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ANTIOXIDANT GLYCOSAMINOGLYCANS EXTRACTED FROM THE SKIN OF "TAMBATINGA", A HYBRID FISH

GLICOSAMINOGLICANOS ANTIOXIDANTES EXTRAÍDOS DA PELE DE TAMBATINGA, UM PEIXE HÍBRIDO

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Abstract Market of "tambatinga" (*Colossoma macropomum* × *Piaractus brachypomus*) has grown in Brazil, but its filleting residue glycosaminoglycans (TbGAGs) are unexplored. This study aimed at analyzing TbGAGs, extracted from skin, on their structural and antioxidant properties. Quantified fresh skin was dehydrated for extraction with papain, in 100mM sodium acetate buffer (pH5)+5mM cysteine/EDTA. Physical-chemical features of TbGAGs were examined by electrophoreses (agarose/polyacrylamide gels) *vs.* standard GAGs stained with toluidine blue or Stains-All; and then structurally by infrared technique. TbGAGs were *in vitro* tested, for antioxidant effects, on the DPPH, total antioxidant capacity (TAC) and ferrous ion chelating assays *vs*. BHT, ascorbic acid and EDTA, respectively. Results of fresh skin (~3.95% fish^{-1}) and TbGAGs extraction (0.37±0.03%) presumed a dermatan (~40kDa) with structural complexity from the molecular analyses. Regarding assays, TbGAGs were concentratio-dependent antioxidants, but there was preponderance on the TAC, although less effective than respective synthetics. Thus, "tambatinga" has skin antioxidant GAGs and analyses from progenitors suggest future studies.

Key Words: freshwater fish, residue, sulfated polymers, oxidation.

Resumo Comércio de "tambatinga" (*Colossoma macropomum* × *Piaractus brachypomus*) no Brasil tem crescido, porém são inexplorados seus glicosaminoglicanos (TbGAGs) de resíduo da filetagem. Este estudo objetivou analisar propriedades estruturais e antioxidantes de TbGAGs de pele extraídos. Pele quantificada fresca foi desidratada para extração com papaína, em tampão acetato de sódio 100mM (pH5)+cisteína/EDTA 5mM. Foram examinadas de TbGAGs as características físico-químicas por eletroforeses (géis de agarose/poliacrilamida) *vs*. glicosaminoglicanos padrões corados com azul de toluidina ou "Stains-All"; e, posteriormente, por técnica de infravermelho estruturalmente. Foram testados *in vitro* os TbGAGs, para efeitos antioxidantes, sobre ensaios DPPH, capacidade antioxidante total (CAT) e quelação de íon ferroso *vs*. BHT, ácido ascóbico e EDTA, respectivamente. Resultados presumiram, de pele fresca (~3,95% peixe^{-1}) e extração de TbGAGs (0,37±0,03%), um dermatam (~40kDa) com complexidade estrutural das análises moleculares. Quanto aos ensaios, TbGAGs foram antioxidantes dependente de concentração, porém houve preponderância sobre a CAT, embora menos eficazes que sintéticos respectivos. Assim, tambatinga possui GAGs antioxidantes de pele e análises sugerem estudos futuros dos progenitores.

Palavras-chave: peixe dulcícola, resíduo, polímeros sulfatados, oxidação.

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Introduction

In the last decades, the commercial activity of native fishes in Brazil has grown substancially and species from the Paraná, São Francisco and Amazon rivers have already been the subject of technological and zootecnical studies aimed at cultivation and development of production chains (Moreira et al., 2001). Between the 80s and 90s, some fish species were introduced to commercial cultivation, such as "pacu" (*Piaractus mesopotamicus* Holmberg, 1887), "tambaqui" (*Colossoma macropomum* Cuvier, 1818), "pirapitinga" (*Piaractus brachypomus* G. Cuvier, 1818), "curimatã" (*Prochilodus* spp.), "piaus" (*Leporinus* spp.) and some hybrids ("tambacu" and "paqui") (Moreira et al., 2001), since fish is a food rich in proteins, lipids, vitamins and carbohydrates that are beneficial to health as diseases-reducing constituints (*e.g.*, cancer and cardiovascular) (Ogawa & Maia, 1999). On the other hand, both industrial and artisanal activities generate solid residues (*e.g.*, fins, heads, gill, spine, scales viscera and skin) causing harmfuls to the environment and opportunities to extract bioactive compounds (*e.g.*, collagen, peptides and sulfated polysaccharides) could take advantage in biotechnology (Oetterer et al., 2014) and for the biochemical knowledge of the Brazilian ichthyofauna (Nogueira et al., 2019; Santiago et al., 2024).

Innumerable sulfated polysaccharides have already been isolated and studied from diverse living-organisms (Pomin & Mourão, 2008; Silva et al., 2012; Arima et al., 2013; Ai et al., 2023). Animals (vertebrates and invertebrates)-derived sulfated polysaccharides (called glycosaminoglycans-GAGs) are evolutionarily conserved (Medeiros et al., 2000; Gandhi & Mancera, 2008) and those found in inedible parts of fishes are of promising use compared to commercially-available GAGs that have infection risks by viruses or prions (Badri et al., 2018). On a glycomic basis, GAGs are highly heterogeneous polyanionics of complex composition varying in sulfation patterns, molecular masses, different linkages and specific saccharide sequences; and polymeric chains associated to a core protein form proteoglycan-structures, playing a biomechanical role on cell-surfaces in the matrix (Badri et al., 2018). Well-defined GAGs classes are heparin (HEP, highly charged), chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA, nonsulfated), keratan sulfate and heparan sulfate (HS) (Gandhi & Mancera, 2008), of which HEP (bovine/porcine lung and intestine), HA (microbial fermentation) and CS (bovine/porcine trachea and shark cartilage) have high global demand (Badri et al., 2018) due to their vast uses as anticoagulant/antithrombotic (Nader et al., 2001), cosmetic (Badri et al., 2018) and anti-inflammatory (Volpi, 2011), respectively. GAGs are composed by anionic chains of linear molecules 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 amounts depending on the organism and extraction approaches (Gandhi & Mancera, 2008; Badri et al., 2018), but, structurally, marine GAGs are different to those present in mammal GAGs (Valcarcel et al., 2017).

Fish filleting produces a large amount of underutilized by-products containing different (composition/bioactivities) GAGs (Arima et al., 2013; Jridi et al., 2019; Nogueira et al., 2019; Gavva et al., 2020; Moura et al., 2021). Skin-derived DSs have been preponderantly identified in a variety of fishes displaying anticoagulant actions, including marine (Dellias et al., 2004; Mansour et al., 2009; Rodrigues et al., 2012) and freshwater (Souza et al., 2007; Rodrigues et al., 2009, 2011; Salles et al., 2017) species, but are few understood as antioxidants. So, GAGs-based antioxidants were found in some inedible parts of a limited number of fishes, such as in cuttlefish *Sepia officinalis* Linnaeus (1758) skin/muscle by Jridi et al. (2019), in Nile tilapia *Oreochromis niloticus* Linnaeus (1758) skin by Nascimento et al. (2021); and, recently, in Bocachico *Prochilodus brevis* Steindachner (1875) gill by Santiago et al. (2024). Aquatic-source antioxidants arise as natural agents to synthetic compounds (*e.g.*, butylatedhydroxytoluene-BHT) known as toxics (Panicker et al., 2014); and to alternatively inhibit the free radical generation from endogenous or exogenous

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origins improving the defense biological system against more complex health problems (*e.g.*, cancer, atherosclerosis, DNA damage and Alzheimer) (Barbosa et al., 210).

Aquaculture of rheophilic fishes has attention gained in Brazilian market and the technological development of hybrids potentially creates new consumption chains for generation of high value-added products (Moreira et al., 2001). The production of "tambatinga", a hybrid fish origined from *C. macropomum* female × *P. brachypomus* male, has already been popularly found in local market of Amazon' states as commercial option of animal-protein source to the population on the basis of skin-free fillet or with it. Studies on this rheophilic hybrid are restricted and based on its cultivation by different systems related to animal performance, potential for growth in agribusiness and phenotypic aspects (Silva et al., 2013). No report on the use of its filleting-discarded materials rich in GAGs has been documented so far.

Fish skin is an external organ formed by epidermis, with mucous glands, and dermis, predominantly fibrous, with average fresh yield of 7.5% (w w^{-1}) for teleost species. It has an extracellular-matrix constituted by collagens, multi-adhesive glycoproteins, elastin and GAGs secreted by epidermal glands ("called mucopolysaccharides) that isolates the body of the fish and protects it against pathogenic agents (Moreira et al., 2001). The present study was designed to use the skin of "tambatinga" as a first-matter for GAGs (TbGAGs) extraction and then provide information on DS-type GAG by biochemical and structural analyses. Furthermore, the antioxidant potential of the TbGAGs was *in vitro* assayed, contribuing to the first report on the composition and bioactivity of GAGs, present in filleting-discarded skin samples, from freshwater hybrid fish produced between *C. macropomum* × *P. brachypomus* in Brazil.

Material and Methods

"tambatinga" fresh skin samples

The fresh skin samples of the hybrid fish were obtained from an artisanal processing unit, located in the state of Ceará, Brazil. According to local information, the fishes came from Maranhão state, Brazil, and were fattened in green-water cultivation and feed with commercial diet. As can be illustrated in figure 1, a total of eleven specimens of "tambatinga" stored on ice (1:1.1 kg ratio) at isothermal boxes intended for filleting operation were locally separated and then the individuals measured and weighed using a rudimentary ichthyometer and a commercial balance on a 1 g precision scale, respectively (Table 1), whose total fish biomass was ~7.970 kg after weighing.

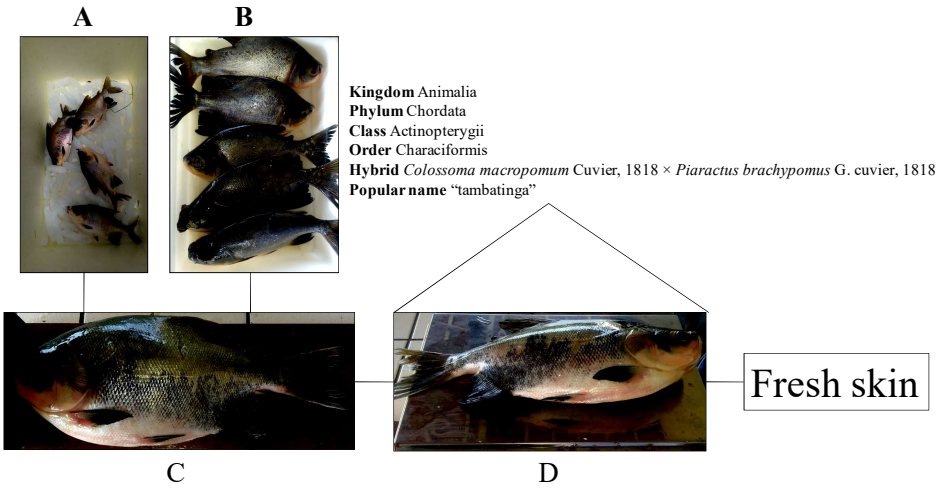

Figure 1. Steps of the procedures performed at an artisanal unit of "tambatinga" filleting. Storage in ice (A), separation (B), measuring (C), weighing (D) and fresh skin removal.

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Table 1. Biometric values of "tambatinga" obtained at artisanal processing unit, Ceará state, Brazil.

Specimen (n)	Total length (cm)	Height (cm)	Weight (g)
11	35.57 ± 2.43	13.34 ± 0.32	724.54 ± 141.99

After biometric technique, each fish was manually washed and then submitted to scaling, evisceration and skin removed with knife (Rodrigues et al., 2009, 2011, 2017) for a total yielding of ~ 43.50 g from the muscle-free fresh tissue. In the Marine Biochemical (MarBio) laboratory located at the Aquaculture Biotechnology Center belonging to the Department of Fisheries Engineering of the Federal University of Ceará (FUC), Brazil, the final yield (%) of fresh skin was based on percentage (w w^{-1}) of the total fish biomass (Moreira et al., 2001). The skin tissue separated from each "tambatinga" body was extensively washed with destillated water for cleaning and then the material was dehydrated in an oven under air circulation (45°C, 48 h) and, subsequently, kept in a closed recipient until TbGAGs extraction (Santiago et al., 2024). The "tambatinga" fish was authorized through our registration with SISGEN/FUC (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).

Extraction of TbGAGs from skin samples and metachromasia analysis

From skin tissue manually obtained and dehydrated (Figure 1), it was further weighed, cut into small peaces and samples of 10 g were incubated in a thermostatic bath with proteolysis by the unspecific action of the crude papain in a digestion rate of 10% (w w^{-1}) in 100 mM sodium acetate buffer (pH 5.0) added of 5 mM EDTA, and 5 mM cysteine, and the extraction process followed at 60°C with duration of 24 h (Rodrigues et al., 2009, 2011), as shown in figure 2*a*.

After incubation period, the mixture was continually filtered using a nylon net and the supernatants were saved and centrifugated (9.560 × *g* for 20 min). TbGAGs that were present in medium were precipitated with 10 mL of 10% cetylpyridinium chloride (CPC) solution at room temperature (25-28°C) for 24 h. The mixtures were then centrifuged at 9.560 × *g* for 20 min. The *pellet*s containing the TbGAGs were washed with 100 mL of 0.05% CPC solution, dissolved (under mechanical stirring for 20 min) in 100 mL of a 2 M NaCl: ethanol (100:15 ratio, v:v) solution, and then sujected to precipitation (24 h, 4°C) with addition of 100 mL of 92.8% ice-cold 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 (9.560 × *g* for 20 min) among the steps, the material was dried using an air drying oven (60°C, 24 h) to obtain the raw mass of TbGAGs. Finally, the extraction yield was calculated according to Eq. (*1*) and expressed as the percentage (w w^{-1} %, n = 3) of the dehydrated matter (g).

$$\text{Yield (\%)} = \text{TbGAGs WDM}^{-1} \times 100 \qquad (1)$$

where: TbGAGs the dry weight of crude GAGs and WDM the raw weight of dehydrated matter.

From TbGAGs sample, a solution was previously prepared and two aliquots (15 or 30 µg, w v^{-1}) were checked for metachromasia in the presence of 1,9-dimethylmetilene (DMB) blue dye used as an indicator of colorimetric reaction based on complex formed (Farndale et al. 1976). The assay was performed following Santiago et al. (2024), in triplicate, using glass tubes and the violet coloring would reveal to be specific for sulfated GAGs in the test sample (Figure 2*b*) (Gavva et al., 2020).

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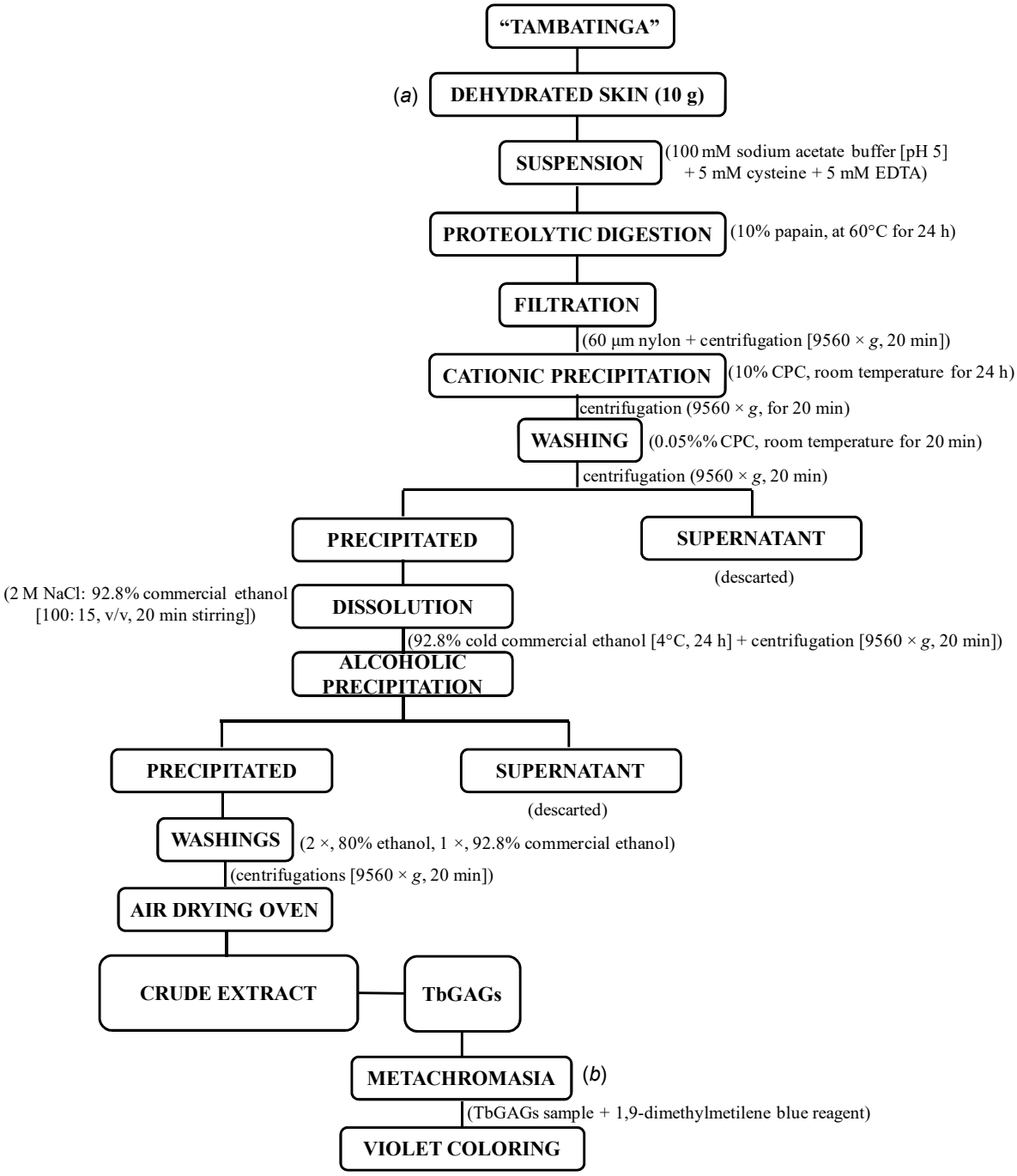

Figure 2. Scheme of obtaining (*a*) and metachromasia (*b*) analysis of GAGs from "tambatinga" skin.

Electrophoretic analyses

By agarose gel

This system initially characterized the TbGAGs from skin samples by charge density as described by Dietrich & Dietrich (1976). An aliquot of 6 µL of the test sample (~ 30 µg) was applied to a 0.5% agarose gel prepared in 0.05 M 1,3-acetate diaminopropane buffer at pH 9.0 and the run was carried out at constant voltage (100 V, 1 h). After that, the gel was treated with 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for ~ 24 h to fix the TbGAGs and then it was dehydrated using an air drying oven (55°C, ~ 6 h).

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By polyacrylamide gel (PAGE)

PAGE examined the TbGAGs from skin samples by apparent molecular mass distribution. Similary, an aliquot of 6 µL of the test sample (~ 30 µg) applied to a 6% polyacrylamide gel using 0.02 M Tris/HCl buffer at pH 8.6 and the run was performed at 500 mA for 1 h based on Santiago et al. (2024).

From both gels, the TbGAGs were stained with 0.1% toluidine blue or Stains-All cationic reagent for 30 min-24 h. After that, the gels were destained 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 applied as standards (Dietrich & Dietrich, 1976; Andrade et al., 2017).

Fourier Transform Infrared (FT-IR) spectroscopy

The TbGAGs from skin samples were structurally analyzed by FT-IR and the spectrum was obtained using a spectrometer (IRPrestige-21 Shimadzu, Japan). For measurement, 10 mg of the test sample were previously prepared in potassium bromide (KBr) *pellets* and the values recorded at a resolution profile of 4 cm^{-1}, with 64 scans min^{-1} at 500-4000 cm^{-1}.

***In vitro* antioxidant assays**

The test sample (TbGAGs) was examined from 0.125 to 4.0 mg mL^{-1} for antioxidant effects at the Seaweed II laboratory located at the Department of Biochemistry and Molecular Biology, FUC, and the *in vitro* assays are described below.

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

The effect of TbGAGs to reduce DPPH was performed according to Blois (1958), with some modifications. In this assay, different concentrations of TbGAGs 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 (%) = $[A_0 - (A - A_b)/A_0] \times 100$, where A_0 = DPPH without sample; A = sample + DPPH; and A_b= sample without DPPH.

Total antioxidant capacity (TAC)

This assay was performed by the formation of the phosphomolybdate complex, based on Prieto et al. (1999). TbGAGs were added to a solution containing ammonium molybdate (4 mM), sulfuric acid (0.6 M), and sodium phosphate (28 mM), 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 (AA) 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 TbGAGs 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.

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

Statistical analyses

All experimental data were expressed as mean ± standard deviation (n = 3). The *in vitro* antioxidant tests were analyzed by one-way ANOVA, followed by Tukey' test, with $p < 0.05$ as statistically significant. The graphical representations of yield and FT-IR were constructed using the GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com) and the Origin software version 8.0 as the Statistical Analysis Software (USA), respectively.

Results and Discussion

"tambatinga" fresh skin yield

The available amount of fresh skin tissue removed and further weighed from eleven filleted individuals of "tambatinga" fish resulted in an important raw material for TbGAGs biochemical analysis. Of all the fish biomass, it was observed that the muscle-free fresh tissue yielded ~ 43.50 g of the individuals sampled from 490 to 960 g for weight, from 33.50 to 41.00 cm for lenght and from 11.50 to 15.00 cm for height, respectively (Table 1). These parameters and the human operation would also determine the fish residue yield, including skin removal (Moreira et al., 2001). On a sensorial basis (Figure 1), skin from "tambatinga" showed an intense and bright body coloring from gradient gray to the original characteristics inherited from progenitors (Moreira et al., 2001) and a firmness of the tissue attesting to its freshness when stored in ice, therefore, a undegraded first-matter and suitable for consumption of fillet with skin (Ogawa & Maia, 1999).

On a mass-to-mass basis, the percentage of fresh skin was ~ 0.54% from the "tambatinga" global biomass used and, per fish, was estimated to be ~ 3.95% of the respective tissue. This fresh skin yield was lowest than for other teleost fishes that varied in this tissue from 5 to 10% (w w^{-1}) depending on the species and body shape (Moreira et al., 2001). So, "tambatinga" fish has a smaller head compared to its progenitors (Silva et al., 2013), which could lead to a greater fillet yield and, consequently, skin residue for GAGs extraction (Arima et al., 2013). However, due to great variation in fish sampling as already mentioned, a more critical study regarding its skin yield is need to support our partial conclusions.

TbGAGs extraction yield

Use of dehydrated "tambatinga"skin allowed us to reduce the constitution water (Ogawa & Maia, 1999) to enzimatically obtain its matrix GAGs (Dellias et al., 2004; Souza et al., 2007; Mansour et al., 2009; Rodrigues et al., 2009, 2011, 2012; Salles et al., 2017) because *in natura* tissues would lead to relatively lower GAGs yields (Nogueira et al., 2019). Thus, skin samples from "tambatinga", when essentially digested with crude papain (60°C, 24 h), followed by both CPC and ice-cold ethanol precipitations, resulted in a crude material rich in GAGs from the tritured fish tissue, as shown in figure 3.

Skin by-product by fish industry has low market-value in fillet (Moreira et al., 2001), but as a valuable source in GAGs compared to other species (Rodrigues et al., 2009, 2012; Arima et al., 2013; Jridi et al., 2019). In this study, the "tambatinga" filleting-discarded skin contained 0.37 ± 0.03% (w w^{-1}) of crude GAGs extract from the dehydrated tissue (Figure 3A), as a satisfactory amount due to high demand of these molecules in international market (Volpi, 2011; Badri et al., 2018). On a raw material-extraction basis, studies on skins (~ 1%, Mansour el. al., 2009; 1.6%, Jridi et al., 2019), scales (0.86%, Moura et al., 2021), visceras (0.18%-pacu and 0.15%-tilapia, Nogueira

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et al., 2019), muscles (8.6%, Jridi et al., 2019) and gills (1.54 ± 0.02%, Santiago et al., 2024) have also been reported as GAGs-important sources. According to Gavva et al. (2020), the fish (*Labeo rohita* / *P. brachypomus*) part (head) there is a difficult to its separation from bony material due to tissue adhering remaining undigested for GAGs obtaining.

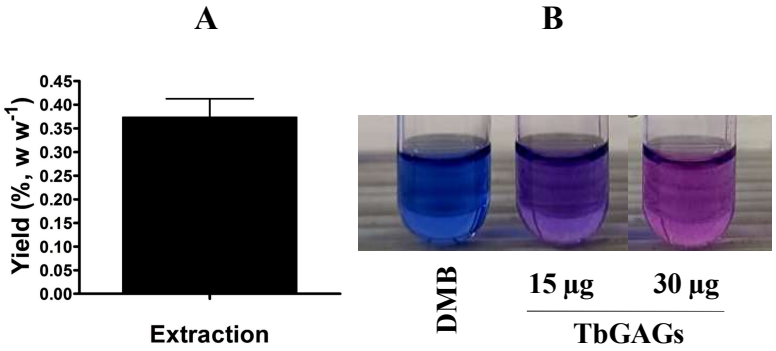

Figure 3. Yield (A) and metachromasia (B) of GAGs extracted from "tambatinga" skin. Extraction yield expressed as the percentage of the dehydrated tissue; Violet property revealing sulfated GAGs.

The "tambatinga" skin-derived GAGs were, at least, 4.1-fold more abundant than in the skin (0.09-0.22%, w w^{-1}) from Nile tilapia *O. niloticus* (Rodrigues et al., 2011, 2017; Nascimento et al., 2021), but yielded 2.70-fold lower than from skins (~ 1%, w w^{-1}) of the ray *Raja radula* (Mansour et al., 2009) and of the cuttlefish *S. officinalis* (Jridi et al., 2019) purchased from fish markets, Tunisia. Furthemore, GAGs represented 7.53 ± 0.79 mg g^{-1} in the dehydrated "tambatinga" skin, an amount of GAGs about 15.43-fold higher than in the *L. rohita* / *P. brachypomus* heads (Gavva et al., 2020), demonstrating that the GAGs vary with other parts of the fishes (Arima et al., 2013). Some authors have associated to GAGs implications related to the aquatic biology of the fishes (Souza et al., 2007; Rodrigues et al., 2012). However, there are limitations to extract GAGs from by-products of the fish industry and investigate their biological roles due to variety of living organisms, such as by extraction time reducing and increasing the process yield of these materials at low-cost, standartization and adulteration-free market (Badri et al., 2018; Gavva et al., 2020). Structural heterogeneity among GAGs from different aquatic species also difficults the biomolecular studies (Medeiros et al., 2000; Souza et al., 2007; Pomin & Mourão, 2008; Arima et al., 2013; Valcarcel et al., 2017).

By metachromatic analysis was demonstrated that the TbGAGs from the skin samples exibited, in the presence of DMB dye by Farndale et al. (1976)' method, a TbGAGs-DMB binding property as showed by violet reaction doubling the amount of the analyzed polymer sample (Figure 3B) (Gavva et al., 2020). In fact, it was revealed the occurence for sulfated GAGs in the "tambatinga" skin extracellular-matrix, since that it is constituded by glands that secrete mucopolysaccharides in the connective tissue as an important role for the natural protection of the bony fishes (Moreira et al., 2001).

Studies on the biochemical knowledge of the species became challenger (Badri et al., 2018; Gavva et al., 2020), not only on the native fishes in Brazil (Souza et al., 2007; Nogueira et al., 2019; Santiago et al., 2024), but also in development of better strategies of cultivation of these aquatic animals at commercial level (Moreira et al., 2001; Rodrigues et al., 2011; Silva et al., 2013). Based on these results, TbGAGs from skin samples were further identified by electrophoretic and FT-IR analyses.

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Identification of the TbGAGs from skin samples

In order to identify the chemical species of GAGs presents in the skin of "tambatinga", electrophoretic (agarose gel / PAGE) analyses were performed (Figure 4). Caracterization by agarose gel of the TbGAGs stained with toluidine blue suggested electrophoretic band migrating as mammalian DS (Figure 4A), revealing the occurence of unique GAG in the skin of the hybrid fish ("tambatinga"). DS has been presumably expressed in the skin of freshwater (*Potamotrygnon motoro* by Dellias et al. (2004); *O. niloticus* by Rodrigues et al. (2011, 2017), Salles et al. (2017) and Nascimento et al. (2021); *Electrophorus electricus* by Souza et al. (2007)) and marine (*Dasyatis americana*, *D. guttata* and *Aetobatus narinari* by Dellias et al. (2004); *C. chrysurus* by Rodrigues et al. (2012)) fishes species. Recently, Santiago et al. (2024) uniquely found CS in the gill of *P. brevis*.

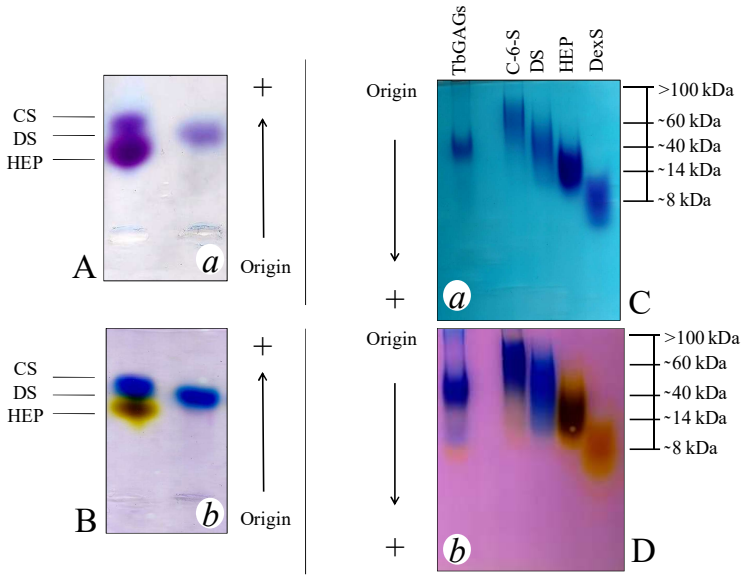

Figure 4. Agarose (A and B) / polyacrylamide (C and D) gels electrophoreses of GAGs extracted from "tambatinga skin and standards chondroitin-6-sulfate (C-6-S,~ 60 kDa) , dermatan sulfate (DS,~ 40 kDa), dextran sulfate (DexS,~ 8 kDa) and/or heparin (HEP,~ 15 kDa) present on gels were stained with 0.1%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

DS-type GAGs in the "tambatinga" skin sample showed to be a highly charged homogeneous band and its characterization occured due to interaction of the diamine buffer with the sulfate groups similar to mammalian GAGs (Dietrich & Dietrich, 1976). This profile found in the skin of "tambatinga" was relevant, since that other authors already found GAGs population from different fish tissue preparations, including skin (DS and HS in *Cyprinus carpio* by Rodrigues et al. (2009); DS and HA in *R. radula* by Mansour et al. (2009)), viscera (CS, DS and HS in *P. mesopotamicus* by Nogueira et al., 2019), scale (CS and DS in *O. niloticus* by Moura et al., 2021) and heads (CS, DS and HS in *L. rohita* / *P. brachypomus* by Gavva et al., 2020).

Stains-All-treated agarose gel (Figure 4B) showed, at initial level, the absence of HA in the GAGs preparation of "tambatinga" skin and the complex was similar to charge/mass from DS standard visualized in a navy blue coloring, because the respective dye reacted with high sensitively to uronic acid residues present in animal GAGs (Santiago et al., 2024).

Molecular mass analysis by PAGE characterized the TbGAGs from skin samples as a DS of ~ 40 kDa when by toluidine blue treatment (Figure 4C), as also estimated to DSs from skins of *O. niloticus* (Rodrigues et al., 2017; Salles et al., 2017) and of *E. electricus* (Souza et al., 2007), respectively. Differences in molecular masses among DSs have been found in the fish skin (Mansour et al., 2009). Staining with Stains-All alone allowed us to analysis the "tambatinga" skin GAGs sample based on standard quality (Figure 4D). Clearly, contaminants could be noted, since

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the sample contained other blots stained in different colors (navy blue and yellow) (Andrade et al., 2017), suggesting the presence of DNA and/or GAGs-complexed matrix proteins (or peptides) in the unfractionated preparation (Gandhi & Mancera, 2008), but this profile confirmed the HA-free skin based on Santiago et al. (2024). Results were favorable to the quality control by transformation industry (Badri et al., 2018; Volpi, 2011; Oetterer et al., 2014) and further fractions of DSs from "tambatinga" skin deserve to be isolated in the pure form (Rodrigues et al., 2011; Salles et al., 2017).

Structural analysis by FT-IR spectroscopy

Information on the structural features of the TbGAGs from skin samples were obtained by FT-IR spectroscopy, in kBr *pellets*, at 500-4000 cm^{-1} (Figure 5). This approach showed to be useful comparing with other natural sources (Pomin & Mourão, 2008; Silva et al., 2012; Ai et al., 2023) to identify fish residues-derived sulfated GAGs (Mansour et al., 2009; Jridi et al., 2019; Santiago et al., 2024).

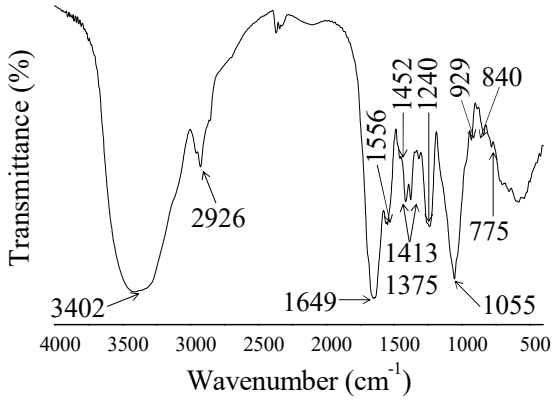

Figure 5. FT-IR spectrum of the "tambatinga" GAGs obtained from skin samples at 500-4000 cm^{-1}.

From spectral window it was qualitatively found the presence of amides A, amide-I and amide-II at the wavenumbers of 3402, 1649 and 1556 cm^{-1}, respectively, typical characteristics for amide groups based on cuttlefish skin-isolated GAGs (Jridi et al. (2019). Bands of FT-IR at 2926, 1375, 1452 and 1055 cm^{-1} were related to CH_2 group, uronic acids (O-C=O) and C-O-C, consequently, which evidenced the acidic region (carboxylated groups) from the sample (Jridi et al., 2019; Santiago et al., 2024). By contrast, other ring vibrations (osidic cycles) not assigned or absence of signals (like 1000-1100 cm^{-1}) on the spectrum were not recorded in this study with basis on GAGs extracted from the skin of the marine ray *R. radula* (Mansour et al., 2009).

Regarding sulfation-related signal, an intense absorption band at 1240 cm^{-1} corresponded to the total sulfation (Silva et al., 2012; Jridi et al., 2019; Santiago et al., 2024) and confirmed the molecular analyses by metachromasia and electrophoreses (Figures 3B, 4). In addition, that presence of band at 1556 cm^{-1} was attributed to C=O stretching in acetyl groups, suggesting acetylated galactosamine residues (Mansour et al., 2009). GAGs-related peaks at 929 and 775 cm^{-1} were also recorded in "tambatinga" skin (Jridi et al., 2019; Santiago et al., 2024).

Conflicting result was to the interpretation on the GAG species present in the "tambatinga" skin sample (Jridi et al., 2019) because the signal at 840 cm^{-1} suffered a chemical shift, since that around 820 (DS) or 850 (CS) cm^{-1} has been identified two different types of GAGs (Mansour et al., 2009; Santiago et al., 2024) as copolymeric structure quite abundant in nature as discussed by Gavva et al.

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(2020). Due to the sulfate groups as substituints (C-O-S) between regions in the spectrum (Figure 5), it was presumed the existence of DS in the skin of "tambatinga" as supported by molecular analyses (Figure 4) (Souza et al., 2007; Mansour et al., 2009; Rodrigues et al., 2011, 2012; 2017; Salles et al., 2017; Nascimento et al., 2021). In-depth studies on the structural biology of the TbGAGs are still need comparing the progenitors of this fish (Medeiros et al., 2000; Dellias et al., 2004; Pomin & Mourão, 2008; Nogueira et al., 2019).

In vitro analysis of the TbGAGs from skin samples on oxidant tests

The results of the *in vitro* tests, using the TbGAGs from skin samples as natural inhibitors and known standards (BHT, ascorbic acid and EDTA) on oxidative processes, are listed in table 2, and to evaluate their antioxidant mechanisms considerating on the two stages: initiation and propagation (Silva et al., 2012; Santiago et al., 2024).

DPPH-scavenging ability

This assay was to firstly examine the TbGAGs from skin samples as scavenged of the free radicals based on absorbance reduced by polymer (Blois,1958). TbGAGs inhibited, by this *in vitro* test, in a dose-dependent manner at range concentration (0.125 to 4 mg mL^{-1}), from 6.58±0.15 to 12.48±0.11% the oxidant reaction, but did not achieve the same potency than the BHT synthetic, on the same weight basis (at 4 mg mL^{-1}), which exhibited ($p < 0.05$) an *in vitro* antioxidant action (94.15±0.25%) of, at least, about 7.54-fold higher than sample (Table 2).

Table 2. Effects of "tambatinga" GAGs from skin samples on DPPH, TAC and FIC assays.

Concentration (mg mL^{-1})	DPPH (%)	FIC (%)	TCA (%)
0.125	7.05 ± 0.44^{a}	1.92 ± 0.40^{a}	1.73 ± 0.11^{a}
0.25	6.58 ± 0.15^{a}	6.99 ± 0.28^{b}	4.37 ± 0.48^{b}
0.5	6.63 ± 0.18^{a}	14.57 ± 0.00^{c}	8.41 ± 0.27^{b}
1	7.21 ± 0.27^{a}	28.11 ± 0.07^{d}	15.26 ± 0.48^{c}
2	10.10 ± 0.15^{b}	38.25 ± 0.57^{e}	30.24 ± 0.06^{d}
4	12.48 ± 0.11^{b}	39.73 ± 0.23^{e}	49.16 ± 0.00^{e}
EDTA (4 mg mL^{-1})	94.15 ± 0.25^{c}	-	-
EDTA 4 mg mL^{-1}	-	99.56 ± 0.00^{f}	-
Ascorbic acid (0.4 mg mL^{-1})	-	-	99.77 ± 0.00^{f}

Different letters indicate significant differences at level of 5% (ANOVA, Tukey' test, $p < 0.05$).

GAGs extracted from different fish-filleting residues can manifest variability of effects by the DPPH assay. Studies performed by Jridi et al. (2019) revealed that the cuttlefish *S. officinalis* skin and muscle from Tunisia market contained antioxidant GAGs, inhibiting by 60 and 65% at 3 and 5 mg mL^{-1}, respectively, by the DPPH method. Nascimento et al. (2021) discovered that the GAGs extracted from skin of *O. niloticus* cultured were capable of demonstrating a maximal potential by 30.26% at 4 mg mL^{-1} by the same assay.

By contrast, such property by DPPH method was not find for GAGs extracted from gills of *P. brevis* by Santiago et al. (2024), when from cultured fishes. These findings attested that the scavenge potential vary with the species, origin of residue and type of GAG acting as proton-donating substrate in the reducing power like BHT property.

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TAC ability

Antioxidant property of the TbGAGs from skin samples by the TCA assay revealed as reducing agents of molybdenum VI to molybdenum V (Prieto et al., 1999). In table 2, TbGAGs, at different concentrations (0.125 to 4 mg mL^{-1}), reacted as a concentration-dependent agent, but revealing a maximum effect by 49.16 ± 0.00% at highest dose ($p < 0.05$). Furthermore, the reference synthetic (ascorbic acid) showed to be higher potentially than sample on the same concentration level, since that led to a double of effectively (%).

From same assay, Nascimento et al. (2021) concluded that the Nile tilapia (*O. niloticus*) GAGs from skin samples inhibited the formation of complex phosphomolybdates with an effectively only of 25.21 ± 0.64% at 4 mg mL^{-1}. According to Santiago et al. (2024), gills from *P. brevis* were a modest source in antioxitant GAGs, reducing by 9.77 ± 0.23% at 4 mg mL^{-1} by the TAC assay, therefore, ~ 5.03-fold lower than the action found in the current study.

Both DPPH and TAC assays indicated that the TbGAGs from skin samples modified the initiation stage during the *in vitro* oxidant events (Silva et al., 2012). In fact, this mechanism by TbGAGs was preponderant by the TAC assay (~3.93-fold higher) preventing the free-radical oxidation from these systems through a chain reaction, therefore, taken advantage of the functionalities for biotechnological use (Nascimento et al., 2021).

FIC ability

TbGAGs from skin samples on the FIC ability was evaluated by disruption of the Fe^{2+}-ferrozine complex visualized by decrease of the purple coloring (Chew et al., 2008). As shown in table 2, at different doses, TbGAGs exhibited FIC power with increase of concentration (0.125 to 4 mg mL^{-1}) from 1.92 ± 0.40 to 39.73 ± 0.23%, respectively ($p < 0.05$), but with a difference reaching only 37.81% within doses. However, EDTA, as a synthetic chelating agent, reduced the process by 99.56 ± 0.00% (at 4 mg mL^{-1}, $p < 0.05$) and its action was more effective at ~ 2.50-fold higher than test sample. This property indicated that the TbGAGs had sulfation sites capable of acting on the propagation phase, but less potent than initiation (Table 2).

To data, GAGs enzymatically extracted from skins or muscle of cuttlefish *S. officinalis* (Jridi et al. (2019) and of Nile tilapia *O. niloticus* (Nascimento et al., 2021) also demonstrated FIC abilities, whose inhibitions were > 90 (at 1 mg mL^{-1}) and 32.22 ± 0.10% (at 2 mg mL^{-1}), respectively. Santiago et al. (2024), studying the PbGAGs from gill samples, observed that they exerted a greater FIC effect only at 2 and 4 mg mL^{-1}, with inhibition rates of 20.61 ± 0.41 and 20.02 ± 0.29%, respectively.

It has been reported that galacturonic acid in GAGs structure displays an important role for the property of FIC (Jridi et al., 2019), as also verified in this study where the galactosamine residues-containing TbGAGs had antioxidant power by capturing ion ions (Figure 5 and Table 2). Mechanistic characterization of the TbGAGs by other tests could reveals the existence of molecular requiriments (sterospecific features, monossacaridic composition, glycosylation sites, aromericity and spartial conformation), not only as a charge/mass function, involved in their antioxidant responses (Valcarcel et al., 2017; Jridi et al., 2019).

As the fish skin GAGs contributes to the matrix structure and other functionalites related to animal biology (Moreira et al., 2001), GAGs-based antioxidants arise as novel natural ingredients of non-terrrestrial origin that integrate the fillet with skin which can also be consumed and taken advantage on their human health-related functionalities in order to reduce or prevent cell-oxidative risks against diverse systemic complications and, consequently, pathologies (*e.g.*, cancer and cardiovascular diseases) (Ogawa & Maia, 1999; Rodrigues et al., 2017).

This investigation demonstrated that the "tambatinga" (*C. macropomum* × *P. brachypomus*), a freshwater rheophilic hybrid fish, as an interesting source in antioxidant GAGs. Use of these GAGs could be explored, not only as a biomarker of biological/population function using its progenitors,

but also as bioactives in biotechnological processes on the basis of by-products discarded from fish filleting (Rodrigues et al., 2009, 2012). Our study brings, for the first time, biological implications on the biochemical of carbohydrates from skin discarded from a hybrid fish developed for the Brazilian market.

Conclusion

The "tambatinga" hybrid fish generates, through artisanal filleting, a quantity of fresh skin for studies on the chemical composition of glycosaminoglycans. This fish by-product subjected to papain-digestion followed by biochemical techniques presumed the presence of unique dermatan sulfate of ~40 kDa apparent molecular mass in the skin-matrix. The molecule showing by infrared spectroscopy structural complexity had antioxidant effects by all the three *in vitro* assays in which it deduced a preponderant mechanism by initiation phase of the oxidative process acting as proton-donating substrate possibly related to a high amount of galacturonic acid residues in its structure, but with actions less potent than the synthetic antioxidants. In-depth investigations suggest at molecular level of dermatan sulfate-associated biomarker regarding its biological function and possible hereditary implications from its progenitors.

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