CHAPTER II

REVIEW OF LITERATURE

2.1 Snake venom: A general consideration

Snake venom is an evolutionary adaptation to immobilize prey, secondarily use in defense. Venoms are highly toxic secretions produced and stored in specialized salivary glands of snakes. It is a unique mixture with reference to their biochemical and pharmacological properties and mostly consists of non-cellular proteins (Kochva, 1987; Meier, 1990). Snake venoms are either colourless or yellowish in colour. The latter colour is due to presence of L-amino acid oxidase (Thomas and Pough, 1979). The content of the solid matter of venoms of Elapidae, Crotalidae and Viperidae has been found between 18 to 52%, 16 to 51%, and 28 to 31%, respectively (Elliot, 1978). Milked venom also contains tissue debris (Mukherjee and Maity, 1998).

2.1.1 Physical properties of snake venom

The snake venom is slightly acidic in nature and specific gravity ranges from 1.03 to 1.07. The relative viscosity of snake venom varies from 1.5 to 2.5. The solubility of Elapidae venom in H₂O is much higher than that of Viperidae and solubility of all venoms increase in physiological saline (Sarkar and Devi, 1968).

2.1.2 Proteins and polypeptides in snake venom

Over 90% of the solid snake venom components are protein and polypeptides responsible for exerting pharmacological effects in victims (Sarkar and Devi, 1968; Stocker, 1990). Proteins/polypeptides of venom can be further divided into enzymes and non-enzymes.
2.1.2.1 A brief account of enzymes present in snake venom

Activity and number of enzymes present in snake venom varies from venom to venom and about 26 enzymes have been identified. Although no single venom contains all of them, at least 10 of these enzymes are present in every snake venom, while the remaining are found in several combinations in different varieties of snakes (Sarkar and Devi, 1968; Mebs, 1970; Lee, 1979; Bieber, 1979; Iwanaga and Suzuki, 1979; Stocker, 1990). A comparative study on the activities of enzymes in venoms of 42 species comprising Colubridae, Elapidae, Viperidae and Crotalidae snake families led to the conclusion that Elapidae venoms are rich in phospholipases, phosphodiesterase, nucleotidase, ATPase and cholinesterase, whereas Russell’s viper and pit viper venoms contain proteases, coagulant, kinin-releasing and arginine-hydrolyzing enzymes (Sarkar and Devi, 1968; Mebs, 1970). Enzymes present in the snake venom play an important role in inducing toxicity following bite. Important enzymes present are protease, acetylcholinesterase, ATPase, AMPase, L-amino oxidase, phospholipase A2, etc. Proteases are responsible for the local effects like haemorrhagic, necrosis and muscular degeneration etc. (Sarkar and Devi, 1968; Soto et al., 1988; Ownby, 1990, Mukherjee and Maity, 1998). The acetylcholinesterase, which is one of the toxic enzymes of cobra venom, acts on acetylthiocholine to liberate the choline and acetate (Guieu et al., 1994). 5’-nucleotidase enzymes are responsible for hydrolysis of terminal phosphate from adenylic acid (AMP). This is a Zn$^{2+}$ and EDTA sensitive enzyme (Sarkar and Devi, 1968; Elliot, 1978; Iwanaga and Suzuki, 1979). Venom ATPase, when injected, is known to give "shock" to the victims due to sudden hydrolysis of ATP (Kini and Gowda, 1982). The L-amino acid oxidase purified from venom of King cobra (Ophiophagus hannah), having a molecular mass of 1,35,000 dalton (by gel filtration) causes aggregation of platelets through the formation of H$_2$O$_2$, and subsequent thromboxane A$_2$ synthesis requiring Ca$^{2+}$ but independent of ADP release (Li et al., 1994). Both procoagulant and anti-coagulant enzymes from snake venom have been isolated which affect different steps of blood coagulation cascade (Seegers and Ouyang, 1979; Teng et al., 1984).
2.1.2.2 Non-enzymatic snake venom proteins

Other than enzymes, snake venom contains numerous non-enzymatic proteins, which play an important role in toxicity of the venom. Various non-enzymatic proteins have been isolated and characterized. Neurotoxins isolated from snake venom have been found to impair the nerve function, mainly neuromuscular transmission (Mebs, 1990). These neurotoxins may be either: (i) Post synaptically active neurotoxins, which block neuromuscular transmission by binding specifically to the AchE receptor. (ii) Presynaptically acting neurotoxins inhibit transmitter release from nerve terminals or enhance release of neurotransmitter (dendrotoxin) (Mebs, 1990). Cardiotoxins are involved in cardiac arrest, muscle contracture, membrane depolarization, cytolysis, myonecrosis, hemolysis and affect on platelets (reviewed by Condrea, 1974; Stocker, 1990). Cytotoxins are low molecular weight toxic polypeptides, that induce various pharmacological effects like hemolysis, cytolysis, depolarization of muscle membrane and specific cardiotoxicity (reviewed by Stocker, 1990). Myotoxins contribute to the digestion of muscle cells or cause significant skeletal muscle necrosis (Ownby, 1990; Brusses et al., 1993). The mode of action of the myotoxin is the lysis of the plasma membrane of skeletal muscle cells. Nerve growth factor activity has been identified in six Viperdae, nine Crotalidae and five Elapidae species, which induces plasma extravasation and histamine release from whole blood cells (Elliot, 1978; Mebs, 1990; Stocker, 1990). Protease inhibitor are low molecular weight polypeptides of Elapidae and Viperidae venoms consisting of 52 to 65 amino acids and cross-linked by 2 or 3 disulfide bridges. They either act as proteinase inhibitors or represent structural analogous of proteinase inhibitors (Iwanaga et al., 1976; Hokama et al., 1976; Ritonja et al., 1983; Jayanthi and Gowda, 1988).

2.1.3 Non-protein components of snake venom

The non-protein components of snake venom can be divided into two category (a) organic constituents and (b) inorganic constituents (Sarkar and Devi, 1968; Bieber, 1979; Stocker, 1990). The organic constituents are carbohydrates
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(glycoproteins), lipids (phospholipids primarily), nucleosides and nucleotides, amino acids, biogenic amines (abundant in Viperidae and Crotalidae venoms) including histamine, serotonin, bufotenine and N-methyl tryptophan etc. The inorganic constituents of snake venoms include $\text{Ca}^{2+}$, $\text{Fe}^{2+}$, $\text{Mn}^{2+}$, $\text{Na}^+$, $\text{Li}^+$, $\text{K}^+$, $\text{Co}^{2+}$, and $\text{Zn}^{2+}$ beside anions like phosphate, sulphate and chloride. All these substances mentioned above are not found in every type of venom and the amount of each varies from species to species (Stocker, 1990).

2.2 Variation in venom composition: Impact on pathogenesis and antivenom treatment

The variation in venom composition is a common phenomenon and plays an important role in pathophysiological symptoms following bite and deserves medical concern. The venom varies greatly due to variation in individual, geographical origin and age of the snakes (Taborska, 1971; Taborska and Kornalik, 1985; Meier, 1986; Minton and Winstein, 1986; Jayanthi and Gowda, 1988; Daltry et al., 1996; Tsai et al., 1996; Mukherjee and Maity, 1998). Gene mutation, which is the primary cause of evolution plays an important role in variation of venom composition between closely related species or even within the same species of snake (Glenn et al., 1983; Yang et al., 1991; Assukuri et al., 1992; Daltry et al., 1996; Fry et al., 2002).

Due to the variation in venom composition, the pathogenesis developed after a bite is complex in nature. Further, the clinical manifestation depends upon the qualitative composition as well as the quantitative distribution of different components of venom proteins (Stocker, 1990; Warrell, 1989; Mukherjee and Maity, 1998). For example, Russell's viper venom from Southern India differs from that of the Western and Northern India in terms of lethal potencies (Jayanthi and Gowda, 1988). Though $\text{Naja naja}$ and $\text{Naja kaouthia}$ are closely related species, but they differ in their venom composition (Mukherjee, 1998; Mukherjee and Maity, 2002). The former venom is more toxic as compared to latter venom and antivenom raised against $\text{Naja naja}$ is hardly effective in neutralizing the pharmacological effects of $\text{Naja kaouthia}$ venom (Mukherjee and Maity, 2002). It has been well documented
that this variation in venom composition affects significantly the neutralizing capacity of antivenom as well (Fry et al., 2003). Therefore the variation in the venom composition should be given proper consideration while producing antivenom, because the antivenom raised against the venom of one population of snakes may be less effective against the venom of another population of snakes, which may be of the same species (Fry et al., 2001).

2.3 Snake venom PLA₂ enzymes: Classification, structure and functions

Phospholipase A₂ (EC: 3.1.1.4) are one of the most studied snake venom enzyme due to their pivotal role in inducing various pharmacological effects. They are abundantly found in nature and human pancreas and of course snake venom is the richest source of this enzyme (Kini and Evans, 1989; Dennis, 1994; Hawgood and Bon, 1991). In addition to digestion of prey, snake venom PLA₂s are involved in many pharmacological effects, such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, haemorrhage, edema etc. Further they attack various vital organs of human victims (Kini, 1997). However, not all PLA₂ enzymes induce all these pharmacological effects; but an individual PLA₂ enzyme exhibit either one or more specific pharmacological effects. For example OHVA-PLA₂ from *Ophiophagus hannah* venom induces myotoxicity, cardiotoxicity, antiplatelet effects (Haung et al., 1993; Haung and Gopalakrishnakone, 1996; Haung et al., 1997). β-Bungarotoxin (β-Btx) a PLA₂ toxin from *Bangurus multicinctus* venom exhibit presynaptic neurotoxicity (Strong et al., 1976) but failed to show postsynaptic neurotoxicity (Yang, 1978).

2.3.1 Classification of PLA₂ enzyme

PLA₂ enzymes can be classified based on various parameters. Balsinde et al. (1999) classified PLA₂ enzymes utilizing their properties into three main types: secretory PLA₂, cytosolic Ca²⁺ dependent PLA₂ and intracellular Ca²⁺ independent PLA₂. Kini (1997) has classified PLA₂ enzymes depending on their structure and mechanism of catalysis (Fig. 2.1). In general they have a molecular weight in the
range of 1,40,000-4,00,000. Based on the molecular weight they are classified into
high molecular weigh and low molecular weight (Kini, 1997).

![Classification of PLA2 enzymes](image)

Fig. 2.1. Classification of PLA2 enzymes (cited from Kini, 1997)

a) **High molecular weight PLA2**: These groups of PLA2 enzymes are intracellular
in origin and specifically hydrolyze plasmogen at sn-2 position. Basically these
groups of enzymes are found in various tissues and snake venom, which is
further divided into Ca$^{2+}$ dependent and Ca$^{2+}$ independent.

b) **Low molecular weight PLA2**: These group of enzymes have a molecular
weight in the range of 13,000-15,000 dalton and are extra cellular or secretory
enzymes. Snake venom and mammalian pancreas are rich source of these
enzymes. They specifically release fatty acid from sn-2 position. Amino acid
sequence of more than 150 proteins are known and they can be classified into
following four groups based on their primary structures.
i) **Group I PLA$_2$ enzymes**: PLA$_2$ enzymes of Elapid and Hydropid snake venom and mammalian pancreas fall under this group. These enzymes typically contain 115-120 amino acid residues and is cystine rich containing 7 disulfide bridges. This group is further subdivided into two, Group I and II, based on the presence or absence of pancreatic loop. PLA$_2$ of Elapid is under Group I and while that of pancreatic is under Group IB PLA$_2$.

ii) **Group II PLA$_2$ enzymes**: This group is isolated from Viperid and Crotalid snake venoms and mammalian cells such as platelets. 120-125 amino acid residues and seven disulfide bridges are present in these enzymes. This enzyme lacks pancreatic loop. This group differs from Group I in having an additional C-terminal, which forms an extra disulfide link with a cystine residue in position 49. In some enzyme it is replaced by lysine. Thus this group can be classified into Asp-49 and Lys-49 enzymes.

iii) **Group III PLA$_2$ enzymes**: This group of PLA$_2$ enzymes has been isolated from bee venom. They contain 130-135 amino acid residues and are glycoprotein in nature. They does not share any homology with Group I and Group II enzymes, but the three dimensional folding shows significant similarities.

iv) **Group IV PLA$_2$ enzymes**: This protein was first isolated as the inhibitor of binding of isradipine, a ligand specific for the L-type Ca$^{2+}$ channel of rat neocortical membrane. This enzyme has two long and short chains containing 77 and 42 residues respectively. This group does not share any significant homology with any other group and is Ca$^{2+}$ dependent.
2.3.2 Structure and mechanism of action of PLA$_2$ enzymes

Fig 2.2. Structure of PLA$_2$ enzyme from *Naja naja naja* (Indian cobra).

Resolution 2.3$^\circ$, R-factor: 0.174 (Fremont et al., 1993).
2.3.2.1 Structure of PLA$_2$ enzyme

Crystal structure of various snake venom PLA$_2$ enzymes have been determined, including rattlesnake venom (Brunie et al., 1985), Chinese pit viper venom (Wang et al., 1996; Tang et al., 1998), Indian cobra venom (Fremonth et al., 1993; Segelke et al., 1998), Thai cobra *Naja kaouthia* venom (Gu et al., 2002) etc. PLA$_2$ enzymes of snake venom belong to the Group I sPLA$_2$ with a highly conserved Ca$^{2+}$ binding loop and a catalytic site. Besides these elements, there are six absolutely conserved disulfide bonds and up to two additional unique disulfide bonds, which contributes to the high degree of stability of these enzymes. Substrate hydrolysis proceeds through the activation and orientation of a water molecule by hydrogen bonding to the active site histidine. Adjacent to this histidine, there is a conserved aspartate residue, which, together with the Ca$^{2+}$-binding loop, acts as a ligand cage for Ca$^{2+}$ (Dennis, 1994). The crystal structures of sPLA$_2$-IB and -IIA have defined conserved active site within a hydrophobic channel lined on one side by the N-terminal helix (Scott et al., 1991). This hydrophobic channel binds a single phospholipid molecule following interfacial binding of the enzyme to the aggregated phospholipid surface (Kudo and Murakami, 2002). However, the relationships between catalytic efficiency of the enzyme and its various pharmacological properties, the identification of pharmacological sites and their characteristic conformations and relationships between conformations and lethalities of these actions are not yet clearly defined. Several attempts have been made to predict the site of toxicity of these enzymes by comparing protein sequences in correlation with their lethalities but it has not yet been understood adequately due to lack of consistency in the correlation (Singh et al., 2001).
2.3.2.2 Mechanism of PLA₂ catalysis

Phospholipase A₂ are esterolytic enzymes, which hydrolyze glycerophospholipids at the sn-2 position of the glycerol backbone liberating free fatty acid and lysophospholipid (Dennis, 1983) (Fig 2.3). They can hydrolyze phospholipid in monomeric, micellar or lipid bilayer phases. PLA₂ exhibits distinct head group preference; the catalytic efficiency is determined by the physical properties of aggregation, such as packing density, phase transition, temperature, liquid crystalline and other parameters. When phospholipid is in bilayer, PLA₂ activity depends on several factors such as curvature of the bilayer, the physical state of lipids and the presence of other molecules, such as ionic or non-ionic detergent (Verheij et al., 1981; Veron and Bell, 1992). The active site of the PLA₂ enzyme consists of histidine residue. Histidine residue assisted by Asp, polarize a bound H₂O, which then attacks the carbonyl group. The Ca²⁺ ion, which is bound to the conserved Ca²⁺ loop, is required to stabilize the tetrahedral transition state (Dennis, 1994). Thus Ca²⁺ plays an important role in catalysis by PLA₂.

Catalytic action of PLA₂ in aqueous phase is postulated to occur as follows. The enzyme binds to substrate molecule to form ES complex, followed by formation of product. The enzyme then either goes back to aqueous phase or binds to another
substrate in the same interface (Kini, 1997). It has been found that the enzyme activity is also dependent on the organization of phospholipids in the membrane (Burack and Biltonen, 1994). Several studies have proposed that the presence of defects in the bilayer structure may act as starting point of enzyme activity (Grainger et al., 1989; Vernon and Bell, 1992).

Recently Hyvonen et al. (2001) studied the structural effects induced by PLA$_2$ hydrolysis on membrane, applying molecular dynamics (MD) system. They documented that the PLA$_2$ hydrolyse bilayers that had a loosened structure as compared to normal intact 1-palmitoyl-2-linoleoyl-sn-glycerol-3-phosphatidylcholine (PLPC) system and increased penetration of H$_2$O molecules. The decreased integrity of the bilayer consisting of the hydrolysis products implies structural perturbation in the hydrolyzed bilayer area. This perturbations may also activate PLA$_2$ by allowing more mobility of the substrate molecules in the membrane-normal direction and accordingly better access to the active site of the enzyme (Apitz-Castro et al., 1982; Jain and de Haas, 1983; Jain and Jahagirdar, 1985; Burack et al., 1993, 1995, 1997; Schefield et al., 1995; Lehtonen and Kinnunen, 1995; Bell et al., 1996; Honger et al., 1996; Grandbois et al., 1998).
2.3.3. Biochemical properties of PLA_{2} enzymes

Table 2.1. Molecular mass of some purified snake venom PLA_{2}.

<table>
<thead>
<tr>
<th>Snake species</th>
<th>Molecular weight (Dalton)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>Vipera russelli</em></td>
<td>11,8000(^{a}) (VRV PL VIIa)</td>
<td>Kasturi and Gowda, 1989</td>
</tr>
<tr>
<td><em>Daboia russelli</em></td>
<td>15,000(^{a}) (DbTx)</td>
<td>Maung-Maung-Thwin et al., 1995</td>
</tr>
<tr>
<td><em>Bothriechis schegelii</em></td>
<td>15,000(^{a})</td>
<td>Angulo et al., 1997</td>
</tr>
<tr>
<td><em>Hydrophis cyanocinctus</em></td>
<td>13,588.1(^{b}) (H1)</td>
<td>Ali et al., 1999</td>
</tr>
<tr>
<td></td>
<td>13,247.2(^{b}) (H2)</td>
<td></td>
</tr>
<tr>
<td><em>Crotalus atrox</em></td>
<td>13,779(^{c})</td>
<td>Tsai et al., 2001</td>
</tr>
<tr>
<td><em>Crotalus m. molossus</em></td>
<td>13,723(^{c})</td>
<td>Tsai et al., 2001</td>
</tr>
<tr>
<td><em>Bothriechis schlegelii</em></td>
<td>13,671(^{c})</td>
<td>Tsai et al., 2001</td>
</tr>
<tr>
<td><em>Porthidium godmani</em></td>
<td>13,836(^{c})</td>
<td>Tsai et al., 2001</td>
</tr>
<tr>
<td><em>Porthidium nummifer</em></td>
<td>13,738(^{c})</td>
<td>Tsai et al., 2001</td>
</tr>
</tbody>
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\(^{a}\) SDS-PAGE  
\(^{b}\) MALDI-MS  
\(^{c}\) Determined by ESI-MS with SE ± 0.01%

As shown in Table 2.1, the molecular mass of snake venom PLA\(_2\) enzymes generally ranges from 11,000 to 15,000 dalton. They have a very rigid tertiary structure arising from the presence of 5-8 disulfide bonds which helps in stability against proteolysis and resistance to denaturation and allows them to retain their activity in the extracellular fluid where they are found (Balsinde et al., 1999). Further, the presence of large number of disulfide bonds in PLA\(_2\) enzymes may attribute them to be thermostable (Vishawanath et al., 1988; Francis et al., 1995). PLA\(_2\) enzymes require Ca\(^{2+}\) in millimolar concentration for its catalytic activity (Balsinde et al., 1999). Adjacent to the histidine residue of the active site, there is a conserved aspartate residue, which, together with the Ca\(^{2+}\)-binding loop, acts as a ligand cage for Ca\(^{2+}\). Ca\(^{2+}\) ion binds to the Ca\(^{2+}\) loop, which is conserved and plays an active role in catalysis (Dennis, 1994). Although Ca\(^{2+}\) enhance the catalytic activity, other divalent ions such as Ba\(^{2+}\), Sr\(^{2+}\) and Mn\(^{2+}\) can substitute Ca\(^{2+}\) ions (Reynolds et al., 1993). The amino acid sequence of many PLA\(_2\) have been
determined and found to be 40-90% identical, however they differ greatly in their enzymatic and pharmacological effects (Rosenberg, 1979; 1986; 1990; Basavarajappa et al., 1993; Doley et al., 2004). Treatment of ρ–bromophenacyl bromide inactivates cobra venom PLA₂ by alkylating 0.5 histidine residue indicating that histidine 48 is conserved among snake venom phospholipase A₂ enzymes and plays an essential role in the catalysis process (Roberts et al., 1977; Tsai et al., 2001; Doley and Mukherjee, 2003; Doley et al., 2004). Some of the PLA₂ enzyme contains aspartate residue at 49 position which is critically involved in the binding of Ca²⁺, however, in several enzymes this residue is replaced by lysine, which does not bind to Ca²⁺ efficiently (Maraganore et al., 1984; Maraganore and Hennrikson, 1986; Scott et al., 1990; Francis et al., 1991). Other chemical inhibitors like Potassium cyanate, o-Methylisourea, Acetic anhydride and Trinitrobenzenesulphonyl (TNBS) modify the Lys residue (Soares and Giglio, 2003). Modification of the Lys residue distorts the binding ability of the PLA₂ enzyme for the substrate, causing a drastic loss in the enzymatic activity (Chang et al., 1994); Nitrophenylsulphenyl chloride (NPSC) modifies the Tyr. and Trp (Soares and Giglio, 2003). Modification of Tyr residues of Bothrops myotoxins have been found to affects lethality, myotoxicity, cytotoxicity and the neuromuscular blocking effect induced by these toxins (Soares et al., 2000a,b; Andriao-Escarso et al., 2000). Chloramine T and Iodoacetic acid modifies the Met. residues. Selective oxidation of Met6 and Met8 of β-bungarotoxin from Bungarus multicinctus by chloramine T did not destroy the two Ca²⁺ binding domains, though it modified the toxin to become less effective for binding Ca²⁺. (Soares and Giglio, 2003).

Most of the reported snake venom PLA₂s are highly stable and resistant to heat, acid and urea but catalytically inactivated at high pH (Bonfim et al., 2001). They have a pH range of 6.9 to 8.0 and optima temperature of 30-55 °C (Rudrammaji and Gowda, 1998; Bonfim et al., 2001). Thermostable PLA₂ enzymes from snake venoms have been reported (Vishawanath et al., 1988; Francis et al., 1995; Ali et al., 1999). CD spectra analysis of thermostable PLA₂ enzymes reveal that the enzymes are of highly compact folded structures, mainly based on the core structure of the disulfide bridges (Dufton et al., 1983; Dufton and Hider, 1983). PLA₂
enzymes of snake venom have a α-helix structure, which is the major secondary structure, this is further confirmed by X-ray crystallography and solution NMR studies (Fremonth et al., 1993; van den Berg et al., 1998; Wang et al., 1996; Han et al., 1997; Perbandt et al., 1997; Segelke et al., 1998; Ali et al., 1999).

2.3.4. Pharmacological properties of PLA₂ enzymes

The fatty acid and lysophospholipids generated during hydrolysis serve as a precursor for lipid-derived mediators with a wide range of biological activities (Gelb et al., 1995, 1999; Tischchfield, 1997; Dennis, 2000), but still the implication of these mode for the pharmacological effects are not clear. The other product of PLA₂, lysophospholipid, may be metabolized to platelet-activating factors which is a potent inflammatory mediator (Kume and Shimize, 1997; Jackson et al., 1998) or to lysophospholipidic acid with mitogenic activities (Fourcade et al., 1998). Snake venom PLA₂ exhibits a wide variety of pharmacological effects despite their similarity in primary, secondary and tertiary structures (Kini, 1997). PLA₂ induce many pharmacological effects which are either, dependent or independent of its catalytic activity and enzymatic activity is partially responsible for, at least, some of these effects (Kini and Evans, 1989). In mechanism where the pharmacological effects are dependent on enzymatic activity, either the hydrolysis of the intact phospholipid or the released product such as lysophospholipid and free fatty acid can cause the pharmacological effect (Kini and Evans, 1989). Whereas in those mechanisms, where they are independent of the enzymatic activity, binding of PLA₂ to the target protein can cause the pharmacological effects by acting as an agonist or an antagonist, or by interfering in the interaction of the target protein with its physiologic ligands (Stefansson et al., 1990; Evans and Kini, 1997; Mounier et al., 2000).

Some snake venom PLA₂ enzyme forms covalent or non-covalent complex with additional venom protein to express their pharmacological effects at full potency, which forms complex. For example, β-Bungarotoxin, crotoxin and Mojave toxin have two subunits (Bon, 1997). Whereas taipoxin and textilotoxin have 3 to 5
subunits (Fohlman, et al., 1976). One of the component is PLA2 and the other subunit(s) is(are) PLA2 like molecule with or without catalytic activity (Kini, 1997). Specific effects of PLA2 enzyme on a particular tissue can be explained by the presence of specific target site on the surface of tissue or cell (Kini and Evans, 1989). These target sites are recognized by specific pharmacological sites of the PLA2 enzymes which are independent or sometime overlapping with the active site of this enzyme (Rosenberg, 1986). It has been proposed that target sites and pharmacological site are complementary to each other in terms of charges, hydrophobicity and Vander waal's contact surface, hence higher affinity (Kini and Evans, 1989). The proposed target sites would be either membrane lipids or proteins (glycoproteins) (Kini, 2003).

Both lethal and non-lethal PLA2 from snake venom have been reported. Some of the reported lethal PLA2 enzymes are CM-II and CM-III (LD50 of 10 ± 3 and 4.4 ± 0.8 μg/g body weight) isolated from Naja naja kaouthia venom (Joubert and Taljaard, 1980), acidic PLA2s of N. naja sputatrix (LD50 of 270 μg/kg) (Tan, 1982), PLA2-H1 (LD50 of 45 μg/kg) isolated from sea snake venom (Ali et al., 1999), Dabio toxin (LD50 of 50 μg/kg) isolated from Daboia russelli (Maung-Maung-Thwin et al., 1995). Whereas PLA2s isolated from Indian cobra (Naja naja naja) are reported to be non-lethal up to a dose of 10 mg/kg body weight (Rudrammaji and Gowda, 1998). Other non-lethal snake venom PLA2s are also reported (Boucheir et al., 1991; Yang et al., 1991; Ali et al., 1999). Basic PLA2s are reported to be more toxic and enzymatically less potent but the acidic PLA2 are less toxic and enzymatically active (Rosenberg, 1986), therefore the lethality of snake venom PLA2 cannot be correlated with the catalytic property (Dhillon et al., 1987).

In presynaptic neurotoxicity, nerve terminal exposed to PLA2 enzyme shows mitochondrial damage and depletion of synaptic vesicle (Gopalakrishnakone and Hawgood, 1984; Cull-Candy et al., 1976; Landon et al., 1980). It has been postulated that during presynaptic blockade PLA2 enzyme first binds to presynaptic site followed by perturbation of the presynaptic membrane by PLA2 near the binding site.
Myotoxin induces myonecrosis that leads to leakage of CPK followed by increase in its level in the plasma of the victim (Mukherjee and Maity, 2002). In addition, myotoxins induce acute muscle cell damage by affecting the integrity of plasma membrane of the target cell, thereby causing hypercontraction and other cellular alteration, leading to the cell death (Gutierrez and Lomonte, 1995). Study has revealed that the catalytic activity of the enzyme has no role to play in the myotoxicity, however, hydrophobic and cationic regions in PLA₂ molecules are responsible for determining the myotoxicity (Kini and Iwanaga, 1986).

Few PLA₂ enzymes exhibit cardiotoxicity (Lee et al., 1977; Fletcher et al., 1981, 1982; Chang et al., 1983) and it has been opined that cardiotoxicity is independent of enzymatic activity. However, the exact mechanism of induction of cardiotoxicity by PLA₂ enzymes has not been well-established (Kini and Evans, 1989).

PLA₂ enzymes are classified into strong, weak and non-anticoagulant enzymes (Verheij et al., 1980; Boffa et al., 1980). Amino acid sequence reveals the presence of anticoagulant region between the 54 and 77 amino acid residue. In strongly anticoagulant PLA₂ enzymes, this region is positively charged. In non-anticoagulant enzyme, lysine is replaced by negatively charged amino acid (Kini and Evans, 1987). According to the model suggested by Kini and Evans (1989), strongly anticoagulant PLA₂ enzyme would bind with high affinity to an unidentified clotting factor, which acts as a target molecule in such a complex, which would interfere the interaction between clotting factors. The enzyme would then hydrolyze phospholipids in the microenvironment. These combine results of binding, hydrolysis and loss of critical phospholipids would slow or stop the normal activation of clotting factor(s), resulting in an anticoagulant effect.

Venom PLA₂ enzymes, which interfere in platelet function, can be classified into two distinct classes- A and B. Class A comprises of platelet affector PLA₂ enzymes which show biphasic effect. These enzymes at a low concentration and short incubation time induce platelet aggregation while at higher concentration and
on prolong incubation inhibit platelet aggregation (Ouyang and Huang, 1984). Class B PLA₂ enzymes cause only the inhibition of platelet aggregation but fail to initiate aggregation (Li et al., 1985; Ouyang et al., 1983). Experimental evidence suggests the presence of distinct target molecule on the platelet surface and separate pharmacological sites exist on the enzyme molecules (Kini and Evans, 1989).

2.3.5. Molecular diversity and evolution of PLA₂ isoenzymes

Occurrence of large number of PLA₂ isoenzymes in snake venom is a common phenomenon (Braganca and Sambray, 1967; Vishwanath et al., 1987, 1988; Takasaki et al., 1990; Ogawa et al., 1992; Subburaju and Kini, 1997; Singh et al., 2000; Shiloah et al., 1973; Sim, 1998). Indian cobra (Naja naja) venom has been reported to contain as many as 14 isoenzymes of PLA₂ (Shiloah et al., 1973) whereas Kini and Gowda (1983) reported 9 PLA₂ isoenzymes. These isoenzymes have been reported to share high identity in their amino acid sequence and similar three-dimensional structure (Heinrikson, 1991; Scott and Sigler, 1994). Recently three PLA₂ isoenzymes (MiPLA₂-2, MiPLA₂-3 and MiPLA₂-4) have been purified from Micropechis ikaheka venom (Gao et al., 2001) which show similar hydrophobic properties but have different charge states. Snake venom PLA₂ isoenzymes may be either acidic, neutral or basic in nature (Jayanthi and Gowda, 1988). Basic PLA₂ are more toxic as compared to acidic and neutral and contributes significantly to the toxicity of venom (Jayanthi and Gowda, 1988; Mukherjee and Maity, 1998). The higher toxicity of the basic PLA₂ might be due to the presence of positive charge, which have been postulated to be responsible for their penetrability in plasma membrane. This may explain why they are more toxic as compared to acidic or neutral PLA₂ (Verheij et al., 1980).

These isoenzymes exhibit different pharmacological effects and often cause problems in purification and determination of their functional specificity (Kini, 1997). Further, they show similar hydrophobicity but have different charge states. Different isoenzymes present in snake venom are not due to the subspecies polymorphism, but exist in a venom sample collected from single snake (Hazlett and Dennis, 1985).
Although there is dearth of knowledge on the biochemical basis of the diversity of PLA$_2$, but it is presumed that the diversity in the venom PLA$_2$ enzymes could result from two types of structural modification:

(i) Pre-translational modification that leads to difference in amino acid sequence.
(ii) Post-translational modification resulting in the alteration in net charge among PLA$_2$ enzymes (Dubourdieu et al., 1987).

According to Kini and Chan (1999), multiple PLA$_2$ isoenzymes found in snake venom are formed by gene duplication and accelerated evolution. Mutation in nature is a random process. Some gene loci mutate more frequently, which are termed as hot spots. However the role of this hot spots in protein evolution is not clearly understood. Examination of three-dimensional structure of PLA$_2$ enzyme reveals that the residue that are located in the surface mutates more frequently. These surface substitutions play a significant role in the evolution of new PLA$_2$ isoenzymes by altering the specificity of targeting to various tissues or cells, resulting in the distinct pharmacological effects.
Fig. 2.4. Scheme for evolution of PLA₂ genes leading to the major present-day PLA₂. Solid vertical lines indicate ancestral lines associated with individual genes. Branch points indicate duplication of a gene. Dashed vertical lines indicate possible radiative events, i.e., evidence for a gene duplication awaits evidence that differences are not the result of speciation in the limited number of species examined. Horizontal lines denote that at the time of emergence of the indicated life forms at least as many PLA₂ genes existed as are intersected by the line. Vertical scaling is not correlated with time. The Solenoglypha are movable front-fanged snakes, and the Proteroglypha are fixed front-fanged snakes. (Tischfield, 1997).
2.4 Medicinal plants in the treatment of snakebite patients including PLA₂ inhibitors

In many parts of the world numerous plant species are used as folk medicines to treat snakebite (Moris, 1991; Martz, 1992; Houghton and Osibogun, 1993). In Papua New Guinea, following plants viz: *Alphitonia incana* (Rhamnaceae), *Cerbera floribunda* (Apocynaceae), *Magnifera minor* (Anacardiaceae), *Maclura sp* (Moraceae), *Melanolepis muttiglandulosa* (Euphorbiaceae), *Osmoxylon micranthum* (Araliaceae) are used for treating bites of two major poisonous snake, the dead adder (*Acanthopsis sp*) and the small-eyed snake (*Micropechis ikaheka*) (Mebs, 2000). In NorthWest region of Columbia, traditional healers attend around 60% of the snakebite patients. Based on field interviews, 101 species of plants used against snakebite were identified (Otero et al., 2000). In ancient Indian books, there are various plants recommended for use in snakebite therapy. Around 50 such plants have been indexed (Biswas and Ghosh, 1977) and many others (not include) have also been popularly used against snakebite by villagers, snake charmers and Ohjas throughout India including many tribal people of North East India.

Mournfully, clinical and pharmacological tests on alkaloid extracted from well-known and reputed medicinal herbs sometimes show distinctly negative results (Jain, 1996). Such observations should prompt us to reassess these herbs carefully and critically. Literature survey shows that there is scanty of works on exploring the antivenom activity of these medicinal plants. However in recent years, much more attention has been paid to pharmacological screening of the medicinal plants used to treat snakebites (Akunyili and Akubue, 1986; Mors et al., 1989; Gomes et al., 1994; 1998; Alkofahi et al., 1997; Mahanta and Mukherjee, 2001). Houghton and Osibogun (1993) reviewed many such flowering plants useful against snakebites. For example, water extracts of root and leaves of *Eryngium creticum*, a perennial globrous herb of Jordan, have been reported to inactive the haemolytic activities of desert viper (*Cerastes cerates*) and scorpion (*Leiurus quinquestiartus*) venoms (Alkofahi et al., 1997). Similarly, Mors and his colleagues (1989) reported that lethality and myotoxicity of American rattlesnake venom could be effectively
neutralized by *Eclipta prostrata* plant extracts. Methanol extract of the stem bark of *Parkia biglobosa* (Mimosaceae) has been shown to protect significantly against the neurotoxic, haemorrhagic and cytotoxic effects of two poisonous snakes of Nigeria (*Naja nigricollis*, and *Echis ocellatus*) (Asuzu and Harvey, 2003). Several plant constituents like flavanoids, quinonoids, xanthene, polyphenols and terpenoids possessed protein binding and enzyme inhibiting properties (Havestean, 1983; Selvanayagam et al., 1996), which also inhibits the PLA₂ activities of both Viper and Cobra venom (Alcaraz and Hoult, 1985). Recently, two Indian medicinal plants (*Hemidesmus indicus* and *Pluchea indica*) were identified for their venom inhibitory activity (Gomes et al., 1994). An organic acid isolated from root extract of an Indian medicinal plant sarsaparilla, locally called “Anantmul” (*Hemidesmus indicus* R.Br) possessed Russell’s viper venom inhibitory activity. This compound responsible for venom inhibitory activity was isolated from the root extract by solvent extraction, silica gel column chromatography and thin layer chromatography. Spectral analysis confirmed the presence of a benzene ring, methoxy group and hydroxyl group; the molecular weight of the compound was 168. This compound (designated as HI-RVIF) significantly antagonized the Russell’s viper venom induced lethality, haemorrhagic, coagulant and anticoagulant activity in experimental rodents. Recently, Mahanta and Mukherjee (2001) reported that the aqueous extract of root of *Mimosa pudica* locally known as “Lajuki late” neutralized the myotoxic and lethality of Cobra *Naja kaouthia* venom. The methanolic root extracts of *Vitex negundo* Linn. and *Emblica officinalis* Gaertn have been found to antagonize significantly the *Vipera russelli* and *Naja kaouthia* venom induced lethal activity, both in *in-vitro* and *in-vivo* studies (Alam and Gomes, 2003).

Since the first report of isolation of a protein from the blood of Habu snake (*Terimerusurus flavoviridis*) by Kihara (1976), research on both venomous and non-venomous snake venom has led to the characterization of series of serum globular proteins which possess the unique ability to neutralize the enzymatic and toxic effects of snake PLA₂ enzymes. Presence of PLA₂ inhibitor proteins (PLIs) has been associated with the resistance of snake to the deleterious effects of their venom PLA₂ effects (Domont et al., 1991; Faure, 2000; Perales and Domont, 2002;
Fortes-Dias, 2002). Some of these inhibitors have also homologous counterparts in mammals and specific mammalian derived inhibitors have been reported (Rocha et al., 2002). Despite the significance of \( \text{PLA}_2 \) inhibitors in antivenom therapy, the most significant consideration of these natural inhibitors is their potential therapeutic use, not only as an alternative to antivenom but also as a potential antagonist for \( \text{PLA}_2 \) activities associated with inflammatory process in human (Lizano et al., 2003). Even if these inhibitors would be an alternative to antivenom this would lead to another limitation: availability of inhibitors. So far inhibitors have been isolated from snake blood but snake themselves would be an insufficient and ecologically unacceptable source of inhibitors for commercial purpose. Further they may elicit immune response which could be deleterious to the patients. Apart from the snake and mammalian origin PLIs, plants are also reported to have these natural inhibitors. A glycoprotein isolated from *Withania somnifera* (Ashwaganda) have been found to be active against scorpion sting envenomation as well as *in-vivo* myotoxic and edematous effect of venom \( \text{PLA}_2 \) (Mishra et al., 2000; Deepa and Gowda, 2002). Screening of plants for the presence of \( \text{PLA}_2 \) inhibitory proteins and other compounds would in near future lead to isolation of potent antivenom compound that will have potential therapeutic use as well.

2.5 An overview of Indian medicinal plants used as folk medicine in the treatment of snakebite patients

*Agyle mermolos*, Linn. (Rutaceae) is a deciduous plant, 6-8 mts in height. Fresh half-ripe fruit is mildly astringent and used to care dysentery, diarrhoea, hepatitis, tuberculosis, and in the treatment of snakebite (Jain, 1991).

*Alstonia scholaris*, Linn. (Apocyanaeace). It is a common resident of India in deciduous and evergreen forest. The bark is bitter in taste, astringent, acrid, thermogenic, digestive, laxative, antihelmenthic, stomachic, cardiotonic. It is useful in fever, malaria, abdominal disorders, skin diseases, ulcers, asthma etc (Sivarajan and Balachandran, 1994). The dried bark is boiled and the extract is used as an anti-dote to snakebite by the tribal people (N. Taye, personal communication).
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*Aristolochia indica*, Linn. (Aristolochiaceae). It is a twining shrub, slender, woody at base. The roots of this plant are butter, acrid, astringent, thermogenic, purative, digestive, antihelminthes, stomachic, cardiotoxin, anti-inflammatory, diuretic and tonic. They are useful in ulcer, inflammation, skin diseases and all type of poisonous bites and stings (Warrier et al., 1994). The tribal people believe that chewing the whole plant or taking its juice can neutralize the snake poison (N. Taye, personal communication).

*Azadirachta indica* A. juss (Maliacea) is known for its several medicinal values. The leaves, seeds, roots and bark of the plant possess bitter active principles in different constituents (Oliver-Bever, 1986). The plant has insecticidal properties (Nwude, 1986; Oliver-Bever, 1986). Moreover, the antifertility (Prakash et al., 1988; Bardhan et al., 1991; Upadhyay et al., 1993) and hypotensive with minimal negative chronotropic effect of *Azadirachta indica* have been reported (Thompson and Anderson, 1978). The bitter principles of *Azadirachta indica* are also known to increase the flow of saliva and gastric juice as a result of which the plant is used as stomachics (Oliver-Bever, 1986). *Azadirachta indica* lowered blood glucose level and attenuated gastric ulcerogenesis (Sen et al., 1992). Garg (2000) has indexed this plant as medicinal plant used against snakebite.

*Calamus rotang*, Linn. (Arecaceae). Climbing palm exhibiting a slender stem ranging from a few millimeters to some centimeter m in diameter, flexible sometimes more or less armed with spines. Roots are astringent, acrid, bitter, expectorant, anti-inflammatory, diuretic and tonic. It is useful in burning, cough, dysentery, and various skin diseases. (Warrier et al., 1994). Fresh root of the plant is crushed and applied on the wound of the snakebite (N. Taye, personal communication).

*Carica papaya*, Linn. (Caricaceae). The papaya is a short-lived, fast-growing, woody, large herb up to 10 or 12 feet in height. The unripe fruit is used as abortificalient. Other uses of the fruit include against bone fracture, indigestion,
ringworm, skin diseases, tooth and gum ache, urine bladder complains and snakebite (Jain, 1991).

*Curucuma aromatica*, (Zingiberaceae) is a perennial tuberous herb native to India and cultivated in the tropics. Rhizomes are bitter, carminative, appetizer, tonic and are useful in various skin diseases and poisonous bite (Warrier, 1994; Mukherjee, 2001).

*Curucuma longa*, (Zingiberaceae). This is a perennial tuberous herb native to India and cultivated in large scale. Oral administration of the powdered rhizomes of *C. longa* had been found to be beneficial in case of Asthma and cough (Jain and Dam, 1979). Paste of the fresh rhizome is applied externally to get relief from the inflammation as well as on the wound caused by the snakebite. In other diseases such as indigestion, insect sting, swelling of body, jaundice, etc this has been used extensively by the Tribal people (N. Taye, personal observation).

*Leucus lavendulaefolia*, Linn. (Laminaceae). It is a common resident of India. The plant is antihistaminic, antipyretic, anticeptic, carminative, febrifuge and wormifuge and is used in anorexia, cough, dyspepsia, fever, helminthic manifestation, jaundice, and other skin diseases. Extract of the plant exhibits strong anti-inflammatory activity on acute and chronic inflammation caused due to snakebite (Sivarajan and Balachandran, 1994; Mukherjee, 2001).

*Murraya koenigii*, Linn. (Rutaceae). The plant is common in India. The leaves are used for many skin diseases, promote appetite and digestion, destroy pathogenic organism, worm troubles, neurosis and poison (Sivarajan and Balachandran, 1994). The tender leaves are made into paste and used as anti-dote to snakebite by the tribal people (Mukherjee, 2001; N. Taye, personal communication).

*Piper longum* Linn. (Piperaceae). This plant is commonly found in India in evergreen forest. It is aromatic slender climber. *Piper nigrum*, Linn. (Piperaceae).
This plant is commonly found in India in evergreen forest as well as cultivated by the local people. The fruit are acrid, bitter in taste. The fresh spike of *P. longum* and dried fruits of *P. nigrum* are chewed and externally applied at the site of snakebite. This has been practiced by the Mishing community from long ago and has been passing from generation to generation as folk medicine to treat snakebite patients (N. Taye, personal communication).

*Terminalia arjuna*, Roxb. (Combretaceae). This is a large evergreen tree. The bark is astringent, sweet, acrid, aphrodisiac, cardiotonic, stypic and urinary astringent. It is useful in fractures, ulcers, cardiopathy, fatigue, asthma, tumors, internal and external haemorrhages (Warrier et al., 1994). The dried bark is boiled and given orally for snakebite by the local healer (Mukherjee, 2001; N. Taye, personal communication).

*Zingiber officinale* Rosc. (Zingiberaceae) is a perennial herb cultivated throughout India. The rhizome is commonly used as species. Rhizome has a pungent aromatic, lemonly and slightly bitter in taste. The rhizome has been used for various diseases like cough, asthma, cholera, scabies, insect sting, snakebite, throatache etc (Jain, 1991; Mukherjee, 2001).