Invitro Antimicrobial and Biocompatible Properties of Sulphated Glycosaminoglycans Extracted from the Skin of Puffer Fish Lagocephalus Inermis

Iswariya. S[1], Dr. S. Shanmugam[2]
[1]*Research Scholar, Department of Microbiology, Presidency College, Chennai 600 005*
[2]*Assistant professor, Department of Microbiology, Presidency College, Chennai 600 005*

Abstract **- The present work was conducted to study the biocompatibility and antimicrobial activity of the glycosaminoglycans (GAGs) extracted from the skin of underutilized puffer fish Lagocephalus inermis. The extracted GAGs showed potent antimicrobial activity against all the tested microorganisms such as Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis and Pseudomonas aeruginosa. The extracted GAGs were also evaluated using NIH3T3 fibroblast cell lines for its biocompatibility. The result suggested that the polysaccharide extracted from the non-edible fish skin was converted to a economically valuable product which will fulfill the emerging needs for the bioactive metabolites in various pharmaceutical applications and also prevents the overexploitation of other endangered species.**

Index Terms **- Antimicrobial activity, Fish skin, Glycosaminoglycans, Puffer fish.**

1.INTRODUCTION

Glycosaminoglycans (GAGs) comprise a complex group of macromolecules with a wide range of biological activities. They are long, linear, sulfated heteropolysaccharide with highly negatively charged molecules [1]. They are composed of repeated disaccharide units comprising of a hexosamine (D-glucosamine or (D-galactosamine) and uronic acid (D glucuronic acid) or L-iduronic acid [2]. These polymers are distributed extensively in marine species and animals such as mammals and invertebrates [3]. GAGs play important roles in physiological processes including blood coagulation, cell growth, host defense mechanism, inflammation, and angiogenesis process [4]. These polysaccharides are mostly isolated from the mammalian source, which was limited due to the chances of disease transmission and religious concern.[5] The increasing demand for the glycosaminoglycans in various fields like cosmetics, pharmaceutics, food industries and biomedical fields, urge the search for an alternative source [6]. Marine sources are found to be a rich in producing various biologically active metabolites, especially glycosaminoglycans from fish and fish processing wastes [7]. The non-edible puffer fish Lagocephalus inermis belongs to the family of Tetraodontidae, contains high amount of glycosaminoglycans in its skin. Nearly more than 10 million tons of puffer fish was caught on every year on the fisherman's net and are discarded without any proper utilization of them. Therefore, the conversion of this kind of bio-waste into biologically active metabolites will prevent the major ecological problems [8,9]. Hence, the present study was conducted to investigate the biocompatibility and antimicrobial properties of the GAGs from the underutilized fish skin against the selected microorganisms.

2.MATERIALS AND METHODS

All the chemicals and reagents (analytical grade) used in these studies were purchased from Merck and Himedia, India. Deionized water was used throughout the studies.

a. PREPARATION OF THE FISH SKIN

The smooth golden puffer fish Lagocephalus inermis was collected from Mallipatinum seashore, Tamilnadu, India and were brought to the laboratory at 4 °C. The skin was removed from the fish and was cut into small pieces and stored at -20 °C until further used.

b. ENZYMATIC EXTRACTION OF THE GLYCOSAMINOGLYCANS

Briefly, five grams of defatted sample were dissolved in 250 ml of 0.2 M sodium phosphate buffer containing 0.1M sodium acetate, cysteine and EDTA mixture. The enzyme Papain at various concentration was added to the mixture and it was left to hydrolyze at different time intervals. After incubation, the mixture was centrifuged at 5000 rpm, the supernatant was collected and finally cetylpyridinium chloride (w/v) was added to the solution to precipitate GAG. The partially purified and lyophilized sulphated glycosaminoglycans (sGAG) were used for further analysis.

c. ANTIMICROBIAL SCREENING

The extracted and partially purified sulfated glycosaminoglycan (sGAG) was tested for antimicrobial activity against the human pathogenic microbial strains such as Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis and Pseudomonas aeruginosa. The cultures were obtained from MTCC, IMTECH, Chandigarh, India

d. PREPARATION OF INOCULUMS

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for the experiments were prepared by transferring loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) and incubated at 37°C for 24h without agitation. The cultures were diluted with fresh MHB to achieve optical densities corresponding to 1x106 colony forming units (CFU/ml) [11].

e. ANTIMICROBIAL SUSCEPTIBILITY TEST

Invitro antibacterial activity of the sGAG was determined by disc diffusion techinique. 60 mg of lyophilized powder was dissolved in 0.6mL of solvent (10mM EDTA) and made into different concentrations of sGAG (0.24, 0.18 0.12 and 0.06 Ml) were known as 100, 75, 50 and 25% respectively. A fresh microbial culture of 0.1ml having 106 CFU was spread on MHA plate with glass spreader. A well was made of 6 mm diameter into the agar medium with a sterile cork borer. Then the well was filled with different concentrations of sGAG and were then incubated at 37°C for 24h in an incubator. After incubation, zone of inhibition was measured and recorded in millimeters (mm). The experiment was done in triplicate and the results were represented as mean ± standard deviation [12].

f. MINIMUM INHIBITORY CONCENTRATION (MIC)

To assess the MIC of the extracted sGAG, the broth dilution test was carried out with the concentration range of 0.7, 1.5, 3.125, 6.25, 12.5 and 25 mg/ml in DMSO respectively. Series of 5ml of nutrient broth tubes were inoculated with 1ml test organism. Different concentrations of sGAG (1ml) were inoculated by the serial dilution method. Nutrient broth tubes with and without sGAG samples were used as controls. All the tubes were incubated at 37°C for 24h, after incubation the absorbance of each sample was measured spectrophotometrically at 600 nm. The lowest concentration with less absorbance was taken as MIC of the extracted sGAG.

g. BIOCOMPATABILTY OF THE EXTRACTED sGAG

Biocompatibility and the Cell viability of the isolated sGAG was performed by using MTT assay. The mouse fibroblast (NIH 3T3) cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune, India. The culture was maintained in Dulbecco's modified eagles medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with penicillin (120 U/ml), streptomycin (75 mg/ml), gentamycin (160 mg/ml) and amphotericin B (3 mg/ml) at 37 °C humidified with 5% CO2 at 37 °C. The samples were added over cultured NIH 3T3 cells in 24 well plates for 48h. Cell viability in terms of metabolic activity was determined calorimetrically by evaluating the uptake of MTT by the cells. Viable cells were determined by the absorbance at 570 nm. Untreated wells served as cell control. Data were normalized to control, for to evaluate the biocompatible potential of isolated sGAG.

h. STATISTICAL ANALYSIS

All data were submitted to Analysis of Variance (ANOVA), and differences between means were evaluated by the Duncan's Multiple Range Test. The data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 10.0 (Chicago, Illinois, USA). All tests were carried out in triplicate

and differences were considered significant at $P < 0.05$.

3. RESULTS

The results in table.I showed that the fish skin sGAG has potent antimicrobial property against the tested organism. In 100% concentration, the highest activity was recorded as 19 mm inhibition zone against E coli, whereas a minimum of 9- and 10-mm inhibition zone were observed against K. pneumoniae and S. aureus respectively. AT 75% concentration, the extracted sGAG showed maximum activity against E. coli with 16mm inhibition zone and the minimum of 6 and 7 mm zone of inhibition was noted against Pneumoniae and S.aureus whereas moderate activity of 13 and 12mm was recorded in P. aeruginosa and B. subtilis respectively. At 50% concentration, the extract showed more or less similar activity in all the strains with the inhibition zone of 9 to 5 mm except S.aureus, which showed lowest inhibition zone of 3mm. At 25% concentration, the extracted sGAG showed maximum of 4mm zone in almost all the strains tested and no activity was found in the S.aureus respectively. In case of control, no activity was recorded against any bacterial strain with respect to the extracted sGAG.

Table I: Antibacterial activities of the isolated sGAG by disc diffusion method

Microorganism	25	50	75	100
E.coli	4	9	16	19
P. aeruginosa	4	7	13	18
Bacillus subtilis	3	7	12	15
K. pneumoniae	2	5	7	10
S. aureus	0	3	6	9

Zone of inhibition (mm) are mean±standard deviation of triplicates. The diameter of agar well plates was 6mm.

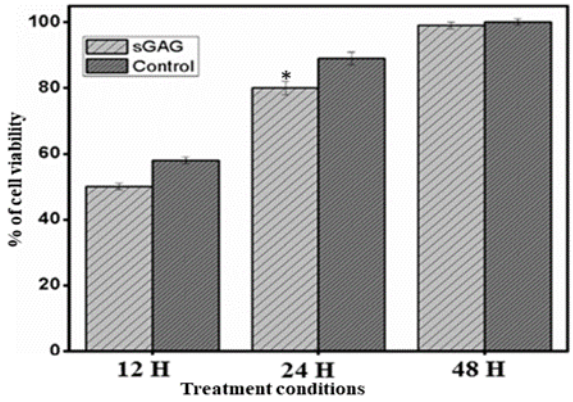
The MIC of the isolated sGAG was assessed by broth dilution test with various concentrations from 0.5 to 25 mg/ml. MIC of the isolated sGAG on bacterial strains such as, E. coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus were determined. The MIC values of the isolated sGAG was given in table II. The results of the isolated sGAG showed that the B. subtilis, Pseudomonas aeruginosa and E. coli were the most sensitive organisms with the lowest MIC value of 1.5 mg/ml followed by Klebsiella pneumoniae and, with MIC of 3.125 mg/ml, whereas S. aureus was less sensitive with MIC of 6.25 mg/ml

Table.II: Minimum inhibitory concentration (MIC) of isolated sGAG

Microorganism	sGAG (mg/ml)
E. coli	1.25
Bacillus subtilis	1.25
Pseudomonas aeruginosa	1.25
Klebsiella pneumoniae	3.125
Staphylococcus aureus	6..25

As in fig.1, the cell adherence and cell proliferation potential of the sGAG was assessed using NIH 3T3 cells which showed significant ($p<0.05$) growth and proliferation of the fibroblast cells. No observable cell death or inhibition of growth was noticed in treated wells after 12, 24 and 48h. All the cells were grown uniformly with normal morphology. This showed that the extracted GAGs from the fish skin was found to be 100% biocompatible and non-toxic, which is suitable for use in biomedical and tissue engineering applications.

Fig.I BIOCOMPATABILTY OF THE EXTRACTED sGAG



MTT assay showed the Percentage of cell viability for sGAG on NIH3T3 fibroblast cell lines. Data represents the mean of three independent experiments. ($p^*<0.05$)

4.DISCUSSION

The sulfated glycosaminoglycans was extracted from the skin of puffer fish by enzymatic digestion and ethanol precipitation. The results clearly showed that the (tables.1and2) extracted GAGs exhibited appreciable antimicrobial activity against the tested organism such as E.coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtilis and

Staphylococcus aureus. The maximum activity of 100% was found to be in an increasing the order from the lower to higher concentration. The extracted SGAG was highly effective against E.coli, Pseudomonas aeruginosa and Bacillus subtilis with the lowest MIC value of 1.25 mg/ml, whereas Staphylococcus aureus showed minimum activity against the extract. In the present study the biocompatibility of the extracted sGAG was assessed by using NIH 3T3 cells lines which showed significant ($p<0.05$) growth and proliferation on the tested fibroblast cells. Interestingly, the extracted GAGs did not induce any significant cytotoxic effect, but rather exhibited higher cell viability of the fibroblast cells. This result enhances the applications of the extracted sGAG for biomedical and pharma industries from the cheapest marine source.

In recent years, great attention was made to study the bioactivity of natural products due to their potential biomedical utilization [12,13]. Only a very few studies have been carried out on the antimicrobial activity of the fish skin. However, many studies are available for the antimicrobial activity of the extracts from the whole-body tissues and bones of the fish or from the fish processing wastes. In this regard, most of the studies rported that the marine sources have potential antimicrobial activities. Shanmugam et al., (2008) stated that the antibacterial activity of the cuttlebone extract of S. aculeata and S. brevimana reported 10 mm zone of inhibition against all the tested nine pathogenic bacterial strains [14]. Fuochi et al., (2017) studied that the crude mucus extract of common stingray, which was strongly inhibit the growth of Gram-negative bacteria such as E. coli, K. pneumoniae, and P. aeruginosa [15].

When compared to the earlier studies, that the non-edible puffer fish Lagocephalus inermis possessing a great economic importance and its skin contains comparatively rich source of biologically active metabolites. The isolation of sulphated glycosaminoglycans from the skin of L. inermis may bring the new insights for the synthesis of more potent drugs and fulfill the emerging needs of various pharmaceutical and industrial applications.

5.CONCLUSIONS

The glycosaminoglycans extracted from the skin of underutilized puffer fish Lagocephalus inermis showed potential antibacterial activity against all the tested human pathogenic bacterial strains. The antimicrobial activity of the isolated glycosaminoglycans indicates the presence of potent bioactive metabolites in them. Furthermore, still, there is a requirement to determine the active compounds present in the extracted glycosaminoglycans, to classify the compounds that might be more effective against these human pathogenic bacterial strains.

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