Chapter 5

Exploring the mechanism of anti-platelet activity of Daboxin P

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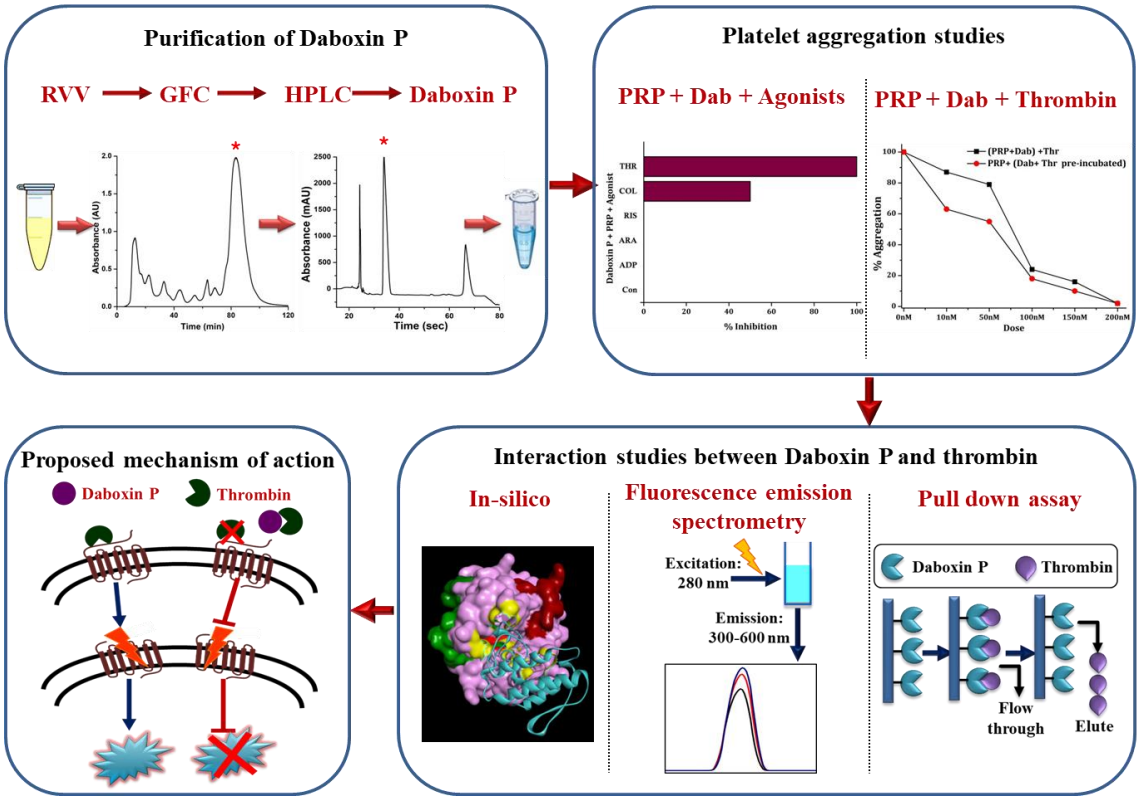
GRAPHICAL ABSTRACT



5.1 INTRODUCTION:

Daboia russelii is one of the Big Four snakes of India contributing to around 43% snakebite incidences [180]. *Daboia russelii* venom is haemotoxic in nature and symptoms observed during envenomation such as persistent and profuse bleeding at the bite site is due to venom protein's interference in the blood coagulation cascade and platelet aggregation pathway after snake bite. Proteomic studies have revealed that phospholipase A_2 (PLA_2) is the most abundant protein family in *Daboia russelii* venom which have shown the ability to manipulate platelet functions by both inhibitory as well

as activatory functions [2,87,129,130]. While on one hand, they can hydrolyze the membrane phospholipids of platelets causing release of factors which aids platelet aggregation, on the other hand, some PLA_2s might act as inhibitors by binding to platelet surface receptors or aggregation agonists thereby interfering with the signalling pathway for platelet shape change, adhesion and aggregation [153].

In the previous chapter, an anti-platelet fraction was characterized which was identified as Dabocetin and Daboxin P. Daboxin P was earlier reported by Sharma et. al. in 2015, which is a strong anticoagulant phospholipase A_2 protein of molecular mass 13.597 kDa [91]. It manipulates the blood coagulation cascade by targeting Factor X and Factor Xa. However, its effect in the platelet aggregation pathway was not explored. In this chapter, we studied the antiplatelet activity of Daboxin P and explored its mechanism of action through interaction studies and in-silico approach.

5.2 RESULTS:

5.2.1 Gel filtration chromatography of crude *Daboia russelii* venom:

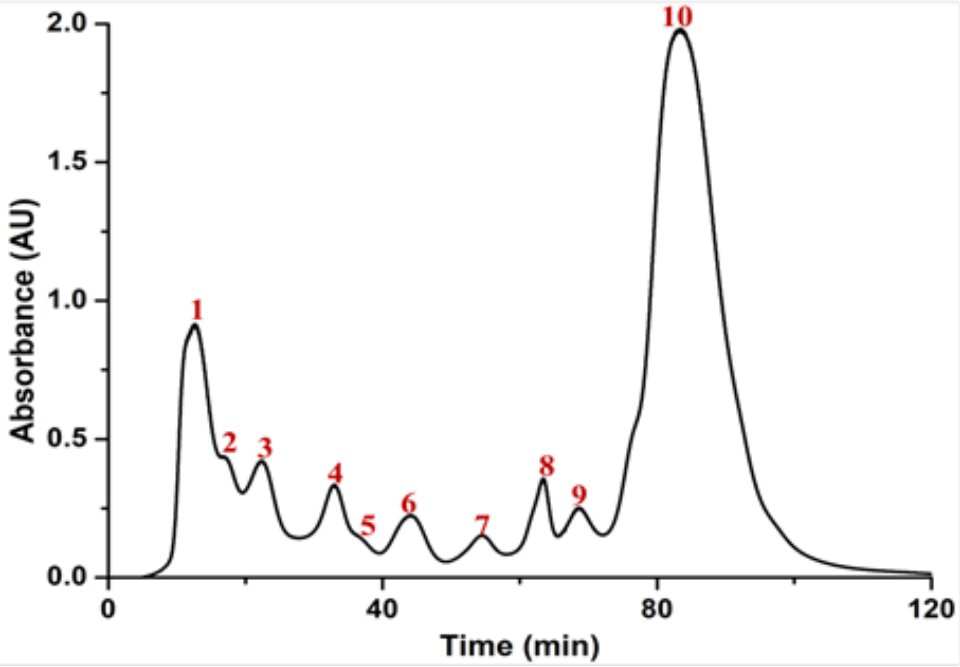


Figure 5.1: Gel filtration chromatiography profile of crude *Daboia russelii* venom: Gel filtration chromatography of crude venom of *Daboia russelii* (10 mg) was performed on Hiload^{TM} 16/600 Superdex 75 prep grade column. Fractionation was carried out at a flow rate of 1 ml/min under isocratic conditions with 50 mM Tris-Cl (pH7.4) and monitored at 215 nm.

Daboxin P was purified from Indian *Daboia russelii* venom using a combination of size exclusion chromatography and RP-HPLC. Gel filtration chromatography revealed a total of 10 peaks (Figure 5.1). The fractions were named as P1 to P10 corresponding to their fraction number and all them were subjected to SDS PAGE and studied for PLA_2 activity and anti-coagulant property.

5.2.2 SDS-PAGE of gel filtration fractions:

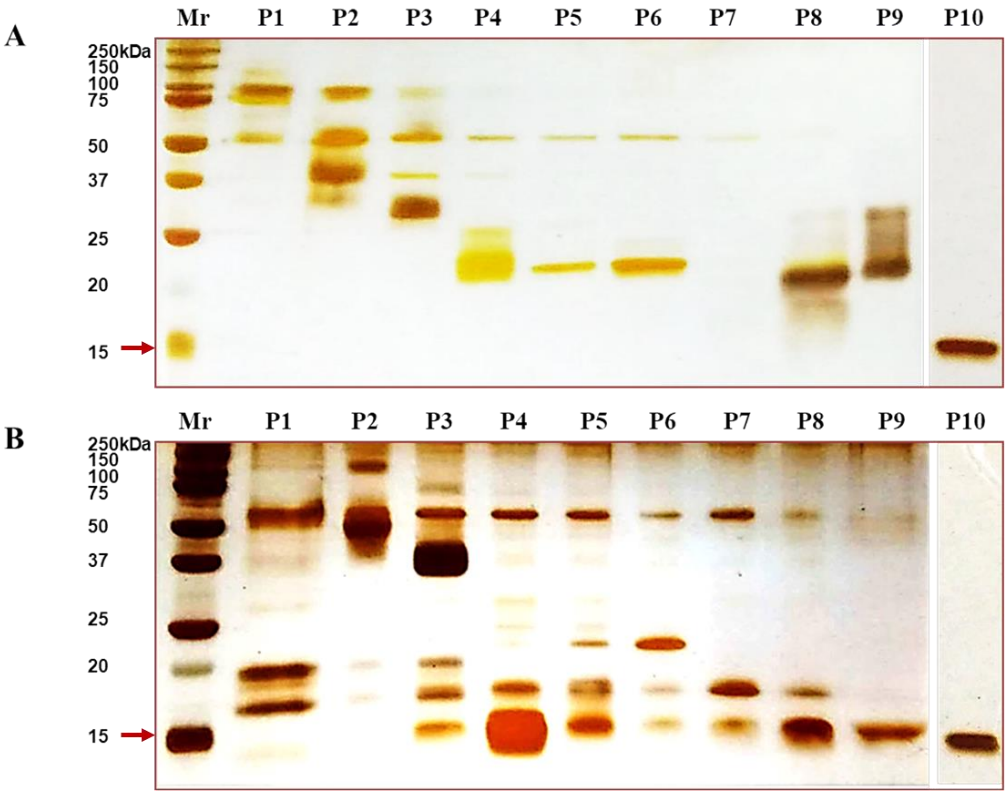


Figure 5.2: Silver stained 12.5% reducing SDS-PAGE of GFC fractions: (A) Non reducing (B) Reducing (Mr: Page ruler plus protein marker, P1-P10: GFC peaks)

Electrophoretic separation of the gel filtration fractions have revealed the presence of different protein bands of varying intensity in the range of ~170 kDa to 15 kDa providing an insight of the various proteins present in this venom (Figure 5.2). In the non-reducing gel, P1 showed the presence of prominent protein bands at 100 and 50 kDa and minor bands at 150 and 75kDa. P2 revealed the presence of protein bands at 100, 50 and 37 kDa. P3 revealed prominent protein bands at 50 and ~30 kDa and faint bands at 100 and 37 kDa. P4 revealed a prominent band at 50 kDa and a smear from 25-20 kDa. P5and P6 both revealed prominent bands at 50 and 20 kDa. P7 showed a band

at 50 kDa. P8 revealed prominent band at 20 kda and faint bands at 50 kDa. P9 revealed prominent band at ~20 kDa and a mear at 25-20 kDa. P10 showed a single band at 15 kDa. In the reducing phase, P1 showed prominent bands at 50, 20 and ~17 kDa and minor bands at 150, 37 and ~19 kDa. P2 showed prominent band at ~100 kDa, smear at 50-37 kDa and faint bands at 20 and ~17 kDa. P3 showed prominent bands at 50 and 37 kDa, minor bands at 20, ~17 and 15 kDa and faint band at 100 kDa. P4 showed prominent bands at 50, ~17 and 15 kDa and faint bands at 37, ~30 and 20 kDa. P5 showed prominent bands at 50 and 15 kDa and minor bands at 25 and 20 kDa. P6 showed prominent bands at 50 and 25 kDa and faint bands at 20 and 15 kDa. P7 showed prominent bands at 50 and 20 kDa and faint band at 15 kDa. P8 showed a prominent band at 15 kDa, a minor band at 20 kDa and a faint band at 50 kDa. P9 showed a prominent band at 15 kDa and a faint band at 50 kDa. P10 showed a prominent single band at 15 kDa.

Different pattern of bands in the non-reducing and their corresponding reducing gels suggest presence of multimeric or dimeric proteins while similar band pattern suggests the presence of monomers (Figure 5.2 A and B). Presence of lower molecular weight protein bands in almost all the fractions correlate to the fact that PLA_2s are the most abundant protein families present in Russell's viper venom.

5.2.3 Phospholipase A_2 (PLA_2) activity of gel filtration fractions:

The gel filtration fractions were screened for phospholipase A_2 (PLA_2) activity by turbidometric method. It was observed that P8, P9 and P10 showed PLA_2 activities with P10 exhibiting the highest activity. The PLA_2 activities of P8, P9 and P10 were 1±1.05 U, 8.45±1.5 U and 25.6±2 U respectively (Figure 5.3).

5.2.4 Recalcification time (RT) of the gel filtration fractions:

The effect of the gel filtration fractions on recalcification time was studied. It was observed Normal clotting time (NCT) of PPP was 150 sec. The fractions P1-P7 were procoagulants as the clotting times were less than that of NCT, P8 had no effect on RT while P9 and P10 were anticoagulants as they prolonged the clotting time more than that of NCT, here, beyond 900 sec (Figure 5.4).

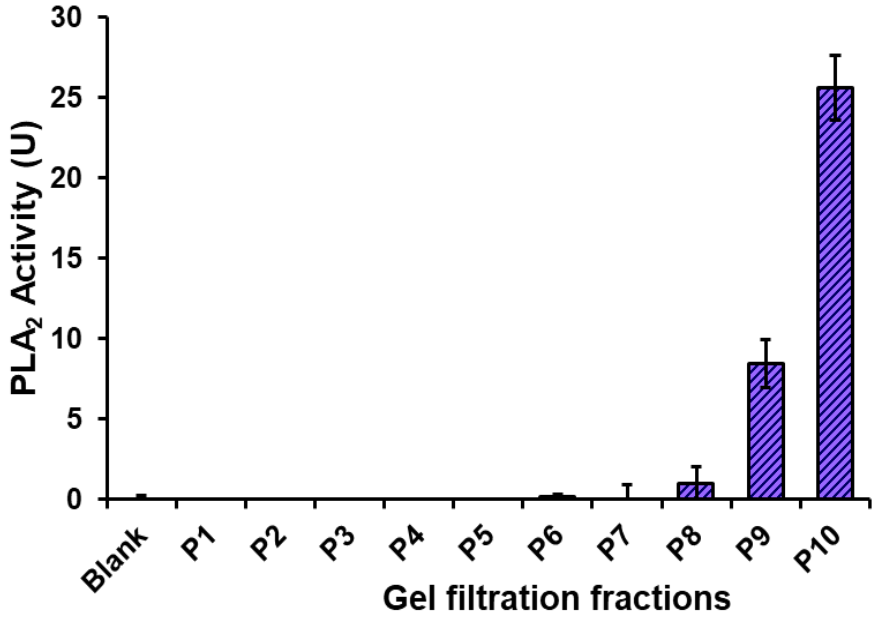


Figure 5.3: PLA_2 activity of the gel filtration fractions: Briefly, 1 µg of each gel filtration fraction (P1-P10) was tested for PLA_2 activity by turbidometric method. One unit (U) PLA_2 activity is defined as decrease in 0.01 OD in 10 min at 740 nm. Results are mean ± SD of three independent experiments.

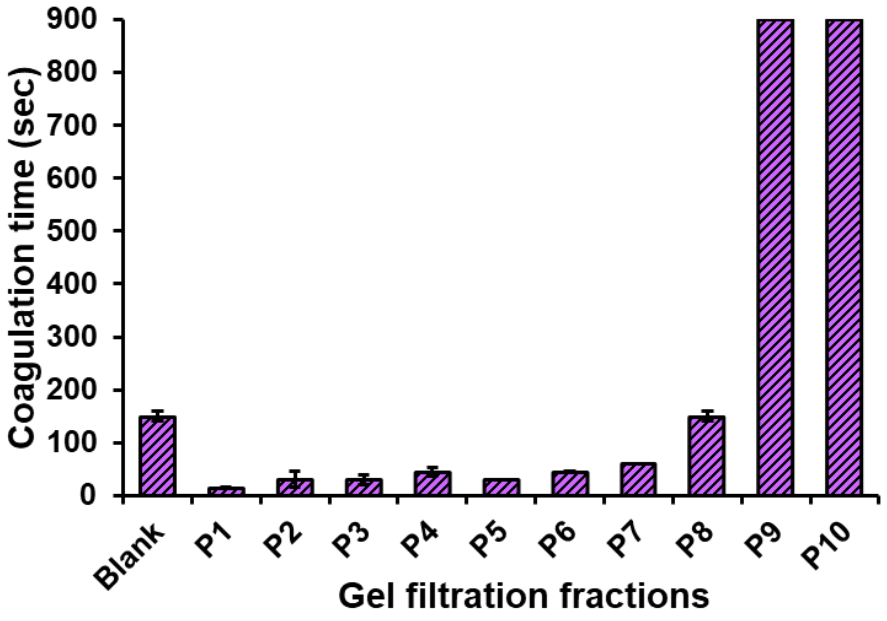


Figure 5.4: Recalcification time of gel filtration fractions: Briefly 1 µg of the gel filtration fractions (P1-P10) were added to PPP followed by initiation of coagulation by addition of $CaCl_2$.which was monitored at 405 nm. The normal clotting time without addition of sample is taken as blank. Results are mean ± SD of three independent experiments.

5.2.5 Reverse phase - high pressure liquid chromatography (RP-HPLC):

The peak 10 was further fractionated using RP-HPLC. The RP-HPLC profile revealed 3 fractions (Figure 5.5). They were named as RP1 – RP3 and each were studied for PLA_2 activity and anti-coagulant property.

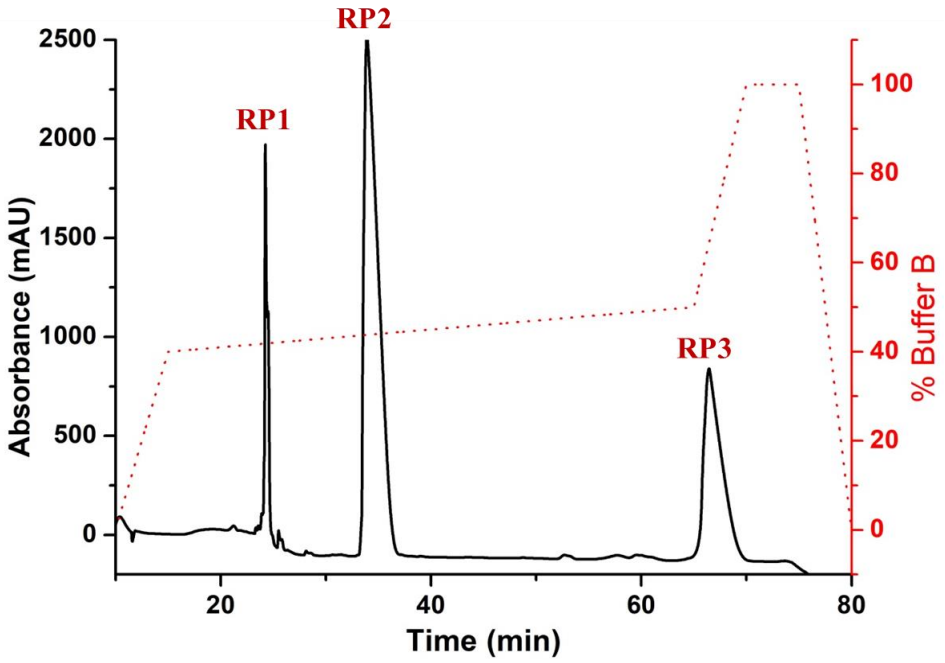


Figure 5.5: RP-HPLC of gel filtration peak 10: RPHPLC profile of 1mg of GFC peak 10 on Acclaim C18 column. Fractionation was carried out at a flow rate of 1ml/min with a gradient of 40-50% of MeCN maintained in 15-65 minutes and monitored at 215 nm.

5.2.6 Phospholipase A_2 (PLA_2) assay of the RP-HPLC fractions:

The RP-HPLC fractions were screened for phospholipase A_2 (PLA_2) activity by turbidometric method. It was observed that RP1 did not show much PLA_2 activity with 0.65 U, RP2 exhibited the highest PLA_2 activity with 25.9±2 U while RP3 exhibited 17.5±1.5 U (Figure 5.6 A).

5.2.7 Recalcification Time (RT) of the RP-HPLC fractions:

The effect of the RP-HPLC fractions on recalcification time was studied. It was observed that RP1 had no effect on clotting time with 140±15 sec. RP2 and RP3 both prolonged the clotting time with RP3 delaying to 500±15 sec and RP2 delaying beyond 900 sec while Normal clotting time (NCT) of PPP was 150±8 sec (Figure 5.6 B).

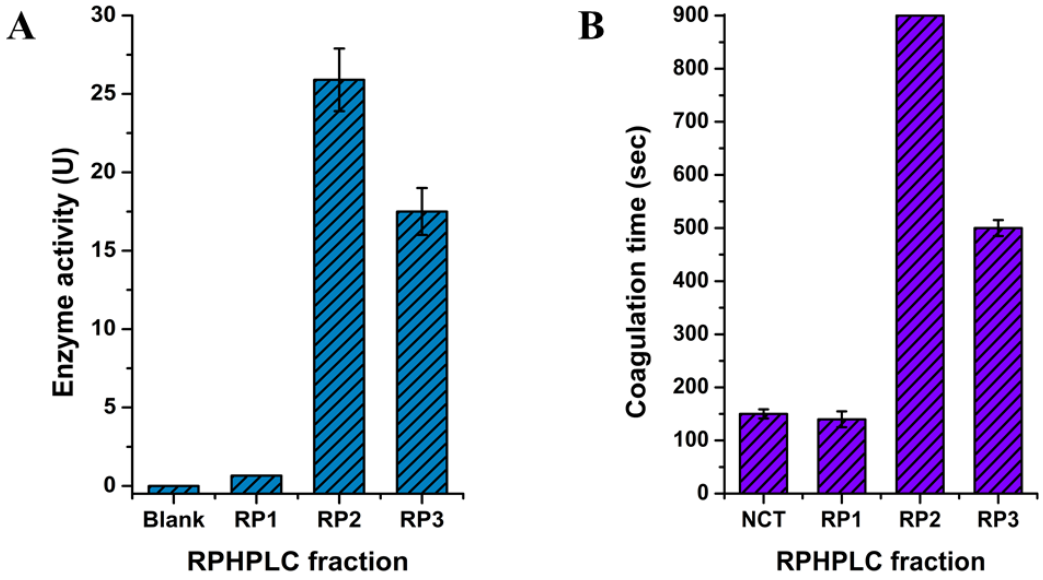


Figure 5.6: Screening for Daboxin P: (A) PLA_2 activity of the RPHPLC fractions of GF10 Briefly, 1µg of each RP-HPLC fraction (RP1-RP3) was tested for PLA_2 activity by turbidometric method. One unit (U) PLA_2 activity is defined as decrease in 0.01 OD in 10 min at 740 nm. **(B) Recalcification time of the RPHPLC fractions of GF10.** Briefly 1µg of the RP-HPLC fractions (RP1-RP3) were added to PPP followed by initiation of coagulation by addition of $CaCl_2$.which was monitored at 405 nm. Results are mean ± SD of three independent experiments.

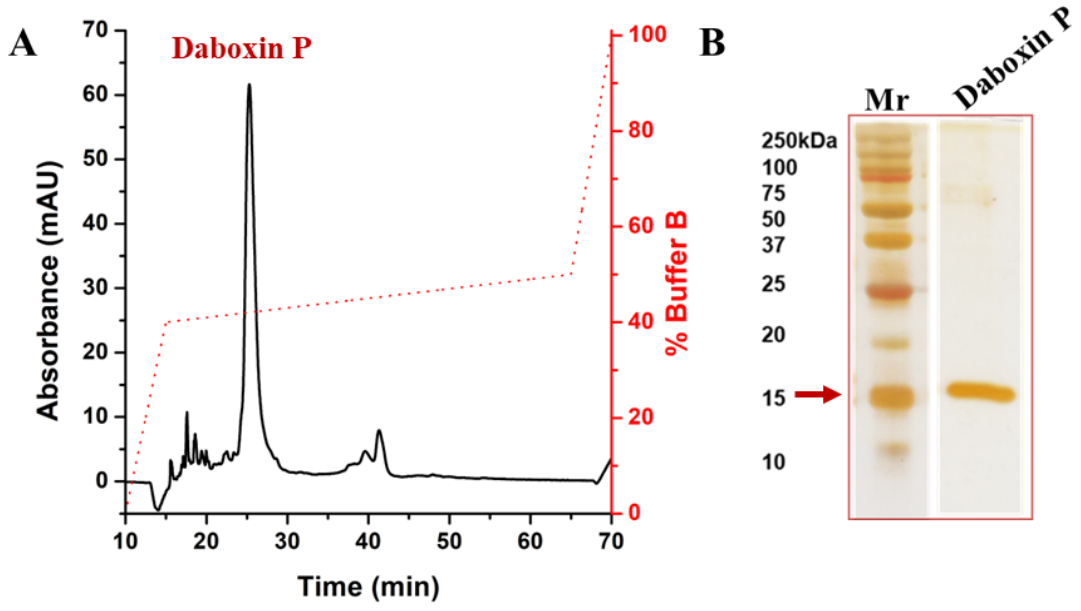


Figure 5.7: Assessment of purity of Daboxin P: (A) Rechromatography pf RP2: RPHPLC profile of 50 µg of RP2 on Acclaim C18 column. Fractionation was carried out at a flow rate of 1 ml/min with a gradient of 40-50% of MeCN maintained in 15-65 minutes and monitored at 215 nm. **(B) 12.5% reducing SDS-PAGE of Daboxin P** (Mr: Page ruler plus protein marker)

5.2.8 Rechromatography of RP2:

Rechromatography of RP2 resulted in a single peak, which suggests the presence of a pure protein (Figure 5.7 A).

5.2.9 SDS PAGE of rechromatography fraction:

The rechromatography was subjected to SDS PAGE under reducing conditions. It expressed as prominent single band of molecular weight ~15 kDa (Figure 5.7 B). This further confirms the presence of a pure protein in the fraction.

5.2.10 Peptide Mass Fingerprinting by LC-MS/MS:

Presence of Daboxin P in RP2 was confirmed by LC-MS/MS analysis. Tryptic digestion of the purified protein yielded 26 distinct peptide fragments. Upon alignment of the peptide fragments obtained, it displayed 71% identity with a Daboxin P (C0HK16.1) and 100% query coverage (Figure 5.8 and Table 5.1).

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Daboxin P   SLLEFGKMILEETGKLAIPSYSSYGCYCGWGGKGTPKDATDRCCFVHDCCYGNLPDCNNKK
This study  SLLEFGKMILEETGKLAIPSYSSYGCYCGWGGK----------------------------

Daboxin P   SKRYRYKKVNGAIVCEKGTSCENRICECDKAAAICFRQNLNTYSKKYMLYPDFLCKGELVC
This study  -------KVNGAIVCEKGTSCENRICECDKAAAICFRQNLNTYSKKYMLYPDFLCK-----
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Figure 5.8: Sequence alignment: Sequence alignment of peptide fragment obtained from LC-MS/MS analysis with Daboxin P (C0HK16.1).

Table 5.1: Peptide sequences of trypsin digested fragments of purified protein obtained by ESI-LC MS/MS

Protein Name Accession No.	Position	Peptide sequence	MW (Da)	Charge (z)
Daboxin P C0HK16.1	1	SLLEFGK	820.431	2
	4	EFGKMILEETGK	1388.70	3
	6	GKMILEETGK	1104.58	2
	7	KMILEETGK	1047.56	2
	8	MILEETGK	962.47	2
	8	MILEETGKL	1075.55	2
	10	LEETGK	675.342	1
	16	LAIPSYSSY	999.495	2

	16	LAIPSYSSYG	1056.51	2
	16	LAIPSYSSYGCYCGWGGK	2056.89	2
	68	RVNGAIVCEK	1117.58	2
	69	VNGAIVCEK	1046.51	2
	69	VNGAIVCEKG	1045.52	2
	69	VNGAIVCEKGTS	1234.59	2
	69	VNGAIVCEKGTSCENR	1792.78	3
	85	ICECDKAAAICFR	1614.72	3
	91	AAAICFR	807.406	2
	98	QNLNTYSK	949.446	2
	98	QNLNTYSKK	1077.54	2
	106	KYMLYPD	928.436	2
	106	KYMLYPDF	1075.50	2
	106	KYMLYPDFL	1188.59	2
	106	KYMLYPDFLCK	1476.72	3
	107	YMLYPDFL	1188.59	2
	107	YMLYPDFLCK	1348.63	3
	108	MLYPDFLCK	1185.55	2

5.2.11 Effect of Daboxin P on platelet aggregation induced by different agonists:

The effect of Daboxin P on the aggregation of PRP induced by various agonists was studied. Although without addition of agonist Daboxin P had no effect on aggregation of PRP, it was observed that at a concentration of 1 μg (163 nM), Daboxin P was effective in inhibiting the platelet aggregation induced by collagen and thrombin (Figure 5.9 A-E and 5.10). However, it did not have any effect on ADP and arachidonic acid-induced platelet aggregation. The percentage of inhibition of thrombin-induced platelet aggregation was significantly higher than that collagen -induced which was 100% and 50% ± 2 respectively (Figure 5.9 F). Aggregation of PMA differentiated K-562 cells in presence of 163 nM Daboxn P induced by various agonist was also evaluated. It was observed that percentage of inhibition of collagen-induced platelet aggregation was 43.8% ± 2 while that thrombin-induced was 48.9% ± 1. However, there was not much significant effect on ADP and arachidonic acid-induced platelet aggregation (Figure 5.11).

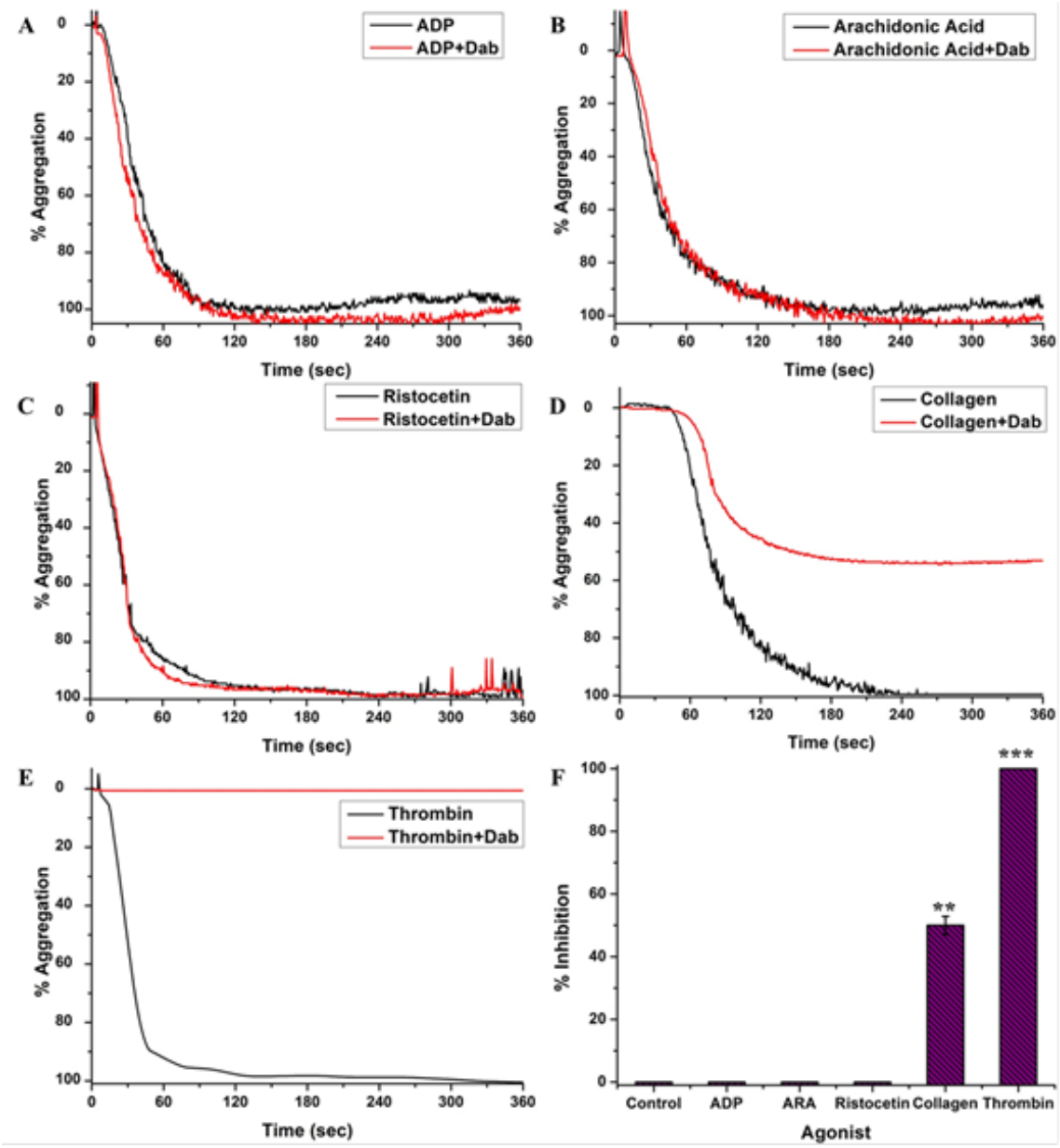

Figure 5.9: Effect of 1µg of Daboxin P on platelet aggregation induced by different agonists: (A) ADP, (B) Arachidonic acid, (C) Ristocetin, (D) Collagen, (E) Thrombin. (Briefly, 1 µg of Daboxin P was pre-incubated with 500 µl of PRP for 3 minutes at 37^{o}C and platelet aggregation induced by different agonists with 6 minutes of continuous stirring at 37^{o}C was recorded. The light transmittance was calibrated with PPP. For each set of experiment, platelet aggregation of only PRP induced by agonists was taken as control.) **(F) Comparative bar graph of inhibition of platelet aggregation** (ADP: adenosine diphosphate, ARA: Arachidonic acid, Dab: Daboxin P). Results are mean ± SD of six independent experiments. *** *p* value <0.001, ** *p* value <0.05 with respect to control.

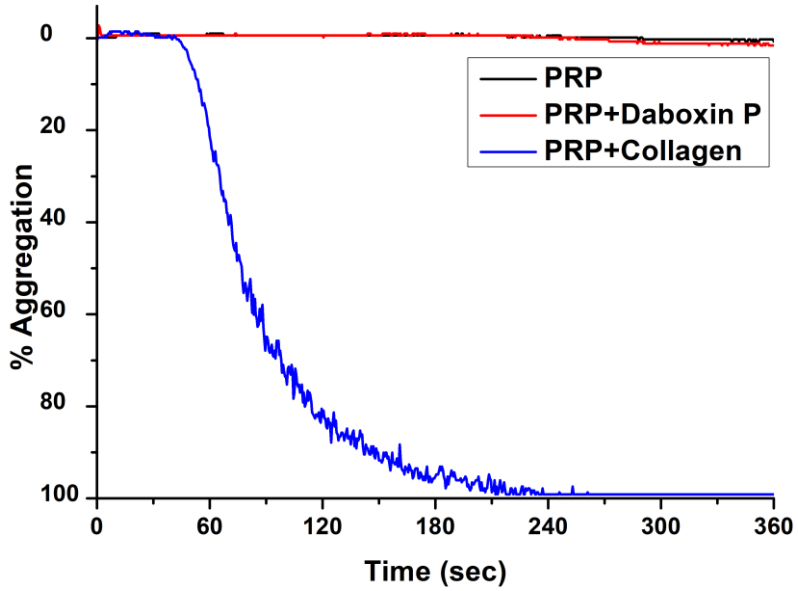

Figure 5.10: Effect of 1µg of Daboxin P on aggregation of PRP: (PRP: Platelet rich plasma)

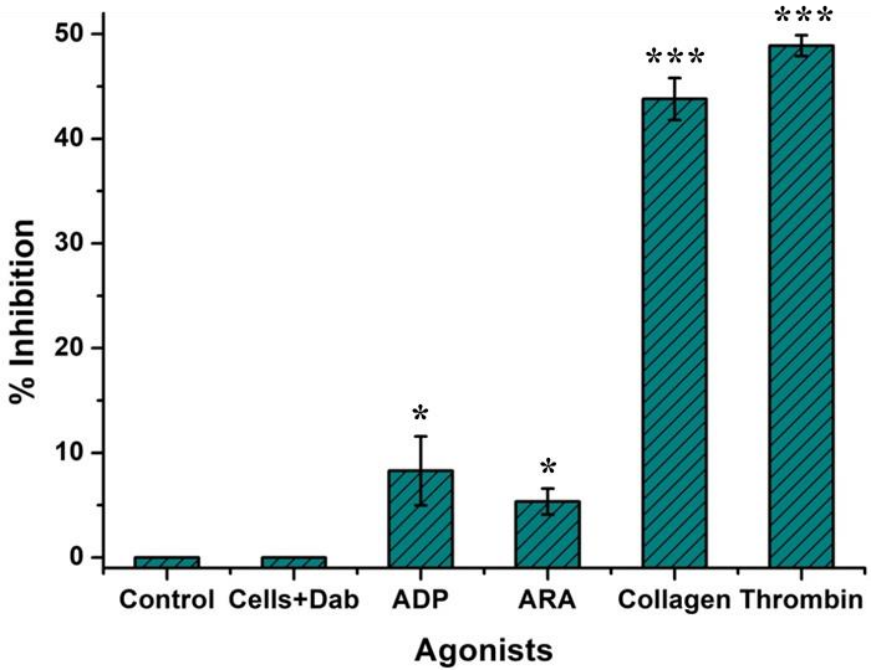

Figure 5.11: Effect of 1µg of Daboxin P on aggregation of differentiated K-562 cells induced by different agonists: Comparative bar graph of percentage inhibition (ADP: adenosine diphosphate, ARA: Arachidonic acid, Dab: Daboxin P).Results are mean ± SD of three independent experiments. *** *p* value <0.001, ** *p* value <0.05 with respect to control, **p* value <0.01

5.2.12 Effect of Daboxin P on platelet aggregation induced by collagen:

Daboxin P dose dependently inhibited the collagen induced platelet aggregation. It was observed that at concentrations of 10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM and 450 nM of Daboxin P, aggregation induced by 2µg/ml of collagen was 73±1.5%, 62±5.0%, 52±0.5%, 47±4.0%, 46% and 45% respectively. When the said doses of Daboxin P, (10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, and 450 nM) were pre-incubated with collagen for 10 minutes prior to initiation of aggregation, the percentage aggregation were observed to be 70±0.5%, 60±3.5%, 52±3.5%, 47±4.0%, 46% and 45% respectively. Daboxin P, when pre incubated with collagen, at lower doses (10 nM and 50 nM) could inhibit aggregation of PRP more in comparison to when Daboxin P was not pre incubated with collagen. However, for higher doses, percentage inhibition was same for both. The inhibition of platelet aggregation was saturated at a dose of 150 nM (Figure 5.12).

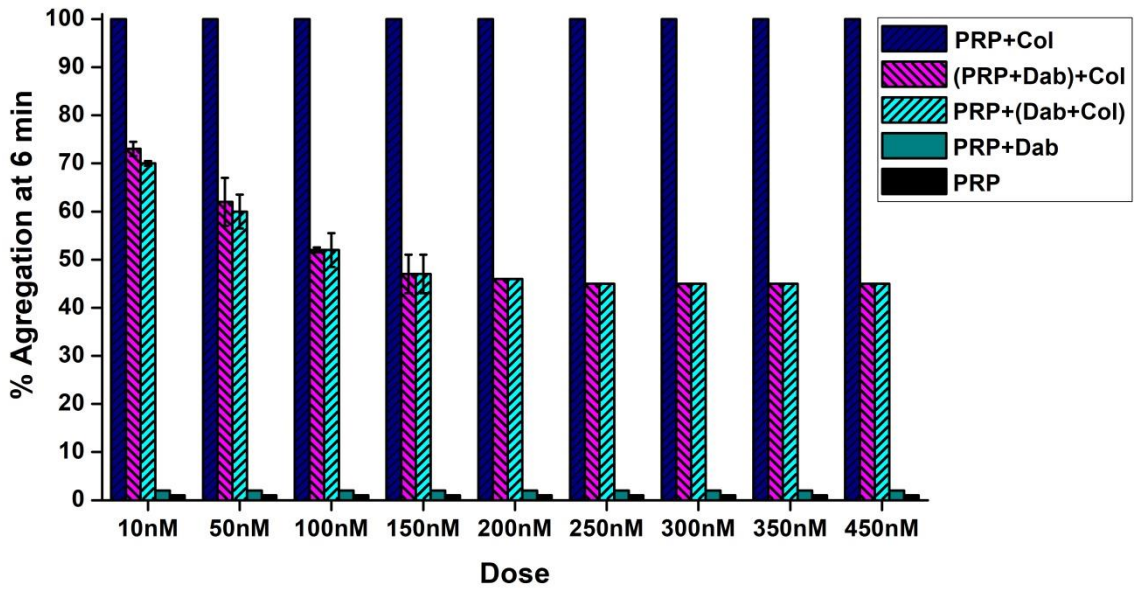

Figure 5.12: Dose dependent effect of Daboxin P on platelet aggregation induced by collagen: (Different doses of Daboxin P was pre-incubated with 500 µl of PRP for 3 minutes at 37℃ and platelet aggregation induced by collagen with 6 minutes of continuous stirring was recorded. In a different set of experiment the doses of Daboxin P pre incubated with 1 µg of collagen for 10 min at 37℃ was used to induce platelet aggregation. Platelet aggregation of only PRP induced by thrombin was taken as control. PRP: Platelet rich plasma, Col: collagen, Dab: Daboxin P)

5.2.13 Effect of Daboxin P on platelet aggregation induced by thrombin:

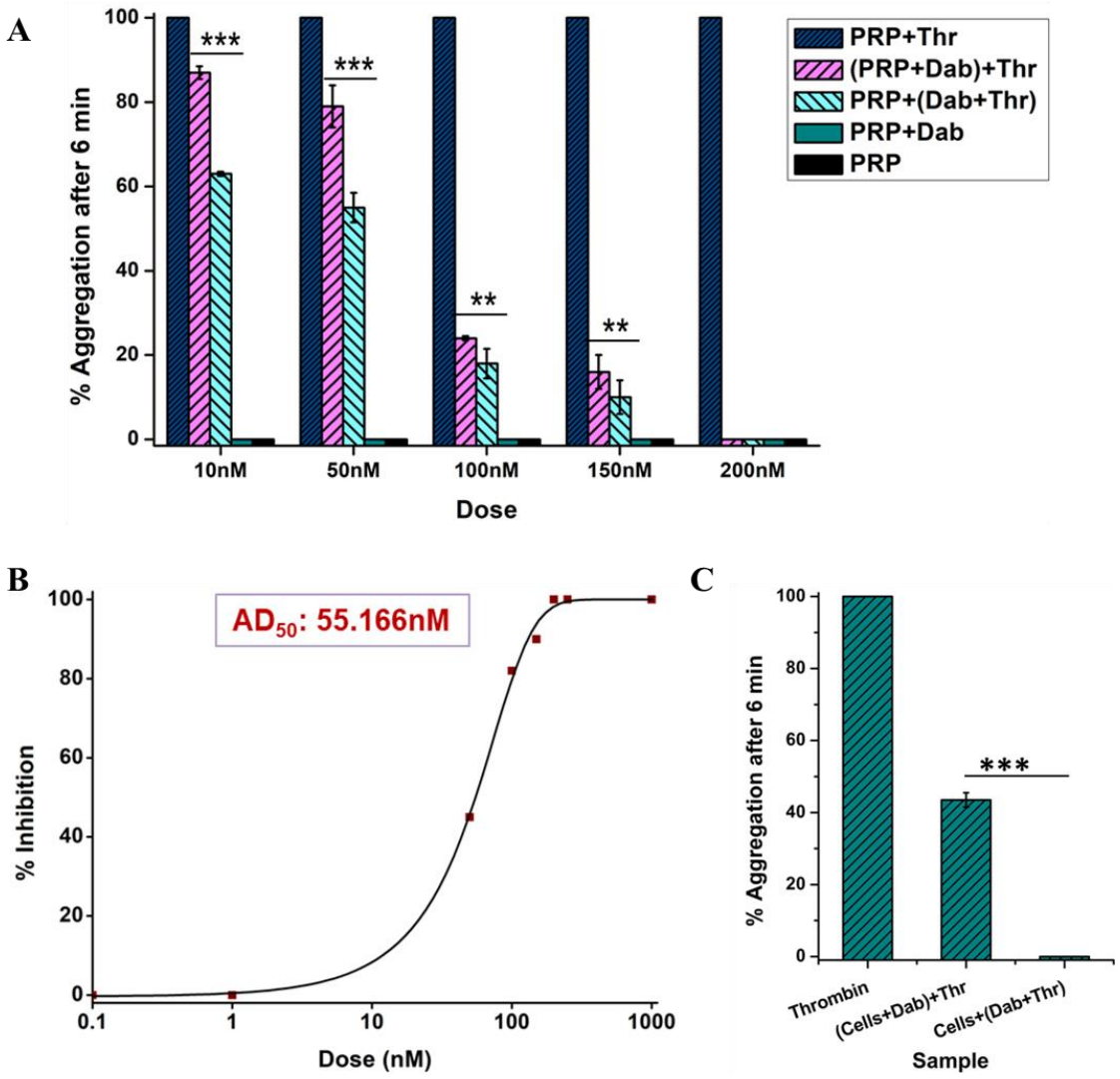

Figure 5.13: Effect of Daboxin P on platelet aggregation induced by thrombin: (A) Comparative dose-dependent effect of Daboxin P on thrombin-induced aggregation of PRP (B) Aggregation Dose 50 (AD_{50} was calculated online using AAT Bioquest) **(C) Effect of Daboxin P on thrombin-induced aggregation of differentiated K-562 cell line** (PRP: Platelet-rich plasma, Thr: thrombin, Dab: Daboxin P, Cells: K-562 cells induced for megakaryocytic differentiation, Dab+Thr: Daboxin P pre-incubated with Thrombin). Results are mean ± SD of six independent experiments. *** *p* value <0.001, ** *p* value <0.05 with respect to control.

Daboxin P on aggregation of PRP: To investigate whether Daboxin P binds with thrombin or the receptors, various doses of Daboxin P were pre-incubated with thrombin for 10 minutes or to the platelets before initiation of aggregation. In both conditions, Daboxin P inhibited platelet aggregation dose-dependently. It was observed

that at a concentration of 10 nM, 50 nM, 100 nM, 150 nM and 200 nM of Daboxin P, aggregation induced by 1.34 nM of thrombin were found to be 87.3±0.5%, 79±1%, 24.3±0.5%, 16±1% and 0% respectively. When these same doses of Daboxin P, (10nM, 50nM, 100nM, 150nM and 200nM) were pre-incubated with thrombin for 10 minutes, the percentage aggregation were 63.3±0.5%, 55±2%, 18.6±1.5%, 10±1.7% and 0% respectively. When the dose was increased to 200 nM concentration of Daboxin P, thrombin-induced platelet aggregation was completely inhibited. But at the lower doses, Daboxin P pre-incubated with thrombin showed higher inhibition than platelets incubated with Daboxin P (Fig 5.13 A). The dose of Daboxin P at which 50% inhibition of thrombin induced platelet aggregation is obtained was found to be 55.166 nM and saturation of inhibition was achieved at the dose 200 nM (Fig 5.13 B).

Daboxin P on aggregation of megakaryotic cells: Daboxin P completely inhibited the thrombin-induced aggregation of K-562 cells when it was pre-incubated with thrombin, whereas when the cells were pre-incubated with Daboxin P, only 43.5% ± 2 aggregation was observed (Figure 5.13 C).

Comparison between effects of Daboxin P on aggregation of washed platelets and PRP: Percentage inhibition of aggregation of washed platelets was observed to be 70±5% while that of PRP was 65±2%. Moreover, when Daboxin P pre-incubated with thrombin was used to initiate aggregation, the percentage inhibition was observed to be 95±3% and 95±5% for washed platelets and PRP respectively (Figure 5.14).

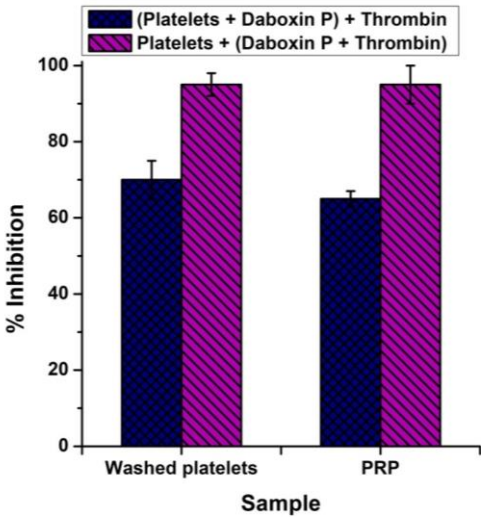

Figure 5.14: Comparative effect of on aggregation of washed platelets and platelet-rich plasma. The effect of 100 nM Daboxin P was studied. (PRP: Platelet Rich Plasma)

5.2.14 Effect of Daboxin P on thrombin mediated calcium mobilization:

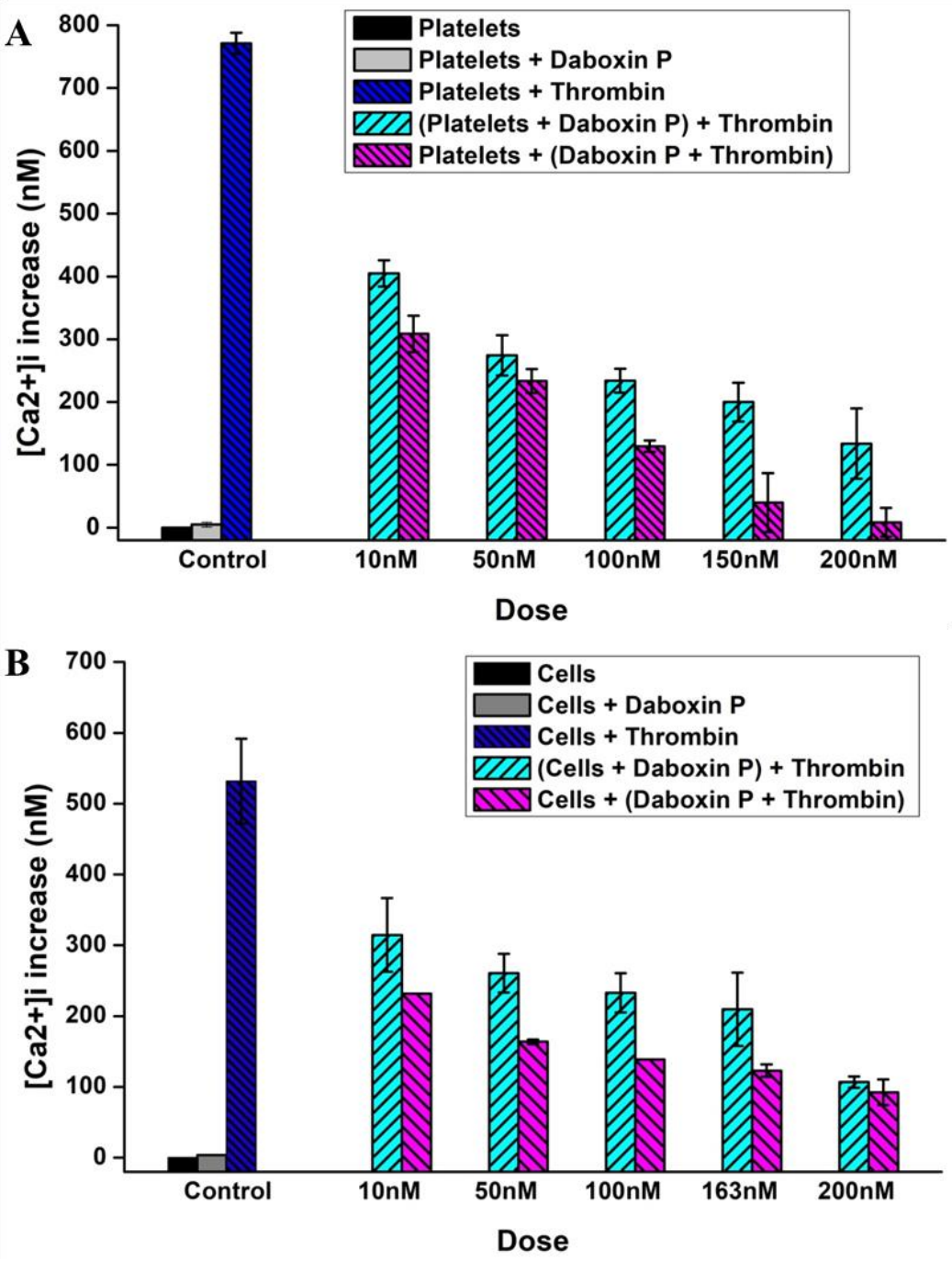

Figure 5.15: Dose dependent effect of Daboxin P on thrombin induced intracellular calcium signal: (A) Platelets (B) Differentiated K-562 cells (The effect of different doses of Daboxin P on thrombin mediated calcium mobilization was studied on platelets using Fura-2AM and on differentiated K-562 cells using Fura-4AM.)

Daboxin P dose dependently reduced the thrombin induced calcium signal (Figure 5.15). At concentrations 10 nM, 50 nM, 100 nM, 150 nM and 200 nM of Daboxin P, intracellular calcium concentration increased to 404.98±21, 274.25±32, 233.93±19, 199.96±31 and 133.81±56 nM respectively, while thrombin-evoked calcium increase was 771±17 nM. Daboxin P pre-incubated with thrombin was significantly more effective in reducing the intracellular calcium signal. When the same doses of Daboxin P were pre-incubated with thrombin and used to stimulate the platelets, the increase in intracellular calcium concentrations were observed to be 308.62±29, 233.38±19, 129.63±9, 39.73±47 and 8.31±23nM respectively (Figure 5.15 A). Similarly, Daboxin P dose dependently reduced the thrombin induced calcium signal in differentiated K-562 cells and Daboxin P pre-incubated with thrombin was found to be more effective in reducing the calcium influx (Figure 5.15 B).

5.2.15 Effect of alkylation of Daboxin P:

Alkylated Daboxin P exhibited no PLA_2 activity (Figure 5.16). The enzyme activity of 0.1 µg Daboxin P was observed to be 4.08 U while that of alkylated Daboxin P was 0. The enzyme activities of the negative control (blank), vehicle control (0.1 µg Daboxin P in ethanol) and positive control (0.1 µg *Daboia russelii* venom) were found to be 0, 3.16 and 4.24 U respectively. The effect of alkylated Daboxin P on thrombin-induced platelet aggregation and thrombin-mediated calcium signal was studied. It was observed in both the cases that there was no significant difference between the effect of alkylated and non-alkylated Daboxin P (Figure 5.17). Percentage inhibition of platelet aggregation by 100nM Daboxin P was 85±2.0% while that by alkylated Daboxin P was 75±7.0%. Besides, both alkylated and non-alkylated Daboxin P when pre-incubated with thrombin inhibited 95±5% platelet aggregation. Likewise, alkylated and non-alkylated Daboxin P increased the intracellular calcium concentration to 395±19 nM and 385±15 nM respectively. Moreover, Daboxin P pre-incubated with thrombin increased the intracellular calcium concentration to 295±8 nM and 302±22 nM under alkylated and non-alkylated conditions respectively.

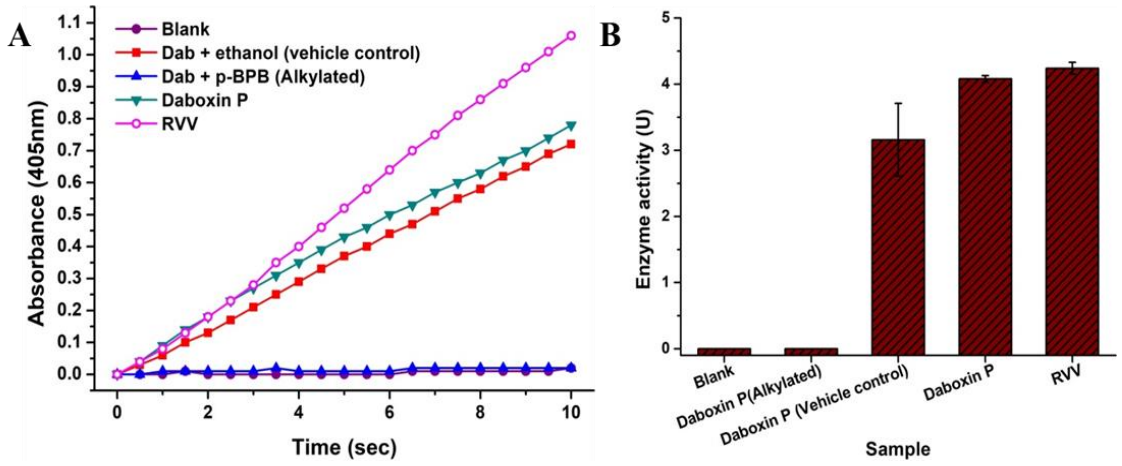

Figure 5.16: Effect of alkylation on phospholipase A_2 activity of Daboxin P: (A) Progressive curve, (B) Comparative bar graph. Daboxin P was alkylated using p-BPB and PLA_2 activity was tested for 0.1 µg of Daboxin P using $sPLA_2$ assay kit (Dab: Daboxin P, p-BPB: p-bromophenacyl bromide, RVV: Russell's viper venom)

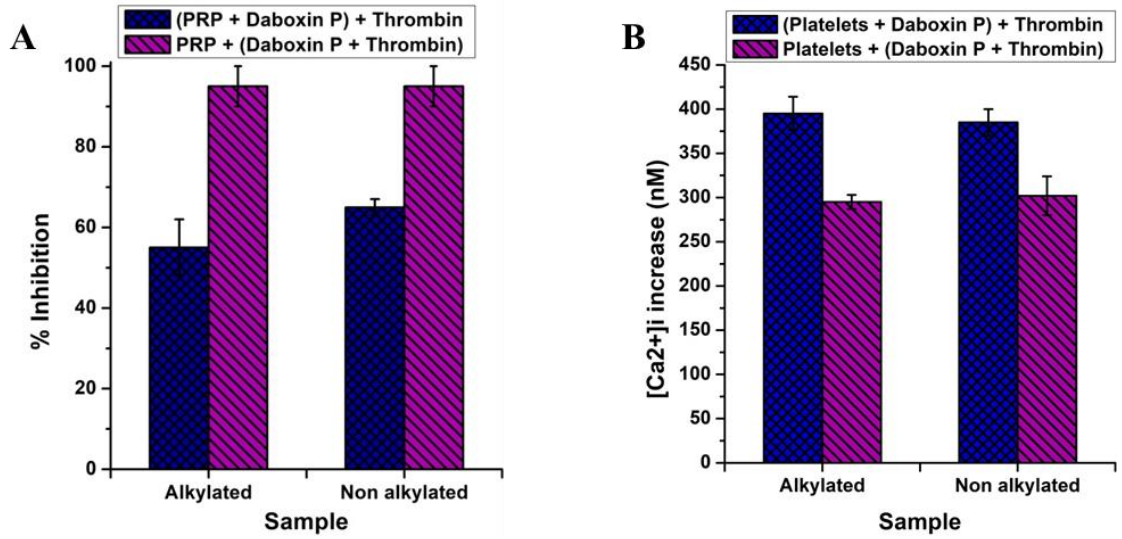

Figure 5.17: Comparative effect of Daboxin P on platelet functions: (A) Thrombin-mediated platelet aggregation, (B) Thrombin-mediated calcium signal. The effect of 100nM (alkylated and non-alkylated) Daboxin P was studied (PRP: Platelet rich plasma)

5.2.16 Interaction studies of Daboxin P and thrombin:

5.2.16.1 In-silico approach:

Molecular docking studies revealed that the site of interaction of Daboxin P on the surface of thrombin is similar to the site of interaction of PAR1 and PAR4. Common residues of thrombin involved in binding PAR1/4 as well as Daboxin P are shown in

yellow (Figure 5.18). The negative binding energies of the interactions suggest feasible binding (Table 5.2). The amino acid residues involved in the interactions between Daboxin P, thrombin, PAR1 and PAR4 were analysed using contact maps. The contact map analysis revealed that Daboxin P binds to fifteen residues of thrombin out of which nine are required for binding PAR1 and PAR4. These residues are Arg60, Glu65, Leu67, Trp90, Lys185, Thr187, Trp188, Asp239 and Cys241. Seven residues of thrombin i.e. Arg60, Glu65, Leu67, Trp90, Lys185, Asp239 and Cys241 are involved in binding to both Daboxin P and extracellular fragment of PAR1. Similarly, seven residues of thrombin i.e. Arg60, Glu65, Leu67, Trp90, Thr187, Trp188 and Cys241 are involved in binding to both Daboxin P and extracellular fragment of PAR4 (Figure 5.19).

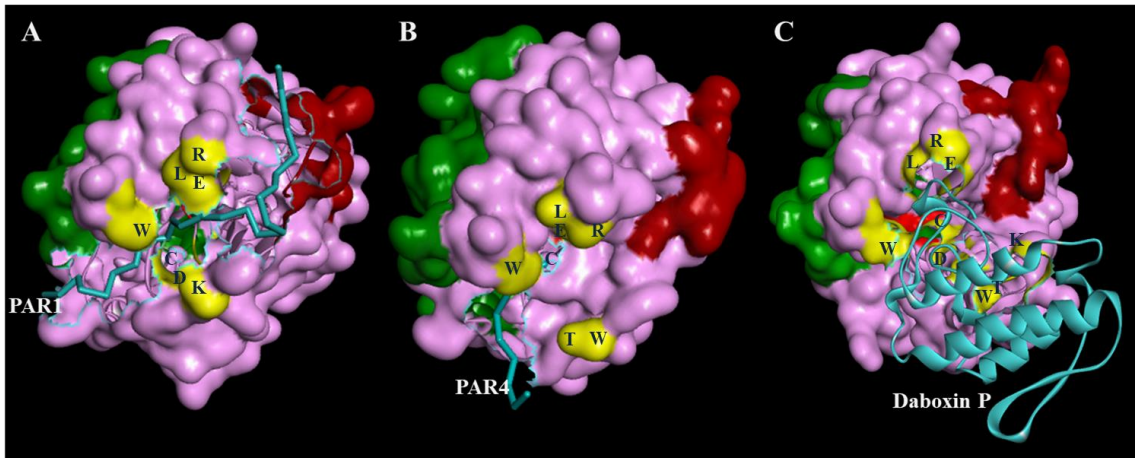

Figure 5.18: In-silico interaction studies between Daboxin P and thrombin: (A) PAR1 - Thrombin, (B) PAR4 - Thrombin, (C) Daboxin P - Thrombin (Thrombin: pink surface representation, Red: exosite I, Green: exosite II, Yellow: common residues of thrombin involved in binding PAR1, PAR4 and Daboxin P, PAR1 and PAR4: Blue stick model, Daboxin P: Blue ribbon.)

Table 5.2: Binding energy of interactions between Thrombin-PAR1, Thrombin-PAR4 and Thrombin-Daboxin P

INTERACTION	BINDING ENERGY (kcal/mol)
Thrombin-PAR1	**-15.6**
Thrombin-PAR4	**-9.4**
Thrombin-Daboxin P	**-8.1**

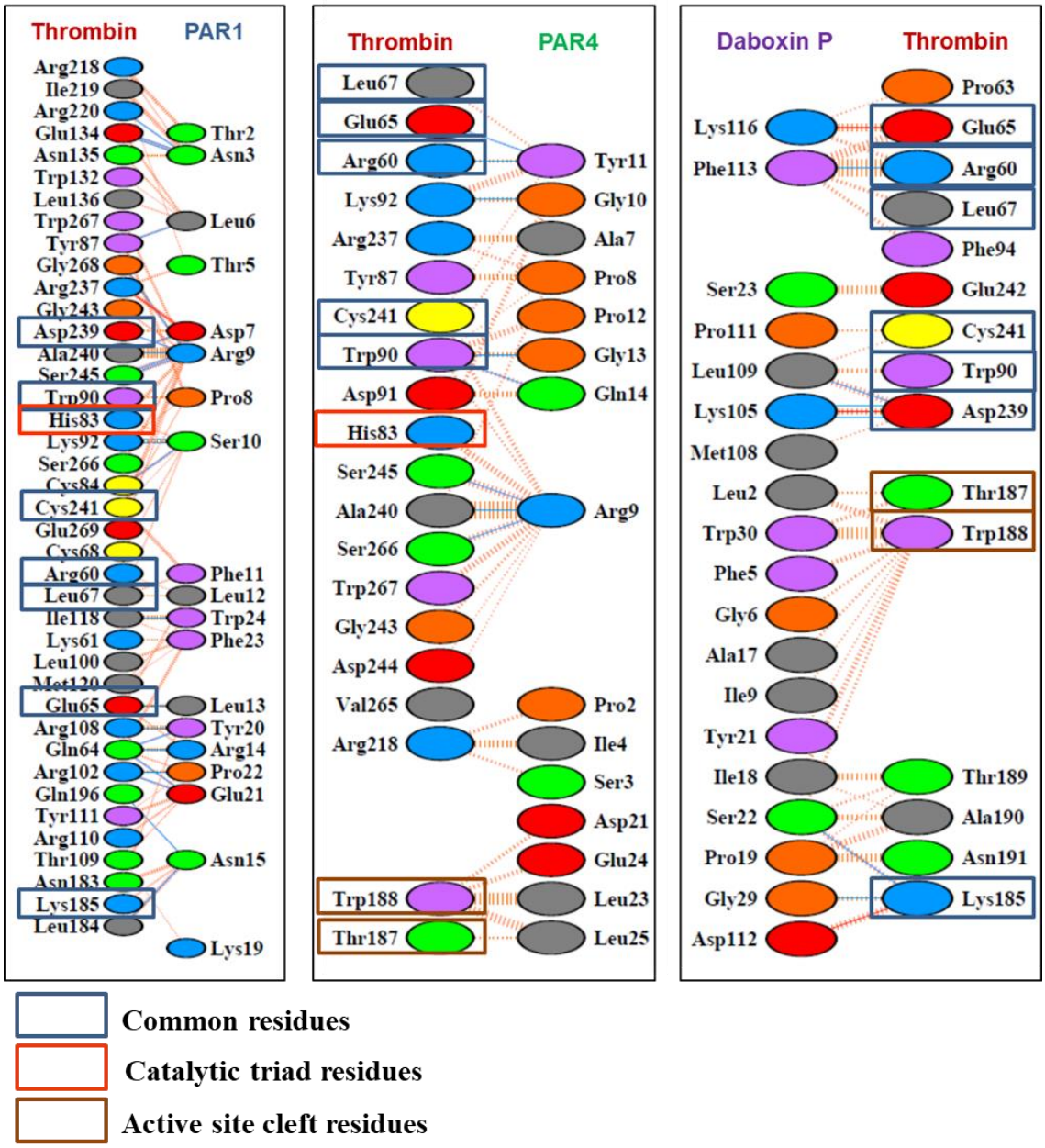

Figure 5.19: Contact map of interacting residues of Thrombin with PAR1/4 and Daboxin P: The amino acid sequences of Daboxin P (C0HK16), Thrombin (P00734), PAR1 (P25116) and PAR4 (Q96RI0) were retrieved from UniProt database and the contact maps of the interacting residues were generated in PDBsum server [122,126]

5.2.16.2 Fluorescence emission spectroscopy:

The fluorescence emission spectrum of thrombin was quenched by Daboxin P. The emission spectra of the mixtures of Daboxin P and thrombin in the ratios 1:2 to 1:5 were lower compared to the emission spectra of individual interacting components and there was gradual decrease with increase in Daboxin P concentration. However, at the

lowest ratio studied, i.e., 1:1, the emission spectrum was higher than that of the individual components (Fig 5.20 A).

5.2.16.3 Affinity column chromatography:

The flow though and elute alongwith thrombin were run on SDS-PAGE. The band for thrombin was observed at 37 kDa in lane 3. The gel showed no protein band in flow through, lane 1 while a prominent protein band was observed at ~ 37 kDa corresponding to the band of thrombin in the eluent, lane 2. Absence of protein band in the flow through suggests interaction between immobilized Daboxin P and thrombin and presence of a band corresponding to the band of thrombin in the eluent suggests disruption of the protein-protein interaction at high salt concentration (Figure 5.20 B).

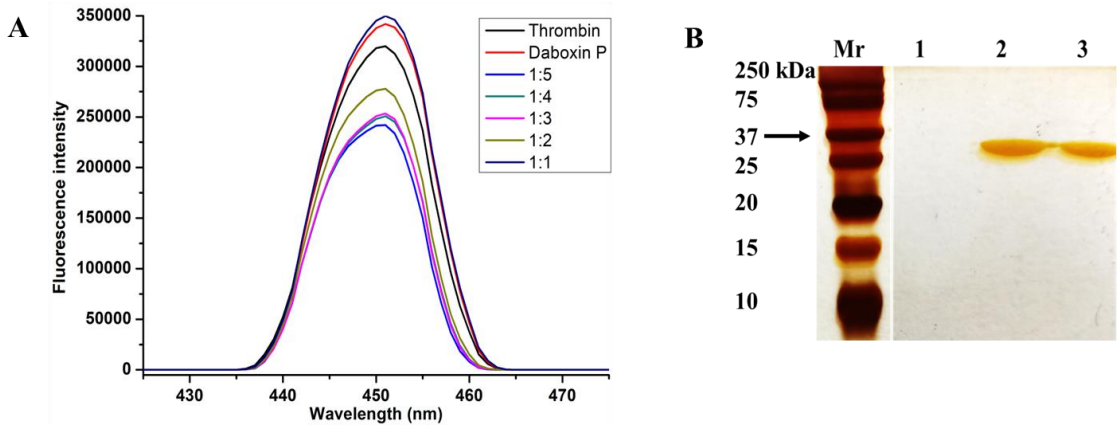

Figure 5.20: Binding studies between Daboxin P and thrombin: (A) Fluorescemce emission spectroscopy: (Briefly, Daboxin P was pre-incubated with thrombin in ratios 1:1, 1:2, 1:3, 1:4 and 1:5 for 20 min at 37℃. The emission spectra of individual components and Daboxin P-Thrombin mixtures were recorded from 280-600nm with an excitation wavelength of 280 nm). **(B) Affinity column chromatography** (SDS PAGE profile of affinity column chromatography fractions: Mr: marker, 1: flow-through 2: elute, 3: thrombin. Briefly, 40µg Daboxin P coupled with CNBr matrix and 6µg of thrombin was allowed to bind followed by elution with an acidic solution. The elution of bound thrombin was confirmed by 12.5% SDS-PAGE)

5.2.17 Inhibition of amidolytic and fibrinogenolytic activities of thrombin by Daboxin P:

Inhibition of amidolytic activity was tested using S2238 substrate. Thrombin was pre-incubated with Daboxin P in the ratio of 1:5 for 1 hour at 37°C followed by the addition of substrate S2238 (200mM). The release of colored product p-nitroaniline (p-NA) was

monitored at 405 nm for 15 minutes at an interval of 10 seconds. For studying inhibition of fibrinogenolytic activity, bovine plasma fibrinogen was used. Similar to the previous experiment, thrombin was pre-incubated with Daboxin and again incubated with fibrinogen for 1 hour. Subsequently, it was run on a 12.5% SDS PAGE. Reactions containing thrombin and substrate were taken as control. It was observed that Daboxin P did not inhibit the amidolytic activity or fibrinogenolytic activity of thrombin (Figure 5.21).

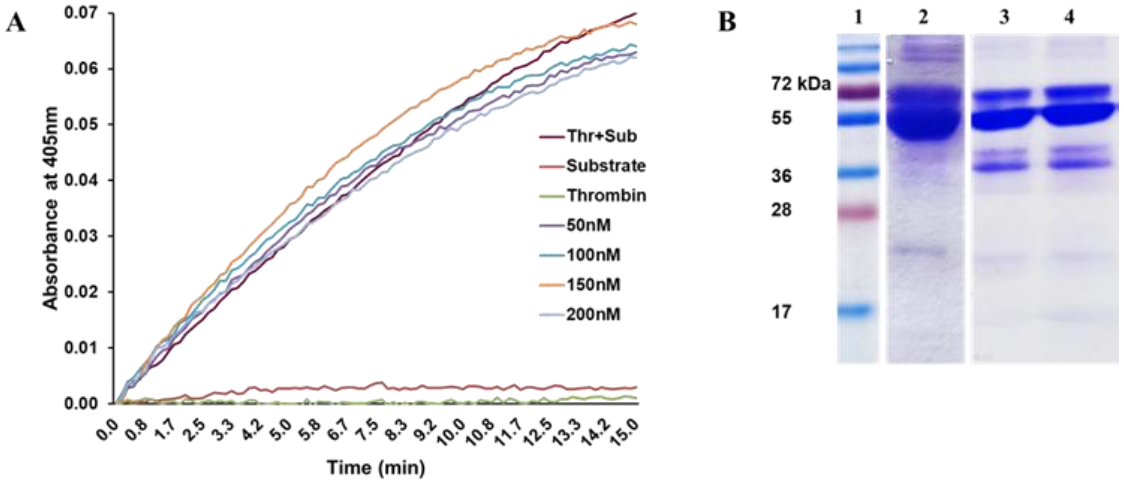

Figure 5.21: Effect of Daboxin P on functions of thrombin: (A) Inhibition of amidolytic activity, (B) Inhibition of fibrinogenolytic activity (Lane 1: Page Ruler Plus protein marker, Lane 2: Fibrinogen, Lane 3: Thrombin + fibrinogen incubated for 1 hour at 37℃, Lane 4: Daboxin P + thrombin incubated for 1 hour before incubation with fibrinogen)

5.2.18 Neutralization study of antiplatelet activity of Daboxin P with polyvalent antivenom:

The polyvalent antivenom could efficiently neutralize the anti-platelet activity of Daboxin P in higher doses. It was observed that in the ratios 1:1 and 1:10, the polyvalent antivenom could neutralize the anti-platelet activity of Daboxin P by 1% and 2±1% respectively. However, loss of anti-platelet activity of Daboxin P was observed in higher ratios like 1:100 and 1:1000, where the polyvalent antivenom was efficient in neutralizing 94±4.0% and 100% of the inhibitory effect of Daboxin P respectively (Figure 5.22).

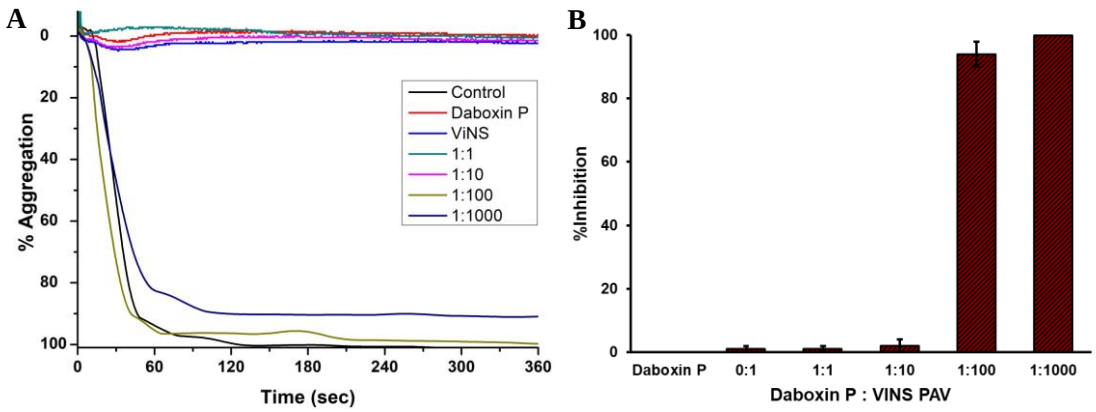

Figure 5.22: Neutralization of antiplatelet activity of 0.1µg Daboxin P by polyvalent antivenom: (A) Progressive curve, (B) Bar graph (Briefly, 0.1ug of Daboxin P was incubated with ViNS PAV in different ratios at 37°C for 1 hour prior to intiation of platelet aggregation by thrombin.)

5.3 DISCUSSION:

Platelets are tiny, non-nucleate cells derived from megakaryocytes whose key role is to stop bleeding by forming aggregates. Aggregation of platelets is mediated by different agonists which activate specific receptors present on platelet surface [26,28]. Various snake venom proteins belonging to different families have been reported to manipulate platelet function either by binding or degrading platelet receptors and modulating the activities of the agonists. For example, botrocetin from *Bothrops jararaca* and bitiscetin from *Bitis arietans* form trimolecular complexes with VWF and GPIb to activate platelets. On the other hand, EMS16 from *Echis multisquamatus* and rhodocetin from *Calloselasma rhodostoma* bind to collagen receptor, $\alpha_2\beta_1$ and bothrojaracin from *Bothrops jararaca* binds to exosites I and II of thrombin, thereby inhibiting platelet activation [45–50]. Snake venom PLA_2 enzymes are also known to inhibit thrombin-induced platelet aggregation [194].

Daboxin P, a PLA_2 enzyme purified from crude venom of Indian *Daboia russelii russelii* also modulates the platelet aggregation induced by various agonists. In this study, Daboxin P was re-purified as described by Sharma et. al and the purity was confirmed by SDS PAGE which displayed a single band of molecular weight ~13kDa. This is in congruence with the previously reported molecular mass of Daboxin P which is 13597.62 ± 1.28 Da [91].

The effect of Daboxin P 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), 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 is considered to be the strongest platelet agonist who activates platelets through proteolysis of the protease-activated receptors (PARs). N-terminal exodomains of PARs are specifically cleaved by this proteolytic enzyme and irreversibly activated when the cleaved fragment acts as tethered ligands binding to an extracellular loop of the same receptor [36,37]. Cleavage of the PARs present on platelet surface leads to signal generation inside the platelets causing granular secretion, increase in cytosolic calcium and platelet shape change 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].

Without addition of any agonist, Daboxin P had no effect on platelet aggregation (Figure 5.10). However, at a concentration of 163 nM, Daboxin P was effective in inhibiting the platelet aggregation induced by collagen and thrombin but it did not have any effect on ADP and arachidonic acid-induced platelet aggregation. This suggests that Daboxin P might not affect the other receptors present on the platelet surface; however, it might possess the ability to manipulate the functions of the receptors for collagen and thrombin, or these agonists themselves.

Similar observations were made when the effect of Daboxin P was studied on K-562 cells. Treatment with phorbol esters induces the human leukaemia cell line, K-562 to differentiate into megakaryocytic lineages which possess platelet surface receptors and respond to agonists like thrombin and ADP [195]. Aggregation of PMA differentiated K-562 cells in presence of 163 nM Daboxn P induced by various agonist was assessed and similar to the platelet aggregation studies in platelet-rich plasma, inhibition of

collagen and thrombin-induced aggregation was observed. Dose-dependent investigation of the effect of Daboxin P on collagen-mediated aggregation revealed that it reduced the aggregation of platelet-rich plasma as well as differentiated K-562 cells. However, the collagen induced platelet aggregation reached saturation at a dose of 150nM Daboxin P with around 45% aggregation (Figure 5.11). Incomplete inhibition might be because of the inability of Daboxin P to inhibit all the collagen receptors [32,196]. Further in-depth studies are required to understand the same. Nonetheless, Daboxin P completely inhibited the thrombin induced platelet aggregation at 200nM Daboxin P and the AD_{50} dose was found to be 55.166nM. It is evident from these experiments that among all the agonists studied, Daboxin P was the most effective in inhibiting platelet aggregation induced by thrombin. Therefore, the mechanism of inhibition of thrombin-mediated platelet aggregation by Daboxin P was subsequently investigated. Several thrombin inhibitors affecting thrombin-mediated platelet aggregation via different modes of action have been reported earlier. For example, Vorapaxar, an oral antiplatelet drug, is a high-affinity thrombin receptor antagonist which selectively antagonises PAR1 [197,198]. SCH-28, a synthetic heparin analogue, on the other hand, inhibits PAR4 activation by binding with thrombin [199]. A snake venom protein, bothrojaracin, has also been reported to inhibit thrombin-mediated platelet aggregation by binding to thrombin [49].

Thrombin consists of a light chain comprising of 36 residues and a heavy chain of 259 residues. The heavy chain contains the catalytic triad (His57, Ser195, Asp102) and a deep canyon-like active site cleft formed by the arrangement of the light and heavy chains [200]. The catalytic triad residues are located at the edge of a negatively charged surface that is surrounded by two positive hot spots called exosites [201]. Recent studies suggest that all of the natural substrates and cofactors of thrombin utilize one or both of the exosites, thereby making thrombin specificity a competition for the three sites: the active site, exosite I and exosite II [202–204]. While the active site bearing the catalytic triad residues (His57, Ser195, Asp102) is responsible for the catalytic activity of thrombin, the exosite interactions ensure the formation of stable thrombin-substrate complexes ensuring the cleavage of peptide bond. Exosite-I has been shown to bind substrates like fibrinogen and the thrombin inhibitor, hirudin whereas exosite-II binds to heparin [202,204,205].

Thrombin activates platelet aggregation by proteolytically activating cell surface receptors (PARs) which is essential to seal small vascular leakages that develop throughout the body every day by the formation of platelet plugs. Based on the above observations we hypothesize that Daboxin P modulated the thrombin-induced platelet aggregation by either binding to thrombin or the PARs present on the platelet surface. The in-vitro platelet aggregation experiments involving platelet-rich plasma and differentiated K-562 cells in this study suggest that when Daboxin P was pre-incubated with thrombin, percentage inhibition was significantly higher as compared to platelets pre-incubated with Daboxin P. Similarly, Daboxin P pre-incubated with thrombin was more effective in reducing the transmembrane calcium influx as depicted by the reduced intracellular calcium signal as compared to thrombin. This further confirms that Daboxin P interacts with thrombin, thereby, inhibiting its role in initiating platelet functions such as cleaving the PARs present on the platelet surface. This hypothesis was supported by in-silico and in-vitro biophysical interaction studies.

Since Daboxin P could bind thrombin and inhibit its ability to activate platelets, we also studied whether it could inhibit other important functions of thrombin-like amidolytic activity and fibrinogenolytic activities. The amidolytic activity was studied using substrate, H-D-Phe-Pip-Arg-pNA·2 HCl (S2238) which is enzymatically cleaved by thrombin to release p-nitroaniline (p-NA) [189]. The fibrinogenolytic activity was studied using bovine plasma fibrinogen. Cleavage of fibrinogen involves its binding to exosite I of thrombin which cleaves two fibrinopeptides, FPA and FPB from the Aα and Bβ chains respectively [205,206]. However, these activities of thrombin were not inhibited by Daboxin P (Figure 5.20). Similar to fibrinogen, PAR1 also requires exosite I for its interaction with thrombin [37]. Consequently, Daboxin P being efficient in inhibiting thrombin's role as a platelet aggregation agonist but ineffective in inhibiting fibrinogenolysis seems contradictory. Nonetheless, it should also be noted that PAR4 interaction with thrombin is independent of exosite I, which might be the probable reason behind Daboxin P's bias in inhibiting thrombin-induced platelet aggregation but not fibrinogenolytic activity [207].

It has been reported earlier that Daboxin P binds to factor X and factor Xa to exhibit its anticoagulatory effect [91]. Similarly, this study demonstrates that Daboxin P binds to thrombin to manifest its antiplatelet property. This finding can be correlated with

symptoms of Russell's viper envenomation where profuse bleeding is observed at the site of injury which might possibly be due to Daboxin P not allowing thrombin to activate platelets and thus preventing clot formation. A similar complex formation of thrombin with another snake venom protein, bothrojaracin was previously reported. Zingali and his group had isolated this 27 kDa protein from the venom of *Bothrops jararaca* that blocks several functions of thrombin including platelet activation (Zingali et al., 1993). Bothrojaracin has been shown to bind both exosite I and exosite II of thrombin without interacting with the catalytic site (Arocas et al., 1996). Although, Daboxin P doesn't bind the catalytic triad residues of thrombin, but it binds the active site cleft residues, probably blocking the active site, thus inhibiting thrombin-mediated platelet aggregation.

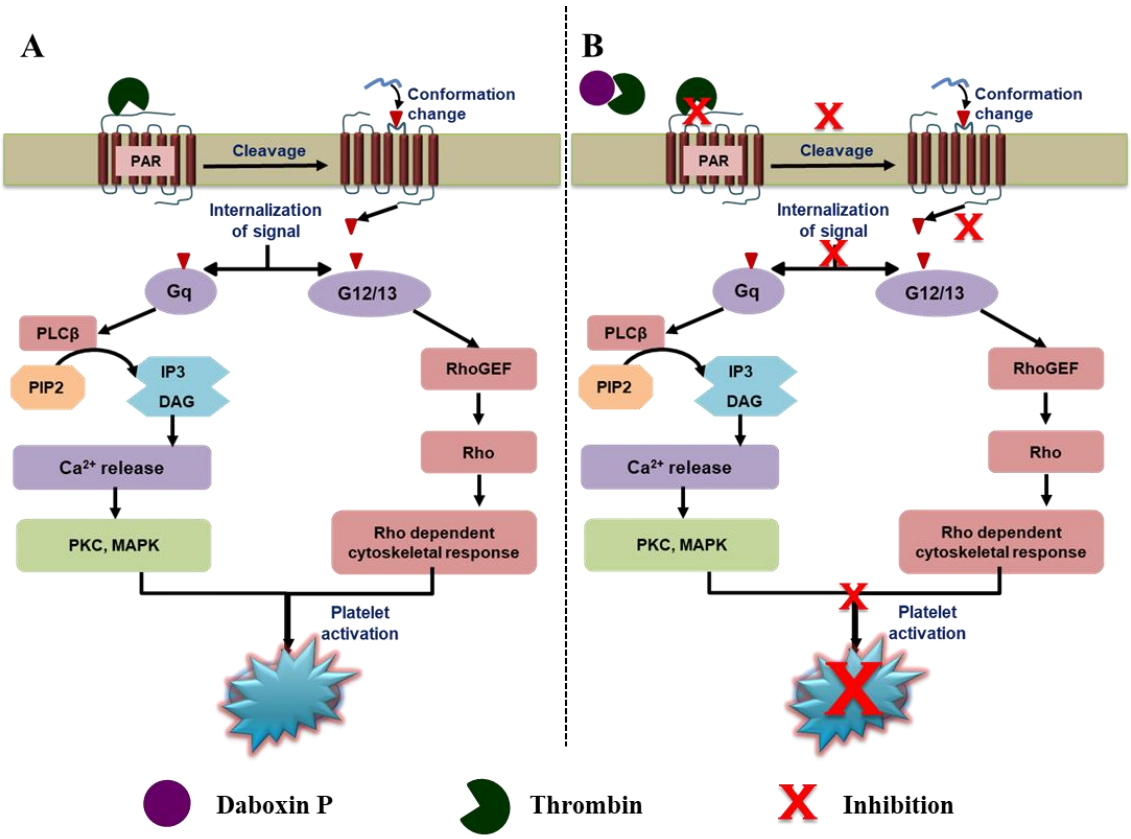

Figure 5.23: Proposed mechanism of anti-platelet activity of Daboxin P: (A) Mechanism of thrombin-induced platelet aggregation. Cleavage of PAR by thrombin initiates a cascade of reactions which leads to platelet activation **(B) Effect of Daboxin P on thrombin-induced platelet aggregation.** Binding of Daboxin P to thrombin inhibits PAR cleavage by thrombin, thus inhibition platelet activation

The present study evaluates the antiplatelet potential of Daboxin P as a probable natural inhibitor of thrombin from snake venom. Based on the in-silico, biochemical and biophysical interaction studies, it can be proposed that Daboxin P inhibits thrombin-mediated aggregation of platelets by interacting with thrombin (Figure 5.23). Since platelet aggregation is followed by an elaborate process of multiple complex signalling pathways activated by various agonists, further studies are required to understand the complete molecular mechanism of Daboxin P in inhibiting platelet aggregation. However, Daboxin P being efficient in inhibiting thrombin's role as a platelet aggregation agonist but ineffective in inhibiting the amidolytic and fibrinogenolytic activities of thrombin is an interesting observation. Therefore, Daboxin P might prove to be useful in engineering better therapeutic interventions for inhibiting thrombin mediated platelet aggregation without affecting other functions of thrombin.