

## Inhibition of secretory PLA<sub>2</sub> – VRV-PL-VIIIa of Russell's viper venom by standard aqueous stem bark extract of *Mangifera indica* L.

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**Abstract.** The aqueous extract of *Mangifera indica* is known to possess anti-snake venom activities. However, its inhibitory potency and mechanism of action on multi-toxic phospholipases A<sub>2</sub>s, which are the most toxic and lethal component of snake venom is still unknown. Therefore, this study was carried out to evaluate the modulatory effect of standard aqueous bark extract of *M. indica* on VRV-PL-VIIIa of Indian Russells viper venom. *Mangifera indica* extract dose dependently inhibited the GIIB sPLA<sub>2</sub> (VRV-PL-VIIIa) activity with an IC<sub>50</sub> value of 6.8±0.3 µg/ml. *M. indica* extract effectively inhibited the indirect hemolytic activity up to 96% at ~40 µg/ml concentration. Further, *M. indica* extract at different concentrations (0-50 µg/ml) inhibited the edema formed in a dose dependent manner. It was found that there was no relieve of inhibitory effect of the extract when examined as a function of increased substrate and calcium concentration. The inhibition was irreversible as evident from binding studies. The *in vitro* inhibition is well correlated with *in situ* and *in vivo* edema inducing activities. As the inhibition is independent of substrate, calcium concentration and was irreversible, it can be concluded that *M. indica* extracts mode of inhibition could be due to direct interaction of components present in the extract with PLA<sub>2</sub> enzyme. In conclusion, the aqueous extract of *M. indica* effectively inhibits svPLA<sub>2</sub> (Snake venom phospholipase A<sub>2</sub>) enzymatic and its associated toxic activities, which substantiate its anti-snake venom properties. Further in-depth studies are interesting to known on the role and mechanism of the principal inhibitory constituents present in the extract, so as to develop them into potent anti-snake venom and as an anti-inflammatory agent.

### INTRODUCTION

Snake bite envenomations are of a significant public health problem causing considerable high morbidity and chronic mortality around the world, particularly in the tropics. It is also of biomedical importance with social and economic impact in developing regions around the world. Snake bite is now recognized as a highly Neglected Tropical Disease (NTD) by the World Health Organization (WHO) (Kasturiratne *et al.*, 2008; Warrell, 2010). The World Health Organization (WHO) estimates that, globally,

at least 4, 21, 000 envenoming and 20,000 deaths occur each year due to snake bite (Kasturiratne *et al.*, 2008). Snake venom is a complex mixture of biologically active components, which are able to interfere with the course of several biological processes in both prey and human (Aird, 2002; Dhananjaya *et al.*, 2010). The pathophysiological effects observed in snake bite is due to the combined action of several enzymatic proteins and peptides, that includes phospholipases A<sub>2</sub>, 5'Nucleotidase, hemorrhagic metalloproteases, proteolytic enzymes, proteins acting on coagulant

components, neurotoxins, cytotoxins and cardiotoxins (Aird, 2002; Koh *et al.*, 2006; Dhananjaya *et al.*, 2010).

The secreted phospholipases A<sub>2</sub> (sPLA<sub>2</sub>s) are the main component of snake venom that have been investigated not only because they possess a wide range of biological effects, but also due to their similarity to mammalian phospholipases (Kini & Chan, 1999). In snake venoms, only two groups of sPLA<sub>2</sub>s (GI and GII) have been identified. Group I (GIA) includes the svPLA<sub>2</sub>s from *Elapinae* and *Hydrophiinae* venoms with 115–120 amino acid residues and these svPLA<sub>2</sub>s are homologous to mammalian pancreatic GIB sPLA<sub>2</sub>. Group II (GIIA and GIIIB) comprises the svPLA<sub>2</sub>s from *Crotalinae* and *Viperinae* venoms with 120–125 amino acid residues and homologous to mammalian non-pancreatic Group II-A sPLA<sub>2</sub> (Burke & Dennis, 2009). Group II PLA<sub>2</sub>s 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 (Lomonte *et al.*, 2009; Nevalainen *et al.*, 2012). 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 sPLA<sub>2</sub>s exhibit a wide spectrum of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, anticoagulant and anticancer effects (Kini, 2003; Doley *et al.*, 2010). Due to the prominent role played by PLA<sub>2</sub>s in the snake envenomation, there is pharmacological interest in search of effective PLA<sub>2</sub> inhibitors (Narendra *et al.*, 2007; Nanda *et al.*, 2007). Further, considering the limitations of anti-serum therapy (Dhananjaya *et al.*, 2011; Girish & Kemparaju, 2011), researches are focusing on development of alternative treatments and in this regard finding inhibitors of the multi-toxic svPLA<sub>2</sub>s from medicinal plants have gained much interest in recent times (Gomes *et al.*, 2012; Carvalho *et al.*, 2013). Several snake venom PLA<sub>2</sub> inhibitors are demonstrated to bring down the toxic and lethal effects in several venoms, thereby helping in management of snake bite (Girish & Kemparaju, 2011). In this context, many

sPLA<sub>2</sub> inhibitors have been isolated from various medicinal plants (Springer, 2001; Nanda *et al.*, 2007; Narendra *et al.*, 2007).

*Mangifera indica* L. (Anacardiaceae) is one of the most popular edible fruit yielding tree that grows in the tropical and subtropical regions of the world. *Mangifera indica* has been traditionally used to treat various diseases (Chopra, 1956; Coe & 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<sup>®</sup>, to treat patients suffering from increased stress (Guevara *et al.*, 1998). The pharmacological studies have indicated that VIMANG<sup>®</sup> has immunomodulatory, analgesic, antinociceptive, antioxidant and anti-inflammatory effects (Makare *et al.*, 2001; Garrido *et al.*, 2004; Ojewole, 2005). In an earlier study, it was shown that the aqueous stem bark extract of *M. indica* L. (Anacardiaceae) inhibited the toxic and lethal effects of Indian Russells viper venom (Dhananjaya *et al.*, 2011). In this study, we report the investigations carried out in regard to evaluation of the modulatory effect of standard aqueous extract of *M. indica* on Group IIB svPLA<sub>2</sub> (i.e. purified VRV-PL-VIIIa phospholipase A<sub>2</sub> enzyme from *Russells Viper* venom) (Kasturi & Gowda, 1989; Srinivasan, 2004), to substantiate its anti-snake venom properties. Also, this study gives an insight on the possible biochemical interaction of extract/components to bring about inhibition/neutralization of svPLA<sub>2</sub>s toxic effects. This study may provide better understanding on the therapeutic molecular interactions of components with anti-ophidian activity to supplement the conventional anti-venom therapy against these multifunctional enzymes.

## MATERIALS AND METHODS

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

### Preparation of extract

*Mangifera indica* stem bark collected in the university campus 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 hour 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 for neutralization assays. The amount of extract is expressed as dry weight.

### 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 Adichunchangiri College of Pharmacy's Institutional Animal Care and Use Committee (IACUC). Animal care and handling were conducted in compliance with the national regulations for animal research.

### Isolation of Group IIB secretory phospholipase A<sub>2</sub> (VRV-PL-VIIIa)

sPLA<sub>2</sub> belonging to group IIB - VRV-PL-VIIIa from the venom *Daboii rusellii pulchela* (Southern region) was purified up to homogeneity as described previously by the method of Kasturi & Gowda (1989), and as modified by Srinivasan (2004). This protein was further used for evaluating the anti-PLA<sub>2</sub> 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.

### Inhibition of Phospholipase A<sub>2</sub> activity

The Phospholipase A<sub>2</sub> assay was carried out according to the method as described by Bhat & 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 VRV-PL-VIIIa (3 mg) was made up to 680 ml with water. To the reaction mixture, 200ml of ether, 100ml of Tris – HCl buffer (0.05M, pH 7.5), and 20ml of CaCl<sub>2</sub> (500 mM) was added. The total reaction mixture was incubated at 37°C

for 60 minutes. After incubation, 0.5 ml of Doles mixture (Isopropanol: Pet ether: 1NH<sub>2</sub>SO<sub>4</sub>, 40:10:1) was added, mixed and centrifuged at 1000 rpm for 3 min. To the organic phase 0.5 ml of CHCl<sub>3</sub>: Pet ether (1:5) was added, mixed and centrifuged at 1000 rpm for 3 min. To the upper phase cobalt reagent [1.35 vol. of Triethanolamine made up to 10 ml with solution A (6 g of CO(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O + 0.8ml glacial acetic acid) and 7 ml of solution B (Saturated Na<sub>2</sub>SO<sub>4</sub>)] was added, mixed and centrifuged 1000 rpm for 3 min. The upper organic phase was carefully transferred and 0.75 ml of  $\alpha$ -nitroso- $\beta$ -naphthol reagent (0.4%  $\alpha$ -nitroso- $\beta$ -naphthol in 96% ethanol) was added. The intensity of the orange colour 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, VRV-PL-VIIIa (3 mg) was preincubated with or without different concentrations of aqueous extract of *M. indica* (0–15  $\mu$ g/ml) at 37°C for 15 minutes. 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%. IC<sub>50</sub> values were calculated using Graphpad version 5.0.

### Effect of substrate and calcium concentration on VRV-PL-VIIIa inhibition by aqueous extract of *M. indica*

Effects of substrate and calcium concentrations on inhibition of VRV-PL-VIIIa (3 mg) at the IC<sub>50</sub> concentration of extract were determined as follows. In general, the reaction mixture containing VRV-PL-VIIIa alone and/or with the IC<sub>50</sub> concentration of aqueous extract of *M. indica*, in 0.05M Tris- HCl buffer, pH 7.5, and 400 mM calcium was used for the PLA<sub>2</sub> assay. In the substrate-dependent assay, substrate concentration in the range of 20 to 120 nM was used as in the final reaction mixture. The calcium-dependent assay was carried out at

concentrations ranging from 0 to 15 mM in the final reaction mixture. After the reaction time, PLA<sub>2</sub> assay was carried out as described above.

#### **Determination of binding characteristics and reversibility of inhibition of VRV-PL-VIIIa**

The reaction mixture containing VRV-PL-VIIIa (3 µg) with the IC<sub>50</sub> concentration of aqueous extract of *M. indica* in 0.05M Tris-HCl buffer, pH 7.5, and 40 mM calcium were 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 cut off of 3000–6000 Dalton) for 24 h with three buffer changes. The PLA<sub>2</sub> activity was assayed before and after dialysis as described above.

#### **Neutralization of Indirect Hemolytic Activity**

Indirect hemolytic activity was assayed as described by Boman & Kaletta (1957). 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 VRV-PL-VIIIa (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 1500×g 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 taken as 100%. Appropriate controls were carried out and the inhibition is expressed as percentage (%). Values are presented as the mean of 4 independent determinations.

#### **Neutralization of edema inducing activity**

The procedure of Yamakawa & Hokama, (1976) as modified by Vishwanath *et al.*, (1987) was followed. VRV-PL-VIIIa (5 mg) was

pre-incubated without or with different concentration of aqueous extract of *M. indica* (0 - 50 µg/ml) in a total volume of 20 ml 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 ml of saline served as control. After 45 min the mice were sacrificed by giving anaesthesia (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 oedema is expressed as the ratio of the weight of oedematous limb to the weight of normal (sham injected) limb x 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 %.

#### **Statistical analysis**

The IC<sub>50</sub> values were calculated using Graph Pad version 5.0. Inhibition percentages were calculated from the difference between inhibitor-treated group and control animals, which received the vehicle. Student's *t*-test for comparisons of unpaired data was used for statistical evaluation.

## **RESULTS AND DISCUSSION**

svPLA<sub>2</sub>s belonging to viperidae family are known to be multi-toxic and lethal (Kini, 2003; Doley *et al.*, 2010). Due to the prominent role played by PLA<sub>2</sub>s in snake envenomation, there is enormous pharmacological interest in search of effective PLA<sub>2</sub> inhibitors (Narendra *et al.*, 2007; Nanda *et al.*, 2007). Further, considering the limitations of anti-serum therapy (Dhananjaya *et al.*, 2011; Girish & Kemparaju, 2011), it is justified that research has been focused on development of alternative treatments. In this context, finding inhibitors of the multi-toxic svPLA<sub>2</sub>s from medicinal plants have gained much interest in recent times (Gomes *et al.*, 2012; Carvalho *et al.*, 2013) Many sPLA<sub>2</sub> inhibitors have been isolated from various medicinal plants

(Springer, 2001; Nanda *et al.*, 2007; Narendra *et al.*, 2007), however, effective and specific inhibitors of sPLA<sub>2</sub> are not available. In these line of studies, the aqueous stem bark extract of *M. indica* was evaluated for its potential to inhibit phospholipase A<sub>2</sub> (PLA<sub>2</sub>) belonging to group IIB i.e. VRV-PL-VIIIa, which was isolated from *Russell's viper* venom as per the previously described method (Kasturi & Gowda, 1989; Srinivasan, 2004).

The sPLA<sub>2</sub> belonging to group IIB i.e. VRV-PL-VIIIa gave a specific activity of around 116.2±8.1, when measured using PC as substrate (Table 1). When pre-incubated with different concentration of extract it was observed that the aqueous extract of *M. indica*, inhibited the enzymatic activity in a concentration dependent manner as shown in Figure 1. It was observed that the extent of inhibition was >95% at the 40 µg/ml of extract used. The IC<sub>50</sub> values calculated by linear XY scattered plot was 6.8±0.3 µg/ml (Table 1). Most of the sPLA<sub>2</sub> inhibitors are known to inhibit the activity either by binding to substrate or by chelating calcium, which is required for activity (Nanda *et al.*, 2007). Also, it was observed that the sPLA<sub>2</sub> inhibitors affect the “Quality of interface” by modifying

the phospholipids bilayer properties, which render the phospholipids inaccessible to the enzyme (Nanda *et al.*, 2007). The steroid inducible inhibitors of PLA<sub>2</sub> like lipocartin I and II were shown to inhibit PLA<sub>2</sub>s by non-specific binding to the membrane phospholipids. It was observed that their inhibition is relieved by increasing the substrate concentration (Davidson *et al.*, 1987). In the experiments, it was observed that, when examined as a function of substrate concentration, there was no relieve of inhibition of the extract that was pre-incubated with PLA<sub>2</sub>, as the substrate concentration was increased from 20 to 120 nM (Fig. 2). This suggests that the inhibition

Table 1. IC<sub>50</sub> value and specific activity of VRV-PL-VIIIa

sPLA <sub>2</sub>	Specific activity <sup>a</sup>	IC <sub>50</sub> <sup>b</sup>
VRV-PL-VIIIa	116.2±8.1	6.8±0.3

<sup>a</sup>nmoles of fatty acid released/mg of protein/min at 37°C.

<sup>b</sup>IC<sub>50</sub> value is defined as the amount of extract (µg/ml) required to inhibit 50% of enzyme activity in the given reaction mixture.

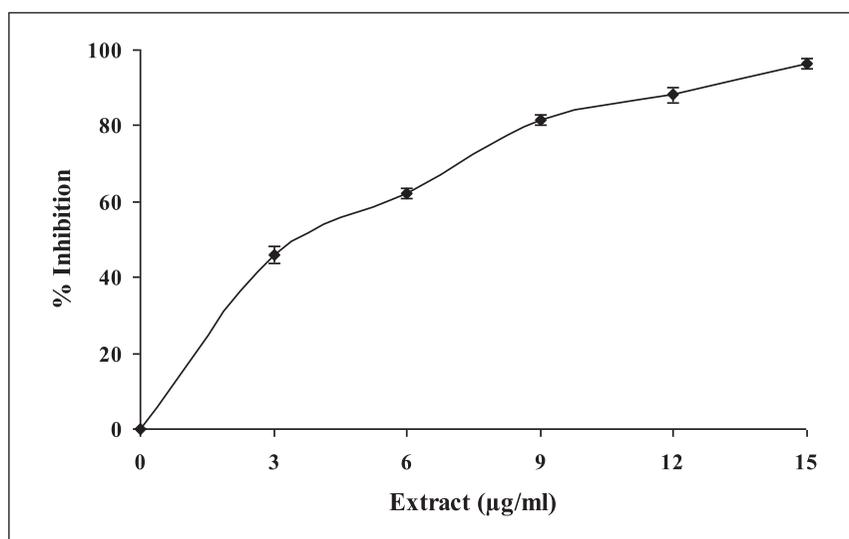


Figure 1. Dose dependent inhibition of *in vitro* PLA<sub>2</sub> (VRV-PL-VIIIa) activity by aqueous stem bark extract of *M. indica*. Briefly, Phosphatidyl choline (PC) corresponding to 1000 nmoles/ml was made up to 680ml with VRV-PL-VIIIa (3 mg), with or without aqueous stem bark extract of *M. indica* at various concentrations (0-15 µg/ml) and was incubated with other reaction mixture at 37°C for 60 min and color developed was read at 540 nm. The results shows ± S.E.M. for n=3.

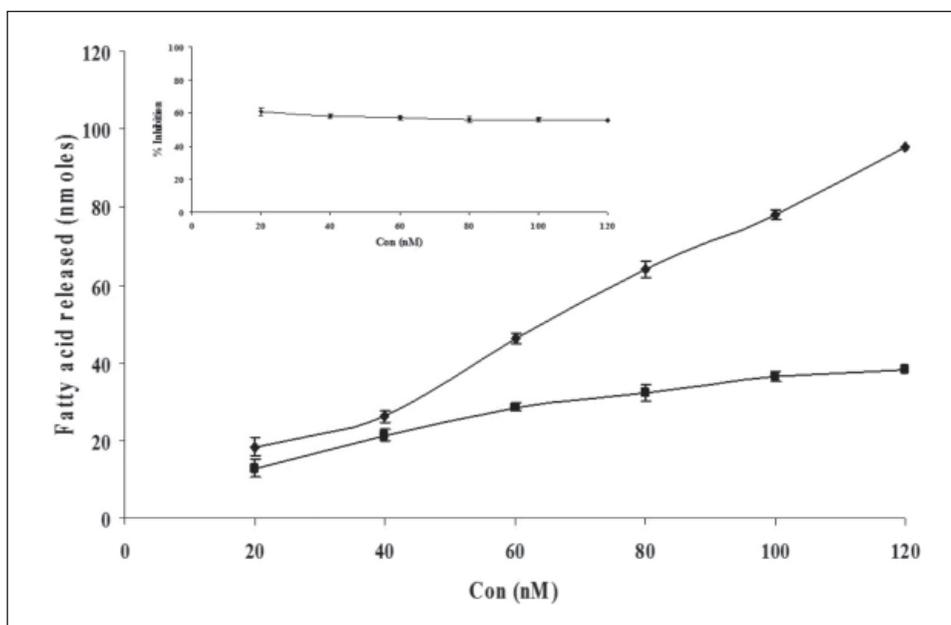


Figure 2. Dose dependent inhibition of indirect hemolytic activity of VRV-PL-VIIIa by aqueous stem bark extract of *M. indica*. VRV-PL-VIIIa (8 mg) in 100  $\mu$ l of phosphate-buffered Saline (PBS) was preincubated with or without aqueous stem bark extract of *M. indica* at various concentrations (0-40  $\mu$ g/ml). The reaction was started by adding erythrocytes, egg yolk and PBS (1:1:8 v/v) incubated for 10 min at 37°C. The released hemoglobin in the supernatant was measured by taking absorbance at 540 nm. The results shows  $\pm$  S.E.M. for n=3.

is independent of substrate concentration. Further, in the calcium dependent activity test, it was observed that an increase in calcium concentration from 2.5 to 15 mM increased VRV-PL-VIIIa enzymatic activity in a dose dependent manner. However, while when IC<sub>50</sub> concentration of *M. indica* extract was used along with varying concentration of calcium, there was no relieve of inhibition (Figure 3), suggesting that the inhibition by *M. indica* extract is independent on calcium concentration. It was reported that some of the PLA<sub>2</sub> inhibitors were shown to mediate displacement of catalytically essential calcium from the enzyme and thus inhibition of enzymatic activity (Pruzanski *et al.*, 1992). In the calcium binding studies experiments, it was found that the PLA<sub>2</sub> enzyme activity in both before and after the dialysis of the enzyme inhibitor mixture was unaltered, i.e. the percentage of inhibitory activity of *M. indica* extract was not decreased upon extensive dialysis, suggesting that the inhibition is irreversible. Also, results support the observation that inhibition by *M. indica*

extract is independent on substrate and calcium concentration. These studies indicate that the inhibition could be due to direct interaction of components or molecules present in *M. indica* extract at active site residues of the sPLA<sub>2</sub> enzyme.

VRV-PL-VIIIa enzyme exhibited indirect hemolytic activity, which is an indirect way of measuring PLA<sub>2</sub> activity using egg yolk and washed erythrocytes is used as substrates. When the effect of aqueous extract of *M. indica* at different concentrations (0-40  $\mu$ g/ml) was tested, it was found that the extract in general effectively inhibited indirect hemolytic activity up to 100% at ~40  $\mu$ g/ml concentration (Figure 4). This *in situ* inhibition activity is well correlated with the inhibitory activity of the *in vitro* PLA<sub>2</sub> enzyme. Therefore, the inhibition of VRV-PL-VIIIa activity by molecules in *M. indica* extract could be attributed to the modulation of the catalytic activity of PLA<sub>2</sub> at the interface itself, i.e. beyond the initial steps of enzyme adsorption and activation, and probably through modifications of the

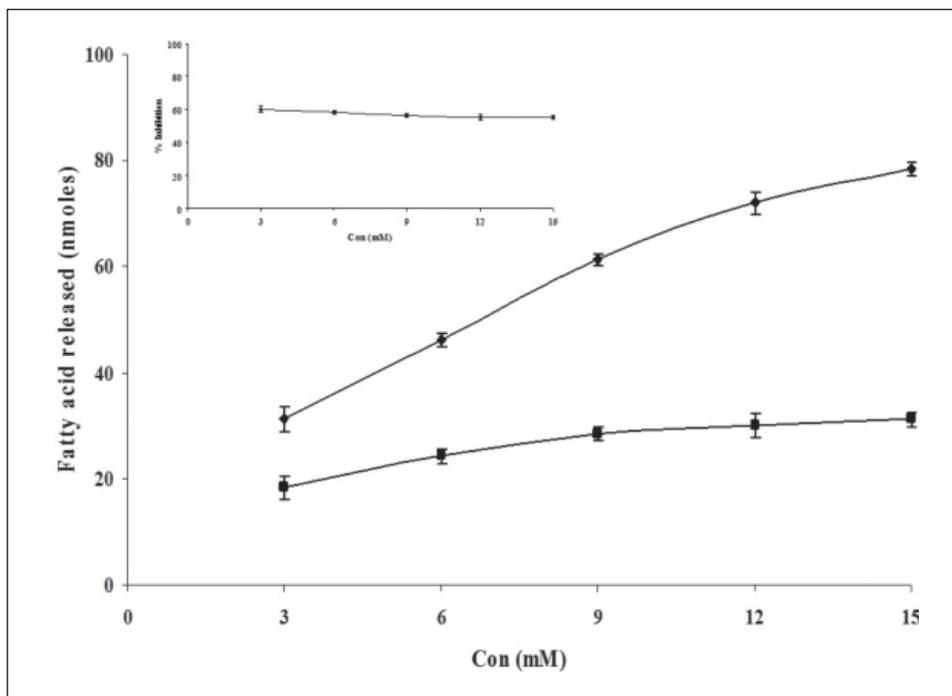


Figure 3. Dose dependent neutralization of edema inducing activity of VRV-PL-VIIIa by aqueous stem bark extract of *M. indica*. The reaction mixture 30  $\mu$ l containing VRV-PL-VIIIa (5  $\mu$ g) was incubated for 30 min with increasing concentration of aqueous stem bark extract (0-50  $\mu$ g/ml) of *M. indica*. Saline (30  $\mu$ l) injected into the mouse foot-pad served as control. Data represents  $\pm$  S.E.M for n=3.

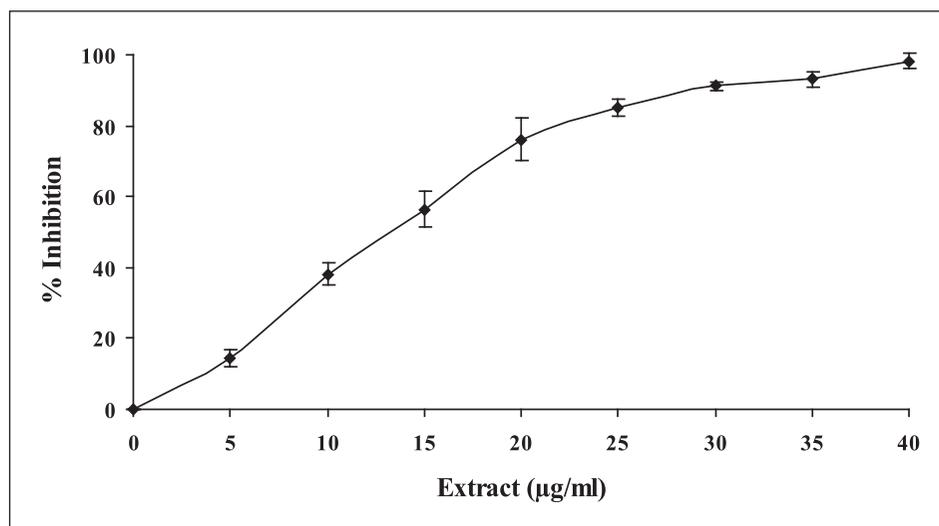


Figure 4. Effect of substrate concentration on inhibition of VRV-PL-VIIIa by aqueous stem bark extract of *M. indica*. The reaction mixture contained VRV-PL-VIIIa enzyme in 0.05 M Tri-HCl buffer pH 7.5, 500 mM CaCl<sub>2</sub>, inhibitors, and increasing concentrations of substrate. The reaction was carried out in the absence (rhombus) and presence of IC<sub>50</sub> concentration of extract (open square) with increasing concentration of substrate. The figure inset represents the percent of inhibition in the presence of IC<sub>50</sub> concentration. Data represents  $\pm$  S.E.M for n=3.

intermolecular organization of the membrane components. It is well known that secretory PLA<sub>2</sub>s cause cell membrane asymmetry by degradation of glycerol phospholipids of the membranes (Nanda *et al.*, 2007).

Many of the snake venom PLA<sub>2</sub>s induces toxic effect like edema when injected into mouse footpad as demonstrated before (Vishwanath *et al.*, 1988). Several svPLA<sub>2</sub> inhibitors are demonstrated to exhibit concomitant inhibition of enzyme activity and edema-inducing activity (Nanda *et al.*, 2007; Mohamed *et al.*, 2010). Since in the study, the aqueous extract of *M. indica* effectively inhibited the *in vitro* PLA<sub>2</sub> activity and *in situ* PLA<sub>2</sub> activity, the inhibitory potential on *in vivo* edema inducing activity of VRV-PL-VIIIa was tested. The edema inducing effect of VRV-PL-VIIIa (4 µg) was more than 178%, when compared to the saline injected mice. Figure 5 shows that aqueous extract of *M. indica*, does dependently (0-40 µg/ml) 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 ear 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 PGE<sub>2</sub> and LTB<sub>4</sub>, when it was stimulated with pro-inflammatory stimuli lipopolysaccharide-interferon gamma (LPS-IFN $\gamma$ ) 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 levels and iNOS, COX-2, TNF- $\alpha$  and TNF R-2 expression in colonic

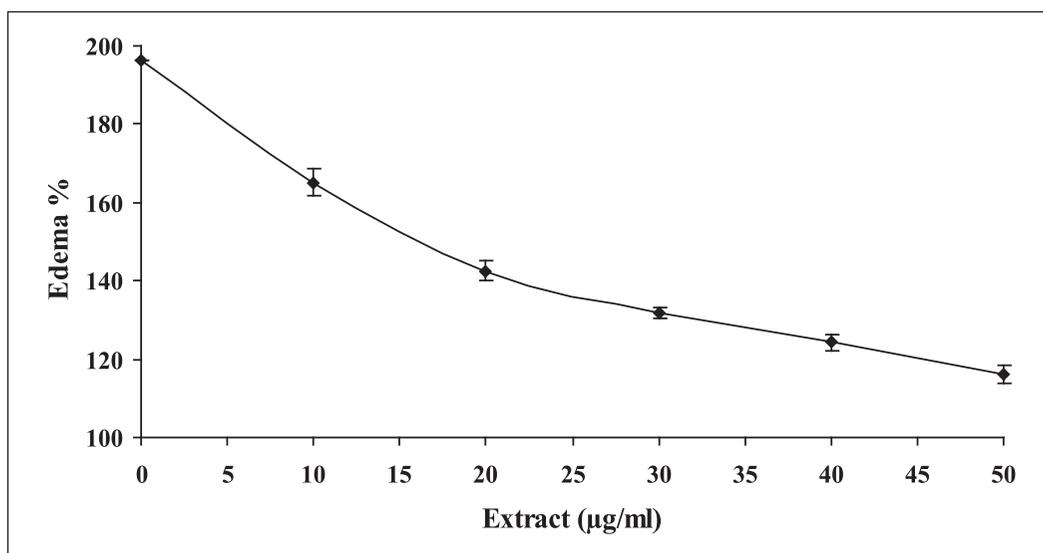


Figure 5. Effect of calcium concentration on inhibition of VRV-PL-VIIIa by aqueous stem bark extract of *M. indica*. The reaction mixture contained VRV-PL-VIIIa enzyme in 0.05 M Tri-HCl buffer pH 7.5, and 3–15mM CaCl<sub>2</sub> in final volume. The reaction was initiated by adding substrate. The reaction was carried out in the absence (rhombus) and presence of IC<sub>50</sub> concentration of extract (open square) with increasing concentration of calcium. The figure inset represents percentage inhibition in the presence of the IC<sub>50</sub> concentration. Data represents  $\pm$  S.E.M for n=3.

tissue, and a decrease in IL-6 and TNF- $\alpha$  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 VRV-PL-VIIIa when incubated with extracts (data not shown), excludes the proteolytic degradation as a potential mechanism (Borges *et al.*, 2000). Considering the binding studies, where it has been observed that the inhibition by the extract is irreversible and also independent on substrate and calcium concentration. The most likely mechanism for anti-PLA<sub>2</sub> activities by this extract could be due to the direct binding of the constituents of the extract with sPLA<sub>2</sub>s 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), and mangiferin (20%) being the predominant one. As with other polyphenols, the phenolic constituents of the extract like phenolic acids (Gallic acid, 3, 4 dihydroxy benzoic acid, benzoic acid) and phenolic esters (Gallic acidmethyl ester, gallic acid propylester, benzoic acid propyl ester), could be also involved in binding with sPLA<sub>2</sub>s, thus bringing about inhibition (Nunez-Selles *et al.*, 2002). In addition, the active constituents of this extract like mangiferin, amento flavone, friedelin, daucosterol and beta-sistosterol (Sanchez *et al.*, 2000) seem to exhibit anti-inflammatory and thus anti-snake effect through quenching of free radicals (Nanda *et al.*, 2007; Narendra *et al.*, 2007; Garrido *et al.*, 2009; Márquez *et al.*, 2012; Dhananjaya *et al.*, 2011; Vyas *et al.*, 2012). This anti-inflammatory activity of the extract was believed to be due to the powerful antioxidant activities exhibited by the constituents like phenolic compounds including mangiferin. It has been demonstrated that VIMANG<sup>®</sup> and mangiferin exhibit inhibitory activity against synovial fluid PLA<sub>2</sub> activities (Garrido *et al.*, 2004).

The aqueous stem bark extract of *M. indica* inhibiting both *in vitro* PLA<sub>2</sub> enzymatic activity and *in vivo* edema

inducing activity of VRV-PL-VIIIa, 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 subchronic toxicity, genotoxicity and irritability, and is classified as a non-toxic product (Sanchez *et al.*, 2000; González *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-snake bite formulation, which contain potent anti-snake venom molecules.

In conclusion, the aqueous extract of *M. indica* effectively inhibited svPLA<sub>2</sub> 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 possess potent anti-PLA<sub>2</sub> agents, that could be developed as a potential therapeutic agent against snake envenomations. This study also substantiates their anti-snake venom properties and further in-depth studies on the role of principal compounds present in the extract responsible for the anti-PLA<sub>2</sub> activity will be interesting, so as to develop them into formulation for tropical application during snakebite, which will supplement the conventional anti-venom therapy and help in management of snakebite.

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