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AN *IN VITRO STUDY* FOR SUSTAINABLE VALORIZATION OF *CHANNA PUNCTATA* FISH-SCALE WASTE INTO BIOACTIVE CHITOSAN FOR ANTI-INFLAMMATORY APPLICATIONS

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ABSTRACT
There are many acute and chronic diseases that are associated with inflammation. There is a great demand to discover new natural and safe anti-inflammatory agents. Chitosan is a biocompatible natural polysaccharide obtained by deacetylation of chitin. Chitosan is widely studied for its various biological activities, including its anti-inflammatory activity. Anti-inflammatory activity of commercial chitosan was compared with the fish chitosan obtained from *Channa punctata*. Different mechanisms of the anti-inflammatory activity of chitosan were examined, by assessing its ability to inhibit proteinase and hyaluronidase, LOX activity (using synthetic substrate for LOX reaction), as well as its ability to stabilize the membrane of erythrocytes, by investigating its effect on hemolysis induced by heating and hypotonicity. The inhibitory activities of both chitosan were compared with the anti-inflammatory drugs diclofenac sodium, aspirin, and indomethacin. In both cases, a concentration-dependent inhibitory effect was observed, with commercial chitosan showing a stronger inhibitory effect at a lower concentration and fish chitosan at a higher concentration. The *in vitro* anti-inflammatory activity of fish extracts was attributed to the anti-inflammatory potential of *Channa punctata* chitosan based on its ability to inhibit certain enzymes and stabilize the cell membrane. The anti-inflammatory mechanism of chitosan is probably linked to the inhibition of certain enzymes involved in the inflammatory response and the stabilization of cell membranes. Thus, we conclude that the biological potency of chitosan is influenced by its source and concentration. Thus, chitosan obtained from fish (*Channa punctata*) holds important anti-inflammatory activity and can be explored as an alternative to the commercially available chitosan obtained from other sources. Valorizing fish processing waste is a sustainable method of producing bioactive chitosan for further in vivo experiments and development into new anti-inflammatory drugs for testing in clinical trials.

KEYWORDS: *Channa punctata*, Chitosan. Anti-inflammatory, Inflammation, Sustainable Valorization,

INTRODUCTION

Inflammation is a complex biological response of protective tissues to harmful biological stimuli (e.g. microorganisms, dead cells, antigenic agents, foreign bodies and other exogenous and endogenous damage) and chemical, physical stimuli (e.g. toxic compounds, mechanical stress, irradiation). While the process is normally protective and essential for homeostasis, excessive or uncontrolled inflammation is an important contributing factor to the pathogenesis of arthritis, cardiovascular disease, diabetes, neurodegeneration and cancer. More recently, several lines of evidence have suggested that low-grade chronic inflammation may be a major driver of the disease process and aging. (1,2,3)

Although nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used for a number of inflammatory disorders, their long-term use may be associated with gastrointestinal ulceration, nephrotoxicity, hepatotoxicity and cardiovascular diseases. Therefore, much research has focused on developing new anti-inflammatory drugs with fewer side effects and a higher degree of biocompatibility. In particular, much attention has focused on finding new anti-inflammatory compounds of natural origin that have low toxicity (4,5). Chitosan is one of the most promising bio-materials as it is biodegradable and biocompatible, non-toxic and exhibits several biological functions. Chitosan is produced by deacetylation of chitin, which is abundant in the shells of crustaceans and the waste from fish processing. Numerous recent reviews have detailed the many areas of biomedical applications of chitosan (6,7,8), including its antimicrobial, antioxidant, wound healing, drug delivery, tissue engineering, and anti-inflammatory properties (Figure 1). Chitosan has anti-inflammatory properties, which probably work by inhibition of inflammatory enzymes and pro-inflammatory cytokines, modulation of oxidative stress, regulation of immunity, and stabilization of the cells' membranes. Also, the ideal physicochemical properties and bioavailability of chitosan have shown important potential for therapeutic applications as chitosan-based formulations and nanoparticles (6).

Channa punctata is a freshwater fish, which is a source of protein, essential amino acids and polyunsaturated fatty acids. The biocompounds from the genus Channa have been assessed for anti-inflammatory, wound healing and radical scavenging properties. Using the snakehead species, TNF-α and other mediators of inflammation have been shown to be

inhibited in the snakehead species used in this study. Therefore, chitosan from snakehead Channa scales would make a good anti-inflammatory and membrane-stabilizing biomaterial (9). Several *in vitro* anti-inflammatory activity assays were completed and are described below. For example, proteinase inhibition assays are used to determine if an active substance inhibits tissue damage during the inflammatory response by proteolytic enzymes secreted by inflammatory cells, such as trypsin, chymotrypsin or elastase (10). Hyaluronidase inhibition assays test the ability of an active substance to interfere with the degradation of the ECM component hyaluronic acid and thus with the associated tissue permeability and leukocyte recruitment (11, 12). LOX (lipoxygenase) inhibition assays test the ability of active principles to inhibit the conversion of arachidonic acid and other polyunsaturated fatty acids (PUFA) to leukotrienes, another group of powerful pro-inflammatory mediators, by lipoxygenases (enzymes that catalyze the oxidation of PUFA (13) (inhibition of LOX is thought to play some role in lysosomal membrane stabilization). Indirect evidence of lysosomal membrane stabilization can be obtained using erythrocyte membrane stabilization assays (hemolysis in vitro by heating or hypotonicity). These assays are often used as screening tests, measuring the anti-inflammatory effects of natural products and other biomaterials (14). Together, both groups of assays allow for the investigation of anti-inflammatory activity through both enzyme inhibition and membrane stabilization pathways (15).

Recently, it has been shown that molecular weight, the degree of deacetylation, crystallinity, and source of the material of chitosan have an important effect on biological activity. Different physicochemical properties have different biological effects, like an anti-inflammatory effect. Due to the abundance, low cost, possible utilization of waste, and bioactivity of fish chitosan biopolymers, fish chitosan can be considered a green alternative to commercial chitosan. Based on their structure and the fact that they are more functional and therapeutic than chitosans obtained from other sources, chitosans from aquatic organisms are considered superior to chitosans obtained from terrestrial sources (7, 8, 16).

Although anti-inflammatory effects of chitosan and its derivatives have been widely reported, other biological activities are also likely to be involved, and these activities depend on chitosan source, molecular weight, degree of deacetylation and preparation method. More recently, the most important factors have been suggested to be structural and physicochemical characteristics of chitosan-based materials, which may have a strong influence on their anti-inflammatory activity. Fish-processing by-products also represent a possible source of sustainably sourced chitosan with good performance in biomedical and bioactive applications, although comparisons between the anti-inflammatory effect of commercial chitosan and fish chitosan combined with complementary in vitro assays are yet to be made. Few assays have been conducted to test for inhibition of enzymes and protection of membranes in chitosan to date, and there has been no research investigating the anti-inflammatory mechanism of chitosan. This study intended to evaluate and compare the in vitro anti-inflammatory potential of commercial chitosan to chitosan extracted from fish (*Channa punctata*) using different experimental tests. The aim of this study was to evaluate the effect of source variability on biological activity and to provide scientific evidence for the development of fish chitosan extract as a green natural anti-inflammatory (17, 18).

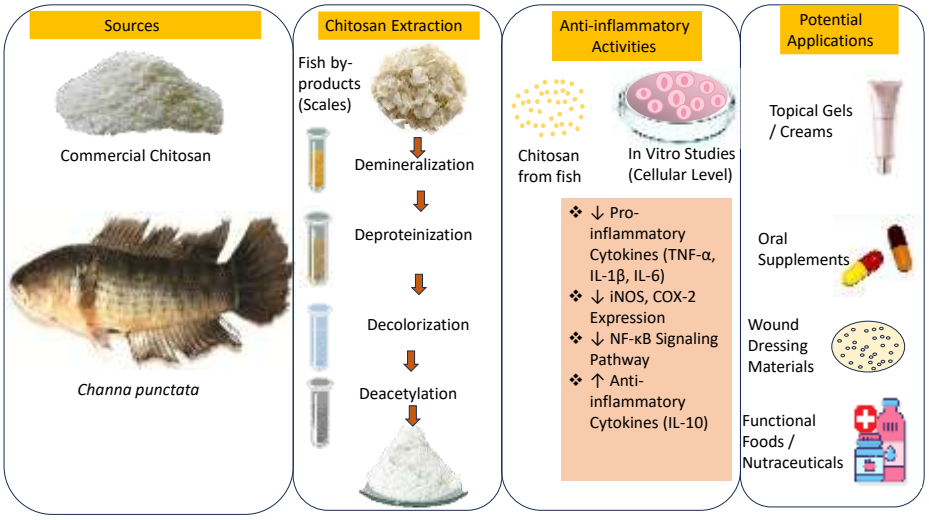

Figure 1: Fish scale waste is valorized through chitin extraction and deacetylation to produce chitosan, a multifunctional biopolymer with potent anti-inflammatory activity and broad biomedical applications.

2.MATERIALS AND METHODS

2.1 Chitosan Samples and Preparation

In the present work commercially available biopolymer chitosan and fish chitosan derived from *Channa punctata* was used. Chitosan was subjected to standard protocols of demineralization, deproteinization and deacetylation(Fig 2). Chitosan samples were dissolved in respective solvents and made respective stock solutions. The stock solutions were diluted to obtain 100, 200, 300, 400, and 500 µg/mL concentrations. In vitro anti-inflammatory activity of chitosan samples was determined by proteinase inhibition, hyaluronidase inhibition, lipoxygenase (LOX) inhibition, and membrane stabilization tests. Except where noted otherwise, experiments were performed in triplicate, and data expressed as mean ± SD.

Figure 2: Chitin derived from fish scales can be converted into chitosan, which demonstrates significant anti-inflammatory activity

2.1 Membrane Stabilization Preparation of Red Blood cells (RBCs) Suspension-
Blood was collected from a healthy human volunteer who had not received any NSAID in the preceding fortnight. Blood was transferred to centrifuge tubes and centrifuged at 3000 rpm for 10 min, after which the sediment was thrice washed with equal volumes of normal saline by centrifugation. The blood volume was estimated and subsequently reconstituted as a 10% v/v suspension of human red blood cells (HRBC) in normal saline.

2.2 Heat-Induced Hemolysis Assay
Chitosan isolated from the fish scale of *C. punctatus* and commercial chitosan were used to perform HRBC membrane stabilization in vitro assay to evaluate anti-inflammatory activity. The HRBC membrane stabilization in vitro assay is one of the most widely used in vitro assay for studying anti-inflammatory activity as the composition of the erythrocyte membrane is similar to that of the lysosomal membrane. Stabilization of HRBC membrane may be due to inhibition of release of inflammatory mediators (19, 20). Now 2 mL of the reaction mixture was placed, containing a test sample (1 mL) of chitosan solution as concentration of 100-500 µg/mL and a 1 mL 10% suspension of HRBC sample prepared using normal saline. In the control case, the test sample was replaced with normal saline. Aspirin was used as the reference anti-inflammatory drug for the above-mentioned HRBC-based anti-inflammatory methods, which have been applied for screening natural biomaterials and anti-inflammatory drugs (20,21).The reaction mixtures are placed in a water bath at 56 °C for 30 minutes. This heat treatment led to membrane rupture and cell lysis. It is used as a model for cell membrane destabilization in inflammatory events (21,22). The tubes were cooled under running water. Later they were centrifuged at 2500 rpm for 5 min. The supernatant was diluted with distilled water and the absorbance was measured at 560 nm using a UV-Visible spectrophotometer. The increase in absorbance indicates the hemolytic property, while the decrease in absorbance indicates the membrane stabilization and anti-inflammatory effect (19, 20) . Haemolysis was calculated as:

Percentage inhibition=(Abs control–Abs sample)X100/Abs control

2.3 Hypotonicity-Induced Haemolysis
Fish scales extract and commercially purchased chitosan solutions (100, 200, 300, 400, 500 µg/mL) were separately mixed with 1 mL of phosphate buffer of pH 7.4, 2 mL of 1.8% NaCl (hyposaline) and 0.5 mL of HRBC suspension. 100 µg/mL diclofenac sodium was used as a positive control. The set up for the other HRBC membrane stabilization studies (23, 24) was made similar to that of previous experiments, and these reaction mixtures were kept in a water bath at 37 °C for 30 min to induce osmotic stress hemolysis. In case the dilutions are treated hypotonic, the cytoplasm of erythrocytes leaks and the membrane ruptures. Hence, any compound that prevents the coagulation is said to have a membrane-stabilizing and anti-inflammatory activity. The supernatants from the test samples were obtained after the incubation by centrifugation at 3000 rpm. One method for hemolysis detection is to measure the release of hemoglobin spectrophotometrically at 560 nm. The stabilization and anti-inflammatory activity correlate inversely with the absorbance.Triplicate sets of tests were carried out for all these experiments. A sample that was equivalent to the control was considered to have 100% hemolysis, and the percentage of protection against hemolysis was calculated as follows:

Percentage protection=100-(OD sample/OD control)x100

2.4 Hyaluronidase Inhibitory Assay
Hyaluronidase inhibitory activity of fish scale chitosan (*Channa punctatus*) and commercially available chitosan was determined according to the method of Sahasrabudhe & Deodhar (1957) with a slight modification. Hyaluronidase is a hydrolytic enzyme that degrades the hyaluronic acid of connective tissues. The inhibition of hyaluronidase is thought to be a major mechanism of anti-inflammatory action (25,26), but excessive hyaluronidase activity also results in tissue damage and inflammation.For this experiment, we prepared 0.1 M acetate buffer (pH 3.6) with 10 µL hyaluronidase

solution (8 mg/mL) and 10 µL different concentrations of chitosan solution (100, 200, 300, 400, 500 µg/mL). Then we incubated the prepared solutions at 37 °C for 20 minutes, and allowed the enzyme and chitosan to interact with each other. The temperature of incubation was similar to that used for the assays of naturally occurring anti-inflammatory agents with hyaluronidase inhibitory activity (27, 28). Next, 20 µL of 12.5 mM calcium chloride was used as an activator. The assay mixture was then incubated at 37 °C for 20 min. Calcium ions are important for hyaluronidase activity and substrate hydrolysis (12, 29).The DMAB reagent was prepared by dissolving 0.4 g of p-dimethylaminobenzaldehyde (DMAB) in 35 mL of glacial acetic acid and then adding 5 mL of 10 N hydrochloric acid. The solution was cooled, and 600 µL of DMAB reagent was added. In the assay, after incubating the reaction mixture at 37 °C for 20 min, DMAB was reacted with the hyaluronic acid degradation products and the chromophore formed was measured spectrophotometrically at 585 nm (30). Thus, the more reduced absorbances were at 585 nm, the more the hyaluronidase activity was inhibited and therefore, the more anti-inflammatory activity was found for the specific chitosan sample.The percentage inhibition of hyaluronidase activity was calculated with the following equation:
%inhibition=100×(absorbance of the control−absorbance of the sample)/absorbance of the control

2.5 Lipoxygenase Inhibition Assay
Lipoxygenase inhibitory activity of chitosan obtained from the fish scale of *Channa punctata* and commercial chitosan was determined according to the method of Wu (1996) with some modifications of Gunathilake et al. (31,32,33).1.0 mL of sodium borate buffer (0.1 M, pH 8.8) mixed with 10 uL of lipoxygenase enzyme (8000 U/mL) was prepared in a quartz cuvette and then incubated at room temperature (30 ± 2 °C) for 5 min after mixing with 10 uL of different concentrations (100, 200, 300, 400 and 500 ug/mL) chitosan solution. The enzymatic reaction was started by the addition of 10 uL linoleic acid (10 mM) substrate and the progress of the reaction was determined by measuring the formation of conjugated dienes from the absorbance at 234 nm of a UV-Visible spectrophotometer. The control reaction was performed by using a phosphate buffer mixture instead of the chitosan solution, and Indomethacin was used as an experimental reference anti-inflammatory drug (31,32). The percentage inhibition of lipoxygenase was calculated using the following equation:

%inhibition=100×(absorbance of the control−absorbance of the sample)/absorbance of the control

2.6 Proteinase Inhibitory Activity
The inhibition of proteinases is important, for example, because proteolytic enzymes released during inflammation contribute to the inflammatory process and damage tissues; thus these enzymes must be inhibited to limit tissue damage. The reaction mixture was prepared by mixing 2 mL of trypsin (0.06 mg), 1 mL of 20 mM Tris-HCl buffer (pH 7.4) and 1 mL of a chitosan solution (100 to 500 µg/mL). After that, the reaction mixture was incubated at 37 °C for 5 min to allow trypsin to bind to the test sample. This technique has been used in other recent studies investigating natural anti-inflammatory compounds using proteinase inhibition assays.After that, 1 mL of 0.8% (w/v) casein solution (substrate) was added to each tube and incubated at 37 °C for the next 20 min, which lead to trypsinized casein low molecular weight peptides. Proteins' hydrolysis inhibition by the inhibitory substances in chitosan was determined by trypsin-casein digestion-based assays which are used for the anti-inflammatory evaluation of natural biomaterials or polymers. Following incubation, 2 mL of 70% perchloric acid solution was added to each tube, thus terminating the enzymatic reaction, and precipitating any undigested protein. The supernatant portion of the centrifuged solution was analyzed spectrophotometrically for residual protein content at 210 nm (buffer solution was used as a blank solution). These lower levels of absorbance suggested that the proteins were more protected from the action of trypsin by the chitosan samples than the control medium, phosphate buffer solution (32, 34, 35). The percentage inhibition of the proteinase activity was calculated as follows:
% inhibition of denaturation=100×(1−A2/A1),
Where A1=absorption of the control sample, and A2=absorption of the test sample.

2.7 Statistical Analysis
The data were expressed as a mean ± standard deviation of three replicates in all experiments. The dose effect (range finding) of the samples was also evaluated mathematically using the experimental data. One-way analysis of variance (ANOVA) and Tukey's post hoc (multiple comparison) analysis were performed using GraphPad Prism where a value of $p < 0.05$ was considered statistically meaningful.

3. RESULTS
3.1 Heat-Induced Hemolysis Assay
When all groups were analyzed, the heat-induced hemolysis assay showed that membrane stabilization of all treatment groups was considerably concentration dependent (one-way ANOVA $p < 0.001$). Tukey's post hoc multiple comparison test indicated that the readings of the commercial chitosan, *C. punctata* chitosan, and aspirin groups differed from one another ($p < 0.05$). Commercial chitosan at 100 µg/mL and 500 µg/mL showed 27.42 ± 3.23% and 72.04 ± 3.40% of stabilization of membrane respectively. *Channa punctata* chitosan showed a meaningful concentration dependent increase in stabilization of membrane (30.65 ± 3.23% at 100 µg/mL and 64.52 ± 2.79% at 500 µg/mL*). Channa punctata* chitosan at 100 to 200 µg/mL produced comparable to slightly better membrane stabilization, whereas at higher concentrations (300 to 500 µg/mL), commercial chitosan showed slightly better inhibition. Aspirin, the control drug, showed a membrane stabilization percentage of observation in the present study, ranging from 15.59 ± 1.86% at 100 µg/mL to 77.42 ± 4.23% at 500 µg/mL. Maximum inhibition was observed at 500 µg/mL. From the results, it can be concluded

that the chitosan extracted from *Channa punctata* has good anti-inflammatory and membrane stabilization activities and has an inhibition activity that is not considerably different from the commercial chitosan at a low concentration (see Figure 3). *Channa punctata* fish scale-derived chitosan has the potential to be developed as a natural anti-inflammatory biomaterial in the pharmaceutical and biomedical fields.

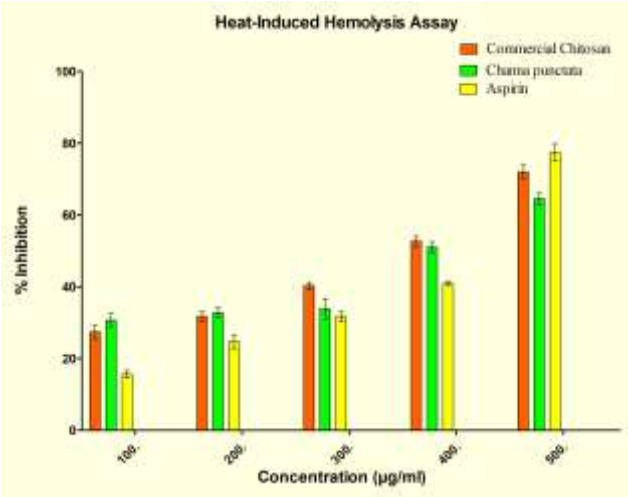

Figure 3: Heat-Induced Hemolysis Assay

3.2 Hypotonicity-Induced Hemolysis

In the hypotonicity-induced hemolysis assay, it was observed that membrane stabilization activity was concentration-dependent in all the treated groups (one-way ANOVA, $p < 0.001$). Tukey's post hoc multiple comparison test showed the meaningful difference of the commercial chitosan, *Channa punctata* chitosan, and diclofenac sodium at the concentration of 0.1 mg/ml to 2 mg/ml ($p < 0.05$).*Channa punctata* chitosan is reported to be better than the already available commercial chitosan in membrane stabilization at 100-400µg/mL concentrations, with membrane stabilization being 23.88 ± 0.78% for 100µg/mL, and 45.99 ± 1.03% for 400µg/mL concentration. Also at 500 µg/mL, the stabilization was 57.86 ± 1.09% which was slightly more than commercial chitosan (56.60 ± 3.27%). For commercial chitosan, stabilization was 10.69 ± 2.88% at 100 µg/mL and 56.60 ± 3.27% at 500 µg/mL. At 100 and 500 µg/mL concentrations of standard drug diclofenac sodium, the membrane stabilization percentage was 15.09 ± 3.27 and 87.42 ± 1.09 respectively (Figure 4). The results of this study suggest that the membrane-stabilizing activity of the chitosan from *Channa punctata* is greater than that of commercial chitosan at most of the concentrations tested. It also shows good dose-dependent anti-inflammatory activity. Although its anti-inflammatory activity is lower than that of diclofenac sodium, the higher protective effect on erythrocyte membranes suggests that chitosan from the fish scale of *Channa punctat*a has the potential to be a natural anti-inflammatory biomaterial.

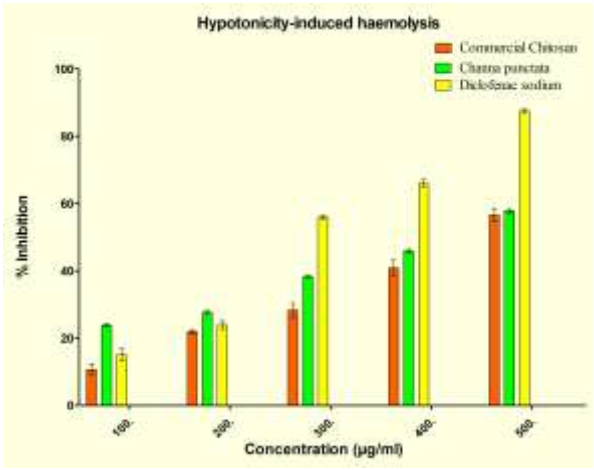

Figure 4: Hypotonicity-Induced Hemolysis

3.3 Hyaluronidase Inhibition Assay

In hyaluronidase inhibition assay, all treatment groups were considerably different among themselves based on concentration (one-way ANOVA, $p < 0.001$). On Tukey's post hoc multiple comparison test, commercial chitosan,

Channa punctata chitosan, and indomethacin were statistically different from each other at most concentrations ($p < 0.05$). The activity of hyaluronidase inhibition also increased gradually due to increase in concentration of *Channa punctata* chitosan. The enzyme inhibition percentage was 28.57 ± 0.89% at 100 µg/mL and 69.64 ± 1.79% at 500 µg/mL of *Channa punctata* chitosan. The hyaluronidase inhibitory activity of *Channa punctata* chitosan ($p < 0.05$) was considerably higher than that of commercial chitosan in the same concentration, from 22.32 ± 0.89% to 62.50 ± 1.55%. The standard drug indomethacin was more effective than the fractions, and it inhibited hyaluronidase activity in a dose-dependent manner, from 37.20 ± 1.03% inhibition at 100 µg/mL to 91.07 ± 1.55% inhibition at 500 µg/mL (Figure 5). *Channa punctata*-derived chitosan had higher hyaluronidase inhibitory activity than the commercial chitosan and had an important anti-inflammatory activity, although the activities were lower than indomethacin, the higher inhibition of hyaluronidase suggests that *Channa punctata*-derived fish scale chitosan is a potential natural biomaterial for pharmaceutical and biomedical use in the future.

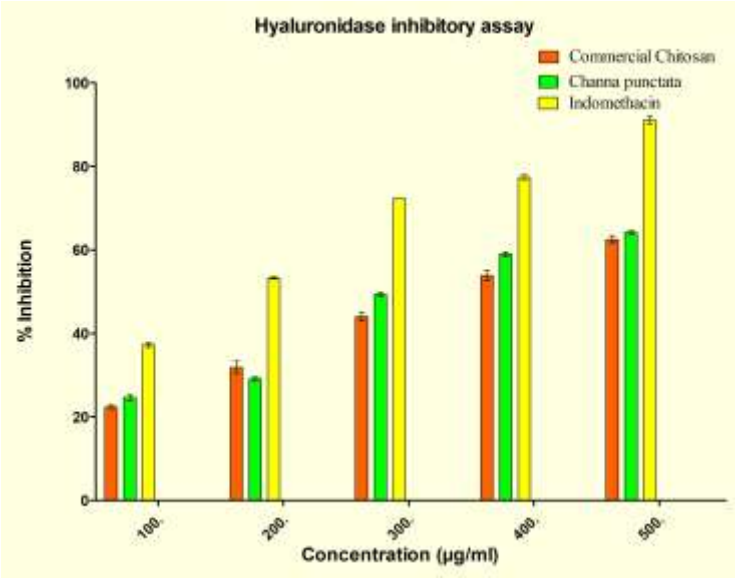

Figure 5: Hyaluronidase Inhibition Assay

3.4 Lipoxygenase (LOX) Inhibition Assay

The LOX inhibition assay result showed a dose-dependent increase in the inhibitory activity among all treatment groups ($p < 0.001$) found by one-way ANOVA. Furthermore, employing the Tukey's post hoc multiple comparison test, a meaningful difference between commercial chitosan, *Channa punctata*-derived chitosan and indomethacin for most of the concentrations was observed ($p < 0.05$). In LOX inhibition activity assay among all the chitosan samples tested, chitosan from *Channa punctata* showed the highest inhibition percentage at all concentrations ranging from 100 to 500 µg/mL. The LOX inhibition percentage was 22.58 ± 0.00% at 100 µg/mL and that was considerably higher than that of commercial chitosan (5.38 ± 3.72%) and indomethacin (14.79 ± 0.47%). The inhibitory percentage increased gradually and was 39.89 ± 1.76%, 58.95 ± 0.62%, 66.88 ± 2.23% and 74.19 ± 3.23% at 200, 300, 400 and 500 µg/mL concentrations respectively. However, the commercial chitosan resulted for the given concentrations as 7.53 ± 1.86%, 23.66 ± 4.98%, 37.63 ± 8.09% and 48.39 ± 3.22% respectively. Although indomethacin is still the most strong inhibitor at higher concentrations (72.85 ± 0.47% and 96.51 ± 0.47% at 400 and 500 µg/mL respectively), *Channa punctata* derived chitosan has a better inhibitory activity than the standard at low concentrations (100-300 µg/mL) (Figure 6). In general, results indicate that chitosan obtained from *Channa punctata* has a considerably higher LOX inhibitory activity than commercial chitosan. The clear concentration-dependent inhibitory effects suggest fish scale-derived chitosan could be used as a sustainable natural anti-inflammatory biomaterial for pharmaceutical and biomedical applications

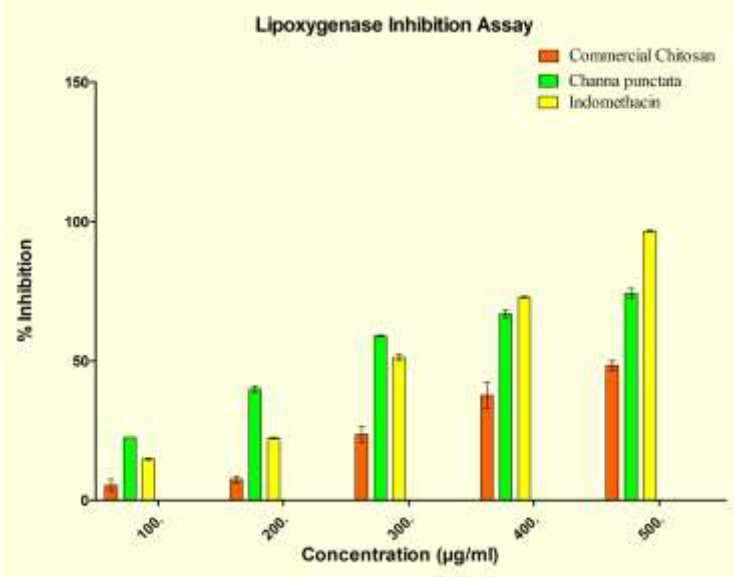

Figure6:Lipoxygenase(LOX)InhibitioncAssay

3.5 Proteinase Inhibitory Activity

The proteinase inhibitory assay showed that the inhibitory activity increased in a concentration dependent manner throughout the treatment groups (one-way ANOVA, $p < 0.001$). Tukey's post hoc multiple comparison test between commercial chitosan, *Channa punctata* chitosan and diclofenac sodium showed important difference in most of the concentrations ($p < 0.05$). The proteinase inhibition in chitosan *Channa punctata* was increased from 9.40 ± 0.46% to 45.22 ± 0.57% at the doses of 100 and 500 µg/mL, thus indicating the dose dependent anti-inflammatory activity of chitosan. Commercial chitosan produced a higher inhibition of proteinase than chitosan from *Channa punctata*. At doses of 100 and 500 µg/mL chitosan, the percent inhibition was 11.32 ± 3.27% and 59.75 ± 1.09%, respectively, with importance at doses greater than 100 µg/mL ($p < 0.05$). Among the four drugs, diclofenac sodium as standard drug showed highest inhibition (27.04 ± 2.88% inhibition at 100 µg/mL, 93.08 ± 1.09% inhibition at 500 µg/mL) (Figure 7).

From the results it was concluded that the proteinase inhibitory activity of *Channa punctata* chitosan was noticeable and dose dependent but less than commercial chitosan and diclofenac sodium drugs. In conclusion, findings support the use of *Channa punctata* chitosan derived from fish scales as a natural anti-inflammatory biomaterial for future pharmaceutical and biomedical applications

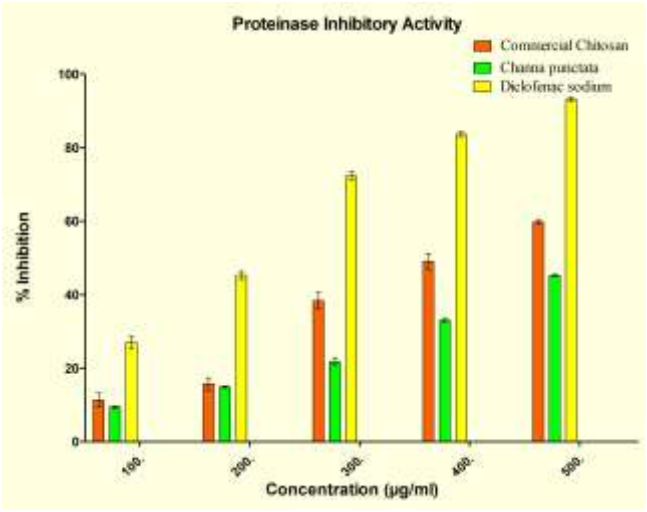

Figure 7: Proteinase Inhibitory Activity

4. DISCUSSION

The above study demonstrated that *Channa punctata* chitosan and commercial chitosan have high and concentration-dependent anti-inflammatory activity. This conclusion was supported by five in vitro tests (heat induced hemolysis, hypotonicity induced hemolysis, inhibition of hyaluronidase, inhibition of lipoxygenase (LOX) and inhibition of

Proteinases). These results suggest that the anti-inflammatory action of chitosan is multifactorial. The anti-inflammatory effect of chitosan may derive from stabilization of cell membranes, inhibition of inflammatory enzymes, inhibition of lipid mediator formation, or inhibition of the enzymatic hydrolysis of extracellular matrix components. The concentration-dependent manner observed in all assays may be due to the increasing availability of chitosan functional groups that ease interaction with a wide range of biological membranes, enzymes, and inflammatory sites.

The heat induced hemolysis assay revealed that *C. punctata* and commercially available chitosan had membrane stabilization property. The erythrocyte membrane stabilization is considered as an indirect model for determining anti-inflammatory property since there is similarity between the structures of the erythrocyte and lysosomal membranes. The stabilization of these membranes can therefore be used to prevent the release of lysosomal enzymes and other mediators of inflammation from the lysosomal compartment that would otherwise cause damage to the tissue and promote inflammation. The comparative membrane stabilization of *C. punctata* chitosan under relatively lower concentrations shows that it is capable of protecting the erythrocyte membrane from thermal stress. The improved activity of commercial chitosan at higher concentrations may have been the result of constant molecular parameters in the commercial chitosan such as degree of deacetylation and molecular weight range. The activity of *C. punctata* chitosan further supports that fish-scale chitosan can achieve and sustain substantial membrane stabilization and inhibition of inflammation through prevention of membrane damage.

The hypotonicity-induced hemolysis test was employed to further support the protective effect of *C. punctata* chitosan on erythrocyte membranes. When erythrocytes are placed in hypotonic solution, they become osmotically swollen due to the excess water influx across the membrane. The membrane stabilizing and anti-inflammatory activity of these compounds are well-documented (36,37). In this study, it was found that *C. punctata* chitosan exhibited a considerably greater membrane stabilization effect than did commercial chitosan over a wide range of concentrations, indicating that the fish chitosan interacts well with the membrane phospholipids and membrane proteins. The concentration-dependent chitosan activity may be due to the ability of the amino residue of protonated chitosan to form electrostatic complexes with negatively charged components of biological membranes (38). It may protect membranes by stabilization and inhibition of the release of inflammatory mediators. Chitosans are also known to downregulate signaling pathways, inflammatory cytokines, and oxidative stress and protect cellular membranes from injury (17). In this respect, the membrane-stabilizing properties of *C. punctata* chitosan point to other possible anti-inflammatory mechanisms, aside from its ability to directly inhibit enzymes.

The enzyme-mediated mechanism was also supported by a hyaluronidase inhibition assay. Hyaluronidase from extracellular matrix digests hyaluronic acid, increasing permeability of tissues and the movement of inflamed mediators (39). Hyaluronidase inhibition may therefore maintain the normal matrix structure and decrease the degradation of hyaluronic acid, thus reducing the edema response to inflammation (40). The more powerful hyaluronidase inhibition of *C. punctata* chitosan compared to commercial chitosan may be due to a better interaction between the fish polymer and hyaluronidase or the substrate. This activity might also be related to the source-specific physicochemical properties such as degree of deacetylation, molecular weight, free amino groups, and surface charge and conformation, influencing the chitosan interaction with biological macromolecules (38,41). The improved activity of *C. punctata* chitosan shows the potential of fish-derived chitosan to affect the breakdown of extravasated matrix and to protect tissue integrity during inflammation.

In this regard, the LOX inhibition assay directly and strongly indicated the possibility of using *C. punctata* chitosan as an anti-inflammatory agent. The LOX enzyme is responsible for the formation of leukotrienes and other lipid mediators involved in vascular permeability and leukocyte migration (42). Thus, inhibiting the activity of LOX might probably inhibit both the lipid-mediated inflammation response and the oxidative inflammation response (43). In the current study, the LOX inhibition activity of *C. punctata* chitosan was generally greater than that of the commercial chitosan, indicating its greater ability to modulate the inflammatory pathway. The reason for the high activity could be explained by the influence of physicochemical characteristics such as the availability of free amino groups and the degree of deacetylation, which resulted in better electrostatic and hydrogen bond interactions between the enzyme and substrate (38,44). Indeed, higher inhibition of LOX by *C. punctata* chitosan than that of the other samples attests that fish chitosan anti-inflammatory activity is attributable not only to membrane stabilization but also to direct modulation of a key enzyme pathway of inflammatory lipid metabolism.

These results in the proteinase inhibition assay supporting the conclusion of a multifactorial, chitosan mediated anti-inflammatory action: the release and/or activation of proteinases at the foci of the inflammation contributes to the fragmentation of extracellular matrix proteins, tissue destruction, cell death, and chronicity of the inflammation. Therefore, the inhibition of unregulated proteinase activity would potentially protect tissues and partially limit the proteolysis associated with inflammatory diseases (45). Although commercially produced chitosan was more strong than *C. punctata* chitosan, the chitosan derived from the arthropod did show a concentration-dependent inhibition effect with considerable proteolytic activity in the system. Commercial chitosan may be more active due to a more uniform degree of deacetylation and molecular-weight distribution (or less batch-to-batch variability), which could ease protein target binding (38). Other possible explanations for the difference in activity have been proposed, including differences in crystallinity, polymer chain organization, surface charge, and the accessibility of functional groups (44). The stronger inhibition by diclofenac sodium is in accordance with the known pharmacological anti-inflammatory activity and modulation of the inflammatory mediators and tissue effects (46).

Combined, the five assay systems suggest a broad anti-inflammatory action for *C. punctata* chitosan, possibly mediated by several different, but complementary mechanisms: membrane stabilizing activity may, for example, protect cellular and lysosomal membranes from disruption, while the hyaluronidase and proteinase inhibitors suggest protection of

extracellular-matrix components against enzymatic degradation. Its high LOX inhibitory activity also points to the possibility that it may act by inhibition of inflammatory lipid mediators in a further pathway, particularly given that, as discussed in detail below, this is a multifactorial biological system with many cellular, enzyme, oxidative, and ECM aspects.

This comparison to commercial material also stresses the importance of source-dependant physicochemical properties of chitosans on their bioactivity. The commercial chitosan was a more effective stabilizer of membranes and proteinase, likely due to its more uniform physicochemical properties. LOX and hyaluronidase inhibition may have been due to the specific character of *C. punctata* chitosan, since it was a more effective stabilizer of membranes and more effective in regard to proteinase inhibition. It also suggests that fish-derived chitosan may have unique structure-activity relationships that could be helpful in a biomedical context, rather than merely being a substitute for chitin from other sources.

The biological activity of chitosan isolated from *C. punctata* may be related to its degree of deacetylation, molecular weight, amino group density, crystallinity, surface charge, molecular conformation, and polymer arrangement. The protonated amino groups of chitosan can electrostatically interact with negatively charged biological macromolecules (membrane phospholipids, enzymes, proteins); hydrogen bonding, hydrophobic interaction, and van der Waals forces may also considerably contribute to biological activity (38,41). Such changes in these properties may affect the binding to enzymes, association with membranes, protein interaction, and anti-inflammatory activity. It can thus be concluded that the biological performance of fish-derived chitosan is most likely determined by a combination of its chemical composition and structural organization.

In conclusion, the results support the use of chitosan derived from the fish scales of *C. punctata* as a multifunctional natural anti-inflammatory biomaterial to stabilize biological cell membranes and inhibit several inflammation-related enzymes, with potential applications for simultaneous targeting of different inflammatory processes. The use of *C. punctata* scales as a source of bioactive chitosan could lead to a more sustainable way of valorizing fish waste and generating biomedical products of commercial interest. Further research is needed to explore the effects of molecular weight, degree of deacetylation, crystallinity, surface properties, and the accessibility of functional groups on the mechanisms of anti-inflammation. This will be helpful for the application of *C. punctata*-derived chitosan in healthcare such as pharmaceuticals, wound-healing, and drug delivery.

5. Conclusion

The present study indicates the membrane stabilizing, lipoxygenase inhibitory, hyaluronidase inhibitory, and proteinase inhibitory activities of *Channa punctata* chitosan. The concentration dependent experiments to confirm the anti-inflammatory activity of *Channa punctata* derived chitosan in different in vitro assays of anti-inflammatory activity, indicate the multi-targeting anti-inflammatory potential of the chitosan. Despite the stronger effect of commercial chitosan on some parameters, the results suggest the potential efficacy of *C. punctata*-derived chitosan as comparable or superior in classical anti-inflammatory mechanisms, thus underlining the role of source-associated physicochemical properties in shaping chitosan's biological profile. Bioactive chitosan can also be obtained from fish scales, which represent a highly sustainable and high-value fish by-product. Chitosan from *C. punctata* appears to be a promising natural source for pharmaceutical, biomedical, wound healing and drug-delivery applications. Further research is needed using *in vivo* models and specific mechanistic studies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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