Chapter 1

GENERAL INTRODUCTION

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Snakes

Snakes have fascinated mankind since the pre-historic period. Humans have used snakes in worship, sport, entertainment, medicine, commercial usage and even in conflicts and war. The essence of this kind of fascination towards these reptiles mainly lies in their venom. Hence, this reptilian creature has played varied roles in humans' thought. Snakes are highly evolved reptiles belonging to the Phylum: ***Chordata***, Order: ***Squamata*** and Suborder: ***Serpentes***. Out of 3,000 species of snakes identified on earth, around 300 species are considered to be poisonous (Fox and Seranno, 2009). However, the number remains progressive as more and more new species are discovered till date. Snakes are widely distributed throughout the world in almost every country between latitudes 50°N and 50°S in the western hemisphere and 65°N (Scandinavia) and 50°S in the eastern hemisphere except in Arctic, New Zealand and Ireland where the ground stays frozen round the year (Deoras, 1965). On land, venomous snakes are found from the sea level up to altitudes of about 4000 m in the Americas and Himalayas. Apart from land, snakes are also widely distributed in salt water bodies. Sea snakes are found in the Indian Ocean and Pacific Ocean between latitudes 30°N and 30°S and can dive to depths greater than 100 m in the oceans (Klauber, 1972; Sclater, 1981; Heatwole, 1999).

Snake venom is the modified saliva that is produced from the venom glands (Jacobson`s glands) situated below the eyes (Chiappinelli, 1985). Snakes inject venom in to their prey through special syringe like apparatuses called fangs during prey possession. In addition, the venom assists in digestion of the prey as well as protection against predators (Cousin and Bon, 1999; Hodgson and Wickramaratna, 2000; Conolly, et al. 1995). However, the amount of venom that snakes inject depend on the species to which they belong, the elapsed time since the last bite, the degree of threat they feel and the size of the prey to which they bite (Hodgson and Wickramaratna, 2000). Venomous snakes are categorized into five major families, *viz*. ***Viperidae***, ***Colubridae***, ***Crotalidae***,

Elapidae and ***Hydrophidae*** based on their morphological characteristics including arrangement of scales, dentition, osteology, myology, sensory organs etc., (Harris, 1985).

The most diverse family of venomous snakes is ***Viperidae***. Vipers have a large, flattened triangular head and the body consists of numerous and heavily keeled scales. The venom fangs are large, which permit deep penetration during envenomation of prey. They have a hinged-fang mechanism, which allows for the venom storage against the roof of the mouth when not in use; opening of the jaw is not always associated with the erection of the fangs. Vipers are found throughout Europe, Africa, Asia, and American continent (Harris, 1985). This family includes Russell's viper, saw-scaled viper, puff adder, gaboon viper, horned viper of Sahara and European vipers.

The ***Crotalidae*** family can be considered as subfamily of *Viperidae*. They differ from vipers in having temperature sensitive deep pits located between the nostrils and the eyes and their heads are usually much wider than their necks. The pits allow the snakes to detect small variations in temperature above ambient temperature from a short distance. This facility evolved to assist in hunting warm-blooded prey, especially at night. They are commonly brown with dark blotches, though some kinds are green. This family includes rattlesnakes, copper head, water moccasins and pit vipers found in the Americas and parts of Southeast Asia (Harris, 1985).

The ***Colubridae***, is the largest of the five families and accounts for nearly two thirds of the snakes in the world comprising 80 to 85% of snakes population. The majority of snakes belonging to this family are non-venomous while, only few of them are venomous. The venom fangs are grooved and mounted at the rear of the upper jaws in contrast to the front as in all other venomous snakes. Some of the venomous Colubrids are African twig snake and Boomslang (Harris, 1985).

The ***Elapidae*** family includes kraits, cobras, mambas and coral snakes. Members of this family typically have a small head with short, fixed fangs mounted at the front of the jaw that fit into grooved slots in the buccal floor when the mouth is

closed. Some members of this family are venomous; however, not all are dangerous to human beings (Harris, 1985).

The ***Hydrophidae*** family comprises of the sea snake. These snakes have evolved several adaptations to allow predominant existence in the water bodies. They have specialized flattened tails used for swimming. Due to their need to breathe air, they have valves over their nostrils, which are closed underwater when they swim. Only a small proportion of bites from these snakes are fatal to human beings because these snakes can control the amount of venom to be injected. They are usually found in the warm waters of the Indian and Pacific Oceans (Harris, 1985; Lee CY, 1979).

Snakebite

Snakebite in the tropical and sub-tropical countries is a serious threat and hence a medical emergency. Rural and sub-urban population and communities in general that comprise of farmers, labourers, snake handlers and workers in the mining areas and quarries are the usual victims of accidental snakebite. Hundreds and thousands of these victims suffer mainly from long-term injury and secondary complications. Global estimate of snakebite victims is found to be approximately 5.4 million per year (Chippaux, 1998). In Asia alone, it contributes to approximately 100,000 deaths (Kasturiratne, 2008).

In the Indian subcontinent, more than 2,00,000 snakebite victims are reported annually and around 35,000 to 50,000 cases of them turn out to be fatal (Brunda, 2007). The exact number of snake species across the world is estimated to be 1776 till date of which the Indian subcontinent harbours 276 snake species (Rajkumar, naturalist). Around 62 species of snakes in the Indian subcontinent are reported venomous, among which, *Naja naja* (common cobra), *Daboia/Vipera russellii* (Russell's viper), *Bungarus caeruleus* (common krait) and *Echis carinatus* (Saw-scaled viper) are endemic and are distributed throughout the subcontinent and hence the snakebite in the region is generally attributed to bite by any one of these four species. Therefore, these four species are popularly called the "**Big fours"** of India. Apart from these snakes, the

other venomous snakes which strike human beings are banded krait (*Bungarus fasciatus*), Indian monocled cobra (*Naja naja kaouthia*), Green pit viper (*Trimeresurus malabaricus*) and Hump nosed pit viper (*Hypnale hypnale*). Banded krait is commonly found in Assam, Bengal, Bihar, and Orissa and also in parts of Madhya Pradesh, Andhra Pradesh and Uttar Pradesh. Monocled cobras are a sub-species most commonly found in north-western parts, parts of Uttar Pradesh, Bihar, Orissa and the Andamans. Hump nosed pit viper is found in southern parts whereas the Green pit viper remains restricted to south-western parts of India. Nevertheless, the biggest and the largest of the venomous snakes, the king cobra (*Ophiophagus hannah*) has been found to be distributed in the western ghats and eastern hilly regions of the country.

Biochemistry of snake venom

Snake venom is a colorless to dark amber, viscous/nearly viscous liquid and is the most complex of all naturally occurring venoms (Cousin and Bon, 1999). Venom of any snake species contains more than 100 different toxic and non-toxic proteins and peptides, non protein toxins, organic, inorganic components and water. Organic components include biogenic amines, carbohydrates such as neutral sugars, amino sugars, Sialic acid and lipids such as cholesterol, monoglycerides, diglycerides and triglycerides. The inorganic components include metal ions such as Ca^{2+}, Cl^{-}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni, P, K^{+}, Na^{+}, S, Zn^{2+} (macro components) and Bi, Au, Mb, Pl, Pb, Se, Ag (micro components). These metal ions are required for the activity of venom enzymes, others are thought to be essential for stabilizing certain proteins while, not all the metal ions are found in every snake venom. The venom components are fairly common and are similar to one another within each family of snakes. However, snake venoms exhibit marked variations in their potency and extent of inducing toxic properties. The variability of venom composition has been considered at several levels: inter family, inter genus, interspecies, and inter subspecies and intra species while, intra species variability may be due to geographical distribution, seasonal and age dependent

change, diet and variation due to sexual dimorphism (Jayanthi and Gowda, 1988; Chipaux et al., 1991; Daltry et al., 1996; Shashidharamurthy et al., 2002).

The proteins and peptides of snake venom are of both enzymatic and non-enzymatic and are among the most toxic components. The protein part contributes to more than 90 to 95% of the dry weight in all the snake venoms. There are over 25 enzymes that have been described and most of them have been characterized in detail. Of the 25 enzymes, 6 to 12 occur in all venoms (Iwanaga and Suzuki, 1979). They include phospholipase A_2, L- amino acid oxidase, phosphodiesterase, proteases including serine and metalloproteinase, 5'-nucleotidase, phosphomonoesterase, deoxyribonuclease, ribonuclease, adenosine triphosphatase, hyaluronidase, NAD-nucleosidase, arylamidase, phosphatase (acid as well as alkaline), esterase, acetylcholinesterase and transaminase (Dowling and Duellman, 1978).

Snakebite Pharmacology

Snake bite results in a wide range of effects, from simple puncture wounds to life-threatening illness and death. Some toxins show effect at the site of the bite; others are only active and cause harmful effects in their respective target tissues and organs after reaching through the blood stream. Irrespective of toxicity, all snake venoms are reported to affect the hemostatic system with different degrees. While Proteases are the major class of enzymes that affect hemostasis, PLA_2 may have role in exhibiting both systemic and local toxicity. Hyaluronidase enzymes are the spreading factors of toxicity at the site of bite and their activity is the hall mark of local toxicity (Girish et al, 2007). Hence the snakebite manifestation can be broadly classified into local and life threatening systemic effects.

a. Local toxicity

Local toxicity is the initial manifestations of snake bites, the symptoms appears within 6-8 minutes in case of *crotalid* and *viperid* bites and it may have onset up to 30 min in case of elapid bite (Paul, 1993). The effects comprise of initial pain with radiation and development of small reddish area at the bite site followed by edema,

hemorrhage, myonecrosis and tissue damage. These symptoms advance resulting in bleeding and tissue softening. Edema is a symptom that shows increased interstitial fluid accumulation at the affected tissues or organs due to the activities of various hydrolytic enzymes (myotoxic PLA_2s, hyaluronidases and SVMPs). This inturn softens the tissue by degrading extracellular matrix and generate pro-inflammatory end products. Myonecrosis may be due to the vascular degeneration and ischemia caused by SVMPs, or a range of venom myotoxic and cytolytic factors which may have direct action on the plasma membrane of muscle cells and are collectively called hemorrhagins. Hemorrhage is the oozing of blood to extravascular space through damaged vascular endothelium (Ownby et al., 1990; Kamiguti et al., 1991, 1996; Gutierrez et al., 2000). Extracellular matrix (ECM) is a diverse, complex structural unit consisting of three classes of molecules namely, structural proteins (collagen, elastin, etc.), glycosaminoglycons (chondroitin, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid) and adhesive glycoproteins **(**fibronectin, laminin and nidogen/entactin). In snake venoms, two groups of enzymes degrade the components of extracellular matrix; hyaluronidases, which primarily degrade the hyaluronic acid and the SVMPs which degrade protein scaffold.

b. Systemic toxicity

Systemic toxins, regarded as the arsenal of elapids target the functioning of the vital organs of the victim involving nervous, muscular and hemostatic systems. Cobra produces symptoms as early as 5 minutes or as late as 10 hours after the bite depending on the amount of venom it has injected while, vipers take slightly longer duration of onset being 20 minutes. However, symptoms may be delayed for several hours. In general, systemic toxicity comprises of neurotoxicity, myotoxicity, cardiotoxicity, nephrotoxicity, altered haemostasis and systemic bleeding.

Neurotoxicity is the selective blockage of neuro-muscular junction by the neurotoxins which results in flaccid paralysis of muscles. The development of cranial nerve palsies is the generally observed symptom of neurotoxin envenomation which is

characterized by ptosis, blurred vision, difficulty in swallowing, slurred speech and weakness in facial muscle. The neurotoxins are of pre-synaptic (β-neurotoxins) and post-synaptic (α-neurotoxins) types (Yang and Chang, 1999; Liu et al., 2001). The α-neurotoxins can block nerve transmission reversibly by competitively binding to the nicotinic acetylcholine receptors located at the post-synaptic membranes of neurons and skeletal muscles preventing the neuromuscular transmission leading to death by asphyxiation. In contrast, the β-neurotoxins act pre-synaptically preventing the release of acetylcholine molecules and hence responsible for high toxicity and ultimately respiratory paralysis. They act by causing the disappearance of acetylcholine containing vesicles, preventing the controlled release of acetylcholine and blocking impulse transmission.

Cardiotoxicity is characterized by the damage of heart muscle by the cardiotoxins which are also referred to as cytotoxins. The main targets of these toxins are excitable cells and cause depolarization and contracture of cardiac, skeletal and smooth muscles, depolarization and loss of excitability of nerves. These toxins are pore-forming agents that lead to the depolarization and degradation of the plasma membrane of skeletal muscle cells. Cardiotoxins I, II, III and IV (Bhaskaran et al., 1994), cardiotoxin-like basic proteins from *Naja atra* venom (Rong et al., 2007), sagitoxin from *Naja saggitifera* (Mir et al., 2008), few PLA_2 enzymes from the venom of *Naja nigricollis* (Fletcher et al., 1982), *Bungarus fasciatus* (Chang et al., 1983), *Ophiophagus hannah* (Huang et al., 1993; Wang et al., 2001) and *Naja naja* (Cher et al., 2005) were reported to exhibit cardiotoxicity.

Snake venom components disturb circulatory system and affect vascular endothelium and as well as blood coagulation cascade. Vasoactive agents derived due to venom induced vascular injury or due to the action of venom SVMPs affect the blood flow (vasoconstriction results in increased blood pressure while, vasodilation reduces the blood pressure and the increased vascular permeability results in reduced

venous return). Altered hemostasis is basically due to activating or inhibiting coagulation factors or platelets. *Viperid* and Australian *elapid* venoms contain procoagulant enzymes which exhibit thrombin-like, fibrinogenase, prothrombin activation, factors V, X, and XIII activation and endogenous plasminogen like activities. Toxins bind to a wide range of platelet receptors and bring about either activation or inhibition of platelets. Anticoagulant venom phospholipases A_2 hydrolyse or bind to procoagulant phospholipids (PS and PE) and inhibit the prothrombinase complex. The SVMPs (hemorrhagins) damage the vascular endothelium, which results in spontaneous bleeding. Bleeding is a common cause of death due to venomous bites of *Viperids*, *Colubrids* and some Australian *Elapids*.

Haemostasis

Haemostasis is an acute phase defence response by the host, which controls the loss of blood following a vascular injury. The process of blood clotting and subsequent clot dissolution and repair of the injured tissue is termed as haemostasis (Bloom, 1990; Davie et al., 1991; Harker, 1997). Haemostasis consists of four major sequential events; vasoconstriction, platelet activation and aggregation, formation of fibrin and clot dissolution (Fibrinolysis).

Platelet activation and aggregation

The endothelial cell monolayer of blood vessels provides an antithrombotic (non adhesive) surface by separating blood from the sub-endothelial matrix proteins and also by synthesising and releasing prostacyclin (PGI_2) and nitric oxide, which both inhibit platelet activation by their ability to increase cAMP and cGMP respectively. Endothelial cells also express CD39 at the luminal surface. CD39 is a nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) that sequentially converts ATP into ADP and AMP, thus eliminating ATP and ADP from the vicinity of the vessel wall and preventing platelet activation (Robson et al., 2005; Marcus et al., 2005). However, upon vessel wall injury, platelets encounter the exposed sub-endothelial matrix, which

contains thrombogenic proteins, particularly collagen. Collagen is one of the major and potent thrombogenic proteins of the subendothelial matrix that cause platelet adhesion and eventual activation. The major collagen receptors on the surface of platelets are the integrin α2β1, GPVI and GPIb-IX-V while, CD36 (GPIV) and p65 are the minor receptors. Following vascular damage, integrin α2β1 and GPVI interact directly and GPIb-IX-V interacts indirectly with collagen. The precise function of the minor collagen receptor remains unclear.

The vWF is predominantly present in subendothelial layer that has been secreted by Weibel–Palade bodies of endothelial cells and remains bound to subendothelial collagen and are also present in the plasma. During endothelial injury, the subendothelial vWF get exposed. In addition, the activated platelets also secrete vWF from their α granules.

Mature vWF forms disulfide-linked multimers up to 20,000 kD in molecular mass, made up of ~275-kDa subunits. At high shear rates, the plasma vWF immediately binds to the exposed collagen through the A3 domain and then induces conformational change in the A2 domain which in turn activates A1 domain (encompassing the Cys509-Cys695 disulfide bond) to bind GPIbα. Hence the platelets are adhered to the collagen indirectly through vWF.

GPVI is a low affinity collagen receptor that binds directly to the exposed collagen. It is an immunoglobulin superfamily protein, which has two extracellular immunoglobulin-like domains, a mucin like core, a transmembrane domain and a short cytoplasmic tail that binds Fyn and Lyn Src kinases. A positively charged Arg residue within the transmembrane domain mediates the association of GPVI with FcR γchain (Watson et al., 2005).

After adhesion, the GPIb-IX-V and GPVI receptors initiate platelet activation through their transmembrane and intracellular signalling. The cytoplasmic region of GP Ibα is associated with filamin (actin binding protein), calmodulin, and 14-3-3ζ, which

provides potential links to relevant signalling proteins such as phosphatidylinositol 3-kinase (PI-3K), focal adhesion kinase (FAK), src-related tyrosine kinases, GTPase-activating protein and tyrosine phosphatases (PTP1b and SHPTP10) (Ozaki et al., 2005; Du, 2007). The GPVI is complexed with FcR γ-chain dimer, which bears an immunoreceptor tyrosine-based activation motif (ITAM) acting as the signal-transducing subunit of the receptor. When the GP VI receptor is cross-linked by binding to collagen, the constitutively bound src kinases phosphorylate the ITAM sequence in the FcR γ-chain allowing the assembly and activation of Syk and initiating activation of a downstream signalling pathway. Essential to this signalling cascade is the formation of the signalosome, composed of various adapter and effector proteins (LAT, SLP-76, Gads), which associates to and activates PLCγ2, thus leading to the liberation of 1, 2 diacylglycerol and inositol 1, 4, 5- triphosphate (Watson SP, et al. 2005).

The intracellular signalling by GPIb and GPVI leads to transient elevations in cytoplasmic Ca^{2+} concentration, protein phosphorylation (PLCγ2, ERK-1/2, Syk), thromboxane A_2 (TxA2) synthesis, change in platelet shape, secretion of their storage granules such as α (growth factors, fibrinogen, vWF, adhesive factors) and dense granules (ATP, ADP, serotonin) and activation of integrin αIIbβ3 (GPIIb-IIIa), the fibrinogen receptor (Ozaki et al., 2005; Du, 2007).

Integrin α2β1, a collagen receptor, plays a complementary role in mediating platelet adhesion to collagen surface since it does not stimulate tyrosine kinase activity which is required for collagen-induced platelet activation. At low shear rate, binding of platelets occur directly via by α2β1 integrin (GPIa-IIa) receptor, which allows further binding of collagen via GPVI receptor. Hence, a two-site–two-step model of platelet activation was proposed (Santoro et al., 1991) in which integrin α2β1 stabilizes interactions with collagen, allowing it to interact with GPVI.

Unvaryingly, platelet activation is also triggered by various agonists. One among them is collagen, which acts via GPVI and thrombin. Once generated after

exposure of tissue factor from the injured vessel wall, it activates platelets through protease-activated receptors (PARs). Human platelets contain two signalling receptors for thrombin; PAR1 and PAR4 (Kahn et al., 1998; Kahn et al., 1999). PAR1 and PAR4 are members of the GTP-binding protein (G-protein)–coupled seven transmembrane receptors family. They are unique since they are activated when thrombin cleaves their amino termini to expose new amino termini that act as intramolecular ligands (Coughlin et al., 1992; Hung et al., 1992). PAR1 mediates rapid platelet responses at low thrombin concentrations whereas PAR4 is activated only at higher thrombin concentrations and appears to be unnecessary when PAR1 function is intact. Recently, GPIb was also shown to be a functional thrombin receptor for platelet aggregation.

Platelet activation also results from rapid, positive feedback mechanisms such as the synthesis of thromboxane A_2 (TXA_2), cyclooxygenase (COX) and TXA_2 synthase from phospholipase A2–released arachidonic acid. TXA_2 is a potent platelet-aggregating and vasoconstriction agent that activates the thromboxane prostanoid receptor (TP receptor) (Hourani and Cusack, 1991). Adenosine diphosphate (ADP) secreted from platelet-dense granules and released from damaged tissue and erythrocytes, binds to three platelet receptors (Daniel et al., 1998): P2X1, an ADP-stimulated calcium channel whose function is unknown; P2Y1, a G-protein–coupled seven-transmembrane domain receptor that activates phospholipase Cβ and induces platelet shape change (Jin et al., 1998) and $P2T_{AC}$ (PTY12), a receptor identified pharmacologically that inhibits platelet adenylyl cyclase (Daniel et al., 1998). Simultaneous stimulation of P2Y1 and $P2T_{AC}$ is required to induce platelet aggregation (Jin and Kunapuli, 1998). Epinephrine stimulates platelets by binding to α2A-adrenergic receptors that are coupled to the G protein Gi and probably acts in synergy with agonists such as thrombin and ADP that stimulate Gq-coupled receptors to induce platelet aggregation.

Despite being considered as secondary agonists of platelets, both TXA_2 and ADP greatly amplify the activation signals and enable strong platelet recruitment at the site of injury, thereby leading to the formation of the hemostatic plug. Binding of any of

the agonist to their respective membrane receptors initiates signalling pathways that alternatively convert αIIbβ3 integrin from low affinity resting state to high affinity activated state (inside-out signalling) for binding of extracellular soluble ligands such as plasma fibrinogen. Subsequently, the divalent fibrinogen functions as a bridge between the activated αIIbβ3 integrin in the neighbouring platelets, which then generates signals (outside-in signalling), thus allowing platelet aggregation to proceed and culminate in forming irreversible aggregates. Fibrinogen binding to activated αIIbβ3 integrin is actually mediated by the γ–chain sequences of fibrinogen. It is also important to note the fact that, platelet activation can also arise due to adrenalin stress or venom derived vasoconstrictors which results in increased shear stress.

a. Agonists of platelet aggregation

Snake venom serine proteases stimulate platelet aggregation process by targeting thrombin receptors. They bring about the proteolytic cleavage of proteinase-activated receptors (PARs) or they bind to the GPIba receptor. PA-BJ from *B. jararaca* venom is found to activate platelets by cleaving PARs (Santos et al., 2000) and Bothrombin isolated and characterized from the same snake venom is found to bind to the GPIba receptor and interfere in platelet aggregation (Nishida et al., 1994) respectively. Many prothrombin activators from snake venoms activate platelets indirectly by generating α-Thrombin. This α-Thrombin generated during the activation process, activates human platelets by cleaving PARs, PAR1 and PAR4 (Covic et al., 2000) and also through binding to GPIba receptor (Mazzucato et al., 1998). C-type lectin-like proteins (CLPs) induce both agglutination and aggregation of platelets. Many of them affect platelets by binding to vWF or receptors such as GPIb, α2β1 and GPVI (Andrews, 2004; Clemetson, 2007).

CLPs that bind to GPVI induce platelet activation by clustering these receptors, thus increasing their affinity for collagen. Convulxin, isolated from *Crotalus durissus terrificus*, is the first toxin that has been found to activate platelets via GPVI (Polgar et al., 1997). Convulxin also binds GPIb, which may play a role in platelet activation (Kanaji et al., 2003).

b. Antagonists of platelet aggregation

The majority of Snake venom metalloproteases (SVMPs) characterized so far have exhibited inhibitory activity on platelet aggregation induced by collagen. This inhibition appears primarily to involve an interaction of the SVMP with platelet collagen receptor α2β1 integrin and GP VI. Both P-I and P-III class SVMPs have been reported to interfere with vWF-dependent platelet activation by binding to and/ or hydrolyzing vWF and GPIb receptor (Ward et al., 1996; Hamako et al., 1998; Hsu et al., 2007). Crotalin, a Metalloprotease-I from *Crotalus atrox* venom inhibits platelet aggregation by cleaving both vWF and GPIb. Jararhagin, a P-III class SVMP from *Bothrops jararacus* venom inhibits Ristocetin-induced platelet aggregation by cleaving vWF (Kamiguti et al., 1996b). Kaouthiagin from *Naja kaouthia* venom, a P-III class SVMP, was shown to bind and cleave vWF (Hamako et al., 1998). Acurhagin, from *Agkistrodon acutus* venom, a P-III SVMP, is found to inhibit collagen and Ristocetin-induced platelet aggregation by cleaving collagen and vWF respectively (Wang and Huang, 2002).

Effects of SVMPs on GPVI receptor have also been reported. SVMPs inhibit platelet aggregation induced by collagen by cleaving or binding to GPVI receptor. Kistomin from *Calloselasma rhodostoma* inhibits aggregation by cleaving GPVI and it also cleaves GPIb (Hsu et al., 2008). Acurhagin and AAV1 from *Agkistrodon acutus* is reported to bind to GPVI receptor (Wang et al., 2005; Wang, 2007). SVMPs are also found to interact with proteins containing vWF-A1 domains, via their Cys-rich domain (Serrano et al., 2006; Pinto et al., 2007).

L-amino acid oxidases (LAAOs) cause both activation and inhibition of platelet aggregation but the effects are contentious. Both the effects are associated with the ability of LAAOs to produce hydrogen peroxide, since the catalases attenuate their effect. The reason for inhibition of platelet aggregation might be in the reduced binding of ADP by platelets exposed to hydrogen peroxide (Belisario et al., 2000). Activation of platelet aggregation is presumably caused by hydrogen peroxide-induced amplification of thromboxane A2 synthesis and therefore, the inducement of platelet

aggregation (Li et al., 1994). On the contrary, some studies imply that hydrogen peroxide production is not the only factor responsible for platelet activation. For example, in the case of NA-LAAO from *Naja atra* venom, it was shown that both hydrogen peroxide production and binding to platelet membrane proteins could be involved (Li et al., 2008). Further, it was propounded that the enzyme binds to the platelet membrane and thereby enhances the sensitivity of platelets to hydrogen peroxide and at the same time, hydrogen peroxide released by the enzyme activates platelets by an unknown mechanism.

CLPs inhibit platelet activation by blocking the binding of vWF/Ristocetin and/or α-thrombin to the GPIb receptor (Lu et al., 2005). Most inhibitory C-type lectins that bind to GPIb are heterodimers. Echicetin from *Echis carinatus sochureki* venom specifically binds platelet GPIb receptor and blocks platelet interactions with vWF and thrombin (Peng et al., 1993). Agkistin from *Agkistrodon acutus* venom (Yeh et al., 2001) and flavocetin-A from the venom of *Trimeresurus flavoviridis* venom (Taniuchi et al., 1995) are also found inhibit platelet aggregation strongly by binding specifically to platelet GPIbα.

Disintegrin bind to $\alpha_{IIb}\beta_3$ integrin on activated platelets through RGD sequence and thus, prevents the interaction with fibrinogen and inhibit platelet aggregation induced by a wide range of agonists such as ADP, α-thrombin, collagen and arachidonic acid (Marcinkiewicz et al., 1997; Kauskot et al., 2008). Trigramin was the first disintegrin discovered and purified from the venom of *Trimeresurus gramineus* venom (Huang et al., 1987). So far, more than 78 disintegrins have been reported from snake venoms (Matsui, et al., 2010). Elegantins from *T. elegans* venom (Williams et al., 1990), Triflavin from *Trimeresurus flavoviridis* venom (Huang et al., 1991), Kistrin from *Agkistrodon rhodostoma* venom are found to contain the RGD sequence in a homologous position and hence inhibit the interaction between fibrinogen and GP IIb/IIIa complex.

Dendroaspin and α-Bungarotoxin are the three-finger toxins that are short-chain neurotoxin analogues from *Dendroaspis jamesonii* and *Bungarus multicinctus* venoms respectively (McDowell et al., 1992; Shiu et al., 2004). Both of them contain disintegrin-like RGD motif on the tip of one of their 'fingers'. These are able to inhibit platelet aggregation and integrin $\alpha_{IIb}\beta_3$-mediated platelet adhesion (Lu et al., 1994).

A 74 kDa 5'-nucleotidase from *Trimeresurus gramineus* venom, a single chain protein is reported to inhibit platelet aggregation induced by ADP, sodium arachidonate and collagen (Ouyang and Huang, 1983). 5'-nucleotidase from *Crotalus atrox* venom, administered *in vivo* to mice, abolished ADP and collagen induced platelet aggregation and increased adenosine concentrations and tail-bleeding time (Hart et al., 2008). 5'-nucleotidase from *Naja naja* venom was also reported to have an anticoagulant effect, presumably by interacting with one or more factors of the intrinsic pathway of blood coagulation (Dhananjaya et al., 2006). A phosphodiesterase from *Bothrops jararaca* venom, NPP-BJ, a homodimeric glycoprotein of 228 kDa, inhibited platelet aggregation induced by ADP whereas, thrombin-induced platelet aggregation was only slightly attenuated (Santoro et al., 2009). NPP-BJ is found to reduce the amount of platelet aggregation induced by ADP, by hydrolysing adenylated nucleotides secreted from platelet dense bodies during platelet activation.

Formation of fibrin

Stabilization of the initially generated loose platelet plug is conferred by the formation of a fibrin mesh (**clot**) that entraps the plug. The eventual fibrin production depends on a series of proteolytic reactions of the blood coagulation pathway in which, an inactive precursor (zymogen) of a proteolytic enzyme is converted to the active enzyme. These enzymes are called proteases or proteinases. Each step in the series is enzyme-catalyzed, and thus, one enzyme molecule can catalyze the formation of a very large number of molecules of product. This cascade is subjected for enormous amplification. The proteases involved in clotting generally require cofactors to regulate

them and enable them to catalyze the reactions they are involved in. The cascade of reactions comprises of two distinct but closely linked pathways, the contact activation pathway (intrinsic pathway) and the tissue factor pathway (extrinsic pathway).

Contact activation pathway (Intrinsic pathway)

The intrinsic pathway is initiated when blood comes in contact with the sub-endothelial connective tissue such as collagen or with negatively charged surface that are exposed as a result of tissue damage. Intrinsic pathway requires the factor XII *(Hageman factor*), factor XI, pre-kallikrein and high molecular weight kininogen for the initial activation. Following other steps, eventually active Factor Xa is generated.

Tissue factor pathway (Extrinsic pathway)

The extrinsic pathway is an alternative route of coagulation cascade, initiated at the site of injury in response to the exposure of tissue factor (factor III) and thus, is also known as the tissue factor pathway. TF is an integral membrane protein with one transmembrane domain. It is normally expressed at very low levels in the endothelial cells, which line the blood vessel and the cells that lie immediately behind the endothelium, chiefly the fibroblasts and smooth muscle cells. Once the vessel wall is damaged, TF in the subendothelial cells comes into contact with the plasma proteins and forms a complex with factor VII to form TF: VII, but this complex has no proteolytic activity. The ‘factor VII’ part in the complex is activated to form VIIa by the factor Xa in a feedback reaction. The proteolytically active TF:VIIa complex, then activates two plasma zymogens: factor IX and factor X . Hence, TF can initiate activation of factor X by alternative routes, one direct, and other is via factors IX and VIII.

The tissue factor pathway is significant in normal haemostasis while, contact activation pathway is responsible for the clotting that occurs when blood or plasma comes into contact with "foreign" surfaces. Coagulation factors are generally indicated

by Roman numerical system. The conversion of a zymogen clotting factor to an enzyme is indicated with a lower-case "a" that is added to the factor name. For example, the proteolytic activation of the zymogen factor II produces the protease factor IIa. A List of coagulation factors and their functions are listed in Table 1.1 and the series of coagulation reactions is represented in Fig 1.1.

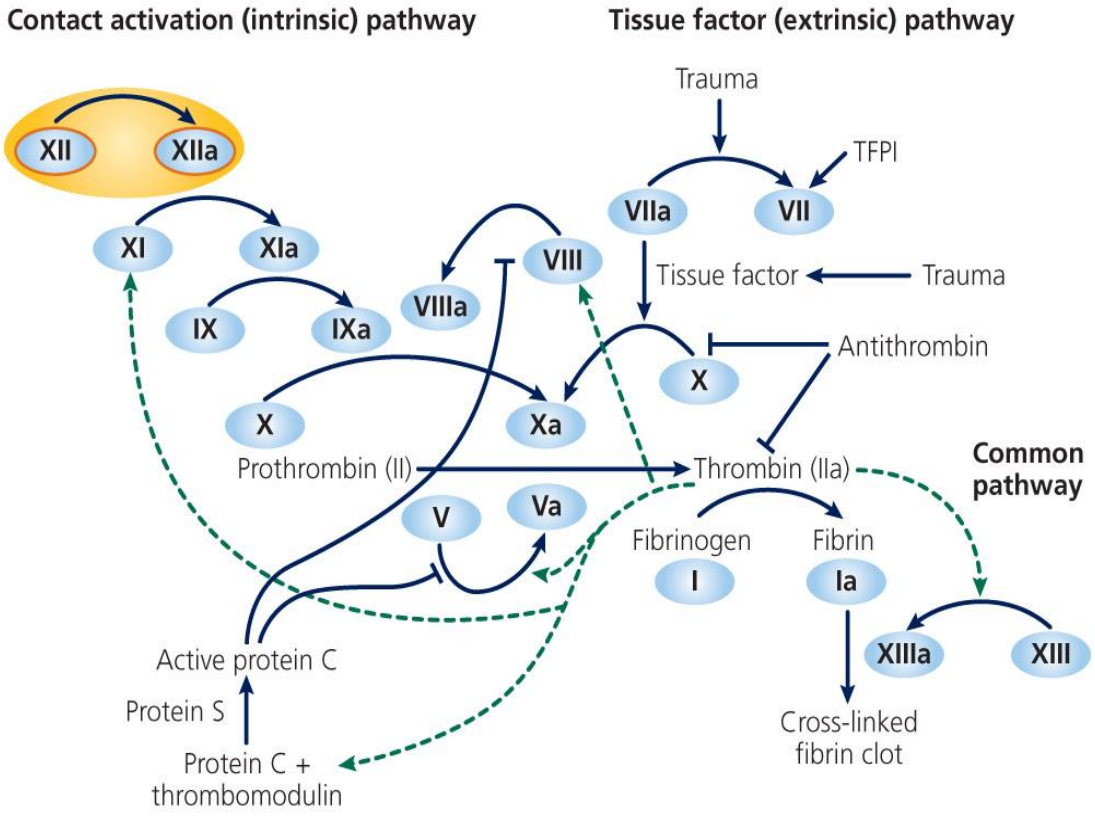

Fig. 1.1: Schematic representation of the blood coagulation cascade

Table 1.1: List of primary coagulation factors involved in blood coagulation cascade

Common name	Trival name (s)	Location	Functions
Prekallikrein (PK)	Fletcher factor	Plasma	Functions with HMWK and factor XII
High molecular weight kininogen (HMWK)	contact activation cofactor; Fitzgerald, Flaujeac Williams factor	Plasma	Co-factor in kallikrein and factor XII activation, necessary in factor XIIa activation of XI, precursor for bradykinin (a potent vasodilator and inducer of smooth muscle contraction
Factor I	Fibrinogen	Plasma, secreted from platelets	Fibrin precursor
Factor II	Prothrombin	Plasma	Protease zymogen (plasma)
Factor III	Tissue factor	Subendothelial surface glycoprotein	Initiator; cofactor for factor VIIa in factor IX and factor X activation
Factor IV	Calcium	Plasma	Metal cation necessary for coagulation reaction
Factor V	Labile factor	Plasma, Platelets	Cofactor for factor Xa in prothrombin activation
Factor VII	Proconvertin, serum prothrombin conversion accelerator (SPCA), cothromboplastin	plasma	Protease zymogen
Factor VIII	Anti-haemophiliac factor A, antihemophilic globulin (AHG)	plasma	Cofactor for factor IXa in factor X activation
Factor IX	Christmas Factor, antihemophilic factor B, plasma thromboplastin component (PTC)	Plasma	Protease zymogen
Factor X	Stuart-Prower Factor	Plasma	Protease zymogen
Factor XI	Plasma thromboplastin antecedent (PTA)	Plasma	Protease zymogen
Factor XII	Hageman Factor	Plasma	Protease zymogen
Factor XIII	Protransglutaminase, fibrin stabilizing factor (FSF), fibrinoligase	Plasma, Platelets	Zymogen of transglutaminase
Thrombomodulin		Endothelial surface	Cofactor for thrombin in protein C activation
Protein C		Plasma	activated to protein C by thrombin bound to thrombomodulin; then degrades factors VIIIa and Va
Antithrombin III	Antithrombin, Heparin cofactor	Plasma	Protease inhibitor, controls activities of thrombin, and factors IXa, Xa, XIa and XIIa
Protein S		Plasma	Cofactor for activated protein C in inactivation of factors Va and VIIIa (plasma)
Tissue factor pathway inhibitor (TFPI)	Extrinsic pathway inhibitor (EPI); lipoprotein-associated coagulation inhibitor (LACI)	platelets, plasma, endothelial surface	Protease inhibitor, reversibly inhibit Xa and thrombin. While Xa is inhibited Xa-TFPI complex can subsequently also inhibit the FVIIa-tissue factor complex

Common pathway

The Common pathway starts at site of formation of Factor Xa, where intrinsic and extrinsic pathways both culminate in Factor Xa formation. Factor Xa activates prothrombin (factor II) to thrombin (factor IIa). The activation of prothrombin occurs on the surface of activated platelets, which requires the formation of a prothrombinase complex. Factor Va binds to specific receptors on the surface of activated platelets and forms the receptor for factor Xa. This membrane-bound complex of factor Va and factor Xa along with Ca^{2+} comprises the prothrombinase complex. This complex catalyzes the proteolytic conversion of prothrombin to thrombin. Thrombin formed then acts on fibrinogen to form fibrin. Thrombin also activates factor XIII to XIIIa, which forms covalent bonds that crosslink fibrin polymers.

The fibrinogen is a hexamer which is a dimeric structure from two trimers of Aα, Bβ and γ chains with a final stoichiometry of $(A\alpha{-}B\beta{-}\gamma)_2$ linked by disulfide bonds. The amino termini of all six chains are close together in the centre of the molecule (the E domain). The COOH termini are in the globular regions at the ends (the D domains). N-terminal sequence of first sixteen amino acids of Aα-chain and the sequence of first fourteen amino acids of Bβ-chain of each fibrinogen molecule is referred as fibrinopeptide A (FPA) and fibrinopeptide B (FPB) respectively. Fibrinopeptides A and B region contain several glutamate and aspartate residues, which impart high negative charge which makes other fibrinogen molecules to repel each other which inturn aid for their solubility in plasma. Thrombin specifically cleaves between Arg16 and Gly17 of Aα chain, and between Arg14 and Gly15 of Bβ chain releasing FPA and FPB respectively to form transient species called fibrin monomer. The fibrin monomer consists of two α, two β, and two γ chains, which polymerizes spontaneously both longitudinally and laterally forming insoluble fibrin polymer. Finally, the fibrin strands are cross-linked by the transglutaminase activity of factor XIIIa that is activated by thrombin. Major cross-links are found mainly between the C-terminal regions of the γ chains, forming longitudinal cross links, and between the C-terminal regions of α chain, forming both longitudinal and lateral cross-links. The

cross-linking reaction itself involves the reaction of the ε-amino group of a Lys residue with the γ-amide of a glutamine residue, forming an "isopeptide" NH-CO bond between the two, with the loss of NH3. Thus, thrombin converts soluble fibrinogen to insoluble fibrin.

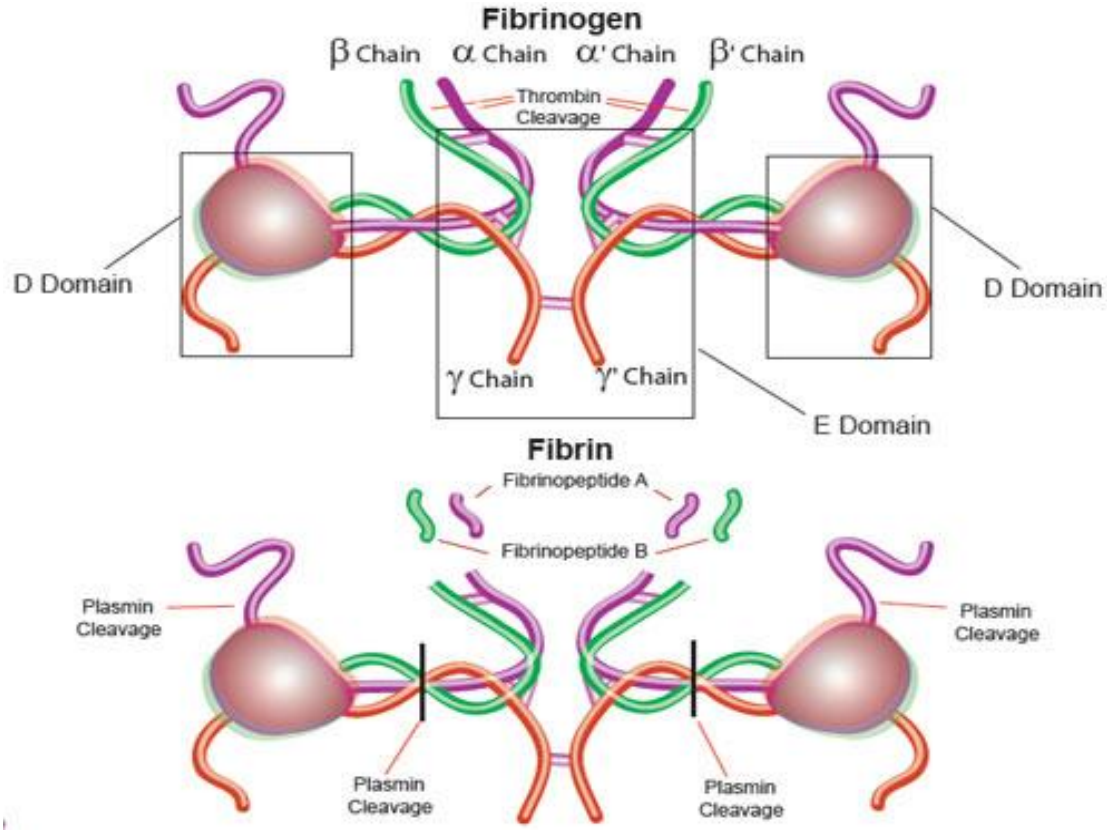

Fig. 1.2: Schematic representation of a fibrinogen molecule and a fibrin monomer

d. Fibrinolysis (Fibrin degradation)

The haemostatic plug containing platelets and fibrin must be dissolved in order to restore vascular effectiveness. Plasminogen is the zymogen form of plasmin, whose primary target is to degrade fibrin in the vasculature. The generation of plasmin occurs specifically on the surface of fibrin, which offers binding sites for plasminogen. Freely circulating plasmin is rapidly inhibited by α_2-antiplasmin. The binding stimulates plasminogen to fibrin initiates activation, but also localizes the action of plasmin to sites of fibrin formation which promotes efficient clot lysis. Plasminogen is activated

by tissue plasminogen activator (t-PA) and urokinase type-plasminogen activator (u-PA). t-PA is the principle activator of plasminogen in blood, while u-PA is the major activator in the extracellular matrix. t-PA is produced by the vascular endothelial cells and is released into the circulation after stimulation. The single-chain t-PA molecule is then converted to a two-chain form by plasmin by cleaving the peptide bond between Arg_{275}-Ile_{276} residues. Binding to fibrin concentrates and correctly orientates the t-PA and plasminogen, as well as inducing conformational changes in the molecules that promote efficient clot lysis. u-PA produced by the epithelial cells that line the excretory ducts can only activate plasminogen in the presence of fibrin, which is deposited on the ducts. However, it neither binds to fibrin nor gets activated by fibrin.

Snake venom enzymes/proteins affecting haemostasis

Hemostasis is a combined process of platelet activation and aggregation, initiation of clot formation and clot dissolution. At the site of vascular injury, platelets adhere to the exposed sub-endothelial macromolecules, mainly collagen. Attached platelets become activated and they aggregate. Simultaneously, blood coagulation factors are activated, leading to fibrin clot formation exactly on platelet surfaces. However, many enzymes/bioactive proteins of snake venoms mentioned below actively interfere and disturb the normal process of hemostasis.

a. Proteases

Snake venom proteases are heterogenous proteins with a wide range of molecular mass between 15 to 380 kDa. Proteases are present in most of the venoms except for the *Hydrophidae* venoms. The isolated and characterized Proteases so far reported are generally classified by the structure into Serine proteases (SVSPs) and Metalloproteinases (SVMPs) and there were very few reports of the presence of thiol proteases and aspartic proteases in the venoms. More than 200 different proteases have been reported to be purified and characterized so far, either completely or partially. The complete amino acid sequences of about 40 out of those proteases have been determined by protein sequencing or deduced from the nucleotide sequence of the cDNA.

Serine proteases

Snake venom serine proteases (SVSP's) are synthesized as zymogens and consists of about 260 amino acid residues and the mature form are single-chain glycoproteins. Depending on the carbohydrate content, their molecular mass ranges between 26 to 67 kDa. Among themselves, they share about 50–80% amino acid sequence identity and display quite diverse substrate specificity. These have both fibrinogenolytic and fibrinolytic activities and the serine proteases showing only fibrinogenolytic activity with fibrinogen clotting activity are called `thrombin-like' proteases (Markland, 1991; Markland, 1998; Pirkle and Theodor, 1990; Pirkle, 1998; Pikle and Stocker, 1991). However, their actions towards fibrinogen as well as the other substrates of thrombin are not exactly identical to those of thrombin. Instead of fibrin(ogen)olytic activity, several venom serine proteases have the activity for releasing bradykinin from kininogen like mammalian kallikrein or kininogenase (Iwanaga et al., 1976; Bjarnason et al., 1983) and are also called 'kallikrein-like' proteases (Bjarnason et al., 1983). In addition, there have been a few reports on the serine proteases from snake venom with a unique activity, such as the activation of factor V (Tokunaga et al, 1998), protein C (Kisiel et al., 1987), plasminogen (Zhang et al., 1995, 1997) or platelets (Serrano et al., 1995).

Metalloproteinases

Snake venom metalloproteinases (SVMPs) are among the most abundant toxins present in many *viperid* venoms and in some *elapid* and *colubrid* venoms (Moura-da-Silva et al., 2007). SVMPs are phylogenetically most closely related to the mammalian ADAM (a disintegrin and metallo-proteinase) family of proteins. SVMPs together with ADAM and the related ADAM with thrombospondin type-1 motif (ADAMTS) family of proteinases constitute the adamalysin/reprolysin/ADAM family or the M12B clan of zinc metalloproteinases (MEROPS classification, http:// merops.sanger.ac.uk/). SVMPs are also termed as reprolysins (Fox and Serrano, 2005) which belong to the metzincin family of zinc-dependent metalloproteinases (Bode et al., 1993). All of them contain characteristic zinc-binding regions, the consensus sequence HEXXHXXGXXH and the Met-turn (Bode et al., 1993). SVMPs are synthesized as multi-domain precursors,

whose enzymatic activity is inhibited by a Cys-switch mechanism (Bjarnason and Fox, 1994). They contain multiple disulfide bonds, which stabilize their structures and functional integrity in the oxidative extracellular environment (Fox and Serrano, 2008).

SVMPs are classified into four major classes, P-I, P-II, P-III and PIV according to their general domain organization (Fox and Serrano, 2008). P-I SVMPs are the simplest class of 20–30 kDa enzymes contains only a metalloproteinase (MP) domain. P-II SVMPs of 30–60 kDa are composed of one MP and one disintegrin (Dis) domain. P-III SVMPs (60–100 kDa) contain a Cys-rich domain in addition to MP and Dis domains. P-II and P-III SVMPs are divided into several subclasses, based on proteolytic processing and dimerization. P-II SVMPs undergo proteolytic processing, being split into an MP domain and a non-enzymatic disintegrin. In the same manner, some P-III SVMPs are processed into a disintegrin-like/Cys-rich containing protein, while the MP domain is degraded. The P-IV class, a P-III structure plus a C-type lectin-like subunit composed of two chains disulphide linked to one another and to the Cys-rich domain (Jia et al., 1996).

All the four class of SVMPs synthesized *in vivo* as multimodular proteins which also comprise of a signal peptide, a pro-domain to the amino terminal end of metalloprotease domain and a spacer between metalloprotease and disintegrin domain. The signal peptide is composed of eighteen amino acids, mostly hydrophobic residues and functionally acts as a protein secretion marker. The pro-domain modulates the enzymatic activity through interactions with the catalytic domain (MP domain), composed of about 200 amino acid residues and it is highly conserved among the SVMP members. MP domain is composed of about 215 amino acids, has metal-dependent endopeptidase activity and is less conserved than the pro-domain. The spacer peptide was first defined as an inter-domain segment with 13–15 residues in length, between the metalloprotease and disintegrin domain.

The disintegrin and disintegrin-like domains may vary in length from 41 to about 100 residues and content of disulfide bonds. Most disintegrins are released from

PII SVMP precursors and have an RGD (Arg-Gly-Asp) motif. There was no RGD (Arg-Gly-Asp) motif found in disintegrin domains of PIII SVMPs. However, alternative sequences such as DCD (Asp-Cys-Asp) or ECD (Glu-Cys-Asp) were found and hence they are called disintegrin-like domains (Paine et al., 1992; Hite et al., 1994; Zhou et al., 1995; Selistre-de-Araujo et al., 1997; Souza et al., 2000). The cysteine-rich domain contains around 112 residues in length and is located C-terminally to the disintegrin domain in PIII SVMPs.

b. Phospholipases A_2

Snake venom phospholipase A_2 (PLA_2) specifically catalyzes the hydrolysis of sn-2 ester bond in glycerophospholipids to give lysophospholipids and fatty acids. The PLA_2s are classified according to their structure into 16 groups (GI to GXVI) and numerous subgroups (Schaloske and Dennis, 2006; Duncan et al., 2008). They can also be grouped in a non- structure-based manner into secretory PLA_2s ($sPLA_2$s), cytosolic PLA_2s, Ca^{2+}-independent PLA_2s, PAF acetylhydrolases and lysosomal PLA_2s. PLA_2s found in snake venoms are $sPLA_2$s (Schaloske and Dennis, 2006). They are 14–18 kDa proteins, possessing five to eight disulfide bonds. They have a His/Asp catalytic diad in their active site and require Ca^{2+} for enzymatic activity. sPLA2s in snake venoms are either GI or GII (Valentin and Lambeau, 2000). GI $sPLA_2$s are found in venoms of Elapidae, and GII in venoms of Viperidae snakes. Although snake venom PLA_2s ($SVsPLA_2$s) share 40–99% amino acid sequence identity and hence, significant similarity in their three-dimensional folding, they also display large differences in their pharmacological activities. These include neurotoxic, cardiotoxic, myotoxic, hemolytic, convulsive, anti-coagulant, anti-platelet, edema inducing and tissue damaging activities (Kini, 2003). This wide spectrum of pharmacological effects correlates with their unique ability to ‘target’ specific organs or tissues and also due to their high affinity binding to specific glycoprotein or glycolipid receptors (Kini, 2003). Upon binding to a target receptor, $sPLA_2$s induce pharmacological effects by mechanisms that are either dependent or independent of their catalytic activity. In dependent type, either hydrolysis of phospholipids itself or the products formed by the enzymatic reaction namely lysophospholipids and fatty acids can cause the pharmacological effect.

c. Amino acid oxidases

Snake venom L-Amino acid oxidases (LAAOs) are widely distributed in *Viperidae* and *Elapidae* snake venoms (Du and Clemetson, 2002). These enzymes are flavoenzymes and catalyze the stereospecific oxidative deamination of L-amino acids to α-keto acids, a process in which ammonia and hydrogen peroxide are formed. LAAOs are homodimeric, FAD- or FMN-binding glycoproteins of molecular mass 110– 150 kDa (Zuliani et al., 2009). Each subunit has three domains, an FAD-binding domain, a substrate-binding domain and a helical domain (Pawelek et al., 2000). Regarding the haemostatic system, LAAOs were reported to influence platelet aggregation and induce hemorrhage, presumably through apoptosis of vascular endothelial cells induced by hydrogen peroxide, one of the products of the reaction that they catalyse (Torii et al., 1997). The role of LAAOs in snake venoms is not fully understood, but since they interfere in platelet aggregation and also can induce apoptotic cell death, they can be considered as toxins (Du and Clemetson, 2002).

d. Nucleases and nucleotidases

Nucleases (DNAses, RNAses, phosphodiesterases) and nucleotidases (5'-nucleotidases, ADPases, ATPases) are distributed widely in snake venoms (Dhananjaya and D'Souza, 2010a, b). These enzymes have been less studied and their pharmacological role in venoms has not been clearly defined. Some of them are reported to have an effect on platelet aggregation. 5'-nucleotidases, that catalyze the hydrolysis of a nucleotide into a nucleoside and a phosphate, cause the degradation of ADP, a platelet aggregation agonist which is released from platelet dense granules. Subsequent generation of adenosine further exerts anti-aggregatory effects on human platelets in vivo, presumably by increasing intra-platelet levels of cAMP (Söderbäck et al., 1987). cAMP inhibits platelet activation by decreasing cytosolic levels of Ca^{2+}, thus inhibiting the release of granules that could lead to activation of additional platelets. An example is a 5'-nucleotidase from *Trimeresurus gramineus* venom, a single chain protein of 74 kDa (Ouyang and Huang, 1983). It inhibits platelet aggregation induced by ADP, sodium arachidonate and collagen. 5'-nucleotidase from *Crotalus atrox* venom, administered *in vivo* to mice, abolished ADP- and collagen induced platelet

aggregation and increased adenosine concentrations and tail-bleeding time (Hart et al., 2008). 5'- nucleotidase from *Naja naja* venom was also reported to have an anticoagulant effect, most probably by interaction with one or more factors of the intrinsic pathway of blood coagulation (Dhananjaya et al., 2006).

e. Disintegrins

Disintegrins are non-enzymatic, Cys-rich small molecular mass proteins consisting of about 41–83 amino acid residues. Most of them are present in *Viperid*, *Crotalid* and few in *Elapid* venoms. Disintegrins are classified into four groups based on their length and number of cysteine residues: short, medium and long monomeric disintegrins, and dimeric homo- and hetero-disintegrins (Marcinkiewicz, 2005). Short disintegrins, composed of 49–51 amino acid residues and eight cysteine residues, medium-size disintegrins, composed of about 70 amino acid residues and 12 cysteine residues; and long disintegrins, composed of 84 amino acid residues and 14 cysteine residues. This classification does not include dimeric disintegrins (13–15 kDa), such as contortrostatin, EMF10, piscivostatin, acostatin, and schistatin .

They are released in the venom by the proteolytic processing of P-II class SVMPs (Kini and Evans, 1992) or synthesized directly from mRNAs (Okuda et al., 2001). The monomeric disintegrins contain characterstic RGD sequence (Arg-Gly-Asp), which binds to integrins, such as fibrinogen, vitronectin and fibronectin receptors on the surface of endothelial cells and fibroblasts. The RGD sequence of the motif is more variable in dimeric disintegrins, giving them the ability to bind more diverse integrins (Calvete et al., 2005). The large number of disulfide bonds provide a specific orientation of the loop carrying RGD (or similar) integrin-binding sequence (Calvete et al., 2005). Integrin-binding sequences enable disintegrins to bind α1, α3 and α5 integrins, thus affecting the cellular processes in which these integrins are involved. Example; platelet aggregation, cell migration, immune response, wound healing, tumour invasion, angiogenesis, inflammation and bone remodelling (reviewed in Marcinkiewicz, 2005; Swenson et al., 2007).

The activity of disintegrins also depends on their C-terminal tail (Marcinkiewicz et al., 1997) since amino acid residues in the C-terminal region of disintegrins are not highly conserved.

f. C-type lectins

C-type lectins are non-enzymatic proteins which were first described as carbohydrate-binding (hence lectin) and calcium-dependent (hence C-type) proteins. They contain a carbohydrate recognition domain (CRD) of 130 amino acids and bind mono- and oligo-saccharides in a Ca^{2+}-dependent manner. Snake venom C-type lectins can be classified into true C-type lectins (CTLs), possessing a carbohydrate recognition domain (CRD) that binds sugars, and C-type lectin-like proteins (CLPs) with CRD-related domains that lack the Ca^{2+}/sugar binding loop and consequently do not bind sugars (Drickamer, 1999). CLPs are more widely present than CTLs in snake venoms (Zelensky and Gready, 2005).

CLPs bind to a wide range of coagulation factors and to platelet receptors and display both anti-coagulant and platelet-modulating activities. Based on their biological activities, CLPs can be divided into four subgroups *viz* (a) FIX-, FX- or α–thrombin-binding CLPs, which are anticoagulants, (b) prothrombin or FX activating SVMPs with their C-type lectin-like domains, which are coagulants (c) platelet aggregation agonists and (d) platelet aggregation antagonists, which affect platelet aggregation by binding to GPIb, either alone or in complex with vWF or to collagen receptors, α2β1 and GPVI (Zingali, 2007; Lu et al., 2005, 2007).

g. Three-finger toxins

Three-finger toxins are non-enzymatic proteins. Their length ranges from 60–74 amino acid residues. They are characterized by specific folding of three β-sheet loops (‘fingers‘) extending from the central core and by four conserved disulphide bridges (Kini and Doley, 2010). Despite their structural similarity, they differ widely in their activities. Being predominantly cardiotoxic and neurotoxic, some of them are also found to act on the hemostatic system (Kini, 2002; Kini and Doley, 2010).

Mechanisms of action of snake venom agents on hemostasis

The components which affect the process of coagulation can be classified as procoagulants and anticoagulants. Procoagulant components enhance the activity of coagulation cascade by activating or inhibiting either one or more factor where as anticoagulants inhibits the cascade respectively. The procoagulants include thrombin-like enzymes (TLEs), prothrombin activators, FV activators, FX activators and Factor VII activators. The anticoagulants include FIX/X binding proteins, protein C activators, thrombin inhibitors, phospholipase A_2; and those acting on fibrin, including fibrinolytic enzymes and plasminogen activators.

Procoagulants

TLEs catalyse the limited cleavage of fibrinogen to release fibrinopeptides (FP) A from fibrinogen Aα-chain, fibrinopeptide B (FPB) from fibrinogen Bβ-chain or both at the amino terminal resulting in the formation of abnormal, "loose" fibrin clots. Since snake venom TLEs does not activate the FXIII, the clot is not stabilized by transglutaminase-catalyzed cross-linking; hence the fibrinolytic system quickly degrades such clots and easily removes from the circulation allowing their clinical use as defibrinogenating agents (Koh, 2006). In contrast to thrombin, TLEs are unaffected by thrombin inhibitors like anti-thrombin III and hirudin but inhibited by most of the serine protease inhibitors.

Snake venom prothrombin activators are metalloproteinases or serine proteinases (Kini, 2005). Based on their structural properties, functional characteristics and co-factor requirements, they have been categorized into four groups, A, B, C and D (Kini et al., 2001). Group A and B activators are metalloproteinases where as Group C and D are serine proteinases. Enzymes in group A function without cofactors while those in group B require Ca^{2+} for their activity. Prothrombin activators of group C and D are structurally and functionally similar to blood coagulation factors. These are found only in venoms of Australian Elapids. Group C prothrombin activators resemble like FVa/FXa complex. They require Ca^{2+} and negatively charged phospholipids for activity, whereas Group D activators require Ca^{2+}, phospholipids and FVa. Group D

prothrombin activators are structurally and functionally homologous to FXa (Joseph et al., 1999).

There are several FV activators which have been reported from *Bothrops atrox, Vipera russelli, Vipera lebetina, Vipera ursine, Naja naja oxiana* and *Naja nigricollis nigricollis* venoms. But, there are only two FV activators belonging to SVSPs that have been isolated and characterized. They are VLFVA from *Vipera lebetina* (Siigur et al., 1998) and RVV-V from *Vipera russelli* (Tokunaga et al., 1988). These two are found to activate factor V to the same extent as that of α-thrombin. The Contortrixobin, a thrombin like SVSP from *Agkistrodon contortrix contortrix* was also reported to activate factor V to a lesser extent than thrombin (Amiconi et al., 2000).

A number of SVMPs which activate FX have been isolated (Tans and Rosing, 2001; Siigur and Siigur, 2006). Among them, RVVX from *Daboia russelli* (Takeya et al., 1992; Takeda et al., 2007) and VLFXA from *Vipera lebetina* (Siigur et al., 2001) belong to class-IIId (P-III) SVMPs are thoroughly investigated. Two similar FX activators, VAFXA-I and VAFXA-II have been purified from the venom of *Vipera a. ammodytes* (Leonardi et al., 2008).

Snake venom proteases which activate only FVII have not been isolated to date but, a few SVSPs such as Oscutarin from *Oxyuranus scutellatus*, a Group C prothrombin activator also activates factor VII (Nakagaki et al., 1992). The exact cleavage of factor VII by Oscutarin is not known but the molecular mass of the degradation products are similar to the endogenous activation of FVII. The activation is potentiated by phospholipids and Ca^{2+}.

Anticoagulants

Both SVSPs and SVMPs have the fibrin(ogen)olytic activity with a few exceptions. These are direct acting endoproteinases and do not release fibrinopeptides A or B. They do not induce fibrin clot formation. Fibrino(geno)lytic SVSPs usually have preferential or specific activity towards the Bβ-chain of fibrinogen and lower activity towards Aα-chain. However, there are exceptions to this generalization and

specificity for the Aα- or Bβ-chains is not absolute as there is substantial degradation of the alternate chain with increasing time of incubation. Swenson and Markland (2005) have described many fibrin(ogen)olytic enzymes which belong to all the structural classes of SVMPs. Fibrin(ogen)olytic SVMPs break down fibrin-rich clots and prevent progression of clot formation. They act directly on fibrin and are not inhibited by blood proteinase inhibitors called as the 'Serpins'. For this reason, fibrin(ogen)olytic SVMPs without hemorrhagic activity have been considered as potential drugs for treating patients with vascular thrombo-embolic diseases (Deitcher and Toombs, 2006; Leonardi et al., 2007).

Bothrojaracin (BJC), a 27 kDa C-type lectin-like thrombin inhibitor from *Bothrops jararaca* venom (Zingali et al., 1993) is shown to prolong fibrinogen-clotting time by inhibiting competitively the binding of thrombin to fibrinogen. It also inhibits thrombin binding to thrombomodulin and decreases the rate of protein C activation (Arocas et al., 1996). It inhibits prothrombin activation by interacting with Proexosite I. In the absence of Phospholipids (PLs), BJC strongly inhibits the zymogen activation by FXa in the presence but not in the absence of FVa. Proexosite I blockage decreases cleavage of prothrombin by FXa-FVa complex or prothrombinase complex. BJC-like proteins are present in other *Bothrops* species and *Lachesis muta* venom.

Hemetexin AB complex was isolated from *Hemachatus hemachatus* venom. It consists of two copies of Hemetexin A and two of Hemetexin B (Banerjee et al., 2005; Banerjee et al., 2007). It inhibits the enzymatic activity of FVIIa and FVIIa/TF complex. Hemetexin A shows only mild anticoagulant activity and Hemextin B is inactive. However, the latter synergistically enhances the activity of Hemetexin A in the complex. Unlike TFPI, Hemetexin AB complex does not require FXa as scaffold and neither binds to the active site of FVIIa (Banerjee et al., 2005).

Protein C (PC) is an anticoagulant protein circulating in the blood as zymogen of 62 kDa. It is activated by α-thrombin on the endothelial surface when it complexed to thrombomodulin, a transmembrane protein on the surface of endothelial cells (Perera

et al., 2000). Activated PC degrades factors V/Va and VIII/VIIIa. Venoms from snake species belonging to the genus *Agkistrodon* contain PC activators and they have been purified from *A. c. contortrix*, *A. bilineatus*, *A. h. halys* and *A. blomhoffi ussuriensis* (Gempeler-Messina and Müller, 2006). A SVSP, ACC-C from *Agkistrodon c. contortrix* is the most thoroughly characterized PC activator (Kisiel et al., 1987). It is a single chain glycoprotein of approximately 40 kDa that selectively cleaves the heavy chain of PC without the need of thrombomodulin (Murakami and Arni, 2005).

Human plasminogen is a zymogen of single-chain glycoprotein with the molecular mass of 92 kDa present in the blood (Forsgren et al., 1987). u-PA and t-PA cleaves plasminogen after Arg561 to generate two-chain plasmin, a key enzyme in fibrinolysis. Only three serine proteases that are specific plasminogen activators (PA) have been isolated from snake venoms till date. They are TSV-PA from *Trimeresurus stejnegeri* (Zhang et al., 1997), Haly-PA from *Agkistrodon halys brevicaudus* (Park et al., 1998) and LV-PA from *Lachesis muta muta* (Hermogenes et al., 2006; Sanchez et al., 2000, 2006). The best characterised, TSV-PA, is a single-chain SVSP, which cleaves plasminogen at the same site as u-PA and t-PA.

Snake venom PLA_2s are either weak or strong anticoagulants (Kini, 2005). $sPLA_2$s from the first group, e.g. CM-I and CMII from *Naja nigricollis* (Kini and Evans, 1995), inhibit extrinsic (TF/FVII) complex formation by hydrolysis of anionic phospholipids, such as phosphatidylserine, that are essential cofactors of coagulation complexes. Strongly anticoagulant sPLA2s on the other hand, e.g. CM-IV from *Naja nigricollis*, inhibit formation of the TF/FVII complex by a combination of enzymatic and non-enzymatic actions (Kini and Evans, 1995). However, CM-IV and ammodytoxins from *Vipera a. ammodytes*, also inhibit prothrombinase complex formation but, without the need to be enzymatically active (Stefansson et al., 1990; Prijatelj et al., 2006). $sPLA_2$s inhibit the formation of prothrombinase complex by binding directly to FXa to prevent its association with FVa (Kerns et al., 1999). It appears that $sPLA_2$s prefer binding to protein rather than phospholipid receptors (Mounier et al., 2001).

The kallikrein-like enzymes from snake venoms release hypotensive bradykinin from kininogen in mammalian plasma. A kallikrein-like serine protease from the *Agkistrodon halys blomhoffi* termed as Halystase is well characterized. This enzyme has higher sequence similarities to kallikrein (42% identity) than thrombin (26%), although it has high similarities to the thrombin-like serine proteases (66-72%) and shows some fibrinogenolytic activity. It cleaves the Bβ–chain and slowly degrades the Aα-chain of fibrinogen to generate a product that is no longer converted to normal fibrin clot by thrombin. Thus, it results in the inhibition of clotting of normal fibrinogen (Matsui et al., 1998).

The venoms of 'big four' of the Indian sub-continent have been extensively characterized for their biochemical and pharmacological properties. In India, extensive research has been done on the pathophysiology of snake venom poisoning. Many enzymatic components which affect normal hemostasis have been isolated and characterized from Indian snake venoms in this regard. A Ca^{2+} and Zn^{2+} dependent non-toxic metalloprotease NN-PF3 purified to homogeneity from the Indian cobra (*N. naja*) venom was reported to be a strong anti-coagulant that increases the recalcification time of human citrated plasma in both dose and time dependent manner (Jagadeesha et al., 2002). *Trimeresurus malabaricus* venom is a strong procoagulant and is reported to reduce the re-calcification time of human citrated plasma to a greater extent. Similarly, it reduces the prothrombin time. Further, *T. malabaricus* venom hydrolyses both Aα and Bβ subunits of human fibrinogen. It is also reported to exhibit thrombin-like activity and induction of fibrin clot formation with purified fibrinogen. On the other hand, *N. naja* venom shows anticoagulant property and prolongs re-calcification time and prothrombin time (Raghavendra gowda et al., 2006).

Hemorrhage, edema and myonecrotic properties of the protease component of the Indian *Daboia/Vipera russellii* venom were reported. It also exhibited pro-coagulant activity and also degraded both Aα and Bβ chains of human fibrinogen (Mahadeswaraswamy et al., 2009). A non-toxic metalloprotease NN-PF3 with bound Ca^{2+} and Zn^{2+} reported earlier by Jagadeesha et al., 2002, from the *N. naja* venom, was

found to exhibit fibrin(ogen)olytic activity. In addition, it hydrolysed blood and plasma clot. The α-polymer of fibrin was preferentially hydrolysed over the α-chain but the β-chain and γ-γ dimer remained untouched. It prolonged the activated partial thromboplastin time, prothrombin time and the thrombin clotting time of citrated human plasma (Kumar et al., 2009). NN-PF3 was further investigated for its mechanism of inhibition of collagen-induced aggregation of human platelets where it exhibited complete inhibition of collagen-induced aggregation by binding to the integrin-α2β1 of the platelets and partial inhibition of ADP and epinephrine-induced aggregation (Kumar et al., 2010). A purified acidic proteolytic enzyme, RVVX with a mol. wt of 79,000 is reported to exhibit factor X activating properties (Jayanthi et al., 1990).

Several phospholipase A_2 enzymes have also been reported in having platelet aggregation inhibiting property. A purified PLA_2, VRV-PL-IIIb was reported to inhibit ADP-induced platelet aggregation in a dose-dependent manner. It was also found to induce edema in the foot pads of mice and was devoid of anticoagulant, myotoxic and direct hemolytic activities (Prasad et al., 1996). An acidic phospholipase A_2 (EC-I-PLA2) from the Indian saw-scaled viper (*Echis carinatus*) venom was reported to be non-lethal when tested on mice models and was devoid of neurotoxicity, myotoxicity, anticoagulant activity and cytotoxicity. However, it inhibited ADP, collagen and epinephrine induced human platelet aggregation *in vitro* and the inhibition was found to be both dose and time dependent (Kemparaju et al., 1999). Three acidic phospholipases A_2 from Indian cobra (*Naja naja*) venom were reported to inhibit platelet aggregation in platelet rich plasma induced separately by ADP, collagen and epinephrine with different potencies (Rudrammaji et al., 2001). NND-IV-PLA_2, an isolated platelet aggregation inhibitor phospholipase A_2 from *Naja naja* (Eastern India) venom was reported to inhibit platelet aggregation induced by ADP, collagen and epinephrine (Satish et al., 2004). Echicetin, a heterodimeric protein from the venom of the Indian saw-scaled viper (*Echis carinatus*) was found to inhibit platelet aggregation by binding to platelet glycoprotein Ib- GPIb (Jasti et al., 2004). *N. naja* venom PLA_2 was reported

to inhibit platelet aggregation in PRP, decreased clot retraction and hence, conciliation of elasticity build up was reported. The report also showed that the PLA_2 and the prothrombin activator from *N. naja* venom had effects on hemostasis and blood clotting by *in vitro* studies (Sundell et al., 2003).

However, the king cobra (*Ophiophagus hannah*) venom has been greatly ignored in the subcontinent till date. *Ophiophagus hannah*, commonly known as king cobra is the largest and most lethal snake belonging to the family *Elapidae*. A single bite of king cobra can deliver up to 400-500 mg of venom and is estimated to be several folds higher than its lethal dose. King cobras are endemic to south-east Asian countries including India, Srilanka, Myanmar, Malaysia, Thailand and Vietnam. They are not found in other parts of the world and therefore restrict the extensive study on the venom of this deadly snake. In the Indian subcontinent itself, the king cobra habitat is restricted only to the Western Ghats and some eastern parts of India. They usually dwell in thick forests located at regions of higher altitudes. Although there are no reports of mortality and morbidity due to king cobra bite in the subcontinent, it is important to note the fact that the chance of survival and eventual treatment after the bite becomes practically very difficult as it can inject the venom that is several folds higher than its LD_{50} value in a single bite which will ensure killing with in few minutes.

Fig. 1.3: King cobra habitat in the south-east Asian countries

The international status on king cobra venom research so far especially in the south-east Asian countries is overwhelming. The lethality of the venom attributing to various deleterious effects and hence co-relating the pathophysiology of envenomation by king cobras on humans has been extensively studied. Likewise, many bio-active proteins, peptides and organic molecules that show varied interference on normal hemostasis are also reported to have been isolated and characterized from king cobra venom.

A convulxin-like C-type lectin called Ophioluxin – a potent platelet agonist having a mass of 85 kDa has been reported from the Indonesian king cobra venom (Du et al., 2002). An approximately 64 kDa L-amino acid Oxidase (LAAO, EC 1.4.3.2) – a flavo enzyme is reported to have been purified from a Chinese king cobra that catalyzes the stereospecific oxidative deamination of the L-isomer of amino acid to give L-keto acid, ammonia and hydrogen peroxide (H_2O_2). It was found to have a potent inhibitory activity on platelet aggregation induced by ADP (jin et al., 2007). A specific blood coagulation factor X activator was reported from the venom of king cobra (China). The enzyme was reported to activate factor X *in vitro* and the effect was absolutely Ca^{2+} dependent. It could not activate prothrombin nor had any effect on fibrinogen and thus appeared to act specifically on factor X (Lee et al., 1995).

However, from the Indian context, very less work has been done. So far, only Gomes and co-workers have reported the isolation of a novel fibrinolytic hexapeptide (Hannapep) from the venom of the Indian King Cobra (*Ophiophagus hannah*). It was reported to exhibit significant fibrino(geno)lytic as well as plasma anti-clotting activity *in vitro*. However, it lacked hemolytic, hemorrhagic, or phospholipase activity (Gomes et al., 1999). Apart from this, neither any bioactive molecule(s) have been isolated and characterized from the Indian king cobra venom that would strongly interfere in the normal blood coagulation cascade nor has a thorough investigation on the biochemical and pharmacological properties of the venom been done.

With reference to the above findings, it can be emphasized that the type, composition biochemical/pharmacological activity of the components of the venom has high degree of diversity and depends on the type of prey and specific regional habitat in

which these snakes sustain their living. Thus it becomes evident that the venom of king cobra from various regions have varied components that causes diversified, lethal / non-lethal systemic effects when envenomated.

Antivenom therapy for snakebite-the global scenario

Though many plant molecules that have been purified and characterized that possess antivenom/neutralizing properties towards toxins and enzymes of snake venoms, their effective usage for treatment of bite victims still remain under clinical trials. Hence, by far, antibodies raised in equines, chicks and rabbits against individual/cocktail of venoms are routinely used for treating snakebite victims. This mode of treatment is regarded and globally accepted as the best method of treating bite victims amidst certain complications though not undermined. However, this questions the efficacy of these snake antivenoms as well as the degree of tolerance required to subdue their non-specificity aspects when administered. Hence, to ensure the efficiency and tolerance of immune therapy, it is necessary to consider antivenom specificity towards the venom components, quantity of venom injected in bite victims and the therapeutic protocol employed during the process of treatment (Chippaux et al., 1998).

Since viper bites show clinical symptoms in a gradual manner than compared to the elapid bites, the severity of the clinical symptoms and the amount of venom injected becomes a questionable aspect if the snake responsible for the bite is unnoticed or unidentified (Reid et al., 1983; Blaylock, 1983).

The severity of the clinical symptoms though may not be precisely judged at that instant, the amount of venom injected can be formidably determined using ELISA techniques that employ kits containing raised mammalian/avian antibodies against various snake venoms (Labrousse et al., 1988; Audebert et al., 1993; sjostrom et al., 1996; Coulter et al., 1980).

The preferential use of polyvalent antivenoms over monovalent antivenoms has gained mileage in the recent years due to a simple fact that the correct identification of the snake responsible for bite usually remains uncertain. Thus, majority of the snake

antivenoms producing firms in the third world countries are found to prefer on the production of polyvalent antivenoms. However, these antivenoms though might show a broad spectrum cross–reactivity with multiple snake venoms, the amount required to neutralize the lethality of the injected venom is found unconvincing in many cases and suffer a set back since higher doses are required due to its poly specificity property. This in turn creates a gradual raging of immune non–specificity against the administered antivenom in the bite victim.

Most of the developing/developed countries currently are producing monovalent antivenoms or polyvalent antivenoms which are a cocktail of multiple monovalent antibodies raised for individual snake venoms. Many Australian monovalent snake antivenoms produced are polyvalent or a mixture of some or all monovalent snake antivenoms (O'Leary et al., 2009). In Thailand, monovalent antivenoms against the four medically important snakes considered by the Thai red cross namely, *naja kaouthia*, *Ophiophagus hannah*, *Bungarus candidus* and *Bungarus fasciatus* have been produced called NPAV (Neuro polyvalent snake antivenom) (Chotwiwatthanakun et al., 2001). The neutralization efficacies of both poly specific and mono specific antivenoms thus produced were shown to be the same. This might be true since the produced polyvalent antivenoms are purified and are added with monovalent purified antivenoms that would enhance the neutralization potencies with lower quantity of IgG hence causing less adverse reactions (Leong et al., 2012).

Antivenom therapy- status in the subcontinent

The antivenom therapy scenario in the Indian subcontinent is different. The available commercial antivenoms for the treatment of snake envenomation in India are predominantly polyvalent and are usually raised against the venom cocktail of the "Big Four" namely *Naja naja*, *Vipera/Daboia russellii*, *Bungarus caerulus* and *Echis carinatus*. However, the commercial antivenoms produced by the major firms are not in the purified form and are the lyophilized antisera produced against the venom cocktail. Hence, the non-specificity of many proteins present in the antisera becomes a questionable criterion. This also reflects on the degree of its effectiveness in

neutralizing the venom lethality. These non-specific proteins present in the snake venom antisera attributes to several adverse reactions and clinical complications (Raweerith et al., 2005). The use of drugs such as adrenaline, hydrocortisone, Promethazine or anti-histamine as a pre-prophylactic measure so as to check the adverse reaction of the antivenom then becomes inevitable. (Premawardhena et al., 1999). Since the polyvalent antivenoms are raised against the cocktail of the "big Four" whole venoms rather than the isolated toxins, one possibility of their less cross-reactivity and neutralizing potencies might be due to the maximum proportion of immunogenicity conferred by the used adjuvants themselves than the venom toxins present.

Though, the snake antivenoms produced in the Indian subcontinent is relatively cheap when compared to the western countries (Theakston et al., 2000), the neutralizing potency of these snake antivenoms is a matter of concern. Apart from this main inadequacy, the other equally important drawback is the non-availability of the snake antivenoms in remote areas and government run rural health centres. Further, the antisera produced against the venom cocktail of big four does not include other equally/more potent venoms of snakes such as pit viper, hump nosed viper, King cobra and so on that are also endemic to the subcontinent. This then raises serious questions on the availability of antivenoms for treatment of bite victims from these snakes during emergency crisis. Since, these snake venoms are not used in the preparation of venom cocktail against which the antivenom is produced, the cross reactivity and hence neutralization of these venoms goes out of question. Thus, the production of polyvalent antivenom in the subcontinent still remains in its dormancy and much needs to be reformed in terms of producing monospecific antivenoms and improving its neutralizing potencies as well as reducing the adverse reactions during treatment. Though, many commercial firms in India produce snake antivenoms against the major endemic snake species namely *Naja naja*, *Echis carinatus*, *Vipera russellii* and *Bungarus caerulus*, the experientially determined doses of these snake antivenoms that needs to be administered were reported to be too high (Theakston et al., 1990; Vijeth et al., 2000).

Aim and scope of the study

In the Indian subcontinent, commendable research has been done on the pathophysiology of snake venom poisoning especially regarding the 'big four' (Vishwanath and Gowda, 1987; Chakraborty et al., 1991, 1993, 2000; Alam and Gomes, 1998a, 1998b; Mukherjee et al., 2000, 2002; Shashidhara murthy et al., 2002; Girish et al., 2004; Doley and Mukherjee, 2003; Jasti et al., 2004; Jabeen et al., 2005; 2006; Bilgrami et al., 2004; Rudrmaji and Gowda, 1998; Sathish et al., 2004). About 15 different laboratories across the subcontinent are involved in snake venom research including the neutralization of venom toxicity. The study of venom toxins help greatly in designing new tools for understanding the physiology and an eventual development of models of highly specific therapeutic agents. With the ultimate view of developing effective therapeutic antivenom to treat the risk of fatal snakebites, snake venoms and their toxins is the subject of extensive analysis of several laboratories all over the world. Although extensive work has been undertaken on the venoms of other poisonous snakes, *Ophiophagus hannah* is less extensively studied.

King cobras (*Ophiophagus hannah*) are endemic to south-east Asian countries that include India, Srilanka, Myanmar, Malaysia, Thailand and Vietnam and are not found in other parts of the world. This fact restricts the extensive study on this venom. So far, some toxins and enzymes have been isolated and characterized from the venom of king cobra from the south-east Asian countries. A protease (Yamakawa and Omori-Satoh, 1988), a hemorrhagin (Tan and Saifuddin, 1989, 1990), a three finger beta cardiotoxin **(**Rajagopalan et al., 2007), alpha neurotoxins (Lin et al., 1999) have been isolated. Very few reports have claimed the successful neutralization of king cobra bite by antivenom (Tin-Myint et al., 1991).

Due to the rapid burst of human population and also deforestation across the subcontinent, the likely chance of humans getting bitten by the king cobra is increasingly high. Despite the fact that, survival rate is very low in king cobra bite victims as it can inject several folds higher doses of venom than the lethal dose in just a single bite, the antivenom may be successful against king cobra bite if administered in

time. Since antivenom (polyvalent / monovalent) is not available against king cobra venom, in order for the possible preparation of the antivenom, it is important to understand the biochemical, pharmacological and immunological properties of this snake venom. Therefore, this study might help in characterizing the king cobra venom in terms of its biochemical, pharmacological and immunological properties and also would help in the production of antivenom. This might also help in validating the king cobra venom neutralizing efficacy of the commercially available antivenom prepared against the big four snakes of the Indian sub-continent.

Hence the objectives of the current study are;

1. Biochemical and pharmacological characterization of the Indian king cobra (*Ophiophagus hannah*) venom.
2. Immunological cross-reactivity of the commercial therapeutic polyvalent antivenoms and rabbit raised monovalent purified anti- *O. hannah* IgG antibodies against king cobra venom.
3. Comparative neutralization of enzymatic and pharmacological properties of king cobra venom by commercial polyvalent and rabbit raised monovalent purified anti- *O. hannah* IgG antibodies.