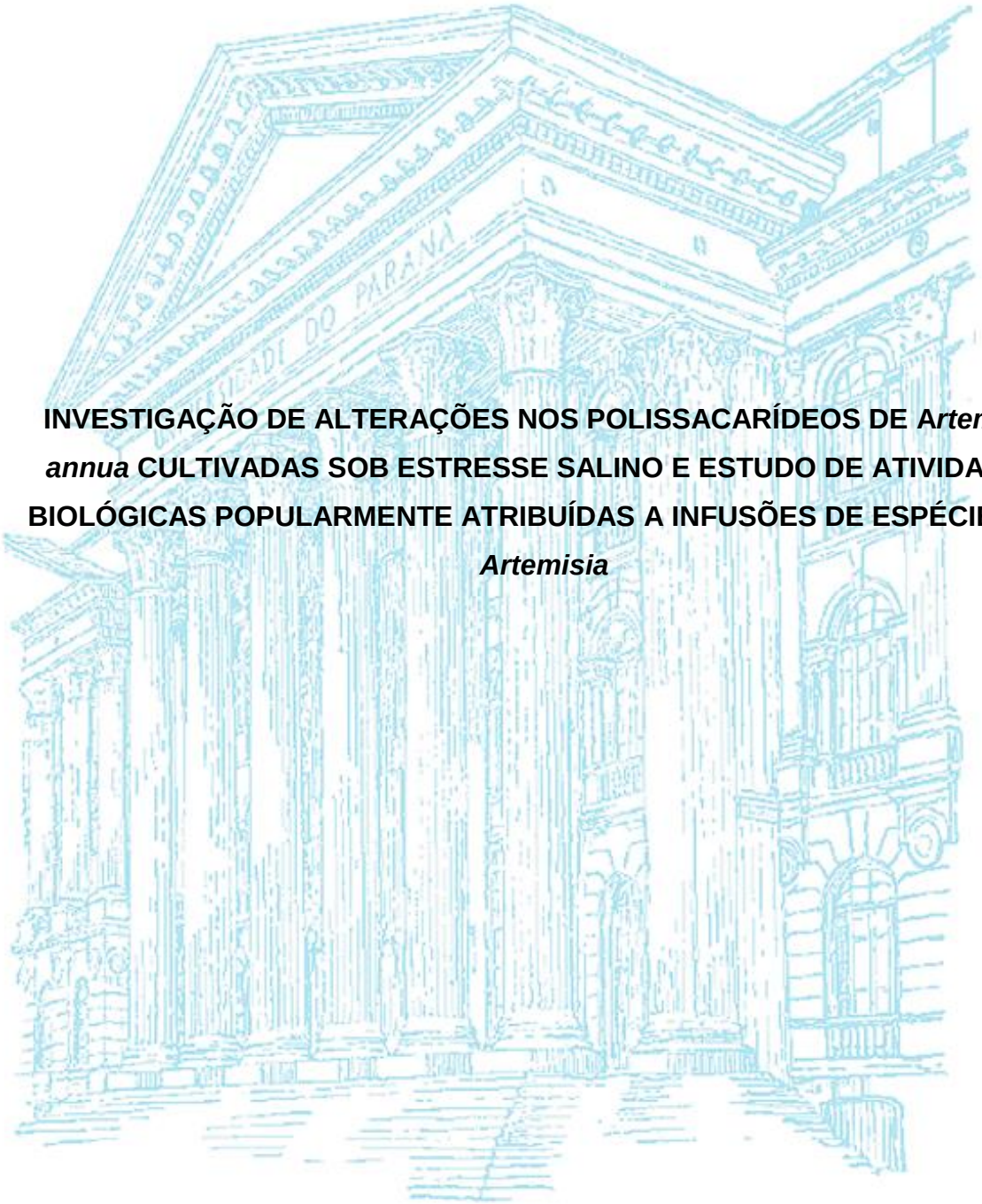
UNIVERSIDADE FEDERAL DO PARANÁ

MARILIA LOCATELLI CORRÊA FERREIRA



INVESTIGAÇÃO DE ALTERAÇÕES NOS POLISSACARÍDEOS DE *Artemisia annua* CULTIVADAS SOB ESTRESSE SALINO E ESTUDO DE ATIVIDADES BIOLÓGICAS POPULARMENTE ATRIBUÍDAS A INFUSÕES DE ESPÉCIES DE *Artemisia*

CURITIBA

2016

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INVESTIGAÇÃO DE ALTERAÇÕES NOS POLISSACARÍDEOS DE *Artemisia annua* CULTIVADAS SOB ESTRESSE SALINO E ESTUDO DE ATIVIDADES BIOLÓGICAS POPULARMENTE ATRIBUÍDAS A INFUSÕES DE ESPÉCIES DE *Artemisia*

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Orientadora: Profª Dra. Carmen Lúcia de Oliveira Petkowicz

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **MARILIA LOCATELLI CORREA FERREIRA**, intitulada: "**Investigação de alterações nos polissacarídeos de *Artemisia annua* cultivadas sob estresse salino e estudo de atividades biológicas popularmente atribuídas a infusões de espécies de Artemisia**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação.

CURITIBA, 09 de Dezembro de 2016.

CARMEN LUCIA DE OLIVEIRA PETKOWICZ
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Dedico esta tese à base fundamental da minha vida:
Minha família!
É por vocês que cheguei até aqui e seguirei sempre em frente!

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“Você não sabe o quanto eu caminhei... Pra chegar até aqui. Percorri milhas e milhas antes de dormir, eu não cochilei. Os mais belos montes escalei..”

NOTA EXPLICATIVA

Esta tese é apresentada em formato alternativo, inserindo-se os manuscritos desenvolvidos no decorrer do doutorado, de acordo com as normas do Programa de Pós-Graduação em Ciências - Bioquímica, da Universidade Federal do Paraná. A tese consta de uma introdução, revisão bibliográfica, objetivos gerais e específicos do trabalho, artigos científicos com os experimentos realizados e conclusões.

RESUMO

As plantas do gênero *Artemisia* são amplamente conhecidas por suas propriedades medicinais. No presente trabalho, três espécies foram estudadas: *A. annua*, *A. vulgaris* e *A. campestris subsp. maritima*. Estas espécies são amplamente estudadas quanto aos seus metabólitos secundários, mas pouco se conhece sobre os compostos do metabolismo primário destas plantas, tais como os polissacarídeos. Neste estudo, diferentes abordagens foram empregadas no estudo dos polissacarídeos destas espécies. Para *A. annua*, foram avaliadas as modificações nos polissacarídeos da parede celular promovidas pela exposição das plantas ao estresse salino. Para as plantas *A. vulgaris* e *A. campestris*, polissacarídeos isolados foram investigados quanto às suas potenciais atividades biológicas. As plantas de *A. annua* foram cultivadas na presença ou ausência de NaCl nas concentrações de 100 e 200 mM por 45 dias. Após colheita, extrações sequenciais usando água fervente (W), EDTA 100 mM (E) e NaOH 6 M, foram realizadas para obtenção dos polissacarídeos da parte aérea e raízes. Pectinas foram os polissacarídeos majoritários nas frações W e E, enquanto a hemicelulose principal extraída com NaOH foi uma O-metil-glucuronoxilana, tanto nas partes aéreas como nas raízes. As principais alterações observadas nas plantas submetidas ao estresse salino foram nos polissacarídeos pécticos, em relação ao grau de metil esterificação, conteúdo de ácidos urônicos e aumento das cadeias laterais das regiões de RG-I. Outras modificações observadas foram alterações no rendimento das frações polissacarídicas e grau de ramificação das hemiceluloses. Para a espécie *A. vulgaris*, a infusão de suas partes aéreas é popularmente indicada no tratamento de desordens hepáticas. Neste trabalho, a infusão (VI) e uma fração polissacarídica composta majoritariamente por inulina (VPI) obtida da infusão das partes aéreas da planta, foram testadas quanto a seu efeito hepatoprotetor *in vivo* em modelo de hepatotoxicidade induzida por tetracloreto de carbono (CCl_4). Camundongos pré-tratados por 7 dias com VI e VPI exibiram notável redução da atividade das enzimas hepáticas no plasma e peroxidação lipídica no fígado, o que indica integridade das membranas dos hepatócitos. Análises histológicas demonstraram que VI e VPI preveniram completamente necrose no fígado causada pelo CCl_4, validando o potencial hepatoprotetor da infusão e do polissacarídeo presente em *A. vulgaris*. Para a espécie *A. campestris subsp maritima*, a qual é popularmente utilizada no tratamento de distúrbios gástricos, uma fração polissacarídica (ACP-E10) foi isolada a partir de extração aquosa fervente das partes aéreas e avaliada quanto ao seu potencial gastroprotetor *in vivo* em modelo de úlcera gástrica induzida por etanol em ratas. A caracterização química de ACP-E10 indicou que esta fração corresponde a uma pectina, com massa molar de 16,600 g/mol, constituída predominantemente por regiões de homogalacturonana (60%) e regiões de ramnogalacturonana tipo-I (RG-I) (29%). As cadeias laterais de RG-I são constituídas por arabinanas e arabinogalactanas do tipo II. O pré-tratamento por via oral dos animais com este polissacarídeo nas doses de 0,3, 3 e 30 mg/kg foi capaz de reduzir de forma significativa a área de lesão induzida pela administração do etanol. O efeito gastroprotetor de ACP-E10 pode ser atribuído ao aumento significativo nos níveis de muco gástrico e glutationa reduzida no estômago causado pelo pré-tratamento com o polissacarídeo. Baseado nos dados obtidos sugere-se que as propriedades biológicas descritas popularmente para as espécies *A. vulgaris* e *A. campestris* possam ser atribuídas ao menos em parte aos polissacarídeos presentes nestas

plantas e também que o estresse salino em *A. annua* afeta tanto o desenvolvimento da planta quanto à composição e organização dos polissacarídeos da parede celular.

Palavras-chave: *Artemisia annua*, *Artemisia vulgaris*, *A. campestris subsp. maritima*, polissacarídeos, parede celular, estresse salino, infusão, efeito hepatoprotetor, CCl_4; inulina, úlcera gástrica, efeito gastroprotetor, pectina.

ABSTRACT

Plants of the *Artemisia* genus are widely known for their medicinal properties. In this study, three species were studied: *A. annua*, *A. vulgaris* and *A. campestris subsp maritima*. These species are widely studied about their secondary metabolites, but little is known about the compounds of the primary metabolism of these plants, such as polysaccharides. In this study, different approaches were employed in the study of the polysaccharides of these species. For *A. annua*, changes in the cell wall polysaccharides induced by the exposure of plants to salt stress were evaluated. For *A. vulgaris* and *A. campestris* plants, isolated polysaccharides were investigated for their potential biological activities. *A. annua* plants were cultivated in the presence or absence of NaCl at concentrations of 100 and 200 mM for 45 days. After harvesting, sequential extractions were performed in aerial parts and roots, using boiling water (W), 100 mM EDTA (E) and 6 M NaOH. Pectins were the major polysaccharide in the fractions W and E, while the main hemicellulosic polymer obtained with 6 M NaOH was an O-methyl-glucuronoxilan, both for aerial parts and roots. The main changes in cell wall of aerial parts of *A. annua* exposed to saline stress were alterations in the fine structure of the pectins, such as degree of methyl esterification, uronic acids content and increase of side chains of rhamnogalacturonana-I (RG-I) regions. Other changes observed were alteration in the yield of polysaccharide fractions and degree of branching of hemicellulose. Regarding to *A. vulgaris*, infusion of its aerial parts is commonly used for treatment of hepatic disorders. In this work, the infusion (VI) and a polysaccharide fraction obtained from the infusion, mainly composed of inulin (VPI) were evaluated for their hepatoprotective effect *in vivo* against carbon tetrachloride (CCl_4)-induced hepatotoxicity. Mice pretreated for 7 days with VPI and VI exhibited notable reductions of activity of hepatic enzymes in plasma and, lipid peroxidation in the liver, indicating integrity of hepatocyte membranes. Histological analysis demonstrated that VI and VPI completely prevented necrosis in the liver caused by CCl_4, validating the hepatoprotective effect of infusion and polysaccharide of *A. vulgaris*. From *A. campestris subsp maritima*, which is popularly used for treatment of gastric disturbance, a polysaccharide fraction (ACP-E10) was isolated with boiling water from the aerial parts of this plant and, it was evaluated for its gastroprotective effect *in vivo* on ethanol-induced ulcer in rats. Chemical characterization of ACP-E10 corresponds to a pectin, with a molar mass of 16,600 g/mol and it consists predominantly of homogalacturonan regions (60%) followed by RG-I regions (29%). Neutral side chains of RG-I are constituted by arabinans and arabinogalactans type II. Oral pretreatment of animals with polysaccharide at doses of 0.3, 3 and 30 mg/kg was able to reduce significantly the area of ulcer induced by ethanol administration. Gastroprotective effect of ACP-E10 may be attributed to the significant increase of reduced glutathione and mucus in the stomach, triggered by pretreatment with polysaccharide. Results obtained in this study suggest that the biological properties popularly described for *A. vulgaris* and *A. campestris* species can be attributed at least in part to the polysaccharides present in these plants and also, that the saline stress in *A. annua* affects both the development of plant and, the composition and organization of cell wall polysaccharides.

Keywords: *Artemisia annua*, *Artemisia vulgaris*, *A. campestris subsp. maritima*, polysaccharides, cell wall, salt stress, infusion; hepatoprotective effect; CCl_4; inulin, gastric ulcer, gastroprotective effect, pectin.

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LISTA DE ABREVIATURAS E SIGLAS

AG-I	- Arabinogalactana tipo I
AG-II	- Arabinogalactana tipo II
AINE/NSAID	- Anti-inflamatório não-esteroidal/*nonsteroidal anti-inflammatory drugs*
ALP	- *Alkaline phosphatase* (Fosfatase alcalina)
ALT	- *Alanine transaminase* (Alanina aminotransferase)
ANOVA	- Análise de variância
AST	- *Aspartate transaminase* (Aspartato aminotransferase)
BHT	- Hidroxitolueno butilado
BSA	- *Bovine serum albumin (soroalbumina bovina)*
CAT	- Catalase
CCl_3•	- Radical triclorometil
CCl_3OO•	- Radical triclorometil peroxil
CCl_4	- Tetracloreto de carbono
CDNB	- 1-cloro-2,4-dinitrobenzeno
CPQBA	- Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas
D_2O	- Água deuterada
DA	- Grau de acetilação
DCFH-DA	- 2,7-diclorofluoresceina diacetato
DILI	- *Drug Induced Liver Injury* (Lesão hepática induzida por fármacos)
DM	- Grau de metil esterificação
DMB	- Azul de dimetilmetileno
dn/dc	- Taxa de variação do índice de refração com relação à concentração
DPPH	- 2,2-difenil-1-picril-hidrazila
DTNB	- 5,5’-dithiobis(2-nitrobenzoic acid)
EDTA	- Ácido Etilenodiaminotetracético
EGase	- Endoglucanase
EXP	- Expansina
GC-MS	- Cromatografia gasosa acoplada à espectrometria de massa

GSH	- Glutationa reduzida
GST	- Glutationa S-transferase
H&E	- Hematoxilina/Eosina
HG	- Homogalacturonana
HM	- *High methoxyl pectin* - Pectina com alto teor de metil esterificação
HPLC	- Cromatografia líquida de alta eficiência
HPSEC	- Cromatografia de exclusão estérica de alta pressão
HSQC	- *Heteronuclear single quantum coherence*
IC_{50}	- Concentração do composto que leva à 50% de inibição do efeito máximo
IL-1β	- Interleucina-1-beta
LM	- *Low methoxyl pectin* - Pectina com baixo teor de metil esterificação
LPO	- Lipoperoxidação
LPS	- Lipopolissacarídeo bacteriano
MALLS	- Detector de espalhamento de luz laser em multiângulos
MAPK	- Proteína quinase ativada por mitógeno
MDA	- Malondialdeído
MPO	- Mieloperoxidase
Mw	- Molar mass
NAG	- *N*-acetIl-β-D-glucosaminidase
NED	- Dicloridrato de N-(1-naftil)-etilenodiamina
NMR	- *Nuclear magnetic ressonance* (ressonância magnética nuclear)
NO	- Óxido nítrico
OD	- Densidade óptica
PAE	- Pectina acetil esterase
PBS	- Solução salina tamponada
PG	- Poligalacturonase
PLL	- Pectina/pectato liase
PME	- Pectina metil esterase
RG-I	- Ramnogalacturonana do tipo I
RG-II	- Ramnogalacturonana do tipo II
RI	- Índice de refração
ROS	- *Reactive Oxygen Species* (espécies reativas de oxigênio)

RPK - Proteína receptora do tipo quinase
Rt - Tempo de Retenção
SEM - Erro padrão da média
SOD - Superóxido dismutase
TCA -Terapias de combinação baseadas em artemisinina
TFA - Ácido trifluoracético
TMSP-*d4* - Sal sódico do ácido 3-(trimetilsilil) propiônico-*d4*
TNF-α - Fator de necrose tumoral-alfa
UA - Ácidos urônicos
VLDL - Lipoproteína de densidade muito baixa
WAK - *Wall associated kinase* (Proteína quinase associada à parede)
WHO - *World Health Organization* (Organização mundial da saúde)
XET/XTH - Xiloglucana endotransglicosilase/hidrolase

Siglas relacionadas às frações polissacarídicas isoladas neste trabalho

Frações polissacarídicas extraídas das partes aérea de plantas de *A. annua*

A-WC - Fração obtida por extração aquosa fervente das plantas controle
A-W100 - Fração obtida por extração aquosa fervente das plantas sob tratamento salino (NaCl 100 mM)
A-W200 - Fração obtida por extração aquosa fervente de plantas sob tratamento salino (NaCl 200 mM)
A-EC - Fração obtida por extração com EDTA 100 mM das plantas controle
A-E100 - Fração obtida por extração com EDTA 100 mM das plantas sob tratamento salino (NaCl 100 mM)
A-E200 - Fração obtida por extração com EDTA 100 mM das plantas sob tratamento salino (NaCl 200 mM)
A-HAC - Fração hemicelulose A da extração com NaOH 6M das plantas controle
A-HA100 - Fração hemicelulose A da extração com NaOH 6M das plantas sob tratamento salino (NaCl 100 mM)
A-HA200 - Fração hemicelulose A da extração com NaOH 6M das plantas sob tratamento salino (NaCl 200 mM)

A-HBC - Fração hemicelulose B da extração com NaOH 6M das plantas controle

A-HB100 - Fração hemicelulose B da extração com NaOH 6M das plantas sob tratamento salino (NaCl 100 mM)

A-HB200 - Fração hemicelulose B da extração com NaOH 6M das plantas sob tratamento salino (NaCl 200 mM)

Frações polissacarídicas extraídas das raízes de plantas de *A. annua*

R-WC - Fração obtida por extração aquosa fervente das plantas controle

R-W100 - Fração obtida por extração aquosa fervente das plantas sob tratamento salino (NaCl 100 mM)

R-W200 - Fração obtida por extração aquosa fervente de plantas sob tratamento salino (NaCl 200 mM)

R-EC - Fração obtida por extração com EDTA 100 mM das plantas controle

R-E100 - Fração obtida por extração com EDTA 100 mM das plantas sob tratamento salino (NaCl 100 mM)

R-E200 - Fração obtida por extração com EDTA 100 mM das plantas sob tratamento salino (NaCl 200 mM)

R-HAC - Fração hemicelulose A da extração com NaOH 6M das plantas controle

R-HA100 - Fração hemicelulose A da extração com NaOH 6M das plantas sob tratamento salino (NaCl 100 mM)

R-NaHA200 - Fração hemicelulose A da extração com NaOH 6M das plantas sob tratamento salino (NaCl 200 mM)

R-HBC - Fração hemicelulose B da extração com NaOH 6M das plantas controle

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R-HB200 - Fração hemicelulose B da extração com NaOH 6M das plantas sob tratamento salino (NaCl 200 mM)

Frações obtidas das partes aéreas de *A. vulgaris*

VI - Infusão da parte aérea de *A. vulgaris*

VPI - Polissacarídeo bruto obtido por infusão da parte aérea de *A. vulgaris*

Frações polissacarídicas das partes aéreas de *A. campestris*

ACP-CR - Fração polissacarídica bruta obtida por extração aquosa fervente

ACP-E0.1 - Fração eluída em membrana de 0.1 µm obtida a partir da fração bruta ACP-CR

ACP-E10 - Fração pura, eluída em membrana de 10 kDa obtida a partir da fração bruta ACP-CR

ACP-E10CX - Fração obtida após carboxi-redução da amostra pura ACP-E10

ACP-R0.1 - Fração retida em membrana de 0.1 µm obtida a partir da fração bruta ACP-CR

ACP-R10 - Fração retida em membrana de 10 kDa obtida a partir da fração bruta ACP-CR

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1 INTRODUÇÃO

O gênero botânico *Artemisia*, pertencente à grande família de plantas Asteraceae (Compositae), tem se destacado devido à diversidade química e biológica de suas espécies e também por sua utilização na medicina popular de ervas (TAN; ZHENG; TANG, 1998).

Dentre as espécies deste gênero que possuem propriedades medicinais destacam-se aqui a *Artemisia annua*, *Artemisia vulgaris* e *Artemisia campestris*, espécies estudadas neste trabalho. Tais plantas têm sido amplamente investigadas com relação a seus metabólitos secundários, para os quais já foram atribuídas inúmeras propriedades biológicas. A espécie *A. annua*, por exemplo, apresenta como principais compostos ativos lactonas sesquiterpênicas, em particular a artemisinina, conhecida por sua rápida e eficiente ação antimalárica (CARBONARA et al., 2012).

O principal consumo destas plantas é na forma de infusão (chá) das partes aéreas, a qual além dos compostos de baixa massa molar também apresenta moléculas de alta massa, dentre as quais estão os polissacarídeos.

Polissacarídeos obtidos de fontes vegetais têm apresentado inúmeras atividades biológicas, dentre as quais podemos citar atividade antiúlcera gástrica (AUSTARHEIM et al., 2012a), antitumoral (BENTO; NOLETO; DE OLIVEIRA PETKOWICZ, 2014), antioxidante (XIONG et al., 2011), imunomoduladora (AMORIM et al., 2016) e hepatoprotetora (LIU et al., 2015). Além da ampla gama de propriedades terapêuticas, outra vantagem dos polissacarídeos vegetais em relação à terapia convencional é a baixa toxicidade e redução dos efeitos colaterais (SCHEPETKIN; QUINN, 2006). As diversas atividades biológicas apresentadas pelos polissacarídeos vegetais devem-se a grande variedade estrutural destes polímeros, uma vez que além da diversidade de monossacarídeos constituintes, estes monômeros podem ser unidos por diferentes tipos de ligações glicosídicas, resultando em uma ampla variedade de estruturas químicas (WANG et al., 2013).

Polissacarídeos vegetais são obtidos em sua maioria, a partir da parede celular das plantas, mas polissacarídeos de reserva, como por exemplo, as frutanas que se encontram armazenadas em vacúolos no citoplasma das células também podem ser isoladas durante os processos de extração dos polissacarídeos. Durante o mestrado, foram estudados os polissacarídeos presentes na infusão das partes

aéreas das espécies *A. absinthium* e *A. vulgaris*. Para *A. absinthium* foi isolada uma arabinogalactana do tipo II e em *A. vulgaris* foi observada a presença de frutanas do tipo inulina como polissacarídeo majoritário da infusão da erva (CORRÊA-FERREIRA; NOLETO; PETKOWICZ, 2014). No entanto, as espécies *A. annua* e *A. campestris* ainda não foram investigadas quanto à composição dos polissacarídeos presentes nas partes aéreas. Para outras espécies do gênero *Artemisia*, polissacarídeos isolados apresentaram atividades biológicas relevantes. Para a espécie *A. capillaris* uma arabinogalactana extraída com água fervente da parte aérea, apresentou efeito antiproliferativo sobre células de carcinoma humano nasofaríngeo (FENG et al., 2013). Xie et al. (2008) isolaram e caracterizaram cinco frações polissacarídicas extraídas com água fervente das folhas de *A. tripartita*. Todas as frações polissacarídicas isoladas continham sulfato (3,4% a 5,5%), sugerindo que os polissacarídeos desta espécie sejam sulfatados. Estes polissacarídeos exibiram potente atividade imunomodulatória e anti-inflamatória *in vitro*, incluindo modulação da função de macrófagos e neutrófilos e também sequestro de espécies reativas de oxigênio (XIE et al., 2008).

A presença de polissacarídeos sulfatados em plantas é rara, sendo encontrados normalmente na parede celular de algas marinhas. A informação contida no artigo de Xie *et al*. (2008) de que a espécie *A. tripartita* apresentava polissacarídeos sulfatados, despertou o interesse em investigar se outras espécies deste gênero também são capazes de produzir polissacarídeos sulfatados. Assim, durante o mestrado, foram investigados os polissacarídeos das partes aéreas de *A. absinthium* e *A. vulgaris* solúveis em água fervente. Nestes estudos não foi detectada a presença de sulfato nos polissacarídeos isolados destas espécies, adquiridas comercialmente.

No entanto, em um artigo de Aquino, Grativol e Mourão (2011) foi demonstrado que uma angiosperma marinha (*Ruppia marítima*) foi capaz de produzir polissacarídeos sulfatados em resposta a um ambiente salino, sugerindo que algumas condições do meio, como por exemplo, o grau de salinidade, possa estimular a produção de polissacarídeos sulfatados em plantas (AQUINO; GRATIVOL; MOURÃO, 2011). Neste trabalho, uma pesquisa realizada pelos autores revelou que angiospermas marinhas e de áreas de mangue, como *Halophila decipiens* e *Avicennia schaueriana*, expostas a diferentes condições de salinidades, apresentavam galactanas e arabinogalactanas sulfatadas, respectivamente. Além

disso, a quantidade de polissacarídeos sulfatados produzidos e o grau de sulfatação foram positivamente correlacionados com a salinidade. Este fato sugere que a presença de polissacarídeos sulfatados em plantas é uma adaptação à vida em ambientes com alta concentração de sal, uma característica que pode ter sido conservada através da evolução a partir das algas marinhas (AQUINO; GRATIVOL; MOURÃO, 2011).

Considerando os relatos da literatura, que sugerem que a salinidade do meio ambiente possa estimular a produção de polissacarídeos sulfatados em plantas (AQUINO; GRATIVOL; MOURÃO, 2011), e que a espécie *Artemisia tripartita* possuía naturalmente polissacarídeos sulfatados (XIE et al., 2008), um dos alvos deste trabalho foi avaliar as modificações dos polissacarídeos da parede celular da espécie *Artemisia annua* em resposta ao excesso de cloreto de sódio (100 e 200 mM) no meio.

Além disso, considerando o potencial biológico observado para os polissacarídeos obtidos de fontes vegetais, e mais especificamente para o apelo medicinal das plantas do gênero *Artemisia*, outros alvos do trabalho foram a investigação de propriedades biológicas dos polissacarídeos obtidos de *A. vulgaris* e *A. campestris*, em modelos animais de hepatoproteção e atividade antiúlcera gástrica, respectivamente.

2 REVISÃO DE LITERATURA

2.1 ESPÉCIES VEGETAIS UTILIZADAS NO TRABALHO

2.1.1 Gênero *Artemisia*

O gênero *Artemisia* é um dos maiores gêneros da família de plantas Asteraceae, o qual inclui cerca de 500 espécies distribuídas atualmente pelo mundo todo, ocorrendo desde o nível do mar até o topo de montanhas, em ambientes áridos e úmidos. As espécies deste gênero concentram-se principalmente na América do Norte, Ásia e Europa e inclui espécies de hábito arbustivo ou herbáceo, com polinização através do vento e ciclo de vida perene, anual ou bienal (ABAD et al., 2012; IVANESCU; MIRON; CORCIOVA, 2015).

As espécies do gênero *Artemisia* são utilizadas principalmente para fins ornamentais, culinários e são bastante conhecidas por seu uso medicinal (ABAD et al., 2012; BORA; SHARMA, 2010). Na medicina popular de plantas, as espécies de *Artemisia* são conhecidas por seu uso no tratamento da malária, hepatite, úlcera, diabetes, câncer, inflamação, infecções bacterianas, virais e fúngicas e também são usadas como ervas anti-helmínticas (IVANESCU; MIRON; CORCIOVA, 2015; TAN; ZHENG; TANG, 1998).

Diversas espécies do gênero produzem óleos voláteis, os quais conferem um odor forte e aromático às plantas. Estes compostos são intensamente explorados na indústria farmacêutica, cosmética e alimentícia, além de serem reconhecidos por suas potenciais atividades biológicas, principalmente antibacteriana, antifúngica e antiviral (ABAD et al., 2012). Os óleos essenciais isolados das plantas apresentam uma composição química complexa, incluindo de 20 a 60 moléculas diferentes, dentre as quais 2 a 3 são majoritárias no extrato. As moléculas encontradas no óleo essencial incluem terpenos, compostos aromáticos derivados de fenólicos e componentes alifáticos (ABAD et al., 2012). Outros metabólitos secundários relatados como compostos bioativos em espécies do gênero *Artemisia* são compostos fenólicos, especialmente conhecidos por sua atividade antioxidante (MELGUIZO-MELGUIZO et al., 2014) e lactonas sesquiterpênicas, com amplo espectro de atividades biológicas, tais como antitumoral, imunomoduladora, antiúlcera e antimicrobiana (FOGLIO et al., 2002; IVANESCU; MIRON; CORCIOVA, 2015).

Além dos compostos do metabolismo secundário das plantas, metabólitos primários como os polissacarídeos tem atraído grande atenção por apresentarem diversas atividades biológicas. Polissacarídeos isolados das sementes de *Artemisia sphaerocephala* apresentam importantes efeitos farmacológicos, tais como antidiabéticos (XING et al., 2009) e atividade hepatoprotetora (REN et al., 2014). A modificação dos polissacarídeos desta espécie como a adição do elemento químico selênio, aumentou significativamente a atividade antitumoral do polímero (WANG et al., 2016a). A sulfatação química de polissacarídeos de *A. sphaerocephala* também promoveu maior capacidade antioxidante do polímero comparado ao polissacarídeo nativo (WANG et al., 2010).

Outras espécies do gênero que possuem propriedades medicinais são a *Artemisia annua*, *Artemisia vulgaris* e *Artemisia campestris*, que foram objeto de

estudo do presente trabalho e que serão abordadas individualmente nas seções subsequentes.

2.1.2 *Artemisia annua*

Artemisia annua L. conhecida popularmente como losna-verde, artemísia, artemísia-chinesa e artemísia-doce, é uma planta herbácea, que atinge de 2-3 m de altura, e apresenta ciclo de vida anual (FIGURA 1). A planta é nativa da Ásia, mas atualmente é cultivada em muitos países. *A. annua* é utilizada na medicina tradicional chinesa no tratamento de febres e calafrios, mas seu principal apelo é devido a sua eficácia no tratamento da malária. O princípio ativo responsável pela ação antimalárica é uma lactona sesquiterpênica, denominada artemisinina, produzida e armazenada nos tricomas glandulares da planta (CARBONARA et al., 2012; JUNG et al., 2004; KOOY; SULLIVAN, 2013). A descoberta da artemisinina rendeu o prêmio Nobel de Medicina do ano de 2015 para a chinesa Youyou Tu, a qual conseguiu isolar juntamente com sua equipe de pesquisadores o composto da parte aérea de *A. annua* que foi eficaz no tratamento da malária (ALVES, 2015).

FIGURA 1 – PARTES AÉREAS DE *A. annua*

A

LEGENDA: (A) Planta de *A. annua* em casa de vegetação
(B) Detalhe das folhas e flores.
FONTE:
A- O autor (2016).

B- Wild Rose College of Natural Healing. Disponível em: <http://wrc.net/encyclopaedia_entry/sweet-annie-artemisia-annua/>. Acesso em 04/10/2016.

Diversos derivados semi-sintéticos da artemisinina foram produzidos, tais como diidroartemisinina, arteméter, artemisiteno e artesunato de sódio, na busca por obter compostos mais ativos. A artemisinina e seus derivados são usados no tratamento da malária em combinação com outros compostos antimaláricos, como por exemplo, artemeter + lumefantrine (Coartem®), com o intuito de evitar surgimento de resistência do *Plasmodium*, agente etiológico da malária, em relação à artemisinina e seus derivados. O uso destas terapias de combinação baseadas em artemisinina (TCAs) é recomendado pela Organização Mundial da Saúde (OMS) para o tratamento de malária causada por *Plasmodium falciparum* (DESROSIERS; WEATHERS, 2016; KOOY; SULLIVAN, 2013). A artemisinina e seus derivados também apresentam efeito antitumoral em diversos tipos de linhagens celulares tumorais (GANGULI et al., 2014; HOU et al., 2008; THANAKETPAISARN et al., 2011).

A quantidade de artemisinina nas folhas e flores de *A. annua* é muito pequena, variando entre 0.01-0.8% do peso seco da erva, dessa forma diversos grupos buscam aumentar o conteúdo de artemisinina na planta, através de alterações nas condições de plantio da erva, tais como submeter as plantas a estresse salino moderado (IRFAN QURESHI et al., 2005; QIAN et al., 2007) e aplicação foliar de compostos como quitosana (LEI et al., 2011) e ácido salicílico (AFTAB et al., 2011).

Além da artemisinina, outros compostos também foram identificados em *A. annua*, dentre os quais podemos citar cumarinas, triterpenóides, monoterpenóides e fenólicos, como os flavonoides, os quais também apresentam propriedades biológicas (BHAKUNI et al., 2001). Como exemplo, os flavonoides casticin e crisosplenol D, isolados de *A. annua* foram capazes de inibir processos inflamatórios tanto *in vitro* quanto *in vivo* (LI et al., 2015). Outro estudo realizado por Melillo de Magalhães et al. (2012) também demonstrou o potencial anti-inflamatório de infusões de *A. annua in vitro* utilizando células Caco-2 estimuladas por um coquetel de citocinas inflamatórias e LPS. Os autores sugerem que os compostos fenólicos presentes nas infusões são os compostos responsáveis pelo efeito anti-inflamatório da erva e que poderiam ser utilizados para potencializar os efeitos da artemisinina no tratamento da malária, reduzindo processos inflamatórios associados à doença.

Apesar dos numerosos estudos acerca da composição química de *A. annua*, não foram encontrados dados na literatura com relação aos polissacarídeos desta espécie.

2.1.3 *Artemisia vulgaris* L.

Artemisia vulgaris é uma planta conhecida popularmente como artemísia, artemígio, artemigem, anador, flor-de-são-joão e absinto-selvagem. É uma planta arbustiva, rizomatosa, de ciclo de vida perene, que pode atingir até 2,5 m de altura (FIGURA 2). A presença do rizoma em *A. vulgaris* a diferencia de espécies semelhantes, tais como *A. annua* e *A. tridentata*, que não possuem este rizoma subterrâneo (BARNEY; DITOMMASO, 2003). Esta espécie é nativa da Ásia, mas atualmente é encontrada em abundância em regiões temperadas e frias, sendo considerada uma planta daninha, a qual infesta viveiros de plantas, áreas de depósitos de resíduos e gramados (BARNEY; DITOMMASO, 2003; PIRES et al., 2009).

FIGURA 2 - Partes aéreas de *Artemisia vulgaris* (A) e detalhe das flores (B).

A **B**

FONTE: Flora finder.com. Disponível em: http://www.florafinder.com/Species/Artemisia_vulgaris.php. Acesso em 04/10/2016

Assim como outras espécies do gênero *Artemisia, A. vulgaris* é conhecida por suas propriedades medicinais, mas também é utilizada para propósitos culinários (MELGUIZO-MELGUIZO et al., 2014). Na medicina popular, *A. vulgaris* é usada no tratamento de doenças inflamatórias, úlcera gástrica, doenças hepáticas, câncer, controle da hipertensão, doenças infecciosas causadas por bactérias, fungos e vírus, como anti-helmíntico, antisséptico, antiespasmódico e recomendado para vários

problemas ginecológicos, tais como tratamento de menstruação irregular (BARNEY; DITOMMASO, 2003; KHAN; GILANI, 2009; MELGUIZO-MELGUIZO et al., 2014).

A composição fitoquímica da parte aérea de *A. vulgaris* é alvo de numerosos estudos, os quais relatam a presença de compostos fenólicos como principais constituintes da fração polar dos extratos da erva, incluindo flavonoides, ácidos hidroxicinâmicos e diversos derivados do ácido quínico (IVANESCU et al., 2010; MELGUIZO-MELGUIZO et al., 2014), os quais possuem alto potencial antioxidante (MELGUIZO-MELGUIZO et al., 2014).

O óleo essencial desta espécie foi extensivamente estudado por vários grupos de pesquisa por contribuir de forma significativa com as atividades biológicas descritas para a erva (BLAGOJEVIĆ et al., 2006; GOVINDARAJ et al., 2008; JUDŽENTIEN; BUZELYTE, 2006). Em relação à composição do óleo essencial, os autores relataram diferenças em relação ao local coletado, clima e órgão da planta (raízes ou partes aéreas) (BLAGOJEVIĆ et al., 2006; JUDŽENTIEN; BUZELYTE, 2006; ZHIGZHITZHAPOVA et al., 2016). Monoterpenos, como 1,8-cineol, β-tujona e cânfora, e os sesquiterpenos: germacreno D e cariofileno são os principais constituintes do óleo essencial obtido desta espécie (GOVINDARAJ et al., 2008; SALEH et al., 2014). Como propriedades biológicas, o óleo essencial de *A. vulgaris* apresentou atividade antimicrobiana (BLAGOJEVIĆ et al., 2006), larvicida contra *Aedes aegypti* (GOVINDARAJ; RANJITHAKUMARI, 2013) e repelente e fumigante contra o inseto *Tribolium castaneum*, um inseto que causa danos a diversas culturas de cereais (WANG et al., 2006).

Outra classe de moléculas importantes na atividade biológica das ervas são os polissacarídeos. Para *A. vulgaris* foi isolada da infusão da erva uma frutana do tipo inulina como componente majoritário (CORRÊA-FERREIRA; NOLETO; PETKOWICZ, 2014). Este polissacarídeo é considerado como um alimento funcional, que afeta os sistemas corporais atuando em benefício da saúde e reduzindo o risco de doenças (ROBERFROID, 2007). Frutanas do tipo inulina também são amplamente conhecidas por seus efeitos prebióticos (ROBERFROID, 2007; TIENGTAM et al., 2015) e, mais recentemente foram relatadas como compostos anti-tumorais, antioxidantes, hepatoprotetores e antiúlcera gástrica (AUSTARHEIM et al., 2012b; LIU et al., 2015; XU et al., 2015).

Alguns usos medicinais de *A. vulgaris* já foram validados por estudos científicos, tais como seu uso como anti-helmíntico (CANER et al., 2008) e

antimicrobiano (BLAGOJEVIĆ et al., 2006). No entanto, estes estudos utilizam extratos metanólicos ou etanólicos e, para a infusão da erva, que é a forma tradicional de uso desta planta, não foi encontrado nenhum trabalho relatando suas propriedades terapêuticas.

2.1.4 *Artemisia campestris* subsp *marítima* Arcangeli

A espécie *Artemisia campestris* consiste de diversas subespécies*:* dentre elas cita-se as *subsp. glutinosa, campestris e maritima* (VALANT-VETSCHERA; FISCHER; WOLLENWEBER, 2003). Neste trabalho a subespécie estudada foi a *subsp. maritima*, encontrada em toda a costa do litoral português, geralmente localizada na duna primária. Conhecida em Portugal como erva-lombrigueira ou madorneira, é um subarbusto perene que alcança até 1 m de altura (ARAUJO *et al*., 2015) (FIGURA 3). Esta subespécie também é encontrada na Tunísia, onde é conhecida por “T’gouft” (MEGDICHE-KSOURI et al., 2015).

Na medicina popular de plantas é usada na forma de decocção das partes aéreas para tratamento de distúrbios gástricos, diarreia, cólicas abdominais, bem como também é reconhecida como antibacteriana, antifúngica, anti-inflamatória e antirreumática (MEGDICHE-KSOURI et al., 2015; SAOUDI et al., 2010).

FIGURA 3 - *Artemisia campestris* subsp. *maritima*. DESTAQUE DAS FOLHAS, FLORES E ARBUSTO.

FONTE: ARAUJO et al. (2015). Flora-On: Flora de Portugal Interactiva, Sociedade Portuguesa de Botânica. Disponível em: <http://www.flora-on.pt.> Acesso em 05/10/2016.

Diversos compostos foram descritos para esta espécie, a qual é rica em flavonoides, taninos, polifenóis, saponinas e óleo essencial (AKROUT et al., 2011). Também já foi relatada a presença de cromonas e cumarinas (VASCONCELOS; SILVA; CAVALEIRO, 1998) e um derivado de acetofenona (RAUTER et al., 1989). Akrout et al. (2011) encontraram como compostos majoritários do óleo essencial obtido de *A. campestris* coletada em uma região montanhosa no sul da Tunísia, monoterpenos hidrocarbonetos (72%), seguido de sesquiterpenos hidrocarbonetos (15%), sesquiterpenos oxigenados (9%) e monoterpenos oxigenados (3%).

Muitas atividades biológicas importantes também têm sido demonstradas para diferentes extratos desta planta. Megdiche-Ksouri et al. (2015) verificaram que os extratos obtidos com metanol e acetato de etila das partes aéreas de *A. campestris* apresentavam atividade antioxidante e antimicrobiana *in vitro*. Estes extratos apresentavam como principais componentes cumarinas, flavonas, flavonois, ácidos fenólicos e sesquiterpenos. Devido à atividade antioxidante e antimicrobiana da erva, os autores sugerem que esta possa ser usada na indústria como conservante de alimentos (MEGDICHE-KSOURI et al., 2015).

Para extratos aquosos de *A. campestris* foram encontrados diversos estudos relatando o efeito benéfico desta planta. Saoudi et al. (2010) relataram que o extrato da planta preparado com água fervente foi capaz de minimizar os efeitos nocivos causados pela administração do extrato de fígado do peixe venenoso *Lagocephalus lagocephalus* em ratos. Aniya et al. (2000) demonstraram o efeito antioxidante *in vitro* e hepatoprotetor *in vivo* de um extrato aquoso de *A. campestris*. A atividade antioxidante do extrato aquoso (infusão) também foi verificada por Akrout et al. (2011), que observaram elevada atividade sequestradora de radicais livres (IC_{50}=0.658 mg/mL). Os autores também avaliaram o efeito antitumoral do óleo essencial, extrato etanol-água e infusão de *A. campestris* sobre células de adenocarcinoma de cólon humano (HT-29). Os extratos mais eficazes foram o óleo essencial e etanol-água (AKROUT et al., 2011). Ghlissi et al. (2016) observaram efeitos benéficos do extrato aquoso (macerado a frio, 24 h) de *A. campestris* em diversos ensaios biológicos. O extrato apresentou atividade antioxidante e antibacteriana *in vitro*, bem como atividade anti-inflamatória (modelo do edema de pata em ratos) e de cicatrização de ferida *in vivo*. A análise fitoquímica deste extrato demonstrou predominância de polifenóis, seguido de flavonoides e taninos (GHLISSI et al., 2016).

Uma vez que os extratos aquosos de *A. campestris* apresentam diversas atividades biológicas, torna-se relevante o estudo de outros compostos bioativos presentes nestes extratos, tais como os polissacarídeos, os quais são obtidos por extração aquosa das plantas. Polissacarídeos são conhecidos por apresentarem diversas propriedades biológicas (RINAUDO, 2008), mas até o momento não foi encontrado nenhum relato na literatura sobre os polissacarídeos de *A. campestris*.

2.2 POLISSACARÍDEOS VEGETAIS

Os polissacarídeos vegetais são moléculas de grande interesse industrial e farmacológico. A celulose, por exemplo, têm sido tradicionalmente usada nas indústrias de papel, têxtil e de construção. Além disso, após transformação química a celulose também pode ser explorada nas indústrias alimentícia, cosmética, biomédica e farmacêutica (LIU; WILLFÖR; XU, 2015). Outra classe de polissacarídeos importantes na indústria alimentícia e farmacêutica são as pectinas, as quais são usadas como agente espessante, gelificante. estabilizantes, emulsionantes e agentes suspensores (FRAEYE et al., 2010; LIU; WILLFÖR; XU, 2015). Além disso, os polissacarídeos vegetais também podem ser aplicados na como biomateriais, na engenharia de tecidos, liberação controlada de fármacos, imobilização de enzimas e como biossensores (DA CUNHA; DE PAULA; FEITOSA, 2009; LIU; WILLFÖR; XU, 2015; RINAUDO, 2008). Polissacarídeos obtidos de fontes naturais oferecem vantagem em relação aos polissacarídeos sintéticos devido ao fato de serem compostos biodegradáveis, biocompatíveis, não tóxicos e mais baratos (LIU; WILLFÖR; XU, 2015).

Os polissacarídeos obtidos de vegetais podem ser classificados como polissacarídeos de reserva, exsudatos ou estruturais. Dentre os polissacarídeos de reserva, o amido e as frutanas são os polímeros majoritários encontrados nas plantas (AVIGAD; DEY, 1997). O amido é encontrado nos plastídeos celulares (cloroplastos nas folhas e amiloplastos nos órgãos não-fotossintéticos). Já as frutanas são encontradas armazenadas em vacúolos citoplasmáticos. Este tipo de polímero se acumula em raízes e tubérculos de plantas das famílias Alliaceae, Liliaceae e Asteraceae, mas também são encontradas em outros órgãos, tais como folhas e caules de gramíneas e ervas das famílias Asteraceae e Liliaceae (AVIGAD; DEY, 1997). As frutanas são polímeros de grande interesse nutricional e terapêutico

por apresentarem funções biológicas relevantes, dentre elas seu potencial em atuar como prebiótico (ROBERFROID, 2007).

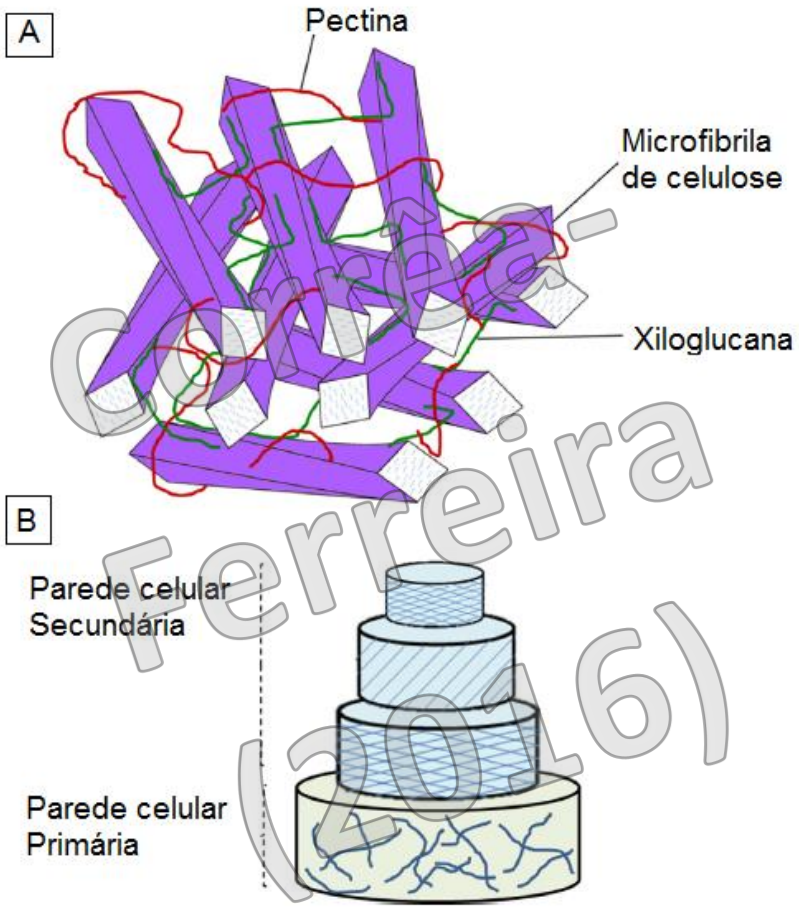
Os polissacarídeos de exsudatos são obtidos a partir de caules de árvores de algumas espécies de plantas, os quais são produzidos em resposta a injuria mecânica ou microbiana (FATHI; MOHEBBI; KOOCHEKI, 2016). Em geral são obtidos polissacarídeos puros, de estrutura química complexa, os quais são de grande interesse na indústria alimentícia, devido ao seu potencial como agente estabilizante, emulsificante, espessante, dentre outras aplicações (DA CUNHA; DE PAULA; FEITOSA, 2009; FATHI; MOHEBBI; KOOCHEKI, 2016). As principais espécies cultivadas para este fim são: *Acacia Senegal* (goma arábica) e *Astragalus spp.* (goma adraganta) (MARTÍNEZ et al., 2015).

Os polissacarídeos estruturais são aqueles encontrados na parede celular dos vegetais, a qual não cumpre apenas um papel estrutural na célula, mas também é responsável por inúmeros processos celulares, tais como controle do crescimento da célula, adesão celular e regulação da entrada e saída de moléculas (ALBERSHEIM et al., 1994; WILLATS et al., 2000). A parede celular ainda constitui uma barreira física contra agentes físicos e biológicos, é fonte de moléculas sinalizadoras (oligossacarinas), e em algumas sementes é local de armazenamento de polissacarídeos de reserva (ALBERSHEIM et al., 1994; BURTON; GIDLEY; FINCHER, 2010).

2.2.1 Polissacarídeos da parede celular vegetal

Os tecidos vegetais jovens que se apresentam em crescimento, possuem apenas uma fina parede celular, denominada de parede celular primária. Já as células diferenciadas, que perdem a capacidade de crescimento, produzem uma parede celular rígida e espessa, denominada de parede celular secundária, a qual é depositada internamente à parede celular primária, em direção ao citoplasma da célula (FIGURA 4B). Estes dois tipos de parede celular diferem entre si em relação à composição e estrutura e por apresentarem distintas funções na célula. Enquanto a parede celular primária, típica de tecidos jovens, permite a expansão e divisão celular, a parede celular secundária é encontrada em tecidos lenhosos, conferindo alta resistência e rigidez (GRANT REID, 1997; MARTINEZ-SANZ; GIDLEY; GILBERT, 2015).

FIGURA 4 - Estrutura esquemática da parede celular.



Correa-Ferreira (2016)

LEGENDA: A – Arranjo esquemático dos polissacarídeos na parede celular primária de dicotiledôneas e monocotiledôneas não-gramíneas.
B – Ilustração da parede celular vegetal, demonstrando a parede celular primária e secundária. Traços azuis representam o arranjo das microfibrilas de celulose em cada camada da parede celular.

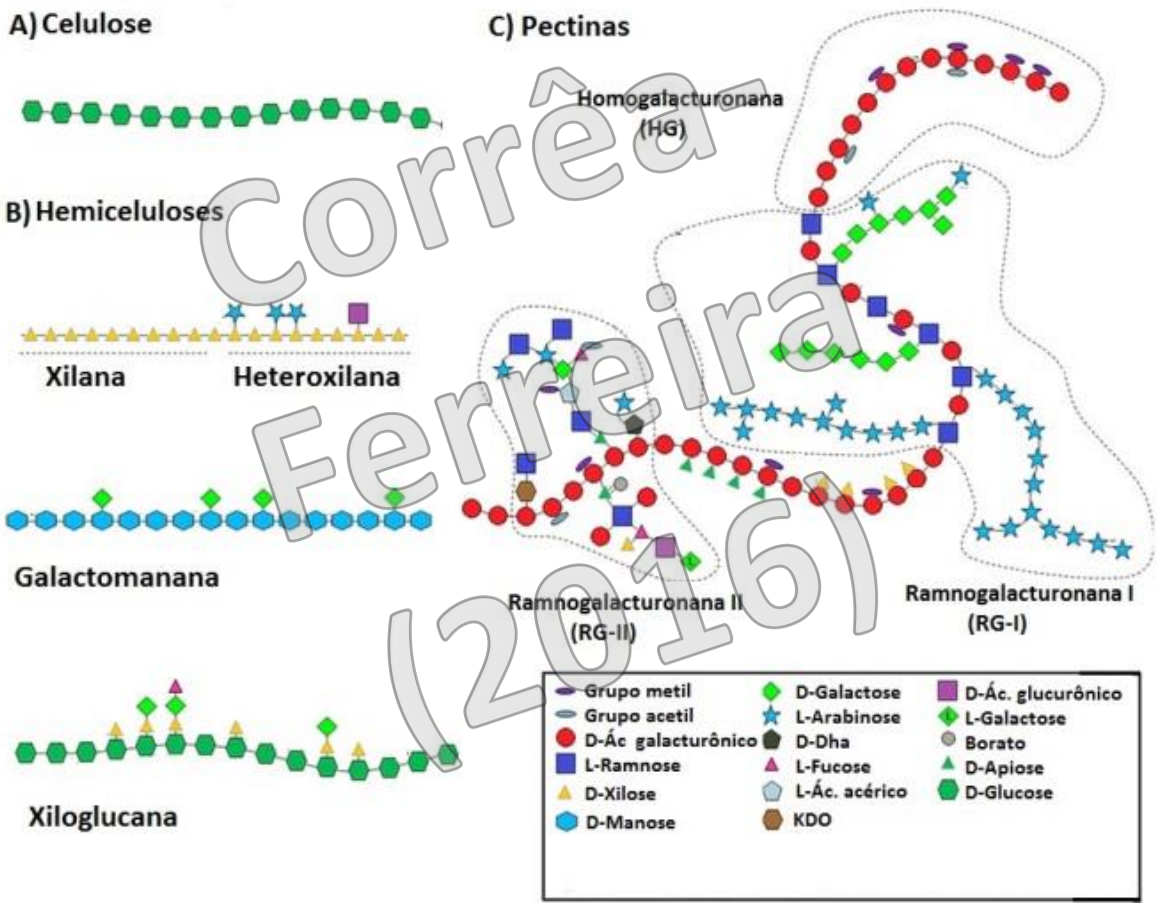
FONTE: Adaptado de Martinez-Sanz, Gidley e Gilbert (2015).

A parede celular primária encontra-se constituída majoritariamente por polissacarídeos (90%) e proteínas (10%). Os polissacarídeos presentes na parede celular são usualmente divididos em celulose, hemicelulose e pectinas (MCNEIL et al., 1984). O domínio celulósico da parede celular, também conhecido como componente semicristalino da parede, é formado por cadeias de β-D-Glc*p* (1→4 (FIGURA 5A) ligadas que formam microfibrilas mecanicamente fortes, através da formação de ligações de hidrogênio entre cadeias de celulose paralelas. As microfibrilas de celulose são o principal componente estrutural da parede celular primária que confere força mecânica ao tecido (MARTINEZ-SANZ; GIDLEY; GILBERT, 2015; WANG et al., 2016c). A celulose constitui o resíduo final insolúvel da parede celular, após a extração dos demais polissacarídeos com agentes

quelantes e álcali, e representa cerca de 20-30% da parede celular primária das plantas (MCNEIL et al., 1984). As microfibrilas de celulose apresentam em sua superfície grupos hidroxila, os quais além de fazerem ligação de hidrogênio entre as microfibrilas adjacentes, também são responsável por interconectar-se a outros componentes da parede, como as hemiceluloses, formando uma estrutura macrofibrilar (MARTINEZ-SANZ; GIDLEY; GILBERT, 2015; TAVARES; BUCKERIDGE, 2015). Os polímeros hemicelulósicos da parede (FIGURA 5B) são extraídos com o uso de álcali diluído e são caracterizados por apresentarem uma cadeia principal de monossacarídeos ligados por ligações do tipo β(1→4). As principais hemiceluloses encontradas em plantas são xiloglucanas, xilanas, mananas, glucomananas e β-(1→3,1→4)-glucanas (SCHELLER; ULVSKOV, 2010). As xiloglucanas são as principais hemiceluloses presentes na parede celular de dicotiledôneas e algumas famílias de monocotiledôneas (não-gramíneas), enquanto as glucuronoarabinoxilanas são as hemiceluloses responsáveis por interligar as microfibrilas de celulose em espécies da família Poaceae (gramíneas) e outras famílias relacionadas (CARPITA; GIBEAUT, 1993). Hemiceluloses que apresentam manose na cadeia, tais como mananas, galactomananas e glucomananas também estão presentes na parede celular, mas em menor quantidade (MARTINEZ-SANZ; GIDLEY; GILBERT, 2015).

As pectinas são polissacarídeos altamente complexos (FIGURA 5C), caracterizados pelo alto conteúdo de ácido galacturônico e extraídos da parede celular com água, agentes quelantes ou ácido. Juntamente com as hemiceluloses, as pectinas constituem o domínio amorfo da parede, no qual as microfibrilas de celulose estão embebidas (HARHOLT; SUTTANGKAKUL; VIBE SCHELLER, 2010; MARTINEZ-SANZ; GIDLEY; GILBERT, 2015). As pectinas contribuem para a flexibilidade e força da parede celular, atuam na regulação da porosidade da parede e seus oligossacarídeos estão envolvidos na sinalização após o ataque de patógenos (CAFFALL; MOHNEN, 2009; HARHOLT; SUTTANGKAKUL; VIBE SCHELLER, 2010). Além de estarem presentes na parede celular primária, são os componentes majoritários na lamela média, região entre as células vegetais, onde agem como moléculas de adesão celular (MARTINEZ-SANZ; GIDLEY; GILBERT, 2015)

FIGURA 5 – Representação esquemática da estrutura química dos polissacarídeos encontrados na parede celular vegetal.



Corrêa-Ferreira (2016)

FONTE: Adaptado de XU et al (2016).

O termo pectina inclui três principais classes estruturais ou também denominados domínios: homogalacturonanas (HG), ramnogalacturonanas do tipo I (RG-I) e ramnogalacturonanas do tipo II (RG-II). O domínio HG e RG-I das pectinas são os mais abundantes nas pectinas e RG-II é o componente minoritário (10%) (HARHOLT; SUTTANGKAKUL; VIBE SCHELLER, 2010). As homogalacturonanas representam o segmento linear das pectinas, que consiste de uma cadeia de α-D-GalA (1→4) ligado. No domínio RG-I, unidades de ramnose intercalam-se com as unidades de ácidos galacturônicos da cadeia principal, na forma de dissacarídeos: α-1,4-D-Gal*p*A-α-1,2-L-Rha*p*. As unidades de ramnose podem apresentar substituições em O-4 por cadeias laterais de açúcares neutros, tais como galactanas, arabinanas e/ou arabinogalactanas (CARPITA; GIBEAUT, 1993; MCNEIL et al., 1984). O domínio RG-II é também um segmento ramificado das pectinas, que apresenta uma cadeia principal de α-D-Gal*p*A (1→4) ligado, com substituições em O-2 ou O-3 por

cadeias laterais formadas por até 12 tipos de açúcares diferentes, dentre os quais açúcares considerados raros, tais como 2-O-metil-L-Fuc e ácido acérico (HARHOLT; SUTTANGKAKUL; VIBE SCHELLER, 2010). A figura 5 apresenta uma representação esquemática da estrutura química dos polissacarídeos encontrados na parede celular vegetal.

A proporção entre os três componentes polissacarídicos da parede celular (celulose, hemicelulose e pectina) varia dependendo da espécie vegetal e órgão da planta estudado. Importantes diferenças são observadas, por exemplo, entre espécies de plantas monocotiledôneas e dicotiledôneas. Nas monocotiledôneas da família Poaceae, a parede celular primária possui significativamente mais hemicelulose e menos pectinas do que dicotiledôneas e monocotiledôneas não-gramíneas (CARPITA; GIBEAUT, 1993). A figura 4A mostra uma representação da estrutura da parede celular de plantas dicotiledôneas.

2.2.2 Modificações dos polissacarídeos da parede celular de plantas em resposta ao estresse abiótico

As plantas estão continuamente expostas a diversos fatores externos adversos ao crescimento, também denominados estresses ambientais, os quais podem ser divididos em abióticos ou bióticos. O estresse biótico é causado por infecções por bactérias, fungos e vírus ou ataque de herbívoros. Dentre os fatores abióticos podemos citar: o estresse de seca, salinidade, térmico (frio ou calor), contaminação por metais pesados e deficiência de nutrientes. Os principais fatores abióticos que influenciam a distribuição geográfica das espécies vegetais e limitam a produtividade agrícola são os estresses de seca, salinidade e térmico (GALL et al., 2015; ZHU, 2016).

Sabe-se que a parede celular desempenha um importante papel na resistência das plantas aos diferentes tipos de estresses ambientais, alterando sua estrutura em resposta às condições ambientais, além disso, também participa na detecção do estresse e transdução do sinal (GALL et al., 2015; VOXEUR; HOFTE, 2016). Estudos recentes têm sugerido que, assim como ocorre com a parede celular fúngica, em resposta a estresses ambientais a integridade da parede celular é alterada, o que leva a ativação de uma cascata de sinalização e posterior adaptação da parede celular, com o intuito de promover a sobrevivência da planta sob

condições desfavoráveis (VOXEUR; HOFTE, 2016). Por este mecanismo, as plantas seriam capazes de detectar alterações na estrutura da parede e desencadear mudanças compensatórias nas suas propriedades (VOXEUR; HOFTE, 2016).

Um exemplo de sinalização do estresse abiótico em nível da parede celular são as proteínas quinases associadas à parede ou “WAKs” (*wall-associated kinases*). Durante o estresse osmótico, que pode ser gerado tanto pela falta de água no solo (estresse de seca) como por excesso de sais (estresse salino), as pectinas (homogalacturonanas) alteram sua conformação, ativando as proteínas WAKs. Quando ativadas, estas proteínas sensoras promovem uma cascata de sinalização através das MAPKs (proteínas quinases ativadas por mitógeno), as quais por sua vez fosforilam diversos substratos, tais como fatores de transcrição que controlam o balanço osmótico, a remodelagem e biossíntese da parede celular (GALL et al., 2015; SEIFERT; BLAUKOPF, 2010). Outro exemplo bastante conhecido de percepção do estresse abiótico pela parede celular envolve as proteínas receptoras do tipo quinase (RPKs), que são proteínas transmembrana que promovem a regulação de inúmeros genes que aumentam a tolerância da planta ao estresse abiótico (TENHAKEN, 2015).

Diversos estudos têm relatado os efeitos de diferentes tipos de estresse abiótico sobre o metabolismo da parede celular. A maioria destes estudos concentra-se em análises do transcriptoma e proteoma e, por sua vez, poucos exploram a alteração da composição química da parede celular. Com relação aos dados de expressão gênica, são observadas alterações na regulação de genes que codificam enzimas responsáveis pela regulação da extensibilidade da parede celular. Exemplos desta classe de genes são os responsáveis pela codificação de expansinas (*EXP*), xiloglucana endotransglicosilase/hidrolase (*XET/XTH*) e endoglucanases (*EGase*) da parede celular (GALL et al., 2015). As expansinas, por exemplo, promovem o afrouxamento da parede e expansão celular, por hidrolisar as ligações entre as microfibrilas de celulose e as hemiceluloses. As enzimas XET/XTH são também responsáveis pelo afrouxamento da parede celular e estão geralmente reguladas positivamente nas plantas sob estresse abiótico, a fim de permitir o crescimento da planta mesmo em condições desfavoráveis. Isto porque durante o estresse osmótico (seca ou salino) o afrouxamento da parede celular mantém a possibilidade das células expandirem-se (TENHAKEN, 2015). A regulação dos genes de afrouxamento e enrijecimento da parede é muito complexa, sendo que em uma

mesma planta, certos tecidos podem apresentar afrouxamento da parede enquanto outros apresentam enrijecimento, a fim de manter o crescimento do vegetal (HOUSTON et al., 2016).

O estudo de plantas transgênicas ou linhagens mutantes são muito úteis na pesquisa da importância de determinados genes na resposta da planta ao estresse. Neste sentido, plantas transgênicas de tomate que superexpressavam o gene *XTH* da espécie *Capsicum*, responsável por promover o relaxamento da parede celular, apresentaram maior tolerância ao estresse salino e de seca do que as plantas controle (CHOI et al., 2011). Os autores sugerem que o aumento da remodelagem da parede celular nas células-guarda dos estômatos possa reduzir a perda de água por transpiração, protegendo o mesofilo do déficit hídrico (CHOI et al., 2011).

Outras enzimas da parede celular que também estão alteradas durante o estresse abiótico, são proteínas que regulam a plasticidade/reologia da parede celular, tais como PME (pectina metil esterase), PG (poligalacturonase), PLL (pectina/pectato liase) e PAE (pectina acetil esterase) (GALL et al., 2015). As enzimas pectina metil esterases favorecem alterações locais que facilmente alteram as propriedades da parede. Isto porque as regiões das homogalacturonanas onde são removidos os grupamentos metil ficam suscetíveis à formação de zonas de junção com o cálcio, alterando rapidamente a viscosidade da parede celular (BURTON; GIDLEY; FINCHER, 2010).

Além da remodelagem da parede celular promovida por enzimas que regulam a extensibilidade da parede (afrouxamento ou enrijecimento), a composição dos polissacarídeos da parede celular primária também é alterada. O conteúdo de celulose, hemicelulose e pectinas se modifica em resposta aos fatores ambientais (AN et al., 2014; DE LIMA et al. 2014). As pectinas têm um papel muito importante na resposta das plantas aos diversos tipos de estresse abiótico. Isto se deve ao fato da importância das pectinas na regulação do crescimento e desenvolvimento da planta. Além disso, as pectinas também são muito importantes na regulação do status hídrico da parede celular, através das cadeias laterais de RG-I e RG-II. O aumento das cadeias laterais de pectinas parece ser um mecanismo comum das plantas na tolerância ao estresse salino e de seca, devido a sua capacidade de ligação a moléculas de água e por ser capaz de formar géis na parede (GALL et al., 2015; LEUCCI et al., 2008; WILLATS et al., 2001). Em plantas de ressurreição, que sobrevivem à dessecação, arabinose encontra-se em alta concentração, sugerindo

um importante papel para as cadeias de arabinanas das pectinas na parede celular de plantas submetidas ao déficit hídrico (GOMEZ et al., 2009; GRIBAA et al., 2013).

Ainda com relação às pectinas, acredita-se que a regulação precisa da quantidade de HG na parede e seu grau de metil esterificação seja capaz de controlar finamente o status hídrico da parede celular em condições de déficit de água (GALL et al., 2015). Já foi relatado também na literatura um aumento da sensibilidade de plantas ao estresse de seca quando estas apresentam redução do conteúdo de pectinas na parede (AN et al., 2014).

Alteração no conteúdo de celulose da parede também foi relatada para plantas submetidas a déficit hídrico. Células de tabaco (*Nicotiana tabacum*) submetidas a crescimento em meio contendo 30% polietileno glicol ou NaCl 428 mM apresentaram substancial inibição da síntese de celulose na parede, fato que segundo os autores facilita as células atingirem uma pressão de turgor maior (IRAKI et al., 1989). A interação entre os componentes hemicelulósicos e a celulose pode também estar alterada durante o estresse abiótico. De Lima et al. (2014) relataram que o estresse salino em plantas de café, promoveu a redução do grau de substituição dos polímeros hemicelulósicos, o que poderia resultar em uma maior interação destes polímeros com a celulose, promovendo enrijecimento da parede.

Em ambiente salino, angiospermas marinhas da espécie *Ruppia maritima* apresentam polissacarídeos sulfatados, os quais completamente desaparecem quando crescidas na ausência de sal, sugerindo uma importância dos polissacarídeos sulfatados na sobrevivência da planta em ambiente salino (AQUINO; GRATIVOL; MOURÃO, 2011). Outras plantas halófitas, que vivem em ambiente salino, tais como *Halophila decipiens* e *Halodule wrightii* também apresentam polissacarídeos sulfatados (AQUINO et al., 2004), característica que não é característica da parede celular de plantas e que pode ser uma modificação induzida pelo ambiente salino.

A parede celular secundária também pode aumentar sua espessura em resposta a estresse abiótico, tanto pela deposição de hemiceluloses quanto pelo aumento de lignina. Em plantas de café, submetidas a estresse térmico, foi encontrado um aumento de monolignóis no resíduo final insolúvel da parede celular, sugerindo um aumento do conteúdo de lignina em resposta ao estresse térmico (LIMA et al., 2013).

Apesar de as mudanças na arquitetura da parede celular em resposta aos diferentes tipos de estresse abiótico variar grandemente entre a espécie vegetal considerada, o órgão da planta, a idade vegetal, a intensidade e duração do estímulo, alguns mecanismos parecem ser comuns à maioria das plantas submetidas a estresse abiótico. Estes mecanismos são a tentativa de manutenção da plasticidade da parede, através do aumento de proteínas que regulam a extensibilidade da parede (expansinas, xiloglucana endotransglicosilase/hidrolase) e também aumento da espessura da parede celular secundária, através da deposição de hemiceluloses e lignina (GALL et al., 2015).

2.2.3 Atividade biológica de polissacarídeos vegetais

A busca por substâncias provenientes de fontes naturais para o tratamento e prevenção de doenças tem se tornado constante nos estudos científicos atuais (WANG et al., 2013). Dentre as moléculas obtidas de fontes naturais, os polissacarídeos são reconhecidos por seu potencial terapêutico (LIU; WILLFÖR; XU, 2016). Comparado a polissacarídeos de origem bacteriana e compostos sintéticos, os polissacarídeos vegetais apresentam baixa toxicidade e poucos efeitos colaterais, o que os torna moléculas biológicas promissoras na busca de novos agentes terapêuticos (LIU; WILLFÖR; XU, 2016; SCHEPETKIN; QUINN, 2006). Diversos tipos de atividade biológica de polissacarídeos têm sido descrita tais como atividade anti-inflamatória, antitumoral, antidiabética, antiviral, antioxidante, hipoglicêmico, antiúlcera gástrica, entre outras (SHI, 2016; WITCZAK, 1997). Acredita-se que grande parte dos efeitos biológicos apresentados por polissacarídeos possam ser atribuídos ao seu potencial como agentes modificadores da resposta biológica, ou seja, modulando a função do sistema imune (LEUNG et al., 2006; SCHEPETKIN; QUINN, 2006).

Polissacarídeos também têm demonstrado potente atividade antioxidante, sendo relatados como sequestradores de radicais livres, agentes redutores e quelantes de ferro (WANG et al., 2013, 2016b). O potencial antioxidante dos polissacarídeos os tornam úteis no tratamento ou prevenção de inúmeras doenças causadas pelo excesso de espécies reativas de oxigênio, tais como doenças hepáticas, cardiovasculares, câncer, doenças autoimunes, neurodegenerativas,

gastrite e úlcera gástrica (COGGER et al., 2004; REPETTO; LLESUY, 2002; WANG et al., 2013).

Estudos científicos têm demonstrado que a atividade biológica dos polissacarídeos é dependente da estrutura química, massa molar e conformação tridimensional dos polímeros, a qual ainda pode ser modulada por modificações químicas nos polissacarídeos (WANG et al., 2013; YANG; ZHANG, 2009). Dessa forma, é crucial determinar a estrutura química do polissacarídeo para poder inferir possíveis aplicações biológicas do polímero isolado.

Neste trabalho as atividades antiúlcera gástrica e de hepatoproteção de polissacarídeos isolados de espécies de *Artemisia* foram investigados. Dessa forma, estes dois modelos de atividades biológicas serão abordados nas próximas duas seções.

2.3 Modelos experimentais utilizados para avaliação da atividade biológica dos polissacarídeos

2.3.1 Lesão hepática induzida por agente químico

O fígado é um órgão responsável por diversas funções fisiológicas importantes, tais como síntese e degradação de proteínas, metabolismo de carboidratos, regulação do metabolismo de lipídios, síntese e excreção da bile e metabolização de drogas provenientes da absorção pelo intestino (HOE; WILKINSON, 1973; SUN; KARIN, 2008). Devido à função de detoxificação de fármacos e xenobióticos, o fígado é um local onde ocorre intenso estresse oxidativo, causado pela metabolização destes compostos no sistema microssomal hepático. Isto porque após metabolização, os compostos podem se tornar mais reativos, interferindo nas funções biológicas (COGGER et al., 2004; NGUYEN; LIGAPPA, 2007). Vários xenobióticos são conhecidos por causar hepatotoxicidade, tais como aditivos alimentares (conservantes, corantes, adoçantes), medicamentos, ingestão excessiva de etanol, drogas de abuso, dentre outros (COGGER et al., 2004; GASPAROTTO et al., 2014).

Dentre os agentes causadores de hepatotoxicidade, a lesão hepática induzida por fármacos (sigla em inglês: DILI – *Drug Induced Liver Injury*) é uma causa comum de lesão hepática, sendo responsável por metade dos casos de falência hepática

aguda (KAPLOWITZ, 2004). Atualmente, cerca de 1200 fármacos já foram relatados como potenciais agentes promotores de toxicidade hepática (RODRIGUES et al., 2016). Exemplos de fármacos que podem causar lesão hepática são o acetaminofeno (paracetamol) e cetoconazol, sendo que a overdose de acetaminofeno é a causa mais comum de injúria hepática aguda podendo levar à falência do órgão (PERIASAMY et al., 2016; RODRIGUES et al., 2016). Devido à importante função do fígado em diversos processos fisiológicos, a insuficiência hepática ou falência do órgão estão relacionadas a altas taxas de morbidade e mortalidade nos pacientes. Dessa forma, a busca de novas drogas para o tratamento ou prevenção de lesão hepática torna-se fundamental.

O agente hepatotóxico tetracloreto de carbono (CCl_4) é amplamente utilizado em laboratório como modelo para selecionar novas drogas com potencial hepatoprotetor (ALTHNAIAN; ALBOKHADAIM; EL-BAHR, 2013). O efeito tóxico do CCl_4 inicia-se com a produção do radical triclorometil (CCl_3•), quando o CCl_4 é metabolizado pelo sistema microssomal do fígado: citocromo P450. O radical formado reage com espécies reativas de oxigênio, produzindo radicais triclorometil peroxil (CCl_3OO•) que se liga as macromoléculas celulares, desencadeando peroxidação lipídica. Este processo irá promover dano às membranas plasmáticas celulares. No fígado, o CCl_4 induz necrose centrilobular, inibição da secreção de lipoproteínas VLDL, resultando em esteatose e ainda inibe a síntese proteica, levando à uma lesão hepática aguda (BOLL et al., 2001; PARK et al., 2010; RECKNAGEL et al., 1989).

Alguns extratos e polissacarídeos obtidos de plantas têm se mostrado como uma alternativa no tratamento e prevenção da lesão hepática induzida por xenobióticos (LIU et al., 2015; ROFIEE et al., 2015; XIAO et al., 2012; YE et al., 2001). O extrato etanólico das partes aéreas da planta *Laggera pterodonta* (família Asteraceae) rico em ácidos dicafeoilquínicos exerceu efeito hepatoprotetor *in vitro* contra diversos agentes químicos, tais como o CCl_4 (WU et al., 2007). Para a espécie *Artemisia vulgaris*, o extrato metanólico das partes aéreas também apresentou efeitos protetores sobre o fígado de camundongos, desafiados com D-galactosamina e lipopolissacarídeo bacteriano (LPS) (GILANI et al., 2005). Para alguns polissacarídeos isolados de fontes vegetais, também foram descritas atividades hepatoprotetoras. A inulina obtida dos tubérculos de dália exerceu efeito hepatoprotetor *in vivo*, em modelo de lesão hepática induzida por CCl_4 (LIU et al.,

2015). Polissacarídeos isolados de *Taraxacum officinale* (Asteraceae) também foram capazes de atenuar o dano hepático em ratos, induzido por CCl_4 (PARK et al., 2010). O mecanismo de ação proposto para os polissacarídeos ou extratos vegetais na proteção do fígado em resposta aos agentes químicos foi associado ao efeito antioxidante dos compostos presentes, os quais reduziriam o estresse oxidativo celular, minimizando os danos celulares. A modulação de mediadores inflamatórios no fígado também foi descrita como mecanismo de ação hepatoprotetora (GILANI et al., 2005; LIU et al., 2015; PARK et al., 2010; WU et al., 2007).

2.3.2 Úlcera gástrica

A úlcera gástrica é uma doença crônica, de alta prevalência mundial e que pode apresentar complicações nos pacientes, tais como sangramentos e perfuração do estômago (KANGWAN et al., 2014; TONETO; OLIVEIRA; LOPES, 2011). A úlcera é uma doença multifatorial, desencadeada pelo desequilíbrio entre os fatores agressores do estômago (suco gástrico e enzimas digestivas) e os fatores protetores (muco e defesas antioxidantes) (ALBAAYIT et al., 2016; SALAMA et al., 2016).

Um dos importantes fatores protetores da mucosa gástrica é a camada de muco, secretada pelas células mucosas do estômago. O muco é constituído por glicoproteínas de alta massa molecular, as quais se combinam com fosfolipídios, bicarbonato e água para compor uma camada mucosa que adere à superfície das células epiteliais do estômago. O muco gástrico é constituído por 95% de água e 5% de glicoproteínas. Esta camada confere proteção às células epiteliais contra agentes agressores físicos e químicos, tais como a lesão causada pela trituração do alimento e o suco gástrico (BUNNET; LINGAPPA, 2007).

Os medicamentos atuais utilizados para tratamento da úlcera gástrica consistem de inibidores da bomba de próton, antagonistas dos receptores H_2 de histamina e citoprotetores. No entanto, mesmo sob tratamento contínuo com estes diferentes medicamentos, pacientes relatam a recorrência da úlcera (KANGWAN et al., 2014). A recorrência da doença tem sido associada com infecções persistentes pela bactéria *Helicobacter pylori*, uso de anti-inflamatórios não-esteroidais (AINES), consumo de etanol e em pacientes fumantes (CHANDRASHEKAR; DHARMESH, 2016; MIWA et al., 2004). Atualmente, pesquisadores buscam novos agentes que possam reconstruir tanto a estrutura da mucosa quanto sua função, a fim de prevenir

a recorrência da úlcera. Este novo conceito de tratamento é denominado de “qualidade na cicatrização da úlcera” (KANGWAN et al., 2014). Dessa forma, a busca por novos compostos eficazes para o tratamento desta doença se faz necessária.

Para avaliação do efeito antiúlcera de novos compostos, diversos modelos animais podem ser utilizados, tais como indução da úlcera por etanol, ácido acético, anti-inflamatórios não-esteroidais ou ainda indução da úlcera por estresse de contenção no frio ou de natação (HARSHA; CHANDRA PRAKASH; DHARMESH, 2016; KANGWAN et al., 2014). Um dos modelos mais utilizados em laboratório é a indução da úlcera por etanol em ratos, a qual é uma metodologia bem estabelecida (ROBERT et al., 1979). A administração aguda de etanol absoluto via oral é capaz de induzir a úlcera dentro de uma hora após a aplicação. O etanol induz a úlcera no estômago por desencadear estresse oxidativo no tecido, devido ao aumento da produção de ânion superóxido, radicais hidroxila e peroxidação lipídica, bem como redução das defesas antioxidantes, como a enzima catalase e níveis da glutationa reduzida (GSH) (ALBAAYIT et al., 2016; REPETTO; LLESUY, 2002). O etanol também é conhecido por destruir a barreira protetora constituída pelo muco gástrico e reduzir o fluxo sanguíneo da mucosa, levando à hipóxia e necrose hemorrágica profunda (SZABO, 1987).

Produtos de fontes vegetais que sejam capazes de prevenir ou tratar a úlcera péptica têm atraído grande atenção na pesquisa. Tanto produtos do metabolismo secundário quanto do metabolismo primário de plantas, têm apresentado efetiva atividade antiúlcera (AUSTARHEIM et al., 2010b; MARIA-FERREIRA et al., 2013; MINOZZO et al., 2016; SIMAS-TOSIN et al., 2014). Os efeitos antiúlcera de compostos naturais são principalmente atribuídos às suas propriedades antioxidantes, aumento da produção de muco, inibição da secreção do suco gástrico e modulação de citocinas inflamatórias (ALBAAYIT et al., 2016; CHANDRASHEKAR; DHARMESH, 2016).

Alguns compostos obtidos de espécies de *Artemisia* têm revelado importante atividade antiúlcera. Lactonas sesquiterpênicas isoladas de um extrato etanólico de *A. annua* reduziram a área de lesão em ratos com úlcera induzida por indometacina e etanol (FOGLIO et al., 2002). Outra espécie, *A. douglasiana* é usada popularmente na Argentina para o tratamento de úlceras. Este uso popular da planta foi confirmado através de ensaios biológicos, onde uma lactona sesquiterpênica isolada

(dehidroleucodina) foi capaz de prevenir úlcera em animais desafiados com etanol e outros agentes necrotizantes (PENISSI; PIEZZI, 1999).

Outros compostos que também têm se destacado na prevenção da úlcera gástrica são os polissacarídeos obtidos de plantas, com destaque para a classe das pectinas. Pectinas isoladas do açafrão foram capazes de prevenir úlcera causada por estresse de natação em ratos (HARSHA; CHANDRA PRAKASH; DHARMESH, 2016). Outra fração péctica obtida das folhas de *Acmella oleracea*, também apresentou efeito antiúlcera em ratos com úlcera induzida por etanol (NASCIMENTO et al., 2013). Pectinas isoladas dos frutos secos de *Prunus domestica* também demonstraram efeito antiúlcera *in vivo* (CANTU-JUNGLES et al., 2014). Estes estudos demonstram que polissacarídeos isolados de fontes vegetais podem ser novas alternativas na busca de compostos com atividade antiúlcera.

3 OBJETIVOS

3.1 OBJETIVO GERAL:

Avaliar o efeito do estresse salino sobre a composição e estrutura dos polissacarídeos da parede celular de plantas de *Artemisia annua* e investigar as atividades biológicas associadas à infusão e polissacarídeos obtidos das espécies *Artemisia vulgaris* e *Artemisia campestris.*

3.2 OBJETIVOS ESPECÍFICOS:

- Cultivar plantas de *A. annua* em diferentes níveis de salinidade do solo;
- Avaliar o efeito do estresse salino sobre parâmetros fisiológicos de plantas de *A. annua*;
- Isolar os polissacarídeos da parede celular da parte aérea e raízes de plantas de *A. annua*, através de extrações sequenciais;
- Caracterizar as frações de polissacarídeos obtidas das extrações sequenciais;
- Identificar modificações dos polissacarídeos da parede celular das partes aéreas e raízes de *A. annua* em resposta ao estresse salino;
- Investigar a composição química da infusão de *A. vulgaris*;
- Avaliar o efeito hepatoprotetor *in vivo* das infusões de *A. vulgaris* e da inulina, polissacarídeo isolado da infusão, utilizando o tetracloreto de carbono (CCl_4) como agente hepatotóxico em camundongos;
- Extrair polissacarídeos das partes aéreas de *A. campestris* através de extração aquosa;
- Purificar e caracterizar quimicamente uma fração de interesse obtida da extração aquosa de *A. campestris;*
- Avaliar o efeito gastroprotetor *in vivo* da fração polissacarídica purificada de *A. campestris*, em modelo de úlcera gástrica induzida por etanol em ratas.

CAPÍTULO 1

EFEITOS DO ESTRESSE SALINO SOBRE OS POLISSACARÍDEOS DA PAREDE CELULAR DE *Artemisia annua*

Effects of salt stress in the cell wall polysaccharides from *Artemisia annua*

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Abstract

Artemisia annua is a medicinal plant, cultivated in several countries mainly for isolation of artemisinin, a potent antimalarial compound. Moderate salt stress has been used to increase the artemisinin synthesis by the plant. Thus, the aim of this study was to evaluate the influence of salt stress on physiological parameters and cell wall polysaccharides from aerial parts and roots of *A. annua.* Plants of *A. annua* were grown in the presence or absence of NaCl (100 and 200 mM) for 45 days. After this time, plants were harvested for growth parameters assessment and polysaccharide extraction. Cell wall polysaccharides were sequentially extracted with hot water, EDTA and NaOH from aerial parts and roots. NaCl treatment affected the development of *A. annua*, reducing the biomass of aerial parts and roots, height of plants and induced high damage of cellular membranes. Salt stress also modified the yield and monosaccharide composition of polysaccharides from cell wall. Pectins were the main cell wall polysaccharide altered under salt stress, both in aerial parts and roots. Aerial parts showed a decrease in pectin content from EDTA fraction, while for roots an increase was observed. The main modifications in the pectic fractions were increase of homogalacturonan domain, neutral side chains of pectins attached to rhamnose units and methylesterification of galacturonic acids. The effects of salt stress were more pronounced in aerial parts than roots, as observed for biomass accumulation and alterations of cell wall polysaccharides.

Keywords: *A. annua*, salt stress, cell wall polysaccharides.

1. Introduction

Artemisia annua L.*,* also known as sweet wormwood, is a medicinal plant, member of Asteraceae family and source of artemisinin, a potent antimalarial compound (WEATHERS et al., 2014). Artemisinin and its derivatives (dihydro-artemisinin, artesunate, artemether, and arteether) in combination with other antimalarial compounds are used as first line treatment of malaria caused by

Plasmodium falciparum. This therapy is known as artemisinin-based combination therapy and it is recommended by World Health Organization (WHO) since 2001 (AFTAB et al., 2016; CARRÀ et al., 2014; TAN et al., 2015). Chemical synthesis of artemisinin is a process with low yields and high costs, therefore, the extraction from *A. annua* plants continues to be the main source of the compound (AFTAB et al., 2016). The yield of artemisinin from *A. annua* is very low, ranging between 0.01-1% (CHEMAT et al., 2017), therefore, many groups have studied different methods to enhance the yield of artemisinin (AFTAB et al., 2016; IRFAN QURESHI et al., 2005; TAN et al., 2015) and refine the extraction and purification process of the compound (CHEMAT et al., 2017). One of the strategies studied to increase the artemisinin biosynthesis/content in *A. annua* is submit the plants to moderate salt stress (AFTAB et al., 2011; IRFAN QURESHI et al., 2005; QIAN et al., 2007). A significant increase in artemisinin content was reported by QIAN et al. (2007), when *A. annua* plants were submitted to 4-6 g/L of NaCl. Yield of artemisinin was 2-3% dry weight in plants submitted to salt stress, while in control plants was approximately 1%. Artemisinin content also enhanced in *A. annua* plants submitted to 100 mM NaCl stress and, this increase was further enhanced when a foliar spray of salicylic acid (1.0 mM) was applied. This condition (100 mM NaCl + 1.0 mM salicylic acid) induces an increase of 52.4% in artemisinin content (AFTAB et al., 2011).

The effects of salt stress on physiological parameters of *A. annua* have been reported. Salt stress reduces plant growth, biomass accumulation, induces oxidative stress, decreases chlorophyll content and inhibits nitrate reductase and carbonic anhydrase activities (AFTAB et al. , 2011; IRFAN QURESHI et al., 2005). However, salt stress may also alter others aspects of plant development, such the composition and architecture of cell wall (DE LIMA et al., 2014; OLIVEIRA et al., 2009; UDDIN et al., 2014), which were not evaluated in *A. annua* plants yet.

The cell wall is a dynamic and complex structure of plant cell, which mediates the interaction between the cell and its environment. Under abiotic stress, the cell wall architecture modify in order to tolerate the new environmental condition (TENHAKEN, 2015). Polysaccharides are the major components of cell wall and, they are usually classified into cellulose, hemicellulose and pectins. Changes in total cell wall sugar content, monosaccharide composition and extractability of the polymers in response to salt stress have been reported for coffee plants (DE LIMA et al., 2014), grapevines (OLIVEIRA et al., 2009) and soya beans (AN et al., 2014). For

the seagrass specie *Ruppia maritima*, sulfated polysaccharides were found in response to high salinity. In the absence of salt, sulfated polysaccharides completely disappeared, suggesting that sulfated polysaccharides in plants is an adaptation to high salt environments (AQUINO; GRATIVOL; MOURÃO, 2011).

Xie et al. (2008) reported the presence of sulfate in polysaccharide fractions obtained from *Artemisia tripartita* collected in the mountain areas of Montana (USA). However, none of the accepted cell wall models describe the presence of sulfated polysaccharides in terrestrial plants (CARPITA; GIBEAUT, 1993; COSGROVE, 2014). Typically, they are found in marine angiosperms (SILVA et al., 2011) and algae (BRITO et al., 2016). Thus, we hypothesize that high salinity might trigger the biosynthesis of sulfated polysaccharides in plants. In order to investigate this hypothesis, plants of *A. annua* were subjected to salt stress and, polysaccharides from cell wall of aerial parts and roots were evaluated regarding to sulfate content, monosaccharide composition and chemical structure and further compared to control plants.

2. Materials and methods

2.1 Plant material, growth condition and salinity treatments

Seeds of *A. annua* from CPQBA/UNICAMP were sown and after 60 days, the seedlings were transferred to 8 kg pots containing a mixture of organic compound, soil and sand (1:1:1). The stress experiment was performed in a greenhouse with daily minimum temperatures 17–20 ºC and maxima of 26–27 ºC.

Stress conditions were imposed after 15 days of acclimation. Plants were treated with 100 and 200 mM NaCl solutions and the control plants received just water. Plants were watered with solutions 3 times a week until soil saturation (tested previously by weighing the pots). Plants were subjected to salt stress for 45 days and 24 h before harvesting, they were kept in the dark to deplete starch. After harvesting, plants from each treatment were immediately frozen at -80 ºC and then lyophilized. The biometric parameters evaluated were plant length and dry mass of aerial parts and roots, using 10 plants per treatment. The plant length was measured from the base of the stem (soil level) to the top of the plant.

2.2 Lipid peroxidation rate

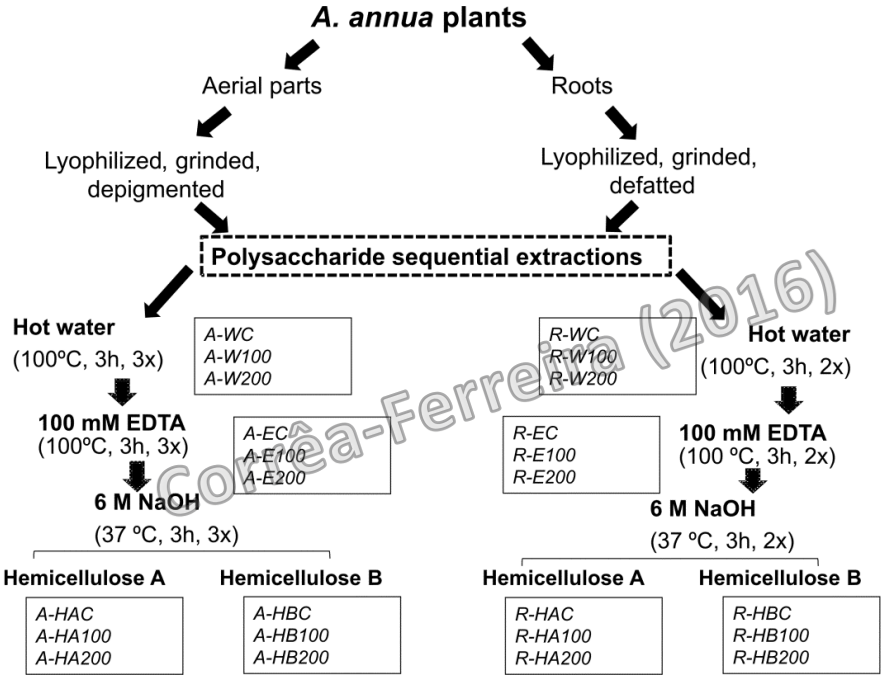
The oxidative damage to leaf lipids was evaluated by the trichloroacetic/thiobarbituric acid method, as described by Cakmak & Horst (1991). Briefly, leaf samples (100 mg fresh weight) were macerated and homogenized in 80 % ethanol (v/v). Homogenates were centrifuged at 3000 g for 10 min. Aliquots of 1 mL of the supernatant were used to quantify the malondialdehyde (MDA) levels using a spectrophotometer. MDA contents were expressed as nmol MDA/mL.

2.3 Sample preparation and polysaccharide extraction

Aerial parts of *A. annua* plants from each treatment were randomly divided in three groups. Samples were cut into small pieces and milled using IKA® A11 basic analytical mill and sieved (32 mesh). Subsequently, aerial parts were depigmented with absolute ethanol in a Soxhlet apparatus. For roots, plants from each treatment were divided in two groups, due to small amount of material. After grinding, the roots were sieved (24 mesh) and defatted using toluene:ethanol (2:1 v/v). Next, the resulting materials from aerial parts and roots were dried and submitted to polysaccharide extractions. Sequential extractions were performed using hot water (W), 100 mM EDTA (E) and 6 M NaOH (FIGURE 1). Fractions from aerial parts were preceded by the letter A and those extracted from the roots by the letter R. Fractions from control plants were identified by letter “C” and fractions from 100 mM NaCl and 200 mM NaCl treatment as “100” and “200”, respectively. Aerial parts were submitted to three extractions with each solvent and for roots just two extractions were performed. After each extraction, the residue was separated by centrifugation (4700 *x g*) and the extract was concentrated and treated with ethanol (4V) for polysaccharide precipitation. Polysaccharides were isolated via centrifugation, treated three times with absolute ethanol and dried under vacuum. Extracts obtained using EDTA were dialyzed for 72 h before treatment with ethanol. Alkaline extractions were performed in the presence of $NaBH_4$. Alkaline extracts were neutralized with acetic acid, kept at 4 ºC overnight and then, the precipitated fractions were separated by centrifugation and named hemicellulose A (HA). The supernatants were dialyzed for 72 h, concentrated and treated with ethanol (4V), resulting in

fractions hemicellulose B (HB). Final insoluble residues were washed with water, ethanol and acetone and dried under vacuum.

FIGURE 1 - SCHEME FOR SEQUENTIAL EXTRACTION OF POLYSACCHARIDES FROM AERIAL PARTS AND ROOTS OF *A. annua*.



Corrêa-Ferreira (2016)

NOTE: W- fraction obtained by boiling water; E- fraction obtained with 100 mM EDTA; HA: Hemicellulose A fraction obtained from 6 M NaOH extraction; HB: hemicellulose B fraction obtained from 6 M NaOH extraction; A- polysaccharide fraction from aerial parts; R- polysaccharide fraction from roots; C- control plants of *A. annua;* 100 – plants submitted to 100 mM NaCl; 200 – plants submitted to 200 mM NaCl.

2.4 Monosaccharide composition

Polysaccharides were hydrolyzed with 2M trifluoroacetic acid at 120 ºC, for 2 h. The hydrolyzate was evaporated to dryness, the residues were reduced with $NaBH_4$ (WOLFROM; THOMPSON, 1963b) and acetylated with pyridine–acetic anhydride (1:1, v/v, overnight) (WOLFROM; THOMPSON, 1963a). Final residues and hemicellulosic fractions were solubilized and partially hydrolyzed with 72% (w/w) aqueous-H_2SO_4 for 1 h on ice, diluted to 8% and kept in boiling water for 15 h. Hydrolyzates were neutralized with $BaCO_3$, the insoluble material was removed and the monosaccharides were reduced and acetylated as above.

The resulting alditol acetates were examined by gas chromatography (GC-MS) using a Varian 4000 chromatograph, linked to a Varian CP-3800 mass spectrometer. The alditol acetates were applied to a VF-5 capillary column (0.25 mm internal

diameter × 30 m). Injector temperature was 250 ºC and initial temperature was 100 ºC to 230 ºC, at a rate of 10 ºC/min, maintained for 35 min. Helium was used as carrier gas (1.0 mL/min). Uronic acids content was estimated with meta-hydroxydiphenyl colorimetric method (BLUMENKRANTZ; ASBOE-HANSEN, 1973) using galacturonic acid as standard, except for hemicellulosic samples, for which it was used glucuronic acid as standard. Insoluble fractions were previously hydrolyzed before colorimetric assay.

2.5 High-performance size-exclusion chromatography (HPSEC)

Isolated polysaccharides were analyzed by HPSEC using a Waters unit coupled to a refractive index (RI), a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector. Four Waters Ultrahydrogel columns (2000; 500; 250; 120) were connected in series and coupled to the multidetection instrument. A solution of 0.1 M $NaNO_2$ and 0.02% NaN_3 was used as eluent at a flux of 0.6 mL/min. Prior to the analyses, the samples (1.5 mg/mL) were filtered through a 0.22 µm cellulose acetate membrane. The data were collected and analyzed by a Wyatt Technology ASTRA program. All the analyses were carried out at 25ºC.

2.6 Nuclear magnetic resonance spectroscopy (NMR)

NMR analyses were recorded at 70 °C using a Bruker Avance III 400 MHz spectrometer equipped with a 5 mm multinuclear inverse detection probe. For 1D and 2D NMR experiments the samples were dissolved in D_2O (99.9%) at 20 mg/mL. Chemical shifts are expressed relative to TMSP-d_4 (3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) as internal standard (δ = 0) or acetone (^{1}H = 2.22 ppm; ^{13}C = 30.2 ppm).

For A-W and A-E fractions, from control and saline treatments, relative integration of signals areas of ^{1}H NMR spectrum was performed. Samples were previously desiccated under vacuum in an Abderhalden equipment containing P_2O_5, accurately weighed (10 mg) and dissolved in D_2O (500 µl), containing TMSp (0.02%). Samples were transferred to NMR tubes and analyzed at 70 °C, using 32 scans. Presaturation of residual HDO was carried out in spectrum acquisition. Integrated

area of signals was relative to TMSp area and triplicates of each fraction (A-W and A-E) from control and saline treatments were performed.

2.7 Sulfate content

The presence of sulfate was evaluated by the 1,9-dimethylmethylene blue (DMB) dye method (ENOBAKHARE; BADER; LEE, 1996). The samples (1 mg/mL) were pipetted in a 96-well plate and the DMB solution (pH 1.5) was added subsequently. The presence of sulfate was determined by the color change of solution compared to an agarose standard chemically sulfated.

2.8 Data analysis

A completely randomized design was used in experiments, with 3 treatments (control, 100 mM NaCl and 200 mM NaCl) and 15 replicates for each treatment. Plants of each treatment were randomly divided in three groups and triplicate of each polysaccharide extraction were performed. Monosaccharide composition of each sample was done at least in duplicate. The data are expressed as mean ± standard error of three independent experiments. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by the Bonferroni's multiple comparison test. Prism 5.0 software (GraphPad, San Diego, CA, USA) was used for the statistical analysis. Values of $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1 Effects of salt stress on the development of *A. annua* plants

Salt stress negatively affected the growth of *A. annua* plants. After 45 days of 100 mM NaCl treatment one plant died and, for the highest NaCl concentration two plants died. Average height of plants treated with 100 and 200 mM NaCl decreased by 28.1 and 44.5%, respectively, compared to control group (FIGURE 2A). Similar results were found by Aftab et al. (2011) for *A. annua* plants submitted to salt stress in which 200 mM NaCl treatment reduced plant shoot length by 37.5%. Aerial biomass decreased in both treatments, in a dose-dependent manner (FIGURE 2B).

Aerial biomass from plants treated with 100 and 200 mM NaCl decreased by 37.4 and 65.2% when compared to control plants, respectively. Aftab et al. (2011) described a reduction of 42.6% in the shoot dry weight in *A. annua* plants submitted to 200 mM NaCl.

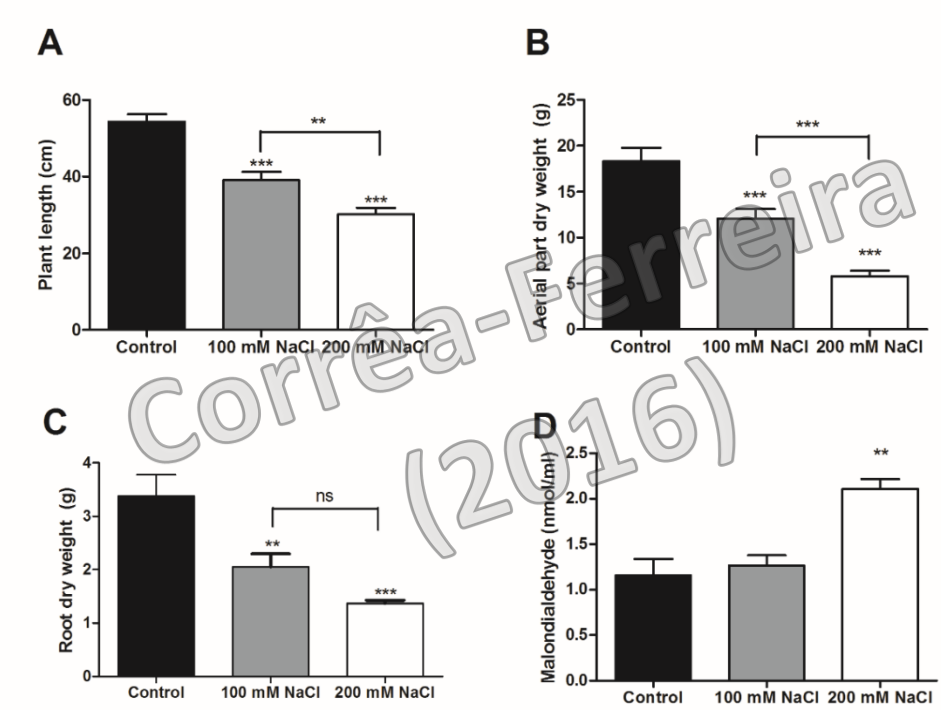
Root biomass was also negatively affected by salinity (FIGURE 2C). Plants exposed to 100 mM NaCl showed a reduction of 40.0% in root biomass while plants growing in 200 mM NaCl exhibited a reduction of 59.6%. On severe stress (200 mM NaCl), aerial parts growth was most affected than root growth. Although roots are directly exposed to salt, they are remarkable robust and usually, their growth are less affected than shoots, allowing to roots explore water in soil (MUNNS, 2002; TENHAKEN, 2015). According to Munns & Tester (2008), this divergence indicates different changes in cell wall properties between roots and aerial parts.

The pronounced reduction in the height and biomass production in *A. annua* plants submitted to salt stress reflects the toxic effects of NaCl on plant metabolism. Salinity affects the plant development firstly through osmotic stress, due to the reduction of osmotic potential of the soil solution, making it difficult for the plant to obtain either water or nutrients (MUNNS; TESTER, 2008). As a result, the reduced turgor pressure in the cell hinders the division and expansion of meristematic cells (GALL et al., 2015; TENHAKEN, 2015). Besides, salt stress also triggers ionic stress, as a result of accumulation of Na^+ ions in the plant cell cytoplasm until it reaches toxic levels (MAHAJAN; TUTEJA, 2005; SHAVRUKOV, 2013). Uptake of Na^+ competes with K^+ absorption, inducing K^+ deficiency in the plant (PARIDA; DAS, 2005). K^+ ion performs key roles at the plant, such as maintenance of the osmotic balance, opening and closing of stomata and it is an essential co-factor for many enzymes (MAHAJAN; TUTEJA, 2005). Therefore, high Na^+/K^+ ratio, as well as high concentration of total salts will cause inactivation of enzymes and protein synthesis, affecting the production of plant biomass.

The damaging effect of salt stress on *A. annua* plants was also observed by the increase in the malondialdehyde (MDA) levels in the leaves. MDA is a product of lipid peroxidation in cellular membranes and largely used as oxidative stress marker (WANKHADE; SANZ, 2013). When plants are submitted to environmental stresses, reactive oxygen species (ROS) accumulate within the cells, generating oxidative damage on cellular structures, such as plasmatic membrane (ALSCHER; ERTURK; HEATH, 2002). Severe stress induced an increased in 81.9% in MDA levels

(FIGURE 2D), indicating high oxidative damage in plant cellular membranes. This increase in MDA content is in agreement with previous report which described an increase of 98% of MDA contents in *A. annua* plants submitted to 200 mM NaCl (AFTAB et al., 2011). Leaf MDA content also increased in potato (*Solanum tuberosum*) submitted to salt stress. MDA content increased in the leaves with the increase of salinity (25-200 mM NaCl) and exposure time to stress (GAO et al., 2014).

FIGURE 2 - EFFECTS OF SALINITY ON GROWTH AND MALONDIALDEHYDE CONTENT IN *A. annua* PLANTS.



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NOTE: (A) Average length of plants; (B) Aerial parts dry weight; (C) Root dry weight; (D) Leaf malondialdehyde content. Results were analyzed using one-way ANOVA test with Bonferroni's multiple comparison test, compared to the control. Symbols: *P<0.05, **P<0.01, *** P<0.001, ns: non-significant. Data represent the means ± standard error (vertical bars) of at least 10 individual plants from each NaCl treatment and control.

3.2 Characterization of polysaccharides from cell wall of the aerial parts and roots of control plants of A*. annua*

Polysaccharides from aerial parts and roots of *A. annua* were sequentially extracted with hot water (W), 100 mM EDTA (E) and 6 M NaOH, to obtain pectic and hemicellulosic polymers. For aerial parts, A-WC fraction displayed the highest yield (17%) followed by A-EC fraction (5.8%), while for roots, alkaline fractions (R-HAC

and R-HBC) exhibited the highest yield (R-HAC: 8.1%; R-HBC: 5.9%), followed by R-WC (6.8%). The yield of final insoluble residue, obtained after all sequential extractions of *A. annua* cell wall was higher for the roots (39%) than that found for aerial parts (7%) (TABLE 1).

Water soluble polysaccharides (A-WC and R-WC fractions) extracted from aerial parts and roots of *A. annua* had similar monosaccharide composition (TABLE 1). Both presented high content of uronic acids and the main neutral sugars were Ara and Gal, suggesting the presence of pectins and arabinogalactans. Pectins are a complex group of polysaccharides, present in large amounts in primary cell wall of plants and mainly characterized by high content of galacturonic acid. Pectic polysaccharides includes three main constituents: homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II), which are probably covalently inter-linked. HG is the linear component of pectin, constituted by a α-D-Gal*p*A (1→4-linked) backbone. RG-I is a ramified portion of pectin, with a backbone constituted by the disaccharide (α-1,4-D-Gal*pA*-α-1,2-L-Rha*p*) repeated. Neutral side chains of arabinans, galactans and arabinogalactans may be found attached to O-4 of Rha units. RG-II is the most complex pectin component, showing a α-D-Gal*pA* (1→4-linked) backbone, substituted with side chains in O-2 or O-3 position with several types of monosaccharides (HARHOLT; SUTTANGKAKUL; VIBE SCHELLER, 2010; RIDLEY; O'NEILL; MOHNEN, 2001; YAPO, 2011).

In order to obtain information about the pectin composition, ratio between the monosaccharides may be calculated. The ratio UA/Rha has been employed to estimate the relative abundance of HG versus RG domains of pectins while the ratio Ara+Gal/Rha has been used to estimate the relative importance of neutral side chains to the RG-I backbone of pectin (RENARD; GINIES, 2009). Roots of *A. annua* displayed higher HG domain in the pectin than aerial parts, as observed by higher UA/Rha ratio (TABLE 1). Regard to RG-I domain, roots displayed more neutral sugars (Ara and Gal) per rhamnose unit of backbone than aerial parts, as demonstrated by higher Ara+Gal/Rha ratio (TABLE 1).

Both W fractions, from aerial parts and roots, displayed a polymodal elution profile, in the HPSEC analysis (data not shown).

TABLE 1 - YIELD AND MONOSACCHARIDE COMPOSITION OF POLYSACCHARIDE FRACTIONS OBTAINED FROM AERIAL PARTS AND ROOTS OF *A. annua* CELL WALL.

	Aerial Parts (A)					Roots (R)				
	A-WC	**A-EC**	**A-HAC**	**A-HBC**	**Residue**	**R-WC**	**R-EC**	**R-HAC**	**R-HBC**	**Residue**
Yield (%)[1]	17.0 ± 0.9	5.8 ± 0.2	1.8 ± 0.5	4.0 ± 1.7	7.1 ± 0.7	6.8 ± 0.2	1.0 ± 0.1	8.1 ± 0.3	5.9 ± 0.2	39.1 ± 0.4
Monosaccharide composition (%)[2]										
Rha	12.4 ± 0.9	14.2 ± 0.6	1.5 ± 0.2	2.1 ± 0.2	1.1 ± 0.1	7.7 ± 0.6	9.8 ± 0.5	2.4 ± 0.04	3.2 ± 0.3	2.3 ± 0.2
Fuc	tr	tr	1.4 ± 0.4	1.4 ± 0.3	tr	1.4 ± 0.2	2.1 ± 1.4	tr	tr	tr
Ara	27.4 ± 0.4	23.1 ± 0.5	2.2 ± 0.3	3.9 ± 0.4	1.7 ± 0.9	19.9 ± 0.1	22.0 ± 5.8	2.3 ± 0.2	4.0 ± 0.2	2.0 ± 0.4
Xyl	3.6 ± 0.1	3.9 ± 0.2	35.5 ± 4.8	27.9 ± 4.1	17.1 ± 0.1	5.1 ± 0.4	3.5 ± 1.0	65.1 ± 0.7	42.0 ± 1.4	26.1 ± 1.6
Man	5.3 ± 0.6	0.85 ± 0.1	21.1 ± 1.5	13.8 ± 0.7	3.6 ± 1.0	7.1 ± 1.1	2.7 ± 0.7	5.6 ± 0.8	16.5 ± 0.9	5.0 ± 0.8
Gal	23.4 ± 0.7	17.3 ± 0.4	7.1 ± 1.1	11.8 ± 1.1	1.9 ± 0.8	24.2 ± 1.6	9.6 ± 0.1	2.8 ± 0.2	6.9 ± 0.4	4.2 ± 1.2
Glc	5.6 ± 0.4	2.0 ± 0.2	25.3 ± 2.6	25.7 ± 0.6	74.4 0.8	8.8 ± 0.1	3.4 ± 0.5	9.5 ± 0.6	12.8 ± 0.5	63.3 ± 3.1
UA[3]	19.6 ± 1.2	34.7 ± 1.0	5.6 ± 0.2	7.8 ± 0.1	tr	25.7 ± 0.1	46.6 ± 2.7	12.0 ± 0.1	14.0 ± 0.3	tr
UA/Rha	1.6 ± 0.2	2.3 ± 0.1				3.3 ± 0.3	4.8 ± 0.5			
(Gal+Ara)/Rha	4.4 ± 0.4	2.5 ± 0.2				5.8 ± 0.6	3.3 ± 0.3			

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NOTE: W- fraction obtained by boiling water; E- fraction obtained with 100 mM EDTA; HA: Hemicellulose A fraction obtained from 6 M NaOH extraction; HB: hemicellulose B fraction obtained from 6 M NaOH extraction; A- polysaccharide fraction from aerial parts; R- polysaccharide fraction from roots; C- control plants of *A. annua*. Results were expressed as mean ± standard error of the mean.
tr: trace.

[1] Based on dry depigmented aerial parts or defatted roots;
[2] Neutral sugars determined by GC-MS;
[3] Uronic acids determined by colorimetric method (BLUMENKRANTZ; ASBOE-HANSEN, 1973).

Correlations in the heteronuclear single quantum coherence (HSQC) of W fractions confirmed the presence of pectins in roots and aerial parts. Identical signals were found in $^{1}H/^{13}C$ HSQC correlation map of the W fraction from roots and aerial parts, and then, only a representative HSQC correlation map is showed (FIGURE 3A). Following assignments were based on literature values (GOLOVCHENKO et al., 2012a, 2012b; KOSTALOVA; HROMÁDKOVÁ; EBRINGEROVÁ, 2013; SHAKHMATOV et al., 2015). The anomeric region displayed chemical shifts typical of the homogalacturonan chain, represented by the signal of 1,4-linked α-D-Gal*p*A at δ 99.3/5.11 and, the methyl esterified units at 100.0/4.95 and 100.0/4.92 ppm.

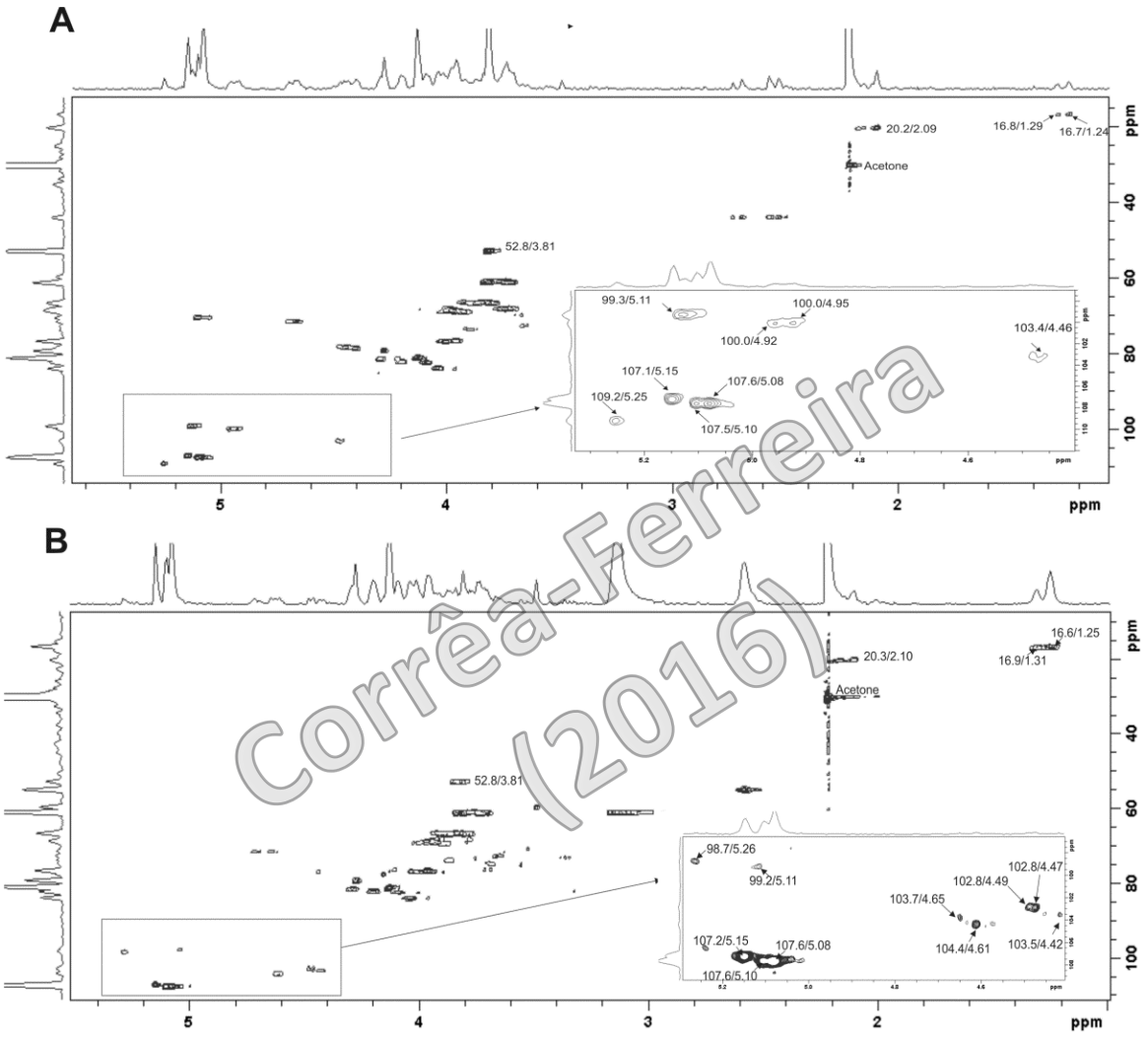
Resonance of the methoxy group carbon was observed at δ 52.8/3.81 ppm and those of the acetyl groups appeared at *δ* 20.2/2.09; 20.4/2.15 and 20.5/2.18.

The signals at δ 107.6/5.08 and 107.5/5.10 were attributed to 1,5 and 1,3,5-linked α-L-Ara*f*. Other signals of arabinose were found in δ 107.1/5.15 and 109.2/5.25 ppm corresponding to 1,3-linked and terminal α-L-Ara*f*, respectively. These signals indicate the presence of arabinans and/or arabinogalactans as side chains of RG-I. It was also identified the signal of β-D-Gal*p* (δ 103.4/4.46), suggesting the presence of galactans and/or arabinogalactans.

The anomeric carbon signal of α-L-Rha*p* was not detected in the HSQC correlation map, however the signals of methyl group of 1,2- and 1,2,4-substituted rhamnose were found at δ 16.7/1.24 and 16.8/1.29 ppm, respectively (SHAKHMATOV et al., 2015), which suggest the presence of the rhamnogalacturonan I (RG-I) in the aqueous fractions.

Pectins were the major components of E fractions (A-EC and R-EC) as indicated by the predominance of uronic acids, arabinose, galactose and rhamnose as monosaccharides. UA/Rha ratio was two-fold higher in roots (4.8) than aerial parts (2.3), indicating predominance of HG domains in pectin from roots (TABLE 1). Roots also exhibited higher Gal+Ara/Rha ratio than aerial parts, as also observed for aqueous fraction, indicating that pectic polymers of E fraction from roots have larger neutral side chains than aerial parts.

FIGURE 3 - $^{13}C/^{1}H$ HSQC OF A-WC FRACTION (A) AND A-EC (B) FRACTION.



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NOTE: Samples were dissolved in D_2O (99.9%) at 20 mg/mL and spectra were recorded at 70 °C. Chemical shifts are expressed relative to acetone (^{1}H = 2.22 ppm; ^{13}C = 30.2 ppm).

The presence of pectins in E fractions from roots and aerial parts was confirmed by NMR analysis. The HSQC of A-EC fraction is showed in FIGURE 3B. For aerial parts, it was observed signals of RG-I domain as evidenced by the signals of C1/H1 of rhamnose at 98.7/5.26 ppm and the C1/H1 of non-esterified galacturonic acid in δ 99.2/5.11. For roots, besides the non-esterified uronic acids, it was also observed the signal of methyl esterified uronic acids at 100.1/4.96 ppm (data not shown). Signals of methyl group of 1,2- and 1,2,4-substituted rhamnose were found at δ 16.7/1.24 and 16.8/1.29 ppm, respectively (SHAKHMATOV et al., 2015).

Signal of acetyl groups were found at δ 20.3/2.11 - 20.4/2.17 and, methyl group at 52.8/3.81 ppm. Arabinan and/or arabinogalactan side chains of RG-I

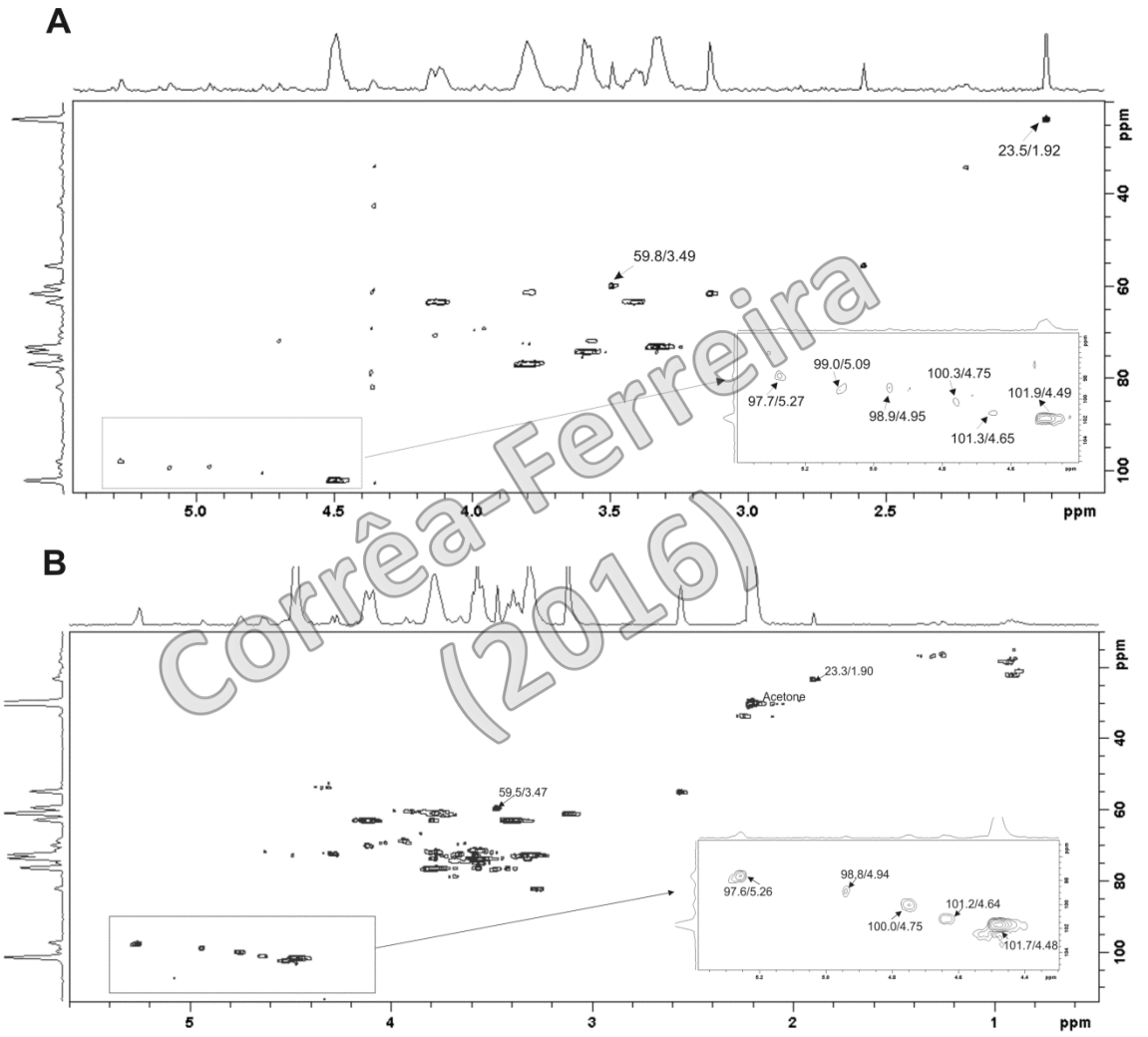
were evidenced by the signals at δ 107.8/5.08, 107.6/5.10 and 107.2/5.15 attributed to C1/H1 of α-L-Ara*f* units. Signals of galactose residues were found in δ 104.4/4.61 and 103.7/4.65 corresponded to C1/H1 of (1→4)-linked-β-D-Gal*p* units from arabinogalactans type I (AG-I) and, in 103.5/4.42, 102.8/4.49 and 102.8/4.47, corresponded to C1/H1 of (1→3,6), (1→6) and (1→3)-linked-β-D-Gal*p* units from arabinogalactans type II (AG-II). Arabinogalactans type II were also found in the cell wall of aerial parts of *Artemisia absinthium* (CORRÊA-FERREIRA; NOLETO; PETKOWICZ, 2014). All above assignments were based on literature values (GOLOVCHENKO et al., 2012a, 2012b; HROMÁDKOVÁ et al., 2014; SHAKHMATOV et al., 2015).

Hemicellulosic fractions extracted with 6 M NaOH were fractionated in hemicellulose A and B (HA and HB, respectively), which displayed similar monosaccharide composition, in the aerial parts as well as in the roots (TABLE 1). Both hemicellulosic fractions exhibited xylose as main monosaccharide, in addition to glucose, mannose, galactose and uronic acids.

HA and HB fractions extracted from aerial parts and roots showed the same correlations in $^{13}C/^{1}H$ in HSQC correlation map, therefore representatives spectra are showed (FIGURE 4A-B). Signals observed in the spectra indicate the presence of a 4-O-methylglucuronoxylan as the main hemicellulosic component of *A. annua* cell wall. $^{13}C/^{1}H$ HSQC of HAC and HBC fractions, either for aerial parts and roots, displayed signals of C1/H1 of 1,4-linked β-D-Xyl*p* at 101.7/4.48 and, at 101.2/4.64 ppm signal from 2,4-substituted β-D-Xyl*p*. The xylan backbone is substituted in O-2 for 4-O-methyl-α-D-Glc*p*A, as evidenced by the signals at 97.6/5.26 ppm of C1/H1 of glucuronic acid and the methyl group at δ 59.5/3.47. The signal at 23.3/1.90 ppm indicates the xylan backbone is acetylated. Signal of arabinose was not found in the $^{13}C/^{1}H$ HSQC of hemicellulosic fractions of *A. annua*, suggesting the glucuronoxylan from *A. annua* cell wall does not show arabinose as substitution. The correlation at 100.0/4.75 ppm may indicate the presence of β-D-Man*p* 1,4-linked, typical of mannans. Signals were assigned based on the HSQC spectrum following sugar composition and literature (GUAN et al., 2015; GUO et al., 2011; PENG et al., 2011). Glucuronoxylans were also found in seeds of *Artemisia sphaerocephala* Krasch (GUO et al., 2011), stems of *Mahonia aquifolium* (KARDOŠOVÁ et al., 2002) and leaves of *Coffea arabica* var Mundo Novo (WENZEL; CORRÊA,

1977). In dicots, as *A. annua*, glucuronoxylans are usually found in the secondary walls (SCHELLER; ULVSKOV, 2010).

FIGURE 4 - $^{13}C/^{1}H$ HSQC OF A-HAC FRACTION (A) AND A-HBC (B) FRACTION.



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NOTE: Sample A-HAC was dissolved in 0.1 M NaOD at 20 mg/mL and spectra were recorded at 70 °C. Chemical shifts are expressed relative to TMPS-*d4* (δ=0). Sample A-HBC was dissolved in D_2O at 20 mg/mL and spectra were recorded at 70 °C. Chemical shifts are expressed relative to acetone (^{1}H = 2.22 ppm; ^{13}C = 30.2 ppm).

Final insoluble residue, obtained after extractions of polysaccharides from cell wall, was also analyzed and it is basically constituted by cellulose, either in aerial parts or roots, showing about 70% of glucose in the residue composition. Cellulose microfibrils are the main structural component of plant cell wall, which provide mechanical strength to plant tissue (MARTINEZ-SANZ; GIDLEY; GILBERT, 2015; WANG; PHYO; HONG, 2016).

The presence of sulfate in the polysaccharides from *A. annua* cell wall was evaluated. Although the presence of sulfate in polysaccharide from leaves of *Artemisia tripartita* had been described (XIE et al., 2008), in the present study, DMB assay showed that polysaccharide fractions isolated from aerial parts and roots of *A. annua* did not contain sulfate in their composition. However, the production of sulfated polysaccharide in halophytic species in response to saline environment suggest a correlation between salinity and sulfated polysaccharides in plants (AQUINO; GRATIVOL; MOURÃO, 2011). Therefore, the presence of sulfated polysaccharides in *A. annua* plants submitted to salt stress was also evaluated.

3.3 Changes in cell wall composition of *A. annua* aerial parts in response to salt stress

Polysaccharides from aerial parts of *A. annua* plants submitted to salt stress were sequentially extracted (FIGURE 1) and the composition was compared to control group.

In response to high-salinity environments, the halophytic specie (*Ruppia maritima*) produces sulfated polysaccharides as an adaptation mechanism to survive in this condition (AQUINO; GRATIVOL; MOURÃO, 2011). The presence of sulfate in the polysaccharides of *A. annua* submitted to salt stress was investigated, but it was not detected sulfate in all fractions tested, indicating that *A. annua* does not produce sulfated polysaccharides in response to salt stress, neither in normal conditions. Instead of, changes observed under salt stress were related to yield and composition of polymers. Severe stress (200 mM NaCl) facilitated the extraction of pectins by hot water, evidenced by the slightly increase of the yield of A-W200 fraction (A-W200: 22%; A-WC: 17%), whereas the amount of pectins extracted with the chelating agent (EDTA) exhibited significantly reduction in the yield (A-E200: 2.5%; A-EC: 5.8%). These data suggest remodeling of cell wall during the salt stress. According to Gall et al. (2015), the cell wall remodeling could allow the plant growth under the stress situation, by contribute in maintain the turgor pressure, which is critical for the plant expansion. Remodeling of polysaccharides from cell wall during salt stress was also reported for coffee leaves, in which yield and composition of pectins

and hemicelluloses were altered in response to salinity (DE LIMA et al., 2014). However, in the present study, the yield of hemicellulosic fractions and final insoluble residue from aerial parts were not altered in response to salt stress.

Monosaccharide composition of all fractions obtained from aerial parts of *A. annua* was affected by salt stress (FIGURE 5A). Regard to aqueous fractions (A-W; A-W100 and A-W200), salinity significantly increased the uronic acids contents ($P<0.001$) and reduced the proportion of rhamnose ($P<0.01$) (FIGURE 5A). The ratio between uronic acids content and rhamnose (UA/Rha) significantly increased in the A-W100 and A-W200 fractions ($P<0.05$) compared to control plants, indicating increase of HG domain of pectins from salt stressed plants (FIGURE 5B). Increase of carboxylated polysaccharides in the cell wall of plants submitted to high salt environment was also reported for rice (*Oryza sativa*) (AQUINO; GRATIVOL; MOURÃO, 2011) and coffee plants (*Coffea arabica*) (DE LIMA et al., 2014), indicating a role of acidic polysaccharides under salt stress condition.

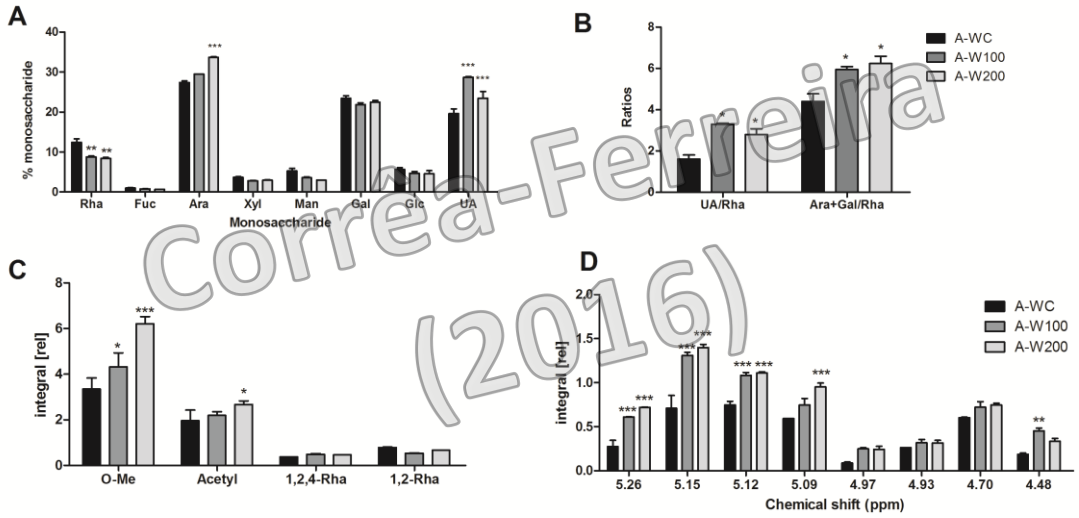
Under severe stress, polysaccharides extracted with hot water also presented significantly increase in arabinose contents ($P<0.001$) and molar ratio of (Gal+Ara)/Rha ($P<0.05$) both in moderate and severe stress comparative to control plants, indicating an increase in neutral side chains of RG-I backbone during salt stress condition. The increase of pectins side chains appears to be a common mechanism of plants in tolerance of salt stress, because their importance is related to their capability in binding water and to form gels in the cell wall, and also, the side chains of pectins may protect the cytosol during drought stress (GALL et al., 2015; LEUCCI et al., 2008; WILLATS et al., 2001).

Regarding to HPSEC analysis, no differences were found between the elution profile of water soluble polysaccharides from control and saline treatments (data not shown).

W fractions were submitted to a detailed analysis, comparing the ^{1}H and $^{13}C/^{1}H$ HSQC NMR spectra of control and salt stressed plants. Qualitative analysis of HSQC indicates that A-W100 and A-W200 were very similar to control (A-WC). All correlations found in HSQC spectrum of A-WC (FIGURE 3A) were also present in the fractions from salt stressed plants. A quantitative analysis was necessary to evidence the differences between the samples, therefore, an integration of signals from ^{1}H spectrum was performed. Integrated

area of signals exhibited is relative to TMSP-d_4 area (FIGURE 5C-D). Regarding the signal at 3.81 ppm from methyl group of galacturonic acid, both A-W100 and A-W200 displayed significantly higher integrated area than A-WC, indicating that under salt stress pectins from *A. annua* are more methylesterified. Highly methylated pectin was found in petioles of an aspen hybrid submitted to 50 mM NaCl (MUSZYŃSKA; JAROCKA; KURCZYNSKA, 2014). Besides, Gall et al. (2015) suggest that the fine regulation of degree of methylesterification of pectins and the amount of HG domain in the cell wall, determines the stiffness and hydration status of pectic matrix during water deficit.

FIGURE 5 - EFFECTS OF SALT STRESS ON THE POLYSACCHARIDES EXTRACTED WITH HOT WATER (W) FROM AERIAL PARTS OF *A. annua*.



Correa-Ferreira (2016)

NOTE: (A) Comparison of monosaccharide composition of aqueous fraction from control (A-W) and saline treatments (A-W100; A-W200); (B) comparison of ratios UA/Rha and Ara+Gal/Rha between W samples from control and saline treatments; (C) Comparison of integrated area of signals from ^{1}H spectrum between W samples from control and saline treatments (O-Me: 3.81 ppm; acetyl: 2.09, 2.15, 2.18 ppm; 1,2,4-Rha: 1.29 ppm; 1,2-Rha: 1.24 ppm); (D) Comparison of integrated area of signals of anomeric region of ^{1}H spectrum between W samples from control and saline treatments. Results are expressed as mean ± standard error of the mean of three independent experiments.
Each value of monosaccharide composition represents the mean of three independent extractions. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni's multiple comparison test. Symbols: * P<0.05, ** P < 0.01 and *** P < 0.001 statistically different from control plants.

Regarding to the signal of acetyl groups (δ 2.19, 2.15, 2.11), salt stressed plants displayed higher integrated area than control plants. The role of acetyl groups in pectins seems to be associated with the cell wall viscosity, prevention of the interaction of pectin with calcium ions and also avoid the

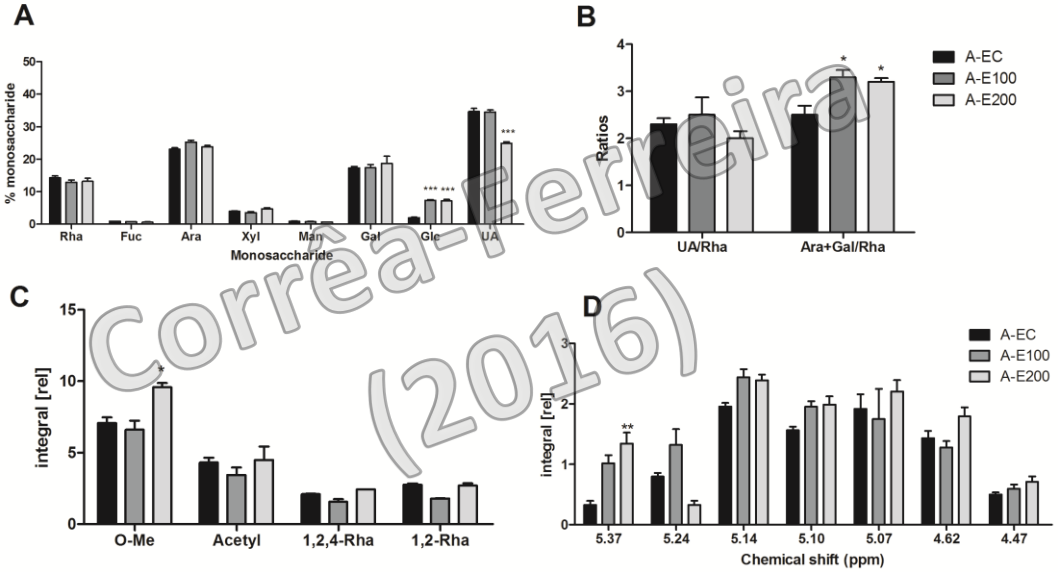
degradation of pectin by microbial hydrolases (CAFFALL; MOHNEN, 2009; SÉNÉCHAL et al., 2014). In the anomeric region of ^{1}H spectrum, important changes were observed. Plants submitted to salt stress showed increase of integrated area of signals corresponding to arabinose signals (δ 5.26, 5.15, 5.12, 5.09) (FIGURE 5D). At 5.12 ppm, signal of 1,4-linked α-D-Gal*p*A is overlaid with arabinose signal. Arabinose is also found in high concentration in resurrection plants that survive desiccation, indicating an important role of arabinans in cell wall of plants submitted to hydric deficit (GRIBAA et al., 2013). Salt stress may be considered a physiological drought, considering the high salt content in the soil make harder for roots to extract water (MAHAJAN; TUTEJA, 2005).

Analysis of fractions extracted with 100 mM EDTA from salt stressed plants also exhibited some changes in monosaccharide composition compared to control plants (FIGURE 6A). It was observed an increase in glucose contents from salt stressed plants and decrease of uronic acids contents. This increase of glucose in salt stressed plants may indicate an increase in the starch content under salt stress, since these fractions also displayed positive reaction with Lugol's solution. For A-E100 and A-E200 fractions was found an intense signal in the ^{13}C/^{1}H HSQC at 99.7/5.37 ppm that was less intense in control group. This signal was attributed to anomeric carbon of α-Glc, from starch molecules (MARIA-FERREIRA et al., 2013). These results were confirmed by the significant increase of integrated area of the signal 5.37 ppm in the ^{1}H spectrum of A-E200 compared to A-EC (FIGURE 6D). The increase of starch in response of salt stress was also observed in potato (GAO et al., 2014), tomato (YIN et al., 2010) and rice (THITISAKSAKUL et al., 2015) submitted to salt stress. In tomato fruits, an increase of ADP-glucose pyrophosphorylase activity, a key enzyme in starch synthesis, was reported along with the increase of starch content.

Uronic acids content reduced in E fraction in response to severe stress ($P<0.001$), but an increase was observed in W fraction, suggesting predominance of slightly bound pectins in salt stressed plants. Regarding to the ratio UA/Rha, no statistical difference was found between the control and salt stress treatments (FIGURE 6B). However, A-E200 displayed increase in the

ratio Ara+Gal/Rha, indicating of increase of side chains of pectins, as observed for aqueous fraction.

FIGURE 6 - EFFECTS OF SALT STRESS ON THE POLYSACCHARIDES EXTRACTED WITH 100 MM EDTA (E) FROM AERIAL PARTS OF *A. annua*.



Correa-Ferreira (2016)

NOTE: (A) Comparison of monosaccharide composition of EDTA fraction from control (A-EC) and saline treatments (A-E100; A-E200); (B) Comparison of ratios UA/Rha and Ara+Gal/Rha between E samples from control and saline treatments; (C) Comparison of integrated area of signals from ^{1}H spectrum between E samples from control and saline treatments (O-Me: 3.81 ppm; Acetyl: 2.11, 2.17 ppm; 1,2,4-Rha: 1.29 ppm; 1,2-Rha: 1.24 ppm); (D) Comparison of integrated area of signals of anomeric region of ^{1}H spectrum between samples from control (A-EC) and saline treatments (A-E100; A-E200). Results are expressed as mean ± standard error of the mean of three independent experiments.
Each value of monosaccharide composition represents the mean of three independent extractions. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni's multiple comparison test. Symbols: * P<0.05, ** P < 0.01 and *** P < 0.001 statistically different from control plants.

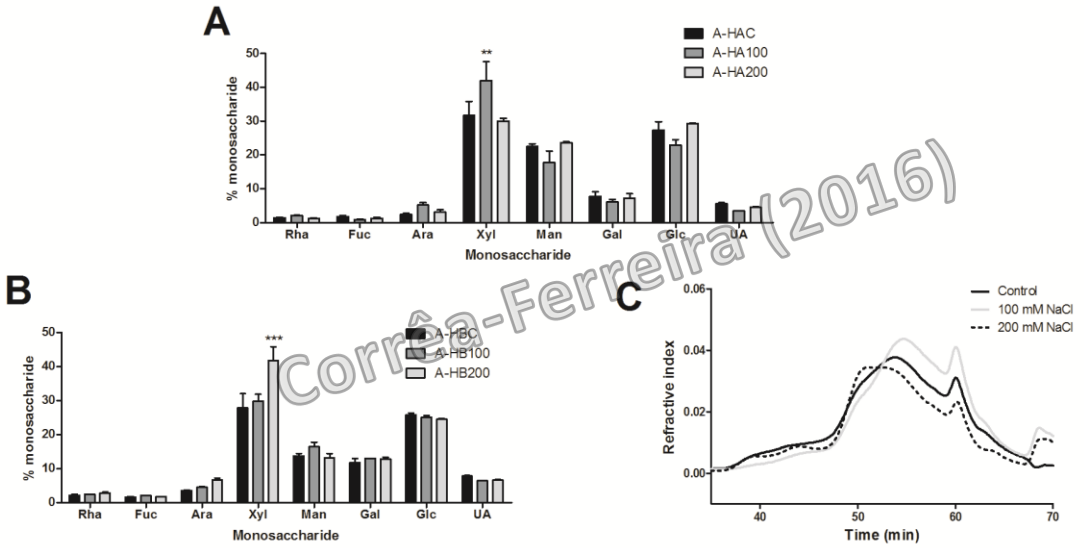
Quantitative analysis of ^{1}H spectrum of A-E fractions indicated that A-E200 exhibited significant increase of methyl groups from uronic acids compared to A-EC (FIGURE 6C). No differences were found in the signals of acetyl groups and CH_3 of Rha units in ^{1}H NMR spectrum among control and saline treatments (FIGURE 6C). In the anomeric region, only the signal at 5.37 ppm showed significant difference between treatments (FIGURE 6D).

Similar chromatograms were obtained on HPSEC analysis for E fractions for control and saline treatments (data not shown).

Hemicellulosic fractions obtained from aerial parts of *A. annua* submitted to salt stress displayed higher levels of xylose than control plants, both in HA

and HB fraction (FIGURE 7A-B), indicating an important role of xylose-containing polysaccharides under salt stress. The increase of xylose suggests that xylan backbone under salt stress displayed lower degree of substitution. The substitutions along the xylan backbone affect their physicochemical properties, such as the interaction with cellulose in the cell wall (CARPITA, 1996; REIS et al., 2003). High degrees of substitutions in hemicellulosic polysaccharides restrict the cross-linking with cellulose microfibrils and, thereafter, low degrees of substitution contribute to the stiffening of the cell wall and allow the cell to resist the internal turgor pressure (CARPITA, 1996; GRIBAA et al., 2013). Stiffening of the cell wall could contribute to enable plants to tolerate salinity stress.

FIGURE 7- EFFECTS OF SALT STRESS ON THE POLYSACCHARIDES EXTRACTED WITH 6 M NaOH FROM AERIAL PARTS OF *A. annua*.



Corrêa-Ferreira (2016)

NOTE: Comparison of monosaccharide composition of hemicellulose A fraction (A) and hemicellulose B fraction (B) between control and saline treatments from aerial parts; (C) comparison of elution profile by HPSEC with RI detection of hemicellulose B fractions.
Each value of monosaccharide composition represents the mean of three independent extractions. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni's multiple comparison test. Symbols: ** $P < 0.01$ and *** $P < 0.001$ statistically different from control plants.

HB fractions from aerial parts exhibited important changes in molar mass distribution of polymers in response to salt stress, mainly in the region near to 50-60 min (FIGURE 7C). The peak eluted around 50 min in A-HB200 fraction is

shifted toward to a lower retention time than A-HBC fraction, indicating the presence of a molecule with higher molar mass. The intensity of the peaks around 50-60 min is also altered in A-HB200, suggesting that salt stress also induces changes in molar mass of cell wall hemicellulosic polymers. The increase of xylose in the polymers may contributed in modify the molar mass distribution pattern.

Comparative analysis of HSQC of hemicellulosic fractions from control and saline treatments demonstrated similar spectra for all samples.

After all sequential extractions, the final insoluble residues of aerial parts from salt stressed plants showed predominance of glucose (70%) and xylose (20%) along with minor components, such as mannose, galactose and arabinose. Statistical analysis did not show any differences between control plants and salt stressed regarding to residue composition.

3.4 Changes in cell wall composition of *A. annua* roots in response to salt stress

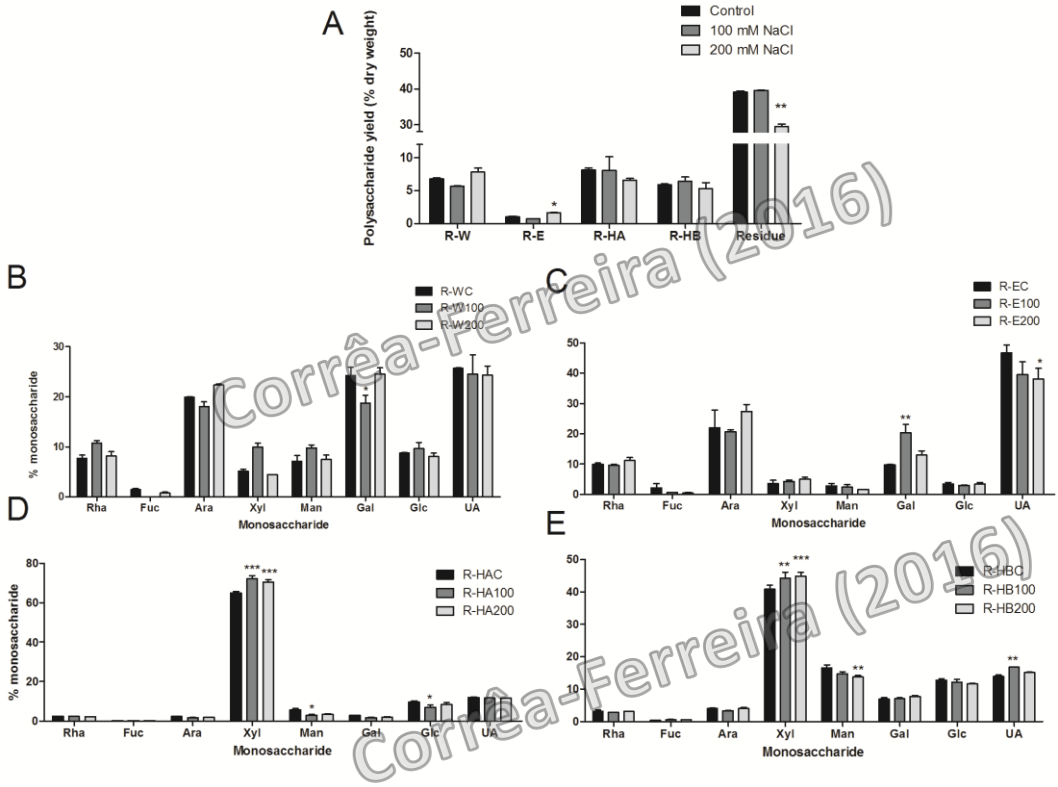
Polysaccharide fractions obtained from roots of *A. annua* grown under salt stress conditions were also evaluated. Pectins were the main components of W and E fractions, and glucuronoxylan was found as the main hemicellulosic polymer. For roots, it was observed pronounced changes in the yield of extracted polymers and minor modifications in the monosaccharide composition of fractions (FIGURE 8). It was observed an increase in the yield of E fraction from 200 mM NaCl treatment ($P<0.05$) (FIGURE 8A), suggesting that increase of pectins might contribute for root growth under salt stress. An et al. (2014) also observed a higher content of pectins in the roots of salt tolerant cultivar of soybean compared with the sensitive cultivar one.

Salinity also induced a decrease in the amount of insoluble cell wall material from roots ($P<0.001$), which is basically constituted by cellulose (~60% Glc). Other studies also observed a reduction in cellulose contents in plants submitted to salt stress (IRAKI et al., 1989; ZHONG; LAUCHLI, 1993). The lower content of cellulose may indicate that high NaCl levels inhibit the incorporation of glucose into cell wall non-cellulosic and cellulosic polysaccharides (ZHONG; LÄUCHLI, 1988).

The presence of sulfate was also investigated in the polysaccharides from *A. annua* roots submitted to salt stress and, as found for aerial parts, sulfated polysaccharides were not produced in response to salinity.

Regard the monosaccharide composition, moderate saline treatment (100 mM) induces a decrease in galactose contents in R-W fraction, which subsequently increase in the R-E fraction (FIGURE 8B-C), indicating remodeling in the cell wall of roots. Content of uronic acids decrease in R-E200 fraction ($P<0.05$) compared to R-EC. Regard to ratios UA/Rha and Ara+Gal/Rha no differences were found between control samples and saline treatments, neither for R-W or R-E fractions (data not shown).

FIGURE 8 - COMPARISON OF YIELD AND MONOSACCHARIDE COMPOSITION OF FRACTIONS OBTAINED FROM SEQUENTIAL EXTRACTIONS OF ROOTS OF *A. annua* SUBMITTED TO SALT STRESS AND CONTROL CONDITION.



NOTE: Yield of fractions (A); comparison of monosaccharide composition of (B) R-W fraction, (C) R-E fractions; (D) R-HA fractions and (E) R-HB fractions.
Each value of monosaccharide composition represents the mean of two independent extractions. Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Bonferroni's multiple comparison test. Symbols: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ statistically different from control plants.

The hemicellulosic fractions from saline treatments, R-HA and R-HB exhibited higher levels of xylose than control plants, as seen for the aerial parts.

This fact reinforces the role of xylose-containing polysaccharides under salt stress. In R-HA100 fraction associated with the increase of xylose, was observed a reduction in mannose and glucose contents ($P<0.05$). In R-HB200, reduction of mannose was also detected, along with increased uronic acids content ($P<0.01$).

4. Conclusion

Salt stress negatively affected the growth, biomass accumulation and damaged the cell membrane of *A. annua.* Aerial parts were more affected by salt stress than roots, as showed by monosaccharide composition alteration and biomass production. The main difference found between the cell wall polysaccharides from aerial parts and roots of *A. annua* was related to the yield of polysaccharides. Pectic fractions had the highest yield among the extractions from aerial parts while hemicellulose was the major component found in the fractions isolated from roots. According to the results, the main changes in cell wall of aerial parts of *A. annua* exposed to saline stress were alterations in the fine structure of the pectins, such as degree of methyl esterification, number of side chains of the rhamnogalacturonan and uronic acids contents. The increased content of xylose in hemicelluloses from aerial parts and roots from plants subjected to salt stress suggest more linear chains which are more prone to associate with cellulose. As a whole, the results point to changes in the cell wall architecture of aerial part of *A. annua* when submitted to salt stress. In this condition, the pectin domain display a looser structure than control plants and the cellulose-hemicellulose domain instead, become stiffer. For roots, a higher content of pectin, lower contents of cellulose and more linear hemicelluloses were the main adaptations in the cell wall during the salt stress. Sulfation of cell wall polysaccharides was not found as a response to salt stress since none of polysaccharide exhibited sulfate groups.

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CAPÍTULO 2

EFEITO HEPATOPROTETOR DA INFUSÃO E DE UMA FRUTANA DO TIPO INULINA ISOLADA DE *Artemisia vulgaris* EM MODELO DE LESÃO INDUZIDA NO FÍGADO POR TETRACLORETO DE CARBONO

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Original article

Inulin-type fructan and infusion of *Artemisia vulgaris* protect the liver against carbon tetrachloride-induced liver injury

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Abstract

Background: Infusions of aerial parts of *Artemisia vulgaris* L. (Asteraceae) are used in herbal medicine to treat several disorders, including hepatosis.
Purpose: Evaluation of *in vivo* hepatoprotective effects of *A. vulgaris* infusion (VI) and inulin (VPI; i.e., the major polysaccharide of VI).
Study Design: The hepatoprotective effect of *A. vulgaris* extracts on carbon tetrachloride (CCl_4)-induced hepatotoxicity and the probable mechanism involved in this protection were investigated in mice.
Methods: A. vulgaris infusion (VI) was prepared according to folk medicine using the aerial parts of the plant. Carbohydrate, protein, and total phenolic content was determined in VI, and its phenolic profile was determined by high-performance liquid chromatography (HPLC). Male Swiss mice were orally pretreated for 7 days with VI or VPI (once per day). On days 6 and 7 of treatment, the mice were intraperitoneally challenged with CCl_4. Liver and blood were collected and markers of hepatic damage in plasma and oxidative stress in the liver were analyzed. Hepatic histology and inflammatory parameters were also studied in the liver. The scavenging activity of VI and VPI were evaluated *in vitro* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.
Results: VI contained 40% carbohydrates, 2.9% proteins and 9.8% phenolic compounds. The HPLC fingerprint analysis of VI revealed chlorogenic, caffeic and dicaffeoylquinic acids as major low-molar-mass constituents. Oral pretreatment with VI and VPI significantly attenuated CCl_4-induced liver damage, reduced the activity of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) in plasma, and prevented reactive oxygen species accumulation and lipid peroxidation in the liver. Comparisons with the CCl_4-treated group showed that VI and VPI completely prevented necrosis, increased the levels of reduced glutathione (GSH), and reduced tumor necrosis factor alpha (TNF-α) level in the liver. VI and VPI also exhibited high radical scavenging activity *in vitro*.

Conclusion: VI and VPI had remarkable hepatoprotective effects *in vivo*, which were likely attributable to antioxidant and immunomodulatory properties. The present findings support the traditional use of *A. vulgaris* infusion for the treatment of hepatic disorders.

Keywords: *Artemisia vulgaris*; infusion; hepatoprotective effect; CCl_4; inulin

Abbreviations: VI, *Artemisia vulgaris* infusion; VPI, polysaccharide from *Artemisia vulgaris* infusion

1. Introduction

Liver diseases are an important problem worldwide. These diseases are caused by several factors such as, alcoholism, xenobiotics, viral infections and drugs (INGAWALE; MANDLIK; NAIK, 2014). Drug-induced liver injury is a common and important cause of liver injury, comprising half of the cases of acute liver failure. Drug metabolism occurs primarily in the liver, and drug metabolites can cause oxidative stress in hepatocytes, leading to cell death (KAPLOWITZ, 2004). Conventional drugs that are used for the treatment of hepatic injury have limited therapeutic actions and sometimes adverse effects (LIU et al., 2015). In this context, some extracts and polysaccharides isolated from plants have been reported as potent hepatoprotective agents against chemically induced liver injury (ROFIEE et al., 2015; XIAO et al., 2012).

In this context, *Artemisia vulgaris* L., commonly known as “mugwort” (family: Asteraceae), is widely known for its medicinal properties. Infusions of its aerial parts are popularly used to treat several disorders, including hepatosis (GOVINDARAJ et al., 2008; SALEH et al., 2014). To date, only one study has reported the hepatoprotective activity of *Artemisia vulgaris*. Gilani et al. (2005) described protective effects of an aqueous-methanolic extract of aerial parts of *A. vulgaris* against D-galactosamine- and lipopolysaccharide-induced hepatitis in mice. However, in folk medicine, infusions of the aerial parts of *A. vulgaris* are used, and their effects on the liver can be different from those of aqueous-methanolic extracts. To our knowledge, the chemical composition of this infusion has not been reported yet.

With regard to the chemical composition of the aerial parts of *A. vulgaris*, the essential oil has been widely studied. The main components were reported

to be 1,8-cineole, β-thujone, caryophyllene, germacrene D, and camphor (GOVINDARAJ et al., 2008; SALEH et al., 2014). Polar extracts of this plant contain mainly phenolic compounds, including flavonoids (isoquercitrin, quercitrin, quercetin, luteolin, and kaempferol), hydroxycinnamic acids (gentisic, caffeic, *p*-coumaric, and ferulic acids), and several quinic acid derivatives (3-caffeoylquinic, chlorogenic, 5-feruloylquinic, 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic, 1,5-dicaffeoylquinic, 1,3-dicaffeoylquinic, 1,4-dicaffeoylquinic and 4,5-dicaffeoylquinic acids) (IVANESCU et al., 2010; MELGUIZO-MELGUIZO et al., 2014). However when an infusion is prepared, using hot water, the volatile oil is lost, leaving metabolites as non-volatile compounds and polysaccharides.

Inulin-type fructan is the major polysaccharide of *A. vulgaris* infusions prepared according to methods of traditional medicine (CORRÊA-FERREIRA; NOLETO; PETKOWICZ, 2014). Structurally, inulin is a linear polymer that is constituted by fructose in β-(2→1)-linkages with a starting α-D-glucose unit. Inulin has been widely studied for its prebiotic effects, but other biological properties have also been described, such as hepatoprotective effects (LIU et al., 2015).

In the present study, we investigated the hepatoprotective and antioxidant effects of an *A. vulgaris* infusion and inulin (i.e., the major polysaccharide of *A. vulgaris*) using carbon tetrachloride (CCl_4) to induce hepatic injury in mice. The low-molar-mass compounds that are present in *A. vulgaris* infusion were also investigated.

2. Materials and methods

2.1 Chemicals

Methanol and glacial acetic acid (HPLC grade) were purchased from J. T. Baker (Mexico City, Mexico). Ultra-pure water was obtained from Milli-Q system. Gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, caffeic acid, acetylsalicylic acid, *p*-coumaric acid, benzoic acid, salicylic acid, cinnamic acid, and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl chlorogenate, 3,4-dicaffeoilquinic acid, 3,5-dicaffeoylquinic acid, 4,5-

dicaffeoylquinic acid, methyl 3,4-dicaffeoylquinate, methyl 3,5-dicaffeoylquinate, and ethyl caffeate were previously isolated from *Moquiniastrum polymorphum* (STRAPASSON et al., 2014). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), tetramethylbenzidine, *p*-nitrophenyl-2-acetamide-*β*-D-glucopyranoside, citric acid, glycine, N-1-napthylethylenediamine dihydrochloride (NED), Triton X-100, reduced glutathione (GSH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), *O*-cresolsulfonphthalein-3',3"-bis(methyliminodiacetic acid sodium salt) (Xylenol orange), Coomassie Brilliant Blue G-250, ethylenediaminetetraacetic acid disodium salt (EDTA), and 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ascorbic acid, hydrogen peroxide, sodium acetate, sodium phosphate, potassium phosphate, and pyrogallol were obtained from Vetec (Rio de Janeiro, Brazil). Aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) detection kits were purchased from Kovalent (São Gonçalo, Brazil). The tumor necrosis factor α (TNF-α) detection kit was purchased from R&D Systems (Minneapolis, MN, USA). The interleukin 1β (IL-1β) detection kit was purchased from eBioscence (San Diego, CA, USA). Sulfuric acid, bovine serum albumin (BSA), and butylated hydroxytoluene (BHT) were obtained from Merck (Darmstadt, Germany). Ethanol, dimethylformamide, and phosphoric acid were purchased from Neon (São Paulo, Brazil). Tris-HCl and *p*-nitrophenol were obtained from Fluka (St. Louis, MO, USA). Carbon tetrachloride (CCl_4) was obtained from Reagen (Rio de Janeiro, Brazil). Folin-Ciocalteu was obtained from Polipur (Porto Alegre, Brazil). Ketamine was obtained from Vetnil Industry and Trade of Veterinary Products (Louveira, Brazil). Xylazine was obtained from Syntec (Cotia, Brazil). Methanol was obtained from Biotec (Pinhais, Brazil). Ammonium ferrous sulfate and *sulfanilamide were obtained from Synth (Diadema, Brazil).*

2.2 Plant material and extract preparation

Aerial parts of *A. vulgaris* were collected in Mandirituba, Paraná, Brazil (April/2014). The plant was identified by Osmar dos Santos Ribas, and a voucher specimen (MBM 388303) of *A. vulgaris* was deposited at the Herbarium of Museu Botânico Municipal de Curitiba.

The *A. vulgaris* infusion was prepared using the aerial parts of the plant according to traditional methods. One teaspoon of herb (1.4 g) was added to 1 cup (200 mL) of boiling water (LORENZI; MATOS, 2008). The material was infused until it reached 40ºC, and then the extract was filtered using filter paper (pore size: 14 um) from Qualy (São José dos Pinhais, Brazil) and freeze-dried. The infusion lyophilized (VI) was used to biological assays and HPLC analyses. A crude polysaccharide fraction rich in inulin-type fructan (VPI), previously isolated and characterized from the *A. vulgaris* infusion (CORRÊA FERREIRA; NOLETO; PETKOWICZ, 2014), was also used in the experiments. Briefly, VPI was obtained after the preparation of *A. vulgaris* infusion according to traditional methods, which was filtered. The extract was concentrated and treated with ethanol (4:1, v/v). The material was kept at 4 ºC overnight and then the polysaccharide (VPI) was isolated by centrifugation (8000 rpm, 20 min), washed with ethanol, and dried under vacuum.

2.3 Characterization of *A. vulgaris* infusion

Total carbohydrates were assayed using the phenol-sulfuric acid method (DUBOIS et al., 1956) with galactose as the standard. Protein content was evaluated according to the Bradford (1976) method with bovine serum albumin (BSA) as the standard. Phenolic content was estimated using Folin-Ciocalteu reagent (adapted from Singleton, et al., 1999) with gallic acid as the standard. The amounts of fructose and fructose-yielding carbohydrates were estimated using a ketose-specific modification of the anthrone method as described by Pollock (1982) with inulin as the standard.

HPLC fingerprint analyses of VI were performed on a Waters HPLC apparatus equipped with 2998 photodiode array detector (PDA). For all of the analyses, a Nucleosil 100-5 C18 column (250 mm $\times$ 4.6 mm, 5 mm particle size) was used. The mobile phase consisted of H_2O with 1% acetic acid (A) and methanol (B), applied in a linear gradient from 95:5 (A:B) to 100 (B), over 60 min, followed by isocratic elution with B for 10 min. The flow rate was 0.8 mL/min at room temperature. The column effluent was monitored at 211, 254, and 330 nm.

Compounds that were previously reported for *Artemisia* species were used as standards (IVANESCU et al., 2010; MELGUIZO-MELGUIZO et al., 2014). The following pure available known compounds, were used as external standards: gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, benzoic acid, salicylic acid, cinnamic acid, kaempferol, methyl chlorogenate, ethyl chlorogenate, 3,4-dicaffeoilquinic acid, 4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, methyl 3,4-dicaffeoylquinate, methyl 3,5-dicaffeoylquinate, and ethyl caffeate. All of the standards were dissolved in methanol before injection into the HPLC system. Phenolic compounds from *A. vulgaris* were identified by comparisons of their retention times (R_t) with those of pure compounds.

2.4 *In vitro* determination of free radical scavenging activity

VI and VPI were evaluated for their scavenging activity against the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). This method was adapted from Chen et al. (2004). Briefly, the samples at different concentrations (1, 3, 10, 30, 100, and 1000 µg/mL) were mixed with DPPH methanolic solution (10 µg/mL). Ascorbic acid (50 µg/mL) and distilled water were used as positive and negative controls, respectively. Absorbance was read at 517 nm.

2.5 Animal treatment and sample collection

Male Swiss mice (*Mus musculus*), weighing 30 ± 4 g, were used for the study. The experiments were approved by the Animal Ethics Committee of the Biological Sciences Sector of the Federal University of Parana (certificate no. 770) and follow the recommendations of Brazilian College of Animal Experimentation (COBEA). The animals were housed in cages at a constant temperature (22ºC ± 2ºC) on a 12 h/12 h light/dark cycle with food and water available *ad libitum*. The animals were acclimatized for 1 week before the experiment began. They were orally pretreated (i.g.) with the compounds or water once per day for 7 days. On days 6 and 7 of treatment, 2 h after oral treatment, they were challenged with CCl_4 (2% in corn oil, 5 mL/kg, i.p.). The animals were divided into six groups (n = 7 per group) according to treatment:

(1) control group (ultrapure water + corn oil), (2) CCl_4 group (ultrapure water + CCl_4), (3) VI group (4 mg/kg VI + CCl_4), (4) 10 mg/kg VPI group (10 mg/kg VPI + CCl_4), (5) 30 mg/kg VPI group (30 mg/kg VPI + CCl_4), and (6) 100 mg/kg VPI group (100 mg/kg VPI + CCl_4). One additional group (7) received only 4 mg/kg VI (i.g.) for 7 days to evaluate the effects of VI alone on liver parameters. The dose of VI (4 mg/kg) that was selected for the biological assays was calculated by considering the consumption of three cups of the *A. vulgaris* infusion per day according to the infusion yield. The dose of inulin (VPI) was based on previous reports on its hepatoprotective and antioxidant effects, in which inulin presented effectiveness at a minimum dose of 100 mg/kg (LIU et al., 2015). From this study a dose-response curve was tested, using lower doses until 10% (10 mg/kg) of the reference (100 mg/kg) and an intermediary dose (30 mg/kg). Twenty hours after the last CCl_4 or corn oil injection, the mice were intraperitoneally anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The abdominal cavity was opened, and blood was collected from the abdominal cava vein using heparinized syringes. The liver was quickly removed, frozen in liquid nitrogen, and stored at -80ºC for further analysis. A portion of the liver (right lobe) was fixed in 10% neutral buffered formalin for histological analysis. The animals were euthanized by puncture of the diaphragm, and the spleen and kidneys were also harvested and weighed.

2.6 Plasmatic analysis

The blood samples were centrifuged at 3,000 × *g* for 5 min. The plasma was then stored at -20ºC. The activity of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) was measured in plasma using commercial kits (Kovalent, São Gonçalo, Brazil) with a Mindray BS-200 (Shenzhen, China) automated device.

2.7 Hepatic oxidative stress measurements

Liver tissue was homogenized in phosphate-buffered saline (PBS; pH 6.5) and centrifuged (10,000 × *g,* 4ºC, 20 min). The supernatant was used to measure the activity of catalase (CAT) (AEBI, 1984), superoxide dismutase (SOD) (GAO et al., 1998), and glutathione *S*-transferase (GST) (HABIG;

PABST; JAKOBY, 1974). The lipid peroxidation levels (LPO) (JIANG; WOOLLARD; WOLFF, 1991) and reduced glutathione (GSH) (SEDLAK; LINDSAY, 1968) were also measured. Most of the oxidative stress parameters are expressed as the amount of protein in the liver samples, determined using the Bradford (1976) method with BSA as the standard. The measurements of ROS in the homogenates were performed using DCFH-DA (2′,7′-dichlorodihydrofluorescin diacetate) as a probe, according to Driver et al. (2000), with slight modifications. Briefly, the homogenates were diluted 1:10 (in 0.1 M potassium phosphate buffer, pH 6.5), mixed with a DCFA solution (56 µM final concentration), and incubated at room temperature for 40 min in the dark. The conversion of DCFH to the fluorescent product (DCF) was measured in a 96-well plate, using a spectrofluorimeter (Biotek Synergy HT), at an excitation wavelength of 485 nm and emission wavelength of 506 nm. The results are expressed as fluorescence units.

2.8 Hepatic histological analysis

Livers were harvested from the mice after euthanasia, stored in a 10% buffered formalin solution for fixation, processed according to routine histological procedures, and embedded in paraffin. The histological sections were stained with hematoxylin and eosin (H&E staining). The sections were analyzed using an optical microscope to evaluate cellular changes, the presence of necrosis, inflammation, and steatosis. The histological alterations in the liver were scored using the histological scoring system adapted from Kleiner et al. (2005). Images were acquired using an Olympus BX41 microscope coupled to an Olympus SC30 camera.

2.9 Measurements of inflammatory parameters

To evaluate inflammatory parameters in the liver, specific enzymes, mediators, and cytokines were measured. The liver samples were homogenized in 0.1% saline-Triton X-100 solution, centrifuged (10,000 × *g*, 4 ºC, 10 min) and the supernatant was used to measure myeloperoxidase (MPO) and *N*-acetyl-β-D-glucosaminidase (NAG) activity. MPO assay was carried out as described by Bradley et al. (1982) with some modifications. Briefly, the supernatants were

incubated with 0.017% hydrogen peroxide solution in 80 mM PBS and 18.4 mM tetramethylbenzidine (in dimethylformamide) for 3 min at 37ºC and put on ice after incubation. The reaction was stopped with sodium acetate (1.46 M, pH 3.0). Absorbance was read at 620 nm in a microplate reader (Biotek Synergy HT). Enzymatic activity is expressed as the optical density (OD) per milligram of wet tissue. NAG activity was measured according to Lloret and Moreno (1995). The results are expressed as nmol/mg wet tissue

The levels of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin 1β (IL-1β) in liver tissues were also determined. The liver samples were homogenized in potassium phosphate buffer (0.1 M, pH 6.5) and centrifuged (10,000 $\times$ *g,* 4 ºC, 10 min). For the NO assay, supernatants of the liver homogenate were mixed with Griess reagent and absorbance was read at 540 nm in a microplate reader (Biotek Synergy HT) (GREEN et al., 1982). The amount of nitrite in the incubation medium was calculated using sodium nitrite as the standard. For the TNF-α and IL-1β assays, the enzyme-linked immunosorbent assay (ELISA) method was used according to the manufacturer's instructions. The results are expressed as picograms per milligram of wet tissue.

2.10 Statistical analysis

The data from each group are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by the Dunnett *post hoc* test. Prism 5.0 software (GraphPad, San Diego, CA, USA) was used for the statistical analysis. For comparisons between mean of two groups Student's t test was used for evaluation of statistical significance. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1 Characterization of *A. vulgaris* infusion

VI was prepared according to traditional consumption habits. The freeze-dried extract yielded 20% VI based on the dry aerial parts. VI displays 40.0 ± 3.1% carbohydrates, 2.9 ± 0.4% protein, and 9.8 ± 1.9% phenolic compounds based on dry weight extract. Among the carbohydrates that were quantified in the *A. vulgaris* infusion by the phenol-sulfuric method, 65% consisted of fructose, based on the anthrone method. The ^{13}C-NMR spectrum of the infusion (FIGURE S1) showed six intense signals that corresponded to fructose ring carbons, linked (2←1), which are typical of inulin-type fructans: C-1 (61.0), C-2 (103.2), C-3 (77.3), C-4 (74.6), C-5 (81.1), and C-6 (62.2) (CHANDRASHEKAR; PRASHANTH; VENKATESH, 2011). We previously isolated polysaccharides from VI (referred to as VPI), which was then characterized. The main polymer was an inulin-type fructan, which comprised 85% of the polysaccharides (CORRÊA-FERREIRA; NOLETO; PETKOWICZ, 2014).

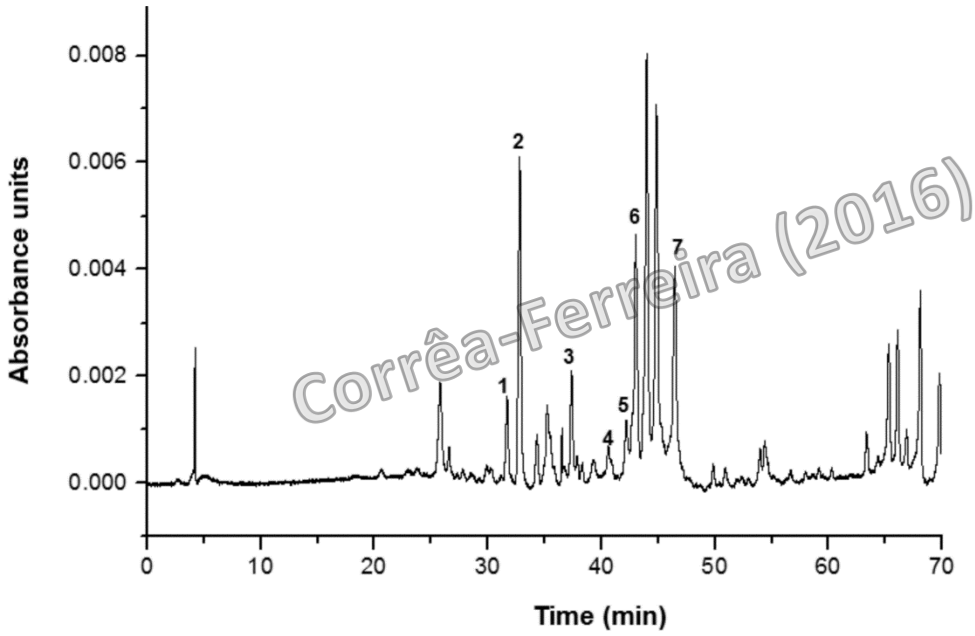
In addition to polysaccharides, low-molar-mass compounds of VI were also investigated by HPLC. The most relevant information was obtained from the chromatogram that was recorded at 330 nm (FIGURE 1), which allowed the identification of chlorogenic acid (**1**, R_t = 31.7 min), caffeic acid (**2**, R_t = 32.8 min), methyl chlorogenate (**3**, R_t = 37.4 min), 3,4-dicaffeoylquinic acid (**4**, R_t = 40.6 min), methyl 3,4-dicaffeoylquinate (**5**, R_t = 42,2), 3,5-dicaffeoylquinic acid (**6**, 43,0 min), and ethyl caffeate (**7**, R_t = 46.4 min).

3.2 Effects of VI and VPI on hepatic biomarkers and general features

No mice died during the experimental period. No differences in body weight or the relative weight of the kidneys or spleen were found among groups (data not shown). However, the relative liver weight significantly increased in the CCl_4-treated groups, even in the groups that were pretreated with VI and VPI (TABLE 1). To evaluate possible hepatocellular damage that was caused by CCl_4, the activity of ALT, AST, and ALP was assessed (FIGURE 2). CCl_4 treatment significantly increased the activity of these enzymes in plasma, indicating intense hepatic damage. Pretreatment with VI and VPI at all doses significantly decreased plasma ALT, AST, and ALP activity ($P < 0.001$) to almost control levels, in which no significant difference from control group was found. VI and VPI pretreatment reduced plasma ALT levels by 90% compared

with the CCl_4-treated group (FIGURE 2A). Pretreatment with VI and VPI decreased AST levels by approximately 63% and 75%, respectively. The lowest dose of VPI (10 mg/kg) was the most effective in decreasing AST activity (by ~85%; FIGURE 2B), suggesting that the effects of the treatment were not dose-dependent. Besides, inulin (VPI) was more efficient in reducing AST levels compared with the infusion (VI).

FIGURE 1 - TYPICAL HPLC CHROMATOGRAM OF A. vulgaris INFUSION.



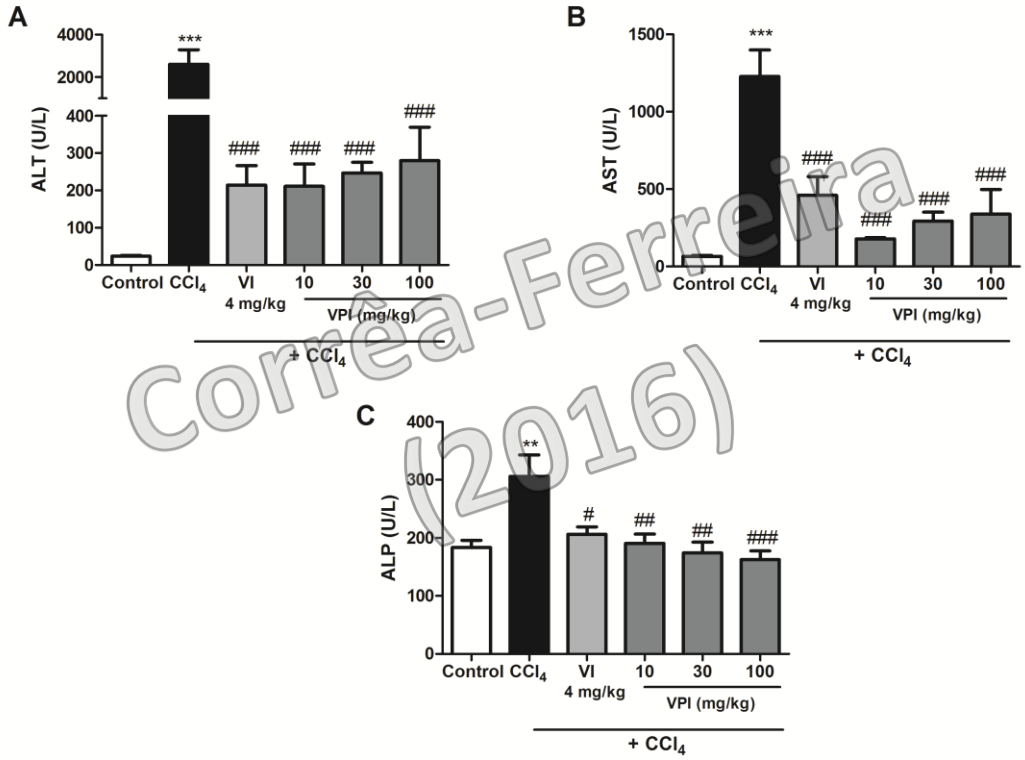
NOTE: Peaks: (1) chlorogenic acid; (2) caffeic acid; (3) methyl chlorogenate; (4) 3,4-dicaffeoylquinic acid; (5) methyl 3,4-dicaffeoylquinate; (6) 3,5-dicaffeoylquinic acid; (7) ethyl caffeate. The chromatogram was recorded at 330 nm. Experimental conditions: see section: *Characterization of A. vulgaris infusion from Material and methods*.

3.3 Histopathological analysis of the liver

The control group exhibited normal architecture of the liver (FIGURE 3A). Liver sections from CCl_4-treated mice presented massive eosinophilic necrosis (FIGURE 3B), mainly surrounding the centrilobular veins (zone 3), and inflammatory infiltration associated with necrotic areas. The liver also presented extensive areas with diffuse microvesicular steatosis (grade 2) and tumefaction (grade 2). Treatment with VI and VPI completely prevented the appearance of centrilobular necrosis. Reversible injuries, such as tumefaction and microvesicular steatosis, significantly decreased to grades 1 and 0, respectively, in VI and VPI-treated groups (TABLE 1). However, with both

treatments, intense inflammatory infiltration was observed in the same area where CCl_4 induced necrosis (FIGURE 3C-F). The infiltrate mostly consisted of lymphohistiocytic cells, but polymorphonuclear leukocytes were also found. Centrilobular inflammatory infiltrate was most expressive in the VI-treated group and less intense in the VPI-treated group.

FIGURE 2 – PLASMA BIOCHEMICAL PARAMETERS IN CCl_4-INDUCED HEPATIC INJURY IN MICE TREATED WITH VEHICLE, VI OR VPI.



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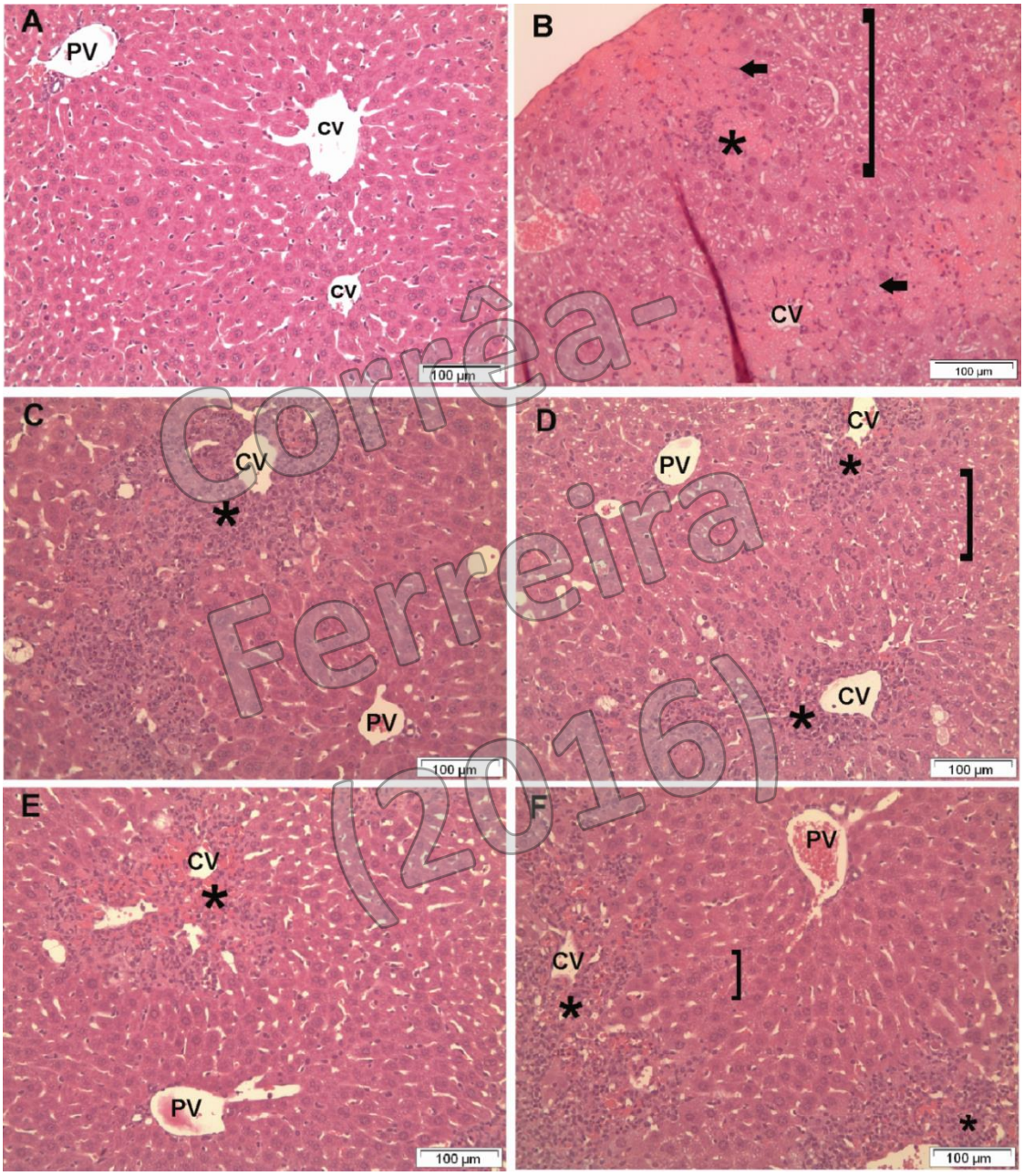
NOTE: (A) ALT, (B) AST, (C) ALP. Groups: Control (ultrapure water + corn oil); CCl_4 (ultrapure water + CCl_4 2%, 5 mL/kg); VI: *A. vulgaris* infusion 4 mg/kg + CCl_4; VPI (10 mg/kg + CCl_4); VPI (30 mg/kg + CCl_4); VPI (100 mg/kg + CCl_4). Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Dunnett post hoc test. Symbols: ** $P < 0.01$ and *** $P < 0.001$ statistically different from control group; # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ statistically different from CCl_4 group.

3.4 Effects of VI and VPI on inflammatory parameters

Histology showed that CCl_4 caused hepatic inflammation, but it did not increase MPO and NAG activity or NO levels (Table 2). However, CCl_4 treatment significantly increased TNF-α levels ($P < 0.01$; Table 2). VPI-treated mice exhibited an increase in NAG activity but no changes in MPO activity compared with controls, indicating that mononuclear cells were the predominant cell types in the inflammatory infiltrate observed in treated livers (FIGURE 3D-

F). VPI at doses of 30 and 100 mg/kg reduced NO levels in the liver compared with the CCl_4-treated group. Although VI-treated mice exhibited intense inflammatory infiltrate around centrilobular veins, it did not affect NAG, MPO, or NO. With regard to cytokines, VI and VPI pretreatment significantly reduced TNF-α levels in liver tissue compared with the CCl_4-treated group and VPI at the highest dose (100 mg/kg) also reduced IL-1β levels in liver tissue (TABLE 2).

FIGURE 3 – PHOTOMICROGRAPHS OF LIVER SECTIONS STAINED WITH HEMATOXYLIN-EOSIN FROM MICE REPRESENTING THE FOLLOWING GROUPS.



Correa-Ferreira (2016)

NOTE: A- Control (ultrapure water + corn oil); B- CCl_4 (ultrapure water + CCl_4 2%, 5 mL/kg); C- VI: *A. vulgaris* infusion 4 mg/kg + CCl_4; D- VPI (10 mg/kg + CCl_4); E- VPI (30 mg/kg + CCl_4); F- VPI (100 mg/kg + CCl_4). Symbols: black arrow: centrilobular necrosis; brackets ( ]): areas with

tumefaction and microvesicular steatosis; asterisks (*): centrilobular inflammatory infiltrate. **CV:** Centrilobular veins; **PV:** Portal veins. Scale bar = 100 µm.

TABLE 1 – HISTOLOGICAL PARAMETERS OBSERVED IN CCl_4-INDUCED HEPATIC INJURY IN MICE.

	Liver(%)[1]	Centrilobular Necrosis	Microvesicular steatosis	Tumefaction	Centrilobular Inflammatory infiltrate	Diagnostic Classification
Control	5.61 ± 0.28 [a]	—	—	0	0	Normal liver
CCl_4	6.52 ± 0.26 [b]	Moderate/ Severe	2 (diffuse)	2 (diffuse)	1	Low hepatitis Severe necrosis
VI 4 mg/kg	6.56 ± 0.13 [b]	—	0	1	3	Severe hepatitis without necrosis
VPI 10 mg/kg	6.50 ± 0.21 [b]	—	0	1	2	Moderate hepatitis without necrosis
VPI 30 mg/kg	6.61 ± 0.22 [b]	—	1	1	2	Moderate hepatitis without necrosis
VPI 100 mg/kg	6.69 ± 0.16 [b]	—	0	1-2	2	Moderate hepatitis without necrosis

Corrêa-Ferreira (2016)

NOTE: Groups: Control (ultrapure water + corn oil); CCl_4 (ultrapure water + CCl_4 2%, 5 mL/kg); VI: *A. vulgaris* infusion 4 mg/kg + CCl_4; VPI (10 mg/kg + CCl_4); VPI (30 mg/kg + CCl_4); VPI (100 mg/kg + CCl_4).

[1] % of wet weight of livers related to the body weight. Symbols: a,b: different letters represented significant differences between groups at $p < 0.05$. Grade of parameters was adapted from Kleiner et al. (2005). Microvesicular steatosis grade (0- <5%; 1- 5-33%; 2- 33-66%; 3- >66%). Tumefaction grade (0- none; 1- few; 2- many). Inflammatory grade: (0 - slight; 1 – moderate; 2 – marked; 3 - very marked.

3.5 Effects of VI and VPI on oxidative stress parameters

To evaluate the protective mechanisms of VI and VPI in CCl_4-induced liver injury, the antioxidant effects of *A. vulgaris* extracts were analyzed using markers of oxidative stress (TABLE 2). CCl_4 treatment increased total ROS and LPO levels in the liver by 97% and 59%, respectively, compared with the control group. Pretreatment with VI and VPI significantly decreased LPO and total ROS

levels compared with the CCl_4-treated group. The enzymes SOD, GST, and CAT were unaffected by CCl_4 treatment compared with the control group, however pretreatment with VPI at all doses reduced SOD activity, and the intermediary dose (30 mg/kg) reduced GST and CAT activity. The hepatic levels of GSH were unaffected by CCl_4, but VI and VPI at the highest dose (100 mg/kg) significantly increased GSH levels in the liver.

TABLE 2 – MEASUREMENT OF INFLAMMATORY PARAMETERS AND HEPATIC OXIDATIVE STRESS IN CCl_4-INDUCED HEPATIC INJURY IN MICE TREATED WITH VEHICLE, VI OR VPI.

	Control	**CCl_4**	**VI 4 mg/kg**	**VPI 10 mg/kg**	**VPI 30 mg/kg**	**VPI 100 mg/kg**
Hepatic inflammatory parameters						
NAG (nmol/mg wet tissue)	34.77 ± 2.68	34.08 ± 1.52	39.66 ± 1.34	40.8 ± 1.46 #	43.86 ± 0.17 ##	40.99 ± 0.66 #
NO (µM nitrite/mg wet tissue)	0.76 ± 0.25	0.79 ± 0.15	0.72 ± 0.15	0.62 ± 0.14	0.42 ± 0.04 #	0.39 ± 0.04 #
MPO (OD/mg of wet tissue)	3.81 ± 0.40	3.53 ± 0.57	3.91 ± 0.18	3.69 ± 0.26	4.09 ± 0.38	3.71 ± 0.30
TNF-α (pg/mg wet tissue)	17.88 ± 1.53	33.70 ± 4.73 **	19.16 ± 3.32 ##	13.02 ± 1.62 ###	11.46 ± 1.44 ###	12.92 ± 2.29 ###
IL-1β (pg/mg wet tissue)	12.98 ± 2.13	12.15 ± 2.38	7.67 ± 0.88	7.09 ± 1.27	7.92 ± 0.58	6.59 ± 0.49 #
Hepatic oxidative stress parameters						
LPO (nmol hydroperoxide/ mg of protein)	8.34 ± 1.21	13.31 ± 2.69 *	8.50 ± 0.90 #	6.61 ± 0.38 ##	7.81 ± 0.51 ##	7.71 ± 0.36 ##
GSH (mg/g tissue)	0.64 ± 0.09	0.62 ± 0.07	0.91 ± 0.05 #	0.85 ± 0.07	0.71 ± 0.05	0.89 ± 0.06 #
GST (mmol/min/mg protein)	16.3 ± 1.65	15.39 ± 1.77	14.75 ± 1.33	12.17 ± 0.51	10.59 ± 0.58 #	13.07 ± 0.66
CAT (µmol/min/mg protein)	512.4 ± 56.87	477.7 ± 53.44	456.50 ± 28.18	379.70 ± 12.6	310.30 ± 22.46 ##	396.90 ± 25.29
SOD (U SOD/mg protein)	32.07 ± 4.57	36.07 ± 4.58	27.93 ± 1.59	25.13 ± 2.35 #	19.80 ± 0.85 ###	24.54 ± 1.41 #
Total ROS (fluorescence units)	12.92 ± 3.21	25.50 ± 8.29 *	5.78 ± 0.77 ###	7.50 ± 0.54 ###	8.28 ± 0.39 ##	8.50 ± 0.77 ##

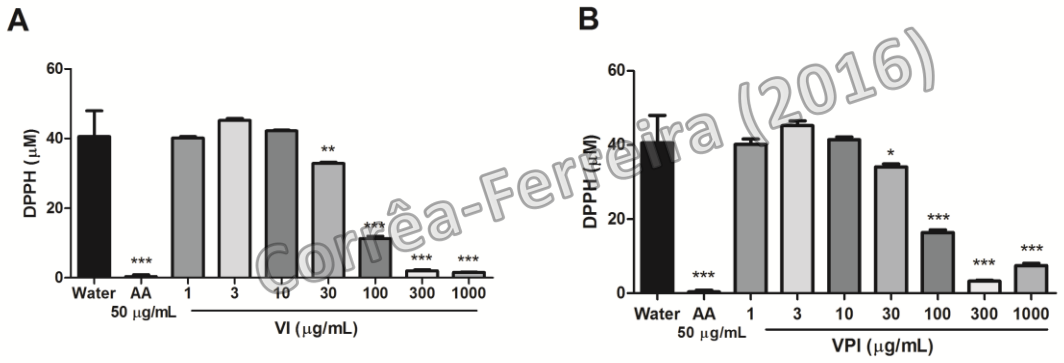
Correa-Ferreira (2016)

NOTE: Groups: Control (ultrapure water + corn oil); CCl_4 (ultrapure water + CCl_4 2%, 5 mL/kg); VI: *A. vulgaris* infusion 4 mg/kg + CCl_4; VPI (10 mg/kg + CCl_4); VPI (30 mg/kg + CCl_4); VPI (100 mg/kg + CCl_4). Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Dunnett post hoc test. Symbols:* P< 0.005, ** P < 0.01 statistically different from control group; # P < 0.05, ## P < 0.01 and ### P < 0.001 statistically different from CCl_4 group.

3.6 Antioxidant potential of VI and VPI *in vitro*

VI and VPI exerted antioxidant effects *in vitro*, determined by the DPPH method, with IC_{50} values of 22.5 and 26.1 µg/mL, respectively (FIGURE 4A, B). These antioxidant effects were concentration-dependent for both compounds, ranging from 30 to 1000 µg/mL. VI reduced DPPH levels in order of 96.1% to the highest concentration and VPI displayed best results at 300 µg/mL, reducing DPPH levels by 91.9%, compared with water. Ascorbic acid (50 µg/mL) used as positive control, reduced DPPH levels by 99.1%.

FIGURE 4 – I*N VITRO* ABILITY OF VI (A) AND VPI (B) TO SCAVENGE THE STABLE FREE-RADICAL DPPH.



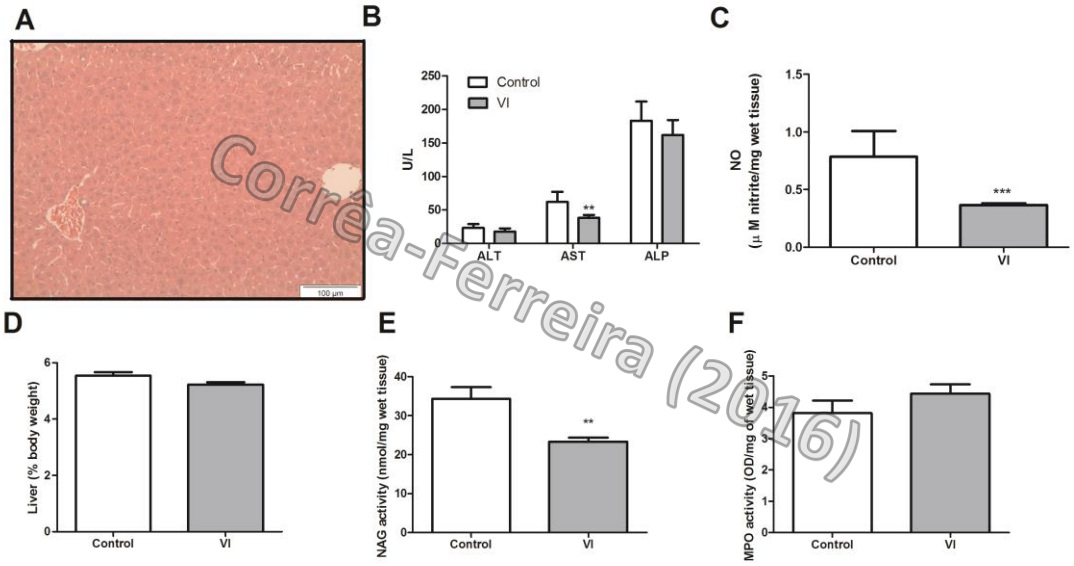
NOTE: Ascorbic acid (AA) 50 µg/mL was used as positive control and distilled water as negative control. * P< 0.05, ** P < 0.01 and *** P < 0.001 statistically different from negative group (distilled water). Values were expressed as mean ± standard error of the mean, analyzed by one-way ANOVA and Dunnett post hoc test.

3.7 Effects of VI on liver and plasmatic parameters

Considering the intense inflammatory infiltrate that was observed in VI-treated mice (FIGURE 3C), the effects of VI on some liver and plasmatic parameters were determined without CCl_4 challenge (FIGURE 5) to evaluate whether inflammatory cells result from a proinflammatory effect of VI or whether it occurs in an attempt to repair CCl_4-induced damage. Treatment with VI for 7 days did not affect plasma ALT and ALP and reduced AST ($P < 0.01$) compared with the control group (FIGURE 5B). The relative liver weight was also maintained (FIGURE 5D). Histology revealed normal architecture of the liver, no alteration in lobular hepatic parenchyma, preserved cytoplasm and nuclei, and normal blood vessels (FIGURE 5A). Inflammatory infiltration was not observed

in the histological analysis, and the absence of inflammation was also confirmed based on the result of NO, NAG, and MPO assays (FIGURE 5C, E, F).

FIGURE 5 – EFFECTS OF VI ON LIVER PARAMETERS.



NOTE: (A) Liver histology of mice treated with VI (Scale bar = 100 μm); (B) Plasma biochemical parameters; (C) NO levels; (D) Percentage of wet weight of livers related to body weight; (E) NAG activity; (F) MPO activity. Groups: Control (ultrapure water + corn oil); VI (*A. vulgaris* infusion 4 mg/kg). Values were expressed as mean ± standard error of the mean, analyzed by Student's t test. Symbols: * P< 0.05 and ** P < 0.01 statistically different from control group.

4. Discussion

In the present study, protective effects of the *A. vulgaris* infusion and its major polysaccharide (inulin) were found in CCl_4-induced liver injury in mice. The toxic effect of CCl_4 is initiated by the production of trichloromethyl radicals ($CCl_3^{\bullet}$) when it is metabolized by the liver microsomal cytochrome P450 oxygenase system. This radical reacts with reactive oxygen species (ROS) that produce trichloromethyl peroxy radicals ($CCl_3OO^{\bullet}$), which bind to macromolecules and trigger lipid peroxidation, resulting in damage to cellular membranes (RECKNAGEL et al., 1989). When such hepatocellular injury occurs, the enzymes ALT and AST, which are usually found in the cytosol of hepatocytes, are released from damaged cells to the plasma (OZER et al., 2008). Elevations of plasma ALP can also indicate hepatobiliary effects of CCl_4 and cholestasis (OZER et al., 2008). The hepatoprotective effects of VI and VPI were confirmed by the histological analysis, reductions of plasma ALT, AST and

ALP and prevention of lipoperoxidation in liver, indicating that hepatocytes had preserved membranes. Interestingly, the lowest dose of VPI (10 mg/kg) was effective in reducing ALT, AST, and ALP activity nearly to healthy control levels. Previous studies found that inulin from dahlia tubers was more effective against CCl_4-induced injury at a dose of 400 mg/kg (LIU et al., 2015). The present study found that the inulin isolated from the *A. vulgaris* infusion provided sufficient protection at a 40-fold lower dose.

The protective mechanisms of VI and VPI in CCl_4-induced liver injury were investigated. One of the mechanisms by which *A. vulgaris* extracts protect the liver is likely through their antioxidant potential, reflected by the high radical scavenging activity of VI and VPI *in vitro* (FIGURE 4), prevention of lipid peroxidation and reduction of total ROS levels in hepatocytes (Table 2). Based on the literature, natural antioxidants may reduce oxidative stress in two ways: (1) directly, by reacting with free radicals and destroying them or (2) indirectly, by regulating enzyme activity, inhibiting the activity or expression of enzymes that are involved in the generation of free radicals (e.g. NAD[P]H oxidase), or enhancing the activity of antioxidant enzymes (e.g. SOD, and CAT) (LÜ et al., 2010). *A. vulgaris* extracts possibly act directly, as scavengers of free radicals that are generated after CCl_4 metabolism, and scavengers of peroxidation products, indicated by the reductions of LPO and total ROS levels in hepatocytes. These actions were not accompanied by changes in the activity of antioxidant enzymes. For some of the doses of VPI, the activity of antioxidant enzymes was even reduced. Gasparotto et al. (2014) also reported a decrease in antioxidant enzymes in rats that were pretreated with the peel and fresh pulp of peaches in a model of CCl_4-induced liver toxicity. The authors suggested that peaches promote antioxidant capacity, thus avoiding cellular oxidative stress. Because the substrates for SOD and CAT (i.e., radicals that are generated by oxidative stress) are unavailable in hepatocytes, increases in enzymatic activity would not be observed (GASPAROTTO et al., 2014). Our data corroborate Gasparotto et al. (2014). VI and VPI exerted preventive effects against cellular ROS accumulation that were associated with a reduction of antioxidant enzymes activity.

Previous studies over the past few years have reported that the hepatoprotective effects of plant extracts can be attributed to their antioxidant

effects (DONG et al., 2015; LIU et al., 2015). Inulin from dahlia tubers exerted hepatoprotective effects *in vivo* through antioxidant actions, protecting biological systems against oxidative stress in the liver caused by CCl_4 (LIU et al., 2015). Among the antioxidant defense mechanisms in cells, GSH has an important protective effect against chemically induced oxidative stress (FRASCHINI; DEMARTINI; ESPOSTI, 2002). The increase in hepatic GSH by VI and VPI (100 mg/kg) may be one of the ways in which *A. vulgaris* protects the liver against CCl_4-induced necrosis. Previous studies reported pronounced necrosis when the GSH content was depleted (WILLIAMS; BURK, 1990). The hepatoprotective effects of some compounds, such as silymarin may be attributable to its ability to increase cellular GSH (FRASCHINI; DEMARTINI; ESPOSTI, 2002).

Another way in which *A. vulgaris* may protect the liver against CCl_4-induced liver injury is through immunomodulatory pathways, including reductions of hepatic TNF-α and IL-1β levels. Hepatotoxins, such as CCl_4, are able to activate hepatic Kupffer cells, resulting in the recruitment of circulating immune cells and production of proinflammatory cytokines, like as TNF-α and IL-1β. These inflammatory mediators can exacerbate liver damage and at high concentrations, TNF-α may induce apoptotic and necrotic injury (DECICCO et al., 1998). The present study corroborates these data, in which CCl_4-treated mice exhibited inflammatory infiltrate around necrotic areas in the liver, associated with an increase in hepatic TNF-α levels. The modulation of inflammatory responses has been described as an important mechanism by which plant compounds exert hepatoprotective effects (DONG et al., 2015; PARK et al., 2010). Pretreatment with polysaccharides from *Taraxacum officinale* (Dandelion) in rats attenuated CCl_4-induced hepatic damage by downregulating the expression of inflammatory mediators in the liver (PARK et al., 2010). However, in the present study, mice that were pretreated with VI and VPI presented more intense inflammatory infiltrate than the CCl_4-treated group. These inflammatory cells may have played a reparative role in tissue, because when VI was administered alone without CCl_4 challenge, it did not induce inflammatory infiltration in liver tissue and did not alter hepatic biomarkers (ALT and ALP) or inflammatory parameters (NAG and MPO). Thus, we may also conclude that at a dose of 4 mg/kg, which is equivalent to consuming three cups

of the *A. vulgaris* infusion per day, the VI extract did not cause signs of hepatotoxicity.

To further identify the components of VI that may be responsible for its hepatoprotective effects, the chemical composition of VI was investigated. Inulin is the major polysaccharide in *A. vulgaris* infusion (CORRÊA-FERREIRA; NOLETO; PETKOWICZ, 2014). This polymer, which was tested in the present study, exerted an important hepatoprotective effect against CCl_4-induced liver injury (LIU et al., 2015). In addition to inulin, low-molar-mass compounds that are present in VI may have contributed to the hepatoprotective effects of the infusion. Phenolic compounds represent 9.8 ± 1.9% of the freeze-dried infusion. To identify these compounds, the HPLC profile of VI was obtained. It was found predominantly caffeic acid derivatives, including caffeic acid and its ethyl ester, chlorogenic acid and its methyl ester, 3,4-dicaffeoylquinic acid and its methyl ester, and 3,5-dicaffeoylquinic acid. To our knowledge, this is the first time that caffeoyl esters have been reported in *A. vulgaris*. Other phenolics were also detected, but could not be identified. 3,4- and 3,5-Dicaffeoylquinic acids, which were found in VI, were also present in the methanolic extract of *A. vulgaris*, as described by Melguizo-Melguizo *et al*. (2014). These dicaffeoylquinic acids were also present in an ethanolic extract of the aerial parts of *Laggera pterodonta* (Asteraceae family). This ethanolic extract exerted hepatoprotective effects in primary cultured neonatal rat hepatocytes against several chemicals, including CCl_4. The neutralization of ROS by nonenzymatic mechanisms may be one of the main mechanisms by which phenolics protect against chemical-induced hepatocyte injury (WU et al., 2007).

The phenolic compounds that are present in VI may be responsible for the high radical scavenging activity of VI (IC_{50} = 22.5 µg/mL) compared with VPI (IC_{50} = 26.1 µg/mL) in the DPPH assay. Caffeic acid derivatives, such as those that were identified in VI, are well known for their potent antioxidant activity. This effect may be attributable to its oxidizable o-diphenolic functionality, which transfer electrons to free radicals and interrupts radical chain reactions (XU; HU; LIU, 2012).

Finally, the protective effects of *A. vulgaris* extracts in the liver are relevant because the CCl_4-induced hepatotoxicity model induces lesions that are similar to those that are caused by conventional drugs such as acetaminophen

(KAPLOWITZ, 2004), which is one of the most popular analgesics used around the world.

5. Conclusion

The present study demonstrated hepatoprotective effects of the *A. vulgaris* infusion (VI) and an inulin-type fructan (VPI) that was isolated from the infusion, against CCl_4-induced liver injury using an *in vivo* model. The results suggest that VI and VPI have antioxidant properties that can prevent chemically induced liver injury. Our findings also suggest that VI and VPI may act through an immunomodulatory pathway that modulates hepatic cytokines (such as TNF-α) and promotes a reparative inflammatory response after CCl_4 administration. Importantly, VI and VPI exerted their effects through oral administration, which is a common form of *A. vulgaris* consumption, thus supporting the traditional use of the *A. vulgaris* infusion for the treatment of hepatic disorders. Additionally, no signs of hepatotoxicity are observed with the consumption of three cups of *A. vulgaris* infusion daily.

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Conflict of interest

The authors declare that they have no conflict of interest.

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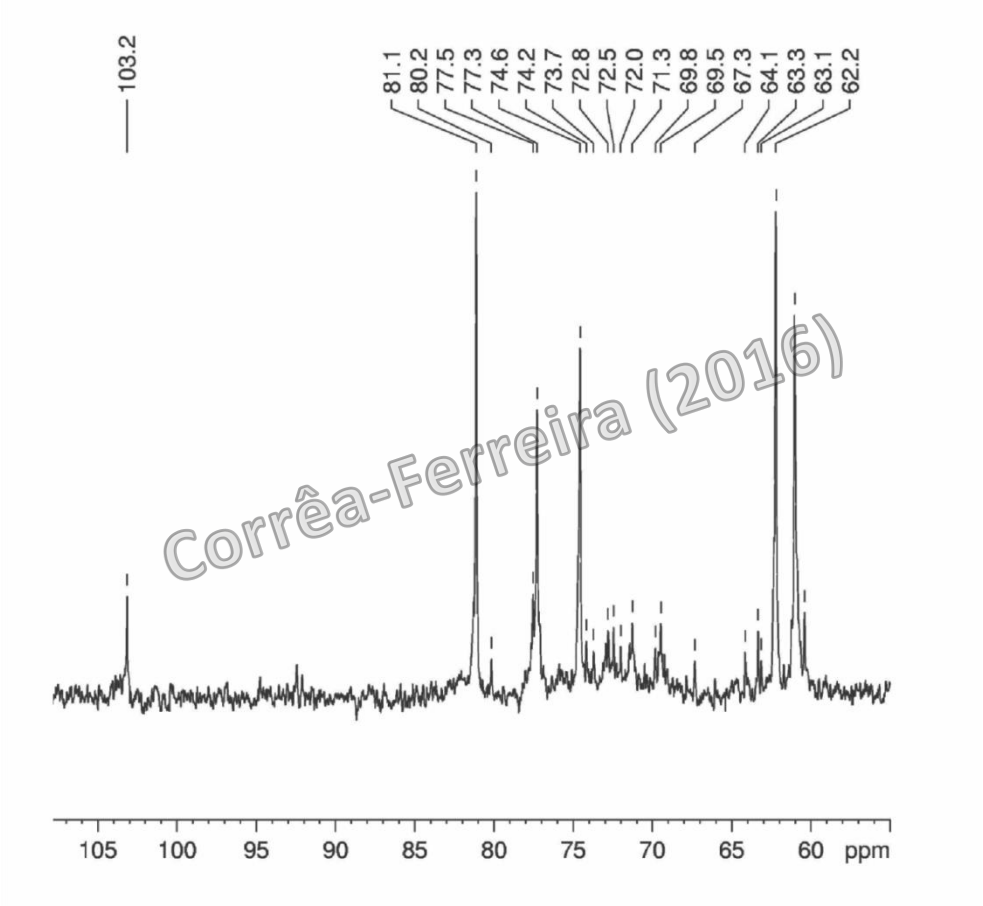
FIGURE S1 - ^{13}C NMR SPECTRUM OF VI.



NOTE: Solvent was D_2O at 70 ºC. Numerical values for chemical shifts (δ) are in ppm.

CAPÍTULO 3

EFEITO GASTROPROTETOR DE UMA FRAÇÃO PÉCTICA ISOLADA DE *Artemisia campestris subsp maritima*

Gastroprotective effects and structural characterization of a pectic fraction isolated from *Artemisia campestris subsp maritima*

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Abstract

In this study, a pectic fraction (ACP-E10) was isolated from aerial parts of *Artemisia campestris subsp maritima*. Results of chemical and spectroscopic analyses demonstrated that ACP-E10 is composed by homogalacturonan (60%) and rhamnogalacturonan-I (RG-I) (29%) regions. Side chains of the RG-I include mainly branched arabinans and arabinogalactans type II. Molar mass of ACP-E10 determined by HPSEC-MALLS was 16,600 g/mol. ACP-E10 was evaluated for its gastroprotective effect against ethanol-induced gastric lesions in rats. Oral pretreatment of animals with ACP-E10 (0.3, 3 and 30 mg/kg) significantly reduced gastric lesions by 77 ± 7.9%, 55 ± 11.1% and 65 ± 11.8%. ACP-E10 also increased mucus and reduced glutathione (GSH) contents in the gastric mucosa. These results demonstrated that pectin from *A. campestris* had significant gastroprotective effects *in vivo*, which were likely attributable to capacity of ACP-E10 in increase the protective defenses of gastric mucosa.

Keywords: *A. campestris subsp. maritima*, pectin, ulcer, gastroprotective effect.

1. Introduction

Peptic ulcer is a prevalent disease that affects the quality of life of approximately 20% of the world population. It is a multifactorial disease triggered by the imbalance between aggressive factors (secretion of gastric acid and digestive enzymes) and, gastro-defensive systems (gastric mucus layer and antioxidant defenses) (ALBAAYIT et al., 2016; SALAMA et al., 2016). Aggravation of ulcer, such as internal bleeding, is a common and clinically significant complication of the disease and, the use of aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) enhances the peptic ulcer bleeding (SUNG et al., 2010).

Recently, natural products from botanical sources have attracted a great deal of studies searching for treatment or prevention of peptic ulcer. Secondary metabolites of plants, such as sesquiterpene lactones, phenolics, saponins and flavonoids have been demonstrated anti-ulcer activity (MINOZZO et al., 2016; VIEIRA JÚNIOR et al., 2015). Besides, compounds from primary metabolism of plants, such as polysaccharides have been studied for their anti-ulcer properties. Polysaccharides extracted from different organs, displaying different structures have been shown efficient protection of gastric mucosa against necrotizing agents (AUSTARHEIM et al., 2012a; MARIA-FERREIRA et al., 2013).

Pectins are acidic polysaccharides extracted from cell wall of plants which have been also demonstrated anti-ulcer properties (MARIA-FERREIRA et al., 2013; NASCIMENTO et al., 2013). Pectins are the major component of the primary cell wall of dicotyledonous and comprise a complex group of polysaccharides which are characterized by the high content of galacturonic acid (Gal*p*A). The term pectin includes several structural domains, among them, three defined structural units are known: homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II), whereas the HG and RG-I are the predominant domains of pectins in plants (CARPITA; GIBEAUT, 1993; HARHOLT; SUTTANGKAKUL; VIBE SCHELLER, 2010). HG is a linear segment of the pectin, that consists of unbranched 1,4-linked α-D-Gal*p*A chain. RG-I is a branched segment, exhibiting rhamnose residues intercalating the galacturonic acid at the backbone (α-1,4-D-Gal*p*A-α-1,2-L-Rha*p*). Rhamnose residues may exhibit substitutions at O-4 by galactans, arabinans and/or arabinogalactans as side chains (CARPITA; GIBEAUT, 1993; MCNEIL et al., 1984).

Artemisia campestris, from Asteraceae family, is a perennial shrub that reaches approximately 1,5 m height, and it is a scarcely aromatic herb (DIB et al., 2016; SEFI et al., 2011). This plant is one of over several species of *Artemisia* genus, which is widely known for its medicinal properties. Traditional consumption of *A. campestris* is the decoction of aerial parts, indicated as antibacterial, anti-inflammatory, gastric disturbances, abdominal cramps, among others disorders (AKROUT et al., 2011; SAOUDI et al., 2010). Some studies have been reported the beneficial effects of *A. campestris* aqueous extracts.

Akrout et al. (2011) demonstrated antitumor and antioxidant activities *in vitro* of the infusion extract. Aqueous extract of the herb also exhibited antibacterial and antioxidant activities *in vitro* and, anti-inflammatory and wound healing effects *in vivo* (GHLISSI et al., 2016). These authors attribute the biological effects of *A. campestris* aqueous extracts to secondary metabolites of plants, such as phenolic compounds. However, water extracts of plants also contain polysaccharides, which may be responsible at least for some extent of the activity of the plant. Until now, no reports were found concerning the composition of the polysaccharides from *A. campestris* aerial parts.

In this study, the aerial parts of *A. campestris* L. *subsp*. *maritima* Arcangeli growing on the beach sands of Costa Nova (Portugal) were submitted to an aqueous extraction in order to obtain polysaccharides, which were chemically characterized and evaluated regarding their anti-ulcer effects in rats.

2. Material and methods

2.1 Plant material

Aerial parts of *A. campestris* L. *subsp maritima* Arcangeli at the flowering stage were collected on the Atlantic beach sands of Costa Nova, near Aveiro (Portugal) in September 2013. Previous plant samples collected in this place were already identified by the botanist Ângelo E. R. Pereira (CAVALEIRO, 1986).

2.2 Extraction and purification of pectic fraction

Aerial parts of *A. campestris* were dried, previously grounded and defatted/depigmented with absolute ethanol in a Soxhlet apparatus, for 48 h. The dry material was milled once again using IKA® A11 basic analytical mill and sieved (32 mesh). The defatted material was extracted with boiling water, under reflux, for 3 h. The aqueous extract was centrifuged (17,226 *x g*, 15 ºC, 20 min) and the supernatant was precipitated with 4 V absolute ethanol (4 ºC, overnight). Polysaccharides were isolated by centrifugation (17,226 *x g*, 15 ºC, 20 min), dissolved in distilled water and submitted to dialysis using 6-8 kDa cut-

off membrane for 48 h against tap water and, subsequently against distilled water for another 48 h. The extract was concentrated and freeze-dried, yielding the crude polysaccharide fraction (ACP-CR). This fraction was dissolved in an appropriate volume of distilled water and submitted to ultrafiltration using a 0.1 µm membrane, yielding a retained (ACP-R0.1) and an eluted fraction (ACP-E0.1). ACP-E0.1 was further filtered using a 10 kDa membrane, to give a retained (ACP-R10) and an eluted fraction in 10 kDa membrane (ACP-E10).

2.3 Monosaccharide composition

Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 ºC, for 2 h. The hydrolyzate was evaporated to dryness, the residues were reduced with $NaBH_4$ (Wolfrom & Thompson, 1963b) and acetylated with pyridine–acetic anhydride (1:1, v/v, overnight) (Wolfrom & Thompson, 1963a). The alditol acetates were analyzed by gas chromatography–mass spectrometry (GC-MS) using a Varian 4000 chromatograph, linked to a Varian CP-3800 mass spectrometer. The alditol acetates were applied to a VF-5 capillary column (0.25 mm internal diameter × 30 m). Injector temperature was 250 ºC, initial temperature 100 ºC to 230 ºC, at a rate of 10 ºC/min, maintained for 35 min. Helium was used as carrier gas (1.0 ml/min). Uronic acids content was estimated with meta-hydroxydiphenyl colorimetric method (BLUMENKRANTZ; ASBOE-HANSEN, 1973) using galacturonic acid as standard.

2.4 Nuclear magnetic resonance spectroscopy (NMR)

1D and 2D NMR experiments (^{13}C, ^{1}H and ^{1}H-^{13}C HSQC) were carried out using a Bruker Avance III 400 MHz spectrometer. The samples were dissolved in D_2O (20 mg/ml) and the analyses were recorded at 70 °C. Chemical shifts are expressed relative to acetone (δ= 30.2) for ^{13}C NMR and TMSP-d_4 (3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt) (δ = 0) for ^{1}H and ^{1}H-^{13}C HSQC NMR spectra.

2.5 High-performance size-exclusion chromatography (HPSEC)

The polysaccharide fractions were analyzed by HPSEC using a Waters unit coupled to a refractive index (RI) and a multi-angle laser light scattering (MALLS) detector (Wyatt Technology Dawn-F). Four Waters Ultrahydrogel columns (2000, 500, 250, 120) were connected in series and coupled to the multidetection instrument. The eluent was a solution of 0.1 mol/L $NaNO_2$ and 0.02% NaN_3, at a flux of 0.6 ml/min. Samples (1.5 mg/ml) were previously filtered through a 0.22 µm cellulose acetate membrane. The data were collected and analyzed by a Wyatt Technology ASTRA program. The analyses were carried out at 25 ºC. Molar mass of ACP-E10 was determined using a refractive index increment dn/dc=0,128 g/ml (VRIESMANN; TEÓFILO; PETKOWICZ, 2011) and Mw was calculated using Astra software.

2.6 Determination of degree of O-methyl esterification and acetylation

Degree of O-methyl esterification (DM) of ACP-E10 was determined by NMR technique, according Grasdalen, Einar Bakoy and Larsen (1988). Degree of acetylation (DA) of the samples was determined using the Hestrin (1949) colorimetric method, using erythritol tetraacetate as standard.

2.7 Carboxy-reduction and methylation analysis of fraction ACP-E10

The carboxyl groups of the uronic acid residues of ACP-E10 were reduced to their corresponding alcohols by $NaBH_4$, using the carbodiimide method (Anderson & Stone, 1985; Taylor & Conrad, 1972), to give a reduced polysaccharide fraction (ACP-E10CX). The reduction of uronic acid residues was accompanied by colorimetric assay (BLUMENKRANTZ; ASBOE-HANSEN, 1973). The monosaccharide composition of API-E10CX was determined as described in section 2.3. The ACP-E10 linkage analysis was done on carboxyl-reduced polysaccharide (ACP-E10CX). The fraction was methylated according to Ciucanu & Kerek (1984). The per-O-methylated product was hydrolyzed with formic acid (45%) for 15 h, at 120 ºC. The hydrolyzate was evaporated to dryness, and the residues were reduced and acetylated to give a mixture of partially O-methylated alditol acetates, which were identified by their typical electron impact breakdown profiles and retention times.

2.8 Animals

Experiments were conducted using female Wistar rats (200-220 g) which were obtained from animal vivarium of the Federal University of Paraná (Curitiba, Brazil). The animals were housed in cages containing softwood and the temperature was maintained at 22 ± 2 ºC under a 12/12 h light/dark cycle, with free access to food and water. The experiments were approved by the Committee of Animal Experimentation of Federal University of Paraná (CEUA/BIO—UFPR, protocol: 899) and conducted in agreement with Guide for the Care and Use of Laboratory Animals" (NIH Publications nº 8023, revised 1985).

2.9 Induction of acute gastric lesions

The gastric lesions were induced by absolute ethanol in rats following the method described by Robert et al. (1979), with minor modifications. The animals (*n* = 6) were fasting for ~18 h, with free access to water and they were orally pretreated by gavage with the compounds or water (vehicle). The animals were divided into five groups and treated with water (1 ml/kg body weight), omeprazole (40 mg/kg), and ACP-E10 (0.3, 3 and, 30 mg/kg). After 1 h, the animals received ethanol by gavage (1 ml/200 g) and then, they were euthanized 1 h after ethanol administration. The stomach were removed, opened throughout the great curvature and photographed. The lesion area was measured as length (mm) × width (mm) using Image Tool 3.0 software. The glandular portion of the stomach was divided in two parts, weighed and, used posteriorly for determination of gastric wall mucus and reduced glutathione contents.

2.10 Determination of gastric wall mucus and reduced glutathione content

The gastric mucus level was determined using the method described by Corne, Morrissey and Woods (1974). The mucus levels were quantified using standard curve of Alcian Blue (6.25–100 g) and the result was expressed in µg of Alcian Blue/g of tissue. Reduced glutathione levels (GSH) in gastric mucosa

were determined by the method of Sedlak and Lindsay (1968). The absorbance was measured in 96-well plate at 415 nm using a microplate reader (Biotek Synergy HT). The GSH contents were quantified using a standard curve of GSH (0.375 - 3 µg) and the result was expressed as µg GSH/g of tissue.

2.12 Statistical analysis

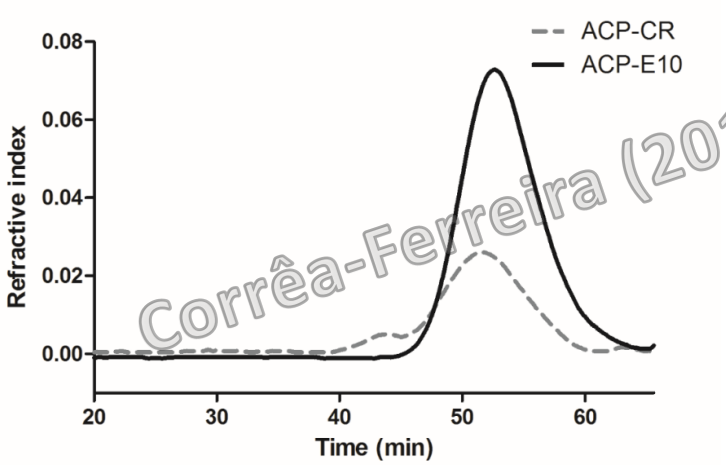
The data of biological assays were analyzed using Prism 5.0 software (GraphPad, San Diego, CA, USA). The results in the graphs are expressed as mean ± standard error of the mean (SEM). Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Values of $*P < 0.05$ were considered statistically significant.

3. Results

3.1 Isolation and purification of polysaccharides present in *A. campestris* aerial parts

Aerial parts of *A. campestris* had 36% lipids, which were removed by alcoholic extraction in Soxhlet apparatus. Crude polysaccharide fraction from *A. campestris* (ACP-CR) yielded 6.9% based on dry defatted aerial parts. Monosaccharide composition of this fraction is shown in table 1. ACP-CR was enriched with uronic acids (61%), followed by galactose (14%) and arabinose (11%), indicating the presence of pectins in this fraction. ACP-CR showed a polymodal elution profile by HPSEC (FIGURE 1) and it was submitted to ultrafiltration process. The fraction eluted in 10 kDa membrane (ACP-E10) had a yield of 1.6% and displayed a monomodal elution profile by HPSEC (FIGURE 1) indicating a pure polysaccharide fraction.

FIGURE 1 - COMPARISON BETWEEN ELUTION PROFILE OF ACP-CR AND ACP-E10 BY HPSEC USING REFRACTIVE INDEX DETECTOR.



Corrêa-Ferreira (2016)

Monosaccharide composition of the fractions obtained from the purification steps of ACP-CR is shown in Table 1. All fractions displayed similar composition with uronic acid as major component, followed by arabinose and galactose. The UA/Rha and (Gal+Ara)/Rha ratios were calculated. These data provide information about the contribution of HG to pectic population and branching of RG-I, respectively (HOUBEN et al., 2011). The pure fraction (ACP-E10) displayed the highest UA/Rha ratio among the fractions from purification steps, indicating that is richer in HG regions than other fractions. The number of neutral sugars (Gal+Ara) per rhamnose unit was similar between the fractions, demonstrating similar side chains lengths.

TABLE 1 - MONOSACCHARIDE COMPOSITION OF FRACTIONS OBTAINED FROM AQUEOUS EXTRACTION OF *A. campestris* AERIAL PARTS.

		Monosaccharide composition (%)[1]							
	Yield (%)[2]	**Rha**	**Ara**	**Man**	**Gal**	**Glc**	**UA[3]**	**UA / Rha**	**(Gal+Ara) / Rha**
ACP-CR	6.9	5.8	11.1	3.1	14.0	4.9	61.0	10.5	4.3
ACP-E0.1	3.0	4.9	12.7	3.7	10.0	4.9	63.8	13.0	4.6
ACP-R0.1	0.7	6.4	8.7	1.3	12.3	2.0	69.3	10.8	3.3
ACP-R10	0.4	8.2	12.9	3.1	18.9	5.3	51.6	6.3	3.9
ACP-E10	1.6	4.1	11.5	4.7	9.3	6.4	64.1	15.6	5.1

Corrêa-Ferreira (2016)

[1] Neutral sugars determined by GC-MS
[2] Based on dry depigmented aerial parts
[3] Uronic acid determined by colorimetric method (BLUMENKRANTZ; ASBOE-HANSEN, 1973).

3.2 Characterization of ACP-E10 fraction

ACP-E10 isolated from *A. campestris* aerial parts showed uronic acids as major monosaccharide (64%) and the neutral monosaccharides are composed by arabinose, galactose, glucose, manose, and rhamnose in a 2.8: 2.3: 1.6: 1.1: 1 molar ratio, indicating that ACP-E10 is composed by pectic polysaccharide. The identity of uronic acid was verified after carboxy-reduction process in ACP-E10 fraction. The carboxy-reduced fraction displayed an increase in the galactose contents (60.9%), confirming that galacturonic acid is the uronic acid present in ACP-E10.

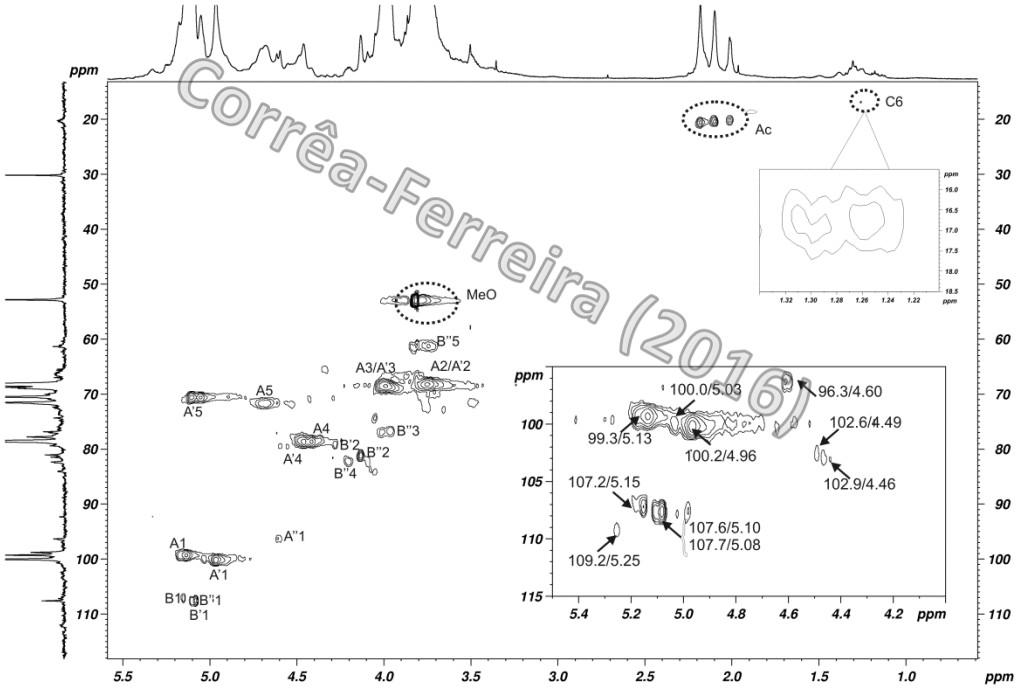
The contribution of HG and RG-I domains for the pectin may be estimated using the equations: HG (%) = GalA (%) – Rha (%) and, RG-I (%) = [GalA (%) – HG(%)] + Rha + Ara + Gal (M'SAKNI et al., 2006). Using these equations, in ACP-E10 the HG domain represents 60% of pectin and RG-I 29%.

The structure of polysaccharide was elucidated by $^{1}H/^{13}C$ HSQC, ^{1}H, ^{13}C NMR spectra and, methylation analysis. Due to the presence of uronic acids in the ACP-E10 fraction, methylation was performed on carboxy-reduced fraction. Signals attribution of NMR was based on previous studies (GOLOVCHENKO et al., 2012; KOSTALOVA; HROMÁDKOVÁ; EBRINGEROVÁ, 2013; SHAKHMATOV et al., 2015; WESTERENG et al., 2008). From HSQC (FIGURE 2) the main signals in the correlation map correspond to Gal*p*A units of the pectin, in their free and methylesterified forms. Intense signals of 1,4-linked α-D-Gal*p*A were observed at δ 99.3/5.13 (C1/H1), 68.2/3.76 (C2/H2), 68.5/3.99 (C3/H3), 78.5/4.41 (C4/H4) and, 71.7/4.68 (C5/H5). From ^{13}C spectrum of ACP-E10 the signal of carboxyl group (C6) of the galacturonic acid was observed at 174.7 ppm (FIGURE 3). The presence of the derivative 2,3,6-Me_3-Gal in the methylation product of the carboxy-reduced fraction confirmed the presence of HG domain in the pectin of *A. campestris*. Signals of methylesterified galacturonic acid were observed at δ 100.2/4.96 (C1/H1), 68.2/3.76 (C2/H2), 68.5/3.99 (C3/H3), 78.5/4.46 (C4/H4), and 70.4/5.11 (C5/H5). The methyl esterified carboxyl group (C6) of the galacturonic acid was observed at 170.7 ppm in the ^{13}C spectrum of ACP-E10. The resonance of the methoxyl group was identified at 52.8/3.81 ppm in HSQC. Degree of O-methyl esterification (DM) calculated for ACP-E10 by ^{1}H NMR spectrum was 69%.

Units of galacturonic acid representing α and β anomers located in the reducing end of the polysaccharide chain are demonstrated by the correlations at 92.4/5.33 and 96.3/4.61 ppm, respectively, in the HSQC spectrum. These signals were also found in the ^{13}C spectrum (FIGURE 3). Gal*p*A as non-reducing end-units of galacturonic acid was also identified in the HSQC at 100.0/5.03 ppm (SHAKHMATOV et al., 2015).

Signals at δ 20.4/2.18, 20.2/2.10 and 19.9/2.01 indicated the presence of acetylation sites in O-2, O-3 and both O-2 and O-3 of galacturonic acid units, respectively (KOSTALOVA; HROMÁDKOVÁ; EBRINGEROVÁ, 2013; RENARD; JARVIS, 1999). Degree of acetylation (DA) of ACP-E10 is 5.5% regard to total galacturonic acid units of the pectin.

FIGURE 2 - ^{1}H-^{13}C HSQC NMR SPECTRUM OF ACP-E10 IN D_2O AT 70 ºC.



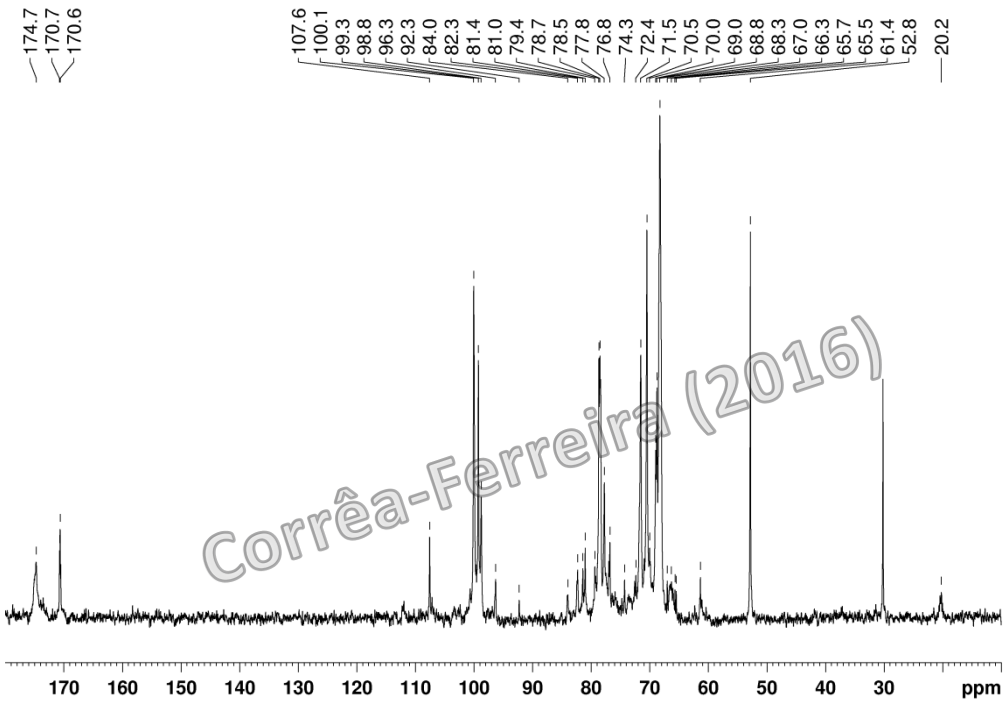
NOTE: Sample was dissolved in D_2O at 20 mg/mL. Chemical shits relative to TMSP-*d*4 (δ=0). Letters A, B and C correspond to GalpA, Ara and Rha units, respectively. The numbers after letters indicate the carbon number of the monosaccharide unit. The insert at right below displays the amplified anomeric region. The insert at right above show amplified signal of C6 of Rha units. **Symbols:** MeO = methoxyl group; Ac = acetyl groups.

The presence of rhamnogalacturonan type I (RG-I) segment in the structure of pectin from *A. campestris* was verified by the signal of anomeric carbon of α-L-Rha*p* in the ^{13}C spectrum of ACP-E10 at 98.8 ppm (FIGURE 3).

Signals of methyl group of 1,2- and 1,2,4-substituted rhamnose were found at δ 16.7/1.24 and 16.8/1.29 ppm, respectively, in the HSQC (FIGURE 2 – insert) (SHAKHMATOV et al., 2015). The proportion between integral intensities of H6 of 1,2- and 1,2,4-substitued rhamnose in the ^{1}H NMR spectrum of ACP-E10 is 35:65, indicating that most of the rhamnose units of RG-I regions in ACP-E10 are branched by side chains.

Arabinose and galactose are present in the side chains of RG-I segment of pectin from *A. campestris*. From methylation analysis, Ara*f* was found to be terminal, 1,5-, 1,3-di-O- and 1,3,5-tri-O-substituted. From HSQC, the correlations at 109.2/5.25, 107.7/5.08, 107.2/5.15 and, 107.6/5.10 ppm confirm the presence of these arabinose units, respectively. Possibly, the arabinose may be present as highly branched arabinans and arabinogalactans.

FIGURE 3 - ^{13}C SPECTRUM OF ACP-E10 IN D_2O AT 70 ºC.



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NOTE: Sample was dissolved in D_2O at 20 mg/mL. Chemical shits relative to acetone (δ=30.2 ppm).

Low intensity signals of β-D-Gal*p* 1,6- and 1,3,6-linked were identified at δ 102.6/4.49 and 102.9/4.46, indicating the presence of arabinogalactan type II (AG-II). From ^{1}H was also identified the signal at 4.69 ppm which may be attributable of 1,3-linked β-D-Gal*p* (Shakhmatov, Atukmaev, & Makarova,

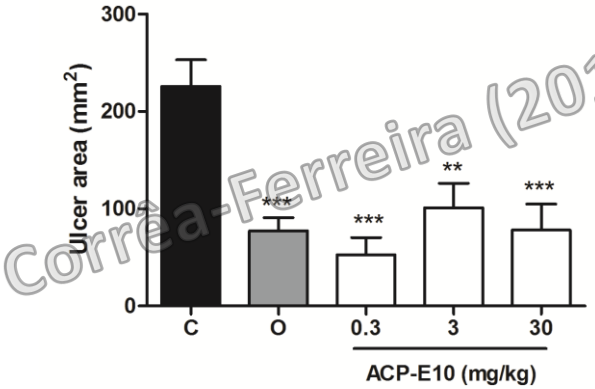
2016). The derivatives 2,4,6- and 2,3,4-Me_3-Gal found in the methylation product confirmed the presence of AG-II as component of the pectin from *A. campestris* aqueous extract.

Molar mass of ACP-E10 was found to be 16,600 g/mol (Mw), which is consistent with a polymerization degree of 85 GalA residues, indicating that ACP-E10 is a small polymer. The presence of the signals of reducing and non-reducing end of GalA units in the HSQC confirmed the low molar mass of ACP-E10.

3.3 Antiulcer activity of ACP-E10

Gastroprotective effects of ACP-E10 were evaluated in an *in vivo* model of acute gastric lesions (FIGURE 4).

FIGURE 4 – EFFECT OF ACP-E10 ON ACUTE GASTRIC LESIONS INDUCED BY ETHANOL IN RATS.



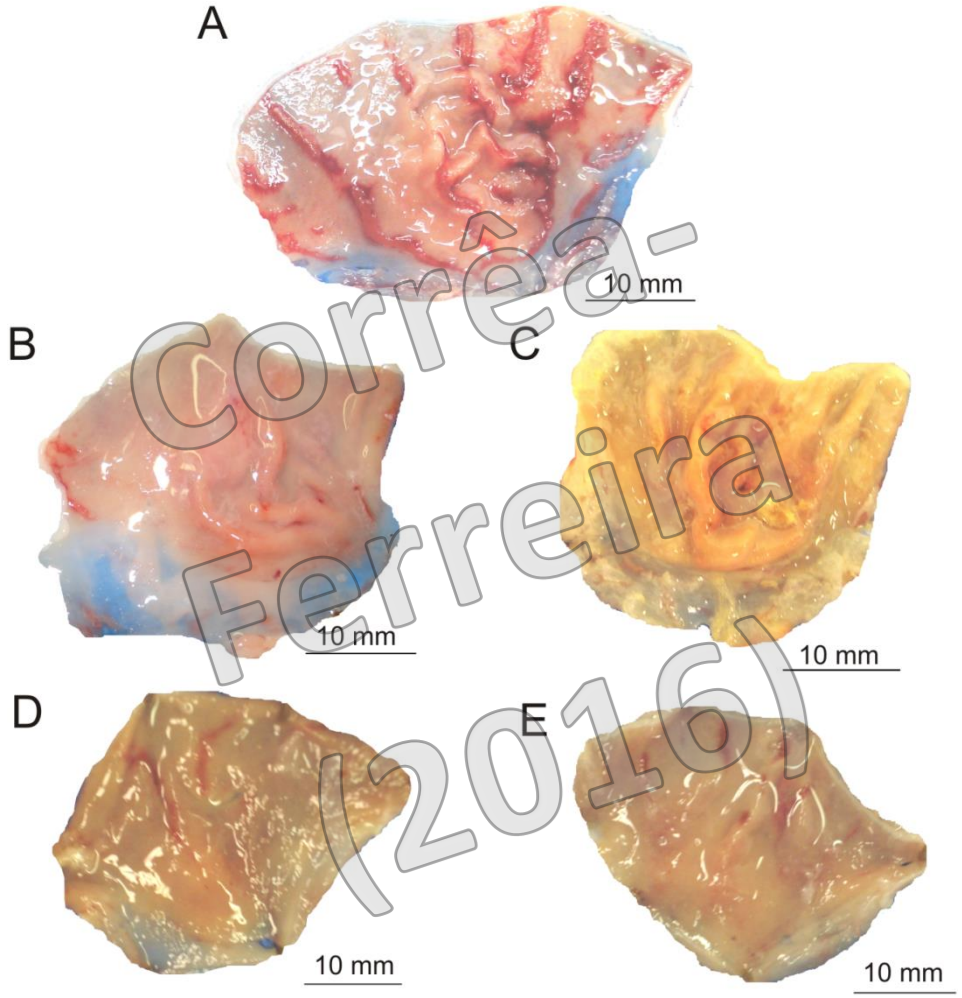
Correa-Ferreira (2016)

NOTE: The animals were orally pretreated with vehicle (C: water 1 ml/kg), omeprazole (O: 40 mg/kg) or ACP-E10 (0.3, 3 and 30 mg/kg), 60 min before oral administration of ethanol (1.0 ml/200 g). Values were expressed as mean ± standard error of the mean (n=6), analyzed by one-way ANOVA and Bonferroni's post hoc test. Symbols: *P<0.05, ** P < 0.01 and *** P < 0.001 when compared to lesioned control group.

Ethanol lesioned control group (C) showed a wide lesion area in the gastric mucosa (225.8 ± 27.3 mm^2) and positive control of the test (O: 40 mg/kg) was effective in reduce the gastric lesions by 66% when compared to the control group. Interestingly, pretreatment of animals with APC-E10 protected the gastric mucosa against the necrotic effects of ethanol, reducing significantly the ulcer area from the lowest to the highest dose in 77% (0.3

mg/kg), 55% (3 mg/kg) and 65% (30 mg/kg) when compared to the ethanol control group. FIGURE 5 shows the macroscopic appearance of the stomachs after ethanol-induced gastric lesions from control and treated groups.

FIGURE 5 – EFFECT OF ORAL ACP-E10 TREATMENT ON MACROSCOPIC APPEARANCE OF THE STOMACHS AFTER ETHANOL-INDUCED GASTRIC LESIONS.



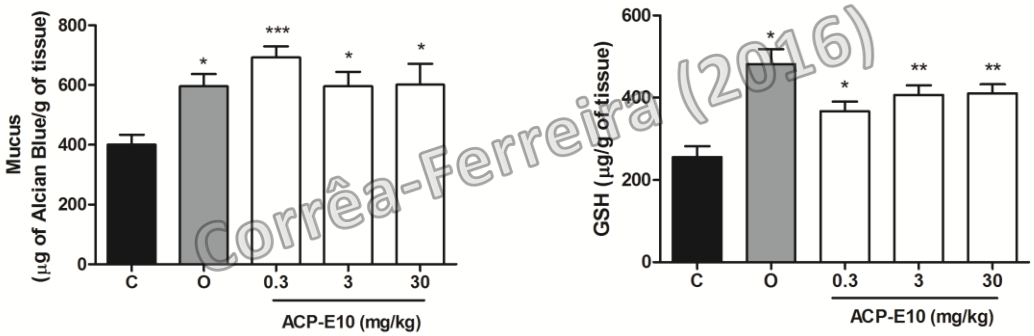
Correa-Ferreira (2016)

NOTE: The animals were orally pretreated with vehicle (C: water 1 ml/kg), omeprazole (O: 40mg/kg) or ACP-E10 (0.3, 3 and 30 mg/kg) 60 min before oral administration of ethanol. The animals were euthanized 1 h after ethanol administration and, the stomachs were removed, opened and photographed. Scale bar = 10 mm.

The administration of ethanol to the animals depleted the mucus barrier in gastric mucosa of lesioned control group, while omeprazole treatment prevented the decrease of gastric mucus levels (C: 401.0 ± 32.3; O: 596.0 ± 40.9 µg of Alcian Blue/g of tissue) (FIGURE 6A). When testing the ACP-E10, all

doses were able to maintain the mucus levels when compared to the control group (0.3 mg/kg: 693.1 ± 36.75; 3 mg/kg: 595.7 ± 48.5; 30 mg/kg: 601.1 ± 70.0 µg of Alcian Blue/g of tissue). Similar to gastric mucus, the administration of ethanol decreased the levels of GSH (255.7 ± 26.5 µg GSH/g tissue) whereas the positive control of the test, omeprazole was able to maintain the GSH levels in 481.6 ± 36.8 µg GSH/g tissue (FIGURE 6B). Likewise, pretreatment with ACP-E10 at all doses, prevented the reduction of GSH levels in the gastric mucosa (0.3 mg/kg: 367.5 ± 23.3; 3 mg/kg: 406.6 ± 23.7; 30 mg/kg: 410.7 ± 22.0 µg GSH/g tissue) when compared to the control group.

FIGURE 6 - EFFECT OF ACP-E10 ON GASTRIC MUCUS AND GSH LEVELS IN ACUTE GASTRIC LESIONS INDUCED BY ETHANOL IN RATS.



Corrêa-Ferreira (2016)

NOTE: The animals were orally pretreated with vehicle (C: water 1 ml/kg), omeprazole (O: 40 mg/kg) or ACP-E10 (0.3, 3 and 30 mg/kg), 60 min before oral administration of ethanol (1.0 ml/200 g). Values were expressed as mean ± standard error of the mean (n=6), analyzed by one-way ANOVA and Bonferroni's post hoc test. Symbols: *P<0.05, ** P < 0.01 and *** P < 0.001 when compared to lesioned control group.

4. Discussion

In the last few years, plenty of attention has been given to molecules isolated from natural products and in fact, some biological activities have been proven. For instance, pectins isolated from a variety of sources have previously been shown to demonstrate some activities, such as anti-cancer (DELPHI; SEPEHRI, 2016), immunomodulatory (AMORIM et al., 2016) and, anti-ulcer activities (CHANDRASHEKAR; DHARMESH, 2016). The biological activity of pectins is determined by their chemical features, such as monosaccharide composition, proportion of HG and RG domains, amount of methyl and acetyl groups and molar mass (SILA et al., 2009; YAMADA, 1994).

In this study, the structure of the pectin isolated from aerial parts of A. campestris subsp. maritima was characterized and its anti-ulcer effect was evaluated. Chemically, ACP-E10 is composed by a pectin rich in HG domain (60%) and also contain RG-I blocks (29%). In pectins from other plants, such as Camellia sinensis and Theobroma cacao, the HG domains also prevail over RG-I regions (ELE-EKOUNA et al., 2011; VRIESMANN; TEÓFILO; PETKOWICZ, 2011).

Neutral side chains of RG-I in pectins may be composed by arabinans, galactans and arabinogalactans. Arabinans are composed by chains of 1,5-linked α-L-Ara*f* units, which may contain Ara*f* units attached to them at O-2, O-3 or both (CARPITA; GIBEAUT, 1993; MCNEIL et al., 1984). Arabinogalactans may be classified in type I (AG-I) or type II (AG-II) according to the monosaccharide linkage between the galactose units (CAFFALL; MOHNEN, 2009). In the present study, pectin from *A. campestris* aerial parts displayed branched arabinans and arabinogalactans type II, which is characterized by 3-, 6-, and 3,6-linked galactose.

Galacturonic acid from homogalacturonan domains of pectin may be esterified with methyl groups at carboxylic group (C6). ACP-E10 displayed more than 50% of the GalA units esterified with methyl groups, indicating a high methoxyl (HM) pectin (Lopes da Silva & Rao, 2006). HM pectins were also described for leaves of *Camellia sinensis* (ELE-EKOUNA et al., 2011). HSQC spectrum of ACP-E10 indicated that acetyl groups (DA=5.5%) are esterifying the GalA units in both O-2 and O-3 positions. The DA found for *A. campestris* pectin is similar to that found for sunflower head pectin (DA=3.4%) (KANG et al., 2015) and lower than found for *Vernonia kotschyana* (DA=11%) (NERGARD et al., 2005), other plant species included in Asteraceae family.

ACP-E10 displays a low molar mass for pectic polymers. The size of pectins may range from 10,000 to 5,000,000 g/mol (AUSTARHEIM et al., 2012b) and ACP-E10 has 16,600 g/mol (Mw). For other pectins isolated from leaves, molar mass values were found vary between 55,000 g/mol for *Cissampelos pareira* (SINGTHONG et al., 2004) and 980,700 g/mol for *Premna microphylla* (CHEN et al., 2014). A pectin obtained from *Vernonia kotschyana* (Asteraceae) roots displays a similar value of molar mass (20,000 g/mol) than that found for pectin of *A. campestris* (NERGARD et al., 2005).

In ACP-E10 minor amounts of glucose and mannose were found in the monosaccharide composition. These monosaccharides may be substituents of AG-II. Other monosaccharides found as AG-II substituents are Rha, Xyl, GlcA and, GalA (FINCHER; STONE, 1983).

In this study, in order to investigate the potential gastroprotective effect of the pectin isolated from *A. campestris*, it were performed an *in vivo* model of gastric lesions induced by ethanol in rats. Ethanol is a well-known necrotic agent that rapidly induces lesion in the gastric mucosa when given orally, leading to hemorrhagic erosion and ulcer formation by decreasing mucus and non-proteic sulphydrilic groups (Szabo, 1987). Interestingly, our results showed that the extent of gastric lesions induced by ethanol was reduced by ACP-E10 even at the lowest dose tested (0.3 mg/kg), demonstrating the potent gastroprotective activity of this compound. Some authors have also reported the protection of the gastric mucosa by pectins isolated from plants. Harsha and collaborators, for instance, demonstrated the gastroprotective activity of pectin isolated from *Curcuma longa* in a dose of 200 mg/kg in a stress model to induce gastric lesion (HARSHA; CHANDRA PRAKASH; DHARMESH, 2016). In the present study, pectin from *A. campestris* exhibited gastroprotective effect at a 660-fold lower dose. A pectin fraction isolated from leaves of *Acmella oleracea* also exhibit anti-ulcer effects in all tested doses (1, 3 and 10 mg/kg) (NASCIMENTO et al., 2013), reinforcing that these compounds could act as gastroprotective agents. In horses, pectin from apple pulp is used in combination with several ingredients (e.g. lecithin, sodium bicarbonate, polar lipids, antioxidants) to treat and prevent gastric ulcer (WOODWARD et al., 2014). Treatment with feed supplements (Egusin®) containing surface-active ingredients (pectin and lecithin) and antacids agents (sodium and calcium bicarbonate) were effective in ameliorate the severity of gastric ulcers in horses submitted to feed-deprivation (WOODWARD et al., 2014). In the present study, it was demonstrated that pectin from *A. campestris* was effective in reduce gastric ulcer in rats without other ingredients, such as antacids.

Usually, the increase of defensive factors of gastric mucosa has been shown better results in prevent the occurrence of peptic ulcer than attenuation of aggressive factors (KHUSHTAR et al., 2016). The gastric mucus gel layer forms a protective barrier in the stomach against acid and lumial pepsin

(ALLEN; FLEMSTRÖM, 2005). As well as mucus, the major non-enzymatic sulphydrilic group of gastric mucosa, GSH, provides protection against oxidative damage in the tissue and it has been shown important protective role against gastric lesions induced by ethanol (HIRAISHI et al., 1991; OLATUNJI; CHEN; ZHOU, 2015). Our results showed that pretreatment with ACP-E10 significantly restored the depleted mucus and GSH levels in all tested doses, demonstrating the ability of this compound in maintaining the protective factors of the mucosa. Pectins extracted from turmeric (HARSHA; CHANDRA PRAKASH; DHARMESH, 2016) and potato (CHANDRASHEKAR; DHARMESH, 2016) were also able to restore the levels of mucus in stomach of animals with gastric lesions. The potential of pectin in maintain the levels of gastric mucus may be attributable to capability of pectin in interacting with the mucin and forming a protective barrier to the gastric mucosa (HARSHA; CHANDRA PRAKASH; DHARMESH, 2016). Recently, it has been proposed that the improvement of quality of ulcer healing can only be achieved through involvement of gastric defense systems and, in this context, natural products have proved to be ideal candidates (KANGWAN et al., 2014).

5. Conclusion

The purified polysaccharide (ACP-E10) from aerial parts of *A. campestris subsp maritima* consists of a pectin with predominant HG domain and the RG-I domain is branched with arabinans and arabinogalactans type II. The pectin displays low molar mass (16,600 g/mol) and presents DM=69% and DA=5.5%. This fraction exhibits potent anti-ulcer effect *in vivo*, using ethanol-induced ulcer model in rats, even at lowest dose (0.3 mg/kg). The underlying mechanisms involved in the anti-ulcerogenic effects of ACP-E10 might be related to its capability of increase the gastro-defensive mechanisms such as gastric mucus and GSH levels. Pectin from *A. campetris subsp. maritima* has a promising potential as an alternative for treatment or prevention of ulcer, considering the ecofriendly extraction process and the properties as solubility in aqueous systems and biocompatibility.

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4 CONCLUSÕES

Com relação ao efeito do estresse salino sobre plantas de *A. annua* pode-se concluir:

- Plantas de *A. annua* submetidas a diferentes níveis de salinidade do solo apresentaram redução no crescimento e biomassa tanto para as raízes quanto para as partes aéreas, sendo que este efeito foi mais pronunciado nas partes aéreas;

- O estresse salino também alterou a composição química dos polissacarídeos presentes na parede celular das plantas de *A. annua*. Estas modificações foram mais intensas nas partes aéreas e a principal classe de polissacarídeos envolvida na resposta ao estresse salino foram as pectinas. Alterações no grau de metil esterificação, cadeias laterais das ramnogalacturonanas e conteúdo de ácidos urônicos foram as principais modificações encontradas nas pectinas de plantas de *A. annua* submetidas ao estresse salino;

- Apesar de estudos na literatura sugerirem que plantas submetidas a ambiente salino pudessem produzir polissacarídeos sulfatados, as plantas de *A. annua* submetidas a estresse salino não apresentaram sulfatação de seus polissacarídeos.

Com relação às atividades biológicas avaliadas neste trabalho com os extratos de *Artemisia*, pode-se concluir:

- A infusão de *A. vulgaris* e o polissacarídeo inulina isolado da infusão apresentaram um importante efeito hepatoprotetor *in vivo* em modelo de lesão hepática induzida por CCl_4;

- A análise da composição química da infusão desta espécie revelou a presença de 40% de carboidratos, 2,9% proteínas e 9,8% de fenólicos. Como principais compostos de baixa massa foram encontrados o ácido clorogênico, cafeico e ácidos dicafeoilquínicos;

- Os prováveis mecanismos de ação dos extratos de *A. vulgaris* na proteção do fígado contra os efeitos tóxicos do CCl_4 foram através de seu efeito antioxidante e modulação de citocinas hepáticas, tais como TNF-α.

- A partir da extração aquosa fervente das partes aéreas de *A. campestris* foi isolada uma fração péctica, de baixa massa molar. A fração é constituída por 60% de regiões de homogalacturonana e 29% de ramnogalacturonana tipo I (RG-I). As cadeias laterais de RG-I incluem arabinanas e arabinogalactanas do tipo II;

- Esta fração péctica apresentou efeito gastroprotetor *in vivo* em modelo de úlcera induzida por etanol em ratas, em todas as doses avaliadas. A redução da área de lesão gástrica promovida pelo tratamento oral com a pectina de *A. campestris* pode ser atribuída pelo aumento dos mecanismos de defesa do estômago, como o aumento da camada de muco e conteúdo de glutationa reduzida.

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