

UNIVERSIDADE FEDERAL DO PARANÁ

PEDRO FELIPE PEREIRA CHAVES

FRUTANAS E FRUTOOLIGOSSACARÍDEOS DE INFUSOS DE *Mikania glomerata*
Spreng E *Baccharis trimera* Less. DC.: CARACTERIZAÇÃO ESTRUTURAL, EFEITOS
HEPATOPROTETORES E SOBRE A CONTRATILIDADE MUSCULAR DE
CAMUNDONGOS

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CAMUNDONGOS

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Orientadora: Prof. Dra. Lucimara M. C. Cordeiro

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Dedico este trabalho aos povos antigos que, mesmo sem ferramentas precisas, descobriram e transmitiram pelo tempo o conhecimento do poder curativo das espécies vegetais.

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“Education is a progressive discovery of our own ignorance.”
– Will Durant

RESUMO

Inúmeras são as espécies vegetais que apresentam finalidade terapêutica e entre elas estão a *Mikania glomerata* Spreng (Guaco) e a *Baccharis trimera* Less. DC (Carqueja). Ambas pertencem à família Asteraceae e são nativas do sul do Brasil, onde são amplamente utilizadas na medicina popular. Estas espécies constam ainda na RENISUS, lista de espécies vegetais voltadas a saúde da população, divulgada pelo Ministério da Saúde Brasileiro em 2009. Mesmo sendo espécies conhecidas e utilizadas, a presença de polissacarídeos nos infusos preparados a partir das espécies não havia sido investigada até o momento. Polissacarídeos podem apresentar vários dos efeitos biológicos atribuídos aos infusos destas espécies, como por exemplo efeito anti-inflamatório, antioxidante, gastroprotetor e hepatoprotetor, entre outros. Estas moléculas também são investigadas quanta muitas outras atividades biológicas, porém seus efeitos nas características contráteis do músculo esquelético não haviam sido explorados. Portanto o presente estudo objetivou caracterizar estruturalmente os polissacarídeos presentes nos infusos obtidos a partir de *M. glomerata* e de *B. trimera*, avaliar seus efeitos no fígado *per se* e em modelo de hepatotoxicidade induzida por tetracloreto de carbono (CCl₄) e avaliar o efeito de polissacarídeos nas características contráteis de músculos esqueléticos de camundongos. Partes aéreas secas de ambas as espécies foram submetidas à infusão que, após serem concentrados e precipitados com etanol geraram frações brutas de polissacarídeos com rendimento de 1,9% (*M. glomerata*) e 1,8% (*B. trimera*). Ambas as frações foram submetidas a purificação e o polissacarídeo principal caracterizado foi uma frutana do tipo inulina (Frações: SMG a partir de *M. glomerata* e SBT de *B. trimera*). A partir da precipitação etanólica com infuso de *M. glomerata*, foi gerada uma fração sobrenadante etanólica com rendimento de 13% (MG-ET), onde foram observados frutooligosacarídeos com grau de polimerização variando de 2 a 10 unidades de frutose. A partir da fração SBT foram purificadas em baixa concentração arabinogalactanas do tipo II com significativo conteúdo de fucose terminal. As frações SMG, MG-ET e SBT foram avaliadas quanto seus efeitos sobre a função hepática *in vivo* e os resultados mostraram que estas não produzem efeito hepatotóxico e ainda podem promover hepatoproteção contra os danos induzidos por CCl₄. Por último a fração composta por inulina (SMG) foi testada quando sua capacidade de promover efeitos nas características contráteis de músculos esqueléticos de camundongos submetidos a endurance e pôde promover alterações na força de contração isométrica máxima e na velocidade máxima de diminuição de força nos músculos EDL. Em conclusão os resultados obtidos com esta tese mostram que os infusos das espécies *M. glomerata* e de *B. trimera* possuem carboidratos complexos bioativos, principalmente inulina, os quais puderam promover efeito protetor ao fígado e também mudanças nas características contráteis do músculo esquelético de camundongos.

Palavras chave: *Baccharis trimera*, *Mikania glomerata*, polissacarídeos, hepatoproteção, músculo esquelético.

ABSTRACT

There are numberless plant species used with therapeutic purposes, including *Mikania glomerata* Spreng (Guaco) and *Baccharis trimera* Less. DC (Carqueja). Both belong to the Asteraceae family and are native to southern Brazil, where they are widely used in folk medicine. These species are also included in RENISUS, a list of health-related plant species published by the Brazilian government in 2009. Although they are known and used species, the presence of polysaccharides in infusions prepared from the species has not been investigated so far. Polysaccharides can present several of the biological effects attributed to the infusions from the species, such as anti-inflammatory, antioxidant, gastroprotective and hepatoprotective effects, among others. These molecules are also investigated for a diverse number of others biological effects, but their effects on contractile characteristics of skeletal muscle had not been explored. Thus, the present study aimed to structurally characterize the polysaccharides present in the infusions obtained from *M. glomerata* and *B. trimera*, to evaluate its effects on the liver *per se* and in the carbon tetrachloride-induced hepatotoxicity model and to evaluate the effect of polysaccharides on the contractile characteristics of mice skeletal muscle. Dried aerial parts of both species were submitted to infusion that was concentrated, precipitated with ethanol and generated crude polysaccharide fractions with yield of 1.9% (*M. glomerata*) and 1.8% (*B. trimera*). Both fractions were purified and the main polysaccharide characterized was an inulin-type fructan (Fractions: SMG from *M. glomerata* and SBT from *B. trimera*). From the ethanolic precipitation with *M. glomerata* infusion, a 13% yield ethanolic supernatant fraction (MG-ET) was generated, where fructooligosaccharides with polymerization degree ranging from 2 to 10 fructose units were observed. From the SBT fraction, low concentration of type II arabinogalactans with significant terminal fucose content was purified. The SMG, MG-ET and SBT fractions were evaluated *in vivo* for their effects on liver function and the results showed that they didn't produce hepatotoxic effect and could promote hepatoprotection against CCl₄-induced injuries. Finally, the inulin fraction (SMG) was tested about its ability to promote effects on the contractile characteristics of skeletal muscles of mice submitted to endurance and was able to promote changes of maximum isometric twitch force and maximum speed of force decrease in EDL muscles. In conclusion, the results obtained with this thesis showed that *M. glomerata* and *B. trimera* infusions contains bioactive complex carbohydrates, mainly inulin, which can promote protective effect on mice liver and also changes in the contractile characteristics of mice skeletal muscle.

Key words: *Baccharis trimera*, *Mikania glomerata*, polysaccharides, hepatoprotection, skeletal muscle.

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LISTA DE SIGLAS E ABREVIATURAS

+dF/dt	- Velocidade máxima do desenvolvimento da força (Força/Tempo)
-dF/dt	- Velocidade máxima de relaxamento (Força/Tempo)
¹³ C RMN	- Ressonância magnética nuclear de carbono 13
δ	- Deslocamento químico
ALT	- Alanina aminotransferase
AST	- Aspartato aminotransferase
ALP	- Fosfatase alcalina
Ara	- Arabinose
BT	- Polissacarídeos brutos provenientes de <i>B. trimera</i>
BTWE	- Fração proveniente de <i>B. trimera</i> eluída com água na coluna cromatográfica
BTSE	- Fração proveniente de <i>B. trimera</i> eluída com NaCl na coluna cromatográfica
CAT	- Catalase
CSA	- Área da seção transversa
D	- Dextrogiro
D ₂ O	- Óxido de deutério
DEPT	- Intensificação sem distorção por transferência de polarização
EDL	- Musculo extensor longo de dedo
<i>f</i>	- Furanosídico
Fmax	- Força tetânica máxima
Fru	- Frutose
Fuc	- Fucose
Gal	- Galactose
GalA	- Ácido galacturônico
GC-MS	- Cromatografia gasosa acoplada à espectrometria de massas
GF	- Glucose + Frutose
Glc	- Glucose
GSH	- Glutathione reduzida
GST	- Glutathione S-transferase
HPSEC	- Cromatografia de exclusão estérica de alta eficiência
HSQC	- Espectroscopia de correlação heteronuclear <i>Single-Quantum</i>
i.p.	- Intraperitoneal

L	- Levogiro
LPO	- Peroxidação lipídica
<i>m/z</i>	- Relação massa-carga
Man	- Manose
MG	- Polissacarídeos brutos provenientes de <i>M. glomerata</i>
MG-ET	- Sobrenadante etanólico proveniente de <i>M. glomerata</i>
<i>p</i>	- Piranosídico
PBT	- Fração precipitada do congelamento e descongelamento proveniente de <i>B. trimera</i>
PMG	- Fração precipitada do congelamento e descongelamento proveniente de <i>M. glomerata</i>
ppm	- Partes por milhão
Rha	- Ramnose
SBT	- Fração sobrenadante do congelamento e descongelamento proveniente de <i>B. trimera</i>
SMG	- Fração sobrenadante do congelamento e descongelamento proveniente de <i>M. glomerata</i>
SOD	- Superóxido dismutase
Tmax	- Força de contração isométrica máxima
AU	- Unidades arbitrárias
Xyl	- Xilose

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1. INTRODUÇÃO

Espécies vegetais que são utilizadas com finalidade terapêutica são denominadas plantas medicinais (ROSSATO et al., 2012). No sul do Brasil diversas plantas medicinais são conhecidas, dentre elas estão o guaco (*Mikania glomerata*) e a carqueja (*Baccharis trimera*), às quais se associa um amplo conhecimento terapêutico que é difundido entre a população (LORENZI; MATOS, 2008; SOUSA et al., 1991). Ambas as espécies citadas pertencem à família Asteraceae e já foi relatado que a utilização dos infusos destas espécies podem promover efeitos sobre a função hepática (GRANDI, 2014; LORENZI; MATOS, 2008). Os membros da família Asteraceae, assim como os de outras famílias, apresentam em seus componentes fitoquímicos moléculas responsáveis por diversas propriedades farmacológicas como por exemplo ácidos fenólicos, flavonoides, alcaloides e polissacarídeos (LORENZI; MATOS, 2008; WILDMAN; KELLEY, 2006).

Os polissacarídeos vêm ganhando destaque, pois, com o aumento exponencial das pesquisas científicas e investigações detalhadas sobre estas moléculas, está sendo possível observar a importância destes polímeros dentro do grupo de compostos bioativos (LIU; WILLFÖR; XU, 2015). Estas moléculas são amplamente investigadas quanto suas propriedades biológicas e apresentam uma alta gama de efeitos biológicos já comprovados (LIU; WILLFÖR; XU, 2015). Entre estes efeitos estão os relacionados ao presente trabalho, como a atividade antioxidante, a hepatoproteção, a atividade prebiótica, a capacidade de promover melhora no desempenho físico e atenuação nos danos oxidativos musculares (CALEFFI et al., 2015; CORRÊA-FERREIRA et al., 2017; KLOSTERHOFF et al., 2018; LIU et al., 2015; ZHONGHUI; XIAOWEI; FANG, 2014). É sabido que as atividades biológicas de polissacarídeos são afetadas por seus parâmetros estruturais, como composição monossacarídica, configuração das ligações glicosídicas, quantidade e tamanho das ramificações e massa molar (YANG; ZHANG, 2009). Desse modo, a elucidação da estrutura química dos polissacarídeos é relevante para avaliar a relação entre os polímeros e as atividades biológicas.

Frações de polissacarídeos provenientes de diferentes espécies vegetais já apresentaram ação sobre a função hepática (KALANTARI et al., 2019; SUN et al., 2013). Entretanto, como a disfunção hepática é um problema causado por vários fatores ainda não há tratamentos específicos para muitos quadros de hepatotoxicidade, mesmo sendo alguns extremamente graves (HAN et al., 2016). O fígado consta entre os órgãos vitais para o

funcionamento do organismo e entre suas funções ele proporciona a neutralização de inúmeras substâncias tóxicas, tornando-o o órgão mais afetado por diversas doenças (YANG et al., 2013). Portanto, combinando o conhecimento sobre plantas medicinais e polissacarídeos biologicamente ativos, podemos investigar novos compostos que sejam capazes de exercer a proteção hepática sem causar disfunções metabólicas no órgão.

Entre as atividades biológicas promovidas por polissacarídeos, uma com baixa abordagem na literatura é a ação sobre o sistema muscular esquelético. Este sistema, quando submetido à estímulos ou treinamentos, sofre adaptações fenotípicas e metabólicas que desempenham papel primordial no rendimento esportivo de atletas e pessoas fisicamente ativas. Neste contexto, existe o interesse na identificação de compostos que possam acelerar a adaptação muscular sem provocar efeitos colaterais significativos (BERRIDGE; BOLON; HERMAN, 2018; POWERS; HOWLEY, 2004). Embora a ação dos polissacarídeos sobre a fadiga já tenha sido investigada (KLOSTERHOFF et al., 2018; WANG et al., 2010; WEI et al., 2017), não foram encontrados trabalhos que analisassem os efeitos destas moléculas nas alterações morfofuncionais do músculo esquelético.

2. REVISÃO BIBLIOGRÁFICA

2.1 PLANTAS DE USO MEDICINAL

A humanidade comumente utiliza plantas no tratamento de enfermidades e seus sintomas, isto devido a facilidade para obtê-las e principalmente pela propagação do conhecimento de seus benefícios através das gerações (ROSSATO et al., 2012). Desde tempos primórdios muitas culturas utilizam plantas para a fabricação de medicamentos, empregando partes ou o vegetal inteiro como matéria prima (ODY, 1993). Nos tempos atuais, espécies vegetais ainda têm desempenhado um papel fundamental na saúde humana, pois mesmo havendo grandes avanços na medicina e na difusão do acesso a fármacos industrializados, existem populações que dependem muito ou exclusivamente destas espécies no tratamento de doenças (AKERELE, 1993).

Preparados brutos elaborados de maneira simples ou complexa (com uma ou mais espécies vegetais) que são utilizadas no tratamento de doenças e seus sintomas, são denominados fitoterápicos. Estes preparados contêm como ingredientes ativos partes ou derivados de vegetais no estado bruto ou processado, além de solventes ou conservantes como excipientes. Fitoterápicos são medicamentos feitos à base de plantas, assim sua utilização terapêutica é de fácil e ampla aceitação pela população, mesmo que frequentemente os princípios ativos responsáveis pela sua ação farmacológica sejam desconhecidos (VANACLOTXA; FOLCARA, 2003).

Os fitoterápicos tiveram sua relevância e interesse aumentados de forma expressiva com o avanço da ciência. É estimado que cerca de 25% de todos os medicamentos comercializados no último século são direta ou indiretamente derivados de vegetais. Ainda, formulações à base de plantas medicinais são comumente populares em países com tradição em sua utilização, como China e Japão e também em alguns países desenvolvidos onde existem legislações específicas para o registo e comercialização de tais formulações, como Alemanha, França e Estados Unidos (CALIXTO, 2000).

No Brasil a utilização de fitoterápicos pela população é difundida e de amplo alcance, desta maneira o Ministério da Saúde divulgou em fevereiro de 2009 a Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS), na qual estão listadas 71 espécies vegetais voltadas para uso na atenção básica à saúde, com indicações para enfermidades e instruções para o preparado a partir destas espécies. A RENISUS, além de auxiliar na

utilização pública das plantas medicinais, visa estimular estudos científicos e pesquisas que sejam base para a elaboração de uma lista de plantas medicinais e fitoterápicos que tenham sua eficácia comprovada para serem disponibilizados à população (BRASIL, 2009). Ambas as espécies de interesse nesta tese, *Baccharis trimera* Less. DC e *Mikania glomerata* Spreng, estão listadas na RENISUS com várias indicações terapêuticas e suas respectivas instruções para consumo.

2.1.1 *Mikania glomerata*

Mikania glomerata Spreng (FIGURA 1) é uma espécie vegetal conhecida popularmente como guaco, cipó-almecega-cabeludo, cipó-catinga, cipó-sucuriçu, coração-de-jesus, erva-cobre, erva-das-serpentes, erva-de-cobra, erva-de-sapo, guaco-de-cheiro, guaco-liso, guaco-trepador e uaco (LORENZI; MATOS, 2008). Em suas características taxonômicas é uma trepadeira sub-lenhosa, de grande porte, perene, com folhas obtusas na base de forma quase deltoide, de cor verde escura e semitorcida, com três nervuras destacadas, carnosas-coriáceas, presas duas a duas ao longo dos ramos volúveis e tem flores reunidas em capítulos congestos resultando em frutos do tipo aquênio. É uma espécie nativa do sul do Brasil, mas devido a popularidade dos seus usos terapêuticos vem sendo cultivada em várias outras regiões. Esta planta medicinal é utilizada na medicina popular do sul do país há muitas gerações, sendo que entre seus usos populares estão, uso como tônico, depurativa, febrífuga, antigripal, estimulante de apetite, para inflamações de boca e garganta, dores reumáticas, como bronco dilatadora, antitussígena e expectorante (BRASIL, 2009; GEMTCHÚJNICOV, 1976; LORENZI; MATOS, 2008). Existe um conhecimento difundido entre a população, que faz uso terapêutico da espécie, que *Mikania glomerata* é contra indicada para pessoas com disfunções hepáticas, porém não foi encontrado nenhum estudo científico indicando a veracidade desta informação (GRANDI, 2014).



FIGURA 1 - ILUSTRAÇÃO DE UM ESPÉCIME DE *Mikania glomerata* Spreng (GRANDI, 2014).

Dentre os constituintes químicos identificados na espécie, há relatos que as cumarinas são os compostos de maior evidência, sendo a 1,2-benzopirona considerada o marcador químico do vegetal (GASPARETTO et al., 2010), porém um trabalho realizado por Melo e colaboradores (2015) não observou a presença destes compostos nos extratos da espécie. Mesmo havendo esta incongruência sobre as cumarinas, elas são relatadas de modo costumeiro nos extratos provenientes de *M. glomerata*, além da presença de outros fitoquímicos como diterpenos, diterpenos ácidos, triterpenos, esteróides, heterósidos flavônicos, lactonas sesquiterpênicas, taninos, derivados do ácido cinâmico e saponinas (OLIVEIRA et al., 1984; VENEZIANI; OLIVEIRA, 1999). No infuso, os principais componentes descritos são as cumarinas e o ácido *O*-cumárico (MAIORANO et al., 2005). A descrição dos polissacarídeos presentes no infuso produzido a partir da espécie ainda não foi relatada.

Quanto às atividades biológicas, extratos aquosos e/ou com solventes orgânicos provenientes da espécie apresentaram os efeitos: analgésico (RUPPELT et al., 1991); anti-hemorrágico (MOURAO et al., 2014); anti-inflamatório (POSSEBON et al., 2018); antialérgico (FIERRO et al., 1999); antidiarreico (SALGADO; RONCARI; MOREIRA, 2005); antiofídico (MAIORANO et al., 2005); antiproliferativo (DALLA NORA et al.,

2010); bactericida (MOREIRA et al., 2016; MORETI et al., 2017); broncodilatador (MOURA et al., 2002) e leishmanicida (LUIZE et al., 2005).

2.1.2 *Baccharis trimera*

Baccharis trimera (Less.) DC (FIGURA 2) é uma espécie vegetal que possui como sinonímia *Molina trimera* Less. e *Baccharis genistelloides* var. *trimera* (Less.) Baker. Ela é conhecida popularmente por carqueja, bacanta, bacórida, cacaia-amarga, cacália-amara, *B. trimera*, *B. trimera*-amarga, *B. trimera*-amargosa, *B. trimera*-graúda, *B. trimera*-verdadeira, quina-de-condamine, tiririca-de-babado, tiririca-de-bêbado, vassoura, vassoura-de-botão, vassoureira e vassourinha (LORENZI; MATOS, 2008). Em suas características taxonômicas é um subarbusto perene, ereto, muito ramificado na base, de caules e ramos verdes com expansões triadas e tem de 50-80 cm de altura. É uma espécie nativa do sul e sudeste do Brasil, principalmente dos altos campos, amplamente utilizada na medicina popular. Entre os usos populares estão o tratamento de esterilidade feminina, da impotência masculina, uso como tônico amargo, febrífuga, vermífuga, para distúrbios do estômago e intestinais, tratamento de úlceras, diabetes, malária, anginas, anemia, diarréias, garganta e principalmente para disfunções hepáticas (BARROSO, 1976; BRASIL, 2009; LORENZI; MATOS, 2008; SOUSA et al., 1991). Esta espécie é uma das mais estudadas dentro do gênero *Baccharis*, assim muitos dos constituintes químicos presentes no vegetal já foram descritos. Nas partes aéreas foram identificados α e β -pinenos, canfeno, carquejol, acetato de carquejila, flavonas eupatorina, lactonas diterpenoides do tipo trans e tetra-nor clerodano, sesquiterpenos, geranilgeraniol, eupatrina, cirsimarina, cirsilol, apigenina, genkwanina, eriodictiol, hispidulina, campferol, quercetina e estigmasterol (MARTINEZ; BESSA; BENITO, 2005; MORS et al., 2000; SOUSA et al., 1991). Nas raízes, diésteres terpênicos e esqualeno. No óleo essencial, carquejol, acetato de carquejila, ledol, β -pineno, álcoois sesquiterpênicos, sesquiterpenos bi e tricíclicos, δ e γ -cadileno, calameno, elemol, eudemol, palustrol, canfeno e nerolidol (MARTINEZ; BESSA; BENITO, 2005; MORS et al., 2000; SOUSA et al., 1991). No infuso, os principais componentes observados foram flavonoides, ácidos fenólicos, terpenos e derivados do ácido cafeico (ABAD; BERMEJO, 2007; RABELO et al., 2017; SILVEIRA RABELO; CALDEIRA COSTA, 2018b). Mesmo tendo diversos componentes descritos, os polissacarídeos presentes no infuso produzido a partir da espécie ainda não foram relatados.



FIGURA 2 - ILUSTRAÇÃO DE UM ESPÉCIME DE *Baccharis trimera* (Less.) DC (GRANDI, 2014).

Quanto as atividades biológicas, extratos aquosos e/ou com solventes orgânicos provenientes da espécie apresentaram os efeitos: anti-adipogênico (NASCIMENTO et al., 2017); anti-artrite (COELHO et al., 2004); anti-hemorragico (JANUÁRIO et al., 2004); anti-helmíntico (OLIVEIRA et al., 2014); anti-inflamatório e analgésico (GENE et al., 1996); antimicrobiano (SILVA et al., 2018); antimutagênico (NAKASUGI; KOMAI, 1998); antioxidante (SABIR et al., 2017); antissecretor (BIONDO et al., 2011); antiviral (ABAD et al., 1999); gastroprotetor (LÍVERO et al., 2016a); hepatoprotetor (LÍVERO et al., 2016b; SOICKE; LENG-PESCHLOW, 1987); hipoglicêmico (OLIVEIRA et al., 2005); leishmanicida (LUIZE et al., 2005) e relaxante vascular (TORRES et al., 2000).

2.2 POLISSACARÍDEOS DE ORIGEM VEGETAL

Os vegetais têm seus constituintes químicos divididos em compostos primários e secundários. Os primários são os componentes essenciais para o crescimento e desenvolvimento do vegetal e os secundários são componentes biossinteticamente derivados dos compostos primários que fornecem vantagens ecológicas ao organismo sem, aparentemente, fazer parte do metabolismo primário do vegetal (BALANDRIN et al., 1985). Dentro dos constituintes definidos como compostos primários encontram-se os carboidratos, sendo estes observados como monossacarídeos, dissacarídeos, oligossacarídeos, polissacarídeos e seus derivados e classificados, de acordo com a sua função, em estruturais ou de reserva (AVIGAD; DEY, 1997).

Os carboidratos estruturais compõem a parede celular, assim formam o alicerce do vegetal promovendo sua estruturação e suporte. Eles são, depois da água, os principais constituintes químicos de quase todas as células e tecidos vegetais. Em consequência, os carboidratos estruturais de todas as plantas formam a mais abundante reserva de compostos naturais disponíveis na Terra e são claramente o mais importante recurso natural renovável (REID, 1997).

A parede celular vegetal é um invólucro que está presente em praticamente todas as células do vegetal, ela é uma estrutura dinâmica cujas características morfológicas, configuração e propriedades são remodeladas de modo contínuo ao longo do desenvolvimento celular. Este remodelamento acontece em resposta ao crescimento e diferenciação do vegetal, função celular e ao ambiente que o vegetal está inserido (CARPITA; GIBEAUT, 1993). Este invólucro em células vegetais jovens é denominado como parede celular primária, pois nesta fase é uma camada delgada e elástica, sendo de fundamental importância no processo de crescimento celular. Na fase final do crescimento e desenvolvimento celular ocorre a formação da parede celular secundária, esta é de maior espessura, força e resistência conferindo rigidez e forma aos tecidos vegetais (ALBERSHEIM et al., 1996). Em ambos os tipos de parede celular, suas estruturações são altamente organizadas, constituídas por complexas moléculas como proteínas, substâncias aromáticas e polissacarídeos. Dentre estas moléculas os polissacarídeos são os que aparecem em maior abundância, constituindo cerca de 90% de toda a estrutura (CARPITA; MCCANN, 2002).

Os polissacarídeos presentes na parede celular vegetal são divididos em pectinas, hemiceluloses e celulose e podem ser encontrados em varias proporções. Nas dicotiledôneas a parede celular primária é, geralmente, composta de 25-40% celulose, 15-25% hemiceluloses, 15-40% pectinas, 5-10% proteínas e proporções muito pequenas de compostos fenólicos (FIGURA 3). Já a parede celular secundária, muito mais espessa, consiste de 40-45% de celulose, 15-35% de hemiceluloses, 15-30% de lignina e quantidades mínimas de pectinas (DEY; BROWNLEADER; HARBORNE, 1997).

Os carboidratos de reserva são acumulados em tecidos diferenciados do vegetal com a função de servirem como substrato energético e metabólico durante o crescimento e desenvolvimento vegetal. Eles geralmente são observados como moléculas simples, sendo a quantidade e localização no organismo diferenciada conforme a espécie vegetal (AVIGAD; DEY, 1997).

Os principais representantes dos carboidratos de reserva são a sacarose, o amido e as frutanas. Eles desempenham um papel fundamental na biossíntese metabólica vegetal, além de poderem atuar na proteção contra o estresse hídrico e termal do organismo (AVIGAD; DEY, 1997).

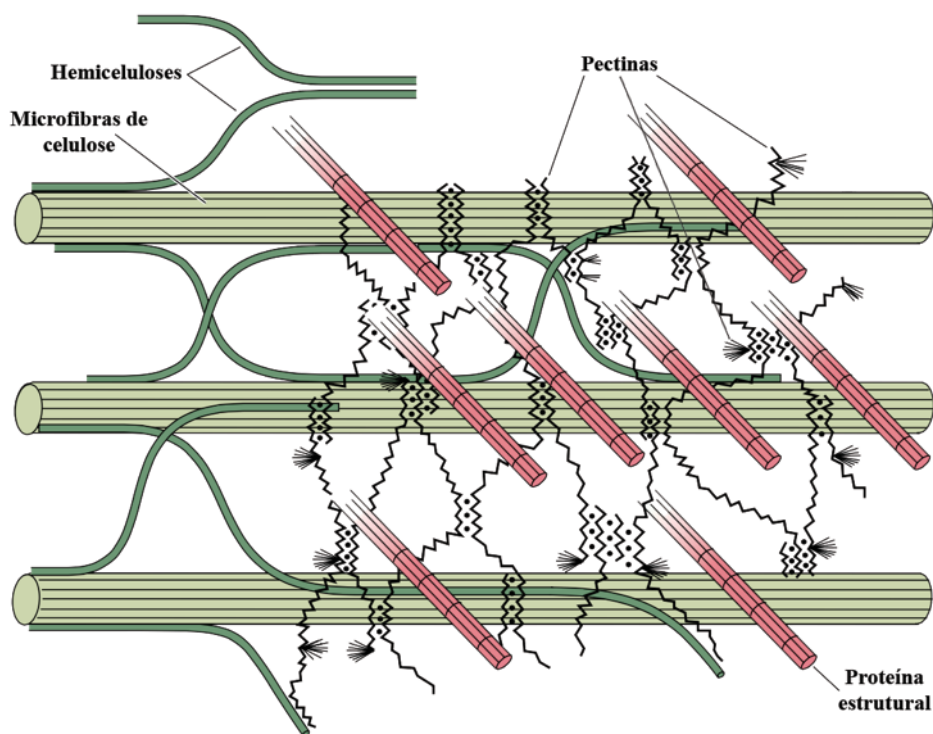


FIGURA 3 - ESQUEMA DA PAREDE CELULAR PRIMÁRIA ADAPTADO DE BRETT E WALDRON (1996).

2.2.1 Frutanas

As frutanas são, depois do amido, os polímeros de reserva mais abundantes encontrados em espécies vegetais. Junto com a função de reserva podem se envolver em processos ligados a proteção física do vegetal, como a tolerância ao estresse (hídrico e térmico) e a osmorregulação (AVIGAD; DEY, 1997; HENDRY, 1987). Estes polímeros são constituídos por unidades de frutose e são geralmente lineares, porém em alguns casos podem apresentar um baixo grau de ramificação e conter unidades de glucose em sua estrutura (RAO et al., 2011). A composição da cadeia, usualmente, varia entre 2 a 50 unidades de frutose, sendo possível observar em determinadas espécies vegetais composições na ordem de 200 unidades. A extremidade inicial é ocupada de modo frequente por uma unidade de α -D-glucose unida por uma ligação do tipo (1 $\rightarrow$ 2) (POLLOCK; CHATTERTON, 1988). Os polímeros compostos de até 10 unidades de frutose são de maneira difusa denominados frutooligossacarídeos (FOS) ou oligofrutoses (HAULY; MOSCATTO, 2002).

Na configuração de sua estrutura, as frutanas podem apresentar diferentes tipos de ligações entre suas unidades monoméricas e de acordo com o tipo de ligação são classificadas em inulina, levana, levana mista, inulina neoseries e levana neoseries (FIGURA 4). Nas inulinas a estrutura do polímero é linear, e são formadas por unidades de D-frutose ligadas β -(2 $\rightarrow$ 1), sendo a unidade inicial do polímero uma α -D-glucose. As levanas se diferem das inulinas somente pelo tipo de ligação, onde as unidades de D-frutose são ligadas β -(2 $\rightarrow$ 6). As levanas mistas apresentam na estrutura do polímero ligações β -(2 $\rightarrow$ 1) e β -(2 $\rightarrow$ 6) entre as unidades de D-frutose e podem ser ramificadas, onde as ramificações podem apresentar as mesmas características da cadeia principal. Nas inulinas neoseries, verifica-se o mesmo tipo de ligação da inulina, no entanto esta possui a unidade de α -D-glucose dentro da cadeia do polímero, apresentando então cadeias de D-frutose em ambas as extremidades da α -D-glucose (nFru-Glc-nFru). As levanas neoseries se diferem das inulinas neoseries somente pelo tipo de ligação, onde as unidades de D-frutose são ligadas β -(2 $\rightarrow$ 6) (VIJN; SMEEKENS, 1999).

Ocasionalmente as frutanas lineares são encontradas com a unidade de α -D-glucose terminal ausente, tornando-as moléculas redutoras. Para estas moléculas os termos inulo-*n*-oses e levan-*n*-oses podem ser utilizados (VAN LAERE; VAN DEN ENDE, 2002).

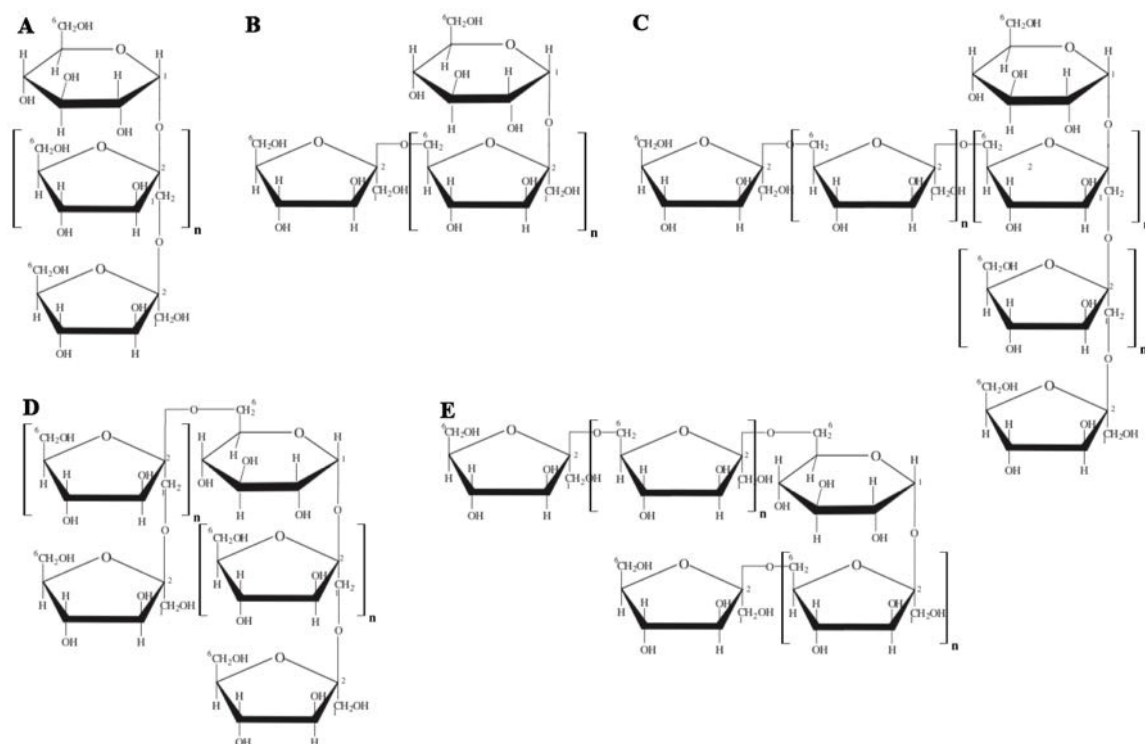


FIGURA 4 - EXEMPLOS DE ESTRUTURAS DE FRUTANAS. INULINA (A), LEVANA (B), LEVANA MISTA (C), INULINA NEOSERIES (D) E LEVANA NEOSERIES (E).

2.2.2 Pectinas

As pectinas são polissacarídeos localizados na parede celular vegetal. As estruturas destas moléculas são observadas em diferentes configurações conforme o tecido e a espécie vegetal, assim torna-se um grupo heterogêneo de polissacarídeos (REID, 1997; TAIZ; ZEIGER, 2010). São polímeros complexos que possuem como principais constituintes os monossacarídeos: ácido galacturônico, ramnose, arabinose e galactose. A presença de unidades de ácido galacturônico em sua estrutura é uma das principais características do grupo das pectinas. Além de serem os polissacarídeos mais solúveis entre os que constituem a parede celular vegetal, podendo ser facilmente extraído com água quente ou quelantes de cálcio (ASPINALL, 1980; BRETT; WALDRON, 1996).

Pectinas possuem alta massa molar e estão localizadas em maior proporção na lamela média da parede celular, onde são cruciais para a adesão celular. Na parede celular primária, elas contribuem para a retenção de água e formação de géis, atuando principalmente em suas propriedades mecânicas. Estas características fazem com que as pectinas tenham influência

no crescimento, na morfologia, no desenvolvimento e também na defesa das espécies vegetais (CARPITA; MCCANN, 2002; COSGROVE, 2005).

Os polissacarídeos que fundamentam o grupo das pectinas são as homogalacturonanas, as ramnogalacturonanas do tipo I e as ramnogalacturonanas do tipo II (FIGURA 5). Outros polímeros como arabinanas, galactanas e/ou arabinogalactanas são frequentemente encontradas em frações pécicas, uma vez que podem constituir as ramificações das ramnogalacturonanas do tipo I (CARPITA; GIBEAUT, 1993; TAIZ; ZEIGER, 2010).

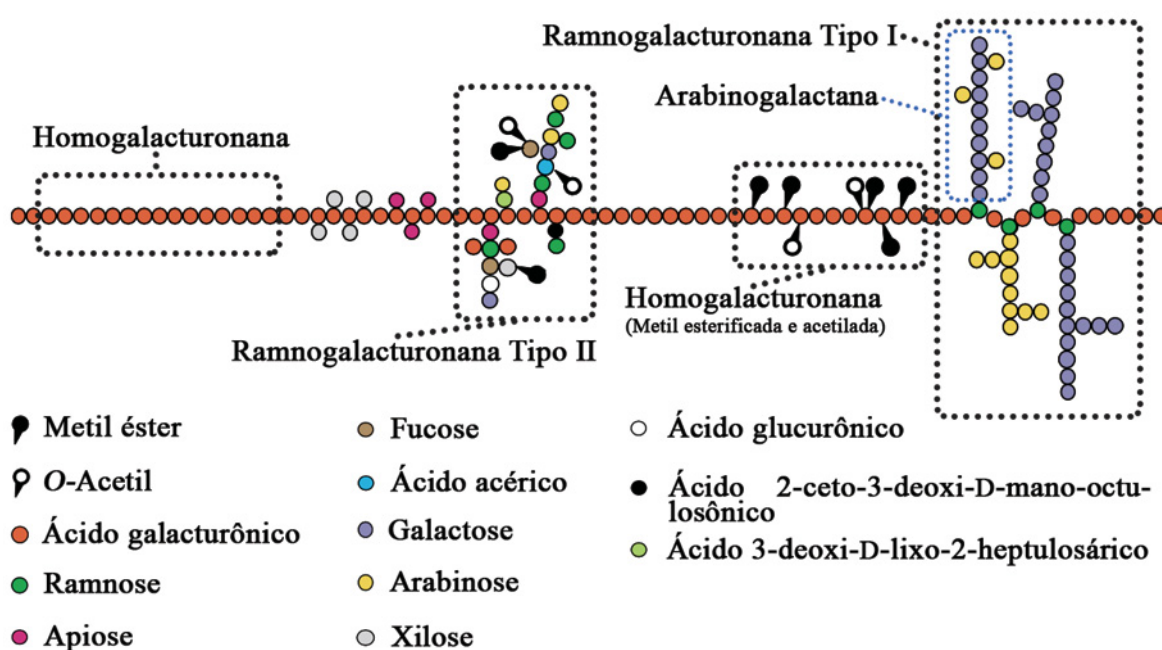


FIGURA 5 - ILUSTRAÇÃO ESQUEMÁTICA DA ESTRUTURA DAS PECTINAS ADAPTADO DE WILLATS E COLABORADORES (2006).

As homogalacturonanas, também denominadas de ácidos poligalacturônicos, são polímeros homogêneos e lineares formados por unidades de ácido D-galacturônico (GalA) ligados α -(1 $\rightarrow$ 4). Elas podem conter em torno de 200 unidades monoméricas e alcançar até 100 nm de comprimento (CARPITA; GIBEAUT, 1993). Algumas das unidades de GalA que constituem estas pectinas podem ser metil-esterificadas e dependendo da espécie vegetal também podem estar parcialmente O-acetiladas. A relação entre o total de unidades GalA com a de unidades GalA metil-esterificadas no polissacarídeo é expressa como grau de esterificação (DE) e de acordo com o DE, as pectinas são divididas em dois grupos: pectinas com alto teor de metil-esterificação (HM – High methoxyl) que possuem DE superior a 50%

e pectinas com baixo teor de esterificação (LM – Low methoxyl) que possuem DE inferior a 50% (RIDLEY; O'NEILL; MOHNEN, 2001; SILVA; RAO, 2006).

Ramnogalacturonanas do tipo I são polímeros altamente ramificados, constituídos por uma cadeia principal alternada entre unidades de ácido D-galacturônico ligados α -(1 $\rightarrow$ 4) e L-ramnose ligados α -(1 $\rightarrow$ 2), onde se ligam cadeias de polímeros e monossacarídeos neutros. As ramificações podem ser de homopolissacarídeos e heteropolissacarídeos, tais como arabinanas, galactanas e arabinogalactanas e também unidades monoméricas distintas, como a D-xilose, D-glucose, D-manose, L-fucose e ácido D-glucurônico (CARPITA; GIBEAUT, 1993; SILVA; RAO, 2006; VORAGEN et al., 1995).

As arabinogalactanas podem constituir as ramificações das ramnogalacturonanas do tipo I, porem com frequência são identificadas de maneira isolada, assim podem ser considerados polissacarídeos pécticos. Conforme a configuração de sua estrutura estes polímeros são classificados em dois grupos: as arabinogalactanas do tipo I que são constituídas por uma cadeia linear formada por unidades piranosídicas de D-galactose ligadas β -(1 $\rightarrow$ 4) com ramificações curtas na posição O-3, formadas por 20-40% de unidades furanosídicas de L-arabinose ligadas α -(1 $\rightarrow$ 5) e as arabinogalactanas do tipo II que são constituídas por uma cadeia linear formada de unidades piranosídicas de D-galactose ligadas β -(1 $\rightarrow$ 3) ou D-galactose ligadas β -(1 $\rightarrow$ 3) e (1 $\rightarrow$ 6) com ramificações nas posições O-3 e/ou O-6, formadas por unidades furanosídicas de L-arabinose ligadas α -(1 $\rightarrow$ 3) e α -(1 $\rightarrow$ 5). Ainda, nas arabinogalactanas do tipo II as ramificações podem conter unidades de ácido D-glucurônico e/ou 4-O-metil-D-glucurônico em sua estrutura (ASPINALL, 1969; CARPITA; GIBEAUT, 1993; CHESSON, 2006; VORAGEN et al., 1995).

Ramnogalacturonanas do tipo II compõem um grupo extremamente heterogêneo. Os polissacarídeos pertencentes a este grupo são pequenos, contudo são os mais complexos dentro das pectinas. São formados em geral por uma cadeia principal contendo 7-10 unidades de ácido D-galacturônico ligados α -(1 $\rightarrow$ 4), com ramificações heteropoliméricas inseridas nas posições O-2 e/ou O-3. Estas ramificações podem conter cerca de 12 monossacarídeos e 20 ligações diferentes, sendo os monossacarídeos mais comuns a L-ramnose, L-arabinose, D-galactose e ácido D-glucurônico. Neste grupo também pode ser identificada a presença de carboidratos raros como, por exemplo, apiose, ácido acérico, ácido 2-ceto-3-deoxi-D-manooctulosônico, ácido 3-deoxi-D-lixo-2-heptulosárico e açúcares metilados (2-O-metil-L-fucose e 2-O-metil-D-xilose) (CARPITA; GIBEAUT, 1993; VINCKEN et al., 2003; YORK et al., 1985).

2.2.3 Atividades Biológicas

Os polissacarídeos isolados a partir de fontes vegetais são utilizados ao longo de décadas em diversas aplicações industriais e através de um atual aumento no interesse científico, com mais e detalhadas investigações, está sendo possível observar a importância destes polímeros dentro do grupo de moléculas bioativas (LIU; WILLFÖR; XU, 2015). Eles se destacam pois além de serem abundantes são considerados mais seguros, possuindo poucos efeitos adversos e baixa toxicidade, principalmente quando comparados com polissacarídeos derivados de fontes não vegetais ou sintéticos (RAMBERG; NELSON; SINNOTT, 2010).

Estas moléculas já foram testadas em uma alta gama de atividades biológicas, e inúmeros trabalhos científicos relatam resultados positivos (LIU; WILLFÖR; XU, 2015). Estes efeitos biológicos são fortemente afetados pelos parâmetros estruturais dos polímeros, como composição monossacarídica, configuração das ligações glicosídicas, quantidade e tamanho das ramificações e massa molar (YANG; ZHANG, 2009).

Muitos destes trabalhos científicos foram desenvolvidos buscando elucidar as características e atividades biológicas de polissacarídeos pécicos e frutanas. Dentre estes estão alguns exemplos como: homogalacturonanas com atividade anti-inflamatória, antiúlcera e gastroprotetora (CIPRIANI et al., 2009a; OLIVEIRA et al., 2017, 2018); ramnogalacturonanas tipo I com atividade anticâncer, antifatiga e gastroprotetora (ADAMI et al., 2018; KLOSTERHOFF et al., 2018; NASCIMENTO et al., 2013); arabinogalactanas com atividade antialérgica, antidiabética, antinocicepitiva e gastroprotetora (CIPRIANI et al., 2009b; DO NASCIMENTO et al., 2015; PETERS et al., 2010; WANG et al., 2015); ramnogalacturonanas tipo II com atividade antiploriferativa de células de adenocarcinoma (AI et al., 2018) e inulinas com atividades anti-influenza, anticâncer, antioxidante, antiúlcera, hepatoprotetora, imunomoduladora e prébiotica (AUSTARHEIM et al., 2012; CALEFFI et al., 2015; CHANDRASHEKAR; PRASHANTH; VENKATESH, 2011; COOPER; CARTER, 1986; CORRÊA-FERREIRA et al., 2017; LEE et al., 2012; LIU et al., 2015).

2.3 HEPATOTOXICIDADE INDUZIDA POR TETRACLORETO DE CARBONO

O fígado é o principal órgão humano associado ao metabolismo biológico e desempenha um papel fundamental para desintoxicação de substâncias nocivas, endógenas e exógenas (YANG et al., 2013). A exposição a níveis elevados destas toxinas pode gerar diversas disfunções metabólicas neste órgão, que variam desde a alteração transitória nas enzimas até lesões e hepatocarcinomas (CHAO et al., 2009; WANG et al., 2016). As disfunções hepáticas podem ser geradas por varias fontes, tais como infecções, ingestão abusiva de drogas, distúrbios autoimunes, efeito adverso de fármacos ou substâncias tóxicas (CHAO et al., 2009; HAN et al., 2016). Dentre as substâncias que apresentam hepatotoxicidade, o tetracloreto de carbono (CCl_4) se destaca por ser um dos mais conhecidos e estudados (RECKNAGEL, 1967).

O CCl_4 em seu estado puro é um líquido incolor, volátil, neutro e de densidade maior que a água. Este composto, no passado, foi amplamente utilizado no tratamento contra helmintíases veterinárias e foi proposto seu uso em humanos, até a descoberta de sua capacidade hepatotóxica (MCVAIL, 1922). A sua ação tóxica acontece através de sua desalogenação redutiva catalisada pelo citocromo P-450 no retículo endoplasmático dos hepatócitos. Esse evento pode levar a uma cascata de mecanismos secundários que são responsáveis pela ruptura da membrana plasmática e morte da célula (RECKNAGEL et al., 1989). Um dos mecanismos aceitos é através da produção de radicais triclorometil ($\text{CCl}_3^{\bullet}$) pelo sistema oxigenase do citocromo P450 microsomal. Esse radical reage com espécies reativas de oxigênio (ROS) produzindo radicais peróxi triclorometil ($\text{CCl}_3\text{OO}^{\bullet}$) que vão se ligar a macromoléculas, desencadeando os danos às membranas hepatocelulares (RECKNAGEL, 1967; RECKNAGEL et al., 1989).

O efeito tóxico e mecanismos de toxicidade conhecidos do CCl_4 , o tornam amplamente utilizado em modelos biológicos *in vivo* que buscam avaliar e desenvolver compostos que possam proteger o fígado contra lesões e disfunções (MCGILL; JAESCHKE, 2018). Nestes modelos geralmente é utilizado o tratamento prévio e/ou em conjunto da substância de interesse com a indução da lesão hepática. Assim é possível medir com clareza a eficiência dos tratamentos utilizando marcadores enzimáticos da lesão, como as enzimas alanina transaminase, aspartato transaminase e fosfatase alcalina (MCGILL; JAESCHKE, 2018; OZER et al., 2008).

A hepatotoxicidade induzida por tetracloreto de carbono já foi utilizada para avaliar a capacidade dos polissacarídeos provenientes de espécies vegetais em proteger o fígado contra lesões. Efeitos positivos foram observados em frações polissacarídicas extraídas de *Amomum villosum* (ZHANG et al., 2013), *Anoectochilus roxburghii* (ZENG et al., 2016), *Astragalus membranaceus* (HAMID et al., 2017), *Atractylodes macrocephalae* (HAN et al., 2016), *Camellia sinensis* (WANG et al., 2014), *Ilex latifolia* (FAN et al., 2014), *Lycium barbarum* (XIAO et al., 2012), *Malus domestica* (YANG et al., 2013), *Ziziphus jujuba* (YUE et al., 2014) e em moléculas purificadas como uma arabinogalactana isolada de *Glycine max* (SUN et al., 2018) e uma frutana isolada de *Artemisia vulgaris* (CORRÊA-FERREIRA et al., 2017).

2.4 ALTERAÇÕES CONTRÁTEIS NO MÚSCULO ESQUELÉTICO

O músculo esquelético (ME) é um tecido singular cujo papel principal é produzir movimento, proteger órgãos vitais e gerar calor. A massa muscular esquelética pode, conforme o organismo, constituir cerca de 50% do peso corporal total e possuir cerca de 700 músculos individuais (BERRIDGE; BOLON; HERMAN, 2018; NAYAK et al., 2015).

A maioria dos músculos tem suas extremidades presas aos ossos através de tendões. O movimento destes é produzido através da ação individual das células, que produzem atividade no músculo inteiro, encurtando seu comprimento e tracionando os tendões. Esse processo é iniciado e controlado por sinalização, através de potenciais de ação, originados no sistema nervoso motor (NAYAK et al., 2015).

A conformação dos MEs é determinada por um arranjo específico de fibras musculares e tecido conectivo associado, sendo sua função estabelecida pelo formato do músculo, pelo ângulo de penação das fibras, pelo volume de sarcômeros e pela proporção do tipo de fibras (FRONTERA; OCHALA, 2015). Existem três tipos principais de fibras musculares, tipo 1, tipo 2A e tipo 2B, cuja determinação é realizada pela isoforma da Miosina de Cadeia Pesada (MHC) nos filamentos contráteis. Estes tipos são encontrados em proporções variáveis no sistema muscular e a maioria dos músculos contém uma mistura dos tipos em diferentes proporções. O tipo 1, também denominado fibra oxidativa lenta, apresenta velocidade de contração e de relaxamento lenta e baixa atividade ATPásica da MHC. Estas células tem uma elevada razão superfície/volume, grande suprimento capilar para troca eficiente de gases, grande quantidade de mitocôndrias e mioglobina, o que torna

a fibra de coloração vermelha. Os músculos que tem predominância das fibras do tipo I são envolvidos em atividades estáveis e contínuas, pois são altamente resistentes à fadiga. Estas fibras são predominantes em atletas com alta capacidade de endurance, como maratonistas. O tipo 2A, também denominado de fibra oxidativa-glicolítica rápida, são intermediárias entre as fibras 1 e 2B. Apresentam velocidade de contração rápida e relaxamento lento além de alta atividade ATPásica da MHC. Como as fibras do tipo 1, as do tipo 2A tem alta razão superfície/volume para troca eficiente de gases, possuem mitocôndrias, mioglobina, o que lhes confere resistência à fadiga, e também enzimas do metabolismo glicolítico para produção de ATP. Estas fibras são altamente adaptáveis ao treinamento e são as fibras que tem a maior capacidade de promover melhoras no desempenho atlético. O tipo 2B, também denominado fibra glicolítica rápida, apresenta alta velocidade de contração e de relaxamento, alta atividade ATPásica da MHC, baixa relação superfície/volume, possuem grande volume de enzimas glicolíticas, poucas mitocôndrias, pouca mioglobina e baixo suprimento de capilares. Os músculos que tem predominância das fibras do tipo 2B possuem a menor taxa de troca de gases e remoção de produtos residuais entre as fibras musculares e possuem predominância do metabolismo energético glicolítico o que as confere coloração branca. É neste tipo de fibra que o ATP pode ser gerado pela metabolização anaeróbica de glicose em ácido láctico. São recrutadas apenas para breves esforços máximos, assim são facilmente fatigáveis. São as fibras predominantes em atletas com capacidade de produzir muita força e potência por períodos curtos de tempo, como levantadores de peso (NAYAK et al., 2015; POWERS; HOWLEY, 2004; RADÁK, 2018).

O ME é um tecido com grande capacidade adaptativa a estímulos externos. Ele pode sofrer alterações na isoforma e o volume das fibras, no volume de mitocôndrias e na quantidade de enzimas de vias metabólicas para otimizar a produção e o consumo de energia e atender as demandas estruturais, funcionais e metabólicas impostas ao organismo. As adaptações induzidas pelo exercício são específicas para cada tipo de treinamento, que pode ser dividido em dois tipos: treinamento de resistência (*endurance*) e treinamento de força (intervalado). Conforme o treinamento, a estimulação de vias de sinalização celular será modificada alterando expressão gênica de proteínas específicas. As vias de adaptação muscular podem ainda serem influenciadas pela ingestão de nutrientes ou compostos reguladores (FRONTERA; OCHALA, 2015; MARGOLIS; RIVAS, 2015).

No presente trabalho a metodologia de treinamento selecionado foi o treinamento de *endurance* (CHEN et al., 2015; KLOSTERHOFF et al., 2018; YEH et al., 2014). O

treinamento é realizado em uma intensidade que permita ao praticante sustentar a atividade por períodos prolongados sem gerar fadiga. A adaptação gerada irá depender de alterações como conversão de fibras glicolíticas em isoformas oxidativas, aumento do volume e densidade de mitocôndrias, aumento da taxa de *turnover* das proteínas do aparato contrátil e das proteínas mitocondriais, além de adaptações sistêmicas como alteração hormonal, alteração da resposta imune e da permeabilidade intestinal, mudanças no equilíbrio metabólico, de fluídos e temperatura, redução dos estoques de glicogênio e aumento nos estoques de lipídios intramusculares e alterações cardiovasculares (FRONTERA; OCHALA, 2015; MACH; FUSTER-BOTELLA, 2017; MARGOLIS; RIVAS, 2015; POWERS; HOWLEY, 2004; SEENE; KAASIK; ALEV, 2011).

A adaptação ao exercício de *endurance* e o aumento da performance atlética é tema de relevância tanto para atletas quanto para pessoas fisicamente ativas. Esses indivíduos utilizam, além de desgastantes sessões de treinamento, dietas personalizadas e/ou administração de fármacos para o aumento na eficiência à adaptação (POWERS; HOWLEY, 2004). Na literatura existe grande acervo de trabalhos que mostram a influência da ingestão de suplementos e fármacos no desempenho e nas alterações relacionadas ao exercício de *endurance*, porém poucos investigaram a influência de polissacarídeos nestes fatores.

Os trabalhos encontrados relataram que polissacarídeos provenientes de fontes vegetais como das espécies *Astragalus membranaceus* (YEH et al., 2014), *Camellia sinensis* (CHI et al., 2015), *Dendrobium officinale* (WEI et al., 2017), *Gynostemma pentaphyllum* (CHI et al., 2008), *Hippophae rhamnoides* (NI et al., 2013), *Lepidium meyenii* (LI et al., 2017), *Lycium barbarum* (NI et al., 2013), *Lycium ruthenicum* (NI et al., 2013), *Malpighia emarginata* (KLOSTERHOFF et al., 2018), *Millettia speciosa* (ZHAO et al., 2015), *Morinda officinalis* (ZHANG et al., 2009), *Nitraria tangutorum* (NI et al., 2013), *Panax ginseng* (WANG et al., 2010) e *Zea mays* (ZHAO et al., 2017) conseguem promover um aumento no desempenho físico de animais, retardando a fadiga, embora os mecanismos de ação sejam desconhecidos. Polissacarídeos também foram capazes de proteger os músculos de animais contra os efeitos do dano oxidativo muscular promovidos por treinamentos intensos, como os provenientes de *Lycium barbarum* (NIU et al., 2008) e *Ganoderma lucidum* (ZHONGHUI; XIAOWEI; FANG, 2014).

3. OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar estruturalmente os carboidratos presentes nos infusos obtidos a partir de *Mikania glomerata* Spreng e de *Baccharis trimera* Less. DC, avaliar seus efeitos no fígado *per se* e em modelo de hepatotoxicidade induzida por tetracloreto de carbono (CCl₄) e avaliar o efeito de polissacarídeos nas características contráteis de músculos esqueléticos de camundongos.

3.2 OBJETIVOS ESPECÍFICOS

- Isolar os carboidratos presentes nos infusos de *M. glomerata* e *B. trimera*.
- Purificar os polissacarídeos.
- Caracterizar a estrutura química fina dos polissacarídeos purificados.
- Investigar os efeitos das frações de carboidratos *in vivo* no fígado *per se* e utilizando o modelo de hepatotoxicidade induzida por CCl₄ em camundongos.
- Investigar o efeito de uma fração polissacarídica nas características contráteis de músculos esqueléticos de camundongos.

4. ARTIGOS CIENTÍFICOS

4.1 ARTIGO I

CHEMICAL CHARACTERIZATION OF CARBOHYDRATES FROM *Mikania glomerata* SPRENG INFUSION AND ITS HEPATOPROTECTIVE EFFECTS

Pedro Felipe Pereira Chaves^a, Eliana Rezende Adami^b, Claudia Rita Corso^b, Letícia Milani^b, Natalia Mulinari Turin Oliveira^b, Liziane Cristine Malaquias da Silva^b, Alexandra Acco^b, Marcello Iacomini^a, Lucimara Mach Côrtes Cordeiro^{a*}

^a *Biochemistry and Molecular Biology Department, Federal University of Paraná, CP 19.046, CEP 81.531-980 Curitiba, PR, Brazil.*

^b *Pharmacology Department, Federal University of Paraná, CEP 81.531-980 Curitiba, PR, Brazil*

ABSTRACT

Plant species which have therapeutic purposes are countless, among them is *Mikania glomerata* Spreng. This plant belongs to the Asteraceae family and is native of the south of Brazil, where its infusion is widely used in folk medicine. Since carbohydrates can present diverse biological effects, the aim of this study was to chemically characterize the carbohydrate content of the *M. glomerata* infusion and evaluate its effects on liver function in mice. Carbohydrates were accessed by ethanolic precipitation. After purification, an inulin type fructan polysaccharide (SMG) was characterized. Fructooligosaccharides ranging from GF2 (m/z 543) to GF10 (m/z 1839) were observed in the ethanolic supernatant (MG-ET). Both fractions showed low amounts of protein, phenolic compounds and flavonoids and high amounts of total carbohydrate. *In vitro* results showed DPPH radical scavenging capacity for both fractions. *In vivo* results indicate that the fractions did not produce alterations in the main parameters analyzed *per se* and, in addition, could attenuate the alterations in alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), catalase (CAT), lipid peroxidation (LPO) and reduced glutathione (GSH) caused by CCl₄ administration. Thus, *Mikania glomerata* infusion shows high carbohydrate content with inulin and fructooligosaccharides as main components. The tested fractions did not produce a hepatotoxic effect *per se* and protected the liver against CCl₄-induced injuries.

Keywords: Inulin; fructooligosaccharides; hepatoprotection; antioxidant; *Mikania glomerata*; Asteraceae.

1. INTRODUCTION

The therapeutic use of medicinal plants is explored by people from virtually all countries and for the population of underdeveloped countries this appliance can be the only resource for treating numerous symptoms and diseases (AKERELE, 1993). The utilization of medicinal plants is widespread in Brazil, where people have easy access to several species. This results from the popular knowledge propagation and from the scientific interest increase in phytomedicine (ROSSATO et al., 2012). In this context the Asteraceae family plays an important role since it is highly diverse in species that show relevant pharmacological effects (BESSADA; BARREIRA; OLIVEIRA, 2015; MEDEIROS-NEVES; TEIXEIRA; VON POSER, 2018; TOYANG; VERPOORTE, 2013).

Mikania glomerata Spreng, commonly known as “guaco”, belongs to the Asteraceae family and is a native plant from south of Brazil (LORENZI; MATOS, 2008; MORETI et al., 2017). The infusion of this species is generally used in folk medicine as depurative, antipyretic, anti-influenza, appetite stimulant, anti-inflammatory, analgesic, anti-allergic, bronchial dilator, antitussive and expectorant (LORENZI; MATOS, 2008). Some of the related activities for the species infusion have been confirmed by scientific studies, such as anti-inflammatory and analgesic (RUPPELT et al., 1991) and bronchial dilatator (MOURA et al., 2002). Apart, there is a folk belief that *M. glomerata* infusion and also decoction is contraindicated for people with hepatic dysfunction, but for our concern there is no literature data justifying that (GRANDI, 2014).

Phytochemical studies of organic or hydroalcoholic extracts from *M. glomerata* identified several coumarins, diterpenes, diterpene acids, triterpenes, steroids, flavonic heterosides, sesquiterpene lactones, cinnamic acid derivatives, saponins and essential oils (GASPARETTO et al., 2010; NAPIMOGA; YATSUDA, 2010). The pharmacological effects exhibited by the species are attributed to some of these compounds, but is not known whether other molecules, such as polysaccharides, are contributing to these effects.

Polysaccharides from different sources can produce various of the biological effects reported for *M. glomerata*, such as anti-influenza (LEE et al., 2012), anti-inflammatory (BEZERRA et al., 2018), analgesic (LEIVAS et al., 2016), anti-allergic (PETERS et al., 2010), antitussive (CHATTOPADHYAY et al., 2011), and also promote an hepatic antioxidant effect (LIU et al., 2015) and hepatoprotection (CORRÊA-FERREIRA et al.,

2017). Thus, here we presented the structural characterization of oligo- and polysaccharides obtained from *M. glomerata* infusion and evaluated its effects on hepatic function *in vivo*.

2. MATERIALS AND METHODS

2.1. Plant material and infusion

Dried aerial parts of *Mikania glomerata* Spreng was bought in local commerce (Chamel Produtos Naturais Lot 10643 Nov/2015) and submitted to a manual separation to remove impurities. The plant material was stored in a sealed plastic container at room temperature until submitted to infusion.

The infusion was carried according to 10/2010 RDC of the Brazilian Government Agency (Agência Nacional de Vigilância Sanitária - ANVISA), that indicates the ideal preparation process of medicinal plants with therapeutically purposes. Plant material was placed in a beaker and boiling distilled water was added at a concentration of 20 g/L, the mixture was slightly stirred, closed and let it rest for approximately 30 min. The mixture obtained from infusion was filtered, yielding an extract and a residue. The extract was submitted to the isolation and fractionation of polysaccharides and the residue was discarded.

2.2. Isolation and fractionation of polysaccharides

The aqueous extract obtained from infusion was concentrated under reduced pressure and the polysaccharides precipitated with three volumes of ethanol 99%. The polysaccharides were collected by centrifugation ($8000 \times g$), dialyzed (Cellulose Spectrumlabs 6-8 kDa cut-off) and freeze-dried, giving fraction MG (FIGURE 1). The ethanolic supernatant was concentrated under reduced pressure and freeze-dried to give MG-ET fraction.

MG was submitted to freeze-thaw process (GORIN; IACOMINI, 1984) and centrifuged ($8000 \times g$), giving cold-water soluble (SMG) and insoluble (PMG) fractions. The yields were calculated based on the weight of initial freeze-dried plant material (461 g).

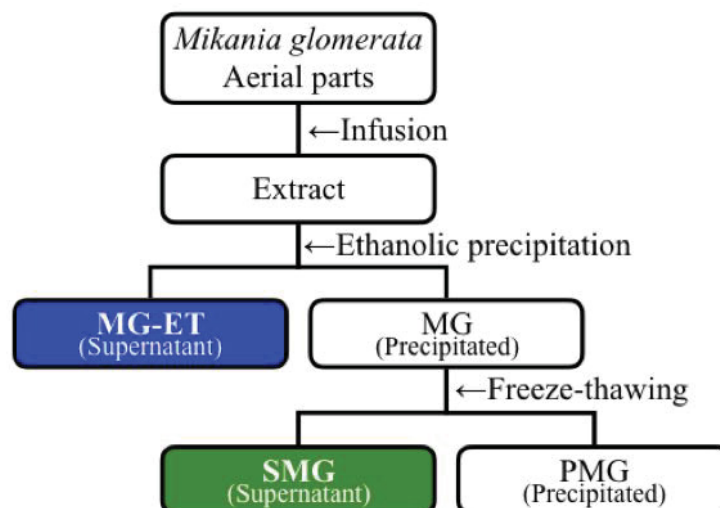


FIGURE 1 - SCHEME OF EXTRACTION AND FRACTIONATION OF POLYSACCHARIDES OBTAINED FROM *Mikania glomerata* SPRENG AERIAL PARTS.

2.3 Determination of homogeneity and relative molecular weight

SMG was solubilized in the eluent (0.1 M aq. NaNO_2 containing 200 ppm aq. NaN_3) at concentration of 1 mg/mL for determination of homogeneity. The sample was filtered through a membrane 0.22 μm (Millipore) and injected (250 μL loop) in a high-performance steric exclusion chromatograph (HPSEC) equipped with four columns in series, with exclusion sizes of 7106 Da (Ultrasphere 2000, Waters), 4105 Da (Ultrasphere 500, Waters), 8104 Da (Ultrasphere 250, Waters) and 5103 Da (Ultrasphere 120, Waters) and a differential refractometer (Waters). The elution volume was at 0.6 mL/min. To obtain the relative M_w , standard dextrans (72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa and 5.0 kDa, from Sigma) were employed to obtain the calibration curve. The relative M_w of the sample was calculated according to the calibration curve.

2.4 Determination of monosaccharide composition

SMG (~2 mg) was hydrolyzed in 500 μL 0.5 M TFA at 80 $^\circ\text{C}$ for 30 min. The TFA was evaporated and the sample was converted to alditol acetates by successive NaBH_4 reduction and acetylation with Ac_2O -pyridine (1:1, v/v, 1 mL) at room temperature for 16 h (WOLFROM; THOMPSON, 1963a, 1963b). The resulting alditol acetates were then extracted with CHCl_3 and were analyzed by GC-MS using a Varian 3800 gas chromatograph

coupled to a Varian Ion-Trap 2000R mass spectrometer (Varian, Palo Alto, CA). The column was DB-225MS (30 m $\times$ 0.25 mm i.d.; Agilent Santa Clara, CA) programmed from 50 to 220 °C at 40 °C/min, with helium as carrier gas, at a flow rate of 1 mL/min. The inlet temperature was 250 °C, and the MS transfer line was set at 250 °C. MS acquisition parameters included scanning from m/z 50 to 550 in electron ionization mode (EI) at 70 eV. Components were identified by their retention times and EI spectra.

The uronic acid contents were determined using the modified *m*-hydroxybiphenyl colorimetric method (FILISETTI-COZZI; CARPITA, 1991), using galacturonic acid as standard.

2.5 Determination of total carbohydrate content

The fractions were analyzed for the total carbohydrate content using the phenol-sulfuric acid method (DUBOIS et al., 1956). The standard curve was made with the predominant monosaccharides in the samples (fructose and arabinose 4:1) and the reading was performed at the wavelength of 485 nm.

2.6 Determination of protein, flavonoid and phenolic content

Protein content was determined according to the Bradford method with bovine serum albumin (BSA) as the standard (BRADFORD, 1976). Phenolic content was estimated by the Folin-Ciocalteu method with gallic acid as the standard (SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS, 1999). Flavonoid content was estimated by the $AlCl_3$ method with rutin as the standard (QUETTIER-DELEU et al., 2000). The results of flavonoid and phenolic content were expressed in mg standard/100 g freeze-dried fraction by comparison with standards treated in the same conditions.

2.7 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR and HSQC spectra were acquired at 70 °C on a AVANCE III 400 NMR spectrometer (Bruker), operating at 9.5 T, observing ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The samples were acquired in D_2O

with chemical shifts expressed as δ ppm, using the resonances of CH_3 groups of acetone (δ_{C} 30.2/ δ_{H} 2.22) as internal reference. All pulse programs were supplied by Bruker.

2.8 Thin Layer Chromatography

Thin layer chromatography (TLC) analysis was performed on a 60G silica gel plate (Merck) with 10 cm of total height. Aqueous solution of fructose (1 mg/mL), sucrose (1 mg/mL), a previously characterized sample, containing fructose, sucrose and fructoligosaccharides from *Chamomilla recutita* Less DC (CHAVES; IACOMINI; CORDEIRO, 2019) (2 mg/mL) and MG-ET (2 mg/mL) were applied spaced 0.5 cm. 6:1 (v/v) *n*-propanol and water was used as eluent and orcinol solution (0.2 g of orcinol in 95 mL of ethanol and 5 mL of sulfuric acid) as developer (SASSAKI et al., 2008).

2.9 Mass spectrometry

MG-ET was solubilized in methanol/water 1:1 (200 $\mu\text{g/mL}$) and directly infused into the mass spectrometer using a syringe pump (5 $\mu\text{L/min}$). The positive high-resolution mass spectroscopy analysis was carried out by electrospray ionization (ESI) at atmospheric pressure ionization (API) in an LTQ OrbiTrap XL (Thermo-Scientific). Nitrogen was used for sample desolvation with sheath gas at a flow rate of 8 AU and auxiliary gas at 2 AU with a source temperature of 300 °C. The ionization was performed following the operational parameters: electrospray voltage at 4 kV, capillary voltage 25 V, tube lens offset 125 V. The spectra were processed and analysed with Thermo Xcalibur 1.0.0.42 software.

2.10 In vitro determination of free radical scavenging activity

Fractions were evaluated for their scavenging activity against the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). This method was adapted from Chen, Wu and Chen (2004). Briefly, samples at different concentrations (3, 10, 30, 100, and 300 $\mu\text{g/mL}$) were mixed with DPPH methanolic solution (10 $\mu\text{g/mL}$). Ascorbic acid (50 $\mu\text{g/mL}$) and deionized water were used as positive and negative controls, respectively. Absorbance was read at 517 nm. The assay was performed in triplicate.

2.11 Animals

Experiments were conducted using female Swiss mice (*Mus musculus*, 25–35 g) from the Federal University of Paraná vivarium. Animals were kept under standard laboratory conditions (12 h light/dark cycles, temperature 22 ± 2 °C) with food and water provided *ad libitum* and acclimatized to the laboratory for at least 48 h before testing. The experiments were performed after approval of the respective protocols by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO – UFPR; approval number 1000). The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NATIONAL RESEARCH COUNCIL (US) COMMITTEE FOR THE UPDATE OF THE GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS, 2011).

2.12 In vivo assays

To investigate if the obtained fractions could damage the liver *per se*, the liver function of mice treated with the fractions was evaluated. The animals were separated into following experimental groups (7 animals per group): a) Control, treated using distilled water; b) SMG, treated using the SMG fraction (100 mg/kg) and c) MG-ET, treated using the MG-ET fraction (10 mg/kg). The treatments were performed orally (gavage) daily for 7 consecutive days and on the day after the last treatment (8th day), the animals were anesthetized and material for the analysis were collected.

To investigate the hepatoprotective effects of the obtained fractions, the carbon tetrachloride (CCl₄)-induced hepatotoxicity model was applied. The animals were separated into following experimental groups (7 animals per group): a) CCl₄, treated with distilled water; b) SIL (positive control), treated with silymarin (100 mg/kg); c) SMG, treated with the SMG fraction (1 mg/kg) and d) MG-ET treated using the MG-ET fraction (10 mg/kg). The treatments were performed orally (gavage) once a day for 7 consecutive days. On the 6th and 7th days of treatment, hepatotoxicity was induced by administering CCl₄ (2% in corn oil, 5 mL/kg) intraperitoneally (i.p) in all groups and after approximately 24 h (8th day), the animals were anesthetized and biological material for further analysis were collected (CORRÊA-FERREIRA et al., 2017).

In both assays the animals were anesthetized with 100 mg/kg ketamine hydrochloride +10 mg/kg xylazine hydrochloride, i.p, samples of blood were collected with heparinized syringes from the abdominal cava vein and, after the euthanasia, liver samples were immediately collected, weighed, frozen (liquid nitrogen) and stored at -80°C for further analysis. The absolute and relative weighing of spleen, kidneys and lung also was checked.

The doses of the fractions used in the CCl_4 -induced hepatotoxicity model were the same that found in a 200 mL cup of infusion and ingested by a individual of 70 kg (1 mg/kg), considering the preparation of infusion according to 10/2010 RDC resolution. Since the SMG fraction is mainly composed of a plant polysaccharide, which is largely known having low toxicity, we opted to 100-fold rise its dose (100 mg/kg) in the *per se* assay.

2.13 Plasmatic analysis

The blood samples were centrifuged at $3000 \times g$ for 5 min. The plasma was analyzed for the ALT, AST and ALP, urea and creatinine levels using commercial kits (Kovalent, São Gonçalo, Brazil) in a Mindray BS-200 (Shenzhen, China) automated device.

2.14 Hepatic oxidative stress measurements

Liver samples were thawed, homogenized in phosphate-buffered saline (PBS; pH 6.5) and centrifuged (10,000 g, 4°C , 20 min). The supernatant was used to measure the enzymatic activities of CAT (AEBI, 1984), and GST (HABIG; PABST; JAKOBY, 1974). The LPO (JIANG; WOOLLARD; WOLFF, 1991) and GSH (SEDLAK; LINDSAY, 1968) levels were also measured from supernatant. The results of CAT, GST and LPO are expressed based in the amount of protein in the liver samples, determined using the Bradford (1976) method with bovine serum albumin (BSA) as the standard. The results of GSH are expressed based in the amount of the liver tissue analyzed.

2.15 Statistical analysis

Data from each group were expressed as mean $\pm$ standard error of the mean. Statistical analyzes were performed using one-way ANOVA followed by the Bonferroni

post-hoc test using the Prism 7.0c software (Graphpad), and $p < 0.05$ was considered significant.

3. RESULTS

3.1 Isolation and characterization of fractions

Polysaccharides were recovered from infusion by ethanolic precipitation, giving fraction (MG) with 1.9% yield. The supernatant containing the low molar mass molecules (MG-ET fraction) was obtained in higher yield (13.1%, FIGURE 1). The single process of freeze-thaw fractionation was carried out, to get the cold-water soluble portion of the MG fraction, with 0.9% yield (SMG fraction).

The SMG and MG-ET were submitted to total carbohydrate, protein, phenolic and flavonoid content analyses (TABLE 1). Both fractions showed low amounts of protein, phenolic compounds and flavonoids. SMG showed higher amounts of total carbohydrate than MG-ET. SMG was then analyzed by HPSEC and presented a homogeneous peak with relative M_w of 6 kDa (FIGURE 2). Its monosaccharide analysis revealed that fructose (97%) was the predominant monosaccharide, together with small amount of arabinose (3%), suggesting the presence of a fructan in the fraction.

TABLE 1 - MAJOR COMPONENTS ANALYSIS OF *Mikania glomerata* FRACTIONS

Fractions	Total carbohydrate (%)	Protein (%)	Phenolic compounds (mg/100 g) ^a	Flavonoids (mg/100 g) ^b
SMG	95.1	4.4	1.2	0.1
MG-ET	35.4	1.1	0.8	0.1

^aGallic acid equivalent. ^b Rutine equivalent.

Proceeding with the characterization process, SMG was submitted to NMR analysis. Its ¹³C NMR spectrum showed signals at δ 60.8 (C-1), δ 103.0 (C-2), δ 77.0 (C-3), δ 74.3 (C-4), δ 80.9 (C-5) and δ 62.0 (C-6) assigned to $\rightarrow$ 1)- β -D-Fruf-(2 $\rightarrow$ units (FIGURE 3). Thus, it is possible to observe that SMG is mainly composed of an inulin-type fructan.

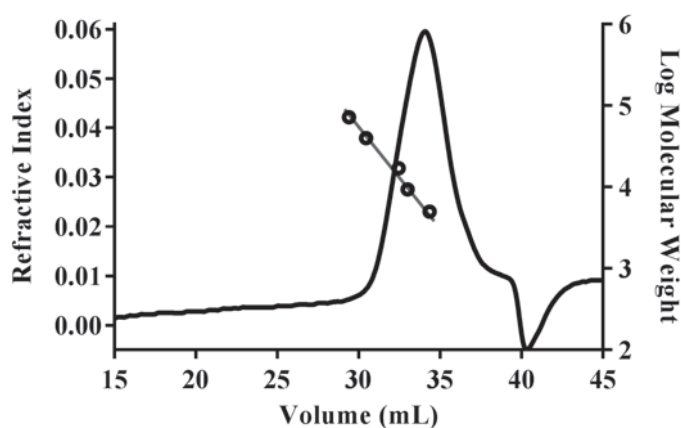


FIGURE 2 - HPSEC ELUTION PROFILE OF SMG FRACTION AND ELUTION VOLUMES OF DEXTRAN STANDARDS (M_w 72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa AND 5 kDa, LEFT TO RIGHT).

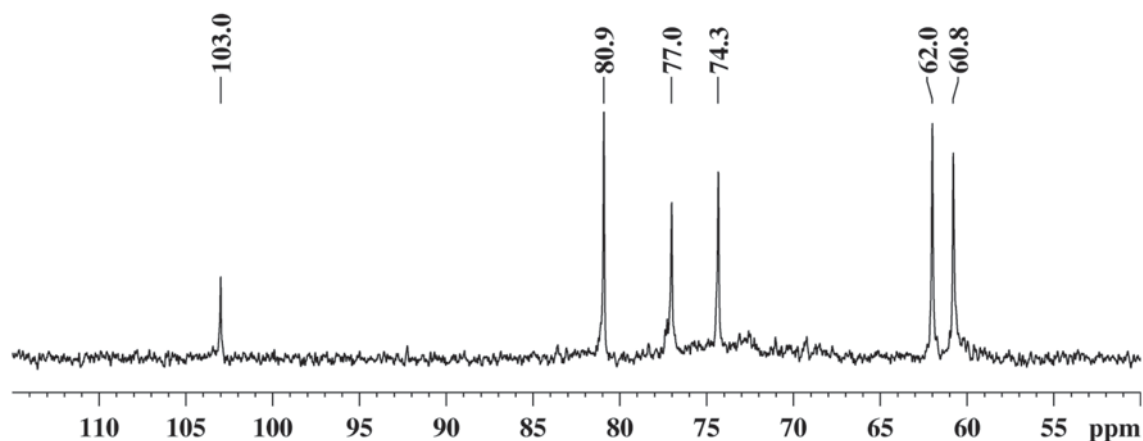


FIGURE 3 - ^{13}C NMR SPECTRUM OF FRACTION SMG IN D_2O AT 70 °C. CHEMICAL SHIFTS ARE EXPRESSED AS δ PPM.

Looking for the presence of fructooligosaccharides (FOS) in the ethanolic supernatant, the MG-ET was submitted to a TLC analysis (FIGURE 4). The analysis showed the presence of bands for MG-ET at same retention factor (R_F) that observed for the FOS containing fraction obtained from *Chamomila recutita* (CHAVES; IACOMINI; CORDEIRO, 2019) indicating the presence of FOS in MG-ET. Bands at same R_F for fructose and sucrose standards were also observed in MG-ET. To verify the degree of polymerization of FOS present in MG-ET, the fraction was submitted to mass spectrometry analysis (FIGURE 5). The spectrum showed ions with mass varying from 381 m/z to 1839 m/z. The first peak (381 m/z) match to sucrose ionized with potassium ion (39 m/z), which

is naturally present in the fraction, and the nine subsequent peaks have a mass difference of 162 m/z, which corresponds to the addition of a fructose residue. This result indicates that MG-ET contains FOS ranging from GF2 to GF10 fructose units (GF = Glucose + n fructose units).

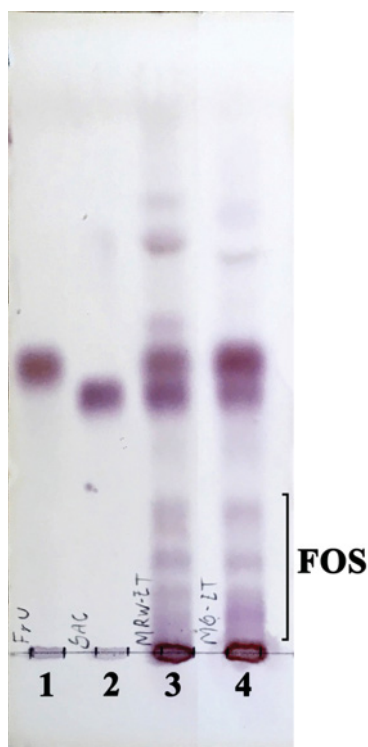


FIGURE 4 - THIN LAYER CHROMATOGRAPHY OF MG-ET FRACTION. FRUCTOSE (1), SUCROSE (2) AND A PREVIOUSLY CHARACTERIZED SAMPLE CONTAINING FRUTOOLIGOSACCHARIDES (3) AS STANDARDS AND MG-ET (4). n -PrOH:H₂O 6:1 WAS USED AS ELUENT AND ORCINOL-H₂SO₄ AS DEVELOPER.

3.2 Free radical scavenging activity

The first activity carried out was the ability of the fractions to sequester the DPPH *in vitro* (FIGURE 6). SMG had higher sequestration capacity than the negative control at all tested concentrations. At 300 μ g/mL, it was able to scavenge 98% of the free radicals, being 8% more effective than the positive control. MG-ET was less effective than SMG, and only at concentrations higher than 30 μ g/mL was significantly different from the vehicle, reaching 89% of scavenging activity at concentration 300 μ g/mL. The positive control (ascorbic acid) at 50 μ g/mL was able to scavenge 90% of the free radicals.

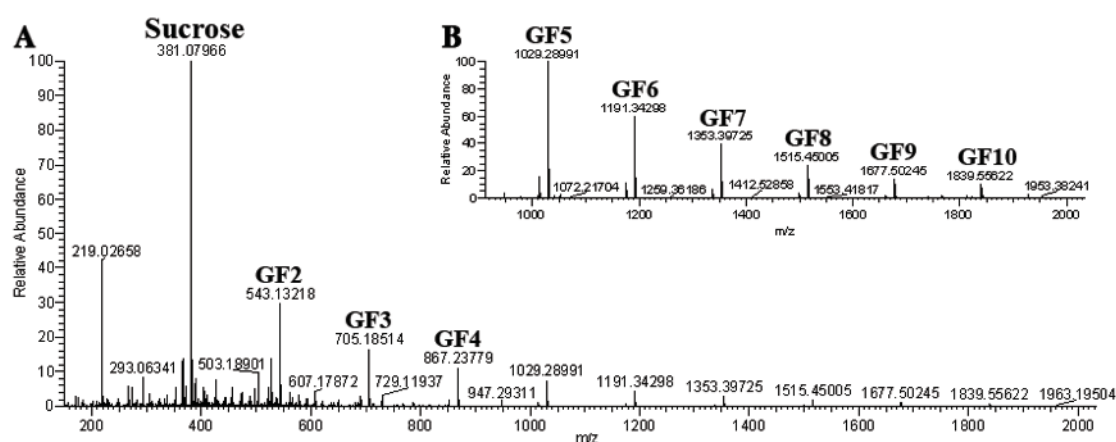


FIGURE 5 - POSITIVE HIGH-RESOLUTION MASS SPECTRUM OF MG-ET FRACTION. FULL SPECTRUM (A) AND ZOOM AT 1000-2000 m/z REGION (B).

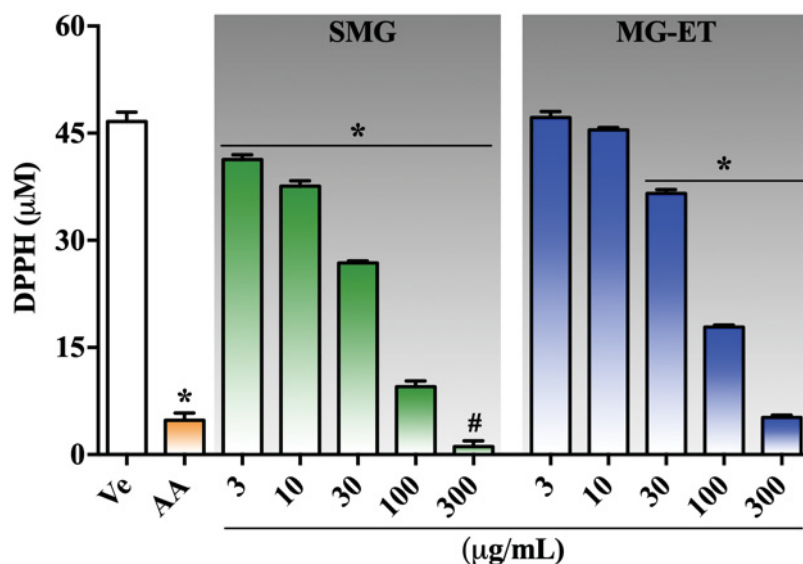


FIGURE 6 - *In vitro* ABILITY OF SMG AND MG-ET FRACTIONS TO SCAVENGE THE FREE-RADICAL DPPH. ASCORBIC ACID (AA, 50 mg/mL) WAS USED AS POSITIVE CONTROL AND DEIONIZED WATER (Ve) AS NEGATIVE CONTROL. VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI *post hoc* TEST. * $p < 0.05$ STATISTICALLY DIFFERENT FROM NEGATIVE CONTROL.

3.3 *In vivo* assays

Fractions obtained from *M. glomerata* infusion were evaluated about their potential to produce liver injury *per se*. Both SMG and MG-ET treated groups did not differ from the control group in ALT, AST and ALP plasma levels (FIGURE 7 A, C, E), as well as in the CAT and GST activity and lipoperoxidation in the liver (FIGURE 8 A, B, D). Both treated

groups showed increased level of hepatic GSH level by 96% and 65% respectively, when compared with control group (FIGURE 8 C). SMG and MG-ET also increase the plasma levels of urea in 28% and 32% respectively, when compared with control group (TABLE 2). All other analyzed parameters in the liver (TABLE 2) as well as the body and organs weight (TABLE 3) of treated groups did not differ from the control group.

To evaluate the hepatoprotective effect of the fractions, the CCl₄-induced hepatotoxicity assay was performed. As expected, the acute administration of the hepatotoxin CCl₄ promoted a great increase in the plasma levels of hepatic enzymes ALT, AST and ALP, when compared to control group (FIGURE 7 B, D, F). In the liver oxidative stress parameters, the CCl₄ caused a decrease in the enzymatic activity of CAT (FIGURE 9 A) and an increase in the lipoperoxidation level (FIGURE 9 B). The GSH content and the activity of GST in the liver were not altered by CCl₄ administration (FIGURE 9 C, D). The CCl₄ also promoted an increase of blood levels of hemoglobin, a change in hematocrit and MCV and promote an increase of liver relative weight (TABLE 2 and TABLE 3).

The pretreatment with SMG and MG-ET reduced the plasma levels of ALT, AST and ALP by 42%, 53%, 88%, and 59%, 58% and 77%, respectively, when compared with the CCl₄ group, reaching the control levels for AST and ALP (Fig 7 B, D and F). In the hepatic oxidative stress parameters, both treatments restored the activity of CAT and decreased the LPO levels similar to control values (FIGURE 9 A, B), and caused an 108% increase in the liver level of GSH (FIGURE 9 C). The activity of GST was not altered by the treatments (FIGURE 9 D). In the hematological analysis and also organs weight results, the pretreatment with SMG and MG-ET could not attenuate the effects of CCl₄ (TABLE 3). SMG also caused an increase in the number of lymphocytes when compared with control and CCl₄ treated-group. The SMG and MG-ET treated groups displayed the same results that the positive control group SIL (silymarin) with no significative differences.

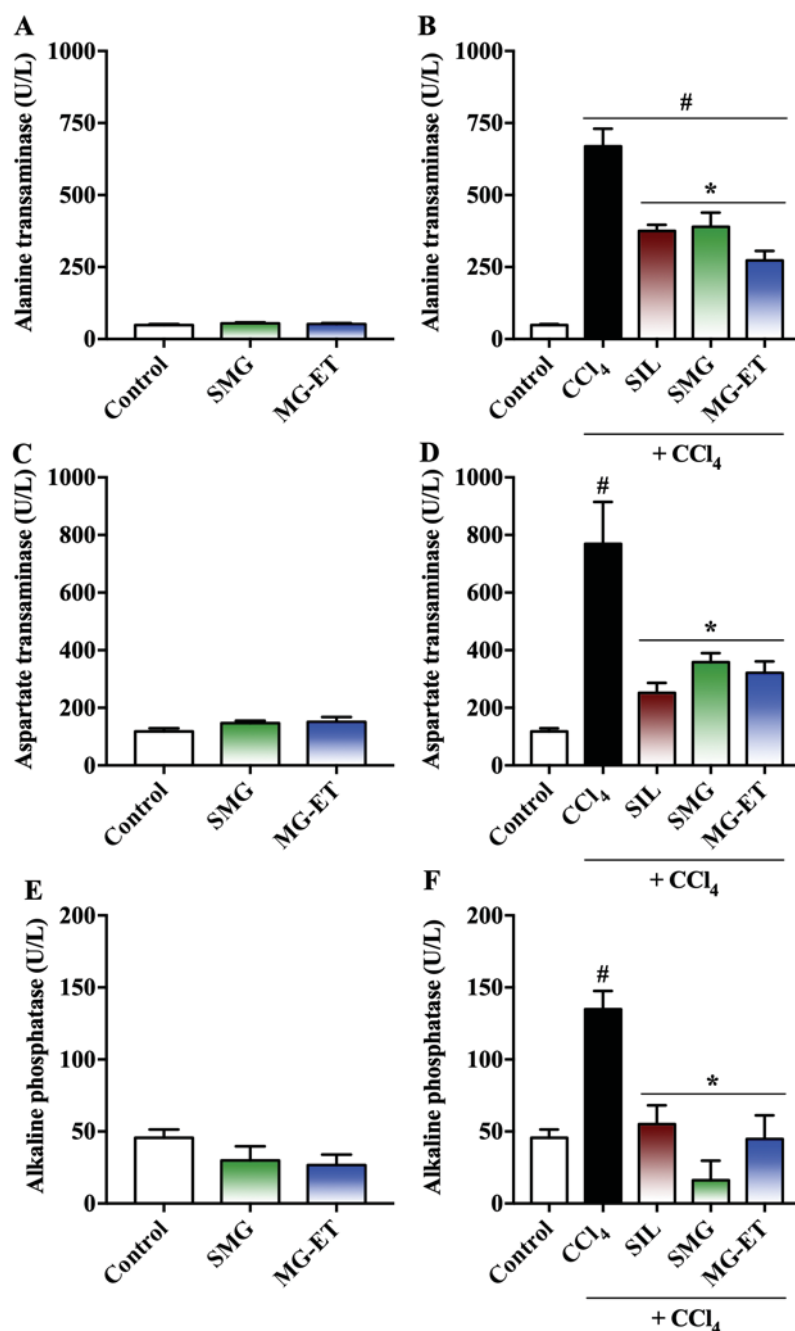


FIGURE 7 - PLASMA BIOCHEMICAL PARAMETERS IN *per se* TREATED MICE (A, C AND E) AND IN CCl₄-INDUCED HEPATIC INJURY MICE (B, D AND F) TREATED WITH VEHICLE, SMG OR MG-ET. ALT (A AND B), AST (C AND D) AND ALP (E AND F). *Per se* ASSAY GROUPS: CONTROL (ULTRAPURE WATER); SMG (100 mg/kg) AND MG-ET (10 mg/kg). CCl₄-INDUCED HEPATIC INJURY ASSAY GROUPS (n=7): CONTROL (ULTRAPURE WATER), CCl₄ (ULTRAPURE WATER + CCl₄); SIL (SILYMARIN 100 mg/kg + CCl₄); SMG (1 mg/kg + CCl₄); MG-ET (10 mg/kg + CCl₄). * $p < 0.05$ STATISTICALLY DIFFERENT FROM CCl₄ GROUP. # $p < 0.05$ STATISTICALLY DIFFERENT FROM Control. VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI *post hoc* TEST.

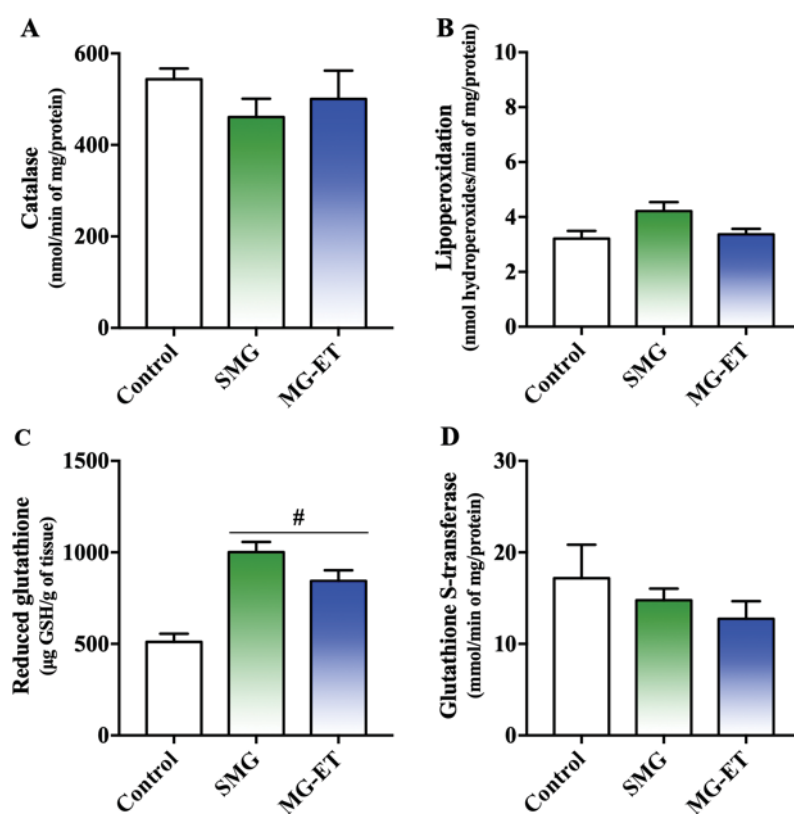


FIGURE 8 - MEASUREMENT OF HEPATIC OXIDATIVE STRESS PARAMETERS IN *per se* TREATED MICE WITH VEHICLE, SMG OR MG-ET. CAT (A), LPO (B), GSH (C) AND GST (D). GROUPS (n=7): CONTROL (ULTRAPURE WATER); SMG (100 mg/kg) AND MG-ET (10 mg/kg). # $p < 0.05$ STATISTICALLY DIFFERENT FROM Control. VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI *post hoc* TEST.

TABLE 2 - HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN *per se* TREATED MICE AND IN CCl₄-INDUCED HEPATIC INJURY MICE TREATED WITH VEHICLE, SMG OR MG-ET.

	<i>Per se</i>			<i>Carbon tetrachloride-induced</i>				
	Control	SMG (100 mg/kg)	MG-ET (10 mg/kg)	Control	CCl ₄	SIL (100 mg/kg)	SMG (1 mg/kg)	MG-ET (10 mg/kg)
Creatinine (mg/dL)	0.23 ± 0.03	0.29 ± 0.05	0.34 ± 0.07	0.28 ± 0.06	0.36 ± 0.02	0.41 ± 0.04	0.34 ± 0.06	0.49 ± 0.12
Urea (mg/dL)	40 ± 2.5	52 ± 2.1 [#]	54 ± 2.9 [#]	55 ± 4.5	42 ± 5.2	37 ± 2.6	44 ± 3.1	39 ± 2.0
WBC (x10 ³ /μL)	4.0 ± 0.93	5.5 ± 0.99	3.5 ± 0.43	4.5 ± 0.37	5.4 ± 0.57	4.1 ± 0.75	9.8 ± 0.59 ^{#*}	7.7 ± 1.10
Lymphocytes (x10 ³ /μL)	3.1 ± 0.8	4.1 ± 0.7	2.4 ± 0.3	3.1 ± 0.3	3.4 ± 0.54	2.9 ± 0.54	6.2 ± 0.5 ^{#*}	4.8 ± 0.6
Monocytes (x10 ³ /μL)	0.09 ± 0.03	0.2 ± 0.05	0.1 ± 0.02	0.21 ± 0.04	0.35 ± 0.07	0.17 ± 0.08	0.4 ± 0.03	0.36 ± 0.06
Granulocytes (x10 ³ /μL)	0.8 ± 0.13	1.2 ± 0.23	0.99 ± 0.13	1.2 ± 0.27	2.4 ± 0.49	1.1 ± 0.19	3.2 ± 0.28 [#]	2.6 ± 0.48
Lymphocytes (%)	73 ± 4.3	74 ± 2.1	68 ± 2.1	70 ± 5.2	60 ± 3.3	70 ± 1.4	63 ± 2.3	63 ± 2.6
Monocytes (%)	3.1 ± 0.31	3.5 ± 0.49	3.3 ± 0.21	4.6 ± 0.71	6.8 ± 0.9	2.5 ± 0.41 [*]	4.4 ± 0.21	4.7 ± 0.3
Granulocytes (%)	23 ± 4.1	23 ± 1.7	28 ± 2.0	26 ± 5	37 ± 4.2	26 ± 2.2	33 ± 2.2	33 ± 2.4
RBC (10 ⁶ /μL)	4.7 ± 0.75	6.9 ± 0.92	6.4 ± 0.82	8.4 ± 0.18	8.9 ± 0.16	9.1 ± 0.15	9.0 ± 0.16	9 ± 0.16
Hemoglobin (g/dL)	6.2 ± 1.1	9.3 ± 1.4	8.5 ± 1.2	11 ± 0.24	13 ± 0.16 [#]	12 ± 0.21 [#]	13 ± 0.16 [#]	12 ± 0.23 [#]
Hematocrit (%)	20 ± 3.6	31 ± 4.4	28 ± 3.7	35 ± 1	40 ± 0.79 [#]	40 ± 0.73 [#]	40 ± 0.66 [#]	39 ± 0.73 [#]

MCV (fL)	43 ± 0.84	44 ± 0.86	44 ± 0.62	14 ± 0.29	44 ± 0.73 [#]	44 ± 0.35 [#]	45 ± 0.56 [#]	44 ± 0.45 [#]
MCH (pg)	13 ± 0.26	13 ± 0.30	13 ± 0.35	14 ± 0.29	14 ± 0.15	14 ± 0.12	15 ± 0.14	14 ± 0.24
RDW (%)	14 ± 0.39	14 ± 0.58	14 ± 0.68	13 ± 0.40	15 ± 0.35	14 ± 0.54	14 ± 0.61	15 ± 0.43
Platelets (10 ³ /μL)	274 ± 44	338 ± 56	328 ± 48	390 ± 40	318 ± 26	275 ± 48	322 ± 30	367 ± 22

Per se assay groups: Control (ultrapure water); SMG (100 mg/kg) and MG-ET (10 mg/kg). CCl₄-induced hepatic injury assay groups: Control (ultrapure water), CCl₄ (ultrapure water + CCl₄); SIL (Silymarin 100mg/kg + CCl₄); SMG (1 mg/kg + CCl₄); MG-ET (10 mg/kg + CCl₄). (n=7). * p <0.05 statistically different from the CCl₄ group.
[#] p <0.05 statistically different from the Control group of respective assays. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni *post hoc* test.

TABLE 3 - BODY AND RELATIVE ORGANS WEIGHT FOR SMG AND MG-ET TREATED AND CONTROL GROUPS.

	<i>Per se</i>			<i>Carbon tetrachloride-induced</i>				
	Control	SMG (100 mg/kg)	MG-ET (10 mg/kg)	Control	CCl ₄	SIL (100 mg/kg)	SMG (1 mg/kg)	MG-ET (10 mg/kg)
Body (g)	27 ± 0.67	28 ± 0.67	28 ± 0.82	30 ± 0.70	28 ± 0.54	28 ± 0.36	28 ± 0.60	27 ± 0.71
Liver (%)	4.80 ± 0.15	5.00 ± 0.21	4.90 ± 0.22	4.4 ± 0.17	7.2 ± 0.27 [#]	6.80 ± 0.20 [#]	6.70 ± 0.24 [#]	6.50 ± 0.21 [#]
Kidney (%)	1.10 ± 0.04	1.10 ± 0.03	1.10 ± 0.03	1.40 ± 0.06	1.40 ± 0.07	1.20 ± 0.03	1.20 ± 0.05	1.20 ± 0.02
Spleen (%)	0.53 ± 0.06	0.46 ± 0.04	0.47 ± 0.03	0.41 ± 0.05	0.44 ± 0.03	0.48 ± 0.02	0.43 ± 0.05	0.45 ± 0.02
Lung (%)	0.69 ± 0.02	0.73 ± 0.05	0.69 ± 0.03	0.60 ± 0.02	0.70 ± 0.03	0.67 ± 0.02	0.68 ± 0.03	0.70 ± 0.03

Per se assay groups: Control (ultrapure water); SMG (100 mg/kg) and MG-ET (10 mg/kg). CCl₄-induced hepatic injury assay groups: Control (ultrapure water), CCl₄ (ultrapure water + CCl₄); SIL (Silymarin 100mg/kg + CCl₄); SMG (1 mg/kg + CCl₄); MG-ET (10 mg/kg + CCl₄). (n=7). * p <0.05 statistically different from the CCl₄ group.
[#] p <0.05 statistically different from the Control group of respective assays. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni *post hoc* test.

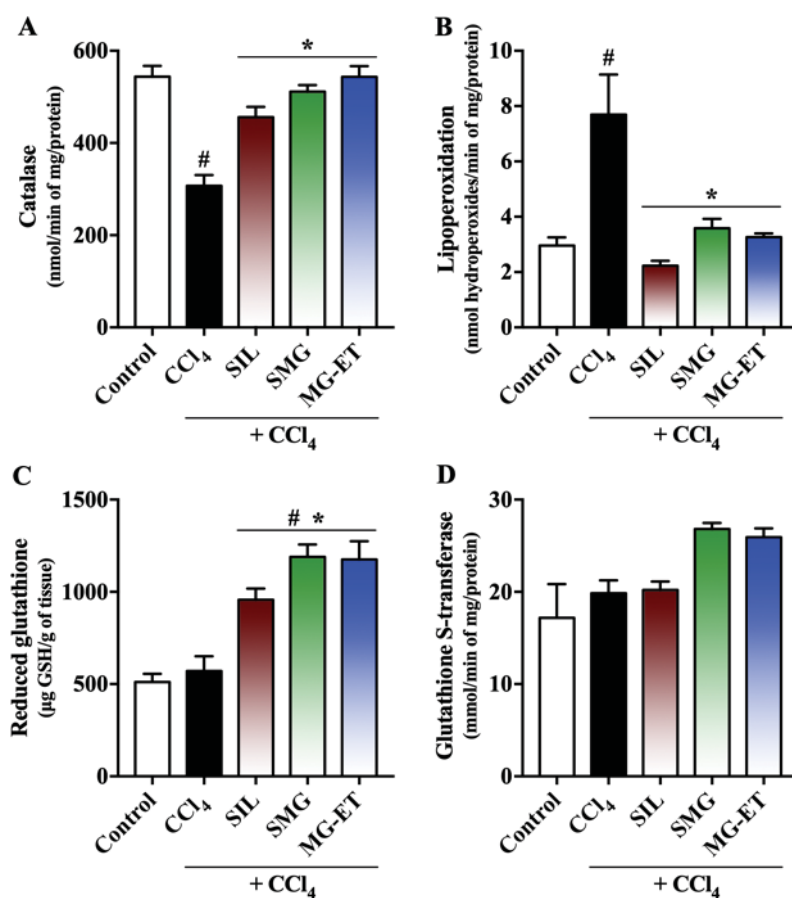


FIGURE 9 - MEASUREMENT OF HEPATIC OXIDATIVE STRESS PARAMETERS IN CCl₄-INDUCED HEPATIC INJURY MICE TREATED WITH VEHICLE, SMG OR MG-ET. CAT (A), LPO (B), GSH (C) AND GST (D). GROUPS (n=7): Control (ULTRAPURE WATER), CCl₄ (ULTRAPURE WATER + CCl₄); SIL (SILYMARIN 100 mg/kg + CCl₄); SMG (1 mg/kg + CCl₄) AND MG-ET (10 mg/kg + CCl₄). *p < 0.05 STATISTICALLY DIFFERENT FROM CCl₄GROUP. # p < 0.05 STATISTICALLY DIFFERENT FROM Control. VALUES WERE EXPRESSED AS MEAN ± STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI *post hoc* TEST.

4. DISCUSSION

The Asteraceae family that include the *Mikania glomerata* species is one of the largest family in the plant kingdom and it is already known that fructan polymers, as storage polysaccharide, are synthesized by these plants (ITAYA et al., 2007; WACK; BLASCHEK, 2006). Structural characterization of inulin-type fructans in Asteraceae has already been performed in *Matricaria maritima* (CÉRANTOLA et al., 2004), *Vernonia kotschyana* (NERGARD et al., 2004), *Echinacea purpurea* (WACK; BLASCHEK, 2006), *Stevia rebaudiana* (LOPES et al., 2015; OLIVEIRA et al., 2011), *Artemisia absinthium* and *A. vulgaris* (CORRÊA-FERREIRA; NOLETO; OLIVEIRA PETKOWICZ, 2014), *Helianthus*

tuberosus (BARHATOVA et al., 2015), *Atractylodes chinensis* (XU et al., 2016), *Smallanthus sonchifolius* (PAREDES et al., 2018), *Chamomilla recutita* (CHAVES; IACOMINI; CORDEIRO, 2019) and dahlia tubers (AFINJUOMO et al., 2019). They produced a linear polymer composed of $\rightarrow 1$)- β -D-Fruf-(2 $\rightarrow$ residues, as that reported herein for *Mikania glomerata* (FIGURE 3). Also, in that studies, the presence of FOS has only been reported for *Matricaria maritima*, *Stevia rebaudiana*, *Smallanthus sonchifolius* and *Chamomilla recutita*.

The main content of SMG fraction is inulin and for our concern there is no evidence in literature that indicate that ingestion of moderate doses of this molecule has toxicity related on liver. Additionally it is widely spread that plant polysaccharides have none or very low toxicity *in vivo* (RAMBERG; NELSON; SINNOTT, 2010). Therefore, because of the popular believe that *M. glomerata* infusion is contraindicated for individuals with hepatic disorders, we tested its fractions *in vivo*. The results showed minor alterations, SMG and MG-ET only increased GSH and urea levels among all 29 parameters analyzed (FIGURE 7 and 8, TABLE 2 and TABLE 3). The altered parameters alone did not indicate hepatotoxicity and thus only these few alterations were observed in the liver and body homeostasis of mice during the experimental period evaluated.

The inulin-type molecule is extensively researched for its prebiotic potential, moreover it is also target of other biological investigation, such as hepatoprotection. Previous studies observed that inulin from dahlia tubers and an inulin-rich fraction from *Artemisia vulgaris* protected the liver from carbon tetrachloride-induced hepatotoxicity, lowering the plasma levels of the enzymes (ALT, AST and ALP) associated with liver injury (CORRÊA-FERREIRA et al., 2017; LIU et al., 2015). The *M. glomerata* fractions presented similar effects, the SMG and MG-ET fractions lowered the plasma levels of the enzymes AST, ALP and ALT in CCl₄-treated mice, indicating hepatoprotection (FIGURE 7). However, it is worth to notice that the inulin fraction SMG provided a protection at a 100-fold lower dose compared to inulin from dahlia tubers and at 10-fold lower dose when compared to inulin-rich fraction from *Artemisia vulgaris*. In the blood analysis although the administration of CCl₄ promoted an increase of hemoglobin and a change in hematocrit and MCV, as well as induced increase of WBC and lymphocytes for fraction SMG (TABLE 3), the hematological results seem to be in the range of reference values for the species (RESTELL et al., 2014; SERFILIPPI; PALLMAN; RUSSELL, 2003; SUCKOW; DANNEMAN; BRAYTON, 2001).

Studies have also demonstrated that inulin protected the liver against isoniazid-, chlorpromazine- and methotrexate-induced hepatotoxicity and also alleviates hepatic steatosis and xenobiotics-induced liver injury in rats fed a high-fat and high-sucrose diet (ABDEL-WAHAB et al., 2017; ALI; JEHANGIR; AYUB, 2016; KALANTARI et al., 2019; SUGATANI et al., 2006).

Hepatoprotection against CCl₄-induced injuries has also been reported for crude fractions of polysaccharides from other plant species, such as *Amomum villosum* (ZHANG et al., 2013), *Anoectochilus roxburghii* (ZENG et al., 2016), *Atractylodis macrocephalae* (HAN et al., 2016), *Camellia sinensis* (WANG et al., 2014), *Ilex latifolia* (FAN et al., 2014), *Lycium barbarum* (XIAO et al., 2012), *Malus domestica* (YANG et al., 2013), *Ziziphus jujuba* (YUE et al., 2014) and for a purified arabinogalactan from *Glycine max* (SUN et al., 2018).

In the studies with inulin fractions the proposed hepatoprotection mechanism in CCl₄-induced liver injury model was linked to their antioxidant effects, since they improved the hepatic oxidative stress parameters *in vivo* and also displayed antioxidant activity *in vitro* (CORRÊA-FERREIRA et al., 2017; LIU et al., 2015). The positive control, silymarin, also acts against liver injury by its efficacy in free radical scavenging, its ability to increase the cellular content of GSH and its capacity to inhibit the hepatic cytochrome P450 detoxification system (CYP) (FRASCHINI; DEMARTINI; ESPOSTI, 2002). In the CCl₄-induced hepatotoxicity model these characteristics play a relevant role, since the toxic effect of CCl₄ is caused by the accumulation of trichloromethyl peroxy radicals, produced by the metabolism of CCl₄ by CYP and reactions with reactive oxygen species, which can attach to macromolecules triggering lipid peroxidation that result in damage to cellular membranes (RECKNAGEL et al., 1989). Fractions SMG and MG-ET also showed antioxidant activity *in vitro* in the DPPH assay (FIGURE 6), as well as improved the CAT and GSH activity, and lowered the lipoperoxidation level in the liver of animals with CCl₄-induced injury, similar to what was observed for the positive control, silymarin (FIGURE 9 A, B, C). Moreover, GSH level increased in animals that received SMG or MG-ET in the *per se* experiment we performed (Fig 8 C). Additionally, this is the proposed mechanism of hepatoprotection shared by the other polysaccharides cited above (FAN et al., 2014; HAN et al., 2016; SUN et al., 2018; WANG et al., 2014; XIAO et al., 2012; YANG et al., 2013; YUE et al., 2014; ZENG et al., 2016; ZHANG et al., 2013). Thus, it seems that the antioxidant capacity of the fractions is an important feature for the hepatoprotection, as

already observed for silymarin, inulin fractions and polysaccharides fractions from other species already studied.

5. CONCLUSION

The present study demonstrates that *Mikania glomerata* infusion presents inulin-type fructan ($\rightarrow 1$)- β -D-Fruf-($2 \rightarrow$ linked) and fructooligosaccharides as main components. The results of *in vivo* experiments indicated that the tested fractions did not produce a hepatotoxic effect *per se* and protected the liver against CCl₄-induced injuries. Our findings also suggest that the tested fractions had comparable hepatoprotective effect to silymarin and the mechanism of action seems to be also mediated by the antioxidant properties of the tested fractions.

ACKNOWLEDGEMENTS

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4.2 ARTIGO II

CHEMICAL CHARACTERIZATION OF POLYSACCHARIDES FROM *Baccharis trimera* (Less.) DC. INFUSION AND THEIR HEPATOPROTECTIVE EFFECTS

Pedro Felipe Pereira Chaves^a, Eliana Rezende Adami^b, Alexandra Acco^b, Marcello Iacomini^a, Lucimara Mach Côrtes Cordeiro^{a*}

^a *Biochemistry and Molecular Biology Department, Federal University of Paraná, CP 19.046, CEP 81.531-980 Curitiba, PR, Brazil.*

^b *Pharmacology Department, Federal University of Paraná, CEP 81.531-980 Curitiba, PR, Brazil.*

ABSTRACT

Baccharis trimera (Less.) DC is a native medicinal plant from South America popularly known as "carqueja". Its infusion is traditionally used in Brazil for the treatment of hepatic disorders. Up to now, only the crude aqueous extract or hydroethanolic fractions, containing the secondary metabolites, have been studied and correlated with their biological action on the liver. Here we report that the polysaccharide inulin is present in the *B. trimera* infusion and also contributes to the hepatoprotective effect. *In vitro*, inulin at 300 µg/mL, was able to scavenge 97% of the DPPH radicals. *In vivo* experiments showed that it protected the liver against CCl₄-induced injuries. The administration of inulin at low dose of 1 mg/kg significantly reduced the blood levels of ALT, AST and ALP, reduced the lipid peroxidation and increased the catalase activity and the levels of reduced glutathione in the liver of CCl₄-treated mice. Moreover, the administration of inulin at 100 mg/kg increased GSH levels in the liver of naïve mice. No signs of toxicity were observed. Thus, inulin present in *B. trimera* infusion protects the liver from the oxidative stress caused by CCl₄ administration and can corroborate with the hepatoprotective effects presented by the species infusion.

Keywords: Inulin; antioxidant; hepatoprotection; *Baccharis trimera*; Asteraceae.

1. INTRODUCTION

Baccharis trimera (Less.) DC. (synonyms: *Baccharis genistelloides* var. *trimera* and *Molina trimera*) is a medicinal plant that belongs to the Asteraceae family. This species grows natively in South of Brazil and also in regions of Paraguay, Argentina and Uruguay (LORENZI; MATOS, 2008; MINTEGUIAGA et al., 2018; MORETI et al., 2017). The therapeutic use of this plant is widespread in folk medicine for treatment of a diverse number of disorders, especially hepatic dysfunctions (BARROSO, 1976; BRASIL, 2009; LORENZI; MATOS, 2008). The hepatoprotective effects of *B. trimera* aerial parts were explored in several pharmacological studies, showing that its extracts can promote liver protection against a broad variety of chemical injury and diseases (LÍVERO et al., 2016b; RABELO et al., 2018; SABIR et al., 2017; SILVEIRA RABELO; CALDEIRA COSTA, 2018a). These effects were linked to the presence of secondary metabolites in the extracts, mainly obtained by hydroethanolic maceration. However, in order to achieve its biological effects, *B. trimera* is mainly consumed as aqueous infusion. It is known that polysaccharides are also solubilized from plant material with this preparation mode. Nevertheless, the potential contribution of polysaccharides for the hepatoprotective effects of *B. trimera* was not explored yet (MARTINEZ; BESSA; BENITO, 2005; SOICKE; LENG-PESCHLOW, 1987).

Plant polysaccharides have been investigated about their numerous biological effects due to their abundance, low adverse effects and low toxicity (LIU; WILLFÖR; XU, 2015; RAMBERG; NELSON; SINNOTT, 2010). Studies demonstrated their relevant pharmacological properties, as anticancer (ADAMI et al., 2018; STIPP et al., 2017), anti-inflammatory (BEZERRA et al., 2018; LEIVAS et al., 2016), immunomodulation (SHI; DONG; DING, 2014; VOGT et al., 2015), antioxidant (SHANG et al., 2018; ZENG et al., 2016), hepatoprotection (CORRÊA-FERREIRA et al., 2017; LIU et al., 2015), among others. Thus, in the present study we report the isolation, chemical characterization and hepatoprotective effects against carbon tetrachloride-induced hepatotoxicity model of polysaccharides from *B. trimera* infusion.

2. MATERIALS AND METHODS

2.1. Plant material and infusion

Dried aerial parts of *Baccharis trimera* Less DC. was bought in local commerce (Chamel Produtos Naturais® Lote11061/Nov2015) and after an inspection, looking for roots and impurities, the plant material was stored in a hermetic container.

The infusion was carried according to 10/2010 RDC of the Brazilian Government Agency ANVISA that indicates the ideal preparation process of medicinal plants to obtain their therapeutic effects (BRASIL, 2009). Plant material was placed in a beaker and boiling distilled water was added in a concentration of 40 g/L, the mixture was slightly stirred, closed and let it rest for 30 min. The mixture obtained from infusion was filtered, yielding an extract and a residue. The extract was submitted to the isolation and purification of polysaccharides and the residue was discarded.

2.2. Fractionation of polysaccharides

The extract obtained from infusion was concentrated under reduced pressure and the polysaccharides precipitated with three volumes of ethanol 99%. The polysaccharides were collected by centrifugation ($8000 \times g$), dialyzed (Cellulose Spectrumlabs 6-8 kDa cut-off) and freeze-dried, giving fraction BT (FIGURE 1). The precipitated fraction (BT) was submitted to freeze-thawing process (GORIN; IACOMINI, 1984), centrifuged ($8000 \times g$) and freeze-dried, giving cold-water soluble (SBT) and insoluble fractions. The yield was calculated based on the weight of initial freeze-dried plant material (512 g).

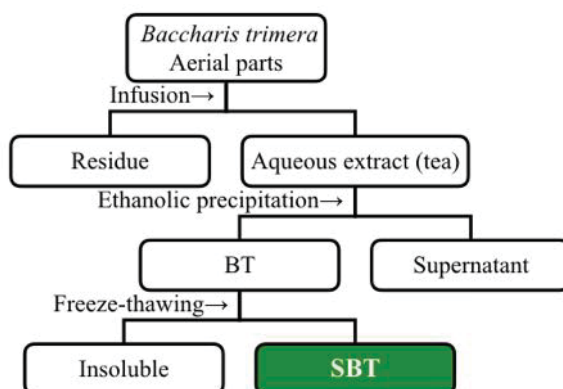


FIGURE 1 - SCHEME OF EXTRACTION AND FRACTIONATION OF POLYSACCHARIDES FROM *Baccharis trimera* INFUSION.

2.3 Determination of homogeneity and relative molecular weight

SBT were solubilized in the eluent (0.1 M aq. NaNO_2 containing 200 ppm aq. NaN_3) at concentration of 1 mg/mL for determination of homogeneity. The sample was filtered through a 0.22 μm membrane (Millipore) and injected (250 μL loop) in a high-performance steric exclusion chromatograph (HPSEC) equipped with four columns in series, with exclusion sizes of 7106 Da (Ultrasphere 2000, Waters), 4105 Da (Ultrasphere 500, Waters), 8104 Da (Ultrasphere 250, Waters) and 5103 Da (Ultrasphere 120, Waters) and a differential refractometer (Waters). The elution volume was at 0.6 mL/min. To obtain the relative M_w , standard dextrans (72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa and 5.0 kDa, from Sigma) were employed to obtain the calibration curve. The relative M_w of the sample was calculated according to the calibration curve.

2.4 Determination of monosaccharide composition

SBT fraction (~2 mg) was hydrolyzed in 500 μL 0.5 M TFA at 80 °C for 30 min. The TFA was evaporated and the sample was converted to alditol acetates by successive NaBH_4 reduction and acetylation with Ac_2O -pyridine (1:1, v/v, 1 mL) at room temperature for 16 h (WOLFROM; THOMPSON, 1963a, 1963b). The resulting alditol acetates were then extracted with CHCl_3 and were analyzed by GC-MS using a Varian 3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer (Varian, Palo Alto, CA). The column was DB-225MS (30 m $\times$ 0.25 mm i.d.; Agilent Santa Clara, CA) programmed from 50 to 220 °C at 40 °C/min, with helium as carrier gas, at a flow rate of 1 mL/min. The inlet temperature was 250 °C, and the MS transfer line was set at 250 °C. MS acquisition parameters included scanning from m/z 50 to 550 in electron ionization mode (EI) at 70 eV. Components were identified by their retention times and EI spectra.

The uronic acid contents were determined using the modified *m*-hydroxybiphenyl colorimetric method (FILISSETTI-COZZI; CARPITA, 1991), using galacturonic acid as standard.

2.5 Determination of protein, flavonoid and phenolic content

Protein content was determined according to the Bradford method with bovine serum albumin (BSA) as the standard (BRADFORD, 1976). Phenolic content was estimated by the Folin-Ciocalteau method with gallic acid as the standard (SINGLETON; ORTHOFER; LAMUELA-RAVENTÓS, 1999). Flavonoid content was estimated by the AlCl_3 method with rutin as the standard (QUETTIER-DELEU et al., 2000). The results of flavonoid and phenolic content were expressed in mg standard/100 g freeze-dried fraction by comparison with standards treated in the same conditions.

2.6 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR and HSQC spectra were acquired at 50-70 °C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The samples were acquired in D_2O with chemical shifts expressed as δ ppm, using the resonances of CH_3 groups of acetone (δ_{C} 30.2/ δ_{H} 2.22) as internal reference. All pulse programs were supplied by Bruker.

2.7 In vitro determination of free radical scavenging activity

SBT was evaluated for its scavenging activity against the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). This method was adapted from Chen, Wu and Chen (2004). Briefly, samples at different concentrations (3, 10, 30, 100, and 300 $\mu\text{g/mL}$) were mixed with DPPH methanolic solution (10 $\mu\text{g/mL}$). Ascorbic acid (50 $\mu\text{g/mL}$) and deionized water were used as positive and negative controls, respectively. Absorbance was read at 517 nm.

2.8 Animals

Experiments were conducted using female Swiss mice (*Mus musculus*, 25–35 g) from the Federal University of Paraná vivarium. Animals were kept under standard laboratory conditions (12 h light/dark cycles, temperature 22 ± 2 °C) with food and water provided *ad libitum* and acclimatized to the laboratory for at least 48 h before testing. The

experiments were performed after approval of the respective protocols by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO – UFPR; approval number 1000). The study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NATIONAL RESEARCH COUNCIL (US) COMMITTEE FOR THE UPDATE OF THE GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS, 2011).

2.9 *In vivo* assays

To investigate the hepatic effects of the polysaccharides, SBT fraction was assayed *per se* and in the carbon tetrachloride (CCl₄)-induced hepatotoxicity model. The animals were separated into following experimental groups (7 animals per group): a) Control, treated with distilled water; b) SBT_{ps}, treated with SBT (100 mg/kg); c) CCl₄, treated with distilled water; d) SIL, treated with silymarin (100 mg/kg) and e) SBT, treated with SBT (1 mg/kg). For the CCl₄-induced hepatotoxicity groups the dose of SBT was chosen based on the portion of the fraction present in a 200 mL cup of infusion, considering the preparation mode according to RDC resolution 10/2010 and ingestion by a 70 kg individual. Since the SBT fraction contains mostly a plant polysaccharide, which is well known produce none to low toxicity, in the *per se* assay (SBT_{ps}) we opted to 100-fold rise its dose.

The treatments were performed orally (gavage) once a day for 7 consecutive days. On the 6th and 7th days of treatment, hepatotoxicity was induced in the groups CCl₄, SIL and SBT by intraperitoneally (i.p.) administration of CCl₄ 2% (corn oil, 5 mL/kg). After approximately 24 h (8th day), the animals were anesthetized with 100 mg/kg ketamine hydrochloride + 10 mg/kg xylazine hydrochloride i.p, samples of blood were collected with heparinized syringes from the abdominal cava vein and liver samples were immediately collected, weighed, frozen (liquid nitrogen) and stored at -80 °C for further analysis (CORRÊA-FERREIRA et al., 2017). The absolute and relative weighing of spleen, kidneys and lung also was checked.

2.10 Hematological and biochemical analysis

Blood samples were submitted to complete blood count and centrifuged at 3000 × *g* for 5 min to obtain the plasma. The plasma was analyzed for the alanine aminotransferase

(ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), creatinine, urea and glucose levels. Analysis were performed using commercial kits (Kovalent, São Gonçalo, Brazil) in a Mindray BS-200 (Shenzhen, China) automated device.

2.11 Hepatic oxidative stress measurements

Liver samples were thawed, homogenized in phosphate-buffered saline (PBS; pH 6.5) and centrifuged ($10,000 \times g$, 4 °C, 20 min). The supernatant was used to measure the enzymatic activities of catalase (CAT) (AEBI, 1984), and glutathione S-transferase (GST) (HABIG; PABST; JAKOBY, 1974). The lipid peroxidation (LPO) (JIANG; WOOLLARD; WOLFF, 1991) and reduced glutathione (GSH) (SEDLAK; LINDSAY, 1968) levels were also measured from supernatant. The results of CAT, GST and LPO are expressed based in the amount of protein in the liver samples, determined using the Bradford (1976) method with bovine serum albumin (BSA) as the standard. The results of GSH are expressed based in the amount of the liver tissue analyzed.

2.12 Statistical analysis

Data from each group were expressed as mean $\pm$ standard error of the mean. Statistical analyzes were performed using one-way ANOVA followed by the Bonferroni *post-hoc* test using the Prism 7.0a software (Graphpad) and $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1 Fractionation and chemical characterization of polysaccharides

The ethanolic precipitation process from *B. trimera* infusion produced a polysaccharide fraction named BT with 1.8% yield. The fraction was then submitted to the purification process of freeze-thawing to get the cold-water soluble portion, producing a fraction named SBT with 1.2% yield (FIGURE 1). SBT was submitted to determination of protein, phenolic compounds and flavonoid content, presenting low amounts of these components, approximately 6%, 1.72 mg/100 g (Gallic acid equivalent) and 0.20 mg/100 g

(Rutine equivalent), respectively. The monosaccharide composition of SBT showed fructose (93%), uronic acids (5%) and arabinose (2%). ^{13}C NMR and HSQC analyses showed that it is mainly composed by an inulin type fructan (FIGURE 2 and 3). The inulin could be detected due to its signals at δ 61.2 (C-1), δ 103.2 (C-2), δ 77.3 (C-3), δ 74.7 (C-4), δ 81.2 (C-5) and δ 62.2 (C-6) and signals from HSQC (FIGURE 3) in δ 61.3/3.97-3.82 (C-1H-1), δ 77.0/4.36 (C-3H-3), δ 74.6/4.13 δ (C-4H-4), δ 81.7/3.86 (C-5H-5) and 61.6/3.89 (C-6H-6) from β -(2 $\rightarrow$ 1) fructosyl-fructose linkages (CHAVES; IACOMINI; CORDEIRO, 2019; CORRÊA-FERREIRA; NOLETO; OLIVEIRA PETKOWICZ, 2014; OLIVEIRA et al., 2011). A low intensity signal, in δ 107.0, was also observed in ^{13}C NMR spectrum, probably from small amounts of an arabinan. The fraction SBT was also submitted to HPSEC and presented a main peak with relative molecular mass of approximately 6.5 kDa (FIGURE 4).

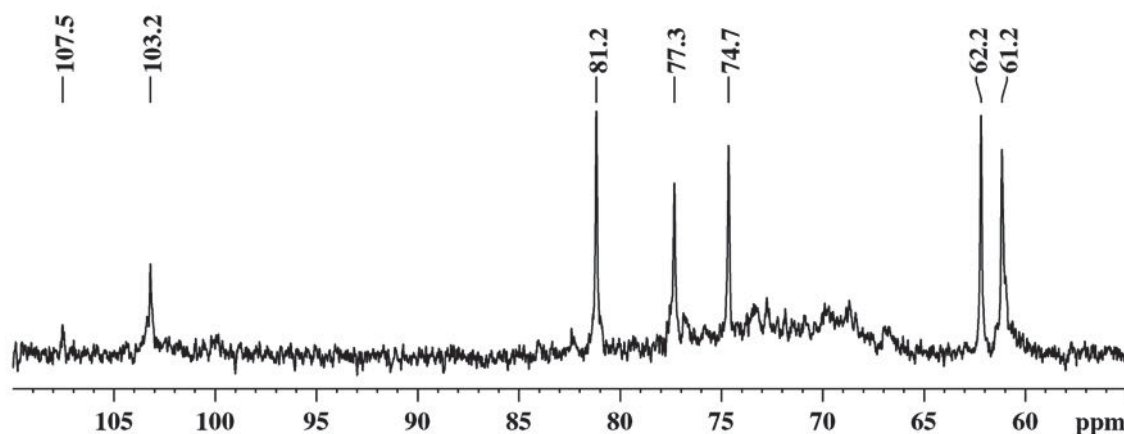


FIGURE 2 - ^{13}C NMR SPECTRUM OF SBT FRACTION IN D_2O AT 70 °C. THE CHEMICAL SHIFTS ARE EXPRESSED AS δ PPM.

Inulin is a known fructan synthesized by members of the Asteraceae family, which include *B. trimera*, and is usually used as storage molecule (ITAYA et al., 2007; WACK; BLASCHEK, 2006). Studies have already reported the presence of inulin in *Matricaria maritima* (CÉRANTOLA et al., 2004), *Vernonia kotschyana* (NERGARD et al., 2004), *Echinacea purpurea* (WACK; BLASCHEK, 2006), *Stevia rebaudiana* (Lopes et al., 2015; de Oliveira et al., 2011), *Artemisia absinthium* and *A. vulgaris* (CORRÊA-FERREIRA; NOLETO; OLIVEIRA PETKOWICZ, 2014), *Helianthus tuberosus* (BARHATOVA et al., 2015), *Atractylodes chinensis* (XU et al., 2016), *Smallanthus sonchifolius* (PAREDES et al., 2018), *Chamomilla recutita* (CHAVES; IACOMINI; CORDEIRO, 2019) and *Dahlia sp.* (AFINJUOMO et al., 2019).

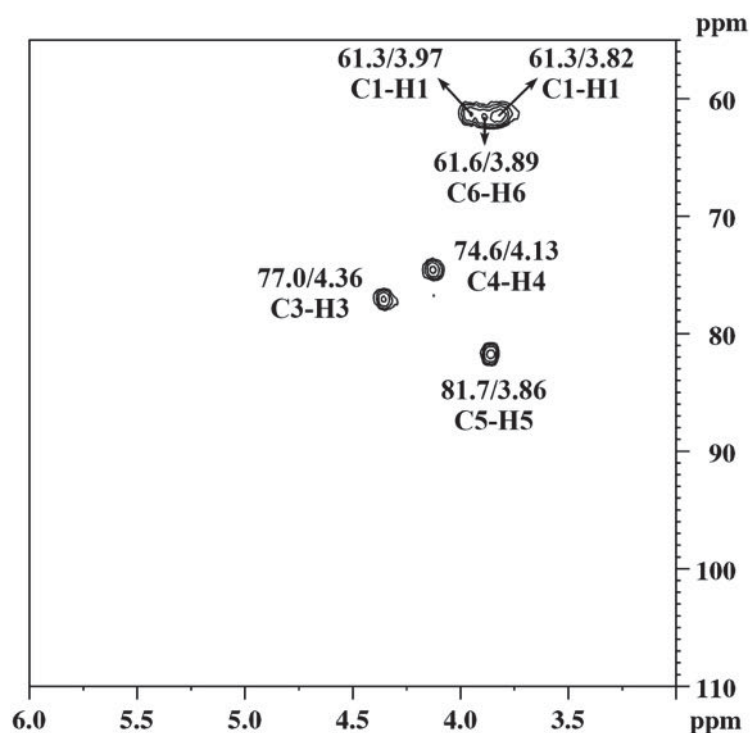


FIGURE 3 - HSQC CORRELATION MAP OF SBT FRACTION IN D_2O AT 70 °C. THE CHEMICAL SHIFTS ARE EXPRESSED AS δ PPM.

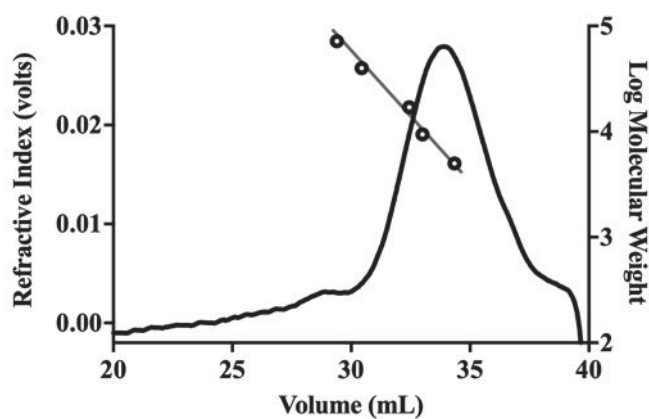


FIGURE 4 - HPSEC ELUTION PROFILE OF SBT FRACTION. REFRACTIVE INDEX DETECTOR. ELUTION VOLUME OF DEXTRAN STANDARDS OF MOLECULAR WEIGHT 72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa AND 5.0 kDa (LEFT TO RIGHT) WERE EMPLOYED TO BUILD THE CALIBRATION CURVE.

3.2 *In vitro* free radical scavenging activity

Antioxidant capacity is a desirable characteristic for compounds that may protect the liver from diseases and chemical toxicity (WALLACE, 1997). Thus, we evaluated the ability

of SBT to sequester the DPPH *in vitro*. DPPH is a stable free radical molecule that in solution can strongly absorb light at 517 nm, as this electron becomes paired off, the molecule changes and absorption vanishes (CHEN; WU; CHEN, 2004; JIN; CHEN, 1998). This characteristics cause DPPH to be frequently used in antioxidant capacity determination of chemical compounds (COTELLE et al., 1996). Fraction SBT presented higher DPPH sequestration capacity than the negative control (Ve) at all tested concentrations. At the higher concentration (300 $\mu\text{g/mL}$), it was able to scavenger 97% of the free radicals, being 7% more effective than the positive control ascorbic acid (FIGURE 5). A recent study have also showed that inulin had significant antioxidant effect *in vitro* in DPPH, ABTS and ferric reducing power assays (SHANG et al., 2018). With this *in vitro* antioxidant activity, the effect of SBT fraction in the liver was further assayed *in vivo*.

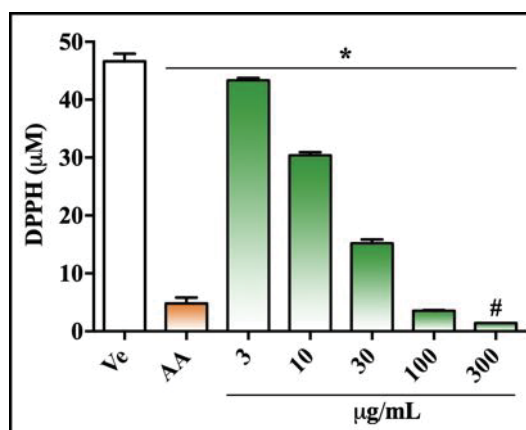


FIGURE 5 - *In vitro* ABILITY OF SBT TO SCAVENGE THE FREE-RADICAL 2,2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH). DEIONIZED WATER (Ve) WAS USED AS NEGATIVE CONTROL AND ASCORBIC ACID (AA, 50 $\mu\text{g/mL}$) AS POSITIVE CONTROL. VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI post hoc TEST. * $p < 0.05$ STATISTICALLY DIFFERENT FROM Ve. # $p < 0.05$ STATISTICALLY DIFFERENT FROM AA.

3.3 *In vivo* assays

To a comprehensive investigation of the hepatic effects of the polysaccharides obtained from *B. trimera* infusion, SBT fraction was evaluated *in vivo* in both alone (*per se*) and in the CCl_4 -induced hepatic injury model.

The results showed that SBT alone did not cause alterations in the plasma levels of ALT, AST and ALP (FIGURE 6), in the hematological and biochemical parameters evaluated (TABLE 1), as well in body and organs weight (TABLE 2). In the hepatic

oxidative stress parameters (CAT, LPO, GSH and GST, FIGURE 7), SBT caused alterations only in the GSH levels, with a 70% increase when compared to Control (FIGURE 7 C). Since SBTps only altered GSH levels among all 29 parameters analyzed and this alteration alone cannot indicate liver toxicity, the results are in conformity with the widely spread knowledge that plant polysaccharides have none or very low toxicity *in vivo* (Ramberg, Nelson, & Sinnott, 2010). Furthermore, the increase of GSH levels could be related to the antioxidant capacity of SBT discussed hereafter.

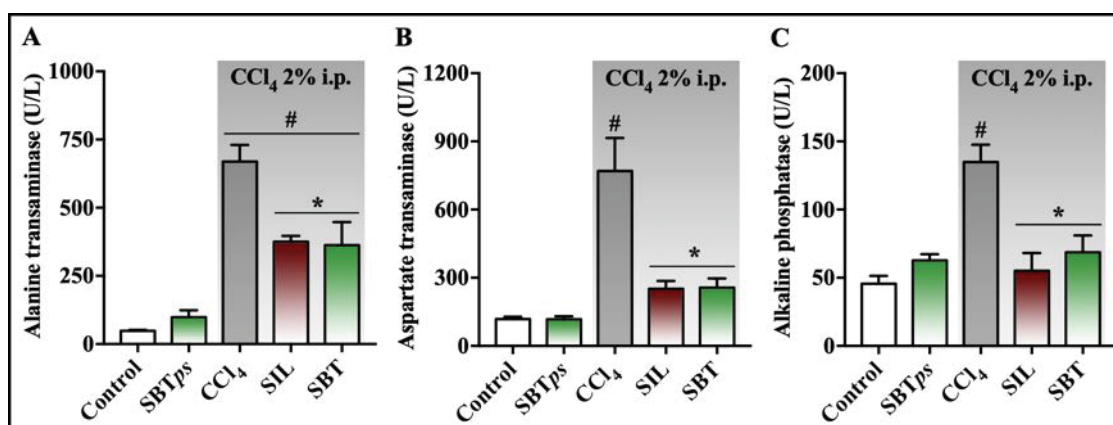


FIGURE 6 - PLASMA BIOCHEMICAL PARAMETERS ALT (A), AST (B) AND ALP (C), IN MICE TREATED WITH SBT *per se*, WITH CCl₄-INDUCED HEPATIC INJURY, AND CONTROLS. GROUPS: Control (ULTRAPURE WATER); SBTps (100 mg/kg *per se*); CCl₄ (ULTRAPURE WATER + CCl₄); SIL (SILYMARIN 100 mg/kg + CCl₄) AND SBT (1 mg/kg + CCl₄) (n=7). * p <0.05 STATISTICALLY DIFFERENT FROM THE CCl₄ GROUP. # p <0.05 STATISTICALLY DIFFERENT FROM THE CONTROL GROUP. VALUES WERE EXPRESSED AS MEAN ± STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI *post hoc* TEST.

TABLE 1 - HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN MICE TREATED WITH SBT *per se* AND WITH CCl₄-INDUCED HEPATIC INJURY AND CONTROLS.

	Control	SBTps (100 mg/kg)	CCl ₄	Sil (100 mg/kg)	SBT (1 mg/kg)
Creatinine (mg/dL)	0.3 ± 0.03	0.3 ± 0.02	0.4 ± 0.03	0.4 ± 0.03	0.4 ± 0.03
Urea (mg/dL)	55 ± 4.5	51 ± 4.3	42 ± 5.2	37 ± 2.6	37 ± 2.2
Glucose (mg/dL)	89 ± 9.9	115 ± 10	81 ± 6.8	119 ± 15	132 ± 12*
WBC (x10 ³ /μL)	4.7 ± 0.44	4.3 ± 1.5	6.0 ± 0.58	4.1 ± 0.75	4.8 ± 0.52
Lymphocytes (x10 ³ /μL)	3.2 ± 0.42	3.3 ± 1.20	3.9 ± 0.42	2.9 ± 0.54	3.2 ± 0.31
Monocytes (x10 ³ /μL)	0.16 ± 0.03	0.13 ± 0.05	0.29 ± 0.05	0.17 ± 0.07	0.11 ± 0.03

Granulocytes (x10 ³ /μL)	1.0 ± 0.15	0.89 ± 0.3	1.8 ± 0.18 [#]	1.1 ± 0.19	1.3 ± 0.17
Lymphocytes (%)	71 ± 3.3	73 ± 1.8	63 ± 1.9	70 ± 1.4	69 ± 2.1
Monocytes (%)	3.9 ± 0.42	3.1 ± 0.14	5.0 ± 0.71	2.7 ± 0.46	3.0 ± 0.29
Granulocytes (%)	27 ± 3.3	24 ± 1.8	34 ± 1.8	26 ± 2.2	28 ± 1.8
RBC (10 ⁶ /μL)	7.6 ± 0.48	7.1 ± 1.3	9.0 ± 0.12	9.1 ± 0.15	9.0 ± 0.16
Hemoglobin (g/dL)	11 ± 0.69	10 ± 1.8	13 ± 0.16	12 ± 0.21	12 ± 0.23
Hematocrit (%)	33 ± 2.0	33 ± 5.1	40 ± 0.58 [#]	40 ± 0.73	40 ± 0.73
MCV (fL)	43 ± 0.84	42 ± 0.59	44 ± 0.41	44 ± 0.35	44 ± 0.33
MCH (pg)	13 ± 0.22	13 ± 0.22	14 ± 0.16	14 ± 0.12	13 ± 0.13
RDW (%)	14 ± 0.28	14 ± 0.53	14 ± 0.4	14 ± 0.54	14 ± 0.54
Platelets (10 ³ /μL)	390 ± 40	260 ± 39	318 ± 26	275 ± 48	345 ± 32

Groups: Control (ultrapure water); SBT_{ps} (100 mg/kg *per se*); CCl₄ (ultrapure water + CCl₄); SIL (Silymarin 100mg/kg + CCl₄) and SBT (1 mg/kg + CCl₄) (n=7). * p <0.05 statistically different from the CCl₄ group. # p <0.05 statistically different from the Control group. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni *post hoc* test.

TABLE 2 - BODY AND RELATIVE ORGANS WEIGHT IN MICE TREATED WITH SBT *per se* AND WITH CCl₄-INDUCED HEPATIC INJURY AND CONTROLS.

	Control	SBT _{ps} (100 mg/kg)	CCl ₄	Sil (100 mg/kg)	SBT (1 mg/kg)
Body (g)	30 ± 0.7	27 ± 0.5	28 ± 0.54	28 ± 0.36	28 ± 1.00
Liver (%)	4.6 ± 0.12	5.1 ± 0.29	7.1 ± 0.18[#]	6.80 ± 0.20[#]	6.7 ± 0.14[#]
Kidney (%)	1.2 ± 0.05	1.1 ± 0.03	1.3 ± 0.04	1.2 ± 0.03	1.2 ± 0.04
Spleen (%)	0.41 ± 0.05	0.43 ± 0.04	0.44 ± 0.03	0.47 ± 0.02	0.44 ± 0.02
Lung (%)	0.65 ± 0.02	0.74 ± 0.01	0.70 ± 0.02	0.67 ± 0.02	0.70 ± 0.04

Groups: Control (ultrapure water); SBT_{ps} (100 mg/kg *per se*); CCl₄ (ultrapure water + CCl₄); SIL (Silymarin 100mg/kg + CCl₄) and SBT (1 mg/kg + CCl₄) (n=7). [#] p <0.05 statistically different from the Control group. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni *post hoc* test.

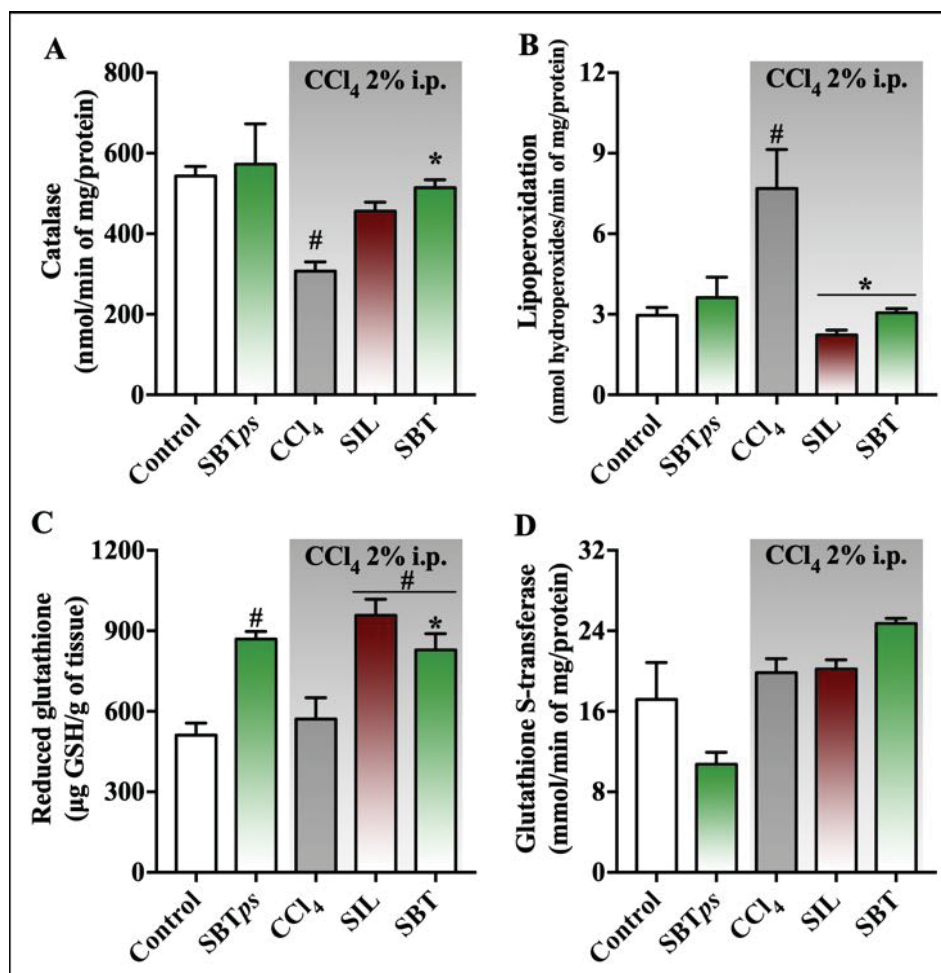


FIGURE 7 - HEPATIC OXIDATIVE STRESS PARAMETERS CAT (A), LPO (B), GSH (C) AND GST (D), IN MICE TREATED WITH SBT *per se* AND WITH CCl₄-INDUCED HEPATIC INJURY AND CONTROLS. GROUPS: Control (ULTRAPURE WATER); SBTps (100 mg/kg *per se*); CCl₄ (ULTRAPURE WATER + CCl₄); SIL (SILYMARIN 100 mg/kg + CCl₄) and SBT (1 mg/kg + CCl₄) (n=7). * p < 0.05 STATISTICALLY DIFFERENT FROM THE CCl₄ GROUP. # p < 0.05 STATISTICALLY DIFFERENT FROM THE Control GROUP. VALUES WERE EXPRESSED AS MEAN ± STANDARD ERROR OF THE MEAN, ANALYZED BY ONE-WAY ANOVA AND BONFERRONI *post hoc* TEST.

CCl₄-induced hepatotoxicity is a robust tool to evaluate the capacity of compounds to protect liver from acute injuries. In this method, the type and extent of injuries can be dose controlled and a wide range of effects, like disruption in hepatocytes and enzymatic alteration can be monitored (WEBER; BOLL; STAMPFL, 2003). As expected, acute administration of 2% CCl₄ (i.p., in corn oil, 5 mL/kg) in mice increased the plasma levels of hepatic enzymes ALT, AST and ALP, when compared to control group (FIGURE 6). In the hepatic oxidative stress parameters, the CCl₄ caused a decrease in the enzymatic activity of CAT (FIGURE 7A) and an increase in the lipoperoxidation level (FIGURE 7B). The GSH content and the activity of GST in the liver were not altered by CCl₄ administration (FIGURE

7C, D). The CCl₄ also promoted an increase in the blood levels of granulocytes, a change in hematocrit and promoted an increase of liver relative weight (TABLE 1, 2).

The pretreatment with SBT decreased the hepatocytes disruption caused by CCl₄ by lowering the plasma levels of ALT (↓ 56%), AST (↓ 67%) and ALP (↓ 49%) when compared with the CCl₄ group, reaching the control levels for AST and ALP (FIGURE 6). In the parameters of hepatic oxidative stress, SBT restored the activity of CAT and decreased the LPO to the values of Control. The fraction promoted a 48% increase in the GSH level, while the activity of GST was not altered by the treatments (FIGURE 7).

Other observed alterations were that SBT promoted an increase in blood glucose (TABLE 1). However, all the hematological results seem to be in the range of reference values for the species (RESTELL et al., 2014; SERFILIPPI; PALLMAN; RUSSELL, 2003; SUCKOW; DANNEMAN; BRAYTON, 2001).

These results indicate an *in vivo* antioxidant effect and for most analyses, SBT showed an outline equal to the positive control silymarin, a known *in vivo* hepatic antioxidant (FRASCHINI; DEMARTINI; ESPOSTI, 2002). Moreover, the pretreatment with SBT or SIL could not attenuate the increase in liver weight induced by CCl₄. Silymarin has a high efficacy in free radical scavenging, ability to increase the hepatic content of GSH, and additionally can inhibit the hepatic cytochrome P450 detoxification system (CYP) (FRASCHINI; DEMARTINI; ESPOSTI, 2002), responsible for generating reactive compounds in liver. Silymarin also display DPPH scavenger activity *in vitro* (DE OLIVEIRA et al., 2015). These features have a crucial role in the hepatic protection promoted by CCl₄, since the hepatic intoxication caused by this haloalkane is mediated by the accumulation of trichloromethyl peroxy radicals, produced by its metabolism by CYP and reactions with reactive oxygen species. These species can attach to macromolecules triggering lipid peroxidation, resulting in hepatocyte disruption (RECKNAGEL et al., 1989). SBT showed a comparable effect with silymarin both *in vitro* and *in vivo*. The fraction promoted DPPH scavenger activity at all tested doses, improved the CAT activity, lowered the lipoperoxidation and improved GSH both with (SBT group) and without (SBT_{ps} group) CCl₄ (FIGURE 5, 7A-C).

Previous studies (CORRÊA-FERREIRA et al., 2017; LIU et al., 2015) have already shown the effects of inulin in hepatoprotection against CCl₄, however, the employed doses were much higher (10 to 100-fold). Thus, we demonstrated that inulin, at low dose of 1 mg/kg, is already enough to protect the liver from injuries. Inulin can also attenuate liver

toxicity caused by other compounds, such as isoniazid, rifampicin, chlorpromazine and methotrexate, can alleviate hepatic steatosis and also hepatic injuries in rats fed with a high-fat and high-sucrose diet (ABDEL-WAHAB et al., 2017; ALI; JEHANGIR; AYUB, 2016; KALANTARI et al., 2019; SUGATANI et al., 2006).

4. CONCLUSION

The present study showed that the main polysaccharide present in *Baccharis trimera* infusion is an inulin-type fructan. *In vitro* and *in vivo* assays indicate that it presented antioxidant effect and protected the liver against CCl₄-induced injuries, with absence of *per se* hepatic toxicity. Our findings also showed that the polysaccharide fraction had comparable hepatoprotective effect to silymarin, largely used as an hepatoprotective compound.

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The authors are grateful to the Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - Grants number 404717/2016-0, 307977/2015-3 and 310332/2015-0) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Finance Code 001) for their financial support; Olair Carlos Beltrame for the blood analyses and the NMR Center of UFPR for recording the NMR spectra.

4.3 ARTIGO III

CHEMICAL CHARACTERIZATION OF ARABINOGALACTANS FROM *Baccharis trimera* (LESS.) DC. INFUSION

Pedro Felipe Pereira Chaves^a, Marcello Iacomini^a, Lucimara Mach Côrtes Cordeiro^a

^a *Biochemistry and Molecular Biology Department, Federal University of Paraná, CP 19.046, CEP 81.531-980 Curitiba, PR, Brazil.*

ABSTRACT

Baccharis trimera (Less.) DC. is a medicinal plant that spontaneously grows in several regions of South America, where is widely used for its therapeutic purposes. The biological effects presented by plants are usually linked to secondary metabolites but is well-established that primary metabolites such as polysaccharides can also promote positive effects. Since chemical characteristic of the polymers play an important role in its biological properties, here the characterization of polysaccharides obtained from *B. trimera* infusion were described. Through purification by enzymatic treatment and chromatographic column two polysaccharide fractions were obtained. A water eluted fraction (BTEW) with 0.06% yield, a relative Mw of 10 kDa and monosaccharide composition of mainly arabinose (41.5%) and galactose (24.6%) and a salt eluted fraction (BTSE) with 0.1% yield, a relative Mw of 7 kDa and also monosaccharide composition of mainly arabinose (60.9%) and galactose (9.3%). The methylation and NMR analysis showed that both fractions are composed by type II arabinogalactans with differences in terminal residues. BTWE presented mainly fucose (11.3%) and arabinose (10.4%) as terminal residues and BTSE mainly galactose (19.9%) and glucose (16%). BTSE also presented signals of 2,4-di-*O*-substituted Rha_p units (1.3 %) and 4-*O*-substituted Gal_pA units (3.2%) indicating that BTSE may be a branch of a type I rhamnogalacturonan. In conclusion type II arabinogalactans with different terminal residues were found in the *B. trimera* infusion.

Keywords: Polysaccharides; type II arabinogalactan; rhamnogalacturonan; *Baccharis trimera*; Asteraceae;

1. INTRODUCTION

Plants used in folk medicine have a fundamental role in the world health, so every year more species are focus of studies for elucidation of their chemical components and to find news molecules with pharmacological applications (AKERELE, 1993; HALBERSTEIN, 2005). In this context *Baccharis trimera* (Less.) DC. emerges (synonyms: *Baccharis genistelloides* var. *trimera* and *Molina trimera*). This species belongs to the Asteraceae family, grows natively in south of Brazil and also in regions of Paraguay, Argentina and Uruguay, where its therapeutic uses is widespread (LORENZI; MATOS, 2008; MINTEGUIAGA et al., 2018; MORETI et al., 2017). Its infusion is used as treatment for diverse number of disorders, such as female sterility, male impotence, ulcers, diabetes, malaria, angina, anemia, diarrhea, throat, stomach and intestinal disorders, used as bitter tonic, antipyretic, anthelmintic and also for hepatic dysfunctions (BARROSO, 1976; BRASIL, 2009; LORENZI; MATOS, 2008). Many of the therapeutic properties presented by vegetal species are attributed to the presence of specific secondary metabolites in the extracts, however it is well-established that primary metabolites, polysaccharides for instance, corroborate with the properties and also can be the active substance (HALBERSTEIN, 2005; LIU; WILLFÖR; XU, 2015; RAMBERG; NELSON; SINNOTT, 2010; SILVEIRA RABELO; CALDEIRA COSTA, 2018a).

Polysaccharides present numberless pharmacological effects, some of them are presented by *B. trimera* extracts, such as anti-ulcer (CIPRIANI et al., 2009a), anti-diabetic (XU et al., 2015), gastroprotective (OLIVEIRA et al., 2018) and hepatoprotective (CORRÊA-FERREIRA et al., 2017). These biological effects are strongly linked to the chemical characteristics of the polymers (FERREIRA et al., 2015; YANG; ZHANG, 2009). Thus, in the present study the chemical characterization of polysaccharides obtained from *B. trimera* infusion were described.

2. MATERIALS AND METHODS

2.1. Obtainment and purification of polysaccharides

Soluble polysaccharide fraction from *Baccharis trimera* Less DC infusion was obtained from our previous work (CHAVES et al., 2019a). Briefly, plant material was

submitted to infusion, the extract was filtered, concentrated and polysaccharides were precipitated with ethanol (FIGURE 1). The polysaccharides were collected by centrifugation and then dialyzed. The dialyzed fraction was submitted to freeze-thaw process, centrifuged and the soluble portion was freeze-dried, giving a cold-water soluble fraction.

The soluble fraction was later treated with 316 U/mg *endo*-inulinase enzyme (Megazyme) in acetic acid/sodium acetate buffer (pH 4.6) for 24 h at 45 °C, dialyzed in a 2 kDa cut-off membrane (Cellulose Spectrumlabs) and freeze-dried. Then it was submitted to anion exchange chromatography column (DEAE Sepharose Fast Flow - GE Healthcare), eluted with water (0.5 mL/min) and freeze-dried to give BTWE fraction (0.06%) (FIGURE 1). The portion retained in the column was recovered with NaCl 1 M, dialyzed in a 2 kDa cut-off membrane (Cellulose Spectrumlabs) and freeze-dried to give BTSE fraction (0.1%). The yields were calculated based on the weight of initial freeze-dried plant material (512 g).

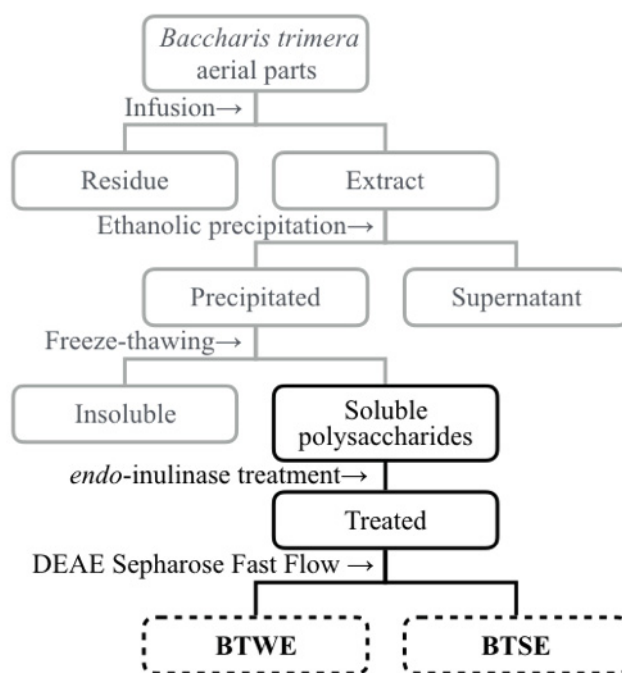


FIGURE 1 - SCHEME OF EXTRACTION AND PURIFICATION OF POLYSACCHARIDES FROM *Baccharis trimera* INFUSION. Note: The gray portion was previously carried out (CHAVES et al., 2019a).

2.2 Determination of homogeneity and relative molecular weight

Fractions were solubilized in the eluent (0.1 M aq. NaNO₂ containing 200 ppm aq. NaN₃) at concentration of 1 mg/mL for homogeneity determination. The sample was filtered

through a membrane 0.22 μm (Millipore) and injected (250 μL loop) in a high-performance steric exclusion chromatograph (HPSEC) equipped with four columns in series, with exclusion sizes of 7106 Da (Ultrahydrogel 2000, Waters), 4105 Da (Ultrahydrogel 500, Waters), 8104 Da (Ultrahydrogel 250, Waters) and 5103 Da (Ultrahydrogel 120, Waters) and a differential refractometer (Waters). The elution flow was at 0.6 mL/min. To obtain the relative M_w , standard dextrans (72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa and 5.0 kDa, from Sigma) were employed to obtain the calibration curve. The relative M_w of the sample was calculated according to the calibration curve.

2.3 Determination of monosaccharide composition

Fractions were hydrolyzed in 500 μL 2 M TFA at 100 $^{\circ}\text{C}$ for 8 h. The TFA was evaporated and the sample was converted to alditol acetates by successive NaBH_4 reduction and acetylation with Ac_2O -pyridine (1:1, v/v, 1 mL) at room temperature for 16 h (WOLFROM; THOMPSON, 1963a, 1963b). The resulting alditol acetates were then extracted with CHCl_3 and analyzed by GC-MS using a Shimadzu GC-2010 Plus gas chromatograph coupled to a Shimadzu GCMS-TQ8040 mass spectrometer. The column was SH-Rtx-5MS (30 m $\times$ 0.25 mmID; Shimadzu) programmed from 50 to 220 $^{\circ}\text{C}$ at 40 $^{\circ}\text{C}/\text{min}$, with helium as carrier gas, at a flow rate of 1 mL/min. The injections were made with an auto sampler system AOC-5000 Plus (Shimadzu). The inlet temperature was 250 $^{\circ}\text{C}$, and the MS transfer line was set at 250 $^{\circ}\text{C}$. MS acquisition parameters included scanning from m/z 50 to 550 in electron ionization mode (EI) at 70 eV. Components were identified by their retention times and EI spectra.

The uronic acid contents were determined using the modified *m*-hydroxybiphenyl colorimetric method (FILISSETTI-COZZI; CARPITA, 1991), using galacturonic acid as standard.

2.4 Methylation analysis

Fractions were submitted to a reduction of carboxyl groups with the carbodiimide method (TAYLOR; CONRAD, 1972). Briefly, using NaBH_4 as the reducing agent, the $-\text{COOH}$ groups of uronic acid residues were reduced to $-\text{CH}_2\text{OH}$. The carboxyl reduced sample was then *O*-methylated according to Ciucanu and Kerek (1984) method, using

powdered NaOH in DMSO-MeI. The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H₂SO₄ (0.5 M, 16 h) and neutralization with BaCO₃. The material was further submitted to reduction and acetylation as described above for monosaccharide composition, except that the reduction was performed using NaBD₄. The products (partially *O*-methylated alditol acetates) were examined with the same system described in item 2.3. The column was hold at 50 °C during injection for 1 min, then programmed to 210 °C at an increase rate of 40 °C /min and sustained at the temperature for 31 min. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (SASSAKI et al., 2005).

2.5 Nuclear magnetic resonance (NMR) spectroscopy

Spectra were acquired at 50-70 °C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ¹³C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The samples were acquired in D₂O with chemical shifts expressed as δ ppm, using the resonances of CH₃ groups from acetone (δ_C 30.2/ δ_H 2.22) as internal reference. All pulse programs were supplied by Bruker.

3. RESULTS AND DISCUSSION

The cold-water soluble fraction obtained from our previous work showed as major compound an inulin type fructan (CHAVES et al., 2019a). The characterization process of the fraction indicated small amounts of arabinose and uronic acids and, given the linear characteristic of inulin, the presence of these monosaccharides could be associated with the presence of other polymers. To proceed the investigation, the fraction had its inulin content removed using an enzymatic treatment (*endo*-inulinase), dialyzed and later subjected to an anion exchange chromatography column. The process generates two fractions, the water eluted portion (BTWE) with 0.06% yield and the salt eluted portion (BTSE) with 0.1% yield, both yields based on the weight of initial freeze-dried plant material. Fractions were then submitted to the structural characterization analysis.

BTWE showed a relative M_w of 10 kDa (FIGURE 2) and contains arabinose and galactose as main monosaccharides, followed by low amounts of mannose, fucose, glucose,

xylose and rhamnose (TABLE 1). Monosaccharide composition indicated the presence of an arabinogalactan as the main component of the fraction, which was then confirmed with the methylation analysis. The main methylated derivatives of carboxyl reduced sample were 2,3,5-Me₃-Ara and 2,3-Me₂-Ara from terminal and 5-*O*-substituted Araf units; 2,3,4-Me₃-Fuc from terminal Fucp units; 2,3,4,6-Me₄-Glc and 2,3,6-Me₃-Glc from terminal and 4-*O*-substituted Glcp units and 2,3,4-Me₃-Gal and 3,4-Me₂-Gal from 6-*O*- and 2,6-di-*O*-substituted Galp units (TABLE 2). Other derivatives observed were from terminal Rhap units; from 3-*O*, 3,5-di-*O* and 2,5-di-*O*-substituted Araf units; from terminal, 2-*O* and 6-*O*-substituted Manp units and from terminal, 4-*O*, 3,6-di-*O*, 4,6-di-*O*-substituted Galp units. Its HSQC-DEPT correlation map showed the anomeric cross signals at δ 108.9/5.25 and δ 107.3/5.07 from terminal and $\rightarrow$ 5)- α -L-Araf-(1 $\rightarrow$ units, at δ 103.1/4.46, δ 102.7/ 4.51 and δ 101.9/4.58 from $\rightarrow$ 6)- β -D-Galp-(1 $\rightarrow$, $\rightarrow$ 2,6)- β -D-Galp-(1 $\rightarrow$ and terminal β -D-Galp, at δ 99.9/5.16 from terminal α -L-Fucp and at δ 99.7/5.18 from terminal α -L-Rhap (FIGURE 3A). BTWE showed, in addition, signals of C6/H6 at δ 16.9/1.29 from α -L-Rhap and δ 15.7/1.23 from α -L-Fucp; inverted DEPT signals at δ 69.4/3.92–69.0/4.03 from 6-*O*-linked β -D-Galp units and at δ 66.6/3.81–66.8/3.87 from 5-*O*-linked α -L-Araf units and also inverted signals at δ 61.7/3.73, δ 61.7/3.81, δ 60.8/3.76 and δ 61.0/3.85 from unsubstituted C-6/H-6 or C-5/H-5 from α -L-Araf-(1 $\rightarrow$ and β -D-Galp-(1 $\rightarrow$. The assignments are in agreement with literature data and together with methylation analysis showed that BTWE is composed mainly by a type II arabinogalactan with mainly terminal substitutions of α -L-Fucp and α -L-Araf units (BILAN et al., 2018; CHAVES; IACOMINI; CORDEIRO, 2019; DAMMAK et al., 2019; GOLOVCHENKO et al., 2012; NASCIMENTO et al., 2013; OLIVEIRA et al., 2017).

Arabinogalactans (AGs) with the main chain formed by (1 $\rightarrow$ 3), (1 $\rightarrow$ 6)-linked galactose can be classified as type II AG, that can be found as branches in *O*-4 of Rhap residues from rhamnogalacturonans or linked to proteins, composing arabinogalactan proteins (CAPEK et al., 2010; CARPITA; GIBEAUT, 1993; CIPRIANI et al., 2009b; PETTOLINO et al., 2012; WANG et al., 2015). These polysaccharides are frequently found in plant aqueous extracts and in species of Asteraceae family the presence of AG was described for *Chamomilla recutita* (CHAVES; IACOMINI; CORDEIRO, 2019), *Artemisia campestris* (CORRÊA-FERREIRA et al., 2018), *Arctium lappa* (CARLOTTO et al., 2016) *Chrysanthemum morifolium* (LIANG et al., 2014), *Vernonia kotschyana* (NERGARD et al., 2005), *Calendula officinalis* (VARLJEN; LIPTÁK; WAGNER, 1989), among others.

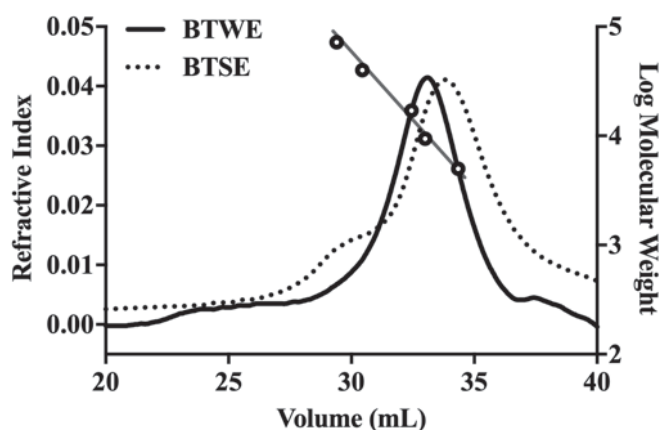


FIGURE 2 - HPSEC ELUTION PROFILE OF FRACTIONS BTWE AND BTSE. REFRACTIVE INDEX DETECTOR. ELUTION VOLUME OF DEXTRAN STANDARDS OF MOLECULAR WEIGHT 72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa AND 5.0 kDa (LEFT TO RIGHT) WERE EMPLOYED TO BUILD THE CALIBRATION CURVE.

TABLE 1 - MONOSACCHARIDE COMPOSITION OF FRACTIONS OBTAINED FROM *B. trimera* INFUSION.

Fractions	Neutral monosaccharides ^a							Uronic acid ^b
	Rha	Fuc	Ara	Xyl	Man	Glc	Gal	
BTWE	3.4	6.1	41.5	3.5	13.3	7.6	24.6	-
BTSE	10.8	4.4	60.9	4.7	4.9	-	9.3	5

^a % of peak area relative to total peak area, determined by GC-MS.

^b Determined using the m-hydroxybiphenyl method (FILISSETTI-COZZI; CARPITA, 1991).

TABLE 2 - LINKAGE TYPES BASED ON ANALYSIS OF PARTIALLY *O*-METHYL ALDITOL ACETATES OBTAINED FROM METHYLATED POLYSACCHARIDE FRACTIONS.

Partially O-methylalditol acetate	BTWE ^a	BTSE ^a	Linkage type ^b
2,3,4-Me ₃ -Rha ^c	1.8	5.1	Rha-(1→
3-Me-Rha	-	1.3	→2,4)-Rha-(1→
2,3,4-Me ₃ -Fuc	11.3	5.3	Fuc-(1→
2,3,5-Me ₃ -Ara	10.4	8.9	Ara-(1→
2,5-Me ₂ -Ara	1.3	1.2	→3)-Ara-(1→
2,3-Me ₂ -Ara	11.2	8.9	→5)-Ara-(1→

2-Me-Ara	2.0	0.7	→3,5)-Ara-(1→
3-Me-Ara	1.0	-	→2,5)-Ara-(1→
2,3,4,6-Me ₄ -Man	2.5	4.2	Man-(1→
3,4,6-Me ₃ -Man	4.4	2.0	→2)-Man-(1→
2,3,4-Me ₃ -Man	3.5	2.3	→6)-Man-(1→
2,3,4,6-Me ₄ -Glc	6.3	16.0	Glc-(1→
2,3,6-Me ₃ -Glc	8.3	4.7	→4)-Glc-(1→
2,3,4-Me ₃ -Glc	2.1	2.6	→6)-Glc-(1→
2,3,4,6-Me ₄ -Gal	5.7	19.9	Gal-(1→
2,3,6-Me ₃ -Gal	2.3	3.2	→4)-Gal-(1→
2,3,4-Me ₃ -Gal	11.9	10.5	→6)-Gal-(1→
2,4-Me ₂ -Gal	0.6	0.8	→3,6)-Gal-(1→
2,3-Me ₂ -Gal	1.1	0.6	→4,6)-Gal-(1→
3,4-Me ₂ -Gal	12.1	1.6	→2,6)-Gal-(1→

^a % of peak area of *O*-methyl alditol acetates relative to total area, determined by GC-MS.

Fraction was carboxyl reduced by Taylor and Conrad (1972) method.

^b Based on derived *O*-methyl alditol acetates.

^c 2,3,4-Me₃-Rha = 2,3,4-tri-*O*-Methylrhamnitol acetate, etc.

Regarding the type II AGs from Asteraceae family cited above, where terminal monosaccharide were mainly arabinose, galactose and glucose. Here, the results showed a high amount of fucose and this is a distinction for the polymer from *B. trimera* (TABLE 2). For plant species from Asteraceae family the high fucose containing type II AG was not described until now, however this terminal Fucp units were found in polysaccharides from *Raphanus sativus* (INABA et al., 2015), *Pereskia aculeata* (MARTIN et al., 2017) and *Arabidopsis thaliana* (TRYFONA et al., 2012).

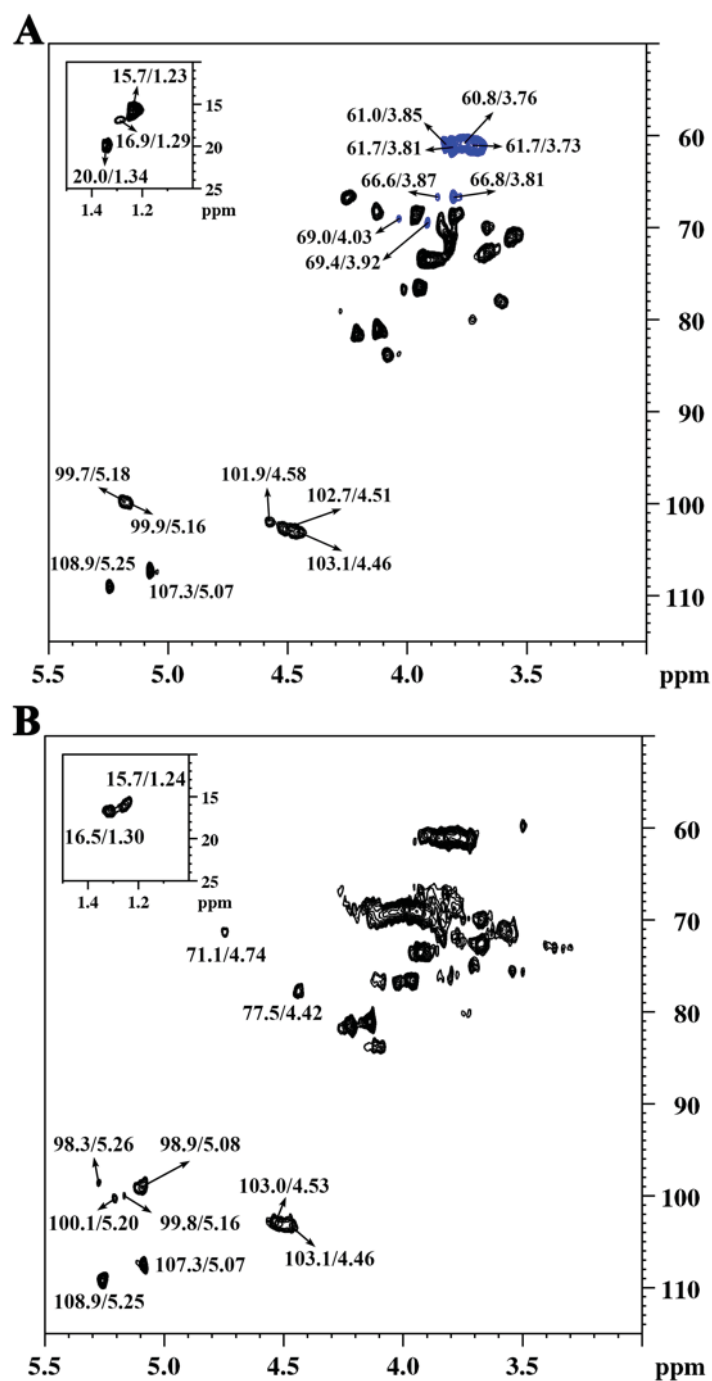


FIGURE 3 - HSQC-DEPT CORRELATION MAP OF BTWE FRACTION (A) AND HSQC CORRELATION MAP OF BTSE FRACTION (B) IN D₂O AT 70 °C. THE CHEMICAL SHIFTS ARE EXPRESSED AS δ PPM. Note: Inverted signals in DEPT experiment are shown in blue color.

Proceeding with the chemical characterization, BTSE showed a main peak in HPSEC analysis with a relative M_w of 7 kDa (FIGURE 2) and presented arabinose, rhamnose and galactose as main monosaccharides, followed by low amounts of uronic acids, mannose, xylose and fucose (TABLE 1). Methylation analysis of carboxyl reduced sample showed

main methylated derivatives 2,3,5-Me₃-Ara and 2,3-Me₂-Ara from terminal and 5-*O*-substituted Araf units; 2,3,4,6-Me₄-Glc and 2,3,6-Me₃-Glc from terminal and 4-*O*-substituted Glcp units and 2,3,4,6-Me₃-Gal and 2,3,4-Me₂-Gal from terminal and 6-*O*-substituted Galp units (TABLE 2). Other derivatives observed were from terminal and 2,4-di-*O*-substituted Rhap units; from 3-*O* and 3,5-di-*O*-substituted Araf units; from terminal, 2-*O* and 6-*O*-substituted Manp units; from 6-*O*-substituted Glcp units and from, 6-*O*, 3,6-di-*O*, 2,6-di-*O*-substituted Galp units. Since fraction was carboxyl reduced, the derivatives 2,3,6-Me₃-Gal and 3,6-Me₂-Gal were attributed to 4-*O* and 2,4-di-*O*-substituted GalpA units. The HSQC correlation map from BTSE showed the anomeric cross signals at δ 108.9/5.25 and δ 107.3/5.07 from terminal and $\rightarrow$ 5)- α -L-Araf-(1 $\rightarrow$ units, at δ 103.1/4.46 and δ 103.0/ 4.53 from $\rightarrow$ 6)- β -D-Galp-(1 $\rightarrow$ and $\rightarrow$ 2,6)- β -D- Galp-(1 $\rightarrow$ units, at δ 100.1/5.20 from terminal α -L-Rhap units, at δ 99.8/5.16 from terminal α -L-Fucp units, at δ 98.9/4.08 from $\rightarrow$ 4)- α -D-GalpA-(1 $\rightarrow$ units and at δ 98.3/5.26 from $\rightarrow$ 2)- α -L-Rhap -(1 $\rightarrow$ units (FIGURE 3B). The correlation map also presented signals of C6/H6 at δ 16.5/1.30 from α -L-Rhap and δ 15.7/1.24 from α -L-Fucp; of C5/H5 at δ 71.1/4.74 and C4/H4 at δ 77.5/4.42 from α -D-GalpA units. The assignments are in agreement with published literature data and methylation analysis (BILAN et al., 2018; CHAVES; IACOMINI; CORDEIRO, 2019; DAMMAK et al., 2019; GOLOVCHENKO et al., 2012; NASCIMENTO et al., 2013; OLIVEIRA et al., 2017).

Results showed that BTSE is composed of a type II arabinogalactan with some differences from the AG found in BTWE fraction. Similarly, the main chain of the AG is composed by (1 $\rightarrow$ 6) β -D-Galp. However, the ratio of terminal monosaccharides changed. In the AG from BTSE it was observed fewer fucose, and higher amounts of galactose and glucose. This AG also seems to be branch of a type I rhamnogalacturonan. Type II AGs are part of the pectic matrix of plant cell wall and this promotes its unique characteristics (CARPITA; GIBEAUT, 1993). Changes of molecular mass, branching ratio, branching length and also terminal monosaccharides can be observed according to the plant species, developmental period, plant parts and, as cited above, these differences could play a crucial role in the biological activities presented by these polymers (CARPITA; GIBEAUT, 1993; FERREIRA et al., 2015; YANG; ZHANG, 2009).

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4.4 ARTIGO IV

INULIN SUPPLEMENTATION CHANGES EXTENSOR DIGITORUM LONGUS (EDL) MAXIMUM ISOMETRIC TWITCH FORCE AND MAXIMUM SPEED OF FORCE DECREASE IN MICE SUBMITTED TO ENDURANCE.

Pedro Felipe Pereira Chaves^a, João Victor Capelli Peixoto^b, Rosalvo Tadeu Hochmuller Fogaça^b, Marcello Iacomini^a, Lucimara Mach Côrtes Cordeiro^a

^a *Biochemistry and Molecular Biology Department, Federal University of Paraná, CP 19.046, CEP 81.531-980 Curitiba, PR, Brazil.*

^b *Physiology Department, Federal University of Paraná, CEP 81.531-980 Curitiba, PR, Brazil*

ABSTRACT

Skeletal muscle adaptation plays a crucial role in the physical performance and endurance. Since inulin is a dietary fiber that can promote diverse health benefits, we tested the hypothesis that inulin supplementation could interfere in the contractile parameters of soleus and EDL from mice submitted to endurance training. The method utilized was the forced swim, where mice were trained for six weeks and then submitted to the *ex vivo* analysis of muscles. Data obtained for soleus muscle from supplemented groups presented no statistical differences from the control. However, EDL muscle from animals supplemented with inulin at 50 and 100 mg/kg presented a significative change in maximum isometric twitch force (Tmax) and maximum speed of force decrease ($-dF/dt$). The results indicated that inulin supplementation could interfere in the contractile adaptation of EDL muscle of mice submitted to endurance.

Keywords: Inulin; skeletal muscle; soleus; extensor digitorum longus.

1. INTRODUCTION

Inulin is a storage polysaccharide found in a wide range of plants and can be found in commonly consumed vegetables, for example, onion, garlic, artichoke, banana, barley and wheat (APOLINÁRIO et al., 2014; VAN LOO et al., 1995). The molecule is a fructan polymer composed mostly or exclusively of β -(2 $\rightarrow$ 1) fructosyl-fructose linkages and this chemical characteristic makes it escape digestion in the upper gastrointestinal tract and reach the large intestine (CHAVES; IACOMINI; CORDEIRO, 2019; GIBSON; ROBERFROID, 1995). For this feature it can be categorized as a dietary fiber (GIBSON; ROBERFROID, 1995; ROBERFROID, 2007).

It is well established in the literature that inulin is a versatile substance with numerous health benefits. Between the systemic improvements observed, it can act as immunomodulator, can regulate absorption of minerals and lipids and also can act in physical condition retarding fatigue (ROBERFROID, 2007; SEIFERT; WATZL, 2007; ZHANG et al., 2009).

Physical condition is a highly relevant topic for athletes and physically active people seeking to enhance their performance and it can be associated to the contractile adaptations of skeletal muscle (POWERS; HOWLEY, 2017). The phenotypic and metabolic adaptations suffered by skeletal muscles occur according to the physical training that they are submitted and can be accelerated or improved with the assistance of diet or pharmaceuticals (COFFEY; HAWLEY, 2007; POWERS; HOWLEY, 2017; SEENE; KAASIK; ALEV, 2011). Despite the plenty interest in identifying compounds that can accelerate muscle adaptation without causing side effects, data of supplementation with dietary fibers linked to muscle contractile alterations are not yet found in literature. Thus, in the present study the contractile measurements of soleus and EDL muscles from mice supplemented with inulin and submitted to endurance training were for the first time described.

2. MATERIALS AND METHODS

2.1. Chemicals

Inulin was previously purified from the infusion of *Mikania glomerata* Spreng plant species (CHAVES et al., 2019b). Ringer's solution chemicals were all from Sigma-Aldrich Co.

2.2 Animals

Male Swiss mice (*Mus musculus*, 25–35 g) were obtained from the Federal University of Paraná vivarium and kept at 12 h light/dark cycles, at temperature of 22 ± 2 °C, with food and water provided *ad libitum*. They were acclimated to the laboratory for 48 h before the beginning of training. The experiments were performed after approval (Approval nº 1129) of the respective protocols by the Committee of Animal Experimentation of the Federal University of Paraná and the study was conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" (NATIONAL RESEARCH COUNCIL (US) COMMITTEE FOR THE UPDATE OF THE GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS, 2011).

2.3 Experiment design

Animals were randomly separated in the following groups, each consisting of eight ($n = 8$) animals: (a) sedentary; (b) trained; (c) treated inulin at 1 mg/kg; (d) treated inulin at 50 mg/kg and (e) treated inulin at 100 mg/kg. Sedentary group was the untrained control and the remaining groups were submitted to endurance training.

Endurance training was adapted from the forced swim method (KLOSTERHOFF et al., 2018; YEH et al., 2014). Animals were submitted to swimming for 6 weeks, with 2 days of rest at the end of each week, in tanks (40×65 cm) with 4 compartments containing water at 32 ± 4 °C until 4/5 of its capacity. In the 1st week, the animals were adapted to the aquatic environment as follows: 1st day they underwent to 30 min of swimming; 2nd day, to 40 min and 3rd to 5th day, to 60 min. In the 2nd and 3rd week, the training was performed for 40 min with 2% of the mice weight coupled to the animal's tail (FIGURE 1). From the 4th to the 6th,

the weight coupled was increased to 5%. The entire trainings were monitored by trained persons, preventing the animals from drowning at any time.

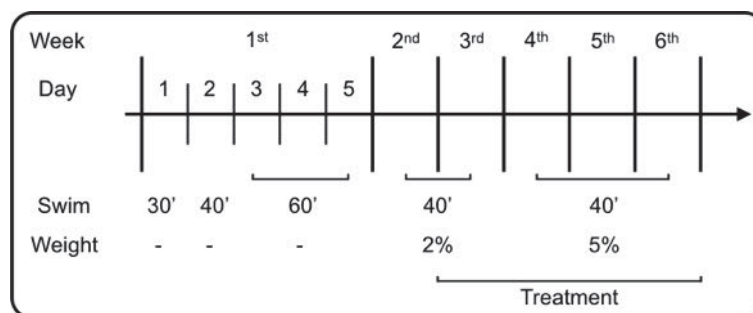


FIGURE 1 - TIMELINE OF ENDURANCE TRAINING, TREATMENT AND WEIGHT LOAD.

Supplementation were performed by gavage from the beginning of 3rd week until the last day of the experiment, one hour before training. Distilled water was used for sedentary and trained groups and inulin at 1, 50 and 100 mg/kg concentration for the supplemented groups.

48 h after the last training the animals were anesthetized (ketamine hydrochloride 100 mg/kg + xylazine hydrochloride 10 mg/kg, ip), the lower limbs were immobilized, one soleus and one EDL were collected and immediately used in the *ex vivo* contractile measurements.

2.4 *Ex vivo* contractile measurements

The method for contractile measurements was previously described by Woodall et al. (2016). Muscles collected were kept in individual chambers with Ringer's solution (NaCl 110 mM; KCl 4 mM; CaCl₂ 2 mM; MgCl₂ 2 mM; TRIZMA® 10 mM and Glucose 11 mM) with pH 7.4, gassed with pure oxygen and maintained at 37 °C. To measure contractions under isometric conditions, one extremity of the tendinous portion of the muscle was fixed to a stationary clip while the other was attached to a force transducer (Fort 10 WPI, Transduction Laboratories Co.). For soleus, optimum muscle length (Lo) was determined from the length at which the isometric twitch force was maximal. For EDL, muscles were stretched to a pre-set load of 33% of maximum tetanic force for each muscle, through a micro manipulator coupled to the stationary clip. The electrical stimulation was performed with one millisecond pulses of suppressive voltage (24 V) in a standard frequency of 0.5 Hz

during seven minutes, directly through two platinum electrodes positioned along the muscle in the chamber. The parameters evaluated were maximum isometric twitch force (T_{max}), maximum speed of force development ($+dF/dt$) and maximum speed of force decrease ($-dF/dt$). For soleus, the results were obtained as mean values of total time (7 min) for each group. For EDL, the results were obtained as mean values of each minute for each group. For both muscles at the end of the seventh minute, the stimulus frequency was increased to 100 Hz, where the maximum tetanic force (F_{max}) and time of resistance to sustained contraction from F_{max} to 50% (RSC) were recorded. The values of T_{max} , $+dF/dt$, $-dF/dt$ and F_{max} were normalized by the muscles cross-sectional area (CSA). CSA was calculated using the formula: $\text{muscle mass}/(L_o(L_f/L_o)\rho)$. L_f/L_o is the ratio of fiber length to optimal muscle length (0.69 for soleus and 0.45 for EDL) and (ρ) the density of muscle (1.06 g/cm^3). The data were acquired using a PowerLab 4/30 acquisition system (AD Instrument) and subsequently analyzed using Lab Chart version 7.3.7 software.

2.5 Statistical analysis.

Data from each group were expressed as mean $\pm$ standard error of the mean. Statistical analyzes were performed using one-way ANOVA followed by Bonfferoni's *post hoc* test or two-way ANOVA followed by Dunnett's *post hoc* test with Graphpad Prism 7.0a software (San Diego, CA, USA). A p-value lower than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

Soleus and EDL muscles of mice were chosen to evaluate the inulin supplementation due to their distinct metabolic profiles. Soleus consists of an average of 22% type I fibers and 78% type IIa fibers, which makes its energy demand predominantly being oxidative, also presenting high resistance to fatigue and low strength development. On the opposite, EDL consists of an average of 12% type I fibers, 28% type IIa fibers and 60% type IIX fibers, which makes its energy demand predominantly glycolytic, with low resistance to fatigue and with high strength development (MORISSETTE et al., 2014).

In the contractile parameters (T_{max} , $+dF/dt$, $-dF/dt$, F_{max} and RSC) evaluated in soleus, as well as in the CSA measures, supplemented groups did not present statistical differences when compared to controls (FIGURE 2 and 3 A-C). Soleus muscle mechanism

of adaptations were linked with the increased oxidative capacity due to mitochondrial biogenesis, vascularization and fiber shift (WAHRMANN; WINAND; RIEU, 2001). Our results indicate that supplementation with inulin did not affect these metabolic bases to promote a contractile change.

EDL from supplemented groups did not presented statistical differences when compared to controls in Fmax and RSC contractile parameters and in the CSA measures (Fig 3 D-F). However, 50 and 100 mg/kg supplemented groups obtained a change in Tmax and -dF/dt when compared to Trained and Sedentary groups, and in +dF/dt when compared to Sedentary group (Fig 4). Both inulin-supplemented groups presented an increase in Tmax force of 36%, 33% and 21% in the first, second and third minute of protocol, respectively, and a decrease in -dF/dt of 28%, 23% and 20% in the first, second and third minute of protocol, respectively, when compared to Trained control group (Fig 4 A, C). When compared to Sedentary control, they presented an increase in Tmax force of 19%, 17% and 13% in the first, second and third minute of protocol, respectively, and a decrease in -dF/dt of 22%, 19% and 22% in the first, second and third minute of protocol, respectively. Still when compared to Sedentary control, an increase in +dF/dt of 25%, 32% and 53% in the third, sixth and seventh minute for 50 mg/kg group and increase of 20% and 30% in the second and third minute for 100 mg/kg group was observed (Fig 4 B). 1 mg/kg supplemented group did not present statistical differences when compared to both controls (Fig 4).

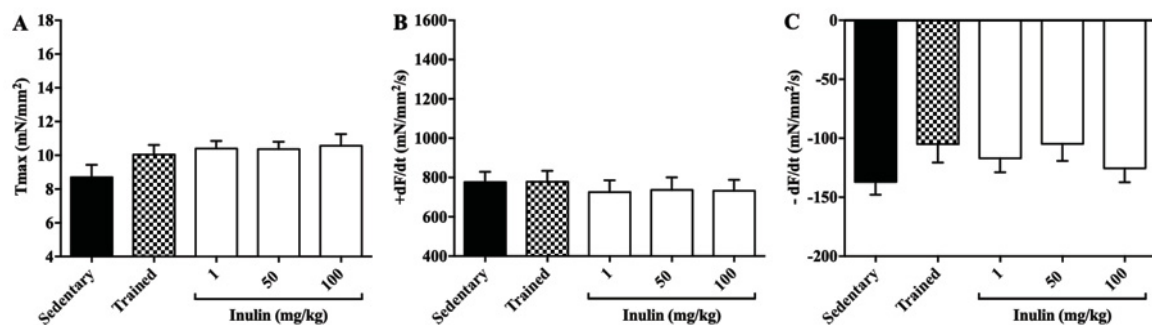


FIGURE 2 - *Ex vivo* CONTRACTILE MEASUREMENTS OF MAXIMUM ISOMETRIC TWITCH FORCE (A), MAXIMUM SPEED OF FORCE DEVELOPMENT (B) AND MAXIMUM SPEED OF FORCE DECREASE (C) IN SOLEUS MUSCLE OF MICE. GROUPS (n = 8): SEDENTARY (DISTILLED WATER); TRAINED (DISTILLED WATER + ENDURANCE TRAINING) AND 1-100 mg/kg (1-100 mg/kg OF INULIN + ENDURANCE TRAINING). VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY UNIDIRECTIONAL ANOVA AND BONFERRONI'S *post hoc* TEST.

Adaptations to endurance reported for EDL includes the change in muscular fiber types ratio, change in capillary supply, increases in the oxidative capacity, changes in key metabolic enzymes involved in fatty acid activation and also change in muscular glycogen reserve (SEENE et al., 2007; SEENE; KAASIK; ALEV, 2011). Even that the changes observed in the present work cannot directly point to most of these adaptations, an increase in muscle strength, as observed (FIGURE 4 A), can be associated to an increase in muscular glycogen reserve (ØRTENBLAD et al., 2011). Similar alterations observed here for EDL, were also described for mice supplemented with distinct compounds, such as the mineral selenium (BODNÁR et al., 2016), the amino acid taurine (GOODMAN et al., 2009) and vitamin D (HAYES et al., 2019).

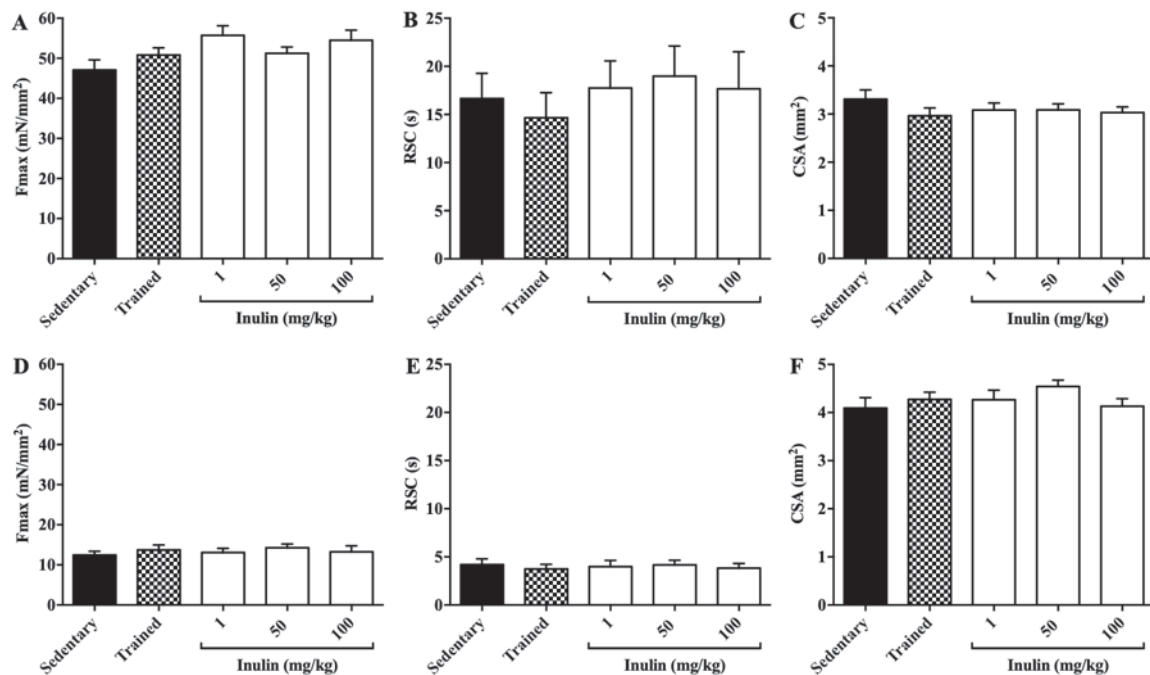


FIGURE 3 - *Ex vivo* MEASUREMENTS OF MAXIMUM TETANIC FORCE (A, D), TIME OF RESISTANCE TO SUSTAINED CONTRACTION (B, E) AND CROSS-SECTIONAL AREA (C, F) IN SOLEUS (A-C) AND EDL (D-F) MUSCLES OF MICE. GROUPS (n=8): SEDENTARY (DISTILLED WATER); TRAINED (DISTILLED WATER + ENDURANCE TRAINING) AND 1-100 mg/kg (1-100 mg/kg OF INULIN + ENDURANCE TRAINING). VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY UNIDIRECTIONAL ANOVA AND BONFERRONI'S *post hoc* TEST.

Skeletal muscle contractile alterations by polysaccharide supplementation have not been reported so far, but several papers describe the activity of structurally diverse polysaccharides on the physical performance of animals. Among them, there is one study on

the antifatigue effect by supplementation with inulin rich fractions (ZHANG et al., 2009). In the paper, the authors suggested that the observed effects were due to the immunostimulant properties of inulin.

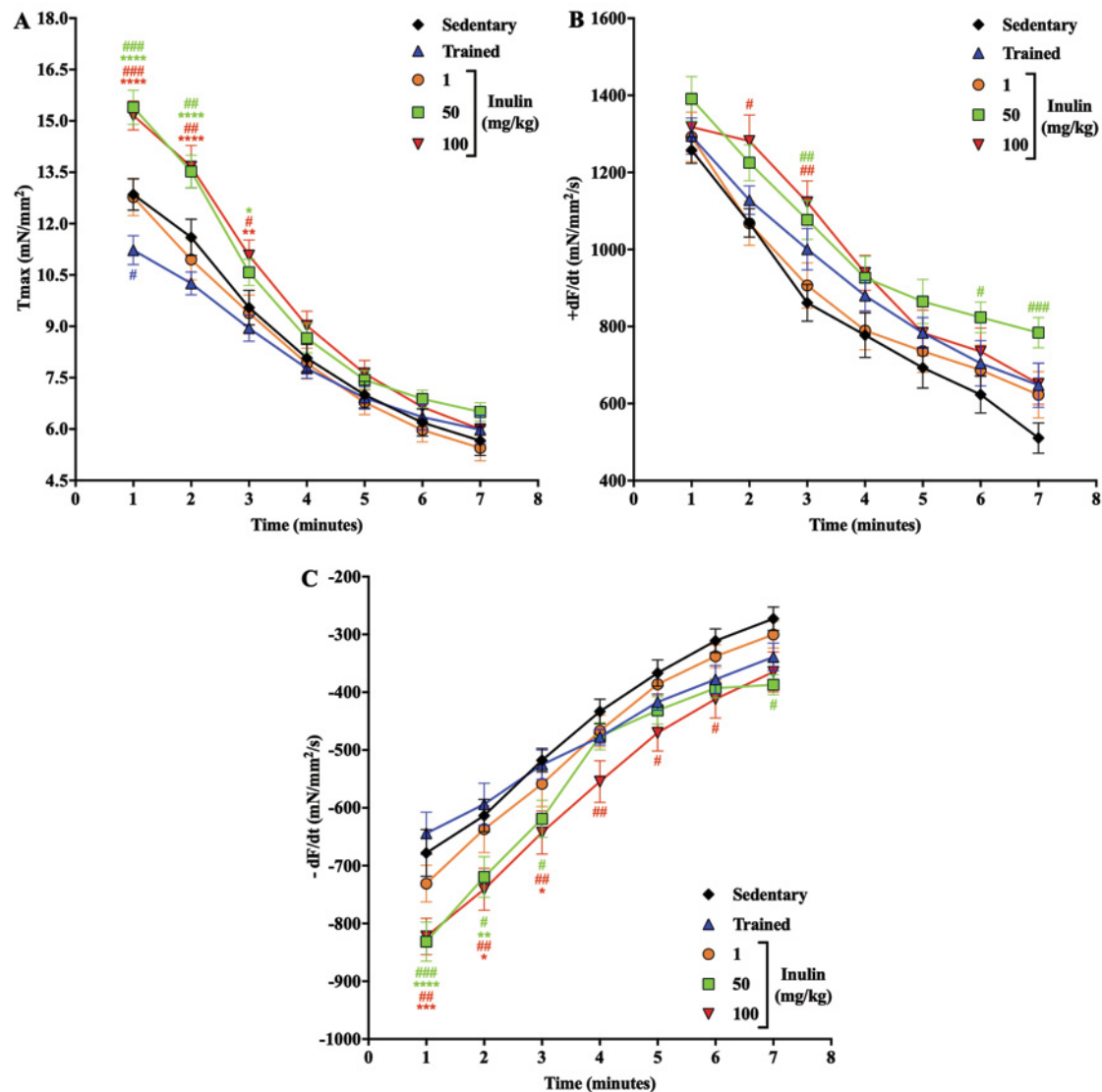


FIGURE 4 - *Ex vivo* CONTRACTILE MEASUREMENTS OF MAXIMUM ISOMETRIC TWITCH FORCE (A), MAXIMUM SPEED OF FORCE DEVELOPMENT (B) AND MAXIMUM SPEED OF FORCE DECREASE (C) IN EDL MUSCLE OF MICE. GROUPS (n=8): SEDENTARY (DISTILLED WATER); TRAINED (DISTILLED WATER + ENDURANCE TRAINING) AND 1-100 mg/kg (1-100 mg/kg OF INULIN + ENDURANCE TRAINING). VALUES WERE EXPRESSED AS MEAN $\pm$ STANDARD ERROR OF THE MEAN, ANALYZED BY BIDIRECTIONAL ANOVA AND DUNNETT'S *post hoc* TEST. SYMBOLS: * P < 0.05, ** P < 0.005, *** P < 0.0005 AND **** P < 0.0001 STATISTICALLY DIFFERENT FROM TRAINED CONTROL GROUP; # P < 0.05, ## P < 0.005 AND ### P < 0.0005 STATISTICALLY DIFFERENT FROM SEDENTARY CONTROL GROUP.

Inulin is a known prebiotic polymer and the administration of prebiotics can be directly linked to an improvement in body immunity (HAULY; MOSCATTO, 2002). It is already described that this improvement can have a positive influence in the physical performance in intense exercises, as immune responses caused through the physical stress can be attenuated (MACH; FUSTER-BOTELLA, 2017). The improvement of the intestinal microbiota can also lead to a improve in the absorption of minerals and selenium, which has showed a significant influence in muscle contractile parameters (BODNÁR et al., 2016; GIBSON; ROBERFROID, 1995). Moreover, polysaccharides may indirectly influence the muscle energy metabolism as a result of their metabolization by gut microbiota, which can lead to the production of short chain fatty acids that can be used in muscle maintenance and activity (GIBSON; ROBERFROID, 1995; POWERS; HOWLEY, 2017).

Comparing Sedentary and Trained controls groups is possible to notice that the statistical differences observed are few, this could be indicative that the intensity or interval of endurance training utilized may be insufficiently to promote a prominent adaptation in mice muscle, even though a slight increase the intensity of the force swim method was made (COFFEY; HAWLEY, 2007; SEENE; KAASIK; ALEV, 2011). Thus, further work is needed to evaluate whether or not inulin supplementation influence mice submitted to endurance promoting changes in the parameters that did not show statistical differences here, as well as to elucidated the mechanisms of action.

4. CONCLUSION

The present study showed that mice supplemented with inulin at 50 and 100 mg/kg caused an increase in EDL maximum isometric twitch force and a decrease of maximum speed of force decrease in endurance training when compared with Trained mice with standard diet. This indicate that a dietary fiber could be interfering in the contractile adaptation of a glycolytic muscle submitted to endurance and for our concern it is the first time which this evidence is reported in literature.

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5. CONSIDERAÇÕES FINAIS

Com os resultados obtidos a partir desta tese foi constatado que os infusos preparados a partir das espécies *Mikania glomerata* (Guaco) e *Baccharis trimera* (Carqueja) possuem carboidratos complexos biologicamente ativos. A partir do infuso proveniente do guaco foi purificada e caracterizada uma frutana do tipo inulina, composta por unidades de ligadas frutose β -(2 $\rightarrow$ 1) e ainda identificados frutooligosacarídeos com grau de polimerização variando de 2 a 10 unidades de frutose. A partir do infuso proveniente da carqueja foi purificada e caracterizada uma frutana com as mesmas características citadas acima e, em baixa quantidade, arabinogalactanas do tipo II. As frações ricas em inulina e a rica em frutooligosacarídeos apresentaram ação sobre a função hepática, promovendo proteção contra os danos induzidos por CCl₄ sem apresentar toxicidade. Ainda, a fração composta por inulina proveniente de *M. glomerata* pôde promover alterações na força de contração isométrica máxima e na velocidade máxima de diminuição de força nos músculos EDL de camundongos.

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ANEXOS



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



Nº 1000

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: 23075.142125/2016-11

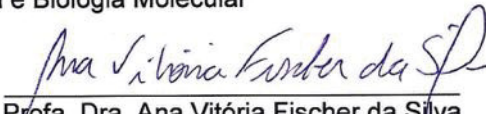
APROVADO/APPROVAL: 13/09/2016 – R.O. 08/2016

TÍTULO: Efeitos dos polissacarídeos dos infusos de carqueja (*Baccharis trimera* Less. DC) e guaco (*Mikania glomerata* Spreng) no modelo de hepatotoxicidade induzida por CCl₄.

TITLE: Effects of polisaccharides from carqueja (*Baccharis trimera* Less. DC) e guaco (*Mikania glomerata* Spreng) infusions on CCl₄ induced hepatotoxicity model.

AUTORES/AUTHORS: Lucimara Mach Cortes Cordeiro, Alexandra Acco, Pedro Felipe Pereira Chaves, Eliana Rezende Adami.

DEPARTAMENTO/DEPARTMENT: Bioquímica e Biologia Molecular


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



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



Nº 1129

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.

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PROCESSO/PROCESS: 23075.218239/2017-12

APROVADO/APPROVAL: 12/12/2017 – R.O. 11/2017

TÍTULO: Efeitos de polissacarídeos complexos na fadiga e nas alterações morfofuncionais e bioquímicas do músculo esquelético de camundongos.

TITLE: Effects of complex polysaccharides on fatigue and morphofunctional and biochemical changes in skeletal muscle of mice.

AUTORES/AUTHORS: Lucimara Mach Cortes Cordeiro, Rosalvo T. H. Fogaça, Aleandra Acco, João Victor Capelli Peixoto, Pedro Felipe Pereira Chaves, Eliana Rezende Adami, Daniel Tourinho Sentone.

DEPARTAMENTO/DEPARTMENT: Bioquímica e Biologia Molecular

Prof. Dra. Katya Naliwaiko
Coordenadora da CEUA