STUDIES ON ANTICOAGULANT PHOSPHOLIPASE A_2 (PLA_2) ISOENZYMES FROM INDIAN RUSSELL'S VIPER (*Daboia russelli*) VENOM

A thesis submitted

in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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December, 2012

Dedicated to my mother

Late Hemaprobha Saikia

"There will never be another like you..
& it's only because of your strong desire
Maa....."

ABSTRACT

ABSTRACT

Snakebite is a serious medical, social and economic problem in many parts of the world, especially in the tropical countries of South America, Africa and Southeast Asia. There are approximately 3000 species of snakes in the world known to date, out of which 410 species are poisonous to human. The number of deaths due to snake bite in India is estimated to be about 15,000-20,000 per year.

Russell's viper (*Daboia russelli*) is one of the most abundant species of venomous snakes in South-East Asia and is responsible for a large number of snakebite morbidity and mortality. An analysis of the snakebite cases in eastern India reveals that death toll from Russell's viper envenomation is the highest in Burdwan district of West Bengal, where 1301 deaths occur per year.

Apart from other components, venom of Russell's viper is known to contain different isoforms of phospholipase A_2 (EC 3.1.1.4). These PLA_2s may be classified as acidic, basic or, neutral on the basis of their elution profile from ion-exchange columns. They hydrolyze glycerophospholipids at the *sn*-2 position of the glycerol backbone releasing lysophospholipids and fatty acids. Venom PLA_2 enzymes in addition to digestion of prey exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, haemolytic, haemorrhagic and edema inducing activities. Each PLA_2 isoenzyme may exert different pathophysiological effects by a wide range of mechanisms in snakebite victims. Therefore, it remains a challenging task for the scientists to elucidate the structure-function relationships of this class of venom protein. Moreover, different PLA_2 enzymes from *Daboia russelli* venom have been purified and characterized, but till date enough information is not available on anticoagulant PLA_2 enzymes from *Daboia russelli* venom of eastern Indian origin.

Circulatory system is one of the major physiological systems targeted by anticoagulant PLA_2s of snake venom. Most of the death from Russell's viper envenomation in eastern India is attributed to prolonged blood coagulation time

of victims. Venom PLA_2s most likely affect blood coagulation through hydrolysis of and/or binding to procoagulant phospholipids. In addition to enzymatic mechanism of anticoagulant effect, strongly anticoagulant PLA_2 enzymes also prolong blood coagulation by mechanism(s) that are independent of phospholipid hydrolysis. However, the mechanism of anticoagulant action of RVV PLA_2 enzymes has still remained obscured. 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. Although during the last two decades significant progress has been made in understanding the structure-function relationship and the mechanism of some of these anticoagulants; however, there are still a large number of questions remained to be answered as more new anticoagulants from snake venom are being discovered.

In ancient Indian books, there are many plants that have been recommended for use in snakebite therapy. Many of such plants have been used traditionally by the tribal populations and rural people of North-East India since time immemorial, but without any scientific validation. Re-evaluation of these plants of north-east India for their anti-snake venom activity, if any, will be a more practical approach towards a better understanding of the medicinal properties of these plants and a projected application of these plants as antidote for snake venom to reduce the snakebite mortality rates.

For the ease of understanding, this thesis is divided into following eight chapters.

Chapter I- This chapter deals the introduction part. This covers a brief introduction about Russell's viper, Russell's viper envenomation cases in different countries of South-east Asia including India, Russell's viper venom phospholipase A_2 enzymes, their classification, structure, functions and evolution. This chapter also contains the aim and objectives of the present study.

Chapter II- This chapter reviews the published literatures on snake venom enzymes with a special emphasis on PLA_2 enzymes from Russell's viper venom and their mechanism(s) of action.

Chapter III- This chapter covers the materials and methods, which includes the materials used, techniques for sample preparation, isolation and purification processes of different PLA_2 isoenzymes from venom of Russell's viper (*Daboia russelli*), and procedures for biochemical, biophysical and pharmacological characterization of purified PLA_2 isoenzymes from Russell's viper venom.

Chapter IV - Chapter VII- These four chapters include results part.

Chapter IV- This chapter includes the first objectives i.e., characterization of isoenzyme pattern of phospholipase A_2 (PLA_2) enzymes of Indian Russell's viper (*Daboia russelli*) venom.

Chapter V- This chapter shows the results of isolation, purification, biochemical and pharmacological characterisation of an acidic anticoagulant phospholipase A_2 (PLA_2) enzyme (RVVA-PLA_2-I) from venom of Russell's viper (*Daboia russelli)* of eastern India origin.

Chapter VI- This chapter describes the results of isolation, purification, biochemical and pharmacological characterisation of a neutral anticoagulant phospholipase A_2 (PLA_2) enzyme (RVVN-PLA_2-I) from Russell's viper venom.

Chapter VII- In this chapter, the results of isolation, purification, biochemical and pharmacological characterisation of a basic anticoagulant phospholipase A_2 (PLA_2) enzyme (RVVB-PLA_2-I) from venom of Russell's viper (*Daboia russelli)* of eastern India origin has been described.

Chapter VIII- This chapter consists of discussion and conclusion part.

On the basis of elution profile of PLA_2 enzymes from ion-exchanges (cation-exchange and anion-exchange columns), presence of twelve (12) basic,

one (01) neutral and six (06) acidic PLA_2s (total 19 PLA_2s) in Russell's viper venom of eastern India origin was identified. To understand the biochemical properties and mechanism of action of different PLA_2 isoenzymes from the same venom, three anticoagulant PLA_2 enzymes possessing different overall charge; i.e., one acidic (RVVA-PLA_2-I), one neutral (RVVN-PLA_2-I) and one basic PLA_2 (RVVB-PLA_2-I) were isolated and purified to homogeneity by a combination of ion exchange, gel filtration and RP-HPLC. By SDS-PAGE and ESI/MS analyses, the molecular mass of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I was determined as of 58.0 kDa (homodimer), 12.8 kDa (monomer) and 6.7 kDa (monomer), respectively. The secondary structure of these PLA_2s was determined by CD spectra which showed strong α-helical contribution which is well consistent with secondary structures of other snake venom PLA_2 enzymes.

In this study, RVVA-PLA_2-I and RVVB-PLA_2-I showed preferential hydrolysis of PC over PS or PE. However, the neutral PLA_2, RVVN-PLA_2-I showed substrate preference towards PS followed by PC and PE. Biochemical characterization of these three purified PLA_2 enzymes reveals that RVVA-PLA_2-I is glycoprotein in nature while RVVN-PLA_2-I and RVVB-PLA_2-I are devoid of any carbohydrate moiety. RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I display their maximum catalytic activities at pH range of 8.0-8.5 and at a temperature 37 °C. The effect of chemical inhibitors, neutralization study with polyvalent antivenom and heat-inactivation of these three anticoagulant PLA_2s revealed that the anticoagulant activity of RVVA-PLA_2-I and RVVB-PLA_2-I is exerted through a dual mechanism (a combination of enzymatic and non-enzymatic way); however, the non-enzymatic mechanism of anticoagulant activity of RVVN-PLA_2-I is yet to understand. The membrane damaging activities of these three PLA_2 enzymes are dependent on their catalytic activity.

The enzymatic mechanism of anticoagulant effect involves hydrolysis of plasma phospholipids essential for blood coagulation process. In addition, RVVA-PLA_2-I and RVVB-PLA_2-I also affected the blood coagulation process non-enzymatically by inhibiting the coagulation factor Xa, even in absence of

phospholipids/Ca^{2+}. Therefore, they slowed down the process of thrombin formation from prothrombin by the action of factor Xa. On the other hand, the non-enzymatic mechanism of anticoagulant action of RVVN-PLA_2-I is yet to be discovered as it does not show any interaction with blood coagulation factor Xa, Va, prothrombin or thrombin. However, further structural characterization of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I is necessary for a better understanding of the correlation between catalytic activity and pharmacological properties. Our finding contradicts earlier reports suggesting higher anticoagulant potency is associated mostly with basic PLA_2 enzymes.

Most of the toxic effects of PLA_2s are due to the hydrolysis of membrane phospholipids and therefore, membrane damaging properties of all the three PLA_2s were studied. RVVA-PLA_2-I and RVVB-PLA_2-I preferentially hydrolyzed phospholipids of erythrocytes membranes compared to liver mitochondrial membranes while RVVN-PLA_2-I showed a preference for hydrolysis of phospholipids of mitochondrial membrane compared to that of erythrocytes membranes. It is very interesting to observe that RVVA-PLA_2-I and RVVB-PLA_2-I preferentially hydrolyzes the phospholipids of erythrocyte membrane compared to mitochondrial membrane even though the latter possesses much greater percentage of PC on its outer leaflet (40.9 %) than the outer leaflet of the former membrane (19 %). Therefore, our result suggests that existence of significantly greater number of these two PLA_2-sensitive domains in the erythrocyte membrane as compared to the mitochondrial membrane might have a relevance to the higher degree of phospholipid hydrolysis of the former membrane. The similar argument may be put forwarded to show that RVVN-PLA_2-I sensitive domains exist more in mitochondrial membranes than the erythrocytes membranes. Moreover, these RVV-PLA_2s could not hydrolyze HT-29 colon adenocarcinoma cell membrane phospholipids within 4 hours of treatment though PC is the most abundant phospholipid in the cell membrane of HT 29 cells. Amongst the tested membranes, the least hydrolysis of HT-29 cells can again be explained on the basis that it is not the overall quantity of PC in a membrane but either the availability of PC in a PLA_2-sensitive membrane and/or physicochemical properties of a membrane are the most important criteria in

order to elicit the RVV-PLA_2s-induced membrane damage. Although, the exact nature of the membrane domain(s) responsible for binding with these PLA_2 enzymes could not be identified; however, our study has provided enough evidences in support of membrane domain hypothesis. Further studies to identify the nature of these membrane domains are in progress.

The RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I did not show any lethality or behavioral changes in BALB/c mice after 48 h of i.p. injection at tested doses. Furthermore, immuno-diffusion test of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I against commercially available polyvanent antivenom against Russell's viper venom documented their very week immunogenic nature.

In this study, four plants which have been used traditionally by the local people of North-East India were screened for anti snake venom effect. Methanol extract of leaves of *A. indica*, *C. sinensis* and *X. Strumarium* and root of *A. marmelos* has showed significant inhibitory activity against crude RVV and also against the three purified phospholipase A_2 (PLA_2) enzymes of RVV. These four plant extracts may be considered as highly promising source for the development of novel anti-snake venom drugs in future. They may be used in combination with antivenom for a better management of snakebite cases.

DECLARATION

I hereby declare that the thesis entitled "**STUDIES ON ANTICOAGULANT PHOSPHOLIPASE A_2 (PLA_2) ISOENZYMES FROM INDIAN RUSSELL'S VIPER (*Daboia russelli*) VENOM**" is an authentic work carried out by me under the supervision of Dr. Ashis K. Mukherjee, Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Assam- 784028. No part of this work had been presented for any other degree or diploma earlier. Due to the unavailability of proper facility at Tezpur University, following experiments/sample analyses were carried at other institutes.

1. ESI/MS analysis of protein samples were done in SAIF, CDRI, Lucknow, India.

2. Circular diachroism (CD) spectroscopy and cell culture (cytotoxicity) study were carried out at Indian Institute of technology, Guwahati, Assam, India.

3. Animal experiments were done at Defence Research Laboratory, Tezpur, Assam, India.

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This is to certify that the thesis entitled **"STUDIES ON ANTICOAGULANT PHOSPHOLIPASE A_2 (PLA_2) ISOENZYMES FROM INDIAN RUSSELL'S VIPER (*Daboia russelli*) VENOM"** submitted to the School of Science and Technology, Tezpur University in partial fulfilment for the award of the degree of Doctor of Philosophy in Science is a record of bonafide research work carried out by Ms. Debashree Saikia, Research Scholar of Department of Molecular Biology and Biotechnology, Tezpur University, Assam, under my supervision and guidance at Department of Molecular Biology and Biotechnology, Tezpur University, Assam- 784028.

All help received by her from various sources have been duly acknowledged.

The results embodied in the thesis have not been submitted to any other University or, Institution for award of any degree or diploma.

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CERTIFICATE OF THE EXTERNAL EXAMINAR AND ODEC

This is to certify that the thesis entitled "**STUDIES ON ANTICOAGULANT PHOSPHOLIPASE A_2 (PLA_2) ISOENZYMES FROM INDIAN RUSSELL'S VIPER (*Daboia russelli*) VENOM**" submitted by Ms. DEBASHREE SAIKIA to Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us and found to be satisfactory.

The Oral Defence Evaluation Committee (ODEC) recommends for the award of the degree of Doctor of Philosophy.

Signature of:

Principal Supervisor

Date:

External examiner

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ACKNOWLEDGEMENT

First and foremost, praises and thanks to the ever-loving God, the Almighty, for His showers of blessings throughout my research to complete the PhD work successfully.

I am immensely pleased to place on record my profound and sincere gratitude and heartfelt thanks to my PhD supervisor Prof. Ashis K. Mukherjee for providing me a wonderful opportunity to work on snake venom PLA_2 enzymes, which are considered as one of the most toxic components of a venom. I love this area of research and enjoyed working with my supervisor. I will always remain indebted to him for introducing me to this field and his valuable guidance throughout my PhD work. I am deeply inspired by his dynamic vision, dedication towards work, sincerity and motivation. I learnt the basics of research from him. His unparallel knowledge over the subject helps me a lot to shape my career. It was a great privilege and honour to work and study under his able guidance.

I am thankful to Prof. M.K. Choudhuri, the honourable Vice-Chancellor of Tezpur University for providing me the opportunity to work in this astonishing university located in my hometown. I always feel fortunate for this opportunity; the institute provided a wonderful campus and environment for research work.

I sincerely acknowledge the financial support provided by Tezpur University (Institutional Fellowship), UGC, New Delhi (Research Fellowship) and CSIR, New Delhi (Senior Research Fellowship) to carry out this work.

I would like to thank the Head, Department of Molecular Biology and Biotechnology, Tezpur University; my Doctoral Committee members: Prof. A.K. Buragohain, Dr. T. Medhi and Dr. R. Doley and all the faculty members of my department for their valuable advice and constant encouragement to pursue the present work.

I also take this opportunity to thank Dr. P Chottoapadhyay from Defence Research Laboratory, Tezpur for his kind help in animal experiments and Prof. S.S. Ghosh from Indian Institute of Technology, Guwahati for providing me help

in cell culture study and CD analysis. I am thankful to Ms. Bashabi Gogoi, Department of English and Foreign Languages for editing the thesis.

I do thank Dr. K. Hazarika, Dr. N Bordoloi, Mr. S. Kakoty, Mr. P. Mudoi, Deepak da and Bania da for all the help they rendered me during my stay.

I am privileged to have lab-seniors and lab-mates like Kishore da, Pallabi ba, Sudhir, Jetendra, Rupamóni, Sourav, Vivek and Pranjal. I would also thank Diganta, Rahul and all the research scholars of our department and Biplob, Druba and Iftikar from Department of Chemical Sciences.

My heartfelt thanks go to my very close friends Clara and Santosh for their help and unconditional support throughout my PhD work. I can't forget the contribution of Ajay. He is like a friend, well wisher, care taker as well as path finder for me. I found him always near to me with all his love and support in times I needed the most. Thanks.

I am thankful to my family. First of all, I am obliged to my beloved mother. I dedicate this work to her. She is no more with us but she is always in my soul. This work is the fruit of the seed planted by my mom. Mom I always feel your unconditional love, care, support at each and every step of my life. I want you as my mom in each and every birth. I am obliged to my father for his love and patience. He takes lot of pain for my work. It is their unceasing prayers, blessing that I am able to complete my work. I am happy to have a younger brother. He helped me a lot during the initial stage of my work and endless support, love and care throughout the life. I am really fortunate to have my little sister (baby). She is always with me with all her love, patience and care. It is she, I find close to me at every step, both good and bad. I just want to say thanks for whatever you did for me and always be with me like my shadow.

Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.

Place: Tezpur
Date: 20.12.2012

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ABBREVIATIONS

Abbreviation	Full form
pBPB	p-bromophenacyl bromide
DTT	Dithiothreitol
FA	Fatty acid
FFA	Free fatty acid
GC	Gas chromatography
GC analysis	Gas chromatographic analysis
IAA	Iodoacetamide
i.p.	intraperitoneally
PC	Phosphatidylcholine
PLA_2	Phospholipase A_2
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PMSF	Phenylmethylsulfonyl fluoride
RVV	Russell's viper venom
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone
TPCK	Tosylphenylalanylchloromethyl ketone

CHAPTER I

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 *Colubroidae* 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*, *Macrovipera*, *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).

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

* 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 poeple 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 russelli*): Systematic classification and distinctive features

Fig 1.1: Indian Russell's viper on the move

[Source: 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].

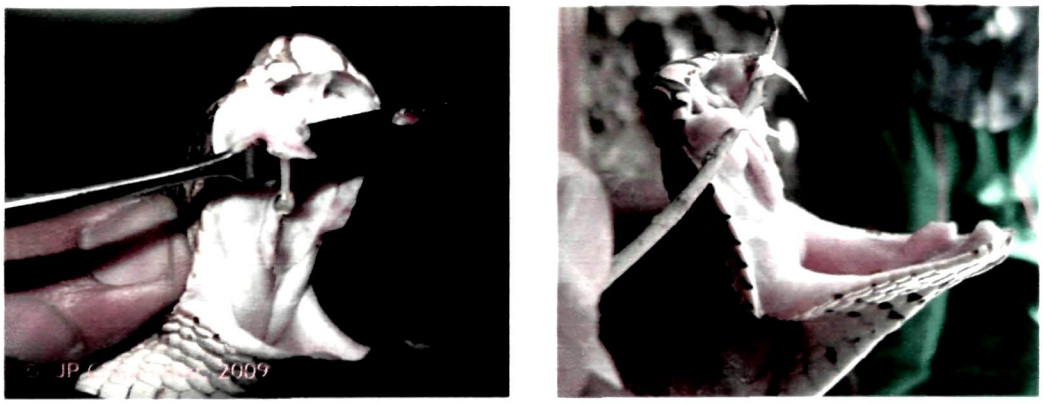

Fig 1.2: Fangs and venom secretion of Russell's viper

Sources:

a. http://en.wikipedia.org/wiki/File:Russel%27s_Viper_Fang_and_Venom.jpg

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 upto 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].

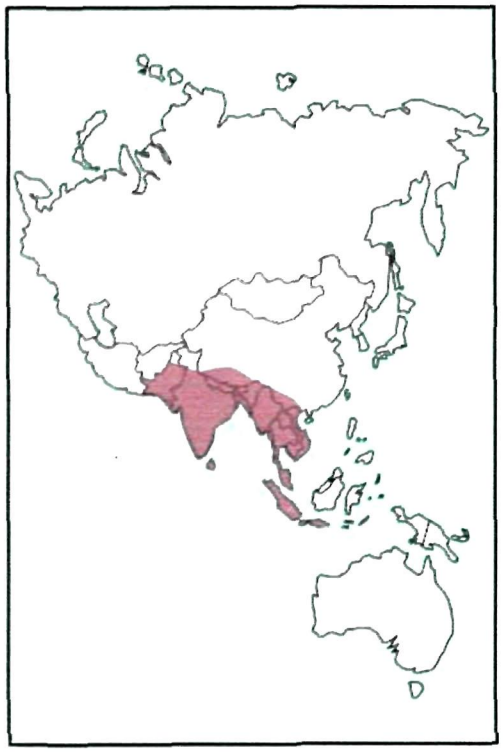

Fig 1.3: Russell's viper habitat in South-east Asia including India.

[Source: 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 Bangle) develops 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]).

Parameters	Percentage of patients exhibiting the symptoms
Edema	100
Intravascular hemolysis	86
Haematemesis	73
Haematuria	86
Haemoptysis	67
Renal failure	23
Hypotension	42
Convulsion	11

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_2 (PLA_2),

d) C-type lectins, and

e) Disintegrins.

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

Phospholipase A_2 (PLA_2s) 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_2 [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 A_2 (PLA_2) enzymes: Classification, structure and functions

Among the snake venom enzymes, Phospholipase A_2 (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 PLA_2 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 PLA_2 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 protiens 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_2s 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_2s 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_2s 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 PLA_2 enzymes are being discovered, and this superfamily has been expanding rapidly. PLA_2 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 PLA_2 enzymes fall under Group I and II [114].

Group I PLA_2 enzymes

This group of PLA_2 enzymes is found in the mammalian pancreas and in venoms from elapid and colubrid snakes. Cobra venom PLA_2 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 PLA_2 enzymes [91].

1. **Group IA**: Group I PLA_2 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 PLA_2 enzymes belong to this group [91].

2. **Group IB**: In mammalian PLA_2 enzymes, there is an additional five amino acid residue extension, which is called the pancreatic loop (residue 62-67). This group of PLA_2 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 *Muicrurus frontalis frontalis* [119]. These enzymes are found abundantly in the pancreatic juice, where they have an important digestive role towards dietary phospholipids [91].

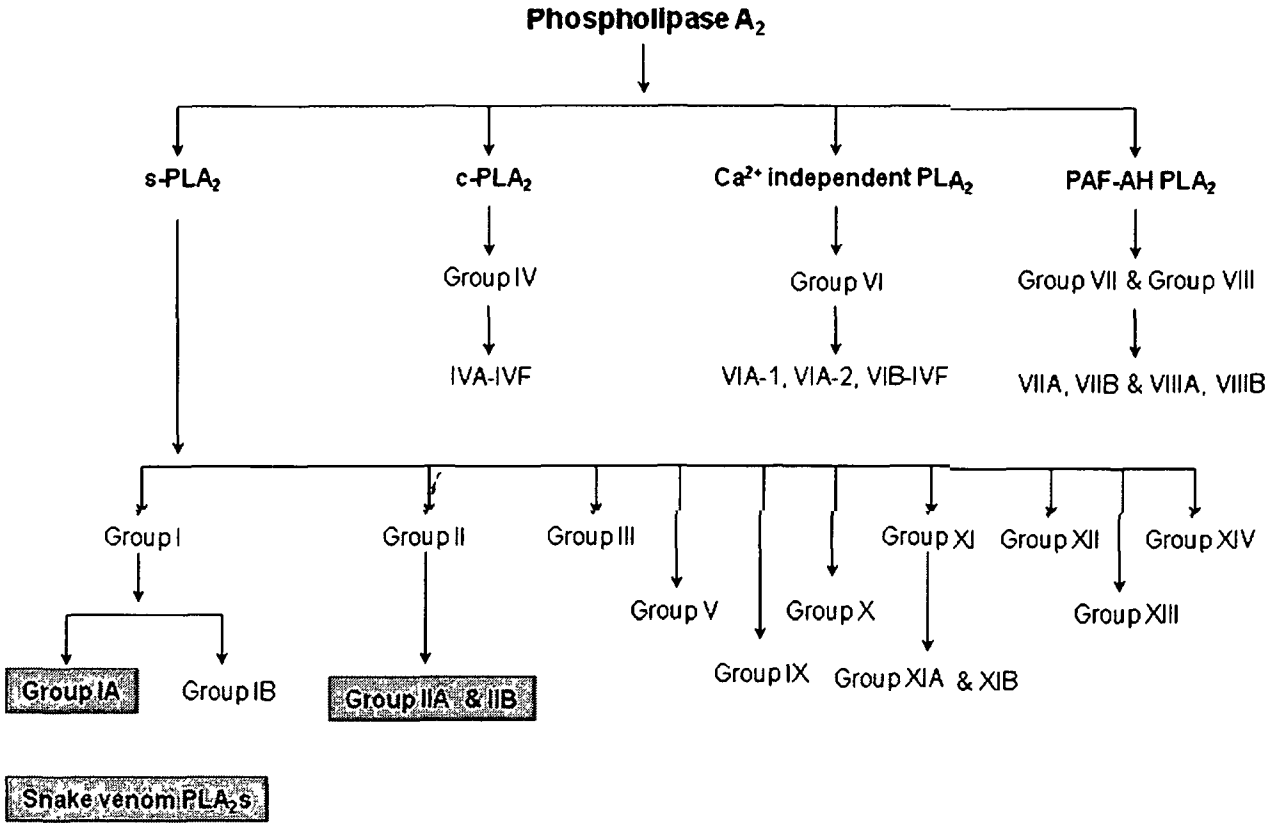

Fig1.4: Classification of Phospholipase A_2 enzymes (Schaloske and Dennis, 2006). s-PLA_2: secretory PLA_2; c-PLA_2: cytosolic PLA_2; PAF-AH PLA_2: platelet activating factor acetylhydrolase PLA_2.

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 133rd 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].

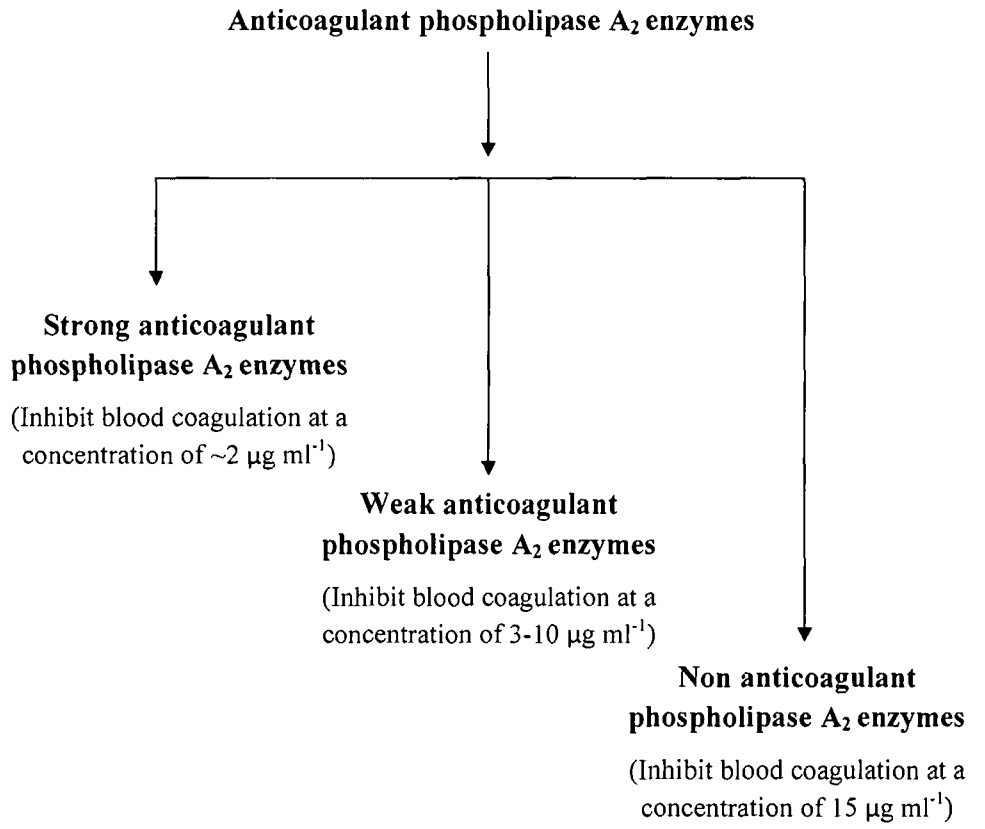

Fig 1.5: Classification of anticoagulant phospholipase A_2 enzymes of snake venom on the basis of their anticoagulant potency [121,122].

A) **Strongly anticagulant 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 blomhoffi* (basic), and *Crotalus durissus terrificus* (basic subunit of crotoxin) enzymes [122,123].

B) **Weakly anticoagulant PLA_2 enzymes,** showed anticoagulnat effetcts between 3.0 and 10.0 μg ml^{-1}. *Naja mossambica* (CM-II), *Naja nigricollis* (acidic), *A. halys blomhoffi* (neutral), *Enhydrina schistosa*

(myotoxin) and *Oxyuranus scutellatus* (taipoxin) belong to this group [122,123].

C) **Non anticoagulant enzymes**, there is another group of PLA_2 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 A_2 enzymes

1.4.2.2.1 Structure of snake venom PLA_2 enzymes

Snake venom PLA_2 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]. PLA_2 enzymes consist of three major α-helices and two antiparallel β-sheets, which are held together by disulfide bridges. The conserved structure in PLA_2 enzymes are the N-terminal helix, calcium binding loop, antiparallel helix, active site and β-wing. The N-terminal segment of PLA_2 enzymes has a highly conserved network of hydrogen bonds and stabilizes the adjacent β-sheet [124]. Some PLA_2 enzymes that retain the N-terminal propeptide (8-mer) lack the catalytic activity, similar to the precursor of pancreatic PLA_2 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 2^{nd}, 4^{th}, 5^{th} and 9^{th} residues. The side chain of the 4^{th} 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].

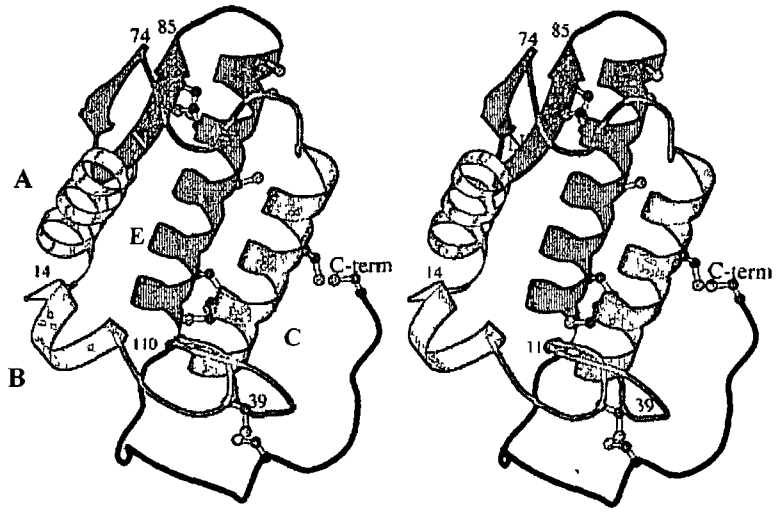

Fig 1.6: This figure shows the Stereo view of the main chain of the Russell's viper toxin (RVV-VD) which represents the secondary structure features of class I/II PLA_2s [127]. The structural data indicate that snake venom PLA_2 enzymes share strong structural similarity to mammalian pancreatic as well as secretory PLA_2 enzymes. They have a core of three major α-helices [A (residues 1-14), C (residues 39-54) and E (residues 92-108)] with two short α-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 β-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 PLA_2 enzymes

PLA_2 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 lysophopholipids [128]. They hydrolyze phospholipids in different phases, such as monomeric, miceller, or lipid bilayer phases.

Phospholipase A_2 preferentially catalyzes reactions at interfaces which is known as "interfacial catalysis". PLA_2 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 PLA_2 enzymes. According to that model, the enzyme (E) binds to the substrate (S) at the ineterface 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 $E^*\text{-}S^{\#}$ complex. Once activated, the PLA_2 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 PLA_2 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].

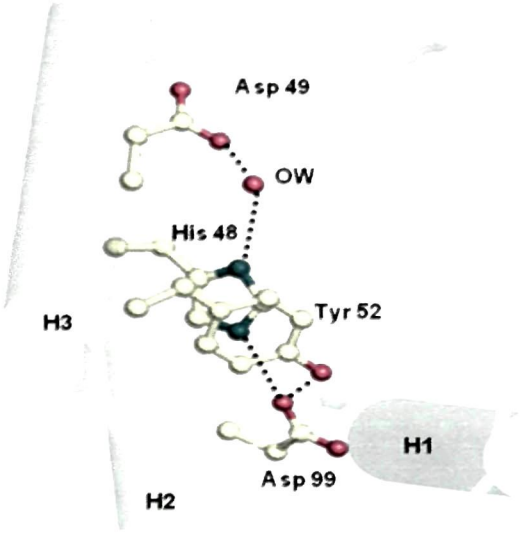

Fig 1.7: The catalytic network in PLA_2 enzyme is showing the above figure. OW indicates a water molecule oxygen atom which serves as the nucleophile. The dotted lines indicate hydrogen bonds. There are three main alpha helices: N-terminal helix H1 (residues 2-12), helix H2 (residues 40-45) and helix 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 PLA_2 enzyme hydrolyze the phospholipid involves the highly specific interaction between the active site histidine, the Ca^{2+} cofactor, conserved water, and the glycerophospholipid substrate. His48 is conserved in $sPLA_2$ enzymes, and its role in phospholipid hydrolysis has been confirmed by chemical modification using p-bromopheacyl bromide (p-BPB) [131,132]. During catalysis, His48, assisted by Asp99, polarize the bound water molecule, which then attacks the *sn*-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].

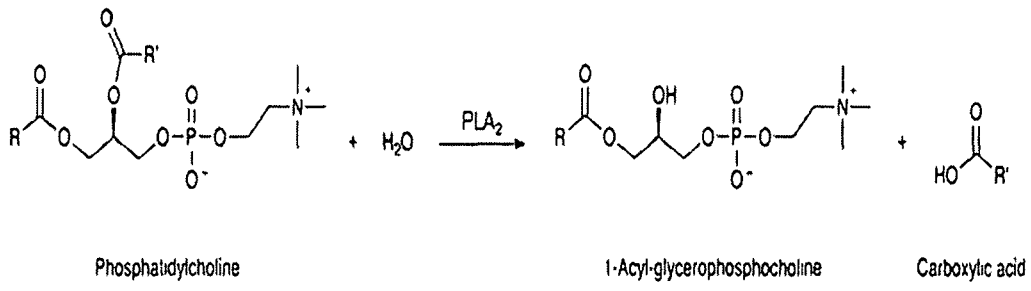

Fig 1.8: An example of mechanism of action of phospholipase A_2 enzymes

1.4.2.4 Mechanism of action of snake venom anticoagulant PLA_2s on blood coagulation

Circulatory system is one of the key physiological systems targeted by anticoagulant PLA_2s 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_2s could be the primary mechanism to account for their anticoagulant effect [93]. In most cases, PLA_2s 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_2s was shown to be dependent on the presence of phospholipids [136], whereas some other PLA_2s can inhibit prothrombinase complex independently from the presence of phospholipids. Furthermore, some of the PLA_2s can bind to blood coagulation factors and thus can inhibit the factor from its normal role in clotting activity [108]. Generally, strong anticoagulant PLA_2s from snake venom interact with blood coagulation factor X or, Xa and thereby, inhibiting the formation of prothrombinae complex which is the most important step of the blood coagulation system (Fig 1.9).

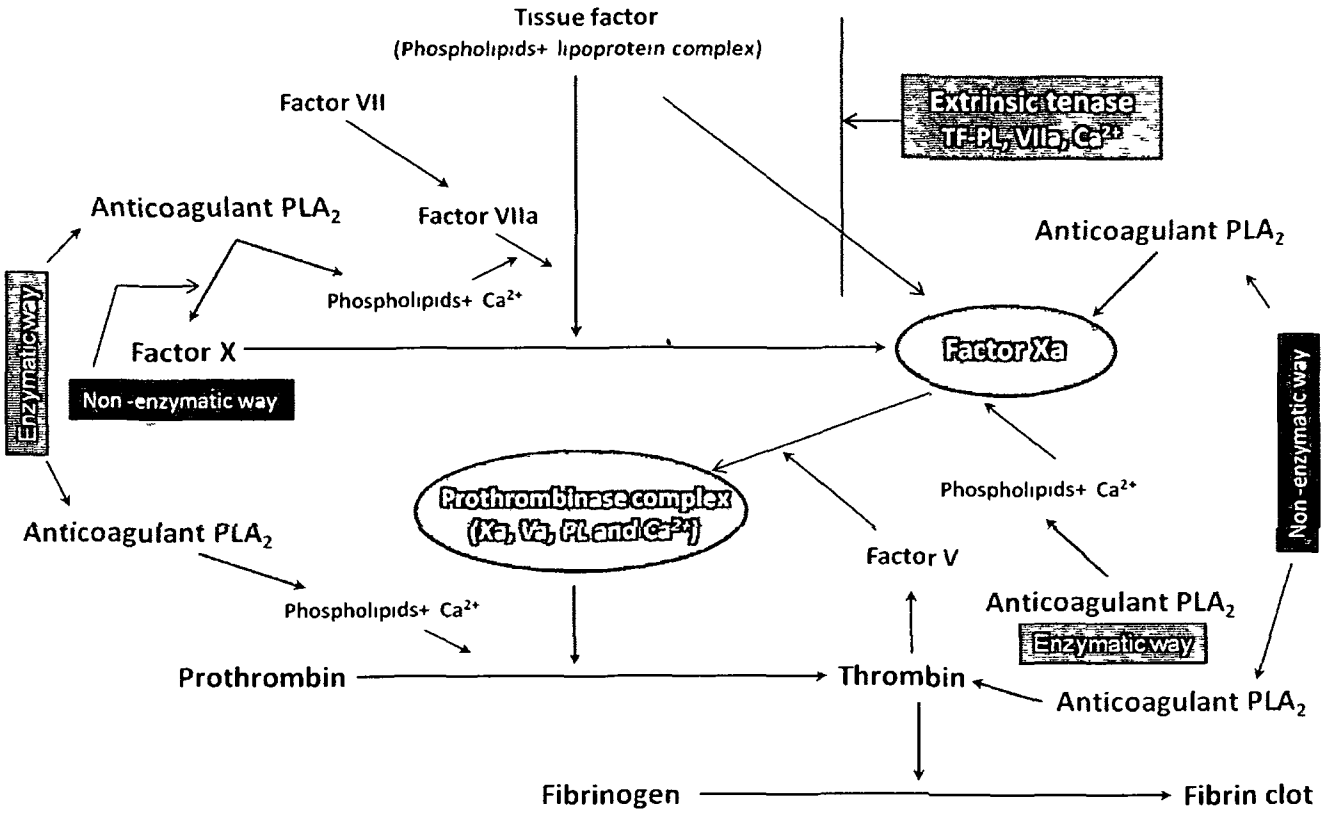

Fig 1.9: Effect of snake venom anticoagulant PLA_2 enzymes in different stages of the extrinsic pathway of blood coagulation.

1.4.2.5 Pharmacological properties of snake venom PLA_2 enzymes

Snake venom PLA_2 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, hemoglobulinurea-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 PLA_2 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 PLA_2 enzymes are also known to inhibit blood coagulation. These anticoagulant PLA_2 enzymes are classified into strong, weak and non anticoagulant enzymes, depending on the dose required to inhibit the blood coagulation [120,122]. Strong anticoagulant PLA_2 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 PLA_2 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 PLA_2 from snake venom have been reported. Basic PLA_2s are reported to be more toxic and enzymatically less potent but the acidic PLA_2s are less toxic and enzymatically active [144], therefore the lethality of snake venom PLA_2 cannot be correlated with the catalytic property [145].

Although a variety of pharmacological effects are induced by PLA_2 enzymes, not all the effects are exhibited by all PLA_2 enzymes. Each enzyme exhibits a special effect. For example, β-bungarotoxin, a PLA_2 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 PLA_2 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 PLA_2 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 PLA_2 enzymes. In some other cases, a single PLA_2 enzyme can exhibit several pharmacological effects. For example, a basic PLA_2 from *Naja nigricollis* venom exhibit anticoagulant effects along with cardiotoxicity, convulsant activity, hemolytic activity and platelet aggregation inhibition [93]. In these cases, their specific effect probably depends on the relative affinities between the PLA_2 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_2 enzymes determine the affinity between the PLA_2 and target proteins. The identification of pharmacological sites helps in

(i) Understanding the structure-function relationships of PLA_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_2 to a specific tissue or cell, they act as 'protein postal addresses' which help in 'delivering' the specific PLA_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_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_2 enzymes and identification of more pharmacological sites [91,92].

1.8 Purification of PLA_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_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 PLA_2 enzymes from snake venom, two factors should be considered:

1. PLA_2 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 PLA_2 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. PLA_2 enzymes interact with each other or with other proteins to form aggregates that are due to protein-protein interaction. In addition, PLA_2 enzymes interact with other venom toxins such as cardiotoxins [166]. These interactions contribute significantly to the enzymatic and pharmacological activity of PLA_2 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 PLA_2 genes

Phylogenetic analysis of mammalian pancreatic and venom PLA_2 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 PLA_2 genes evolved separately after species diversification [168,169]. Typically genes encoding Group I PLA_2 enzymes are about 4 kb, which follows a typical structural organization comprised of four exons interrupted by three introns (which resembles the human pancreatic PLA_2

gene). However, the size of the intron 3 in the venom PLA_2 gene is smaller than the pancreatic counterpart [167,170]. The smaller size of intron 3 is attributed to adaptive evolution of snake venom PLA_2 enzymes, and intron 3 and 4 might code for addition of pharmacological properties [167]. The characteristic feature of the mammalian PLA_2 enzyme is the presence of pancreatic loop, which is encoded by exon 3. In some snake venom PLA_2 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 PLA_2 enzymes. The loss of this loop has been interpreted as providing an adaptive advantage for the development of toxic properties among the venom PLA_2 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 PLA_2 enzymes [170]. In snake venom PLA_2 enzymes, the loss of the pancreatic loop has followed a Darwinian type of accelerated evolution, while the mammalian PLA_2 enzyme has undergone natural evolution, retaining the pancreatic loop and showing a reduced rate of mutation in gene [91].

Group II PLA_2 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 PLA_2 gene [173].

Snake venoms contain a large number of PLA_2 isoenzymes that exhibit different physiological functions, although they share similarities in their amino acid sequence and three-dimensional structure [91,93]. Venom PLA_2 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_2s 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_2s (PLA_2s) 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_2s 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.

CHAPTER II

2 REVIEW OF LITERATURE

2.1 Snake venom enzymes

Snakes use their venoms as offensive weapons in incapacitating and immobilizing their prey (the primary function), as defensive tools against their predators (the secondary function) and to aid in digestion [130]. Thus, snake venom is one of the most incredible and unique adaptations of animal evolution, which has evolved to have a strong balance in their toxic and digestive components [93]. Toxins have evolved to specifically target various critical points in the physiological systems of prey animals, mainly circulatory and neuromuscular system, blood coagulation as well as homeostasis of victim [108,177,178].

Biochemically, snake venoms are complex mixtures of pharmacologically active proteins and polypeptides. All of them in concert help in immobilizing the prey. A large number of protein toxins have been purified and characterized from snake venoms [78] and snake venoms typically contain from 30 to over 100 protein toxins [130]. Some of these proteins exhibit enzymatic activities, whereas several others are non-enzymatic proteins and polypeptides. Based on their structures, they can be grouped into a small number of toxin super-families. The members in a single family show remarkable similarities in their primary, secondary and tertiary structures but they often show distinct pharmacological effects [93,94,100,130,179]. More than 20 enzymes have been detected in snake venoms, and 12 are found in all venoms [63]. Although no venomous snake has all of these toxins [63,180], most snakes employ between six to twelve of these enzymes in their venom (www.chm.bris.ac.uk.). Each of these enzymes has its own special function. Some aid in the digestive process, while others specialize in paralysing the prey.

Snake venoms are rich sources of water soluble enzymes [181]. Many of these enzymes are hydrolases and possess a digestive role, for instance proteinases, exo- and endopeptidases, phosphodiesterases, and phospholipases. Various toxins that have been identified in snake venom are: proteolytic enzymes; phosphomonoesterase; arginine ester hydrolase; phosphodiesterase; Thrombin-like enzyme; acetylcholinesterase; collagenase; RNase; hyaluronidase; DNase; phospholipase A_2 (A); 5'-Nucleotidase; phospholipase B; L-Amino acid oxidase; phospholipase C; lactate dehydrogenase; adenosine triphosphatase (www.chm.bris.ac.uk.). However, higher catalytic efficiency, thermal stability and resistance to proteolysis make these enzymes attractive models for biochemists, enzymologists and structural biologists [130].

2.2 Russell's viper venom enzymes

Russell's viper venom is characterized by the presence of RVV-V [182-185], RVVX [186-188], VRH-1 proteases [189], trypsin inhibitors [190] and PLA_2s. Neurotoxic VRVPL-V [102], VRV-PL-VIIIa (inducer of lung hemorrhage [191]) and VRV-PL-VI (inducer of hemorrhage in the pituitary and thyroid glands [192]) have also been reported. In 1990, Jayanthi and Gowda [193] demonstrated a synergistic interaction between protease, RVVX and a trypsin inhibitor leading to increased toxicity of RVVX.

2.3 Phospholipase A_2 (PLA_2): A major enzyme of Russell's viper venom

Phospholipase A_2 is known to be one of the major components of snake venom [194,195] including Russell's viper (*Daboia russelli*) venom. It catalyses the hydrolysis of the sn-2 ester bond in glycerophospholipids in a calcium-dependent manner and releases non-esterified fatty acids and lysophospholipids [114]. *In vivo*, the sn-2 position of phospholipids frequently contains polyunsaturated fatty acids,

and when released, these can be metabolized to form various eicosanoids and related bioactivelipid mediators [196]. The remaining lysophospholipid can also have important roles in biological processes [197]. In addition to digestion of prey, PLA_2 seems to be involved in many of the pharmacological effects induced by snake envenomation [52,198-200]. Phospholipase A_2s of different snake venoms differ widely in their spectrum of toxic actions and some of them have very intrinsic toxicity but may modulate the activity of other toxins in the venom [108].

The presence of PLA_2 isoforms in *Daboia russellii* snake venom has been demonstrated by different investigators [102,191]. All the hydrolytic enzymes including PLA_2 in *D russelii* venom exhibits diverse biological activities [160,201]. They are known to contribute to the clinical manifestations encountered in envenomed victims [202]. The Russell's viper PLA_2s are known to cause degeneration of muscle fibers, hemorrhage in lungs [190], necrosis in kidney and liver, and hemorrhage in pituitary and thyroid glands [191]. Although different phospholipase A_2 enzymes from *Daboia russelli* venom have been purified and characterized, yet there are many more anticoagulant proteins (PLA_2s) have to be isolated, identified and characterized from *Daboia russelli* venom of Indian origin.

2.3.1. Membrane damaging property of the snake venom PLA_2s

Most of the toxic effects of snake venom PLA_2 are dependent on hydrolysis of cellular or subcellular membrane phospholipids, and/or generation of phospholipid hydrolysis breakdown products which are themselves lytic and cause considerable membrane damage [94,97,203]. Kinetic studies of $sPLA_2$ in the scooting mode establish that these enzymes bind to the intact membrane surface as a prelude to loading of the active site with a single phospholipid molecule, more specifically they bind with the phosphatidylcholine (PC) present in the outer leaflet of the membrane for the lipolysis reaction [204]. It is becoming apparent that

different isoforms of venom PLA_2 or secretary PLA_2 can display dramatically different affinities for biomembranes, composed of different phospholipid polar head groups and fatty acyl chains resulting in their differential membrane damaging activity [97,140,205]. This specificity of phospholipases has been used extensively to explore the physical structure of phospholipids in biological membranes [206].

The primary target of PLA_2 is cell membrane dysfunction through hydrolysis of membrane phospholipids [160] including hydrolysis of erythrocyte membrane [203]. Management of viper bite patients with massive intravascular hemolysis is a severe problem [207]. Therefore, endeavour must be taken for the primary target of PLA_2 is cell membrane dysfunction through hydrolysis of membrane phospholipids directed towards stabilizing the erythrocyte membrane against the hydrolytic action of these venoms [207]. Like disruption of erythrocyte membrane, venom PLA_2 can damage lysosomes [208] and encapsulated lysosomal enzymes may be released following venom toxicity, especially after *Vipera russelli* envenomation [209]. Lysosomes are a structurally heterogeneous group of organelles containing many hydrolytic enzymes concerned with the degradation of metabolites [210]. Several of these enzymes, which are released into the surrounding environment following the physical or chemical disruption of lysosomes, may provoke inflammation and tissue injury [211].

Myonecrosis may be due to an indirect action as a consequence of vessel degeneration and ischemia caused by a direct effect of myotoxic phospholipase A_2s (PLA_2s) homologues upon plasma membranes of muscle cells [200,212]. Recently, some authors have shown the bactericidal effect induced by PLA_2s isolated from viper (Bothrops) snake venoms [95,212,213]. Samy et al., 2010 [212] reported a PLA_2 enzyme named EcTx-I that could be able to damage severely the multi-drug resistant *B. pseudomallei* (KHW) bacterial membrane at a low dose (15 mg ml^{-1}).

Another bacteria *E. aerogenes* also lost membrane integrity as well as accumulated debris on the cell wall surface after treatment by EcTx-I (30 mg ml^{-1}).

It is exemplary to mention that although our understanding of the mode of hydrolysis of different membrane phospholipids by $sPLA_2$ is increasing, there is still a dearth of report on membrane phospholipids hydrolyzing property of PLA_2s from *Daboia russelli* venom and such studies will advance our understanding of the mechanism of membrane damage and subsequent toxicity by PLA_2 from Russell's viper venom.

2.3.2 Anticoagulant property of snake venom PLA_2s

Many scientist report the anticoagulant property of snake venom PLA_2 enzymes that could inhibit blood coagulation [120-122,163]. A simple 'dissection approach' was used to identify the specific stage of the coagulation cascade that is inhibited by anticoagulant PLA_2 enzymes [136,214]. In general, the effects of an anticoagulant on three commonly used clotting time assays viz., prothrombin time, Stypven (Russell viper venom) time and thrombin time, are studied to identify the stage in the extrinsic coagulation cascade. The anticoagulant will prolong clotting times when the cascade is initiated 'upstream' of the inhibited step, whereas it will not affect the clotting times when the cascade is initiated 'downstream' of the inhibited step. Since the above clotting assays specifically initiate the coagulation cascade at three different stages, it is easier to pinpoint the specific step(s) that is (are) inhibited by the anticoagulant [123,136,214].

In the prothrombin time assay, clotting of the plasma is initiated at the tenase complex upon the addition of Ca^{2+} and thromboplastin, which contains tissue factor and phospholipids (TF-PL). In the Stypven time assay, activation of factor X and V by protease in the Russell's viper venom initiate the formation of the

prothrombinase complex on the phospholipid surface (PL) upon addition of Ca^{2+}. On the other hand, in thrombin time assay, thrombin is directly added to the plasma, and facilitates clotting by hydrolysis of fibrinogen to give a fibrin clot.

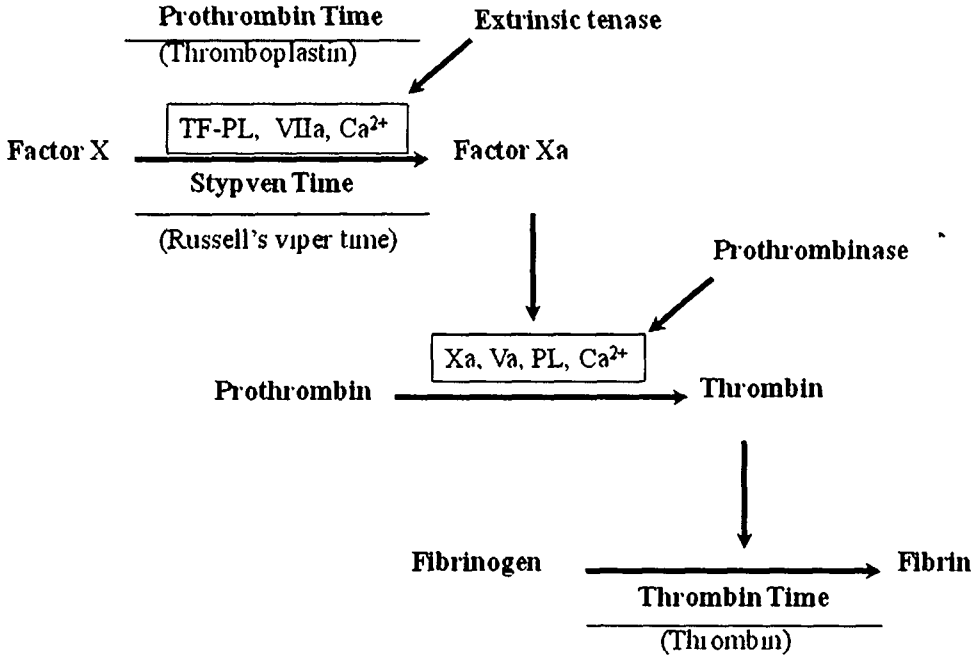

Fig 2.1: Schematic representation showing the selective activation of the extrinsic coagulation pathway by prothrombin, Stypven and the thrombin time clotting assays [123].

PLA_2 enzymes exert their anticoagulant activity by affecting any one of these clotting time. The weakly anticoagulant PLA_2 enzymes prolong the prothrombin time but not the Stypven time and thrombin time clotting assays. In contrast, strong anticoagulant PLA_2s prolong prothrombin time and Stypven time, but not the thrombin time clotting assays [123,136].

In *in vivo* condition, Russell's viper venom is coagulant in nature because of the predominance of the coagulant proteins [215]; though crude RVV contains both coagulant as well as anticoagulant proteins. Nonetheless, anticoagulant PLA_2 enzymes also play an important role in the pathogenesis of the victims [216].

2.4 Enzymatic activity and pharmacological properties of PLA_2s

PLA_2 enzymes, because of their inherent phospholipid-hydrolyzing activity, can cause membrane damage and hence a change in the environment of some membrane-bound proteins, most likely that of the target protein [93]. PLA_2 enzymes induce their pharmacological effects through mechanisms that are either dependent on or independent of their enzymatic activity. In the mechanisms that are dependent on enzymatic activity, either the hydrolysis of intact phospholipids or the released products such as lysophospholipids and fatty acids can cause the pharmacological effect [217]. The inherent enzymatic activity can cause membrane damage and hence a change in the environment of membrane-bound proteins, particularly that of the target protein. These protein-protein mechanisms are sometimes dependent on, and other times independent of their enzymatic activity, playing important roles in determining the specific function of $sPLA_2$s [218-220]. *Viperidae* snake venoms contain several toxic group IIA PLA_2s ($SVPLA_2$), which may act as presynaptic neurotoxins [221] and may interfere with blood coagulation by possessing strong anticoagulant properties [143,220,222].

In the mechanisms that are independent of enzymatic activity, binding to the target protein can cause the pharmacological effect by acting as an agonist or an antagonist, or by interfering in the interaction of the target protein with its physiologic ligands [217]. However, even in this case, the enzymes would hydrolyze the phospholipids in the neighborhood of their target proteins, but this hydrolysis is inconsequential to the observed pharmacological effect [141,157,223].

Some pharmacological effects are due to a combination of both enzymatic and nonenzymatic mechanisms [142]. Therefore, it is critical to determine the role of enzymatic activity in inducing the pharmacological effects.

2.5 Enzymatic activity and anticoagulant activity of PLA_2s

There are lots of controversies regarding the association/dissociation of enzymatic activity of PLA_2s with its anticoagulant potency [123]. Some experiments suggested that catalytic activity is essential for anticoagulant effects. For example, in all snake venom PLA_2 enzymes, His48 is conserved and plays a crucial role in the hydrolysis of phospholipids. Modification of His48 by alkylation leads to the complete loss of enzymatic activity. PLA_2 enzymes from *N nigricollis*, *H hemachatus* and *N atra* venoms upon alkylation using p-bromophenacyl bromide lost their enzymatic activity with concomitant dramatic loss of anticoagulant effects [122,224,225]. In sharp contrast, some other experiments suggested that catalytic activity is not essential for exerting the anticoagulant effects and there is some other non-enzymatic mechanism for its anticoagulant effect. For example, modification of Lys residues led to loss of anticoagulant effects with very little loss of enzymatic activity [224,225]. In contrast, modification of carboxylate groups led to loss of enzymatic activity with very little loss of anticoagulant effects [226]. These results indicate that there is no correlation between enzymatic activity and anticoagulant activity.

The contradictory results and controversial conclusions with regard to the role of enzymatic activity and the mechanism of anticoagulant effect of PLA_2 enzymes were due to the following factors.

1. **Difference in mechanism of anticoagulant effects**. All anticoagulant PLA_2 enzymes may not have the same mechanism. Thus, it may not be

possible to extrapolate the results from one enzyme to all others without sufficient understanding.

2. **Different coagulation times**. Anticoagulant effects were determined using different coagulation times in whole plasma. Although the coagulation time assays start blood clotting at different stages, the difference in the number of stages each coagulation time assays encompass was not taken into consideration.

3. **Phospholipid requirements for coagulation complexes**. Various coagulation complexes are formed through interaction between different protein factors. During interpretation of the results, no consideration was given to difference in the phospholipid requirements of different coagulation complexes.

2.6 Purification of phospholipase A_2 (PLA_2s) enzymes from Indian Russell's viper (*Daboia russelli*) venom

Daboia russelli PLA_2 isoenzymes are acidic, neutral or basic in nature [50]. Basic PLA_2 are more toxic as compared to acidic and neutral and contributes significantly to the toxicity of the whole venom [50,83]. The higher toxicity of the basic PLA_2 may be attributed to the presence of positive charge, which has been postulated to be responsible for their penetrability in plasma membrane [122]. These isoenzymes of RVV display a wide range of pharmacological effects and often cause problems in purification. A combination of different chromatographic techniques viz ion exchange chromatography, gel filtration chromatography, HPLC etc are used commonly for their purification. Till date many PLA_2 enzymes are purified and characterized from Indian Russell's viper venom by using different

chromatographic techniques and characterized by different scientists. Few of them are listed below-

Jayanthi et al. in the year 1989 [227] isolated and purified a neurotoxic phospholipase A_2 from *Vipera russelli* venom and named as VRV PL-V. They purified this PLA_2 in a single step by CM-Sephadex C-25 column chromatography. VRV PL-V is a basic PLA_2 with a mol. wt of approximately 10,000 Da. The lethal potency of VRV PL-V was greater than that of the crude *Vipera russelli* venom. That purified PLA_2 had anticoagulant property and induced edema in the foot pad of mouse.

One major basic phospholipase A_2 (VRV PL-VIIIa) was purified from *Vipera russelli* venom by Kasturi and Gowda, 1989 [102] which constitutes 24 % of the whole venom. Purification was done by CM-Sephadex C-25 column chromatography followed by gel filtration on Sephadex G-50 that had a molecular weight of 11,800 Da. In addition, this enzyme contributes 45 % of the total PLA_2 activity of the venom, but it is least toxic compared to other purified basic PLA_2 enzymes prepared from *V. russelli* venom. It showed neurotoxic symptoms and damaged vital organs such as lung, liver and kidney. They reported that VRV PL-VIIIa induces myonecrosis when injected i.m. into the thigh muscle of mice and edema when injected into the foot pads.

Gowda et al., 1994 [104] reported about the most basic phospholipase A_2 (PLA_2), VRV-PL-VIIIa which was purified from (Sri Lankan) *Vipera russelli* venom. It was a major component of the *Vipera russelli* venom, contributing over 40 % to the whole venom PLA_2 activity. The purification of VRV-PL-VIIIa was done by electrophoresis and by reverse phase high-pressure liquid-chromatography (RP-HPLC). That PLA_2 was a single polypeptide with an apparent mol. wt of

13,000 Da. VRV-PL-VIIIa induced multiple toxic effects viz. neurotoxicity, myotoxicity, edema and hemorrhage [191].

Prasad et al, 1996 [105] found major variation of phospholipases A_2 in the venom samples of India viz. southern regional venom is rich in basic, toxic PLA_2 while this activity showed a dramatic decrease as one moves towards west, north and eastern regions of India.

Tsai et al., 1996 [53] performed a study for comparing the different PLA_2s present in four commercially available venom of Russell's viper subspecies by HPLC fractionation and partial sequence analysis.

A potent toxin with phospholipase A_2 (PLA_2) and hemolytic activity *in vitro* was purified and characterized by Chakraborty et al, 2000 [106] from the crude venom of Russell's viper of eastern India. The purified protein (RVV-PFIIc') was the most toxic PLA_2 so far reported from the Indian subcontinent and also possessed anticoagulant activity as it enhanced the prothrombin induced plasma clotting time *in vitro*.

Tsai et al., 2007 [107] studied various isoforms of venom phospholipases A_2 (PLAs) from two *Daboia* species at their geographic boundary (Myanmar and eastern India). They fractionated the crude venoms through Superdex G-75 column on a FPLC system. Then they lyophilized PLA containing fractions and further purified by reversed-phase HPLC. Four venom PLAs were purified and identified from each of DrK (eastern India) and DsM (northern Myanmar).

Moreover, Maity et al., 2007 [228] reported a basic toxin (low molecular weight multifunctional cytotoxic PLA_2) from Russell's viper venom of eastern India. This 7.2 kDa PLA_2 (RVV-7) had been purified to homogeneity after partial unfolding by 4M urea followed by filtration through Centricon-30 membrane. They

reported that RVV-7 possess different functional sites inspite of its low molecular weight. Coexistence of different functional sites within a small protein like RVV-7 might accommodate complex clinical manifestations of Russell's viper ophidism. Earlier also many myotoxins or cytotoxins from viper venoms were reported to be phospholipase A_2 [98,229].

2.7 Role of medicinal plants in snakebite treatment

Till date, the most effective and accepted therapy for snakebite patients is immediate administration of specific or polyvalent antivenom following envenomation; however, this therapy carries an associated risk of anaphylaxis and serum reactions [230,231]. Monospecific or polyvalent antivenom raised against individual Russell's viper venoms have been noted to be highly effective for restoring blood coagulation functions and stopping spontaneous hemorrhage [57,231]. These antivenoms rapidly neutralize circulating venom and protect animals and humans from the lethal effects of various components of Russell's viper venom. But, these antivenoms had not proved satisfactory in restoring patient's renal function [38]. Further, due to geographical variation in venom composition of snakes, antivenom raised against the venom of a snake from a particular geographical origin may not be able to neutralize or prevent local effects of envenomation by snakes from other geographical locations [83,232,233]. Therefore, in addition to administration of antivenom, there should be alternative therapy for the snakebite patients.

Plants as a source of many biologically active products are nature's best gift to human beings and are of great interest to the pharmaceutical industries. The study of how people of different cultures use plants in particular ways has led to the discovery of important 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.

Based on field interviews, 101 species of plants used against snakebite were identified [234]. In ancient Indian books, there are various plants recommended for use in snakebite therapy. Around 50 such plants have been indexed [235] and many others (not included there) have also been popularly used against snakebite by villagers, snake charmers and Ojhas throughout India including many tribes of North East India. Sometimes unenthusiastically, pharmacological tests on alkaloid extracted from well known and reputed medicinal plants show distinctly negative results [236]. Nonetheless, in recent years much attention has been paid to pharmacological screening of the medicinal plants to treat snakebite. For example,

Alam and Gomes, in the year 2003 [237] reported the snake venom neutralization property of two Indian medicinal plants *Vitex negundo* and *Emblica officinalis* by using their root extracts.

Likewise, Chatterjee et al, 2004 [238] showed the anti-snake venom activity of ethanolic seed extract of *Strychnos nux vomica linn.* The neutralization *of Daboia russellii* and *Naja kaouthia* venom by lupeol acetate isolated from the root extract of Indian sarsaparilla *Hemidesmus indicus* was also reported by Chatterjee et al., 2006 [239].

Girish et al in the year 2004 [240] published the neutralization property of *Mimosa pudica* root extract against hyaluronidase and protease activities from Indian snake venoms.

Shirwaikar et al, 2004 [241] gave the report of the neutralization potential of *viper russelli russelli* (Russell's viper) venom by ethanol leaf extract of *Acalypha indica.*

Furthermore, Ushanandini et al, 2006 [242] reported about the inhibition property of *Tamarindus indica* (leguminosae) seed extract on the pharmacological as well as the enzymatic effects (viz. PLA_2, protease, hyaluronidase, L-amino acid oxidase and 5'-nucleotidase enzyme activities) induced by *V. russelli* venom.

Previous study from our laboratory has demonstrated that aqueous root extract of *Mimosa pudica*, a plant used by the local people of North-east India to treat snakebite patients, was effective to neutralize the lethality and toxic enzymes of *Naja kaouthia* venom [243]. This has prompted us to screen the anti-toxic activity of some more medicinal plants which are popularly used by the snake charmers of the region as well as by the local healers (traditionally known as Bejs) for treating poisonous snakebites. During this study, we have isolated "AIPLAI" from the leaves extract *of A. indica*, which was proved to be a potential candidate for the development of anti snake venom drugs [244].

CHAPTER III

3 MATERIALS AND METHODS

3.1 Materials

Crude Russell's viper (*Daboia russelli*) venom (RVV) in crystallized form was purchased from Calcutta Snake Park, Kolkata. Dried venom samples were kept in a desiccator at room temperature. CM Sephadex C-50, DEAE Sephadex A-50, Sephadex G-50 (fine grade) were obtained from Pharmacia Fine Chemicals (Sweden). Desalting columns (desalting column pack DS05) were purchased from Bangalore Genei, Bangalore, India. TPCK, TLCK, PMSF, p-BPB, EDTA, DTT, IAA, PC, PE and PS were obtained from Sigma-Aldrich, St. Louis, USA. Horse radish peroxidase, Tetramethyl benzidine/H_2O_2, molecular weight marker, range 10-250 kDa (Lot No 00077637) were obtained from Bangalore Genei, Bangalore, India. Blood coagulation factors Xa and Va were obtained from Merck Calbiochem, India. Thrombin and prothrombin were obtained from Himedia, India and Merck, India respectively. The human colon adenocarcinoma cell (HT-29) was procured from National Centre for Cell Sciences (NCCS), Pune, India. The "*In Vitro* Toxicology Assay Kit (XTT based)" was purchased from Sigma-Aldrich, India. The kits for estimation of total protein, triglycerides, total cholesterol, urea, uric acid, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) were procured from Coral Clinical Systems, Goa, India. The kits for the measurement of serum levels of glucose, alkaline phosphatise (ALP), and creatine phosphokinase (CPK-MB) were obtained from Erba Diagnostics (Mumbai, India) whereas kit for estimation of serum lactate dehydrogenase (LDH) was obtained from Fisher scientific (Mumbai, India). Thromboplastin reagent for prothrombin time determination (Liquiplastin®) was obtained from Tulip diagnostics, India. Fresh goat blood and fresh tissues (liver, heart and lungs) were collected from known local slaughter houses. Polyvalent antivenom (B. No 2505003) was purchased from Bharat Serum and Vaccines Limited, Ambernath, India. All other reagents of analytical grade were purchased from Sigma-Aldrich, USA. In the present study, different medicinal plants screened

for anti-PLA_2-activity were selected on the basis of previous survey reports from this laboratory [243,244] as well as from the information gathered from the traditional healers as well as from local people of Assam. Herbarium sheets for each plant were prepared and kept in the department.

3.1.1 Animals and housing conditions

All the BALB/c mice of both sexes weighted between 20-30 g were born in the laboratory breeding colony of the Central Facility of Animal House, Defence Research Laboratory, Tezpur, Assam and were pathogen free. General conditions of captivity were maintained in simulated atmospheric conditions of north east India (temperature 33-36 °C, relative humidity ≥ 75 %). Animal food was obtained from Pranav Agrotech, Delhi, India. In captivity, seasonal variations of physiological functions are entrained by altering six-month periods of summer like long photoperiod (14 h of light/day). The physiological status of animals was supposed to be stabilized. The general conditions of captivity were applied as described above and animals were maintained in social groups before and after experimenting. All experimenting protocols using animals were performed according to the "Principles of Laboratory Animal care" (NIH publication 85-23, revised 1985) and approved by the Tezpur University Animal Ethical Committee.

3.2 Methods

3.2.1 Characterization of isoenzyme pattern of PLA_2s of crude Russell's viper (*Daboia russelli*) venom of eastern India origin

3.2.1.1 Fractionation of crude RVV by cation exchanger (CM Sephadex C-50) to identify the basic PLA_2s

20 mg of crude *Daboia russelli* venom was dissolved in 2.0 ml of 20 mM K-phosphate buffer, pH 7.0 and centrifuged at 10,000 x g for 5 min at 4 °C in a cooling centrifuge (C-24BL, REMI). The clear supernatant was applied to a CM Sephadex C-50 (20 mm x 60 mm) column pre-equilibrated with 20 mM K-

phosphate buffer, pH 7.0 and eluted stepwise using K-phosphate buffers of various molarities and pH values at room temperature (~23 °C). The increasing molarities and pH values were (a) 20 mM K-phosphate pH 7.0, (b) 50 mM K-phosphate pH 7.0, (c) 50 mM K-phosphate pH 7.5, (d) 75 mM K-phosphate pH 7.5, (e) 100 mM K-phosphate pH 8.0, (f) 180 mM K-phosphate pH 8.0, (g) 230 mM K-phosphate pH 8.0, (h) 260 mM K-phosphate pH 8.0 and (i) 300 mM K-phosphate pH 8.0. Flow rate was adjusted to 24 ml h^{-1} and 1.0 ml fraction was collected in each tube. The protein content, phospholipase A_2 activity and anticoagulant activity of each fraction were estimated as described in sections 3.2.6.1, 3.2.6.3 and 3.2.7.1, respectively. In this process, six basic coagulant PLA_2s were pooled and named as CMII, CMIII, CMIV, CMV, CMVI and CMVII; and six basic anticoagulant PLA_2s were pooled and they were named as CM-AC-I, CM-AC-II, CM-AC-III, CM-AC-IV, CM-AC-V and CM-AC-VI.

3.2.1.2 Fractionation of CM Sephadex flow through anion exchanger (DEAE Sephadex A-50) to identify the neutral and acidic PLA_2s

Non-retained proteins from CM Sephadex C-50 column (containing neutral and acidic proteins of the crude RVV) eluted with wash buffer 20 mM K-phosphate buffer, pH-7.0 were pooled (CMI) and then loaded to an anion exchanger DEAE Sephadex A-50 (20 mm X 60 mm) column pre-equilibrated with 20 mM K-phosphate buffer, pH 7.0. After washing the column with (a) 20 mM K-phosphate pH 7.0, bound proteins were eluted stepwise using phosphate buffers of increasing molarities and decreasing pH values at room temperature (~23 °C). The increasing molarities and decreasing pH values were (b) 20 mM K-phosphate pH 6.5, (c) 20 mM K-phosphate pH 6.0, (d) 20 mM K-phosphate pH 5.5, (e) 100 mM K-phosphate pH 5.0 and (f) 120 mM K-phosphate pH 5.0. Flow rate was maintained as 22 ml h^{-1} and 1.0 ml fraction was collected in each tube.

Proteins eluted with equilibration buffer 20 mM K-phosphate buffer, pH-7.0, contained the neutral proteins. The protein content, phospholipase A_2 and anticoagulant activities of each fraction were estimated as described in sections

3.2.6.1, 3.2.6.3 and 3.2.7.1, respectively. The neutral proteins fraction showing PLA_2 activity was isolated and named as CMIDEI.

Proteins eluted with K-phosphate buffers of increasing molarities and decreasing pH values (b-f) contained the acidic proteins. A total of six acidic PLA_2 fractions were pooled and named as CMIDEII, CMIDEIII, CMIDEIV, CMIDEV, CMIDEVI and CMIDEVII.

3.2.2 Purification of an acidic anticoagulant PLA_2 (RVVA-PLA_2-I) from RVV

3.2.2.1 Fractionation of CMIDEV by gel filtration chromatography by using Sephadex G-50 column

Peak CMIDEV, one of the acidic fraction eluted with 100 mM K-phosphate buffer, pH-5.0, obtained from the fractionation of *Daboia russelli* venom on DEAE Sephadex A-50 anion exchanger (as described in the section 3.2.1.1) was desalted on pre-packed desalting column (Bangalore Genei) and then fractionated through a Sephadex G-50 column (1 cm X 64 cm) previously equilibrated with the 20 mM K-phosphate buffer, pH 7.0. Elution was carried out with the equilibration buffer at room temperature (~23 °C). The flow rate was adjusted to 20 ml h^{-1} and 1.0 ml fraction was collected in each tube. The protein content, phospholipase A_2 activity and anticoagulant activity of each fraction were estimated as described in sections 3.2.6.1, 3.2.6.3 and 3.2.7.1 respectively. Three fractions were pooled as anticoagulant PLA_2 namely, GFI, GFII and GFIII. Among them, the fraction GFI showed the highest PLA_2 as well as anticoagulant activity. This fraction was lyophilized and stored at -20 °C until further use. Gel filtration column was calibrated with the following protein molecular weight markers aprotinin (6500), cytochrome c (12,400), carbonic anhydrase (29,0000), bovine serum albumin (66,000) and blue dextran (2,000,000).

3.2.2.2 Fractionation of CMIDEV-GFI by RP- HPLC

The gel filtration fraction GFI was further purified on a reverse phase C_{18}-μ-Nova pak column (4.6 x 250 mm, 300 Å, 5μ) coupled to a HPLC system (Waters, Milenium-2000). Briefly, about 50 μg protein of GFI was dissolved in 20 μl of solvent A (0.1 % v/v TFA in 5 % v/v acetonitrile) and then applied on a reverse phase C_{18} HPLC column, previously equilibrated with solvent A. Column was washed with solvent A for 5 min and then bound proteins were eluted at a flow rate of 1.0 ml min^{-1} using a gradient of 5-95 % solvent B (0.1 % v/v TFA in 95 % v/v acetonitrile) from 5 to 37 min. Detection was monitored at 280 nm and individual fraction was collected manually. Each peak was screened for protein content, PLA_2 and anticoagulant activities. The peak showing PLA_2 activity as well as anticoagulant property was pooled, dried in vacuum and stored at -20 °C until further use. This purified acidic anticoagulant PLA_2 was named as RVVA-PLA_2-I.

3.2.3 Purification of a neutral anticoagulant PLA_2 (RVVN-PLA_2-I) from crude RVV

3.2.3.1 Fractionation of CMIDEI by gel filtration chromatography by using Sephadex G-50 column

The neutral fraction, CMIDEI (obtained from fractionation of *Daboia russelli* venom on CM Sephadex C-50 followed by DEAE Sephadex A-50 anion exchanger (as described in the section 3.2.1.1) was desalted on a pre-packed desalting column (Bangalore Genei, Bangalore). It was vacuum concentrated at -20 °C in a MAXI dry plus (Heto Lab Equipment, Denmark) and then applied to a Sephadex G-50 gel filtration column (1 cm X 64 cm) previously equilibrated with the 20 mM K-phosphate buffer, pH 7.0. Elution was carried out with the equilibration buffer at room temperature (~23 °C). The flow rate was adjusted to 20 ml h^{-1} and 1.0 ml fraction was collected in each tube. The protein content, phospholipase A_2 activity and anticoagulant activity were estimated as described in sections 3.2.6.1, 3.2.6.3 and 3.2.7.1 respectively. Three gel filtration fractions were pooled as anticoagulant PLA_2s and among them the third fraction showed

highest PLA_2 as well as anticoagulant activity. This pooled fraction was named as GFIII. The GFIII was lyophilized, and stored in aliquots at -20 °C until further use.

3.2.3.2 Fractionation of CMIDEI-GFIII by RP- HPLC

The gel filtration fraction GFIII was further purified on a reverse phase C_{18}-μ-Nova pak column (4 μm, 3.9 mm x 150 mm, Waters) attached to a HPLC system (described in section 3.2.2.4). This purified neutral anticoagulant PLA_2 was named as RVVN-PLA_2-I.

3.2.4 Purification of a basic anticoagulant PLA_2 (RVVB-PLA_2-I)

3.2.4.1 Fractionation of CM-AC-IV by gel filtration chromatography by using Sephadex G-50 column

Peak CM-AC-IV (see section 3.2.1.1) was desalted on a pre-packed desalting column (Bangalore Genei, Bangalore), and then vacuum concentrated at -20 °C in a MAXI dry plus (Heto Lab Equipment, Denmark). This fraction was separated in a Sephadex G-50 gel filtration column (1 cm x 64 cm) previously equilibrated with 20mM K-phosphate buffer. The protein content, phospholipase A_2 activity and anticoagulant activity of the gel filtration fractions were estimated as described in sections 3.2.6.1, 3.2.6.3 and 3.2.7.1 respectively. The gel filtration fractions (Peak GFIII) showing highest PLA_2 as well as anticoagulant activity were pooled, the protein content of pooled fraction was determined, lyophilized and stored in aliquots at -20 °C until further use.

3.2.4.2 Fractionation of CM-AC-IV-GFIII by RP- HPLC

The basic PLA_2 enzyme of gel filtration (fraction GFIII) was further purified on a reverse phase C_{18}-μ-Nova pak column (4 μm, 3.9 mm x 150 mm, Waters) by using HPLC system (Waters, Milenium-2000) following the protocol as described in section 3.2.2.4. This purified basic anticoagulant PLA_2 was named as RVVB-PLA_2-I. The fraction was lyophilized and stored in aliquots at 20 °C until further use.

3.2.5 Criteria of purity and determination of molecular weight of the purified anticoagulant PLA_2s of RVV

3.2.5.1 Gel filtration chromatography

Molecular weight of the purified PLA_2 enzyme in its native state was determined by gel filtration chromatography on Sephadex G-50 gel filtration column (1 cm X 64 cm) previously equilibrated with the 20 mM K-phosphate buffer, pH 7.0. About 50 µg of purified protein dissolved in 0.5 ml of equilibrium buffer was loaded into the column and eluted with the same buffer at room temperature (~23 °C). The flow rate was adjusted to 20 ml h^{-1} and 1.0 ml fraction was collected in each tube. Elution of the protein was monitored at 280 nm using an UV/Vis Spectrophotometer (Thermo scientific UV10, England). Gel filtration column was equilibrated with the following protein molecular weight markers (Da): aprotinin (6500), cytochrome c (12,400), carbonic anhydrase (29,0000), bovine serum albumin (66,000) and blue dextran (2,000,000). The molecular weight of the unknown proteins was calculated from the standard curve obtained by plotting log molecular weight Vs relative elution volume of the known protein.

3.2.5.2 Reverse-phase high performance liquid chromatography (RP-HPLC)

RP-HPLC of purified PLA_2 enzymes was performed on a reverse-phase phase C_{18}-µ-Nova pak column by using HPLC system (Waters, Milenium-2000). About 50 µg of purified protein was pre-incubated with 20 µl of solvent A (0.1% v/v TFA in 5 % v/v acetonitrile: Millipore water) for 30 min at room temperature (~23 °C) and passed through Spartan 3 nylon filters before application to RP-HPLC. Protein was eluted with a linear gradient from 5-95 % (v/v) acetonitrile containing 0.1 % (v/v) TFA over 37 min of time and detection was monitored at 220 nm. The flow rate was adjusted to 0.5 ml min^{-1}. Protein peaks were collected and assayed for PLA_2 and anticoagulant activity as described in the sections 3.2.6.3 and 3.2.7.1 respectively.

3.2.5.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The homogeneity of the preparation of the PLA_2 enzymes was checked by SDS-PAGE with or without reduction of protein with β-mercaptoethanol [245]. Briefly, 50 µg of crude venom and 20 µg of each purified proteins (PLA_2) were loaded into different wells of separating gels of different concentration (12.5 %, 15 % and 18 %) containing 5 % glycerol. Electrophoresis was carried out at a constant current of 15 mA until the dye front (bromophenol blue) reached the bottom of the gel. Before staining, proteins were fixed by incubating the gel in 20 % TCA for 30 min at 4 °C followed by washing the gel several times in distil water. Protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 in methanol: acetic acid: water (4:1:5 v/v/v) and destained with methanol: acetic acid: water (4:1:5 v/v/v). Destained gels were scanned in Biospectrum® 500 Imaging system, India. Mobility of the purified protein was compared with the protein molecular weight Markers; 10 kDa-250 kDa. Molecular weight of the unknown protein was calculated by calculating the Rf value of the protein and dye front.

3.2.5.4 Electrospray ionization-mass spectrometry (ESI/MS)

Molecular weight was also determined by electrospray ionization-mass spectrometry (ESI/MS). The electrospray mass spectra were recorded on a Micromass Quattro II triple quadrupole mass spectrometer. The sample was dissolved in 50 µl Millipore water and 10 µl of sample was diluted with 500 µl [1:1 (v/v); methanol: water containing 0.3 % (v/v) formic acid] solvent system. Then the sample was introduced into the ESI source through a syringe pump at the rate of 5 µl min-1. ESI capillary was set at 3.5 kV and the cone voltage was 40 V. The spectra were collected in 6 scans and the data was represented in the average spectra of 4-6 scans.

3.2.6 Biochemical Characterization

3.2.6.1 Estimation of protein content

The protein content was estimated by Folin-Lowry method [246] using BSA as a protein standard. The protein content of the unknown samples was calculated from the standard curve obtained by the plotting of absorbance Vs concentration of BSA (1.0 mg ml^{-1}).

3.2.6.2 Estimation of carbohydrate content

Total carbohydrate was quantitated by phenol-sulphuric acid method as described by Dubois et al. [247] using D-glucose as a standard. The optical density of the reaction mixture was measured at 490 nm against a reagent blank. The carbohydrate content of the unknown samples was calculated from the standard curve obtained by plotting absorbance Vs concentration of D-glucose (0.1 mg ml^{-1} in H_2O).

3.2.6.3 Assay of phospholipase A_2 (PLA_2) activity

Phospholipase A_2 activity was measured by using two methods viz. turbidometric method and titrametric method (see the description below).

3.2.6.3.1 Turbidometric method

Phospholipase A_2 activity of crude venom/purified protein was assayed by the turbidometric method of Joubart and Taljaard [248] with the following modifications as desribed by Doley and Mukherjee [179]. One egg yolk was suspended in 250 ml of 0.9 % (w/v) NaCl containing 0.02 % (w/v) sodium azide. The egg yolk suspension was stored in 4 °C and used within 3 days of preparation. Before experiment, 1.0 ml of suspension was mixed with 10.0 ml of 0.1 M Tris-HCl, pH-8.0 and the absorbance of the resulting mixture was adjusted to 1.0 at 740 nm with the same buffer. For the PLA_2 enzyme assay, a fixed amount of crude RVV/ fraction/ purified protein was mixed with 2.0 ml of the reaction mixture and the decrease in turbidity after 10 min was monitored at 740

nm against the reagent blank. One unit of PLA_2 activity is defined as decrease in 0.01 absorbance in 10 min at 740 nm.

3.2.6.3.2 Titrametric method

Titrametric method for estimation of PLA_2 activity of crude RVV/purified protein samples was performed by following the procedure of Deems and Dennis [249]. Briefly, 1.0 µg of PLA_2 was incubated with 1.0 mM of phospholipid suspension (phospholipid substrate viz. phophatidylcholine/ phosphatidylserine/ phosphatidylethanolamine; individual phospholipids was suspended in 100 mM Tris-HCl, pH 8.0 at a final concentration of 1 mM and sonicated for about 5 min at 4 °C with a Labsonic® M (Sartorius) sonicator) for 10 min at 37 °C in the final volume of 3.0 ml, adjusted with 100 mM Tris-HCl buffer, pH 8.0. Following incubation for 10 min, the reaction mixture containing phospholipids hydrolysis products (treated with PLA_2 as well as control) was titrated with 0.1 N NaOH with one drop of phenopthelin as an indicator. One unit of enzymatic activity (U) is defined as one microlitre of 0.1 N NaOH required to neutralize the reaction mixture. Fatty acid liberated from reaction mixture without treatment with venom protein was considered as control.

3.2.6.4 Assay of protease activity

Casienolytic activity and plasma protease activity was evaluated by the original method of Ouyang and Teng [250] and modified by Doley and Mukherjee [179]. Briefly, 1.0 ml of 1 % (w/v) of casein/plasma protein in 0.1 M K-phosphate buffer, pH 8.0 was incubated with specific amount of crude RVV/purified proteins for 90 min at 37 °C and the reaction was stopped by addition of 0.5 ml of 10 % (w/v) ice-cold TCA. The reaction mixture was centrifuged and the supernatant was transferred to a fresh tube. Then 2.0 ml of 2 % (w/v) Na_2CO_3 in 0.1 N NaOH was added and incubated for 10 min at room temperature followed by the addition of 0.5 ml of Folin-Ciocalteu's reagent (1:2 dilution). After 30 min, absorbance was measured at 660 nm. Caseinolytic/ plasma protein hydrolysis activity of the crude venom/purified PLA_2, if any, was

calculated from the standard tyrosine curve. One unit (U) of the protease activity, if any, is defined as n mole equivalent of tyrosine formed per minute.

3.2.6.5 Assay of acetylcholinesterase activity

For assaying acetylcholinesterase activity of crude venom/purified proteins of *Daboia russelli* venom the method of Ellman et al. [251] was followed. Briefly, 10 µg of crude RVV/purified protein was added to the reaction mixture containing 300 µl of 10 mM acetylcholine iodine in a final volume of 3.0 ml, adjusted with 70 mM Tris-HCl buffer, pH 8.0. This was followed by addition of 10.0 µl of 5,5-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent), freshly prepared in 100 mM Tris-HCl buffer, pH 7.4 containing 15 mg Na_2CO_3,and change in absorbance at 412 nm after 1 min was recorded against a reagent blank. One unit (U) of AchE activity, if any, is defined as micromole of thiocholine formed per minute under the reaction conditions.

3.2.6.6 Assay of adenosine monophosphatase (AMPase) activity

AMPase or, 5′ -nucleotidase activity assay was done by the method of Campbell [252] as modified by Mukherjee et al. [27]. The typical reaction mixture contained 100 mMTris-HCl buffer, pH 8.0, 100 mM $MgCl_2$, 3 mM5′-adenosine monophosphate and reaction was initiated by addition of 100 µg crude RVV or 50 µg purified proteins. After incubation for 30 min at 37 °C, 0.5 ml of 10 % (w/v) ice-cold TCA was added to stop the reaction and the tubes were centrifuged at 300 rpm for 15 min. For the estimation of liberated Pi, 0.2 ml of supernatant was transferred to another tube containing 0.25 ml of 1 % (w/v) ammonium molybdate in 5 N H_2SO_4 and 0.25 ml 1% (w/v) *p*-methyl amino phenol sulphate containing 1.5% (w/v) sodium bisulphate. The final volume was adjusted to 5.0 ml with Millipore water. Absorbance was read at 660 nm after 30 min of incubation at room temperature against a reagent blank. One Unit (U) of enzyme activity, if any, is defined as µg of Pi liberated per 30 min at 37 °C.

3.2.6.7 Assay of adenosine triphosphatase (ATPase) activity

ATPase activity of crude venom/purified proteins (if any) was determined by the method of Esnouf and Williams [253]. Briefly, 5.0 µg of venom protein in 800 µl of reaction mixture was incubated for 30 min at 37 °C. The reaction mixture (1.0 ml) consists of 2 mM $MgSO_4$, 50 mM KCl, 50 mMTris-HCl buffer, pH 8.0 and 1mM ATP. The reaction was terminated by the addition of 0.2 ml of 10 % (w/v) ice-cold TCA and mixture was centrifuged at 3000 rpm for 10 min. The filtrate (0.2 ml) was transferred to a fresh tube containing 0.25 ml of 1 % (w/v) ammonium molybdate and 0.25 ml of 1 % (w/v) metol and final volume was adjusted to 5.0 ml of with distilled water. The reaction was allowed to stand for 30 min at room temperature and then the absorbance was measured at 735 nm against a reagent blank. The ATPase activity of the crude RVV/purified proteins was calculated from the standard curve of inorganic phosphate (Pi). One unit (U) of the ATPase activity, if any, is defined as the amount of enzyme required to liberate 1 µmole of inorganic phosphate in 30 min at 37 °C.

3.2.6.8 Enzyme kinetics

3.2.6.8.1 Substrate specificity of PLA_2 enzymes

To determine the substrate specificity and phospholipid head-group preference of the purified anticoagulant PLA_2 enzymes, different commercially available phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) were used at a final concentration of 1.0 mM and incubated with 20 mM PLA_2 at 37 °C for desired time periods. The PLA_2 activity was assayed by titrimetric method as described in the section 3.2.6.3.2 using palmitic acid as free fatty acid standard [140].

3.2.6.8.2 Effect of substrate concentration on PLA_2 activity

Effect of substrate concentration on PLA_2 activity was determined by following the procedure of Doley and Mukherjee [179] by titrametric method.

Graded concentrations of substrate (PC/ PS/ PE) ranging from 0.1-2.5 mM were incubated with 1.0 µg of purified protein for 5 min at 37 °C and then PLA_2 activity was assayed by the titrametric method as described in the section 3.2.6.3.2.

3.2.6.8.3 Effect of enzyme concentration on PLA_2 activity

To determine the effect of enzyme concentration on catalytic activity, graded concentrations of PLA_2 (0.1-1.0 µg ml^{-1}) were added to the reaction mixture and liberation of fatty acids (PLA_2 activity) was assayed by titrametric method as described in the section 3.2.6.3.2.

3.2.6.8.4 Determination of K_m and V_{max} for the enzyme catalyzed reactions

A constant amount of enzyme was incubated with graded concentrations of purified phospholipid suspension for 10 min at 37 °C. The kinetics of enzyme catalysed reaction (K_m and V_{max} values) of purified enzymes were calculated using Lineweaver-Burk plot [254]. Phospholipase activity was estimated by using the preferred substrate of specific PLA_2 (PC/ PS/ PE) as described in the section 3.2.6.3.2. By plotting the values of 1/v as a function of 1/s, a straight line was obtained that intersect the vertical line at a point which is the $1/V_{max}$ (since 1/[S] =0, therefore $1/v= 1/V_{max}$). Extension of the straight line results in intersecting the horizontal axis (1/[S]) at the point which is $-1/K_m$.

3.2.6.8.5 Determination of temperature optimum for PLA_2 catalyzed reactions

To study the effect of temperature on the PLA_2 activity of purified proteins, the reaction mixture (egg yolk phospholipid and 100 mM Tris-HCl, pH 8.0) was incubated with a constant amount of purified PLA_2 at different temperature (25- 75 °C) for 10 min followed by assay of PLA_2 activity by turbidimetric method (see section 3.2.6.3.1).

3.2.6.8.6 Determination of pH optimum for PLA_2 catalyzed reactions

To determine the optimum pH for the catalytic activity of the purified PLA_2 enzymes, the PLA_2 assay was carried out as described in the section 3.2.6.3.2 by using buffers of different pH values. The different pH values were obtained as follows 0.1 M sodium acetate buffer, pH 5.5-6.5; 0.1 M phosphate buffer, pH 7.0- 7.5 and 0.1 M Tris-HCl buffer, pH 8.0-9.5. From a line graph, the optimum temperature for PLA_2 activity was calculated.

3.2.6.8.7 Circular dichroism

Circular dichroism (CD) measurement was performed by using a Jasco J-715 spectropolarimeter (Tokyo, Japan). Protein samples (native and heated at 75 °C for 45 min) were dissolved a room temperature (~ 23°C) in Millipore water at a final concentration of ~ 1mg ml^{-1}. CD spectra were recorded using a quartz cuvette with an optical path length of 0.5 mm. Each CD spectrum represents an average of 5 scans in the range of 195-250 nm, collected at 0.2 nm intervals, with a spectral band width of 0.5 nm and 4 sec integration time. The CD spectra were corrected by subtraction of water blank and expressed in molar ellipticity [θ] (degree cm^2 $dmol^{-1}$), using 113 as mean residue molecular weight [140,255].

3.2.6.8.8 Heat-inactivation study of PLA_2 enzymes

Purified PLA_2 enzymes were dissolved in 20 mM K-phosphate buffer, pH 7.0 (100 nM) and incubated for different time periods (0 - 60 min) at 75 °C. The reaction mixture was cooled immediately in ice-bath and then the required volume was withdrawn and catalytic activity and pharmacological activities of the heated and native PLA_2 enzymes were analysed. The activity of native enzyme (without heating) was considered as control and other values were compared with that.

3.2.6.8.9 Chemical modification of PLA_2 enzymes by pBPB

The active sites of purified PLA_2 enzymes were chemically modified by the method of Doley and Mukherjee [179]. Briefly, PLA_2 enzymes (100 nM)

were incubated with pBPB in 25 mM Tris-HCl buffer pH 8 (at a final concentration of 2.0 mM) at 37 °C for 30 min. The reaction mixture was desalted through desalting column to remove the excess reagent. The modified PLA_2 enzymes were assayed for catalytic activity and other pharmacological activities and these activities were compared with control enzyme (PLA_2 without incubation with pBPB).

3.2.6.8.10 Chemical modification of PLA_2 enzymes by other inhibitors

The different inhibitors viz. TPCK, TLCK (serine and cysteine inhibitor), PMSF (serine protease inhibitor), EDTA (metal chelator), DTT (reducing agent for disulphide bridge) and IAA (alkylating reagent/ cysteine inhibitor) were also tested for chemical modification of active site of purified enzymes. For the chemical modification study, 100 nM of purified PLA_2 enzyme was incubated with different inhibitors (at 2.0 mM final concentration) at 37 °C for 30 min. After incubation the modified enzymes were assayed for PLA_2 activity and other pharmacological properties and compared with activities of control PLA_2. The control PLA_2 represents enzyme that was not incubated with any inhibitor and this activity was considered as 100 % activity.

3.2.6.8.11 Fluorescence spectrophotometric measurements of phospholipids-PLA_2 and coagulation factors-PLA_2 interactions

Interaction of different phospholipids with purified PLA_2 was determined by using a fluorescence spectrometer (LS55, Perkin Elmer). Briefly, either a pure phospholipid (PC/PS/PE) or a mixture of phsopholipids [PC:PS:PE (1:1:1)] were suspended in 20 mM Tris-HCl, pH 8.0 buffer at a final concentration of 1 mM and sonicated for about 5 min at 4 °C with a Labsonic® M (Sartorius) sonicator. Graded concentrations of PLA_2 (dissolved in 20 mM Tris-HCl, pH 8.0) was mixed with a fixed concentration of either PC, PS or PE or a mixture of PC: PS: PE (1:1:1), either in presence or, absence of Ca^{2+} and fluorescence spectra were obtained at an excitation wavelength 280 nm, excitation and emission slits 5 nm, temperature 30 °C. Wavelength shifts were measured by

taking the midpoint at two third height of the spectrum. The maximum fluorescence of free protein (I_0) was also measured.

To measure the interaction of PLA_2 with blood clotting factors viz. factor Xa, factor Va, prothrombin and thrombin, the former protein was mixed with any one of the coagulation factors at a time to get the different molar ratio of PLA_2: coagulation factor either in presence or absence of Ca^{2+} and change in the fluorescence intensity at 280 nm excitation was measured [216]. To assure the reproducibility, all the binding experiments were done in triplicate.

3.2.6.8.12 Study of inhibitory effect of PLA_2s on prothrombin activation by factor Xa: amidolytic activity assay of thrombin generation

The effect of PLA_2 on inhibition of factor Xa was assessed by inhibition of factor Xa mediated thrombin formation from prothrombin in presence of PLA_2 enzymes. The thrombin formation was assayed by amidolytic activity of thrombin towards its chromogenic synthetic substrate (N-p-tosyl-Gly-Pro-Arg p-nitroanilide acetate salt) which was carried out with a microplate reader (Thermo Electron Corporation, Multiskan ascent, Type 354). Briefly, 200 µl of assay buffer (20 mM K-phosphate buffer, pH-7.4) containing 10 µl of 10 mM stock of chromogenic substrate was incubated with 100 nM of either purified PLA_2s at 37 °C for 20 minutes, and the formation of p-nitroaniline was monitored at 405 nm [216,256]. The amount of substrate hydrolyzed was calculated from a standard curve of p-nitroaniline.

The inhibition of prothrombin activation was also analyzed by 15 % SDS-PAGE [245]. Briefly, 5 µg prothrombin was incubated with factor Xa, Ca^{2+} ions and either in presence or absence of 100 nM of purified PLA_2 at 37 °C for 3 h. The hydrolysis products of prothrombin were analyzed by 15 % SDS-PAGE under non-reducing condition.

3.2.6.8.13 Effect of PLA_2 on amidolytic activity of factor Xa

Factor Xa (50 µM) dissolved in 20 mM K-phosphate buffer, pH 7.4 was incubated with various concentrations (50-100 nM) of purified anticoagulant

PLA_2 (either in presence or absence of Ca^{2+}) for 10 min at room temperature (~23 °C). The factor Xa amidolytic activity was measured by transferring an aliquot of the mixture to a cuvette containing 50 µM of F3301 (CH_3OCO-D-CHA-Gly-Arg-*p*-nitroanilide; chromogenic substrate for factor Xa). The hydrolysis of F3301 was measured at 405 nm. The reaction mixture without factor Xa was considered as negative control whereas reaction mixture with factor Xa and F3301 but without PLA_2 served as positive control. The amidolytic activity of factor Xa in absence of PLA_2 was considered as 100 % activity and other values were compared with that.

3.2.7 Pharmacological Characterization

3.2.7.1 Assay of plasma clotting activity

3.2.7.1.1 Ca- clotting time

The plasma clotting activity of the purified enzymes was assayed by the method of Angulo et al. [257]. Briefly, blood from goat / healthy human donor was collected in 3.8 % of trisodium citrate and then platelet poor plasma (PPP) was prepared by centrifuging (2,500 x g) twice the anticoagulated blood for 15 min at 4 °C and used within 4 h of collection. To assay the re-calsification time, specific amount of crude RVV/ purified PLA_2s (in a final volume of 30 µl) was added to 300 µl of PPP pre-incubated at 37 °C. The reaction mixture was incubated for 3 min at 37 °C and then 40 µl of 250 mM $CaCl_2$ was added. The PLA_2 induced clotting time of plasma was recorded with the help of a stop watch, based on the first appearance of fibrin thread/clot. As a control, plasma aliquot was incubated with 30 µl of phosphate buffer saline (PBS) and the clotting time was determined identically.

3.2.7.1.2 Prothrombin time test

Prothrombin time (PT) of goat PPP was determined by the method of Quick et al. [258] using commercial kit (Lot No. 331901). For this assay 0.1 ml of PPP was taken in a clean and dry test tube and pre incubated for 5 min at 37°C

in a water bath. Various amounts of crude RVV/ purified protein were added and incubated in a water bath for 5 min at 37 °C followed by addition of 0.2 ml of Liquiplastin reagent and appearance of the first fibrin clot was noted using a stop watch. Addition of 0.9 % (w/v) NaCl, instead of PLA_2 served as control.

3.2.7.1.3 Gas-chromatography analysis of liberated fatty acids from plasma phospholipids

The fatty acids released after treatment of 0.3 ml of goat plasma with 100 nM of PLA_2 for 3-20 min were extracted and methylated as describe by Mukherjee et al. [205]. Briefly, 5.0 ml of methanolic sodium hydroxide solution was added to a measured amount of lipid extract (in a round bottomed flask) and the mixture was refluxed for half an hour. Then 5.0 ml of deionised water and 1.0 ml of concentrated HCl (11.65 N) were added, mixed well and the mixture was extracted with 10.0 ml of petroleum ether. The process was repeated twice and the sample was dried in a rotary vacuum evaporator. To these FFAs, 10.0 ml of methyl alcohol and few drops of concentrated H_2SO_4 were added. The mixture was again refluxed for 3 hrs followed by dilution with 10.0 ml of water and then re-extracted with 10.0 ml of petroleum ether. The extraction procedure was repeated twice. All the petroleum-ether extracted fractions were pooled and allowed to dry in a dessicator for analysis by GC.

After that GC analysis of the dry sample (fatty acid methyl esters) was done as described by Saikia et al. [216]. The dry sample (fatty acid methyl esters) was dissolved in a minimum quantity of chloroform and analyzed on a GC-MS (Varian 3800, Saturn 2000) system. The samples were injected using a split ratio of 100:1 into a fused silica GC column CP- Sil 8 CB low bleed (30 m x 0.25 mm x 0.25 μm) coupled with a CP-Sil 5 C low bleed /MS (30m x 0.25 mm x 0.25 μm) column with helium as the carrier gas. The system was equipped with flame ionization detector. The initial oven temperature was 120 °C and a temperature program of 8 °C per min began at injection and continued to a final oven temperature of 270 °C, which was hold isothermal for 3 min. The injector port and detector temperature were set at 250 °C. The mass spectrometric data

were acquired in electron ionization mode (70eV). The unknown methylated fatty acids were identified by matching both retention time and MS of the unknown compound with those of authentic standards (Saturn 2000 MS library search). The fatty acids were quantitated by measuring and comparing the GC peak area for released (unknown) fatty acids with the GC chromatogram of a standard (known) fatty acid.

3.2.7.2 Direct and indirect haemolytic activity assay

Haemolysis was assayed by the semi quantitative method of PLA_2 assay [259]. Direct haemolytic activity of crude RVV/purified $PLA_2$was tested by incubating 10 µg of crude venom protein or, 100 nM of purified PLA_2 with 5 % (v/v) of goat blood erythrocytes suspension in 100 mM phosphate buffer, pH 7.4 in a final volume of 3.0 ml. After incubating for 60 min at 37°C, the tubes were placed in ice to stop the reaction. The reaction mixture was then centrifuged and released haemoglobin was measured spectrophotometrically at 540 nm. For indirect haemolysis assay, 10 µg of egg yolk suspension as added to the reaction mixture (as prepared for direct haemolytic assay) as a source of phospholipids and haemolysis was induced by addition of PLA_2 enzyme. Total haemolysis (100 %) was achieved by adding deionised water (Millipore) instead of venom protein to the erythrocyte suspension. Haemolysis was expressed as percent total haemolysis. To check the haemolytic effect of purified PLA_2 enzymes on PC, PS and PE enriched human erythrocyte; the method Diaz et al. [260] was followed. Briefly, 300 µl of 5 % (v/v) washed erythrocytes suspension was pre-treated with 5 mM of either PC, PS or PE for 1 h at 4 °C. The pre-treated cells were then incubated with 100 nM of PLA_2 enzyme for a period of 3 h at 37 °C either in presence or absence of 1.5 mM $CaCl_2$ and percentage of haemolysis was determined as described above.

3.2.7.3 *In-vitro* tissue damaging activity assay

For the assay of *in vitro* tissue damaging activity, procedure of Dutta and Bhattarcharyya [261], as modified by Doley and Mukherjee [179] was followed. Briefly, fresh chicken liver/heart/lungs tissues were washed with 0.9 % NaCl to

remove adhered blood clots, cut into small uniform sized pieces, and patted with dry tissue paper. For each experiment, 300 ± 10 mg of tissues were pre-incubated with 1.0 ml of 0.2 M K-phosphate buffer, pH 7.4 for 45 min at 37 °C. The tissues were then washed twice with the same buffer and incubated with either crude RVV (1μg ml^{-1}) or, purified PLA_2 (100 nM) in a final volume of 2.0 ml of 0.2 M K-phosphate buffer, pH 7.4 for 5 h at 37 °C. After incubation, the reaction mixture was centrifuged for 5 min at 3,000 rpm and absorbance of the supernatant was read at 540 nm (Thermo scientific UV10, England). The percentage of haemoglobin released i.e., the *in vitro* tissue damaging activity of crude RVV/purified PLA_2s was calculated with respect to tissue incubated with 0.1 % (v/v) Triton X-100 (100 % activity).

3.2.7.4 Assay of antibacterial activity of the PLA_2 enzymes

Antibacterial/ bactericidal activity of crude Russell's viper venom and purified PLA_2 enzymes against *Escherichia coli* (DH5α) (Gram negative), *Bacillus subtilis* (Gram positive) and *Pseudomonus auregonisa,* M and NM strains bacteria was assessed as described by Mukherjee, 2007. To study the effect of the purified PLA_2 enzymes on bacterial membrane phospholipids hydrolysis, mid-logarithmic culture ($O.D._{630nm}$ ~ 0.3) of bacteria were centrifuged at 10,000 X g for 15 min to pellet the cells and then incubated with 10 μg of PLA_2 enzyme at 37 °C upto 18 h. Lysis of bacterial cells (if any) was measured as an decrease in optical density at 630 nm (OD630) by a spectrophotometer (Thermo scientific UV10, England) after incubation [262]. A control was run in parallel where instead of crude RVV or PLA_2 enzyme, PBS was added and bacteria were incubated under identical conditions.

3.2.7.5 Isolation of chicken liver mitochondria

Mitochondria were isolated from fresh chicken liver as described by Valente et al. [263]. All the operations were carried out at 4 °C, unless otherwise stated. Briefly, liver tissues were homogenized in 0.25 M sucrose containing 20 mM Tris-HCl, pH 7.4 and 1 mM EDTA (isolating buffer), centrifuged in Sorvall refrigerated centrifuge (Sorvall®RC 5B plus) for 10 min at 460 x g to sediment

the nuclei. The supernatant was transferred to another tube and centrifuged at 12,500 x g for 7 min. In appearance, the centrifuged tube has three distinct layers. The middle layer containing the mitochondria was dislodged very gently, re-suspended in 4 ml of isotonic buffer (20 mM Tris-HCl, pH 7.4 containing 0.3 M sucrose) to give a final protein concentration of 80-100 mg ml^{-1}.

3.2.7.5.1 Assay of membrane damaging activity

Isolation of mitochondria from fresh chicken liver at 4 °C was described above. For the assay of PLA_2 induced mitochondrial swelling and membrane damage, mitochondrial suspension containing 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissues) in 2.0 ml of assay buffer (20 mM Tris-HCl, pH 7.4, containing 0.3 M sucrose) were incubated with 100 nM of purified PLA_2, either in the presence or absence of 2 mM Ca^{2+}, at 37 °C for the desired time period. Mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 520 nm for 30 min [140]. One unit of swelling is defined as a decrease in 0.01 absorbance/min of mitochondrial suspension at 520 nm by added PLA_2 enzyme (100 nM) as compared to control (mitochondrial suspension without PLA_2) under the experimental condition. The basic test system without added PLA_2 was served as a control. Qualitative and quantitative analyses of liberated fatty acids and lysophospholipids from the mitochondrial membranes due to the action of PLA_2 were performed by gas-chromatography as described below. A control was also set up where the mitochondria were treated under the identical condition except the addition of PLA_2.

For the estimation of RVV-PLA_2 induced haemolysis and erythrocyte phospholipids hydrolysis, goat blood was collected in 3.8 % tri-sodium citrate and erythrocytes were separated by centrifuging the citrated blood at 1000 X g for 15 min, washed twice with isotonic K-phosphate buffer, pH 7.4 and suspended in the same buffer at a concentration of 5 % (v/v). Purified PLA_2 enzyme at a final concentration of 100 nM was added to 3.0 ml of the above said erythrocytes suspension and the haemolysis and erythrocyte phospholipids

hydrolysis by PLA_2 were measured as described by Doley et al. [140]. For determining the role of exogenously added phospholipids in haemolytic activity (indirect haemolytic activity), either PC (final concentration of 1 mM) or egg yolk phospholipids (0.1 % v/v) was added to the erythrocyte suspension prior to the addition of PLA_2. The reaction was initiated by the addition of PLA_2 followed by measuring the haemolysis as stated above [140]. For each of the experiment isotonic saline instead of PLA_2 was used as a control.

Erythrocytes or mitochondria, without or without PLA_2 treatment for various time periods were centrifuged at 10,000 x g and 1.0 ml of supernatant was used for the extraction and quantification of total lipid and fatty acids from the reaction mixture [140]. The total lipid and fatty acids were also extracted from control (untreated) erythrocytes and mitochondria incubated under similar experimental conditions. For the estimation of phospholipids/lysophospholipids released from the membrane, the total lipid extracted from the supernatant was digested with concentrated HNO_3 in a Kjeldahl flask until a white precipitate was formed and then the liberated Pi was estimated calorimetrically as described earlier [140,264]. Briefly, the digest was dissolved in water and final volume was adjusted to 10.0 ml. To this, 5.0 ml of ammonium molybdate was added and mixed well. After that the reading was taken at different time periods (5-15 min) at 640 nm by using a spectrophotometer (Thermo scientific UV10, England). The reading was plotted against time and the reading at zero minute was find out by extrapolation and thus calculated the unit of inorganic phosphorous (p_i) formed.

3.2.7.5.2 Analysis of fatty acids released from membranes by GC-MS

The total lipid released from the membranes (mitochondrial and erythrocyte) with or without PLA_2 treatment was extracted and from this total lipid, liberated fatty acids were extracted and methylated as described in the section 3.2.7.1.3. The dry sample (fatty acid methyl esters) was dissolved in a minimum quantity of chloroform and analyzed on a GC-MS (Varian 3800, Saturn 2000) system as described in the section 3.2.7.1.3. The unknown

methylated fatty acids were identified by matching the retention time as well as MS of the unknown compound with those of authentic standards (Saturn 2000 MS library search). The fatty acids were quantitated by measuring and comparing the GC peak area for released (unknown) fatty acids with the GC chromatogram of a standard (known) fatty acid.

3.2.7.5.3 Enzyme immunoassay to determine the binding of RVV-PLA_2s with membrane

Supernatant obtained from the incubation of intact mitochondria or erythrocytes with purified PLA_2 (native, heated or chemically modified) at 4 °C for 30 min was tested for free (unbound) toxin concentration by ELISA using horse polyclonal antibodies against RVV and rabbit anti-horse IgG peroxidase conjugate [140,264] as described in the section 3.2.8.2. A standard curve of PLA_2 was plotted by adding graded concentration of RVV-PLA_2s (30-200 nM per well) in the wells of the ELISA plate and the concentration of unbound PLA_2 was determined from this curve by using an ELISA plate reader (Thermo Electron Corporation, Multiskan ascent, Type 354). Binding of native PLA_2 with membrane was taken as 100 % binding and then values were calculated. Each experiment was repeated thrice to assure the reproducibility.

3.2.7.6 Cytotoxicity assay on tumour cells

Cytotoxicity was assessed on adenocarcinoma tumour cell line HT29 [264]. Various dilutions of PLA_2 enzymes (2.0-10.0 μg ml^{-1}), dissolved in the cell culture medium (DMEM with 10 % serum), were added to cells and then aliquoted in a 96-well plate at a concentration of 1×10^4 cells ml^{-1}. The final volume of cell culture was adjusted to 100 μl $well^{-1}$. The cells were allowed to grow at 37 °C (in a 5 % CO_2 atmosphere) for 4 h as well as 24 h post-incubation with crude RVV/purified PLA_2 followed by addition of 20 μl $well^{-1}$ of water soluble XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) solution (1 mg ml^{-1}). After 4 h incubation at 37 °C and growth conditions for each cell line, the absorbance at 490 nm was recorded using a microplate reader (Tecan, model-infinite®200 PRO). Cytotoxicity was expressed

as percentage cell death which was determined by comparing absorbance with values obtained from the standard curve of control cells treated with XTT Solution under identical conditions. The cytotoxicity assay for the cell line was done in quadrupled. Control experiments were also run in parallel where cell cultures were treated with toxin-free growth medium (negative control) and medium without cells (blanks). The control and crude RVV/PLA_2 treated cells were also observed under a compact high performance inverted light microscope (Nikon ECLIPSE, TS100, Tokyo) for any visible morphological changes.

3.2.7.7 *In vivo* animal experiments

3.2.7.7.1 Determination of toxicity on mice

The toxicity experiment was done on BALB/c mice at Defence Research Laboratory, Tezpur. The acute toxicity was determined as per protocol of OECD/OCED guideline 425 [265] and as approved by the Tezpur University animal ethical committee. For toxicity assessment of PLA_2 in rodents, purified PLA_2 enzyme (0.08-0.4 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB/c mice (20–30 g). The mice were assigned to control group (Group I, n=6) and one PLA_2 treated group (Group II, n=6) for each PLA_2. The animals were observed at regular intervals up to 48 h post-injection for any behavioural changes viz., body weight, food and water intake, faecal and urination, grip strength, ear twist, rectal temperature etc., effect on circulatory system, and/or death. Bleeding time along with clotting time was also checked before sacrificing the animals. Control animals (placebo) were injected with 0.1 ml of PBS only.

Mice were sacrificed 48 h post injection, blood was collected immediately by venipuncture and the serum was used for the assay of different parameters viz. total protein, glucose, cholesterol, triglycerides, uric acid and urea level in serum as well as different enzyme activity viz. LDH (lectate dehydrogenase), ALP (alkaline phosphatase), CPK (creatine phosphokinase), SGOT (serum glutamic oxalocetic transminase) and SGPT (serum glutamic pyruvic transminase) by using commercial kits in an auto analyzer. To study the

effect of PLA_2 enzymes on different blood cells, RBC and WBC count of treated and control animals was done by an automatic cell counter (Automated hematology cell counter- MS 4(s), Milet Schloesing Laboratories).

3.2.7.7.2 Histopathological examination

Histopathological study of the tissues was done in order to evaluate the pathogenic alterations, if any, induced by i.p. injection of PLA_2 in mice. After 48 h of injection, the animals were euthanized by deep anesthesia with diethyl ether. Heart, lung, liver and kidneys were excised, cut into small pieces, washed in PBS, pH 7.4 to and then treated in 10 % formaldehyde, and processed for embedding into paraffin (ParaplastTM resin). The materials were then dehydrated by passing through increasing concentrations of ethanol (50-100 %). The resulting blocks were sliced in 5 μm thick sections and then stained with hematoxyline and eosine. The tissues were examined under a light microscope (LEICA DM 3000) for PLA_2-induced pathological evaluation.

3.2.8 Immunological Characterization

3.2.8.1 Immunological cross-reactivity

Immunological cross reaction between crude RVV/purified PLA_2 enzyme(s) against commercial polyvalent antivenom (raised against the venom of *Naja naja*, *Daboia russelli*, *Bungarus Caeruleus* and *Echis Carinatus*) was studied by gel immunodiffusion test as described by Ouchterlony and Nilnsan [266]. Briefly, 1 % (w/v) agarose was prepared in PBS containing 0.02 % sodium azide (w/v). Wells of suitable diameter were prepared on the plate using a puncture, one in the centre and four surrounding the central well. Central well was filled with 10 μl of 2 mg ml^{-1} commercial antivenom and four peripheral wells were filled with either crude RVV or purified anticoagulant PLA_2 of different dilutions. The Petri-plates were placed in a humid box and kept at 37 °C. The appearance of antigen-antibody complex surrounding the central well was detected by visual monitoring up to 48 h. A control was also set where instead of crude RVV/PLA_2, 20 mM K-phosphate buffer, pH-7.4 was added.

After the formation of precipitin line, the gel was extensively washed for 24 h with several changes of normal saline, dried and stained with 1 % (w/v) Coomassive brilliant blue-250 to visualize the precipitating bands.

3.2.8.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA was also used for evaluating the antigenic cross-reactivity of purified PLA_2 enzymes with commercial polyvalent antivenom. The walls of 96-well ELISA plate (Micro Test IIITm flexible assay plate, Becton and Dickinson and Co, Oxnard, USA) were coated with 0.5 mg $well^{-1}$ of crude RVV/purified PLA_2(s) in 50 mM sodium bicarbonate pH 9.6 (100 µl, standard volume) and kept at 4 °C overnight. After washing with 0.05 % Tween/saline, the wells were blocked with 2 % (w/v) BSA in PBS (phosphate buffered saline, pH 7.4) for 1 h at room temperature. Polyvalent antivenom were added and incubated for 1 h at 37 °C at 1:200, 1:400, 1: 1,000 dilutions. The plates were then washed with wash buffer (PBS with 0.05 % (v/v) Tween 20) and incubated with anti-horse IgG-ALP conjugated secondary antibodies (Sigma-Aldrich, St Louis, MO, USA), diluted 1:4000, 1:10,000 for 1 h at 37 °C. The wells were washed with 0.05 % Tween 20 /PBS and the bound antibodies were detected by incubating with 100 µl of tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) as substrates and the color reaction was developed for 1 h at 37 °C. Then 1 N H_2SO_4 was added to stop the reaction and absorbance was measured at 405 nm in an ELISA reader (Thermo Electron Corporation, Multiskan ascent, Type 354). To assure the reproducibility, all ELISA tests were performed in triplicate.

3.2.9 Preparation of plant extract

Plant extracts were prepared according to the method of Mahanta and Mukherjee [243]. Fresh leaves/roots/bark were shade dried and made to coarse powder. 2.0 g of powder of leaves/roots/bark was taken in a beaker and soaked with 100 ml of H_2O with continuous stirring for 2 h at room temperature (~23 °C). The extract was filtered through a muslin cloth and filtrate was concentrated at 40 °C under vacuum. The dried extract was dissolved in normal saline (0.9 %) at a concentration of 1 mg ml^{-1} and kept at 4 °C until further use. However, for

alcohol/chloroform extraction, the mixture was continuous stirred for 2 h at room temperature (~23 °C). After filtration through muslin cloth, the filtrate was concentrated and dried. The residue was suspended in normal saline at a concentration of 1 mg ml^{-1} and stored at 4 °C until further use.

3.2.10 Neutralization of catalytic activity and pharmacological properties of purified PLA_2 enzymes

3.2.10.1 By polyvalent antivenom

For neutralization of PLA_2 activity, commercial polyvalent antivenom was incubated with crude RVV/purified PLA_2s at different ratios (1:100, 1:200 and 1:500, w/w) at 37 °C for 30 min, followed by assay of catalytic activity and pharmacological properties. Activity of PLA_2 enzyme without polyvalent antivenom was served as control (100 % activity) and other values were compared with that.

3.2.10.2 By plant extract

For neutralization of PLA_2 activity, graded amounts of plant extract (10-100 mg ml^{-1}) was pre-incubated with a fixed amount of (1 μg ml^{-1}) of crude RVV/purified PLA_2s for 30 min at 37 °C. Then PLA_2 activity as well as pharmacological properties were assayed. PLA_2 activity without plant extract was served as control (100 % activity) and other values were compared with that.

3.2.11 Storage stability of PLA_2 enzymes at 4 °C

Stability of aqueous solution (1.0 mg ml^{-1}) of purified PLA_2 enzymes were checked by keeping the PLA_2(s) at 4 °C for a specified period of time (0-28 days). A suitable aliquot was withdrawn at a regular interval to assay the catalytic as well as pharmacological properties of PLA_2 enzymes. These values at 0 day were considered as 100 % activity and other values were compared with that.

CHAPTER IV

Isoenzyme pattern of phospholipase A_2 (PLA_2) enzymes of venom of Russell's viper (*Daboia russelli*) of eastern India origin

4 Results

4.1 Separation of basic phospholipase A_2 (PLA_2) enzymes by fractionation of crude *Daboia russelli* venom through a cation exchanger

The fractionation of crude RVV through cation exchanger renders the presence of a total of twelve basic PLA_2 enzymes. These isoenzymes were progressively eluted from the cation exchanger on the basis of their overall positive charge at the pH 7.0. Amongst the isolated twelve basic PLA_2, six enzymes were shown to possess strong anticoagulant activity and were designated as CM-AC-I to CM-AC-VI (Fig 4.1). The remaining six PLA_2 enzymes were devoid of anticoagulant activity and named as CM-II to CM-VII (Fig 4.1). A summary of isolation of basic phospholipase A_2 (PLA_2) enzymes from crude *Daboia russelli* venom is shown in the Table 4.1.

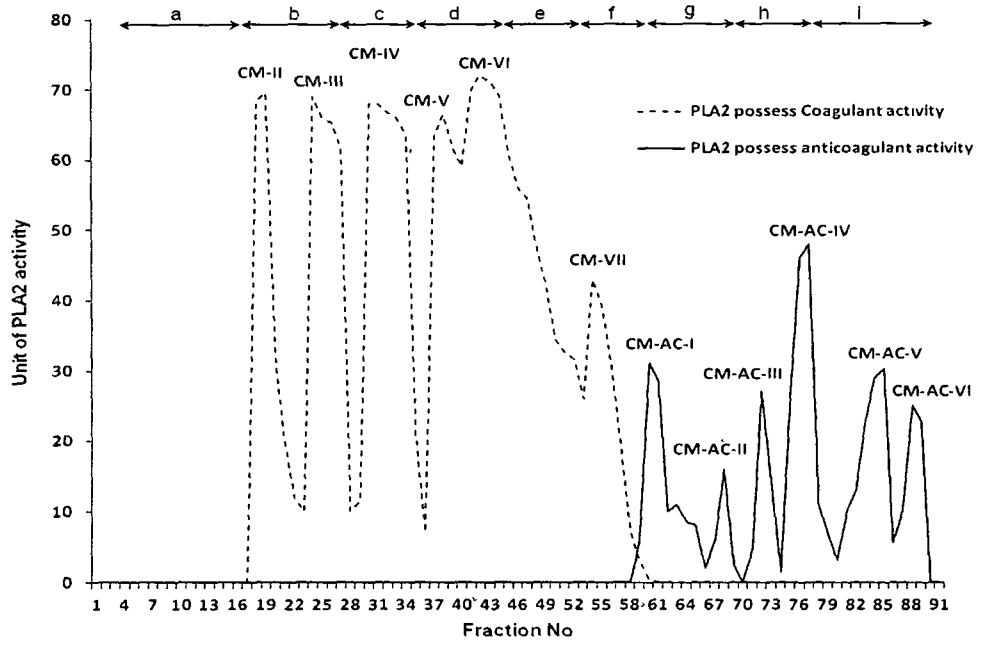

Fig 4.1: CM Sephadex C-50 chromatography profile of crude Russell's viper (*Daboia russelli*) venom. The column (20 mm X 60 mm) was pre-equilibrated with 20 mM K-phosphate buffer, pH 7.0 and loaded with 20.0 mg venom dissolved in 2.0 ml of the same buffer. The bound proteins were eluted stepwise using K-phosphate buffers of various molarities and pH values at room temperature (~23 °C). The increasing molarities and pH values were (a) 20 mM, pH 7.0, (b) 50 mM, pH 7.0, (c) 50 mM, pH 7.5, (d) 75 mM, pH 7.5, (e) 100 mM, pH 8.0, (f) 180 mM, pH 8.0, (g) 230 mM, pH 8.0, (h) 260 mM, pH 8.0 and (i) 300 mM, pH 8.0, at 23 °C. Fraction volume was 1 ml and flow rate was adjusted to 24 ml h^{-1}. The anticoagulant PLA_2s were detected after washing the column with 230 mM K-phosphate buffer, pH 8.0. Dotted line represents the PLA_2s with coagulant activity. Fractions number 1-17 were eluted with wash buffer and showed showing anticoagulant activity. These fractions were pooled and named CM-I.

Table 4.1: A Summary of isolation of basic PLA_2 enzymes of crude *Daboia russelli* venom through CM Sephadex C-50 column. Values are represented as mean ± S.D. of four experiments.

Peaks	% recovery of protein	PLA_2 specific activity* (Unit/mg of protein)	Anticoagulant specific activity** (Unit/mg of protein)	% Haemolysis	
				Direct	Indirect
Crude venom	100	$7.9 \pm 0.63 \times 10^3$	ND	1.6 ± 0.5	55.7 ± 0.5
Basic fractions					
CM-II	2.2	$8.4 \pm 0.21 \times 10^4$	ND	ND	44.3 ± 1.1
CM-III	1.1	$7.3 \pm 0.11 \times 10^4$	ND	ND	56.6 ± 2.1
CM-IV	0.9	$1.2 \pm 0.52 \times 10^4$	ND	1.0 ± 0.5	55.3 ± 2.3
CM-V	1.1	$5.7 \pm 0.71 \times 10^3$	ND	ND	48.8 ± 2.2
CM-VI	0.9	$7.5 \pm 0.31 \times 10^3$	ND	1.1 ± 0.5	51.9 ± 1.3
CM-VII	1.5	$3.4 \pm 0.25 \times 10^3$	ND	1.3 ± 0.8	46.6 ± 2.7

Continued in next page

Table 4.1 continue.

Peaks	% Recovery of protein	PLA_2 specific activity* (Unit/mg of protein)	Anticoagulant specific activity** (Unit/mg of protein)	% Haemolysis	
				Direct	Indirect
CM-AC-I	0.5	$6.4 \pm 0.51 \times 10^3$	$1.1 \pm 0.63 \times 10^4$	1.5 ± 0.9	53.1 ± 2.2
CM-AC-II	0.6	$1.3 \pm 0.11 \times 10^3$	$1.6 \pm 0.63 \times 10^4$	1.3 ± 1.1	52.5 ± 2.1
CM-AC-III	1.1	$3.8 \pm 0.28 \times 10^3$	$0.8 \pm 0.63 \times 10^4$	ND	50.7 ± 2.2
CM-AC-IV	1.5	$5.2 \pm 0.46 \times 10^4$	$7.1 \pm 0.63 \times 10^4$	1.1 ± 0.7	48.8 ± 3.2
CM-AC-V	0.7	$2.1 \pm 0.18 \times 10^4$	$1.1 \pm 0.63 \times 10^4$	0.7 ± 0.4	49.9 ± 21
CM-AC-VI	0.5	$4.1 \pm 0.63 \times 10^3$	$2.3 \pm 0.63 \times 10^4$	ND	45.3 ± 0.21

ND: Not Detected

* One unit of PLA_2 activity is defined as decrease in 0.01 absorbance in 10 min at 740 nm.

**One unit of anticoagulant activity is defined as increase in time (s) of clotting compared to clotting time of control plasma.

4.2 Fractionation of CM Sephadex C-50 unbound proteins through an anion exchanger to separate the neutral and acidic PLA_2 isoenzymes

The non-retained fractions of CM Sephadex C-50 column (eluted with 20 mM K-phosphate buffer, pH 7.0) were pooled together and named as CMI. For the purpose of isolation of the neutral and acidic isoenzymes, CMI was re-fractionated through an anion exchanger DEAE Sephadex A-50 column. This fractionation reveals the presence of one neutral PLA_2, named as CMIDEI and six acidic PLA_2s, namely CMIDEII to CMIDEVII (Fig 4.2). None of these neutral and acidic PLA_2 fractions showed anticoagulant activity in *in vitro* condition. A summary of isolation of neutral and acidic phospholipase A_2 (PLA_2) enzymes from RVV is shown in the Table 4.2.

Fig (Flow chart) 4.3 represents the flow chart of all the acidic, neutral and basic PLA_2 isoenzymes isolated from RVV by a combination of ion-exchange (cation exchange followed by anion exchange) chromatography. A total of 19 PLA_2 isoenzymes (twelve basic, one neutral and six acidic in nature) were identified from venom of Russell's viper of eastern India origin.

A comparison of the catalytic activity of the PLA_2 isoenzymes from RVV reveals that the acidic and neutral PLA_2 enzymes are catalytically more active as compared to their basic counterparts. However, crude RVV contains highest quantity/amount of basic PLA_2 enzymes followed by acidic and neutral PLA_2 enzymes (Fig 4.4). The basic PLA_2 isoenzymes constitute 61.0 % of the total RVV PLA_2s, whereas the acidic and the neutral anticoagulant PLA_2 isoenzymes constitute 32.5 % and 6.4 % of the total venom PLA_2s respectively (Fig 4.4).

All the PLA_2 isoenzymes (acidic, neutral and basic) of RVV have shown significant indirect haemolytic activity though they did not show considerable direct haemolytic activity against washed goat or human washed erythrocytes (Table 4.1 & Table 4.2).

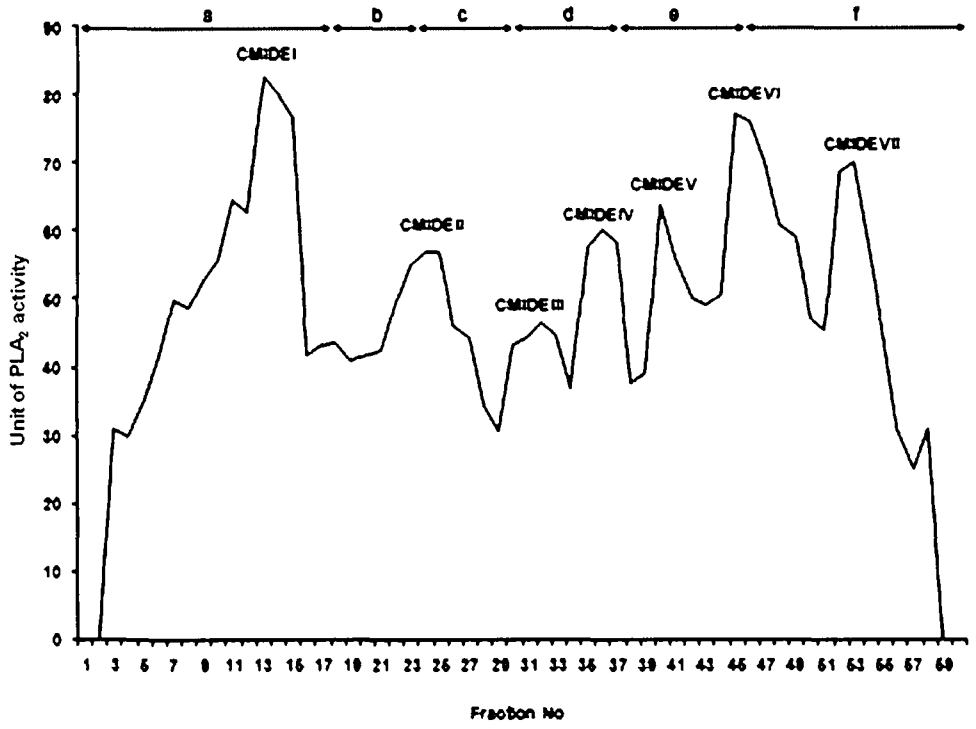

Fig 4.2: Fractionation of unbound proteins from CM Sephadex C-50 column (CMI) to DEAE Sephadex A-50 to separate the neutral and acidic PLA_2 enzymes of RVV. The column (20 mm x 60 mm) was equilibrated with 20 mM K-phosphate buffer (pH 7.0) and elution was carried out stepwise with K-phosphate buffers of various molarities and pH values at 23 °C - a) 20 mM, pH 7.0, (b) 20 mM, pH 6.5, (c) 20 mM, pH 6.0, (d) 20 mM, pH 5.5, (e) 100 mM, pH 5.0, (f) 120 mM, pH 5.0. Fraction volume was 1 ml and flow rate was adjusted to 22 ml h^{-1}.

Table 4.2: A Summary of isolation of neutral and acidic PLA_2 enzymes of crude *Daboia russelli* venom by fractionation of CMI through DEAE Sephadex A- 50 column. Values are represented as mean ± S.D. of four experiments.

Peaks	% Recovery of protein	PLA_2 specific activity* (Unit/mg of protein)	Anticoagulant specific activity** (Unit/mg of protein)	% Haemolysis	
				Direct	Indirect
Flow-through of CM Sephadex C-50 column					
CMI	4.1	$8.4 \pm 0.68 \times 10^3$	ND	1.5 ± 0.1	55.4 ± 1.8
Neutral PLA_2					
CMIDEI	0.98	$1.9 \pm 0.17 \times 10^4$	ND	0.9 ± 0.1	45.5 ± 1.5
Acidic PLA_2s					
CMIDEII	0.91	$2.3 \pm 0.22 \times 10^4$	ND	ND	46.0 ± 2.1
CMIDEIII	0.22	$1.6 \pm 0.14 \times 10^4$	ND	ND	46.1 ± 1.1
CMIDEIV	0.35	$1.8 \pm 0.16 \times 10^4$	ND	0.8 ± 0.2	42.1 ± 1.6
CMIDEV	0.49	$3.8 \pm 0.31 \times 10^4$	ND	1.6 ± 0.1	51.2 ± 2.4

Table 4.2 continue.

Peaks	% Recovery of protein	PLA_2 specific activity* (Unit/mg of protein)	Anticoagulant Specific activity** (Unit/mg of protein)	% Haemolysis	
				Direct	Indirect
CMIDEVI	0.51	$0.9 \pm 0.08 \times 10^4$	ND	ND	47.4 ± 1.2
CMIDEVII	0.61	$1.7 \pm 0.15 \times 10^4$	ND	1.2 ± 0.4	52.3 ± 2.3

ND: Not Detected

* One unit of PLA_2 activity is defined as decrease in 0.01 absorbance in 10 min at 740 nm.

**One unit of anticoagulant activity is defined as increase in time (s) of clotting compared to clotting time of control plasma.

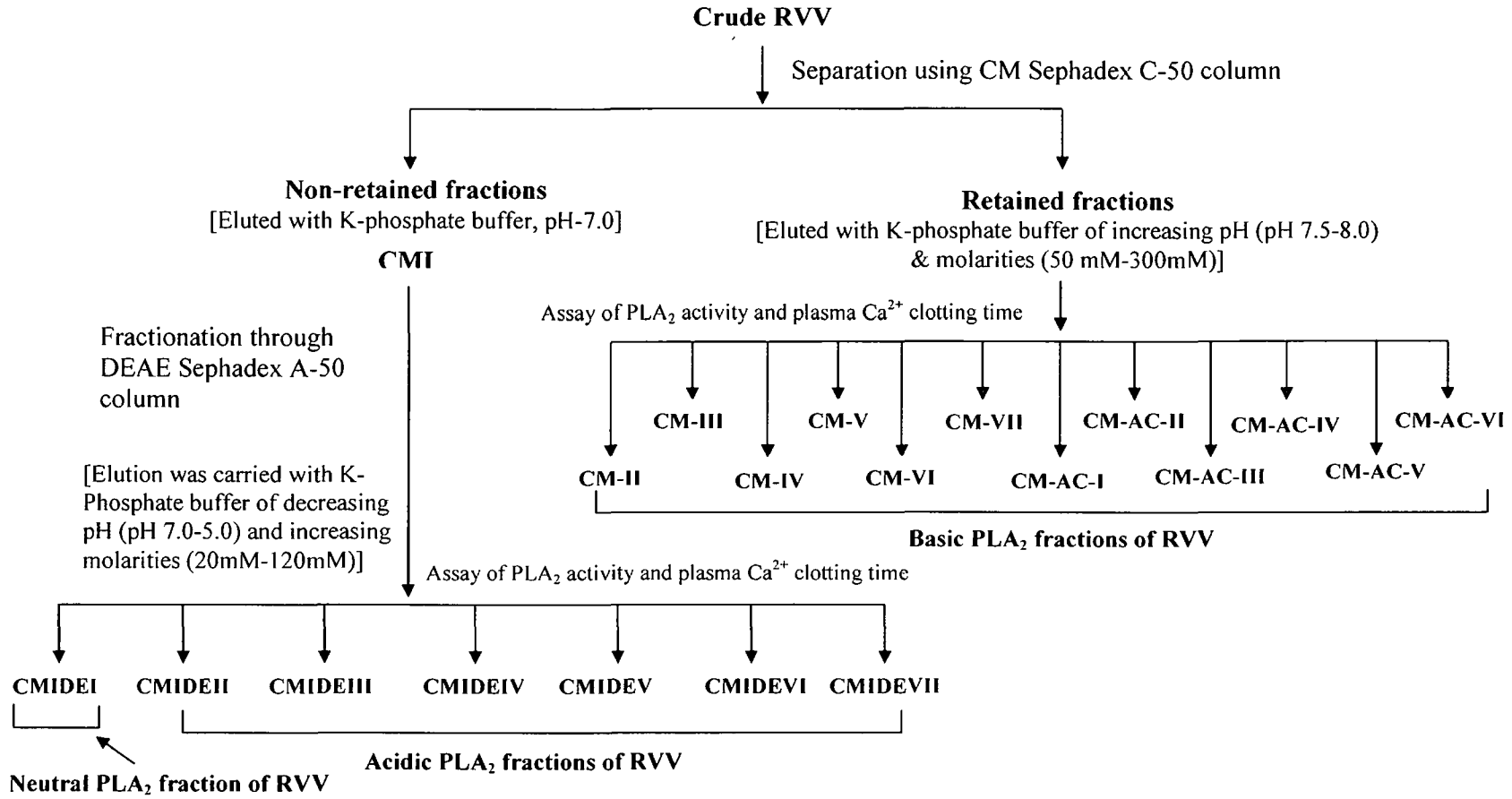

Fig 4.3: A flow chart showing the presence of different PLA_2 isoenzymes in venom of Russell's viper venom of eastern India origin. Fractionation was carried out as described in chapter –III (Materials and Methods), section 3.2.1.

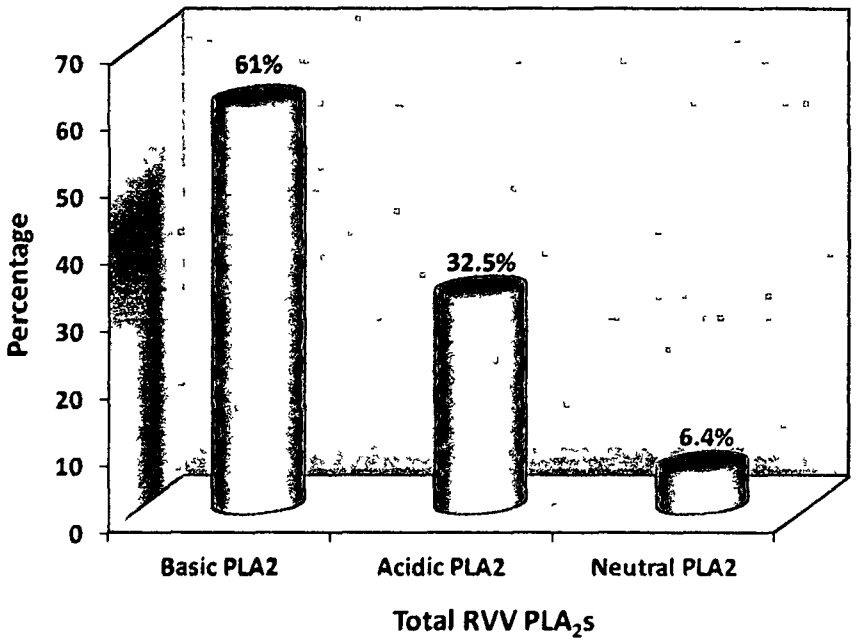

Fig 4.4: Percent contribution of basic, acidic and neutral anticoagulant PLA_2s in crude Russell's viper (*Daboia russelli*) venom.

CHAPTER V

Isolation, purification, biochemical and pharmacological characterisation of an acidic anticoagulant phospholipase A_2 (PLA_2) enzyme (RVVA-PLA_2-I) from venom of Russell's viper (*Daboia russelli*) of eastern India origin

5 Results

5.1 Purification of an acidic anticoagulant phospholipase A_2

5.1.1 Fractionation of crude RVV through cation exchanger followed by fractionation of CMI through anion exchanger

Crude RVV was fractionated through a cation exchanger CM Sephadex C-50 column and the non-retained fractions of this column were pooled and named as CMI (as described in the section 4.2). CMI was further fractionated through an anion exchanger DEAE Sephadex A-50 column that resolved into seven peaks; one was neutral in nature (named as CMIDEI) and other six were acidic in nature namely CMIDEII, CMIDEIII, CMIDEIV, CMIDEV, CMIDEVI and CMIDEVII (as described in the section 4.2 and Fig 4.2)

5.1.2 Fractionation of CMIDEV through gel filtration column Sephadex G-50

The peak CMIDEV, eluted with 100 mM K-phosphate, pH- 5.0 (Fig 4.2) showing the highest PLA_2 activity among the acidic fractions, was selected for further purification through a Sephadex G-50 (1 cm x 64 cm) gel filtration column. This was resulted in the separation of CMIDEV into three peaks viz. GFI to GFIII (Fig 5.1A). The fraction GFI showed highest PLA_2 as well as anticoagulant activity. A summary of purification of the gel filtration fractions (GFI to GFIII) of CMIDEV is shown in Table 5.1.

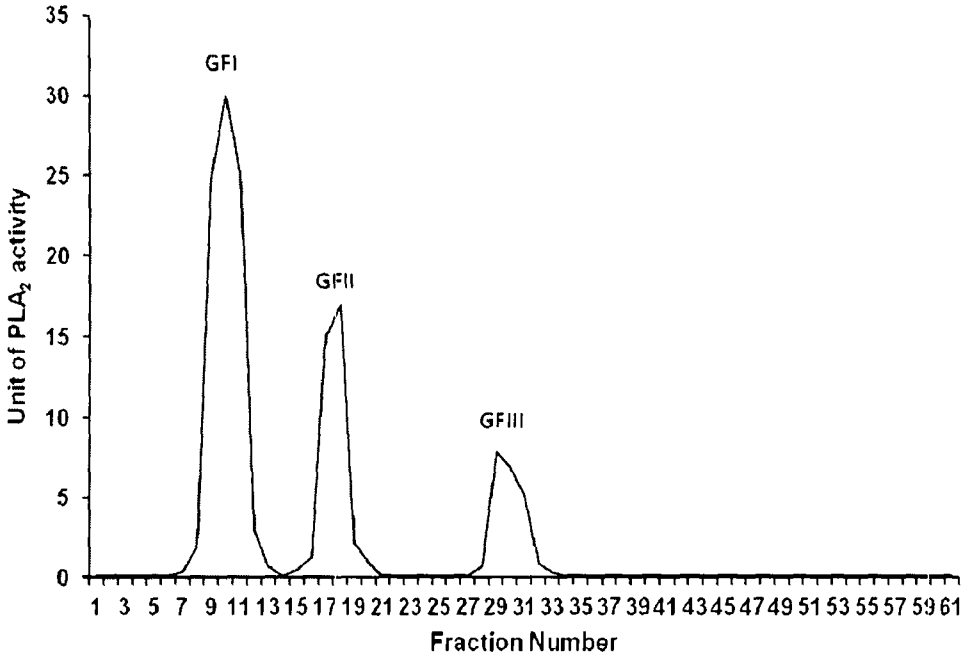

Fig 5.1A: Fractionation of CMIDEV through Sephadex G-50 gel filtration column (1 cm x 64 cm). Gel filtration fractionation was done as described in the section 3.2.2.1. Each protein peak was screened for PLA_2 activity.

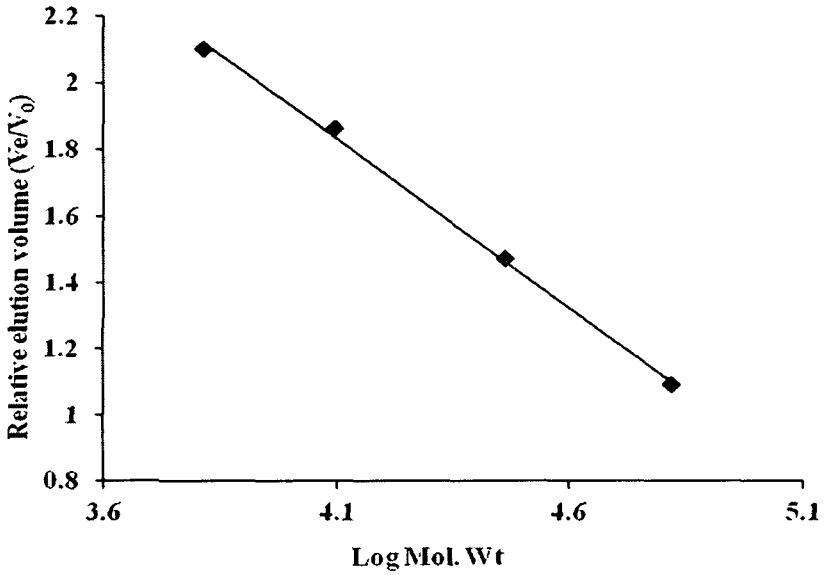

Fig 5.1B: Calibration curve of Sephadex G-50 column chromatography. The column was equilibrated with following molecular weight markers; aprotinin (6,500 Da), cytochrome C (12,400 Da), carbonic anhydrase (29,000 Da), BSA (66,000 Da) and blue dextran (2,00,000 Da).

Table 5.1: PLA_2 and anticoagulant activity of the three peaks eluted from Sephadex G-50 gel filtration column after the fractionation of CMIDEV. Values are from a typical experiment.

Peaks	% recovery of protein	PLA_2 specific activity	Anticoagulant Specific activity
GF-I	0.12	6.0×10^4	1.9×10^3
GF-II	0.05	2.1×10^4	0.6×10^3
GF-III	0.08	1.9×10^4	0.35×10^3

5.1.3 Purification of an acidic PLA_2 by RP-HPLC of GFI

The gel filtration fraction GFI showing highest PLA_2 and anticoagulant activity against PPP was further purified using RP-HPLC where it was eluted as a single sharp peak with a retention time of 12.7 min (Fig 5.2). This fraction displayed the PLA_2 as well as anticoagulant activities.

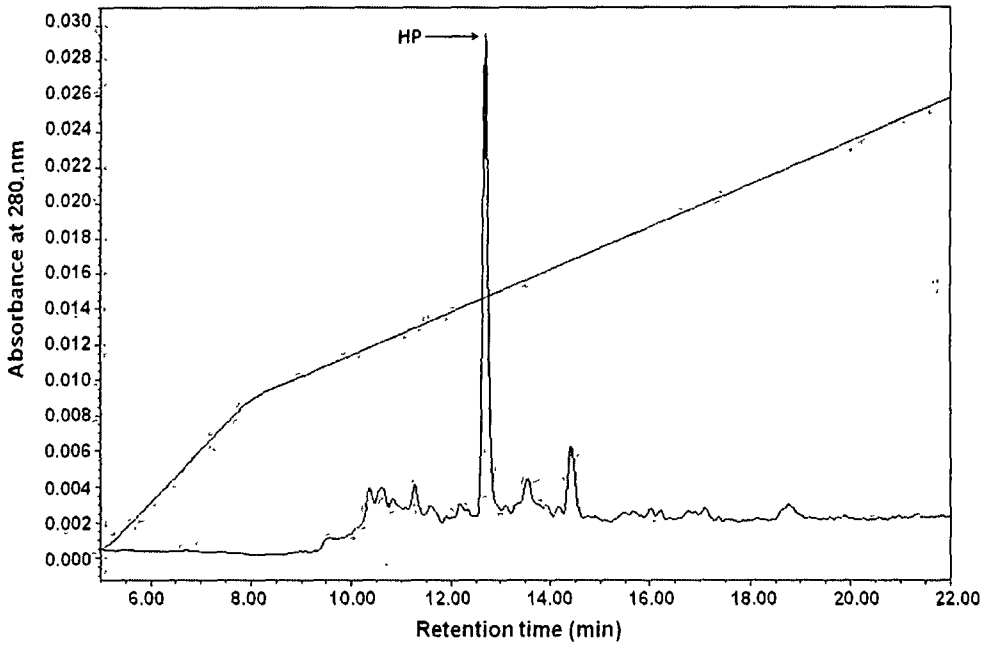

Fig 5.2: Separation of gel filtration fraction GFI on a Waters reverse-phase HPLC C_{18}-μ Nova Pak column. Solvents A and B were 0.1% (v/v) TFA in 5.0% (v/v) acetonitrile, and 0.1% TFA in 95% (v/v) acetonitrile, respectively. Column was washed with solvent A for 5 min and then bound proteins were eluted at a flow rate of 1 ml min^{-1} using a gradient of 5-95% solvent B (0.1% v/v TFA in 95% v/v acetonitrile) from 5 to 37 min. Detection was monitored at 280 nm and individual fraction was collected manually. The peak was screened for protein content, anticoagulant and PLA_2 activity. The protein (PLA_2 enzyme) was eluted with a retention time 12.7 min.

Table 5.2: Summary of purification of the acidic anticoagulant phospholipase A_2 (RVVA-PLA_2-I). Values are from a typical experiment. ND, not detected.

Fraction	Total protein (mg)	Protein yield (%)	PLA_2 activity		Anticoagulant activity		Purification fold	
			Total activity (Units)	Specific activity (Units / mg)	Total activity (Units)	Specific activity (Units/ mg)	PLA_2 activity	Anticoagulant activity
Crude RVV	20.0	100.0	1.3×10^5	1.2×10^4	ND	ND	1	-
CM-Cellulose	0.84	4.1	1.1×10^3	2.5×10^4	ND	ND	2.0	-
DEAE Sephadex	0.98	0.49	1.9×10^3	3.8×10^4	ND	ND	3.2	-
Gel filtration	0.235	0.12	7.2×10^2	6.0×10^4	23.8	1.9×10^3	4.9	1
RP-HPLC	0.014	0.1	5.9×10^2	8.4×10^4	36.8	5.3×10^3.	7.0	2.8

Yield of this protein was 0.1 % of the original venom load and was named as RVVA-PLA_2-I (Russell's viper venom acidic phospholipase A_2-I). A summary of purification of RVVA-PLA_2-I is shown in Table 5.2.

Up to a protein concentration of 25.0 μg ml^{-1}, no detectable protease, acetylcholine and adenosine monophosphate activity was associated with RVVA-PLA_2-I preparation documenting the purity of this PLA_2 isolation.

5.1.4 Assessment of purity and determination of molecular mass of RVVA-PLA_2-I

5.1.4.1 Gel filtration chromatography and SDS-PAGE

The molecular mass of the PLA_2 was checked by gel filtration chromatography, SDS-PAGE and ESI/MS analyses. By molecular sieve chromatography, the molecular mass of this acidic PLA_2 was estimated as 61.0 kDa. About 20 μg of RVVA-PLA_2-I gave a sharp, Coomassie Brilliant blue positive band on 12.5 % SDS-PAGE under both reduced and non-reduced conditions (Fig. 5.3). By SDS-PAGE, this protein appeared as a single band of 28.5 kDa after reduction with β-mercaptoethanol, but in absences of reducing agent and heating, it migrated as a single band of an apparent mass of 58.0 kDa (Fig 5.3) indicating that the purified PLA_2 is a homodimmer.

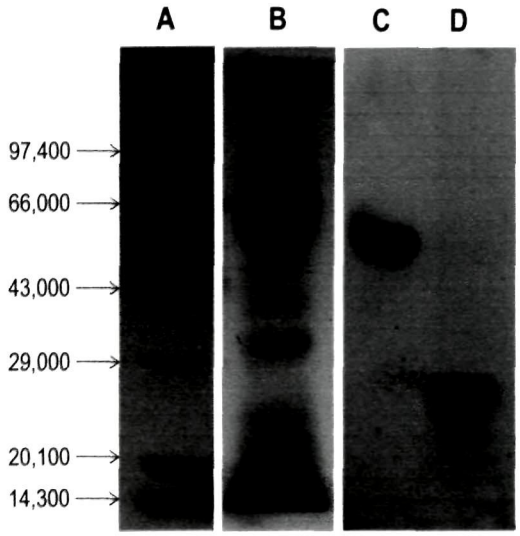

Fig 5.3: SDS-polyacrylamide gel electrophoresis. Purified RVVA-PLA_2-I (both reduced and non-reduced) and crude *D. russelli* venom were separated by 12.5% SDS-PAGE: Lane A) molecular weight marker; lane B) crude RVV (30 µg); lane C) 20 µg RVVA-PLA_2-I (non-reduced condition); lane D) 20 µg RVVA-PLA_2-I (reduced condition). Experiment was done as described in the section 3.2.5.3.

5.1.4.2 ESI/MS

Molecular mass of the purified acidic PLA_2 was determined as 28.588 kDa by ESI/MS (Fig 5.4).

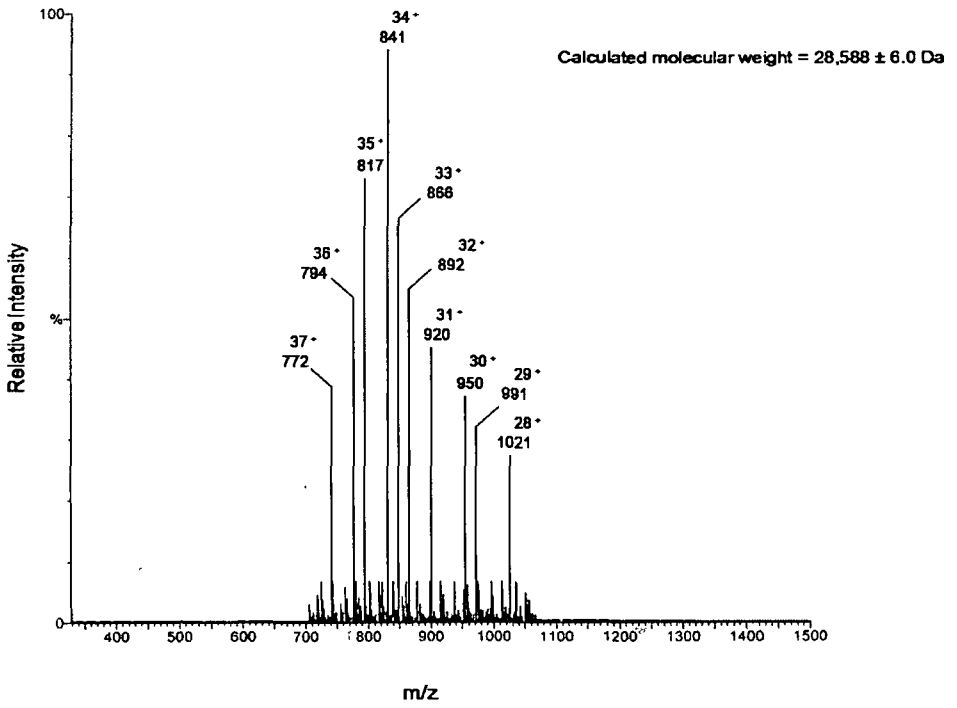

Fig 5.4: ESI/MS of RVVA-PLA$_2$-I for molecular mass determination. Experiment was done as described in the section 3.2.5.4.

5.2 Biochemical Characterisation

5.2.1 PLA$_2$ specific activity

RVVA-PLA$_2$-I showed PLA$_2$ activity against egg yolk phospholipids as substrate (Table 5.2).

5.2.2 Dose-dependent PLA$_2$ activity

The dose-dependent study demonstrated that the PLA$_2$ activity (hydrolysis of phospholipids) was increased linearly up to enzyme concentration

of 0.6 μg ml^{-1}; however, beyond this concentration, saturation in enzyme activity was observed (Fig 5.5).

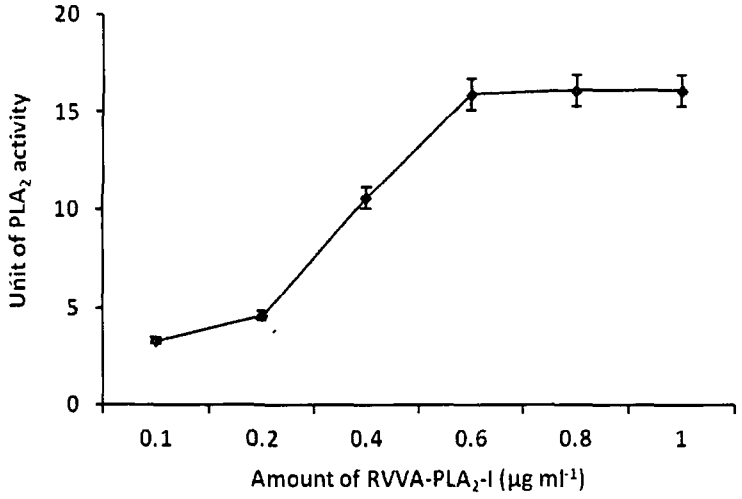

Fig 5.5: Dose-dependent catalytic activity of RVVA-PLA_2-I. Values represent mean ± S.D. of four experiments. Experiment was done as described in the section 3.2.6.8.3 by using different concentration of RVVA-PLA_2-I (0.1-1.0 μg ml^{-1}).

5.2.3 Carbohydrate content

The total carbohydrate content of RVVA-PLA_2-I, as determined by phenol-sulphuric acid method was found to be 14.5 microgram of glucose per mg of the enzyme.

5.2.4 Substrate Specificity of RVVA-PLA_2-I

To check the substrate specificity of RVVA-PLA_2-I, commercially available phospholipids with different head groups viz., PC, PS and PE were

used as substrates. The assay reveals that RVVA-PLA_2-I has highest specificity towards PC followed by PS and then PE (Table 5.3).

Table 5.3: Substrate specificity of the acidic anticoagulant PLA_2 (RVVA-PLA_2-I). Values are mean ± S.D. of four determinations. Experiment was done as described in the section 3.2.6.8.1.

Phospholipid Substrate (Final concentration 1 mM)	PLA_2 Specific activity (Unit* $mg^{-1}min^{-1}$)
Phosphotidylcholine (PC)	$9.6 \times 10^5 \pm 0.8$
Phosphotidylserine (PS)	$1.2 \times 10^5 \pm 1.1$
Phosphotidylethanolamine (PE)	$6.8 \times 10^3 \pm 1.4$

*Unit is defined as μg of FFA released min^{-1} at 37 °C.

5.2.4.1 Effect of substrate concentration on catalytic activity of RVVA-PLA_2-I

Effect of substrate concentration on catalytic activity of RVVA-PLA_2-I shows that (Fig 5.6) with an increase in the PC concentration, a corresponding enhancement of phospholipid hydrolysis was detected. A saturation in enzyme activity was reached at 2.0 mM PC (Fig 5.6).

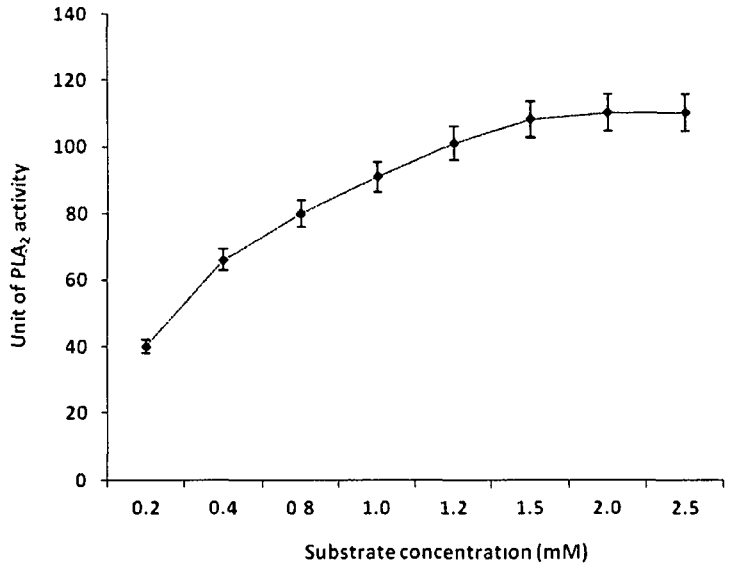

Fig 5.6: Effect of substrate concentration on PLA_2 activity of RVVA-PLA_2-I (0.5 µg ml^{-1}). Effect of substrate concentration on PLA_2 activity of RVVA-PLA_2-I was determined as described in the section 3.2.6.8.2. Results are expressed as mean ± S.D. of four determinations.

5.2.5 Determination of kinetics (Km and Vmax) of PC hydrolysis

By plotting the values of 1/v as a function of 1/[S], a straight line was obtained (Fig 5.7), that intersect the vertical line at a point which is the 1/Vmax (Since 1/[S] = 0. We have 1/v =1/Vmax). If we extend the straight line then the horizontal axis (1/[S]) intersect at the point which is -1/Km. The straight line was obtained with the following regression equation (Fig 5.7).

$y = 0.0228x + 0.0346$

When y=0, then x = -0.0346/0.0228 or, x= -1.52 i.e., -1/Km = 1.52,

i.e., Km = 0.65 mM or, 0.65 x 10^{-4} M i.e., Km = 0.65 x 10^{-4} M

1/Vmax = 0.0346 i.e., Vmax = 28.9 x 10^{-3} mM μg^{-1} or, 28.9 x 10^{-3} μM μg^{-1} or, 28.9 μmol mg^{-1}

The apparent Km and Vmax value of enzyme catalyzed reaction are 0.65 x 10^{-4} M and 28.9 μmol min^{-1} mg^{-1}, respectively.

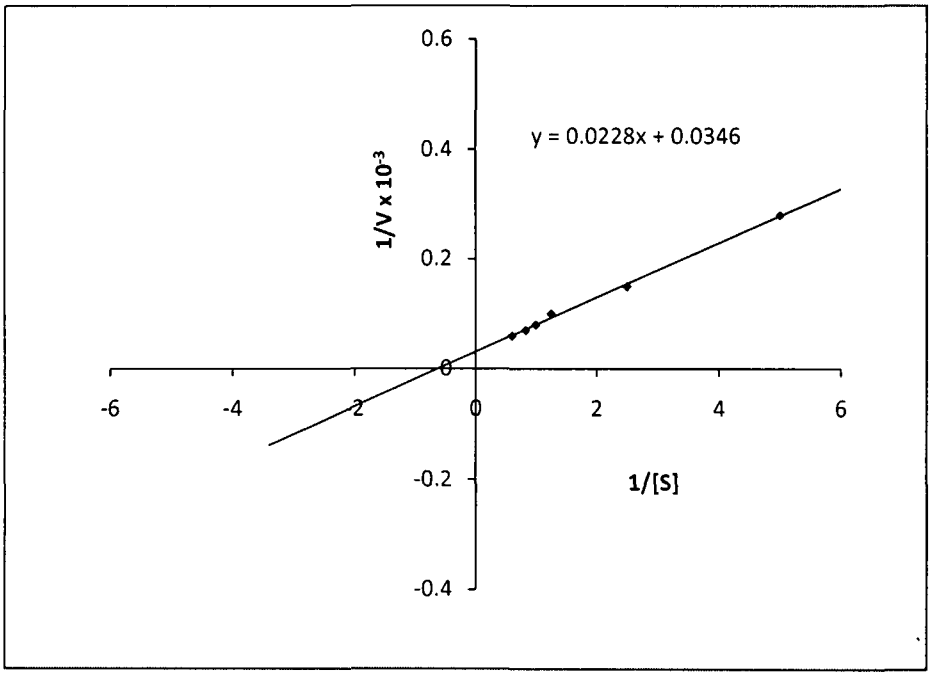

Fig 5.7: Determination of Km and Vmax of RVVA-PLA$_2$-I using Lineweaver-Burk plot. Purified PC (0.1-5.0 mM concentration) was used as substrate. Values are mean ± S.D. of four determinations. Experiment was done as described in the section 3.2.6.8.4.

5.2.6 Optimum temperature

The optimum temperature for enzymatic activity of RVVA-PLA_2-I was assayed at a temperature range of 20-75 °C using PC as substrate (Fig 5.8). RVVA-PLA_2-I showed optimum catalytic activity at temperature 37 °C (34.5 units).

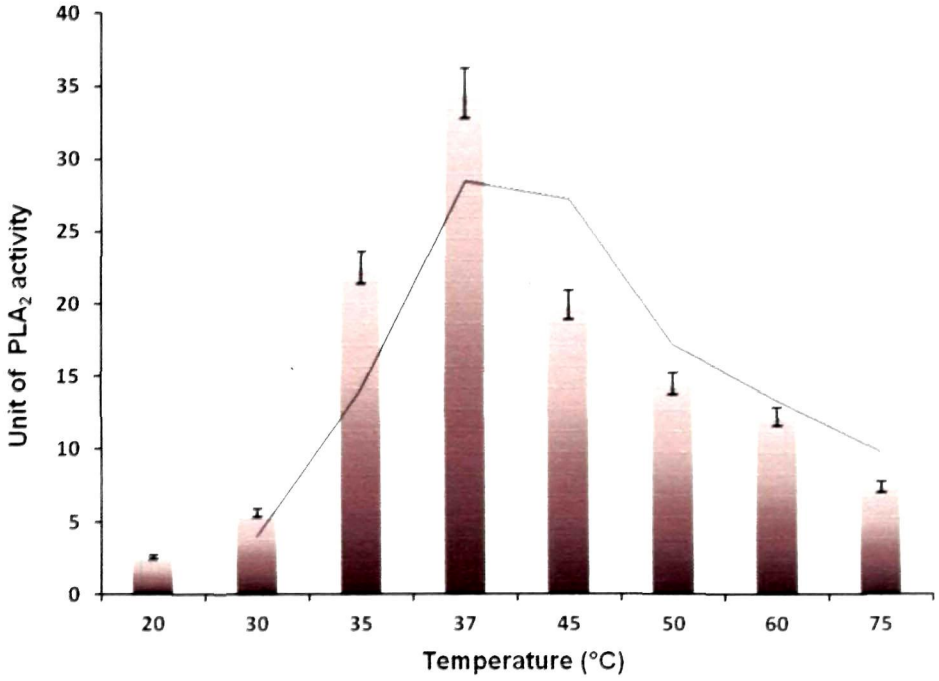

Fig 5.8: Effect of temperature on catalytic activity of RVVA-PLA_2-I. PLA_2 activity of RVVA-PLA_2-I at different temperature was done as described in the section 3.2.6.8.5. Results are expressed as mean ± S.D. of four determinations.

5.2.7 Optimum pH

The optimum pH for enzymatic activity of RVVA-PLA_2-I was assayed at a pH range of 5.5-9.0 using PC as a substrate (Fig 5.9). RVVA-PLA_2-I showed optimum catalytic activity at pH 8.0 (34.5 units). At pH 7.5, the enzyme activity was 39.6 % of the activity shown at pH 8.0 (Fig 5.9). RVVA-PLA_2-I did not show any catalytic activity at a pH lower and higher than 7.0 and 8.5, respectively.

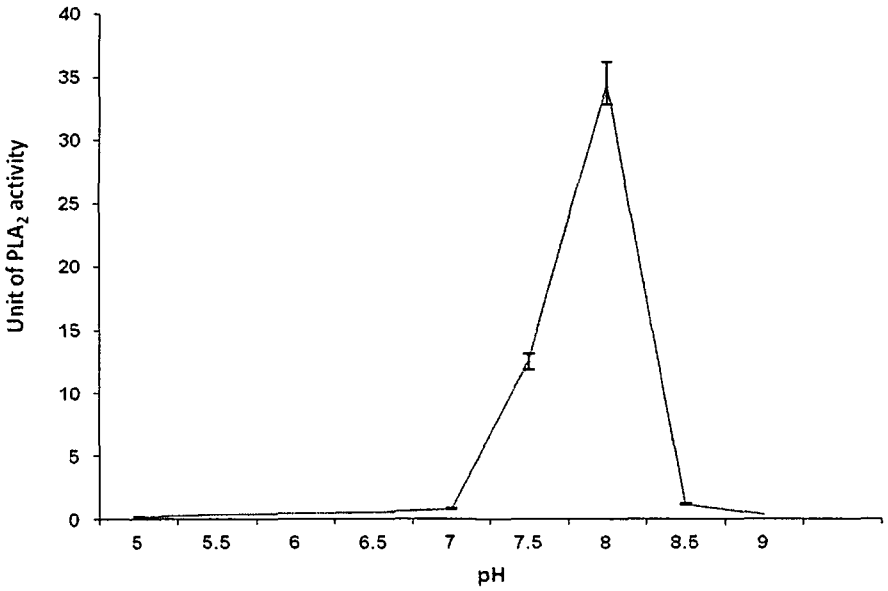

Fig 5.9: Effect of pH on catalytic activity of RVVA-PLA_2-I. PLA_2 activity of RVVA-PLA_2-I at different pH values were done as described in the section 3.2.6.8.6. The various pH values were obtained as follows: 0.1 M sodium acetate, pH 5.0-6.5; 0.1 M K-phosphate, pH 7.0-7.5 and 0.1 M Tris-HCl, pH 8.0-9.0. Results are expressed as mean ± S.D. of four determinations.

5.2.8 Determination of secondary structure: Circular dichroism spectroscopy

CD spectra of native RVVA-PLA_2-I demonstrated defined minima at 215 and 218 nm, indicating a strong α-helical contribution to the CD signal (Fig 5.10). The CD signal was not much affected after 45 min of heating of RVVA-PLA_2-I at 75 °C (Fig 5.10).

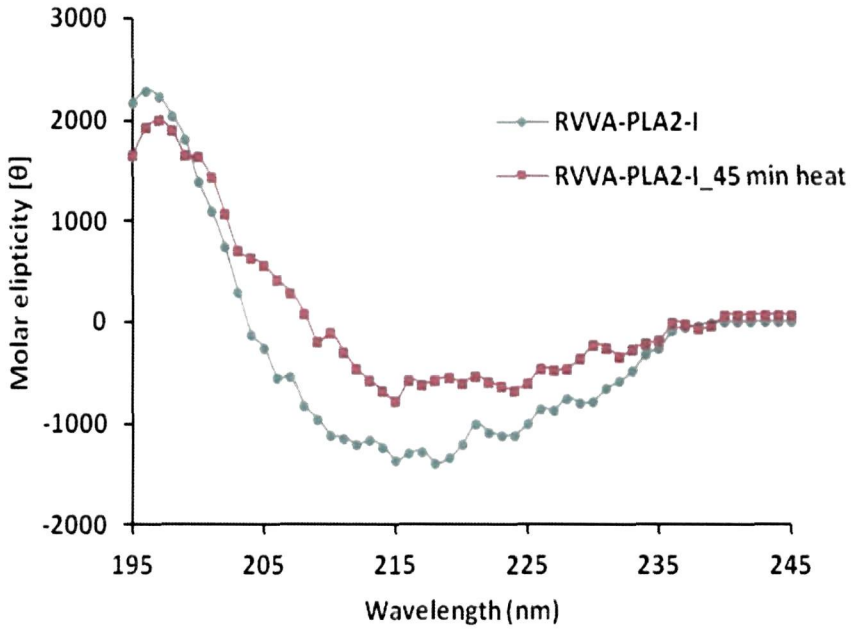

Fig 5.10: CD spectra of RVVA-PLA_2-I (100 nM). CD measurement was performed as described in the section 3.2.6.8.7. The CD signals are expressed as mean residue ellipticity [θ] (degree cm^2 $dmol^{-1}$), using 113 as the mean residue molecular weight.

5.3 Pharmacological Characterization

5.3.1 Anticoagulant activity

The dose-dependent anticoagulant activity of RVVA-PLA_2-I is shown in Fig. 5.11. RVVA-PLA_2-I even at a concentration of 0.3 μg ml^{-1} prolonged the normal clotting time of platelet poor goat plasma. It was observed that with an increase in concentration of RVVA-PLA_2-I up to 1.0 μg ml^{-1}, its anticoagulant activity (as determined by Ca-clotting time of PPP) was also enhanced linearly; however, a further increase in the concentration of RVVA-PLA_2-I (beyond 1.0 μg ml^{-1}) did not result in any increase in Ca-clotting time of PPP and a saturation curve was obtained (Fig 5.11).

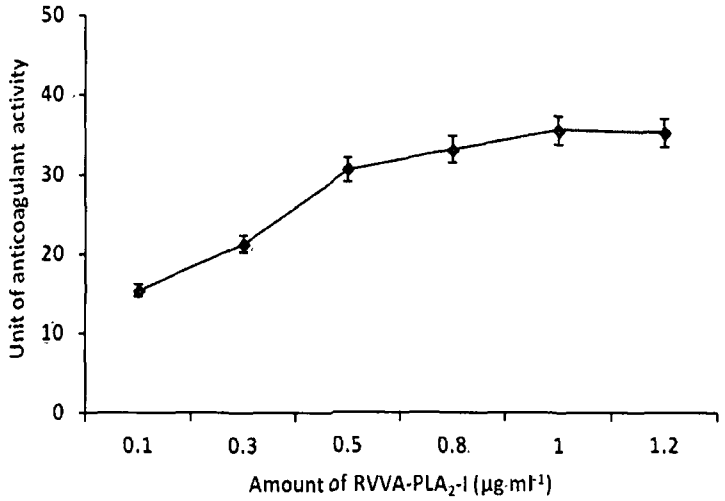

Fig 5.11: Dose dependent study of anticoagulant activity of RVVA-PLA_2-I (Ca-clotting time). This study was done as described in the section 3.2.7.1.1 by using different concentrations of RVVA-PLA_2-I (0.1-1.2 μg ml^{-1}). Values represent the mean ± S.D. of four experiments.

Effect of pre-incubation time of PPP with RVVA-PLA_2-I (0.5 μg ml^{-1}) on the anticoagulant activity demonstrated that 3-5 min was the optimum pre-incubation time necessary for exerting maximum anticoagulant activity. As depicted in Fig 5.12, it was observed that beyond 5 min pre-incubation of PPP with PLA_2 had no additional effect in increasing the Ca-clotting time of plasma (Fig 5.12).

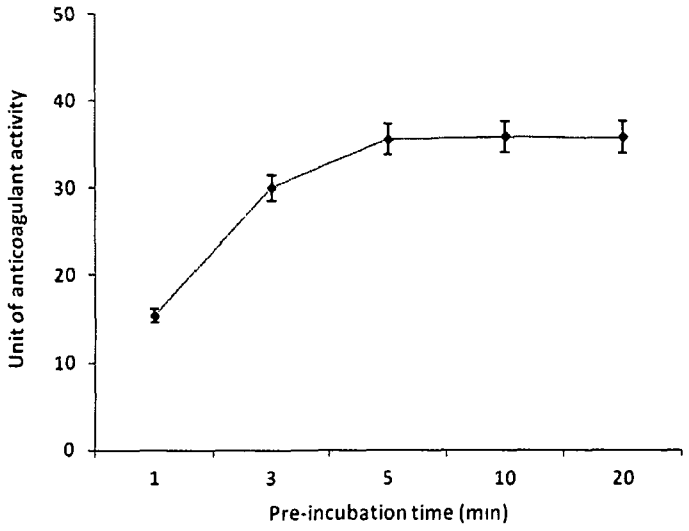

Fig 5.12: Effect of pre-incubation time on anticoagulant activity of RVVA-PLA_2-I (Ca-clotting time). This study was done as described in the section 3.2.7.1.1 for different time period (1-20 min). Values represent the mean ± S.D. of four experiments.

5.3.1.1 Prothrombin time test

To check the effect of RVVA-PLA_2-I on the inhibition of extrinsic pathway of blood coagulation, anticoagulant activity of this PLA_2 on PPP of goat blood was assayed using commercial prothrombin time test kit. RVVA-PLA_2-I was effective in delaying the coagulation time of PPP of goat (specific activity

was 1.6 x10^3 ± 0.9 unit mg^{-1} of protein) as compared to the coagulation time of control plasma.

5.3.1.2 Plasma phospholipids hydrolysis and FFAs release

Our study shows that with an increase in the pre-incubation time of plasma (1-5 min) with PLA_2, a concomitant increase in hydrolysis of plasma phospholipids was observed (Fig. 5.12). However, after 5 min of pre-incubation, no significant enhancement of plasma phospholipids hydrolysis could be detected (Fig 5.12). The GC-analysis of FFAs released post hydrolysis of plasma phospholipids with RVVA-PLA_2-I (0.5 μg ml^{-1}) at different time intervals also supported the above observation (Fig 5.13)

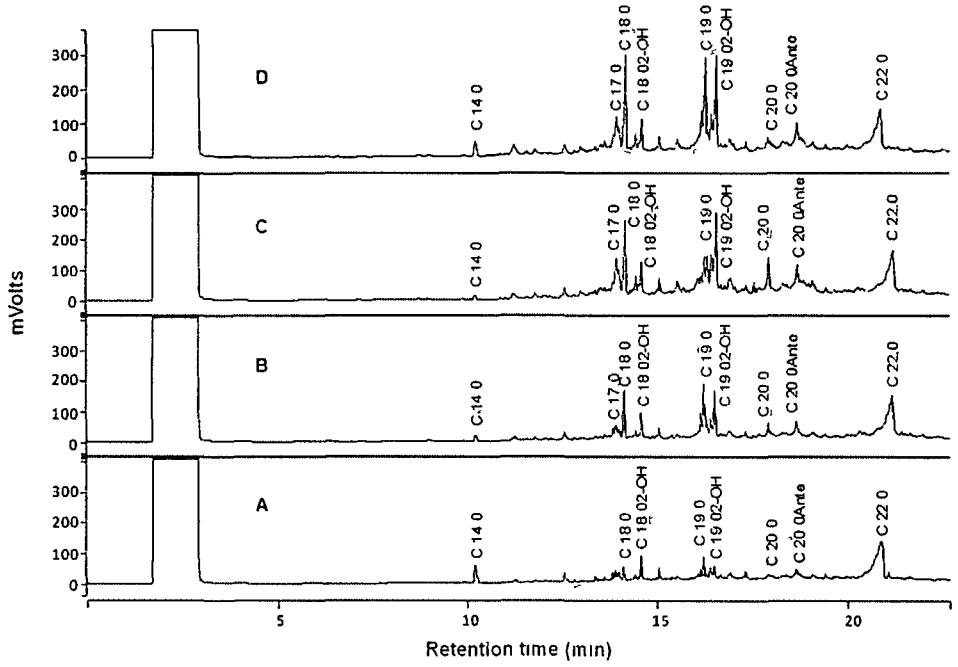

Fig 5.13: GC analysis of plasma phospholipids hydrolysis by RVVA-PLA_2-I: A) control B) 3 min, C) 5 min, D) 10 min post incubation with RVVA-PLA_2-I. This study was done as described in the section 3.2.7.1.3. Data shows a typical experiment and repetition of experiment demonstrated similar result.

5.3.1.3 Binding of RVVA-PLA_2-I with different phospholipids

Initially, the excitation of fluorescence of free PLA_2 (0.4 μM) was done at 280 nm and emission maximum were observed at 331.5 nm. Fluorescence intensity of PLA_2 after mixing with different phospholipids bearing different polar head groups exhibited different results. A large increase in the fluorescence intensity of PLA_2 in presence of PC was observed (Fig 5.14) and addition of 2 mM Ca^{2+} further enhanced the fluorescence intensity (Fig 5.15). Addition of PS and PE also resulted in an increase in the fluorescence intensity of RVVA-PLA_2-I but to a lesser extent than the intensity exhibited in presence of PC (Fig 5.14). When the binding experiment was performed with an equimolar mixture of PC: PS: PE (final concentration 50 μM; Fig 5.14), the emitted fluorescence intensity was also increased but it was definitely less than the fluorescence intensity shown in presence of PC alone (Fig 5.15).

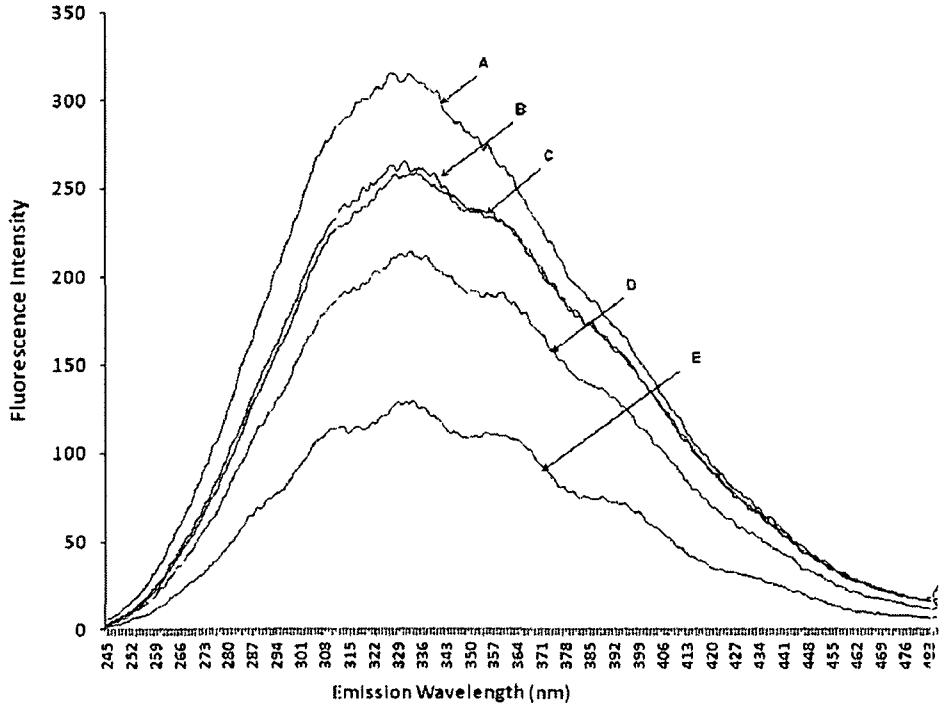

Fig 5.14: Interaction of RVVA-PLA$_2$-I (100 nM) with PC (50 µM), PS (50 µM) and PE (50 µM). A) RVVA-PLA$_2$-I and PC; B) RVVA-PLA$_2$-I and PC, PS and PE; C) RVVA-PLA$_2$-I and PS; D) RVVA-PLA$_2$-I and PE; E) RVVA-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.11.

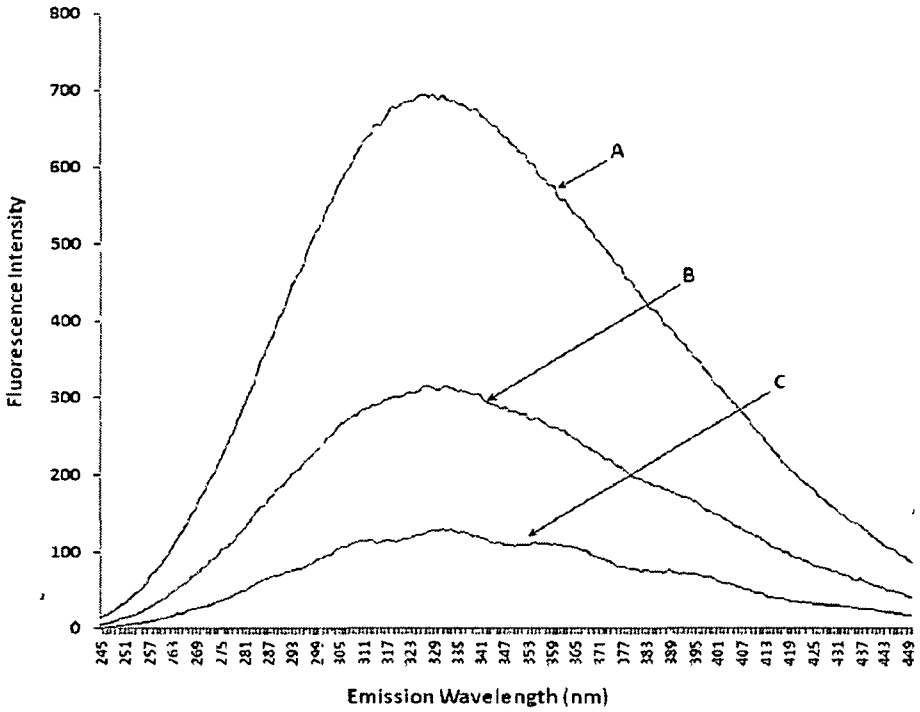

Fig 5.15: Interaction of RVVA-PLA$_2$-I (100 nM) with PC (50 µM) in presence of 2 mM Ca^{2+} A) RVVA-PLA$_2$-I, PC and Ca^{2+}; B) RVVA-PLA$_2$-I and PC; C) RVVA-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.11.

A comparison of PC binding property of native Vs heated-PLA$_2$ by spectroflurometric study revealed that phospholipid (PC) binding efficiency of the heat-inactivated PLA$_2$ was drastically reduced (69%) after 60 min of heating as compared to the PC binding potency of native PLA$_2$ (Table 5.13 and Fig 5.16).

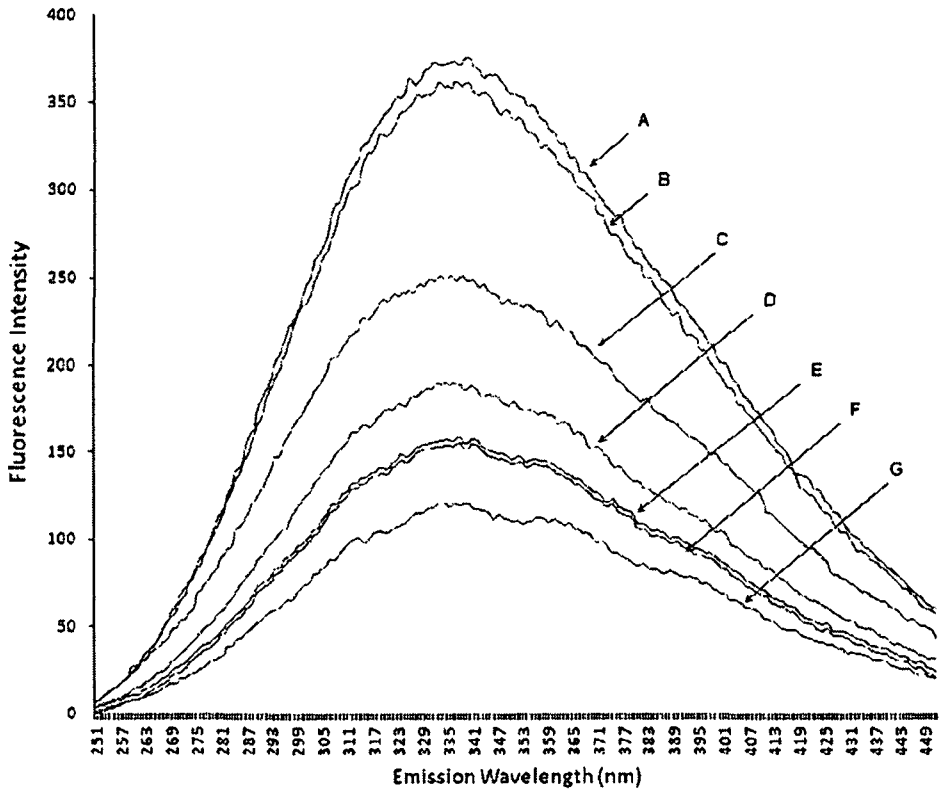

Fig 5.16: Spectroflurometric study of effect of heating on the binding of RVVA-PLA$_2$-I (100 nM) to phospholipid (PC, concentration 50 µM). A) RVVA-PLA$_2$-I and PC, B) heated RVVA-PLA$_2$-I (for 10 min) and PC, C) heated RVVA-PLA$_2$-I (for 20 min) and PC, D) heated RVVA-PLA$_2$-I (for 30 min) and PC, E) heated RVVA-PLA$_2$-I (for 60 min) and PC, F) RVVA-PLA$_2$-I, G) heated RVVA-PLA$_2$-I (for 60 min) without PC.

5.3.1.4 Binding of RVVA-PLA$_2$-I with blood coagulation factors

A significant decrease in the fluorescence signal of RVVA-PLA$_2$-I in presence of coagulation factor Xa was recorded (Fig 5.17). However, in a sharp contrast to this observation, no change in the fluorescence intensity of RVVA-PLA$_2$-I post incubation with activated factor Va, prothrombin and thrombin was observed indicating the acidic PLA$_2$ under study does not interact with these coagulation factors (Fig 5.18).

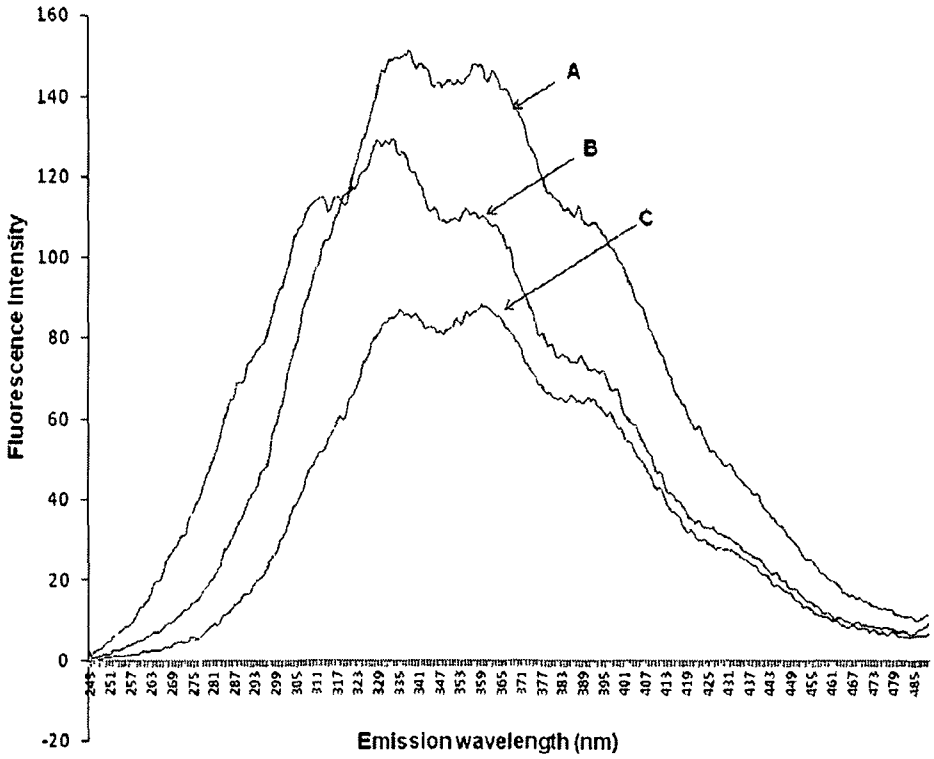

Fig 5.17: Fluorescence spectra showing interaction of RVVA-PLA$_2$-I (100 nM) with activated factor X (50 µM), (A) factor Xa, (B) RVVA-PLA$_2$-I, (C) activated factor X in presence of RVVA-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.11.

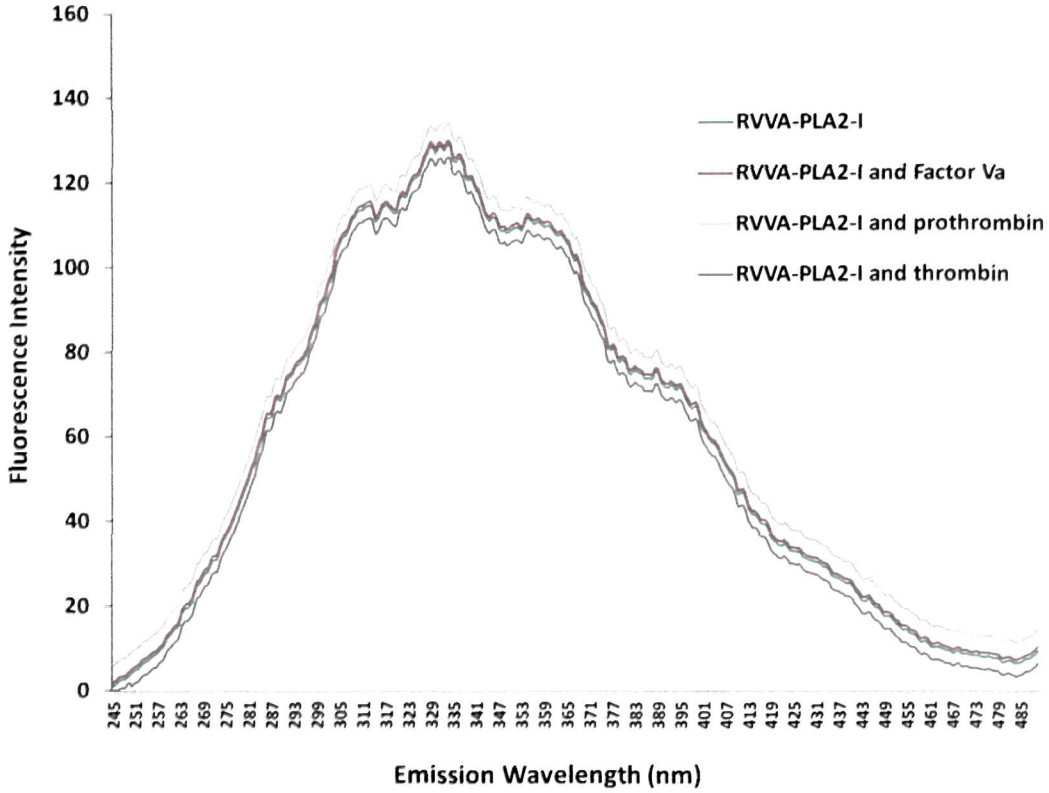

Fig 5.18: Fluorescence spectra showing interaction of RVVA-PLA$_2$-I (100 nM) with activated factor V (50 µM), prothrombin (50 µM) and thrombin (50 µM). Experiment was done as described in the section 3.2.6.8.11.

5.3.1.5 Prothrombin inhibition assay

By amidolytic activity assay of thrombin, it was observed that generation of thrombin from prothrombin by factor Xa in presence of RVVA-PLA$_2$-I was decreased significantly ($p < 0.05$) as compared to the thrombin generation in presence of factor Xa in absence of this acidic PLA$_2$ (Fig 5.19).

RVVA-PLA$_2$-I mediated inhibition of prothrombin activation was also analysed by 15% SDS-PAGE. In absence of RVVA-PLA$_2$-I, prothormbin was converted to thrombin by factor Xa (Fig 5.20). In a sharp contrast, presence of

RVVA-PLA$_2$-I in the reaction mixture inhibited the factor Xa to hydrolyse prothrombin to thrombin (Fig 5.20).

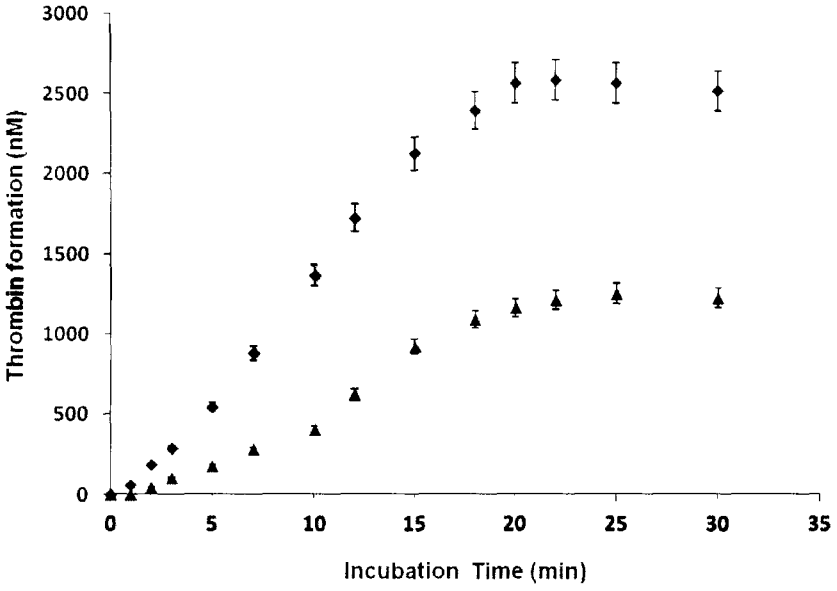

Fig 5.19: Inhibition of factor Xa -induced prothrombin activation by RVVA-PLA$_2$-I. Generation of thrombin (as determined by amidolytic activity assay of thrombin) from prothrombin (5 µg) in presence of factor Xa (50 µM) (♦); and in presence of RVVA-PLA$_2$-I (100 nM) treated factor Xa (50 µM) (▲). Values are mean ± S.D. of triplicate determinations. Experiment was done as described in the section 3.2.6.8.12.

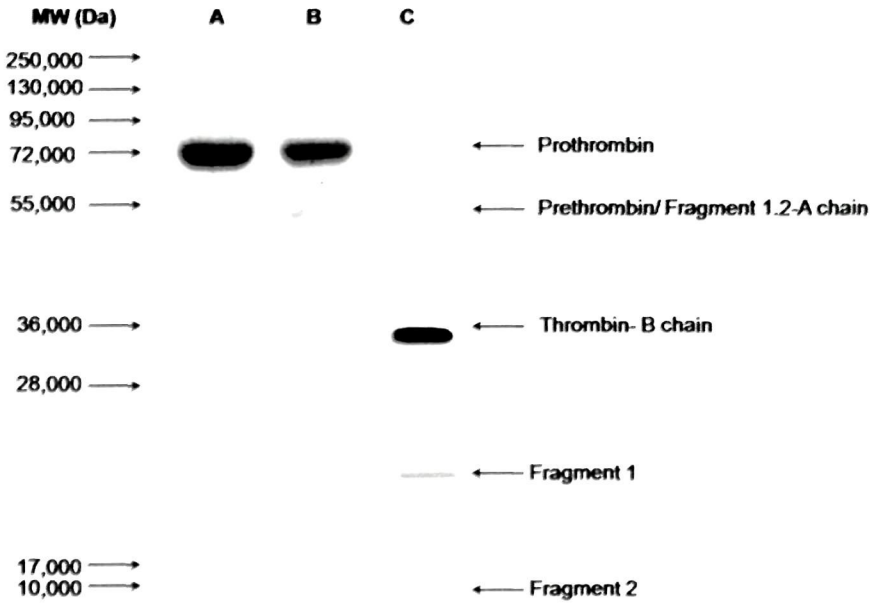

Fig 5.20: The inhibition of prothrombin activation as analyzed by 15% SDS-PAGE: Lane A) prothrombin (5 µg); lane B) prothrombin (5 µg) incubated with factor Xa (50 µM), Ca^{2+} ions (10 µM) and RVVA-PLA_2-I (100 nM); lane C) prothrombin (5 µg) treated with factor Xa (50 µM) and Ca^{2+} ion(10 µM) only. Experiment was done as described in the section 3.2.6.8.12.

5.4 Direct and indirect haemolytic activity of RVVA-PLA$_2$-I

RVVA-PLA$_2$-I did not show any significant direct haemolytic activity on washed goat/human washed erythrocytes, but showed an appreciable haemolytic activity in presence of egg yolk phospholipids which is a good source of PC (Fig 5.21 and Table 5.4). Erythrocytes pre-incubated with neutral phospholipid PC and 1.5 mM Ca^{2+} was highly susceptible to lysis induced by RVVA-PLA$_2$-I. On the other hand, pre-incubation with PS and PE had no influence on haemolytic activity of RVVA-PLA$_2$-I, irrespective of the presence or absence of 1.5 mM Ca^{2+} in the reaction medium (Fig 5.21).

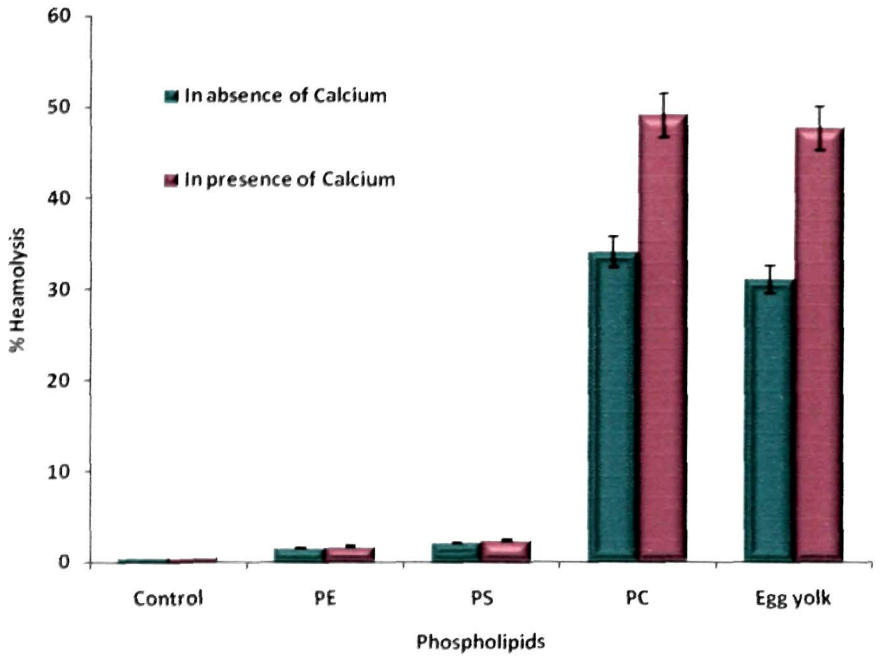

Fig 5.21: Effect of RVVA-PLA$_2$-I (100 nM) on erythrocytes exogenously supplemented with different phospholipids (PC/ PS/ PE at a final concentration of 1.0 mM and egg yolk at a concentration of 1 µg ml^{-1}) either in presence or absence of 1.5 mM Ca^{2+}. Experiment was done as described in the section 3.2.7.2. Lysis is expressed as percentage, considering 100 % haemolysis of erythrocytes when incubated with Milli Q water. Erythrocyte

suspension without exogenous supply of phospholipid(s) served as a control. Each result represents mean ±S.D. of four individual experiments.

5.5 *In-vitro* tissue damaging activity of RVVA-PLA$_2$-I

The *in-vitro* damage of liver, heart and lung tissues by RVVA-PLA$_2$-I (100 nM) at 37 °C was significantly less as compared to tissue damaging activity by crude Russell's viper venom (Table 5.4).

Table 5.4: *In vitro* tissue damaging activity of crude RVV and RVVA-PLA$_2$-I (% Hb released from 300 ± 10 mg tissue by 100 nM protein post 5 h of incubation at 37 °C)*. Values represent mean ±S.D. of four experiments.

Tissues of	Crude RVV (1 µg ml^{-1})	RVVA-PLA$_2$-I (100 nM)
Liver	4.5 ± 0.3^a	1.1 ± 0.3^b
Heart	11.8 ± 0.9^a	6.1 ± 0.7^b
Lungs	14.8 ± 0.7^a	0.1 ± 0.1^b

*100 % activity was achieved by treatment of tissues with 0.1% (v/v) triton X-100.

Values in the same row with different superscripts are significantly different ($p<0.05$).

5.6 Stability of RVVA-PLA$_2$-I at 4 °C

RVVA-PLA$_2$-I (kept in 20 mM K-phosphate buffer, pH 7.0) retained its catalytic and anticoagulant activity even after 28 days (when stored at 4 °C) of its isolation from crude RVV. However, after 28 days of storage, RVVA-PLA$_2$-I lost its original catalytic and anticoagulant activity up to 30 % and 54 % respectively (Fig 5.22).

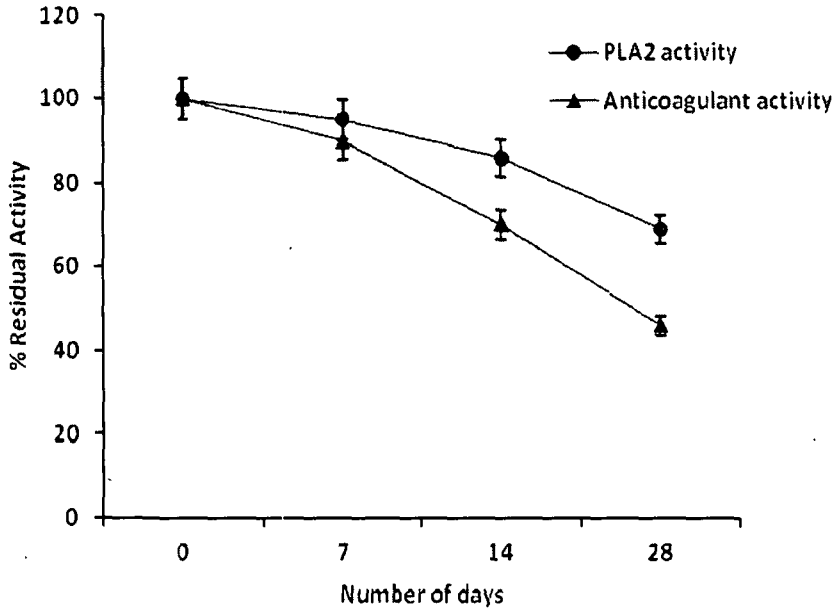

Fig 5.22: Stability of RVVA-PLA$_2$-I (at 4 °C). Storage stability was determined as described in the section 3.2.11. Values represent the mean ± S.D. of four experiments.

5.7 Membrane phospholipids hydrolysis by RVVA-PLA$_2$-I

5.7.1 Mitochondrial membrane phospholipids hydrolysis

RVVA-PLA$_2$-I demonstrated dose-dependent swelling of chicken liver mitochondria (Fig 5.23) and this effect was more pronounced in presence of 1.5 mM Ca^{2+} (Table 5.5).

With an increase in incubation time of mitochondria with RVVA-PLA$_2$-I, mitochondrial damage was significantly enhanced (Table 5.5). It was observed that RVVA-PLA$_2$-I hydrolyzed the outer membrane of mitochondria without any lag phase which was evident from the release of FFA and Pi from the membranes post incubation with this anticoagulant PLA$_2$ enzyme (Table 5.5).

Addition of Ca^{2+} pronounced the mitochondrial membrane hydrolysis effect of RVVA-PLA$_2$-I as was evident from the fact that the extent of phospholipids hydrolysis induced by this enzyme in presence of Ca^{2+} was about 1.4 fold higher compared to phospholipids hydrolysis in absence of Ca^{2+} after 15 min of incubation (Table 5.5).

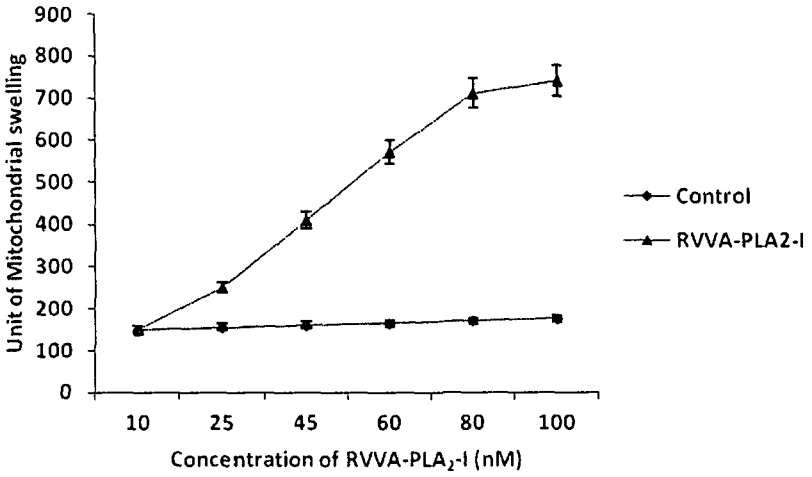

Fig 5.23: Dose dependent hydrolysis of mitochondrial membrane phospholipids by RVVA-PLA$_2$-I. Experiment was done as described in the section 3.2.7.5.1 by using different concentration of RVVA-PLA$_2$-I (10-100 nM). Values represent the mean ± S.D. of four experiments.

To gain further insight into the mode of attack of RVVA-PLA$_2$-I on the mitochondrial membrane, the GC-analysis of liberated fatty acids from membrane revealed that straight chain saturated fatty acids such as $C_{16\,0}$ and iso fatty acids like $C_{17\,0}$, $C_{18\,0}$, and $C_{19\,0}$ were the most prominent FFA released within the initial 30 min of attack by RVVA-PLA$_2$-I (Fig 5.24). With an increase in the incubation of mitochondria with the acidic PLA$_2$, a corresponding increase in membrane phospholipids hydrolysis was observed (Fig 5.24).

Addition of Ca^{2+} did not alter the fatty acid release pattern from the mitochondrial membrane; however, presence of this cation potentiated the membrane hydrolytic activity of RVVA-PLA$_2$-I resulting in release of higher quantity of fatty acids from the mitochondrial membrane by the action of PLA$_2$ enzyme under study (Table 5.5).

Table 5.5: RVVA-PLA$_2$-I induced swelling and phospholipids hydrolysis of intact mitochondrial membrane either in presence or absence of 2 mM Ca^{2+}. About 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissue) from chicken liver was incubated with 100 nM of RVVA-PLA$_2$-I at 37 °C for different time periods. The measured Pi value was obtained from acid treatment of a sample of the supernatant. Experiment was done as described in the section 3.2.7.5.1. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	Mitochondrial swelling (U/min)*		Phospholipids hydrolysis				Ratio of saturated/unsaturated FFA
			µg FFA released		µg of P_i released		
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
0 (control)	0	0	0	0	0		0
15	550 ± 2.5^a	700 ± 1.1^a	132.9 ± 0.9^a	159.5 ± 0.5^a	15.7 ± 2.6^a	17.1 ± 3.5^a	5.1 ± 0.5^a
30	900 ± 1.2^b	1150 ± 2.5^b	799.4 ± 1.2^b	1019.1 ± 1.3^b	77.8 ± 1.9^b	85.0 ± 4.2^b	7.1 ± 0.2^b
60	1350 ± 2.5^c	1650 ± 0.5^c	1503.8 ± 1.6^c	1954.9 ± 1.8^c	226.5 ± 1.3^c	263.4 ± 3.2^c	8.9 ± 0.8^c

*Mitochondrial swelling was measured spectrophotometrically and one unit of swelling is defined as a decrease in 0.01 absorbance/min of mitochondrial suspension at 520 nm by 100 nM of RVVA-PLA$_2$-I.

Values (for different time periods) with different superscripts in same column are significantly different ($p<0.05$).

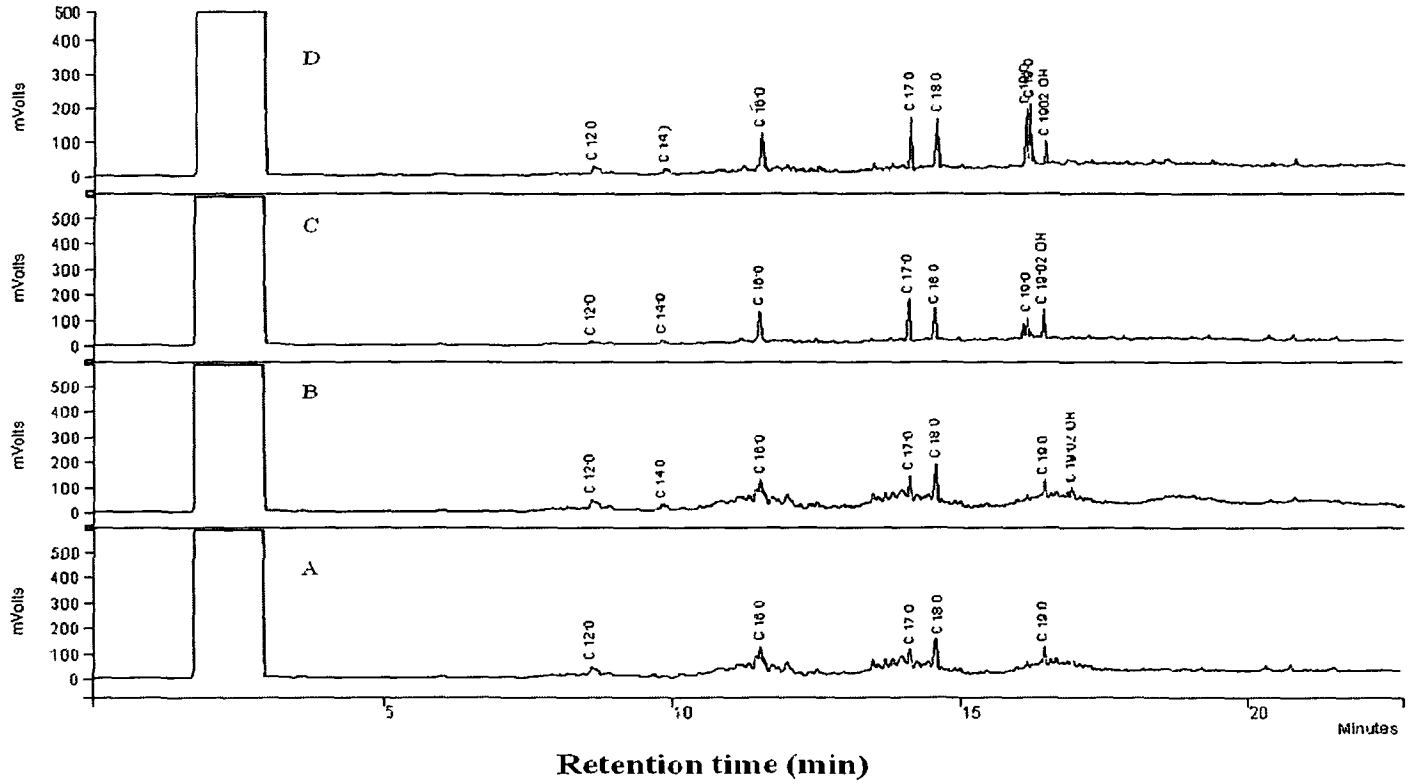

Fig 5.24: Liberation of FAs from intact mitochondrial membranes by RVVA-PLA$_2$-I (GC analysis).(A) Control, (B) 15 min, (C) 30 min, (D) 60 min post incubation of mitochondrial membranes with RVVA-PLA$_2$-I. Experiment was done as described in the section 3 2.7.5.2. Data shows a typical experiment and repetition of experiment demonstrated similar result.

5.7.2 Effect of RVVA-PLA$_2$-I on erythrocytes membrane phospholipids hydrolysis

Table 5.6 displays the effect of pre-incubation time of RVVA-PLA$_2$-I on haemolysis and liberation of FFAs from washed erythrocytes. Either with an increase in the incubation time of RBC with purified PLA$_2$ (Table 5.6) or increasing the concentration of PLA$_2$ (Fig 5.25), a concurrent enhancement of release of FFAs and Pi from the RBC membrane was observed suggesting an increase in membrane phospholipids hydrolysis by added PLA$_2$. It is worthy to mention that during the initial 30 min of attack, haemolysis could not be observed although the PLA$_2$ was able to release the FFA and lysophospholipids from intact RBC membrane. In contrast, addition of egg-yolk phospholipids (a source of PC) to the erythrocytes suspension resulted in initiation of haemolysis within 15 min of incubation and after 120 min, about 47.6 % of total RBC were haemolysed. Approximately 12.5-fold increase in FFA release from RBC membrane was observed post 120 min of incubation with RVVA-PLA$_2$-I as compared to post 15 min of incubation under the identical condition (Table 5.6).

The GC analysis of erythrocytes membrane phospholipids hydrolysis by RVVA-PLA$_2$-I (Fig 5.26) also supported the FFA release pattern as shown in Table 5.7. It was observed that RVVA-PLA$_2$-I had a specific preference for releasing the saturated straight chain fatty acids such as $C_{14:0}$, $C_{18:0}$, and iso fatty acids for example, $C_{18:0}$ and $C_{19:0}$ from the intact RBC membrane post 30 min of incubation. However, a quantitative as well as qualitative differences in fatty acids release pattern from membrane was detected after 120 min of incubation of RBC with RVVA-PLA$_2$-I, since additional FFAs of chain length $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{19:0}$ and few unidentified fatty acids (>C_{20}) could also be detected by GC (Fig 5.26).

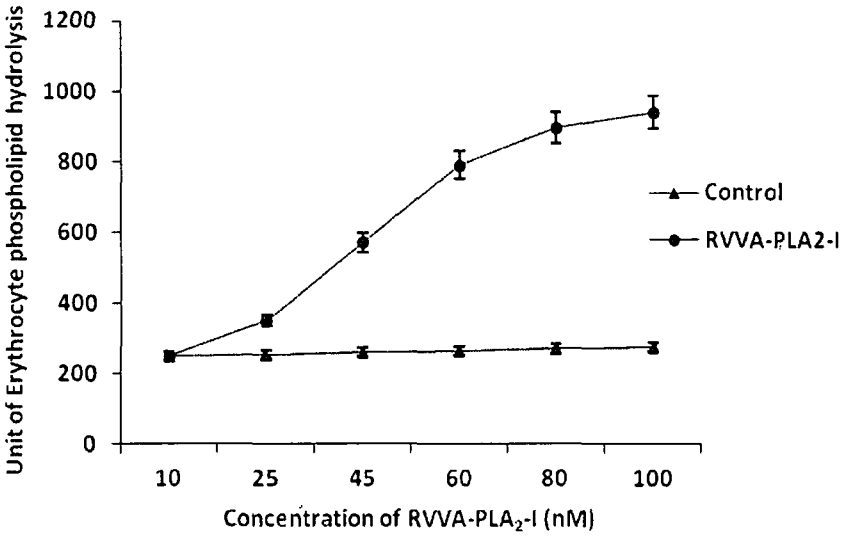

Fig 5.25: Dose dependent hydrolysis of erythrocyte membrane phospholipids by RVVA-PLA$_2$-I. Experiment was done as described in the section 3.2.7.5.1 by using different concentration of RVVA-PLA$_2$-I (10-100 nM). Values represent the mean ± S.D. of four experiments.

Table 5.6: RVVA-PLA$_2$-I induced haemolysis and phospholipids hydrolysis of goat washed erythrocytes. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVA-PLA$_2$-I at 37 °C and haemolysis (direct and indirect) and erythrocyte phospholipids hydrolysis were determined. Indirect phospholipids hydrolysis was achieved in presence of PC (1 mM). The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	% haemolysis		Phospholipids hydrolysis		Ratio of saturated/unsaturated FFA
	Direct	Indirect	µg FFA released	µg of P_i released	
Control	0	0	0	0	0
15 min	0	0	202.5 ± 2.1^a	28.4 ± 0.9^a	0.68 ± 0.3^a
30 min	0	34.5 ± 0.45^b	1015.3 ± 2.5^b	163.2 ± 1.2^b	0.86 ± 0.4
60 min	0.6 ± 0.03^c	42.1 ± 0.45^c	2045.1 ± 1.4^c	253.2 ± 1.7^c	1.64 ± 0.8
120 min	1.9 ± 0.09^d	47.6 ± 0.12^d	2531.9 ± 1.9^d	302.3 ± 1.1^d	1.91 ± 0.1^d

Values (for different time periods) with different superscripts in same column are significantly different ($p<0.05$).

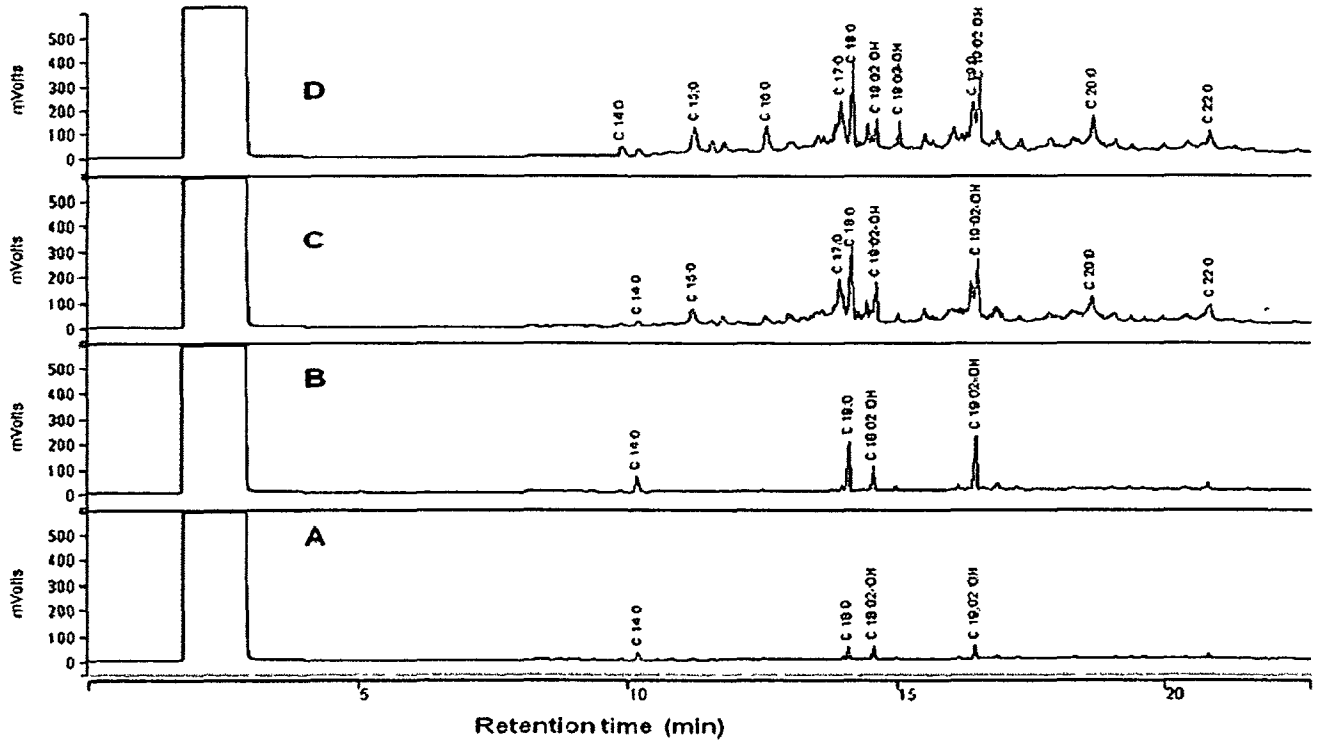

Fig 5.26: Kinetics of erythrocyte membrane phospholipids hydrolysis by RVVA-PLA$_2$-I. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVA-PLA$_2$-I (in presence of 2 mM Ca^{2+}) at 37°C for different time periods. The liberated fatty acids were analyzed by GC-MS as described in the section 3.2.7.5.2. A) control, B) 30 min, C) 60 min, D) 120 min after incubation of erythrocytes membranes with RVVA-PLA$_2$-I.

5.7.3 Binding study of RVVA-PLA$_2$-I with membrane phospholipids

The membrane binding property of native and heat-inactivated PLA$_2$ was further confirmed by ELISA experiment which revealed that about 65 ± 2 % (mean ± S.D., n=3) of RVVA-PLA$_2$-I (heated for 60 min at 75 °C) could bind to intact mitochondrial (Fig 5.27) or erythrocytes suspensions (Fig 5.28) as compared to binding of native (unheated) PLA$_2$ enzyme. In contrast, native and histidine-modified PLA$_2$ were captured by intact erythrocytes and mitochondria to the same extent (P > 0.05) (Table 5.13).

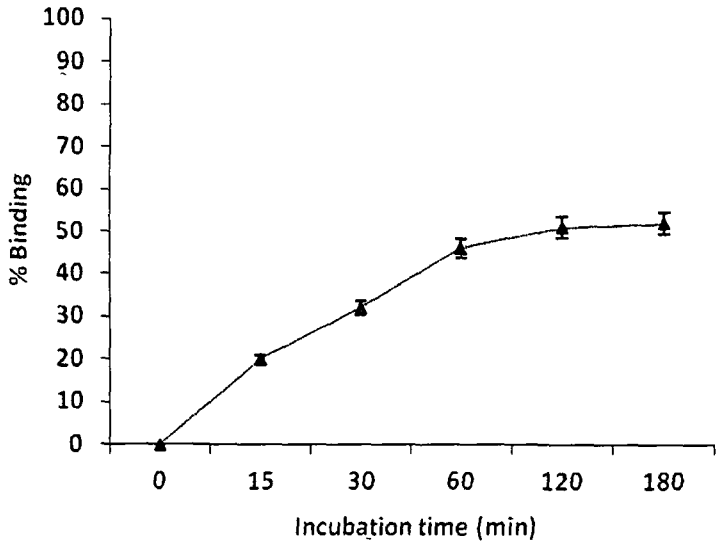

Fig 5.27: Binding of heat inactivated RVVA-PLA$_2$-I (heated for 60 min at 75 °C) with mitochondrial membranes. Values are ± S.D. of four experiments. Experiment was done as described in the section 3.2.7.5.3. Binding of native PLA$_2$ with membrane was considered as 100 % binding and then other values were calculated.

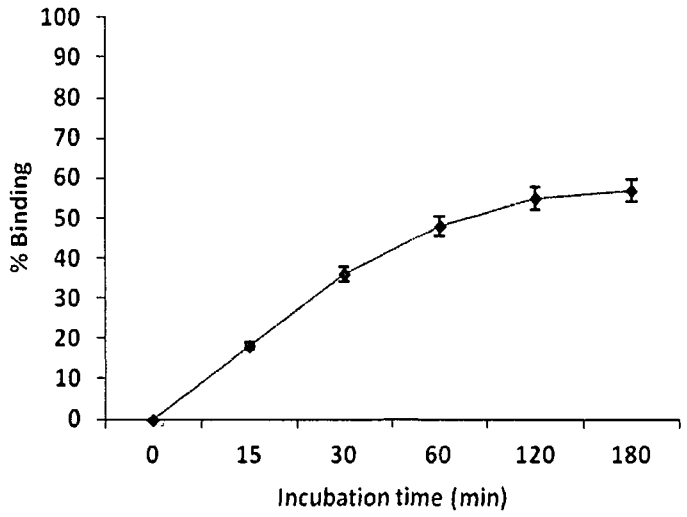

Fig 5.28: Binding of heat inactivated RVVA-PLA$_2$-I (heated for 60 min at 75 °C) with erythrocyte membranes. Values are ± S.D. of four experiments. Experiment was done as described in the section 3.2.7.5.3. Binding of native PLA$_2$ with membrane was taken as 100 % binding and then other values were calculated.

5.8 Effects of chemical inhibitors, antivenom and temperature on catalytic and pharmacological properties of RVVA-PLA$_2$-I

Different serine inhibitors viz. TPCK, TLCK and PMSF did not affect the catalytic activity and pharmacological properties of RVVA-PLA$_2$-I (Table 5.7). However, modification of enzyme with pBPB (a modifier of histidine residue) drastically reduced the catalytic, anticoagulant as well as the membrane damaging activities of RVVA-PLA$_2$-I indicating the presence of histidine in the active site of the PLA$_2$ enzyme (Table 5.7). The metal chelator EDTA (2.0 mM final concentration) significantly inhibited the catalytic, anticoagulant and membrane phospholipids hydrolyzing activity of PLA$_2$. Nevertheless, the inhibition of anticoagulant activity of the enzyme was more pronounced as

compared to inhibition of mitochondrial and erythrocyte membrane phospholipids hydrolysis properties of RVVA-PLA_2-I (Table 5.7). It was observed that DTT inhibited the catalytic, anticoagulant and membrane damaging activity of RVVA-PLA_2-I almost to the same extent. Nevertheless, IAA exerted more inhibition of catalytic and membrane hydrolysing activity compared to anticoagulant activity of RVVA-PLA_2-I (Table 5.7).

As shown in table 5.8, commercially available polyvalent antivenom at a ratio of 1:500 neutralized the catalytic activity and anticoagulant activity of RVVA-PLA_2-I to 24.6 % and 45.2 % respectively of its original activity (Table 5.7). The polyvalent antivenom neutralized the mitochondrial and erythrocyte membrane phospholipids hydrolyzing activity of RVVA-PLA_2-I in a dose-dependent manner (Table 5.7).

Heat inactivation study showed that heating the enzyme at 75 °C for 60 min could inhibit 29.2 % of catalytic activity of RVVA-PLA_2-I whereas under the same experimental condition, it inhibited 34.5 % of anticoagulant activity of the purified PLA_2 enzyme (Table 5.7). Similarly, heat affects the membrane phospholipids hydrolysis activity of RVVA-PLA_2-I as shown in the Table 5.7. Similarly, PC binding capacity of RVVA-PLA_2-I was also affected by heating the enzyme at 75 °C (Table 5.7).

Table 5.7: Effects of heating, antivenom and chemical inhibitors (at a final concentration of 2.0 mM) on catalytic, anticoagulant and membrane phospholipids hydrolysis activities of RVVA-PLA$_2$-I (100 nM). Values are mean ± S. D. of triplicate determinations. MM: mitochondrial membrane, EM: erythrocyte membrane.

Treatment	% residual activity				PC Binding (%)
	PLA$_2$	Anticoagulant	MM hydrolysis	EM hydrolysis	
Control	100	100	100	100	100
Heating at 75°C					
10min	97.0 ± 4.8	95.0 ± 4.7	97.5 ± 4.8	95.9 ± 4.7	97.5 ± 4.8
20min	94.5 ± 4.7	92.0 ± 4.6	95.1 ± 4.7	91.8 ± 4.6	91.4 ± 4.6
30min	90.5 ± 4.5	87.1 ± 4.3	89.0 ± 4.5	88.5 ± 4.4	86.6 ± 4.3
45min	85.4 ± 4.3	78.6 ± 3.9	79.4 ± 3.9	74.4 ± 3.7	75.1 ± 3.8
60min	70.8 ± 3.5	65.5 ± 3.2	69.5 ± 3.4	67.2 ± 3.3	69.0 ± 3.4
Antigen: antivenom (w:w)					
1:100	90.1 ± 4.5	93.1 ± 4.6	89.9 ± 4.5	85.8 ± 4.3	-
1:200	84.4 ± 4.2	78.1 ± 3.9	76.8 ± 3.8	66.7 ± 3.3	-
1:500	75.4 ± 3.8	54.8 ± 2.7	55.3 ± 2.7	51.6 ± 2.6	-
Chemicals/ inhibitors					
TPCK	97.8 ± 1.9	100	96.0 ± 1.8	96.5 ± 4.7	-
TLCK	94.9 ± 1.7	99.7 ± 1.9	97.2 ± 1.9	100	-
pBPB	9.7 ± 0.5	8.9 ± 0.4	15.4 ± 0.8	18.5 ± 0.9	-
PMSF	97.6 ± 1.8	88.8± 1.4	97.7 ± 1.8	95.2 ± 1.7	-
DTT	63.0 ± 3.1	59.9 ± 0.9	55.5 ± 1.7	61.5 ± 1.7	-
IAA	56.7 ± 2.8	71.2± 1.1	60.1 ± 3.0	56.6 ± 2.8	-
EDTA	30.6 ± 1.5	18.3 ± 0.9	35.2 ± 1.8	41.9 ± 2.1	-

5.9 Other Pharmacological properties

5.9.1 Antibacterial activity

At a concentration of 25 μg ml^{-1}, RVVA-PLA_2-I did not exhibit any antibacterial activity against tested Gram positive bacteria viz. *B subtilis* or Gram negative bacteria viz. *E coli* and *P. auregonosa* after 24 h of incubation at 37 °C (Fig 5.29).

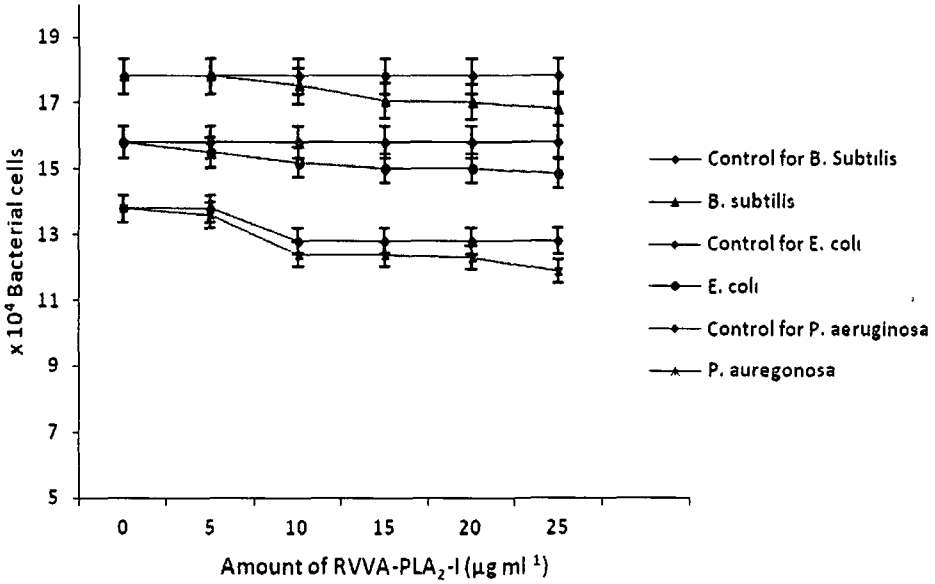

Fig 5.29: Bactericidal activity of RVVA-PLA_2-I on *B.subtilis*, *E.coli* and *P. auregonosa*. Varying amount of RVVA-PLA_2-I (5-25 μg ml^{-1}) was incubated with 18.4 x 10^4 cells of each bacterium for 24 h at 37 °C and change in optical density was recorded at 630 nm. 1 OD at 630 nm= 10^6 cells. Each point represents the mean ± S.D. of four experiments. Only media (without RVVA-PLA_2-I) served as control for each experiment. Experiment was done as described in the section 3.2.7.4.

5.9.2 Cytotoxicity assay

Our study demonstrated that RVVA-PLA_2-I had a minor cytotoxic effect on HT 29 cells (Fig 5.30). RVVA-PLA_2-I, at a dose of 10 μg ml^{-1} could cause lyses of 12 % of total HT29 cells 4 h post incubation. A further incubation time did not result in any further significant enhancement of cell lysis as after 24 h incubation, it shows 15.6 % lyses of total HT29 cells. In a sharp contrast, crude RVV at the same dose caused more than 18 % of cell-death post 4 h incubation and almost all the HT 29 cells were dead post 24 h incubation with crude RVV (Fig 5.30). The light microscopic observation of RVVA-PLA_2-I treated HT 29 cells post 4 h and 24 h (Fig 5.31) incubation did not reveal any gross morphological change.

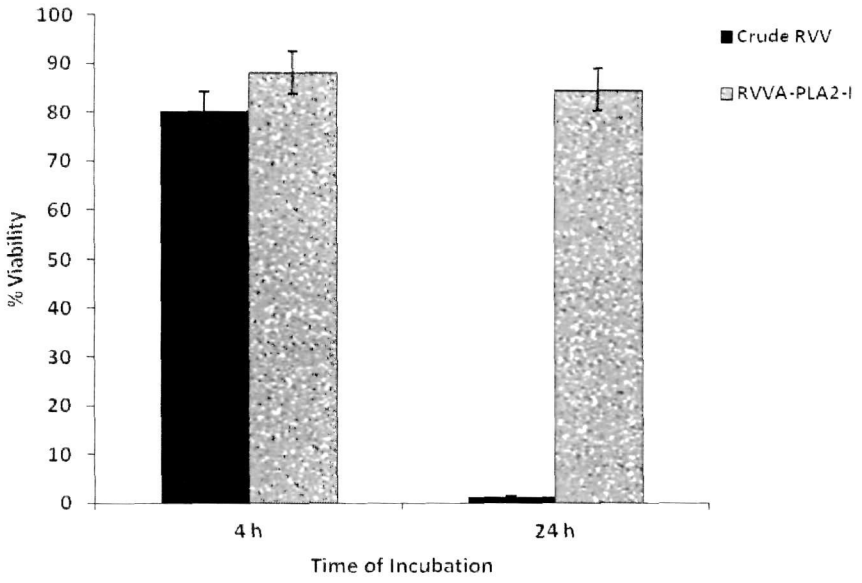

Fig 5.30: A comparison of cell cytotoxicity of crude RVV and RVVA-PLA_2-I on HT 29 cells. The incubation was carried out at 37 °C, 5 % CO_2 for the indicated time period. Experiment was done as described in the section 3.2.7.6. Values are mean ± S. D. of triplicate determinations.

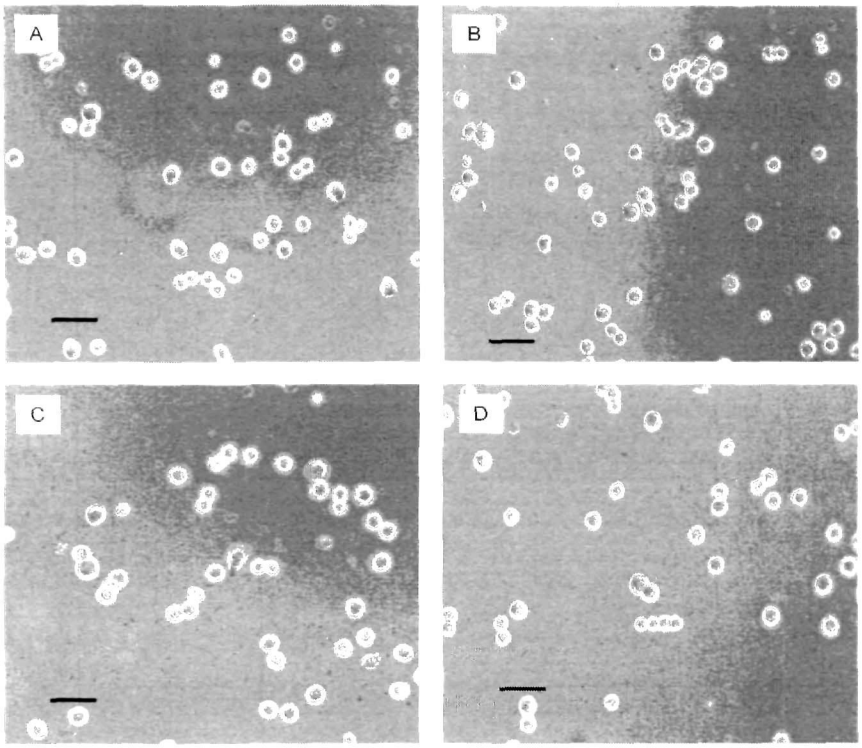

Fig 5.31: Light microscopic observation of the effect of RVVA-PLA$_2$-I on HT 29 cells (photographs were taken at a compact high-performance inverted microscope, Nikon ECLIPSE, TS100, Tokyo). A) Control (4 hr) and b) HT 29 cells treated with 10.0 μg ml^{-1} RVVA-PLA$_2$-I post 4 hr of incubation. C) Control (24 hr) and D) HT 29 cells treated with 10.0 μg ml^{-1} RVVA-PLA$_2$-I post 24 hr of incubation. The incubation was carried out at 37 °C, 5 % CO_2 for the indicated time period. Magnifications 100x: (A-D) bar=50 μm.

5.10 *In-vivo* toxicity assay in BALB/c mice model

5.10.1 Lethality and *in-vivo* toxicity

RVVA-PLA_2-I was non lethal to experimental BALB/c mice at a concentration of 0.2 mg kg^{-1} body weight and did not show any sign of haemostatic disorder. Further, no behavioural changes in treated mice were observed after 48 h (Table 5.8).

5.10.2 Effect of *in vitro* blood coagulation

The i.p. administration of RVVA-PLA_2-I at a dose of 0.2 mg kg^{-1} body weight prolonged the *in vitro* tail bleeding time in mice. The *in vitro* coagulation time of PPP from RVVA-PLA_2-I treated mice was enhanced as compared to coagulation time of PPP from control group of mice (Table 5.9).

5.10.3 Effect on blood cells

The *in vivo* effect of RVVA-PLA_2-I on haematological parameters of mice blood viz. RBC, WBC and haemoglobin counts showed that the WBC and the RBC content as well as the Hb content were decreased significantly in the blood of RVVA-PLA_2-I treated mice as compared to the control group of mice (Table 5.10).

Table 5.8: Behavioural Changes, if any, in mice 48 h after the administration of RVVA-PLA$_2$-I at a dose of 0.2 mg kg^{-1}

Values are mean ± S.D. of six determinations.

Group of mice	Parameters									
	Body weight (gm)		Grip Strength (sec)		Rectal temperature (F)		Faecal tendency (times per 15 min)		Urination (times per 15 min)	
	Initial*	Final**	Initial*	Final**	Initial*	Final**	Initial*	Final**	Initial*	Final**
Control	32.4 ± 1.2	32.6 ± 1.3	67.9 ± 3	32.5 ± 1.4	94.2 ± 0.9	94.1 ± 0.4	3 ± 1.4	5 ± 0.8	2 ± 1.0	4 ± 1.1
RVVA-PLA$_2$-I treated	31.6 ± 1.4	32.1 ± 1.1	42.9 ± 2.3	21.6 ± 2.1	94.1 ± 0.5	93.6 ± 0.8	3 ± 0.7	4 ± 1.0	3 ± 0.8	5 ± 1.5

*Initial: determined at the onset of experiment.

**Final: determined after 48 h of RVVA-PLA$_2$-I administration.

Table 5.9: The *in vitro* clotting time of blood and tail bleeding time in mice post 48 h injection of RVVA-PLA$_2$-I at a dose of 0.2 mg kg^{-1}

Values are mean ± S.D. of six determinations.

Group of mice	Plasma clotting time (sec)	Tail bleeding time (sec)
Control	168.5 ± 1.5	45 ± 0.6
RVVA-PLA$_2$-I treated	314.8 ± 2.1[a]	72 ± 1.2[a]

Level of significance [a] $p < 0.001$

Table 5.10: The effect of RVVA-PLA$_2$-I on different haematological parameters of PLA$_2$ treated mice.

Values are mean ± S.D. of six determinations.

Group of mice	Haematological parameters		
	WBC (m/mm^3)	RBC (m/mm^3)	Haemoglobin (Hb) (g/dL)
Control	11.46 ± 1.1	5.73 ± 0.9	6.45 ± 1.2
RVVA-PLA$_2$-I treated	5.99 ± 0.7	2.84 ± 0.5	4.33 ± 0.9
% decreased	52.27 %	43.28 %	67.13 %

5.10.4 Effect of RVVA-PLA$_2$-I on serum parameters of mice

Table 5.11 shows the effect of RVVA-PLA$_2$-I on serum parameters of treated mice. Administration of RVVA-PLA$_2$-I resulted in a significant increase ($p < 0.05$) in the level of intracellular enzymes such as ALP, CPK-MB, SGOT, SGPT, and triglycerides in the serum of treated mice as compared to the control group of mice. On the other hand, the serum level of LDH was significantly decreased in RVVA-PLA$_2$-I treated mice as compared to control. However, there was no change in the cholesterol and glucose levels in the serum of RVVA-PLA$_2$-I treated mice as compared to control mice (Table 5.11).

Table 5.11: Effect of RVVA-PLA$_2$-I on different parameters of serum of albino mice. RVVA-PLA$_2$-I (0.2 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB/c mice (n=6). Mice were sacrificed after 48 hours of injection, blood was collected immediately by venipuncture and the serum was used for the assay of different parameters. Values are mean ± S.D. of six determinations.

	Total protein (g/L)	Glucose (g/L)	CPK-MB (U/L)	LDH (U/L)	ALP (U/L)	SGOT (U/L)	SGPT (U/L)	Cholesterol (g/L)	Triglycerides (g/L)
Control	270.0 ± 0.7	2.11 ± 1.2	27.5 ± 0.65	1281.5 ± 0.9	27.5 ± 0.8	144.0 ± 2.2	50.0 ± 0.91	0.7 ± 0.4	0.2 ± 0.04
Treated	180.5 ± 0.5[a]	2.53 ± 1.5	303.0 ± 1.6[a]	1054.5 ± 1.1[a]	79.3 ± 1.2[a]	191.7 ± 2.6[a]	135.0 ± 1.4[a]	0.8 ± 0.3	1.0 ± 0.05[a]

Significance of difference [a] $p < 0.001$

5.10.5 Histopathological study

Again, in histological study of different tissues, there was not found any significant change in the RVVA-PLA_2-I treated tissues as compared to control tissues (Fig 5.32).

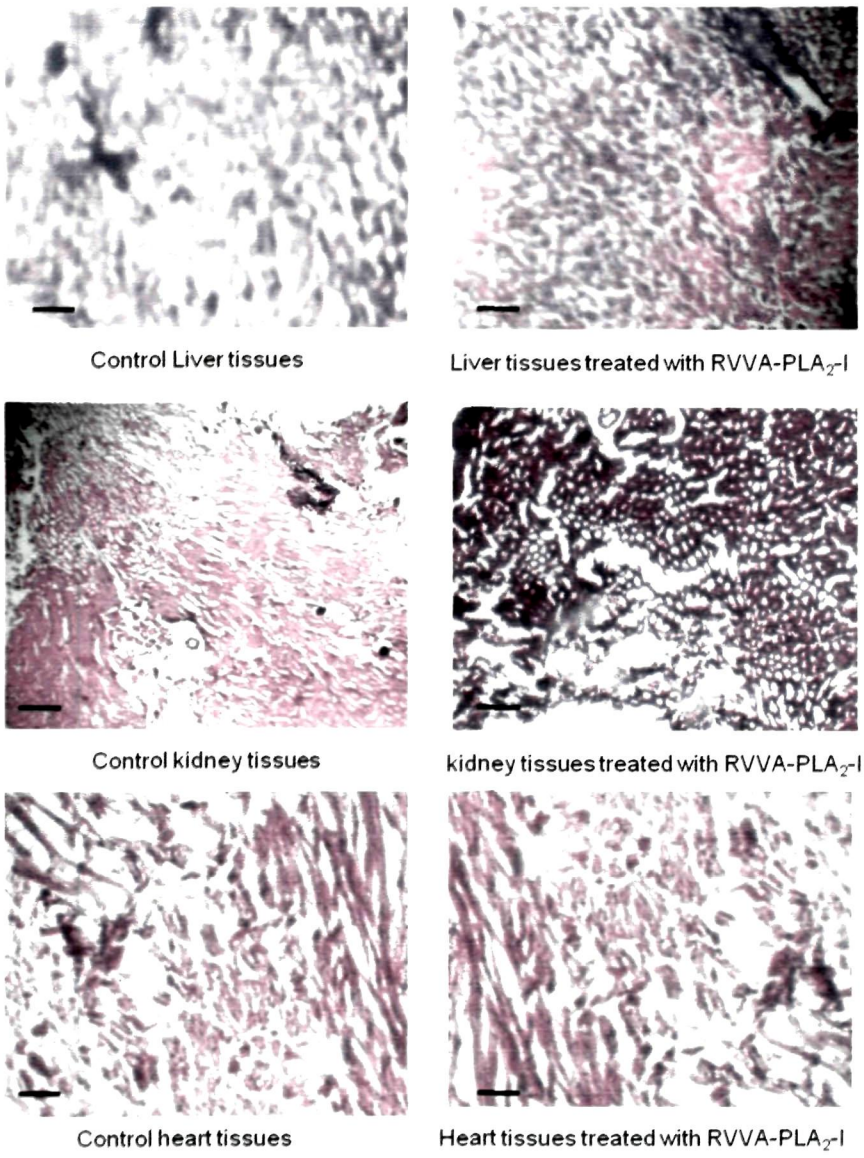

Fig 5.32: Light microscopic observation of the effect of RVVA-PLA_2-I on different organs of treated mice. Tissues treated with PBS were served as control. Hematoxyline-eosine (H&E) staining; Magnifications (100x): bar=50 μm.

5.11 Immunological cross reactivity

5.11.1 Immunodiffusion

Immunodiffusion technique was used to determine the presence of antibodies against RVVA-PLA_2-I in commercially available polyvalent antivenom. By immunodiffusion test, RVVA-PLA_2-I did not show cross reactivity with commercially available antivenom at a ratio of 1:100 (antigen: antibody, w/w) even after 48 h of incubation (Fig 5.33 C). Whereas in the case of crude RVV, a single, sharp precipitating band was observed in the immunodiffusion plate after 24 h of incubation indicating the presence of anti-RVV antibodies in the polyvalent antivenom (Fig 5.33 B).

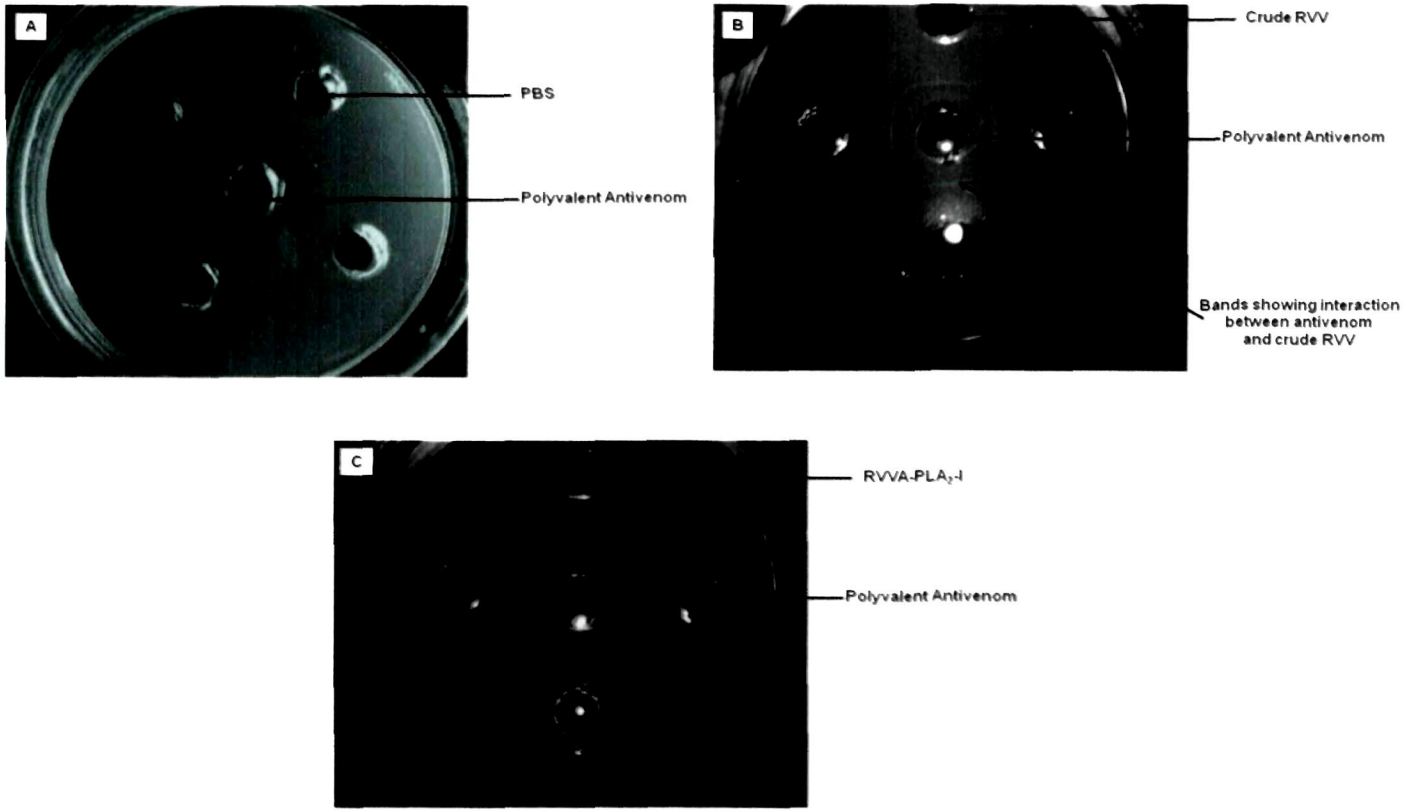

Fig 5.33: Immunodiffusion test to determine cross reactivity of crude RVV (Fig B) and RVVA-PLA$_2$-I (Fig C) against commercial polyvalent antivenom used for the treatment of snakebite patients. For control PBS was used replacing the venom proteins (Fig A). Experiment was done as described in the section 3.2.8.1. Data shows a typical experiment and repetition of experiment demonstrated similar result.

5.12 Screening of Indian medicinal plants for anti snake venom activity

Four Indian plants were selected for assessing their anti-PLA_2 activity assay which was based on prior knowledge on their traditional use for the treatment of snakebite patients by local practitioners. These four plants were found to show activity against PLA_2 as well as anticoagulant activity. The selected plants and other related information pertaining to snakebite treatment are listed in the table 5.12.

Table 5.12: Screening of medicinal plants for anti-PLA_2 activity of crude RVV and anti-anticoagulant activity of RVVA-PLA_2-I from North East India.

Sl. No.	Name	Local Name	Parts used
1.	*Azadirachta indica*	Maha neem	Leaves
2.	*Camellia sinensis*	Sah (tea plant)	Leaves
3.	*Aegle marmelos*	Bell	Root
4.	*Xanthium strumarium*	Agora	Leaves

5.12.1 Neutralization by *Azadirachta indica*

One PLA_2 inhibitor was purified from *Azadirachta indica* leaves extract (methanol extract) and was named as AIPLAI (Mukherjee et al, 2008). Fig 5.34 shows the effect of AIPLAI (*A. indica* PLA_2 inhibitor), the methanol extract of *A. indica* leaves on catalytic activity of both crude RVV and RVVA-PLA_2-I as well as anticoagulant activity of RVVA-PLA_2-I. The AIPLAI purified from *A. indica* leave extract at a dose of 50 $\mu g\ ml^{-1}$ demonstrated equal inhibition of

catalytic (27 % inhibition) and anticoagulant (29 % inhibition) activity RVVA-PLA_2-I (Fig 5.34).

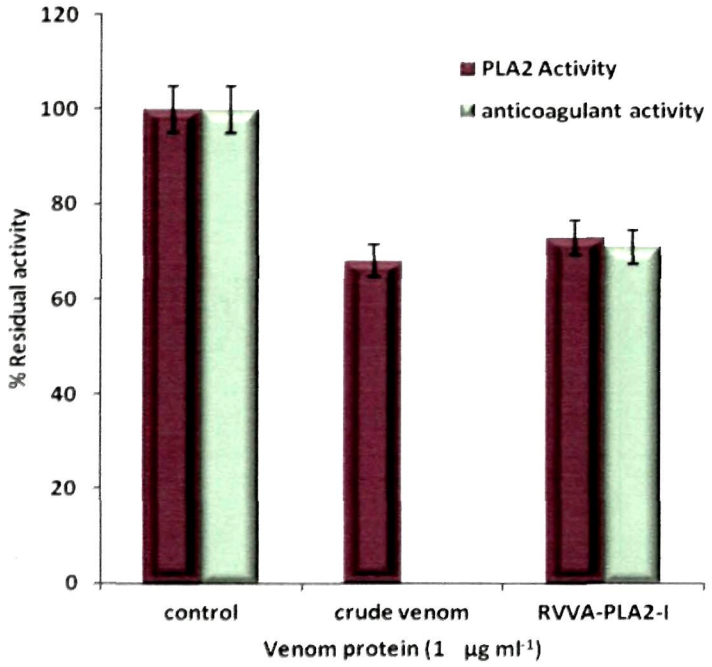

Fig 5.34: Effect of AIPLAI (at a dose of 50 µg ml^{-1}) on neutralizing the catalytic and anticoagulant activity of crude RVV/ RVVA-PLA_2-I (at a final concentration 1 µg ml^{-1}). Crude RVV does not show anticoagulant activity, so for crude RVV; the effect of anticoagulant activity was not checked. Values are expressed as the mean ± S.D. of four experiments.

5.12.2 Neutralization by *Camellia sinensis, Xanthium strumarium and Aegle marmelos*

Among the different solvent extracts, methanol extracts of the leaves of *C. sinensis* and *X. strumarium* and the roots of *A. marmelos* were found to be most potent in inhibiting the PLA_2 activity of both crude Russell's viper venom and RVVA-PLA_2-I. The effect of water, chloroform and methanol extract of these three plants on the catalytic activity of crude RVV and RVVA-PLA_2-I and

anticoagulant activity of RVVA-PLA_2-I are listed in the Table 5.13 and Table 5.14 respectively.

Moreover, in a dose dependent study, amongst all the tested doses, 50 μg ml^{-1} was found as the optimal dose for inhibition of enzymatic activity (Fig 5.35).

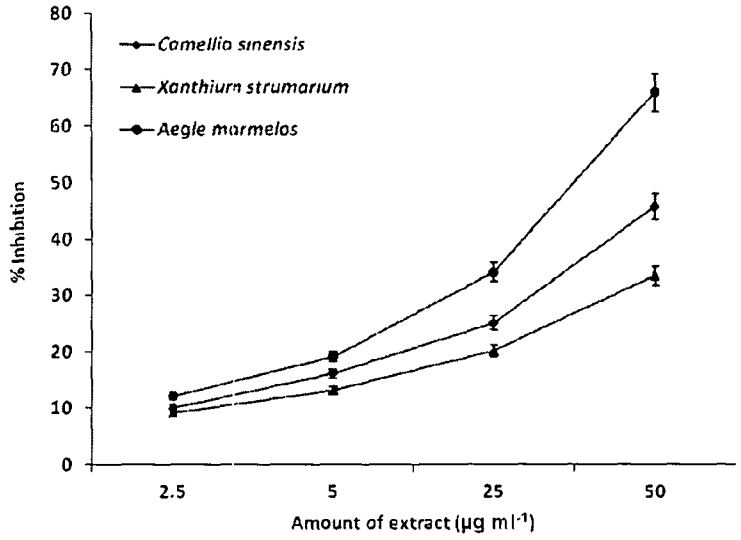

Fig 5.35. Dose-dependent inhibition of PLA_2 activity of crude Russell's viper venom (final concentration 1 μg ml^{-1}) by methanol leave extract of *Camellia sinensis*, *Xanthium strumarium* and root extract of *Aegle marmelos*. Values represent mean of three experiments.

These plant extracts (at a dose of 100 μg ml^{-1}) showed significant inhibition of different pharmacological activities of CRVV and RVVA-PLA_2-I (Table 5.15). Methanol extracts of these three plants neutralized the anticoagulant potency to a maximum extent followed by neutralization of *in vitro* tissue damaging property and catalytic activity of the PLA_2 enzymes (Table 5.15). The RVVA-PLA_2-I induced membrane phospholipids hydrolysis was antagonized by the methanol leaf extract (at the dose of 100 μg ml^{-1}) showing more significant protection than even snake venom antiserum (Table 5.15).

Table 5.13: Percent inhibition of PLA_2 activity of crude RVV and RVVA-PLA_2-I (final concentration 1 µg ml^{-1}) by leaves extract of *Camellia sinensis, Xanthium strumarium* and root extract of *Aegle marmelos.* Preparation of plant extract and inhibition study was done as described in the section 3.2.10.2. PLA_2 activity without plant extract was treated as control (100 %). Results are mean ± S.D. of four determinations.

Amount of plant extract (µg)		% inhibition of PLA_2 activity					
		Camellia sinensis		*Xanthium strumarium*		*Aegle marmelos*	
		Crude RVV	RVVA-PLA_2-I	Crude RVV	RVVA-PLA_2-I	Crude RVV	RVVA-PLA_2-I
Water Extract	5	2.1 ± 0.1	0.8 ± 0.04	1.1 ± 0.05	0.3 ± 0.02	3.6 ± 0.18	4.5 ± 0.23
	10	5.1 ± 0.3	2.2 ± 0.11	1.9 ± 0.09	1.2 ± 0.06	9.5 ± 0.47	10.1 ± 0.51
	50	12.5 ± 0.6	8.3 ± 0.41	8.2 ± 0.41	7.1 ± 0.36	19.1 ± 0.95	16.4 ± 0.82
	100	21.9 ± 1.1	14.2 ± 0.7	15.6 ± 0.78	11.1 ± 0.55	29.5 ± 1.47	22.6 ± 1.12
Chloroform	5	1.09 ± 0.05	0.5 ± 0.025	0.8 ± 0.04	0.2 ± 0.01	2.1 ± 0.10	1.9 ± 0.09
	10	3.05 ± 0.15	2.1 ± 0.1	1.5 ± 0.07	0.9 ± 0.05	7.1 ± 0.35	5.7 ± 0.28

	50	7.8 ± 0.4	6.4 ± 0.32	7.8 ± 0.39	5.6 ± 0.28	16.3 ± 0.81	14.2 ± 0.71
	100	19.9 ± 0.9	15.8 ± 0.8	10.2 ± 0.51	9.6 ± 0.48	23.1 ± 1.15	21.1 ± 1.05
Methanol Extract	**5**	5.6 ± 0.5	3.1 ± 0.16	2.2 ± 0.11	4.5 ± 0.23	11.6 ± 0.58	9.6 ± 0.48
	10	23.1 ± 1.2	15.7 ± 0.78	16.0 ± 0.8	8.6 ± 0.43	29.4 ± 1.47	22.1 ± 1.1
	50	**45.7 ± 2.3**	**35.2 ± 1.8**	**33.3 ± 1.7**	**24.8 ± 1.24**	**65.9 ± 2.4**	**45.6 ± 2.1**
	100	52.9 ± 2.6	38.6 ± 1.9	39.2 ± 2.1	29.9 ± 1.5	71.0 ± 2.6	48.8 ± 2.3

Table 5.14: Percent inhibition of anticoagulant activity of RVVA-PLA$_2$-I (final concentration 1 µg ml^{-1}) by leaves extract of *Camellia sinensis*, *Xanthium strumarium* and root extract of *Aegle marmelos*. Preparation of plant extract and inhibition study was done as described in the section 3.2.10.2. PLA$_2$ activity without plant extract was treated as control (100 %). Results are mean ± S.D. of four determinations.

Amount of plant extract (µg)		% inhibition of anticoagulant activity of RVVA-PLA$_2$-I by		
		Camellia sinensis	*Xanthium strumarium*	*Aegle marmelos*
Water Extract	5	1.1 ± 0.05	0.45 ± 0.02	0.21 ± 0.01
	10	5.1 ± 0.25	2.5 ± 0.12	8.20 ± 0.41
	50	15.0 ± 0.75	10.1 ± 0.51	17.1 ± 0.85
	100	21.1 ± 1.05	15.4 ± 0.77	24.5 ± 1.21
Chloroform Extract	5	1.5 ± 0.07	0.78 ± 0.03	0.23 ± 0.01
	10	8.7 ± 0.43	5.7 ± 0.28	4.5 ± 0.23
	50	19.8 ± 0.99	15.1 ± 0.75	13.6 ± 0.68
	100	23.4 ± 1.17	17.8 ± 0.89	19.5 ± 0.97
Methanol Extract	5	5.1 ± 0.25	3.2 ± 0.16	7.8 ± 0.36
	10	21.1 ± 1.05	15.3 ± 0.75	27.7 ± 1.32
	50	**39.0 ± 1.15**	**29.3 ± 1.46**	**40.5 ± 1.75**
	100	41.4 ± 1.91	32.2 ± 1.61	44.5 ± 2.1

Table 5.15: A comparison of anti-PLA_2 activity of polyvalent antivenom and methanolic extracts of leaves of *C. sinensis* and *X. strumarium* and roots of *A. Marmelos*. Crude RVV/ RVVA-PLA_2-I (1 μg ml^{-1}) were incubated either with plant extract (100 μg ml^{-1}) or with polyvalent antivenom in a ratio of 1:100 (in a final volume of 1.0 ml) at 37 °C for 30 min and then assayed for residual catalytic and pharmacological properties of the enzymes. Values are mean ± S.D. of triplicate determinations. Values in the same row with different superscripts are significantly different ($p<0.05$). ND: Not determined.

Pharmacological properties	% Inhibition							
	Polyvalent antivenom		*C. sinensis*		*X. strumarium*		*A. marmelos*	
	Crude *D russelli* venom	RVVA-PLA_2-I	Crude *D. russelli* venom	RVVA-PLA_2-I	Crude *D. russelli* venom	RVVA-PLA_2-I	Crude *D russelli* venom	RVVA-PLA_2-I
PLA_2 activity	25.6 ± 1.1[a]	15.6 ± 0.8[a]	45.7 ± 1.4[b]	35.2 ± 1.8[b]	33.3 ± 1.7[c]	24.8 ± 1.2[c]	65.9 ± 1.4[d]	45.6 ± 1.6[d]
Anticoagulant activity	ND	21.9 ± 1.3[a]	ND	54.0 ± 1.1[b]	ND	49.3 ± 1.4[c]	ND	61.5 ± 1.7[d]
Indirect haemolytic activity	31.1 ± 0.5[a]	26.9 ± 0.5[a]	34.4 ± 1.0[b]	45.0 ± 1.0[b]	29.3 ± 0.9	31.3 ± 0.9	39.5 ± 1.3[d]	46.5 ± 1.3[d]
In vitro tissue damaging activity								
Liver	43.4 ± 0.5[a]	40.8 ± 0.8[a]	49.3 ± 1.1[b]	55.4 ± 1.4[b]	44.2 ± 0.6[c]	48.5 ± 1.6[c]	62.3 ± 0.5[d]	72.3 ± 1.1[d]
Heart	50.1 ± 1.6[a]	46.0 ± 1.2[a]	52.7 ± 1.5[b]	61.2 ± 0.5[b]	46.1 ± 1.3[c]	52.1 ± 0.8[c]	59.1 ± 1.1[d]	67.1 ± 0.9[d]
Lungs	48.8 ± 1.4[a]	51.1 ± 0.7[a]	61.3 ± 1.2[b]	73.3 ± 1.5[b]	51.0 ± 0.9[c]	64.2 ± 0.5[c]	57.5 ± 1.2[d]	75.4 ± 1.3[d]
Mitochondrial membrane phospholipids hydrolysis	ND	23.2 ± 1.1[a]	ND	41.1 ± 1.0[b]	ND	36.3 ± 1.2[c]	ND	53.3 ± 1.3[d]
Erythrocytes membrane phospholipids hydrolysis	ND	33.3 ± 1.2[a]	ND	42.9 ± 1.7[b]	ND	38.8 ± 0.9[c]	ND	64.1 ± 1.2[d]

CHAPTER VI

Purification, biochemical and pharmacological characterisation of a neutral anticoagulant phospholipase A_2 enzyme (RVVN-PLA_2-I) from venom of Russell's viper (*Daboia russelli)* of eastern India origin

6 Results

6.1 Purification of a neutral anticoagulant phospholipase A_2

6.1.1 Fractionation of CMIDEI through gel filtration column Sephadex G-50

Fractionation of CMI (non-retained fractions of crude RVV on CM cellulose C-50 column) through an anion exchanger DEAE Sephadex A-50 was resulted into its separation into seven peaks (as described in the section 4.1.2. and Fig 4.2). The first peak CMIDEI (eluted with 20 mM K-phosphate, pH- 7.0) containing the neutral proteins showed significant PLA_2 activity and was selected for purification of a neutral PLA_2 from RVV (Fig 4.2). The fractionation of CMIDEI through Sephadex G-50 gel-filtration column (1cm x 64 cm) resulted in the separation of proteins into three peaks (GFI to GFIII) as shown in Fig 6.1. The fraction GFIII showed highest PLA_2 as well as anticoagulant activity (Table 6.1).

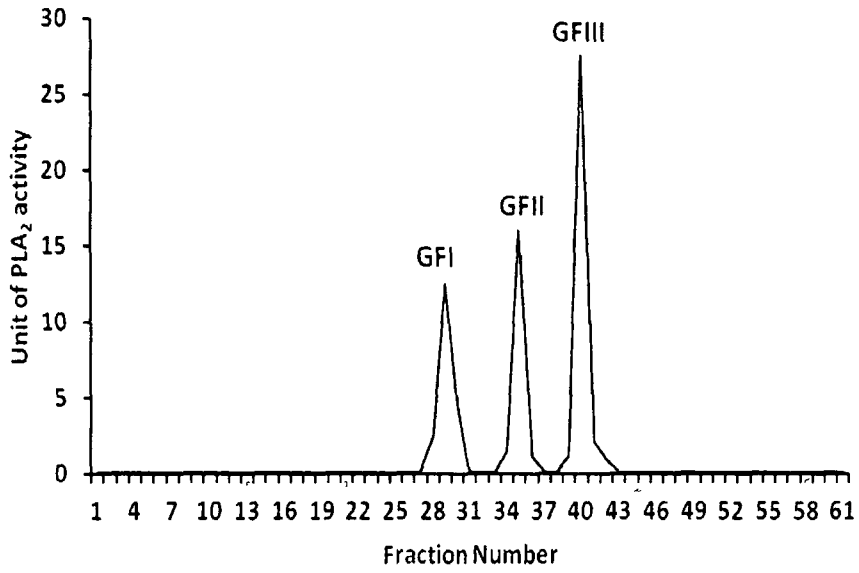

Fig 6.1: Gel filtration of CMIDEI through Sephadex G-50 column. Gel filtration was done as described in the section 3.2.3.1.

Table 6.1: PLA_2 and anticoagulant activity of peaks eluted from Sephadex G-50 gel filtration column after the fractionation of CMIDEI. Values are from a typical experiment.

Peaks	% recovery of protein	PLA_2 specific activity	Anticoagulant Specific activity
GFI	0.07	1.6×10^4	1.5×10^3
GFII	0.08	1.1×10^4	0.9×10^3
GFIII	0.14	3.0×10^4	4.4×10^4

6.1.2 Purification of a neutral PLA_2 enzyme by RP-HPLC of GFIII

GFIII was further purified by RP-HPLC. It was eluted as one major peak at a retention time of 17.4 min (Fig 6.2). This fraction displayed the PLA_2 as well as anticoagulant activities.

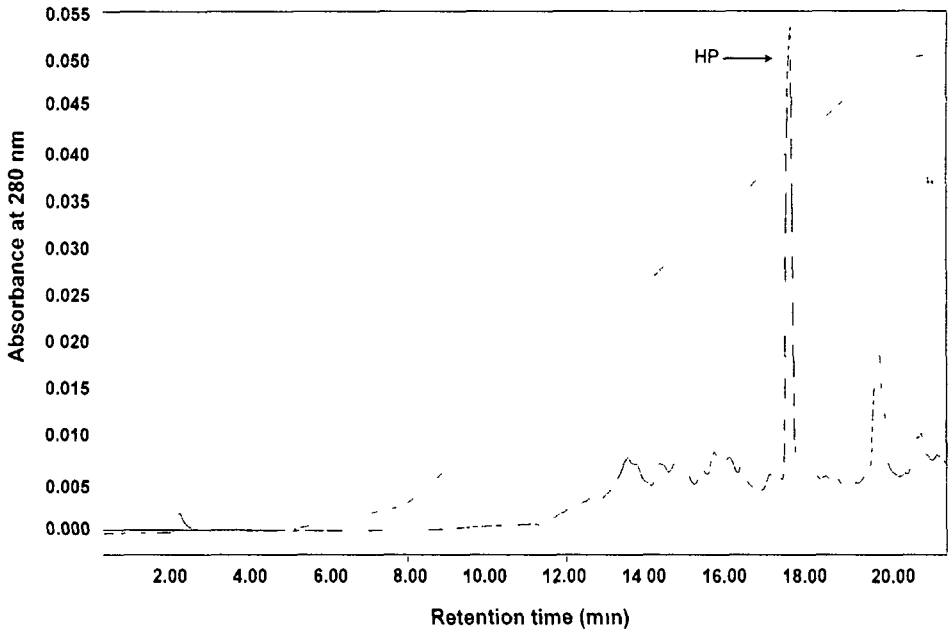

Fig 6.2: **Reverse-phase HPLC of fraction GFIII** (third gel filtration faction of CMIDEI) on a Waters reverse-phage HPLC C_{18}-μ Nova Pack column as described in the section 3.2.3.2. Solvents A and B were 0.1% (v/v) TFA in 5.0% (v/v) acetonitrile, and 0.1% TFA in 95% (v/v) acetonitrile, respectively. Each peak was screened for protein content, anticoagulant and PLA_2 activity. The protein (PLA_2 enzyme) was eluted with a retention time 17.4 min.

This anticoagulant neutral PLA_2 was named as RVVN-PLA_2-I (Russell's viper neutral phospholipase A_2-I). The yield of this neutral PLA_2 was 0.04 % of the original venom load. A summary of purification of RVVN-PLA_2-I is shown in Table 6.2.

Table 6.2: Summary of purification of a neutral anticoagulant phospholipase A_2 (RVVN-PLA_2-I) from RVV. Values represent a typical experiment. ND, not detected.

Fraction	Total protein (mg)	Protein yield (%)	PLA_2 activity		Anticoagulant activity		Purification fold	
			Total activity (Units)	Specific activity (U/ mg)	Total activity (Units)	Specific activity (Units/ mg)	PLA_2 activity	Anticoagulant activity
Crude RVV	20.0	100.0	1.3×10^5	1.2×10^4	ND	ND	1	-
CM-Cellulose	0.84	4.1	1.1×10^3	2.5×10^4	ND	ND	2.0	-
DEAE Sephadex	0.17	0.9	4.9×10^3	3.1×10^4	ND	ND	2.4	-
Gel filtration	0.03	0.15	14.0×10^2	4.9×10^4	1.8×10^3	6.0×10^4	3.8	1
RP-HPLC	0.008	0.04	9.1×10^2	12.4×10^4	7.2×10^2	9.0×10^4	9.5	1.5

RVVN-PLA_2-I did not show any detectable protease, ATPase, AMPsae activities, even at a concentration of 25.0 μg ml^{-1} that reveals the purity of this PLA_2 isolation.

6.1.3 Assessment of purity and determination of molecular mass of RVVN-PLA_2-I

6.1.3.1 Gel filtration chromatography and SDS-PAGE

The molecular mass of RVVN-PLA_2-I was checked by gel filtration chromatography, SDS-PAGE and ESI/MS analyses. From the calibration curve of the gel filtration column (Fig 5.1B), the molecular mass of GFIII was found 13.0 kDa. About 20 μg of RVVN-PLA_2-I gave a sharp, Coomassie Brilliant blue positive band on 15 % SDS-PAGE under both reduced and non-reduced conditions (Fig. 6.3). By SDS-PAGE, this protein migrated as a single band of 12.8 kDa both in presence and absence of reducing agent and heating, which indicates that the purified PLA_2 is a monomer.

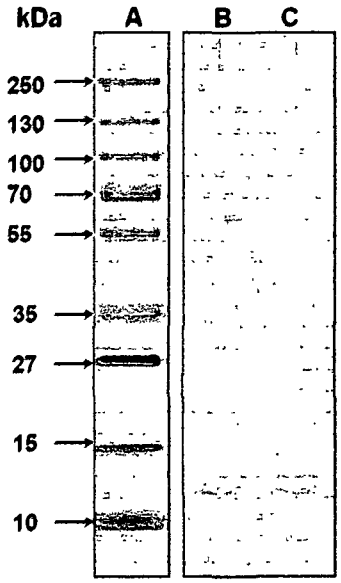

Fig 6.3: 15% SDS-polyacrylamide gel electrophoresis: Lane A) molecular weight marker; lane B) 20 µg RVVN-PLA_2-I (non-reduced condition); lane C) 20 µg RVVN-PLA_2-I (reduced condition). Experiment was done as described in the section 3.2.5.3.

6.1.3.2 ESI/MS analysis of RVVN-PLA_2-I

The molecular mass of RVVN-PLA_2-I was determined as 12.792 KDa by electron spray ionization-mass spectrophotometry (ESI/MS) (Fig 6.4).

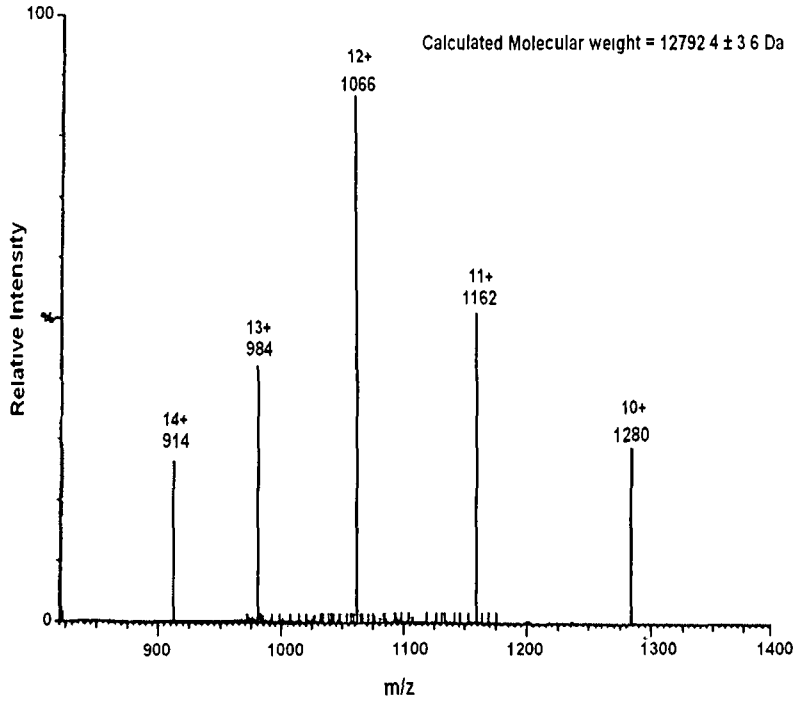

Fig 6.4: ESI/MS of RVVN-PLA$_2$-I for molecular mass determination. Experiment was done as described in the section 3.2.5.4.

6.2 Biochemical Characterisation of RVVN-PLA$_2$-I

6.2.1 PLA$_2$ specific activity

Significant PLA$_2$ activity was showed by RVVN-PLA$_2$-I when the activity was checked by using egg yolk phospholipids as substrate (Table 6.2).

6.2.2 Substrate Specificity of RVVN-PLA_2-I

When substrate specificity of RVVN-PLA_2-I was determined on commercially available phospholipid substrates with different polar head groups viz., PC, PS and PE, it was found that the enzyme has the highest specificity towards PS followed by PC and it demonstrated lowest activity against PE (Table 6.3).

Table 6.3: Substrate Specificity of RVVN-PLA_2-I. Substrate specificity of RVVN-PLA_2-I towards purified phospholipids was determined as described in section 3.2.6.7.1. Results are mean ± S.D. of four determinations.

Phospholipid Substrate (Final concentration 1 mM)	PLA_2 Specific activity (Unit* $mg^{-1}min^{-1}$)
Phosphatidylserine (PS)	$6.0 \times 10^5 \pm 1.4$
Phosphatidylcholine (PC)	$1.5 \times 10^5 \pm 1.1$
Phosphatidylethanolamine (PE)	$9.0 \times 10^3 \pm 0.8$

*One unit of PLA_2 activity is defined as the increase in release of 1 µg of palmitic acid (FFA) released per min.

6.2.3 Effect of substrate (PS) concentration on catalytic activity of RVVN-PLA_2-I

Effect of substrate (PS) concentration on catalytic activity of RVVN-PLA_2-I showed that with an increase in the substrate concentration up to 2.0 mM, the phospholipid hydrolysis was enhanced proportionatley. However, a

saturation (steady state kinetics) in enzyme activity was observed at 2.0 mM PS concentration (Fig 6.5).

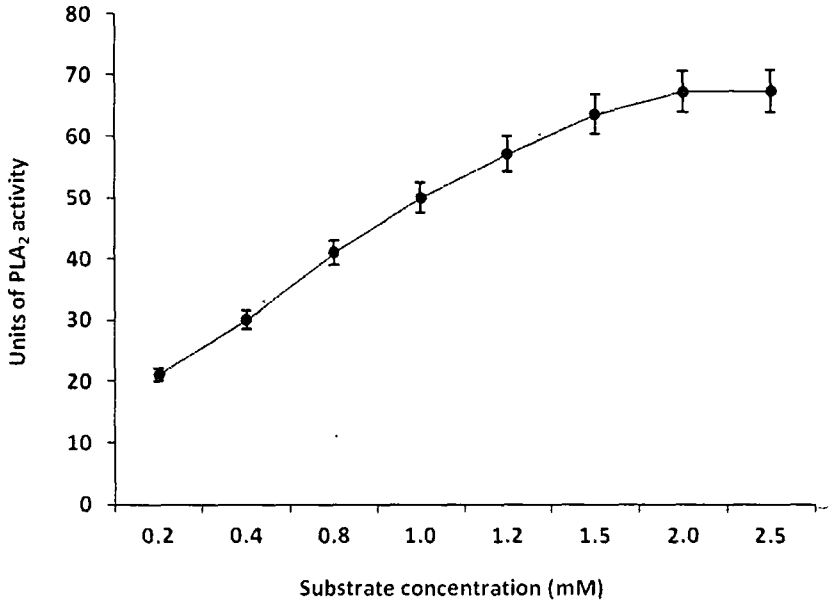

Fig 6.5: Effect of PS concentration on catalytic activity of RVVN-PLA$_2$-I (0.6 µg ml^{-1}). Effect of substrate concentration on PLA$_2$ activity of RVVN-PLA$_2$-I was determined as described in section 3.2.6.8.2. Results are expressed as mean ± S.D. of four determinations.

6.2.4 Effect of enzyme concentration

It was found that an increase in the enzyme concentration resulted in a concomitant increase in substrate (PS) hydrolysis by RVVN-PLA$_2$-I and saturation was observed at 0.6 µg ml^{-1} concentration of RVVN-PLA$_2$-I (Fig 6.6).

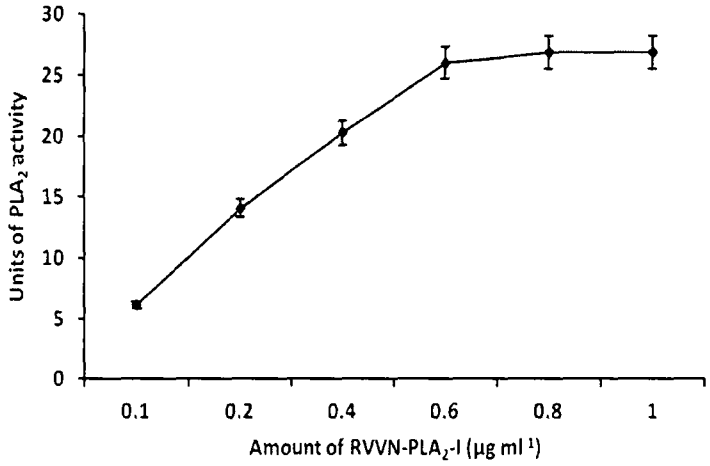

Fig 6.6: Effect of enzyme concentration on catalytic activity of RVVN-PLA$_2$-I. Effect of enzyme concentration on catalytic activity of RVVN-PLA$_2$-I was determined as described in the section 3.2.6.8.3. Results are expressed as mean ±S.D. of four determinations.

6.2.5 Carbohydrate content

By phenol-sulphuric acid method, RVVN-PLA$_2$-I was found to be non-glycoprotein in nature.

6.2.6 Determination of Km and Vmax of PS hydrolysis

The Michaelis constant, Km and maximum velocity, Vmax of the RVVN-PLA$_2$-I catalysed reaction were determined by using Lineweaver-Burk representation. By plotting the values of 1/v as a function of 1/[S], a straight line was obtained (Fig 6.7), that intersect the vertical line at a point which is the 1/Vmax (Since 1/[S] =0. We have 1/v=1/Vmax). If we extend the straight line, then the horizontal axis (1/[S]) intersect at the point which is -1/Km.

From the graph we have the equation, y = 0.0559x + 0.0773

When y=0, then x = -0.0773/0.0559 or, x= -1.38 i.e., -1/Km = 1.38,

i.e., Km = 0.72 mM or, 0.72 x 10^{-4} M i.e., Km = 0.72 x 10^{-4} M

1/Vmax = 0.0773 i.e., Vmax = 12.93 x 10^{-3} mM μg^{-1} or, 129.3 x 10^{-3} μM μg^{-1} or, 129.3 μmol mg^{-1}

The apparent Km and Vmax value of enzyme catalyzed reaction are 0.72 x 10^{-4} M and 129.3 μmol min^{-1} mg^{-1} respectively.

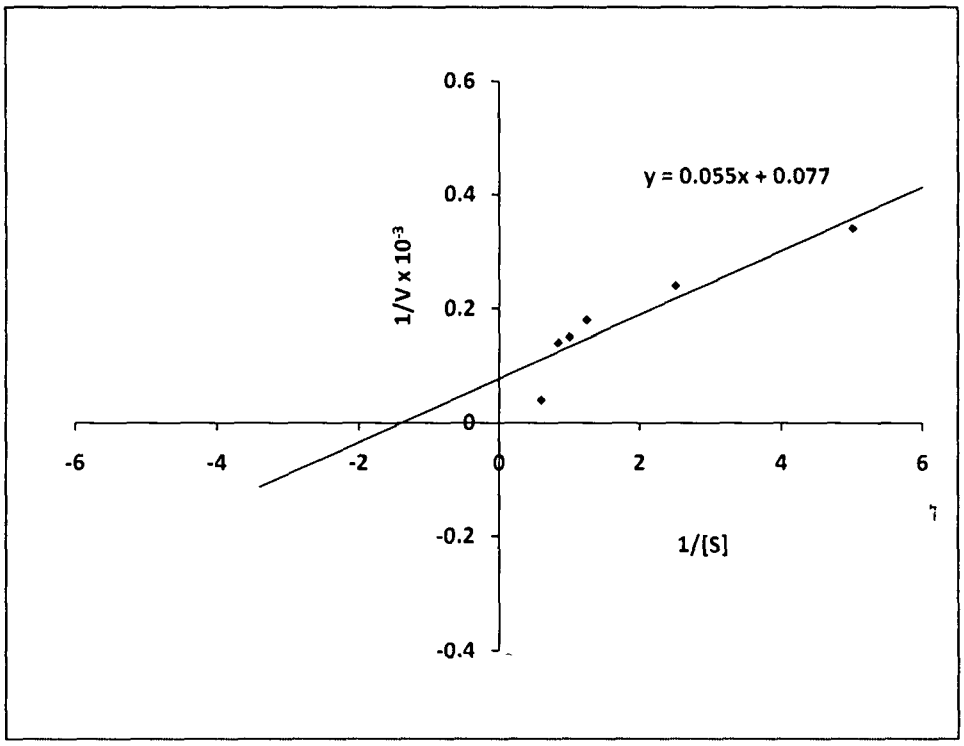

Fig 6.7: Determination of Km and Vmax of RVVN-PLA$_2$-I using Lineweaver-Burk plot. Purified PS was used as substrate (0.1-5.0 mM concentration). Experiment was done as described in the section 3.2.6.8.4. Values are mean ± S.D. of four determinations.

6.2.7 Optimum temperature

The enzymatic activity of RVVN-PLA_2-I was checked at different temperature range of 20-75 °C using PS as substrate (Fig 6.8). RVVN-PLA_2-I showed optimum catalytic activity at temperature 37 °C (11.5 units).

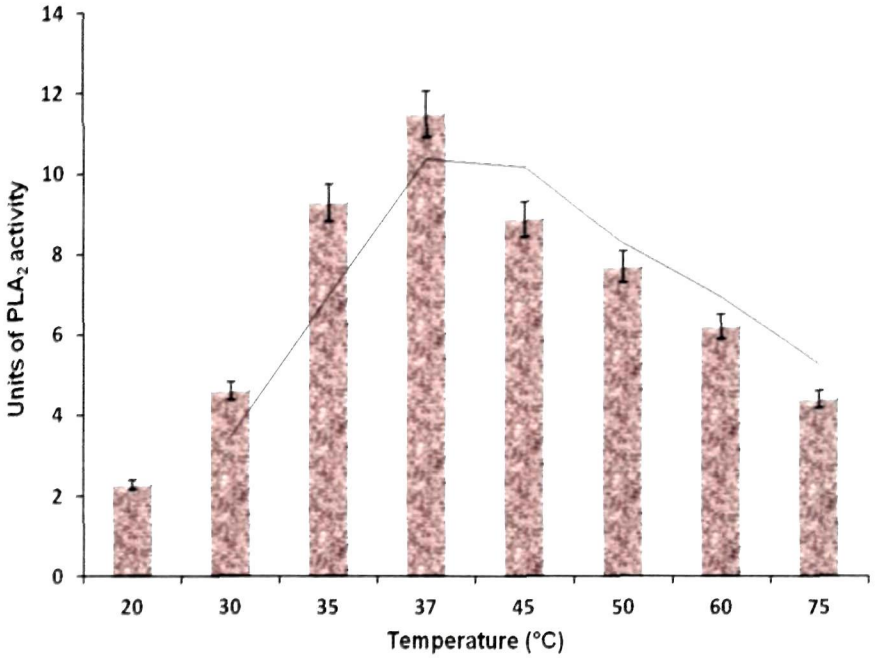

Fig 6.8: Effect of temperature on catalytic activity of RVVN-PLA_2-I. PLA_2 activity of RVVN-PLA_2-I at different temperature was done as described in the section 3.2.6.8.5. Results are expressed as mean ± S.D. of four determinations.

6.2.8 pH Optima

The optimum pH for enzymatic activity of RVVN-PLA_2-I was assayed at a pH range of 5.5-9.0 using PS as a substrate (Fig 6.9). RVVN-PLA_2-I showed maximum catalytic activity at pH 8.0 (9.5 units). At pH 7.5 and pH 7.0, the enzyme activity was 73 % and 21 % respectively, of the activity shown at pH 8.0 (Fig 6.9). RVVN-PLA_2-I did not show any catalytic activity at a pH lower than 6.5 or higher than 8.5.

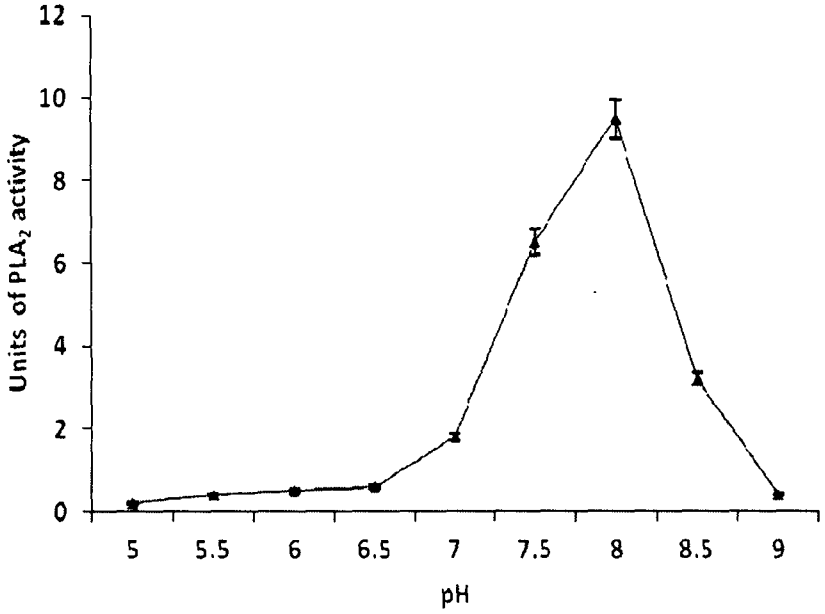

Fig 6.9: Effect of pH on catalytic activity of RVVN-PLA_2-I. PLA_2 activity of RVVN-PLA_2-I at different pH values were done as described in the section 3.2.6.8.6. The various pH values were obtained as follows: 0.1 Sodium acetate, pH 5.0-6.5; 0.1M K-Phosphate, pH 7.0-7.5 and 0.1 M Tris-HCl, pH 8.0-9.0. Results are expressed as mean ± S.D. of four determinations.

6.2.9 Secondary structure determination by Circular dichroism

Structural investigation through circular diachroism spectroscopy revealed that RVVN-PLA$_2$-I has a high content of alpha helix and beta-turn structures (54.6 % and 29.7 % respectively) along with random coils (15.7 %). CD spectra of native RVVN-PLA$_2$-I indicates a strong α-helical contribution to the CD signal (Fig 6.10) as it demonstrated defined minima at 216 nm (Fig 6.10). The CD spectra of heat-inactivated RVVN-PLA$_2$-I showed a minute deviation in CD signal after 45 min of heating the enzyme at 75 °C suggesting secondary structure is least affected by heating (Fig 6.10).

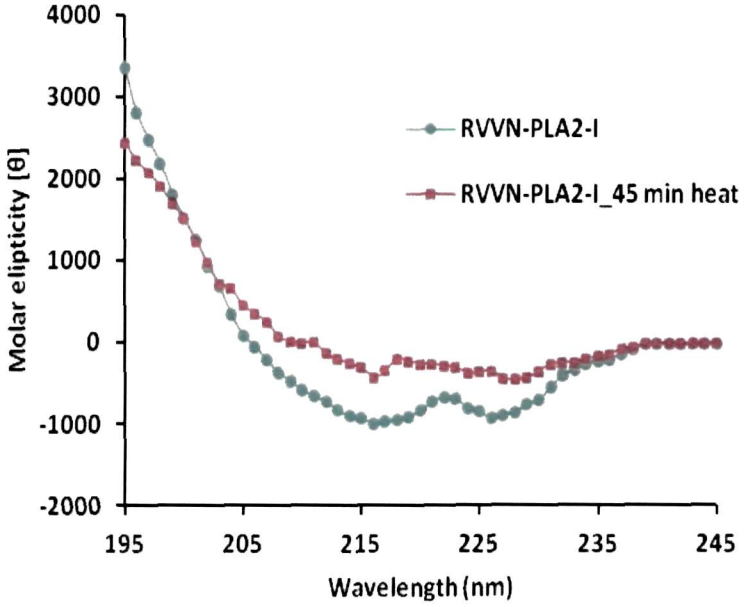

Fig 6.10: CD spectra of RVVN-PLA$_2$-I. CD measurement was performed as described in the section 3.2.6.8.7. The CD signals are expressed as mean residue ellipticity [θ] (degree cm^2 dmol^{-1}), using 113 as the mean residue molecular weight.

6.3 Pharmacological Characterization of RVVN-PLA_2-I

6.3.1 Anticoagulant activity

6.3.1.1 Plasma clotting time

RVVN-PLA_2-I demonstrated dose-dependent anticoagulant activity in *in vitro* condition. Though with initial concentration (0.1-0.3 μg ml^{-1}) of PLA_2, anticoagulant activity did not increased significantly, however, with an increase in concentration of RVVN-PLA_2-I up to 1.0 μg ml^{-1}, its anticoagulant activity (as determined by Ca-clotting time of PPP) was enhanced linearly. Nevertheless, at an enzyme concentration beyond 1.0 μg ml^{-1}, a saturation in enzyme activity was observed (Fig 6.11).

The effect of pre-incubation time of RVVN-PLA_2-I (0.5 μg ml^{-1}) with PPP on the anticoagulant activity demonstrated that 5 min was the optimal pre-incubation time for exerting maximum anticoagulant activity; beyond 5 min of pre-incubation, no further increase in Ca-clotting time of plasma was observed (Fig 6.12).

6.3.1.2 Prothrombin time test

RVVN-PLA_2-I was hardly effective in delaying the coagulation time of PPP of goat (specific activity was 0.9 $x10^3$ ± 0.8 unit mg^{-1} of protein) as compared to the coagulation time of control plasma when assayed by using commercial prothrombin time test kit.

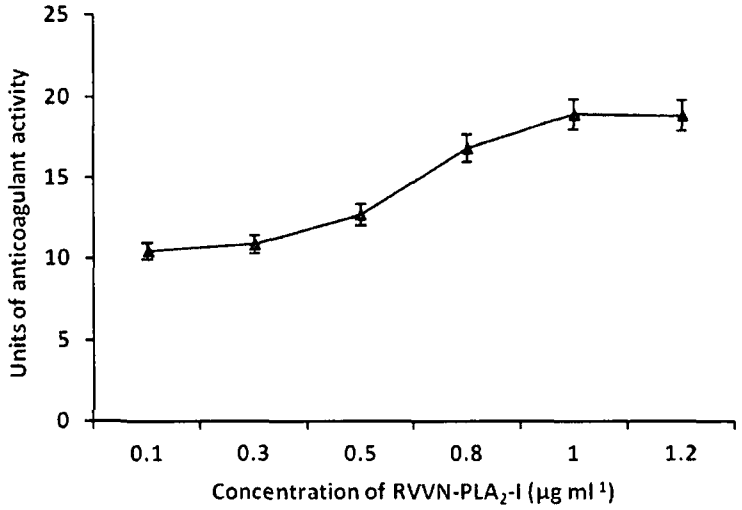

Fig 6.11: Dose dependent study of anticoagulant activity of RVVN-PLA$_2$-I (Ca-clotting time). This study was done as described in the section 3.2.7.1.1 by using different concentrations of RVVN-PLA$_2$-I (0.1-1.2 µg ml^{-1}). Values represent the mean ± S.D. of four experiments.

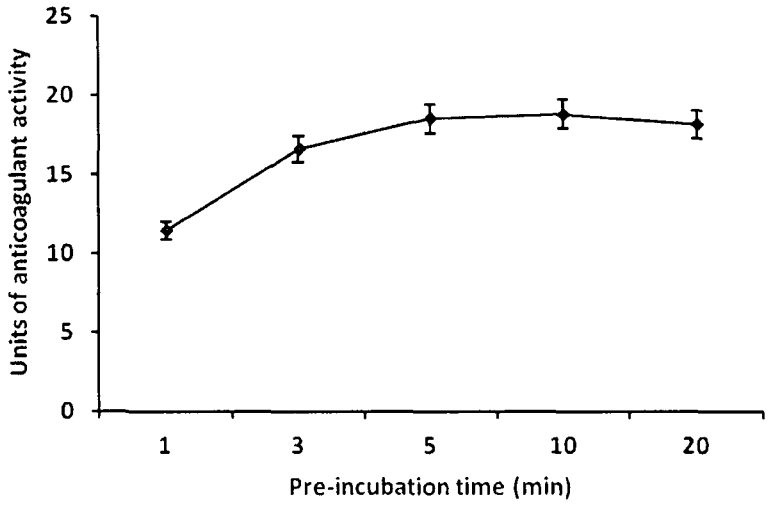

Fig 6.12: Effect of pre-incubation time on anticoagulant activity of RVVN-PLA$_2$-I (Ca-clotting time). This study was done as described in the section 3.2.7.1.1 for different time of pre-incubation (1-20 min) of plasma with RVVN-PLA$_2$-I (0.5 µg ml^{-1}). Values represent the mean ± S.D. of four experiments.

6.3.1.3 Effect of RVVN-PLA$_2$-I on plasma phospholipids hydrolysis and FFAs release

With an increase in the pre-incubation time of plasma with PLA$_2$, a concomitant increase in plasma phospholipids hydrolysis was observed up to 5 min. Beyond 5 min of pre-incubation, no significant enhancement of plasma phospholipids hydrolysis could be detected (Fig 6.12). The GC-analysis of FFAs released from plasma phospholipids by the action of RVVN-PLA$_2$-I (0.5 μg ml^{-1}) at different time intervals supported the above observation (Fig 6.13).

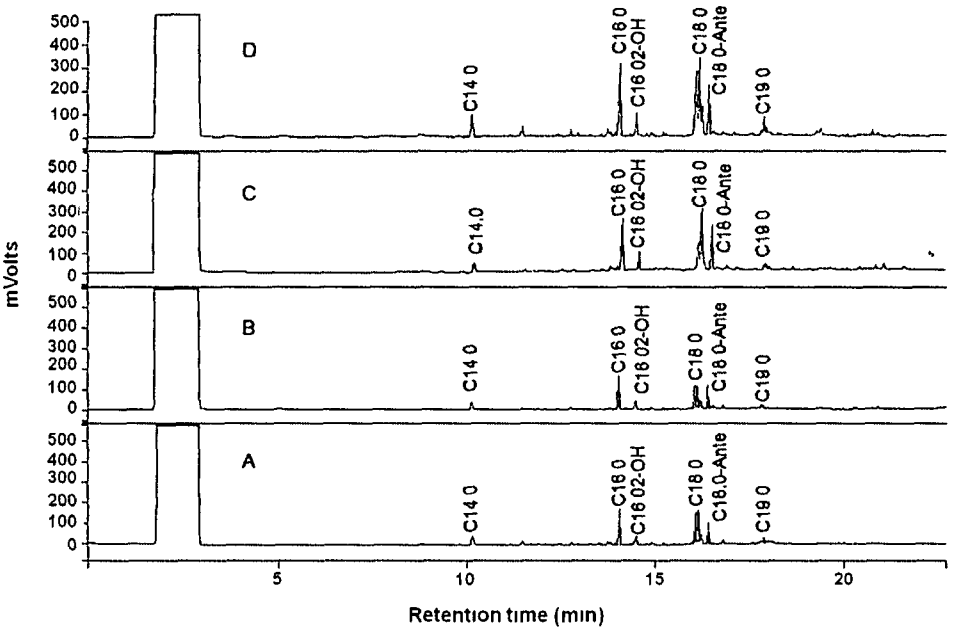

Fig 6.13: GC Analysis of plasma phospholipids hydrolysis by RVVN-PLA$_2$-I: A) control B) 3 min, C) 5 min, D) 10 min post incubation with RVVN-PLA$_2$-I. This study was done as described in the section 3.2.7.1.3. Data shows a typical experiment and repetition of experiment demonstrated similar result.

6.3.1.4 Binding of RVVN-PLA_2-I with different phospholipids

The excitation of fluorescence of free RVVN-PLA_2-I (100 nM) was done at 280 nm and emission maximum were observed at 343.5 nm (Fig 6.14). As shown in Fig 6.14 fluorescence intensity of RVVN-PLA_2-I post mixing with different phospholipids bearing different polar head groups exhibited different results. A large increase in fluorescence intensity (about 2.8 fold increase) of RVVN-PLA_2-I in presence of PS was observed and addition of 2 mM Ca^{2+} further enhanced the fluorescence intensity (Fig 6.14). In contrast, a minor decrease in fluorescence intensity of PLA_2 was observed after addition of PE; addition of PC did not influence the fluorescence intensity of protein. It is to be noted that neither PC nor PE influenced the shifting in emission spectrum of PLA_2. When the binding experiment was performed with an equimolar mixture of PC: PS: PE (final concentration 50 μM), the fluorescence intensity changed with a red shift of 5 nm (emission maxima 348.5 nm).

A slight decrease in the fluorescence intensity with a 10 nm blue shift of the heat-inactivated RVVN-PLA_2-I (heated for 60 min at 75 °C) was observed (Fig 6.14). However, a significant decrease in the fluorescence intensity of the heated RVVN-PLA_2-I post mixing with PS revealed that phospholipid (PS) binding efficiency of the heat-inactivated protein was drastically reduced compared to the PS binding potency of native PLA_2 (Table 6.12 and Fig 6.14).

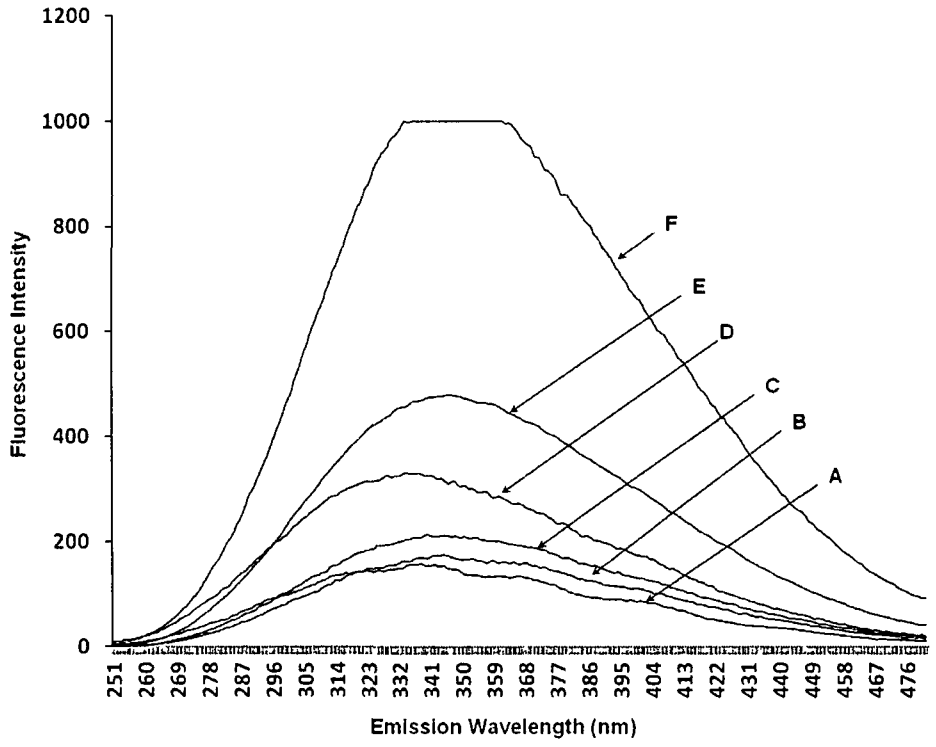

Fig 6.14: Fluorescence spectra showing interaction of RVVN-PLA$_2$-I (100 nM) with phospholipids (50 μM), A. Heated RVVN-PLA$_2$-I (heated for 60 min at 75 °C), **B.** RVVN-PLA$_2$-I, **C.** RVVN-PLA$_2$-I and PC, PE and PS, **D.** Heated RVVN-PLA$_2$-I and PS, **E.** RVVN-PLA$_2$-I and PS, **F.** RVVN-PLA$_2$-I and PS and Ca^{2+}.

6.3.1.5 Interaction of RVVN-PLA$_2$-I with blood coagulation factor Xa, factor Va, prothrombin and thrombin

There was not any change observed in the fluorescence signal of RVVN-PLA$_2$-I in presence of coagulation factor Xa (Fig 6.15), factor Va, prothrombin and thrombin was observed (Fig 6.16). This indicated that there was lack of interaction of RVVN-PLA$_2$-I with these coagulation factors.

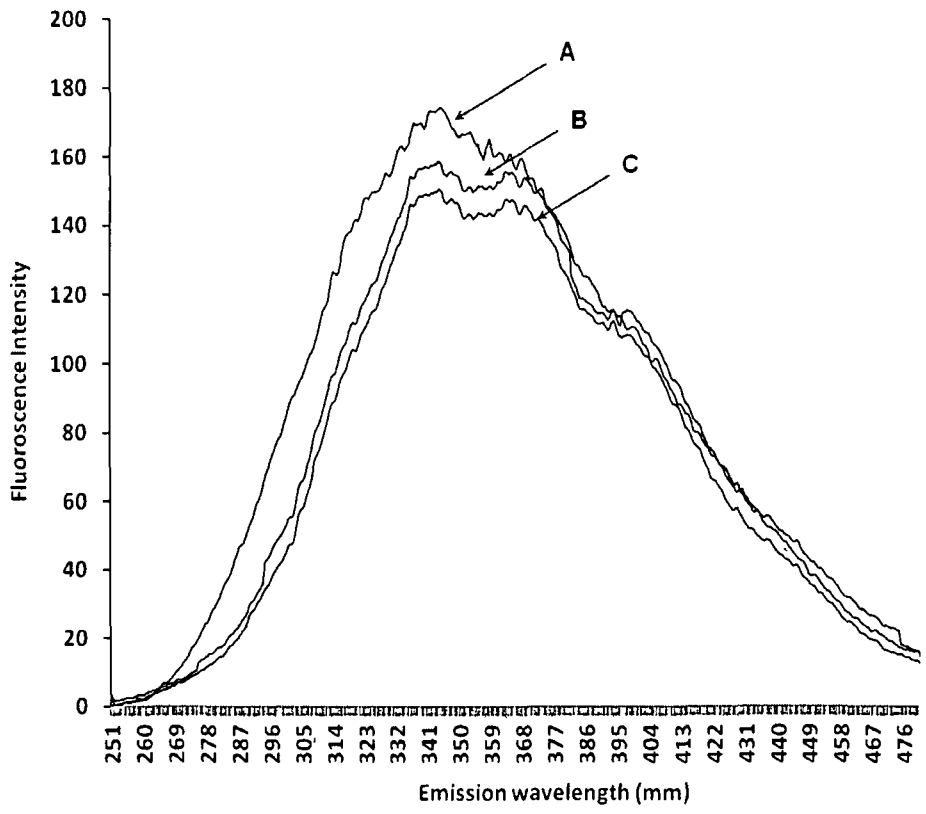

Fig 6.15: Fluorescence spectra showing interaction of RVVN-PLA$_2$-I (100 nM) with factor Xa (50 µM) A. Factor Xa, **B.** RVVN-PLA$_2$-I and Factor Xa, **C.** RVVN-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.11.

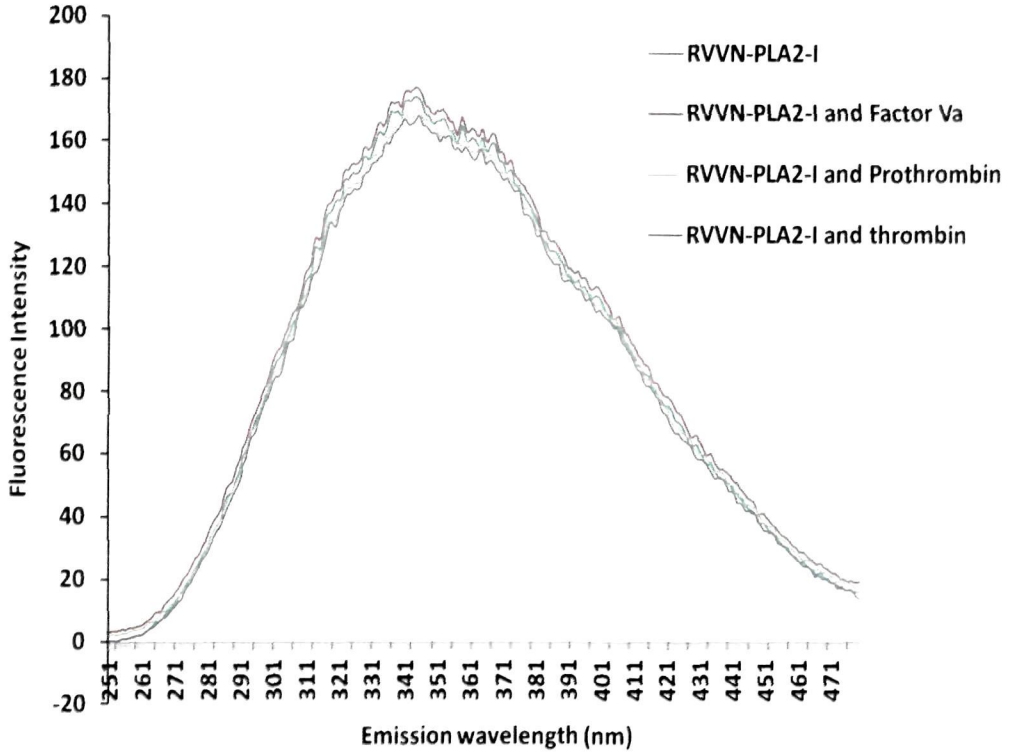

Fig 6.16: Fluorescence spectra showing interaction of RVVN-PLA$_2$-I (100 nM) with activated factor V (50 µM), prothrombin (50 µM) and thrombin (50 µM). Experiment was done as described in the section 3.2.6.8.11.

6.3.2 Direct and indirect haemolytic activity

RVVN-PLA$_2$-I did not exert considerable direct haemolytic activity on washed goat/human erythrocytes; however it showed an appreciable haemolytic activity in presence of egg yolk phospholipids/PS (Fig 6.17 and Table 6.6). It was also noted that erythrocytes pre-incubated with PS and 1.5 mM Ca^{2+} were highly susceptible to lysis by RVVN-PLA$_2$-I (Fig 6.17). Substitution of PS by PC also affected the haemolysis but to a lesser extent that in presence of PS. Nevertheless, pre-incubation of erythrocytes with PE did not influence the

haemolytic activity of RVVN-PLA_2-I even when 1.5 mM Ca^{2+} was added in the reaction medium (Fig 6.17)

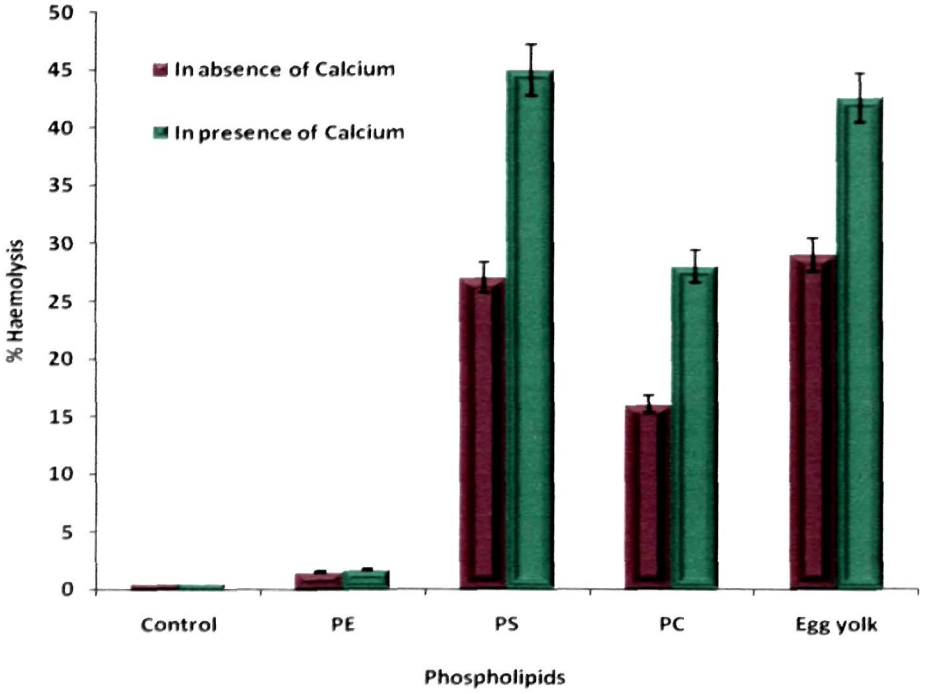

Fig 6.17: Haemolytic effect of RVVN-PLA_2-I (100 nM) in presence of different phospholipids (PC/PS/PE at a final concentration of 1.0 mM and egg yolk at a concentration of 1 µg ml^{-1}) and in presence or absence of 1.5 mM calcium. Experiment was done as described in the section 3.2.7.2. Lysis is expressed as percentage, taking 100 % absorbance of erythrocyte suspension incubated with Milli Q water. Erythrocyte suspension without exogenous supply of phospholipid(s) served as a control. Each result represents mean ±S.D. of four individual experiments.

6.3.3 *In-vitro* tissue damaging activity

In *in-vitro* condition, RVVN-PLA_2-I exerted significantly less heart tissue damaging activity as compared to crude RVV (Table 5.6). However,

RVVN-PLA_2-I was not detrimental to lung and liver tissues under identical experimental conditions (Table 6.4).

Table 6.4: *In vitro* tissue damaging activity of RVVN-PLA_2-I (% Hb released from 300 ± 10 mg tissue by 100 nM protein post 5 h of incubation at 37 °C)*. Values represent mean ±S.D. of four experiments

Tissues of	RVVN-PLA_2-I
Liver	0
Heart	10.1 ± 0.7
Lungs	0

*100% activity was achieved by treatment with 0.1% (v/v) triton X-100.

6.3.4 Stability of RVVN-PLA_2-I after storage at 4 °C

It was observed that after 28 days of isolation of RVVN-PLA_2-I from crude RVV (while kept in 20 mM K-phosphate buffer, pH 7.0 and stored at 4 °C), it lost 36 % and 56 % of its original catalytic and anticoagulant activity, respectively (Fig 6.18).

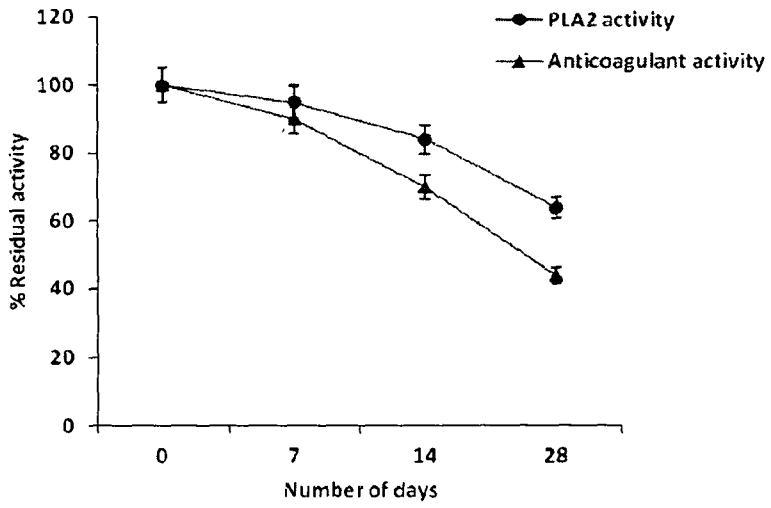

Fig 6.18: Assessment of catalytic and anticoagulant activity of RVVN-PLA$_2$-I after storage at 4 °C for different time periods. Storage stability was determined as described in the section 3.2.11. Values are expressed as mean ± S.D. of four typical experiments.

6.4 Membrane phospholipids hydrolysis by RVN-PLA$_2$-I

6.4.1 Effect of RVVN-PLA$_2$-I on mitochondrial membranes phospholipids hydrolysis

The RVVN-PLA$_2$-I showed swelling of chicken liver mitochondria in a dose dependent manner (Fig 6.19) and this effect was more pronounced in presence of Ca^{2+} (Table 6.5).

With an increase in incubation time of mitochondria with RVVN-PLA$_2$-I, the mitochondrial damage was enhanced concomitantly (Table 6.5). RVVN-PLA$_2$-I hydrolyzed the membrane of intact mitochondria with an initial lag phase of 15 min which was evident from the release of FFA and Pi from the membranes post incubation with this neutral anticoagulant PLA$_2$ enzyme from RVV (Table 6.5).

Addition of Ca^{2+} enhanced the membrane phospholipids hydrolysis effect of RVVN-PLA_2-I and the extent of phospholipids hydrolysis in presence of Ca^{2+} was about 1.5 fold higher compared to phospholipids hydrolysis in absence of Ca^{2+} post 15 min of incubation (Table 6.5).

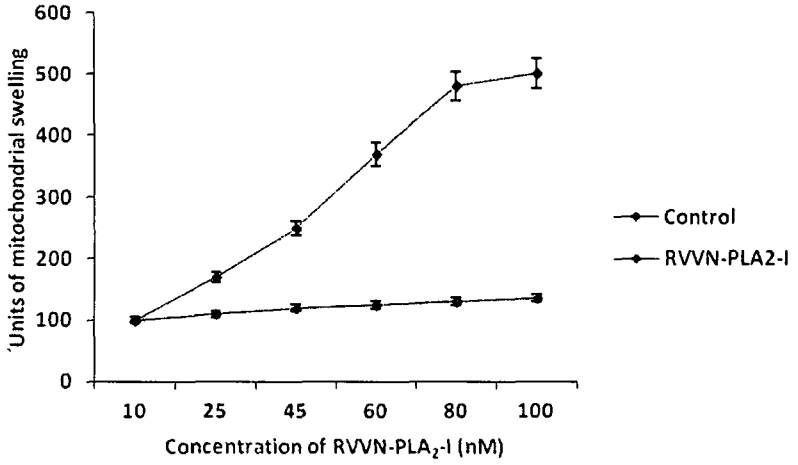

Fig 6.19: Dose-dependent swelling of chicken liver mitochondria by the effect of RVVN-PLA_2-I. Experiment was done as described in the section 3.2.7.5.1 by using different concentrations of RVVN-PLA_2-I (10-100 nM). Values are ± S.D. of triplicate determinations.

To gain further insight into the mode of attack of RVVN-PLA_2-I on the mitochondrial membrane, GC-analysis of liberated fatty acids from membrane revealed that $C_{14\,0}$, $C_{15\,0}$ anteiso, $C_{16\,0}$ $C_{18\,1}$, $C_{18\,0}$ iso, $C_{19\,0}$, $C_{20\,0}$, $C_{20\,1}$ and few unidentified fatty acids (>C_{20}) could be detected from the mitochondrial membranes post 60 min of incubation with PLA_2 (Fig 6.20). Addition of Ca^{2+} did not alter the fatty acid release pattern from the mitochondrial membrane; however, this metal ion potentiated the membrane hydrolytic activity of RVVN-PLA_2-I resulting in release of higher quantity of fatty acids from the mitochondrial membrane by the action of PLA_2 enzyme (Table 6.5).

Table 6.5: RVVN-PLA$_2$-I induced swelling and phospholipids hydrolysis of intact mitochondrial membrane either in presence or absence of 2 mM Ca^{2+}. About 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissue) from chicken liver was incubated with 100 nM of RVVN-PLA$_2$-I at 37 °C for different time periods. The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	Mitochondrial swelling (U/min)*		Phospholipids hydrolysis				Ratio of saturated/unsaturated FFA
			µg FFA released		µg of P_i released		
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
0 (control)	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
30	950 ± 4.5^a	1100 ± 3.1^a	1055 ± 2.7^a	1146.9 ± 1.3^a	31.4 ± 2.9^a	48.0 ± 1.4^a	N.D.
60	1150 ± 2.3^b	1450 ± 2.2^b	1376.3 ± 1.8^b	1651.6 ± 2.6^b	101.0 ± 2.5^b	131.2 ± 1.6^b	1.03

*Mitochondrial swelling was measured spectrophotometrically and one unit of swelling is defined as a decrease in 0.01 absorbance/min of mitochondrial suspension at 520 nm by 100 nM of RVVN-PLA$_2$-I.

Values (for different time periods) with different superscripts in same column are significantly different ($p<0.05$).

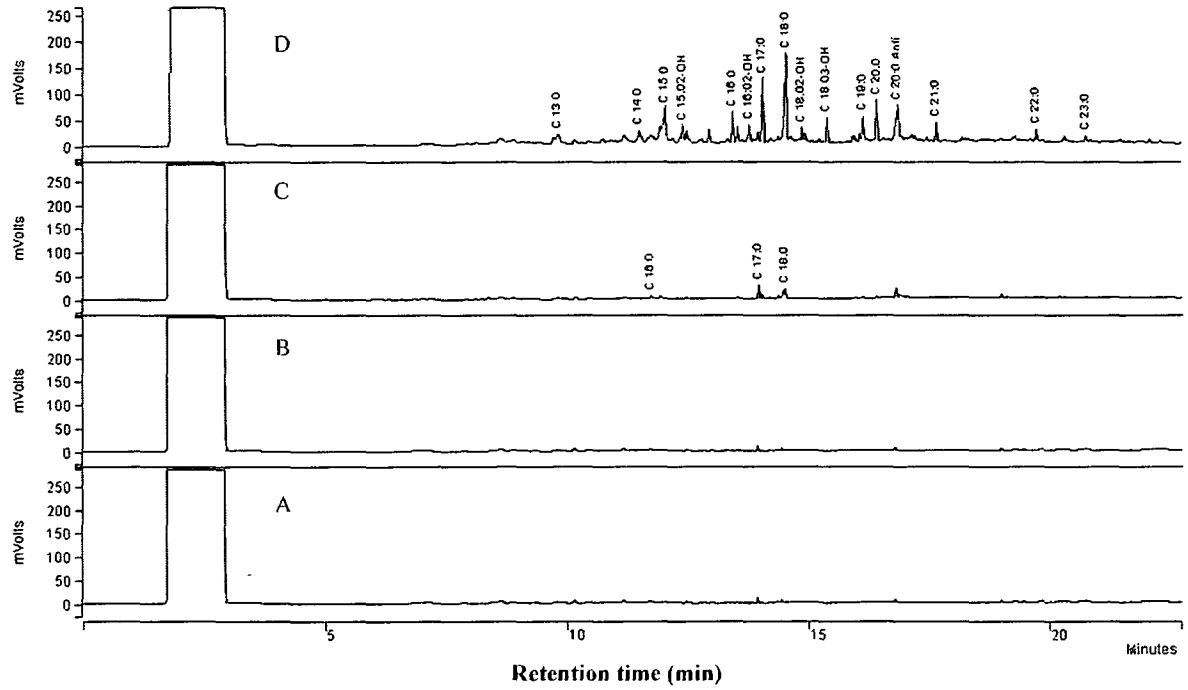

Fig 6.20: Gas Chromatographic analysis of mitochondrial membrane phospholipids hydrolysis by RVVN-PLA$_2$-I. A) Control, B) 15 min, C) 30 min, D) 60 min post incubation of mitochondrial membranes with RVVN-PLA$_2$-I. Experiment was done as described in the section 3.2.7.5.2. Data shows a typical experiment and repetition of experiment demonstrated similar result.

6.4.2. Effect of RVVN-PLA$_2$-I on erythrocyte membranes phospholipids hydrolysis

The effect of pre-incubation time of RVVN-PLA$_2$-I on haemolysis and liberation of FFAs from washed erythrocytes was showed in Table 6.6. With an increase in incubation time of RBC with purified PLA$_2$ (Table 6.6) or increasing the concentration of PLA$_2$ (Fig 6.21), the extent of phospholipids hydrolysis was enhanced concomitantly as was evident from the increased release of FFA and P_i from the RBC membrane. Interestingly, during the initial 30 min of attack, no haemolysis was observed although the PLA$_2$ was able to release the FFA and lysophospholipids from intact RBC membrane. On the contrary, addition of PS to the erythrocytes suspension resulted in initiation of haemolysis within 15 min of incubation and after 120 min, about 42.6 % of total RBC were haemolysed (Table 6.6). Approximately 9-fold increase in FFA release was detected post 120 min of incubation of RBC with RVVN-PLA$_2$-I compared to 15 min of incubation under the identical condition (Table 6.6).

The GC analysis of erythrocytes membrane phospholipids hydrolysis by RVVN-PLA$_2$-I also corroborated well with the FFA release pattern as shown in Fig 6.21 and Table 6.6. It was observed that RVVN-PLA$_2$-I had shown a specific preference for releasing the $C_{17:0}$, $C_{18:0}$, and $C_{20:0}$ anti fatty acids from the intact RBC membrane post 30 min of incubation. However, a quantitative as well as qualitative differences in fatty acids release pattern from membrane was detected after 120 min of incubation of RBC with the PLA$_2$; additional FFAs of chain length $C_{14:0}$, $C_{20:0}$ could also be detected by GC (Fig 6.22).

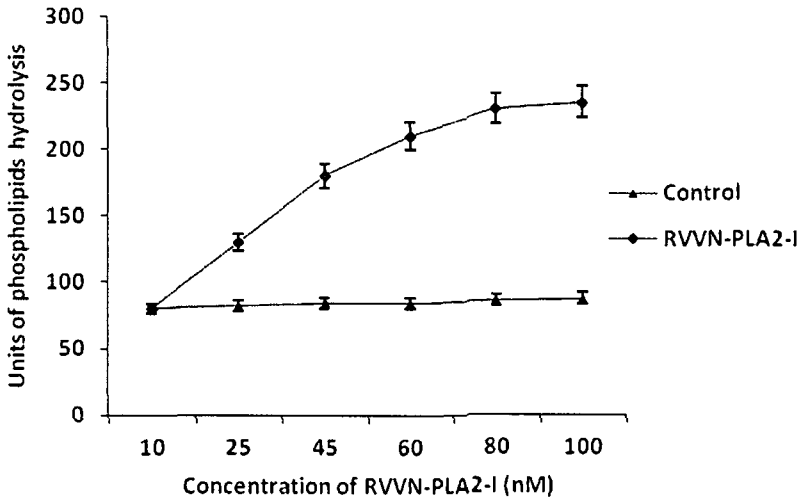

Fig 6.21: Dose dependent study of erythrocyte membrane phospholipids hydrolysis activity of RVVN-PLA$_2$-I. Experiment was done as described in the section 3.2.7.5.1 by using different concentrations of RVVN-PLA$_2$-I (10-100 nM). Values are ± S.D. of triplicate determinations.

Table 6.6: RVVN-PLA$_2$-I induced haemolysis and phospholipids hydrolysis of goat washed erythrocytes. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVN-PLA$_2$-I at 37 °C and haemolysis (direct and indirect) and erythrocyte phospholipids hydrolysis were determined. Indirect phospholipids hydrolysis was achieved in presence of PS (1.0 mM). The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	% haemolysis		Phospholipids hydrolysis		Ratio of saturated/unsaturated FFA
	Direct	Indirect	µg FFA released	µg of P_i released	
0 (control)	0	0	0	0	0
15 min	0	0	225.0 ± 1.2^a	17.3 ± 0.9^a	N.D.
30 min	0	32.7 ± 0.8^b	506.2 ± 2.3^b	38.9 ± 1.9^b	1.5
60 min	0.4	40.1 ± 0.4^c	1069.0 ± 1.4^c	87.3 ± 1.1^c	1.5
120 min	1.3	45.6 ± 0.2^d	1225.0 ± 2.1^d	121.0 ± 1.8^d	1.8

Values (for different time periods) with different superscripts in same column are significantly different ($p<0.05$).

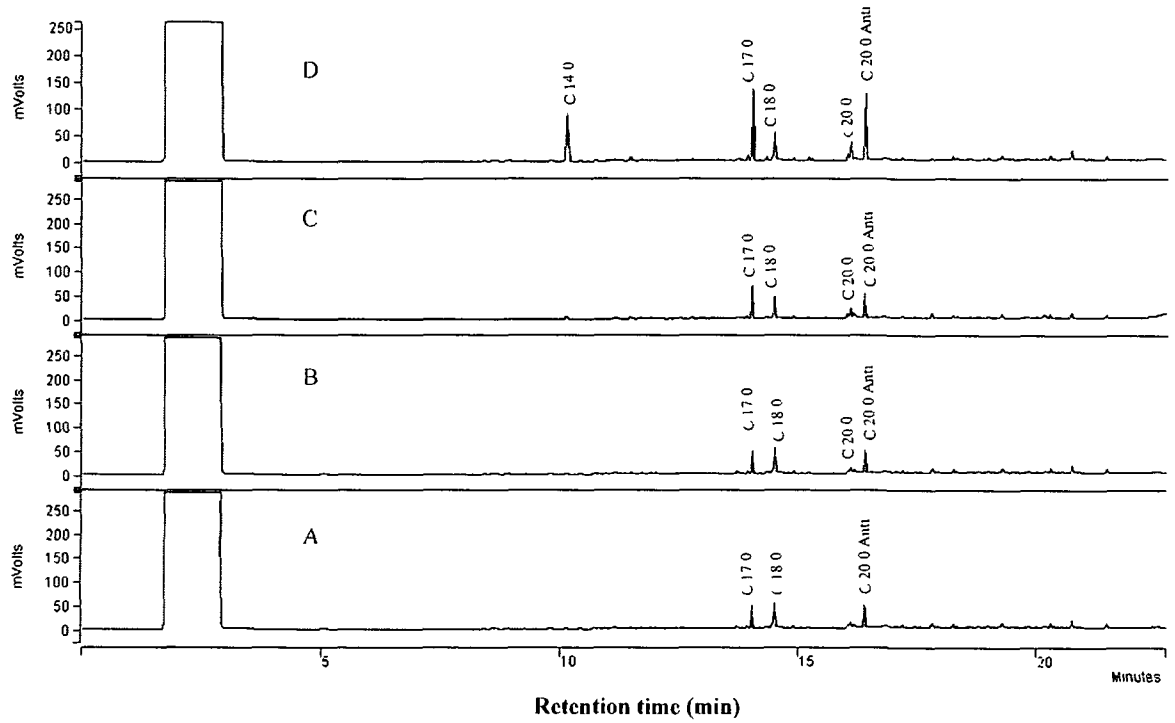

Fig 6.22: Gas chromatographic analysis of erythrocyte membrane phospholipids hydrolysis by RVVN-PLA$_2$-I. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVN-PLA$_2$-I (in presence of 2 mM Ca^{2+}) at 37°C for different time periods. The liberated fatty acids were analyzed by GC-MS as described in the section 3.2.7.5.2. A) control, B) 30 min, C) 60 min, D) 120 min after incubation of erythrocytes membranes with RVVN-PLA$_2$-I.

6.4.3 ELISA: Binding study of RVVN-PLA$_2$-I with membrane phospholipids

ELISA experiment was also done to study the membrane binding property of native and heat-inactivated PLA_2 which revealed that about 57.3 ± 1.1 % (mean ± S.D., n=3) of RVVN-PLA_2-I (heated for 60 min at 75 °C) could bind to intact mitochondrial (Fig 6.23) or, erythrocytes (Fig 6.24) suspensions as compared to binding of native (unheated) PLA_2 enzyme. This suggests that heat inactivated RVVN-PLA_2-I partially lost its membrane binding property.

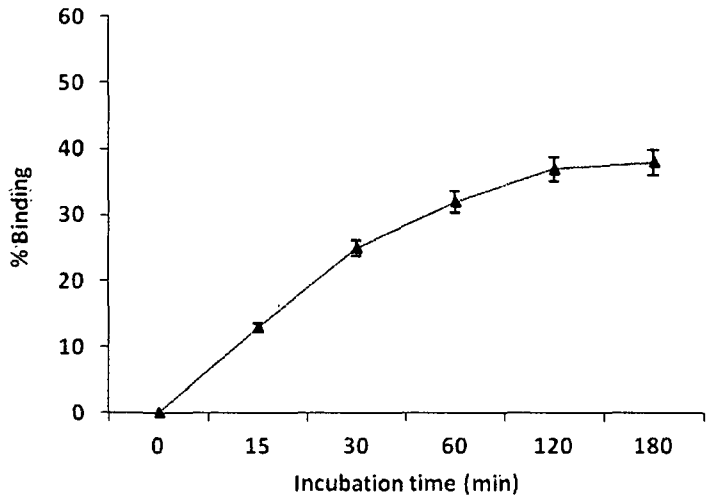

Fig 6.23: Binding of heat inactivated RVVN-PLA$_2$-I (heated for 60 min at 75 °C) with intact mitochondrial membranes. Experiment was done as described in the section 3.2.7.5.3. Values are ± S.D. of four experiments. Binding of native PLA_2 with membrane was considered as 100 % binding and then other values were calculated.

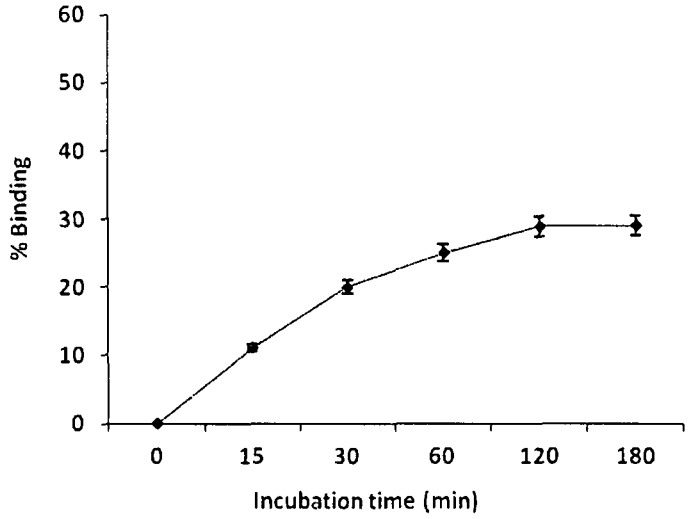

Fig 6.24: Binding of heat inactivated RVVN-PLA$_2$-I (heated for 60 min at 75 °C) with erythrocyte membranes. Experiment was done as described in the section 3.2.7.5.3. Values are ± S.D. of four experiments. The control value was considered as 100 % and then the other values were calculated.

6.5 Effects of chemical inhibitors, antivenom and heating on catalytic and pharmacological properties of RVVN-PLA$_2$-I

Table 6.7 shows the effect of various chemical inhibitors, polyvalent antivenom and heating on modulation of catalytic, anticoagulant and membrane damaging activities of RVVN-PLA$_2$-I. Chemical modification of histidine residue resulted in a significant inhibition (91.5 %) of catalytic activity; however, 19 % of anticoagulant activity was retained (Table 6.7). Catalytic, anticoagulant and membrane damaging activities of the purified enzyme were not inhibited by serine inhibitors like PMSF, TLCK and TPCK. Interestingly EDTA, DTT and IAA inhibited the catalytic activity of PLA$_2$ more than its anticoagulant activity (Table 6.7).

Commercial polyvalent antivenom raised in horse inhibited the catalytic, anticoagulant and membrane hydrolysing activities of RVVN-PLA_2-I almost in a parallel manner (Table 6.7).

To explore the relationship between catalytic site and other pharmacological site(s) we also investigated the effect of heating on catalytic, anticoagulant and membrane damaging properties of RVVN-PLA_2-I. Heating at 75 °C for different time periods suggesting similar effect of heating on catalytic, anticoagulant and membrane damaging activities of RVVN-PLA_2-I (Table 6.7).

Table 6.7: Effects of heating, antivenom and chemical inhibitors (at a final concentration of 2.0 mM) on catalytic, anticoagulant and membrane phospholipids hydrolysis action of RVVN-PLA$_2$-I. Values are mean ± S. D. of triplicate determinations. MM: mitochondrial membrane, EM: erythrocyte membrane.

	% residual activity				PC Binding (%)
	PLA$_2$	**Anticoagulant**	**MM hydrolysis**	**EM hydrolysis**	
Control	100	100	100	100	100
Heating at 75°C					
10 min	100	92.3 ± 2.4	84.0 ± 4.2	82.0 ± 4.0	96.5 ± 4.8
20 min	97.0 ± 1.3	88.3 ± 1.3	80.4 ± 3.2	78.0 ± 2.9	61.4 ± 3.1
30 min	94.0 ± 2.8	84.3 ± 1.1	75.0 ± 1.0	75.0 ± 1.0	50.6 ± 2.5
45 min	88.2 ± 1.4	78.3 ± 2.1	69.4 ± 3.2	70.4 ± 3.2	46.1 ± 2.3
60 min	83.1 ± 3.1	69.3 ± 1.4	63.4 ± 3.2	66.4 ± 2.1	42.0 ± 2.1
Antigen: antivenom (w:w)'					
1:100	92.8 ± 1.4	87.1± 2.3	80.1 ± 1.0	83.2 ± 2.2	-
1:200	87.7 ± 1.1	79.7± 1.7	75.3 ± 1.8	79.1 ± 1.9	-
1:500	79.6 ± 1.3	73.7± 1.1	72.4 ± 0.9	75.3 ± 1.3	-
Chemicals/ inhibitors					
TLCK	99.2 ± 1.1	99.0 ± 1.9	N.D.	100	-
TPCK	97.8 ± 1.1	97.1 ± 2.8	96.0 ± 1.2	94.5 ± 1.5	-
PMSF	97 ± 2.1	100	N.D.	N.D.	-
pBPB	8.5 ± 0.3	19.2 ± 2.8	29.0 ± 1.45	25.0 ± 1.25	-
DTT	42.2 ± 1.5	55.2 ± 1.5	N.D.	N.D.	-
IAA	37.7 ± 2.3	52.2 ± 1.5	50.0 ± 2.4	46.6 ± 2.3	-
EDTA	32.3 ± 1.6	45.5 ± 2.5	33.0 ± 1.2	31.5 ± 1.6	-

6.6 Other Pharmacological properties

6.6.1 Antibacterial activity

RVVN-PLA_2-I (at a concentration of 25 μg ml^{-1}) did not show any antibacterial activity against Gram positive bacteria viz. *B. subtilis* or Gram negative bacteria viz. *E. coli* and *P auregonosa* when tested by time course experiment (Fig 6.25).

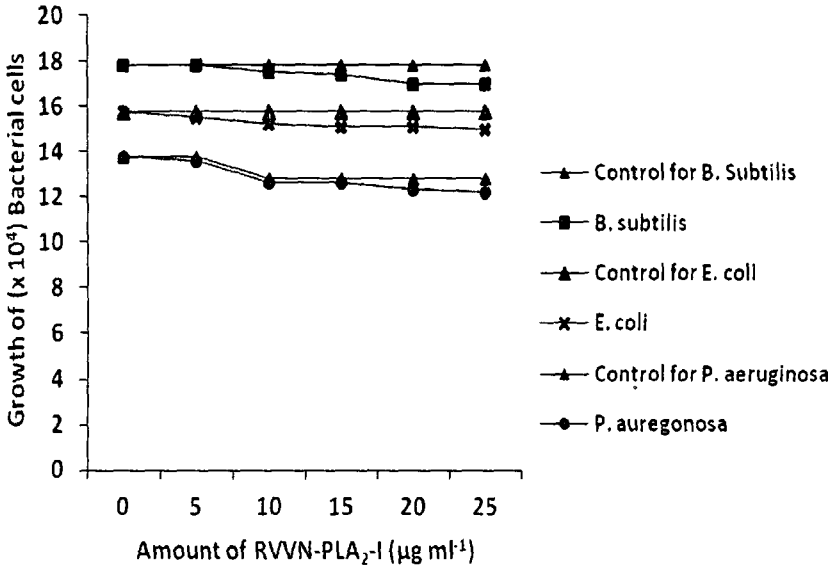

Fig 6.25: Bactericidal activity of RVVN-PLA_2-I on *B.subtilis*, *E.coli* and *P. auregonosa*. Varying amount of RVVN-PLA_2-I (5-25 μg ml^{-1}) was incubated with 18.4 x 10^4 cells of each bacterium for 24 h at 37 °C and change in optical density was recorded at 630 nm. 1 OD at 630 nm= 10^6 cells. Each point represents the mean ± S.D. of four experiments. Only media without RVVN-PLA_2-I served as control for each experiment. Experiment was done as described in the section 3.2.7.4.

6.6.2 *In vitro* cytotoxicity assay on mammalian cells

Fig 6.26 shows the cytotoxic effect of RVVN-PLA_2-I and crude RVV on HT 29 (human colon adenocarcinoma) cells. RVVN-PLA_2-I (at a dose of 10 µg ml^{-1}) could cause lysis of 8.8 % of total HT 29 cell after 4 h of incubation at 37 °C; and after 24 h incubation, it showed 11.6 % lysis of total HT 29 cells. The light microscopic observation of RVVN-PLA_2-I treated HT 29 cells (post 4 h and 24 h incubation) also supported the above observation (Fig 6.25) as no gross morphological change in cells had been detected 24 h after the treatment of RVVN-PLA_2-I.

The crude RVV, on the other hand, was significantly cytotoxic to HT-29 cells and 99 % cells were died after 24 h of incubation with crude RVV (10 µg ml^{-1}) [Fig 6.26].

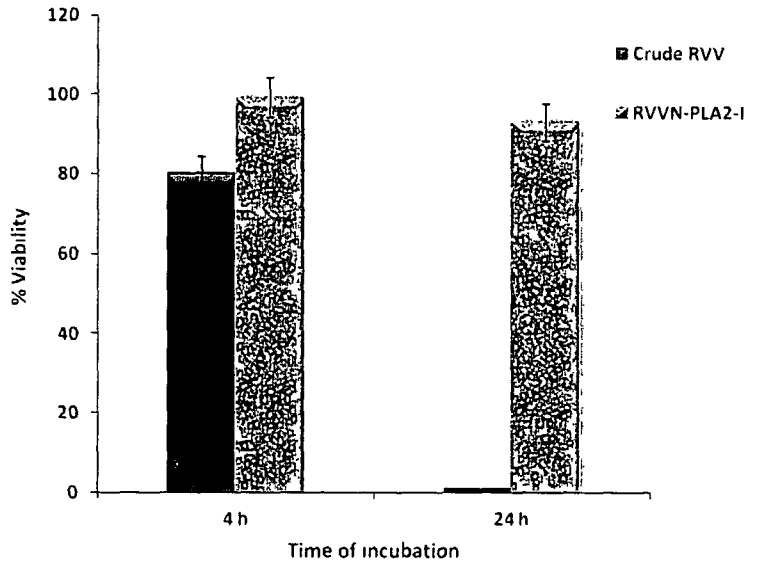

Fig 6.26: Cell cytotoxicity assay: the effect of crude RVV and RVVN-PLA_2-I on HT 29 cells. The incubation was carried out at 37 °C, 5 % CO_2 for the indicated time period. Experiment was done as described in the section 3.2.7.6. Values are mean ± S. D. of triplicate determinations.

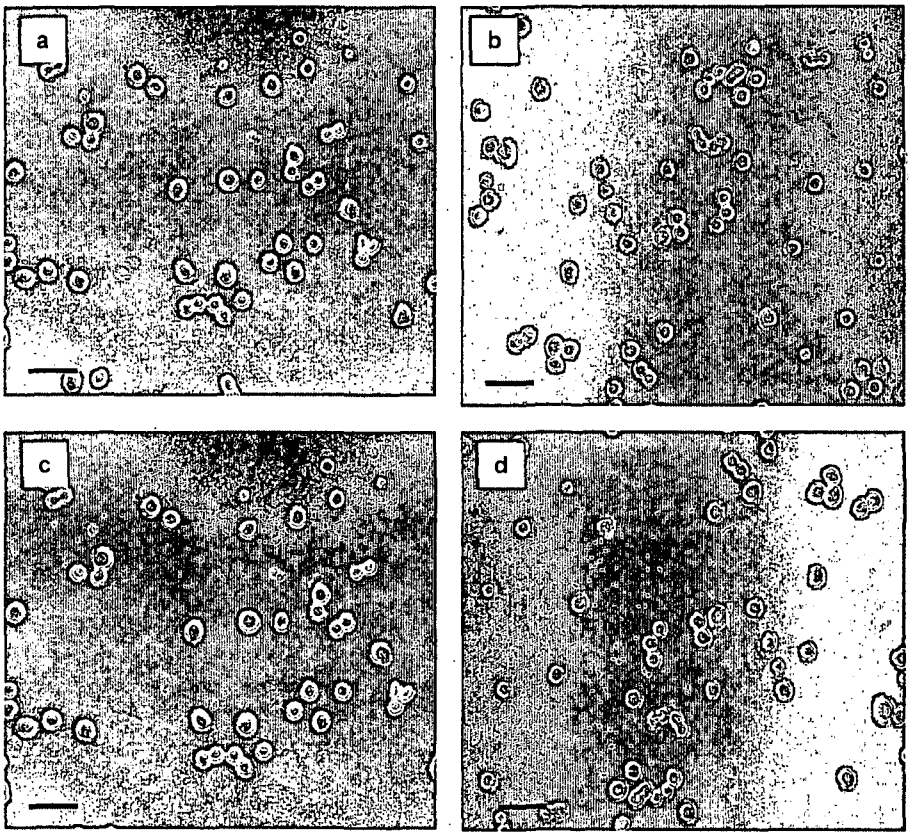

Fig 6.27: Light microscopic observation of the effect of RVVN-PLA$_2$-I on HT 29 cells. A) Control (4 hr) and b) HT 29 cells treated with 10.0 μg ml^{-1} RVVN-PLA$_2$-I post 4 hr of incubation. C) Control (24 hr) and D) HT 29 cells treated with 10.0 μg ml^{-1} RVVN-PLA$_2$-I post 24 hr of incubation. The incubation was carried out at 37 °C, 5 % CO_2 for the indicated time period. Magnifications, 100x: (a-d) bar=50 μm. Photographs were taken at a compact high-performance inverted microscope, Nikon ECLIPSE, TS100, Tokyo.

6.7 *In-vivo* toxicity assay

6.7.1 Lethality and *in-vivo* toxicity in mice

RVVN-PLA_2-I was not lethal to BALB/c mice at a concentration of 0.08 mg kg^{-1} body weight and did not show any sign of haemostatic disorder and behavioural changes in mice up to 48 h (Table 6.8).

6.7.2 Anticoagulant effect

The i.p. injection of RVVN-PLA_2-I (at a dose of 0.08 mg kg^{-1}) prolonged the *in vitro* tail bleeding time in mice as compared to the control group of mice. The *in vitro* coagulation time of PPP from RVVN-PLA_2-I treated mice was enhanced as compared to Ca-clotting time of plasma from the control group of mice (Table 6.9).

6.7.3 Effect on blood cells

Table 6.10 shows the *in vivo* effect of RVVN-PLA_2-I on haematological parameters of blood. It was observed that administration of RVVN-PLA_2-I resulted in significant decrease in erythrocytes and leucocytes count and Hb content in the blood of RVVN-PLA_2-I treated mice as compared to the control group of mice (Table 6.10).

Table 6.8: Behavioural Changes, if any, in mice 48 h after the administration of RVVN-PLA_2-I at a dose of 0.08 mg kg^{-1}

Values are mean ± S.D. of six determinations.

Group of mice	Parameters									
	Body weight (gm)		Grip Strength (sec)		Rectal temperature (F)		Faecal tendency (times per 15 min)		Urination (times per 15 min)	
	Initial*	Final**	Initial*	Final**	Initial*	Final**	Initial*	Final**	Initial*	Final**
Control	32.4 ± 0.5	32.6 ± 1.1	67.9 ± 1.5	32.5 ± 0.9	94.2 ± 1.8	94.1 ± 1.5	3 ± 0.6	5 ± 0.8	2 ± 10.5	4 ± 0.6
RVVN-PLA_2-I treated	25.1 ± 1.1	26.24 ± 1.2	23.1 ± 1.6	18.3 ± 1.4	94.1 ± 1.3	94.3 ± 1.7	5 ±0.5	4 ± 0.5	3 ± 0.5	3 ± 0.3

*Initial: determined at the onset of experiment.

**Final: determined after 48 h of RVVN-PLA_2-I administration.

Table 6.9: The *in vitro* clotting time of blood and tail bleeding time in mice post 48 h injection of RVVN-PLA$_2$-I at a dose of 0.08 mg kg^{-1}

Values are mean ± S.D. of six determinations.

Group of mice	Plasma clotting time (sec)	Tail bleeding time (sec)
Control	168.5 ± 1.5	45 ± 0.6
RVVN-PLA$_2$-I treated	319.8 ± 2.4[a]	64 ± 1.1[a]

Level of significance [a] $p < 0.001$

Table 6.10: Effect of RVVN-PLA$_2$-I on different haematological parameters of treated mice. Values are mean ± S.D. of six determinations.

Group of mice	Haematological parameters		
	WBC (m/mm^3)	RBC (m/mm^3)	Haemoglobin (Hb) (g/dL)
Control	11.46 ± 0.5	5.73 ± 0.6	6.45 ± 0.9
RVVN-PLA$_2$-I treated	7.68 ± 0.3	3.58 ± 0.5	5.14 ± 0.6
% decreased	32.99 %	37.53 %	20.31 %

6.7.4 Effect on serum parameters

Administration of RVVN-PLA_2-I resulted in a significant increase ($p < 0.05$) in ALP, CPK-MB, SGOT, SGPT and triglycerides levels in the serum of treated mice as compared to control group of mice; however the LDH and total protein levels were decreased in the serum of treated mice. The cholesterol, and glucose level in the serum of the treated mice were remain unchanged as compared to control (Table 6.11). Table 6.11 shows the effect of RVVN-PLA_2-I on serum parameters of treated BALB/c mice.

Table 6.11: Effect of RVVN-PLA$_2$-I on different parameters of serum of BALB/c mice. RVVN-PLA$_2$-I (0.08 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB/c mice (n=6). Mice were sacrificed after 48 hours of injection, blood was collected immediately by venipuncture and the serum was used for the assay of different parameters. Values are mean ± S.D. of six determinations.

	Total protein (g/dL)	Glucose (g/dL)	CPK-MB (U/L)	LDH (U/L)	ALP (U/L)	SGOT (U/L)	SGPT (U/L)	Cholesterol (g/dL)	Triglycerides (g/dL)
Control	270.0 ± 0.7	2.11 ± 1.2	27.5 ± 0.65	1281.5 ± 0.9	27.5 ± 0.8	144.0 ± 2.2	50.0 ± 0.91	0.7 ± 0.4	0.2 ± 0.04
Treated	184.0 ± 0.4[a]	2.79 ± 1.6	47.0 ± 0.9[a]	798.0 ± 2.1[a]	84.7 ± 1.1[a]	216.0 ± 2.1[a]	118.0 ± 1.5[a]	0.83 ±1.1	0.65 ± 0.05[a]

Significance of difference [a] $p < 0.001$.

6.7.5 Histopathological study

In histopathological study, any significant change was not observed in the tissues of RVVN-PLA_2-I treated mice organs as compared to control tissues (Fig 6.28).

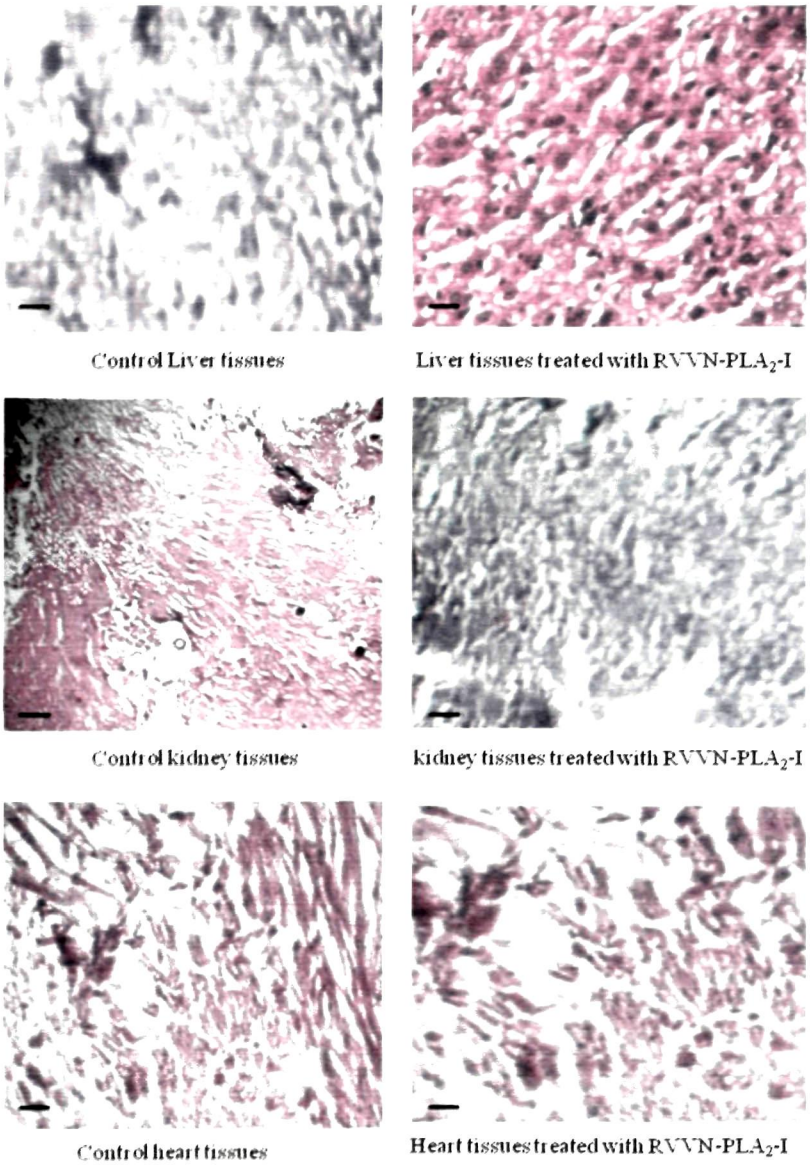

Fig 6.28: Light microscopic observation of the effect of RVVN-PLA_2-I on different organs of treated mice. Tissues from mice treated with PBS were served as control. Hematoxyline-eosine (H&E) staining; Magnifications 100x: bar=50 μm.

6.8 Immunological cross reactivity

6.8.1 Immunodiffusion

RVVN-PLA_2-I did not show cross reactivity towards commercially available polyvalent antivenom at a ratio of 1:100 (antigen: antibody, w/w). In immunodiffusion test, no visible antigen (RVVN-PLA_2-I)-antibody (polyvalent antivenom) precipitate band was observed even after 48 h of incubation (Fig 6.29). PBS was used as control in this immunodiffusion assay in place of venom proteins (Fig 5.33 A).

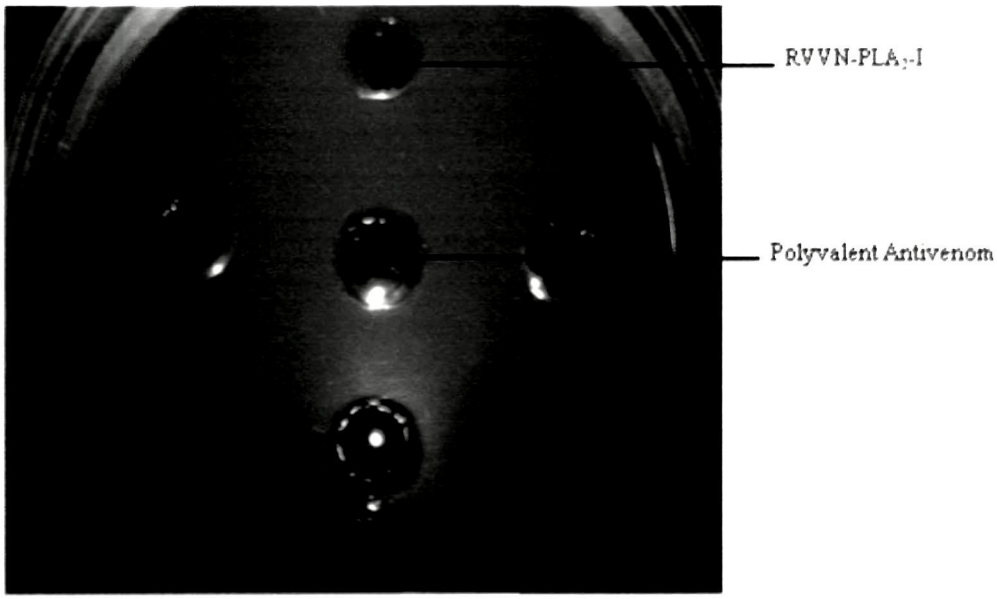

Fig 6.29: Immunodiffusion test to determine cross reactivity of RVVN-PLA_2-I against commercial polyvalent antivenom used for the treatment of snakebite patients. Experiment was done as described in the section 3.2.7.7.1. Data shows a typical experiment and repetition of experiment demonstrated similar result.

6.9 Neutralization by plant extracts

6.9.1 Neutralization by AIPLAI

AIPLAI (*A. indica* PLA_2 inhibitor, the methanol extract of *A. indica* leaves) affect both the catalytic as well as the anticoagulant activity of RVVN-PLA_2-I. The AIPLAI at a dose of 50 μg ml^{-1} demonstrated 41.8 % and 48.0 % inhibition of catalytic anticoagulant activity of RVVN-PLA_2-I, respectively (Fig 6.30).

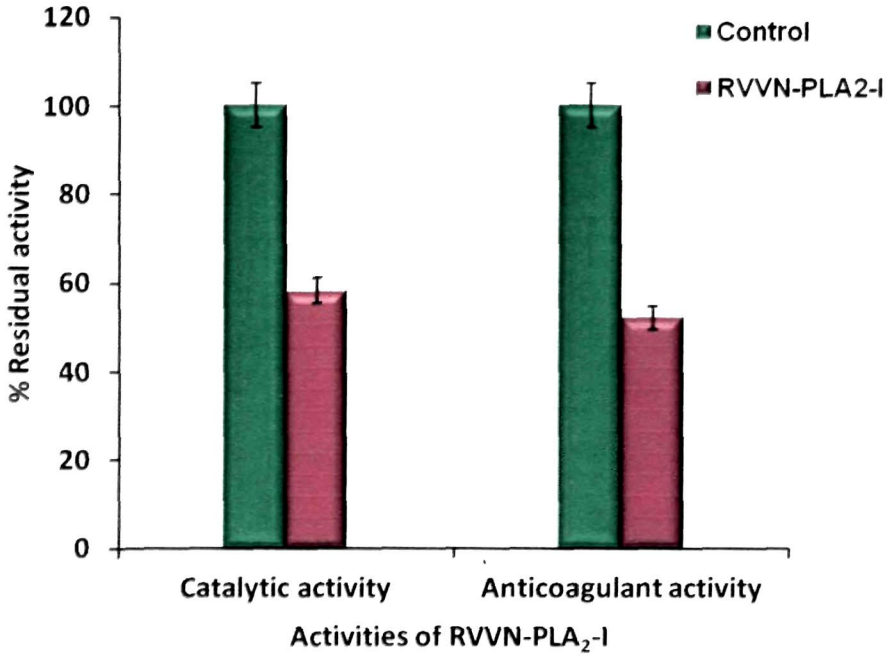

Fig 6.30: Effect of AIPLAI (50 μg ml^{-1}) on the catalytic and anticoagulant activity of RVVN-PLA_2-I (1 μg ml^{-1}). Values are expressed as the mean ± S.D. of four experiments.

6.9.2 Neutralization by *Camellia sinensis*, *Aegle marmelos* and *Xanthium strumarium*

Methanol extracts of the leaves of *C. sinensis* and *X. strumarium* and the roots of *A. marmelos* were found to be most potent in inhibiting the PLA_2 activity of RVVN-PLA_2-I among the different tested solvent extracts. Moreover, in a dose dependent study, amongst all the tested doses, 50 $\mu g\ ml^{-1}$ was found as the optimal dose for inhibition of enzymatic activity (Fig 5.35).

The effect of water, chloroform and methanol extract of these three plants on the catalytic and anticoagulant activity of RVVN-PLA_2-I are listed in the Table 6.12 and Table 6.13 respectively.

Table 6.14 shows the inhibition of catalytic as well of pharmacological properties of RVVN-PLA_2-I after the treatment with methanol extract of leaves of *C. sinensis* and *X. strumarium* and the roots of *A. marmelos.* Further, it shows a comparison of inhibition cause by polyvalent antivenom and by the plant extracts (Table 6.14).

Table 6.12: Percent inhibition of PLA_2 activity of RVVN-PLA_2-I (final concentration 1 μg ml^{-1}) by different extract of *Camellia sinensis*, *Aegle marmelos* and *Xanthium strumarium*. Preparation of plant extract and inhibition study was done as described in the section 3.2.10.2. PLA_2 activity without plant extract was treated as control (100 %). Results are mean ± S.D. of four determinations.

Amount of plant extract (μg)		% inhibition of PLA_2 activity of RVVN-PLA_2-I by		
		Camellia sinensis	*Xanthium strumarium*	*Aegle marmelos*
Water Extract	5	0.21 ± 0.01	0	2.3 ± 0.12
	10	1.3 ± 0.06	0.01 ± 0.001	7.1 ± 0.36
	50	7.8 ± 0.39	4.4 ± 0.22	11.4 ± 0.57
	100	11.2 ± 0.57	5.1 ± 0.26	15.6 ± 0.78
Chloroform Extract	5	3.5 ± 0.17	0.6 ± 0.03	3.1 ± 0.16
	10	8.1 ± 0.41	3.9 ± 0.19	9.7 ± 0.48
	50	16.4 ± 0.82	13.6 ± 0.68	21.2 ± 1.06
	100	19.8 ± 0.99	16.6 ± 0.83	24.4 ± 1.22
Methanol Extract	5	4.1 ± 0.21	2.1 ± 0.11	7.7 ± 0.38
	10	12.7 ± 0.64	6.6 ± 0.33	16.1 ± 0.81
	50	**29.5 ± 1.47**	**20.8 ± 1.04**	**34.5 ± 1.73**
	100	33.9 ± 1.68	25.7 ± 1.28	41.5 ± 2.06

Table 6.13: Percent inhibition of anticoagulant activity of RVVN-PLA_2-I (final concentration 1 µg ml^{-1}) by different extract of *Camellia sinensis*, *Aegle marmelos* and *Xanthium strumarium*. Preparation of plant extract and inhibition study was done as described in the section 3.2.10.2. PLA_2 activity without plant extract was treated as control (100 %). Results are mean ± S.D. of four determinations.

Amount of plant extract (µg)		% inhibition of anticoagulant activity of RVVN-PLA_2-I by		
		Camellia sinensis	*Xanthium* strumarium	*Aegle marmelos*
Water Extract	5	0.78 ± 0.04	0.31 ± 0.02	2.21 ± 0.06
	10	4.7 ± 0.23	3.1 ± 0.16	8.20 ± 0.41
	50	12.1 ± 0.61	10.0 ± 0.55	15.1 ± 0.75
	100	19.0 ± 0.95	17.1 ± 0.86	23.7 ± 1.18
Chloroform Extract	5	2.1 ± 0.11	3.7 ± 0.18	4.5 ± 0.22
	10	10.7 ± 0.54	8.7 ± 0.44	16.1 ± 0.81
	50	20.5 ± 1.01	19.1 ± 0.75	28.6 ± 1.43
	100	25.3 ± 1.21	23.1 ± 0.96	34.1 ± 1.71
Methanol Extract	5	6.1 ± 0.31	4.2 ± 0.21	9.8 ± 0.48
	10	18.1 ± 0.91	16.3 ± 0.81	23.7 ± 1.15
	50	**34.7 ± 1.2**	**29.0 ± 1.5**	**49.1 ± 1.7**
	100	39.4 ± 1.4	33.1 ± 1.6	54.5 ± 1.9

Table 6.14: A comparison of anti-PLA_2 activity of polyvalent antivenom and methanolic extracts of leaves of *C. sinensis* and *X. strumarium* and roots of *A. Marmelos*. RVVN-PLA_2-I (1 μg ml^{-1}) was incubated either with plant extract (100 μg ml^{-1}) or with polyvalent antivenom in a ratio of 1:100 (in a final volume of 1.0 ml) at 37 °C for 30 min and then assayed for residual catalytic and pharmacological properties of RVVN-PLA_2-I. Values are mean ± S.D. of triplicate determinations. Values in the same row with different superscripts are significantly different ($p<0.05$).

Pharmacological properties	% Inhibition of RVVN-PLA_2-I by			
	Polyvalent antivenom	*C. sinensis*	*X. strumarium*	*A. marmelos*
PLA_2 activity	7.2 ± 0.8^a	29.5 ± 1.7^b	20.8 ± 1.4^c	34.5 ± 1.7^d
Anticoagulant activity	24.9 ± 1.3^a	34.7 ± 1.2^b	29.0 ± 1.5^c	49.1 ± 1.7^d
Indirect haemolytic activity	27.5 ± 0.5^a	41.0 ± 1.0^b	33.3 ± 0.9^c	48.5 ± 1.3^d
In vitro tissue damaging activity				
Liver	37.7 ± 0.6^a	51.5 ± 1.1^b	43.3 ± 1.6^c	64.6 ± 0.6^d
Heart	42.0 ± 0.9^a	55.2 ± 0.8^b	53.1 ± 0.8^c	61.1 ± 1.1^d
Lungs	48.8 ± 1.1^a	59.6 ± 1.2^b	56.4 ± 0.7^c	71.3 ± 0.9^d
Mitochondrial membrane phospholipids hydrolysis	19.9 ± 1.1^a	39.9 ± 1.0^b	31.3 ± 1.2^c	45.1 ± 1.3^d
Erythrocytes membrane phospholipids hydrolysis	16.8 ± 0.9^a	44.8 ± 1.5^b	36.6 ± 1.2^c	56.9 ± 0.9^d

CHAPTER VII

A basic anticoagulant phospholipase A_2 enzyme (RVVB-PLA_2-I) isolated and purified from *Daboia russelli* venom: its biochemical and pharmacological characterisation

7 Results

7.1 Purification of a basic anticoagulant phospholipase A_2

7.1.1 Fractionation of CM-AC-IV by Sephadex G-50 gel filtration column

As described in the section 4.1, fractionation of crude RVV on a cation exchanger, CM-Sephadex C-50 column (20 mm x 60 mm), was resulted in the separation of RVV proteins into twelve basic PLA_2 fractions. However, only six out of those twelve protein peaks were found to possess anticoagulant activity (Fig 4.1). The fourth peak namely CM-AC-IV (eluted with 260 mM K-phosphate buffer, pH 8.0) showed the highest PLA_2 and anticoagulant activity among the basic anticoagulant fractions. Therefore, CM-AC-IV was selected for the purification of a basic PLA_2 enzyme.

As shown in Fig. 7.1, gel filtration of CM-AC-IV on a Sephadex G-50 column (1 cm x 64 cm) resulted in separation of CM-AC-IV into three peaks viz. GFI, GFII and GFIII. The PLA_2 as well as anticoagulant activities of these gel filtration peaks are shown in Table 7.1. Among the three peaks, GFIII showed maximum PLA_2 as well as anticoagulant activity.

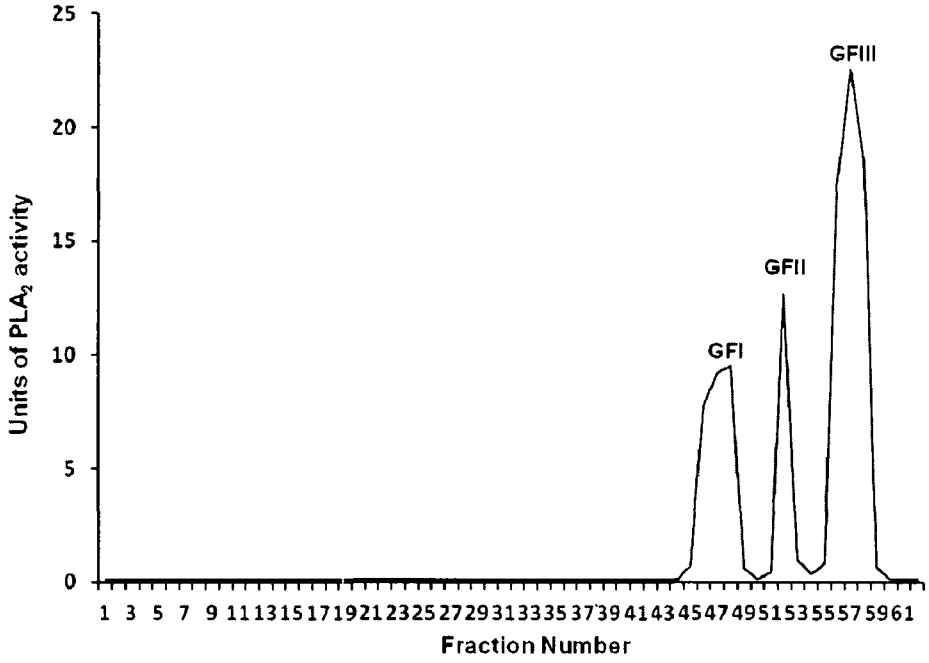

Fig 7.1: Fractionation of CM-AC-IV on Sephadex G-50 column (1 cm x 64 cm) to seperate the basic PLA_2 enzymes. Gel filtration fractionation was done as described in the section 3.2.4.1. Each protein peak was screened for PLA_2 activity.

Table 7.1: The PLA_2 as well as anticoagulant activity of the three gel filtration peaks of CM-AC-V on Sephadex G-50 column. Values are from a typical experiment.

Peaks	Recovery of protein (% of total venom load)[a]	PLA_2 specific activity (U/mg of protein)[b]	Anticoagulant Specific activity (U/mg of protein)[c]
GF-I	0.15	4.1×10^4	6.6×10^4
GF-II	0.12	5.7×10^4	6.2×10^4
GF-III	0.25	8.8×10^4	9.0×10^4

[a]Protein content was estimated by the method of Lowry et al., 1951.

[b]One unit of PLA_2 activity is defined as decrease in 0.01 absorbance at 740 nm as compared to control.

[c]One unit of anticoagulant activity is defined as protein/PLA_2 induced decrease in 1 sec clotting of platelet poor plasma (PPP) compared to clotting time of normal plasma under the same experimental condition.

7.1.2 RP-HPLC of GFIII: Purification of a basic PLA_2

Among the three peaks of the gel filtration column, the third peak, GFIII showed the highest PLA_2 as well as anticoagulant activity. Therefore, GFIII was further purified by using RP-HPLC and it resulted in a sharp single peak with a retention time of 15.9 min, showing the purity of the protein (Fig 7.2).

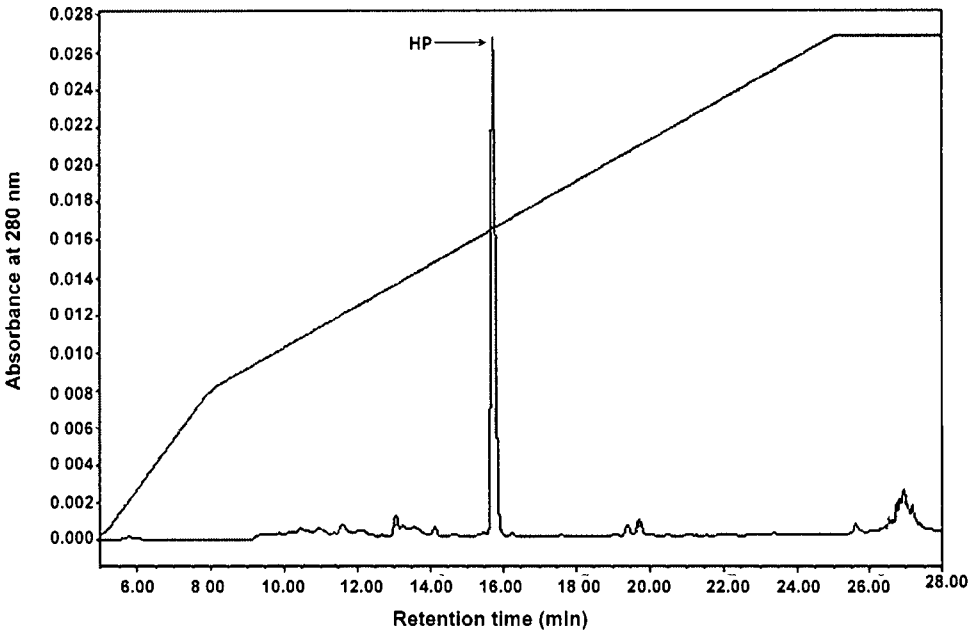

Fig 7.2: **Reverse-phase HPLC of fraction GFIII** (third gel filtration faction of CM-AC-IV) on a Waters reverse-phage HPLC C_{18}-μ Nova Pak column as described in the section 3.2.4.2. Solvents A and B were 0.1% (v/v) TFA in 5.0% (v/v) acetonitrile, and 0.1% TFA in 95% (v/v) acetonitrile, respectively. The protein was eluted with a retention time 15.9 min.

This purified protein was found to possess PLA_2 as well as anticoagulant activity and was named as RVVB-PLA_2-I (Russell's viper venom basic phospholipase A_2-I). Yield of RVVB-PLA_2-I was 0.15 % of the original venom load. A summary of purification of RVVB-PLA_2-I is shown in Table 7.2.

At a protein concentration of 25 μg ml^{-1}, RVVB-PLA_2-I was devoid of other enzymatic activity such as protease, ATPase, AMPsae suggesting the purity of preparation.

Table 7.2: Summary of purification of the basic anticoagulant phospholipase A_2 (RVVB-PLA_2-I) from Russell's viper venom.

Values are from a typical experiment.

	Total protein (mg)	Protein yield (%)	PLA_2 activity		Anticoagulant activity		Purification fold	
			Total activity (unit)	Specific activity (unit / mg)	Total activity (Unit)	Specific activity (unit / mg)	PLA_2 activity	Anticoagulant activity
Crude RVV	20.0	100.0	1.3×10^5	1.2×10^4	ND	ND	1	-
CM-AC-IV	0.30	1.5	1.6×10^4	5.3×10^4	2.2×10^4	7.3×10^4	4.0	1
Gel filtration	0.05	0.26	4.5×10^3	8.8×10^4	4.6×10^3	9.0×10^4	6.8	1.2
RP-HPLC	0.03	0.15	4.8×10^3	1.6×10^5	3.9×10^3	1.3×10^5	12.3	1.8

7.1.3 Assessment of purity and determination of molecular mass of RVVB-PLA_2-I

7.1.3.1 Gel filtration chromatography and SDS-PAGE

The molecular mass of the PLA_2 was checked by gel filtration, SDS-PAGE and ESI/MS analyses. The molecular mass of GFIII was calculated as 7.0 kDa from the calibration curve (Fig 5.1B) of the gel filtration column.

About 20 µg of RVVB-PLA_2-I gave a sharp, Coomassie Brilliant blue positive band on 18% SDS-PAGE under both reduced and non-reduced conditions (Fig 7.3). Both in reduced as well as non-reduced condition, this protein appeared as a single band of 6.7 kDa indicating purified PLA_2 is a monomer.

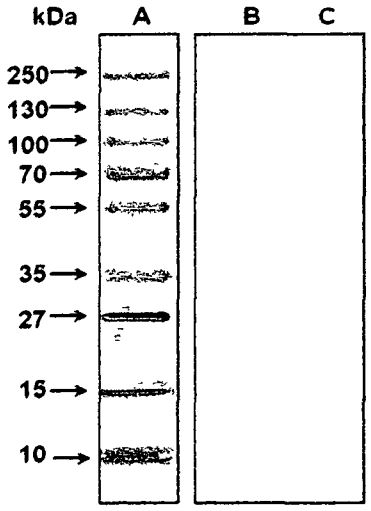

Fig 7.3: 18% SDS-polyacrylamide gel electrophoresis: Lane A) molecular weight marker; lane B) 20 µg RVVB-PLA_2-I (non-reduced condition); lane C) 20 µg RVVB-PLA_2-I (reduced condition). Experiment was done as described in the section 3.2.5.3.

7.1.3.2 ESI/MS analysis of RVVB-PLA$_2$-I

The molecular mass of RVVB-PLA$_2$-I was determined as 6.763 Da by electron spray ionization-mass spectrophotometry (ESI/MS) (Fig 7.4).

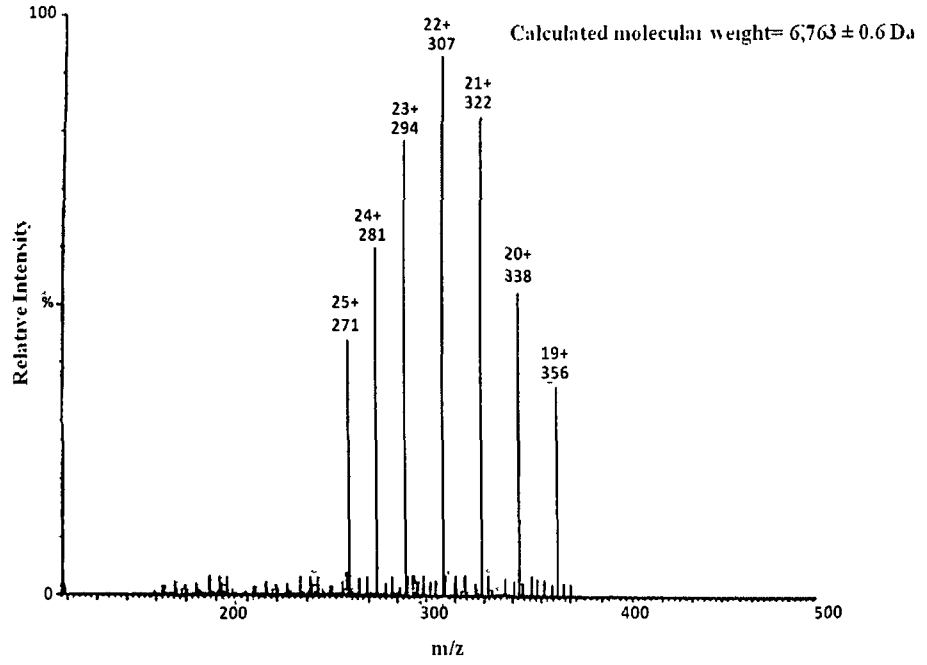

Fig 7.4: ESI/MS of RVVB-PLA$_2$-I for molecular mass determination. Experiment was done as described in the section 3.2.5.4.

7.2 Biochemical Characterisation of RVVB-PLA$_2$-I

7.2.1 PLA$_2$ activity and substrate specificity

RVVB-PLA$_2$-I demonstrated significant PLA$_2$ activity towards egg yolk phospholipids substrate (Table 7.2). The substrate specificity of RVVB-PLA$_2$-I towards different commercially available phospholipid substrates ps viz., PC, PS and PE showed that RVVB-PLA$_2$-I has a distinct preference for hydrolysis of PC followed by PS and PE was least hydrolyzed (Table 7.3).

7.2.2 Carbohydrate content

RVVB-PLA$_2$-I was found to be devoid of any carbohydrate content as checked by the phenol-sulphuric acid method.

Table 7.3: Substrate specificity of the basic anticoagulant PLA$_2$ (RVVB-PLA$_2$-I). Values are mean ± S.D. of four determinations. Experiment was done as described in the section 3.2.6.8.1.

Phospholipid Substrate (Final concentration 1 mM)	**PLA$_2$ Specific activity (Unit* mg^{-1}min^{-1})**
Phosphotidylcholine (PC)	$7.9 \times 10^5 \pm 1.1$
Phosphotidylserine (PS)	$5.6 \times 10^5 \pm 1.2$
Phosphotidylethanolamine (PE)	$8.7 \times 10^3 \pm 1.4$

*One unit of PLA$_2$ activity is defined as the increase in release of 1 μg of palmitic acid (FFA) released per min.

7.2.3 Effect of substrate (PC) concentration on catalytic activity of RVVB-PLA_2-I

It was observed that with an increase in substrate (PC) concentration from 0.2 to 1.2 mM, RVVB-PLA_2-I induced phospholipid hydrolysis was also enhanced linearly. However, beyond this concentration of PC, the rate of hydrolyis was decreased slightly and a saturation in enzyme activity was observed at 2.0 mM PCconcentration (Fig 7.5).

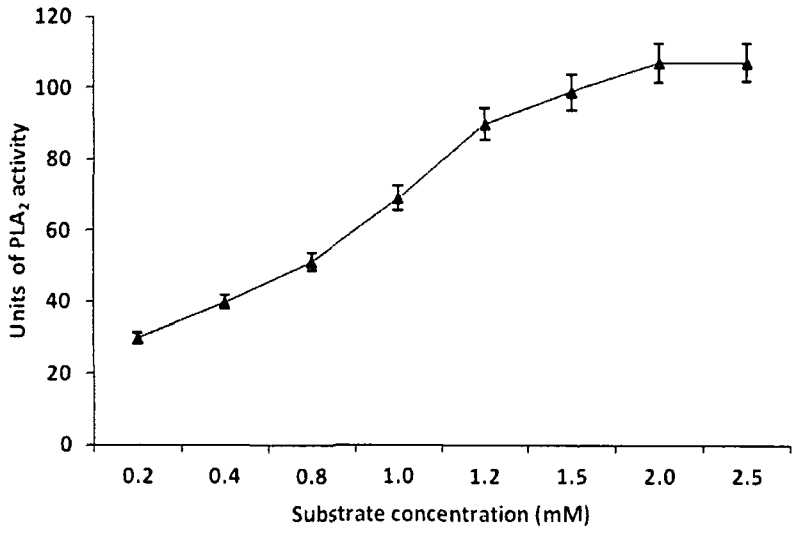

Fig 7.5: Effect of substrate (PC) concentration on PLA_2 activity of RVVB-PLA_2-I (50 mM). Effect of substrate concentration on PLA_2 activity of RVVB-PLA_2-I was determined as described in section 3.2.6.8.2. Results are expressed as mean ± S.D. of four determinations.

7.2.4 Effect of enzyme concentration

With an increase in enzyme concentration up to 0.6 μg ml^{-1}, a parallel increase in PC hydrolysis was observed. A further increase in enzyme concentration beyond 0.8 μg ml^{-1}, a saturation in enzyme activity was observed (Fig 7.6).

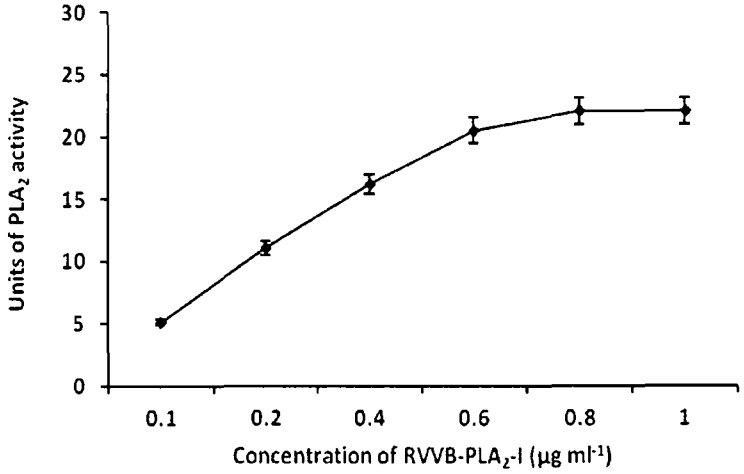

Fig 7.6: Effect of enzyme concentration on catalytic activity of RVVB-PLA_2-I. Effect of enzyme concentration on catalytic activity of RVVB-PLA_2-I was determined as described in the section 3.2.6.8.3. Results are expressed as mean ± S.D. of four determinations.

7.2.5 Determination of kinetics (Km and Vmax) of PC hydrolysis by RVVB-PLA_2-I

Using Lineweaver-Burk representation, the Michaelis constant Km and maximum velocity Vmax of the RVVB-PLA_2-I catalysed reaction were determined. By plotting the values of 1/v as a function of 1/[S], a straight line with the following regression equation was obtained.

$y = 0.0652x + 0.0617$

When y=0, then x = -0.0617/0.0652 or, x= -0.946 i.e., -1/Km = 0.946,

i.e., Km = 1.06 mM or, 1.06×10^{-4} M i.e., Km = 1.06×10^{-4} M

1/Vmax = 0.0617 i.e., Vmax = 16.21×10^{-3} mM μg^{-1} or, 162.1×10^{-3} μM μg^{-1} or, 162.1 μmol mg^{-1}

The apparent Km and Vmax value of enzyme catalyzed reaction are 1.06×10^{-4} M and 162.1 μmol mg^{-1} min^{-1} respectively.

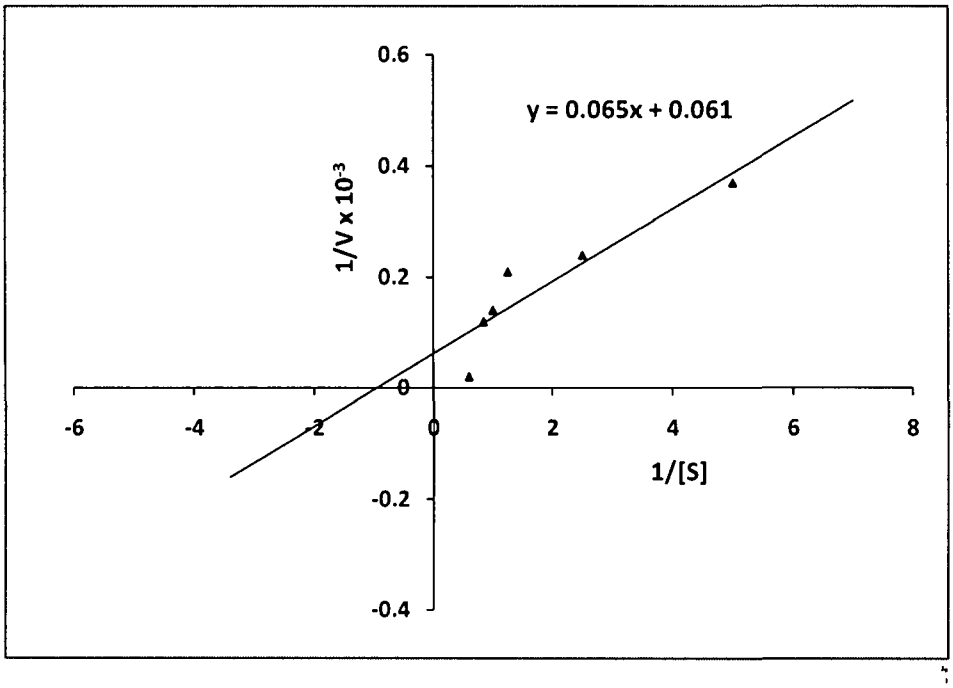

Fig 7.7: Lineweaver-Burk plot for the determination of Km and Vmax values of RVVB-PLA$_2$-I towards PC (0.1-5 mM). Experiment was done as described in the section 3.2.6.8.4. Values are mean ± S.D. of four determinations.

7.2.6 Optimum temperature

To determine the optimum temperature for the enzymatic activity of RVVB-PLA_2-I, PLA_2 activity was assayed at a temperature range of 20-75 °C using PC as substrate (Fig 7.8). RVVB-PLA_2-I showed highest catalytic activity at temperature 37 °C (27.5 units).

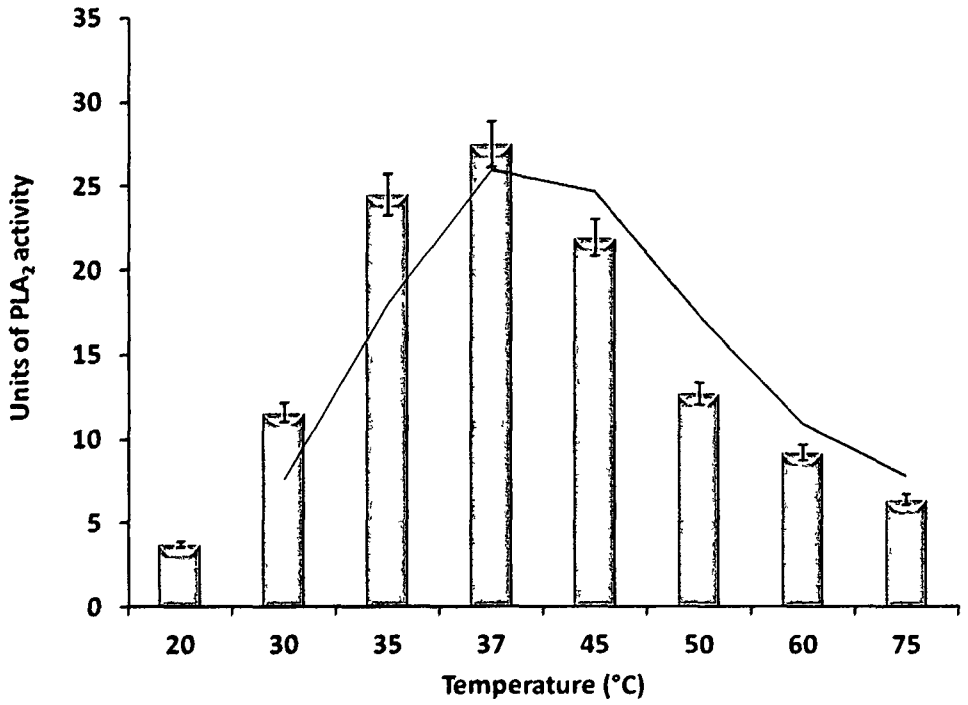

Fig 7.8: Effect of temperature on catalytic activity of RVVB-PLA_2-I. PLA_2 activity of RVVB-PLA_2-I at different temperature was done as described in section 3.2.6.8.5. Results are expressed as mean ± S.D. of four determinations.

7.2.7 pH Optima

The optimum pH requirement for efficient hydrolysis of PC by RVVB-PLA_2-I was determined at pH 8.0 (17.5 units). At a pH lower than 7.0 and higher than 8.5, RVVB-PLA_2-I could not show catalytic activity (Fig 7.9).

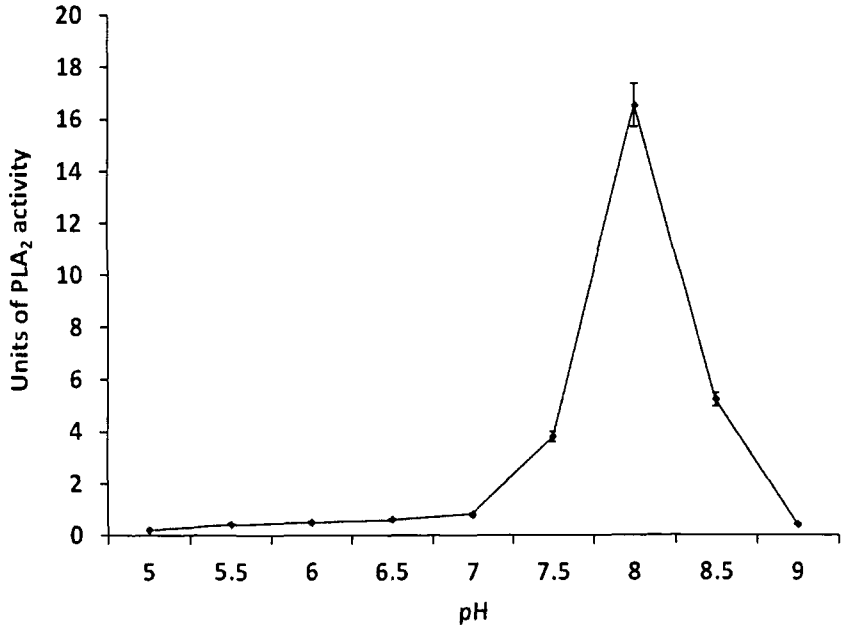

Fig 7.9: Effect of pH on catalytic activity of RVVB-PLA_2-I. PLA_2 activity of RVVB-PLA_2-I at different pH values were done as described in the section 3.2.6.8.6. The various pH values were obtained as follows: 0.1 sodium acetate, pH 5.0-6.5; 0.1M K-phosphate, pH 7.0-7.5 and 0.1 M Tris-HCl, pH 8.0-9.0. Results are expressed as mean ± S.D. of four determinations.

7.2.8 Determination of secondary structure of RVVB-PLA$_2$-I: Circular dichroism spectroscopy

Examination of RVVB-PLA$_2$-I by CD spectroscopy showed defined minima at 217 nm (Fig 7.10) and indicated a high content of alpha-helical structures (52.2 %), beta-turns (33.0 %), and random coils (14.7 %). This result suggested that the predominant secondary structure of RVVB-PLA$_2$-I consisted of alpha-helices (Fig 7.10). Moreover, RVVB-PLA$_2$-I is highly thermostable as the CD spectra was not much effected after heating the PLA$_2$ enzyme at 75 °C for 45 min (Fig 7.10).

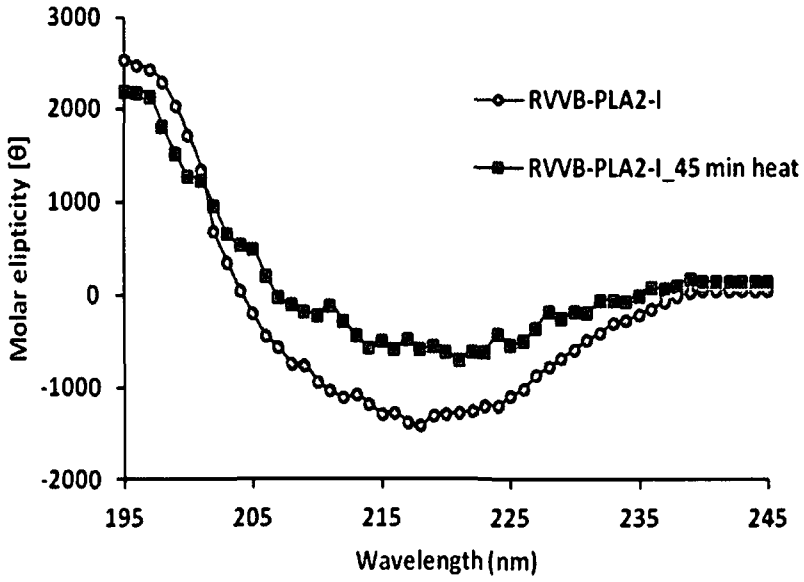

Fig 7.10: CD spectra of RVVB-PLA$_2$-I (100 nM). CD measurement was performed as described in the section 3.2.6.8.7. The CD signals are expressed as mean residue ellipticity [θ] (degree cm^2 dmol^{-1}), using 113 as the mean residue molecular weight.

7.3 Pharmacological Characterisation of RVVB-PLA_2-I

7.3.1 Anticoagulant activity

7.3.1.1 Ca^{2+} clotting time of plasma and determination of plasma phospholipids hydrolysis by GC analysis

RVVB-PLA_2-I showed dose-dependent anticoagulant activity in *in vitro* condition. With an increase in concentration of RVVB-PLA_2-I up to 1.0 μg ml^{-1}, its anticoagulant activity (as determined by Ca-clotting time of PPP) was also enhanced linearly. However, a further increase in the enzyme concentration resulted in saturation of anticoagulant activity of this basic PLA_2 enzyme (Fig 7.11).

An identical result was obtained with increasing in pre-incubation time of PPP with RVVB-PLA_2-I (0.5 μg ml^{-1}) on the anticoagulant activity. It was observed that 3-5 min was the optimal pre-incubation time for exerting maximum anticoagulant activity and beyond this period, no further increase in anticoagulant activity (increase in Ca-clotting time of plasma) was noted (Fig 7.12).

The GC analysis of plasma phospholipids hydrolysis by RVVB- PLA_2-I (0.5 μg ml^{-1}) reinforced the above observation. It was observed that after 5 min pre-incubation of this enzyme with PPP, there was no significant enhancement of plasma phospholipids hydrolysis (Fig 7.13).

7.3.1.2 Prothrombin time test

RVVB-PLA_2-I significantly enhanced the coagulation time of PPP as compared to the coagulation time of control plasma when assayed by prothrombin time test with a specific activity of 7.8 $x10^4$ ± 2.1 unit mg^{-1} of protein.

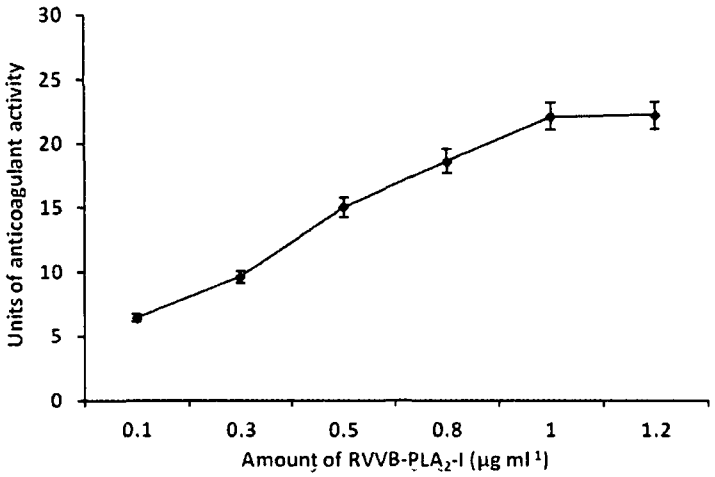

Fig 7.11: Dose dependent study of anticoagulant activity of RVVB-PLA$_2$-I (Ca-clotting time). This study was done as described in the section 3.2.7.1.1 by using different concentration of RVVB-PLA$_2$-I (0.1-1.2 µg ml^{-1}). Values represent the mean ± S.D. of four experiments.

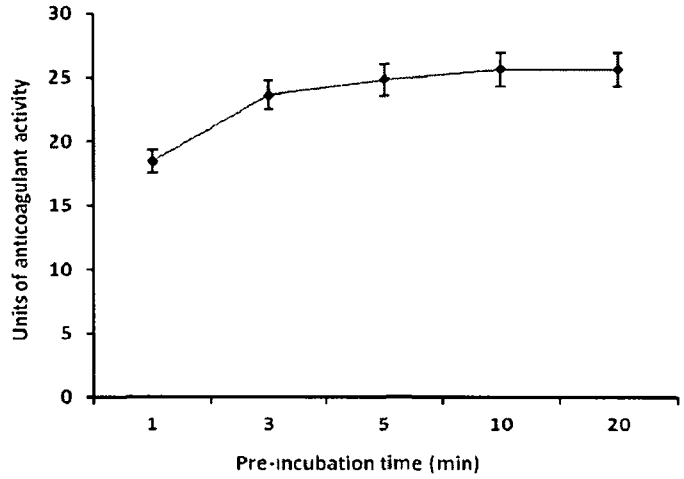

Fig 7.12: Effect of pre-incubation time on anticoagulant activity of RVVB-PLA$_2$-I (Ca-clotting time). This study was done as described in the section 3.2.7.1.1 for different time of pre-incubation of plasma with RVVB-PLA$_2$-I. Values represent the mean ± S.D. of four experiments

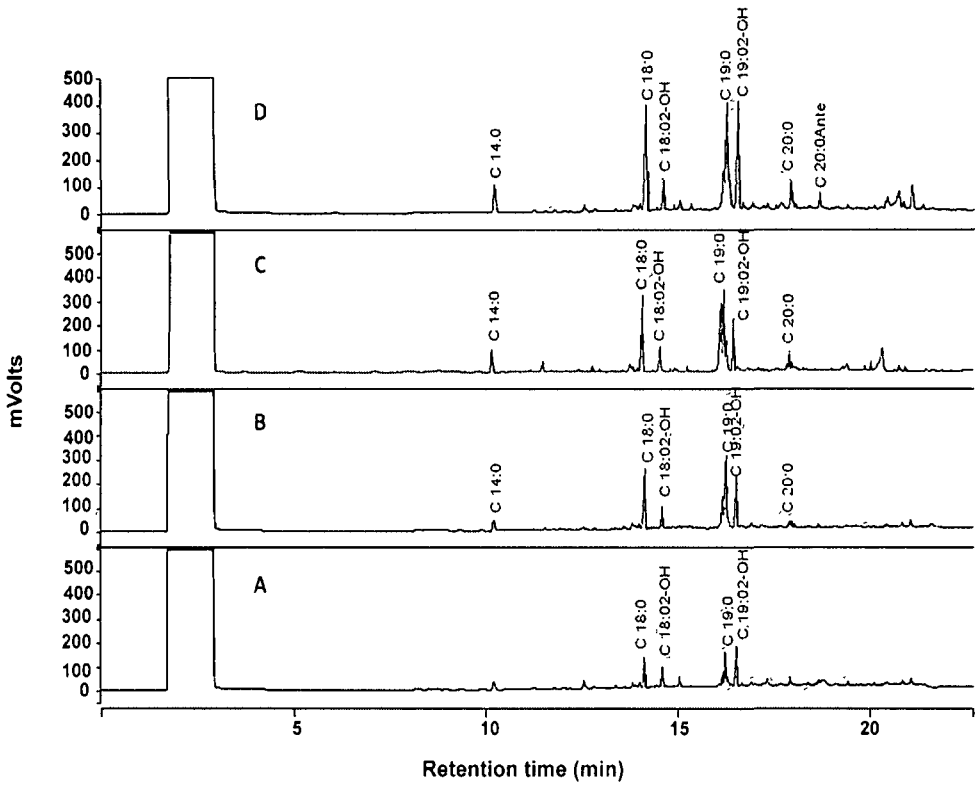

Fig 7.13: Gas chromatographic analysis of plasma phospholipids hydrolysis by RVVB-PLA$_2$-I: A) control B) 3 min, C) 5 min, D) 10 min post incubation with RVVB-PLA$_2$-I. This study was done as described in the section 3.2.7.1.3. Data shows a typical experiment and repetition of experiment demonstrated similar result.

7.3.1.3 Binding of RVVB-PLA$_2$-I with phospholipids

Initially, the excitation of fluorescence of free PLA$_2$ (0.4 μM) was done at 280 and emission maximum were observed at 348.5 nm. Fluorescence intensity of RVVB-PLA$_2$-I post mixing with different phospholipids bearing different polar head groups exhibited different results; a large increase in fluorescence intensity of this PLA$_2$ in presence of PC was observed (Fig 7.14

and Fig 7.15) and addition of 2 mM Ca^{2+} further enhanced the intensity (Fig 7.15). Addition of PS also resulted in an increase in intensity; however, this intensity was less than that observed for PC. In a sharp contrast, addition of PE resulted in decrease in fluorescence intensity of RVVB-PLA_2-I (Fig 7.14).

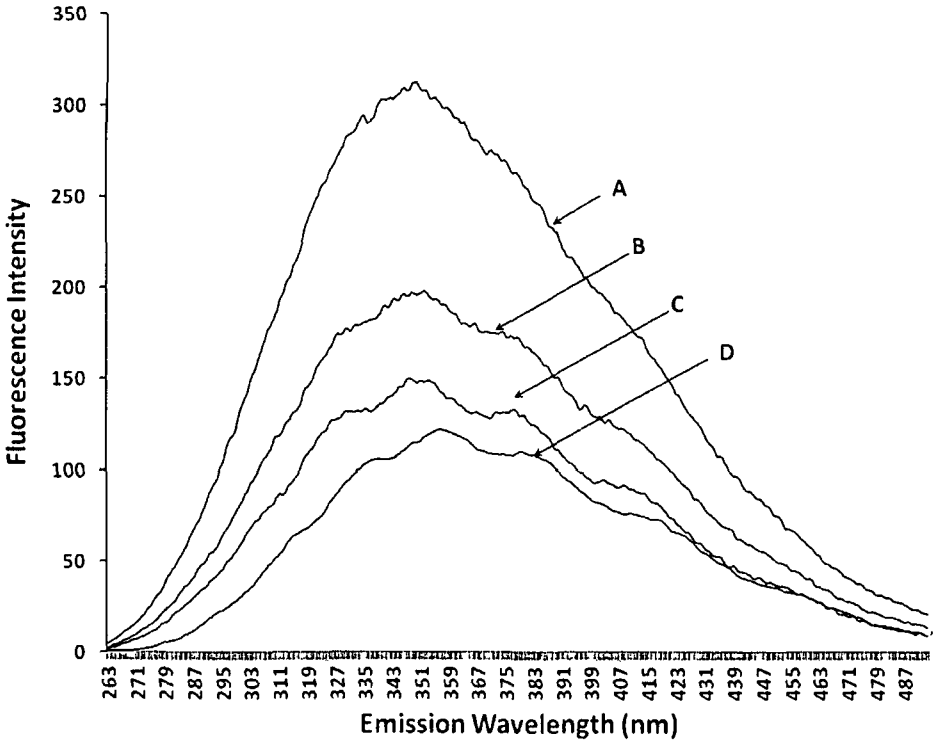

Fig 7.14: Interaction of RVVB-PLA_2-I (100 nM) with different phospholipids (50 µM): A) RVVB-PLA_2-I and PC; B) RVVB-PLA_2-I and PS; C) RVVB-PLA_2-I; D) RVVB-PLA_2-I and PE. Experiment was done as described in the section 3.2.6.8.11.

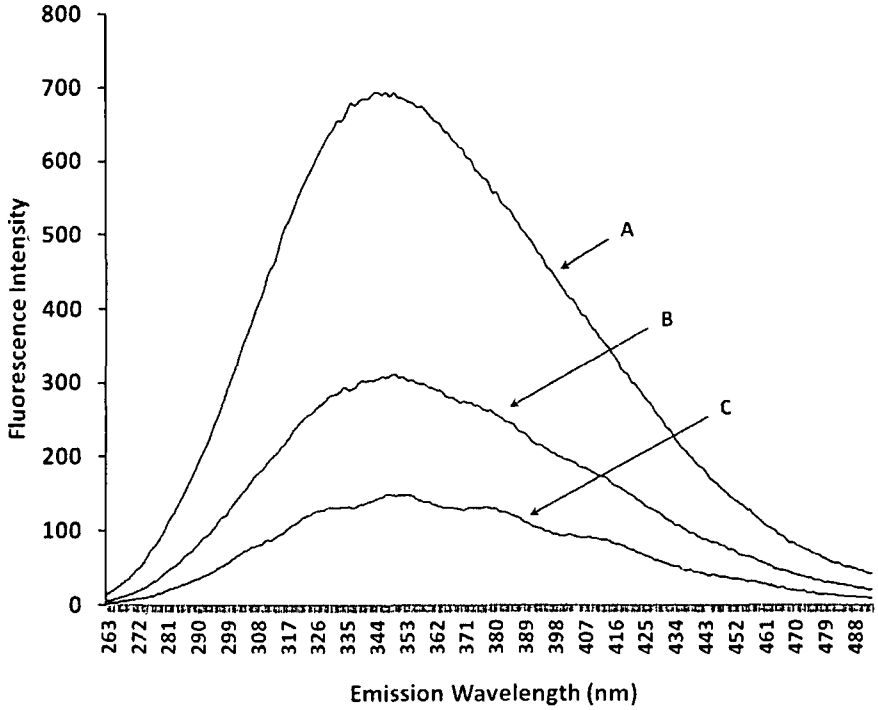

Fig 7.15: Interaction of RVVB-PLA$_2$-I (100 nM) with PC (50 μM) in presence of 2 mM Ca^{2+}: A) RVVB-PLA$_2$-I, PC and Ca^{2+}; B) RVVB-PLA$_2$-I and PC; C) RVVB-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.11.

From the spectroflurometric study of PC binding property of native and heated-PLA$_2$ revealed that phospholipid (PC) binding efficiency of the heat-inactivated RVVB-PLA$_2$-I was drastically reduced (72 %) after 60 min of heating at 75 °C compared to the PC binding potency of native PLA$_2$ (Table 7.13).

7.3.1.4 Binding of RVVB-PLA$_2$-I with blood coagulation factors

Incubation with RVVB-PLA$_2$-I with activated coagulation factor X resulted in a significant increase in the fluorescence signal suggesting interaction between these two proteins (Fig 7.16).

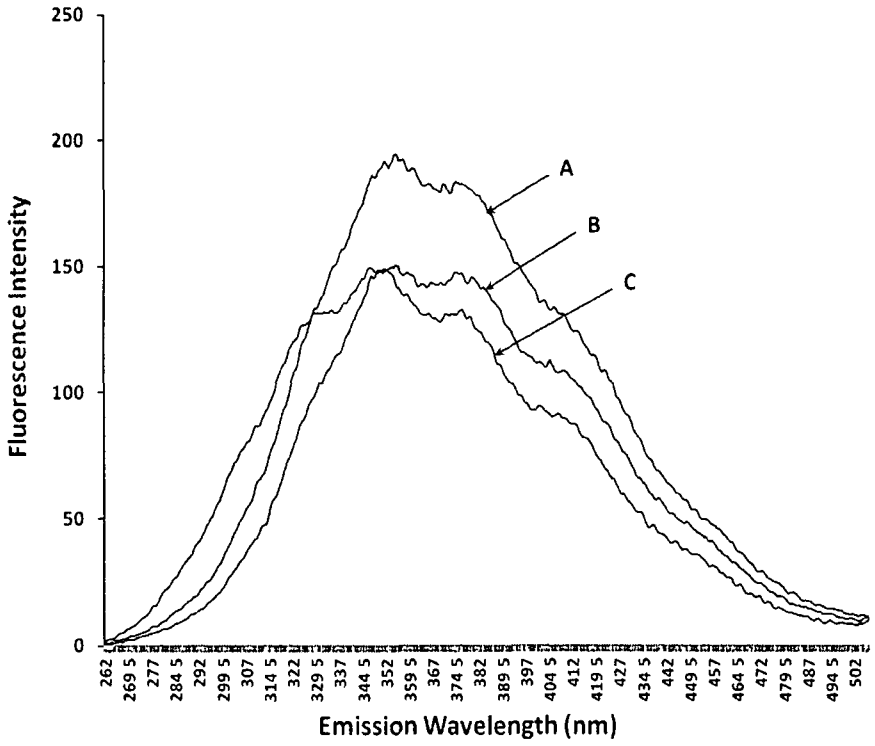

Fig 7.16: Interaction of RVVB-PLA$_2$-I (100 nM) with the blood coagulation factor Xa (50 µM): A) RVVB-PLA2-I Factor Xa, B) Factor Xa, C) RVVB-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.11.

Incubation with RVVB-PLA$_2$-I with blood coagulation factors Va, prothrombin and thrombin did not result in change in its fluorescence intensity, indicating no interaction of RVVB-PLA$_2$-I with these factors (Fig 7.17).

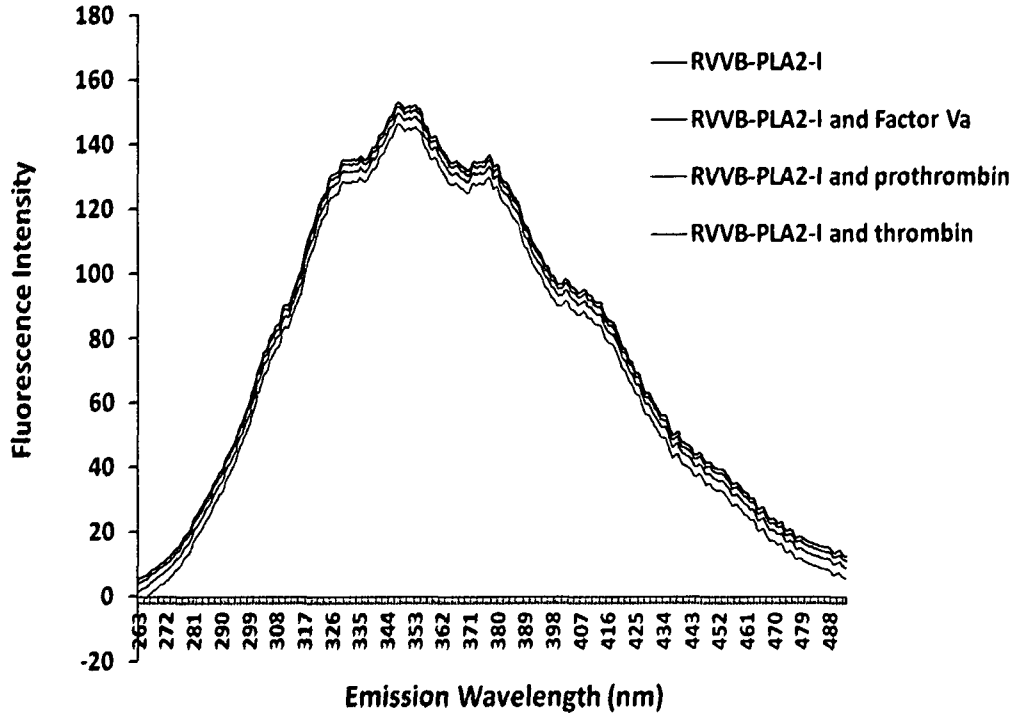

Fig 7.17: Fluorescence spectra showing interaction of RVVB-PLA$_2$-I (100 nM) with activated factor Va (50 µM), prothrombin (50 µM) and thrombin (50 µM). Experiment was done as described in the section 3.2.6.8.11.

7.3.1.5 Prothrombin inhibition assay

From the amidolytic activity assay of thrombin, it was observed that the generation of thrombin from prothrombin (5 µg) in presence of RVVB-PLA$_2$-I (100 nM) treated factor Xa (50 µM) was significantly ($p<0.05$) low as compared to the thrombin generation in presence of factor Xa (50 µM) [Fig 7.18].

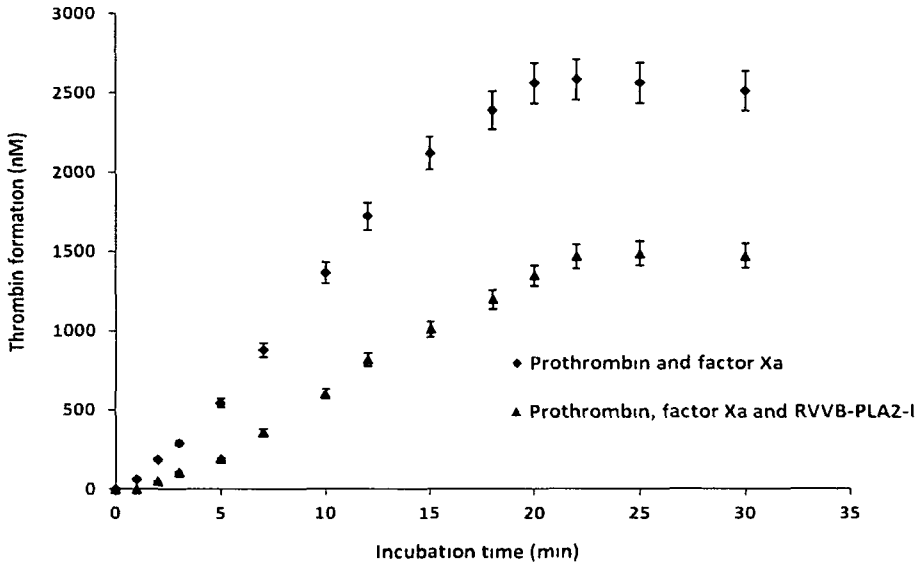

Fig 7.18: Generation of thrombin from prothrombin by Xa in presence and absence of RVVB-PLA$_2$-I. Experiment was done as described in the section 3.2.6.8.12. Values are mean ± S.D. of triplicate determinations.

The inhibition study of prothrombin activation by RVVB-PLA$_2$-I was also analysed by 15 % SDS-PAGE. Prothrombin was incubated with factor Xa, Ca^{2+} ions and either in presence or absence of 100 nM of purified PLA$_2$ at 37 °C for 3 h. As shown in Fig. 7.19, prothormbin was hydrolysed completely by factor Xa into thrombin and its fragments. In contrast, presence of factor Xa inhibited the hydrolysis of prothrombin (Fig 7.19).

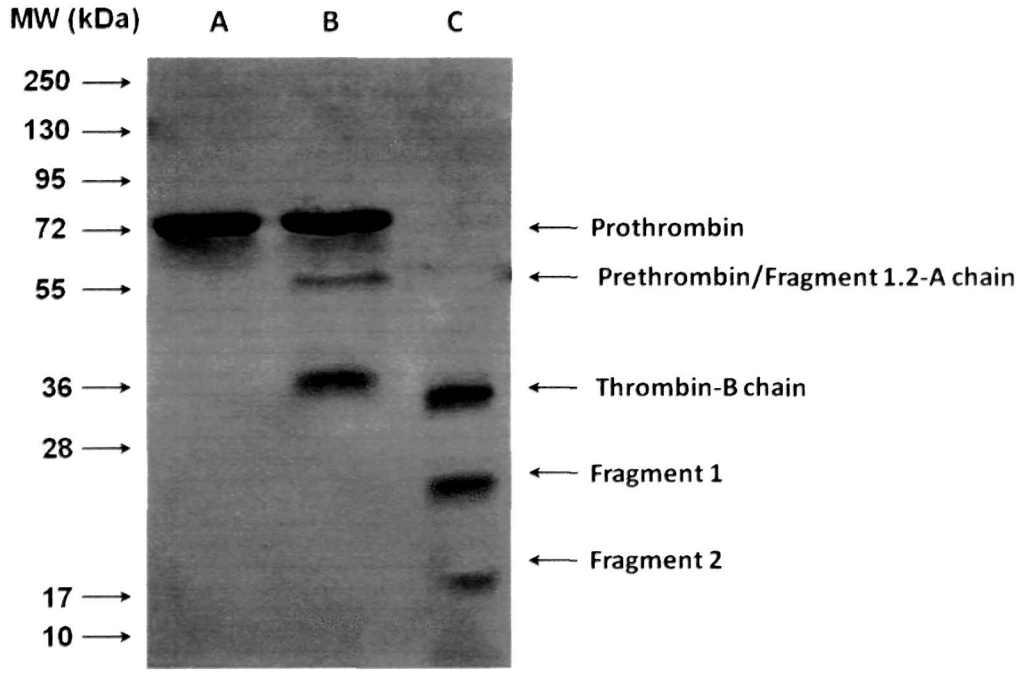

Fig 7.19: The inhibition of prothrombin activation as analyzed by 15% SDS-PAGE: Lane A) prothrombin (5 µg); lane B) prothrombin (5 µg) incubated with factor Xa (50 µM), Ca^{2+} ions (10 µM) and RVVB-PLA_2-I (100 nM); lane C) prothrombin (5 µg) treated with factor Xa (50 µM), Ca^{2+} ion (10 µM) in absence of RVVB-PLA_2-I. Experiment was done as described in the section 3.2.6.8.12.

7.3.2 Direct and indirect haemolytic activity

RVVB-PLA_2-I showed an appreciable haemolytic activity in presence of egg yolk phospholipids (Fig 7.20 and Table 7.7) though it did not show any significant direct haemolytic activity on washed goat/human erythrocytes. Erythrocytes pre-incubated with neutral phospholipid PC and 1.5 mM Ca^{2+} were highly susceptible to lysis induced by RVVB-PLA_2-I, but pre-incubation with PS

and PE had no influence on haemolytic activity of RVVB-PLA_2-I, irrespective of the presence or absence of 1.5 mM Ca^{2+} in the reaction medium (Fig 7.20).

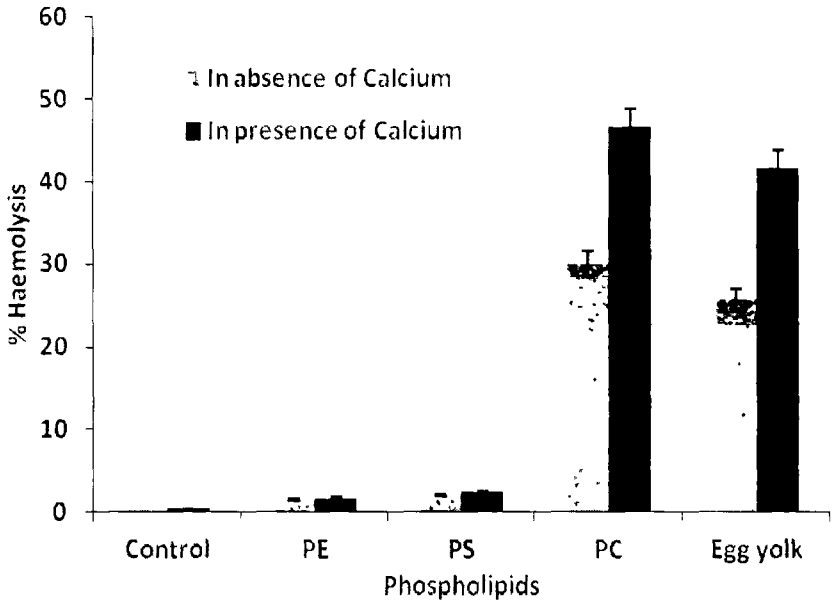

Fig 7.20: Effect of RVVB-PLA_2-I on erythrocytes enriched with different phospholipids in presence or absence of 1.5 mM calcium. Experiment was done as described in the section 3.2.7.2. Lysis is expressed as percentage, taking 100 % absorbance of erythrocyte suspension incubated with Milli Q water. Erythrocyte suspension without phospholipid served as control. Each result represents mean ± S.D. of four individual experiments.

7.3.3 *In-vitro* tissue damaging activity

In *In-vitro* condition, RVVB-PLA_2-I did not induce damage to tested tissues suggesting it is devoid of tissue haemorrhagic activity (Table 7.4).

Table 7.4: *In vitro* tissue damaging activity of RVVB-PLA$_2$-I. (% Hb released from 300 ± 10 mg tissue by 100 nM of RVVB-PLA$_2$-I post 5 h of incubation at 37 °C)*. Values represent mean ±S.D. of four experiments.

Tissues of	RVVB-PLA$_2$-I
Liver	1.01 ± 0.11^a
Heart	0.48 ± 0.02^b
Lungs	0.36 ± 0.01^c

*100% activity was achieved by treatment with 0.1% (v/v) triton X-100.

Values with different superscripts are significantly different ($p<0.05$).

7.3.4 Stability of RVVB-PLA$_2$-I at 4 °C

While studying the storage stability of RVVB-PLA$_2$-I, it was found that after 28 days of storage at 4 °C, RVVB-PLA$_2$-I lost 32 % and 52 % of its original catalytic and anticoagulant activity, respectively (Fig 7.21).

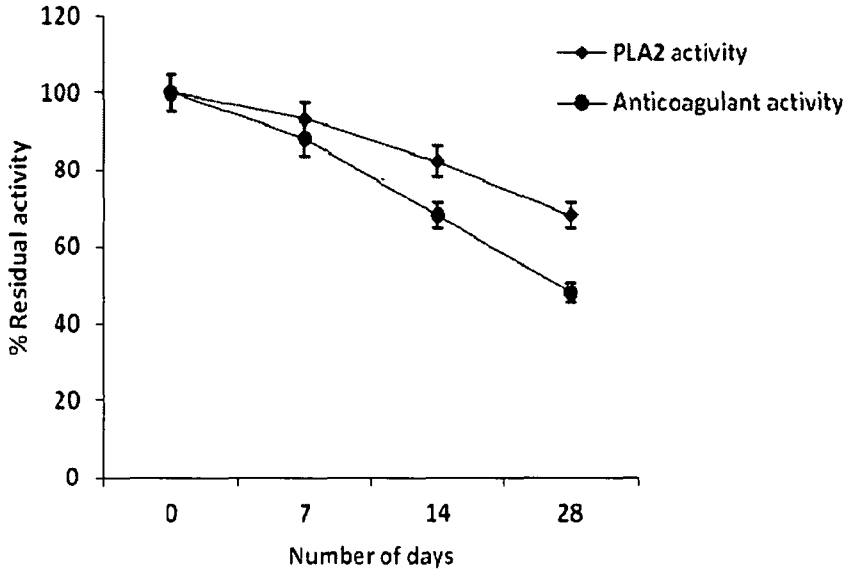

Fig 7.21: Loss of activity (catalytic and anticoagulant) of RVVB-PLA$_2$-I post storage of 28 days at 4 °C. Storage stability was determined as described in the section 3.2.11. Values are expressed as mean ± S.D. of four typical experiments.

7.3.5 Mitochondrial membrane phospholipids hydrolysis and FFAs release

RVVB-PLA$_2$-I demonstrated dose-dependent swelling of chicken liver mitochondria (Fig 7.22) and this effect was more pronounced in presence of Ca^{2+} (Table 7.5). The mitochondrial damage was enhanced concomitantly with an increase in incubation time of mitochondria with RVVB-PLA$_2$-I (Table 7.5). It was observed that RVVB-PLA$_2$-I hydrolyzed the outer membrane of mitochondria without any lag phase which was evident from the release of FFA and Pi from the membranes post incubation with this anticoagulant PLA$_2$ enzyme (Table 7.5). Addition of Ca^{2+} enhanced the membrane hydrolysis effect of RVVB-PLA$_2$-I and the extent of phospholipids hydrolysis induced by this

enzyme in presence of Ca^{2+} was about 1.31 fold higher compared to phospholipids hydrolysis in absence of Ca^{2+} post 15 min of incubation.

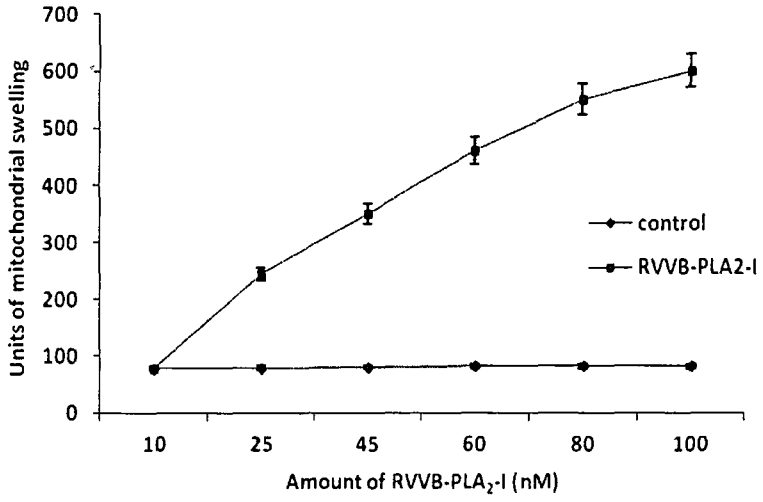

Fig 7.22: Dose dependent study of mitochondrial swelling of RVVB-PLA_2-I. Experiment was done as described in the section 3.2.7.5.1 by using different concentrations of RVVB-PLA_2-I (10-100 nM). Values represent the mean ± S.D. of four experiments.

To gain further insight into the mode of attack of RVVB-PLA_2-I on the mitochondria, GC-analysis of liberated fatty acids from membrane revealed that $C_{15\,0}$, $C_{16\,0}$, $C_{17\,0}$, $C_{18\,0}$ and $C_{19\,0}$ iso fatty acids were the prominent FFA released (Fig 7.23). With increase in pre-incubation time, FFA increases quantitatively. Addition of Ca^{2+} did not alter the fatty acid release pattern from the mitochondrial membrane; however, this metal ion potentiated the membrane hydrolytic activity of RVVB-PLA_2-I resulting in release of higher quantity of fatty acids from the mitochondrial membrane by the action of PLA_2 enzyme.

Table 7.5: Russell's viper basic PLA_2-induced swelling and phospholipids hydrolysis of membrane of intact mitochondria either in presence or absence of 2 mM Ca^{2+}. About 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissue) from chicken liver was incubated with 100 nM of RVVB-PLA_2-I at 37 °C for different time periods. The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	Mitochondrial swelling (U/min)*		Phospholipids hydrolysis				Ratio of Saturated/Unsaturated FFAs
			µg FFA released		µg of P_i released		
	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	
0 (control)	0	0	0	0	0	0	0
15	650 ± 1.5^a	900 ± 0.8^a	236.4 ± 1.1^a	309.6 ± 1.4^a	16.7 ± 1.2^a	22.4 ± 1.0^a	N.D.
30	1650 ± 0.5^b	1900 ± 1.0^b	1270.1 ± 0.9^b	1536.6 ± 1.2^b	71.9 ± 1.6^b	95.6 ± 1.3^b	2.98
60	2250 ± 1.4^c	2450 ± 1.5^c	2040.5 ± 1.7^c	2885.8 ± 1.5^c	146.9 ± 1.4^c	211.0 ± 0.6^c	4.2

*Mitochondrial swelling was measured spectrophotometrically and one unit of swelling is defined as a decrease in 0.01 absorbance/min of mitochondrial suspension at 520 nm by 100 nM of RVVB-PLA_2-I.

Values (for different time periods) with different superscripts are significantly different ($p<0.05$).

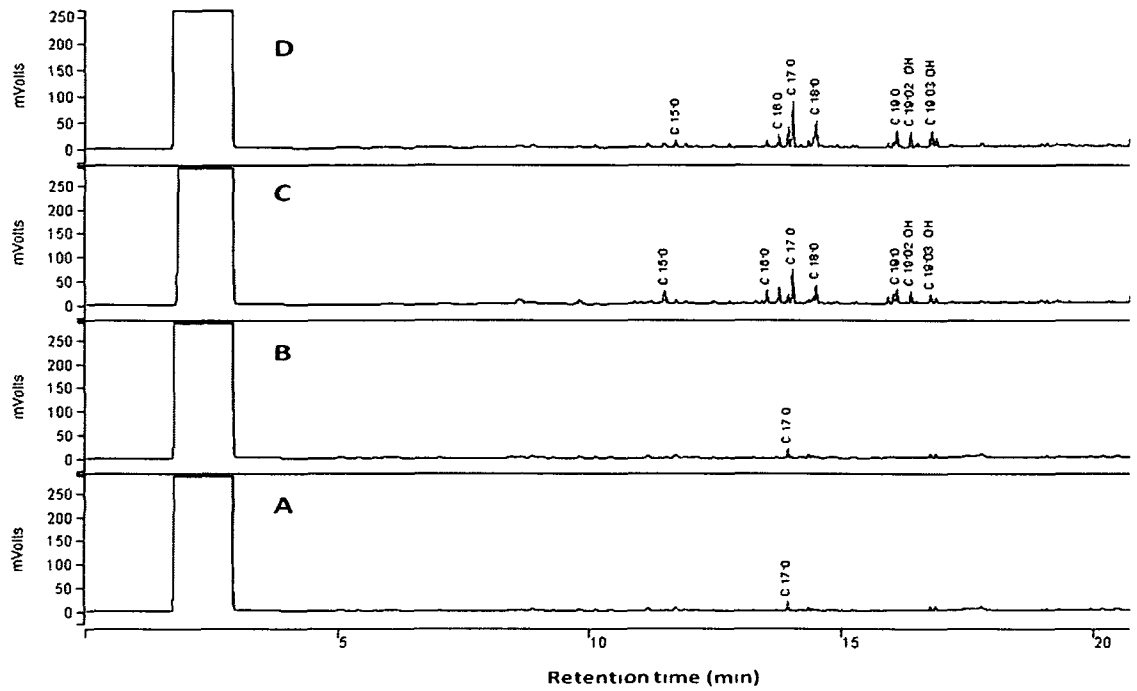

Fig 7.23: Gas Chromatographic analysis of mitochondrial membrane phospholipids hydrolysis by RVVB-PLA$_2$-I. A) Control, B) 15 min, C) 30 min, D) 60 min post incubation of mitochondrial membranes with RVVB-PLA$_2$-I. Experiment was done as described in the section 3.2.7.5.2. Data shows a typical experiment and repetition of experiment demonstrated similar result.

7.3.6 Effect of RVVB-PLA$_2$-I on erythrocyte membranes phospholipids hydrolysis

Table 7.6 displays the effect of pre-incubation time of RVVB-PLA$_2$-I on haemolysis and liberation of FFAs from washed erythrocytes. With an increase in incubation time of RBC with purified PLA$_2$ or increasing the concentration of PLA$_2$ (Fig 7.24), the extent of phospholipids hydrolysis was enhanced concomitantly as was evident from the increased release of FFA and Pi from the RBC membrane. Interestingly, though no haemolysis was observed during the initial 30 min of attack, but the PLA$_2$ was able to release the FFA and lysophospholipids from intact RBC membrane. In a sharp contrast, addition of egg-yolk phospholipids to the erythrocytes suspension resulted in initiation of haemolysis within 30 min of incubation and after 120 min, about 48.8 % of total RBC were haemolysed (Table 7.6). Approximately 7-fold increase in FFA release was detected post 120 min of incubation of RBC with RVVB-PLA$_2$-I compared to 15 min of incubation under the identical condition (Table 7.6).

The GC analysis of erythrocytes membrane phospholipids hydrolysis by RVVB-PLA$_2$-I also corroborated well with the FFA release pattern as shown in Table 7.6. It was observed that this PLA$_2$ had shown a specific preference for releasing the C16:0, C18:0, C19:0 fatty acids from the intact RBC membrane post 30 min of incubation. However, a quantitative increase in fatty acids release pattern from membrane was detected after 120 min of incubation of RBC with the PLA$_2$ (Fig 7.25).

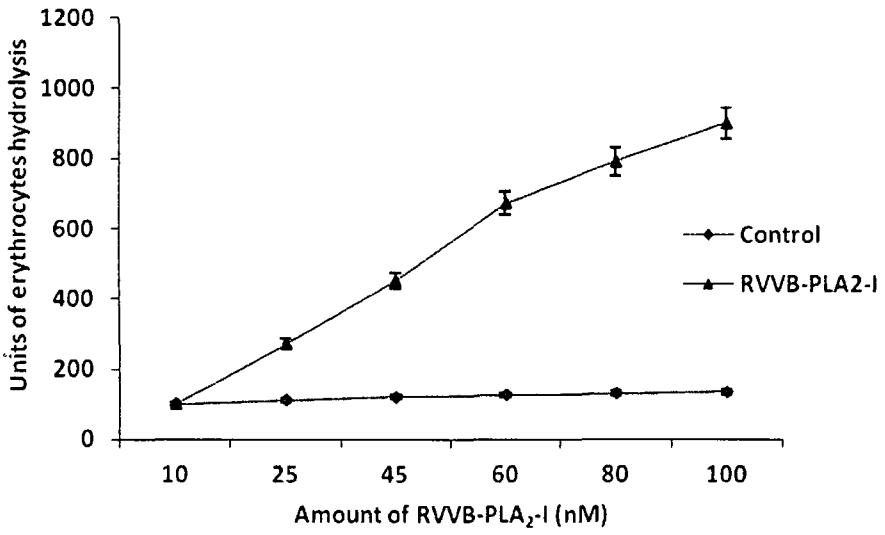

Fig 7.24: Dose dependent study of erythrocyte membrane phospholipids hydrolysis activity of RVVB-PLA$_2$-I. Experiment was done as described in the section 3.2.7.5.1 by using different concentration of RVVB-PLA$_2$-I (10-100 nM). Values represent the mean ± S.D. of four experiments.

Table 7.6: RVVB-PLA$_2$-I induced haemolysis and phospholipids hydrolysis of goat washed erythrocytes. The 5 % (v/v) erythrocyte suspension was incubated with 100 nM of RVVB-PLA$_2$-I at 37 °C and haemolysis (direct and indirect) and erythrocyte phospholipids hydrolysis were determined. Indirect phospholipids hydrolysis was achieved in presence of PC (1 mM). The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	% hemolysis		Phospholipids hydrolysis by 1.0 µg of PLA$_2$ ml^{-1} of 5% (v/v) RBC suspension		Ratio of Saturated/Unsaturated FFAs
	Direct	Indirect	µg FFA released	µg of P$_i$ released	
Control	0	0	0	0	0
15 min	0	0	485.7 ± 1.2^a	14.3 ± 0.7^a	N.D
30 min	0	17.1 ± 1.1^b	1433.8 ± 1.6^b	42.2 ± 1.9^b	N.D
60 min	0.8 ± 0.04^c	39.8 ± 0.5^c	2850.9 ± 1.5^c	95.5 ± 1.7^c	N.D
120 min	2.8 ± 0.14^d	48.8 ± 0.8^d	3389.4 ± 1.5^d	136.6 ± 1.1^d	N.D

Values (for different time periods) with different superscripts are significantly different ($p<0.05$).

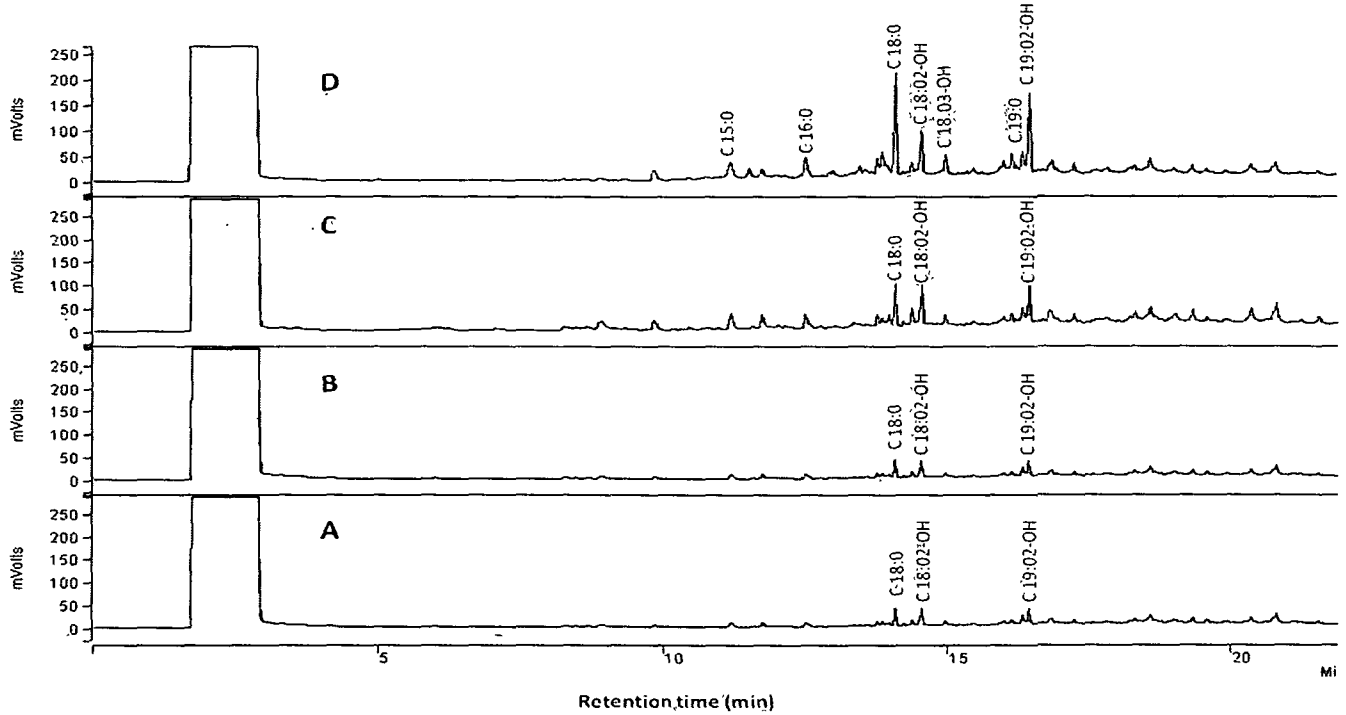

Fig 7.25: Gas chromatographic analysis of erythrocyte membrane phospholipids hydrolysis by RVVB-PLA$_2$-I. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVB-PLA$_2$-I (in presence of 2 mM Ca^{2+}) at 37°C for different time periods. The liberated fatty acids were analyzed by GC-MS as described in the section 3.2.7.5.2. A) control, B) 30 min, C) 60 min, D) 120 min after incubation of erythrocytes membranes with RVVB-PLA$_2$-I.

7.3.7 Binding study by ELISA

The binding property of native and heat-inactivated PLA_2 with mitochondrial and erythrocytes membranes was also studied by ELISA experiment. From this experiment, it was revealed that about 62.7 ± 1.4 % (mean ± S.D., n=3) of RVVB-PLA_2-I (heated for 60 min at 75 °C) could bind to intact erythrocytes or mitochondrial suspensions as compared to binding of native (unheated) PLA_2 enzyme (Fig 7.26 and Fig 7.27).

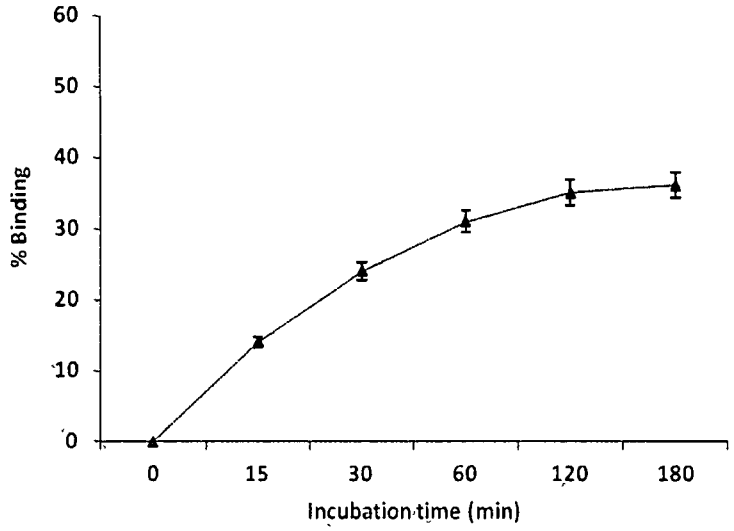

Fig 7.26: Binding of heat inactivated RVVB-PLA_2-I with mitochondrial membranes. Experiment was done as described in the section 3.2.7.5.3. Binding of native PLA_2 with membrane was considered as 100 % binding and then values were calculated. Values are ± S.D. of four experiments.

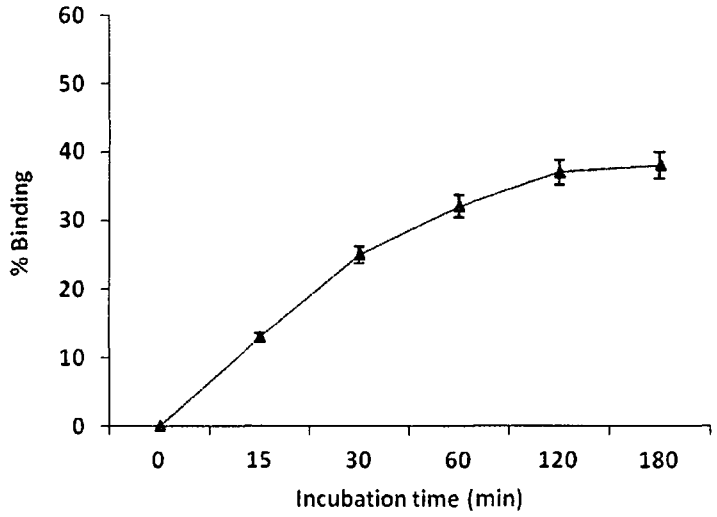

Fig 7.27: Binding of heat inactivated RVVB-PLA$_2$-I with erythrocyte membranes. Experiment was done as described in the section 3.2.7.5.3. The control value was considered as 100 % and then values were calculated. Values are ± S.D. of four experiments.

7.3.8 Effects of different chemical inhibitors, antivenom and heat inactivation study of RVVB-PLA$_2$-I

7.3.8.1 Effects of chemical modification on PLA$_2$ activity

Serine inhibitors viz. TPCK, TLCK and PMSF did not have any effect on the PLA$_2$ activity of RVVB-PLA$_2$-I. However, in the presence of pBPB, only 9.3 % of its catalytic activity was remained. EDTA reduced its PLA$_2$ activity upto 38.8 %. DTT and IAA reduced the catalytic activity of RVVB-PLA$_2$-I upto 59.5 % and 62.3 % respectively (Table 7.8). Polyvalent antivenom at a ratio of 1:500 (antigen: antibody) reduiced 79.0 % of the PLA$_2$ activity of the purified enzyme.

7.3.8.2 Effects of chemical modification on pharmacological activities

At 2.0 mM final concentration, p-BPB inhibited the mitochondrial and erythrocyte membrane phospholipids hydrolysis activity of RVVB-PLA_2-I upto 84.6 % and 81.5 % respectively, but the anticoagulant activity was reduced to 57.2 % of the original activity (Table 7.7). TLCK, TPCK and PMSF were ineffective in modulating any of the tested pharmacological properties of the purified protein. Treatment with 2.0 mM of either EDTA or DTT resulted in significant inhibition of all the tested pharmacological properties of the protein. Polyvalent antivenom: RVVB-PLA_2-I at 1:500 (w/w) ratio completely abolished the anticoagulant activity of RVVB-PLA_2-I, but other activities were neutralized to an extent (Table 7.7).

7.3.8.3 Effect of heating

Heating the purified protein RVVB-PLA_2-I at 75 °C for 10 min at pH 8.0 did not influence the catalytic activity, while the anticoagulant activity was reduced to 55.7 % of its original activity. on the other hand, 85.3 % of the enzymatic activity was retained even after 60 min of heating at 75 °C, but the anticoagulant and membrane phospholipids hydrolyzing activity were completely abolished after 45 min of heating at 75 °C (Table 7.7).

Table 7.7: Effects of heating, antivenom and inhibitors on PLA_2, anticoagulant and membrane phospholipids hydrolysis action of RVVB-PLA_2-I. Values are mean ± S. D. of triplicate determinations. MM: mitochondrial membrane, EM: erythrocyte membrane.

	% residual activity				PC Binding (%)
	PLA_2	Anticoagulant	MM hydrolysis	EM hydrolysis	
Control	100	100	100	100	100
Heating at 75°C					
10 min	100	55.7 ± 2.7	75.3 ± 1.7	75.0 ± 0.7	95.7 ± 0.8
20 min	95.6 ± 2.8	23.3 ± 1.2	55.1 ± 1.8	50.0 ± 2.5	57.4 ± 1.1
30 min	86.2 ± 1.6	3.5 ± 0.2	16.0 ± 0.8	9.5 ± 0.5	42.6 ± 1.5
45 min	78.8 ± 1.3	0	0	0	36.1 ± 0.9
60 min	73.3 ± 2.2	0	0	0	28.0 ± 2.1
Antigen: antivenom (w:w)					
1:100	91.4 ± 2.6	71.3 ± 2.5	87.2 ± 1.3	82.8 ± 1.1	-
1:200	85.2 ± 1.1	52.1 ± 0.6	56.8 ± 2.8	53.7 ± 0.7	-
1:500	79.0 ± 1.9	32.1 ± 0.6	25.3 ± 1.2	21.6 ± 1.1	-
Chemicals/ inhibitors					
TPCK	97.4 ± 1.8	100	96.0 ± 1.1	94.5 ± 0.7	-
TLCK	94.6 ± 2.1	97.5 ± 1.5	99.2 ± 0.9	100	-
pBPB	9.3 ± 0.5	57.2 ± 0.9	17.4 ± 0.8	19.5 ± 0.9	-
PMSF	96.2 ± 0.8	100	97.7 ± 2.8	95.2 ± 1.7	-
DTT	60.5 ± 2.0	46.5 ± 1.0	65.5 ± 1.7	75.5 ± 1.7	-
IAA	37.7 ± 1.8	73.0 ± 1.2	60.1 ± 1.1	56.6 ± 0.8	-
EDTA	28.8 ± 3.1	39.1 ± 1.6	37.8 ± 1.2	45.5 ± 1.8	-

7.3.9 Antibacterial activity

It was checked whether this PLA_2 contain any antibacterial activity against Gram positive or Gram negative bacteria or not by using *B. Subtilis*, *E.coli* and *P. auregonosa* MM strains. RVVB-PLA_2-I did not show any antibacterial activity against these even at a concentration of 25 μg ml^{-1} when tested by time course experiment (Fig 7.28).

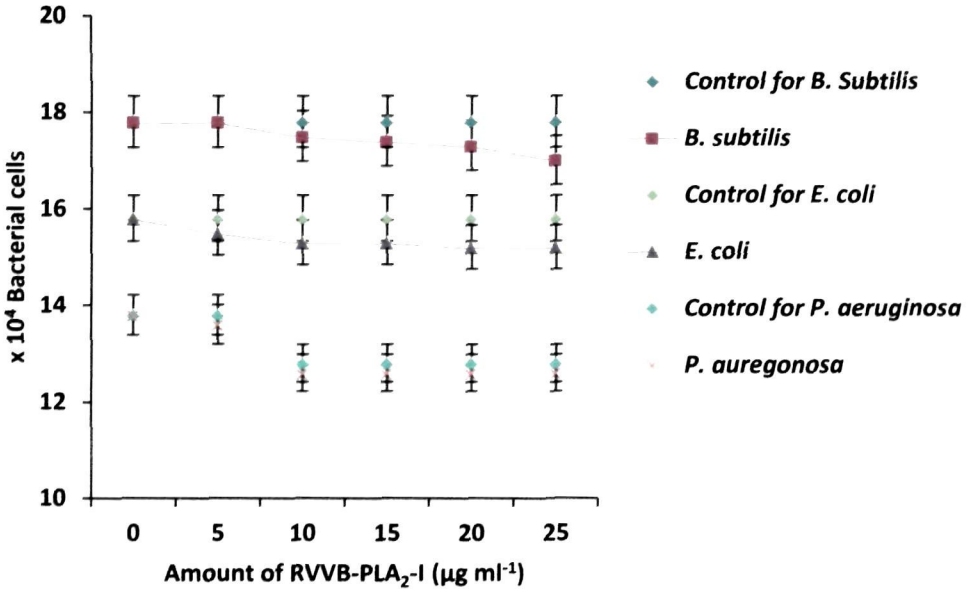

Fig 7.28: Antibacterial activity study of RVVB-PLA_2-I on *B. subtilis*, *E. coli* and *P. auregonosa*. Varying amount of RVVB-PLA_2-I (5-25 μg ml^{-1}) was incubated with 18.4 x 10^4 cells of each bacterium for 24 h at 37 °C and change in optical density was recorded at 630 nm. 1 OD at 630 nm= 10^6 cells. Each point represents the mean ± S.D. of four experiments. Only media without RVVB-PLA_2-I served as control for each experiment. Experiment was done as described in the section 3.2.7.4.

7.3.10 *In vitro* cytotoxicity assay on HT 29 cells

RVVB-PLA$_2$-I did not show any considerable toxic effect on HT 29 cells at the tested doses. RVVB-PLA$_2$-I, at a dose of 10 μg ml^{-1} could cause lyses of 9 % of total HT 29 cell 4 h post incubation and further incubation time did not result any further significant enhancement of cell lyses as after 24 h of incubation RVVB-PLA$_2$-I caused only 12.8 % cell lyses (Fig 7.29). The light microscopic observation of RVVB-PLA$_2$-I treated HT 29 cells post 4 h and 24 h incubation also shows the same result (Fig 7.30).

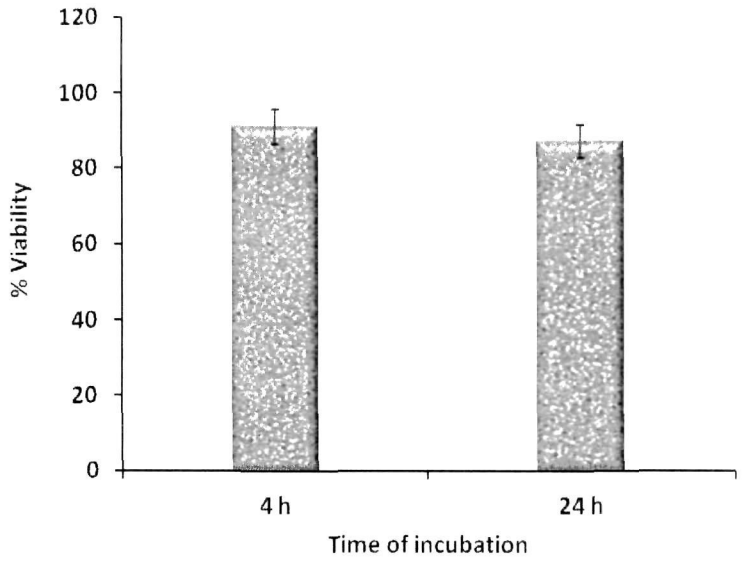

Fig 7.29: Cell cytotoxicity assay: showing the effect of RVVB-PLA$_2$-I on HT 29 cells at 4 h and 24 h incubation. The incubation was carried out at 37 °C, 5 % CO_2 for the indicated time period. Experiment was done as described in the section 3.2.7.6. Values are mean ± S. D. of triplicate determinations.

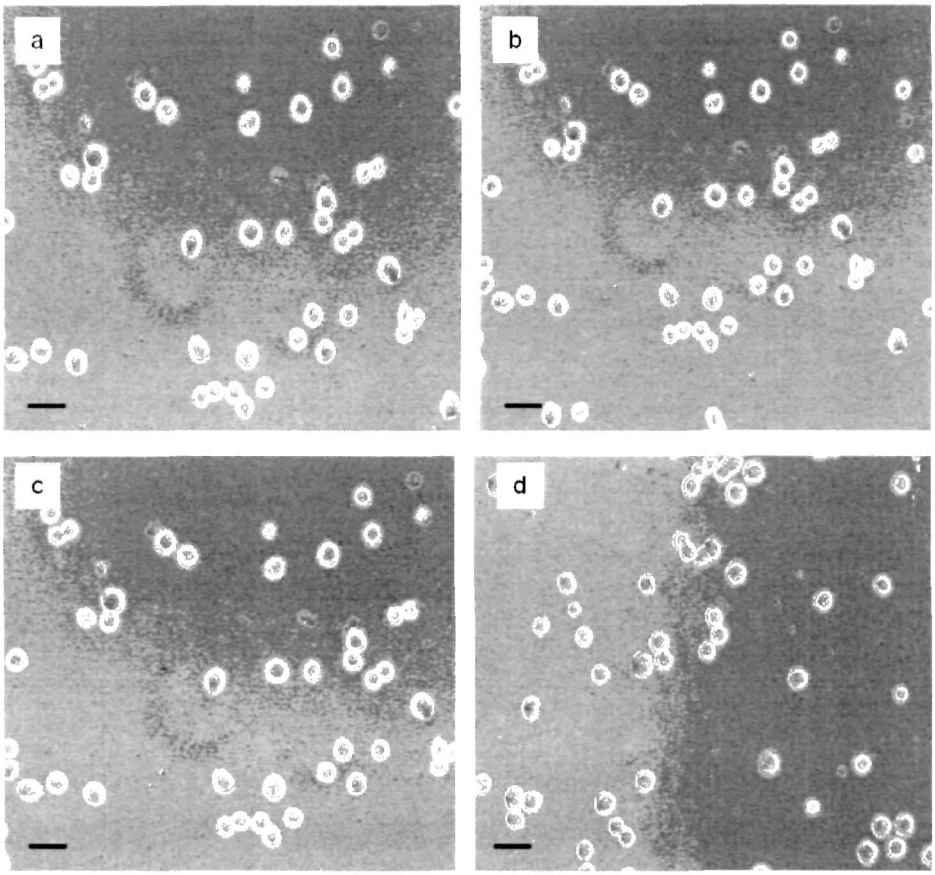

Fig 7.30: Light microscopic observation of the effect of RVVB-PLA$_2$-I on HT 29 cells (photographs were taken at a compact high-performance inverted microscopes, Nikon ECLIPSE, TS100, Tokyo). A) Control (4 hr) and b) HT 29 cells treated with 10.0 µg ml^{-1} RVVB-PLA$_2$-I post 4 hr of incubation. C) Control (24 hr) and D) HT 29 cells treated with 10.0 µg ml^{-1} RVVB-PLA$_2$-I post 24 hr of incubation. The incubation was carried out at 37 °C, 5 % CO_2 for the indicated time period. Magnifications 100x: (a-d) bar=50 µm.

7.4 *In-vivo* toxicity assay

7.4.1 Lethality and *in-vivo* toxicity

RVVB-PLA_2-I was not lethal to albino mice at a concentration of 0.4 mg kg^{-1} body weight and did not show any sign of haemostatic disorder. Further, no behavioural changes in treated mice were observed up to 48 h (Table 7.8).

7.4.2 Anticoagulant effect

In contrast, the i.p. administration of RVVB-PLA_2-I at a dose of 0.4 mg kg^{-1} body weight prolonged the *in vitro* tail bleeding time in mice and *in vitro* coagulation time of PPP from RVVB-PLA_2-I treated mice was significantly enhanced as compared to the control group of mice (Table 7.9).

7.4.3 Effect on blood cells

The *in vivo* effect of RVVB-PLA_2-I on haematological parameters of blood viz. RBC, WBC and haemoglobin counts showed there was no change in the WBC count of treated mice; nonetheless, the RBC count and the Hb content of blood were decreased 48 h post administration of RVVB-PLA_2-I in mice as compared to the control group of mice (Table 7.10)

Table 7.8: Behavioural Changes, if any, in mice 48 h after the administration of RVVB-PLA_2-I at a dose of 0.4 mg kg^{-1}

Values are mean ± S.D. of six determinations.

Group of mice	Parameters									
	Body weight (gm)		Grip Strength (sec)		Rectal temperauture (F)		Faecal tendency (times per 15 min)		Urination (times per 15 min)	
	Initial*	Final**	Initial*	Final**	Initial*	Final**	Initial*	Final**	Initial*	Final**
Control	32.4 ± 1.1	32.6 ± 0.8	67.9 ± 1.2	32.5 ± 0.9	94.2 ± 0.2	94.1 ± 0.5	3 ± 1.0	5 ± 0.9	2 ± 0.4	4 ± 1.2
RVVB-PLA_2-I treated	31.6 ± 0.9	32.1 ± 0.7	42.9 ± 1.3	21.6 ± 0.5	94.1 ± 0.3	93.6 ± 0.5	3 ± 0.5	4 ± 1.0	3 ± 0.6	5 ± 0.5

*Initial: determined at the onset of experiment.

**Final: determined after 48 h of RVVB-PLA_2-I administration.

Table 7.9: The *in vitro* clotting time of blood and tail bleeding time in mice post 48 h injection of RVVB-PLA$_2$-I at a dose of 0.4 mg kg^{-1}

Values are mean ± S.D. of six determinations.

Group of mice	Clotting time (sec)	Bleeding time (sec)
Control	168.5 ± 1.5	45.0 ± 0.6
RVVB-PLA$_2$-I treated	378.8 ± 1.9[a]	76.5 ± 2.1[a]

Level of significance [a] $p < 0.001$

Table 7.10: Effect of RVVB-PLA$_2$-I on different haematological parameters of PLA$_2$ treated mice.

Values are mean ± S.D. of six determinations.

Group of mice	Haematological parameters		
	WBC (m/mm^3)	RBC (m/mm^3)	Haemoglobin (Hb) (g/dL)
Control	11.46 ± 0.5	5.73 ± 0.3[a]	6.45 ± 0.3[a]
RVVB-PLA$_2$-I treated	10.99 ± 0.6	3.84 ± 0.4[b]	5.33 ± 0.5[b]
% decreased	4.1 ± 0.3	32.9 ± 0.3	17.4 ± 0.5

7.4.4 Effect on serum parameters

Table 7.11 shows the effect of RVVB-PLA_2-I on serum biochemical parameters of treated mice. The i.p. administration of RVVB-PLA_2-I did not show significant change in ALP, CPK-MB, SGOT, SGPT and LDH levels in the serum of treated mice as compared to control group of mice. Likewise, the cholesterol, triglycerides, glucose, and total protein levels were remain unchanged as compared to control (Table 7.11).

Table 7.11: Effect of RVVB-PLA$_2$-I on different parameters of serum of albino mice after 48 h of i.p injection at a dose of 0.4 mg/kg body weight of mice. Values are mean ± S.D. of six determinations.

	Total protein (g/L)	Glucose (g/L)	CPK-MB (U/L)	LDH (U/L)	ALP (U/L)	SGOT (U/L)	SGPT (U/L)	Cholesterol (g/L)	Triglycerides (g/L)
Control	270.0 ± 0.7	2.11 ± 1.2	27.5 ± 0.65	1281.5 ± 0.9	27.5 ± 0.8	144.0 ± 2.2	50.0 ± 0.91	0.7 ± 0.4	0.2 ± 0.45
RVVB-PLA$_2$-I Treated	220.5 ± 0.5	1.46 ± 1.5	30.0 ± 1.6	1254.5 ± 1.1	29.3 ± 1.2	142.7 ± 2.6	48.4 ± 1.4	0.5 ± 1.3	0.3 ± 0.5

7.4.5 Histopathological study

Histological study of different tissues of RVVB-PLA_2-I treated mice supports the above study (Table 7.12) as there was not found any significant change in the RVVB-PLA_2-I treated tissues as compared to control tissues (Fig 7.31).

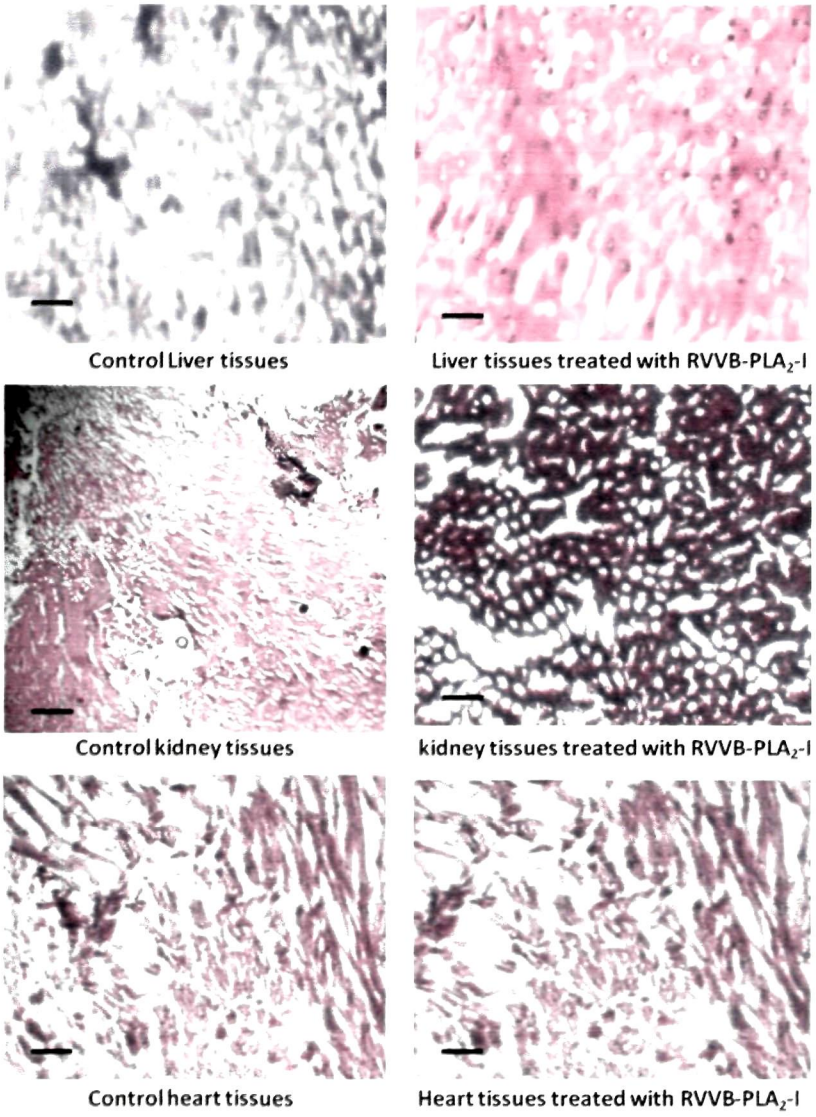

Fig 7.31: Light microscopic observation of the effect of RVVB-PLA_2-I on different organs of treated mice. Tissues treated with PBS were served as control. Hematoxyline-eosine (H&E) staining; Magnifications 100x: bar=50 µm.

7.5 Immunological cross reactivity

7.5.1 Immunodiffusion

Like other two PLA_2s, RVVB-PLA_2-I also did not show cross reactivity towards commercially available polyvalent antivenom at a ratio of 1:100 (antigen: antibody, w/w). No visible antigen (RVVB-PLA_2-I) – antibody (polyvalent antivenom) precipitate band was observed in the immunodiffusion test (Fig 7.32).

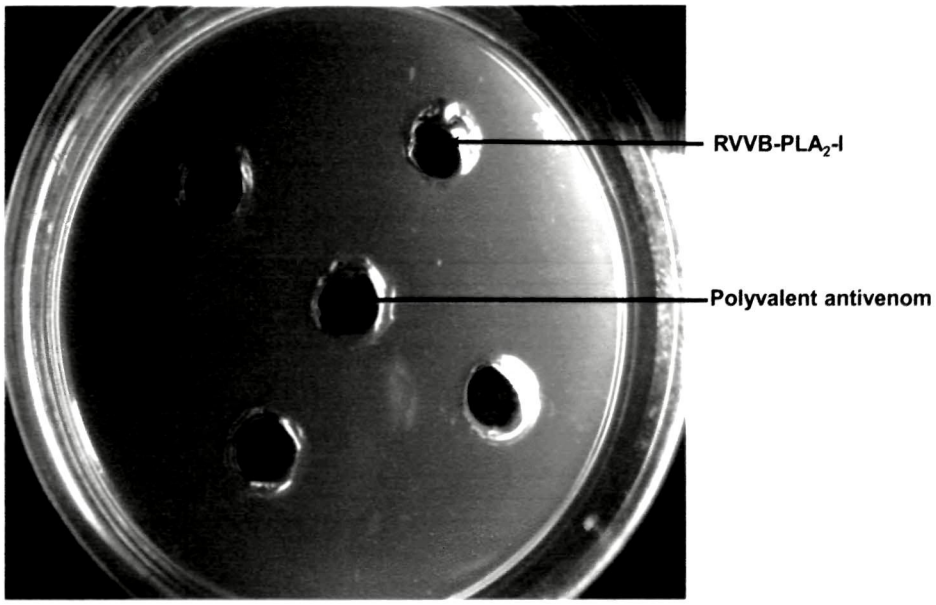

Fig 7.32: Immunodiffusion: No visible antigen (RVVB-PLA_2-I) -antibody precipitate band was observed. Experiment was done as described in the section 3.2.7.7.1. Data shows a typical experiment and repetition of experiment demonstrated similar result.

7.6 Neutralization by plant extracts

7.6.1 Neutralization by AIPLAI

Fig 7.33 shows the effect of AIPLAI (*A. indica* PLA_2 inhibitor), the methanol extract of *A. indica* leaves, on the catalytic as well as anticoagulant activity of RVVB-PLA_2-I. The AIPLAI (50 μg ml^{-1}) demonstrated 25 % inhibition of catalytic activity and 29.5 % inhibition anticoagulant activity RVVB-PLA_2-I (Fig 7.33).

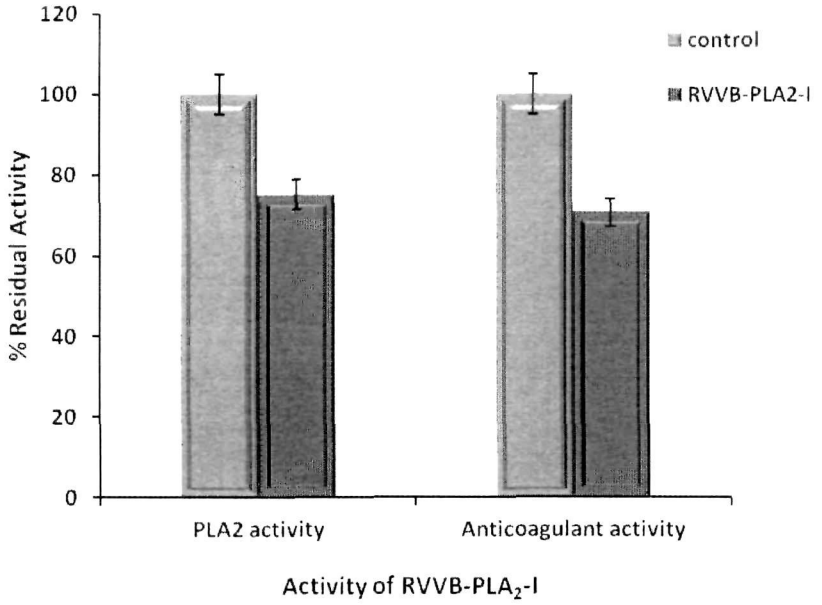

Fig 7.33: Effect of AIPLAI (50 μg ml^{-1}) on the catalytic and anticoagulant activity of RVVB-PLA_2-I (1 μg ml^{-1}). Values are expressed as the mean ± S.D. of four experiments.

7.6.2 Neutralization by *Camellia sinensis*, *Aegle marmelos* and *Xanthium strumarium*

Among the tested solvent extracts, methanol extracts of the leaves of *C. sinensis* and *X. strumarium* and the roots of *A. marmelos* were found to be most potent in inhibiting the PLA_2 as well as anticoagulant activities of RVVB-PLA_2-I (Table 7.12 and Table 7.13). Moreover, methanol extracts of these plants at a concentration of 50 $\mu g\ ml^{-1}$ is found to be most potent against the PLA_2 activity of crude RVV (Fig 5.35) as well as RVVB-PLA_2-I (Table 7.12 and 7.13).

Methanol extract of leaves of *C. sinensis* and *X. strumarium* and the roots of *A. marmelos* showed significant inhibition of different pharmacological activities of RVVB-PLA_2-I (Table 7.14). Further, Table 7.14 shows a comparison of inhibition cause by polyvalent antivenom and by these three plant extracts.

Table 7.12: Percent inhibition of PLA_2 activity of RVVB-PLA_2-I (1 μg ml^{-1}) by different extract of *Camellia sinensis*, *Xanthium* strumarium and *Aegle marmelos*. Preparation of plant extract and inhibition study was done as described in the section 3.2.10.2. PLA_2 activity without plant extract was treated as control (100 %). Results are mean ± S.D. of four determinations.

Amount of plant extract (μg)		% inhibition of PLA_2 activity of RVVB-PLA_2-I by		
		Camellia sinensis	*Xanthium* strumarium	*Aegle marmelos*
Water Extract	5	0.21 ± 0.01	0	2.21 ± 0.06
	10	1.3 ± 0.06	0.01 ± 0.001	8.20 ± 0.41
	50	7.8 ± 0.39	4.4 ± 0.22	15.1 ± 0.75
	100	11.2 ± 0.57	5.1 ± 0.26	23.7 ± 1.18
Chloroform Extract	5	3.5 ± 0.17	0.6 ± 0.03	4.5 ± 0.22
	10	8.1 ± 0.41	3.9 ± 0.19	16.1 ± 0.81
	50	16.4 ± 0.82	13.6 ± 0.68	28.6 ± 1.43
	100	19.8 ± 0.99	16.6 ± 0.83	34.1 ± 1.71
Methanol Extract	5	4.1 ± 0.21	2.1 ± 0.11	9.8 ± 0.48
	10	12.7 ± 0.64	6.6 ± 0.33	23.7 ± 1.15
	50	**29.5 ± 1.47**	**20.8 ± 1.04**	**49.1 ± 1.7**
	100	33.9 ± 1.68	25.7 ± 1.28	54.5 ± 1.9

Table 7.13: Percent inhibition of anticoagulant activity of RVVB-PLA_2-I (1 µg ml^{-1}) by different extract of *Camellia sinensis*, *Aegle marmelos* and *Xanthium strumarium*. Preparation of plant extract and inhibition study was done as described in the section 3.2.10.2. PLA_2 activity without plant extract was treated as control (100 %). Results are mean ± S.D. of four determinations.

Amount of plant extract (µg)		% inhibition of anticoagulant activity of RVVB-PLA_2-I by		
		Camellia sinensis	*Aegle marmelos*	*Xanthium* strumarium
Water Extract	5	0.78 ± 0.04	0.31 ± 0.02	2.21 ± 0.06
	10	4.7 ± 0.23	3.1 ± 0.16	8.20 ± 0.41
	50	12.1 ± 0.61	10.0 ± 0.55	15.1 ± 0.75
	100	19.0 ± 0.95	17.1 ± 0.86	23.7 ± 1.18
Chloroform Extract	5	2.1 ± 0.11	3.7 ± 0.18	4.5 ± 0.22
	10	10.7 ± 0.54	8.7 ± 0.44	16.1 ± 0.81
	50	20.5 ± 1.01	19.1 ± 0.75	28.6 ± 1.43
	100	25.3 ± 1.21	23.1 ± 0.96	34.1 ± 1.71
Methanol Extract	5	6.1 ± 0.31	4.2 ± 0.21	9.8 ± 0.48
	10	18.1 ± 0.91	16.3 ± 0.81	23.7 ± 1.15
	50	**34.7 ± 1.2**	**29.0 ± 1.5**	**49.1 ± 1.7**
	100	39.4 ± 1.4	33.1 ± 1.6	54.5 ± 1.9

Table 7.14: A comparison of anti-PLA_2 activity of polyvalent antivenom and methanolic extracts of leaves of *C. sinensis* and *X. strumarium* and roots of *A. Marmelos*. RVVB-PLA_2-I (1 μg ml^{-1}) were incubated either with plant extract (100 μg ml^{-1}) or with polyvalent antivenom in a ratio of 1:100 (in a final volume of 1.0 ml) at 37 °C for 30 min and then assayed for residual catalytic and pharmacological properties of RVVB-PLA_2-I. Values are mean ± S.D. of triplicate determinations. Values in the same row with different superscripts are significantly different ($p<0.05$).

Pharmacological properties	% Inhibition of RVVB-PLA_2-I by			
	Polyvalent antivenom	***C. sinensis***	***X. strumarium***	***A. marmelos***
PLA_2 activity	7.2 ± 0.8^a	18.6 ± 1.2^b	23.8 ± 1.6^c	28.5 ± 1.9^d
Anticoagulant activity	24.9 ± 1.3^a	44.7 ± 2.2^b	34.0 ± 2.5^c	59.1 ± 2.3^d
Indirect haemolytic activity	17.5 ± 0.5^a	28.0 ± 1.1^b	37.3 ± 2.1^c	45.5 ± 2.4^d
In vitro tissue damaging activity				
Liver	42.5 ± 0.6^a	46.5 ± 1.8^b	44.2 ± 1.4^c	61.1 ± 2.6^d
Heart	45.0 ± 0.9^a	52.2 ± 2.1^b	48.8 ± 1.9^c	57.7 ± 2.1^d
Lungs	51.4 ± 1.1^a	60.1 ± 2.7^b	57.4 ± 2.7^c	65.5 ± 2.7^d
Mitochondrial membrane phospholipids hydrolysis	19.9 ± 1.1^a	33.6 ± 1.3^b	25.5 ± 2.2^c	48.2 ± 2.5^d
Erythrocytes membrane phospholipids hydrolysis	16.8 ± 0.9^a	41.4 ± 1.4^b	37.2 ± 1.1^c	52.9 ± 2.9^d

CHAPTER VIII

8 Discussion

8.1 Importance and justification of the present study

Phospholipase A_2 (PLA_2) enzymes are esterases that hydrolyze the sn-2 ester of glycerophospholipids and constitute one of the largest families of lipid hydrolyzing enzymes [267]. These PLA_2 enzymes are one of the toxic components of snake venom which have been extensively studied because of their pivotal role in various pharmacological activities in victims as well as to elucidate the structure-function relationship among this class of puzzling enzymes [91,93]. During the last decade, numerous reports on characterizing the biochemical and pharmacological properties including structure-function relationships of PLA_2 enzymes from Russell's viper (*Daboia russelli*) venom of Indian origin have been published. The reason behind this study was dearth of knowledge on different properties of strong anticoagulant PLA_2 enzymes from Indian Russell's viper venom, particularly their mechanism of anticoagulant action and membrane damaging activity.

The rationale of the present study is mainly of four folds.

1. Considering the presence of several phospholipase A_2 isoenzymes in venom of Russell's viper, an effort has been given in the present study to identify the presence of acidic, basic and neutral PLA_2 isoenzymes (depending on their overall charge) in *Daboia russelli* venom of eastern India origin.

2. Different PLA_2 enzymes present in single venom exert their pharmacological activities by different mechanism of action. Therefore, in order to understand the structure-function properties and mechanism of action, three different (one acidic, one neutral and one

basic PLA_2), catalytically active, anticoagulant PLA_2 isoenzymes have been purified from the venom of *Daboia russelli* of eastern India origin. Furthermore, biochemical and pharmacological properties of these three PLA_2 isoenzymes enzymes were compared. This comparison has provided valuable information to know their role in pathogenesis post Russell's viper envenomation.

3. Thirdly, the mechanisms of anticoagulant action as well as differential membrane damaging activities of the three PLA_2s were compared to elucidate the relationship between overall charge on PLA_2 molecules and their two most important pharmacological activities.

4. Finally, the pharmacological re-evaluation of few medicinal plants of North-east India for their inhibitory activities against the crude and PLA_2 enzyme (s) of *Daboia russelli* venom and against their strong anticoagulant activity had been done in an effort to provide an alternative to antivenom therapy against Russell's viper bite.

8.2 Presence of PLA_2 isoenzymes in *Daboia russelli* venom shows functional diversification of venom-gland PLA_2 isoezymes

To be acquainted with the number of acidic, neutral and basic PLA_2 isoenzymes present in crude venom sample of *Daboia russelli* venom, venom was fractionated by using a cation exchanger, CM-Sephadex C-50 column followed by an anion exchanger, DEAE Sephadex A-50 column and eluted with buffers of different pH and molarities. Due to positive charge on basic PLA_2 enzymes, they were retained by the cation exchanger and eluted with buffers of increasing molarities and pH values. Conversely, neutral and acidic PLA_2 isoenzymes were not retained by the cation exchanger and were eluted in a single peak with the wash buffer (K-phosphate buffer, pH 7.0). Then to know the

number of neutral and acidic isoenzymes present in *Daboia russelli* venom, non-retained fractions of CM-Sephadex C-50 column was fractionated on an anion exchanger, DEAE Sephadex A-50 column. Because of the net negative charge on acidic PLA_2 isoenzymes, they bound with the anion exchanger and strength of binding was proportional to the net negative charge on them. These enzymes were progressively eluted from the column with the buffers of increasing molarities and decreasing pH values. On the other hand, neutral PLA_2 isoenzymes could not be retained by either of the anion exchangers and were eluted in a single peak with the wash buffer (K-phosphate buffer, pH 7.0).

The present study documented that the venom of *Daboia russelli* of eastern India origin contains a total of 19 PLA_2 isoenzymes- six acidic, one neutral and twelve basic in nature. Earlier report suggested presence of 13 PLA_2 isoenzymes in the venom of *Daboia russelli* (eastern Indian origin) as determined by gel filtration of crude venom [27]. Therefore, sequential separation of venom components through ion-exchange resins (cation exchange followed by anion exchange) was found to be superior for the identification of PLA_2 isoenzymes as compared to gel-filtration alone. These PLA_2 isoenzymes with similar protein folds and three-dimensional structures have been evolved to exhibit diverse biological properties of snake venom [108]. Gene duplication is a ubiquitous feature of genome evolution and has been viewed as the mechanism for evolution of new gene functions [268]. The PLA_2 multigenes are thought to have been formed by gene duplication, starting from a single ancestral gene, and the newly formed genes have acquired new functions by proper nucleotide substitutions at the non-synonymous sites [269]. Therefore, it may be anticipated that functional diversification of venom-gland PLA_2 isoezymes has been brought about by accelerated evolution of their genes. Such evolution seems to be in no way explained by the neutral theory [175,269]. PLA_2 enzymes induce distinctly different pharmacological effects and show a wide range of toxicity and catalytic efficiency in hydrolyzing phospholipids. Thus, both catalytic and functional

properties of PLA_2 enzymes are altered during evolution. Consequently, these natural substitutions in PLA_2 enzymes are not neutral or near-neutral [270]. Accelerated evolution is considered to be universal in snake venom-gland isoezymes. These studies suggest that accelerated evolution has occurred in snake venom PLA_2s possibly to acquire new physiological functions. Such an accelerated evolution is proposed to result in the production of diverse pharmacological properties [91,271]. Positive Darwinian selection may play a role in the evolution of different venom PLA_2 isoenzymes [100].

8.3 Purification of three PLA_2 enzymes from *Daboia russelli* venom

In this study, we purified one acidic (RVVA-PLA_2-I), one neutral (RVVN-PLA_2-I) and one basic (RVVB-PLA_2-I) phospholipase A_2 enzyme by following a combination of ion exchange (cation and anion exchange), gel filtration chromatography and RP-HPLC.

8.3.1 Molecular mass and homogeneity

RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I constituted only about 0.1 %, 0.04 % and 0.15 % of the total RVV proteins, respectively implying they are minor components of RVV. However, all the three PLA_2 enzymes of RVV demonstrated strong anticoagulant activity suggesting their important role in pathogenesis post Russell's viper envenomation. Percent yield of some other PLA_2s purified from Russell's viper venom in different laboratories are shown in the table 8.1. In this present study, we used three (for the basic PLA_2) and four steps (acidic and neutral PLA_2) for purification of PLA_2 isoenzymes. However, other workers have used mostly single step or sometime two steps for purification of venom PLA_2 enzymes (Table 8.1).

The molecular mass of snake venom PLA_2 is reported mostly in the range of 11–15 kDa [108,130,222,272]. However, molecular mass of RVVA-

PLA_2-I was determined as 58.0 kDa. There is no other report of occurrence of 58.0 kDa homodimeric snake venom PLA_2 except one example of 29.0 kDa monomeric, non-anticoagulant PLA_2 from the venom of *Vipera russelli* of Myanmar origin [51].

The molecular mass of RVVN-PLA_2-I and RVVB-PLA_2-I was determined by gel filtration as 13.0 kDa and 7.0 kDa, respectively. These values are very close to the molecular mass of these proteins determined by SDS-PAGE. The purity and molecular mass of RVVN-PLA_2-I and RVVB-PLA_2-I were further confirmed by ESI/MS and were found as 12.8 kDa and 6.7 kDa respectively. Molecular weight of the neutral PLA_2 is quite consistent with the molecular weight of previously reported PLA_2 enzymes from snake venoms [Table 8.1,92,273]. However, RVVB-PLA_2-I is a very low molecular mass PLA_2 present in RVV. Till date, there is only one report of occurrence of such a low molecular mass (7.2 kDa) cytotoxic PLA_2 from the venom of Russell's viper of Indian origin [228].

Due to the predominance of pro-coagulant proteins and peptides, in *in vitro* condition, crude RVV failed to show anticoagulant activity. That is the reason why the acidic PLA_2 (RVVA-PLA_2-I) and the neutral PLA_2 (RVVN-PLA_2-I) of this study with strong anticoagulant activities, could be separated from the coagulant components of crude RVV; only after the gel-filtration step [216].

Table 8.1: Percent yield and molecular mass of different PLA_2 enzymes purified from Russell's viper venom.

Name of the isolated PLA_2	RV species	Step(s) of purification	Yield of PLA_2 (%)	Molecular mass (Da)	Reference
VRV PL-VIIIa (Basic)	*Vipera russelli*	Single	24	11,800	[102]
Daboiatoxin (dbTx)	*Daboia russellii siamensis*	Three	12.3	15,000	[52]
VRV-PL-IIIb (Basic)	*Vipera russelli*	Two	2.3	14,800	[105]
RVV-PFIIc'	*Vipera russelli*	Two	11.5	15,300	[106]
RVV-7 (Basic)	*Daboia russelli russelli*	Single	7.5	7,200	[228]
Drs-PLA_2 (Basic)	*Daboia russellii siamensis*	Two	27.4	13,679	[274]

8.4 Biochemical characterization of the three purified RVV-PLA$_2$s

Biochemical characterization of these three purified PLA_2 enzymes reveals that RVVA-PLA_2-I is glycoprotein in nature while the other two PLA_2s viz. RVVN-PLA_2-I and RVVB-PLA_2-I do not contain carbohydrate moiety and therefore, they are non-glycoprotein in nature. The carbohydrate moiety attached to a PLA_2 molecule assists in protein folding, stabilises the natural conformation and protects the protein against degradation. Moreover, the presence of carbohydrate moiety in the PLA_2s may also be helpful for receptor-ligand interactions [100,275]. However, glycosylation in PLA_2 molecule is very rare [108].

RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I display their optimum catalytic activity at pH range of 8.0-8.5 and at a 37 °C although the overall charge on these three PLA_2s are different. The pH optima of these *D. russellli* PLA_2s are higher than the reported pH optima for the acidic PLA_2s (6.9) isolated from the Indian cobra *N. n. naja* venom [276]. Nevertheless, this value is found to be in a close agreement with the pH optima of many other snake venom PLA_2s such as VRV-PL-VIIIa from *Vipera russelli,* optimum pH 7.2 [102]; Bj IV from *Bothrops jararacussu* venom, optimum pH 8.2 [277], trimorphin purified from the venom of *Trimorphodon biscutatus lambda,* showing an apparent peak of activity at pH 7.5 [278].

Determination of *in vitro* head-group specific phospholipid hydrolyzing capacity of phospholipase enzymes has great relevance in elucidating the catalytic efficiency of the enzyme and to explain some of their pharmacological effects on the target cell membranes [93,97,179,216,279]. In this study, the RVVA-PLA_2-I and RVVB-PLA_2-I showed preferential hydrolysis of PC over PS or PE. However, the neutral PLA_2 (RVVN-PLA_2-I) showed preference towards PS followed by PC and PE for hydrolysis. This is in contrast to the earlier

reports demonstrating enhanced hydrolysis of PE over PC or PS by PLA_2 from *N. n. kaouthia, N. n. atra* [279] and *D. russelli* venom [160]. This may be due to geographical and species specific variation in the substrate specificity of PLA_2 isoenzymes.

The Km values showed by RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I are quite lower than the earlier reported Km values of VRV-PL-IIIa (2.92×10^{-4} M, [102]), VRV-PL-IIIb (1.6×10^{-3} M, [105]) purified from *Vipera russelli* and one PLA_2 purified from Russell's viper of Myanmar origin (2.3×10^{-2} M, [51]). The lower Km values indicate the high binding affinity of these three PLA_2s towards their specific phospholipid substrates.

Like many other venom PLA_2s, these three purified PLA_2 enzymes of *Daboia russelli* venom are highly thermostable in nature because heating barely affects the catalytic activity or secondary structure of these enzymes [117,140,160]. CD spectra of native and heated PLA_2s also suggested their stability against heat denaturation.

The secondary structures of these three PLA_2s are quite similar and they indicate a strong α-helical contribution to the CD signal. Alpha helix is the major secondary structure element for a large number of PLA_2s from snake venom [140,255,280,281]. The thermostability is due to compact folding of the enzyme, which is based on core structure of disulfide bridges [140,280,282]. The presence of six conserved disulfide bonds along with one or, two variable disulfide bonds in snake venom PLA_2 molecule [283] is mainly responsible for the retention of tertiary structure in PLA_2s.

8.5 Pharmacological characterization of three purified PLA_2s of RVV

8.5.1 All the three PLA_2s in the present study represent the strong anticoagulant activity

All the three PLA_2 enzymes viz. RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I purified from RVV are anticoagulant in nature. Venom PLA_2 enzymes have been classified into strong, weak and non-anticoagulant enzymes [108,121,122]. The RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I displayed anticoagulant effect at a concentration of 0.3-0.6 μg ml^{-1} which was lower than recommended concentration (~ 2.0 μg ml^{-1}) required for the strong anticoagulant PLA_2 to prolong the normal clotting time of plasma [108]. Therefore, it is reasonable to assume that all the three purified PLA_2s viz. RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I belong to the group of strong anticoagulant PLA_2s. Further, our finding contradicts an earlier report suggesting higher anticoagulant potency is associated mostly with basic PLA_2 enzymes [122]. According to Kini [108], it is not the overall basic charge but the nature of charge in the anticoagulant site(s) determines the anticoagulant potency of PLA_2 enzymes. In case of strong anticoagulant PLA_2 enzymes, it has been postulated that the anticoagulant region is positively charged, but negatively charged in weakly and non-anticoagulant enzyme [108]. Therefore, it might be hypothesized that although overall net charge on RVVA-PLA_2-I (anionic in nature) is negative whereas RVVN-PLA_2-I is a neutral enzyme (on the basis of charge); however, some positive residues on these enzyme may play a significant role in demonstrating their strong anticoagulant effect [108].

8.5.2 Mechanism of action of the anticoagulant activity

8.5.2.1 Correlation between catalytic and anticoagulant activity

The understanding of structure-function relationships in PLA_2 enzymes is complicated and contradictory data have been presented to explain the anticoagulant action of venom PLA_2 enzymes. For example, there are reports demonstrating a correlation between catalytic activity and anticoagulant property of PLA_2 molecules [95,121,122,179]. In a sharp contrast, some of the study suggested that non-enzymatic mechanism might play a key role in inhibiting the different blood coagulation factors by venom PLA_2 and therefore, nullifying the role of PLA_2 activity in anticoagulant process [141,216,220]. Present study supports that anticoagulant activity of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I is exerted by a dual mechanism. The anticoagulant potency is mainly dependent on the catalytic activity of that particular PLA_2 enzyme, i.e. its potency to hydrolyse the essential plasma phospholipids required for the coagulation process. However, anticoagulant activity is also attributed partly by a non-enzymatic mechanism. In case of RVVA-PLA_2-I and RVVB-PLA_2-I, the non-enzymatic mechanism operates by binding with coagulation factor Xa and thus inhibiting the activation of prothrombin to thrombin. Whereas in case of the neutral PLA_2 (RVVN-PLA_2-I), the non-enzymatic mechanism of anticoagulant action is yet to be known as it doesn't show any interaction with blood coagulation factor Xa, thrombin or, prothrombin.

It is well known that phospholipids play a crucial role in the formation of several coagulation complexes. In this present study, the acidic and the basic PLA_2 (RVVA-PLA_2-I and RVVB-PLA_2-I) hydrolyse preferentially PC, the natural precursor for PS; followed by hydrolysis of PS, which is known to be the most active phospholipids involved in the blood clotting process [94]. However, the neutral PLA_2 (RVVN-PLA_2-I) preferentially hydrolyse PS which

is responsible for showing the strong anticoagulant potency of this enzyme. Moreover, GC analysis suggested low hydrolysis of plasma phospholipids by all these three anticoagulant PLA_2s which might lead us to anticipate that destruction of phospholipids surface would be the primary mechanism to account for anticoagulant effect of these enzymes [216]. Those results support the role of enzymatic activity in anticoagulant action by RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I [179,216,224]. The above hypothesis is supported by the observations that very low but specific plasma phospholipids hydrolysis is the characteristics feature of strongly anticoagulant PLA_2s whereas non specific, non-anticoagulant PLA_2 enzymes randomly hydrolyse the plasma phospholipids [94]. Therefore, higher amounts of phospholipids hydrolysis are required before non-specific PLA_2s show any significant anticoagulant effect [94]. It is noteworthy to mention that non-anticoagulant PLA_2 enzymes from *Naja naja naja* [284] *Crotalus atrox* [285] and *Crotalus adamanteus* [286] and weakly anticoagulant PLA_2 from *N. kaouthia* [97] preferentially hydrolyses PC over PS and PE, whereas anticoagulant PLA_2 from *Vipera berus* has a specificity towards PS hydrolysis [287]. In this study, RVVA-PLA_2-I and RVVB-PLA_2-I are strong anticoagulant PLA_2s and showed preferential hydrolysis of PC over PS or PE. This novel finding leads to conclude that strong anticoagulant activity may not always be restricted to only those PLA_2 enzymes showing preferential hydrolysis of PS.

Further, the anticoagulant activity was parallelly enhanced with an increase in the initial concentration of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I supporting anticoagulant potency is dependent on the catalytic activity of these PLA_2 enzymes. Nevertheless, the metal chelator EDTA significantly inhibited the catalytic as well as anticoagulant activity of these PLA_2 enzymes by chelating the Ca^{2+} ions require [288-290] to enhance these activities. Moreover, alkylation of histidine residue, the most conserved amino

acid amongst PLA_2 enzymes [97,140,291], resulted in parallel inhibition of catalytic and anticoagulant activity of three PLA_2 enzymes under study reinforcing a correlation between catalytic and anticoagulant activities. Besides, inhibition study with medicinal plant extracts and heat-inactivation study also supported the correlation between catalytic and anticoagulant activity of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I.

8.5.2.2 Non-enzymatic mechanism of anticoagulant activity: Interaction of PLA_2s with plasma phospholipids and coagulation factor Xa

The non-enzymatic mechanism of anticoagulant action of the three PLA_2s from RVV possessing different overall net charge is evident from the fact that beyond a concentration of 0.6 $\mu g\ ml^{-1}$ of any of these three PLA_2s, no further plasma phospholipids hydrolysis was observed. However, anticoagulant activity of all these three PLA_2s was found to be increased up to a PLA_2 concentration of 1.0 $\mu g\ ml^{-1}$. Moreover, the heat inactivation study in case of the acidic (RVVA-PLA_2-I) and basic PLA_2 (RVVB-PLA_2-I) showed distinctly that anticoagulant activities of these two enzymes are not completely dependent on their catalytic activities. Therefore, these studies signify that apart from the contribution of catalytic activity in anticoagulant process, some non-enzymatic mechanism may run in parallel to exert strong anticoagulant action.

One of the most important key factors influencing the anticoagulant potency is the penetrating property of PLA_2 enzymes [108,203]. Penetratability is the ability of the PLA_2 enzyme to insert itself into the phospholipid membrane and hydrolyse phospholipids [93]. Since phospholipids in plasma membranes are packed at higher density compared to the phospholipid vesicles which are used in *in vitro* enzyme studies, PLA_2 enzymes with higher penetratability cause more significant damage to the membrane than those with lower penetratability [93]. Most basic PLA_2 enzymes, but not all, have higher penetratability compared to

neutral and acidic isoenzymes [121,122], which is probably because of the abundance of positive residues. Present study contradicts those reports postulating penetratability is the property associated with basic PLA_2s of venom [121,122]. Nevertheless, RVVA-PLA_2-I, the acidic PLA_2 strongly binds with PC vesicles even in absence of Ca^{2+} suggesting its high penetrating ability which in turn reflects its strong anticoagulant activity [216,291]. It has been suggested by Kini and Evans [94] that anticoagulant region lies between residues 54 and 77 of PLA_2 molecule; this region is positively charged in strongly anticoagulant PLA_2 enzymes even if the overall charge on the enzyme is different. It is to be noted that all the three PLA_2 of this study i.e., RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I differ from *N. naja naja* phospholipase (group I PLA_2) which has an absolute requirement of Ca^{2+} for its binding to lipids [292]. However, our result does not corroborate with the report of Prigent-Dachary et al. [291], who suggested that strong anticoagulant PLA_2 should always have a greater binding with PS. A correlation between decrease in percent binding with plasma phospholipid PC and a parallel decrease in catalytic as well as anticoagulant activity after heating of any of the PLA_2s under study reinforces the hypothesis that binding followed by hydrolysis of plasma phospholipids particularly PC are the essential steps for inducing the strong anticoagulant activity by these PLA_2s [216,293].

It has been suggested that anticoagulant phospholipases may inhibit blood coagulation cascade non-enzymatically by competing with clotting factors for the lipid surface [108,141,294]. However, strongly anticoagulant PLA_2 enzymes in addition to hydrolysis of plasma phospholipids may bind to coagulation factor X and/or factor Xa and/or by inhibition of thrombin induces anticoagulant activity [108,294]. By Intrinsic fluorometric study, an interaction of RVVA-PLA_2-I/RVVB-PLA_2-I with coagulation factor Xa was observed in absence of phospholipids/Ca^{2+} documenting these PLA_2 may block the formation

of prothrombinase complex even in a phospholipids/Ca^{2+}-independent manner. The non-enzymatic, Ca^{2+}-independent mechanism of inhibition of blood coagulation by RVVA-PLA_2-I is further evident from the fact that addition of EDTA resulted in decrease of about 62 % of anticoagulant activity although the catalytic activity is reduced to 80 % of its original activity. Further, RVVA-PLA_2-I and RVVB-PLA_2-I bind with blood coagulation factor Xa at a site other than their chromogenic substrate binding site resulting in no interference in the amidolytic activity of factor Xa although their prothrombin activation property after addition of PLA_2 were lost. This result is in accordance with the report of Stefansson et al [141] showing a basic PLA_2 from *N. Nigricollis* venom (CM-IV) inhibits the prothrombinase complex in absence of phospholipids without affecting the amidolytic activity of factor Xa.

There was not any change observed in the fluorescence signal of RVVN-PLA_2-I in presence of blood coagulation factor Xa/ thrombin/ prothrombin. Furthermore, addition of phospholipids viz., PC, PS or PE did not influence the fluorescence intensity. The data may suggest absence of interaction between this neutral PLA_2 and coagulation factor Xa either in presence or absence of plasma phospholipids; however, further studies are necessary to reveal the fact.

8.5.3 Mechanism of hydrolysis of membrane phospholipids by the RVV-PLA_2 enzymes

8.5.3.1 Correlation between catalytic activity and membrane damaging activity

Lysis of artificial membranes by PLA_2s from various snake venoms e.g., *Bothrops pirajai* and *Crotalus durissus terrificus* has been shown to be independent of their enzymatic activity, since both native as well as catalytically inactivated enzymes are fully able to disrupt membranes [95]. However, we have

presented contradictory evidences suggesting that mitochondrial and erythrocyte membranes hydrolyzing property of these three Russell's viper venom PLA_2 enzymes (RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I) is dependent on their catalytic activity which is similar to membrane damaging activity of PLA_2 enzymes (NK-PLA_2-I and NK-PLA_2-II) isolated from *N. kaouthia* venom [140]. The current investigation shows that the different membranes studied in the present investigation have different structural organisation as reflected by their distinctly different sensitivity to these RVV-PLA_2s. Nonetheless, this membrane damaging activity is dependent on Ca^{2+} ion because in presence of the metal chelator EDTA, RVV-PLA_2s lost their membrane damaging as well as their catalytic activity in a parallel manner. The degree of membrane phospholipids hydrolysis increases with the increase in the pre-incubation time of the membranes with the RVV-PLA_2s, documenting that the membrane damage is dependent on their catalytic activity.

Majority of the PLA_2 enzymes are reported to be devoid of direct haemolytic activity [140,295,296]. None of these three purified PLA_2s from RVV show considerable haemolytic activity on washed goat/ human erythrocytes. However, significant haemolytic activity shown by all the three PLA_2s in presence of exogenously added egg yolk (which is a source of PC) and 1.5 mM Ca^{2+} was due to the formation of phospholipids hydrolysis products like lysophospholipids and free fatty acids from egg-yolk phospholipids/ PC and these products caused further damage to the membrane [297]. The same hypothesis can be put forwarded to explain the enhanced susceptibility of the erythrocytes in presence of egg yolk phospholipids/ PC/ PS and Ca^{2+}, because amongst the tested phospholipids, PC is the most favoured substrate for the acidic and basic PLA_2s while PS is preferred by the neutral PLA_2 for binding followed by hydrolysis. The spectroflurometric study suggested the role of Ca^{2+} in enhancing the binding of these PLA_2s with PC, confirming these three are

Ca^{2+} -dependent PLA_2s for membrane damage. Furthermore, inhibitor studies with specific amino acids modifying reagents, neutralization study with polyvalent antivenom, and the heat-inactivation data have suggested a correlation between the catalytic and membrane phospholipids hydrolysis property of these RVV-PLA_2s. Therefore, although there is enough controversy regarding the role of enzymatic activity in the pharmacological effects of snake venom PLA_2 enzymes [94,97,108,140,216]; however, present study provides enough evidences that at least in case of membrane damaging activity; the catalytic (enzymatic) mechanism of phospholipids hydrolysis plays a great role.

The heat-inactivation study also lead us to conclude that the binding of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I to membrane PC followed by its hydrolysis are essential steps for inducing membrane damage [97,140,216]. This hypothesis was based on the observation that a parallel inhibition of membrane PC binding, catalytic activity as well as membrane damage in case of heat-inactivated RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I was noted. This phenomenon may be explained by the fact that heat treatment partially distorted the PLA_2 structure which was evidenced by a comparison of secondary structure of native and heat-inactivated PLA_2 enzymes. This partial unfolding of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I could not be regained even after cooling to room temperature resulting in a reduction in PC binding property as well as catalytic activity of heat-denatured PLA_2s as compared to native enzymes. ELISA experiments also suggested that heat inactivated enzyme partially lost its membrane binding property. However, modification of histidine residue of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I did not affect its membrane binding property suggesting histidine is involved with phospholipids hydrolysis only and does not play any role in binding of PLA_2s [122] with membranes. However, this result is in contrast of the findings of Tatulian [298] where His48 modification affected PLA_2 structure,

its membrane binding affinity, and the effects of PLA_2 on the membrane structure.

8.5.3.2 Differential hydrolysis of mitochondrial and erythrocyte membrane phospholipids: does it reflect the differences in PLA_2-sensitive phospholipids composition of biomembranes?

Membrane surface properties, including membrane fluidity, curvature, surface charge, and membrane-induced structural changes in the enzyme, determine the strength of interaction, cooperatively of membrane binding, and the extent of PLA_2 activation [97,140]. Increase in volume of mitochondria due to an influx of fluid; it occurs in hypotonic solutions due to osmotic pressure and in isotonic solutions (here, as sucrose solution) as a result of altered permeability (here, increase/swelling of the organelle with a concomitant decrease in the light scattered by the mitochondrial suspension) of the membranes of respiring mitochondria [140]. Snake venom PLA_2 induced injury to mitochondria brings about a change in the mitochondrial volume [140,298].

The preferential release of short chain fatty acids from mitochondria and erythrocytes membrane during the initial phase of attack by RVVA-PLA_2-I is correlated with their preference for short chain fatty acids at the *sn-2* position. It is presumed that even if PLA_2 binds uniformly across the solid and fluid domains of membrane enriched in the long and short chain fatty acids respectively, enzyme shows a preference for short chain fatty acids, which are in fluid phase [300]. This is due to the lower surface area density of the lipid head groups in short chain fatty acids that are present in fluid phase [301]. Similarly, RVVN-PLA_2-I and RVVB-PLA_2-I also showed preferential release of short chain fatty acids from erythrocyte membranes. However, mitochondrial membrane phospholipid hydrolysis by RVVN-PLA_2-I and RVVB-PLA_2-I was detected only after an initial lag phase of 30 min following the addition of PLA_2.

$sPLA_2$s display large variations in their affinities for membranes with differing surface properties [140]. Since RVVB-PLA_2-I has significantly low affinity for mitochondrial membrane compared to erythrocyte membranes, as revealed by ELISA experiment, binding is extremely low during the initial phase of attack.

Our study has shown that RVVA-PLA_2-I and RVVB-PLA_2-I preferentially hydrolyze PC over PS and PE. It is very interesting to observe that RVVA-PLA_2-I and RVVB-PLA_2-I preferentially hydrolyze the phospholipids of erythrocyte membrane compared to mitochondrion membrane even though the latter possesses much greater percentage of PC on its outer leaflet (40.9%) than the outer leaflet of the former membrane (19%) [302]. Therefore, our result suggests the existence of significantly greater number of RVVA-PLA_2-I and RVVB-PLA_2-I sensitive regions in erythrocytes membrane as compared to mitochondrial membrane might have a relevance to the higher degree of phospholipids hydrolysis of the former membrane [97,140,264]. These specific domains or venom PLA_2 susceptible region(s) may result from the presence of different fatty acids, more particularly the short-chain fatty acids in these regions of the membrane [97]. Therefore, the presence of a large number of venom PLA_2-sensitive phospholipid compositions, rather than only PC content of that particular membrane, may determine the extent of membrane damage induced by a particular PLA_2 [97,264]. However, the neutral PLA_2 (RVVN-PLA_2-I) preferentially hydrolyse the membrane phospholipids of mitochondria as compared to that of erythrocytes membranes; though this neutral PLA_2 shows preferential hydrolysis of PS over PC. Therefore, PC hydrolysis also plays an important role in this case.

Furthermore, RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I could hydrolyze the erythrocyte membrane phospholipids within 15 min of incubation which is in sharp contrast to the previous reports published from our laboratory.

It was demonstrated that two phospholipase A_2 enzymes viz. NK-PLA_2-I and NK-PLA_2-II from *Naja kaouthia* venom showed a lag phase for binding with erythrocyte membrane and subsequent membrane hydrolysis [140]. Besides, *N. kaouthia* PLA_2 enzymes preferentially hydrolyze the membrane phospholipids of mitochondria as compared to that of erythrocytes membranes [140] which are similar to the result of RVVN-PLA_2-I of the present study. It is interesting to note that RVVA-PLA_2-I, RVVB-PLA_2-I and *N. kaouthia* PLA_2s have shown a preference for hydrolyzing PC over PS or PE [140,207,264]; while RVVN-PLA_2-I showed preferential hydrolysis of PS over PC or PE. However, RVVA-PLA_2-I is acidic in nature; RVVN-PLA_2-I is neutral in nature and RVVB-PLA_2-I (from *D. russelli* venom) as well as NK-PLA_2-I and NK-PLA_2-II from *N kaouthia* venom are basic in nature [140]. Therefore, the differences in overall net charge in a venom PLA_2 molecule may attribute to differential binding and subsequent hydrolysis of phospholipids of a particular membrane [206].

Evidence has been accumulating regarding the presence of nanometer range small-scale structures and lipid domains in the lipid bilayer and such organizational heterogeneity of lipids microdomains may have structural and functional significance [303-305]. The phospholipids-binding domains with secondary binding sites for specific proteins are well known, and this binding is often tightly regulated [306]. The differences in the membrane phospholipid hydrolysis by a venom PLA_2 can be supported by our previous observation that the phospholipids constituent of microsomal membranes are less hydrolyzed as compared to lysosomal membrane by the action of *Vipera russelli* venom basic PLA_2 [207,208]. Further, it has been suggested that PLA_2 may be particularly active at domain interfaces which are the sites of structural defects and hence good points of attack for these enzymes [140].

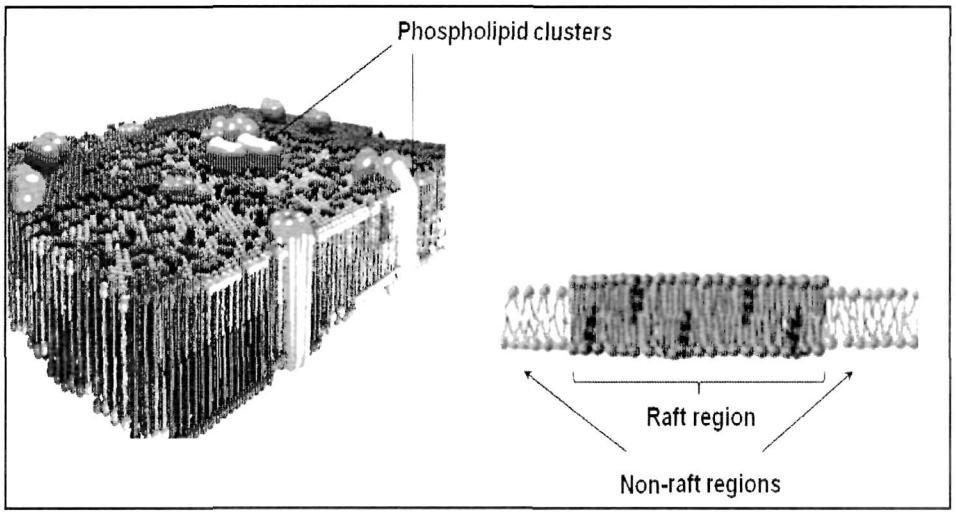

Fig 8.1: The picture of membranes allows for lateral heterogeneities, cluster and raft/domain formation within the membrane plane. The asymmetrical distribution of membrane lipids along with some other components of membrane constitutes "Membrane Domain" and PLA_2s are sensitive to these domains. Picture generated by H. Seeger, NBI Copenhagen [307].

The mechanism by which snake venom phospholipase A_2 sensitive phospholipid domains are formed in mitochondrial or erythrocyte membrane is not very clear. Although no adequate description of the nature of such membrane lipid domains in terms of their abundance, composition or dynamics has been provided; however, it may be assumed that the physicochemical properties of individual phospholipids, circumstantial effects of membrane proteins, phospholipids/cholesterol ratio, and vitamin E content of the membrane contribute significantly to the formation of such domains [97,140,208,305].

A comparison of ratio of saturated / unsaturated fatty acids released from the intact erythrocytes and mitochondrial membranes after treatment with RVVA-PLA_2-I and RVVB-PLA_2-I suggested that these enzymes have distinct

preferences for hydrolyzing phospholipids containing saturated fatty acids at *sn*-2 position in mitochondrial membrane. In a sharp contrast, during the initial phase of attack the PLA_2 prefers hydrolysis of unsaturated fatty acids in erythrocytes membrane and therefore, the ratio of saturated/unsaturated fatty acids was less than 1.0 up to 30 min of incubation. A further increase in incubation time resulted in a greater hydrolysis of erythrocytes phospholipids containing saturated fatty acids compared to phospholipids containing unsaturated fatty acids at sn-2 position and the ratio of saturated/unsaturated fatty acids was higher than 1.0 post 60 min of incubation. In a sharp contrast, RVVN-PLA_2-I shows preferential hydrolysis of saturated fatty acids in erythrocytes membrane and unsaturated fatty acids in mitochondrial membrane in the initial phase of attack. These data clearly indicate the differential hydrolysis of erythrocytes and mitochondrial membrane phospholipids by three different PLA_2s viz. RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I possessing different overall net charge.

The difference in the release of saturated and unsaturated fatty acids from the membranes may be explained by the fact that in case of mitochondrial membrane, RVVA-PLA_2-I and RVVB-PLA_2-I preferentially binds to one of the PLA_2-sensitive domains of the membrane resulting in quantitative increase in release of fatty acids from that membrane with respect to time [140,264]. However, RVVA-PLA_2-I and RVVB-PLA_2-I attack different phospholipids domains/ region(s) of erythrocyte membrane and therefore, this kinetics of erythrocyte membrane phospholipids hydrolysis favours the release of quantitatively as well as qualitatively different FAs with respect to time. The neutral PLA_2 (RVVN-PLA_2-I) attack mitochondrial membrane with an initial lag phase while this PLA_2 preferentially binds to one of the PLA_2-sensitive domains of the erythrocyte membrane that resulted quantitative increase in release of fatty acids from that membrane with respect to time [140,264]. The ratio of

saturated/unsaturated fatty acids released from erythrocyte membrane may lead us to assume that during the initial phase of attack, RVVA-PLA_2-I and RVVB-PLA_2-I hydrolyzes a particular domain of erythrocyte membrane consisting of mostly the unsaturated fatty acids. Slowly with an increase in time, the reaction products build up which may help in PLA_2 binding and attacking another domain of the same membrane enriched in more saturated fatty acids than unsaturated fatty acids. Interestingly, this result contradicts with the finding of Shukla and Hanahan [206] showing the acidic PLA_2 purified from the venom of *Agkistrodon halys blomhoffi* hydrolyses only one domain of PC in intact erythrocytes. These differences in erythrocyte membrane phospholipids hydrolysis pattern of acidic PLA_2s from two different snake venoms lead us to conclude that not only the overall acidic charge, but the charge at a particular region, known as interfacial binding surface (IBS) of PLA_2 may determine its competence for binding with different domains of a membrane [308]. This reinforces the presence of venom PLA_2-specific sensitive regions in a particular membrane. It may be presumed that by virtue of possessing arrays of phospholipase A_2 isoenzymes, injected venom can induce greater damage to membranes and subsequent toxicity to the cells.

8.5.3.3 Why do these PLA_2s fail to hydrolyze HT-29 cell membrane phospholipids?

None of the PLA_2s in the present study at the tested dose (10 $\mu g\ ml^{-1}$) did not show any cytotoxicity against HT29 colon adenocarcinoma cells even though PC is the most abundant phospholipids present in the outer cell membrane of HT-29 cells [309]. Lomonte et al [310] have shown the cytotoxic effect of Myotoxin II from *Bothrops asper* on HT-29 cells at a dose of 50-100 $\mu g\ ml^{-1}$ (micromolar range) whereas in this study, cytotoxicity have examined at a dose of 10 $\mu g\ ml^{-1}$ (nanomolar concentration) to avoid any non-specific

binding of PLA_2 to cultured cells [140]. Furthermore, considering the proportion of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I present in RVV, cytotoxicity at a very high dose was not performed. Amongst the tested membranes, the least hydrolysis of HT-29 cells can again be explained on the basis that it is not the overall quantity of PC in a membrane but either the availability of PC in a PLA_2-sensitive membrane and/or, (b) physicochemical properties of a membrane are the most important in order to elicit these three RVV-PLA_2s-induced membrane damage [97]. It has been observed that some of the components of the membrane such as cholesterol/phospholipids ratio and vitamin E (α-tocopherol) content may influence its fluidity which in turn modulates the activity of venom PLA_2 enzymes [208,311].

8.5.4 PLA_2 specific tissue damaging activity

Although the total phospholipid compositions of tissues like heart, lung and liver are very similar [312]; however, percent damage (*in vitro*) inflicted to heart tissues by RVVA-PLA_2-I and RVVB-PLA_2-I enzyme is significantly higher ($P< 0.05$) as compared to damage to the other tested tissues. On the other hand, RVVN-PLA_2-I causes damage to liver tissues to a much more extent than the heart or kidney tissues. The reason for the organ/subcellular organelle preference of these purified PLA_2s is not clearly understood [25]. It has been suggested that PLA_2s may be particularly active at domain interfaces that are sites of structural defects and hence good points to attack by PLA_2 enzymes [313]. Further, it may be assumed that differences in the biochemical nature of the vascular wall in the different organs/tissues like phospholipid/cholesterol ratio, presence of specific phospholipids, vitamin E content of that membranes etc, may have some relevance to differential membrane hydrolysis [207,208]. Moreover, mutational theory states that the substitutions of surface residues play a significant role in the evolution of new PLA_2 isoenzymes by altering the

specificity of targeting to various tissues, organs or cells [100]. Thus, differential membrane specificity of PLA_2 enzymes depends on the surface residues of enzymes.

8.5.5 Antibacterial activity

There are several reports on snake venom PLA_2 enzymes possessing antibacterial activity against Gram-positive and Gram-negative bacteria [212,314-318]. Antibacterial peptides isolated from snake venom contain cationic site(s), flanked by hydrophobic residues, which might disturb the phospholipid bilayer integrity, initiating a rapid cell death process [94,319].

The bactericidal activity of PLA_2 enzymes in snake Gram-positive bacteria requires recognition of anionic sites and subsequent hydrolysis of the phospholipid membrane [91]. In Gram-negative bacteria, it requires the synergistic action of a bactericidal/permeability increasing protein and depends on the hydrolytic activity [91,320,321]. The antibacterial site of snake venom PLA_2 enzymes was determined by comparing the amino acid sequences and by chemical modification [322,323]. The overall basicity and presence of a cluster of basic residue on the surface of the N-terminal helix is responsible for the bactericidal action of PLA_2 enzymes [321,324,325].

In the present study, none of the three PLA_2s of RVV up to a dose of 10 $\mu g\ ml^{-1}$ shows antibacterial activity against the tested Gram-positive and Gram-negative bacteria. Since RVVB-PLA_2-I is basic in nature, therefore; it might be assumed that this PLA_2 bearing positive charge on its surface should show antibacterial property. However, our study suggests that not the overall positive charge but the specific charge in a particular region of phospholipase A_2 molecule may be responsible for their penetrability in the bacterial cell wall [93].

Moreover, further studies are obligatory to decipher the antibacterial property of snake venom PLA_2s.

8.5.6 The three PLA_2s from RVV are non-lethal to mice

RVVA-PLA_2-I (at a dose of 0.2 mg kg^{-1} i.p.) did not show lethality in BALB/c mice after 48 hours injection suggesting that at the tested dose, it is devoid of lethality in mice. However, there was some rational for calculating the above dose. The rational for calculating the above dose was RVVA-PLA_2-I represents 0.1% (w/w) of total RVV proteins. On an average an adult Russell's viper may inject 225-250 mg of venom (total amount in a bite) to its victim (personal communication to Prof. A.K. Mukherjee, MBBT department, Tezpur University by Mr. D. Mitra, in-charge, Calcutta Snake Park, Kolkata). Therefore, a maximum of 0.2 to 0.25 mg of RVVA-PLA_2-I would be injected in a victim by the bite of an adult Russell's viper corresponding to nanomolar concentration of RVVA-PLA_2-I in the blood of an adult victim. Since RVVA-PLA_2-I was found to be non-toxic to mice at a much higher dose than this, therefore, it may be suggested that RVVA-PLA_2-I does not contribute to lethality of RVV. Similarly, RVVN-PLA_2-I and RVVB-PLA_2-I were also devoid of lethality in mice at the tested dose of 0.04 mg kg^{-1} and 0.35 mg kg^{-1} body weight (i.p.) respectively. It is noteworthy to mention that non-toxic PLA_2 enzymes from snake venom demonstrating phospholipids hydrolysis activity have been reported and the exact contribution of these PLA_2s in snake venom-induced pathogenesis in victims is poorly understood [179,255,264]. However, evidence has been presented from our laboratory to show that non-covalent interaction of relatively non-toxic PLA_2s from *N. kaouthia* venom with that of weak neurotoxin like molecules (kaouthitoxins) from the same venom resulted in marked synergism to potentiate the cytotoxicity of PLA_2-kaouthiotoxin complex without altering the biological properties of PLA_2 enzymes [326]. The proteome analysis of *D.*

russelli siamensis venom revealed the presence of complex of PLA_2 with other unidentified venom proteins [54]. Presence of such PLA_2-interacting component(s) from *D. russelli russelli* venom remains to be elucidated.

The decrease RBCs number as well as the Hb content in blood of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I treated mice as compared to control group of mice suggested that these PLA_2s cause lysis of erythrocytes in *in vivo* condition. As these PLA_2s showed significant indirect haemolytic activity *in vitro* on washed erythrocytes in presence of exogenously added phospholipids; therefore, we can assume that by the hydrolysis of plasma phospholipids, these PLA_2s exert *in vivo* haemolytic activity in mice.

Every living cell contains its complement of enzymes, most of which are intracellular and are released into the circulation only after breakdown of the cell or impairment of its membrane post envenomation [27]. Elevated CPK-MB level suggested damage to the skeletal muscle and indicative of myonecrosis, whereas increased SGOT, SGPT and ALP levels in the serum of treated mice indicated damage to the liver tissues reinforcing RVVA-PLA_2-I and RVVN-PLA_2-I targets cardiac and hepatic tissues. In contrast, the basic PLA_2 (RVVB-PLA_2-I) did not show any significant change to the serum parameters of treated mice documenting that it does not target the liver and cardiac muscles. Therefore, it may be assumed that different PLA_2 isoenzymes present in Russell's viper venom show differential toxicity towards different tissues and organs. This differential toxicity plays an important role towards the toxicity of the whole venom. Different isoenzymes of a venom may show their toxicity in combination with other components of the venom [326], though some of them are non toxic alone.

8.6 Weak immunogenic and non-toxic nature, and anticoagulant property of these PLA_2s suggest their therapeutic application in blood coagulation disorders

All the three RVV-PLA_2s viz. RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I were found to be very weak immunogenic in nature as they did not show visible antigen-antibody precipitate band against commercial antivenom. Host antibody response to toxin molecules is a major obstacle to the use of immunotoxins as efficacious agents in the treatment of human cancer and other diseases [327]. As these antigens (PLA_2s) will give weak immune response to antibody, this will be helpful in the development of novel anticoagulants in future. Snake venom molecules affecting blood coagulation have great potential as therapeutic agents for human diseases [10]. Venoms from vipers are rich sources of different enzymes that strongly affect the haemostatic mechanism [328]. Anticoagulant molecules from snake venom can be used for the development of drugs to treat or, to prevent unwanted clot formation i.e., in some cardiovascular and haematological disorders [329,330]. Different toxins that affect blood circulation have been isolated and characterized from various snake venoms [331-333]. For example, some snake venom molecules that dissolve blood clots both *in vitro* and *in vivo* include afaacytin from horned viper (*Cerastes cerastes*) venom [334], atroxase from western diamondback rattlesnake venom [335] and fibrinogenase from *Vipera lebetina* (*Microvipera lebetina*, EMBL Reptile Database) venom [336]. Weak immunogenic and non-toxic nature as well as anticoagulant property of these three purified RVV-PLA_2s may helpful in therapeutic use in blood coagulation disorders in future; however further study will require for this.

8.7 Assessment for neutralization potency of plant extract in neutralizing the catalytic and pharmacological properties of RVV-PLA_2s

Medicinal plants constitute an effective source of both traditional and modern medicines, herbal medicines have genuine utility and about 80 % of rural population of India depends on it as primary health care.

Neutralization of catalytic and/or pharmacological properties of crude venom and/or any of its toxic constituents such as PLA_2 enzymes by plant extract reflects the presence of anti-snake venom compounds in the plant [337,338]. The result of this present study indicates the presence of anti-PLA_2 compounds in four plants commonly used by traditional medical practitioners against snakebite. The AIPLAI (*A. indica* PLA_2 inhibitor) purified from *A. indica* leave extract [244] and the methanol extract of leaves of *C. sinensis*, *X. strumarium* and roots of *A. marmelos* and these showed significant inhibition of PLA_2 activity of both crude RVV and three purified PLA_2s as well as inhibition of different pharmacological activities of crude RVV and all the three PLA_2s. These extracts also inhibited the tested pharmacological activities of crude RVV and all the three PLA_2s. It is noteworthy to mention that most of the anti-snake venom compounds were isolated from the methanol extracts of plants [239,244]. Our study also supports the presence of anti-PLA_2 compounds in tested plants. Since neutralization of toxic effect of PLA_2 enzymes of snake venom would be a step forward in the management of snakebite cases therefore, anti-PLA_2 compounds may be isolated and purified from the methanolic extracts of these plants. To the best of our knowledge, this is the first report describing the inhibitory activity of these four tested plant extracts against any toxic component of snake venom.

Most of the plant extracts which are reported to possess snake venom neutralizing potential capacity; that may be due to the presence of some phytochemical constituents in those plants. Several chemical constituents like alkaloids, flavonoids, sitosterol or glucoside, lupeol, gymnemagenin, phenolics, pentacyclic triterpenes like oleanoic acid, ursolic, tannins, taraxasterol, α and β amyrin are found to be present in varying proportions in those plants. They have also been previously reported for antisnake venom activity [99]. All these classes of chemical compounds are capable of interacting with macromolecular targets (enzymes or receptors) and can effectively inhibit the toxic effect of snake venoms [339]. The results of the present study confirmed that the plant extracts possess potent Russell's viper venom neutralizing capacity. Therefore, we can assume that some phytochemical constituents may present in these four plant extracts and we will try to isolate and purify those components in our future study. This will definitely be helpful for mankind for overcome the limitations of currently available polyvalent antivenom therapy, particularly in the developing countries.

8.8 Future prospects of study of snake venom phospholipase A_2s

Snake venom PLA_2 enzymes are small enzymes and different isoforms of the same PLA_2 may be present in single venom. They cause havoc by interfering in the normal physiological processes of the victim and different isoenzymes induce a variety of pharmacological effects. They provide a great challenge to protein chemists as subtle and complex puzzles in structure-function relationships. A better understanding of these molecules of snake venom will contribute to our knowledge of protein-protein interactions, protein targeting and protein engineering and to the development of better targeted delivery systems for the treatment of some diseases. Snake venom PLA_2 enzymes exhibit a wide variety of pharmacological effects by their specific interaction with and binding

to target proteins. Strongly anticoagulant PLA_2 enzymes specifically bind to blood coagulation factor Xa and interfere in the formation of the prothrombinase complex. They exhibit their strong anticoagulant effect either by non enzymatic way or, in a combination of both enzymatic and non enzymatic mechanism. The electrostatic interaction with factor Xa is through some specific arrangement of positively charged residues in PLA_2 molecule. Identifying the sites of interaction of the anticoagulants at the level of the specific amino acid residues involved can ultimately lead to the development of peptides and peptide mimetics, which will be useful in the treatment of cardiovascular and cerebrovascular diseases.

These three PLA_2s of RVV attack preferentially different membranes depending on the presence of PLA_2 specific membrane phospholipids domains. Although, the exact nature of the membrane domain(s) responsible for binding with these PLA_2 enzymes could not be identified; however, our study has provided enough evidences in support of membrane domain hypothesis. Further studies to identify the nature of these membrane domains are in progress which will give fairly good indications of the differential mode of attack of snake venom PLA_2s on different membranes.

Further research in identifying the target proteins of PLA_2s, if any, as well as to study the nature of different membrane domains will help us to determine the details of the mechanisms of the different pharmacological effects of these PLA_2 enzymes at the cellular and molecular levels. The studies in these areas will result in new, exciting and innovative opportunities and avenues in the future, both in finding answers to toxicity of PLA_2 enzymes as well as in developing proteins with novel functions.

CONCLUSION

Conclusion

In this present investigation, isoenzyme pattern of phospholipase A_2 enzymes of *Daboia russelli* venom was studied. This study reveals the presence of twelve basic, one neutral and six acidic PLA_2s i.e., a total of 19 PLA_2 enzymes in Russell's viper venom of east India origin.

Three PLA_2 enzymes of different nature (i.e., one acidic, one neutral and one basic in nature) were isolated and purified to homogeneity by combination of ion exchange, gel filtration and RP-HPLC. RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I has a molecular mass of 58.0 kDa (homodimer), 12.8 kDa (monomer) and 6.7 kDa (monomer) respectively and were determined by SDS-PAGE and ESI/MS analysis. The secondary structure of these three PLA_2s showed strong α-helical structure which is well consistent with secondary structure of other snake venom PLA_2 enzymes. Further, heat inactivation study as well as secondary structure of all these three PLA_2s from RVV documented that they are thermostable in nature like most of the earlier reported PLA_2s of snake venom.

The understanding of structure-function relationships in PLA_2 enzymes is complicated, and contradictory results are presented to explain the anticoagulant action of venom PLA_2 enzymes. However, this present study advocates that anticoagulant activity of PLA_2s is mainly attributed to the enzymatic hydrolysis of pro-coagulant phospholipids and partly by binding to the phospholipids of plasma by a non-enzymatic mechanism. Therefore, it may be concluded that strong anticoagulant effect of the PLA_2 under study is contributed by both enzymatic and non-enzymatic mechanisms.

RVVA-PLA_2-I and RVVB-PLA_2-I preferentially hydrolysed phospholipids of erythrocytes membranes compared to liver mitochondrial

membranes whereas in case of RVVN-PLA_2-I this preference was reversed. None of these PLA_2s could hydrolyze HT-29 colon adenocarcinoma cell membrane phospholipids within 4 hours of treatment suggesting a differential mode of attack on membrane phospholipids by these three PLA_2s purified from Russell's viper venom. These PLA_2s show distinct preference for hydrolyzing those phospholipid domain(s) of intact membranes which are specific towards particular PLA_2s.

The GC analysis of saturated/unsaturated fatty acids release pattern from intact mitochondrial and erythrocytes membranes after the addition of RVVA-PLA_2-I and RVVB-PLA_2-I suggested the existence of a significantly greater number of RVVA-PLA_2-I and RVVB-PLA_2-I sensitive domains in erythrocyte membrane as compared to mitochondrion membrane while RVVN-PLA_2-I sensitive domains exist more in mitochondrial membrane than the erythrocytes. Further studies to identify the nature of these PLA_2 sensitive membrane domains are in progress.

None of the PLA_2s in the present study at tested dose showed lethality in mice even after 48 hours injection suggesting that they are devoid of any lethal effects in mice. Moreover, immunodiffusion test documented very week immunogenic nature of RVVA-PLA_2-I, RVVN-PLA_2-I and RVVB-PLA_2-I.

In this study, four medicinal plants which have been used traditionally by the local people of North-East India were screened for anti snake venom effect. Methanol extract of leaves of *A. indica*, *C. sinensis* and *X. Strumarium* and root of *A. marmelos* has showed significant inhibitory activity against crude RVV and also against the three purified phospholipase A_2 (PLA_2) enzymes of *Daboia russelli* venom. AIPLAI (*A. indica* PLA_2 inhibitor) purified from *A. indica* leave extract and also the other three plant extracts are highly promising source for the development of novel anti-snake venom drug in future.

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PUBLICATIONS

Publications in journal

1. **Saikia, D.**, Bordoloi, N.B., Chattopadhyay, P., Chocklingam, S., Ghosh, S.S. & Mukherjee, A.K. Differential mode of attack on membrane phospholipids by an acidic phospholipase A_2 (RVVA-PLA_2-I) from *Daboia russelli* venom. *Biochimica et Biophysica Acta* 1818, 3149-3157, 2012.

2. **Saikia, D.**, Thakur, R. & Mukherjee, A.K. An acidic phospholipase A_2 (RVVA-PLA_2-I) purified from *Daboia russelli* venom exerts its anticoagulant activity by enzymatic hydrolysis of plasma phospholipids and by non-enzymatic inhibition of factor Xa in a phospholipids/Ca^{2+} independent manner. *Toxicon* 57, 841-850, 2011.

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Publications in conference/symposium

1. **Saikia, D.** and Mukherjee, A.K.: "The mechanism of anticoagulant action of a neutral phospholipase A_2 enzyme purified from Russell's viper (*Daboia russelli*) venom of eastern India origin". National conference on toxinology: "Snakebite management", 10-12th December, 2012, Mysore.

2. **Saikia, D.** and Mukherjee, A.K.: "An acidic phospholipase A_2 purified from venom of *Daboia russelli* of eastern India origin exerts its anticoagulant activity by dual mechanism". Proceeding of 1st National conference on animal, microbial, plant toxins and snakebite management, biotoxins in health and disease, 11-12th December, 2010, Kolkata.

3. Das, D., Kalita, R.D., **Saikia, D.**, Mukherjee, A.K. and Doley, R.: "Green medicine for Snakebite". Proceeding of 1st National conference on animal, microbial, plant toxins and snakebite management, biotoxins in health and disease, 11-12th December, 2010, Kolkata.

4. **Saikia, D.** and Mukherjee, A.K.: "Correlation between catalytic activity and membrane phospholipids hydrolysing property of a neutral PLA_2 purified from Indian Russell's viper (*Daboia russelli*) venom". Proceeding of snake symposium, 18-19th December, 2009, Tezpur University.

5. Mukherjee, A.K. and **Saikia, D.**: "Mechanism of anticoagulant action of a neutral PLA_2 purified from Indian Russell's viper (*Daboia russelli)* venom". Proceeding of snake symposium, 18-19th December, 2009, Tezpur University.

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1. **Saikia, D.**, Doley, R. and Mukherjee, A.K. Pharmacological reassessment of three ethnomedicinal plants of North-East India for inhibition of PLA_2 enzymes purified from Indian monocled cobra and Russell's viper venoms.

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Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage www elsevier com/locate/bbamem

Differential mode of attack on membrane phospholipids by an acidic phospholipase A_2 (RVVA-PLA_2-I) from *Daboia russelli* venom

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ARTICLE INFO

Article history
Received 7 April 2012
Received in revised form 20 July 2012
Accepted 8 August 2012
Available online 16 August 2012

Keywords
Acidic phospholipase A
Erythrocyte
HT 29 cell
Membrane damage
Mitochondrion
Russell's viper venom PLA

ABSTRACT

An acidic phospholipase A_2 (RVVA PLA -I) purified from *Daboia russelli* venom demonstrated dose-dependent catalytic, mitochondrial and erythrocyte membrane damaging activities. RVVA PLA -I was non lethal to mice at the tested dose, however it affected the different organs of mice particularly the liver and cardiac tissues as deduced from the enzymatic activities measured in mice serum after injection of this PLA_2 enzyme. RVVA PLA -I preferentially hydrolyzed phospholipids (phosphatidylcholine) of erythrocyte membrane compared to the liver mitochondrial membrane. Interestingly, RVVA PLA_2-I failed to hydrolyze membrane phospholipids of HT 29 (colon adenocarcinoma) cells which contain an abundance of phosphatidylcholine in its outer membrane within 24 h of incubation. The gas chromatographic (GC) analysis of saturated unsaturated fatty acids release patterns from intact mitochondrial and erythrocyte membranes after the addition of RVVA PLA_2-I showed a distinctly different result. The results are certainly a reflection of differences in the outer membrane phospholipid composition of tested membranes owing to which they are hydrolyzed by the venom PLA_2s to a different extent. The chemical modification of essential amino acids present in the active site, neutralization study with polyvalent antivenom and heat inactivation of RVVA PLA -I suggested the correlation between catalytic and membrane damaging activities of this PLA_2 enzyme. Our study advocates that the presence of a large number of PLA_2 sensitive phospholipid domains composition rather than only the phosphatidylcholine (PC) content of that particular membrane may determine the extent of membrane damage by a particular venom PLA_2 enzyme.

1. Introduction

Russell's viper (*Daboia russelli*) is regarded as one of the most medically important venomous snakes in many South-east Asian countries including India [1]. Depending on the zoogeographic origins of Russell's vipers, their venom composition may vary as a result of which envenomation by this snake displays an intriguing variation in the clinical manifestation [2,3].

Russell's viper venom is known to contain different isoforms of phospholipase A_2 (EC 3.1.1.4) that hydrolyzes glycerophospholipids at the *sn*-2 position of the glycerol backbone releasing lysophospholipids and fatty acids [4–6]. PLA_2s from venoms in addition to digestion of prey exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrosis, anticoagulation, hypotension, haemolysis, haemorrhage and oedema inducing activities [7]. Different isoforms of PLA_2, which may be acidic, basic or neutral in nature on the basis of their elution profile from ion exchange columns, can exist in a single venom [3] and each PLA_2 may exert different pathophysiological effects by a wide range of mechanisms in snakebite victims [2,3,6]. Therefore, it remains a challenging task for the scientists to elucidate the structure–function relationships of this class of protein.

Most of the toxic effects of snake venom PLA$_2$ are dependent on the hydrolysis of cellular or sub cellular membrane phospholipids and/or generation of phospholipid hydrolyzed breakdown products which are themselves lytic and can cause considerable membrane damage [7–9]. Kinetic studies of PLA_2 in the scooting mode establish that these enzymes bind to the intact membrane surface as a prelude to loading of the active site with a single phospholipid molecule, more specifically they bind to the phosphatidylcholine (PC) present in the outer leaflet of the membrane for the lipolysis reaction [5,10]. It is now becoming apparent that the different isoforms of venom PLA_2 can display dramatically different affinities for biomembranes composed of different phospholipid polar head groups and fatty acyl

Abbreviations: pBPB, p-bromophenacyl bromide; DTT, Dithiothreitol; FA, Fatty acid; IAA, Iodoacetamide; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; RVV, Russell's viper venom; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone; TPCK, Tosyl phenylalanyl chloromethyl ketone.

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0005-2736/$ – see front matter
http://dx.doi.org/10.1016/j.bbamem.2012.08.005

chains resulting in their differential membrane damaging activity [5 8 11] This specificity of phospholipases has been extensively used to explore the physical structure of phospholipids in biological membranes [12] It is exemplary to mention that only a few reports on membrane hydrolyzing property of PLA s from RVV are available [13 14] Therefore more precise studies are necessary to advance our understanding of the mechanism(s) of membrane damage and subsequent toxicity of PLA$_2$ enzymes from RVV

Perusal of literature has showed that limited attempts have been made to explore the biochemical and pharmacological properties of acidic PLA$_2$ from snake venom Recently we reported the purification and biochemical characterization of an acidic strong anticoagulant PLA$_2$ (RVVA PLA$_2$ I) from Russell s viper venom [15] In this study we are reporting the membrane damaging activity of RVVA PLA$_2$ I from *D russelli* venom Further our study provides an insight into the membrane damaging activity of this acidic PLA$_2$ and suggests that it has a distinct preference for hydrolyzing specific phospholipid domain(s) in intact mitochondrial and erythrocyte membranes whereas its effect on HT 29 adenocarcinoma cells are minimal which may be due to the absence of RVVA PLA$_2$ I sensitive phospholipid domains in HT 29 cells

2. Materials and methods

2 1 Materials

D russelli venom was purchased from Calcutta Snake Park Kolkata CM Sephadex C 50 DEAE Sephadex A 50 and Sephadex G 50 (fine grade) were obtained from Pharmacia Fine Chemicals Sweden All other reagents of analytical grade were purchased from Sigma India The human colon adenocarcinoma (HT 29) cell was procured from National Centre for Cell Sciences (NCCS) Pune India The In Vitro Toxicology Assay Kit (XTT based) was purchased from Sigma Aldrich India Polyvalent antivenom was purchased from Bharat Serum and Vaccines Limited Ambernath The kits for estimation of total protein triglycerides total cholesterol urea uric acid serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were procured from Coral Clinical Systems (Goa India) The kits for the measurement of serum levels of glucose alkaline phosphatase (ALP) and creatine phosphokinase (CPK MB) were obtained from Erba Diagnostics (Mumbai India) whereas the kit for estimation of serum lactate dehydrogenase (LDH) was obtained from Fisher scientific (Mumbai India) The animal food was obtained from Pranav Agrotech Delhi India The RVVA-PLA$_2$ I an acidic PLA$_2$ was purified from crude RVV as described previously [15]

2 2 Methods

2 2 1 Phospholipase A$_2$ activity

For the screening purpose PLA$_2$ activity of crude RVV and RVVA PLA$_2$-I was determined as described by Doley and Mukherjee [16] using egg yolk phospholipids as substrate One unit of PLA$_2$ activity was defined as the amount of protein which produces a decrease in 0 01 absorbance in 10 min at 740 nm To determine the substrate specificity and phospholipid head group preference of RVVA PLA$_2$ I different commercially available phospholipids such as PC PS and PE at a final concentration 1 mM were incubated with 100 nM enzyme at 37 °C for desired time periods and PLA$_2$ activity was assayed by titrametric method using palmitic acid as fatty acid standard [5]

2 2 2 Membrane damaging activity

Isolation of mitochondria from fresh chicken liver at 4 C was described previously [5] For the assay of RVVA PLA$_2$ I induced mitochondrial swelling and membrane damage mitochondrial suspension containing 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissues) in 2 0 ml of assay buffer (20 mM Tris–HCl pH 7 4 containing 0 3 M sucrose) were incubated with 100 nM of RVVA-PLA$_2$-I either in the presence or absence of 2 mM Ca^{2+} at 37 C for the desired time period Mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 520 nm for 30 min [5] One unit of swelling is defined as a decrease in 0 01 absorbance per min of mitochondrial suspension at 520 nm by added RVVA-PLA$_2$-I (100 nM) as compared to control (mitochondrial suspension without RVVA-PLA$_2$-I) under the experimental condition The basic test system without added RVVA-PLA$_2$-I was served as a control Qualitative and quantitative analyses of liberated fatty acids and lysophospholipids from the mitochondrial membranes due to the action of RVVA-PLA$_2$-I were performed by gas-chromatography as described below A control was also set up where the mitochondria were treated under the identical condition except the addition of RVVA PLA$_2$ I

For the estimation of RVVA-PLA$_2$-I induced haemolysis and erythrocyte phospholipid hydrolysis goat blood was collected in 3 8% tri-sodium citrate and erythrocytes were separated by centrifuging the citrated blood at 1000 *g* for 15 min washed twice with isotonic K-phosphate buffer pH 7 4 and suspended in the same buffer at a concentration of 5 % (v v) RVVA PLA$_2$ I at a final concentration of 100 nM was added to 3 0 ml of the above said erythrocyte suspension and the haemolysis and erythrocyte phospholipid hydrolysis by RVVA-PLA -I were measured as described previously [5 8] For determining the role of exogenously added phospholipids in haemolytic activity (indirect haemolytic activity) either PC (final concentration of 1 mM) or egg yolk phospholipids (0 1% v v) were added to the erythrocyte suspension prior to the addition of RVVA PLA$_2$ I The reaction was initiated by the addition of PLA$_2$ followed by measuring the haemolysis as stated above [5] For each of the experiment isotonic saline instead of RVVA PLA$_2$ I was used as a control

Erythrocytes or mitochondria after treatment with RVVA-PLA$_2$-I were centrifuged at 10 000 × g and 1 0 ml of supernatant was used for the extraction and quantification of total lipid and fatty acids from the reaction mixture [5] For the estimation of phospholipids lysophospholipids released from the membrane the total lipid extracted from the supernatant was digested with concentrated HNO_3 in a Kjeldahl flask until a white precipitate was formed and then the liberated Pi was estimated calorimetrically as described by Doley et al [5]

To study the effect of heating on catalytic and membrane damaging activities RVVA-PLA$_2$-I solution (100 nM) was heated at 75 C for different time periods (5 60 min) cooled immediately in ice bath and then the required volume was withdrawn for the spectrofluorometric study assay of catalytic and membrane damaging activities of this enzyme

2 2 3 Gas chromatography analysis of liberated fatty acids from membranes

The total lipid released from the membranes after the treatment of RVVA PLA$_2$ I was extracted and from this total lipid liberated fatty acids were extracted and methylated as described by Mukherjee et al [11] The dry sample (fatty acid methyl esters) was dissolved in a minimum quantity of chloroform and analysed on a GC–MS (Varian 3800 Saturn 2000) system The samples (1 0 µl) were injected using a split ratio of 100 1 into a fused silica GC column CP Sil 8 CB low bleed (30 m × 0 25 mm × 0 25 µm) coupled with a CP-Sil 5 CB low bleed MS (30 m 0 25 mm × 0 25 µm) column with helium as the carrier gas The system was equipped with flame ionization detector The initial oven temperature was 120 °C and a temperature programme of 8 °C per min began at injection and continued to a final oven temperature of 270 °C which was hold isothermal for 3 min The injector port and detector temperature were set at 250 °C The mass spectrometric data were acquired in electron ionization mode (70 eV) The unknown methylated fatty acids were identified by matching both retention time and MS of the unknown compound with those of authentic standards (Saturn 2000 MS library search) The fatty acids were quantitated by measuring and

comparing the GC peak area for released (unknown) fatty acids with the GC chromatogram of standard (known) fatty acids

2.2.4 Modification and neutralization of RVVA PLA 1 with inhibitors and commercial antivenom

For RVVA-PLA_2 I inhibition study different inhibitors such as TPCK TLCK PMSF p-BPB EDTA DTT IAA and commercial polyvalent antivenom raised in horse against crude RVV were pre-incubated with a constant amount of RVVA-PLA -I (100 nM) at 37 °C for 30 min [5,15] The mixture was then assayed for the catalytic and membrane damaging activities of the RVVA-PLA_2-I in the corresponding assay system

2.2.5 Enzyme immunoassay to determine the binding of RVVA PLA 1 with membranes

Supernatant obtained from the incubation of intact mitochondria or erythrocytes with purified RVVA-PLA_2-I (native heated or chemically modified) at 4 °C for 30 min was tested for free (unbound) toxin concentration by ELISA using horse polyclonal antibodies and rabbit anti-horse IgG peroxidase conjugate [5] A standard curve of PLA_2 was plotted by adding graded concentration of RVVA PLA_2 I (30–200 nM per well) in the wells of the ELISA plate and the concentration of unbound RVVA-PLA_2-I was determined from this curve by using an ELISA plate reader (Thermo Electron Corporation Multiskan ascent Type 354) Each experiment was repeated thrice to assure the reproducibility

2.2.6 Measurement of interaction of RVVA-PLA_2-I with PC

The dose-dependent interaction of PC with RVVA-PLA_2 I was measured by using a fluorescence spectrometer [15] Briefly PC was suspended in 20 mM Tris–HCl pH 8.0 buffer at a final concentration of 0.1–1.0 mM and sonicated for about 5 min at 4 °C with a Labsonic® M (Sartorius) sonicator To this 100 nM of RVVA PLA_2 I (dissolved in 20 mM Tris–HCl pH 8.0) was mixed and fluorescence spectra were obtained at an excitation wavelength of 280 nm excitation and emission slits of 5 nm (at room temperature 23 °C) Wavelength shifts were measured by taking the midpoint at two thirds the height of the spectrum The maximum fluorescence of free protein (I_0) was also measured

2.2.7 Cytotoxicity assay on tumour cells

Cytotoxicity was assessed on human adenocarcinoma tumour cell line (HT29) as described by us with the following modifications [8] Briefly human colon adenocarcinoma (HT 29) cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) penicillin (50U ml^{-1}) streptomycin (50 mg ml^{-1}) at 5% CO_2 in a humidified incubator at 37 °C For cytotoxicity assays 1×10^4 cells were seeded in 96 well plate and left overnight for attachment Then crude RVV (10 μg ml^{-1}) or RVVA PLA-I (10 μg ml^{-1} equivalent to 172 nM) diluted in DMEM media were added to the cells and incubated for either 4 h or 24 h Cell viability was measured by using in vitro Toxicology Assay Kit (XTT based) according to the manufacturer's protocol and the percentage viability was calculated as the ratio of treated cells to the control (cells without addition of CRVV/RVVA PLA_2 I) cells Cells were also observed under the inverted phase contrast microscope for any visible morphological changes

2.2.8 Determination of lethality and toxicity on mice

All the BALB/c mice weighed between 20 and 30 g were born in the laboratory breeding colony of the Central Facility of Animal House Defence Research Laboratory Tezpur Assam and were pathogen free General conditions of captivity were maintained in simulated atmospheric conditions of North East India (temperature 33–36 °C relative humidity ~75%) In captivity seasonal variations of physiological functions are entrained by altering six month periods of summer like long photoperiod (14 h of light day) The general conditions of captivity were applied as described above and animals were maintained in social groups before and after experimenting All experimenting protocols using animals were performed according to the Principles of Laboratory Animal care (NIH publication 85-23 revised 1985) and approved by the Institutional Animal Ethical Committee

The acute toxicity was determined as per protocol of OECD OCFD guidelines 425 [17] For toxicity assessment of RVVA-PLA_2-I-in rodents RVVA-PLA_2-I (0.2 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB c mice The mice were assigned as a control group (Group I n=6) and one RVVA-PLA_2-I treated group (Group II n=6) The animals were observed at regular intervals for up to 48 h after the injection for any behavioural changes viz body weight food and water intake faecal and urination grip strength ear twist rectal temperature etc effect on circulatory system and or death Bleeding time along with clotting time was also recorded before sacrificing the animals Control animals (placebo) were injected with 0.1 ml of PBS only

From mice sacrificed after 48 h of injection blood was collected immediately by venipuncture and the serum used for the assay of different parameters viz total protein glucose cholesterol triglycerides uric acid and urea levels as well as different enzymatic activities viz LDH ALP (alkaline phosphatase) CPK (creatine phosphokinase) SGOT and SGPT by using commercial kits following the instructions of manufacturers To study the effect of RVVA PLA_2-I on blood cells RBC and WBC counts were done by an automatic cell counter (Automated haematology cell counter – MS 4(s) Melet Schloesing Laboratories)

2.2.9 Statistical analysis

The statistical analysis of the data was done by Student's *t* test using the software SigmaPlot 11.0 for windows (version 7.0) The value of $p \leq 0.05$ was considered as significant

3 Results

3.1 Effect of RVVA-PLA_2-I on membrane phospholipid hydrolysis

With an increase in incubation time of mitochondria with RVVA-PLA_2-I a significant damage to mitochondria was observed which was further pronounced in the presence of Ca^{2+} Table 1) A deeper insight into the mode of attack of RVVA-PLA_2-I on the mitochondrial membrane was revealed by GC-analysis of liberated fatty acids which showed that straight chain saturated fatty acids such as $C_{16:0}$ $C_{17:0}$ $C_{18:0}$ and $C_{19:0}$ were the most prominent fatty acids released from the membrane within the initial 30 min of attack by RVVA PLA_2 I (Fig 1) Further with an increase in the incubation time of mitochondria with the RVVA-PLA_2-I a corresponding increase in membrane phospholipid hydrolysis was observed (Fig 1)

The RVVA-PLA_2-I at a concentration of 100 nM did not exert any appreciable haemolytic activity directly on washed erythrocytes however exogenous addition of egg yolk phospholipids (which is a source of PC) to the erythrocyte suspension enhanced the haemolytic activity of RVVA PLA_2 I to a significant extent (Table 2) Either with an increase in the concentration of RVVA-PLA_2-I (data not shown) or an increase in the incubation time of PLA_2 with the erythrocytes (Table 2) a concomitant enhancement in the release of fatty acids and measured Pi from the erythrocyte membranes was observed It is worthy to mention that during the initial 30 min of attack haemolysis could not be detected although RVVA-PLA_2 I was able to release the FAs and lysophospholipids from the intact erythrocyte membranes In contrast the addition of egg yolk phospholipids to the erythrocyte suspension resulted in the initiation of haemolysis within 30 min of incubation and after 120 min about 47.6% of total erythrocytes were haemolysed (Table 2)

The GC analysis of erythrocyte membrane phospholipid hydrolysis by RVVA-PLA_2-I had shown a specific preference for releasing the unsaturated straight chain fatty acids such as fatty acids for example

Table 1
RVVA PLA$_2$ I induced swelling and phospholipid hydrolysis of intact mitochondrial membrane either in the presence or in the absence of 2 mM Ca^{2+}. About 100 mg equivalent of mitochondria (mitochondria obtained from 100 mg wet weight of tissue) from chicken liver was incubated with 100 nM of RVVA PLA$_2$ I at 37 °C for different time periods. The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± SD of triplicate determinations.

Incubation time (min)	Mitochondrial swelling (Δ A/min)		Phospholipid hydrolysis				Ratio of saturated/unsaturated FA
			μg FA released		μg of measured Pi		
	−Ca^{2+}	+Ca^{2+}	−Ca^{2+}	+Ca^{2+}	−Ca^{2+}	+Ca^{2+}	
0 (control)	0	0	0	0	0	0	0
15	550 ± 2.5	700 ± 1.1	1329 ± 0.9	1595 ± 0.5	157 ± 2.6	170 ± 1.5	5.1 ± 0.5
30	900 ± 1.2	1150 ± 2.5	7994 ± 1.2	10191 ± 1.3	775 ± 1.9	850 ± 1.2	7.1 ± 0.2
60	1350 ± 1.5	1650 ± 0.5	15038 ± 1.6	19549 ± 1.8	1265 ± 1.3	1634 ± 2.2	8.9 ± 0.8

Mitochondrial swelling was measured spectrophotometrically and one unit of swelling is defined as a decrease in 0.01 absorbance/min of mitochondrial suspension at 520 nm by 100 nM of RVVA PLA$_2$ I.

$C_{18:1\text{-}OH}$ and $C_{18:0\text{-}OH}$ from the intact erythrocyte membrane after 30 min of incubation. This result corroborates well with our previous report showing preferential release of unsaturated fatty acids from the erythrocyte membrane during the initial stage of attack by a PLA_2 (NK PLA_2 I) from *N. kaouthia* venom [18]. However, after 120 min of incubation of erythrocytes with RVVA-PLA_2-I additional saturated FAs of chain lengths $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{19:0}$, $C_{20:0}$ and $C_{22:0}$ and unsaturated fatty acid such as $C_{18:0}$ were detected. Nevertheless a few saturated and unsaturated fatty acids (C_{20}) liberated from the membrane due to action of RVVA-PLA_2-I could not be identified (Fig. 2). The TLC analysis of phospholipids and lysophospholipids released from the erythrocyte or mitochondrial membrane after the treatment with RVVA PLA$_2$ I demonstrated the release of most of the FAs from the membrane PC pools (Supplementary Fig. S1).

A comparison of ratios of saturated/unsaturated fatty acids released from the intact mitochondrial membrane (Table 1) after the addition of RVVA PLA_2 I suggested that the enzyme has a distinct preference for hydrolyzing phospholipids containing saturated fatty acids at *sn*-2 position in mitochondrial membrane. But in sharp contrast during the initial phase of attack this acidic PLA_2 prefers hydrolysis of unsaturated fatty acids in the erythrocyte membrane (Table 2). Therefore the ratio of saturated/unsaturated fatty acids released from the erythrocyte membrane was less than 1.0 for up to 30 min of incubation with RVVA-PLA_2-I. Increase in incubation time beyond 30 min resulted in a change in RVVA-PLA_2-I induced fatty acids release pattern from the erythrocyte membrane owing to a greater hydrolysis of phospholipids containing saturated fatty acids compared to unsaturated fatty acids at sn-2 position (Table 2).

3.2 Effect of inhibitors and antivenom on catalytic and membrane damaging activities

Different irreversible modifiers of serine residue viz. TPCK (inhibitor of chymotrypsin like serine protease), TLCK (inhibitor of trypsin-like serine protease) and PMSF (inhibitor of serine proteases) at 2.0 mM concentration did not affect the catalytic and membrane damaging

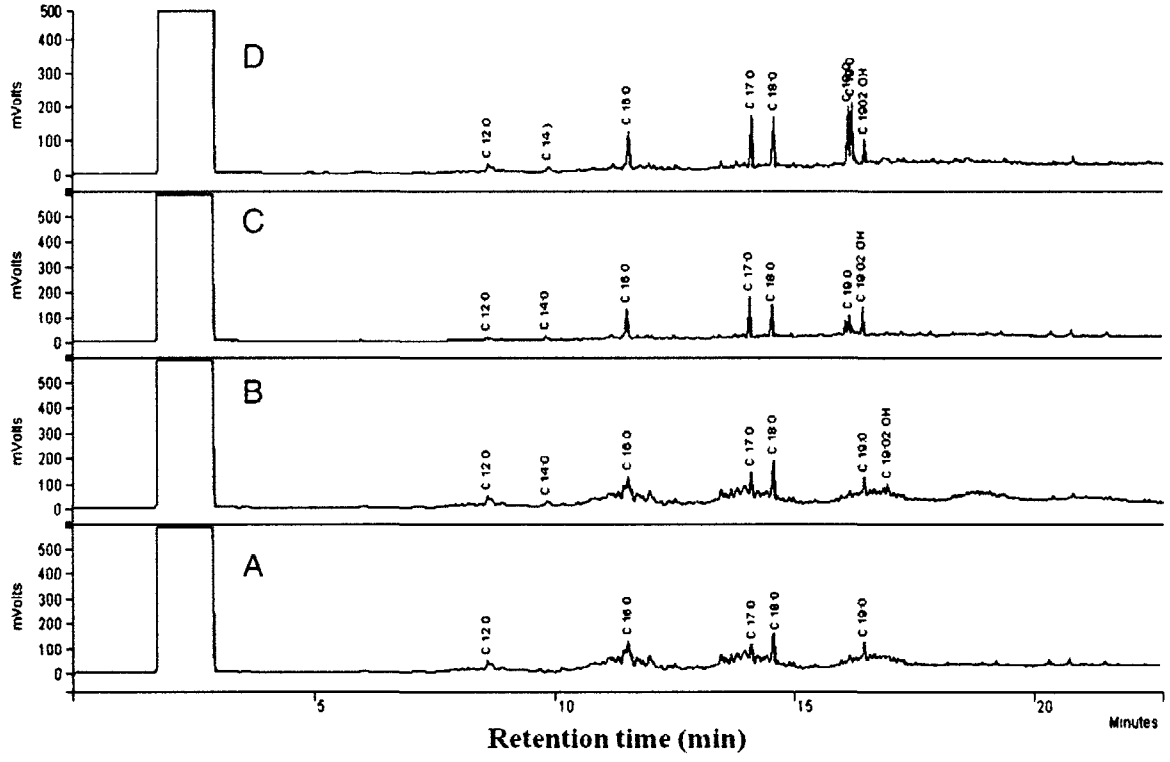

Fig. 1 Kinetics of mitochondrial membrane phospholipid hydrolysis by RVVA PLA$_2$ I. About 100 mg equivalents of liver mitochondria (mitochondria obtained from 100 mg wet weight of tissue) were incubated with 100 nM of RVVA PLA$_2$ I (in the presence of 2 mM Ca^{2+}) at 37 °C for different time periods. The liberated fatty acids were analysed by GC–MS as described in the text. A) control, B) 15 min, C) 30 min and D) 60 min after incubation of mitochondrial membranes with RVVA PLA$_2$ I.

Table 2
RVVA PLA_2-I induced haemolysis and phospholipid hydrolysis of goat washed erythrocytes. The 5% (v/v) erythrocyte suspension was incubated with 100 nM of RVVA PLA_2-I at 37 °C and haemolysis (direct and indirect) and erythrocyte phospholipid hydrolysis were determined. Indirect phospholipid hydrolysis was achieved in the presence of egg yolk phospholipids which is a source of PC. The measured Pi value was obtained from acid treatment of a sample of the supernatant. Values are mean ± S.D. of triplicate determinations.

Incubation time (min)	% haemolysis		Phospholipid hydrolysis		Ratio of saturated/unsaturated FA
	Direct	Indirect	μg FA released	μg of measured Pi	
Control	0	0	0	0	0
15 min	0	0	202.5 ± 2.1	28.4 ± 0.9	0.68 ± 0.3
30 min	0	34.1 ± 0.45	1015.3 ± 2.5	163.2 ± 1.2	0.86 ± 0.4
60 min	0.6 ± 0.03	42.1 ± 0.45	2045.1 ± 1.4	253.2 ± 1.7	1.64 ± 0.8
120 min	1.9 ± 0.09	47.6 ± 0.12	2531.9 ± 1.9	302.3 ± 1.1	1.91 ± 0.1

activities of RVVA-PLA_2-I. Therefore based on our experimental result we propose that serine residue perhaps does not have a contribution to the above mentioned activities of RVVA-PLA_2-I (Table 3). However the modification of enzyme with pBPB an inhibitor of histidine residue, drastically reduced the catalytic as well as the membrane damaging activities of RVVA-PLA_2-I indicating a correlation between catalytic and membrane damaging activities of this enzyme (Table 3). Furthermore, as shown in Table 3, EDTA significantly inhibited the catalytic and membrane phospholipid hydrolyzing activity of RVVA-PLA_2-I probably by chelating the Ca^{2+} ion required for the enzymatic activity [15]. The observed significant reduction (37–44%) in catalytic and membrane damaging activities of RVVA-PLA_2-I post treatment with DTT under the experimental condition was due to partial reduction of disulfide bonds present in this PLA_2 molecule. Presence of 7–8 intra-molecular disulfide bridges has been reported in PLA_2 molecules from snake venom [5–8]. Furthermore, IAA (a sulfhydryl-reactive alkylating reagent used to modify the cysteine residue) at the tested concentrations inhibited the catalytic and membrane damaging activities of RVVA-PLA_2-I almost to the same extent which may be due to the modification of cysteine residues of this PLA_2 responsible for the intramolecular disulfide bond formation (Table 3).

3.3 Binding with phospholipids

The spectrofluorometric study revealed the concentration dependent binding of PC with RVVA-PLA_2-I (Supplementary Fig. S2). Binding efficiency of the heat-inactivated RVVA-PLA_2-I had drastically reduced (69%) compared to the PC binding potency of native RVVA-PLA_2-I (Table 3 and Supplementary Fig. S3). In the present study RVVA-PLA_2-I showed equal binding affinity for both mitochondrial and erythrocyte membranes (data not shown). The membrane binding property of heat-inactivated RVVA-PLA_2-I, as evaluated by the ELISA experiment, revealed the binding of about 65 ± 2% (mean ± S.D., n = 3) of heated RVVA-PLA_2-I (heated for 60 min at 75 °C) to intact erythrocyte or mitochondrial membranes as compared to 100% binding of native (unheated) RVVA-PLA_2-I. This suggested the partial loss of membrane binding property of heat-inactivated enzyme. In contrast histidine-modified RVVA-PLA_2-I was captured by intact erythrocytes and mitochondria to the

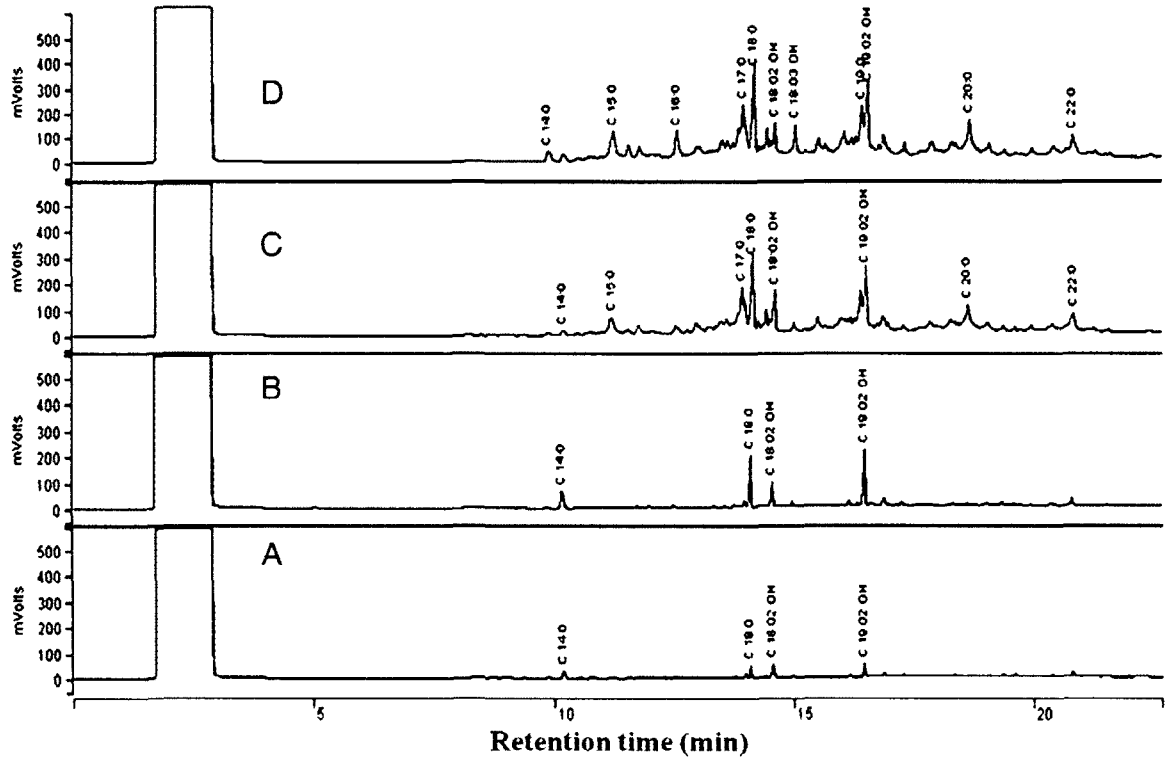

Fig. 2 Kinetics of erythrocyte membrane phospholipid hydrolysis by RVVA PLA_2-I. The 5% v/v erythrocyte suspension was incubated with 100 nM of RVVA PLA_2-I (in the presence of 2 mM Ca^{2+}) at 37 °C for different time periods. The liberated fatty acids were analysed by GC–MS as described in the text. A) control, B) 30 min, C) 60 min and D) 120 min after incubation of erythrocyte membranes with RVVA PLA_2-I.

Table 3
Effects of heating, antivenom and inhibitors (at a final concentration of 2 mM) on catalytic and membrane phospholipid hydrolytic activities of RVVA-PLA$_2$-I (100 nM). Values are mean ± S.D. of triplicate determinations. MM: mitochondrial membrane, EM: erythrocyte membrane.

	% residual activity			Binding with PC (%)
	PLA$_2$	MM hydrolysis	EM hydrolysis	
Control	100	100	100	100
Chemicals/inhibitors	97.8 ± 1.9	96.0 ± 1.8	96.5 ± 4.7	-
TPCK	94.9 ± 1.7	97.2 ± 1.9	100	-
TLCK	9.7 ± 0.5	12.1 ± 0.8	18.5 ± 0.9	-
pBPB	97.6 ± 1.8	97.7 ± 1.8	95.2 ± 1.7	97.0 ± 1.3
PMSF	63.0 ± 3.1	55.5 ± 1.7	61.5 ± 1.7	
DTT	56.7 ± 2.8	60.1 ± 2.0	56.6 ± 2.8	
IAA	20.6 ± 1.5	23.2 ± 1.8	25.9 ± 2.1	-
EDTA				
Antigen: antivenom (w/w)				
1:100	90.1 ± 1.5	89.9 ± 0.5	85.8 ± 1.3	-
1:200	84.4 ± 1.2	76.8 ± 1.8	66.7 ± 2.3	-
1:500	75.4 ± 0.8	55.3 ± 1.7	51.6 ± 2.6	-
Heating at 75 °C				
10 min	97.0 ± 1.8	97.5 ± 0.8	95.9 ± 1.7	97.5 ± 1.8
20 min	94.5 ± 1.7	95.1 ± 1.7	91.8 ± 1.6	91.4 ± 1.6
30 min	90.5 ± 1.5	89.0 ± 0.5	88.5 ± 2.4	86.6 ± 2.3
45 min	85.4 ± 2.3	79.4 ± 1.9	74.4 ± 0.7	75.1 ± 0.8
60 min	70.8 ± 1.5	69.5 ± 1.4	67.2 ± 1.3	69.0 ± 1.4

same extent ($p > 0.05$) as that of the native RVA-PLA$_2$-I, implying that the modification of histidine residue(s) of RVVA-PLA$_2$-I did not affect its membrane binding property (Table 3).

3.4. Cytotoxicity assay

The cytotoxicity assay showed that the RVVA-PLA$_2$-I did not have any cytotoxic effect on HT-29 cells at the tested doses. RVVA-PLA$_2$-I at a dose of 10 μg ml^{-1} (final concentration of 172 nM) was not detrimental to HT-29 cells after 24 h of treatment (data not shown). In contrast, crude RVV at the same dose (10 μg ml^{-1}) killed more than 18% of cells after 4 h of treatment and almost all the HT-29 cells were dead after 24 h of treatment (data not shown). The inverted phase contrast microscopic observation of RVVA-PLA$_2$-I treated HT-29 cells did not reveal any gross morphological changes after 24 h of treatment (data not shown). Analysis of cell membrane phospholipid hydrolysis revealed that the RVVA-PLA$_2$-I failed to hydrolyze the membrane phospholipids of HT-29 cells after 4 h of treatment and about 350 ± 19 μg (mean ± S.D., n = 3) of FAs was released after 24 h of treatment. This suggests that the amount of phospholipids hydrolyzed by RVVA-PLA$_2$-I in treated HT-29 cells was significantly less ($p < 0.001$) as compared to the phospholipids hydrolyzed in the mitochondrial and erythrocyte membranes.

3.5. In-vivo toxicity assay

Mortality or behavioural changes were not observed in mice after 48 h i.p. injection of RVVA-PLA$_2$-I at a dose of 0.2 mg kg^{-1} body weight. However, RVVA-PLA$_2$-I prolonged the bleeding time and the coagulation time of PPP in RVVA-PLA$_2$-I treated mice as compared to the control mice (data not shown). After the treatment of mice with RVVA-PLA$_2$-I, no changes were observed in WBC count. Nevertheless, there was a significant decrease ($p < 0.01$) in the RBC count and the Hb content in the blood of treated mice as compared to the control mice (data not shown) suggesting RVVA-PLA$_2$-I caused lysis of erythrocytes in in-vivo condition.

Table 4 shows the effect of RVVA-PLA$_2$-I on serum parameters of treated mice. Administration of RVVA-PLA$_2$-I resulted in a significant increase ($p < 0.05$) in serum enzymes such as ALP, CPK-MB, SGOT, SGPT, and triglycerides. On the other hand, the serum level of LDH was significantly decreased in RVVA-PLA$_2$-I treated mice as compared to control. However, there was no change in the cholesterol and glucose levels in the serum of RVVA-PLA$_2$-I treated mice as compared to control mice (Table 4).

4. Discussion

4.1. Correlation between catalytic activity and membrane damaging activity of RVVA-PLA$_2$-I

Lysis of artificial membranes by PLA$_2$s from various snake venoms e.g., *Bothrops pirajai* and *Crotalus durissus terrificus* has been shown to be independent of their enzymatic activity, since both native and catalytically inactivated enzymes are fully able to disrupt membranes [19]. Conversely, we have presented contradictory evidences showing that mitochondrial and erythrocyte membrane hydrolyzing properties of Russell's viper acidic PLA$_2$ (RVVA-PLA$_2$-I) are dependent on its catalytic activity which is similar to membrane damaging activity of PLA$_2$ enzymes (NK-PLA$_2$-I and NK-PLA$_2$-II) isolated from *Naja kaouthia* venom [5]. The current investigation shows that the different membranes studied in the present investigation have different structural organisations as reflected by their distinctly different sensitivities towards RVVA-PLA$_2$-I. Nonetheless, this membrane damaging activity is dependent on Ca^{2+} ion because in the presence of the metal chelator EDTA, RVVA-PLA$_2$-I lost its membrane damaging as well as its catalytic activity in a parallel manner. The degree of membrane phospholipid hydrolysis increases with the increase in the pre-incubation time of the membranes

Table 4
Effect of RVVA-PLA$_2$-I on different parameters of serum of albino mice. RVVA-PLA$_2$-I (0.2 mg kg^{-1} body weight) dissolved in 0.1 ml of PBS was injected intraperitoneally into a group of six BALB c mice (n = 6). Mice were sacrificed after 48 h of injection, blood was collected immediately by venipuncture and the serum was used for the assay of different parameters. Values are mean ± S.D. of six determinations.

	Total protein (g/L)	Glucose (g/L)	CPK-MB (U/L)	LDH (U/L)	ALP (U/L)	SGOT (U/L)	SGPT (U/L)	Cholesterol (g/L)	Triglycerides (g/L)
Control	270.0 ± 0.7	2.11 ± 1.2	27.5 ± 0.65	1281.5 ± 0.9	27.5 ± 0.8	144.0 ± 2.2	50.0 ± 0.91	0.7 ± 0.4	0.2 ± 0.04
Treated	180.5 ± 0.5[a]	2.53 ± 1.5	303.0 ± 1.6[a]	1054.5 ± 1.1[a]	79.3 ± 1.2[a]	191.7 ± 2.6[a]	135.0 ± 1.4[a]	0.8 ± 0.3	1.0 ± 0.05[a]

Significance of difference
[a] $p < 0.001$

with RVVA-PLA$_2$-I documenting that the membrane damage is partially dependent on the catalytic activity of RVVA-PLA$_2$-I

The indirect haemolysis exhibited by RVVA PLA$_2$ I was due to the formation of phospholipid hydrolysis products like lysophospholipids and FAs from egg yolk phospholipids/PC and these products caused further damage to the membrane [20] The same hypothesis can be put forward to explain the enhanced susceptibility of the erythrocytes in the presence of egg yolk phospholipids PC and Ca^{2+} because amongst the tested phospholipids PC is the most favoured substrate for RVVA-PLA$_2$ I for binding followed by hydrolysis [15] The spectroflurometric study has also shown that the presence of Ca^{2+} enhanced the binding of RVVA-PLA$_2$-I with PC [15] confirming this is a Ca^{2+} -dependent PLA$_2$ Furthermore inhibitor studies with specific amino acid modifying reagents neutralization studies with polyvalent antivenom and the heat inactivation data in the present study have suggested a correlation between the catalytic and membrane phospholipid hydrolysis properties of RVVA-PLA$_2$-I However there is enough controversy regarding the role of enzymatic activity in the pharmacological effects of snake venom PLA$_2$ enzymes and it may be suggested that both enzymatic and non-enzymatic processes have contributed in the pharmacological process [5 8 15]

The heat-inactivation study also leads us to conclude that the binding of RVVA-PLA$_2$ I to membrane PC followed by its hydrolysis is an essential step for inducing membrane damage [5 6] because a parallel inhibition of membrane PC binding catalytic activity as well as membrane damage in case of heat-inactivated RVVA PLA$_2$-I was observed owing to the fact that heat treatment partially distorted PLA$_2$ structure (Supplementary Fig S3) This partial unfolding of RVVA PLA$_2$ I could not be regained after cooling (Saikia D and Mukherjee A K unpublished observation) resulting in a reduction in PC binding property and activity of heat-denatured RVVA-PLA$_2$ I

4 2 Differential hydrolysis of mitochondrial and erythrocyte membrane phospholipids does it reflect the differences in PLA sensitive phospholipid composition of biomembranes?

Membrane surface properties including membrane fluidity curvature surface charge and membrane induced structural changes in the enzyme determine the strength of interaction cooperatively of membrane binding, and the extent of PLA$_2$ activation [5 8] Snake venom PLA$_2$ induced injury to mitochondria brings about a change in the mitochondrial volume [5 21] The preferential release of C_{16} to C_{18} FAs from the mitochondrial and erythrocyte membranes during the initial phase of attack by RVVA PLA I is correlated with its preference for short chain fatty acids at the *sn 2* position It is presumed that even if PLA$_2$ binds uniformly across the solid and fluid domains of membrane enriched in the long and short chain fatty acids respectively enzyme shows a preference for short chain fatty acids which are in fluid phase [22] This is due to the lower surface area density of the lipid head groups in short chain fatty acids that are present in fluid phase [23]

Our previous study has shown that RVVA PLA$_2$ I preferentially hydrolyzes PC over PS and PE [15] It is very interesting to observe that RVVA PLA$_2$ I preferentially hydrolyzes the phospholipids of erythrocyte membrane compared to mitochondrial membrane even though the latter possesses much greater percentage of PC on its outer leaflet (40 9%) than the outer leaflet of the former membrane (19%) [24] Therefore our result suggests that existence of significantly greater number of RVVA PLA$_2$ I sensitive regions in the erythrocyte membrane as compared to the mitochondrial membrane might have a relevance to the higher degree of phospholipid hydrolysis of the former membrane [5 8] These specific domains or venom PLA$_2$ susceptible region(s) may result from the presence of different fatty acids more particularly the short-chain fatty acids in these regions of the membrane Therefore the presence of a large number of venom PLA$_2$-sensitive phospholipid compositions rather than only PC content of that particular membrane may determine the extent of membrane damage induced by a particular PLA$_2$ [8]

Furthermore RVVA PLA$_2$ I could hydrolyze the erythrocyte membrane phospholipids within 15 min of incubation which is in sharp contrast to our previous observation where two phospholipase A$_2$ enzymes *viz* NK-PLA$_2$-I and NK-PLA$_2$-II from *N kaouthia* venom showed a lag phase for binding with the erythrocyte membrane and subsequent membrane hydrolysis [5] Besides *N kaouthia* PLA$_2$ enzymes preferentially hydrolyze the membrane phospholipids of the mitochondria as compared to that of the erythrocyte membranes [5] It is interesting to note that all these three PLA$_2$s from snake venom have shown a preference for hydrolyzing PC over PS or PE [5 13] however NK PLA$_2$-I and NK PLA$_2$ II from *N kaouthia* venom are basic in nature [5] whereas RVVA PLA$_2$-I from *D russelli* venom is an acidic PLA$_2$ enzyme Therefore the differences in overall net charge in a venom PLA$_2$ molecule may attribute to differential binding and subsequent hydrolysis of phospholipids of a particular membrane [12]

Evidence has been accumulating regarding the presence of nanometer range small-scale structures and lipid domains in the lipid bilayer and such organizational heterogeneity of lipid microdomains may have structural and functional significance [25 27] The phospholipid-binding domains with secondary binding sites for specific proteins are well known and this binding is often tightly regulated [28] The differences in the membrane phospholipid hydrolysis by a venom PLA$_2$ can be supported by our previous observation that the phospholipid constituents of microsomal membranes are less hydrolyzed as compared to those of lysosomal membranes by the action of *Vipera russelli* venom basic PLA [13 14] Further it has been suggested that PLA$_2$ may be particularly active at domain interfaces which are the sites of structural defects and hence good points of attack for these enzymes [5] The mechanism by which snake venom phospholipase A$_2$ sensitive phospholipid domains is formed in the mitochondrial or erythrocyte membrane is not very clear Although no adequate description of the nature of such membrane lipid domains in terms of their abundance composition or dynamics has been provided however it may be assumed that the physicochemical properties of individual phospholipids circumstantial effects of membrane proteins phospholipid cholesterol ratio and vitamin E content of the membrane contribute significantly to the formation of such domains [5 8 14 27]

The difference in the release of saturated and unsaturated fatty acids from the membranes may be explained by the fact that in the case of mitochondrial membrane RVVA PLA$_2$ I preferentially binds to one of the PLA$_2$-sensitive domains of the membrane resulting in quantitative increase in the release of fatty acids from that membrane with respect to time [5] In a contrast RVVA-PLA$_2$-I attacks different phospholipid domains/region(s) of erythrocyte membrane and therefore this kinetics of erythrocyte membrane phospholipid hydrolysis favours the release of quantitatively as well as qualitatively different FAs with respect to time The ratio of saturated/unsaturated fatty acids released from the erythrocyte membrane may lead us to assume that during the initial phase of attack RVVA PLA$_2$-I hydrolyzes a particular domain of erythrocyte membrane consisting of mostly the unsaturated fatty acids Slowly with an increase in time the reaction products build up which may help in PLA$_2$ binding and in attacking another domain of the same membrane enriched in more saturated fatty acids rather than unsaturated fatty acids Interestingly this result contradicts with the finding of Shukla and Hanahan [12] showing the acidic PLA$_2$ purified from the venom of *Agkistrodon halys blomhoffi* hydrolyzing only one domain of PC in intact erythrocytes These differences in erythrocyte membrane phospholipid hydrolysis pattern of acidic PLA$_2$s from two different snake venoms lead us to conclude that not only the overall acidic charge but also the charge at a particular region known as interfacial binding surface (IBS) of PLA$_2$ may determine its competence for binding with different domains of a membrane [29] This reinforces the presence of venom PLA$_2$-specific sensitive regions in a particular membrane It may be presumed that

by virtue of possessing arrays of phospholipase A_2 isoenzymes, injected venom can induce greater damage to membranes and subsequent toxicity to the cells.

4.3. Why does RVVA-PLA$_2$-I fail to hydrolyze HT-29 cell membrane phospholipids?

The RVVA-PLA$_2$-I at the tested dose (10 μg ml^{-1}) did not show any cytotoxicity against HT29 colon adenocarcinoma cells even though PC is the most abundant phospholipids present in the outer cell membrane of HT-29 cells [30]. Lomonte et al. [31] have shown the cytotoxic effect of Myotoxin II from *Bothrops asper* on HT-29 cells at a dose of 50–100 μg ml^{-1} (micromolar range) whereas we have examined the cytotoxicity at a dose of 10 μg ml^{-1} (nanomolar concentration) to avoid any non-specific binding of PLA_2 to cultured cells [5]. Furthermore, considering the proportion of RVVA-PLA$_2$-I present in RVV, we did not perform cytotoxicity at a very high dose of this PLA_2. Amongst the tested membranes, the least hydrolysis of HT-29 cells can again be explained on the basis that it is not the overall quantity of PC in a membrane but either the availability of PC in a PLA_2-sensitive membrane and/or physicochemical properties of a membrane are the most important criteria in order to elicit the RVVA-PLA$_2$-I-induced membrane damage [8]. It has been observed that some of the components of the membrane such as cholesterol/phospholipid ratio and vitamin E (α-tocopherol) content may influence its fluidity which in turn modulates the activity of venom PLA_2 enzymes [14,32].

4.4. The RVVA-PLA$_2$-I is non-lethal but affects the liver and cardiac tissues of experimental animals

RVVA-PLA$_2$-I (at a dose of 0.2 mg kg^{-1} i.p.) does not show any mortality to the mice even after 48 h of injection suggesting that it is devoid of any lethal effects in mice. The RVVA-PLA$_2$-I represents 0.1% (w/w) of total RVV protein [13] and on average an adult Russell's viper may inject 225–250 mg of venom (total amount in a bite) to its victim (personal communication from Mr. D. Mitra, in-charge, Calcutta Snake Park, Kolkata). Therefore, a maximum of 0.2 to 0.25 mg of RVVA-PLA$_2$-I would be injected in a victim by the bite of an adult Russell's viper and this dose corresponds to the nanomolar concentration of RVVA-PLA$_2$-I in the blood of an adult victim. Since RVVA-PLA$_2$-I was found to be non-toxic to mice at a much higher dose than this, therefore, it may be suggested that the PLA$_2$ under study does not contribute to lethality of RVV. It is noteworthy to mention that non-toxic PLA_2 enzymes from snake venom demonstrating phospholipid hydrolysis activity have been reported and the exact contribution of these PLA_2s in snake venom is poorly understood [16,33]. However, evidence has been presented from our laboratory to show that non-covalent interaction of relatively non-toxic PLA_2s from *N. kaouthia* venom with that of weak neurotoxin like molecules (kaouthitoxins) from the same venom resulted in marked synergism to potentiate the cytotoxicity of PLA_2–kaouthiotoxin complex without altering the biological properties of PLA_2 enzymes [19]. The proteome analysis of *D. russelli siamensis* venom revealed the presence of complex of PLA_2 with other unidentified venom proteins [34]. Presence of such PLA_2-interacting component(s) from *D. russelli russelli* venom remains to be elucidated.

Increase in the level of most of the tested enzymes in the serum of RVVA-PLA$_2$-I treated mice suggests that it might be affecting different organs, particularly the liver and cardiac tissues of mice. Every living cell contains its complement of enzymes, most of which are intracellular and are released into the circulation only after breakdown of the cell or impairment of its membrane post-envenomation [2]. Elevated CPK-MB level suggested damage to the skeletal muscle and is indicative of myonecrosis, whereas increased SGOT, SGPT and ALP levels in the serum of treated mice indicated damage to the liver tissues reinforcing RVVA-PLA$_2$-I targets cardiac and hepatic tissues.

5. Conclusion

In conclusion, RVVA-PLA$_2$-I preferentially hydrolyzed phospholipids of erythrocyte membranes compared to liver mitochondrial membranes. Interestingly, this PLA_2 could not hydrolyze HT-29 colon adenocarcinoma cell membrane phospholipids after 24 h of treatment suggesting a differential mode of attack on membrane phospholipids by RVVA-PLA$_2$-I. The GC analysis of saturated/unsaturated fatty acid release pattern from intact mitochondrial and erythrocyte membranes after the addition of RVVA-PLA$_2$-I suggested the existence of a significantly greater number of RVVA-PLA$_2$-I sensitive domains in the erythrocyte membrane as compared to the mitochondrial membrane. Although, the exact nature of the membrane domain(s) responsible for binding with this acidic PLA_2 from Russell's viper could not be identified; however, our study has provided enough evidences in support of membrane domain hypothesis. Further studies to identify the nature of these membrane domains are in progress.

Acknowledgements

Authors thank Basabi Gogoi, Department of English and Foreign Languages, Tezpur University, for editing the manuscript. Ms. D. Saikia and Dr. N. Bordoloi were recipients of CSIR Senior Research Fellowship and Institutional fellowship, respectively. This work was supported partially by Research Grants to A.K.M. from the University Grants Commission (UGC), New Delhi.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.08.005.

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Toxicon 57 (2011) 841–850

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

An acidic phospholipase A_2 (RVVA-PLA_2-I) purified from *Daboia russelli* venom exerts its anticoagulant activity by enzymatic hydrolysis of plasma phospholipids and by non-enzymatic inhibition of factor Xa in a phospholipids/Ca^{2+} independent manner

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ARTICLE INFO

Article history:
Received 25 December 2010
Received in revised form 12 February 2011
Accepted 15 February 2011
Available online 26 February 2011

Keywords:
Anticoagulant activity
Acidic phospholipase A_2
Factor Xa inhibition
Phospholipids hydrolysis
Inhibition of prothrombin activation
Russell's viper venom

ABSTRACT

A homodimeric acidic PLA_2 (RVVA-PLA_2-I) of 58.0 kDa molecular weight purified from Russell's viper (*Daboia russelli*) venom demonstrated dose-dependent catalytic, strong anticoagulant and indirect hemolytic activities and inhibited blood coagulation cascade in both enzymatic and non-enzymatic mechanisms. In *in vitro* condition, RVVA-PLA_2-I showed preferential hydrolysis of phosphatidylcholine with a K_m and V_{max} values of 0.65 mM and 28.9 μmol min^{-1}, respectively. Biochemical study and GC-analysis of plasma phospholipids hydrolysis by PLA_2 revealed that anticoagulant activity of RVVA-PLA_2-I was partly attributed by the enzymatic hydrolysis of pro-coagulant phospholipids PC, followed by PS. The spectrofluorometric and gel-filtration analyses documented binding of RVVA-PLA_2-I with activated factor *X* and PC; however, it does not bind with factor Va, prothrombin and thrombin. Therefore, this anticoagulant PLA_2 inhibits the blood coagulation cascade non-enzymatically by binding with coagulation factor Xa, even in the absence of phospholipids and Ca^{2-} and thus slows down the blood coagulation by partially inhibiting the prothrombin activation. Chemical modification of essential amino acids present in the active site, neutralization with *Azadirachta indica* leaves extract (AIPLAI) and heat-inactivation study reinforce the association of catalytic and anticoagulant activity of RVVA-PLA_2-I and also throw a light on its non-enzymatic mechanism of anticoagulant action.

1. Introduction

Russell's viper (*Daboia russelli*), one of the major venomous snakes found in Indian subcontinent, is responsible for a large number of snakebite morbidity and mortality (Mukherjee et al., 2000). Phospholipase A_2 (EC 3.1.1.4) is one of the major components of Russell's viper venom that hydrolyzes glycerophospholipids at the sn-2 position of the glycerol backbone releasing lysophospholipids and fatty acids (Dennis, 1983; Doley and Mukherjee, 2003). In addition to the digestion of prey, venom PLA_2 exhibits wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, hemorrhage and edema inducing activities (Soares et al., 2001; Murakami and Arni, 2003; Mukherjee, 2007; Samy et al., 2007). A single venom including RVV may contain several isoforms of PLA_2 which are acidic, basic or, neutral in nature (Jayanthi and Gowda, 1988), and each PLA_2 may exert different pathophysiological effects by different mechanisms in snakebite victims (Mukherjee et al., 2000; Kini, 2006). However, studies have presented contradictory data showing pharmacological properties of PLA_2 may be dependent,

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0041-0101/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2011.02.018

independent or partially dependent on its catalytic activity (Kini and Evans, 1989, Doley and Mukherjee, 2003, Doley et al, 2004, Mukherjee, 2007) Therefore, this remains a challenging task for the scientists to elucidate the structure–function relationships in this class of protein

Circulatory system is one of the physiological systems targeted by anticoagulant PLA_2s from snake venom (Lima et al, 2005, Doley and Mukherjee, 2003) Most of the death from Russell's viper envenomation in eastern India is attributed to prolonged blood coagulation time of victims (Mukherjee et al, 2000) 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_2s could be the primary mechanism to account for their anticoagulant effect (Kini, 2006, Mukherjee, 2007) It is worthy to mention that mechanism of anticoagulant action of PLA_2 from RVV, particularly the acidic PLA_2 has still remained obscured 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 (Schoni, 2005, Kini, 2006)

Literature survey documents that limited attempts have been made to characterize biochemical and pharmacological properties of acidic PLA_2s from Russell's viper venom Our preliminary study has shown that anticoagulant acidic PLA_2 isoenzymes comprise about 32% of total PLA_2 present in RVV of eastern India origin (Saikia, D and Mukherjee, A K, unpublished observation) Therefore, it is likely that this group of PLA_2 may contribute significantly in the overall toxicity of RVV In this study we report for the first time the biochemical properties of a high molecular weight potent anticoagulant acidic PLA_2 from *Daboia russelli* venom Further, our study provides an insight into the mechanism of anticoagulant activity of this acidic PLA_2, and suggests this PLA_2 exerts its anticoagulant action in both enzymatic and non-enzymatic mechanisms

2. Materials and methods

2.1 Materials

Venom of *Daboia russelli* was obtained from Calcutta Snake Park, Kolkata CM Sephadex C-50, DEAE Sephadex A-50 and Sephadex G-50 (fine grade) were obtained from Pharmacia Fine Chemicals, Sweden The thrombin and prothrombin were obtained from Himedia, India and Merck India, respectively All other reagents of analytical grade were purchased from Sigma–Aldrich, USA

2.2 Isolation and purification of an acidic anticoagulant PLA_2

Crude *Daboia russelli* venom (20 mg protein) was dissolved in 2.0 ml of 20 mM K-phosphate buffer, pH 7.0 and centrifuged at 10,000 × g for 5 min in an MPW-350 centrifuge The clear supernatant was applied to a CM Sephadex C-50 (20 mm × 60 mm) column pre-equilibrated with 20 mM K-phosphate buffer, pH 7.0 and the column was washed with two volumes of elution buffer to elute the non-bound proteins This flow-through fraction was applied to a DEAE Sephadex A-50 (20 mm × 60 mm) column previously equilibrated with 20 mM K-phosphate buffer, pH 7.0 Elution was carried at room temperature (~23 °C) at a flow rate of 24 ml h^{-1} with K-phosphate buffers of decreasing pH and increasing molarities One ml fraction was collected in each tube and each fraction was assayed for both anticoagulant as well as PLA_2 activity The fractions showing significant PLA_2 as well as anticoagulant activity were pooled, desalted on pre-packed desalting column (Bangalore Genei, Bangalore), concentrated at −20 °C in a MAXI dry plus (Heto Lab Equipment, Denmark) and then applied to a Sephadex G-50 gel-filtration column (1 cm × 64 cm) previously equilibrated with 20 mM K-phosphate buffer, pH 7.0 Elution was carried out with the equilibration buffer at room temperature (~23 °C) The flow rate was adjusted to 20 ml h^{-1} and 1 ml fraction was collected in each tube Protein content of the individual fraction was estimated by the method of Lowry et al (1951) using bovine serum albumin as standard This fractionation resulted into two major and one minor anticoagulant PLA_2 fractions and the first peak showed highest anticoagulant as well as PLA_2 activity The fractions of first peak (GFI) were pooled, lyophilized and stored at −18 °C temperature

The gel-filtration fraction GFI was further purified on a reverse phase C_{18}-μ-Nova pack column by using HPLC system (Waters, Milenium-2000) Briefly, about 20 μg protein of GFI was dissolved in 20 μl of solvent A (0.1% v/v TFA in 5% v/v acetonitrile) and then applied on a reverse phase HPLC column previously equilibrated with solvent A Column was washed with solvent A for 5 min and then bound proteins were eluted at a flow rate of 1 ml min^{-1} using a gradient of 5–95% solvent B (0.1% v/v TFA in 95% v/v acetonitrile) from 5 to 37 min Detection was monitored at 280 nm and individual fraction was collected manually Each peak was screened for protein content, anticoagulant and PLA_2 activity The peak showing anticoagulant PLA_2 activity was pooled, dried in vacuum and stored at −18 °C until further use

2.3 Determination of molecular weight by SDS-PAGE and ESI/MS

The homogeneity of preparation of the PLA_2 was checked by 12.5% SDS-PAGE with or without reduction of protein with β-mercaptoethanol (Laemmli 1970) Molecular weight was further determined by electrospray ionization mass-spectrometry (ESI/MS) The electrospray mass spectra were recorded on a Micromass Quattro II triple quadrupole mass spectrometer The sample was dissolved in 50 μl Millipore water and 10 μl of sample was diluted with 500 μl [1 1 (v/v), methanol water containing 0.3% (v/v) formic acid] solvent system Then the sample was introduced into the ESI source through a syringe pump at the rate of 5 μl min^{-1} ESI capillary was set at 3.5 kV and the cone voltage was 40 V The spectra were collected in 6 scans and the data represented an average spectra of 4–6 scans

2.4 Phospholipase A_2 activity

For screening purpose PLA_2 activity of crude RVV as well as various chromatographic fractions was determined as described by Doley and Mukherjee (2003) using egg yolk

phospholipids as substrate One unit of PLA_2 activity was defined as the amount of protein which produces a decrease in 0 01 absorbance in 10 min at 740 nm To determine the substrate specificity and phospholipids head-group preference of the acidic anticoagulant PLA_2, different commercially available phospholipids such as PC, PS and PE (final concentration 1 mM) were incubated with 20 mM enzyme at 37 °C for desired time periods and PLA_2 activity was assayed by titrametric method using palmitic acid as a free fatty acid standard (Doley et al 2004)

2 5 Anticoagulant activity assay

Platelet pool plasma (PPP) from goat blood was prepared by centrifuging the citrated blood twice at 2500 × g for 15 min at 4 °C and used within 4 h The methods for the assay of re-calcification time of plasma and prothrombin time test (by Quick one stage procedure) were described previously (Doley and Mukherjee 2003) One unit of anticoagulant activity is defined as crude venom/purified PLA_2 induced 1 s increase in clotting of PPP compared to clotting time of normal plasma (Mukherjee et al 2000)

2 6 Biochemical characterization

The activity for protease, acetylcholinesterase, and ATPase in PLA_2 preparation was assayed as described earlier (Mukherjee et al , 2000, Mukherjee and Maity, 2002) The kinetic parameters (K_m and V_{max}) of purified PLA_2 were determined by Lineweaver-Burk plot using different concentrations (3 0–70 mM) of PC as substrate and by plotting the values of 1/v as a function of 1/S To study the effect of heating on catalytic, anticoagulant and membrane damaging activity, purified PLA_2 solution (1 mg ml^{-1}) was heated at 75 °C for different time periods (5–60 min), cooled immediately in ice-bath and then the required volume was withdrawn for the assay of PLA_2, plasma-clotting time and membrane damaging activities of PLA_2 enzyme

2 7 Gas-chromatography analysis of liberated fatty acids from plasma phospholipids

The FFAs released post treatment of PLA_2 were extracted and methylated as describe by Mukherjee et al (2010) The dry sample (fatty acid methyl esters) was dissolved in a minimum quantity of chloroform and analyzed on a GC–MS (Varian 3800, Saturn 2000) system The samples were injected using a split ratio of 100 1 into a fused silica GC column CP-Sil 8 CB low bleed (30 m × 0 25 mm × 0 25 μm) coupled with a CP-Sil 5 C low bleed/MS (30 m × 0 25 mm × 0 25 μm) column with helium as the carrier gas The system was equipped with flame ionization detector The initial oven temperature was 120 °C and a temperature program of 8 °C per min began at injection and continued to a final oven temperature of 270 °C, which was hold isothermal for 3 min The injector port and detector temperature were set at 250 °C The mass spectrometric data were acquired in electron ionization mode (70 eV) The unknown methylated fatty acids were identified by matching both retention time and MS of the unknown compound with those of authentic standards (Saturn 2000 MS library search)

2 8 Inhibition of catalytic and anticoagulant activities of PLA_2 with inhibitors

The PLA_2 inhibitor (AIPLAI) was isolated from the methanol extract of *Azadirachta indica* using a Waters reverse phase C_{18}-μ-Nova pack HPLC column (3 9 × 150 mm) as described by Mukherjee et al (2008) For PLA_2 inhibition study, graded amounts of different inhibitors such as tosyl phenylalanyl chloromethyl ketone (TPCK), N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethanesulfonyl fluoride (PMSF), p-bromophenacyl bromide (pBPB) ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), iodoacetamide (IAA), and *Azadirachta indica* PLA_2 inhibitor (AIPLAI) were pre-incubated with fixed concentration of acidic anticoagulant PLA_2 at 37 °C for 30 min The mixture was then assayed for the inhibition catalytic and anticoagulant activities of the PLA_2, if any, in the corresponding assay system

2 9 Measurement of interaction of PLA_2 with phospholipids and coagulation factors

Measurement of interaction of different phospholipids with purified PLA_2 was performed by using a fluorescence spectrometer (LS55, Perkin Elmer) Briefly, either a pure phospholipid (PC/PS/PE) or a mixture of phsopholipids (PC PS PE, 1 1 1) were suspended in 20 mM Tris-HCl, pH 8 0 at a final concentration of 1 mM and sonicated for about 5 min at 4 °C with a Labsonic® M (Sartorius) sonicator To this, graded concentrations of PLA_2 (native or heated at 75 °C for various time period) dissolved in 20 mM Tris-HCl, pH 8 0 were mixed either in presence or absence of Ca^{2+} and fluorescence spectra were obtained at an excitation wavelength 280 nm, excitation and emission slits 5 nm, temperature 30 °C Wavelength shifts were measured by taking the midpoint at two third height of the spectrum The maximum fluorescence of free protein (I_0) was also measured

To measure the interaction of PLA_2 with blood clotting factors viz factor Xa, factor Va, prothrombin and thrombin, the former protein was mixed with any one of the coagulation factors at a time to get the different molar ratio of PLA_2 coagulation factor either in presence or absence of Ca^{2+} and change in the fluorescence intensity at 280 nm excitation was measured To assure the reproducibility, all the binding experiments were done in triplicate

In *in vitro* condition, interaction of RVVA-PLA_2-I with factor Xa was also checked by gel-filtration of individual PLA_2, factor Xa and reconstituted PLA_2 factor Xa (1 1) complex on a Sephadex G-50 (1 × 64 cm^2) gel-filtration column equilibrated with 20 mM K-phosphate buffer, pH 7 2 Elution was carried out with the same buffer at room temperature (~23 °C) at a flow rate of 24 ml/h and 1 0 ml fraction was collected in each tube Protein content of individual tube was determined spectrophotometrically at 660 nm (Lowry et al 1951) The gel-filtration column was calibrated with the following molecular weight marker proteins-aprotinin (6500) cytochrome C (12,400), carbonic anhydrase (29,000), bovine serum albumin (66,000) and blue dextran (200000)

2.10 Prothrombin inhibition assay

The prothrombin complex (factor Xa, factor Va phospholipids and Ca^{2+} ions) was reconstituted either in presence or absence of 10 nM of purified PLA_2 (Joseph et al 1999) The prothrombin (5 μg) activation was indirectly determined by measuring the formation of thrombin from prothrombin using the chromogenic substrate for thrombin (Berger et al, 2008) The amount of thrombin generated by activation of prothrombin was determined from the concentration of p-nitroaniline which was released from 0.2 mM of N-p-tosyl-Gly-Pro-Arg p-nitroanilide acetate salt (chromogenic substrate for thrombin) by the action of thrombin The activation of prothrombin (thrombin generation) by factor Xa in absence of purified PLA_2 was considered as 100% activity and from this value, present inhibition of prothrombin activation by anticoagulant PLA_2 was calculated

The inhibition of prothrombin activation was also analyzed by 15% SDS-PAGE (Laemmli 1970) Briefly, 5 μg prothrombin was incubated with factor Xa, Ca^{2+} ions and either in presence or absence of 10 nM of purified PLA_2 at 37 °C for 3 h The hydrolysis products of prothrombin were analyzed by 15% SDS-PAGE under non-reducing condition

2.11 Effect of the PLA_2 on amidolytic activity of factor Xa

Factor Xa (25 nM) dissolved in 20 mM K-phosphate buffer, pH 7.4 was incubated with various concentrations (1–8 μM) of purified acidic anticoagulant PLA_2 (either in presence or absence of Ca^{2+}) for 10 min at room temperature (~23 °C) The factor Xa amidolytic activity was measured by transferring an aliquot of the mixture to a cuvette containing 250 μM of F3301 (CH_3OCO-D-CHA-Gly-Arg-*p*-nitroanilide, chromogenic substrate for factor Xa) The hydrolysis of F3301 was measured at 405 nm The reaction mixture without factor Xa was taken as negative control whereas reaction mixture with factor Xa and F3301 but without PLA_2 served as positive control The amidolytic activity of factor Xa in absence of PLA_2 was considered as 100% activity and other values were compared with that

3. Results

3.1 Purification of an acidic anticoagulant PLA_2

The flow-through of CM Sephadex C-50 contained neutral and acidic proteins of RVV (at pH 7.0) Fractionation of these proteins through an anion exchanger DEAE Sephadex A-50 resulted in their separation into seven peaks (supplementary Fig S1) The peak-V (eluted with 100 mM K-phosphate, pH-5.0) showed both anticoagulant and PLA_2 activity By fractionation through gel-filtration column, it was separated into three fractions (supplementary Fig S2) The fraction GFI showing strong anticoagulant as well as PLA_2 activity was further purified using RP-HPLC where it was eluted as a single sharp peak with a retention time of 12.5 min (Fig 1A) This peak was found to be homogenous by SDS-PAGE (see below) This PLA_2 was named as RVVA-PLA_2-I (RVV acidic PLA_2-I) The summary of purification of RVVA-PLA_2-I is given in Table 1

3.2 Assessment of purity and determination of molecular mass

The molecular mass of the PLA_2 was determined by SDS-PAGE and ESI/MS analysis About 20 μg of RVVA-PLA_2-I gave a sharp, Coomassie Brilliant blue positive band on 12.5% SDS-PAGE under both reduced and non-reduced conditions (Fig 1B) By SDS-PAGE this protein appeared as a single band of 28.5 kDa after reduction with β-mercaptoethanol, but in absences of reducing agent and heating it migrated as a single band of an apparent mass of 58.0 kDa (Fig 1B) indicating purified PLA_2 is a homodimmer By molecular sieve chromatography, the molecular mass of this acidic PLA_2 was estimated as 61.0 kDa Molecular mass of the purified acidic PLA_2 was determined as 28.57 kDa by ESI/MS (Fig 2)

3.3 Substrate specificity and biochemical characterization

Among the tested phospholipid substrates, RVVA-PLA_2-I showed highest specific activity toward PC (1.1×10^6 U mg^{-1}) followed by PS (2.4×10^5 U mg^{-1}) and then PE (6.0×10^3 U mg^{-1}) The K_m and V_{max} values of RVVA-PLA_2-I toward PC were determined as 0.65 mM and 28.9 μmol min^{-1}, respectively RVVA-PLA_2-I was glycoprotein in nature and showed optimum catalytic activity at pH range of 8.0–8.5 However, it did not show any proteolytic, ATPase and AchEase activity at a dose of 750 mM The dose-dependent study demonstrated that the PLA_2 activity increased linearly up to concentration of 30 mM, and beyond this concentration, saturation in enzyme activity was detected (data not shown)

3.4 Anticoagulant activity

In *in vitro* condition, crude RVV did not show the anticoagulant activity nevertheless, RVVA-PLA_2-I at a concentration of 25 mM prolonged the normal clotting time of platelet poor plasma It was observed that with an increase in the concentration of RVVN-PLA_2-I up to 100 mM, its anticoagulant activity (as determined by Ca-clotting time of PPP) was enhanced linearly, however, a further increase in the concentration of this PLA_2 did not show any significant increase in Ca-clotting time of PPP and a saturation was obtained (supplementary Fig S3) A concomitant increase in plasma phospholipids hydrolysis with an initial increase in the pre-incubation time of plasma with PLA_2 was observed and after 10 min of pre-incubation, no further significant enhancement ($p < 0.05$) of plasma phospholipids hydrolysis could be detected (data not shown) The GC-analysis of FFAs released post hydrolysis of plasma phospholipids by RVVA-PLA_2-I at different time intervals also supported the above observation (supplementary Fig S4)

3.5 Binding of RVVA-PLA_2-I with factor Xa and inhibition of prothrombin activation

A significant decrease in fluorescence signal of coagulation factor Xa in presence of PLA_2 (3.5 μM) was recorded (Fig 3) Interestingly, RVVA-PLA_2-I at a dose of 10.0 μM did

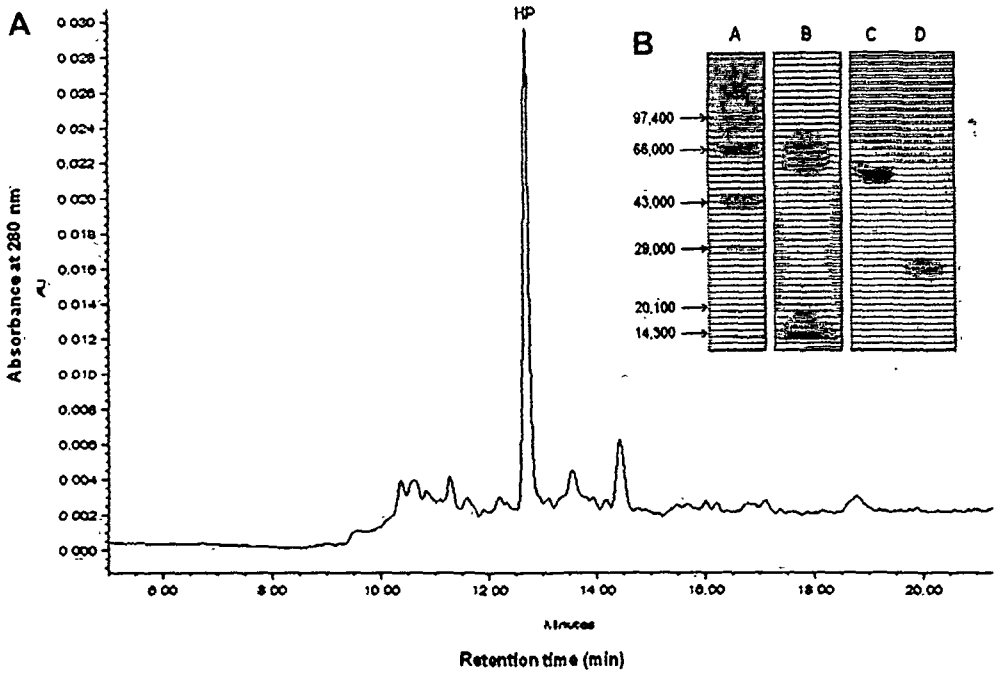

Fig. 1. A. Reverse phase HPLC of gel-filtration fraction GFI on a Waters reverse-phage HPLC C_{18}-μ Nova Pak column. Solvents A and B were 0.1% (v/v) TFA in 5.0% (v/v) acetonitrile, and 0.1% TFA in 95% (v/v) acetonitrile, respectively. Elution was performed at a flow rate of 1 ml/min using the gradient as described in the text. The unabsorbed fraction was not a protein and was from the buffer as confirmed from a blank run. The protein was eluted with a retention time 12.7 min. B. The 12.5% SDS-PAGE of purified RVVA-PLA_2-I (both reduced and non-reduced) and crude *Daboia russelli venom* : Lane A) molecular weight markers (Phosphorylase b 97,400 Da; Bovine Serum Albumin 66,000 Da; Ovalbumin 43,000 Da; Carbonic Anhydrase 29,000 Da; Soyabean Trypsin Inhibitor 20,100 Da; Lysozyme 14,300 Da; Aprotinin 6500 Da); lane B) crude RVV (30 μg); lane C) 20 μg RVVA-PLA_2-I (non-reduced condition); lane D) 20 μg RVVA-PLA_2-I (reduced condition).

not affect the amidolytic activity of the factor Xa and vice-versa (data not shown). However, we could not detect any change in the fluorescence intensity of RVVA-PLA_2-I post incubation with activated factor Va, prothrombin and thrombin indicating absence of interaction of acidic PLA_2 with these coagulation factors. Further, gel-filtration of RVVA-PLA_2-I: factor Xa complex also revealed the binding of these two components (data not shown).

Factor Xa could activate prothrombin, to generate thrombin as was revealed from the amidolytic assay for thrombin (Fig. 4) and SDS-PAGE analysis of prothrombin degradation products (Fig. 5). However, formation of thrombin from prothrombin by factor Xa was drastically reduced in presence of RVVA-PLA_2-I. Since RVVA-PLA_2-I is devoid of proteolytic activity therefore, it leads us to anticipate that this acidic PLA_2 non- inhibited the factor Xa in a non-enzymatic mechanism (Figs. 4 and 5).

3.6. *Effect of inhibitors and antivenom on catalytic and anticoagulant activity*

Different serine inhibitors viz. TPCK, TLCK and PMSF did not affect the catalytic and anticoagulant activity of RVVA-PLA_2-I suggesting serine does not play any role in these activities of PLA_2 (Table 2). However, modification of enzyme with pBPB drastically reduced the catalytic as well as the anticoagulant activity of RVVA-PLA_2-I indicating presence of histidine in the active site of the PLA_2 enzyme (Table 2). Further, EDTA significantly inhibited the catalytic and anticoagulant activity of PLA_2 nevertheless, the inhibition of catalytic activity was more pronounced as compared to inhibition of the anticoagulant property of PLA_2 (Table 2). It might be noted that DTT inhibited the catalytic and anticoagulant activity of RVVA-PLA_2-I almost at the same extent although IAA exerted more inhibition of catalytic activity

Table 1
Summary of purification of the acidic anticoagulant phospholipase A_2 (RVVA-PLA_2-I) from RVV. Data represents a typical experiment. ND: Not detected.

Fraction	Protein yield (%)	PLA_2 activity		Anticoagulant activity		Purification fold	
		Total activity (unit)	Specific activity (unit/mg)	Total activity (Unit)	Specific activity (unit/mg)	PLA_2 activity	Anticoagulant activity
Crude RVV	100	1.3×10^5	1.2×10^4	ND	ND	1	-
CM-Cellulose	4.1	1.1×10^3	2.5×10^4	ND	ND	2.0	-
DEAE Sephadex	0.49	1.9×10^3	3.8×10^4	ND	ND	3.2	-
Gel-filtration	0.12	7.2×10^2	6.0×10^4	23.8	1.9×10^3	4.9	1
RP-HPLC	0.1	5.9×10^2	8.4×10^4	36.8	5.3×10^3	7.0	2.8

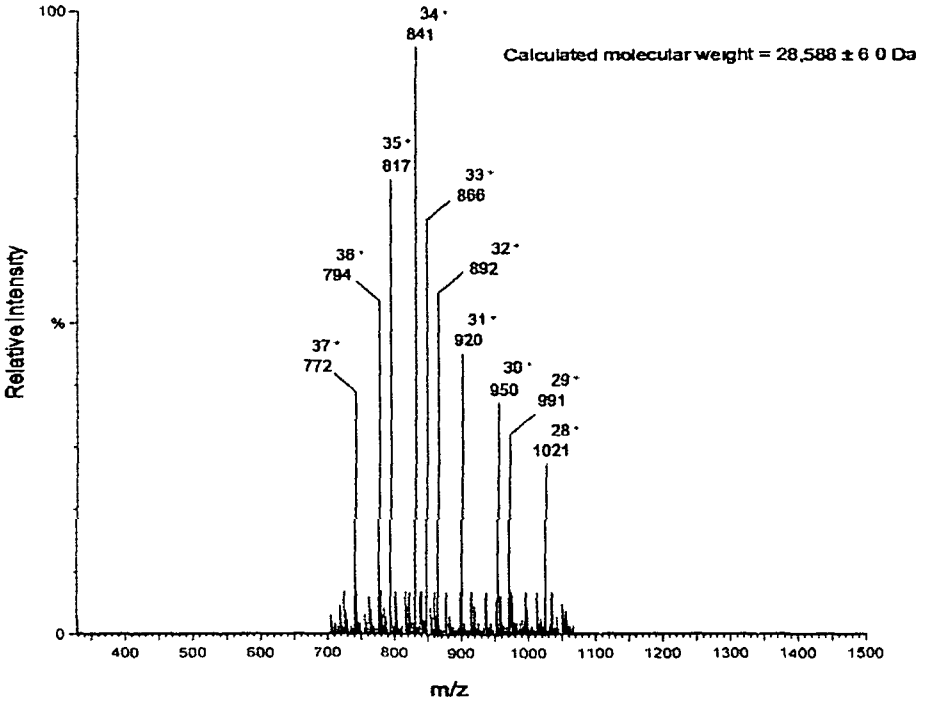

Fig 2 ESI/MS of RVVA-PLA_2-I for molecular mass determination

compared to anticoagulant activity of RVVA-PLA_2-I (Table 2) The AIPLAI purified from *Azadirachta indica* leave extract at a dose of 50 μg ml^{-1} demonstrated equal inhibition of catalytic (73% inhibition) and anticoagulant (71% inhibition) activity RVVA-PLA_2-I (data not shown)

3 7 *Binding with phospholipids*

The excitation of fluorescence of free PLA_2 (0 4 μM) was done at 280 nm and emission maximum were observed at 331 5 nm Fluorescence intensity of PLA_2 post mixing with different phospholipids bearing different polar head groups exhibited different results, a large increase in fluorescence intensity of PLA_2 in presence of PC was observed (Fig 6) and addition of 2 mM Ca^{2+} further enhanced the fluorescence intensity (data not shown) Addition of PS and PE also resulted in an increase the fluorescence intensity of RVVA-PLA_2-I but to a lesser extent that observed in case of PC (Fig 6) When the binding experiment was performed with an equimolar mixture of PC PS PE (final concentration 50 μM), the emitted fluorescence intensity was found to be less than the fluorescence intensity of PLA_2 shown in presence of PC alone (Fig 6) A comparison of PC binding property of native and heated-PLA_2 by spectroflurometric study revealed that phospholipid (PC) binding efficiency of the heat-inactivated PLA_2 was drastically reduced (69%) compared to the PC binding potency of native PLA_2 (Table 2)

4. Discussion

This acidic phospholipase A_2 in the present study constituted about 0 1% of the total RVV protein however, it demonstrated strong anticoagulant activity suggesting its important role in pathogenesis post Russell's viper envenomation Further, data suggested this $^{*}PLA_2$ occurs as a dimmer in RVV The molecular weight of snake venom PLA_2 is reported mostly in the range of 11–15 kDa (Kini, 2006, Valentin and Lambeau, 2000) There is no other report of occurrence of 58 0 kDa homodimeric snake venom PLA_2 except one example of 29 kDa monomeric, non-anticoagulant PLA_2 from the venom of *Vipera russelli* of Myanmar origin (Aye-Kyaw et al , 1994) Due to the predominance of pro-coagulant proteins and peptides in *in vitro* condition crude RVV failed to show anticoagulant activity and RVVA-PLA_2-I was separated from crude venom only after the gel-filtration step

4 1 *RVVA-PLA_2-I is a strong anticoagulant PLA_2 from RVV*

Venom PLA_2 enzymes have been classified into strong, weak and non-anticoagulant enzymes (Kini, 2006, Boffa et al , 1980, Verheij et al , 1980) The RVVA-PLA_2-I displayed anticoagulant effect at a concentration of 0 3 μg ml^{-1} which was far lower than recommended concentration (~2 μg ml^{-1}) required for the strong anticoagulant PLA_2 to prolong the normal clotting time of plasma Therefore it is reasonable to assume that RVVA-PLA_2-I belongs to the group of strongly anticoagulant PLA_2s (Kini 2006) Further, our finding contradicts the report of Verheij et al (1980) suggesting higher anticoagulant potency is associated mostly with basic PLA_2 enzymes However, it is not the overall basicity but the nature of charge in the anticoagulant site determines the anticoagulant potency of PLA_2 enzymes according to Kini (2006) In strongly anticoagulant PLA_2

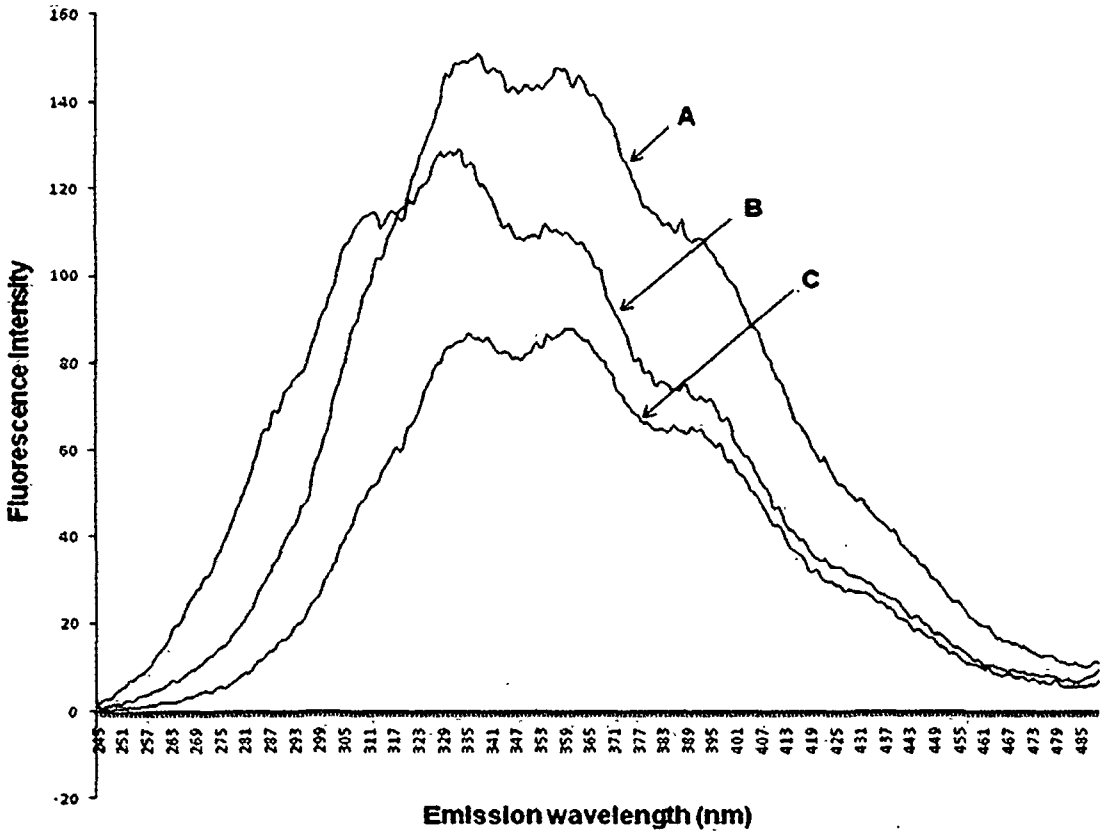

Fig. 3. Fluorescence spectra showing interaction of RVVA-PLA_2-I with activated factor X. (A) factor Xa, (B) RVVA-PLA_2-I, (C) activated factor X in presence of RVVA-PLA_2-I.

enzymes the anticoagulant region is positively charged, but negatively charged in weakly and non-anticoagulant enzyme. Therefore, it might be reasonable to assume that although overall net charge of RVVA-PLA_2-I is negative (anionic in nature); however, some positive residues on enzyme may play a significant role in demonstrating its strong anticoagulant effect (Kini, 2006).

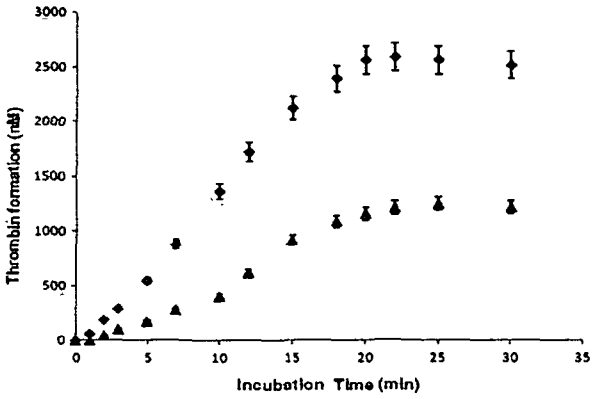

Fig. 4. Inhibition of factor Xa -induced prothrombin activation by RVVA-PLA_2-I. Generation of thrombin (as determined by amidolytic activity assay of thrombin) from prothrombin (5 μg) in presence of factor Xa (25 nM) (◆); and in presence of RVVA-PLA_2-I (10 nM) treated factor Xa (25 nM) (▲). Values are mean ± S.D. of triplicate determinations.

4.2. Correlation between catalytic and anticoagulant activity of RVVA-PLA_2-I

The understanding of structure–function relationships in PLA_2 enzymes is complicated and contradictory data have been presented to explain the anticoagulant action of venom PLA_2 enzymes. For example, there are reports demonstrating a correlation between catalytic activity and anticoagulant property of PLA_2 molecules (Boffa et al., 1980; Verheij et al., 1980; Soares et al., 2001; Doley and Mukherjee, 2003). In a sharp contrast, it has also been reported that a non-enzymatic mechanism might play a key role in inhibiting the different blood coagulation factors by venom PLA_2 and therefore, nullifying the role of PLA_2 activity in anticoagulant process (Stefansson et al., 1990; Faure et al., 2007). Present study supports that anticoagulant activity of RVVA-PLA_2-I is mainly dependent on its catalytic activity and also partly attributed through a non-enzymatic mechanism by binding with coagulation factor Xa and thus inhibiting the activation of prothrombin to thrombin.

It is well known that phospholipids play a crucial role in the formation of several coagulation complexes. Therefore, preferential hydrolysis of PC, the natural precursor for PS; followed by hydrolysis of PS, which is known to be the most active phospholipids in clotting process and low hydrolysis of plasma phospholipids by RVVA-PLA_2-I might lead us to

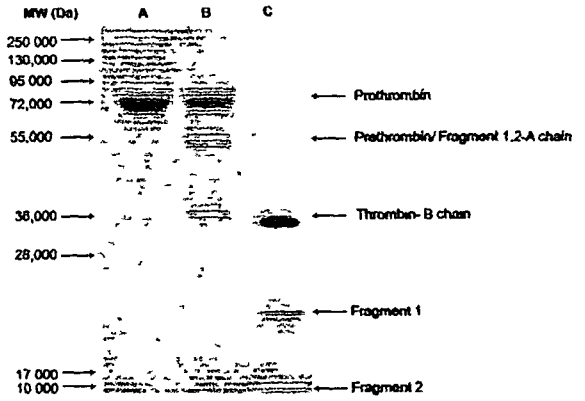

Fig 5 The inhibition of prothrombin activation as analyzed by 15% SDS PAGE Lane A) prothrombin (5 μg) lane B) prothrombin (5 μg) incubated with factor Xa (25 mM) Ca^{2+} ions (10 mM) in presence of RVVA PLA_2 I (10 mM) lane C) prothrombin (5 μg) treated with factor Xa (25 mM) Ca^{2+} ion(10 mM) in absence of RVVA-PLA_2-I

anticipate that destruction of phospholipids surface would be the primary mechanism to account for anticoagulant effect of this enzyme Those results support the role of enzymatic activity in anticoagulant action by RVVA-PLA_2-I (Condrea et al, 1981 Doley and Mukherjee 2003) The above hypothesis is supported by the observations that very low but specific plasma phospholipids hydrolysis is the characteristics feature of strongly anticoagulant PLA_2s whereas non-specific, non-anticoagulant PLA_2 enzymes randomly hydrolyze the plasma phospholipids (Kini and Evans, 1989) Therefore, higher amounts of phospholipids hydrolysis are required before non-specific PLA_2s show any significant anticoagulant effect It is noteworthy to mention that non-anticoagulant PLA_2 from *Naja naja naja* (Roberts et al, 1978) *Crotalus atrox* (Tinker and Wei, 1979) and *Crotalus adamanteus* (Pattnaik et al, 1976) and weakly anticoagulant PLA_2 from *Naja Kaouthia* (Mukherjee 2007) preferentially hydrolyzes PC over PS and PE whereas anticoagulant PLA_2 from *Vipera berus* has a specificity toward PS (Boffa et al 1972) In this study, RVVA-PLA_2-I a strong acidic anticoagulant PLA_2 showed preferential hydrolysis of PC over PS or PE This novel finding leads to conclude that strong anticoagulant activity may not always be restricted to only those PLA_2 showing preferential hydrolysis of PS

Further the anticoagulant activity was parallel enhanced with an increase in the initial concentration of RVVA-PLA_2-I supporting anticoagulant potency is dependent on the catalytic activity of PLA_2 enzyme Nevertheless, the metal chelator EDTA significantly inhibited the catalytic as well as anticoagulant activity of this PLA_2 by chelating the Ca^{2+} ions require to enhance theses activities Moreover, alkylation of histidine residue, the most conserved amino acid amongst PLA_2 enzymes (Pungent-Dachary et al 1980, Doley et al 2004, Mukherjee, 2007) resulted in parallel inhibition of catalytic and anticoagulant activity of RVVA-PLA_2-I, reinforcing a correlation between these two activities Besides, inhibition study with AIPLAI isolated from *Azadirachta indica* leaves and heat-inactivation study also supported the correlation between catalytic and anticoagulant activity of RVVA-PLA_2-I

4 3 *Non-enzymatic mechanism of anticoagulant activity interaction of RVVA-PLA_2-I with plasma phospholipids and coagulation factor Xa*

The non enzymatic mechanism of anticoagulant action of RVVA-PLA_2-I is evident from the fact that beyond 30 mM concentration of PLA_2, no further plasma phospholipids hydrolysis was observed, however, anticoagulant activity was increased up to a concentration of 100 mM PLA_2

One of the most important key factors influencing the anticoagulant potency is the penetrating property of PLA_2 enzymes (Condrea et al, 1980, Kini, 2006) Present study contradicts many earlier reports postulating penetrability is the property associated with basic PLA_2s of venom (Boffa et al 1980, Verheij et al, 1980) Nevertheless, RVVA-PLA_2-I, an acidic PLA_2 strongly binds with PC vesicles even in absence of Ca^{2+} suggesting its high penetrating ability which in turn reflects its strong anticoagulant activity (Pungent-Dachary et al, 1980) It has been suggested by Kini and Evans (1989) that anticoagulant region lies between residues 54 and 77 of PLA_2 molecule, this region is positively charged in strongly anticoagulant PLA_2 enzymes even if the overall charge on the enzyme is different It is to be noted that RVVA-PLA_2-I differs from *Naja naja* phospholipase (group I PLA_2) which has an absolute requirement of Ca^{2+} for its binding to lipids (Roberts et al, 1977) However, our result does not corroborate with the report of Pungent-Dachary et al (1980), who suggested that strong anticoagulant PLA_2 should always have a greater binding with PS A correlation between decrease in percent binding with plasma phospholipid PC and a parallel decrease in catalytic and anticoagulant activity post heating of RVVA-PLA_2-I reinforces the hypothesis that binding followed by hydrolysis of plasma phospholipids particularly PC are the essential steps for inducing anticoagulant activity by this PLA_2 (Zingali, 2007)

It has been suggested that anticoagulant phospholipases may inhibit blood coagulation cascade non-enzymatically

Table 2
Effects of heating and inhibitors on PLA_2 and anticoagulant activity of RVVA-PLA_2-I Values are mean ± S D of triplicate determinations

	PLA_2 activity (% residual activity)	Anticoagulnat activity (% residual activity)
Control	100	100
Heating at 75 °C		
10 min	97 0 ± 1 8	95 0 ± 1 7
20 min	94 5 ± 1 7	92 0 ± 0 6
30 min	90 5 ± 1 5	87 1 ± 1 4
45 min	85 4 ± 2 3	78 6 ± 0 9
60 min	70 8 ± 1 5	65 5 ± 1 2
Chemicals/inhibitors		
TPCK	97 8 ± 1 9	100
TLCK	94 9 ± 1 7	99 7 ± 1 9
pBPB	97 + 0 5	15 4 + 0 4
PMSF	97 6 ± 1 8	88 8 ± 1 4
DTT	63 0 ± 3 1	59 9 ± 0 9
IAA	56 7 ± 2 8	71 2 ± 1 1
EDTA	20 6 ± 1 5	38 3 ± 0 9

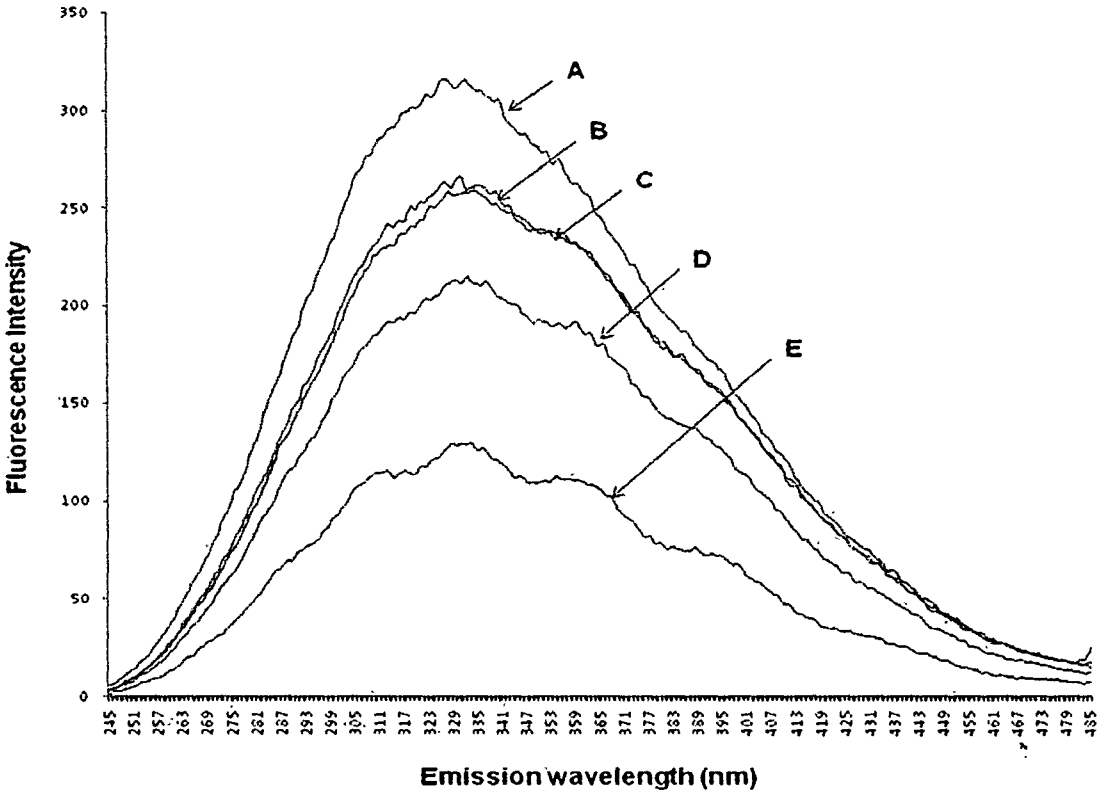

Fig. 6. Fluorescence spectra showing interaction of RVVA-PLA$_2$-I with phospholipids. A) RVVA-PLA$_2$-I and PC; B) RVVA-PLA$_2$-I and PC, PS and PE (1:1: 1); C) RVVA-PLA$_2$-I and PS; D) RVVA-PLA$_2$-I and PE; E) RVVA-PLA$_2$-I.

by competing with clotting factors for the lipid surface and strongly anticoagulant PLA$_2$ enzyme binds to coagulation factor X and/or factor Xa or by inhibition of thrombin (Stefansson et al., 1990; Kini, 2006; Osipov et al., 2010). By Intrinsic fluorometric as well as by gel-filtration study, an interaction between RVVA-PLA$_2$-I and coagulation factor Xa was observed in absence of phospholipids/Ca^{2+} documenting this PLA$_2$ may block the formation of prothrombinase complex even in a phospholipids/Ca^{2+} independent manner. The non-enzymatic, Ca^{2+}-independent mechanism of inhibition of blood coagulation by RVVA-PLA$_2$-I is further evident from the fact that it retains about 38% of anticoagulant activity although the catalytic activity is reduced to 80% of its original activity post addition of EDTA. Further, RVVA-PLA$_2$-I binds with factor Xa at a site other than its chromogenic substrate binding site resulting in no interference in the amidolytic activity of factor Xa although its prothrombin activation property post binding with PLA$_2$ was lost. This result is in accordance with the report of Stefansson et al. (1990) showing a basic PLA$_2$ from *Naja Nigricollis* venom (CM-IV) inhibits the prothrombinase complex in absence of phospholipids without affecting the amidolytic activity of factor Xa.

In conclusion, RVVA-PLA$_2$-I exerts its anticoagulant effect by a dual mechanism of action by enzymatically hydrolyzing the plasma phospholipids as well as by binding with those phospholipids essential for the blood coagulation process. This PLA$_2$ interfered with the blood coagulation process non-enzymatically by inhibiting the coagulation factor Xa, even in absence of phospholipids/Ca^{2+}, and thereby slowing down the process of thrombin formation from prothrombin by the action of factor Xa. However, further structural characterization of RVVA-PLA$_2$-I is necessary for better understanding of its catalytic and pharmacological properties.

Conflict of interest

There is no conflict of interest.

Acknowledgments

Authors thank SAIF, CDRI Lucknow for ESI/MS analysis and Dr. N. Bordoloi for GC-analysis of plasma phospholipids hydrolysis products. Ms. D. Saikia and Ms. R. Thakur were recipients of UGC fellowship and DST-INSPIRE fellowship, respectively. This work was supported partially by Research Grants to A.K.M. from University Grants Commission (UGC), New Delhi.

Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.toxicon.2011.02.018.

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JPP JOURNAL OF PROTEINS AND PROTEOMICS
2(1), January-June 2011, pp. 31-40

MEDICAL AND DIAGNOSTIC APPLICATIONS OF SNAKE VENOM PROTEOMES

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Abstract: Snake venom is a highly toxic secretion produced and stored in specialized salivary glands of snakes which constitutes a vast array of biologically-active compounds, such as enzymes, proteins, peptides and low molecular weight compounds. These substances target an immense number of receptors and membrane proteins as well as coagulation proteins with high affinity, selectivity and potency, and can serve as potential drugs or scaffolds for drug design. During the recent years, much attention has been given to understand the mechanism of action of complex venom proteins for the development of novel drugs and therapeutic agents to treat life-threatening diseases such as cardiovascular diseases, cancer, thrombosis, arthritis, microbial infections and hypertension etc. Further, snake venom components have found uses in the diagnosis of haemostatic disorders. This paper reviews the various biomedical applications of snake venom proteins in terms of therapeutic and diagnostic values.

Key words: anticancer drug; antimicrobial agent; diagnostic reagents from snake venom; medical application of venom toxins; thrombolytic agents.

Introduction

The Indian subcontinent apart from being a rich source of flora and fauna is also home to a rich diversity of ophidian species. Only 242 species of snakes have been identified in India so far which includes 57 poisonous or harmful species. Snake vénom is an evolutionary adaptation to immobilize, incapacitate as well as to digest their prey, which is also used as a defense mechanism by the snakes when encountered by enemies. In the recent years, snake venom and its components has gathered enormous interest by researchers across the globe. Scientists over the past few decades have postulated that despite of the harmful and life-threatening affects of snake venom, its component may provide highly specific research tools for the development of novel life-saving medicines and drugs against some common and life threatening diseases (Stocker, 1990) besides providing insight to basic coagulation process.

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A Brief Account of Snake Venom Composition

Over 90% of the solid snake venom components are pharmacologically active proteins and polypeptides, responsible for exerting pharmacological effects in victims (Sarkar and Devi, 1968; Stocker, 1990). Non-protein ingredients of venom include carbohydrate and metals (often in the form of glycoprotein and metalloprotein enzymes), lipids, free amino acids, nucleotides and biogenic amines.

The proteins/ polypeptides constituting snake venom can further be divided into enzymatic and non-enzymatic toxins, which in turn may be coagulant, anticoagulant or fibrinolytic in nature. Non enzymatic toxins are found to be predominant in cobra venom, whereas viper venom is found to be composed mainly of enzymatic proteins (Sarkar and Devi, 1968; Stocker, 1990). Snake venom toxins may have more than one specific activity, and therefore they may play multiple roles in the overall effect of envenoming. Considering this fact, the isolation and characterization of individual venom

components constitutes the mainstay of toxinology, as a key strategy to scrutinize and to analyze the complex series of events involved in envenoming. The number of enzymes and their specific activities varies from venom to venom and about 26 such enzymes have been identified. Although no single venom contains all of them, at least 10 of these enzymes are present in every snake venom, while others are found in several combinations in different varieties of snakes (Stocker, 1990; Sarkar and Devi, 1968; Mebs, 1970). A comparative study on the activities of enzymes in venom of 42 species comprising Colubridae, *Elapidae, Viperidae and Crotalidae snake families* led to the conclusion that Elapidae venoms are rich in phospholipases and phosphodiesterase (Mukherjee *et al.*, 2000; Mukherjee and Maity, 1998a; Mukherjee and Maity, 1998b; Doley and Mukherjee, 2003; Doley *et al.*, 2004; Mukherjee, 2007), whereas Viperidae venom contain proteases, coagulant, kinin-releasing and arginine ester hydrolyzing enzymes (Stocker, 1990; Kini 1997; Mukherjee *et al.*, 2000).

The variation in venom composition is a common phenomenon and plays an important role in pathophysiological symptoms following envenomation and therefore deserves dedicated medical concern. The venom composition varies greatly due to variation in individual, geographical origin, age and diet of the snakes (Mukherjee and Maity, 1998a; Daltry *et al.*, 1996; Tsai *et al.*, 1996). The mutational changes in the gene which is the primary basis of evolution also contributes significantly to the venom variation that occurs between closely related species or even within a species (Daltry *et al.*, 1996; Assakura *et al.*, 1992; Fry *et al.*, 2003).

Biomedical and Therapeutic Application of Snake Venom Components

Investigations over the past few decades, have shown that the myriad of proteins found in venoms of different snakes have the potential to be developed as a drug for the treatment of a number of medical concerns such as cardiovascular ailments, thrombosis, arthritis, cancer and many other diseases (Stocker, 1990; Lipps, 1999; Toombs, 2001; Marsh and Williams, 2005). Besides, many of them are now successfully employed as conventional diagnostic tools for the assessment of different coagulation factors and proteins involved in the coagulation cascade. Venom toxins have developed highly specific molecular targets, which make them valuable for drug usage in terms of limiting potential side effects. Studies about these protein toxins and their mechanism of action have contributed to the knowledge about the various molecular mechanisms involved in the physiological processes and in the development of novel therapeutic agents for the treatment of various life threatening diseases.

The search for lead compounds for the development of new therapeutic agents has long included a focus on snake venoms. The descriptions below and Table 1 lists some of the snake venom proteins which have found a place in the biomedical industry.

(a) Novel Drugs from Snake Venom Showing Anticancer and Anti-tumor Activity

Cancer, which has long been known to the human as a life threatening disease is characterized by uncontrolled growth and spread of abnormal cells. Medical science despite of its constant research for the development of effective drugs against cancer has not been fully successful in dealing with this deadly disease.

A major problem faced by the physicians is various drugs and chemotherapeutic agents usually used for the treatment of cancer are unable to distinguish between tumor cells and other healthy cells, causing undesirable side effects, sometimes leading to death. The venom-derived toxins seem to act only on certain types of cells and have shown differential lytic activity against various cell lines and subcellular organelles (Doley *et al.*, 2004). Recent research has been directed with special emphasis on potential application of purified proteins and crude snake venoms that might disrupt the normal sequence of events leading to the spread of tumor. In 1933, Calmette and his colleagues first time reported the anti-carcinogenic activity of cobra (*N. naja*) venom (Iwaguchi *et al.*, 1985) and thereafter, many reports have established the anticancer potential of different species of Elapidae, Viperidae and Crotalidae snake venoms

Table 1
Therapeutic Applications of Snake Venom and Snake Venom Components

Snake venom component	*Example*	*Source*	*Biological functions*	*Applications*	*Reference*	*Comments*
Snake venom thrombin-like enzymes	Ancrod	*Agkistrodon rhodostoma*	Converts fibrinogen into non-clottable form of fibrin i e Therapeutic difibrination	Treatment of ischaemic stroke, HATT syndrome, deep vein thrombosis and peripheral occlusive diseases, alternative to heparin in cardiopulmonary bypass	[2]	Marketed as Viprinex TM
Plasminogen activating enzymes	TSV-PA	*Trimeresurus stejnegeri*	Dissolution of fibrin clot via activation of plasminogen to plasmin	Treatment of vascular diseases such as myocardial infarction, pulmonary embolism, stroke, deep vein thrombosis and other vascular thromboses, cancer	[39] [50]	Potential to be developed as a fibrinolytic drug In Phase II clinical trials
Direct fibrinolytic enzymes	Alfimeprase (recombinant form of Fibrolase)	*Agkistrodon contortrix contortrix*	Dissolution of fibrin/ whole blood clot directly (plasmin-like activity)			
Disintegrins	Contortrostatin	*A contortrix contortrix*	Blocks integrins during tumor progression	Applicable as antitumor agents	[51]	Not yet available in market
	Rhodostomin	*Calloselesma rhodostoma*	Inhibits angiogenesis		[55]	-do-
	Salmosin	*A hays brevicaudus*			[21]	-do-
Platelet glycoprotein IIb/IIIa antagonists	Integrilin (Eptifibatide)	*Sistrurus miliarius barbouri*	Inhibits platelet aggregation	For reducing the risk of acute cardiac ischemic events in patients with unstable angina	[57]	Marketed as Integrilin® by Millenium Pharmaceuticals
Thrombin inhibitors	Bothrojaracin	*Bothrops jaraca*	Anticoagulant		[58]	Not yet marketed
Plasmin inhibitors	Textilinin-1	Pseudonaja textilis	inhibitor of plasmin-catalysed fibrinolysis	Anti-bleeding agent in the treatment of chronic inflammatory diseases such as rheumatoid arthritis and arteriosclerosis Restriction of cell proliferation during cancer	[9]	Marketed by QRXPHARMA, but withdrawn due to concerns over side effects
Angiotensin-converting enzyme (ACE) inhibitor	Captopril	Bothrops jaraca	blocks the conversion of angiotensin I to angiotensin II of the RAAS system	Treatment of hypertension, congestive heart failure, also investigated for use in treatment of cancer	[45]	Marketed by Bristol-Myers Squibb under the trade name Capoten

(Iwaguchi *et al.*, 1985; Yang *et al.*, 2005a; Debnath *et al.*, 2007; Gomes *et al.*, 2007).

It has been suggested that fibrin deposition plays an important and distinct roles at different stages of tumor growth and dissemination. Blood vessel thrombus due to fibrin accumulation may lead to myocardial infarction or other cardiovascular diseases. Moreover, occurrence of venous thromboembolism is a common complication in cancer patients. Although the degree of malignancy is not related to the extent of fibrin deposition, but the amount of fibrin deposited is a characteristic of a particular type of tumor (Markland, 1990). In addition to fibrin, there are strong evidences to show that platelets also play an important role in spreading the tumor metastasis and this is probably by shielding tumor cells in platelet thrombi (Markland, 1990). Fortunately, many of snake venoms are enriched in fibrinolytic enzymes (Trikha *et al.*, 1994; Leonardi *et al.*, 2007); therefore, attempts have been made to inhibit the tumor growth and invasion by interfering with fibrin deposition and/ or platelet aggregation with the help of fibrinolytic enzymes.

Infusion of a purified 33.7 kDa monomeric fibrinogen clotting snake venom enzyme crotalase to different animals resulted in a benign state of defibrinogenation (Markland, 1990). There was neither fibrin deposition in the internal organs nor any intravascular coagulation was observed. In order to suppress the tumerogenecity of mouse B16 melanoma cell lines, the cells were treated with purified crotalase *in vitro* and then subcutaneously transplanted into C57 BL/6 mice (Markland, 1990). Interestingly, although crotalase did not show cytotoxicity against B16 melanoma cells, but treatment with crotalase resulted in inhibition of the growth of cancer cells. It has been suggested that by inhibiting the fibrin gel formation around a tumor cell, crotalase could create a more favorable condition for immunogenic attack of cancer cells by macrophages or other effector cells. Thrombin-like venom enzymes may help to stop metastasis of breast cancer as well as ovarian cancer as it can inhibit tumor dissemination and angiogenesis (Trikha *et al.*, 1994).

Osteosarcoma is a very malignant bone tumor which has a high metastatic potential and usually lead to poor prognosis. In the metabolic cascade, the adhesion of tumor cells to the endothelium or, extracellular matrix is an essential step. Rhodostomin, a peptide isolated from the venom of *Calloselesma rhodostoma*, can block the osteosarcoma cell line (ROS 17/2.8) of rat (Yang *et al.*, 2005a). It is known that Rhodostomin may serve as a potent anti-metastatic agent in blocking the thrombin enhanced cell adhesion potential. Further investigation of anti-adhesion therapy with venom components perhaps leads to the prevention of potential metastasis in certain cancer patients (Yang *et al.*, 2005a). Salmosin, is yet another example of a peptide isolated from the venom of *Agkistrodon hays brevicaudus*, which exhibits anti-tumor and anti-angiogenic effects (Kang *et al.*, 1999).

Among the various toxins of the venom, cytotoxins, the primary target for which is the cell membrane, are among the most prominent components of cobra venom (Mukherjee and Maity, 1998a; Mukherjee and Maity, 2002; Feofanov *et al.*, 2005; Izidoro *et al.*, 2006) .For example, cytotoxins CT1 and CT2 from *Naja oxiana*, CT3 from *N. kaouthia* and CT1 from *N. haje* are demonstrated to possess this property with respect to human lung adenocarcinoma A_{549} and promyelocytic leukemia HL60 cells (Feofanov *et al.*, 2005). It was demonstrated further that an L-amino acid oxidase isolated from *Bothrops pirajai* snake venom possessed cytotoxic activity and in *in vitro* condition it showed antitumor activity against S180 tumor, human breast (SKBR-3), acute T cell leukemia (Jurkat) cancer, and Erlich ascitic tumor (EAT) cell lines (Izidoro *et al.*, 2006). Similarly, in a study done by Debnath and his colleagues, it has been shown that venoms of Indian monocellate cobra (*N. kaouthia*) and Russell's viper (*Daboia russelli*) possessed anticancer activity and in *in vitro* condition prevented the proliferation of malignant cells, at non-toxic concentrations (Debnath et al, 2007). Similarly, Yang and his colleagues in 2005 also demonstrated the induction of apoptosis in human leukemia K562 cells by cardiotoxin III isolated from *N. n. atra* venom. In fact, this was the first report on the mechanism of the anticancer effect of CTX III on human leukaemia K562 cells

through a mitochondrial mediated pathway (Yang *et al.* 2005b).

(b) Snake Venom Proteins for the Prevention/ treatment of Thrombosis and Vascular Occlusive Diseases

Therapeutic defibrinogenation with snake venom enzymes has been attempted to treat peripheral arterial occlusion diseases in which surgical revascularization could not be satisfactorily achieved (Furukawa and Ishimaru, 1990). Batroxobin (Funk *et al.*, 1971) and Ancrod (Burkhart *et al.*, 1992) are thrombin-like enzymes; purified from the venom of *Bothrops moojeni* and *Agkistrodon rhodostoma,* respectively, have found tremendous application in this aspect. Intravenous or subcutaneous injection of Arvin®, (a commercial preparation of Ancrod) into human causes a specific, dose-dependent reduction of the plasma fibrinogen level; therefore, Arvin is used in the treatment and prevention of vascular occlusive diseases (Furukawa and Ishimaru, 1990). Similarly, batroxobin (Defibrinase R) is currently used for controlled depletion of fibrinogen and is a selective antithrombic agent on deep vein thrombosis peripheral arterial diseases (Itoh *et al.*, 1988).

Unlike snake venom thrombin like enzymes, fibrinolytic enzymes isolated from snake venom do not lead to defibrinogenation but can digest fibrin clots directly; thereby suggesting potential application of fibrinolytic enzymes for the treatment of thrombotic ailments such as strokes, heart attacks and other diseases associated with the formation of thrombus. Of the many fibrinolytic enzymes identified so far, Fibrolase, a direct-acting fibrinolytic metalloproteinase enzyme isolated from southern copperhead venom (*Agkistrodon contortrix contortrix*), has been used to digest occlusive blood clots in animal models (Retzios and Markland, 1988). In 1997, Sanchez and colleagues reported the preparation of a superior thrombolytic agent possessing both thrombolytic and antiplatelet properties, by conjugating fibrolase to a peptide which inhibits platelet aggregation (Sanchez *et al.*, 1997). Recombinant fibrolase called Alfimeprase was also been produced which seemed to be a potent thrombolytic agent (Toombs, 2001). However, its failure at the phase III clinical trials lead to the abolishment of its further implications as a thrombolytic drug. We have also isolated a low molecular weight (~12 kDa) direct fibrinolytic, non-toxic enzyme from venom of *Daboia russelli. In vitro* studies demonstrated that this non-cytotoxic peptide has tremendous potential to dissolve fibrin as well as artificial blood clot thus advocating the future application of this peptide as thrombolytic agent (Mukherjee, A. K. and Thakur, R., unpublished observation). Plasminogen activators are yet another class of fibrinolytic enzymes which result in the breakdown of thrombus by activating inactive plasminogen to plasmin which in turn degrades fibrin. TSV-PA is once such enzyme purified from *Trimeresurus stejnegeri* (Parry *et al.*, 1998).

(c) Other Biomedical Application of Venom Proteins

Besides having potential therapeutic importance in handling diseases associated with thrombosis such as cardiovascular diseases, and cancer, snake venom proteins are also found to have other therapeutic applications. For example, snake venom neurotoxins, mainly α-Btx, played a leading role in myasthenia gravis research (Samson *et al.*, 2001). Crotamine, myotoxin a, and homologous peptides are also seem to be useful candidates to study the muscle cell membrane and their sodium channels (Hong and Chang, 1985). Dendrotoxin (a toxin derived from *Dendroaspic* venom) and their homologues, are becoming extremely valuable tools to characterize K^+ channels which are involved in the regulation mechanisms of cell excitability and synaptic transmission (Harvey and Anderson, 2004).

Antagonists and inhibitors of different coagulation and membrane proteins isolated from snake venom, has hypothesized for its further biomedical applications in the field of hemastatic disorders and ischemic ailments. For example, Inhibitors of angiotensin converting enzymes such as captropril isolated from *B. jararaca* and marketed as captopen, serves for the control of hypertension and congestive heart failure (Smith and Vane, 2003). Another example is of bothrojaracin, which is a potent thrombin inhibitor and as such a strong anticoagulant

(Zingali *et al.*, 2003). Textilin-1 inhibits plasmin catalysed fibrinolysis, thereby suggesting its application as a potent anti-bleeding agent (Flight *et al.*, 2009). Platelet glycoprotein IIb/IIIa antagonists such as Integrilin which inhibits platelet aggregation, is widely used for reducing the risk of acute cardiac ischemic events in patients with unstable angina (Zeymer, 2007).

Recent studies have shown that snake venom can be explored as a promising source for analgesics (Mancin *et al.*, 1998; Pu *et al.*, 1995). Crotamine (~5kDa) from *Crotalus durissus terrificus* is 30 fold more potent than morphine as analgesic (Mancin *et al.*, 1998). Hannalgesin from *Ophiophagus Hannah* (Pu *et al.*, 1995) and Cobrotoxin b from *Naja naja atra* (Chang *et al.*, 1997) are potent analgesics isolated, that do not cause neurological or muscular deficit.

Diagnostic Applications of Snake Venom and its Components

As shown in Table 2, Snake venoms contain a vast array of components, many of which have found extensive applications in the diagnosis of haemostatic disorders (Marsh and Williams, 2005). For example, Russell's viper venom contains toxins which are used to assay blood clotting factors V, VII, X, platelet factor 3 and lupus anticoagulants as well as coagulation proteins such as fibrinogen and prothrombin.

RVV contains a potent activator of factor X (RVV-X) and RVV-X® (Pentapharm) has been employed in a number of clotting assays, notably for the measurement of factor X itself, for distinguishing between factor VII and factor X and in lupus anticoagulant (LA) assay (Takeya *et al.*, 1992). RVV-X enzyme has also been used to assay platelet factor 3 (Hardisty and Hutton, 1966). Due to their unique characteristics, RVV enzymes have been used for the improvement of the detection of Von-Willebrand disease as well. The dilute Russell's viper venom time (dRVVT) occupies a particular niche in the assay of LA as it is quick, sensitive and inexpensive. RVV-V, an enzyme which is commercially marketed by pentapharm for the measurement of Factor V and study of factor V activation was also isolated and purified from *Daboia russelli* venom (Kisiel, 1979).

Yet another diagnostically important compound is the Reptilase® reagent which is the trademark for a lyophilized preparation of pure Batroxobin from *Bothrops atrox* venom, used for measuring "reptilase time", a very popular screening test which is always performed in parallel with thrombin time (Funk *et al.*, 1971). Batroxobin and possibly several other fibrinogen coagulant snake venom enzymes may be used for quantitative determination of fibrinogen in plasma (Funk *et al.*, 1971).

A potent, fast acting Protein C activator (Protac®, Pentapharm) from the venom of the Southern copperhead snake, *Agkistrodon c. contortrix* (Stocker *et al.*, 1987) has been identified which is extremely useful in activating Protein C for assay by direct chromogenic method (Nathan *et al.*, 1987), an indirect chromogenic activated partial thromboplastin time (Stocker *et al.*, 1988); and a functional clotting assay (Martinoli and Stocker, 1986). Many other proteins isolated serve for measuring vWF level or prothrombin levels in plasma as well (Usami *et al.*, 1993; Weigner *et al.*, 1980).

Disintegrins are yet another diagnostically important component from snake venom which prevent platelet aggregation via inhibition of surface glycoprotein receptor activity (Ouyang *et al.*, 1992). Because of the presence of this property, disintegrins offer a unique opportunity for the study of platelet-platelet and platelet-endothelium interactions (Marsh and Williams, 2005). One of the most studied disintegrins include Triflavin from *Trimeresurus flavoviridis* (Huang *et al.*, 1991). Disintegrins may also find a role in imaging both thrombi and ĕmboli and it has been shown that Bitistatin from *Bitis arietans* venom is the most promising in this regard (Knight *et al.*, 1996).

Conclusion

It is obvious that these proteins or peptides, derived from snake venom could produce potentially huge medical benefits for mankind. During the last decade, many of the snake venom proteins and toxins showing great promise for medical application have been isolated, purified and characterized; however, further studies for many of the isolated proteins are required for

Table 2
Diagnostic Applications of Snake Venom and Snake Venom Components

Snake venom component	*Example*	*Source*	*Biological functions*	*Applications*	*Reference*	*Comments*
Prothrombin activators	Ecarin	*Echis carinatus*	Activation of prothrombin	Detection of abnormal type of prothrombin	[54]	Marketed by Pentapharm as Ecarin
Thrombin like enzymes	Batroxobin	*Bothrops moojeni*	Release of fibrinopeptide A of fibrinogen	Deplete fibrinogen and make plasma unclottable	[11]	Commercially available as Reptilase
Factor X activators	RVV-X	*Daboia russelli*	Activation of Factor X	Measurement of Factor X	[49]	Marketed as Pefachrome® FXa by Pentapharm
Factor V activators	RVV-V	*Daboia russelli*	Activation of factor V	Measurement of Factor V and study of factor V activation	[23]	Marketed by Pentapharm
C-type lectin	Botrocetin	*Bothrops jaraca*	Agglutination of platelets in the presence of vWF	Used as a diagnostic of von Willebrand disease and Bernard-Soulier syndrome	[53]	Botrocetin
Protein C activators	ACC-C	*Agkistrodon c contortrix*	Activation of Protein C	Quantitative determination of Protein C and S in human plasma	[47]	Protac®

better understanding of the structure-function relationship and mechanism of action of these venom proteins in order to develop novel drugs and diagnostic reagents

Acknowledgements

D Saikia and R Thakur were recipients of fellowship from University Grants Commission, New Delhi and DST-INSPIRE fellowship, respectively

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Toxicon 51 (2008) 1548–1553

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

Isolation of a snake venom phospholipase A_2 (PLA_2) inhibitor (AIPLAI) from leaves of *Azadirachta indica* (*Neem*): Mechanism of PLA_2 inhibition by AIPLAI *in vitro* condition ☆

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ARTICLE INFO

Article history:
Received 24 December 2007
Received in revised form
20 March 2008
Accepted 20 March 2008
Available online 25 March 2008

Keywords:
Snake venom
Cobra venom
Viper venom
Neem
Azadirachta Indica
Phospholipase A_2-inhibitor
Non-competitive inhibition

ABSTRACT

A compound (AIPLAI (*Azadirachta indica* PLA_2 inhibitor)) purified from the methanolic leaf extract of *A. indica* (*Neem*) inhibits the cobra and Russell's viper venoms (RVVs) phospholipase A_2 enzymes in a dose-dependent manner. Inhibition of catalytic and tested pharmacological properties of cobra venom (*Naja naja* and *Naja kaouthia*) PLA_2 enzymes by AIPLAI is significantly higher ($P<0.05$) compared to the inhibition of PLA_2 enzymes of crude RVV (*Daboia russelli*) when tested under the same condition. Kinetic study reveals that in *in vitro* condition, AIPLAI inhibits the purified *N. kaouthia* PLA_2 enzymes in a non-competitive manner. The AIPLAI is quite stable at room temperature. The present study shows that AIPLAI holds good promise for the development of novel anti-snake venom drug in future.

1. Introduction

Snakebite is a global problem, especially in tropical countries like India. It has been estimated that 5 million people are bitten by venomous snakes annually around the world, thereby resulting in about 100,000 fatalities (Chippaux, 1998). A perusal of the literature shows that cobra and viper are the two important categories of snakes in India responsible for maximum snakebite mortality (Bhat et al., 1991; Mukherjee, 1998; Mukherjee et al., 2000; Mukherjee and Maity, 2002).

PLA_2 enzymes are one of the most toxic proteins present in snake venom; in addition to the digestion of prey, they exhibit many pharmacological effects by disturbing the normal physiological process of victims (Kini, 2003; Doley and Mukherjee, 2003; Doley et al., 2004; Mukherjee, 2007). The most effective and accepted therapy for snakebite patients is immediate administration of specific or polyvalent antivenom following envenomation; however, this therapy carries an associated risk of anaphylaxis and serum reactions (Russell et al., 1985; Gilon et al., 1989). Further, due to geographical variation in venom composition of snakes, antivenom raised against the venom of a snake from a particular geographical origin may not be able to neutralize or prevent local effects of envenomation by snakes from other geographical locations (Gillissen et al., 1994; Mukherjee and Maity, 1998; Shashidharamurthy and Kemparaju, 2007). Therefore, in addition to administration of antivenom, there should be alternative therapy for the snakebite patients.

☆ *Ethical statement*: The animal experiments were done as per the guidelines of experiments on animals as set up by the Indian Council of Medical Research and Tezpur University.

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doi:10.1016/j.toxicon.2008.03.021

Numerous plant species are used as folk medicine to treat venomous snakebite in India, but without any scientific validation Therefore, this type of treatment remains questionable and needs thorough scientific investigation Our previous study has demonstrated that aqueous root extract of *Mimosa pudica*, a plant used by the local people to treat snakebite patients, was effective to neutralize the lethality and toxic enzymes of *Naja kaouthia* venom (Mahanta and Mukherjee, 2001) This has prompted us to screen the anti-phospholipase A_2 activity of some medicinal plants of the region popularly used by the snake charmers as well as by the local healers (traditionally known as *Bejs*) for treating poisonous snakebites The present study shows that methanolic leaf extract of *Azadirachta indica* (*Neem*) is effective against the PLA_2 enzymes of cobra (*Naja naja* and *N kaouthia*) and Russell's viper (*Daboia russelli*) venom (RVV) samples *A indica* has been used as antidote (folk medicine) to treat poisonous snakebite in the rural areas of the Indian subcontinent However, to the best of our knowledge, this is the first report describing the inhibitory activity of *neem* extract against any toxic component of snake venom

2. Materials and methods

Venom samples of *N naja*, *N kaouthia* and *D russelli* were obtained from a commercial venom dealer in Kolkata, India Various plant parts used against snakebite were collected from local areas and taxonomically identified A voucher specimen of each sample was deposited in the department Actual field survey was conducted among the rural and tribal people of Assam to know the plant parts they use against snakebite, procedures for the preparation of medicine from these parts and treatment methodology References have also been collected from books and pamphlets on medicinal plants of Assam and North-East India For the preparation of the plant (root/ leaf/stem) extracts, fresh leaves/roots/bark were shade dried, made into coarse powder, and 2 0 g of powder of leaves/roots/bark was taken in a beaker and soaked with 100 ml of either distilled H_2O or methanol or chloroform with continuous stirring for 2 h at room temperature The extract was filtered through a muslin cloth and filtrate was concentrated at 40 °C under vacuum The dried extract was dissolved in normal saline (0 9% NaCl) at a concentration of 1 mg/ml and kept at 4 °C until further use (Mahanta and Mukherjee, 2001)

Separation of methanolic extract (leaves) was carried out by high-performance liquid chromatography (Waters) coupled with a reverse-phase C_{18}-μ-Nova pak HPLC column (3 9 × 150 mm) Elution was carried out with an isocratic gradient of methanol and acetonitrile at a flow rate of 1 ml/min Elution was monitored at 210 nm and individual peak was screened for PLA_2 inhibition activity The active compound (fraction-I) was re-fractionated in the same column under identical condition, dried under vacuum at −20 °C and kept in a desiccator for further characterized This fraction was named AIPLAI (*A indica* PLA_2 inhibitor)

The molecular mass of the pure fraction was determined by matrix-assisted laser desorption/ionization-time of flight mass spectroscopy (MALDI-TOF-MS) Briefly, the MALDI-TOF-MS was run in a Micromass TofSpec 2E instrument using a nitrogen 337 nm laser (4 ns pulse) At least 40–50 shots were summed up The matrix used was sinapinic acid dissolved in CAN 0 1% TFA-water The infrared (IR) spectrum of the purified dried compound was recorded using KBr pellet in a Nicolet Impact 410 FTIR spectrophotometer Sample was prepared by dispersing the solid uniformly in a matrix of dry nujol (KBr) mull, compressed to form an almost transparent disk The spectra showing the functional groups were used to study the composition of the PLA_2 inhibitor Absorption spectra were plotted using a built-in plotter IR spectra were collected from 500–4000 wave numbers (cm^{-1}) The ^{1}H-NMR spectra of HPLC-purified active fraction (PLA_2 inhibitor) were recorded in C_6D_6 at 300 mHz by a FT-NMR spectrometer

The two most abundant *N kaouthia* PLA_2 enzymes (NK-PLA_2-I and NK-PLA_2-II) were purified as described previously (*Doley and Mukherjee, 2003*; *Doley et al*., 2004) Phospholipase A_2 activity of crude venom samples as well as purified enzymes was determined by the egg yolk method (Doley et al., 2004, Mukherjee 2007) Direct hemolytic activity was tested by incubating 10 μg of crude venom protein or 100 nM of purified PLA_2 enzyme with 5% (v/v) of erythrocytes suspension in 100 mM phosphate buffer, pH 7 4, in a final volume of 3 ml After incubating for 60 min at 37 °C, the reaction mixture was centrifuged and released hemoglobin was measured spectrophotometrically at 540 nm (Mukherjee and Maity, 2002, Doley and Mukherjee, 2003) For indirect hemolytic assay, 10 μg of egg yolk suspension was added to the reaction mixture (as prepared for direct hemolytic assay) as a source of phospholipids Total hemolysis (100%) was achieved by adding 2 7 ml of deionized water (Millipore) instead of venom protein to the erythrocyte suspension in phosphate buffer For the determination of coagulant, anticoagulant, edema and *in vitro* chicken-liver tissue-damaging activity of crude venom as well as the purified PLA_2 enzymes, the procedures as described by Doley and Mukherjee (2003) were followed

For determining the effect of purified *N kaouthia* PLA_2 enzymes on chicken liver mitochondrial swelling, our previously described method was followed (Doley et al., 2004) One unit of mitochondrial swelling is defined as decrease in 0 01 OD at 520 nm after 30 min per mg of mitochondria Basic test system without PLA_2 enzyme served as control

Neutralization of catalytic and pharmacological properties of phospholipase A_2 enzymes (either of crude venom samples or those purified from *N kaouthia* venom sample) was studied by incubating a fixed concentration of venom/pure enzyme with increasing concentrations of leaf extract/AIPLAI/ anti-NK-PLA_2-I antibodies for 30 min at 37 °C followed by assay of residual enzymatic activity and pharmacological properties (Mahanta and Mukherjee, 2001) Enzymatic or any tested pharmacological property of venom PLA_2 enzymes without any inhibitor served as control and was considered to have 100% activity

For determining the effectiveness of inhbitor to inhibit the catalytic activity of PLA_2 enzymes, the inhibitor

constant (K_i) was measured by incubating a constant amount of NK-PLA_2 enzyme with different concentrations of substrate (phosphatidylcholine) either in the presence or absence of the inhibitor (AIPLAI). K_i was then calculated from the value of apparent V_{max} as obtained from Lineweaver–Burk plot by using the following formula

$$K_i = V_{max}\,(\text{apparent})/V_{max} - V_{max}\,(\text{apparent})X[I]$$

where K_i is the inhibition constant; V_{max} (apparent) is the V_{max} in the presence of PLA_2 inhibitor (fraction-I); V_{max} is the maximum velocity of enzyme catalyzed reaction in absence of inhibitor; [I] is the inhibitor concentration (mM).

Results are represented as mean ± S.D. of at least three experiments. Statistical analysis was done by Student's "*t*" test. A probability level of $p<0.05$ was considered statistically significant.

3. Results and discussion

Neutralization of catalytic and/or pharmacological properties of crude venom and/or any of its toxic constituents such as PLA_2 by plant extract reflects the presence of anti-snake venom compounds in the plant (Alcaraz and Hoult, 1985; Soares et al., 2005). Isolation, purification and characterization of active compounds from plants possessing anti-snake venom activity might have a rational, because many of these compounds may prove to be the potential candidates for the development of novel anti-snake venom drugs in future.

Out of the 13 plants screened for possessing of compound(s) active against cobra and RVV PLA_2 enzymes, none of the plants, except the leaves of *A. indica* locally known as *Neem*, showed positive results (Table 1). Among the different solvents extracts, methanol extract of *A. indica* leaves showed the most promising results in inhibiting the catalytic activity of PLA_2 enzymes of crude venom samples. It is interesting to note that most of the anti-snake venom compounds were isolated from the methanol extracts of plants (Selvanayagam and Gnanavendhan, 1996; Alam et al., 1996; Chatterjee et al., 2006).

In a dose-dependent study it was observed that the dried methanol extract of *A. indica* leaves at a final concentration of 2.5 μg/ml could not inhibit the PLA_2 activity (1.0 μg/ml) of any tested venom significantly (Fig. 1); however, at 50.0 μg/ml methanol extract, the PLA_2 activity of crude cobra venoms (*N. naja* and *N. kaouthia*) was neutralized to 52% and 49%, respectively, whereas the PLA_2 activity of crude RVV was inhibited to 32% of their original activity (Fig. 1). This result indicates the differential inhibition of PLA_2 enzymes of cobra and RVVs by methanol leaf extract of *A. indica*.

Fractionation of methanol extract of *A. indica* leaves by RP-HPLC using C_{18} column resulted in its separation into seven fractions (Fig. 2). When individual fraction was screened for anti-PLA_2 activity, only fraction-I with a retention time of 1.061 neutralized the PLA_2 activity of venom samples. When fraction-I was re-fractionated in the same RP-HPLC column under identical condition, it resulted in elution of active compound in a single peak (data not shown). This compound was named as AIPLAI.

Table 1
Screening of medicinal plants for anti-PLA_2 activity against crude *N. naja*, *N. kaouthia* and *D. russelli* venom samples

Medicinal plants	Plant part used	Anti-PLA_2 activity		
		Water extract	Methanol extract	Chloroform extract
Agyl marmolos	Leaves	–	–	–
Alostonia scholaris	Bark	–	–	–
Aristolochia indica	Leaves	–	–	–
Azadirachta indica	Leaves	++	+++	+
Calamus rotang	Root	–	–	–
Carica papaya	Fruit	–	–	–
Gronton aromatica	Rhizome	–	–	–
Curcuma longa	Rhizome	–	–	–
Leucas lavendulaefolia	Leaves	–	–	–
Murrya koenigii	Leaves	–	–	–
Piper longum	Spike	–	–	–
Piper nigrum	Fruit	–	–	–
Terminalia arjuna	Bark	–	–	–

+ Sign indicates ability to neutralize the PLA_2 activity, whereas – sign indicates inability to neutralize the PLA_2 enzymes of *N. naja*, *N. kaouthia* and *D. russelli* venom samples by tested plant extracts.

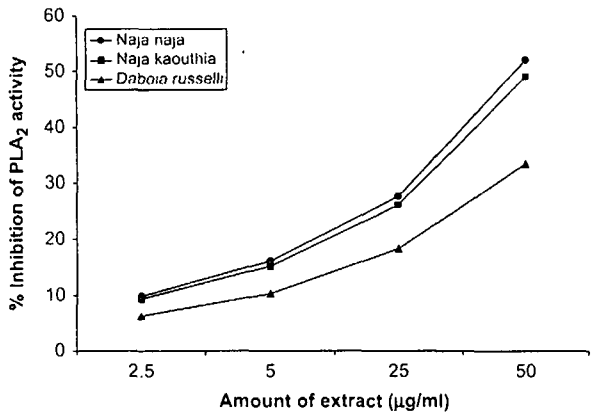

Fig. 1. Dose-dependent inhibition of PLA_2 activity of cobra and Russell's viper crude venom samples (final concentration 1 μg/ml) by methanol leave extract of *A. indica*. Values represent mean of three experiments

The mass spectrum of AIPLAI showed the [M^+] and [M+H] peaks at *m/z* 1052.52 and 1053.53, respectively. This analysis also shows that isolated fraction-I (AIPLAI) is pure in nature. The molecular mass of AIPLAI is much higher than the mass of the previously reported compounds isolated so far from *neem*. For example, different tetrnortriterpenoids isolated from uncrushed green leaves of *A. indica* are shown to have the *m/z* at 469.25 (Govindachari et al., 1999), *m/z* at 452.2593 and 554.2914 (Siddiqui et al., 2000), nimonolide having *m/z* at 484 (Gopalakrishnan et al., 2002); Azadirachtin A, B and H isolated from *neem* seed kernel are shown to have *m/z* at 721 [M+H^+], 663 [M^++H] and 645 [M], respectively

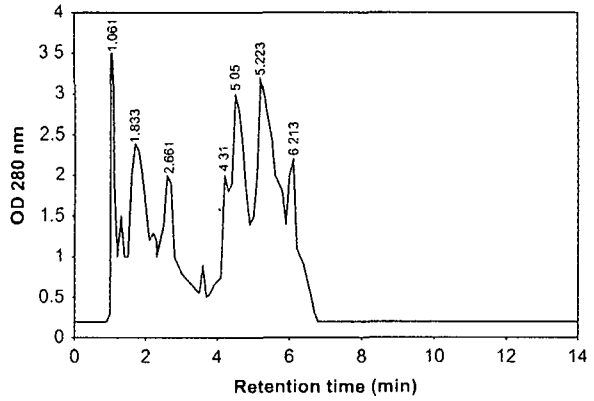

Fig. 2. RP-HPLC profile of methanol leave extract of *A. indica*. Data represent a typical experiment.

(Sharma et al., 2003). Thus, AIPLAI is a novel compound possessing anti-PLA_2 activity isolated from *neem* leaf; however, the actual chemical nature and structure of the AIPLAI remains to be elucidated.

The IR spectrum of AIPLAI displayed peaks at ~3400 cm^{-1} (hydroxyl), 2977, 2934 and 1406 cm^{-1} (aliphatic chains at C–H stratching modes), 1597 cm^{-1} (C=C) and 1195–632 cm^{-1}(aromatic). The ^{1}H-NMR spectrum (300 mHz, C_6D_6) of AIPLAI showed doublet (δ_H 1.40 and 1.42) representing CH_3-aromatic group, singlet (δ_H 1.45) representing CH_2 attached with aromatic ring, multiplate (δ_H 3.45)-C=C–CH_2–phenyl, multiplates at δ_H 3.60 and 3.64 representing R–CO–CH_2–CO–R (ketonic group), multiplate at δ_H 3.77 and 3.78 representing the probable structure of R–CO–CH_2–CO–phenyl, multiplate δ_H 3.52 represents R–CO–CH_2–CO–NR_2, multiplate δ_H 3.51 SR–CH_2–SR, multiplate δ_H 3.57 R–CO–CH_2–SR and multiplate δ_H 3.8 and 4.25 CH_3–CH_2–COOR (ester). Further, because of the absence of five quarternary methyl signals at 0.82, 1.17, 1.19, 1.24 and 1.31 in the ^{1}H-NMR spectrum, fraction-I is presumably non-terpenoidal in nature (Siddiqui et al., 2000).

As shown in Table 2, AIPLAI neutralized the PLA_2 activity as well as tested pharmacological properties of all the three venom samples, but to a significantly different extent ($p<0.05$). AIPLAI inhibited the anticoagulant activity to the maximum extent followed by neutralization of tissue hemorrhagic activity of PLA_2 enzymes (Table 2).

Table 3 shows a comparison of PLA_2 inhibitory activity of AIPLAI Vs anti-NK-PLA_2-I antibodies. The latter shows a higher potency to inhibit all the tested properties of *N. kaouthia* PLA_2 enzymes, except the inhibition of anticoagulant activity.

Kinetics study shows that inhibition of catalytic activity of NK-PLA_2-I (Fig. 3) and NK-PLA_2-II (data not shown) by AIPLAI is non-competitive in nature because both the slop and the 1/[v] intercept of the Lineweaver–Burk plot increases in the presence of PLA_2 inhibitor (AIPLAI), but the intercept on the 1/[s] axis (−1/km) does not change, indicating that km value of PLA_2 enzyme for the substrate is not altered in the presence of AIPLAI but

Table 2
Inhibition of catalytic pharmacological properties of PLA_2 enzymes (1 μg/ml) of crude venom samples by isolated pure compound AIPLAI (100 μg/ml)

Properties	% Inhibition by AIPLAI		
	N. naja	*N. kaouthia*	*D. russelli*
PLA_2 activity	28.0[a]	27.0[a]	23.0[b]
Indirect hemolytic activity	34.0[a]	36.0[a]	31.0[b]
Anticoagulant activity	86.0[a]	90.0[a]	N.D.
Edema induction	24.0[a]	24.0[a]	18.0[b]
In vitro tissue damaging activity			
Liver	48.2[a]	50.2[a]	43.0[b]
Heart	46.1[a]	46.0[a]	39.0[b]
Lung	39.0[a]	40.0[a]	33.0[b]

Property exhibited by crude venom PLA_2 enzymes in absence of AIPLAI was considered as 100% activity and other values were compared with that. Values are mean of triplicate determinations. Values in the same row with different superscripts are significantly different ($p<0.05$).
N.D., not determined.

Table 3
A comparison of anti-PLA_2 activity of AIPLAI and rabbit anti-NK-PLA_2-I antibodies. Purified PLA_2 enzymes from *N. kaouthia* venom were incubated either with AIPLAI or with anti- NK-PLA_2-I antibodies in a ratio of 1:100 (in a final volume of 1.0 ml) at 37 °C for 30 min and then assayed for residual catalytic and pharmacological properties of the enzymes

Property	% Inhibition of activity			
	NK-PLA_2-I		NK-PLA_2-II	
	AIPLAI	AB	AIPLAI	AB
PLA_2 activity	14.2[a]	41.0[b]	10.0[a]	42.0[b]
Anticoagulant activity	85.0[a]	21.0[b]	64.0[a]	26.0[b]
Indirect hemolytic activity	21.0[a]	86.0[b]	18.2[a]	72.0[b]
Edema induction	10.2[a]	13.7[a]	9.0[a]	12.0[a]
In vitro tissue damaging activity				
Liver	40.2[a]	100[b]	39.0[a]	96[b]
Heart	43.2[a]	100[b]	39.0[a]	93.1[b]
Lung	36.0[a]	100[b]	21.0[a]	94.0[b]
Mitochondrial swelling	68.0[a]	98.0[b]	59.0[a]	100[b]

Property exhibited by purified NK-PLA_2 enzymes in the absence of AIPLAI or NK-PLA_2-I antibodies was considered as 100% activity, and other values were compared with that. Values are mean of triplicate determinations. Values in the same row for the same PLA_2 enzyme with different superscripts are significantly different ($p<0.05$).
AIPLAI: *A. indica* PLA_2 inhibitor.
AB: anti-NK-PLA_2-I antibodies.
[c] Isolated from chicken liver.

Vmax decreases. The non-competitive nature of AIPLAI was further confirmed by the fact that inhibition produced by this compound on PLA_2 enzyme did not change with an increase in the substrate concentration (unpublished observation). Therefore, it may be assumed that AIPLAI binds at a site on the PLA_2 enzymes other than the substrate-binding site resulting in inactivation of catalytic site. The K_i (inhibitor constant) for AIPLAI was calculated as 0.5 and 0.55 mM for NK-PLA_2-I and NK-PLA_2-II, respectively.

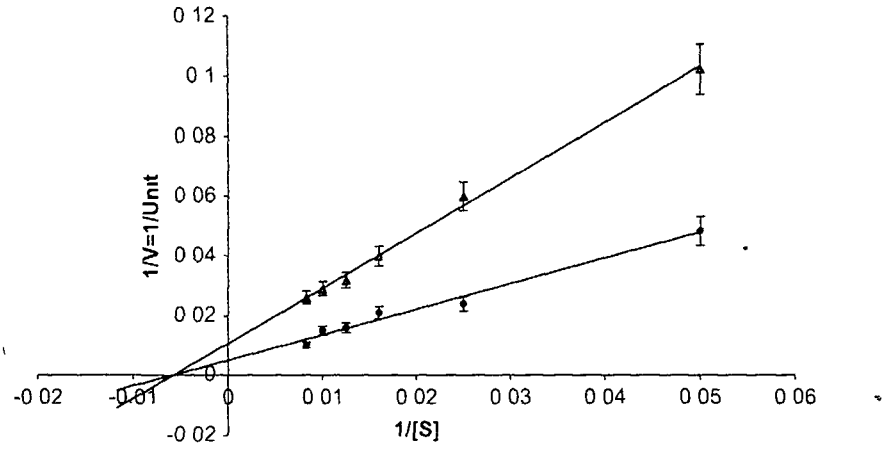

Fig 3 Kinetic study of interaction between NK-PLA_2-I and AIPLAI

The purified anti-PLA_2 compound (AIPLAI) is quite stable at room temperature, and 12% of its PLA_2 inhibitory activity is lost post storage for 6 months (in the desiccator) at room temperature This property of AIPLAI should be considered as highly advantageous in overcoming one of the major limitations of currently available commercial antivenoms as the anti-snake venom vials 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 traveling a long exhaustive journey Late antivenom therapy may not be useful in saving the lives of these patients (Mukherjee 1998) Since the anti-PLA_2 compound (AIPLAI) isolated in the present study need not be stored in refrigerated condition, it can easily be kept in the rural health centers where the maximum incidences of snakebites are reported

In conclusion, AIPLAI is a highly promising candidate for the development of novel anti-snake venom drug in future Further *in vivo* study with suitable animal models to explore the true potential of AIPLAI in inhibiting the snake venom PLA_2 enzymes and elucidation of chemical structure of AIPLAI are our next goals

Acknowledgments

Authors thank Dr P K Das Department of English and Foreign Languages, Tezpur University for editing the manuscript and Head SAIF Shillong for FT-NMR analysis of the sample Mr R Doley and Ms D Saikia were the recipients of the UGC projects fellowships This work was supported by Research Grants to A K M from University Grants Commission (UGC) New Delhi

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Corrigendum

Sl. No.	Chapter/ Page No/ Paragraph/ Section/ Line	Mistake	Corrigendum/Should be read as
1	Chapter IV, Page 8, section 4.1	"at the pH 7.0...."	"at pH 7.0...."
2	Chapter IV, Page 85, second paragraph, section 4.2	"Fig (flow chart) 4.3 represents the flow chart of...."	"Fig 4.3 represents the flow chart of...."
3	In the chapter V, VI and VII	FFAs	FAs
5	References: Ref No. 60	Sarkar, N.K. & Devi, A., 1968. "In: *Venomous*....." New York.167, 1968	Sarkar, N.K. & Devi, A. "In: *Venomous*....." New York.167, 1968.
6	References: Ref No. 77	Daltry, et al. 1996. "Diet.....". *Nature* 379, 537–540, 1996	Daltry, et al.. "Diet.....". Nature 379, 537–540, 1996
7	References: Ref No. 106	Chakrabarty et al. 2002. "Purification and.....". *Mol Cell Biochem* 237, 95-102, 2002.	Chakrabarty et al. "Purification and.....". Mol Cell Biochem 237, 95-102, 2002.
8	References: Ref No. 251	Ellman et al., 1961. "A new.....". *Biochem Pharmacol* 7, 88-90, 1961.	Ellman et al. "A new.....". *Biochem Pharmacol* 7, 88-90, 1961.

Debashree Saha.

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Addendum (Figure)

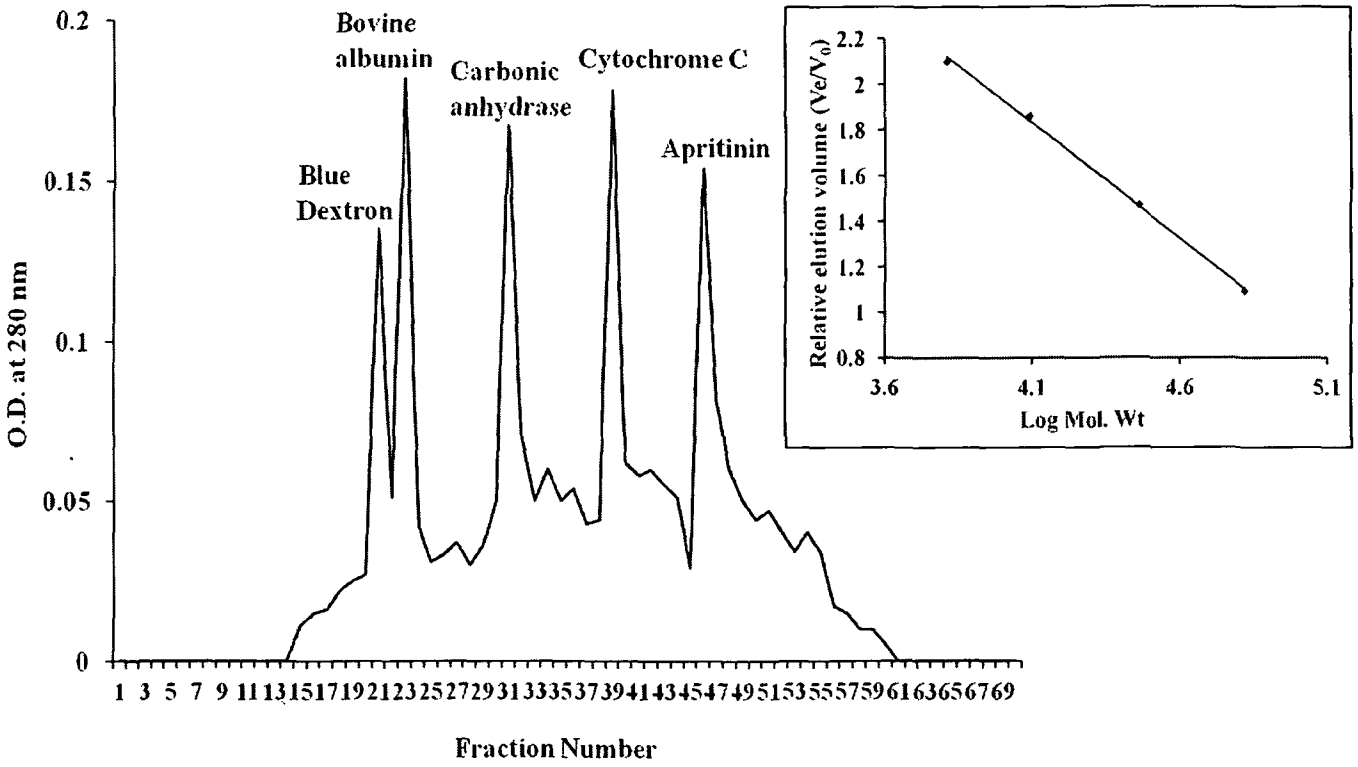

Fig: The elution profile of the molecular weight standards from Sephadex G-50 column. The column was equilibrated with following molecular weight markers; A. blue dextran (2,00,000 Da); B. BSA (66,000 Da); C. carbonic anhydrase (29,000 Da); D. cytochrome C (12,400 Da) and E. aprotinin (6,500 Da). **Inset:** Calibration curve of Sephadex G-50 column chromatography.

Debashree Saikia.

(Signature of the student)