Chapter 7

Conclusion and Future Prospects

7.1 Conclusion

In the current study the venom composition of Indian *Daboia russelii* from four different geographical locations of India, viz: West Bengal, Tamil Nadu, Kerala and Karnataka were analysed by electrophoretic and chromatographic techniques. The electrophoretic profile of all the four venoms displayed the presence of both high and low molecular mass proteins with different band intensities. The gel filtration and Rp-HPLC profile of the four venoms also presented substantial difference in their elution pattern. Moreover, all the four crude venoms displayed differential level of procoagulant and PLA_2 activity. Thus, considerable variations in the venom composition of the four crude venoms were observed both qualitatively and quantitatively which suggests the differential level of protein expression in their venom glands. The commercially available Indian polyvalent antivenom produced against the "Big four" snake venoms could differentially neutralize the procoagulant and PLA_2 activity of the four venoms. Antivenomics studies revealed the presence of N-toxins in the four venoms and P-toxins in RvTN and RvKA. Thus, the inefficacy of the polyvalent antivenom to neutralize and immunodeplete the four crude venoms could be due to the presence of these P and N-toxins. Hence, the present study emphasizes the need to consider compositional variation and non-immunogenicity of the venom proteins based on geographical location for developing regiospecific antivenom.

Further the study was focussed towards deciphering the complete proteome profile of the *Daboia russelii* venom procured from Irula by tandem mass spectrometry and analyse some of its crucial biochemical and biological activities. From the exhaustive investigation of the MS/MS spectra 63 different proteins belonging to 12 snake venom protein families were identified. This is the first report on the proteome profile of the Indian *Daboia russelii* venom by high throughput proteomics approach and the presence of snake venom protein families like 5'-NUC (4.7%), CRISPs (10.9%),

KSPI (4.7%), disintegrin (3.1%), VEGF (3.1%) and VNGF (1.6%) in its proteome. In addition to these, relatively abundant protein families like PLA_2 enzymes (23.4%), SvSPs (17.2%), SvMPs (9.4%), LAAO (7.8%), Snaclecs (9.4%) and PDE (3.1%) along with a hypothetical protein (1.6%) were also observed. Moreover, the proteome profile of Indian *Daboia russelii* displayed profound compositional dissimilarity from the venom profile of *Daboia russelii siamensis* from Myanmar and *Daboia russelii pulchella* from Sri Lanka (123,396). Myanmar viper comprised of 6 protein families, namely, SvSPs, SvMPs, PLA_2, LAAO, VEGF and Snaclecs. The absence of CRISP, VNGF, KSPI, 5'-NUC, PDE and the presence of daboiatoxin (the major lethal PLA_2 enzyme) in the Myanmar viper revealed its compositional variation from the Indian viper. On the other hand, the proteome profile of Sri Lankan viper unveiled the presence of 11 protein families, viz., PLA_2, Snaclec, SvSPs, SvMPs, LAAO, KSPI, VNGF, 5'-NUC, CRISPs, PDE and PLB Unlike the Indian viper it was devoid of VEGF and disintegrins and contained PLB in its proteome.

The last part of the study was directed towards the purification of a major anticoagulant PLA_2 enzyme from this Indian viper venom followed by its complete biochemical, biophysical and biological characterization. The purified major protein, named daboxin P accounts 24% of the total protein of the crude venom and has a molecular mass of 13597.62 ± 1.28 Da. Its primary structure is composed of 121 amino acids with 14 cysteine residues and secondary structure showed distinctive α-helical pattern while the 3D model displayed conserved structural scaffold, characteristics of PLA_2 enzymes. Sequence homology search revealed its close sequence similarity with snake venom PLA_2 enzymes with unique amino acid substitutions at 5 positions which make it unique from the already reported PLA_2 enzymes while phylogenetic analysis displayed its close contiguity to strong anticoagulant and FXa binding PLA_2 enzymes. Daboxin P was non-cytotoxic to the tested normal and cancerous mammalian cell lines and was devoid of antibacterial, proteolytic, haemolytic and fibrinogenolytic activities. It exhibited profound PLA_2 activity and delayed the plasma clotting time of RT, PT and APTT and ST but not TT. Although daboxin P did not inhibit the amidolytic activity of the activated serine proteases but it inhibited the activation of FX by both the Xase complexes even in the absence of phospholipids and post His48 alkylation. Furthermore, it also inhibited

prothrombin activation by the prothrombinase complex when pre-incubated with FXa followed by addition of FVa. This suggests its non-enzymatic action by protein–protein interaction apart from enzymatic action by phospholipid hydrolysis.The protein-protein interaction of daboxin P with FX and FXa was physically validated by fluorescence emission spectroscopy and affinity column chromatography.

Docking analysis revealed plausible interaction of the Ca^{2+} binding loop, helix C, anticoagulant region and C-terminal region of daboxin P with the heavy chain of FXa. Some of the residues involved in the interaction are reported to be crucial for binding of FVa on FXa (533). Hence, it was hypothesized that interaction of daboxin P with some of these crucial residues on FXa might hinder the binding of FVa on FXa, thus obstructing the formation of prothrombinase complex. Additionally, amino acid residues like Asp4 and Asp7 of the light chain and Asp185a, Lys186, Lys134 of the heavy chain of FXa are reported to be crucial for binding of TF/FVIIa complex on FXa (534). Noteworthy that, the crystal structure of FX is not available and as FX differs from FXa only in having the activation peptide (52 amino acid residues) hence, it was proposed that the interaction of daboxin P with some of these residues on FXa which are in vicinity of FVIIa-TF binding region, might create a steric hindrance impeding the association of the tenase complex with FX as well. Based on this mechanism, the purified protein has been named as Daboxin P i.e., *Daboia russelii* FX inhibitor PLA_2 enzyme. This is the first report of a PLA_2 enzyme from the Indian viper venom which targets both FX and FXa for its anticoagulant action.

7.2 Future prospects of the current study

1) The present study evaluates the need to analyse the venom composition of *Daboia r. russelli* venom from different geographical regions of India by modern high throughput proteomic techniques and advocates the need to design regiospecific antivenom for better efficacy and safety. It also emphasizes a comparative study of the venom composition of *Daboia russelii* from other geographical regions of India like Orissa, Maharashtra, Andhra

Pradesh, Gujarat and Punjab where the prevalence of Russell's viper envenomation is high.

2) Comparative venom analysis should be succeeded by neutralization and antivenomics studies using the commercial polyvalent antivenom which would provide a clear understanding about its efficacy and specificity towards the venom proteins. This relative study might, in turn, compel the antivenom producers to re-design the present procedure of antivenom production and make necessary modifications for its better therapeutic application in dealing with envenomated victims.

3) The present study reports for the first time the proteome profile of Indian *Daboia russelii* venom by tandem mass spectrometry which unveils the presence of 63 different proteins belonging to 12 snake venom protein families. Some of these proteins identified were found to be unique and not reported earlier from this Indian viper venom. As such it would be interesting to isolate and characterize such proteins which might unveil some of the unexplored aspects of this venom.

4) The study also emphasizes the need to carry out similar high throughput proteomic studies on *Daboia russelii* venom from other geographical regions of India. Such analysis would help in comparative analysis and might, in turn, unfold the variation in venom composition precisely at the proteome level.

5) Moreover, we also accentuate the need for transcriptomic study of the venom gland of this snake, a comparative analysis of which with the existing proteomic profile would provide a holistic outlook of its venomics as a whole.

6) Further, co-crystallization of daboxin P, the major anticoagulant PLA_2 enzyme from the venom of Indian *Daboia russelii* (Irula) with FX/FXa can be attempted which would provide a better understanding of the interaction with its anticoagulant targets.

7) Site directed mutagenesis can be performed to confirm the amino acid residues which were found to be critical for daboxin P-FXa interaction by docking study.

8) And last but not the least, since daboxin P is a natural inhibitor of FX/FXa so further *in-vivo* experiments can be designed to explore any possibilities to propose it as a template for anticoagulant drug design in future, if any.