Chapter 4

Identification and characterization of anti-platelet protein from *Daboia russelii* venom

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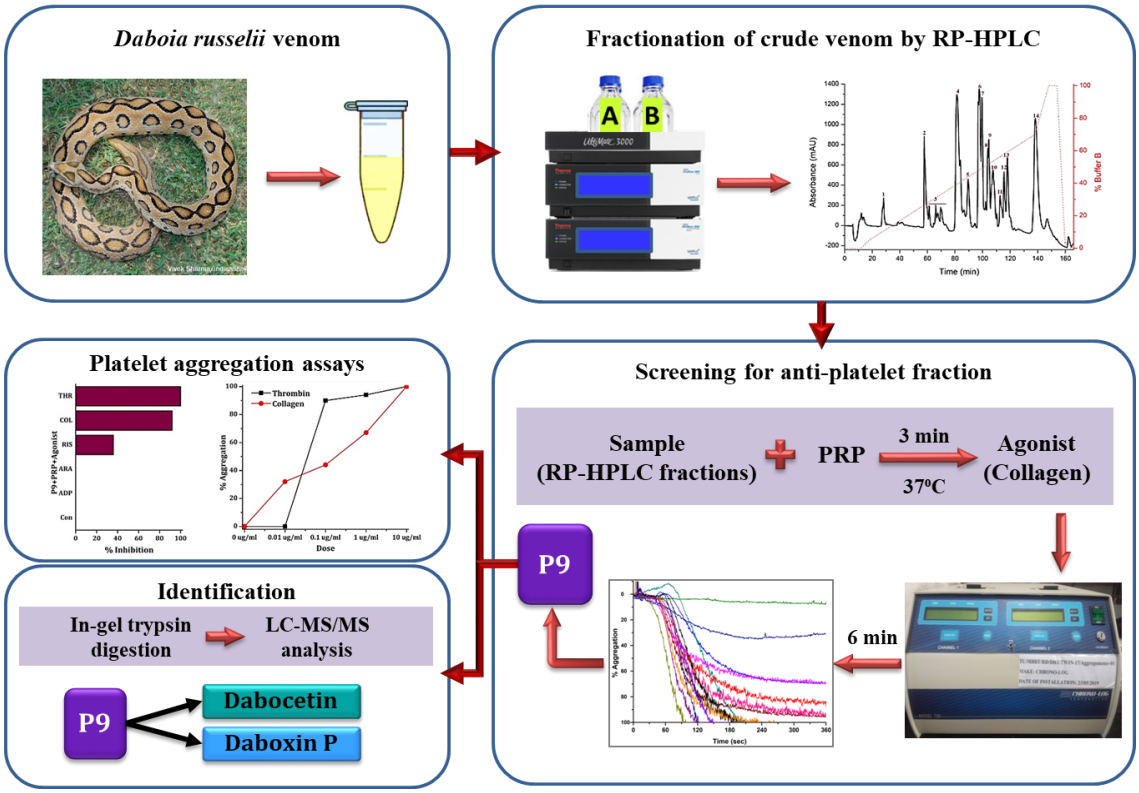
GRAPHICAL ABSTRACT



4.1 INTRODUCTION:

Snake venom is a rich concoction of pharmacologically active proteins and polypeptides with therapeutic potential. They act independently or synergistically for inducing various pharmacological effects in prey/victims [1–3]. A common target of snake venom proteins is the haemostatic system of the prey/victim, which is symptomized by haemotoxic conditions post envenomation. Such haemotoxic conditions are generally observed in cases of bites by snakes belonging to the Viperidae family. In such cases, a combination of symptoms such as anticoagulant activity,

consumption coagulopathy, thrombocytopenia, and vessel wall damage can result in severe blood loss, often becoming a common cause of fatality [179]. Over the years, a number of snake venom toxins that affect blood circulation have been studied which has led to the development of pharmacological tools or drugs for treatment of various conditions like coagulopathic abnormalities, cardiovascular diseases etc. and also as diagnostic tools [1–3,7,12].

Daboia russelii, belonging to family Viperidae is a medically important snake of the Indian sub-continent, distributed throughout Pakistan, India, Nepal, Bhutan, Bangladesh and Sri Lanka [2,81,83]. In India, during 2000-19, *Daboia russelii* alone was responsible for 43% of total snakebite incidences [180]. The venom of *Daboia russelii* is haemotoxic in nature and symptoms observed during envenomation include persistent and profuse bleeding at the bite site as well as at other sites such as gingival sulci, nose, gastrointestinal tract, conjunctivae and skin among others [81]. Studies have shown that *Daboia russelii* venom proteins interfere with the haemostatic system and a number of proteins affecting the blood coagulation cascade have been studied. For instance, RVV-X is a procoagulant heterocomplex comprising of snaclec and snake venom metalloproteinase that activate Factor X and Factor IX. While Daboxin P is an anticoagulant PLA_2 known to inhibit coagulation Factor X and Factor Xa [16,91]. However, not much work has been done to explore proteins affecting the platelet aggregation pathway from *Daboia russelii* venom and their detailed mechanism of action. In this chapter, identification and characterization of an anti-platelet protein is discussed.

4.2 RESULTS:

4.2.1 Fractionation of *Daboia russelii* venom by RP-HPLC:

Crude *Daboia russelii* venom was subjected to fractionation using Jupiter C18 column which resulted in separation into 14 fractions (Figure 4.1). The fractions were named as P1 to P14 corresponding to their fraction number and were screened for anti-platelet activity.

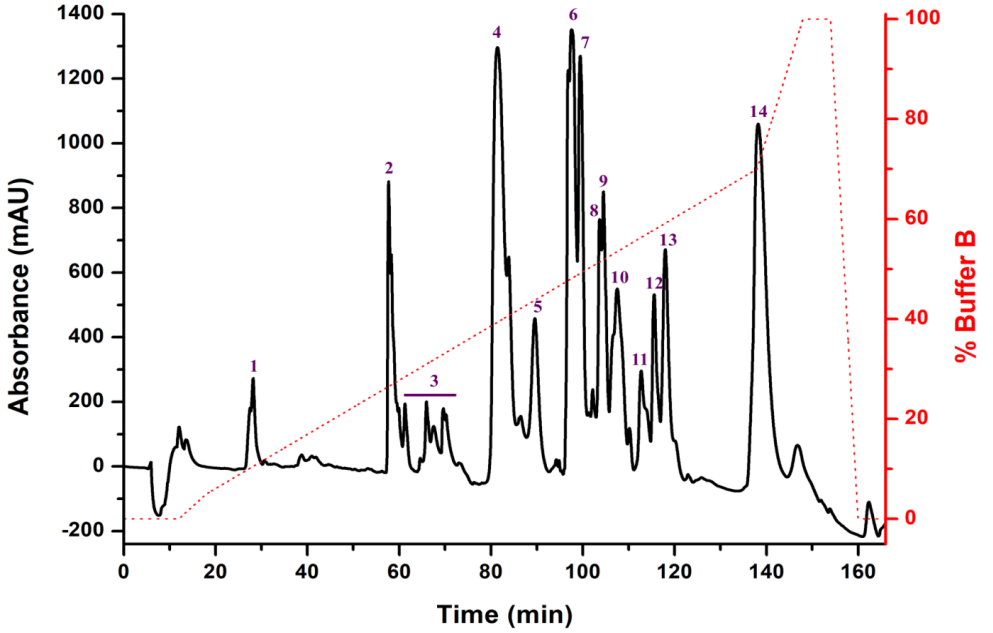


Figure 4.1: Fractionation of crude *Daboia russelii* venom: RP HPLC profile of 2mg crude *Daboia russelii* venom on Jupiter C18 column (4.6*250mm). Fractionation was carried out at a flow rate of 0.6 ml/min with a gradient of 5-70% of MeCN maintained in 15-140 minutes and monitored at 215 nm.

4.2.2 Screening of RP-HPLC fractions for anti-platelet activity:

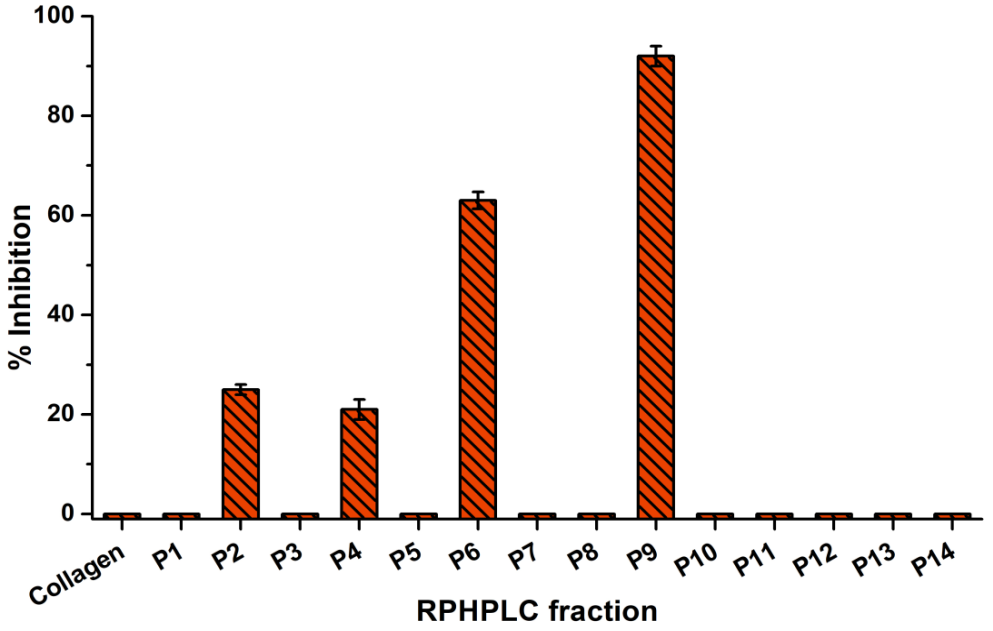


Figure 4.2: Effect of RP-HPLC fractions on platelet aggregation induced by collagen: (Briefly, 1µg of each RP-HPLC fraction was assessed for their effect on collagen-induced platelet aggregation. The results are mean ± SD of three individual experiments.)

The effect of 1µg of each RP-HPLC fraction on aggregation of platelet rich plasma (PRP) mediated by collagen was studied. It was observed that the fractions P2, P4, P6 and P9 inhibited 25±1%, 21±2%, 63±1.7% and 92±2.8% collagen induced platelet aggregation respectively (Figure 4.2). The fraction with the most anti-platelet activity was found to be P9.

4.2.3 Platelet aggregation studies of anti-platelet fraction (P9):

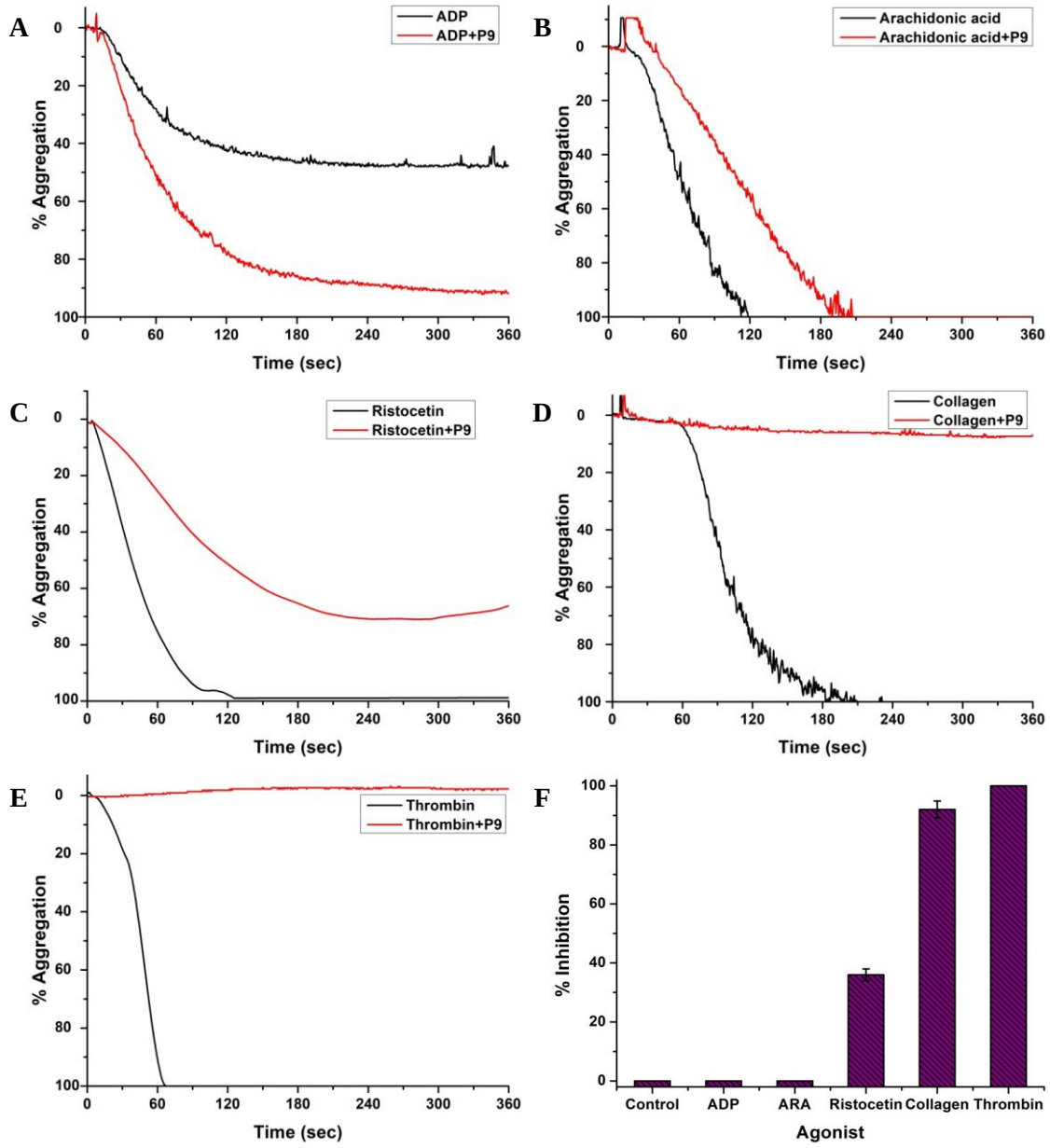


Figure 4.3: Effect of 1µg of P9 on platelet aggregation induced by various agonists: (A) Adenosine diphosphate (ADP), (B) Arachidonic acid (ARA), (C) Ristocetin, (D) Collagen, (E) Thrombin (F) Comparative bar graph.

4.2.3.1 Effect of P9 on platelet aggregation induced by various agonists:

The effect of P9 (1 µg) on platelet aggregation induced by various agonists was studied. It was observed that P9 could not inhibit platelet aggregation induced by ADP and arachidonic acid. However, platelet aggregation induced by ristocetin, collagen and thrombin were inhibited by 36±2%, 92±2.8% and 100% respectively (Figure 4.3).

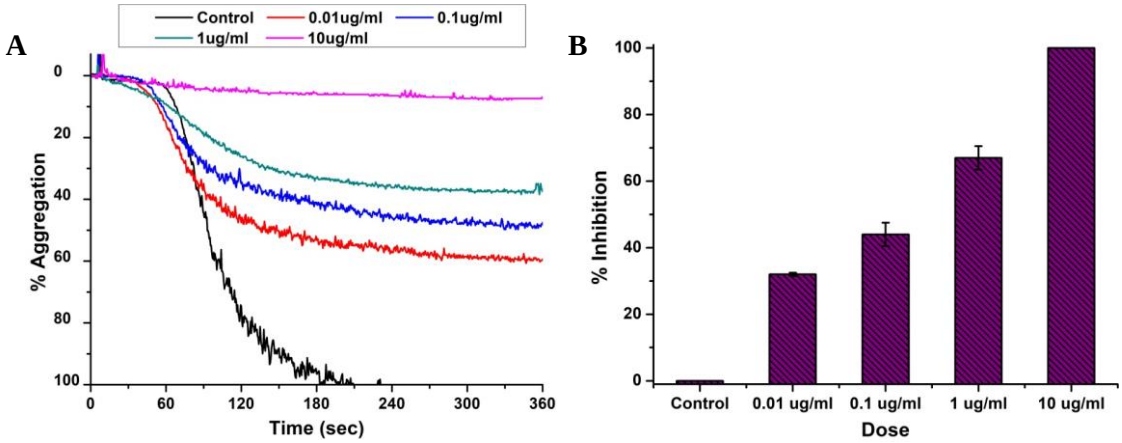


Figure 4.4: Dose dependent effect of P9 on platelet aggregation induced by collagen: (A) Progressive curve (B) Bar graph (Briefly, different doses of P9 (0.01µg/ml, 0.1 µg/ml, 1 µg/ml and 10µg/ml) were assessed for their effect on collagen-induced platelet aggregation. For bar graph, the results are mean ± SD of three individual experiments.)

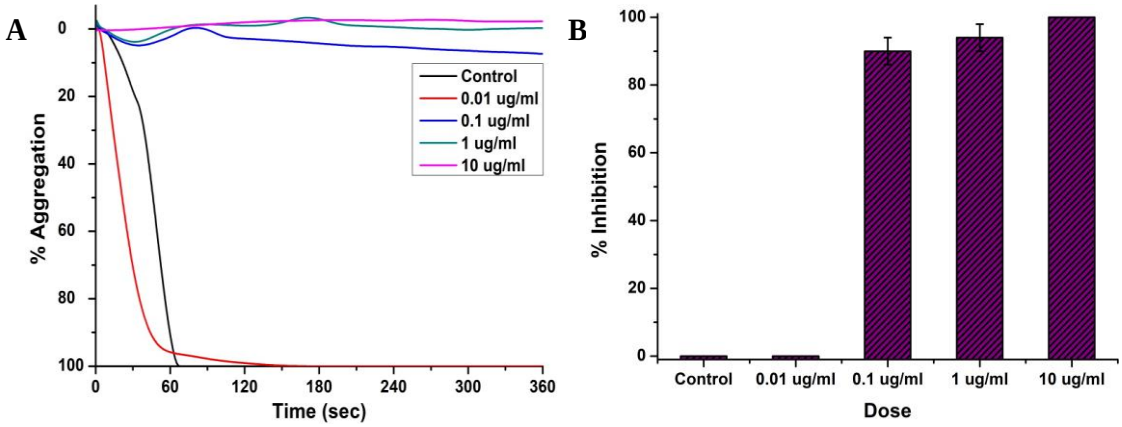


Figure 4.5: Dose dependent effect of P9 on platelet aggregation induced by thrombin: (A) Progressive curve (B) Bar graph (Briefly, different doses of P9 (0.01µg/ml, 0.1 µg/ml, 1 µg/ml and 10µg/ml) were assessed for their effect on thrombin-induced platelet aggregation. For bar graph, the results are mean ± SD of three individual experiments.)

4.2.3.2 Dose dependent effect of P9 on platelet aggregation induced by collagen:

P9 inhibited platelet aggregation induced by collagen in a dose dependent manner. Percentage inhibition of collagen induced platelet aggregation by 0.01, 0.1, 1 and 10 µg/ml of P9 was 32±0.5%, 44±3.5%, 67±3.5% and 100±0% respectively (Figure 4.4).

4.2.3.3 Dose dependent effect of P9 on platelet aggregation induced by thrombin:

P9 inhibited platelet aggregation induced by thrombin in a dose dependent manner. Percentage inhibition of thrombin induced platelet aggregation by 0.01, 0.1, 1 and 10 µg/ml of P9 was 0%, 90±4%, 97±4% and 100±0% respectively (Figure 4.5).

4.2.4 Partial biochemical characterization of P9:

4.2.4.1 SDS-PAGE:

SDS-PAGE of P9 displayed a single band of molecular weight ~15kDa in reducing condition while in the non-reducing condition; an additional band of ~30kDa was observed (Figure 4.6A).

4.2.4.2 Coagulation assays:

P9 slightly delayed the coagulation time of goat PPP. RT at concentrations, 0.01µg/ml, 0.1µg/ml, 1µg/ml and 10µg/ml were 135±10, 165±19, 180±9 and 195±5 sec respectively while NCT was 105±0 sec. P9 did not have much effect on PT and APTT. At concentrations 0.01µg/ml, 0.1µg/ml, 1µg/ml as well as for NCT PT was 21±0 sec. However, at a concentration of 10µg/ml, it was 24±2 sec. At concentrations, 0.01µg/ml, 0.1µg/ml and 1µg/ml APTT was 18±2 sec and at 10µg/ml it was 21±2 sec while the NCT was 18±0 sec (Figure 4.6B).

4.2.4.3 Phospholipase A_2 (PLA_2) assay:

P9 exhibited PLA_2 activity in a dose dependent manner. At concentrations 0.1, 1.0 and 10µg/ml PLA_2 activity of P9 was found to be 2.9±0.3, 10.4±3.4 and 30.0U, however, at a lower dose 0.01µg/ml, no PLA_2 activity was observed (Figure 4.6C).

4.2.4.4 Haemolytic assays:

P9 exhibited slight haemolytic activity. At concentrations of 1µg/ml and 10µg/ml, P9 displayed 0.1±0.1% and 0.2±0.12% direct haemolytic activity respectively. However, at lower doses, 0.01µg/ml and 0.1µg/ml, no direct haemolysis of RBC was observed.

Again, at concentrations, 0.01µg/ml, 0.1µg/ml, 1µg/ml and 10µg/ml indirect haemolytic activity was observed to be 1.55±0.21, 1.68±0.21, 1.81±0.36, 2.21±0.17 and 5.1±0.28 respectively (Figure 4.6D).

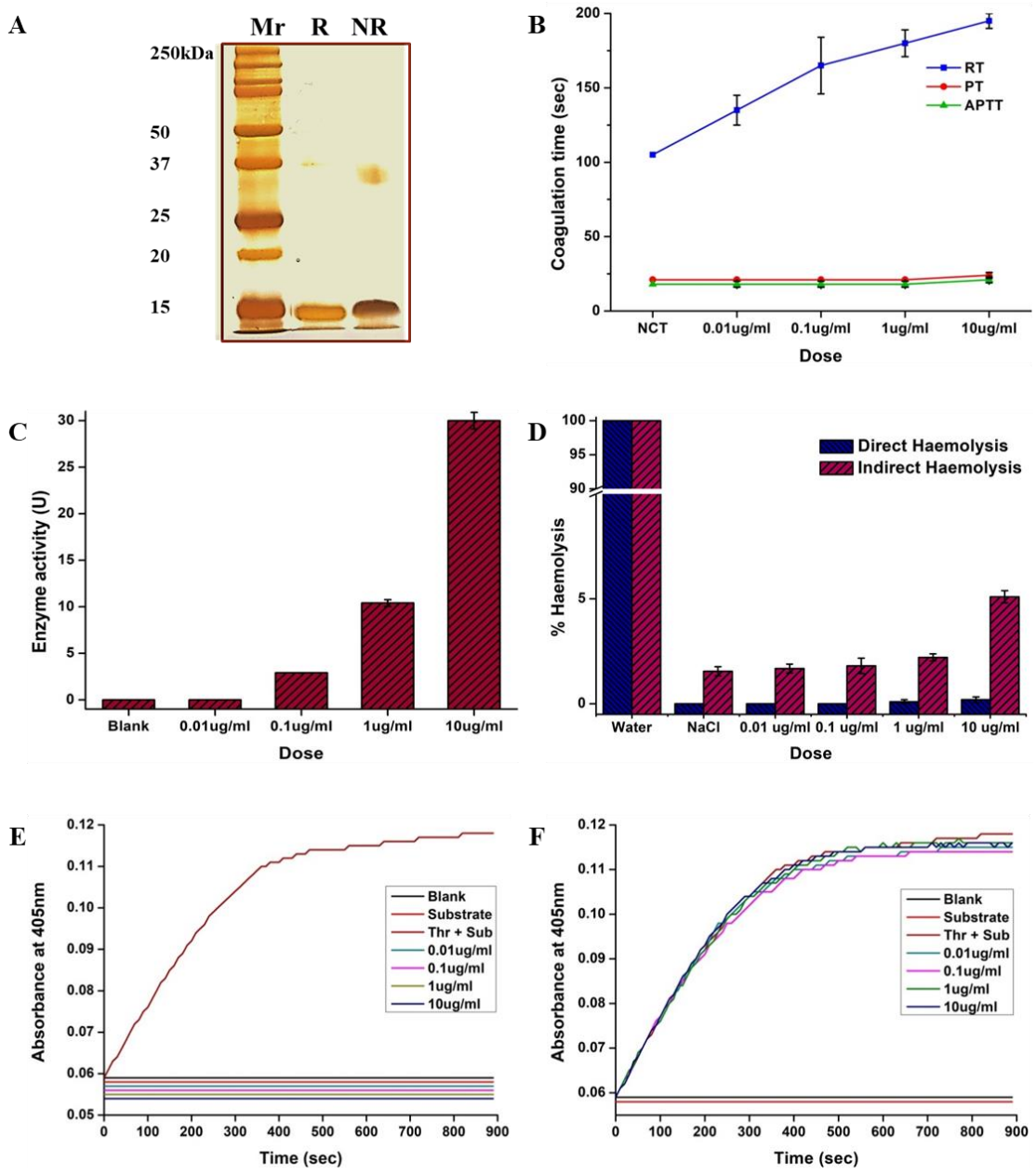


Figure 4.6: Partial biochemical characterization of the anti-platelet fraction (P9): (A) Silver stained 12.5% SDS-PAGE (Mr: Precision plus protein marker, R: reduced, NR: non-reduced) **(B) Coagulation assays** (NCT- Normal Clotting Time, RT- Recalcification Time, APTT- Activated Partial Thromboplastin Time, PT- Prothrombin Time) **(C) Phospholipase A_2 activity (**PLA_2 activity was determined using turbidometric method) **(D) Haemolysis assays** (Percentage haemolysis by water is considered as 100%) **(E) Thrombin like activity (F) Inhibition of amidolytic activity of thrombin** (Thr: Thrombin, Sub: Substrate S2238).

4.2.4.5 Amidolytic Activity:

The amidolytic activity of P9 was assessed for doses 0.01, 0.1, 1 and 10µg/ml using S2238 substrate. It was observed that with increase in doses there was no release of colored product *p*-nitroaniline (p-NA) when monitored at 405 nm suggesting that P9 does not possess amidolytic activity (Figure 4.6E).

4.2.4.6 Inhibition of amidolytic of thrombin:

Inhibition of amidolytic activity of thrombin by P9 was assessed for doses 0.01**,** 0.1, 1 and 10µg/ml. It was observed P9 did not reduce the release of colored product *p*-nitroaniline (p-NA) by thrombin (Figure 4.6F).

4.2.5 Identification of P9:

LC-MS/MS analysis of P9 and sequence similarity of the obtained fragments suggest the presence of a phospholipase A_2 enzyme, Daboxin P and two subunits of snake venom C-type lectin like protein, Dabocetin alpha and Dabocetin beta. The obtained peptide sequence showed 100% query coverage (Figure 4.7 and Table 4.1).

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Daboxin P    SLLEFGKMILEETGKLAIPSYSSYGCYCGWGGKGTPKDATDRCCFVHDCCYGNLPDCNPKSDRYKYKRVNGAIVCE
This study   SLLEFGKMILEETGKLAIPSYSSYG-------------------------------------------VNGAIVCE

Daboxin P    KGTSCENRICECDKAAAICFRQNLNTYSKKYMLYPDFLCKGELKC
This study   KGTSCENR------AAAICFRQNLNTYSKKYMLYPDFLCK-----

Dabocetin-α  MGRFISVSFGLLVVFLSLSGTGADCPSDWSSHEGHCYKVFKLLKTWEDAEKFCTQQANGWHLASIESVEEANFVAQL
This study   ------------------------------------------KTWEDAEKF-------------------FVAQL

Dabocetin-α  ASETLTKSKYHAWIGLRDQSQRQQCSSHWTDGSAVSYETVTKYTKCFGLNKETKYHEWITLPCGDKNPFICKSWVLH
This study   ASETLTK--YHAWIGLR-----QQCSSHWTDGSAVSYETVTK------------YHEWITLPCGDK-----------

Dabocetin-β  MGRFISVGFGLLVVFLSLSGTGAKQDCLSDWSFYEGYCYKVFNEKKTWEDAEKFCNEQVNGGYLVSFRSSEEMDF
This study   -------------------------------SFYEGYCYK-----KTWEDAEKF--------------SSEEMDF

Dabocetin-β  VIRMTFPIFRFDFFWIGLRDFWRDCYWRWSDGVNLDYKAWSREPNCFVSKTTDNQWLRWNCNDPRYFVCKSRVSC
This study   VIRMTFPIFR------------------WSDGVNLDYK------------TTDNQWLR-----------------
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Figure 4.7: Identification of the anti-platelet fraction: Sequence alignment of peptide fragments obtained from LC-MS/MS analysis with Daboxin P (C0HK16.1), Dabocetin- α (ADK22821.1) subunit and Dabocetin-β subunit (ADK22822.1).

Table 4.1: Peptide sequences of trypsin digested fragments of P9 obtained by ESI-LC MS/MS (MW: Molecular weight, Da: Dalton, z: charge)

Protein Name (Accession No.)	Position	Peptide sequence	MW (Da)	Charge (z)
Daboxin P (C0HK16.1)	1	MRTLWIVAVCLIGVEGSLLEFGK	2605.35	3
	16	GSLLEFGK	849.462	2
	17	SLLEFGK	820.440	2
	24	MILEETGK	951.45	2
	32	LAIPSYSSYG	1056.51	2
	84	RVNGAIVCEKGTSCENR	1949.87	3
	85	VNGAIVCEKG	1045.52	2
	85	VNGAIVCEKGTSCENR	1793.76	2
	107	AAAICFR	807.412	2
	114	QNLNTYSKK	966.476	2
	115	NLNTYSK	838.418	2
	122	KYMLYPDFLCK	1476.72	3
	123	YMLYPDFLCK	1364.61	3
Dabocetin alpha subunit (ADK22821.1)	44	KTWEDAEK	1005.47	2
	45	TWEDAEKF	1024.45	2
	73	FVAQLASETLTK	1306.71	2
	74	VAQLASETLTK	1159.63	2
	77	LASETLTK	861.479	2
	87	YHAWIGL	858.441	2
	87	YHAWIGLR	1014.54	2
	100	QQCSSHWTDGSAVSYETVTK	2269.99	3
	109	GSAVSYETVTK	1140.56	2
	132	YHEWITLPCGDK	1517.70	3
Dabocetin beta subunit (ADK22822.1)	32	SFYEGYCYK	1215.48	2
	44	KTWEDAEK	1005.47	2
	45	TWEDAEKF	1024.45	2
	69	SSEEMDFVIR	1254.55	3
	70	SEEMDFVIR	1076.51	2
	79	MTFPIFR	926.471	2
	104	WSDGVNLDYK	1195.55	2
	105	SDGVNLDYK	1009.47	2
	107	GVNLDYK	807.408	2
	126	TTDNQWLR	1032.49	2

4.3 DISCUSSION:

Daboia russelii envenomation causes multitude of pathophysiological abnormalities in its victims including aberrations in the haemostatic system [2,130,134]. Platelets, being a crucial part of the haemostatic system, are often targets of snake venom proteins which manipulate platelet functions by modulating the activities of the agonists or their receptors [11,38]. In this chapter, we attempted to identify and study the fraction with maximum anti-platelet activity from this venom. Using RP-HPLC the crude venom was fractionated and the fractions were screened for anti-platelet activity. The fraction most efficient in inhibiting collagen induced platelet aggregation was fraction number 9 (P9) and it was further studied.

The effect of P9 on the aggregation of platelet-rich plasma induced by various agonists was studied according to Born et. al. [115]. The agonists studied were adenosine diphosphate (ADP), acrachidonic acid (ARA), ristocetin, collagen and thrombin. Collagen is the most thrombogenic factor of the subendothelium, which upon vascular injury, binds to platelet surface receptors, GPVI and $\alpha_2\beta_1$. This initiates a cascade of signals resulting in various events required for platelet aggregation like morphological change, secretion of granular contents and presentation of procoagulant surface [181–183]. Thrombin activates platelets through proteolysis of the protease-activated receptors (PARs) which leads to signal generation inside the platelets causing granular secretion, increase in cytosolic calcium level and causes changes in platelet shape thereby, activating platelets [183,184]. ADP acts on platelet G-protein coupled receptors, $P2Y_1$ and $P2Y_{12}$ triggering an additional release of ADP to amplify activation of platelets following a positive feedback loop [185,186]. Arachidonic acid is converted to Thromboxane A_2 (TXA_2) by COX1 and thromboxane synthase. TXA_2 acts via G-protein coupled receptors, TPα and TPβ receptors and engages in platelet recruitment and their aggregation to a primary platelet plug [187]. Ristocetin, a vancomycin-like antibiotic from *Nocardia lurida*, induces binding of vWF to the GPIb-IX-V complex [188]. Without addition of any agonist, P9 had no effect on platelet aggregation. However, at a concentration of 1μg/ml, it was effective in inhibiting the platelet aggregation induced by ristocetin, collagen and thrombin but it did not have any effect on ADP and arachidonic acid-induced platelet aggregation. P9 dose-dependently inhibited collagen and thrombin mediated platelet aggregation.

Since snake venom proteins are usually multi-functional, we studied P9 for a few other functions contributing to the haemotoxic nature of *Daboia russelii* venom. P9 slightly delayed the coagulation time beyond the NCT suggesting it to be a mild anticoagulant. It exhibited PLA_2 activity and indirect haemolytic activity. Amidolytic activity of P9 and inhibition of amidolytic activity of thrombin by P9 was studied using chromogenic substrate, S2238, which on being cleaved releases colored product p-nitroaniline (p-NA), monitored at 405nm [189]. It was observed that P9 neither exhibited thrombin like amidolytic activity nor inhibited amidolytic activity of thrombin. SDS-PAGE profile P9 under reducing condition displayed a single band at ~15kDa, however, an additional band prominent band at 27 kDa was observed under the non-reducing condition. This suggests that P9 is a mixture of more than one protein. Based on LC-MS/MS analysis and sequence similarity, the anti-platelet fraction, P9 was found to be Dabocetin and Daboxin P.

Dabocetin was earlier isolated from the venom of *Daboia siamensis* which belongs to snake venom C-type lectin (snaclec) family with no effect on coagulation cascade, but affecting ristocetin-induced platelet aggregation [154]. However, studies on the detailed structure and the interaction mechanism between dabocetin and platelets were not performed. Daboxin P, on the other hand is a well- studied PLA_2 enzyme purified from Indian *Daboia russelii* venom [91]. It was reported to exhibits strong anticoagulant activity under in-vitro and in-vivo conditions and inhibited the activation of factor X to factor Xa by both enzymatic and non-enzymatic modes of action. Nonetheless, its role in platelet aggregation is yet to be explored.

Snaclecs have been reported to affect haemostasis by binding to a wide array of coagulation factors, platelet membrane receptors and other proteins critical in haemostasis. Many snaclecs from snake venom affecting platelets have been studied. For instance, botrocetin and bitiscetin form trimolecular complexes with vWF and GPIb, while snake convulxin, stejnulxin and ophioluxin engage the collagen receptor, GPVI to activate platelets [11]. Echicetin on the other hand specifically binds platelet receptor GPIb, thus blocking platelet interactions with vWF and thrombin [190]. However, a few proteins have more than one binding site and may interact with more than one receptor. Examples of such proteins include aggretin and bilinexin, both of which bind GPIb and α2β1 receptors in order to activate platelets [191,192]. Similarly,

the members of phospholipase A_2 family are also known to induce wide range of pharmacological effects including myotoxicity, neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathic, haemorrhage, edema, tissue damage and convulsion during envenomation [193]. PLA_2 enzymes are known to affect platelet functions either by hydrolyzing the membrane phospholipids of platelets causing release of factors which aids platelet aggregation or by binding to platelet surface receptors or aggregation agonists thereby interfering with the signalling pathway for platelet shape change, adhesion and aggregation [153]. Thus, presence of Dabocetin, a snaclec and Daboxin P, a PLA_2 explains the anticoagulant and anti-platelet characteristics of P9. Further in-depth studies of these proteins can be undertaken to understand their mechanisms of action in victims and exploited to design therapeutic interventions against cardiovascular diseases. Dabocetin was already being reported as a platelet aggregation inhibitor by Zhong et.al. in 2006 [154]. Therefore, it would be interesting to investigate whether Daboxin P also possess this property. In the subsequent chapter, the anti-platelet potential of Daboxin P and its mechanism of action are described.