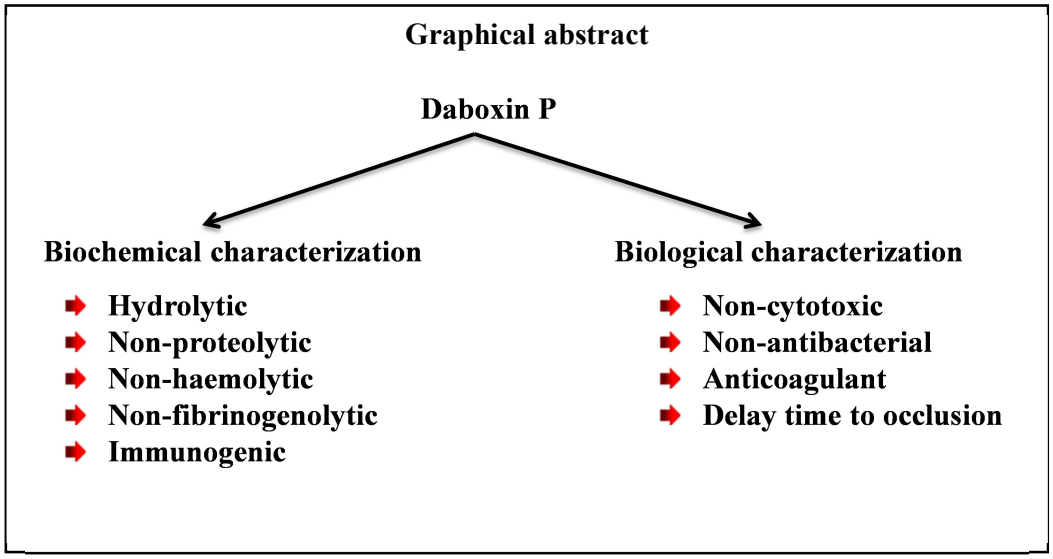
Chapter 5

Biochemical and biological characterization of the purified protein



5.1 INTRODUCTION

Snake venom is a rich repertoire of various isoforms of PLA_2 enzymes with potent pharmacological activities (38). Unlike the mammalian PLA_2 enzymes which are their structural homologs, these enzymes display very complex structure-function chemistry with wide range of pathological activities apart from prey digestion (81). It is reported that a single snake venom PLA_2 enzyme is capable of exhibiting various pathological activities like neurotoxicity, myotoxicity, edema inducing, haemorrhage, anticoagulation, etc. in the prey or the victim (46). The subtle differences in their activities is mainly attributed to the presence of various pharmacological sites (responsible for non-enzymatic activity via protein-protein interaction) which are distinct from the catalytic site (responsible for enzymatic activity via protein-phospholipid interaction) (292). The amino acid substitutions in the exposed regions of these isoforms are responsible for the diverse pharmacological sites (180).

In the previous chapter, the purification and biophysical properties of daboxin P, a major protein from the venom of Indian *Daboia russelii* have been discussed. Its primary structure revealed it to be a calcium dependent catalytic PLA_2 enzyme with the conserved Ca^{2+} binding loop and His48, Asp49, Tyr52, Tyr73 and Asp99-residues. Moreover, the presence of positively charged amino acid residues in the anticoagulant region suggests it to be a strong anticoagulant PLA_2 enzyme. As the primary structure of daboxin P suggests its role in pathology during Russell's viper envenomation hence, the present chapter has discussed some of the biochemical and pharmacological activities of daboxin P under *in-vitro* and *in-vivo* conditions.

5.2 MATERIALS

5.2.1 Chemicals and reagents

Western blotting: Westran clear signal (0.45 µm, 30 cm x 3M) was bought from GE Healthcare Life Sciences (Chicago, USA). Anti-Horse IgG (whole molecule)−Alkaline Phosphatase antibody produced in rabbit and BCIP/NBT [5-Bromo-4-chloro-3-indoyl-phosphate (BCIP) /nitroblue tetrazolium (NBT)] solution premixed were purchased from Sigma-Aldrich (Missouri, USA). Tween-20 was purchased from Merck (Darmstadt, Germany).

Antivenoms: The polyvalent antivenom (Snake venom antiserum i.p.) was purchased from Vins Bioproduct limited (batch # 01AS15007), (Telangana, India).

Others: Thrombin and 2, 4-dibromoacetophenone (para-Bromophenacyl bromide) were purchased from Sigma-Aldrich (Missouri, USA). The bacterial strains, *E. coli* and *S. aureus* were a generous gift from the Microbiology laboratory of Dr. Manabendra Mandal, Department of Molecular Biology and Biotechnology, Tezpur University.

All other reagents and chemicals used are described in material section 2.2.2 and 3.2.2 of Chapter 2 and 3 respectively.

5.2.2 Animals

The C57BL/6 male (9–11 weeks old, 24–28 g) mice were obtained from *InVivos* (Singapore).The animals were kept under optimal housing conditions in well ventilated clean cages. They were supplied with proper food and water obtained from

the centre. The temperature of the animal house was set at 25± 3˚C with alternative cycles of light/dark conditions after every 12 h. Proper care was taken for the health and cleanliness of the mice by trained professionals. The *in-vivo* experiments were performed as per the guidelines approved by Institutional Animal Care and Use Committee (IACUC), National University of Singapore, Protocol number 041/12 (enclosed in Appendix-V).

5.3 METHODS

5.3.1 PLA_2 activity and determination of Km and Vmax of daboxin P

The hydrolytic activity of daboxin P was determined by the $sPLA_2$ assay kit, Cayman Chemical according to the instructions of the manufacturer as described in section 3.3.8 of Chapter 3.

For determining the Km and Vmax of daboxin P, 0.01 µg of daboxin P was incubated with various concentration of substrate (diheptanoyl thio-PC) and its hydrolysis was quantified at 414 nm. The Michaelis-Menten's curve was plotted by non-linear curve fit using Origin (OriginLab) and the Lineweaver burk plot was plotted using Excel MS office 2007.

5.3.2 Alkylation of histidine residue of daboxin P

The alkylation of the histidine residue at the 48^{th} position of the primary sequence of daboxin P was carried out according to the method developed by Maduwage and co-workers (470) (Figure 5.1). Briefly, 100 µg of p-BPB was dissolved in 30 µl of 100% ethanol. Following this, 150 µg of daboxin P was treated with 15 µl of the dissolved p-BPB and incubated at room temperature for 20 h. For vehicle control, 150 µg of daboxin P was treated with 15 µl of 100% ethanol and incubated under the same conditions. After incubation, the samples were desalted by dialyzing in 20 mM of Tris-Cl pH 7.4 at 4^oC for 3 times. The dialysed samples were lyophilized in pre-conditioned lyophilizer at -80^oC. The alkylated daboxin P was analysed on ESI-MS for the change in mass. Following this, the samples were reconstituted in appropriate buffer to carry out the PLA_2 assay.

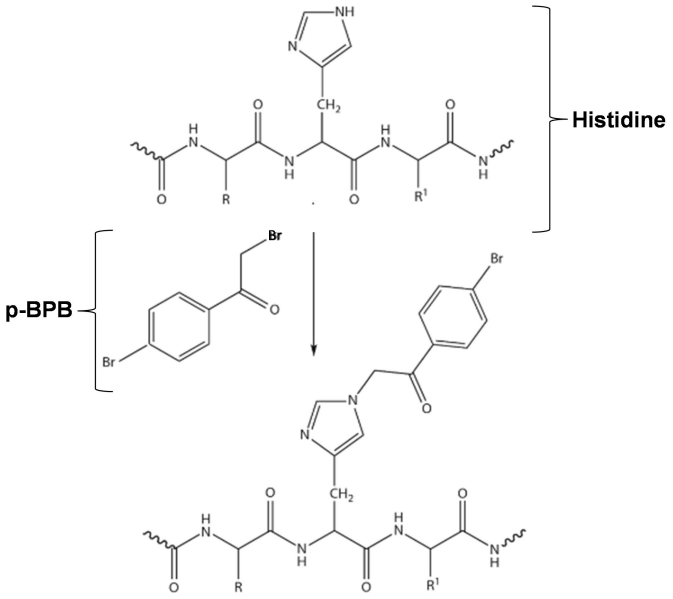


Figure 5.1: Pictorial demonstration of the mechanism of histidine residue alkylation by p-BPB (Adapted from Lundblad R.L; 2011 (471))**.** p-BPB is a α-halo carboxylic acid which binds to the His48 of the PLA_2 enzymes due to its hydrophobic nature. It binds to N1 atom of the imidazole ring of the histidine residue.

5.3.3 Proteolytic activity of daboxin P

Different amount of daboxin P were pre-incubated with 1% casein for 90 min at 37°C. The detailed procedure for the proteolytic experiment has been described in section 3.3.9 of Chapter 3.

5.3.4 Direct and indirect haemolytic activity of daboxin P

The direct and indirect haemolytic activity daboxin P were determined as described in section 3.3.7 of Chapter 3.

5.3.5 *In vitro*-anticoagulant activities of daboxin P

For the evaluation of the anticoagulant potency of daboxin P under *in-vitro* conditions, PP human plasma was used. Briefly, fresh whole blood was collected in a sterilized tube containing 3.2% of tri-sodium citrate in the ratio of 1:9 (citrate:blood) from healthy human volunteers (402). The whole blood was centrifuged at 3,000 rpm for 20 min and the supernatant containing the PP plasma was aliquot and stored at -20°C.

The recalcification time, prothrombin time and activated partial thromboplastin time of daboxin P were assayed as described in section 2.3.6.2 of Chapter 2 and 3.3.5.2 and 3.3.5.3 of Chapter 3.

5.3.5.1 Stypven time of daboxin P

The anticoagulant effect of daboxin P on stypven time was estimated using RVV-X as the reagent. Briefly, in a reaction volume of 300 µl, different concentration of daboxin P were pre-incubated with 75 µl of PP plasma for 3 min at 37^{o}C (472). Subsequently, 75 µl of RVV-X (10 ng/ml) was added and incubated for 2 min. The clot formation was initiated by addition of 25 mM of $CaCl_2$ and recorded using Coastat-1 Coagulation Analyser.

5.3.5.2 Thrombin time of daboxin P

The effect of different concentration of daboxin P on the thrombin time was assessed using PP plasma at 37^{o}C (472). Briefly, 50 µl of 10 u/ml of thrombin was added to initiate the clot formation and was monitored using Coastat-1 Coagulation Analyser.

5.3.6 Fibrinogenolytic activity of daboxin P

The fibrinogenolytic activity of different amount of daboxin P was determined as described in section 3.3.6 of Chapter 3.

5.3.7 *In-vivo* anticoagulant activity of daboxin P

The anticoagulant activity of daboxin P under *in-vivo* conditions was determined using $FeCl_3$-induced carotid artery thrombosis model (473-475). C57BL/6 male mice weighing ~24–28 g (9–11 weeks old) were used for the experiment. Briefly, the male mice were injected intraperitoneally with 75 mg/kg of ketamine and 1 mg/kg of medetomidine. To the anesthetized mice (n=6), 10 mg/kg of Daboxin P was injected into the tail vein. After treatment, the right carotid artery was exposed using blunt dissection. The flow of blood from the carotid artery was monitored using a doppler flow probe connected to a perivascular flow module (Transonic System Inc., New York, USA). To initiate thrombus formation, a piece of filter paper (2x2 mm) soaked in 10% $FeCl_3$ solution was placed on the surface of the exposed carotid artery for 3 min. As negative control, saline treated mice (n=5) were considered for the

experiment. Following $FeCl_3$ treatment, the Time-to-occlusion (TTO) for each case was measured. TTO is defined as the time taken for the blood flow to reach zero after the application of $FeCl_3$. Each tested mice was observed for a maximum time of 60 min after the application of $FeCl_3$. TTO was recorded as 60 min, if no occlusion occurred by this time. The protocol for the experiment was approved by Institutional Animal Care and Use Committee, National University of Singapore and was performed as per the guidelines of the animal ethical committee (enclosed in Appendix-V).

5.3.8 Antibacterial activity of daboxin P

The antibacterial activity of different concentrations of daboxin P were analysed using gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Esherichia coli*) using the Agar diffusion well method developed by Valgas and colleagues (476). Briefly, freshly prepared Luria Bertani Agar (LBA) was poured over perti dishes and allowed to solidify under sterile conditions in a Laminar air flow cabinet, Reico Equipment and Instrument Pvt. Ltd. (West Bengal, India). The solidified LBA plates were spread with appropriate dilutions of freshly cultured *E. coli* and *S. aureus*. Subsequently, the plates were punched uniformly at 5 different positions with a sterile gel puncher to make up the diameter of each well up to 0.5 cm. As positive control, 50 µl of filter sterilized ampicillin (25 mg/ml) was added while 50 µl of 20 mM of Tris-Cl buffer, pH 7.4 was added to another well as the negative control. To the rest of the three wells different amount of the crude venom (1, 5, 10 µg) were added. The LBA plates were incubated at 37^{o}C for 16 h in static incubator, Scigenics Biotech Pvt. Ltd. (Tamil Nadu, India). Next day the plates were observed for the zone of clearance.

5.3.9 Cytotoxicity study of daboxin P

The cytotoxic effect of daboxin P was tested on both normal (HEK-293) and cancerous cell lines (MCF-7) using the MTT based colorimetric method as described in section 3.3.12 of Chapter 3.

5.3.10 Inhibition study of daboxin P

For the neutralization of the PLA_2 activity of daboxin P, different amount of polyvalent antivenom (Vins Bioproduct limited) were pre-incubated with 0.01 µg of

daboxin P for 1 h at 37°C. Following incubation, the PLA_2 activity was measured as described in section 3.3.8 of Chapter 3. For neutralization of the recalcification time of daboxin P, different amount of polyvalent antivenom (Vins Bioproduct limited) were pre-incubated with 0.1 µg of daboxin P for 1 h at 37°C. Following incubation, the clotting time was determined as described in section 2.3.6.2 of Chapter 2.

5.3.11 Western blotting

The immunoreactivity of daboxin P towards the Vins polyvalent antivenom was determined by Western blotting using the Bio-Rad mini protean® system (California, USA) (477). Briefly, 7 µg of daboxin P was treated with βME and boiled for 3 min at 100°C. The treated sample was loaded onto a 12.5% glycine SDS-PAGE and electrophoresis was carried out as described in section 2.3.2 of Chapter 2. After the electrophoretic run the gel was dipped in transfer buffer, Tris buffered saline (TBS) (25 mM Tris-Cl, pH 7.4, 192 mM glycine, 20% methanol, 1.28 mM SDS) for 15 min along with the PVDF membrane which was initially activated by soaking in 100% methanol for 2 min. Subsequently, the blotting sandwich was prepared containing 2 layers of each of sponges and blotting paper, all previously soaked in the transfer buffer. To this blotting cassette the membrane and the gel were inserted in the right orientation and electroblotting was carried out at a constant voltage of 100 v, 300 mA for 90 min using the Bio-Rad electrophoresis apparatus (California, USA). Following the run, the membrane was carefully retrieved from the blotting cassette and immediately blocked with Tris buffered saline tween, TBST (20 mM Tris, 137 mM NaCl and 500 µl of Tween 20) containing 10% of skimmed milk with gentle shaking for 1 h. Skimmed milk prevents non-specific binding of the primary antibody (to be added in the subsequent step) to the membrane. Following this, the blot was treated with 10 ml of primary antibodies (1:4000) (polyvalent antivenom) prepared in blocking buffer and incubated at 4°C overnight with continuous shaking. Next day, the membrane was thoroughly rinsed with TBST twice to remove excess blocking buffer. Consequently, it was treated with secondary antibody (anti-horse IgG-alkaline phosphatase) at a dilution of 1:1000 for 2 h. The blot was thoroughly washed with TBST for 2-3 times to minimize the background noise created by the non-specific binding of the secondary antibody. Consequently, 1 ml of pre-mixed BCIP-NBT solution was added to the blot and observed for the band development. After the

formation of the bands, the reaction was stopped with 1% acetic solution. The blot was air dried and documented using ChemiDoc^{TM}XRS+, Biorad (California, United States).

5.4 RESULTS

5.4.1 PLA_2 activity of daboxin P

The Km and Vmax for the PLA_2 activity of daboxin P on diheptanoyl thio-PC were calculated to be 6.6 mM and 1.14 mmol/(min*mg) respectively which were determined by Lineweaver Burk-plot (Figure 5.2 A, B & C). Daboxin P exhibited hydrolytic activity on diheptanoyl thio-PC in a dose dependent manner with a specific activity of 37.53±1.52 µmol/(min*mg) (Figure 5.2 D).

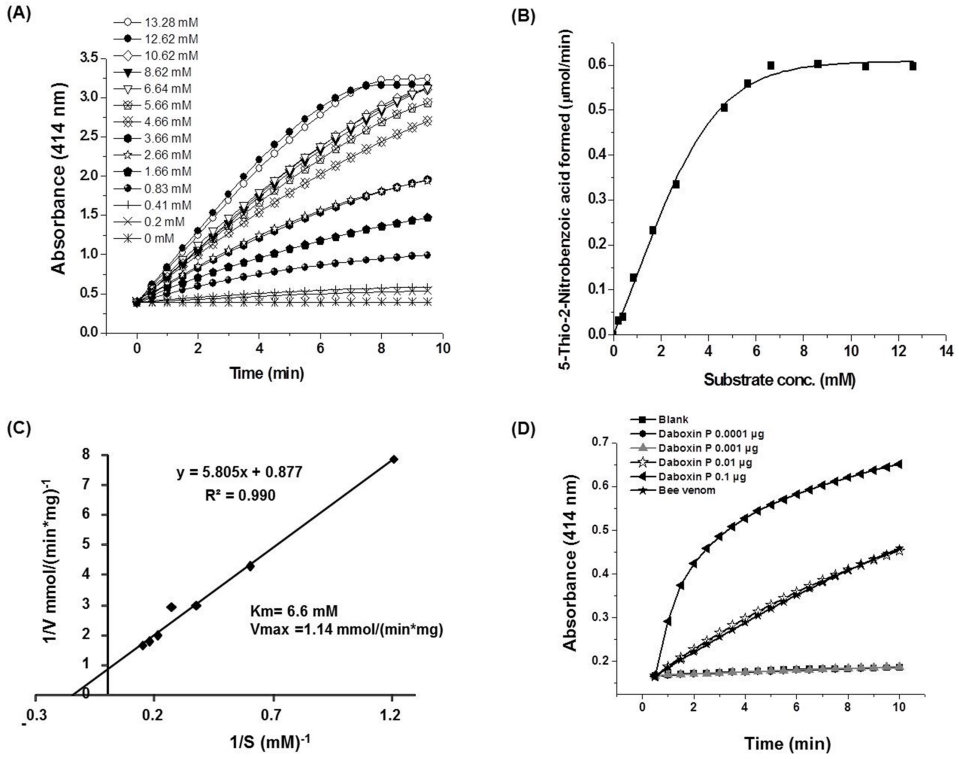


Figure 5.2 PLA_2 activity of daboxin P. (A): Progress curve for the PLA_2 activity of 0.01 µg of daboxin P at different substrate concentration. **(B**): Michaelis-Menten's curve for the $sPLA_2$ activity. **(C):** Lineweaver-Burk plot for determination of Km and Vmax. **(D):** The progressive curve of diheptanoylthio-PC cleavage by different concentrations of daboxin P. Bee venom PLA_2 enzyme is considered as the positive control.

5.4.2 Alkylation of histidine residue of daboxin P

Alkylation of the histidine residue of daboxin P with p-BPB has led to the complete loss of its PLA_2 activity on diheptanoyl thio-PC. At 0.01 µg, the unmodified daboxin exhibited 37.53 ± 1.52 µmol/(min*mg) of PLA_2 activity however, after alkylation the same amount of the protein did not show any PLA_2 activity (Figure 5.3).

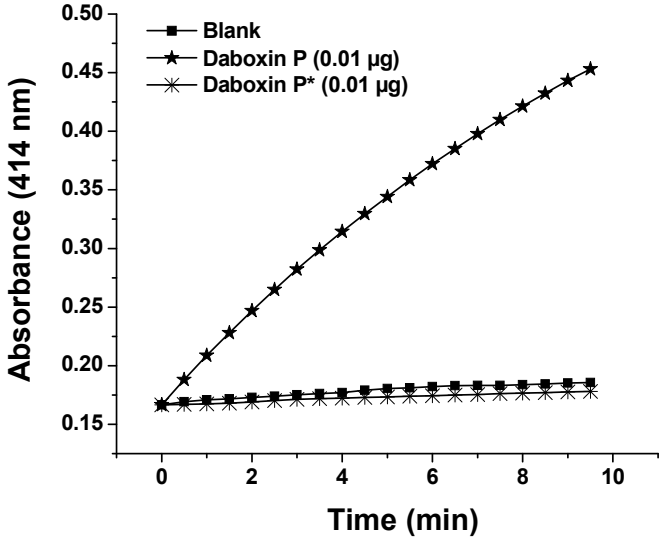


Figure 5.3: Progress curve for PLA_2 activity of modified and unmodified daboxin P. The PLA_2 effect of modified and unmodified daboxin P (0.01 µg) on diheptanoyl thio-PC was quantified at 414 nm using UV-Vis MultiSkan Go spectrophotometer. Histidine modified daboxin P is represented with asterisk sign *.

5.4.3 Proteolytic activity of daboxin P

It was observed that, 5 µg of crude venom of *Daboia russelii* exhibited 1.3 x 10^{-2} µg/min/ml of caseinolytic activity; however, the same amount of daboxin P did not display any proteolytic activity on casein (Figure 5.4).

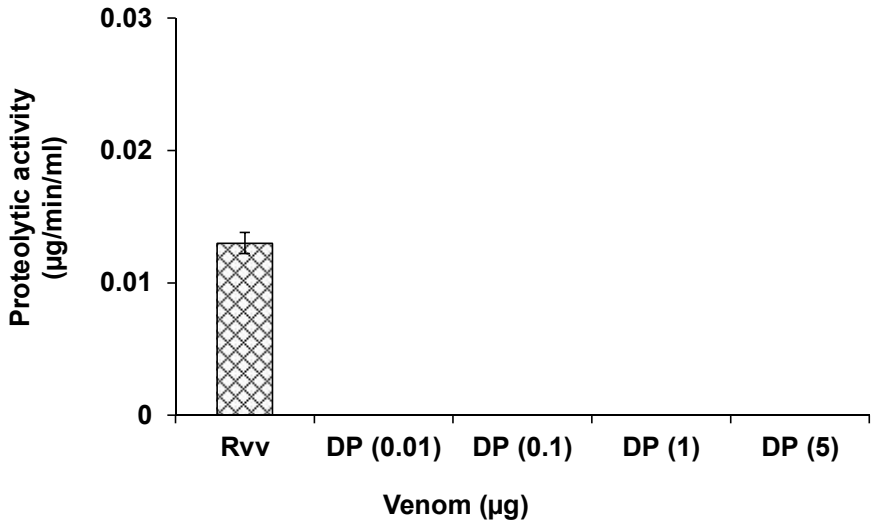


Figure 5.4: Proteolytic activity of daboxin P. Different amount of daboxin P was pre-incubated with 1% casein for 90 min at 37°C. Crude *Daboia russelii* venom (5 µg) was considered as the positive control for the assay.The results are mean ± SD of three independent experiments.

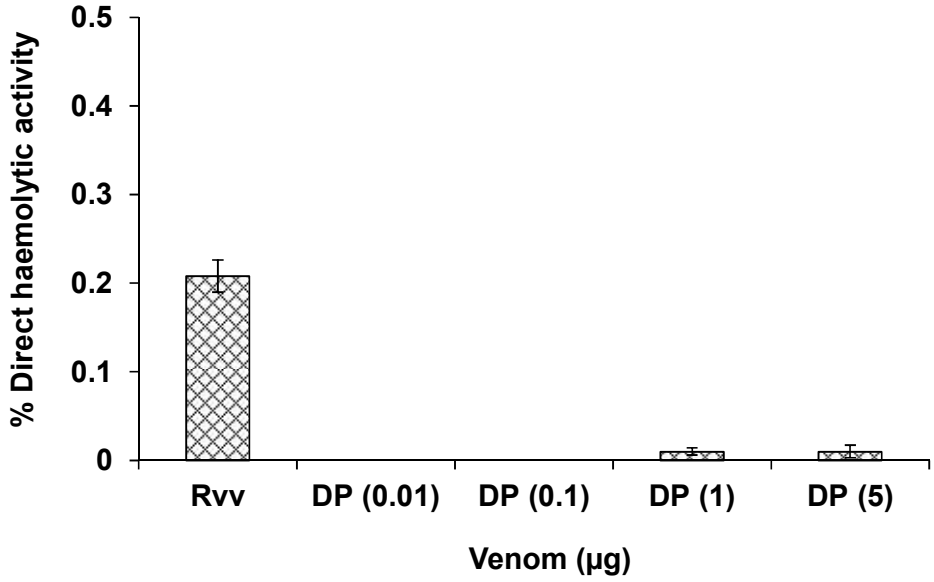


Figure 5.5: Direct haemolytic activity of daboxin P. Different amount of daboxin P were pre-incubated with 10% RBC for 1 h at 37°C. As positive control 5 µg of crude *Daboia russelii* venom was taken. The results are mean ± SD of three independent experiments.

5.4.4 Direct haemolytic activity of daboxin P

Daboxin P exhibited mild direct haemolytic activity on erythrocyte suspension; however, the effect was not dose dependent. Five micrograms of daboxin P displayed 0.01 ± 0.0071% of direct haemolytic activity while the same amount of the crude venom showed 0.208 ± 0.018% of haemolytic activity which was considered as the positive control for the experiment (Figure 5.5).

5.4.5 Indirect haemolytic activity of daboxin P

Daboxin P displayed indirect haemolytic activity on erythrocyte suspension in the presence of egg yolk in a dose dependent manner. It was observed that 5 µg of daboxin P exhibited 77.5 ± 2.98% of indirect haemolytic activity in presence of egg yolk phospholipid while the same amount of the crude venom of *Daboia russelii* showed 20 ± 2.1% of indirect haemolysis under same experimental condition (Figure 5.6).

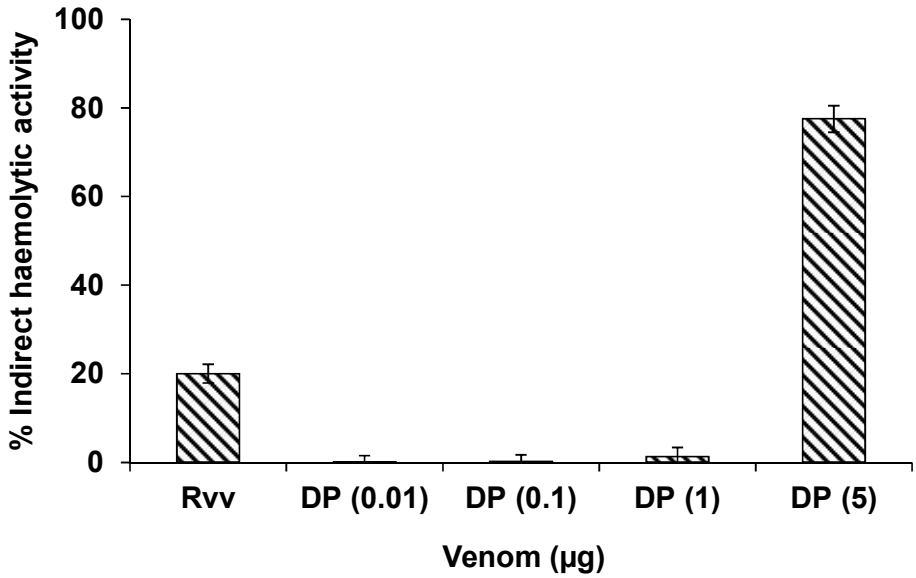


Figure 5.6: Indirect haemolytic activity of daboxin P. Different amount of daboxin P were pre-incubated with 10% RBC and egg yolk suspension for 1 h at 37^{o}C. As positive control 5 µg of crude *Daboia russelii* venom was taken. The results are mean ± SD of three independent experiments.

5.4.6 *In-vitro* anticoagulant activity of daboxin P

5.4.6.1 Recalcification time of daboxin P

Daboxin P prolonged the recalcification time of human plasma in a dose dependent manner (Figure 5.7). At a concentration of 0.01 µM, it prolonged the recalcification time of plasma beyond 600 s which is 3.28 fold higher than the NCT (183 ± 5.5 s).

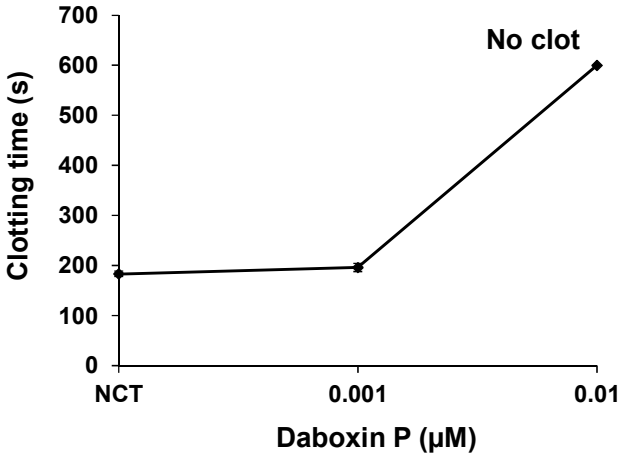


Figure 5.7: Recalcification time of daboxin P. Different concentrations of daboxin P were pre-incubated with 150 µl of plasma at 37°C for 2 min. $CaCl_2$ (50 mM) was added to initiate clot formation. The results are mean ± SD of three independent experiments.

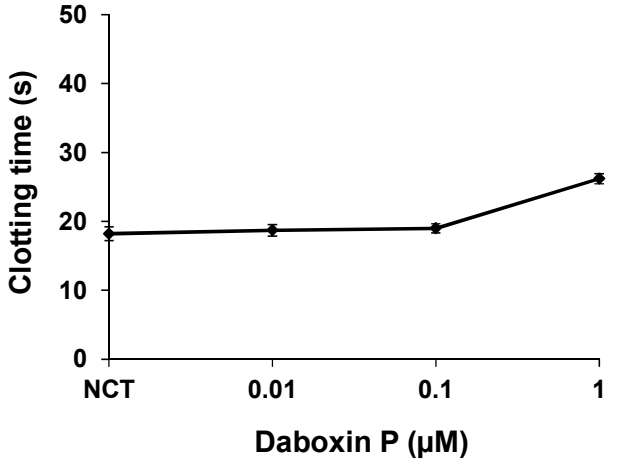


Figure 5.8: Effect of daboxin P on the prothrombin time. Different concentrations of daboxin P (0.01, 0.1 & 1 µM) were pre-incubated with PP plasma at 37°C for 2 min. PT reagent (50 µl) was added to initiate the clot formation. The results are mean ± SD of three independent experiments.

5.4.6.2 Prothrombin time of daboxin P

Daboxin P did not prolong the prothrombin time of plasma (Figure 5.8). At 1 µM, it delayed the clotting time to 26.2 ± 0.73 s which is only 1.44 fold higher compared to NCT (18.2 ± 1 s).

5.4.6.3 Activated partial thromboplastin time of daboxin P

Daboxin P delayed the activated partial thromboplastin time of PP plasma in a dose dependent manner (Figure 5.9). At 1 µM, it prolonged the clotting time to 130 ± 5.56 s which is 3.09 fold higher than the NCT (42 ± 3.34 s).

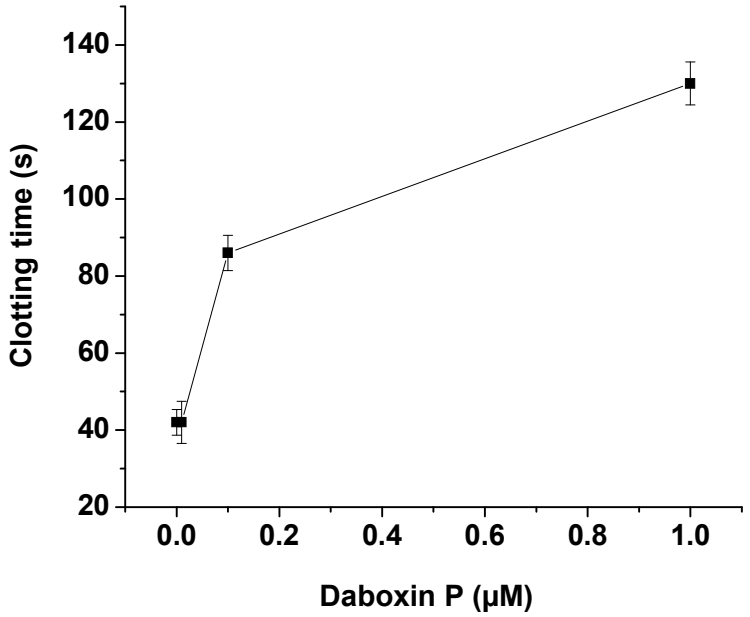


Figure 5.9: Effect of daboxin P on the activated partial thromboplastin time of PP plasma. Different concentrations of daboxin P were pre-incubated with PP plasma and APTT reagent for 3 min at 37°C. Clot formation was initiated by addition of 25 mM $CaCl_2$. The results are mean ± SD of three independent experiments.

5.4.6.4 Stypven time of daboxin P

Daboxin P also exhibited anticoagulant effect on stypven time in a dose dependent manner (Figure 5.10). At 1 µM concentration, it delayed the stypven time to 126.33 ± 4.34 s which is 6.05 fold higher than the NCT (20.85 ± 3.2 s).

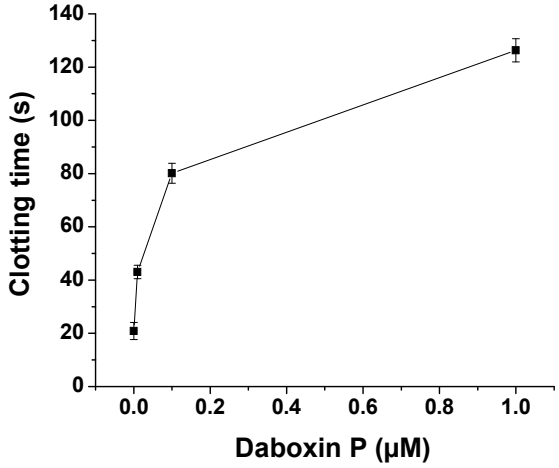


Figure 5.10: Effect of daboxin P on the stypven time of PP plasma. Different concentrations of daboxin P were pre-incubated with PP plasma for 3 min at 37°C followed by incubation with RVV-X (10 ng/ml) for 2 min. Clot formation was initiated by addition of 25 mM of $CaCl_2$. The results are mean ± SD of three independent experiments.

5.4.6.5 Thrombin time of daboxin P

The thrombin time of PP plasma with 5 µg of daboxin P was found to be 14 ± 1.3 s while that with 20 mM Tris-Cl (pH 7.4) was 13.8 ± 1.5s. No noteworthy change in thrombin time was observed with daboxin P compared to NCT signifying no inhibitory effect on thrombin (Figure 5.11).

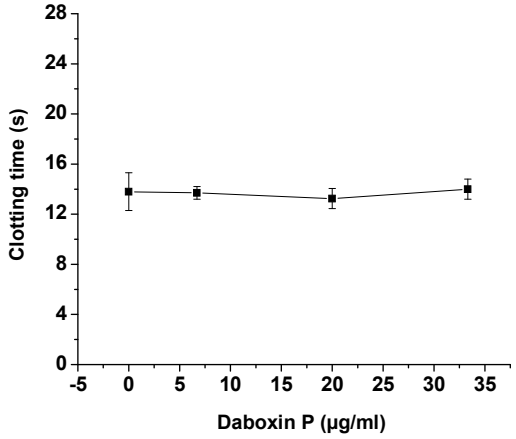


Figure 5.11: Thrombin time of daboxin P. Different concentration of daboxin P was pre-incubated with PP plasma for min at 37°C. Clot formation was initiated by addition of thrombin. The results are mean ± SD of three independent experiments.

5.4.6.6 Fibrinogenolytic activity of daboxin P

It was observed that, daboxin P did not show any proteolytic effect on the three bands (α, β and γ) of fibrinogen even after an incubation period of 24 h at 37°C up to 5 µg (Figure 5.12). However, under the same experimental conditions, 1 µg of crude *Daboia russelii* venom degraded α band completely and partially degraded the β-band of fibrinogen.

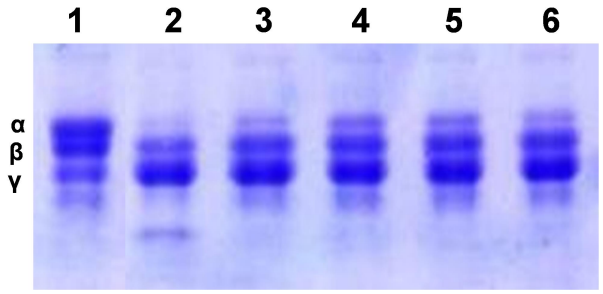


Figure 5.12: Assessment of the fibrinogenolytic activity of daboxin P on 12.5% glycine SDS-PAGE. Lane 1: Tris-Cl (50 mM Tris-Cl, pH 7.5) treated fibrinogen considered as negative control; **Lane 2:** crude *Daboia russelii* venom (1 µg) treated fibrinogen considered as positive control. Fibrinogen treated with different amount of daboxin P. **Lane 3** 0.01 µg, **Lane 4:** 0.1 µg, **Lane 5:** 1 µg and **Lane 6:** 5 µg.

5.4.7 *In-vivo* anticoagulant activity of daboxin P

Apart from *in-vitro* anticoagulant activity, daboxin P also exhibited anticoagulant effect under *in-vivo* conditions when tested on the mouse $FeCl_3$ induced carotid artery thrombosis model (Figure 5.13). The saline treated mice displayed a TTO of 8.29 ± 2.61 min however; 10 mg/kg of daboxin P delayed the TTO in treated mice to 35.18 ± 21.58 min. Thus, the dealy in thrombus formation after daboxin P treatment was 4-fold higher than the thrombus formation in saline treated mice.

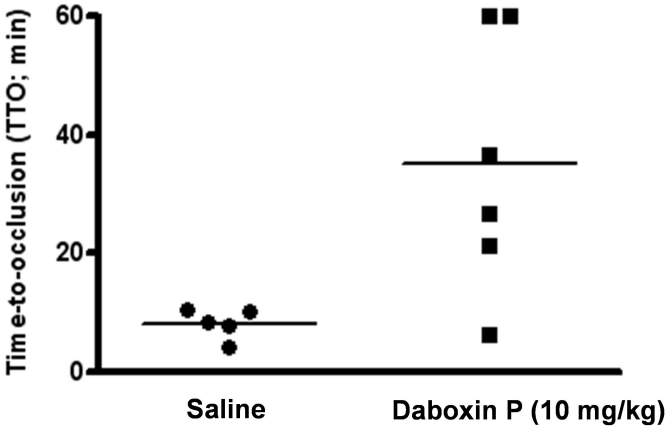


Figure 5.13: Effect of daboxin P on the time to occlusion (TTO) in the carotid artery of mice treated with $FeCl_3$. C57BL/6 mice were injected (i.p.) with daboxin P (10 mg/kg). Saline treated mice were considered as negative control. Each data-point in the graph represents the TTO for a single mouse. Maximum occlusion time was considered for 60 min after $FeCl_3$ treatment.

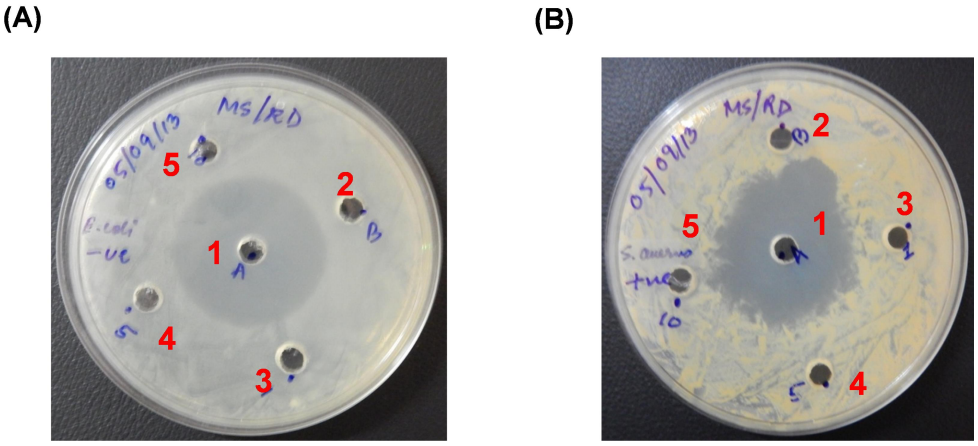


Figure 5.14: Antibacterial activity of daboxin P by Agar diffusion well method. Different amount of daboxin P was incubated with **(A):** gram-negative (*E. coli*) and **(B):** gram-positive bacteria (*S. aureus*) for 16 h at 37°C. On each plate; **Well 1:** ampicillin (50 µg/ml) treatment (positive control), **Well 2:** 20 mM Tris-Cl pH 7.4 (negative control), **Well 3:** 1 µg of daboxin P, **Well 4:** 5 µg of daboxin P, **Well 5:** 10 µg of daboxin P**.**

5.4.8 Antibacterial activity of daboxin P

Daboxin P did not show any antibacterial effect on both gram negative (*E. coli*) and gram positive (*S. aureus*) bacteria when tested using Agar diffusion well method

(Figure 5.14). Ampicillin, the positive control displayed a clear zone of inhibition with a diameter of 3.5 and 3.0 cm for *E. coli* and *S. aureus* respectively. However, no zone of inhibition was observed on the agar plate for both the bacteria even up to 10 µg of daboxin P under the same experimental conditions.

5.4.9 Cytotoxicity of daboxin P

Daboxin P did not affect the viability of both the normal (HEK-293) and cancerous (MCF-7) mammalian cell lines significantly (Figure 5.15 to 5.18). It was observed that even after an incubation period of 24 h with 5 µg/ml of daboxin P, no change in cell morphology was observed for both the cell lines (Figure 5.15 and 5.17). However, for HEK-293 cells the cell viability was abruptly reduced from 100% to 75.94 ± 2.58% at 0.1 µg/ml. However, beyond this concentration there was not much change in viability up to 5 µg/ml (68.07 ± 1.34%) (Figure 5.16). On the other hand, daboxin P did not exnibit any effect on MCF-7 cells (Figure 5.17 and 5.18).

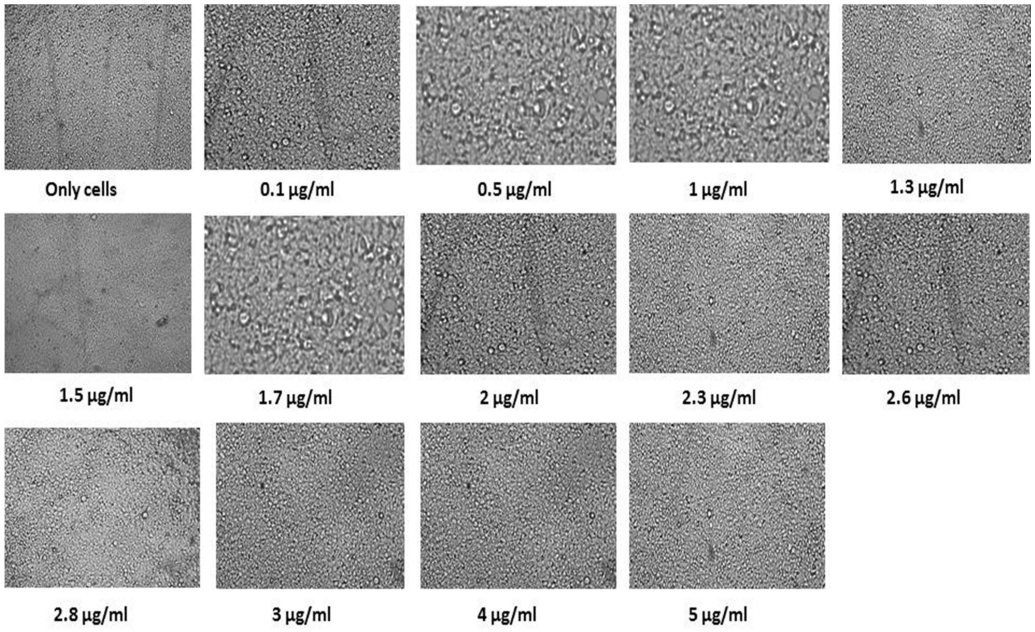


Figure 5.15: Microscopic images of the daboxin P treated HEK293 cell lines. Different concentrations of daboxin P were treated with HEK-293 cell lines for 24 h at 37^{o}C in CO_2 (5%) incubator. Photographs were taken at 10X magnification under Inverted microscope (AxioVert A1., Zeiss).

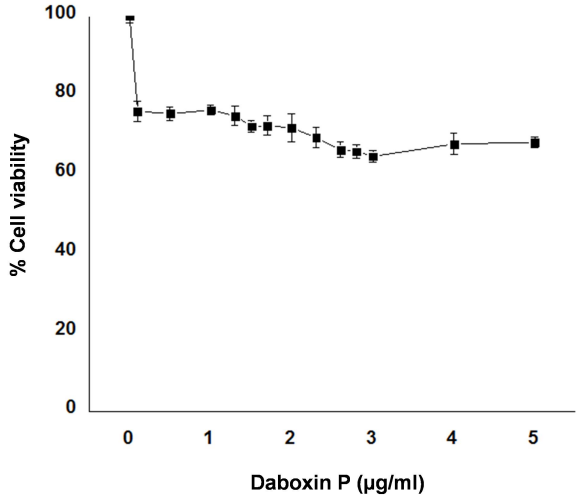


Figure 5.16: Assessment of cytotoxic effect of daboxin P on HEK293 cell lines using MTT based colorimetric assay. Percentage cell viability of the treated cells was quantified at 590 nm using UV-Vis MultiSkan Go spectrophotometer (Thermo Scientific). Percentage cell viability was calculated by considering the cells without venom treatment as 100% viable.

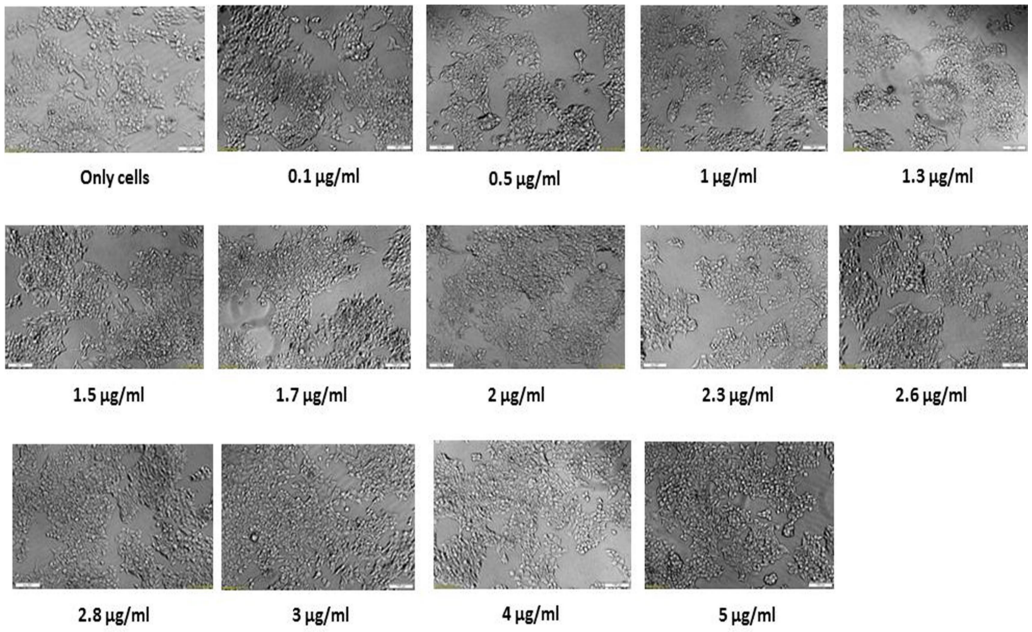


Figure 5.17: Microscopic images of the daboxin P treated MCF-7 cell lines. Different concentrations of daboxin P were treated with MCF-7 cell lines for 24 h at 37^{o}C in CO_2 (5%) incubator. Photographs were taken at 10X magnification under Inverted microscope (AxioVert A1., Zeiss).

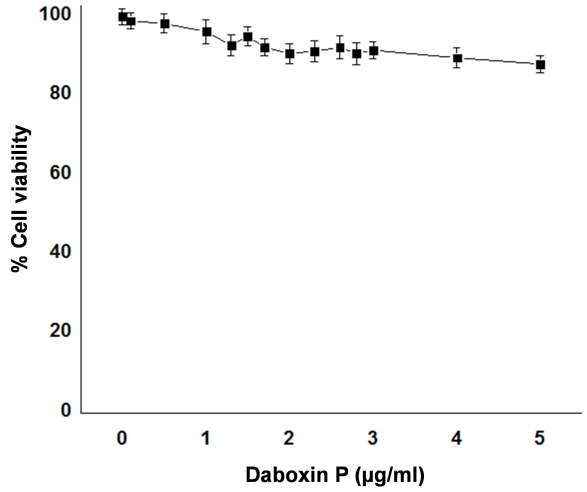


Figure 5.18: Assessment of cytotoxic effect of daboxin P on MCF-7 cell lines using MTT based colorimetric assay. Percentage cell viability of the treated cells was quantified at 590 nm using UV-Vis MultiSkan Go spectrophotometer (Thermo Scientific). Percentage cell viability was calculated by considering the cells without treatment as 100% viable.

5.4.10 Neutralization study

The Vins polyvalent antivenom could neutralize the PLA_2 activity of daboxin P on diheptanoyl thio-PC in a dose dependent manner (Figure 5.19). It was observed that the polyvalent antivenom could not neutralize PLA_2 activity of daboxin P up to 1:1 ratio. However, from 1:10 ratio there was gradual loss in activity of daboxin P and at 1:2000 ratio, only 10.12 ± 2.1% of the residual PLA_2 activity of daboxin P remained beyond which the neutralization reached saturation.

Similarly, the Vins polyvalent antivenom could neutralize the recalcification time of daboxin P in a dose dependent manner (Figure 5.20). The recalcification time of PP plasma with 0.1 µg of daboxin P was observed to be 19.1 ± 0.5 min which is 6 fold higher than the NCT (3.04 ± 0.34 min). It was observed that, at 1:1 ratio, the recalcification time was reduced to 16.82 ± 0.4 min only. However, at 1:250 ratio the clotting time was lowered to 2.97 ± 0.2 min which was close to the NCT of the experiment.

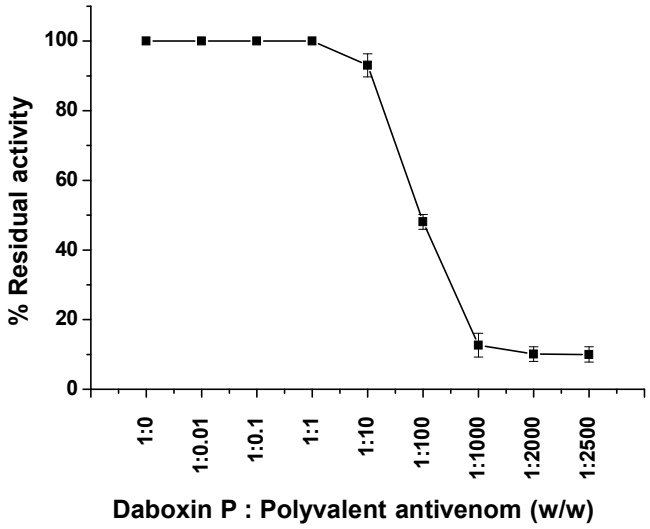


Figure 5.19: Neutralization of the PLA_2 activity of daboxin P by Vins polyvalent antivenom. Different amount of the polyvalent antivenom (0.0001, 0.001, 0.01, 0.1, 1, 10, 20, 25 µg) was pre-incubated with 0.01 µg of daboxin P for 1 h at 37°C. The amount of neutralization was quantified by estimating the hydrolysis of diheptanoyl thio-PC at 414 nm using UV-Vis Multiskan spectrophotometer.

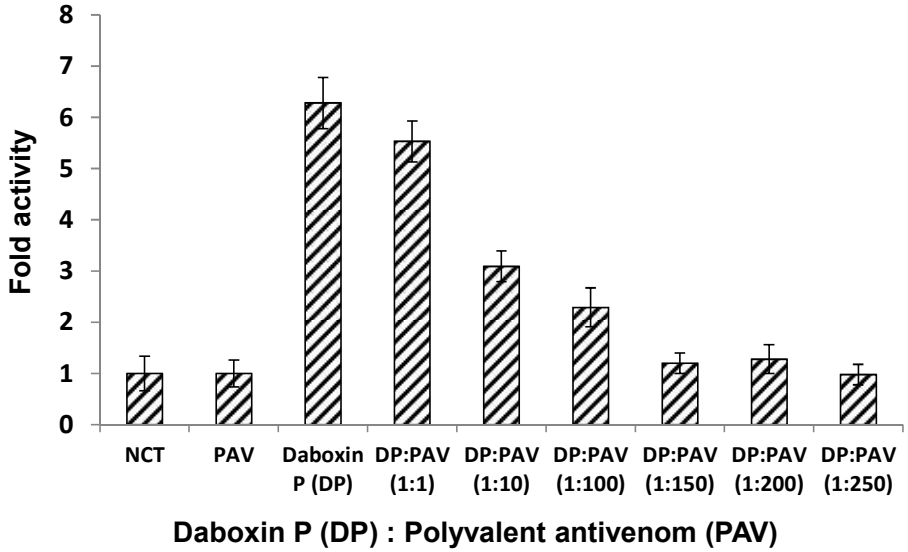


Figure 5.20: Neutralization of recalcification time of daboxin P by Vins polyvalent antivenom. Different amount of polyvalent antivenom (0.1, 1, 10, 15, 20, 25 µg) was pre-incubated with 0.1 µg of daboxin P for 1 h at 37°C. The clotting time was initiated by addition of 50 mM $CaCl_2$. Clotting time of PP plasma treated with daboxin P and polyvalent antivenom was calculated considering NCT as 1.

5.4.11 Western blotting

Daboxin P displayed a single protein band of ~12 kDa on 12.5% glycine SDS-PAGE which was transferred to PVDF membrane. The western blot showed a single bright band corresponding to ~12 kDa suggesting immunoreactivity of daboxin P towards the Vins polyvalent antivenom (Figure 5.21).

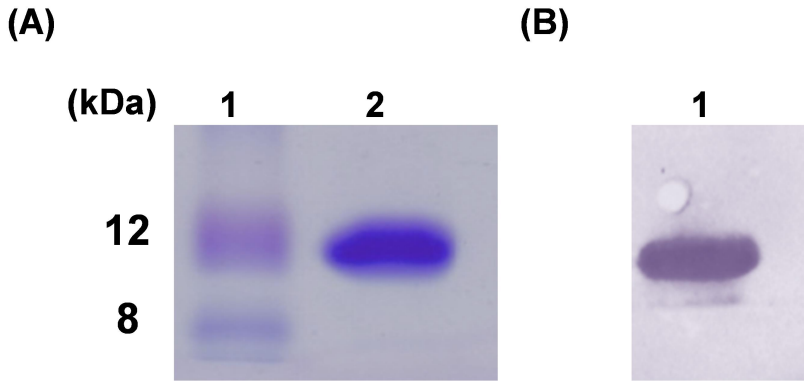


Figure 5.21: Assessment of immunoreactivity of daboxin P towards Vins polyvalent antivenom by western blotting. (A): 12.5% glycine SDS-PAGE of daboxin P. Lane 1: PageRuler^{TM} prestained protein marker (220-8 kDa); **Lane 2:** 7 µg of daboxin P. **(B): Western blot of daboxin P Lane 1:** immunoblot of 7 µg of daboxin P after treatment with anti horse IgG-Alkaline phosphatase and development with BCIP/NBT solution.

5.5 DISCUSSION

The hydrolysis of membrane phospholipids by the PLA_2 enzymes releases lysophospholipids and fatty acids that participate in various biological activities causing further cell damage and inflammatory responses (240,241,478,479). Snake venom PLA_2 enzymes exhibit a myriad of pharmacological activities with potent clinical implications that are either dependent or independent of the enzymatic activity. This includes myotoxicity, neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathic, haemorrhage, edema, tissue damage and convulsion (46). Single snake venom PLA_2 enzyme is capable of displaying a plethora of pharmacological activities due to the presence of various pharmacological sites that are distinct from the catalytic site (292). For example daboiatoxin, a major

PLA_2 enzyme isolated from the venom of *Daboia russelii siamensis* from Myanmar, is reported to exhibit myonecrotic, neurotoxic, edema inducing, indirect haemolysis and cytotoxic activities (426). Similarly, crotoxin, a major protein component from *C. durissus terrificus* exhibits a number of biological activities like neurotoxicity, myotoxicity, cytotoxicity myoglobinuria, inhibition of inflammatory response, platelet aggregation initiation etc. (352,480-488).

In the present study, daboxin P has been identified as one of the major proteins in the venom of Indian *Daboia russelii* which was purified by a collective approach of chromatographic techniques. Primary sequence analysis reveals that it belongs to group II A PLA_2 enzymes due to the presence of Cys50 and Cys133 disulphide bond and extended C-terminal end (discussed in Chapter 4). Biochemical characterization revealed its potent hydrolytic activity on diheptanoyl thio-PC in the presence of Ca^{2+} ions validating its Ca^{2+} dependent catalytic properties. However, chemical modifications of histidine residue at the active site of PLA_2 enzymes by alkylating agent like p-BPB or methylation with methyl-*p*-nitrobenzenesulfonate, affect their enzymatic activity (489). p-BPB, a bulky α-halo carboxylic acid is reported to bind to the N1 atom of the imidazole ring of histidine residue (471,490). This alkylation prevents the interaction of the histidine molecule with the phospholipids molecules bound to the substrate binding site thus, curtailing the enzymatic activity of these enzymes. In the present study, it was observed that alkylation of daboxin P with p-BPB led to the complete loss of its enzymatic activity on diheptanoyl thio-PC which confirms the crucial role of the histidine residue at the 48^{th} position of daboxin P in its catalytic activity.

Apart from enzymatic activity, daboxin P also exhibited mild direct haemolytic with potent indirect haemolytic activity. PLA_2 enzymes are reported to exhibit significant level of indirect haemolysis compared to direct haemolysis (323,491). This is well explained by the presence of free phospholipids (lecithin) in egg yolk which are first hydrolysed by the venom PLA_2 enzymes to lysolecithin and fatty acid. These lysolecithin then facilitate the breakdown of the RBC phospholipids thus enhancing the catalytic reaction. However, in case of direct haemolysis where there is no egg

yolk, the accessibility of the PLA_2 enzymes into the RBC membrane becomes difficult.

Unlike the crude *Daboia russelii* venom which has shown proteolytic and fibrinogenolytic activity, daboxin P did not exhibit any proteolytic effect on casein or fibrinogen under the same experimental conditions. This rule out the association of any low molecular weight protease with daboxin P. Usually PLA_2 enzymes are not reported with any proteolytic activity unless they are complexed with some proteases. For example, β-bungarotoxin (covalent association of PLA_2 enzyme with serine protease inhibitor) isolated from *Bungarus multicinctus* and taicatoxin (oligomeric complex of PLA_2 enzyme with α-neurotoxin like peptide and a serine protease inhibitor) isolated from *Oxyuranus scutellatus scutellatus* (492,493).

A number of PLA_2 enzymes like Vip Tx-II (*Daboia russellii russelii)*, VRV-PL-V (*Daboia russellii pulchella*), NN-XIb-PLA2 (*Naja naja*), etc. are reported to exhibit antibacterial activity on both gram positive and gram negative bacteria (2,331,333). The antibacterial activity of most of these enzymes is found to be either dependent or independent of their enzymatic activity. Moreover, some catalytically inactive (Lys49) PLA_2 enzymes like myotoxin II has been also reported with potential antibacterial activity due to the presence of basic/hydrophobic amino acids near their C-terminal loop (115^{th}-129^{th} residues) (326). However, in the present study, daboxin P has not shown any inhibitory effect on the growth of both gram positive and gram negative bacteria when tested using Agar diffusion well method. This could be due to the presence of considerable dissimilarity in the amino acid residues between the two proteins in the C-terminal segment.

Although the crude venom of *Daboia russelii* has displayed prominent cytotoxic effect on HEK-293 and MCF-7 cells in a dose dependent manner but the same concentrations of daboxin P did not show any cytotoxic effect. This suggests that the cytotoxic effect of the crude venom is not contributed by daboxin P. However, there are reports of some snake venom PLA_2 enzymes with cytotoxic activity on cell lines. BnSP-6 is a Lys49 PLA_2 enzyme isolated from the venom of *Bothrops pauloensis* which induces both early and late apoptosis in mammalian cancerous cell lines by up-

regulation of apoptotic genes and down regulation of anti-apoptotic genes (494). Bth-Tx-I, a basic myotoxic PLA_2 enzyme isolated from the venom of *Bothrops jararacussu* is reported to cause cytotoxic effect on mammalian cancerous cell lines by affecting the G0/G1 phase of the cell cycle and by induction of apoptosis (3).

Nonetheless, daboxin P has shown considerable inhibitory effect on the clotting time of PP plasma. Apart from profoundly delaying the RT assay, it has also delayed the APTT of PP plasma which is a characteristic clotting assay for the intrinsic pathway and mildly affected the PT assay which is characteristic clotting assay for the extrinsic pathway. Moreover, daboxin P has also delayed the stypven time considerably, which is a specific blood coagulation assay performed with RVV-X as the thromboplastin reagent for determining the deficiency of FX in plasma. However, daboxin P did not exhibit any inhibitory effect on the TT of PP plasma. This suggests that daboxin P exerts its anticoagulant effect upstream of the common pathway as it does not target thrombin and fibrinogen but prolongs the clotting time of PT, APTT and ST. Apart from exhibiting anticoagulant effect under *in–vitro* conditions, daboxin P has also exhibited anticoagulant activity under *in-vivo* conditions where it has significantly delayed the thrombus formation by $FeCl_3$ in carotid artery of treated mice. This suggests that it interacts with one or more physiological factors of the coagulation cascade to exert its anticoagulant effect even under *in-vivo* conditions that have caused the delay in the thrombus formation.

Daboxin P was found to be highly antigenic to the commercially available polyvalent antivenom suggesting its immunogenicity to elicit immune response during immunization of animals, a step in antivenom production. The neutralizing ability of the polyvalent antivenom was further confirmed when the anticoagulant and PLA_2 activity of daboxin P were considerably reduced upon incubation with it. This suggests the efficacy of the tested polyvalent antivenom towards neutralizing the two most potent activities of this major protein of Indian *Daboia russelii* venom.

Thus, the present study highlights some of the most crucial biochemical and biological activities of daboxin P, a major anticoagulant PLA_2 enzyme from the venom of Indian *Daboia russelii*. It has shown PLA_2 and haemolytic activity in a dose

dependent manner. Moreover, it has exhibited profound anticoagulant activity under both *in-vitro* and *in-vivo* conditions. Daboxin P has delayed the clotting time of RT, PT, APTT along with ST in a dose dependent manner. However, no effect was observed on the TT and fibrinogen. This suggests that it might exhibit anticoagulant effect upstream of the common pathway. Nonetheless, daboxin P did not exhibit any inhibitory effect on the growth of prokaryotic or eukaryotic cells. Furthermore, the commercially available Vins polyvalent antivenom was immunoreactive to it and could neutralize its PLA_2 and anticoagulant activity at higher concentration.