1.1 Introduction

Snakes, a topic of feared, revered, and often misunderstood, and have been a source of legend and nightmare since time immemorial. Living snakes are found in every continent except Antarctica, and in most islands. Fifteen families of snakes are currently recognized, comprising 456 genera and over 2,900 species [1,2]. They range in size from the tiny, 10 cm-long thread snake to Pythons and Anacondas of up to 7.6 metres (25 ft) in length. Snakes are remarkable animals, successful on land, in the sea, in forests, in grasslands, in lakes, and in deserts.

Snake venom is one of the most amazing and unique adaptations of animal evolution. The evolution of snakes dates back to some 70 million years in the Cretaceous period as fossils readily identifiable as snakes (though often retaining hind limbs) first appear in the fossil record during the Cretaceous period [3]. Since then, they independently evolved their own venom apparatus in ophidian evolution, at the base of the Colubroidea radiation [4,5,6]. All the known advanced snake species are venomous and most of them are found in the superfamily Colubroidea that also includes the families Elapidae (incl. Hydrophiidae; Cobras, Kraits, Coral Snakes, Sea Snakes) and Viperidae (Vipers and Pit vipers) [6].

India has a vast potential and rich diversity of snake fauna, of which only 278 species have been identified [7]. Among those kraits (Genus: Bungarus), coral snakes (Genera: Calliophis, Sinomicrurus), cobras (Genus: Naja), king cobra (Genus: Ophiophagus), sea snakes (Laticauda, Kerilia, Enhydrina, Hydrophis, Lapemis, Astrotia, Pelamis), vipers (Daboia, Macrovierea, Echis) and pit vipers (Gloydius, Hypnale, Trimeresurus, Protobothrops, Ovophis) are venomous or, harmful to human beings [7]. The Indian subcontinent boasts of housing approximately 10 percent of the total snake species found in the world (www.iloveindia.com/wildlife/indian-snakes/index.html; 8, http://ildoutdoors.in/
author/admin/page/2). From warm seas to semi-deserts, swamps, lakes and even the Himalayan glaciers, one can find snakes in almost all the habitats in India. The snakes of India range from Worm Snakes, about 10 cm in length, to the King Cobra, measuring up to 6 m. Russell’s viper, one of the deadliest snakes, which is known by a number of other names, like Daboia, Tic Polonga, etc. It is a highly poisonous snake of the Viperidae family, scientifically known as *Daboia russelli*. The Indian Russell’s viper (*Daboia russelli*) feeds mainly on rodents which are commonly associated with human habitations; it therefore is a common cause of snake bite and many people die each year following bites by this species.

Despite the harmful and life-threatening affects, snake venom has an important place in scientific discoveries owing to the fact that venom toxins provide highly specific research tools which may lead to the development of novel life-saving medicines and drugs from venoms [9,10]. In recent years, the subject of snake venom has been receiving much more interest from the standpoint of biochemistry, toxicology, pathophysiology, pharmacology, immunology and biomedical research.

Venomous snakebites can be deadly for victims as venoms are highly toxic in nature. However research on snake venom proteins shows that they contain natural components of medical importance, so that venom, one of nature’s deadliest toxins, can be transformed into curative agents of various diseases i.e., drugs [10]. Snakes use venom to alter biological functions, and that’s what medicine does too, this is why venoms have always been of interest to medical researchers. Venoms are exquisitely complex, composed of as many as a hundred different peptides, enzymes, and toxins. Not only are the venoms of every snake species different, there are also subtle variations within each species. There are differences between [venoms of] juveniles and adults, and even among different geographic regions. These differences may be due to different evolutionary pressures, like different ancestry, prey, and environments.
1.2 Snake envenomation: The snakebite problem

Snake venom poisoning is a well-rounded examination of the many facets of serpent life, including natural history, environmental considerations, serum toxicology, clinical indications, legal concerns and even common misconceptions and mythology. From time immemorial, people have gathered pain and distress due to envenomation, as a result of which snakes and their venoms have been shrouded with myth and superstitions. The health hazards to children, agricultural workers and hunters, posed by venomous snakes in tropical countries are very real, and it is perhaps understandable that, in countries where snakebite is endemic, snakes and snake-like creatures are usually killed on sight. This attitude will persist until the local people can be convinced of the crucial ecological role of snakes in controlling rodents and other pests.

Snake bite is a common medical emergency and the epidemiological features vary from region to region. Snake envenomation is an occupational hazard, more so in tropical India, where farming is a major source of employment. There are approximately 3000 species of snakes in the world known to date, out of which 410 species are poisonous to human [11,12,13]. It has been estimated that 5 million snake-bite cases occur worldwide every year, causing about 100,000 deaths [14]. On an average, nearly 2,00,000 persons fall prey to snake-bite per year in India and 35,000-50,000 of them die every year [15,16]. Snake bite is a neglected tropical disease and snakebite epidemiology is not properly represented [17]. The true global incidences of envenomation and their severity remain largely misunderstood [17], except for a few countries where these accidents are rare or are correctly reported [18]. Very few countries possess a reliable epidemiological reporting system capable of providing precise data on snakebites. Instead, scientific reports and publications have to be used to assess the magnitude of the problem posed by snakebites. These data thus obtained are generally more precise and reliable but often cover limited geographical areas or deal with species aspects [19].
1.2.1 Epidemiology of snakebite in Asia

Snake bite is a common and frequently devastating environmental and occupational disease and one of the most neglected public health issues in poor rural communities living in the tropics [20]. Because of serious misreporting, the true worldwide burden of snake bite is not known. South Asia is the world’s most heavily affected region [21], due to its high population density, widespread agricultural activities, numerous venomous snake species and lack of functional snake bite control programs [22,23]. Despite increasing knowledge of snake venoms’ composition and mode of action, good understanding of clinical features of envenoming and sufficient production of antivenom by Indian manufacturers, snake bite management remains unsatisfactory in this region [22]. Field diagnostic tests for snake species identification do not exist and treatment mainly relies on the administration of antivenoms that do not cover all of the important venomous snakes of the region. Care-givers need better training and supervision, and national guidelines should be fed by evidence-based data generated by well-designed research studies. Poorly informed rural populations often apply inappropriate first-aid measures and vital time is lost before the victim is transported to a treatment centre, where cost of treatment can constitute an additional hurdle [24]. The deficiency of snake bite management in South Asia is multi-causal and requires joint collaborative efforts from researchers, antivenom manufacturers, policy makers, public health authorities and international funders [22,23].
**Table 1.1: Frequency and statistics of snakebite** (Global evaluation of snakebites).

<table>
<thead>
<tr>
<th>Landmasses</th>
<th>Population (x106)</th>
<th>Total number of bites</th>
<th>No. of envenomations</th>
<th>No. of fatalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>730</td>
<td>25000</td>
<td>8000</td>
<td>30</td>
</tr>
<tr>
<td>Middle East</td>
<td>160</td>
<td>20000</td>
<td>15000</td>
<td>100</td>
</tr>
<tr>
<td>USA and Canada</td>
<td>310</td>
<td>45000</td>
<td>6500</td>
<td>15</td>
</tr>
<tr>
<td>Central and South America</td>
<td>400</td>
<td>300000</td>
<td>150000</td>
<td>5000</td>
</tr>
<tr>
<td>Africa</td>
<td>760</td>
<td>1000000</td>
<td>500000</td>
<td>20000</td>
</tr>
<tr>
<td>Asia</td>
<td>3500</td>
<td>4000000</td>
<td>2000000</td>
<td>100000</td>
</tr>
<tr>
<td>Oceania</td>
<td>20*</td>
<td>10000</td>
<td>3000</td>
<td>200</td>
</tr>
<tr>
<td>Total</td>
<td>5840</td>
<td>5400000</td>
<td>2682500</td>
<td>125345</td>
</tr>
</tbody>
</table>

* Population at risk

Source: indjst.org/archive/vol.2.issue.9-10/oct09meenakshisun-30.pdf

### 1.2.2 Epidemiology of snakebite in India

South Asia is by far the most snakebite affected region [19,21]. India has the highest number of deaths due to snake bites in the world with 35,000-50,000 people dying per year according to World Health Organization (WHO) direct estimates [7,19,21].
Russell’s viper is the major cause of snakebite morbidity and mortality in many Southeast Asian countries including Thailand, Myanmar, India, Sri Lanka, China, Taiwan, and Indonesia [25]. The problem is particularly severe and acute in the eastern zone of India. An analysis of the snakebite cases in eastern India reveals that death toll from Russell's viper envenomation is highest in the Burdwan district of West Bengal [26,27], where 1301 deaths occur per year [28].

1.2.3 Epidemiology of snakebite in other countries

In Pakistan, 40,000 bites are reported annually, which result in up to 8,200 fatalities [21,29]. In Nepal, more than 20,000 cases of envenoming occur each year, with 1,000 recorded deaths [30]. Sri Lanka is one of the highest-risk countries in terms of snakebite fatality [31] with about 37,000 people suffering from snakebite every year [32]. A postal survey conducted in 21 of the 65 administrative districts of Bangladesh estimated an annual incidence of 4.3 per 100,000 population and a case fatality of 20 % [33]. However, existing epidemiological data remain fragmented and the true impact of snake bites is very likely to be underestimated. Surveys in rural Sri Lanka showed that hospital data record less than half of the deaths due to snakebite [31,32]. In Nepal, a review of district hospital records showed that national figures underestimated the incidence of snake bite by one order of magnitude [34]. The highest figures reported in Asia so far come from a community-based survey conducted in southeast Nepal in 2002, which revealed annual incidence and mortality rates of 1,162/100,000 and 162/100,000, respectively [35]. Figures of a similar magnitude were recently also obtained in a nation-wide community-based survey in Bangladesh [22]. In Thailand, the incidence of snakebites is estimated at 13 per 100,000 persons and the death rate is 0.04 per 100,000 persons [36].

Snakebites are more frequent in young men, and generally occur on lower limbs. The incidence of snake bites is higher during the rainy season and during periods of intense agricultural activity [37]. Snake bite incidence and mortality
also increase sharply during extreme weather conditions such as floods. Viperid snakes are represented by 26 species belonging to the true vipers (subfamily Viperinae) and pit vipers (Crotalinae). Among the true vipers, Russell’s viper (*Daboia russelii*) is associated with the highest morbidity and mortality [22]. In Anuradhapura District of Sri Lanka, up to 73 % of all admitted snake bites are attributed to this species [38] whose distribution extends from north to the Indus valley of Pakistan and Kashmir, to the foothills of the Himalayas in Nepal and Bhutan and to Bangladesh in the east [22].

1.3 **Indian Russell’s viper (*Daboia russelii*): Systematic classification and distinctive features**

![Fig 1.1: Indian Russell’s viper on the move](http://www.treknature.com/gallery/photo98478.htm)
1.3.1 Systematic classification

**Phylum**: Chordata  
**Group**: Vertebrata  
**Subphylum**: Gnathostomata  
**Class**: Reptilia  
**Subclass**: Diapsida  
**Order**: Squamata  
**Suborder**: Ophidia  
**Infra order**: Xenophidia  
**Family**: Viperidae  
**Subfamily**: Viperinae  
**Genus**: Daboia  
**Species**: russelli

1.3.2 Distinctive features

Medium-sized to large; strongly keeled scales; distinctive bright chain pattern; large triangular head. The bright symmetrical spots on Russell’s viper's backs make them easy to recognise [39].

1.3.3 Description

Russell’s Vipers are heavy, rough-scaled snakes with vertical eye pupils and generally a very bright pattern. The body colour is usually brown or yellowish and the pattern is composed of dark, round spots edged with white and black [7]. The underside is white in the western, partly speckled in the south-eastern and heavily speckled in the north-eastern race. Colour variation is common, and the best recognition characters are the short, fat body, the
triangular-shaped head and very regular chain like pattern [7]. Russell’s vipers resemble the fat, harmless common sand boas which however have shorter and blunter tails and irregular body patterns (http://www.goa-world.com/goa/rahulsnakepit/Snakes How to catch, take care and release snakes.htm). Russell’s Vipers are one of the “Big Four” dangerous snakes of India [39,40]. Average length of Daboia russelli is about 120 cm (4 ft) and grows to a maximum length of 166 cm (5.5 ft) [39].

1.3.4 Fang structure of Viperidae snakes including Russell’s viper

Many advanced snakes use fangs- specialized teeth associated with a venom gland [4, 41] to inject venom into prey for immobilization or attacker for self defence. Various front- and rear-fanged groups are recognized, according to whether their fangs are positioned anterior (for example cobras and vipers) or posterior (for example grass snakes) in the upper jaw [42,43,44]. Fangs can occupy various positions on the upper jaw, but are always located on the maxilla and never on any other tooth-bearing bone [45].

![Fig 1.2: Fangs and venom secretion of Russell’s viper](http://en.wikipedia.org/wiki/File:Russel%27s_Viper_Fang_and_Venom.jpg)

Sources:


b. http://www.flickr.com/photos/42573699@N03/3972411624/in/photostream/
The fangs of a viper, however, unlike those of a cobra, are hinged and movable. A sheath and muscles fold the fangs along the jaw and swing them into position when the snake wants to use them [46].

1.3.5 Distribution

*Daboia russelli* is found in Asia throughout the Indian subcontinent (hills and plains throughout India found up to 3,000 m above sea level), much of Southeast Asia including Sri Lanka, Pakistan, Myanmar, Southern China and Taiwan [47]. It has been found 2756m (9040ft) above sea level [7].

![Fig 1.3: Russell’s viper habitat in South-east Asia including India.](http://danger.mongabay.com/survival/afm/e.html)
The snake was classified into 5 sub-species based on the differences of coloration and markings, *Daboia russellii russellii* (India and Pakistan), *Daboia russellii pulchella* (Sri Lanka), *Daboia russellii siamensis* (Thailand, Myanmar and China), *Daboia russellii formosensis* (Taiwan) and *Daboia russellii limitis* (Indonesia) [25].

1.3.6 Pathophysiological and clinical symptoms of Russell’s viper envenomation in human victims

In South East Asia, Russell's viper is responsible for more snakebite deaths than any other venomous snake (http://www.iloveindia.com/wildlife/indian-snakes/russels-viper.html). It is highly irritable and when threatened, coils tightly, hisses, and strikes with lightning speed (http://www.iloveindia.com/wildlife/indian-snakes/russels-viper.html). While sluggish most of the time, the snake will strike and hold on when objects come into effective biting range (http://www.engin.umich.edu/~cre/web_mod/viper/introduction_2.htm). On average an adult Russell’s viper may inject 225-250 mg of venom to its victim (personal communication to Prof. A.K. Mukherjee from Mr. D. Mitra, in-charge, Calcutta Snake Park, Kolkata) and the effects begin. Its hemotoxic venom is a very potent coagulant, which damages tissue as well as blood cells. There are two times during the year when the number of bites increase, these correspond with the times when the rice fields are being planted and harvested (http://www.engin.umich.edu/~cre/web_mod/viper/introduction_2.htm). Once bitten, people experience a wide variety of symptoms including pain, swelling at the bite area, blistering, vomiting [48], dizziness, systematic bleeding/blood incoagubility [27] and kidney failure [49]. The severity of the symptoms depends on the age and the size of the victim. The snakes live in the rice fields, many farmers are bitten and their rural location hinders them from seeking immediate medical attention. Therefore, study on the effect of snake venom on blood clotting mechanism would lead to a better understanding on how does the venom interferes with the normal haemostasis of
victim; and this knowledge in turn will further be useful in the proper treatment of envenomed patients.

Depending on the zoogeographic origins of Russell's vipers, their venom composition may vary [27,50-53], as a result of which Russell's viper envenomation displays a fascinating variation in the clinical manifestation [27,38,50,52,54]. For example, Russell's viper patients in Sri Lanka develop features of neurotoxicity [27,55,56] whereas in Burma, patients exhibit increased capillary permeability and facial edema [25,52]. The patients from eastern India (Burdwan district of West Bengal) develop the symptoms such as hypotension, shock, etc. which develop as a consequence of increased vascular permeability, vasodilation and cytotoxicity which are due to the presence of basic PLA$_2$, proteases [25,27,57] and ATPase [58] in Russell's viper venom (RVV). The majority of the deaths from Russell's viper bite in Burdwan are attributed to prolonged blood coagulation time, renal failure and intravascular hemolysis [27]. Incoagulable blood is caused by defibrination resulting from consumption of the components of the haemostatic system [25,27]. This may lead to bleeding causing haemostasis and haemoptysis of Russell's viper bite victims. Therefore once the patients' blood has become defibrinated and incoagulable, the activity of hemorrhagins, which damage the vascular wall endothelium [59], may lead to spontaneous systematic bleeding from vital organs [25].
Table 1.2: Clinical features of Russell's viper envenomation in Burdwan, eastern India (Source: Mukherjee et al., 2000 [27]).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Percentage of patients exhibiting the symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
<td>100</td>
</tr>
<tr>
<td>Intravascular hemolysis</td>
<td>86</td>
</tr>
<tr>
<td>Haematemesis</td>
<td>73</td>
</tr>
<tr>
<td>Haematuria</td>
<td>86</td>
</tr>
<tr>
<td>Haemoptysis</td>
<td>67</td>
</tr>
<tr>
<td>Renal failure</td>
<td>23</td>
</tr>
<tr>
<td>Hypotension</td>
<td>42</td>
</tr>
<tr>
<td>Convulsion</td>
<td>11</td>
</tr>
</tbody>
</table>

1.4 Snake venom: In a broad-spectrum concern

Venom is a prey-immobilizing substance in snakes that is used secondarily as a defence system. Since venoms serve in both immobilization and digestion, they have evolved to have a strong balance in their toxic and digestive components. Snake venom contains a large number of biologically active proteins and polypeptides that are usually similar in structure but not identical to that of prey physiological systems. These molecules are produced by specialized glands, which are evolutionarily related to salivary glands, and are toxic to prey [4].

The snake venom is slightly acidic in nature and specific gravity ranges from 1.03 to 1.07. The relative viscosity of snake venom varies from 1.5 to 2.5. The solubility of Viperidae venom in water is much lesser than that of Elapidae and solubility of all venoms increase in physiological saline [60].
Almost all venoms are composed of approximately 90-95% proteins and polypeptides, including amino acids, nucleotides, free lipids, carbohydrates and other 5% composed of non-proteins part [5,6,61,62]. More than 20 enzymes have been detected in snake venoms, and 12 are found in all venoms, although their level differs markedly [63]. The enzyme levels of viperid and crotalid venoms fall in the range 80 to 95% of the total dry matter, whereas the corresponding range for elapid venoms is 25% to 70% [64]. The enzyme content of hydrophid venoms is at the lower end of the elapid range.

Snake venoms is complex mixtures of enzymatic and non-enzymatic toxic proteins including phospholipases A$_2$, myotoxins, hemorrhagic metalloproteases and other proteolytic enzymes, coagulant components, neurotoxins, cytotoxins and cardiotoxins, among others [65]. Ophidian envenomations are characterized by prominent local tissue damage, i.e. hemorrhage, necrosis and edema, alterations in the blood coagulation system as well as systemic neurotoxic effect. Additive or synergistic effects of active enzymes and toxins present in the venoms are responsible for this complex pathological picture [66-68].

Snake venom protein constituents may present different biological activities that affect physiological process of their prey such as neurotransmission, the complement system and homeostasis [9,12,69]. These venoms can act in more than one system at the same time and they may present antigenic effect [69,70]. Viperidae family venom molecules are good examples, such as in homeostasis, where they act as pro- and anticoagulant factors, and also inducers and inhibitors of platelet aggregation [69,71,72]. Due to their diversity, the proteins from the Viperidae family members are classified as:

a) Serine proteases,

b) Metalloproteases,
c) Phospholipase A<sub>2</sub> (PLA<sub>2</sub>),

d) C-type lectins, and

e) Disintegrins.

However, only the initial three groups display enzymatic activity [61,71].

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>s) are described as responsible for some of the envenomation symptoms, which involve not only the hemostatic system, with an anticoagulant and an antiplatelet profile, but also inflammatory and myotoxic effects [73]. Local inflammation and pain are important features of Viperidae and Elapidae snakebite envenomations that are rich in myotoxic nociceptive events induced by PLA<sub>2</sub> [73].

1.4.1 Variation in snake venom composition and its impact on pathogenesis

Variation in venom composition is ubiquitous among venomous snakes, occurring at all taxonomic and biological levels [74]. Although it is generally accepted that the primary function of snake venom is to facilitate immobilization and/or digestion of prey, the extent to which adaptive processes drive the evolution of snake venom diversity has been widely debated. Several authors have supported an ‘overkill’ hypothesis of venom evolution, which postulates that, due to the apparent high toxicity of many snake venoms and the large doses injected, variation in venom composition is unlikely to be subject to natural selection for lethality to prey, and that venom diversity largely results from neutral evolutionary processes [75,76]. By contrast, other authors argue that snake venom composition is subject to strong natural selection, and that venom diversity results from adaptation to specific diets [77,78].

Snake venom composition may exhibit variations associated with the geographical origin, habitat, seasonal variation, diet, age and gender [74,79-81].
This variation plays an important role in pathophysiological symptoms following snakebite and deserves medical concern. Due to the variation in venom composition, the pathogenesis developed after a bite is complex in nature. It is not only dependent on the qualitative composition of venom, but also on the quantitative distribution of different components of particular venom [25, 82, 83]. Therefore, biochemical characterization of snake venom from a particular geographical location is of great importance to know the pharmacological, toxicological and clinical action of envenomation [83].

It has been well documented that this variation in venom composition significantly affects the neutralizing capacity of antivenom as well [84]. Therefore, the variation in the venom composition should be given proper consideration while producing antivenom, because the antivenom raised against the venom of one population of snake may be less effective against the venom of another population of snakes, which may be of the same species of snake but from a different geographical location [85]. The clinical manifestations of Russell’s viper bites differ in each geographic region [10, 25, 86]. Variation in venom composition may explain the diversity of signs and symptoms in RV bite victims.

1.4.2 Snake venom phospholipase A2 (PLA2) enzymes: Classification, structure and functions

Among the snake venom enzymes, Phospholipase A2 (EC: 3.1.1.4) are the most fascinating group of proteins due to their pivotal role in inducing various pharmacological effects on snakebite victims, despite similarity in primary, secondary and tertiary structures and common catalytic properties [87, 88]. The first PLA2 enzymes were purified from the venom of *Naja naja* and *Naja tripudians* and were named as hemolysins due to their ability to haemolysed red blood cells indirectly [89]. Since then, hundreds of snake venom PLA2 enzymes have been purified and characterized. To date, amino acid
sequence of over 300 PLA$_2$ enzymes have been reported from snake venom. PLA$_2$ enzymes share 40 to 99 \% identity in their amino acid sequences and hence significant similarity in their three-dimensional folding [90,91]. However, they differ greatly in pharmacological properties [92]. Thus, the functional differences among PLA$_2$ enzymes cannot be easily correlated to their structural differences.

The structure-function relationship and the mechanism of this group of small proteins are subtle, complex and intriguing challenges to biochemists, molecular biologists, toxinologists, pharmacologists and physiologists. They occur ubiquitously in nature as both intracellular and extracellular forms and human pancreas and snake venom are the richest source of PLA$_2$ enzymes [93]. In general, mammalian PLA$_2$ enzymes are non toxic and do not induce potent pharmacological effects. In contrast, snake venom PLA$_2$ enzymes are among the major toxic proteins of the venom and play an important role in immobilization and capture of prey [91]. In addition to the digestion of prey, PLA$_2$ exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, haemorrhage and edema inducing activities [94-99]. This diverse pharmacological profile has been acquired through an accelerated evolutionary process [100,101]. A single venom including RVV may contain several isoforms of PLA$_2$ which are acidic, basic or, neutral in nature [50], and each PLA$_2$ may exert different pathophysiological effects by different mechanisms in snakebite victims [27,97,102]. In general, PLA$_2$ enzymes and their complexes are among the most toxic and potent pharmacologically active components of snake venoms. Therefore, much effort has been put into characterization of snake venom PLA$_2$ enzymes, and they are the best studied families of snake venom proteins [93]. A high degree of homology in the amino acid sequence and enzyme active sites has been displayed by diversity of snake venom PLA$_2$. There is a dearth of knowledge on the biochemical basis of diversity of Phospholipase A$_2$ enzymes [103].
Among the different isoenzymes of PLA$_2$s present in RVV, basic phospholipase A$_2$ is much more toxic as compared to acidic or, neutral PLA$_2$ and contributes significantly to the toxicity of venom [50,83]. Indian Russell’s viper venom has been reported to contain as many as 13 isoenzymes [27]. Although different PLA$_2$ enzymes from *Daboia russelli* venom have been purified and characterized [102,104-107] but till date not much information are available regarding the mechanism of anticoagulant action of PLA$_2$ enzymes from *Daboia russelli* venom of Indian origin.

Circulatory system is one of the physiological systems targeted by anticoagulant PLA$_2$s of snake venom [108]. Mukherjee et al. [27] have reported that most of the death from Russell’s viper envenomation in eastern India is attributed to prolonged blood coagulation time of victims. Venom PLA$_2$s most likely affect blood coagulation through hydrolysis of and/or binding to procoagulant phospholipids [109]. Strongly anticoagulant PLA$_2$ enzymes also affect blood coagulation by mechanisms that are independent of phospholipid hydrolysis [108]. However, the mechanism of anticoagulant action of RVV PLA$_2$ enzymes has still remained obscure. Studies on such anticoagulants contribute to our understanding of ‘vulnerable’ sites in the coagulation cascade which may further help us to design novel strategies to develop anticoagulant therapeutic agents and new functional diagnostic test kits in the field of hemostasis [108,110].

**1.4.2.1 Classification of Phospholipase A$_2$ (PLA$_2$) enzymes**

Balsinde et al [111] classified PLA$_2$ enzymes based on their properties into three main types: Secretory PLA$_2$, cytosolic Ca$^{2+}$ dependent PLA$_2$ and intracellular Ca$^{2+}$ independent PLA$_2$. Kini [93] has classified PLA$_2$ enzymes depending on their structure and mechanism of catalysis. Intracellular and secretory PLA$_2$ enzymes have been classified into fourteen groups based on various parameters such as structure, amino acid sequence, catalysis, and
expression [112]. A number of new PLA₂ enzymes are being discovered, and this superfamily has been expanding rapidly. PLA₂ enzymes that share high sequence homology are classified under the same group. The active site of these enzymes has a histidine residue, and they share a common mechanism for cleaving the sn-2 ester bond of phospholipids [113]. However, based on the amino acid sequence, three dimensional structure, and disulfide bonding pattern, snake venom PLA₂ enzymes fall under Group I and II [114].

**Group I PLA₂ enzymes**

This group of PLA₂ enzymes is found in the mammalian pancreas and in venoms from elapid and colubrid snakes. Cobra venom PLA₂ enzymes were the first to be characterized under this group. These enzymes typically contain 115-120 amino acids residues with 7 disulfide bridges, and the disulfide bond between the 11th and 77th Cys residues is unique to this group. This group is further subdivided on the basis of characteristic surface loop present in snake and mammalian PLA₂ enzymes [91].

1. **Group IA**: Group I PLA₂ enzymes in snake venoms have a characteristic surface loop called the elapid loop that connects the catalytic α-helix and the β-wing. In general, most of the elapid venom PLA₂ enzymes belong to this group [91].

2. **Group IB**: In mammalian PLA₂ enzymes, there is an additional five amino acid residue extension, which is called the pancreatic loop (residue 62-67). This group of PLA₂ enzymes are mainly found in mammalian pancreas. However, Group IB enzymes have also been reported in some snake venoms, such as *Oxyuranus scutellatus* [115], *Pseudonaja textilis* [116], *Notechis scutus* [117], *Ophiophagus Hannah* [118] and *Muicurus frontalis frontalis* [119]. These enzymes are found abundantly in the pancreatic juice, where they have an important digestive role towards dietary phospholipids [91].
Fig1.4: Classification of Phospholipase A$_2$ enzymes (Schaloske and Dennis, 2006). s-PLA$_2$: secretory PLA$_2$; c-PLA$_2$: cytosolic PLA$_2$; PAF-AH PLA$_2$: platelet activating factor acetylhydrolase PLA$_2$. 
Group II PLA$_2$ enzymes

PLA$_2$ enzymes from Viperidae and crotalidae snake venoms fall under group II. These enzymes contain 120-125 amino acid residues and 7 disulfide bridges. They lack the pancreatic or elapid loop and differ from Group I in having an extended C-terminal tail. The 133$^{\text{rd}}$ cysteine residue near the active site is unique to Group II. Most of the enzymes in this group contain an aspartate residue in position 49 which is critically involved in the binding of Ca$^{2+}$ and thus known as Asp-49. In some enzymes, it is replaced by lysine, serine, asparagines or, arginine. Therefore, this group can be classified as K49, S49, N49 or, R49 [91].

1.4.2.1.1 Classification of PLA$_2$ enzymes depending on their ability to prolong blood clotting time

The anticoagulant activity was first attributed to venom PLA$_2$ enzymes by Boffa and Boffa in the year of 1976 [120]. PLA$_2$ enzymes have been classified into three groups depending on their anticoagulnat potency viz. strong, weak and nonanticoagulnat enzymes [121,122].
Fig 1.5: Classification of anticoagulant phospholipase A$_2$ enzymes of snake venom on the basis of their anticoagulant potency [121,122].

A) **Strongly anticoagulant PLA$_2$ enzymes**, inhibit blood coagulation at low concentrations (~2.0 µg ml$^{-1}$). They include *Naja nigricollis* (basic), *N. m. mossambica* (CM-III), *Vipera berus orientale*, *D. russelli*, *Agkistrodon halys blomhoffii* (basic), and *Crotalus durissus terrificus* (basic subunit of crotoxin) enzymes [122,123].

B) **Weakly anticoagulant PLA$_2$ enzymes**, showed anticoagulant effects between 3.0 and 10.0 µg ml$^{-1}$. *Naja mossambica* (CM-II), *Naja nigricollis* (acidic), *A. halys blomhoffii* (neutral), *Enhydrina schistosa*
(myotoxin) and *Oxyuranus scutellatus* (taipoxin) belong to this group [122,123].

**C) Non anticoagulant enzymes**, there is another group of PLA₂ enzymes which have little effect on the clotting times even at concentrations higher than 15.0 µg ml⁻¹. This group includes *N. m. mossambica* (CM-I), *N. naja*, *N. melanoleuca* (DEI and DEIII), *A. halys blomhoffi* (acidic), *Hemachatus hemachatus* (DEI), *Bitis gabonica*, *Crotalus admanteus*, *C. durissus terrificus* (crotoxin), *Vipera aspis* (V. aspis B), *Notechis scutatus* (notexin and II-5), and *Bungarus multicinctus* (β-bungarotoxin) [122,123].

1.4.2.2 Structure and mechanism of action of snake venom phospholipase A₂ enzymes

1.4.2.2.1 Structure of snake venom PLA₂ enzymes

Snake venom PLA₂ enzymes are small proteins (~13-14 kDa) with 115-133 amino acid residues. They have fourteen conserved Cys residues that form seven disulfide bridges and stabilize the tertiary structure [124]. PLA₂ enzymes consist of three major α-helices and two antiparallel β-sheets, which are held together by disulfide bridges. The conserved structure in PLA₂ enzymes are the N-terminal helix, calcium binding loop, antiparallel helix, active site and β-wing. The N-terminal segment of PLA₂ enzymes has a highly conserved network of hydrogen bonds and stabilizes the adjacent β-sheet [124]. Some PLA₂ enzymes that retain the N-terminal propeptide (8-mer) lack the catalytic activity, similar to the precursor of pancreatic PLA₂ enzymes. The N-terminal helix between residues 1 and 12 contributes significantly to the hydrophobic channel. The side chains of the residues in the helix form the opening of the channel, especially from the 2nd, 4th, 5th and 9th residues. The side chain of the 4th residue is functionally important, as it anchors the N-terminal helix to the enzyme [124].
Ca$^{2+}$ is the most important cofactor for catalysis. During catalysis, Ca$^{2+}$ binds to the enzyme at the conserved Ca$^{2+}$ binding loop that lies between residues 25 and 33 with a consensus sequence. The oxygen atom from Asp49, along with three carbonyl oxygen atoms and two water molecules, form the pentagonal bipyramidal cage for Ca$^{2+}$ [125]. Two long helices (from residues 37 to 54, known as catalytic helices, and from residues 90 to 109) are oriented antiparallel and held together by disulfide bridges. The conserved side chains of these helices assist in the coordination of the primary Ca$^{2+}$ and form the deeper contour of the hydrophobic channel [90]. His 48 is the crucial active site residue that is responsible for the catalysis and is supported by hydrogen bonds from Tyr52 to the side chain of the opposite helix (Asp99). This network, together with close coupling of Asp49 and His48, defines the active geometry of PLA$_2$ enzymes. All PLA$_2$ enzymes have two distinct β-sheets that form the β-wing. This β-wing connects the major helices and protrudes out from the main structure into the solvent. The extended C-terminal end is the characteristic feature of Group II PLA$_2$ enzymes and is cross-linked to the main structure by two disulfide bridges [126].
Fig 1.6: This figure shows the Stereo view of the main chain of the Russell's viper toxin (RVV-VD) which represents the secondary structure features of class I/II PLA$_2$ [127]. The structural data indicate that snake venom PLA$_2$ enzymes share strong structural similarity to mammalian pancreatic as well as secretory PLA$_2$ enzymes. They have a core of three major $\alpha$-helices [A (residues 1-14), C (residues 39-54) and E (residues 92-108)] with two short $\alpha$-helices [B (17-22) and D (58-66)], a distinctive backbone loop that binds catalytically important calcium ions (residues 25-37), and two strands of an antiparallel $\beta$-sheet (residues 74-85) follow. The C-terminal segment forms a semicircular ‘banister’, particularly in viperid and crotalid PLA$_2$ enzymes, around the Ca$^{2+}$-binding loop. The loops and most of the secondary structure elements are firmly attached to each other through a network of seven disulfide bridges.
1.4.2.3 The catalytic mechanism of snake venom PLA₂ enzymes

PLA₂ enzymes are esterolytic enzymes which are unique calcium-dependent hydrolytic enzymes that are highly water soluble and hydrolyze water-insoluble phospholipids, liberating free fatty acids and lysophospholipids [128]. They hydrolyze phospholipids in different phases, such as monomeric, micellar, or lipid bilayer phases.

Phospholipase A₂ preferentially catalyzes reactions at interfaces which is known as “interfacial catalysis”. PLA₂ contains a hydrophobic channel that provides the substrate with direct access from the phospholipids aggregate (micelle or, membrane) surface to the bound enzyme’s active site. Hence, on leaving its micelle to bind to the enzyme, the substrate need not become solvated and then desolvated. In contrast, soluble and dispersed phospholipids must surmount these significant kinetic barriers in order to bind to the enzyme. In 2006, Winget and co-workers [126] proposed a model for interfacial catalysis of venom PLA₂ enzymes. According to that model, the enzyme (E) binds to the substrate (S) at the interface as the E* form. The enzyme-substrate (E*-S) is the interface-bound form. An additional anion activating step occurs at this stage, where an anionic phospholipid interacts at the interface of the enzyme, forming the E*-S# complex. Once activated, the PLA₂ can catalyze the formation of the enzyme-product (E*-P) complex and then release the product. After release of the product, the E* diffuses in a scooting mode to bind another substrate (S*) for the next cycle of catalysis [129].

The active site of the PLA₂ molecule is a semicircular cavity at the end of the hydrophobic channel (Fig 1.7). It consists of four residues: His48, Asp49, Tyr52 and Asp99. A conserved water molecule plays an essential role in the catalysis and is connected to the side chains of the active site residues His48 and Asp49 through hydrogen bonds [130].
Fig 1.7: The catalytic network in PLA2 enzyme is showing the above figure. OW indicates a water molecule oxygen atom which serves as the nucleophile. The dotted lines indicate hydrogen bonds. There are three main alpha helices: N-terminal helix H1 (residues 2-12), helix H2 (residues 40-45) and helix H3 (residues 90-108). The alpha helices H2 and H3 are antiparallel and are at the core of the protein [130].

The mechanism by which PLA2 enzyme hydrolyze the phospholipid involves the highly specific interaction between the active site histidine, the Ca2+ cofactor, conserved water, and the glycerophospholipid substrate. His48 is conserved in sPLA2 enzymes, and its role in phospholipid hydrolysis has been confirmed by chemical modification using p-bromophenacyl bromide (p-BPB) [131,132]. During catalysis, His48, assisted by Asp99, polarize the bound water molecule, which then attacks the sn1-2 bond of the bound phospholipid to form a tetrahedral oxyanion intermediate [90,91]. An alternative mechanism has also
been proposed, whereby two more water molecules are involved in the formation and breakdown of the tetrahedral intermediate [133]. In both the mechanisms, Ca$^{2+}$ ion, coordinated by the oxygen atom of Asp99, serves as an electrophile during catalysis [90,133,134]. Overall, catalysis by sPLA$_2$ enzymes can be summarized [135] as follows:

1. Binding of Ca$^{2+}$ and substrate;
2. General base-mediated catalysis;
3. Formation and breakdown of tetrahedral intermediate, and
4. Release of the reaction product [135].

![Diagram of mechanism of action of phospholipase A$_2$ enzymes](image)

**Fig 1.8**: An example of mechanism of action of phospholipase A$_2$ enzymes

### 1.4.2.4 Mechanism of action of snake venom anticoagulant PLA$_2$s on blood coagulation

Circulatory system is one of the key physiological systems targeted by anticoagulant PLA$_2$s from snake venom (Kini, 2006). Most of the death from Russell’s viper envenomation in eastern India is attributed to prolonged blood coagulation time of victims [27]. Since plasma phospholipids play a crucial role in the formation of several coagulation complexes; therefore, it might be
endorsed that the obliteration of phospholipid surface by venom PLA$_2$s could be the primary mechanism to account for their anticoagulant effect [93]. In most cases, PLA$_2$s affect blood clotting through the hydrolysis of pro-coagulant phospholipids or by binding to them. Other PLA$_2$ are described as weak anticoagulant enzymes and inhibit the extrinsic complex. The anticoagulant activity of some PLA$_2$s was shown to be dependent on the presence of phospholipids [136], whereas some other PLA$_2$s can inhibit prothrombinase complex independently from the presence of phospholipids. Furthermore, some of the PLA$_2$s can bind to blood coagulation factors and thus can inhibit the factor from its normal role in clotting activity [108]. Generally, strong anticoagulant PLA$_2$s from snake venom interact with blood coagulation factor X or, Xa and thereby, inhibiting the formation of prothrombinase complex which is the most important step of the blood coagulation system (Fig 1.9).
Fig 1.9: Effect of snake venom anticoagulant PLA$_2$ enzymes in different stages of the extrinsic pathway of blood coagulation.
1.4.2.5 Pharmacological properties of snake venom PLA₂ enzymes

Snake venom PLA₂ enzymes exhibit a wide variety of pharmacological effects despite their similarity in primary, secondary, and tertiary structures such as neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant effects, platelet aggregation initiation, platelet aggregation inhibition, hemolytic activity, hemoglobinurea-inducing activity, internal hemorrhage, convulsant activity, hypotensive activity, edema-inducing activity, organ or tissue damage (Liver, kidney, lungs, testis, pituitary damage), cell migration and cell proliferation, and bactericidal activity.

Most of the PLA₂ enzymes are devoid of direct hemolytic activity [137,138]; however, in presence of exogenously added phospholipids, they exhibit potent hemolytic activity that is due to the hydrolysis products namely, lysophospholipid and free fatty acids, which are lytic by themselves [139,140].

Snake venom PLA₂ enzymes are also known to inhibit blood coagulation. These anticoagulant PLA₂ enzymes are classified into strong, weak and non anticoagulant enzymes, depending on the dose required to inhibit the blood coagulation [120,122]. Strong anticoagulant PLA₂ enzymes inhibit the activation of factor X to Xa by both enzymatic and non enzymatic mechanisms, and they inhibit the activation of prothrombin to thrombin by non enzymatic mechanism [141-143]. On the other hand, weakly anticoagulant PLA₂ enzymes inhibit the activation of factor X to Xa by an enzymatic mechanism, and they do not inhibit the activation of prothrombin to thrombin [141].

Both lethal and non-lethal PLA₂ from snake venom have been reported. Basic PLA₂s are reported to be more toxic and enzymatically less potent but the acidic PLA₂s are less toxic and enzymatically active [144], therefore the lethality of snake venom PLA₂ cannot be correlated with the catalytic property [145].
Although a variety of pharmacological effects are induced by PLA₂ enzymes, not all the effects are exhibited by all PLA₂ enzymes. Each enzyme exhibits a special effect. For example, β-bungarotoxin, a PLA₂ containing toxin, induces a presynaptic effect [146], but fails to show any postsynaptic effect [147]. It also fails to induce myotoxicity [148] and anticoagulant effects [121,122]. Thus, β-bungarotoxin appears to affect only the presynaptic site and hence is ‘targeted’ to the presynaptic site. The example of *Vipera russelli* isoenzymes provides a stronger indication of ‘targeting’. When the isoenzymes are injected intraparitonially, they exhibit various toxic effects in experimental animals: one specifically induces hemorrhage in liver and kidney [149], the second damages the lungs, causing hemorrhage [102], whereas the third kills the mice through neurotoxic symptoms [92]. This indicates the susceptibility of a specific system, tissue or organ to a particular PLA₂ enzyme. Such susceptibility can be explained by the presence of specific ‘target sites’ on the surface of target cells or, tissues [94]. These target sites are recognized by specific ‘pharmacological sites’ on the PLA₂ molecule. These pharmacological sites are independent of, but sometimes overlapped with the active site of the enzyme. It was proposed that the target sites and pharmacological sites are complementary to each other in terms of charges, hydrophobicity and van der Waal’s contact surfaces; hence the high affinity [94]. High affinity between the target and pharmacological sites determines the specific pharmacological effects of PLA₂ enzymes. In some other cases, a single PLA₂ enzyme can exhibit several pharmacological effects. For example, a basic PLA₂ from *Naja nigricollis* venom exhibit anticoagulant effects along with cardiotoxicity, convulsive activity, hemolytic activity and platelet aggregation inhibition [93]. In these cases, their specific effect probably depends on the relative affinities between the PLA₂ enzyme and various target sites as well as the relative possibilities of exposure to a specific site.
1.5 Pharmacological Sites

PLA$_2$ enzymes bind to target proteins through their specific pharmacological sites [94]. The presence of pharmacological sites is supported by chemical modification studies [131,150,151], studies using polyclonal and monoclonal antibodies, and analyses of interaction of inhibitors [152,153]. Various chemical methods to modify specific amino acid residues have been used to identify these structural features. Despite systematic efforts, it has been difficult to pinpoint the residues or segments of PLA$_2$ enzymes which determine the pharmacological effects. As an alternative, various laboratories have used theoretical methods to identify specific pharmacological sites [92].

1.6 Anticoagulant Region

By a systematic and direct comparison of the amino acid sequences of strong, weak and non-anticoagulant enzymes, Kini and Evans [154] identified the anticoagulant region to be between residues 54 and 77. The major difference between the strong anticoagulant and weakly or non-anticoagulant PLA$_2$ is the replacement of the residues in the anticoagulant region. Negatively charged Glu54 is replaced by neutral residue, positively charged Lys54 is replaced by negatively charged Glu, uncharged Gly57 is substituted by negatively charged Glu, positively charged Lys75 is replaced by Ser or, Thr, and positively charged Lys77 is replaced by negatively charged Glu or, Asp [155]. However, according to Kini and Evans [155], not the overall basicity but the nature of the charge in the anticoagulant site determines the anticoagulant potency of PLA$_2$ enzymes. In strongly anticoagulant PLA$_2$ enzymes, the anticoagulant region is positively charged, but negatively charged in weakly and non-anticoagulant enzyme. All chemical modification studies have supported this prediction [108], as have site-directed mutagenesis studies [156,157] and synthetic peptide studies [92,157].
1.7 Importance of identification of pharmacological sites

The pharmacological sites of PLA\textsubscript{2} enzymes determine the affinity between the PLA\textsubscript{2} and target proteins. The identification of pharmacological sites helps in

(i) Understanding the structure-function relationships of PLA\textsubscript{2} enzymes;

(ii) Developing strategies to neutralize the toxicity and pharmacological effects by targeting these sites; and

(iii) Developing prototypes of novel research tools and pharmaceutical drugs.

As these sites help in targeting the PLA\textsubscript{2} to a specific tissue or cell, they act as ‘protein postal addresses’ which help in ‘delivering’ the specific PLA\textsubscript{2} to the specific cell. These ‘addresses’ could be exploited to develop a delivery system which is targeted to a specific tissue or cells. Since PLA\textsubscript{2} enzymes affect almost every vital organ or tissue, one could get ‘addresses’ to many types of cells and tissues. Such a possibility should provide a strong impetus for studying structure-function relationships of PLA\textsubscript{2} enzymes and identification of more pharmacological sites [91,92].

1.8 Purification of PLA\textsubscript{2} enzymes from snake venom

Occurrence of large number of isoenzymes in snake venom is a common phenomenon. These isoenzymes exhibit different pharmacological effects and often cause problems in purification and determination of their functional specificity [93]. The enzyme preparation quality is very crucial for functional characterization and structure-function studies. Snake venom is a mixture of proteins that differ in their molecular weights as well as ionic charges. Among these proteins, particularly PLA\textsubscript{2} enzymes, share the same molecular but differs in ionic charges. Therefore, a combination of chromatographic steps should be
used for their purification. During the purification of PLA₂ enzymes from snake venom, two factors should be considered:

1. PLA₂ enzymes exist as isoenzymes, and a single venom may contain more than one isoenzyme. For example, *Naja naja*, *Daboia russelli*, *Trimeresurus flavoridis*, *Austrelaps superbus*, and *Pseudechis australis* venoms contain more than ten isoenzymes [158-162]. These isoenzymes show similarities in their molecular weight, isoelectric point, and even in N-terminal amino acid sequence, and therefore they might co-elute during purification. Thorough/exact methods should be used in determining the homogeneity of PLA₂ enzymes [161,163-165], and sophisticated methods including capillary electrophoresis (high number of theoretical plates) and high resolution mass spectrometry may be used in determining the homogeneity of the preparation.

2. PLA₂ enzymes interact with each other or with other proteins to form aggregates that are due to protein-protein interaction. In addition, PLA₂ enzymes interact with other venom toxins such as cardiotoxins [166]. These interactions contribute significantly to the enzymatic and pharmacological activity of PLA₂ enzymes. Therefore, a combination of different purification steps such as gel filtration followed by ion-exchange or, vice-versa and reverse-phase HPLC should be employed.

1.9 Origin and evolution of PLA₂ genes

Phylogenetic analysis of mammalian pancreatic and venom PLA₂ genes reveals that they have a common origin from a nontoxic ancestral gene, however, human and group I genes have followed a common pathway of evolution [167], but group II PLA₂ genes evolved separately after species diversification [168,169]. Typically genes encoding Group I PLA₂ enzymes are about 4 kb, which follows a typical structural organization comprised of four exons interrupted by three introns (which resembles the human pancreatic PLA₂
gene). However, the size of the intron 3 in the venom PLA2 gene is smaller than the pancreatic counterpart [167,170]. The smaller size of intron 3 is attributed to adaptive evolution of snake venom PLA2 enzymes, and intron 3 and 4 might code for addition of pharmacological properties [167]. The characteristic feature of the mammalian PLA2 enzyme is the presence of pancreatic loop, which is encoded by exon 3. In some snake venom PLA2 enzymes this loop is present, for example king cobra [118], Brazilian coral snake [119] and Australian elapid venoms; however, it is absent from most of the other snake venom PLA2 enzymes. The loss of this loop has been interpreted as providing an adaptive advantage for the development of toxic properties among the venom PLA2 enzymes [171]. Therefore, it was postulated that with the loss of the pancreatic loop from exon 3, additional toxicity and enhanced enzymatic activity has been acquired by some snake venom PLA2 enzymes [170]. In snake venom PLA2 enzymes, the loss of the pancreatic loop has followed a Darwinian type of accelerated evolution, while the mammalian PLA2 enzyme has undergone natural evolution, retaining the pancreatic loop and showing a reduced rate of mutation in gene [91].

Group II PLA2 enzymes, though sharing similarities in catalytic activity, are structurally different from Group I enzymes. The gene organization of Group II is also different from that of Group I, as it has five exons and four introns [172], similar to human synovial PLA2 gene [173].

Snake venoms contain a large number of PLA2 isoenzymes that exhibit different physiological functions, although they share similarities in their amino acid sequence and three-dimensional structure [91,93]. Venom PLA2 isoenzymes are products of multiple genes and are known to evolve through gene duplication, followed by accelerated evolution to acquire diverse physiological functions [91].
By comparing the large number of nucleotide and protein sequences of PLA$_2$ enzymes, Lynch in the year 2007 [174] concluded that in Group I PLA$_2$ enzymes, gene duplication and diversification occurred after speciation. In contrast, functional diversification in Group II occurred before the diversification of the species [174]. Thus, the accelerated evolution of exons and surface substitutions plays a significant role in the evolution of new isoenzymes by altering target specificity. Accelerated evolution continued until it acquired a stable function and then evolved with lowered mutation rates, favoring functional conservation [175]. However, in the case of a unique sea snake (*Aipysurus eydouxii*), the venom has been found to be evolving at a much slower rate than other terrestrial and sea snake venoms, and PLA$_2$ enzymes also followed a decelerated mode of evolution [91].

1.10 Anticoagulant PLA$_2$ enzymes from snake venom: Potential candidates for the development of novel drugs against cardiovascular diseases

Over the last several decades, research on snake venom toxins has not only provided new tools to decipher molecular details of various physiological processes, but also inspiration to design and develop a number of therapeutic agents. Blood circulation, particularly thrombosis and haemostasis, is one of the major targets of several snake venom proteins [108]. Snake venom is a veritable gold mine of bioactive molecules [10], capable of binding to a wide variety of pharmacological targets, including the blood coagulation cascade. Among the snake venoms, anticoagulant proteins have contributed to our understanding of molecular mechanisms of blood coagulation and provided potential new leads for the development of drugs to treat or, to prevent unwanted clot formation. Although significant progress has been made in understanding the structure-function relationship and the mechanism of some of these anticoagulants, there are still a number of questions to be answered as more new anticoagulants from snake venom are being discovered.
1.11 Role of medicinal plants in snakebite treatment

Plants are a source of many biologically active products and now-a-days, they are of great interest to the pharmaceutical industries. The study of how the people of different cultures use plants in particular ways has led to the discovery of important plant based new medicines. In India, numerous plant species are used as folk medicine to treat venomous snakebite, but without any scientific validation. Therefore, this type of treatment remains questionable and needs thorough scientific investigation.

Till now, antivenom is the only effective medicine used for the treatment of snakebite patients. Other than the various side effects of antivenom, one of the major limitations of currently available commercial antivenoms is that vials of the anti-snake venom must have to be stored under refrigerated condition (at 4-8 °C). It is to be noted that due to lack of this facility, the majority of primary health centers in the rural tropics fail to keep this life-saving drug (personal observation). As a result of this, in the rural tropics, often snakebite patients arrive at district (town) hospitals for treatment hours after being bitten and after travelling a long exhaustive journey. Late antivenom therapy may not be useful in saving the lives of these patients [176]. Medicinal plants have been overtaken in the treatment of snake bites by serum therapy and are rarely considered efficacious remedies in biomedicine. Nevertheless, rural inhabitants rely on plant medical material and the attention of highly regarded local traditional healers when threatened by snakebite poisoning. Screening of different medicinal plants which are used by the local people as anti-snakebite medicine and by examining whether the plant extracts showing anti-Russell’s viper venom activity, if any, will open a new horizon for the treatment of Russell’s viper envenomed patients.
1.12 Aims and objectives of the present study

The present study has been taken up to study the different isoenzymes of PLA$_2$s present in the Russell’s viper (Daboia russelli) venom of eastern India origin. The goal of the present work entails the isolation, purification and then the biochemical as well as pharmacological characterisation of the anticoagulant PLA$_2$ enzymes of crude RVV. We also elucidated their mechanism of action through which they affect the blood coagulation system of victims. Accordingly, the following objectives have been taken up under the present investigation.

1. To display/study the isoenzyme pattern of phospholipase A$_2$s (PLA$_2$s) from the venom of Indian Russell’s viper (Daboia russelli) of eastern India origin.

2. Isolation and/or purification of different (neutral, acidic and basic) anticoagulant PLA$_2$s from Indian Russell’s viper (Daboia russelli) venom.

3. Biochemical and pharmacological characterization of the isolated/purified phospholipase A$_2$ (PLA$_2$) enzymes.

4. Pharmacological screening of medicinal plants of North-East India to ascertain their inhibitory activity against anticoagulant phospholipase A$_2$ enzymes of Daboia russelli venom.