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Comparative Evaluation of Anti-Inflammatory Properties of Chitosan Extracted from the Scales of *Labeo rohita* and *Anabas testudineus: An In Vitro Study*

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Abstract

Inflammation is a natural physiological process involved in many acute and chronic diseases and there is a great need for developing new safer, natural anti-inflammatory agents. Chitosan is a biocompatible polysaccharide, produced by chitin deacetylation, and has been extensively studied due to its several biological activities, including anti-inflammatory properties. The *in vitro* anti-inflammatory effect of commercial chitosan was compared with that of fish chitosan sourced from two species: *Labeo rohita*, and *Anabas testudineus*. The modes of action of the anti-inflammatory activity of the chitosan were determined by measuring its ability to inhibit proteinase and hyaluronidase, as well as its Lipoxygenase (LOX) inhibition activity (assessed by LOX reaction with a synthetic substrate) and its membrane stabilising activity (determined by the polysaccharide's ability to reduce heat-induced and hypotonicity-induced hemolysis). In this study, anti-inflammatory drugs such as diclofenac sodium, aspirin and indomethacin were used as positive control. A concentration-dependent inhibition of inflammation-related processes was found in all chitosan samples. For commercial chitosan, greater inhibition was achieved at a lower concentration, whereas fish chitosan had the same or greater inhibition at a higher concentration. The *in vitro* anti-inflammatory potential of the fish-based extracts was attributed to *L. rohita* and *A. testudineus* chitosan based on enzyme inhibition and membrane stabilisation measurements. The proteinase inhibitory activity increased dose-dependently, with commercial chitosan showing the highest inhibition among the fish-derived chitosans (59.75% at 500 µg/mL), followed by *L. rohita* (40.25%) and *A. testudineus* (32.94%), as compare to positive control. The anti-inflammatory activity of chitosan is likely caused by a combination of the inhibition of inflammation-related enzymes and the stabilisation of cellular membranes against inflammatory damage.This study suggests that the source and concentration of chitosan influence its biological activities, and that chitosan from fish, especially *L. rohita*, has noteworthy anti-inflammatory properties with the potential to act as a promising alternative to commercial chitosan. The valorisation of fish-processing waste as a sustainable source of bioactive chitosan could provide a substrate for subsequent *in vivo* studies and set the stage for clinical trials on new anti-inflammatory drugs.

Major Findings: This study suggests that the source and concentration of chitosan influence its biological activities, and that chitosan from fish, especially *L. rohita*, has noteworthy anti-inflammatory activity with the potential to act as a promising alternative to commercial chitosan.

Keywords: *Anabas testudineus*, Anti-inflammatory, Chitosan, Inflammation, *In vitro*, *Labeo rohita*

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1. Introduction

Inflammation is a natural and complex biological response to harmful biological stimuli (e.g. microorganisms, dead and/or damaged cells, antigenic agents, foreign particles, and other exogenous insults), as well as chemical, physical stimuli (e.g. toxic compounds, mechanical stress, and irradiation). While inflammation is essential for homeostasis, chronic or dysregulated inflammation contributes to the pathogenesis of arthritis, cardiovascular disease, diabetes, neurodegeneration, and cancer. More recently, strong evidence that low-grade chronic inflammation plays a central role in disease progression and ageing has come to light[1-3].

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are widely used for the treatment of inflammation. However their long-term use is associated with various side effects such as gastrointestinal ulceration, nephrotoxicity, hepatotoxicity and cardiovascular disorders. Hence much effort has focused on finding new anti-inflammatory drugs with reduced side effects and biocompatibility, particularly through the use of natural products which may have lower toxicity[4,5]. Chitosan is one of the most promising natural biomaterials available. Chitosan is biodegradable, biocompatible, non toxic and it has several biological functions. It is derived from the deacetylation of chitin which is naturally abundant in the shells of crustaceans and fish processing residues. Many recent reviews report its broad array of biomedical applications, including antimicrobial, antioxidant, wound healing, drug delivery, tissue engineering, and anti-inflammatory activity[6-8]. The anti-inflammatory properties of chitosan are due to its ability to inhibit the activity of inflammatory enzymes and pro-inflammatory cytokines, regulate oxidative stress, modulate immune cell activity, and stabilise cell membranes. In addition, given their ideal physicochemical properties and bioavailability, chitosan-based formulations and nanoparticles offer improved therapeutic effects[6].

Labeo rohita is one of the most economically important freshwater fish species in South Asia. The scale discard from this species can be employed towards the production of chitosan due to its high concentration of chitin[9]. The chitin in the scales can be converted to chitosan (Figure 1). Chitosan as a biocompatible, biodegradable, and nontoxic substance from the scales of the Indian major carps *L. rohita* has been observed to possess potential biomedical qualities[10]. Several recent studies have shown important antioxidant effects of *L. rohita* chitosan and also metabolic protection including enhancement of antioxidant defense systems. Because inflammatory pathways and oxidative stress are linked, the antioxidant activity of *L.rohita* chitosan may also contribute to its anti-inflammatory effects[11]. *Anabas testudineus* (the climbing perch or papuyu fish), a freshwater fish found throughout South and Southeast Asia, has been suggested as an alternative source of chitinous fish-scale waste for the commercial production of chitosan. Chitosan can be extracted from fish scales, including *A. testudineus* scales after demineralisation, deproteinisation and deacetylation. It has been shown that chitosan can be obtained from processing waste and is a good biomaterial[12]. Moreover, chitosan derived from *A. testudineus* scales was shown to be biologically active in wound healing and wound regeneration in animal models of diabetes. The anti-inflammatory activity of chitosan

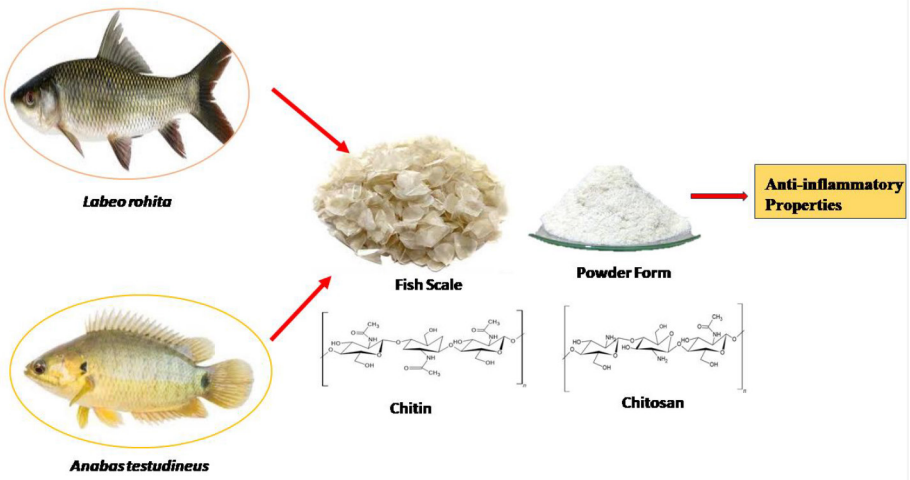


Figure 1. Fish scale-derived chitin is transformed into chitosan, which exhibits potent anti-inflammatory activity.

derived from *A. testudineus* may be associated with stabilisation of erythrocytes and lysosomal membranes and reduction of release of inflammatory mediators, including histamine, proteases and pro-inflammatory cytokines. The availability of *A. testudineus* scales has thus opened a new source of chitosan for biomedical and anti-inflammatory applications[13].

There are a variety of *in vitro* tests used to assess the anti-inflammatory activity, for example the proteinase inhibition assay which studies how a compound prevents proteolytic tissue damage from inflammatory proteases such as trypsin, chymotrypsin and elastase, which are responsible for tissue degradation during inflammation[14]. Hyaluronidase inhibition assays are used to test the active agents' ability to prevent the degradation of the extracellular matrix component hyaluronic acid, and subsequent enhancement of vascular permeability and recruitment of leukocytes[15,16]. LOX inhibition assays are also performed to test whether the active agents can prevent the synthesis of leukotrienes by the LOX, which oxidize polyunsaturated fatty acids to form these strong pro-inflammatory mediators[17]. Indirect evidence for lysosomal membrane stabilisation is obtained from erythrocyte membrane stabilisation assays (heat-induced and hypotonicity-induced hemolysis models), which reduce the release of inflammatory mediators and tissue damage. These assays are frequently used as screening methods to measure anti-inflammatory activity of natural products and biomaterials[18]. In combination, these assays allow for the assessment of the anti-inflammatory effects via enzyme inhibition and membrane stabilisation pathways[19] (Figure 2).

It has been recently shown that the molecular weight, deacetylation degree, crystallinity, and source of material of chitosan have an important effect on its biological activity. Different physicochemical properties will have different biological effects, such as anti-inflammatory effect. Chitosan from fish has been recognised as a valuable and green alternative to commercial chitosan, due to its availability, cost-effectiveness, and waste utilisation potential, with promising bioactivity. Chitosan from aquatic animals has been reported to have better functionality and therapeutic potential due to its structural properties, which depend on the source[7,8,20].

Although many studies show the anti-inflammatory properties of chitosan and its derivatives, these compounds have also been reported to have different biological activities based on the source of chitosan, molecular weight, degree of deacetylation, and preparation of the polymer. Most recently, it has been shown that the structural and physicochemical properties of chitosan-based materials are key determinants of

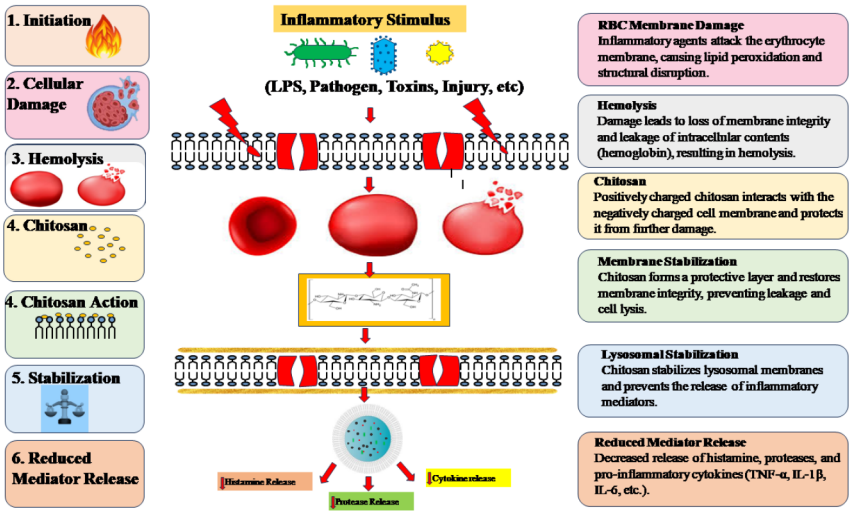


Figure 2. Proposed mechanism of anti-inflammatory activity of chitosan. Inflammatory stimuli cause erythrocyte membrane damage, resulting in hemolysis and release of inflammatory mediators. Chitosan interacts with cell membranes and stabilises erythrocyte and lysosomal membranes, thereby reducing the release of histamine, proteases, and pro-inflammatory cytokines. This membrane-stabilising effect ultimately reduces inflammation, decreases tissue damage, and improves healing.

the anti-inflammatory activity. Additionally, fish-processing by-products provide a possible sustainable chitosan resource, and have shown potential promise in biomedical and bioactive applications. However, comparative studies on the *in vitro* anti-inflammatory effects of commercial chitosan and fish chitosan with complementary models have yet to be conducted. To date, there have only been a few studies that have used enzyme inhibition and membrane protection assays in chitosan, but these have not yet fully explored the anti-inflammatory mechanisms of chitosan. The aim of this study was to evaluate and compare the *in vitro* anti-inflammatory activity of commercial chitosans and chitosan extracted from different fish sources using different experimental models. The aim of this study is to investigate the effect of source variability on biological activity, in order to provide scientific evidence for the development of fish chitosan as a sustainable natural anti-inflammatory extract[21,22].

2. Materials and Methods

2.1 Chitosan Samples and Preparation

Commercial available biopolymer chitosan and fish chitosan obtained from *L. rohita* and *A. testudineus* were used in the present work. The chitosan obtained was prepared as per standard protocols for its demineralisation, deproteinisation and deacetylation. Each chitosan sample was dissolved in a corresponding solvent to obtain the stock solution, and the obtained stock solutions were further diluted to 100, 200, 300, 400, and 500 µg/mL. For the *in vitro* tests of anti-inflammatory activity, proteinase inhibition, hyaluronidase inhibition,LOX inhibition, and membrane stabilisation tests were carried out. All experiments were performed in triplicate and expressed as mean ± Standard Deviation (SD).

2.1 Membrane Stabilisation Preparation of Red Blood Cells (RBC) Suspension

Blood was drawn from a healthy human volunteer who had not taken any NSAID for at least 2 weeks before the experiment. Blood was transferred into centrifuge tubes. The centrifuge tubes were centrifuged at 3000rpm for 10 min. The sediment was washed three times with an equal volume of normal saline. Volume of blood was estimated and then reconstituted as a 10% v/v suspension with normal saline of Human Red Blood Cells (HRBC).

2.2 Heat-Induced Hemolysis Assay

Chitosan obtained from fish scales of *L. rohita*,*A. testudineus*and commercial chitosan was used in an *in vitro* HRBC membrane stabilisation study to evaluate anti-inflammatory activity. HRBC membrane stabilisation assay is one of the most common *in vitro* anti-inflammatory assays as the composition of the erythrocyte membrane is similar to the lysosomal membrane and stabilisation of the HRBC membrane suggests that the test sample has the capacity to inhibit the release of inflammatory mediators[23,24]. In brief, the 2mL of reaction mixture was prepared by combining 1mL of test sample (chitosan solution at 100-500 µg/mL) with 1mL 10% HRBC suspension prepared with normal saline. In place of the test sample, the normal saline was used in the control group. Aspirin was used as the reference anti-inflammatory drug, and various HRBC-based anti-inflammatory protocols like the aforementioned have been widely used to screen natural biomaterials and anti-inflammatory agents[24,25]. The reaction mixtures were then placed in a water bath at 56°C for 30 minutes. The heat treatment disrupts the membranes to lyse the cells, causing cellular material to spill out of erythrocytes, and is considered a model for membrane destabilisation in inflammatory processes[25,26]. Post-incubation, the tubes were cooled under running water and centrifuged at 2500rpm for 5 min. The supernatants were diluted with distilled water, and the absorbance was measured at 560nm using Ultra Violet (UV)-Visible spectrophotometer. Absorbance increase indicates the hemolytic property, while an absorbance decrease indicates the stabilisation of the membranes and anti-inflammatory effect[23,24]. Haemolysis was calculated as follows:

$$\text{Percentage inhibition} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{X100/Abs control}}$$

2.3 Hypotonicity-Induced Haemolysis

Three solutions of chitosan (100, 200, 300,400 and 500 µg/mL), prepared from fish scales and commercial chitosan, were added separately to 1mL of phosphate buffer, adjusted to pH 7.4, followed by 2mL of hyposaline solution and 0.5mL of HRBC suspension. Diclofenac sodium (100 µg/mL) was used as the positive control and other HRBC membrane stabilisation studies[27,28] have used similar setups as described above. These reaction mixtures were incubated for 30 min at 37°C to induce osmotic stress-

induced hemolysis. In the case of hypotonic treatment of dilutions, the fluid present in erythrocytes leaks out and the membrane ruptures, causing hemolysis. Thus, any compound that prevents the coagulation is said to have membrane-stabiliing and anti-inflammatory activity. All the samples were centrifuged at 3000 rpm after incubation and the supernatant was collected. One approach uses spectrophotometric measurement of hemoglobin released by hemolysis at 560nm, observing that increased stabilisation and anti-inflammatory activity correspond to reduced absorbance.

All the experiments were carried out in triplicate and, in each case, the percentage hemolysis was taken as 100% if the sample produced hemolysis equal to that of control. The percentage protection against hemolysis was calculated as follows:

$$\text{Percentage protection} = 100 - (\text{OD sample}/\text{OD control}) \times 100$$

2.4 Hyaluronidase InhibitoryAssay

Hyaluronidase inhibitory activity of fish scale chitosans (*L.rohita and A.testudineus*) and commercially available chitosan was determined according to the method of Sahasrabudhe and Deodhar with slight modifications. Hyaluronidase is an important hydrolytic enzyme that degrades hyaluronic acid of connective tissues. The inhibition of hyaluronidase is considered an important indicator of anti-inflammatory activity because excessive hyaluronidase activity causes tissue damage, triggering an inflammatory response[29,30]. The 0.1M acetate buffer pH 3.6 was prepared and 10µL of hyaluronidase solution (8 mg/mL) was mixed with 10µL of the chitosan solution of different concentrations (100-500 µg/mL), preparing the assay itself for the reactions to take place. Samples were incubated for 20 min at 37°C to allow enzyme and chitosan to interact. This temperature is similar to those recently used when studying natural anti-inflammatory substances with hyaluronidase inhibition tests[31-33]. Next, 20µL of 12.5mM calcium chloride was added as an activator and incubated at 37°C for another 20 min. Calcium ions was required for the activity of hyaluronidase and to enable hydrolysis of the substrates[16,33]. To prepare the p-dimethylaminobenzaldehyde (DMAB) reagent, 0.4g of DMAB was dissolved in 35mL of glacial acetic acid and 5mL of 10N hydrochloric acid. The solution was cooled to room temperature, to which 600µL of DMAB reagent was added. The resulting mixture was incubated at 37°C for 20 min to form the chromophore. DMAB reacted with the hyaluronic acid degradation products, and the resulting colored chemical was analysed by spectrophotometry at 585nm[34]. The lower the absorbance readings were at 585nm, the more hyaluronidase activity was inhibited, and thus the greater the anti-inflammatory activity of that chitosan sample.

The percentage inhibition of hyaluronidase activity was calculated using the following equation:

$$\%\text{inhibition} = 100 \times (\text{absorbance of the control} - \text{absorbance of the sample})/\text{absorbance of the control}$$

2.5 Lipoxygenase Inhibition Assay

Lipoxygenase inhibitory activity of chitosan from fish scales of *L. rohita* and *A. testudineus*, and commercial chitosan was determined according to the procedure of Wu (1996) with some modifications of Gunathilake *et al*[35-37]. In brief, 1.0mL of sodium borate buffer (0.1M; pH 8.8) containing 10µL of lipoxygenase enzyme (8000 U/mL) was prepared and mixed with 10µL of various concentrations (100, 200, 300, 400 and 500 µg/mL) of chitosan solution in a quartz cuvette at room temperature (30 ± 2°C) and the mixture was incubated for 5 min, then 10µL of linoleic acid (10mM) substrate was added to initiate the enzymatic reaction. The formation of conjugated dienes through the absorbance at 234nm in a UV-Visible spectrophotometer. A control reaction was performed by using a mixture of phosphate buffer (instead of the chitosan solution) as a blank, with Indomethacin serving as an experimental standard anti-inflammatory drug[35,36]. The lipoxygenase percentage inhibition was determined using the following equation:

$$\%\text{inhibition} = 100 \times (\text{absorbance of the control} - \text{absorbance of the sample})/\text{absorbance of the control}$$

2.6 Proteinase Inhibitory Activity

Proteinase inhibition is an important indicator of anti-inflammatory activity since proteolytic enzymes are released during inflammation and contribute to inflammatory response and tissue breakdown. Inhibiting enzymes would reduce inflammation and tissue breakdown. The reaction mixture was 2mL, containing 0.06mg of trypsin, 1mL of 20mM Tris-HCl buffer (pH 7.4), and 1mL of a chitosan solution (100 to 500 µg/mL). The reaction mixture was then incubated at 37°C for 5 min to allow binding of the trypsin to the test sample.

This methodology is similar to recent studies examining natural anti-inflammatory compounds with proteinase inhibition assays.

After this time 1mL of 0.8% (w/v) casein solution (substrate) was added to each tube and placed back in the incubator (37°C) to allow another 20 min incubation. This way trypsin generated casein low molecular weight peptides. The proteolytic activity was inhibited by the presence of the inhibitory compounds present in the chitosan. Trypsin-casein digestion-based proteinase inhibition assays are commonly used for evaluating potential anti-inflammatory activity of natural biomaterials and polymers. After incubation, 2mL of a 70% perchloric acid solution was added to stop the enzymatic reaction, and the undigested proteins were precipitated. The supernatants of the centrifuged solutions were analyzed spectrophotometrically at 210nm for remaining protein content (buffer solution was used as blank). The decline in absorbance suggested that proteins were more stable against trypsin degradation under the influence of the chitosan samples, thereby indicating an enhancement of their anti-inflammatory potential. The phosphate buffer solution was used as a control[36-39]. The percentage inhibition of proteinase activity was calculated using the following formula :

$$\%\text{inhibition of denaturation} = 100 \times (1 - A2/A1),$$

Where A1=absorption of the control sample, and A2=absorption of the test sample.

2.7 Statistical Analysis

All experiments were performed in triplicate, and all data are expressed as mean ± standard deviation. Further, the experimental results were mathematically analysed for the dose effect of the samples. Data were analysed using One-Way Analysis of Variance (ANOVA) followed by Tukey's post hoc tests using the GraphPad Prism software. Results were considered statistically meaningful at $p < 0.05$.

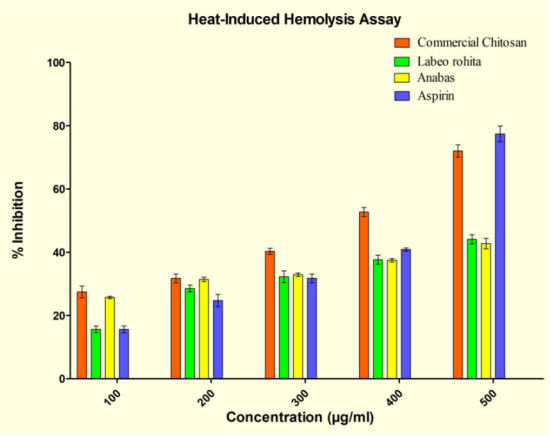


Figure 3. Heat-induced hemolysis assay.

3. Results

3.1 Heat-Induced Hemolysis Assay

Chitosan from fish scales was screened for this anti-inflammatory activity using the heat induced hemolysis assay, which is the ability of an agent to stabilise the erythrocyte (red blood cell) membrane against breakage produced by heat. Since the erythrocyte and lysosomal membranes are structurally similar, membrane stabilization is taken as index of anti-inflammatory potential. The percentage inhibition (mean ± SD; n = 3) by commercial chitosan, *L. rohita* chitosan, *A. testudineus* chitosan and standard drug aspirin (100, 200, 300, 400 and 500 µg/mL) were statistically analysed using one-way Analysis of Variance (ANOVA) and Tukey's post hoc multiple comparison test using the level of importance fixed at $p < 0.05$. In the heat-induced hemolysis assay, all the treatments showed an important concentration dependent increase in membrane stabilisation activity (one-way ANOVA, $p < 0.001$). The Tukey post hoc test also showed the commercial chitosan, the *L. rohita* chitosan, the *A. testudineus* chitosan and the aspirin group were statistically different from each other at almost all concentrations ($p < 0.05$). In comparison to *Labeo* and *Anabas*chitosans from 100 to 500 µg/mL concentration, the commercial chitosan showed more stability with the percentage of inhibition from 27.42 ± 3.23% to 72.04 ± 3.40%. The percentage inhibition of membrane stabilisation of *L. rohita* chitosan was from 15.59 ± 1.86% to 44.09 ± 2.48% and *Anabas* chitosan was from 25.70 ± 0.65% to 42.71 ± 2.88%. The maximum percentage inhibition of membrane stabilisation (77.42 ± 4.23%) was seen for aspirin at the highest concentration, indicating maximum anti-inflammatory activity(Figure3). Chitosan derived from fish exhibited membrane stabilising activity that increased in a dose-dependent manner but was considerably lower than commercial chitosan and aspirin. Commercial chitosan also demonstrated meaningful protection against heat-induced membrane damage and inflammation. Otherwise, *L. rohita* and

A. testudineus chitosan demonstrated concentration dependent anti-inflammatory activity and hence has an interesting potential for medical applications.

3.2 Hypotonicity-Induced Hemolysis

The anti-inflammatory effect of fish scale chitosan was studied using hypotonicity induced hemolysis based on the erythrocyte membrane stabilising potential against osmotic pressure from the surrounding medium. Commercial chitosan, *L. rohita* chitosan, *A. testudineus*chitosan and diclofenac sodium (standard) were screened against various concentrations i.e. 100, 200, 300, 400 and 500 µg/mL. All values are means ± standard deviations (n = 3). The statistical importance of differences was evaluated by one-way analysis of variance with Tukey's post hoc multiple comparison test at $p < 0.05$. A meaningful increase in the stabilisation of the cell membrane was observed in a dose dependent manner in all treatments when subjected to hypotonicity-induced hemolysis (one-way ANOVA, $p < 0.001$). The membrane stabilisation effects of commercial chitosan, *L. rohita* chitosan, *A.* chitosan and diclofenac sodium were considerably different according to Tukey's post hoc test ($p < 0.05$) at most of the doses. *A. testudineus* chitosan has a more strong membrane protective activity in comparison with fish chitosans. The rate of inhibition by *A. test udineus* chitosan was 21.70 ± 0.92%, 29.54 ± 2.52%, 37.57 ± 2.53% and 43.01 ± 2.36% at 100, 200, 300 & 400 µg/mL respectively. The *L. rohita* chitosan was also able to inhibit the cell membrane stabilisation from 17.61 ± 1.89% at 100 µg/mL to 54.09 ± 2.50% at 500 µg/mL. The inhibition rates by the commercial chitosan were 10.69 ± 2.50% at the lowest concentration of 100 µg/mL and 56.60 ± 2.83% at 500 µg/mL; all compared to the sodium diclofenac which registered the highest stabilisation by 87.42 ± 0.94% at 500 µg/mL(Figure 4). From the obtained data of its erythrocyte membrane-protective activity, it may be inferred that all of the chitosan films evaluated in this study possess meaningful membrane stabilising activity in a dose dependent manner, yet showed a lower activity than the reference drug diclofenac sodium.

3.3 Hyaluronidase Inhibition Assay

Antiinflammatory activity of fish scale chitosan was evaluated by the hyaluronidase inhibition assay. The hyaluronidase is an enzyme that causes the hydrolysis of hyaluronic acid, a polysaccharide during conditions of inflammation. The hyaluronidase activity was inhibited for different concentrations (100, 200, 300, 400, 500 µg/mL) of commercial chitosan, *L. rohita* chitosan, *A. test udineus* chitosan and indomethacin. All data are expressed as means ± SD (n = 3) and were analysed by one-way ANOVA followed by Tukey's post hoc multiple comparison test. Differences were considered meaningful at $p < 0.05$. In the hyaluronidase inhibition assay, the enzyme inhibitor activity was considerably higher in all groups in a dose-dependent manner (one-way ANOVA $p < 0.001$). Tukey's post hoc was used for multiple comparisons. The experimental result showed important differences ($p < 0.05$) for commercial chitosan, *L.rohita* chitosan, *Anabas* chitosan and indomethacin, for all concentrations. The chitosans obtained from commercial sources had the highest hyaluronidase inhibition percentage, ranging from 22.32 ± 0.89% at 100 µg/mL to 62.50 ± 1.55% at 500 µg/mL concentration. *Labeorohita* chitosan gave the next highest values at 18.94 ± 0.63% inhibition at 50 µg/mL and 59.82 ± 1.79% inhibition at 400 µg/mL, which were still above those of commercial at 400 µg/mL (55.36 ± 0.89% inhibition). Similarly, the inhibition levels obtained from all concentrations of *Anabas* chitosan (from 16.93 ± 0.89% inhibition at 50 µg/mL to 53.73 ± 0.60% inhibition at 500 µg/mL) were medium. The inhibition values for indomethacin were always the highest (91.07 ± 1.55% inhibition at 500 µg/mL) (Figure 5). In conclusion, the chitosan samples obtained showed high dose-dependent hyaluronidase inhibition and good anti-inflammatory activity but, however, much lower than indomethacin. Thus, chitosan isolated from fish scales can be considered as a potential green and biocompatible biomaterial for pharmaceutical and biomedical applications.

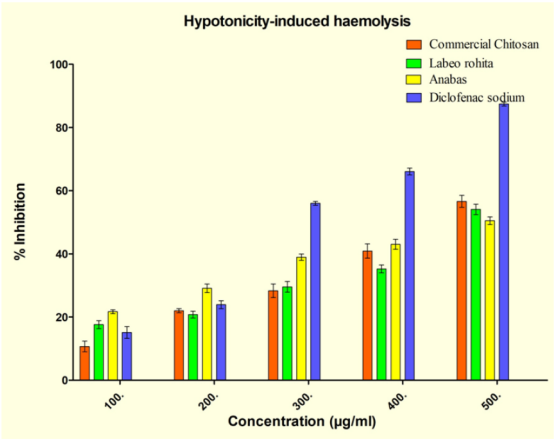


Figure 4. Hypotonicity-induced hemolysis.

3.4 Lipoxygenase (LOX) Inhibition Assay

All chitosan samples showed anti-inflammatory activity in a dose dependent manner. The inhibition of the LOX enzyme was maximum at a concentration of 100 µg/mL for *L. rohita* and *A. Testudineus* chitosan samples compared to the commercial chitosan sample. Indomethacin maximally inhibited the LOX activity. 200-500 µg/mL of *L. rohita* chitosan inhibited the LOX activity more considerably (one way ANOVA, $p < 0.05$) than the commercial chitosan and *Anabas* chitosan at each concentration of *L. rohita* chitosan in all the time points. The LOX inhibition was found to be 67.11 ± 0.52%, 48.39 ± 3.22% and 61.08 ± 1.61% of the plant extracts at 500 µg/mL concentration with indomethacin being the most strong with 96.51 ± 0.47% inhibition at 500 µg/mL (Figure 6). It was found that the inhibitory effect of *L. rohita* chitosan alone was higher than commercial chitosan ($p < 0.05$) after Tukey's post hoc test. These findings suggest that *L.rohita* chitosan possesses dose-dependent anti-inflammatory action and can be used as a natural lipoxygenase pathway inhibitor.

3.5 Proteinase Inhibitory Activity

The data for the proteinase assay showed a meaningful difference between the groups ($p < 0.001$), indicated by the dose-dependent increase in proteinase inhibitory activity. The post hoc multiple comparison Tukey tests of the individual comparison groups also demonstrated important differences between commercial chitosan, *L. rohita* chitosan, and *A. testudineus*chitosan with diclofenac sodium at most concentrations ($p < 0.05$). Commercial chitosan had the highest proteinase inhibition among the extracts with 11.32 ± 3.27% inhibition at 100 µg/mL and 59.75 ± 1.09% inhibition at 500 µg/mL. Chitosan extracted from the fish *L. rohita* increased in inhibition from 4.40 ± 1.09% to 40.25 ± 1.09%. Dose dependent anti-inflammatory activity was also recorded with *A.testudineus* chitosan, where the percentage inhibitions ranged from 6.62 ± 0.43% to 32.94 ± 0.99%. Among the drug molecules studied, diclofenac sodium was found to inhibit the proteinases in a dose dependent manner with the percentage of inhibition being 27.04 ± 2.90% (100 µg/mL) to 93.08 ± 1.09% (500 µg/mL) (Figure 7). Although the fish chitosan samples were less effective than commercial chitosan and diclofenac sodium in inhibiting the proteinases, the observed increase in inhibitory effect with increasing concentrations of fish chitosan suggests that the latter may be pursued as a drug candidate and as a natural anti-inflammatory biomaterial.

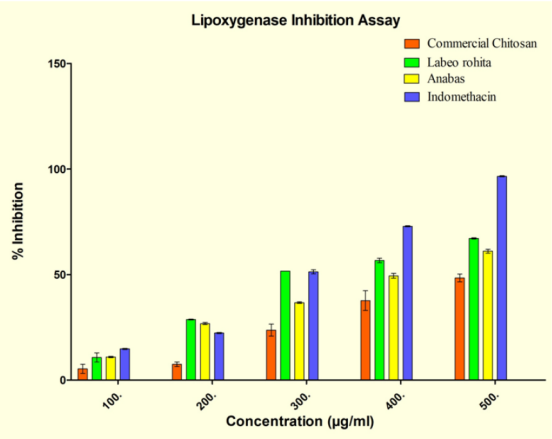


Figure 6. Lipoxygenase (LOX) inhibition assay.

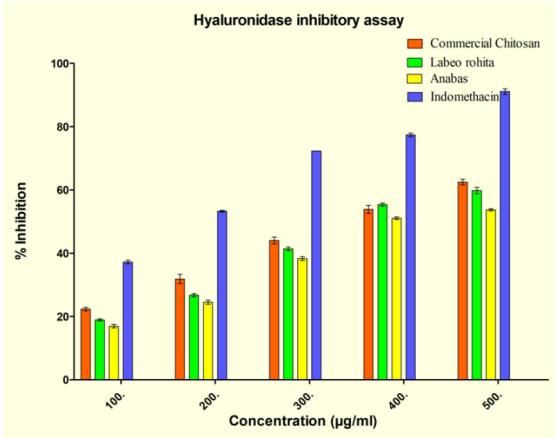


Figure 5. Hyaluronidase inhibition assay.

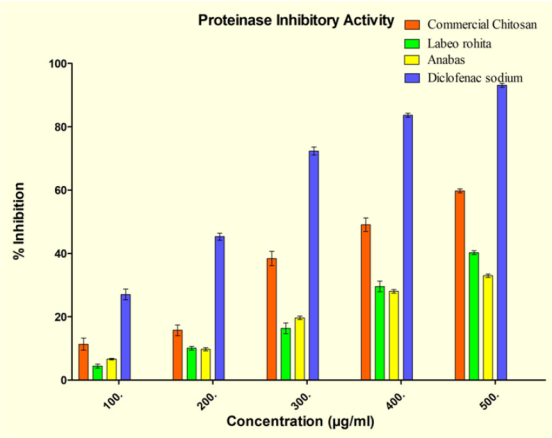


Figure 7. Proteinase inhibitory activity.

4. Discussion

The *in vitro* anti-inflammatory activities of commercial and fish chitosan samples were measured in terms of inhibition of NO production, inhibition of superoxide generation, inhibition of NF-kB expression, and inhibition of IL-8 secretion. All chitosan samples showed anti-inflammatory activity, the effectiveness of which was concentration-dependent. Membrane stabilisation is one of its anti-inflammatory effects, in that it decreases the release of pro-inflammatory mediators from damaged cell membranes. The erythrocyte membrane is widely used as a model biomembrane to investigate biological membrane properties. In a few hemolysis tests, erythrocyte membrane stabilisation was determined by subjecting the erythrocyte to hypotonicity and heat-induced hemolysis assays to further confirm the dose dependency of chitosan membrane stabilisation[40]. The similarity of the erythrocyte membrane to lysosomal membrane may also increase the inhibition of the release of lysosomal enzymes induced by the inflammatory stimuli[41]. *Labeo rohita* chitosan among the fishes was found to exert a relatively higher enzyme-targeted anti-inflammatory activity in particular on the LOX pathway. Another possibility for anti-inflammatory action is the inhibition of lipoxygenase LOX, which catalyze the conversion of arachidonic acid to leukotrienes that act as mediators of inflammation, vascular permeability and leukocyte recruitment[42]. This may explain why fish chitosans inhibit the production of LOX-mediated lipid mediators and reduce the activation of oxidative inflammatory pathways in the above experimental models[43]. High activity of *L. rohita* chitosan may relate to physicochemical properties (which vary according to chitosan source) which affect chitosan's affinity for inflammatory enzymes and other biological compounds. Degree of deacetylation, molecular weight distribution, degree of amino moiety, surface charge, molecular conformation, and electrostatic and hydrogen-bonding interactions between chitosan and biological macromolecules can influence chitosan's biological activity. Biological activity of chitosan may be influenced by the degree of availability of amino groups which may increase the interaction of chitosan with their target and improve biological activity[44]. The good physicochemical properties of *L. rohita* chitosan compared to other sources of chitosan may have contributed to the higher LOX inhibition potency[45]. Apart from LOX inhibition, the membrane-stabilising effect of *L. rohita* and *A. Testudineus* chitosans, which protected erythrocyte membranes from heat and osmotic induced hemolysis, suggested that other mechanisms are likely responsible for the anti-inflammatory activity. The hyaluronidase- and proteinase-inhibitory activities of the chitosans further suggested the involvement of other mechanisms in addition to the identified enzymes. Although *A. testudineus* chitosan was found to have strong bioactivities, the weak activity seen in the studies could be due to extraction conditions and source dependent physicochemical properties of the chitosan, such as degree of deacetylation, molecular structure, and crystallinity and other physicochemical properties of polymer which affects interaction of chitosan with the biomolecules[45]. The important biological activity of fish-derived chitosans, particularly that of *L. rohita*, stresses the impact of source-dependent physicochemical properties on chitosan's functional characteristics. Collectively, these findings further broaden the potential of fish scale chitosans as a source of sustainable multifunctional biomaterials for anti-inflammatory pharmaceutical and biomedical applications. Inhibition of hyaluronidase is a key anti-inflammatory mechanism, since the enzyme hydrolyzes hyaluronic acid, a major component of the Extracellular Matrix (ECM), resulting in increased permeability of tissue and diffusion of inflammatory mediators[46]. The anti-inflammatory activity of the fish chitosan may be associated with its anti-hyaluronidase activity, being able to maintain the homeostasis of the extracellular matrix as well as to inhibit the enzymatic degradation of hyaluronic acid and the edema associated with inflammatory processes[47]. Among the chitosans, the one produced with the substrate *L. rohita* showed higher anti-inflammatory activities and could be associated with the better physicochemical characteristics of the substrate for the action of the enzyme, such as higher degree of deacetylation, greater availability of amino groups and more appropriate molecular weight distribution[45]. The improved bioactivity of *L. rohita* chitosan may also be due to a stronger interaction with negatively charged sites in inflammatory biological macromolecules and their interacting enzymes, due to the presence of positively charged amino groups of chitosan[48]. The anti-inflammatory activity of *A. testudineus* chitosan was lower than *L. rohita* chitosan, but the functional structure of chitosan may help attach to the inflammatory biological target without losing the activity of the chitosan molecule[21]. Other factors that might contribute to variances in inhibitory potency include extraction efficiency, degree of deacetylation, crystalline structure, molecular arrangement, and protein-binding capacity of the chitosan source, which can all affect the biological activity of chitosan[44]. For

example, experiments on hyaluronidase and proteinase inhibition show that chitosan may affect inflammation status in addition to membrane stabilisation. Increased hyaluronidase activity leads to excessive hyaluronic acid degradation and extracellular matrix disintegration. Similarly, increased proteinase activity at the site of inflammation promotes degradation of components of the extracellular matrix, leading to tissue injury as well as prolonged inflammation. Dual inhibition of these enzymes may help maintain tissue integrity and prevent prolonged inflammation. The inhibition of the two major tissue based enzymes by fish chitosans, particularly *L. rohita*, indicates that it is able to inhibit the activities of those enzymes responsible for tissue-matrix degradation and inflammatory tissue damage. The observed increase in the inhibition of proteinases with the increased concentration of chitosan supports that chitosan is involved in inhibition of the inflammatory enzyme activity which is one of the anti-inflammatory mechanisms of chitosan and its derivatives[21]. Chitosan's anti-inflammatory properties may be due to inhibition of proteinases. Proteolytic enzymes are released or activated by inflammation and degrade extracellular matrix components causing tissue damage and inflammation by exerting positive feedback on the inflammatory response. Inhibiting proteolytic activity may help to limit tissue destruction and the degradation of other cells and components of extracellular matrix[49]. However, the higher proteinase inhibitory activity of commercial chitosan as compared to the chitosan generated in this study may be due to improved control of physicochemical parameters, such as molecular weight and degree of deacetylation, allowing for better interactions with specific biological targets[45]. Amongst the fish species studied, *L. rohita* showed maximum activity, which can be attributed to its specific structural features. The lower activity of *A. testudineus* chitosan may be attributed to source-dependent differences in extraction yield, degree of deacetylation, crystallinity, polymer conformation and ability to bind to proteins. These physicochemical properties may influence the accessibility and reactivity of functional groups that mediate biological activities[44]. The relatively higher proteinase inhibition for diclofenac sodium is congruent with the well-characterized anti-inflammatory pharmacodynamics as well as the modulatory effects on inflammatory mediators and processes[50]. The potential for inhibition of the inflammatory response by the commercial and fish-derived chitosans indicates that chitosans can inhibit inflammatory processes through mechanisms which are distinct from those of the commercially available NSAID. Other anti-inflammatory mechanisms by which chitosan and its derivatives may exert anti-inflammatory effects are: Inhibition of inflammatory mediators, immune modulation, antioxidant activity, stabilisation of cellular membranes, and tissue injury prevention by inhibiting enzymes. Thus, the combination of hyaluronidase- and proteinase-inhibitory activities demonstrated in the present study additionally provides support for the multi-factorial mechanism of chitosan-mediated anti-inflammatory activity. In general, the commercially available chitosan showed the greatest and most reproducible inhibitory potency. *Labeo rohita* as fish source exhibited the most potential to exhibit enzyme-inhibiting activity and thus anti-inflammatory potential. The better performance of *L. rohita* suggests that the chitosan prepared from fish scales could become a source of biologically active products and provide sustainable valorization of fish-processing waste material[22,44]. These studies combined infer that fish-derived chitosans such as that from *L. rohita* could have promise as eco-friendly raw materials for anti-inflammatory drugs and biomedical products. They also suggest that the ability of chitosan to stabilise the membrane of erythrocytes in response to treatment with cell membrane-destabilising agents such as heat and osmotic stress is influenced by the type of cellular stress and the manner of interaction of the polymer with the erythrocyte membrane. Since the erythrocyte membrane and lysosomal membrane are similar in structure, erythrocyte stabilization has been accepted as the major criterion for the ability of a compound to inhibit lysosomal enzyme discharge and inflammatory mediator release during inflammation[51]. Under hypotonic conditions erythrocytes swell and burst (osmotic hemolysis) when water enters the erythrocyte cytoplasm; This lysis can be inhibited by membrane-stabilising agents[52]. In the present study, chitosans from *L.rohita* and *A. testudineus* were able to protect the membrane under stress conditions, although the degree of protection was dependent on the type of stress (heat shock or hypotonicity). This could be due to differences in polymer-membrane interaction, molecular flexibility, charge density, deacetylation degree of the polymer, and the ability of chitosan chains to interact with membrane proteins and phospholipids. This membrane stabilisation could be due to the electrostatic interaction between the positively charged amino groups of the protonated chitosan molecule and the negatively charged phospholipids and proteins on the membrane, in such a way that preservation of the integrity of the membrane occurs[45]. This would prevent the

disruption of the membrane and the liberation of the various intracellular and lysosomal inflammatory substances[48]. In addition to direct membrane-stabilising action, the anti-inflammatory action of chitosan may be due to its ability to inhibit pro-inflammatory cytokines, inflammatory signaling pathways and oxidative stress, and to stabilise the membranes of cells undergoing inflammation[21]. This activity is consistent with the activity of chitosan and other polysaccharides of plant origin in the inhibition of inflammation through stabilisation of biological membranes and prevention of release of inflammatory mediators from damaged cells[53]. Overall, the differences noted from both hemolysis assays show that although *L. rohita* and *Anabas chitosans* belong to the same class of biopolymers, they show dissimilar behavior in terms of membrane protective properties, favouring their potential of being natural, multifunctional anti-inflammatory biomaterials.

5. Conclusion

Thus, it has been found in the present investigations that the chitosan obtained from the fish scales exhibits good anti-inflammatory property as observed by the five *in vitro* anti-inflammatory assays viz., heat-induced hemolysis, hypotonicity-induced hemolysis, proteinase inhibition, hyaluronidase inhibition and LOX inhibition with an increasing concentration. The results of the present study show that commercial chitosan had the maximum anti-inflammatory effect and fish scale chitosan of *L. rohita*had the maximum anti-inflammatory effect in most tests followed by *A. testudineus*. This study concludes that the anti-inflammatory effects of fish-derived chitosan are the result of the stabilisation of biological membranes, the inhibition of inflammatory enzymes and a reduction of tissue damage caused by the inflammatory process. The chitosan of *L. rohita* fish scales has characteristics that indicate a good anti-inflammatory biomaterial for future pharmaceutical and biomedical applications.

6. References

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Author Contribution

Kavita Verma: Designed and conducted the experiments, analysed data, and wrote the manuscript; Yoganchal Mishra: Conducted the experiments and analysed the outcomes; Priyanka: Conducted the experiments and analysed the outcomes; Neelam Yadav: Conceived, designed and coordinated the study, analysed the results, and edited the manuscript. All authors contributed to editing and reviewing the final manuscript.

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