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The anti-inflammatory activity of standard aqueous stem bark extract of *Mangifera indica* L. as evident in inhibition of Group IA $sPLA_2$

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ABSTRACT

The standard aqueous stem bark extract is consumed as herbal drink and used in the pharmaceutical formulations to treat patients suffering from various disease conditions in Cuba. This study was carried out to evaluate the modulatory effect of standard aqueous bark extract of *M. indica* on Group IA $sPLA_2$. *M. indica* extract, dose dependently inhibited the GIA $sPLA_2$ (NN-XIa-PLA_2) activity with an IC_{50} value 8.1 µg/ml. *M. indica* extract effectively inhibited the indirect hemolytic activity up to 98% at ~40 µg/ml concentration and at various concentrations (0-50 µg/ml), it dose dependently inhibited the edema formation. When examined as a function of increased substrate and calcium concentration, there was no relieve of inhibitory effect on the GIA $sPLA_2$. Furthermore, the inhibition was irreversible as evidenced from binding studies. It is observed that the aqueous extract of *M. indica* effectively inhibits $sPLA_2$ and it is associated inflammatory activities, which substantiate their anti-inflammatory properties. The mode of inhibition could be due to direct interaction of components present in the extract, with $sPLA_2$ enzyme. Further studies on understanding the principal constituents, responsible for the anti-inflammatory activity would be interesting to develop this into potent anti-inflammatory agent.

Key words: secretory phospholipase A_2, anti-inflammatory, pro-inflammatory, inhibition, drug, plants.

INTRODUCTION

Phospholipases A_2 (PLA_2) (PLA_2, EC 3.1.1.1.4) are a family of key enzymes, that cleaves fatty acids at the *sn*-2 position of glycerol phospholipids to liberate free fatty acid and lysophospholipid (Nanda et al. 2007, Burke and Dennis 2009). The released free fatty acid i.e., arachidonic acid, is known to liberate potent and short-lived pro-inflammatory mediators such as prostaglandins, thromboxanes, prostacyclins and leukotrienes; by the action of cyclooxygenase (COX) and lipooxygenase (LOX) (Nanda et al. 2007, Vadas and Pruzanski 1984, Diaz and Arm 2003). The other $sPLA_2$ action product -lysophospholipid that is liberated has also been demostrated to serve as a substrate for the synthesis of pro-inflammatory platelet activating factors (PAF) (Burke and Dennis 2009, Diaz and Arm 2003). Thus, it can be concluded that the release of archidonic acid

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from membrane lipids is the first regulatory and obligatory step in the synthesis of pro-inflammatory mediators in inflammatory. This arachidonic acid metabolic pathway is known to be regulated mainly by secretory phospholipase A_2, suggesting the importance of enzyme in inflammation related processes (Nanda et al. 2007). In snake venoms, only two groups of $sPLA_2$s (GI and GII) have been identified. Group I (GIA) includes the $svPLA_2$s from *Elapinae* and *Hydrophiinae* venoms with 115–120 amino acid residues and these $svPLA_2$s are homologous to mammalian pancreatic GIB $sPLA_2$. Group II (GIIA and GIIB) comprises the $svPLA_2$s from *Crotalinae* and *Viperinae* venoms with 120–125 amino acid residues and homologous to mammalian non-pancreatic Group II-A $sPLA_2$ (Burke and Dennis 2009). Group II PLA_2s 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 subgroups described above exhibit a wide variety of physiological and pathological effects including the inflammatory one (Doley et al. 2010, Kini 2003).

An increased level of $sPLA_2$ are found in inflammatory exudates such as synovial fluid, pleural fluid, ascitis fluid (Bomalaski and Clark 1993) and also in plasma after an inflammatory challenge (Wright et al. 1990). In addition, an excess level of $sPLA_2$s is found to be associated with many physiopathological processes such as in cerebral illnesses, cardiovascular disorders, cancers, asthma, respiratory distress syndrome, and progression of tonsillitis (Mallat et al. 2010, de Luca et al. 2011, Chalbot et al. 2011, Farooqui et al. 1999). Furthermore, an increased level of $sPLA_2$ activity is also observed in some brain tumors, in chronic neurological disorders associated with neurodegenerative diseases such as neural trauma, Alzheimer's, and Parkinson's diseases (Farooqui et al. 1999, Sun et al. 2004). Several studies have demonstrated that the $sPLA_2$ inhibitors suppress inflammatory processes, which provides a strong evidence for their role in pathogenesis in inflammation (Nanda et al. 2007, Moses et al. 2006, Balsinde et al. 1999, Granata et al. 2003, Kim et al. 2004, Rosenson et al. 2010). It has been observed that effective inhibitors of $sPLA_2$ are known to suppress the inflammation and its associated aspects (Nanda et al. 2007, Meyer et al. 2005, Narendra Sharath Chandra et al. 2007). Considering the role of $sPLA_2$s in the inflammatory process, there is considerable pharmacological interest in finding potent and specific $sPLA_2$ inhibitors from diverse sources (Nanda et al. 2007, Narendra Sharath Chandra et al. 2007). In addition, considering the drawbacks and severe side effects exhibited by the current anti-inflammatory therapies (non-steroidal anti-inflammatory drugs) targets either LOX or COX-1/2 enzymes (Vane and Botting 1998), Therefore, it appears rational to develop or find effective inhibitors of $sPLA_2$ which could deplete the downstream pro-inflammatory metabolites of arachidonic acid as well as PAF. Many plant extracts and its constituents are reported for their anti-inflammatory activity through inhibition of $sPLA_2$s (Nanda et al. 2007, Narendra Sharath Chandra et al. 2007, Springer 2001); effective and specific inhibitors of $sPLA_2$ are not available to date. Many studies have shown that $sPLA_2$s (of Group I) have a very definite role in pathogenesis of inflammation (Granata et al. 2003, Kim et al. 2004, Rosenson et al. 2010), therefore, it would be beneficial to find plant extract/compounds exhibiting inhibitory activity against group I $sPLA_2$, this would be of potential therapeutic relevance in many inflammatory diseased state (Scott et al. 2010, Dessì et al. 2013, Magrioti and Kokotos 2013, Slesser et al. 2013).

Mangifera indica L. (Anacardiaceae), also known as mango, aam, grows in the tropical and sub tropical regions and is one of the most popular edible fruit yielding trees in 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 aqueous stem bark extract of *M. indica* obtained by decoction has been traditionally used for the treatment of menorrhagia, scabies, diarrhea, syphilis, deabetes, cutaneous infections and anemia, as reported in the Naprolert database (Coe and Anderson 1996, Shah et al. 2010). This standard aqueous stem bark extract has been used in pharmaceutical formulations in Cuba under the brand name VIMANG®, to treat patients suffering from increased stress (Guevara et al. 1998). Pharmacological studies have indicated that VIMANG® has immunomodulatory, analgestic, antinociceptive, antioxidant and anti-inflammatory effects (Makare et al. 2001, Garrido et al. 2004). Previously, the standard aqueous extract was demonstrated to inhibit the edema induced by carrageen and formalin in mice, rats and guinea-pigs (Garrido et al. 2001, Ojewole 2005). Even though, *M. indica* extract is known to possess anti-inflammatory activities, no study has been conducted with regard to $sPLA_2$ enzyme (main regulatory enzyme in arachidonic acid pathway) inhibition in support of its anti-inflammatory effects. Therefore, in this study, for the first time we report the investigations carried out to evaluate the modulatory effect of standard aqueous extract of *M. indica* on Group IA $sPLA_2$ (i.e., purified NN-XIa-PLA_2 phospholipase A_2 enzyme from *Naja naja* venom), to substantiate their anti-inflammatory properties. Furthermore, this study provides an insight on the possible biochemical interaction of extract/components to bring about inhibition of $sPLA_2$s and its inflammatory process.

MATERIALS AND METHODS

Venom of *Naja naja* (Indian Cobra) 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 campus of the university, which 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 in accordance 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 Wistar albino mice weighing about 20-25 g were obtained from the central animal house facility. All protocols of animal experiments were approved by the Sri Adichunchangiri 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.

Isolation of Group IA Secretary Phospholipase A_2 (NN-XIa-PLA_2)

$sPLA_2$ belonging to the group IA- NN-XIa-PLA_2 from the venom of *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.

Inhibition of Phospholipase A_2 Activity

The Phospholipase A_2 assay was carried out according to the method described by Bhat and Gowda (1989). Phosphatidyl choline (PC) was diluted with petroleum ether (60 – 80 °C) to a

concentration of 1000 nmoles/50 ml. The reaction mixture containing NN-XIa-PLA_2 (5 µg) was made up to 680 ml with water. To the reaction mixture, 200 µl of ether, 100 µl of Tris – HCl buffer (0.05M, pH 7.5), and 20 µl of $CaCl_2$ (500 mM) was added. The total reaction mixture was incubated at 37 °C for 60 min. After incubation, 0.5 ml of Doles mixture (Isopropanol: Pet ether: $1NH_2SO_4$, 40:10:1) was added, mixed and centrifuged at 1000 rpm for 3 min. To the organic phase 0.5 ml of $CHCl_3$: 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 $CO(NO_3)2.- 6H_2O$ + 0.8 ml glacial acetic acid) and 7 ml of solution B (Saturated Na_2SO_4)] was added, mixed and centrifuged at 1000 rpm for 3 min. The upper organic phase was carefully transferred and 0.75 ml of a-nitroso-b-naphthol reagent (0.4% a-nitroso-b-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 the standard linolenic acid curve. Enzyme activity was expressed as nmol of fatty acid released/min/mg of protein.

For inhibition studies, NN-XIa-PLA_2 (5 µg) was preincubated with or without different concentrations of aqueous extract of *M. indica* (0 - 20 µg/ml) at 37 °C for 15 minutes. Appropriate controls were carried out and further experiments were conducted as described above. The inhibition is expressed as percentage (%) taking activity of venom alone as 100%. IC_{50} values were calculated using Graphpad version 5.0.

Effect of Substrate and Calcium Concentration on NN-XIa-PLA_2 Inhibition by Aqueous Extract of *M. Indica*

Effects of substrate and calcium concentrations on the inhibition of NN-XIa-PLA_2 (5 µg) at IC_{50} concentration of extract were determined. In general, the reaction mixture containing NN-XIa-PLA_2 alone and/or with the IC_{50} 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_2 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 with concentrations ranging from 0 to 15 mM in the final reaction mixture. After the reaction time, PLA_2 assay was carried out as described above.

Determination of Binding Characteristics and Reversibility of Inhibition by *M. Indica*

The reaction mixture containing NN-XIa-PLA_2 (5 µg) with the IC_{50} concentration of aqueous extract of *M. indica* in 0.05M Tris- HCl buffer, pH 7.5, and 40mM calcium were pre-incubated for 15 min. Following the reaction mixture was dialyzed against 1000 ml of 100mM 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) for 24 h with three buffer changes. The PLA_2 activity was assayed before and after dialysis as described above.

Neutralization of Indirect Hemolytic Activity

Indirect hemolytic activity was assayed as described by Boman and 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 NN-XIa-PLA_2 (4 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 of distilled water is taken as 100% hydrolysis. 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 and Hokama (1976) as modified by Vishwanath et al. (1987) was followed. NN-XIa-PLA_2 (6 µg) was preincubated without and/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 the 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 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 edema 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 of protein into footpads of mice and sacrificing them at regular periods of time, provided time-course curve of edema inducing activity. Edema ratio was calculated and expressed as %.

Statistical Analyses

The IC_{50} 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

$sPLA_2$ are groups of enzymes that are the main regulating enzymes in arachidonic acid pathway-derived pro-inflammatory mediators release, and thus play an important role in inflammatory processes (Nanda et al. 2007, Vadas and Pruzanski 1984). Considering the drawbacks and the severe side effects exhibited by current anti-inflammatory therapies that include the non-steroidal anti-inflammatory drugs that inhibit either of the LOX or COX-1/2 enzymes (Vane and Botting 1998). It appears rational to develop effective inhibitors of $sPLA_2$ that could deplete the downstream pro-inflammatory metabolites of arachidonic acid as well as PAF (Nanda et al. 2007). It has been observed that effective inhibitors of $sPLA_2$ are known to suppress the inflammation and its associated process (Nanda et al. 2007), thus there is renound interest among the researchers around the world for the search of new potent and specific $sPLA_2$ inhibitors from different sources. Many $sPLA_2$ inhibitors have been isolated from various medicinal plants (Nanda et al. 2007, Narendra Sharath Chandra et al. 2007, Springer 2001), however, effective and specific inhibitors of $sPLA_2$ are still not available. In these line of studies, the aqueous steam bark extract of *M. indica* is evaluated for its anti-inflammatory potential by inhibiting inflammatory phospholipase A_2 (PLA_2) belonging to group IA i.e., NN-XIa-PLA_2, which is isolated from *Naja naja* venom as per the previously described method of Rudrammaji and Gowda (1989). The $sPLA_2$ belonging to group IA i.e., NN-XIa-PLA_2 gave a specific activity of around 183.6±4.6, when measured using PC as substrate (Table I). When preincubated with different concentrations 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 result shows that the extent of inhibition was >95% at 40 µg/ml of extract used. The IC_{50} values

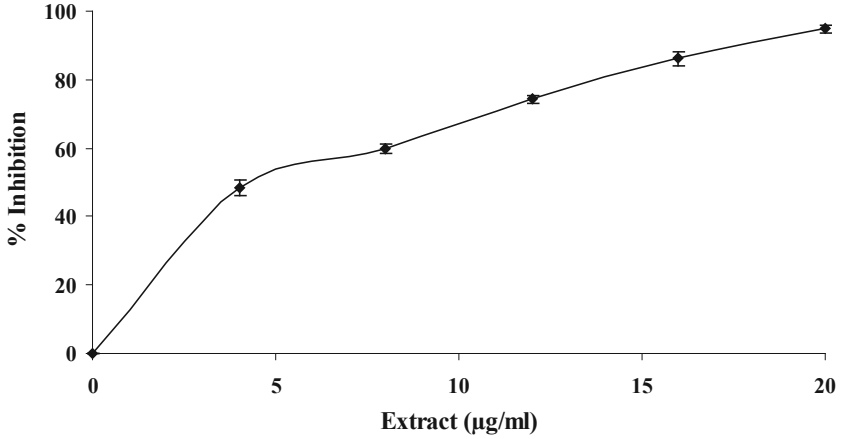

Figure 1 - Dose dependent inhibition of *in vitro* PLA_2 (NN-XIa-PLA_2) activity by aqueous stem bark extract of *M. indica*. Briefly, Phosphatidyl choline (PC) corresponding to 1000 nmol/ml was made up to 680 µl with NN-XIa-PLA_2 (5 µg), with or without aqueous stem bark extract of *M. indica* at various 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.

calculated was 8.1 µg/ml (Table I). Most of the $sPLA_2$ 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). Furthermore, it was observed that the $sPLA_2$ inhibitors affect the "Quality of interface" by modifying the phospholipids bilayer properties which render the phospholipids inaccessible to the enzyme. The steroid inducible inhibitors of $sPLA_2$ like lipocartin I and II are shown to inhibit by non-specific binding to the membrane phospholipids. It is also observed that their inhibition is relieved by increasing the substrate concentration (Davidson et al. 1987). In our experiments, we observed that, when examined as a function of substrate concentration, there was no relieve of inhibition of the extract preincubated, and also when the substrate concentration was increased from 20 to 120 nM (Fig. 2). This suggests that the inhibition is independent of substrate concentration. Furthermore, in the calcium dependent activity test, it was observed that an increase in calcium concentration from 2.5 to 15 mM, increased the NN-XIa-PLA_2 enzymatic activity in a dose dependent manner. However, while when IC_{50} concentration of *M. indica* extract was preincubated and then used along with varying concentrations of calcium, there was no relieve of inhibition (Fig. 3), suggesting that the inhibition by *M. indica* extract is independent on calcium concentration. All these studies show that inhibition by aqueous extract of *M. indica* is independent on substrate and calcium concentration. Furthermore, it is reported that some of the $sPLA_2$ inhibitors have been known to mediate displacement of catalytically essential calcium from the enzyme and consequently inhibition of enzymatic activity (Pruzanski et al. 1992). In the calcium binding experiments, it was found that the PLA_2 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

TABLE I
IC_{50} value and specific activity of NN-XIa-PLA_2.

$sPLA_2$	Specific activity [a]	IC_{50} [b]
NN-XIa-PLA_2	183.6±4.6	8.1 µg/ml

[a] nmol of fatty acid released/mg of protein/min at 37 °C.
[b] IC_{50} value is defined as the amount of extract (µg/ml) required to inhibit 50% of enzyme activity in the given reaction mixture.

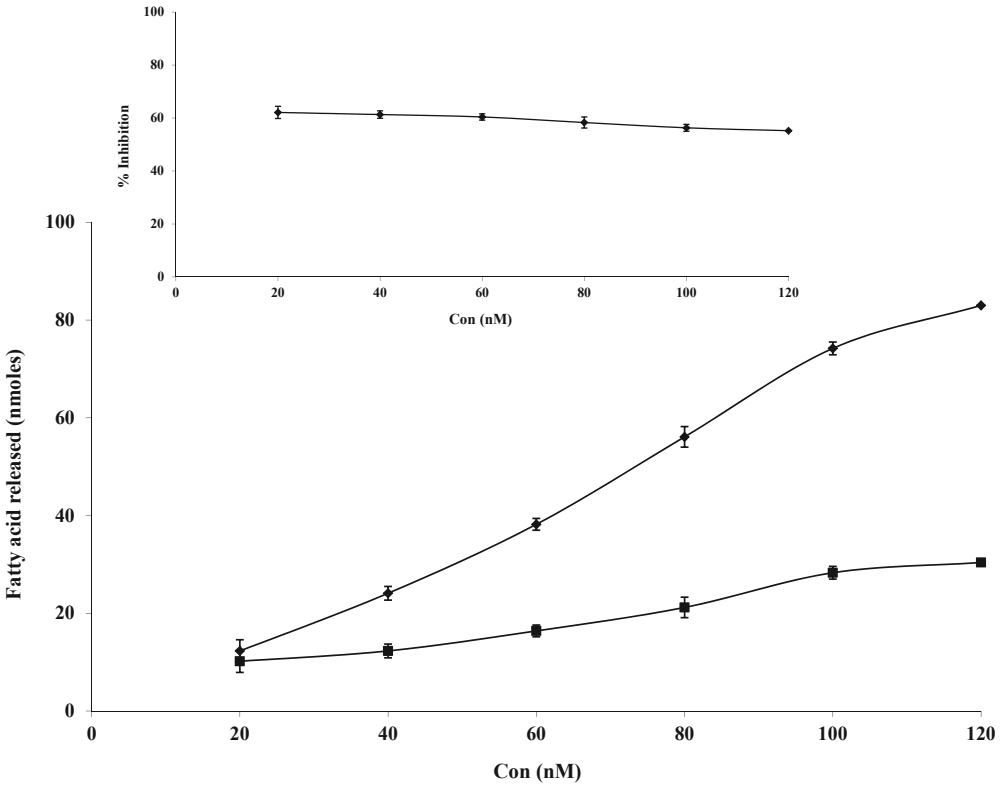

Figure 2 - Dose dependent inhibition of indirect hemolytic activity of NN-XIa-PLA$_2$ by aqueous stem bark extract of *M. indica*. NN-XIa-PLA$_2$ (4 µg) in 100 µl of phosphate-buffered Saline (PBS) was preincubated with or without aqueous stem bark extract of *M. indica* at various concentrations (0-40 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.

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 of the sPLA$_2$ enzyme.

NN-XIa-PLA$_2$ enzyme exhibited indirect hemolytic activity, which is an indirect way of measuring PLA$_2$ activity using egg yolk and washed erythrocytes which is 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$_2$ enzyme. Therefore, the inhibition of NN-XIa-PLA$_2$ activity by molecules in *M. indica* extract could be attributed to the modulation of the catalytic activity of PLA$_2$ 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 sPLA$_2$s cause cell membrane asymmetry by degradation of glycerol phospholipids of the membranes (Granata et al. 2003).

Many of the inflammatory sPLA$_2$s induce edema (inflammatory reactions) when injected into mouse footpad as demonstrated before

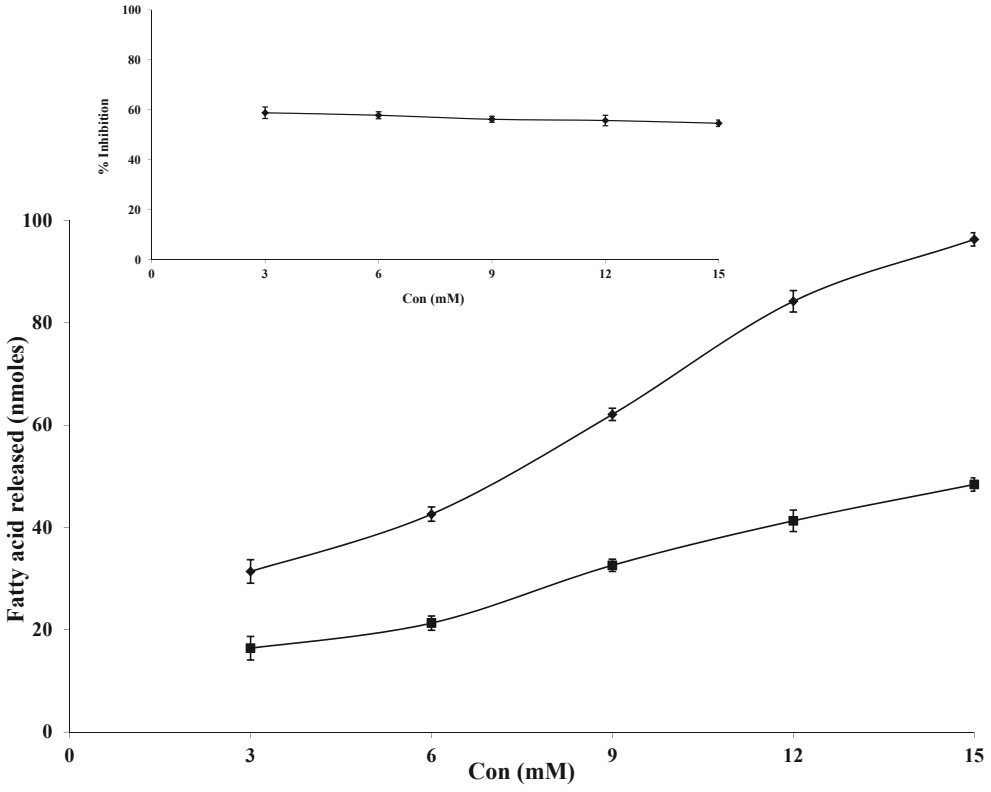

Figure 3 - Dose dependent neutralization of edema inducing activity of NN-XIa-PLA$_2$ by aqueous stem bark extract of *M. indica*. The reaction mixture 30 µl containing NN-XIa-PLA$_2$ (6 µgs) was incubated for 30 min with increasing concentration of aqueous stem bark extract (0-50 µg/ml) of *M. indica*. Saline (30 µl) injected into the mouse foot pad served as control. Data represents ± S.E.M for n=3.

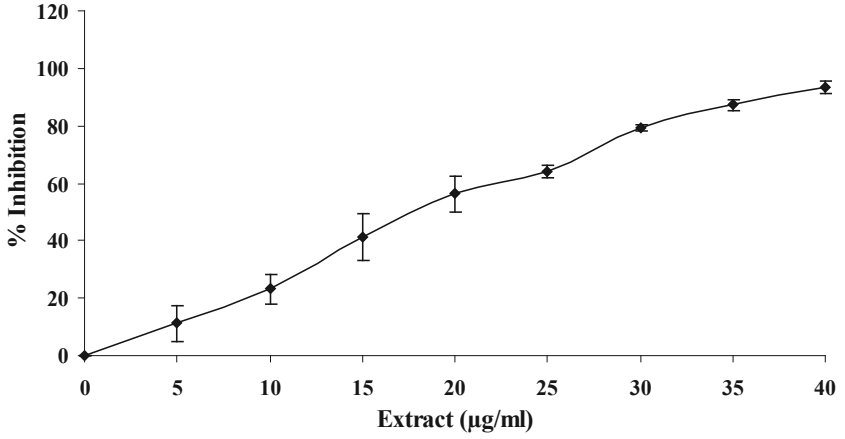

Figure 4 - Effect of substrate concentration on inhibition of NN-XIa-PLA$_2$ by aqueous stem bark extract of *M. indica*. The reaction mixture contained NN-XIa-PLA$_2$ enzyme in 0.05 M Tri-HCl buffer pH 7.5, 500 mM $CaCl_2$, inhibitors, and increasing concentrations of substrate (0-120 nM). The reaction was carried out in the absence (rhombus) and presence of IC_{50} concentration of extract (open square) with increasing concentration of substrate. The figure inset represents the percent of inhibition in the presence of IC_{50} concentration. Data represents ± S.E.M for n=3.

(Vishwanath et al. 1988). Several $sPLA_2$ 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, the aqueous extract of *M. indica* effectively inhibited the *in vitro* $sPLA_2$ activity and *in situ* $sPLA_2$ activity, the inhibitory potential of *in vivo* edema inducing activity of NN-XIa-PLA_2 was tested. The edema inducing effect of NN-XIa-PLA_2 (6 µg) was more than 175%, when compared to the saline injected mice. Figure 5 shows that aqueous extract of *M. indica* at different concentrations (0-50 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 footpads of mice. 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.) 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. 2001, Magrioti and Kokotos 2013). Furthermore, the extract 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_2 and LTB_4, when it was stimulated with pro-inflammatory stimuli lipopolysaccharide-interferon gamma (LPS-IFNg) 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 reduced TBARS levels and iNOS, COX-2, TNF-α and TNF R-2 expression in colonic tissue. 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-XIa-PLA_2 when incubated with extracts (data not shown), excludes the proteolytic degradation as a potential mechanism (Borges et al. 2000). Furthermore, considering the results of binding

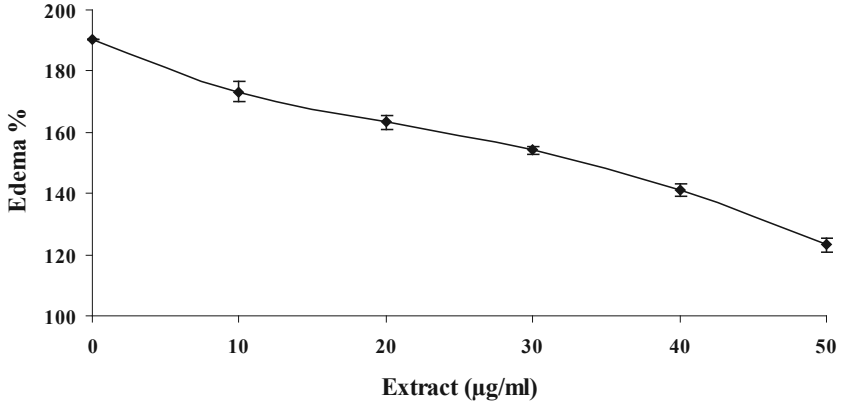

Figure 5 - Effect of calcium concentration on inhibition of NN-XIa-PLA_2 by aqueous stem bark extract of *M. indica*. The reaction mixture contained NN-XIa-PLA_2 enzyme in 0.05 M Tri-HCl buffer pH 7.5, and 0–15mM $CaCl_2$ in final volume. The reaction was initiated by adding substrate. The reaction was carried out in the absence (rhombus) and presence of IC_{50} concentration of extract (open square) with increasing concentration of calcium. The figure inset represents percentage inhibition in the presence of the IC_{50} concentration. Data represents ± S.E.M for n=3.

studies, the inhibition is known to be of irreversible nature. In addition, inhibiton is observed to be of independent of substrate and calcium concentration. The most likely mechanism for anti-inflammatory activities by this extract is probably due to the direct binding of the constituents of the extract with $sPLA_2$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 also be involved in binding with $sPLA_2$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) seems to exhibit anti-inflammatory effect through quenching of free radicals, as they are involved in the mechanism of inflammatory process (Nanda et al. 2007, Garrido et al. 2004, 2009, Márquez et al. 2012, Vyas et al. 2012). Previously, it was demonstrated and concluded that the standardized aqueous extract of *M. indica* (VIMANG®) can be classified as a dual inhibitor, as it inhibits both COX and LOX pathways of arachidonate metabolism (Garrido et al. 2004). 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® and mangiferin, exhibit inhibitory activity against synovial fluid PLA_2 activities (Garrido et al. 2004).

The aqueous stem bark extract of *M. indica* inhibiting both *in vitro* $sPLA_2$ enzymatic activity and *in vivo* edema inducing activity of NN-XIa-PLA_2, 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 (Garrido et al. 2009, Márquez et al. 2012, González et al. 2007). Thus, *M. indica* bark extract can be developed for topical application, as it is non-toxic and an effective anti-inflammatory formula contains potent anti-inflammatory molecules.

CONCLUSIONS

The aqueous extract of *M. indica* effectively inhibited $sPLA_2$ and its associated inflammatory 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. In addition, 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-inflammatory agents, which could be developed as a potential therapeutic agent against inflammatory related diseases. This study also substantiates their anti-inflammatory properties. Further studies on compounds responsible for the anti-inflammatory activity would be interesting, as these are highly attractive candidates for clinical development as a new class of anti-inflammatory agents.

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RESUMO

O extrato aquoso padrão da casca do caule é consumido como bebida à base de plantas e usado em formulações farmacêuticas para o tratamento de pacientes que sofrem de várias doenças em Cuba. Este estudo foi conduzido para avaliar o efeito modulador do extrato aquoso padrão da casca do caule de *M. indica* sobre o Grupo IA $sPLA_2$. Extrato de *M. indica* inibiu, de forma dependente de dose, a atividade do GIA $sPLA_2$ (NN-XIa-PLA_2) com IC_{50} de 8,1 μg/ml. O extrato da *M. indica* inibiu efetivamente a atividade hemolítica indireta em até 98% em uma concentração de 40 μg/ml, e em várias concentrações (0-50 μg/ml), esse extrato inibiu a formação de edema de maneira dependente de dose. Quando examinado como uma função de aumento de concentrações de substrato e cálcio, não houve diminuição do efeito inibitório sobre GIA $sPLA_2$. Adicionalmente, a inibição foi irreversível, como evidenciado em estudos de ligação. Foi observado que o extrato aquoso da *M. indica* inibe efetivamente $sPLA_2$ e está associado a atividades anti-inflamatórias, o que corrobora suas propriedades anti-inflamatórias. A inibição pode ser devida a uma interação direta dos componentes presentes no extrato com a enzima $sPLA_2$. Mais estudos sobre o entendimento dos principais constituintes responsáveis pela atividade anti-inflamatória seriam de interesse para o desenvolvimento destes componentes como potenciais agentes anti-inflamatórios.

Palavras-chave: fosfolipase A_2 secretória, anti-inflamatório, pró-inflamatório, inibição, drogas, plantas.

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