192). It is reported to convert prothrombin into meizothrombin and degrade fibrinogen and fibrin thus aiding the process of coagulation (192). Basparin A (70 kDa), a P-III class

metalloprotease is also a group A prothrombin activator isolated from the venom of *Bothrops asper* (193). It is reported to convert prothrombin to meizothrombin and inhibits collagen induced platelet aggregation (193). Carinactivase-1 is a group B prothrombin activator isolated from the venom of *Echis carinatus* (194). It is composed of two subunits, one a 62-kDa metalloprotease homologous to ecarin and the other subunit is a 25 kDa polypeptide similar to IX/X-bp isolated from habu snake. Its metalloprotease domain is reported to cleave prothrombin to meizothrombin in the presence of Ca^{2+} ions facilitating coagulation (194).

Snake venom serine protease (SvSP): The group C and D prothrombin activators belong to serine proteases where the former requires Ca^{2+} ions and phospholipids for its action while the later requires Ca^{2+} ions, phospholipids and FVa for its activity (187,190,191). Both the C and D prothrombin activators cleave prothrombin at Arg(274)-Thr(275) and Arg(323)-Ile(324) and activate it to α-thrombin (187). Pseutarin C (250 kDa) is a group C prothrombin activator isolated from the venom of *Pseudonaja textilis*. It is composed of subunits similar to FXa and FVa that facilitates the catalytic activity of the complex thus enhancing the cleavage of prothrombin to thrombin leading to coagulation (195). Tocarin D is a group D prothrombin activator isolated from the venom of *Tropidechi carinatus* (191,196). It is composed of catalytic subunit similar to FXa, thus a functional homolog of FXa participating in the coagulation of blood (191,196). Factor X activator belonging to serine protease superfamily have been reported from the venom of *Ophiophagus hannah* (62 kDa) and *Bungarus fasciatus* (70 kDa) (197,198). These proteins are reported to cleave the zymogenic form of FX at its heavy chain facilitating its activation to FXa (197,198). Oscutarin is a factor VII activator isolated from the venom of *Oxyuranus scutellatus scutellatus* (coastal Taipan), is reported to convert inactivated FVII to activated FVIIa in the presence of Ca^{2+} ions and phospholipids (187). RVV-V and VLFVA are FV activators isolated from the venom of *Daboia russelii* (Russell's viper) and *Vipera lebetina* respectively (199,200). They are reported to cleave inactivated coagulation factor FV after Arg-1545 converting it to activated FVa (201). CR-serpinase (45.5 kDa) is an antagonist of serine proteinase inhibitor (serpins) isolated from the venom of *Causus rhombeatus* (night adder) (202). It is reported to inactivate antithrombin – III (a thrombin inhibitor) by cleaving it at its active site (Arg-393 and Ser-394) in the

presence of heparin (187,202). Bothrombin (35.0 kDa) isolated from the venom of *Bothrops jararaca* is reported to activate platelet aggregation by binding to platelet receptor GPIbα and cleaves fibrinogen releasing fibrinopeptide A but does not generate fibrinopeptide B (203).

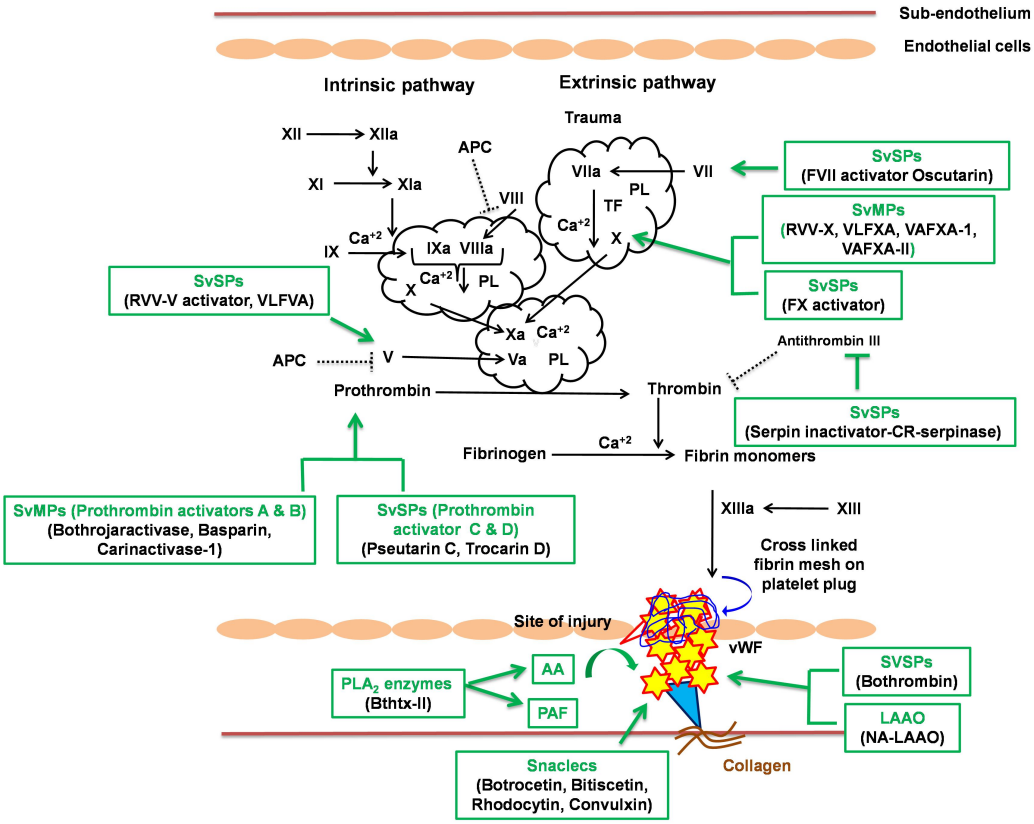

Figure 1.4: Schematic representation of the various snake venom procoagulant proteins affecting the haemostatic system.

Snake venom C-type lectins (Snaclec): Botrocetin (25 kDa), a heterodimeric snaclec is isolated from the venom of *Bothrops jararaca* (204,205). It is reported to enhance the affinity of the A1 domain of vWF for the platelet glycoprotein Ib (GPIb) receptor thus facilitating platelet agglutination and aggregation (206). Bitiscetin, a heterodimeric complex of alpha (16 kDa) and beta (13 kDa) subunits connected by disulphide bonds is isolated from the venom of *Bitis arietans* (207). It is reported to induce platelet agglutination and aggregation by binding vWF to GPIb receptor of platelets (207).

Convulxin, a dimeric protein $(\alpha\beta)_4$ in solution, is isolated from the venom of *Crotalus durissus terrificus* (tropical rattlesnake). It is reported to cause platelet aggregation and activation induced by collagen (208). It can bind to GPIV receptor of platelet from eight sites which aid in platelet aggregation and binding to GPIb receptor help in platelet activation (208-210). Rhodocytin or aggretin, a heterodimeric snaclec isolated from the venom of *Calloselasma rhodostoma* (Malayan pit viper) is reported to induce platelet aggregation by binding to receptors GPIb, CLEC-2 and $\alpha_2\beta_1$ of platelet (211-213).

L-amino acid oxidase (LAAO): NA-LAAO isolated from the venom of *Naja atra* is reported to induce platelet activation by binding to platelet membrane proteins thereby increasing the sensitivity of the platelets to hydrogen peroxide (H_2O_2) released during the reaction of oxidative deamination (214).

1.4.2 Anticoagulant toxin families

PhospholipaseA_2 enzymes: PLA_2 enzymes from both elapidea and viperidea families are reported to exhibit a potential anticoagulant effect on various components of the coagulation system both enzymatically and non-enzymatically. Enzymatically these proteins hydrolyse the phospholipids which are important for the formation of the coagulation complexes thus inhibiting the clotting process. Non-enzymatically they interact with various coagulation factors and inhibit their activation or interaction with other factors of the coagulation cascade thus hindering the clot formation. The anticoagulant and antiplatelet snake venom PLA_2 enzymes isolated from snake venoms are described in details in sections 1.5.7.6 and 1.5.7.7.

Snake venom serine protease (SvSP): Protac (37 kDa) and Acc-C (40 kDa), protein C activator are trypsin-type serine protease isolated from the venom of *Agkistrodon contortrix contortrix* (southern copperhead) (215,216). They are reported to convert the inactivated form of protein C to its activated form by cleaving its heavy chain (216). The activated protein C then exerts its anticoagulant effect by the proteolytic degradation of FVa and FVIIIa (215). The thrombin like enzymes also known as the defibrinogenating enzymes like Ancrod isolated from *Calloselasma rhodostoma* venom, batroxobin isolated from *Bothrops moojeni* venom and crotalase isolated from

Crotalus adamanteus venom exhibited strong proteolytic effect on fibrinogen (217). Ancrod is reported to cleave A-fibrinopeptides while batroxobin and crotalase cleave both fibrinopeptides A and B. The cleavage lowers the level of fibrinogen in blood plasma leading to the accumulation of fibrinogen-fibrin degradation products (217). Due to lack of cross-linking, the fibrin formed is exposed to degradation by fibrinolytic enzymes thus making the blood non-coagulable (184,217). TSV-PA is a plasminogen activator isolated from the venom of *Trimeresurus stejnegeri* (218). It is reported to cleave plasminogen after the residue Arg-561 to release plasmin, a key enzyme involved in the dissolution of the fibrin clot thus preventing blood from clotting (218).

Snake venom metalloprotease (SvMP): Barnettlysin-I, Bar-I (23.386 kDa) is a non-hemorrhagic metalloprotease (P-I class) isolated from the venom of *Bothrops barnetti* (Barnett´s pitviper) (219). It is reported to degrade the α-chains of fibrin and fibrinogen and inhibited the aggregation of platelets induced by vWF and collagen (219). On the other hand, the haemorrhagins disrupt the components of the endothelium and sub-endothelium like type IV collagen, laminin, fibronectin etc. and interfere the interaction of the integrins with their ligands thus, destroying the integrity of the basement membrane (187,220). Bothropoidin (49.558 kDa) is a haemorrhagic metalloproteinase (P-III class) isolated from the venom of *Bothrops pauloensis* (221). It is reported to exhibit a proteolytic effect on fibrinogen, fibrin, collagen and fibronectin and inhibited platelet aggregation induced by collagen and ADP (221).

L-amino acid oxidase (LAAO): LAAO enzymes are reported to affect the haemostatic system of the vertebrates by various mechanisms including platelet aggregation inhibition, haemorrhage of endothelial cells apart from interacting with certain coagulation factors (187,222). M-LAO, isolated from the venom of *Agkistrodon halys blomhoffii* (Japanese mamushi) delayed the clotting time of intrinsic pathway by inhibiting the activity of FIX (223). Apoxin I isolated from the venom of *Crotalus atrox* (western diamondback rattlesnake) is reported to cause haemorrhagic effect on endothelial cells by inducing apoptosis via H_2O_2 released during the oxidative deamination of L-amino acid (222).

Nucleotidase and Phosphodiesterase: The breakdown of adenosine diphosphate, ADP (a platelet aggregation inducer) into nucleoside and phosphate by nucleotidase is considered as the main reason for the anticoagulant potency of nucleotidase (187). 5'-NUC, a nucleotidase isolated from the venom of *Trimeresurus gramineus* and *Crotalus atrox* inhibit platelet aggregation induced by ADP and collagen (224,225). On the other, NPP-BJ, a phosphodiesterase isolated from the venom of *Bothrops jararaca* (228 kDa) inhibited platelet aggregation induced by ADP (226).

Three-finger toxin (3FTx): Anticoagulant 3FTx such as Hemextin AB and ringhalexin have been reported from the venom of *Hemachatus haemachatus* (African Ringhals cobra). Hemextin AB is a non-competitive inhibitor of FVIIa-soluble Tissue factor (227). The complex of hemextin A and hemextin B act synergistically where hemextin B increases the anticoagulant potency of hemextin A for FVIIa (227). On the other hand, ringhalexin is a mixed-type inhibitor of FVIIa thus inhibiting the activation of FX to FXa by the extrinsic pathway (228). However, mambin, a short chain neurotoxin, inhibit platelet aggregation induced by ADP and prevent the binding of glycoprotein platelet receptor GP IIb-IIIa to fibrinogen. The RGD motif present in the third loop of this protein is reported to be involved in interacting with glycoprotein receptor GP IIb-IIIa of platelet to inhibit aggregation (229).

Serine protease inhibitor (SPI): Fasxiator, a Kunitz-type serine protease inhibitor is a FXIa inhibitor isolated from the venom of *Bungarus fasciatus* (Banded krait) (230). It is reported to delay the activated partial thromboplastin time significantly without affecting the prothrombin time (230). Its mutated recombinant form, rFasxiatorN17R,L19E is found to be a competitive slow-type inhibitor of FXIa which exhibits ~10 times stronger anticoagulant activity on human plasma and prolongs the ferric chloride-induced carotid artery thrombosis in mice models (230).

Snake venom C-type lectins (Snaclec): Bothrojaracin (27 kDa) is an antithrombotic agent isolated from the venom of *Bothrops jararaca* (231,232). It is reported to affect various functions of thrombin by binding to exosite 1 and 2 with high affinity (232). In addition to this, it also binds to prothrombin thus inhibiting its conversion to thrombin by the prothrombinase complex (232,233). Coagulation factor IX/X binding

protein (FIX/X-bp) is a potent anticoagulant protein isolated from the venom of *Trimeresurus flavoviridis* (234). It binds to the Gla-domains (γ-carboxyglutamic acid) of factors IX and X in the presence of Ca^{2+} ions thus interfering the interaction of these coagulation factors to the phospholipids (234). Apart from this, specific FIX binding protein (FIX-bp) and FX binding protein (FX-bp) are also reported from the venom of *Trimeresurus flavoviridis* and *Deinagkistrodon acutus* respectively (179,235). FIX-bp bind to Gla-domain of FIX while FX-bp bind to the Gla domain of FX in the presence of Ca^{2+} ions thus inhibiting the plasma clotting induced by IXa and Xa respectively (179,235).

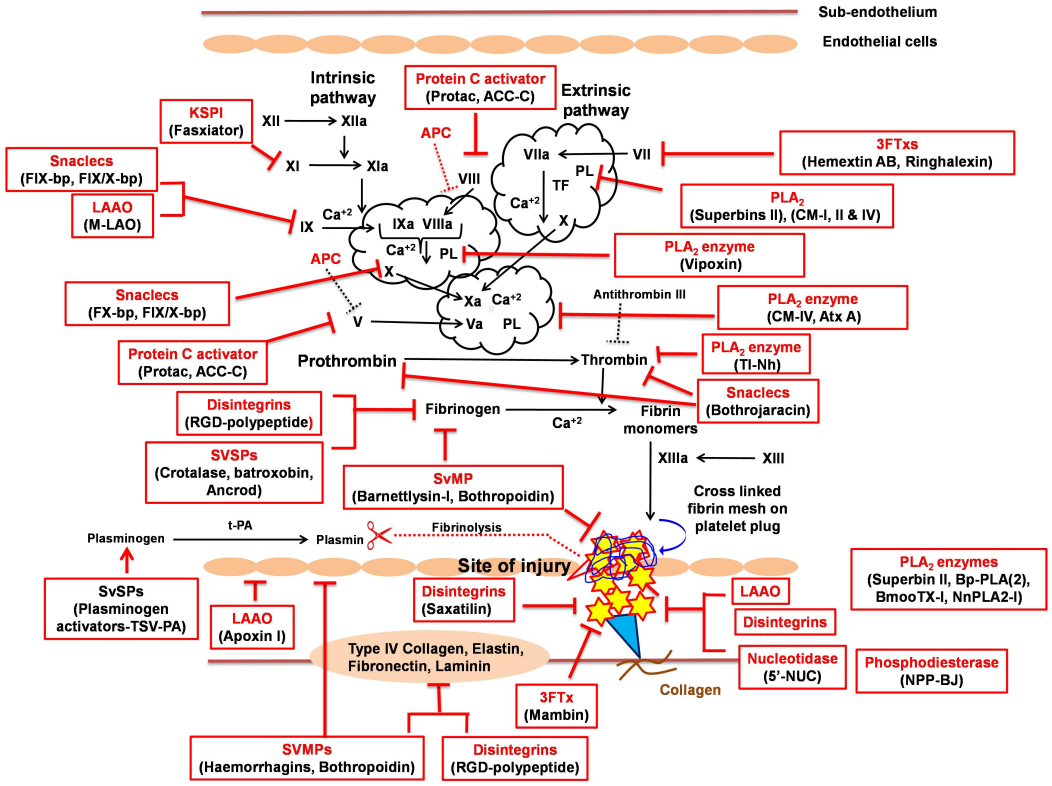

Figure 1.5: Schematic representation of the various snake venom anticoagulant proteins affecting the haemostatic system.

Disintegrin: Saxatilin, a novel disintegrin isolated from the venom of *Gloydius saxatilis* (Korean snake) is reported to inhibit the activation and aggregation of platelets. It is also reported to exhibit thrombolytic effects on ferric chloride-induced

carotid artery thrombosis model in mice (236). The low molecular mass disintegrins with RGD containing tripeptide are reported to inhibit the fibrinogen binding to platelet receptors GPIIb/IIIa thus interfering the blood clot formation (237). Disintegrins in viper venoms are also reported as hemorrhagins affecting the components of the sub-endothelium (237). The high molecular mass disintegrins with RGD-polypeptides (50-90 KDa) from viper venoms are also reported to integrin receptors thus inhibiting cell to matrix or cell to cell interactions (238).

1.5 Snake venom PLA_2 enzyme

1.5.1 Phospholipase A_2 enzymes in general

The superfamily of phospholipase (E.C 3.1.1.4) is composed of water soluble hydrolytic enzymes that catalyse the esterolytic reaction on the membrane glycerophospholipids at the water-lipid interface (46). Based on the site of catalysis, these enzymes are classified into PLA_1, PLA_2, PLB, PLC and PLD (239). The PLA_1 and PLA_2 cleave at the *sn*-1 and *sn*-2 position of the acyl chain respectively. On the other hand, PLB cleaves at both the *sn*-1 and *sn*-2 position. While the PLC cleaves before the phosphate group liberating diacylglycerol and phosphate containing head group while PLD cleaves after the phosphate group releasing phosphatidic acid and alcohol.

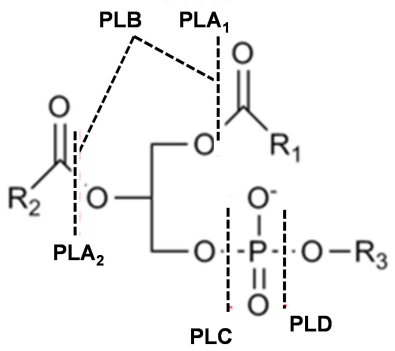

Figure 1.6: Schematic representation of various sites of phospholipid hydrolysis by different phospholipase enzymes.
(Adapted from https://commons.wikimedia.org/wiki/File:Phospholipases2.png)

Out of these, the members of PLA_2 enzymes are most extensively studied due to their relatively higher abundance in organisms both at the intracellular and extracellular level and their vital involvement in various physiological functions (46). These enzymes cleave the glycerol backbone of the glycerophospholipids on the carbon atom at the *sn*-2 position liberating lysophospholipids and fatty acids (46).

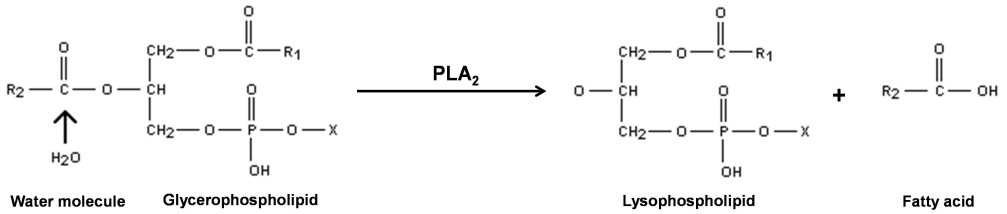

Figure 1.7: Hydrolysis of glycerophospholipid by PLA_2 enzymes at the *sn*-2 position of the glycerol backbone.

Hydrolysis damages the integrity of the membrane phospholipid affecting its fluidity, packing and selective permeability to various molecules thus, hindering its overall cellular functions (240). On the other hand, lysophospholipids, the lytic products further facilitate membrane damage by enhancing the penetrability of the PLA_2 enzymes and act as potential inflammatory mediators (241). While the non-esterified fatty acids especially the arachidonic acid participates in the production of secondary messengers like leukotrienes, thromboxanes and prostaglandins leading to platelet activation and various inflammatory responses (240).

The PLA_2 enzymes are found in various organisms including mammals, snakes, insects and arachnids and can be present as extracellular or intracellular components (242-246). They are abundantly found as the secretory ($sPLA_2$), cytosolic ($cPLA_2$), calcium independent ($iPLA_2$) and platelet activating factor acetylhydrolase (PAF-AH) PLA_2 enzymes (240) (Figure 1.8).

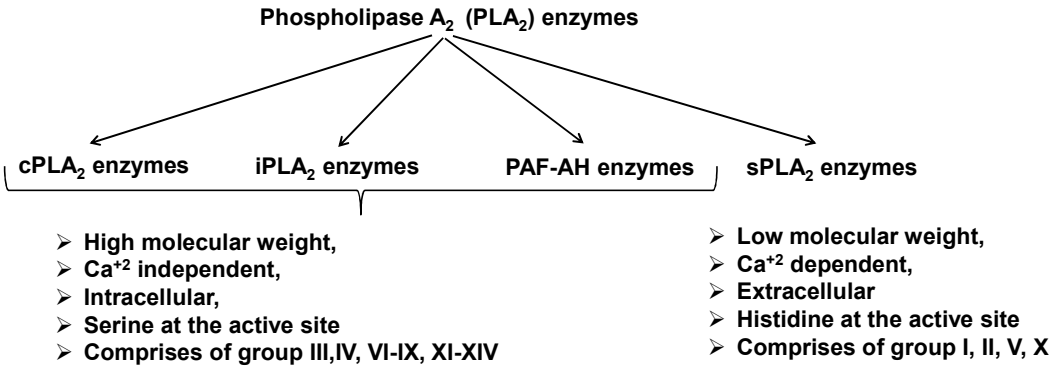

Figure 1.8: Schematic representation of different types of PLA_2 enzymes found in various living system (240,247).

1.5.2 Classification of PLA_2 enzymes

The $sPLA_2$ enzymes are mostly found in the venoms of bee, scorpions, lizards and snake along with various body fluids of the mammalian system like tears, inflammatory exudates, pancreatic juice, synovial fluid etc. (240,248,249). Apart from digestion of dietary phospholipids, the mammalian $sPLA_2$ enzymes play a crucial role in various physiological events of an organism including fertilization, cell proliferation, cell migration, smooth muscle contraction, hypersensitization, inflammatory and anti-inflammatory reactions and regulate signalling pathways involving prostaglandins and leukotrienes, immunity against bacterial infections, etc. (250-255). Nonetheless, mammalian $sPLA_2$ enzymes are also known to participate in various pathological events like asthma, rheumatoid arthritis, osteoarthritis along with inflammatory diseases (256,257). On the other hand, the $sPLA_2$ enzymes in the venomous animals mostly play a crucial role in prey immobilization and prey digestion along with other venom components (258,259).

Based on the amino acid sequence of the primary structure, 3D scaffold, mode of catalysis and site of expression, the members of intracellular and secretory PLA_2 enzymes are categorized into 14 groups (I-XIV) (Figure 1.8) (260). Out of these, group I, II, V and X are closely related and categorized along with the secretory PLA_2 enzymes while group III, IV, VI-IX, XI-XIV are categorized as intracellular high molecular weight PLA_2 enzymes (Figure 1.8). However, snake venom PLA_2 enzymes

are classified under group I and group II only based on primary sequence, 3D structure as well as number and position of disulphide bonds (247).

$SvPLA_2$ enzymes from elapid, hydrophids and colubrids along with the mammalian pancreatic PLA_2 enzymes are categorized under group I PLA_2 enzymes. These are low molecular weight proteins with 115-120 amino acids and 14 cysteine residues. These cysteine residues correspond to 7 disulphide bonds formed between **Cys11-Cys77**, Cys27-Cys126, Cys29-Cys45, Cys44-Cys105, Cys51-Cys98, Cys61-Cys91, Cys84-Cys96 (247). In addition to this, a few members of this group consists of a typical surface loop known as the elapid loop (that connects the catalytic α helix with the β-wing) therefore grouped as IA. On the other hand, mammalian pancreatic PLA_2 enzymes grouped as IB, consists of a pancreatic loop (62^{nd}-67^{th}) which is an extension of 5 amino acid residues that distinguishes them from group IA enzymes. This group IB PLA_2 enzymes are secreted by the pancreas as zymogens due to the presence of a propeptide sequence consisting of 8 amino acid residues. At the time of maturation/activation, the propeptide sequence is cleaved by trypsin. The activated enzyme plays a critical role in the digestion of the dietary phospholipids and is abundantly found in the pancreatic juice. Surprisingly a few $SvPLA_2$ enzymes (from *Ophiophagus hannah*, *Pseudonaja textilis*, *Oxyuranus scutellatus, Micrurus frontalis frontalis* etc.) are also reported as group IB enzymes owing to the presence of the propeptide sequence, which is retained in a few snakes while is cleaved in others, post maturation (261-264).

The $SvPLA_2$ enzymes from viperid and crotalid snakes are categorized into Group II (A and B) (265,266). They are also low molecular weight secretory PLA_2 enzymes with 120-133 amino acids and 14 cysteine residues (240,267). This group is distinguished from group I PLA_2 enzymes due to the presence of a long C-terminal tail with 6 amino acid residues and the formation of the 7^{th} disulphide bond between **Cys-50 and Cys-133** (266). Moreover, the members of this group lack the elapid loop or the pancreatic loop which is a characteristic feature of Group IA and IB PLA_2 enzymes respectively (240,265). Based on the amino acid substitution at the 49^{th} position of the group II $SvPLA_2$ enzymes, they can be classified as Asp49, Lys49, Ser49, Asn49 or Arg49 (268-272). It was reported that the substitution of the aspartate

residue for any of these amino acid residues at the 49th position led to the partial or complete loss of catalytic activity (273).

Moreover, based on the site of tissue specific expression, this group can be further sub-divided into **IIA** which are expressed in the venom gland of Rattle snake and viper snake and in the human synovial fluid; **IIB** are reported from the venom gland of Gaboon viper venom, while **IIC** PLA_2 enzymes are expressed in the testes of rat and mouse; **IID** are expressed in the pancreas and spleen of human and mouse; **IIE** enzymes are found in the tissues of brain, heart and uterus of human and mouse and last but not the least, **IIF** PLA_2 enzymes are reported to be expressed in the testis and embryo of mouse (247).

1.5.3 Evolution of the PLA_2 gene

Although the venom and mammalian pancreatic PLA_2 enzymes have evolved from a common nontoxic ancestral gene, considerable variation have been reported in their evolutionary pattern (274-276). The mammalian and group I genes followed a similar evolution pathway while the group II PLA_2 genes evolved after species diversification pathway (274-276). Moreover, the structural organization for both group I and group II genes are reported to be considerably different where group I genes are composed of 4 exons and 3 introns while group II genes consisted of 5 exons and 4 introns (277). Although the group I gene organization is similar to the pancreatic PLA_2 gene but the smaller size of intron 3 and the loss of propeptide sequence in most of the snake venoms following maturation have attributed to their additional pharmacological activities, which is considered to be on the account of adaptive evolution (240,276). In addition to this, the loss of the pancreatic loop encoded by exon 3 in most of the snake venoms (except in King cobra, Brazilian coral snake, Australian elapid snake) is also considered as an adaptive trait leading to enhanced enzymatic and toxic activities (262,265,278,279). On the other hand, the group II PLA_2 enzymes exhibited similarities with the gene organization of human synovial fluid PLA_2 enzyme where the exon 1 and exon 2 encode for 5'UTR and the signal peptide respectively while the last three exons coded for the mature protein (277,280).

Most of the time single snake venom is reported with different array of PLA_2 isoenzymes (38,281-283). Although these enzymes share a common 3D structure and catalytic scaffold but they are often capable of exhibiting plethora of pharmacological activities (240). This diversity in function is mostly credited due to their evolution from multiple genes which are subjected to the selection pressure of gene duplication and accelerated point mutation (277,280,284-289). The most susceptible mutational hotspots in these molecules are found to be the protein coding regions (compared to UTRs), exons (compared to introns) and the surface exposed residues where the rate of substitution is reported to be 2.6 to 3.5 times higher than the buried residues (280,284,287,288,290,291). Kini and Evans proposed that the diverse biological activities of PLA_2 isoforms occur due to the presence of pharmacological site which are responsible for non-enzymatic activity via protein-protein interaction. These sites are found to be well differentiated from the catalytic site that are responsible for enzymatic activity via protein-phospholipid interaction (292).

1.5.4 Structure of the PLA_2 enzyme

The primary structure of the members of the $sPLA_2$ enzymes show 40-99% of sequence similarity due to which they share a highly conserved 3D structure comprising of three major α-helices and two antiparallel β-sheets stabilized by conserved disulphide bonds (240,267). The conserved regions include the N-terminal helix, calcium binding loop, two antiparallel helices, active site, β-wing and the C-terminal loop (240). The first 12 residues of the N-terminal helix form the hydrophobic channel with their side chains forming the channel opening (267). The calcium binding loop (25^{th}-33^{rd}) is composed of highly consensus sequence of Tyr-Gly-Cys-Tyr-Cys-Gly-X-Gly-Gly residues where the Ca^{2+} ions bind during catalysis (293). Banumathi and co-workers reported that a pentagonal bipyramidal cage is formed by the oxygen atom of Asp49 and the three carbonyl groups of Tyr/Phe28, Gly30 and Gly32 in the presence of water molecules to capture the Ca^{2+} ions (293). The core of the hydrophobic channel is formed by the two long α-helices (37^{th}-54^{th} and 90^{th}-109^{th}) oriented opposite to each other (267). The active site of the PLA_2 enzyme is composed of His48 residue along with Tyr52, Asp99 and the Asp49 crucial for Ca^{2+} binding (240). The β-wing composed of two β-sheets connects the hydrophobic core and move outward into the solvent (240). The extended C-terminal

loop, with 6 amino acid residues and the position of the 7th disulphide bond, formed between Cys50 and Cys133 (C-terminal loop) are the only characteristic features of group II PLA_2 enzymes that distinguish them from group I PLA_2 enzymes (266).

1.5.5 Catalytic mechanism of sPLA$_2$ enzymes

The PLA_2 enzymes can differentially hydrolyse membrane phospholipid with different polar head groups like phosphatidylcholine, phosphahtidylethanolamine, phosphatidylserine, etc. both at monomeric and aggregated levels (240). However, the rate of catalysis is found to be more pronounced (10, 000 fold higher) on aggregated forms of phospholipids like micelles, liposomes or as bilayers compared to the monomeric forms (255). The catalytic efficiency of these enzymes is largely determined by various physical parameters of the phospholipids like their packing density, temperature, transition phase apart from the preference for different polar head groups (240,294). Based on the type of binding of the PLA_2 enzymes at the lipid-water interface, the catalysis of the phospholipids can be explained by the hopping or the scooting mode (295,296). In the hopping mode, the enzyme binds and unbinds from the interface at each turnover cycle while in the scooting mode, the enzyme remains bound to the interface between each catalytic cycle. The overall catalytic efficiency of each mode is determined by various physical parameters like temperature, packing of the phospholipids, binding kinetics, catalysis and desorption (240,295,296).

Verheiji and co-workers were the first to evaluate the catalytic mechanism of PLA_2 enzymes based on their structural comparison with serine proteases (255). They reported it to be a general base mediated catalysis in the presence of water molecule as the nucleophile (255). During the process of catalysis, calcium binding loop along with the Ca^{2+} ions play a very crucial role in the formation of the enzyme-substrate complex and the stabilization of the intermediate states formed during the reaction. The calcium binding loop forms a part of the hydrophobic channel which aid in the binding of the phospholipids onto the PLA_2 enzyme (293). The bound Ca^{2+} ions not only help in placing the phospholipids correctly on the active site of the enzyme but also help in the electrostatic interaction of the enzyme-substrate complex. Once the substrate is positioned correctly onto the enzyme, the bound water molecules on the

interface get polarized by His48 along with Asp99, Tyr52 and Tyr73 (267,297). This polarized nucleophilic water molecules then attack the *sn*-2 bond (already polarized by electrophiles containing Ca^{2+} ions and oxygen atom of Asp49) of the glycerol backbone by general base mediated catalysis and form a tetrahedral oxyanion intermediate (stabilized by Ca^{2+} ions) (267,297-299). This leads to the formation and breakdown of this tetrahedral intermediate and finally release the lytic products (298).

1.5.6 Snake venom PLA_2 enzyme

The first PLA_2 enzymes were purified from the venom of two elapid snakes, *Naja naja* and *Naja tripudians* in the year 1944. These were named as haemolysins due to their indirect haemolytic effect on red blood cells (300). Since then hundreds of PLA_2 enzymes have been isolated and characterized from snake venom. These enzymes are known to be one of the most abundant and potentially toxic venom components in almost all venomous snakes. $SvPLA_2$ enzymes are reported to exits in multiple isoforms in the venom gland of a single snake (38,239,301,302). Moreover, they can exist as monomers or as complexes interacting with other PLA_2 enzymes or with different venom components both covalently and non-covalently in the snake venom. Ammodytoxin and notexin, two pre-synaptic neurotoxins, isolated from the venom of *Vipera ammodytes ammodytes* and *Notechis scutatus scutatus* respectively are examples of monomeric PLA_2 enzymes (303-305). On the other hand, β-bungarotoxin pre-synaptic neurotoxin isolated from the venom of *Bungarus* species is an example of heterodimeric covalent complex. The subunit A, similar to group I PLA_2 enzyme is connected via a disulphide bond to its subunit B which is similar to Kunitz-type serine protease inhibitor and dendrotoxins (47,306). Nevertheless, a number of PLA_2 enzyme complexes are also reported with non-covalent interactions like trimucrotoxin, crotoxin, taipoxin etc. Trimucrotoxin, a pre-synaptic neurotoxin, isolated from the venom of *Trimeresurus mucrosquamatus* is a homodimeric complex of 14 kDa group II PLA_2 enzymes (307). Crotoxin isolated from the venom of *Crotalus durissus terrificus* is a heterodimeric neurotoxin (308). One of its subunit named as crotapotin (CA) is acidic, non-toxic and non-enzymatic in nature while its other subunit is basic, weakly toxic PLA_2 enzyme (CB) (308,309). Although the CA subunit is inactive but in the native form (CA-CB) as a complex, it enhances the neurotoxic activity of CB. On the other hand, taipoxin, a heterotrimeric PLA_2

complex isolated from the venom of *Oxyuranus scutellatus scutellatus* is composed of three subunits namely α (most toxic, group IA), β (non-toxic, group IA) and γ (moderately toxic, group IB) and is reported to block neuromuscular functioning (310,311).

1.5.7 Biological activities of snake venom PLA_2 enzymes

In spite of having sequence similarity and conserved structure, the PLA_2 enzymes exhibit a plethora of pharmacological activities on the prey or the victim due to the presence of different pharmacological sites other than the common catalytic site (46). The most profound biological activities reported so far from $SvPLA_2$ enzymes include neurotoxicity (pre-synaptic and post-synaptic), myotoxicity (local and systemic), cardiotoxicity, platelet aggregation initiation or inhibition, anticoagulant, haemolytic, antibacterial, edema inducing, internal haemorrhage, hypotension, tissue and organ damaging activities (46).

1.5.7.1 Neurotoxicity

Snake venom PLA_2 enzymes are reported to exhibit both pre-synaptic (inhibit neurotransmitter release from the nerve terminal) and post-synaptic (inhibit neurotransmitter action on the muscles) neurotoxic effect (240). Several hypotheses were proposed to explain the neurotoxic effect of PLA_2 enzymes like their hydropathy profile or hydrophobicity in the region from 80-110th residues (312). However, the most recent and convincing mechanism was proposed by Curin-Serbec and co-workers where the antibodies raised against the C-terminal loop of ammodytoxin A (AtxA) (*Vipera ammodytes ammodytes*) inhibited the neurotoxic effect of the enzymes (313). Further site directed mutagenesis studies conducted by Pungercar and co-workers on AtxA revealed that the substitution of Phe124, an aromatic amino acid residue at the exposed region of the C-terminal loop by aliphatic amino acid residue like Ile124 lowered the neurotoxic effect by several folds (314). This suggested the significance of the Phe124 residue in execution of the neurotoxic effect by PLA_2 enzymes (314). On the other hand, chemical modification of Trp residues of Daboia neurotoxin III (DNTx-III) (*D. russelii*) lowered the twitches in *Rana hexadactyla* sciatic nerve gastrocnemius muscle preparations (315). This suggests the importance of aromatic residues in AtxA and DNTx-III for neurotoxic activity.

1.5.7.2 Myotoxicity

Snake venom PLA_2 enzymes are often reported with both local myonecrosis and systemic myotoxic effects, the severity of which can be estimated by quantifying the release of creatine phosphokinase into the plasma of the victim (316-318). The catalytic activity of these enzymes are observed to be insignificant for the execution of myotoxic effects (317). Kini and Iwanaga proposed the importance of cationic sites (+00+++00+) at the amino terminal end of the hydrophobic helix E and hydrophobic regions for executing the myotoxic effect (319). Lomonte and co-workers proposed the crucial role of C-terminal cationic and the hydrophobic segment with Lys36 and Lys38 for the activity (320). On the other hand, chemical modification of His, Lys and Tyr residues in PhTX-I, a basic PLA_2 enzyme isolated from *Porthidium hyoprora* venom altered its myotoxic effect (321). This observation signifies the crucial role of both catalytic and pharmacological sites for displaying myotoxic effect in this enzyme (321). In another study, Asp49 and Lys49 PLA_2 enzymes from *Bothrops asper* venom are reported to act synergistically to induce myotoxicity and cell death in C2C12 myotubes along with increase in intracellular Ca^{2+} ions levels (322). The catalytically active Asp49 PLA_2 enzymes are reported to create anionic patches with the hydrolysed products to interact with Lys49 myotoxins electrostatically and exert cumulative myotoxic effects (322).

1.5.7.3 Cardiotoxicity

Although the exact mechanism of cardiotoxic effect by PLA_2 enzymes is not yet clear but it is proposed to be independent of the enzymatic activity (323). Huang and co-workers reported an acidic PLA_2 enzyme, OHV A-PLA2 from the venom of *Ophiophagus hannah* which was proposed to exhibit the cardiotoxic effect in rat heart by raising the influx of intracellular Ca^{2+} ions (324). Two basic PLA_2 enzymes (Lys-49 and Asp-49) isolated from the venom of *Agkistrodon piscivorus piscivorus* were reported to affect the functioning of a preparation of phrenic nerve-diaphragm and isolated ventricle strip of heart (325). The cardiotoxic effects of these enzymes were found to be comparable with that of catalytically active Asp-49 PLA_2 enzymes from *Naja nigricollis*, *Hemachatus haemachatus* and *Naja naja atra*. This suggests that the toxic effects are independent of the catalytic activity of the enzymes (325).

1.5.7.4 Antimicrobial activity

A number of SvPLA$_2$ enzymes are reported with the bactericidal activity which is found to be either dependent or independent of the catalytic activity. PLA$_2$ enzymes with Lys49 from *Bothrops asper* are reported to display antibacterial activity suggesting the role of non-enzymatic mechanism for antimicrobial activity (326). Weiss and co-workers proposed overall basicity and cluster of basic residues on the surface of N-terminal helix of PLA$_2$ enzymes to be important for antibacterial activity (327,328). For the bactericidal activity on gram-positive bacteria, recognition of the anionic site and phospholipid hydrolysis were crucial while for gram-negative bacteria along with phospholipid hydrolysis cumulative effect of the bactericidal/permeability increasing protein were reported to be important (329,330). VRV-PL-V from the venom of *Daboia russellii pulchella*, NN-XIb-PLA2 and NN-XIa-PLA2, from the venom of *Naja naja* are reported to exhibit potent antibacterial activity against both gram-positive and gram-negative bacteria (2,331,332). In the case of VRV-PL-V the antimicrobial activity persisted even after its modification with para-Bromophenacyl bromide (p-BPB) suggesting the existence of properties similar to bactericidal/membrane permeability-increasing protein (331). However, treatment of NN-XIb-PLA2 and NN-XIa-PLA2 with p-BPB lowered their antibacterial activity suggesting their dependence on the catalytic activity (2,332). In addition to this, Viperatoxin (VipTx-II) isolated from the venom of *Daboia russelii* (Indian Russell's viper) is reported to exhibit antimicrobial effect (*Staphylococcus aureus* and *Burkholderia pseudomallei* (KHW & TES), *Proteus vulgaris* and *P. mirabilis*) by pore formation and membrane damage (333).

1.5.7.5 Edema inducing

The edema-inducing activity of PLA$_2$ enzymes were first reported by Vishwanath and co-workers in *Trimeresurus flavoviridis* (Habu snake) venom (334). The manifestation of the edema formation is due to the enzymatic action of these enzymes where the arachidonic acid released during the phospholipid hydrolysis participates in inflammatory pathways generating leukotrienes, prostaglandins and thromboxane A2. These eicosanoids then increase vascular permeability and cause the formation of edema at the site of envenomation (335,336). PLA-A and PLA-B, (Asp49 PLA$_2$ enzymes) isolated from *Trimeresurus flavoviridis* venom displayed edema formation

with PLA-B exhibiting stronger activity compared to PLA-A (337). Both sequences were reported to be identical to each other except at position 79 with PLA-A having an aspartate residue while PLA-B with an asparagine residue. The beta-turn segment at this position was proposed to be crucial for edema-inducing activity for both the enzymes (337). Thus, apart from the enzymatic activity, structural conformations also seem to be crucial for explicating the inflammatory responses.

1.5.7.6 Platelet aggregation initiation or inhibition

Some PLA_2 enzymes are also reported to cause platelet aggregation initiation or inhibition induced by various agonists like collagen or ADP causing delay in clot formation (338). Most of the acidic PLA_2 enzymes reported so far are known to inhibit platelet aggregation induced by ADP or collagen (339,340). The platelet deaggregation and platelet binding properties of NnPLA2-I, an acidic PLA_2 enzyme isolated from the venom of *Naja naja* is reported to be lowered upon alkylation of the active site histidine residue suggesting the importance of catalytic action for exhibiting antiplatelet activity (341). Apart from this, several other acidic PLA_2 enzymes like Bp-PLA(2) from *Bothrops pauloensis* and BmooTX-I from *Bothrops moojeni* have also reported reduction in platelet aggregation inhibition induced by collagen or ADP upon treatment with p-BPB (342,343). Superbins II purified from the venom of *Austrelaps superbus* also exhibited anticoagulant effect on the extrinsic tenase complex and inhibited platelet aggregation induced by collagen (282). Thus, catalytic activity of these enzymes seems to play a crucial role in platelet aggregation inhibition; however, the exact mechanism of action is not clear. On the other hand, the ability of the PLA_2 enzymes to activate platelet aggregation is mostly attributed to the release of the lytic products like arachidonic acid and/or platelet aggregation factor (PAF) which in turn facilitate blood coagulation at the site of injury (143,344).

1.5.7.7 Anticoagulation

The ability of the $SvPLA_2$ enzymes to inhibit blood coagulation was first reported by Boffa & Boffa in *Vipera berus* venom (345). Since then a number of PLA_2 enzymes have been isolated from the various venomous snake with potent anticoagulant activity. Most of these anticoagulant PLA_2 enzymes are reported to act either enzymatically by hydrolysing the procoagulant phospholipids required for the

formation of the coagulation complexes or non-enzymatically by targeting FXa in prothrombinase complex inhibiting the conversion of prothrombin to thrombin or thrombin directly affecting the fibrin clot formation (180,182,283,341,346-348). Verheij and co-workers categorized the anticoagulant PLA_2 enzymes into strong, weak and non-anticoagulant based on the concentration required to delay the plasma clot formation (349). The strong anticoagulant PLA_2 enzymes exhibit anticoagulant effect at a concentration lower than ~2 µg/ml while the weak anticoagulant PLA_2 enzymes display at 3-10 µg/ml whereas the non-anticoagulant PLA_2 enzymes show above 15 µg/ml. On the other hand, Kini & Evans have classified PLA_2 enzymes into these three groups based on the amino acid composition at the anticoagulant region (54^{th} to 77^{th} residue based on Renetseder numbering system) (292,350). They proposed the importance of positively charged amino acid residues at the anticoagulant region for determining the anticoagulant potency of these enzymes. The strong anticoagulant PLA_2 enzymes are proposed to have positive amino acid residues in this region while the weak or non-anticoagulant enzymes contain neutral or negatively charged residues. Most of the anticoagulant PLA_2 enzymes reported so far are basic in nature while only a few of them are reported to be neutral or acidic in nature (181,351-357).

Three basic PLA_2 enzymes, CM-I, II and CM-IV with anticoagulant activity were isolated from the venom of *Naja nigricollis* (346). CM-I and CM-II exhibited anticoagulant effect on the extrinsic tenase complex by phospholipid hydrolysis. On the other hand, CM-IV exhibited anticoagulant effect on both extrinsic tenase complex and prothrombinase complex both enzymatically and non-enzymatically (182). It inhibited the prothrombinase complex enzymatically by phospholipid hydrolysis and non-enzymatically by competing with FVa for binding to FXa thus, interfering the formation of the prothrombinase complex (182,353). Ammodytoxin A, a PLA_2 enzyme isolated from the venom of *Vipera ammodytes ammodytes* inhibits the prothrombinase complex by binding to FXa and by phospholipid hydrolysis (358). Vipoxin, a heterodimeric PLA_2 complex isolated from the venom of *Vipera ammodytes meridionalis* is reported to inhibit the intrinsic tenase complex by phospholipid hydrolysis (347). TI-Nh isolated from the venom of *Naja haje* is reported to be a strong anticoagulant PLA_2 enzyme delaying the thrombin time. It

exhibits its anticoagulant activity by inhibiting the fibrinogenolytic, amidolytic and platelet aggregating properties of α-thrombin but does not show any effect on prothrombin time or activated partial thromboplastin time (187,359).

1.6 Indian *Daboia russelii* venom proteomics

1.6.1 *Daboia russelii*

Daboia russelii commonly known as the Russell's viper (honoured in the name of Patrick Russell, a renowned herpetologist known as the Father of Indian Ophiology) is one of the most venomous snake and member of the "Big Four" snakes of India. It is widely prevalent throughout the Indian sub-continent including some parts of Southeast Asia (9) (Figure 1.9). Morphologically it has a stout body with a triangular head, vertical pupil, short tail and large nostrils (9). They are nocturnal in nature and mostly dwell in areas with thick grasses, scrub, rocky hillocks and in mangrove forests (9). The scientific classification of *Daboia russelii* is shown in table 1.5

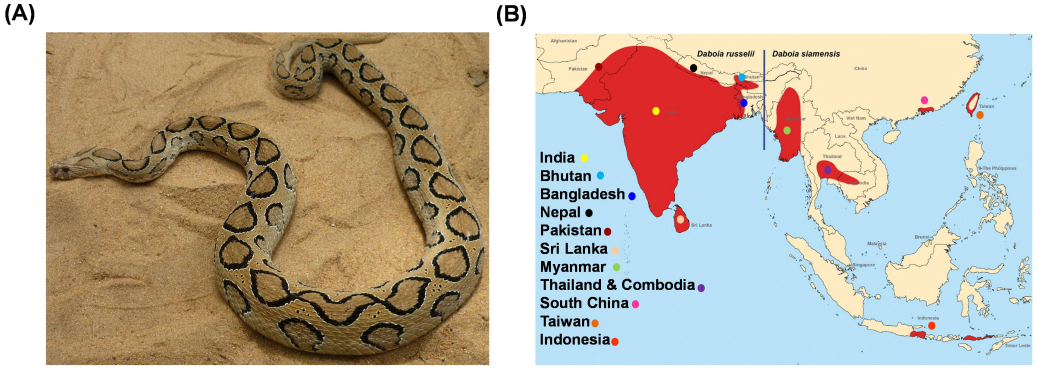

Figure 1.9: *Daboia russelii* snake and its distribution. (A): *Daboia russelii* from Irula snake catcher's society, Tamil Nadu. **Photo source:** Dr. J.C. Menon. **(B):** *Daboia russelii* is prevalent throughout the Indian sub-continent and parts of Southeast Asia **(**Adapted from Antonypillai, C.N. et. al. 2010 (360). Photo **c**ourtesy: D. J. Williams based on WHO species maps http://apps.who.int/bloodproducts/snakeantivenoms/database).

Based on the morphological difference in coloration and rows of dorsal spots the species of *Daboia russelii* was classified into 6 subspecies namely, *Daboia russelii russelii* found in India, Pakistan and Bangladesh, *Daboia russelii pulchella* in Sri

Lanka, *Daboia russelii siamensis* in Myanmar, Thailand, Combodia and southern parts of China, *Daboia russelii limits* in Java, Komodo, Flores and Lomblen while *Daboia russelii formosensis* in Taiwan and *Daboia russelii sublimits* in Javan (361). However, based on mitochondrial gene phylogenetic analysis along with morphological and basic colouration pattern, Thrope and co-workers have reclassified these snakes into two broad species namely, *D. russelii* (includes the former subspecies like *D. r. pulchella*, *D. r. russelii* and *D. r. nordicus* from northern India) and *D. siamensis* (includes *D. r. limitis*, *D. r. sublimitis* and *D. r. formosensis*) (361).

Table 1.5: Scientific classification of *Daboia russelii*

Kingdom	Animalia
Phylum	Chordata
Sub-phylum	Vertebrata
Class	Reptilia
Order	Squamata
Sub-order	Serpentes
Family	Viperidae
Sub-family	Viperinae
Genus	Daboia (Gray 1842)
Species	russelii

Human population close to forests, cultivation area and tea gardens are mostly reported to encounter this deadly snake. In India it is mostly prevalent in Punjab, Gujarat, Himachal Pradesh, Madhya Pradesh, Maharashtra, Uttar Pradesh, Bihar, West Bengal, Tamil Nadu, Andhra Pradesh and Kerala (http://reptile-database.reptarium.cz/species?genus=Daboia&species=russelii). It is responsible for most of the snakebite fatalities in India along with the cobras (9). The most profound signs/symptoms of *Daboia russelii* envenomation include pain, swelling, blistering, bleeding at the site of bite along with many severe systemic pathologies like myonecrosis, neurotoxicity, coagulopathy, renal failure etc. (95). Venom induced consumptive coagulopathy (VICC) is one of the most manifested pathological

condition observed in *Daboia russelii* envenomation where the RVV-X and RVV-V activators present in the venom activate FX and FV (362-364). This, in turn, leads to activation of prothrombin to thrombin which then facilitate the conversion of fibrinogen to fibrin (362). This triggers fibrinolysis and subsequently the fibrin degradation products accumulate. Consequently, in a very short span of time the body gets deprived of FX, FV and fibrinogen thereby causing blood incoagulable. This might lead to severe bleeding disorders in patient including to mucosal and intracranial haemorrhage (362). An-Chun Cheng and co-workers reported the down regulation of activated protein C (APC) (FVa and FVIIIa inhibitor) by DrKIn-I, a Kunitz-type APC inhibitor from Russell's viper which further enhance the process of VICC (45). Acute renal failure (ARF) or acute kidney injury (AKI) is yet another most frequently observed pathology in victims where the glomerular filtration rate of kidneys get severely lowered causing the victim to be put on dialysis (365,366). Apart from these effects, few Russell's viper envenomated victims are also reported with abdominal pain, hypopituitarism with deficiency of gonadotrophins, thyrotrophin and corticotrophin, ischemic stroke and bilateral blindness (367-370).

1.6.2 Research so far on Indian *Daboia russelii* venom

Alike other venomous snakes, the venom composition of Indian *Daboia russelii* is also reported to vary considerably with different geographical locations (371,372). The variation in venom composition and toxicity of Indian *Daboia russelii* venom was first reported by Jayanthi and Gowda in the year 1988. They compared the venom form three different locations of India viz., southern, northern and western parts (371). The chromatographic and electrophoretic analysis of the three venom samples revealed the presence of three extra proteins (66 kDa, 39 kDa and 9 kDa) in northern and western samples which were absent in the southern sample (371). Moreover, the venom samples from north and west revealed the abundance of acidic PLA_2 enzymes with higher proteolytic and trypsin inhibitor activity compared to the venom from south which was rich in basic PLA_2 enzymes. Moreover, the venoms from north and west were found to be more lethal compared to the southern venom (371). In another study, Prasad and co-workers reported the variation in *Daboia russelii* venom from different geographical locations of India in terms of chromatographic and electrophoretic profile along with biochemical activities (372). The venom from

eastern India was reported to be most toxic followed by western, southern and northern India (372). The polyclonal antibodies prepared against the Russell's viper venom of the southern region displayed differential level of cross-reactivity with the other venoms (372).

Apart from venom variation, research on Indian *Daboia russelii* is mostly directed towards isolation and characterization of individual venom proteins from some of the well characterized protein families. This includes 9 PLA_2 enzymes, 6 SvMPs, 3 LAAO, 1 each of SvSP and PDE (52,181,357,373-378). Apart from these proteins, apoptosis stimulating and pro-angiogenic peptides were also reported from the eastern *Daboia russelii* (379,380). List of venom proteins isolated from the Indian *Daboia russelii* venom with their biological functions have been shown in table 1.6.

Table 1.6: List of proteins isolated and characterized from Indian *Daboia russelii* venom.

Protein family	Protein	Characteristics
$SvPLA_2$	VRV-PL-IIIb (14-15kDa)	PLA_2 activity, induce edema in foot pad of mice, inhibit ADP induced platelet aggregation, devoid of anticoagulant, myotoxic and direct haemolytic activities (37)
	VRV-PL-V (10 kDa)	PLA_2 activity, neurotoxic, LD50 value is 4.1 mg/kg body weight, edema inducing, hemorrhage and hemolytic activity, antibacterial activity against gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative bacteria (*Escherichia coli*, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Salmonella paratyphi*) (331,381-383)
	VRV-PL-VI (12 kDa) basic protein	order of hydrolysis is phosphatidylethanolamine > phosphatidylcholine > phosphatidylserine > phosphatidylinositol, edema inducing, LD50 value (i.p.) of 3.5 µg/g body weight in mice (384)
	VRV-PL-VIIIa (13 kDa) basic protein	PLA_2 activity, indirect haemolytic, neurotoxic, edema inducing, myotoxic, haemorrhage, anticoagulant, antibacterial activities against gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative (*Escherichia coli*, *Vibrio cholerae*, *Klebsiella pneumoniae* and

		Salmonella paratyphi) bacteria (37,385-390)
	RVVA-PLA2-I (58 kDa)	exhibit strong anticoagulant activity by phospholipid hydrolysis and inhibiting the activity of FXa in presence of Ca^{2+} ions, indirect haemolytic, erythrocyte & mitochondrial membrane damaging (181,391)
	DPLA2 (14 kDa)	PLA_2 activity, anticoagulant, pre-synaptic neurotoxic (373)
	Neupholipase (13 kDa) Neutral protein	indirect haemolytic & anticoagulant activity, preferentially hydrolyses phosphatidylserine followed by phosphatidylcholine and least phosphatidylethanolamine, non-lethal to BALB/c mice, damaged liver of treated-mice (356)
	RVV-PFIIc' (15.3 kDa)	PLA_2 activity, anticoagulant (delay Prothrombin time), LD50 (i.p.) of 0.1 mg/kg body weight in mice, haemolytic activity (357)
	Viperatoxin-II (VipTx-I, VipTx-II) (15 kDa)	antimicrobial activity, low cytotoxic effects on human (THP-1) cells at higher concentrations (333)
SvMPs	Daborhagin-K (65 kDa)	PIII metalloprotease, induce dermal haemorrhage in mice (375)
	VRR-12 (12 kDa)	induces skeletal muscle and intestinal haemorrhage, no proteolytic and esterolytic activity (392)
	VRH-1 (22 kDa)	proteolytic activity and intradermal injection induced severe lung haemorrhage but no skin haemorrhage (393)
	VRR-73 (73 kDa)	haemorrhagic, fibrinolytic & arginine esterolytic activity (394)
	RVBCMP (15 kDa)	dose-dependent procoagulant activity on platelet-poor human plasma, exhibits caseinolytic, α-fibrinogenolytic and liver tissue haemorrhage (376)
	Rusviprotease (26.8kDa)	similar to group A prothrombin activators i.e., cleave prothrombin independent of co-factor into meizothrombin and exhibit α-fibrinogenase activity (395)
SvSP	RVV-V (Russell's viper venom FV activator) (~28 kDa)	destabilizes Aβ-amyloid aggregate, exhibit potential for prevention and propagation of the Alzheimer disease caused by β-amyloid plaque (377)
LAAO	L1 & L2 (60-63 kDa)	prefer hydrolysis of hydrophobic amino acids with maximum catalytic efficiency for L-Phe (374)
	DrLAO (60-64 kDa)	inhibit ADP and collagen induced platelet aggregation (52)
PDE	DR-PDE (100.4 kDa)	no 5'-nucleotidase, alkaline phosphatase and protease activities, inhibits ADP induced platelet aggregation (378)

Apoptosis stimulating	Ruviprase (4.4236 kDa)	anticoagulant (inhibit thrombin uncompetitively by binding to its active site and exhibit mixed type inhibition on FXa for prothrombin activation), in-vivo anticoagulant activity, nontoxic (380)
Pro-angiogenic peptide	RVVAP (3.9 kDa)	angiogenesis and endothelial cell migration at a concentration of 50 ng/ml, non-cytotoxic to U87-MG, HeLa and HT-29 cells, above a concentration of 500 ng/ml it induced chromosomal aberrations and delayed cell cycle kinetics in chinese hamster ovary cell lines (379)

1.7 Gap in the study

Ironically most of the proteins reported so far in the Indian *Daboia russelii* venom are only partially characterized in terms of sequence and mechanism of action. Moreover, no data is available regarding the complete proteomic or transcriptomic profile of this medically important snake of India. For example in 2009, Risch and co-workers analysed the complete protein profile of *Daboia russelli siamensis* from Myanmar by 2-dimensional electrophoresis followed by tandem mass spectrometry and biochemical assays (396). This snake is reported to be composed of 6 protein families namely SvMPs, LAAO, Snaclecs, VEGF along with relative maximum abundance of SvSPs followed by PLA_2 enzymes (396). The unfolded profile of venom proteins were reported to be in agreement with the clinical pathologies of the Russell's viper envenomation like coagulopathy, edema, hypotension, necrotic and tissue damaging effects (396). Hence, deciphering the complete proteome of the Indian *Daboia russelii* venom would also provide comprehensive information regarding its probable pharmacological profile along with the major and unique proteins. Moreover, no major anticoagulant protein has been reported so far from Indian Russell's viper. Thus, identification and characterization of such major toxins might help understand some of the most crucial pathological effects of this snake. Moreover, elucidation of the structure-function relationship of such major proteins will shed light into their physiological targets and decipher the mechanism of action.

1.8 Aims and objectives of the study

As such it is important to re-visit the variation in the venom composition of Indian *Daboia russelii* from different geographical locations and analyse the impact of compositional variation on the efficacy of the available polyvalent antivenom. Although several proteins have been reported from Indian *Daboia russelii* however, venom proteome analysis has not been carried out. Thus, venom composition of this medically important snake needs to be deciphered by using high throughput proteomic techniques which would not only unfold the complete toxin profile but also help in identifying the major proteins available in this venom. Hence, unveiling the venom composition and characterization of the major protein will provide better understanding of this medically important Indian snake and aid in its better clinical management post-envenomation.

Based on this review of literature and breach of information, the objectives of this thesis have been framed as follows;

Objectives

1. **Analysis of crude *Daboia russelii* venoms from different geographical locations of India.**
2. **Proteomics of *Daboia russelii* venom (Irula) and identification of a major protein.**
3. **Purification of a major protein from the venom of *Daboia russelii* (Irula) and its biophysical characterization.**
4. **Biochemical and Biological characterization of the purified protein.**
5. ***In-silico* structural elucidation and mechanism of the purified protein.**