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Anti-Inflammatory Properties of Memecylaene: A Novel Compound Isolated From *Memecylon malabaricum*.

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ABSTRACT

To evaluate the anti-inflammatory properties of a novel compound Memecylaene, isolated from Memecylon malabaricum(Cl.) in acute and chronic inflammatory models. Memecylaene was tested for anti-inflammatory activity in albino rats. The in vivo anti-inflammatory activity was studied using acute and sub-acute animal models. The biochemical parameters such as antioxidant enzyme activities from ganuloma, lipid peroxidation inhibition in the liver of granuloma induced rats; as well as mucopolysaccharides from the granuloma were carried out. The in vitro effect of memecyleane was evaluated through the inhibition of snake venom PLA'2s which are known to induce inflammation. Memecylaene exhibited significant anti-inflammatory activity in acute and sub-acute models of inflammation with significant ($P<0.001$) reduction of paw edema and granuloma tissue compared to treatment with standard drug diclofenac sodium. Acute oral toxicity study indicates that the molecule is safe as drug. Memecylaene treatment significantly increased the antioxidant enzyme activities (CAT, SOD and GPx ($P<0.05$). Inhibition of lipid peroxides in liver and mucopolysaccharides in granuloma tissue was found. Memecylaene inhibited the PLA2's V and VIII of Russell viper venom with an IC50 value of 12.58µg and10µg. Memecylaene showed anti-inflammatory activity in both the models of inflammation which is attributed to their antioxidant and phospholipase A2 inhibitory activities. Thus, the study validated the scientific rationale of ethno medicinal use of M. malabaricum to inflammatory associated diseases and unveils its mechanism of action.

Keywords: Inflammation, Memecylaene, Memecylon malabricum, Russell viper Phospholipase A2

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INTRODUCTION

Inflammation is a complex physiological response to a variety of stimuli such as tissue injuries and infection. In general, inflammation is classified into acute and chronic, based on the onset of immune response. Acute phase of inflammation is the initial response of the body to the harmful stimuli, resulting from the increased outflow of blood plasma proteins and granulocytes to the site of injury [1]. The cells at the site of injury are then activated to release several mediators, for example, Thromboxanes (TX), Prostaglandins (PGs), Prostacyclins (PCs), Leukotrienes (LTs) and Cytokines. These mediators elicit the inflammatory response from acute to the chronic phase. In chronic inflammation a continuous flow of injured cells occur at the site [2]. During this process reactive oxygen species (ROS) are generated, due to the incomplete reduction of O_2 in electron transfer reactions [3]. To regulate the redox imbalance and oxidative damage, mammalian cells possess elaborate defense mechanisms involving the antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) [4].

Phospholipases A_2 (PLA_2) is a super family of polypeptides play a key role in the metabolism of phospholipids. The PLA_2 catalyze the hydrolysis of Sn2-ester bond of membrane phospholipids and release unsaturated fatty acid, arachidonic acid (AA) and lysophospholipid(LP). Arachidonic acid serves as a precursor in the biosynthesis of eicosanoids such as prostaglandins, leukotrienes, lipoxins. The lysophospholipid is a precursor for platelet activating factor (PAF). These products are proinflammatory under normal physiological condition [5].

The physiological events leading to the excess production of eicosanoids is linked to diseases such as cancer and cardiovascular disorders. Several findings point to the importance of PLA_2 in inflammatory reactions. Inhibition of PLA_2 enzyme by endogenous inhibitors, natural compounds is of potential therapeutic relevance in many inflammatory disease states. The existence of different types of PLA_2 in inflammatory diseases drew attention to the importance of finding the selective and specific inhibitors for the enzyme.
Non-steroidal anti-inflammatory drugs (NSAIDs) make up one of the largest groups of drugs used against pain and inflammation. Currently available NSAIDs, anti-inflammatory agents are associated with many side effects and limit their use. It is reported that about 34-46% of the users of NSAIDs usually sustain some gastrointestinal damage due to the inhibition of the protective cyclooxygenase enzyme in gastric mucosa [6].

Nearly 80% of global population depends on phytochemicals or plant source to get their ailments treated. It is well known that Indian Ayurvedic medicines are effectively used to cure many chronic diseases and disorders. However, several of the plants used in ancient folk and traditional medicines are often not subjected to scientific validation. Hence, experimental evidence for the claimed benefits or medicinal property is lacking. Therefore it is necessary that the valuable therapeutic properties should be subjected to scientific testing. Further it may lead to the isolation of active principle(s). *Memecylon malabaricum* is one such plant whose beneficial pharmacological properties have not been subjected to scientific investigations. In the present investigation it is focused on to evaluate the novel compound memecylaene, 4,9,14,19-Tetramethyl-1,6,11,16-tetraoxacycloeicos-3,8,13,19-tetraene isolated from *Memecylon malabaricum*(Cl.) for its anti-inflammatory properties.

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MATERIALS AND METHODS

Memecylaene was isolated from *Memecylon malabaricum* leaves by adopting various chromatographic methods. The structure of the compound was elucidated by utilizing various spectroscopic techniques comprising NMR, MS, IR and UV (patent filed, CBR No: 9452, Application No. 3437/CHE/2012). Diclofenac sodium was obtained from Zydus Cadilla Ltd. Carrageenan was purchased from Sigma-Aldrich, USA. All other chemicals were purchased from Sisco Research Laboratories, Mumbai. *Vipera russelli* venom was obtained from IRULA snake catchers (Madras).

Animals

Wistar albino rats (150 – 200 grams) of either sex were used in the study. They were randomly selected from central animal facility, JSS Medical College, Mysore. They were randomly distributed into groups and housed in cages (3 per cage). The animals were maintained under standard conditions at 24 ± 1°C with relative humidity (65±10%) and 10 h light: 14 h dark cycles each day for one week before and during the experiments. All animals were fed with the normal rodent pellet diet (Amrut, India) and water *ad libitum*. The study protocol was approved by Institutional Animal Ethical Committee.

Acute oral toxicity study

The acute oral toxicity test was performed following the OECD guidelines for testing chemicals with minor modifications [7]. The animals were divided into five groups (n = 3 per group). Group I served as control received the vehicle only (Sunflower oil). Group II, group III, group IV and group V served as test, received Memecylaene at doses of10mg/Kg, 50mg/Kg, 500mg/Kg and 2000mg/kg body weight via gastric intubation for seven days. All the experimental animals were observed for mortality and clinical signs of toxicity. The weight of each animal was recorded at 24 h interval. On day 8, the overnight fasted animals were euthanized and subjected to gross pathological examination of all the major internal organs such as liver, kidney, stomach and intestines.

Anti-inflammatory activity

The anti-inflammatory activity was evaluated using carrageenan induced rat hind paw edema method [8]. A mark was made on right hind paw just below the tibiotarsal junction and the paw was dipped in the mercury column of the plethysmometer up to the mark to ensure constant paw volume The animals fasted for 24 h were divided into control, standard and three test groups each consisting of six rats. The first group of rats was treated with sunflower oil, served as control, second group was administered orally with a dose of 10mg ⁄ kg of diclofenac sodium served as standard, third, fourth and fifth group were treated with memecylaene in graded doses of 10, 20, and 30 mg ⁄ kg respectively. After one hour each animal was injected with 0.1 mL of freshly prepared suspension of carrageenan solution (1% in 0.9% saline) into the sub plantar region of the right hind paw of rats. The volume of hind paw was measured using mercury plethysmometer both in control and in animals treated with standard and test compounds at Zero h, 1 h, 2 h, 3 h and 4th h after

carrageenan challenge. The anti-inflammatory activity was expressed as a percentage inhibition of the inflammation in treated animals in comparison with the control group [9].

$$\%\text{inhibition of edema} = \left[1 - \frac{Vt}{Vc}\right] X100$$

Where Vc and Vt are the mean relative changes in the volume of paw edema in the control and test groups, respectively. Potency of the tested compound Memecylaene was calculated relative to Diclofenac sodium, reference standard treated group according to the following equation.

$$\text{Potency} = \left[\frac{\%\text{ edema inhibition of test compound trated group}}{\%\text{ edema inhibition of diclofenac sodium trated group}} \right]$$

Cotton pellet induced granuloma in rats (sub acute model)

The effect of memecylaene was evaluated on sub acute model of inflammation using the method of Winter and Porter [10]. The rats were grouped into three of control, standard and test, containing six each. The cotton pellets, weighing exactly 10 mg each, were sterilized in an autoclave for 20 minutes at 120°C under 15 lb pressures. Four pellets were inserted sub cutaneously into the axilla and groin region on both sides of every rat under light ether anesthesia. The control, standard and the test groups were orally administered with sunflower oil, 10 mg/Kg diclofenac sodium, and 10 mg/Kg of Memecylaene in sunflower oil respectively for 7 consecutive days. On the day 8, the animals were sacrificed, the liver was excised and lipid peroxides were estimated in the liver homogenate. The cotton pellets were removed surgically along with the granuloma tissues. The pellets were then dried in incubator at 37ºC for 3 days and weighed after cooling. Difference in the initial and final weight of cotton pellets were noted and taken as the measure of granuloma formation. Activity of SOD, GPx and CAT as well as the amount of mucopolysaccharides were carried out in the granuloma tissue of freshly operated animals.

Assay of antioxidant enzymes and Mucopolysaccharides

Granuloma tissue from control, standard and test groups was homogenized (10% w/v) in ice cold 50mM phosphate buffer (pH 7.4), centrifuged at 10,000rpm for 20 min. at 4ºC and supernatant was used to assay the enzyme activities.

Activity of the enzyme, SOD was measured using pyrogallol (2mM) auto oxidation in tris buffer by the method of Marklund S, Marklund G [11]. The GPx activity was measured by the indirect assay method using glutathione reductase. Cumene hydroperoxides (1mM) and glutathione (0.25mM) were used as substrates and coupled oxidation of NADPH by glutathione reductase (0.25U) in tris buffer(50mM, pH 7.6) was monitored at 340nm [12]. The CAT activity was measured using H2O2 (3%) as the substrate in phosphate buffer [13]. Mucopolysaccharides were estimated by orcinol method [14].

Assay of PLA_2 by indirect hemolytic activity

Protein concentration in the venom was calculated, using bovine serum albumin fraction V as standard (0-75µg) [15]. A semi quantitative indirect hemolytic assay was employed [16]. Briefly, packed human erythrocytes, egg yolk and phosphate buffer saline was mixed (1:1:8 V/V). 1ml of this suspension was incubated with 60µg enzyme for 10 min at 37^0C. The reaction was stopped by adding 10ml of ice cold phosphate buffer saline and centrifuged at 4^0C for 10 min at 800Xg. The amount of hemoglobin released in the supernatant was measured at 540 nm. The assay was also carried out in presence of various doses of memecylaene.

Purification of phospholipase A_2 from *V. russelli* venom

The PLA2, VRV-PL V and VRV-PL VIII were purified by the method of Jayanthi et al [17]. Russell's viper venom from IRULA snake catchers from Madras was fractionated on CM-Sephadex –C-25 column by varying pH and ionic strength of Phosphate buffer (pH7-8; 0.02M to 0.3M). Purity of the PLA_2's was confirmed by electrophoresis (data not shown).

Lipid peroxidation

Lipid peroxide content in the liver homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS) [18]. Tissue homogenate (10% w/v in 50mM phosphate buffer, pH 7.4) was boiled in TCA (10%) and TBA (0.34%) for 15min, cooled and centrifuged. Optical density of the supernatant was read at 535nm.

Statistical Analysis

Statistical analysis was carried out using Statistical Package for Social Science (SPSS,version 12.0). The experimental results were expressed as the mean ± Standard Deviation (SD). Group comparisons were performed using One Way ANOVA followed by Waller-Duncan Post Hoc test. A p value of 0.05 was considered statistically significant.

RESULTS

Acute oral toxicity test

There were no deaths related to treatment or signs of toxicity developed in both the control and experimental animals used in acute toxicity studies. During this study, no significant difference in body weights of animals was observed.

Anti-inflammatory activity

Memecylaene, a terpene derivative, isolated from *Memecylon malabaricum* reduced the edema activity in sub-acute carragenan induced paw edema in rats in a dose dependent manner [table 1]. Memecylaene at 10, 20, and 30mg/Kg body weight reduced the edema by 77 %, 81% and 84% respectively at 3^{rd}h and decreased thereafter. The standard drug diclofenac sodium at 10 mg/Kg also reduced the edema by 63%, under similar conditions.

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The potency of the reductuion of paw edema by memecylaene when compared to diclofenac sodium is 1.21 at the corresponding dose. Increasing the memecylaene dose also increased the potency of the reduction of edema [Table 1].

Table 1: Study of diclofenac sodium and memecylaene (at different doses) on carrageenan-induced paw edema in rats. Values are expressed as mean±SD. n=6 ,[a, b] p<0.001 as compare to control (one- way anova)

Treatment groups	Mean paw edema(cm)±SD	% inhibition	Potency
Control	4.59±0.76	0	
Diclofenac sodium 10mg/Kg	1.69±0.28[a]	63	
Memecylaene 10mg/Kg	1.066±0.15[b]	77	1.21
Memecylaene 20mg/Kg	1.26±0.40[b]	81	1.28

Cotton pellet induced granuloma

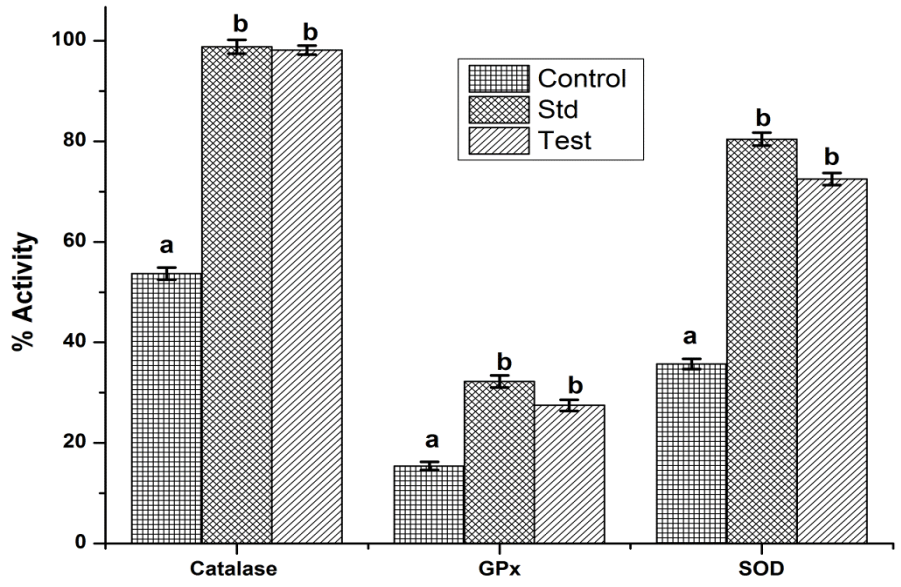
Memecylaene inhibited the granuloma by 37% at the concentration of 10 mg/Kg, on the other hand diclofenac sodium inhibited the granuloma by 29% at the same dose compared with the control group [Table 2]

Table 2: Studies of memecylaene on cotton pellet induced granuloma in rats. Standard, Diclofenac sodium and test compound memecylaene at dose of 10 mg Kg-1 b.w. Values are mean ± S.D., n=6 , [a, b] p<0.001 as compare to control (one- way anova)

Treatment groups	Weight of granuloma (mg)	Inhibition (%)
Group I – Control (Sunflower oil-500µl)	47.8 ± 3.10[a]	0
Group II Standard 10mg Kg^{-1}	33.9 ± 2.60[b]	29
Group III Memecylaene.10mg Kg^{-1}	30.3± 2.00[b]	37

Influence of Memecylaene on antioxidant enzymes

Figure 1: Effect of Memecylaene treatment on CAT, GPx and SOD in the granuloma of normal and experimental animals. Values are means from 3 independent experiments, each performed in duplicate. Means with no common superscript letters differ significantly (P<0.05).



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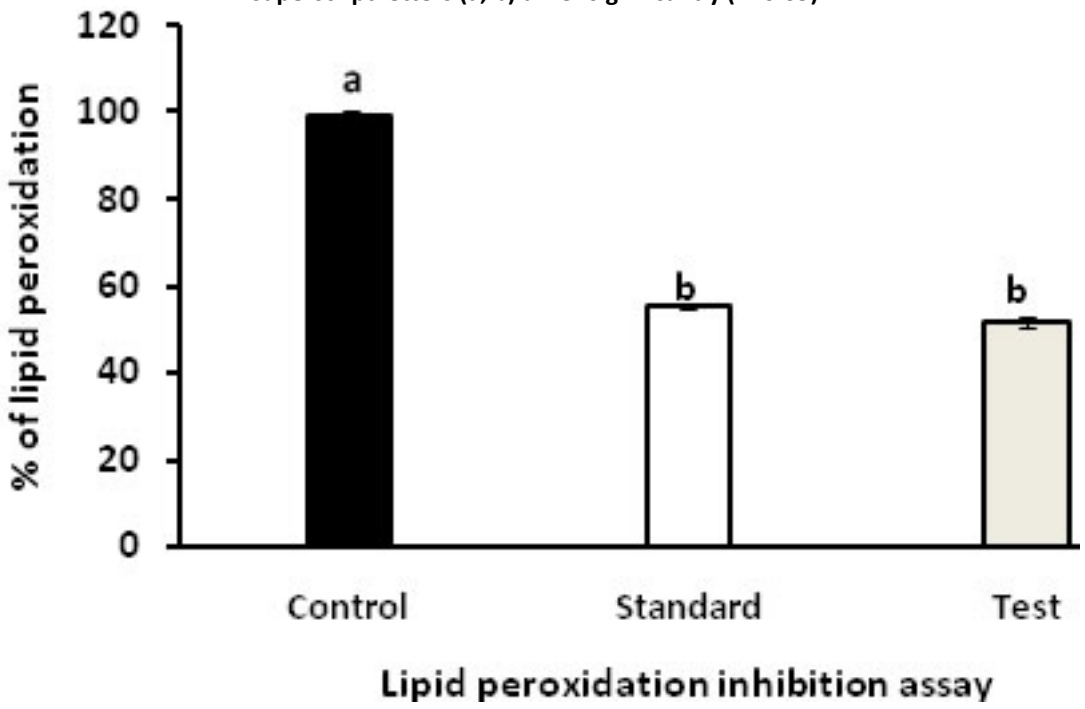
The antioxidant enzyme, Catalase activity was increased to a similar extent both by diclofenac sodium and memecylaene. On the other hand, Glutathione peroxidase activity was increased by more than 18% by memecylaene when compared to diclofenac sodium. Superoxide dismutase activity was also increased by 4% by memecylaene when compared to diclofenac sodium [Fig: 1]

Malondialdehyde levels in the liver of cotton pellet induced granuloma rats

In oxidative stress model of cotton pellet induced granuloma rats, memecylaene produced a significant (P<0.05) reduction in (Malondialdehyde) MDA level 52.% in comparison to control, on the other hand standard drug decreased MDA level to 55% [Fig: 2].

Figure 2: Comparative study of diclofenac sodium and memecylaene on hepatic MDA level in rats. Values are means from 3 independent experiments, each performed in duplicate. Means with no common superscript letters (a, b) differ significantly (P<0.05).



Mucopolysaccharide level in the granuloma

Granuloma formed due to cotton pellet when treated with Memecylaene produced a significant (P<0.01) reduction in mucopolysaccharide level by 67% when compared to the control. On the other hand standard drug reduced the mucopolysaccharide only by 49% [Fig: 3].

Phospholipase assay

Memecylaene inhibited the VRVPL-VIII and V in a dose dependent manner with an IC_{50} value of 10µg and 12.8µg respectively. Inhibition of PLA_2's was dose dependent [Fig: 4].

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Figure 3: Effect of Memecylaene on mucopolysaccharide level in the granuloma. Values are means from 3 independent experiments, each performed in duplicate. Means with no common superscript letters differ significantly (P<0.05).

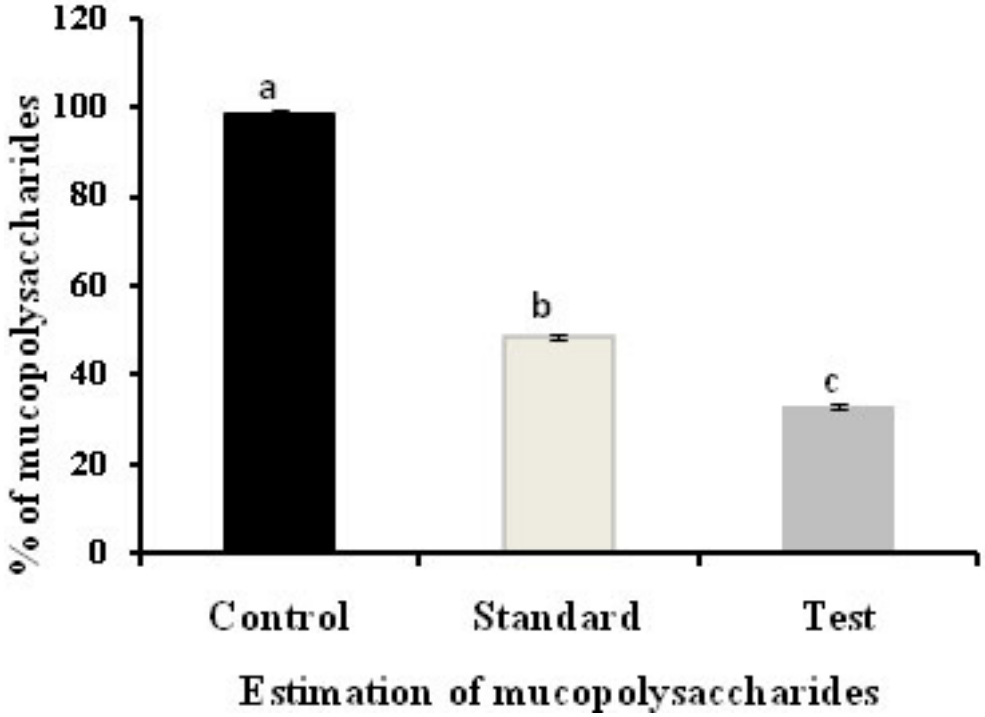
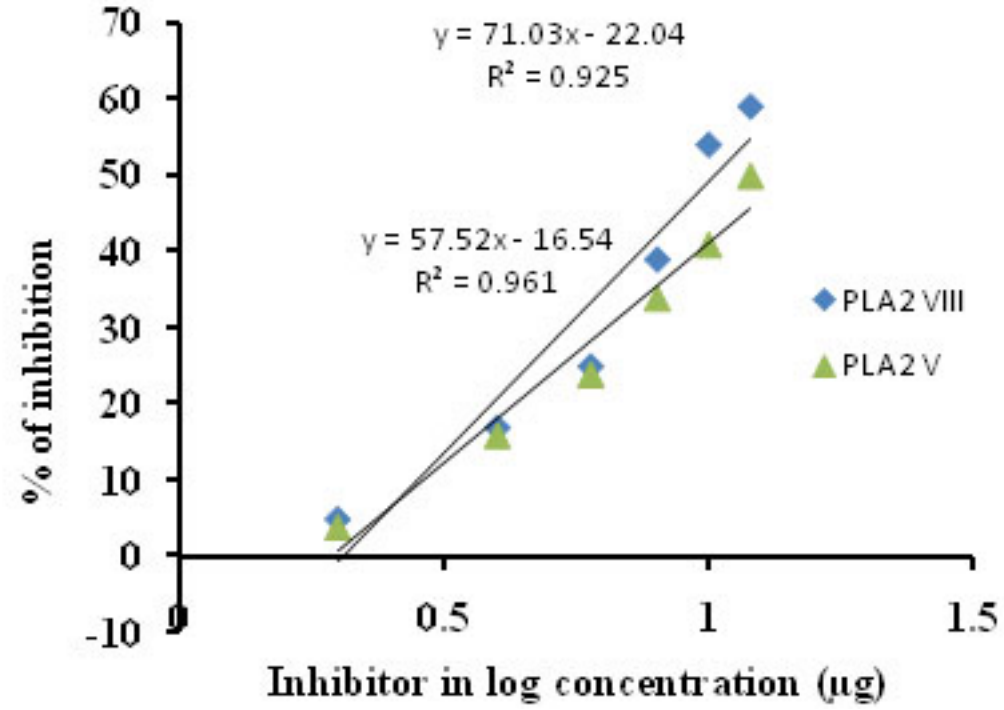


Figure 4: Inhibition of PLA_2's VRVPL-VIII and V by memecylaene. Values are means from 3 independent experiments, each performed in duplicate.



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DISCUSSION

Memecylon malabaricum is an indigenous medicinal plant used in ethno medicine for the treatment of bacterial infections, [19] inflammation and skin diseases including psoriasis and herpes, [20] most of these maladies are associated with inflammation. The compound memecylaene is isolated from the plant *Memecylon malabaricum.*The investigations are carried out to examine the anti-inflammatory property of the molecule.

The carrageenan induced edema in rat paw in a dose dependent manner. The edema induced is characterized by biphasic event with the involvement of different pharmacologically important mediators. Initial 2 hours of carrageenan injection releases chemical mediators such as histamine, 5-hydroxytrypatamine and bradykinin. In the second phase, kinin and prostaglandin derivatives are detected as mediators of late phase inflammation [21].

The memecylaene inhibited the edema continuously and hence reverses the edema in the course of 4 to 5 hours; also memecylaene inhibited inflammatory PLA_2 of snake venom in a dose dependent manner. The putative results suggest memecylaene not only inhibits the release of mediators such as (Histamine, 5-hydroxy tryptamine) but also appears to inhibit the release of kinins and prostaglandins.

Antiinflammatory activity in cotton pellet induced granuloma was greatly inhibited by memecylaene when compared to diclofenac sodium. This is further supported by decreased formation of mucopolysaccharides by memecylaene in granuloma. Also cotton pellet induced granuloma in the memecylaene treated group exhibited increased level of SOD, GPx and CAT which are fairly comparable to the levels of these enzymes under the influence of diclofenac sodium except for GPx whose activity was increased more than 18% and SOD by 4% compared to diclofenac sodium. The high activity of GPx and SOD are known to control reactive oxygen species. Hence memecylaene appears to be a potential candidate in controlling ROS. The MDA levels in the liver of cotton pellet induced granuloma rats were controlled by memecylaene as much as diclofenac sodium. Therefore memecylaene appears to control both inflammation as well as ROS formation. This validates the use of *Memecylon malabaricum* in the treatment of diseases associated with inflammation as well as diseases arise due to ROS formation.

The Russell viper venom PLA_2 VRVPL-V and VRVPL-VIII are known to induce presynaptic neurotoxicity and myotoxicity in experimental mice [22]. VRVPL-VIII induces lung hemorrhage in rats [23]. All these maladies are associated with PLA_2 activity. Memecylaene inhibits both the PLA_2's in a dose dependent manner. Therefore it tempts to suggest a role for Memecylon malabaricum as a candidate for the treatment of snake bite.

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Disclosure Statement:

No competing financial interests exist.

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