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## ORIGINAL ARTICLE

# The standard aqueous stem bark extract of *Mangifera indica* L. inhibits toxic PLA<sub>2</sub> – NN-XIb-PLA<sub>2</sub> of Indian cobra venom



Bhadrapura Lakkappa Dhananjaya<sup>a,b,\*</sup>, Shivalingaiah Sudarshan<sup>a</sup>,  
Yashad Dongol<sup>c</sup>, Sunil S. More<sup>d</sup>

<sup>a</sup> Venom Research Unit, Adichunchanagiri Biotechnology and Cancer Research Institute (ABCRI), Balangadharanatha Nagara, Mandya District, Karnataka 571 448, India

<sup>b</sup> Toxinology/Toxicology and Drug Discovery Unit, Center for Emerging Technologies, Jain Global Campus, Jain University, Kanakapura Taluk, Ramanagara 562112, Karnataka, India

<sup>c</sup> Department of Biochemistry, KIST Medical College, Lalitpur, Kathmandu, Nepal

<sup>d</sup> School of Basic and Applied Sciences, Dayananda Sagar University, K.S. Layout, SM Hills, Bangalore 560078, India

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## KEYWORDS

Drug;  
Antivenom;  
Anti-snake venom;  
Pro-inflammatory;  
Inhibition;  
Extract

**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 A<sub>2</sub>s 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-PLA<sub>2</sub> of Indian cobra venom. The *in vitro* sPLA<sub>2</sub>, *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 sPLA<sub>2</sub> (NN-XIb-PLA<sub>2</sub>) activity with an IC<sub>50</sub> 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-PLA<sub>2</sub>. 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 PLA<sub>2</sub> enzyme. The aqueous extract of

\* Corresponding author at: Toxinology/Toxicology and Drug Discovery Unit, Center for Emerging Technologies, Jain Global Campus, Jain University, Kanakapura 562112, Karnataka, India. Tel.: +91 80 2757 7200 (Office), mobile: +91 8197324276; fax: +91 80 2757 7199.

E-mail address: [chandu\\_greeshma@rediffmail.com](mailto:chandu_greeshma@rediffmail.com) (B.L. Dhananjaya).

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*M. indica* effectively inhibits svPLA<sub>2</sub> enzymatic and its associated toxic activities, which substantiate their anti-snake venom properties. Further in-depth studies on the role and mechanism of the principal constituents present in the extract, responsible for the anti-PLA<sub>2</sub> activity will be interesting to develop them into potent antsnake component and also as an anti-inflammatory agent.

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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 A<sub>2</sub>, 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 A<sub>2</sub> (svPLA<sub>2</sub>s) 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 PLA<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 GIIB) 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 and 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 (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 sPLA<sub>2</sub>s 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 PLA<sub>2</sub>s in the snake envenomation, there is enormous pharmacological interest in search of sPLA<sub>2</sub> inhibitors (Narendra Sharath Chandra et al., 2007; Nanda et al., 2007; Sivaramkrishnan 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 svPLA<sub>2</sub>s from medicinal plants have gained much interest (Carvalho et al., 2013; Gomes et al., 2012). In this context, although many sPLA<sub>2</sub> 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 sPLA<sub>2</sub> 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, antinociceptive, antioxidant 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, antihepatotoxic, anticancer and larvicidal activities (Garrido-Suárez et al., 2014; Morffi et al., 2012; Rajakumar et al., 2015; Singh et al., 2015; Awad El-Gied et al., 2015; Fahmy et al., 2016; Khurana et al., 2016). 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 *Russell's viper* venom (Dhananjaya et al., 2011); however, the extract's effect on multi-toxic and lethal PLA<sub>2</sub>s 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 IA svPLA<sub>2</sub> (i.e. purified NN-XIb-PLA<sub>2</sub> phospholipase A<sub>2</sub> 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 svPLA<sub>2</sub>s 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 A<sub>2</sub> (NN-XIb-PLA<sub>2</sub>) from the venom *Naja naja*

sPLA<sub>2</sub> belonging to group IA – NN-XIb-PLA<sub>2</sub> 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 A<sub>2</sub> activity by standard aqueous stem bark extract of *M. indica* L.

The Phospholipase A<sub>2</sub> 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-PLA<sub>2</sub> (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 CaCl<sub>2</sub> (500 mM) were added. The total reaction mixture was incubated at 37°C for 60 min. After incubation, 0.5 ml of Doles mixture (Isopropanol:Pet ether:NH<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 ml 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 + 0.8 ml 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 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-PLA<sub>2</sub> (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%. IC<sub>50</sub> values were calculated using Graphpad version 5.0.

### 2.5. Effect of substrate and calcium concentration on NN-XIb-PLA<sub>2</sub> inhibition by standard aqueous stem bark extract of *M. indica* L.

Effects of substrate and calcium concentrations on inhibition of NN-XIb-PLA<sub>2</sub> (3 µg) at IC<sub>50</sub> concentration of extract were determined as follows. In general, the reaction mixture containing NN-XIb-PLA<sub>2</sub> alone and/or with the IC<sub>50</sub> 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 PLA<sub>2</sub> 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, PLA<sub>2</sub> 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-PLA<sub>2</sub> (3 µg) with the IC<sub>50</sub> 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 PLA<sub>2</sub> 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-PLA<sub>2</sub> (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-PLA<sub>2</sub> (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 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 was used for statistical evaluation.

## 3. Results and discussion

Snake venom PLA<sub>2</sub>s belonging to elapidae family are known to be multi-toxic and lethal (Doley et al., 2010; Kini, 2003). Due to the prominent role of PLA<sub>2</sub>s play in snake envenomation, there is enormous pharmacological interest in search of PLA<sub>2</sub> inhibitors (Narendra Sharath Chandra et al., 2007; Nanda et al., 2007). Further, considering the limitations of antiserum therapy (Dhananjaya et al., 2011; Girish and Kemparaju, 2011), it is justified that research has to focus on developing alternatives and in this regard finding inhibitors of the multi-toxic svPLA<sub>2</sub>s from medicinal plants have gained much interest in the recent past (Carvalho et al., 2013; Gomes et al., 2012). Although many sPLA<sub>2</sub> inhibitors have been isolated from various medicinal plants (Springer, 2001; Nanda et al., 2007; Narendra Sharath Chandra et al., 2007), however,

still effective and specific inhibitors of sPLA<sub>2</sub> are not available. In these line of studies, the aqueous steam bark extract of *M. indica* is evaluated for its potential to inhibit phospholipase A<sub>2</sub> (PLA<sub>2</sub>) belonging to group IA i.e. NN-XIb-PLA<sub>2</sub>, which was isolated from *R. viper* venom as per the previously described method (Rudrammaji and Gowda, 1998).

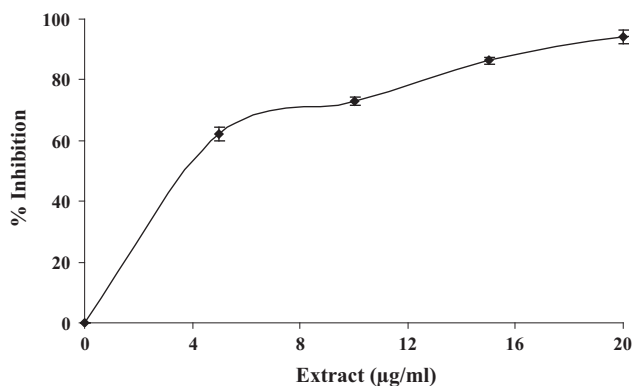
The sPLA<sub>2</sub> belonging to group IA i.e. NN-XIb-PLA<sub>2</sub> gave a specific activity of around 172.4 ± 3.1, when measured using PC as substrate (Table 1). When pre-incubated with different concentration of extract it was observed that the aqueous extract of *M. indica*, inhibited the enzymatic activity in a concentration dependent manner as shown in Fig. 1. The results show 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 were 7.6 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. Also, it is observed that the sPLA<sub>2</sub> inhibitors affect the "Quality of interface" by modifying the phospholipid bilayer properties which render the phospholipids inaccessible to the enzyme. The steroid inducible inhibitors of PLA<sub>2</sub> like lipocortin I and II are shown to inhibit PLA<sub>2</sub>s by nonspecific binding to the membrane phospholipids. It is observed that their inhibition is relieved by increasing the substrate concentration (Davidson et al., 1987). In this study, it was observed that, when examined as a function of substrate concentration, there was no relieve of inhibition of the extract pre-incubated, as the substrate concentration was increased from 20 to 120 nM (Fig. 2). This suggests that the inhibition 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 NN-XIb-PLA<sub>2</sub> 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 (Fig. 3), suggesting that the inhibition by *M. indica* extract is independent on calcium concentration. These all studies show that inhibition by aqueous extract of *M. indica* is independent on substrate and calcium concentration. Further, it is reported that some of the PLA<sub>2</sub> inhibitors are shown to mediate displacement of catalytically essential calcium from the enzyme and thus inhibition of enzymatic activity (Przanski et al., 1992). In the calcium binding studies experiments, it was found that the PLA<sub>2</sub> enzyme activity both before and after the dialysis of the enzyme inhibitor mixture was unaltered i.e., the % of inhibitory activity of *M. indica* extract was not decreased upon extensive dialysis, suggesting that the inhibition is irreversible, supporting 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/molecules present in *M. indica* extract at active site residues

**Table 1** IC<sub>50</sub> value and specific activity of NN-XIb-PLA<sub>2</sub>.

sPLA <sub>2</sub>	Specific activity <sup>a</sup>	IC <sub>50</sub> <sup>b</sup>
NN-XIb-PLA <sub>2</sub>	172.4 ± 3.1	7.6 µg/ml

<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.

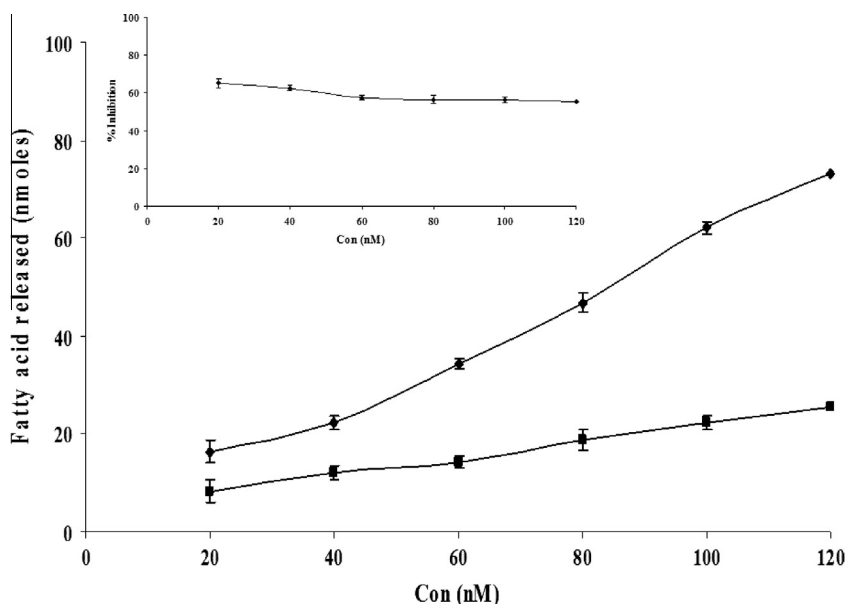


**Figure 1** Dose dependent inhibition of *in vitro* PLA<sub>2</sub> (NN-XIb-PLA<sub>2</sub>) activity by aqueous stem bark extract of *M. indica*. Briefly, Phosphatidyl choline (PC) corresponding to 1000 nmoles/ml was made up to 680 µl with NN-XIb-PLA<sub>2</sub> (5 µg), with or without aqueous stem bark extract of *M. indica* at various concentrations (0–20 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 show ±S.E.M. for *n* = 3.

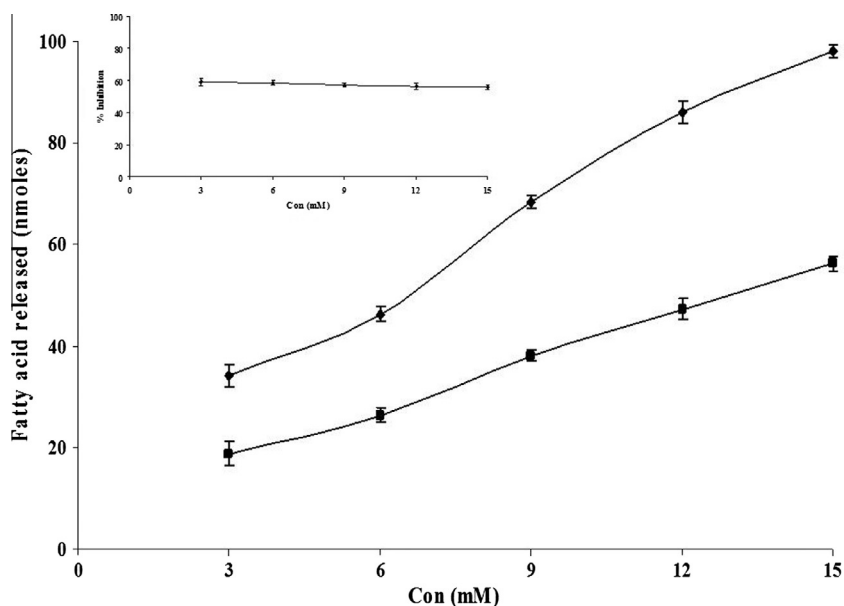
of the sPLA<sub>2</sub> enzyme. NN-XIb-PLA<sub>2</sub> enzyme exhibited indirect hemolytic activity, which is an indirect way of measuring PLA<sub>2</sub> activity using egg yolk and washed erythrocytes are used as substrates. When the effect of aqueous extract of *M. indica* at different concentrations (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* PLA<sub>2</sub> enzyme. Therefore, the inhibition of NN-XIb-PLA<sub>2</sub> 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, probably through modifications of the 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.

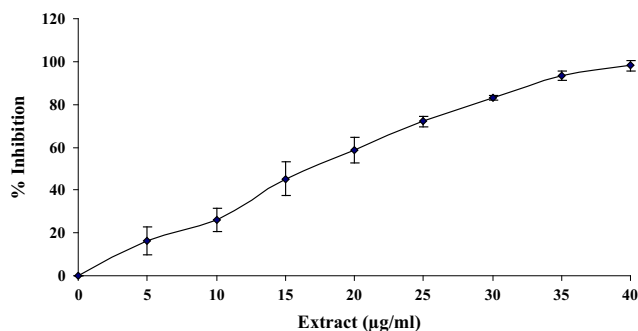
Many of the snake venom PLA<sub>2</sub>s induce toxic effect such as edema when injected into mouse footpad as demonstrated before (Vishwanath et al., 1988). Several svPLA<sub>2</sub> inhibitors are demonstrated to exhibit concomitant inhibition of enzyme activity and edema-inducing activity (Nanda et al., 2007; Mohamed et al., 2010). Since in our study, as 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 NN-XIb-PLA<sub>2</sub> was tested. The edema inducing effect of NN-XIb-PLA<sub>2</sub> (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)



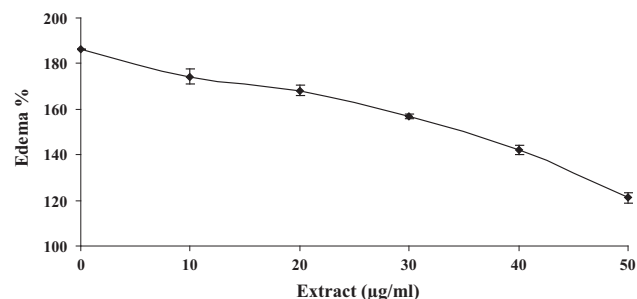
**Figure 2** Dose dependent Inhibition of indirect hemolytic activity of NN-XIb-PLA<sub>2</sub> by aqueous stem bark extract of *M. indica*. NN-XIb-PLA<sub>2</sub> (5 µg) in 100 µl of phosphate-buffered Saline (PBS) was preincubated with or without aqueous stem bark extract of *M. indica* at various concentrations (0–40 µg/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 show ±S.E.M. for *n* = 3.



**Figure 3** Dose dependent neutralization of edema inducing activity of NN-XIb-PLA<sub>2</sub> by aqueous stem bark extract of *M. indica*. The reaction mixture 30  $\mu$ l containing NN-XIb-PLA<sub>2</sub> (6  $\mu$ 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 represent  $\pm$  S.E.M. for  $n = 3$ .



**Figure 4** Effect of substrate concentration on inhibition of NN-XIb-PLA<sub>2</sub> by aqueous stem bark extract of *M. indica*. The reaction mixture contained NN-XIb-PLA<sub>2</sub> 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 represent  $\pm$  S.E.M. for  $n = 3$ .



**Figure 5** Effect of calcium concentration on inhibition of NN-XIb-PLA<sub>2</sub> by aqueous stem bark extract of *M. indica*. The reaction mixture contained NN-XIb-PLA<sub>2</sub> enzyme in 0.05 M Tri-HCl buffer pH 7.5, and 3–15 mM 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 represent  $\pm$  S.E.M. for  $n = 3$ .

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 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 elec-

trophoretic pattern of NN-XIb-PLA<sub>2</sub> 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-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), mangiferin (20%) being the predominant one. As with other polyphenols, the phenolic

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 sPLA<sub>2</sub>s, 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; 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 such as phenolic compounds including mangiferin. It has been demonstrated that VIMANG® 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 NN-XIb-PLA<sub>2</sub>, 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; 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-snakebite 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 possesses potent anti-PLA<sub>2</sub> 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-PLA<sub>2</sub> 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-PLA<sub>2</sub> activity can be carried out to develop them into potent anti-snake component and also as an anti-inflammatory agent.

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#### References

- Aird, S.D., 2002. Ophidian envenomation strategies and the role of purines. *Toxicon* 40, 335–393.
- Awad El-Gied, A.A., Abdelkareem, A.M., Hamedelniei, E.I., 2015. Investigation of cream and ointment on antimicrobial activity of *Mangifera indica* extract. *J. Adv. Pharm. Technol. Res.* 6 (2), 53–57.
- Bhat, M.K., Gowda, T.V., 1989. Purification and characterization of a myotoxic phospholipase A<sub>2</sub> from Indian cobra (*Naja naja naja*) venom. *Toxicon* 27, 861–873.
- Boman, H.G., Kaletta, U., 1957. Chromatography of rattle snake venom, a separation of three phosphodiesterases. *Biochim. Biophys. Acta* 24, 619–631.
- Borges, M.H., Soares, A.M., Rodrigues, V.M., Andrio-Escarso, S.H., Diniz, et al, 2000. Effects of aqueous extract of *Casearia sylvestris* (Flacourtiaceae) on actions of snake venom and bee venoms and on activity of phospholipase A<sub>2</sub>. *Comp. Biochem. Physiol. Biochem. Mol. Biol.* 127, 21–30.
- Burke, J.E., Dennis, E.A., 2009. Phospholipase A<sub>2</sub> structure/function, mechanism, and signaling. *J. Lipid Res.* 50, S237–S242.
- Carvalho, B.M., Santos, J.D., Xavier, B.M., Almeida, J.R., Resende, L.M., et al, 2013. Snake venom PLA<sub>2</sub>s inhibitors isolated from Brazilian plants: synthetic and natural molecules. *Biomed. Res. Int.* 2013, 153045.
- Chopra, R.N., Nayar, S.L., Chopra, I.C., 1956. Glossary of Indian Medicinal Plants. CSIR Publication, New Delhi.
- Coe, F.G., Anderson, G.J., 1996. Screening of medicinal plants used by the Garifuna of eastern Nicaragua for bioactive compounds. *J. Ethnopharmacol.* 53, 29–50.
- Davidson, F.F., Dennis, E.A., Powell, M., Glenney Jr., J.R., 1987. Inhibition of phospholipase A2 by “lipocortins” and calpactins. An effect of binding to substrate phospholipids. *J. Biol. Chem.* 262, 1698–1705.
- Dhananjaya, B.L., Vishwanath, B.S., D’Souza, C.J., 2010. Snake venom nucleases, nucleotidases and phosphomonoesterase. In: Mackessy, S.P. (Ed.), *Handbook of Venoms and Toxins of Reptiles*. CRC Press/Taylor and Francis, Boca Raton, Florida, USA, pp. 155–172.
- Dhananjaya, B.L., Zameer, F., Girish, K.S., D’Souza, C.J., 2011. Anti-venom potential of aqueous extract of stem bark of *Mangifera indica* L. against *Daboia russelii* (Russell’s viper) venom. *Indian J. Biochem. Biophys.* 48 (3), 175–183.
- Dhananjaya, B.L., Shivalingaiah, S., 2016. The anti-inflammatory activity of standard aqueous stem bark extract of *Mangifera indica* L. as evident in inhibition of Group IA sPLA<sub>2</sub>. *An. Acad. Bras. Cienc.* 88 (1), 197–209.
- Doley, R., Zhou, X., Kini, R.M., 2010. Snake venom phospholipase A<sub>2</sub> enzymes. In: Mackessy, S.P. (Ed.), *Handbook of Venoms and Toxins of Reptiles*. CRC Press, Boca Raton, FL, USA, pp. 173–205.
- Fahmy, S.R., Amien, A.I., Abd-Elgleel, F.M., Elaskalany, S.M., 2016. Antihepatotoxic efficacy of *Mangifera indica* L. polysaccharides against cyclophosphamide in rats. *Chem. Biol. Interact.* 244, 113–120.
- Garrido, G., González, D., Lemus, Y., Delporte, C., Delgado, R., 2006. Protective effects of a standard extract of *Mangifera indica* L. (VIMANG) against mouse ear edemas and its inhibition of eicosanoid production in J7774 murine macrophages. *Phytomedicine* 13, 412–418.
- Garrido, G., Gonzalez, D., Lemus, Y., Garcia, D., Lodeiro, L., Quintero, G., Delporte, C., Nunez-selles, A.J., Delgado, R., 2004.

- In vivo and in vitro anti-inflammatory activity of *Mangifera indica* L. extract (VIMANG). *Pharmacol. Res.* 50, 143–149.
- Garrido, G., Rodeiro, I., Hernández, I., García, G., et al, 2009. In vivo acute toxicological studies of an antioxidant extract from *Mangifera indica* L. (Vimang). *Drug Chem. Toxicol.* 32, 53–58.
- Garrido-Suárez, B.B., Garrido, G., García, M.E., Delgado-Hernández, R., 2014. Antihyperalgesic effects of an aqueous steam bark extract of *Mangifera indica* L.: role of mangiferin isolated from the extract. *Phytother. Res.* 28, 1646–1653.
- Girish, K.S., Kemparaju, K., 2011. Overlooked issues of snakebite management: time for strategic approach. *Curr. Top. Med. Chem.* 11, 2494–2508.
- Gomes, A., Bhattacharya, S., Mukherjee, S., Inn-ho-Tsai, Gomes, A., 2012. Inhibition of toxic actions of phospholipase A<sub>2</sub> isolated & characterized from the Indian Branded Krait (*Bungarus fasciatus*) venom by synthetic herbal compounds. *Indian J. Med. Res.* 136, 40–45.
- González, J.E., Rodríguez, M.D., Rodeiro, I., Morffi, J., Guerra, E., et al, 2007. Lack of in vivo embryotoxic and genotoxic activities of orally administered stem bark aqueous extract of *Mangifera indica* L. (Vimang). *Food Chem. Toxicol.* 45, 2526–2532.
- Guevara, G.M., Tamayo, B.D., Gonzalez, L.S., Paez, B.E., Ocegüera, J., Nunez-Selles, A.J., 1998. Ethnopharmacologic study of *Mangifera indica* L. extract (QF-808). In: Abstract of 1<sup>st</sup> International Congress Pharmacology'98. ORTOP, La Habana, p. 45.
- Kasturiratne, A., Wickremasinghe, A.R., de Silva, N., Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Laloo, D. G., de Silva, H.J., 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS Med.* 5 (11), e218. <http://dx.doi.org/10.1371/journal.pmed.0050218>.
- Kini, R., Chan, Y., 1999. Accelerated evolution and molecular surface of venom phospholipase A<sub>2</sub> enzymes. *J. Mol. Evol.* 48, 125–132.
- Kini, R.M., 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A<sub>2</sub> enzymes. *Toxicon* 42, 827–840.
- Koh, D.C.I., Armugam, A., Jeyaseelan, K., 2006. Snake venom components and their applications in biomedicine. *Cell. Mol. Life Sci.* 63, 3030–3041.
- Khurana, R.K., Kaur, R., Lohan, S., Singh, K.K., Singh, B., 2016. Mangiferin: a promising anticancer bioactive. *Pharm. Pat. Anal.* 5 (3), 169–181.
- Lomonte, B., Angulo, Y., Sasa, M., Gutiérrez, J.M., 2009. The phospholipase A<sub>2</sub> homologues of snake venoms: biological activities and their possible adaptive roles. *Protein Pept. Lett.* 16, 860–876.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Makare, N., Bodhankar, S., Rangari, V., 2001. Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice. *J. Ethnopharmacol.* 78, 133–137.
- Márquez, L., García-Bueno, B., Madrigal, J.L., Leza, J.C., 2012. Mangiferin decreases inflammation and oxidative damage in rat brain after stress. *Eur. J. Nutr.* 51, 729–739.
- Márquez, L., Pérez-Nievas, B.G., Gárate, I., García-Bueno, B., et al, 2010. Antiinflammatory effects of *Mangifera indica* L. extract in a model of colitis. *World J. Gastroenterol.* 16, 4922–4931.
- Mohamed, R., Dharmappa, K.K., Tarannum, S., Jameel, N.M., et al, 2010. Chemical modification of ascorbic acid and evaluation of its lipophilic derivatives as inhibitors of secretory phospholipase A(2) with anti-inflammatory activity. *Mol. Cell. Biochem.* 345, 69–76.
- Morffi, J., Rodeiro, I., Hernández, S.L., González, L., Herrera, J., Espinosa-Aguirre, J.J., 2012. Antimutagenic properties of *Mangifera indica* L. steam bark extract and evaluation of its effects on hepatic CYP1A1. *Plant Foods Hum. Nutr.* 67, 223–228.
- Nanda, B.L., Nataraju, A., Rajesh, R., Rangappa, K.S., Shekar, M. A., Vishwanath, B.S., 2007. PLA<sub>2</sub> mediated arachidonate free radicals: PLA<sub>2</sub> inhibition and neutralization of free radicals by anti-oxidants—a new role as anti-inflammatory molecule. *Curr. Top. Med. Chem.* 7, 765–777.
- Narendra Sharath Chandra, J.N., Ponnappa, K.C., Sadashiva, C.T., Priya, B.S., Nanda, B.L., Gowda, T.V., Vishwanath, B.S., Rangappa, K.S., 2007. Chemistry and structural evaluation of different phospholipase A<sub>2</sub> inhibitors in arachidonic acid pathway mediated inflammation and snake venom toxicity. *Curr. Top. Med. Chem.* 7 (8), 787–800.
- Nevalainen, T.J., Cardoso, J.C., Riikonen, P.T., 2012. Conserved domains and evolution of secreted phospholipases A(2). *FEBS J.* 279, 636–649.
- Nunez-Selles, A.J., Velez, H.T., Agüero, J., Gonzalez, J., Naddeo, F., De Simone, F., Rastrelli, L., 2002. Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from Mango (*Mangifera indica* L.) stem bark aqueous decoction used in Cuba as a nutritional supplement. *J. Agric. Food Chem.* 50, 762–766.
- Ojewole, J.A., 2005. Antiinflammatory, analgesic and hypoglycemic effects of *Mangifera indica* linn. (Anacardiaceae) stem-bark aqueous extract. *Methods Find. Exp. Clin. Pharmacol.* 2, 547–554.
- Pruzanski, W., Greenwald, R.A., Street, I.P., Laliberte, F., Stefanski, E., Vadas, P., 1992. Inhibition of enzymatic activity of phospholipases A<sub>2</sub> by minocycline and doxycycline. *Biochem. Pharmacol.* 44, 1165–1170.
- Rajakumar, G., Rahuman, A.A., Roopan, S.M., Chung, I.M., Anbarasan, K., Karthikeyan, V., 2015. Efficacy of larvicidal activity of green synthesized titanium dioxide nanoparticles using *Mangifera indica* extract against blood-feeding parasites. *Parasitol. Res.* 114, 571–581.
- Rudrammaji, L.M., Gowda, T.V., 1998. Purification and characterization of three acidic, cytotoxic phospholipase A<sub>2</sub> from Indian cobra (*Naja naja*) venom. *Toxicon* 36, 921–932.
- Sanchez, G.M., Re, L., Giuliani, A., Nunez-Selles, A.J., Davison, G. P., Leon-Fernandez, O.S., 2000. Protective effects of *Mangifera indica* L. extract, mangiferin and selected antioxidants against TPA-induced biomolecules oxidation and peritoneal macrophage activation in mice. *Pharmacol. Res.* 42, 565–573.
- Shah, K.A., Patel, M.B., Patel, R.J., Parmar, P.K., 2010. *Mangifera indica* (Mango). *Pharmacogn. Rev.* 4, 42–48.
- Singh, R., Singh, S.K., Maharia, R.S., Garg, A.N., 2015. Identification of new phytoconstituents and antimicrobial activity in stem bark of *Mangifera indica* (L.). *J. Pharm. Biomed. Anal.* 105, 150–155.
- Sivaramakrishnan, V., Ilamathi, M., Ghosh, K.S., Sathish, S., Gowda, T.V., Vishwanath, B.S., Rangappa, K.S., Dhananjaya, B.L., 2016. Virtual analysis of structurally diverse synthetic analogs as inhibitors of snake venom secretory phospholipase A<sub>2</sub>. *J. Mol. Recognit.* 29, 22–32.
- Springer, D.M., 2001. An update of inhibitors of human 14 kDa type II s-PLA<sub>2</sub> in development. *Curr. Pharm. Des.* 7, 181–198.
- Vishwanath, B.S., Fawzy, A.A., Franson, R.C., 1988. Edema inducing activity of phospholipase A<sub>2</sub> purified from human synovial fluid and inhibition by aristolochic acid. *Inflammation* 12, 549–561.
- Vishwanath, B.S., Kini, R.M., Gowda, T.V., 1987. Characterization of three edema inducing phospholipase A<sub>2</sub> enzymes from habu (*Trimeresurus flavoviridis*) venom and their interaction with the alkaloid aristolochic acid. *Toxicon* 25, 501–515.
- Vyas, A., Syeda, K., Ahmad, A., Padhye, S., Sarkar, F.H., 2012. Perspectives on medicinal properties of mangiferin. *Mini Rev. Med. Chem.* 12, 412–425.
- Warrell, D.A., 2010. Snake bite. *Lancet* 375, 77–88.
- Yamakawa, M.M., Hokama, N.Z., 1976. Fractionation of Sakishima habu (*T. elegans*) venom and lethal hemorrhagic and edema forming activity of the fractions. In: Ohsaka, A., Hayashi, K., Sawai, Y. (Eds.), . In: *Animal, Plant and Microbial Toxins*, vol. 1. Plenum Press, New York, p. 97.