Phospholipase A2 enzymes are biologically active components of snake venom which have been extensively studied because of their pivotal role in various biological activities and to elucidate the structure-function relationship (Kini, 1997). During the last decade, there are numerous reports published on the biochemical and pharmacological characterization including structure-function relationship of PLA2 isoenzymes from N. naja venom of Indian origin (Achyutan et al., 1980; Rudrammaji and Gowda, 1998; Bhat and Gowda, 1989, Mukherjee and Maity, 1998). However, there is a dearth of knowledge on the PLA2 enzymes from the venom of N. kaouthia, particularly of Indian origin. Although, Mukherjee and Maity (2002) reported the difference in biochemical properties, lethality and pathophysiology in the venom samples of Naja naja and Naja kaouthia of the same geographical origin, however, they did not mention in detail about the number of PLA2 isoenzymes present in the venom of these two closely related snakes, biochemical and pharmacological properties of purified PLA2 enzymes, particularly from N. kaouthia venom. Therefore, the purpose of the present study is three fold. First, an effort has been given to compare and characterize the PLA2 isoenzyme present in the venom of adult male N. naja and N. kaouthia snakes of eastern India origin to exclude the influence of age, sex and geographical variation in the venom composition. Secondly, two novel, most catalytically active phospholipase A2 isoenzymes viz. NK-PLA2-I and NK-PLA2-II have been isolated, purified and characterized from venom sample of N. kaouthia of Indian origin. Finally, the pharmacological reassessment of medicinal plants of north-east India for their inhibitory activity against the PLA2 enzyme(s) of N. kaouthia venom has been done in an effort to provide for an alternative to antivenom therapy for snakebite.
To compare and identify the number of PLA2 isoenzymes present in *N. naja* and *N. kaouthia* venom samples, elution pattern of PLA2 isoenzymes through a cation and anion exchangers under the identical condition, was studied. Fractionation of both the venom samples on a cation exchanger viz. CM-Sephadex C-50 reveal presence of seven basic PLA2 isoenzymes in both the venom samples. Since the basic PLA2 isoenzymes are positively charged, therefore they are retained by the cation exchanger and eluted with buffers of increasing molarity and pH values. On the other hand, neutral or acidic PLA2 isoenzymes are not retained by the cation exchanger and are eluted in a single peak with the equilibration buffer. However, it is worthy to be mentioned here that although both of the venom samples possess identical number of basic PLA2 isoenzymes, but they differ in their net positive charge on the enzyme molecule resulting in differences in elution profile from a cation exchanger. Further basic PLA2 isoenzymes of *N. naja* and *N. kaouthia* venom samples also differ quantitatively. The total protein content as well as the catalytic activity of the basic PLA2 enzymes from the *N. naja* venom sample are higher than the basic enzymes from *N. kaouthia* venom sample, which may explain the observed higher lethality of *N. naja* venom (*LD*<sub>50</sub> 0.4 ± 0.08 mg/kg body weight of mice) than that of *N. kaouthia* venom (*LD*<sub>50</sub> 0.7 ± 0.09 mg/kg body weight of mice) (Mukherjee and Maity, 2002). Since, basic PLA2 enzymes are more toxic than the acidic or neutral PLA2 enzymes, therefore, the former group of enzymes shows higher potency in inducing pharmacological effects (Boffa et al., 1980; Verheij et al., 1980; Kini and Evans, 1989; Kanashiro et al., 2002). The higher toxicity of the basic PLA2 s are due to the higher penetrability, probably due to abundance of positive charges on the PLA2 molecule (Kini, 2003).

To investigate the number of acidic PLA2 isoenzymes present in either venom sample, both the venom samples were fractionated on a DEAE Sephadex A-50 anion exchanger. Because of net negative charge on acidic PLA2 isoenzymes, therefore, they are retained by the anion exchanger and are eluted only with the buffers of increasing molarity and decreasing pH values. Fractionation reveals the
presence of three acidic PLA2 isoenzymes in *N. naja* and two acidic PLA2 isoenzymes in *N. kaouthia* venom samples. Assay of enzymatic activity shows that acidic PLA2 isoenzymes are catalytically more active in hydrolyzing phospholipids than those of the basic PLA2 enzymes from the same venom, which is in close agreement with other findings (Rosenberg, 1986; Rudrammaji and Gowda, 1998; Ketelhut et al., 2003).

Therefore, the present study documents that venom of *N. naja* of eastern India origin possesses a total of 10 PLA2 isoenzymes—seven basic and three acidic, whereas nine PLA2 isoenzymes including seven basic and two acidic PLA2 isoenzymes are present in the venom sample of *N. kaouthia* from the same geographical origin. Differences in the quantitative and qualitative distribution of PLA2 isoenzymes in *N. naja* and *N. kaouthia* venom samples reinforces the difference in venom composition between these two species of Indian cobra (Mukherjee and Maity, 2002). Further, since the commercial polyvalent antivenom available in India contain antibodies against venom of *N. naja* and not against *N. kaouthia*; this prompts us to give proper consideration for preparing appropriate antivenom for treating the cobra bite patients.

Till date, amino acid sequence of about 280 PLA2 enzymes have been determined (Tan et al., 2003) and they are found to share common homology. But these isoenzymes show different potencies in inducing pharmacological activities. Neither the mechanism by which venom phospholipase A2 enzymes show their pharmacological effects, nor the role of enzymatic activity in inducing these effects is clearly understood (Rosenberg, 1986; Fletcher et al., 1981; Kini and Evans, 1988; 1989). Therefore, a complicated and subtle interrelationship exists between these two properties of PLA2 enzymes. To investigate this relationship further, in the present study, two major phospholipase A2 isoenzyme from *N. kaouthia* venom were isolated, purified and characterized.
Fractionation of crude *N. kaouthia* venom on CM-Sephadex C-50 ion-exchanger column resulted in separation of venom components into eight fractions, based on the net surface charge on the PLA$_2$ enzyme. Screening of these fractions for phospholipase A$_2$ activity reveals that highest enzymatic activity was associated with the unbound acidic fraction CM I, followed by basic fraction CM II. Therefore, these two fractions were selected for further purification and characterization. CM I was further fractionated by gel filtration chromatography followed by RP-HPLC for the purification of NK-PLA$_2$-I, while NK-PLA$_2$-II was purified from the basic fraction CM II by RP-HPLC. NK-PLA$_2$-I and NK-PLA$_2$-II constitute about 6% and 3.5% of the total venom proteins respectively.

Both the purified PLA$_2$ enzymes at a concentration of 30 μg showed a single band in SDS-PAGE indicating the purity of the preparation. NK-PLA$_2$-I displayed an apparent subunit molecular mass of 13.6 kDa and 19.26 kDa under reduced and non-reduced conditions respectively showing it is dimer in nature. The molecular weight of NK-PLA$_2$-II under both reduced and non-reduced condition was determined as 13.1 kDa documenting this protein's existence as a monomer (Selistre et al., 1996). The molecular weight of native NK-PLA$_2$-I and NK-PLA$_2$-II, as determined by gel filtration, were 12.9 kDa and 12.4 kDa respectively, which are very close to the molecular weight of these proteins determined by SDS-PAGE. The purity and molecular mass of NK-PLA$_2$-I and NK-PLA$_2$-II were further confirmed by MALDI-MS which revealed a protonated molecular ion [MH$^+$] at m/z 13,786.205 and 13,346.19 Da respectively, which is similar to the mass determined by SDS-PAGE. MALDI-MS normally yields single charged states, but a lower intensity, doubly charged [MH$^{2+}$] peak at m/z 7193.1025 and 6673 Da were also noticed. PLA$_2$s also displayed such doubly charged peaks as seen in case of PLA$_2$ enzyme from the venom of the sea snake *Hydrophis cyanocinctus* (Ali et al., 1999). The molecular mass of NK-PLA$_2$-I and NK-PLA$_2$-II from the venom of *N. kaouthia* are quite consistent with the molecular masses of PLA$_2$ enzymes from many other snake venoms such as H1 and H2 PLA$_2$s (from Sea snake *Hydrophis cyanocinctus*).
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venom) having molecular mass of 13.588 and 13.247 kDa respectively (Ali et al., 1999); NN-12c-PLA2, NN-12d-PLA2, and NN-12e-PLA2 having molecular mass of 14.5, 13.3 and 13.7 kDa respectively isolated from Naja naja naja venom (Rudrammaji and Gowda, 1998); MiPLA2 2,3,4 having molecular mass of 13.95, 13.88 and 14.01 kDa respectively isolated from Micropechis ikaheka venom (Gao et al., 2001); Notechis II-1 having molecular mass of 13.233 kDa; Notechis II-2 having molecular mass of 13.013 kDa; and Notechis II-5 having molecular mass of 13.676 kDa isolated from Notechis scutatus venom (Halpert et al., 1976). Further, absence of protease, 5’-nucleotidase, ATPase, and acetylcholiesterase activities in these two enzymes clearly indicate that these purified proteins are not contaminated with other venom enzymes.

Biochemical characterization of either enzyme reveals that NK-PLA2-I is glycoprotein in nature while NK-PLA2-II is devoid of any carbohydrate moiety. The specific activity of NK-PLA2-I and NK-PLA2-II are 94,700 ± 1500 (mean ± S.D.) and 88,100 ± 1100 (mean ± S.D.) (Units/mg protein) respectively when egg yolk phospholipid was used as source of substrate. NK-PLA2-I displays maximum catalytic activity in the pH range of 7.5-8.5 whereas, NK-PLA2-II shows sharp pH optima at 9.0, demonstrating that these two enzymes require different pH optima for exerting maximum catalytic activity. The pH optima of N. kaouthia PLA2s are higher than the reported pH optima for acidic PLA2s (pH 6.9), isolated from the Indian cobra N. n. naja venom (Rudrammaji and Gowda, 1998), but in close agreement with the pH optima of many other snake venom PLA2s, such as VRV-PL-VIIa, optimum pH 7.2 (Kasturi and Gowda, 1989) and Bj IV, optimum pH 8.2 (Bonfim et al., 2001).

Determination of in-vitro head-group specific phospholipid hydrolyzing capacity of phospholipases has great relevance in elucidating the catalytic efficiency of the enzyme and to explain some of their pharmacological effects on the target cell membranes (Kini, 1997; Fletcher and Rosenberg, 1997). Present experiments
showed preferential hydrolysis of PC over PS or PE by both NK-PLA$_2$-I and NK-PLA$_2$-II; however, the rate of hydrolysis of PC by NK-PLA$_2$-I is much higher than NK-PLA$_2$-II. This is in contrast to the earlier reports demonstrating enhanced hydrolysis of PE over PC or PS by PLA$_2$s from *N. n. kaouthia*, *N. n. atra* (Fletcher and Rosenberg, 1997) and *D. russelli* venom samples (Vishwanath et al., 1988). This may be due to geographical and/or species specific variation in the substrate specificity of PLA$_2$ isoenzymes. The $K_m$ and $V_{\text{max}}$ values for NK-PLA$_2$-I is $0.988 \times 10^{-4}$ M and $1.4 \times 10^{-2}$ $\mu$mol mg$^{-1}$ while the same values for NK-PLA$_2$-II is $0.926 \times 10^{-4}$ M and $1.032 \times 10^{-2}$ $\mu$mol mg$^{-1}$ when PC is used as a source of substrate. The $K_m$ values of either enzyme is quite lower than the reported $K_m$ value of NN-I$_{2c}$-PLA$_2$, NN-I$_{2d}$-PLA$_2$ and NN-I$_{2e}$-PLA$_2$ (3.2 $\times$ $10^{-4}$, 4.2 $\times$ $10^{-4}$ and 3.8 $\times$ $10^{-4}$ M respectively) from venom of Indian cobra *N. n. naja* (Rudrammaji and Gowda, 1998). NH$_2$–terminal amino acid sequence (determined up to 10 amino acid residue) of both the *N. kaouthia* PLA$_2$s are identical (NIYQFKNNIQ). A comparison of the PLA$_2$ enzyme sequence listed in the Gene bank protein database indicates that both PLA$_2$ sequences share substantial homology with sequences of some of the already described snake venom phopholipase A$_2$s. The similarity of N-terminal amino acid sequence with that of already sequenced snake venom PLA$_2$s reinforces that the purified proteins from the venom of *N. kaouthia* belong to PLA$_2$ family.

The optimum temperature range for exerting maximum catalytic activity of NK-PLA$_2$-I is 37-40 °C while that for NK-PLA$_2$-II is 37±1 °C. Heat-inactivation study shows that heating the PLA$_2$ enzymes at 100 °C for 20 min has hardly any effect on their catalytic activity or secondary structure, documenting that like many other venom PLA$_2$s, *N. kaouthia* PLA$_2$s are highly thermostable in nature (Vishwanath et al., 1988; Francis et al., 1995). The CD spectra gives further evidence of its thermostability. The CD spectra of NK-PLA$_2$-I and NK-PLA$_2$-II are quite similar. The far-UV CD spectrum of native NK-PLA$_2$-I demonstrated defined minima at 210 and 222 nm, while NK-PLA$_2$-II displayed almost identical spectra having a minima at 210 nm and 222.5 nm, indicating a strong $\alpha$–helical contribution to the CD signal. $\alpha$–
helix is the major secondary structure element for a large number of PLA₂s from snake venoms (Ali et al., 1999; Dufton et al., 1983). NK-PLA₂-I and NK-PLA₂-II were both highly thermostable and did not begin to lose their secondary structure until heating at 100 °C for 45 min, which is in close agreement with the earlier reports (Dufton et al., 1983; Vishwanath et al., 1988; Francis et al., 1995; Soares et al., 2001b). The thermostability is due to compact folding of the enzyme, which is based on core structure of the disulfide bridges (Dufton et al., 1983; Dufton and Hider, 1983).

It has been reported that a single histidine residue, present at position 48 is conserved among cobra venom PLA₂ enzymes and plays a significant role in phospholipid hydrolysis (Kini, 1997; Ali et al., 1999; Fuly et al., 2000; Gao et al., 2001). However, exception is noticed for a dimer toxic PLA₂ from venom of Vipera ammodytes meridionalis, where His-48 residue is replaced by Gln-48 (Komori et al., 1996; Perbandt et al., 1997). Treatment of N. kaouthia PLA₂ enzymes with ρ-BPB results in a dramatic loss of enzymatic activity, presumably due to alkylation of active site histidine residue. Although ρ-BPB bears little resemblance to a phospholipid molecule, but the hydrophobic tail of this reagent could mimic a fatty acid chain which helps this inhibitor to bind at the active-site histidine residue of PLA₂s and preventing the binding of physiological substrate at the active site of the enzyme (Roberts et al., 1977). A significant protection against this activation by Triton X-100 (30 mM) and Ca²⁺ (10 mM) might be evidenced for either a surface dilution effect of reagents or by sequestering ρ-BPB into the apolar micellar core and thus lowering the reagent concentration available for reaction with enzyme (Roberts et al., 1977). Although Roberts et al. (1977) reported the almost complete inhibition of PLA₂ enzyme from N. n. naja venom by α-bromo-2-acetophenone and α-chloroacetophenone, but the catalytic activity of NK-PLA₂-I and NK-PLA₂-II were least affected by these reagents suggesting absence of a hydrophobic site on the N kaouthia PLA₂ enzymes. Further, phospholipid hydrolytic activity of the purified enzymes are not inhibited by serine-protease inhibitors like PMSF, TLCK and
TPCK, indicating absence of active serine group in the catalysis process (Roberts et al., 1977). EDTA on the other hand, inhibits the phospholipid hydrolysis by chelating the metal ions required for enzymatic activity (Kini, 1997). But inhibitory effect of DTT on PLA2 activity of either enzymes might be due to reduction of intramolecular disulfide bridges required for maintaining the three dimensional structure of the active enzyme.

Although NK-PLA2-I and NK-PLA2-II have shown significant phospholipid hydrolysis activity, but in contrast to crude venom, they do not display lethality in rodents, a fact which is in close agreement with many of the previous reports describing non-toxic but catalytically active PLA2 molecules from snake venom (Bouchier et al., 1991; Yang et al., 1991). In general, basic PLA2 are more toxic as compared to acidic or neutral PLA2 and have a significant contribution in the overall toxicity, membrane hydrolysis and edema-inducing activity of the venom (Bhat et al., 1991; Mukherjee et al., 1998a,b; Mukherjee and Maity, 1998). Therefore, presence of higher amount of basic PLA2, including other toxic enzymes, low molecular weight membrane active polypeptides as well as non-enzymatic toxins in crude venom may be responsible for its higher lethality as compared to NK-PLA2-I or NK-PLA2-II (Mukherjee and Maity, 2002).

Anticoagulant PLA2 were isolated from elapidae and other snake venoms (Evans et al., 1980; Angulo et al., 1997). It may be reasonable to assume that hydrolysis of essential phospholipids of coagulation complex by PLA2 inhibits the coagulation process (Evans and Kini, 1997). However, there is enough controversy concerning the role of enzymatic activity in the anticoagulant and other pharmacological effects of PLA2 (Kini and Evans, 1988, 1998; Chwetzott, 1989; Gao et al., 2001). It has been suggested that both enzymatic and non-enzymatic process have contributed in the pharmacological process (Kini and Evans, 1988; Kini, 1997). Present study documents the significant role of catalytic activity of N.
kaouthia PLA$_2$s on the anticoagulant effect. Our hypothesis can be supported by the following observations:-

1. The anticoagulant activity was enhanced either with the increase in the amount of PLA$_2$s or with an increase in the pre-incubation time of PLA$_2$ enzymes with the platelet poor plasma supporting that anticoagulant potency is parallel with the catalytic activity of PLA$_2$ enzymes.

2. Further, parallel inhibition of anticoagulant activity along with catalytic activity of either enzyme by p-BPB clearly indicates that the catalytic activity of *N. kaouthia* PLA$_2$s has a role to play in the anticoagulant process.

3. However, for unexplained reason, EDTA not only failed to inhibit, but apparently enhanced the anticoagulant potency of NK-PLA$_2$-I. This can be correlated with the effect of EDTA on the anticoagulant activity of *Vipera berus* PLA$_2$ (Boffa et al., 1972). The catalytic activity of NK-PLA$_2$-I being more active than NK-PLA$_2$-II, therefore the anticoagulant potency of former is higher under identical condition.

Majority of the PLA$_2$ enzymes are reported to be devoid of direct hemolytic activity (Jiang et al., 1989a, 1989b; Fletcher et al., 1991; Mukherjee and Maity, 1998, 2002). In contrast to the crude venom, neither PLA$_2$ demonstrated significant hemolytic activity on washed human erythrocytes; NK-PLA$_2$-I could induce only 0.23% hemolysis whereas NK-PLA$_2$-II fails to induce direct hemolysis. It seems that large number of low molecular weight membrane active polypeptides of crude venom along with PLA$_2$ may be responsible for hydrolysis of erythrocyte phospholipids and subsequent hemolysis (Harvey et al., 1982; Jiang et al., 1989a; Fletcher et al., 1991; Mukherjee and Maity, 1998, 2002). However, significant hemolytic activity shown by either PLA$_2$ in the presence of exogenously added PC and 1.5 mM Ca$^{2+}$ is due to hydrolysis of PC resulting formation of phospholipid hydrolysis products, like lysophospholipids and free fatty acids which are lytic by
themselves (Condrea et al., 1964). Since NK-PLA₂-I hydrolyze PC to a much higher extent than NK-PLA₂-II, therefore, it may be assumed that the indirect hemolytic activity of the former PLA₂ is higher as compared to the latter PLA₂. Since treatment of either enzyme with ρBPB, polyvalent antivenom and heat-inactivation have the same effect on their catalytic as well as indirect hemolytic activities, therefore, it is suggested that indirect hemolytic activity of _N. kaouthia_ PLA₂s are dependent on catalytic activity (Kini and Evans, 1988). It is to be noted here that for this reason indirect hemolytic activity is also used as a semiquantitative method of PLA₂ assay.

Unlike specific PLA₂ enzymes, non-specific enzymes lack specific pharmacological sites and therefore fail to bind specifically to the target site or target cell. However, when incubated with organ or tissue in vitro condition, these nonspecific enzymes could induce pharmacological effects similar to that of the specific PLA₂ enzymes (Kini and Evans, 1989). Present study indicates that _in-vitro_ condition, tissue damaging property of NK-PLA₂-I and NK-PLA₂-II is specific in nature because, nanomolar concentration of the either enzyme used in the current investigation completely eliminated the possibility of non-specific binding to the target tissue membrane and subsequent hydrolysis of membrane phospholipids at high enzyme concentration. However, it is difficult to distinguish between _N. kaouthia_ PLA₂ induced release of hemoglobin from the tissues due to haemolysis and haemolysis followed by RBC release. But from the present data, it seems that _in-vitro_ tissue damaging activity of NK-PLA₂-I and NK-PLA₂-II are independent of their haemolytic property because these two pharmacological properties are inhibited to a different extent by inhibitors and antivenoms under the identical test conditions. But rupturing of blood capillaries of tissues was necessary for the release of haemoglobin and it may be contributed by phospholipid hydrolysis property of PLA₂ enzymes (Datta and Bhattacharyya, 1999). Although, NK-PLA₂-I and NK-PLA₂-II even at a concentration of 25 μg/ml failed to show any proteolytic activity towards conventional substrates like casein, BSA, plasma albumin, globulin and fibrinogen, but it might require to investigate some other natural substrates of
NK-PLA$_2$-I and NK-PLA$_2$-II for protease activity because, in-vitro release of haemoglobin from tissues was partially inhibited by serine and chymotrypsin like serine protease inhibitors e.g PMSF and TPCK respectively. pBPB also could inhibit the in-vitro tissue damaging activity partially suggesting that the catalytic activity may be partially responsible.

Although the total phospholipid compositions of tissues like heart, lung and liver are similar (Fletcher et al., 1981), however, percent hydrolysis of heart tissue by either PLA$_2$ enzyme is significantly higher as compared to other tissues. This observation is well consistent with our previous reports that phospholipid constituents of microsomal membranes are less hydrolyzed as compared to lysosomal membranes by the action of Vipera russelli basic PLA$_2$ (Mukherjee et al., 1997; Mukherjee et al., 1998a,b). The reason for the organ/subcellular organelle preference of the purified PLA$_2$s is not clearly understood (Warrell, 1989). It has been suggested that PLA$_2$ may be particularly active at domain interfaces that are sites of structural defects and hence good point of attack by PLA$_2$ enzyme (Jorgensen et al., 2002). Further, it may be assumed that differences in the biochemical nature of the vascular wall in different organs/tissues like phospholipid/cholesterol ratio, presence of specific phospholipids, vitamin E content of that membranes etc, may have some relevance to differential membrane hydrolysis (Simionescu, 1983; Kini, 1997; Mukherjee et al., 1997, 1998). Moreover, membrane specificity of PLA$_2$ enzymes may also be explained on the basis of mutational theory, which states that mutation in the surface residues could alter the function of the protein as the surface residues play a critical role in protein-protein interaction with receptor/acceptor proteins present in the membrane (Kini and Chan, 1999).

Edema inducing activity of purified PLA$_2$ enzyme was first reported by Vishwanath et al. (1985) and till date, several snake venom PLA$_2$ enzymes are reported to induce edema in experimental animals. Edema induced by NK-PLA$_2$-I and NK-PLA$_2$-II was rapid and a saturation level was reached after 45 min followed
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by a gradual diminishing of edematous condition, which is in contrast to the edema induced by *B. schleheleii* myotoxin I, that lasted for 6 h (Angulo et al., 1997). However, the minimum edema-inducing dose of either *N. kaouthia* PLA2 enzyme (MED 2.21 µg and 4.14 µg for NK-PLA2-I and NK-PLA2-II respectively) was quite similar to those of acidic PLA2s from Indian cobra *N. n. naja* venom (MED in the range of 2.5 to 2.7 µg) (Rudrammaji and Gowda, 1998), but significantly higher than that of Daboitoxin (DbTx) from venom of *Daboia russelli siamensis* (0.05 µg) (Maung-Muang-Thwin et al., 1995). The edema induced by PLA2 may be due to processes which promotes cell membrane dysfunction by- (i) loss of membrane phospholipid and/or (ii) the generation of phospholipid break down products such lysophospholipid and free fatty acids including arachidonic acid (Vishwanath et al., 1988). In the secondary phase, transformation of the released arachidonic acid into prostagladins and leukoterines leads to increase in vascular permeability and subsequent edema formation (Ali et al., 1999). The role of catalytic activity of PLA2 enzyme in inducing edema is still controversial. Basavarajappa et al. (1993) reported the presence of separate edema-inducing site on the PLA2 molecule distinct from the catalytic site. For example, *B. asper* myotoxin II possesses low PLA2 activity but induces severe edema in the footpad of mice (Angulo et al., 1997). In a sharp contrast to these findings, various other researchers, for example Vishwanath et al. (1987), Bhat et al. (1991), Fuly et al. (2002) have demonstrated a direct relationship between the catalytic activity and edema-inducing property of snake venom PLA2s. Our current study supports the latter hypothesis because alkylation of active site histidine residue or treatment of either *N. kaouthia* PLA2 enzyme with polyvalent antivenom has equal inhibitory effect on the catalytic activity as well as edema inducing potency of the enzymes.

NK-PLA2-I and NK-PLA2-II hydrolyze the mitochondrial membrane phospholipids PC–pools in a dose dependent and highly characteristics manner. The reaction reached a steady level after 45 min, and the extent of phospholipid splitting by NK-PLA2-I after 60 min was about 6 fold more than that induced by NK-PLA2-II under
the identical condition. The presence of mM amount of Ca\(^{2+}\) in the reaction mixture did not enhance the PLA\(_2\)-induced membrane hydrolysis to a much higher extent, indicating that presence of Ca\(^{2+}\) is not an absolute requirement for the \(N. \text{kaoouthia}\) PLA\(_2\) induced mitochondrial membrane phospholipid hydrolysis reactions. It has been reported that lysis of artificial membranes by PLA\(_2\)s from various snake venom e.g. \textit{Bothrops pirajai}, \textit{Crotalus durissus terrificus} have shown to be independent of their enzymatic activity, since both native as well as catalytically inactivated enzymes are fully able to disrupt the membranes (Soares et al., 2001a; 2001b). In contrary to this observation, present study provide strong evidence that membrane hydrolyzing properties of \(N. \text{kaoouthia}\) PLA\(_2\)s are dependent on their catalytic activity and there exists distinct catalytic and membrane binding sites in PLA\(_2\)s. The following observations support our hypothesis:

1. With an increase in the incubation time of membranes with PLA\(_2\)s, degree of phospholipid hydrolysis increases concomitantly; documenting membrane hydrolysis is dependent on the catalytic activity of the enzyme.

2. Chemical modification of histidine residue, which is most conserved amino acid among all PLA\(_2\)s, results significant inhibition of catalytic as well as membrane hydrolyzing activities without interfering the affinity of the modified PLA\(_2\)s towards the membranes. In this respect, \(N. \text{kaoouthia}\) PLA\(_2\)s differ from \(N. \text{nigricollis}\) and \textit{Vipera berus} PLA\(_2\)s, because the \(N. \text{nigricollis}\) PLA\(_2\), inactivated at the histidine residue has a lower affinity for the membrane phospholipids compared to native enzyme and that \(V. \text{berus}\) PLA\(_2\) has a higher affinity for phospholipids (Dachary et al., 1980).

3. Modification of serine or cysteine residue results drastic reduction of membrane binding as well as phospholipid hydrolysis action, but does not interfere catalytic activity of either PLA\(_2\). sPLA\(_2\) have a common interfacial-binding surface that is located on the flat external surface surrounding the
active site slot. It has already been shown that presence of Trp on the putative interfacial binding surface of human Group V and *N. n. naja* PLA$_2$s plays an important role in the binding of PLA$_2$s to PC vesicles and the outer plasma membrane (Han et al., 1999; Demel et al., 1975). Present study document that serine and cysteine also forms a part of putative interfacial binding surface in *N. kaouthia* PLA$_2$ molecules and hence modification of these two residues might result significant loss of affinity as well as penetrability of the PLA$_2$ into the phospholipid bilayer of the membranes.

4 Heating PLA$_2$s at 100 °C for different time periods or incubation with anti-NK-PLA$_2$-I IgG results differential inhibition of their catalytic and membrane-hydrolyzing properties reinforcing catalytic site is separated from the membrane-binding region in the *N. kaouthia* PLA$_2$s.

However, one may argue that chemical modification of PLA$_2$s or their interaction with antibodies may induce structural changes in the PLA$_2$ molecule, resulting in different properties of modified enzymes. CD spectrum and electrophoretic analyses of native and chemically modified enzymes and recognition of modified PLA$_2$s by anti-NK-PLA$_2$-I antibodies eliminate the chances of structural changes as a consequence of these treatments (Doley et al, 2004). Further advance studies are required for a deeper understanding of the relationships between the structural and dynamical properties of different membrane phospholipids and mechanism of activation of phospholipase A$_2$ at the interfaces for their application in liposome based drug delivery.

There are several reports on snake venom PLA$_2$ enzymes possessing antibacterial activity against Gram positive and Gram negative bacteria (Diaz et al., 1991; Paramo et al., 1998; Lomonte et al., 1999; Soares et al., 2001b). In case of human sPLA$_2$, the presence of cationic charge allows the enzyme to penetrate the anionic cell wall of Gram positive bacteria (Beers et al., 2002). Identically,
antibacterial peptides isolated from snake venom contain cationic site(s), flanked by hydrophobic residues, which might disturb the phospholipid bilayer integrity, initiating a rapid cell death process (Kini and Evans, 1989; Calderon and Lomonte, 1989). Further, it was observed that the membrane damaging activity in case of Myotoxin II, isolated from Bothrops asper snake venom, depends on its amphiphilic character (Lomonte et al., 1994). It has been suggested that bactericidal activity of PLA$_2$ are dependent on their interaction with essential membrane components common to both eukaryotes and prokaryotes; for example, in case of Gram-negative bacteria, these membrane components are identified as lipopolysaccharide moieties (Paramo et al., 1998). In the present study, none of the PLA$_2$ up to a dose of 10 $\mu$g/ml shows bactericidal activity against Gram positive or Gram negative bacteria. Since NK-PLA$_2$-II was base eluted from the cation exchanger, therefore it might be assumed that this pLA$_2$ molecule bears positive charge on its surface and should show antibacterial property. However, it might be inferred from our observation that not the overall positive charge but the specific distribution of the positive charge in the phospholipase A$_2$ molecules may be responsible for their penetrability in the bacterial cell wall (Kini, 1997). Moreover, further studies are essential to decipher the antibacterial properties of PLA$_2$ molecules.

Acute skeletal muscle damage is a frequent manifestation in envenomations induced by snakes of the families Elapidae and Viperidae (White, 1995; Warrell, 1996). Histological and ultrastructural studies of the effect of venom PLA$_2$s on skeletal muscle reveal a common series of pathological alterations which include: (1) plasma membrane disruption, (2) formation of 'delta-lesions', wedge shaped areas of degeneration located at the periphery of muscle fibers, (3) hypercontraction of myofilaments, (4) mitochondrial swelling together with formation of flocculent densities and rupture of mitochondrial membranes, (5) disruption of intracellular membrane systems, i.e. sarcoplasmic reticulum and T tubules, and (6) pycnosis of nuclei (Harris and Maltin, 1982; Gutierrez et al., 1984a; Harris and Cullen, 1990;
Mebs and Ownby, 1990). Acute muscle degenerative events are followed by an acute inflammatory reaction associated with pain, edema and recruitment of polymorphonuclear leucocytes and macrophages (Harris and Maltin, 1982; Gutierrez et al., 1984a, 1990; Chacur et al., 2003). Finally, a regenerative process ensues, with activation of myogenic satellite cells and fusion of myoblasts to form myotubes within the space delimited by the remaining basal lamina of necrotic muscle cells (Gutierrez et al., 1984b; Gutierrez and Ownby, 2003). The present study reveals that both NK-PLA$_2$-I and NK-PLA$_2$-II induce degeneration of the muscle cells when injected to the thigh muscle of mice. The rise in the activity of intracellular enzymes in the serum is a clear indication of cell damage, that results in leakage of cytosolic and membrane bound enzymes of the organ in circulation (Mukherjee and Maity, 1998; Sahu et al., 1991). Thus the myotoxicity of *N. kaouthia* PLA$_2$s may be related to their membrane damaging activity of muscle tissues (Fletcher and Jiang, 1993; Fletcher et al., 1991).

Comparison of the charge density distribution has demonstrated that presence of hydrophobic and cationic regions in PLA$_2$ molecules are responsible for displaying their myotoxicity (Kini and Iwanaga, 1986). It has been seen in case of myotoxic K49 PLA$_2$ that the cationic regions interacts with negatively charged lipids, forming a cytolytic motif (Lomonte et al., 1994; Gutierrez and Lomonte, 1997) which penetrates and disorganizes membranes (Murakami and Arni, 2004). Further, it has been observed that myotoxic PLA$_2$s from crotaline snake venoms and crotoxin isolated from *Crotalus durissus terrificus* have a set of Try residue located in the C-terminal region of molecule. This Try may contribute to the hydrophobic-cationic combination proposed to play a role in myotoxicity (Francis, et al 1991; Soares, et al., 2001a). A multidomain membrane protein of 180 kDa, named M-type receptor, having a tandem repeat of eight domains homologous to C-type lectin carbohydrate recognition regions, was characterized in the plasma membrane of myocytes and other cell types that binds to PLA$_2$ enzyme (Lambeau et al., 1990). On the other hand, the demonstration of the existence of lipid domains and rafts within plasma
membranes suggest that regions enriched in particular glycerophospholipids or glycolipids may function as acceptors of PLA$_2$s (Gutierrez and Ownby, 2003). However, further studies are essential to explore the role of cationic site or involvement of specific amino acid residue(s) of $N. kaouthia$ PLA$_2$ in inducing myotoxicity.

Formation of single precipitin band between anti-NK-PLA$_2$-I antibodies raised in rabbits and NK-PLA$_2$-I clearly indicates the polyvalent nature of the antibodies (Basavarajappa et al., 1993). Further, western blotting experiment demonstrated that anti-NK-PLA$_2$-I antibodies strongly cross-reacted with both NK-PLA$_2$-I as well as NK-PLA$_2$-II, documenting these enzymes are immunogenic in nature and are closely related (Calderon, et al., 1993). Anti-NK-PLA$_2$-I IgG at 1:100 (w/w) antigen:antibody ratio, could neutralize the catalytic activity of NK-PLA$_2$-I and NK-PLA$_2$-II up to 68.34% and 48.34% respectively of their original catalytic activity. The differential inhibition of two enzymes may be explained by the fact that the antibodies were raised against the NK-PLA$_2$-I enzyme; therefore, the extent of inhibition of catalytic activity and other pharmacological properties of NK-PLA$_2$-I enzyme by anti-NK-PLA$_2$-I antibodies was higher as compared to the inhibition against NK-PLA$_2$-II enzyme. The separation of the catalytic site from the pharmacological sites in snake venom PLA$_2$ molecules using polyclonal/monoclonal antibodies and plant alkaloids have been reported (Jayanthi et al., 1989; Kasturi and Gowda, 1991; Bhat et al., 1991). Poor inhibition of catalytic activity as compared to pharmacological properties of either enzyme by anti-NK-PLA$_2$-I IgG suggests that $N. kaouthia$ PLA$_2$ enzymes possess distinct, perhaps overlapping sites for catalytic activity and pharmacological effects and this hypothesis is in close agreement with those proposed by Rosenberg, (1986) and Kini and Iwanaga (1986). Differential inhibition of catalytic activity and pharmacological effects of PLA$_2$s by anti-PLA$_2$ antibodies might be due to the fact that the pharmacological site of NK-PLA$_2$-I and NK-PLA$_2$-II is a poor epitope for antibody production and recognition. Furthermore, heat-inactivation study in the current investigation
reinforces our hypothesis because, nearly half of the original catalytic activity of either enzyme was lost when heated for 45 min at 100 °C, but the tested pharmacological properties of the enzymes were completely abolished under the identical heating condition.

Snake venom is a collection of many enzymes (Stocker, 1990; Tsai et al., 1996; Mukherjee and Maity, 1998). Some of which are very toxic in nature (Mukherjee and Maity, 1998, Mukherjee, 1998) and responsible for the most pharmacological effects of the envenomation. Neutralization of these enzyme activities by the plant extracts reflects the presence of anti-snake venom compounds in those plants. Isolation and purification of the active compound(s) responsible for inhibition of enzyme activities might be useful, because they may have a potential application in future as therapeutic agents against snake envenomation. Further, since majority of these natural compounds are stable at room temperature (Finar, 1989), therefore they can be stored even at room temperature and can easily be supplied to rural areas, where maximum snakebites take place. It should be pointed out that antivenom vials (currently available for snakebite therapy) have to be stored under refrigerated condition (at 4 °C to 8 °C). Since majority of rural hospitals or health centers in India or other developing countries lack proper refrigerated storage condition (personal observation), therefore antivenom vials are not kept in these centres. As a result of which, in the rural tropics, it is often late when snakebite patients arrive at district (town) hospitals for treatment hours after bite travelling long exhaustive journey; and late antivenom therapy may not be useful to save the life of the patients (Mukherjee, 1998; Mukherjee et al., 2000).

In the present investigation, out of the 13 medicinal plants tested for anti-PLA2 activity, only the leave extract of *Azadiracta indica* was found to possess anti-PLA2 activity. The methanol extract of the leaves could neutralize the catalytic activity of crude venom as well as the purified enzymes significantly as compared to water or chloroform extracts. *Azadiracta indica* has been extensively used for anti-
inflammatory, antipyretic, analgesic, immunostimulant, diuretic, hypoglycaemic, cardiovascular, antimicrobial, antiviral, antimalarial and anthelmintic (Dhawan and Patnaik, 1993; Sivarajan and Balachandran, 1994). Many constituents from this plant, for example, diterpenoids, triterpenoids, polyphenolics, sulphurous compounds, and polyacetate derivatives are responsible for showing many of these medicinal properties of *A. indica* (Dev Kumar and Sukh Dev, 1993). Several plant constituents like flavonoids, quinonoid, xanthene, polyphenols and terpenoids possess protein binding and enzyme inhibiting properties (Havsteen, 1983; Selvanayagam et al., 1996). Which also inhibit snake venom phospholipase A2 activities of both viper and cobra venom (Alcaraz and Hoult, 1985). Although *A. indica* has been used as antidote (folk medicine) to snakebite in the rural tropics of Indian subcontinent, but till date, neither the plant was scientifically screened to explore the antivenom property nor a single antivenom compound has been isolated and characterized from this plant. Result of the present investigation documents that methanol soluble active compounds like triterpenoids and others, present in the leaf of *A. indica*, may be responsible for neutralization of PLA2 activity (Siddiqui et al., 2001; Alam and Gomes, 2003). Interestingly, all the tested plant extracts except *A. indica*, used as folk medicine to treat snakebite patients, failed to show inhibitory activity against the *N. kaouthia* PLA2 enzymes. However, their role in inhibiting the toxic effects of other components of snake venom cannot be ruled out. It should be mentioned here that folk medicines are administered in the form of mixture, comprising of different plant constituents, which may act synergistically in neutralizing the toxic effects of snake venom (A.K. Mukherjee, personal communication). Therefore, whatever every plant used by local people as antidote against snakebite, must be examined systematically, carefully and critically to evaluate their actual antivenom potency. This will further assist us in overcoming the limitations of currently available polyvalent antivenom therapy, particularly in the developing countries.
Conclusion

In the present investigation, PLA$_2$ isoenzyme pattern of two Indian cobras, *N. naja* and *N. kaouthia* venom samples of eastern India origin were compared and two major phospholipase A$_2$ isoenzymes were purified to homogeneity from the venom of Indian monocled cobra (*Naja kaouthia*). Further, some of the Indian medicinal plants were screened for anti-PLA$_2$ activity.

Comparison of the PLA$_2$ isoenzymes of *Naja kaouthia* and *Naja naja* venom samples reveals that 10 PLA$_2$ isoenzymes are present in *Naja naja* whereas 9 PLA$_2$ isoenzymes are present in *Naja kaouthia* venom. Therefore, these two venom samples differ with respect to qualitative and quantitative distribution of PLA$_2$ isoenzymes.

The two major phospholipase A$_2$ isoenzymes viz. NK-PLA$_2$-I and NK-PLA$_2$-II from *Naja kaouthia* venom were purified to homogeneity by combination of ion-exchange, gel filtration and RP-HPLC. NK-PLA$_2$-I had a molecular weight of 13.6 kDa and 19.26 kDa under reduced and non-reduced conditions whereas NK-PLA$_2$-II displayed sharp band of 13.1 kDa under both reduced and non-reduced conditions in SDS-PAGE indicating NK-PLA$_2$-I is dimmer and NK-PLA$_2$-II is monomer in nature. The molecular mass of NK-PLA$_2$-I and NK-PLA$_2$-II was further confirmed by MALDI-MS, which revealed a protonated molecular ion [MH$^+$] at m/z 13,786.205 and 13,346.19 Da which is similar to the mass determined by SDS-PAGE respectively. NH$_2$-terminal amino acid sequence (determined up to 10 amino acid residue) of both the *N. kaouthia* PLA$_2$s are identical (NIYQFKNNIQ) and share 90% homology with that of already characterized snake venom PLA$_2$s. The CD spectra of NK-PLA$_2$-I and NK-PLA$_2$-II showed strong $\alpha$-helical structure, which is well consistent with other snake venom PLA$_2$ enzymes. Further, heat inactivation study
as well CD spectra of both the enzymes revealed that they are thermostable in nature.

Both the enzymes, at a dose of 10 mg/kg body weight of mice did not display any sign of lethality. Pharmacological characterization reveals that none of PLA₂s shows any direct hemolytic activity on washed human or goat erythrocyte unless phospholipid hydrolysis products were present in the incubation medium but both the PLA₂s displayed strong anticoagulant, myotoxicity, edema-induction, damage of liver mitochondria and tissues of heart, liver and lung in in-vitro condition. although NK-PLA₂-I is more potent than NK-PLA₂-II in inducing mitochondrial swelling, but both these PLA₂s have displayed remarkable similarity in inducing a prompt and marked swelling of mitochondria without any lag phase. Further, it is observed that NK-PLA₂-I and NK-PLA₂-II hydrolyze the mitochondrial membrane phospholipids PC-pools in a dose dependent and highly characteristic manner. NK-PLA₂-I is more potent in exhibiting anticoagulant, indirect hemolytic and in-vitro liver tissue damaging activity as compared to NK-PLA₂-II, where as reverse is true for induction of edema, myotoxicity and indirect hemolytic activity. The tissue damaging activities of either enzyme is specific and heart tissue damaging activity of NK-PLA₂-I is detected as more as compared to NK-PLA₂-II.

Chemical modification study reveals that like other snake venom PLA₂ enzymes, histidine residue is also present in the active site of both the enzymes. Pharmacological activities, of *N. kaouthia* PLA₂s, e.g. like anticoagulant, edema and indirect hemolytic activities were significantly neutralized by treatment with ρBPB, indicating that catalytic activity plays a significant role in inducing these effects. However in-vitro tissue damaging activity was partially inhibited by ρBPB, reinforcing that PLA₂ enzymes have a separate pharmacological site for displaying tissue damaging activity other than the catalytic site. Furthermore, heat-inactivation study in the current investigation reinforces our hypothesis because, nearly half of the original catalytic activity of either enzyme was lost when heated for 45 min at
100 °C, but the tested pharmacological properties of the enzymes were completely abolished under the identical heating condition.

Immunological study reveals that both the enzymes are immunogenic in nature and are closely related. Antibodies were raised against NK-PLA₂-I in rabbit, but the catalytic activity and pharmacological effects of PLA₂s were inhibited in differential manner by anti-PLA₂ antibodies suggesting that the pharmacological site of NK-PLA₂-I and NK-PLA₂-II is a poor epitope for antibody production and recognition.

In the present study, medicinal plants used by the tribal people of NorthEast were scientifically screened for the anti-snake venom compound(s). Interestingly, all the tested plant extracts except A. indica, used as folk medicine to treat snakebite patients, failed to show inhibitory activity against the N. kaouthia PLA₂ enzymes. However, their role in inhibiting the toxic effects of other components of snake venom cannot be ruled out because folk medicine are administered in mixture of different plants. Therefore, each and every plant used as antidote must be examined critically which will help to over come the limitation of antivenom therapy.