International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 3, Issue 4, 2012, pp 768 -776
http://www.bipublication.com

# AN ACIDIC POSPHOLIPASE$A_2$ OF SOUTH INDIAN COBRA ( *Najanaja* ) VENOM IS A PLATELET AGGREGATION INHIBITOR

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[Received - 05/10/2012, Accepted - 02/11/2012]

**ABSTRACT**

A platelet aggregation inhibitor phospholipase $A_2$ (NND-IV-$PLA_2$) was isolated from *NajaNaja* (southern India) venom was purified by cation and anion exchange chromatography. NND-IV-$PLA_2$ is the most catalytically active enzyme isolated from the Indian cobra venom. The acidic $PLA_2$profile of southern regional Indian cobra venom is distinctly different from that of the western and eastern regional venom. However the acidic $PLA_2$s from all the regions follow the pattern of increasing catalytic activity with increase in the acidic nature of the $PLA_2$isoform. NND-IV-$PLA_2$ is a class B1 platelet aggregation inhibitor and inhibits platelet aggregation induced by ADP, collagen and epinephrine. Modification of the active site of Histidine abolishes both catalytic activity and platelet aggregation inhibition activities, while aristolochic acid an acidic $PLA_2$ inhibitor has only a partial effect on the two activities.

**Keywords:** platelet aggregation inhibitor, Acidic PospholipaseA2, aristolochic acid, NBD-PC.

## INTRODUCTION

Asia is the habitat of various species of venomous snakes' including Elapidae, Hydrophilidae and Viperidae. According to statistics, the rate of snake bite and envenomation in Asia has been the highest among the world [11] .The characterization of snake venom components is important because a suitable medical treatment depends on a better understanding of the site and mode of action of the venom components. Phospholipases A2 (PLA2s, EC3.1.1.4) are a group of enzymes that catalyze the Ca2'-dependent hydrolysis of the 2-acyl ester bond in 3-sn-phospholipid. Secreted forms of the enzyme are abundant in the mammalian pancreas and in snake and bee venoms. Amino acid sequences of many PLA2s have been determined, with most being about120 amino acids long and having 14 Cys residues forming seven di sulfide bonds. Overall these proteins are closely related (>45% identity), with key residues that are required for catalysis and structure to be conserved [2-81).

In this paper we present the isolation and characterization of the catalytically highly active

acidic NND-IV $PLA_2$s, from *Najanaja* (southernIndia) venom. The biological properties of this $PLA_2$s are distinctly different from any of the $PLA_2$s isolated from western and eastern region. NND-IV-$PLA_2$s exhibits platelet aggregation inhibition, which is dependent on its catalytic activity making it a Class B platelet aggregation inhibitor. In this paper we are reporting the presence of Class B platelet aggregation inhibitor $PLA_2$ from *Najanaja* (SouthernIndia) venom.

The initial inventory of platelet aggregation inhibitors (1) dealt with those isolated from snakevenoms. The updated inventory by kiniand chow (ISTH2000)includes in addition to several new inhibitors platelet aggregationfrom snake venoms, those that were isolated from other animal sources. They are proteins orglycoproteins with their molecular weight ranging from 5000 to several tens of thousands. Various factors inhibit platelet aggregation by different mechanisms. A large number of these inhibitorsdo not exhibit any enzymatic activity. In contrast, some of them exhibit enzymatic activities, suchas phospholipase A2 (PLA2), proteinase and nucleotidase and Metalloproteinases. In general, the mechanism of inhibitionof platelet aggregation is well understood for several groups of non-enzymatic proteins. However,further research is required to delineate the mechanism of inhibition by some of theenzymes.

**Inhibitors with enzymatic activity**

Several proteins with enzymatic activity inhibit platelet aggregation. Some of these enzymesinhibit aggregation by indirect mechanisms either by the formation of a product, which inhibitsplatelet aggregation or by physical destruction of the agonist or its receptor. In these cases, themechanisms appear to be simple and are directly dependent on the respective enzymatic activity. *Nucleotidases*. ADP is a potent inducer of platelet aggregation that is secreted from the plateletsas dense granules by various agonists. Logically, hydrolysis of ADP should lead to inhibition.Thus ADPases and 5'-nucleotidases inhibit platelet aggregation Such inhibitors havebeen isolated from *Trimeresurusgramineus*), *Agkistrodonacutus*and *Viperaaspis.*It was concluded that the removal of ADP and possibly the generation of adenosine was responsiblefor the inhibitory effect of.*Phospholipase A2 enzymes*. Some PLA2 enzymes induce platelet aggregation, whereas othersinhibit it. In addition, there are a few PLA2 enzymes that both initiate as well as inhibit plateletaggregation, though under different conditions. However, not all PLA2 enzymes affect platelet aggregation. PLA2 enzymes that affect platelet aggregation are classified into three major classes. An effort had been made to verify the platelet aggregation inhibition.

## MATERIALS AND METHODS

Najanaja venom was milked lyophilized from western ghats of south india(hassan). CM-Sephadex C-25, Sephadex G-50, DEAE-Sephadex A-50, parabromophenacyl bromide (pBPB), Linoleic acid and egg Phosphatidyl Choline (PC) were from Sigma chemical company (St. Louis, USA). Aristolochic acid was purchased from Biomol, USA. 1-acy1-[-2-[12-[7- nitro-2, 1,3 benzoxadiazol-4-yl) amino]-dodecanoyl] phosphatidylcholine (NBD-PC) was purchased from Avanti polar lipids, USA. All other reagents were of analytical grade. Fresh human blood samples were collected from healthy volunteers from the Department of Biochemistry, University of Mysore, India, who were non-smokers and were not on drugs ten days prior to blood withdrawal. Male Swiss Wistar mice weighing 20-25 g were used for pharmacological studies.

**CM-Sephadex C-25 column chromatography**

Southern regional Indian Cobra (Najanaja) venom was fractionated on a CM-Sephadex C-25 column. A solution containing 200 mg of lyophilized whole venom in 1 ml of 0.02 M Sodium phosphate buffer, pH 7.0 was loaded on a CM-Sephadex C-25 column (4 cm X 20 cm) equilibrated in the same buffer. The column was eluted by a stepwise gradient of phosphate buffers of various molarities

and pH as indicated in Fig.1. The flow rate was adjusted to 30 ml per hour and 3 ml fractions were collected. Protein elution was monitored at 280 nm spectrophotometrically. Peak fractions were pooled, desalted, lyophilized and stored at -20$^0$C.

**DEAE Sephadex A-50 column chromatography**

The NN-I peak was further fractionated on DEAE Sephadex A-50 column (2 cm X 20 cm). The column was equilibrated with 0.01 M tris-HCI buffer (pH .0). A solution of 15 mg of the NN-I fraction in equilibration buffer was loaded onto the column. The column was eluted with stepwise NaCI gradient as indicated in Fig. 2. Two ml fractions were collected at a flow rate of 20 ml/h. Elution was monitored at 280 nm. The $PLA_2$ fractions were pooled individually, desalted, lyophilized and stored at -20$^0$C

**Phospholipase $A_2$ assay and positional specificity**

$PLA_2$ activity was assayed by estimating the free fatty acid released by the action of the enzyme on egg phosphatidyl choline (PC) as described earlier [7]. The reaction mixture contained 1 µmol of egg PC, 0.5 ml of ether, 50 mMTris-HCI buffer (pH 7.5), 40 µmol of $Ca^{2+}$ and 0.1 µg of enzyme in a final volume of 1 ml. The enzyme activity was expressed as micromole equivalents of linoleic acid released per minute per milligram protein.To determine the positional specificity of the purified phospholipase, phosphatidyl choline labeled with fluorescent fatty acid at Sn2 position (NBD-PC) was used as substrate. Mixed micelles of egg PC and NBD-PC was used as substrate. Mixed micelles of egg PC and NBD-PC were prepared as described previously [12]. The fluorescence emission of 2 ml of the mixed micelles was monitored at 530 nm after excitation at 460 nm. This served as control. The phospholipase reaction was followed by adding 2 µg of NND-I-$PLA_2$ to the mixed micelles. Change in fluorescence emissionintensity was followed for 15 min at room temperature using Shimadzu RF-5310 PC Spectroflurophotometer.

**Chemical modification of Histidine residue**

Histidine residue was modified using Para bromophenacy1 bromide (pBPB) as described by Yang and King [21]. A 0.5ml solution of 500 µg of NND-IV-PLA2 in 50 mMTris-HCl buffer, pH 7.5 was incubated with 30 µl of 40 mMpBPB dissolved in acetone. The reaction was allowed to proceed for 45 min at room temperature and stopped by acidifying with glacial acetic acid to pH 4.0. Excess reagent was removed by ultrafiltration through Amicon 10 Kda cut off centricons.

Circular Dichroism of NND-IV-$PLA_2$

Native and Histidine modified NND-IV-$PLA_2$ were dissolved in 10mM TrisHCl buffer pH 7.5 to give a concentration of 0.4 mg/ml. these were taken in quartz cuvettes of 1 cm path length. Spectra were recorded between 195 nm and 260 nm on a Jasco J-810 Spectropolarimeter instrument. The bandwidth was 1 nm and response time was 1 s. The final spectrum was cumulative of three scans.

**Interaction of aristolochic acid NND-IV- $PLA_2$**

Aristolochic acid binding to NND-IV-$PLA_2$ was followed by spectrofluorimetry. A 2 ml solution containing 3 nmoles of NND-IV_$PLA_2$ in 50 mMTris-HCl buffer, pH 7.5 was incubated with different molar ratios of aristolochic acid ranging from 1:1 to 1:100 mole/mole of $PLA_2$:Aristolochic acid for 5 min. fluorescence emission spectra of $PLA_2$ alone or mixture of PLA2 and aristolochic acid was recorded after excitation at 280 nm. The effect of aristolochic acid on the $PLA_2$ activity and platelet aggregation inhibitory acitivity was studied by incubating NND-IV-$PLA_2$ with varying concentration of aristolochic acid for 5 min before the assays.

**Platelet aggregation inhibitory activity**

Platelet aggregation inhibition studies were done according to the method of Born [19]. Nine volumes of fresh blood were drawn into one volume of 0.11 m trisodium citrate. Platelet rich plasma (PRP) was prepared by recentrifuging the citrated human blood at 90X g for 20 min.

Homologous platelet poor plasma (PPP) was prepared by recentrifugation of the remaining blood for 15 min at 500 X g at room temperature. The platelet count of PRP was adjusted to around 3,00,000 platelets per µl. The PRP was preserved at $37^0$ C and used within 2 h of preparation. Platelet aggregation was monitored in a Chrono-log dual channel aggregometer. For each assay 0.45 ml of PRP was taken in the sample cell kept stirred at 1000 rpm with a magnetic stirrer and an equal volume of PPP was taken in the reference cell. Aggregation was then induced by adding ADP (76 µM) or Collagen (2µg/ml) or Epinephrine (22µM) as agonists to PRP. For concentration dependent aggregation inhibition. PRP was preincubated with different concentrations of $PLA_2$ separately for 1 min followed by induction with the agonists. The aggregation was monitored for at least 3 min. the IC50, defined as the concentration of the inhibitor, which causes 50% inhibition of aggregation induced by various agonists, was calculated. The time dependence of inhibition was checked by preincubating PRP with 0.2µg of PLA2 for different time intervals before induction by collagen as before.

Washed platelets (WP) were prepared according to the method described by Gerrard [20]. Briefly, 5 ml of PRP was applied onto a Sepharose 2B column (1 cm X 10 cm), previously equilibrated with Calcium free Tyrode's buffer. Elution was carried out with same buffer at a flow rate of 20ml/h. 0.5 ml fractions were collected. Platelets that elute at the void volume were monitored by changing opacity of the fractions. The platelets were suitably adjusted with Tyrodes buffer to match the experiment with PRP. The aggregation inhibition studies washed platelets were done with collagen as the agonist and as described for PRP. In another set of experiment washed platelets was supplemented with 25, 50, and 75µl of plasma and incubated for one minute before adding NND-IV-$PLA_2$.

**RESULTS**

Najanaja (southern) venom upon stepwise fractionation on a CM Sephadex C-25 column resolved into 18 peaks (Fig.1). Eleven of these peaks exhibited $PLA_2$activity and were numbered from NN-I to NN-XI according to the scheme adopted previously [7]. The unbound fraction (NN-I) accounted for 14.5% of total protein and 88.52% of the total phospholipase activity loaded onto the column. Fraction-I, (NN-I) was subjected to anion exchange chromatography on DEAE Sephadex A-50 column, which resolved it into four well separated peaks designated NND-I to NND-IV (Fig 2). The fourth peak (NND-IV) was the largest fraction and accounted for 42.8% of the total protein and 78.31% of the total phospholipase activity recovered from the DEAE column. It was observed that the specific activity of the four $PLA_2$s increased with the increase in the acidity (retention in the DEAE column) of the molecule.

Positional specificity was determined using phosphatidyl choline labeled with a fluorescent fatty acid at a Sn2 position as substrate. Mixed micelles of NBD-PC and egg PC show fluorescence emission at 530 nm when excited at 460 nm. The fluorescence of NBD-PC is higher in the hydrophobic milieu of miscelles. Liberation of the labeled fatty acid into the aqueous medium results in a decrease in fluorescence due to lower quantum yield in water. Incubation of NBD-PC miscelles with NND-IV-PLA resulted in a time dependent decrease in the fluorescence emission of the reaction mixture confirming the cleavage of fluorescent labeled fatty acid from Sn2 position (Fig.4).

The specific activity of the acidic isoforms (NND-I to NND-IV) increased with increase in the retention time of the molecule in DEAE column. NND-I was the least active with specific activity of 0.5 µmoles fatty acid released/min/mg while NND-IV was the most active isoform. NND-IV-$PLA_2$ was non-toxic to mice up to an i.p. dose of 20 mg/Kg body weight. The animals did not exhibit signs/symptoms of neurotoxicity. NND-

IV- PLA$_2$ had no effect on prothrombin time and on serum levels of LDH and CPK activities. Edema induced was not significant even at the highest dose of 50 µg tested.

NND-IV- PLA$_2$ exhibited a very potent platelet aggregation inhibitory activity. Inhibition was dose dependent. Aggregation induced by all the three agonists (ADP, collagen and epinephrine) was inhibited but with different potencies (Fig 3A to 3C). The IC$_{50}$ values forinhibitions were found to be 0.5 µg for epinephrine, 0.3 µg for collagen and more than 15 µg for ADP. The extent of inhibition showed a positive dependence on time of incubation of the platelets with NND-IV-PLA$_2$ (Fig. 3D). When washed platelets were used the inhibition of collagen-induced aggregation reduced to 15% at IC$_{50}$ concentration of NND-IV-PLA$_2$. Normal inhibition however could be recovered by supplementing washed platelets with plasma.

The relationship between enzyme activity and pharmacological action was examined by chemical modification of the active site histidine residue and by interaction with aristolochic acid, a known PLA$_2$ inhibitor [22, 23]. Chemical modification of histidine by pBPB resulted in complete loss of both catalytic activity and platelet aggregation inhibitory activity. There was no significant difference in the circular dichroism spectra of native and histidine modified NND-IV-PLA$_2$ (Fig.4) Aristolochic acid interacted with NND-IV-PLA$_2$ in a dose dependentmanner and completely quenched the fluorescent emission at 348 nm (Fig.5B). However the inhibition of enzyme activity was only partial. Inhibition of enzyme activity increased with increasing concentration of aristolochic acid and reached a maximum value of 30% at a mole to mole ratio of 1:30, NND-IV-PLA$_{2:}$ Aristolochic acid. Similar observation was also made in platelet aggregation inhibition. The maximum loss of this activity was 24% (Fig. 5A).

**Fig.1.** CM-Sephadex C-25 Column chromatography.

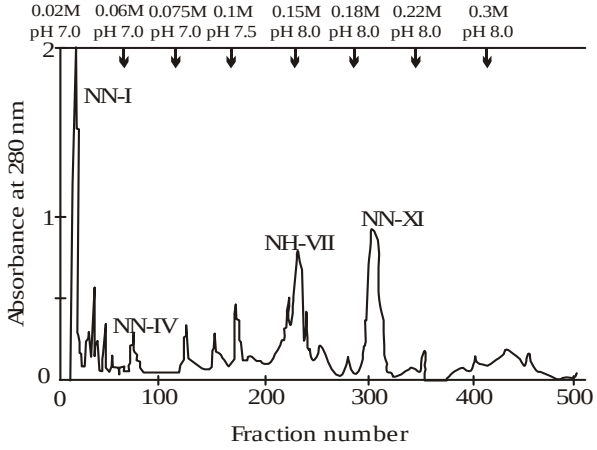



**Fig.2**.DEAE Sephadex A-50 Column chromatography

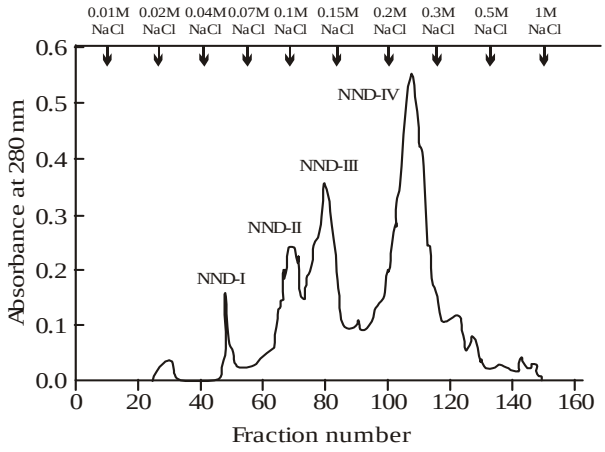
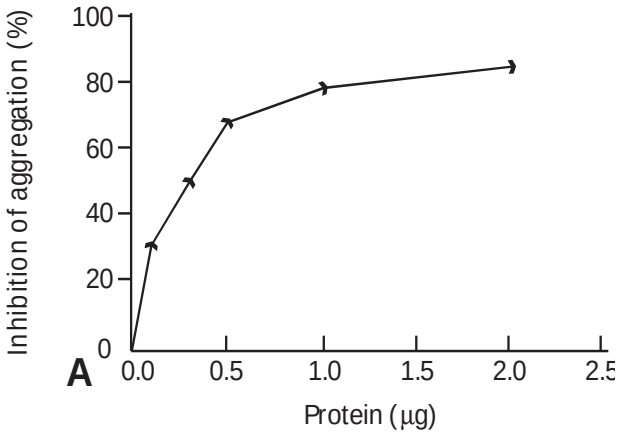
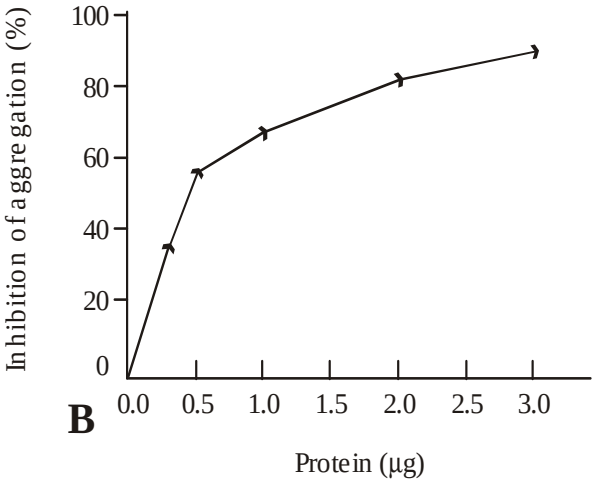



**Fig-3** Effect of Acidic Pla2 on platelet aggregation





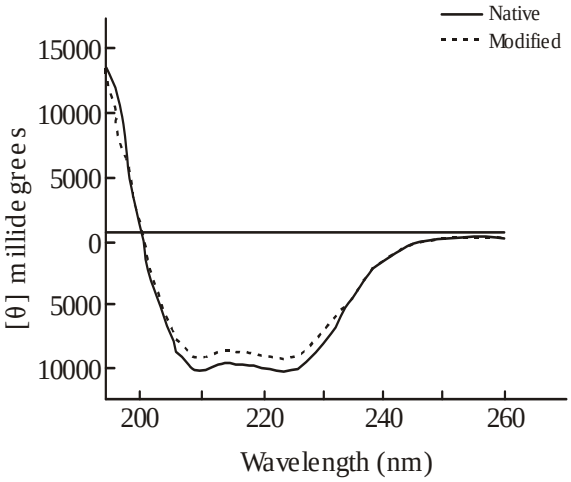
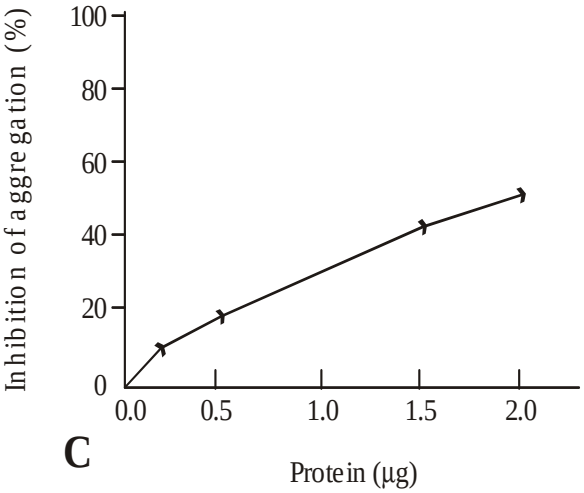




**Fig-5;** Effect of aristolochic acid with acidic Pla$_2$



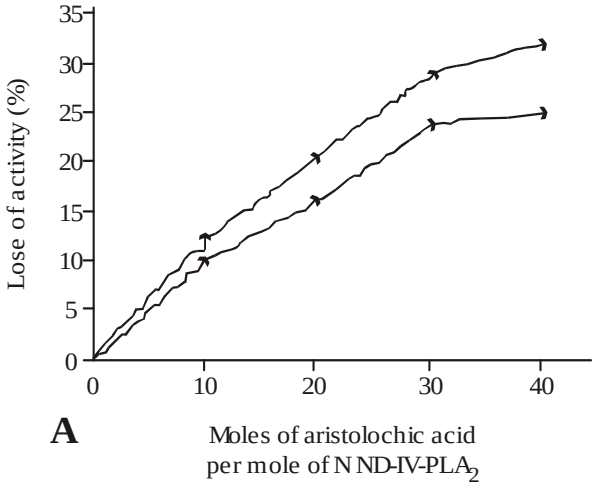
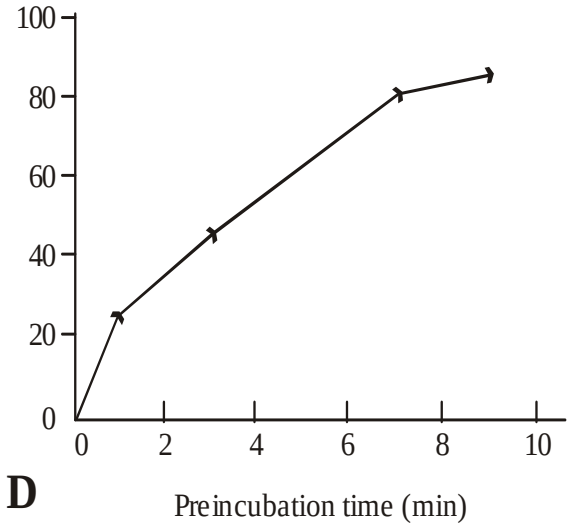




**Fig-6;** Action of Acidic Pla2 on egg PC and NBD-PC



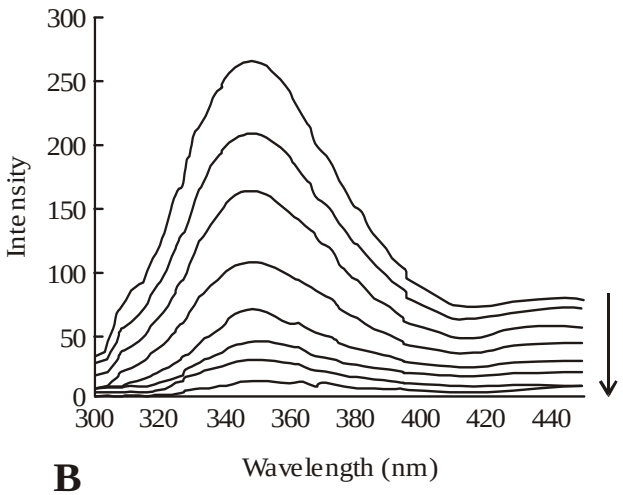




**Fig-4;** Circular Dichroism spectra Pla2

## DISCUSSION

Acidic $PLA_2$ are generally considered less toxic with few or no pharmacological activities. Some of the pharmacological properties reported in acidic NND-IV- $PLA_2$s are cardiotoxic [4], myotoxic [5], edema inducing activities [16]. NND-IV- NND-IV- $PLA_2$ was non-toxic to mice similar to the acidic NND-IV- $PLA_2$s isolated from western regional venom [9]. It did not induce either myotoxicity or anticoagulant activity. However, while NND-IV- NND-IV- $PLA_2$ was non edematic, all the acidic NND-IV- $PLA_2$s isolated from western regional venom induced edema [9].Platelet aggregation is one of the important defensive sequence of events in thrombosis and hemostasis. NND-IV- NND-IV- $PLA_2$ exhibited a potent platelet aggregation inhibitory activity. Both NN-$I_{2e}$ [28] and NND-IV- NND-IV- $PLA_2$ exhibited platelet aggregation inhibition induced by all the three agonists tested, ADP, collagen and epinephrine. However the potency of inhibition was different. Inhibition of aggregation of washed platelets was not to the same extent as PRP. NND-IV- NND-IV- $PLA_2$ required addition of plasma to achieve the same level of inhibition as PRP. This is probably because of the presence of plasma phospholipids which act as substrates to NND-IV- NND-IV- $PLA_2$ generating products which mediate inhibition of aggregation [5]. The inhibition observed when washed platelets alone were used suggests that NND-IV- NND-IV- $PLA_2$ might also be acting directly on the platelets. Class B platelet aggregation inhibitors are further divided into B1 and B2 respectively depending on whether inhibition is dependent or is independent of enzyme activity [6]. Chemical modification of active site histidine residue by pPBP completely abolished the enzyme activity as well as the platelet aggregation inhibitory activity. The histidine modified NND-IV- NND-IV- $PLA_2$ did not show any significant changesin the secondary structure compared to the unmodified form as seen by circular dichorism. Therefore the loss of enzyme activity is solely due to the alkylation of the active site histidine and not due to change in conformation as a result of modification. This result is also supported by the increase in platelet aggregation inhibition with increase in the incubation time of platelets with NND-IV- NND-IV- $PLA_2$ and by the does dependent inhibition of both enzyme activity and platelet aggregation inhibition activity by aristolochic acid, a known NND-IV- $PLA_2$ inhibitor

## CONCLUSSION

In conclusion our investigation clearly demonstrates that NND-IV- $PLA_2$ is a highly catalytically active $PLA_2$ with platelet aggregation inhibitory activity, isolated from southern regional Indian cobra venom. This is the first report of isolation and characterization of an acidic Class B1 platelet aggregation inhibitor $PLA_2$ from this region. NND-IV- $PLA_2$ is distinct in both catalytic and pharmacological potencies from any of the acidic $PLA_2$ isolated from Indian cobra venom.

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