Revista Brasileira de Ciências Agrárias
ISSN: 1981-1160
agrarias.prppg@ufrpe.br
Universidade Federal Rural de Pernambuco
Brasil

Caetana Salles, Tayane; Gurgel Rodrigues, José Ariévilo; Gomes Barcellos, Priscila; Farias Amaral, Gerlânia; Fernandes Araújo, Ianna Wivianne; de Souza Mourão, Paulo Antônio

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Revista Brasileira de Ciências Agrárias, vol. 12, núm. 1, 2017, pp. 98-104
Universidade Federal Rural de Pernambuco
Pernambuco, Brasil

Agrária - Revista Brasileira de Ciências Agrárias
ISSN (on line) 1981-0997
v.12, n.1, p.98-104, 2017
Recife, PE, UFRPE. www.agraria.ufrpe.br
DOI:10.5039/agraria.v12i1a5412
Protocolo 5412 - 29/03/2016 • Aprovado em 28/12/2016

Inhibition of thrombin generation by dermatan sulfate isolated from the skin of *Oreochromis niloticus*

Tayane Caetana Salles[1], José Ariévilo Gurgel Rodrigues[1], Priscila Gomes Barcellos[1], Gerlânia Farias Amaral[2], Ianna Wivianne Fernandes Araújo[2], Paulo Antônio de Souza Mourão[1]

[1] Universidade Federal do Rio de Janeiro, Hospital Universitário Clementino Fraga Filho Laboratório de Tecido Conjuntivo, Rua Rodolpho Paulo Rocco, 255, Ilha do Fundão, CEP 21941-590, Rio de Janeiro-RJ, Brasil. E-mail: tayanecaetana@yahoo.com.br; arieviloengpesca@yahoo.com.br; pricila_gomes17@yahoo.com.br; pmourao@hucff.ufrj.br
[2] Universidade Federal do Ceará, Campus do Pici, Departamento de Engenharia de Pesca, Laboratório de Processamento do Pescado, Av. Mister Hull, 2977, Bloco 840, Campus do Pici, CEP 60356-001, Fortaleza-CE, Brasil. Fortaleza. Brasil. E-mail: gerlaniafarias@hotmail.com; iwfaraujo@gmail.com

ABSTRACT

Oreochromis niloticus has skin anticoagulant glycosaminoglycans (GAGs), but their effects on thrombin generation (TG) are unknown. This study partially characterized skin GAGs and analyzed as inhibitors of TG. Papain-extraction yield of 0.1% contained two fractions separated by DEAE-cellulose chromatography, differing on charge density and carboxylated groups by a combination of agarose/polyacrylamide electrophoresis and sequential toluidine blue/stains-all staining, presenting molecular sizes ca. 40 kDa. Depolymerization of the fractions with chondroitin ABC lyase showed dermatan sulfate (DS) as the unique GAG by agarose analysis. Both activated partial thromboplastin time (APTT) and prothrombin time tests only showed anticoagulation by fractions and mammalian DS by APTT (0.61, 0.47 and 1.72 IU, respectively) against heparin (193 IU). Fractions acted concentration-dependent on both intrinsic/extrinsic pathways in TG using 60-fold diluted human plasma, with more than 50% inactivation (41.6 and 83.3 µg), whereas DS and heparin entirely abolished at low amounts. DS from *O. niloticus* skin blocks *in vitro* TG in human plasma.

Key words: Cichlidae; freshwater fish; sulfated glycans; thrombosis *in vitro*; waster

Inibição da geração de trombina por dermatam sulfato isolado da pele de Oreochromis niloticus

RESUMO

Oreochromis niloticus possui glicosaminoglicanos (GAGs) anticoagulantes de pele, porém desconhecidos são seus efeitos sobre geração de trombina (GT). Caracterizou-se parcialmente GAGs de pele e analisou-se como inibidores de GT. Rendimento de 0,1% da extração com papaína, conteve duas frações separadas por cromatografia de DEAE-celulose, e por combinação de eletroforese de agarose/poliacrilamida e coramento sequencial azul de toluidina/"stains-all", diferiram quanto à densidade da carga e grupos carboxilados, apresentando tamanhos moleculares ca. 40 kDa. Depolimerização das frações com condroitinase ABC mostrou, por análise em agarose, dermatam sulfato (DS) como GAG único. Ambos os testes do tempo de tromboplastina parcial ativada (TTPA) e do tempo de protrombina mostraram anticoagulação, das frações e DS de mamífero, somente pelo TTPA (0,61; 0,47 e 1,72 UI, respectivamente) contra ao da heparina (193 UI). Frações atuaram sobre ambas vias intrínsica/extrínsica dependente de concentração na GT usando plasma humano diluído 60 vezes, com inativação mais que 50% (41,6 e 83,3 µg), enquanto em quantidades baixas DS e heparina aboliram totalmente. DS da pele de *O. niloticus* bloqueia GT *in vitro* no plasma humano.

Palavras-chave: Cichlidae; peixe dulcícola; glicanos sulfatados; trombose *in vitro*; resíduo

Introduction

GAGs comprise a variable family of highly complex glycans produced from vertebrate and invertebrate tissues (Volpi & Maccari, 2002; Arima et al., 2013; Pomin, 2014; Sayan et al., 2016). They are heteropolysaccharides consisting of repeated units of alternating residues of *N*-acetylgalactosamine or glycosamine linked to a non-nitrogenated sugar, like uronic acid (D-glycuronic acid or L-iduronic acid) or a galactose, making themselves typical chains are known as mucopolysaccharides, and their association to proteoglycans form the matrix' principal elements playing several physiological and pathological events (Tovar et al., 2005; Volpi, 2011; Oliveira et al., 2015). Chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate and heparin (HEP) are the most GAGs found, varying not only the hexosamine and non-aminated sugar residue but also the degree and position of sulfated groups, as well as the type of glycoside bond. Hyaluronic acid does not contain sulfation and may occur freely in tissue (Volpi, 2011; Kang et al., 2015; Oliveira et al., 2015).

GAGs have been traditionally used as health food ingredients and medications (Volpi, 2011); however, the decontrolled fishing of sharks (Arima et al., 2013) and lobsters (Sayan et al., 2016) and the risk of viral contamination from the pig as another source of commercially-important GAGs (Mourão, 2015), as well as the price and limited supply of GAGs produced by fermentation from bacteria or extracted from avian (Arima et al., 2013) indicate the need to look for alternative sources of glycans with both economic and environmental benefits (Cardozo et al., 2007; Volpi, 2011).

GAGs from fish tissues have been isolated as a highly valuable platform for the valorization of residues obtained from food processing for the development of novel commercially-important biomaterials (Arima et al., 2013). DS has been predominantly found in the skin of several marine fish species than other GAGs types (Dellias et al., 2004; Souza et al., 2007; Mansour et al., 2009).

The anticoagulant potential of GAGs isolated from the skin of fishes has been still few studied. DSs isolated from the ventral skin of four Brazilian species of rays (*Dasyatis americana*, *D. gutatta, Aetobatus narinari* and *Potamotrygon motoro*), Dellias et al. (2004) reported only those from *D. americana* and *D. gutatta* exhibited anticoagulation mediated by HCII (IC_{50} = 1-3 mg mL^{-1}). This anticoagulant mechanism was also proposed for skins DSs from the electric eel *Electrophorus electricus* (Souza et al., 2007). Skin DS from the ray *Raja radula* showed a significant factor Xa inhibition (IC_{50} = 0.6 mg mL^{-1}) (Mansour et al., 2009). Teleost fishes also exhibited GAGs with *in vitro* anticoagulant actions (2-5 IU) (Rodrigues et al., 2009; Rodrigues et al., 2012; Krichen et al., 2017).

The employment of classical coagulation methods, such as the activated partial thromboplastin time (APTT) and the prothrombin time tests, indicate only a limited value on the total amount of thrombin formed. Because this limitation in terms of precision toward direct measurement of thrombin generation (TG) potential in a plasma sample, TG-based methods more precisely represent the haemostatic status (Castoldi & Rosing, 2011; Jun et al., 2014) and provide relevant information to analyze substitutes to HEP by preventing thrombosis *in vitro* (Glauser et al., 2009; Rodrigues et al., 2016), which it induces bleeding and thrombocytopenia (Mourão, 2015). No contribution of fishes GAGs concerning their effects on TG assays has been described so far.

Oreochromis niloticus (Linnaeus, 1758) is a popular freshwater fish in world aquaculture for meet human nutrition (Araújo et al., 2010). This African tropical species, belonging to the Cichlidae family, has greater growth performance in intensive systems and its commercial production to agribusiness had a rapid expansion in recent decades, and generating side-products from fish meat processing, such as skin tissue (Moreira et al., 2001). GAGs previously isolated from this fish species revealed anticoagulation (4-23 IU) less potent than HEP (193 IU) (Rodrigues et al., 2011).

The current study analyzed physical-chemical characteristics of complex GAGs present in the skin of *O. niloticus* by a combination of agarose/polyacrylamide gel electrophoresis and sequential staining with toluidine blue and stains-all; it also whether these GAGs inhibit an *in vitro* TG continuous system in 60-fold diluted human plasma.

Material and Methods

Fish skin samples and sulfated GAGs analyses

Skin tissue samples of *O. niloticus* were obtained from experimentally cultured specimens (Weigh: 495.6 ± 58.13 g; Total length: 29.90 ± 0.87 cm, n = 5) from the Farm of the Federal University of Ceará, Brazil. All the fishes were cultured in ponds (4 fishes.m^{2-1}) and fed (twice per day) with a commercial diet containing 28% crude protein at the end of the cultivation. The water quality parameters temperature (29 ± 2 °C), pH (7.5 ± 0.7), dissolved oxygen (5 ± 0.4 mg L^{-1}) and ammonia (0.01 ± 0.3 mg L^{-1}) showed normal levels for tilapia culture (Moreira et al., 2001; Araújo et al., 2010).

The dehydrated skin tissue samples (40 °C, 24 h) were conserved in plastic bags and then conducted at the Connective Tissue Laboratory, Federal University of Rio de Janeiro, Brazil, for analyses. Tilapia skin dehydrated and cut into small pieces (19.5 g) was subjected to papain digestion (60 °C, 24 h) in 100 mM sodium acetate buffer (pH 5.0) containing cysteine and EDTA (both 5 mM), as previously published elsewhere (Rodrigues et al., 2011) and the yield expressed as the percentage (%) of the dehydrated matter. The total extract (18.5 mg) was dissolved in 10 mL of 50 mM sodium acetate buffer (pH 5.0) and applied to a DEAE-cellulose column (1.2 × 12 cm) equilibrated with the same buffer. The fractionation was conducted using a stepwise ranging from 0 to 1.5 M NaCl at 0.25 M intervals in the same buffer. Fractions of 2.5 mL were collected and analyzed for GAGs using the metachromatic assays ($A_{525\ nm}$) containing dimethyl methylene blue with an Amersham Bioscience Ultrospec 3100 spectrophotometer at 525 nm, as also described (Rodrigues et al., 2011). The metachromatic fractions were further dialyzed and freeze dried.

Detection of GAGs on agarose (Dietrich & Dietrich, 1976) and polyacrylamide (Rodrigues et al., 2013) gels by sequential staining with toluidine blue and stains-all (Volpi & Maccari, 2002) was also performed by comparison with the

electrophoretic mobility of standard compounds low molecular weight dextran sulfate (~ 8 kDa), chondroitin-4-sulfate (~ 40 kDa), chondroitin-6-sulfate (~60 kDa) (Rodrigues et al., 2013), dermatan sulfate (DS) and/or heparan sulfate (HS) (Rodrigues et al., 2011). Subsequently, fractions were also characterized by a new agarose analysis after enzymatic digestions (12 h, 37 °C) with chondroitin AC and ABC lyases in order to demonstrate the GAG species in respective fractions as described by Dellias et al. (2004) and Souza et al. (2007).

Activated partial thromboplastin time (APTT) and prothrombin (PT) tests

The anticoagulant effects of the fractions were assessed by both *in vitro* APTT and PT tests, using normal citrated human plasma (10 different donors, University Hospital Clementino Fraga Filho, FURJ) according to the manufacturers' specifications, for measure a possible anti-clotting effect in a coagulometer Amelung KC4A before of the *in vitro* TG assay. For APTT assay, a mixture of 100 µL of plasma and concentration of GAGs (1 mg mL^{-1}) was incubated with 100 µL of APTT reagent (kaolin bovine phospholipid reagent). After 2 min of incubation at 37°C, 100 µL of 25 mM $CaCl_2$ was added to the mixtures, and the clotting time was recorded. Regarding the PT assay, a mixture of 100 µL of plasma and concentration of GAGs (1 mg mL^{-1}) was incubated for 1 min at 37 °C. After that, 100 µL of PT reagent was added to the mixtures, and the clotting time was recorded using same coagulation equipment. Unfractionated heparin (UHEP) with 193 international units per mg (IU mg^{-1}) of polysaccharide was used as the standard on both tests. All the tests were performed in triplicate.

TG-based coagulation assay

This assay was carried out in a microplate format, containing: 10 µL of APTT reagent (contact-activator system) or PT reagent (830 µg well-$plate^{-1}$, factor tissue-activator system) + 30 µL of 0.02 M Tris-HCl/PEG buffer (pH 7.4) + 10 µL of GAGs (*O. niloticus* fractions: 0, 4.1, 41.6 or 83.3 µg well-$plate^{-1}$; UHEP: 2 or 4 µg well-$plate^{-1}$; or mammalian DS: 4 or 8.3 µg well-$plate^{-1}$) + 60 µL of 20 mM $CaCl_2$/0.33 mM chromogenic substrate S2238 (10:50 ratio, $v.v^{-1}$). The *in vitro* reaction was triggered at 37 °C by addition of plasma (diluted 60-fold well-$plate^{-1}$, ten µL), and the absorbance (405 nm) was recorded every 1 min for 60 min using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The *in vitro* inhibitory response of TG by GAGs was analyzed by lag phase, peak thrombin (PTh) and time to peak (TPeak) (Rodrigues et al., 2016).

Statistical analyses

The APTT and PT values were expressed as mean ± S.E.M., and analysis of variance (ANOVA) was performed, followed by Tukey's test for unpaired data, with $p < 0.05$ as statistically significant.

Results and Discussion

Tilapia skin reveals a little amount of matrix' GAGs

Skin samples of *O. niloticus* were subjected to proteolytic incubation (60 °C, 24 h) to obtain and partially characterize GAGs. Based on the dehydrated tissue, a total GAGs extraction yield of approximately 0.1 ± 0.05% was revealed to be 10-fold lower than that of the overall extract (1%) from the skin tissue of the ray *R. radula* using same papain method (Mansour et al., 2009). This amount of GAGs contained in the *O. niloticus* skin contrasted with a previously described study from Arima et al. (2013), who digested (55 °C, three h) defatted dry scale tissue with protease N Amano G, yielding a very scarce amount of total GAGs. This combined information allowed the assumption that some GAGs in this Cichlidae species varies depending on the tissue of origin. Therefore, these findings demonstrate that the abundance of fish GAGs vary according to the species and tissue for use as raw material supplies for the world's biopolymers industry (Rodrigues et al., 2009; Kang et al., 2015). It is described that the nature of first matter and the protocol of extraction are determining factors for some polysaccharides (Cardozo et al., 2007; Rodrigues et al., 2012).

Combined physical-chemical analyses reveal unique DS

The analysis by anion-exchange chromatography (DEAE-cellulose) of the crude GAG confirmed the separation into two fractions (F I and F II), eluted at 0.5 and 0.75 M of NaCl, respectively, as demonstrated by Rodrigues et al. (2011), who previously reported *O. niloticus* skin GAGs isolation. According to the metachromasy assay, 0.5 M NaCl fraction (F I), which was obtained at the beginning of the stepwise, accounted about 8-fold greater the total material recovered from the column (9.8 mg yield) than F II (1.2 mg yield), which required a 0.75 M salt concentration for the elution of sulfated GAGs. Total fraction yield was 3.66-fold greater compared with that found for this same fish species by Rodrigues et al. (2011). On elution with stepwise of NaCl, the concentration of GAGs in *O. niloticus* skin was also greater than that of freshwater fish *Cyprinus carpio* (5.7 mg) (Rodrigues et al., 2009), but lower than those found in the deep-sea fishes *Lycodes nakamurae* and *L. toyamensis* (13 and 18 mg, respectively), when sulfated GAGs fractions were obtained by stepwise of LiCl (Arima et al., 2013).

As the separation by DEAE-cellulose of a complex mixture of sulfated glycans is not always possible (Tovar et al., 2005), our studies further extended to physical-chemically analyze by electrophoretic techniques the degree of purity and molecular mass of the *O. niloticus* fractions, as illustrated in Figure 1.

Agarose analysis revealed, by treatment with the cationic dye toluidine blue, an electrophoretic profile of sulfated GAGs (Figure 1Aa). On the basis this result, 0.5 M NaCl fraction (F I), which had greater yield from the DEAE-cellulose column, showed strong homogeneous band co-migrating as porcine DS on gel, similar to other studied fishes DS-type GAGs (Dellias et al., 2004; Souza et al., 2007; Rodrigues et al., 2012). On the contrary, fraction F II, eluted at 0.75 M, appeared virtually due to its relatively lower charge density in the chemical structure than F I, which revealed great metachromasy (Rodrigues et al., 2009).

Although clearly individualized by polyanionic character, both fractions suggested same structural conformation due to their interaction with the 1,3-diaminopropane/acetate buffer along their electrophoretic migrations on the gel (Dietrich &

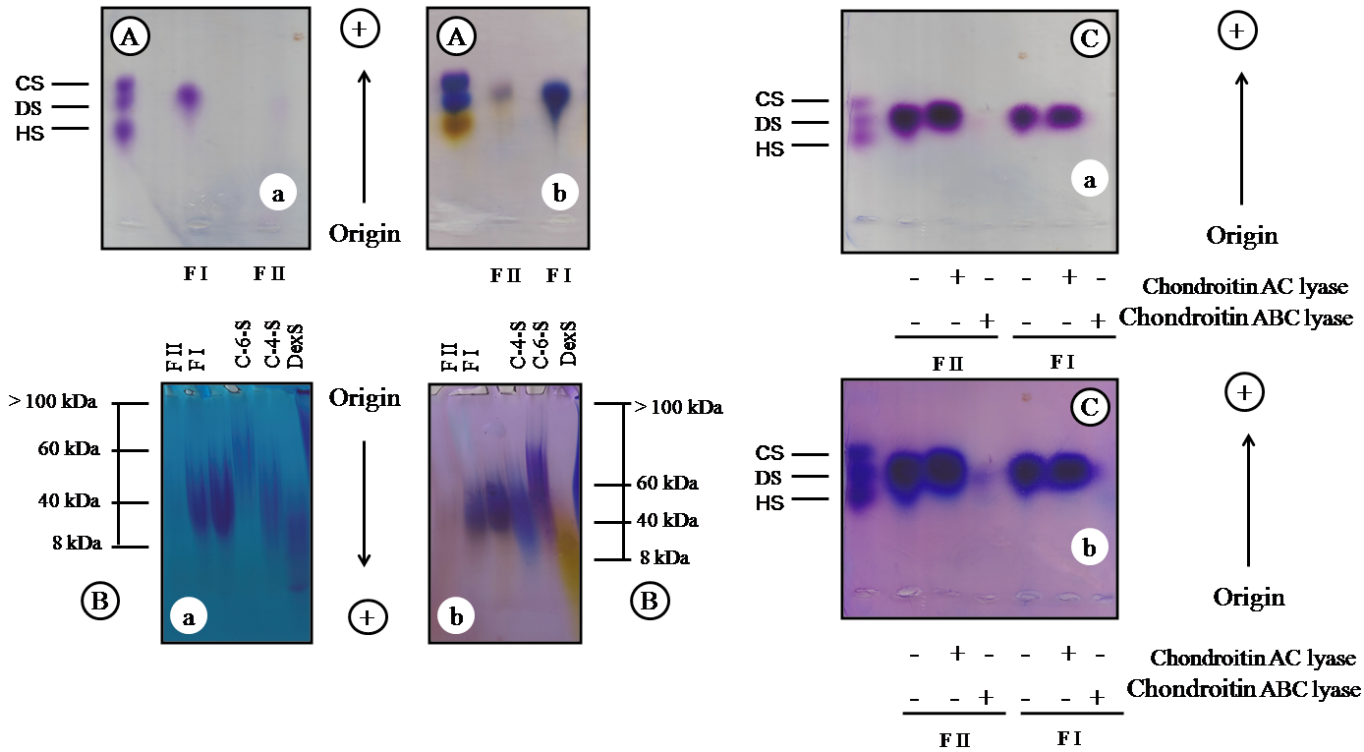

Figure 1. Agarose (A, C) and polyacrylamide (B) gels electrophoreses of *Oreochromis niloticus* GAGs fractions, obtained by DEAE-cellulose, and standards GAGs chondroitin-6-sulfate (C-6-S, 60 kDa), chondroitin-4-sulfate (C-4-S, 40 kDa), dextran sulfate (DexS, 8 kDa), dermatan sulfate (DS), heparan sulfate (HS) and/or before (-) and after (+) chondroitin AC and ABC lyase digestions, present on gels were stained with 0.1% toluidine blue (a) or stains all (b)

Dietrich, 1976). Similarly, the staining of the fraction F II after its separation by polyacrylamide analysis with toluidine blue led to a weak revelation of the band containing sulfated GAGs. On the basis this procedure, the fractions exhibited average molecular mass distribution as C-4-S (ca. 40 kDa) (Figure 1Ba) (Souza et al., 2007). Taking to literature data, it was speculated a possible mixture of *O. niloticus* skin DS and CS, which were not separated by DEAE-cellulose using different ionic strength (Tovar et al., 2005).

To clarify this hypothesis, samples of the fractions (at high concentrations) were subjected to enzymatic digestions with particular lyases (chondroitin AC and ABC) and examined by comparison with the known porcine DS standard. Fractions evaluated by chondroitin AC lyase were resistant to action this enzyme after incubation at 37 °C for 12 h. By contrast, treatment with chondroitin ABC lyase totally degraded both fractions, revealing DS as the unique sulfated GAG species contained in these preparations (Figure 1Ca); therefore, free of CS contamination (Dellias et al., 2004). Overall, DS exhibiting an average molecular mass of 40 kDa (Figure 1Ba) was present in the *O. niloticus* skin as an attractive report on freshwater fishes because this GAG is the most abundant express in marine fish skin tissues (Dellias et al., 2004; Souza et al., 2007; Mansour et al., 2009). More recently, DS was also found in the shell from the Norway lobster *Nephrops norvegicus* (Sayan et al., 2016).

Since fraction F II showed poor detection of GAGs by staining with toluidine blue (Figure 1Aa), this result also led us to more precisely examine this GAGs preparation associated with stains-all, as shown in Figure 1Ab. This approach allowed improved visualization of standards and both fractions containing complex GAGs, especially F II due to its small amount of available material for analysis (Volpi & Maccari, 2002). It was interesting to note that combined toluidine blue/stains-all treatment (both cationic dyes) also revealed a unique GAG band for each fraction on gel free of contamination with hyaluronic acid within complex GAGs preparations. Diverse classes of fishes GAGs can be found from the same or different tissue (Dellias et al., 2004; Mansour et al., 2009; Arima et al., 2013; Krichen et al., 2017).

Therefore, both low molecular size and low charge density of F II did not limit the complex formed with the acidic residues (D-glycuronic acid or L-iduronic acid) of the compound (Figure 1Ab; Figure 1Bb). The observation was also supported by treatment with chondroitin ABC lyase because no band was observed on agarose gel after stains-all staining (Figure 1Cb). The purity of GAGs is an essential step in the course of their extraction to quality control of commercially available products (Volpi & Maccari, 2002).

Anticoagulation by *O. niloticus* GAGs is better reflected on an *in vitro* TG protocol than typical APTT and PT tests

The *in vitro* effects of *O. niloticus* GAGs on both standard APTT and PT clotting assays are summarized in Table 1, and were compared with UHEP (193 IU mg^{-1}, ca. 14 kDa) and mammalian DS.

On a weight-to-weight basis, normal human plasma treated with a high concentration (1 mg mL^{-1}) of both fractions (F I and F II) only marginally inactivated the intrinsic coagulation pathway in 1.6 (53.50 ± 0.30 s, 0.61 IU mg^{-1}) and 1.25-fold (42.00 ± 0.04 s, 0.47 IU mg^{-1}) ($p < 0.05$), respectively, compared with plasma control (33.5 ± 0.08 s), whereas UHEP and mammalian DS still delayed the plasma clotting time at

Table 1. *In vitro* anticoagulant effects of fractions obtained by anion-exchange chromatography (DEAE-cellulose) from the skin of *Oreochromis niloticus* compared with mammalian DS and UHEP

Fractions	NaCl (M)	APTT (s)* (1 mg mL^{-1}***)	PT (s)** (1 mg mL^{-1}***)	$T_1.T_0^{-1}$&	IU mg^{-1}#
F I	0.50	53.50 ± 0.30$^{\Delta}$	11.20 ± 0.01	1.60	0.61
F II	0.75	42.00 ± 0.04	10.20 ± 0.02	1.25	0.47

NaCl – Sodium chloride; *Activated partial thromboplastin time (APTT); **Prothrombin time (PT); ***SPs concentration to prolong the APTT or PT in seconds; &Ration for prolong the APTT test; #Anticoagulant effect expressed in international units (IU) per mg of GAGs (IU mg^{-1}); DS (1.72 IU mg^{-1}: APTT [250 µg mL^{-1}]: 37.80 ± 1.92 s$^{\Delta\Delta}$; PT [1 mg mL^{-1}]: 11.93 ± 0.17 s) or UHEP (193.00 IU mg^{-1}: APTT [2.5 µg mL^{-1}]: 42.15 ± 0.6 s$^{\Delta\Delta}$; PT [100 µg mL^{-1}]: 20.13 ± 0.6 s); Controls: 33.5 ± 0.08 s and 10.02 ± 0.01 s for APTT and PT tests, respectively (n = 3, $p < 0.05^{\Delta}$ or $p < 0.01^{\Delta\Delta}$ vs. control).

concentrations of 2.5 µg mL^{-1} (42.15 ± 0.6 s) and 250 µg mL^{-1} (37.80 ± 1.92 s, 1.72 IU mg^{-1}), respectively, as revealed by *in vitro* APTT test. Krichen et al. (2017) studied GAGs isolated from the skins of smooth hound (*Mustelus asterias*) and grey triggerfish (*Balistes capriscus*) and found APTT' results by prolonging 1.3 and 1.23-fold, respectively, in comparison with the plasma control.

Likewise, samples of skin tissue of the marine fish *Chloroscombrus chrysurus* (Rodrigues et al., 2012) and the freshwater fish *C. carpio* (Rodrigues et al., 2009) yielded GAGs that virtually coagulation inhibited by APTT method (2.67→3.30 and 4.89 IU mg^{-1}, respectively) than UHEP. Unexpectedly, our APTT values did not achieve at same level of effects than those previously revealed related to the degree of sulfation by *O. niloticus* GAGs (4.72→23.80 IU mg^{-1}), with intrinsic coagulation inhibition of about 39-fold (97.43%) greater in terms of IU than the current study (Rodrigues et al., 2011). On this basis, a highly charged DS (25 kDa) isolated from the skin of the ray *R. radula* also displayed a level of 73.25% inhibition (at 1 mg mL^{-1}) greater than the tilapia GAGs (Mansour et al., 2009).

It was speculated that the *O. niloticus* exposure to severe management would lead to a stress-related response (Moreira et al., 2001), without affecting both levels of yield and charge density of its skin mucus (Figure 1Aa), but resulting in a negative impact on their anticoagulant actions as measured by *in vitro* APTT test (Table 1) (Rodrigues et al., 2012). Dellias et al. (2004) and Souza et al. (2007) assumed that the anticoagulation of fish skin DSs does not merely occurs a consequence of their charge density, but also related to both different composition and arrangements of the disulfated disaccharide units present their polysaccharide chains. Further evaluation by PT test allowed the assumption that *O. niloticus* skin GAGs had no extrinsic coagulation inhibition even in the normal plasma treated on a mass-to-mass basis compared with UHEP and mammalian DS that did not exhibit substantial effects in this standard assay (Table 1).

Given the modest actions of the GAGs obtained from *O. niloticus* on standard coagulation assays (Table 1), could they act with more precision on an *in vitro* TG method? In order to follow this proposition increasing concentrations of the GAGs (4.1→83.3 µg well-plate^{-1}) added to 60-fold diluted human plasma were assessed for their inhibitions on the formation of thrombin by addition of each activator (cephalin or thromboplastin for the intrinsic and extrinsic pathways, respectively) recorded continually at 37 °C for 60 min, as shown in Figure 2, using in parallel UHEP and mammalian DS as two references.

All the classes of sulfated polysaccharides were able to inhibit TG in both of the coagulation pathways under our *in vitro* conditions as revealed by different dose-response curves based on the maximum absorbance of amydolytic activity of thrombin that decreased rapidly until a plateau was reached (controls: 17→23 min) (Rodrigues et al., 2016). Fractions displayed their most inhibitory actions in both intrinsic and extrinsic pathways using concentrations at 41.6 and 83.3 µg well-plate^{-1} as analyzed by the PTh and TPeak parameters, inducing more than 50% inhibition of PTh (49.20 →77.85%) compared with control (TPeak: 17 and 23 min), although with an amount of GAGs 41.6 and 20.31-fold greater than UHEP and mammalian DS, which completely abolished TG on this *in vitro* system at 2→4 or 4.1→8.3 µg well-plate^{-1}, respectively, as properties already demonstrated by these polysaccharides using TG assays from other studies (Glauser et al., 2009; Jun et al., 2014; Rodrigues et al., 2016).

Results demonstrated, even at low levels of polysaccharides, that the *in vitro* dynamic role of anticoagulation on our TG assay using diluted human plasma was in coherency with the TG parameters analyzed after 60 min, when in increasing concentrations of polysaccharides; it contrasting to the conventional clotting tests that were sensitive at only high polysaccharide concentration (Table 1) (Castoldi & Rosing, 2011; Jun et al., 2014). No activator response of TG in plasma in the absence of cephalin (negative control) was observed *in vitro* during 60 min.

The difference in the degree of *in vitro* inactivation by the different classes of polyanionic suggested to a distinct mode of action of each polymer added to plasma collected from Brazilian for display anticoagulation. It is recognized that UHEP (that has AT-binding pentasaccharide sequence which does not present in other SPs) shows inhibitory mechanism on the coagulation system by AT, and by preventing the formation of the factor X- and prothrombin-activating complexes (Mourão, 2015) because UHEP modifies TG in plasma as also revealed for Chinese (Jun et al., 2014), whereas mammalian DS has HCII-dependent anticoagulant mechanism (Mourão, 2015).

Some inhibitory roles on the coagulation have been proposed for GAGs isolated from fishes (Dellias et al., 2004; Souza et al., 2007; Mansour et al., 2009) and other polysaccharides from different sources (Glauser et al., 2009; Mourão, 2015; Rodrigues et al., 2013; Rodrigues et al., 2016). It seemed that *O. niloticus* GAGs may display their *in vitro* inhibitory actions by stereospecific features independent of their degree of sulfation and/or molecular size (Mourão, 2015) since these physical-chemical features did not limit the levels of anticoagulant responses between both fractions (Figures 1 and 2) in comparison with the classical assays, which were of limited value (Table 1) (Castoldi & Rosing, 2011). On the basis of application, our *in vitro* TG assay could perhaps be an additional tool for biochemically explore *O. niloticus* matrix' GAGs role against stress-related responses under intensive production conditions (Moreira et al., 2001; Araújo et al., 2010).

Overall, *O. niloticus* features DS may constitute a novel glycan from the freshwater origin as a promising prophylactic candidate on TG and coagulation parameters to studies of

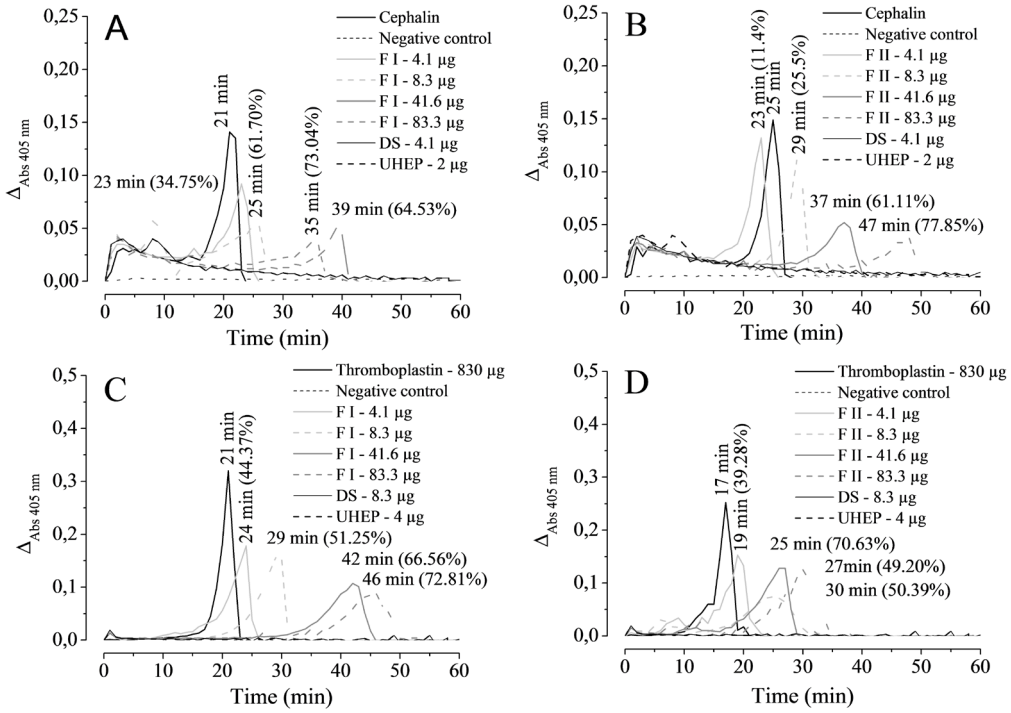

Figure 2. Effect of different concentrations of GAGs fractions (F I and F II), obtained by DEAE-cellulose, from *Oreochromis niloticus* skin on cephalin (A and B)- or thromboplastin (C and D)-triggered TG in 60-fold diluted human plasma using chromogenic method by a continuous detection system (37 °C, 60 min)

alternative anticoagulants to UHEP. Due to the importance of this species in world aquaculture and the generally discarded wasters from its food processing (Moreira et al., 2001), its skin is a rich source in unique DS (ca. 40 kDa) with therapeutic implications in the coagulation, when an increase of plasma prothrombin's referential values increase the TG after activation by both intrinsic and extrinsic pathways (Castoldi & Rosing, 2011). A more detailed analysis regarding its anticoagulant mechanisms is needed as an essential step to antithrombotic drug development (Tovar et al., 2005; Jun et al., 2014; Mourão, 2015).

Conclusion

The skin of Nile tilapia, *Oreochromis niloticus*, reveals dermatan sulfate-type glycosaminoglycans (ca. 40 kDa) displaying experimental thrombosis inhibition by both intrinsic and extrinsic pathways independently of charge and molecular size, when in 60-fold diluted human plasma using continuous method of thrombin generation *in vitro*, but with less efficacy than heparin and mammalian dermatan sulfate.

Acknowledgements

We thank the funding from CAPES/PNPD, FAPERJ, CNPq, and MCT.

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