CHAPTER 3

Comparative proteomic and biochemical profiling of Indian green pit vipers

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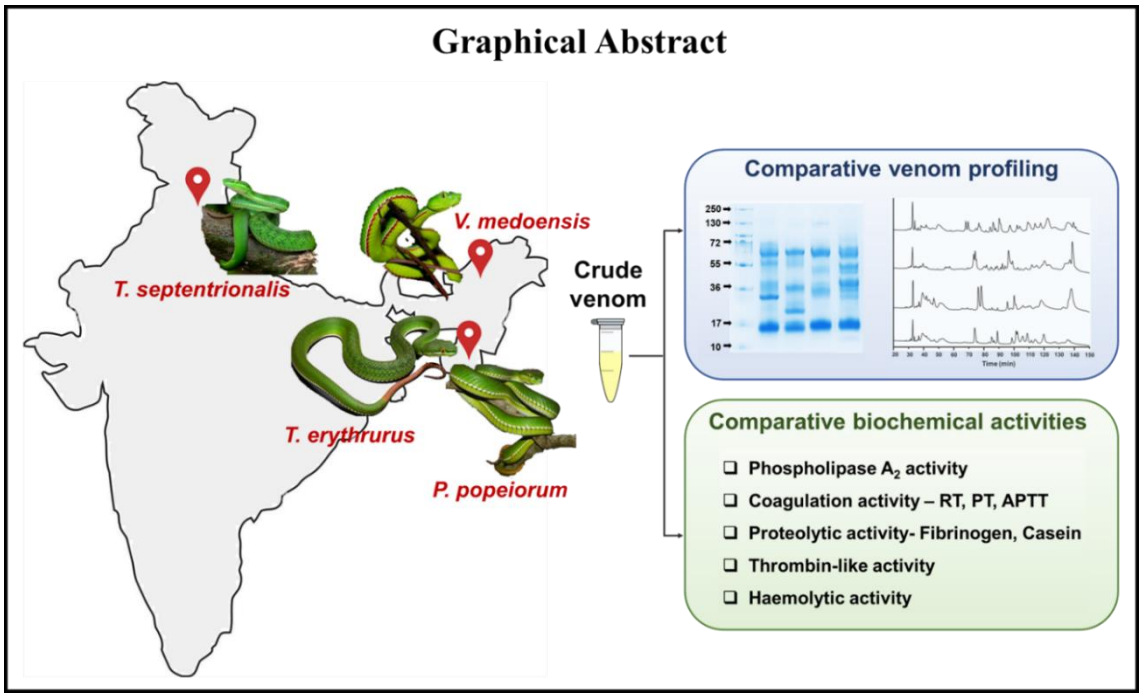

3.1. Introduction

Snake venom is a complex mixture of various bioactive molecules which induce an array of physiological alterations in prey as well as human victims. The high complexity of venom has been largely accredited to the process of gene duplication and trophic adaptation of snakes which facilitated transition from mechanical to chemical method of prey acquisition [1-3]. However, the composition of venom remains highly variable at taxonomic levels viz. family, genus, species and within same species [4]. Other than phylogeny, various factors like sex, geography, season, age and prey preference has been reported to influence variation in venom composition [4-11]. Thus, snakes having high phenotypic similarities might possess a very diverse venom composition. Notably, interspecific and intraspecific venom variation in a particular geographical region presents serious challenges of antivenom inefficacy in treatment of snakebite envenomated patients [12, 13]. Along with inefficacy concern, the

administration of non-specific toxins in the form of antivenom further increases the chances of adverse reactions in patients [14]. Consequently, the studies of inter and intraspecific venom variability among morphologically indistinguishable snakes of a particular geographical region is utmost important as this knowledge might aid in improvement of antivenom quality leading to better treatment of snakebite victims [4, 15].

The majority of snakebite in India is reported to be caused by "Big-Four" snakes, however, the northern and north-east India abode various species of pit vipers, banded kraits, black krait, monocled cobra etc. which are known to cause significant mortality and morbidity in victims. Among them, the most prevalent are the green pit vipers, which represent the largest group of venomous snakes belonging to the *Trimeresurus* complex of Asiatic pit vipers, having a typical bamboo-green coloured body. Although epidemiological reports of Indian green pit vipers are scarce in the literature, they have been reported to cause haematological abnormalities in patient causing morbidity leading to socio-economic burden to patient's family [16, 17]. Despite their predominance in this geographical region, there exists a dearth of knowledge regarding the venom composition, variation, and pathophysiological effects of the green pit vipers of Indian subcontinent. Thus, in the present study, profiling of crude Indian green pit viper venom was carried out to reveal their venom composition and understand their venom variation.

3.2. Results

3.2.1. Comparative analysis of SDS-PAGE profiles

The electrophoretic profiles of the crude venoms, analysed by gel electrophoresis under reducing conditions, revealed that the majority of protein bands were in the molecular weight range of 15-72 kDa in all the species studied (Figure 3.1). Markedly, 4-5 clusters of protein bands at approximately 15 kDa, 30-37 kDa, 50-55 kDa, and 60-75 kDa can be seen in all the lanes based on comparison with the molecular mass of the standard protein ladder. Presence of such protein bands broadly gives an idea of occurrence of major snake venom protein families in the venom. Moreover, the banding pattern across the species show some prominent differences in the number of bands present as well as in their intensities. For example, in addition to the common cluster of protein bands, *T. erythrurus* shows an additional faint band at 130 kDa, and *P. popeiorum* possess a prominent band at ~25 kDa, which are absent in all other venoms. Likewise, a

dense band at 15 kDa is present in all the species; however, the intensity of the band varies to a great extent among the species.

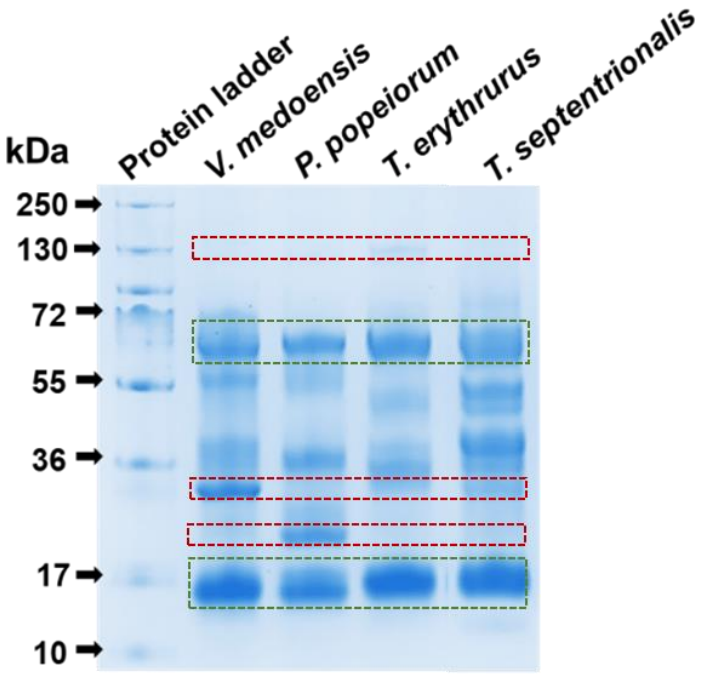

Figure 3.1. Electrophoretic profile of crude venoms of green pit vipers. Crude venom (20 µg) was run on 10% Tris-Tricine gel in reducing condition. PageRuler plus prestained protein marker was used as standard protein ladder (10-250 kDa). The green box indicates the protein bands which are present in all the species and the red box indicates the bands showing differences in banding pattern across species.

3.2.2. Comparative analysis of chromatographic profiles

The variation in venom composition of crude venoms was further studied using reverse phase chromatography (Figure 3.2). The elution pattern of different peaks clearly showed qualitative as well as quantitative differences among the species, along with some similarities (Figure 3.2). It is noticeable that under similar chromatographic conditions, all the peaks were eluted from 30 to 150 minutes in a gradient range of 20-80%; however, the number of peaks and their intensities vary to a great extent across the species.

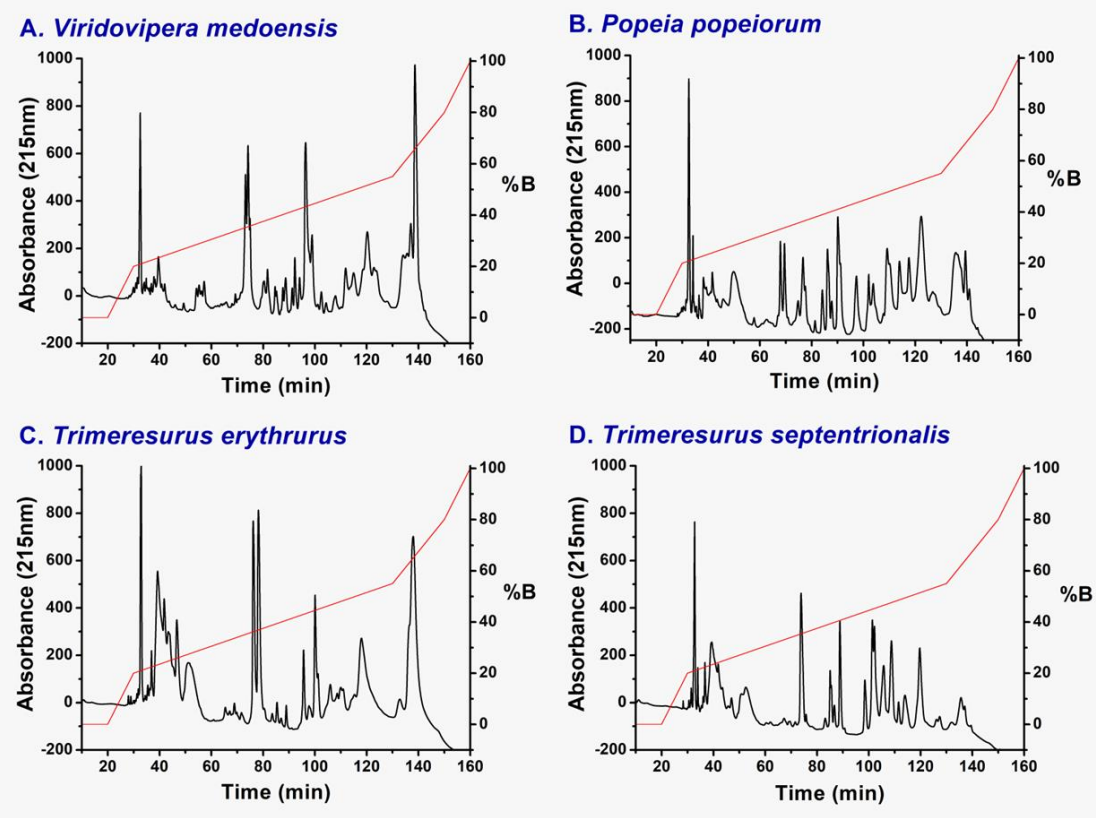

Figure 3.2. Reverse phase chromatographic profiles of green pit vipers. Each crude venom (2 mg) was loaded on Symmetry C18 column pre-equilibrated with Milli Q water and 0.1% TFA. Elution of protein was carried out at a flow rate of 1 ml/min with 80% Acetonitrile in Milli Q water containing 0.1% TFA. Gradient used for separation was 20-55% for 100 minutes followed by 55-80% for 20 minutes.

For ease of comparison, the elution profiles were divided into five zones and labelled as I-V (Figure 3.3). Zone I contains a sharp peak that is present in all the species with variable size; however, an extra small peak is also present in Zone I of *P. popeiorum* and *T. septentrionalis* venom. Zones II and IV contain a wide group of smaller and larger peaks which differ considerably among all the four species. Zone III shows a very dissimilar pattern across the species. Notably, *T. septentrionalis* and *T. erythrurus* contain one and two sharp peaks respectively, while *V. medoensis* contains a complex of three or more proteins and *P. popeiorum* contains 4-5 peaks with varied elution time. The two peaks present in *T. erythrurus* were nearly double the size of corresponding peaks present in *V. medoensis*. Zone V is characterized by the presence of a protein complex with variable peak area in *V. medoensis, P. popeiorum,* and *T. erythrurus*, which is almost absent in *T. septentrionalis*. The protein complex of zone V contains a sharp peak which was nearly three times more abundant in *P. popeiorum* and *T. erythrurus* as compared to *V. medoensis*.

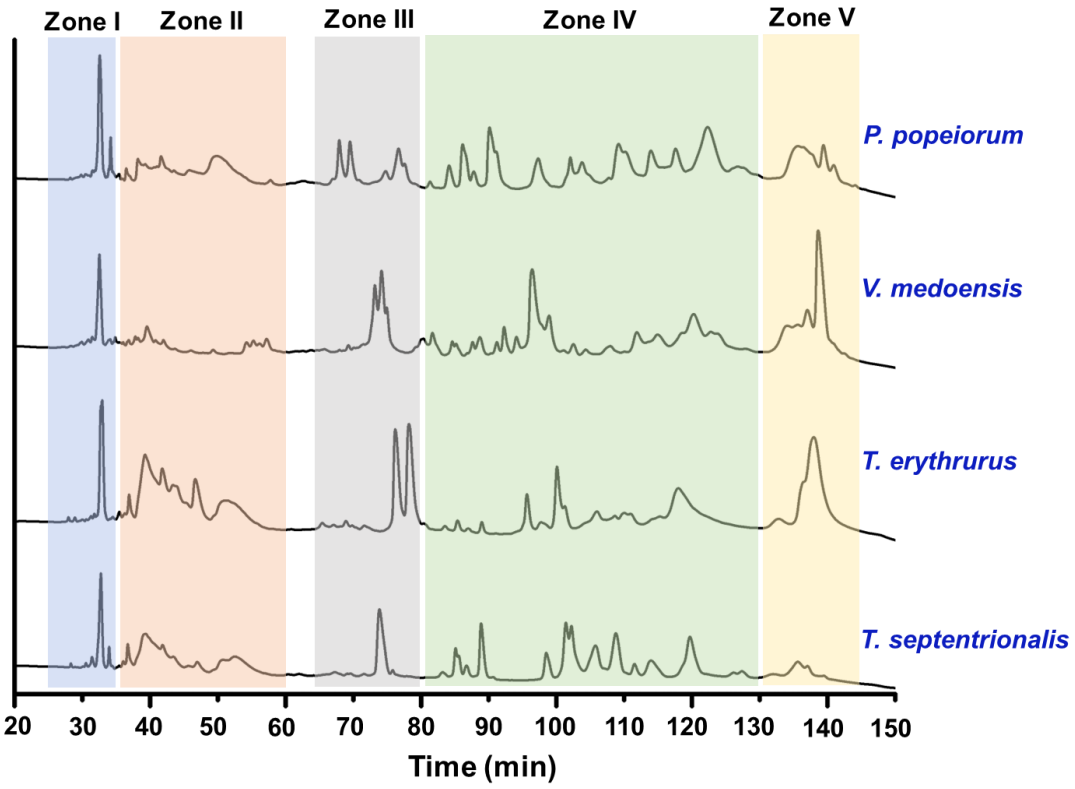

Figure 3.3. Comparative Rp-HPLC chromatogram of green pit vipers. Chromatographic condition was kept same for all the samples. Based on distribution of peaks, the profile was divided into five zones: I (25-35 minutes), II (35-60 minutes), III (65-80 minutes), IV (80-130 minutes) and V (130-145 minutes).

3.2.3. Phospholipase A_2 activity

The crude venoms of green pit vipers under study showed PLA_2 activity in a dose-dependent manner (Figure 3.4, Table 3.1). Noticeably, the PLA_2 activity of *T. septentrionalis* venom (specific activity 12.72 ± 0.164 µmol/min/µg) was found to be the highest among all the green pit viper venoms studied, whereas, *V. medoensis*, *P. popeiorum*, and *T. erythrurus* show comparable PLA_2 activity with specific activities of 10.33 ± 0 µmol/min/µg, 9.75 ± 0.042 µmol/min/µg, and 11.19 ± 0.071 µmol/min/µg respectively, under the same experimental condition. Further studies involving comparison with *Daboia russelii* revealed its PLA_2 activity (specific activity of 14.14 ± 0.092 µmol/min/µg) to be significantly higher than *V. medoensis* and *P. popeiorum* at 1 µg/ml dose.

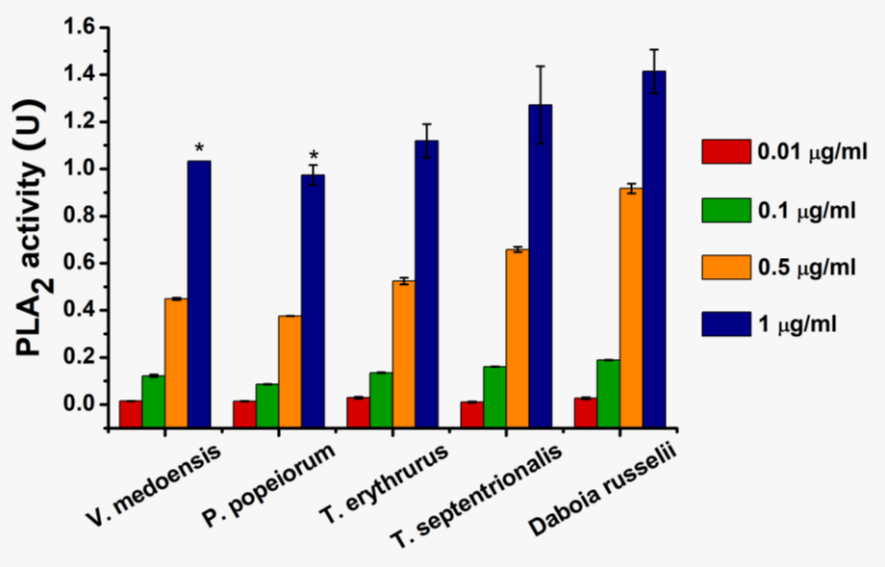

Figure 3.4. Phospholipase A_2 activity of crude venom of green pit vipers (*Popeia popeiorum*, *Viridovipera medoensis*, *Trimeresurus erythrurus* and *T. septentrionalis*). PLA_2 activity of the crude venoms were assessed using $sPLA_2$ assay kit in a dose-dependent manner. Enzyme activity (U) is expressed as micromoles of product formed per minute. Each data represents mean ± SD of three independent experiments. * *p* value < 0.05 with respect to *Daboia russelii*.

3.2.4. Coagulation activity

The effect of crude venom of the green pit vipers on the coagulation of platelet poor plasma (PPP) was assessed by observing the recalcification time (Figure 3.5A). At a concentration of 1 μg/ml, *Trimeresurus septentrionalis* showed a decrease in clotting time by 43 ± 5.7s as compared to the normal clotting time (NCT) of 170s. At a higher dose of 10 μg/ml, the clotting time was further decreased to 23s in *T. septentrionalis* and 33s in *T. erythrurus*, which was similar to the clotting time of *D. russelii*, suggesting the procoagulant nature of venoms. *Viridovipera medoensis* and *P. popeiorum* also showed similar coagulant activity at a still higher dose of 20 μg/ml. No significant change was observed in prothrombin time of plasma treated with crude venoms except for *T. septentrionalis*, which showed a decrease in PT compared to NCT (Figure 3.5B), however, APTT was found to decrease with respect to NCT in all the crude venoms (Figure 3.5C).

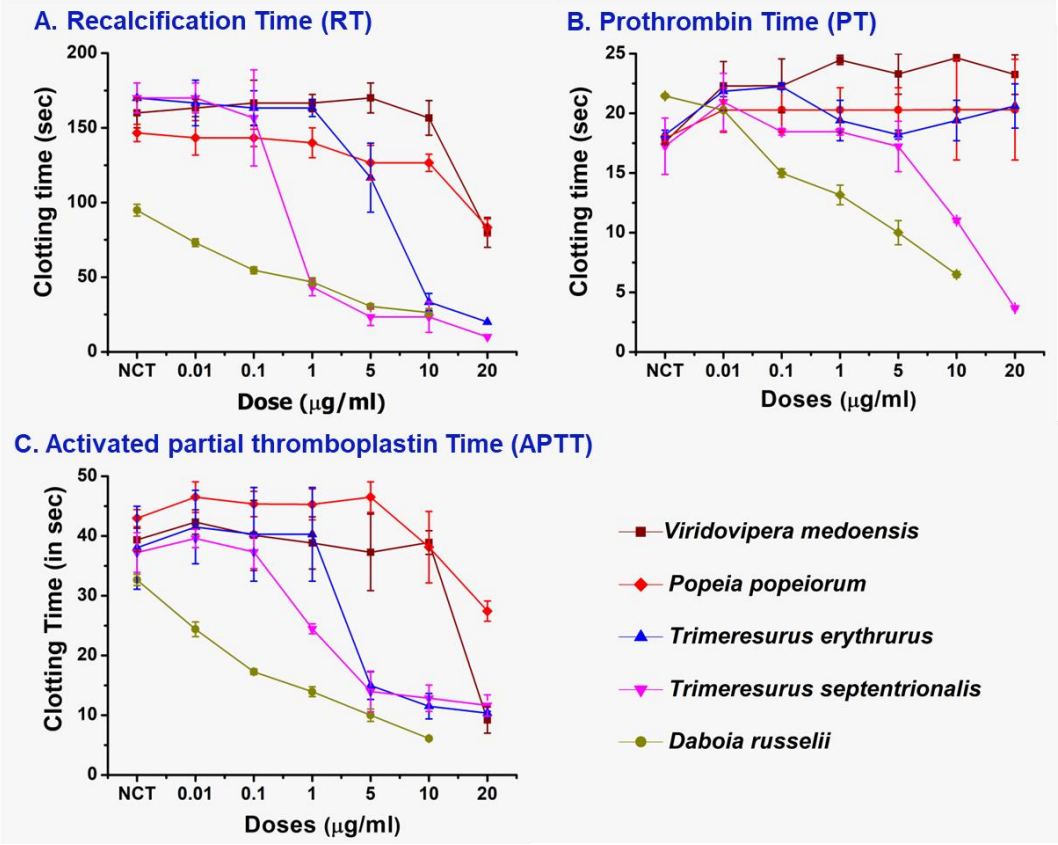

Figure 3.5. Coagulation activity of crude venom of green pit vipers. (A) Recalcification time (RT), (B) Prothrombin time (PT), (C) Activated partial thromboplastin time (APTT). NCT (Normal Clotting Time) represents clotting time of plasma in presence of Tris buffer (20 mM, pH 7.4). Each point represents mean ± SD of three independent experiments.

3.2.5. Thrombin-like activity

The crude venoms of green pit viper under study showed thrombin-like activity at a minimum dose of 10 µg/ml (Figure 3.6, Table 3.1). *Trimeresurus septentrionalis* possesses the highest activity (specific activity 6.256 ± 0.828 µmol/min/µg) followed by *T. erythrurus* and *P. popeiorum* i.e., 4.270 ± 0.194 µmole/min/µg and 2.094 ± 0.166 µmole/min/µg respectively. However, *V. medoensis* showed very less activity i.e., 0.607 ± 0.083 µmole/min/µg compared to others. However, the activity of Thrombin (0.5 nM in reaction) was found to be significantly higher than all the venom except *T. septentrionalis* at 10 µg/ml. At a dose of 20 µg/ml, *T. erythrurus* and *T. septentrionalis* showed approximately 2U higher activity than Thrombin.

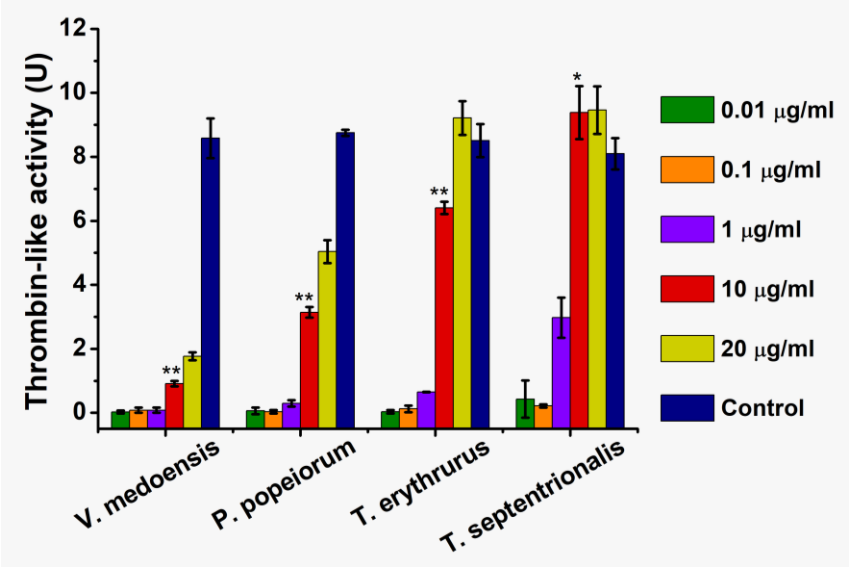

Figure 3.6. Thrombin-like activity of crude venom of green pit vipers. Enzyme activity (U) was expressed in micromoles of product formed per minute. Control represents the enzyme activity of Thrombin. Each data represents mean ± SD of three independent experiments. * *p* value < 0.05 and ** *p* value < 0.01 with respect to Control (Thrombin).

3.2.6. Proteolytic activity

The crude venoms of the species under investigation showed proteolytic activity by hydrolysis of fibrinogen and casein very effectively. Proteolysis of fibrinogen was observed clearly on Coomassie stained gel (Figure 3.7A). Upon incubation with fibrinogen for 1 hour, the proteolytic enzymes in crude venom first cleave the Aα chain followed by hydrolysis of the Bβ chain on further increment of the dose (Figure 3.7A). *Viridovipera medoensis* visibly showed the highest fibrinogenolytic activity by degrading Aα band at a dose of 1 μg/ml, while the other three venoms showed similar degradation pattern at 5 μg/ml dose.

The crude venoms also exhibited caseinolytic activity dose-dependently (Figure 3.7B). The comparative caseinolytic activity of the crude venoms is shown in Table 3.1. Notably, *T. erythrurus* is shown to possess the highest caseinolytic activity of 45.11 ± 0.011 U/mg, which was significantly more than the activity shown by Russell's viper venom. Other venoms showed caseinolytic activity in a decreasing order of *V. medoensis*, *T. septentrionalis*, and *P. popeiorum* respectively (Table 3.1).

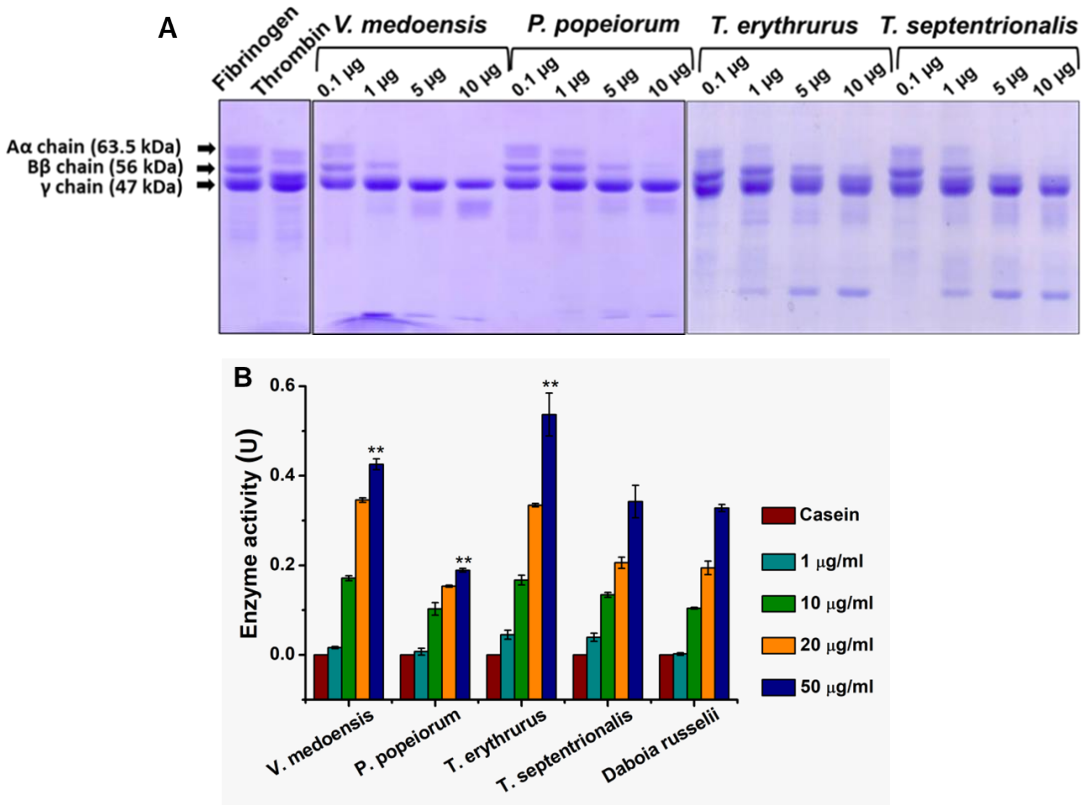

Figure 3.7. Proteolytic activity of crude venom of green pit vipers. (A) Fibrinogenolytic activity. Pattern of band degradation was observed in SDS-PAGE profile of fibrinogen after incubation with different doses of crude venoms. **(B) Caseinolytic activity.** Caseinolytic activity (U) was expressed as n mole equivalent of tyrosine formed per min per ml. Each data represents mean ± SD of three independent experiments. ** *p* value < 0.01 with respect to *Daboia russelii.*

3.2.7. Haemolytic activity

Crude venom of the green pit viper species showed a direct effect on hydrolysis of goat RBC at higher doses (Figure 3.8A). At a dose of 20 µg/ml, *V. medoensis* showed the highest haemolysis percentage of 36.2 ± 0.007 with respect to the control (haemolysis by distilled water). The comparative haemolytic activity of other venoms is shown in Table 3.1. When indirect haemolytic activity was investigated in the presence of egg yolk using goat RBC, the haemolysis was higher than the direct haemolytic activity (Figure 3.8B). Similarly, *V. medoensis* exhibited the highest indirect activity, which was also much higher than the direct activity. At 20 µg/ml dose, *V. medoensis* shows 59.5 ± 0.678% haemolysis followed by 43.8 ± 1.102%, 36.5 ± 1.13%, and 21.3 ± 0.624% for *P. popeiorum*, *T. septentrionalis* and *T. erythrurus*, respectively. However, the haemolytic activity of green pit viper venoms was found to be significantly much less than that of crude Russell's viper venom in both direct and indirect assays.

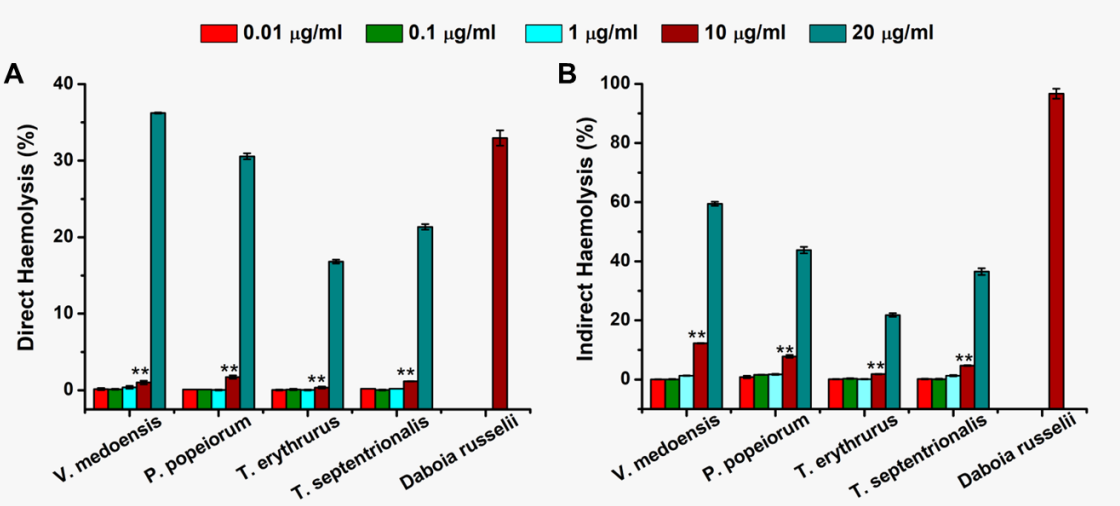

Figure 3.8. Haemolytic activity of crude venom of green pit vipers. Haemolytic activity of crude venom was assessed on 10% RBC suspension. Haemolytic percentage of distilled water was considered as 100%. **(A)** Direct haemolysis for different doses of crude venom **(B)** Indirect haemolysis for different doses of crude venom. ** *p* value < 0.01 with respect to *Daboia russelii*.

Table 3.1. *In-vitro* enzymatic activity of Indian green pit viper venoms

Enzymatic activity	***Viridovipera medoensis***	***Popeia popeiorum***	***Trimeresurus erythrurus***	***Trimeresurus septentrionalis***
PLA_2 activity (U/µg)	10.33 ± 0	9.75 ± 0.042	11.19 ± 0.071	12.72 ± 0.164
Caseinolytic activity (U/mg)	16.19 ± 0.002	7.2 ± 0.007	45.11 ± 0.011	39.33 ± 0.009
Thrombin-like activity (U/µg)	0.607 ± 0.083	2.094 ± 0.166	4.270 ± 0.194	6.256 ± 0.828
Percent direct haemolysis (for 20 µg/ml)	36.2 ± 0.076	30.6 ± 0.392	16.8 ± 0.256	21.3 ± 0.367
Percent indirect haemolysis (for 20 µg/ml)	59.5 ± 0.678	43.8 ± 1.102	21.8 ± 0.624	36.5 ± 1.13
Minimum procoagulant dose (µg/ml)	10	10	5	1
Minimum fibrinogenolytic dose (µg)	1	5	5	5

3.3. Discussion

Green pit vipers represent a large group of morphologically indistinguishable snakes which are responsible for majority of snakebite incidences in south-east Asian countries like Thailand, Vietnam, and southern China [18-20]. Although non-fatal, their venom can cause several haemotoxic anomalies, leading to severe morbidity in the patients [21, 22]. Due to similarity in climatic and geographic conditions, the north-eastern India presents quite similar range of floral and faunal diversity with their neighbouring south-east Asian countries. As a result of this, prevalence of green pit vipers is observed in northern and north-eastern states of India. However, no study commencing the venom dynamics of Indian green pit vipers has been reported till now. The present study was undertaken to characterize the crude venom of Indian green pit vipers and to assess the interspecific venom variation among them in terms of venom composition and enzymatic activities.

Analysis of crude venoms by electrophoresis revealed multiple clusters of protein bands ranging from 15-72 kDa, depicting complex venom composition of a typical Viperidae snake. The wide-ranging electrophoretic profile corresponding to diverse protein families directly relates to the broad range of pathophysiological effects observed in victims of green pit vipers. Similar observations have been previously made in the case of *Popeia nebularis* venom, where the SDS-PAGE profile showed prominent bands including SVPs, SVSPs, PLA_2s, and disintegrins, which were later confirmed by mass-spectrometric profiling and correlated with pathophysiological manifestations like swelling, pain, coagulopathy, haemorrhage, and thrombocytopenia [23]. Further, the SDS-PAGE profile also revealed similarities and dissimilarities in the venom composition of green pit vipers under study, marked by the presence of some unique and common bands of variable band intensities. The presence of a prominent band at ~15 kDa with comparable intensities in all the crude venom indicates the abundance of PLA_2s in the venom. Moreover, unique band at 130 kDa in *T. erythrurus* and ~25 kDa in *P. popeiorum* suggests the presence of distinctive proteins, which might provide with additional toxicity to these venoms.

The compositional variations and similarities among the crude venoms of Indian green pit vipers were further evaluated by comparing the elution profiles obtained from reverse phase chromatography. The disparities in elution profiles in terms of both the

number of peaks as well as their abundance indicates variation in venom composition among the crude venoms. The variations in the height of Rp-HPLC fractions between crude venoms are primarily caused by differences in the expression levels of proteins [24]. However, proteomic analysis is crucial to further confirm such disparities in protein expression as well as to get an insight into the complex protein profile responsible for various pathophysiological effects in prey/victims. Interspecific variation in venom composition has been previously reported in literature [4, 25-27]. However, various environmental factors like sex, geography, season, age, and diet have also been reported to immensely influence variations in venom composition [4-11].

Furthermore, the crude venoms of Indian green pit vipers were assessed for *in vitro* enzymatic assays in order to determine various toxic effects of venom on prey/victim as well as their amplitude of variation. Phospholipase A_2 is one of the major snake venom enzymatic families and plays an important role in the immobilization and digestion of prey. In addition to this, they are responsible for a wide range of clinical conditions, including myotoxicity, neurotoxicity, cardiotoxicity, coagulopathy, platelet aggregation inhibition, haemolysis, haemorrhage as well as organ/tissue damage in bite victims [28]. The crude venom of green pit vipers showed *in vitro* PLA_2 activity in a dose dependent manner, illustrating the presence of enzymatically active PLA_2s in the venom. The presence of enzymatically active PLA_2s suggests their significant role in inducing pathophysiological effects in patients and in the overall toxicity of venom. Indirect haemolytic activity on red blood cells (RBCs) further confirms the presence of enzymatically active PLA_2s in the crude venom. The PLA_2s cleave the phospholipid lecithin present in egg yolk, thereby forming lysolecithin, which eventually helps in the breakage of the erythrocyte membrane in the indirect haemolysis. However, the PLA_2s in venom are mostly present as multiple isoenzymes exhibiting different pharmacological profiles, and are not necessarily enzymatically active [29, 30]. Among the green pit vipers studied, *T. septentrionalis* showed maximum PLA_2 activity at a given dose, indicating a higher amount of enzymatically active PLA_2s in the venom, however, it was less than the PLA_2 activity of Russell's viper venom.

Green pit vipers are known to instigate haemostatic alterations leading to prolonged coagulopathy in victims [21, 22, 31]. The crude venoms of Indian green pit vipers were also assessed for its ability to affect coagulation cascade. It was observed

that the venom decreased the recalcification time (RT) of platelet poor plasma with increasing dose indicating the procoagulant nature of venom. Moreover, the venom showed decrease in activated partial thromboplastin time (APTT) but not prothrombin time (PT) suggesting their potential target to be the intrinsic pathway of blood coagulation cascade. However, *T. septentrionalis* showed procoagulant activity at a minimum dose of 1 µg/ml affecting both PT and APTT, indicating its strong procoagulant activity. The procoagulant nature of venoms was observed to be consistent with other species of the *Trimeresurus* radiation [32, 33]. Bourke and his team recently studied the coagulation in *T. albolabris* venom and reported that the venom could clot the plasma and fibrinogen in a pseudo-procoagulant manner, forming significantly weak clots which was measured using a Thrombelastogram haemostasis analyser [34]. These unstable clots dissolve readily giving an overall anticoagulant effect in envenomated patients as reported in various case studies [20-22, 31].

Viper venoms mostly contain thrombin-like enzymes (TLEs), belonging to the broad family of snake venom serine proteases (SVSPs). SVTLEs function like thrombin by cleaving either α or β bands of fibrinogen, releasing fibrin monomers which polymerize to form a weak thrombus. However, unlike thrombin, SVTLEs lack the ability to activate factor XIII and hence the thrombus gets dissolved. This leads to a clinical condition called consumptive coagulopathy [35], which has been observed frequently in patients with green pit viper bites [21]. Several thrombin-like proteins (TA-2, albolabrase, purpurase, chitribrisin) have been isolated and characterized from *Trimeresurus* venoms in the last few decades [36-39]. All these proteins range in molecular weight between 30-38 kDa and cleave fibrinogen to release either fibrinopeptide A or B or both. The presence of protein bands in the above-mentioned range in SDS-PAGE profile (Figure 2.2) led us to investigate the thrombin-like potential of the crude venoms. *In vitro* assessment of thrombin-like activity was performed using chromogenic substrate specific for thrombin. It was observed that the crude venoms of Indian green pit vipers showed significant elevation in hydrolysis of substrate unveiling their thrombin-like property. *Trimeresurus septentrionalis* showed highest thrombin-like activity followed by *T. erythrurus, P. popeiorum,* and *V. medoensis*. The presence of thrombin-like activity in crude venom is attributed to SVTLEs, which is suggested to be responsible for clinical conditions leading to consumptive coagulopathy and the detection of fibrin degradation products in bite patients [21, 40].

Snake venom proteases are ubiquitously observed in Viperidae family targeting various components of haemostatic system of prey. To assess the presence of proteases in Indian green pit vipers, fibrinogen and casein was used as substrate for proteolytic cleavage. The crude venoms were observed to cleave Aα and Bβ band of fibrinogen, thereby showing fibrinogenolytic activity. Further, crude venoms also showed caseinolytic activity in dose dependent manner, with *T. erythrurus* to show highest activity, which was even more than Russell's viper venom. Proteolytic activity of snake venoms is typically attributed to snake venom serine proteases (SVSPs) and snake venom metalloproteases (SVMPs), which are usually observed in SDS-PAGE in the molecular weight range of 25-55 kDa. Presence of protein bands in above-mentioned range in electrophoretic profile further confirms the presence of snake venom proteases in Indian green pit vipers. Various SVSPs causing proteolytic activity have been isolated from green pit viper venom, most of which are functionally analogous to thrombin [35, 37, 39]. A 23.4 kDa serine protease named malabarase has been isolated from *C. malabaricus* venom, which, along with fibrinogenolytic activity, displays proteolysis of casein [41]. Malabarin, a zinc-containing metalloprotease isolated from the same venom, shows procoagulant activity by degradation of fibrinogen to fibrin monomers leading to fibrin clot formation [42]. Malabarin preferentially cleaves Aα bond of fibrinogen followed by Bβ bond from N-terminal similar to the crude venoms in the present study. The functional similarity of crude venoms with these proteins indicates presence of homologous proteins in the green pit viper venom. Besides proteolytic activity, SVMPs have major roles in haemorrhage, tissue necrosis, inducing edema, which can be associated with swelling of bite site in patients and local tissue injury along with severe pain [40, 43, 44].

One of the essential components of haemostatic system is red blood cells (RBCs), which along with activated platelets and fibrin polymers, results in formation of clot, eventually preventing blood loss. Various snake venom proteins, specifically PLA_2s and cytotoxins are known to cause rupture of RBCs leading to excessive blood loss in patients. Crude venoms of Indian green pit vipers were also examined for haemolytic activity in goat RBCs and the results showed venom induced haemolysis of RBCs, however, the activity was observed to be much less than Russell's viper venom. Higher indirect haemolytic activity is due to the presence of enzymatically active PLA_2s in the crude venom (discussed above). The relatively lower direct haemolytic activity of crude

venom compared to indirect, indicates the weaker potential of crude venoms to directly induce lysis of phospholipid membrane of RBCs.

Thus, the present study unveils the biochemical profile of Indian green pit vipers, indicating the presence of proteins targeting various components of haemostatic system of prey and their correlation with various pathophysiological manifestations observed in envenomated victims. Among the studied species, *T. septentrionalis* venom exhibited highest activity in maximum number of *in vitro* enzymatic assays suggesting it to be most potent among them. Further, the green pit vipers of Indian subcontinent, although morphologically indistinguishable, showed substantial variation in venom composition depicted by their protein profile and biochemical activities. This study highlights interspecific venom variation, which can be attributed to the phylogenetic distance among the species as well as on other ecological factors.