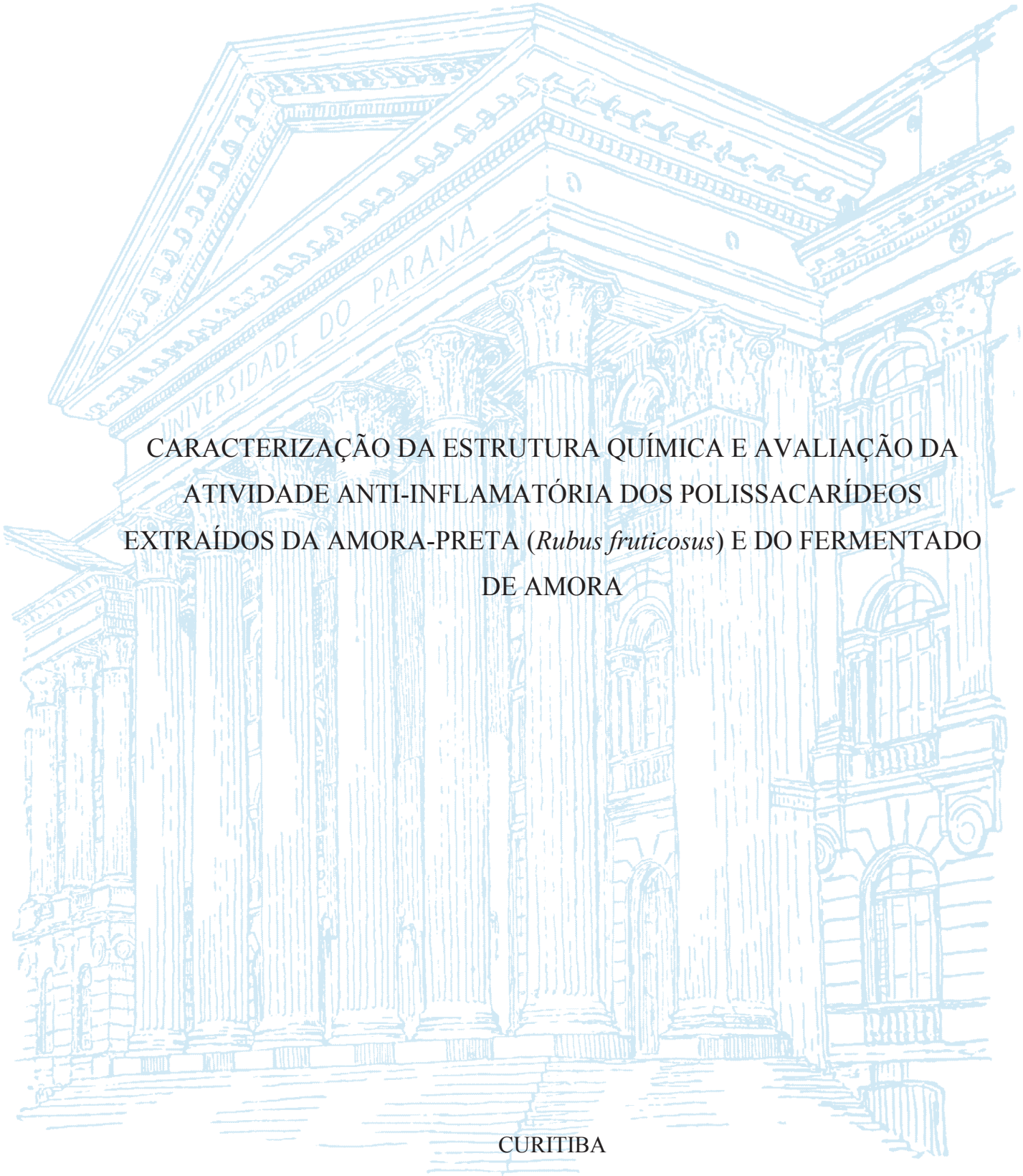
UNIVERSIDADE FEDERAL DO PARANÁ

ADRIANA RUTE CORDEIRO CAILLOT



CARACTERIZAÇÃO DA ESTRUTURA QUÍMICA E AVALIAÇÃO DA ATIVIDADE ANTI-INFLAMATÓRIA DOS POLISSACARÍDEOS EXTRAÍDOS DA AMORA-PRETA (*Rubus fruticosus*) E DO FERMENTADO DE AMORA

CURITIBA

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CARACTERIZAÇÃO DA ESTRUTURA QUÍMICA E AVALIAÇÃO DA ATIVIDADE ANTI-INFLAMATÓRIA DOS POLISSACARÍDEOS EXTRAÍDOS DA AMORA-PRETA (*Rubus fruticosus*) E DO FERMENTADO DE AMORA

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Orientador: Prof. Dr. Guilherme Lanzi Sassaki

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **ADRIANA RUTE CORDEIRO CAILLOT**, intitulada: **CARACTERIZAÇÃO DA ESTRUTURA QUÍMICA E AVALIAÇÃO ANTI-INFLAMATÓRIA DOS POLISSACARÍDEOS EXTRAÍDOS DA AMORA-PRETA (*RUBUS FRUTICOSUS*) E DO FERMENTADO DE AMORA**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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Curitiba, 18 de Maio de 2018.

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Com amor:

Aos meus pais Maria e João,

Ao meu marido Henrique,

Aos meus queridos irmãos, e suas famílias.

Dedico

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RESUMO

Nos últimos anos fermentados de frutas vêm ganhando espaço no mercado nacional e internacional devido a diversas evidências científicas relacionadas aos seus benefícios para a saúde. Neste trabalho foi analisada a amora-preta bem como o fermentado de amora. A fração bruta de polissacarídeos da amora-preta BFPs foi analisada por RMN e GC-MS. BFPs apresentou como principais polissacarídeos uma homogalacturonana (HG) formada por unidades de *α*-D-Gal*p*A(1→4)-ligadas, com grau de metil esterificação de 72%, caracterizada como uma pectina altamente metil esterificada (HM) e uma arabinogalactana do tipo II (AG II) formada por uma cadeia principal de unidades de *β*-D-Gal*p*(1→3)-ligadas, substituídas em *O*-6 por cadeias laterais constituída por unidades de *β*-D-Gal*p*(1→6)-ligadas substituídas em *O*-3 por *α*-L-Ara*f*. Os efeitos anti-inflamatórios das frações de polissacarídeos foram avaliados na linhagem celular RAW 264.7. As frações reduziram significativamente a produção de óxido nítrico (NO) e citocinas pró-inflamatórias (TNF-α e IL-1β) em células tratadas com LPS. BFPs também foi avaliada em camundongo no modelo de septicemia induzida pela cirurgia de ligadura e perfuração do ceco (CLP). Os resultados mostraram que a administração oral e subcutânea de BFPs diminuiu a letalidade dos animais durante a sepse. Além disso, diminuiu os níveis de citocinas pró-inflamatórias, ureia, creatinina e bilirrubina, bem como a diminuição na infiltração de neutrófilos em órgãos como fígado, pulmões e rins. O fermentado de amora produzido pela fermentação dos açúcares naturais presentes no suco de amora também teve alguns dos seus polissacarídeos caracterizados. Foram obtidas três frações de polissacarídeos principais: BWPs, obtida por precipitação com etanol e processo de congelamento-descongelamento, a qual posteriormente foi submetida ao tratamento de Fehling, gerando uma fração solúvel (BWPFs) e outra insolúvel (BWPFp). Todas as frações foram caracterizadas por GC-MS e RMN. Os principais polissacarídeos identificados foram: uma manana, AG II e uma ramnogalacturonana de tipo I (RG I). A manana apresentou cadeia principal constituída por unidades de *α*-D-Man*p*(1→6)-ligadas; a AG II apresentou uma cadeia principal unidades de *β*-D-Gal*p*(1→6)-ligadas, substituída em *O*-3 por unidades não-redutoras de *α*-L-Ara*f*; a RG I por [4)-*α*-D-Gal*p*A-(1→2)-*α*-L-Rha*p*-(1→]$_n$. Os efeitos anti-inflamatórios das frações de polissacarídeos foram avaliados na linhagem celular RAW 264.7. As frações reduziram marcadamente a produção de óxido nítrico (NO) e citocinas pró-inflamatórias (TNF-α e IL-1β) em células tratadas com LPS. As frações também foram avaliadas *in vivo* (sepse). Os resultados mostraram que a administração oral e subcutânea das frações diminuíram a letalidade dos animais durante a sepse em até 65%. Além disso,

diminuiu os níveis de citocinas pró-inflamatórias, ureia, creatinina e bilirrubina. Também ocorreu uma diminuição significativa da infiltração de células de defesa no fígado, pulmões e rins, segundo a avaliação por técnicas de imuno-histoquímica.

Palavras-chave: Amora-preta. Fermentado de amora. Polissacarídeos. RMN. CG-MS. Atividades biológicas.

ABSTRACT

In recent years fermented fruits have been gaining ground in the domestic and international market due to a growing body of scientific evidence related to their health benefits. In this work blackberry was analyzed as well as the fermented blackberry. The crude fraction of polysaccharides obtained from blackberry (BFPs) was analyzed by NMR and GC-MS. BFPs presented as main polysaccharides a homogalacturonan (HG) formed by *α*-D-Gal*p*A-(1→4)-linked units, with a methyl esterification degree of 72%, characterized as a highly methyl esterified pectin (HM), and arabinogalactan (1→6), substituted in the *O*-6 by side chains consisting of *β*-D-Gal*p*-(1→6)-linked units which in turn are substituted at*O*-3 by *α*-L-Ara*f*. The anti-inflammatory effects of the polysaccharide fractions were evaluated with RAW 264.7 cell model. Fractions significantly diminished the production of nitric oxide (NO) and pro-inflammatory cytokines (TNF-α and IL-1β) in LPS-treated cells. BFPs were also evaluated in mice in the septicemia model induced by cecal ligation and puncture (CLP). The results showed that oral and subcutaneous administration of BFPs decreased lethality in the animals during sepsis. In addition, it decreased the levels of pro-inflammatory cytokines, urea, creatinine and bilirubin, as well the infiltration of macrophages in organs such as liver, lungs and kidneys. Fermented blackberry produced by the fermentation of the natural sugars present in the blackberry juice also had some of its polysaccharides characterized. Three major polysaccharide fractions were obtained: BWPs, obtained by ethanol precipitation and freeze-thaw process, which was subsequently submitted to Fehling treatment, generating a soluble fraction (BWPFs) and an insoluble fraction (BWPFp). All fractions were characterized by GC-MS and NMR. The main polysaccharides identified were mannan, AG II and type I rhamnogalacturonan (RG I). The mannan presented a main chain consisting of *α*-D-Man*p*(1→6)-linked units, the AG II was formed by a *β*-D-Gal*p*(1→6)-linked main chain, substituted in *O*-3 by non-reducing units *α*-L-Ara*f* and RG I was formed by [→4-*α*-D-Gal*p*A-(1→2)-*α*-L-Rha*p*-(1→]$_n$. The anti-inflammatory effects of the polysaccharide fractions were evaluated in the RAW 264.7 cell line. Fractions markedly reduced the production of nitric oxide (NO) and pro-inflammatory cytokines (TNF-α and IL-1β) in LPS-treated cells. Fractions were also evaluated *in vivo* (sepsis). The results showed that oral and subcutaneous administration of the fractions decreased the lethality of the animals during sepsis by up to 65%. In addition, it decreased the levels of pro-inflammatory cytokines, urea, creatinine and bilirubin. There was also a significant decrease in leukocyte infiltration to the liver, lungs and kidneys, according to immunohistochemistry assays.

Keywords: Blackberry. Blackberry wine. Polysaccharides. NMR. CG-MS. Biological activities.

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LISTA DE SIGLAS E ABREVIATURAS

AcOEt - Acetato de etila

AG I - Arabinogalactana do tipo I

AG II - Arabinogalactana do tipo II

Ara - Arabinose

CLP - Ligação e punção cecal (cecal ligation and puncture)

CP - Polissacarídeo bruto

$CuSO_4$- Sulfato de cobre

D_2O - Óxido de deutério

DEPT - Intensificação sem distorção por transferência de polarização

ELISA - Enzyme-linked immune sorbent assay

Gal - Galactose

GalA - Ácido galacturônico

GC-MS - Cromatografia gasosa acoplada à espectrometria de massas

Glc - Glucose

HG -Homogalacturonana

HPSEC - Cromatografia de exclusão estérica de alta eficiência

HSQC - Espectroscopia de correlação heteronuclear *Single-Quantum*

i.p. - Intraperitoneal

IFN-γ - Interferon-gama

IL-10 - Interleucina 10

IL-1β - Interleucina 1β

LPS - Lipopolissacarídeo

Man - Manose

MeOH- Metanol

MTT - (3-(4,5-dimetiltiazol-2-yl)-2,5-difenil brometo de tetrazolina)

$NaBH_4$ - Borohidreto de sódio

$NaNO_2$ - Nitrito de sódio

PBS - *Phosphatebuffered saline*

RG I - Ramnogalacturonana do tipo I

RGII - Ramnogalacturonana do tipo II

Rha - Ramnose

RMN - Ressonância magnética nuclear

TFA - Ácido trifluoroacético

TLC - Cromatografia de camada delgada

TNF-α - Fator de necrose tumoral

δ - Deslocamento químico

SUMÁRIO

CAPÍTULO I

No capítulo I, estão às considerações gerais compostas por: Introdução, Objetivo e Revisão Bibliográfica, bem como as Referências usadas no desenvolvimento do capítulo.

1 INTRODUÇÃO

Tecnologias de vinificação estão entre as mais antigas conhecidas pelos seres humanos, com base nas escavações arqueológicas indicando-se a existência de vinho há mais de 7500 anos (MCGOVERN *et al.*, 1996). O vinho tem sido anunciado como um agente terapêutico sendo, um auxiliar importante para a dieta humana, pois possui compostos bioativos (SHAHIDI, 2009).Os frutos, além das uvas, utilizados para a produção de vinho incluem maçãs, morangos, cerejas, amoras entre outras frutas (AABY *et al*., 2005). A amora-preta tem sido incluída nesta perspectiva de mercado, visto que, além do seu sabor agradável, seus frutos são fonte importante de vitaminas, minerais e componentes oriundos do metabolismo secundário, como os polifenóis. A amora-preta tem sido bastante estudada devido ao fato de seus metabólitos apresentarem atividade antioxidante, anti-inflamatória, antitumoral e antiviral (AABY *et al*., 2005; BOWEN-FORBES *et al*., 2010; DANAHER *et al*., 2011).

No entanto, a amora-preta apresenta um período de vida pós-colheita bastante curto devido à elevada atividade respiratória e deterioração causada por microrganismos presentes na própria fruta (ANTUNES *et al*., 2003). Sendo assim, ela se mostra muito versátil do ponto de vista tecnológico para a produção de fermentado (vinho de amora). O fermentado de amora é constituído principalmente por carboidratos, alcoóis, ácidos orgânicos, compostos fenólicos, compostos voláteis e aromáticos (ésteres, aldeídos e cetonas) e vitaminas. Dentre os carboidratos são encontrados os polissacarídeos que são provenientes das amoras e da levedura (*Sacharomices cerevisae*) utilizada no processo de fermentação (BOULET *et al*., 2007). Embora já tenha sido relatada a presença de polissacarídeos nos vinhos de uvas, ainda não encontramos estudos relacionados aos polissacarídeos dos fermentados de amora. Esses polissacarídeos podem estar relacionados com diversos benefícios para a saúde, visto que estudos feitos com polissacarídeos extraídos de plantas, frutas e fungos já mostraram a relação com atividades biológicas, dentre elas, antioxidante (PRISTOV *et al*., 2011), imunomoduladora (DING *et al*., 2012), antinociceptiva (NASCIMENTO *et al*., 2015), anticomplemento (JIN *et al*., 2017), gastroprotetora (CANTU-JUNGLES *et al*., 2014), efeitos antineoplásicos (STIPP *et al*., 2017), anticoagulante (RÓMAN OCHOA *et al*., 2017) e atividades anti-inflamatórias (BEZERRA *et al*., 2018; POPOV*et al*., 2011).

Portanto, devido ao crescente interesse por bebidas fermentadas produzidas a partir de amora-preta, e também à escassez de estudos relacionados à caracterização dos polissacarídeos da amora-preta e do fermentado de amora, nesse trabalho foi realizada a

caracterização estrutural de polissacarídeos de ambos. Além disso, avaliamos a atividade anti-inflamatória dos polissacarídeos, pela avaliação dos efeitos inibitórios na produção de NO, IL-1β e TNF-α em macrófagos RAW 264.7 induzidos por LPS (*in vitro*) e pela avaliação da proteção contra a sepse *in vivo*, em um modelo murino de ligadura e punção cecal (CLP), bem pelos seus efeitos sobre a migração de neutrófilos.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Com base no que foi exposto anteriormente, este trabalho tem por objetivo caracterizar quimicamente os polissacarídeos presentes na amora-preta (*Rubus fruticosus*) e no fermentado de amora, e identificar uma possível ação farmacológica deles durante a sepse, analisando seu efeito na inflamação sistêmica.

2.2 OBJETIVOS ESPECÍFICOS

- Extrair os polissacarídeos presentes na amora-preta e no fermentado de amora.
- Determinar a composição dos monossacarídeos presentes na amora-preta e no fermentado de amora.
- Caracterizar a estrutura química fina das moléculas encontradas empregando técnicas químicas, espectrométricas e espectroscópicas.
- Avaliar a capacidade das amostras estudadas de inibirem mediadores inflamatórios, como a interleucina-1β, TNF-α e óxido nítrico.
- Avaliar os efeitos anti-inflamatórios das frações através do modelo de septicemia induzida pela cirurgia de ligadura e perfuração do ceco (CLP).
- Produção artesanal de vinho bordo (*Vitis labrusca*), avaliação da produção de etanol e de ácidos orgânicos durante o processo de produção do vinho.
- Caracterização dos polissacarídeos e atividade biológica do vinho bordo.

3 REVISÃO BIBLIOGRÁFICA

3.1 AMORA-PRETA

A amoreira-preta é uma planta que pertence à família *Rosaceae* e ao gênero *Rubus*. É nativa do Brasil e de maior ocorrência em lugares com maior altitude e temperaturas mais frias, como na região Sudeste (Minas Gerais, São Paulo, Rio de Janeiro) e Sul do país (Paraná, Santa Catarina, Rio Grande do Sul) (ANTUNES, 2002). Segundo Grandi *et al*. (1989), é uma planta utilizada na medicina popular, sendo que as folhas, caules, flores e frutas são usados para dores de garganta, dentes, rins, bexiga, em casos de bronquite e diabetes, sendo utilizados também como vermífugo e diurético. Estudos científicos atestam que o uso da planta como um todo pode trazer benefícios. Gomar *et al.* (2014), realizaram um estudo com as folhas da amoreira, usando o extrato hidroalcoólico e concluíram que ocorreu um efeito preventivo sobre o aprendizado e comprometimento da memória em ratos com neuropatia diabética. Já as amoras (fruto da amoreira) são apreciadas por consumidores não só por sua alta qualidade nutricional, mas também por seus benefícios à saúde física e mental (ANTUNES, 2002; IVANOVIC *et al*., 2014).

FIGURA 1. AMORA-PRETA (*Rubus fruticosus*) DURANTE SEU ESTÁGIO DE MATURAÇÃO ATÉ A COLHEITA.
Fonte: O Autor, 2017.

Os frutos da amoreira-preta pesam de 4 a 7 g, são formados por um conjunto de unidades separadas ou drupeletes, que ao amadurecer, tornam-se pretos ou roxo escuros (FIGURA 1). Eles têm sabor doce e um pouco ácido, com paladar peculiar, porém seu comércio *in natura* é baixo. Pelo fato de ser uma fruta pequena e sensível, o que dificulta o transporte e armazenamento, além de possuir um curto prazo de validade, em função de sua elevada taxa respiratória (HUMMER, 2010; FERRI; SAGGIN, 2014). A amora-preta faz

parte do grupo das chamadas “pequenas frutas”, apresentando elevados conteúdos de vitaminas e sais minerais (Tabela 1). Além dos altos teores de fibras, a amora é uma fonte importante de compostos fenólicos, como ácidos fenólicos, taninos, flavonoides e antocianinas (ELISIA *et al*., 2007).

TABELA 1- COMPOSIÇÃO DA AMORA-PRETA (*Rubus* sp.).

Composição	Quantidades
Conteúdo energético (Kcal/100g)	52,5±2,5
Água (g/100g)	90±3
Proteínas (g/100g)	1,5
Fibras (g/100g)	4,1 ±0,6
Cinzas (g/100g)	0,33±0,14
Carboidratos (g/100g)	9,5±3,5
Lipídeos (g/100g)	0,55±0,25
Cálcio (mg/100g)	32
Fósforo (mg/100g)	21
Potássio (mg/100g)	196
Magnésio (mg/100g)	20
Ferro (mg/100g)	0,57
Selênio (mg/100g)	0,60
Vitamina C (mg/100g)	21

Fonte: HIRSCH *et al*., 2012.

A amora-preta é uma fonte de compostos com atividade antioxidante, que possuem ação inibitória sobre radicais livres (CAVANAGH *et al*., 2004). Estudos *in vitro* revelaram amoras como possuindo potentes atividades antioxidantes, antiproliferativas e anti-inflamatórias (ZIA-UL-HAQ *et al*, 2014), tendo efeito anticarcinogênico, com capacidade de inibir o crescimento de linhagens celulares tumorais de mama, cólon e próstata(STONER *et al*, 2006; WANG *et al*, 2008; SEERAM *et al*., 2006)e antimutagênico (TATE *et al*., 2003).Estas atividades biológicas relacionadas à amora-preta são atribuídas ao seu elevado teor de fitoquímicos, como os ácidos fenólicos, as antocianinas, os flavonoides, estilbenoides, taninos e ligninas (DAÍ *et al*., 2007; HAGER *et al*., 2008; SEERAM *et al*., 2006). Além destes compostos a amora-preta contém pectina em abundância, uma fibra solúvel que ajuda a reduzir os níveis de colesterol no sangue, atuando na prevenção de enfermidades cardiovasculares e circulatórias (NESS; POWLES, 1997). Ainda, esta fruta atenua os riscos e sintomas do diabetes e diminui o risco de doenças coronarianas (AZOFEIFA *et al*., 2016; HERTOG *et al*., 1997; SARKAR *et al*., 2016).

3.2 FERMENTADO DE AMORA

A partir da fermentação alcoólica de frutas é obtido o vinho. O vinho é um produto da fruta, mas a fermentação produz uma variedade de mudanças químicas no mosto, sendo assim o vinho está longe de ser suco com etanol adicionado (AABY *et al*., 2005). Desde a antiguidade o vinho é produzido pelo homem, fazendo parte da vida na antiga Grécia e também em Roma. Atualmente sabe-se que as origens do vinho ultrapassam em muito os três mil anos de história que nos separam dos primeiros habitantes da Grécia. Atualmente no Brasil, a produção de vinho é regulamentada por um conjunto de normas, segundo a Lei n°. 7.678, decretada pelo Congresso Nacional em 8 de novembro de 1988:

> Art. 3º Vinho é a bebida obtida pela fermentação alcoólica do mosto simples de uva sã, fresca e madura.
>
> Parágrafo único. A denominação vinho é privativa do produto a que se refere este artigo, sendo vedada sua utilização para produtos obtidos de quaisquer outras matérias-primas (BRASIL, 1988).

Bebidas fermentadas com graduação alcoólica entre 4 e 14% em volume, produzidas a partir de outras frutas sendo ou não utilizado açúcar no mosto recebem, a denominação de **fermentado de frutas**, segundo o Decreto n° 2.314, de 04 de setembro de 1997, do Congresso Nacional (BRASIL, 1997).O processo de fermentação modifica a composição do mosto, onde o açúcar da fruta ou o açúcar adicionado é consumido e há uma formação de etanol e outros produtos secundários, o que torna a composição do fermentado mais complexa, dando ao produto final características diferentes de outros produtos derivados da fruta (FLANZY, 2000). Estudos realizados com bebidas alcoólicas, derivadas da fermentação de amoras têm mostrado que a amora-preta é uma matéria-prima adequada para a produção de fermentados, com uma capacidade antioxidante *in vitro* comparável aos vinhos tintos de uva. Em comparação com os fermentados produzidos de maçã e mirtilo, o fermentado de amora apresentou a maior quantidade de compostos fenólicos e maior atividade antioxidante (ORTIZ *et al*., 2013).

O fermentado de amora tradicionalmente tem sido usado na medicina popular para tratamento de anemia, por ser uma comprovada fonte de ferro (PETRAVIC-TOMINAC *et al*., 2013), previne doenças cardiovasculares e circulatórias, pois é fonte de antioxidantes naturais (KOCA;KARADENIZ, 2009), como os compostos fenólicos (ORSER, YANG, 1966; JOHNSON, GONZALEZ, 2012; AROZERENA *et al*., 2012), as antocianinas, os

carotenóides (DEIGHTON *et al*., 2000; FERREIRA *et al*., 2010) e os polifenóis (MOYER *et al*., 2002; ROMMEL, 1990).Os ácidos fenólicos podem eliminar os radicais livres e extinguir espécies reativas de oxigênio e, portanto, proporcionar meios eficazes de prevenção e tratamento de doenças mediadas por radicais livres (SHAHIDI, 2009). Outros estudos têm investigado a composição dos fermentados de amora, dando ênfase à cor e pigmentos (ORSER, YANG, 1966; AROZARENA *et al*., 2012), bem como os efeitos do processamento e do armazenamento na composição de antocianinas e na modificação do aroma (ROMMEL, 1990). Além dos compostos já descritos na literatura, o fermentado de amora possui muitos outros constituintes ainda não estudados que podem também ser responsáveis por atividades biológicas, como os polissacarídeos. Em produtos como o fermentado de amora, a determinação da composição dos carboidratos, bem como dos polissacarídeos, podem ser importantes quando considerados fatores como o tipo de amora e o processo de fermentação utilizado.

As técnicas usadas para a produção do fermentado de amora são bastante semelhantes ao vinho, sendo realizado da seguinte maneira. O processo de produção do fermentado (FIGURA 2) inicia-se com a colheita, recepção e lavagem das amoras, esmagamento e trituração, adição de açúcar, adição ou não de leveduras, fermentação, filtragem, engarrafamento e rotulagem, envelhecimento e distribuição.

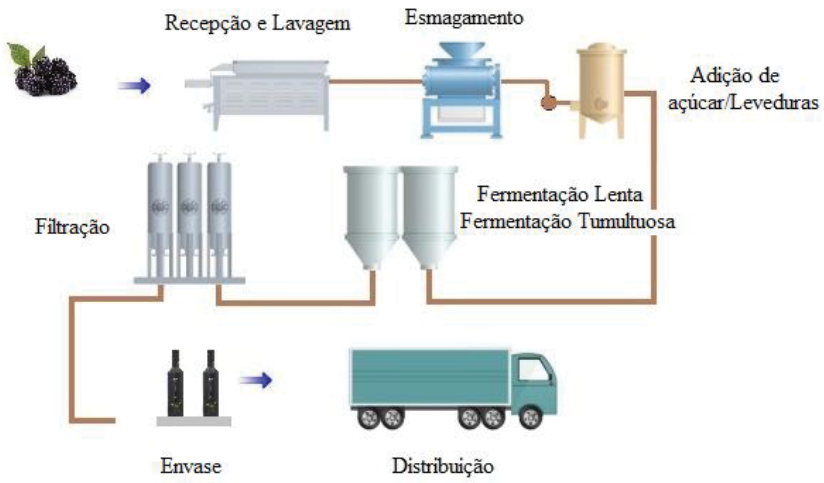

FIGURA 2. PROCESSO DE PRODUÇÃO DO FERMENTADO DE AMORA

FONTE: O autor.

3.3 A IMPORTÂNCIA DOS CARBOIDRATOS ESTRUTURAIS

Os principais constituintes químicos em tecidos de plantas e células vegetais, depois da água, são os carboidratos. Devido aos carboidratos formarem a parede celular, que é a rede de suporte estrutural da célula vegetal, representam aproximadamente 90% da parede primária (REID, 1997; CAFFALL; MOHNEN, 2009). A parede celular é um envoltório que protege a célula, é uma estrutura dinâmica e é essencial para a sobrevivência das plantas.

A parede celular da planta apresenta uma composição altamente organizada de diferentes polissacarídeos (CARPITA; McCANN, 2000), levando a uma heterogeneidade dos polissacarídeos constituintes das paredes celulares de plantas (FIGURA 3), os quais são divididos em três grupos: celulose, pectinas e hemiceluloses.

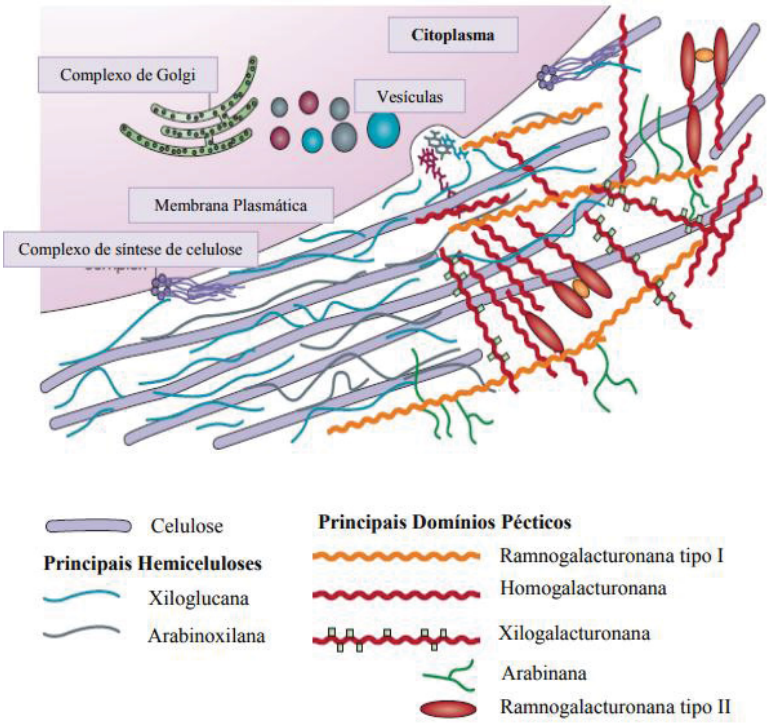

FIGURA 3. ILUSTRAÇÃO ESQUEMÁTICA DA ESTRUTURA DA PAREDE CELULAR PRIMÁRIA VEGETAL
FONTE: Adaptado de COSGROVE, 2005.

A celulose principal constituinte da parede das células vegetais é um homopolímero linear e bastante rígido que consiste em unidades de D-glucopiranose. Estas unidades estão ligadas entre si por ligações glicosídicas β-(1→4) (KLEMM *et al*., 1998).

As hemiceluloses são polissacarídeos da parede celular que estão associados à celulose e à lignina (ASPINAL, 1969). Vários tipos de hemiceluloses são encontradas na parede celular vegetal, sendo as principais: xilanas, mananas e xiloglucanas. As xilanas possuem

cadeias lineares constituída por unidades de *β*-D-xilose (1→4)-ligadas podendo ser acetiladas nas posições *O*-2 ou *O*-3. As xilanas podem ser encontradas como arabinoxilanas (AX), glucuronoarabinoxilanas (GAX), glucuronoxilanas (GX) ou homoxilanas quando não possuem ramificações. As mananas são componentes estruturalmente importantes da parede celular, além de serem encontradas como polissacarídeos de reserva em alguns vegetais, na forma de galactomananas (GMs) e galactoglucomananas (GGMs). As GMs são molécula lineares que possuem cadeia principal constituída por unidades alternadas de D-glucose e D-manose com ligações do tipo *β*-(1→4), enquanto as GGMs possuem pequenas cadeias laterais de unidades de D-galactose (CAFFALL e MOHNEN, 2009). Xiloglucanas são constituídas por uma cadeia principal de unidades de *β*-D-glucose (1→4)-ligadas, com numerosos resíduos de *α*-D-xilose ligados na posição *O*-6 das unidades de glucose. Além disso, alguns destes resíduos de xilose podem ser substituídos com *α*-L-arabinose ou *β*-D-galactose, e algumas vezes, a unidade de galactose pode estar substituída com *α*-L-fucose (CARPITA e McCANN, 2000).

As pectinas (FIGURA 4) têm função no crescimento, na morfologia, no desenvolvimento, na defesa da planta, e são amplamente usadas na indústria como geleificantes e estabilizantes. As pectinassão uma família de polissacarídeos ricos em ácido galacturônico (GalA) incluindo homogalacturonana (HG), ramnogalacturonana tipo I (RGI), ramnogalacturonana tipo II (RGII) e xilogalacturonana (XGA), arabinana, galactana e arabinogalactanas (AGs) tipo I e II (CARPITA e McCANN, 2000; MOHNEN, 2009). As últimas três classes de polímeros descritas constituem as substâncias pécticas neutras.

Os polissacarídeos são biomoléculas com um grande potencial biotecnológico por apresentarem uma grande diversidade estrutural e comprovadas propriedades biológicas. Eles desempenham papéis importantes na comunicação célula-célula, adesão celular e reconhecimento molecular no sistema imunológico (DWEK, 1996). As propriedades biológicas de polissacarídeos são dependentes de uma variedade de parâmetros estruturais, como composição monossacarídica, tipo e configuração da ligação glicosídica, pontos de ramificação, peso molecular e a presença de substituição por grupos polares e apolares (PEREIRA *et al*., 2002).

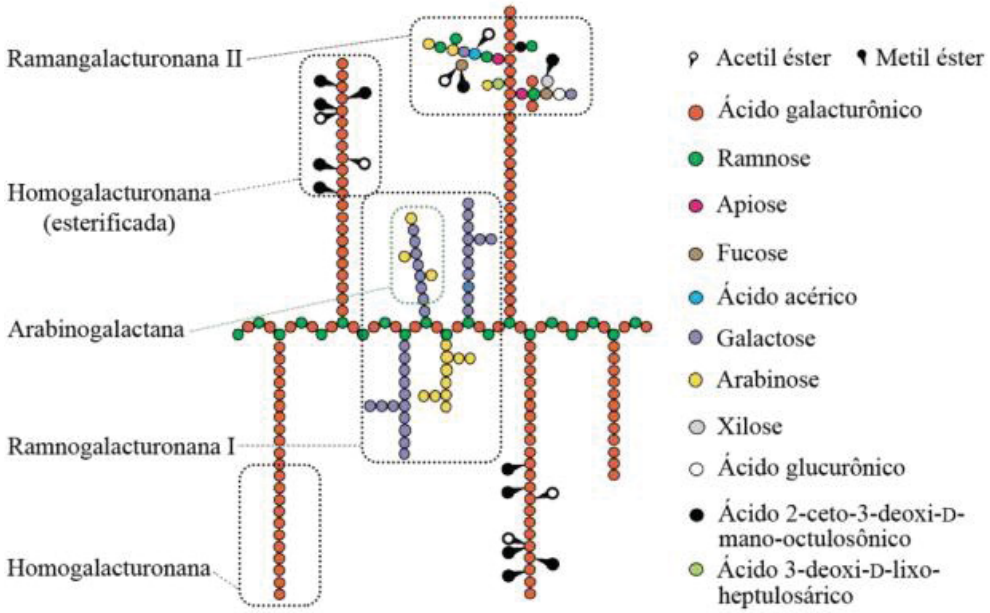

FIGURA 4. ILUSTRAÇÃO ESQUEMÁTICA DA ESTRUTURA DAS PECTINAS.
FONTE: Adaptado de WILLATS *et al*., 2006.

Uma ampla gama de polissacarídeos é capaz de interagir com o sistema imunológico para regular as partes específicas da resposta do hospedeiro e pode ser classificada como moduladores imunológicos. As atividades biológicas mais comuns atribuídas aos polissacarídeos de diferentes fontes são atividade antibacteriana (SHAO *et al*., 2017), antiviral (SHI *et al*., 2017; YUE*et al*., 2017), anticoagulante (OCHOA *et al*., 2017), antitumoral (DING *et al*., 2007; STIPP *et al*., 2017), antiúlcera (NERGARD *et al*., 2005), hipoglicêmico (WANG *et al*., 2017) anti-inflamatória (DU *et al*., 2016; BEZERRA *et al*., 2018), entre outras.

3.3.1 Polissacarídeos presentes no fermentado de amora

Os polissacarídeos em vinhos são principalmente de duas fontes, originadosdas paredes celulares das bagas de uva após a degradação por enzimas pécticas durante a maturação da baga e os vários tratamentos de vinificação, ou das paredes celulares das leveduras usadas na fermentação alcoólica. Eles formam um dos principais grupos de macromoléculas nos vinhos, juntamente com polifenóis e proteínas (BOULET *et al*., 2007).As pectinas estão entre os polissacarídeos vegetais encontrados no vinho, como as homogalacturonanas (HG), às vezes metiladas/acetiladas, e as regiões de alta densidade de cadeias laterais são conhecidas como rhamnogalacturonanas (RG) de tipo I e II (CAFFALL;

MOHNEN, 2009). RG I consiste de ramnose (Rha) e ácido galacturônico (GalA) e representa uma proporção muito pequena de pectinas à base de uva. No vinho tinto, o polissacarídeo péctico mais abundante é o RG II, formado na uva durante a maturação e extraído durante os estágios iniciais da vinificação.

As arabinogalactanas-proteínas (AGP) são glicoproteínas e também estão localizadas nas paredes celulares da planta e extraídas durante a vinificação (GUADALUPE, MARTÍNEZ-PINILLA, GARRIDO, CARRILLO, & AYESTARÁN, 2012). Os polissacarídeos provenientes de células de levedura são liberados durante a fermentação alcoólica e envelhecimento do vinho, quando as paredes celulares se quebram. Eles foram identificados como glucanas, quitina, manoproteínas (MP) e mananas (ESCOT *et al*., 2001; BEZERRA *et al*., 2018). Até o presente momento não foram encontrados trabalhos com a caracterização dos polissacarídeos do fermentado de amora.

3.4 PROCESSO INFLAMATÓRIO E POLISSACARÍDEOS

Inflamação é uma resposta adaptativa complexa e altamente regulada,sendo desencadeada por uma variedade de estímulos, incluindo patógenos, agentes mecânicos e químicos e condições como infecção e lesão tecidual (MEDZHITOV, 2008). Os macrófagos são umas das principais células imunes do sistema de fagócitos mononucleares. Elesdesempenham um papel importante na iniciação, manutenção e resolução da inflamação (KOH; DIPIETRO, 2011). Na inflamação, os macrófagos têm três funções principais; apresentação de antígenos, fagocitose e imunomodulação. Esta última por meio da produção de várias citocinas e fatores de crescimento sendo ativados e desativados no processo inflamatório. Os macrófagos são ativados por estímulos como interferon-gama (IFN-γ), lipopolissacarídeos bacterianos (LPS), citocinas pró-inflamatórias (TNF-α, IL-6 e IL-1β), proteínas da matriz extracelular e outros mediadores químicos (FUJIWARA; KOBAYASHI, 2005). Durante a inflamação, os macrófagos se acumulam em grande quantidade dentro do tecido afetado. Este acúmulo de macrófagos e outras células imunes no local de lesão ou infecção são cruciais para combater as infecções e restaurar a homeostase dos tecidos (HELMING, 2011).

A inibição da inflamação por remoção ou desativação de mediadores e células efetoras inflamatórias permite ao hospedeiro reparar os danos nos tecidos. Os macrófagos ativados são desativados por citocinas anti-inflamatórias. Macrófagos ativos secretam excessivas quantidades de mediadores inflamatórios como óxido nítrico (NO), prostaglandina-E2 (PGE2)

e citocinas pró-inflamatórias, incluindo TNF-α, IL-6 e IL-1β, as quais foram associados a várias doenças inflamatórias. A ativação desregulada de algumas enzimas inflamatórias, como óxido nítrico sintase induzível (iNOS), catalisando a reação que converte L-arginina e oxigênio para óxido nítrico, citrulina e ciclo oxigenase-2 (COX-2), gerando prostaglandina-E2 a partir de ácido araquidônico, demonstraram desempenhar papéis significativos na progressão de processos oncogênicos (MURAKAMI; 2007). Portanto, suprimira superprodução de mediadores inflamatórios (NO, PGE2, TNF-α, IL-6 e IL-1β) e controlar as ativações anormais de iNOS e COX-2 é importante para o tratamento e prevenção da inflamação e doenças relacionadas.A inflamação e a disfunção imune estão intimamente relacionadas com a patogênese da sepse (HELMING, 2011).

A sepse é definida como uma disfunção orgânica potencialmente fatal que é causada por uma resposta desregulada do hospedeiro à infecção, podendo induzir lesões renais agudas e falhas de vários órgãos, como fígado, pulmões e cérebro. A sepse inicia uma resposta imune complexa que varia ao longo do tempo, com a ocorrência concomitante de mecanismos pró-inflamatórios e anti-inflamatórios (VENET; MONNERET, 2017).

A resposta imune inata inicia o processo inflamatório na sepse. Ela é mediada pelos receptores de reconhecimento, como os receptores *Toll-like* (TLR) e o cluster de diferenciação (CD14), que reconhecem os patógenos ou seus produtos chamados de PAMPs (padrões moleculares associados a patógenos). Os TLRs reconhecem os peptideoglicanos das bactérias que uma vez ativados, desencadeiam uma cascata de sinalização intracelulares que culmina com a translocação nuclear do NF-kB, que promove a expressão gênica de moléculas pró-inflamatórias, como (TNF-α) e (IL-1β) e também anti-inflamatória como IL-10 (HENKIN, 2009; RUSSEL, 2006; COHEN, 2002; BALDWIN, 2001). Então, o TNF-α e IL-1β ativam a resposta imune adaptativa que é responsável pela amplificação da imunidade inata (FIGURA 5). Além da inflamação, os microrganismos também ativam a cascata da coagulação, aumentando os fatores pró-coagulantes e reduzindo os anticoagulantes (AIRD, 2003). Sendo iniciada por meio da expressão do fator tecidual (FT) na superfície das células endoteliais e monócitos.

Com expressão de FT, ativa-se o fator VII, resultando no complexo fator VIIa + FT, esse por sua vez converte o fator X em Xa. O fator Xa + Va convertem a protrombina em trombina, resultando na clivagem do fibrinogênio em fibrina, acarretando translocação nuclear do NF-kB. Fatores anticoagulantes endógenos como a proteína C, a proteína S, antitrombina III e o inibidor da via do fator tecidual (TFPI – Tissue Factor Pathway Inhibitor)

modulam a coagulação, aumentando a fibrinólise e removendo os microtrombos (HENKIN, 2009).

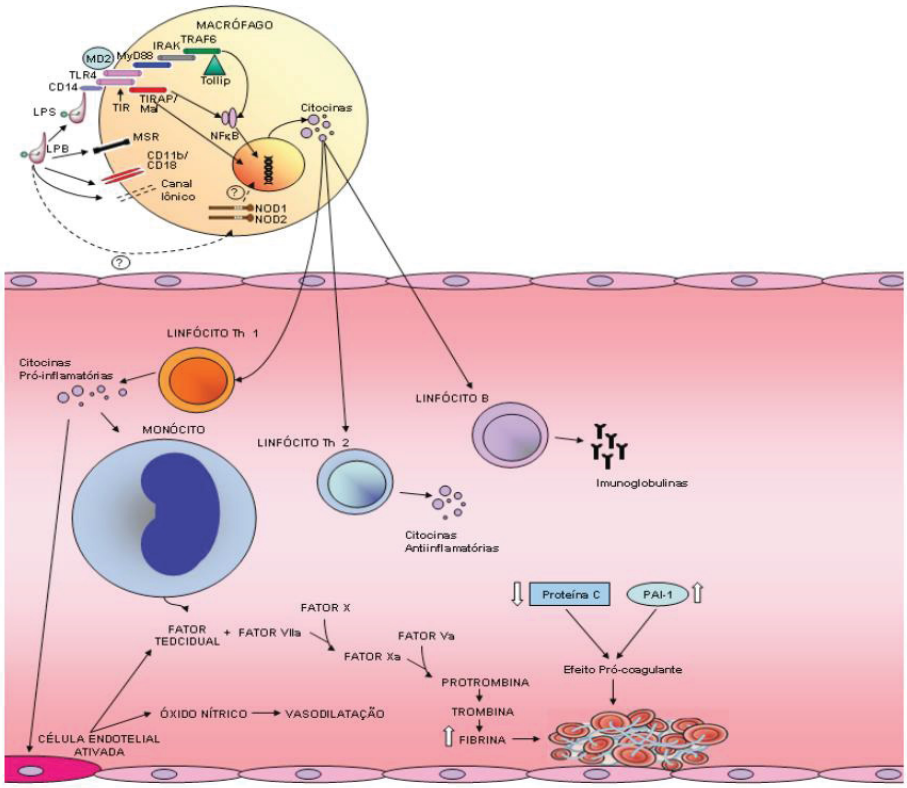

FIGURA 5. MECANISMOS FISIOPATOLÓGICOS DA SEPSE.
FONTE: Adaptado HENKIN *et al*., 2009

Para o tratamento da sepse são sugeridas frequentemente novas medicações que visam interferir na cascata inflamatória e da coagulação, todavia sem uma resposta convincente na maior parte das vezes. A estratégia básica subjacente à imunomodulação é identificar aspectos da resposta do hospedeiro que podem ser aprimorados ou suprimidos de forma a aumentar ou complementar uma resposta imune desejada (INNGJERDINGEN *et al*., 2005).

Em experimentos biológicos realizados com camundongos, pode-se induzir o processo inflamatório causando injúria aos tecidos, seguido de avaliação do aumento da permeabilidade dos vasos sanguíneos e da migração dos leucócitos. A administração de drogas como dexametasona, anteriormente a essas lesões, diminui esses efeitos mostrando uma atividade anti-inflamatória (VINEGAR *et al*., 1979). Atividade anti-inflamatória promovida por polissacarídeos isolados de várias espécies de fungos, algas, vegetais e vinhos tem sido descrita na literatura. Polissacarídeos pécticos extraídos da parte aérea da planta *Comarum palustre* e polissacarídeos ácidos isolados da alga *Turbinaria ornata* demonstraram

atividade anti-inflamatória. A avaliação desta atividade foi realizada por meio do modelo de edema de pata induzida por carragenina em ratos e do teste de permeabilidade vascular em camundongos, sendo que estes polissacarídeos inibiram o edema, e a adesão de leucócitos peritoneais *in vitro* (POPOV *et al*., 2005; ANANTHI *et al*. 2010). Extratos ricos em *β*-glucanas e outros polissacarídeos obtidos de *Geastrum saccatum* (DORE *et al*., 2007), *Ganoder matsugae* (LIN *et al*., 2006) e do basídiomiceto *Pleurotus pulmonarius* (SMIDERLE *et al*. 2008) apresentaram efeito anti-inflamatório, reduzindo a migração de leucócitos e a liberação de mediadores pró-inflamatórios. Uma fucomanogalactana isolada do basidiocarpo do fungo *Lentinus edodes*, apresentou propriedades anti-inflamatórias nos testes de permeabilidade capilar peritoneal e infiltração leucocitária promovida por ácido acético em camundongos (CARBONERO *et al*. 2008).

Do suco de morango e da amora foram isolados polissacarídeos que apresentaram forte potencial anti-inflamatório via modulação do perfil de secreção de citocinas pró-/anti-inflamatórias secretadas por culturas de macrófagos estimuladas por LPS. O tratamento com os polissacarídeos diminuiu os níveis das citocinas pró-inflamatórias, incluindo IL-1β e IL-6, e aumentou o nível da citocina anti-inflamatória IL-10 (LIU *et al*. 2012). Ovodova (2009) caracterizou galacturonana que foi responsável pelo aumento da sobrevivência de camundongos, submetidos a uma dose letal de LPS, sendo este efeito mediado pela diminuição na produção de IL-1β (pró-inflamatória), aumento de IL-10 (anti-inflamatória), e pela diminuição da migração de neutrófilos para a cavidade peritoneal. Em camundongos submetidos à sepse, sendo tratados com fucogalactanas de cogumelos comestíveis *Agaricus bisporus* e selvagens *Lactarius rufus.* Observou-se redução da mortalidade dos camundongos em 62,5% além daprevenção do acúmulo de neutrófilos no íleo e a diminuição dos níveis séricos de TNF-α e IL-1β (RUTHES *et al*., 2012).

Em amostras de vinhos foram identificadas a presença de manana, AG II, RG I e RG II. A mistura de polissacarídeos e as frações isoladas inibiram a produção de citocinas inflamatórias (TNF-α e IL-1β) e mediador anti-inflamatório (NO) em células RAW 264.7 estimuladas com LPS (BEZERRA *et al*., 2018). Esses estudos fornecem informações importantes de como essas moléculas possuem ação anti-inflamatória.

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CAPÍTULO II

Este capítulo é compost pelo manuscrito "*In vitro* and *in vivo* anti-inflammatory potential of crude polysaccharide from blackberry fruit (*Rubus fruticosus*)". O manuscrito será submetido a revista Food Hydrocolloids.

In vitro and in *vivo* anti-inflammatory potential of crude polysaccharide from blackberry fruit (*Rubus fruticosus*)

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ABSTRACT

Blackberry fruit polysaccharides (BFPs) were extracted through ethanol precipitation and analyzed by Nuclear Magnetic Resonance (NMR) and Gas Chromatography-Mass Spectrometry (GC-MS). Major polysaccharides were identified as homogalacturonans with units of *α*-D-Gal*p*A (1→4)-linked. The degree of methyl-esterification was 72%, characterizing this polymer as a high-methoxyl (HM) pectin. Also identified were a Type II Arabinogalactan (AG II) formed by a main chain of *β*-D-Gal*p* (1→3)-linked, substituted in *O*-6 by side chains of *β*-D-Gal*p* (1→6)-linked and 3-*O*-substituted by non-reducing end units of *α*-L-Ara*f*. The anti-inflammatory effect of polysaccharide fraction was evaluated in RAW 264.7 cell line. Fraction markedly reduced nitric oxide (NO) and pro-inflammatory cytokines (TNF-α and IL-1β) production in LPS-treated cells. Furthermore, BFPs were evaluated *in vivo* on the lethality induced by cecal ligation and puncture. Cytokine levels and biochemistry profile were determined and correlated to mortality data. The results showed that both BFPs oral and subcutaneous administration decreased lethality during sepsis. Also, levels of pro-inflammatory cytokines, urea, creatinine and bilirubin decreased. Immuno histochemical analysis showed an significant improvement of liver, lung and kidney tissues.

Keywords: Polysaccharide; Sepsis; Inflammation.

1. INTRODUCTION

Blackberries are consumed globally fresh or processed as pulp, juices, jams or desserts and are appreciated for their color, flavor and high content of natural bioactive compounds, such as vitamins, phytosterols, carotenoids, organic acids, fiber and polysaccharides which may have beneficial effects on human health (Rao & Snyder, 2010). Polysaccharides from natural sources are non-toxic substances and have wide variety of pharmacological activities, such as antioxidant (Pristov, Mitrović, & Spasojević, 2011), immunomodulatory (Ding, Zhu, & Gao, 2012), antinociceptive (Nascimento, et al., 2015), anti-complementary (Togola, et al., 2008), gastroprotective (Cantu-Jungles, et al., 2014), antineoplastic (Stipp, et al., 2017) and anti-inflammatory (Popov, et al., 2011; Cordeiro Caillot, et al., 2018). Hence, in the present study we focused on crude polysaccharides extracted from *Rubus fruticosus* to investigate their anti-inflammatory properties. Inflammation is a complex adaptive response, tightly regulated, involving both signals that initiate and maintain inflammation and signals that shut the process down (Koh & DiPietro, 2011). Macrophages are a major component of the mononuclear phagocyte system. When an inflammatory process occurs, macrophages become activated and may secrete excessive amounts of inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines that have been associated with various inflammatory diseases (Fujiwara & Kobayashi, 2005). Therefore, suppressing the overproduction of inflammatory mediators (NO, TNF-α and IL-1β) is important for the treatment and prevention of inflammation and related diseases. Sepsis, considered a systemic inflammatory disorder, represents a state of over production of pro-inflammatory mediators which frequently occurs after various noxious injuries which includes pathological changes (Bone, Grodzin, & Balk, 1997).

The objective of this study was to demonstrate the anti-inflammatory activity of crude polysaccharides of blackberry fruit in LPS-stimulated murine RAW 264.7 macrophage *in vitro,* their protection against sepsis *in vivo*, in a murine model of cecal ligation and puncture (CLP), and their effects on neutrophil migration.

2. MATERIALSAND METHODS

2.1 PLANT MATERIAL

Fruits of *Rubus fruticosus* blackberry (Fig. 1A) were collected in Ponta Grossa (25°09′14″ S; 49°59′06″ W), State of Paraná (PR), Brazil. A voucher specimen was deposited

in the UPCB (Herbarium of the Federal University of Paraná), registration number 90661, 90662 and 90663.

2.2 POLYSACCHARIDES EXTRACTION AND PURIFICATION

Fruits (500 g) were freeze-dried and milled. Dried fruit was defatted with chloroform-methanol (2:1) in order to remove lipids, pigments and other hydrophobic material. Polysaccharides were extracted from the residue with water at 100 °C for 2 h (×5, l L each) obtaining fraction BFA. The aqueous extracts were combined and concentrated under reduced pressure. The polysaccharides were recovered by ethanol (3 vol.), collected by centrifugation (2800 G, 20 min at 4 °C) and freeze-dried, generating fraction BFP. A freeze-thaw treatment was applied to fraction BFP to generate cold-water soluble fraction BFPs (Fig. 1B). In this procedure, the sample was frozen and then thawed at room temperature. Insoluble polysaccharides from fraction BFPi were recovered by centrifugation (2800 G, 20 min at 4 °C).

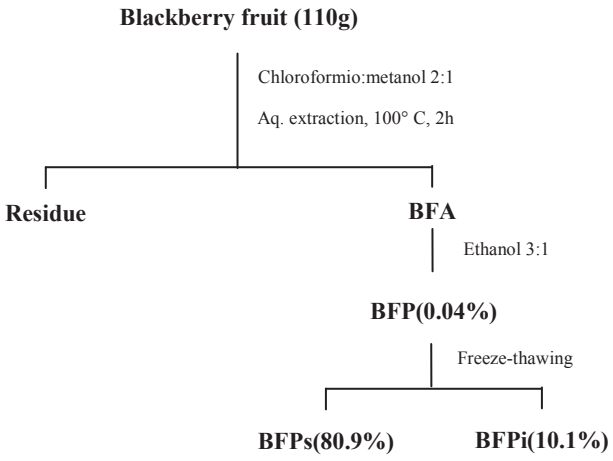

FIGURE. 1. Scheme of extraction and fractionation of polysaccharides from blackberry.

2.3 MONOSACCHARIDE ANALYSIS

BFPs (~5 mg) was hydrolyzed in 1 mL of trifluoroacetic acid (TFA) 2 M at 100 ºC for 12 hours and then evaporated to dryness. Samples were then solubilized in 400 µL D_2O for NMR analysis (Sassaki et al., 2014).

2.4 METHYLATION ANALYSIS

BFPs was methylated using NaOH (10 mg), DMSO/Iodomethane (0.5 mL), adapted from the method of Ciucanu & Kerek (1984). Per-O-methylated polysaccharides were submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with formic acid (45%, 12 h) (BIERMANN; MCGINNIS, 1989). The latter was dried and reduced with $NaBD_4$ and acetylation was performed. The partially *O*-methylated alditol acetates (PMAA) were examined by GC-MS (Varian Saturn 3800 Gas Chromatograph coupled to a Varian 4000 ion trap mass spectrometer), using a DB-1-MS capillary column (30 m x 0.25 mm), injector 250 °C, oven start at 50 °C (held 1 minute) to 185 °C (40 °C/min) held 10 minutes and then to 210 °C (40 °C/min) held for 15 minutes, He being the carrier gas (1 mL/min). Chromatogram peaks were identified by their typical electron impact fragmentation profiles and retention times (Sassaki, Gorin, Souza, Czelusniak & Iacomini, 2005). BFPs was also submitted to carboxy-reduction according to Taylor & Conrad (1972), resulting in BFPs-CR sample.

2.5 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

BFPs (10 mg) was dissolved in 0.5 mL D_2O with internal standard 10 µL 3-trimethylsilyl-$^2H_4$-propionic acid sodium salt (TMSP) 1% and nuclear magnetic resonance analysis was performed using a Bruker Avance *III* NMR spectrometer operating at 14.1 Tesla (600.13 MHz for ^1H) equipped with an inverse 5 mm probe head (QXI) at 343 K incorporating Fourier transform. 2D NMR experiments ^1H-^1H (COSY and TOCSY) and (^1H-^{13}C multiplicity-edited HSQC), which was carried out by correlation via double inept transfer with decoupling during acquisition using sensitivity improvement trim pulses as compiled in the pulse program hsqcedetgpsisp2.2 using 6993 Hz (^1H) and 24900 Hz (^{13}C) widths and a recycle delay of 1.080 s. 2D correlation maps were recorded using quadrature detection in the indirect dimension and 24 scans per series of 1024 x 320 W data points, with zero filling in F1 (2048), prior to Fourier transformation (Sassaki et al., 2014).

2.6 CELL CULTURE AND POLYSACCHARIDE TREATMENT

Murine macrophages cells line RAW 264.7 were incubated under ideal conditions (5% CO_2 at 37°C) in 24-well culture plates (BD Falcon, San Jose, CA, USA) and cultured in

DMEM culture medium (Invitrogen, San Diego, CA, USA) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 20 mM sodium bicarbonate. Murine RAW 264.7 macrophages were treated with different concentrations of BFPs (0.1, 1.0, 10 and 100 µL/mL) and then after one hour induced with LPS (2 µg/mL).

2.7 CELL VIABILITY ANALYSIS

Murine Raw 264.7 macrophage were plated in 24 well plates ($4.8x10^5$ cells/well) and treated with different concentrations of BFPs (0.1, 1.0, 10 and 100 µg/mL). Cell viability was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-bromo-diphenyltetrazolium) method (Mosmann, 1983). After 24 hours 350 µL/well of a 5 mg/mL MTT solution was added. After 4 h of incubation, cell supernatant was removed and 500 µl of DMSO (Dimethyl sulfoxide) were added for cell lyse and crystals solubilization, prior measurement on a microplate reader at wavelength of 570 nm.

2.8 MEASUREMENT OF TNF-α, IL-1β AND NO PRODUCTION *in vitro* AND *in vivo*

RAW 264.7 cells (4.8×10^5 cells/100 µL) were treated by LPS (2µg/mL) with or without BFPs (0.1, 1.0, 10 and 100 µl/mL) for 24 h. 100 µl of the culture supernatant was aspirated and submitted to cytokine and NO assays. Cytokine Interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α) levels were assessed using an immunoenzymatic assay (ELISA) kit (eBioscience), according to the manufacturer recommendations. Nitrite concentration was used to access NO production. Griess reagent (50 µl) was added to 50 µl of collected supernatant and absorbance was read at 545 nm. Griess method (Griess, 1879) is based on the determination of nitrite ions obtained by quantitative reduction (greater than 90%) of nitrate ions present in the sample. For tests *in vivo* the levels of IL-1β, TNF-α and Interleukin-10 (IL-10) in serum were determined with mouse ELISA kits (Elabscience).

2.9 ANIMALS

The studies were performed using male albino *Swiss* mice (3 months old, weighing 30-40g) from the UFPR (Paraná/Brazil). The animals were maintained under standard conditions (12 h light/dark cycle and controlled temperature 22 ± 2 °C). Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available *ad libitum*. All experimental procedures were

previously approved by the Institutional Ethics Committee of the University (authorizations numbers 1018 and 1099) in appendix A.

2.10 WRITHING TEST

Mice were treated with different doses of BFPs subcutaneously (0.1, 1 and 10 mg/kg) or orally (3, 7 and 10 mg/kg). The BFPs was suspended in saline solution prior injection or ingestion. The non-treated group received only the vehicle (saline solution 10 mL/kg), subcutaneous or oral.

2.11 SEPSIS INDUCTION BY CECAL LIGATION AND PUNCTURE (CLP)

Sepsis was induced by cecal ligation and puncture (CLP) in mice, as previously described (Fink & Heard, 1990). Mice were randomly grouped into eleven clusters of 8 mice: (1) sham or False operated mice, (2) CLP + Saline (positive control), (3) CLP + BFPs 0.1 mg/kg; (4) CLP + BFPs 1 mg/kg; (5) CLP + BFPs 10 mg/kg, all with subcutaneous treatment. Groups with oral treatment: (6) sham or false operated mice, (7) CLP + Saline (positive control), (8) CLP + BFPs 3 mg/kg; (9) CLP + BFPs 7 mg/kg; (10) CLP + BFPs 10 mg/kg. The mice average weight was 35 g, thus, 25 μL of each BFPs solution was found appropriate to be administered orally, avoiding regurgitation. As positive control, commercially available dexamethasone was administered subcutaneously (11) CLP + dexamethasone 0.5 mg/kg. Then, mice were anesthetized by intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (20 mg/kg), and a small abdominal midline incision was made. The cecum was carefully exposed and 50% of the distal moiety was ligated. The cecum was then punctured thrice with a sterile 16-gauge needle and squeezed to extrude the fecal material from the wounds; the abdominal incision was closed in two layers. Each mice received 1 mL sterile saline subcutaneously administered for fluid resuscitation. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. After surgery food and water were given *ad libitum* throughout the experiment. The survival rate was monitored for 7 days, each 12 h. During this period, solution saline (vehicle) and drugs were subcutaneous and orally administered daily.

In another experimental set, 30 min (for subcutaneous administration) and 1 h (for oral administration) before surgery, mice were treated with: (a) BFPs 1 mg/kg (subcutaneous administration); (b) BFPs 10 mg/kg (oral administration); (c) Saline solution (subcutaneous

administration); (d) Saline solution (oral administration) and (e) dexamethasone (0.5 mg/kg). After 6 h post-operation, mice were sacrificed. Their lung, liver and kidneys were collected for histological assays and their blood for biochemical analysis.

2.12 BLOOD LEUKOCYTE MEASUREMENT

Blood was collected by cardiac puncture. Total leukocytes, mononuclear (MN) and polymorphonuclear (PMN) leukocytes were counted by an automated system, according to the manufacturer's recommendations (Mindray BC-2800 vet, São Paulo, SP, Brazil). Additionally, slides were prepared and stained with May-Grunwald-Giemsa for analysis of mononuclear and polymorphonuclear leukocytes morphology.

2.13 PLASMA BIOCHEMISTRY

Plasma samples were prepared by centrifugation at 3000 g for 10 min and levels of Bilirubin (direct, indirect and total), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA) and urea activity were analyzed by an automated system, according to the manufacturer's recommendations (Mindray BS-200, São Paulo, SP, Brazil).

2.14 HISTOLOGIC ANALYSIS

Samples of lungs, liver and kidney tissue sections were fixed in ethanol solution (70% - at room temperature - for 6 h), then in ALFAC solution (alcohol 80%, formaldehyde 15% and acetic acid glacial 5% - for 16h). After fixation, samples were dehydrated in a graded series of increasing ethanol strength before paraffin embedding. Sections (4 μm) were stained with hematoxylin and eosin and analyzed under microscopy.

2.15 CYTOKINE LEVELS DETERMINATION

Plasma samples were prepared by centrifugation at 8000 g for 10 min and stored at -80 °C. Analyses of tumor necrosis factor alpha (TNF-α), interleukins IL-1β and IL-10 concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (elabscience) according to the manufacturer´s recommendation.

2.16 STATISTICAL ANALYSIS

Data are expressed as means. Statistical analyses were performed using GraphPad Prisma® 5.0 (La Jolla, CA) software and one-way analysis of variance ANOVA followed by Turkey test. Significant differences were considered at $p< 0.05$. Histological analysis was performed by qualitative observations.

3. RESULTS AND DISCUSSION

3.1MONOSACCHARIDE OBTAINMENT AND COMPOSITION OF THE CRUDE POLYSACCHARIDE

Blackberry fruits (500 g) were freeze-dried, yielding moisture of approximately 80%. The dried berries (110 g) were defatted and extracted with water under reflux conditions. The extract was treated with EtOH to provide a crude precipitate of polysaccharides. The latter was submitted to freezing-thawing until no more precipitate appeared, resulting in a soluble BFPs (5.3%). The monosaccharide composition of BFPs was obtained by NMR analysis, revealing galacturonic acid (60.1%), arabinose (16.3%), galactose (11.3%), glucose (8.7%) and rhamnose (3.6%) (Table S1, Supporting Information). The galacturonic acid monosaccharide had a higher concentration.

3.1.1 NMR spectroscopy of BFPs

The NMR ^{1}H and ^{13}C chemical shifts were obtained by combination of COSY, TOCSY and HSQC experiments. The $^{1}H/^{13}C$ correlation map of BFPs (Fig. 2) suggest the presence of a pectic structure, with units of *α*-D-Gal*p*A(1→4)-linked, with $^{13}C/^{1}H$ correlations at δ 100.9/4.9 attributed to C-1/H-1, δ 79.6/4.4 (C-4/H-4), 71.0/5.01 (C-5/H-5) and 54.0/3.78 (CO_2CH_3) typical of →4)-6-*O*Me-*α*-D-Gal*p*A-(1→ units. Besides δ 100.7/5.06 (C-1/H-1), 79.3/4.39 (C-4/H-4), 71.6/4.67 (C-5/H-5) of non-methyl-esterified →4)-*α*-D-Gal*p*A-(1→ residues (Ovodova et al., 2009; Popov et al., 2011; Nascimento et al., 2013, Cordeiro et al., 2018; Bezerra et al., 2018). Due to the presence of methyl-esterified *α*-D-Gal*p*A units, the degree of methyl esterification was determined by NMR spectroscopy. According to integration of the cross peak area of C-5/H-5 from 6-*O*Me-D-Gal*p*A and D-Gal*p*A units on HSQC map correlation the degree of methyl-esterification was 72%, characterizing the

polymer as a high-methoxyl (HM) pectin. In the spectra, besides the signals of a partially methyl-esterified (1→4)-linked-*α*-D-galacturonic acid (substituted and unsubstituted anomeric carbons at δ 100.9/4.9 and δ 100.7/5.06, respectively), it was also observed $^{13}C/^{1}H$ correlations of C-1/H-1 at δ 109.3/5.20, 108.7/5.05 and 108.1/5.14 of *α*-L-Ara*f* units, and correlations of C-1/H-1, C-3/H-3 and C-6/H-6 of 3,6-linked β-D-Gal*p* units at δ 103.1/4.49, 80.2/3.85 and 68.1/3.85; 3.77 respectively (Delgobo, Gorin, Jones, & Iacomini, 1998; Nascimento et al., 2013; Nascimento et al., 2015 Cordeiro Caillot et al., 2018; Bezerra et al., 2018). The results obtained from NMR analysis indicates that BFPs is a highly methyl-esterified homogalacturonan branched by side chains of arabinans, which corroborates with type II arabinogalactans (AG II).

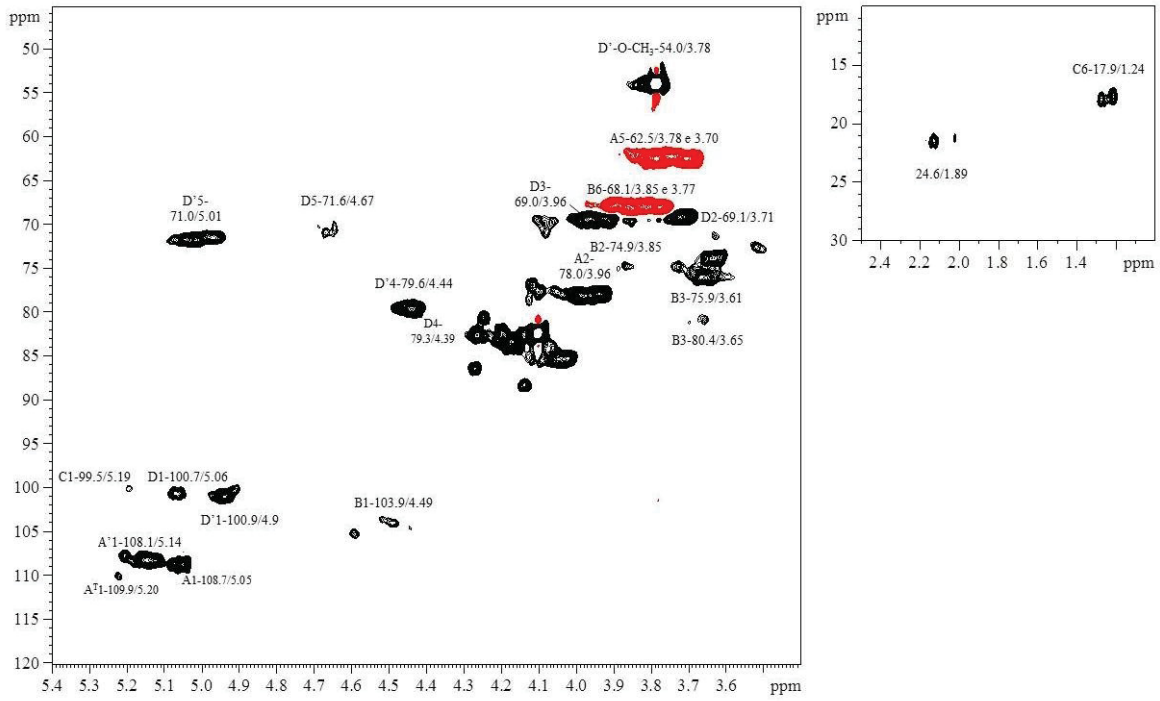

FIGURE 2. 2D multiplicity-edited ^{1}H-^{13}C HSQC NMR correlation map of fraction BFPs, in D_2O, acquired at 70 °C . A (*α*-L-Ara*f*); B (*β*-D-Gal*p*); C (*α*-L-Rha*p*); D (*α*-D-Gal*p*A).

3.1.2 Methylation analysis

Methylation analysis (Table S3, Supporting Information) was performed with the BFPs and the carboxy-reduced BFPs (BFPs-CR). After carboxy-reduction (Taylor & Conrad, 1972), the BFPs galacturonic acid units were converted to galactose units. On BFPs methylation analysis 10.4% of 4-*O*-substituted galactopyranosyl units were detected,

compared with 51.8% in BFP-CR. Besides, it was observed 2,3,5-Me_3-Ara*f* (7.1%) and nonreducing end-units of 2,3-Me_2-Ara*f* (13.1%), 2,5-Me_2-Ara*f* (3.9%), 2,3,4-Me_3-Rha*p* (1.8%), 3-Me-Rha*p* (4.1%), 2,3,4,6-Me_4-Gal*p* (3.5%), 2,3,4-Me_3-Gal*p* (7.7%), 2,3-Me_2-Gal*p* (3.7%) and 2,3,6-Me_3-Glc*p* (2.9%). Methylation analysis confirmed the NMR data, which indicates a homogalacturonan branched by side chains of type II AG.

3.2 BIOLOGICAL EXPERIMENTS

3.2.1 Cellular anti-inflammatory activity

Macrophages are important immune system cells and the body's first line of defense against infection (Alamuru-Yellapragada, Kapadia, & Parsa, 2017). After treatment with BFPs (0.1, 1, 10 and 100 μg/mL) for 24 h, cell viability was detected using MTT assay (Fig. 3A). Compared to control group, there was no significant difference at the doses tested. Next, the effect of BFPs on NO concentration following LPS (2 μg/mL) stimulation was assessed to investigate the polysaccharides anti-inflammatory activity. Without LPS, the level of NO in macrophages was low and with LPS was 56% higher. The samples treated with BFPs (0.1, 1, 10 and 100 μg/mL) significantly decreased LPS-induced NO production in a dose-dependent manner by 46.8%, 54.9%, 58.0% and 58.0% (Fig. 3B). Activated macrophages can produce pro-inflammatory cytokines, such as TNF-α and IL-1β, that play an important role in the initiation, development and resolution of inflammation (Koh & DiPietro, et al., 2011). The effects of BFPs on TNF-α and IL-1β secretion were investigated by culturing the RAW 264.7 macrophages with a combination of LPS (2 μg/mL) and BFPs samples (0.1, 1, 10 and 100 μg/mL). As shown in Fig. 3C and 3D, BFPs dose-dependently inhibited LPS-induced TNF-α and IL-1β production, compared with the LPS control with maximum inhibition of TNF-α (48.4%, 47.9%, 60.6% and 64.6%) and IL-1β (36.8%, 42.8%, 53.8% and 53.9%). The results indicate that BFPs treatment in LPS-stimulated cells significantly inhibited the levels of NO, TNF-α and IL-1β in a dose-dependent manner (Fig. 3B-C and D).

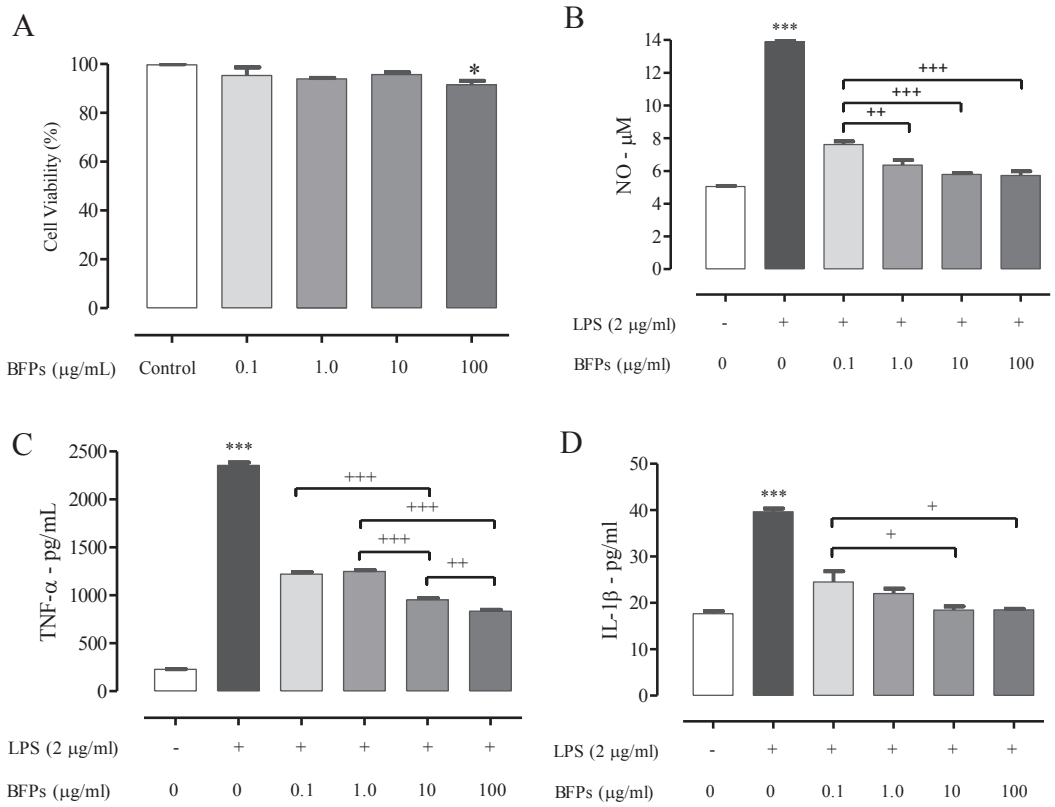

FIGURE 3. Cell viability of polysaccharides presents in blackberry treated with RAW 264.7 cells in the presence of LPS. The viability of cells without sample and LPS has been taken as reference (100%) - positive control (A). NO concentration in the culture supernatant was determined by Griess assay using sodium nitrite standard curve (B). TNF-α concentration in the culture supernatant was determined by ELISA using standard curve (C). IL-1β concentration in the culture supernatant was determined by ELISA using standard curve (D).

All data are presented as mean ± SD from three sets of independent experiments. *p< 0.05 represents significant difference with cells treated with LPS alone (positive control) compared with fractions. +p <0.05 represents significant difference beetwen fractions.

3.2.2 Effects of BFPs on CLP-induced sepsis in mice

In order to evaluate the therapeutic potential of BFPs in vivo, we conducted a mice sepsis model. Sepsis is considered a systemic inflammatory disorder caused by a dysregulated host response to infection (Bone, Grodzin, & Balk, 1997). As previously described, treatment included subcutaneous administration (s.a) and oral administration (o.a). Survival curves were analyzed in six groups each for s.a (fig.4A) and o.a (fig.5A). The lethality was markedly delayed in mice treated, subcutaneously, with SPI and their areas under the curve were increased to 6.204, 14.796 and 10.512 after administrating 0.1, 1 and 10 mg/kg, respectively.

At the end of the period, the overall survival in these BFPs groups was 13%, 67% and 50%. The mice treated, orally, with BFPs demonstrated their areas under the curve were increased to 9.324, 10.992 and 10.512 after administrating 3, 7 and 10 mg/kg, respectively. At 7 days after surgery, the overall survival in these groups was 25%, 38% and 50%. Mice treated with vehicle started to die at 48 h after CLP, with a death rate reaching 75%. The overall mortality at the end of the observation period was 100%, and the area under the curve was 4.200 (arbitrary units). No death was observed in the sham-operated mice and its corresponding area under the lethality curve was 16.800 (arbitrary units). Dexamethasone-treated mice showed a significant improvement in survival, with an overall survival rate of 50% and the area under the curve was 12.336 (arbitrary units). All results showed a significant difference between them.

3.2.3 Measuring blood leukocyte cells and plasma biochemical profile

The doses of 1mg/kg (subcutaneous administration) and 10 mg/kg (oral administration) were used for biochemical and histopathological analyses. Six hours after CLP, mice were analyzed for the effects of sepsis. Sepsis comprises hemodynamic changes and alterations in hematological parameters, such as leucopenia and leukocytosis (Angus & Van der Poll, 2013). These alterations are not present in the sham group. However, treatment with BFPs s.a decreased the level of blood cells in 55.9% in post-CLP mice (Fig. 4B), and treatment with BFPs o.a decreased in 46.7% (Fig. 5B). Polymorphonuclear (PMN) leukocytes present on blood in septic mice s.a and o.a decreased 47.3 % and 44.7%, respectively. Also, mononuclear (MN) leukocytes present on blood in septic mice s.a and o.a decreased 46.0%, 40.7%, respectively (Fig. 4C and 5C). Organ failure assessment for sepsis focuses on respiratory, hepatic, renal and hematological systems, among others (Haak & Wiersinga, 2017). The septic positive control mice exhibited significantly increased levels of leukocytes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, urea and creatinine in blood when compared with the sham. Subcutaneous administration of BFPs treatment reduced the levels of CLP-induced ALT 60.5 % (Fig. 4D), AST 53.7 % (Fig. 4E) bilirubin direct 76.0%, indirect 79.0%, total 72.1% (Fig. 4F), urea 52.2% (Fig. 4G) and creatinine 69.2% (Fig. 4F). The oral administration showed a decrease of ALT 51.6% (Fig. 5D), AST 64.5% (Fig. 5E), bilirubin direct 65.8%, indirect 43.8%, total 61.0% (Fig. 5F), urea 41.4% (Fig. 5G) and creatinine 53.8% (Fig. 5F). The treatment by subcutaneous administration was more effective when compared to oral administration.

Further, to assess whether the administration of BFPs globally attenuated CLP-induced inflammation, we measured the levels of pro-inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10) factors in the blood serum at 6 h after the treatment. The results suggest that the levels of TNF-α, IL-1β and NO markedly decreased in the LPS-challenged mice pre-treated with BFPs, as well as the levels of IL-10, compared with positive control (Fig. S1, Supporting Information).

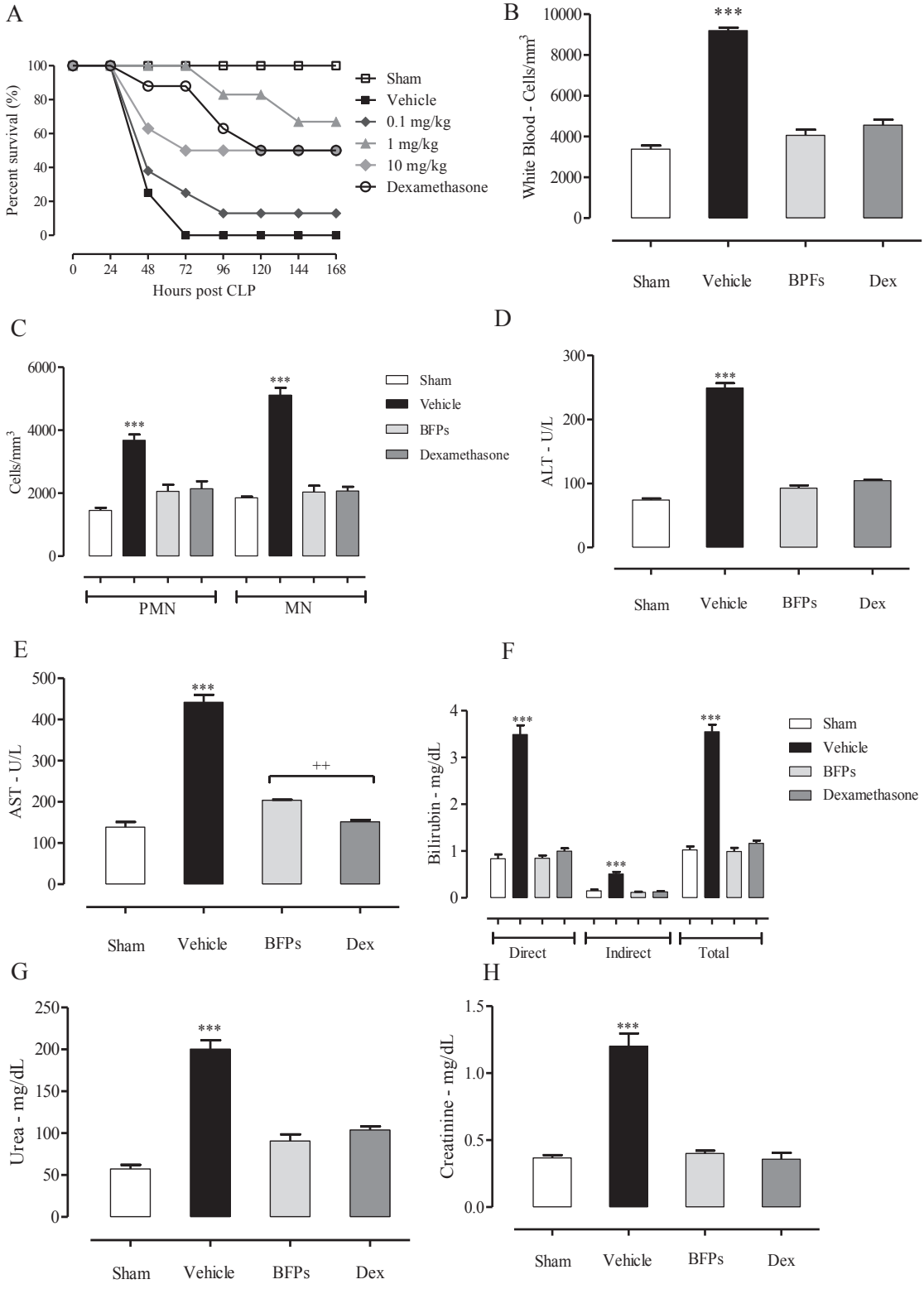

FIGURE 4. Effect of polysaccharide BPFs on survival rate of subcutaneousadministration (A). The effect of the BFPs (1 mg/kg) treatment on white blood cells (B), differential cell counting (C), ALT plasma levels (D), AST plasma levels (E), Bilirubin plasma levels (F), urea plasma levels (G) and creatinine plasma levels (H).Points or bars represent the mean ± mean standard errors (n = 8). Symbols: * Statistically group *P < 0.05.

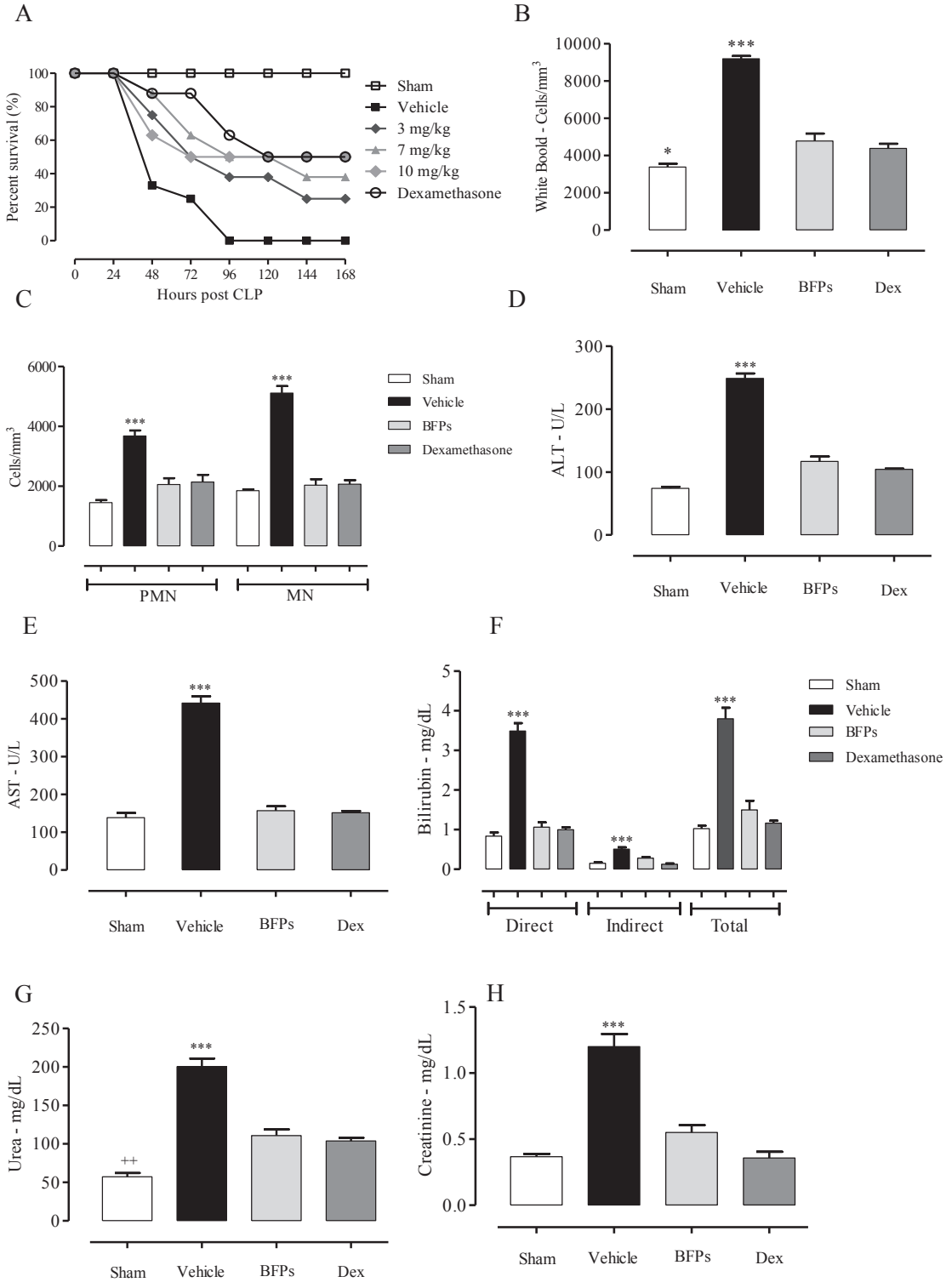

FIGURE 5. Effect of polysaccharide BPFs on survival rate of oral administration (A). The effect of the BFPs (10 mg/kg) treatment on white blood cells (B), diferencial cell counting (C), ALT plasma levels (D), AST plasma levels (E), Bilirubin plasma levels (F), urea plasma levels (G) and creatinine plasma levels (H). Points or bars represent the mean ± mean standard errors (n = 8). Symbols: * Statistically group *P < 0.05.

3.2.4 Histopathology

In order to confirm that BFPs could protect the animals against sepsis, the histopathology of mice livers, lungs and kidney were immunohistochemically evaluated. In livers, sepsis led to severe hepatocyte swelling and inflammatory cells infiltration in positive control (Fig. 6B and 6F). In the Sham group, liver structure was nearly normal and there were no hepatocyte necrosis, hemorrhage or inflammatory cells infiltration (Fig. 6A and 6E). In the Sham group the lung structure was intact: no alveolar hemorrhage, normal alveolar septum, normal pulmonary capillaries and no inflammatory cells (Fig. 6H - 6L). In positive control (Fig. 6I - 6M) the lung structure was significantly disrupted, with alveolar hemorrhage, pulmonary interstitial edema, alveolar collapse and infiltration of a large amount of inflammatory cells.

Kidney injury manifested with increasing urea and creatinine levels (Fig. 4G and 4H) (Angus & Van der Poll, 2013). Inflammatory cells infiltration and glomerulonephritis were observed. Hemorrhagic areas were visible in addition to increased edema and leukocyte aggregation; infiltration was severe in positive control (Fig. 6P and 6T). After BFPs treatment, liver injury was significantly attenuated: hepatocytes were nearly normal and there was no inflammatory cells infiltration (Fig. 6C and 6F). Decreased neutrophil infiltration in lung (Fig. 6J and 6N) and histopathological changes were obvious in the BFPs-treated kidneys (Fig. 6R and 6U). Recently, polysaccharides have received increasing scientific attention due to their possible anti-inflammatory effects (Bezerra, et al., 2018; Cordeiro Caillot et al., 2018; Gao, et al., 2018). The present study showed that both the subcutaneous and oral administration of BFPs were potential bioactive treatments for protection against inflammation.

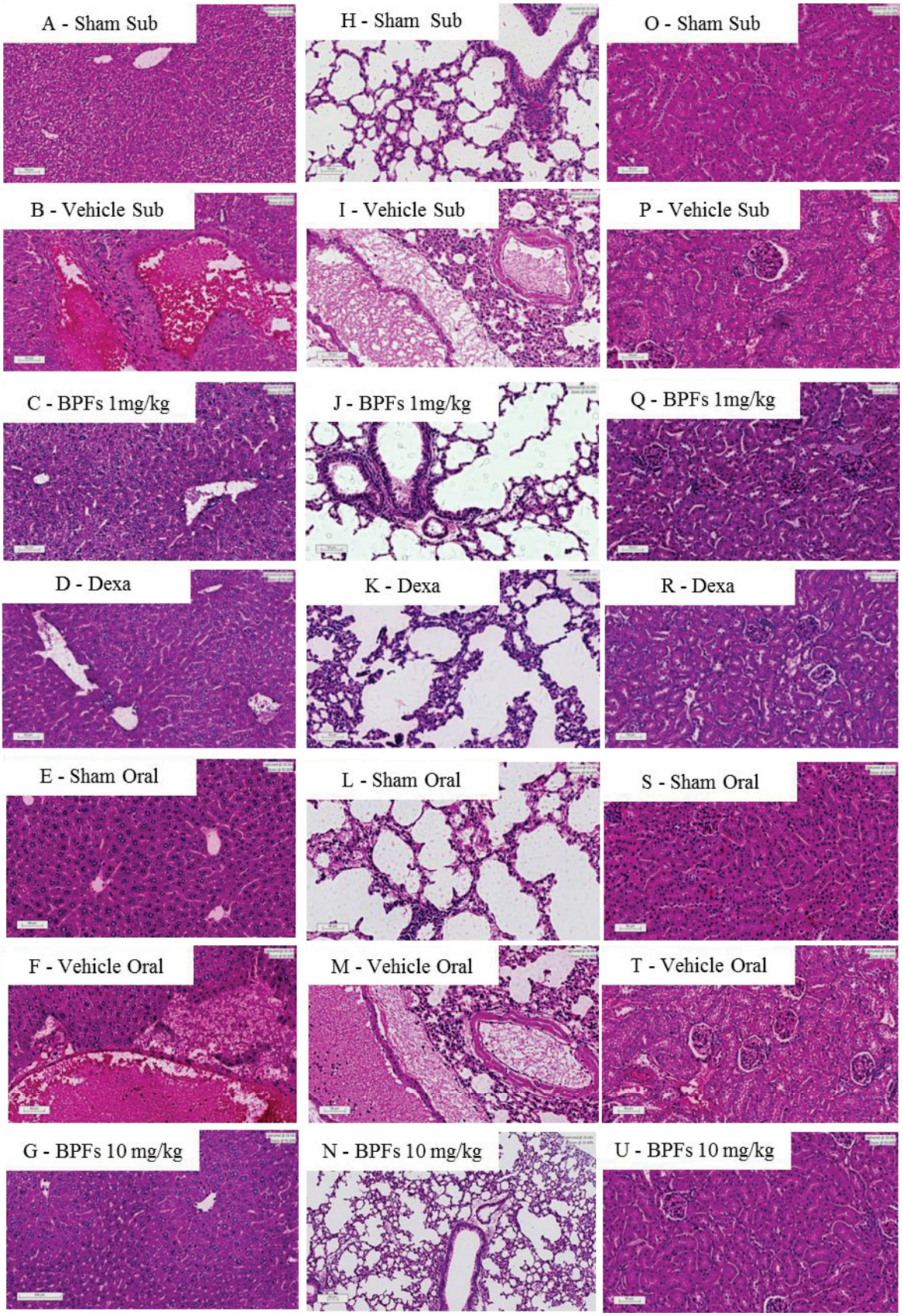

FIGURE 6. Histopathological changes induced by CLP in the liver tissue (6 h after CLP) and the effects of BFPs. Sections were prepared and stained with haematoxylin and eosin (20 magnification). Subcutaneous treatment: In liver - (A) sham, (B) vehicle, (C) BFPs-treatment

(1 mg/kg) and (D) dexamethasone. In lungs - (H) sham, (I) vehicle, (J) BFPs-treatment (1 mg/kg) (K) dexamethasone. In kidneys - (O) sham, (P) vehicle, (Q) BFPs-treatment (1 mg/kg) (R) dexamethasone. Treatment oral: In liver - (E) sham, (F) vehicle and (G) BFPs-treatment (10 mg/kg). In lungs - (L) sham, (M) vehicle and (N) BFPs-treatment (10 mg/kg). In kidneys - (S) sham, (T) vehicle and (U) BFPs-treatment (10 mg/kg).

4. CONCLUSIONS

This study demonstrated the *in vitro* and *in vivo* dose-dependent effect of BFPs on decreasing inflammatory cytokines. Previous studies indicated a relationship between polysaccharides commonly found in plants with low cytotoxicity and anti-inflammatory activities. Thus, they have been shown to be important components regarding maintenance of human immune health. The results of this study showed that BFPs was not cytotoxic at the concentrations evaluated and the protective effects exerted by BFPs were attributable to the reduction of inflammatory cytokines. Finally, the conducted experiments confirmed that the treatment with BFPs reduced the organ dysfunction/injury in a CLP model.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

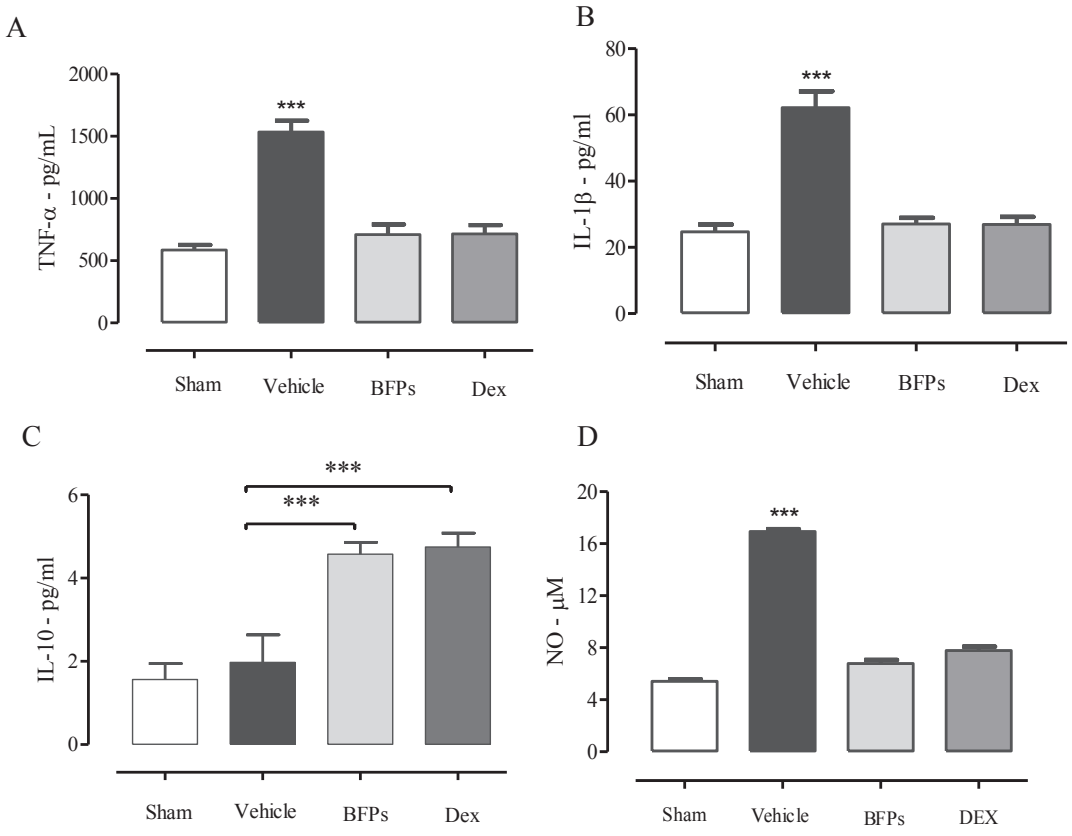

FIGURE S1. (A) TNF-α concentration in blood serum was determined by ELISA using standard curve. (B) IL-1β concentration in blood serum was determined by ELISA using standard curve. (C) IL-10 concentration in blood serum was determined by ELISA using standard curve.(D) NO concentration in blood serum was determined by Griess metod using standard curve.

All data are presented as means ± SD from three sets of independent experiments. *p< 0.05 represent significant difference with mice treated with saline solution alone (positive control) compared with BFPs-treatment and Sham (negative control). +p < 0.05 represent significant difference beetwen of fractions.

Table S1. Monosaccharide composition of fractions obtained from blackberry fruit.

	Monosaccharidecomposition (%)						
Fraction	**(%)**	**Rha**	**Ara**	**Xyl**	**Gal**	**Glc**	**Gal*A***
BFPs	77.6	3.6	16.3	0.9	11.3	8.7	60.1
BFPs *	77.6	3.2	15.6	1.4	13.5	8.8	57.3**

Notes: Rha (Rhamnose); Ara (Arabinose); Xyl (Xylose)* Analysis for CG Uronic acids, ** Determined using colorimetric method Filisetti-Cozzi (1991).

Table S2. The NMR signals of fractions obtained from blackberry fruit.

Resídues	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
Type II Arabinogalactan						
→3,6)-β-D-Gal*p*-(1→	103.9/4.49	74.9/3.85	80.4/3.70	-	75.9/3.61	68.1/3.85;3.77
→5)-α-L-Ara*f*-(1→	108.1/5.14	-	-	-	-	-
→3)-α-L-Ara*f*-(1→	108.7/5.05	-	81.1/4.11	-	-	-
α-L-Ara*f*Terminals	109.9/5.20	-	-	-	74.7/3.84	62.1/3.78;3.70
Type I Ramnogalacturanan						
→2)-α-L-Rha*p*-(1→	99.5/5.19	77.0/4.01	-	-	-	17.9/1.24
→4)-α-D-GalA*p*-(1→	100.7/5.06	69.1/3.71	69.0/3.96	79.3/4.39	71.6/4.67	* 175.6
Homogalacturanan						
→4)-α-D-GalA*p*-(1→	100.7/5.06	69.1/3.71	69.0/3.96	79.3/4.39	71.6/4.67	* 175.6
→4)-α-D-GalA*p*-(1→	100.9/4.9	-	-	79.6/4.44	71.0/5.01 $-CO_2-CH_3$	-

References: Nascimento *et al*., 2013; Delgobo *et al*., 1998, 1999; Renard *et al*., 1998; Carpita e Gibeaut, 1993.

Table S3. Profile of partially *O*-methylated alditol acetates of BFPs, BFPs-CR obtained on methylation analysis.

***O*Me-alditol acetate**	**BFPs**	**BFPs-CR**	**Structure**
2,3-Me_2-Ara	26.8	13.1	→5)-Ara*f*-(1→
2,5-Me_2-Ara	8.1	3.9	→3)-Ara*f*-(1→
2,3,5-Me_3-Ara	14.4	7.1	Ara*f*-(1→
2,3,4-Me_3-Rha	3.7	1.8	Rha*p*-(1→
3-Me-Rha	8.2	4.1	→2,4-Rha*p*-(1→
2,3,4,6-Me_4-Gal	7.3	3.5	Gal*p*-(1→
2,3,4-Me_3-Gal	7.3	7.7	→6)-Gal*p*-(1→
2,3-Me_2-Gal	7.6	3.7	→4,6)-Gal*p*-(1→
2,3,6-Me_3-Gal	10.4	51.8	→4)-Gal*p*-(1→
2,3,6-Me_3-Glc	5.9	2.9	→4)-Glc*p*-(1→

Appendix I

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

Nº 1018

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.149858/2016-79

APROVADO/APPROVAL: 08/11/2016 – R.O. 10/2016

TÍTULO: Caracterização estrutural e estudo farmacológico de polissacarídeos extraídos dos vinhos de uva, fermentados de amora e da fruta amora preta.

TITLE: Structural characterization and pharmacological study of polysaccharides extracted from grape and blackberry wines and blackberry fruit.

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DEPARTAMENTO/DEPARTMENT: Bioquímica e Biologia Molecular

Profa. Dra. Ana Vitória Fischer da Silva
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Ministério da Educação
UNIVERSIDADE FEDERAL DO PARANÁ
Setor de Ciências Biológicas
Comissão de Ética no Uso de Animais
(CEUA)

Nº 1099

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.189085/2017-44

APROVADO/APPROVAL: 19/09/2017 – R.O. 08/2017

TÍTULO: Caracterização estrutural e estudo farmacológico de polissacarídeos extraídos da amora preta e da planta *Casearia sylvestris*.

TITLE: Structural characterization and pharmacological study of polysaccharides extracted from blackberry fruit and plant *Casearia sylvestris*.

AUTORES/AUTHORS: Guilherme Lanzi Sassaki, Elaine Caroline Kiatkoski, Adriana Rute Cordeiro Caillot, Iglesias de Lacerda Bezerra.

DEPARTAMENTO/DEPARTMENT: Bioquímica e Biologia Molecular

Profa. Dra. Katya Naliwaiko
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CAPÍTULO III

Neste capítulo encontra-se o manuscrito intitulado "Structural characterization of blackberry wine polysaccharides and immunomodulatory effects on LPS-activated RAW 264.7 macrophages". Publicado na revista **Food Chemistry***v.257*, p. 143-149, 2018.

Structural characterization of blackberry wine polysaccharides and immunomodulatory effects on LPS-activated RAW 264.7 macrophages

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Abstract

Three polysaccharide fractions were isolated from blackberry wine. The crude extract BWPs was obtained with ethanol precipitation and freeze-thawing process, it was then submitted to Fehling treatment, giving soluble BWPFs and insoluble BWPFp fractions. These fractions were characterized by Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR). Major polysaccharides were identified for each fraction: mannan, type II arabinogalactan and type I rhamnogalacturonan for BWPs, a mannan formed by a major chain of α-Man*p*(1→6)-linked units, *O*-2 substituted with *α*-D-Man*p*(1→2)-linked side chains for BWPFp and a AG II formed by a major chain of *β*-D-Gal*p*(1→3)-linked, substituted at *O*-6 by side chains of the *β*-D-Gal*p*(1→6)-linked, which then are substituted at *O*-3 by non-reducing units of *α*-L-Ara*f*and a RG I, formed by [→4)-*α*-D-Gal*p*A-(1→2)-*α*-L-Rha*p*-(1→]$_n$ for BWPFs. Anti-inflammatory effects of polysaccharide fractions were evaluated in RAW 264.7 cells. Fractions markedly reduced nitric oxide (NO) and pro-inflammatory cytokine production (TNF-α and IL-1β) in LPS-treated cells.

Keywords: Blackberry wine; Polysaccharides; Anti-inflammatory activity; NMR; GC-MS.

1. Introduction

Vinification technologies are among the oldest known to humans. In recent years, non grape fruit wines have caught the attention of alcoholic beverages consumers due to scientific evidence relating health benefits and habitual consumption of a wide variety of fruits and their products (Vasantha Rupasinghe, Joshi, Smith, & Parmar, 2017). These benefits have added value to these wines and consequently increased economic importance of this beverage segment. Emerging fruit wines include those of blueberries, cherries, strawberries, and blackberries. Blackberry wine is produced by yeast fermentation of natural sugars present in blackberry juice. It is recognized as a natural source of essential minerals and bioactive phytochemicals that can play an important role in health promotion and disease prevention (Petravić-Tominac, et al., 2013). Information regarding blackberry wines is very scarce. Previous studies investigate mainly the mineral composition, phenolic compounds and polyphenols (Amidzic Klaric, Klarić, Velić, & Vedrina Dragojevic, 2011 and Amidzic Klaric, Klarić, & Mornar, 2011). In addition to promoting beneficial effects as antioxidants, phenolic compounds can act as inhibitors of carbohydrate-utilizing enzymes and as potential inhibitors of inflammation (Arozarena, et al., 2012; Johnson, Mejia, Fan, Lila, & Yousef, 2013). Health benefits and sensory properties attributed to fermented berry products are mainly attributed to high amounts of polyphenolic compounds present in this fruit (Lim, Hwang, & Shin, 2012). Also, phenolic compounds from the beverage had an impact to attenuate development of obesity and fasting blood glucose in C57BL/6J mice (Johnson, Wallig, Luna Vital, & Mejia, 2016). In addition to the compounds already described in the literature, blackberry wine has many other constituents that have not been structurally elucidated and can also be responsible for biological activities, such as polysaccharides.

Polysaccharides present in blackberry wine are derived from yeast cell walls used in the fermentation process or blackberry fruits (Boulet, Williams, & Doco, 2007). Polysaccharides from different sources have shown therapeutic effects including anti-tumor, anti-ulcer, anti-complementary, anti-coagulant, hypoglycemic agents and anti-inflammatory activities (Jin, Liu, Zhong, Sun, & Zhang, 2017;Leivas, Nascimento, Barros, Santos, Iacomini & Cordeiro, 2016; Nergard, et al., 2005; Ochoa, Iacomini, Sassaki, & Cipriani, 2017; Wang et al., 2017). Macrophages are immune cells implicated in the initiation of inflammatory responses, secreting several pro-inflammatory mediators, including nitric oxide (NO) and pro-inflammatory cytokines, like tumor necrosis factor (TNF-α) and interleukin 1-β (IL-1β) (Lee & Park 2015). Therefore, this study reports fractionation and structural characterization of

blackberry wine polysaccharides and evaluate their inhibitory effects on NO, IL-1β and TNF-α production in Lipopolysaccharide (LPS)-induced RAW 264.7 macrophages.

2. Materials and methods

2.1. Polysaccharide source

The blackberry wine (750 mL) was obtained after fermentation of mature blackberry fruits (*Rubus fruticosus*) in October of 2010 and it was produced by Adega Porto Brazos (Ponta Grossa, Paraná, Brazil).

2.2. Polysaccharide extraction and purification

After concentration under reduced pressure, blackberry polysaccharides were precipitated by addition of cold EtOH (3x vol.) and stand at 4 °C overnight. After centrifugation (8.000 rpm at 4 °C, 20 min) the precipitate was dissolved in water and dialyzed (6 - 8 kDa cut-off membrane) against distilled water for 72 h, originating polysaccharide fraction blackberry wine polysaccharide (BWP). This fraction was again solubilized in water (150 mL) and submitted to freeze-thawing process thrice (Gorin & Iacomini, 1984), which resulted in water soluble, blackberry wine polysaccharide-soluble (BWPs) and an insoluble, blackberry wine polysaccharide-insoluble (BWPi) fraction. The former was submitted to Fehling treatment, resulting in two fractions: supernatant, blackberry wine polysaccharide-Fehling supernatant (BWPFs) and precipitate, blackberry wine polysaccharide-Fehling precipitate (BWPFp).

2.3. Homogeneity and molecular weight analysis

BWPs, BWPFs and BWPFp samples homogeneity and isolated BWPFp sample average molar mass (Mw) were determined by high-performance size-exclusion chromatography (HPSEC) using refractive index and multi-angle laser light scattering detectors. Polysaccharide permeation was performed in serial array of columns with exclusion sizes of 7×10^6 (Ultrahydrogel 2000), 4×10^5 (Ultrahydrogel 500), 8×10^4 (Ultrahydrogel 250) and 5×10^3 (Ultrahydrogel 120) Daltons using 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min as eluent. Samples were filtered through a 0.22 μm membrane (Millipore) and injected (100 μL, loop) at a concentration of 1 mg/mL. Specific refractive

index increment (dn/dc) was determined for polysaccharide purified BWPFp and data were analysed and processed in ASTRA software (Wyatt Technologies). Final graphics were drawn using GraphPad Prism software version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

2.4. Monosaccharide analysis

Polysaccharides (~5 mg) were hydrolyzed using 2 M trifluoroacetic acid (TFA) (1 mL) at 100 ºC for 12 hours and then evaporated to dryness. Samples were then solubilized in 400 µl D_2O for NMR analysis (Sassaki, et al., 2014).

After NMR analysis, the solution was reduced with $NaBH_4$ (2 mg) at room temperature overnight, neutralized after addition of acetic acid (500 µL) and evaporated to dryness. Resulting boric acid was removed as trimethyl borate by co-evaporation with MeOH in a nitrogen stream. Resulting alditols were converted to acetyl esters with Ac_2O-pyridine (1:1 v/v, 0.5 mL) at 100 °C for 1 h and extracted with $CHCl_3$. Organic phase was washed thrice with 5% $CuSO_4$, once with distilled water and dried at room temperature. Acetylated samples were analyzed by GC-MS (Varian Saturn 3800 Gas Chromatograph coupled to a Varian 4000 ion trap mass spectrometer) using a DB-225-MS capillary column (30 m x 0.25 mm) with injector at 250 °C and oven programed from 50 °C (initial) to 230 °C at 40 °C/min, He being the carrier gas (1 mL/min) and dissolved in acetone(Sassaki, Souza, Serrato, Cipriani, Gorin, & Iacomini, 2008).Uronic acid contents of polysaccharides were determined by the colorimetric *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991), using galacturonic acid standard.

2.5. Methylation analysis

Polysaccharides BWPs, BWPFs and BWPFp were methylated using NaOH/DMSO-MeI as described by Ciucanu & Kerek (1984). Per-*O*-methylated polysaccharides were submitted to methanolysis with 3% HCl-MeOH (80 °C, 2 h) followed by hydrolysis with formic acid (45%, 12 h) (Biermann, 1988). The latter was dried and reduced with $NaBD_4$ and acetylation was performed as described above. The partially *O*-methylated alditol acetates (PMAA) were examined by GC-MS (Varian Saturn 3800 Gas Chromatograph coupled to a Varian 4000 ion trap mass spectrometer), using a DB-225-MS capillary column (30 m x 0.25 mm), injector 250 °C, oven start at 50 °C (held 1 minute) to 185 °C (40 °C/min) held 10

minutes and then to 210 °C (40 °C/min) held for 15 minutes, He being the carrier gas (1 mL/min). Chromatograms peaks were identified by their typical electron impact fragmentation profiles and retention times (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). BWPFs was also submitted to carboxy-reduction according to Taylor & Conrad (1972), resulting BWPFs-CR sample.

2.6. NMR spectroscopy

Polysaccharides (10 mg) were dissolved in 0.5 mL D_2O with internal standard 10 µL 3-trimethylsilyl-$^2H_4$-propionic acid sodium salt (TMSP) 1%. Nuclear magnetic resonance analyses were performed using a Bruker Avance*III* NMR spectrometer operating at 14.1 Tesla (600.13 MHz for ^1H) equipped with an inverse 5 mm probe head (QXI) at 303 K. 1D ^1H and ^{13}C NMR were collected after 90° pulse calibration for each sample. ^1H and ^{13}C chemical shifts were determined by 2D NMR experiments. 2D ^1H-^{13}C multiplicity-edited HSQC was performed by correlation via double inept transfer with decoupling during acquisition using sensitivity improvement trim pulses as compiled in the pulse program hsqcedetgpsisp2.2 using 6993 Hz (^1H) and 24900 Hz (^{13}C) widths and a recycle delay of 1.080 s. 2D correlation maps were recorded using quadrature detection in the indirect dimension and 24 scans per series of 1024 x 320 W data points, with zero filling in F1 (2048), prior to Fourier transformation (Sassaki, et al., 2014).

2.7. Cell culture and reagents

Murine macrophage cells (RAW 264.7) were grown in Dulbecco´s modified Eagle´s medium (DMEM) with 4.5 $g.L^{-1}$ glucose supplemented with 10% fetal bovine serum and 20 mM sodium bicarbonate (Cultilab, Campinas, SP, Brazil). All cultures were performed in culture plates (Falcon BD, San Jose, CA, USA). Lipopolysaccharide from *Escherichia coli* O55:B5 and the other reagents were purchased from Sigma (St Louis, MO, USA).

2.8. Cell viability assay

Cell viability was determined by MTT (3- (4,5-Dimethylthiazol-2-yl) -2,5-bromo diphenyl tetrazolium) method (Mosmann, 1983), where reduction of crystal tetrazolium forming through mitochondrial enzymes is possible only in viable cells. Cells were plated in

24 well plates (4.8×10^5 cells/well) and treated with different concentrations of BWPs, BWPFs and BWPFp polysaccharides (0.1, 1.0, 10 and 100 μg/mL), dissolved in DMEM. After 24 hours, 350 μL/well of the 5 mg/mL MTT solution was added. After 4 h of incubation, cell supernatant was removed and 500 μL of DMSO (Dimethylsufoxide) were added for cell lysis and crystal solubilization. Absorbance then was recorded at 570 nm wavelength.

2.9. Measurement of cytokine and NO production

RAW 264.7 cells (4.8×10^5 cells/100 μL) were plated, induced with LPS previously dissolved in DMEM (2 μg/mL), then 1 h later cells were treated with BWPs, BWPFs and BWPFp fractions (0.1, 1.0, 10 and 100 μg/mL). After 24 h, supernatant was collected and submitted to cytokine and NO assays. Cytokines IL-1β and TNF-α levels were assessed using an immunoenzymatic assay (ELISA) kit (eBioscience), according to the manufacturer instructions. Samples were analyzed in triplicate and optical density was determined at 450 nm. In order to determine total NO concentration, Griess reagent (50 μL) was added to 50 μL of collected supernatant and absorbance at 545 nm wavelength was recorded. Griess method (Griess, 1879) is based on the determination of nitrite ions obtained by quantitative reduction (greater than 90%) of nitrate ions present in the sample. Therefore, the sum of nitrite and nitrate ion concentrations is measured. Nitrate concentration is deduced by subtracting the original nitrite from sample of the total nitrite concentration (sum of these NO_2 with reduced NO_3). A standard solution of $NaNO_3$ was used for calibration.

2.10. Statistical analysis

Data are expressed as mean ± S.D. Statistical analyses were performed using GraphPad Prisma® software version 5.0 and one-way analysis of variance ANOVA following by Tukey's test. Significant differences were considered at $p < 0.05$.

3. Results and discussion

3.1. Polysaccharide isolation and chemical analysis

Blackberry wine was concentrated and precipitated with excess ethanol (3:1 v/v), resulting in fraction containing polysaccharides which was named BWP (220 mg 0.04% v/w).

BWP fraction was submitted to freezing-thawing, resulting in cold water-insoluble (BWPi) and a cold water-soluble (BWPs) fractions (Fig. 1).

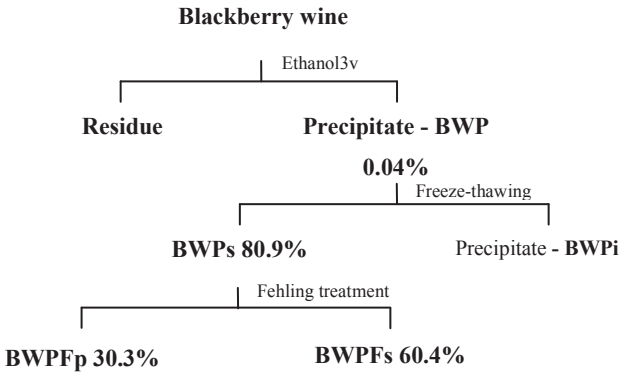

Figure1. Scheme of extraction the polysaccharides from blackberry wine.

Monosaccharide composition of BWPs fraction showed mannose, galactose, galacturonic acid, glucose, arabinose, rhamnose, xylose in a 12:7:5:4:4:2:1 ratio (w/w) (Table S1, Supporting Information). Fraction BWPs was submitted to NMR analysis. 2D ^{1}H-^{13}C multiplicity-edited HSQC NMR correlation maps are shown on Figure 2 (Table S2, Supporting Information), suggesting the presence of a pectic structure, with signals in the region at δ 103.5/4.45 (C-1/H-1), 71.0/3.50 (C-2/H-2), 80.4/3.70 (C-3/H-3 substituted), 68.4/4.12 (C-4/H-4), 75.2/3.69 (C-5/H-5) and 69.4/3.92-4.04 (C-6/H-6 substituted; negative phase), typical of →3,6)-*β*-D-Gal*p*-(1→, besides δ 107.3/5.20 (C-1/H-1) 82.8/4.27 (C-2/H-2) of the →2)-*α*-L-Ara*f*-(1→ and δ 107.5/5.06 (C-1/H-1), 81.1/4.11 (C-3/H-3) of the →3)-*α*-L-Ara*f*-(1→ and signals of the *α*-L-Ara*f*terminal atδ 109.3/5.24 (C-1/H-1), 74.7/3.67 (C-5/H-5), and 65.6/3.74;3.97 (C-6/H-6) typical signals indicating presence of type II arabinogalactan (AG II) (Carpita& Gibeaut, 1993; Delgobo, Gorin, Tischer, & Iacomini, 1999; Delgobo, Gorin, Jones, & Iacomini, 1998; Stipp, Bezerra, Corso, Reis Livero, Lomba, Caillot, et al., 2017). Furthermore, it was possible to observe the presence of typical signals of a type I rhamnogalacturonan (RG I), with main signals at δ 99.3/4.99 (C-1/H-1), 68.2/3.82 (C-2/H-2), 68.5/3.89 (C-3/H-3), 80.0/4.37 (C-4/H-4), 77.4/4.41 (C-5/H-5), typical of →4)-*α*-D-Gal*p*A-(1→ non-methyl esterified,also containing signals at δ 70.9/4.69 (C-5/H-5) and 53.0/3.84 (-CO_2-CH_3) typical of (1→4)-linked 6-*O*Me-*α*-D-Gal*p*A units, these later belonging to the homogalacturonan region and/or rhamnogalacturonan II (RG II) (Ovodova, et al., 2009; Popov, et al., 2011; Renard, Lahaye, Mutter, Voragen, & Thibault, 1997).Presence of C-1/H-1 and C-6/H-6 of Rha*p* units (1→2)-linked was shown by resonances at δ 98.5/5.24 and

16.1/1.29, respectively (Renard, Lahaye, Mutter, Voragen, & Thibault, 1997). These results are in agreement with methylation analysis, confirming that the polysaccharide is a rhamnogalacturonan formed by large sequences of →4)-*α*-D-Gal*p*A-(1→ units, interspersed with *α*-L-Rha*p* units (Delgobo, Gorin, Tischer, & Iacomini, 1999; Delgobo, Gorin, Jones, & Iacomini, 1998). Since wine is fermented by yeasts, residual exopolysaccharides from this microorganism may be found. 2D ^{1}H-^{13}C multiplicity-edited HSQC NMR correlation maps showed signals from →2,6)-*α*-D-Man*p*-(1→ backbone, which were observed at C-1/H-1 region in δ 98.1/5.09, 100.5/5.28 corresponding to units of →2)-*α*-D-Man*p*-(1→ 102.1/5.03 and *α*-D-Man*p*-(1→ with signals at δ 103.1/5.13 corresponding to C-1/H-1 units of *α*-D-Man*p* (1→3) (Vinogradov, Petersen, & Bock, 1998).

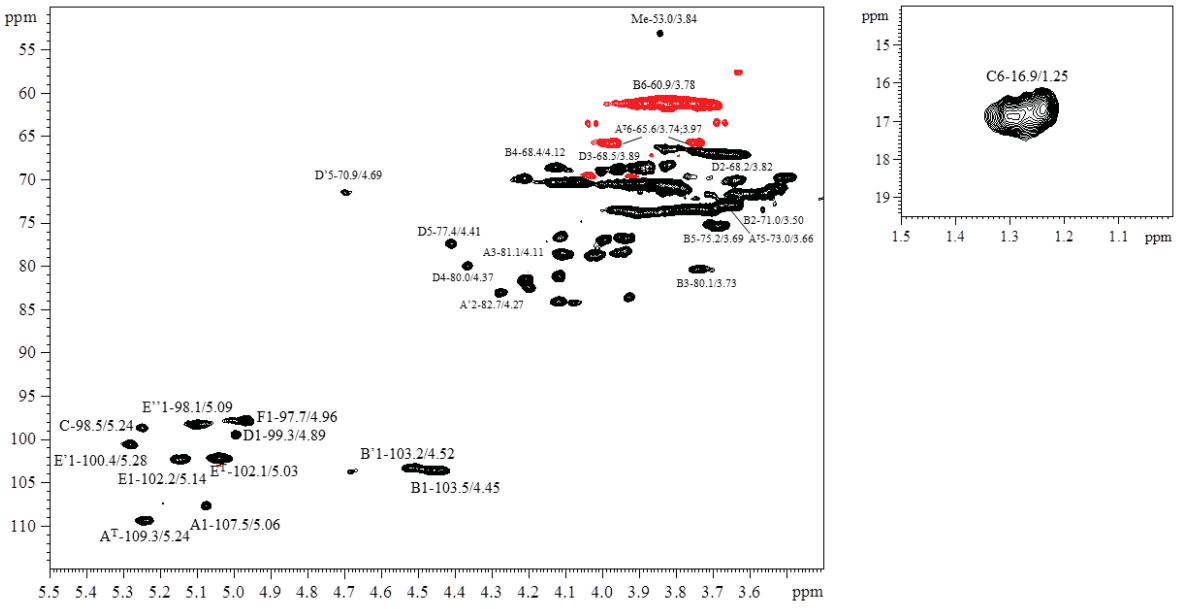

Figure 2. 2D multiplicity-edited ^{1}H-^{13}C HSQC NMR correlation map of fraction BWPs,acquired at 30 °C using D_2O as solvent. A (*α*-L-Ara*f*); B (*β*-D-Gal*p*); C (*α*-L-Rha*p*); D (*α*-D-Gal*p*A); E (*α*-D-Man*p*); F (*α*-D-Glc*p*).

BWPs fraction was also submitted to Fehling treatment (Jones & Stoodley, 1965), resulting in a supernatant (BWPFs) and a precipitate (BWPFp) fractions (Fig. 1). BWPFs fraction showed galactose, arabinose, galacturonic acid, rhamnose and glucose at a 9:3:2:1:1 ratio (w/w). HPSEC-MALLS analysis showed a heterogenic profile. 2D ^{1}H-^{13}C multiplicity-edited HSQC NMR correlation map of BWPFs fraction (Fig. 3) (Table S2, Supporting Information) suggests the presence of type II arabinogalactan and type I rhamnogalacturonan, which are also present in the crude BWPs fraction (Fig.2). Methylation analysis was

performed with BWPFs fraction and its carboxy-reduced form, namely BWPFs-CR (Table S3, Supporting Information). Carboxy-reduction was required since under the conditions of semi-methylated alditols, units of uronic acids are not possible to be analyzed. Methylation analysis of BWPFs fraction showed 13.7% of non-reducing terminals of Ara*f*, as evidenced by the presence of methylated derivative 2,3,5-Me_3-Ara*f*. Gal*p* non-reducing terminals are also present, due to the presence of 2,3,4,6-Me_4-Gal*p* derivative (6.6%). Other derivatives found in BWPFs were 2,4,6-Me_3-Gal*p* (5.8%), 2,3,4-Me_3-Gal*p* (11.7%) and 2,4-Me_2-Gal*p*(33.7%), indicating that the main chain of polysaccharide present in BWPFs is composed by Gal*p* 3-*O*-, 6-*O*- and 3,6-di-*O*-substituted Gal*p* units, the non-reducing terminals of Ara*f* probably are linked to the main chain at positions *O*-3 and *O*-6 of some Gal*p* units frequently observed for type II arabinogalactan (Carpita& Gibeaut, 1993). Comparing the results of methylation analysis of native sample BWPFs and BWPFs-CR, it is possible to observe an increase in the proportion of 2,3,6-Me_3-Gal*p* derivative in 7.03% after the carboxy-reduction procedure, indicating the presence of residues of Gal*p*A 1→4 linked. In addition, the presence of derivative 3-Me-Rha*p* (6.5%) indicate that Rha*p* residue 2,4-di-*O*-substituted derivative is present. These type I rhamnogalacturonans are formed by repetition of disaccharide unit [→4)-*α*-D-Gal*p*A-(1→2)-*α*-L-Rha*p*-(1→]$_n$, often having the C-4 of Rha*p* units substituted by arabinans, galactans and/or arabinogalactans (Carpita & Gibeaut, 1993).

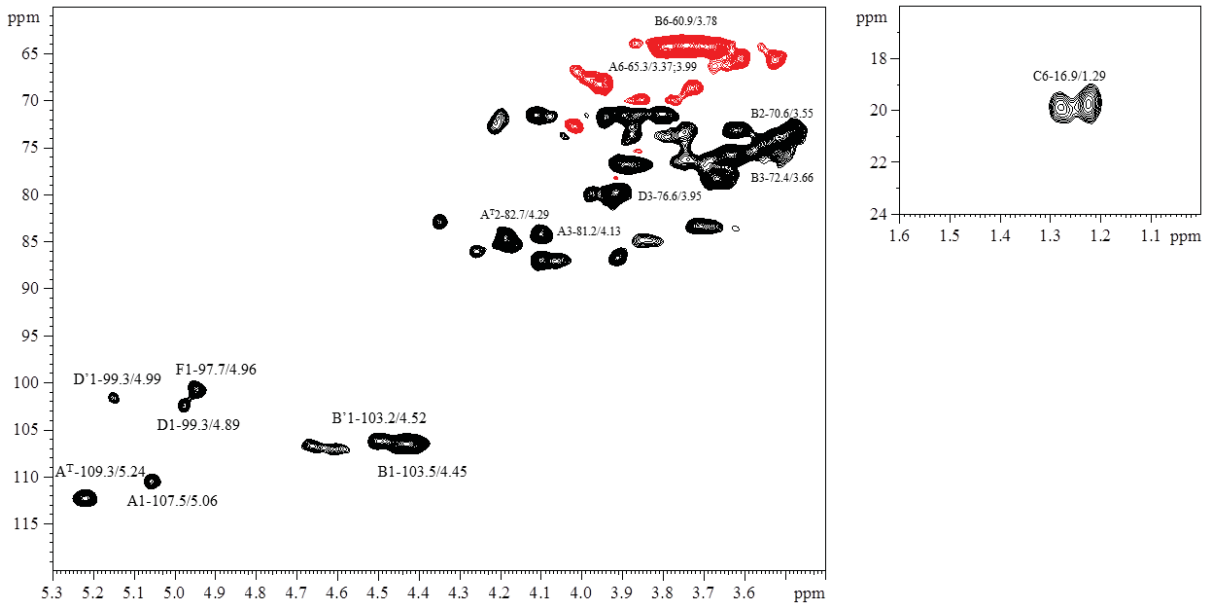

Figure 3. 2D multiplicity-edited ^{1}H-^{13}C HSQC NMR correlation map of fraction BWPFs, acquired at 30 °C using D_2O as solvent. A (*α*-L-Ara*f*); B (*β*-D-Gal*p*); C (*α*-L-Rha*p*); D (*α*-D-Gal*p*A); F (*α*-D-Glc*p*).

BWPFp fraction was analyzed by HPSEC-MALLS and showed a homogeneous profile with a Mw 122.700 g/mol (dn/dc = 0.135) (Fig. 4-B). Monosaccharide composition showed exclusively mannose. 2D ^{1}H-^{13}C multiplicity-edited HSQC of BWPFp (Fig. 4-A) have signals in anomeric region at δ 98.1/5.09 corresponding to C-1/H-1 of units →2,6)-*α*-D-Man*p*-(1→ beyond δ 78.6/4.01 (C-2/H-2), 65.7/3.66;3.99 (C-6/H-6) and 100.5/5.28 (C-1/H-1), 78.4/4.09 (C-2/H-2), 66.4/3.80 (C-4/H-4), 61.0/3.74;3.87 (C-6/H-6) of units →2)-*α*-D-Man*p*-(1→ and signals corresponding to the C-1/H-1 of units →3)-*α*-D-Man*p*-(1→ 102.2/5.13, 70.5/4.05 (C-2/H-2), 70.3/3.87 (C-3/H-3) and non-reducing terminal *α*-D-Man*p*-(1→ with δ 102.1/5.03 (C-1/H-1), 69.7/4.20 (C-2/H-2), 78.0/3.94 (C-3/H-3) and 71.2/3.7 (C-5/H-5), suggesting a mannan with a main chain of *α*-Man*p*(1→6) linked (Vinogradov, Petersen, & Bock, 1998). Methylation analysis corroborated the results above, showing that about 32.2% of BWPFp fraction are non-reducing terminals as indicated by the presence of 2,3,4,6-Me$_4$-Man derivative. Other derivatives found were 2,3,4-Me$_3$-Man (3.2%), 3,4-Me$_2$-Man (32.1%), 2,4,6-Me$_3$-Man (10.7%) and 3,4,6-Me$_3$-Man (21.5%), proving that the main polysaccharide is a mannan with a main chain of *α*-D-Man*p* (1→6)-linked, *O*-2 substituted *α*-D-Man*p* (1→2)-linked side chains. One of the side chains being substituted at *O*-3 by *α*-D-Man*p* (1→3)-linked ends. Based on these results, mannan structure was established (Fig. 5) and in order to discuss structural details, two digit numbering of sugar residues in mannan structure was proposed. The first digit indicates side chain number and the second refers to sugar position in oligosaccharide counting from the reducing end.

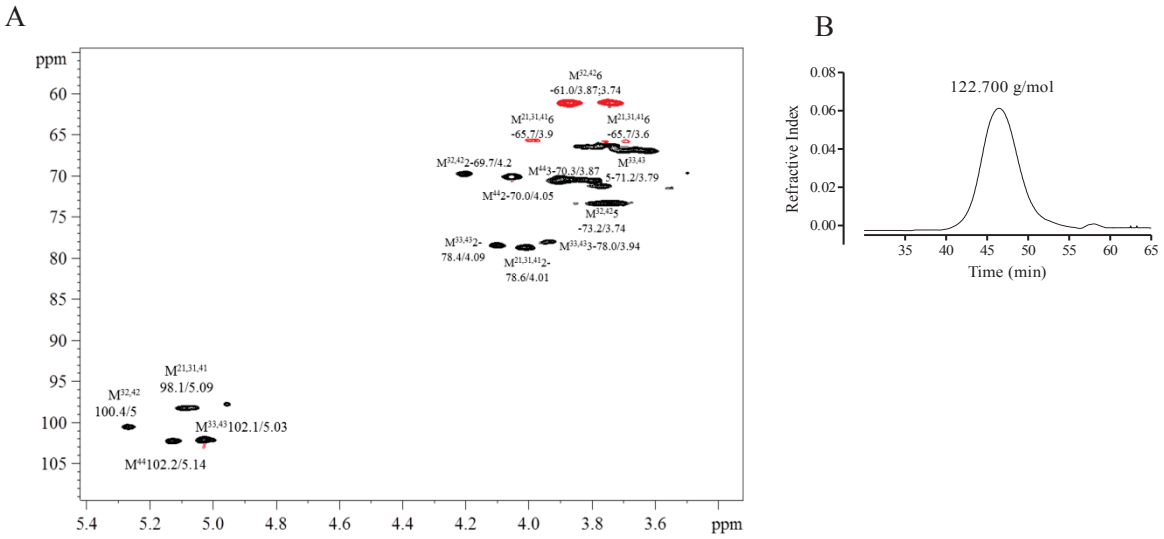

Figure 4. (A) 2D multiplicity-edited ^{1}H-^{13}C HSQC NMR correlation map of fraction BWPFp, acquired at 30 °C using D_2O as solvent. M^{33,43}(*α*-D-Man*p*-(1→), M^{44}(→3)-*α*-D-Man*p*-(1→),

$M^{32,42}$(→2)-α-D-Man*p*-(1→), $M^{21,31,41}$(→2,6)-α-D-Man*p*-(1→) (Vinogradov, Petersen, & Bock, 1998)and (B) elution profiles of BWPFp.

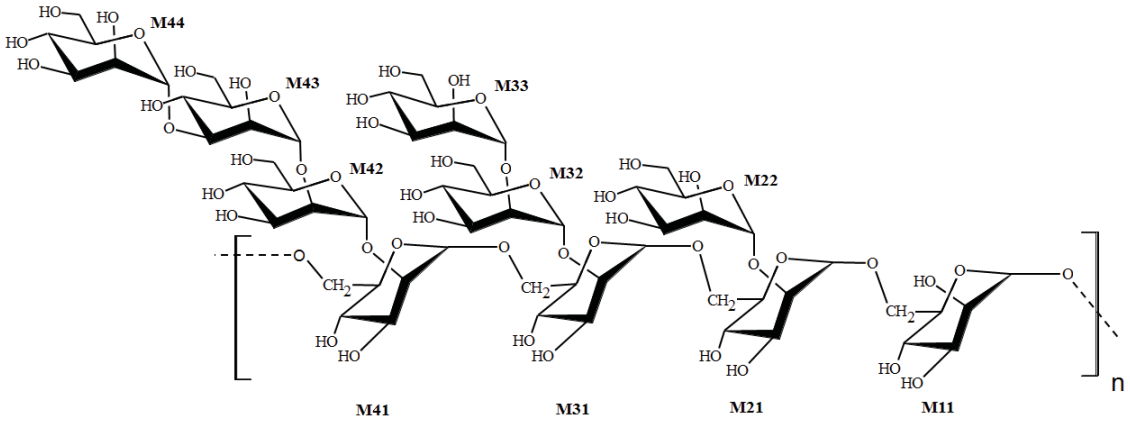

Figure 5. Proposed structure for mannan. $M^{33,43}$(α-D-Man*p*-(1→), M^{44}(→3)-α-D-Man*p*-(1→), $M^{32,42}$(→2)-α-D-Man*p*-(1→), $M^{21,31,41}$(→2,6)-α-D-Man*p*-(1→).

3.2. Anti-inflammatory activity

Inflammation is an adaptive response that is triggered by a variety noxious stimuli and conditions, such as infection and tissue injury (Medzhitov, 2008). Macrophages play an important role in initiation, maintenance, host defense, promotion and resolution of inflammation (Koh & DiPietro, 2011). They are activated by stimuli such as bacterial lipopolysaccharides (LPS), that stimulates immune responses by interacting with the membrane receptor CD14 to induce the generation of cytokines (Meng & Lowell, 1997). In this study, four concentrations (0.1, 1.0, 10 and 100 μg/mL) of BWPs, BWPFs and BWPFp fractions were investigated for cell viability using the MTT assay. These concentrations showed cell viability above 92%, thus allowing for all fractions to being used for further investigations (Fig. 6A). Once macrophages are activated in the presence of LPS, a naturally high content of pro-inflammatory molecules, such as cytokines and NO, are produced. Fractions effects on NO production are shown in Fig. 6B. RAW 264.7 cells were treated with different BWPs, BWPFs and BWPFp concentrations (0.1, 1.0, 10, and 100 μg/mL) and LPS (2 μg/mL). LPS-induced NO production was significantly decreased by all fractions in a dose-dependent manner ($p < 0.05$): BWPs (44-64%), BWPFs (45-54%), BWPFp (45-59%). Pro-inflammatory cytokines, just like NO, are important mediators of inflammatory responses. TNF-α, and IL-1β cytokines up-regulate inflammatory responses and stimulate the production

of acute phase reactants. These cytokines play an important role in inflammatory process. Therefore, reducing levels of these cytokines might be an effective strategy to treat inflammatory diseases (Dinarello, 2010). Consequently, inhibition of pro-inflammatory cytokines induced by LPS stimulation in RAW 264.7 macrophages were investigated. Results indicate that BWPs, BWPFs and BWPFp fractions markedly reduce inflammatory cytokines (TNF-α and IL-1β) in LPS-treated murine RAW 264.7 macrophages. It suggests that these fractions could possibly modulate inflammation. Pro-inflammatory cytokines TNF-α and IL-1β significantly increased in inflammation group control. Polysaccharides BWPs, BWPFs and BWPFp fractions were capable to significantly reduce TNF-α production by macrophages, achieving 55-56%, 51-62% and 43-55% of inhibition, respectively (Fig. 6C). IL-1β production significantly decreased in BWPs (34-62%), BWPFs (36-54%) and BWPFp (33-56%) presence, being the inhibitory effect concentration-dependent in all treatments groups (Fig. 6D). We performed assays regarding the phenol content of the polysaccharides sample using the Folin Ciocalteau method, and found that on the 1000, 100, 10 and 1 μg/mL polysaccharides solutions, the polyphenols content, measured as galic acid equivalents, were of 9, 5, 1.3 and ~ 0 μg/mL respectively, thus The data in the literature regarding macrophage activation by plant/fruit extract containing polyphenols (Hooshmand, Kumar, Zhang, Johnson, Chai & Arjmandi, 2015) suggest that only at concentrations between 100 - 1000 μg/mL these compounds could significantly affect anti-inflammatory assays using LPS elicited RAW 264.7 cells. Thus we can conclude that is unlikely that the observed biological activities demonstrated by the polysaccharide treatments could be somewhat influenced by their contents of polyphenols.

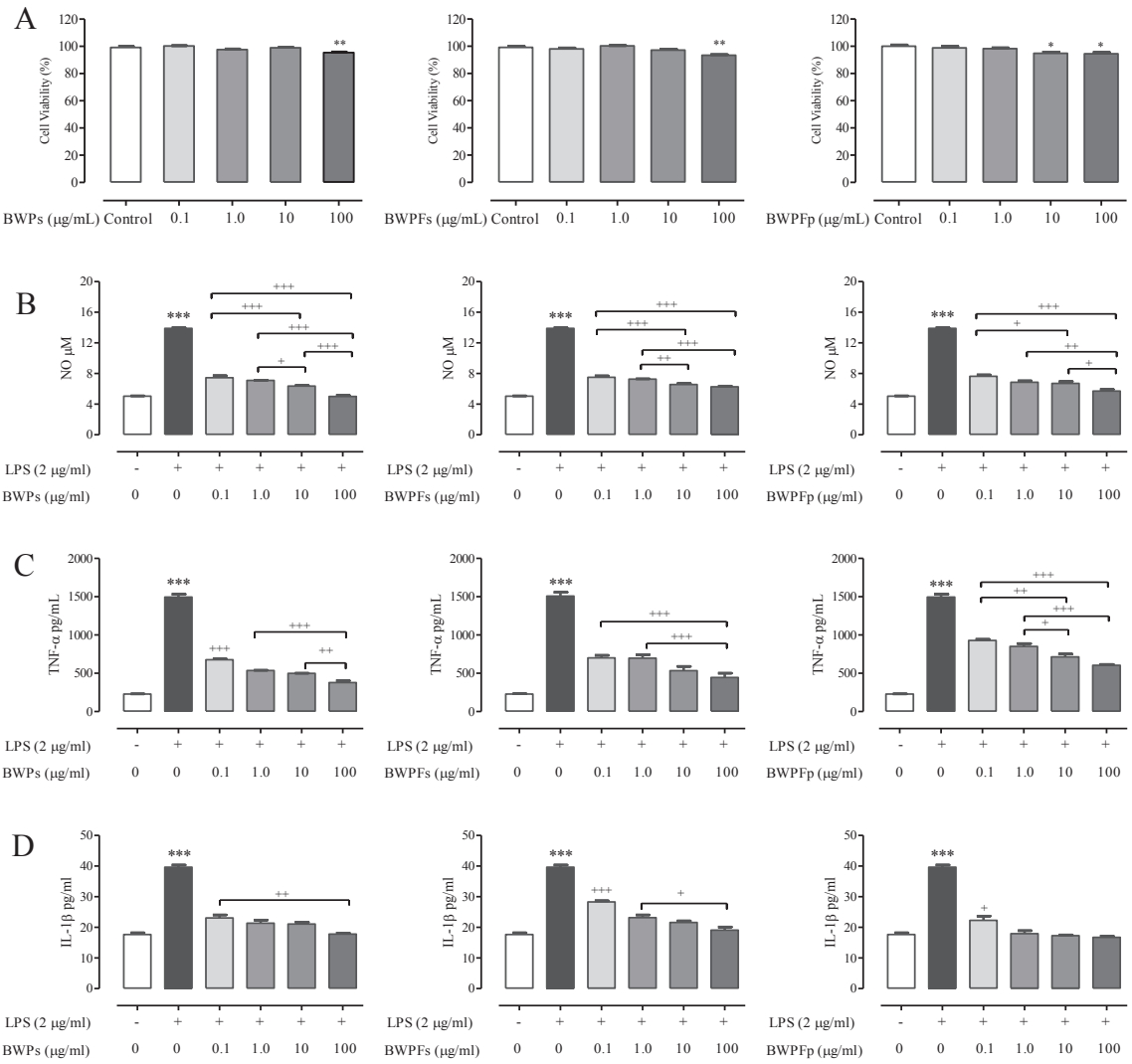

Figure 6. (A) Cell viability of polysaccharides present in blackberry wine on RAW 264.7 cells in the presence of LPS. The viability of cells without sample and LPS has been taken as reference (100%) - positive control. (B) NO concentration in the culture supernatant was determined by the Griess assay. (C) TNF-α concentration in the culture supernatant was determined by ELISA using standard curve. (D) IL-1β concentration in the culture supernatant was determined by ELISA using standard curve.

All data are presented as means ± SD from three sets of independent experiments. *p< 0.05 represent significant difference with cells treated with LPS alone (positive control) compared with fractions. +p < 0.05 represent significant difference between of fractions.

Natural polysaccharides isolated from several plants, fruits, beverages, fungi, bacteria, marine algae and among others have been shown to profoundly affect the immune system both *in vivo* and *in vitro* (Li, Qian, Xu, Chen, Wang, Nie, et al., 2015; Leivas, Nascimento, Barros, Santos, Iacomini, &Cordeiro, 2016; Shu, Liu, Ma, Gao, He, Cao, et al., 2017; Jose

&Kurup, 2017). This study demonstrated that blackberry wine polysaccharides decreased both NO production and pro-inflammatory cytokines, indicating that they may be considered as promising candidates for immunomodulatory compounds.

4. Conclusions

It was demonstrated that blackberry wine contains a mannan (40.8%) with M_w 122.700 g/mol. Moreover, a type II arabinogalactan (36.2%) containing a backbone with high portions of 6-*O*-linked and 3,6-*O*-linked Gal*p* chains rather than 3-*O*-linked Gal*p* was also present. It was found as a type I rhamnogalacturonan (19.1%) formed by the disaccharide unit $[\rightarrow 4)$-*α*-D-Gal*p*A-$(1\rightarrow 2)$-*α*-L-Rha*p*-$(1\rightarrow]_n$. These results bring new insights into wine polysaccharide composition. Also, this study is the first to demonstrate the anti-inflammatory effects of the extract prepared from blackberry wine on LPS-induced RAW 264.7 macrophages. In conclusion, anti-inflammatory effect of blackberry wine polysaccharide extract was demonstrated by an effective decrease of NO, TNF-α and IL-1βproduction by LPS-stimulated macrophages without cytotoxicity. Further studies on the mechanisms involved in NO and pro-inflammatory cytokine inhibitions *in vivo* are underway.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Structural characterization of blackberry wine polysaccharides and immunomodulatory effects on LPS-activated RAW 264.7 macrophages

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Table S1. Monosaccharide composition of fractions obtained from blackberry wine.

	Monosaccharidecomposition (%)							
Fraction	**Rha**	**Ara**	**Xyl**	**Fuc**	**Man**	**Gal**	**Glc**	**GalA*** **
BWPs*	5.65	12.59	2.87	-	33.61	19.41	12.83	13.02
BWPs**	6.14	11.82	2.83	-	33.90	19.54	12.62	13.15
BWPFs*	7.81	15.35	-	-	Tr	56.49	5.95	10.12
BWPFs**	7.88	15.89	-	-	-	56.70	5.76	10.16
BWPFp*	-	-	-	-	96.22	-	3. 78	-
BWPFp**					98.03		1.97	

* Determined using NMR (Sassaki, 2014). ** Determined using GC-MS ***Uronic acids, determined using the m-hydroxybiphenyl method.

Table S2. The NMR signals of fractions obtained from blackberry wine.

Resídues	C1/H1	C2/H2	C3/H3	C4/H4	C5/H5	C6/H6
Type II Arabinogalactan						
→3,6)-β-D-Gal*p*-(1→	103.5/4.45	71.0/3.50	80.4/3.70 (substituted)	68.4/4.12	75.2/3.69	69.4/3.92;4.04 (DEPT)
→2)-α-L-Ara*f*-(1→	107.3/5.20	82.8/4.27	-	-	-	-
→3)-α-L-Ara*f*-(1→	107.5/5.06	-	81.1/4.11	-	66.1/3.93	-
α-L-Ara*f*Terminals	109.3/5.24	-			74.7/3.67	65.6/3.74;3.97
Type I Ramnogalacturanan						
→2)-α-L-Rha*p*-(1→	98.5/5.24	77.0/4.01	-	-	-	16.1/1.29
→4)-α-D-GalA*p*-(1→	99.3/4.99	68.2/3.82	68.5/3.89	80.0/4.37	77.4/4.41	175.6
Mannan						
→2,6)-α-D-Man*p*-(1→	98.1/5.09	78.6/4.01	78.9/3.93	-	-	65.7/3.66;3.99
→2)-α-D-Man*p*-(1→	100.5/5.28	78.4/4.09	-	66.4/3.80	73.7/3.87	61.5/3.74;3.87
α-D-Man*p*-(1→	102.1/5.03	69.7/4.20	78.0/3.94	66.8/3.68	71.2/3.76	61.3/3.72
→3)-α-D-Man*p*-(1→	102.2/5.13	70.5/4.05	70.3/3.87	-	-	-

References: Delgobo *et al*., 1998, 1999; Renard*et al*., 1998; Vinogradov, E. *et al*., 1998; Carpita e Gibeaut, 1993.

Table S3. Profile of partially *O*-methylated alditol acetates of BWPs, BWPFs (BWPFs-CR) and BWPFp obtained on methylation analysis.

***O*Me-alditolacetate**	**BWPs**	**BWPFs**	**BWPFs-CR**	**BWPFp**	**Structure**
2,5-Me_2-Ara	3.9	6.9	6.4	-	→3)-Ara*f*-(1→
3,5-Me_2-Ara	0.8	1.7	0.7	-	→2)-Ara*f*-(1→
2,3,5-Me_3-Ara	7.4	13.7	12.8	-	Ara*f*-(1→
3,4-Me_2-Rha	12.4	7.1	6.6		→2)-Rha*p*-(1→
2,3,4,6-Me_4-Gal	2.2	7.1	7.4	-	Gal*p*-(1→
2,4,6-Me_3-Gal	-	6.2	5.8	-	→3)-Gal*p*-(1→
2,3,4-Me_3-Gal	12.8	36.2	33.7	-	→6)-Gal*p*-(1→
2,4-Me_2-Gal	7.3	12.6	11.8	-	→3,6)-Gal*p*-(1→
2,3,6-Me_3-Gal	-	-	7.1	-	→4)-Gal*p*-(1→
2,3,6-Me_3-Glc	5.8	9.2	8.4	3.2	→4)-Glc*p*-(1→
2,3,4,6-Me_4-Man	5.8	-	-	32.2	Man*p*-(1→
3,4-Me_2-Man	15.9	-	-	32.1	→2,6)-Man*p*-(1→
3,4,6-Me_3-Man	9.7	-	-	21.5	→2)-Man*p*-(1→
2,4,6-Me_3-Man	9.6	-	-	10.7	→3)-Man*p*-(1→

CAPÍTULO IV

O capítulo IV é compost pelo manuscrito, “Polysaccharides from blackberry wine and their protective effect against murine sepsis”.

Polysaccharides from blackberry wine and their protective effect against murine sepsis

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ABSTRACT

The present study evaluated the blackberry wine polysaccharides anti-inflammatory activity using a clinically relevant model of sepsis induced by cecal ligation and puncture (CLP). Blackberry wine polysaccharides previously studied presented anti-inflammatory effects on macrophage cells. We investigated the effects of polysaccharides from fractions BWPs, BWPFp and BWPFs in different concentrations on sepsis in mice. Polysaccharides treatment attenuated the migration of polymorphonuclear cells caused by sepsis. In immune histochemical analysis also attenuated injuries in liver, lung and renal dysfunction. In addition, administration of BWPs, BWPFp and BWPFs increased survival rates (60%) at the end of the observation period (7 days). Also, treatment reduced the development of systemic toxicity and mortality caused by sepsis. This study provides evidence that polysaccharides from blackberry wine attenuate the degree of sepsis in mice.

Keywords: Polysaccharide. Sepsis. Anti-inflammatory.

1. INTRODUCTION

Sepsis represents the systemic inflammatory response to infection affecting multiple organ systems (GANTNER; NICHOL, 2012). Infection is defined as an inflammatory response to microorganisms or the invasion of normally sterile host tissue by those organisms (AIRD, 2003). The interaction between pathogens and host cells results in the initiation of inflammatory and coagulation cascades. Inflammation is a complex and highly regulated adaptive response (MEDZHITOV, 2008).

The innate immune response is responsible for the initial inflammatory process in sepsis. It is mediated by recognition receptors, such as Toll-like receptors (TLRs) and CD14, which recognize pathogens or their products, called PAMPs (molecular patterns associated with pathogens). TLRs recognize the peptidoglycan of the bacteria and, once activated, trigger an intracellular signaling cascade that culminates in nuclear translocation of NF-kB, which promotes the gene expression of pro-inflammatory molecules, such as tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β), and also anti-inflammatory cytokines, such as interleukin 10 (IL-10) (BALDWIN, 2001; COHEN, 2002; RUSSEL, 2006). Polysaccharides from blackberry wine exert their immune function mainly via macrophages. Simultaneously, decrease NO (an inflammatory mediator), and pro-inflammatory cytokines including TNF-α and IL-1β (CORDEIRO CAILLOT *et al*., 2018)

Cell culture data does not always accurately predict the results of an animal model. Therefore, is also necessary to study the *in vivo* effect of the blackberry wine polysaccharides. In cell culture studies, only one type of cell is present, whereas multiple pro-inflammatory cytokines producer cell types are present in a whole animal system. Considering previous research, the objective of this study was to reveal effects of blackberry wine polysaccharides on systemic inflammatory response (sepsis). Cordeiro Caillot *et al*. (2018) isolated and purified three polysaccharide fractions from blackberry wine, BWPs, BWPFs and BWPFp. These fractions were characterized by Gas Chromatography-Mass Spectrometry (GC-MS) and Nuclear Magnetic Resonance (NMR). BWPs fraction presented typical signals of a pectic structure, indicating the presence of a type II arabinogalactan (AG II), type I rhamnogalacturonan (RG I) and mannan as major polysaccharides; BWPFs fraction presented only AG II and RG I; and BWPFp fraction, mannan. In this work, these three fractions were analyzed *in vivo* in the CLP-sepsis model.

2. MATERIALS AND METHODS

2.1 POLYSACCHARIDES SOURCE

Blackberry wine (750 ml) was obtained from the Adega Porto Brazos, at Paraná state, Brazil. Fractions BWPs, BWPFs and BWPFp were obtained, isolated and characterized according to Caillot et al., 2018.

2.2 ANIMALS

The research was performed using male albino Swiss mice (3 months old, weighing 30-40g) from the UFPR (Paraná/Brazil). The animals were maintained under standard conditions (12 h light/dark cycle and controlled temperature 22 ± 2 °C). Standard pellet food (Nuvital®, Curitiba/PR, Brazil) and water were available *ad libitum*. All experimental procedures were previously approved by the Institutional Ethics Committee of the University (authorization numbers 1018 and 1099, appendix I).

2.3 WRITHING TEST

Mice were treated with different doses of BWPs, BWPFs and BWPFp subcutaneously (0.1, 1 and 10 mg/kg) and orally (3, 7 and 10 mg/kg) administrated. Fractions were suspended in saline solution prior injection or ingestion. The non-treated group received only the vehicle (saline solution 10 mL/kg), 100 µL subcutaneously or 20 µL orally.

2.4 SEPSIS INDUCTION BY CECAL LIGATION AND PUNCTURE (CLP)

Sepsis was induced by cecal ligation and puncture (CLP) in mice, as previously described (FINK; HEARD, 1990). Mice were randomly grouped into eleven clusters of 8 mice, for each fraction: (1) sham or False operated mice, (2) CLP + Saline (positive control), (3) CLP + (BWPs, BWPFs or BWPFp) 0.1 mg/kg; (4) CLP + (BWPs, BWPFs or BWPFp) 1 mg/kg; (5) CLP + (BWPs, BWPFs or BWPFp) 10 mg/kg, all with subcutaneous administration. Groups with oral treatment were similar: (6) sham or False operated mice, (7) CLP + Saline (positive control), (8) CLP + (BWPs, BWPFs or BWPFp) 3 mg/kg; (9) CLP + (BWPs, BWPFs or BWPFp) 7 mg/kg; (10) CLP + (BWPs, BWPFs or BWPFp) 10 mg/kg. The weight mice ranged from 30-40 g, thus, 20 µL of each fraction (BWPs, BWPFs or BWPFp)

were orally administered to avoid regurgitation. Commercial dexamethasone was another positive control, subcutaneously administered; (11) CLP + dexamethasone 0.5 mg/kg. Mice were anesthetized by intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (20 mg/kg), and a small abdominal midline incision was made. The cecum was carefully exposed and 50% of the distal moiety was ligated. The cecum was then punctured thrice with a sterile 16-gauge needle and squeezed to extrude the fecal material from the wounds; the abdominal incision was closed in two layers. Each mouse received 1 mL of sterile saline subcutaneously administered for fluid resuscitation. Sham-control animals were treated identically, but no cecal ligation or puncture was carried out. After surgery food and water were given *ad libitum* throughout the experiment. The survival rate was monitored for 7 days, each 12 h. During this period, saline solution (vehicle) and drugs were daily subcutaneous and orally administered.

In another experimental set, 30 minutes (subcutaneous administration) and 1 h (oral administration) before surgery, mice were treated with; (a) (BWPs, BWPFs or BWPFp) 1 mg/kg (subcutaneous administration); (b) (BWPs, BWPFs or BWPFp)10 mg/kg (oral administration); (c) Solution Salina (subcutaneous administration); (d) Solution Salina (oral administration) and (e) dexamethasone (0.5 mg/kg). After 6 h post-operation, mice were sacrificed. Their lung, liver and kidneys were collected for histological assays and their blood for biochemical analyses.

2.5 BLOOD LEUKOCYTES MEASUREMENT

Blood was collected by cardiac puncture. Total leukocytes and mononuclear (MN) and polymorphonuclear (PMN) leukocytes were counted by an automated system, according to the manufacturer's recommendations (Mindray BC-2800 vet, São Paulo, SP, Brazil). Additionally, slides were prepared and stained with May-Grunwald-Giemsa for leukocyte mononuclear and polymorphonuclear morphology analyses.

2.6 PLASMA BIOCHEMISTRY

Plasma samples were prepared by centrifugation at 3000 g for 10 min and levels of Bilirubin (direct, indirect and total), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA) and urea activity were analyzed by an automated system, according to the manufacturer's recommendations (Mindray BS-200, São Paulo, SP, Brazil).

2.7 HISTOLOGIC ANALYSIS

Samples of lungs, liver and kidney tissue sections were fixed in ethanol solution (70% - at room temperature - for 6 h), then in ALFAC solution (alcohol 80%, formaldehyde 15% and acetic acid glacial 5% - for 16h). After fixation, samples were dehydrated in a graded series of increasing ethanol strength before paraffin embedding. Sections (4 μm) were stained with hematoxylin and eosin and analyzed under microscopy.

2.8 CYTOKINE LEVELS DETERMINATION

Plasma samples were prepared by centrifugation at 8000 g for 10 min and stored at -80 °C. Analyses of tumor necrosis factor alpha (TNF-α), interleukins IL-1β and IL-10 concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits (elabscience) according to the manufacturer´s recommendation.

2.9 STATISTICAL ANALYSIS

Data are expressed as means. Statistical analyses were performed using GraphPad Prisma® 5.0 (La Jolla, CA) software and one-way analysis of variance ANOVA followed by Turkey test. Significant differences were considered at $p< 0.05$. Histological analysis was performed by qualitative observations.

3. RESULTS

3.1. SURVIVAL ANALYSES OF BWPs, BWPFp AND BWPFs SUBCUTANEOUSLY AND ORAL ADMINISTRATED

Survival rates were analyzed in six groups each for subcutaneous administration (s.a) and oral administration (o.a), as shown in fig.1A-C (s.a) and fig.2A (o.a). Mice treated only with vehicle started to died between 24 and 48 h after sepsis induction (CLP). The overall mortality in this group at the end of the observation period (7 days) was 100%, and the area under the curve was 5.664 (arbitrary units). Mice treated subcutaneously with polysaccharides of blackberry wine, tested at doses of 0.1, 1 and 10 mg/kg the lethality was markedly delayed.

Demonstrated, with BWPs areas under the curve were 12.216, 13.212, 12.636, a survival rate of 50 %, 75 % and 67 %; with BWPFs, areas under the curve were 12.468, 15.792, 15.504 a survival rate of 63 %, 88 % and 88 %; and BWPFp areas under the curve were a survival rate 9.012, 11.604, 11.916 of and 25 %, 63 % and 63 % at 7 days after surgery. Due to the amount of sample obtained in BWPFs and BWPFp the oral test was performed only with the BWPs, which had higher yield (data not shown). The lethality was delayed in mice treated, orally, with BWPs and their areas under the curve were increased to9.348, 10.704 and 13.980 after administrating 3, 7 and 10 mg/kg, respectively. At the end of the period, the overall survival in these BWPs groups was 25%, 38% and 63%. No death was observed in the sham-operated mice and its corresponding area under the lethality curve was 16.800 (arbitrary units). Dexamethasone, used as positive control, showed a significant improvement in survival (area under curve 12.336), with an overall survival rate of 50% at the end of the observation period.

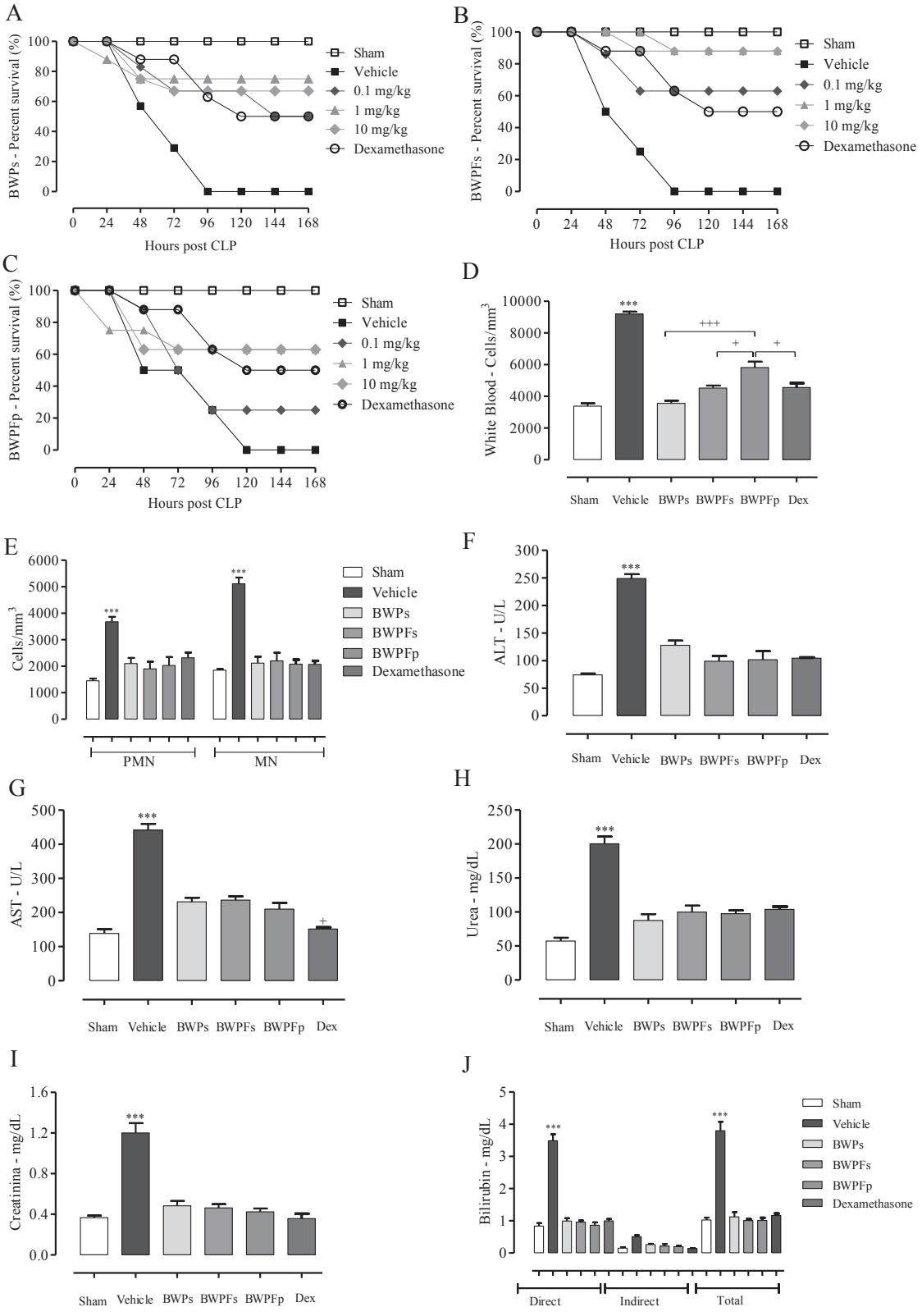

FIGURE 1. Effect of polysaccharides (A) BWPs (B) BWPFs and (C) BWPFp on survival rate in sepsis (subcutaneous administration). (D) The effects of the BWPs, BWPFs and BWPFp treatment on white blood cells, (E) differential cell counting on the plasma levels of ALT (F),

AST (G), urea (H), creatinine (I) and bilirubin (J). Points or bars represent the mean ± mean standard errors (n = 8). Symbols: * Statistically group *P < 0.05.

3.2 BLOOD LEUKOCYTE MEASUREMENT AND PLASMA BIOCHEMISTRY

Based on the results obtained from the survival rate, we choseBWPs, BWPFs and BWPFp treatments via subcutaneous administration (1mg/kg) and oral administration of BWPs (10 mg/kg) for biochemical and histopathological analyses. BWPs, BWPFs and BWPFp (s.a) treatments decreased the blood cell levels in 61.2%, 49.4% and 33.3% in post-CLP mice (Fig. 1D); and BWPs (o.a) treatment decreased it in 33.1% (Fig. 2B). BWPs, BWPFs and BWPFp subcutaneously administered decreased the number of polymorphonuclear (PMN)(42.1%, 55.2%, 39.4%) and mononuclear (MN) (55.5%, 56.4%, 59.2%) leukocytes present on blood in septic mice (Fig. 1E). BWPs oral administration decreased PMN (47.3%) and MN (58.3%) - Fig. 2C, all results compared to positive control.

Septic positive control mice exhibited significantly increased levels of leukocytes in blood, an increase in alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, urea and creatinine levels when compared to sham. BWPs, BWPFs and BWPFp treatment via subcutaneous administration reduced CLP-induced ALT (45.5%, 62.4%, 60.3%) (Fig. 1F), AST (48.1 %, 46.8%, 54.6%) (Fig. 1G) bilirubin direct (70.0%, 70.6%, 76.9%), indirect (52.3 %, 71.4%, 68.5%), total (70.1%, 73.8%, 75.0%) (Fig. 1H), urea (53.4%, 39.8%, 50.5%) (Fig. 1I) and, creatinine (61.5%, 69.2%, 65.3%) (Fig. 1J) levels. BWPs oral administration presented a decrease in ALT 30.5% (Fig. 2D), AST 39.6% (Fig. 2E) bilirubin direct 57.3%, indirect 28.5%, total 47.2% (Fig. 2F), urea 54.3% (Fig. 2G), creatinine 57.6% (Fig. 2F). Subcutaneous administration was more effective when compared to oral administration.

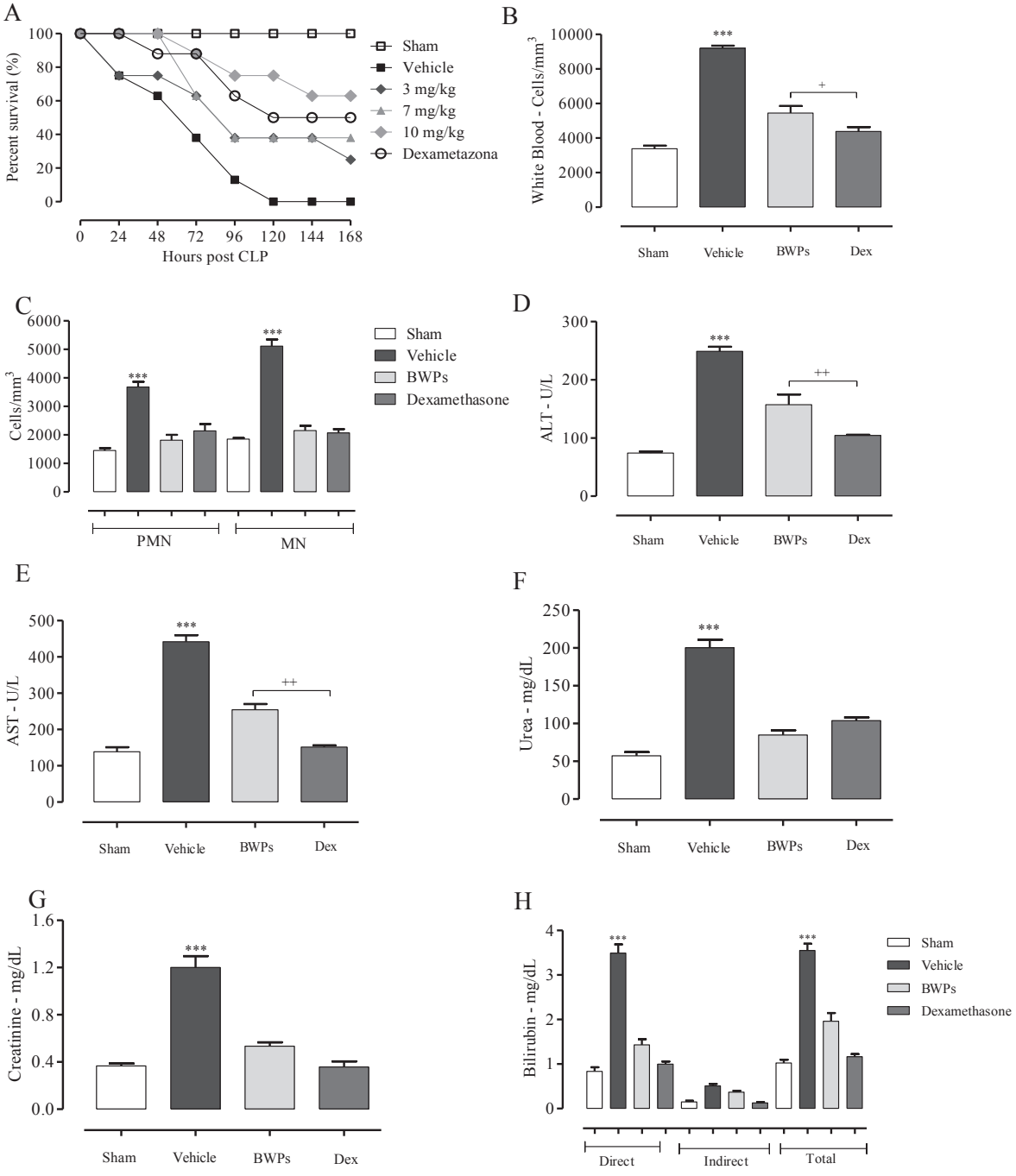

FIGURE 2. Effect of polysaccharides (A) BWPs survival rate in sepsis (oral administration). (B) BWPs treatment effects on white blood cells, (C) differential cell counting on the plasma levels of ALT (D), AST (E), urea (F), creatinine (G) and bilirubin (H). Points or bars represent the mean ± mean standard errors (n = 8). Symbols: * Statistically group *P < 0.05.

3.3 CYTOKINES ANALYSIS

To assess the effect of fractions from blackberry wine on inflammatory response following CLP, cytokines concentration in plasma (systemically) were detected 6 h after

surgery. Concentration of pro-inflammatory cytokines (TNF-α and IL-1β) were dramatically reduced in plasma in mice under sepsis with polysaccharides-treatment (Fig. 3A-C and 4A-C). However, polysaccharides increased anti-inflammatory cytokine (IL-10) production in plasma in septic mice (Fig. 4A-C and E-G). Sepsis induced NO production was significantly decreased by all fractions (Fig. 3D and 4D).

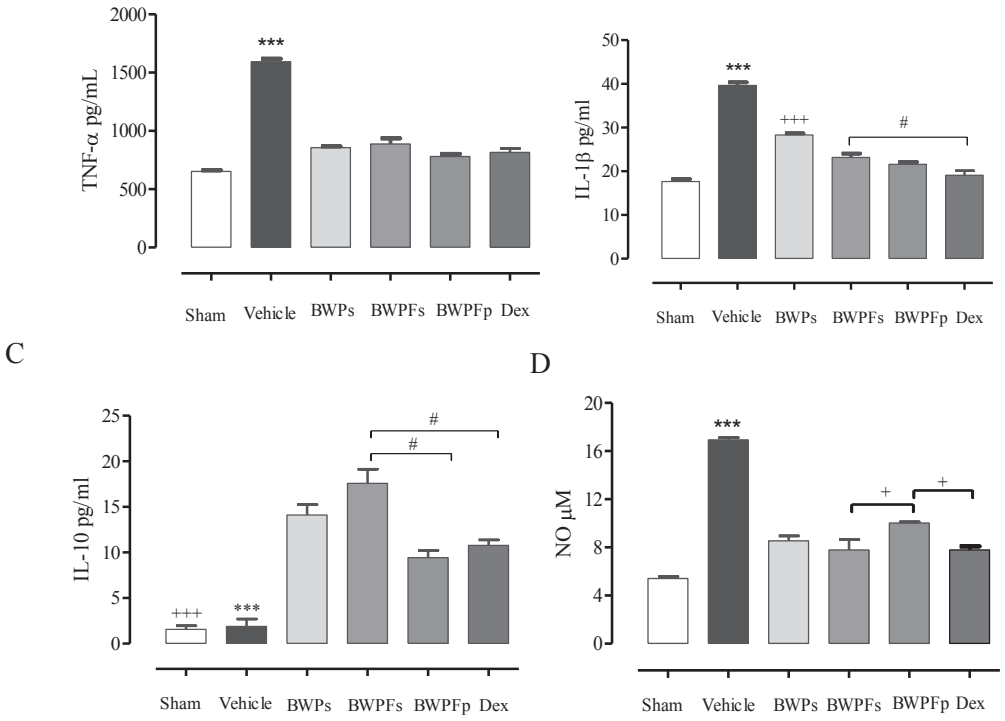

FIGURE 3. Effect of polysaccharides on plasma cytokines concentration. (A) TNF-α, (B) IL1-β, (C) IL-10 and (D) NO in subcutaneous administration.

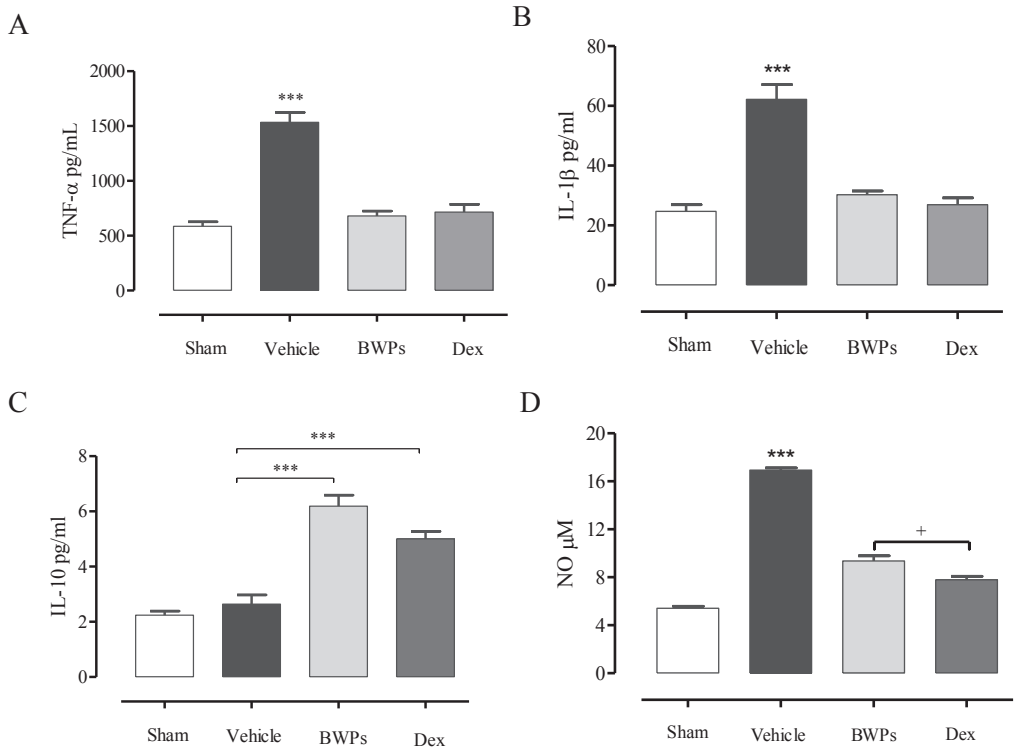

FIGURE 4. Effect of polysaccharidesin plasma cytokines concentration. (A) TNF-α, (B) IL-1β, (C) IL-10 and (D) NO in oral administration.

3.4 HISTOPATHOLOGY

Mice in the negative control (sham) group displayed normal tissue histology. Liver (Fig. 5A and 6A) showed normal hepatocytes architecture; liver structure was normal and there were no hepatocyte necrosis and no inflammatory cells infiltration. Lung (Fig. 5G and 6D) structure was intact: no alveolar hemorrhage, normal alveolar septum, normal pulmonary capillaries and no inflammatory cells. Kidney (Fig. 5M and 6G) did not presented injury and/or hemorrhage. In the positive control the liver showed degenerated hepatocytes and inflammatory cells infiltration (Fig. 5B and 6B). The lung structure was significantly disrupted, and infiltration of a large amount of neutrophil were observed (Fig. 5H and 6E), whilst the kidney showed increased necrosis and capsular inflammation (Fig. 5N and 6H). BWPs, BWPFs and BWPFp subcutaneous administration and BWPs oral administration significantly attenuated the liver injury: hepatocytes were nearly normal and inflammatory cells infiltration decreased (Fig. 5C-E and 6C). Lung injury was markedly attenuated, there was no obvious alveolar hemorrhage, only a few inflammatory cells infiltrated in the lung (Fig. 5I-K and 6F) and kidney injury was attenuated (Fig. 5O-Q and 6I).Furthermore, blood biochemistry for liver and kidney injury were also measured. Liver normal function was

damaged following CLP, as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels increased significantly (Fig. 2M and N); renal function was also compromised in septic mice, as indicated by increased urea and blood creatinine levels (Fig. 1G,H and 2E,F). Interestingly, blackberry wine polysaccharides post treatment protected tissues in both liver and kidney (Fig. 2M-P).

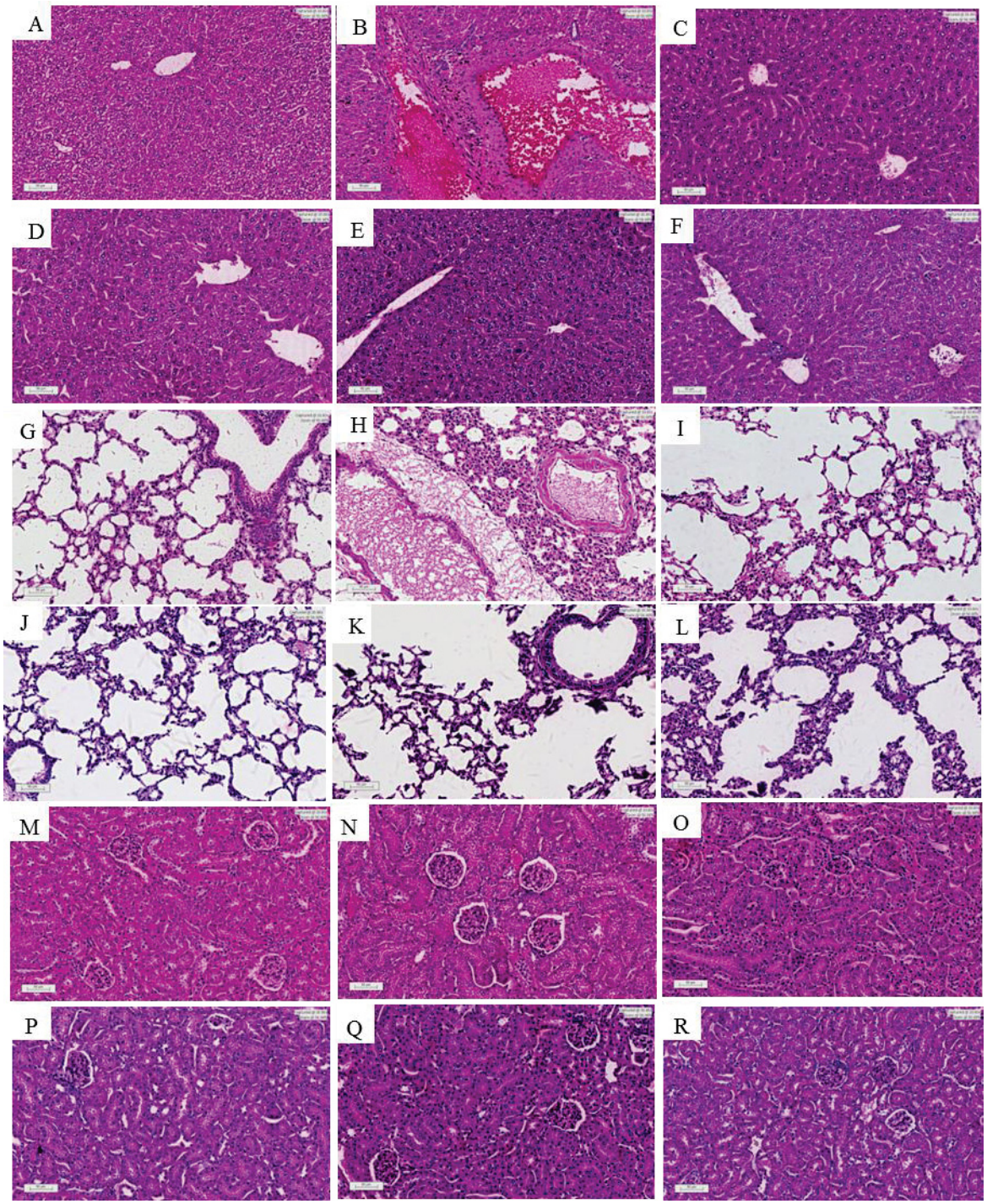

FIGURE 5. Histopathological changes induced 6 h after CLP and the effects of blackberry winepolysacarides fractions subcutaneously administered. Sections were prepared and stained

with haematoxylin and eosin (20 magnification). Liver (A) Sham, (B) positive control, (C) BWPs (1 mg/kg), (D) BWPFs (1 mg/kg), (E) BWPFp (1 mg/kg), (F) Dexamethasone (5 mg/kg). Lung (G) Sham, (H) positive control, (I) BWPs (1 mg/kg), (J) BWPFs (1 mg/kg), (K) BWPFp (1 mg/kg), (L) Dexamethasone (5 mg/kg). Kideny (M) Sham, (N) positive control, (O) BWPs (1 mg/kg), (P) BWPFs (1 mg/kg), (Q) BWPFp (1 mg/kg), (R) Dexamethasone (5 mg/kg).

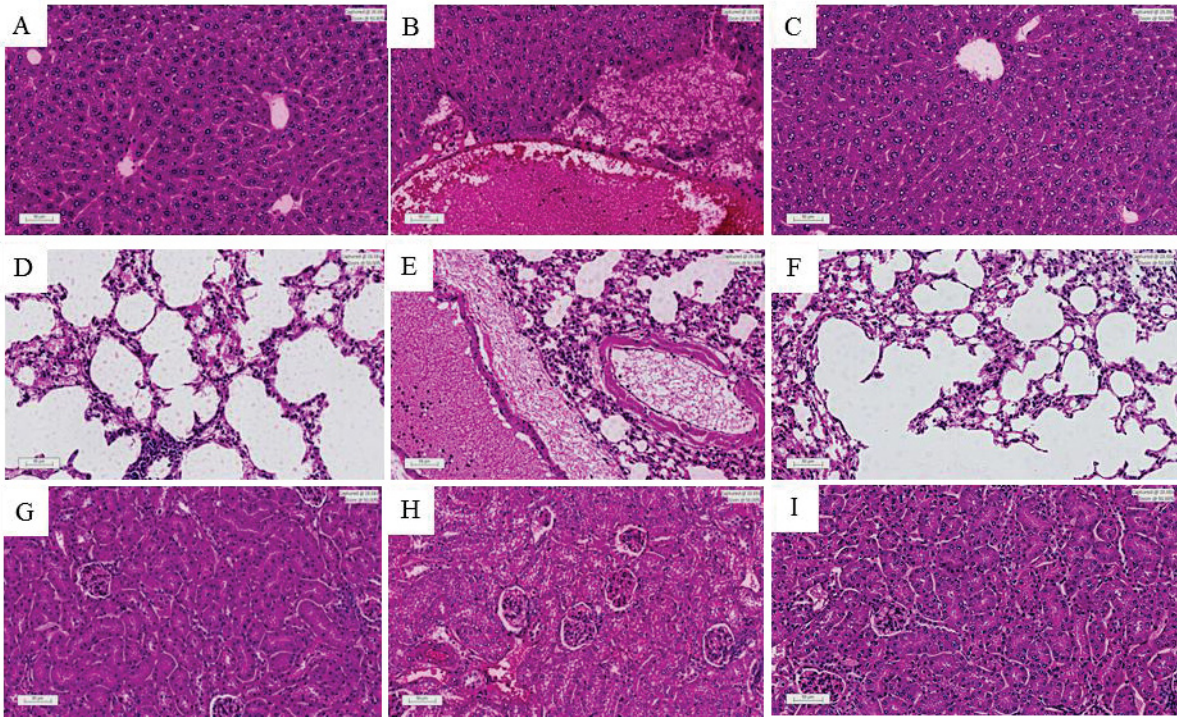

FIGURE 6. Histopathological changes induced 6 h after CLP and the effects of blackberry winepolysacarides fractions orally administered. Sections were prepared and stained with haematoxylin and eosin (20 magnification). Liver (A) Sham, (B) positive control, (C) BWPs (10 mg/kg). Lung (D) Sham, (E) positive control, (F) BWPs (10 mg/kg). Kideny (G) Sham, (H) positive control, (I) BWPs (10 mg/kg).

4. DISCUSSION

There are many reports of polysaccharides, biological response modifiers, for example anti-inflammatory activity (CORDEIRO CAILLOT *et al*., 2018; ANANTHI *et al*., 2010; BEZERRA *et al*., 2018; DORE *et al*., 2007; POPOV *et al*., 2005;). In this study, a polymicrobial sepsis was induced by cecal ligation and puncture (CLP) what may lead to host inflammatory responses. Considering that sepsis is defined as a potentially fatal organic

dysfunction that is caused by a dysregulated host response to infection, it can induce acute kidney injury and failure of various organs, such as liver and lungs. Sepsis initiates a complex immune response that varies over time, with the concomitant occurrence of pro-inflammatory and anti-inflammatory mechanisms (BRENT, 2017; GANTNER; NICHOL, 2012; VENET; MONNERET, 2017).

New medications are often suggested for sepsis treatment. They aim to interfere in the inflammatory and coagulation cascade. Conventional treatments depend on the use of antibiotics and anti-inflammatory medication against infection to control inflammation.The basic strategy underlying immunomodulation is to identify aspects of host response that can be enhanced or suppressed in order to enhance or complement a desired immune response (ANNANE, 2011; INNGJERDINGEN *et al*., 2005).

Our work corroborates with recent studies on natural anti-inflammatory polysaccharides from *Eucommia ulmoides* (LI *et al*., 2017), green and black teas (SCOPARO *et al*., 2013), *Trebouxia* sp (RATTMANN *et al*., 2013), *Ilex paraguariensis* (DARTORA *et al*., 2013), *Agaricus bisporus* and *Lactarius rufus* (RUTHES *et al*., 2012). These polysaccharides have demonstrated potential for antiseptic drugs development.

According to Cordeiro Caillot *et al*., (2018) after the discovery of blackberry wine polysaccharides, with defined chemical structure and confirmed anti-inflammatory activity in macrophages (*in vitro*). We sought to confirm these polysaccharides antisepsis effects using the CLP model due its capacity to mimic the human sepsis caused by pathogens derived from the intestinal tract (OTERO-ANTON *et al*., 2001). It was observed what the mice pre-treated with blackberry wine polysaccharides in different concentrations, 30 min or 1 hour before CLP, presented functional markers significantly improved: ALT, AST, urea, creatinine and bilirubin production were decreased. All mice submitted to CLP had the septic shock within 48 h. However, for the animals treated with polysaccharides, the survival rate was significantly higher. The polysaccharides tested were able to decrease significantly TNF-α and IL-1β serum levels. Studies have demonstrated that some cytokines, especially TNF-α and IL-1β, are strongly associated with sepsis syndrome (HENKIN, *et al*., 2009).To further confirm that these polysaccharides could protect the animals against sepsis, we checked the histology of liver, lung and kidney tissues of treated mice. It was observed a significant improvement of these tissues. We concluded that blackberry wine polysaccharides present protective effects against sepsis and may corroborate for the development of new polysaccharide-based therapeutics against sepsis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Appendix I

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

Nº 1018

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.149858/2016-79

APROVADO/APPROVAL: 08/11/2016 – R.O. 10/2016

TÍTULO: Caracterização estrutural e estudo farmacológico de polissacarídeos extraídos dos vinhos de uva, fermentados de amora e da fruta amora preta.

TITLE: Structural characterization and pharmacological study of polysaccharides extracted from grape and blackberry wines and blackberry fruit.

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DEPARTAMENTO/DEPARTMENT: Bioquímica e Biologia Molecular

Profa. Dra. Ana Vitória Fischer da Silva
Coordenadora da CEUA

CAPITULO V

Durante a realização desse trabalho houve a oportunidade de se trabalhar com uma matriz semelhante, assim realizamos a produção artesanal de vinho, visando aplicar as metodologias analíticas para a quantificação da produção dos polissacarídeos, etanol e compostos secundários formados durante o período de fermentação do vinho. Os resultados foram compilados e são apresentados a seguir no artigo "Wine fermentation processe valuation through NMR analysis: polysaccharide characterization and biological activity".

Wine fermentation process evaluation through NMR analysis: polysaccharide characterization and biological activity

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ABSTRACT

Winemaking is a simultaneously saccharification and fermentation process. During the fermentation process, ethanol concentration is one of the key parameters which indicates the wine quality and is directly linked to the consumption of carbohydrates. In this work it was possible to determine the best fermentation time, in which the wine retains its highest concentration of ethanol and polysaccharides. Polysaccharides isolated by ethanol precipitation were quantified by NMR analysis. Mannan and type II arabinogalactan were the main polysaccharides, followed by type I and II rhamnogalacturonanand dextrin. This study provides a promising instruction for improving wine features and quality control.

Keywords: wine, polysaccharides, ethanol, quantification, NMR.

1. INTRODUCTION

The grape variety Ives Noir (popularly known as Bordo), from *Vitis labrusca* originating in United States, is cultivated in tropical regions and adapted to hot and wet climates with high luminosity and abundant irrigation, which contributes to the synthesis and accumulation of sugars in the berries (CAMARGO *et al*., 2005). The production of this grape is mainly destined to the elaboration of juices and ordinary Wine Bordo (CAMARGO; OLIVEIRA, 2001).The wine is obtained from the complete or partial fermentation of fresh Ives Noir (Bordo) grapes or grape must, containing water, alcohols, acids, sugars, mineral salts, pigments and aromatic compounds (CHEYNIER *et al*., 2010). Ethanol (EtOH) is the second largest component (after water) in wine, with concentrations ranging from 10% to 20% v/v (PINNEY, 2012). The EtOH content is one of the most important parameters for process and quality control in wine industries (COLLINS*et al*., 1997). Therefore, it is essential to know when to finalize the wine fermentation to obtain the ideal concentration of alcohol to have a quality wine.

The ethanol proportion in alcoholic beverages and its interactions with other matrix components (e.g., carbohydrates, acids, and tannins) can also have significant effects on the behavior of volatile compounds responsible for aroma and flavor (GERBAUD *et al*., 1997). Among these matrix components, carbohydrates have important influence on several stages of the winemaking process and are in part responsible for the organoleptic properties of wines (GERBAUD *et al*., 1999). Among carbohydrates, polysaccharides from grapes and yeast are the most important. They come from grape berries and are released by yeast either during fermentation or by enzymatic autolysis during aging on yeast lees (DOCO; BRILLOUET, 1993; VIDA *et al*., 2003). Polysaccharides are interesting for enology due to their positive effects on the final quality of the wine (DOCO *et al*., 2003; FORNAIRON-BONNEFOND *et al*., 2002). Many of these polysaccharides have been evaluated as biological response modifiers, presenting anti-inflammatory activity, for example (XIONG *et al*., 2017;DU*et al*., 2016; KOMURA*et al*., 2010). Besides the importance of polysaccharides and ethanol concentration in wine making, to identify and characterize sensory and biological properties of the wine components is essential to optimize the wine production process and improve the quality of the product that will be marketed. To unravel wine polysaccharides anti-inflammatory characteristics, the polysaccharides were cultured with RAW 264.7 macrophages and pro-inflammatory cytokine secretions, including TNF-α and IL-1β and nitric oxide (NO), were accessed (LEE; PARK,2015). Nowadays one of the main targets of

the wine sector is to improve wine quality, elaborating wines that satisfy consumer demand and expanding the offer of quality wines that bring health benefits.

2. MATERIALS AND METHODS

2.1 WINEMAKING

Vitis labrusca grapes (Fig. 1) were used in the winemaking process. The grapes were selected and washed, then 44kg was putted in an anaerobic 70L fermentation reactor with 11 kg of sugar. Whole grapes were placed in layers of 4kg of grapes and 1 kg of sugar cane until 70% of the reactor space was occupied. During the fermentation process, the reactor was kept in a room with stable temperature of about 20ºC. Every 5 days the reactor was opened for sampling. 40 mL aliquots were collected from the top wine layer. In the 25th day of process, the wine was filtered, bottled, corked and pasteurized. The pasteurization process was composed of a preheating stage of 60ºC for 15 minutes. After the soluble CO_2 extraction, the bottles were corked and heated for another cycle of 75ºC for 10 minutes. The bottles were then cooled at room temperature in a cool water bath. The yeast microorganism *Saccharomyces cerevisiae,* responsible for the fermentation, was obtained from the grapes through a high sucrose content environment selection.

2.2 EXTRACTION AND PURIFICATION OF WINE POLYSACCHARIDES

Polysaccharides were precipitated by addition of cold EtOH (3 vol.) and separated by centrifugation (8.000 rpm at 4°C, 20 min). The sediment was dissolved in H_2O and dialyzed against water for 72 h to remove the remaining low-molecular weight compounds, giving rise to a crude WP polysaccharide fraction. For the purification, the fraction was frozen and then allowed to thaw at room temperature (GORIN; IACOMINI, 1984), resulting in soluble and insoluble fractions, which were separated by centrifugation, as described above. The insoluble fraction was not analyzed in this study due to its low yield and solubilization. The water-soluble fraction was treated with Fehling solution (JONES; STOODLEY, 1965) and the soluble fraction was isolated from the insoluble Cu^{2+} complex by centrifugation under the same conditions described above. The fractions were neutralized with HOAc, dialyzed against water and deionized with mixed ion exchange resins and then freeze dried.

2.3 MONOSACCHARIDE ANALYSIS

WP (2 mg) was hydrolyzed with 1 M TFA at 100 °C for 14 h, evaporated and the residue dissolved in water (2 mL). The resulting monosaccharide mixture was examined by thin layer chromatography (TLC) silica-gel 60 (Merck), with ethyl acetate:aceticacid:n-propanol:water (4:2:2:1, v/v), then stained with orcinol-sulfuric acid (Sassaki et al., 2008; Skipski, 1975). The monosaccharides were reduced with 2 mg $NaBH_4$ yielding alditols which were acetylated in Ac_2O-pyridine (1:1, v/v, 0.5 mL) at room temperature for 12 h (WOLFROM; THOMPSON, 1963a,b). The resulting alditol acetates were extracted with $CHCl_3$ and analyzed by gas chromatography–mass spectrometry (GC-MSVarian, Saturn 2000R, Ion-Trap detector), using a DB-225-MS column (30 m × 0.25 mm × 0.25μm), programmed from 50 to 220 ◦C at 40 ◦C/min, with He as carrier gas. Components were identified by their typical retention times and electron ionization (EI 70 eV) spectra. The uronic acids content of fractions were determined using the colorimetric m-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991). Carboxy-reduction of WP was carried out by the carbodiimide method (TAYLOR; CONRAD, 1972), using $NaBH_4$ as the reducing agent, having its uronic acid carboxyl groups reduced to primary alcohols.

2.4 METHYLATION ANALYSIS

Per-*O*-methylation of each isolated polysaccharide (10 mg) was carried out using NaOH-Me_2SO-MeI (CIUCANU; KEREK, 1984). The process of isolation of the products by neutralization (HOAc), dialysis and evaporation was repeated, and the methylation was complete. The per-*O*-methylated derivatives were hydrolyzed with 72% (v/v) aq. H_2SO_4 (0.5 mL, v/v, 1 h, 0 °C), followed by dilution to 8% (v/v). The solution was kept at 100 °C for 17 h, then neutralized with $BaCO_3$, filtered and evaporated to dryness (SAEMAN*et al*., 1954). The hydrolysate was reduced with $NaBD_4$ (sodium boro deuteride), acetylated, giving rise to partially *O*-methylated alditol acetates and analyzed by GC–MS, as previously, but with a final temperature of 215 °C. They were identified by their typical retention times and electron impact spectra (SASSAKI *et al*., 2005).

2.5 NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

1D and 2D NMR spectra were obtained with a 600 MHz Bruker spectrometer using 5 mm direct or inverse probe heads (Avance III and HD, Bruker, Billerica, Massachusetts, USA). Analyses of 2D-NMR and HSQCed (Heteronuclear Single Quantum Coherence edited spectroscopy) experiments were performed at 30 ºC in deuterium oxide (D_2O). Chemical shifts of the polysaccharides were expressed in δ (ppm) relative to trimethylsilyl propionic acid (TMSP). The ratio of the polysaccharide units were calculated by integrating the area of specific anomeric signals corresponding to each cross-peak assignment on the 2D-HSQCed spectra.

2.6 DETERMINATION OF HOMOGENEITY AND MOLAR MASS

The homogeneity and molar mass of the purified polysaccharides were determined by high performance steric exclusion chromatography (HPSEC), using a refractive index (RI) detector. A series of four columns were used, with exclusion sizes of 7 x 10^6 Da (Ultrahydrogel 2000, Waters), 4 x 10^5 Da (Ultrahydrogel 500, Waters), 8 x 10^4 Da (Ultrahydrogel 250, Waters) and 5 x 10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M $NaNO_3$, containing 0.5 g/L NaN_3. The solutions were filtered through a 0.22 µm pore size membrane (Millipore) and loaded (100 µL, loop) at a concentration of 1 mg/mL. The molar mass of the polymer was estimated using Astra software 4.70.

2.7 WINE ETHANOL CONCENTRATION (Ec) CALCULATION BY NMR ANALYSIS

During the 25 days of the fermentation process, samples were collected every 5 days (encoded W5, W10, W15, W20 and W25) and stored in the freezer until analysis. ^1H NMR analysis was used for the calculation of the ethanol concentration in wine. Samples were prepared with 100 µL of wine each (W5, W10, W15, W20 and W25) with the addition of 100 µL of TMSP 1% and 400 µL of D_2O. The integrals obtained in the NMR analyses of the samples were then used for the calculation, based on the calibration curve. The calibration curve was performed by fixing the D_2O volumes to 400 µL and the values of 1% TMSP in 100 µL and the ethanol values in 100 µL with changing of 1%, 2%, 3%, 5%, 10%, 15% and 20%.

2.8 WINE POLYSACCHARIDE CONCENTRATION (Pc) CALCULATION BY NMR ANALYSIS

Polysaccharide concentration of the fractions W5, W10, W15, W20 and W25 were analyzed by 2D multiplicity-edited ^{1}H-^{13}C HSQC NMR using the integral value of each peak.Samples were prepared as stated previously.

2.9 CELL CULTURE AND POLYSACCHARIDE TREATMENT

WPs were evaluated for their anti-inflammatory activity *in vitro*. RAW 264.7 macrophage were cultured in DMEM culture medium (Invitrogen, San Diego, CA, USA) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 20 mM sodium bicarbonate in a humidified atmosphere of 5% CO_2 at 37 °C. RAW 264.7 cells were treated with different concentrations of WP fractions (0.1, 1.0, 10 and 100 μL/mL) and induced with LPS (2 μg/mL)after one hour.

2.10 CELL VIABILITY ASSAY OF POLYSACCHARIDES IN RAW 264.7 CELLS

The cytotoxicity of WPs on RWA 264.7 macrophage was determined by MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-bromo diphenyl tetrazolium) method (MOSSMANN, 1983), where reduction of crystal tetrazolium forming through mitochondrial enzymes is possible only in viable cells. Cells were plated in 24-well plates ($4.8x10^5$ cells/well) and containing different concentrations (0.1, 1.0, 10 or 100 μg/mL) of WPs. After 24 hours 350 μL/well of the 5 mg/mL MTT solution was added. After 4 hours incubation, cell supernatant was removed and 500 μL of DMSO (Dimethyl sulfoxide) were added for cell lyse and crystals solubilization. Plates were scanned at 570 nm.The cytotoxicity of WPs on RAW 264.7 macrophages in each well was evaluated using its cell viability (%).

2.11 NO AND CYTOKINES PRODUCTION MEASUREMENT

Exponential phase RAW 264.7 macrophages were inoculated at a density of 4.8 × 105 cells/100 μL, induced with LPS (2μg/mL) and treated with WPs (0.1, 1.0, 10 and 100 μL/mL). After 24h, supernatant was aspirated and submitted to cytokine and NO assay. In order to determine total NO concentration, Griess reagent (50 μL) was added to 50 μL of

collected supernatant and the absorbance was measured at 545 nm (Griess, 1879). Interleukin 1-β (IL-1β) and tumor necrosis factor-α (TNF-α) cytokines levels were assessed by an immunoenzymatic assay (ELISA) kit (eBioscience). WPs was analyzed in triplicate and the optical density was determined at 450 nm.

2.12 STATISTICAL ANALYSIS

Results were expressed as mean ± standard error from wine sample triplicates. Each sample (W5, W10, W15, W20 and W25) was tested in quintuplicate. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's test. Data were considered different at a significance level of $p < 0.05$. Graphs design and statistical analyses were performed using excel version 2010 for Windows and GraphPad Prisma® software version 5.0.

3. RESULTS AND DISCUSSION

3.1 POLYSACCHARIDES STRUCTURAL ANALYSIS

750 mL of each wine were concentrated and polysaccharides recovered by ethanol precipitation followed by centrifugation and dialysis against water (Fig. 2). The solution was then freeze-dried, giving rise to fractions of polysaccharides 0.06% (WP).^{13}C-NMR analysis of the WP fraction showed characteristic signs of polysaccharides and the anomeric region showed a complex profile, with many peak overlaps signals due to mixture of polysaccharides (Fig. 3).

Polysaccharides fractionation and purification were carried out by a freeze-thawing procedure (GORIN; IACOMINI, 1984), resulting in the cold water-soluble fraction. Therefore, this fraction was submitted to complexation with copper, which resulted in a soluble (WFS) and an insoluble (WFP) fractions.

WFS fraction showed a heterogeneous profile (Data not showed). The monosaccharide presented galactose (47.4%), arabinose (20.1%), rhamnose (9.3%), glucose (13.2%) and galacturonic acid (10.0%). Methylation analysis was performed with the carboxy-reduced WFS and the uronic units were converted in to reduced form.

Methylation analysis of WFS showed a polysaccharide by the presence of non-reducing end units of Gal*p*, Ara*f* and the derivatives 2,4-Me_2-Gal*p*, 2,4,6-Me_3Gal*p* and 2,3,4-Me_3-Gal*p*. We suggest that the main chain is composed of *β*-D-Gal*p* (1→3)-linked, due the

presence of high amounts of 2,4,6-Me_3Gal*p*. According to the methylation data, branching points may be situated mainly at *O*-6 position of galactose residues. Side chains may be substituted at *O*-3 position of non-reducing end units of *α*-L-Ara*f*.

In order to resolve the peak overlaps, edited HSQC experiment was performed. WFS showed cross peaks at δ 102.8/4.52 C-1/H-1 of *β*-D-Gal*p* units (Fig. 3). Signals of C-3/H-3 at δ 81.1/3.85 and C-6/H-6 at δ 60.8/3.75 are from →3,6)-*β*-D-Gal*p*-(1→ units, whereas the resonances at δ 80.0/3.71 (C-3/H-3) and δ 69.9/3.75 (C-6/H-6) are from *β*-D-Gal*p* units 3-*O*- and 6-*O*-substituted, respectively (CIPRIANI *et al*., 2009; CIPRIANI*et al*., 2008; DELGOBO *et al*., 1999; FRANSEN *et al*., 2000; GORIN; MAZUREK, 1975). Typical signals of nonreducing end of *α*-L-Ara*f* of C-1/H-1 and C-5/H-5 were observed at δ 107.2/5.08 and δ 74.2/4.15, respectively (DELGOBO *et al*., 1998; RENARD *et al*., 1997). Therefore, the NMR result agreed with the methylation analysis, suggesting that the polysaccharide WFS is a type II arabinogalactan, formed by a (1→3)-linked *β*-D-Gal*p* main chain, substituted at *O*-6 by (1→6)-linked *β*-D-Gal*p* side chains and substituted by nonreducing end-units of arabinose 3-*O*-substituted *α*-L-Ara*f* chains.

Carboxy-reduced derivatives from WFS (Fig. 3) presented Gal*p*A (1→4)-linkages, due the derivative 2,3,6-Me_3-Gal*p* and non-reducing end units of Rha*p* (Sassaki et al., 2005). The presence of 3,4-Me_2-Rha*p* indicates *O*-2-substitution, which is often found in type I rhamnogalacturonan (CARPITA; GIBEAUT, 1993). Convergingly, $^{1}H/^{13}C$ HSQC spectrum contain signals at 100.9/5.08 attributed to C-1/H-1 and δ 77.5/4.45 (C-4/H-4), indicating the sequence of (1→4)-linked *α*-GalA residues. WFS showed cross peaks at δ 68.3/3.82 (C-2/H-2), 70.5/3.97 (C-3/H-3), 69.8/5.02 (C-5/H-5), and 170.5 (C-6) (OVODOVA *et al*., 2009; Popov et al., 2011). Rha*p* units (1→2)-linked were shown by resonances at δ 99.6/5.11 (C-1/H-1) and 16.2/1.25 (C-6/H-6) (RENARD *et al*., 1997). The NMR data and methylation analyses suggest that the WFS also presents a type I rhamnogalacturonan, formed by large sequences of →4)-6-*O*Me-*α*-D-Gal*p*A-(1→ units, interspersed with a few *α*-L-Rha*p* units.

WFP monosaccharide composition showed exclusively mannose and absence of residual protein. The HPSEC analysis showed a homogeneous peak (Fig. 2B), with Mw 60.060 g/mol (dn/dc = 0.272). In order to characterize the glycosidic linkages of the isolated polysaccharide, methylation analysis was performed. The analysis showed a branched polysaccharide due the presence of non-reducing end units, as 2,3,4,6-Me_4-Man*p* and the derivatives 3,4-Me_2-Man*p*, 2,3,4-Me_2-Man*p* and 3,4,6-Me_3-Man*p*. Some mannose units were 6-*O* and 2,6-di-*O*-substituted (KOMURA *et al*., 2010). According to the methylation data, the

presence of the derivative 3,4,6-Me_3-Man*p* indicates thatthe branching points may be situated mainly at *O*-2 position, suggesting (1→2)-linked-Man*p* units.

The $^{1}H/^{13}C$ HSQC spectrum of WFP (Fig. 4) contains signals at δ 97.4/5.09 (C-1/H-1), δ 77.2/3.93 and 77.8/4.02 (C-2/H-2) typical of →2,6)-*α*-D-Man*p*-(1→. Also, the presence of signals at δ 98.5/4.88, 99.7/5.27, 101.3/5.03 and 101.4/5.13 (C-1/H-1) and δ 79.2/4.08 (C-2/H-2) typical of →2)-*α*-D-Man*p*-(1→ (KOBAYASHI *et al*., 1995; VINOGRADOV, PETERSEN; BOCK, 1998). In addition, signals at δ 68.4/4.20 and 69.3/4.05 (C-3/H-3), 65.4/3.78 and 66.9/3.62 (C-4/H-4), 69.6/3.87 and 72.4/3.75 (C-5/H-5) and 60.2/3.88 and 60.3/3.74 (C-4/H-4) (Kobayashi et al., 1995; Komura et al., 2010; Vinogradov et al., 1998). Results from NMR and methylation analysis in agreement suggest that WFP is a mannan formed by large sequences *α*-D-Manp (1→6) linked and side chains *O*-2 substituted for *α*-D-mannan (1→2) linked.

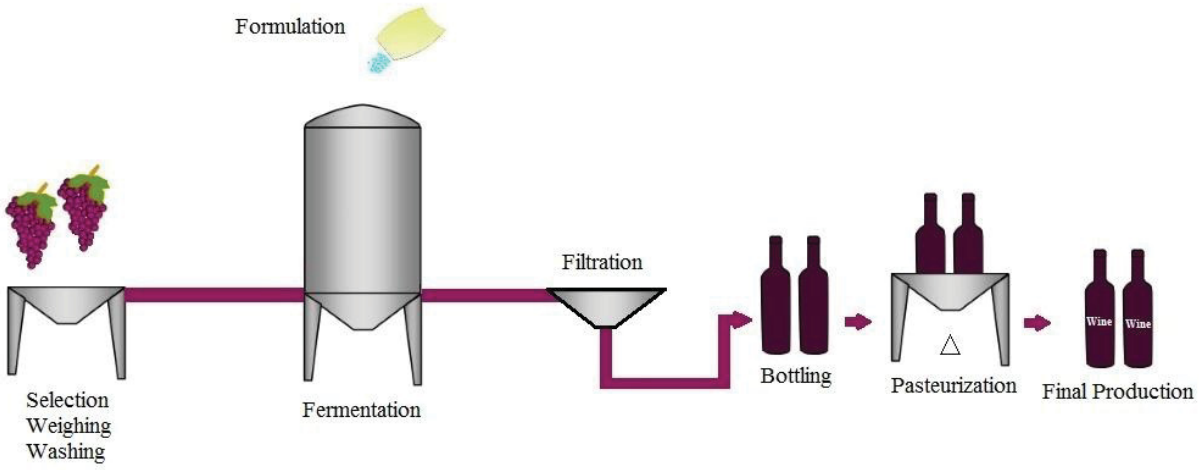

FIGURE 1. Diagram of the wine production process

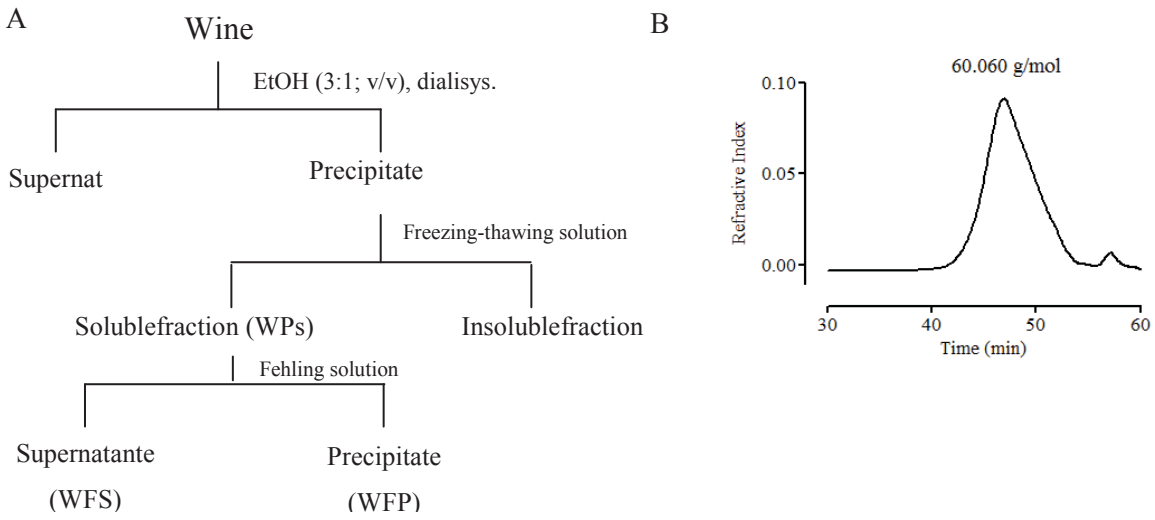

FIGURE 2.Scheme of extraction and purification of polysaccharides (A). Elution profiles of WFP (B).

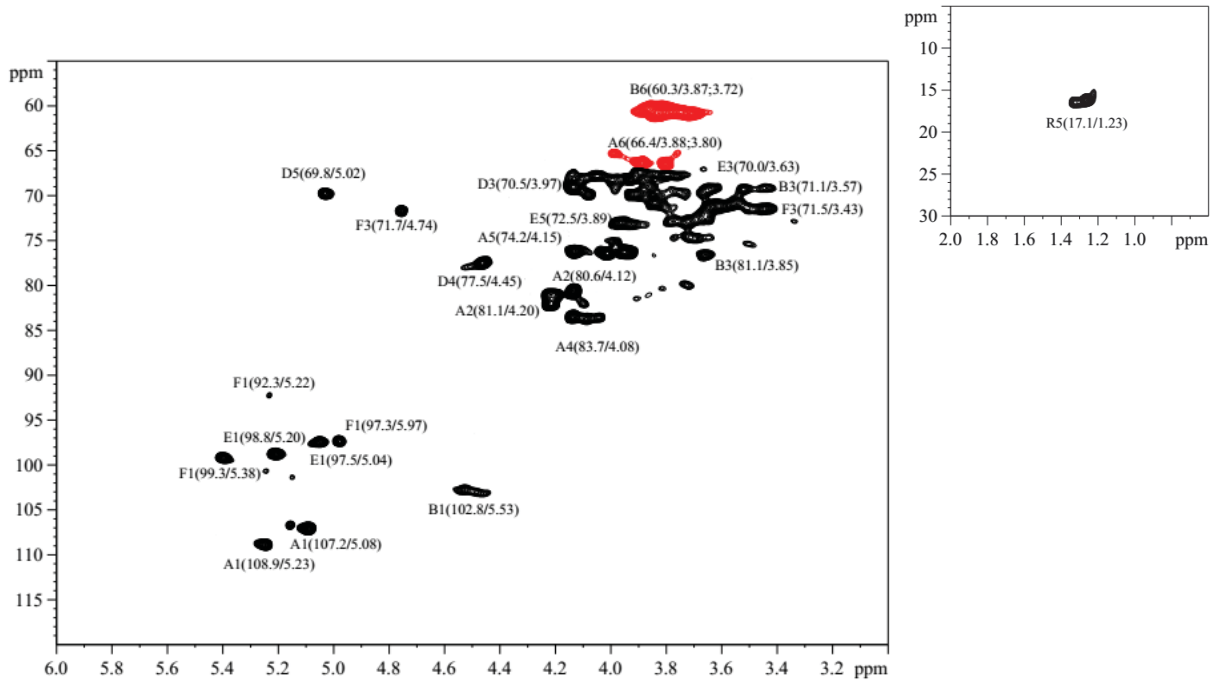

FIGURE 3.$^{1}H/^{13}C$ HSQC NMR map of correlation of WFS. Solvent D_2O at 30 ºC; numerical values are in δ ppm. A (*α*-L-Ara*f*); B (*β*-D-Gal*p*); C (*α*-L-Rha*p*); D (*α*-D-Gal*p*A); E (*α*-D-Man*p*); F (*α*-D-Glc*p*). The letters are followed by the carbon number of the monosaccharide unit.

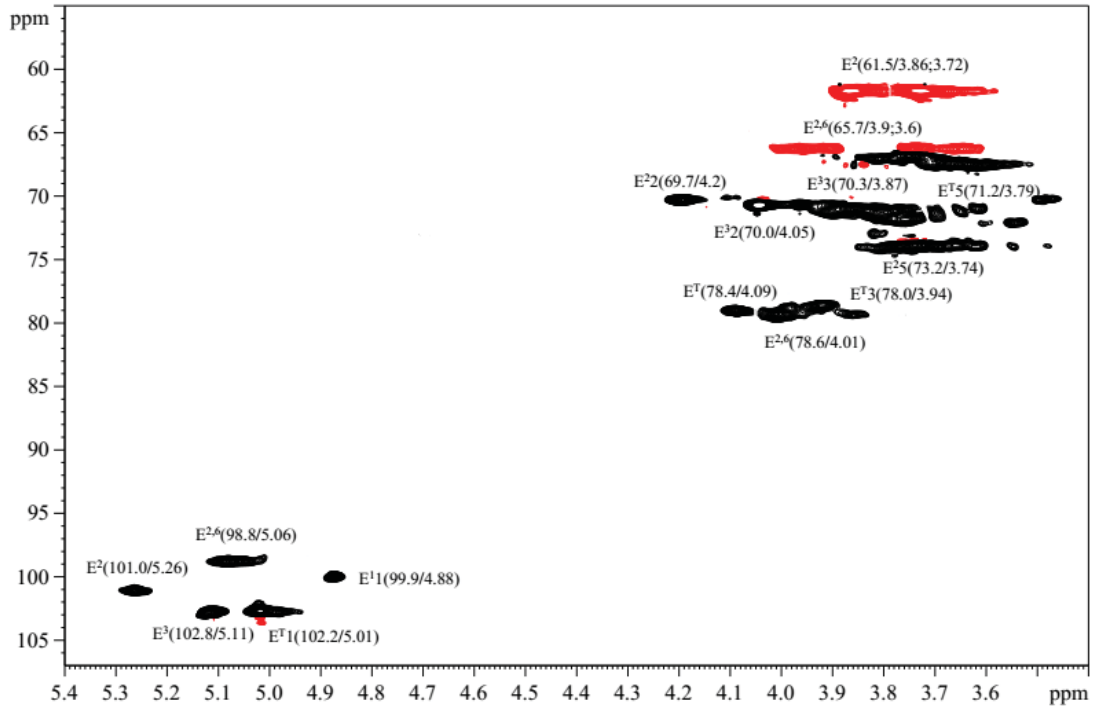

FIGURE 4.^{1}H/^{13}C HSQC NMR spectrum of WFP. Solvent D_2O at 30 ºC; numerical values are in δ ppm. E (Man*p*).

3.2 ETHANOL CONCENTRATION (Ec) IN WINE

Ethanol production by fermentation involves 12 reactions in an orderly sequence (LIMA *et al*., 2001) and consists of the transformation of sugar into alcohol and carbonic gas under the action of yeasts. The determination of Ec is critical for wine quality control (FLETCHER; VAN STADEN, 2003). The values obtained for the Ec were calculated by NMR, providing an estimate of the variation of ethanol formation during the 25 days of production, correlating with the consumption of the carbohydrates present in the medium. Analyzing the^{1}H-NMR spectrum (Fig.5) we can observe an increase in the ethanol concentration in 5 - 20 days of fermentation, which varies from 2 to 18° GL, and its decrease for 15° GL after 25 days. The exact concentration of ethanol in each of the fermentation phases was then determined. Ethanol and CO_2 are excretion products, without metabolic utility for yeast in anaerobiosis (LIMA *et al*., 2001). For wine quality the amount of ethanol is in the range between 9 and 15º GL (MATAIX; LUQUE DE CASTRO, 2000).The volatile compounds are important factors that affect the wine flavor quality. Ethanol concentrations above 15º GL become toxic to the enzymes involved in the fermentation (NICULESCU *et al*., 2003).

Volatile compounds are responsible for the olfactory characteristics of wines. Low concentrations of these components are important for their quality. Methanol has a toxicity problem, in general, all wines contain methanol, in doses ranging from 50 to 300 mg/L on average. However, several studies point out that wines made from *Vitis labrusca* actually have more methanol in their composition than wines made with *Vitis vinifera* (MIELE *et al*., 2016). The main reason for this difference is due to the greater presence of pectin polysaccharides in species *Vitis labrusca* due to the methanol present in the wine being generated by the hydrolysis of the methoxyl groups of the pectin of the grape during the fermentation. The wine produced from the 15th fermentation day 87 mg/L, followed by 129 mg/L and 297 mg/L, on the 20th and 25thdays, respectively. Values within the limits of Brazilian legislation that is 350 mg/L (Brazil, 1998). During the course of winemaking and in the finished wine, acetic, lactic and succinic acid have a significant role in the quality. From the results obtained with the concentration ratio between acetic, lactic and succinic acid, which was 0.1 -0.8 g/L , 0.1 - 1.0 g/L and 0.05 - 2.9 g/L in wine (SIRÉN *et al*., 2015).. It was observed that when the samples were at acid pH these had a higher δ when compared to the basic pH because of the acids present, they were in their protonated form. The data of δ are shown in the table 1.

Table 1. The NMR signals obtained from wine.

Compound	H1	H2	H3’	pH acid	pH basic
Ethanol	1.2	3.6	-	-	-
Methanol	3.3	-	-	-	-
Lactic acid	1.3	4.1	-	1.3/4.1	1.2/4.0
Acetic acid	2.0	-	-	2.0	1.8
Succinic acid	2.6	-	-	2.6	2.3
Glucose	5.2	-	-	-	-
Sucrose	5.4*	-	4.2**	-	-

* H1 (Glc) **H3 (Fru)

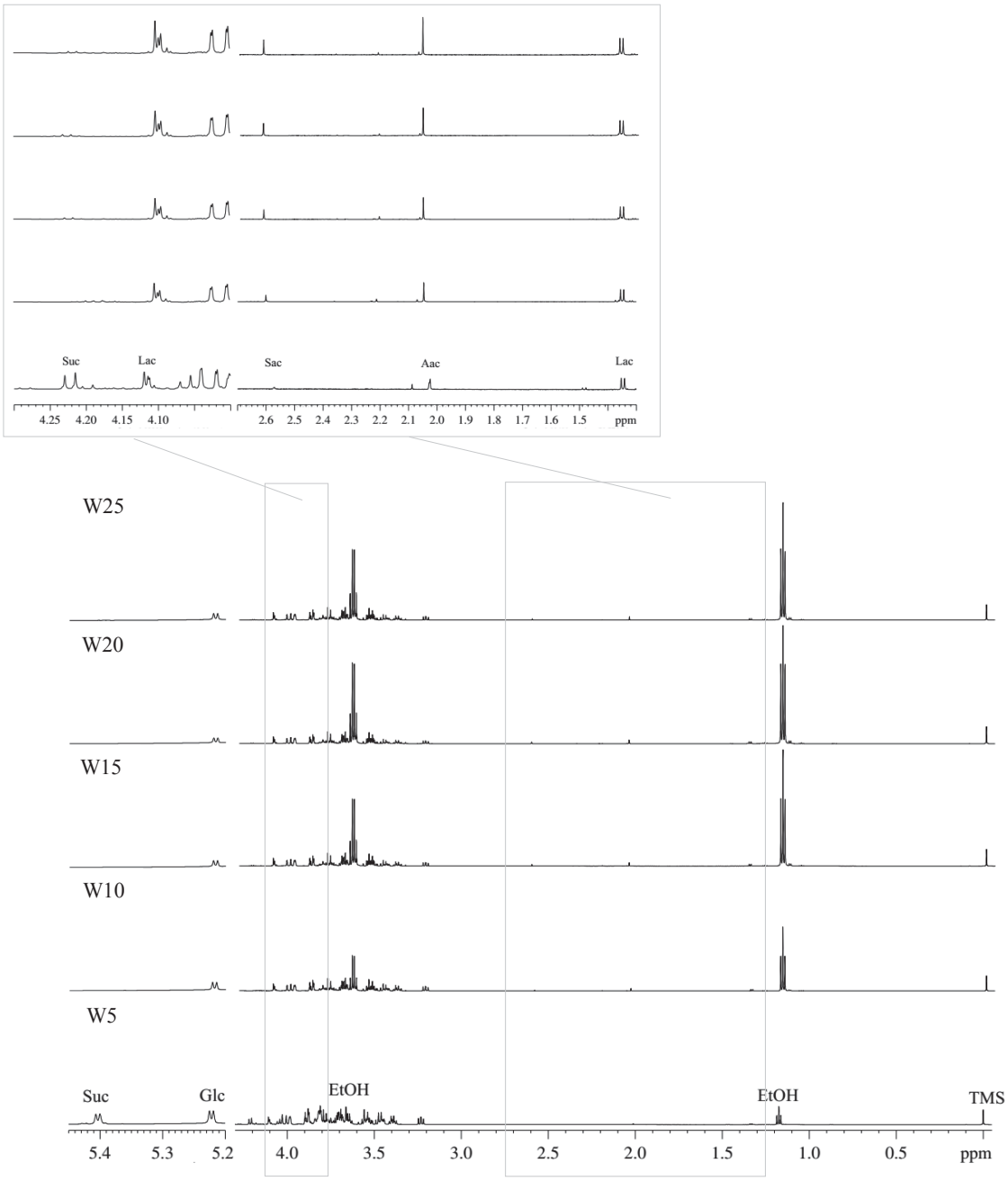

FIGURE 5.^{1}H HSQC spectrum of W5, W10, W15, W20 and W25. Solvent D_2O at 30 ºC; numerical values are in δ ppm.

Subtitle: Ethanol (EtOH); Lactic acid (Lac); Acetic acid (Aac); Succinic acid (Sac) Glucose (Glc); Sucrose (Suc).

3.3 POLYSACCHARIDE CONCENTRATION (Pc) IN WINE

Polysaccharides constitute one of the main groups of wine macromolecules and are derivatives both from the grapes and cell walls of yeast used in the fermentation process

(BOULET *et al*., 2007). Based on chemical structure, the main polysaccharides found in wine are type II arabinogalactan, type I and II rhamnogalacturonan, and mannan. Arabinogalactans represent an important part of the total soluble polysaccharides: between 100-200 mg/L in red wines (DOCO *et al*., 2000). The rhamnogalacturonans are complex, acidic polysaccharides with a low degree of polymerization (~30) with average content in red wines between 100-150 mg/L. Mannan produced by yeast *Saccharomyces cerevisae* are abundant in wines (150 mg/L) (DOCO*et al*., 2000). However, its quantity depends on the vinification process (DOCO *et al*., 2003) and the yeast strain used (ESCOT*et al*., 2001). Some values found in this wine are 198 mg/mLfor type II arabinogalactan, 51 mg/mL for rhamnogalacturonans and 197 mg/mL for manana. The values obtained for Pc were calculated by NMR, providing an estimate of the variation during the 25 days of production, according to the graph shown in Figure 6. We can observe that the concentration of dextrin decreases from 37% to 0.9% with the ethanol increase, confirming the consumption of this polysaccharide by the yeasts during the fermentation process. The mannan production was 25% in day 5 and 39% in the last day. Mannan should show a significant increase, having its peak of production on the 15th day, with an amount of 50%. Type II arabinogalactan in fifth day was 17% and in the last day 39%. However, type I and II rhamnogalacturonan presented low variation during the 25 fermentation days,ranging from 11% in the fifth day to 10% in the last day (fig.6).

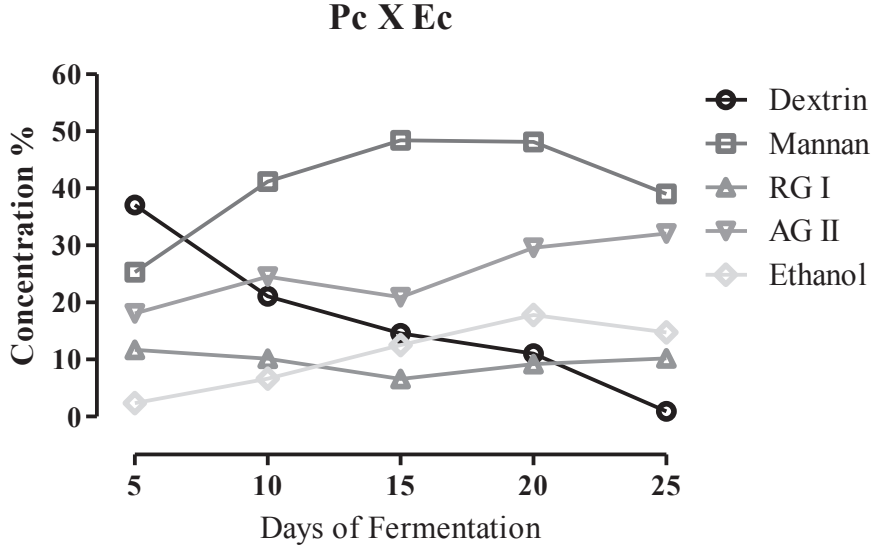

FIGURE 6. Graph of the concentration variation of the polysaccharides, on the different days of fermentation.

3.4. ANTI-INFLAMMATORY ACTIVITY

Inflammation is an essential protective process that helps to preserve the integrity of an organism and leads to the release of a large number of inflammatory mediators (ACHOUI *et al*., 2010; CHEN *et al*., 2010). Macrophages play a central role in managing many different immune-pathological phenomena, such as the overproduction of the pro-inflammatory cytokine, tumor necrosis factor (TNF)-α and inflammatory mediators with nitric oxide (NO) (LUNDBERG, 2000).

Cytotoxicity was evaluated by measuring cell viability after 24 h of exposure by the MTT assay. None of the concentrations (0.1, 1, 10 and 100ug/mL) reduced cell viability below 95% of the control (Fig. 7A), and thus were not considered cytotoxic up to 100 µg/mL. When LPS was administered to RAW 264.7 macrophages, NO and cytokines production levels increased dramatically (positive control). Results showed that WPs inhibited cytokines and NO production significantly. NO decreased in a dose-dependent manner ($p < 0.05$):(46-53%) (Fig. 7B). Inflammatory cytokines (TNF-α and IL-1β) reduced TNF-α and IL-1β production significantly, 14-65% (Figure 7C) and 31-47% (Fig. 7D), respectively.

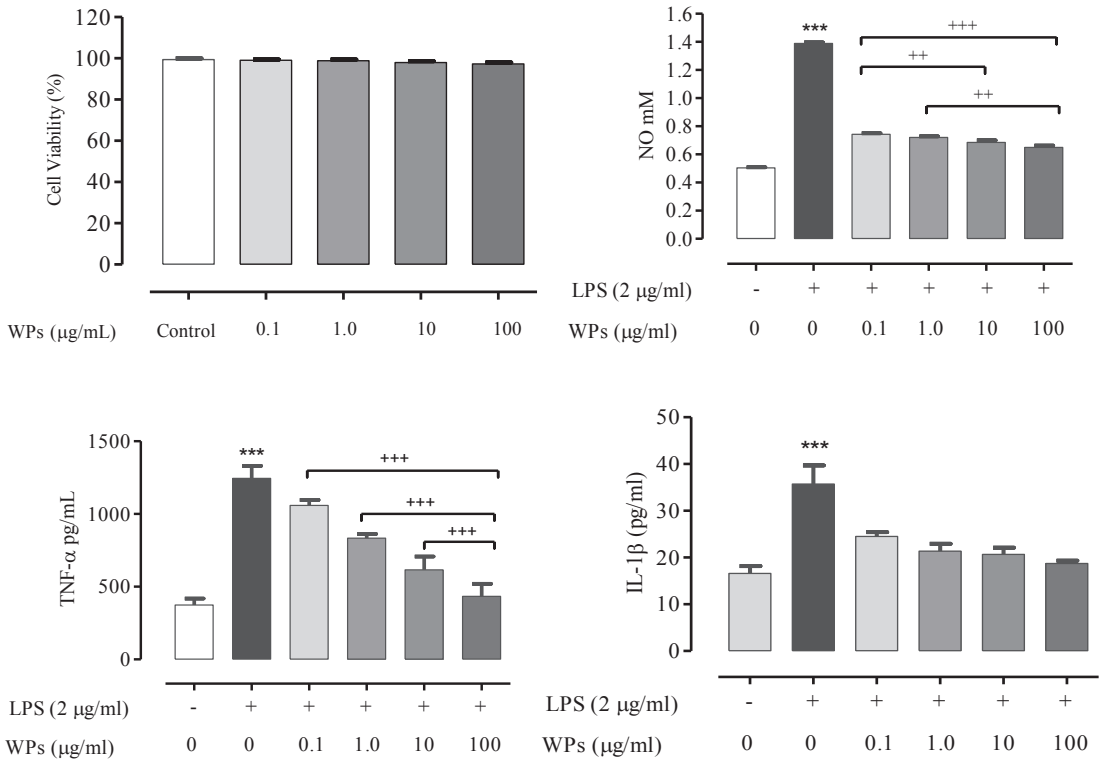

FIGURE 7. (A)WPs effects treated with different concentrations 0.1, 1.0, 10, and 100 µg/mLon cell viabilities of RAW264.7 macrophages. The viability of cells without sample

and with Lipopolysaccharide (LPS) at 2 μg/mL has been taken as reference (100%) - was selected as a positive control. (B) NO concentration in the culture supernatant was determined by Griess assay using sodium nitrite standard curve. (C) TNF-α concentration in the culture supernatant was determined by ELISA using standard curve. (D) IL-1β concentration in the culture supernatant was determined by ELISA using standard curve.

All data are presented as means ± SD from three sets of independent experiments. * $p < 0.05$ represent significant difference compared with cells treated with LPS alone and $p < 0.05$ represent significant difference compared with control group.

4. CONCLUSION

The wine production was monitored by NMR analysis, being possible to determine the better fermentation time, where the wine retains its highest concentration of ethanol and a higher formation of compounds. It was possible to reach 17% of ethanol in 20 days. The present study showed, based on the chemical structure, that the polysaccharides found in wine produced from *Vitis labrusca* of the variety Bordo are: type II arabinogalactan, formed by a (1→3)-linked *β*-D-Galp main chain, substituted at *O*-6 by (1→6)-linked *β*-D-Gal*p* side chains, respectively and nonreducing end-units of arabinose 3-*O*-substituted *α*-L-Ara*f* chains; type I rhamnogalacturonan, formed by sequences of →4)-6-*O*Me-*α*-D-Gal*p*A-(1→ units, interspersed with a few *α*-L-Rha*p* units; and mannan, formed by sequences *α*-D-Man*p* (1→6) linked and side chains *O*-2 substituted for *α*-D-mannan (1→2) linked. Anti-inflammatory results showed that WPs not only greatly decreased the pro-inflammatory cytokine secretion ratios, including TNF-α and IL-1β, but also reduced release levels of nitric oxide (NO). These results indicated that the crude fraction WPs possess anti-inflammatory activity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CAPÍTULO VI

Neste capítulo encontram-se as considerações finais, composta de considerações finais, resultados complementares, conclusão geral e referências da tese.

1. CONSIDERAÇÕES FINAIS

1.1 COMPARAÇÃO ENTRE AS FRAÇÕES POLISACARÍDICAS

Na presente pesquisa foram estudadas as amostras de uma espécie de amora-preta (*Rubus fruticosus*) e o fermentado de amora (produzido na adega Porto Brazos). Conforme descrito anteriormente nos artigos, cada material estudado passou por processos de extração e purificação, para análise dos polissacarídeos. Da amora-preta foi obtida a fração aquosa polissacarídica PBFs (Blackberry fruit polysaccharides soluble) e do fermentado de amora foram obtidas três frações polissacarídicas BWPs (Blackberry wine polysaccharides soluble), BWPFs (Blackberry wine polysaccharides Fehling soluble) e BWPFp (Blackberry wine polysaccharides Fehling precipitate).

O polissacarídeo majoritário presente na fração PBFs, obtido por extração aquosa a partir da amora-preta (*Rubus fruticosus*) foi uma homogalacturana (HG) com alto grau de metil esterificação aproximadamente 72%, bem como uma arabinogalactana do tipo II. Formada por uma cadeia principal de unidades de β-D-Gal*p* (1→3)-ligadas, substituída em *O*-6 por cadeias laterais de β-D-Gal*p* (1→6)-ligadas. As cadeias laterais são substituídas em *O*-3 por terminais não redutores de α-L-Ara*f*. Esta arabinogalactana do tipo II provavelmente está ligada na posição *O*-4 de algumas unidades de ramnose, de uma ramnogalacturonana do tipo I, formada pela repetição da unidade dissacarídica [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→].

Três frações de polissacarídicas foram obtidas do fermentado de amora-preta. O extrato bruto de BWPs foi obtido por meio da precipitação com etanol e pelo processo de congelamento-descongelamento, sendo então submetido ao tratamento com Fehling, fornecendo BWPFs solúveis e frações insolúveis de BWPFp. Os principais polissacarídeos foram identificados para cada fração: manana, arabinogalactana tipo II e ramnogalacturonano tipo I para BWPs, uma manana formada por uma cadeia principal de unidades ligadas a α-Man*p* (1→6), *O*-2 substituído por cadeias laterais ligadas α-D-Manp (1→2) para BWPFs e uma AG II formada por uma cadeia principal de β-D-Gal*p* (1→3) ligada, substituída em *O*-6 pelas cadeias laterais da β-D-Gal*p* (1→6) ligados, que então são substituídos em *O*-3 por unidades não redutoras de α-L-Ara*f* e um RG I, formado por [→ 4) -α-D-Gal*p*A- (1→2) - α-L-Rha*p*- (1→. Em BWPFp uma manana formada por uma cadeia principal de unidades ligadas a α-Man*p* (1→6), *O*-2 substituído por cadeias laterais ligadas α-D-Man*p* (1→2).

Para analisar farmacologicamente as frações, foi realizada a avaliação da atividade anti-inflamatória com macrófagos RAW 264.7 (Tabela 1) com as frações PBFs e BWPs

(BWPs escolhida por conter todos os polissacarídeos presentes também nas outras frações obtidas do fermentado).

TABELA 1- ANÁLISE DA ATIVIDADE ANTI-INFLAMATÓRIA *in vitro* DA AMORA-PRETA (*Rubus* sp.) E DO FERMENTADO DE AMORA.

Fração	**Concentração μg/ml**	**NO** %	**TNF-α** %	**IL-1β** %
BFPs	0,1	46,9	48,5	36,9
	1,0	55,0	48,0	42,6
	10	58,2	60,7	53,8
	100	58,1	64,7	53,9
BWPs	0,1	44,8	56,9	34,7
	1,0	49,1	51,4	36,8
	10	54,9	43,3	36,8
	100	64,1	64,5	45,3

A avaliação da atividade anti-inflamatória das frações polissacarídicas PBFs e BWPs, em camundongos, quando analisada em vivo, demonstram que foram capazes de reduzir a mortalidade (Tabela 2) dos animais frente à sepse, bem como a diminuição dos marcadores bioquímicos.

TABELA 2- ANÁLISE DA ATIVIDADE ANTI-INFLAMATÓRIA *in vivo* DA AMORA-PRETA (*Rubus* sp.) E DO FERMENTADO DE AMORA.

Fração	**Concentração** mg/kg	**Sobrevivência** (oral)%	**Concentração** mg/kg	**Sobrevivência** (Subcutânea)%
BFPs	3,0	25	0,1	13
	7,0	38	1,0	67
	10	50	10	50
BWPs	3,0	25	0,1	50
	7,0	38	1,0	88
	10	63	10	88

Este estudo fornece evidências de que os polissacarídeos da amora preta (*Rubus fruticosus*) e fermentado de amora atenuam o grau de sepse em camundongos, observamos e comparando os resultados podemos concluir que a via subcutânea foi mais efetiva, abrindo caminho para novos estudos para a ação farmacologia destes compostos por outras vias.

2. RESULTADOS COMPLEMENTARES

Os frutos da amora preta foram liofilizados e moídos (500 g). O fruto seco foi desengordurado com clorofórmio-metanol (2:1) como o objetivo da remoção lipídios e

pigmentos. Os polissacarídeos foram extraídos do resíduo com água a 100 °C durante 2 h (5 vezes, l L cada). Os extratos aquosos foram obtidos combinados e concentrados sob pressão reduzida. Os polissacarídeos foram recuperados por etanol (3 vol.). Recolhidos por centrifugação (5000 rpm, 20 min a 4 ° C) e liofilizados, dando fração PBF (estuda no capitulo II dessa tese) (Cordeiro et al., 2012). O resíduo da extração aquosa foi submetido à extração com KOH 5% durante 100 °C durante 2 h (× 3, lL cada), sob refluxo, obtendo-se o extrato alcalino (BK), em seguida foi neutralizado e precipitado com etanol 3:1. A fração obtida POK foi submetida a fracionamento por congelamento-degelo obtendo-se as frações Pks solúvel e Pkp insolúvel. O fluxograma do processo de purificação utilizado encontra-se na FIGURA 1B (capitulo II). Os processos de purificação das extrações foram acompanhados pela análise da composição monossacarídica, bem como por análises de HPSEC, CG-MS e RMN.

Na composição monossacarídica a fração PKs obtida da extração alcalina apresentou um perfil heterogêneo quando analisado por HPSC (FIGURA 1A), tem como principal monossacarídeo a galactose 34.6 % seguida de ramnose 19.2%, arabinose 19.2%, glucose 9.5% e xilose 4.9%. O mapa de correlação de 2D - HSQC (RMN ^{1}H-^{13}C) da fração PKs, (FIGURA 2) contém sinais em δ 103.5/4.46 (C-1/H-1), 71.3/3.48 (C-2/H-2), 81.4/3.97 (C-3/H-3), 75.1/3.61 (C-5/H-5) e 66.1/3.68(C-6/H-6 substituído; invertido no DEPT), típicos de unidades de →3,6)-β-D-Gal*p*-(1→, e sinais em δ 106.3/5.01 (C-1/H-1) de *α*-L-Ara*f* presentes em arabinogalactanas do tipo II. O espectro também apresenta sinais em δ 101.2/4.31 (C-1/H-1) atribuídos a *β*-D-Xyl provavelmente referente a uma xilana (1→4) (VIGNON; GEY, 1998) e sinais em δ 98.3/4.9 (C-1/H-1), 16.3/1.14 (C-1/H-1) de *α*-L-Rha*p* que pode estar relacionada a uma ramnogalacturonana.

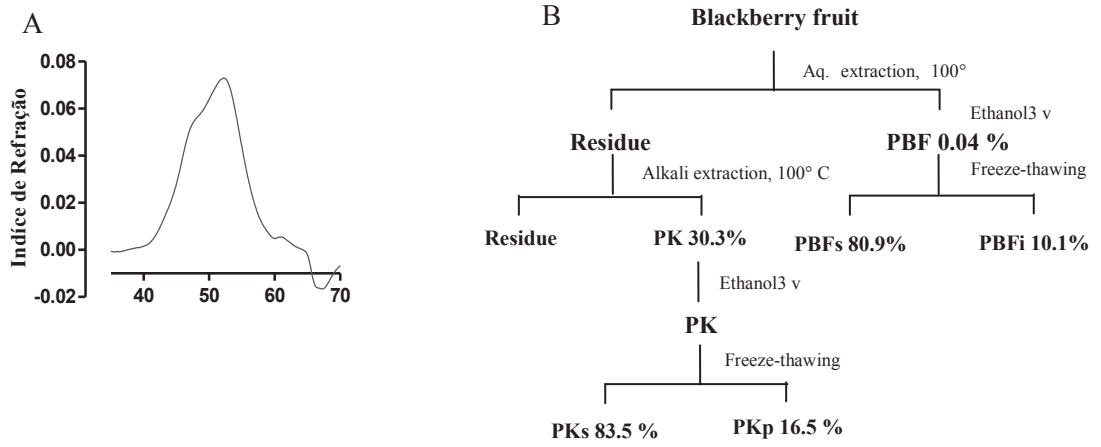

FIGURA 1. (A) HPSC FRAÇÃO PKs (B) FLUXOGRAMA DA EXTRAÇÃO E FRACIONAMENTO DOS POLISSACARÍDEOS DA AMORA-PRETA.

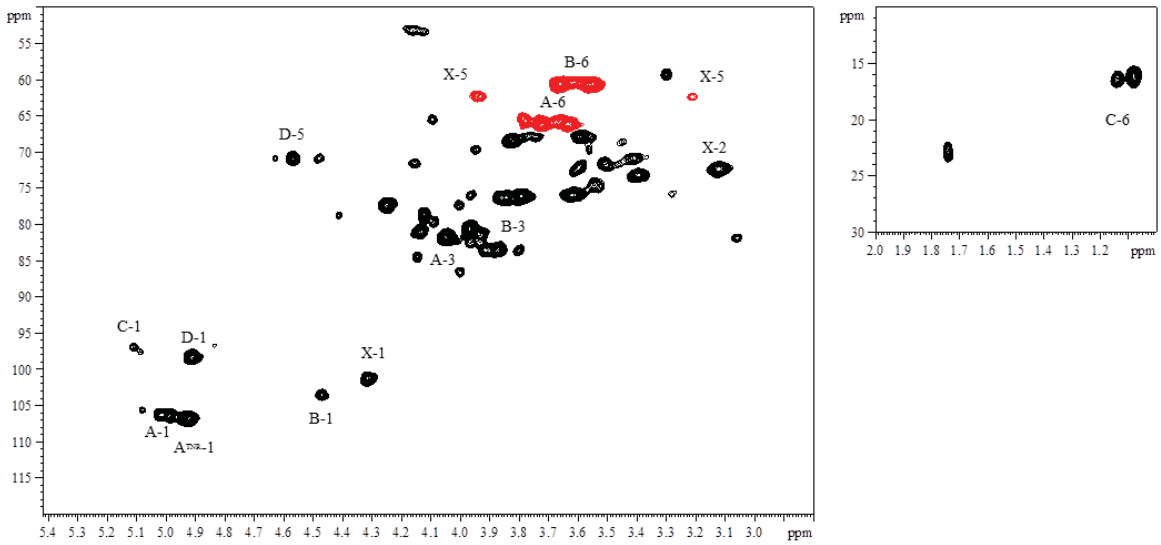

FIGURA 2. MAPA DE CORRELAÇÃO DE ^{1}H-^{13}C HSQC DA FRAÇÃOPKs - 30 °C USANDOD_2O COMO SOLVENTE. A (*α*-L-Ara*f*); B (*β*-D-Gal*p*); C (*α*-L-Rha*p*); D (*α*-D-Gal*p*A); X (*α*-D-Xyl).

3. CONCLUSÃO GERAL

Após a análise dos resultados obtidos nesta pesquisa, pode-se concluir que:

- A amora-preta pode ser considerada uma fonte de polissacarídeos com atividade anti-inflamatória. A fração aquosa bruta PBFs apresentou como principais polissacarídeos homogalacturonana altamente metil esterificada com unidades de *α*-D-Gal*p*A-1,4-ligadas e grau de metil esterificação igual a 72%. Além de AG II formada por uma cadeia principal de ligações *β*-D-Gal*p* (1→3), substituídas em cadeias laterais *O*-6 por cadeias laterais ligadas em *β*-D-Gal*p* (1→6) substituídas em *O*-3 por *α*-L-Ara*f*. A fração alcalina apresentou AG II, ramnogalacturonanas e xilana (1→3) ligada como principais polissacarídeos (necessitando ainda de mais estudos).

- O fermentado de amora é um dos produtos feitos a partir da amora preta com maior valor agregado de mercado. Dele foram caracterizadas três frações de polissacarídeos. BWPs a qual foi obtida da precipitação com etanol seguida de congelamento-descongelamento, foi submetida ao tratamento de Fehling, obtendo-se as frações solúvel BWPFs e insolúvel BWPFp. Os principais polissacarídeos identificados para cada fração foram:BWPs -manana formada por uma cadeia principal de unidades ligadas a α-Man*p*(1→6), *α*-D-Man*p* substituído com *O*-2 (1→2), AG II formado por uma cadeia principal de ligações *β*-D-Gal*p* (1→3), substituídas em *O*-6 por cadeias laterais da *β*-D-Gal*p* (1→6), cadeias laterais ligadas em *O*-3

por não-redução de *α*-L-Ara*f* e RG I , formadas por [(4)-*α*-D-Gal*p*A-(1→2)-*α*-L-Rha*p*-(1→]$_n$, em BWPs foi identificada a AG II e a RG I já no precipitado BWPFp encontrou-se como polissacarídeo principal a manana.

- Os efeitos anti-inflamatórios das frações polissacarídicas PBFs (amora preta) e BWPs, BWPFs e BWPFp (fermentado de amora) foram avaliadas na linhagem celular RAW 264.7. As frações reduziram marcadamente a produção de óxido nítrico (NO) e citocinas pró-inflamatórias (TNF-α e IL-1β) em células tratadas com LPS. Além disso, nas análises*in vivo*onde os camundongos foram submetidos a CLP os resultados mostraram que a administração oral e subcutânea dos polissacarídeos apresentou um aumento na sobrevivência dos camundongos com sepse, quando comparados com o controle.Levando também a diminuiçãodos níveis de citocinas pró-inflamatórias, bem como dos marcadores ureia, creatinina e bilirrubina. Na avaliação histoquímica apresentou uma melhora significativa dos tecidos fígados, pulmões e rim.

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