Rev. Bras. Eng. Pesca (2024) 15 (1): 08-20
DOI: 10.18817/repesca.v15i1.3619

ARTIGO *REVISTA BRASILEIRA DE ENGENHARIA DE PESCA*

IN VITRO EVALUATION OF ANTIOXIDANT ACTION OF CHONDROITIN SULFATE FROM THE GILLS OF *Prochilodus brevis*

AVALIAÇÃO *IN VITRO* DE AÇÃO ANTIOXIDANTE DE CONDROITIM SULFATO DAS BRÂNQUIAS DE *Prochilodus brevis*

Lorena Martins Santiago[1], José Ariévilo Gurgel Rodrigues[1*], Ismael Nilo Lino de Queiroz[1], Thaís de Oliveira Costa[2], Johnny Peter Macedo Feitosa[3], Sandra de Aguiar Soares[3], Carminda Sandra Brito Salmito-Vanderley[4] & Ianna Wivianne Fernandes de Araújo[1*]

[1]Departamento de Engenharia de Pesca, Universidade Federal do Ceará - UFC
[2]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará - UFC
[3]Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará - UFC
[4]Faculdade de Veterinária, Universidade Estadual do Ceará - UECE

*e-mails: arieviloengpesca@yahoo.com.br , iwfaraujo@gmail.com

Recebido: (25/02/2024) / Publicado: (18/07/2024)

Abstract *Prochilodus brevis* is a Brazilian rheophilic fish of local market, but its bioactive glycosaminoglycans (PbGAGs) composition is unknown. This study analyzed PbGAGs from gills on yield, physical-chemical features and *in vitro* antioxidant effects. Tritured gills were incubated with papain, in 100 mM sodium acetate buffer (pH 5)+cysteine/EDTA, both 5 mM, to obtain PbGAGs. After extraction, PbGAGs were characterized by agarose/polyacrylamide gels electrophoreses using known markers stained in toluidine blue or Stains-All and then by infrared technique. Antioxidant actions were *in vitro* evaluated by DPPH, total antioxidant capacity (TAC) and ferrous ion chelanging (FIC) assays using BHT, ascorbic acid and EDTA as references. Extraction yielded 1.54±0.02% and biochemical analyses indicated chondroitin-4/6-sulfate similar to mammalian derived of sulfation pattern and size of ~ 40 kDa. PbGAGs had no effect on DPPH assay, but exerced antioxidation greater in FIC than TAC one, alghough significantly less actives than synthetic agents. Thus, gills from *P. brevis* are a freshwater source containing antioxidant chondroitin sulfate.

Key-words: rheophilic fish, by-product, polyanions, reducing power.

Resumo *Prochilodus brevis* é um peixe reofílico brasileiro de mercado local, porém é desconhecida sua composição de glicosaminoglicanos (PbGAGs) bioativos. Este estudo teve como objetivo analisar a ação de PbGAGs sobre rendimento, características físico-químicas e efeitos antioxidantes *in vitro* nas brânquias de *P. brevis*. Brânquias trituradas foram incubadas com papaína, em tampão acetato de sódio 100 mM (pH 5) + cisteína/EDTA, ambos 5 mM, para obter PbGAGs. Após extração, PbGAGs foram caracterizados por eletroforeses em géis de agarose/poliacrilamida usando marcadores conhecidos corados em azul de toludina ou "Stains-All" e, posteriormente, por técnica de infravermelho. Ações antioxidantes foram avaliadas *in vitro* pelos métodos DPPH, capacidade antioxidante total (CAT) e quelante de íon ferroso (QIF) usando BHT, ácido ascórbico e EDTA como referências. Extração rendeu 1,54±0,02% e análises bioquímicas indicaram condroitim-4/6-sulfato semelhante ao derivado de mamífero de grau de sulfatação e tamanho de ~ 40 kDa. PbGAGs não tiveram efeito sobre ensaio DPPH, mas exerceram maior antioxidação no ensaio QIF que na CAT, embora menos ativos significativamente que agentes sintéticos. Assim, brânquias de *P. brevis* são uma fonte dulcícola contendo condroitim sulfato antioxidante.

Palavras-Chave: peixe reofílico, subproduto, polianiônicos, poder redutor.

Introduction

The Brazilian ichthyofauna is made up of numerous aquatic species (*e.g.*, bocachico, "pintado", "dourado", "piapara", "tambaqui" and "pacu") and some of them have been used for decades through extractive fishing or cultivated for human consumption. On the other hand, the fishing of native species as commercial activity and the problems associated with the inadequate descard of fish processing residues, even if artisanal, generate concerns regarding the rational exploitation of the species and the use of their organic residues (Moreira et al., 2001). On a basis of sustainable use, freshwater fish-residual materials (*e.g.*, fins, heads, gill, viscera and skin) would be potential sources to extract industrially-important compounds (*e.g.*, collagen, peptides and sulfated polysaccharides) as a promissing alternative and for the biochemical knowledge of the species (Oetterer et al., 2014; Nogueira et al., 2019).

Sulfated polysaccharides have already been isolated from different origins (Wasserman et al., 1972; Regnault & Durand, 1998; Rodrigues et al., 2016; Valcarcel et al., 2017; Moura et al., 2021). Those from animals are called glycosaminoglycans (GAGs) and are biosynthetized not only by invertebrates (Medeiros et al., 2000), but also in vertebrates (Volpi & Maccari, 2002; Mansour et al., 2009; Oliveira et al., 2015a, 2015b; Andrade et al., 2017) featuring anionic macromolecules of heterogeneous composition and complexes in terms of sulfation patterns, molecular masses, different linkages and specific saccharide sequences along backbone structure (Valcarcel et al., 2017). The main GAG is heparin (HEP) commercially extracted from mammalian connective tissues (bovine and porcine) due to its anticoagulant and antithrombotic properties in clinical practice (Badri et al., 2018). Other animals-derived GAGs are chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA, nonsulfated), keratan sulfate and heparan sulfate (HS), whose biopolymers show variable structures depending on the tissue of origin (Oliveira et al., 2015a). GAGs are formed by chains of linear molecules consisting of repeating disaccharide units of aminosugar (D-galactose or D-glucosamine) and uronic acid (L-iduronic or D- glucuronic acid) or galactose sugar and their chemical percentages varying among organisms and extraction protocols (Oliveira et al., 2015a; Valcarcel et al., 2017; Badri et al., 2018). Aquatic sources are safer platforms to obtain GAGs without infection risk (*e.g.*, viruses or prions) than those from terrestrial vertebrates (Badri et al., 2018).

DS is ubiquous or preponderant in some by-products generated in the fish filleting industry and well-known as anticoagulant agent (Souza et al., 2007; Salles., 2017; Valcarcel et al., 2017; Bougatef et al., 2018). GAGs from fish processing discard have also been described as anti-inflammatory (Valcarcel et al., 2017), antibacterial from cuttlefish (*Sepia officinalis*) skin and muscle (Jridi et al., 2019) and osteogenic from *Labeo rohita* and *Piaractus brachypomus* head (Gavva et al., 2020), but they have been few investigated as antioxidants. Jridi et al. (2019) enzimatically extracted GAGs from cuttlefish *Sepia officinalis* skin/muscle and discovered their antioxidant actions by various *in vitro* assays. Skin from Nile tilapia (*Oreochromis niloticus*) yielded to be a nonterrestrial waste source of *in vitro* antioxidant GAGs (Nascimento et al., 2021). The search by new antioxidant alternatives is justified due to routine use of synthetic compounds (*e.g.*, butylatedhydroxytoluene-BHT) known for its toxic effects (Panicker et al., 2014). Antioxidants is a term designed for agents that neutralize the free radical production from endogenous or exogenous sources protecting the natural biological system against diseases-related factors (*e.g.*, cancer, atherosclerosis, DNA damage and Alzheimer) (Barbosa et al., 210).

Bocachico (*Prochilodus brevis* Steindachner, 1875), as populary known in the semiarid region of Brazil, is a freshwater rheophilic fish (Characiform, Prochilodontidae) found in various river basins of northeast Caatinga area. This species is an important ecological component for these ecosystems, however, its predatory fishing in the reproductive period affects the population survival and knowledge of your reproductive biology in terms of management and conservation (Nascimento et al., 2012). *Prochilodus* species have local market (Moreira et al., 2001) and studies on the

cultivation and seminal cryopreservation have already been developed in experimental phase (Nascimento et al., 2021). No description on its GAGs has been reported in the literature so far.

As the fish gill is a respiratory organ (formed by arches and filaments) located into opercular cavity playing an important role related to exchange of gases and ions and its evolutive adaptability offers a diffusional barrier to ecological fluctuations in teleosts (Baldisserotto, 2002) and is constituted by connective tissue of epithelial layer rich in mainly collagen and mucopolysaccharides as matrix components prevening loss of water and acting as osmotic agents regulating the diffusion of small molecules and ions (Wasserman et al., 1972), current research was to examine the gills of *P. brevis* as a raw source in GAGs (PbGAGs) and then partially describe them by biochemical and structural techniques. Antioxidant assays were also performed to explore their potential on *in vitro* tests, contributing thus with the biotechnological studies on the composition and bioactivity of GAGs present in cartilage by-products from native Brazilian species.

Material and Methods

P. brevis specimens and gills removal

A total of sixteen adult individuals of *P. brevis* males were provided by the Laboratory of Biotechnology of Fish Reproduction located at the State University of Ceará (SUC), Brazil. All the fishes were mantained in tank (0.02 fish L^{-1}) where were fed (twice per day) with a commercial diet containing 28% crude protein at level of 1% from the stoched biomass and the average water quality parameters temperature (25.5°C), pH (7), dissolved oxygen (7 mg L^{-1}) and ammonia (0.25 mg L^{-1}) recorded at the time of fish capture.

In the Marine Biochemical (MarBio) laboratory installed at the Aquaculture Biotechnology Center belonging to the Department of Fisheries Engineering of the Federal University of Ceará (FUC), Brazil, slaughtered fishes were received in ice (1:1.1 kg - biomass:ice ratio) at isothermal boxe and then the specimens frozen at -20°C until use. The use of bocachico fish was authorized through our registration with SISGEN/FUC (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) and approved by the Animal Ethical Committee of the SUC (protocol nº 09664402/2019).

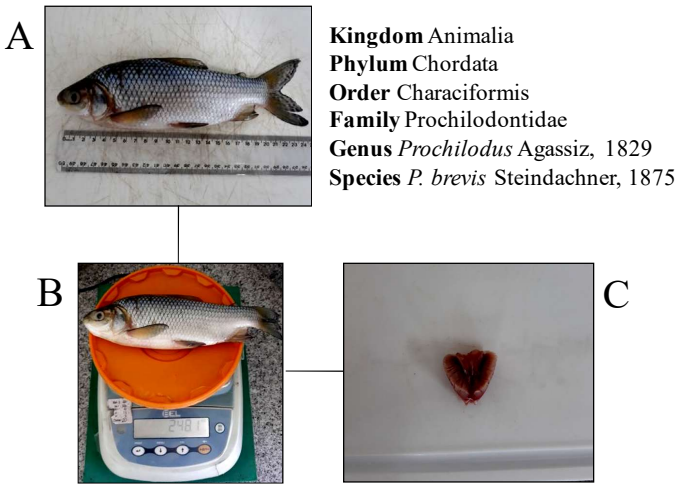

Figure 1. Specimen of *P. brevis* sujected to procedures of measurement (A) followed by weighing (B) and fresh gills removed from its opercular cavity (C).

The animals taken to the MarBio laboratory were measured (25.50 ± 21.00 cm) (Figure 1A) and weighed (182.80 ± 101.60 g) (Figure 1B) using a rudimentary ichthyometer and a balance on a 1 g precision scale, respectively. Each fish was manually treated by scaling, evisceration and gill removed with knife, clamp and chicken cutter (Figure C). After these procedures, gill separated from each animal head was extensively washed with destillated water for cleaning blood and then the tissue was dehydrated in an oven under air circulation (45°C, 48 h) and, subsequently, kept in a closed recipient until PbGAGs extraction.

Extraction of PbGAGs from gill samples and metachromasia checking

Dehydrated gills from the bocachico fishes (*P. brevis*) were cut into small peaces and the GAGs extracted following a previously performed protocol for fish skin GAGs (Salles et al., 2017; Nascimento et al., 2021). Briefly (Figure 2*a*), tritured samples (10 g) were suspended in 100 mL (w/v) of 100 mM sodium acetate buffer (pH 5.0) containing 10% crude papain, 5 mM EDTA, and 5 mM cysteine, and incubated at 60°C for 24 h.

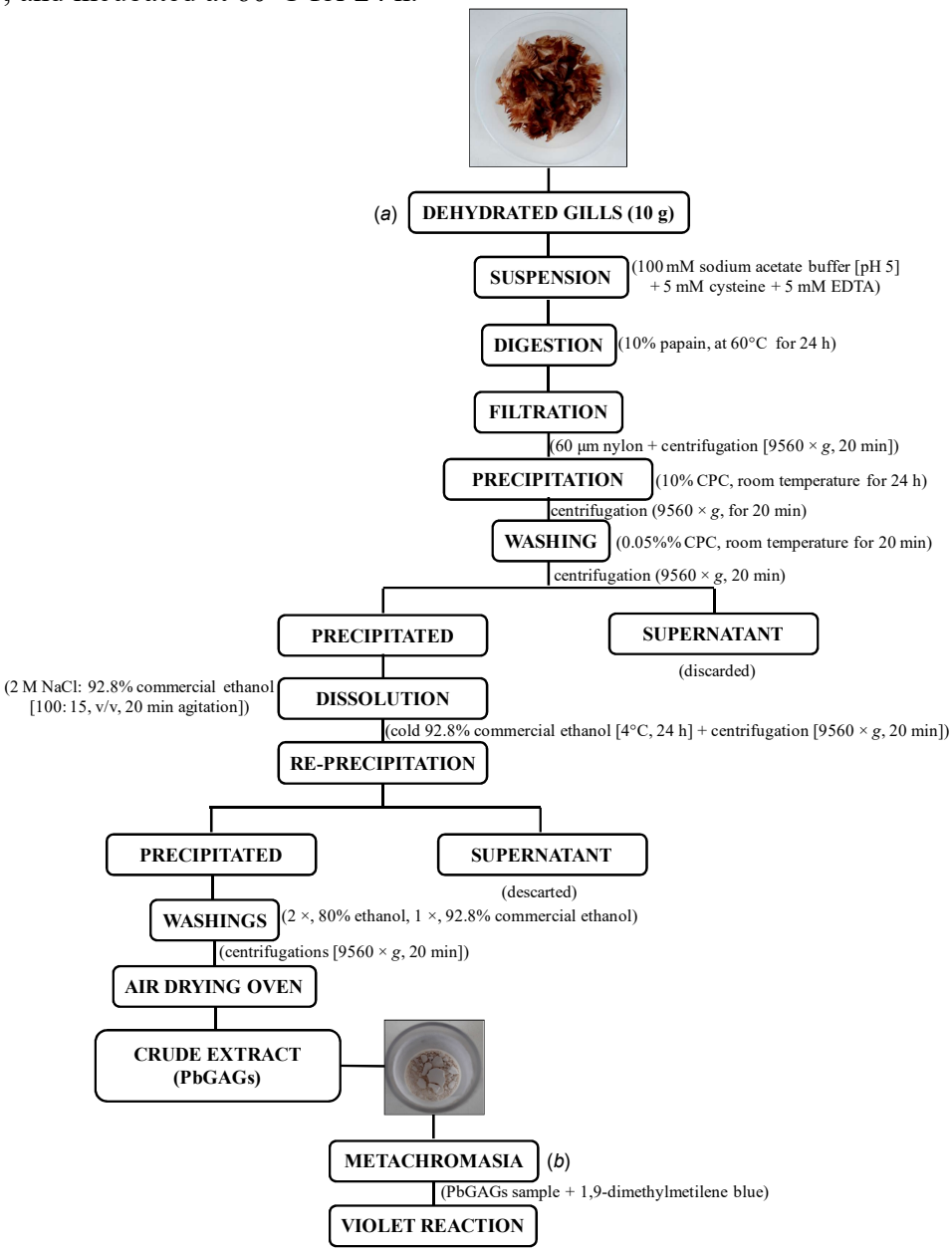

Figure 2. Scheme of obtaining (*a*) and metachromasia (*b*) checking of GAGs from the bocachico gill, *P. brevis*.

The incubation mixture was then filtered using a nylon screen and the supernatants were saved and centrifugated (9.560 × *g* for 20 min). PbGAGs that were present in solution were precipitated with 10 mL of 10% cetylpyridinium chloride (CPC) solution at room temperature for 24 h. The mixtures were then centrifuged at 9.560 × *g*, for 20 min. The *pellet*s containing the PbGAGs were washed with 100 mL of 0.05% CPC solution, dissolved (under mechanical agitation for 20 min) in 100 mL of a 2 M NaCl:ethanol (100:15 ratio, v:v) solution, and then re-precipitated for 24 h at 4°C with addition of 100 mL of cold 92.8% commercial ethanol. The precipitate obtained was centrifugated (9.560 × *g* for 20 min), washed twice with 100 mL of 80% ethanol, and once with the same volume of 92.8% commercial ethanol. After each centrifugation step (9.560 × *g* for 20 min), the PbGAGs were then dried using an air drying oven (60°C, 24 h) and the yield of crude extract containing PbGAGs was expressed as the percentage (w/w %, n = 3) of the dehydrated matter.

Then, the PbGAGs preparation was checked for metachromasia using 1,9-dimethylmetilene (DMB) blue dye as an indicator of reaction by complex formed in violet indicating sulfation (Farndale et al. 1976). The sample of PbGAGs (9 µg) was assassed in triplicate using glass tubes and the color visualized to be specific for sulfated polyanions against the reagent (Figure 2*b*).

PbGAGs identification by electrophoreses

Agarose gel

The Dietrich & Dietrich (1976)' method was applied to examine the polydispersion pattern and charge density of PbGAGs from gill samples. For this, sample (12 µg) was applied to a 0.5% agarose gel prepared with 0.05 M 1,3-acetate diaminopropane buffer (pH 9.0) and the run was carried out at constant voltage (100 V, 1 h). After the run, the PbGAGs present in the gel were fixed with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for 24 h and then dehydrated in air drying oven (50°C, 6 h).

Polyacrylamide gel

The Rodrigues et al. (2016)' protocol was used to analyze the apparent molecular mass distribution of PbGAGs. For this, the sample (12 µg) was applied to a 6% polyacrylamide gel using 0.02 M Tris/HCl buffer (pH 8.6) and the run was performed at 500 mA for 1 h.

The PbGAGs from gill samples present in both gels were revealed with 0.1% toluidine blue or Stains-All cationic dye and, subsequently, the gels were decolorized with a solution containing absolute ethanol, distilled water and acetic acid or using distilled water only. As known markers of molecular mass, chondroitin-6-sulfate (C-6-S,~ 60 kDa), chondroitin-4-sulfate (C-4-S,~ 40 kDa), sulfated dextran (DexS,~8 kDa), dermatan sulfate (DS,~40 kDa) and/or UHEP (~15 kDa) were used as references (Andrade et al., 2017; Volpi & Maccari, 2002).

Fourier Transform Infrared (FT-IR) spectroscopy

The spectrum of PbGAGs was obtained by FT-IR using a spectrometer (IRPrestige-21 Shimadzu, Japan). For measurement, 10 mg of PbGAGs sample were pressed in potassium bromide (KBr) *pellets*. The measurements were performed at a resolution of 4 cm^{-1}, with 64 scans min^{-1} at 500-4000 cm^{-1}.

***In vitro* antioxidant assays**

All the antioxidant assays of PbGAGs from gill samples were performed at the Seaweed II laboratory located at the Department of Biochemistry and Molecular Biology, FUC, and the *in vitro* tests described below.

1,1-diphenyl-2-picryl-hydrazil (DPPH) scavenging effect

The possible effect of PbGAGs to reduce DPPH was performed according to Blois (1958), with some modifications. In this assay, different concentrations of PbGAGs (0.125 to 4.0 mg mL^{-1}) were added to the methanol solution of DPPH (75 M). After 30 min, absorbance was measured at 517 nm. All reactions were performed in triplicates and BHT was used as a reference.

The DPPH scavenging effect was calculated using the following equation: scavenging activity (%) = [A0− (A − Ab)/A0] × 100, where A0 = DPPH without sample; A = sample + DPPH; and Ab= sample without DPPH.

Total antioxidant capacity (TAC)

This assay was performed by the formation of the phosphomolybdate complex, based on Prieto et al. (1999). PbGAGs (0.125 to 4.0 mg mL^{-1}) were added to a solution containing 4 mM ammonium molybdate, 0.6 M sulfuric acid, and 28 mM sodium phosphate, and were incubated at 95°C for 90 min. Absorbance was measured at 695 nm. All reactions were performed in triplicate and a 200 g mL^{-1} sample of ascorbic acid (A. A.) was used as a positive control and considered as 100% TAC.

The data were expressed as a percentage of TAC using the following formula: TAC (%) = $[(A_{sample}-A_{blank}) / (A_{ascorbic\ ac}-A_{blank})] \times 100$.

Ferrous ion chelating (FIC) effect

This assay was based on methodology of Chew et al. (2008), with modifications. For this, different concentrations of PbGAGs (0.125 to 4.0 mg mL^{-1}) were added to 0.1 mM ferrous sulfate ($FeSO_4$) and 0.25 mM ferrozine acid (3- (2-pyridyl) -5,6-diphenyl-1,2,4-triazine -p, p-disulfonic). The tubes were shaken 1 min, incubated 10 min and the absorbance measured at 562 nm. All reactions were performed in triplicates and EDTA was used as a positive control.

Data were expressed as a percentage of chelating effect according to the following formula: FIC effect (%) = $[A_0− (A − Ab)/A_0] \times 100$, where A_0= $FeSO_4$ + Ferrozine without sample; A = sample + $FeSO_4$ + Ferrozine; and Ab = sample without $FeSO_4$ + Ferrozine.

Statistical analyses

The PbGAGs extraction yield of *P. brevis* gill was expressed as mean ± standard deviation (n = 3). Regarding *in vitro* antioxidant assays, data were analyzed by one-way ANOVA, followed by Tukey' test, with $p < 0.05$ as statistically significant. The graphical representations were also constructed using GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com).

Results and Discussion

Yield of PbGAGs from gill samples

Digestion of dehydrated *P. brevis* gills to extract GAGs from the tritured waste samples was effective. After protease incubation (papain, at 60°C) combined with both CPC and 92.8% ethanol precipitations, material was obtained and yielded 1.54 ± 0.02% (w/w), as shown in figure 3A. On the basis of this yield was speculated that *P. brevis* gills are a good source in GAGs because there is a high demand of these molecules for commercial use (Nader et al., 2001; Volpi, 2011).

Bocachico gills, as a by-product generated by fish industry that does not have any market value and is underutilized (Moreira et al., 2001), was susceptible to enzymatic action for 24 h to isolate matrix GAGs when also compared to other studies on the basis of raw material, such as skins (~ 1%, Mansour el. al., 2009; 1.6%, Jridi et al., 2019; and 0.22 ± 0.00%, Nascimento et al., 2021), scales (0.86%, Moura et al., 2021), visceras (0.18%-pacu and 0.15%-tilapia, Nogueira et al., 2019), muscles (8.6%, Jridi et al., 2019) and heads (488 ± 9.06 and 434 ± 50.70 µg g^{-1} of dry weight, Gavva et al., 2020).

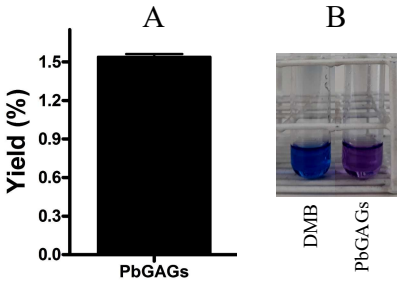

Figure 3. Yield (A) and metachromasia (B) of GAGs extracted from *P. brevis* gills. Yield was calculated as percentage (%) with basis of the dehydrated tissue; Violet reaction in the presence of DMB indicating sulfation.

Based on gills-derived GAGs, studies have been restricted in the literature, as those isolated from the freshwater fish *Cyprinus carpio* (Wasserman et al., 1972) and from the crab *Carcinus maenas* (Regnault & Durand, 1998), whose authors evaluated their composition and some biological responses. There are certain limitations concerning GAGs from animal sources and the difficulty in consolidation of extraction methods due to variety of living organisms, as well as risk-free sources (*e.g.*, viruses and adulteration) as industrial options (Badri et al., 2018).

Regarding PbGAGs from gill samples obtained in figure 3A, they were then tested in the presence of DMB dye and revealed a PbGAGs-DMB binding ability by metachromatic reaction as observed in violet color from the analyzed polymer sample (Figure 3B). The occurence of sulfated polyanions in the *P. brevis* gill extracellular matrix suggested that it has GAGs in the connective tissue (Regnault & Durand, 1998), since that the Farndale et al. (1976)' method has high specificity for sulfated GAGs. As the gills make part of waste generated from the fish filleting operation without added-value (Moreira et al., 2001), on the other hand, its biochemical composition will offer new frontiers for the knowledge of the species, since that studies on the native fishes in Brazil are still scarces and concentrated for Amazon' species (Souza et al., 2007; Nogueira et al., 2019) and/or tilapia cultured in the country (Salles et al., 2017; Nogueira et al., 2019; Moura et al., 2021).

From these observations, GAGs from Bocachico gills were examined for their partial characteristics by electrophoretic and FT-IR analyses.

Physical-chemical identification

Electrophoretic identification of PbGAGs from gill samples was conducted by two gels: agarose or polyacrylamide (Figure 4). Procedure in 0.5% agarose gel prepared in 1,3-diaminopropan buffer system revealed, by toluidine blue staining, an intense metachromatic homogeneous band comigrating as mammalian CS for PbGAGs and its separation occured by fact that the diamine interacted with the sulfate radicals present in backbone structure (Dietrich & Dietrich, 1976), as illustrated in figure 4A.

The unique presence of CS in *P. brevis* gills suggested as a pioneer report in relation to other aquatic sources (*e.g.*, *C. carpio* and *C. maenas*) that identified GAGs population (HA, CS, HS and KS) from gills crude preparations checked or not by electrophoresis (Wasserman et al., 1972; Regnault & Durand, 1998). Taking with literature data, DS has been found as the GAG preponderant or unique in the skin of fishes (Souza et al., 2007; Salles et al., 2021), while viscera rich in CS, DS and HS (Nogueira et al., 2019), scale containing CS and DS chains (Moura et al., 2021) and head revealing CS, DS and HS (Gavva et al., 2020).

Sample-impurity presence (Figure 2) was then checked with Stains-All alone as visualized in figure 4B. Clearly, PbGAGs were strongly stained on gel with basis in navy blue color as standard CS showing similar characteristics in terms of charge/mass; in addition, absence of band related to HA, since that the dye form a complex with high sensitively to groups of uronic acid residues (Volpi & Maccari, 2002).

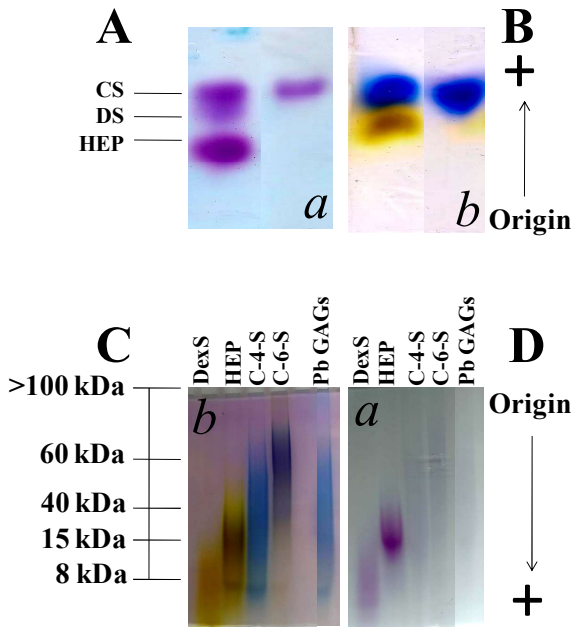

Figure 4. Agarose (A and B) / polyacrylamide (C and D) gels electrophoreses of *P. brevis* GAGs extract and standards chondroitin-6-sulfate (C-6-S,~60 kDa) , dermatan sulfate (DS,~40 kDa), dextran sulfate (DexS,~ 8 kDa) and/or heparin (HEP,~15 kDa) present on gels were stained with 0.1% toluidine blue (*a*) or (*b*) Stains-All.

Gill samples from bocachico (*P. brevis*) presented, at least on initial level, a contaminant-free fish CS (Figures 4A, B). This study also explored its molecular weight by polyacrylamide gel analysis because the structural variety of GAGs became them difficult to precisaly characterize polymers by their colors only (Andrade et al., 2017). On this view, figure 4D showed a separation of molecular masses between PbGAGs and known standards as observed by their respective electrophoretic mobilities, after toluidine blue treatment (Souza et al., 2007; Salles et al., 2017). It was noted to the sample of PbGAGs comigrated as a CS of ~ 40 kDa in central portion of gel (Wasserman et al., 1972), although showing discrete blots with the respective standard.

The here analyzed sample of PbGAGs, also stained with Stains-All alone, showed a electrophoretic profile by polyacrylamide gel of more intense band with CS-like molecular mass distribution and confirmed the HA-free material based on figure 4C. Therefore, this molecular environment would favors the quality control by transformation industry (Badri et al., 2018; Volpi, 2011; Oetterer et al., 2014) *vs*. other aquatic sources that revealed a gills GAGs mixture (Wasserman et al., 1972; Regnault & Durand, 1998).

Structural analysis of PbGAGs by FT-IR

Spectral analysis of PbGAGs from the gill samples, prepared in KBr *pellet*, was revealed between 4000 to 500 cm^{-1} and offered qualitative signals corresponding to sulfated GAGs, as illustrated in figure 5. Values of FT-IR at 3396, 1635 and 2922 cm^{-1} indicated OH stretching, OH bend and CH_2 group, respectivley (Oliveira et al., 2015b; Jridi et al., 2019). The absorption bands at 1307-1375 and 1411 cm^{-1} were attributed to uronic acid (-COO) of the PbGAGs backbone structure (Oliveira et al., 2015b; Jridi et al., 2019). The signal at 1560 cm^{-1} was related to acetyl groups and it denoted the presence of acetylated galactosamine residues (Mansour el. al. 2009). In the spectral window showed an intense signal at 1238 cm^{-1} for total sulfation (S=O) (Mansour el. al. 2009; Oliveira et al., 2015b; Jridi et al., 2019) and other discrete at 929 cm^{-1} ($-SO_3$). More relevant it was evidenced at 819 and 854 cm^{-1} (C-O-S) which indicated for C-6-S and C-4-S, respectively, where

this last one suggested in greater concentration in the sample (Oliveira et al., 2015b). Absorption values at 721 and 594 cm^{-1} were also recorded in PbGAGs.

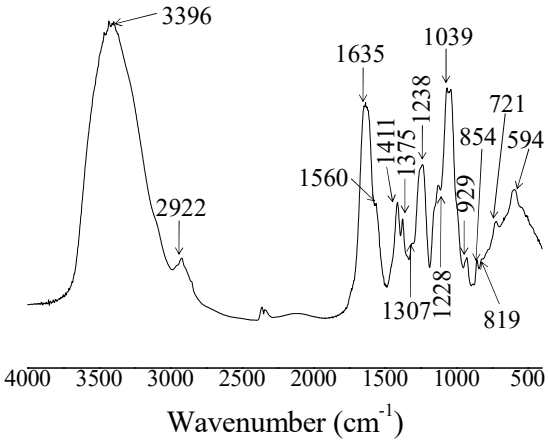

Figure 5. FT-IR spectrum of the *P. brevis* GAGs obtained from gills at 500-4000 cm^{-1}.

Combined observations in this report led us to corroborate that gill residues discarded from *P. brevis* filleting were rich for CS in different proportions and the papain method as methodology applied to isolate it resulted a product with apparent purity from the physical-chemical and structural analyses based on Oliveira et al. (2015b) studying tilapia bone residues as an alternative source in CS.

From our results, PbGAGs from gill samples expressed features appreciable for antioxidation analyses considerating classical three assays: DPPH-scavenging, TAC (both initial phase) and FC (propagation phase) tests; and their *in vitro* effects are described as following.

Analysis by DPPH-scavenging assay

This assay is characterized by an initial mechanism by which the antioxidant agent inhibits oxidative reactions and the radicals are scavenged based on absorbance reduced by analyzed polymer (Blois,1958). In this study, the possible *in vitro* effects of PbGAGs on DPPH radical were evaluated in this assay. However, PbGAGs from gill samples, at a range concentration (0.125 to 4 mg mL^{-1}), did not scavenge the DPPH radical; therefore, no inhibiting the process even a higher dose of sample than that of BHT on the same weight basis required to achieve significant antioxidant effect (95.03 ± 0.63% inhibition, at 4 mg mL^{-1}).

Other waste sources rich in GAGs manifested reductor power by DPPH method. For instance, cuttlefish *S. officinalis* skin and muscle (Jridi et al., 2019) possessed *in vitro* effects on DPPH radical by 60 and 65% at 3 and 5 mg mL^{-1}, respectively. Even *O. niloticus* skin, the *in vitro* antioxidant effects by its GAGs on DPPH free radicals were observed with a maximal potential by 30.26% at 4 mg mL^{-1} (Nascimento et al., 2021). From our findings, it was concluded that the PbGAGs from gill samples showed no scavenge ability because them were not proton-donating substrates in this assay *vs*. the BHT property.

Antioxidant assays: TCA x FIC

Extract containing PbGAGs from gill samples was further analyzed and compared by TAC and FIC methods and the results are demonstrated in table 1. Overall, different concentrations used of sample (from 0.125 to 4 mg mL^{-1}) virtually inhibited the oxidative processes on both *in vitro* tests and higher doses of PbGAGs required to achieve with lower effects than respective controls (ascorbic acid and EDTA).

Table 1. Effects of *P. brevis* GAGs from gill samples on TAC and FIC assays.

Concentration (mg mL^{-1})	FIC (%)	TCA (%)
0.125	5.70 ± 1.42^{a}	0.70 ± 0.46^{a}
0.25	6.82 ± 0.22^{a}	0.75 ± 0.11^{a}
0.5	13.91 ± 0.38^{b}	2.65 ± 0.54^{b}
1	16.82 ± 0.29^{c}	3.47 ± 0.38^{b}
2	20.61 ± 0.41^{d}	6.08 ± 0.17^{c}
4	20.02 ± 0.29^{d}	9.77 ± 0.23^{d}
EDTA (4 mg mL^{-1})	99.56 ± 0.00^{e}	-
Ascorbic acid (0.4 mg mL^{-1})	-	99.77 ± 0.00^{e}

Different letters among the lines indicate significative difference at level of 5% (ANOVA, followed by Tukey' test, $p < 0.05$).

Regarding TCA assay, PbGAGs from gill samples discretally acted as reducing agents of molybdenum VI to molybdenum V (Prieto et al., 1999). The dose-dependent relationship was considered 10-fold lower at concentration than that of ascorbic acid (99.77 ± 0.00%, at 0.4 mg mL^{-1}) used as standard that totally almost abolished the *in vitro* oxidant event. Using the same method, Nascimento et al. (2021) observed that tilapia GAGs from skin samples reduced, the formation of complex phosphomolybdates, with a maximum value of 25.21 ± 0.64% at a concentration of 4 mg mL^{-1}; therefore, 2.58-fold higher in terms of effect in the same concentration used in this study.

Comparing with the FIC method (Table 2), PbGAGs from gill samples exerted a greater chelating effect, concentration-dependent, but only at 2 and 4 mg mL^{-1} (20.61 ± 0.41 and 20.02 ± 0.29% inhibitions, respectively) with similar significantly responses than at lower concentrations (5.70 ± 1.42 and 6.82 ± 0.22% inhibitions, respectively, $p > 0.05$). This behaviour was detected by color reduction produced by an interruption of the complex formed [ferrozine + ferrous ion - Chew et al. (2008)]. By contrast, the sample revealed less potent effect than the standard EDTA ($p < 0.05$) used as positive control, which led to an inhibitory level of 99.37 ± 0.00% at 4 mg mL^{-1}. It could represent that the Pb gill (bocachico) had GAGs capable of acting with a major ability on the propagation phase than intiation one during the *in vitro* oxidation as also suggested by Nascimento et al. (2021) testing the GAGs extracted from the tilapia skin on both assays.

Results in the present study could be related to the existence of a greater ability of active sites in relation to the iron chelating capacity; therefore, PbGAGs deserve further investigation into their mechanism of action using other tests (Jridi et al., 2019). Bioactivities of GAGs would involve sterospecific features, monossacaridic composition, glycosylation sites, aromericity and spartial conformation, not only as a consequence of their charges (Volpi, 2011; Valcarcel et al., 2017).

As the *P. brevis* GAGs from gills apparently demonstrated high degree of purity and satisfactory charge density (Figures 3 and 4), they could be implicated on their matrix functions (Moreira et al., 2001); however, some individuals presented gills in different stages of conservation under fronzen and degraded GAGs could be influenced their *in vitro* antioxidant properties, although considering well-conserved molecules in phylogenetic terms (Medeiros et al., 2000).

The presence of GAGs in connective tissues would participate of the biology in teleost fishes. This aquatic vertebrate group has matrix cells that mucous secrete in response to water quality fluctuations by which they are suject (Moreira et al., 2001). It has been believed that anticoagulant glycoprotein (a carbohydrate-protein macromolecular complex) found in carp gills (*C. carpio*) might be relation with the maintaining of normal blood flow that involves a rich blood channel system and correlations with the mammalian lungs containing HEP, which would correspond to fish

gills, could be the same functional significance (Wasserman et al., 1972). Even a study performed with the crab gill (*C. maenas*), Regnault and Drurand (1998) examined this organ when the animals were submitted to stress by prolonged air exposure and observed a significant change in relation to GAG population under this condition, leading to implications on the gill tissue homeostasis.

In summary, gill from bocachico *P. brevis*, a freshwater rheophilic fish, is descrited for first time as a source in antioxidant GAGs. In-depth studies could open opportunities in terms of molecular markers focusing biological function/populational identity and the use of this species as platform to explore other by-products often discarded from fish filleting on the basis of biotechological applications. Our study contributed to the partial knowledge of its carbohydrate composition extracted from gills of a tropical species cultured and that occurs in rivers of semiarid region of Brazil.

Conclusion

The tropical species *Prochilodus brevis* that occurs in the semiarid area of Brazil generates gills as a by-product discarded from filleting operation that could open frontier to the biochemical knowledge of other native fishes. The raw material sujected by papain-assisted extraction methodology combined with classical biochemistry analyses revealed that it is a valuable source in an unique chondroitin-4/6-sulfate type glycosaminoglycan of ~40 kDa aparent molecular size with similar properties to mammalian derived. The molecule isolated from *P. brevis* gills suggested as an useful tool for other biological studies since that it also showed *in vitro* antioxidant actions, but with significantly less potency than traditional synthetic compounds.

References

Andrade, J. P. S., Oliveira, C. P., Tovar, A. M. F., Mourão, P. A. d. S., & Vilanova, E. (2017). A color-code for glycosaminoglycans identification by means of polyacrylamide gel electrophoresis stained with the cationic carbocyanine dye Stains-All. *Electrophoresis*, *39*(4), 666-669.

Badri, A., Williams, A., Linhardt, R. J., & Koffas, M. A. (2018). The road to animal free glycosaminoglycan production: current efforts and bottlenecks. *Current Opinion in Biotechnology*, *53*, 85-92.

Baldisserotto, B. (2002). Fisiologia de peixes aplicada à piscicultura. Santa Maria: Ed.. UFSM, 212p.

Barbosa, K. B. F., Costa, N. M. B., Alfenas, R. C. G., De Paula S. O., Minim, V. P. R., & Bressan, J. (2010). Estresse oxidativo: conceito, implicações e fatores modulatórios. *Revista de Nutrição*, *23*(4), 629-643.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*(4617), 1199-1200.

Bougatel, H., Krichen, F., Capitani, F., Amor, I. B., Maccari, F., Mantovani, V., Galeotti, F., Volpi, N., Bougatef, A., & Sila, A. (2018). Chondroitin sulfate/dermatan sulfate from corb (*Sciaena umbra*) skin: Purification, strucutural analysis and anticoagulant effect. *Carbohydrate Polymers*, *196*, 272-278.

Chew, Y. L., Lim, Y. Y., Omar, M., & Khoo, K. S. (2008). Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT-Food Science and Technology*, *41*(6), 1067-1072.

Dietrich, C. P., & Dietrich, S. M. C. (1976). Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Analytical Biochemistry*,*70*(2), 645-647.

Farndale, R. W., Buttle, D. J., & Barrett, A. J. (1986). Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochimica et Biophysica Acta*, *883*(2), 173-177.

Gavva, C., Patel, K., Kudre, T., Sharan, K., & Nandini, C. D. (2020). Glycosaminoglycans from fresh water fish processing discard - Isolation, structural characterization, and osteogenic activity. *International Journal of Bilogical Macromolecules*, *145*(24), 558-567.

Jridi, M., Nasri, R., Marzougui, Z., Abdelhedi, O., Hamdi, M., & Nasri, M. (2019). Characterization and assessment of antioxidant and antibacterial activities of sulfated polysaccharides extracted from cuttlefish skin and muscle. *International Journal of Biological Macromolecules*, *123*, 1221-1228.

Mansour, M. B., Majdoub, H., Bataille, I., Roudesli, M. S., Hassine, M., Ajzenberg, N., Chaubet, F., & Maaroufi, R. M. (2009). Polysaccharides from the skin of the ray *Raja radula*. Partial characterization and anticoagulant activity. *Thrombosis Research*, *123*(4), 671-678.

Medeiros, G. F., Mendes, A., Castro, R. A. B., Baú, E. C., Nader, H. B., & Dietrich, C. P. (2000). Distribution of sulfated glycosaminoglycans in the animal kingdom: widespread occurrence of heparin-like compounds in invertebrates. *Biochimica et Biophysica Acta*, *1475*(3), 287-294.

Moura, H. C., Novello, C, R., Balbinot-Alfaro, E., Düsman, E., Barddal, H. P. O., Almeida, I. V., Vicentini, V. E. P., Prentice-Hernández, C., & Alfaro, A. T. (2021). Obtaining glycosaminoglycans from tilapia (*Oreochromis niloticus*) scales and evaluation of its anticoagulant and cytotoxic activities. *Food Research International*, *140*, 110012.

Moreira, H. L. M., Vargas, L., Ribeiro, R. P., & Zimmermann, S. (2001). *Fundamentos da moderna aqüicultura*. Canoas: Ulbra, 200p.

Nader, H. B., Pinhal, M. A. S., Baú, E. C., Castro, R. A. B., Medeiros, G. F., Chavante, S. F., Leite, E. L., Trindade, E. S., Shinjo, S. K., Rocha, H. A. O., Tersariol, I. L. S., Mendes, A., & Dietrich, C. P. (2001). Development of new heparin-like compounds and other antithrombotic drugs and their interactions with vascular endothelial cells. *Brazilian Journal of Medical and Biological Research*, *34*(6), 699-709.

Nascimento, M. M., Nascimento, W. S., Chellappa, N. T., & Chellappa, S. (2012). Biologia reprodutiva do curimatã comum, *Prochilodus brevis* (Characiformes: Prochilodontidae) no açude Marechal Dutra, Rio Grande do Norte, Brasil. *Biota Amazonia*, *2*(2), 31-43.

Nascimento, R. P., Pereira, V.A., Almeida-Monteiro, P. S., Sales, Y. S., Araújo, I. W. F., Rodrigues, J. A. G.; Costa, T. O., Oliveira, A. G., Montenegro, A. R., & Salmito-Vanderley, C. S. B. (2021). Use of glycosaminoglycans from *Oreochromis niloticus* skin as an antioxidant supplement for milt cryopreservation of Brazilian bocachico. *Seminas: Ciências Agrárias*, *42*(5), 2959, 2021.

Nogueira, A. V., Rossi, G. R., Iacomini, M., Sassaki, G. L., Trindade, E. S., & Cirpiani, T. R. (2019). Viscera of fishes as raw material for extraction of glycosaminoglycans of pharmacological interest. *International Journal of Biological Macromolecules*, *121*, 239-248.

Oetterer, M., Galvão, J. A., & Sucasas, L. F. A. *Sustentabilidade na cadeia produtiva do pescado: aproveitamento de resíduos*. In: Galvão, J. A. & Oetterer, M. Qualidade e processamento de pescado. Rio de Janeiro: Elsevier, 2014, p. 97-118.

Oliveira, G. B., Vale, A. M., Santos, A. C., Moura, C. E. B., Rocha, H. A. O., & Oliveira, M. F. (2015a). Composition and significance of glycosaminoglycans in the uterus and placenta of mammals. *Brazilian Archives of Biology and Technology*, *58*(4), 512-520.

Oliveira, A. P.V., Feitosaa, V. A., Oliveira, J. M., Coelho, A. L., Vieira, L. A. P., Silva, F. A. R., Sobrinho, F. A. A. F., Duarte, E. B., Souza, B. W., & Filho, M. S. M. S. (2015b).

Characteristics of chondroitin sulfate extracted of tilapia (*Oreochromis niloticus*) processing. *Procedia Engineering*, *200*, 193-199.

Panicker, V. P., George, S., & Krishna, D. (2014). Toxicity study of butylated hydroxyl toluene (BHT) in rats. *World Journal of Pharmacy and Pharmaceutical Sciences*, *3*, 758-763.

Regnault, M., & Durand, F. (1998). Glycosaminglycans in gills of an intertidal grab (*Carcinus maenas*): changes in the gag population in response to prolonged air exposure. *The Journal of Exparimental Zoology*. *281*, 554-564.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Analytical Biochemistry*, *269*(2), 337-341.

Rodrigues, J. A. G., Queiroz, I. N. L., Quinderé, A. L. G., Benevides, N. M. B., Tovar, A. M. F., & Mourão, P. A. S. (2016). Extraction and structural properties of *Acanthophora muscoides* (Rhodophyceae) extracellular matrix sulfated polysaccharides and their effects on coagulation. *Acta Scientiarum. Technology*, *38*(3), 273-282.

Salles, T. C., Rodrigues, J. A. G., Barcellos, P. G., Amaral, G. F., Araújo, I. W. F., & Mourão, P.A.S. (2017). Inhibition of thrombin generation by dermatan sulfate isolated from the skin of *Oreochromis niloticus*. *Brazilian Journal of Agricultural Sciences, 12*(1), 98-104.

Souza, M. L. S., Dellias, J. M. M., Melo, F. R., & Silva, L. C. F. (2007). Structural composition and anticoagulant activity of derman sulfate from the skin of the electric eel, *Electrophorus electricus* (L.). *Comparative Biochemistry and Physiology*, Part B. *147*(3), 387-394.

Valcacel, J., Novoa-Carballal, R., Pérez-Martins, R. I., Reis, R. L., & Vázquez, J. A. (2017). Glycosaminoglycans from marine sources as therapeutic agents. *Biotechnology Advances*, *35*(6), 711-725.

Volpi, N., & Maccari, F. (2002). Detection of submicrogram quantities of glycosaminoglycans on agarose gels by sequential staining with toluidine blue and Stains-All. *Electrophoresis*, *23*(24), 4060-4066.

Volpi, N. (2011). Anti-inflammatory activity of chondroitin sulphate: new functions from an old natural macromolecule. *Inflammopharmacol*, *19*(6), 299-306.

Wasserman, L., Ber, A., & Allalouf, D. (1972). Acidic glycosaminoglycans composition of the gills of *Cyprinus carpio*. *Comparative Biochemistry and Physiology*. *42*(4), 669-677.