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Fish gills of *Thunnus albacares*: A novel source of chondroitin sulphate glycosaminoglycans

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Abstract
Fish gills of *Thunnus albacares* have been chosen as a unique source of glycosaminoglycans (GAGs) in this study. Chemical and physical characterization were carried out by ATR – FTIR spectroscopy, DEAE - Cellulose anion exchange chromatography, SAX – HPLC, and NMR techniques to determine the nature of GAG isolated. The results showed that the isolated GAGs (8.5mg/g yield) were mostly Chondroitin sulphate (CS) type. SAX HPLC chromatogram of isolated GAG and CS A standard showed comparable peaks A at 4.9, B at 7.8 and C at 9.99 min. FTIR spectra, IR (KBr) υ max in cm^{-1} presented characteristic peaks of –CONH vibration of amide group, coupling of C–O stretching vibration, S=O stretching vibrations and –C–O–S molecules confirmed the presence of CS type GAG in *T. albacares*gills.^{1}H NMR spectra in D_2Oalso revealed the presence of CS moiety. All these results substantiate *T. albacares* gills as an excellent source of CS type GAG.

Keywords: Fish gills, Chondroitin sulphate glycosaminoglycans, DEAE anion exchange chromatography, SAX -HPLC, ATR-FTIR, NMR spectroscopy

1. Introduction
Glycosaminoglycans (GAGs) are a complex, linear, sulphated, negatively charged, specific group of heteropolysaccharides in the cell–ECM (extracellular matrix) linked to core proteins to form proteoglycans [1, 2]. They are involved in an extensive array of biological functions [3,4]. GAGs have requisite roles in the regulation of physiological processes like, cellular proliferation, differentiation, matrix assembly, homeostasis, and cellular behaviour. The structural diversity of glycosaminoglycans poses significant challenges in the field of glycobiology. Proteoglycans found on the surface of all higher animal cells, in the extracellular matrix of connective tissues and in basement membranes function as structural molecules and as scaffold structures, binding a wide variety of protein ligands through GAG-protein and protein-protein interactions [5]. Chondroitin sulphate (CS) could be a major category of GAGs and a vital element of extracellular matrix in holding the structural responsibility of the tissue hence widely explored for regeneration studies. Chondroitin sulphates are unbranched polysaccharides of variable length with repeating D-glucuronic acid and D-N-acetyl-galactosamine units combined together by β (1 → 4) and β (1 → 3) glycosidic links, and the N-acetyl-galactosamine is substituted with a sulphate at either its 4′(CS-A) or 6’ (CS-C) position, with almost one sulphate per disaccharide unit. These molecules bind and regulate a few distinct proteins, including chemokines, cytokines, growth factors, morphogens, and adhesion molecules on the cell surface and in the ECM. Novel documented areas of interest for different potential use of CS are as antiviral, anti-infective and in tissue regeneration and engineering. CS based colloidal gel preparations conjointly established to shorten the wound healing period.

The rising interest in the application of glycosaminoglycans is one of the main reasons for exploring and optimizing the extraction of various classes of GAGs from different fish species. They are widely used especially in the biomaterials industry and an increasing number of new applications have been found for GAGs as tissue-engineering material, biodegradable film-forming material, and micro-encapsulating drug delivery agent. On the other hand, these bioactivities are reliant on their fine structure with diverse monosaccharide units and sulphation configurations. This study aims at isolation and characterization of GAGs from fish gills of *Thunnus albacares*.

2. Materials and methods

2.1 Isolation of GAG from fish gills

2.1.1 Preparation of acetone dry powder

Gills of *Thunnus albacares* was used for acetone dry powder preparation [6]. Washed, shade dried small pieces of gills were placed in chilled acetone 1:5 w/v (20.0 ml acetone / g tissue) at 0ºC for 72 hours. Then filtered samples were dried under vacuum, treated with diethyl ether: acetone (3:1) at 37ºC for an hour. After drying the samples were re-extracted with diethyl ether under same conditions to obtain the acetone dry powder.

2.1.2 Papain enzyme digestion

The acetone dry powder prepared were digested with papain (20U/g dry weight) in 0.2M sodium acetate buffer, pH 7 comprising 2mg/ml cysteine – HCl at 65ºC for 72hours [7]. Controls were prepared as comparable amount of papain in sodium acetate buffer.

2.1.3 Extraction of GAG

Enzyme digested samples were centrifuged at 5000 x g for 5 min at room temperature and precipitated with 4 to 5 volumes of 95% (v/v) ethanol, maintained at -10 ºC for 24 hrs. [8, 9]. The crude precipitate obtained were centrifuged, dissolved in deionized Milli Q water to obtain the GAG rich supernatant (mixture of GAG and papain). GAGs and papain fraction thus retrieved were separately lyophilized and crude GAG yield was determined.

2.2 Chemical composition analysis

The amount of hexuronic acid, hexosamines, sialic acid, hydroxyl proline, fucose, protein and sulphated GAG of isolated fish gills GAG were calculated spectrophotometric ally. GAG dry powder was dissolved in Milli Q water and used for the following estimations.

2.2.1 Estimation of uronic Acid

Estimation of hexuronic acid in isolated GAGs was done using carbazole reaction [10], employing glucuronolactone as standard. Absorbance was measured at 530nm.

2.2.2 Estimation of hexosamines

The GAGs were hydrolysed using 6M HCl at 100^0C for 4 hours and to 1.0 ml of hydrolysed sample was used for estimation of hexosamines [11]. OD was measured at 530nm.

2.2.3 Estimation of sialic acid

Estimation of sialic acid was carried out by the thiobarbituric acid (TBA) method [12]. Optical density measured at 580nm.

2.2.4 Estimation of hydroxyproline

The acid hydrolysed sample was diluted to 2.0 ml with distilled water and was used as sample and freshly prepared hydroxyproline as standard [13]. OD was taken at 588 nm.

2.2.5 Estimation of fucose

0.5ml of sample was made up to 1 ml with distilled water, added 4.5 ml cold sulphuric acid reagent to the tubes and cooled in an ice bath [14]. Added 0.1 ml of cysteine reagent. Separate aliquots of each sample heated with H_2SO_4 reagent but without the addition of cysteine reagent were also set. The absorption due to methyl pentoses were determined by subtracting the $OD_{396}$from OD_{427} of the samples analysed without cysteine reagent from the OD_{396} – OD_{427} of the samples analysed with cysteine reagent.

2.2.6 Estimation of protein

Protein estimation was doneusing bovine serum albumin (BSA) as standard [15]. Optical density was measured at 670 nm.

2.2.7 Estimation of sulphated GAGs

Sulphated GAGs of *Thunnus albacares*gills were determined using 1, 9-dimethyl methylene blue (DMMB) dye-binding assay [16]. Absorbance was measured at 656 nm against chondroitin sulphate standards.

2.3 Structural characterization

2.3.1 DEAE - Cellulose anion exchange chromatography

Anion exchange resins are appropriate for GAGs since they possess high negative charge. The crude glycosaminoglycans (200 mg extract) were fractionated by anion exchange chromatography [17], on DEAE (Diethyl amino ethyl) – cellulose, column (15 x 2.5 cm) equilibrated with 0.05 M sodium acetate buffer (pH 5.5). 3.0 ml fractions were gathered and subjected to DMMB dye binding assay for GAGs.

2.3.2 SAX – HPLC analysis of glycosaminoglycan

Analytical strong anion exchange (SAX) HPLC was implemented to detect and quantitate the constitutive disaccharide in *T. albacares* gills GAG sample obtained by digestion using chondroitinase ABC [18]. The results were monitored at 232nm using a dual Z absorbance detector (UV/VIS Model 484) and analysed with EMPOWER 2 chromatography software.

2.3.3 ATR – FTIR spectroscopy-Fourier Transform Infra-Red spectrum analysis

The functional groups present in the isolated GAGs from *T. albacares* gills were determined using FT-IR spectroscopy. FT-IR spectroscopy of purified GAG sample were performed by attenuated total reflectance (ATR) spectroscopy [19], which examined diverse amino and hydroxyl groups of sample molecules. The samples were analysed from 450 to 4000 cm^{-1}.

2.3.4 Nuclear magnetic resonance (NMR) spectroscopy

The GAGs from the *T.albacares* gills were subjected to NMR analysis to assess the purity of the isolates. Samples (100 mg each) were dissolved in 0.5 ml of 99.8% deuterium oxide (D_2O) after micro- filtration through 0.45 µm nylon filters. Further the samples were lyophilized twice from 99.8% D_2O and dissolved in the same solvent (0.8 ml) in a 5 mm NMR tube for analysis. The phase correction was performed using an automatic (zero and first order) phase correction procedure after Fourier transform (FT).

2.4 Statistical analysis

All statistical analysis was executed in the IBM SPSS Statistics 22.0 Software. Data of the results are presented as the mean ± SEM from triplicate experiments.

3. Results

3.1 Glycosaminoglycans isolation

GAGs were obtained from defatted fish gills by subsequent proteolysis and precipitation with ethanol. High ADP content was presented by *T. albacares.* The GAGs extracted and purified from the gills were subsequently lyophilized to attain

a snowy white hygroscopic GAG. Glycosaminoglycans thus successfully isolated was quantified and reported as GAG yield in mg/g tissue. The yield of acetone dry powder and GAG obtained from dry defatted fish gills were quantitated as 78.78±0.22 and 8.5±0.22 (mg/g) respectively.

3.2 Chemical composition analysis of GAGs

The composition analysis was carried out by estimating hexosamines, uronic acid, sialic acid, hydroxyproline, fucose, protein and sulphated GAG (Table 1.). Significant amount of hexosamine and uronic acid were obtained in the *T. albacares* gills assessed. The protein and hydroxyproline estimates indicated that the samples were free of collagen or any further residual proteins. The concentrations of isolated GAGs were evaluated using the metachromatic dye dimethyl methylene blue and uronic acid by carbazole method and results indicated significant amount of sulphated GAG and uronic acid in *T. albacares*. Sulphated GAG, hexosamines and uronic acid were present in *T. albacares* whereas sialic acid, hydroxyl proline, fucose and protein were absent in the GAG isolate of *T. albacares* fish gills.

Table 1: Analysis of GAG composition of extract from gills of *T. albacares* (mg/g)

Sl.	Composition	*T. albacares*
1	Hexosamines	41.60±0.01
2	Uronic acid	36.06 ±0.74
3	Sialic acid	ND
4	Hydroxyproline	ND
5	Fucose	ND
6	Protein	ND
7	Sulphated GAG	79.43 ±0.93

ND- not detected

All values are expressed as mean ± standard deviation, n=3.

3.3 DEAE Cellulose anion exchange chromatography

Characteristic chromatograms of fish gills glycosaminoglycans are presented in Fig.1.The mainstream of the GAG was detected between 0.6 and 1.0 M NaCl, resulting in a single specific peak. A minor second peak in the chromatogram denotes the existence of unsulphated types apart from the charged fraction.

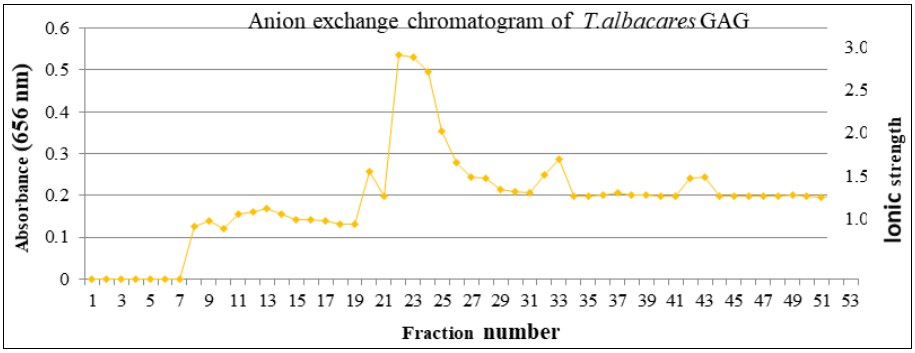

Fig 1: Anion-exchange chromatogram of GAGs from the gills of *T. albacares*

3.4 Glycosaminoglycan analysis by SAX – HPLC

The analytical anion exchange HPLC results Fig.2. (a and b) indicated similar pattern in the case of *T. albacares* fish gills GAGs and that of CS A standard

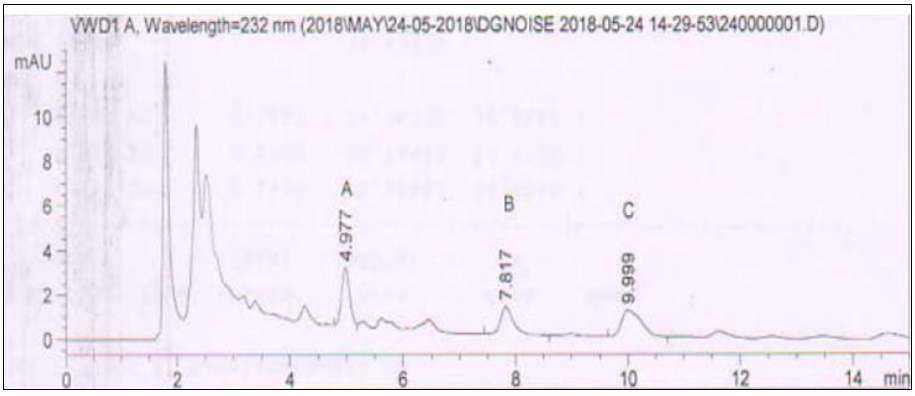

Fig 2a: SAX HPLC pattern of standard CS A

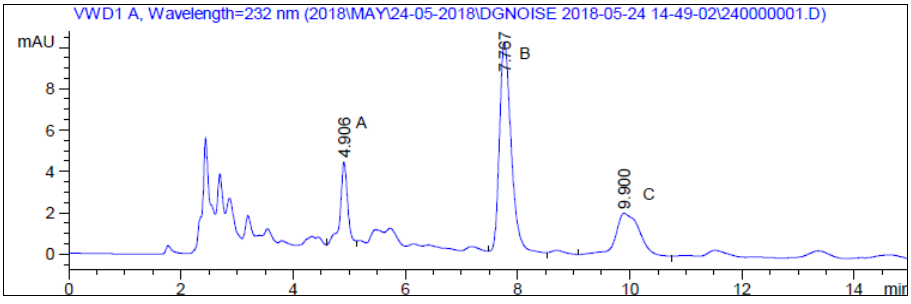

Fig2b: SAX HPLC pattern GAG isolated from gills of *Thunnus albacares*

Fig.2.a and b depict the SAX HPLC chromatogram of CS a standard and of GAG isolated from gills of the sample fish respectively showing peak A at 4.9, peak B at 7.8 and peak C at 9.99 min.

3.5 Spectroscopic analysis -*ATR – FTIR spectroscopy*

The IR spectra of the all the GAG samples were categorized by a broad band above 3000 cm^{-1} and dominant absorption around 1650 and 1050cm^{-1} (Fig. 3 a and b). The results of IR spectra of isolated GAG from the sample exhibited closer peak intensities of comparable conformation to the chondroitin sulphate A standard on comparison.

3.5.1 IR spectrum of CSA (Standard)

The IR spectrum of CSA shows a broad absorption peak centered at 3425 cm^{-1} which represents the characteristic stretching peaks of N-H and O-H functionalities. The carbonyl group (C=O) presented a peak at 1648 cm^{-1}. The peaks around 2928 cm^{-1} and 2852 cm^{-1} can be attributed to the C-H stretching vibrations of CH_2 groups. The peak corresponding to the sulphate group is seen at 852cm^{-1}. The FTIR spectrum of standard Chondroitin sulphate A and sample are given as Fig 3.a and 3.b.

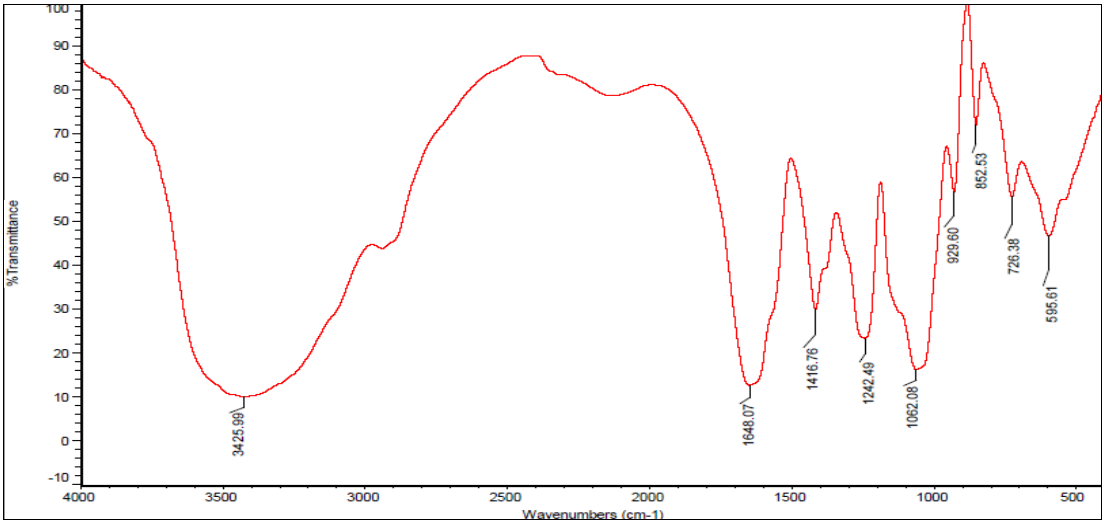

Fig 3a: IR spectrum of Chondroitin sulphate A (CSA: Standard)

3.5.2 IR spectrum of GAG from *T. albacares*

The IR spectra of GAGs from the *T. albacares* gills gave similar peaks as that of standard CSA. The merged broad peak corresponding to N-H and O-H group was observed at 3397 cm^{-1}. The carbonyl group was present at 1631 cm^{-1}. The CH_2 bending mode of vibration presented a peak at 1408 cm^{-1}. The C-OH vibration showed a strong peak at 1101 cm^{-1}. All other peaks were in good correlation with the standard bands. IR (KBr) υ max in cm^{-1}: 3397, 1631, 1408, 1101, 624.

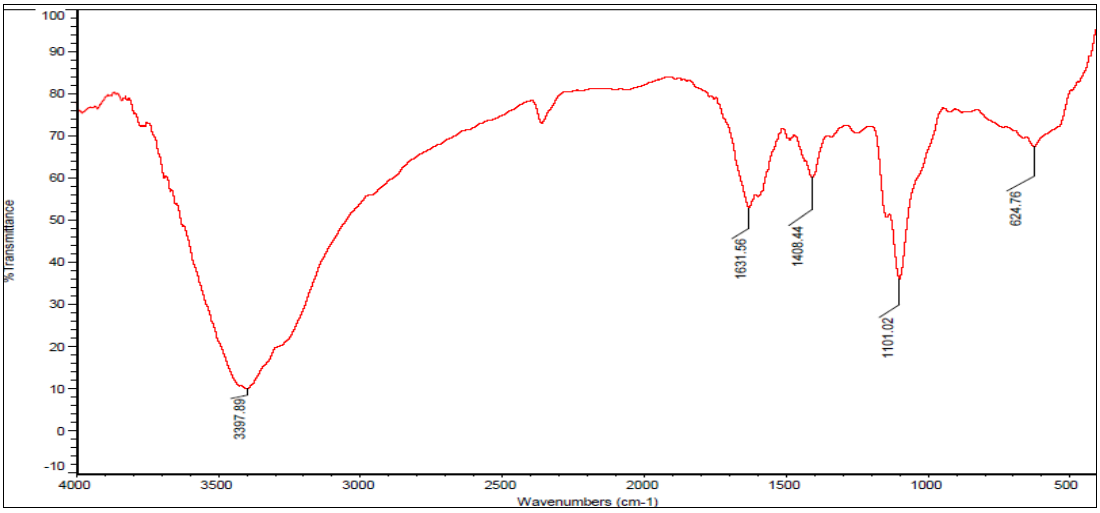

Fig 3: b. IR spectrum of GAG isolated from gills of *T. albacares*

The FTIR spectral data of –CONH vibration of amide group, coupling of C–O stretching vibration, S=O stretching vibrations and –C–O–S molecules endorses that the noticeable GAG types in the isolated GAG sample of *T. albacares* gills are of the chondroitin sulphate type. The sample analysed confirmed to be in pure form without any residual protein contaminants ensured by the absence of absorption band at 1540cm^{-1} sensed in the IR spectrum.

3.6 Proton NMR spectroscopy

Nuclear magnetic resonance (^{1}H NMR) spectroscopic analysis was carried out for the characterization of GAG obtained from the fish gills of *Thunnus albacares*. NMR spectroscopy ^{1}H NMR spectra obtained are comparable with the authentic

chondroitin sulphate type glycosaminoglycan. The spectra were taken in D_2O. The solvent peak for D_2O centred at 4.80 ppm. The NMR peaks were distributed into three ranges, from 1.5 to 2.4 ppm (range A), from 3.0 to 4.1 (range B) and from 5.0 to 5.5 ppm (range C), signifying the methyl protons and the sugar ring protons; pertinent for the portrayal of GAG sample.

The proton NMR spectrum of the standard CSA (purchased sample) is given in Fig 4.a. The characteristic methyl protons of acetamide moiety were visible at 2.00 ppm. The protons from hexuronic acids and hexosamine units presented a group of peaks between 3.33 to 5.00 ppm. The broad peak present in the spectrum is due to the solvent D_2O. In this spectrum both -NH and -COOH protons were not present in notable intensity.

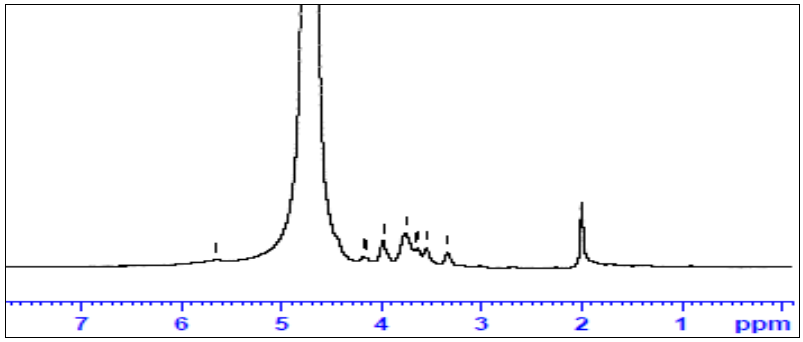

Fig 4a: ^{1}H NMR spectrum of CSA (Standard)

The proton NMR spectrum of GAG isolated from tuna fish gills is depicted in Fig.4. b. In this spectrum, the methyl protons of acetamide side chain were visible at 2.00 ppm as a singlet. The protons of hexuronic acids and hexosamine units presented a group of peaks which ranges from 3.33 to 5.00 ppm. The broad peak present in the spectrum is again of the D_2O peak. The peaks shown below 1.5 ppm may be attributed due to the presence of some impurities.

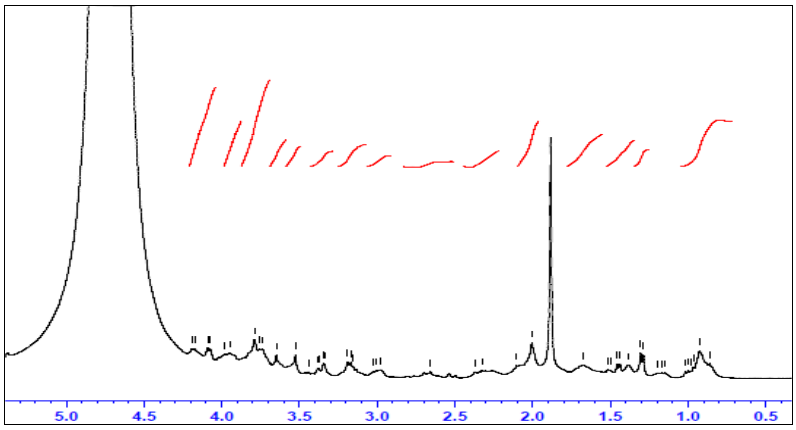

Fig 4b: ^{1}HNMR spectrum of GAG from *T. albacares*

The characteristic signals (^{1}H) for the frequently befalling sugar residues have been identified in ^{1}H NMR spectroscopy. ^{1}H NMR spectra of all the sample analysed was in the range 15.50 to 1.114 ppm acquired in steps of 0.001 ppm and the 6 range was limited from 5.198 to 0.842 ppm since the ^{1}H NMR peaks of GAGs remains inside this array.

4. Discussion

Marine organisms are a rich source of a variety of structurally novel and biologically active metabolites [20] and own pronounced pharmacological activities and other properties applied in biomedical area. Some of these pharmacological activities are attributed to the presence of sulphated polysaccharides, particularly the GAGs [21, 22, 23].

Chemical composition analysis of the isolated GAGs from the fish gills indicated the presence of a reasonable number of sulphated GAGs confirmed by the DMMB method and hexosamine and uronic acid estimations. The GAG yield was found to be 8.5mg/g in the dry defatted fish gills of *T.albacares* and was in accordance with Zhang *et al*., 2014 [24] where the GAGs recovery was 6 to 7 mg from 1 gram of salmon head powder. CS content ranged from 0.011% for cod up to 0.34% for monkfish [25]. In the present study, the colour change in the DMMB assay by GAG sample compared to CS standard confirmed that the sample is of chondroitin type and it is pure and reveals the presence of sulphated GAGs.

DEAE cellulose anion-exchange chromatography has the advantage that the polysaccharide solution need not be concentrated before application on the column, and it is useful for isolating low-molecular-weight glycosaminoglycans. HPLC techniques involve charge-based separation of GAG fragments using an amine-based or a strong anion exchange (SAX) column coupled to a high- performance liquid chromatography (HPLC) instrument after treating with chondroitinase [26, 27, 28] which revealed the structure of GAGs.

ATR-FTIR spectroscopy is a predominantly beneficial technique that may analyse the molecular configuration at a sample surface and deliver evidence on the intra- and/or inter-molecular chemical bonds [29]. In the current study, fish gills glycosaminoglycans from *T. albacares* was analysed using FTIR spectroscopy and the data were assessed in an analytical tool competent in perceiving distinctive vibrational styles of separate chemical groups and bonds. The extracted and purified GAGs produced spectra indicated the presence of

methyl groups, carboxyl groups and sulphate groups. Comparable characteristic peaks of –CONH was observed at 1646 cm^{-1} for standard CS and same peaks of isolated GAG was reported in samples of buffalo tracheal, nasal, and joint cartilages at 1647, 1650, 1650 cm^{-1} respectively [30]. Characteristic peaks of –CONH vibration of amide group coupling of C–O stretching vibration at 1641 cm^{-1} were obtained for CS in samples extracted from chicken keel cartilage [31].

Absorption band at 825.78 and 885.25 cm^{-1} attributable to the C–O–S axial and equatorial bending vibration were found characteristics of 4-sulphate and 6-sulphate of D-galactosamine of the CS extract from shark fin cartilage, chicken keel cartilage, crocodile hyoid and sternal cartilage by FTIR spectroscopy [33]. From the results obtained, the isolated GAG is mainly CS type. Chondroitin sulphate has been concerned with innumerable physiological roles comprising cell division and morphogenesis [34, 35], central nervous system function [36] and signal transduction [37].

Nuclear magnetic resonance (NMR) spectroscopy is a potent non-destructive and theoretically an excellent analytical technique. NMR spectroscopy is currently used for structural characterization of glycosaminoglycans (GAGs). It is used for the determination of the content and purity of a sample as well as its molecular structure.

Conformational arrangements or dynamical activities of GAGs in solution regulate the functions of GAGs. ^{1}HNMR spectra chemical shift data of fish gills GAG at 500 MHz confirmed specifically the occurrence of GalNAc and glucuronic acid through the presence of protons at specific spectral region 3.30 to 5.00 ppm [38] on characterization of oligosaccharides from the chondroitin/dermatan sulphates. Thus, the spectral assignments of ^{1}H NMR confirmed the presence of CS type GAG in fish gills of *T. albacares* as it encloses peaks which might be consigned to CS type GAG and these conclusions also agree to the published results [39,40]. Similar work asserted the occurrence of CS type GAG from the processing discards of *Labeorohita* and *Piaractus brachypomus*[41] and from chain cap of articular cartilage [42]. The physical and chemical analysis revealed that the GAG isolated from the gills of *T. albacares* are mainly CS type.

5. Conclusion

Fish gills from *T. albacares* were screened for GAG for its usage as an innovative material ought to be of interest in the pharmaceutical, nutraceuticals, and cosmetic industries. Furthermore, the isolation, characterization and purification process encompass papain digestion, ethanol precipitation, DEAE ion-exchange chromatography and HPLC analysis. The GAGs yield was in the range 8.5 mg/g dry weight of defatted fish gills. The chemical and physical characterization confirmed that the chief component of GAG in the gills of *T. albacares* gills is chondroitin sulphate type with characteristics sulphation pattern. The gills of *T.albacares* is thus a rich source of GAGs especially of the chondroitin sulphate type, exhibiting characteristic sulphation pattern which requires further structural elucidation. These findings extend to the tissue source for commercial GAGs preparation by utilising the abundant fish discard gills in the fish processing industry.

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