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Original Article

HUMAN SECRETORY PHOSPHOLIPASE A_2 ($sPLA_2$) INHIBITION BY AQUEOUS EXTRACT OF *MACROTYLOMA UNIFLORUM* (SEED) AS AN ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Objective: *Macrotyloma uniflorum* (Horse gram) is an important legume widely consumed in the tropical south Asian countries including India. The present investigation is the elucidation of anti-inflammatory activity of *M. uniflorum* as it has several medicinal properties. The *M. uniflorum* was evaluated for inhibition of human secretory phospholipase A_2 ($sPLA_2$) as a function of anti-inflammatory activity.

Methods: The total phenols, antioxidant (DPPH scavenging), Anti-lipid peroxidation, PLA_2 inhibition and lipoxygenase (5 & 15-LOX) inhibition activity of aqueous extracts of *M. uniflorum* coat and pulp were assayed by *in vitro* method. The aqueous extract of *M. uniflorum* seed coat was subjected to inhibit PLA_2 enzymes from human inflammatory fluids (Human Synovial Fluid and Human Pleural Fluid) and snake venoms (*Naja naja* and *Vipera russllii*) using [14]C labeled *E. coli* by *in vivo* method. A further effect of substrate and calcium concentration on inhibition of VRV-PLA_2 in presence and absence of *M. uniflorum* coat extract were assayed.

Results: Aqueous coat extract of *M. uniflorum* shows higher phenolics and biological activity and inhibited all $sPLA_2$ enzymes in concentration dependent manner. The IC_{50} values are found to be in the range of 11.42-20.88µg and IC_{50} values for 5-LOX and 15-LOX is 25.92µg and 32.47µg respectively. The extract effectively neutralized indirect hemolytic activity and showed similar potency in neutralizing the *in vivo* $sPLA_2$ induced mouse paw edema.

Conclusion: These findings suggest that, the active compound/s in extracts of *M. uniflorum* individually or synergistically responsible for observed $sPLA_2$ inhibition.

Keywords: HSF, Human Synovial Fluid, HPF, Human Pleural Fluid, Anti-inflammatory, $sPLA_2$, secretory Phospholipase A_2, VRV-PLA_2, *Vipera russelli* PLA_2, NN-PLA_2, *Naja naja* PLA_2, COX-1/2, Cyclo-oxygenase-1/2, LOX, Lipoxigenase, Lipid peroxidation.

INTRODUCTION

Inflammation is a localized reaction that produces redness, warmth, swelling and pain result in infection, irritation or injury. It contributes to all disease process including immunity, vascular pathology and leads to several undesirable consequences such as systemic shock, circulatory collapse and local tissue injury in many organs [32, 19]. Inflammation is mainly mediated by secretory phospholipase A_2 ($sPLA_2$). Several snake venom PLA_2 (EC 3.1.1.4) enzymes are responsible for local tissue damage such as edema and hemorrhage. An elevated level of $sPLA_2$ enzymes are detected in many inflammatory disorders [13, 14] and play a key role by releasing arachidonic acid and lysophospholipid, which are rate-limiting precursors for the production of pro-inflammatory lipid mediators. Cyclooxigenase-1/2 (COX-1/2) and lipoxygenase (LOX) catalyze arachidonic acid into pro-inflammatory mediators such as prostaglandins, thrombaxanes, and leukotrienes respectively. Lysophospholipid is further converted into platelet activation factor (PAF) by acetyltransferase [29]. Further, phospholipase A_2 (PLA_2) plays a crucial role in a number of diverse cellular as well as chemical and metabolic injuries. Apart, they involved in phospholipid digestion and metabolism, host defense, signal transduction and provide precursors for eicosanoid generation.

Present anti-inflammatory therapies include the non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit either Cyclo-oxygenase-1/2 (COX-1/2) or Lipoxygenase (LOX) catalysis. The specific inhibitors of COX-1/2 and LOX enzymes have severe side effects such as intestinal ulceration, bleeding and cardiovascular complications [12]. In addition to these problems COX-1/2 or LOX inhibitors cannot regulate the production of leukotrienes or PAF that continue to cause inflammation [8]. It appears rational that effective inhibitors of $sPLA_2$ could deplete the sources of arachidonic acid and, therefore, its downstream metabolites and PAF would not affect the homeostasis of COX-1/2 and LOX enzymes.

Therefore, the great demand for natural products for PLA_2 inhibition rather than NSAIDs to treat inflammatory disorders. The literature survey showed that several endogenous and exogenous agents have been reported to inhibit $sPLA_2$ enzymes [31]. In addition, several laboratories are synthesizing compounds to inhibit $sPLA_2$ [26]. But none of them are made into the market, which demands researchers to look for new specific $sPLA_2$ inhibitors.

Since a couple of years, the constant effort is made to bring out potent PLA_2 inhibitors from a plant source. The aristolochic acid, lipoic acid, mangiferin, ellagic acid, genistein, oleanolic acid, ursolic acid, pongamia, anisic acid, ascorbic acid and derivatives of tricyclic dipyrido diazepin one, ascorbic acid, lipoic acid are evaluated for PLA_2 inhibition from our laboratory [31, 26, 10, 11]. The present study, the legumes are a very interested source to identify/isolate the bioactive molecules for $sPLA_2$ inhibition. The legumes are nutraceuticals provide both nutrients as well as pharmaceuticals. Among legumes, *Macrotyloma uniflorum* (Horse gram) is an important legume, highly consumed/used in Asian countries. The few metabolites from *M. uniflorum* have shown the ability to interfere with the complex network of biochemical pathways connected to inflammatory processes [25]. The *M. uniflorum* is reported that protective effect on hyper uricemic and nephrotoxicity, diuretic, increase appetite, remove stone from kidney, cure hiccough, eye troubles, piles, enlargement of the spleen, pain in the liver, menstrual derangements, Antihepatotoxic activity [28], Anti-tumor efficacy [20], anti-themintic activity [24], hemolytic activity [16] and hepatoprotective activity[21]. Therefore, the *M. uniflorum* is selected for the present study.

MATERIALS AND METHODS

Materials

^{14}C-oleic acid was obtained from Perkin Elmer Life Sciences Inc. Boston, MA, USA. Fatty acid-free bovine serum albumin (BSA) fraction V was purchased from PAA Laboratories, GmbH Haidmannweg, Austria. Scintillation cocktail (Ultima Gold) was obtained from Packard

Bioscience, USA. *Escherichia coli* [lyophilized cells of strain W (ATCC 9637)], Diphenyl Picryl Hydrazyl radical (DPPH˙), thiobarbituric acid, gallic acid, Dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich Chemical Laboratories, St. Louis, MO, USA. Human pleural fluid and synovial fluid were obtained from Chest Disease Hospital (Sanitorium), Mysore and Dr. Hegde's Orthopedic Clinic, Mysore, India. Lyophilized powder of snake venoms (*Naja naja* and *Vipera russllii*) were purchased from Hindustan Park, Kolkata and Irula Co-operative Society Ltd., Chennai, India. The legumes were purchased from local provision store, Mysore, India. Swiss Wister mice weighing 20-25 g were obtained from Central Animal House Facility, University of Mysore, India. Ethical committee approved the use of laboratory animals (sanction order No: UOM/IAEC/38/2011). Animal care and handling were conducted in compliance with the national regulations for animal research. All other chemicals and reagents used in this study were of analytical grade or better.

Methods

Preparation of extracts and phytochemical analysis

The good quality *M. uniflorum* seeds were purchased from local provision store; cleaned and soaked overnight. Then the seed coat and pulp were separated manually, air-dried and pulverized in classical method. The powder of coat and pulp (75g each) were packed in Whatman No.1 filter paper and subjected to soxhlet extraction with organic solvents such as hexane, benzene, chloroform-acetone, ethanol, methanol and water in the ratio of 75g/500 ml (w/v). The extracts of organic solvents were concentrated to powder by flash evaporator, and water sample was reduced to powder by lyophilization. The yield was calculated and expressed as % w/w. The phytochemical analysis of different solvent extracts of *M. uniflorum* was done by methods described in Talari Samatha *et.* al [36]. The concentration of total phenolics and tannins in *M. uniflorum* extracts were estimated by the methods of Singleton [27] and Makkar [33] respectively.

Estimation of antioxidant activity

Antioxidant activity of *M. uniflorum* was determined using 2, 2-Diphenyl-1-picryl hydrazyl radical (DPPH˙) as described by Blios [4]. Briefly, 100µg extracts of *M. uniflorum* (coat & pulp) were mixed with 5 ml of 0.1 mM methanolic solution of DPPH and incubated at 20° C for 20 min in the complete dark. The control was prepared as above without extract and methanol was used for the baseline correction. Change in the absorbance of the samples was measured at 517 nm. Radical scavenging activity was expressed as percentage activity using the following formula.

$$\% \text{ radical scavenging activity} = \frac{\text{Control absorbance-sample absorbance}}{\text{Control absorbance}} \text{ X } 100$$

Lipid peroxidation activity

Lipid peroxidation activity was estimated according to Buege JA *et al* [6]. The albino Swiss Wister strain rat's perfused liver was isolated, and 10% (w/v) homogenate was prepared using a potter elvehjam homogenizer under ice cold (0-4°C) condition. The homogenate was centrifuged at 1,500 g for 5 min and clear supernatant was used for lipid peroxidation activity. The *M. uniflorum* extract (100µg) was mixed with 1.0 ml of 0.15M KCl and 0.5 ml of rat liver homogenate. The peroxidation assay was initiated by adding 100 µl of 0.2 mM ferric chloride. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 2 ml of ice-cold HCl (0.25N) containing 4% TCA and 0.38% TBA. The reaction mixture was heated at 80° C for 60 min. The samples were cooled and centrifuged; the absorbance of the supernatant was measured at 532 nm. An identical experiment was performed in the absence of extract to determine the amount of lipid peroxidation obtained in the presence of inducing agents. The anti-lipid peroxidation activity (ALP) is expressed as % ALP by using the following formula:

% ALP = 1-sample absorbance/control absorbance X 100

Phospholipase A_2 assay

The secretory PLA_2 from human synovial fluid (HSF) and human pleural fluid (HPF) was purified by the modified method as described by Vishwanath *et al.,* [34]. The $sPLA_2$ activity will be measured using [14]C-oleate labeled autoclaved *E. coli* [22]. The reaction mixture 350 µl contained 100 mM Tris-HCl buffer (pH 7.4), 5 mM calcium and 3.18 x 10^9 autoclaved *E. coli* cells (corresponds to 10,000cpm and 60nM lipid phosphorus). The reaction components will be mixed in the following order: buffer, calcium, enzyme, and water. The reaction will be initiated by adding 30 µl of *E. coli* substrate and incubated at 37°C for 60 min. The reaction will be terminated by adding 100 µl of 2N HCl and 100µl of fatty acid-free BSA (10%) to entrap free fatty acids released. The tubes are vortexed and centrifuged at 20,000 g for 5 min. An aliquot (140 µl) of supernatant containing released [14]C-oleic acid is mixed with scintillation cocktail and [14]C radiation will be measured in Packard Scintillation analyzer. Enzyme activity will be expressed as a number of moles of free fatty acid released/min/mg of protein at 37° C.

Inhibition of $sPLA_2$ activity

The reaction mixture 350 µl contained 10 mM Tris-HCl buffer pH 7.4, 5 mM calcium and the amount of protein (PLA_2 enzyme) was chosen such that 60-70% hydrolysis of substrate. Inhibition was carried out by adding aqueous coat extract of *M. uniflorum* in 0-75µg (w/v). The reaction was initiated by adding 30 µl of *E. coli* substrate and incubated at 37°C for 60 min. Oleanolic acid, a known inhibitor of PLA_2 is used as the positive control. IC_{50} concentration of extract was calculated using software 'GraphPad version 5.0 USA'.

Effect of substrate and calcium concentration on $sPLA_2$ inhibition

The total reaction mixture of 350 µl containing VRV-PLA_2 alone and with IC_{50} concentration of aqueous coat extract of *M. uniflorum* in 100 mM Tri-HCl buffer, pH 7.4 and 5 mM calcium. Substrate-dependent assay was carried by the addition of various concentration of substrate (20-120 nM) and the calcium-dependent assay was carried with calcium concentration ranging from 2.5-15 mM. The assay was carried out as described above.

Inhibition of 5-Lipoxygenase

The Poly Morpho Nuclear Leukocytes (PMNLs) 5-lipoxigenase (5-LOX) assay was performed according to the method published by Aharony and Stein [1]. 1 ml of standard reaction mixture contained 100 mM phosphate buffer pH 7.4, 50µg of DDT, 200µg of ATP, 300µg of $CaCl_2$, 150µg of arachidonic acid and PMNLs lipoxygenase (5µg). The reaction was carried out at room temperature. Absorbance was read for every 30 seconds up to 2 min at 234 nm using Shimadzu spectrophotometer. The enzyme activity was expressed as µgole of 5-HETE formed/min/mg protein. The 5-lipoxigenase was subjected to the inhibition by aqueous *M. uniflorum* coat extract. The Quercetin, a known inhibitor of LOX was used as a positive control.

Inhibition of 15-lipoxygenase

The enzyme assay was performed according to the method of Axelrod [3]. The standard reaction mixture for the 15-LOX assay (1.0 ml final volume) contained 100µg linoleic acid and soybean 15-lipoxygenase enzyme (5µg) in 200 mM borate buffer (pH 9.0). The reaction was carried out at room temperature. The absorbance was measured continuously for 3 min at 234 nm. The enzyme activity was expressed as µgole of 13-HPODE formed/min/mg protein. For inhibition study, different concentrations of extract were pre-incubated with 15-LOX for 5 min. The reaction was initiated by adding linoleic acid to respective assay mixtures. Quercetin, a known inhibitor of LOX, was used as a positive control.

Neutralization of indirect hemolytic activity

The indirect hemolytic activity of *M. uniflorum* was measured as described by Boman and Kaletta [5]. The substrate was prepared by suspending 1 ml of packed fresh human RBC and 1 ml fresh hen egg yolk in 8 ml of PBS. Aqueous coat extract of *M. uniflorum* was pre-incubated with 30µg of PLA_2 for 30 min at 37°C. The substrate (1 ml) was added to the pre-incubated sample and allowed to react for 45 min at 37 °C. The reaction was stopped by adding 9 ml of ice-cold PBS and the suspension was mixed and centrifuged at 1,500 g for 20 min. The released hemoglobin was read at 530 nm. The substrate with $sPLA_2$ enzyme served as positive control.

Neutralization of edema-inducing activity

The procedure of Yamakawa *et al.,* [35] as modified by Vishwanath *et al.*, [21] was followed. Mice weighing 20–25g were injected with 5µg of PLA_2 enzyme alone or mixed with different concentrations of *M. uniflorum* extract in a total volume of 20 µl saline into their intraplantar surface of the right hind footpad. The respective left footpad received 20 µl of saline or vehicle and served as controls. The mice were sacrificed after 45 min 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. Percentage increase of sham-injected control compared to the uninjected limb.

Statistical analysis

Inhibition percentage was calculated from the difference between extract/inhibitor treated group and control animals, which received the vehicle. Student's t-test for comparisons of unpaired data was used for statistical evaluation. The experimental results were presented as mean±SD of three determinations. The IC_{50} concentration was calculated using GraphPad version 5.0 USA. The statistical significance between different $sPLA_2$s at a given concentration was analyzed by Duncan's multiple range tests at $P<0.05$ using ANOVA of SPSS statistical software.

RESULTS AND DISCUSSION

The chemical substances and antioxidants derived from plants are potential interest in therapeutic intervention for chronic inflammatory disorders. They act either by inhibiting pro-inflammatory enzymes (PLA_2, COX and LOX) or by inhibition of cytokines (IL-1β, TNF-α) release and inhibition of mast cell degranulation that are known contributors to chronic inflammatory disorders. The $sPLA_2$ enzyme catalyze rate-limiting step in the production of pro-inflammatory eicosanoids and free radicals. The PLA_2 catalyzed reaction is considered to be a significant pathway for reactive oxygen species (ROS) it turn activates PLA_2 as well as lipid peroxidation and thereby augment the chronic inflammatory diseases to several folds [30]. Hence, the PLA_2 inhibition is legitimate in the neutralization of inflammation. The antioxidants such as flavonoids, retinoids and vitamin E and their potential benefit in the intervention of inflammatory reactions arise if it inhibits the key enzyme PLA_2 along with neutralizing the free radical generation reaction.

The legume *M. uniflorum* is reported to have anti-inflammatory activity, but the mechanism is unclear. The PLA_2 enzyme is subjected to inhibition as a function of anti-inflammatory activity. Initially, different solvent extracts of *M. uniflorum* were prepared and checked for VRV-PLA_2 (*Vipera russellii* snake venom PLA_2) inhibition. The aqueous extracts of *M. uniflorum* inhibit VRV-PLA_2 to a greater extent (fig. 1a). Further, seed coat and pulp of *M. uniflorum* seed were separated, and the respective extracts are subjected to inhibition. The aqueous extracts of the seed coat and seed pulp inhibited the VRV-PLA_2 to 87.56% and 52.1% respectively (fig. 1b).

The total phenolics and antioxidant activity of extracts of *M. uniflorum* (100µg concentration) were estimated by Lowry's and DPPH methods respectively. The seed coat showed higher concentration of phenolics (83.4%) than pulp (30.2%). The seed coat showed 94.5% of anti-oxidant activity whereas pulp showed 56.9%. The aqueous extract of coat showed anti-lipid peroxidation activity to 85.1% and pulp showed up to 60.8% (fig. 1b). The aqueous extract of seed coat inhibits VRV-PLA_2 to the higher extent and it directly correlates to antioxidant activity and total phenolics. The phytochemical analysis of *M. uniflorum* showed that the ethanol and aqueous extracts contain higher concentration of flavonoids, alkaloids, tannins, saponins, phenols and quinones compared to non-polar solvent extracts (table 1). The aqueous extract of seed coat showed a higher concentration of phytochemicals than pulp. The extracts were dialyzed and subjected for the antioxidant activity and anti-lipid peroxidation activity and it has nil activity for the same.

To substantiate the potency of *M. uniflorum* coat extract, the secretory PLA_2 enzymes from different sources were subjected to inhibition. The PLA_2 enzyme NN-PLA_2 from *Naja naja* snake venom and human PLA_2s (HPF-PLA_2 and HSF-PLA_2) from Human Pleural Fluid (HPF) and Human Synovial Fluid (HSF) were checked. The seed coat extract inhibited entire $sPLA_2$ enzymes at 60µg concentration in concentration dependent manner. The extent of inhibition was found to be>90% for HPF and HSF-PLA_2;>80% for VRV-PLA_2 and NN-PLA_2 (fig. 2a). Percentage of inhibition varies significantly among the four different sPLA2 enzymes as tested by ANNOVA. Though $sPLA_2$ enzymes exhibit more than 70% homology, many inhibitors inhibit these enzymes differentially due to differential binding affinities [14]. The IC_{50} values of coat extract for these $sPLA_2$ ranges from 11.42-20.88µg (table 2) at 95% confidence limit. The oleanolic acid is taken for a positive control whose IC50 values ranges from 3.08 to 7.78µg for those same PLA_2 sources [10]. Since aqueous extract of *M. uniflorum* inhibited all $sPLA_2$ enzymes taken up in this study. Further investigation was carried out with VRV-PLA_2 enzyme to understand the mechanism of inhibition.

Many inhibitors inhibit the $sPLA_2$ activity either by binding to the substrate or by chelating the calcium. The steroid inducible $sPLA_2$ inhibitors, lipocortin I and II have shown to inhibit by non-specific binding and affecting the 'quality of interface' of membrane phospholipid [9]. Inhibition of VRV-PLA_2 by aqueous extract of *M. uniflorum* (seed coat) was examined as a function of substrate concentration. The activity of VRV-PLA_2 in the absence or presence of IC_{50} concentration of extract increases linearly with increasing substrate concentration. The maximal activity was observed with 120 nmol of phospholipids. The percentage of inhibition was approximately 32% (fig. 3a) and remained constant over the entire range of substrate concentration used.

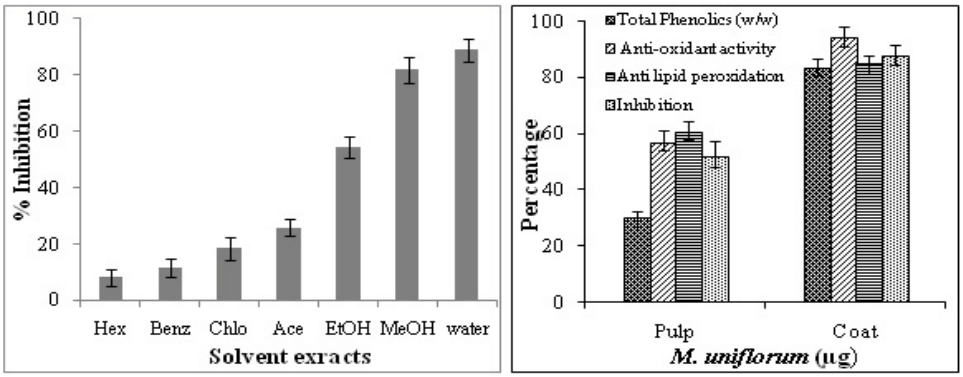

Fig. 1: a) Inhibition of VRV-PLA_2 by different solvent extracts of *M. uniflorum.* The 350 ml reaction mixture contained 5 mM Ca, 10 mM Tris-HCl buffer, 30 µl of substrate and extracts (100µg each). b) Estimation of total phenolics (% w/w), antioxidant activity, anti-lipid peroxidation and inhibition of PLA_2 enzyme by aqueous extracts of *M. uniflorum* at 100µg. Data represents mean±SD (n=3)

The possibility of PLA_2 inhibition by aqueous extract is due to metal ion chelating was determined as a function of calcium concentration. PLA_2 inhibition was checked by increasing calcium concentration from 2.5-15 mM. The VRV-PLA_2 activity was increased linearly, but

did not change the extent of inhibition (fig. 3b). These data suggests that the active molecule/s present in the aqueous extract of *M. uniflorum* may inhibit $sPLA_2$ directly and cause irreversible inhibition.

The most NSAIDs target the either COX-1/2 or LOX enzymes or both the enzymes. Therefore, the aqueous extract of *M. uniflorum* was checked for the two of the inflammatory enzymes Poly Morpho Nuclear Lymphocytes LOX (PMNLs 5-LOX) and soybean LOX (15-LOX). The activity of 5-LOX and 15-LOX was measured in terms of amounts of formation of 5-HETE and 13-HPODE respectively. The aqueous extract of *M. uniflorum* inhibited both the LOX enzymes in concentration dependent manner. IC_{50} values of extract of *M. uniflorum* coat for PMNLs 5-LOX and 15-LOX is 25.92µg (fig. 4a) and 32.47µg (fig. 4b) respectively.

The *in situ* hemolytic activity is an indirect way of measuring PLA_2 activity using egg yolk phospholipids dispersed as micelles together with washed erythrocytes. Aqueous extract inhibited the indirect hemolytic activity in concentration manner (table 3). Since, certain bioactive molecule/s might have bind to the enzyme irreversibly, the enzyme inhibition was irrespective of the nature of substrate provided for its activity.

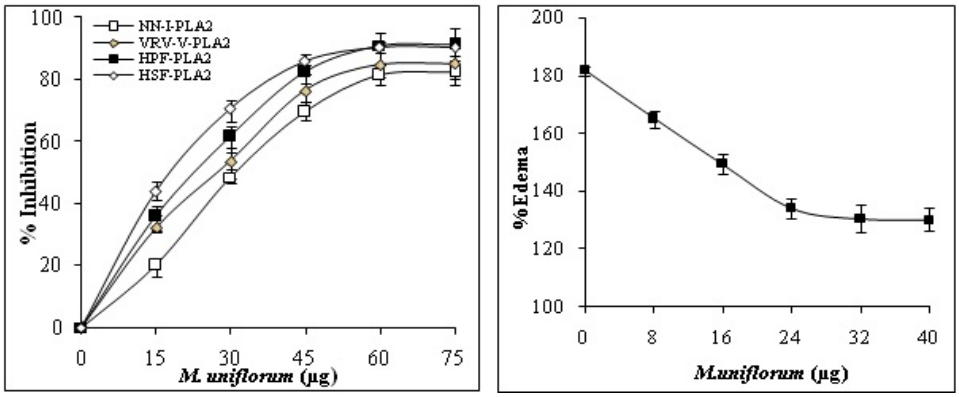

Fig. 2: a) Inhibition of different inflammatory PLA_2 enzymes by aqueous extract of seed coat of *M. uniflorum* at different concentration. The 350 µl reaction mixture contained PLA_2 enzymes (HSF-PLA_2, HPF-PLA_2, VRV-PLA_2 and NN-PLA_2), 5 mM Ca, 100 mM Tris-HCl buffer and *M. uniflorum* seed coat. b) Neutralization of $sPLA_2$ (VRV-PLA_2) edema-inducing activity by *M. uniflorum* coat extract. Data represents mean±SD (n=3)

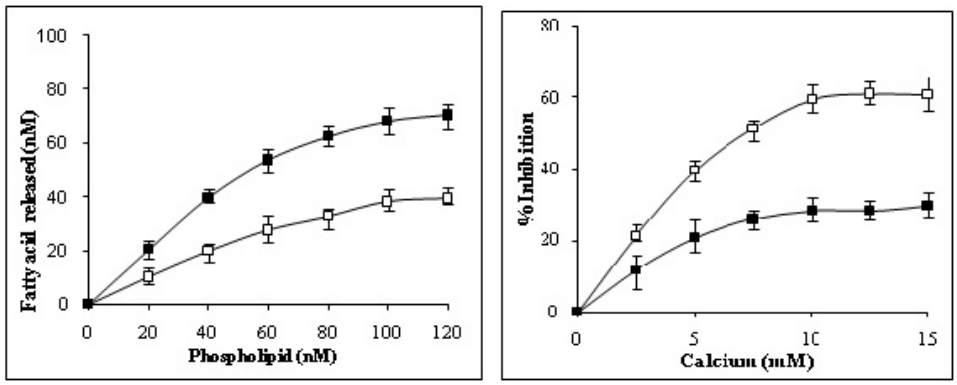

Fig. 3: Effect of substrate and calcium concentration on inhibition of VRV-PLA_2 in the presence (□) and absence (■) of *M. uniflorum* coat extract. The reaction mixture contains VRV-PLA_2 enzyme, 100 mM Tri-HCl buffer pH-7.4 and IC_{50} concentration of coat extract. Assay was carried by various concentration of a) substrate (20-120nM) and b) the calcium concentration (2.5-15 mM). Data represents mean±SD (n=3)

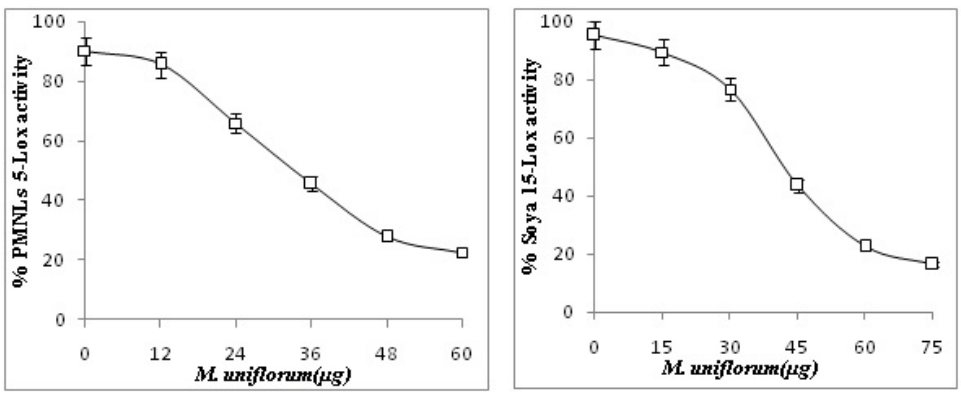

Fig. 4: Inhibition of 5-LOX and 15-LOX by aqueous coat extract of *M. uniflorum*. 1 ml of reaction mixture contained, a) 100 mM phosphate buffer pH 7.4, DDT (50µg), ATP (200µg), $CaCl_2$ (300µg), arachidonic acid (150µg) and 5-LOX (5µg). b) linoleic acid (100µg) and 15-LOX (5µg) in 200 mM borate buffer pH 9.0. Absorbance measured at 234 nm by µgole of 5-HETE and 13-HPODE formed/min/mg protein

Table 1: Photochemical analysis of *M. uniflorum* extracts

Phytochemicals	Hexane		Benzene		Acetone		chloroform		Methanol		ethanol		Aqueous	
	pulp	coat	pulp	coat	Pulp	Coat	pulp	coat	pulp	Coat	pulp	coat	pulp	Coat
Tannins	-	-	-	-	-	-	-	-	-	+	-	+	+	+
Saponins	-	-	-	-	-	-	-	-	+	-	+	-	+	+
Alkaloids	-	-	-	-	+	+	+	+	-	+	-	+	-	+
Phenolics	-	+	-	+	+	+	+	+	+	+	+	+	+	+
Terpenoids	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Cardiac glycosides	-	-	-	-	+	+	+	+	-	+	-	+	-	-
Flavanoids	-	-	-	-	-	-	-	-	-	-	-	+	+	+
Anthraquinone	-	-	-	-	-	-	-	-	+	-	+	-	+	+

The different solvents extracts of *M. uniflorum* (both coat and pulp) were filtered decolorized using activated charcoal and dried using evaporator. The 100µg of extracts were re-dissolved in the respective solvents and used for analysis. (+) present; (-) absent

Table 2: Inhibition of $sPLA_2$ activity by coat extract of *M. uniflorum*

Source of $sPLA_2$	Specific activity* (nmol/mg/min at 37°C)	IC_{50} (µg)#
VRV-PLA_2	148.0	11.42
NN-PLA_2	132.0	13.18
HSF-PLA_2	77.64	16.36
HPF-PLA_2	82.5	20.88

*n moles of fatty acid released/mg of protein/min at 37 °C. #IC_{50} value is defined as the amount of inhibitors (µg) required to inhibit 50 % of enzyme activity in the given reaction mixture.

Table 3: Inhibition of hemolytic activity of $sPLA_2$ by coat extract of *M. uniflorum*

Source of $sPLA_2$	Hemolytic activity at different conc. of coat extract (µg)		% of inhibition
	0	40	
NN-PLA_2	92.5	31.5	61
VRV-PLA_2	90.58	24.8	65.78
HPF-PLA_2	95.5	24	71.5
HSF-PLA_2	93.94	28.6	65.34

Aqueous extract of *M. uniflorum* was pre-incubated with 30µg of PLA_2 for 30 min at 37 °C. The reaction is initiated by adding 1 ml substrate, incubated for 45 min at 37 °C and stopped by adding ice-cold PBS. Centrifuge at 1,500 rpm for 20 min, read the released hemoglobin at 530 nm.

CONCLUSION

The above data summarized that extract of *M. uniflorum* exhibited very good *in vitro* $sPLA_2$ inhibition and *in vivo* anti-inflammatory activity by inhibiting edema induction in dose dependent manner. The $sPLA_2$ inhibition by the aqueous extract is independent of calcium and substrate concentration. Significantly, the aqueous extract of *M. uniflorum* inhibited the pro-inflammatory enzymes like 5-LOX and 15-LOX in concentration dependent manner. But, the extract used in the assay is crude there is always a possibility of cumulative effect which may be the other reason for $sPLA_2$ inhibition. However, several important questions remain open and further investigations are necessary to understand the mechanism underlying the effects of the extract and their active compound/s responsible for the anti-inflammatory activity.

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CONFLICT OF INTERESTS

Declare None

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