Anti-inflammatory activity of cyclo-oxygenase2 inhibitory anionic protein fraction from *Lamellidens marginalis* (Lamarck)

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Aqueous extract of freshwater mussel, *Lamellidens marginalis* is known to possess potent antioxidant and anti-inflammatory activity. Here, we have made an attempt to purify anti-inflammatory protein from *Lamellidens marginalis* extract (LME). Aqueous LME was prepared, and total protein was precipitated by 60% ammonium sulfate followed by purification through ion exchange chromatography. Isolated fractions were studied for anti-inflammatory activity in *in vitro* and *in vivo* experimental models. Active fractions were characterized by SDS PAGE and HPLC. Protein recovered from ammonium sulfate precipitation showed four distinct peaks in diethyl-aminoethyl cellulose ion exchange chromatography when eluted with stepwise salt gradient. Protein fraction eluted in 0.5 M sodium chloride solution showed maximum specific activity and anti-inflammatory activity in acute model and adjuvant induced chronic inflammation model. This fraction also showed cyclo-oxygenase 2 (COX2) enzyme inhibitory activity in *in-vitro* system. In SDS-PAGE 0.5 M NaCl fraction showed multiple bands after Coomassie brilliant blue staining and three distinct peaks in HPLC. In this study, we identified an anti-inflammatory protein fraction with high anionic property which could be attributed to inhibition of COX2 enzyme activity.

**Keywords:** Arthritis, Bivalve, COX2, Freshwater mussel, Inflammation, Protein purification

Inflammation is the body’s first response against invading pathogens which could be described by four major symptoms like dolor (pain), calor (heat), rubor (redness), and tumor (swelling). These appear superficially in case of acute inflammation. Aggravation and persistence of that acute inflammation may lead to the development of chronic inflammation,¹ which is an underlying mechanism of many diseases like osteoporosis, arthritis, cancer etc. Anti-inflammatory drugs are commonly used to control inflammation and related diseases. Nonsteroidal anti-inflammatory drugs, though has been used for hundreds of years, showed several side effects like ulcer, cardiovascular problems, etc. Recent development in immune selective anti-inflammatory derivatives, known as ‘biologicals’, are not only costly but also have other limitations such as their undefined interactions with immune system and lack of detail pharmacokinetic study. Steroidal drugs like glucocorticoids are also in use, though it showed cardiovascular problems and drug dependencies. Hence, search for new anti-inflammatory molecules from natural products still continue to progress²-⁸.

Aquatic animals are one of the infinite sources of anti-inflammatory molecules. Several anti-inflammatory proteins and peptides have already been identified from different aquatic invertebrates and vertebrates. Many of them are found to be conserved throughout different species and are the important part of innate immunity. Peptidoglycan recognition proteins isolated from Rainbow trout⁹, a high molecular weight protein purified from homogenate of marine polychaete (*Mastobranchus indicus*)¹⁰, and a novel lectin, HGA-2, isolated from the sea cucumber *Holothuria grisea*¹¹ are some of the examples of such proteins with anti-inflammatory activity.

Mussels are commonly used against inflammation all over the world. Studies revealed that high level n3 polyunsaturated fatty acids and some proteins in mussel are one of the important factors responsible for their anti-inflammatory activity.¹² Miller et al.¹³ proposed that anti-inflammatory activity of glycogen extract from *Perna canaliculus* is associated with...
some protein moiety as the activity was lost when the extract was treated with potassium hydroxide or proteinase K. Mani and Lawson\textsuperscript{14} also described loss of anticytokine activity of Tween 20 extract of the Perna mussel after treatment with proteinase K. These findings indicate that the activity of the extract is associated with the protein. In addition, antioxidant polyphenolic proteins were isolated from different marine mussels as well\textsuperscript{15}.

In our previous study, we have reported antioxidant activity of aqueous extract of \textit{Lamellidens marginalis}\textsuperscript{16}. Estari \textit{et al.}\textsuperscript{17} reported antibacterial activity of this freshwater bivalve in \textit{in vitro} models.

In the present study, we explored \textit{L. marginalis} for its anti-inflammatory activity associated mainly with its protein component in \textit{in vitro} and \textit{in vivo} experimental models of inflammation.

**Materials and Methods**

**Reagents**

FCA, carrageenan, DEAE cellulose and indomethacin were purchased from Sigma, USA. Hydroxyproline (MP Biomedical, France), rat IL6, TNFα, CINC1 kit (R&D, USA), Acetic acid (SRL, India), xylene (SRL, India), Cyclooxygenase 2 (Cayman Chemical, USA), Hematin (Himedia, India) were used in experiments.

**Animals**

Swiss male albino mice weighing 20±2 g and Wistar strain male albino rats were used in the experiments. Animals were obtained from animal facility, CNCI, Kolkata, and housed under a controlled environment (RT: 23±2°C, relative humidity: 60±5%, 12 h day/night cycle) with balanced diet and water \textit{ad libitum}. All animal experiments were approved by the animal ethical committee, Department of Physiology, University of Calcutta and were in accordance with the guideline of the committee for the purpose of control and supervision of experiments on animal (IAEC Ref no:820/04/ac/CPCSEA.2010).

**Collection of sample and preparation of extract**

Live adult fresh water mussels of both sexes were collected from local market of Kolkata, India and the species was identified as \textit{Lamellidens marginalis} (Lamarck) (Voucher specimen no: M26322/5) from mollusca section of Zoological Survey of India, New Alipore, Kolkata, India. Foot pad portion of mussel was taken and aqueous homogenate (LME) was prepared using phosphate buffer (0.01 mM, pH 7.4; 1 mM phenyl methyl sulfonyl fluoride). It was then centrifuged (10000 rpm), supernatant (LME) was collected, stored at 2-4°C and used within 3-4 days for experiment. Protein content of LME was determined\textsuperscript{18} and expressed as gram of protein per 100 g of tissue.

**Study of anti-inflammatory activity of LME in \textit{in vitro} models**

**Human Red Blood Cell (HRBC) membrane stabilizing activity**

Fresh whole human blood was collected, centrifuged at 3000 rpm for 10 min and packed cells were washed thrice with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline.

The principle involved here is stabilization of human red blood cell membrane against heat induced membrane lysis. The assay mixture contains 1 mL phosphate buffer saline, 0.5 mL HRBC suspension [10% v/v] with 0.5 mL of extracts of various concentrations (16-65 µg protein), normal control (phosphate buffer, 0.01 mM, pH 7.4 instead of extract) and lysis control (distilled water instead of extract to produce 100% hemolysis) were incubated at 57°C for 30 min and centrifuged respectively. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm\textsuperscript{19}.

The percentage of hemolysis of HRBC membrane was calculated as follows:

\[
\% \text{ Hemolysis} = \left( \frac{\text{Optical density of test sample}}{\text{Optical density of control}} \right) \times 100
\]

The percentage of HRBC membrane stabilization was calculated as follows:

\[
\% \text{ Protection} = 100 – \left[ \frac{\text{Optical density of test sample}}{\text{Optical density of control}} \right] \times 100
\]

**In vitro cyclo-oxygenase2 (COX2) inhibitory activity**

COX2 inhibitory activity was determined by spectrophotometrically at 610 nm depending on the principle of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) oxidation. In brief, COX2 enzyme was incubated with LME (65-330 µg protein) for 5 min in 37°C with 0.5 mM heme solution at pH 8.0. After incubation 100 µM arachidonic acid and 10µM TMPD was added and was further incubated for 5 min and absorbance was taken at 610 nm and percent
inhibition in TMPD oxidation was calculated considering control as 100% oxidation\textsuperscript{20}.

**Lethality testing**

Minimum lethal dose (MLD) of *Lamellidens marginalis* extract was determined in male Swiss albino mice (20±2 g). Mice (n=6) was subjected to different dose of crude extract through i.p. route and mortality was recorded up to 24 h of treatment. The minimum dose of crude extract required for death of all 6 animals within 24 h was considered as minimum lethal dose\textsuperscript{21}.

**Effect of LME in \textit{in vivo} model of inflammation**

*Carrageenan induced acute inflammation*

Anti-inflammatory activity of LME was assessed in carrageenan induced paw edema model of acute inflammation\textsuperscript{22}. In short, the initial right hind paw volume of the mice was measured using digital calipers. 0.02 mL of 2.5% (w/v) carrageenan was injected into the sub-plantar region of the right hind paw. Right hind paw diameter was measured at one hour interval up to 5 h after carrageenan injection, and the change in paw diameter was determined. LME (75 and 150 mg/kg) was administered intraperitoneally (i.p.) 1 h prior to carrageenan injection.

At the end of the experiment paw was isolated and myeloperoxidase enzyme activity of paw homogenate were measured from all group of animals\textsuperscript{23}.

*Xylene induced inflammation*

Anti-inflammatory activity of LME was assessed in xylene induced ear edema model of acute inflammation\textsuperscript{24}. About 30 µl of xylene was given on the anterior and posterior surfaces of the right ear lobe of mice. LME (75 and 150 mg/kg; i.p.) were administered 1 h prior to giving xylene. After one hour the animals were sacrificed by cervical dislocation, and the right and left ears of each animal were removed. The left ear was considered as control. Circular sections were taken with a cork borer (diameter of 7 mm) and weighed. At the end of the experiment, ear tissues were isolated and myeloperoxidase enzyme activity of ear homogenate was measured from all group of animals\textsuperscript{23}.

**Assessment of tissue Myeloperoxidase (MPO) activity**

The activity of tissue MPO was assessed according to Bradley \textit{et al.}\textsuperscript{25}. The tissue samples of normal and treated groups were homogenized (w/v) in 50 mM potassium phosphate buffer (pH 7.2) containing 1% hexadecyl trimethyl ammonium bromide (HTAB). The homogenates were centrifuged at 10000 rpm for 15 min, supernatant was separated and assayed for MPO activity. Samples (50 µL) were mixed with phosphate buffer (500 µL) containing 1 mM O-dianisidine dihydrochloride and 0.001% hydrogen peroxide. Absorbance was kinetically measured at 480 nm, 30 s intervals up to 2 min. MPO activity was expressed as Unit of MPO per mg of tissue protein where 1 unit of MPO activity was defined as 0.1 absorbance change at 480 nm\textsuperscript{26}.

**Purification of protein fraction from LME**

*Lamellidens marginalis* extract (LME) was prepared in 0.01 M phosphate buffer at pH 7.4 and the soup was brought to 60% ammonium sulfate [(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}] saturation. Solution was then centrifuged and the precipitate was reconstituted in 0.01 M phosphate buffer. It was then dialyzed against 0.01 M phosphate buffer to remove excess (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} from solution.

It was further subjected to ion exchange chromatography using DEAE cellulose column (20×80 mm) and phosphate buffer (0.01 M, pH 7.4). Fractions were collected at room temperature (25±2ºC) by stepwise salt (0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 M of NaCl) gradient. Fraction collected was subjected to protein estimation. The entire fractions were tested for their \textit{in vitro} cyclo-oxygenase2 enzyme inhibitory activity and \textit{in vivo} anti-inflammatory activity in carrageenan model\textsuperscript{22,24}. The fraction with highest specific activity was selected for antiarthritic activity study.

**Characterization of protein component from LME**

Active protein fraction *Lamellidens marginalis* protein fraction 4 (LMPF4) (0.5 M fraction) from LME was characterized by 12% SDS PAGE\textsuperscript{27} and HPLC (60: 40 methanol: water solvent in C18 column 4×250 mm, flow rate: 0.5 mL/min). Detection of protein peaks in HPLC was done at 280 nm and the retention time of the eluted fractions were measured.
Antiarthritis activity of protein fraction in adjuvant induced chronic inflammation model

Adjuvant induced arthritis model was induced in Wistar strain male albino rats. The day of arthritis induction was considered as 0 day. On the 1st day after induction of arthritis animals were divided into the following groups: Group I, normal control; Group II, arthritic control; Group III, LMPF4; and Group IV, standard. Animals of Group I and II were treated with 0.5 mL of 0.9% saline (i.p.); and Group III and IV were treated with LMPF4 (100 µg/Kg, i.p.) and standard drug Indomethacin (1 mg/kg) perorally (p.o.), respectively from the next day of arthritis induction. All the treatments were carried out for next 13 days. All groups of experimental animals were provided with normal diet and water ad libitum.

On 14th day urine was collected from all groups of animals for 24 h in fasting condition and on the next day animals were anesthetized and blood was collected from hepatic portal vein without anticoagulant. Serum was separated by centrifugation (2500 rpm × 25 min) and stored at −20ºC until assessment of different biochemical parameters (except interleukins). Paw and joints were separated and weighed for physical parameter assessment.

Arthritis severity was assessed from arthritis score index, urinary parameters (hydroxyproline, glucosamine), and serum markers (cytokine-induced neutrophil chemoattractant 1 (CINC1); prostaglandin E2 (PGE2); acid phosphatase (ACP); and alkaline phosphatase (ALP) level).

Arthritic score was determined on a blinded manner considering edema and erythema of the toes, right paw and ankle of arthritis and treated group of rats in a scale of 0-4, where 0 meant no inflammation; 1, erythema and edema of toes; 2, edema of paws; 3, edema of ankles; and 4, restricted movement of the limbs. The score was recorded on 1st, 5th, 10th, and 15th day respectively. Urinary hydroxyproline was measured according to Neuman & Logan; glucosamine, according to Elson & Morgan; serum phosphatases, as explained by Michell et al.; and interleukins by ELISA following manufacturer’s instructions.

Fig. 1—Study of anti-inflammatory activity of LME in in vitro models (A) HRBC membrane stabilizing activity; and (B) In vitro cyclo-oxygenase2 enzyme inhibitory activity.

Statistical analysis

All the results were expressed as mean±SD, n = 6. Level of significance was determined by one-way ANOVA followed by Tukey’s post hoc test. P <0.05 was considered as significant.

Results

Lethality

The protein content of the Lamellidens marginalis extract (LME) was 1.136 ±0.21 g% (wet weight) and the LME treatment was lethal at the dose of 300 mg/kg body wt. after intra-peritoneal injection in mice.

Anti-inflammatory activity

In vitro HRBC membrane stabilizing and cyclo-oxygenase inhibitory activity

LME showed HRBC membrane stabilizing activity against heat induced hemolysis in a dose dependent manner (16-65 µg protein) (Fig. 1A). Similarly, it exhibited cyclo-oxygenase2 (COX2) enzyme inhibitory activity dose dependently (65-330 µg protein). The IC50 value of LME for cyclo-oxygenase inhibitory activity was 0.325 mg protein of LME (Fig. 1B).

In vivo carrageenan induced acute inflammation

LME treatment (150 and 75 mg/kg, i.p.) decreased carrageenan induced paw edema dose dependently by 39.47 and 32.89%, respectively as compared to positive control animals after the 4th h of carrageenan injection (Fig. 2 A-C). Significant effect of LME treatment against carrageenan induced paw edema was also reflected in the assessment of MPO enzyme activity from paw homogenate, where treated group showed significant 74.42, 43.98% protection, respectively as compared to positive control animals (Fig. 2D).
In vivo xylene induced inflammation

LME treatment (150 and 75 mg/kg, i.p.) decreased xylene induced ear edema dose dependently by 29.46 and 19.14% as compared to positive control animals (Fig. 3A). Significant effect of LME treatment against xylene induced ear edema was also reflected in the assessment of MPO enzyme activity from ear homogenate, where treated group showed significant 51.50% and 33.26% protection respectively as compared to positive control animals (Fig. 3B).

Purification, activity assessment and characterization of protein fraction from LME

Total protein of LME was precipitated by 60% (NH₄)₂SO₄ which further produced four distinct peaks (LMPF1, LMPF2, LMPF3, LMPF4) after purification through DEAE cellulose ion exchange chromatography (Fig. 4A). All the peaks showed dose dependent in vitro cyclo-oxygenase 2 enzyme inhibitory activity (Fig. 5A) among which LMPF4 showed maximum activity (IC₅₀: 4.312 µg protein) (Fig: 4D). LMPF1, LMPF2, LMPF3, LMPF4 were then further assessed in in vivo

Fig. 2—Effect of LME in (A) in vivo model of carrageenan induced acute inflammation (A1, A2) morphology of left paw of carrageenan control and LMPF4 treated animals respectively, (A3, A4) morphology of right paw of carrageenan control and LMPF4 treated animals respectively; (B) on change in paw diameter per hour against carrageenan induced acute inflammation; (C) on change in paw diameter on the 4th hour against carrageenan induced acute inflammation; and (D) on tissue MPO activity in carrageenan induced acute inflammation.

Fig. 3—Effect of LME in in vivo model of xylene induced ear edema. Effect of LME (A) on difference in ear weight; and (B) on tissue MPO activity in xylene induced acute inflammation.

In vivo xylene induced inflammation
carrageenan induced inflammation model with different doses. LMPF4 fraction (1.25 µg/kg, i.p.) showed significant 20.43% inhibitory activity against carrageenan induced paw edema (Fig. 5B). LMPF4 showed maximum specific activity (231.90) and yield (1.36%) among all the other fractions (Fig. 4D).

LMPF4 fraction showed several major and minor banding patterns in SDS PAGE after CBB staining (Fig. 4B). LMPF4 produce four sharp peaks in HPLC C-18 column, with retention time 3.639 (Peak 1), 5.408 (Peak 2), 18.062 (Peak 3), and 19.209 (Peak 4) (Fig. 4C).
Antiarthritis activity of LMPF4 in adjuvant induced chronic inflammation model

Assessment of arthritis from arthritis score index

Arthritis score index data showed development of arthritis in group II rats where significantly increased edema, erythema and restriction of movement of limbs were observed as compared to the normal control group. LMPF4 treated group III rats and indomethacin treated group IV rats showed significant restoration of changes in physical characteristics as compared with group II arthritis rats (Fig. 6A).

Paw weight of arthritis group II rats showed significant increase on 15th day after FCA injection, as compared with normal group I rats. LMPF4 treated group III and indomethacin treated group IV rats showed significant decrease in paw weight by 35.89, and 45.77% respectively on the 15th day after FCA injection, as compared with arthritis group II rats.

Assessment of arthritis from urinary parameters

Urinary hydroxyproline and glucosamine level was increased significantly in arthritis group II animals as compared with normal control group I animals after 14th day of FCA injection. LMPF4 treated group III, and indomethacin treated group IV rat showed significant decrease in hydroxyproline and glucosamine level as compared with arthritis group II animals (Fig. 6B).

Assessment of arthritis from serum parameters

Serum CINC1, PGE2 level, ACP and ALP level was increased significantly in arthritis group II animals as compared with normal control group I animals after 14th day of FCA injection. LMPF4 treated group III, and indomethacin treated group IV showed significant decrease in serum CINC1, PGE2, ACP and ALP level as compared with arthritis group II animals. No significant change in serum ALP level was observed in indomethacin treated Group IV animals as compared with arthritis group II animals (Fig. 6C).

Discussion

Aqueous *Lamellidens marginalis* extract was prepared and assessed for HRBC membrane stabilizing activity, cyclo-oxygenase 2 enzyme inhibitory activity in *in vitro* models and anti-inflammatory activity against carrageenan induced acute inflammation, xylene induced inflammation in *in vivo* models. These are well accepted models used widely for screening of anti-
inflammatory agents. Human red blood cell membrane is considered equivalent to the membrane of lysosomes, which has active participation in regulation of inflammation. Anosike et al. explain the role of membrane stabilization in regulation of inflammation. In the present study, LME showed significant dose dependent inhibition of cyclo-oxygenase 2 associated in different disease. Cyclo-oxygenase2 is the enzyme that causes production of proinflammatory prostaglandin E2 from membrane phospholipids through arachidonic acid pathway. PGE$_2$ further contribute significantly in the development of inflammation associated in different disease. Nonsteroidal anti-inflammatory drugs act as cyclo-oxygenase 2 inhibitor to control inflammation.

In the present study, LME showed significant dose dependent inhibition of cyclo-oxygenase 2 enzyme activity in $in$ $vitro$ model. IC$_{50}$ value for cyclo-oxygenase2 enzyme inhibitory activity was found to be 0.325 mg protein of LME. These two $in$ $vitro$ studies indicated that LME might have some potential to control inflammation by modulating inflammatory cells and mediators. Thus, it was further studied for anti-inflammatory activity in $in$ $vivo$ models of inflammation which involve both inflammatory cells like neutrophils, containing lysosomes and inflammatory mediators like histamine, serotonin, and COX2. The minimum lethal dose of LME in i.p. route in mice to determine the non toxic treatment dose of LME for $in$ $vivo$ experiments was found to be 300 mg of protein/kg body wt. Hence, equal or less than half MLD doses were selected for acute inflammation in i.p. route $viz.$ 150 and 75 mg/kg dose. Carrageenan induced paw edema model and xylene induced ear edema model of inflammation were developed in male swiss albino mice in the present study. These acute models of inflammation are widely in use for the rapid screening of anti-inflammatory agents.

Carrageenan develops inflammation through the pathway of arachidonic acid metabolism, by plasma extravasation, increased tissue water and plasma protein exudation along with neutrophil extravasation. On the other hand, xylene causes swelling of ear in the mice and degree of inflammation developed could be calculated from the ear weight differences of mice. In these two acute inflammation models intraperitoneal LME treatment 1 h prior to carrageenan injection significantly decreased the peak value of edema formation in paw at 4$^{th}$ hour and significantly decreased ear weight after xylene application as compared to the saline treated animals.

In addition, LME treatment showed significant inhibition of tissue myeloperoxidase enzyme activity at inflammatory site as assessed from paw and ear homogenate of carrageenan injected mice and xylene applied mice. Since myeloperoxidase is the indicator of neutrophil infiltration at the site of inflammation, inhibition of MPO activity indicated reduction of neutrophil infiltration, thereby development of inflammation. These results also indicate that LME possess some anti-inflammatory agents, possibly some protein molecule that can control acute inflammation and is effective when applied through intra peritoneal route. To confirm this, we purified protein fractions from LME and studied their anti-inflammatory activity. Serhan et al. reported that persistence of acute inflammation and aggregation of inflammatory cells and mediators at the site of inflammation may cause aggravation of tissue damage and development of chronic inflammation which is an underline mechanism of many fatal diseases. Thus, clearance of inflammatory cell and mediators are also essential along with the prevention of edema to recover tissue homeostasis to normal.

As discussed earlier, MPO activity of tissue is one of the important markers to assess neutrophil infiltration at inflammatory site, thereby release of inflammatory mediators, reduction of tissue MPO level is the indicator of less cellular infiltration. In our experiments, LMPF4 significantly prevents development of carrageenan induced acute inflammation as assessed from paw edema as well as tissue MPO levels, and thereby indicating its potential to prevent chronic inflammation. It was thus further assessed for activity against adjuvant induced chronic inflammatory arthritis model in rat. LMPF4 (100 µg/Kg ×13 days), at the dose active against acute inflammation showed significant restoration of parameters of chronic inflammatory arthritis severity as shown by significant protection in arthritis score, paw weight, urinary hydroxyproline, glucosamine level, serum PGE$_2$, CINC1 and lysosomal phosphatases compared to arthritis animals. Thus, the semi pure fraction
References


