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Anti-Inflammatory effect of ethyl acetate extracts and pure molecules of ethno medicinal plants

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Abstract

Aim/Objective: To evaluate the anti-inflammatory activity of ethyl acetate extracts and pure molecules of roots of *M. malabaricum*, *A. serpyllifolia*, and *L. aspera*

Materials and Methods: Edema was induced by injecting subcutaneously into the sub plantar tissue of the left hind paw of each Wistar rat (150 to 200 g [N=30]), 0.1 mL of 1% carrageenan suspension in saline. Rats were treated with drug vehicle (1% Sodium CMC) and served as normal control and those treated with ibuprofen as standard. Ethyl acetate extracts of *M. malabaricum*, *A. serpyllifolia*, *L. aspera* and pure molecules MM-1, AS-1, LA-1 obtained from ethyl acetate extract were tested for carrageenan induced paw edema. The drug effects were estimated by comparing the maximal edema response during 6 h and the total edema response as the area under the time course curve.

Results: Results showed that the ibuprofen (62.23 ± 1.88), crude extract of *M. malabaricum* (48.55 ± 1.27 , 56.52 ± 0.38 and 59.92 ± 0.78), *A. serpyllifolia* (40.1 ± 2.16 , 48.25 ± 1.65 and 55.42 ± 1.91) and *L. aspera* (41.68 ± 1.08 , 54.74 ± 0.88 and 59.68 ± 0.79) significantly inhibited the maximal edema response during the 6 h of the carrageenan-induced rat paw acute inflammation. The total edema response (AUC) was inhibited by 71.6 ± 1.99 , 62.31 ± 2.73 , and 63.21 ± 1.61 , 59.2 ± 1.96 , respectively over 6 h.

Conclusion/Future directions: The results suggested that the standard drug Ibuprofen significantly inhibited paw edema, whereas the ethyl acetate extract of *M. malabaricum*, *A. serpyllifolia* and *L. aspera* produced significant and dose dependent effect in reducing paw edema at the 2 phases of inflammation when compared to the drug vehicle treated group.

Keywords: *M. malabaricum*, *A. serpyllifolia*, and *L. aspera*, paracetamol, methanol extract

Introduction

Inflammation is a natural defense essential immune response mechanism of the mammalian body. This defense mechanism enables the body to survive during infection or injury and maintain tissue homeostasis in noxious conditions [1]. Inflammation is a complex biological response of vascular tissues disturbance due to pathogens, irritants, harmful stimuli, etc. Inflammation is known to be responsible for life-long lasting life threatening conditions such as rheumatoid arthritis, osteoarthritis, multiple sclerosis, psoriasis, etc. [2]

Non-steroidal anti-inflammatory drugs are generally used for the treatment of inflammatory diseases. However, the adverse effects associated with these drugs include increased risk of blood clot resulting in cardiovascular diseases. The development of anti-inflammatory drugs from the natural products is being considered and preferred in the current generation as they are rich eminent sources with diversified chemicals and phytoconstituents. [Error! Bookmark not defined., Error! Bookmark not defined.]

In India, the Science of Ayurveda had provided a system of medical treatment and most of the remedies for treating illnesses were taken from plants. During the last few decades, much work has been done in the field of natural products. Moreover, natural products have also served as lead molecules for the development of novel synthetic drugs, for example atropine for tropicamide, quinine for chloroquine, cocaine for procaine and tetracaine, etc. India is known as the 'Emporium of Medicinal Plants' due to the occurrence of several thousands of medicinal plants in different bioclimatic zones [3]. As the medicinal value of Indian traditional medicine cannot be ignored, researchers are gradually becoming interested in identification of active principles in their extracts with intensive follow-up study of their mechanisms of action.

Experimental Models used for Testing of Anti-inflammatory Activity

The inflammatory process involves a cascade of events that can be elicited by numerous stimuli, e.g. infectious agents, ischemia, antigen-antibody interactions, chemical, thermal or mechanical injury. The response is accompanied by the clinical signs of erythema, edema, hyperalgesia and pain inflammatory responses which occur in 3 distinct phases with different mechanisms.

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An acute transient phase characterized by local vasodilation and increased capillary permeability; a Sub-acute phase characterized by infiltration of leukocytes and phagocytic cells; and a chronic proliferative phase where tissue degeneration and fibrosis occur^[4].

In vivo methods for testing acute and sub-acute inflammation are as follows:

- UV – erythema in guinea pigs
- Vascular permeability
- Oxazolone induced ear edema in rats and mice
- Croton oil induced ear edema in rats and mice
- Paw edema in rats

Experimental Models for Evaluation of Anti-Inflammatory Activity

The various models available for testing anti-inflammatory activity with reasonable accuracy, minimum time and test compound consumption are described below:

Acute models of inflammation

Carrageenan induced edema model

Acute hind paw edema was induced either in mice or in rats by injecting 0.05 ml to 0.1 of 1% w/v Carrageenan which reaches a peak level at 3-5 h of carrageenan injection. Although edema can be induced by other phlogistic agents like dextrin, formaldehyde, 5-hydroxytryptamine, histamine bradykinin and prostaglandin E1 etc., for routine screening, acute carrageenan induced edema test was employed^[5, 6].

U.V. light induced erythema model

Exposure to U.V. radiation also induces acute erythema which is used as model for anti-inflammatory activity testing.

Chronic models of inflammation

Cotton pellet method

Chronic inflammation was induced by the implantation of sterile cotton pellets (50 mg \pm 1 mg) on the back or axilla of the rats aseptically. The peak effect was reached within 7 days^[7, 8].

Granuloma pouch method

Pouch on the back of the rat was produced by injecting 20 ml of air and 1.0 ml of 1% croton oil in olive oil or 0.5 ml of turpentine oil in the subcutaneous tissues in between the shoulder blades. The effect was seen after 7 days^[9, 10].

Formaldehyde induced arthritis

Arthritis was induced by injecting 0.1 mL of 2% formaldehyde solution into the subplanta region of one of the hind paws of rat on the first and third day of the 10 days' experiment^[11, 12].

Adjuvant induced arthritis

Chronic arthritis in rats was induced by injection of 0.5 mg of killed *Mycobacterium tuberculosis* (Difco) suspended in 0.1 ml of liquid paraffin into one of the hind paws. The effect was observed till 40 days of irritant injection^[13].

Plants with Anti-Inflammatory Activity

Inflammatory diseases including rheumatic diseases are very common and afflict people all over the world. Although, these diseases are present since ages, no substantial progress has

been made to cure permanently. Consequences such as toxicity, recurrence of symptoms after the discontinuation of treatment are associated with the current synthetic drugs. The greatest disadvantage of the presently available potent synthetic drugs lies in their toxicity and reappearance of symptoms after discontinuation of treatment. Screening and development of drugs for anti-inflammatory activity is an ongoing process with a hope to discover antirheumatic drugs from indigenous plants. The literature survey reveals that the plant species of about 96 genera belonging to 56 families have exhibited anti-inflammatory activity^[13, 14].

Objectives/aim

- To evaluate the anti-inflammatory activity of ethyl acetate extracts of roots of *M. malabaricum*, *A. serpyllifolia*, and *L. aspera*
- To evaluate the anti-inflammatory activity (*in-vivo*) of pure compounds MM-1 from *M. malabaricum*, AS-1 from *A. serpyllifolia*, and LA-1 from *L. aspera* on Carrageenan-induced rat paw inflammation

Materials and methods

Preparation of Extracts and Isolation of Phytoconstituents

Dried root powders of *M. malabaricum*, *A. serpyllifolia* and *L. aspera* were extracted. The Root materials were shade dried and were extracted in a Soxhlet apparatus successively with ethyl acetate and methanol. The solvent was removed by the process of distillation and the crude extract was dried under vacuum and stored in a desiccator prior to chromatographic separation. The extracts were subjected to hepatoprotective activity, the extract producing significant activity was column chromatographed.

Preliminary Extract Screening

The extract was preliminarily screened using thin layer chromatography (TLC) and nuclear magnetic resonance (NMR) to know the types of compounds present in the extracts. TLC was developed in n-hexane: ethyl acetate solvent systems of different polarities and then plates were visualized under Iodine vapor exposure, UV short wave 254 nm and long wave 366 nm. Plates were sprayed with a solution of visualizing reagent 10% H₂SO₄ in methanol followed by heating in an oven at 110 °C for up to five minutes. The compounds develop various colors with this reagent.

Chromatographic Techniques

The Column chromatography was done by standard procedure silica gel (400 g, finer than 200#, ACME) was used as adsorbent. The column was eluted with n-hexane, ethyl acetate and finally with methanol. Thin-layer chromatography was simultaneously used to identify and further separate compounds from the fraction using the same solvent system. The developing reagent is 10% H₂SO₄ in methanol. The dried and powdered roots of *M. malabaricum*, *A. serpyllifolia*, *L. aspera* were extracted using Soxhlet apparatus with ethyl acetate. Ethyl acetate extracts were tested for carrageenan induced paw edema. The extracts which produced significant anti-inflammatory effect were subjected to column chromatography. The pure molecules MM-1, AS-1, LA-1 obtained from ethyl acetate extract have been studied for anti-inflammatory effects on carrageenan induced paw

edema.

Selection of Animals

Wistar albino rats of either sex weighing between 150-200 g were obtained from National Institute of Nutrition, Hyderabad, Andhra Pradesh, India. The animals were housed under standard environmental conditions (temperature of 25 ± 2 °C with an alternating 12 h light-dark cycle and relative humidity of $50 \pm 15\%$), a week before the start and also during the experiment as per the rules and regulations of the Institutional Animal Ethics committee and by the Regulatory body of the government (Regd no. 516/01/A/CPCSEA). They were fed with standard laboratory diet (supplied by Ratan Brothers, India) and water *ad libitum* during the experiment. The rats were given doses orally with extracts at different dose levels 18 h and 2 h prior to the induction of carrageenan subcutaneously (SC) into the subplantar tissue of the hind paw of each rat, 0.1 ml of 1% Carrageenan suspension.

Carrageenan Suspension

Suspension of Carrageenan sodium salt (Sigma-Aldrich Chemicals Ltd.), 1% was prepared by sprinkling 100 mg of Carrageenan powder on 10 mL of saline (0.9%) solution and set aside to soak for 1 h. A homogeneous suspension was then obtained by thorough mixing with a magnetic stirrer.

Sodium Carboxy Methyl Cellulose Suspension

Stock suspension of Sodium carboxy methyl cellulose (CMC) was prepared by triturating 1g Sodium CMC in 100 ml of distilled water and used for suspending the test and standard drugs.

Induction of Paw edema

Wistar rats (150-200 g, [n=30] purchased from Mahaveer enterprises Ltd, Hyderabad) were used. Edema was induced by injecting, subcutaneously into the sub plantar tissue of the left hind paw of each rat, 0.1 ml of 1% Carrageenan suspension in saline.

The right hind paws of the same rats were administered with 0.1 ml of saline alone in the same manner as control. Before the induction of edema, the thickness of the both paws of each rat between lower and upper surface was measured using an instrument consisting of a graduated micrometer combined with a constant loaded lever system (Figure 1) to magnify the small changes in paw thickness during the course of the experiment. The measurements were then taken at 1h intervals after the induction of the edema for up to 6 h. Edema was

monitored as the percentage increase in paw thickness in the carrageenan injected paw. To assess the effect of saline on the edema produced, the percentage increase in paw thickness produced in the saline injected paw was subtracted from that of carrageenan injected left paw [15]. The percentage increase in paw thickness was plotted against the time (h) and the maximal edema response induced during the 6 h was determined. The total edema response as the area under the time course curves (AUC) was also determined.

The drug effects were estimated by comparing the maximal edema response during 6 h in the drug as extract treated group with that of vehicle treated group as control. Group I normal rats treated with drug vehicle (1% Sodium CMC) and served as normal control and Group II rats were treated with Ibuprofen 2.5×10^{-5} moles/kg body weight. All the doses were administered orally according to the body weight of the animals.

Evaluation of the Model

To evaluate this model, the percentage increase in paw thickness was plotted against the time (h) and the maximal edema response induced during the 6 h was determined. The results showed the ability of the model in detecting that the time course changes in the paw size was associated with carrageenan induced rat paw edema. The paw edema was constantly increased during 4 h and reached peak of edema at 4th hour. At the 5th and 6th hour, the edema gradually reduced.

Assessment of drug effects

For screening purposes, drugs (extract or compounds) in sodium CMC were always pre-dosed to rats prior to the induction of carrageenan paw edema. The drug actions were evaluated by comparing the maximal paw edema response during 6 h (monitored as % increase in paw thickness) in the drug treated groups with that produced in the drug vehicle (control) treated group. The total (area under the time course curve, AUC, calculated using Trapezoid Rule) edema response as the area under the time course curve, produced in the drug treated groups was also compared with that from the control group.

The pure molecules isolated from *M. malabaricum*, *A. serpyllifolia*, and *L. aspera* at single dose were administered orally in sodium CMC suspension 18 h and 2 h prior to the induction of edema by carrageenan injection and monitored the edema progression.

Extracts were administered orally in the following order	
Group III	Received ethyl acetate extract of <i>M. malabaricum</i> 200 mg/kg
Group IV	Received ethyl acetate extract of <i>M. malabaricum</i> 400 mg/kg
Group V	Received ethyl acetate extract of <i>M. malabaricum</i> 800 mg/kg
Group VI	Received ethyl acetate extract of <i>A. serpyllifolia</i> 200 mg/kg
Group VII	Received ethyl acetate extract of <i>A. serpyllifolia</i> 400 mg/kg
Group VIII	Received ethyl acetate extract of <i>A. serpyllifolia</i> 800 mg/kg
Group IX	Received ethyl acetate extract of <i>L. aspera</i> 200 mg/kg
Group X	Received ethyl acetate extract of <i>L. aspera</i> 400 mg/kg
Group XI	Received ethyl acetate extract of <i>L. aspera</i> 800 mg/kg
Pure molecules were administered in the following order	
Group XII	Received MM-1 from <i>M. malabaricum</i> at 2.5×10^{-5} moles/kg
Group XIII	Received AS-1 from <i>A. serpyllifolia</i> at 2.5×10^{-5} moles/kg
Group IV	Received LA-1 from <i>L. aspera</i> at 2.5×10^{-5} moles/kg

Results

Crude Ethyl acetate Extracts

Results showed that the ibuprofen and crude extract of *M. malabaricum* significantly inhibited the maximal edema response by 62.23 ± 1.88 , 48.55 ± 1.27 , 56.52 ± 0.38 and 59.92 ± 0.78 , respectively during the 6 h of the carrageenan-induced rat paw acute inflammation. The total edema response (AUC) was inhibited by 71.6 ± 1.99 and 55.85 ± 3.52 , 69.51 ± 1.55 and 71.03 ± 4.19 respectively over 6 h when compared to the control group treated with drug vehicle at first and second phases of inflammation.

Crude extract of *A. serpyllifolia* significantly inhibited the maximal edema response by 40.1 ± 2.16 , 48.25 ± 1.65 and 55.42 ± 1.91 during the 6 h of the carrageenan-induced rat paw acute inflammation. The total edema response (AUC) was inhibited by 54.06 ± 2.18 , 59.21 ± 2.84 and 68.21 ± 2.68 over 6 h when compared to the control group treated with drug vehicle at first and second phases of inflammation.

The results showed that the crude extract of *L. aspera* significantly inhibited the maximal edema response by 41.68 ± 1.08 , 54.74 ± 0.88 and 59.68 ± 0.79 during the 6 h of the carrageenan-induced rat paw acute inflammation. The total edema response (AUC) was inhibited by 56.62 ± 1.61 , 60.86 ± 1.82 and 66.61 ± 1.73 over 6 h when compared to the control group treated with drug vehicle at first and second phases of inflammation. The results of the standard and the ethyl acetate extracts of the studied plants are presented in Table 1, Figures 2 and 3.

The results suggested that the standard drug Ibuprofen significantly inhibited paw edema, whereas the ethyl acetate extract of *M. malabaricum*, *A. serpyllifolia* and *L. aspera* produced significant and dose dependent effect in reducing paw edema at the 2 phases of inflammation when compared to the drug vehicle treated group.

Pure Molecules

The results showed that the ibuprofen, pure compounds MM-1, AS-1, and LA-1, significantly inhibited the maximal edema response by 62.23 ± 1.88 , 55.45 ± 6.01 , 53.21 ± 1.86 , and 50.08 ± 1.66 , respectively during the 6 h of the carrageenan-induced rat paw acute inflammation. The total edema response (AUC) was inhibited by 71.6 ± 1.99 , 62.31 ± 2.73 , and 63.21 ± 1.61 , 59.2 ± 1.96 , respectively over 6 h. The pure molecules exhibited significant reduction in reducing paw edema when compared to the control group at all evaluated intervals of time. The results of percentage inhibition of the maximal paw edema and total AUC paw during 6 h is depicted in Table 2 and Figure 4.

The results suggested that both standard Ibuprofen and pure molecules MM-1, LA-1 significantly inhibited paw edema compared to drug vehicle treated group. The pure molecule MM-1 exhibited almost 97% equipotency compared to standard drug treated group. To confirm the exact potency, it is still required to carry out experiment with lower and higher dose of the pure molecule. As there is no sufficient pure molecule, it was not possible to conduct the experiment with other doses. The pure molecule AS-1 exhibited almost 90% equipotency compared to standard drug treated group. The exact potency was not determined due to lack of availability of the pure molecule. The pure molecule LA-1 exhibited greater effect when compared to the standard drug. The exact potency was not determined due to dearth of the pure

molecule. The significant activity exhibited by the pure molecules might be due to the sterol content (Reference). However, the exact mechanism behind the activity should be identified.

Discussion

The most widely used and industrially accepted experimental model to evaluate anti-inflammatory activity is Carrageenan induced paw edema in rat's acute inflammatory model. Edema formation in paw is the result of synergism between various inflammatory mediators that increase vascular permeability and blood flow [16].

Carrageenan, the experimental inflammation produced significant edema in the left hind paw of the rats and the paw edema in rats was significantly ($p < 0.01$) reduced by the standard drug, ibuprofen (2.5×10^5 moles/kg). The methanolic extract of *M. malabaricum*, *A. serpyllifolia* and *L. aspera* roots at three different doses produced significant ($p < 0.05$) reduction at 400 and 800 mg/kg, whereas at the dose of 200 mg/kg has not produced significant reduction when compared to drug vehicle treated control group.

The development of edema after the injection of carrageenan has been described as biphasic event. The initial phase which occurs between 0 and 2.5 h has been attributed to the action of histamine, serotonin and bradykinin on vascular permeability [17]. The edema volume reaches its maximum approximately at 3h post treatment and then begins to decline. The late phase, which is also a complement dependent reaction, has been shown to be a result of over production of prostaglandins in tissues [18].

The ethyl acetate extracts from the three different plants inhibited the edema at the first hour by acting in both the earlier and the later phases. This indicates that the extracts could inhibit different aspects and chemical mediators of inflammation (histamine, serotonin, bradykinin and prostaglandins). Pharmacological investigations clearly indicated that anti-inflammatory activity of many plants has been attributed to their flavonoid and sterol contents [19]. Several flavonoids isolated from the medicinal plants have been discovered to possess significant anti-inflammatory activity [20].

The results clearly indicated that the pretreatment with the selected plant extracts and ibuprofen have contained the increase in paw edema due to the phlogistic agent. All the 3-ethyl acetate extracts of *M. malabaricum*, *A. serpyllifolia* and *L. aspera* produced significant reduction of paw edema than methanolic extracts. The exact bioactive principle responsible for the reduction in paw edema due to carrageenan remains to be explored. Furthermore, it is difficult, at this stage to draw any logical conclusion on the mechanism of anti-inflammatory action of such a diverse mixture of chemical compounds contained in the plants of *M. malabaricum*, *A. serpyllifolia* and *L. aspera*.

Conclusion

The results clearly suggest that pretreatment with *M. malabaricum*, *A. serpyllifolia* and *L. aspera* plant extracts and ibuprofen have contained the increase in paw edema due to the phlogistic agent. All the 3-ethyl acetate extracts of selected plants produced significant reduction of paw edema than methanolic extracts. The exact bioactive principle responsible for the reduction in paw edema due to

carrageenan remains to be explored. Furthermore, it is difficult, at this stage to draw any logical conclusion on the mechanism of anti-inflammatory action of such a diverse mixture of chemical compounds contained in the plants of *M. malabaricum*, *A. serpyllifolia* and *L. aspera*. Further studies have to be conducted on chronic inflammation (Adjuvant induced arthritis model) to establish the mechanism of action for the extracts which exhibited significant activity.

Table 1: The anti-inflammatory effect of ethyl acetate extracts and standard group used in the study

Treatment Group	Percentage inhibition of the maximal paw edema during 6 h	Percentage inhibition of total AUC paw edema during 6 h
Group I	0.0 ± 4.22	0.0 ± 6.19
Group II	62.23 ± 1.88**	71.6 ± 1.99**
Group III	48.55 ± 1.27*	55.85 ± 3.52*
Group IV	56.52 ± 0.38**	69.51 ± 1.55**
Group V	59.92 ± 0.78 **	71.03 ± 4.19**
Group VI	40.1 ± 2.16**	54.06 ± 2.18**
Group VII	48.25 ± 1.65**	59.21 ± 2.84**
Group VIII	55.42 ± 1.91**	68.21 ± 2.68**
Group IX	41.68 ± 1.08*	56.62 ± 1.61*
Group X	54.74 ± 0.88**	60.86 ± 1.82**
Group XI	59.68 ± 0.79**	66.61 ± 1.73**

Table 2: The anti-inflammatory effect of pure molecules and standard group used in the study

Treatment Group	Percentage inhibition of the maximal paw edema during 6 h	Percentage inhibition of total AUC paw edema during 6 h
Group XII	55.45 ± 6.01**	62.31 ± 2.73**
Group XIII	53.21 ± 1.86**	63.21 ± 1.61**
Group XIV	50.08 ± 1.66*	59.2 ± 1.96**

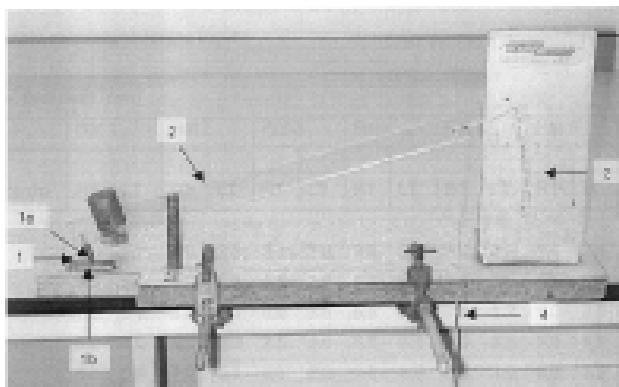


Fig 1: Zeitin's Constant Loaded Lever (Paw thickness measuring device)

1. Place where the paws use to be kept to measure the thickness; 2. Constant loaded lever; 3. Graduated scale numbered between 1 – 10 and divided by 0.5 equal to 20 divisions; Thread to pull down the lever with right leg in order to facilitate to keep the paw in between pointer 1a and basement 1b.

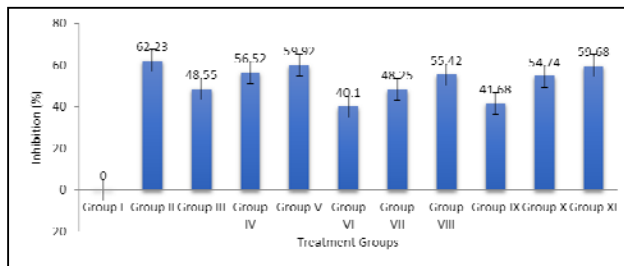


Fig 2: Percentage inhibition of the maximal paw edema during 6 h between the treatment groups and standard group

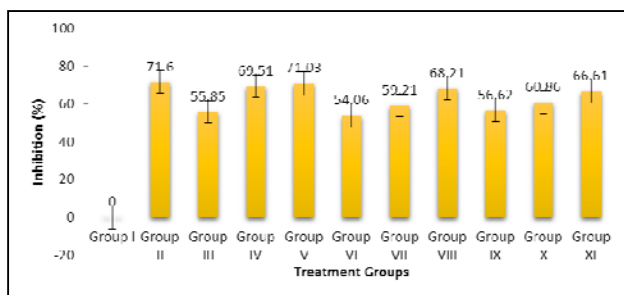


Fig 3: Percentage inhibition of total AUC paw edema during 6 h between the treatment groups and standard group

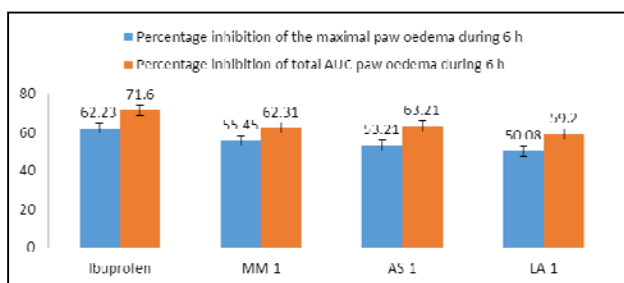


Fig 4: Anti-inflammatory effect of pure molecules MM-1, AS-1, LA-1 in comparison with Ibuprofen

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