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ALCOHOLIC PRECIPITATE OF *Oreochromis niloticus* SKIN GLYCOSAMINOGLYCANS REVEALS ANTIOXIDANT POTENTIAL

PRECIPITADO ALCOÓLICO DE GLYCOSAMINOGLICANOS DA PELE DE *Oreochromis niloticus* REVELA POTENCIAL ANTIOXIDANTE

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Abstract *Oreochromis niloticus* skin contains anticoagulant glycosaminoglycans-OnGAGs, but their obtaining as antioxidants at lower cost require studies. This research examined OnGAGs, by eco-friendly method, on yield, physical-chemical features and *in vitro* antioxidant effects. Tritured skin was digested with papain, in 100 mM sodium acetate buffer (pH 5)+5 mM cysteine/EDTA, to obtain OnGAGs. Then, OnGAGs precipitated and washed with alcohol were verified by agarose/polyacrylamide gels electrophoreses stained with toluidine blue or Stains-All. Infrared characterized OnGAGs extract. Antioxidant potentials were tested by DPPH, total antioxidant capacity and ferrous ion chelanging-FIC assays using BHT, ascorbic acid and EDTA as respective standards. Alcoholic precipitate yielded 2.32 ± 0.19% and revealed dermatan-unrelated OnGAGs, with relatively reduced charge and size ~40kDa from the molecular analyses. OnGAGs had concentration-dependent antioxidation in all assays evaluated, but there was a preponderant response as powerful as EDTA by FIC method (~100% inhibition, 4 mg mL^{-1}) with contaminants. OnGAGs from skin recovered with alcohol had antioxidant effects, but toxicity studies are need due to low purity.

Key-words: Cichlidae, glycans, organic solvent, oxidation.

Resumo Pele de *Oreochromis niloticus* contém glicosaminoglicans anticoagulantes-OnGAGs, mas estudos a menor custo requerem a obtê-los como antioxidantes. Esta pesquisa examinou OnGAGs, por método "eco-amigável", sobre rendimento, características físico-químicas e efeitos antioxidantes *in vitro*. Pele triturada foi digerida com papaína, em tampão acetato de sódio 100 mM (pH 5)+cisteína/EDTA 5 mM, para obter OnGAGs. Após, OnGAGs precipitados e lavados com álcool foram verificados por eletroforeses em géis de agarose/poliacrilamida corados com azul de toludina ou "Stains-All". Infravermelho caracterizou extrato de OnGAGs. Potenciais antioxidantes foram testados pelos métodos DPPH, capacidade antioxidante total e quelante de íon ferroso-QIF usando BHT, ácido ascóbico e EDTA como padrões respectivos. Precipitado alcoólico rendeu 2,32±0,19% e revelou OnGAGs diferente ao dermatam, com carga relativamente reduzida e tamanho ~40kDa pelas análises moleculares. OnGAGs possuiram antioxidação dependente de concentração em todos os ansaios, porém existiu uma resposta preponderante tão potente quanto EDTA pelo método QIF (inibição ~100%; 4 mg mL^{-1}) com contaminantes. OnGAGs de pele recuperados com álcool detiveram efeitos antioxidantes, mas estudos de toxicidade são necessários pela baixa pureza.

Palavras-Chave: Cichlidae, glicanas, solvente orgânico, oxidação.

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ARTIGO REVISTA BRASILEIRA DE ENGENHARIA DE PESCA

Introduction

Fish industry respond to an economic sector that generates much attention in several countries over the world. On the other hand, it discard large amounts of fish by-products (*e.g.*, fins, heads, viscera and skin) leading to critical health and environmental problems (Moreira et al., 2001; Oetterer et al., 2014). As an eco-friendly solution there is the alternative use of these residual materials, which are frequently discarded due to the lack of adequade destination (Moreira et al., 2001), as natural reservoirs of active biologically molecules for biotecnological applications (Valcarcel et al., 2017). By transformation of these fish processing raw wastes could obtain human and animal health-related compounds, including, *e.g.*, collagen, peptides and sulfated polysaccharides (SPs) (Oetterer et al., 2014).

SPs are complex polyanionics found in a variety of living organisms (Medeiros et al., 2000; Pomin, 2012; Ai et al., 2023) since bacteria to animals, but not occuring in higher plants, fungi and protists (Badri et al., 2008). Those from animals (invertebrate and vertebrate) are well-known and called glycosaminoglycans (GAGs), which are usually found in all species that have tissue organization (Medeiros et al., 2000; Oliveira et al., 2015). Structurally, GAGs are biological molecules of hydrophilic (anionic) character consisting of repeating disaccharide units of aminosugar (D-galactose or D-glucosamine) and uronic acid (L-iduronic or D- glucuronic acid) or galactose sugar. The GAGs chondroitin sulfate (CS), dermatan sulfate (DS) hyaluronic acid (HA, nonsulfated), heratan sulfate, heparin (HEP) and heparan sulfate (HS) are the most recognized by glycobiologists, of which only HA does not forms proteoglycan in nature (Gandhi & Ricardo, 2008; Oliveira et al., 2015). Comparing HEP and HS, they also show great similarity molecular (Gandhi & Ricardo, 2008), but HEP, commercially extracted from porcine and bovine intestines, is the main GAG explored because its clinical applications as anticoagulant and antithrombotic in the treatment of coagulation-related pathologies; however, it is also known by its adverse consequences (*e.g.*, thrombocytopenia, pathogenic particles, and hemorrhagic effect) (Nader et al., 2001), and new potential substitutes to it have been searched for decades (Mourão, 2015). On an industrial scale, GAGs require quality and purity for use (Volpi, 2011). On their functionalities, GAGs have already been reported with, for instance, anticoagulant (Mansour et al., 2009; Krichen et al., 2018; Nogueira et al., 2019), antioxidant and antibacterial (Jridi et al., 2019) effects.

Oxidative damage is origined by imbalance between two main causes: free radical generation and scavenging capacity from the natural biological systems. As a result, the endogenous antioxidant mechanisms, or those caused by exogenous sources, became the biological system vulnerable to free radical actions, leading to severe dysfunctions to biomolecules, arising thus cell injury and irreversible consequences including oxidative stress-associated diseases, *e.g.*, carcinogenesis, atherosclerosis, DNA damage and degenerative events (Barbosa et al., 210). Studies on the natural products from macroalga (Alves et al., 2012), seagrass (Silva et al., 2012), fish waste (Jridi et al., 2019; Santiago et al., 2024) and cyanobacteria (Ai et al., 2023) have indicated as rich sources in antioxidant SPs. Still on this context, new antioxidant agents became crucial in view of commun use of sysnthetic antioxidants, such as butylatedhydroxytoluene-BHT, which it induce toxic reactions (Panicker et al., 2014).

Some SPs, from different sources, were already demonstrated with great potential against free radicals, where in their heterogeneous structures revealed the existence of active sulfation sites capable of neutralizing oxidative processes, including *in vitro* and/or *in vivo* assays. From seagrass *Halodule wrightii*, Silva et al. (2012) discovered that its SPs extracted with proteolysis followed by sequential acetone precipitation resulted in a cetonic fraction rich in antioxidant SPs by DPPH radical and TAC methods. Study with the macroalga *Gracilaria caudata* (Rhodophyta), Alencar et al. (2019) isolated its SPs by papain digestion combined with cetylpyridinium chloride precipitation and observed *in vitro* effects by ferreous ion chelanting (FIC) and total antioxidant capacity (TAC)

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ARTIGO REVISTA BRASILEIRA DE ENGENHARIA DE PESCA

methods, and *in vivo* experimentaly using rats. More recently, the investigation of GAGs obtained from cuttlefish *Sepia officinalis* skin and muscle (Jridi et al., 2019) and from Nile tilapia *Oreochromis niloticus* skin (Nascimento et al., 2021), using papain combined with cetylpyridinium chloride precipitation, yielded to be antioxidants by, at least, in three *in vitro* protocols. Recently, gills of *Prochilodus brevis* fish were reported to be GAGs with *in vitro* antioxidant effects (Santiago et al., 2024). However, reports on fish waste-derived GAGs *in vitro* tested regarding their antioxidant potentials have been few explored in the literature.

O. niloticus (Linnaeus 1758) is a fishwater species belonging to the Cichlidae family and populary known as tilapia. This african fish has been commercially cultured worldwide as a good animal protein source of appreciable quality due to its nuttritional value and for the development of various high value-added products. As about 50% of this fish after filleting operation is composed by waste and skin represents by 5.1% (w/w) (Moreira et al., 2001), studies on the biotechnological interest of GAGs recovered from such inedible parts of tilapia have been satisfactory as anticoagulant from skin (Rodrigues et al., 2011a; Salles et al., 2017) and viscera (Nogueira et al., 2019); and antioxidant from skin (Nascimento et al., 2021) as supplement for cryopreservation of tropical fish semen (Pereira et al., 2020; Nascimento et al., 2021). However, the used protocol for these studies was of high cost and laborious for *O. niloticus* skin-derived GAGs production.

The objective of this research was to analyze an eco-friendly protocol that combined proteolysis and alcoholic precipitation for *O. niloticus* skin GAGs (OnGAGs) obtaining; then examining their molecular features by classical biochemical techniques; and finally, by their *in vitro* antioxidant effects by mean of three conventional assays in order to primarily explorar both initial/propagation phases of the oxidation process.

Material and Methods

Acquisition of *O. niloticus* specimens and skin preparation

Specimens of monosex Nile tilapia (50 individuals) were obtained from the Aquaculture station (Figure 1A) of the Federal University of Ceará (FUC). After collected from the tank, fishes were subjected to Santiago et al. (2024)' slaughter procedure, followeed by packed in ice (1:1 kg - biomass:ice ratio) using isothermal boxes and then taken to the Marine Biochemical (MarBio) laboratory located at Aquaculture Biotechnology Center, Department of Fisheries Engineering, FUC. The use of tilapia fish was authorized through our registration with SISGEN - AA4816B (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) and approved by the Ethical Committee of the same institution (CEUA - nº 6974061020).

Figure 1. General view of the experimental farm of Aquaculture - FUC (A), procedures of measurement (B) followed by weighting (C) and skin removed from Nile tilapia (D).

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
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In MarBio laboratory, the animals were measured (18.90 ± 1.69 cm) (Figure 1B) and weighted (183.10 ± 52.20 g) (Figure 1C) with a rudimentary ichthyometer and a balance on a 1 g precision scale, respectively. Further step was to manually treat the fishes by scaling, evisceration and skin removal procedures with knife and clamp for muscle-free material obtaining (Figure D). Next, the fish skin separated from residual meat was extensively washed with distilled water, dehydrated in an oven under air circulation (45°C, 24 h) and, subsequently, stored in an appropriated recipient until experimental analyzes.

Extraction of OnGAGs

After the fish skin treatment, the dehydrated *O. niloticus* tissue in air drying oven (45°C, 24 h) was cut into small peaces and the GAGs extraction performed according to the Rodrigues et al. (2009, 2011a)' combined methods (Figure 2). Initially, tritured sample (20 g) was rehydrated in 200 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. The incubation mixture was then filtered using a nylon screen and the supernatants were saved and centrifugated (9.560 × *g* for 20 min). OnGAGs that were present in solution were precipitated with 92.8% ice-cold alcohol in a ration of 3:1 at -20°C for 48 h. The mixture was then centrifuged at 9.560 × *g*, for 20 min. The *pellet* containing the OnGAGs was washed twice with 50 mL of 80% alcohol, and once with the same volume of 92.8% alcohol. After each centrifugation (9.560 × *g* for 20 min), the OnGAGs were then dried using an air drying oven (60°C, 24 h) and the yield of crude extract containing OnGAGs was expressed as the percentage (w/w %, n = 3) of the dehydrated matter.

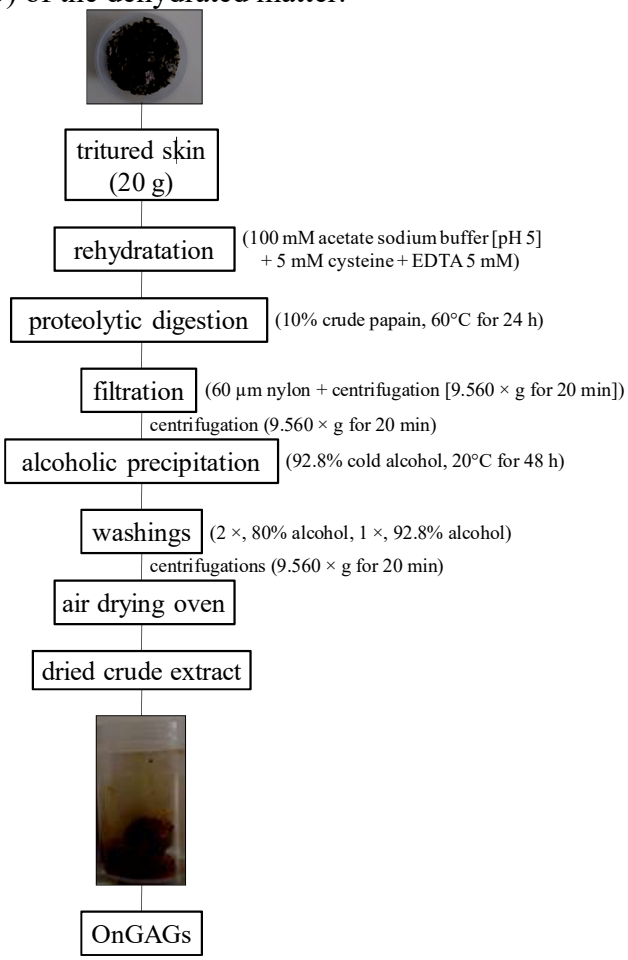

Figure 2. Scheme of obtaining of GAGs from the Nile tilapia, *O. nilticus*.

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ARTIGO REVISTA BRASILEIRA DE ENGENHARIA DE PESCA

Biochemical characterization by electrophoreses

Agarose gel

This analysis was carried out following the Dietrich & Dietrich (1976)' method. It was performed to examine the polydispersion pattern and charge density of OnGAGs. For this, sample 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 OnGAGs 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

This procedure was based on Rodrigues et al. (2016) to analyze the apparent molecular mass distribution of OnGAGs. For this, the sample 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 OnGAGs present in both gels were revealed with 0.1% toluidine blue or Stains-All cationic reagent 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 OnGAGs was obtained by FT-IR using a spectrometer (IRPrestige-21 Shimadzu, Japan). For each measurement, 10 mg of OnGAGs 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

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

The effect of OnGAGs to reduce DPPH was performed according to Blois (1958), with some modifications. In this assay, different concentrations of OnGAGs (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). OnGAGs (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 (%) = $[(A_{sample}-A_{blank}) / (A_{ascorbic\ ac}-A_{blank})] \times 100$.

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
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ARTIGO REVISTA BRASILEIRA DE ENGENHARIA DE PESCA

Chelating ferrous (CF) effect

This assay was based on methodology of Chew et al. (2008), with modifications. For this, different concentrations of OnGAGs (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: CF effect (%) = [A0−(A − Ab)/A0] × 100, where A0= $FeSO_4$ + Ferrozine without sample; A = sample + $FeSO_4$ + Ferrozine; and Ab = sample without $FeSO_4$ + Ferrozine.

Statistical analyses

Values of GAGs extraction yield of *O. niloticus* skin were 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 OnGAGs from skin samples

The analysis of GAGs extraction yield of *O. niloticus* skin on the basis of tritured fish tissue, when sujected to proteolysis (papain, at 60°C) and material precipitated with ice-cold alcohol for 48 h, showed an amount of 2.32 ± 0.19% (w/w), as shown in table 1.

Table 1. Yield of SPs extracted from *O. niloticus* skin cultured *vs.* other sources.

Source	Crude SPs	Protocol/time	Tissue	Yield*	Reference
O. niloticus (cultivation)	OnGAGs	papain+cold alcohol precipitation/48 h	tritured	2.32±0.19%	this study
Hypnea musciformis (Flecheiras beach)	SPs	papain+cetylpiridinium choride precipitation/24 h	tritured	49.05±0.38%	Rodrigues et al. (2011b)
	SPs	hot water (80°C)+alcohol precipitation/4 h		4.28±0.26%	
Acanthophora muscoides (Pacheco beach)	SPs	papain+cetylpiridinium choride precipitation/24 h	tritured	17.00% 4.65% 1.82%	Rodrigues et al. (2016)
Oreochromis niloticus (cultivation)	OnGAGs	papain+cetylpiridinium choride precipitation/24 h	tritured	0.15%	Rodrigues et al. (2011a)
Oreochromis niloticus (cultivation)	OnGAGs	papain + cetylpiridinium choride precipitation / 24 h	tritured	0.10±0.05 %	Salles et al. (2017)
Sepia officinalis (market)	GAGs	papain + cetylpiridinium choride precipitation / 24 h	ground	1.60±0.1%	Jridi et al. (2019)
Oreochromis niloticus (cultivation)	OnGAGs	papain + cetylpiridinium choride precipitation / 24 h	tritured	0.22±0.00%	Nascimento et al. (2021)

Yield was calculated as percentage (%) with basis of the dehydrated tissue.

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
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This result was, at least, 23.2-fold higher than those of samples of dehydrated skin studying the same species by other authors (Rodrigues et al., 2011a, Salles et al., 2017; Nascimento et al., 2021), when the OnGAGs were obtained by papain method combined with cationic precipitation. Compared with other sources (Table 1), *O. niloticus* had a low concentration in skin SPs than macroalgae species (*G. caudata* [24.96%, Alencar et al., 2019], *Hypnea musciformis* and *Acanthophora muscoides*) of different tissue structures and belonging to "vegetable" kingdom (Rodrigues et al., 2011b, 2016). In fact, fish skins are a *poor* source in matrix GAGs *vs.* other raw tissues (*e.g.*, muscle - Jridi et al., 2019; gill - Santiago et al., 2024), but similar yields of GAGs containing-crude extracts were found between *in natura* viscera removed from Nile tilapia (0.18%) and Pacu (0.15%) (Nogueira et al., 2019), when applyning papain method. Krichen et al. (2018) obtained with Alacalase method 7.78% of GAGs from Atlantic bluefin tuna (*Thunnus thynnus*) skin. Combined protocols, organic solvents, tissues of origin and first-matter pretreatments are factors that can influence on the yield of SPs (Rodrigues et al., 2011b; Ai et al., 2023). The development of alternative animal-GAGs production approaches has been challenged for industrial use (Badri et al., 2008).

Fish skin has a structure formed by epidermis, with mucous glands, and dermis, predominantly fibrous (Moreira et al., 2001) with its extracelluar matrix constituted by collagens, multi-adhesive glycoproteins, elastin and GAGs (Oliveira et al., 2015). Such matrix components also interact with cells displaying roles related to physiology and pathological events (Oliveira et al., 2015). In particular, GAGs are produced by epidermal glands and the mucus formed ("called mucopolysaccharides) isolates the body of the fish and protects it against pathogens (Moreira et al., 2001). The biological role of fish GAGs have been more discussed (Santiago et al., 2024).

On this compositional basis, alternative use of alcohol solvent as a precipitant agent led to a low quality of OnGAGs extract, since that the raw material showed impurities as illustrated in figure 2. It has been reported that the use of this organic solvent shows low efficiency to recover bioactive SPs and matrix compounds and pigments naturally found in tilapia skin could also be coprecipitated due to its polar character based on other studies (Rodrigues et al., 2011b; Ai et al., 2023).

Considerating these questions, crude extract of OnGAGs was further analyzed by electrophoretic and FT-IR procedures.

Physical-chemical analyses of OnGAGs

Study on the physical-chemical features of OnGAGs involving electrophoretic procedures is shown in figure 3. As expected, by agarose gel (Figure 3A), it indicated by staining with toluidine blue the presence of a single homogeneous band comigrating as DS based on Rodrigues et al. (2011a) and Salles et al. (2017), who formaly OnGAGs-rich fractions characterized, confirming in this research an unique DS in *O. niloticus* skin; but, with extract showing relatively reduced metachromasia contrasting that of high charge density for OnGAGs isolated by Nascimento et al. (2021). On the basis of this result, it was possible to infer that the diamine of the electrophoretic buffer interacted with the OnGAGs-containing alcoholic precipitate as characterized for animal GAGs (Dietrich & Dietrich, 1976; Santiago et al., 2024).

These combined observations led us to partially conclude that the alcoholic precipitate revealed low sulfate composition in OnGAGs, since that the charges of the GAGs interacted with the water and the organic solvent (alcohol) modified the dieletric constant in the medium to precipitate the polysaccharides. This ability proved by polarity of organic solvent was also discussed for other sources rich in SPs (*e.g.*, seaweeds) and checked or not by agarose gel (Rodrigues et al., 2009, 2011b; Ai et al., 2023).

To analyze sample-impurity presence (Figure 2), OnGAGs were stained with Stains-All alone and the result is in figure 3B. As can be seen, Stains-All strongly reacted with the sample of OnGAGs on agarose gel by increase of the intensity of a purple/blue polydisperse band from DS to

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
DOI: 10.18817/repesca.v16i3.3608

HEP, suggesting the detection of other elements in OnGAGs structure; therefore, cationic dye (Stains-All) sensively complexed with other nonsulfated compounds in a high limit of detection revealed no with toluidine blue alone, as visualized in figures 3A, B.

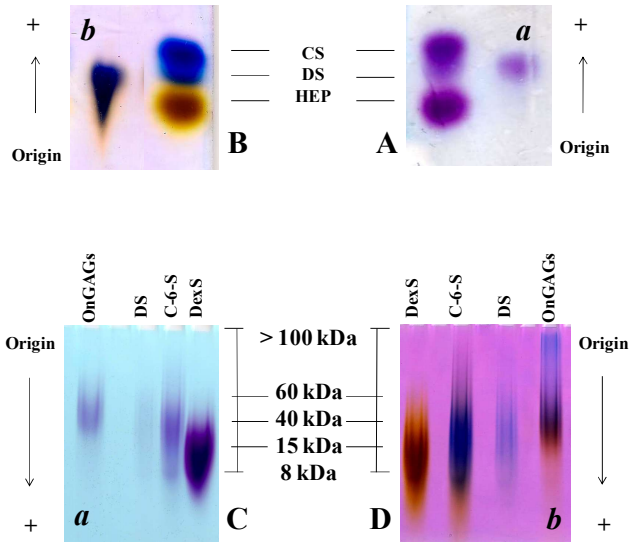

Figure 3. Agarose (A and B) / polyacrylamide (C and D) gels electrophoreses of *O. niloticus* GAGs extract and standards chondroitin-6-sulfate (C-6-S,~ 60 kDa) , dermatan sulfate (DS,~ 40 kDa), dextran sulfate (DexS,~ 8 kDa) and/or heparin (HEP,~ 15 kDa) present on gels were stained with 0.1% toluidine blue (*a*) or (*b*) Stains-All.

The identification of contaminants in preparation of OnGAGs, at least on initial level, is important (Figure 3B), because in fish skin there are diverse natural products in high levels (Moreira et al., 2001), including other GAGs depending on the species (Krichen et al., 2018). Nogueira et al. (2019) identified a mixture of CS, DS and HS in *in nature* visceras from tilapia and pacu. This scenario difficults the quality control by polymer industry (Volpi & Maccari, 2002; Volpi, 2011).

In order to explore this fact, our study was also combined with the electrophoretic analysis of molecular weight by polyacrylamide gel, because is impossible to precisaly identify molecules by their colors only (Andrade et al., 2017; Santiago et al., 2024) and DSs from fish skin vary their sizes (Mansour et al., 2009). On this perspective, figure 3C demonstrated that the OnGAGs and standards used as known markers were separated by their molecular masses based on respective electrophoretic mobilities, after toluidine blue treatment (Krichen et al., 2018). As expected, the sample of OnGAGs comigrated as a DS of ~ 40 kDa, which showed mobility in central portion of gel (Salles et al., 2017), although discrete in purple color due to its origin (Andrade et al., 2017).

As the molecular basis of OnGAGs was confirmed by charge and size of DS (Rodrigues et al., 2011a; Salles et al., 2017; Nascimento et al., 2021), the here analyzed sample also evidenced an electrophoretic profile containing a mixture of colors and second component of high molecular size (> 100 kDa) that did not migrate on gel, when the material was visualized after Stains-All staining alone (Figure 3D). It was surprising because seemed HA present in examined polymer sample (Andrade et al., 2017). This nonsulfated GAG was not identified in a previous report (Salles et al., 2017) or recovered peptides by alcohol action based on Andrade et al. (2017).

Collectivelly, our observations suggested the presence of various biological molecules, such as nonSPs, peptides, DNA and/or other tilapia skin-alcohol coprecipitated products, attached in

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
DOI: 10.18817/repesca.v16i3.3608

OnGAGs structure, since that the standard GAGs used mantained their identities during the electrophoretic procedures (Figure 3).

Structural features of OnGAGs alcoholic precipitate

To verify the chemical identity of the sample, OnGAGs obtained by precipitation with ice-cold alcohol were characterized by FT-IR spectroscopy and the spectrum is shown in figure 4. It was also compared with the study of Pereira et al. (2021) that extracted OnGAGs by proteolysis and cationic precipitations with cetylpiridium chloride/alcohol and found a GAG DS in Nile tilapia skin.

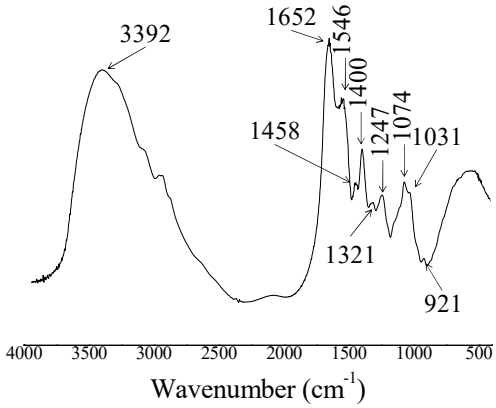

Figure 4. FT-IR spectrum of the *O. niloticus* GAGs obtained with alcoholic precipitation at 500-4000 cm^{-1}.

FT-IR spectrum of OnGAGs, between 4000 to 500 cm^{-1}, revealed that the sample, obtained under our conditions (Figure 2), indicated the presence of signals at 3392 and 1652 cm^{-1} (OH stretching and OH bend, respectivley) (Alves et al., 2012; Costa et al., 2012; Silva et al., 2012; Krichen et al., 2018; Jridi et al., 2019). The occurence of bands at 1321 and 1400-1458 cm^{-1} were attributed to uronic acid making part of the OnGAGs backbone structure (Jridi et al., 2019). The spectral value at 1546 cm^{-1} was observed to acetyl groups in analyzed polymer sample, denoting the occurence of acetylated galactosamine residues (Mansour el. al. 2009). Although showing the evidence of total sulfation at 1247 cm^{-1} (S=O) (Mansour el. al. 2009; Alves et al., 2012; Costa et al., 2012; Silva et al., 2012; Jridi et al., 2019) and at 921 cm^{-1} ($-SO_3$), the spectrum did not reveal band related to C-O-S (820 or 850 cm^{-1}) as indicative for GAGs DS/CS (Pereira et al., 2021). Absorption values at 1031 and 1074 cm^{-1} were also noted and corresponded to the C-O-C and C-OH ring vibrations in the osidic cycles, respectively (Mansour el. al. 2009).

Such divergence was the result of the 92.8% alcohol solvent used as alternative to cationic one (Pereira et al., 2021) and confirmed reduced sulfation by biochemical analyses (Figure 3), since that fish skin-derived GAGs have already been precipitates with higher alcohol percentagens, showing some chemical variations in the samples (Mansour et. al. 2009). In fact, the spectrum of OnGAGs represented DS-unrelated alcohol insoluble material, which was extracted and precipitated from the fish skin (Figure 2). It suggested that other contaminants were not specifically identified, since that pigments and DNA molecules are susceptible to oxidation/degradation and more refined techniques are need for better extract characterize observed in figure 2. However, the FT-IR spectrum excluded the possible presence of HA in sample and those results found from the polyacrylamide gel could be interpreted as a component of HA-unrelated high molecular mass (Figure 3) based on Salles et al. (2017) and Santiago et al. (2024).

Studies on the fish skin composition have revealed DS as the GAG most found (Mansour et al., 2009; Salles et al., 2017; Jridi et al., 2019; Krichen et al., 2018). Our findings supported for a

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
DOI: 10.18817/repesca.v16i3.3608

sulfated polymer rich in uronic acid; therefore, characterized as acid polysaccharide, but as far from DS molecule as partially identified by FT-IR (Pereira et al., 2021).

Consideranting that the oxidative event involves three stages (initiation, propagation and termination) (Silva et al., 2012), the alcoholic precipitate of OnGAGs was then explored on a basis of application using classical three assays: DPPH-scavenging, TAC (both initial phase) and FC (propagation phase) methods; and its *in vitro* effects are shown in figures 5, 6 and 7.

DPPH-scavenging assay

The *in vitro* effects of OnGAGs on DPPH radical are represented in figure 5. OnGAGs were able to scavenge the DPPH radical in a dose-dependent property (0.125 to 4 mg mL^{-1}) and showed a similar inhibition rate up to 0.5 mg mL^{-1} (~ 7%). Increasing concentration of sample revealed significant values of reduction, whose majority effect was only at concentration of 4 mg mL^{-1} (19.88 ± 0.47%). As reference, BHT had an effectiveness of 99 ± 0.072% ($p < 0.05$) using the same concentration of OnGAGs.

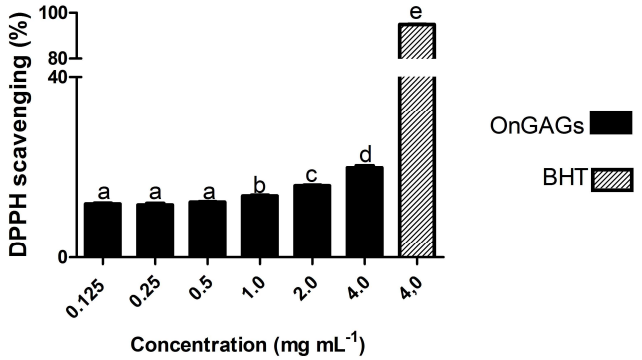

Figure 5. Effects of *O. niloticus* GAGs precipitated with alcohol on DPPH free radicals. Different letters among the bars indicate significative difference at level of 5% (ANOVA, followed by Tukey' test, $p < 0.05$).

Previous studies demonstrated that SPs-rich extracts from seagrass *H. wrightii* (Silva et al., 2012), macroalga *Hypnea musciformis* (Alves et al., 2012) and cuttlefish *S. officinalis* skin and muscle (Jridi et al., 2019) had *in vitro* effects on DPPH radical by 41.4% (at 0.5 mg mL^{-1}), 9.88% (at 5 mg mL^{-1}) and 60-65% (at 3 and 5 mg mL^{-1}), respectively. Regarding *O. niloticus* skin, the *in vitro* antioxidant response by OnGAGs, precipitated with cold alcohol, on DPPH free radicals presented a modest effect and corresponded to a difference of 1.52-fold lower in scavenge ability compared to OnGAGs-rich extract (30.26% inhibition, at 4 mg mL^{-1}), obtained by proteolysis combined with both cationic and alcohol precipitations, in another study (Nascimento et al., 2021). By contrast, GAGs extracted from *P. brevis* gills had no *in vitro* effect by the DPPH method (Santiago et al., 2024). These comparative analyses suggested that both GAGs-containing materials from *O. niloticus* skin showed scavenge ability because them were proton-donating substrates in this assay (Ai et al., 2023; Jridi et al., 2019).

TAC assay

Analysis by mean of TAC method of the sample of OnGAGs recovered with ice-cold alcohol in this study is demonstrated in figure 6. Based on our findings, OnGAGs-containing alcoholic precipitate reduced, concentration dependently, the assay-induced oxidant process, but only with a significant rate of 30.11 ± 1.79% (at 4 mg mL^{-1}) *vs*. the standard ascorbic acid (A. A.) that totally suppressed the oxidant event at concentration of 20-fold lower than that of OnGAGs obtained from

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
DOI: 10.18817/repesca.v16i3.3608

fish skin. This profile of antioxidant action was similar to that of OnGAGs observed in DPPH assay (Figure 5).

Some authors have reported SPs isolated from different origins with low potential by TAC assay, as those isolated from seagrass *H. wrightii* (Silva et al., 2012), cyanobacteria (Ai et al., 2023) and gills of *P. brevis* fish (Santiago et al., 2024), similar to this study (Figure 6). However, this profile could not exclude for a high TAC, as was that found by Alencar et al. (2019), who reported a relevant dose-dependent TAC by a crude SP from the macroalga *G. caudata*, enzimatically extracted, precipitated (cetylpiridium chloride+alcohol) and sequencially washed (alcohol and acetone) with organic solvents, which showed a value of 89.10% at 4 mg mL^{-1}.

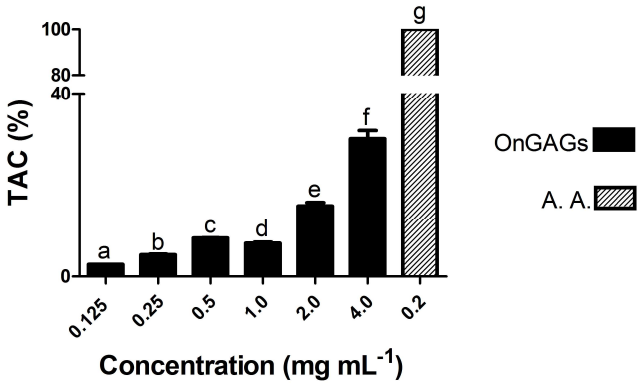

Figure 6. Effects of *O. niloticus* GAGs precipitated with alcohol on TAC. Different letters among the bars indicate significative difference at level of 5% (ANOVA, followed by Tukey' test, $p < 0.05$).

Therefore, the TAC of the seaweed SP was very good than our study and other aquatic resources, including that of OnGAGs previously obtained by proteolysis (papain) and cationic+alcohol precipitations by Nascimento et al. (2021) using the same method.

As the OnGAGs of this study showed as an antioxidant tool on the initiation phase (Silva et al., 2012), they were also *in vitro* tested on FIC ability (propagation phase).

FIC assay

The figure 7 shows that the OnGAGs recovered with ice-cold alcohol resulted in a bioactive precipitate with FIC ability, which was observed by color reduction produced by an interruption of the complex formed [ferrozine + ferrous ion - Chew et al. (2008)]; therefore, OnGAGs acted as iron chelating agents in dose-dependent manner (0.125 to 4 mg mL^{-1}), displaying high FIC effect with inhibition levels ($p < 0.05$) of 58.77 ± 0.40 and 99.37 ± 0.36% at concentrations of 2 and 4 mg mL^{-1}, respectively, of sample. This important effect was as potent as the EDTA antioxidant ($p > 0.05$), which produced an inhibitory response of 99.37 ± 0.00% at 4 mg mL^{-1}.

Findings of this study were considerably more significant than those observed by Alves et al. (2012), who obtained a FIC level of 8% with a concentration of 5 mg mL^{-1} of SPs extracted from the red seaweed *H. musciformis*. With the red seaweed *G. caudata*, Alencar et al. (2018) also identified a chelation action by SP lower than that found in this study, where the maximum antioxidant potential observed was in the order of 69.80% (at 4 mg mL^{-1}). On the other hand, these values contrasted those found by Nascimento et al. (2021), who first described the FIC actions by OnGAGs and observed that at concentrations of 1.0, 2.0 and 4.0 mg mL^{-1} they inhibited by 29.43 ± 0.40, 32.22 ± 0.10 and 31.10 ± 0.59%, respectively; therefore, at least, ~ 3.19-fold lower than the FIC actions this current investigation. Recently, Santiago et al. (2024) found an inhibitory effect of only 20.02 ± 0.29% at 4 mg mL^{-1}, for GAGs extracted from *P. brevis* gills, by the FIC assay.

Rev. Bras. Eng. Pesca (2025) 16(3): 65-80
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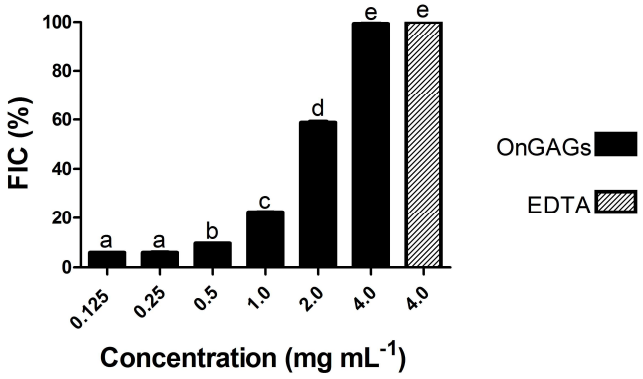

Figure 7. Effects of *O. niloticus* GAGs precipitated with alcohol on FIC. Different letters among the bars indicate significative difference at level of 5% (ANOVA, followed by Tukey' test, $p < 0.05$).

Based on three *in vitro* assays used, it was possible to confirm that the antioxidant actions by OnGAGs precipitated with ice-cold alcohol were preponderant in FIC assay than DPPH and TAC methods. Results were consistents with those found by Nascimento et al. (2021) where speculated the existence of a greater ability of active sites in relation to the iron chelating capacity; therefore, OnGAGs acted preferentially in the propagation phase of the oxidation process (Santiago et al., 2024) and deserve further investigation into their mechanism of action using other tests, including *in vitro* (Ai et al., 2023; Jridi et al., 2019) and *in vivo* assays (Alencar et al., 2018).

Due to the fact that this study suggested a greater affinity of active sites with iron, inhibiting the production of hydroxyl radicals in the system (Alencar et al., 2019), it is believed that the presence of sulfate radicals may be related to the chelation effects, sequestration or electron-donating ability of antioxidant OnGAGs (Ai et al., 2023; Jridi et al., 2019; Santiago et al., 2024). Structural studies of OnGAGs could reveal the requeriments related to their actions in the oxidant system. It has been reported that the bioactivities of these molecules involve sterospecific features, monossacaridic composition, glycosylation sites, aromericity and spartial conformation, not only as a consequence of their charges (Pomin, 2012; Mourão, 2015).

For the low quality of OnGAGs extract (Figure 2), the presence of contaminants is not discard in material and would contribute for cobioactivity, since that in tilapia skin matrix has elements with health-related beneficits (Moreira et al., 2001; Salles et al., 2017), although use of papain would remove carbohydrates-complexed proteins during preparation of OnGAGs (Rodrigues et al., 2011; Salles et al., 2017; Santiago et al., 2024). From these partial conclusions, OnGAGs coprecipitated with other elements in presence of ice-cold alcohol characterized color that would influency the acceptability of products (Krichen et al., 2018). Studies on the toxic effects of alcoholic OnGAGs deserve be analyzed using, for instance, fish semen (Pereira et al., 2020; Nascimento et al., 2021).

Conclusion

Nile tilapia (*Oreochromis niloticus*) skin digested with papain followed by ice-cold alcohol alternative precipitation revealed uronic acid rich glycosaminoglycans of relatively reduced charge and molecular size of ~ 40 kDa, but dermatan sulfate-unrelated extract based on infrared analysis. Hyaluronic acid-free extract exerced *in vitro* antioxidant effects considerating all the *in vitro* assays tested, but there was a preponderant action for ferreous ion chelanting as EDTA standard. However, finding demonstrated that the presence of contaminants was not discard in material and would contribute for coantioxidation. Our study revealed that the alternative use of ice-cold alcohol

yielded antioxidant glycosaminoglycans in a relatively lower cost, but with a level of purity not appreciable for biotecnological applications and their toxicity must be evaluated in future.

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