The standard aqueous stem bark extract of Mangifera indica L. inhibits toxic PLA₂ – NN-XIb-PLA₂ of Indian cobra venom

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Abstract The aqueous extract of Mangifera indica is known to possess diverse medicinal properties, which also includes anti-snake venom activities. However, its inhibitory potency and mechanism of action on multi-toxic snake venom phospholipases A₂ are still unknown. Therefore, the objective of this study was to evaluate the modulatory effect of standard aqueous bark extract of M. indica on NN-XIb-PLA₂ of Indian cobra venom. The in vitro sPLA₂, in situ hemolytic and in vivo edema inhibition effect were carried out as described. Also the effect of substrate and calcium concentration was carried out. M. indica extract dose dependently inhibited the GIA sPLA₂ (NN-XIb-PLA₂ activity with an IC₅₀ value of 7.6 µg/ml. M. indica extract effectively inhibited the indirect hemolytic activity up to 98% at ~40 µg/ml concentration. Further, M. indica extract (0–50 µg/ml) inhibited the edema formed in a dose dependent manner. When examined as a function of increased substrate and calcium concentration, there was no relieve of inhibitory effect of M. indica extract on the NN-XIb-PLA₂. Further, the inhibition was irreversible as evident from binding studies. The in vitro inhibition is well correlated with in situ and in vivo edema inhibiting activities of M. indica. As the inhibition is independent of substrate and calcium and was irreversible, it can be concluded that M. indica extract mode of inhibition could be due to direct interaction of components present in the extract with the PLA₂ enzyme. The aqueous extract of

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1. Introduction

Snakebite envenomation is a significant public health problem causing considerable high morbidity and chronic mortality around the world, particularly in the tropical and subtropical regions (Kasturiratne et al., 2008; Warrell, 2010). It is also of biomedical importance with social and economic impact on developing regions around the world. Snakebite is now recognized as a highly Neglected Tropical Disease (NTD) by the World Health Organization (WHO) (Kasturiratne et al., 2008; Warrell, 2010) and it estimates that, globally, at least 4,21,000 envenoming and 20,000 deaths occur each year due to snakebite (Kasturiratne et al., 2008). The high mortality and morbidity of snakebite are due to its venom, which consists of a complex mixture of biologically active components, that interfere with the course of several biological processes in both prey and human (Aird, 2002; Dhananjaya et al., 2010). Therefore, the pathophysiological effects observed in snakebites are due to the combined action of several enzymes, proteins and peptides, which include phospholipase A₂, hemorrhagic metalloproteases and other proteolytic enzymes, coagulant components, neurotoxins, cytotoxins and cardiotoxins, among others (Aird, 2002; Koh et al., 2006; Dhananjaya et al., 2010).

The snake venom phospholipase A₂ (svPLA₂) are the main multi-toxic and lethal component of snake venom and have been investigated because they not only have a wide range of biological effects, but also due to their similarity to mammalian phospholipases (Kini and Chan, 1999). In snake venoms, only two groups of secretory PLA₂ (GI and GII) have been identified. Group I (GIA) includes the svPLA₂s from Elapinae and Hydrophiinae venoms with 115–120 amino acid residues and these svPLA₂s are homologous to mammalian non-pancreatic GIB sPLA₂. Group II (GIIA and GIIB) comprises the svPLA₂s from Crotalinae and Viperinae venoms with 120–125 amino acid residues and homologous to mammalian non-pancreatic Group II-A sPLA₂ (Burke and Dennis, 2009). Group II PLA₂s are in turn divided into different subgroups on the basis of amino acid residue in the 49th position: catalytically active D49 enzymes, catalytically inactive or with low activity K49, S49, N49 or R49 forms (Nevalainen et al., 2012; Lomonte et al., 2009).

The above described subgroups exhibit a wide variety of physiological and pathological effects. In addition to their possible role in the digestion of prey, snake venom sPLA₂s exhibit a wide spectrum of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, anticoagulant and anticancer effects (Doley et al., 2010; Kini, 2003). Due to the prominent role played by PLA₂s in the snake envenomation, there is enormous pharmacological interest in search of sPLA₂ inhibitors (Narendra Sharath Chandra et al., 2007; Nanda et al., 2007; Sivaramakrishnan et al., 2016). Further, considering the limitations of antiserum therapy (Dhananjaya et al., 2011; Girish and Kemparaju, 2011), researches are focusing on the development of alternative treatments and in this regard finding inhibitors of the multi-toxic svPLA₂s from medicinal plants have gained much interest (Carvalho et al., 2013; Gomes et al., 2012). In this context, although many sPLA₂ inhibitors have been isolated from various medicinal plants (Springer, 2001; Nanda et al., 2007; Narendra Sharath Chandra et al., 2007) that have been demonstrated to bring down the toxic and lethal effects of several venoms (Girish and Kemparaju, 2011), however, still effective and specific inhibitors of sPLA₂ are not available.

Mangifera indica L. (Anacardiaceae) is one of the most popular edible fruit yielding trees that grow in the tropical and subtropical regions of the world. M. indica has been traditionally used to treat various diseases (Chopra et al., 1956; Coe and Anderson, 1996; Shah et al., 2010; Dhananjaya et al., 2011). The standard aqueous stem bark extract of M. indica has been used in pharmaceutical formulations in Cuba under the brand name VIMANG®, to treat patients suffering from increased stress (Guevara et al., 1998). The pharmacological studies have indicated that VIMANG® has immunomodulatory, analgesic, anti-inflammatory and anti-inflammatory effects (Makare et al., 2001; Garrido et al., 2004; Ojewole, 2005; Dhananjaya and Shivalingaiah, 2016). Recently, it has been demonstrated for its potential antibacterial, anti-hyperalgesic, anti-arthritic, antimutagenic, antihypertotoxic, anticancer and larvicidal activities (Garrido-Suarez et al., 2014; Morfi et al., 2012; Rajakumar et al., 2015; Singh et al., 2015; Awad El-Gied et al., 2015; Falmy et al., 2016; Khurana et al., 2016). In an earlier study, it was shown that the aqueous steam bark extract of M. indica L. (Anacardiaceae) inhibited the toxic and lethal effects of Indian Russell’s viper venom (Dhananjaya et al., 2011); however, the extract’s effect on multi-toxic and lethal PLA₂s was not exemplified. Therefore, the objective of this study was to carry out investigations in regard to evaluating the modulatory effect of standard aqueous extract of M. indica on Group I A svPLA₂ (i.e. purified NN-XIb-PLA₂ phospholipase A₂ enzyme from Naja naja venom). Also, studies were carried out to get an insight into the possible biochemical interaction of extract/components to bring about inhibition/neutralization of svPLA₂s toxic effects. This study may provide better understanding on the therapeutic molecular interactions of components with antiophidian activity to supplement the conventional antivenom therapy against these toxic and lethal multifunctional enzymes.

2. Materials and methods

Venom from Naja naja was purchased from Irula Co-operative Society Ltd., Chennai, India. All other reagents and chemicals
used were of analytical grades purchased from Sisco Research Laboratories (SRL), Bangalore, India.

2.1. Preparation of standard aqueous stem bark extract of *M. indica* L.

*M. indica* stem bark was collected in the university campus and was authenticated at the university herbarium centre, Department of Botany, University of Mysore, Mysore, India, where a voucher specimen (UOM/DOSB/PL/82) was deposited. The stem bark extract of *M. indica* was prepared by decoction for 1 h according to the method of Garrido et al. (2004) as described earlier (Dhananjaya et al., 2011). The fine brown powder obtained after desiccation was dissolved in saline and used for neutralization assays. The amount of extract is expressed as dry weight.

2.2. Animals

Swiss Wister albino mice weighing about 20–25 g were obtained from the central animal house facility. All protocols of animal experiments have been approved by the Sri Adichunchanagiri College of Pharmacy – Institutional Animal Care and Use Committee (IACUC). Animal care and handling were conducted in compliance with the national regulations for animal research.

2.3. Isolation of secretory phospholipase A2 (NN-XIb-PLA2) from the venom *Naja naja*

sPLA2 belonging to group IA – NN-XIb-PLA2 from the venom *Naja naja* (Southern region) was purified up to homogeneity as described previously by the method of Rudrammaji and Gowda (1998). This protein was further used for evaluating the anti-inflammatory potential of aqueous extract of *M. indica*. The protein concentration was estimated according to the method of Lowry et al. (1951) using BSA as protein standard.

2.4. Inhibition of Phospholipase A2 activity by standard aqueous stem bark extract of *M. indica* L.

The Phospholipase A2 assay was carried out according to the method as described by Bhat and Gowda (1989). Phosphatidyl choline (PC) was diluted with petroleum ether (60–80 °C) to get a concentration of 1000 nmoles/50 ml. The reaction mixture containing NN-XIb-PLA2 (3 µg) was made up to 680 ml with water. To the reaction mixture, 200 µl of ether, 100 µl of Tris–HCl buffer (0.05 M, pH 7.5), and 20 µl of CaCl2 (500 mM) were added. The total reaction mixture was incubated at 370°C for 60 min. After incubation, 0.5 ml of Dole mixture (Isopropanol:Pet ether:NH4SO4, 40:10:1) was added, mixed and centrifuged at 1000 rpm for 3 min. To the organic phase 0.5 ml of CHCl3:Pet ether (1:5) was added, mixed and centrifuged at 1000 rpm for 3 min. To the upper phase cobalt reagent [1.35 ml of Triethanolamine made up to 10 ml with solution A (6 g of CO(NO3)2·6H2O + 0.8 ml glacial acetic acid) and 7 ml of solution B (Saturated Na2SO4)] was added, mixed and centrifuged 1000 rpm for 3 min. The upper organic phase was carefully transferred and 0.75 ml of α-nitroso-β-naphthol reagent (0.4% α-nitroso-β-naphthol in 96% ethanol) was added. The intensity of the orange color is directly proportional to the amount of cobalt present. After 30 min, 2 ml of ethanol was added to dilute the contents and absorbance was read at 540 nm. The amount of free fatty acid released was estimated using standard linolenic acid curve. The enzyme activity was expressed as nmoles of fatty acid released/min/mg of protein.

For inhibition studies, NN-XIb-PLA2 (3 µg) was preincubated with or without different concentrations of aqueous extract of *M. indica* (0–15 µg/ml) at 37 °C for 15 min. Appropriate controls were carried and further experiments were carried out as described above. The inhibition is expressed as percentage taking activity of venom alone as 100%. IC50 values were calculated using Graphpad version 5.0.

2.5. Effect of substrate and calcium concentration on NN-XIb-PLA2 inhibition by standard aqueous stem bark extract of *M. indica* L.

Effects of substrate and calcium concentrations on inhibition of NN-XIb-PLA2 (3 µg) at IC50 concentration of extract were determined as follows. In general, the reaction mixture containing NN-XIb-PLA2 alone and/or with the IC50 concentration of aqueous extract of *M. indica*, in 0.05 M Tris–HCl buffer, pH 7.5, and 400 mM calcium was used for the PLA2 assay. In the substrate-dependent assay, substrate concentration in the range of 20-120 nM was used as in the final reaction mixture. The calcium-dependent assay was carried out of concentrations ranging from 0 to 15 mM in the final reaction mixture. After the reaction time, PLA2 assay was carried out as described above.

2.6. Determination of binding characteristics and reversibility of inhibition by standard aqueous stem bark extract of *M. indica* L.

The reaction mixture containing NN-XIb-PLA2 (3 µg) with the IC50 concentration of aqueous extract of *M. indica* in 0.05 M Tris–HCl buffer, pH 7.5, and 40 mM calcium was pre-incubated for 15 min. Then the reaction mixture was dialyzed against 1000 ml of 100 mM Tris–HCl buffer, pH 7.5, containing 0.2 mM Ca at 4 °C in dialysis tubing (with a molecular weight cutoff of 3000–6000) for 24 h with three buffer changes. The PLA2 activity was assayed before and after dialysis as described above.

2.7. Neutralization of indirect hemolytic activity by standard aqueous stem bark extract of *M. indica* L.

Indirect hemolytic activity was assayed as described by Boman and Kaletta (1957), using packed human erythrocytes (blood group A). The human erythrocytes used for the study were sourced from previously published work, which had ethical approval from ethical committee of the University of Mysore (UOM) for the withdrawal of blood (Dhananjaya et al., 2011). Further, the ethics committee approval was obtained for the use of human material in our current work. The written informed consent from the donor was obtained for the use of this sample in this research. The substrate for the indirect hemolytic assay was prepared by suspending 1 ml of packed
fresh human red blood cells and 1 ml fresh hen’s egg yolk in 8 ml of phosphate buffered saline (PBS). Aqueous extract of *M. indica* (0–40 μg/ml) was pre-incubated with or without NN-XIb-PLA2 (8 μg), which showed 100% hemolytic activity, for 30 min at 37 °C. To the pre-incubated sample, 1 ml of substrate was added and allowed to react for 45 min at 37 °C. The reaction was stopped by adding 9 ml of ice-cold PBS. The suspension was mixed and centrifuged at 1500g for 20 min. The released hemoglobin was read at 530 nm. A sample with venom alone served as positive control. The hydrolysis of erythrocyte caused by the addition of 9 ml distilled water was taken as 100%. Appropriate controls were carried out and the inhibition is expressed as percentage (%). Values are presented as the mean of four independent determinations.

2.8. Neutralization of edema inducing activity by standard aqueous stem bark extract of *M. indica* L.

The procedure of Yamakawa and Hokama (1976) as modified by Vishwanath et al. (1987) was followed. NN-XIb-PLA2 (5 μg) was pre-incubated without or with different concentration of aqueous extract of *M. indica* (0–50 μg/ml) in a total volume of 20 μl saline. The reaction mixture was injected into intra plantar surface of right hind footpad of mice weighing 20–25 g. The left footpad that received 20 μl of saline served as control. After 45 min, the mice were sacrificed by giving anesthesia (Pentobarbitone, 30 mg/kg, i.p.) and both hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as the ratio of the weight of edematous limb to the weight of normal (sham injected) limb × 100. Minimum edema dose is defined as the microgram of protein causing an edema ratio of 120%. Injecting a fixed dose protein into mice footpads and sacrificing them at regular period of time obtained time course curve of edema inducing activity. Edema ratio was calculated and expressed as %.

2.9. Statistical analysis

The IC50 values were calculated using Graph Pad version 5.0. Inhibition percentages were calculated from the difference for 30 min at 37 °C. To the pre-incubated sample, 1 ml of substrate was added and allowed to react for 45 min at 37 °C. The reaction was stopped by adding 9 ml of ice-cold PBS. The suspension was mixed and centrifuged at 1500g for 20 min. The released hemoglobin was read at 530 nm. A sample with venom alone served as positive control. The hydrolysis of erythrocyte caused by the addition of 9 ml distilled water was taken as 100%. Appropriate controls were carried out and the inhibition is expressed as percentage (%). Values are presented as the mean of four independent determinations.

### Table 1

| sPLA2     | Specific activitya | IC50b |
|-----------|--------------------|-------|
| NN-XIb-PLA2 | 172.4 ± 3.1        | 7.6 μg/ml |

*a* nmoles of fatty acid released/mg of protein/min at 37 °C. *b* IC50 value is defined as the amount of extract (μg/ml) required to inhibit 50% of enzyme activity in the given reaction mixture.
of the sPLA₂ enzyme. NN-XIb-PLA₂ enzyme exhibited indirect hemolytic activity, which is an indirect way of measuring PLA₂ activity using egg yolk and washed erythrocytes are used as substrates. When the effect of aqueous extract of *M. indica* at different concentrations (0–40 µg/ml) was tested it was found that the extract in general effectively inhibited indirect hemolytic activity up to 98% at 40 µg/ml concentration (Fig. 4). This *in situ* inhibition activity is well correlated with the inhibitory activity of the *in vitro* PLA₂ enzyme. Therefore, the inhibition of NN-XIb-PLA₂ activity by molecules in *M. indica* extract could be attributed to the modulation of the catalytic activity of PLA₂ at the interface itself, i.e., beyond the initial steps of enzyme adsorption and activation, probably through modifications of the intermolecular organization of the membrane components. It is well known that secretory PLA₂s cause cell membrane asymmetry by degradation of glycerol phospholipids of the membranes.

Many of the snake venom PLA₂s induce toxic effect such as edema when injected into mouse footpad as demonstrated before (Vishwanath et al., 1988). Several svPLA₂ inhibitors are demonstrated to exhibit concomitant inhibition of enzyme activity and edema-inducing activity (Nanda et al., 2007; Mohamed et al., 2010). Since in our study, as the aqueous extract of *M. indica* effectively inhibited the *in vitro* PLA₂ activity and *in situ* PLA₂ activity, the inhibitory potential on *in vivo* edema inducing activity of NN-XIb-PLA₂ was tested. The edema inducing effect of NN-XIb-PLA₂ (6 µg) was more than 176%, when compared to the saline injected mice. Fig. 5 shows that aqueous extract of *M. indica* at different concentrations (0–40 µg/ml), dose dependently inhibited the edema formation, when co-injected with enzyme. In addition, *M. indica* extract at the tested dose alone did not cause edema when injected into mice footpads. The neutralization of edema inducing activity is known to be well correlated with the *in vitro* enzymatic activity inhibition. It has been demonstrated that the standard extract of *M. indica*, administered orally (50–200 mg/kg body wt.) had reduced edema induced by arachidonic acid (AA) and phorbol myristate acetate (PMA) in mice (Garrido et al., 2006). In addition, the extract was demonstrated to inhibit the edema induced by carrageen and formalin in mice, rats and guinea-pigs (Garrido et al., 2004; Ojewole, 2005). Further, it has been shown to reduce the tumor necrosis factor alpha (TNF alpha) serum levels in both arachidonic acid (AA) and phorbol myristate acetate (PMA)
induced models of inflammation in mice (Garrido et al., 2004). It was demonstrated that the extract inhibited the induction of PGE2 and LTB4, when it was stimulated with pro-inflammatory stimuli lipopolysaccharide-interferon gamma (LPS-IFNγ) or calcium ionophore A23187 in J774 macrophage cell lines (Garrido et al., 2006). Recently, it has been shown that the aqueous stem bark extract of *M. indica* administration had reduced TBARS concentration and iNOS, COX-2, TNF-α and TNF-R-2 expression in colonic tissue, and a decrease in IL-6 and TNF-α serum levels was also observed (Márquez et al., 2010).

Although the mechanism of action of the extract is unclear, the finding that no visible change was detected in electrophoretic pattern of NN-XIb-PLA2 when incubated with extracts (data not shown), excludes the proteolytic degradation as a potential mechanism (Borges et al., 2000). Further, considering our binding studies, where it has been observed that the inhibition is irreversible and also independent on substrate and calcium concentration, the most likely mechanism for anti-PLA2 activities by this extract could be due to the direct binding of the constituents of the extract with sPLA2s active site. The extract is known to contain a well defined and standardized mixture of components such as polyphenols, terpenoids, steroids, fatty acids and microelements (Nunez-Selles et al., 2002; Shah et al., 2010), mangiferin (20%) being the predominant one. As with other polyphenols, the phenolic

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**Figure 3** Dose dependent neutralization of edema inducing activity of NN-XIb-PLA2 by aqueous stem bark extract of *M. indica*. The reaction mixture 30 µl containing NN-XIb-PLA2 (6 µg) was incubated for 30 min with increasing concentration of aqueous stem bark extract (0–50 µg/ml) of *M. indica*. Saline (30 µl) injected into the mouse foot-pad served as control. Data represent ± S.E.M. for *n* = 3.

**Figure 4** Effect of substrate concentration on inhibition of NN-XIb-PLA2 by aqueous stem bark extract of *M. indica*. The reaction mixture contained NN-XIb-PLA2 enzyme in 0.05 M Tri-HCl buffer pH 7.5, 500 mM CaCl2, inhibitors, and increasing concentrations of substrate. The reaction was carried out in the absence (rhombus) and presence of IC50 concentration of extract (open square) with increasing concentration of substrate. The figure inset represents the percent of inhibition in the presence of IC50 concentration. Data represent ± S.E.M. for *n* = 3.

**Figure 5** Effect of calcium concentration on inhibition of NN-XIb-PLA2 by aqueous stem bark extract of *M. indica*. The reaction mixture contained NN-XIb-PLA2 enzyme in 0.05 M Tri-HCl buffer pH 7.5, and 3–15 mM CaCl2 in final volume. The reaction was initiated by adding substrate. The reaction was carried out in the absence (rhombus) and presence of IC50 concentration of extract (open square) with increasing concentration of calcium. The figure inset represents percentage inhibition in the presence of the IC50 concentration. Data represent ± S.E.M. for *n* = 3.
constituents of the extract such as phenolic acids (Gallic acid, 3, 4 dihydroxy benzoic acid, benzoic acid) and phenolic esters (Gallic acid methyl ester, gallic acid propylester, benzoic acid propyl ester), could be also involved in binding with sPLA2s, thus bringing about inhibition (Shah et al., 2010). In addition, the active constituents of this extract such as mangiferin, amento flavone, friedelin, daucosterol and beta-sitosterol (Sanchez et al., 2000) seem to exhibit anti-inflammatory and thus anti-snake effect through quenching of free radicals (Nanda et al., 2007; Narendra Sharath Chandra et al., 2007; thus anti-snake effect through quenching of free radicals). This anti-inflammatory activity of the extract was believed to be due to the powerful antioxidant activities exhibited by the constituents such as phenolic compounds including mangiferin. It has been demonstrated that VIMANG® and mangiferin, exhibit inhibitory activity against synovial fluid PL2 activities (Garrido et al., 2004).

The aqueous stem bark extract of *M. indica* inhibiting both in vitro PL2 enzymatic activity and in vivo edema inducing activity of NN-Xlb-PL2, suggests a strong correlation between lipolytic activity and pro-inflammatory activity inhibition. It is to be noted that this standard aqueous stem bark extract of *M. indica* has been tested in a broad set of toxicological studies with satisfactory results, including acute and sub-chronic toxicity, genotoxicity, and irritability and is classified as a non-toxic product (Sanchez et al., 2000; Gonzalez et al., 2007; Garrido et al., 2009). Thus, it can be viewed that *M. indica* bark extract can be developed for topical application, as it is non-toxic and can be developed for effective anti-snakebite formulation, which contain potent anti-snake venom molecules. In conclusion, the aqueous extract of *M. indica* effectively inhibited svPLA2 and its associated toxic activities. The inhibition is irreversible and also independent on substrate and calcium concentration, suggesting that the constituents of the extract might possibly directly interact to bring about inhibition. Also it was found that there is a strong correlation between lipolytic activity and pro-inflammatory activity inhibition. Therefore, the study suggests that the extract possesses potent anti-PLA2 agents that could be developed as a potential therapeutic agent against snake envenomation. This study also substantiates their anti-snake venom properties. Further in-depth studies on compounds present in the extract that are responsible for the anti-PLA2 activity will be interesting, as these are highly attractive candidates for formulation development for topical application during snakebite. This supplements the conventional antivenom therapy and helps in management of snakebite. Further in-depth studies on the role and mechanism of the principal constituents present in the extract, responsible for the anti-PLA2 activity can be carried out to develop them into potent anti-snake component and also as an anti-inflammatory agent.

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