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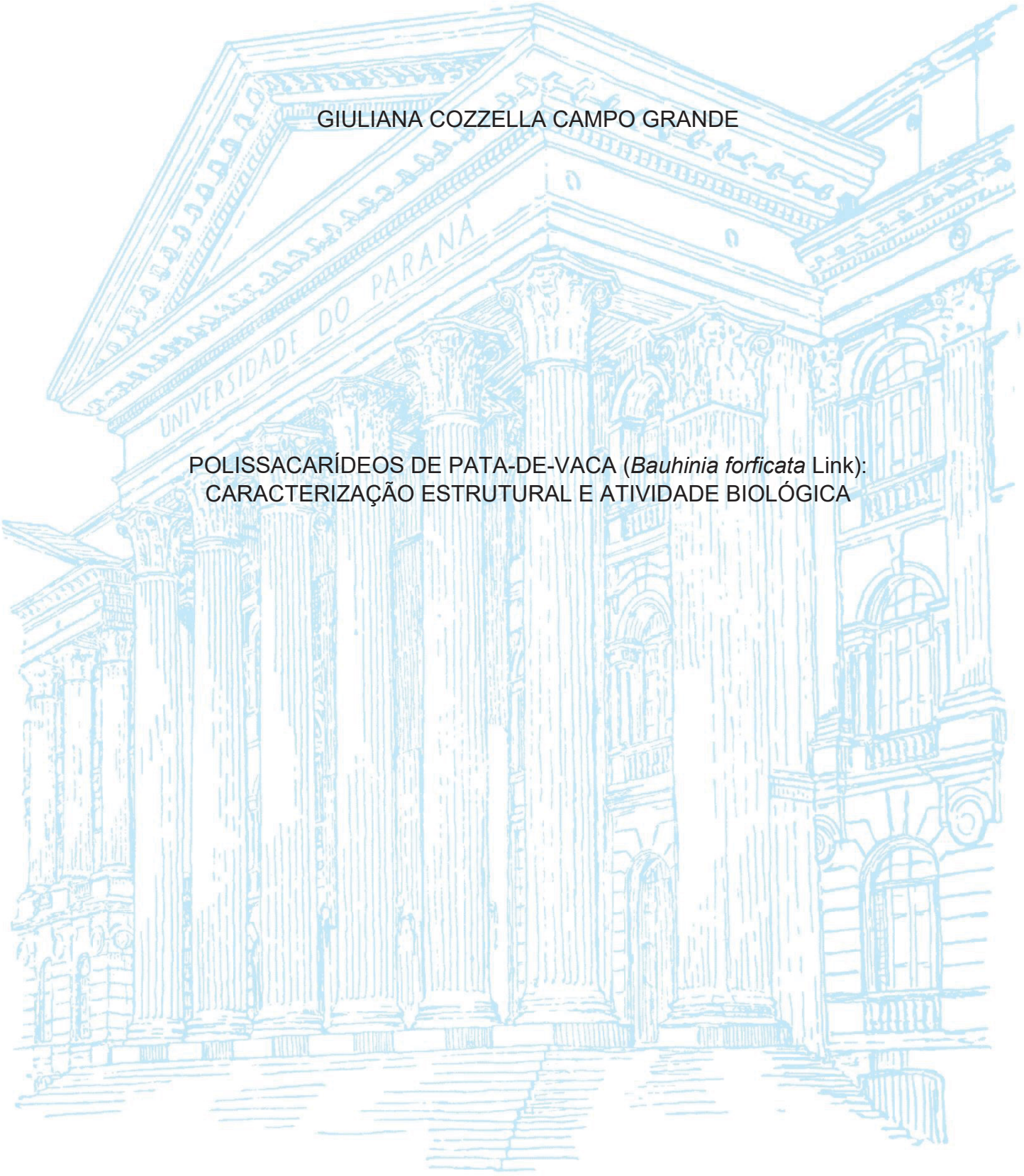
GIULIANA COZZELLA CAMPO GRANDE




# POLISSACARÍDEOS DE PATA-DE-VACA (*Bauhinia forficata* Link): CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE BIOLÓGICA

CURITIBA

2023

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# POLISSACARÍDEOS DE PATA-DE-VACA (*Bauhinia forficata* Link): CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE BIOLÓGICA

Tese apresentada ao Programa de Pós-Graduação em Ciências (Bioquímica), Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Ciências (Bioquímica).

Orientador: Prof. Dr. Thales Ricardo Cipriani

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## TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **GIULIANA COZZELLA CAMPO GRANDE** intitulada: **POLISSACARÍDEOS DE PATA-DE-VACA (*Bauhinia forficata* Link): CARACTERIZAÇÃO ESTRUTURAL E ATIVIDADE BIOLÓGICA**, sob orientação do Prof. Dr. THALES RICARDO CIPRIANI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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

CURITIBA, 16 de Junho de 2023.

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28/06/2023 22:50:00.0
THALES RICARDO CIPRIANI
Presidente da Banca Examinadora

Assinatura Eletrônica
26/06/2023 14:30:32.0
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26/06/2023 15:12:12.0
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## NOTA EXPLICATIVA

Esta tese está estruturada em formato alternativo, conforme as normas do Sistema de Bibliotecas (SiBi) da Universidade Federal do Paraná (UFPR). A tese contém introdução, revisão bibliográfica, justificativa, objetivos, artigos científicos, conclusões, referências e anexos. Os artigos científicos incluem introdução, materiais e metodologias, resultados e discussão, referências e material suplementar.

## DEDICATÓRIA

Dedico este trabalho à minha família: à minha mãe Andréa, meu irmão Luís e ao meu namorado, João Paulo, que são minhas bases de apoio e foram minhas maiores fontes incentivo ao longo da minha jornada acadêmica.

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Há de vir o tempo no qual uma pesquisa diligente durante longos períodos revelará coisas que hoje estão ocultas [...]. E por isso esse conhecimento só se desdobrará ao longo de sucessivas eras. Virá um tempo no qual nossos descendentes ficarão espantados com o fato de que não sabíamos de coisas que para eles serão tão evidentes [...]. Muitas descobertas estão reservadas para épocas ainda por vir, quando a lembrança sobre nós estará apagada. Nosso universo é um caso lamentavelmente ínfimo, a menos que encerre coisas que cada época terá de investigar [...]. A natureza não revela seus mistérios de uma só vez.

Sêneca, Questões naturais, livro 7, século I

‘’Há verdadeiramente duas coisas diferentes: saber e crer que se sabe. A ciência consiste em saber; em crer que se sabe reside a ignorância.’’

Hipócrates

## RESUMO

*Bauhinia forficata* Link é uma planta medicinal popularmente conhecida como pata-de-vaca e possui diversas aplicações biológicas, entre elas tratamento da inflamação e como complementar no tratamento do diabetes. Este trabalho teve como objetivo geral extrair e isolar uma fração rica em polissacarídeos das folhas de *B. forficata*, os caracterizar estruturalmente e avaliá-los quanto a sua contribuição para a atividade hipoglicemiante e antidiabética *in vivo* e para a atividade anti-inflamatória *in vitro*. A partir do extrato aquoso feito com as folhas da planta foram obtidas duas frações: BFSGD (BFSF), sobrenadante do processo de congelamento e degelo a temperatura ambiente, e TCA-S, sobrenadante do tratamento da fração BFSGD com ácido tricloroacético (TCA), com a finalidade de desproteinização. Em relação à fração BFSGD, ela apresentou perfil de eluição quase homogêneo na análise de homogeneidade por HPSEC-RI e nas análises de dosagens espectrofotométricas apresentou 50,4% de açúcares totais, 15,1% de proteínas, e 15,0% de compostos fenólicos. A análise de composição monossacarídica por GC apresentou 27,4% de arabinose, 23,7% de galactose, 16,1% de ramnose, 13,8% de glucose, 6,2% de manose, 2,5% de xilose, 0,3% de fucose e 10,0% de ácido galacturônico, este último determinado por método colorimétrico. Através das análises de RMN e metilação, foi possível concluir que os polissacarídeos presentes na fração BFSGD são homogalacturonana, arabinana, amido e arabinogalactanas tipos I e II. A fração BFSGD, nas doses de 10, 30, 100 e 300 mg/kg, foi analisada quanto ao seu potencial hipoglicemiante pelo modelo animal de diabetes induzido por estreptozotocina em ratos. Foram avaliados os efeitos agudo (3 h) e crônico (21 dias) da fração sobre os animais. A fração não promoveu alteração na glicemia em comparação ao grupo controle, tratado apenas com salina, no período do experimento. Após o período do teste e eutanásia dos animais, o sangue foi coletado e foram realizadas análises de parâmetros bioquímicos e hematológicos. Dentre os parâmetros avaliados, observou-se diferença estatística em relação aos níveis de bilirrubina conjugada, ocorrendo diminuição de 63,7% na dose de 10 mg/kg e de 54,6% na dose de 30 mg/kg, e foi observada diminuição dos níveis de enzima AST: a fração na dose de 10 mg/kg foi capaz de reduzir em 43,7% sua concentração em comparação ao grupo controle. Os estudos histológicos de fígado demonstraram que a fração, nas doses de 30 e 100 mg/kg, foi capaz de amenizar significativamente o aparecimento e a intensidade de injúria tecidual, em 67,0% e 69,4%, respectivamente. Esses resultados indicam efeito hepatoprotetor exercido pela fração BFSGD. Em relação à TCA-S, a fração apresentou perfil de eluição homogêneo em HPSEC-RI, 81,6% de açúcares totais, 12,6% de compostos fenólicos e 4,7% de proteínas totais e é constituída de 38,2% de arabinose, 20,0% de galactose, 17,6% de ramnose, 15,1% de glucose, 6,3% de manose, 1,4% de xilose e 1,3% de ácido galacturônico. As análises de RMN e metilação demonstraram que a fração é constituída principalmente por arabinogalactanas tipos I e II, arabinana e amido. O amido foi removido de TCA-S através de tratamento com α-amilase, e a fração livre de amido foi renomeada TCA-Sα. A avaliação do potencial imunomodulatório de TCA-Sα, realizado através de células THP-1, demonstrou que a fração apresenta propriedade imunoestimulatória nas concentrações 50, 100 e 500 µg/mL, aumentando a concentração de todas as

citocinas quantificadas no sobrenadante do cultivo celular (TNF-α, IL-1β e IL-10), e efeito imunomodulador na presença de LPS, na concentração de 50 µg/mL. A razão entre as concentrações das citocinas pró e anti-inflamatórias, estimuladas pelo controle positivo (LPS) sozinho e por TCA-Sα junto com LPS foi calculada para cada concentração. A diminuição dos valores das razões em todas as concentrações do grupo TCA-Sα + LPS em comparação com ao controle positivo pode sugerir que a fração polissacarídica tenha efeito imunomodulador quando incubada simultaneamente com LPS.

Palavras-chave: *Bauhinia forficata* Link, pata-de-vaca, hipoglicemiante, anti-inflamatória, planta medicinal.

## ABSTRACT

*Bauhinia forficata* Link is a medicinal plant popularly known as cow's paw and has several biological applications, including treatment of inflammation and as an adjutant in the treatment of diabetes. The work's main objective was to extract and isolate a fraction rich in polysaccharides from the leaves of *B. forficata*, structurally characterize them and evaluate their contribution to hypoglycemic and antidiabetic activity *in vivo* and to anti-inflammatory activity *in vitro*. From the aqueous extract prepared with the leaves of the plant, two fractions were obtained: BFSF (BFSGD), supernatant from the freeze-thawing process at room temperature, and TCA-S, supernatant from the treatment of the BFSF fraction with trichloroacetic acid (TCA), with the purpose of deproteinization. In relation to the BFSF fraction, it presented an almost homogeneous elution profile in the homogeneity analysis by HPSEC-RI and in the analysis of spectrophotometric determinations it presented 50.4% of total sugars, 15.1% of proteins, and 15.0% of compounds phenolics contents. Analysis of monosaccharide composition by GC showed 27.4% arabinose, 23.7% galactose, 16.1% rhamnose, 13.8% glucose, 6.2% mannose, 2.5% xylose, 0.3% fucose and 10.0% galacturonic acid, the latter determined by colorimetric method. By NMR and methylation analysis, it was possible to conclude that the polysaccharides present in the BFSF fraction are homogalacturonan, arabinan, starch and types I and II arabinogalactans. The BFSF fraction, at doses of 10, 30, 100 and 300 mg/kg, was analyzed for its hypoglycemic potential through the animal model of streptozotocin-induced diabetes in rats. The acute (3 h) and chronic (21 days) effects of the fraction on the animals were evaluated. The fraction did not change blood glucose levels compared to the control group, treated only with saline, during the experiment. After the period of testing and euthanasia of the animals, blood was collected and biochemical and hematological analyzes were performed. Among the evaluated parameters, there was a statistical difference in the levels of conjugated bilirubin, with a decrease of 63.7% in the 10 mg/kg treated group and 54.6% in the 30 mg/kg group, and a decrease in of AST enzyme levels: the fraction at the dose of 10 mg/kg was able to reduce its concentration by 43.7% when compared to the control group. Histological liver studies demonstrated that the fraction, at doses of 30 and 100 mg/kg, was capable of significantly reducing the appearance and intensity of tissue injury, in 67.0% and 69.4%, respectively. These results indicate a hepatoprotective effect promoted by BFSF fraction. Regarding TCA-S fraction, it showed a homogeneous elution profile in HPSEC-RI, 81.6% of total sugars, 12.6% of phenolic compounds and 4.7% of total proteins and consists of 38.2% arabinose, 20.0% galactose, 17.6% rhamnose, 15.1% glucose, 6.3% mannose, 1.4% xylose and 1.3% galacturonic acid. NMR and methylation analyzes demonstrated that the fraction is mainly composed of types I and II arabinogalactans, arabinan and starch. The starch was removed from TCA-S through α-amylase treatment, and the starch-free fraction was renamed TCA-Sα. The evaluation of the immunomodulatory potential of TCA-Sα, carried out using THP-1 cells, demonstrated that the fraction has immunostimulatory properties at concentrations 50, 100 and 500 μg/mL, by increasing all the cytokines quantified in the cell culture supernatant (TNF-α, IL-1β and IL-10), and immunomodulatory effect in the presence of LPS, at a concentration of 50 μg/mL. The ratio between the concentrations of pro- and anti-inflammatory cytokines, stimulated

by the positive control (LPS) and by TCA-Sα plus LPS, was calculated for each concentration. The decrease in ratio values in all the concentrations of the TCA-Sα + LPS group in comparison with LPS alone may suggest that the polysaccharide fraction has an immunomodulatory effect when simultaneously incubated with LPS.

Keywords: *Bauhinia forficata* Link, pata-de-vaca, hypoglycemic, anti-inflammatory, medicinal plant.

## LISTA DE FIGURAS – REVISÃO BIBLIOGRÁFICA

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## LISTA DE ABREVIATURAS E SIGLAS

δ - Deslocamento químico

ALT - Alanina aminotransferase

AG-I - Arabinogalactana tipo I

AG-II - Arabinogalactana tipo II

ALP - *alkaline phosphatase*

AST - Aspartato aminotransferase

Ara - Arabinose

$CH_3I$ - Iodeto de metila

$D_2O$ - Óxido de deutério

DEPT-135 - *Distortionless enhancement by polarization transfer*

DMSO - Dimetilsulfóxido

*f* - Furanosídico

Fuc - fucose

Gal - Galactose

GalA - Ácido galacturônico

GC-MS - *Gas chromatography - mass espectrometry*

Glc - Glucose

HDL-C - *high density lipoprotein cholesterol*

HPSEC - *High performance size exclusion chromatography*

$H_2SO_4$ - Ácido sulfúrico

HSQC - *Heteronuclear single quantum correlation spectroscopy*

i.p. - Intraperitoneal

LDL-C - *low density lipoprotein cholesterol*

Man - Manose

Mw - *Molecular weight*

$NaBD_4$ - Boroidreto de sódio deuterado

$NaBH_4$ - Boroidreto de sódio

$NaN_3$ - Azida

$NaNO_2$ - Nitrito de sódio

NaOH - Hidróxido de sódio

*p* - Piranosídico

ppm - Partes por milhão

p/v - Peso por volume

RG I - Ramnogalacturonana tipo I

RG II - Ramnogalacturonana tipo II

Rha - Ramnose

RID - Detector de índice de refração

rpm - Rotação por minuto

TCA - Ácido tricloroacético

TFA - Ácido trifluoroacético

tR - Tempo de retenção

v/v - Volume por volume

VLDL-C - *very-low density lipoprotein cholesterol*

Xyl - Xilose

## SUMÁRIO

## 1 INTRODUÇÃO

Plantas medicinais são aquelas que contém em um ou mais de seus órgãos moléculas que possam exercer atividade terapêutica ou que seus precursores sejam elegíveis para semissíntese químico-farmacêutica (ROSSATO *et al*., 2012). O uso de plantas medicinais está intimamente atrelado à evolução humana e registros mostraram que foram os primeiros recursos terapêuticos utilizados em praticamente todas as civilizações antigas. Os primeiros relatos de utilização de plantas medicinais como cedro, alcaçuz, papoula e mirra, foram encontrados na Mesopotâmia e datam de aproximadamente 2600 a.C. (BRANDELLI, 2017). O ‘’Ebers Papyrus’’ – ou Papiro Ebers –, tratado médico que relaciona mais de 700 drogas de origens vegetal, animal e mineral, foi escrito no Egito antigo e data de 1500 a.C. O documento prevê o uso terapêutico de óleos vegetais e descreve diversos produtos naturais à base de funcho, coentro, genciana, sene e losna, os quais ainda são utilizados atualmente. Ao longo dos séculos, diversos outros documentos farmacopeicos, que incluem plantas e outras fontes, foram redigidos conforme novas descobertas e usos medicinais de produtos naturais (NEWMAN; CRAGG; SNADER, 2000).

Atualmente, a utilização de plantas medicinais pela população é significativa: em países em desenvolvimento, 85% da população faz o uso de plantas medicinais para atender cuidados básicos em saúde, como terapia alternativa ou complementar à medicina tradicional (ROSA; CÂMARA; BERIA, 2011; MAGNO-SILVA; ROCHA; TAVARES-MARTINS, 2020). Somado a isso, o uso de terapias alternativas tem recebido grande interesse recentemente, uma vez que fármacos sintéticos podem apresentar inconvenientes como ineficiência, efeitos colaterais indesejados e/ou dificuldade de acesso. Em consequência disso, a pesquisa e o interesse por novas plantas e novos compostos biologicamente ativos vêm crescendo significativamente no mundo (RATES, 2001; DUTRA *et al*., 2016).

O Brasil possui a maior biodiversidade do mundo, representando aproximadamente 20-22% da biodiversidade de todo o mundo e 45.000 espécies vegetais conhecidas, tornando a área de pesquisa de plantas medicinais e elucidação dos seus princípios ativos um campo de investigação muito relevante no país. Isso pode ser ratificado pelo alto número de publicações sobre o tema por cientistas brasileiros: entre 2011 e 2013 foram publicados mais de 10.000 artigos científicos

sobre o assunto. Em contrapartida, o mercado de fitoterápicos ainda é muito pequeno no país, representando menos de 5% de todos os medicamentos comercializados (DUTRA *et al*., 2016).

A inserção da Política Nacional de Práticas Integrativas e Complementares (PNPIC) viabiliza e regulamenta o uso de tratamentos alternativos à medicina tradicional através do Sistema Único de Saúde (SUS), e essa política inclui a fitoterapia. Em 2009 foi elaborada a RENISUS (Relação de Plantas Medicinais com Interesse ao SUS), a qual elenca 71 espécies vegetais com potencial de gerar produtos de interesse ao SUS e ao MS (Ministério da Saúde) (BRASIL, 2009). Dentre as espécies da lista está citada a *Bauhinia forficata* Link, a qual é empregada pela população no tratamento de diferentes enfermidades e é o objeto de estudo deste trabalho.

Conhecida na medicina popular como pata-de-vaca, *Bauhinia forficata* Link é uma planta medicinal utilizada na forma de chá principalmente devido à sua conhecida propriedade hipoglicemiante. A planta também possui outras aplicações terapêuticas, e é usada como purgativa, diurética, antidiarreica, depurativa, tônica renal, auxiliar em moléstias da pele, para tratamento de úlcera, hipertensão, inflamação, entre outras (LÓPEZ; SANTOS, 2015).

Diversos metabólitos secundários de *B. forficata* foram identificados anteriormente, e entre eles destacam-se os flavonoides glicosilados, flavonoides livres e fitoesteróis, também muito presentes em outras espécies do gênero *Bauhinia*, sendo que na espécie *B. forficata* foi relatada a presença predominante de β-sitosterol e kanferol-3,7-diramnosídeo (SILVA *et al*., 2000; PIZZOLATTI *et al*., 2003). Quando um chá é preparado por infusão ou decocção, diversos outros compostos hidrossolúveis são extraídos, e, portanto, passíveis de ingestão, incluindo metabólitos primários, como os polissacarídeos. Sendo assim, os polissacarídeos constituem uma importante classe de moléculas bioativas que pode ser utilizada no tratamento de diversas condições clínicas (SIMÕES, 2003). Na literatura existem estudos que demonstram que os polissacarídeos apresentam atividade antioxidante (SCHNEIDER *et al.*, 2020; NERGARD *et al.*, 2005), hepatoprotetora (CHAVES *et al.*, 2020a; CHAVES *et al*., 2020b), imunomodulatória (CAILLOT *et al.*, 2018; ABREU *et al.*, 2021) anti-inflamatória (MARIA-FERREIRA *et al.*, 2021; OLIVEIRA, *et al.*, 2017; NASCIMENTO *et al.,* 2015; NASCIMENTO *et al*., 2017a; BEZERRA *et al*., 2018)

gastroprotetora (JUNGLES *et al*., 2014; CIPRIANI *et al*., 2006), antiviral (CHEN; HUANG, 2018), antitumoral (ZAVADINACK *et al*., 2021; MILHORINI *et al*., 2022) antidepressiva (WANG *et al*., 2010), anticoagulante (BARDDAL *et al*., 2020; ROMÁN *et al*., 2017), antinociceptiva (CHAVES, *et al*., 2020c; MARIA-FERREIRA *et al*., 2020), antidiabética (MA *et al*., 2018; ZHANG *et al*., 2014), entre diversas outras.

Fundamentado no fato de *B. forficata* ser uma planta medicinal extensivamente utilizada pela população brasileira, e que os polissacarídeos, conforme descrito anteriormente, podem exercer atividade anti-inflamatória e hipoglicemiante, este trabalho tem como objetivo principal extrair e caracterizar estruturalmente os polissacarídeos presentes nas folhas de pata-de-vaca, até então desconhecidos, e verificar a sua contribuição para essas ações biológicas.

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 *Bauhinia forficata* Link

Conhecida popularmente como pata-de-vaca, a *Bauhinia forficata* Link (FIGURAS 1 e 2), nativa do sudeste brasileiro, também pode ser encontrada sob as sinonímias populares casco-de-vaca, mororó, pata-de-boi e unha-de-vaca. Como características botânicas, é uma árvore semidecídua de 5 a 9 metros de altura. Suas folhas são coriáceas e divididas até acima do meio, com aspecto bilobado que remete a pata de uma vaca, de onde deriva seu nome popular. Suas flores brancas são características que a difere da espécie *Bauhinia variegata*, que possui flores cor-de-rosa e é morfologicamente muito semelhante a *B. forficat*a. Seus frutos são vagens achatadas e deiscentes. É comumente utilizada na arborização urbana e pode ser encontrada em áreas montanhosas do nordeste do Brasil (LORENZI; MATOS, 2008; FORTUNATO, 1986).

FIGURA 1 – *Bauhinia forficata* Link.

Fonte: LORENZI, 1992.

FIGURA 2 – Flores de *Bauhinia forficata* Link.

Fonte: https://en.wikipedia.org/wiki/ Bauhinia_forficata.

Na medicina popular, as folhas são comumente empregadas como auxiliares no tratamento de diabetes por apresentarem atividade hipoglicemiante, sendo que o primeiro ensaio clínico que avaliou e confirmou esta propriedade foi realizado em 1929 (JULIANE, 1929). O preparo do chá varia com o efeito que se deseja obter, porém, para tratamento de diabetes, recomenda-se a ingestão do chá das folhas três vezes ao dia (uma vez em jejum e as outras duas antes das principais refeições) preparado por decocção em água por 3 minutos com a medida de uma colher de sobremesa das folhas trituradas para uma xícara média (LORENZI; MATOS, 2008). A planta também dispõe de outras atividades biológicas, como purgativa, diurética, antidiarreica, depurativa, tônica renal, auxiliar em moléstias da pele, para tratamento de úlcera, hipertensão, inflamação entre outras (LÓPEZ; SANTOS, 2015).

A família Fabaceae, a qual pertence *B. forficata*, é uma das maiores famílias de angiospermas do mundo, abrangendo mais de 19.000 espécies. As plantas dessa família são comumente utilizadas para a produção de produtos naturais, como temperos, venenos, tintas, e para propósitos medicinais (AHMAD; ANWAR; HIRA, 2016). Estudos demonstram que diversas plantas dessa família possuem propriedades biológicas, como anti-inflamatória (BURNETT *et al*., 2007),

hepatoprotetiva, hipoglicemiante (RAY; SHARATCHANDRA; THOKCHOM, 2006) (*Acacia catechu*) antioxidante, imunomodulatória (NDIAYE *et al*., 2012) (*Pisum sativum*), entre outras.

Estudos farmacológicos a respeito do potencial antidiabético da pata-de-vaca foram feitos com diferentes tipos de extratos das folhas, explorando múltiplos parâmetros relativos ao diabetes. Pinafo *et al*. (2019) verificaram a atividade antidiabética e antioxidante do tratamento com extrato etanólico comercial de folhas de *B. forficata* por via oral, avaliando os níveis de glicemia, perfil lipídico, glicogênio hepático e estresse oxidativo em ratos expostos a bisfenol A. Os autores observaram efeitos redutores de glicemia e de colesterol total e LDL, minimização da redução dos depósitos de glicogênio no fígado e atividade antioxidante, promovidos pelo extrato. Cunha *et al*. (2010) avaliaram a atividade hipoglicemiante de extrato hidroalcóolico de folhas de pata-de-vaca, via oral, com alto teor de flavonoides, entre eles quercetina e kampferol, em ratos com diabetes induzida por estreptozotocina (STZ). Foi observada redução significativa da concentração de glicose plasmática pelo tratamento com o extrato de *B. forficata*. Pepato *et al*. (2002) investigaram o efeito do tratamento com decocto de folhas de pata-de-vaca sobre parâmetros metabólicos utilizando o modelo de diabetes induzida por estreptozotocina em ratos e verificaram atenuação significativa nos níveis de glucose sérica e urinária e ureia urinária em relação ao grupo diabético controle.

É interessante destacar as diferenças no processo de obtenção das frações testadas quanto a atividade hipoglicemiante no trabalho publicado por Pepato *et al*. (2002) e neste presente trabalho. No primeiro, o decocto foi preparado fervendo 150 g de folhas frescas em 1 litro de água por 5 minutos seguido de resfriamento e filtração por papel, e foi verificada a atividade farmacológica hipoglicemiante crônica desempenhada por este extrato. Neste trabalho, a fração BFSGD, testada quanto ao potencial hipoglicemiante, também foi extraída por decocção, mas em condições diferentes de temperatura, tempo de fervura e material original (100 g de folhas secas em 1 litro de água destilada, por 3 minutos após a fervura). Somado a isso, outros processos de purificação foram feitos em sequência à extração, como precipitação com etanol gelado, diálise com membrana de limite de exclusão de 6-8 kDa e congelamento e degelo a temperatura ambiente, tornando a fração avaliada por Pepato *et al*. (2002) diferente da fração testada nesse trabalho. A fração BFSGD foi

preparada visando uma alta proporção de polissacarídeos e uma baixa quantidade de metabólitos secundários em sua composição química. Já a fração obtida por Pepato *et al.* (2002), de acordo como seu modo de preparo, era rica em metabólitos secundários, com baixa quantidade de polissacarídeos em sua composição química.

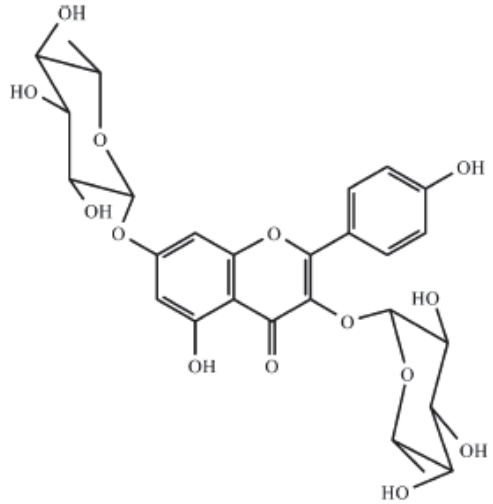
Em relação à sua composição química, outros estudos já foram desempenhados com a finalidade de elucidar os metabólitos secundários constituintes da planta, sendo eles principalmente flavonoides glicosilados, cianoglicosídeos, fitoesterois, e outros derivados fenólicos, como o β-sitosterol, kampferol-3-7-diramnosídeo, e derivados da quercetina, kaempferitrina e kaempferol (PIZZOLATTI *et al*., 2003; SILVA *et al*., 2000).

Sousa e colaboradores (2004) relacionaram o efeito hipoglicemiante de *B. forficata* com o flavonóide kampferol-3,7-O-(α)-diramnosídeo (FIGURA 3). Em linhas gerais, a partir das folhas da planta, os pesquisadores extraíram e isolaram a molécula e a testaram quanto ao seu potencial antidiabético, utilizando modelo animal de indução de diabetes com aloxano em ratos. O tratamento oral com o flavonoide em diferentes doses foi capaz de reduzir significativamente a hiperglicemia dos ratos diabéticos em função do tempo (t=0, 1, 2 e 3 h), com o pico do efeito determinado em 2 horas após a administração da fração teste. Com base nesse resultado, os autores sugerem que é provável que o kaempferol-3,7-O-(α)-diramnosídeo seja a molécula responsável pelo efeito conhecido da planta.

Em virtude da constatação que flavonoides apresentam propriedades hipoglicemiantes, a maioria dos estudos de avaliação de atividade antidiabética de *B. forficata* foram conduzidos com extratos ricos em flavonoides, e muitos deles apresentaram resultados promissores, o que sugere que esses metabólitos secundários, que na planta apresentam função de defesa, possam ser os principais responsáveis pela sua atividade. Uma possibilidade de mecanismo de ação seria considerando a relação entre diabetes e inflamação e a capacidade dos flavonoides de proteger o organismo contra radicais livres, sendo assim plausível afirmar que o consumo dessas moléculas ou de alimentos e plantas ricos em flavonoides possa reduzir o risco de diabetes (VINAYAGAM; XU, 2015). Por mais que já tenham sido publicados diversos artigos na literatura científica com o intuito de investigar a atividade antidiabética e hipoglicemiante de *B. forficata*, utilizando diferentes modelos e formas de preparo de extratos, ainda não existe um estudo com a finalidade de

apurar a influência dos polissacarídeos sobre essa atividade, e por isso, este é um dos objetivos desse trabalho.

FIGURA 3 – kampferol-3,7-O-(α)-diramnosídeo isolado das folhas de *B. forficata*.




Fonte: SOUSA *et al*. (2004).

## 2.2 Carboidratos de plantas

Os carboidratos estruturais são um dos componentes químicos dos tecidos e células vegetais encontrados em maior abundância nas plantas, uma vez que constituem a parede celular e a matriz extracelular, estruturas de suporte das plantas (REID, 1997). Existem como monossacarídeos, dissacarídeos, oligossacarídeos, polissacarídeos e seus derivados, como flavonoides glicosilados e glicoproteínas, e a natureza das ligações entre monossacarídeos em carboidratos complexos e a estrutura de cada unidade sacarídica influenciam a fisiologia vegetal (AVIGAD; DEY, 1997).

### 2.2.1 Parede Celular

A parede celular é uma estrutura macromolecular complexa, altamente organizada e essencial para a sobrevivência das plantas. Ela proporciona resistência e forma para a célula vegetal, participa da comunicação célula-célula, protege contra o ataque de predadores, atua na manutenção da pressão osmótica celular, no transporte de moléculas e controla o crescimento celular (CARPITA; RALPH;

MCCANN, 2015). É composta de polissacarídeos, proteínas e compostos aromáticos e alifáticos. A estrutura e composição da parede celular é constantemente modificada ao longo da vida da célula, de acordo com o estágio de vida da planta, com o ambiente que ela está inserida (CAFFALL; MOHNEN, 2009), entre espécies e com a função desempenhada pelo tecido vegetal (REID, 1997; PETTOLINO *et al.*, 2012).

Podem ser encontrados três estratos nas paredes das células vegetais: a parede primária, uma camada fina e formada enquanto a célula ainda está em crescimento; a parede secundária, uma camada espessa que se forma dentro da parede celular primária após o total crescimento da célula; e a lamela média, mais externa, formando uma interface entre células adjacentes (CARPITA; RALPH; MCCANN, 2015).

As paredes primárias são estruturas de 1 a 3 μm de espessura e são compostas principalmente por polissacarídeos e água. À medida que as células meristemáticas aumentam em tamanho e comprimento, mais materiais são incorporados na parede celular primária. Quando as células vegetais perdem a capacidade de crescer e se dividem, podem se diferenciar em células de diferentes tipos, alguns dos quais têm paredes celulares que são muito espessas. Esses espessamentos são provocados por depósito de material entre a parede celular primária e a membrana plasmática da célula. A parede das células com espessamento passa a ser denominada de secundária e é importante em conferir rigidez aos tecidos vegetais, independente de turgor celular, como ocorre em paredes primárias (CARPITA; RALPH; MCCANN, 2015; REID, 1997). Nas paredes secundárias, ocorre a deposição de lignina, uma macromolécula polifenilpropanoide hidrofóbica, que confere à célula dureza e resistência à água (CARPITA; RALPH; MCCANN, 2015). Esse processo é conhecido como lignificação, e é seguido por morte celular e pelo desaparecimento do conteúdo citoplasmático (REID, 1997).

Como mencionado anteriormente, polissacarídeos são os componentes majoritários da parede celular e são categorizados em pectinas, hemiceluloses e celulose. Apesar de serem conhecidas muitas informações a respeito dos polissacarídeos constituintes de parede celular que permitem saber os mais comuns, os polissacarídeos da parede são heterogêneos em relação a sua estrutura, tamanho e consequentemente a suas propriedades físico-químicas e biológicas (PETTOLINO *et al*., 2012). Nas dicotiledôneas, a composição da parede celular é 25 a 40% de

celulose, 15 a 25% de hemicelulose, 15 a 40% de pectinas, 5 a 10% de proteínas e uma pequena quantidade de compostos fenólicos (DEY; BROWNLEADER; HARBORNE, 1997).

As pectinas são uma mistura de polissacarídeos heterogêneos, ramificados e altamente hidratados, ricos em ácido galacturônico. Ocorrem naturalmente em frutas e plantas, e possuem aplicação comercial na produção de geleias pela sua capacidade de formação de gel (REID, 1997). Fisiologicamente, regulam a adesão célula-célula na lamela média, reconhecem a presença de organismos simbióticos, patógenos e insetos, determinam a porosidade da parede celular e fornecem superfícies carregadas que modulam o pH e o balanço iônico da parede celular (CARPITA; RALPH; MCCANN, 2015). Os polímeros que representam essa classe são as homogalacturonanas (HG), as xilogalacturonanas (XGA), ramnogalacturonanas tipo I (RG-I), ramnogalacturonanas tipo II (RG-II) e arabinogalactanas tipos I (AG-I) e II (AG-II) (CAFFALL; MOHNEN, 2009; RIDLEY; O'NEILL; MOHNEN, 2001).

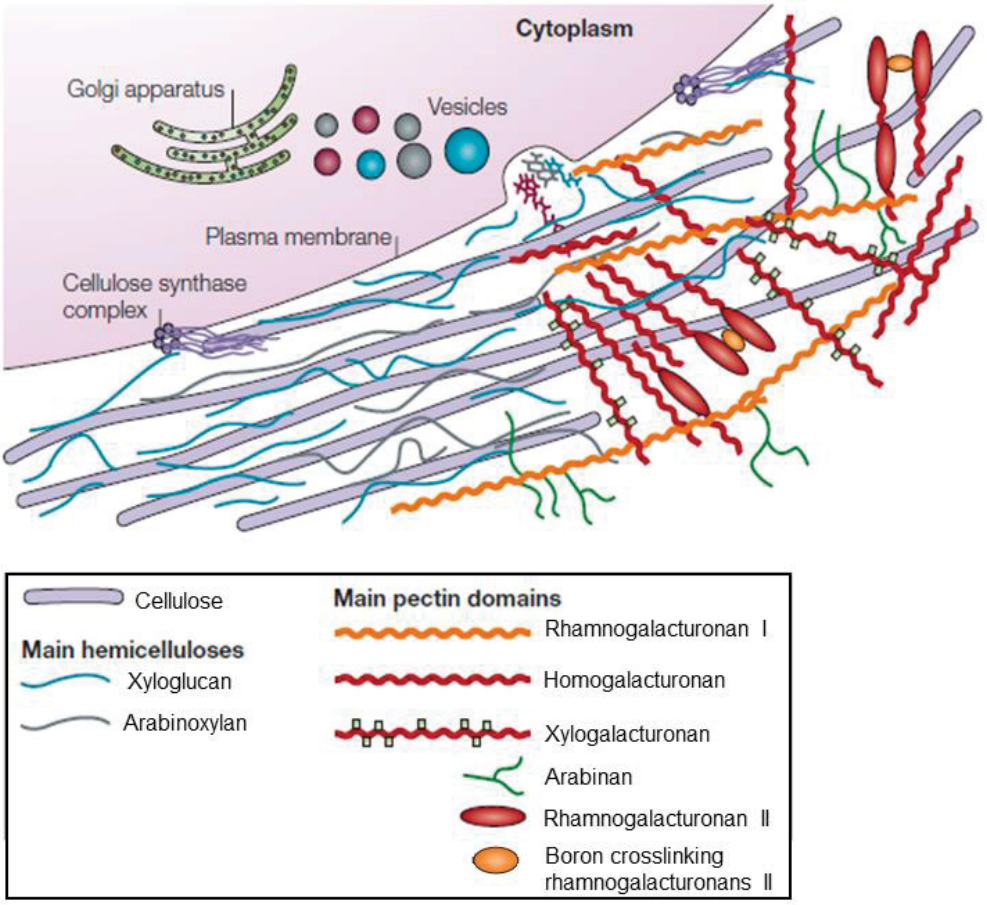
A celulose é o principal polissacarídeo da parede celular vegetal e representa 15-30% da massa seca de paredes celulares primárias e em porcentagem muito maior nas paredes secundárias. Se encontra na forma de microfibrilas, que são associações entre muitas cadeias de glucana β(1→ 4) ligadas de forma intermolecular ao longo das suas extensões por ligações de hidrogênio (CARPITA; RALPH; MCCANN, 2015).

As hemiceluloses são polissacarídeos que estão intimamente organizados com a celulose na estrutura da parede celular, formando uma película sobre as microfibrilas através de ligações de hidrogênio, e as ligam de modo a formar uma rede (CARPITA; RALPH; MCCANN, 2015). As hemiceluloses são representadas pelas xilanas, mananas, glucomananas, galactomananas, xiloglucanas, entre outras estruturas (CAFFALL; MOHNEN, 2009).

Estruturalmente, a parede celular primária é formada pelas microfibrilas de celulose embebidas em uma matriz polissacarídica de pectinas e hemiceluloses (FIGURA 4). Na maioria das espécies de plantas, a principal hemicelulose que envolve as microfibrilas de celulose é a xiloglucana, enquanto hemiceluloses como arabinoxilanas e mananas são encontradas em menor proporção. Os principais polissacarídeos pécticos que constituem a matriz péctica incluem ramnogalacturonana tipo I e homogalacturonana, e menores quantidades de

xilogalacturonana, arabinana, arabinogalactana tipo I e ramnogalacturonana tipo II (COSGROVE, 2005). Carpita e Gibeaut (1993) descreveram dois modelos de parede celular primária para angiospermas: paredes do tipo I, as quais apresentam grande quantidade de pectina e hemicelulose e estão presentes em dicotiledôneas e monocotiledôneas, com exceção das gramíneas; e paredes tipo II, que estão presentes nas gramíneas, e contém pouca pectina e mais hemicelulose em sua composição.

FIGURA 4 – Estrutura geral de parede celular primária tipo I.




Fonte: Adaptado de Cosgrove (2005).

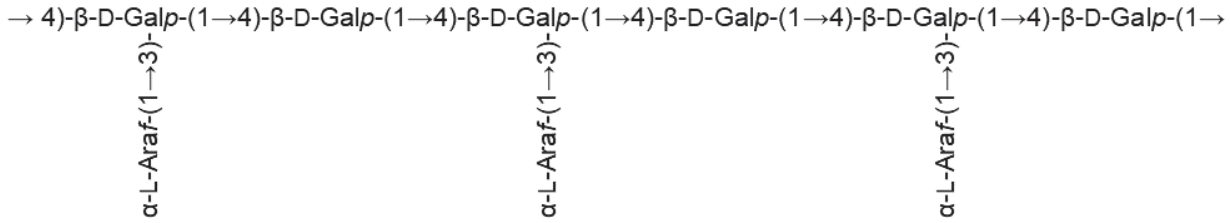
## 2.2.2 Arabinogalactanas

Arabinogalactanas são polímeros constituídos de arabinose e galactose. De acordo com Aspinall (1973), as arabinogalactanas podem ser classificadas em dois grupos com base nas diferenças de ligações glicosídicas entre as unidades de galactose que constituem a cadeia principal desses polissacarídeos. As

arabinogalactanas do tipo I possuem cadeia principal formada por β(1→4)-galactana e as do tipo II, cadeia principal de β(1→3) e β(1→6)-galactanas. São encontradas em diversas estruturas vegetais, como folhas (CARLOTTO *et al*., 2016; NASCIMENTO *et al*., 2017a), frutos (CANTU-JUNGLES *et al*., 2014), gomas (TANAKA *et al*., 2010) caules (CARLOTTO *et al*., 2020), entre outras.

As arabinogalactanas do tipo I (AG-I) (FIGURA 5) são encontradas apenas em frações pécticas, geralmente associadas com resíduos de ramnose na posição O-4 das ramnogalacturonanas tipo I (CAFFALL; MOHNEN, 2009) e podem apresentar unidades arabinosil ligadas na posição O-3 da galactana (CARPITA; RALPH; MCCANN, 2015).

FIGURA 5 – Estrutura de arabinogalactanas tipo I.



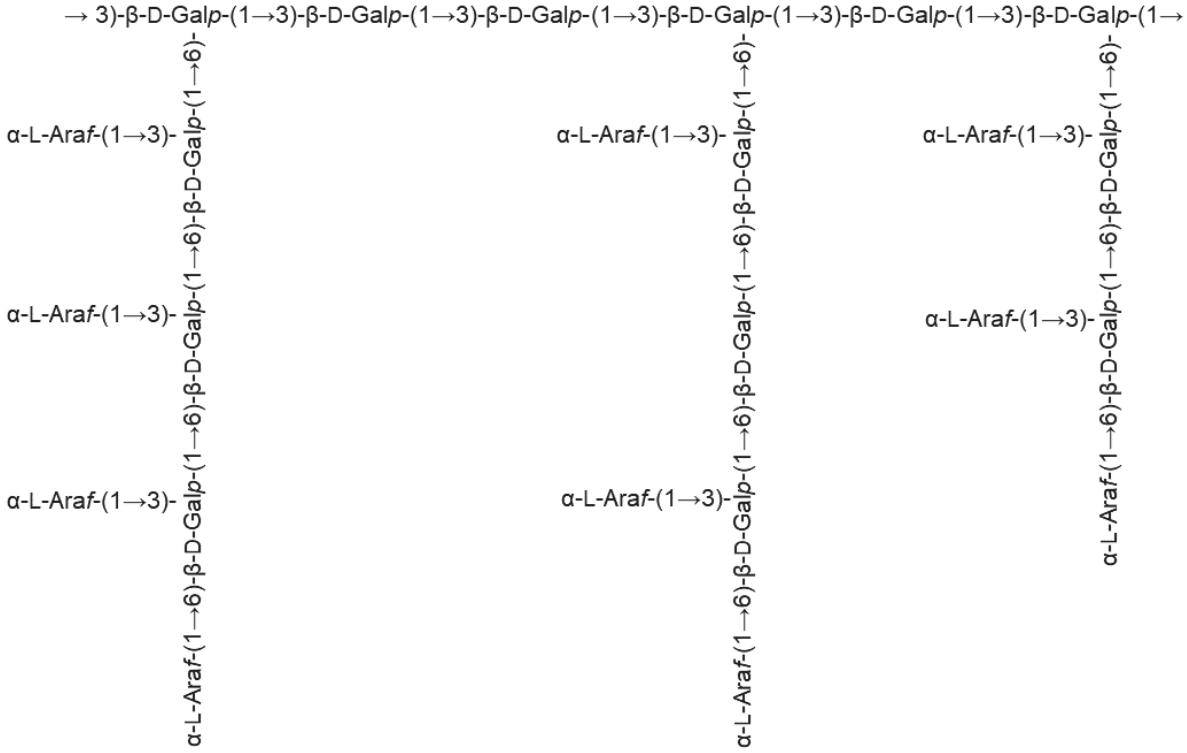

Fonte: Carpita e Gibeaut (1993).

As arabinogalactanas do tipo II (AG-II) (FIGURA 6) são um grupo diverso de cadeias curtas de β(1→3) e β(1→6)-galactanas conectadas entre elas por pontos de ramificação β(1→3, 1→6). A maior parte das posições O-3 e O-6 das galactoses restantes estão preenchidas com grupos t-arabinosil (CARPITA; GIBEAUT, 1993; CARPITA; RALPH; MCCANN, 2015). Quanto à composição monossacarídica desses polissacarídeos, o teor de arabinose varia entre 10 e 80% em relação à proporção de açúcares neutros e pode haver presença de resíduos ácidos, como D-Glc*p*A e o seu respectivo 4-metil-éster e D-Gal*p*A (STEPHEN, 1983).

As AG-II podem ainda estar associadas a proteínas ricas em hidroxiprolina, formando as arabinogalactana-proteínas (AGP), que podem estar localizadas em vesículas derivadas do Golgi, na membrana plasmática e na parede celular. Embora nenhuma função clara tenha sido descrita para AGPs, elas estão presentes apenas em tipos específicos de células, em estágios de desenvolvimento, e em resposta a

estímulos específicos (CARPITA; GIBEAUT, 1993; CARPITA; RALPH; MCCANN, 2015).

FIGURA 6 – Estrutura de arabinogalactanas tipo II.

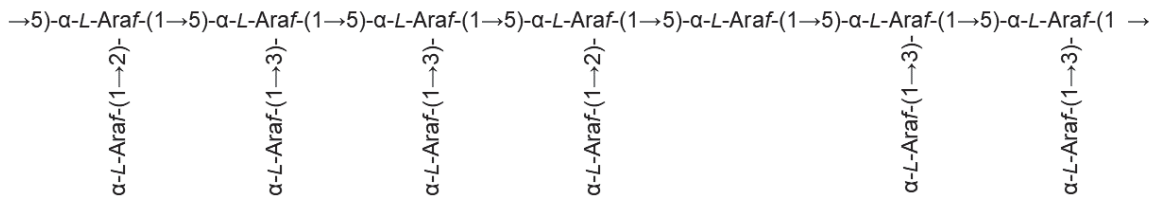



Fonte: Carpita e Gibeaut (1993).

## 2.2.3 Arabinanas

Estruturalmente, as arabinanas (FIGURA 7) consistem em cadeias de unidades de arabinose unidas por ligações α(1→5), substituídas preferencialmente nas posições O-3 e O-2. De modo geral, as arabinanas são encontradas associadas a galactanas ou pectina e podem ser extraídas de sementes, frutas, cascas e madeiras (STEPHEN, 1983).

FIGURA 7 – Estrutura de arabinanas




Fonte: Carpita e Gibeaut (1993).

### 2.2.4 Homogalacturonanas

As homogalacturonanas (HG) são polissacarídeos lineares de resíduos de α-D-Gal*p*A, unidos por ligação do tipo α-(1→4). As unidades de ácido galacturônico podem estar quimicamente modificadas, como metilesterificadas na carboxila C-6 e O-acetiladas em C-2 ou C-3 (RIDLEY; O'NEILL; MOHNEN, 2001). Existem dois tipos mais conhecidos de homogalacturonanas modificadas estruturalmente: as xilogalacturonanas, que são homogalacturonanas substituídas na cadeia principal na posição C-3 por unidades de D-xilose; e as ramnogalacturonanas tipos I e II (CARPITA; RALPH; MCCANN, 2015; CAFFALL; MOHNEN, 2009).

### 2.2.5 Atividades biológicas de polissacarídeos de plantas

O uso medicinal de polissacarídeos extraídos de fontes naturais é vantajoso devido aos seus notáveis efeitos farmacológicos, ausência de toxicidade e ampla disponibilidade (PAWAR; KAMAT; CHOUDHARY, 2015). Sendo assim, os polissacarídeos de origem vegetal são uma importante classe de compostos naturais bioativos. Na literatura científica, algumas ações biológicas desempenhadas por polissacarídeos já foram descritas, como antitumoral (MILHORINI *et al*., 2022; CARLOTTO *et al*., 2020), imunomodulatória (ABREU *et al*., 2021; CAILLOT *et al*., 2018; BEZERRA *et al*., 2018) anti-inflamatória (MARIA-FERREIRA *et al*., 2020), anticoagulante (BARDDAL *et al*., 2020; ROMÁN *et al*., 2017), hipoglicemiante (MUTAILIFU *et al*., 2022; YANG *et al*., 2022; XIE *et al*., 2022), entre outras. A atividade destes polissacarídeos está associada às suas características físico-químicas e estruturais, como a composição monossacarídica, tipo de ligação glicosídica entre unidades monoméricas, ramificação, tamanho e massa molecular (SRIVASTAVA; KULSHRESHTHA, 1989).

### 2.2.6 Atividade hipoglicemiante de polissacarídeos

Em estudos anteriores já foi documentada a atividade antidiabética exercida por polissacarídeos. Ma, Yuan e Zhuang (2018) avaliaram o potencial antidiabético de polissacarídeos (administrados por via intraperitoneal) extraídos de tubérculos de jícama (*Pachyrrhizus erosus*), obtidos através de extração aquosa, pelo modelo de diabetes induzida por estreptozotocina em ratos. Os autores observaram diminuição do nível de glucose em jejum, regulação dos níveis de proteína glicada sérica, triglicerídeos totais e colesterol total. Também, por análise histológica, foi verificada a capacidade protetora, promovida pelos polissacarídeos, de estruturas teciduais de pâncreas, fígado e rins de danos provocados por diabetes.

Pan e colaboradores (2017), utilizando modelo de diabetes tipo 2 induzido por dieta rica em gorduras e estreptozotocina em ratos, avaliaram o efeito de polissacarídeos da seda do milho administrados por via oral, obtidos por extração aquosa a quente, e observaram melhora dos parâmetros de perda de peso, glucose sanguínea, nível de insulina sérico e tolerância à glucose, além da regulação de níveis de proteína glicada e perfil lipídico e redução significativa de ácidos graxos não esterificados.

### 2.2.7 Atividade anti-inflamatória de polissacarídeos

A atividade anti-inflamatória e imunomodulatória desempenhada por polissacarídeos é sustentada por diversos artigos publicados na literatura. Oliveira *et al*. (2017) isolaram, caracterizaram e testaram a atividade anti-inflamatória *in vitro* de polissacarídeos pécticos isolados das folhas de *Sedum dendroideum*, conhecido popularmente como bálsamo, e verificaram o efeito anti-inflamatório na presença de agentes pró-inflamatórios em razão da redução da secreção de TNF-α e IL-1β e estímulo de produção de citocinas anti-inflamatórias, como a IL- 10, em células THP-1 estimuladas com LPS.

Tamiello *et al*. (2018) extraíram e caracterizaram uma arabinogalactana tipo II de frutos de *Syzygium jambos* e testaram a propriedade imunomodulatória desse polissacarídeo em modelo *in vitro,* também utilizando macrófagos THP-1, na presença

e ausência de lipopolissacarídeo (LPS) como estímulo pró-inflamatório. Os resultados obtidos demonstraram que a AG-II da fruta aumentou a secreção das citocinas TNF-α, IL-1β e IL-10 na ausência de LPS, de maneira concentração dependente, e atenuou a resposta inflamatória na presença de LPS na maior concentração testada, demostrando potencial imunomodulador deste polissacarídeo.

Em 2020, Maria-Ferreira e colaboradores avaliaram a atividade anti-inflamatória de polissacarídeos isolados das folhas de *Handroanthus albus*, popularmente conhecido como ipê amarelo, através dos modelos animais de nocicepção induzida por formalina e por glutamato, e inflamação induzida por ácido acético em camundongos. A fração polissacarídica, contendo principalmente arabinogalactana tipo II, promoveu atividade antinociceptiva, reduzindo o número de contorções abdominais e anti-inflamatória, minimizando a infiltração de leucócitos na cavidade peritoneal.

## 2.3 Diabetes

De acordo com a Federação Internacional do Diabetes (2021), o diabetes afeta mais de meio bilhão de pessoas no mundo. Estima-se que, no ano de 2045, 783 milhões de pessoas sofram de diabetes, representando 12,2% da população mundial. É uma doença metabólica crônica, caracterizada por hiperglicemia, na qual o corpo não produz insulina ou não consegue empregar adequadamente a insulina que produz. Na prática clínica, é classificada em dois tipos: tipo I ou autoimune, na qual ocorre destruição parcial ou total das células β nas ilhotas de Langerhans no pâncreas, com a consequente inabilidade de produzir insulina; e tipo II, que se caracteriza pela insuficiência de secreção e/ou ação da insulina (KUMAR; COTRAN; ROBBINS, 1992).

A insulina é um hormônio peptídico produzido pelas células β pancreáticas e age por meio de receptores na membrana plasmática, estimulando a captação de moléculas de glucose pelos músculos e tecido adiposo. No fígado, a insulina ativa a enzima glicogênio-sintase, canalizando a glucose-6-fosfato em síntese de glicogênio (NELSON; COX, 2011). A falta da insulina ou a resistência das células em responder a ela provocam altos níveis de glucose sanguínea e, ao longo do tempo, a

hiperglicemia crônica ocasiona diversas complicações, como distúrbios microvasculares (retinopatia, nefropatia e neuropatia), macrovasculares (derrame, infarto do miocárdio e doença arterial periférica), insuficiência cardíaca e doença hepática (ROHM *et al*., 2022; KUMAR; COTRAN; ROBBINS, 1992).

### 2.3.1 Relação entre diabetes e inflamação

No processo fisiopatológico do diabetes é observada a inflamação crônica de tecidos que são alvo da insulina, como o adiposo, hepático, muscular e pancreático, estabelecendo uma relação entre processos inflamatórios e disfunções metabólicas. Os macrófagos pró-inflamatórios e seu recrutamento, acúmulo e ativação nesses tecidos são os principais fatores que impulsionam a inflamação crônica na doença (ROHM *et al*., 2022).

Há vários artigos publicados nos últimos anos que fornecem a base da relação entre inflamação e diabetes. Hotamisligil, Shargill e Spiegelman (1993) e Yuan *et al*. (2001) observaram que o tecido adiposo na obesidade expressa altos níveis de TNF-α. Essa citocina provoca intolerância à glucose (FEINGOLD *et al*., 1989) e ativa moléculas de sinalização intracelular que resulta em ação inefetiva da insulina (ARKAN *et al*., 2005; HIROSUMI *et al*., 2002). No entanto, a neutralização dessa citocina melhora a intolerância à glucose e a sensibilidade das células à insulina (HOTAMISLIGIL; SHARGILL; SPIEGELMAN, 1993). Outro componente inflamatório essencial para essa associação é o inflamassomo, um complexo proteico multimérico do sistema imune inato que pode ser ativado por nutrientes como a glucose (MAEDLER *et al*., 2002; MARTINON; MAYOR; TSCHOPP, 2009). O açúcar se liga em receptores TLR-4 (*toll-like receptor* 4) e interage com vias bioquímicas responsáveis pela regulação da ativação da caspase-1, enzima responsável pela proteólise de precursores de citocinas pró-inflamatórias como a interleucina 1β, aumentando a concentração de IL-1β ativa no tecido (OKLA *et al*., 2018; ZHOU *et al*., 2010). Somado a isso, existem estudos que relacionam a influência do aumento da quantidade de IL-1β e a perda da massa e morte celular de células β pancreáticas (DINARELLO; DONATH; MANDRUP-POULSEN, 2010). Outro fator importante para essa relação foi a descoberta de que macrófagos acumulam no tecido adiposo ao longo do desenvolvimento de obesidade, descrito por Weisberg *et al*. (2006) e Xu *et*

*al.* (2003). A ativação dessas células leva à polarização pró-inflamatória que, conforme discutido anteriormente, induz a liberação de uma série de citocinas inflamatórias e contribui para a diminuição da sinalização da insulina.

Existe uma quantidade significativa de medicamentos antidiabéticos disponíveis, mas a maioria deles não atinge níveis ótimos de controle da glucose sanguínea. Além disso, o controle da glicemia por si só nem sempre é suficiente para prevenir as complicações a longo prazo do diabetes e a sua progressão não é retardada por esses medicamentos. Isso destaca a importância de outras potenciais opções terapêuticas, como drogas imunomoduladoras por exemplo, destinadas a tratar ou a inibir a progressão e as complicações de caráter inflamatório da doença (ROHM *et al*., 2022).

### 2.3.2 Modelo animal de avaliação do potencial antidiabético

Com o avanço científico, diversos modelos animais foram desenvolvidos para entender melhor a patogênese do diabetes e para testar, validar e introduzir no mercado novas drogas para o tratamento da doença. O diabetes pode ser induzido por manipulações farmacológicas, cirúrgicas ou genéticas em modelos animais. Com relação à indução do diabetes com fármacos, uma das drogas mais utilizadas é a estreptozotocina (STZ), um agente alquilante antineoplásico que desempenha toxicidade em células β pancreáticas (FRÖDE; MEDEIROS, 2008). A STZ possui estrutura molecular similar à glucose e é transportada para o interior da célula através dos receptores GLUT2, muito presentes na superfície das células β, não sendo reconhecido por outros transportadores de glucose, e sua ação citotóxica é mediada por espécies reativas de oxigênio que levam à morte celular por necrose (SZKUDELSKI, 2001; MYTHILI *et al*., 2004).

Em ratos adultos, a dose mais comum para induzir o diabetes insulino-dependente, ou seja, do tipo I, é 60 mg/kg de STZ (GASPARIN *et al*., 2021; LEÃO *et al*., 2022), administrada pelas vias intravenosa, intraperitoneal ou subcutânea, em jejum de 8 a 12 horas. Os animais são considerados diabéticos se, após 2 dias da administração do fármaco, o nível de glucose sanguínea detectado estiver entre 200 e 300 mg/dL. Um modelo de diabetes tipo II também pode ser induzido com STZ,

através do tratamento com a droga nos primeiros dias de vida do animal. Uma discreta hiperglicemia basal é observada entre 8 e 10 semanas depois do tratamento, além de redução da sensibilidade à glucose das células β e resposta prejudicada ao teste de tolerância à glucose (FRÖDE; MEDEIROS, 2008).

Esse modelo já foi empregado anteriormente para a avaliação do potencial antidiabético de polissacarídeos. Zhou *et al*. (2015), a partir de uma fração polissacarídica isolada de *Rehmannia glutinosa* (Gaertn.) DC., uma planta medicinal chinesa tradicionalmente utilizada para o tratamento de diabetes e suas complicações, estudaram o efeito hipoglicemiante da administração oral da fração polissacarídica purificada através do modelo de diabetes induzido por STZ em ratos e concluíram que houve redução significativa do nível de glucose sanguínea, colesterol total, triglicerídeos e aumento de insulina plasmática, oferecendo uma opção terapêutica para o tratamento de diabetes tipo I. De forma semelhante, Xiang, Sun-Waterhouse e Cui (2021), a partir de cogumelos comestíveis, extraíram e obtiveram polissacarídeos com capacidade hipoglicemiante, e avaliaram parâmetros como peso corporal, glucose em jejum, insulina sérica, citocinas e produtos de estresse oxidativo em animais diabéticos induzidos com STZ.

## 2.4 Inflamação

A inflamação é uma resposta adaptativa iniciada por um estímulo nocivo, como infecção ou lesão tecidual, com o objetivo de restaurar a homeostase. Pode ser classificada em aguda ou crônica e seus sinais cardinais são conhecidos como rubor, calor, edema, dor e a perda de função (KUMAR; ABBAS; ASTER, 2013).

De maneira concisa, a inflamação aguda se inicia com a entrega coordenada de componentes sanguíneos, como plasma e leucócitos, ao sítio onde houve estímulo nocivo. O reconhecimento inicial de infecção se dá pelos macrófagos e mastócitos residentes da região danificada, que produzem diversos mediadores inflamatórios, como quimiocinas e citocinas como TNF-α, IL-1β e aminas vasoativas, com objetivo de promover a exsudação de proteínas plasmáticas e leucócitos que normalmente são restritos aos vasos sanguíneos até o local de injúria, e o endotélio ativado permite a passagem de neutrófilos para a região. Os neutrófilos, agora ativados por contato

direto com o patógeno ou pela ação das citocinas, tentam eliminar os agentes invasores liberando conteúdos tóxicos presentes em seus grânulos, como espécies reativas de oxigênio e nitrogênio, por exemplo. Uma resposta inflamatória aguda bem-sucedida culmina na eliminação dos agentes infecciosos e é seguida de uma fase de reparo, mediada por macrófagos recrutados ou residentes do local. Essa fase se caracteriza pela presença de moléculas anti-inflamatórias, como a interleucina IL-10, que são cruciais para a transição da inflamação à resolução, e o resultado é a remoção de células mortas e início da remodelação tecidual (MEDZHITOV, 2008).

Se a resposta de inflamação aguda falhar em eliminar o estímulo nocivo, o processo inflamatório persiste, passando para o estado crônico, e adquire novas características. A inflamação crônica tem maior duração e caracteriza-se pelo influxo de macrófagos e linfócitos, e fibrose, também conhecido como processo de cicatrização (KUMAR; ABBAS; ASTER, 2013).

### 2.4.1 Modelo *in vitro* de avaliação de atividade anti-inflamatória com células THP-1

As células THP-1 são uma linhagem de monócitos humanos isolados do sangue periférico de paciente com leucemia monocítica, e é utilizada em pesquisas de imunologia e toxicologia. É interessante para investigação *in vitro* da função dos macrófagos, uma vez que, após serem submetidas à diferenciação com forbol 12-miristato 13-acetato (PMA), as células adquirem características fenotípicas e funcionais muito similares àquelas de macrófagos humanos (LUND *et al*., 2016). As células THP-1, após sofrerem exposição à estímulos pró-inflamatórios, como o lipopolissacarídeo (LPS), um componente da parede celular de bactérias gram-negativas, passam a expressar marcadores clássicos de macrófagos, aumentam a sua habilidade fagocítica e secretam mediadores pró-inflamatórios, como o fator de necrose tumoral (AUWERX, 1991; SCHWENDE *et al*., 1996).

Com relação ao uso dessa linhagem celular para análise de atividade imunomodulatória de polissacarídeos, existem artigos publicados que demonstram a aplicabilidade desse modelo. Abreu *et al.* (2021) analisaram a atividade imunomodulatória de manogalactana, de (1→6)-β-D-glucana e (1→3)-β-D-glucana isoladas de corpos frutíferos de *P. eryingii* em células THP-1, mensurando a secreção

das citocinas IL-1β, IL-10 e óxido nítrico (NO). Tamiello *et al*. (2018) investigaram o efeito da arabinogalactana tipo II isolada de *Syzygium jambos* na concentração de TNF-α, IL-1β e IL-10 na presença e ausência de LPS, utilizando células THP-1. De forma semelhante, Nascimento *et al*. (2017b) analisaram a influência de uma pectina modificada, extraída de pimenta doce, na secreção de TNF-α, IL-1β e IL-10 por macrófagos THP-1.

## 3 JUSTIFICATIVA

A *Bauhinia forficata*, planta integrante da RENISUS, é amplamente utilizada na medicina popular, principalmente devido a sua propriedade hipoglicemiante. Na literatura científica, existem estudos que abordam essa propriedade antidiabética da pata-de-vaca, avaliando diferentes extratos, principalmente ricos em polifenóis, e atualmente atribui-se a atividade hipoglicemiante da planta à molécula de kampferol-3-7-diramnosídeo, presente em suas folhas. No entanto, é sabido que polissacarídeos de diferentes fontes e com diferentes estruturas podem exercer atividade antidiabética e hipoglicemiante. Considerando que ainda não se conhecem os polissacarídeos da *B. forficata*, este trabalho busca descrever a estrutura dos polissacarídeos presentes no chá medicinal das folhas da planta e investigar se a fração que contém essas moléculas é capaz de contribuir para a conhecida propriedade hipoglicemiante da planta e/ou na melhora de diversos parâmetros relacionados a fisiopatologia do diabetes. Além disso, considerando a relação entre diabetes e inflamação descrita na seção anterior e que outras opções terapêuticas, como imunomoduladores, podem minimizar as complicações do diabetes a longo prazo, essa pesquisa investigou se a fração rica em polissacarídeos possui atividade anti-inflamatória.

# 4 OBJETIVOS

## 4.1 Objetivo geral

A pesquisa teve como objetivo geral elucidar a estrutura dos polissacarídeos presentes nas folhas de *B. forficata*, extraídos durante o preparo do chá medicinal e submetidos a procedimentos de purificação, além de avaliar se há contribuição dessas moléculas para a indicação popular da planta contra diabetes e inflamação.

## 4.2 Objetivos específicos

a) Extração aquosa dos polissacarídeos das folhas de *B. forficata*;

b) Isolamento de uma fração majoritariamente composta por polissacarídeos a partir do extrato aquoso das folhas de *B. forficata*;

c) Caracterização estrutural dos principais polissacarídeos presentes no extrato aquoso das folhas das folhas de *B. forficata*;

d) Avaliação da atividade anti-inflamatória *in vitro* da fração rica em polissacarídeos obtida a partir do extrato aquoso das folhas de *B. forficata*;

e) Avaliação *in vivo* da atividade hipoglicemiante e antidiabética da fração rica em polissacarídeos obtida a partir do extrato aquoso das folhas de *B. forficata*.

# 5 ARTIGOS

**ARTIGO I**

# A polysaccharide rich fraction from *Bauhinia forficata* Link leaves presents hepatoprotective effect and contributes to the plant's antidiabetic property

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Keywords: polysaccharides; *Bauhinia forficata*; cow's paw; hepatoprotective effect; phytotherapy.

Highlights

- A crude polysaccharide fraction was obtained from *Bauhinia forficata* Link leaves through hot aqueous extraction and purification processes;
- This fraction, named BFSF was structurally characterized and showed to be constituted mainly by type I and II arabinogalactans, and homogalacturonan;
- When evaluated against its hypoglycemic potential by estreptozotocin-induced diabetes in rats' model, BFSF fraction demonstrated no acute or chronic effect in glycemia levels.
- BFSF showed hepatoprotective effect by lowering indirect and total bilirubin, AST enzyme levels and by ameliorating cytological aspects of liver tissue, analyzed by histological analysis.

## ABSTRACT

*Bauhinia forficata* Link, popularly known as cow's paw, is a Brazilian native plant with many medicinal properties. Its leaves tea is popularly consumed as a coadjutant in the treatment of diabetes, this activity being attributed to its polyphenols. Considering that previous studies in the recent years have suggested that polysaccharides extracted from different natural sources hold antidiabetic and hypoglycemic properties, and that the structure of polysaccharides from cow's paw leaves is unknown up to this date, the aim of this study was to obtain a crude polysaccharide fraction from the aqueous extract of *B. forficata* leaves and test this fraction in the streptozotocin-induced diabetes model in rats. The fraction was obtained by hot water extraction, followed by precipitation of high molecular weight molecules with cold ethanol, and freeze-thawing process, generating an insoluble (BFIF) and a soluble fraction (BFSF). Structural characterization of polysaccharides and biological evaluation was proceeded only with BFSF fraction. It was observed that BFSF showed an almost homogeneous elution profile in HPSEC-RI analysis and was mainly constituted of arabinose (27.4%), galactose (23.7%), rhamnose (16.1%), glucose (13.8%), and galacturonic acid (10.0%). NMR analysis confirmed that the polysaccharides present in BFSF were type I and type II arabinogalactans and homogalacturonan. When tested for its hypoglycemic and antidiabetic potential, BFSF did not show significative effect in reducing acute or chronic glycemia of diabetic rats. However, it was able to reduce direct bilirubin plasmatic levels in 63.7% at the dose of 10 mg/kg and in 54.6% at 30 mg/kg, and AST in 43.7% at 10 mg/kg, suggesting that it can promote hepatoprotective effect. By histological analysis, it was verified that BFSF at doses of 30 and 100 mg/kg could significantly reduce liver damage, diminishing the histological score in 67.0% and 69.4%, respectively. These findings indicate that, although BFSF fraction does not interfere with glycemic levels, it can prevent liver damage caused by diabetes, thus indirectly contributing to the antidiabetic activity of the cow's paw tea, due to its hepatoprotective action.

## 1 Introduction

Diabetes affects more than half a billion people worldwide and it is estimated that 783 million people will suffer from diabetes by the year 2045, representing 12.2% of the world's population, making it a worrying and growing disease. It is a chronic metabolic disorder characterized by hyperglycemia, whereas the body does not produce insulin or is unable to properly employ the insulin it produces, and its progression can lead to several aggravations, such as atherosclerosis, nephropathy, hepatic complications and so on. Diabetes treatment is mainly done using insulin or oral hypoglycemic medications like sulphonylureas and biguanides (International Federation of Diabetes, 2021), but these drugs can induce many unwanted side effects, such as hypoglycemia and liver damage, and they are not effective in preventing occurrence of complications caused by disease development. However, there are adjutant treatments that can be employed, including phytotherapy, as an attempt to reduce undesirable effects from standard medications and to promote a protective effect against disease evolution (TROJAN-RODRIGUES *et al*., 2012). In this regard, it is advantageous to seek therapeutic options that can deliver the expected beneficial outcome with as few negative consequences as possible. An interesting choice would be the use of medicinal plants, which are easily accessible and in general non-toxic alternatives.

*Bauhinia forficata* Link or cow's paw, as the plant is best known in folk medicine, is a medicinal plant native of Brazilian flora, from Southeast region (LORENZI; MATOS, 2008). The plant is used as a purgative, diuretic, antidiarrheal, depurative, for ulcer treatment, hypertension, inflammation, among others (LOPÉZ; SANTOS, 2015). The most common and well-known use of cow's paw leaves is as complementary in the treatment of diabetes because of their hypoglycemic effect. Previous studies have designated polyphenols as the main pharmacological compounds of *B. forficata* responsible for its antidiabetic activity. For instance, Sousa *et al.* (2004) associated the hypoglycemic effect of *B. forficata* with the flavonoid kaempferol-3,7-O-(α)-dirhamnoside isolated from the leaves of the plant. The antidiabetic potential was tested through model of alloxan-inducted diabetes in rats, and the oral treatment with the flavonoid at different doses was able to significantly reduce hyperglycemia in diabetic rats as a function of time. Based on this result, the

authors suggested that this molecule is likely to be the one responsible for the plant's hypoglycemic property.

Polysaccharides are macromolecules known for their many biological effects and have been extensively studied in the past years as possible therapeutic options to treat diabetes. For example, Zhou *et al.* (2015) isolated and purified a polysaccharide fraction from *Rehmannia glutinosa* (Gaertn.) DC. and studied its hypoglycemic effect through the streptozotocin-induced diabetes model in rats and observed a significant reduction of important parameters in diabetes physiopathology, providing a therapeutic option for the treatment of type I diabetes. Ma, Yuan and Zhuang (2018) evaluated the antidiabetic potential of polysaccharides extracted from *Pachyrrhizus erosus* obtained through aqueous extraction using the same animal model, at dose of 100 mg/kg. The authors observed a decrease in the level of fasting glucose, regulation of the levels of serum glycated protein, total triglycerides and total cholesterol. In addition, by histological analysis, it was verified that the polysaccharide fractions obtained from the material were able to protect pancreas, liver and kidneys tissues from damage caused by diabetes. The medicinal use of polysaccharides extracted from natural sources is advantageous due to their absence of toxicity and great availability, and to their notable biological effects (PAWAR; KAMAT; CHOUDHARY, 2015).

To enlighten the contribution of *B. forficata* polysaccharides to the antidiabetic activity of the plant, the main objective of this study was to obtain a polysaccharide rich fraction using the tea's most common preparation method in folk medicine, structurally characterize the polysaccharides present in this fraction, and evaluate the fraction's antidiabetic and hypoglycemic activities using streptozotocin-induced diabetes model in rats.

## 2 Materials and Methods

### 2.1 Material of study

*B. forficata* Link leaves were acquired at local warehouse (Sasaki Alimentos) in Curitiba, State of Paraná, Brazil, in April 2019, produced by Chamel *Produtos Naturais*, under lot number 12675.

## 2.2 Extraction and preparation of crude polysaccharide fraction BFSF

Dried leaves of *B. forficata* (1 kg) were subjected to extraction through decoction, in 1:10 (w/v) of leaves:distilled water proportion, under boiling for 3 minutes (LORENZI; MATOS, 2008). This procedure was repeated three times with the same leaves for yield enhancement. The resultant extract was reduced to small volume (~300 mL) under reduced pressure and treated with cold ethanol 99% (v/v) in a 3:1 proportion (900 mL of ethanol to 300 mL of aqueous extract) aiming high molecular weight molecules precipitation, such as polysaccharides. The precipitate fraction was separated from the supernatant by centrifugation at 8000 rpm for 15 minutes at 4 °C and dialyzed against tap water, for 2 days, using 6-8 kDa cut-off membrane, to eliminate residual small molecules. The precipitate fraction, the one containing polysaccharides, was resuspended in distilled water at 10% (w/v) and submitted to freeze-thawing process (GORIN; IACOMINI, 1984) until no more precipitate was formed, giving rise to a supernatant fraction, entitled BFSF (BF stands for *Bauhinia forficata* and SF for Soluble Fraction in cold water) and a precipitated one, named BFIF (IF after Insoluble Fraction in cold water). BFSF was chosen to proceed with structural characterization and biological evaluation due to its higher water solubility and yield in comparison with the precipitated fraction.

## 2.3 Characterization of the BFSF fraction

### 2.3.1 HPSEC and molecular weight analyses

For high performance size exclusion chromatography (HPSEC) analysis, BFSF was solubilized in 0.1 M $NaNO_2$ and 0.2 g/L $NaN_3$, same solution used as eluent, to a final concentration of 1 mg/mL and then filtered through cellulose acetate membranes with a porosity of 0.22 µm. The injection volume of sample in the equipment was 100 µL with a controlled flow of 0.6 mL/min. The analysis was executed on a Waters chromatograph connected with four columns in series packed with Ultrahydrogel® (2000, 500, 250, 120; with exclusion sizes of $7x10^6$, $4x10^5$, $8x10^4$, $5x10^3$ g/mol, respectively; Milford, MA, USA), and attached to a Waters 2410 differential refractometer (RI) detector. The result was analyzed using the Wyatt Technology software ASTRA version 4.70.07. Determination of weight average molar mass (Mw) of BFSF fraction was performed using its specific refractive index increment

(dn/dc), which was obtained using the same equipment with the columns uncoupled. BFSF fraction was prepared at concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL, filtered through a 0.22 µm membrane, injected (500 µL loop) and analyzed at 25 °C using the RI detector only. The chromatograms were analyzed using the Wyatt Technology software ASTRA version 4.70.07.

2.3.2 Monosaccharide composition

Approximately 2 mg of BFSF were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 8 h. Then, the hydrolyzed material was evaporated to dryness and the residue was resuspended in distilled water and reduced with $NaBH_4$ for 18 h. The reaction was interrupted by neutralization with glacial acetic acid and the boric acid formed was removed as trimethyl borate by repeated addition and evaporation of methanol (WOLFROM; THOMPSON, 1963). With a pyridine:acetic anhydride mixture (1:1 v/v; 1 mL), the alditols were acetylated overnight at room temperature and the reaction was interrupted by addition of 1 mL of distilled water. Acetylated alditol acetates were extracted with chloroform and subsequently washed with 5% aqueous $CuSO_4$ (w/v) solution to remove residual pyridine. Lastly, the material was filtered through cotton embedded in chloroform, the organic phase was collected and dried under steam of $N_2$. The alditol acetates were analyzed by gas chromatography (GC) (Thermo Scientific Trace GC 3), using a DB-225 column (30 m x 0.25 mm), programmed from 50 up to 210 °C at a 40 °C/min rate, with helium as carrier gas. The alditol acetates were identified by comparison of their retention times with those of alditol acetates prepared from standard monosaccharides (Sigma-Aldrich).

To verify the presence and identity of the uronic acid, the hydrolyzed sample, before being treated with $NaBH_4$, was examined by silica-gel 60 thin layer chromatography (TLC; Merck). The plate was developed with ethyl acetate:n-propanol:acetic acid:$H_2O$ (4:2:2:1, v/v) and stained with orcinol–$H_2SO_4$ (SASSAKI *et al.*, 2008). Uronic acids content was quantified by colorimetric *m*-hydroxybiphenyl method according to Filisetti-Cozzi and Carpita (1991). A standard curve of galacturonic acid was built, with the absorbance recorded at 525 nm. Results were expressed as µg of galacturonic acid per 100 µg of BFSF sample.

### 2.3.3 Colorimetric determinations of phenolic compounds, total sugar and protein contents

Phenolic compounds content of BFSF was determined by Singleton, Orthofer and Lamuela-Raventos method (1999), with gallic acid as standard, and absorbance was measured at 720 nm. Total sugar content of BFSF was determined according to Dubois *et al*. (1956) method. A standard curve of a mixture of arabinose, galactose, rhamnose and galacturonic acid (1:1:1:1 w/w; 1 mg/mL) was built and the absorbance was determined at 525 nm. Proteins were quantified according to Bradford (BRADFORD, 1976) method, using bovine serum albumin (BSA) as the standard. Absorbance was read at 595 nm. Results of all analytes are expressed as µg per 100 µg of BFSF sample.

All colorimetric assays were performed in microplates and the absorbance was measured by spectrophotometry using a microplate reader (Epoch, BioTek, USA). All determinations were made in triplicate.

### 2.3.4 Linkage analysis

Once uronic acids presence was identified in BFSF, the fraction was carboxyl-reduced by the carbodiimide method (TAYLOR; CONRAD, 1972), using $NaBH_4$ as reducing agent. The carboxyl-reduced sample was dialyzed with a 3.5 kDa cut-off membrane against tap water for 2 days, freeze-dried and submitted to another cycle of carboxyl-reduction.

Carboxyl-reduced and native BFSF fractions were then methylated according to Ciucanu and Kerek (1984), using DMSO-MeI and powdered NaOH. Hydrolysis was conducted with formic acid 45% (v/v) at 100 °C for 6 h and after evaporation to dryness, the sample was hydrolyzed again with TFA 2 M at 100 °C for 16 h. Reduction was carried out using $NaBD_4$ for 18 h, and acetylation was conducted with addition of pyridine and acetic anhydride (1:1; 1 mL), originating partially O-methylated alditol acetates. The derivatized sample was analyzed by GC-MS (Shimadzu, model QP2020NX, using a quadrupole detector), using a VF-5MS column (30 m x 0.25 mm), at 100 °C, programmed at 10 °C/min to 220 °C, then 250 °C and 280 °C (held for 3 min at each temperature, with a total runtime of 30 min), with helium as carrier gas at 2 mL/min. The partially O-methylated alditol acetates were identified by their

characteristic retention times and electron ionization mass spectra, relative to partially O-methylated alditol acetates prepared from standard monosaccharides (Sigma-Aldrich), according to Sassaki *et al.* (2005).

### 2.3.5 NMR analysis

The NMR analysis was performed using a 400 MHz Bruker-Avance III spectrometer (Bruker, Germany). BFSF was solubilized in $D_2O$ (600 µL), sonicated for 15 minutes, centrifuged at 8000 rpm for 5 min, and the supernatant analyzed at 50 °C. The $^{13}C/^{1}H$ correlations observed in the HSQC spectrum were expressed as chemical shifts (δ) in ppm relative to the resonance of the anomeric $^{13}C/^{1}H$ of arabinose at δ 109.5/5.25 (CARLOTTO *et al*., 2019). The correlation map was analyzed in Topspin software version 4.1.3 and signals were attributed according to literature data available.

## 2.4 Animals

Experiments were conducted with adult male Whistar rats (220-300 g), supplied by the UFPR Biological Sciences Sector Vivarium. Animals were kept in 12 h light/dark cycle, at controlled temperature (22 ± 2 °C), with air exhaustion and with free access to food and water, except when kept fasting 12 h prior to streptozotocin (STZ) administration. All protocols were analyzed and approved by UFPR Animal Use Ethics Committee (CEUA / BIO-UFPR; approval number 1422).

## 2.5 Antidiabetic effect evaluation of BFSF

After 12 h of fasting, diabetes was induced in rats by a single injection of STZ at 60 mg/kg, solubilized in citrate buffer (10 mM, pH 4.5), intraperitoneally (i.p.). STZ is described as a useful inducer of type I diabetes for experimental models of hypoglycemic activity studies (FRÖDE; MEDEIROS, 2008). Hyperglycemia was confirmed 3 days after STZ injection, collecting 5 µL of blood from the tail vein, adding to glucose oxidase impregnated strips (Accu-Check ActiveTM, Roche), and analyzed using a digital glucometer (Accu-CheckTM, Roche). Animals with glycemia equal to or greater than 250 mg/dL were considered diabetic and kept in the experiment. Rats were randomly divided into 5 groups (4 groups treated with BFSF fraction and a control group), with a minimum of 8 animals per group.

Ten days after STZ administration, different BFSF doses (10, 30, 100 and 300 mg/kg; orally by gavage; 500 µL), chosen considering polysaccharide fraction doses used before in other studies (SEEDEVI *et al*., 2020; DE PAULA *et al.*, 2005), were evaluated for potential acute hypoglycemic activity by measuring blood glucose level at 0, 30, 60, 120 and 180 minutes after BFSF administration. The control group received only vehicle (saline; aq. NaCl 0.9% w/v). To assess the chronic effect, treatments were maintained with doses of 10, 30 and 100 mg/kg for 21 days and blood glucose was measured every other day before the daily treatment. The high dose of 300 mg/kg were not used in the chronic assay because of the amount of BFSF fraction available and the number of animals in the experiment. On the last day of the experiment, animals were euthanized by decapitation. Blood samples were collected in different centrifuge tubes, containing or not anticoagulant, for further biochemical and hematological analysis. Animal body weight was measured every week throughout the experiment.

2.6 Biochemical and blood count analysis

The blood was collected in heparinized centrifuge tubes and immediately sent to the clinical laboratory of the Veterinary Hospital of the Federal University of Paraná to evaluate biochemical parameters. The biochemical parameters analyzed in the plasma were: triglycerides, total cholesterol, HDL-C (high-density lipoprotein cholesterol), LDL-C (low-density lipoprotein cholesterol), VLDL-C (very low-density lipoprotein cholesterol), ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), total protein, albumin, globulin, total bilirubin, direct bilirubin, indirect bilirubin, creatinine and urea. LDL-C was calculated by Friedewald's formula.

The hematological parameters analyzed were WBC (white blood cell), Lym (lymphocytes), Mon (monocytes), Gran (granulocytes), RBC (red blood cell), hemoglobin, HCT (hematocrit level), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW (red cell distribution width), platelets, MPV (mean platelet volume), PDW (platelet distribution width), PCT (plateletcrit).

To determine insulin content in serum, whole blood was collected into centrifuge tubes with no anticoagulant and the sample was left to clot at room

temperature for 30 min. Then, the clotted blood was centrifuged at 4500 rpm for 15 min. Insulin quantification was performed by ELISA (enzyme-linked immunosorbent assay), according to the manufacturer's kit instructions (Merck Millipore, St. Charles, Missouri, USA).

### 2.7 Histological studies

A small piece of the left lobe of the liver of each animal was collected, fixed in formalin (10% v/v) solution for 48 h at room temperature, and embedded in paraffin wax. All sections were stained with hematoxylin-eosin dye. The histological parameters analyzed were trabecular disorganization, inflammatory infiltrate, cytoplasmic vacuolation, nuclear vacuolation, megalocytosis, apoptosis and necrosis in hepatocytes. Histological score analysis was based on lesion frequency, being 0-absent, 1-mild, 2-moderate or 3-severe (Table S2), and each parameter was multiplied by a severity factor, according to what was previously established on literature (TERCIOLO *et al*., 2019).

### 2.8 Statistical analysis

Statistical analysis of acute and chronic treatment with BFSF and body weight were performed by two-way analysis of variance (ANOVA) (two parameters: time and BFSF treatment). Data were analyzed by Shapiro-Wilk normality test and Tukey's test of multiple comparison. All other parameters were analyzed by Shapiro-Wilk normality test, one-way ANOVA, followed by Tukey's test of multiple comparison. Results are expressed as mean ± standard error of mean (SEM) and the difference was considered significant when $*p < 0.05$ or $**p < 0.01$. All data was processed using GraphPad Prism® version 8.00.

## 3 Results and Discussion

### 3.1 Extraction and structural characterization of BFSF

Dried leaves of *B. forficata* (1 kg) were submitted to aqueous extraction, followed by purification processes, as described in the methods section, resulting in a crude polysaccharide fraction, entitled BFSF, with a yield of 0.95% (w/w) in relation to the dried leaves. BFSF was analyzed through HPSEC-RI, showing an almost

homogeneous elution profile (Figure 1), with a major peak in 57.9 min. The weight average molar mass (Mw) of BFSF, calculated by its dn/dc value (0.118), was 1.08 x $10^5$ g/mol.

In relation to its monosaccharide composition (Figures S1 and S2), BFSF is constituted of arabinose (27.4%), galactose (23.7%), rhamnose (16.1%), glucose (13.8%), galacturonic acid (10.0%), mannose (6.2%), xylose (2.5%) and fucose (0.3%). Furthermore, total sugar content was determined as 50.4%, protein was 15.1% and phenolic compounds 15.0%. Therefore, BFSF is a complex fraction constituted of many different compounds rather than a purified fraction constituted exclusively by a polysaccharide. However, since its carbohydrate content is higher than other constituents, BFSF was considered a polysaccharide rich fraction.

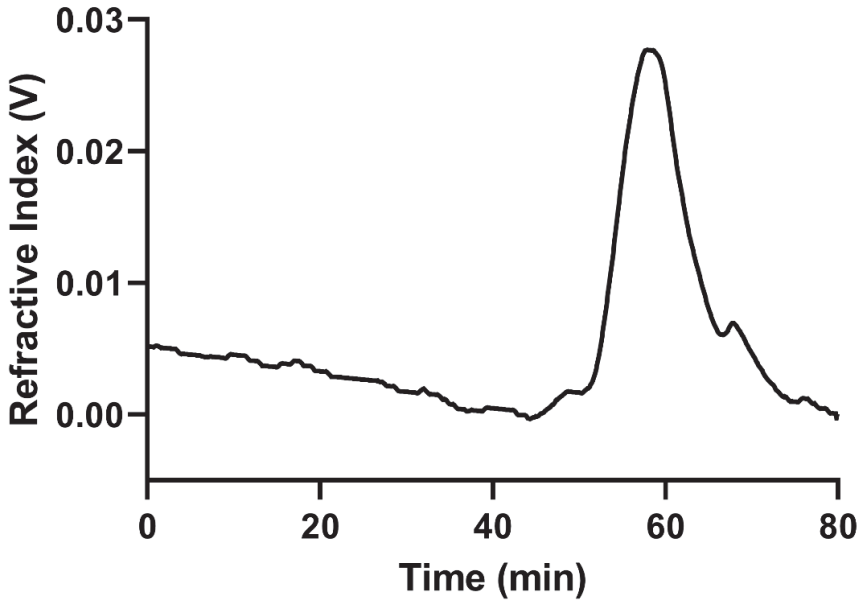


**Figure 1: Elution profile of BFSF in HPSEC-RI.**

Table 1: Profile of partially O-methylated alditol acetates and their respective linkage type of BFSF fraction obtained by methylation analysis.

| O-Me-alditol acetate | Linkage type | % |
|---|---|---|
| 2,3,5-$Me_3$-Ara*f* | Ara*f*-(1→ | 4.6 |
| 2,3,4-$Me_3$-Rha*p* | Rha*p*-(1→ | 19.4 |
| 2,3,4-$Me_3$-Ara*p* | Ara*p*-(1→ | 2.5 |
| 2,5-$Me_2$-Ara*f* | →3)-Ara*f*-(1→ | 1.8 |
| 2,3-$Me_2$-Ara*f* | →5)-Ara*f*-(1→ | 5.0 |
| 2,3-$Me_2$-Xyl*p* | →4)-Xyl*p*-(1→ | 2.0 |
| 2,3,4,6-$Me_4$-Glc*p* | Glc*p*-(1→ | 11.0 |
| 2,3,4,6-$Me_4$-Gal*p* | Gal*p*-(1→ | 5.5 |
| 2,3,6-$Me_3$-Glc*p* | →4)-Glc*p*-(1→ | 4.2 |
| 2,3,6-$Me_3$-Gal*p* | →4)-Gal*p*-(1→ | 5.9 |
| 2,3,4-$Me_3$-Glc*p* | →6)-Glc*p*-(1→ | 8.8 |
| 2,3,4-$Me_3$-Gal*p* | →6)-Gal*p*-(1→ | 6.8 |
| 2,3-$Me_2$-Glc*p* | →4,6)-Glc*p*-(1→ | 1.1 |
| 2,3-$Me_2$-Gal*p* | →4,6)-Gal*p*-(1→ | 1.0 |
| 3,6-$Me_2$-Glc*p* | →2,4)-Glc*p*-(1→ | 9.7 |
| 3,6-$Me_2$-Gal*p* | →2,4)-Gal*p*-(1→ | 9.0 |
| 2,4-$Me_2$-Gal*p* | →3,6)-Gal*p*-(1→ | 1.5 |

Note: EI-MS spectrum of each methylated derivative of native BFSF is shown on Fig. S3.

The glycosidic linkage analysis of BFSF showed numerous partially O-methylated alditol acetates derivatives (Table 1; Figure S3), what is characteristic of a complex polysaccharide fraction. The methylation analysis of the BFSF fraction showed a high amount of non-reducing terminals in relation to branch points, which is conflicting with a polysaccharide structure, since in these molecules the non-reducing terminals are in equal number to branch points. Nevertheless, it was possible to identify the presence of methylated derivatives that still provides useful information about the linkage types between the monosaccharides that build up the main polysaccharides. Presence of type I arabinogalactan (AG-I) was confirmed by the derivatives 2,3,6-$Me_3$-Gal*p* (5.9%) and 3,6-$Me_2$-Gal*p* (9.0%), from the main chain constituted by β-D-Gal*p*-(1→4)-linked units and to the branching point in the main chain at the O-2 position, probably by non-reducing terminals of arabinose (PETTOLINO *et al.*, 2012). Type II

arabinogalactan (AG-II) was identified by the presence of the 2,3,4-$Me_3$-Gal*p* (6.8%) and 2,4-$Me_2$-Gal*p* (1.5%) methylated derivatives, which are from a main chain of β-D-Gal*p*-(1→6)-linked units, partially substituted in O-3 (ASPINALL, 1973; CARPITA; GIBEAUT, 1993; PETTOLINO *et al*., 2012). Moreover, the 2,3-$Me_2$-Ara*f* (5.0%) derivative confirms the presence of a (1→5)-linked arabinan (PETTOLINO *et al.,* 2012). Also, it is suggestive that starch is present in the BFSF fraction, due to the appearance of the methylated derivative 2,3,6-$Me_3$-Glc*p* (4.2%), corresponding to (1→4)-linked Glc*p* units.

Since galacturonic acid was detected in BFSF and considering that uronic acids are unsuitable for analysis by GC-MS, the fraction was submitted to carboxyl-reduction to transform acidic monosaccharides into their corresponding neutral monosaccharides. The only difference between native and carboxyl-reduced BFSF was an increase of the methylated derivative 2,3,6-$Me_3$-Gal*p* from 5.9% to 10.4%, suggesting there is also a small amount of homogalacturonan (HG) in BFSF. HG are linear polysaccharides constituted of α-D-Gal*p*A units, joined together by α-(1→4) bonds. Units of galacturonic acid in the polysaccharide structure can be methyl esterified at the C-6 carboxyl and can be O-acetylated at C-2 or C-3. These chemical modifications may vary between plant species (RIDLEY; O'NEILL; MOHNEN, 2001).

The methylated derivatives 2,3,4,6-$Me_4$-Glc*p* (11.0%) and 2,3,4-$Me_3$-Rha*p* (19.4%) are non-reducing terminals that most likely are not derived from a polysaccharide structure, but probably from glycosylated secondary metabolites, especially considering the result obtained from total phenolic compounds determination (15.0%). For instance, kampferol-3-7-dirhamnoside (SOUSA *et al.,* 2004), a phenolic compound with an aromatic nucleus linked to two rhamnose residues, have been described before in literature as a secondary metabolite of *B. forficata*, and its presence in BFSF fraction could explain the high proportion of terminal units of rhamnose that was observed in methylation analysis. Also, this possibility has already been described by Carlotto *et al.* (2020). They observed a high content of non-reducing terminals of rhamnose and 17% of total phenolic compounds in a polysaccharide fraction isolated from *ipê-roxo* barks. In another study, it was suggested that these sugar terminals units can be linked to phenolic compounds such as condensed tannins and stilbene glucosides (LE NORMAND *et al.,* 2014).

The $^{13}C/^{1}H$ correlation map (HSQC-DEPT) of the BFSF (Figure 2) showed correlations of C-1/H-1 of α-L-Ara*f* at δ 109.5/5.25 and of β-D-Gal*p* at δ 103.5/4.45 (CAPEK *et al.,* 2010; BRECKER *et al.,* 2005). At δ 82.4/4.04 was observed the C-3/H-3 correlation of 3-O-substituted β-D-Gal*p* units (DELGOBO *et al.,* 1998). Furthermore, the correlation at δ 77.0/3.95 was attributed to the C-4/H-4 of 4-O-substituted β-D-Gal*p* residues (DELGOBO *et al.,* 1998). These correlations suggest the presence of type II and type I arabinogalactans (AG-II and AG-I) in BFSF, respectively. The correlations observed in δ 66.5/3.91 and 67.0/3.87 ppm are related to C-5/H-5 of →1)-α-L-Ara*f*-(5→ units (SCHNEIDER; IACOMINI; CORDEIRO, 2019; SHAKHMATOV *et al.*, 2014), suggesting the presence of arabinan in BFSF. Still in the anomeric region, correlations at δ 100.6/4.75 and δ 99.6/5.00 are characteristic of C-1/H-1 of methylesterified and non-methylesterified α-D-Gal*p*A units, respectively. The correlation at δ 53.3/3.80 belongs to the methyl group of the esterified residues. These correlations suggest the presence of a homogalacturonan in BFSF fraction. Degree of esterification was calculated by integration of signals of C-1/H-1 of methylesterified and non-methylesterified and resulted in 39.8%. The C-6/H-6 correlation of non-reducing end-units of α-L-Rha*p* can be seen at δ 16.7/1.27 (OVODOVA *et al*., 2009; POPOV *et al.*, 2011; RENARD *et al*., 1998).

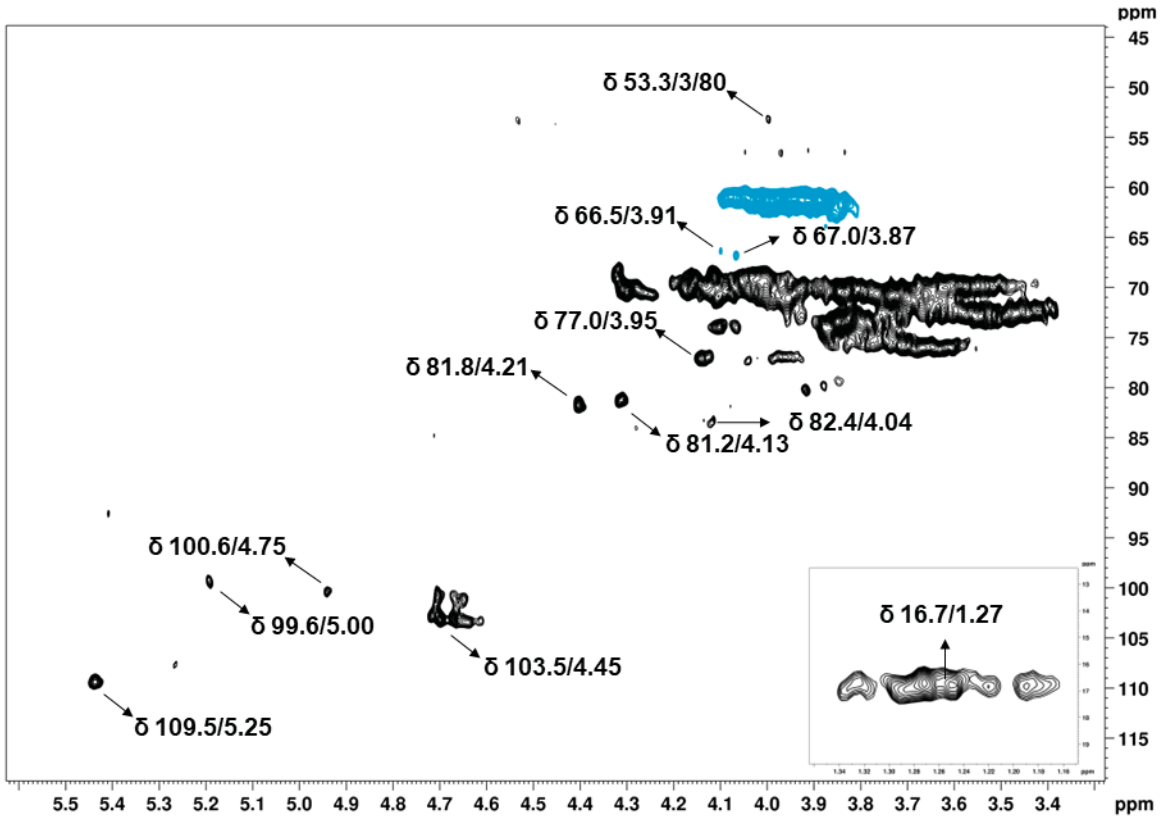


**Figure 2: $^{13}C/^{1}H$ HSQC-DEPT correlation map of BFSF.** The sample was solubilized in $D_2O$ and analysis was performed at 50 °C, in a 400 mHz spectrometer. Chemical shifts are expressed as ppm. Blue signals are from $-CH_2$.

*B. forficata* leaves are commonly used in folk medicine, in the form of an herbal tea, as an adjutant in diabetes treatment. Since polysaccharides from other plants have presented antidiabetic and hypoglycemic effects, as reported in literature, the contribution of the polysaccharide fraction BFSF to the known antidiabetic property of *B. forficata* was evaluated.

3.2 Evaluation of antidiabetic activity of BFSF

To understand the acute impact of BFSF fraction on blood glucose levels, STZ-induced diabetic rats were treated with different doses of BFSF fraction (10, 30, 100 and 300 mg/kg) or saline, orally by gavage. Blood glucose was monitored for 3 h, measured at times 0, 30, 60, 120 and 180 min after treatments. From these data, a glycemic curve was built, and data was obtained about BFSF acute effect on glycemia, as shown in Figure 3. No influence of BFSF on blood glucose levels was observed in

comparison with the control group, treated only with saline, within the period of experimentation, suggesting that there is no immediate effect from BFSF in glycemia of rats with type I diabetes. In agreement with this finding, Pepato *et al*. (2002) also reported no acute alteration in glycemia levels when treating diabetic rats induced with STZ with *B. forficata* decoction.

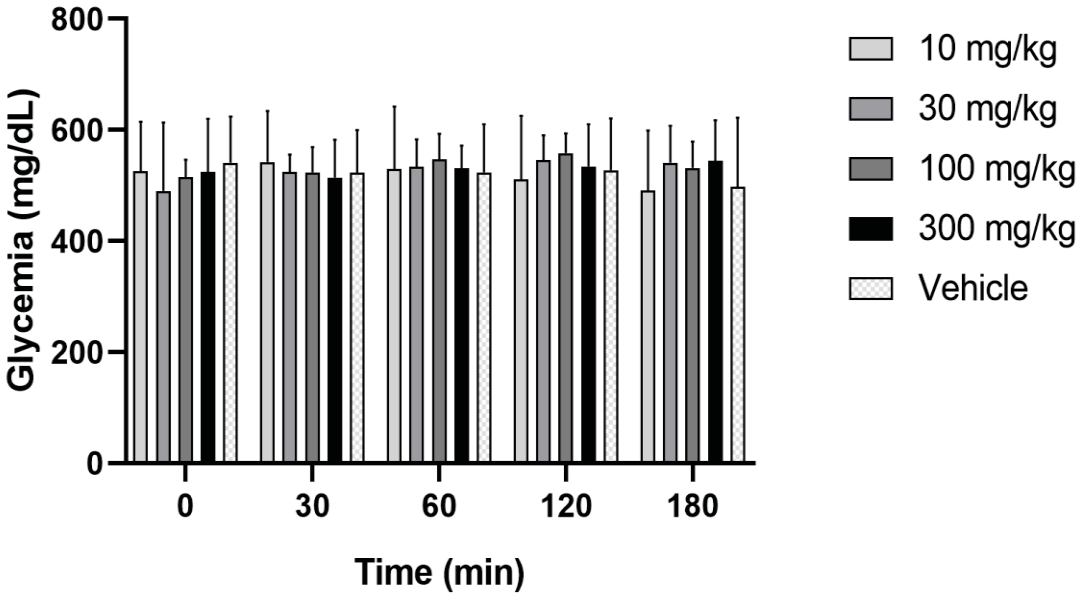


**Figure 3: Acute effect on glycemia on diabetic rats treated with BFSF at 10, 30, 100 and 300 mg/kg or vehicle (saline).** Results are expressed as mean ± SEM (n = 8). Comparison between groups were performed by two-way ANOVA (blood glucose levels; time) followed by Tukey's test of multiple comparison.

To verify long-term effects on blood glucose levels of diabetic rats, BFSF treatment, at doses of 10, 30 and 100 mg/kg, was maintained for 21 days (Figure 4). Although there is a slight tendency of glycemia reduction on groups treated with BFSF at 10 mg/kg and 30 mg/kg, these results are not statistically relevant when comparing the treatments with control group over time. Salgueiro *et al*. (2016) also did not observe a significant hypoglycemic effect when treating mice for 21 days with cow's paw tea, prepared with 1 mg of dried leaves per 1 mL of water, in contrast to what was previously reported by Pepato *et al*. (2002), that observed a chronic hypoglycemic effect when treating animals with *B. forficata* decoction, prepared with 150 g of fresh leaves in 1 L

of water. Thus, the proportion (leaves per water) and the state (dried or fresh) of the leaves can influence the characteristic of the compounds that are extracted.

Regarding evaluation of *B. forficata* hypoglycemic activity, Jorge *et al*. (2004) tested the antidiabetic effect of a butanoic fraction obtained from the plant leaves, containing kaempferitrin as predominant compound, in alloxan-induced diabetic rats, and observed a significant hypoglycemic effect between 1 and 3 h after oral administration in comparison with untreated diabetic control group and suggested that this reduction is promoted by insulin-mimetic effect, altering intrinsic activity of glucose transporter. Franco *et al*. (2020) tested fractions obtained from liquid-liquid partition of an ethanolic macerate from the leaves of *B. forficata* and water, hexane, dichloromethane, ethyl acetate and n-butanol, analyzing many important parameters related to diabetes, including enzyme inhibition and antioxidant activity assays. They observed that some of these rich-polyphenol and flavonoid fractions could significantly inhibit α-amylase, α-glucosidase and lipase and significantly reduce ROS production. Cunha *et al*. (2010) showed a decrease of 48.17% in plasma glucose levels of STZ-induced diabetic rats in comparison with vehicle group when testing a fraction with high flavonoid concentration obtained by percolation using ethanol-water (1:2) followed by step of oven-dry at 50 °C for 26 h. Also, as mentioned before, hypoglycemic property was observed by Sousa *et al*. (2004), that extracted and isolated kaempferitrin from the plant leaves and tested it for its antidiabetic potential, using the same animal model used in the present study. In conclusion, all these scientific studies have in common the acquisition of a fraction mainly constituted of phenolic compounds, polyphenols and flavonoids, and as they all observed an interesting and expressive effect in diabetes-related parameters, it is plausible to consider that such molecules act directly and beneficially to ameliorate diabetes condition. The results of our experiments indicate that polysaccharides, major components in BFSF, do not play a role in reducing blood glucose in STZ-induced diabetic rats. Since *B. forficata* is very well-known for its effects of blood glucose minimization and there are other studies which demonstrate that polyphenols could be responsible for its hypoglycemic activity (SOUSA *et al*., 2004), our results reinforce that these molecules are the ones responsible for reducing glycemia alone.

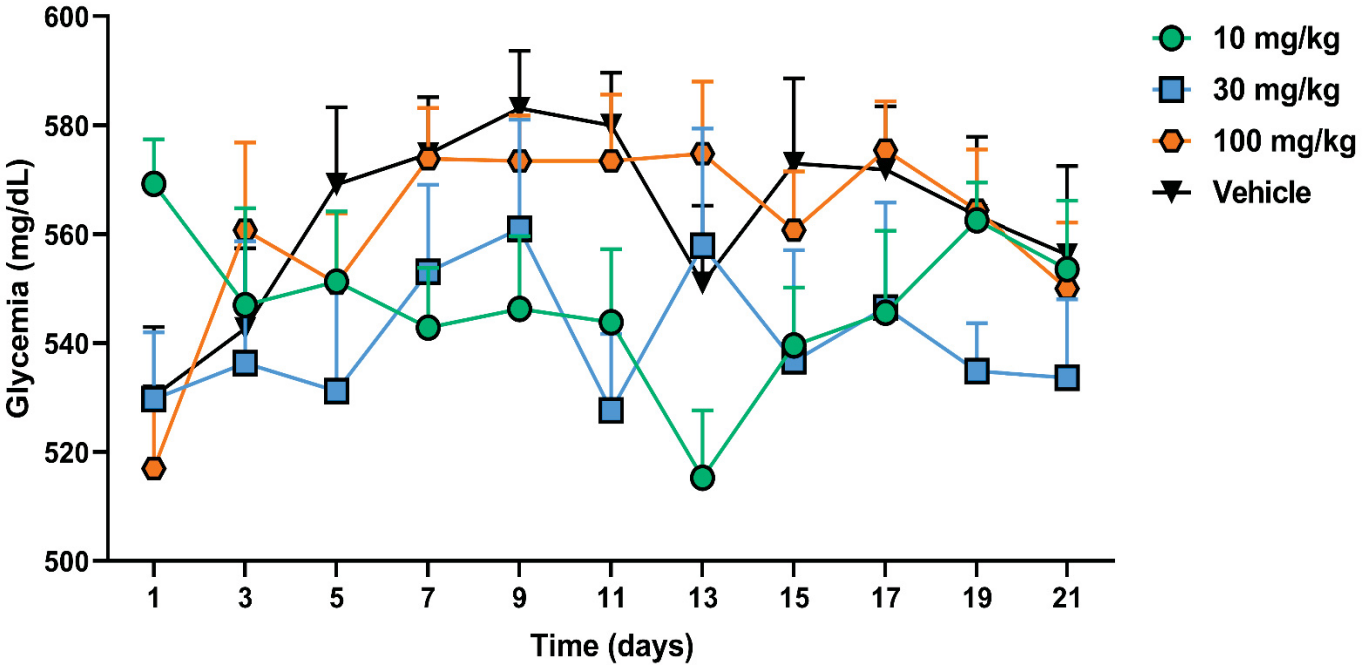


**Figure 4: Chronic effect on glycemia on diabetic rats treated with BFSF at 10, 30 and 100 mg/kg or vehicle (saline).** Results are expressed as mean ± SEM (n = 8). Comparison between groups were performed by two-way ANOVA (blood glucose levels; time) followed by Tukey's test of multiple comparison.

Since polysaccharides are non-toxic, safe, widely distributed in nature and accessible biomolecules with several biological effects, they could be a good alternative for pharmaceuticals that can provoke undesirable side-effects. It is known from previous work that polysaccharides extracted from natural sources may have hypoglycemic and antidiabetic activity. For example, Liu *et al*. (2018) isolated an arabinogalactan from *Phyllostachys heterocycla* and observed positive effect on inhibition of glucose absorption on Caco-2 cells. Also, Liu *et al*. (2017) tested a rhamnogalacturonan extracted from the edible vegetable okra for antidiabetic activity and found reduced blood glucose levels and glucose tolerance in groups treated with this polysaccharide. In this regard, it was plausible to investigate the effects of polysaccharides from *B. forficata* leaves, especially as it is a plant so widely used in folk medicine for this purpose. Although there are antidiabetic and hypoglycemic polysaccharides from plants with structure and composition similar to those extracted, purified and characterized from *B. forficata* leaves in this study, these compounds do

not play this role and do not contribute to hypoglycemic effect of cow's paw when analyzed through the animal model of type I diabetes described in this study. Still, the accomplishment of experiments evaluating hypoglycemic activity with other compounds isolated from *B. forficata* are encouraged, in order to investigate their contribution to this biological property of the plant.

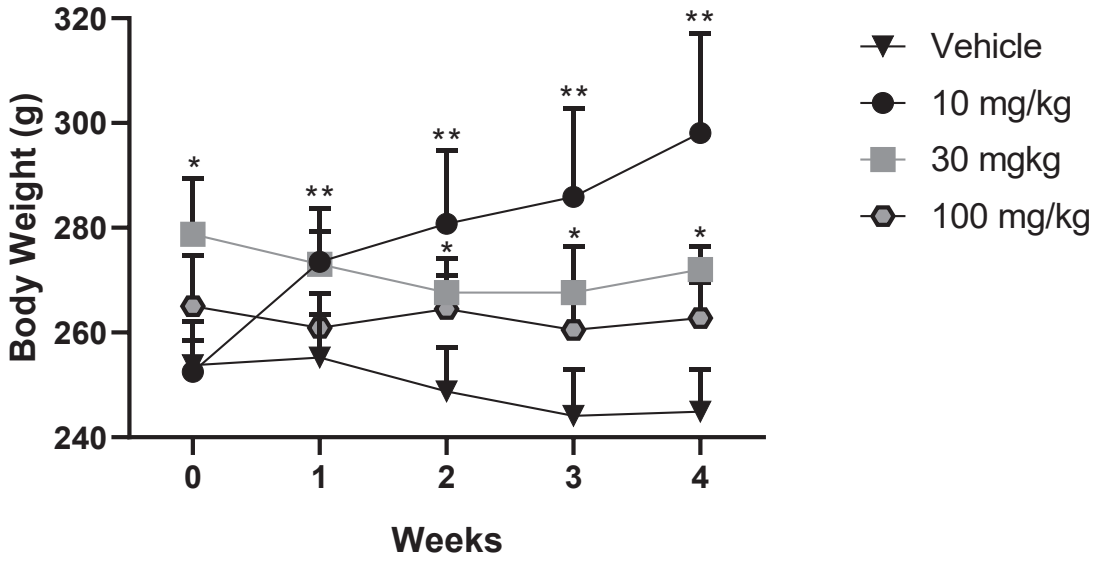


**Figure 5: Effect of the chronic BFSF treatment at 10, 30 and 100 mg/kg on diabetic rats body weight**. Animals were weighed once every week during the time of the experiment. *p < 0.05 and **p < 0.01 when compared to the vehicle group (saline). Results are expressed as mean ± SEM (n = 8). Comparison between groups were performed by two-way ANOVA (body weight; time) followed by Tukey's test of multiple comparison.

As to the animal body weight, the treatments with 10 and 30 mg/kg BFSF significantly increased the parameter, while with 100 mg/kg BFSF there was no significant difference when compared to the vehicle group (Figure 5). These weight gain trend in animals treated with *B. forficata* preparations were also observed in the study conducted by Pinafo *et al*. (2019), in which the authors evaluated whether a commercial extract prepared with cow's paw leaves would have an influence on the metabolic disruption caused by the treatment of animals with BPA (bisphenol A; 2,2-

bis(4-hydroxyphenyl)propane), a compound that interferes with several metabolic processes.

Diabetes is a complex and multifactorial disease. Its origins depend on genetic, environmental and behavioral factors and the disease progression leads to several complications. Therefore, it is essential for patients with diabetes to carry out laboratory monitoring of biochemical parameters such as biomarkers of renal, hepatic and metabolic dysfunction. To estimate BFSF effects on such parameters, several biochemical analyses were performed after the chronic treatment of the diabetic rats with BFSF, and the results are represented in Figure 6 and Figure S4. The descriptive data is gathered in Table S1.

Among all the biochemical markers tested, BFSF fraction was able to reduce direct bilirubin and AST levels. Regarding conjugated bilirubin, when compared to the vehicle group, it was observed a decrease of 63.7% at dose of 10 mg/kg and 54.6% at 30 mg/kg. Direct or conjugated bilirubin relates to conjugation between bilirubin and glucuronic acid in the liver. Higher concentration of direct bilirubin can suggest liver damage or biliary obstruction (KUMAR; COTRAN; ROBBINS, 1992). AST level was 43.7% lower in the group treated with BFSF at 10 mg/kg when compared to the vehicle group. This could suggest a hepatoprotective effect of BFSF, but further investigations are still necessary. BFSF didn’t interfere with any of the hematological components evaluated (Table S1).

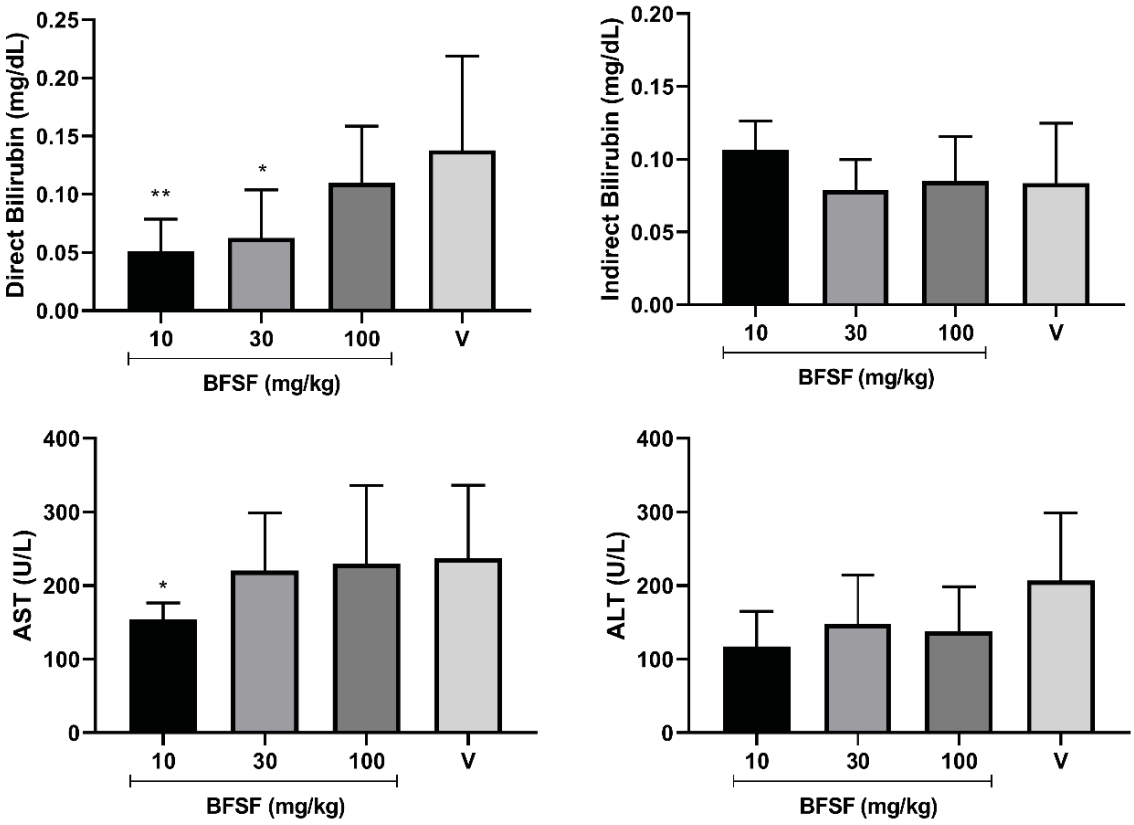


**Figure 6: Effect of the chronic BFSF treatment at 10, 30 and 100 mg/kg on direct and indirect bilirubin, ALT and AST liver enzymes.** *$p < 0.05$ and **$p < 0.01$ when compared to the vehicle group (saline). Results are expressed as mean ± SEM ($n > 8$). Comparison between groups were performed by one-way ANOVA followed by Tukey's test of multiple comparison.

Liver disease has been reported in type I diabetic patients. Elevated aminotransferases activities are more frequently observed among people with diabetes in comparison with general population (ARKKILA *et al*., 2001). Prevalence of hepatic steatosis between type I diabetes patients was investigated before and found that 11.3% of the patients between 8 months and 15 years old and 24% of adults between 58 and 72 years old presented fatty liver (REGNELL; LERNMARK, 2012).

To comprehend the influence of BFSF fraction on hepatic tissue, a small piece of the liver's left lobe of each animal was analyzed by histological studies, collected on the last day of the pharmacological study (21$^{st}$ day of experiment). From the occurrence, frequency and severity of tissue damage parameters, a histological score

was calculated (Figure 8), that takes into consideration appearance of inflammatory infiltrate, trabecular disorganization, cytoplasmic vacuolation, nucleic vacuolation, megalocytosis, apoptosis and necrosis of hepatocytes. Each parameter has a different influence on the final score. All observations of analyzed histological slides of all groups are described in Table S2.

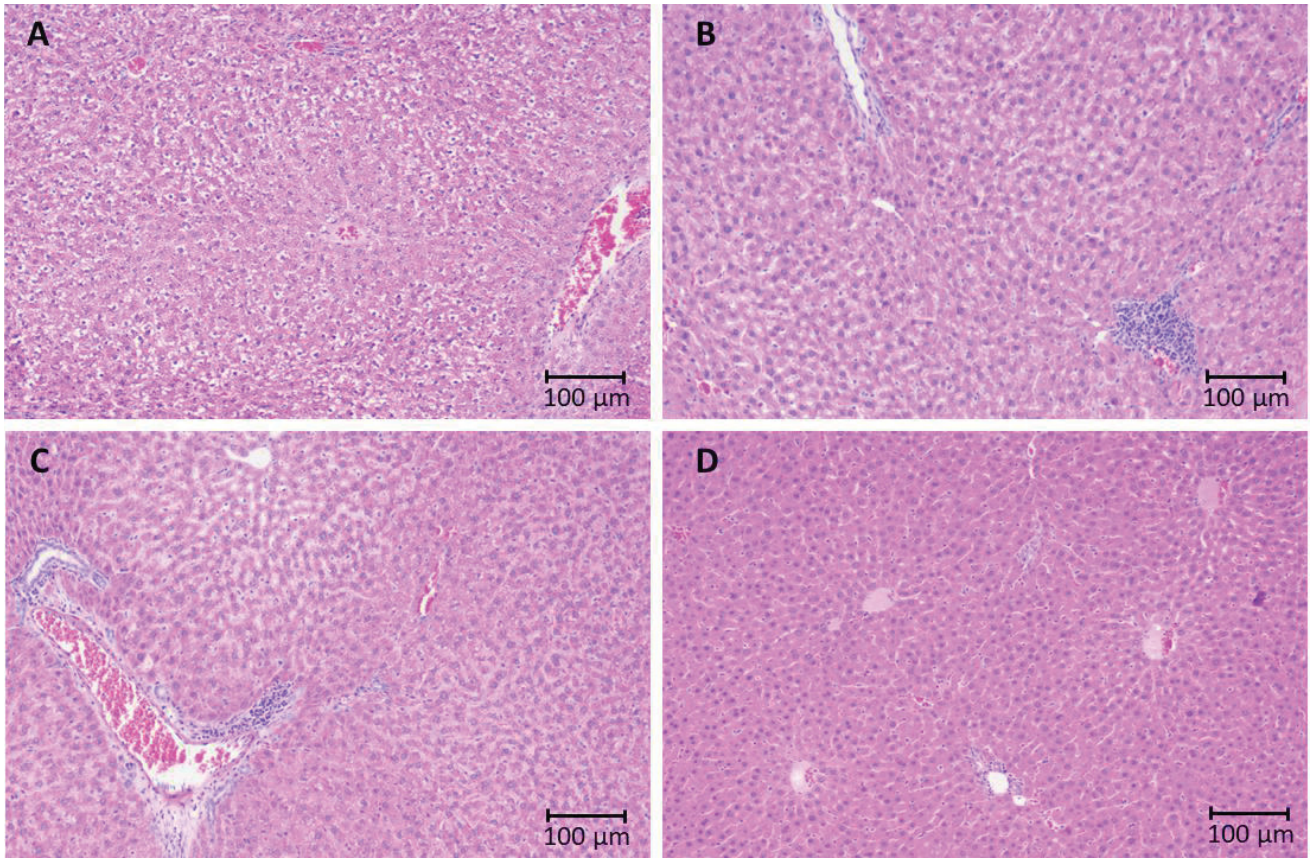


**Figure 7: Effect of BFSF on histological parameters of diabetic rat liver.** Hematoxylin and eosin-stained sections of liver. Animals were treated orally by gavage every day for 21 days with saline as negative control (A), or BFSF at 10 (B), 30 (C) or 100 (D) mg/kg.

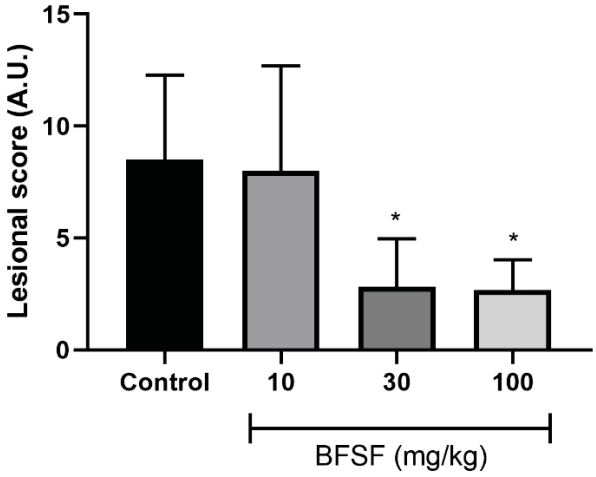


**Figure 8: Histological score of liver damage after chronic treatment with BFSF in diabetic rats.** *p < 0.05 when compared to the control group (saline). Results are expressed as mean ± SEM (n = 6). Comparison between groups were performed by one-way ANOVA followed by Tukey's test of multiple comparison.

The histological analysis of liver sections from all treated groups showed a clear hepatic damage by diabetes induced by STZ injection (Figure 7). As shown in Figure 7A, liver from rats in control diabetic group had a damaged hepatic architecture and cytological alterations such as cytoplasmic vacuolation, inflammatory infiltrate (not shown) and multifocal necrosis were observed. In the treated group with BFSF at 10 mg/kg (Figure 7B), a largely damaged tissue was detected, with hepatocytes exhibiting accentuated cytoplasmatic vacuolization, some necrotic cells presenting pyknosis and karyolysis, and lymphocytic infiltration. In fact, histological score from this group did not demonstrate any difference in comparison to control group (Figure 8). However, although some tissue damage in treated groups with BFSF at 30 and 100 mg/kg (Figures 7C and 7D, respectively) is still observed, cellular alterations analyzed were far more discrete and sometimes absent in comparison to control group. It is possible to verify in the histological images that in these groups the intrinsic cellular architecture of the liver was either restored or their disruption was prevented by BFSF treatment. Regarding the histological score, BFSF at 30 mg/kg was able to reduce 67.0% extent of injury, and at 100 mg/kg, 69.4%. These findings show that treatment with BFSF fraction, at doses of 30 and 100 mg/kg, significantly reduced severity and extension of tissue damage. This result, along with reduction of biochemical parameters related to

liver health presented before, shows that BFSF fraction had a positive effect on hepatic tissue, thus indicating a hepatoprotective effect.

Salgueiro *et al.* (2016) tested an aqueous extract produced using 1 mg/mL of *B. forficata* Link subsp. Pruinosa (Vogel) Fortunato & Wunderlin and investigated its effects against oxidative stress and liver damage in diabetic mice. Animals were also diabetic induced with a STZ injection (150 mg/kg) and treatment with *B. forficata* leaves tea was maintained for 21 days. They did not observe a chronic hypoglycemic effect from the aqueous extract, which the authors attribute to the absence of kaempferol-3,7-O-(α)-dirhamnoside, but they did observe hepatoprotective activity promoted by the tea, by different approaches from the ones used in our study. They suggest that treatment with the plant tea can modulate the liver oxidative damage that occurs in diabetic mice by increasing catalase activity, reducing liver DCF-RS (dichlorofluorescein reactive species) and TBA-RS (thiobarbituric acid reactive species) levels. Pinafo *et al*. (2019) assessed the effects of a commercial alcoholic *B. forficata* leaves extract on Wistar rats exposed to BPA (bisphenol A; 2,2-bis(4-hydroxyphenyl)propane), a compound that interferes with several metabolic processes. BPA elevated hepatic malondialdehyde (MDA) concentration and reduced catalase activity, indicating liver oxidative stress. Treatment with *B. forficata* reduced MDA levels and did not reduce catalase activity, protecting the liver mainly by its antioxidant property. These studies converge and support the hepatoprotective property performed by the plant tea. The test fraction BFSF is, among other biomolecules, composed mainly of carbohydrates. Although is not possible to affirm the hepatoprotective effect observed is from polysaccharides present in BFSF, it is likely that the polysaccharides contributed to it, since they build up the majority of the fraction's composition. For that, research that aims to test purified polysaccharide fractions isolated from *B. forficata* leaves is encouraged.

Polysaccharides have been reported for its great biological properties, including hepatoprotective one. For example, Chaves *et al.* (2020) isolated an inulin type fructan from *Baccharis trimera*, commonly known as ''carqueja'' and showed that the polysaccharide was able to protect liver tissue against $CCl_4$-induced injuries, and at 1 mg/kg it was able to significantly diminish ALT, AST and ALP levels. Wang et al. (2020) tested an acidic polysaccharide isolated from *Panax notoginseng* residue, mainly constituted of a backbone of GalA (1→4) linked units, and branches of

arabinose and rhamnose terminals and arabinan (1→5) chains, and observed normalization of ALT, AST and MDA levels. More specifically to hepatoprotective effect performed by arabinogalactans, Sun *et al.* (2018) tested a type II arabinogalactan isolated from black soybean against its hepatoprotective potential by carbon tetrachloride-induced liver injury, in mice. Treatment with AG significantly attenuated the increase of ALP, ALT and AST levels, caused by $CCl_4$, aside from elevating levels of antioxidant enzymes and non-enzyme antioxidants and reducing liver peroxidation.

There are studies whose main objective is to investigate how polysaccharides can act as hepatoprotective. Qu *et al*. (2020), in their review paper about hepatoprotective effect of polysaccharides, stated hepatoprotective polysaccharides mechanisms mainly by three different ways: reducing inflammation, oxidative stress and/or apoptosis, affecting various biochemical pathways. Concerning structure and activity relationship between polysaccharides and hepatoprotective property, the authors pointed those polysaccharides containing higher uronic acids content, complex monosaccharide compositions, lower molecular weights and higher degree of branching perform better effect. BFSF presented expressive hepatoprotective effect and as to its structural parameters, has high proportions of galactose, arabinose, rhamnose, glucose, and in minor extension xylose, fucose and mannose. It also presented 10% galacturonic acid and a relative molecular weight of 1.08 $x10^5$ g/mol. Thus, BFSF possesses features that meet several requirements established by the author. Still, experiments are required in order to establish a mechanism responsible for the hepatoprotective effect herein observed by BFSF fraction.

## 4 Conclusions

In this study was prepared a fraction (BFSF), mostly constituted of polysaccharides among other compounds, from *Bauhinia forficata* Link leaves, a popularly used medicinal plant especially as coadjutant in diabetes treatment. By analyses of structural characterization of polysaccharides was demonstrated BFSF fraction is mainly constituted of type I and type II arabinogalactans and type I rhamnogalacturonan. This fraction was tested against its potential contribution to the already known antidiabetic property of the plant. Although BFSF did not ameliorate acute or chronic glycemic levels in diabetic animals, an hepatoprotective activity

promoted by the fraction was observed, when compared to the negative control group, by diminishing direct bilirubin and AST levels, and presenting a significant effect on attenuating liver damage. Despite more research being necessary, the findings of the present study are interesting to better understand the effect of the plant in diabetic patients.

## 5 Authors Contributions

Giuliana Cozzella Campo Grande – Investigation, Methodology, Writing-Original draft; Genilza da Silva Mello – Investigation, Methodology; Philippe Rodrigues Benedetti – Investigation, Methodology; Matheus Vinicius Ferreira – Investigation, Methodology; Jaderson Pedro: Investigation, Methodology; Carlos Alves de Jesus: Investigation, Methodology; Joice Maria da Cunha: Investigation, Methodology, Conceptualization; Thales Ricardo Cipriani - Conceptualization, Project administration, Funding acquisition, Supervision and Writing – Review and editing.

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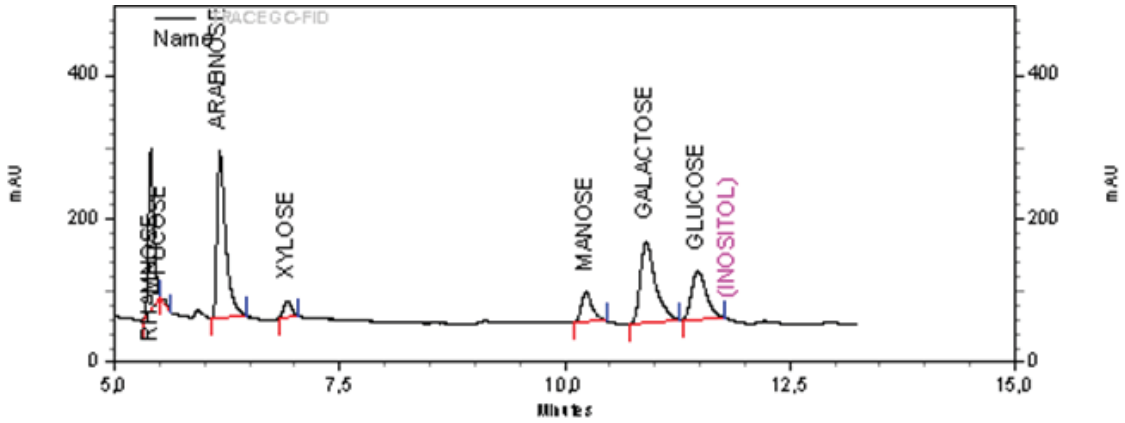
## 7 Supplementary data




| Pk# | Nome | Tempo ret. | Área | Area Percent |
|---|---|---|---|---|
| 1 | RHAMNOSE | 5,400 | 9061242 | **17,971** |
| 2 | FUCOSE | 5,498 | 157361 | **0,312** |
| 3 | ARABNOSE | 6,170 | 15354941 | **30,453** |
| 4 | XYLOSE | 6,917 | 1404184 | **2,785** |
| 5 | MANOSE | 10,238 | 3493308 | **6,928** |
| 6 | GALACTOSE | 10,900 | 13237313 | **26,253** |
| 7 | GLUCOSE | 11,480 | 7713942 | **15,299** |
| | INOSITOL | | | |

**Figure S1: Monosaccharide composition chromatogram through GC analysis of the alditol acetates obtained from the BFSF fraction.**

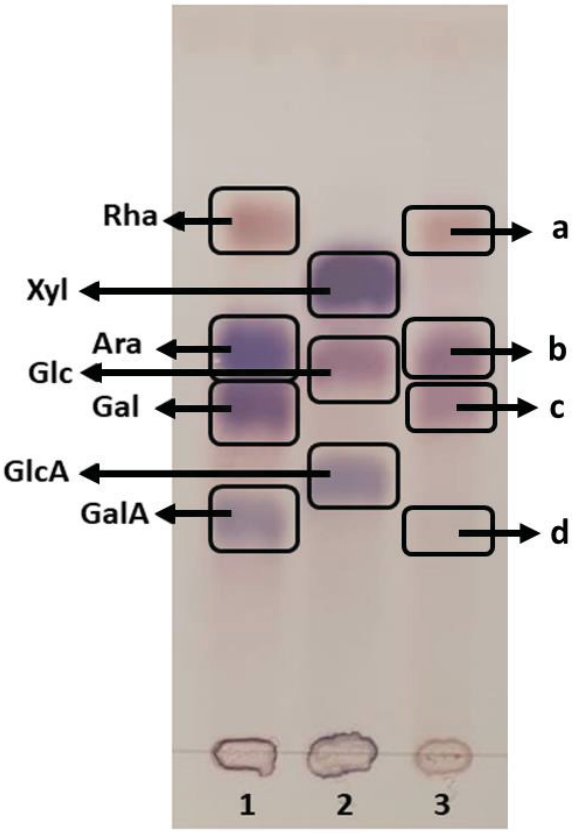
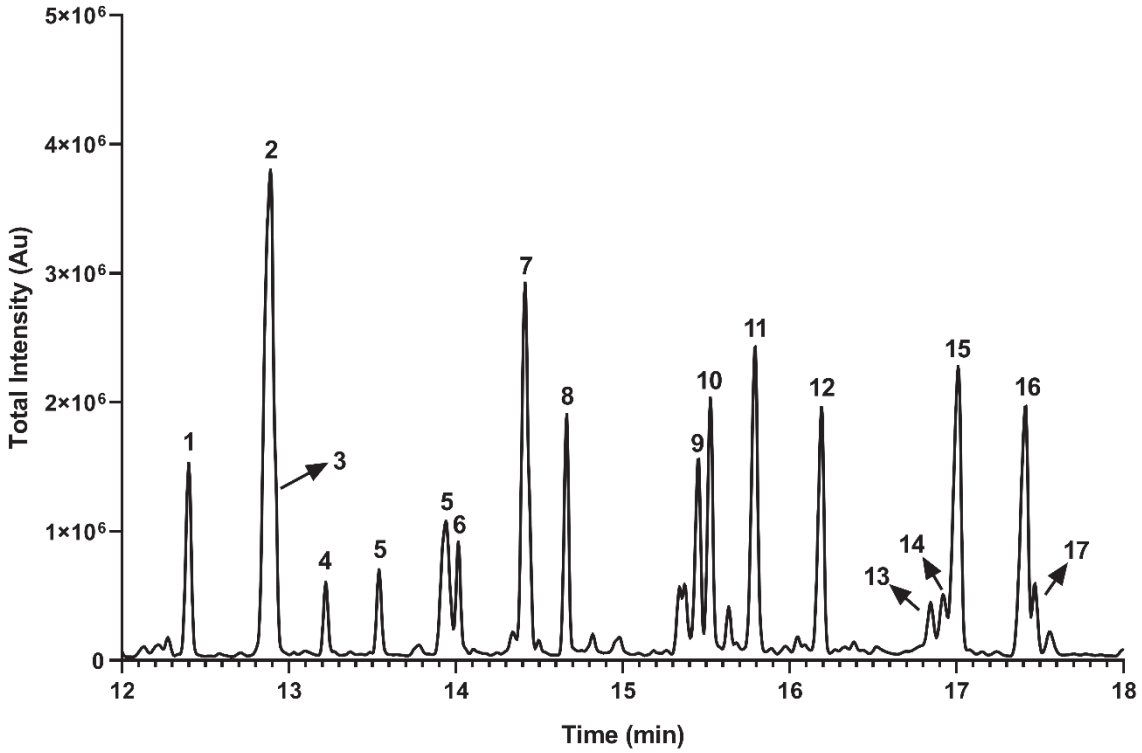
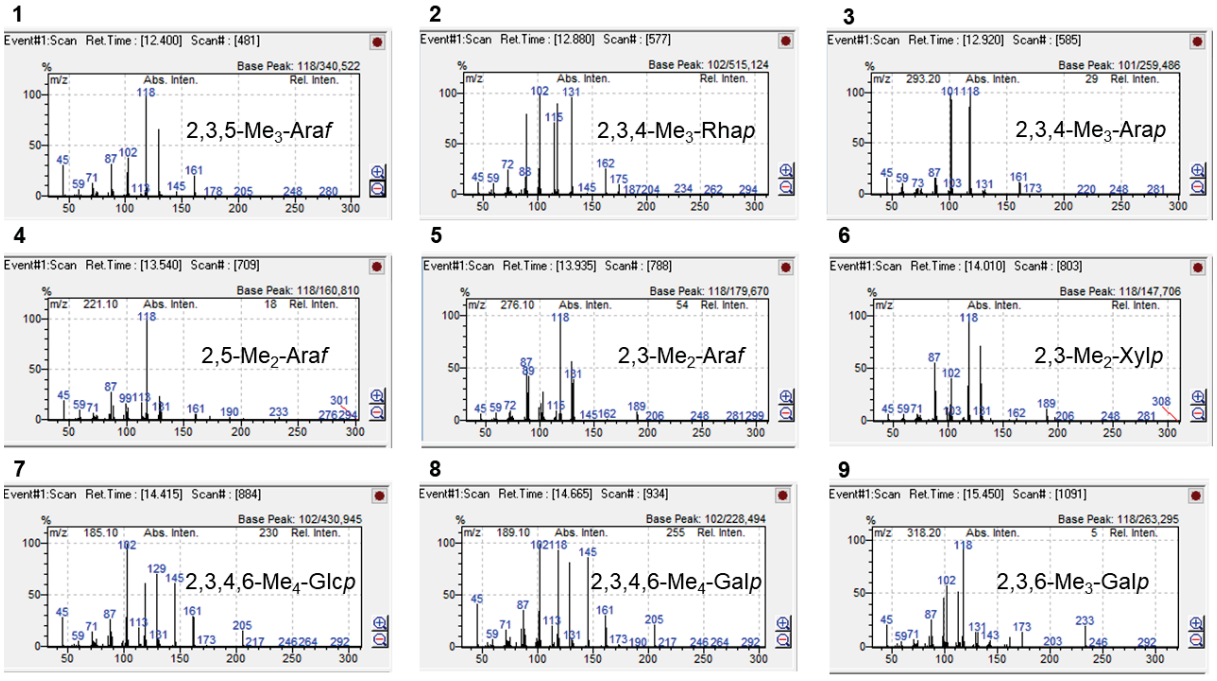



**Figure S2: Thin-layer chromatography of hydrolyzed (TFA 2 M, 100 °C for 8 h) BFSF fraction**. The Retention factor (Rf) for each monosaccharide standard (1 and 2) and each band from BFSF (3) were calculated measuring the distance traveled by monosaccharides divided by the distance traveled by the solvent (both measured from the origin). Rf values: Galacturonic acid: 0.34; Galactose: 0.52; Arabinose: 0.61; Rhamnose: 0.80; Xylose: 0.72; Glucose: 0.57; Glucuronic Acid: 0.41; BFSF: (a) 0.78, (b) 0.61, (c) 0.52 and (d) 0.33. Comparing Rf values and band patterns with those of monosaccharide standards, BFSF is mainly constituted of rhamnose, arabinose, galactose and galacturonic acid.

**A**


Total Intensity (Au)
5×10⁶
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
12
13
14
15
16
17
18
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

**B**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6
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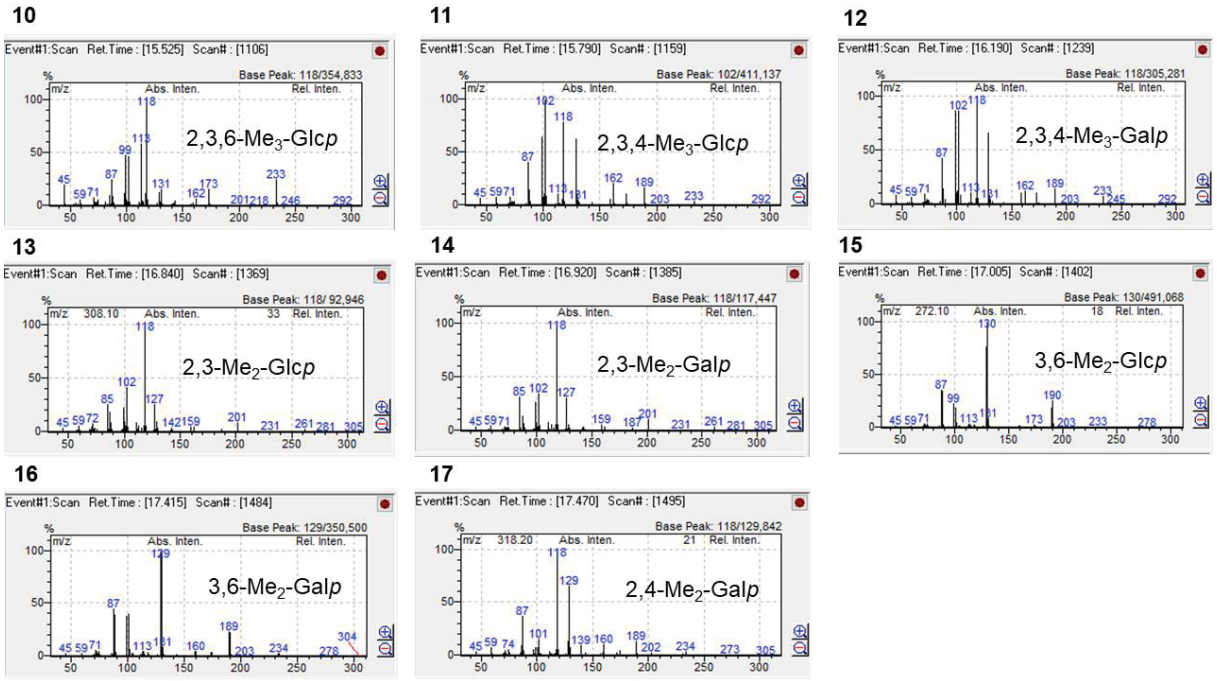


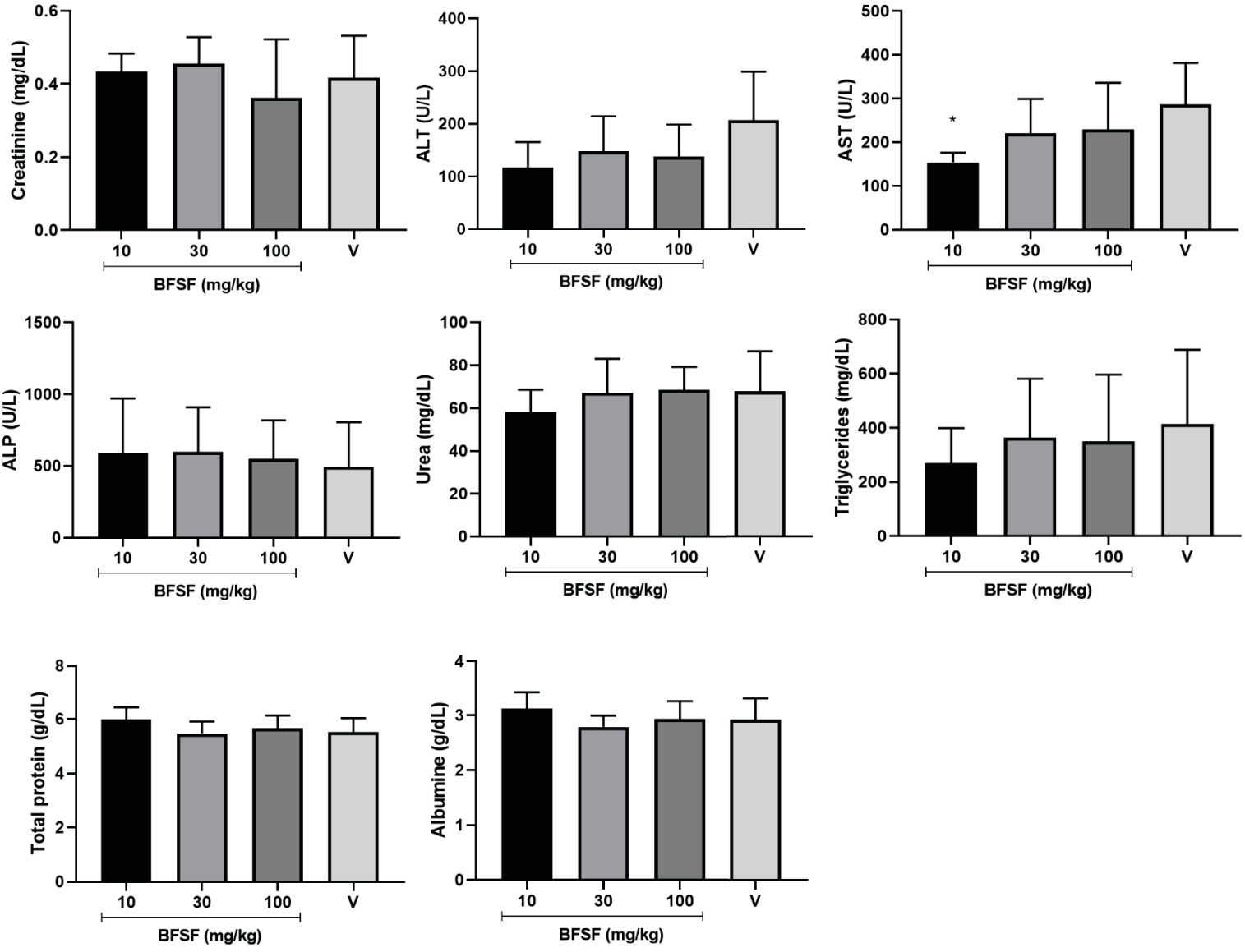

**Figure S3: Chromatogram (A) and mass spectra (B) of the GC-MS analysis of the partially O-methylated alditol acetates of BFSF fraction.**

Table S1: Biochemical and hematological parameters analyzed in plasma or blood of rats treated with BFSF for 21 days.

| | BFSF | | | |
|---|---|---|---|---|
| | **10 mg/kg** | **30 mg/kg** | **100 mg/kg** | **Saline** |
| **ALT (U/L)** | 117.3 ± 16.94 | 148.1 ± 23.30 | 138.2 ± 21.36 | 206.7 ± 32.60 |
| **AST (U/L)** | 154.3 ± 7.81* | 220.6 ± 26.07 | 229.9 ± 40.09 | 274.0 ± 37.92 |
| **ALP (U/L)** | 592.3 ± 120.1 | 599.3 ± 103.6 | 549.2 ± 95.4 | 495.8 ± 72.9 |
| **Creatinine (mg/dL)** | 0.43 ± 0.01 | 0.45 ± 0.02 | 0.36 ± 0.05 | 0.41 ± 0.02 |
| **Urea (mg/dL)** | 58.3 ± 3.4 | 67.2 ± 5.3 | 68.6 ± 3.8 | 67.8 ± 4.7 |
| **Triglycerides (mg/dL)** | 271.3 ± 42.6 | 364.1 ± 76.7 | 349.4 ± 93.3 | 414.8 ± 64.4 |
| **Total cholesterol (mg/dL)** | 96.2 ± 5.4 | 94.4 ± 5.3 | 151.7 ± 36.0 | 116.1 ± 11.4 |
| **Total protein (g/dL)** | 5.99 ± 0.15 | 5.47 ± 0.15 | 5.66 ± 0.16 | 5.52 ± 0.12 |
| **Albumine (g/dL)** | 3.12 ± 0.10 | 2.79 ± 0.07 | 2.94 ± 0.11 | 2.93 ± 0.09 |
| **Globuline (g/dL)** | 2.87 ± 0.09 | 2.94 ± 0.17 | 2.72 ± 0.10 | 2.70 ± 0.08 |
| **Direct bilirubin (mg/dL)** | 0.05 ± 0.01** | 0.06 ± 0.01* | 0.11 ± 0.02 | 0.14 ± 0.02 |
| **Indirect bilirubin (mg/dL)** | 0.11 ± 0.01 | 0.08 ± 0.01 | 0.08 ± 0.01 | 0.08 ± 0.01 |
| **Total bilirubin (mg/dL)** | 0.17 ± 0.01 | 0.16 ± 0.02 | 0.19 ± 0.01 | 0.11 ± 0.01 |
| **HDL-C (mg/dL)** | 46.26 ± 2.55 | 44.77 ± 2.08 | 44.90 ± 1.68 | 46.83 ± 2.25 |
| **VLDL-C (mg/dL)** | 47.24 ± 5.45 | 60.73 ± 10.93 | 52.12 ± 6.73 | 87.98 ± 12.45 |
| **LDL-C (mg/dL)** | 88.13 ± 6.50 | 64.93 ± 9.77 | 78.35 ± 4.23 | 65.07 ± 6.06 |
| **WBC** | 3000 ± 170.1 | 3533 ± 405.5 | 4433 ± 578.3 | 3600 ± 845.8 |
| **Lym** | 2113 ± 132.9 | 2500 ± 321.5 | 3000 ± 416.3 | 2438 ± 561.2 |
| **Mon** | 62.50 ± 18.30 | 66.67 ± 33.33 | 100.00 ± 0.00 | 87.50 ± 29.50 |
| **Gran** | 825.0 ± 70.1 | 966.7 ± 88.2 | 1333.0 ± 318.0 | 1075.0 ± 278.9 |
| **RBC (/dL)** | $8.3x10^6 \pm 1.1x10^5$ | $9.1x10^6 \pm 1.9x10^5$ | $5.8x10^6 \pm 2.5x10^6$ | $7.6x10^6 \pm 3.7x10^5$ |
| **Hemoglobine** | 14.0 ± 0.12 | 14.7 ± 0.11 | 13.7 ± 0.37 | 12.9 ± 0.5 |
| **HCT (%)** | 41.3 ± 0.6 | 44.0 ± 0.6 | 40.7 ± 1.0 | 36.9 ± 1.9 |
| **MCV (fL)** | 49.7 ± 0.4 | 48.6 ± 1.5 | 49.5 ± 0.6 | 48.3 ± 0.3 |
| **MCH (pg)** | 16.8 ± 0.1 | 16.1 ± 0.2 | 16.6 ± 0.2 | 16.7 ± 0.2 |
| **MCHC (g/dL)** | 33.8 ± 2.9 | 33.4 ± 0.5 | 33.5 ± 0.1 | 35.3 ± 0.7 |
| **RDW (%)** | 11.2 ± 0.2 | 11.3 ± 0.5 | 10.7 ± 0.2 | 10.7 ± 0.1 |
| **Platelets ($x10^3$/µL)** | 342.2 ± 36.1 | 325.3 ± 22.5 | 335.0 ± 34.3 | 261.0 ± 60.0 |
| **MPV (fL)** | 6.8 ± 0.2 | 6.7 ± 0.3 | 6.7 ± 0.1 | 7.1 ± 0.1 |
| **PDW (%)** | 15.7 ± 0.2 | 15.4 ± 0.3 | 15.5 ± 0.1 | 15.6 ± 0.1 |
| **PCT (%)** | 0.23 ± 0.02 | 0.22 ± 0.02 | 0.22 ± 0.02 | 0.18 ± 0.04 |

Subtitle: ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), HDL-C (high-density lipoprotein cholesterol), LDL-C (low-density lipoprotein cholesterol), VLDL-C (very low-density lipoprotein cholesterol), WBC (white blood cells), Lym

(lymphocytes), Mon (monocytes), Gran (granulocytes), RBC (red blood cells), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW (red cell distribution width), MPV (mean platelet volume), PDW (platelet distribution width), PCT (plateletcrit). *p < 0.05 and **p < 0.01 when compared to the vehicle group.



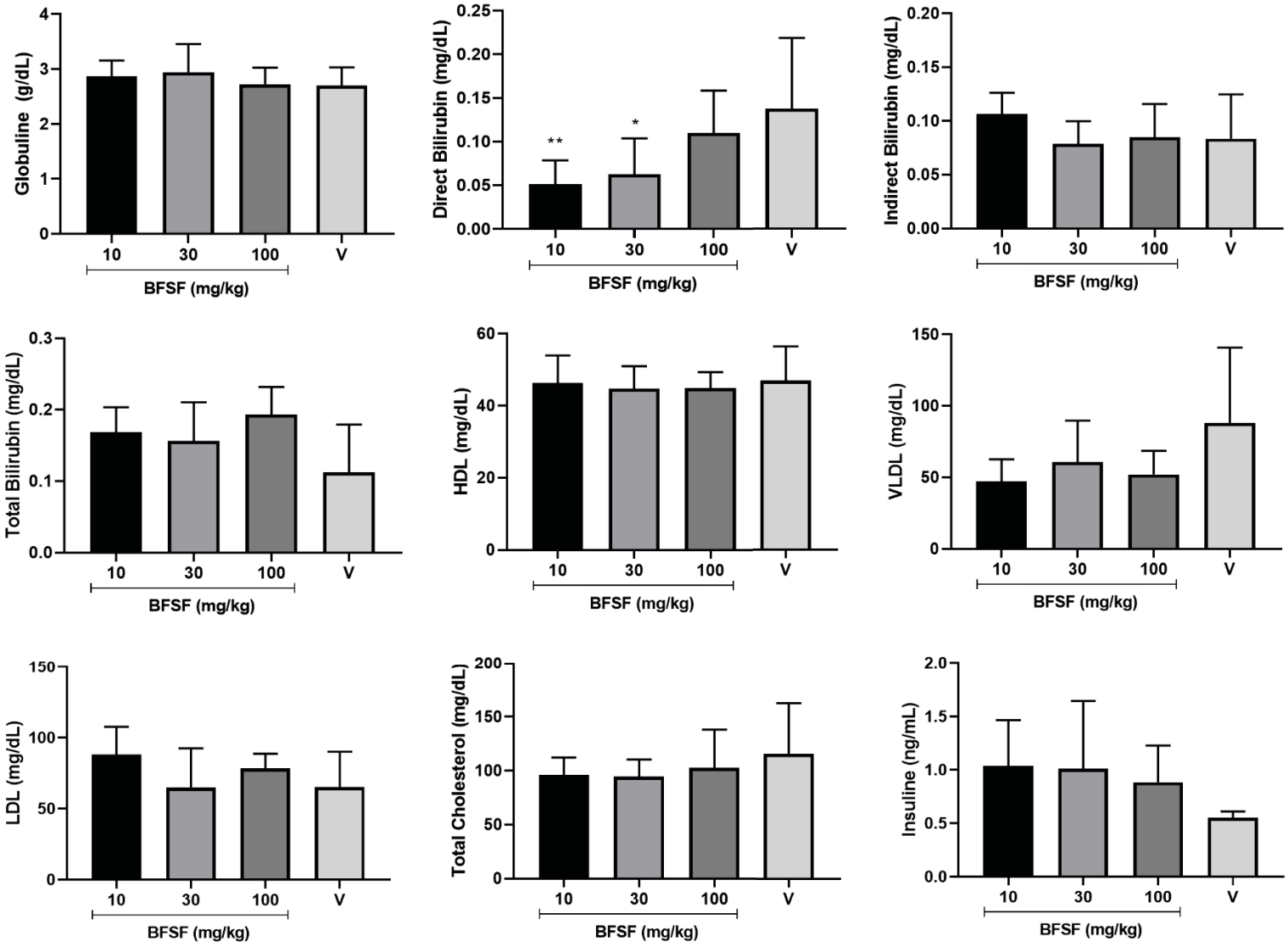



**Figure S4: Effect of the chronic BFSF treatment at 10, 30 and 100 mg/kg on biochemical parameters.** *p < 0.05 and **p < 0.01 when compared to the vehicle group (saline). Results are expressed as mean ± SEM (n > 8). Comparison between groups were performed by one-way ANOVA followed by Tukey's test of multiple comparison.

Table S2: Histological score of liver damage after chronic treatment with BFSF in diabetic rats.

| Group | Animal | TD (x1) | II (x1) | CV (x1) | NV (x1) | M (x2) | A (x2) | N (x3) | Total | GS | Mean GS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 1 | 0 | 1 | 1 | 0 | 2 | 0 | 0 | 6 | 51 | 8.5 |
| | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 7 | | |
| | 3 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 8 | | |
| | 4 | 3 | 0 | 3 | 0 | 1 | 0 | 2 | 14 | | |
| | 5 | 2 | 1 | 2 | 0 | 2 | 0 | 1 | 12 | | |
| | 6 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 4 | | |
| **2** | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 6 | 49 | 8.1 |
| | 2 | 0 | 2 | 2 | 0 | 1 | 0 | 1 | 9 | | |
| | 3 | 3 | 1 | 3 | 0 | 2 | 0 | 2 | 16 | | |
| | 4 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 6 | | |
| | 5 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | | |
| | 6 | 2 | 1 | 3 | 0 | 0 | 0 | 1 | 9 | | |
| **3** | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 17 | 2.8 |
| | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | | |
| | 3 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | | |
| | 4 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | | |
| | 5 | 2 | 1 | 2 | 0 | 0 | 0 | 0 | 5 | | |
| | 6 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 6 | | |
| **4** | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 16 | 2.6 |
| | 2 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | | |
| | 3 | 1 | 0 | 2 | 0 | 1 | 0 | 0 | 5 | | |
| | 4 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | | |
| | 5 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 3 | | |
| | 6 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | | |

Subtitle: Group 1: control group; Groups 2, 3 and 4: treated group with BFSF at 10, 30 and 100 mg/kg, respectively. NOTE: The following criteria were analyzed in liver samples: trabecular disorganization (TD); inflammatory infiltrate (II); cytoplasmic vacuolation (VC); nuclear vacuolation (NV); megalocytosis (M); apoptosis (A); and necrosis (N). The score for each criterion is given according to the extent or frequency of the observed lesion, scored as 0 (absence), 1 (mild), 2 (moderate) and 3 (severe). Each criterion has a severity factor that will multiply the score according to the importance of the lesion, being factor 1 for trabecular disorganization, inflammatory infiltrate, cytoplasmic vacuolation and nuclear vacuolation; factor 2 for megalocytosis and apoptosis; and factor 3 for necrosis, represented in parentheses in the following table; GS: group score.

## CONSIDERAÇÃO ENTRE ARTIGOS I e II

Uma vez que não foi observado efeito hipoglicemiante com a fração BFSGD (BFSF) no modelo de diabetes avaliado e considerando a relação entre o processo fisiopatológico do diabetes com a inflamação dos tecidos alvo de insulina, a propriedade imunomodulatória da fração TCA-S (fração obtida da desproteinização de BFSGD) foi explorada em células THP-1.

**ARTIGO II**

# Structural characterization of a polysaccharide rich fraction obtained from *Bauhinia forficata* Link (cow's paw) leaves and evaluation of its effect on THP-1 macrophages

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Keywords: *Bauhinia forficata* Link; cow's paw; immunomodulatory; phytotherapy.

Highlights

- A polysaccharide rich fraction was obtained from *Bauhinia forficata* Link leaves through hot aqueous extraction and purification processes;
- This fraction, named TCA-S was structurally characterized and showed to be constituted mainly of type I and II arabinogalactans, arabinan and starch;
- When evaluated against its immunomodulatory potential, TCA-Sα (fraction TCA-S after treatment with α-amylase for starch removal) demonstrated an immunostimulatory effect at all concentrations tested by increasing cytokine release by THP-1 macrophages and an immunomodulatory effect when cells were simultaneously incubated with proinflammatory agent LPS.

## ABSTRACT

*Bauhinia forficata* Link is a medicinal plant popularly known as cow's paw and it is used for many medicinal purposes, including to treat inflammation. Although there are many published studies that aimed to elucidate compounds from the plant leaves, especially polyphenols and secondary metabolites, there is no information about its polysaccharides. So, this study intended to extract, purify and structurally characterize the water-soluble polysaccharides from *B. forficata* leaves, as well as evaluate their effect on THP-1 cells. From the aqueous extract, followed by purification processes, a polysaccharide fraction named TCA-S was obtained, constituted mainly of arabinose and galactose. By NMR and methylation analyses, the polysaccharide fraction showed type II and type I arabinogalactans, arabinan and starch. TCA-S was then submitted to starch removal process and renamed as TCA-Sα. TCA-Sα showed absence of cytotoxicity by neutral red and crystal violet assays at all concentrations tested on THP-1 macrophages. The evaluation of the immunomodulatory potential of TCA-Sα on THP-1 cells showed that the fraction has immunostimulatory properties at concentrations 50, 100 and 500 µg/mL in relation to all cytokines quantified in the cell culture supernatant (TNF- α, IL-1β and IL-10), and immunomodulatory effect in the presence of LPS, at a concentration of 50 µg/mL. In addition, the ratio between the concentrations of pro and anti-inflammatory cytokines stimulated by the positive control (LPS) and by TCA-Sα together with LPS was calculated for each concentration. The decrease in ratio values at all concentrations of the TCA-Sα + LPS group compared to LPS alone may suggest that the polysaccharide fraction has an overall anti-inflammatory effect when incubated simultaneously with LPS.

## 1 Introduction

Brazil has the greatest biodiversity in the world and its population often resorts to the use of medicinal plants for the treatment of clinical conditions (DUTRA *et al.,* 2016). Consequently, the country represents a great source of natural and biologically active compounds, arousing great interest in the search for new bioactive molecules and their biomedical applications. In this context, the Fabaceae family plays an important role since many of its species show relevant pharmacological effects (AHMAD; ANWAR; HIRA, 2016).

*Bauhinia forficata* Link is a medicinal plant that belongs to Fabaceae family and it is popularly known as cow's paw. Native to Southeast Brazil, it is a semideciduous tree, 5 to 9 meters tall, with leathery leaves divided up to the middle, with a format that refers to the appearance of a cow's paw, which characterizes its popular synonymy (LORENZI; MATOS, 2002; FORTUNATO, 1986). In folk medicine, the plant leaves are best known as coadjutant in treatment of diabetes but is also used for other therapeutical purposes, such as purgative, diuretic, depurative, hypertension, treatment of inflammation, among others (LÓPEZ; SANTOS, 2015).

Previous studies have demonstrated that *B. forficata* biological activities are mainly attributed to molecules derived from the plant secondary metabolism (SOUSA *et al*., 2004). Although secondary metabolites are extracted in tea preparation, primary metabolites, such as water-soluble polysaccharides, are also obtained in such extractions and can have a lot of beneficial biological effects. Polysaccharides are a relevant class of biomolecules since they have a variety of biological activities (SIMÕES, 2003) that are intrinsic to their structural parameters, such as monosaccharide composition, linkage type and chain branching (SRIVASTAVA; KULSHRESHTHA, 1989). In scientific literature, biological properties of polysaccharides isolated from plants have been investigated and confirmed, such as hepatoprotective (CHAVES *et al*., 2020a), treatment of gut disorders (OLIVEIRA *et al*., 2022) antioxidant (SCHNEIDER *et al*., 2020), gastroprotective (CARLOTTO *et al*., 2019), anxiolytic (CHAVES *et al*., 2020b), antidiabetic (MA; YUAN; ZHUANG, 2018), including others. In addition, many polysaccharides isolated from plants have been reported to contain anti-inflammatory activity, as the ones isolated from *jambo* fruit (*Syzygium jambos* (L.) Alston) (TAMIELLO *et al*., 2017), *tamarillo* (*Solanum betaceum*)

(NASCIMENTO *et al*., 2015), tree stonecrop (*Sedum dendroideum*) (OLIVEIRA *et al*., 2017) and *jambu* (*Acmella oleracea*) (MARIA-FERREIRA *et al*., 2021).

Considering that there is no data in scientific literature published to this date about the structure of water-soluble polysaccharides isolated from *B. forficata* leaves and that the plant tea is commonly used for treating numerous illnesses, including inflammation, this study main objectives were to extract (under conditions that simulate the tea preparation as in folk medicine), isolate and structurally characterize the polysaccharides that build up the cell wall of the plant's leaves, and evaluate their potential to the anti-inflammatory activity of the plant, by *in vitro* model using THP-1 human macrophages.

## 2 Materials and Methods

### 2.1 Material of study

1 kg of *B. forficata* leaves were acquired in local warehouse in Curitiba, State of Paraná, Brazil, in April 2019, produced by Chamel *Produtos Naturais*, under lot number 12675.

### 2.2 Extraction and purification of a polysaccharide fraction

Dried leaves of *B. forficata* (1 kg) were submitted to hot water extraction (100 °C) through the process of decoction, in 1:10 (w/v) proportion of leaves:distilled water. The leaves were maintained under boiling for three minutes (LORENZI; MATOS, 2002), cooled to room temperature and filtered by cotton. The extraction was repeated three times with the same leaves to improve its yield. All aqueous extracts were combined and reduced to small volume (~300 mL) by evaporation under reduced pressure. The resultant concentrated extract was then treated with cold ethanol 99% (v/v) in a 3:1 proportion of ethanol:extract, aiming precipitation of high molecular weight molecules. The precipitated fraction was separated from the supernatant by centrifugation at 8000 rpm for 15 minutes at 4 °C and dialyzed against tap water, for 2 days, using 6-8 kDa cut-off membranes to remove small molecules and excess ethanol. The precipitated fraction was resuspended in distilled water 10% (w/v) and submitted to freeze-thawing (GORIN; IACOMINI, 1984), originating a supernatant

fraction, intitled BFSF (BF stands for *Bauhinia forficata* and SF for Soluble Fraction in cold water) and a precipitated one, named BFIF (IF after Insoluble Fraction in cold water). This procedure was repeated until no more precipitate formation was observed. Finally, due to the high protein content (15.1%), observed by Coomassie blue dye method of protein dosage (BRADFORD, 1976), the BFSF fraction was submitted to treatment with trichloroacetic acid (TCA) for protein precipitation (OLIVEIRA; MARQUES; AZEREDO, 1999), giving rise to TCA-P (precipitated; 50.6%) and TCA-S (supernatant; 42.0%) fractions. The flowchart of extraction and purification of the polysaccharide fraction TCA-S is shown in Figure 1.

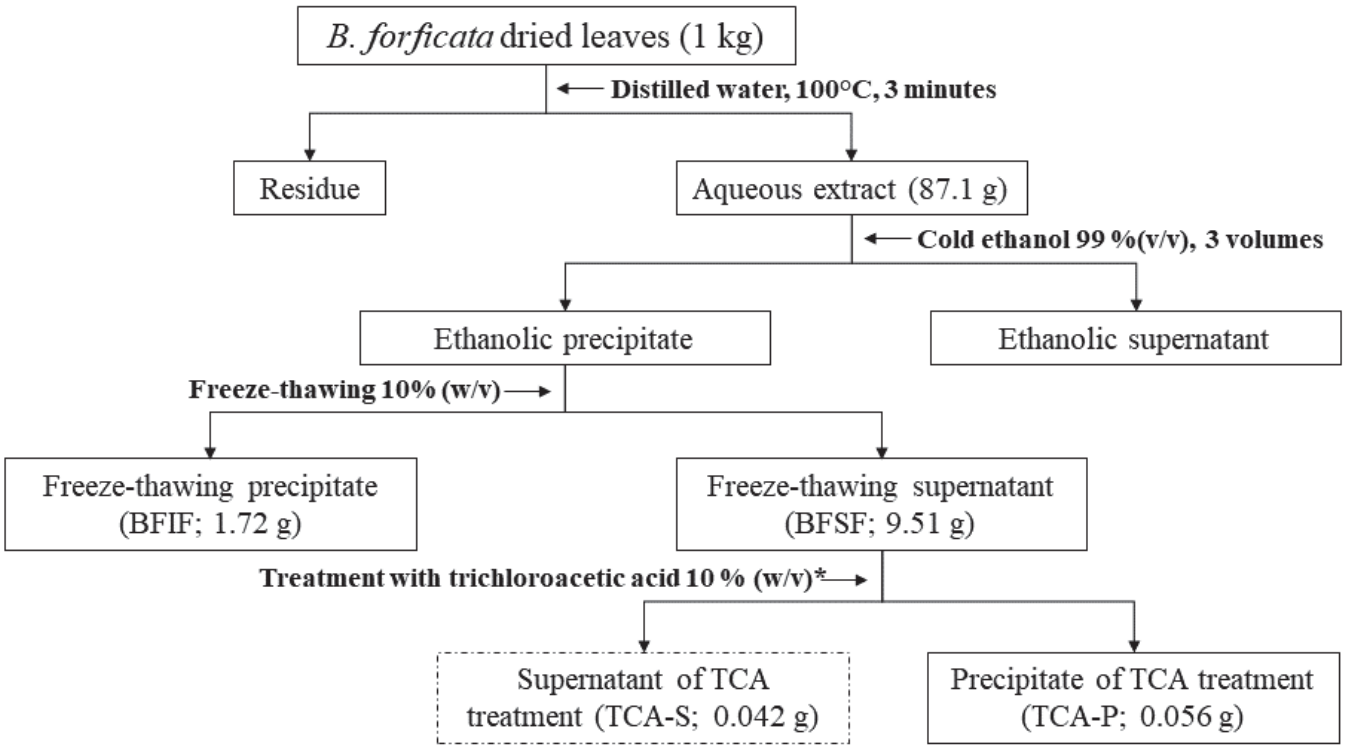


**Figure 1: Extraction and purification scheme to obtain TCA-S fraction from *B. forficata* leaves.** *Treatment with trichloroacetic acid was made using only 100 mg of BFSF.

### 2.2.1 Homogeneity analysis and *Mw* determination

Determination of the elution profile of TCA-S was performed by high performance size exclusion chromatography (HPSEC). TCA-S was solubilized in 0.1 M sodium nitrite and 0.2 g/L azide solution to a final concentration of 1 mg/mL and then filtered through a cellulose acetate membrane with 0.22 µm porosity. The volume of

sample injection into the equipment was 100 µL. Eluent was the same solution used as solvent, with a controlled flow rate of 0.6 mL/min. Homogeneity analysis was performed on a Waters chromatograph connected with four columns in series packed with Ultrahydrogel® (2000, 500, 250, 120; with exclusion sizes of $7x10^6$, $4x10^5$, $8x10^4$, $5x10^3$ g/mol, respectively, Milford, MA, USA), attached to a Waters 2410 differential refractometer (RI) and to a multiangle laser light scattering (MALLS; Wyatt Technology) detectors. The weight average molar mass (Mw) of TCA-S fraction was determined using its specific refractive index increment (dn/dc), which was obtained using the same equipment with the columns uncoupled. TCA-S fraction was prepared at concentrations 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL, filtered through a 0.22 µm membrane, injected (500 µL loop) and analyzed at 25 °C using the RI detector only. The chromatograms were analyzed using the Wyatt Technology software ASTRA version 4.70.07.

## 2.3 Structural characterization of polysaccharides from TCA-S fraction

### 2.3.1 Monosaccharide Composition

TCA-S (2 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 8 h. After complete evaporation of TFA, the residue was solubilized in distilled water and reduced with $NaBH_4$ for 18 h. The reaction was interrupted by neutralization with glacial acetic acid and the resultant boric acid formed was removed as trimethyl borate by washing repeatedly with methanol (WOLFROM; THOMPSON, 1963). The alditols generated by reduction were then acetylated using a pyridine:acetic anhydride mixture (1:1 v/v; 1 mL) at room temperature, for 16 h, and the reaction was stopped by addition of 1 mL of distilled water. The solution was partitioned between chloroform, and the organic phase was collected and washed with 5% (w/v) aqueous $CuSO_4$ solution to remove residual pyridine. The excess of $CuSO_4$ solution and water was removed by addition of anhydrous sodium sulfate. Lastly, the material was filtered through cotton and the alditol acetates were analyzed by GLC (Thermo Scientific Trace GC 3), using a DB-225 column (30 m x 0.25 mm), programmed from 50 up to 210 °C at a 40 °C/min rate, with helium as carrier gas. The alditol acetates were identified by comparison of their retention times with those of alditol acetates prepared from monosaccharide standards (Sigma-Aldrich).

To verify the presence and identity of the uronic acid, the hydrolyzed sample, before being treated with $NaBH_4$, was examined by silica-gel 60 thin layer chromatography (TLC; Merck). The plate was developed with ethyl acetate:n-propanol:acetic acid:$H_2O$ (4:2:2:1, v/v) and stained with orcinol–$H_2SO_4$ (SASSAKI *et al.*, 2008). Uronic acids content was measured using colorimetric *m*-hydroxybiphenyl method according to Filisetti-Cozzi and Carpita (1991). A standard curve of galacturonic acid was built, with the absorbance recorded at 525 nm. Results were expressed as µg of galacturonic acid per 100 µg of TCA-S fraction.

2.3.2 Spectrophotometric determinations of phenolic compounds, protein content and total sugar contents

Phenolic compounds content of TCA-S was determined by Singleton, Orthofer and Lamuela-Raventos method (1999), using Folin-Ciocalteu 2 N solution. A calibration curve of gallic acid was built as a standard, the experiment was performed in a microplate and the absorbance was measured by spectrophotometry (Epoch, BioTek, USA) at 720 nm. All measurements were made in triplicate and result is expressed as µg of gallic acid equivalent per 100 µg of sample.

Proteins were quantified according to Bradford (BRADFORD, 1976). A standard curve was built using bovine serum albumine (BSA) and protein content was determined by absorbance measurement at 595 nm. The result is expressed as µg protein per 100 µg of TCA-S sample.

Total sugar content was determined as described by Dubois *et al*. (1956). A calibration curve was built with a mixture of arabinose, galactose and galacturonic acid, in equal proportion of each monosaccharide standard (1:1:1; 1 mg/mL) and absorbance was recorded at 490 nm. Measurements were performed in triplicate and the result is expressed as µg of sugar per 100 µg of TCA-S sample.

2.3.3 Methylation analysis

TCA-S fraction was methylated using DMSO-MeI and powdered NaOH, according to Ciucanu and Kerek (1984). The step of methylation was repeated twice to ensure complete methylation of the sample. The methylated material was hydrolyzed with formic acid 45%(v/v) at 100 °C for 6 h and after acid evaporation, the

sample was again hydrolyzed with TFA 2 M for 16 h, following evaporation to dryness. $NaBD_4$ was used as reduction agent and then acetylation step was completed exactly as described in monosaccharide composition methodology, giving partially O-methylated alditol acetates, that were analyzed by GC-MS (Shimadzu, model QP2020NX) using a quadrupole detector, in a VF-5MS column (30 m x 0.25 mm) at 100 °C. The experiment temperature was programmed at a rate of 10 °C/min until 220 °C, 250 °C and 280 °C (held for 3 min at each temperature, with a total runtime of 30 minutes), using helium as carrier gas (2 mL/min). The partially O-methylated alditol acetates were identified by their characteristic retention times and electron ionization mass spectra, compared to those of partially O-methylated alditol acetates prepared from standard monosaccharides (Sigma-Aldrich), according to Sassaki *et al*. (2005).

2.3.4 NMR spectroscopy

TCA-S was solubilized in $D_2O$ and left overnight under stirring at 50 °C, then sonicated for 15 min and centrifuged at 8000 rpm for 5 min to remove insoluble particles. The spectrum was recorded on a 400 MHz Bruker model Avance III spectrometer (Bruker, Germany) with a 5 mm inverse probe, at 50 °C. The $^{13}C/^{1}H$ correlations observed in HSQC spectrum were expressed as chemical shifts (δ) in ppm relative to the resonance of the anomeric $^{13}C/^{1}H$ correlation of arabinose at δ109.5/5.25 (CARLOTTO *et al.,* 2019). The correlation map was analyzed in Topspin software version 4.1.3 and signals were attributed according to literature data available.

2.4 Effect of TCA-Sα fraction on THP-1 macrophages

2.4.1 Cells

THP-1 is a monocytic cell line from a leukemic patient, and it was chosen once it is a widely used *in vitro* model for evaluation of immune system modulation, due to their acquirement of morphology and differentiation hallmarks that resemble those of primary human monocyte-derived macrophages (CHANPUT; MES; WICHERS, 2014). Cells were obtained from BCRJ (Cell Bank of Rio de Janeiro).

2.4.2 Starch removal from TCA-S

TCA-S was treated with α-amylase (cat. number: A3403; Sigma-Aldrich) aiming its withdrawal. The sample was solubilized in distilled water (5 mL) and pH was neutralized with diluted NaOH (10%; w/v). Then, the solution was warmed to 60°C and α-amylase was added in a 1:100 proportion (100 U/mL) under stirring for 2 h. Confirmation of starch removal was made with lugol's solution and nmr analysis (Figure S3). Then, the solution was submitted to ethanolic precipitation to recover high mass molecules, centrifuged at 8000 rpm for 15 min to separate supernatant from precipitate, and dialyzed against tap water using 6-8 kDa cut-off membrane. After this process, the sample was renamed TCA-Sα. Finally, TCA-Sα was freeze-dried for further testing.

2.4.3 Detection of LPS contamination

The possible presence of lipopolysaccharides (LPS) in the sample was evaluated according to the method described by Santana-Filho *et al*. (2012). This assay is based on detection of 3-hydroxyl fatty acids methyl esters, chemical markers of LPS. A calibration curve was built using LPS from *Escherichia coli* O111:B4 (Sigma) at different concentrations (20, 25, 50, 100, 200 and 500 ng/μL; Linear equation: y=1717.8 + 10929; y axis as peak area and x axis as LPS concentrations; $R^2$= 0.9961). TCA-Sα (300 ng) was solubilized in 400 μL of methanol and then submitted to methanolysis with 0.6 M MeOH-HCl (100 μL), at 80 °C for 20 h. To the methanolized material, 0.5 mL of distilled water was added, and the solution was partitioned with 1 mL of hexane. The organic phase was collected and dried under gentle stream of $N_2$. The residue was acetylated with 1:1 pyridine:acetic anhydride (100 μL), at 100 ºC for 1 h and then the solvent was evaporated. The dried residue was solubilized in acetone (10 μL) and submitted to analysis by GC-MS. Analysis of chromatograms and mass spectra was made with GC-MS Solution (Shimadzu Corporation) software, 4.20 version.

2.4.4 Cell culture and macrophage differentiation

THP-1 cells were cultured in RPMI-1640 culture medium (GIBCO Laboratories), supplemented with 10% fetal bovine serum (FBS), 1 U/mL penicillin, 1 μg/mL streptomycin and 25.5 μg/mL amphotericin, in a humidified incubator at 37 °C and 5% $CO_2$. Cells were split twice a week. The cells grown to a density of 8 x $10^5$ cells/mL were used in experiments of viability and cytokine measurements.

The mature macrophage state (phenotype M1) was achieved through cell treatment with 10 ng/mL (~16 nM) of phorbol 12-myristate 13-acetate (PMA) for 48 h, inducing THP-1 monocytes to differentiate. After differentiation, fresh medium, free of PMA, was replaced. Subsequently, the differentiated THP-1 macrophages were incubated for 24 h at 5% $CO_2$, at 37 °C, for resting time.

2.4.5 Cytotoxicity of TCA-Sα on THP-1 cells

To measure cytotoxic activity of TCA-Sα on differentiated THP-1 cells, crystal violet and neutral red methods were used. THP-1 cells were seeded in 96 well plates, differentiated to M1 phenotype and then exposed to TCA-Sα at 2, 10, 50, 100 or 500 µg/mL for 24 h. After this period, medium was refreshed and cells were incubated with neutral red dye, for assessment of cellular viability, whereas absorbance was recorded at 550 nm (BORENFREUND; PUERNER,1985). Sequentially, dye was eluted, and cells were stained with crystal violet, for cellular proliferation evaluation. Absorbance was then recorded at 570 nm (GILLIES; DIDIER; DENTON, 1986). Saponin 0.1 % (cat. number: 84510; Sigma-Aldrich) was used as positive control. Cytotoxicity of TCA-Sα was expressed in %, and values were normalized with negative control group (cells with culture medium without FBS, only).

2.4.6 Cytokine measurements

Differentiated THP-1 macrophages were treated with fresh medium containing TCA-Sα at 50, 100 or 500 µg/mL, medium as negative control, LPS as positive control (1 µg/mL) or TCA-Sα plus LPS. After this, cells were incubated at 37 °C in a 5% $CO_2$ atmosphere for 18 h, optimal exposure time for maximal cytokine secretion by THP-1 cells stimulated with LPS (CHANPUT *et al.*, 2010). By the end of this period, supernatants were collected and stored at -80 °C for cytokine secretion measurements. The concentrations of TNF-α, IL-1β and IL-10 cytokines were quantified by InvitroGen ELISA kits specific for human cytokines, according to the manufacturer's instructions.

2.4.7 Statistical analysis

Data is expressed as mean ± standard error of mean (SEM) and analyzed by one-way analysis of variance (ANOVA). Dunnett's multiple comparison test was

chosen as post hoc test. Treatment with TCA-Sα was considered significantly different from control group when $p < 0.05$. All analysis were made and all graphs were constructed using Graphpad Prism version 8.0.1 for Windows (GraphPad Software, San Diego, CA, USA).

## 3 Results and Discussion

### 3.1 Extraction, purification and structural characterization of TCA-S fraction

From hot aqueous extraction of dried leaves of *B. forficata*, followed by purification processes, such as treatment with cold excess ethanol, freeze-thawing and precipitation of proteins with TCA, a polysaccharide rich fraction, entitled TCA-S (supernatant of treatment with trichloroacetic acid), was obtained. Considering its greater water solubility and higher total sugar content amongst all fractions obtained from purification processes, only TCA-S was structurally characterized and evaluated towards its biological activity.

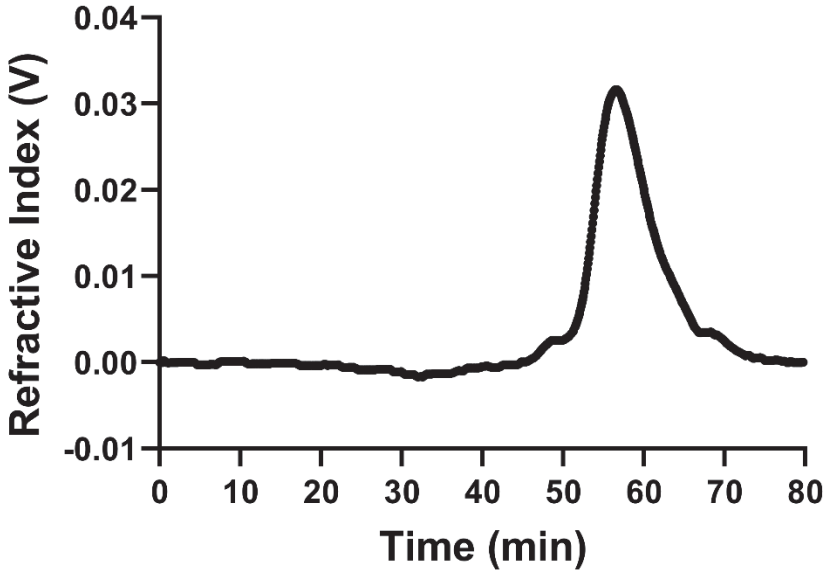


**Figure 2: HPSEC-RI elution profile of the polysaccharide fraction TCA-S.**

The elution profile of TCA-S fraction in HPSEC-RI (Figure 2) was homogeneous, with a molecular weight of $8.7 \times 10^4$ g/mol, calculated using its specific

dn/dc of 0.198. This fraction showed 81.6% of sugar content and in its monosaccharide composition (Figure S1), arabinose (38.2%), galactose (20.0%), rhamnose (17.6%), glucose (15.1%), mannose (6.3%), xylose (1.4%), identified by GC, and galacturonic acid (1.3%), quantified by the colorimetric method of Filisetti-Cozzi and Carpita (1991). Identification of the uronic acid as galacturonic acid was made through thin layer chromatography, using glucuronic and galacturonic acids as monosaccharide standards. In addition, total phenolic compounds and total protein contents were determined, resulting in 12.6% and 4.7%, respectively.

Table 1: Profile of partially O-methylated alditol acetates and their respective linkage type of TCA-S fraction obtained by methylation analysis.

| O-Me-alditol acetate | Linkage type | % |
|---|---|---|
| 2,3,5-$Me_3$-Ara*f* | Ara*f*-(1→ | 0.7 |
| 2,3,4-$Me_3$-Rha*p* | Rha*p*-(1→ | 2.7 |
| 2,3,4-$Me_3$-Ara*p* | Ara*p*-(1→ | 0.4 |
| 2,5-$Me_2$-Ara*f* | →3)-Ara*f*-(1→ | 2.0 |
| 2,3-$Me_2$-Ara*f* | →5)-Ara*f*-(1→ | 5.1 |
| 2,3-$Me_2$-Xyl*p* | →4)-Xyl*p*-(1→ | 3.5 |
| 2,3,4,6-$Me_4$-Glc*p* | Glc*p*-(1→ | 6.4 |
| 2,3,4,6-$Me_4$-Gal*p* | Gal*p*-(1→ | 5.1 |
| 2,3,6-$Me_3$-Gal*p* | →4)-Gal*p*-(1→ | 10.7 |
| 2,3,6-$Me_3$-Glc*p* | →4)-Glc*p*-(1→ | 13.1 |
| 2,3,4-$Me_3$-Glc*p* | →6)-Glc*p*-(1→ | 11.8 |
| 2,3,4-$Me_3$-Gal*p* | →6)-Gal*p*-(1→ | 10.4 |
| 3,6-$Me_2$-Glc*p* | →2,4)-Glc*p*-(1→ | 13.8 |
| 3,6-$Me_2$-Gal*p* | →2,4)-Gal*p*-(1→ | 9.6 |
| 2,4-$Me_2$-Gal*p* | →3,6)-Gal*p*-(1→ | 4.5 |

Note: EI-MS spectrum of each methylated derivative of TCA-S is shown on Fig. S2.

Glycosidic linkage profile in TCA-S was investigated by methylation analysis and the partially O-methylated alditol acetates derivatives obtained are compiled in Table 1 and Figure S2. The glycosidic bonds referring to type I arabinogalactan (AG-I) were identified by the derivatives 2,3,6-$Me_3$-Gal*p* (10.7%) and 3,6-$Me_2$-Gal*p* (9.6%) related to the main chain constituted by β-D-Gal*p*-(1→4)-linked units and to the branching point in the main chain at the O-2 position, probably by non-reducing terminals of arabinose (PETTOLINO *et al*., 2012). The methylated derivatives 2,3,4-$Me_3$-Gal*p* (10.4%) and 2,4-$Me_2$-Gal*p* (4.5%) are associated with type II

arabinogalactan (AG-II), which is composed of a main chain of β-D-Gal*p*-(1→6)-linked units, partially substituted at O-3 (ASPINALL, 1973; CARPITA; GIBEAUT, 1993; PETTOLINO *et al*., 2012). Moreover, the 2,3-$Me_2$-Ara*f* derivative (5.1%) suggests the presence of a (1→5)-linked arabinan (PETTOLINO *et al*., 2012). Also, it is suggestive that starch is present in the TCA-S fraction, due to the appearance of the methylated derivative 2,3,6-$Me_3$-Glc*p* (13.1%), corresponding to (1→4)-linked Glc*p* units. According to methylation analysis, AG-I, AG-II, arabinan and starch are the main polysaccharides in TCA-S fraction. Furthermore, methylation analysis also suggests the presence of other polysaccharides, in minor proportions, such as linear xylan, indicated by the presence of 2,3-$Me_2$-Xyl*p* (3.5%) (PETTOLINO *et al*., 2012).

The $^{13}C/^{1}H$ correlation map (HSQC-DEPT) of the TCA-S fraction (Figure 3) showed, in the anomeric region, C-1/H-1 correlations of α-L-Ara*f* units at δ 109.5/5.25 ppm and β-D-Gal*p* at δ 103.4/4.45 ppm (OVODOVA *et al*., 2009; POPOV *et al*., 2011; RENARD *et al*., 1998). At δ 81.9/3.88 ppm, the correlation of C-3/H-3 of 3-O-substituted β-D-Gal*p* units was observed, and at δ 69.6/4.22 ppm, C-6/H-6 correlations of 6-O-substituted β-D-Gal*p* units (DELGOBO *et al*., 1998). These correlations suggest the presence of type II arabinogalactan (AGII) in the fraction. The correlation observed at δ 77.5/3.95 ppm is characteristic of C-4/H-4 of 4-O-substituted β-D-Gal*p* units (DELGOBO *et al*., 1998), suggesting the presence of type I arabinogalactan (AGI) in TCA-S fraction. Still, the correlation in δ 99.5/5.01 ppm is characteristic of C-1/H-1 and in δ 16.7/1.29, of C-6/H-6 of α-L-Rha*p* units (NASCIMENTO *et al*., 2013; RENARD *et al*., 1998). The correlation at δ 100.6/4.74 ppm was attributed to the C-1/H-1 of α-D-Glc*p* units (NOWAK *et al*., 2019).

Finally, the correlations observed in δ 107.7/5.08, δ 81.4/4.13, δ 81.7/4.21 and δ 66.9/3.98 and 67.0/4.05 ppm are related to C-1/H-1, C-2/H-2, C-4/H-4 and C-5/H-5 of →1)-α-L-Ara*f*-(5→ units (SCHNEIDER; IACOMINI; CORDEIRO, 2019; SHAKHMATOV *et al*., 2014), suggesting the presence of arabinan in TCA-S.

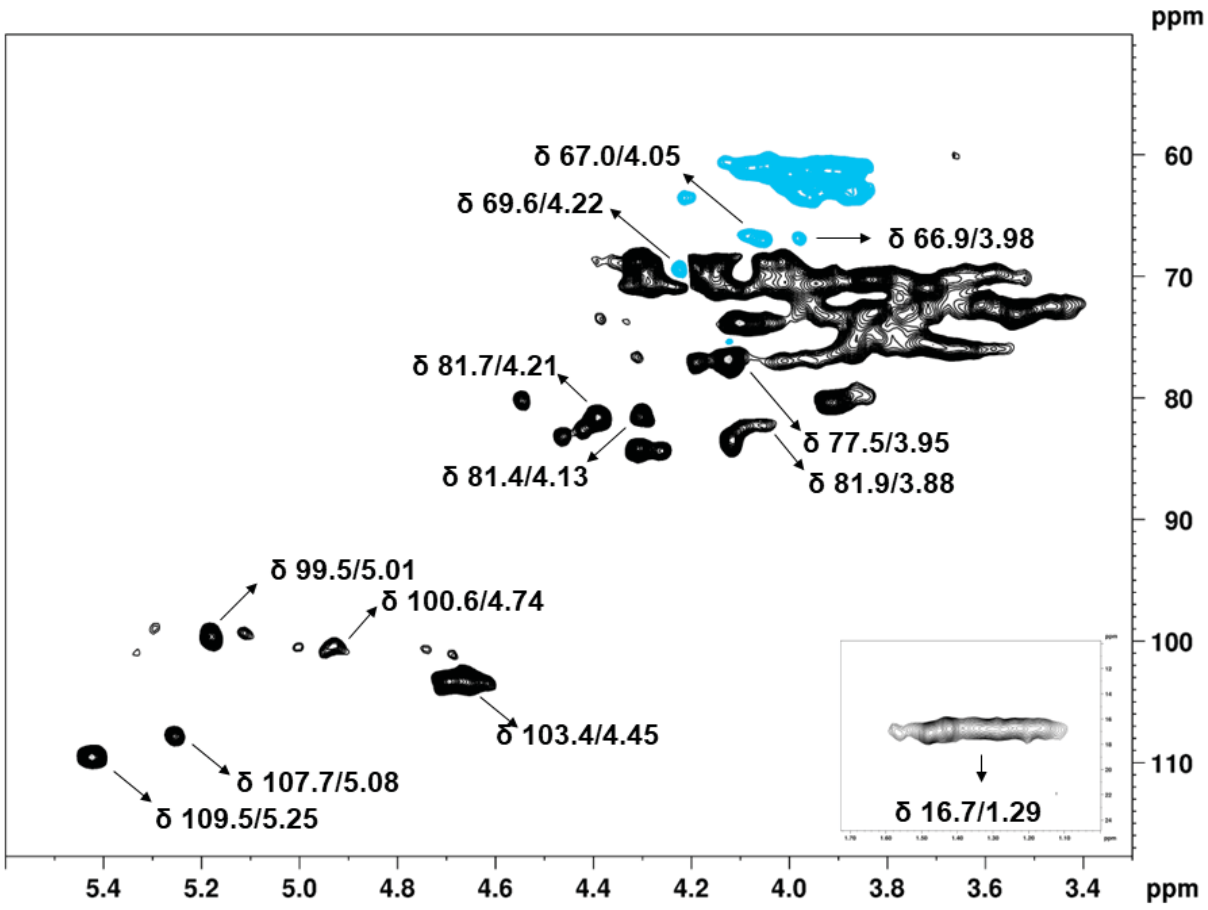


**Figure 3: $^{1}H/^{13}C$ HSQC-DEPT correlation map of TCA-S.** Sample was solubilized in $D_2O$ and analysis was performed at 50 °C, in a 400 mHz spectrometer. Chemical shifts are expressed as ppm. Blue signals are from $-CH_2$.

From data obtained on NMR and methylation analyses, TCA-S is mainly constituted of type I and type II arabinogalactans, arabinan and starch.

AGI and AGII are polysaccharides whose structural characteristics are backbones consisting of β-D-Gal*p*-(1→4)-linked units and β-D-Gal*p*-(1→3)-, (1→6)- and (1→3,6)-linked units, respectively (ASPINALL, 1973). Moreover, arabinans structurally consist of chains of arabinose units joined together by α-(1→5) bonds, preferentially substituted at the O-3 and O-2 positions.

3.2 Evaluation of the immunomodulator potential of TCA-Sα

Once starch was detected in TCA-S by methylation analysis and confirmed using lugol's solution, α-amylase treatment was performed aiming starch removal, originating the fraction tested in immunomodulatory studies, named TCA-Sα, free of starch. Confirmation of starch was made by lugol's solution test and by nmr (Figure S3). Regarding detection of LPS by GC-MS in the sample (Figure S4), no contamination was detected, so effects on THP-1 macrophages demonstrated in this study can be attributed exclusively to TCA-Sα fraction. Immunomodulatory activity of TCA-Sα, a polysaccharide rich fraction isolated from *B. forficata* leaves, was evaluated by analysis of cytokine secretion (TNF-α, IL-1β and IL-10) by THP-1 macrophages. While TNF-α and IL-1β are proinflammatory cytokines, IL-10 is considered as an anti-inflammatory one.

First, to define the non-toxic concentrations of TCA-Sα, THP-1 macrophages were treated with the test fraction and viability was evaluated by neutral red and crystal violet assays. None of the concentrations of TCA-Sα tested interfered with neutral red uptake (Figure 4A) nor diminish cellular density of THP-1 cells (Figure 4B). Considering the absence of cytotoxicity, the influence of TCA-Sα on cytokine secretion by THP-1 cells was analyzed in the presence and absence of LPS.

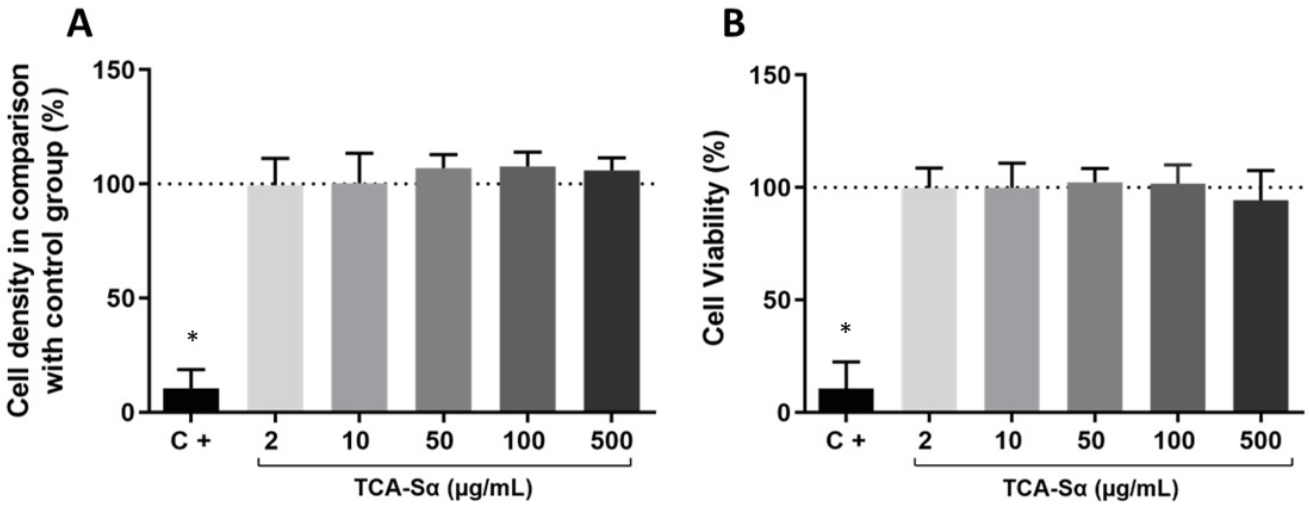


**Figure 4: THP-1 cell proliferation by neutral red (A) and cell viability by crystal violet assay (B), when treated with TCA-Sα.** Saponin was used as positive control. The results are expressed as mean ± SEM and represented by a set of at least three independent

experiments. Negative control group is represented by the dotted line. * Different from negative control group.

As expected, the positive control LPS induced the secretion of all cytokines assessed in this study (Figure 5). TCA-Sα also stimulated cytokine secretion by THP-1 cells. TCA-Sα at 50, 100 and 500 µg/mL was able to significantly increase TNF-α production (Figure 5A), but when incubated along with LPS, TCA-Sα at concentration of 50 µg/mL interestingly reduced this cytokine secretion when compared to positive control group, indicating a potential immunomodulatory effect. In relation to IL-1β measurement (Figure 5B), TCA-Sα fraction, at any concentration tested, induced cytokine secretion by THP-1 cells, and it did not affect LPS-induced secretion. Lastly, all TCA-Sα concentrations tested (Figure 5C) significantly increased IL-10 secretion when compared to the negative control group. Moreover, TCA-Sα did not affect LPS-induced secretion of IL-10.

The ratio between pro-inflammatory and anti-inflammatory cytokines induced by LPS alone and by LPS plus TCA-Sα at all concentrations was calculated (OLIVEIRA *et al*., 2017). The ratios TNF-α/IL-10 and IL-1β/IL-10 for positive control alone were respectively, 8.09 and 1.05. For the polysaccharide fraction plus LPS, they were 4.94 and 1.10 at 50 µg/mL, 5.31 and 0.90 at 100 µg/mL, and 4.04 and 0.74 at 500 µg/mL of TCA-Sα. The decrease of the value of pro/anti-inflammatory cytokine ratios relative to treatment with polysaccharide fraction at all concentrations in comparison to the ratios derived from cells treated with LPS alone, may suggest an overall anti-inflammatory effect from the polysaccharides when incubated simultaneously with LPS.

Altogether, these results indicate that the polysaccharide fraction both stimulates cytokine secretion and modulates their secretion when incubated concomitantly with LPS, exhibiting an immunomodulatory effect. This characteristic behavior was already observed before for other plant polysaccharides (TAMIELLO *et al*., 2017; NASCIMENTO *et al*., 2017).

Natural polysaccharides, including those from plants, are great alternatives for medicinal purposes once they present in general absence of toxicity and great availability, besides their notorious biological properties, as citated before (PAWAR; KAMAT; CHOUDHARY, 2015; SCHEPETKIN; QUINN, 2006). Plant polysaccharides

occur in many different forms; they can be neutral or acidic, have different branch degrees or be assembled by one up to many different types of monosaccharides, and they have been extensively researched for their ability to influence in cytokine release (PAULSEN, 2001). In addition, it is known that their chemical structure can influence biological activity. As an example, Nascimento *et al*. (2017) observed a difference in polysaccharide performance when evaluating cytokine secretion by THP-1 macrophages, of a structurally modified pectin from sweet pepper, caused by acid hydrolysis, when compared to the native pectin. Schepetkin and Quinn (2006), in their review paper about plant polysaccharides and their relationship with macrophage immunomodulation, stated by analyzing studies aiming investigation of plant polysaccharides on macrophages responses, that most of them activate macrophages and increase the concentration of chemokines and cytokines.

Concerning immunoactivity of arabinogalactans, there are studies that demonstrate different responses performed by them. Freysdottir *et al*. (2016) verified the immunoenhancing activity of a polysaccharide fraction mainly constituted of galacturonic acid, galactose and arabinose from *Achillea millefolium*, by observation of increased secretion of the proinflammatory cytokines TNF-α and IL-1β in THP-1 cells stimulated with LPS. Yao *et al*. (2018) observed a modulation of cytokine secretion promoted by a type II arabinogalactan from *Carthamus tinctorius* L. on splenocytes, increasing concentrations of TNF-α and IL-1β, in a concentration-dependent way, when compared to negative control group. Tamiello *et al*. (2017) isolated a type II arabinogalactan from *jambo* fruits and evaluated its response on cytokine secretion by THP-1 macrophages, in the presence and absence of LPS, in the same model as employed in this study. They verified that AGII enhanced, in a concentration-dependent way, TNF-α, IL-1β and IL-10 secretions. However, it was observed a depletion of these cytokines in LPS presence, therefore attenuating the inflammatory response induced by LPS. Similarly, to the results obtained by Tamiello *et al*. (2017), TCA-Sα fraction, composed mainly by type I and II arabinogalactans, increased cytokine liberation in absence of LPS and minimized inflammatory response induced by LPS, through decrease of TNF-α at concentration of 50 µg/mL and lowering pro-/anti-inflammatory ratios. Nascimento *et al*. (2017) investigated the effects of a pectic fraction from sweet pepper on cytokine secretion by THP-1 cells and found that it induced TNF-α, IL-1β and IL-10 secretion. Also, at the highest concentration evaluated,

different from what was observed in this study, they observed that, when the fraction was incubated along with inflammatory agent LPS, it could attenuate inflammation by reducing the secretion of proinflammatory cytokines and increasing anti-inflammatory ones. Perhaps this could mean that a lot of factors can influence arabinogalactans immunoactivity, such as source, monosaccharide proportion, branching and concentration.

Zhang *et al*. (2014) demonstrated the immunomodulating activity from these molecules, associating their structural domains, especially galactose-rich regions, with ability to promote this biological effect. Also, the arabinogalactans β-(1→3) and β-(1→6) core were described before by Lai *et al*., (2015) as being responsible for immune activity, such as inducer of dendritic cells (LAI *et al*., 2015).

The cytokine release profile observed in this study, caused by the treatment of a fraction rich in arabinogalactans, may have some interesting therapy applications. It has been described before that cytokines can contribute to cancer immunotherapy. For example, IL-10, in a context of chronic infections and cancer, demonstrated to inhibit CD8+ T cell apoptosis induced by antigen, therefore prolonging cytotoxic activity of lymphocytes; TNF-α, among its mechanisms of antitumoral effect, is considered as a mediator of anti-tumor immune responses, and studies revealed that immunotherapies have shown decreased anti-tumor efficacy when coadministered with TNF-α antagonists (VAN HORSSEN *et al*., 2006; BERRAONDO *et al*., 2019). Another application for the stimulatory cytokine secretion profile is as modulator of intestinal epithelium cell restitution. After an injury, the intestinal mucosal epithelium is rapidly re-established and there are previous studies that show that cytokines, including TNF-α and IL-10, have the ability of promoting intestinal epithelium restitution by stimulating epithelial proliferation (ANDREWS *et al*., 2018). Considering that TCA-Sα displayed an immunostimulatory-like property, we suggest that these could be a potential application for polysaccharides from *B. forficata* leaves. However, it certainly needs further investigation to confirm such activity and possible related benefits.

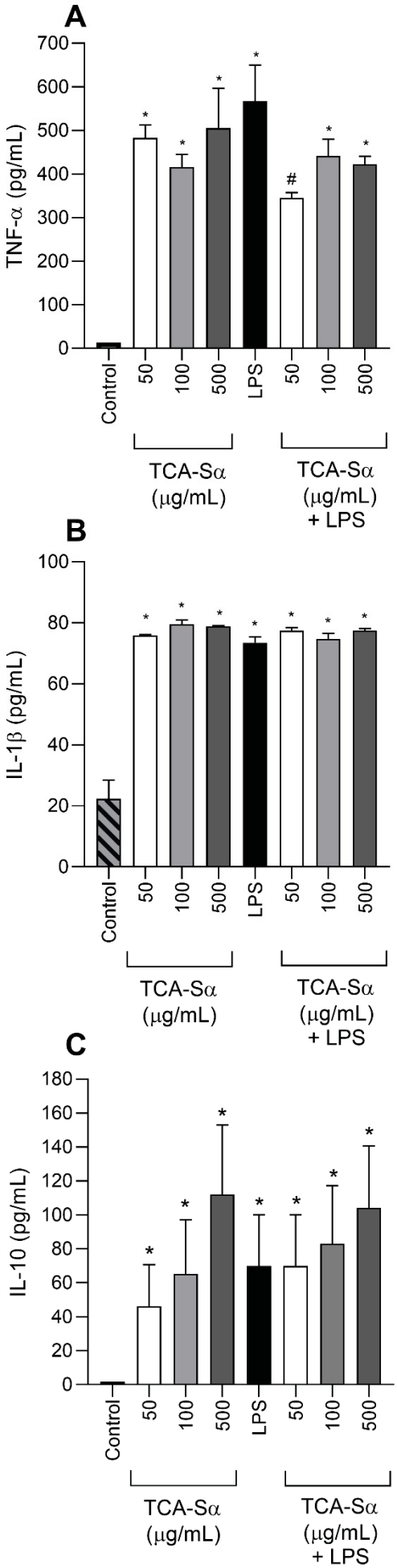
A
TNF-α (pg/mL)
700
600
500
400
300
200
100
0
Control
50
100
500
LPS
50
100
500
TCA-Sα (μg/mL)
TCA-Sα (μg/mL) + LPS
B
IL-1β (pg/mL)
100
80
60
40
20
0
C
IL-10 (pg/mL)
180
160
140
120
100
80
60
40
20
0

**Figure 5: Effect of TCA-Sα on TNF-α (A), IL-1β (B) and IL-10 (C) secretion by THP-1 macrophages.** Cells were incubated with fresh medium as negative control, LPS (1 μg/mL) as positive control, TCA-Sα or TCA-Sα + LPS. Statistical significance is considered when $p < 0.05$. * Different from negative control group; # Different from LPS positive control group.

## 4 Conclusion

Type I, type II arabinogalactans and a small amount of arabinan and starch were characterized in TCA-S fraction, obtained by hot aqueous extraction of *B. forficata* leaves. Through evaluation of the effects of TCA-Sα (TCA-S after starch removal) fraction on cytokine secretion by THP-1 human macrophages, the fraction displayed a stimulant effect on cytokine secretion at all concentrations tested, in the absence of LPS, and in the presence of LPS, a modulator effect. This is the first record in literature to investigate the composition and structure of polysaccharides from *B. forficata* leaves, which contributes to data about identification and potential application of plant compounds.

## 5 Acknowledgments

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## 6 Authors Contributions

Giuliana Cozzella Campo Grande – Investigation, Methodology, Writing-Original draft; Jessica Boschini D'Agostin – Investigation, Methodology; Genilza da Silva Mello – Investigation, Methodology; Philippe Rodrigues Benedetti – Investigation, Methodology; Arquimedes Paixão de Santana Filho – Investigation, Methodology; Fernanda Fognanoli Simas: Conceptualization, Investigation, Methodology, Resources; Thales Ricardo Cipriani - Conceptualization, Project administration, Funding acquisition, Supervision and Writing – Review and editing.

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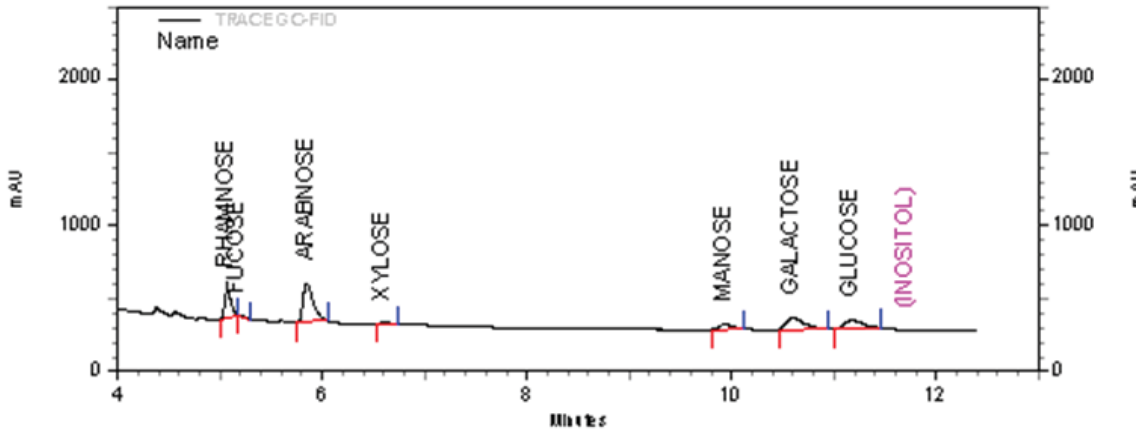
## 8 Supplementary Material

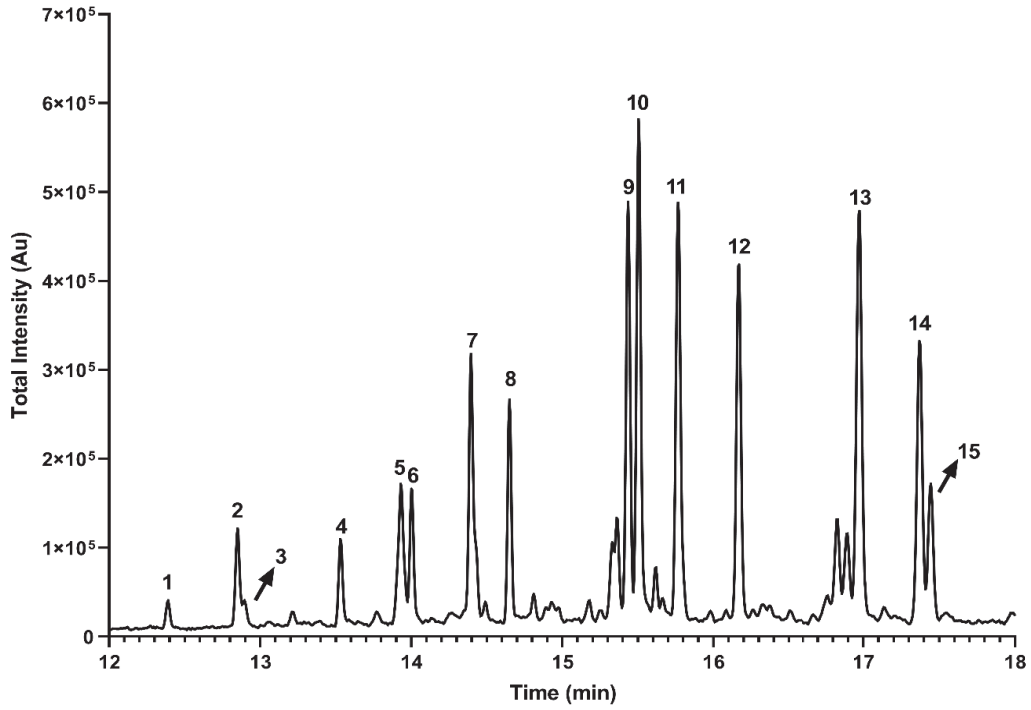



TRACE GC-FID
Results (System
(24/5/2021
08:50:30)
(Reprocessed))

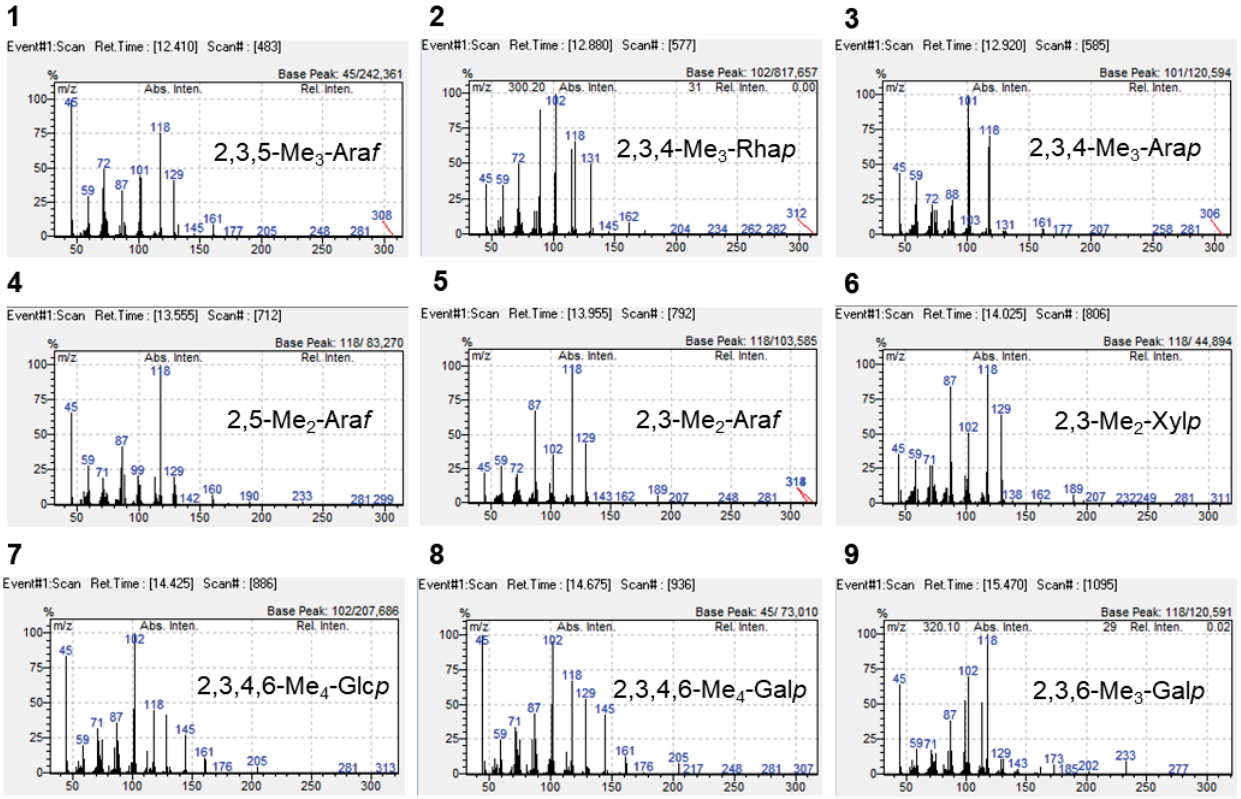
| Pk# | Nome | Tempo ret. | Área | Area Percent |
|---|---|---|---|---|
| 1 | RHAMNOSE | 5,068 | 8485488 | 17,897 |
| 2 | FUCOSE | 5,173 | 24624 | 0,052 |
| 3 | ARABNOSE | 5,845 | 18354720 | 38,713 |
| 4 | XYLOSE | 6,598 | 652880 | 1,377 |
| 5 | MANOSE | 9,938 | 3025204 | 6,381 |
| 6 | GALACTOSE | 10,588 | 9617937 | 20,286 |
| 7 | GLUCOSE | 11,168 | 7251733 | 15,295 |

**Figure S1: Monosaccharide composition chromatograms through GC analysis of the alditol acetates obtained from the TCA-S fraction.**

**A**


Total Intensity (Au)
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

**B**


1
2,3,5-Me3-Araf
2
2,3,4-Me3-Rhap
3
2,3,4-Me3-Arap
4
2,5-Me2-Araf
5
2,3-Me2-Araf
6
2,3-Me2-Xylp
7
2,3,4,6-Me4-Glcp
8
2,3,4,6-Me4-Galp
9
2,3,6-Me3-Galp

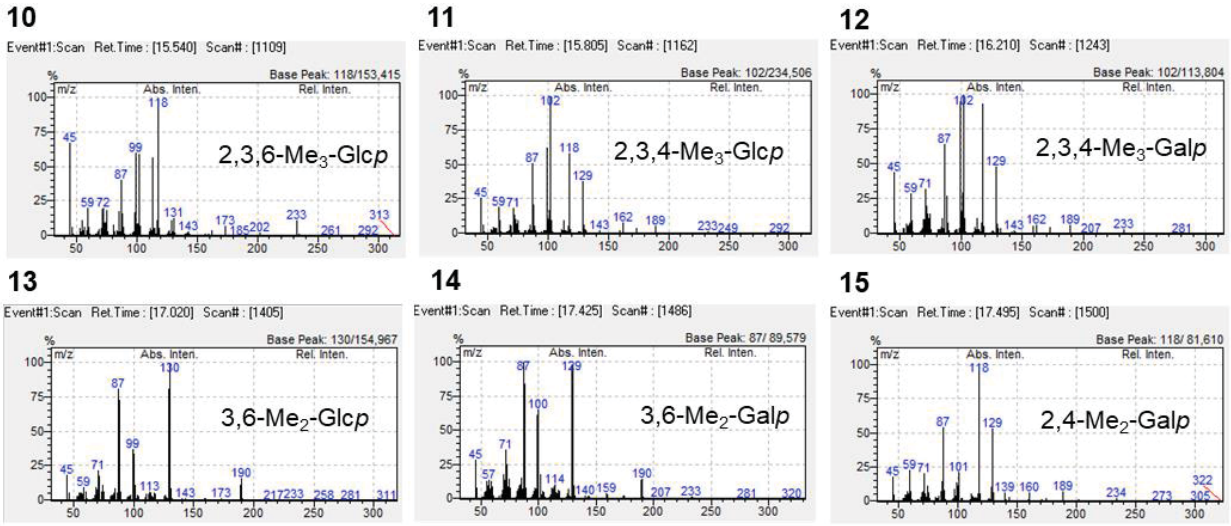



**Figure S2: Chromatogram (A) and mass spectra (B) of the GC-MS analysis of the partially O-methylated alditol acetates of TCA-S.**

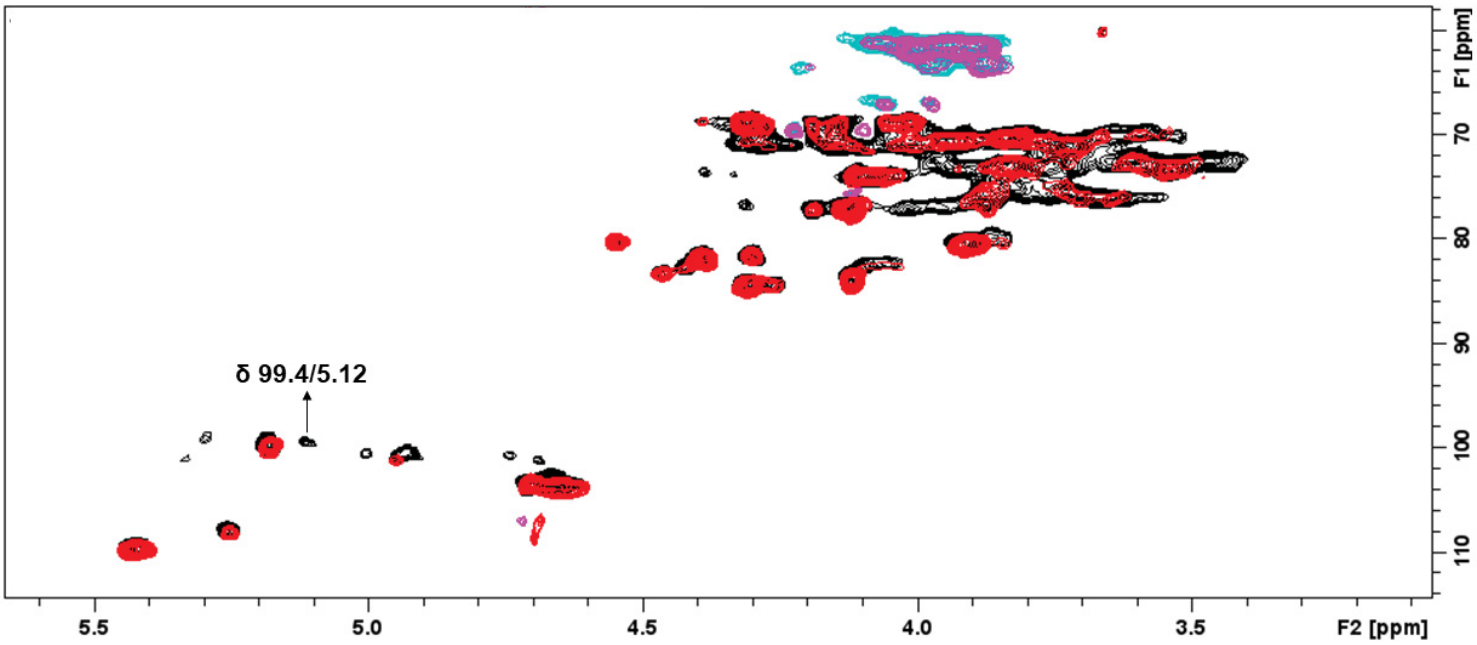



**Figure S3: Superposition of $^{1}H/^{13}C$ HSQC-DEPT correlation maps of TCA-S (black and blue signals) and TCA-Sα (red and pink signals).** Samples were solubilized in $D_2O$ and analysis was performed at 50 °C, in a 400 mHz spectrometer. Chemical shifts are expressed as ppm. Blue and pink signals are from $-CH_2$. Signals present in TCA-S fraction and absent in TCA-Sα were attributed to starch. In anomeric region, δ 99.4/5.12 is from C-1/H-1 units of α-D-Glc*p,* common signal in starch structure (NOWAK *et al*., 2019).

**A**


**B**


**Figure S4: A: Chromatogram and fragmentation profile obtained by GC-MS analysis of standard of LPS. Mass spectra was filtered using m/z 257 ion, characteristic of 3-OH fatty acids, according to Santana-Filho et al. (2013). B: Chromatogram obtained from GC-MS analysis for detection of LPS in TCA-Sα. Mass spectra was filtered using m/z 257, and no contamination by LPS was detected in the sample.**

## 6 CONCLUSÕES

A partir das folhas de *Bauhinia forficata* Link, foram obtidas duas frações: BFSGD (BFSF no Artigo I), sobrenadante do processo de congelamento e degelo, constituída de homogalacturonana, arabinana, amido e arabinogalactanas tipos I e II, e que foi analisada quanto ao seu potencial hipoglicemiante e antidiabético pelo modelo de diabetes induzido por estreptozotocina em ratos. A fração não apresentou resultado estatisticamente relevante em relação à redução de glicemia a curto e longo prazo, mas demonstrou efeito hepatoprotetor ao reduzir significativamente os marcadores bioquímicos bilirrubina direta e a enzima AST nas doses de 10 e 30 mg/kg, e através de estudos histopatológicos, ao apresentar redução do dano ao tecido hepático nas doses de 30 100 mg/kg. Outra fração, obtida a partir de BFSGD, denominada TCA-Sα, constituída de arabinogalactanas tipos I e II e arabinana, foi testada quando à sua atividade imunomodulatória em células THP-1 e apresentou propriedade imunoestimulatória e imunomodulatória, na ausência e presença de LPS, respectivamente.

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# ANEXO I

**MINISTÉRIO DA EDUCAÇÃO**
UNIVERSIDADE FEDERAL DO PARANÁ
SETOR DE CIÊNCIAS BIOLÓGICAS
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

**Nº 1422**

**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**: 23075.055916/2021-61

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**TÍTULO:** Polissacarídeos de *Bauhinia forficata* (pata-de-vaca): caracterização estrutural e atividade biológica.

**TITLE:** *Bauhinia forficata* (pata-de-vaca) polysaccharides: structural characterization and biological activity.

**AUTORES/AUTHORS:** Thales Ricardo Cipriani, Giuliana Cozzella Campo Grande, Joice Maria da Cunha, Carlos Henrique Alves Jesus.

**DEPARTAMENTO/DEPARTAMENT:** Bioquímica e Biologia Molecular

Prof. Dr. Breno Castello Branco Beirão
**Coordenador da CEUA**

Documento assinado eletronicamente por **ISELEN ABREU FLORENTINO IVANOSKI**, **Institucional**, em 28/10/2021, às 14:17, conforme art. 1º, III, "b", da Lei 11.419/2006.

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