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Antioxidant and Anti-inflammatory Activities of the Methanolic Leaf Extract of Traditionally Used Medicinal Plant *Mimusops elengi* L.

Ganesh Gadamsetty[1], Saurabh Maru[2] and Sarada N.C[1]

[1] *Chemistry Division, School of Advanced Sciences, VIT University, Vellore-14, Tamil Nadu, India*

[2] *Department of Pharmacy, Pharmacology division, Shri G S Institute of Technology and Science, Indore, India*

Abstract

Mimusops elengi (M. elengi) is the most traditionally used medicinal plant to treat several pathologies. The main aim of this study is to evaluate the *in vitro* antioxidant (DPPH, ABTS radical scavenging), *in vitro* anti-inflammatory (HRBC membrane stabilization) and *in vivo* anti-inflammatory activity (inhibition of carrageenan induced paw edema in rat) for methanolic extracts of leaves. In *in vitro* antioxidant method the methanolic extracts of leaf showed higher free radical scavenging activity compared to standards with IC_{50} of 10.25µg/ml (DPPH) and 13.5µg/ml (ABTS). These extract was able to inhibit inflammation by *in vitro* about 70-75% and *in vivo* about 44-47% (Paw edema method) anti-inflammatory assays compared to standard produced 50.04% at 200mg/kg extract for 6h period. The results indicate that *M. elengi* leaves show good anti-inflammatory and anti-oxidant properties. The phenolics, flavonoids may play an important role in the antioxidant and anti-inflammatory activities of *M. elengi*.

Key Words: Anti-oxidant; Anti-inflammatory; carrageenan rat paw edema; HRBC membrane stabilization; *Mimusops elengi*

INTRODUCTION

Inflammation is a basic mechanism in which the body responds to infection, irritation or other injury of the body cells and tissues, and the key feature being redness, warmth, swelling and pain [1]. Inflammations are mainly as acute and chronic inflammations. Most of the present anti-inflammatory drugs inhibit the production of cyclooxygenase (COX) enzymes, COX-1 and COX-2 which synthesize prostaglandins and thromboxane, inflammatory mediators [2]. Steroidal and Non-steroidal anti-inflammatory drugs (NSAID's) are widely used drugs for treating inflammation, and long term use of NSAID's cause gastric erosions and stomach ulcers. In addition to this, some herbs are used as anti-inflammatory agents [3]. Previously, it was reported that reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion, and peroxynitrite radicals will participate in the process of inflammation [4]. These ROS will produce in excess will injure cellular bio molecules such as nucleic acids, proteins, carbohydrates and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation[5]. Therefore, the compounds showing scavenging activities towards these ROS may expect to have therapeutic potentials towards inflammatory diseases.

Yet, it is still the challenge of the pharmaceutical chemist to develop more effective and less toxic agents to treat inflammatory diseases. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. The research into plants with alleged folkloric use as pain relievers, anti-inflammatory agents, should therefore be viewed as a fruitful and logical research strategy in the search for new anti-inflammatory drugs.

Mimuosops elengi L.(sapotaceae) is a small to large evergreen tree found and distributed throughout the greater parts of India. It is commonly known as Spanish cherry, bullet wood in English and Bakul in Sanskrit. Different parts of this plant are used in the indigenous system of medicine for treatment of different ailments. In Ayurveda, the bark, flowers, fruit and seeds are of great value for treating various diseases such as cardiotonic, alexipharmic, stomachiac, astringent cooling, anthelmintic, tonic, and febrifuge properties [6]. The bark and fruits of this plant are used in the treatment of diarrhoea and dysentery and decoction of the bark is used as a gargle [7]. Rinsing mouth with bark decoction is believed to strengthen the gums, reduce inflammation, prevent bleeding of gums, and to stop bad breath caused by pyorrhea and dental caries [8]. Different parts of the plant have also been reported for anti-microbial, anti-ulcer, anti-anxiety, anti-oxidant, Hyperglycemic, anti- hyperlipidemic, anti-helminthic, anti-inflammatory, analgesic, anti-pyretic[9], cytotoxic[10] and anti-tumour activities[11].

Several triterpenoids, steroids, steroidal glycosides, flavonoids and alkaloids have been identified and reported from this plant [12]. Recently, other research group have tested *in vitro* anti-inflammatory activity of leaves, which showed promising results [13]. However to the best of our knowledge, the leaves of *M. elengi* were not scientifically done for its *in vivo* anti-inflammatory activity. Hence we have investigated the *in vitro* and *in vivo* anti-inflammatory effects of methanolic extracts of leaves. Furthermore, we have also evaluated the antioxidant scavenging activities of these extracts.

MATERIALS AND METHODS

Plant material and Extraction

M. elengi leaves were collected in the month of January, 2010 at VIT University (12°58' North, 79°09' East), Vellore, Tamil Nadu, India. The plant was identified and authenticated by Prof. P. Jayaraman, Director, National Institute of Herbal Science, Chennai, India. The leaves were dried under shade, pulverized and extracted (40gm) with 400 ml Hexane to remove chlorophyll and oily substances and followed by 100% methanol at 70 °C using soxhlet extraction. The extracts were concentrated to dryness at 50°C to 54°C under reduced pressure using a rotary evaporator (BUchi, Switzerland). The extracts were kept under 4° C until further use.

Preliminary Phytochemical Screening

The extract was screened for the presence of various phytochemical constituents employing standard screening tests [14].

Drugs and Chemicals

The chemicals used were 1,1-dipheny l-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), Folin ciocalteau reagent (FC Reagent), λ-Carrageenan were procured from Sigma (St Louis, MO). Sodium carbonate, Sodium chloride, Ascorbic acid, Gallic acid, Ammonium per sulphate, Aluminium trichloride was purchased from SD-Fine Chem limited. Indomethacin and Diclofenac potassium were obtained from Cipla pharmaceuticals. All other chemicals and solvents were of AR grade and obtained from standard sources.

Experimental Animals

Wistar rats (150-200gm) of either sex were obtained from Animal house, VIT University, housed at 20±2°C, with a 12:12 h light and dark cycle and given food and water *ad libitum*. The animals fasted for 12 h before the experiment, with free access to water. All the procedures were approved by the Institute Animal Ethics Committee and the experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) given on animal experimentation.

Determination of total phenolic content

The total phenolic content was determined by FC method [15]. To 20 µl of the test sample (1 mg/mL) 40 µl of FC reagent and 100 µl of Na_2CO_3 (25% w/v) were added and made up to 2 mL and incubated at 45°C in dark for 30 min. In blank, FC reagent was replaced with distilled water and absorbance of developed blue colour was measured at 765 nm using UV-Visible spectrophotometer (Jasco V-670). A calibration curve was prepared using standard solution of gallic acid. The results were expressed in mg of gallic acid equivalents per gram of extract. All determinations were carried out in triplicates.

Determination of total flavonoid content

Briefly, 1mL of extract (1mg/mL) in methanol was mixed with 1 mL of 20% aluminium trichloride in ethanol and a drop of acetic acid was added and then diluted with ethanol to 25 mL and left at room temperature for 30 min. The absorption of the reaction mixture was measured at 415 nm. A calibration curve was prepared using standard quercitin under same conditions. The results were expressed in mg of Quercetin equivalents per gram of extract [15].

DPPH radical scavenging activity

The extract was dissolved in methanol by using sonicator bath. 500 µL of the sample solutions of varying concentrations (2-20 µg/ml) were mixed with 500 µL of freshly prepared methanolic solution of 0.2 mM DPPH. The solution in the test tubes were shaken well and kept in dark for 15 min at room temperature. The reduction in the colour was measured at 517 nm. The control solution consisted of a mixture of appropriate concentrations of methanol and 500 µL DPPH. The blank solution contains 500µL of sample and 500µL of methanol. Ascorbic acid was used as a standard. Results were expressed as percentage of inhibition of the DPPH radical. All determinations were done in triplicates. Percentage of inhibition of the DPPH radical was calculated according to the following equation [16].

$$\text{Inhibition of DPPH (\%)} = (A_c - A_s/A_c) \text{ X } 100$$

Where, A_c = Absorbance of control

A_s = Absorbance of samples (or) standard.

ABTS radical cation decolourisation assay

This assay was carried out by the method explained by Subhasree et al., with minor modifications [17]. 7 mM of ABTS solution was reacted with freshly prepared 2.45 mM ammonium persulphate solution and kept in dark for 12-16 h, to produce a dark coloured solution containing ABTS radical cations. The initial absorbance was measured at 734 nm. This stock solution was diluted to give a final absorbance value of about 0.7 (±0.02). 0.1 mL of different concentrations (2-20 µg/mL) of test samples was mixed with 1 mL of ABTS working standard in a microcuvette. The decrease in absorbance was measured; the final absorbance at seventh minute was noted. Gallic acid was used as a standard at different concentrations. Results were expressed in percentage of inhibition of ABTS radical. All determinations were done in triplicates.

The percentage inhibition was calculated according to the formula:

$$\text{Inhibition of ABTS (\%)} = (A_c - A_s/A_c) \text{ X } 100$$

Where, A_c = Absorbance of control

A_s = Absorbance of samples (or) standard.

in Vitro Anti-Inflammatory Activity - HRBC Method

Preparation of human red blood cells (HRBC) solution

Blood was collected from a healthy human donor not consuming any steroidal drugs for past two weeks and was centrifuged (3000gX5m) and the supernatant part was carefully pipetted out. The packed cells were re suspended with equal volume of normal saline (pH 7.4) and centrifuged again. The process was repeated until the supernatants were clear. A 10% HRBC suspension was then prepared with normal saline and used immediately.

Effect of extracts on HRBC stability

4.5 mL of reaction mixture consisting of 2 mL hypotonic saline, 1 mL of sodium phosphate buffer (0.15 M, pH 7.4) and 1 mL of extract were dissolved in normal physiological saline. Then 0.5 mL of 10% HRBC was also added. Two controls were performed, one with 1.0 mL of isotonic saline instead of extract and the second control with 0.5 mL of isotonic saline instead of red blood cells. The mixture was incubated at 56°C for 30 min. The tubes were cooled under running water for 20 min and the mixture was

centrifuged at 3000 rpm. The supernatants were separated and the absorbance of the supernatants read at 560nm [18]. The percentage membrane stabilizing activity was determined using the following equation [19]. The control represents 100% HRBC lysis. Diclofenac potassium was the standard drug used:

% Membrane stability = $100 – (A_s – A_{c2} / A_{c1}) X 100$

Where, A_s = Absorbance of standard (or) samples

A_{c1} & A_{c2} = Absorbance of control 1 and control 2 respectively.

Acute toxicity studies

The acute toxicity was performed according to OECD guidelines [20]. The wistar rats either sex were used for this study. The animals were divided into four groups each group with three animals (n=3). The animals were fasted overnight and extracts were given orally to rats at a dose of 100, 300 and 1000 mg/kg body weight. The animals were observed continuously for its behavioural changes for first four hours and for mortality at the end of 24h. We have examined the animals daily till 10^{th} day for any behavioural change or mortality.

***in vivo* Anti-inflammatory activity – Carrageenan induced rat paw edema method**

Wistar rats (150-200gm) of either sex were used. Animals were weighed and randomized in 5 groups (n=6). Before treatment, the volume of the right paw of each animal was determined using a plethysmometer (UGO Basile, 7140). All the animals were starved for 12h. To ensure uniform hydration, the rats received 5 mL of water by stomach tube. Group I served as control (V_c) and did not receive any drug. Group II received the standard Indomethacin (10 mg/kg, p.o) and Group III, IV, and V received crude extract in three different doses (100, 200, 300 mg/kg, p.o). Thirty minutes later, the rats were challenged by a subcutaneous injection of 0.1 mL of 1% w/v freshly prepared solution of λ-carrageenan in saline into the plantar side of the left hind paw. The paw was marked with ink at the level of the lateral malleolus and immersed in water reservoir of digital Plethysmometer up to that mark to measure the paw volume. The paw volume (V_t) was measured at 2, 4, 6, 12 and 24 h immediately after carrageenan injection in control, extract treated and indomethacin treated groups [21].

The percentage of inhibition of each group was determined using the following formulae:

$$\% \text{ Inhibition} = (V_c - V_t / V_c) X 100$$

Where, V_c = Mean variation of edema for the control group

V_t = Mean variation of edema for treated groups with plant extracts or standard drugs.

Statistical evaluation of data

The results were presented as the mean % ± Standard Error of Mean (SEM). Statistical differences between the treated and control groups were evaluated by one way ANOVA by SPSS version 9.05 software and followed by Dunnetts t-test. The values were considered significant when, $p < 0.01$.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of leaf extracts showed the presence of bioactive components like Saponins, Flavonoids, Phenols, Tannins, Terpenoids and trace amounts of carbohydrates and amino acids

The leaves were extracted with hexane to remove chlorophyll and oil substances and followed by methanol. The crude extract was analysed for total phenolic and flavonoid contents and further its antioxidant activity was analysed by using two different radical scavenging methods, namely DPPH and ABTS free radical scavenging assays. The total phenolic content was expressed in mg of gallic acid equivalents (GAE) per gram of plant sample and was found to be 40.3±1.32 mg/g plant extracts, where these results were similar to that of previous reports [22]. The content of flavonoids was expressed in mg of quercetin equivalents (QE) per gram of plant sample and was found to be 52.7±0.49 mg/g. The phenolic compounds will exhibit significant free radical scavenging activities, because of the presence of hydrogen or electron donating agents, and metal ion chelating properties.

The scavenging properties of leaf extract were measured in terms of their ability to bleach the stable radicals $DPPH^{\bullet}$, $ABTS^{\bullet+}$. These scavenging assays were carried out at six different concentrations. In this study, the antioxidant activity of the extracts was evaluated on the basis of two methods, the analysis of their scavenging effects with regard to the $DPPH^{\bullet}$, $ABTS^{\bullet+}$. The results for the antioxidant activity of the methanolic extract of *M. elengi* leaves were reported in Figure 1 which indicates that these extract exhibit free radical scavenging activity in a concentration dependent manner with reference to the standards ascorbic acid and gallic acid. The antioxidants in extracts reduced the purple colored DPPH free radical to a yellow colored diphenylpicrylhydrazine which is measured at 517nm spectrophotometrically. The extent of the reaction will depend on the hydrogen donating ability of the antioxidants. The IC_{50} value of the leaf extract was 10.25µg/ml, where as the standard ascorbic acid was found to be 3.48µg/mL.

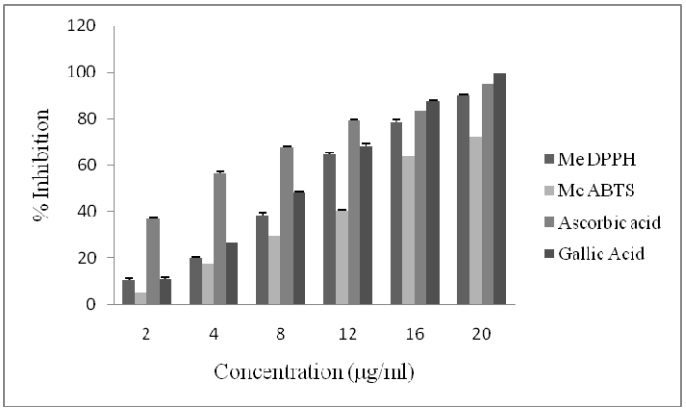

Figure 1- DPPH and ABTS radical scavenging effects of methanolic Leaf extracts of *Mimusops elengi*

The reduction capability of ABTS radical was determined by the decrease in its absorbance at 734nm which is induced by antioxidants [23]. The ability of the extract to scavenge ABTS radical cation was expressed in Figure 1 at a concentration range of 2-20µg/ml by comparison with standard. The methanol extracts showed most potent and concentration dependent ABTS scavenging activity with IC_{50} values of 13.5µg/ml, whereas the standard gallic acid has an IC_{50} of 8.94µg/mL respectively. It is well known

that antioxidant properties are very often related to the presence of an easily oxidizable portion of the molecule. The presence of a hydroxyl group on a hydrocarbon chemical makes the compound much easier to be oxidized [24]. Phenolic components of the methanolic extracts of *M. elengi* are probably involved in their free radical activity and the presence of these compounds would certainly contribute to the antioxidant activity.

The *in vitro* anti-inflammatory activity of *M. elengi* extracts was investigated by HRBC membrane stabilization method. The RBC membrane stability test is based on the finding that a number of non-steroidal anti-inflammatory agents inhibit heat induced lysis of erythrocytes, presumably by stabilizing the membrane of the cell. The percentage protection of erythrocyte membrane by *M. elengi* leaf extracts were given in Figure 2. The erythrocyte membrane may be considered a model of the lysosomal membrane which plays an important role in inflammation [25]. The compounds which prevent the lysis of membrane caused by the release of hydrolytic enzymes contained within the lysosomes may relieve some symptoms of inflammation [26]. When the human RBC is subjected to hypotonic stress, the haemoglobin release from RBC, will be prevented by anti-inflammatory drugs because of the membrane stabilization. It has been demonstrated that certain herbal preparations were capable of stabilizing the red blood cell membrane and this may be indicative of their ability to exert anti-inflammatory activity [27]. The mode of action of the extracts or drugs, may bind to the erythrocyte membranes with subsequent alteration of the surface charges of the cells. It has been reported that certain saponins and flavonoids exerted profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins and saponins possess ability to bind cations, thereby stabilizing erythrocyte membranes and other biological macromolecules [28]. It was noted that methanolic extract of leaves which showed positive tests for phenolics and flavonoids exhibited highest membrane stabilizing activity of 69.13±0.78 compared to that of standard Diclofenac potassium which exhibited 87.89±0.69% protection at 100 µg/mL concentrations respectively.

Results of acute toxicity study showed that there was no mortality or any significant change in the behaviour of the mice recorded up to the dose of 1000mg/kg of the plant extract. Based on the results of the preliminary toxicity testing, the doses of the extract for further studies were decided to be 100, 200 and 300 mg/kg body weight of the rats.

The anti-inflammatory activity of *M. elengi* methanolic extracts of leaves (MEL) in acute experimental model was reported in Figure 3 which explained that the results are comparable to that of a standard drug Indomethacin. Before injection of carrageenan, the basal values ranged between 0.39 and 0.65 ml (0.50±0.039 ml). Edema inhibition in the compound or drug treated groups was calculated with reference to the control group values and the percentage inhibition of all groups were shown in Figure 4. Extracts at oral doses of 100 mg/kg does not show much significant effect. Methanol leaf at 200 and 300 mg/kg showed a maximum inhibition of about 33.03% ($p<0.01$) and 32.2 % ($p<0.01$) in carrageenan induced rat paw edema, whereas indomethacin produced at 10 mg/kg produced 33.2% of inhibition after 12 hr of carrageenan injection. Carrageenan induced rat paw edema model is a suitable experimental animal model for evaluating the anti-inflammatory effects of natural products.

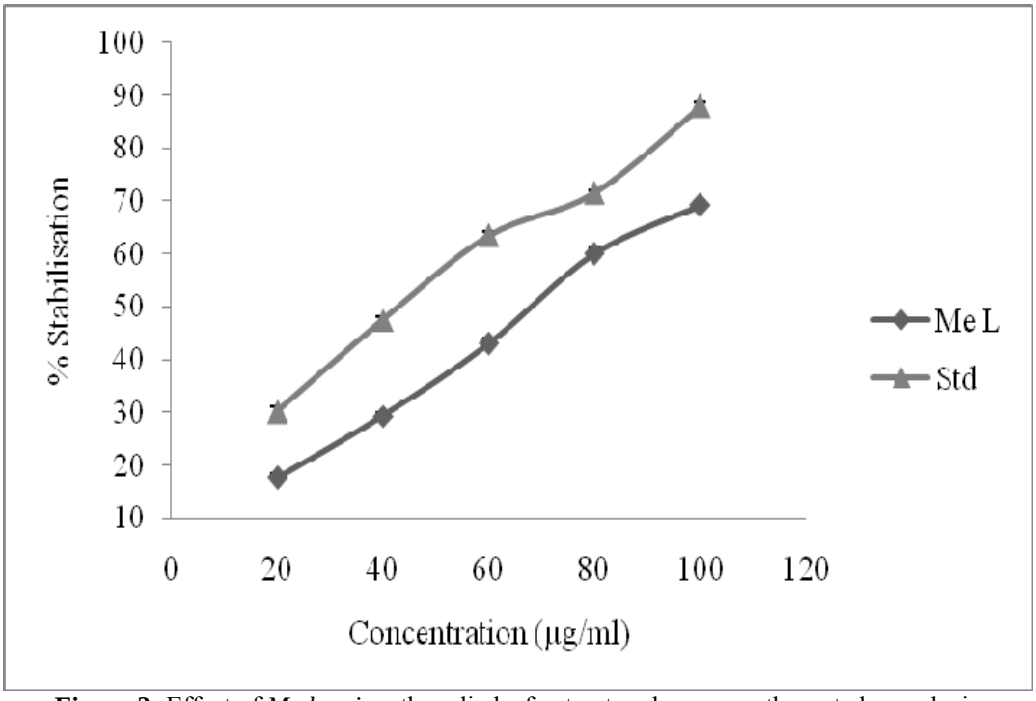

Figure 2- Effect of *M.elengi* methanolic leaf extract on human erythrocyte haemolysis

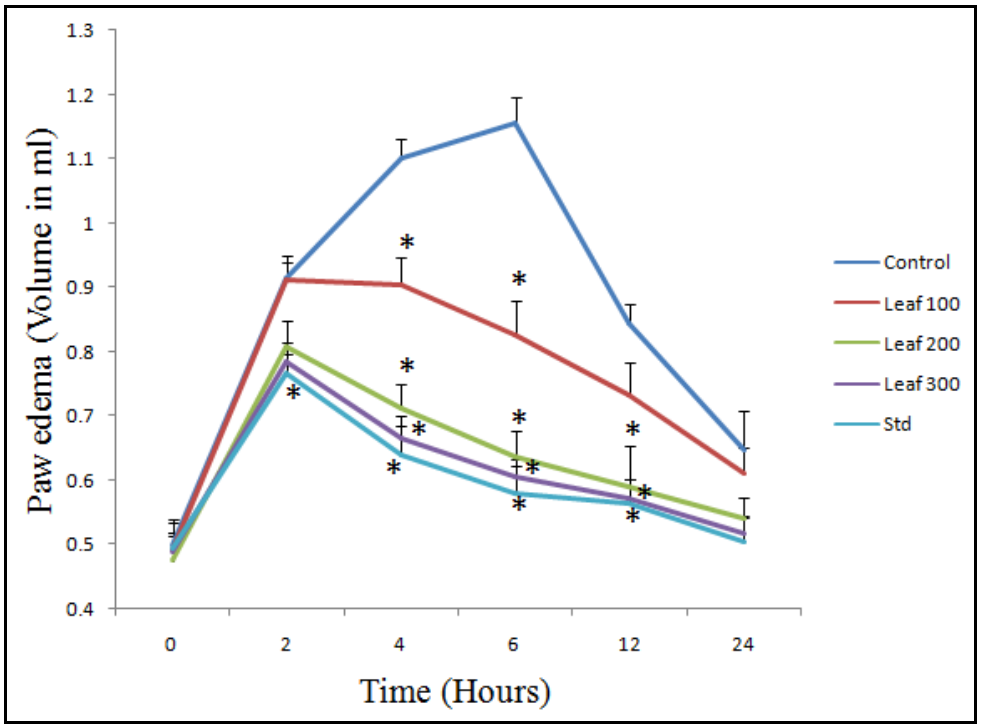

Figure 3- Effect of the Methanol Leaf extracts of *Mimusops elengi* on rat paw edema induced by carrageenan. Each value represents as mean±SEM (n=6, *P<0.01 as compared with control group)

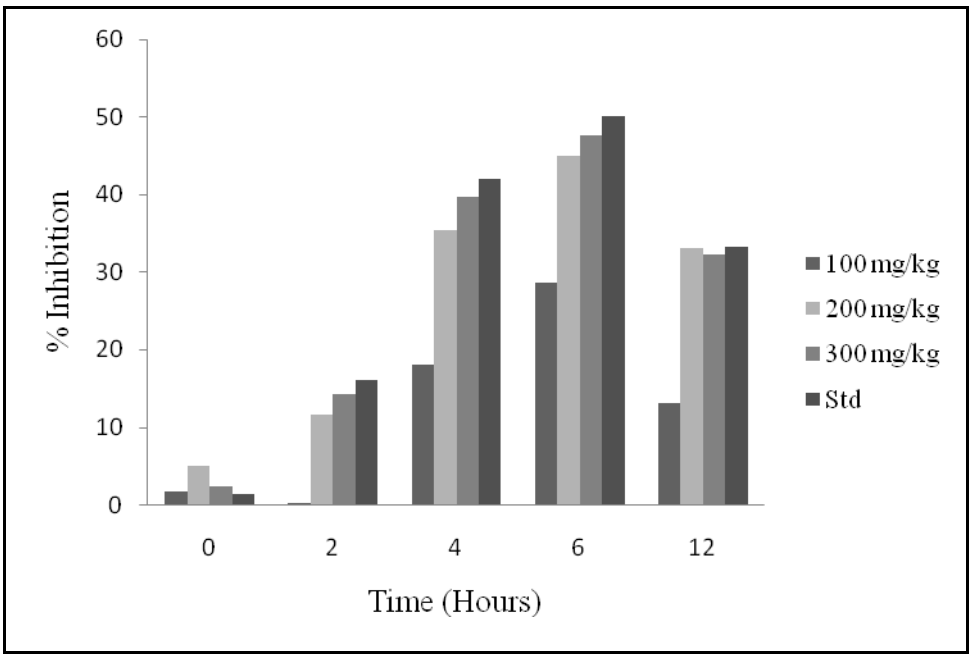

Figure 4 - Percentage inhibition of Methanol Leaf extracts of *Mimusops elengi* on rat paw edema induced by carrageenan

Generally inflammation occurs through two distinct phases and is a biphasic, the first phase (up to 1 h) involves the release of serotonin and histamine and in the second phase (after 1 h) is mediated by prostaglandin, the cylooxygenase products, and the continuity between the two phases is provided by bradykinins [29]. The extract at medium dose (200 mg/kg) was effective in inhibiting paw edema. The crude plant extracts are more pharmacologically active than their isolated active principles [30]. Many reports have shown that plant flavonoids possess potent anti-inflammatory and anti-oxidant properties [31]. The anti-inflammatory activity is may be due to inhibition of enzymes responsible for the production of chemical mediators of inflammation and arachidonic acid metabolism [32].

CONCLUSION

On interest, the methanolic extract was able to significantly reduce inflammation and also have an ability to scavenge free radicals which plays a major role in many metabolisms. These findings suggest that the traditional use of this plant is mostly justified. The further isolation and identification of the individual constituents present in the various fractions is currently under investigation in our laboratory.

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