Chapter 1

General introduction and review of literature

Chapter 1

General introduction and review of literature

Snakes are one of the most fascinating and perplexing creatures, which have intrigued the curiosity of mankind since the inception of humanity. The mystery revolving around this creature has left civilizations around the world enchanted and enthralled making them either sacred or evil in their beliefs. The confusion between venomous and non-venomous snakes, dry bites, fear psychosis among people and succumbing to faith healers for recovery have only added to the mysteries and myths. Envenomation by venomous snakes is a medical emergency and a potentially lethal multi-organ disease concerning mostly the tropical and sub-tropical countries of the world. The fascination for snakes doesn't end with common people; it extends to the research fraternity as well. The present day toxinologists have been intrigued by the snake venom composition and the endless opportunities it presents for research, channelizing it into a noble cause for humanity. Snake venom, having evolutionarily adapted to kill prey rapidly, contains complex mixtures of toxins which exert multitude of effects on their victims by targeting various physiological systems. These pathophysiological actions on the physiological system of victims make snake venom an oxymoronic angelic devil and a blessing in disguise, as snake venom proteins which have the ability to kill, also bear the potential to be used as therapeutic agents for management of various life-threatening diseases and conditions.

1.1 Snake venom proteins:

Snake venom is an assortment of pharmacologically active proteins and polypeptides [1–3]. Snake venom proteins, which evolved as an aid of "offense and defence" for snakes facilitating immobilization and digestion of prey, as well as defence against threats, comprises of the major snake venom protein families. Based on their functions and three dimensional structures, they can be broadly classified into enzymatic and non-enzymatic protein superfamilies. The enzymatic families include phospholipase A_2 (PLA_2), snake venom serine protease (SVSP), snake venom metalloprotease (SVMP), L- amino oxidase (LAAO), acetylcholine esterase, hyaluronidase, phosphodiesterase,

nucleotidase and nucleosidase. On the other hand, the non-enzymatic families comprise of snake C- type lectin like proteins (Snaclec), disintegrins, cysteine rich secretory peptide (CRISP), three finger toxins (3FTX), kunitz type serine protease inhibitor (KSPI), vascular endothelial growth factor (VEGF) and vascular nerve growth factor (VNGF) [4]. For inducing various pharmacological effects on their victims, these snake venom proteins interact with a wide variety of mammalian proteins and can disrupt the central and peripheral nervous systems, the blood coagulation cascade, the cardiovascular and neuromuscular systems, and the general homeostasis state [5]. Based on their site of action, they can also be classified as neurotoxic, haemotoxic, myotoxic or cytotoxic [6]. The local effects include burning, bursting, or throbbing pain followed by local swelling and tissue necrosis, while on the systemic level, snake venom can cause various toxic effects like neurotoxicity, myotoxicity, cardiotoxicity, nephrotoxicity, coagulopathy, and circulatory shock [7]. These mortal conditions caused by venom, have led an expedition amidst the research community to harness these life-threatening toxins from snake venom and manipulate them into life-saving therapeutics.

1.2 Snake venom protein affecting haemostasis:

Haemostasis is a complex mechanism that maintains integrity of the blood circulatory system ensuring blood remains in a fluid state unless trauma or injury occurs, and limiting blood loss during injury. It is maintained by a delicate balance between blood clot formation upon vascular injury and its subsequent dissolution to restore normal blood flow. It is an important physiological process involving an intricate interplay between blood cells, plasma factors and the vessel wall [8]. The stages of haemostasis can be broadly categorised as (a) primary haemostasis or platelet aggregation forming the platelet plug, (b) secondary haemostasis or blood coagulation and (c) tertiary haemostasis or fibrinolysis [9,10].

A common target of snake venom proteins is the haemostatic system of the prey/victim, which is symptomized by haemotoxic conditions post envenomation. The anticoagulant proteins of venom cause delayed blood coagulation leading to persistent and profuse bleeding at the bite site as well as from the gums and internal organs. The procoagulant proteins, on the other hand, cause excess clot formation leading to thrombosis and consumptive coagulopathy [11]. These proteins disrupt haemostasis by targeting

various proteins and factors for such pathological manifestations. It is believed that for almost every factor involved in coagulation or fibrinolysis there exists a venom protein that can manipulate it by activation or inhibition [11]. Some of them affect the blood coagulation cascade, whereas others affect platelet aggregation. Over the years, a number of toxins that affect blood circulation through varied mechanisms have been identified from various snake venoms [12]. For example, procoagulant proteins which activate the factors involved in coagulation have been isolated from the venom of *Ohiophagus hannah* (Factor X), *Bungarus fasciatus* (Factor X), *Oxyuranus scutellatus* (Factor VII), *Daboia russelii* (Factor X and V), *Vipera lebetina* (Factor X and V) etc [13–17]. Likewise, snake venom proteins like Bothrojaractivase isolated from *Bothrops jararaca*, Basparin A from *Bothrops asper*, Pseutarin C from *Pseudonaja textilis* and Tocarin D from *Tropidechi carinatus* enhances the conversion of prothrombin to thrombin leading to coagulation [18–21]. On the contrary, thrombin like enzymes like Ancrod isolated from *Calloselasma rhodostoma* venom, Batroxobin from *Bothrops moojeni* and Crotalase from *Crotalus adamanteus* exhibit strong proteolytic effect on fibrinogen [22]. Furthermore, coagulation factor inhibitors like Ringhalexin and Hemextin AB isolated from the venom of *Hemachatus haemachatus* and M-LAO from *Agkistrodon halys blomhoffii*, delays coagulation time by inhibiting the activation of factor X to factor Xa and the activities of factor VII and IX respectively [23–25].

1.3 Snake venom proteins affecting platelet aggregation:

Platelets are tiny, anucleate cytoplasmic fragments with diameter 1 to 4 mm, which are derived from megakaryocytes [26]. Although anucleate, they possess many functional features of whole cells and are metabolically active. Under normal physiological conditions, platelets circulate the vasculature in an inactive, non-adhesive state. But when they come in contact with damaged vessel, they rapidly change their own characteristics [27]. Platelets are significant for hemostasis as they form aggregates or plug on encounter with areas of vascular damage, allowing them to limit blood loss. Platelet aggregation is a dynamic process that requires a co-ordinated series of events involving platelet membrane receptors specifically triggered by different agonists [28].

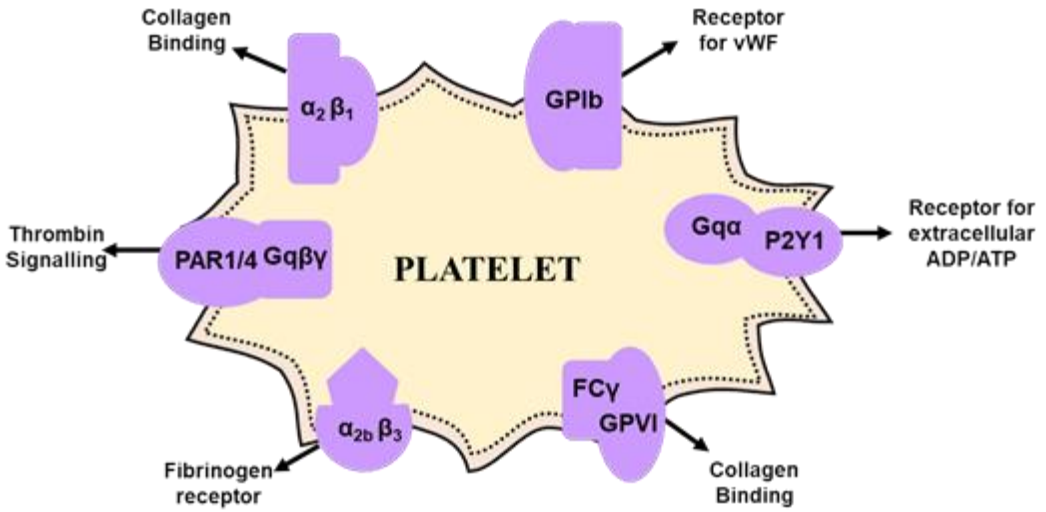

Figure 1.1: Overview important platelet receptors and their functions

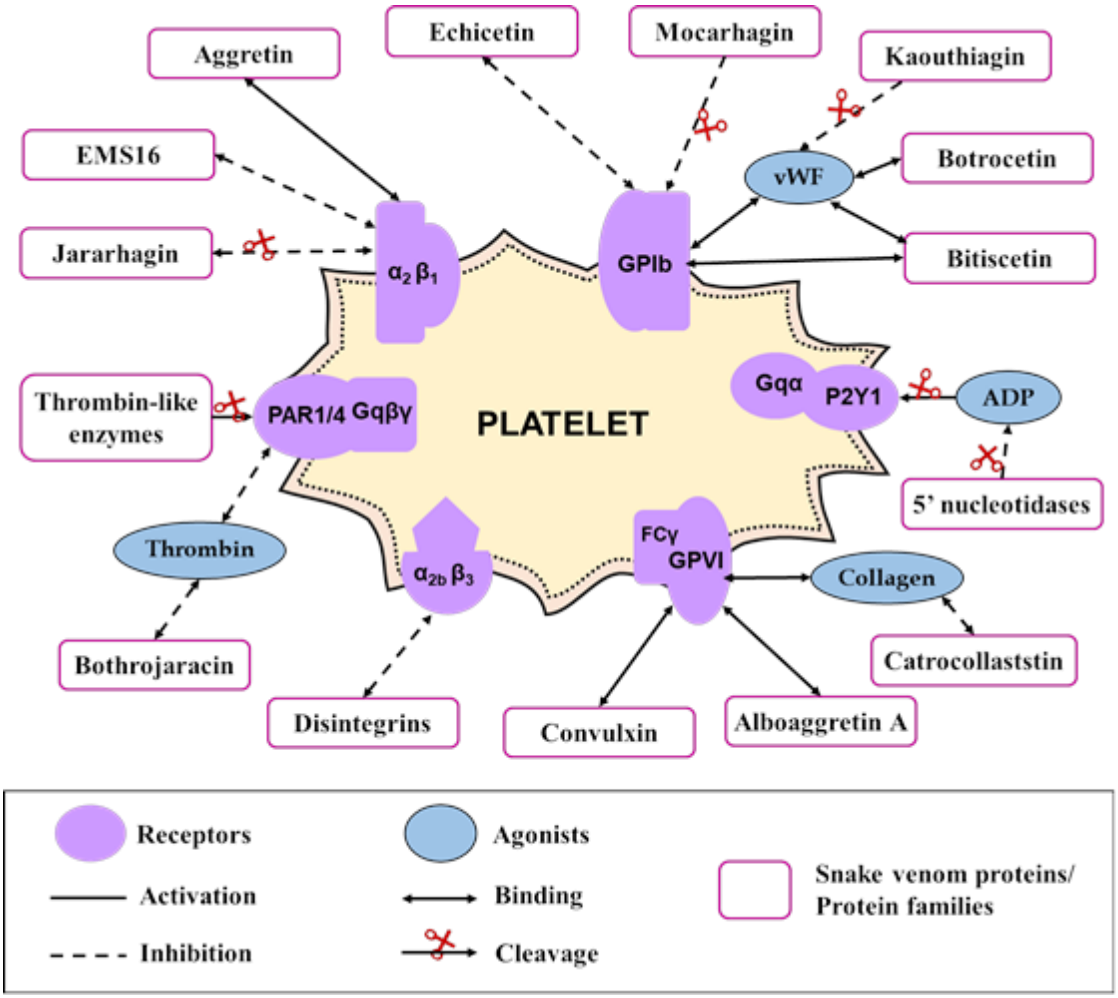

Figure 1.2: A few examples of snake venom proteins affecting different platelet receptors

Platelet receptors are important for normal functioning of the platelets (Fig. 1.1). They either activate the platelets or function as adhesion molecules which interact with the injured endothelium and other platelets. For instance, under conditions of high shear stress, the initial recruitment of platelets is mediated by the interaction of von Willebrand factor (vWF) with the glycoprotein (GP)Ibα subunit of the GPIb-V-IX complex [29,30]. However, under low shear stress, platelets predominantly adhere to collagen by binding to glycoprotein (GP)VI and $\alpha_2\beta_1$ receptors [31]. In addition to releasing secondary mediators such as thromboxane A_2 (TxA_2), GPVI also plays a vital role in collagen-mediated exposure of procoagulant phospholipids at the platelet surface, allowing efficient generation of thrombin [32–34]. Thrombin triggers platelet aggregation in humans through the coordinated actions of the Protease activated receptors (PAR), PAR1 and PAR4 receptors [26,35,36]. PARs are transmembrane GPCRs, the N-terminal exodomains of which are specifically cleaved by thrombin and irreversibly activated when the cleaved fragment acts as tethered ligands binding to an extracellular loop of the same receptor [36,37].

Various snake venom proteins belonging to different families have been reported to manipulate platelet function by various mechanisms (Fig. 1.2). They might either bind the platelet receptors, aiding platelet aggregation [11,38]. For instance, a fibrinogen clotting serine protease, bothrombin, isolated from the venom of *Bothrops jararaca* causes aggregation platelets in presence of exogenous fibrinogen by binding the receptor GPIbα [39]. While in other cases, snake venom proteins might aid binding between agonists and receptors by interacting with the both. For example, botrocetin, a heterodimeric snaclec from the venom of *Bothrops jararaca,* reported to cause thrombocytopenia in mice, facilitates platelet agglutination and aggregation by enhancing binding between vWF and its glycoprotein receptor GPIb [40–42]. Another snaclec, bitiscetin from *Bitis arietans* aids in platelet aggregation by forming trimolecular complexes with vWF and GPIb similar to botrocetin [43]. Moreover, multi-functional snake venom proteins such as, Bothropstoxin-II, a PLA_2 from *Bothrops jararacussu,* has been reported to induce platelet aggregation by triggering multiple signalling pathways [44].

Similarly, there are reports of snake venom proteins that inhibit platelet aggregation either by binding or degrading platelet receptors and modulating the activities of the

agonists. EMS16 and rhodocetin, for example, isolated from *Echis multisquamatus* and *Calloselasma rhodostoma* respectively, are reported to inhibit platelet aggregation by binding to collagen receptor, $\alpha_2\beta_1$ [45–48]. Bothrojaracin from *Bothrops jararaca*, on the other hand, binds to the agonist, thrombin, thereby not allowing it to interact with its receptor [45–50]. Again, Barnettlysin-I, a snake venom metalloproteinase from *Bothrops barnetti* has shown to inhibit aggregation of platelets induced by vWF and collagen. It inhibits vWF mediated aggregation by cleaving both vWF and its receptor by cleaving the receptors, and additionally, collagen-induced activation is inhibited by cleaving the α2-A domain of $\alpha_2\beta_1$ integrin, required for collagen-binding [51]. Manipulation of these snake venom proteins affecting platelet functions can help design therapeutic interventions against cardiovascular diseases as antiplatelet drugs or antithrombotics.

1.4 Cardiovascular diseases:

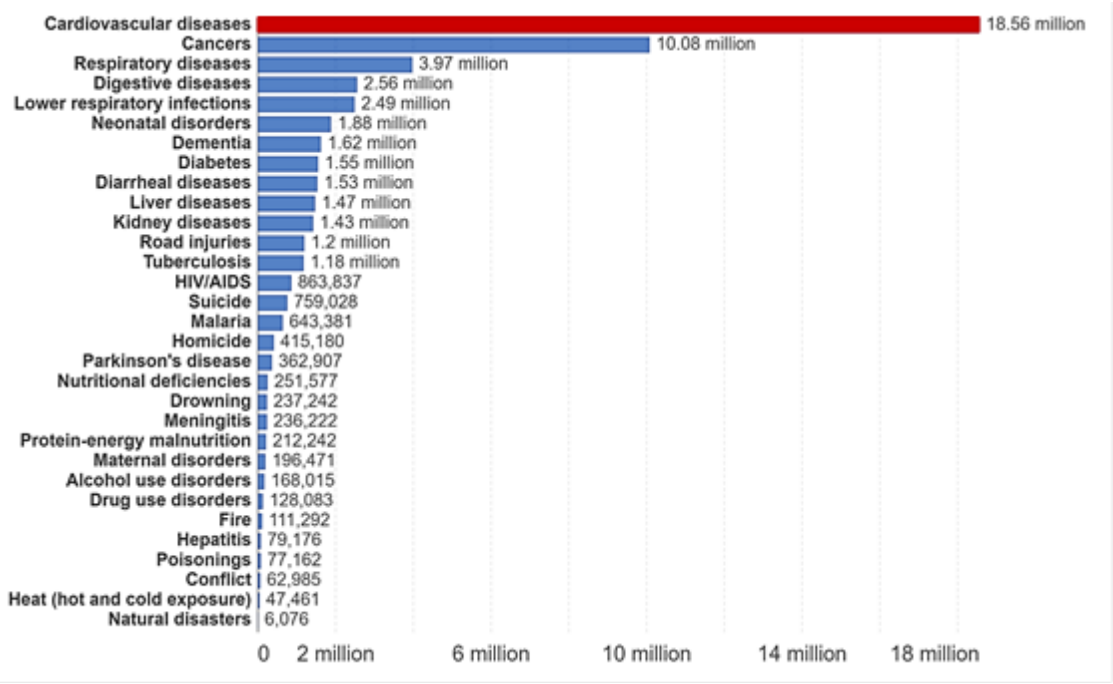

Figure 1.3 Major causes of death according to Global Burden of Diseases Study, 2019 (Source: OurWorldInData.org/causes-of-death, Global Burden of Disease (2019)

Cardiovascular disease (CVD), the largest single cause of mortality worldwide, is a cluster of diseases and injuries that affect the cardiovascular system [52]. These are most commonly diseases of the heart, brain and the blood vessels [53,54]. The severity

of this disease was rightly highlighted by the Global Burden of Disease (GBD) Study 2019. GBD is a multinational collaborative research undertaken in order to estimate disease burden for every country in the world, produce standard epidemiological measures and allow consistent comparison over time from 1990 to 2019 [55]. According to the study, CVD continues to remain the global leading cause of disease burden across decades. The condition being prevalent in developed as well as low and middle income countries, it contributes to an estimated 25% of total deaths globally [52,55]. The prevalent cases of total CVD nearly doubled from 271 million in 1990 to 523 million in 2019, and the number of deaths due to CVD steadily increased from 12.1 million in 1990 to 18.56 million in 2019 (Fig 1.3).

The importance of cardiovascular diseases as a globally prevelant disease is also reflected in the research effort worldwide that aims to reduce the death rate due to CVDs as well as the prevalence of the disease. Cardiovascular research goes back to as far as the 26th century BC China, when it was concluded that the blood flowing in the circulatory was controlled by the heart. The modern day cardiovascular research, however, is a conglomeration of various scientific fields including electrics, mechanics, surgical procedures and drug treatments. As such, the major discoveries of these fields like electrocardiography, echocardiography, angiography, angioplasty, pacemakers, open heart surgery, transplantation, thrombolytics etc. form the basis of contemporary cardiology [56]. However, the usage of biological technologies such as genetics, molecular biology, cell biology, immunobiology and genomics potentially hold the key to the future of CVD research. Furthermore, emphasis has been given on discovery of novel early stage biomarkers for prediction and prevention using proteomics approaches and microRNA profiling as well as designing of protein drugs for targeted treatment of various cardiovascular conditions [54,57].

CVDs have been associated with dysfunctional coagulation pathway or its components and individuals with coronary artery diseases have been proven to be more prone to the risk of cardiovascular deaths due to activation of the coagulation process [58]. Moreover, occurrence of abnormal clotting, thrombosis of atherosclerotic plaques and their rupture are often seen triggering the onset of cardiovascular events, which subsequently results in heart attacks or strokes [59,60]. Since any aberration in haemostasis can potentially contribute to the pathogenesis of several cardiovascular

conditions, the search for therapeutic interventions with the ability to manipulate the haemostatic system continues. The present management for CVDs mainly include pharmacotherapy with a wide range of drugs with various mechanism of actions [54]. But they are often accompanied by various drug therapy problems including adverse drug reactions, the need for additional drug therapy, ineffective drug therapy, drug therapy related loss of life etc. [61,62]. Thus, the search for more effective drugs, with fundamentally new mechanism of action and free of the existing limitations continues.

1.5 Snake venom toxins and drugs discovery in light of cardiovascular diseases:

The search for more effective therapeutic interventions against cardiovascular diseases has ventured into snake venom research since snake venom proteins interact with a wide variety of mammalian proteins and can disrupt the blood coagulation cascade, the cardiovascular system, and the general homeostasis state [5]. In the past few decades, several drugs in use were either isolated or derived from snake venom proteins [63]. Captopril was the first drug designed on the basis of a bioactive component from snake venom to be clinically approved [64]. It is a synthetic analogue of bradykinin-potentiating peptide isolated from the venom of *Bothrops jararaca*. It was first designed in the year 1975 and was commercially available six years later. It was the founder member of a family of angiotensin-converting enzyme (ACE) inhibitor drugs used in the treatment of clinical hypertension [65,66]. Since the success of captopril, snake venom toxins have become a significant natural repertoire of bioactive molecules providing lead compounds for development of new drugs [67]. Many toxins have been explored and developed into drugs for the treatment of cardiovascular conditions, such as hypertension, thrombosis, thrombocutopenia, cardiac failure etc. For instance, Tirofiban and Eptifibatide are antiplatelet drugs derived from disintegrins echistatin and barbourin respectively which are inhibitors of platelet receptor GPIIb/IIIa [5,68,69]. Furthermore, numerous snake venom toxin-based drugs are presently in clinical trials at various stages of development with promising horizons of application. A few examples of snake venom derived drugs that have been clinically approved and those in clinical trials are listed in Table 1.1.

Table 1.1 Examples of drugs derived from snake venom toxins for cardiovascular conditions that has been clinically approved or under clinical trials (BPF: Bradykinin potentiating factor, Dis: Disintegrin, SVSP: Snake venom serine protease, SVMP: Snake venom metallopotease, Snaclec: Snake C-type lectin like protein, NP: Natriuretic peptide, PM: Peptidomimetic)

Drug (Protein/ Peptide)	Snake venom component	Source	Mechanism of action	Applications	Ref.
CLINICALLY APPROVED DRUGS					
Captopen (Captopril)	BPF	*Bothrops jararaca*	ACE inhibitor	Hypertension, cardiac failure	[65]
Vasotec (Enalapril)	BPF	*Bothrops jararaca*	ACE inhibitor	Hypertension, cardiac failure	[70]
Aggrastat (Tirofiban)	Dis	*Echis carinatus*	Antagonist of fibrinogen binding to the GPIIb/IIIa	Acute coronary syndrome	[71]
Integrilin (Eptifibatide)	Dis	*Sistrurus miliarius barbouri*	Prevents binding of ligands to GPIIb/IIIa	Acute coronary syndrome	[72]
Reptilase (Batroxobin)	SVSP	*Bothrops atrox*	Cleaves Aα chain of fibrinogen	Internal and external haemorrhages, thrombosis	[73]
Defibrase (Moojenin)	SVMP	*Bothrops moojeni*	Cleaves Aα and Bβ chains of fibrinogen	Cerebral infarction, ischaemia, angina, microcirculation dysfunctions	[74]
POTENTIAL DRUGS IN CLINICAL AND PRE-CLINICAL STUDIES					
Anfibatide	Snaclec	*Agkistrodon acutus*	Platelet receptor GPIb antagonist	Myocardial infarction	[75]
Cenderitide	NP	*Dendroaspis angusticeps*	Activator of membrane receptors,	Heart failure	[76]
Alfimeprase	SVMP	*Agkistrodon contortrix contortrix*	Fibrinolytic activity	Acute peripheral arterial occlusion	[77]
Ximelagatran	PM	*Naja spp.*	Direct thrombin inhibitor	Prevention of venous thromboembolic events	[78,79]
Ancrod	SVSP	*Calloselasma rhodostoma*	De-fibrinogenating agent	Acute ischemic stroke, thrombosis, thrombocytopenia	[22,80]

1.6 Need for the proposed study

Daboia russelii, commonly known as the Russell's viper, was first mentioned by Patrick Russell, who wrote about it in his 1796 work *"An account of Indian serpents, collected on the coast of Coromandel".* It has a stout body with flattened and triangular head, blunt and rounded snout and a short tail [81]. The body color is typically yellowish to brown, adorned with distinctive bright chain patterns composed of dark spots with black ring and intensified white rim around the edges run the length of the body [82]. The venom of *Daboia russelii* is haemotoxic in nature and symptoms observed post its envenomation include persistent and profuse bleeding at the bite site as well as at other sites such as gingival sulci, nose, gastrointestinal tract, conjunctivae and skin among others. Prolonged poisoning of Russell's viper venom has also been shown to cause thrombocytopenia [81].

Daboia russelii is distributed throughout Pakistan, India, Nepal, Bhutan, Bangladesh and Sri Lanka [2,81,83]. It is one of the deadliest snakes across the Indian sub-continent. It is also one of the most extensively studied snakes in the world. Advances in proteomic approaches have assisted toxinologists in undertaking research to study its venom composition and correlate it with the pathophysiological symptoms observed post envenomation [84,85]. These investigations, alongwith comparative analyses, have also helped in better understanding of the similarities and differences among the venom profiles and their pathological implications. Such similarities and differences are a result of variation in various aspects such as diet, growth stage, geography, habitat etc. [86]. Since the only available antidote for snakebite is polyvalent antivenom, multiple studies assessing neutralization potency and immunological cross-reactivity of commercially available antivenom towards *Daboia russelii* venom have been performed [85,87]. These assessments have pointed towards various limitations, such as adverse reactions and inefficacy, owing to variation in venom composition [88,89]. As such, an inclusive understanding of venom profiles from venomous snakes belonging to different geographical locations becomes crucial for designing effective antidotes [90]. *Daboia russelii,* being a category I medically important snake across most of the Indian sub-continent, numerous research endeavours have been undertaken to study and compare its venom composition. However, more venom profiling of this species from lesser explored regions needs to be done for the formulation of better alternatives to the

current antivenom therapy for efficient treatment against *Daboia russelii* bites. Apart from these, research towards isolation and characterization of toxicologically and therapeutically important proteins has been carried out. *Daboia russelii* venom being haemotoxic in nature, numerous proteins have been studied under anticoagulant and procoagulant milieu (Table 1.2). However, their exact mechanisms of action remain unclear and their ability to modulate platelet hyperreactivity (antiplatelet potential) remains largely unexplored.

Table 1.2: *Daboia russelii* venom proteins affecting haemostasis

Protein	Family	Function	Ref.
Daboxin-P	PLA_2	Strong anticoagulant activity by targeting factor X and factor Xa	[91]
$DPLA_2$	PLA_2	Anticoagulant activity	[92]
RVVA-PLA_2I	PLA_2	Anticoagulant activity by enzymatic hydrolysis of plasma phospholipids and by non-enzymatic inhibition of factor Xa	[93]
VRV-PL-VIIIa	PLA_2	Anticoagulant activity by inhibiting factor Xa and prothrombinase activity	[94]
Neupholipase	PLA_2	Anticoagulant activity by preferential hydrolysis phosphatidylserine	[95]
RVV-PFIIc’	PLA_2	Anticoagulant activity by targeting the extrinsic pathway of the coagulation cascade	[96]
RVV-V	SVSP	Procoagulant activity by targeting Factor V	[15]
RVV-X	SVMP	Procoagulant activity by targeting Factor X and IX	[17]
Daborhagin-K	SVMP	Induces dermal haemorrhage	[97]
VRR-12	SVMP	Induces skeletal muscle and intestinal haemorrhage	[98]
VRH-1	SVMP	Induces severe lung haemorrhage	[99]
VRR-73	SVMP	Haemorrhagic activity	[100]
RVBCMP	SVMP	Procoagulant possessing alpha-fibrinogenase and tissue haemorrhagic activity	[101]
Rusviprotease	SVMP	Procoagulant activity by targeting alpha-fibrinogen and prothrombin	[102]
Ruviprase	Peptide	Potent anticoagulant activity by non-enzymatic inhibition of thrombin and factor Xa	[103]

In this study, profiling the proteome of *Daboia russelii* venom from Tanore, Rajshahi, Bangladesh in order to gain in-depth understanding of the venom composition as well as to identify proteins with anti-platelet potential is undertaken. The present study also explores the structure-function relationship of the identified anti-platelet protein in order to understand its mechanisms of action.

1.7 Objectives:

The objectives of this thesis have been framed as follows:

1. *Proteomics and partial characterization of crude Daboia russelii venom*
2. *Identification and characterization of anti-platelet protein from Daboia russelii venom*
3. *Exploring the mechanism of anti-platelet activity of the isolated protein*