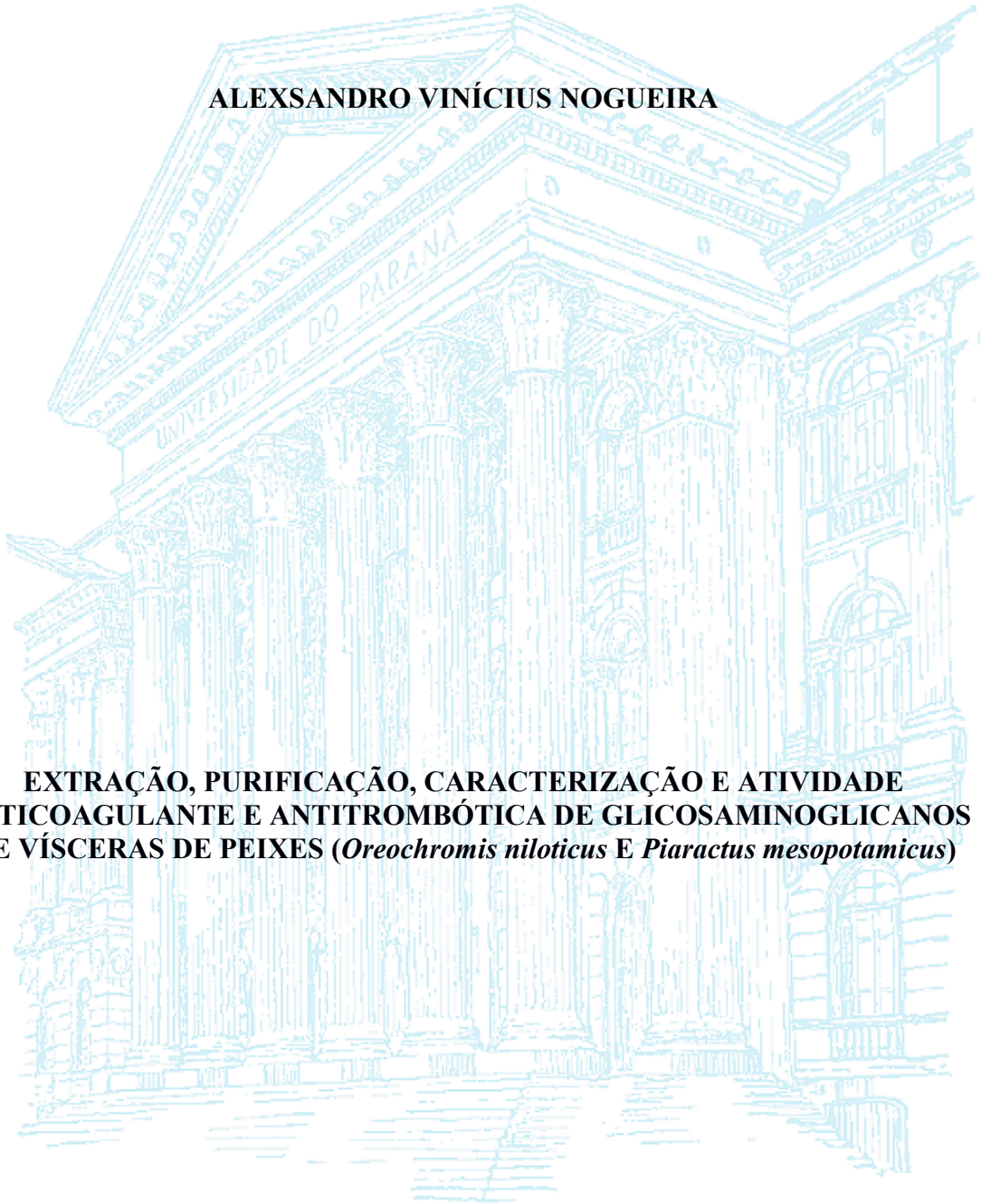
UNIVERSIDADE FEDERAL DO PARANÁ

ALEXSANDRO VINÍCIUS NOGUEIRA



EXTRAÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO E ATIVIDADE ANTICOAGULANTE E ANTITROMBÓTICA DE GLICOSAMINOGLICANOS DE VÍSCERAS DE PEIXES (*Oreochromis niloticus* E *Piaractus mesopotamicus*)

CURITIBA

2018

ALEXSANDRO VINÍCIUS NOGUEIRA

EXTRAÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO E ATIVIDADE ANTICOAGULANTE E ANTITROMBÓTICA DE GLICOSAMINOGLICANOS DE VÍSCERAS DE PEIXES (*Oreochromis niloticus* E *Piaractus mesopotamicus*)

Tese apresentada como requisito parcial para obtenção do Título de Doutor em Ciências – Bioquímica, no curso de Pós-Graduação em Ciências-Bioquímica, do Departamento de Ciências Bioquímica e Biologia Molecular, do Setor de Ciências Biológicas, da Universidade Federal do Paraná,

Orientador: Prof. Dr. Thales Ricardo Cipriani

Coorientadores: Prof. Dr. Guilherme L. Sassaki
Prof. Dr. Marcello Iacomini

CURITIBA

2018

Universidade Federal do Paraná. Sistema de Bibliotecas.
Biblioteca de Ciências Biológicas.
(Giana Mara Seniski Silva – CRB/9 1406)

Nogueira, Alexsandro Vinícius
Extração, purificação, caracterização e atividade anticoagulante e antitrombótica de glicosaminoglicanos de vísceras de peixes (*Oreochromis niloticus* e *Piaractus mesopotamicus*. / Alexsandro Vinícius Nogueira. – Curitiba, 2018.
94 f.: il. ; 30cm.

Orientador: Thales Ricardo Cipriani
Coorientador: Marcello Iacomini

Tese (doutorado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica.

1. Tilápia (Peixe) 2. Pacu (Peixe) 3. Anticoagulantes 4. Trombose 5. Glicosaminoglicanos I. Título II. Cipriani, Thales Ricardo III. Iacomini, Marcello IV. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica.

CDD (20. ed.) 574.192

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
Setor CIÊNCIAS BIOLÓGICAS
Programa de Pós Graduação em CIÊNCIAS (BIOQUÍMICA)
Código CAPES: 40001016003P2

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **ALEXSANDRO VINICIUS NOGUEIRA**, intitulada: "**EXTRAÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO E ATIVIDADE ANTICOAGULANTE E ANTITROMBÓTICA DE GLICOSAMINOGLICANOS DE VÍSCERAS DE PEIXE (*Oreochromis niloticus* E *Piaractus mesopotamicus*)** ", após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 31 de Outubro de 2017.

THALES RICARDO CIPRIANI
Presidente da Banca Examinadora (UFPR)

DIOGO RICARDO BAZAN DUCATTI
Avaliador Interno (UFPR)

LUCIMARA MACH CORTES CORDEIRO
Avaliador Interno (UFPR)

MARCELO ANDRADE DE LIMA
Avaliador Externo (UNIFESP)

MARIA FERNANDA DE PAULA WERNER
Avaliador Externo (UFPR)

RESUMO

Glicosaminoglicanos (GAGs) são uma importante família de polissacarídeos aniônicos. Cada tipo de GAG apresenta diferente densidade de carga negativa em sua estrutura e isso faz com que eles apresentem propriedades estruturais e biológicas distintas. Com exceção do ácido hialurônico, todos os GAGs ocorrem nos tecidos como proteoglicanos, glicoconjugados encontrados principalmente na superfície celular e na matriz extracelular. Os GAGs estão presentes em todo o reino animal e em peixes eles já foram confirmados por alguns pesquisadores. Em 2015 o Brasil foi responsável pela produção de 219 mil toneladas de tilápia do Nilo (*Oreochromis niloticus*) e cerca de 13 mil toneladas de pacu (*Piaractus mesopotamicus*). Junto com essa produção existe a geração de resíduos que podem se tornar matéria prima para a extração e isolamento de GAGs com potenciais aplicações. Com isso, nosso objetivo foi extrair, isolar, caracterizar e determinar possíveis atividades farmacológicas de GAGs obtidos de vísceras dos peixes Tilápia do Nilo (T) e Pacu (P), criados na região oeste do Paraná. As vísceras foram deslipidificadas e submetidas à proteólise, seguida de filtração. Após, o filtrado foi tratado com etanol para a precipitação da fração bruta de GAGs os quais foram fracionados por cromatografia de troca aniônica e, então, submetidos à tratamento com condroitinase. Foram obtidas as frações P-1.0R e T1.0R (constituídos de dermatam sulfato), P-0.75R e T-0.75R (constituídos por mistura de dermatam sulfato e heparan sulfato) e P-0.75B, P-0.75C e T-0.75B (constituídos por mistura de dermatam, condroitim e heparam sulfato). Uma vez que diversos GAGs comumente apresentam atividade anticoagulante, esta propriedade foi investigada para as frações de GAGs obtidas a partir de tilápia e pacu. Todas as frações foram capazes de prolongar o aPTT em ensaio realizado com plasma ovino, demonstrando assim efeito anticoagulante, com as amostras de tilápia apresentando melhores efeitos. Também foi demonstrado que a atividade anticoagulante das frações está relacionada à inibição da atividade das enzimas α-trombina e fator Xa da cascata de coagulação, de maneira dependente de antitrombina e cofator II da heparina. Além disso, foi verificado efeito antitrombótico *in vivo* para todas as frações de GAGs, com T-0.75A apresentando a melhor atividade, inibindo em quase 100% a formação de trombo, na dose de 1 mg/kg. Desta forma, neste estudo foi comprovada a presença de diferentes GAGs nas vísceras de tilápia e pacu, que apresentam atividades anticoagulante e antitrombótica, mostrando que as vísceras destes peixes podem ser utilizadas para a obtenção de compostos com potencial aplicabilidade.

Palavras-chave: Tilápia do Nilo, Pacu, Condroitim sulfato, Dermatam sulfato, Heparam sulfato

ABSTRACT

Glycosaminoglycans (GAGs) are an important family of anionic polysaccharides. Each type of GAG presents a different density of negative charge in its structure and this is related to their different biological structures and properties. Except for hyaluronic acid, all GAGs are present in tissues in the form of proteoglycans, which are glycoconjugates found in the cell surface and in the extracellular matrix. GAGs are present throughout the animal kingdom, and in fish they have already been confirmed by some researchers. In 2015, Brazil was responsible for the production of 219 thousand tons of Nile tilapia (*Oreochromis niloticus*) and about 13 thousand tons of pacu (*Piaractus mesopotamicus*). With this production there is the generation of waste that can become raw material for the extraction and isolation of GAGs with potential applications. Therefore, the objective of this work was to extract, isolate, characterize and determine possible pharmacological activities of GAGs obtained from viscera from Nile tilapia (T) and Pacu (P), grown in the western region of Paraná. The viscera were delipidified and submitted to proteolysis, followed by filtration. Then, the filtered part was treated with ethanol to precipitate the crude GAGs fraction, which had been obtained by anion exchange chromatography and treated with chondroitinase. Fractions P-1.0R and T-1.0R (consisting of dermatan sulfate), P-0.75R and T-0.75R (consisting of a mixture of dermatan sulfate and heparan sulfate) and P-0.75B, P-0.75C and T-0.75B (consisting of a mixture of dermatan, chondroitin and heparan sulfate) were obtained. Due to GAGs commonly present anticoagulant activity, this property was evaluated for all GAGs fractions obtained from tilapia and pacu. All fractions were able to increase aPTT, in a test performed with ovine plasma, demonstrating anticoagulant activity, with the samples from tilapia showing better effects. It was also demonstrated that the anticoagulant activity of the fractions is related to inhibition of the enzymes of the coagulation cascade, α-thrombin and factor Xa, in a way dependent of antithrombin and heparin cofactor II. In addition, *in vivo* antithrombotic effect of all GAGs fractions was observed, with T-0.75A showing the best activity, inhibiting almost 100% thrombus formation at a dose of 1 mg/kg. Thus, in this study the presence of different GAGs in the viscera of tilapia and pacu, which present anticoagulant and antithrombotic activities, has been proven, showing that the viscera from these fish species can be used to obtain compounds with potential applicability.

Keywords: Nile Tilapia, Pacu, Chondroitin sulfate, Dermatan sulfate, Heparan sulfate

LISTA DE FIGURAS

INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

ARTIGO I

EXTRACTION AND CHARACTERIZATION OF GLYCOSAMINOGLYCANS FROM VISCERA OF THE FISHES *Oreochromis niloticus* AND *Piaractus mesopotamicus*

ARTIGO II

ANTICOAGULANT AND ANTITHROMBOTIC ACTIVITIES OF GLYCOSAMINOGLYCANS FROM VISCERA OF THE FISHES *Oreochromis niloticus* AND *Piaractus mesopotamicus*

LISTA DE TABELAS

INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

ARTIGO I

EXTRACTION AND CHARACTERIZATION OF GLYCOSAMINOGLYCANS FROM VISCERA OF THE FISHES *Oreochromis niloticus* AND *Piaractus mesopotamicus*

ARTIGO II

ANTICOAGULANT AND ANTITHROMBOTIC ACTIVITIES OF GLYCOSAMINOGLYCANS FROM VISCERA OF THE FISHES *Oreochromis niloticus* AND *Piaractus mesopotamicus*

LISTA DE ABREVIATURAS, SIGLAS SÍMBOLOS

Frações obtidas do Pacu

P-0.75AR	-	Fração eluida com NaCl 0,75M, submetida a recromatografia, eluida com 0,5 e 0,525 M e tratada com condroitinase AC
P-0.75B		Fração eluida com NaCl 0,75M, submetida a recromatografia, eluida com 0,55 a 0,6 M
P-0.75C		Fração eluida com NaCl 0,75M, submetida a recromatografia, eluida com 0,65 a 0,7 M
P-1.0R		Fração eluida com NaCl 1,0 M e tratada com condroitinase AC

Frações obtidas da Tilápia do Nilo

T-0.75AR	Fração eluida com NaCl 0,75M, submetida a recromatografia, eluida com 0,5 a 0,65 M e tratada com condroitinase AC
T-0.75B	Fração eluida com NaCl 0,75M, submetida a recromatografia, eluida com 0,675 e 0,7 M
T-1.0R	Fração eluida com NaCl 1,0 M e tratada com condroitinase AC

Termos associados à estrutura dos glicosaminoglicanos

G-ANS	-	Ácido glucurônico ligado a glucosamina N-sulfatada
G-ANAc	-	Ácido glucurônico ligado a glucosamina N-acetilada
I2S	-	Ácido Idurônico 2-sulfato
ANAc	-	Glucosamina N-acetilada
ANS	-	Glucosamina N-sulfatada
A*	-	Glucosamina 3,4,6-sulfato
G-A4S	-	Ácido glucurônico ligado a galactosamina 4-sulato
G-A6S	-	Ácido glucurônico ligado a galactosamina 6-sulato
A4S	-	Galactosamina 4-sulato
A6S	-	Galactosamina 6-sulato
A4S-I	-	Galactosamina 4-sulato liagada a ácido idurônico
I	-	Ácido idurônico

SUMÁRIO

1 INTRODUÇÃO

Glicosaminoglicanos (GAGs) são uma família importante de polissacarídeos aniônicos, constituídos por unidades dissacarídicas repetitivas de hexosamina (glucosamina ou galactosamina) e ácido urônico (ácido glucurônico ou ácido idurônico), ou galactose (em queratam sulfato) (NAKANO *et al.*, 2010; RUDD *et al.*, 2009). Cada tipo de GAG apresenta diferente densidade de carga negativa em sua estrutura e isso faz com que eles apresentem propriedades estruturais e biológicas distintas (GAMA e HSIEH-WILSON, 2005; TINGBØ *et al.*, 2012).

Os GAGs mais comuns são condroitim sulfato (CS – *condroitin sulfate*), dermatam sulfato (DS – *dermatan sulfate*), ácido hialurônico (HA – *hyaluronic acid*), Queratam sulfato (KS – *keratan sulfate*), heparina (*heparin*) e heparam sulfato (HS – *heparan sulfate*) (Tabela 1) (NAKANO *et al.*, 2010).

TABELA 1 – COMPOSIÇÃO ESTRUTURAL DOS GAGs

GAGs	Unidades dissacarídicas		Grupos Ácidos	Variação da Massa Molecular
	Monossacarídeo não nitrogenado	Hexosamina		
CS	→4)-β-D-GlcA-(1→	→3)-β-D-GalNAc-(1→	Carboxil Sulfato	CS-4 40 - 80 kDa CS-6 5 - 50 kDa
DS	→4)-β-D-GlcA-(1→ →4)-α-L-IdoA-(1→	→3)-β-D-GalNAc-(1→	Carboxil Sulfato	11 - 25 kDa
HA	→4)-β-D-GlcA-(1→	→3)-β-D-GlcNAc-(1→	Carboxil	10^2 - 10^4 kDa
KS	→3)-β-D-Gal-(1→	→4)-β-D-GlcNAc-(1→	Sulfato	6 - >50 kDa
Hep	→4)-β-D-GlcA-(1→ →4)-α-L-IdoA-(1→	→4)-α-D-GlcN-(1→	Carboxil Sulfato	5 - 40 kDa
HS	→4)-β-D-GlcA-(1→ →4)-α-L-IdoA-(1→	→4)-α-D-GlcN-(1→	Carboxil Sulfato	5 - 50 kDa

Fonte: Adaptado de NAKANO *et al.* (2010).

Com exceção do ácido hialurônico, todos os GAGs ocorrem nos tecidos como proteoglicanos (PGs) (LI e VLODAVSKY, 2009; SAMPAIO *et al.*, 2006). PGs são caracterizados como glicoconjugados, macromoléculas da superfície celular ou da matriz extracelular que apresentam núcleo proteico no qual uma ou mais cadeias de GAGs podem se ligar covalentemente. Além de representar o principal constituinte de todas as matrizes extracelulares, os GAGs apresentam significantes alterações em seu conteúdo, síntese e distribuição, durante os processos de crescimento de órgãos em neonatais, lesões agudas e degeneração de tecidos devido à idade (LI e VLODAVSKY, 2009; ROTH *et al.,* 2008).

Os GAGs sulfatados são ausentes nos reinos Plantae, Fungi e Protista. Porém, no reino animal estão presentes em todas as espécies que apresentam organização tecidual, sendo heparam sulfato e condroitim sulfato os mais abundantes, seguidos de heparina e dermatam sulfato (FIGURA 1) (SAMPAIO *et al.*, 2006).

FIGURA 1: DISTRIBUIÇÃO DE GAGs NO REINO ANIMAL (*Fonte:* Adaptado de SAMPAIO *et al.*, 2006).

Como mostrado na figura 1, existem GAGs em peixes, e este fato é comprovado por alguns pesquisadores, com variações quanto à quantidade de determinado GAG e variações no grau de sulfatação.

Sabendo da importância dos GAGs para a elaboração de medicamentos, principalmente relacionados à distúrbios de coagulação, o estudo de novas fontes para a obtenção destes polissacarídeos apresenta grande relevância científica.

2 REVISÃO BIBLIOGRÁFICA

2.1 Hemostasia

Hemostasia é um mecanismo fisiológico fundamental para todos os vertebrados, que envolve dois processos complementares, a formação do coágulo ou trombo, que serve para bloquear o dano no vaso e parar o sangramento, e o processo de dissolução do trombo, ou fibrinólise, que elimina os elementos formadores de coágulo assim que o reparo do endotélio vascular tenha sido concluído. Estes processos envolvem os vasos sanguíneos, as plaquetas, as proteínas da cascata de coagulação e da fibrinólise e os anticoagulantes naturais. Desta forma a hemostasia tem como objetivo minimizar a perda de sangue, restaurar a integridade vascular e preservar a vida (BROOKS *et al.*, 2011; COLMAN, 2006; EYRE e GAMLIN, 2010; GENTRY, 2004; REIS *et al.*, 2003).

2.1.1 Sistema de Coagulação Sanguínea

A formação do coágulo envolve um grande número de interação entre proteases plasmáticas e seus cofatores. Estas interações convertem pró-trombina à enzima trombina, que por sua vez converte o fibrinogênio, solúvel, em fibrina, insolúvel (FRANCO, 2001). A Tabela 2 apresenta a nomenclatura e a origem dos fatores envolvidos na coagulação.

TABELA 2: NOMENCLATURA E ORIGEM DOS FATORES DE COAGULAÇÃO DO SANGUE

Fator de coagulação	Nome comum	Local de síntese
I	Fibrinogênio	Fígado
II	Protrombina	Fígado
III	Fator tecidual	Tecidos em geral
IV	Íons cálcio	Tecidos em geral
V	Pró-acelerina	Fígado
VII	Pró-convertina	Fígado
VIII	Anti-hemofílico	Endotélio
IX	Fator Christmas	Fígado
X	Fator de Stuart	Fígado
XI	Pró-transglutaminase	Fígado
XII	Antecedente da tromboplastina	Fígado
XIII	Fator de Hageman	Fígado
Proteína C		Endotélio
Proteína S		Endotélio

Fonte: Adaptado de Murray *et al.* (2007).

2.1.1.1 Modelo clássico da coagulação

Este modelo foi descrito em 1964 por Macfarlane e também por Davie e Ratnoff, onde a coagulação é divida em uma via extrínseca (ativada por um fator externo ao sangue, quando o vaso é lesado) e uma via intrínseca (ativada diretamente por exposição do sangue a uma superfície negativamente carregada, como o vidro). Ambas as vias tem como objetivo a ativação do fator X (FRANCO, 2001) (FIGURA 6).

Na via extrínseca o fator tissular (TF) é exposto ao plasma e o fator VII é ativado a fator VIIa, que por sua vez ativa o fator X diretamente. Já a via intrínseca ativa o fator XII a fator XIIa devido ao estímulo provocado pelo contato do plasma com uma superfície carregada negativamente (ativação por contato). Contudo, para que o fator XII seja ativado também se fazem necessárias a presença da pré-calicreína (uma serino-protease) e do cininogênio de alta massa molecular (um cofator não enzimático). Em seguida o fator XIIa ativa o fator XI a XIa que por sua vez ativa o fator IX. O fator IXa na presença do fator VIIIa ativa o fator X. O fator Xa desencadeia a formação de

trombina a partir da protrombina, e esta desencadeia a formação de fibrina a partir de fibrinogênio (HOFFMAN e MONROE, 2001; VOGLER e SIEDLECKI, 2009).

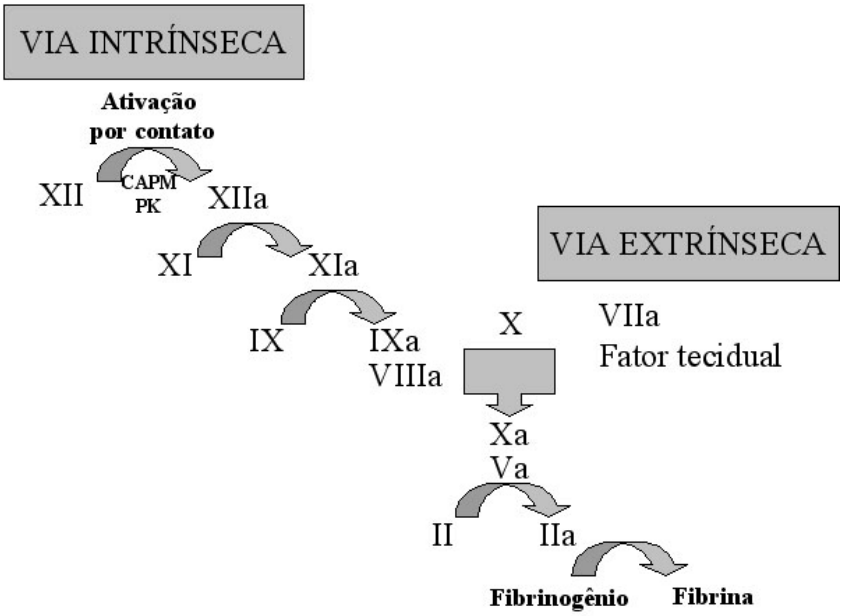


FIGURA 2: MODELO CLÁSSICO DA COAGULAÇÃO (*Fonte:* Adaptado de FRANCO, 2001). CAPM - cininogênio de alta massa molecular (um cofator não enzimático); PK - pré-calicreína (uma serino-protease).

2.1.1.2 Modelo de coagulação baseado em superfície celular

Devido a algumas descobertas, o modelo de coagulação baseado na célula foi proposto em 2001 por Hoffman e Monroe. Neste modelo o processo de coagulação é descrito em três fases sobrepostas: iniciação, amplificação e propagação (FIGURA 7).

Ao decorrer da iniciação o fator tecidual (TF), exposto pelo endotélio vascular danificado, liga-se ao fator VIIa circulante, formando um complexo TF/VIIa. Este complexo converte o fator IX em IXa e o fator X em Xa. O fator IXa amplifica a ativação do fator X e ajuda na ativação das plaquetas. O fator Xa liga-se e ativa o fator V, formando o complexo Xa/Va, que por sua vez forma a trombina a partir da protrombina (EYRE e GAMLIN, 2010; HOFFMAN e MONROE, 2001; VINE, 2009).

Na amplificação ocorre a ativação das plaquetas. Elas primeiramente se ligam às proteínas do meio extravascular que se encontram no plasma, devido à lesão vascular, sendo desta forma, parcialmente ativadas. Além disso, a trombina, formada na fase de

iniciação, amplifica o sinal anticoagulante e aumenta a adesão das plaquetas, ativando-as completamente. A trombina também ativa o fator V, VIII e XI e o fator XIa ativa o fator IX. Por fim os fatores ativados Va, VIIIa e IXa ligam-se a superfície das plaquetas ativadas (GENTRY, 2004; HOFFMAN e MONROE, 2001).

Durante o processo de propagação, ocorre a formação do complexo tenase, que é a interação dos fatores VIIIa e IXa. Este complexo amplifica a ativação do fator X, que complexa-se com o fator Va, convertendo uma grande quantidade de protrombina em trombina, sendo a trombina responsável pela conversão do fibrinogênio em fibrina. Além disso, altas concentrações de trombina ativam o fator XIII, que é um estabilizador das redes de fibrina, por meio da formação de ligações covalentes cruzadas entre as moléculas de fibrina, formando um coágulo estável (CURRY e PIERCE, 2007; HOFFMAN e MONROE, 2001).

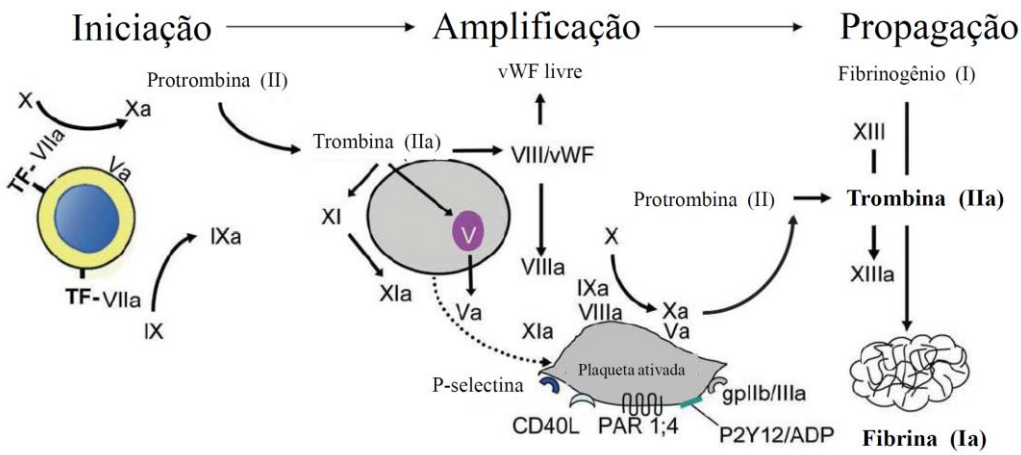


FIGURA 3: MODELO DE COAGULAÇÃO BASEADO NA CÉLULA (*Fonte:* Adaptado de CATERINA *et al.*, 2013).

2.1.2 Sistema Anticoagulante (Fase de Finalização)

Após a coagulação cumprir seu papel, ela deve ser controlada para que o coágulo não se estenda para áreas não lesionadas. Para isso existe o mecanismo de anticoagulação natural, que tem como objetivo reestabelecer o equilíbrio hemostático atuando em múltiplos níveis, indo da inibição enzimática à modulação da atividade dos fatores de coagulação. Os principais componentes do mecanismo anticoagulante são a

antitrombina III, o cofator II da heparina, o complexo proteína C-proteína S, e o inibidor da via do fator tissular (CATERINA *et al.*, 2013; SAGRIPANTI e CARPI, 1998; SOARES *et al.*, 2006).

2.1.2.1 Inibidor da via do fator tissular

A inibição do início da coagulação se dá pela inibição do complexo TF/VIIa, que é feita pelo inibidor da via do fator tissular (TFPI). TFPI, uma protease com massa molecular variando de 34-41 kDa, apresenta três domínios moleculares denominados domínios tipo Kunitz (K-1; K-2; K-3). Em um primeiro momento ocorre a inibição reversível do fator Xa, a partir da interação deste fator ao domínio K-2. Esta interação aumenta a afinidade do domínio K-1 pelo complexo TF/VIIa e desta forma, o complexo é inibido. Já o domínio K-3 não possui uma ligação direta com a inibição do complexo TF/VIIa, mas serve como sítio de ligação para heparina e outros glicosaminoglicanos e está envolvido na ligação do TFPI à lipoproteínas e à superfície celular (CATERINE *et al.*, 2013; CRAWLEY e LANE, 2008; MINE *et al.*, 2002).

2.1.2.2 Antitrombina III (AT)

A AT é uma glicoproteína de 58 kDa de massa molecular, presente no plasma em concentrações de 150 µg/mL. A AT é uma serpina, ou seja, uma proteína inibidora de serino-proteases. Ela inibe a ação da trombina e dos fatores IXa e Xa. (PROST, 1986; ROSENBERG e ROSENBERG, 1984). Na presença de heparina o efeito inibitório da AT é aumentado em ~600 vezes em relação ao FXa, ~2000 vezes em relação à trombina e $\sim 10^6$ vezes em relação ao FIXa. Esses aumentos dramáticos na inibição dessas três enzimas constituem a base para o uso clínico da heparina como um anticoagulante desde a década de 1940 (DESAI, 2005).

2.1.2.3 Cofator II da heparina (HCII)

O HCII é uma glicoproteína de aproximadamente 66 kDa, homóloga à antitrombina, sendo também membro da família das serpinas. Sua ação é direcionada exclusivamente à inibição da trombina, sem exercer atividade inibitória nas outras proteases envolvidas na cascata de coagulação. A atividade de inibição da trombina pelo HCII é muito lenta, demorando cerca de 5 minutos para reduzir em 50% a atividade da trombina quando o HCII está presente numa concentração de 1 μmol/L. Porém, na presença de heparina, heparam sulfato ou dermatam sulfato, a atividade do HCII é potencializada incrivelmente, sendo que na mesma condição anterior, mas com o acréscimo de heparina, o HCII demora cerca de 50 milissegundo para reduzir a atividade da trombina em 50% (RAGG, 1986; TOLLEFSEN, 2007; TOLLEFSEN e PESTKA, 1985).

2.1.2.4 Proteína C e proteína S

A proteína C (PC) é uma glicoproteína com 62 kDa de massa molecular, circulante no plasma humano em concentração de aproximadamente 4 μg/mL. A proteína C é ativada pela ação da trombina, e exerce sua função anticoagulante inibindo dois fatores da coagulação, o fator VIIIa e o Va (CATARINE *et al.*, 2013; ROSENBERG e ROSENBERG, 1984).

A proteína S (PS), com 69 kDa de massa molecular, age como um cofator da proteína C, potencializando sua atividade, a partir da formação do complexo PC-PS (SOARES *et al.*, 2006; WALKER, 1980).

2.2 Trombose

Quando ocorre um desequilíbrio na hemostasia o principal efeito desta desordem é a trombose, que é caracterizada pela formação de coágulos no sistema circulatório, na ausência de lesão vascular, podendo ocorrer tanto nas veias quanto nas artérias. Trombose arterial aguda é a principal causa dos casos de infarto do miocárdio e o tromboembolismo venoso é o terceiro maior responsável por morte associada a problemas cardiovasculares (MACKMAN, 2008).

2.2.1 Tromboembolismo Venoso

É o caso mais comum de morte evitável entre pacientes hospitalizados. Inclui duas manifestações clínicas, a trombose venosa profunda e a embolia pulmonar. São estimadas cerca de 100 mil mortes por ano por causa de tromboembolismo venoso nos Estados Unidos (AGNELLI e SONAGLIA, 2000; HEIT, 2008).

O trombo responsável pela trombose venosa profunda comumente inicia-se nas veias das panturrilhas, de onde se estende para as veias mais próximas ou pode se desprender da parede do vaso e ser carregado até os pulmões, onde irá bloquear a passagem de sangue, causando a embolia pulmonar (KEARON, 2003).

Alguns dos fatores de risco clínico para tromboembolismo venoso podem ser a idade (acima de 40 anos), obesidade, varizes, imobilização prolongada e desidratação além do uso de medicamentos como contraceptivos orais e para terapia de reposição hormonal. Também existem alguns fatores inerentes, tais como a resistência à proteína C ativada, deficiência da antitrombina, deficiência nas proteínas C e S, e fatores adquiridos, como a síndrome do anticorpo antifosfolipídio e níveis de fator VIII elevados, que podem induzir o tromboembolismo venoso (BOMBELI e SPAHN, 2004; PIAZZA e GOLDHABER, 2006).

2.2.2 Trombose Arterial

Comumente ocorre após a erosão ou a ruptura de uma placa aterosclerótica e através de trombos mediados por plaquetas. A trombose arterial pode causar injúria isquêmica em tecidos com um leito terminal vascular, como pulmões. Sendo, isquemia cardíaca e acidente vascular cerebral (AVC) as mais severas manifestações clínicas da trombose arterial. Os fatores de risco para trombose arterial são basicamente os mesmos que para tromboembolismo venoso (PREVITALI *et al.*, 2011).

2.3 Agentes anticoagulante e antitrombótico

Apesar dos Glicosaminoglicanos (GAGs) não serem encontrados naturalmente na corrente sanguínea sabe-se que eles apresentam grandes efeitos anticoagulante e antitrombótico quando utilizados como fármacos. O principal exemplo disso é a heparina que é utilizada no tratamento de tromboses e doenças relacionadas à coagulação. Ela apresenta grande efeito anticoagulante indireto, aumentando consideravelmente a atividade da antitrombina e do cofator II da heparina (PATEL *et al.*, 2007; PETITOU *et al.*, 2003).

A heparina comercial é obtida do tecido de animais utilizados no consumo humano, principalmente, do intestino suíno e do pulmão bovino (LINHARDT, 2003). Porém, nos anos 90 a heparina bovina passou a não ser mais utilizada na Europa e nos Estados Unidos, devido ao medo de contaminação com o príon responsável pela encefalopatia espongiforme bovina, popularmente conhecida como doença da vaca louca, o que representou uma ameaça ao mercado de heparina devido à potencial escassez da matéria prima (KORT *et al*., 2005).

Apesar de sua ampla utilização, a heparina tem algumas limitações que dificultam sua aplicação clínica, como suas características farmacocinéticas e biofísicas. As heparinas não fracionadas (*unfractionated heparin*, UFH) quando administradas por via endovenosa apresentam interação não específica com proteínas circulantes. Por consequência a AT compete com outras proteínas plasmáticas na ligação com a UFH e, desta forma, o efeito anticoagulante torna-se imprevisível, necessitando de monitoramento laboratorial frequente do paciente. Um fator complicante do uso de heparina é a hemorragia, que surge após a utilização de altas concentrações. Quanto às propriedades biofísicas, UFH não tem a capacidade de inibir fatores de coagulação ligados à superfície dos vasos, tais como trombina ligada à fibrina e FXa ligado a fosfolipídios (PATEL *et al.*, 2007).

Além disso, o uso prolongado de heparina pode levar ao desenvolvimento de trombocitopenia, a qual pode ser do tipo I – que é uma resposta não imunogênica à terapia, causando uma leve diminuição no nível de plaquetas livres no sangue e apenas a interrupção do tratamento com heparina é o suficiente para que o número de plaquetas no sangue volte ao normal; ou pode ser do tipo II – a qual é uma resposta imune causada principalmente por um anticorpo de imonoglobulina G que se liga a plaquetas na presença de heparina, o que resulta na ativação de plaquetas, agregação plaquetária e aumento na geração de trombina, levando a complicações que ameaçam os membros e a

vida do paciente (BRIEGER *et al.*, 1998; MENAJOVSKY, 2005; THONG e KAM, 2005).

Devido estas evidências, novas alternativas para UFHs são necessárias. Com este intuíto, desde a década de 1990 foram desemvolvidas as heparinas de baixa massa molecular (*low molecular weight heparin*, LMWH), que são derivadas da clivagem enzimática ou química de UFH, ligam-se menos fortemente às proteínas do plasma e dos tecidos, aumentando sua biodisponibilidade à AT (COHEN, 2000). Além disso, quando LMWHs são utilizadas é observada uma menor propensão a hemorragias, quando comparadas a UFH, utilizando-se a mesma atividade anticoagulante específica (CALABRESE *et al.*, 2002).

Após o surgimeto das LMWHs foram feitos testes em pacientes com trombose venosa profunda. Um grupo de pacientes foi tratado com UFH intravenosa e outro grupo com LMWH subcutânea, sendo ambas as heparinas de origem suína. Os pacientes tratados com UFH tiveram que permanecer no hospital de 7 a 10 dias para monitoramento de possíveis efeitos colaterais, enquanto os pacientes tratados com LMWH receberam as primeiras doses no hospital, e puderam continuar com o tratamento em casa. Estes estudos forneceram a base para a alteração do plano de tratamento desta enfermidade em hospitais (BRATT *et al*., 1986; KAKKAR, 2004).

Por outro lado, a heparina não é o único GAG que apresenta efeito anticoagulante e antitrombótico. Um exemplo é o dermatam sulfato que potencializa o efeito do cofator II da heparina, aumentando sua atividade em mais de 1000 vezes (TOLLEFSEN, 2010; HALLDÓRSDÓTTIR *et al.*, 2006; MAIMONE e TOLLEFSEN, 1990; THELIN *et al.*, 2013). Além disso, o condroitim sulfato extraído de fontes alternativas vem sendo testado e demonstra bons efeitos anticoagulante (MOU *et al*., 2017) e antitrombótico (GUI *et al.*, 2015).

Heparam sulfato, que tem grande similaridade estrutural com a heparina também apresenta estes efeitos e já foi comprovado que as células endoteliais vasculares produzem HS com alta afinidade pela antitrombina, aumentando seu efeito anticoagulante (SHWORAK et al., 2010). Além disso, HS extraído de diferentes fontes apresenta boa atividade anticoagulante (SARAVANAN e SHANMUGAM, 2011).

2.4 Glicosaminoglicanos

Glicosaminoglicanos (GAGs) são uma família importante de polissacarídeos aniônicos, constituídos por unidades dissacarídicas repetitivas de hexosamina (glucosamina ou galactosamina) e ácido urônico (ácido glucurônico ou ácido idurônico), ou galactose (em queratam sulfato) (NAKANO *et al.*, 2010; RUDD *et al.*, 2009). Cada tipo de GAG apresenta diferente densidade de carga negativa em sua estrutura e isso faz com que eles apresentem propriedades estruturais e biológicas distintas (GAMA e HSIEH-WILSON, 2005; TINGBØ *et al.*, 2012).

Os GAGs mais comuns são condroitim sulfato (CS – condroitin sulfate), dermatam sulfato (DS – *dermatan sulfate*), ácido hialurônico (HA – *hyaluronic acid*), Queratam sulfato (KS – *keratan sulfate*), heparina (*heparin*) e heparam sulfato (HS – *heparan sulfate*) (Tabela 1) (NAKANO *et al.*, 2010).

2.4.1 Condroitim Sulfato e Dermatam Sulfato

O condroitim sulfato (CS) é um polissacarídeo composto por unidades dissacarídicas de →4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→ que se repetem ao longo da cadeia, podendo haver sulfatação nas posições C-4 ou C-6 da N-acetilgalactosamina (GalNAc) (FIGURA 2A). As moléculas de CS são divididas em CS-4 e CS-6, onde CS-4 é rico em dissacarídeos O-sulfatados na posição C-4 das unidades de GalNAc e CS-6 é rico em dissacarídeos O-sulfatados na posição C-6 das unidades de GalNAc. Contudo, o grau de sulfatação e a posição do grupo sulfato varia de tecido para tecido e de espécie para espécie. A massa molecular também varia, sendo que para CS-4 a massa molecular pode variar de 40 a 80 kDa e para CS-6 pode variar de 5 a 50 kDa (NAKANO *et al.*, 2010; RUDD *et al.*, 2009).

Por sua vez, o dermatam sulfato (DS) apresenta resíduos de ácido α-L-idurônico ou ácido β-D-glucurônico, ligados à galactosamina, constituindo unidades dissacarídicas de →4)-ácido urônico-(1→3)-β-D-GalNAc-(1→ (FIGURA 2B). Pode apresentar O-sulfatação em C-4 e C-6 da galactosamina e em C-2 do ácido idurônico,

sendo desta forma relativamente mais sulfatado que o condroitim sulfato. Sua massa molecular varia de 11 a 25 kDa (MAIMONE e TOLLEFSEN, 1990; TROWBRIDGE e GALLO, 2002).

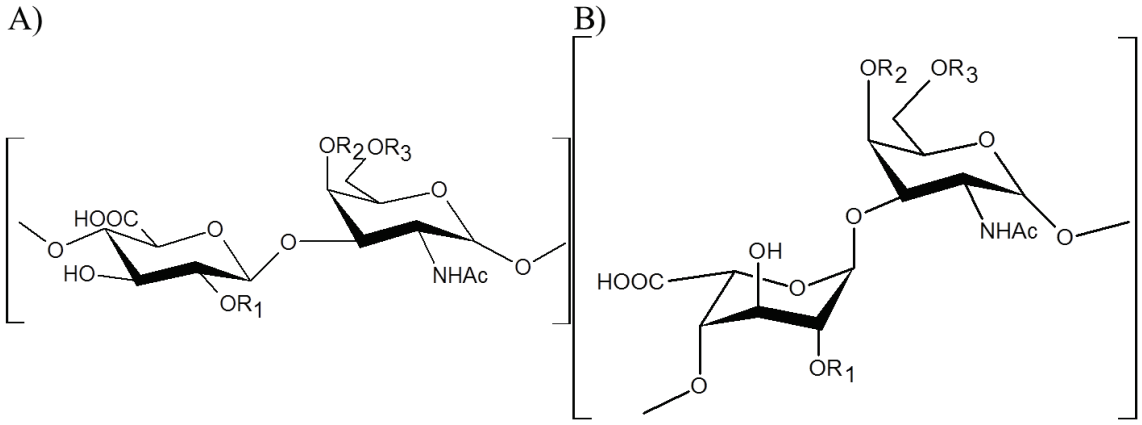


FIGURA 4: UNIDIDADE DISSACARÍDICA REPETITIVA DE CS E DS, →4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→ (A). UNIDADE DISSACARÍDICA REPETITICA DE DS, →4)-α-L-IdoA-(1→3)-β-D-GalNAc-(1→ (B). R1 = SO_3^-/H, R2 = SO_3^-/H, R3 = SO_3^-/H. *Fonte:* O AUTOR

CS é o GAG mais abundante nas articulações e contribui para a lubrificação das juntas neste local, além de ativar enzimas na matriz extracelular. Clinicamente CS é utilizado no tratamento de osteoartrite, onde ele melhora a mobilidade do joelho e diminui a dor (ROTH *et al.*, 2008; VERGÉS *et al*., 2005; VOLPI, 2006).

DS liga-se a diversos PGs que possuem diferentes efeitos, afetando a função de fatores de crescimento, se depositando nos espaços entre as fibras de colágeno estabilizando a matriz extracelular contra estresse mecânico. Além disso, o DS ocorre como GAG circulante no sangue e se liga a vários tipos de proteínas que participam da cascata de coagulação. (DU *et al*., 2007; ROTH *et al.*, 2008; SUGAHARA *et al*., 2003).

2.4.2 Ácido Hialurônico

O ácido hialurônico (HA) é o único glicosaminoglicano não sulfatado, sendo composto de dissacarídeos repetitivos de →4)-β-D-GlcA-(1→3)-β-D-GlcNAc-(1→

(FIGURA 3). Ele tem massa molecular muito grande quando comparado aos outros GAGs, com 100 a 10.000 kDa (KAKEHI *et al.*, 2003; NAKANO *et al.*, 2010).

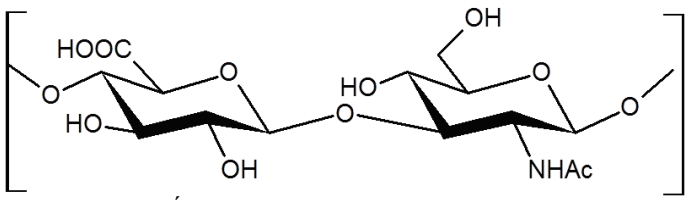


FIGURA 5: UNIDADE DISSACARÍDICA REPETITIVA DE HA, →4)-β-D-GlcA-(1→3)-β-D-GlcNAc-(1→. *Fonte:* O AUTOR

O HA é produzido na face interna da membrana plasmática e representa mais de 50% do conteúdo da matriz extracelular da pele, sendo responsável por sua estabilidade, controla o conteúdo de água do tecido, lubrificação, integridade estrutural (ROTH *et al.*, 2008). Injeções locais de HA restauram as propriedades físicas do fluido sinovial e é empregado no tratamento de osteoartrite (GOSSEC e DOUGADOS, 2004).

2.4.3 Queratam Sulfato

O queratam sulfato (KS) é constituído por unidades repetitivas de →3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→ (FIGURA 4), que são normalmente O-sulfatadas em C-6 de um ou ambos os monossacarídeos constituintes. Sua massa molecular pode variar de menos de 6 kDa a mais de 50 kDa dependendo do organismo e do tecido de onde for extraído (BHAVANANDAN e MEYER, 1967; HIRANO *et al.*, 1961; MATHEWS e CIFONELLI, 1965; PEÑA *et al.*, 1998; SENO *et al.*, 1965).

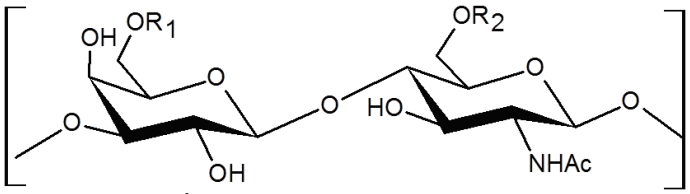


FIGURA 6: UNIDADE DISSACARÍDICA REPETITIVA DE KS, →3)-β-D-Gal-(1→4)-β-D-GlcNAc-(1→. R1 = SO_3^-/H, R2 = SO_3^-/H. *Fonte:* O AUTOR

KS está presente em grandes quantidades na matriz extracelular das córneas, ossos e cérebro. Seu tamanho e padrão de sulfatação varia com a idade, tipo de célula, órgão. (BROWN *et al*., 1998; CHRISTNER *et al.,* 1979; KRUSIUS *et al*., 1986; ROTH *et al.*, 2008). Como o principal tecido que contem KS é a cartilagem, sua presença no soro, urina ou líquido sinovial pode ser usado como marcador para quantificar os danos da cartilagem em doenças degenerativas (VOLPI, 2006).

2.4.4 Heparina e Heparam Sulfato

A heparina é constituída por unidades de α-L-IdoA ou β-D-GlcA e α-D-GlcN 1→4 ligadas (FIGURA 5). O ácido urônico consiste normalmente de 90% de ácido idurônico e 10% de ácido glucurônico. É altamente substituída com resíduos N-sulfato na posição C-2 e O-sulfato na posição C-6 dos resíduos de glucosamina e na posição C-2 dos resíduos de ácido idurônico, apresentando cerca de 2,7 grupos sulfatos por unidade dissacarídica. A massa molecular da heparina varia de 5 a 40 kDa, tendo como massa molecular média aproximadamente 18 kDa (CASU, 2005; HILEMAN *et al.*, 1998; LINHARDT, 2003; ROSENFELD *et al.*, 1991; TORRI e GUERRINI, 2008).

O heparam sulfato (HS) é muito similar à heparina, mas apresenta características que tornam sua estrutura única. Assim como a heparina, o HS é um heteropolímero linear constituído por unidades de α-L-IdoA ou β-D-GlcA e α-D-GlcN 1→4 ligadas (Figura 5). É composto predominantemente por ácido glucurônico, podendo apresentar níveis substanciais de ácido idurônico. O HS apresenta cerca de 1 grupo sulfato por unidade dissacarídica, sendo que a sulfatação pode ocorrer na forma de O-sulfato na posição C-2 do ácido idurônico e/ou na forma N-sulfato na posição C-2 e O-sulfato na posição C-6 dos resíduos de glucosamina. A massa molecular do HS varia de 5 a 50 kDa, tendo como massa molecular média 29 kDa (HILEMAN *et al.*, 1998; KREUGER *et al.* 2006; SAMPAIO *et al*., 2006; SASISEKHARAN *et al*., 2002; STRINGER e GALLAGHER, 1997).

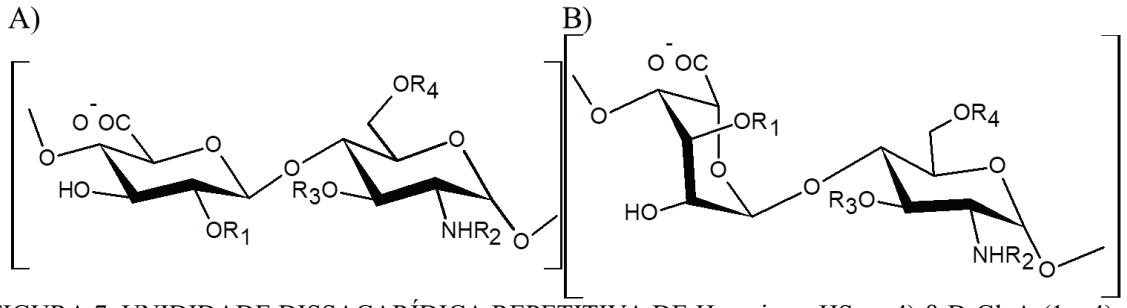


FIGURA 7: UNIDIDADE DISSACARÍDICA REPETITIVA DE Heparina e HS. →4)-β-D-GlcA-(1→4)-α-D-GlcN-(1→ (A). →4)-α-L-IdoA-(1→4)-α-D-GlcN-(1→ (B). R1 = SO_3^-/H, R2 = SO_3^-/H, R3 = SO_3^-/Ac, R4 = SO_3^-/H. *Fonte:* O AUTOR

A heparina é produzida nos grânulos dos mastócitos, onde interage com histamina, proteases e mediadores inflamatórios. É encontrada somente em alguns tecidos de vertebrados e invertebrados, principalmente nas mucosas dos pulmões e intestinos. Apesar de ter grande efeito anticoagulante quando utilizado como fármaco, endogenamente a heparina não é encontrada na corrente sanguínea e, até o momento, não se sabe exatamente qual sua função biológica (CARLSSON e KJELLÉN, 2012; GUNAY e LINHARDT, 1999; SAMPAIO *et al*., 2006).

HS, ao contrário da heparina, é produzido em todos os tecidos na forma de diferentes tipos de PGs e se encontra na face externa da membrana plasmática das células e na matriz extracelular. Está envolvido em uma grande gama de processos: na interação proteína-proteína, como correceptor para o fator de crescimento, protege proteínas da degradação, regula o transporte através de membranas basais e medeia a internalização de proteínas nas células (KREUGER *et al*., 2006; LI e VLODAVSKY, 2009).

2.5 Glicosaminoglicanos encontrados em peixes

A figura 1 mostra que os GAGs estão presentes em diversos grupos de animais, sendo os peixes um destes grupos. A comprovação de que GAGs estão presentes em peixes já foi feita por alguns pesquisadores.

Peña *et al.* (1998) isolaram queratam sulfato de larvas de *bonefish* (*Albula* sp.), que foi extraído por digestão enzimática das larvas com papaína, seguido de

precipitação com etanol (1,25 e 3 volumes) com 1% de acetato de sódio. O precipitado final foi estruturalmente caracterizado por análises de RMN, HPLC, TLC.

Flengsrud *et al.* (2010) conseguiram obter uma heparina parcialmente purificada do intestino e guelras de salmão (*Salmo salar*), com potencial atividade anticoagulante tanto *in vitro* quanto *in vivo*. Intestino ou guelras foram tratados com papaína, filtrados e os eluídos foram aplicados em coluna de troca aniônica. Os materiais obtidos foram então caracterizados por RMN, cromatografia de exclusão de tamanho, entre outros métodos.

Rodrigues *et al.* (2011) utilizaram a pele de tilápia do Nilo (*Oreochromis niloticus*) para extrair GAGs. Inicialmente foi feita proteólise da pele com papaína, precipitação dos GAGs com cloreto de cetilpiridínio a 10%, e lavagem e precipitação com etanol. Este material foi então aplicado em coluna de troca aniônica DEAE-celulose. O fracionamento dos GAGs foi feito por eluição com soluções de NaCl nas concentrações de 0,50 e 0,75 M. Os GAGs foram identificados por eletroforese em gel de agarose e em ambas as frações foi verificada apenas dermatam sulfato.

Tingbø *et al.* (2012) observaram a presença de ácido hialurônico, condroitim sulfato/dermatam sulfato e heparam sulfato no músculo esquelético de bacalhau do atlântico (*Gadus morhua* L.). O músculo foi submetido a proteólise com Pronase, seguido de tratamento com Benzonase, para degradação de DNA e RNA. Por fim os GAGs foram isolados em coluna de troca aniônica DEAE-Sephacel, os quais foram submetidos a degradação enzimática para análise quantitativa dos dissacarídeos por HPLC. Os autores verificaram que dependendo da dieta do animal, a quantidade de GAGs presente no tecido pode ser alterada.

Rodrigues *et al.* (2012) isolaram GAGs da pele de palombeta (*Chloroscombrus chrysurus*) e guaiúba (*Ocyurus chrysurus*). A pele foi submetida a proteólise com papaína, e os GAGs foram precipitados com cloreto de cetilpiridínio a 10%. Posteriormente este precipitado foi lavado e submetido a nova precipitação com etanol. O material obtido foi aplicado em coluna de troca aniônica DEAE-celulose. O fracionamento dos GAGs foi feito por eluição com soluções de NaCl nas concentrações de 0,50, 0,75 e 1,00 M. Os GAGs foram identificados por eletroforese em gel de agarose, onde foi possível confirmar a presença de dermatam sulfato na pele de palombeta nas frações eluídas com 0,75 e 1.00 M.

Arima *et al.* (2013) avaliaram a quantidade e diferença na composição de condroitim sulfato, dermatam sulfato e ácido hialurônico em diferentes tecidos de várias

espécies de peixes como, escamas de tilápia do Nilo, cabeça de cavala do Atlântico, estômago, intestino e átrio de atum do Pacífico. Para extração dos GAGs foi realizada proteólise do material deslipidificado com protease N Amano G. Posteriormente este material foi aplicado em coluna de troca aniônica DEAE-cellulose, que foi eluída com soluções de LiCl nas concentrações de 0,15, 0,5, 1,0, e 2,0 M. Essas frações foram degradadas enzimaticamente para análise quantitativa dos dissacarídeos por HPLC. A partir destes resultados foi verificado que dependendo do tecido e da espécie da qual o GAG é extraído, existem variações quanto à quantidade e grau de sulfatação do GAG.

Gui *et al.* (2015) utilizaram o crânio e a espinha dorsal de esturjão (híbrido de *Acipenser baerii* × *Acipenser schrenckii*) para extração de condroitim sulfato. O material foi deslipidificado com etanol 95% por 2 h. Após, o material foi submetido a proteólise com alcalase, seguido por tratamento com ácido tricloroacético (concentração final 5%), centrifugado e o sobrenadante foi precipitado com etanol (75% v/v) e centrifugado. O precipitado etanólico foi ressuspenso em água e misturado a solução de cloridrato de cetilpiridina (concentração final 1%), novamente. O precipitado foi então solubilizado em NaCl 2,5 M (1,6% m/v), precipitado com etanol (75% v/v), centrifugado e, este precipitado final, foi ressuspenso em agua, dialisado e liofilizado. Este material foi caracterizado como CS por RMN e HPLC.

3 JUSTIFICATIVA

Como já relatado, os GAGs estão presentes em peixes e a produção mundial total de peixes foi de 167 milhões de toneladas em 2014, destes 47,1 milhões de toneladas são de aquicultura de interior. O Brasil foi responsável por 474 mil toneladas (FAO, 2016). Em 2015 o Brasil produziu 219 mil toneladas de tilápia do Nilo (*Oreochromis niloticus*) e cerca de 13 mil toneladas de pacu (*Piaractus mesopotamicus*) (Dados IBGE, acessado 2017).

No Paraná a piscicultura é realizada em praticamente todo o Estado, sendo a principal região produtora a região oeste, com cerca de 50% da produção total do Estado. A principal espécie cultivada é a tilápia do Nilo, que compreende 84% do total de peixes produzidos (EMATER, 2017). A produção no ano de 2015 foi de aproximandamente 63.065 t de tilápia e 1.926 t de pacu (IBGE, 2017). Desta forma, existe a produção de uma grande quantidade de resíduos (vísceras), que não são aproveitados. Contudo, esse material que a princípio é descartado ou utilizado como ração pode servir de matéria prima para a extração e isolamento de glicosaminoglicanos com potenciais aplicações.

4 OBJETIVOS

4.1 Objetivo geral

Extrair, caracterizar e determinar possíveis atividades farmacológicas de glicosaminoglicanos obtidos de vísceras dos peixes Tilápia do Nilo (*Oreochromis niloticus*) e Pacu (*Piaractus mesopotamicus*), criados na região oeste do Paraná.

4.2 Objetivos específicos

- Determinar se as vísceras dos peixes tilápia do Nilo e pacu são fontes de glicosaminoglicanos;
- Extrair e caracterizar GAGs das vísceras dos peixes;
- Identificar os GAGs;
- Avaliar os GAGs quanto às suas propriedades anticoagulante e antitrombótica tanto *in vitro* quanto *in vivo*;

ARTIGO I

Viscera of fishes as raw material for extraction of glycosaminoglycans of pharmacological interest

Viscera of fishes as raw material for extraction of glycosaminoglycans of pharmacological interest

Alexsandro V. Nogueira [a], Gustavo R. Rossi [b], Marcello Iacomini [a], Guilherme L. Sassaki [a], Edvaldo S. Trindade [b], Thales R. Cipriani [a,*]

[a] Department of Biochemistry and Molecular Biology, Federal University of Paraná, CP 19046, CEP 81531-980, Curitiba, PR, Brazil

[b] Department of Cell and Molecular Biology, Federal University of Paraná, CEP 81531-980, Curitiba, PR, Brazil

* Corresponding author.

E-mail address: trcipriani@ufpr.br (T. R. Cipriani)

Abstract

World fisheries and aquaculture production totaled 167 million tons in 2014. This high fish production generates a lot of waste that could be used as raw material for extraction of substances of pharmacological interest. In this work, we extract and characterize glycosaminoglycans (GAGs) present in the viscera of Nile tilapia (*Oreochromis niloticus*) and pacu (*Piaractus mesopotamicus*), which are among the most vastly produced fishes in inland aquaculture in Brazil. Moreover, the anticoagulant activity of the GAGs fractions was evaluated. GAGs were obtained from total defatted viscera, after proteolysis, precipitation with ethanol, anion exchange chromatography and treatment with chondroitinase. Chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) were identified by agarose gel electrophoresis and NMR analyses. CS, DS and HS were identified in equivalent fractions obtained from both fishes, and all GAGs fractions showed anticoagulant activity.

Keywords: Dermatan sulfate; Chondroitin sulfate; Heparan sulfate; *Oreochromis niloticus*; *Piaractus mesopotamicus*; Anticoagulant activity.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), world fisheries and aquaculture production totaled 167 million tons in 2014, from which 47.1 million tons were from inland aquaculture. Brazil was responsible for the production of 474 thousand tons in inland aquaculture (FAO, 2016). In 2015, Brazil was responsible for the production of 219 thousand tons of Nile tilapia (*Oreochromis niloticus*) and about 13 thousand tons of pacu (*Piaractus mesopotamicus*) (IBGE, 2017). About 50% of the fish mass is residue (head, fins, skin and viscera), and about 15% of these are viscera. This residual material is often discarded for lack of a suitable destination (Souza, 2001). This large amount of waste can be used as raw material for the extraction of substances of pharmacological interest, such as glycosaminoglycans (GAGs).

GAGs are an important biological family of anionic polysaccharides consisting of repeating disaccharides units of aminosugar (D-galactosamine or D-glucosamine) and uronic acid (L-iduronic or D-glucuronic acid) or galactose (in keratan sulfate) (Nakano, Betti, & Pietrasik, 2010; Rudd et al., 2009). The most common GAGs are chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronic acid (HA), keratan sulfate (KS), heparin (Hep) and heparan sulfate (HS) (Nakano, Betti, & Pietrasik, 2010).

GAGs are absent in plantae, fungi and protist kingdoms. However, in the animal kingdom they are present in all species that have tissue organization, and HS and CS are the most abundant (Sampaio et al., 2006). In fishes, GAGs have been confirmed by some researchers. Peña, Williams, & Pfeiler (1998) isolated KS from bonefish (*Albula* sp.) larvae. Flengsrud, Larsen, & Ødegaard (2010) obtained Hep from Atlantic salmon (*Gadus morhua* L.) intestines. Tingbø et al. (2012) observed the presence of HA, CS/DS and HS in skeletal muscles of Atlantic cod (*Gadus morhua* L.). Arima et al. (2013) evaluated the amount and compositional difference of GAGs in various tissues of many species of fish and observed that different tissues and species present variation in the type and amount of GAG.

Despite GAGs are not found naturally in the bloodstream, it is known that they have great anticoagulant and antithrombotic effects when used as drugs. The main example of this is heparin, which is used in the treatment of thrombosis and coagulation-related diseases. It has a great indirect anticoagulant effect, significantly increasing the activity of antithrombin (AT) and heparin cofactor II (HCII) (Patel,

Berry, & Chan, 2007; Petitou et al., 2003). Commercial heparin is obtained from porcine and bovine intestines (Keire et al., 2015).

On the other hand, heparin is not the only GAG that has antithrombotic and anticoagulant effects. DS enhances the anticoagulant effect of HCII, increasing its activity by more than 1,000 times (Tollefsen, 2010; Halldórsdóttir, Zhang, & Tollefsen 2006; Maimone e Tollefsen, 1990; Thelin et al., 2013). CS has been tested and demonstrates good anticoagulant (Mou et al., 2017) and antithrombotic (Gui et al., 2015) effects. HS, which has great structural similarity with heparin, also has these effects (Saravanan, & Shanmugam, 2011). It has already been proved that vascular endothelial cells produce HS with high affinity for AT, increasing its anticoagulant effect (Shworak et al., 2010).

Considering the large amount of residue generated from fishing and fish farming and the potential of this residue to generate products of commercial interest, the aim of the present investigation was to verify if the viscera of the fishes Nile tilapia (*O. niloticus*) and pacu (*P. mesopotamicus*) are suitable as raw material for extraction of GAGs, and to evaluate if the extracted GAGs have anticoagulant activity.

2. Materials and Methods

2.1. Materials

The fish were supplied by *Big Peixe* (Cascavel, PR, Brazil). Alcalase (2.4 L FG; 1.7 g/mL of protein; 2.4 U/g) was supplied by LNF Latino Americana (Bento Gonçalves – RS – Brazil). Chondroitinase AC came from *Flavobacterium heparinum*, expressed in *Escherichia coli*, with specific activity ≥200 U/mg protein (Sigma-Aldrich).

2.2. GAGs extraction

Total viscera from *Oreochromis niloticus* (Nile tilapia) and *Piaractus mesopotamicus* (pacu) (6 and 5 kg, respectively) were fragmented and the lipids removed with acetone (4 times) and finally with a mix of chloroform:methanol 2:1 v/v, under stirring at room temperature. The material was then subjected to proteolysis with alcalase (1 mL/100 g of material/1 L of buffer) for 48 h under stirring in 0.1 M

phosphate buffer pH 8.2, at 50 °C. The material was then filtrated, the eluate treated with 10% trichloroacetic acid to precipitate proteins, and centrifuged at 12,000 g at 4 °C, for 30 minutes. The resulting supernatant was treated with 1 M NaCl and 3 volumes of ethanol and kept at -20 °C overnight. The precipitate was recovered by centrifugation at 12,000 g at 4 °C, for 30 minutes, solubilized in distilled water and dialyzed through a 3.5 kDa cut-off membrane against distilled water. The material was finally freeze-dried to give the crude extract of GAGs from pacu (PCE) and tilapia (TCE).

2.3. Fractionation of GAGs

GAGs were fractionated by anion exchange chromatography using a DEAE Sepharose Fast Flow resin in a column with 25 cm x 2.7 cm i.d., which was stabilized with distilled water until pH 5.0. After stabilization, PCE or TCE (1 g) was solubilized in distilled water (10 mL) and applied in the column. As mobile phases, distilled water and different concentrations of NaCl (0.15; 0.25; 0.5; 0.75; 1.0; 2.0 and 4.0 M) were used, which were collected until negative reaction for carbohydrates by the phenol–H_2SO_4 method (Dubois et al., 1956). The 0.75 fractions of both fishes were rechromatographed using DEAE Sepharose Fast Flow resin in a column with 12.5 cm x 2.1 cm i.d., using water and NaCl at concentrations of 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75 and 1.0 M as mobile phases. All fractions were dialyzed through a 3.5 kDa cut-off membrane against distilled water to remove salts, and freeze-dried.

2.4. Analysis of nuclear magnetic resonance (NMR)

NMR analyses were performed in a 600 MHz Bruker model Avance III spectrometer, equipped with a QXI inverse probe of 5 mm. The samples were dissolved in D_2O and then analyzed at 70 ºC. The chemical shifts of ^{1}H and ^{13}C were referenced in relation to 0.001% of TMSP-d_4 (2,2,3,3- tetradeuterium-3-trimethylilsilylpropionate) as internal standard ($\delta = 0$). 1D ^{1}H-NMR analyses were performed after 90° (p1) pulse calibration by evolution until 360° using a start p1 of 4 μs plus increment of 2 μs (p1 6.4-7.0 μs), calculation of offset (1,885.0-1,885.6 Hz) to obtain a spectrum width of 4,795 Hz, using 16 scans to give a signal/noise ratio (S/N) of at least 1,000:1 for the

anomeric region (90° pulse, relaxation delay = 4.0 s, number of time domain points = 65,536 and acquisition time = 6.832 s). Integration of H-1 areas was performed without tube spinning and respecting an HDO signal with a medium half line varying from 1.0-1.2 Hz and TMSP 0.8-1.0 Hz. Presaturation of residual HDO was carried out with the pulse program zgpr, which included presaturation during relaxation delay, using a relaxation delay = 4.0 s, number of time domain points = 65,536, and acquisition time = 6.832 s. 2D $^{1}H/^{13}C$-NMR analyses (HSQC), via double inept transfer with decoupling during acquisition, using sensitivity improvement trim pulses in inept transfer and shaped pulses for all 180 degree pulses on the ^{13}C channel (hsqcetgpsisp2.2 on Bruker spectrometers) were performed as described by Torri & Guerrini (2008). The spectral widths for HSQC were 3,595 Hz (^{1}H) and 5,031 Hz (^{13}C), experiments being recorded for quadrature detection in the indirect dimension, using 24 scans per series of 1 K x 320 W data points with zero filling in F1 (2 K) prior to Fourier transformation.

All chemical shifts were signaled based on comparison with standards of Hep, HS, CS, DS and literature data: Gargiulo et al. (2009), Lauder et al. (2011), Maccari, Galeotti, & Volpi (2015), Mansour et al. (2009), Mucci, Schenetti, & Volpi (2000), Naggi et al. (2016), Pfeiler et al. (2002), Sakai et al. (2003), Saravanan & Shanmugam (2011) (table S1).

2.5. Agarose gel electrophoresis

Electrophoresis was performed on 0.55% agarose gel in 0.05 M PDA (1,3-diaminopropan) buffer pH 9.0 (Dietrich & Dietrich, 1976). The samples (5 mg/mL) and standards of CS, DS and HS (1 mg/mL) were solubilized in distilled water and 5 µL of them were applied in the gel. Electrophoresis time was 2 h, at 80 mA, at 5 ºC. The gel was then treated with 0.1% cetavlon for 2 h to precipitate GAGs, dried and stained with of 0.1% toluidine blue solution and clarified with 1% acetic acid in 50% ethanol.

2.6. Chondroitinase AC treatment

Fractions treated with chondroitinase AC were solubilized in 25 mM phosphate buffer, pH 6.5, with 150 mM NaCl. Reactions were performed with 0.5 U chondroitinase AC for 50 mg of sample, at 37 °C, for 48 h. The enzyme was then

inactivated with high temperature (80 °C for 10 min), and the material ultrafiltered through a 10 kDa cut-off membrane (regenerated cellulose membrane, Millipore) to get retained (not degraded) and eluted (degraded) material. All fractions were dialyzed through a 3.5 kDa cut-off membrane against distilled water to remove salts, and freeze-dried.

2.7. Anticoagulant activity

The anticoagulant activity was determined *in vitro* by aPTT (activated partial thromboplastin time) using SYNTHETIC PHOSPHO kit - HEMOSIL (IL/USA), a COAG-A-MATE XM coagulometer (Organon Teknika Corporation), and a pool of citrated sheep plasma. Plasma (50 µL) was incubated at 37 °C with saline or GAGs (at 0.25 mg/mL) (50 µL) for 1 min. Then, rabbit cephalin (50 µL) was added. After 2.5 min, 25 mM $CaCl_2$ (50 µL) was added and the clotting time measured. Results were expressed as T_1/T_0, which is the ratio between clotting time in the presence (T_1) and absence of GAGs (T_0) in the incubation mixture ± standard error of the mean (SEM) (n = 2).

3. Results and discussion

3.1. GAGs extraction from fish viscera

The flowchart for GAGs extraction is presented in figure 1.

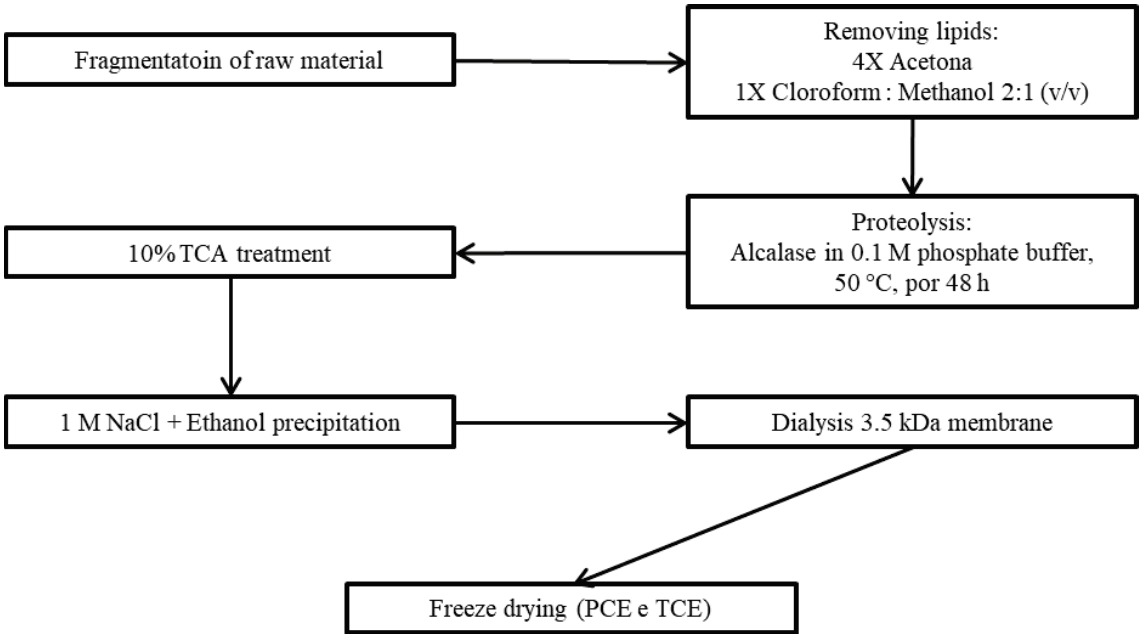


Figure 1: Flowchart of GAGs extraction from fish viscera; PCE – crude extract from pacu; TCE – crude extract from Nile tilapia.

Crude extracts from pacu (PCE) and Nile tilapia (TCE) yielded 0.18% and 0.15%, respectively (table 1). These yields were obtained from total *in natura* viscera, and correspond to freeze-dried materials. Analyses of NMR were performed (data not shown) and signals of GAGs were detected in both PCE and TCE.

Table 1: Yield of crude extracts of GAGs

Pacu	**Amount (g)**	**Yield* (%)**
Total viscera	6117	-
Defatted viscera	585	9.57
PCE	11	0.18
Nile tilapia	**Amount (g)**	**Yield* (%)**
Total viscera	5399	-
Defatted viscera	432	8.02
TCE	8	0.15

PCE – crude extract of GAGs from pacu; TCE – crude extract of GAGs from Nile tilapia;
* Yield was calculated from the total *in natura* viscera.

3.2. Fractionation of PCE and TCE

Considering the anionic characteristic of GAGs, they were fractionated by anion exchange chromatography. Elution with water and crescent concentrations of NaCl solutions (0.15 to 4.0 M) generated eight fractions (table 2).

Table 2: Yield of the fractions obtained after anion exchange chromatography of PCE and TCE

Fractions	Yield (g)	
	PCE (7.05 g)[a]	TCE (6.07 g)[a]
H_2O	1.4	1.7
0.15 M NaCl	2.5	2.1
0.25 M NaCl	0.7	0.6
0.5 M NaCl	1.1	0.9
0.75 M NaCl		0.2
1.0 M NaCl	0.1	0.06
2.0 M NaCl	0.04	0.02
4.0 M NaCl	0.00	0.00
Total[b]	6.0 (85%)	5.6 (92%)

[a] Total sample chromatographed.
[b] Total material recovered.

The fractions eluted with 2.0 M NaCl showed a very low yield (0.04 and 0.02 g from pacu (P) and Nile tilapia (T), respectively) and those eluted with 4.0 M NaCl did not yield any material. The fractions eluted with 0.15 to 1.0 M NaCl, which probably contain sulfated GAGs, were analyzed by agarose gel electrophoresis (figure 2). Fractions eluted with 0.75 M NaCl from both fishes (P-0.75 and T-0.75) showed bands corresponding to CS, DS and HS, whereas fractions eluted with 1.0 M NaCl (P-1.0 and T-1.0) showed bands corresponding to CS and DS. These GAGs were not present in the other fractions.

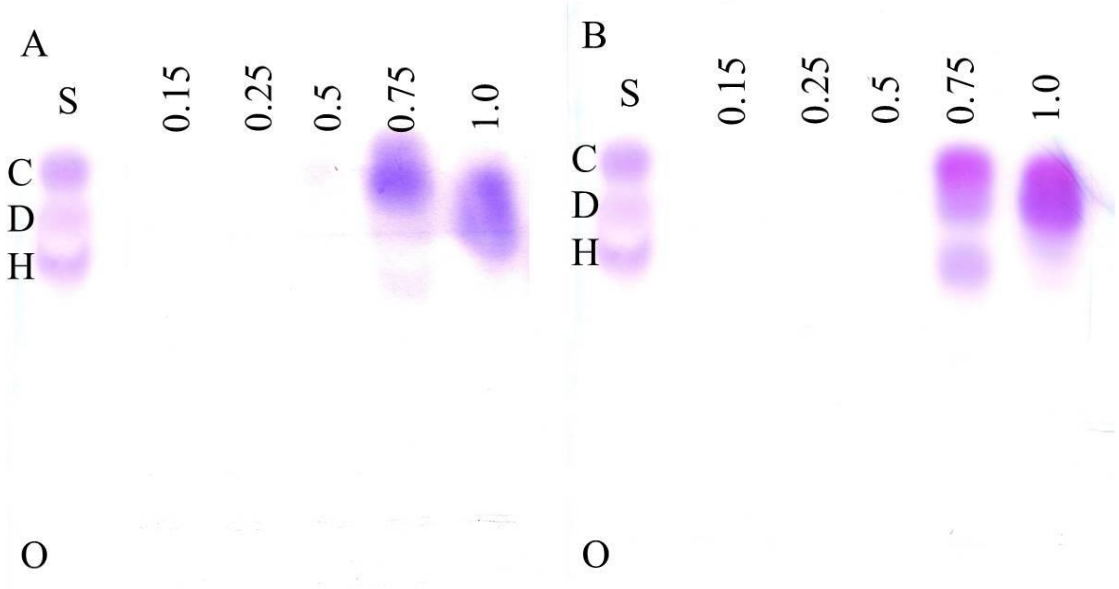


Figure 2: Agarose gel electrophoresis for analysis of sulfated GAGs from pacu (A) and Nile tilapia (B). A) Fractions from PCE. B) Fractions from TCE. O – Origin; S – Standards; C – Chondroitin sulfate; D – Dermatan sulfate; H – Heparan sulfate; 0.15 to 1.0 were the NaCl molar concentrations used as mobile phase in the fractionation by anion exchange chromatography.

HSQC analyses of P-0.75 and T-0.75 (figures 3A and S1A) showed typical $^{1}H/^{13}C$ correlations, in the anomeric region, of glucuronic acid linked to glucosamine N-acetylated (G-ANAc – 4.51/105.7 ppm), iduronic acid 2-sulfate (I2S – 5.20/103.0 ppm), glucosamine N-acetylated (ANAc – 5.30/100.7 ppm) and glucosamine N-sulfated (ANS – 5.36/100.2 ppm), referent to HS and/or Hep (correlations 2 to 5), and the correlations of glucuronic acid linked to galactosamine 4-sulfate (G-A4S – 4.44/106.4 ppm), glucuronic acid linked to galactosamine 6-sulfate (G-A6S – 4.48/106.9 ppm), galactosamine 4-sulfate (A4S – 4.59/103.7 ppm), galactosamine 6-sulfate (A6S – 4.59/103.7 ppm), referent to CS and DS (correlations 7 to 10), and galactosamine 4-sulfate linked to iduronic acid (A4S-I – 4.68/104.8 ppm) and iduronic acid (I – 4,90/105,8 ppm), referent to DS (correlations 11 and 12). And, HSQC analysis of P-1.0 and T-1.0 (figures 3B and S1B) showed $^{1}H/^{13}C$ correlations for CS and DS.

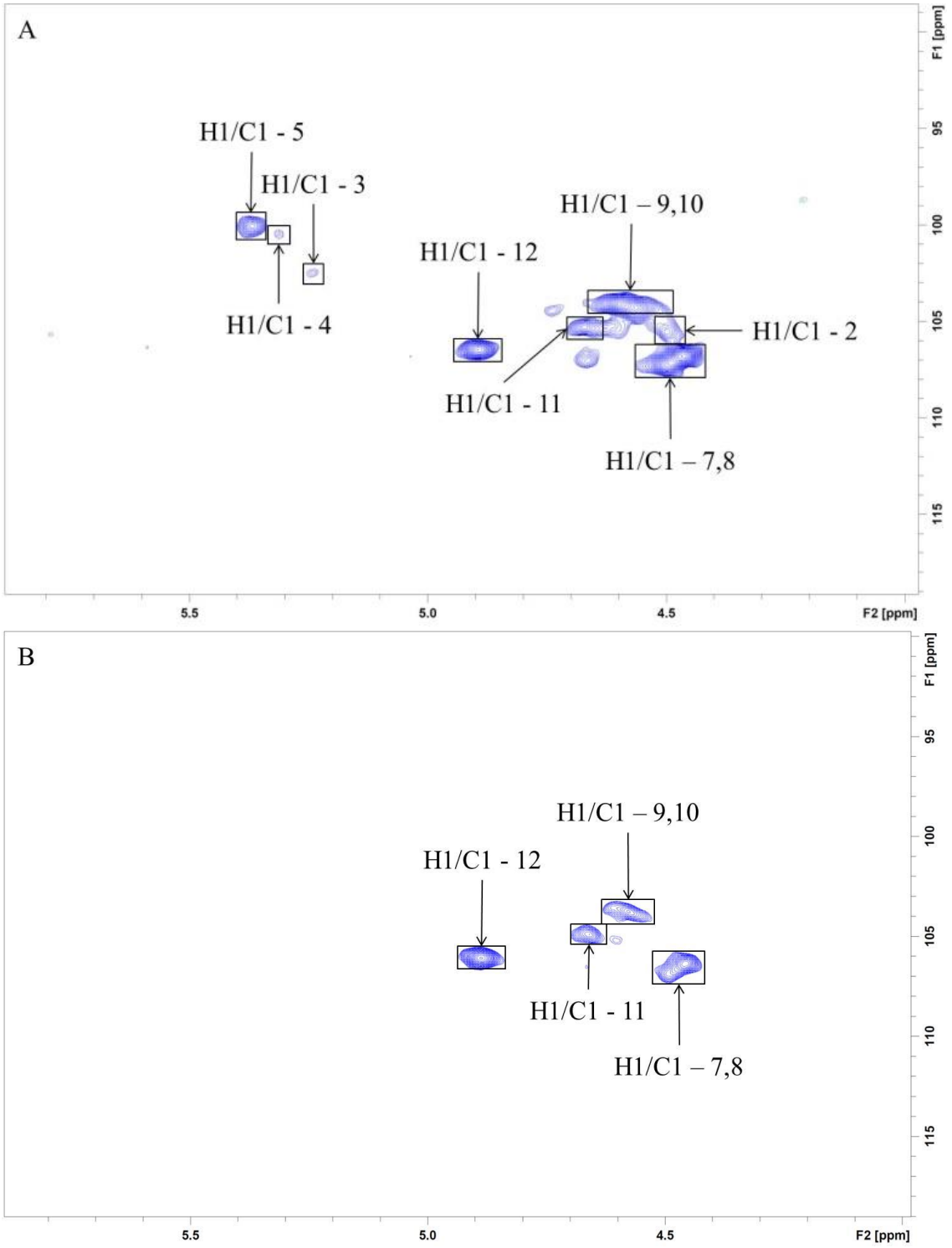


Figure 3: HSQC of the anomeric regions of P-0.75 (A), P-1.0 (B). 2 – G-ANAc; 3 – I2S; 4 – ANAc; 5 – ANS; 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate

According to the results of agarose gel electrophoresis (figure 2) and HSQC (figures 3 and S1) analyses, the fractionation method used allowed to obtain two fractions with GAGs from both fishes. P-0.75 and T-0.75, which contain CS, DS and HS or Hep, and P-1.0 and T-1.0, which contain CS and DS.

The yields of P-0.75 and T-0.75 were 2.3% and 3.3% and of P-1.0 and T-1.0 were 1.4% and 1.0%, taking into account the crude extracts PCE and TCE. When compared to literature, it was possible to observe that Rodrigues et al. (2011) obtained a yield of 10% of DS extracted from skin of Nile tilapia. Arima et al. (2013), in an extensive study on the amount of GAGs (CS, DS and HA) in different tissues of different fishes, verified variable yields, which reached a maximum of 1.3% depending on the species and the tissue. Gui et al. (2015) achieved 19% and 22% yield of CS in extracts of skull and spinal sturgeon. It is important to observe that in these works the authors extracted GAGs from tissues which have larger amount of a specific GAG. In the present study, we used total fish viscera, which is composed of many internal organs. This could explain the variety and the yield of GAGs obtained in this work compared to other works.

3.3. Rechromatography

In order to separate the GAGs present in P-0.75 and T-0.75, a new anion exchange chromatography was performed, using water and NaCl at 0.5, 0.525, 0.55, 0.575, 0.6, 0.625, 0.65, 0.675, 0.7, 0.725, 0.75 and 1.0 M as mobile phases. Using the phenol–H_2SO_4 method (Dubois et al., 1956), carbohydrates were detected in the fractions collected with 0.5 to 0.7 M NaCl, which were analyzed by agarose gel electrophoresis (figure 4).

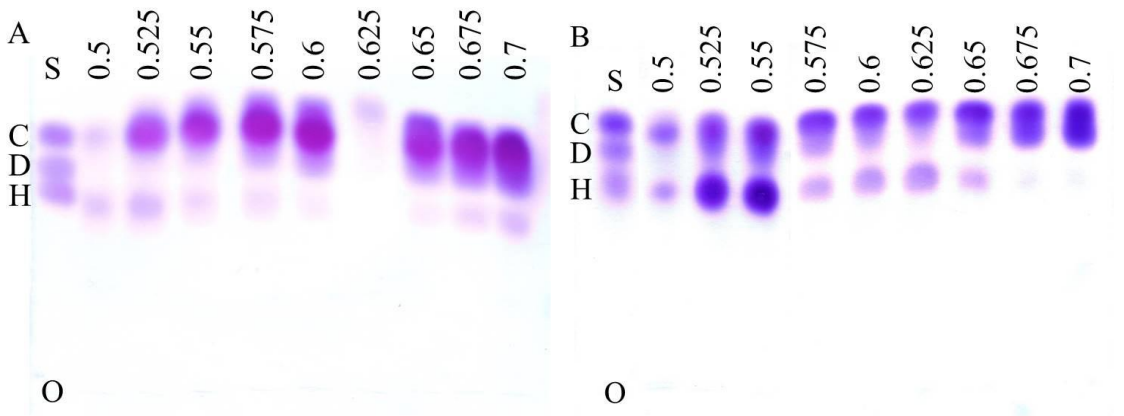


Figure 4: Agarose gel electrophoresis for analysis of sulfated GAGs from P-0.75 (A) and T-075 (B). O – Origin; S – Standards; C – Chondroitin sulfate; D – Dermatan sulfate; H – Heparan sulfate; 0.5 to 0.7 were the NaCl molar concentrations used as mobile phase in the fractionation by anion exchange chromatography.

The new chromatography did not entirely separate CS, DS and HS/Hep, but the fractions obtained could be grouped according to their electrophoretic pattern: groups P-0.75A (0.5 and 0.525 fractions), P-0.75B (0.55 to 0.6 fractions) and P-0.75C (0.65 to 0.7 fractions) from pacu, and T-0.75A (0.5 to 0.65 fractions) and T-0.75B (0.675 and 0.7 fractions) from Nile tilapia (table 3).

Table 3: Yield of fractions obtained after anion exchange chromatography of fractions 0.75.

Fractions	Yield (mg)	
	Pacu (230 mg)[a]	Nile tilapia (210 mg)[a]
0.75A	95.5	164.2
0.75B	98.2	42.9
0.75C	35.8	-

[a] Total sample rechromatographed.

HSQC analyses showed typical $^{1}H/^{13}C$ correlations of HS/Hep (correlations 1 to 5), CS and DS (correlations 7 to 12) in P-0.75A, P-0.75B, T-0.75A (figures 5A, 5B and S2A). P-0.75C and T-0.75B fractions showed only $^{1}H/^{13}C$ correlations of CS and DS (correlations 7 to 12) (figures 5C and S2B), although HS/Hep appeared in the electrophoresis analysis (figure 4). Furthermore, P-0.75A fraction showed more intense correlations of HS/Hep (correlations 4 - glucosamine N-acetylated and 5 - glucosamine N-sulfated in 5.30/100.7 and 5.36/100.2 ppm for H1/C1 and in 3.89/56.9 and 3.27/62.0 ppm for H2/C2) and P-0.75B showed more intense correlations of DS (correlations 11 - galactosamine 4-sulfate linked to iduronic acid and 12 - iduronic acid in 4.68/104.8 and

4.90/105.8 ppm for H1/C1 and 4.65/78.8 and 4.10/82.9 ppm for H4/C4). These results showed that the rechromatography was helpful to partially separate GAGs.

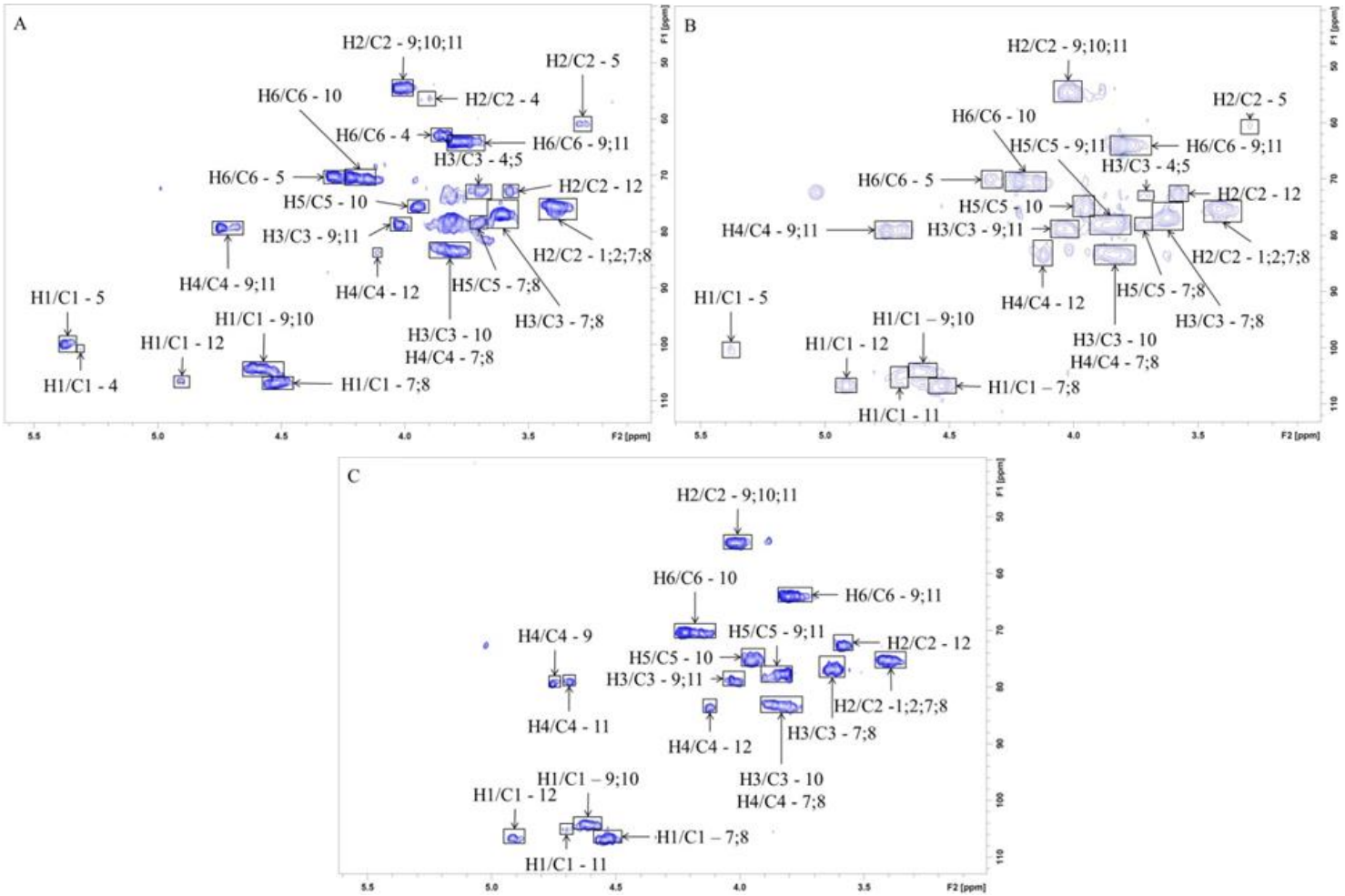


Figure 5: HSQC of P-0.75A (A), P-0.75B (B), P-0.75C (C). 1 – G-ANS; 2 – G-ANAc; 3 – I2S; 4 – ANAc; 5 – ANS; 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 1 - G-ANS – Glucuronic acid linked to glucosamine N-sulfated; 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate

3.4. Chondroitinase AC treatment

P-0.75A, P-1.0, T-0.75A and T-1.0 fractions were treated with chondroitinase AC, followed by ultrafiltration through a 10 kDa cut-off membrane, giving eluted fractions (P-0.75AE, T-0.75AE, P-1.0E and T-1.0E) and retained fractions (P-0.75AR, T-0.75AR, P-1.0R and T-1.0R). P-0.75AE and T-0.75AE yields corresponded to 30% and P-0.75AR and T-0.75AR to 70%, while P-1.0E and T-1.0E yields were insignificant (about 7%).

HSQC analysis showed typical $^{1}H/^{13}C$ correlations of reducing end-units (5.18/94.2 and 4.68/98.0 ppm) in P-0.75AE (figure 6B) and T-0.75AE (figure S3B), which are from low molecular weight fragments obtained after degradation of CS by chondroitinase AC. Moreover, correlations of H4/C4Δ at 5.91/109.7 and 5.84/110.2 ppm, corresponding to the lyase activity (4,5-unsaturated glucuronic acid of C4S and C6S), were also observed (Silva et al., 2015).

On the other hand, HSQC analysis of P-0.75AR (figure 6A) and T-0.75AR (figure S3A) showed $^{1}H/^{13}C$ correlations of DS (correlations 7 to 12). In these spectra, it was also possible to determine the presence of HS, due to the low intensity of H1/C1 correlation of IdoA in 5.20/103.0 ppm (correlation H1/C1 - 3), and H6/C6 correlation of glucosamine 6-sulfated in 4.27/69.8 ppm (correlation H6/C6 - 5), which are markers of Hep. In Hep, IdoA is predominant, while in DS, GlcA is predominant. Furthermore, in HS the glucosamine residues are predominantly N-acetylated and in Hep they are N-sulfated. Moreover, Hep is composed mainly by the disaccharide →4)-α-L-IdoA-2-O-sulfate(1→4)-α-D-Glc-N,6-sulfate(1→ while HS by the →4)-β-D-GlcA-(1→4)-α-D-GlcNAc(1→, that can be either N-acetylated or N-sulfated (Casu, 1989; Gallagher & Walker, 1985; Meneghetti et al., 2015; Rosenberg & Lam, 1979). The low intensity of typical correlations of IdoA and glucosamine 6-sulfated leads us to believe that HS and not Hep was extracted from pacu and Nile tilapia viscera.

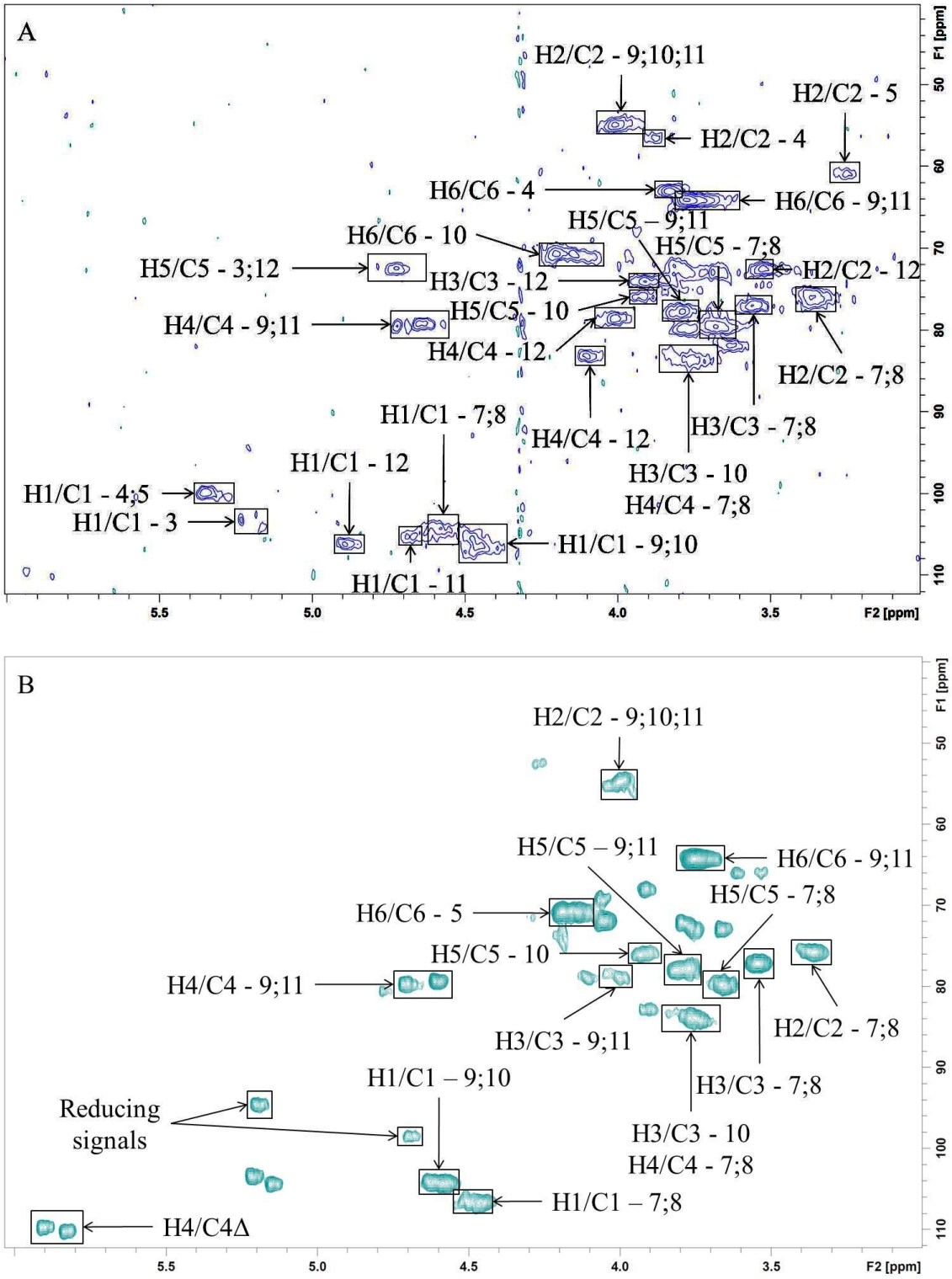


Figure 6: HSQC of P-0.75AR (A), P-0.75AE (B). 1 – G-ANS; 2 – G-ANAc; 3 – I2S; 4 – ANAc; 5 – ANS; 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 1 - G-ANS – Glucuronic acid linked to glucosamine N-sulfated; 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate

In relation to P-1.0R and T-1.0R fractions, both showed the same $^{1}H/^{13}C$ correlations on HSQC spectra (figures 7A and 7B, respectively), corresponding to DS (correlations 7 to 12). P-1.0E and T-1.0E fractions did not show $^{1}H/^{13}C$ correlations of reducing end-units and 4,5-unsaturated glucuronic acids that are formed by enzymatic degradation of CS (data not shown). Therefore, P-1.0 and T-1.0 fractions corresponded to purified DS.

Some works have shown the presence of these GAGs in different tissues of marine or freshwater fishes. Gui et al. (2015) characterized CS from sturgeon skull and sturgeon backbone. He et al. (2014) optimized the extraction of CS from fish bones. Panagos et al. (2014) characterized hyaluronic acid, chondroitin and DS from lumpsucker fish. Maccari, Galeotti, & Volpi (2015) characterized CS from monkfish, spiny dogfish, codfish, salmon and tuna, and showed the existence of a variety in the amount of this GAG in the bones of the different fishes. Zhang, Xie, & Linhardt (2014) isolated and characterized different types of CS from the head of red salmon. Rodrigues et al. (2011) purified DS from Nile tilapia skin. Zhang et al. (2009) characterized CS and HS from zebrafish in different ages and observed that there is a dynamic modification of the major GAGs during zebrafish development. Flengsrud, Larsen, & Ødegaard (2010) characterized a partially purified Hep from intestine and gills of Atlantic salmon. Tingbø et al. (2005) found different GAGs (chondroitin, DS, HS and keratan sulfate) in skeletal muscles of Atlantic cod and spotted wolffish and observed that there is a different proportion of the GAGs between the fishes available. Arima et al. (2013) determined that chondroitin, DS and hyaluronic acid are present in tissues (skin, gill, stomach, intestines, head, spine, fin, dorsal fin and tail fin) of various fishes (Japanese jack mackerel, Atlantic mackerel, Pacific bluefin tuna, golden threadfin bream, Nile tilapia, eelpout, broadbanded thornyhead, rough snailfish, yellowfin sole, squid and gonatid squid), in different proportions.

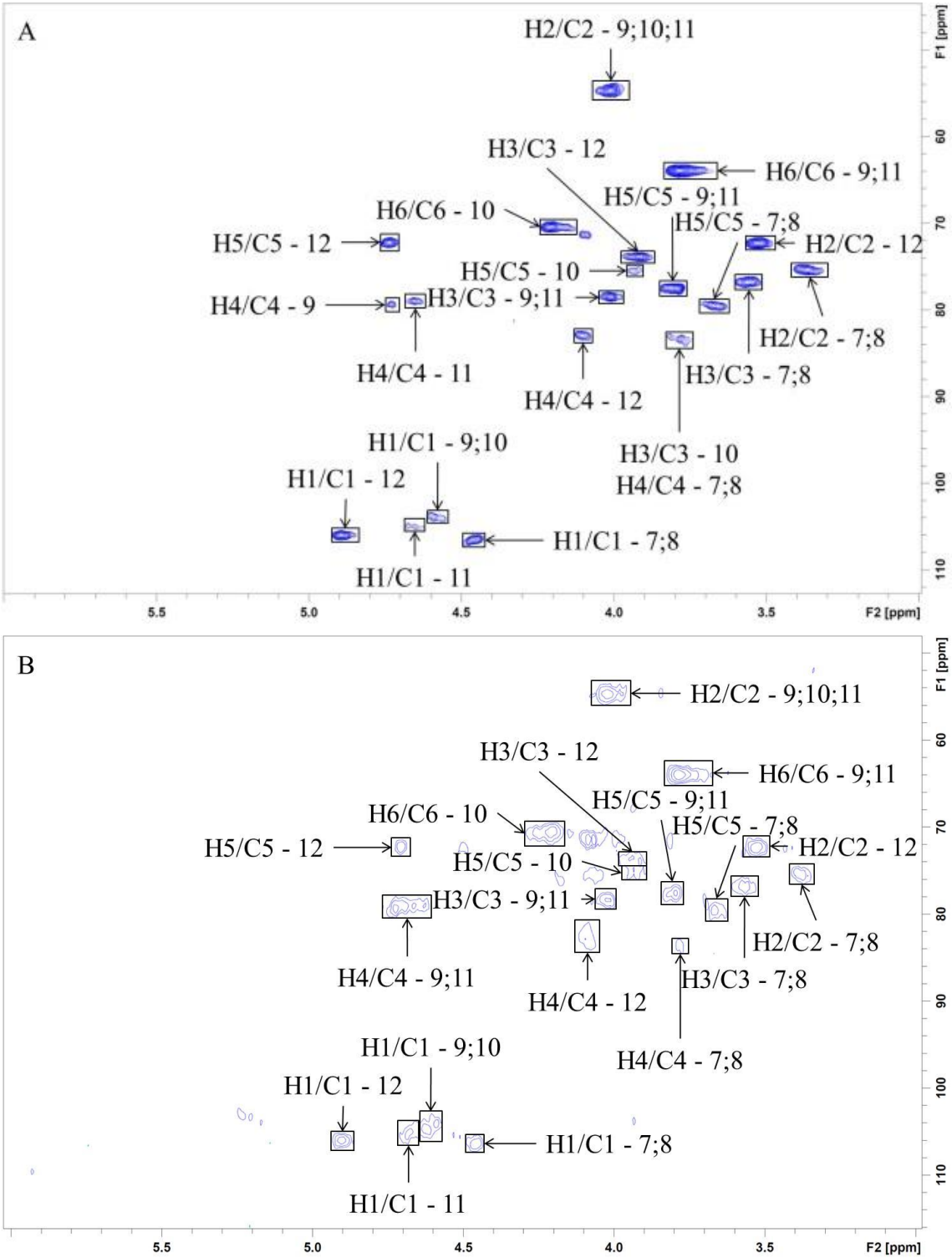


Figure 7: HSQC of P-1.0R (A) and T-1.0R (B). 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 1 - G-ANS – Glucuronic acid linked to glucosamine N-sulfated; 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate

3.5. Anticoagulant activity

Anticoagulant activity is frequently observed in GAGs and this property makes them molecules of pharmacological interest. This activity was determined here for GAGs fractions from pacu and Nile tilapia, at 0.25 mg/mL, using aPTT assay. All samples tested were able to increase aPTT, showing anticoagulant activity (figure 8).

The Nile tilapia samples had the best activity, where T-1.0R, T-0.75AR and T-0.75B increased normal clotting time at 2.8, 3.3 and 2.3 times, respectively. The pacu samples P-1.0R, P-0.75AR, P-0.75B and P-0.75C increased normal coagulation time at 2.4, 2.3, 1.5 and 1.7 times, respectively. aPTT in the absence of GAGs samples was 25.6 s (normal clotting time).

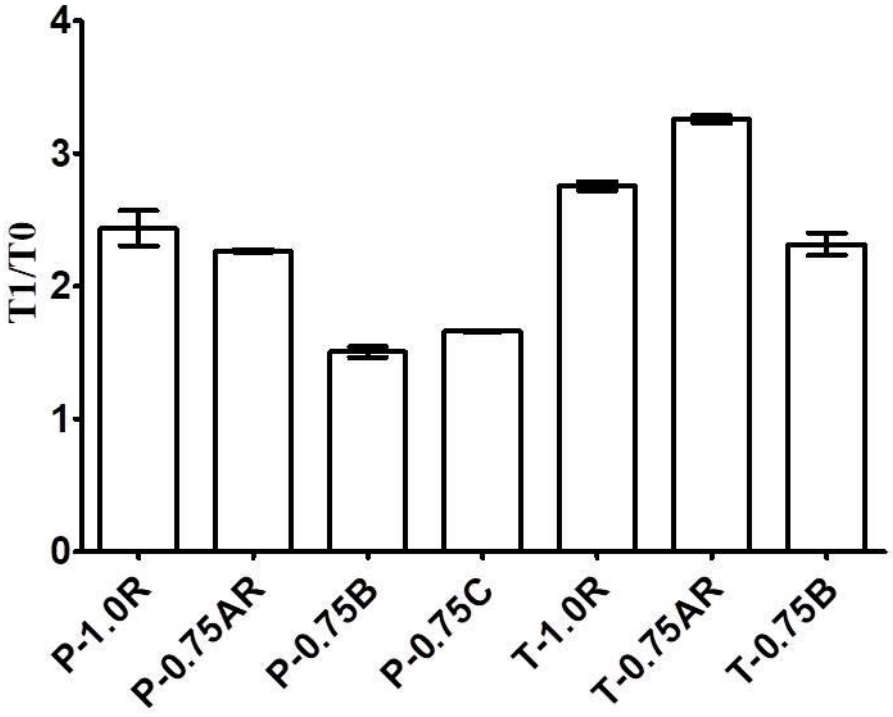


Figure 8: Anticoagulant activity was determined by aPTT assay for the pacu and Nile tilapia samples. Plasma (50 µL) was incubated at 37 °C with saline or GAGs (at the concentration 0.25 mg/mL) (50 µL) for 1 min. Then, rabbit cephalin (50 µL) was added. After 2.5 min, 0.025 M $CaCl_2$ (50 µL) was added and the clotting time measured. Results were expressed as T1/T0, which is the ratio between the clotting time in the presence (T1) and absence of GAGs (T0) in the incubation mixture ± standard error of the mean (SEM) (n = 2). In the absence of GAGs, aPTT value was 25.6 s.

When compared with other works, the GAGs samples evaluated in this study showed good anticoagulant activity. Rodrigues et al. (2009) studied fractions with HS from skin of

common carp (*Cyprinus carpio*), which increased clotting time at 1.4 and 2.3 times (at 1.25 mg/mL). Rodrigues et al. (2011) extracted DS from skin of Nile tilapia, which increased clotting time at 1.3 and 4.1 times (at 1.0 mg/mL). Salles et al. (2017) also extracted DS from skin of Nile tilapia, which increased clotting time at 1.6 and 1.2 times (at 1.0 mg/mL). And, Krichen et al. (2017) extracted a mixture of HA, CS and DS from skins of smooth hound and grey triggerfish, which increased clotting time at 1.7 and 1.2 times (at 0.1 mg/mL).

4. Conclusions

Despite the low yield, the proposed method allowed the extraction and identification of different GAGs from viscera of the fishes Nile tilapia and pacu. The fractions obtained from both fishes showed a very similar profile of GAGs, according to electrophoresis and NMR analyses. CS, DS and HS were identified from P-0.75 an T-0.75 fractions, pure DS from P-1.0 and T-1.0 fractions, and all fractions showed anticoagulant activity. Therefore, this study indicates that the residue generated from fish farming can be used to obtain products of commercial interest, which can add value to fish production.

Acknowledgments

The authors would like to thank the Brazilian agencies *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq – Grant number 449176/2014-2) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for financial support; *Big Peixe* for the supply of the fishes; *Centro de Desenvolvimento de Testes e Ensaios Farmacêuticos* (CTEFAR), from Federal University of Santa Maria, for the supply of ovine plasma; and UFPR-RMN Center.

References

Arima K, Fujita H, Toita R, Imazu-Okada A, Tsutsumishita-Nakai N, Takeda N, Nakao Y, Wang H, Kawano M, Matsushita K, Tanaka H, Morimoto S, Nakamura A, Kitagaki M, Hieda Y, Hatto R, Watanabe A, Yumura T, Okuhara T, Hayashi H, Shimizu K, Nakayama K, Masuda S, Ishihara Y, Yoshioka S, Yoshioka S, Shirade S, Tamura J. (2013). Amounts and compositional analysis of glycosaminoglycans in the tissue of fish. *Carbohydrate Research*, 366, 25-32.
doi: 10.1016/j.carres.2012.11.010

Casu, B. (1989). Structure of heparin and heparin fragments. *Annals of the New York Academy of Sciences*. 556, 1–17.

Dietrich, C. P., & Dietrich, S. M. C. (1976). Electrophoretic Behaviour of Acidic Mucopolysaccharides in Diamine Buffers. *Analytical biochemistry*, 647, 645-647.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28, 350-356.
doi: 10.1021/ac60111a017

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Retrieved from http://www.fao.org/fishery/sofia/en. Accessed in August, 2017.

Flengsrud, R., Larsen, M. L., & Ødegaard, O. R. (2010). Purification, characterization and *in vivo* studies of salmon heparin. *Thrombosis Research*, 126, e409-e417.
doi: 10.1016/j.thromres.2010.07.004.

Gallagher, J. T., & Walker, A., (1985). Molecular distinctions between heparan sulphate and heparin. Analysis of sulphation patterns indicates that heparan sulphate and heparin are separate families of N-sulphated polysaccharides. *Biochemical Journal*. 230, 665–674.

Gargiulo, V., Lanzetta, R., Parrilli, M., & Castro, C. (2009). Structural analysis of chondroitin sulfate from *Scyliorhinus canicula*: A useful source of this polysaccharide. *Glycobiology*, 19, 1485-1491.
doi: 10.1093/glycob/cwp123.

Gui, M., Song, J., Zhang, L., Wang, S., Wu, R., Ma, C., & Li, P. (2015). Chemical characteristics and antithrombotic effect of chondroitinsulfates from sturgeon skull and sturgeon backbone. *Carbohydrate Polymers*, 123, 454-460.
doi: 10.1016/j.carbpol.2015.01.046.

Halldórsdóttir, A. M.; Zhang, L.; Tollefsen, D. M. (2006). N-Acetylgalactosamine 4,6-O-sulfate residues mediate binding and activation of heparin cofactor II by porcine mucosal dermatan sulfate. *Glycobiology*, 16, 693-701.
doi: 10.1093/glycob/cwj117

He, G., Yin, Y., Yan, X., & Yu, Q. (2014). Optimisation extraction of chondroitin sulfate from fish bone by high intensity pulsed electric fields. *Food Chemistry*, 164, 205-210.
doi: 10.1093/glycob/cwj117

IBGE, Retrieved from http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?c=3940&z=t&o=21. Accessed in August of 2017.

Keire, D., Mulloy, B., Chase, C., Al-Hakim, A., Cairatti, D., Gray, E., et al. (2015).Diversifying the global heparin supply chain: Reintroduction of bovine heparin inthe United States? Retrieved from http://www.pharmtech.com/diversifying-global-heparin-supply-chain-reintroduction-bovine-heparin-united-states. Accessed: 05.03.18.

Krichen, F.; Ghlissi, Z.; Amor, I. B.; Sayari, N.; Kalleld, R.; Gargouri, J.; Sahnoun, Z.; Boudawara, T.; Bougate, A. (2017). *In vitro* and *in vivo* anti-coagulant activity and toxicological studies of marine sulfated glycosaminoglycans. *Experimental and Toxicologic Pathology,* 69, 45-53.
doi: 10.1016/j.etp.2016.11.002.

Lauder, R. M., Huckerby, T. N., Nieduszynski, I. A., & Sadler, I. H. (2011). Characterisation of oligosaccharides from the chondroitin/dermatan sulphates: ^{1}H and ^{13}C NMR studies of oligosaccharides generated by nitrous acid depolymerisation. *Carbohydrate Research*, 346, 2222-2227.
doi: 10.1016/j.carres.2011.06.033.

Maccari, F., Galeotti, F., & Volpi, N. (2015). Isolation and structural characterization of chondroitin sulfatefrom bony fishes. *Carbohydrate Polymers*, 129, 143-147.
doi: 10.1016/j.carbpol.2015.04.059.

Maimone, M. M., & Tollefsen, D. M. (1990). Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. *The Journau of Biology Chemistry*. 265, 18263-18271.

Mansour, M. B., Dhahri, M., Bertholon, I., Ollivier, V., Bataille, I., Ajzenberg, N., et al. (2009). Characterization of a novel dermatan sulfate with high antithrombin activity from ray skin (*Raja radula*). *Thrombosis Research*, 123, 887-894.
doi: 10.1016/j.thromres.2008.09.009.

Meneghetti, M. C. Z., Hughes, A. J., Rudd, T. R., Nader, H. B., Powell, A. K., Yates E. A., & Lima. M. A. (2015) Heparan sulfate and heparin interactions with proteins. *Journal of the Royal Society Interface*. 12, 20150589.
doi: 10.1098/rsif.2015.0589.

Mou, J., Wang, C., Li, W., Yang, J. (2017). Purification, structural characterization and anticoagulant properties of fucosylated chondroitin sulfate isolated from *Holothuria mexicana*. *International Journal of Biological Macromolecules*, 98, 208-215.
doi: 10.1016/j.ijbiomac.2017.01.123.

Mucci, A., Schenetti, L., & Volpi, N. (2000). 1H and 13C nuclear magnetic resonance identification and characterization of components of chondroitin sulfates of various origin. *Carbohydrate Polymers*, 41, 37-45.
doi: 10.1016/S0144-8617(99)00075-2.

Naggi, A., Gardinia, C., Pedrinola, G., Mauria, L., Urso, E., Alekseeva, A., et al. (2016). Structural peculiarity and antithrombin binding region profile of mucosal bovine and porcine heparins. *Journal of Pharmaceutical and Biomedical Analysis*, 118, 52-63.
doi: 10.1016/J.JPBA.2015.10.001.

Nakano, T., Betti, M., & Pietrasik, Z. (2010). Extraction, isolation and analysis of chondroitin sulfate glycosaminoglycans. *Recent Patents on Food, Nutrition & Agriculture*, 2, 61-74.

Panagos, C. G., Thomson, D., Moss, C., Bavington, C. D., Ólafsson, H. G., & Uhrín, D. (2014). Characterisation of hyaluronic acid and chondroitin/dermatan sulfate from the lumpsucker fish*, C. lumpus*. *Carbohydrate Polymers*, 106, 25-33.
doi: 10.1016/j.carbpol.2014.01.090.

Patel, S., Berry, L. R., Chan, A. K. C. (2007). Covalent antithrombin-heparin complexes. *Thrombosis Research*, 120, 150-160.
doi: 10.1016/j.thromres.2006.08.003.

Peña, M., Williams, C., & Pfeiler, E. (1998). Structure of keratin sulfate from bonefish (*Albula* sp.) larvae deduced from NMR spectroscopy of keratanase-derived oligosaccharides. *Carbohydrate Research*, 309, 117-124.

Petitou, M., Casu, B., Lindahl, U. (2003). 1976-1983, a critical period in the history of heparin: the Discovery of the antithrombin binding site. *Biochimie*, 85, 83-89.

Pfeiler, E., Toyoda, H., Williams, M. D., & Nieman, R. A. (2002). Identification, structural analysis and function of hyaluronan in developing fish larvae (leptocephali). *Comparative Biochemistry and Physiology*, 132, 443-451.

Rodrigues, J. A. G., Quinderé, A. L. G., Queiroz, I. N. L., Coura, C. O., Araújo, G. S., & Benevides, N. M. B. (2011). Purification, physical and chemical characterization, and anticoagulant activity of glycosaminoglycans isolated from the skin of Nile tilapia (*Oreochromis niloticus*). *Acta Scientiarum. Technology*, 33, 233-241.
doi: 10.4025/actascitechnol.v33i3.9400

Rodrigues, J. A. G., Vanderlei, E. S. O., Queiroz, I. N. L., Quinderé, A. L. G., Benevides, N. M. B. (2009) Purification and anticoagulant activity of glycosaminoglycans isolated from the skin of common carp, *Cyprinus carpio*. *Revista Ciência Agronômica*, 40, 381-387.

Rosenberg, R. D., & Lam, L. (1979). Correlation between structure and function of heparin. *Proceedings of the National Academy of Sciences USA*. 76, 1218–1222.

Rudd, T. R., Skidmore, M. A., Guimond, S. E., Cosentino, C., Torri, G., Fernig, D. G., et al. (2009). Glycosaminoglycan origin and structure revealed by multivariate analysis of NMR and CD spectra. *Glycobiology*, 19, 52-67.
doi: 10.1093/glycob/cwn103.

Sakai, S., Kim, W. S., Lee, I. S., Kim, Y. S., Nakamura, A., Toida, T., & Imanari, T. (2003). Purification and characterization of dermatan sulfate from the skin of the eel, *Anguilla japonica*. *Carbohydrate Research*, 338, 263-269.

Salles, T. C., Rodrigues, J. A. G., Barcellos, P. G., Amaral, G. F., Araújo, I. W. F., Mourão, P. A. S. (2017). Inhibition of thrombin generation by dermatan sulfate isolated from the skin of *Oreochromis niloticus*. *Agrária*, 12, 98-104.
doi:10.5039/agraria.v12i1a5412

Sampaio, L. O., Tersariol, I. L. S., Lopes, C. C., Bouças, R. I., Nascimento, F. D., Rocha, H, A., & Nader, H. B. (2006). Heparins and heparan sulfates. Structure, distribution and protein

interaction. In: Verli, U. (Ed), (pp. 1-24). Insights into carbohydrates structure and biological function. Transworld Research Network.

Saravanan, R., & Shanmugam, A. (2011). Is isolation and characterization of heparan sulfate from marine scallop *Amussium pleuronectus* (Linne.) an alternative source of heparin?. *Carbohydrate Polymers*, 86, 1082-1084.
doi: 10.1016/j.carbpol.2011.05.015.

Shworak, N. W., Kobayashi, T., Agostini, A., Smits, N. C. (2010). Anticoagulant Heparan Sulfate: To Not Clot—Or Not?. *Progress in Molecular Biology and Translational* **Science**, 93, 153-177.
doi: 10.1016/S1877-1173(10)93008-1.

Silva, C., Novoa-Carballal, R., Reis, R. L., Pashkuleva, I. (2015). Following the enzymatic digestion of chondroitin sulfate by a simple GPC analysis. *Analytica Chimica Acta*, 885, 207-213.
doi: 10.1016/j.aca.2015.05.034.

Souza, M. L. R. (2001). Industrialização, comercialização e perspectivas. In.:Moreira, H. L. M., Vargas, L., Ribeiro, R. P., & Zimmermann, S. (Eds), (pp. 149-189). Fundamentos da moderna aquicultura. ULBRA.

Thelin, M. A., Bartolini, B., Axelsson, J., Gustafsson, R., Tykesson, E., Pera, E., Oldberg, A., Maccarana, M., Malmstrom, A. (2013). Biological functions of iduronic acid in chondroitin/dermatan sulfate. *FEBS journal*, 280, 2431-2446.
doi: 10.1111/febs.12214.

TingbØ, M. G., Kolset, S. O., Ofstad, R., Enersen, G., & Hannesson, K. O. (2005). Sulfated glycosaminoglycans in the extracellular matrix of muscle tissue in Atlantic cod (*Gadus morhua*) and Spotted wolffish (*Anarhichas minor*). *Comparative Biochemistry and Physiology*, 140, 349-357.
doi: 10.1016/j.cbpc.2004.09.032.

TingbØ, M. G., Pedersen, M. E., GrØndahl, F., Kolset, S. O., Veiseth-Kent, E., Enersen, G., & Hannesson, K. O. (2012). Type of carbohydrate in feed affects the expression of small leucine-rich proteoglycans (SLRPs) glycosaminoglycans (GAGs) and interleukins in skeletal muscle of Atlantic cod (*Gadus morhua* L.). *Fish & Shellfish Immunology*, 32, 582-589.
doi: 10.1016/j.fsi.2012.06.025.

Tollefsen, D. M. (2010). Vascular Dermatan Sulfate and Heparin Cofactor II. *Progress in Molecular Biology and Translational Science*, 93, 351-372.
doi: 10.1016/S1877-1173(10)93015-9.

Torri, G., & Guerrini, M. (2008). Quantitative 2D NMR analysis of glycosaminoglycans. In: Holzgrabe, U., Wawer, I., & Diehl B. (Eds.), (pp. 407-428). NMR spectroscopy in pharmaceutical analysis. Elsevier.

Zhang, F., Xie, J., & Linhardt, R. J. (2014). Isolation and structural characterization of glycosaminoglycans from heads of red salmon (*Oncorhynchus nerka*). *Jacobs Journal of Biotechnology and Bioengineering*, 1, 1-6.

Zhang, F., Zhang, Z., Thistle, R., McKeen, L., Hosoyama, S., Toida, T., et al. (2009). Structural characterization of glycosaminoglycans from zebrafish in different ages. *Glycoconjugate Journal*. 26, 211-218.
doi: 10.1007/s10719-008-9177-x.

Supplementary data

Title: Extraction and characterization of glycosaminoglycans from viscera of the fishes Oreochromis niloticus and Piaractus mesopotamicus
Journal:
Author: Alexsandro V. Nogueira, Marcello Iacomini, Guilherme L. Sassaki, Thales R. Cipriani*

Table S1: Chemical shift of carbon and hydrogen of standards based on bibliography data

	Monosaccharaides Unities		Heparin/Heparan Sulfate					
			H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
1	Glucuronic acid	G-ANS	4.60/105.4	3.38/77.0	-	-	-	-
2		G-ANAc	4.51/105.7		-	-	-	-
3	Iduronic acid	I2S	5.20/103.0	4.33/79.6	4.23/73.1	4.11/79.6	4.77/73.0	-
4	Glucosamine	ANAc	5.30/100.7	3.89/56.9	3.67/73.3	3.75/80.0	4.02/72.7	3.84/63.5*
5		ANS	5.36/100.2	3.27/62.0	3.74/73.0	3.82/80.2	4.10/72.5	4.27/69.8*
6		A*	5.45/100.2	3.45/60.4	-	-	-	-
			Chondroitin Sulfate/Dermatan Sulfate					
			H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
7	Glucuronic acid	G-A4S	4.44/106.4	3.36/75.5	3.56/75.4	3.75/84.2	3.68/77.8	-
8		G-A6S	4.48/106.9	3.37/76.6	3.55/76.8	3.78/83.4	3.67/79.4	-
9	Galactosamine	A4S	4.59/103.7	4.01/54.2	4.00/78.5	4.73/79.3	3.80/77.6	3.78/63.9
10		A6S			3.83/83.0	4.16/71.3	3.93/75.6	4.20/70.4
11		A4S-I	4.68/104.8	4.02/54.9	4.02/78.3	4.65/78.8	3.83/73.4	3.79/63.8
12	Iduronic acid	I	4.90/105.8	3.52/72.2	3.91/73.9	4.10/82.9	4.70/72.2	-

The chemical shifts were determined with this bibliography data - Guagiulo, Lanzetta, Parrilli, & Castro (2009), Lauder, Huckerby, Nieduszynski, & Sadler (2011), Maccari, Galeotti, & Volpi (2015), Mansour *et al*. (2009), Mucci, Schenetti, & Volpi (2000), Naggi *et al*. (2016), Pfeiler, Toyoda, Williams, & Nieman (2002), Sakai *et al*. (2003), Saravanan, & Shanmugam (2011).
1 - G-ANS – Glucuronic acid linked to glucosamine N-sulfated; 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 – I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 6 - A* – Glucosamine 3,4,6-sulfate; 7 – G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 – G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 – A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate.
* H6/C6 of glucosamine refers to C6 acetylated (3.84/63.5) and C6 sulfated (4.27/69.8).

Supplementary data

Title: Viscera of fishes as raw material for extraction of glycosaminoglycans
Journal: Fish and Fisheries
Author: Alexsandro V. Nogueira, Marcello Iacomini, Guilherme L. Sassaki, Thales R. Cipriani*

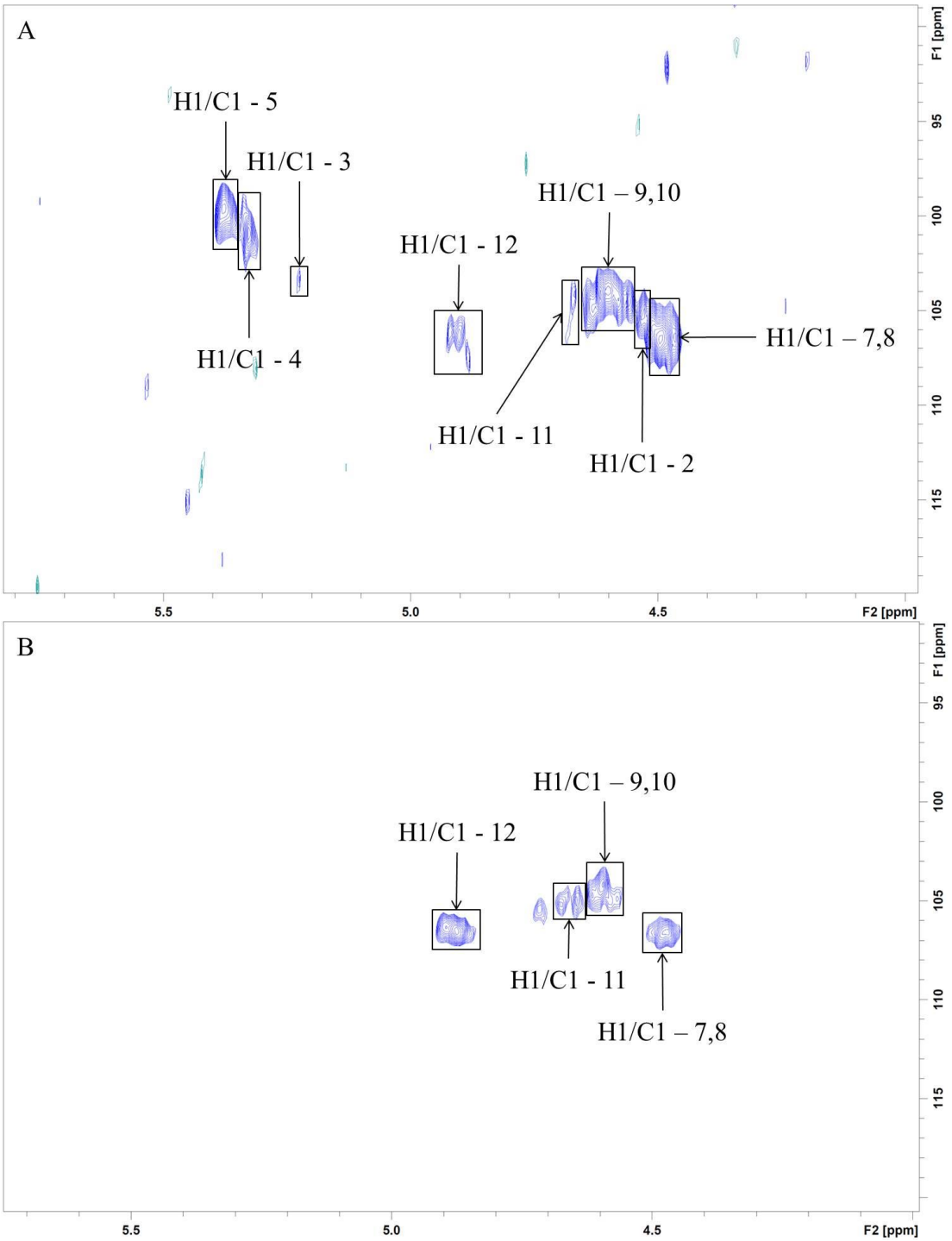


Figure S1: HSQC NMR spectra of the anomeric regions of. T-0.75 (A), T-1.0 (B). 2 – G-ANAc; 3 – I2S; 4 – ANAc; 5 – ANS; 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate.

Supplementary data

Title: Viscera of fishes as raw material for extraction of glycosaminoglycans
Journal: Fish and Fisheries
Author: Alexsandro V. Nogueira, Marcello Iacomini, Guilherme L. Sassaki, Thales R. Cipriani*

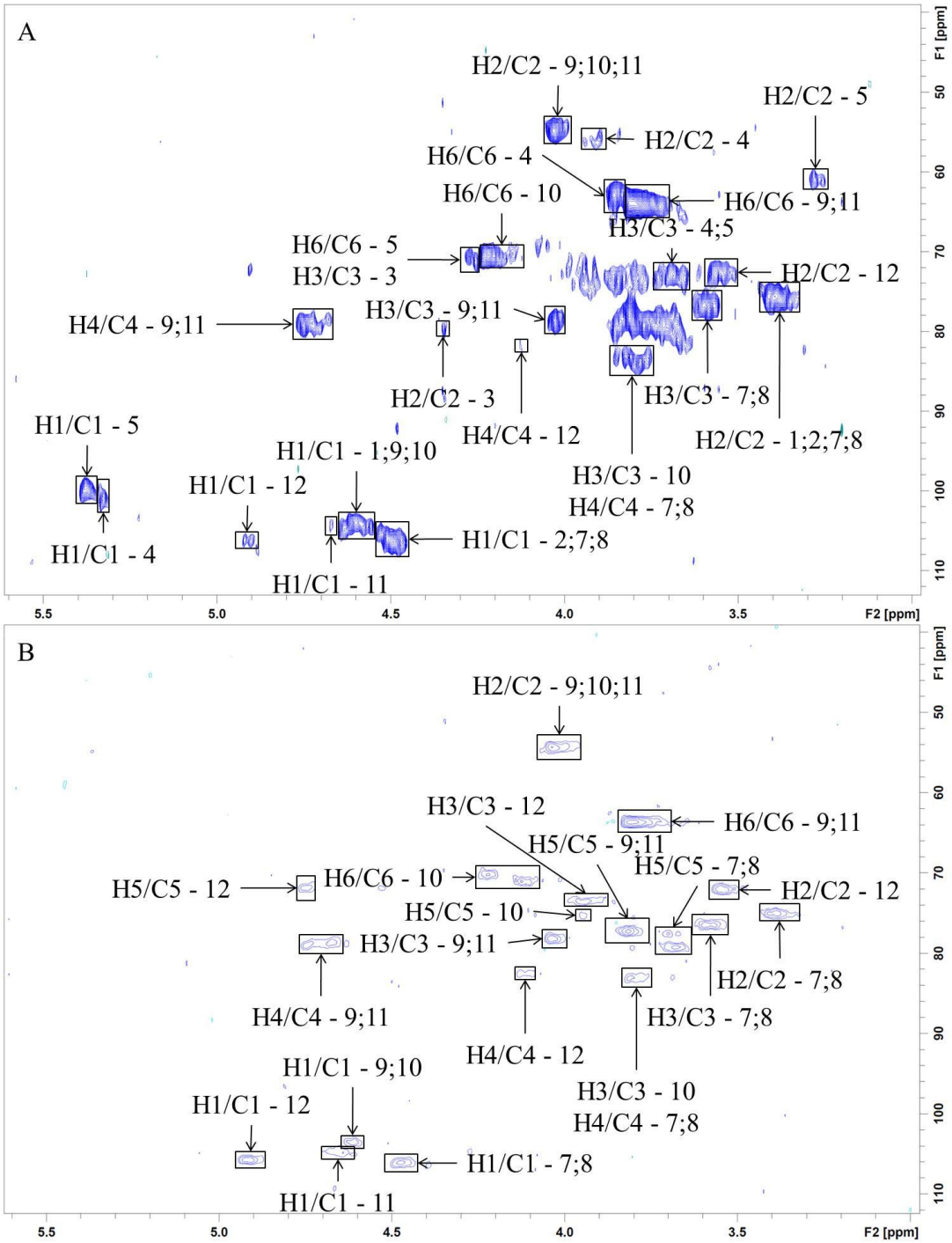


Figure S2: HSQC NMR spectra of T-0.75A (A) and T-0.75B (B). 1 – G-ANS; 2 – G-ANAc; 3 – I2S; 4 – ANAc; 5 – ANS; 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 1 - G-ANS – Glucuronic acid linked to glucosamine N-sulfated; 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate.

Supplementary data

Title: Viscera of fishes as raw material for extraction of glycosaminoglycans
Journal: Fish and Fisheries
Author: Alexsandro V. Nogueira, Marcello Iacomini, Guilherme L. Sassaki, Thales R. Cipriani*

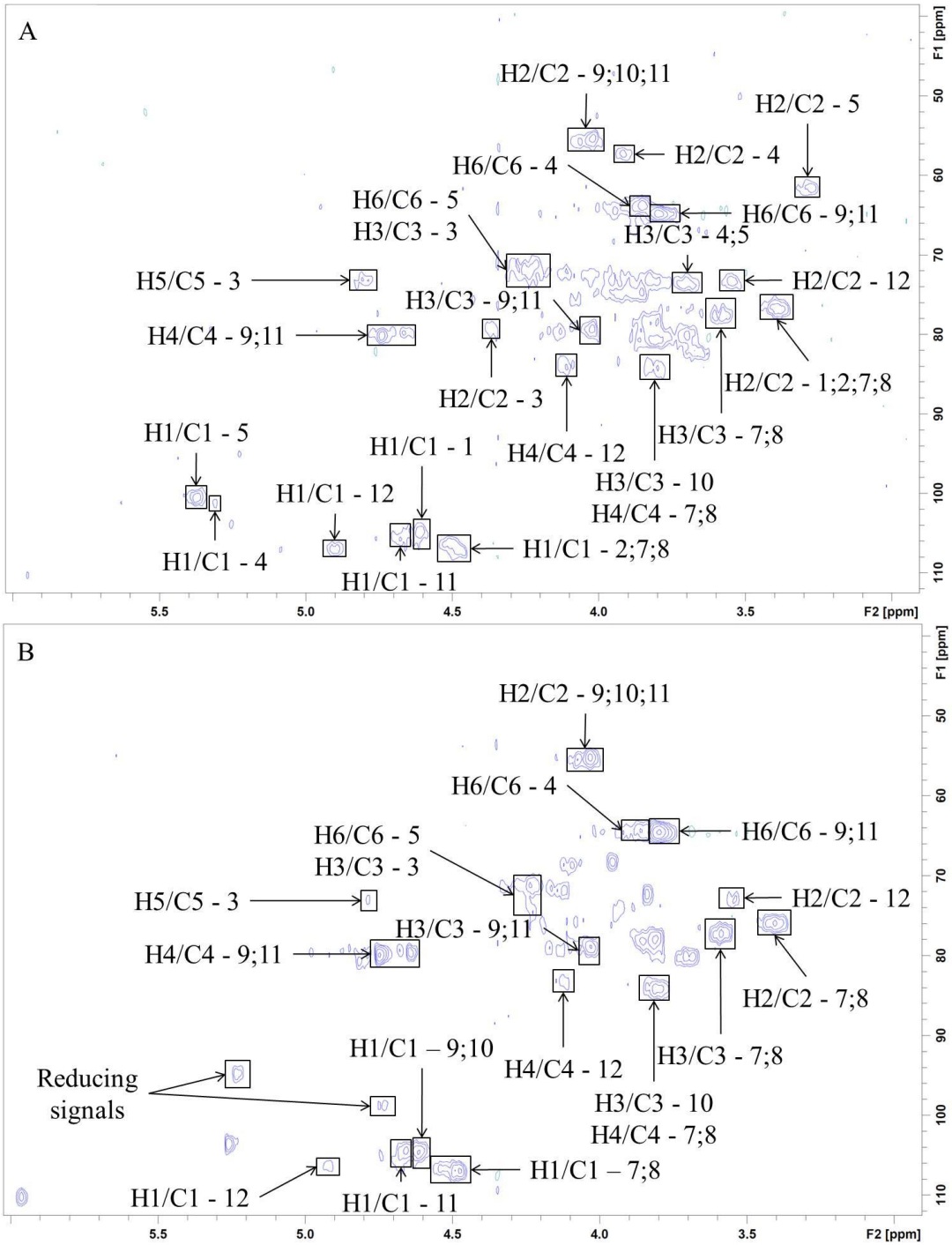


Figure S3: HSQC NMR spectra of T-0.75AR (A) and T-0.75AE (B). 1 – G-ANS; 2 – G-ANAc; 3 – I2S; 4 – ANAc; 5 – ANS; 7 – G-A4S; 8 – G-A6S; 9 – A4S; 10 – A6S; 11 – A4S-I; 12 – I. T (Nile tilapia); P (pacu). 1 - G-ANS – Glucuronic acid linked to glucosamine N-sulfated; 2 - G-ANAc – Glucuronic acid linked to glucosamine N-acetylated; 3 - I2S – Iduronic acid 2-sulfate; 4 - ANAc – Glucosamine N-acetylated; 5 - ANS – Glucosamine N-sulfated; 7 - G-A4S – Glucuronic acid linked to galactosamine 4-sulfate; 8 - G-A6S – Glucuronic acid linked to galactosamine 6-sulfate; 9 - A4S – Galactosamine 4-sulfate; 10 - A6S – Galactosamine 6-sulfate; 11 - A4S-I – Galactosamine 4-sulfate linked to iduronic acid; 12 - I – iduronic acid from dermatan sulfate

ARTIGO II

Anticoagulant and antithrombotic activities of glycosaminoglycans from viscera of the fishes *Oreochromis niloticus* and *Piaractus mesopotamicus*

Anticoagulant and antithrombotic activities of glycosaminoglycans from viscera of the fishes *Oreochromis niloticus* and *Piaractus mesopotamicus*

Alexsandro Vinícius Nogueira, Marcello Iacomini, Helyn Priscila de Oliveira Barddal, Guilherme Lanzi Sassaki, Thales Ricardo Cipriani*

Department of Biochemistry and Molecular Biology, Federal University of Paraná, CP 19046, CEP 81531-980, Curitiba, PR, Brazil

* Corresponding author.

E-mail address: trcipriani@ufpr.br (T. R. Cipriani)

Abstract

Currently, heparin is the main anticoagulant agent used for prevention and treatment of thromboembolic problems. However, other glycosaminoglycans (GAGs) have anticoagulant and antithrombotic effects. In this work, the anticoagulant and antithrombotic effects of seven GAGs samples, extracted from total viscera of the fishes *Oreochromis niloticus* (Nile tilapia; T) and *Piaractus mesopotamicus* (Pacu; P), were evaluated: P-1.0R and T-1.0R fractions (constituted of dermatan sulfate), P-0.75AR and T-0.75AR (constituted of a mix of dermatan and heparan sulfate), and P-0.75B , P-0.75C and T-0.75B (constituted of a mix of dermatan, chondroitin and heparan sulfate). All samples showed anticoagulant activity when tested in aPTT assay, but those from Nile tilapia showed the best activities. All samples also inhibited α-thrombin in the presence of antithrombin and heparin cofactor II, and factor Xa in the presence of antithrombin. Moreover, they showed *in vivo* antithrombotic effect, with the sample T-0.75AR showing the best activity, reaching almost 100% of thrombosis inhibition at a dose of 1 mg/kg body weight. This study demonstrates that GAGs samples from Nile tilapia and pacu have anticoagulant and antithrombotic effects, and that the samples from Nile tilapia have, in general, the best activities, despite the similar composition of GAGs between the samples.

Keywords: Dermatan sulfate; Heparan sulfate; Chondroitin sulfate

1. Introduction

Hemostasis is a fundamental physiological mechanism for all vertebrates, which involve two complementary processes, clot formation, which blocks the damage in the vessel and stops bleeding, and the clot dissolution processes or fibrinolysis, which eliminates the clot when the vascular endothelium repair was completed. These processes involve vessels, platelets, coagulation and fibrinolysis proteins and natural anticoagulants [1,2,3,4,5].

When there is an imbalance in hemostasis, the main consequence is thrombosis, which is characterized by clot formation in the circulatory system. Acute arterial thrombosis is the main cause of the myocardial infarction and venous thromboembolism is the third largest responsible for death associated with cardiovascular problems [6].

Currently, heparin is the main anticoagulant agent used for prevention and treatment of thromboembolic problems. It has a high indirect anticoagulant effect, increasing the effect of the serpins antithrombin (AT) and heparin cofactor II (HCII) [7,8]. However, heparin is not the only glycosaminoglycan (GAG) that has anticoagulant and antithrombotic effects. Dermatan sulfate (DS), for example, potentiates the effect of HCII more than 1000 times [9,10,11,12], and chondroitin sulfate (CS), extracted of different sources, have demonstrated good anticoagulant [13] and antithrombotic [14] effects.

Heparan sulfate (HS), which has great structural similarity with heparin, also has potential to act as an anticoagulant and antithrombotic agent. Endothelial cells produce HS with high affinity for AT, enhancing the AT effects on coagulation [15], and HS extracted of different sources has shown anticoagulant activity [16,17].

In a previous work [18], seven GAGs samples, extracted from total viscera of the fishes *Piaractus mesopotamicus* (Pacu; P) and *Oreochromis niloticus* (Nile tilapia; T), were characterized: P-1.0R and T-1.0R (constituted of DS), P-0.75AR and T-0.75AR (constituted of a mix of DS and HS), and P-0.75B , P-0.75C and T-0.75B (constituted of a mix of DS, CS and HS) (Table 1). Now, the anticoagulant and antithrombotic effects of them were evaluated.

2. Materials and Methods

2.1. Materials

GAGs samples obtained from total viscera of the fishes *Piaractus mesopotamicus* (Pacu; P) and *Oreochromis niloticus* (Nile tilapia; T), according to a previous work [18]: P-

1.0R and T-1.0R (constituted of DS), P-0.75AR and T-0.75AR (constituted of a mix of DS and HS), and P-0.75B, P-0.75C and T-0.75B (constituted of a mix of DS, CS and HS). Sheep plasma donated by Centro de Desenvolvimento de Testes e Ensaios Farmacêuticos (CTEFAR), from Federal University of Santa Maria, RS, Brazil.

2.2. Anticoagulant activity

The anticoagulant activity was determined *in vitro* by aPTT (activated partial thromboplastin time), using SYNTHETIC PHOSPHO kit - HEMOSIL (IL/USA), a COAG-A-MATE XM coagulometer (Organon Teknika Corporation), and a pool of citrated sheep plasma. Plasma (50 μL) was incubated at 37 °C with saline, GAGs (1.5; 1.0; 0.5; 0.25; 0.125; 0.0625 and 0.03125 mg/mL) or heparin (0.007; 0.008; 0.009 and 0.01 mg/mL) (50 μL) for 1 min. Then, rabbit cephalin (50 μL) was added. After 2.5 min, 25 mM $CaCl_2$ (50 μL) was added and the clotting time measured. The results were expressed as aPTT mean (s) ± standard error of the mean (SEM) (n = 2).

2.3. Inhibition of α-thrombin and factor Xa (FXa)

The assays were performed in 96-well plates. The final concentrations of the reactants included 100 nM AT or 15 nM HCII, 6 nM α-thrombin or 8 nM FXa and 1×10^{-4} to 10 μg/mL of GAGs in 75 μL of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/mL polyethylene glycol 8000, pH 7.4). α-Thrombin or FXa was last added to initiate the reaction. After 1 min of incubation at 37 °C, 25 μL of chromogenic substrate S-2238 for α-thrombin or S-2222 for FXa (Chromogenix AB) were added (100 μM – final concentration), and absorbance at 405 nm recorded for 16 min (Multimode microplate reader, Infinite M200, Tecan). α-Thrombin and FXa activities in the absence of AT and HCII and in the presence of GAGs (10 μg/mL) were also measured. The activities obtained in absence of AT, HCII and GAGs were considered 100%. All enzymatic activities considered the initial velocity of the reaction, which was calculated from the slope of the linear plot of absorbance, which is proportional to the product concentration, as a function of time. The results were expressed as α-thrombin or FXa residual activity means ± SEM (n = 3).

2.4. Animals

Experiments were conducted on female Wistar rats (170–200 g) from the colony of Federal University of Paraná, Curitiba, Brazil. They were maintained under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2°C), with standard pellet food and water *ad libitum*. The animals were anesthetized with an intramuscular injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (16 mg/kg). The Institutional Ethics Committee of Federal University of Paraná approved all the procedures adopted in this study (authorization number 835).

2.5. Venous thrombosis

Thrombus formation was induced by promoting a combination of stasis and hypercoagulability [19,20]. Rats were anesthetized and their right carotid artery cannulated for injection of vehicle (phosphate buffered saline − PBS; 0.136 M NaCl, 0.0268 M KCl, 0.0081 M Na_2HPO_4, 0.00147 M KH_2PO_4, pH adjusted for 7.2 with 1 M HCl), GAG sample and thromboplastin. The abdominal vena cava was dissected, and loose sutures were placed between the right renal vena and femoral veins, and in the left renal vena. PBS or GAG sample was infused into the right carotid artery and allowed to circulate for 5 min. Thrombus formation was then induced by injection of thromboplastin (5 mg/kg body weight), and 20 s later by stasis of a 0.7 cm segment of the abdominal vena cava, tightening the sutures. After 20 min, the thrombus formed inside the occluded segment was then pulled out, washed with PBS, freeze-dried for 24 h, and weighed. For each group ($n \geq 6$), the thrombus weight mean ± standard error of the mean (SEM) was determined and expressed as percentage of thrombosis, with 100% representing absence of any inhibition of thrombus formation (thrombus weight with PBS administration).

2.6. Statistical

Results are expressed as the mean ± standard error of the mean (SEM) and the statistical significance of the results was determined using one-way analysis of variance (ANOVA), followed by Tukey's test. Data were considered different at a significance level of

$p < 0.05$. The graphs were drawn and the statistical analyses performed using the GraphPad Prism version 5.03 for Windows.

3. Results and discussion

Table 1: Composition of the samples extracted from viscera of the fishes pacu (P) and Nile tilapia (T).

Samples	P	T
1.0R	Dermatan sulfate	Dermatan sulfate
0.75AR	Dermatan sulfate and Heparan sulfate	Dermatan sulfate and Heparan sulfate
0.75B	Dermatan sulfate, Condroitin sulfate and Heparan sulfate	Dermatan sulfate, Condroitin sulfate and Heparan sulfate
0.75C	Dermatan sulfate, Condroitin sulfate and Heparan sulfate	-

3.1. In vitro *anticoagulant activity*

All GAGs samples were able to increase the aPTT, showing anticoagulant activity in a dose-dependent manner (Fig. 1).

When the anticoagulant activity of the samples was determined at the same concentration (0.25 mg/mL), P-1.0R, P-0.75AR, P-0.75B and P-0.75C increased the aPTT at 2.4, 2.3, 1.5 and 1.7 times respectively, whereas T-1.0R, T-0.75AR and T-0.75B increased the aPTT at 2.8, 3.3 and 2.3 times respectively. Thus, the sample T-0.75AR from Nile tilapia, which is constituted of DS and HS showed the best anticoagulant activity.

Heparin, the positive control of the test, showed a very intense dose-response anticoagulant effect. The lower potency of the GAGs samples tested may mean greater safety of use, with a lower risk of bleeding caused by a dosing error.

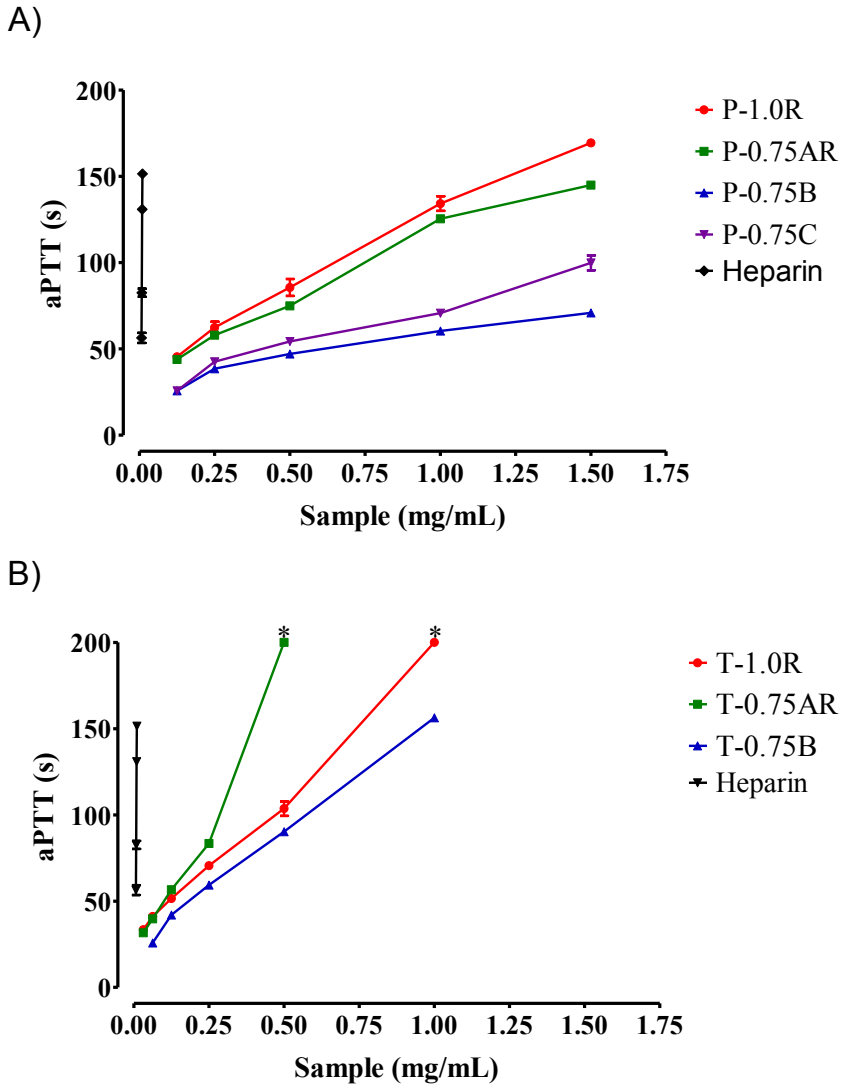


Fig. 1. Anticoagulant activity was determined in aPTT assays for the pacu (A) and Nile tilapia (B) GAGs samples. Plasma (50 µL) was incubated at 37 °C with saline, GAGs (1.5; 1.0; 0.5; 0.25; 0.125; 0.0625 and 0.03125 mg/mL) or heparin (0.007; 0.008; 0.009 and 0.01 mg/mL) (50 µL) for 1 min. Then, rabbit cephalin (50 µL) was added. After 2.5 min, 25 mM $CaCl_2$ (50 µL) was added and the clotting time measured. The results were expressed as aPTT mean (s) ± standard error of the mean (SEM) (n = 2). The aPTT with saline was 25.6 s. * aPTT > 300 s.

Anticoagulant activity of GAGs samples from other fishes have been determined. Salles et al. [21] tested two DS samples from skin of Nile tilapia, which increased the aPTT time at 1.6 and 1.25 times, at 1 mg/mL. Rodrigues et al. [22] studied fractions (FI and FII, both DS) from skin of Nile tilapia, which increased the aPTT at 1.29 and 4.08 times, at 1.0 mg/mL. Rodrigues et al. [23] showed that fractions (FIII and FIV, both HS) from skin of *Cyprinus carpio* increased the aPTT at 1.39 and 2.31 times, at 1.25 mg/mL. Krichen et al. [24] showed that samples (mix of hyaluronic acid, CD and DS) from skin of smooth hound and grey triggerfish increased the aPTT at 1.66 and 1.24 times, at 0.1 mg/mL. Dellias et al. [25] evaluated the anticoagulant activity of four DS samples from skin of different species of

rays, and the best result was to the sample of *Dasyatis americana*, which increased the aPTT to 100 s, at 1.0 mg/mL. Souza et al. [26] studied the anticoagulant activity of the DS from skin of electric eel. At a concentration of 1 mg/mL, the aPTT time was prolonged to about 120 s.

So, it was possible to conclude that the GAGs samples studied here had a good anticoagulant activity when compared to other GAGs samples obtained from different fishes.

3.2. Effect of the GAGs samples on inhibition of α-thrombin and FXa

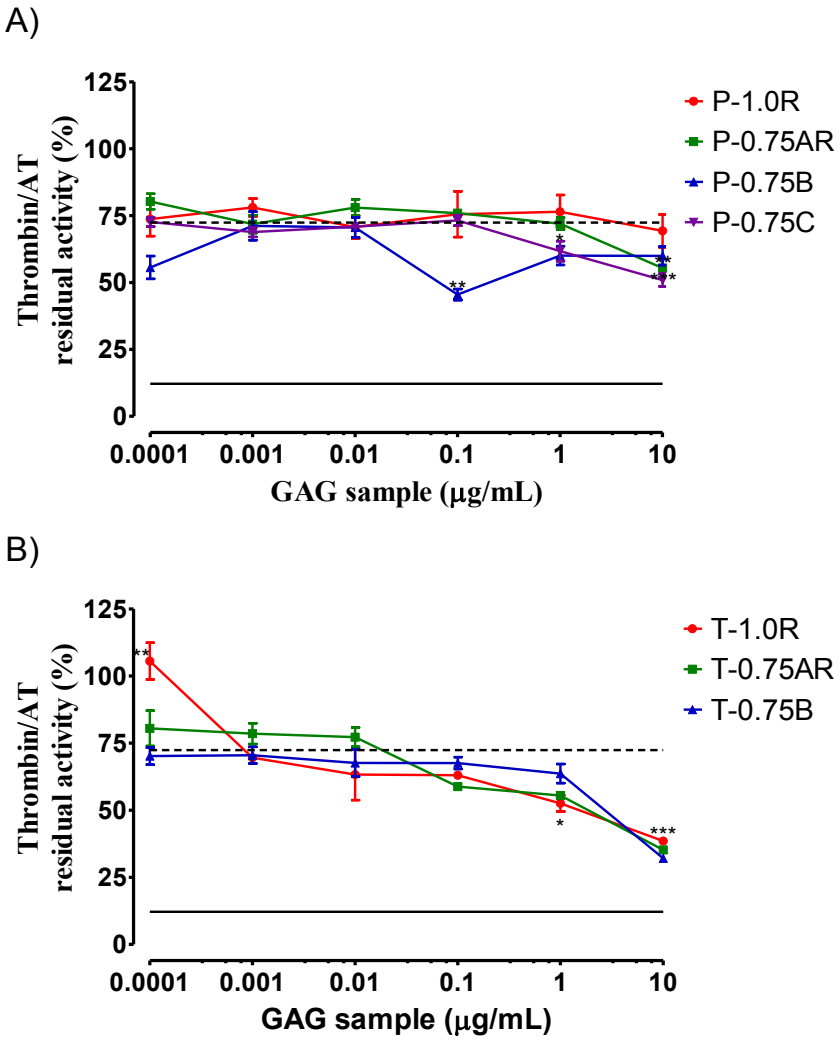


Fig. 2. Effect of the GAGs samples from Pacu (A) and Nile tilapia (B) on inhibition of α-thrombin by AT. 100 nM AT, GAGs and 6 nM α-thrombin were incubated for 1 min at 37 °C, specific chromogenic substrate was added, and the α-thrombin residual activity determined from the slope of absorbance curves recorded at 405 nm (means ± SEM, n = 3 with). Data were considered different at a significance level of $p < 0.05$*, $p < 0.01$** and $p < 0.001$*** compared to the dashed line (---), which represent the effect of AT on α-thrombin in the absence of GAGs. 100% represents the α-thrombin activity in absence of AT and GAGs. Heparin 1 µg/mL (continuous line —) was used as control.

Heparin, the main GAG used as anticoagulant and antithrombotic drug, acts inhibiting blood coagulation mainly by binding to AT and HCII, increasing the effect of these natural anticoagulants on α-thrombin and of AT on FXa [7,8]. The figures 2-4 show the effect of the GAGs from pacu and Nile tilapia on α-thrombin and FXa activities.

According to figure 2, AT decreased the α-thrombin activity to 72% in the absence of GAGs (dashed line), which represents its intrinsic inhibitory effect. In the presence of P-0.75AR, P-0.75B and P-0.75C (10 µg/mL), the α-thrombin residual activity was of about 55%, and with T-1.0R T-0.75AR and T-0.75B (10 µg/mL) it was of about 35%. The P-1.0R did not show effect at the concentrations tested.

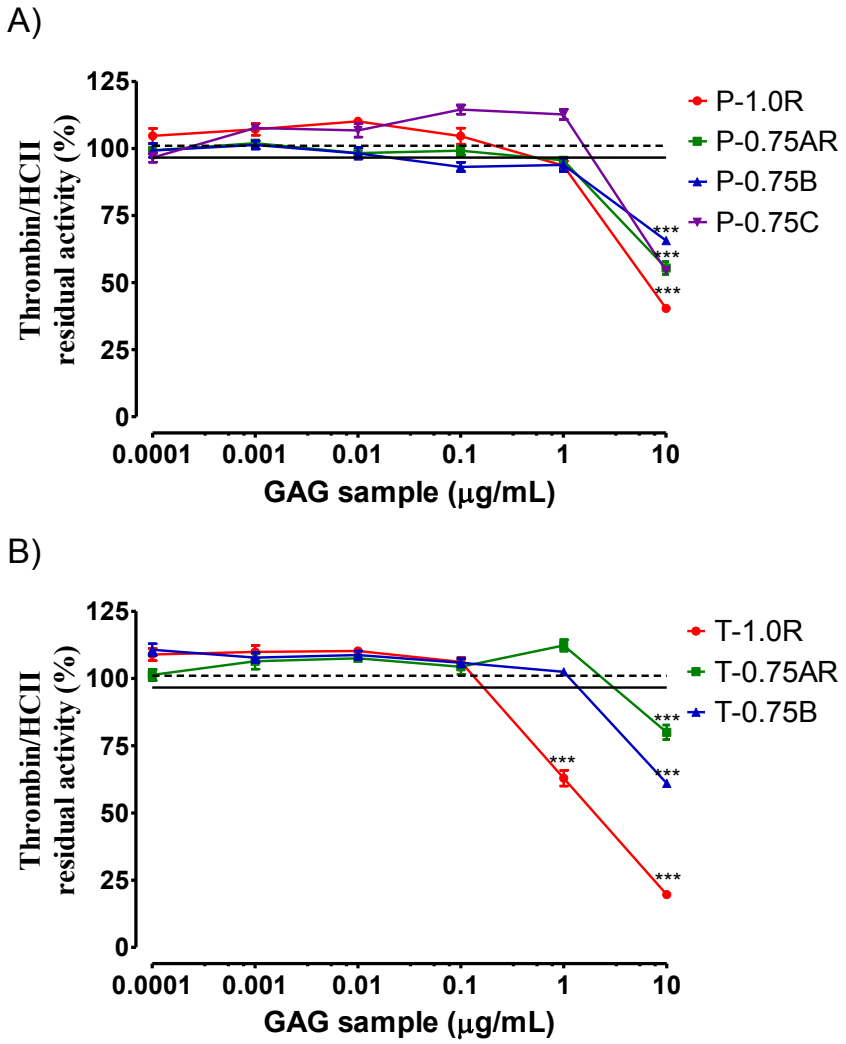


Fig. 3. Effect of the GAGs samples from Pacu (A) and Nile tilapia (B) on inhibition of α-thrombin by HCII. 15 nM HCII, GAGs and 6 nM α-thrombin were incubated for 1 min at 37 °C, specific chromogenic substrate was added, and the α-thrombin residual activity determined from the slope of absorbance curves recorded at 405 nm (means ± SEM, n = 3 with). Data were considered different at a significance level of $p < 0.05$*, $p < 0.01$** and $p < 0.001$*** compared to the dashed line (---), which represent the effect of HCII on α-thrombin in the absence of GAGs. 100% represents the α-thrombin activity in absence of HCII and GAGs. Heparin 1 µg/mL (continuous line —) was used as control.

Different from that was observed with AT, HCII did not inhibit the α-thrombin in the absence of GAGs (dashed line). However, in the presence of P-1.0R, P-0.75AR, P-0.75B and P-0.75C (10 μg/mL), the α-thrombin residual activity was decreased to 40%, 55%, 66% and 54% respectively, while with T-1.0R T-0.75AR and T-0.75B (10 μg/mL) it was decreased to 20%, 80% and 61% respectively (Fig. 3).

In the absence of AT or HCII, all GAGs samples did not were able to inhibit α-thrombin (residual activity was close to 100%), showing that their inhibitory effect on α-thrombin is dependent of serpins.

According to figure 4, AT decreased the FXa activity to 93% in the absence of GAGs (dashed line), which represents its intrinsic inhibitory effect. In the presence of P-1.0R, P-0.75AR, P-0.75B and P-0.75C (10 μg/mL), the FXa residual activity was decreased to 69%, 28%, 54% and 64% respectively, while with T-1.0R T-0.75AR and T-0.75B (10 μg/mL) it was decreased to 45%, 18% and 26% respectively. Different from that was observed with α-thrombin, for some GAGs samples (P-0.75AR, T-1.0R and T-0.75AR) the inhibitory effect on FXa was observed from the lowest concentrations tested. In the absence of AT, all GAGs samples did not were able to inhibit FXa (residual activity was close to 100%), showing that their inhibitory effect on FXa is dependent of serpin.

T-0.75AR, which showed the best anticoagulant activity on aPTT assay (Fig. 1), showed the best inhibitory effect on FXa and, in addition, inhibited α-thrombin.

Heparin at 1 μg/mL (continuous line —), showed a high inhibitory effect on α-thrombin and FXa, in the presence of AT (Fig. 2 and Fig. 4), but it practically did not inhibit α-thrombin in the presence of HCII. This result was already expected and can be explained by the higher affinity of heparin for AT than for HCII. Unlike heparin, the GAG sample T-1.0R, at 1 μg/mL, was able to inhibit α-thrombin in the presence of HCII. This suggests that different GAGs can be used how anticoagulants in different situations, such as in cases of deficiency of AT.

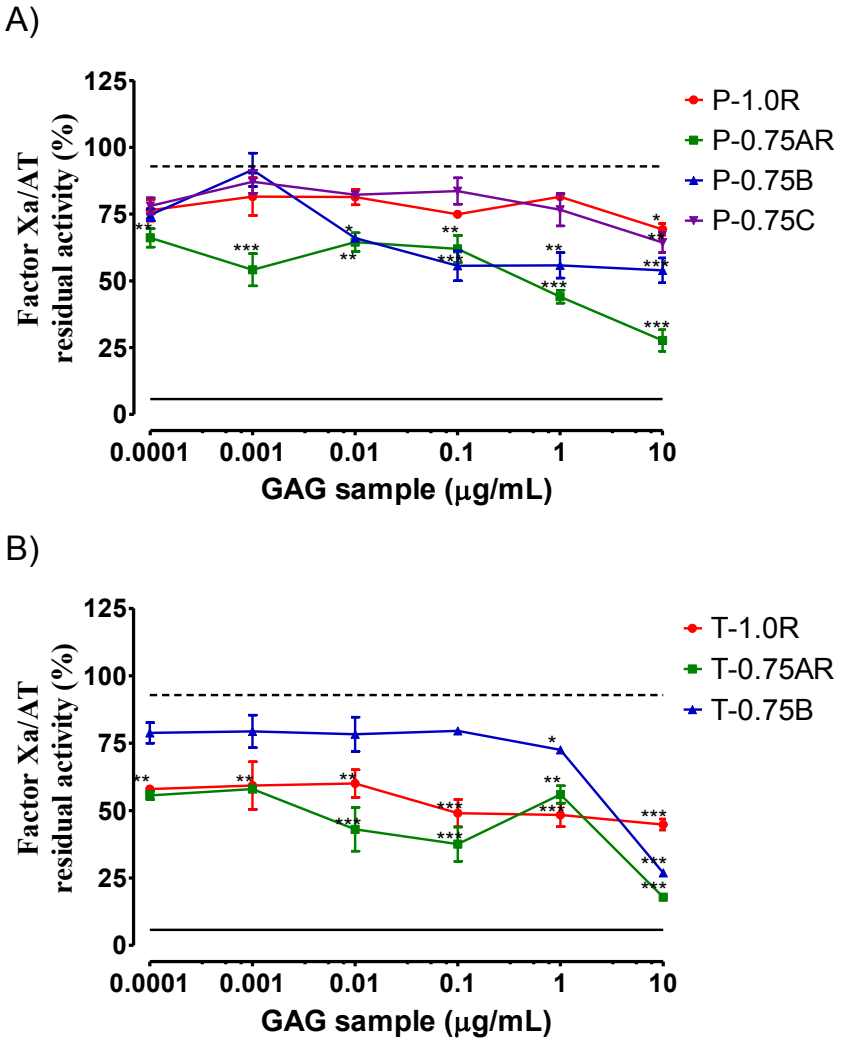


Fig. 4. Effect of the GAGs samples from Pacu (A) and Nile tilapia (B) on inhibition of FXa by AT. 100 nM AT, GAGs and 8 nM FXa were incubated for 1 min at 37 °C, specific chromogenic substrate was added, and the FXa residual activity determined from the slope of absorbance curves recorded at 405 nm (means ± SEM, n = 3 with). Data were considered different at a significance level of $p < 0.05$*, $p < 0.01$** and $p < 0.001$*** compared to the dashed line (---), which represent the effect of AT on FXa in the absence of GAGs. 100% represents the FXa activity in absence of AT and GAGs. Heparin 1 µg/mL (continuous line —) was used as control.

Besides, the samples P-1.0R and T-1.0R, which are constituted only by DS, showed the lowest effects on FXa. Other studies have shown low anti-Xa activity of DS in presence of AT, with high DS concentrations being required to have an activity (>100 µg/mL) [27,28]. On the other hand, the other samples, which have HS in their composition, showed higher anti-Xa activity in the presence of AT. It is known that HS has a specific pentasaccharide motif with high affinity for AT [15].

In relation to the inhibitory effect on α-thrombin in the presence of HCII, the samples constituted only by DS (P-1.0R and T-1.0R) showed the highest activities. It is known that DS has affinity for HCII, which gives him anticoagulant effect [9,11,29]. Mansour et al. [27] reported a DS from ray skin that inhibited the α-thrombin activity in the presence of HCII in

50%, at a concentration of about 1 μg/mL. Souza et al. [26] showed that the DS from skin of the electric eel decrease the α-thrombin activity in the presence of HCII to 50%, at a concentration of about 0.1 μg/mL. And, Dellias et al. [25] studied four DS samples from different species of rays, and showed that they decreased the α-thrombin activity to 50%, in the presence of HCII, at concentrations of 1.0 μg/mL, 3.0 μg/mL, 40 μg/mL and more that 100 μg/mL.

3.3. In vivo *antithrombotic activity*

The antithrombotic effect of the GAGs samples was also evaluated (Fig. 5). P-1.0R, P-0.75B and P-0.75C had similar effects at a dose of 3 mg/kg, with significant antithrombotic activities of 82%, 78% and 83% respectively. At the same dose, P-0.75AR inhibited 49% the thrombus formation, and at 5 mg/kg, the antithrombotic activity reached 96% (Fig. 5A).

For the Nile tilapia samples (Fig. 5B), T-1.0R and T-0.75B showed significant antithrombotic activities of 30% and 50% respectively, at a dose of 3 mg/kg. T-0.75AR, which showed the higher anticoagulant activity (Fig 1), also showed the higher antithrombotic activity between all the samples tested, inhibiting 94% the thrombus formation at a dose of 1 mg/kg. Heparin, the positive control of the test, showed antithrombotic effect from 0.1 mg/kg.

Despite the existence of several studies showing the anticoagulant activity of fish GAGs, studies describing antithrombotic activity were not found.

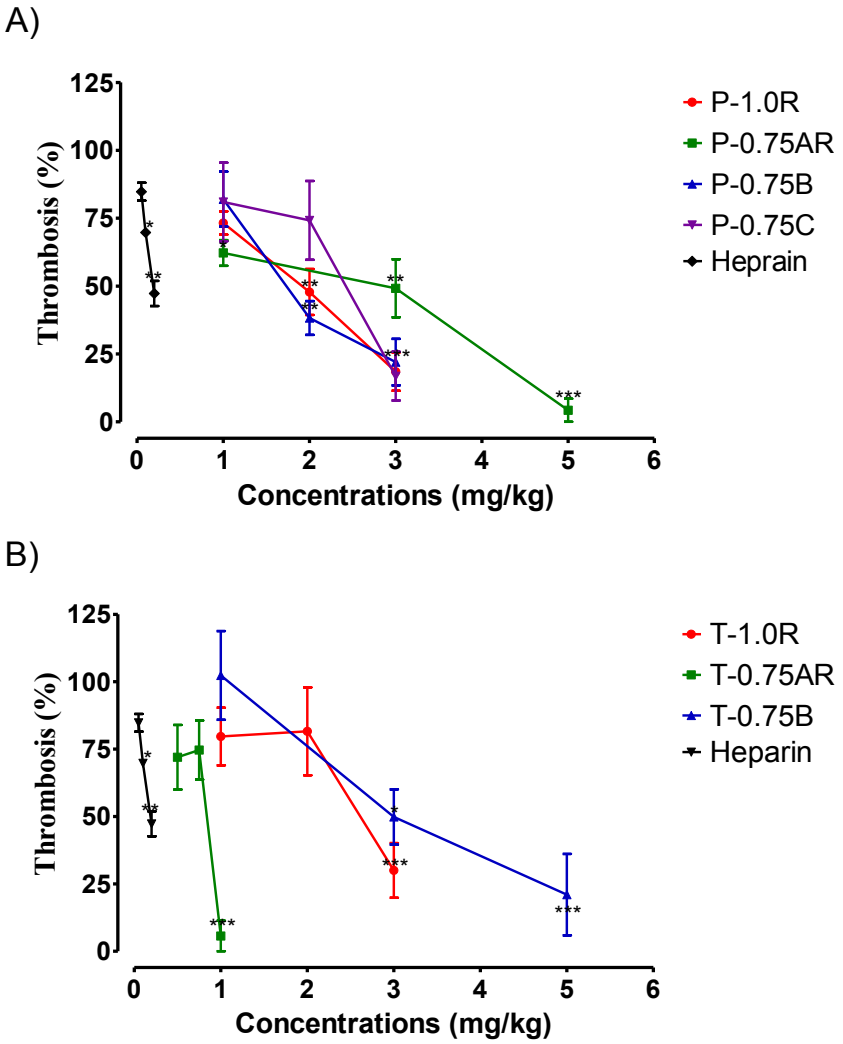


Fig. 5. Venous antithrombotic effect after intravascular administration of GAGs samples from pacu (A) and Nile Tilapia (B). Thrombus formation was induced by promoting a combination of stasis and hypercoagulability. Different doses of the samples were administered in the right carotid artery and allowed to circulate for 5 min. Thromboplastin (5 mg/kg body weight) was then injected, and 20 s later, 0.7 cm of an isolated segment of the abdominal vena cava was tied off. After stasis for 20 min, the thrombus formed on the interior was pulled out, freeze-dried and weighed. Results are expressed as % of thrombosis (mean ± SEM, $n \geq 6$ with $p < 0.05$*, $p < 0.01$** and $p < 0.001$*** compared to 100%), with 100% representing absence of any thrombosis inhibition (thrombus weight in the absence of GAGs, which corresponded to a thrombus of 3.4 ± 0.2 mg).

4. Conclusions

This study demonstrated that the waste generated from fish production (viscera) contains pharmacologically active substances. GAGs samples from viscera of the fishes Nile tilapia and pacu showed anticoagulant and antithrombotic activities. Despite the similar GAGs composition, the samples from Nile tilapia showed, in general, the higher effects.

Acknowledgments

The authors would like to thank the Brazilian agencies *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq – Grant number 449176/2014-2) and *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for financial support; *Big Peixe* for the supply of the fishes; *Centro de Desenvolvimento de Testes e Ensaios Farmacêuticos* (CTEFAR), from Federal University of Santa Maria, for the supply of ovine plasma; and UFPR-RMN Center.

References

[1] M.B. Brooks, T. Stokol, J.L. Catalfamo, Comparative Hemostasis: Animal Models and New Hemostasis Tests. Clin. Lab. Med. 31 (2011) 139-159.

[2] R.W. Colman, Are hemostasis and thrombosis two sides of same coin? J. Exp. Med. 203 (2006) 493-495.

[3] L. Eyre, F. Gamlin, Haemostasis, blood platelets and coagulation, Anaesth. Intensive Care. 11 (2010) 144-146.

[4] P.A. Gentry, Comparative aspects of blood coagulation, Vet. j. 168 (2004) 238-251.

[5] C.V. Reis, L.M. Vieira, L.M.S. Dusse, E.F. Coelho, M.L. Freitas, M.R. Diniz, C.C. Costa, M.G. Carvalho, Evaluation of coagulation, fibrinolysis and protein C in risk patients and presenting coronarian diseases, J. Bras. Patol. Med. Lab. 39 (2003) 7-13.

[6] N. Mackman, Triggers, targets and treatments for thrombosis, Nature. 451 (2008) 914-918.

[7] S. Patel, L.R. Berry, A.K.C. Chan, Covalent antithrombin-heparin complexes. Thromb. Res. 120 (2007) 150-160.

[8] M. Petitou, B. Casu, U. Lindahl, 1976-1983, a critical period in the history of heparin: the Discovery of the antithrombin binding site, Biochimie. 85 (2003) 83-89.

[9] D.M. Tollefsen, Vascular Dermatan Sulfate and Heparin Cofactor II, Prog. Mol. Biol. Transl. Sci. 93 (2010) 351-372.

[10] A.M. Halldórsdóttir, L. Zhang, D.M. Tollefsen, N-Acetylgalactosamine 4,6-O-sulfate residues mediate binding and activation of heparin cofactor II by porcine mucosal dermatan sulfate, Glycobiology. 16 (2006) 693-701.

[11] M.M Maimone, D.M Tollefsen, Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity, J. Biol. Chem. 265 (1990) 18263-18271.

[12] M.A. Thelin, B. Bartolini, J. Axelsson, R. Gustafsson, E. Tykesson, E. Pera, A. Oldberg, M. Maccarana, A. Malmstrom, Biological functions of iduronic acid in chondroitin/dermatan sulfate, FEBS J. 280 (2013) 2431-2446.

[13] J. Mou, C. Wang, W. Li, J. Yang, Purification, structural characterization and anticoagulant properties of fucosylated chondroitin sulfate isolated from *Holothuria mexicana*, Int. J. Biol. Macromol. 98 (2017) 208-215.

[14] M. Gui, J. Song, L. Zhang, S. Wang, R. Wu, C. Ma, P. Li,. Chemical characteristics and antithrombotic effect of chondroitinsulfates from sturgeon skull and sturgeon backbone, Carbohydr. polym. 123 (2015) 454-460.

[15] N.W. Shworak, T. Kobayashi, A. Agostini, N.C. Smits, Anticoagulant Heparan Sulfate: To Not Clot—Or Not?, Prog. Mol. Biol. Transl. Sci. 93 (2010) 153-177.

[16] R. Saravanan, A. Shanmugam, Is isolation and characterization of heparan sulfate from marine scallop *Amussium pleuronectus* (Linne.) an alternative source of heparin? Carbohydr. Polym. 86 (2011) 1082-1084.

[17] A.S. Brito, R.S. Cavalcante, L.C.G.F. Palhares, A.J. Hughes, G.P.V. Andrade, E.A. Yates, H.B. Nader, M.A. Lima, S.F. Chavante, A non-hemorrhagic hybrid heparin/heparan sulfate with anticoagulant potential, Carbohydr. Polym. 99 (2014) 372- 378.

[18] A.V. Nogueira, G.R. Rossi, M. Iacomini, G.L. Sassaki, E.S. Trindade, T.R. Cipriani, Extraction and characterization of glycosaminoglycans from viscera of the fishes *Oreochromis niloticus* and *Piaractus mesopotamicus*. 'Unpublished results'.

[19] C.N. Berry, D. Girard, S. Lochot, C. Lecoffre, Antithrombotic actions of argatroban in rat models of venous, 'mixed' and arterial thrombosis, and its effects on the tail transection bleeding time, Br J Pharmacol, 113 (1994) 1209-1214.

[20] G.M.T. Vogel, D.G. Meuleman, F.G.M. Bourgondiën, P.M.J. Hobbelen, Comparison of two experimental thrombosis models in rats effects of four glycosaminoglycans, Thromb. Res. 54 (1989) 399-410.

[21] T.C. Salles, J.A.G. Rodrigues, P.G. Barcellos, G.F. Amaral, I.W.F. Araújo, P.A.S. Mourão, Inhibition of thrombin generation by dermatan sulfate isolated from the skin of *Oreochromis niloticus*, Agrária.12 (2017) 98-104.

[22] J.A.G. Rodrigues, A.L.G. Quinderé, I.N.L. Queiroz, C.O. Coura, G.S. Araújo, N.M.B. Benevides, Purificação, caracterização físico-química e atividade anticoagulante de glicosaminoglicanos isolados da pele de tilápia do Nilo (*Oreochromis niloticus*), Acta Sci. Technol. 33 (2011) 233-241.

[23] J.A.G. Rodrigues, E.S.O. Vanderlei, I.N.L. Queiroz, A.L.G. Quinderé, N.M.B. Benevides, Purification and anticoagulant activity of glycosaminoglycans isolated from the skin of common carp, *Cyprinus carpio*, Rev. Ciênc. Agron. 40 (2009) 381-387.

[24] F. Krichen, Z. Ghlissi, I.B. Amor, N. Sayari, R. Kalleld, J. Gargouri, Z. Sahnoun, T. Boudawara, A. Bougate, In vitro and in vivo anti-coagulant activity and toxicological studies of marine sulfated glycosaminoglycans, Exp. Toxicol. Pathol. 69 (2017) 45-53.

[25] J.M.M. Dellias, G.R. Onofre, C.C. Werneck, A.M. Landeira-Fernandez, F.R. Melo, W.R.L. Farias, L.-C.F. Silva, Structural composition and differential anticoagulant activities of dermatan sulfates from the skin of four species of rays, *Dasyatis americana*, *Dasyatis gutatta*, *Aetobatus narinari* and *Potamotrygon motoro*, Biochimie. 86 (2004) 677-683.

[26] M.L.S. Souza, J.M.M. Dellias, F.R. Melo, L.C.F. Silva, Structural composition and anticoagulant activity of dermatan sulfate from the skin of the electric eel, *Electrophorus electricus* (L.), Comp. Biochem. Physiol., Part B. 147 (2007) 387-394.

[27] M.B. Mansour, M. Dhahri, I. Bertholon, V. Ollivier, I. Bataille, N. Ajzenberg, M. Hassine, M. Jandrot-Perrus, F. Chaubet, R.M. Maaroufi, Characterization of a novel dermatan sulfate with high antithrombin activity from ray skin (*Raja radula*), Thromb. Res. 123 (2009) 887-894.

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

[29] H.-Y Du, S.-L. Ji, H.-F. Song, Q.-N. Ye, J.-C. Cao, The relationship between the structure of dermatan sulfate derivatives and their antithrombotic activities, Thromb. Res. 119 (2007) 377-384.

CONCLUSÕES

Em relação a extração de GAGs das visceras de peixes *Oreochromis niloticus* (tilápia do Nilo) e *Piaractus mesopotamicus* (Pacu), o método utilizado mostrou ser efetivo, permitindo a obtenção de fraçõem com dermatan sulfato isolado (P-1.0R e T-1.0R), mixturas de dermatan sulfato e heparan sulfato (P-0.75AR e T0.75AR) e mixturas de dermatan sulfato, condroitin sulfato e heparan sulfato (P-0.75B, P-0.75C e T-0.75B).

A metodologia empregada para a identificação dos GAGs a partir de espectros de RMN e eletroforese em géis de agarose provou ser suficiente para a descrição e determinação dos GAGs presentes nas vísceras dos peixes utilizados neste trabalho.

As frações com GAGs apresentaram efeitos anticoagulante e antitrombótico e, a pesar da similaridade no conteúdo entre os peixes, as amostras provenientes da tilápia do Nilo apresentarem efeitos melhores que as amostras de pacu, o que prova que mesmo sendo moléculas semelhantes elas ainda possuem diferenças estruturas as quais não conseguimos identificar até o momento.

Portanto, este estudo mostrou que o grande resíduo gerado a partir da produção de peixes (vísceras) pode ser utilizado para a obtenção de moléculas com potencial aplicação farmacológica, podendo agregar ainda mais valor à piscicultura.

REFERÊNCIAS

AGNELLI, G.; SONAGLIA, F. Prevention of venous thromboembolism. **Thrombosis Research**, v. 97, p. V49-V62, 2000.

ARIMA, K.; FUJITA, H.; TOITA, R.; IMAZU-OKADA, A.; TSUTSUMISHITA-NAKAI, N.; TAKEDA, N.; NAKAO, Y.; WANG, H.; KAWANO, M.; MATSUSHITA, K.; TANAKA, H.; MORIMOTO, S.; NAKAMURA, A.; KITAGAKI, M.; HIEDA, Y.; HATTO, R.; WATANABE, A.; YUMURA, T.; OKUHARA, T.; HAYASHI, H.; SHIMIZU, K.; NAKAYAMA, K.; MASUDA, S.; ISHIHARA, Y.; YOSHIOKA, S.; YOSHIOKA, S.; SHIRADE, S.; TAMURA, J. Amounts and compositional analysis of glycosaminoglycans in the tissue of fish. **Carbohydrate Research**, v. 366, p. 25-32, 2013.

BERRY, C. N.; GIRARD, D.; LOCHOT S.; LECOFFRE C. Antithrombotic actions of argatroban in rat models of venous, 'mixed' and arterial thrombosis, and its effects on the tail transection bleeding time. **British Journal of Pharmacology**, v. 113, p. 1209-1214, 1994.

BHAVANANDAN, V. P.; MEYER, K. Studies on keratosulfates: methylation and partial acid hydrolysis of bovine corneal keratosulfate. **The Journal of Biological Chemistry**, v. 242, p. 4352-4359, 1967.

BOMBELI, T.; SPAHN, D. R. Updates in perioperative coagulation; physiology and management of thromboembolism and haemorrhage. **British Journal of Anaesthesia**, v. 93, p. 275-287, 2004.

BRIEGER, D. B.; MAK, K.; KOTTKE-MARCHANT, K.; TOPOL, E. J. Heparin-induced thrombocytopenia. **Journal of the American College of Cardiology**, v. 31, p. 1449-1459, 1998.

BRITO, A. S.; CAVALCANTE, R. S.; PALHARES, L. C. G. F.; HUGHES, A. J.; ANDRADE, G. P. V.; YATES, E. A.; NADER, H. B.; LIMA, M. A.; CHAVANTE, S. F. A non-hemorrhagic hybrid heparin/heparan sulfate with anticoagulant potential. **Carbohydrate Polymers**, v. 99, p. 372- 378, 2014.

BRATT, G.; TORNEBOHM, E.; WIDLUND, L.; LOCKNER, D. Low molecular weight heparin (KABI 2165, fragmin): pharmacokinetics after intravenous and subcutaneous administration in human volunteers. **Thrombosis Research**, v. 42, p. 613-620, 1986.

BROOKS, M. B.; STOKOL, T.; CATALFAMO, J. L. Comparative Hemostasis: Animal Models and New Hemostasis Tests. **Clinics in Laboratory Medicine**, v. 31, p. 139-159, 2011.

BROWN G. M.; HUCKERBY T. N.; BAYLISS M. T.; NIEDUSZYNSKI I. A. Human aggrecan keratan sulfate undergoes structural changes during adolescent development. **The Journal of Biological Chemistry**, v. 273, p. 26408-26414, 1998.

CALABRESE, G. C.; RECONDO, E. F.; RECONDO, M. E. F. D. Antithrombin and first complement protein recognize the same active heparin fraction. **Thrombosis Research**, v. 105, p. 537-541, 2002.

CARLSSON, P.; KJELLÉN, L. Heparin Biosynthesis. In: LEVER, R.; MULLOY, B.; PAGE, C. P. **Heparin – A Century of Progress**, Springer, 2012, p. 23-42.

CASU, B. Structure and Active Domains of Heparin. In: GARG, H. G.; LINHARDT, R. J.; HALES C. A. **Chemistry and Biology of Heparin and Heparan Sulfate**, Elsevier, 2005, p. 1-28.

CASU, B. Structure of heparin and heparin fragments. **Annals of the New York Academy of Sciences**, v. 556, p. 1-17, 1989.

CATERINA, R. D.; HUSTED, S.; WALLENTIN, L.; ANDREOTTI, F.; ARNESEN, H.; BACHMANN, F.; BAIGENT, C.; HUBER, K.; JESPERSEN, J.; KRISTENSEN, S. D.; LIP, G. Y. H.; MORAIS, J.; RASMUSSEN, L. H.; SIEGBAHN, A.; VERHEUGT, F. W. A.; WEITZ, J. I. General mechanisms of coagulation and targets of anticoagulation (Section I): Position paper of the ESC working group on thrombosis - task force on anticoagulants in heart disease. **Journal of Thrombosis and Haemostasis**, v. 109, p.569-579, 2013.

CHRISTNER J. E; DISTLER J. J.; JOURDIAN G. W. Biosynthesis of keratan sulfate: purification and properties of a galactosyltransferase from bovine cornea. **Archives of Biochemistry and Biophysics**; v. 192, p. 548-558, 1979.

COHEN, M. The role of low-molecular-weight heparins in arterial diseases: optimizing antithrombotic therapy. **Thrombosis Research**, v. 100, p. V131-V139, 2000.

COLMAN, R. W. Are hemostasis and thrombosis two sides of same coin?. **The Journal of Experimental Medicine**, v. 203, p. 493-495, 2006.

CRAWLEY, J. T. B.; LANE, D. A. The haemostatic role of tissue factor pathway inhibitor. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 28, p. 233-242, 2008.

CURRY, A. N. G.; PIERCE, J. M. T. Conventional and near-patient tests of coagulation. **Continuing Education in Anaesthesia, Critical Care & Pain**, v. 7, p. 45-50, 2007.

DELLIAS, J. M. M.; ONOFRE, G. R.; WERNECK, C. C.; LANDEIRA-FERNANDEZ, A. M.; MELO, F. R.; FARIAS, W. R. L.; SILVA, L.-C.F. Structural composition and differential anticoagulant activities of dermatan sulfates from the skin of four species of rays, *Dasyatis americana*, *Dasyatis gutatta*, *Aetobatus narinari* and *Potamotrygon motoro*. **Biochimie**, v. 86, p. 677-683, 2004.

DESAI, U. R. Antithrombin Activation and Designing Novel Heparin Mimics. In: GARG, H. G.; LINHARDT, R. J.; HALES C. A. **Chemistry and Biology of Heparin and Heparan Sulfate**, Elsevier, 2005, p. 483-512.

DIETRICH, C. P.; DIETRICH, S. M. C. Electrophoretic Behaviour of Acidic Mucopolysaccharides in Diamine Buffers. **Analytical biochemistry**, v. 647, p. 645-647, 1976.

DU, H.-Y.; JI, S.-L.; SONG, H.-F.; YE, Q.-N.; CAO, J.-C. The relationship between the structure of dermatan sulfate derivatives and their antithrombotic activities. **Thrombosis Research**, v. 119, p. 377-384, 2007.

DUBOIS, M.; GILLES, K. A.; HAMILTON, J. K.; REBERS, P. A.; SMITH, F. Colorimetric method for determination of sugars and related substances. **Analytical Chemistry**, v. 28, p. 350-356, 1956.

EMATER: Empresa de Assistência Técnica e Extensão Rural. Disponível em: <http://www.emater.pr.gov.br/modules/conteudo/conteudo.php?conteudo=71>. Acesso em Setembro, 2017.

EYRE, L.; GAMLIN, F. Haemostasis, blood platelets and coagulation. **Anaesthesia and Intensive Care Medicine**, v. 11, p. 144-146, 2010.

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Disponível em: http://www.fao.org/fishery/sofia/en. Acesso em Agosto, 2017.

FLENGSRUD, R.; LARSEN, M. L.; ØDEGAARD, O. R. Purification, characterization and *in vivo* studies of salmon heparin. **Thrombosis Research**, v. 126, p. e409-e417, 2010.

FRANCO, R. F. Overview of coagulation, anticoagulation and fibrinolysis. **Medicina, Ribeirão Preto**, v. 34, p. 229-237, 2001.

GALLAGHER, J. T.; WALKER, A. Molecular distinctions between heparan sulphate and heparin. Analysis of sulphation patterns indicates that heparan sulphate and heparin are separate families of N-sulphated polysaccharides. **Biochemical Journal**, v. 230, p. 665-674, 1985.

GAMA, C. I.; HSIEH-WILSON, L. C. Chemical approaches to deciphering the glycosaminoglycan code. **Current Opinion in Chemical Biology**, v. 9, p. 609-619, 2005.

GARGIULO, V.; LANZETTA, R.; PARRILLI, M.; CASTRO, C. Structural analysis of chondroitin sulfate from *Scyliorhinus canicula*: A useful source of this polysaccharide. **Glycobiology**, v. 19, p. 1485-1491, 2009.

GENTRY, P. A. Comparative aspects of blood coagulation. **The Veterinary Journal**, v. 168, p. 238-251, 2004.

GOSSEC L; DOUGADOS M. Intra-articular treatments in osteoarthritis: from the symptomatic to the structure modifying. **Ann Rheum Disease Journal**; v. 63, p. 478-482, 2004.

GUI, M.; SONG, J.; ZHANG, L.; WANG, S.; WU, R.; MA, C.; LI, P. Chemical characteristics and antithrombotic effect of chondroitinsulfates from sturgeon skull and sturgeon backbone. **Carbohydrate Polymers**, v. 123, p. 454-460, 2015.

GUNAY, N. S.; LINHARDT, R. J. Heparinoids: structure, biological activities and therapeutic applications. **Planta Medica**, v. 65, p. 301-306, 1999.

HALLDÓRSDÓTTIR, A. M.; ZHANG, L.; TOLLEFSEN, D. M. N-Acetylgalactosamine 4,6-O-sulfate residues mediate binding and activation of heparin cofactor II by porcine mucosal dermatan sulfate. **Glycobiology**, v. 16, p. 693-701, 2006.

HE, G.; YIN, Y.; YAN, X.; YU, Q. Optimisation extraction of chondroitin sulfate from fish bone by high intensity pulsed electric fields. **Food Chemistry**, v. 164, p. 205-210, 2014.

HEIT, J. A. The epidemiology of venous thromboembolism in the community. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 28, p. 370-372. 2008.

HILEMAN, R. E.; FROMM, J. R.; WEILER, J. M.; LINHARDT, R. J. Glycosaminoglycan-protein interaction: definition of consensus sites in glycosaminoglycans binding proteins. **BioEssays**, v. 20 p. 156-167, 1998.

HIRANO, S.; HOFFMAN, P.; MEYER, K. The structure of keratosulfate of bovine cornea. **The Journal of Organic Chemistry**, v. 26, p. 5064-5069, 1961.

HOFFMAN, M.; MONROE III, D. M. A cell-based model of hemostasis. **Journal of Thrombosis and Haemostasis**, v.85, p. 958-965, 2001.

IBGE, Disponível em: http://www.sidra.ibge.gov.br/bda/tabela/listabl.asp?c=3940&z=t&o=21 Acessado em Agosto, 2017.

KAKEHI, K.; KINOSHITA, M.; YASUEDA, S. Hyaluronic acid: separation and biological implications. **Journal of Chromatography B**, v. 797, p. 347-355, (2003).

KAKKAR, A. K. Low- and ultra-low-molecular-weight heparins. **Best Practice & Research Clinical Hematology**, v. 17, p. 77-87, 2004.

KEARON, C. Natural history of venous thromboembolism. **Circulation**, v. 107, p. I22-I30, 2003

KEIRE, D., MULLOY, B., CHASE, C., AL-HAKIM, A., CAIRATTI, D., GRAY, E., HOGWOOD, J., MORRIS T., MOURÃO, P., SOARES, M. L. C., SZAJEK, A. (2015). Diversifying the global heparin supply chain: Reintroduction of bovine heparin inthe United States? Retrieved from http://www.pharmtech.com/diversifying-global-heparin-supply-chain-reintroduction-bovine-heparin-united-states. Accessed: 27.04.16.

KORT, M.; BUIJSMAN, R. C.; BOECHEL, C. A. A. Synthetic heparin derivatives as new anticoagulant drugs. **Drug Discovery Today**, v. 10, p. 769-779, 2005.

KREUGER, J.; SPILLMANN, D.; LI, J.; LINDAHL, U. Interactions between heparan sulfate and proteins: the concept of specificity. **The Journal of Cell Biology**, v. 174, p. 323-327, 2006.

KRICHEN, F.; GHLISSI, Z.; AMOR, I. B.; SAYARI, N.; KALLELD, R.; GARGOURI, J.; SAHNOUN, Z.; BOUDAWARA, T.; BOUGATE, A. *In vitro* and *in vivo* anti-coagulant activity and toxicological studies of marine sulfated glycosaminoglycans. **Experimental and Toxicologic Pathology**, v. 69, p. 45-53, 2017.

KRUSIUS T.; FINNE J.; MARGOLIS R. K.; MARGOLIS R. U. Identification of an O-glycosidic mannose-linked sialylated tetrasaccharide and keratan sulfate oligosaccharides in the chondroitin sulfate proteoglycan of brain. **The Journal of Biological Chemistry**; v. 261, p. 8237-8242, 1986.

LAUDER, R. M.; HUCKERBY, T. N.; NIEDUSZYNSKI, I. A.; SADLER, I. H. Characterisation of oligosaccharides from the chondroitin/dermatan sulphates: ^{1}H and ^{13}C NMR studies of oligosaccharides generated by nitrous acid depolymerisation. **Carbohydrate Research**, v. 346, p. 2222-2227, 2011.

LI, J.; VLODAVSKY, I. Heparin, heparan sulfate and heparanase in inflammatory reactions. **Thrombosis and Haemostasis**, v. 102. P. 823-828, 2009.

LINHARDT, R. J. Heparin: structure and activity. **Journal of Medicinal Chemistry**, v. 46, p. 2551-2564, 2003.

MACCARI, F.; GALEOTTI, F.; VOLPI, N. Isolation and structural characterization of chondroitin sulfatefrom bony fishes. **Carbohydrate Polymers**, v. 129, p. 143-147, 2015.

MACKMAN, N. Triggers, targets and treatments for thrombosis. **Nature**, v. 451, p. 914-918, 2008.

MAIMONE, M. M.; TOLLEFSEN, D. M. Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity. **The Journau of Biology Chemistry**, v. 265, v. 18263-18271, 1990.

MANSOUR, M. B.; DHAHRI, M.; BERTHOLON, I.; OLLIVIER, V.; BATAILLE, I.; AJZENBERG, N.; HASSINE M.; JANDROT-PERRUS M.; CHAUBET F.; MAAROUFI R. M. Characterization of a novel dermatan sulfate with high antithrombin activity from ray skin (*Raja radula*). **Thrombosis Research**, v. 123, p. 887-894, 2009a.

MANSOUR, M. B.; MAJDOUB, H.; BATAILLE, I.; ROUDESLI, M. S.; HASSINE, M.; AJZENBERG, N.; CHAUBET, F.; MAAROUFI, R. M. Polysaccharides from the skin of the ray *Raja radula*. Partial characterization and anticoagulant activity. **Thrombosis Research,** v. 123, p. 671-678, 2009b.

MATHEWS, M. B.; CIFONELLI, J. A. Comparative biochemistry of keratosulfates. **The Journal of Biological Chemistry**, v. 240, p. 4140-4145, 1965.

MENAJOVSKY, L. B. Heparin-induced thrombocytopenia: clinical manifestations and management strategies. **The American Journal of Medicine**, v. 118, p. 21S-30S, 2005.

MENEGHETTI, M. C. Z.; HUGHES, A. J.; RUDD, T. R.; NADER, H. B.; POWELL, A. K.; YATES E. A.; LIMA. M. A. Heparan sulfate and heparin interactions with proteins. **Journal of the Royal Society Interface**, v. 12, p. 20150589, 2015.

MINE, S.; YAMAZAKI, T.; MIYATA, T.; HARA, S.; KATO, H. Structural mechanism for heparin-binding of the third kunitz domain of human tissue factor pathway inhibitor. **Biochemistry**, v. 42, p. 78-85, 2002.

MOU, J.; WANG, C.; LI, W.; YANG, J. Purification, structural characterization and anticoagulant properties of fucosylated chondroitin sulfate isolated from *Holothuria mexicana*. **International Journal of Biological Macromolecules**, v. 98 , p. 208-215, 2017.

MUCCI, A.; SCHENETTI, L.; VOLPI, N. 1H and 13C nuclear magnetic resonance identification and characterization of components of chondroitin sulfates of various origin. **Carbohydrate Polymers**, v. 41, p. 37-45, 2000.

MURRAY, R. K.; GRANNEER, D. K.; RODWELL, V. W. **Harper: bioquímica ilustrada**. MxGraw-Hill, 2007, 27. ed.

NAGGI, A.; GARDINIA, C.; PEDRINOLA, G.; MAURIA, L.; URSO, E.; ALEKSEEVA, A.; CASU B.; CASSINELLI G.; GUERRINI M.; IACOMINI M.; BAIGORRIA V.; TORRI G. Structural peculiarity and antithrombin binding region profile of mucosal bovine and porcine heparins. **Journal of Pharmaceutical and Biomedical Analysis**, v. 118, p. 52-63, 2016.

NAKANO, T.; BETTI, M.; PIETRASIK, Z. Extraction, isolation and analysis of chondroitin sulfate glycosaminoglycans. **Recent Patents on Food, Nutrition & Agriculture**, v. 2, p. 61-74, 2010.

PANAGOS, C. G.; THOMSON, D.; MOSS, C.; BAVINGTON, C. D.; ÓLAFSSON, H. G.; UHRÍN, D. Characterisation of hyaluronic acid and chondroitin/dermatan sulfate from the lumpsucker fish*, C. lumpus*. **Carbohydrate Polymers**, v. 106, p. 25-33, 2014.

PATEL, S.; BERRY, L. R.; CHAN, A. K. C. Covalent antithrombin-heparin complexes. **Thrombosis Research**, v. 120, p. 150-160, 2007.

PEÑA, M.; WILLIAMS, C.; PFEILER, E. Structure of keratan sulfate from bonefish (*Albula* sp.) larvae deduced from NMR spectroscopy of keratanase-derived oligosaccharides. **Carbohydrate Research**, v. 309, p. 117-124, 1998.

PETITOU, M.; CASU, B.; LINDAHL, U. 1976-1983, a critical period in the history of heparin: the Discovery of the antithrombin binding site. **Biochimie**, v. 85, p. 83-89, 2003.

PFEILER, E.; TOYODA, H.; WILLIAMS, M. D.; NIEMAN, R. A. Identification, structural analysis and function of hyaluronan in developing fish larvae (leptocephali). **Comparative Biochemistry and Physiology**, v. 132, p. 443-451, 2002.

PIAZZA, G.; GOLDHABER, S. Z. Acute pulmonary embolism: Part I: Epidemiology and diagnosis. **Circulation**, v. 114, p. e28-e32, 2006.

PREVITALI, E.; BUCCIARELLI, P.; PASSAMONTI, S. M.; MATINELLI, I. Risk factors for venous and arterial thrombosis. **Blood Transfusion**, v.9, p. 120-138, 2011.

PROST, D. Heparin fractions and analogues: a new therapeutic possibility for thrombosis. **Trends in Pharmacological Sciences**, v. 7, p. 496-500, 1986.

RAGG, H. A new member of the plasma protease inhibitor gene family. **Nucleic Acids Research**, v. 14, p. 1073-1088, 1986.

REIS, C. V.; VIEIRA, L. M.; DUSSE, L. M. S.; COELHO, E. F.; FREITAS, M. L.; DINIZ, M. R.; COSTA, C. C.; CARVALHO, M. G. Evaluation of coagulation, fibrinolysis and protein C in risk patients and presenting coronarian diseases. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 39, p.7-13, 2003.

RODRIGUES, J. A. G.; QUINDERÉ, A. L. G.; QUEIROZ, I. N. L.; COURA, C. O.; ARAÚJO, G. S.; BENEVIDES, N. M. B. Purification, physical and chemical characterization, and anticoagulant activity of glycosaminoglycans isolated from the skin of Nile tilapia (*Oreochromis niloticus*). **Acta Scientiarum. Technology**, v. 33, p. 233-241, 2011.

RODRIGUES, J. A. G.; VANDERLEI, E. S. O.; QUEIROZ, I. N. L.; QUINDERÉ, A. L. G.; BENEVIDES, N. M. B. Purification and anticoagulant activity of glycosaminoglycans isolated from the skin of common carp, *Cyprinus carpio*. **Revista Ciência Agronômica**, v. 40, p. 381-387, 2009.

RODRIGUES, J. A. G.; VANDERLEI, E. S. O.; QUINDERÉ, A. L. G.; FONTES, B. P.; QUEIROZ, I. N. L.; BENEVIDES, N. M. B. Glicosaminoglicanos isolados da pele de palombeta (*Chloroscombrus chrysurus*) e guaiúba (*Ocyurus chrysurus*): características e implicações biológicas. **Acta Scientiarum. Biological Sciences**, v. 34, p. 141-148, 2012.

ROSENBERG, R. D.; LAM, L. Correlation between structure and function of heparin. **Proceedings of the National Academy of Sciences USA**, v. 76, p. 1218-1222, 1979.

ROSENBERG, R. D.; ROSENBERG, J. S. Natural anticoagulant mechanism. **The Journal of Clinical Investigation**, v. 74, p.1-6, 1984.

ROSENFELD, L.; PRIOR, M. T.; GIRARDI, L. M. Comparison of the separation of bovine heparin by strong anion exchange and by gel filtration chromatography. **Thrombosis Research**, v. 64, p. 203-211, 1991.

ROTH, M.; PAPAKONSTANTINOU, E.; KARAKIULAKIS, G.. Biological function of glycosaminoglycans. In: Garg, H. G.; Cowman, M. K.; Hales, C. A. **Carbohydrate chemistry, biology and medical applications**. Elsevier, 2008, p. 209-226.

RUDD, T. R.; SKIDMORE, M. A.; GUIMOND, S. E.; COSENTINO, C.; TORRI, G.; FERNIG, D. G.; LAUDER R. M.; GUERRINI M.; YATES E. A. Glycosaminoglycan origin and structure revealed by multivariate analysis of NMR and CD spectra. **Glycobiology**, v. 19, p. 52-67, 2009.

SAGRIPANTI, A.; CARPI, A. Natural anticoagulants, aging, and thromboembolism. **Experimental Gerontology**, v.33, p. 891-896, 1998

SAKAI, S.; KIM, W. S.; LEE, I. S.; KIM, Y. S.; NAKAMURA, A.; TOIDA, T.; IMANARI, T. Purification and characterization of dermatan sulfate from the skin of the eel, *Anguilla japonica*. **Carbohydrate Research**, v. 338, p. 263-269, 2003.

SALLES, T. C.; RODRIGUES, J. A. G.; BARCELLOS, P. G.; AMARAL, G. F.; ARAÚJO, I. W. F.; MOURÃO, P. A. S. Inhibition of thrombin generation by dermatan sulfate isolated from the skin of *Oreochromis niloticus*. **Agrária**, v.12, p. 98-104, 2017.

SAMPAIO, L. O.; TERSARIOL, I. L. S.; LOPES, C. C.; BOUÇAS, R. I.; NASCIMENTO, F. D.; ROCHA, H, A.; NADER, H. B. Heparins and heparan sulfates. Structure, distribution and protein interaction. In: Verli H. INSIGHTS INTO CARBOHYDRATE STRUCTURE AND BIOLOGICAL FUNCTION. **Transworld Research Network,** Kerala, 2006, p. 1-24.

SARAVANAN, R.; SHANMUGAM, A. Is isolation and characterization of heparan sulfate from marine scallop *Amussium pleuronectus* (Linne.) an alternative source of heparin?. **Carbohydrate Polymers**, v. 86, p. 1082-1084, 2011.

SASISEKHARAN, R.; SHRIVER, Z.; VENKATARAMAN, G.; NARAYANASAMI, U. Roles of heparan-sulphate glycosaminoglycans in cancer. **Nature Reviews Cancer**, v. 2, p. 521-528, 2002.

SENO, N.; MEYER, K.; ANDERSON, B.; HOFFMAN, P. Variation in keratosulfates. **The Journal of Biological Chemistry**, v. 240, p. 1005-1010, 1965.

SHWORAK, N. W.; KOBAYASHI, T.; AGOSTINI, A.; SMITS, N. C. Anticoagulant Heparan Sulfate: To Not Clot—Or Not?. **Progress in Molecular Biology and Translational Science**, v. 93, p. 153-177, 2010.

SILVA, C.; NOVOA-CARBALLAL, R.; REIS, R. L.; PASHKULEVA, I. Following the enzymatic digestion of chondroitin sulfate by a simple GPC analysis. **Analytica Chimica Acta**,v. 885, p. 207-213, 2015.

SOARES, A. L.; LASMAR, M. C.; GARCIA, M. L.; NOVELLI, B. A.; LAGES, G. F. L.; DUSSE, L. M. S.; VIEIRA, L. M.; FERNANDES, A. P. S. M.; SOUSA, M. O.; CARVALHO, M. G. Natural anticoagulation evaluation in type 2 diabetes. **Jornal Brasileiro de Patologia e Medicina Laboratorial**, v. 42, p. 67-75, 2006.

SOUZA, M. L. R. Industrialização, comercialização e perspectivas. In.:Moreira, H. L. M., Vargas, L., Ribeiro, R. P., & Zimmermann, S., **Fundamentos da moderna aquicultura**. ULBRA, 2001, p. 149-189.

SOUZA, M. L. S.; DELLIAS, J. M. M.; MELO, F. R.; SILVA, L. C. F. Structural composition and anticoagulant activity of dermatan sulfate from the skin of the electric eel, *Electrophorus electricus* (L.). **Comparative Biochemistry and Physiology Part B**, v. 147, p. 387-394, 2007.

STRINGER, S. E.; GALLAGHER, J. T. Heparan Sulphate. **The International Journal of Biochemistry & Cell Biology**, v. 29, p. 709-714, 1997.

SUGAHARA K; MIKAMI T; UYAMA T; MIZUGUCHI S; NOMURA K; KITAGAWA H. Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate. **Current Opinion in Structural Biology**, v. 13, p. 612-620, 2003.

THELIN, M. A.; BARTOLINI, B.; AXELSSON, J.; GUSTAFSSON, R.; TYKESSON, E.; PERA, E.; OLDBERG, A.; MACCARANA, M.; MALMSTROM, A. Biological functions of iduronic acid in chondroitin/dermatan sulfate. **FEBS journal**, v. 280, p. 2431-2446, 2013.

THONG, C. L.; KAM, P. C. A. Heparin-induced thrombocytopenia. **Current Anaesthesia & Critical Care**, v. 16, p.143-150, 2005.

TINGBØ, M. G.; KOLSET, S. O.; OFSTAD, R.; ENERSEN, G.; HANNESSON, K. O. Sulfated glycosaminoglycans in the extracellular matrix of muscle tissue in Atlantic cod (*Gadus morhua*) and Spotted wolffish (*Anarhichas minor*). **Comparative Biochemistry and Physiology**, v. 140, p. 349-357, 2005.

TINGBØ, M. G.; PEDERSEN, M. E.; GRØNDAHL, F.; KOLSET, S. O.; VEISETH-KENT, E.; ENERSEN, G.; HANNESSON, K. O. Type of carbohydrate in feed affects the expression of small leucine-rich proteoglycans (SLRPs) glycosaminoglycans (GAGs) and interleukins in skeletal muscle of Atlantic cod (*Gadus morhua* L.). **Fish & Shellfish Immunology**, v. 32, p. 582-589, 2012.

TOLLEFSEN, D. M. Heparin cofactor II modulates the response to vascular injury. **Arteriosclerosis, Thrombosis, and Vascular Biology**, v. 27, p. 454-460, 2007.

TOLLEFSEN, D. M. Vascular Dermatan Sulfate and Heparin Cofactor II. **Progress in Molecular Biology and Translational Science**, v 93, p. 351-372, 2010.

TOLLEFSEN, D. M.; PESTKA, C. A. Heparin cofactor II activity in patients with disseminated intravascular coagulation and hepatic failure. **Blood**, v. 66, p. 769-774, 1985.

TORRI, G.; GUERRINI, M. Quantitative 2D NMR analysis of glycosaminoglycans. In: Holzgrabe, U.; Wawer, I.; Diehl, B. **NMR spectroscopy in pharmaceutical analysis**. Elsevier, 2008, p. 407-428.

TROWBRIDGE, J. M.; GALLO, R. L. Dermatan sulfate: new functions from an old glycosaminoglycan. **Glycobiology**, v.12, p. 117R-125R, 2002.

VERGÉS J; MONTELL E; HERRERO M; PERNA C; CUEVAS J; PEREZ M; MOLLER I. Clinical and histopathological improvement of psoriasis with oral chondroitin sulfate: a serendipitous finding. **Dermatology Online Journal**, v. 11 p. 1-5, 2005.

VINE, A. K. Recent advances in haemostasis and thrombosis. **Retina: The Journal of Retinal and Vitreous Diseases**, v.29, p. 1-7, 2009.

VOGEL, G. M. T.; MEULEMAN, D. G.; BOURGONDIËN, F. G. M.; HOBBELEN, P. M. J. Comparison of two experimental thrombosis models in rats effects of four glycosaminoglycans. **Thrombosis Research**, v. 54, p. 399-410, 1989.

VOGLER, E. A.; SIEDLECKI, C. A. Contact activation of blood-plasma coagulation. **Biomaterials**, v. 30, p. 1857-1869, 2009.

VOLPI N. Therapeutic applications of glycosaminoglycans. **Current Medicinal Chemistry**; v. 13, p. 1799-1810, 2006.

WALKER, F. J. Regulation of activated protein C by a new protein. **The Journal of Biological Chemistry**, v. 255, p. 5521-5524, 1980.

YUMURA T; OKUHARA T; HAYASHI H; SHIMIZU K; NAKAYAMA K; MASUDA S; ISHIHARA Y; YOSHIOKA S; YOSHIOKA S; SHIRADE S; TAMURA J. Amounts and compositional analysis of glycosaminoglycans in the tissue of fish. **Carbohydrate Research,** v. 366, p. 25-32, 2013.

ZHANG, F.; XIE, J.; LINHARDT, R. J. Isolation and structural characterization of glycosaminoglycans from heads of red salmon (*Oncorhynchus nerka*). **Jacobs Journal of Biotechnology and Bioengineering**, v. 1, p. 1-6, 2014.

ZHANG, F.; ZHANG, Z.; THISTLE, R.; MCKEEN, L.; HOSOYAMA, S.; TOIDA, T.; LINHARDT R. J.; PAGE-MCCAW P. Structural characterization of glycosaminoglycans from zebrafish in different ages. **Glycoconjugate Journal**, v. 26, p. 211-218, 2009.

ANEXO 1

Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

UFPR
UNIVERSIDADE FEDERAL DO PARANÁ

Nº 835

CERTIFICADO

A Comissão de Ética no Uso de Animais (CEUA) do Setor de Ciências Biológicas da Universidade Federal do Paraná, instituído pela PORTARIA Nº 787/03-BL, de 11 de junho de 2003, com base nas normas para a constituição e funcionamento da CEUA, estabelecidas pela RESOLUÇÃO Nº 01/03-BL, de 09 de maio de 2003 e considerando o contido no Regimento Interno da CEUA, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado, estão de acordo com os princípios éticos estabelecidos pelo Colégio Brasileiro de Experimentação Animal (COBEA) e exigências estabelecidas em "*Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care)*".

CERTIFICATION

The Ethics Animal Experiment Committee of the Setor de Ciências Biológicas of the Federal University of Paraná, established by the DECREE Nº 787/03-BL on June 11th 2003, based upon the RESOLUTION Nº 01/03-BL from May 9th 2003, and upon the CEUA internal regiment, CERTIFIES that the procedures using animals in the research project specified below are in agreement with the ethical principals established by the Experimental Animal Brazilian Council (COBEA), and with the requirements of the "Guide for the Care and Use of Experimental Animals (Canadian Council on Animal Care)".

PROCESSO: 23075.038875/2014-10 **APROVADO**: 11/11/2014 – R.O. 09/2014

TÍTULO: Extração, purificação, caracterização e atividade biológica de glicosaminoglicanos isolados de viceras de peixes (*Oreochromis niloticus* e *Piaractus mesopotamicus*)

AUTORES: Thales Ricardo Cipriani, Alexsandro Vinícius Nogueira, Daiana Drehmer

DEPARTAMENTO: Bioquímica

Prof. Dr. Aleksander Roberto Zampronio
Coordenador da CEUA