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(RESEARCH ARTICLE)

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An investigation on the antimicrobial activity of shrimp exoskeleton extracted glycosaminoglycans (GAGs) against *Escherichia coli* and *Staphylococcus aureus*

Abhisubesh Vijayakumar, Jayanthi Jayaprakash *, Bharathi Ravikrishan, Sharmila Sankar, Ankesh Yadav, Rebecca Vaishali John and Ragunathan Manikavalli Gurunathan

Department of Advanced Zoology and Biotechnology, Guru Nanak College, Chennai- 600042, Tamil Nadu, India.

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Abstract

The present study was aimed to extract the Glycosaminoglycans (GAGs) from the shrimp head waste and carry out analysis for any antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*. Glycosaminoglycans (GAGs) are linear acidic polysaccharides with repeating disaccharide units. GAGs are important for cell signaling, which controls a wide range of metabolic activities including regulation of cell growth, promotion of cell adhesion, wound repair, proliferation, anti-coagulation and antimicrobial activity. In the present study, the shrimp heads were washed, dried and pulverized. From this pulverized shrimp head waste, extraction of GAG protein was achieved. The total protein content in the biological sample was estimated. Antimicrobial study of GAGs complex was accomplished by using Minimal Inhibitory Concentration, Minimal Bactericidal Concentration, Well Diffusion Method against E. *coli* and *Staphylococcus aureus*. It was observed that *Staphylococcus aureus* has the highest antibacterial activity when compared to *Escherichia coli.*

Keywords: Glycosaminoglycans; Minimal Inhibitory Concentration; Minimal Bactericidal Concentration; Well Diffusion Method

1. Introduction

Anionic, linear polysaccharides made up of periodic disaccharide units are known as glycosaminoglycans (GAGs). They are essential for many ongoing biological processes occurring in the extracellular matrix [1]. Animal life is largely attached to a substrate; thus it responds to ecological demand by producing bioactive metabolites [2]. Seafood forms an important part in the fisheries sector with regard to employment, livelihood, food security and human nutrition. There is also an increased worldwide concern of over exploitation of natural seafood resources processing discards which are considered as waste. The utilization of processing discards for the production of commercially viable products can have enormous economic implications in a developing country like India. Scientists have recently been paying more and more attention to marine organisms and the secondary metabolites that they produce. The study of marine natural products promotes a multidisciplinary approach among scientists who study biology, pharmacology, and chemistry [3].

Monetarily the shrimp production is said to be the second most important commodity trade and with an increase in shrimp output which has resulted in more discarded waste, which turns out to be an environmental hazard. One of the main byproducts of the fishing business is waste from the processing of shrimp. In order to extract the bioactive compounds from shrimp waste, it is typically treated using hydrolysis. Hydrolysis may be used in industrial settings with ease, and it has even been used to create extremely nutritious protein hydrolysate. It is regarded as one of the procedures in the extraction of other biologically active compounds, like chitin and carotenoids. Cephalothorax, carapace, and tail are the three main components of shrimp processing debris. The head hydrolysate contains a high

* Corresponding author: Jayanthi Jayaprakash

concentration of critical amino acids and contains 44% protein. Shrimp head waste has rich source of protein and essential amino acids [4].

For biological scientists, GAGs are a fascinating group of molecules that have attractive role in medical cell biology, immunology and biochemistry. GAGs like heparin are widely used as an infection anticoagulant for over half a century. They are lengthy, negatively charged linear acidic polysaccharides that are typically linked to matrix proteins [5]. The extracellular space in animal tissues is filled with the gel-like substance known as extracellular matrix(ECM), which serves as a porous channel for the transport of oxygen and nutrients to individual cells. Due to the sulphate or carboxyl group, they have a significant negative charge. GAGs can be primarily divided into galactoaminoglycans (GalGs) and glucosaminoglycans based on the presence of hexosamine units (GlcGs) [6].

One of the most crucial elements of ECM is glycosaminoglycans. Hyaluronic acid and chondroitin sulphate are thought of as glucosamine polymers. The purpose of the amino monosaccharide glucosamine is to lessen joint injury. Due to their significant structural variability, which produces various GAG chains with comparable chemical properties, glycosaminoglycans (GAGs) study is a difficult task. At the same time, it is crucial to comprehend their function and behavior in biological systems. GAGs are highly polar and attract water and therefore they are used as lubricants [7].

Their molecular makeup can be used to determine how they work in the body. GAGs were previously thought to have only two functions: to hydrate cells and to serve as structural scaffolding. Today's research indicates that GAGs are important for cell signaling, which controls a wide range of metabolic activities. Some of these processes include regulation of cell growth, promotion of cell adhesion, wound repair, proliferation, anti-coagulation and so on [8].

An agent that kills microorganisms or stops their grow this said to possess antimicrobial activity. In diagnostic laboratories, Minimum Inhibitory Concentrations (MIC) are used to confirm microbial resistance to an antibiotic. The simplest laboratory test for determining an antimicrobial agent's activity against an organism is the MIC test. The minimum antimicrobial concentration required to suppress bacterial growth that can be seen as a zone of clearance is known as the Minimum Inhibitory Concentration. MIC values are used to assess the efficacy of new antimicrobial medications as well as the drug susceptibilities of bacteria. The development of antibiotic drug resistance can be determined by the Minimum inhibitory concentrations [9].

Agar dilution and tube dilution methods are the most common methods that are employed in the MIC. This method is targeted for the testing susceptibility to antibiotic agents and act as preservatives and disinfectants. The extracted Glycosaminoglycans was subjected for antimicrobial activity against *E. coli* and *Staphylococcus aureus* [10]. In the present study, Glycosaminoglycans was extracted from shrimp head waste and used for determination of antimicrobial activity against *E. coli* and *Staphylococcus aureus*.

2. Material and methods

The shrimp head waste was collected from the Saidapet market, Chennai, Tamil Nadu, India. The sample was washed thoroughly with running tap water, till the unwanted particles are been removed. After washing, it was air dried at room temperature. Then the sample was kept at hot air oven for two hours at 80ºC. The dried sample was left at room temperature for an hour. Then the sample was made to ground to powder for the extraction of glycosaminoglycan (Figure 1).

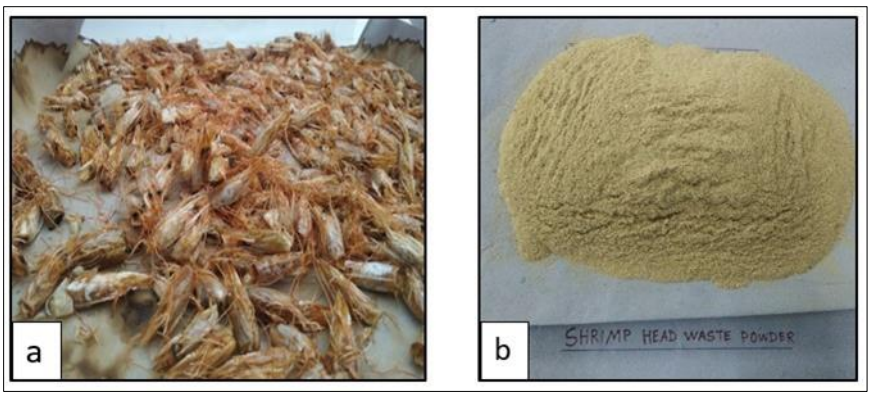

Figure 1 (a) Dried Shrimp head (b) Powdered Shrimp head

2.1. Extraction of glycosaminoglycan protein from shrimp head waste

The GAG protein was extracted from shrimp head waste using the protocol with slight modification [11]. Accordingly, 10 grams of the fine powdered sample was taken and defatted with 20 ml of acetone. Then it is filtered and further defatted with 10 ml of petroleum ether. The defatted sample was mixed with 50 ml of 0.4 M sodium sulphate and incubated at 55ºC for 1 hour and the pH was maintained at 11. After the incubation period, aluminum sulphate crystals were added to lower the pH to 7.7, and the mixture was then heated at 95 °C for 15 minutes. After cooling, it was centrifuged for 25 minutes at 3000 rpm. After centrifugation, 10 ml of 3% cetyltrimethyl ammonium bromide (CTAB) in 0.8 M sodium chloride were added to the supernatant. To obtain the crude GAG complex, this suspension was centrifuged at 3000rpm for 90 minutes at 4°C after incubating at 37°C for 24 hours. To get the crude GAGs complex, the precipitate was dried in vacuum desiccators after being washed with absolute ethanol [12].

2.2. Estimation of protein by Lowry's method

The "Lowry Assay" Protein estimation by Folin's Reaction has been the most widely used method to estimate the amount protein in biological samples. Different concentrations (0.2, 0.4, 0.6, 0.8 and 1ml) of Bovine Serum Albumin (BSA) as a standard solution and 0.5 and 1 ml of test sample were taken in a labeled test tube. Make all the test tube volume up to 2ml by adding distilled water. Add 2.5 ml of Lowry's reagent in all the Test tube and kept in dark for 10 minutes. After incubation add 0.01 ml of Folin's reagent in all test tubes and kept in dark for 10 minutes. After the incubation take the reading of the samples against blank in the UV Spectrophotometer at 650 nm [13].

2.3. Minimal inhibitory concentration (MIC)

Serial dilution of the extracted GAG sample was performed by adding 100 µl of GAG extract into 5ml of the Luria Bertani broth in test tubes (10 nos serially). After adding GAG sample into the test tube, 100 µl of the diluted sample was transferred into the next tube after mixing into a proper suspension and serial dilution was carried out till the last tube. 100 µl of the suspension from the last tube was discarded to maintain the constant volume in all the tubes. Similarly a second set of ten test tubes were also prepared. A loop full of inoculum of *E. coli and Staphylococcus* was added to the two sets of serially diluted test tubes. The samples were then incubated at room temperature for 24 hours and the results were recorded [14].

2.4. Minimal bactericidal concentration (MBC)

Plate count agar was prepared and after dissolving on hot plate, it was autoclaved. After cooling the agar to 70ºC, it was poured into 10 Petri plates aseptically and allowed to solidify. 10µl of broth exposed to the sample was lawn cultured into agar plates. The same was repeated with *Staphylococcus* also. The plates were incubated at 37˚C for 24 hrs [15].

2.5. Well diffusion method

Mueller Hinton agar is the popular culture medium for assessing bacterial antibiotic susceptibility test. For preparation of this media, 1.9 gm of Mueller Hinton agar was dissolved in 50 ml of distilled water and autoclaved at 121ºC for 15 minutes. After cooling, it was poured into sterile petri plates aseptically and allowed to cool. The two bacteria (*E. coli* and *Staphylococcus*) were lawn cultured in these MHA plates and wells were made in the agar. 10µl, 20µl, 30µl, 40µl and 50µl of the extracted Glycosaminoglycans protein dissolved in DMSO was added to the wells in one plate and 60µl, 70µl, 80µl, 90µl, and 100µl in another plate. The plates were incubated for 24 hours at 37˚C. Then the zone of inhibition was measured in millimeter [16].

3. Results

GAGs and proteoglycans have been linked to a number of pathological processes, including cancer biology, inflammation and infection, and amyloid disorders [17]. The shrimp head waste was used for the isolation of GAG protein by Holick *et al*., (1985) methodology with slight modification. Recent data reveals that they contain specified monosaccharide sequences and that they are potentially dense molecules [18]. Regarding chain length, disaccharide content, and sulfation pattern, GAG proteins show variation within and between species. The crude GAG complex was purified as dry powder and was suspended in PBS buffer (7.2 pH), by vigorous stirring. The GAG suspension was observed under microscope at 40X (Figure 2a and 2b). The exoskeleton makes up 50% of the entire shrimp and forms the majority of the waste produced during shrimp processing [19].

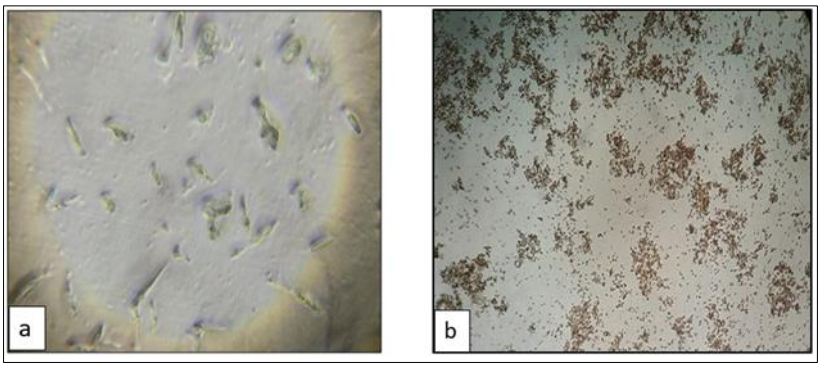

Figure 2 Microscopic observation of GAG protein a) with buffer b) Without buffer

From the above figure, it is evident that the isolated crude GAG proteins get imbedded when buffer was added and it shrinks in the absences of buffer. This confirms the gelatinous property of the extracted biomolecules. With molecular weights of 100,000–100,000,000, hyaluronic acid polymers are extraordinarily big and can remove a lot of water (https://themedicalbiochemistrypage.org/glycosaminoglycans-and-proteoglycans/).

3.1. Antibacterial activity by agar well diffusion method

Certain sulfated glycans isolated from land or marine mammals or invertebrates have therapeutic effects against diseases caused by harmful microbes like bacteria, viruses, fungi, and protozoan parasites because of their inherent structural characteristics [20]. The antibacterial activity can be impacted by partial digestion, structural changes, self-association, and molecular interactions, particularly with GAGs [21]. 1mg crude protein extract was suspended in 10 ml of PBS buffer and was used as the sample for protein estimation. Bovine serum protein was used as the standard. From the results it was estimated that the concentration of the crude protein extract in the shrimp head waste sample was determined to be 205 g/mg.

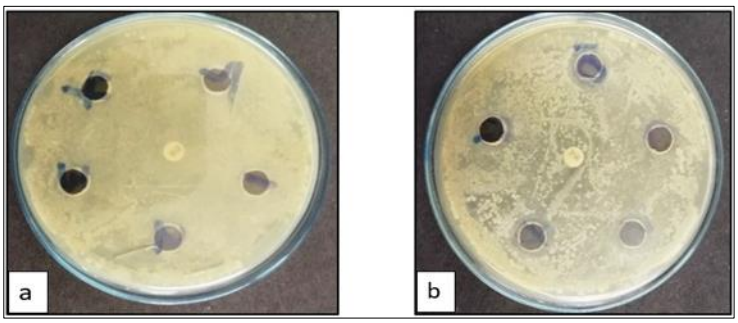

Figure 3 Antibacterial activity by agar well diffusion method (a) *E. coli* (b) *Staphylococcus aureus*

The antibacterial activity against *E. coli* and *Staphylococcus aureus* was performed by MH agar and tetracycline was used as control and the zone of inhibition was tabulated (Figure 3 and Table 1). The *Staphylococcus aureus* exhibited the maximum antibacterial activity, when compared to *E. coli*.

Table 1 Antibacterial Activity

S.No.	Sample	Zone of Inhibition (mm)				
		10 µl	**20 µl**	**30 µl**	**40 µl**	**50 µl**
1.	*E.coli*	Nil	Nil	Nil	Nil	Nil
2.	*Staphylococcus aureus*	2mm	5mm	5mm	6mm	6mm
3.	*Control- tetracycline*	2mm				

3.2. Minimal Inhibition Concentration and Minimal Bactericidal Concentration

The minimal inhibition concentration assay was performed in order to confirm the antibacterial activity. By recording the O.D. of the *Staphylococcus aureus* overnight co-culture with GAG, the dilution of 10^{-6} tube confirmed the growth inhibition activity. Hence the MIC was calculated as 10^{-6} (Table.2).

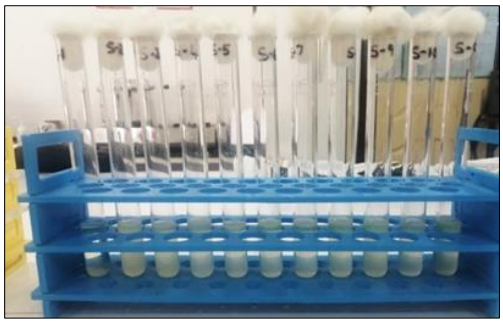

Figure 4 Minimal bactericidal concentration (MBC): *Staphylococcus aureus*

Table 2 OD values of overnight incubated *Staphylococcus aureus* co-culture with GAG

S. No.	Dilution	Optical density
1.	10-1	0.00
2.	10-2	0.00
3.	10-3	0.00
4.	10-4	0.00
5.	10-5	0.00
6.	10-6	0.23
7.	10-7	0.36
8.	10-8	0.48
9.	10-9	0.53
10.	10-10	0.59

To confirm the minimum bacterial growth concentration, the overnight co-culture of *Staphylococcus aureus* with GAG were streaked on to the MH agar. The colonies were counted and CFU was calculated as 52 CFU/10µl (Fig.5).

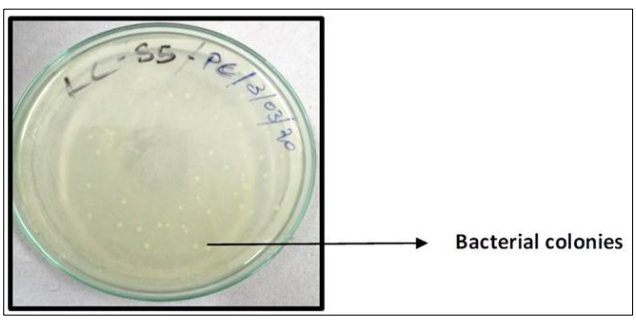

Figure 5 Minimal inhibitory concentration (MIC)

4. Discussion

In the present study the glycosaminoglycans was extracted from shrimp head waste and the antimicrobial activity against *Staphylococcus aureus* and *E. coli* was carried out. To verify the antibacterial activity, the minimum inhibition concentration experiment was done. The growth inhibition activity of the dilution of 10^{-6} tube was validated by measuring the O.D. of the co-culture of overnight incubated *Staphylococcus aureus* with GAG. Thus, the MIC was determined to be 10^{-6}. MH agar was used to test the antibacterial activity against *E. coli* and *Staphylococcus aureus* and to identify the zone of inhibition. When compared to *E. coli*, *S.aureus* showed more antibacterial activity.

In recent studies, the antibacterial activities of heparin and heparin-like glycosaminoglycans (GAGs) that were isolated from the cuttlefish *Euprymnaberryi* were examined. It was observed that these GAGs showed effective activity against bacterial strains which include *Shigella flexineri*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. In addition they showed activity against fungi strains of *Candida albicans, Aspergillus fumigatus, Fusarium sp., Cryptococcus neoformans, Microsporium sp*., and other fungi at 25, 50, 75, and 100% concentrations. Heparin and heparin-like (GAG) samples, both unprocessed and refined, were effective against all kinds of pathogenic bacteria. *Microsporium sp*. (crude) and *Fusarium sp*. (purified) were not affected by the antifungal activity in the crude and purified samples, respectively, in any concentration. The other fungi strains are all active and present everywhere. It was discovered that the extract of heparin and heparin-like GAGs had greater action at 100% concentration than at lower concentrations. In general, the rising concentration indicated that the extract was becoming more active. To determine the active components of the heparin and heparin-like extract that exhibit good antibacterial activity, more study is being done [22].

In previous study the bioavailability and antibacterial efficacy of glycosaminoglycans (GAGs) isolated from the skin of the underused puffer fish *Lagocephalus inermis*. *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus subtilis* were only a few of the bacteria that the isolated GAGs demonstrated significant antibiotic activity against. Using NIH3T3 fibroblast cell lines, the biocompatibility of the isolated GAGs was also investigated. The findings indicated that the non-edible fish skin polysaccharide was transformed into an economically useful product that can satisfy the growing demand for bioactive metabolites in a variety of medicinal applications and prevent the overexploitation of other endangered species [23].

In earlier studies the effective therapeutic agents against cancer, antimicrobial peptides (AMPs) currently offer selectivity and few side effects. Finally, they evaluated the interaction of the cationic peptide with the two most anionic molecules on the cell surface, heparan sulphate (HS) and chondroitin sulphate (CS), using molecular dynamic simulation to gain a better understanding of the peptide's mode of action on cytotoxic activities. The outcomes of the in- silico investigation demonstrated that the interaction between the HS and CS had a completely distinct amino acid profile. The findings of this inquiry demonstrated that a cationic peptide with strong anticancer action was successfully expressed by the P170 expression method. Molecular docking studies further revealed the pattern of peptide interaction with negatively charged membrane molecules [24].

Some studies show that the wound healing agent was created by chemically altering the glycosaminoglycan (GAG) biopolymers hyaluronic acid (HA) and chondroitin sulphate (CS) to improve the loading and release of nitric oxide (NO). GAGs including alkylamines, terminal primary amines, and intermediate nitric oxide-release kinetics effectively eradicated *Staphylococcus aureus* and *Pseudomonas aeruginosa* microorganisms with three different antibiotic resistance profiles. It was discovered that nitric oxide loading of the GAGs reduced mouse TLR4 activation, indicating that the drug has anti-inflammatory properties. Based on the adhesion and proliferation profiles of the GAG derivatives as well as their antibacterial properties, the most promising wound healing candidates were chosen for additional in vivo investigations. Active nitric oxide release and the biopolymer backbone were both credited with the benefits of quick wound healing and lower bacterial burden [25].

In particular, the survival of some organisms on test tube walls and the development phase of the inoculum were demonstrated to have a significant impact on the minimum bactericidal concentration of oxacillin for *Staphylococcus aureus*. Reproducibility was considerably enhanced by attention to technical details, and log-phase cultures of all strains demonstrated higher than 99.9% death in 24 hours at or near the lowest inhibitory concentration, including eight strains that were considered tolerable. In some strains, the paradoxical characteristic of having more surviving in concentrations greater than the minimal bactericidal concentration was evident. In order to establish clinical correlations and to examine the endpoint criteria, a reference minimum bactericidal concentration approach must be widely acknowledged [26].

5. Conclusion

The objective of the current study is to extract glycosaminoglycans from shrimp head debris. The GAGs' antibacterial efficacy against *Staphylococcus aureus* and *E. coli* was investigated. Additionally, it has dual benefits as the sustainability of the byproducts utilization of processing discards and diminishing the waste with aspects of environmental pollution as well. As a result, the MIC was found to be 10^{-6}. To determine the zone of inhibition and evaluate the antibacterial activity against *E. coli* and *Staphylococcus aureus* MH agar was employed. *Staphylococcus aureus* has the highest antibacterial activity when compared to *E. coli.*

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest

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