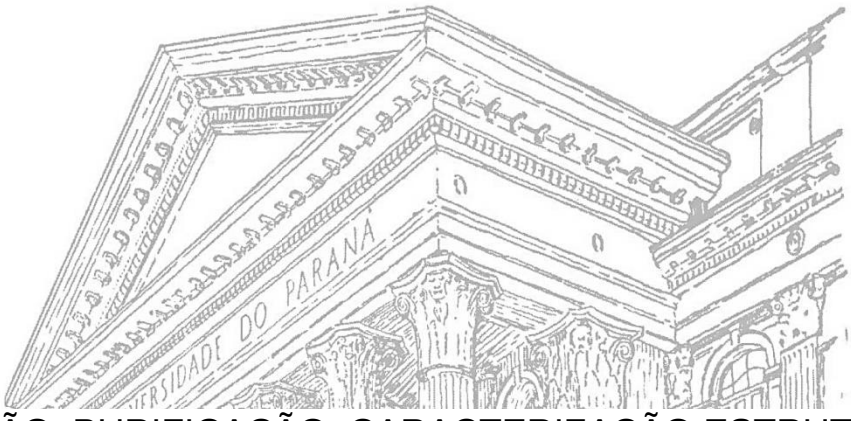
UNIVERSIDADE FEDERAL DO PARANÁ

RAFAEL ROBERTO KLOSTERHOFF



EXTRAÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE ANTIOXIDANTE E ANTIFADIGA DE PECTINAS OBTIDAS DA ACEROLA (*Malpighia emarginata*).

CURITIBA

2017

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EXTRAÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE ANTIOXIDANTE E ANTIFADIGA DE PECTINAS OBTIDAS DA ACEROLA (M*alpighia emarginata*).

Dissertação apresentada ao Programa de Pós-Graduação em Ciências–Bioquímica, do Departamento de Ciências Bioquímica e Biologia Molecular, do Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requisito parcial para obtenção do Título de Mestre em Ciências – Bioquímica.

Orientador: Profª. Drª. Lucimara M. C. Cordeiro

Co-orientador: Prof. Dr. Marcello Iacomini

CURITIBA

2017

Universidade Federal do Paraná
Sistema de Bibliotecas

Klosterhoff, Rafael Roberto
Extração, purificação, caracterização estrutural e atividade antioxidante e antifadiga de pectinas obtidas da acerola (*Malpighia emarginata*). / Rafael Roberto Klosterhoff . – Curitiba, 2017.
88 f.: il. ; 30cm.

Orientador: Lucimara M. C. Cordeiro
Coorientador: Marcello Iacomini

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

1. Acerola 2. Pectina 3. Antioxidantes I. Título II. Cordeiro, Lucimara Mach C. III. Iacomini, Marcello IV. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Bioquímica.

CDD (20. ed.) 574.192482

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

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 Dissertação de Mestrado de **RAFAEL ROBERTO KLOSTERHOFF** intitulada: **EXTRAÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE ANTIOXIDANTE E ANTI-FADIGA DE PECTINAS OBTIDAS DA ACEROLA (*Malpighia emarginata*)**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação.

CURITIBA, 30 de Março de 2017.

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NOTA EXPLICATIVA

Esta dissertação tem os principais resultados apresentados em formato de artigo científico. Na dissertação consta uma introdução, revisão bibliográfica, dois artigos científicos e considerações finais. Este formato está de acordo com as normas do Programa de Pós-Graduação em Ciências - Bioquímica, da Universidade Federal do Paraná.

Dedico este trabalho a minha mãe, Dona Fátima,
minha hermana, Camila
e ao meu amor, Fabiola,
os três pilares da minha vida,
sem vocês não teria conseguido!

AGRADECIMENTOS

Agradeço primeiramente a minha família que forneceu todo suporte necessário para que eu conseguisse me dedicar a este projeto, Dona Fátima, Seu Ademar, Fabiola, Camila, Tia Ivone, Tio Miguel, Tio Anacleto, e todos os outros familiares que auxiliaram nessa trajetória acadêmica;

Agradeço a minha orientadora, a professora Drª Lucimara e ao meu co-orientador, o professor Drº Marcello Iacomini por me aceitarem no grupo e abraçarem a idéia do meu projeto.

Aos demais professores que colaboraram para que o trabalho fosse realizado, professoras Sheila Winnischofer, Sílvia Cadena e Alexandra Acco e aos professores Roberto Andreatini e Rosalvo Fogaça, meu profundo agradecimento.

Aos alunos integrantes nos trabalhos, em especial para a Ju Bark, Paloma, Aninha, Luiz, João, Cláudia e ao Nícolas, este trabalho não teria acontecido sem vocês.

Um agradecimento especial para a Geórgia, que me acompanha desde longínquos tempos de iniciação científica, banca do meu TCC (nunca esquecerei a mensagem errônea que lhe enviei momentos antes da apresentação), mas acima de tudo por ser a amiga de longas conversas sobre infinitos temas, projetos, angústias, lamentações.

A Kahlile devo um agradecimento especial, por me aguentar reclamando incansávelmente de tudo e por compartilhar das minhas dúvidas e angústias.

Aos demais orientados da professora Lucimara, muito obrigado, de alguma maneira cada um de vocês contribuiu para minha formação acadêmica, Thaísa, Carol Leivas, Camila Tamiello e Pedro.

A turma do cafézinho, em especial para o Alex, ter a bancada na janela de frente para o cafézinho me proporcionou muitas overdoses de cafeína, discussões sobre política, esportes, religião, mercado financeiro, animes, games, e uma pitadinha de ciência.

Aos professores Guilherme Sassaki e Thales Cipriani, vocês certamentes são exemplos de profissionais que almejo me tornar.

Por último devo agradecer a turma dos mal-acabados, sem vocês confesso que seria muito mais difícil. Desejo que nossas amizades prosperem independentemente da distância. Arquimedes, Lauro, Kimura, Bernardo, Daniel, Diegão, Luizinho, Cícero e Adamara, vocês são INCRÍVEIS!

À toda equipe do RMN pelas análises.

À Elisangela pelas análises de HPSEC.

À Rosane e ao Lauro pelas análises em GC-MS.

Ao Programa de Pós-Graduação em Ciências-Bioquímica da UFPR.

Ao CNPq pelo suporte financeiro e ao PRONEX Carboidratos.

À todos, MUITO OBRIGADA!

“No que diz respeito ao empenho, ao compromisso, ao esforço, à dedicação, não existe meio termo. Ou você faz uma coisa bem feita ou não faz.”.

Ayrton Senna

RESUMO

Malpighia emarginata é uma planta frutífera tropical, encontrada naturalmente nas ilhas do Caribe e na América do Sul, seu fruto comestível é conhecido como Acerola ou *Barbados Cherry*. Seus polissacarídeos foram obtidos por extrações aquosas, submetidas ao processo de congelamento e descongelamento e por ultrafiltração. Foram realizadas análises de composição monossacarídica, HPSEC, metilação e obtidos espectros de RMN da fração purificada (ACWS-01E). Os resultados apresentaram uma pectina rica em arabinana, com massa molar de 6.1 x 10^4 g/mol e constituída principalmente por uma homogalacturonana altamente metil-esterificada (DM= 86%) e arabinanas ramificadas. Estas últimas estão ancoradas em regiões de ramnogalacturonana do tipo I. A cadeia principal das arabinanas são constituídas por unidades de α-Ara*f* (1→5)-ligadas ramificadas somente em *O*-3. A potencial atividade antioxidante intracelular da fração ACWS-01E contra o *stress* oxidativo induzido pelo H_2O_2 em linhagem celular de fibroblasto murino (3T3) foi determinada atráves da sonda DCFH-DA. O tratamento com a fração ACWS-01E reduziu significativamente o efeito citotóxico induzido pelo H_2O_2, assim como os níves de ROS. Estes resultados sugerem que ACWS-01E protegeu e aumentou a viabilidade das células NIH-3T3 após toxicidade induzida pelo H_2O_2 atráves da diminuição dos níveis intracelulares de ROS. Uma nova extração foi realizada para obter quantidade suficiente de amostra para avaliar atividades biológicas *in vivo*, da fração denominada ACWS. Esta nova fração apresentou 93% de carboidratos totais, massa molar relativa de 7,5 × 10^4 g/mol, ácido galacturônico, arabinose, galactose, xilose e ramnose numa relação molar de 52,1:32,4:7,2:4,8:3,5 e teve sua estrutura confirmada por análises de RMN. A atividade antifadiga da fração ACWS foi avaliada utilizando o *Weight load swim test*. ACWS foi administrada via oral em doses de 50 mg/kg, 100 mg/kg e 200 mg/kg durante 28 dias. Foram determinados parâmetros bioquímicos plasmáticos, respiração de fibras musculares esqueléticas permeabilizadas e dosados níveis de GSH e lipoperoxidação (LPO) em diferentes áreas do cérebro (córtex pré-frontal, hipocampo, estriado e hipotálamo). ACWS prolongou o tempo de natação, aumentou os níveis plasmáticos de glucose, triglicérideos, lactato e os níveis de GSH no hipocampo em todas as doses testadas. A capacidade respiratória mitocondrial do músculo esquelético foi aumentada nas doses média e alta de ACWS. Este estudo fornece uma forte evidência de que a suplementação de polissacarídeos pécticos da *M. emarginata* tem atividade antifadiga, pode modificar a cinética da mobilização de substratos energéticos (carboidratos e gorduras) e a capacidade respiratória do músculo esquelético, bem como o status antioxidante no hipocampo dos animais.

Palavras-chave: *Malpighia emarginata*, pectina, atividade antioxidante intracelular, atividade antifadiga.

ABSTRACT

Malpighia emarginata is a tropical fruit plant, found naturally in the Caribbean islands and South America that produces an edible fruit known as acerola or Barbados Cherry. Its polysaccharides were obtained by aqueous extraction, submitted to a freezing and thawing process and ultrafiltration. A homogeneous fraction (ACWS-01E) was analyzed by sugar composition, HPSEC, methylation and NMR spectroscopy analyses. The results showed an arabinan-rich pectic polysaccharide, with 6.1 x 10^4 g/mol and formed mainly by a high methyl esterified (DM= 86%) homogalacturonan and branched arabinan. This latter is anchored in type I rhamnogalacturonan regions. The main chain of arabinan consisted of (1→5)-linked α-Ara*f*, branched only at *O*-3. The potential ACWS-01E intracellular antioxidant activity against H_2O_2-induced oxidative stress in murine fibroblast cell line (3T3) was determined by DCFH-DA assay. The treatment with ACWS-01E significantly reduced H_2O_2-induced cytotoxic effect and the levels of ROS. These findings suggested that ACWS-01E protected and improved NIH 3T3 cell viability from H_2O_2-induced toxicity by decreasing intracellular levels of ROS. A new extraction was performed to obtain sufficient amount of sample of ACWS fraction to perform biological activities in vivo. This fraction presented 93% of total carbohydrate, relative molecular weight of 7.5 × 10^4 g/mol, galacturonic acid, arabinose, galactose, xylose and rhamnose in 52.1:32.4:7.2:4.8:3.5 molar ratio and had its structure confirmed by NMR analysis. The anti-fatigue activity of ACWS was evaluated using the weight load swim test on trained mice. ACWS was orally administered at doses of 50mg/kg, 100mg/kg and 200mg/kg for 28 days. Plasma biochemical parameters, respiration of permeabilized skeletal muscle fibers, and GSH levels and lipoperoxidation (LPO) in the brain (pre-frontal cortex, hippocampus, striatum and hypothalamus) were determined. ACWS could lengthen the swimming time, increase the plasma levels of glucose, triglycerides, lactate, and the GSH levels in the hippocampus at all tested doses. The mitochondrial respiratory capacity of the skeletal muscle was increased at middle and high ACWS dose. This study provides strong evidence that *M. emarginata* pectic polysaccharide supplementation have anti-fatigue activity, can modify the kinetics of energy substrates (carbohydrate and fat) mobilization and the respiratory capacity of the skeletal muscle, as well the antioxidant status in the hippocampus of ACWS treated animals.

Keywords: Pectin; *Malpighia emarginata*; intracellular antioxidant activity, anti-fatigue activity.

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ARTIGO 1

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Solventes e reagentes

$BaCO_3$	-	Carbonato de bário
DMSO	-	Dimetilsulfóxido
D_2O	-	Água deuterada
EtOH	-	Etanol
H2O2	-	Peróxido de hidrogênio
H_2SO_4	-	Ácido sulfúrico
HCl	-	Ácido clorídrico
MeI	-	Iodometano
MeOH	-	Metanol
MTT	-	Brometo de (3-metil-[4-5-dimetiltiazol-2-il]-2,5 difeniltetrazólio)
$NaBD_4$	-	Borohidreto de sódio deuterado
NaOH	-	Hidróxido de sódio

Frações obtidas dos frutos da acerola *(Malpighia emarginata)*

ACW	-	Extrato polissacarídico obtido após extração aquosa da acerola
ACWP	-	Fração precipitada do congelamento e degelo, após extração aquosa da acerola
ACWS	-	Fração sobrenadante do congelamento e degelo, após extração aquosa da acerola
ACWS-01E	-	Fração eluída na ultrafiltração em membrana de 0,1 μm do sobrenadante da extração aquosa da acerola
ACWS-01E-CR	-	Fração caboxireduzida da fração eluída na ultrafiltração em membrana de 0,1 μm do sobrenadante da extração aquosa da acerola
ACWS-01R	-	Fração retida na ultrafiltração em membrana de 0,1 μm do sobrenadante da extração aquosa da acerola

Termos associados à estrutura de polissacarídeos

f	-	Furanosídica
p	-	Piranosídica
AG	-	Arabinogalactana
AG-I	-	Arabinogalactana tipo I
AG-II	-	Arabinogalactana tipo II
AGP	-	Arabinogalactana proteína
APGA	-	Apiogalacturonanas
Ara	-	Arabinose
ArGa	-	Arabinogalacturonanas
DE / DM	-	Grau de metil-esterificação
Gal	-	Galactose
GalA	-	Ácido galacturônico

GGA	-	Galactogalacturonanas
Glc	-	Glucose
HG	-	Homogalacturonana
HM	-	Pectinas com grau de esterificação superior a 50% (*High methoxyl*)
LM	-	Pectinas com grau de metil esterificação menor que 50% (*Low methoxyl)*
M_w	-	Massa molecular
RG-I	-	Ramnogalacturonana tipo I
RG-II	-	Ramnogalacturonana tipo II
Rha	-	Ramnose
UA	-	Ácido Urônico
XGA	-	Xilogalacturonanas
Xyl	-	Xilose

Métodos analíticos

^{13}C-NMR	-	Ressonância magnética nuclear de carbono treze
^{1}H-NMR	-	Ressonância magnética nuclear de hidrogênio
DCFH-DA	-	*2-7- dichloro-fluorescin-diacetate*
DPPH	-	*2,2-diphenyl-1-picrylhydrazyl*
FRAP	-	*Ferric ion reducing antioxidant power*
GC-MS	-	Cromatografia gasosa acoplada à espectrometria de massa
HAT	-	*Hydrogen atom transfer*
HPSEC	-	*High pressure size exclusion chromatography* (Cromatografia de exclusão estérica de alta performance)
HSQC	-	*Heteronuclear Single Quantum Coherence* (coerência heteronuclear simples quântica)
MALLS	-	Detector de espalhamento de luz laser em multiângulos
NMR	-	*Nuclear magnetic resonance* (Ressonância magnética nuclear)
ORAC	-	*Oxygen radical absorbance capacity*
RI	-	Indice de refração
SET	-	*Single electron transfer*
TRAP	-	*Total radical-trapping antioxidant parameter*

Atividade biológica

FBS	-	Soro fetal bovino
GSH	-	Glutationa redutase
HD	-	Grupo alta dosagem
LD	-	Grupo baixa dosagem
LPO	-	Lipoperoxidação
MD	-	Grupo média dosagem
NIH 3T3	-	Linhagem celular de fibroblastos murinos
NTC	-	Grupo controle não treinado
ROS	-	*Reactive oxygen species* (Espécies reativas de oxigênio)
TC	-	Grupo controle treinado
WLST	-	*Weight load swim test*

Análises Estatísticas

ANOVA - Análise de variancia
SD - *Standard derivation* (Desvio padrão)

SUMÁRIO

1 INTRODUÇÃO

Acerola (*Malpighia emarginata*) é uma fruta comestível nativa das ilhas do Caribe, encontrada em países da América Central e do Sul (SCHRECKINGER et al., 2010). De acordo com a Tabela Brasileira de Composição de Alimentos (TACO) a acerola tem 90,5% de umidade, carboidratos totais representam 8,0%, proteínas 0,9%, lipídios 0,2% e as cinzas 0,4% em 100 gramas do fruto *in natura*. Os carboidratos totais englobam tanto carboidratos de reserva energética, quanto carboidratos estruturais, sendo que destes 8% de carboidratos, 1,5% deles são fibras dietéticas. Os polissacarídeos constituintes das fibras dietéticas da acerola são desconhecidos, uma vez que nenhum estudo foi publicado com a caracterização química de suas estruturas (NEPA, 2011).

A heterogeneidade dos polissacarídeos de plantas requer uma caracterização química refinada para que sua estrutura seja estabelecida. Quando isolados e caracterizados, esses polissacarídeos são frequentemente estudados quanto às suas atividades biológicas, apresentando diversas aplicações biomédicas propostas na literatura (HAMAKER; TUNCIL, 2014).

Os polissacarídeos são apontados como importantes agentes antioxidantes, tanto em testes *in vitro* quanto *in vivo*, desempenhando um papel crítico na defesa dos organismo vivos contra os danos oxidativos (DING et al., 2016; WANG et al., 2013, 2016a; XU et al., 2017). Além dissofd, a atividade antifadiga de polissacarídeos vem se consolidando com resultados promissores em diversos estudos (CHEN et al., 2015; CHI et al., 2012, 2015; LI et al., 2016; TAN et al., 2012; WEI et al., 2017; ZHANG et al., 2009; ZHAO et al., 2015).

Portanto estudos que elucidem a estrutura química fina dos polissacarídeos que constituem as fibras dietéticas da acerola são necessários para novas aplicações, tanto industriais quanto biomédicas para o fruto, agregando ainda mais valor a este produto. Propomos além disso, testar as atividades antioxidante e antifadiga dos polissacarídeos extraídos da acerola, visto que são escassos na literatura estudos onde o potencial antioxidante intracelular dos polissacarídeos é investigado, assim como, nenhum estudo envolvendo um polissacarídeo extraído de

algum fruto foi investigado quanto ao seu potencial antifadiga até o presente momento.

2 REVISÃO BIBLIOGRÁFICA

2.1. ACEROLA (*Malpighia emarginata*)

Acerola (*Malpighia emarginata*) também conhecida popularmente como cereja das antilhas, *Barbados Cherrys* ou *West Indian cherry* é uma fruta comestível nativa das ilhas do Caribe, encontrada em países da América Central e do Sul (SCHRECKINGER et al., 2010). O fruto da aceroleira, é uma drupa, carnosa, podendo variar o tamanho de 1 a 4 cm e o peso de 2 a 15 gramas (Figura 1). A coloração varia de acordo com a maturação, indo do verde ao roxo, devido sobretudo à degradação da clorofila e síntese de antocianinas (SCHRECKINGER et al., 2010).

No Brasil, seu cultivo intensificou-se na década de 80, sendo constatada considerável expansão da área cultivada. Apresenta boa adaptação climática e facilidade de cultivo contando, ainda, com um grande apelo popular, despontando ser uma fruta com um imenso mercado a ser explorado. O Brasil é o principal produtor, consumidor e exportador do fruto, tendo o Japão como seu maior destino, seguido pelos Estados Unidos e Europa (DELVA; SCHNEIDER, 2013).

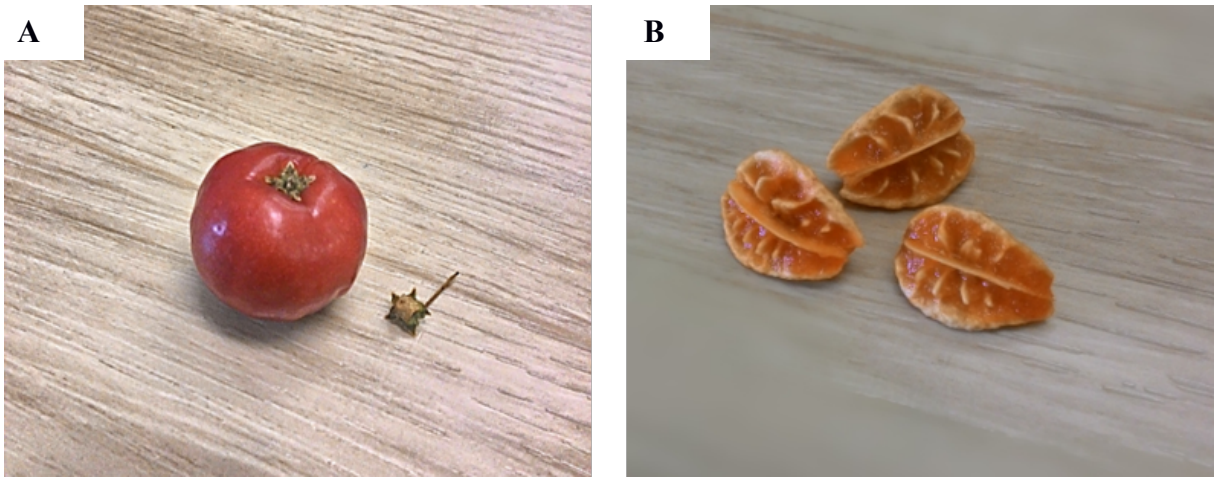
O uso popular medicinal da acerola se deve principalmente às grandes quantidades de vitamina C presentes no fruto e grande potencial antioxidante, por isso é recomendada popularmente para tratamento ou prevenção de diversas doenças causadas por *stress* oxidativo (DELVA; SCHNEIDER, 2013). Já foram descritas atividades hepatoprotetora e antioxidante dos frutos da acerola (DÜSMAN et al., 2014; NUNES et al., 2011; PAZ et al., 2015), além de atividades hipoglicemiante e diminuição dos triglicerídeos plasmáticos após consumo de sucos naturais da acerola (BARBALHO et al., 2011; DIAS et al., 2014; LEFFA et al., 2014, 2015).

De acordo com a Tabela Brasileira de Composição de Alimentos a acerola tem umidade de 90,5%, carboidratos totais representam 8,0%, proteínas 0,9%, lipídios 0,2% e as cinzas 0,4%, Os carboidratos totais englobam tanto carboidratos de reserva energética, quanto carboidratos estruturais, e quando se observa quanto desses carboidratos são fibras dietéticas obtemos 18,8% (1,5% do fruto *in natura*) (NEPA, 2011). Este fruto exótico com alto valor nutricional, apresenta além da

vitamina C, outros compostos bioativos como antocianinas, ácidos fenólicos, carotenoides. Ao contrário dos compostos de baixa massa molecular que são bem estudados, a estrutura da fibra dietética presente na acerola permanece desconhecida, embora tenha grande representatividade na composição do fruto desidratado (BATAGLION et al., 2015; DA SILVA et al., 2014; DE ROSSO; MERCADANTE, 2005; MEZADRI et al., 2008; VERA DE ROSSO et al., 2008).

A definição publicada pela comissão do *Codex Alimentarius* em 2009 chegou a um consenso de que fibras dietéticas são polímeros de carboidratos com dez ou mais unidades, os quais não são hidrolisados por enzimas endógenas do intestino humano (FAO, 2009). Neste contexto muitos polissacarídeos podem ser classificados como fibra dietética, assim como celulose, hemiceluloses, pectinas, gomas, mucilagem e frutanas. Estes polissacarídeos compõe a parede celular (Figura 2) das plantas, uma rede altamente organizada de polissacarídeos, como pectinas, hemiceluloses e celulose (CARPITA; GIBEAUT, 1993).

FIGURA 1 - FRUTO DA ACEROLEIRA (*Malpig$_h$ia emarginata*) (**A**) E SEMENTE DO FRUTO (**B**).



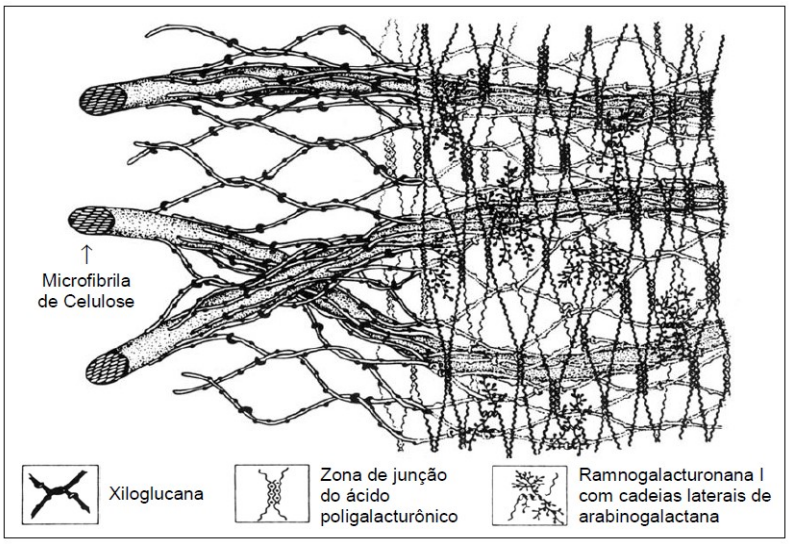
FONTE: O autor (2017)

2.2. PECTINAS

As pectinas são consideradas os polissacarídeos mais complexos estruturalmente e apresentam diversas funções no metabolismo das plantas, relacionadas ao crescimento, morfologia, desenvolvimento e defesa das plantas.

Estão presentes na lamela média e na parede celular primária (CAFFALL; MOHNEN, 2009).

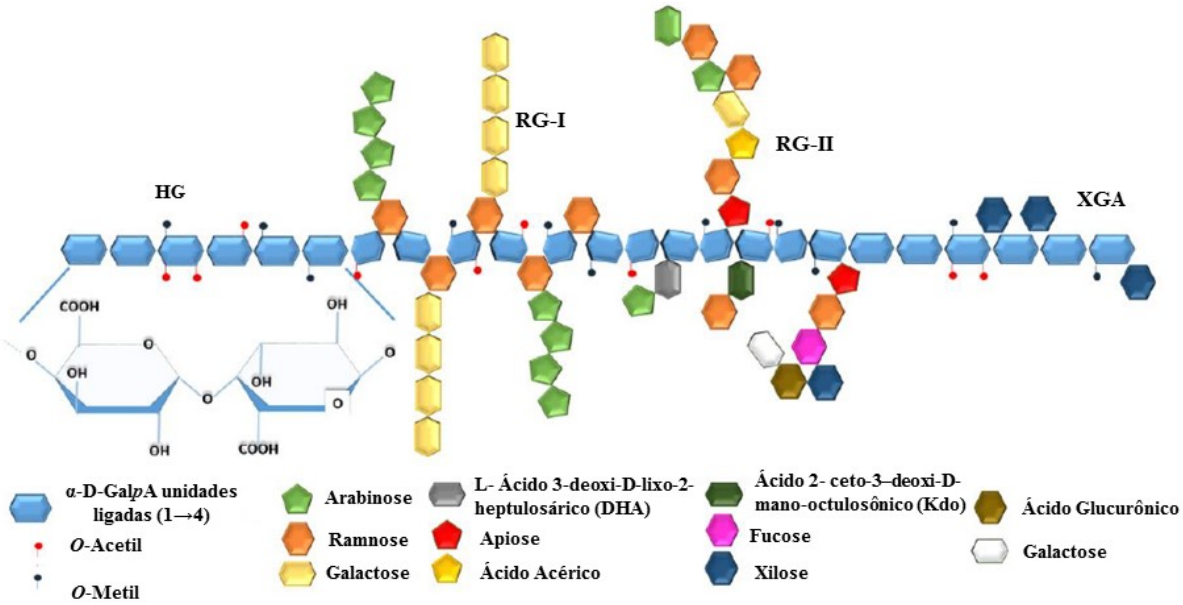
FIGURA 2 - ESQUEMA PROPOSTO DA PAREDE CELULAR PRIMÁRIA



FONTE: Adaptado de Carpita e Gibeaut (1993)

O ácido galacturônico (Gal*p*A) é o monossacarídeo mais abundante nas pectinas, seguido por galactose (Gal) e arabinose (Ara), porém mais de 17 monossacarídeos já foram observados em pectinas. Os monossacarídeos são distribuídos em regiões características, e não de maneira aleatória, são pelo menos sete as regiões pécticas descritas na literatura, nomeadas como homogalacturonana (HG), ramnogalacturonana do tipo I (RG-I) e ramnogalacturonana do tipo II (RG-II) as mais relatadas, mas também são encontradas xilogalacturonanas (XGA), apiogalacturonanas (APGA), galactogalacturonanas (GGA), e arabinogalacturonanas (ArGA) (YAPO, 2011). Um modelo esquemático pode ser observado na FIGURA 3, onde a cadeia de HG (*smooth regions*) são substituídas por regiões ramificadas de RG (*hairy regions*).

FIGURA 3 - MODELO ESQUEMÁTICO DE PECTINAS



FONTE: Adaptado de (ALBUQUERQUE; COELHO; TEIXEIRA, 2016)

As HGs representam aproximadamente 55-70% do total de pectinas, sendo o principal elemento estrutural das pectinas. São homopolímeros constituídos de unidades α-D-Gal*p*A ligadas (1→4), as quais podem ser metil-esterificadas no C6 e acetil-esterificadas nas posições *O*-2 e/ou *O*-3. (YAPO, 2011). O grau de esterificação (DE) das pectinas faz referência a proporção de grupos carboxílicos metil-esterificados, classificando em pectinas de alta metoxilação (HM- *High methoxyl*) e de baixa metoxilação (LM – *Low methoxyl*). Pectinas HM apresentam 50% ou mais de grupos carboxílicos metil-esterificados, enquando as LM apresentam menos que 50%.

As RG-I fazem parte das "*hairy regions*" das pectinas, geralmente são heteropolissacarídeos altamente ramificados, constituídos por uma cadeia principal de unidades alternantes de α-D-Gal*p*A ligadas (1→4) e unidades de α-L-Rha*p* ligadas (1→2), podendo ser substituídas nas posições *O*-4 e/ou *O*-3, por uma única unidade glicosil neutra, ou por cadeias laterais poliméricas, tais como (1→5)-α-L-arabinanas, (1→4)-β-D-galactanas, arabinogalactanas tipo I (AG-I), arabinogalactanas tipo II (AG-II) (MOHNEN, 2008; YAPO, 2011).

São denominadas arabinanas polissacarídeos compostos por arabinose (Ara), principalmente na forma furanosídica, α-(1→5)-L-Ara*f* ligadas, podendo também estarem substituídas em *O*-2 e/ou *O*-3 por cadeias laterais de unidades Ara*f*.

Arabinanas frequentemente estão ancoradas nas RG-I na posição *O*-4 e/ou *O*-3 das ramnoses (REID, 1997).

Arabinogalactanas (AG) são subdivididas de acordo com o tipo de ligação das unidades de galactose que compõe sua cadeia principal, arabinogalactanas do tipo I (AG-I) e arabinogalactanas do tipo II (AG-II) (CARPITA; GIBEAUT, 1993). As AGs que apresentam cadeia principal (1→4)-β-D-galactanas são denominas AG-I, e frequentemente apresentam unidades de arabinose em *O*-3 das galactoses e estão ancoradas nas RG-I, compondo as "*hairy regions*", por isso também são chamadas de arabinogalactanas pécticas. Por sua vez, as AG-II são amplamente mais distribuidas que as AG-I, formadas por curtas cadeias de (1→3) e (1→6)-β-D-galactanas concectadas umas às outras por ramificações em *O*-3 e *O*-6, e pode chegar a apresentar até 80% de arabinose ligada nas posições *O*-3 e *O*-6 (ALBERSHEIM et al., 1996; CARPITA; GIBEAUT, 1993). Monossacarídeos ácidos, como o ácido glucurônico, ácido 4-*O*-metil-glucurônico e o ácido galacturônico também foram relatados presentes nas AG-II, assim como proteínas, constituindo as proteoglicanas denominas arabinogalactanas-proteínas (AGP) (CAFFALL; MOHNEN, 2009).

2.3. ATIVIDADE BIOLÓGICA DE POLISSACARÍDEOS

Os polissacarídeos de plantas são frequentemente estudados quanto às suas atividades biológicas, apresentando diversas aplicações biomédicas já apresentadas na literatura, como ação anticoagulante (OCHOA et al., 2017; SANTANA et al., 2016), ação anti-inflamatória (LEIVAS et al., 2016; PEREIRA et al., 2012; WANG et al., 2015), antinociceptiva (COURA et al., 2017; DO NASCIMENTO et al., 2013, 2015; NASCIMENTO et al., 2016) , gastroprotetora (BADANAVALU CHANDRASHEKAR; DHARMESH, 2016; HARSHA; CHANDRA PRAKASH; DHARMESH, 2016; NASCIMENTO et al., 2017), hepatoprotetora (WANG et al., 2016b; XU et al., 2017; ZHANG et al., 2017), ação no sistema imune (BO et al., 2016; HOLDERNESS et al., 2011; OHTA et al., 2014), além de atividade antioxidante e antifadiga, as quais serão descritas nas seções posteriores

2.3.1. Atividade antioxidante

Os polissacarídeos emergiram como importantes agentes antioxidantes, tanto em testes *in vitro* quanto *in vivo*, desempenhando um papel crítico na defesa dos organismo vivos contra os danos oxidativos (WANG et al., 2016a). Diversos modelos *in vitro* são utilizados para avaliar o potencial antioxidante das moléculas. Podemos dividi-los em três grupos distintos: métodos de transferência de átomos de hidrogênio (HAT – *Hydrogen atom transfer*), métodos de transferência de um único elétron (SET – *Single electron transfer*) e métodos em cultivos celulares, onde a defesa antioxidante intracelular é investigada (WANG et al., 2016a).

Como exemplos de métodos HAT, os mais utilizados e conhecidos na literatura são: capacidade de absorção do radical oxigênio (ORAC – *Oxygen radical absorbance capacity*) e o parâmetro antioxidante de captura radical total (TRAP – *Total radical-trapping antioxidant parameter*). Métodos SET que detectam a capacidade de transferência de um elétron e redução de qualquer composto, resultando na mudança de cor aferível por espectrofotômetro, incluem o DPPH (*2,2-diphenyl-1-picrylhydrazyl*) como o método mais utilizado, assim como o FRAP (*Ferric ion reducing antioxidant power*). E por último com relação aos métodos de cultivos celulares a quantidade de alternativas para investigar a defesa oxidante das células é enorme, porém a utilização de sondas intracelulares que fluorescem na presença de radicais livres emergem como importante ferramenta de metodologia para detectar os compostos que agem na defesa antioxidante celular (WANG et al., 2013, 2016a; YAZDANI, 2015).

Para monitorar os níveis de espécies reativas de oxigênio (ROS – *Reactive oxygen species*) em sistemas *in vitro*, a detecção por sondas tem atraído o interesse por se tratar de um modelo mais reprodutivo em sistemas biológicos do que os métodos HAT e SET (WANG et al., 2016a; YAZDANI, 2015). A sonda DCFH-DA (2-7- dichloro-fluorescin-diacetate) é um excelente sensor para detecção de ROS não específicos para análises preliminares da atividade biológica em linhagens celulares (YAZDANI, 2015). A seguir será apresentado alguns trabalhos que utilizaram esta sonda (DCFH-DA) para investigar a capacidade antioxidante de polissacarídeos.

QI et al., (2014) observaram que o extrato aquoso do fruto *Goji berry* (*Lycium barbarum*), predominantemente composto por polissacarídeos, diminuiu significativamente a apoptose celular e os níveis de ROS em células epiteliais da

retina humana (SRV01/04) após dano induzido por peróxido de hidrogênio (H_2O_2). JIA et al., (2015) estudaram o polissacarídeo purificado extraído da planta oriental *Gynostemma pentaphyllum,* e observaram a capacidade do polissacarídeo composto por glucose e manose de proteger as células PC12 contra o efeito citotóxico causado pelo H_2O_2. Os polissacarídeos da planta medicinal chinesa *Rhizoma dioscoreae* foram capazes de atenuar os níves intracelulares de ROS induzidos por H_2O_2 em células do cordão umbilical (HUVECs) (JIN et al., 2015). E por último CHOWDHURY et al., (2014) observaram redução significativa dos níveis de ROS após adição de H_2O_2 em fibroblastos humanos (WI38) quando realizado tratamento com polissacarídeo rico em fucose extraído de uma espécie de bactéria (*Bacillus megaterium*). Com isso, podemos observar que polissacarídeos de diversas fontes apresentam efeito protetor intracelular contra danos caudados por ROS em diversas linhagens celulares.

2.3.2. Atividade antifadiga

A fonte de energia imediata para a contração muscular é adenosina trifosfato (ATP), e seus níveis de armazenamento intracelulares são pequenos (5-6 mM) e caso haja uma máxima contração muscular, seu estoque seria esgotado dentro de 2 segundos (WESTERBLAD; BRUTON; KATZ, 2010). Desta maneira outras vias metabólicas devem ser ativadas para evitar a depleção de ATP. Estas vias podem ser divididas em vias anaeróbica e aeróbica, das quais a primeira é predominantemente ativada em exercícios de alta intensidade e curta duração enquanto a segunda em exercícios de longa duração e baixa intensidade (SAHLIN; TONKONOGI; SÖDERLUND, 1998).

A fadiga é definida pela diminuição do rendimento e incapacidade de manutenção das contrações musculares e pode ser categorizada em duas vertentes: fadiga periférica e central. A fadiga periférica está associada a uma diminuição da força muscular causada por um processo distinto ao sistema nervoso central, enquanto fadiga central é o termo para a fadiga causada por fatores que residem dentro do sistema nervoso central, cérebro, medula espinhal e neurônios motores (AMENT; VERKERKE, 2009; PHILLIPS, 2015). São múltiplos os fatores que levam

uma pessoa a entrar em estado de fadiga, os quais estão relacionados às características de intensidade e volume, específicas de cada exercício. As avalições destas duas variáveis são imprescindíveis quando se investiga os causadores do aparecimento da fadiga (PHILLIPS, 2015).

Exercícios mais intensos, onde a demanda por adenosina trifosfato (ATP) é grande e rápida, o metabolismo anaeróbico predomina na resíntese do ATP atráves da degradação da fosfocreatina (PCr) e da quebra do glicogênio muscular até lactato e íons hidrogênio (WESTERBLAD; BRUTON; KATZ, 2010). O metabolismo oxidativo de carboidratos e de lípidios é o responsável pela ressíntese de ATP em exercícios de alto volume (maior duração) e baixa intensidade, sendo o glicogênio muscular o principal fornecedor de carboidrato para o metabolismo aeróbico, enquanto os ácidos graxos livres (FFA) a serem oxidados são provenientes dos triglicerídeos estocados no músculo e no tecido adiposo (SPRIET; WATT, 2003).

Os polissacarídeos de plantas quando testados com o objetivo de retardar o aparecimento da fadiga mostraram promissores resultados, quando utilizados principalmente dois modelos experimentas, o *Forced Swim Test* (FST) e o *Weight Load Swim Test* (WLST). O FST é um modelo comportamental, onde os animais são colocados para nadar num determinado período (15-30 min) e os tempos que estes animais permaneceram flutuando ou nadando são quantificados, e quando o animal permanece nadando mais tempo que o controle, a molécula é apontada como agente antifadiga. Por outro lado, no WLST são acrescidos pesos ao rabo dos animais e eles são colocados para nadar sem um tempo limítrofe, e sim até eles afundarem e não retornarem a superfície após um período pré-determinado (5-10 seg), este momento é anotado e estabelecido como tempo suportado até a exaustão. Serão apresentados a seguir estudos onde o método utilizado para verificar a atividade antifadiga foi o WLST (TANAKA et al., 2003).

Apenas dois estudos apresentam a caracterização química fina dos polissacarídeos testados. WEI et al. (2017) testaram uma glucomanana extraída do *Dendrobium officinale*, planta medicinal chinesa, e observou aumento no tempo para exaustão, enquanto CHEN et al. (2015) testaram uma fucoidana, extraída da alga *Laminaria japanica* e também observaram atividade antifadiga.

Outros estudos apresentaram somente a composição monossacarídica dos polissacarídeos testados. LI et al. (2016) relataram atividade antifadiga de polissacarídeos extraídos do *Lepidium meyenni*, um tubérculo original da região dos Andes, conhecido como Maca peruana, que apresentou predominantemente na composição monossacarídica glucose, galactose, arabinose e xilose. CHI et al. (2015) estudaram um polissacarídeo extraído do chá verde *Ziyang*, com glucose, arabinose e galactose na composição monossacarídica, e relataram que o polissacarídeo foi hábil em prolongar o tempo de nado até a exaustão. Polissacarídeo contendo glucose, galactose e arabinose na sua composição foi extraído da planta *Gynostemma pentaphyllum* e testado por CHI et al. (2012) apresentando atividade antifadiga. Extratos aquosos de *Millettiae speciosae*, *Radix Rehmanniae* e raízes de *Morinda officinalis* foram testados por ZHAO et al. (2015), TAN et al. (2012) e ZHANG et al. (2009), respectivamente, e todos eles apresentaram atividade antifadiga.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Caracterizar estruturalmente polissacarídeos presentes na acerola (*Malpighia emarginata*) e avaliar os possíveis efeitos antioxidante e antifadiga dos polissacarídeos em modelos *in vitro* e *in vivo*.

3.2. OBJETIVOS ESPECÍFICOS

Para atingir o objetivo geral acima descrito, os seguintes objetivos específicos foram propostos:

- Extrair os polissacarídeos da acerola por extrações aquosas;
- Purificar polissacarídeos extraídos;
- Caracterizar estruturalmente os polissacarídeos purificados;
- Investigar o potencial antioxidante dos polissacarídeos purificados em modelos *in vitro*;
- Investigar o efeito antifadiga de polissacarídeos em modelo *in vivo*.

ARTIGO I

Structure and intracellular antioxidant activity of pectic polysaccharide from acerola (*Malpighia emarginata*)

Submetido na revista *International Journal of Biological Macromolecules*

Structure and intracellular antioxidant activity of pectic polysaccharide from acerola (*Malpighia emarginata*)

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ABSTRACT

Malpighia emarginata is a tropical fruit plant, found naturally in the Caribbean islands and South America that produces an edible fruit known as acerola or Barbados Cherry. Its polysaccharides were obtained by aqueous extraction, submitted to a freezing and thawing process and ultrafiltration. A homogeneous fraction (ACWS-01E) was analyzed by sugar composition, HPSEC, methylation and NMR spectroscopy analyses. The results showed an arabinan-rich pectic polysaccharide, with 6.1 x 10^4 g/mol and formed mainly by a high methyl esterified (DM = 86%) homogalacturonan and branched arabinan. This latter is anchored in type I rhamnogalacturonan regions. The main chain of arabinan consisted of (1→5)-linked α-Ara*f*, branched only at *O*-3. The potential ACWS-01E intracellular antioxidant activity against H_2O_2-induced oxidative stress in murine fibroblast cell line (3T3) was determined by DCFH-DA assay. The treatment with ACWS-01E significantly reduced H_2O_2-induced cytotoxic effect and the levels of ROS. These findings suggested that ACWS-01E protected and improved NIH 3T3 cell viability from H_2O_2-induced toxicity by decreasing intracellular levels of ROS.

Keywords: Pectin; *Malpighia emarginata*; intracellular antioxidant activity.

1 INTRODUCTION

Malpighia emarginata is a tropical fruit plant, found naturally in the Caribbean islands and South America, that produces an edible fruit known as acerola or Barbados Cherry. In folk medicine, acerola possess a great attractiveness because of its very high vitamin C content. Besides, due to its antioxidant activity its ingestion is recommended for the management of various diseases caused by oxidative stress (DELVA; SCHNEIDER, 2013). Pharmacological activities have been reported for acerola fruits (DÜSMAN et al., 2014; NUNES et al., 2011; PAZ et al., 2015), natural juices (BARBALHO et al., 2011; DIAS et al., 2014; LEFFA et al., 2014, 2015) and industrial pulps (ARAÚJO et al., 2015) and their beneficial effects are attributed to low molecular weight compounds.

This exotic fruit has a high nutritional value providing significant amounts of bioactive compounds besides vitamin C, like anthocyanins, phenolic acids, carotenoids and dietary fiber (BATAGLION et al., 2015; DA SILVA et al., 2014; DE ROSSO; MERCADANTE, 2005; MEZADRI et al., 2008; VERA DE ROSSO et al., 2008). Unlike the low molecular weight compounds which are well known, the structure of dietary fibers present in acerola remains unknown, although they represent approximately 10% of the dry fruit (NEPA, 2011). The definition published by the Codex alimentarius commission in 2009 reaches a consensus that dietary fiber are carbohydrate polymers with ten or more units, which are not hydrolyzed by endogenous enzymes in the human intestine (FAO, 2009). In this context, many polysaccharides are included in this definition, as well as cellulose, hemicelluloses, pectins, gums, mucilages and fructans, which exhibit different physicochemical properties (HAMAKER; TUNCIL, 2014; VERSPREET et al., 2016).

Along with different physicochemical properties of polysaccharides, comes diversity of their biological activities. Therefore, the importance of scientific studies of structural characterization of polysaccharides is wide. Data from literature shows that a variety of polysaccharides exhibit antioxidant capacity, *in vitro* and *in vivo* and that many diseases, such as asthma, chronic obstructive pulmonary disease, inflammation, diabetes, myocardial infarction, and cardiovascular diseases, are linked to oxidative stress. However, more studies are necessary to elucidate the action of several purified polysaccharides (WANG et al., 2016a).

Thus, the present study reports the structural characterization of the main pectic polysaccharide present in acerola's soluble dietary fiber, as well as its intracellular antioxidant activity in cell culture.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL

Ripe fruits of acerola (*Malpighia emarginata*) were purchased in a local market in Curitiba, state of Paraná, Brazil.

2.2 EXTRACTION AND PURIFICATION OF POLYSACCHARIDES

The seeds were manually removed from the fruit and the pulp, along with the peel, were freeze-dried and milled. The nonpolar compounds of the dried pulp were extracted using chloroform-methanol (1:1) as solvent using Soxhlet apparatus.

Polysaccharides were extracted from the residue with boiling water under reflux for 2 h (×6, 1 L each). The aqueous extracts were combined and concentrated under reduced pressure. The polysaccharides were precipitated with ethanol (3 vol.), and freeze-dried, giving fraction ACW. This was further submitted to a freezing and thawing process (GORIN; IACOMINI, 1984). In this procedure the sample was frozen and thawed at room temperature followed by centrifugation, originating the cold-water soluble (ACWS) and insoluble (ACWP) fractions. After, fraction ACWS was purified by ultrafiltration through membrane with 0.1 μm cut-off, yielding fractions eluted (ACWS-01E) and retained (ACWS-01R) on the membrane.

Polysaccharides yields were expressed as percentages based on the weight of dried acerola subjected to extraction (48g).

2.3 HOMOGENEITY, MOLECULAR WEIGHT, MONOSACCHARIDE COMPOSITION AND TOTAL URONIC ACID ANALYSIS DETERMINATION

Homogeneity, molecular weight, monosaccharide composition and total uronic acid analyses were performed as previously published protocols (CANTU-JUNGLES et al., 2017; DO NASCIMENTO; IACOMINI; CORDEIRO, 2016).

Briefly, the homogeneity and molecular weight (Mw) of the soluble polysaccharides were determined by High Performance Steric Exclusion chromatography (HPSEC). The analyses were performed at 25 ºC on a Waters chromatograph equipped with four Ultrahydrogel column connected in series (2000, 500, 250, 120; with size exclusion of $7x10^6$ Da, $4x10^5$ Da, $8x10^4$ Da and $5x10^3$ Da), coupled to a Waters 2410 differential refractometer (RI) detector and a DSP-F Wyatt Technology Multiangle Laser Light Scattering (MALLS) detector. The specific refractive index increment (*dn/dc*) was determined and the results were processed with software ASTRA provided by the manufacturer.

Neutral monosaccharide compounds and their ratios were determined after hydrolysis of the polysaccharides with trifluoracetic acid, then the hydrolysates were converted to alditol acetates by sodium borohydride reduction, and acetylation with acetic anhydride-pyridine (1:1, v/v). These were analyzed by GC-MS (Varian Saturn 2000R–3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225 column (30 m x 0.25 mm i.d.) programmed from 50 to 210 ºC at 40 ºC /min, with He as carrier gas.

Uronic acid contents were determined using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

2.4 REDUCTION OF URONIC ACIDS AND METHYLATION/GC-MS ANALYSIS

Uronic acids were reduced using carboxyl reduction method of Taylor & Conrad (TAYLOR; CONRAD, 1966) prior to glycosyl linkage analysis. Carboxyl-reduced samples were *O*-methylated according to the method of Ciucanu and Kerek (CIUCANU; KEREK, 1984), using powdered NaOH in DMSO-MeI. The per- *O*-methylated polysaccharide was then submitted to methanolysis in 3.5% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 12 h, at 100 °C) and

neutralization with $BaCO_3$. The material was then submitted to reduction and acetylation as described above for sugar composition, except that the reduction was performed using $NaBD_4$. The products (partially *O*-methylated alditol acetates) were examined by capillary GC-MS. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (SASSAKI et al., 2005).

2.5 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

^{13}C {^1H} and HSQC NMR spectra were acquired at 70 ºC on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^1H at 400.13 MHz and ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The water-soluble samples were acquired in D_2O. Chemical shifts were expressed as δ ppm relative to CH_3 signal from acetone at δ 30.2 as internal reference. Chemical shifts were expressed as δ ppm relative to CH_3 signal from acetone at δ 30.2 and 2.22 for ^{13}C and ^1H signals, respectively, as internal reference.
The degree of methyl esterification (DE) was determined by ^1H NMR spectroscopy according (GRASDALEN; EINAR BAKOY; LARSEN, 1988). Briefly, the fraction was deuterium-exchanged three times by freeze-drying with D_2O solutions, finally dissolved in D_2O, transferred into 5-mm NMR tube. The ^1H NMR spectra were acquired at 70°C, with 256 scans, on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^1H at 400.13 MHz. Chemical shifts were expressed as δ ppm, using the resonances of HDO at δ 4.22 as internal reference.
All pulse programs were supplied by Bruker.

2.6 CELL CULTURE

NIH 3T3 murine fibroblast cell line was kindly provided by Prof. Mari C. Sogayar (Cell and Molecular Therapy Center - NUCEL/NETCEM, Faculty of Medicine, University of São Paulo - FMUSP). The cell line was cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100µg/mL) and was maintained at 37°C in a 5% CO_2 atmosphere.

2.7 CELL VIABILITY ASSAY

Cell viability was measured by colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Briefly, 5 ×10^3 cells/well were cultured on 96-well plates and incubated for 24 hours at 37 °C in a 5% CO_2 atmosphere. Cells were then treated with 10, 100 and 1000 µg/mL of ACWS-01E for 24, 48 and 72 hours. Polysaccharide-containing culture medium was removed after treatment and replaced with fresh medium plus 20 µL of MTT (5 mg/mL) for 3 hours, protected from light, at 37 ºC. The medium was then removed and 200 µL of dimethyl sulfoxide (DMSO) were added in order to solubilize the formazan formed on viable cells. Absorbance was measured spectrophotometrically at 545 nm. Results were expressed as percentage of control (vehicle used to dissolve the extract - milliQ water) assigned as 100% of viability.

2.8 TREATMENT PROCEDURE

For treatment, 5 × 10^3 cells/well were cultured in 96-well plates and incubated for 24 hours at 37 °C. The cells were then treated with 10µg/mL of ACWS-01E for 48 hours. Following time treatment, medium was removed and cells were exposed to 100 µM of H_2O_2 for 15 minutes. MTT method was performed as described above as well as the measure of intracellular levels of reactive oxygen species (ROS).

2.9 INTRACELLULAR ROS MEASUREMENT

The intracellular levels of ROS were assessed using DCFH-DA (2', 7'-dichloro-fluorescin diacetate, Sigma). Cells were seeded on black 96-well plates, treated as described above, and then incubated with 5 mM of DCFH-DA at 37 ºC for 30 minutes. Fluorescence was then measured on a spectrofluorometer (TECAN, Infinite M200) with a 405 nm excitation filter and a 520 nm emission.

2.10 DATA ANALYSIS

All data are expressed as mean ± SD of three independent experiments in sextuplicate and analysis were performed on GraphPad Prism software using a one-way analysis of variance (ANOVA) followed by Tukey's. Difference of $p < 0.05$ was considered to be statistically significant.

3 RESULTS

3.1 EXTRACTION, PURIFICATION AND CHARACTERIZATION OF POLYSACCHARIDES FROM ACEROLA (*M. emarginata*)

Polysaccharides were extracted from deffated acerola with water at 100 ºC and recovered from the extracts by EtOH precipitation and centrifugation, giving fraction ACW (8% yield). It was subjected to freezing and thawing procedure, yielding the cold-water soluble fraction (ACWS) in higher yield (6.6%) than cold-water insoluble one (ACWP, 0.2% yield). Monosaccharides analysis of ACWS revealed 59.6% of uronic acids, and arabinose (35.0%) as main neutral sugar (Table 1), indicating the presence of arabinan-rich pectic polysaccharides. On HPSEC analysis, this fraction presented three peaks (Fig. 1). In order to separate them, ACWS was submitted to ultrafiltration, resulting in eluted (ACWS-01E) and retained (ACWS-01R) fractions on the membrane. Both peaks were still observed in ACWS-01R, while only a single peak was observed in ACWS-01E, which presented a molecular weight of 6.1×10^4 g/mol (*dn/dc* = 0.139).

Data from the monosaccharide composition (Table 1) showed a great similarity between ACWS-01E, ACWS-01R and ACWS, with arabinose and uronic acid as main sugars present. The identity of uronic acid was revealed by carboxyl-reduction. The increase of galactose in the carboxyl-reduced ACWS-01E (ACWS-01E-CR) indicates the presence of GalA. ^{13}C NMR spectra of ACWS-01E and ACWS-01R (Figure 2 A, B), were also similar, indicating the presence of polysaccharides with similar structures, but with different molecular weights. Thus, detailed structural characterization was performed only with ACWS-01E, which presented a single peak on HPSEC analysis.

In order to achieve the sugar linkages of ACWS-01E, the fraction was carboxyl-reduced and submitted to methylation analysis (Table 2). Among the partially *O*-methylated derivatives, the 2,3,6-Me_3-Gal-ol acetate and 2,3-Me2-Ara-ol acetate were the main observed ones, from (1→4)-linked Gal*p* and (1→5)-linked Ara*f* units, respectively. These arise probably from an homogalacturonan (HG) and arabinan, respectively, and are in agreement with monosaccharide composition data. The arabinan main chain carried some branches at *O*-3 due to the presence of 2-Me-Ara-ol acetate. Other derivatives of galactose and arabinose were also present in small amounts, such as 2,3,4,6-Me_4-Gal-ol, 2,6-Me_2-Gal-ol, 2,3-Me_2-Gal-ol, 2-Me-Gal-ol, 2,3,5-Me_3-Ara-ol, 2,3,4-Me_3-Ara-ol and 2,5-Me_2-Ara-ol acetates, indicating the presence of arabinogalactan. Rhamnogalacturonan was also present, due to the 3,4-Me_2-Rha-ol and 3-Me-Rha-ol acetate derivatives. The derivative 2-Me-Xyl-ol acetate indicates the presence of 2,4-di-*O*-substituted Xyl*p* units, which may be linked to galacturonic acid at position 3, represented by the derivative 2,6-Me_2-Gal. This derivative suggest the presence of branched HG regions, already reported in the literature for soybean (NAKAMURA et al., 2002), apple, tomato (ZANDLEVEN et al., 2006), and *Arabidopsis thaliana* (ZANDLEVEN et al., 2007).

In addition, two-dimensional HSQC correlation map of ACWS-01E was obtained (Fig. 3), and the chemical shifts are arranged in Table 3. They were determined by analysis of COSY, TOCSY and according to the literature (CANTU-JUNGLES et al., 2015). The degree of methyl esterification of homogalacturonan was determined by ^{1}H NMR according Grasdalen, Bakoy and Larsen (GRASDALEN; EINAR BAKOY; LARSEN, 1988), giving a value of 86%, characterizing it as a high methoxyl (HM) pectin.

Finally, the NMR results, monosaccharide composition and methylation analyses showed that ACSW-01E had an arabinan-rich pectic polysaccharide, formed mainly by homogalacturonan and arabinan anchored in type I rhamnogalacturonan regions.

3.2 EFFECTS OF ACWS-01E ON NIH 3T3 CELL LINE VIABILITY

In order to evaluate the cytotoxic effects of ACWS-01E on NIH 3T3 cells, the MTT method was performed on the presence of increasing concentration (10, 100

and 1000 µg/mL) of the polysaccharide during 24, 48 and 72 hours of treatment. As Fig. 4 shows, at 10 and 100 µg/mL ACWS-01E presented no significant effects on cell viability when compared to negative control group. On the other hand, ACWS-01E at high concentration (1000 µg/mL) caused a significant time-dependent decrease in the number of viable cells. Compared to control (100%), cell viability fell to 81.7%, 61.1% and 52.6% on 24, 48 and 72 hours of treatment, respectively. Noting that ACWS-01E presented no effects on NIH 3T3 cells viability using both 10 and 100 µg/mL concentrations, the following experiments were carried only with the lowest (10 µg/mL) concentration during 48 h of treatment.

3.3 CYTOPROTECTIVE ACTIVITY OF ACWS-01E AGAINST H_2O_2-INDUCED TOXICITY

A concentration-response curve of H_2O_2 on different times of exposure was firstly established (Fig. 5A). The concentration of 100 µM H_2O_2 and treatment time of 15 minutes was chosen to promote an oxidative stress on NIH 3T3 cells, since it was the lowest concentration in the shortest time to promote a statistically significant decrease in cell viability (positive control).

Then, the protective effect of ACWS-01E at 10 µg/mL against the H_2O_2 induced toxicity was tested. Cells were treated with the polysaccharide for 48 hours prior to H_2O_2 addition. Results are presented on Fig. 5B. In the group exposed only to H_2O_2, cell viability declined to 57.8% when compared to negative control cell condition. In contrast, the pretreatment of cells with ACWS-01E for 48 hours protected the cells against the toxic effects of H_2O_2, resulting in cell viability of 73%. In order to find out if ACWS-01E exhibit antioxidant capacity, intracellular ROS level was evaluated.

3.4 ANTIOXIDANT CAPACITY OF ACWS-01E AFTER TREATMENT *IN VITRO*

Intracellular ROS levels on NIH 3T3 cells was measured using the intensity of DCFH-DA fluorescence when treated only with ACWS-01E (10 µg/mL) for 48 hours. However, no statistical changes on its levels were detected, comparing to vehicle control (Supplementary Figure 1). On the other hand, when NIH 3T3 cells

were pre-treated with ACWS-01E (10 µg/mL) for 48 hours and then exposed to H_2O_2 (100 µM), it was possible to observe a decreased on the percentage of ROS levels, when compared to cells exposed only to H_2O_2 (Fig. 6).

4 DISCUSSION

The pectic polysaccharide extracted from acerola is formed by high methoxyl homogalacturonan, consisting of linear α-(1→4) linked galacturonic acid, with rhamnogalacturonan type I insertions carrying mainly arabinans, with little amounts of arabinogalactans. The main chain of arabinan present in acerola consisted of (1→5)-linked α-Ara*f*, branched only at *O*-3. Saulnier and Brillouet (SAULNIER; BRILLOUET, 1988) reported on grape berries pulp an arabinan with the same branching profile to that found herein for acerola. In other fruits, arabinans were found branched at both *O*-2 and *O*-3, such as in apple (ASPINALL; FANOUS, 1984), buriti (CANTU-JUNGLES et al., 2015), prickly pear (HABIBI; MAHROUZ; VIGNON, 2005) and olive pomace (CARDOSO et al., 2002). Most of reports about arabinans cited their presence in seeds, as for example *Cassia fastuosa* (PETKOWICZ et al., 1998), *Gleditsia triacanthos* (NAVARRO et al., 2002), *Prunus dulcis* (DOURADO et al., 2004), Nata karanja (MANDAL et al., 2011), flexseed kernel (DING et al., 2015) and quinoa (CORDEIRO et al., 2012) and in roots of marshmallow (PETER CAPEK, RUDOLF TOMAN, ALŻBETA KARDOŚOVA, 1983), parsnip (SIDDIQUI; EMERY, 1990) and *Echinacea pallida* (THUDE et al., 2005).

Polysaccharides from a variety of sources, such as plants, fungi, bacteria, algae and animals have their antioxidant potential described in the literature (WANG et al., 2016), either for their ability to sequester free radicals or to prevent oxidative damage in living organisms. To monitor ROS levels in *in vitro* systems, their detection by fluorescent probes has attracted the interest of several researchers because it is a more reproductive model in biological systems than the current methods that observe the scavenger ability of the compounds, thus becoming the method of choice an excellent sensor for detection of non-specific ROS in preliminary analysis of biological activity in cell lines (YAZDANI, 2015).

In this study, we observed the potential of the pretreatment with pectin extracted from acerola in reducing the cytotoxicity induced by H_2O_2 (100 µM) both in

cell viability and in the amounts of intracellular ROS detected by the intensity of fluorescence generated by DCFH-DA. In this methodology, we could observe that the polysaccharide somehow contributed to a better antioxidant intracellular defense of the cells when exposed to oxidizing agents, in a distinct way from just sequester ROS, as observed in tests more like DPPH.

Studies with polysaccharides from different sources can be found in the literature with potential antioxidant activity obtained by a similar protocol employed in the present study. A polysaccharide obtained by aqueous extraction of goji berry fruit (*Lycium barbarum*) significantly reduced H_2O_2-induced (200 μM) apoptosis and the levels of ROS in human lens epithelial cell lines (SRA01/04) after pretreatment for 24 hours (QI et al., 2014). Jia, et al. (JIA et al., 2015) purified a polysaccharide from “Jiao-Gu-Lan” a medicinal oriental plant (*Gynostemma pentaphyllum*) mainly composed of glucose and mannose and showed that its pretreatment for 24 hours significantly protected PC12 cells from cytotoxic effect induced by ROS (JIA et al., 2015). Moreover, another study pointed out that polysaccharides extracted from traditional Chinese medicine, *Rhizoma dioscoreae* greatly attenuated the intracellular ROS levels induced by H_2O_2 in Human umbilical vein endothelial cells (HUVECs) (JIN et al., 2015). Chowdhury et al. (ROY CHOWDHURY et al., 2014) determined the potential antioxidant activity of bacterial polysaccharides against H_2O_2 (300 μM)-induced stress in human lung fibroblast cells (WI38) and obtained a significant reduction of ROS levels in the group treated for one hour with the fucose-rich polysaccharide.

5. CONCLUSIONS

In summary, an arabinan-rich pectic polysaccharide was extracted from acerola and showed potential intracellular antioxidant activity. This is a pioneering study involving this type of molecule and biological model. The results showed that the pectin was able to protect the cell against cytotoxicity generated by H_2O_2 through the reduction of intracellular ROS levels. Confirmatory studies are needed to elucidate signaling pathways and by what mechanisms of action this molecule exerts its biological activity.

Acknowledgements

This research was supported by Projeto Universal (Process 477971/2012-1) provided by CNPq foundation (Brazil) and by PRONEX-Carboidratos. The authors are grateful to NMR Center of UFPR and to Professor Dr. Guilherme L. Sassaki and Dr. Arquimedes Santana-Filho for recording the NMR spectra.

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FIGURAS

FIGURA 1 - HPSEC ELUTION PROFILES OF ACWS, ACWS-01E AND ACWS-01R. REFRACTIVE INDEX DETECTOR (RI)

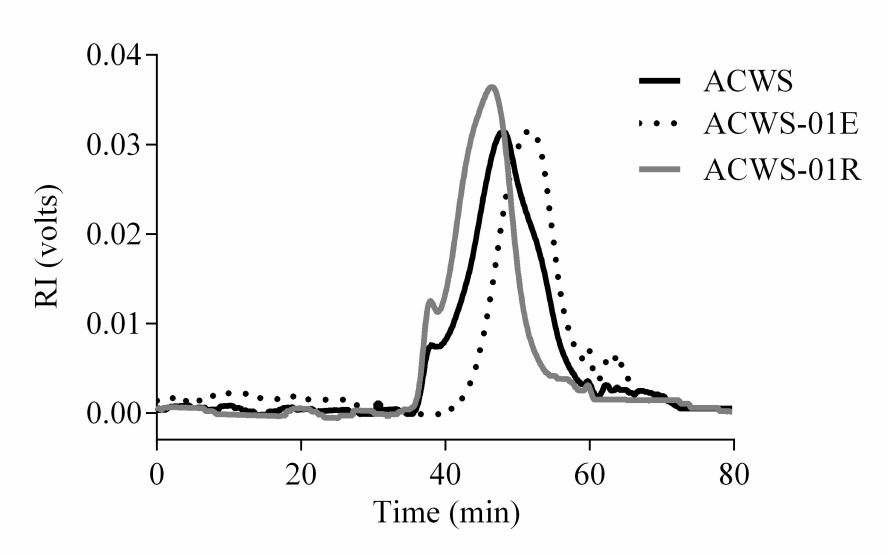


FIGURA 2 - ^{13}C NMR SPECTRA OF ACWS-01R (**A**) AND ACWS-01E (**B**) FRACTIONS IN D_2O AT 70 ºC

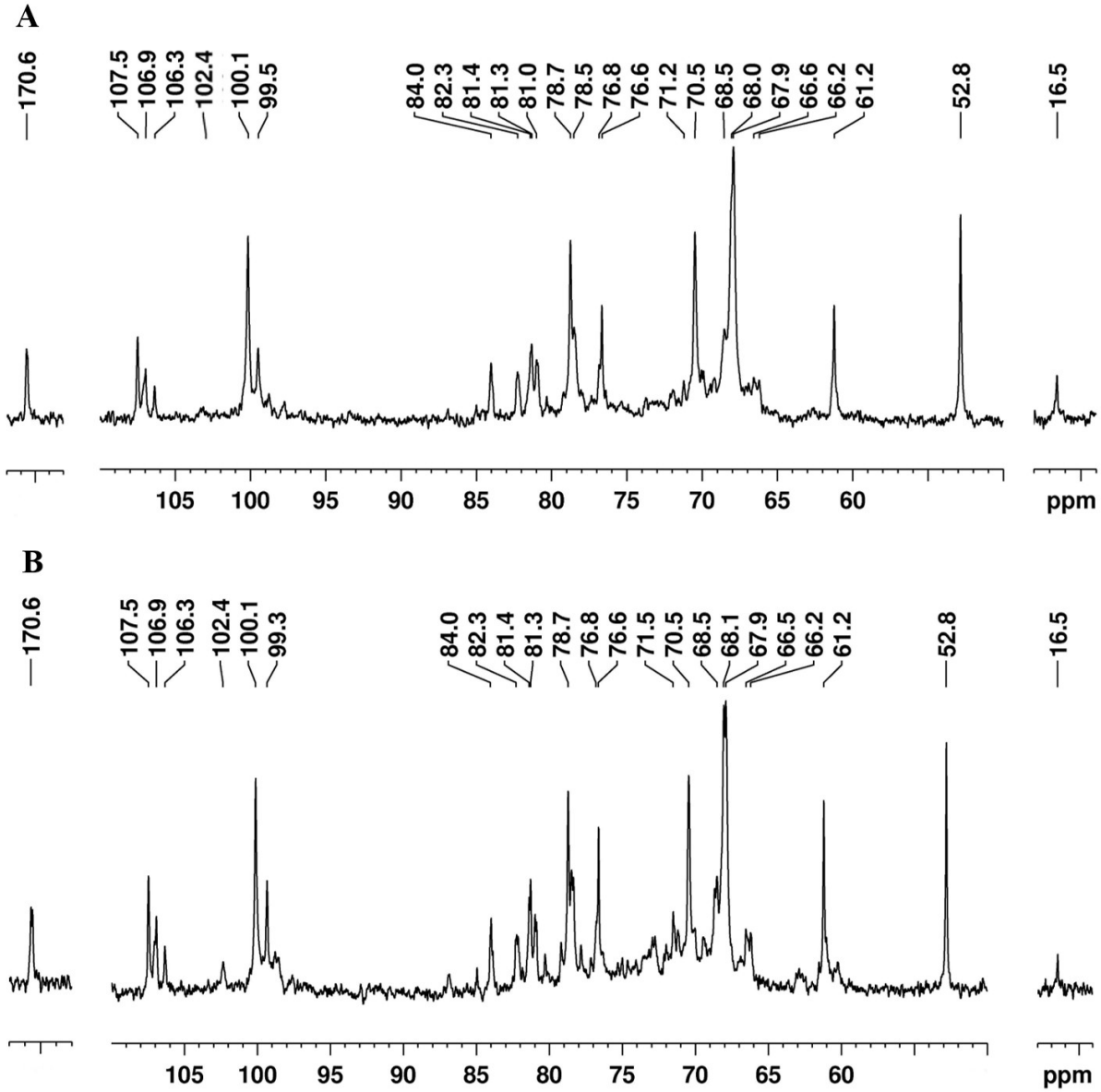
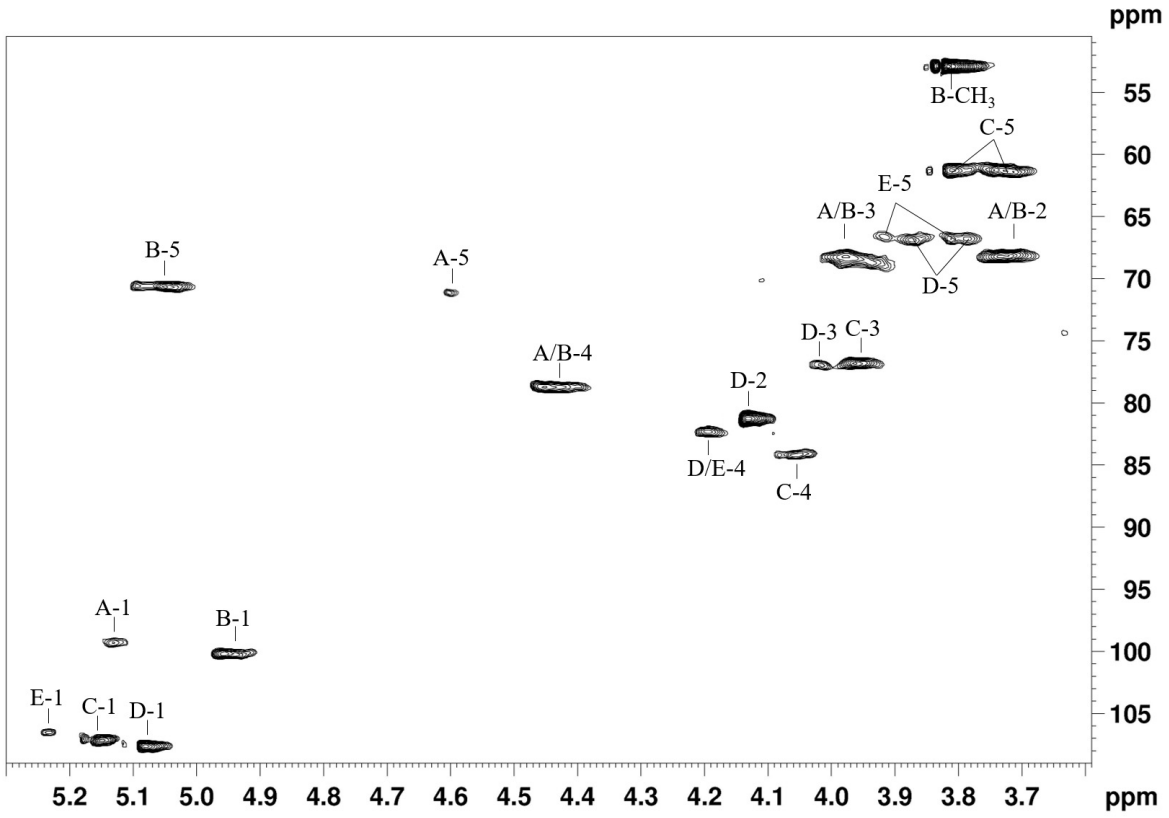
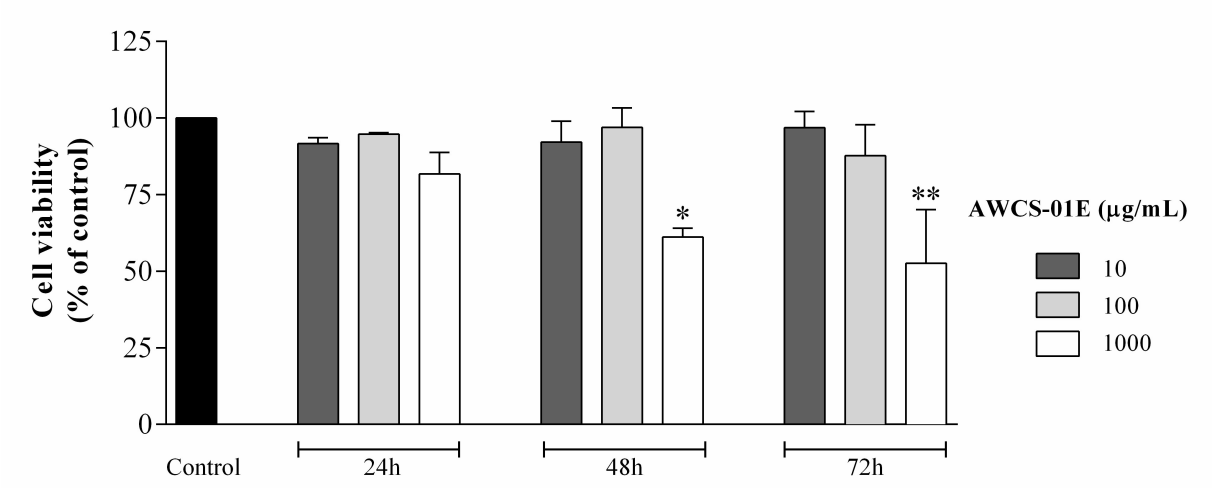


FIGURA 3 - $^{1}H/^{13}C$ HSQC CORRELATION MAP OF ACWS-01E. SOLVENT D_2O AT 70ºC.



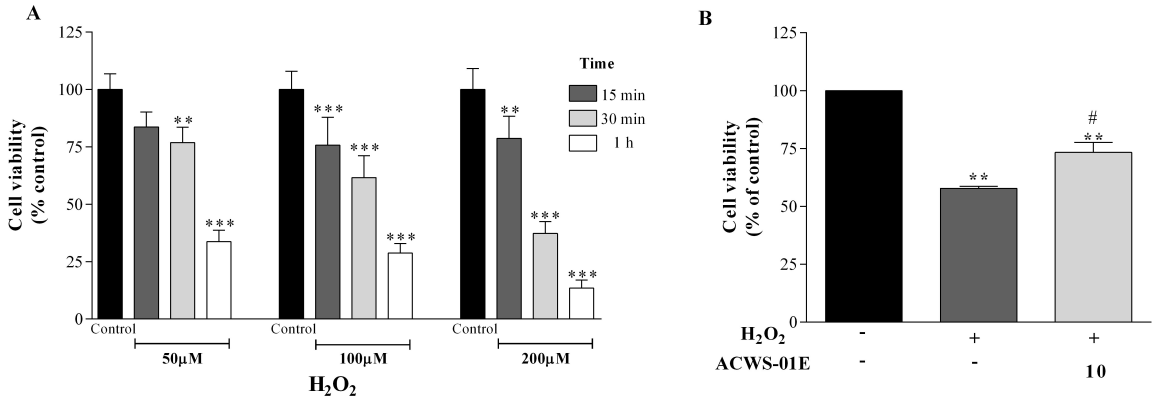
Numerical values are in δ ppm. The letter represents the sugar unit according Table 3, followed by its corresponding carbon and hydrogen number.

FIGURA 4 - EFFECT OF ACWS-01E ON NIH 3T3 CELL VIABILITY



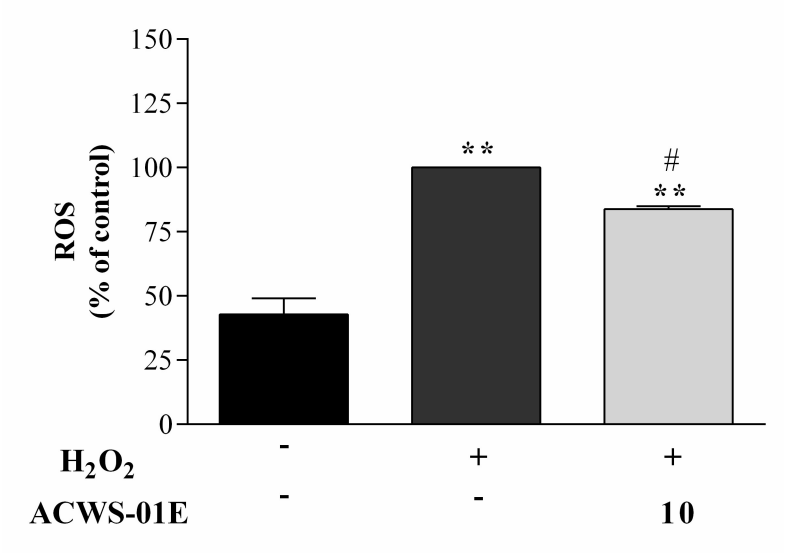
Cells were treated with ACWS-01E (10-1000 µg/mL) or vehicle control (milliQ water) for 24, 48 and 72 hours. The proportion of viable cells was measured by MTT assay. Data are presented as mean±SD from three independent experiments, each in sextuplicate. One-way analysis of variance (ANOVA) followed by Tukey's was performed and * p <0.05, **p<0.01 considered significantly different from negative control.

FIGURA 5- CYTOPROTECTIVE EFFECT OF ACWS-01E ON NIH 3T3 CELL VIABILITY AFTER H_2O_2-INDUCED TOXICITY



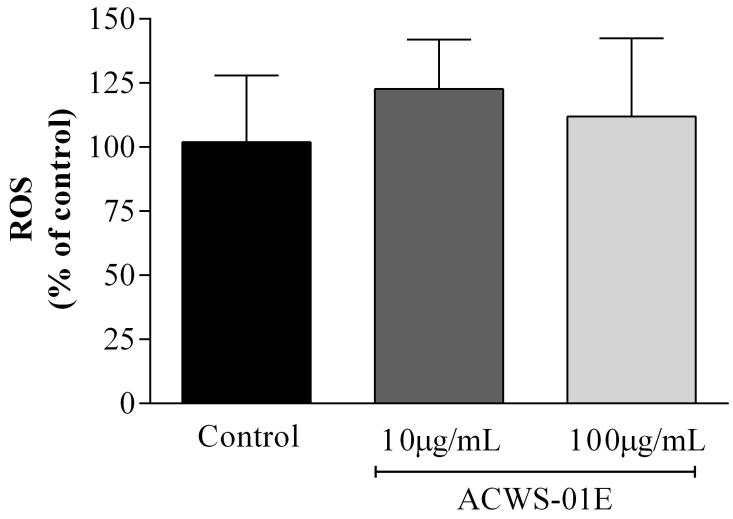
(**A**) Concentration-response curve of H_2O_2 on different times of exposure (**B**) Cells were treated with either ACWS-01E (10µg/mL) or vehicle control (milliQ water) for 48 hours prior to H_2O_2 (100µM) addition for 15 minutes. The proportion of viable cells was measured by MTT assay. Data are presented as mean±SD from three independent experiments, each in sextuplicate. One-way analysis of variance (ANOVA) followed by Tukey's was performed and * p <0.05, **p<0.01 considered significantly different from vehicle control and #p<0.05 considered significantly different from H_2O_2-treated condition.

FIGURA 6 - ANTIOXIDANT EFFECTS OF ACWS-01E ON NIH 3T3 CELL LINE



Cells were pre-treated with ACWS-01E (10µg/mL) or vehicle control (milliQ water) for 48 hours prior to H_2O_2 (100µM) addition for 15 minutes. The levels of ROS were measured by addition of DCFH-DA probe. Data are presented as mean±SD from three independent experiments, each in sextuplicate. One-way analysis of variance (ANOVA) followed by Tukey's was performed and * p <0.05, **p<0.01 considered significantly different from vehicle control and #p<0.05 considered significantly different from H_2O_2-treated condition.

FIGURA S1 -INTRACELLULAR ROS LEVELS ON NIH 3T3 CELLS TREATED ONLY WITH ACWS-01E FOR 48 HOURS



The levels of ROS were measured by addition of DCFH-DA probe. Data are presented as mean±SD from three independent experiments, each in sextuplicate. One-way analysis of variance (ANOVA) followed by Tukey's was performed and treated groups were compared with vehicle control.

TABELA 1 - MONOSACCHARIDE COMPOSITION OF FRACTIONS OBTAINED FROM ACEROLA (*M. emarginata*)

	Monosaccharide composition (%)[a]				
Fractions	Uronic acid[b]	Ara	Gal	Xyl	Rha
ACWS	59.6	35.0	2.2	1.9	1.3
ACWS-01R	59.0	33.4	3.6	2.3	1.8
ACWS-01E	50.0	39.0	6.5	2.6	1.9
ACWS-01E-CR	3.7	25.3	56.8	2.2	3.8

[a] % of peak area relative to total peak areas, determined by GC-MS.

[b] Determined spectrophotometrically using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991)

TABELA 2 - LINKAGE TYPES BASED ON ANALYSIS OF PARTIALLY *O*-METHYL ALDITOL ACETATES OBTAINED FROM METHYLATED FRACTION ACWS-01E-CR FROM ACEROLA (*M. emarginata*)

Partially *O*-methylalditol Acetate	**Linkage type[b]**	**ACWS-01E-CR[a]**
2,3,4,6-Me_3-Gal	Gal*p*-(1→	8.1
2,3,6-Me_3-Gal	→4)-Gal*p*-(1→	34.7
2,3-Me_2-Gal	→4,6)-Gal*p*-(1→	8.0
2,6-Me_2-Gal	→3,4)-Gal*p*-(1→	2.2
2-Me-Gal	→3,4,6)-Gal*p*-(1→	1.3
2,3,5-Me_3-Ara	Ara*f*-(1→	9.1
2,3,4-Me_3-Ara	Ara*p*-(1→	2.6
2,3-Me_2-Ara	→5)-Ara*f*-(1→	21.3
2-Me-Ara	→3,5)-Ara*f* -(1→	3.5
2,5-Me_2-Ara	→3)-Ara*f*-(1→	1.5
3,4-Me_2-Rha	→2)-Rha*p*-(1→	2.6
3-Me-Rha	2,4)-Rha*p*-(1→	1.1
2-Me-Xyl	→3,4)-Xyl*p*-(1→	4.1

[a] The percentage of peak area of *O*-methylalditol acetates relative to total area, determined by GC–MS.
[b] Based on derived *O*-methylalditol acetates.

TABELA 3 - NMR CHEMICAL SHIFTS OF FRACTION ACWS-01E OBTAINED FROM ACEROLA (*M. emarginata*)

Unit	Nucleus	1	2	3	4	5	6	CH_3
→4)-α-Gal*p*A-(1→	**^{13}C**	99.3	68.0	68.3	78.7	71.7	nd[b]	-
A	**^{1}H**	5.15	3.74	3.99	4.46	4.66	-	-
→4)-α-6Me-Gal*p*A-(1→	**^{13}C**	100.1	68.0	68.3	78.7	70.6	170.6	52.8
B	**^{1}H**	4.95	3.74	3.99	4.46	5.06	-	3.82
α-Ara*f*-(1→	**^{13}C**	107.1	nd	76.7	84.2	61.3	-	-
C	**^{1}H**	5.14	nd	3.96	4.05	3.73/3.80	-	-
→5)-α-Ara*f*-(1→	**^{13}C**	107.6	81.2	76.9	82.3	66.8	-	-
D	**^{1}H**	5.08	4.13	4.01	4.19	3.78/3.87	-	-
→3,5)-α-Ara*f*-(1→	**^{13}C**	106.5	nd	nd	82.3	66.6	-	-
E	**^{1}H**	5.23	nd	nd	4.19	3.81/3.91	-	-

[a] In ppm relative to the signal of internal acetone in D_2O, at 2.22 ppm (^{1}H) or at 30.2 ppm (^{13}C).
[b] nd = Not determined.

ARTIGO II

Anti-fatigue activity of pectin arabinan-rich from acerola (*Malpighia emarginata*)

Anti-fatigue activity of an arabinan-rich pectin from acerola (*Malpighia emarginata*)

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ABSTRACT

A fraction composed of an arabinan-rich pectin was extracted from acerola fruit (*Malpighia emarginata*) and named ACWS. This fraction presented 93% of total carbohydrate, relative molecular weight of 7.5×10^4 g/mol, galacturonic acid, arabinose, galactose, xylose and rhamnose in 52.1:32.4:7.2:4.8:3.5 molar ratio and had its structure confirmed by NMR analysis. The anti-fatigue activity of ACWS was evaluated using the weight load swim test on trained mice. ACWS was orally administered at doses of 50 mg/kg, 100 mg/kg and 200 mg/kg for 28 days. Plasma biochemical parameters, respiration of permeabilized skeletal muscle fibers, and GSH levels and lipoperoxidation (LPO) in the brain (pre-frontal cortex, hippocampus, striatum and hypothalamus) were determined. ACWS could lengthen the swimming time, increase the plasma levels of glucose, triglycerides, lactate, and the GSH levels in the hippocampus at all tested doses. The mitochondrial respiratory capacity of the skeletal muscle was increased at middle and high ACWS dose. This study provides strong evidence that *M. emarginata* pectic polysaccharide supplementation have anti-fatigue activity, can modify the kinetics of energy substrates (carbohydrate and fat) mobilization and the respiratory capacity of the skeletal muscle, as well the antioxidant status in the hippocampus of ACWS treated animals.

Keywords: Pectin, acerola, weight load swim test, anti-fatigue.

1 INTRODUCTION

Fatigue is defined as a decrease performance and inability to maintain muscle contractions and can be categorized into two strands: peripheral and central fatigue. Peripheral fatigue is associated with a decrease in muscle strength caused by a process distal to the neuromuscular junction and central fatigue is the term for fatigue caused by factors residing within the central nervous system, brain, spinal cord and motor neurons (AMENT; VERKERKE, 2009).

There are several factors that may led to the peripheral fatigue, among the most discussed are the availability of energy for muscle contractions with emphasis on macronutrient kinetics, intra and extracellular pH alterations and thermal regulation (AMENT; VERKERKE, 2009).

One of the reasons for central fatigue is the damage caused by oxidative stress in the brain. Physical exercise causes an increase in the generation of reactive oxygen species (ROS) in the brain, an organ highly sensitive to the damage caused by ROS, mainly due to the number of molecules susceptible to oxidation, such as neurotransmitters, glutamate, iron and membranes rich in polyunsaturated fatty acids. As a defense mechanism, adaptive response to this stress in brain is the increase of mitochondria number and the antioxidant defense (SACHDEV; DAVIES, 2008; VIÑA et al., 2012).

Polysaccharides are the most abundant group of biopolymers in Nature. Several biological activities have already been attributed to these natural compounds (LIU; WILLFÖR; XU, 2015). They can be employed as protective agents against oxidative stress (WANG et al., 2016a), in reducing the effect of aging (BEST et al., 2015; DING et al., 2016), altering macronutrient metabolism (BÄCKHED et al., 2004; WANG et al., 2017; ZHOU et al., 2015), releasing intestinal hormones (CANI; DEWEVER; DELZENNE, 2004; HUANG et al., 2011; PHUWAMONGKOLWIWAT; HIRA; HARA, 2014; REIMER et al., 2010; ZHOU et al., 2008), modulating the immune system (BRUFAU et al., 2017; FERREIRA et al., 2015; POPOV; OVODOV, 2013), modulating the intestinal microbiota (FLINT et al., 2012; SHI et al., 2015), etc.

Among the biological activities studied for polysaccharides, the anti-fatigue effect has attracted interest after positive effects have been obtained by several studies with different polysaccharide sources (CHEN et al., 2015; CHI et al., 2012,

2015; LI et al., 2016; TAN et al., 2012; WEI et al., 2017; ZHANG et al., 2009; ZHAO et al., 2015). In this work we aimed to investigate the anti-fatigue activity in the weight load swim test of an arabinan-rich pectic polysaccharide obtained from acerola (*Malpighia emarginata*), a tropical fruit, found naturally in the Caribbean islands and South America (DELVA; SCHNEIDER, 2013). Plasma biochemical parameters, respiration of permeabilized skeletal muscle fibers, and GSH levels and lipoperoxidation (LPO) in the brain (pre-frontal cortex, hippocampus, striatum and hypothalamus) were also determined.

2 MATERIALS AND METHODS

2.1 EXTRACTION OF POLYSACCHARIDES FROM ACEROLA FRUIT

Ripe fruits of acerola were purchased in a local market in Curitiba, state of Paraná, Brazil. The seeds were manually removed, and the pulp (with the peel) was freeze-dried and milled. The nonpolar compounds were extracted using chloroform-methanol (1:1) as solvent using Soxhlet apparatus.

Polysaccharides were exhaustively extracted from deffated pulp with boiling water under reflux for 2 h (×6, 1 L each). The extracts were pooled, concentrated under reduced pressure and the polysaccharides precipitated with ethanol (3 vol.), collected by centrifugation (8000 rpm, 10 min), dialyzed (12-14 kDa cut-off Spectra-Por® membrane dialysis) and lyophilized. Freeze-thaw process was performed (GORIN; IACOMINI, 1984), giving cold-water soluble (ACWS) and insoluble fractions. Polysaccharides yields were expressed as percentages based on the weight of dried acerola subjected to extraction (100 g).

2.2. HOMOGENEITY, MOLECULAR WEIGHT, NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY, MONOSACCHARIDE COMPOSITION AND TOTAL SUGAR CONTENT.

The homogeneity and relative molecular weight of ACWS was determined according (LEIVAS; IACOMINI; CORDEIRO, 2015).

^{13}C NMR spectrum was acquired in a 400 MHz spectrometer (Bruker AVANCE III), with a 5-mm multinuclear inverse probe, operating at 9.5 T, observing ^{13}C nucleus at 100.61 MHz. Sample was dissolved in D_2O, and analyzed at 70 ºC. Chemical shifts (δ) were expressed in ppm relative to CH_3 signal from acetone at δ 30.2 as internal reference.

Neutral monosaccharides were determined after hydrolysis with 2 M trifluoracetic acid for 8 hours. The acid was evaporated and the sample was dissolved in 0.5 M NH_4OH (200 µL), held at 50 ºC for 5 min in sealed tubes. The NH_4OH was evaporated and the sample was finally dissolved in 500µL D_2O for NMR analysis. Relative monosaccharide quantification was performed by integration of the anomeric cross peaks and normalized to percentage values (SASSAKI et al., 2014). Uronic acid content was determined using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

The total sugar content was determined using the phenol-sulfuric acid colorimetric method (DUBOIS et al., 1956).

2.3 ANTI-FATIGUE ACTIVITY OF ACWS

2.3.1 Animals and Experimental Design

The experiments were conducted using male Swiss mice (25-30 g) that were housed at 22 ± 2 °C under a 12 h light/ dark cycle (lights on at 7:00 a.m.). The animals were kept in poly-propylene cages (41 cm × 34 cm × 16 cm (height)) with food and water *ad libitum*.

Mice were randomly divided into five groups, each consisting of six mice:

Group 1 – Non-Trained Controls (NTC) – Mice were given distilled water for 28 days, did not perform training sessions and did not participate in the exhaustion test;

Group 2 – Trained Control (TC) – Mice were given distilled water, trained for 6 weeks and participated in the exhaustion test;

Group 3 – Low Dose (LD) – Mice were treated with ACWS (50 mg/kg/day) for 28 days, trained for 6 weeks and participated in the exhaustion test;

Group 4 – Middle Dose (MD) – Mice were treated with ACWS (100 mg/kg/day) for 28 days, trained for 6 weeks and participated in the exhaustion test;

Group 5 – High Dose (HD) – Mice were treated with ACWS (200 mg/kg/day) for 28 days, trained for 6 weeks and participated in the exhaustion test;

All of the experiments were approved by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA number 997).

2.3.2. Training Protocol, Treatment and Weight Load Swim Test

Before swimming protocol starts, the animals were set for three days in the room where the training would be performed. The training procedure used in this study followed that of Yeh et al. (YEH et al., 2014), and can be seen in Fig. 1. Mice swam 30 min on the first day, 45 min on the second day and 60 min between day 3-5. This time was maintained from weeks 2-6, 5 days /week. At week 2, was loaded 1% of weight to mice's tail, in weeks 3-4 was loaded 2% and weeks 5-6 was loaded 3%. The swimming exercise was performed in round plastic tanks (40 cm diameter × 65 cm height) filled with water up to 40 cm and kept at 28 ± 2 ºC. To avoid circadian variations in the physical activity, all sessions were performed between 10:00 a.m. and 12:00 a.m.

The animals were weighed once a week to adjust the weights that would be coupled to the tail in order to progressively increase the exercise intensity and force the mouse to keep the legs moving continuously (WEI et al., 2017). The treatments with ACWS were performed for 28 days (day 15-43) every day at 09:00 a.m. and fed by gavage.

The weight load swim test (WLST) was based in Chen et al (CHEN et al., 2015) study, which consisted in the addition of weights in the tails of the mice corresponding to 5% of body weight. The time for exhaustion was determined when there was loss of movement coordination and failure to return to the surface in 7 seconds. The exhaustion exercise performed on the last day occurred in the same period of the day (10:00 a.m. and 12:00 a.m.) of the trainings. (CHEN et al., 2015; YEH et al., 2014; ZHANG et al., 2009). At the end of session, mice were removed from the water, dried and anesthetized after 30 minutes to perform a cardiac puncture for blood collection.

2.4 BIOCHEMICAL ANALYSES

Blood samples were collected in heparinized tubes by cardiac puncture during the anesthesia effect. Plasma was prepared by blood centrifugation at 4000 rpm at 4 ºC for 10 minutes. The levels of lactate (LAC), triglycerides (TGC), glucose (GLU), urea (URE), alanine amino transferase (ALT), aspartate amino transferase (AST), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ GT) and creatine kinase (CK) were analyzed by commercial kits (Bioclin, Quibasa Química Básica Ltda, Belo Horizonte, Brazil).

2.5 RESPIRATION OF PERMEABILIZED MUSCLE fiBERS

The gastrocnemius muscle was removed immediately after the cardiac puncture and placed in Petri dish containing a preservation solution (MES-K^+ 50 mM, Taurine 20 mM, Dithiothreitol 0.5 mM, $MgCl_2$ 6.56 mM, ATP 5.77 mM, Phosphocreatine 15 mM, Imidazole 20 mM, pH adjusted with KOH at 0 ºC) on the ice (FONTANA-AYOUB; FASCHING; GNAIGER, 2014). The connective tissue was removed and the myofibers (approximately 10 mg) were placed in saponin solution (0.05 mg/mL) for 30 minutes over constant stirring. Saponin is a detergent agent that has high cholesterol affinity. As plasma membranes contain approximately 7 times more cholesterol than mitochondrial membranes, small concentrations of saponin are sufficient to selectively permeate only the plasma membranes (PERRY et al., 2013). Then, the samples were dried on filter paper and transferred to the cameras of high-resolution Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) containing respiration medium, at 30 ºC and under gentle agitation. The medium consisted of EGTA 0.5 mM, $MgCl_2$ 3 mM, Lactobionic Acid 60 mM, Taurine 20 mM, KH_2PO_4 10 mM, HEPES 20 mM, D-Sucrose 110 mM, BSA (1g/L) (FASCHING; RENNER-SATTLER; GNAIGER, 2016). The following additions were made: Malate 1.5 mM plus glutamate 19 mM as oxidizable substrates for Complex I, ADP 4.8 mM, Rotenone 0.7 µM and Antimycin A 12 µM as inhibitors of complex I and III, respectively. The results are expressed as the oxygen flow per mg of tissue [Flux of O_2 (pmols/seg/mg tissue)].

2.6 EVALUATION OF OXIDATIVE STRESS PARAMETERS IN THE MOUSE BRAIN

2.6.1 Brain samples

The pre-frontal cortex, hippocampus, striatum and hypothalamus were dissected, frozen in liquid nitrogen, and stored at -80 °C until further analysis. The brain samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) in a 1:10 dilution. One part of the homogenate was used to determine reduced glutathione (GSH) levels, and the other was centrifuged at 8000 rpm in a micro-high-speed refrigerated centrifuge (VS-15000 CFNII, Vision Scientific, Daejeon, South Korea) for 20 min. The supernatant was used to evaluate lipid peroxidation (LPO) (KANAZAWA et al., 2016).

2.6.2 Evaluation of GSH levels

To evaluate GSH levels, 100 µL of the homogenate obtained above was mixed with 80 µL of 12.5% trichloroacetic acid and centrifuged at 6000 rpm for15 min at 4 °C. Afterward, 20 µL of the supernatant was mixed with 280 µL of Tris buffer (0.4 M, pH 8.9) and 5 µL of DTNB (5,5′-dithiobis-[2-nitrobenzoic acid] according to the Sedlak and Lindsay protocol (SEDLAK; LINDSAY, 1968), with minor modifications. Absorbance was read at 415 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The individual values were interpolated in a standard curve of GSH (0.375–3 µg) to verify the linearity of the reaction (r^2 must be >0.99). The results are expressed as µg/g of tissue (KANAZAWA et al., 2016).

2.6.3 Evaluation of LPO

The method described by Jiang et al (JIANG; HUNT; WOLFF, 1992) was performed with minor modifications. Firstly, 100 µL of the brain sample supernatant were suspended in 100 µL of methanol, vortexed, and centrifuged at 5000 rpm for 5 min at 4 °C. Afterward, 100 µL of the supernatant was added to 900 µL of FOX2 reagent (4mM BHT, 250 *µ*M $FeSO_4$, 250 mM H_2SO_4, and 100 mM xylenol orange).

Samples were then vortexed and incubated for 30 min at room temperature in the dark. Absorbance was read at 560 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA). The results are expressed as μmol of hydroperoxides/ mg of tissue (KANAZAWA et al., 2016).

2.7 STATISTICAL ANALYSIS

For all of the experiments, one-way analysis of variance (ANOVA) was used, with the exception of respiration of permeabilized muscle fibers, being used two-way analyses of variance, followed by the Newman-Keuls *post-hoc* test if significant main effects or interactions were found in the ANOVA. The data are expressed as mean ±SEM. Values of <0.05 were considered statistically significant.

3 RESULTS

3.1 EXTRACTION AND CHEMICAL CHARACTERIZATION OF FRACTION ACWS FROM ACEROLA (*M. emarginata*)

Aqueous extraction of deffated acerola followed by freeze-thawing treatment produced a polysaccharide fraction (ACWS) in 7.2% yield. Its total sugar content was 93% and on gel permeation chromatography eluted as single peak (Fig. 2), with relative molecular weight of 7.5×10^4 g/mol. Monosaccharides composition revealed the presence of uronic acids (52.1%), arabinose (32.4%), galactose (7.2%), xylose (4.8%) and rhamnose (3.5%). The ^{13}C NMR spectrum (Fig. 3) showed anomeric carbon signals of α-L-Ara*f* units at δ 107.6, 107.0 and 106.4. Signals at δ 100.0/170.5 and 99.2/174.7 were assigned to C-1/C-6 of esterified and unesterified α-D-Gal*p*A units, respectively, while methyl carbons of esterified carbonyls in Gal*p*A appeared at δ 52.8. The presence of C-6 of Rha*p* units could be seen at δ 16.6 (CANTU-JUNGLES et al., 2015; CORDEIRO et al., 2012). A detailed description can be seen in Klosterhoff et al. (KLOSTERHOFF et al., 2017). Thus, ACWS obtained herein is similar to that previously extracted by our lab (KLOSTERHOFF et al., 2017) and presented an arabinan-rich pectin, with high methyl esterified homogalacturonan and arabinan anchored in type I rhamnogalacturonan regions.

3.2 EFFECT ON ACWS ON WEIGHT LOAD SWIM TEST (WLST)

The time that mice resist to fatigue after the swimming test is commonly used as an indicator of anti-fatigue activity of several molecules (BOGDANOVA et al., 2013; TANAKA et al., 2003). We observed that all ACWS supplemented groups had statistically higher swimming times compared with trained control group (Fig. 4), which showed a time of exhaustion of 53 ± 11.02 min. The LD group had mean time until the fatigue of 95 ± 08.56 min, while MD and HD of 151 ± 20.25 min, and 129 ± 9.60 min, respectively.

3.3 PLASMA BIOCHEMICAL PARAMETERS

Several biochemical parameters were analyzed in order to elucidate metabolic alterations involved in anti-fatigue activity of ACWS. After 30 minutes of exhaustion, the levels of plasma glucose were statistically higher in the treated groups (Fig. 5A). LD presented 153.2 ±11.53 mg/dL, MD 183.6 ±9.99 mg/dL and HD 154.2 ±11.93 mg/dL when compared with TC who presented 115.4 ±4.00 and NTC 94.2 ±8.78 mg/dL.

The plasma levels of triglycerides (Fig 5B) in treated groups (LD 177.3 ±10.72 mg/dL, MD 201.1 ±10.5 mg/dL and HD 175.1 ±6.85 mg/dL) are statistically different and superior to control groups (NTC 116.4 ±5.17 mg/dL and TC 114.4 ±7.66 mg/dL).

Plasma lactate levels (Fig. 5C) were investigated as a marker of anaerobic metabolism, and the obtained values for treated groups (LD 57.3 ± 7.34 mg/dL, MD 61.3 ± 9.80 mg/dL and HD 56.2 ± 6.40 mg/dL) are higher and statistically different to the control groups (NTC 26.3 mg/dL ± 3.55 and TC 23.8 mg/dL ± 2.94).

Physical exercises cause breakdown of muscle cells and one way to infer the damage is to quantify the levels of a muscle tissue specific enzyme (CK) in the plasma. We observed that all treated and TC groups had a statistically significant increase in CK levels when compared to NTC (Fig 5D). Thus, it means that the training had the necessary intensity to generate muscle injuries. No statistical differences were observed in the non-specific tissue injury marker (LDH), hepatic injury markers (AST, ALT and γ-GT) and urea levels in plasma (Tab. S1).

3.4 RESPIRATION OF PERMEABILIZED MUSCLE FIBERS

In long-term exercises with low intensity the oxidative pathway is the main source of energy to support the activity. For this reason, we evaluated the oxygen consumption by myofibers and differences between the groups (Fig. 6) were observed. In the basal condition the oxygen uptake was statistically the same among all groups. However, after malate and glutamate addition, two groups had statistically lower intakes (NTC 9.92 pmols/seg/mg and LD 5.81 pmols/seg/mg) when compared to the TC group (7.49 pmols/seg/mg). With ADP addition, the oxygen uptake values were close to the maximum. In this state, the HD (13.37 pmols/seg/mg, $p< 0.0001$), MD (11.98 pmols/seg/mg, $p <0.05$) and NTC (11.58 pmols/seg/mg, $p<0.05$) groups were statistically superior to the trained control. In the inhibitions condition of complex 1 (with Rotenone) and complex 3 (with Antimycin A) no differences between the groups were observed.

3.5 EVALUATION OF OXIDATIVE STRESS PARAMETERS IN THE MOUSE BRAIN

The parameters related to oxidative stress in the brain show that the treatment with ACWS was able to increase the tissue levels of GSH in the hippocampus (Fig. 7), the main intracellular antioxidant defense. However, no statistical difference between the groups were observed in LPO, a marker of membrane injury caused by free radicals. The other studied brain areas, pre-frontal cortex, striatum and hypothalamus did not show statistical differences for both GSH and LPO (Fig. S1).

4 DISCUSSION

The ability of polysaccharides to prolong the time to exhaustion gained prominence after positive results have recently been published (CHEN et al., 2015; CHI et al., 2012; LI et al., 2016; TAN et al., 2012; WEI et al., 2017; ZHANG et al., 2009; ZHAO et al., 2015). Herein we present the first report of a polysaccharide extracted from an edible fruit that was able to increase the time for exhaustion in WLST. The arabinan-rich pectin extracted from acerola fruit after oral

supplementation for 28 days prolonged the swimming time (Fig. 4) at all tested doses. Regarding structure-function relationship, only a glucomannan and a sulfated fucoidan have been tested in WLST. The glucomannan was extracted from the stem of *Dendrobium officinale*, a Chinese medicinal herb (WEI et al., 2017), and the sulfated fucoidan from the algae *Laminaria japanica* (CHEN et al., 2015). Both polysaccharides increased the time of exhaustion the mice in the WLST and showed anti-fatigue activity. However, several polysaccharide fractions demonstrated positive anti-fatigue results in WLST. Li et al, (2016) reported anti-fatigue activity for two polysaccharide fractions extracted from *Lepidium meyenii*, a tubercle from Andes region, where glucose was the major monosaccharide for both fractions. Chi et al. (CHI et al., 2015) presented a polysaccharide extracted from Ziyang green tea with glucose, arabinose and galactose as main monosaccharides. Three polysaccharide fractions were extracted from the herb *Gynostemma pentaphyllum* and tested by Chi et al. (CHI et al., 2012), and only the polysaccharide that had glucose, galactose and arabinose (0.18:0.72:1.00 molar ratio) in its composition was able to prolong the time for exhaustion after WLST. Aqueous extracts containing polysaccharides from rhizome of *Millettiae speciosae*, and roots of *Rehmanniae glutinosa* and *Morinda officinalis* were tested by Zhao et al. (ZHAO et al., 2015), Tan et al. (TAN et al., 2012) and Zhang et al. (ZHANG et al., 2009), respectively, and exhibited anti-fatigue activity.

In addition to the time of swimming to exhaustion, we analyzed some parameters related to peripheral fatigue. The increase in plasma glucose levels in treated groups (Fig. 4A) was also increased after WLST in the studies of Chen et al. (CHEN et al., 2015) and Zhao et al. (ZHAO et al., 2015) in which polysaccharides where also orally administered for a long period of time. In intense exercise, glucose production rise seven- to eight fold while glucose utilization is only increased three- to four fold. Thus, blood glucose levels increase during intense exercise and also immediately further at exhaustion and persists for up to 1 hour (PETER ADAMS, 2013).

Another macronutrient, besides glucose, which provides energy for exercise are fats. We observed increased triglyceride levels (Fig. 4B) in treated groups when compared to the NTC ant TC group. Increased triglyceride levels at rest indicate that

more lipolysis occurred during exercise, according to Phillips et al. (PHILLIPS et al., 1996).

It is widely known in the literature that anaerobic metabolism is used in high intensity exercise. It is expected that animals that have prolonged swim times and at high intensities, have high plasma lactate levels, which can lead to a decreased muscle pH and to fatigue (WESTERBLAD; BRUTON; KATZ, 2010). Surprisingly, we found elevated lactate levels in treat groups (Fig. 5C) and was contrary to those observed in the literature by Zhang et al. (ZHANG et al., 2009), Tan et al. (TAN et al., 2012) and Zhao et al. (ZHAO et al., 2015) who reported lower lactate levels in plasma of treated animals and argued that such levels contributed to prolong the swimming time.

In order to close the metabolic scenario, we applied a methodology that evaluates the respiratory capacity of the skeletal muscle. The most common mitochondria function is the ATP synthesis via oxidative phosphorylation. This process can indirectly be determined by oxygen consumption measuring. Consequently, variation in the response after availability of certain substrates is often interpreted as reflecting changes in oxidative phosphorylation and in the regulation of cellular energy homeostasis (PERRY et al., 2013). We observed in Figure 6 that the HD group had the higher oxygen flow after ADP addition when compared to both TC and NTC controls even the muscle having gone to exhaustion. In the MD, we observed that the supplementation could maintain the oxygen flow at the same levels of the group that did not perform the WLST, and was statistically superior to the TC group. While the TC and LD groups presented reduction of oxygen flow when compared to the NTC group. These results show that the treatment, especially at the high dose, is able to alter the mitochondrial oxidative capacity positively, something of extreme importance to prolong the time of exercise and to delay the fatigue.

In this study, besides checking the parameters related to peripheral fatigue, we directed to the brain in search of potential mechanisms that may be involved in the anti-fatigue effect of the acerola polysaccharide. Interestingly, we observed a significant increase in GSH concentrations in the hippocampus of all treated groups when compared to the TC and NTC. Stone et al. (STONE et al., 2015) conducted a study where mice swam 5 times a week during 30 minutes for 28 days, and showed that only the exercise, without any supplementation increased the antioxidant status

in the animals' hippocampus (STONE et al., 2015). GSH is the main antioxidant defense, in this way a new hypothesis of the mechanism of action of the polysaccharide in the anti-fatigue activity is opened. This being the first report in the literature of an action related to central fatigue attributed to administration of polysaccharide in weight load swim test protocol.

5 CONCLUSION

The arabinan-rich pectin extracted from acerola showed anti-fatigue activity in weight load swim test, increasing swimming time at all doses (50 mg/kg, 100 mg/kg and 200 mg/kg). It also modified the energy substrate (carbohydrate and fat) kinetics for exercise, and increased the respiratory capacity of the skeletal muscle. We report for the first time a polysaccharide action on central fatigue, where we observed an increase in GSH levels in mice hippocampus. Mechanistic studies are needed to elucidate how the polysaccharide acts systematically in the body.

Acknowledgements

This research was supported by Projeto Universal (Process 477971/2012-1) provided by CNPq foundation (Brazil) and by PRONEX-Carboidratos. The authors are grateful to NMR Center of UFPR for recording the NMR spectra.

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FIGURAS

FIGURA 1 - TIMELINE OF TRAINING PROTOCOL, TREATMENT AND WEIGHT LOAD SWIM TEST

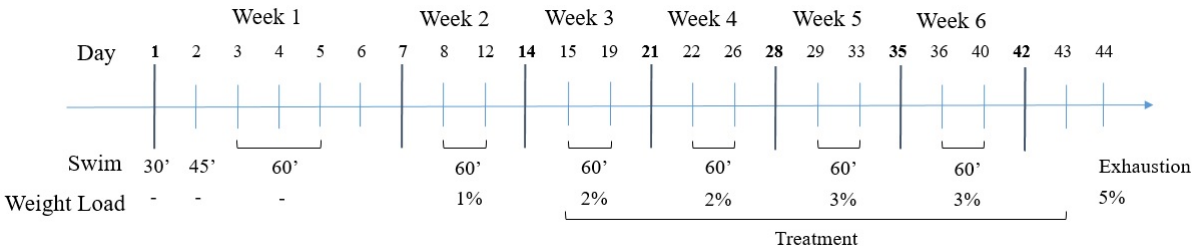


FIGURA 2 - ELUTION PROFILE OF ACWS ON HPSEC. REFRACTIVE INDEX DETECTOR

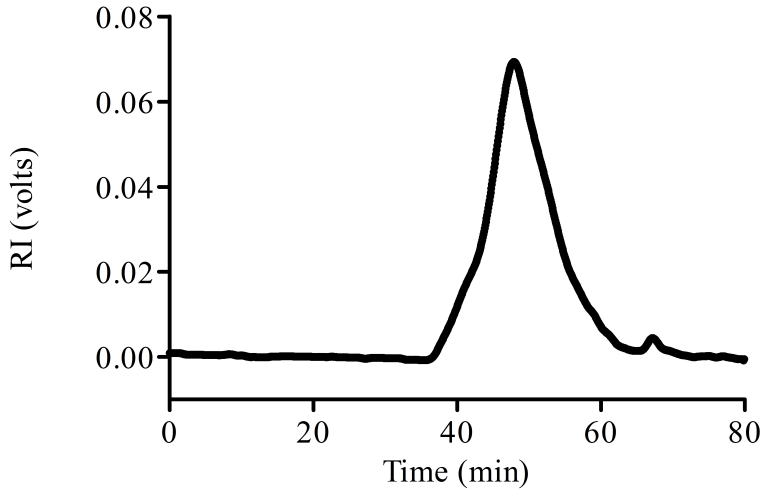


FIGURA 3 - ^{13}C NMR SPECTRUM OF FRACTION ACWS IN D_2O AT 70 ºC

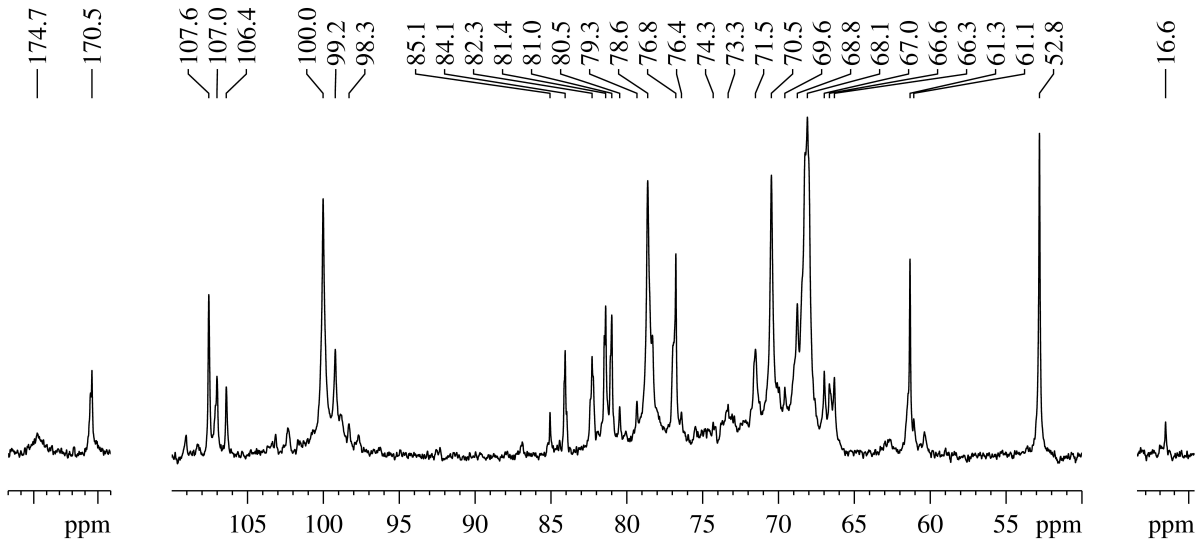
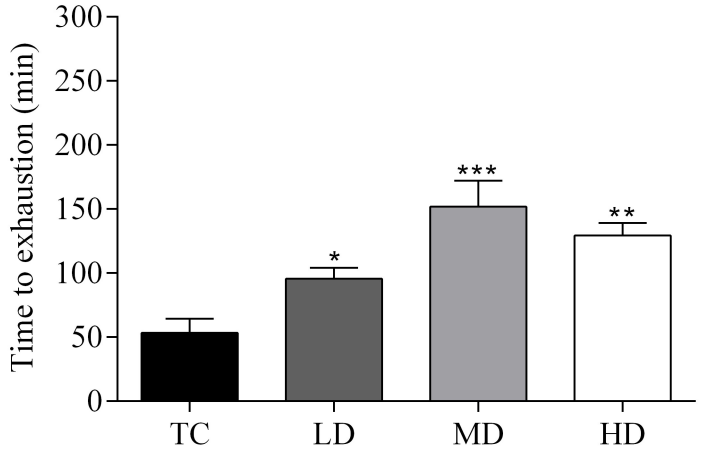
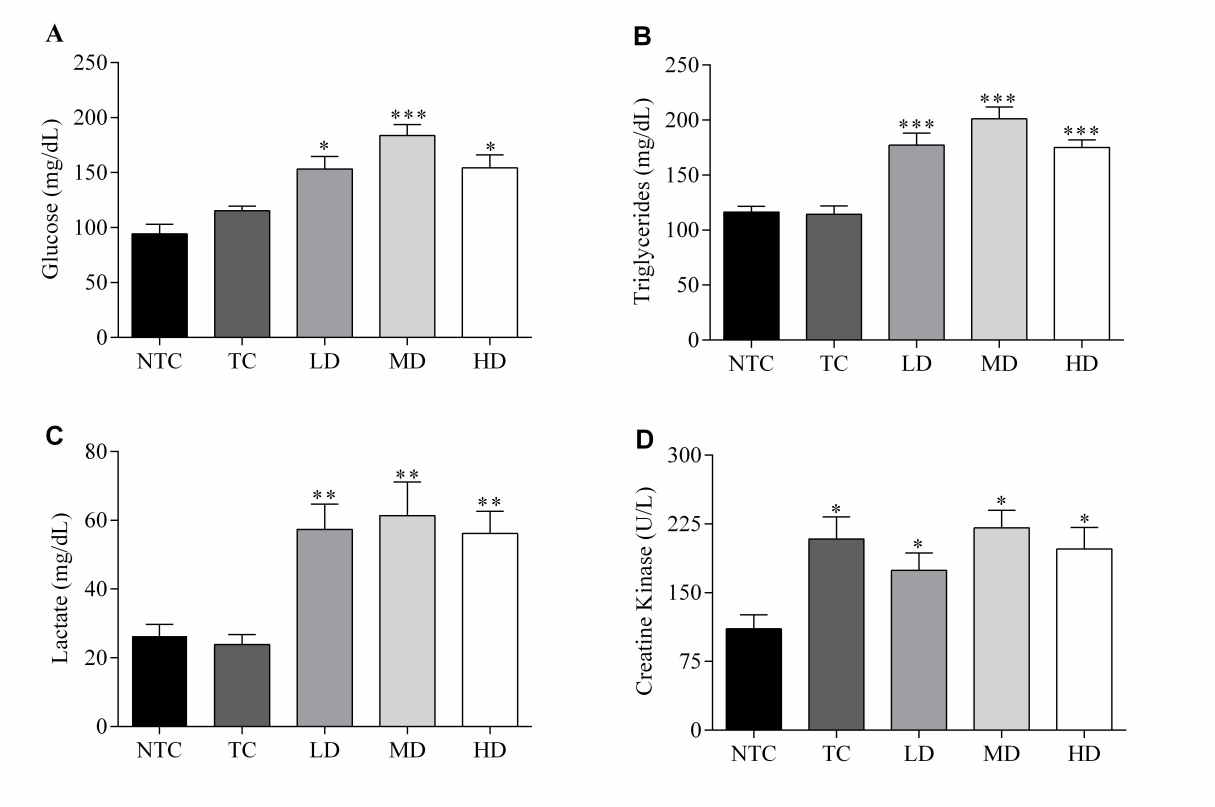


FIGURA 4 - EFFECT OF ACWS ON WEIGHT LOAD SWIM TEST



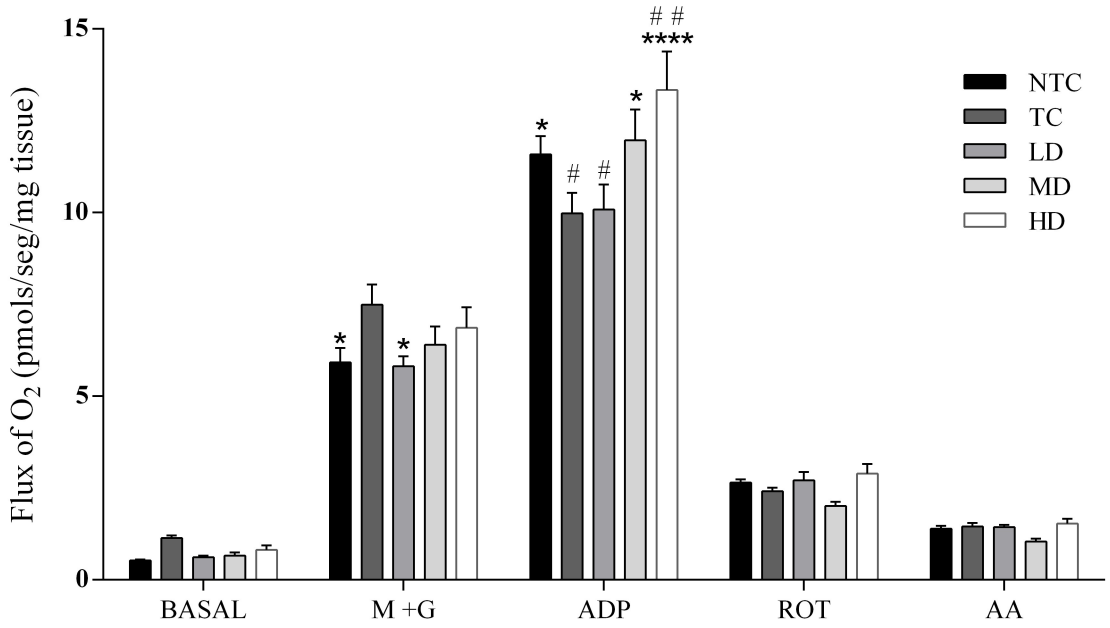
*p < 0.05, **p <0.01, ***p < 0.001, compared with trained control (one-way ANOVA followed by Newman-Keuls *post-hoc* test, n = 5)

FIGURA 5 - EFFECT OF ACWS ON PLASMA GLUCOSE (A), TRIGLYCERIDES (B), LACTATE (C) AND CREATINE KINASE (D) LEVELS



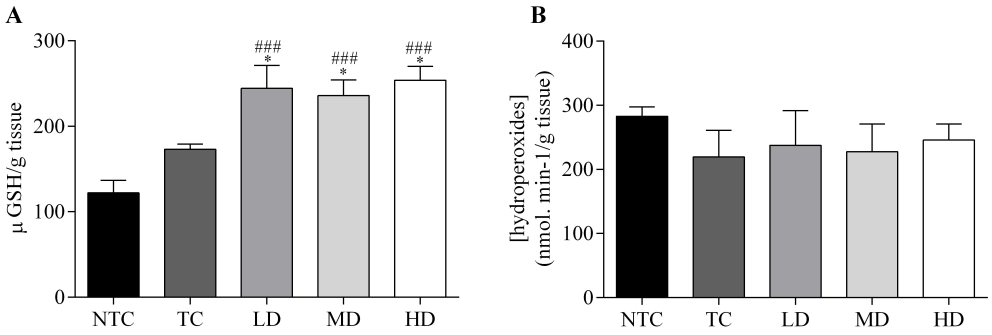
*p < 0.05, **p <0.01, ***p < 0.001, in A, B and C was compared with trained control and (D) was compared with non-trained control (one-way ANOVA followed by Newman-Keuls *post-hoc* test, n = 5).

FIGURA 6 - O_2 FLUX IN PERMEABILIZED SKELETAL MUSCLE FIBERS



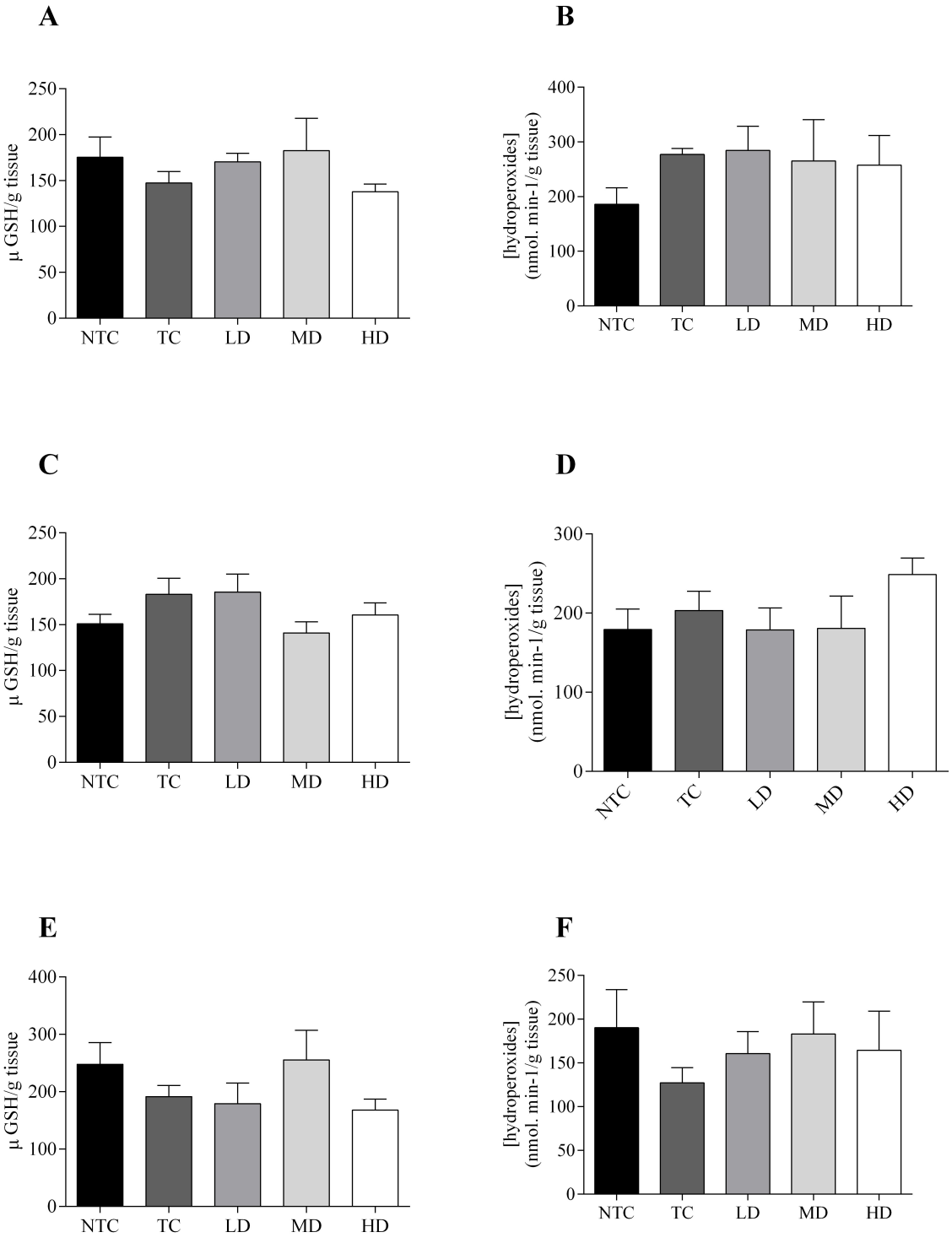
*p < 0.05, ****p < 0.0001 were compared with trained control (TC), and #p < 0.05, ##p < 0.01 were compared with non-trained control (two-way ANOVA followed by Newman-Keuls *post-hoc* test). M: Malate, G: Glutamate, ROT: Rotenone, AA: Antimycin A.

FIGURA 7 - EFFECT OF ACWS ON GSH LEVELS (A) AND LPO (B) IN THE HIPPOCAMPUS



*p < 0.05, compared with TC and ###p < 0.001 were compared with NTC (one-way ANOVA followed by Newman-Keuls *post-hoc* test, n = 5).

FIGURA S1 - EFFECT OF ACWS ON GSH LEVELS (A) AND LPO (B) IN THE PRE FRONTAL CORTEX, ON GSH LEVELS (C) AND LPO (D) IN THE STRIATUM, ON GSH LEVELS (E) AND LPO (F) IN THE HYPOTHALAMUS



There was no statistical difference (one-way ANOVA followed by Newman-Keuls *post-hoc* test).

TABELA S1 - EFFECT OF ACWS ON THE BIOCHEMICAL PARAMETERS

	URE (mg/dL)	LDH (U/L)	AST (U/L)	ALT (U/L)	GGT (U/L)
NTC	55.6 ±2.7	1090 ±82.1	161.7 ±10.6	26.7 ±2.4	14.4 ±3.2
TC	62.8 ±1.9	910.9 ±62.7	160.8 ±21.1	26.1 ±2.9	22.1 ±1.6
LD	61.5 ±2.5	674 ±38.9	174.2 ±18.9	27.0 ±5.4	18.2 ±2.0
MD	64.0 ±3.4	743.3 ±71.0	164.3 ±18.9	29.2 ±4.2	14.2 ±2.3
HD	60.1 ±3.4	777.9 ±59.4	181.5 ±13.0	30.7 ±5.0	15.6 ±2.1

There was no statistical difference (one-way ANOVA followed by Newman-Keuls *post-hoc* test).

CONCLUSÕES

Ao longo dessa dissertação foram abordados os seguintes aspectos: 1) caracterização da estrutura química de polissacarídeos presentes na acerola (*Malpighia emarginata*); 2) avaliação da atividade antioxidante da fração purificada da acerola; 3) avaliação da atividade antifadiga da principal fração polissacarídicas extraída da acerola.

As seguintes conclusões podem ser propostas:

A acerola apresentou homogalacturonana altamente metil-esterificada contendo inserções de ramnogalacturonana do tipo I com cadeias laterais de arabinana.

O tratamento com a fração purificada (ACWS-01E) da acerola diminuiu o efeito citotóxico gerado por H_2O_2 em fibroblastos murinos (NIH 3T3), através da redução dos níveis de espécies reativas de oxigênio intracelulares.

A principal fração (ACWS) extraída da acerola prolongou o tempo de nado até a exaustão em camundongos treinados após o *weight load swim test*. Alterações nas cinéticas dos macronutrientes (carboidratos e lipídios) foram observadas, assim como maior respiração mitocondrial de fibras musculares permeabilizadas, assim como aumento dos níveis de GSH no hipocampo dos camundongos.

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