UNIVERSIDADE FEDERAL DO PARANÁ

GEORGIA ERDMANN DO NASCIMENTO

POLISSACARÍDEOS DE ALGUNS FRUTOS COMESTIVEIS DA FAMÍLIA SOLANACEAE: CARACTERIZAÇÃO ESTRUTURAL, REOLOGIA, AVALIAÇÃO DA ATIVIDADE IMUNOMODULADORA E ANTINOCICEPTIVA

CURITIBA

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

Coorientadora: Profa. Dra. Sheila Maria B. Winnischofer

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de GEORGIA ERDMANN DO NASCIMENTO intitulada: POLISSACARÍDEOS DE ALGUNS FRUTOS COMESTÍVEIS DA FAMÍLIA SOLANACEAE: CARACTERIZAÇÃO ESTRUTURAL, REOLOGIA E AVALIAÇÃO DA ATIVIDADE IMUNOMODULADORA E ANTINOCICEPTIVA, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação.

CURITIBA, 26 de Janeiro de 2017.

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

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“Suba o primeiro degrau com fé.

Não é necessário que você veja toda a escada, apenas dê o primeiro passo”.

Martin Luther King Jr

RESUMO

A família Solanaceae tem grande importância econômica, com destaque às espécies com grande representatividade na alimentação humana. Entre essas espécies encontram-se o tomate (*Solanum lycopersicum*), o pimentão (*Capsicum annuum*) e o tamarillo (*S. betaceum*). Os polissacarídeos constituem o principal macronutriente desses frutos e são nutricionalmente importantes, constituindo as fibras dietéticas. Na indústria alimentícia os polissacarídeos são utilizados como ingredientes tecnologicamente funcionais, como por exemplo: geleificantes, espessantes e estabilizantes. Fisiologicamente podem apresentar efeitos imunomodulatórios dependentes das suas estruturas químicas. O presente trabalho apresenta a caracterização estrutural de polissacarídeos extraídos dos frutos do pimentão e das mucilagens que envolvem as sementes do tamarillo e do tomate. Também apresenta a avaliação do comportamento reológico da principal fração péctica presente na polpa do tamarillo, assim como a análise da atividade imunomudolatória de uma arabinoxilana isolada do tomate em modelos de dor *in vivo* e o efeito na secreção das citocinas TNF-α, IL-1β e IL-10 em modelo *in vitro* de uma pectina do pimentão antes e após modificação da sua estrutura. Os polissacarídeos foram obtidos por extrações aquosas e alcalinas. Foram fracionados por congelamento/degelo, tratamentos com α-amilase e com solução de Fehling e também ultrafiltração. As frações obtidas foram caracterizadas por técnicas espectrométricas, espectroscópicas (RMN) e cromatográficas (GC-MS e HPSEC). A partir da mucilagem do tamarillo foram caracterizadas (a) homogalacturonanas altamente metoxiladas provavelmente contendo inserções de ramnogalacturonanas do tipo I com cadeias laterais constituídas principalmente por arabinogalactanas tipo I; (b) uma arabinana linear (1→5)-ligada e (c) uma heteroxilana. A análise comparativa com os polissacarídeos da polpa mostrou que os polissacarídeos da mucilagem diferem no rendimento, comprimento das cadeias laterais das pectinas e no grau de ramificação das xilanas. O estudo do comportamento reológico da fração péctica presente na polpa do tamarillo mostrou que em água as soluções com diferentes concentrações da pectina (3, 5 e 8%) apresentam pouca viscosidade aparente, porém são positivamente afetadas pelo aumento da concentração. Essas soluções possuíram comportamento pseudoplástico, semelhante a líquidos, e obedecem à regra de Cox-Merz, podendo ser descritos pelo modelo de Ostwald-de Waele. Em condições que favorecem a formação de gel, a solução com apenas 1% da pectina tem comportamento de uma solução concentrada, enquanto que com o aumento para 2 e 3% de pectina, as soluções apresentam comportamento de gel, com as curvas de fluxo melhor descritas pelo modelo de Hershel-Bulkley. Esses géis também apresentaram termoestabilidade a variações de temperatura entre 5 a 80 °C. A partir da mucilagem do tomate foi purificada uma arabinoxilana (fração PTOK), formada por uma cadeia principal de unidades de β-D-Xyl*p* (1→4)-ligadas, pouco ramificada, nas posições *O*-2 e *O*-3, por unidades terminais de Ara*f* ou Xyl*p*. A administração intraperitonial da fração PTOK (10 mg/kg) mostrou redução significativa de aproximadamente 80% na contorção abdominal induzida por 0,6% de ácido acético e de cerca de 90% na fase inflamatória da nocicepção induzida por 2,5% de formalina em camunondongos, indicando que o efeito desse polissacarídeo na dor é através de mecanismos anti-inflamatórios. Em relação aos polissacarídeos do pimentão, foi obtida uma fração péctica (ANWS) com Mw estimada de 367 kDa constituída principalmente por uma homogalacturonana altamente metoxilada (DM = 85%), e aproximadamente 31% de arabinogalactanas do tipo I e II ancoradas em ramnogalacturonana do tipo I. Desta fração foi purificada uma arabinogalactana do tipo II e a caracterização estrutural detalhada foi realizada. Por fim, a fração ANWS (dose de 300 μg/mL) foi capaz de estimular a secreção das citocinas TNF-α, IL-1β e IL-10 por células THP-1 diferenciadas em macrófagos. Na presença

de LPS, a fração reduziu os níveis de secreção de TNF-α e de IL-1β, e aumentou o nível de secreção de IL-10, além de reduzir as razões TNF-α/IL-10 e IL-1β/ IL-10. A fração foi ainda submetida à hidrólise parcial com HCl 0,1 M a 90 °C por 16 h, resultando na remoção das cadeias laterais da pectina, diminuição do grau de metil-esterificação (DM = 17%) e do peso molecular (Mw = 36 kDa). A fração modificada (ANWS-M) também foi avaliada quanto seu efeito na secreção de citocinas pelas células THP-1 diferenciadas em macrófagos. Os resultados demonstraram que as diferenças no DM, peso molecular e a presença de cadeias laterais podem contribuir para diferente estimulação celular e consequente diferente efeito imubomodulatório pelas pectinas testadas.

Palavras-chave: Solanaceae. *Solanum betaceum*. *Solanum lycopersicum*. *Capsicum annuum*. Homogalacturonana. Arabinogalactanas. Arabinana linear. Arabinoxilana. Reologia. Efeito imunomudulador. Macrófagos THP-1.

ABSTRACT

The Solanaceae family has great economic importance, especially on species with great representativeness in human food. Among these species are the tomato (*Solanum lycopersicum*), green sweet pepper (*Capsicum annuum*) and tamarillo (*S. betaceum*). Their main macronutrients are the polysaccharides, which are nutritionally important and constitute the dietary fibers. In food industry, the polysaccharides are used as technologically functional ingredients, as for example: gelling agents, thickeners and stabilizers. Physiologically may have immunomodulatory effects depending on the chemical structure. This paper presents a structural characterization of polysaccharides extracted from green sweet pepper fruits and mucilage of tamarillo and tomato fruits. Also, this showed an evaluation of the rheological behavior of the main tamarillo pulp pectic fraction. Moreover, the immunomodulatory activity of an arabinoxylan isolated from tomato was evaluated in *in vivo* models, as well as effects on TNF-α, IL-1β e IL-10 secretion *in vitro* model of sweet pepper pectin, before and after structure changes. The polysaccharides were extracted by aqueous and alkali extractions. They were fractionated by freeze/thawing, α-amylase and Fehling´s solution treatments, and ultrafiltration process. The obtained fractions were characterized by spectrometric, spectroscopic (NMR), chromatographic (GC-MS and HPSEC) techniques. From tamarillo mucilage were characterized (a) a highly methyl-esterified homogalacturonan, possibly with inserts of type I rhamnogalacturonans containing type I arabinogalactan side chain; (b) a linear α-arabinan (1→5)-linked; and (c) a heteroxylan. Comparative analysis with pulp polysaccharides showed differences in yield, in the length of pectins side chains, and in the degree of branching of the xylans. Rheological behavior of aqueous dispersions of tamarillo pulp pectin at different concentrations (3, 5, and 8%) showed low apparent viscosities, but positively affected by concentration increase. These dispersions also showed shear-thinning and liquid-like behaviors. They were well fitted using the Ostwald-de Waele model and obey the Cox-Merz rule. Under conditions that favor gel formation, dispersion of pectin at 1% showed shear-thinning and concentrated solution behaviors. The pectin increase to 2% and 3% gave pronounced shear-thinning and gel like behaviors. Their flow curves profiles were better fitted using the Hershel-Bulkley model. These gels also presented thermostability at 5 to 80° C. From tomato mucilage was isolated an arabinoxylan (PTOK fraction, 10 mg/kg), formed by (1→4)-linked β-D-Xyl*p* units, carrying a low proportion of branching, at *O*-2 and *O*-3 position, with side chains constituted by single Ara*f* or Xyl*p* units. Intraperitoneal PTOK administration in mice significantly reduced around 80% the number of abdominal constrictions induced by 0.6% acetic acid and around 90% the inflammatory phase of nociception induced by 2.5% formalin, indicating that the polysaccharide effect on pain is through anti-inflammatory mechanisms. Regarding sweet pepper polysaccharides, a pectic fraction (ANWS) with estimated molecular weight of 367 kDa was obtained and constituted mainly of highly methoxylated homogalacturonan (DM = 85 %) and approximately 31% of type I and type II arabinogalactan anchored in type I rhamnogalacturonan. Moreover, a type II arabinogalactan was purified and its detailed structural characterization was performed. Finally, the ANWS fraction (at 300 μg / mL) could stimulate the TNF-α, IL-1β and IL-10 cytokine secretion by THP-1 macrophages. In the presence of LPS, the fraction reduced the levels of TNF-α and IL-1β and increased IL-10 secretion, as well as decreased TNF-α / IL-10 and IL-1β / IL-10 ratios. Further, the fraction was submitted to partial hydrolysis with 0.1 M HCl at 90 °C for 16 h, removing the pectin side chains and decreasing the degree of methyl esterification (DM = 17%) and molecular weight (Mw = 36 kDa). The modified fraction (ANWS-M) was also evaluated for its effect on cytokine secretion by THP-1 macrophages.

The results demonstrated that differences on DM, molecular weight and presence of side chains are important structural features to different cell stimulation and consequent different immunomodulatory effect of tested pectins.

Keywords: Solanaceae. *Solanum betaceum*. *Solanum lycopersicum*. *Capsicum annuum*. Homogalacturonans. Arabinogalactans. Linear arabinan. Arabinoxylan. Rheology. Immunomulatory effect. THP-1 macrophages.

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

ARTIGO II

RHEOLOGICAL BEHAVIOR OF HIGH METHOXYL PECTIN FROM THE PULP OF TAMARILLO FRUIT (*Solanum betaceum*)

ARTIGO I1I

ARABINOXYLAN FROM MUCILAGE OF TOMATOES (*Solanum lycopersicum* L.): STRUCTURE AND ANTINOCICEPTIVE EFFECT IN MICE MODELS

ARTIGO IV

NEW FINDINGS ON GREEN SWEET PEPPER (*Capsicum annuum*) PECTINS: RHAMNOGALACTURONAN AND TYPE I AND II ARABINOGALACTANS

ARTIGO V

THE INFLUENCE OF SWEET PEPPER PECTIN STRUCTURAL CHARACTERISITCS ON CYTOKINE SECRETION BY THP-1 MACROPHAGES

LISTA DE TABELAS

ARTIGO II

RHEOLOGICAL BEHAVIOR OF HIGH METHOXYL PECTIN FROM THE PULP OF TAMARILLO FRUIT (*Solanum betaceum*)

ARTIGO III

ARABINOXYLAN FROM MUCILAGE OF TOMATOES (*Solanum lycopersicum* L.): STRUCTURE AND ANTINOCICEPTIVE EFFECT IN MICE MODELS

ARTIGO IV

NEW FINDINGS ON GREEN SWEET PEPPER (*Capsicum annuum*) PECTINS: RHAMNOGALACTURONAN AND TYPE I AND II ARABINOGALACTANS

LISTA DE ABREVIATURAS, SIGLAS SÍMBOLOS

Solventes e reagentes

Ac_2O	-	Anidrido acético
$BaCO_3$	-	Carbonato de bário
CDTA		Ácido ciclohexileno 1,2 dinitrilotetra acético
EtOH	-	Etanol
H_2SO_4	-	Ácido sulfúrico
HCl	-	Ácido clorídrico
HOAc	-	Ácido acético
KOH	-	Hidróxido de potássio
LPS	-	Lipolissacarídeo
Me_2SO	-	Dimetilsulfóxido
Me_2SO-d_6,	-	Dimetilsulfóxido deuterado
MeOH	-	Metanol
MTT	-	Brometo de (3-metil-[4-5-dimetiltiazol-2-il]-2,5 difeniltetrazólio)
$NaBD_4$	-	Borohidreto de sódio deuterado
$NaBH_4$	-	Borohidreto de sódio
NaN_3	-	Azida de sódio
$NaNO_2$	-	Nitrito de sódio
NaOH	-	Hidróxido de sódio
PBS	-	Solução salina tamponada
PMA	-	12-miristato 13-acetato de forbol
RPMI	-	Meio Roswell Park Memorial Institute
TFA	-	*Trifluoroacetic acid* (Ácido trifluoacético)

Frações obtidas dos frutos do tamarillo (*Solanum betaceum*)

STW-A	-	Fração STW após tratamento com α-amilase

Frações obtidas da mucilage do tomate (*Solanum lycopersicum*)

PTOK	-	Fração precipitada após congelamento e degelo de TOK
STOK	-	Fração sobrenadante após congelamento e degelo de TOK
TOK	-	Extrato polissacarídico obtido após extração alcalina
TOW	-	Extrato polissacarídico obtido após extração aquosa a quente

Frações obtidas dos frutos do pimentão (*Capsicum annuum*)

ANW	-	Extrato polissacarídico obtido após extração aquosa a quente
ANWP	-	Fração precipitada após congelamento e degelo de ANW
ANWS	-	Fração sobrenadante após congelamento e degelo de ANW
ANWS-50E	-	Fração eluída após ultrafiltração em membrana de 50 kDa de ANWS-SF
ANWS-50R	-	Fração retida após ultrafiltração em membrana de 50 kDa de ANWS-SF

ANWS-M	-	Fração ANWS modificada após hidrólise com HCl 0,1M
ANWS-PF	-	Fração precipitada de Fehling obtida a partir de ANWS
ANWS-SF	-	Fração sobrenadante de Fehling obtida a partir de ANWS

Termos associados à estrutura de polissacarídeos

f	-	Furanosídica
p	-	Piranosídica
AG	-	Arabinogalactana
AG-I	-	Arabinogalactana tipo I
AG-II	-	Arabinogalactana tipo II
Ara	-	Arabinose
AXG	-	Arabinoxiloglucana
DA	-	Grau de acetilação
DE / DM	-	Grau de metil-esterificação
Fuc	-	Fucose
Gal	-	Galactose
GalA	-	Ácido galacturônico
Glc	-	Glucose
GlcA	-	Ácido glucurônico
HG	-	Homogalacturonana
HM	-	Pectinas com grau de esterificação superior a 50%
kDa	-	Kilodaltons
LM	-	Pectinas com grau de metil esterificação menor que 50%
Man	-	Manose
M_w	-	Massa molecular
RG-I	-	Ramnogalacturonana tipo I
RG-II	-	Ramnogalacturonana tipo II
Rha	-	Ramnose
UA	-	Ácido Urônico
Xyl	-	Xilose

Métodos analíticos

^{13}C-NMR	-	Ressonância magnética nuclear de carbono treze
^{1}H-NMR	-	Ressonância magnética nuclear de hidrogênio
DEPT	-	*Distortionless Enhancement by Polarization Transfer*
ELISA	-	*Enzyme-linked immunosorbent assay*
GC-MS	-	Cromatografia gasosa acoplada à espectrometria de massa
HPSEC	-	*High pressure size exclusion chromatography* (Cromatografia de exclusão estérica de alta performance)
HSQC	-	*Heteronuclear Single Quantum Coherence* (coerência heteronuclear simples quântica)
MALLS	-	Detector de espalhamento de luz laser em multiângulos
MHz	-	Megahertz
NMR	-	*Nuclear magnetic resonance* (Ressonância magnética nuclear)
ppm	-	Partes por milhão

Termos associados a análise reológica

G″	-	Módulo de perda ou viscoso
G´	-	Módulo de armazenamento ou elástico
K	-	índice de consistência
n	-	Índice de comportamento de fluxo
Pa	-	Pascal
pH	-	Potencial hidrogênico
w/w (m/m)	-	Relação entre massa e massa
δ	-	Deslocamento químico expresso em ppm
η	-	Viscosidade
η*	-	Viscosidade complexa
η_a	-	Viscosidade aparente
τ	-	Tensão de cisalhamento
τ_o	-	Tensão inicial
γ	-	Deformação
$\dot{\gamma}$	-	Taxa de cisalhamento

Termos associados a atividade biológica

ID_{50}		Dose capaz de reduzir a resposta biológica em 50%
i.p.	-	Intraperitoneal
IFN-γ	-	Interferon gama
IL-10	-	Interleucina 10
IL-1β	-	Interleucina 1 beta
NF-κB	-	*Nuclear factor-kappa B* (fator nuclear kappa B)
THP-1	-	Célula monocítica humana isolada de paciente com leucemia aguda
TNF-α	-	*Tumor necrosis factor* – α (Fator de necrose tumoral alfa)

Análises Estatísticas

ANOVA	-	Análise de variância

SUMÁRIO

1. INTRODUÇÃO

Os carboidratos estão presentes na maioria dos alimentos e são essenciais para a manutenção da vida. Dentre os polissacarídeos, o amido é o principal carboidrato fornecendor de energia para o organismo. Porém, os polissacarídeos consumidos na alimentação humana são diversos quanto a função e a composição. Os polissacárideos não amiláceos intrínsecos da parede celular das plantas, apesar de resistentes à digestão e absorção pelo intestino delgado, são biologicamente importantes, sendo os constituintes principais das fibras dietéticas (ENGLYST *et al.*, 2007).

Na indústria alimentícia os polissacarídeos são utilizados como um ingrediente tecnologicamente funcional seguro e estável, que visa melhorar a palatabilidade, textura, entre outras propriedades dos produtos alimentícios (STEPHEN, 1995). Dependendo das suas características físico-químicas, os polissacarídeos presentes naturalmente nos alimentos ou incorporados em formulações alimentícias podem apresentar diversas funcionalidades biológicas, com destaque às propriedades benéficas sob os processos gastrointestinais, por exemplo, na absorção e motilidade; e sistêmicos, entre eles, reduzindo o risco de desenvolvimento de doenças crônicas como: doença arterial não coronariana, hipertensão arterial, diabetes melito e melhora do sistema imunológico. Efeitos sistêmicos atribuídos principalmente como resultado indireto da ação regulatória dos ácidos graxos de cadeia curta produzidos pela microbiota. Porém, apesar de não absorvidos, estudos têm demonstrado que os polissacarídeos podem também interagir diretamente com células do sistema imune quando ingeridos oralmente (WISMAR *et al.*, 2010; MCDOLE *et al.*, 2012; COURTS, 2013; SUH *et al.*, 2013) e assim apresentar efeitos imunomodulatórios dependentes das suas estruturas químicas.

A família Solanaceae é conhecida por apresentar representantes de grande importância econômica, apresentando diversas espécies com elevado grau de representatividade na alimentação humana, como por exemplo a batata (*Solanum tuberosum*), tomate (*S. lycopersicum*), pimentas em geral, o pimentão (*Capsicum annuum*) e muitos outros frutos. Dentre os frutos exóticos encontrados nessa família, recentemente foram caracterizados os polissacarídeos da polpa do tamarillo (*S.betaceum*). Da polpa desta fruta foi obtida uma fração péctica com grande rendimento e também foram isoladas uma galactoarabinoglucuronoxilana e uma arabinogalactana do tipo I, com significante efeito antinociceptivo através de

mecanismos anti-inflamatórios (DO NASCIMENTO *et al.*, 2013; DO NASCIMENTO *et al.*, 2015).

Tendo em vista a importância da família Solanaceae e dos polissacarídeos na alimentação humana, este estudo visa caracterizar a estrutura química fina dos polissacarídeos presentes em representantes dessa família, assim como avaliar o comportamento reológico, componente importante para a indústria, e testar diferentes moléculas quanto à atividade antinociceptiva e imunomoduladora em modelos *in vivo* e *in vitro,* respectivamente.

REVISÃO BIBLIOGRÁFICA

2.1. ESTRUTURA QUÍMICA DE POLISSACARÍDEO DE PLANTAS

Nutricionalmente os polissacarídeos de plantas podem ser divididos em (a) disponíveis ou (b) resistentes à digestão e absorção pelo intestino delgado com completa ou partial fermentação no intestino grosso pela microbiota, constituindo a principal classe de polímeros englobada dentro das definições de fibras dietéticas (ENGLYST *et al.*, 2007).

Nas plantas os polissacarídeos podem ter papel de reserva energética (tais como, o amido e as frutanas) e estrutural, constituindo a parede celular vegetal. A parede celular é altamente organizada (FIGURA 1), sendo os polissacarídeos estruturais divididos em pectinas, hemiceluloses e celulose (CARPITA e GIBEAUT, 1993).

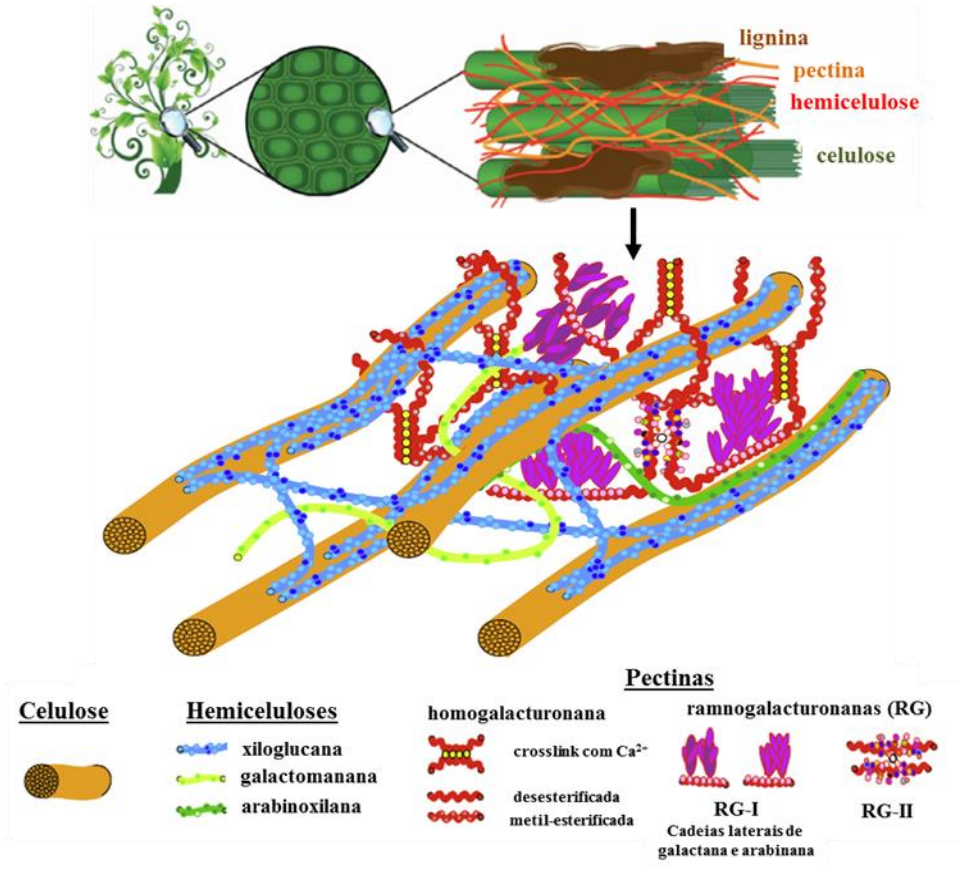

FIGURA 1 - REPRESENTAÇÃO ESQUEMÁTICA DA ESTRUTURA DA PAREDE CELULAR PRIMÁRIA VEGETAL DE DICOTILEDÔNEAS. Nota: A celulose é organizada em microfibrilas, enquanto as hemiceluloses e pectinas reticulam as microfibrilas de celulose. Fonte: Adaptado de TURUMTAY (2015) e LOQUÉ *et al.* (2015)

A composição da parede celular primária das dicotiledôneas, de fundamental importância no processo de expansão celular é, tipicamente, 25-40% celulose, 15-25% hemicelulose, 15-40% material péctico e 5-10% proteínas e proporções muito pequenas de compostos fenólicos. Enquanto a parede celular secundária, que se encontra entre a primária e a membrana plasmática da célula, e confere rigidez aos tecidos vegetais, é muito mais espessa que a parede primária e consiste de 40-45% de celulose, 15-35% de hemicelulose, 15-30% de lignina e traços de pectina (DEY *et al.*, 1997).

A estrutura química dos polissacarídeos pécticos e hemicelulósicos estudados neste trabalho será abordada a seguir.

2.1.1. Pectinas

O termo "pectina" surgiu em referência à palavra grega *pektikos*, que significa "congelar, solidificar ou coalhar" (NUSSINOVITCH, 1997), engloba diferentes tipos de polissacárideos que apresentam uma heterogeneidade significativa na sua estrutura química, que pode variar por espécies de frutos, estágios de desenvolvimento e método de extração. Presentes na lamela média e parede celular primária das plantas, as pectinas são consideradas os polissacarídeos mais complexos estrutural e funcionalmente, possuem função no crescimento, na morfologia, no desenvolvimento, na defesa da planta e também servem como polímeros geleificantes e estabilizantes em diversos produtos alimentícios e da indústria cosmética, além de possuírem efeitos positivos na saúde humana e diversos usos biomédicos (CAFFALL e MOHNEN, 2009).

As pectinas tem como o monossacarídeo mais abundante o ácido galacturônico (Gal*p*A), seguido por galactose (Gal) e arabinose (Ara). Porém podem ser compostas por até 17 diferentes monossacarídeos. Seus monossacarídeos não são distribuídos aleatoriamente nas estruturas das pectinas, mas sim concentrados em diferentes elementos estruturais (ou domínios). Pelo menos sete tipos de pectinas são descritos na literatura, nomeadamente, homogalacturonanas (HG), ramnogalacturonanas tipo I (RG-I), ramnogalacturonanas tipo II (RG-II), xilogalacturonanas (XGA), apiogalacturonanas (APGA), galactogalacturonanas (GGA), e arabinogalacturonanas (ArGA), sendo as três primeiras as principais pectinas reportadas (MOHNEN, 2008; BURTON *et al.*, 2010; YAPO, 2011). Tradicionalmente, a complexa interação entre os diferentes tipos de pectinas é suportada pelo modelo esquemático

apresentado na FIGURA 2A, no qual as cadeias de HGs (regiões lineares ou *smooth regions*) são interrompidas por porções ramificadas de RG (regiões cabeludas ou *hairy regions*), porém outros modelos têm sido propostos na literatura, cuja representação mostra HGs constituindo as cadeias laterais das RG-I, FIGURA 2B (VINCKEN *et al.*, 2003; YAPO, 2011).

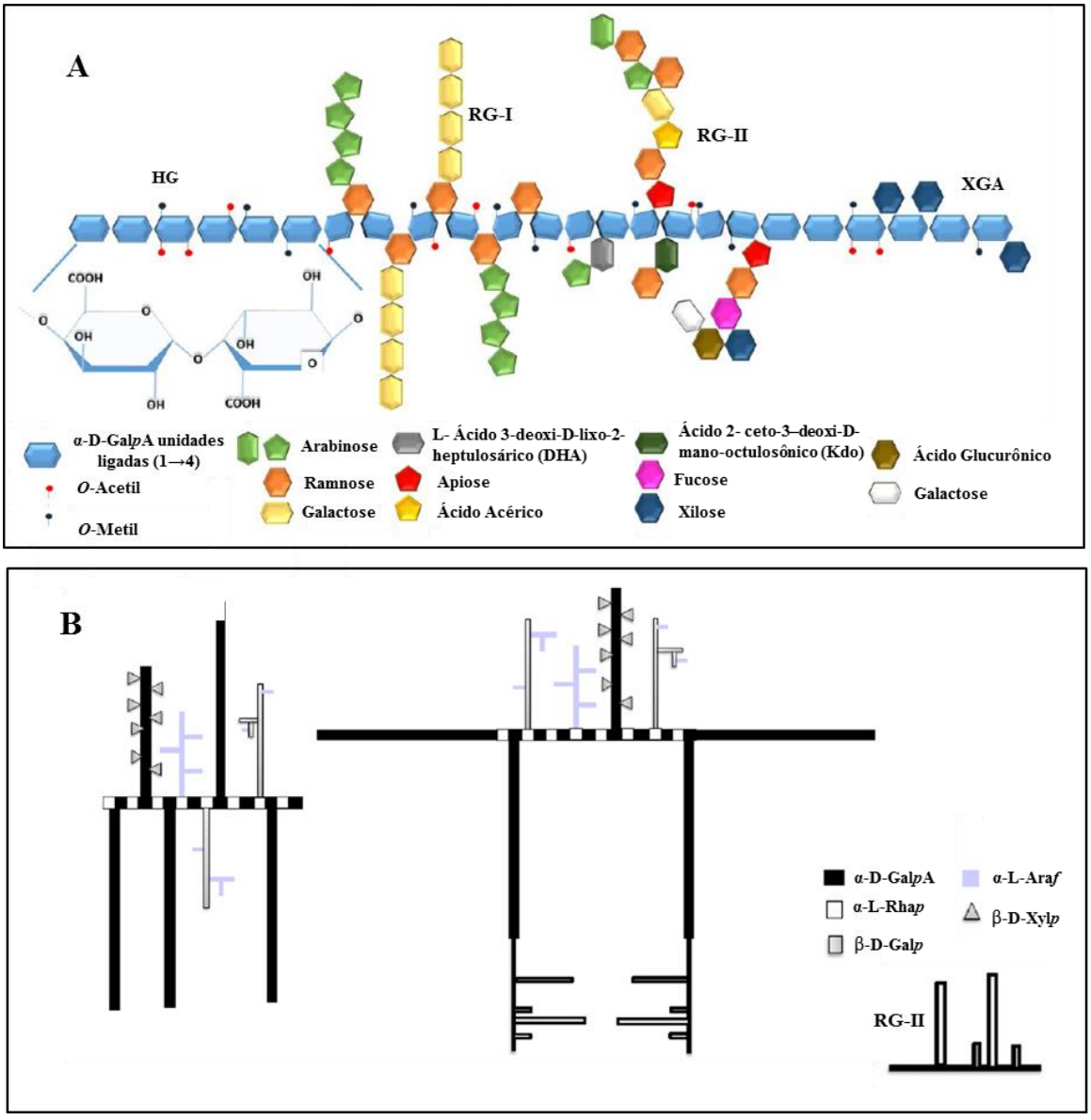

FIGURA 2- MODELOS ESQUEMÁTICOS DO COMPLEXO DE PECTINAS. (A) MODELO TRADICIONAL COM CADEIA PRINCIPAL FORMADA POR HG INTERCALADA COM RG-I RAMIFICADA E RG-II. (B) MODELOS NÃO TRADICIONAIS MOSTRANDO QUE HG PODEM FAZER PARTE DAS CADEIAS LATERAIS DE RG-I. Fonte: (A) Adaptado de ALBUQUERQUE (2016) e (B) Adaptado de YAPO (2011).

As homogalacturonanas (HG) são o principal elemento estrutural de pectinas da parede celular representando de 55-70% da quantidade total de pectinas (YAPO, 2011). Essas pectinas são homopolímeros formados por unidades de α-D-Gal*p*A ligadas (1→4) que podem ser parcialmente metil-esterificadas no C6 e acetil-esterificadas nas posições *O*-2 e/ou *O*-3, dependendo da origem vegetal (YAPO, 2011). O grau de metil-esterificação e de acetilação das HGs tem profundo impacto nas propriedades funcionais das pectinas, sendo responsáveis pelo comportamento de geleificação das pectinas (WILLATS *et al.*, 2006). Geralmente pectinas nativas apresentam alto grau de esterificação e baixo grau de acetilação (VORAGEN *et al.*, 1995).

A proporção de grupos carboxílicos metil-esterificados nas pectinas é expressa como grau de esterificação (DE). As pectinas de alta metoxilação (HM – *High methoxyl pectin*) apresentam 50% ou mais dos seus grupos carboxílicos esterificados, enquanto que as de baixa metoxilação (LM – *Low methoxyl pectin*) possuem menos de 50% destes grupos esterificados (ROLIN, 1993). As unidades de GalA não metiladas no C6 são negativamente carregados e podem interagir ionicamente com Ca^{2+} se mais de 10 unidades de GalA não metil-esterificados estiverem coordenados, dando origem às zonas de junção, muitas vezes referido como modelo da caixa de ovo (*egg - box*) (FIGURA 3A), mecanismo pelo qual pectinas LM são capazes de formar um gel estável com outras moléculas de pectinas (RIDLEY *et al.*, 2001; COSGROVE, 2005; WILLATS *et al.*, 2006). Enquanto pectinas HM formam géis pela formação de ligações cruzadas entre unidades de Gal*p*A através de ligações de hidrogênio e por forças hidrofóbicas entre os grupos metoxil presentes (FIGURE 3B) (VORAGEN *et al.*, 1995; O´NEILL *et al.*, 2001; WILLATS *et al.*, 2006).

As ramnogalacturonanas tipo I (RG-I) são heteropolissacarídeos geralmente altamente ramificados. As RG-I são formadas por uma cadeia principal constituída por unidades alternantes de α-D-Gal*p*A ligadas (1→4) e unidades de α-L-Rha*p* ligadas (1→2), parcialmente substituída nas posições em *O*-4 e/ou *O*-3 (menos presente) nas unidades de L-Rha*p* por uma única unidade neutra glicosil ou por cadeias laterais poliméricas de diferentes tipos, tais como (1→5)-α-L-arabinanas, (1→4)-β-D-galactanas, arabinogalactanas tipo I (AG-I), arabinogalactanas tipo II (AG-II) e, possivelmente, galactoarabinanas (MOHNEN, 2008; YAPO, 2011).

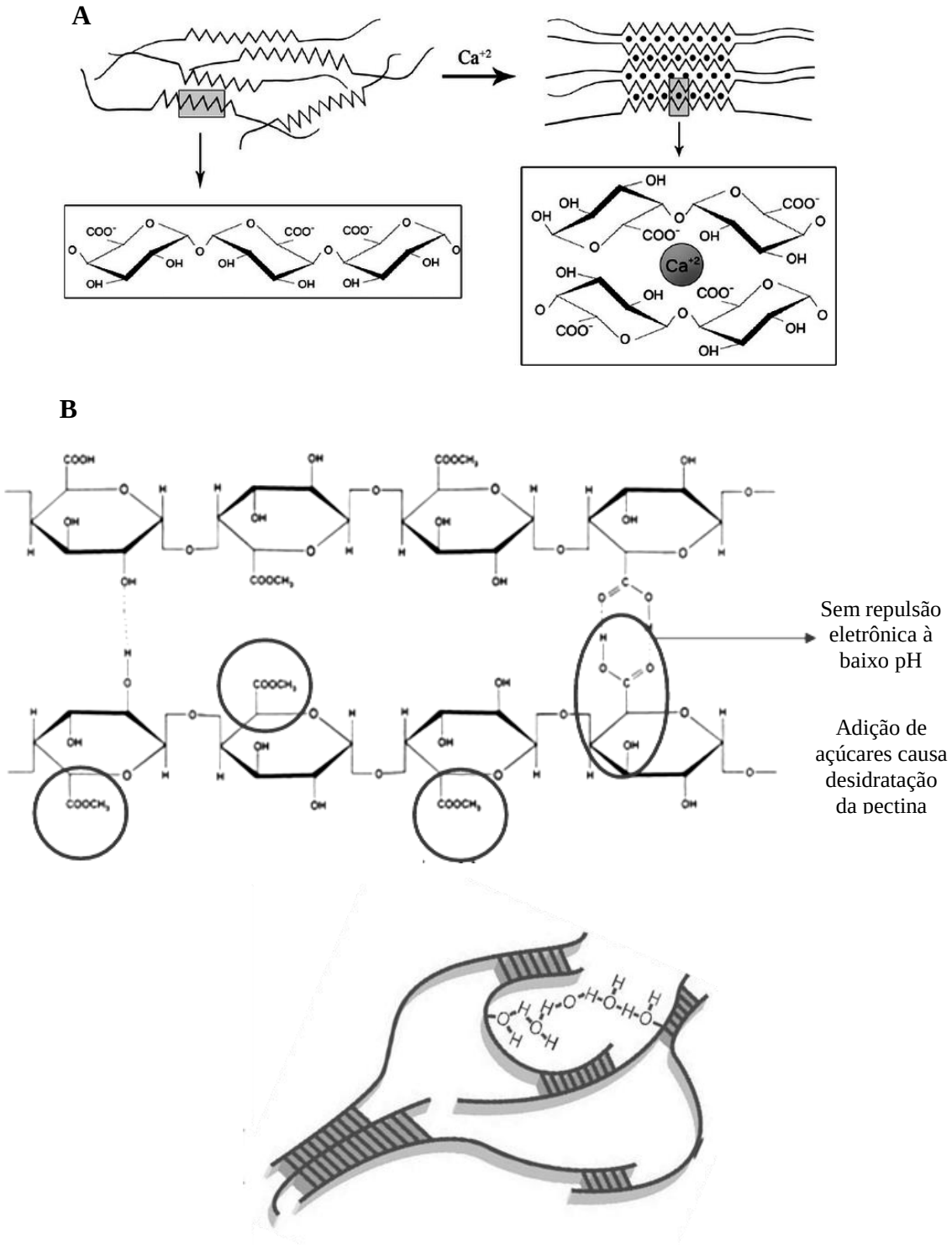

FIGURA 3 - MECANISMO DE GELEIFICAÇÃO DE PECTINAS. OS GRUPOS CARBOXILICOS DAS PECTINAS LM FORMAM COMPLEXOS COM CÁTIONS POLIVALENTES (A), ENQUANTO PECTINAS HM FORMAM GEL POR DESIDRATAÇÃO E MUDANÇA DE CARGA (B). Fonte: Adaptado de KIRTIL et al (2014) e BOOMINATHAN (2012)

2.1.2. Arabinogalactanas

Arabinogalactanas (AG) ocorrem em dois tipos estruturais diferentes, de acordo com o tipo de ligação das unidades de galactose que compõem a cadeia principal. As arabinogalactanas (AG) compõem, em parte, as cadeias laterais das RG-I, sendo assim considerados polissacarídeos pécticos. No entanto, AG também podem existir independentemente na parede celular. As arabinogalactanas de plantas superiores foram classificadas por Aspinall (1973) em dois grandes grupos ao constatar diferenças nas ligações químicas envolvidas na formação da cadeia principal destas macromoléculas. As que apresentavam cadeia principal de (1→4) β-D-galactanas foram denominadas arabinogalactanas tipo I (AG-I), enquanto as que tinham cadeia principal formada por (1→3) e (1→6) β-D-galactanas foram classificadas como arabinogalactanas tipo II (AG-II).

As AG-I, também chamadas de pécticas, apresentam 20 – 40% de unidades de L- Ara*f* ligadas α-(1→5) conectadas na posição *O*-3 das galactoses, constituindo cadeias laterais curtas (CARPITA e GIBEAUT, 1993). Já as AG-II são mais complexas e altamente ramificadas, com um amplo grupo de cadeias curtas de (1→3) e (1→6) β-D-galactanas conectadas umas às outras por pontos de ramificação em *O*-3 e *O*-6 e apresentam a maior parte das posições *O*-3 e *O*-6 restantes ocupadas por unidades de L- Ara*f* em ligações (1→3) e/ou (1→5), que são geralmente terminadas por unidades de L- Ara*f* e em algum grau por L-Ara*p* (CARPITA e GIBEAUT, 1993; VORAGEN et al., 1995).

Arabinogalactanas tipo II são aparentemente mais distribuídas nos tecidos das plantas em comparação com as AG-I (CLARKE *et al.*, 1979). Apesar de apresentarem uma estrutura geral semelhante, a estrutura fina das AG-II varia muito de espécie para espécie (CARPITA e GIBEAUT, 1993; ALBERSHEIM *et al.*, 1996). A composição monossacarídica geralmente apresenta variações, podendo o conteúdo de arabinose atingir até 80%. Também podem ser observados monossacarídeos ácidos, como o ácido glucurônico, ácido 4-*O*-metil-glucurônico e ácido galacturônico (STEPHEN, 1983). As AG-II podem estar envolvidas estruturalmente com pectinas ou não. Elas podem também estar ligadas a proteínas, constituindo uma classe de proteoglicanas denominadas arabinogalactanas-proteínas (AGP) (PONDER e RICHARDS, 1997; CAFFALL e MOHNEN, 2009).

2.1.3. Hemiceluloses

As hemiceluloses são polissacarídeos que ocorrem em íntima associação com a celulose, especialmente em tecidos lignificados (ASPINALL, 1973). A função principal das hemiceluloses em tecidos vegetais é unir microfibrilas de celulose, fortalecendo assim a parede celular (COSGROVE, 2005). Por estarem comumente associadas à celulose, hemiceluloses são normalmente extraídas da parede celular vegetal por meio de extrações alcalinas (ASPINALL, 1969).

O termo hemiceluloses é atribuído a um grupo heterogêneo de polissacarídeos formados por uma cadeia principal de glucose, manose ou xilose ligados β-(1→4) em configuração equatorial no C-1 e C-4 (Figura 4A) (SCHELLER e ULVSKOV, 2010), sendo a classificação de cada polímero dada de acordo com o monossacarídeo majoritário presente na cadeia principal. São hemiceluloses: xilanas (homoxilanas, heteroxilanas neutras e ácidas), glucomananas, mananas, galactomananas, xiloglucanas, calose (β-glucanas com ligações 1→3), (1→3,1→4) β-glucanas (ASPINALL, 1980). As estruturas das xiloglucanas e heteroxilanas ácidas (glucuronoarabinoxilana e glucuronoxilana) estão representadas na Figura 4B.

Em frutos de dicotiledôneas, classe onde está inserida a família Solanaceae objeto desse estudo, as xiloglucanas constituem as hemiceluloses mais abundantes (YAPO e KOFFI, 2008; OCHOA-VILLARREAL *et al.*, 2012). Porém as xiloglucanas encontradas nas solanáceas diferem do encontrado na maioria das dicotiledôneas. Em dicotiledôneas, as xiloglucanas normalmente possuem ramificações em *O*-6 em 75% das unidades de β-Glc*p* (1→4) ligadas, e 50% das unidades de α-D-Xyl*p* substituídas subsequentemente por unidades de β-D-Gal*p* (1→2) ligadas ou pelo dissacarídeo α-L-Fuc*p*-(1→2)-β-D-Gal*p*. Enquanto que em solanáceas somente 40% das unidades de β-Glc*p* (1→4) possuem ligação glicosil *O*-6 na cadeia lateral com uma unidade de α-D-Xyl*p*, e 60% das unidades de α-D-Xyl*p* são substituídas subsequentemente por ligações em *O*-2 com unidades de α-L-Ara*f*, sendo chamadas, portanto, de arabinoxiloglucanas (AXGs). AXGs de algumas solanáceas, como no bagaço da batata, resíduo da extração do amido. também contém substituintes de β-D-Gal*p* em *O*-2 de algumas de unidades de α-D-Xyl*p* (YORK *et al.*, 1996).

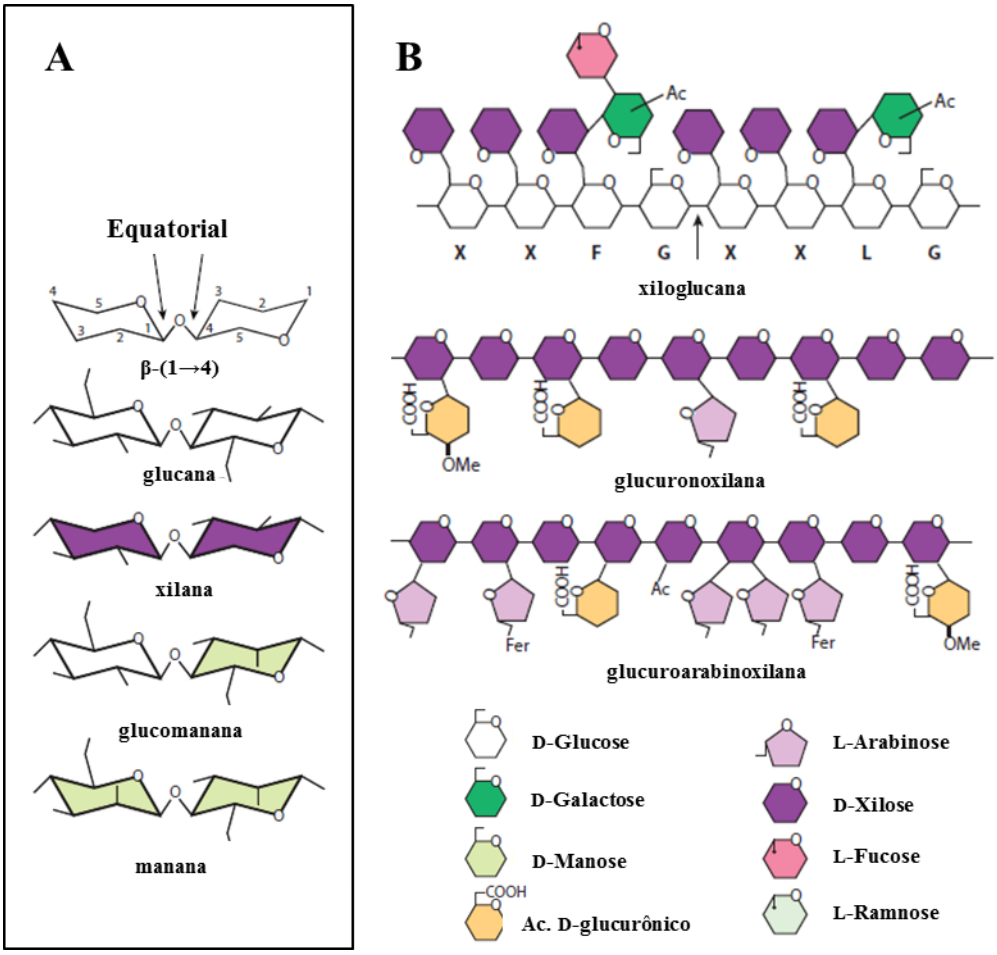

FIGURE 4 - POLISSACARÍDEOS HEMICELULÓSICOS DE PLANTAS. (A) HEMICELULOSES SÃO CARACTERIZADAS PELA LIGAÇÃO β-(1→4) EM CONFIGURAÇÃO EQUATORIAL NO C-1 E C-4. (B) REPRESENTAÇÃO ESTRUTURAL DE XILOGLUCANA E HETEROXILANAS. Fonte: Adaptado de SCHELLER e ULVSKOV (2010)

As xilanas apresentam grande variedade estrutural, mas normalmente apresentam uma cadeia principal formada por unidades de β-D-Xyl*p* (1→4) ligadas, substituídas em *O*-2 e/ou *O*-3 por diferentes grupos ou cadeias laterais. Essas cadeias laterais consistem principalmente de ácido α-D-glucurônico, ácido 4-*O*-metil-α-glucurônico e ocasionalmente unidades de alguns açúcares neutros (α-L-Ara*f*, α-D-Xyl*p* ou α-D-Gal*p*). Entre os grupos laterais mais comuns estão os grupos acetil, os ácidos fenólicos, e os ácidos ferúlico e coumárico, dependendo da espécie em estudo (EBRINGEROVÁ e HROMÁDKOVÁ, 1999). A presença

de grupos acetil em xilanas pode não ser determinada quando elas são extraídas com álcali, o qual remove estes grupamentos (VIERHUIS *et al.*, 2001).

Heteroxilanas neutras ou arabinoxilanas foram extensivamente estudadas na parede celular de endosperma de cereais (monocotiledôneas). Elas contém somente unidades de L-Ara*f*, geralmente (1→3) ligadas, ou (1→3) e (1→2) ligadas nas unidades de xiloses em arabinoxilanas mais substituídas ou com maiores extensões de cadeias laterais de L-Ara*f* ligadas à outros substituintes (MCNEIL *et al.*, 1984). O grau de substituição da cadeia principal por unidades de Ara*f* determina o nível de solubilidade da xilana e a habilidade de se ligar à celulose (IZYDORCZYK *et al.*, 1998; HAN, 2000). Em dicotiledôneas, as xilanas são principalmente ácidas, onde a cadeia principal encontra-se substituída por unidades de ácido 4-*O*-metil-D-glucurônico ou ácido D-glucurônico (HABIBI *et al.*, 2005; CIPRIANI *et al.*, 2008; ANGONE *et al.*, 2009; SENGKHAMPARN *et al.*, 2009; DO NASCIMENTO *et al.*, 2013).

2.2. REOLOGIA

A reologia pode ser definida como a ciência da deformação e do escoamento da matéria, ou seja, é o estudo da maneira segundo a qual os materiais respondem à aplicação de uma determinada tensão ou deformação (TONELI *et al.*, 2005).

Propriedades como a cremosidade, suculência, suavidade e dureza sentidas quando consumimos os alimentos são de natureza reológica. A estabilidade e aparência dos alimentos frequentemente dependem das características reológicas de seus componentes. Na fabricação e processamento de produtos alimentícios as propriedades reológicas também servem como um meio de controlar e monitorar um processo industrial (STEFFE, 1996; SHARMA *et al.*, 2000; MCCLEMENTS, 2008).

Os polissacarídeos são largamente utilizados como matéria-prima no desenvolvimento de produtos alimentícios, principalmente pelas propriedades reológicas de suas soluções, sendo utilizados para melhorar a viscosidade, textura, características sensoriais e tempo de prateleira dos produtos alimentares, oferecendo resistência aos processos físicos indesejavéis como a cristalização, sedimentação e desagregação mecânica (OSMAN, 1975; HOUSKA *et al.*, 1998; MUDGIL e BARAK, 2013). As propriedades reológicas de sistemas

polissacarídicos, no contexto da tecnologia de alimentos, fornecem informações que podem influenciar na seleção do equipamento de fabricação e o modo de processamento. Dentre os polissacarídeos, as pectinas, gomas e mucilagens, constituem um grupo de substâncias com expressivo interesse pela indústria de alimentos, devido a sua capacidade de atuar como agentes geleificantes, estabilizantes e espessantes.

2.2.1. Propriedade dos fluídos

O comportamento reológico estende-se através de dois extremos idealizados, desde a mecânica de fluidos newtonianos (fluidos perfeitos) até a elasticidade de Hooke (sólidos perfeitos) (BIRD *et al.*, 1982). Os sólidos ideais são materiais de forma definida, que se deformam elasticamente por uma tensão externa, no qual a energia requerida para a deformação é completamente recuperada quando as tensões são removidas. Por outro lado, os fluidos ideais, tais com líquidos e gases, deformam-se irreversivelmente e escoam, sendo a energia requerida para a deformação dissipada dentro do fluido na forma de calor não podendo ser recuperada simplesmente pela remoção das tensões (STANLEY *et al.*, 1996). Na reologia de sólidos, a propriedade de maior interesse é a elasticidade ao passo que em fluídos a propriedade mais importante é a viscosidade. Entre os dois comportamentos extremos existem os materiais que se comportam ora como líquidos ora como sólidos, dependendo da tensão, da frequência ou da temperatura a que são expostos. Estes materiais são denominados de viscoelásticos (BARNES *et al.*, 1989).

Quando fluidos ideais ou newtonianos são deformados, a tensão de cisalhamento (τ – "*shear stress*") gerada é diretamente proporcional à taxa de cisalhamento ou de deformação ($\dot{\gamma}$ - "*shear rate*"). A resistência que o fluido oferece ao escoamento é caracterizada como a sua viscosidade newtoniana (η) (BARNES *et al.*, 1989), seguindo desse modo a lei de Newton expressa pela equação abaixo:

$$\tau = \eta . \dot{\gamma}$$

Onde: τ = tensão de cisalhamento (Pa)

η = viscosidade (Pa.s)

$\dot{\gamma}$ = taxa de cisalhamento (s^{-1})

Desta maneira, a viscosidade pode ser matematicamente expressa como sendo a tensão dividida pela taxa de cisalhamento.

Os fluidos newtonianos, por definição, possuem uma relação estritamente linear entre tensão e a taxa de cisalhamento, com a linha passando pela origem. Porém, na maioria dos fluidos a viscosidade varia dependendo da taxa de cisalhamento e, desta maneira, eles são denominados de não newtonianos. Dentre eles, destacam-se os fluidos pseudoplásticos, dilatantes e plásticos (SCHRAMM, 2006). A FIGURA 5 apresenta o comportamento de fluxo para fluidos. O coeficiente de viscosidade para esses fluidos não newtonianos é chamado de viscosidade aparente (η_a).

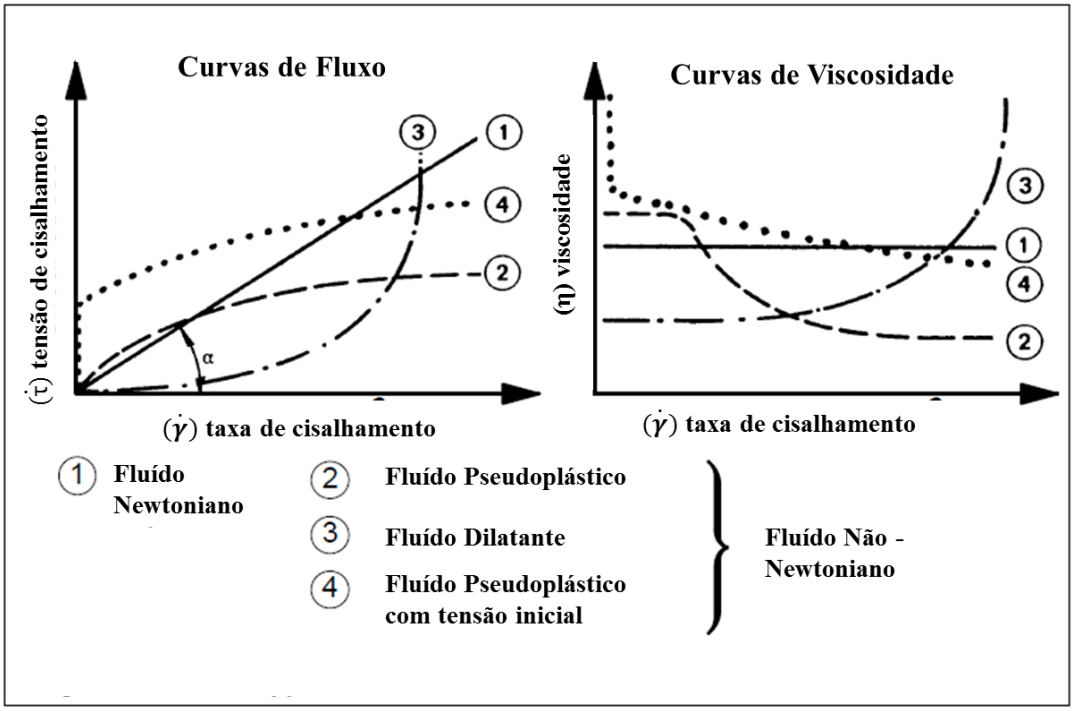

FIGURE 5 - COMPORTAMENTOS DE FLUXO DE FLUÍDOS NEWTONIANOS E NÃO-NEWTONIANOS. Fonte: Adaptado de SCHRAMM (2006)

A maioria das soluções concentradas de polissacarídeos apresenta comportamento pseudoplástico. Os fluídos pseudoplásticos são caracterizados pela diminuição na viscosidade aparente com o aumento da taxa de cisalhamento. Em repouso, as partículas ou cadeias poliméricas das soluções apresentam um estado desordenado, resultando em uma ordem interna irregular, caracterizadas por uma considerável resistência interna ao fluxo, ou seja,

uma alta viscosidade. Com o aumento das taxas de cisalhamento a viscosidade diminui devido à tendência das moléculas de se orientar na direção da força aplicada. Este fato se deve ao rompimento de agregados e entrelaçados moleculares, que por sua vez permitirão um escoamento com maior facilidade (SCHRAMM, 2006).

A descrição do comportamento reológico não-Newtoniano dos materiais é feita através de modelos empíricos que relacionam como a tensão de cisalhamento varia com a taxa de cisalhamento (HOLDSWORTH, 1971). Dentre os modelos matemáticos existentes, Ostwald-de Waelle, também conhecido como a Lei da potência ou *Power law*, descreve a maioria dos fluidos em alimentos, devido ao comportamento pseudoplástico destes (STEFFE, 1996). Porém, muitos alimentos apresentam uma relação não linear entre a tensão e taxa de cisalhamento e necessitam de uma tensão inicial (τ_o) para começar a se movimentar, sendo matematicamente descritos pelo modelo de Herschel–Bulkley (STEFFE, 1996; RAO, 2007). As equações de Ostwald-de Waelle e Herschel–Bulkley estão apresentadas, abaixo:

$$\tau = K\dot{\gamma}^n \quad \text{(Ostwald-de Waelle)}$$

$$\tau = \tau_0 + K\dot{\gamma}^n \quad \text{(Herschel–Bulkley)}$$

Onde: τ = tensão de cisalhamento (Pa)

τ_o= tensão de cisalhamento (Pa)

K = índice de consistência ($Pa.s^m$)

$\dot{\gamma}$ = taxa de cisalhamento (s^{-1})

n = índice de comportamento de fluxo (adimensional)

As curvas de fluxo em regime estacionário são os principais meios para caracterizar o comportamento reológico de fluidos de importância na indústria de alimentos. A viscosidade é a propriedade de todos os fluidos independentemente se eles exibem ou não comportamento elástico. No entanto, muitos fenômenos não podem ser descritos simplesmente em função da viscosidade (característica de fluídos) e o seu comportamento elástico (característica de sólidos) deve ser levado em consideração. Para avaliar soluções viscoelásticas é necessário verificar o comportamento reológico em sistemas dinâmicos onde a amostra é submetida a tensões oscilatórias (SCHRAMM, 2006).

O caráter de sólido e de líquido é verificado através dos valores de G′ (módulo de armazenamento ou elástico) e G″ (módulo de perda ou viscoso), respectivamente. Estes módulos são expressos em Pascal (Pa), onde G′ significa que a energia de tensão é temporariamente armazenada durante o teste, porém pode ser recuperada posteriormente, e G″ indica que a energia usada para iniciar o fluxo é irreversivelmente perdida, sendo transformada em calor de cisalhamento (SCHRAMM, 2006). Se uma substância for puramente viscosa seu módulo de armazenamento (G′) será igual a zero, e se ela for puramente elástica seu módulo de perda (G″) será igual a zero. Porém, a maioria das substâncias apresenta tanto G′ quanto G″ (SCHRAMM, 2006). Quando o módulo elástico (G′) é superior ao módulo viscoso (G″), e ambos são independentes da frequência, o material tem caráter de sólido ou gel forte. Um gel fraco (ou solução concentrada) apresenta valores de G″ maiores do que G′ em baixas frequências e, em altas frequências, ocorre uma inversão dos módulos, com G′ maior do que G″. Em soluções diluídas, o espectro dinâmico apresenta valores de G″ significativamente maiores do que G′ em toda a faixa de frequências, especialmente em baixas frequências (FIGURA 6).

É possível ainda analisar as interações dos polímeros das soluções utilizando-se a regra de Cox e Merz. Para isto comparam-se graficamente os dados obtidos nos experimentos de regime estacionário com os dados de regime oscilatórios, sobrepondo a viscosidade aparente (η_a) com a viscosidade complexa (η^*). Esta última definida através da razão entre o módulo de cisalhamento complexo (G*) e a frequência. Caso a viscosidade complexa em função da frequência for consideravelmente maior que a viscosidade aparente em relação à taxa de cisalhamento, pode-se afirmar que existe uma estrutura de gel na solução (COX e MERZ, 1958).

O comportamento térmico das soluções também pode ser um importante fator do ponto de vista tecnológico. A variação de temperatura pode influenciar nas interações hidrofóbicas e ligações de hidrogênio das moléculas tendo efeito direto nas propriedades viscoelásticas (DA SILVA e GONÇALVES, 1994). Outros fatores que podem influenciar no comportamento reológico são a concentração do polímero, massa molar, falta de flexibilidade, taxa de cisalhamento adotada, bem como características do solvente (RINAUDO, 2005).

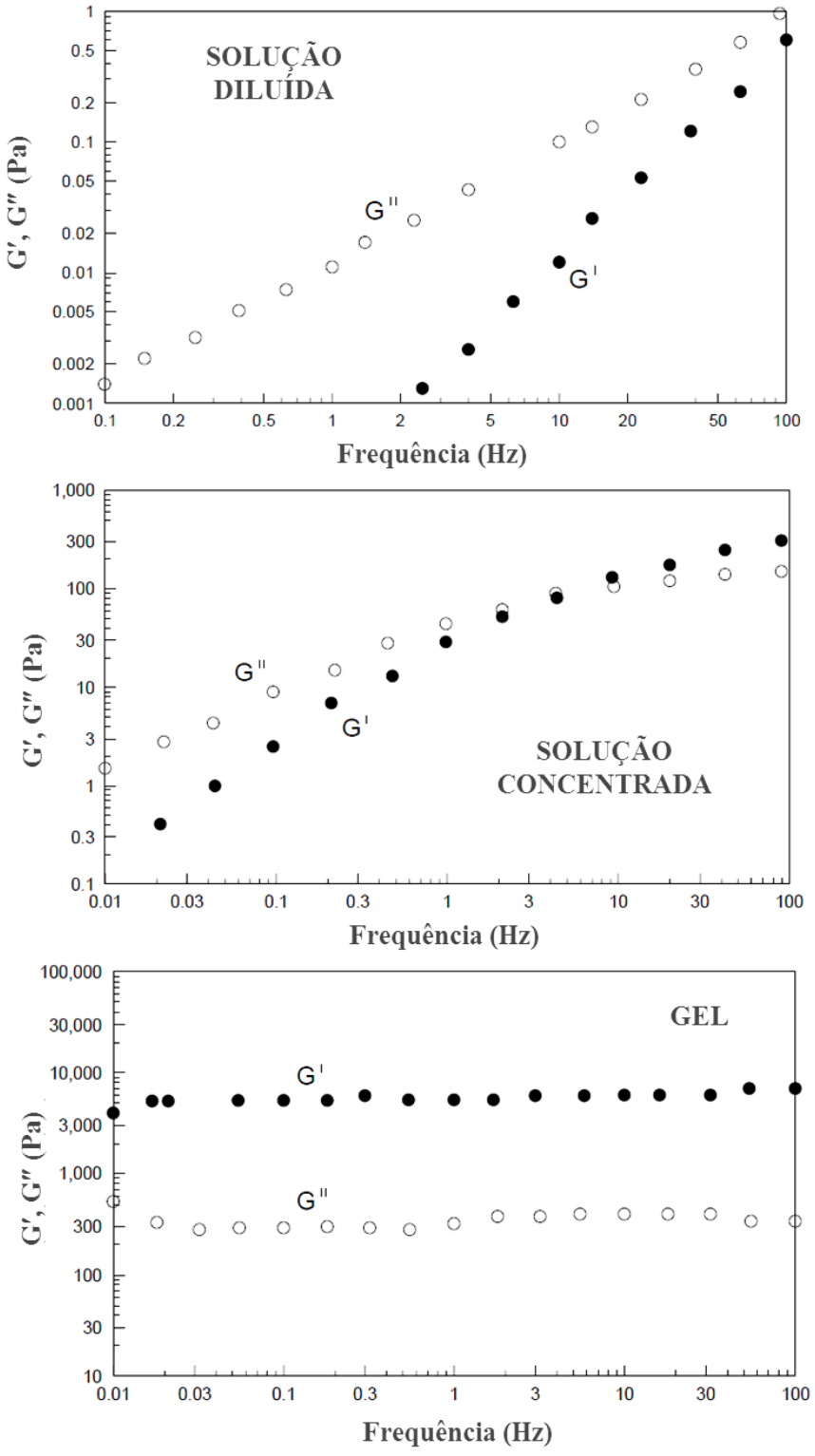

FIGURE 6 - REPRESENTAÇÃO DO COMPORTAMENTO REOLÓGICO EM SISTEMAS OSCILÁTÓRIOS DE SOLUÇÕES DILUÍDAS, SOLUÇÕES CONCENTRADAS E DE GÉIS. Fonte: Adaptado de STEFFE (1996)

2.3. ATIVIDADE IMUNOMODULADORA DE POLISSACARÍDEOS DA DIETA

O consumo regular de fibras dietéticas, através de uma dieta rica em frutas, verduras e legumes é relacionado com a prevenção de processos inflamatórios vinculados a fisiopatologia de diferentes doenças crônicas, como síndrome metabólica, obesidade, diabetes mellitus do tipo 2, câncer e doenças cardiovasculares (AJANI *et al.*, 2004).

Embora os mecanismos não estejam completamente esclarecidos, classicamente os benefícios para saúde observados para as fibras dietéticas, são considerados consequência dos seus efeitos indiretos no lúmem intestinal, como o aumento da viscosidade do bolo fecal associada com a diminuição dos níveis de LDL e glucose sanguínea, além da ação prebiótica aumentando a produção de ácidos graxos de cadeia curta pela microbiota intestinal (ANDOH *et al.*, 1999; MEIJER *et al.*, 2010; BROWNLEE, 2011; LANDBERG, 2012; MUDGIL e BARAK, 2013). Contudo, alguns trabalhos têm demonstrado que oligossacarídeos e polissacarídeos podem ser capazes de atravessar a barreira epitelial intestinal e interagir diretamente com os componentes dos sistemas inflamatório e imune presentes na lâmina própria intestinal e da circulação sistêmica (WISMAR *et al.*, 2010; MCDOLE *et al.*, 2012; COURTS, 2013; SUH *et al.*, 2013). Por exemplo, HONG e colaboradores (2004) observaram que glucanas (1→3)- ligadas, isoladas da cevada e de leveduras marcadas com flurescência, foram fagocitadas por macrófagos e transportadas para o baço, linfonodos e medula óssea. Além disso, os autores demonstraram que o mecanismo da atividade citotóxica para células tumorais atribuída para essas estruturas, após ingestão oral em camundongos, ocorre via o reconhecimento dos fragmentos dessas glucanas, por receptores presentes em granulócitos marginalizados da medula óssea.

Os potenciais mecanismos pelos quais os polissacarídeos podem exercer seus efeitos imunomodulatórios após ingestão oral foram sumarizados por VOS *et al.* (2007) (FIGURA 7). Além dos efeitos dependentes da microbiota, as células do epitélio intestinal poderiam estabelecer interações bidirecionais com os polissacarídeos e as células do sistema imune subjacente (macrófagos e células dendríticas), e participar na resposta inflamatória da mucosa de duas formas: modulando seletivamente a permeabilidade da monocamada epitelial e, assim, a exposição de células imunes a antígenos; e sintetizar e liberar, elas mesmas, mediadores inflamatórios. Os macrófagos e células dendríticas poderiam projetar extensões dendríticas trans-epiteliais (TEDs) em direção ao lúmen com o objetivo de detectar e potencialmente absorver material luminal. Como também, as células M (*microfold cells*),

localizadas nas placas de Peyer, poderiam captar os polissacarídeos do lúmen, afetando a resposta imunológica de células como os macrófagos intestinais.

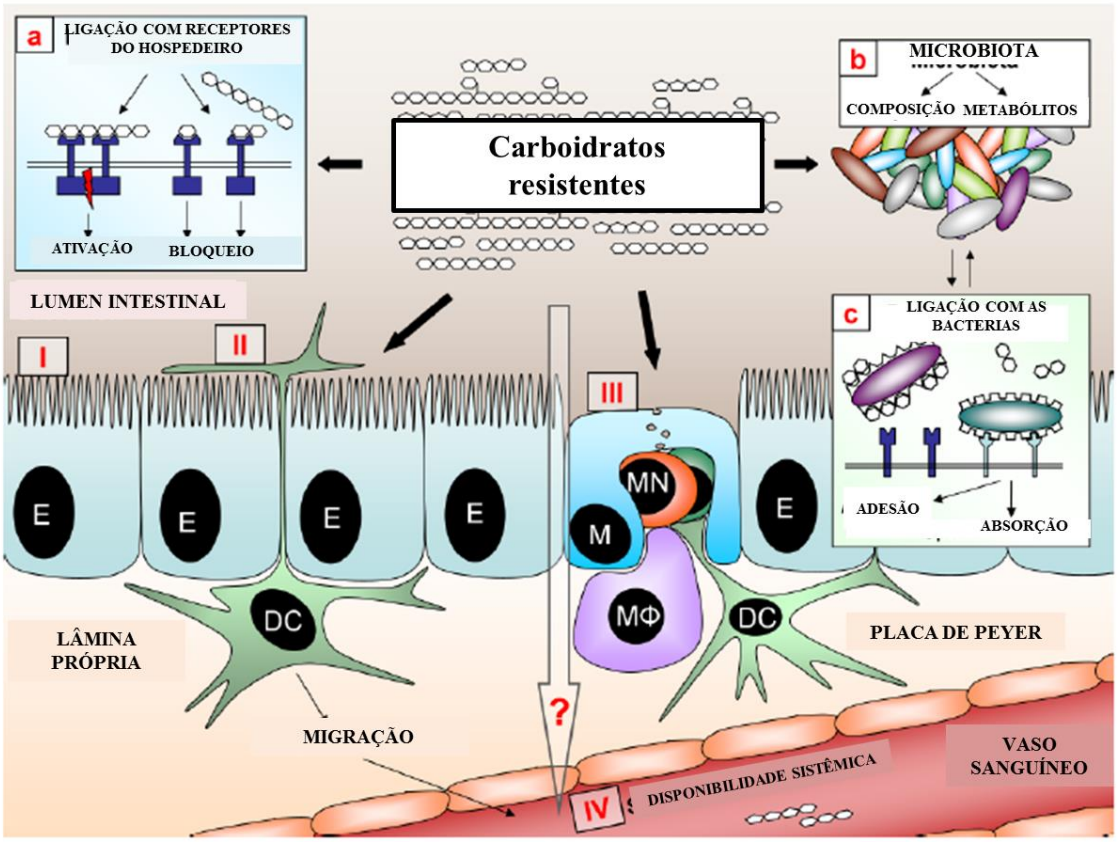

FIGURE 7 - ESQUEMA COM OS POTENCIAIS MECANISMOS DA IMUNOMODULAÇÃO POR POLISSACARÍDEOS RESISTENTES QUANDO INGERIDOS VIA ORAL. Nota: (A) os polissacarídeos se ligariam diretamente com receptores do hospedeiro ativando ou bloqueando a resposta imunológica; (B) os polissacarídeos modulariam a composição da microbiota e a produção de metabólitos bacterianos; (C) os polissacarídeos se ligariam às bactérias alterando a adesão dessas à mucosa e absorção; (I) As células epiteliais intestinais (E) poderiam interagir com os polissacarídeos através de receptores e com a adesão bacteriana modulada pelos polissacarídeos; (II) células dendríticas (DC) presentes na lâmina própria poderiam absorver amostra dos polissacarídeos do lúmen intestinal e migrar para linfonodos mesentéricos, resultando em efeitos imunomodulatório localmente na mucosa e sistemicamente; (III) os polissacarídeos podem modular absorção de bactérias e antígenos solúveis pelas células M ou serem absorvidos por essas células, afetando outras células subjacentes, incluindo células mononucleares (MN), macrófagos (MΦ), e DC; (IV) tem sido descrito que oligossacarídeos da dieta são secretados pela urina em crianças e adultos. Fonte: Adaptado de VOS (2007)

Dentre os estudos que avaliaram especificamente a atividade anti-inflamatória de polissacarídeos destacam-se estudos utilizando modelos murinos. Tais como, o estudo com

extratos aquosos de polissacarídeos obtidos de sementes de *Semen sterculiae lychnophorae* que demonstrou diminuição do edema de orelha e da formação de tecido granulomatoso (WU *et al.*, 2007); e de extratos de lótus (*Nelumbo nucifera*) que aumentou a produção de IL-10 e IL-6 pelos esplenócitos (LIAO *et al.*, 2011). Além desses, Popov e colaboradores (2011) avaliaram a administração oral da pectina do pimentão, extraída com solução que simula o meio gástrico e verificaram diminuição na produção de TNF-α e aumento da produção de IL-10 após estimulação por LPS no sangue dos animais, e ainda o aumento do tempo de sobrevida após aplicação de dose letal de LPS. Também observaram que pectinas cítricas com grau de metil-esterificação <50%, após administração oral, preveniram a progressão de colite induzida por ácido acético, diminuíram a adesão peritoneal de granulócitos, produção de EROs e de TNF-α, além de aumentar a produção de IL-10, enquanto que a pectina com alto grau de metil-esterificação (>50%) apenas preveniu a progressão da colite (POPOV *et al.*, 2013). Outras estruturas, como glucomananas obtidas da orquídea *Cyrtopodium cardiochilum* inibiram o aumento da permeabilidade vascular induzida por ácido acético e aumentaram a atividade fagocítica (BARRETO e PARENTE, 2006), e arabinoxilanas de farinha de trigo também foram capazes de aumentar a atividade fagocítica de macrófagos e a produção de IL-2, assim como retardar a reação de hipersensibilidade após injeção intradermal de eritrócitos de carneiro (CAO *et al.*, 2011).

Já entre os estudos que avaliaram atividade em modelos *in vitro*, podem-se citar os que mostraram que β-glucanas de diferentes fontes apresentaram atividade antiinflamatória através da modulação na produção de citocinas por células do sistema imune, tais como interleucinas TNF-α, IFN-γ e NO, como também de prostaglandina E2 (SCHEPETKIN e QUINN, 2006; SATITMANWIWAT *et al.*, 2012; DU *et al.*, 2015). O efeito dos polissacarídeos na inflamação também foi avaliado em outros tipos celulares, sendo que em células Caco-2, os extratos polissacarídicos da planta medicinal *Radix astragali* diminuíram a produção de TNF-α, IL-1β e IL-8 e mantiveram a integridade da monocamada após inflamação induzida por LPS (WANG *et al.*, 2013). Em modelos de co-cultura de células, Tanoue e colaboradores (2008) avaliaram o efeito de fucoidana de algas marrons na inflamação, utilizando as células Caco-2 e RAW 264.7 (células epitelial de adenocarcinoma coloretal humano e macrófagos murinos, respectivamente). Eles observaram que o polissacarídeo sulfatado foi capaz de suprimir a expressão do gene de IL-8 nos macrófagos estimulados por LPS. O tratamento com polissacarídeos de diferentes fontes alimentares levou a uma diminuição na produção de citocinas pro-inflamatórias por células Caco-2 e

células dendríticas, sendo os efeitos imunomodulatórios observados influenciados pelas diferenças estruturais dos polissacarídeos e dependentes da ligação com receptores do tipo *Toll-like* (BERMUDEZ-BRITO *et al.*, 2015). DAGUET e colaboradores (2016) avaliaram os produtos da fermentação de uma arabinogalactana obtida do exsudato da árvore de Acácia, produzidos pela microbiota de pacientes com doença inflamatória intestinal, sobre as linhagens de THP-1 (linhagem de monócitos humanos) e Caco-2 em modelo de co-cultura e verificaram um significante aumento da resistência elétrica transepitelial (TEER) nas células Caco-2, diminuição da atividade de NF-κB e aumento da produção de IL-10.

Embora haja estudos avaliando a atividade antiinflamatória de polissacarídeos na literatura, muitos utilizam extratos brutos ou falham nas metodologias de fracionamento e de caracterização química dos polissacarídeos. A presença de contaminantes como polifenóis e proteínas, ou mesmo misturas de diferentes polissacarídeos podem interferir na atividade biológica individual dos polissacarídeos, dificultando desse modo, a relação entre estrutura e função (FERREIRA *et al.*, 2015). Parâmetros estruturais, como composição monossacarídica, tipo e configuração da ligação glicosídica, tamanho dos pontos de ramificação e peso molecular podem resultar em diferença na atividade antiinflamatória e imunomodulatória (FERREIRA *et al.*, 2015; YANG e ZHANG, 2009).

2.4. FAMÍLIA SOLANACEAE

A família Solanaceae A. L. Jussieu é extremamente diversificada e ampla. Com aproximadamente 96 gêneros e 2.300 espécies, possui distribuição cosmopolita, sendo a América do Sul um dos principais centros de diversidade e endemismo (HUNZIKER, 1979; D'ARCY, 1991; BOHS, 1994; HUNZIKER, 2001). Seus membros diferem grandemente em termos habitat (dos desertos a florestas tropicais úmidas) e da morfologia das flores e frutos, variando de árvores a pequenas ervas (KNAPP, 2002). Dentre os gêneros, o mais representativo é o *Solanum L*. que contem quase metade do total de espécies. No Brasil, a família Solanaceae é bem representada, ocorrendo 34 gêneros e 466 espécies, sendo 222 destas exclusivas do país (STEHMANN *et al.*, 2015).

A família Solanaceae é conhecida principalmente pela sua grande importância econômica, apresentando diversas espécies com elevado grau de representatividade na alimentação humana, como exemplos a batata (*Solanum tuberosum*), o tomate (*S.*

lycopersicum), a berinjela (*S. melongena*), o jiló (*S. gilo*), pimentas em geral, o pimentão (*Capsicum annuum*) e muitos outros frutos. Outros membros, como a mortal beladona (*Atropa beladona*), o agridoce (*S. dulcamara*), o meimendro (*Hyoscyamus niger*) e a trombeta (*Datura stramonium*), são mais conhecidos por seus venenos, enquanto *Petunia* spp., *Brugmansia* spp. (trompetes selva) e *Nicotiana* spp. são cultivadas como plantas ornamentais (HAWKES, 1999; SOUZA e LORENZI, 2005).

Na literatura, destacam-se os estudos relacionados à presença de substâncias químicas da classe dos alcalóides, principalmente alcalóides tropânicos e ale-esteróides que ocorrem em muitos gêneros, devido ao seu grande interesse na indústria farmacêutica. Muitas espécies são largamente utilizadas para fins medicinais, alucinógenos e para estudos de biotecnologia e engenharia genética (BARENDSE e VAN DER WEERDEN, 1999; HAWKES, 1999). Porém poucos trabalhos envolvem o estudo de polissacarídeos presentes nas plantas pertencentes a essa família.

Em relação às espécies mais utilizadas na alimentação humana foi encontrado no tubérculo da batata após extração do amido (*Solanum tuberosum*) uma arabinogalactana do tipo I, com a presença incomum de unidades de β-D-Gal*p* (1→3)-ligadas (HINZ *et al.*, 2005) e uma xiloglucana com substituintes de β-D-Gal*p* em *O*-2 em algumas de unidades de α-D-Xyl*p* (RING e SELVENDRAN, 1981), além de um grande conteúdo de ramnogalacturonanas (RG) altamente ramificadas principalmente por cadeias laterais de galactanas (RING e SELVENDRAN, 1978; ISHII, 1981; JARVIS *et al.*, 1981; RYDEN e SELVENDRAN, 1990). O desenvolvimento de técnicas de extração destas RG, após a extração do amido pela indústria, tem recebido atenção devido a sua potencial utilização como fibra dietética (LÆRKE *et al.*, 2007; THOMASSEN *et al.*, 2011; KHODAEI e KARBOUNE, 2013).

Já no fruto do tomate (*Solanum lycopersicum*) predominam pectinas com grande linearidade geral, baixo grau de ramificação de ramnogalacturonanas do tipo I (RG-I) e alto grau de metoxilação (HOUBEN *et al.*, 2011). Ramificação esta, formada por unidades de β-Gal*p* (1→4)-ligadas e por unidades de α-Ara*f* (1→5)-ligadas, e grau de metil-esterificação de aproximadamente 88%, maior que o encontrado em pectinas cítricas que é de 81% (SHARMA *et al.*, 1997). Seymour e colaboradores (1990) compararam os polissacarideos pécticos do tomate solúveis em CDTA e em carbonato de sódio, e encontraram que ambas as frações possuiam uma cadeia principal de ramnogalacturonana, porém menos ramificada no primeiro caso, e as cadeias laterais eram compostas principalmente por unidades de β-Gal*p*

(1→4)-ligadas e por unidades de α-Ara*f* (1→5)-ligadas. Nesse mesmo estudo a principal hemicelulose encontrada foi uma xiloglucomanana, além de pequenas quantidades de xilana complexada com pectina. E duas frações de galactoglucomanana e glucuronoxilana complexadas também foram purificadas por Prakash e colaboradores (2012). Enquanto em células de cultura de tomate, foi caracterizada uma arabinoxiloglucana (AXG) constituída principalmente por unidades de β-D-Glc*p* (1→4)-ligadas e substituídas em *O*-6 por várias cadeias laterais, as quais são compostas por α-D-Xyl*p* e α-L-Ara*f*-(1→2)-α-D-Xyl*p*, contendo também nas cadeias laterais o dissacarídeo β-D-Gal*p*-(1→2)-α-D-Xyl*p* e o trissacarídeo β-D-Ara*f*-(1→3)-α-L-Ara*f*-(1→2)-α-D-Xyl*p* (YORK *et al.*, 1996).

Mondal e colaboradores (2009) demonstraram a presença de um heteropolissacarídeo contendo unidades de GalA metil esterificadas em frutos de pimentão (*Capsicum annuum*). Mimetizando o meio gástrico, Popov e colaboradores (2011) isolaram um polissacarídeo denominado como capsicuman, contendo uma homogalacturonana parcialmente substituída com grupos metil e acetil, o qual apresentou, após administração oral em camundongos e estimulação intraperitoneal com lipopolissacarídeo (LPS), diminuição dos níveis de TNF-α e aumento da produção de IL-10 no sangue. Potente atividade anticomplemento foi encontrada para o extrato bruto solúvel em água obtido de frutos de pimentão, e também partir de suas frações purificadas por cromatografia de troca iônica (PAIK *et al.*, 2003).

Entre as frutas exóticas, o goji (*Lycium barbarum*) apresenta ramnogalacturonanas com diferentes graus de ramificação como principais polissacarídeos, mas também foi encontrada uma glucana, uma 4-*O*-metil-glucuronoxilana e há indicação da presença de uma xiloglucana, uma manana e arabinogalactanas-proteínas (REDGWELL *et al.*, 2011). Atividades protetoras contra o estresse oxidativo e antitumoral foram verificadas em diversos estudos realizados com extratos polissacarídeos do fruto de goji (ZHANG *et al.*, 2005; LI e ZHOU, 2007; MING *et al.*, 2009; ZHANG *et al.*, 2011; JIN *et al.*, 2013). No fruto do fisális (*Physalis alkekengi)*, uma fração polissacarídica foi purificada contendo Ara:Gal:Glc:GalA, na proporção de 2.6:3.6:2:1, apresentou atividade hipoglicemiante (TONG *et al.*, 2008) e imunomoduladora (LI *et al.*, 2011), já no seu cálice quatro frações polissacarídicas foram estudadas (uma constituída principalmente por arabinose, outra por xilose, glucose e frutose, a terceira por ramnose, manose e frutose e a última por ramnose, frutose, glucose e galactose), todas demonstrando atividade antioxidante (GE *et al.*, 2009).

Os polissacarídeos da polpa do fruto do tamarillo (*Solanum betaceum*) foram estudados recentemente por DO NASCIMENTO (2013). Foram caracterizadas uma fração péctica com alto rendimento (6% em relação ao peso seco) contendo uma homogalacturonana altamente metil-esterificada, com inserções de RG-I contendo cadeias laterais constituídas principalmente por AG-I, uma arabinana linear (1→5)-ligada, além de uma AG-I , contendo uma cadeia principal formada por unidades de β-D-Gal*p* (1→4) ligadas, parcialmente substituídas em *O*-3 por unidades de α-L-Ara*f* e uma galactoarabinoglucuronoxilana, ambas apresentando efeito antinociceptivo através de mecanismos anti-inflamatórios (DO NASCIMENTO *et al.*, 2013; DO NASCIMENTO *et al.*, 2015).

3. OBJETIVOS

3.1. OBJETIVO GERAL

Caracterizar estruturalmente polissacarídeos presentes nos frutos e na mucilagem de tomate (*Solanum lycopersicum*) e do fruto do pimentão (*Capsicum annuum*), além de dar continuidade ao estudo dos polissacarídeos dos frutos do tamarillo (*S. betaceum*), caracterizando os polissacarideos presentes na mucilagem que envolve as sementes. Realizar a análise do comportamento reológico e avaliar os possíveis efeitos antinociceptivo e imunomoduladores em modelos *in vivo* e *in vitro*, respectivamente, dos polissacarídeos.

FIGURE 8. MATERIAIS USADOS NO ESTUDO. (A) TAMARILLO (*Solanum betaceum*). (B) TOMATE (*S. lycopersicum)* E (C) PIMENTÃO (*Capsicum annuum). Fonte: O AUTOR*

3.2. OBJETIVOS ESPECÍFICOS

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

- Extrair os polissacarídeos da mucilagem que envolvem as sementes dos frutos dos tamarillo e do tomate, e do fruto do pimentão por extrações aquosas e alcalinas;
- Purificar os polissacarídeos extraídos;
- Caracterizar estruturalmente os polissacarídeos obtidos;
- Comparar estruturalmente os polissacarídeos obtidos nos extratos aquosos e alcalinos dos frutos do tamarillo (polpa e mucilagem);

- Realizar análises reológicas com a fração péctica de maior rendimento da polpa do tamarillo;
- Investigar o potencial antinociceptivo dos polissacarídeos purificados em modelos *in vivo* de uma arabinoxilana isolada da mucilagem do tomate;
- Investigar o efeito imunomodulador de polissacarídeos pécticos isolados no fruto do pimentão em modelo *in vitro* utilizando células THP-1 diferenciadas em macrófagos.

ARTIGO I

A comparative study of mucilage and pulp polysaccharides from tamarillo fruit (*Solanum betaceum* Cav.)

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A comparative study of mucilage and pulp polysaccharides from tamarillo fruit (*Solanum betaceum* Cav.)

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ABSTRACT

A comparative study of mucilage (locular tissue) and pulp polysaccharides from ripe tamarillo fruits (*Solanum betaceum* Cav.) was carried out. After aqueous and alkaline extractions and various purification steps (freeze-thaw and a-amylase - EC 3.2.1.1 treatments, Fehling precipitation and ultrafiltration through 50 kDa cut-off membrane), the obtained fractions from mucilage were analyzed by sugar composition, HPSEC, and NMR spectroscopy analyses. The results showed that the mucilage of tamarillo contains a highly methoxylated homogalacturonans mixed with type I arabinogalactans, a linear (1→5)-linked α-L-arabinan, and a linear (1→4)-β-D-xylan. A comparison with polysaccharides extracted from the pulp revealed that differences were observed in the yield and in the ratio of extracted polysaccharides. Moreover, structural differences between pulp and mucilage polysaccharides were also observed, such as in the length of side chains of the pectins, and in the degree of branching of the xylans.

Keywords*:* Arabinan; arabinogalactan; mucilage; pectins; *Solanum betaceum*; tamarillo; xylan.

ARTIGO II

Rheological behavior of high methoxyl pectin from the pulp of tamarillo fruit (*Solanum betaceum*)

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Rheological behavior of high methoxyl pectin from the pulp of tamarillo fruit (*Solanum betaceum*)

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ABSTRACT

Rheological behavior of a high methyl-esterified pectic fraction (STW-A) from tamarillo was evaluated at different concentrations in water and with sucrose (50% w/w, pH 3). STW-A dispersions at 3, 5, and 8% (w/w) showed low apparent viscosities, shear-thinning and liquid-like behaviors. They were well fitted using the Ostwald-de Waele model and obey the Cox-Merz rule. The viscosity and the viscoelastic behavior were greatly modified by the presence of sucrose. STW-A at 1% (+ sucrose) showed shear-thinning and concentrated solution behavior. Pronounced shear-thinning and gel like behaviors were obtained with STW-A at 2% and 3% (+ sucrose). Their flow curves profiles were better fitted using the Hershel-Bulkley model and not followed the Cox and Merz rule. Temperature sweeps (5 to 80°C) showed that STW-A formed thermostable gels. Altogether, our results suggested tamarillo can be a new source of pectin with potential applications as thickeners/gelling agents depending on solvent or applied processes.

Keywords: *Solanum betaceum*, tamarillo, pectin, gel, rheology.

1. Introduction

Pectins have widely been used as high-value functional ingredient in innumerable food products for their beneficial health properties. Among the several health benefits of pectins is its effect in all gut processes (e.g. digestion, absorption, gastrointestinal motility and its control, gastrointestinal immunity and prebiotic effects), which in turn may have impact on cardiovascular/systemic health and on anti-cancer treatment (DIKEMAN *et al.*, 2006; BROWNLEE, 2011; MAXWELL *et al.*, 2012; VIEBKE *et al.*, 2014; WICKER *et al.*, 2014). Regarding their functional properties, pectins are major contributors to textural quality of fruit and vegetable products also affecting their palatability. When incorporated in foods such as jams and jellies, they reduce syneresis, increase viscosity and enhance gel strength (THAKUR *et al.*, 1997; SAHA e BHATTACHARYA, 2010). Such properties are affected by pectin concentration, solvent properties, temperature and structural features of pectins like degree of methylesterification (DM) and molecular weight (WILLATS *et al.*, 2006; YAPO, 2011).

The degree of methylesterification is a particularly important parameter since LM and HM pectins have distinct gel formation mechanisms. A pectin gel is formed when portions of homogalacturonan are cross-linked to form a three dimensional crystalline network in which water and solutes are trapped. In LM pectins, the gel is formed in the presence of divalent cations, particularly Ca^{2+}, according to the well-known 'egg-box' model, over a wide range of pHs and the content of soluble solids. Thereby, LM pectins are mainly used in low or sugar-free, and low-acid products. Otherwise, the gelling mechanism in HM pectins is governed by hydrophobic interactions and hydrogen bonding which increase the interconnection between the homogalacturonan chains contributing to form a rigid three dimensional network that traps the water within it. For this process to happen they require acidic conditions and high content of soluble solids (>50%; MAY, 1990; THAKUR *et al.*, 1997; TSOGA *et al.*, 2004; BRACCINI *et al.*, 2005) and for this reason are used in jam, jellies, acid milk products, bakery and confectionery (MAY, 1990).

Formulas for gelling formation of HM pectins generally suggest a limit of 65% sugar (sucrose) and acidity of around pH 3. Although the ideal concentration of pectin is not known, it is traditionally suggested a minimum of 1% (MAY, 1990). A high DM is also desirable since homogalacturonan regions protected by methyl groups are able to participate in hydrogen bonding and hydrophobic inter-actions between adjacent pectin molecules (CAMERON *et al.*, 2015). An optimum degree of methyl esterification for pectin gelation

under conditions of low water activity is about 70% (MORRIS *et al.*, 1980). Although most plant tissues contain pectin, the commercial production is based almost entirely on just a few sources such as citrus peel and apple pomace stemming from the juice industry (THAKUR *et al.*, 1997).

Tamarillo is a small Andine native tree also known as tomato tree belonging to the Solanaceae family. Tamarillo types are distinguished according to their fruit skin colors: solid deep-purple, orange, yellow, or red-and-yellow. In countries like Colombia, Malaysia and New Zealand it is a commercial crop for international export, differently from Brazil, where all types of tamarillo are found but only in home gardens or small crops (PANTOJA *et al.*, 2009).

It is an exotic fruit with high levels of micronutrients, and bioactive components such as anthocyanins, carotenoids, flavonoids (BOBBIO *et al.*, 1983; MERTZ *et al.*, 2009; OSORIO *et al.*, 2012; ACOSTA-QUEZADA *et al.*, 2015), type I arabinogalactan and acidic xylan polysaccharides (DO NASCIMENTO *et al.*, 2013; DO NASCIMENTO *et al.*, 2015). The tamarillo crops have attracted increasing interest in the last few years for their edible, juicy, and flavorful fruit which has a characteristic acidic taste. The fruit is consumed fresh or used in various culinary preparations, such as sauces, jellies, ice creams, juices and liqueurs (ACOSTA-QUEZADA *et al.*, 2015).

The present work seeks to evaluate the rheological behavior of the HM pectic fraction associated to type I arabinogalactan isolated from the pulp of the yellow tamarillo fruit in different concentrations in water and under appropriate conditions for gel formation in HM pectins, with sucrose content fixed at 50% and pH 3.

2. Materials and methods

2.1. Pectin sample

STW-A fraction used in this study was extracted and characterized as previously described by DO NASCIMENTO *et al.* (2015). Briefly, the pulp without seeds and mucilage was freeze-dried and defatted with chloroform–methanol (1:1). Polysaccharides were extracted from the residue with water at 100°C for 2 h (×7, 1 l each). Polysaccharides from aqueous extracts were precipitated with ethanol (3 vol.). Freeze–thaw treatment was applied to give cold-water soluble fraction (STW). In order to remove starch, STW was extensively

treated with α-amylase (from *Bacillus licheniformis*, Sigma A3403, 100 units/ml, 24 h at 37°C), yielding STW-A fraction. It was then characterized by monosaccharide analysis, degree of methylesterification (DE), homogeneity (HPSEC) and ^{13}C-RMN.

2.2. Preparation of pectin for rheological analysis

STW-A at 3, 5 and 8% (w/w) was dispersed in deionized water by magnetic stirring for 16 h at 25°C. STW-A at 1, 2 and 3% (w/w) containing sucrose fixed at 50% (w/w) and at pH 3 was also prepared, following VRIESMANN *et al.* (2010). In this procedure, weighted amounts of STW-A were dispersed in deionized water by stirring for 2 h at 25◦C. Sucrose in the adequate proportion was then added. After, they were heated at 92°C with continuous stirring for 15 min and cooled to room temperature. Their pH was then adjusted to 3 with a saturated solution of citric acid and the volumes were corrected with water to achieve the appropriate concentration. The samples were stored under refrigeration (4°C) for16 h and then analyzed.

2.3. Rheological measurements

Rheological measurements were carried out at a HAAKE MARSII rheometer, at 20◦C with a cone-plate (C60/2◦TiL) measurement system. The temperature was controlled by a circulating water bath (DC5, Haake) coupled to a Peltier temperature control device (TC81, Haake). A 1 mm measurement gap was used. Before starting the experiments, the exposed sample edge was covered with a thin layer of low viscosity mineral oil and a sample hood (POM222-1903) was used to prevent evaporation losses during measurements. Before all rheological analyses, samples were placed on the plate for 300 s, in order to allow samples temperature equilibrium. Flow curves were evaluated in the CR mode (controlled shear rate) by applying an increasing shear rate (0.01–500 s^{-1}) during 300 s. The shear stress (τ) was then measured as a function of shear rate. The data of flow curves were evaluated and fitted according to the rheological models of Ostwald–de Waele ($\tau = K\dot{\gamma}^n$) and Herschel–Bulkley ($\tau = \tau_0 + K\dot{\gamma}^n$), where τ is the shear stress (Pa), *K* is the consistency index (Pa.s^m), $\dot{\gamma}$ is the shear rate (s^{-1}), *n* is the flow behavior index (dimensionless) and τ_0 is the yield stress (Pa) (RAO, 2007).

In order to determine the stress corresponding to the linear viscoelastic region, stress sweeps measurements (0.01–100 Pa) at constant frequency (1 Hz) were performed. Fixed

stresses (τ) used at frequency sweeps were 0.05, 0.3 and 1.0 Pa for STW-A at 3, 5 and 8%, respectively; and 0.1, 0.1, and 1.0 Pa for STW-A at 1, 2, and 3% with sucrose, respectively. The mechanical spectra obtained were characterized by values of G′, G″ Pa) as a function of frequency (*f*) in the range of 0.02–10 Hz. G′ is the elastic modulus, related to the solid response of the material and G″ is the viscous modulus, related to the fluid response of the material (ZHONG e DAUBERT, 2013).

To study the influence of the temperature on the STW-A gels, the temperature sweeps were performed with STW-A at 2 and 3% with sucrose, with a heating (5–80°C) and subsequent cooling (80–5°C) at a rate of 2°C/min, at a frequency of 1 Hz and stress of 1 Pa. The software RheoWin 4 Data Manager was employed to obtain the rheological and statistical parameters. All the analyses were performed in triplicates with replicates at two different days. Graphics show the mean values and corresponding standard error of the mean (SEM). Data of frequency sweeps were compared using one-way analysis of variance (ANOVA) and Tukey's tests were applied to verify the differences between mean at the same frequency (0.1, 1, or 10 Hz). Data were considered different at as significant level of $p < 0.05$

3. Results and discussion

In previous work (DO NASCIMENTO *et al.*, 2015), some structural aspects of fraction STW-A obtained after aqueous extraction, freeze–thaw and α-amylase treatments were described. STW-A was composed of rhamnose (2.1%), arabinose (27.4%), xylose (3.5%), mannose (5.8%), galactose (23.4%), glucose (3.0%) and uronic acids (35.0%). Its HPSEC-MALLS profile suggested that it is a heterogeneous fraction and, according to its monosaccharide composition and ^{13}C-NMR analysis, STW-A was characterized as a high methylesterified (HM) homogalacturonan (with a degree of methylesterification of 95%), associated with a type 1 arabinogalactan. Since this fraction formed viscous dispersions and HM pectins have wide industrial application, this research was interested in its rheological properties.

According to flow behavior analysis (Fig. 1A), the apparent viscosity improved with increasing STW-A concentration in water. This phenomenon is typical for polysaccharide systems meaning that zero shear viscosity value becomes higher as the polymer concentration rises and their viscosity decreases as the shear rate increases (MAY, 2000; SILVA e RAO,

2006). STW-A at 3% (w/w) presented the lowest apparent viscosity (< 0.2 Pa.s) and a shear-thinning behavior despite the almost linear shear stress increase with shear rate and viscosity values showing meaningless variations with increasing shear rates (Fig. 1A and B). This behavior was similar to other HM pectin dispersions in concentration below 4%, as those from mango pulp (IAGHER *et al.*, 2002), pitaya peel (MUHAMMAD *et al.*, 2014) and citrus pectin (SOUSA *et al.*, 2015). HM pectins from apple pomace (MIN *et al.*, 2011) and cacao pod husks (VRIESMANN e PETKOWICZ, 2013) at 5% also showed this behavior. In STW-A, at 5% and 8% (w/w), the shear-thinning behavior can be observed (Fig. 1A), especially after shear rate at 10 s^{-1}, where apparent viscosities decreased. Similar viscosity to STW-A at 5% was observed by GANNASIN *et al.* (2015) for hydrocolloid fractions at 2% extracted from red tamarillo fruit (composed of LM pectic polysaccharides and arabinogalactan-protein).

The apparent viscosity was greatly enhanced by the presence of sucrose (50% w/w) in the solution at pH 3. The apparent viscosity of STW-A at 3%, in presence of sucrose, was more than 100,000-fold higher than its aqueous dispersion (Fig. 2A). STW-A at 1% with sucrose showed a shear-thinning behavior (Fig. 2A and B), similar to the behavior observed in the dispersions without sucrose. Pronounced shear-thinning behaviors were observed with STW-A at 2% and 3% (w/w) with sucrose. In such concentrations, polysaccharide aggregation probably occurs to form a three-dimensional network structure, therefore requiring more energy to break the network structure (PHILIPS e WILLIAMS, 2000; BRACCINI *et al.*, 2005).

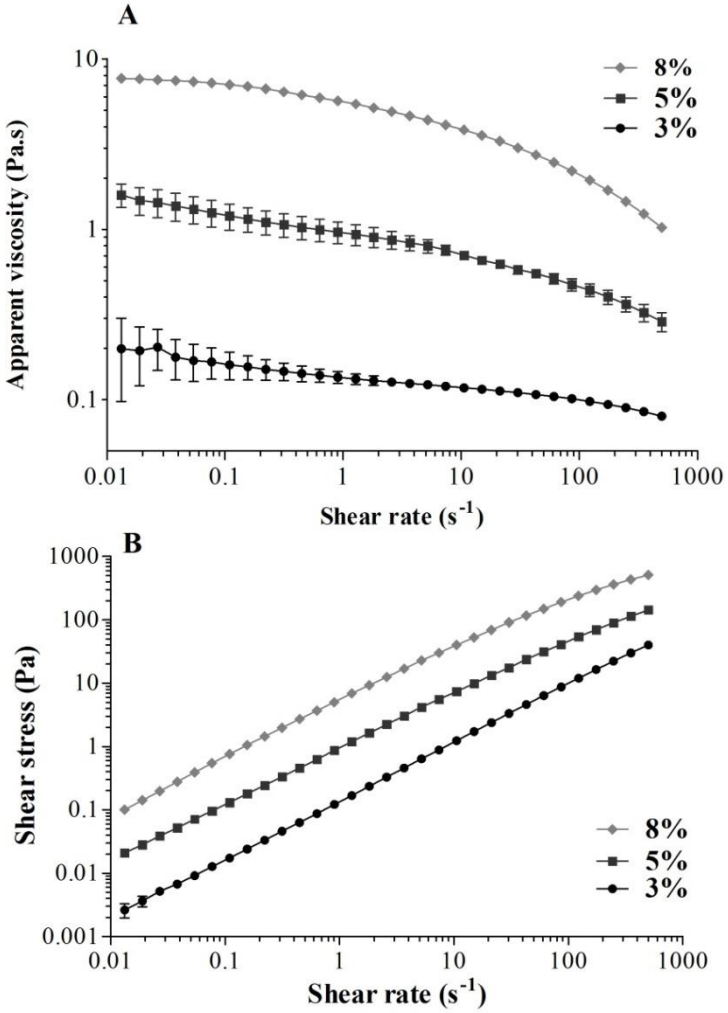

Figure 1 - Influence of shear rate on the apparent viscosity (A) and shear stress (B) of aqueous STW-A solution from tamarillo pulp at 3, 5 and 8%.

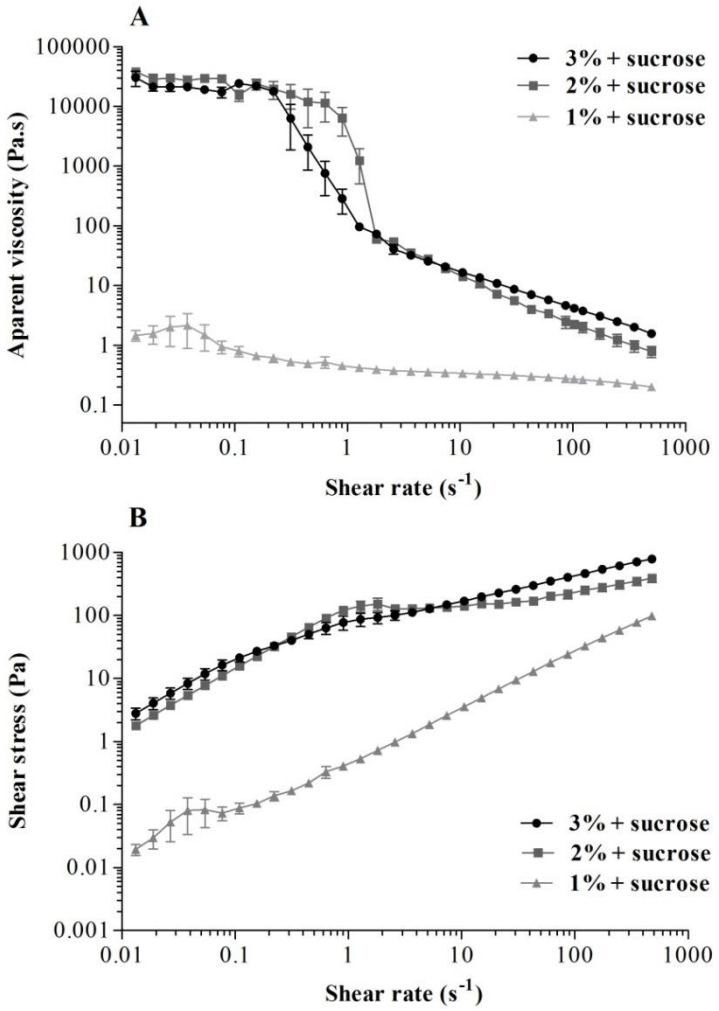

Figure 2 - Influence of shear rate on the apparent viscosity (A) and on shear stress (B) of aqueous STW-A dispersion from tamarillo pulp at 1, 2 and 3% and with sucrose at 50% (w/w), at pH 3.

The experimental data of all flow curves were well fitted to both Ostwald–de Waele and Hershel–Bulkley models with high regression coefficients (R^2) values (≥ 0.990). The Hershel–Bulkley model provides the parameters of flow behavior (n) and the consistency (K) indexes as well as Ostwald–de Waele model. The former also brings a yield stress (τ_0) value as an additional parameter. When $n < 1$ the fluid is shear-thinning; $n >$ the fluid is dilatant; and $n = 1$ is a special case of a Newtonian, while K is related with the increase in the viscosity of solutions. Materials which present an yield stress (τ_0) remain rigid at shear rates under τ_0, while in the presence of values above τ_0 they flow as a power-law fluid (RAO, 2007). Positive values of τ_0 were only obtained for samples at 2 and 3% with sucrose (Table 1). We concluded that for both samples, the Hershel–Bulkley model was better fitted than the Ostwald–de Waele model.

Table 1 - Rheological parameters based on flow curves of STW-A samples

STW-A fraction	Rheological parameter			
	τ_0	K [Pa.s^m]	n	R^2
3%[a]	-	0.176	0.876	0.9999
5%[a]	-	1.516	0.737	0.9996
8%[a]	-	11.220	0.625	0.9984
1% + sucrose[a]	-	0.593	0.831	0.9998
2% + sucrose[b]	71.955	21.590	0.473	0.9908
3% + sucrose[b]	62.320	43.979	0.470	0.9979

K (consistency coefficient), *n* (flow behavior index), R^2 (regression coefficient). Parameters obtained from fitting to the the Ostwald-de Waele model [a] and to the Hershel-Bulkley model [b]

All tested samples showed values of flow behavior index (*n*) lower than 1 (Table 1), in accordance to the previously observed shear-thinning profiles. The increase in the STW-A concentration and the presence of sucrose were accompanied by an increase in the shear-thinning behavior, demonstrated by a decrease in values of n. Likewise, the consistency coefficient (*K*) also augmented with the increase of STW-A concentration and in the presence of sucrose. MARCOTTE *et al.* (2001) also fitted their data using the Ostwald–de Waele model, showing similar *n* values for commercial citrus peel pectin (DM = 70%) at 3 and5% at 20°C dispersed in water, but with higher *K* values (0.93 and 5.92, respectively) than those observed for samples of STW-A in the same concentrations. The Ostwald–de Waele model is possibly the most widely employed model for non-Newtonian liquids and it is extensively used to describe the flow properties of liquids in theoretical analysis as well as in practical engineering application (VLIET e LYKLEMA, 2005). The values of yield stress (τ_0) for STW-A at 2 and 3% with sucrose were 71.9 and 62.3, respectively (Table 1), and did not show any significant difference ($p > 0.05$). These data demonstrated that the gel structure needs to be disrupted before it can flow and then presents a shear-thinning behavior upon further deformation as it occurs with many other food gels, such as ketchup and margarine (VLIET e LYKLEMA, 2005; ZHONG e DAUBERT, 2013).

The analysis of viscoelastic properties of aqueous STW-A dispersions showed liquid-like behaviors in all tested concentrations (Fig. 3A). The viscous modulus (G″) was higher

than the elastic modulus (G′) at all frequency range analyzed, with both moduli increasing with the augmentation of the frequency. The liquid-like behavior has also been observed for other HM pectins when dispersed in water. For example, for pectin dispersions from apple pomace at 7% (with DMs of 58 and 69%; MIN et al., 2011), from cacao pod husk at 5% (DM = 56.6% and degree of acetylation = 17%; VRIESMANN e PETKOWICZ, 2013) and from red tamarillo fruit at 2% (GANNASIN *et al.*, 2015).

STW-A dispersions at 2 and 3% with sucrose showed gel-like behaviors, with G′ values higher than G″ in all frequency range tested and both moduli were weakly frequency dependent (Fig. 3B). VRIESMANN *et al.* (2010) evaluated the dependence of the frequency of a gel formed by a HM pectin (DM = 53%) and starch extracted from cupuassu pulp at 2%, sucrose at 55% (w/w) and at 25°C. Comparing their data with those obtained in the present research, one can observe that the difference between the moduli and the magnitude of their values were greater for cupuassu pulp (G′ and G″ values at 0.1 Hz near to 4000 and 300 Pa, respectively) than those for STW-A gel at 2% with 50% sucrose content at 20°C (G′ and G″ values at 0.1 Hz equal to 1915 and 249 Pa, respectively).

The STW-A gel at 1% with sucrose showed concentrated solution behavior with G′ values higher than G″ at small frequencies with crossover of moduli at higher frequencies. Our data also showed that the gel strength was not significantly ($p < 0.05$) altered when the STW-A concentration increased from 2 to 3% (Table 2).Otherwise, a significant ($p < 0.05$) increase in the gel strength was observed when the STW-A concentration rose from 1 to 2% (Table 2). These results were in accordance to the flow curves (Fig. 2B) and suggested that most of the junction zones had already been formed at 2% of polysaccharide and only a few more could be added at 3%. This behavior was also observed in other polysaccharides, such as peach gum (SIMAS-TOSIN *et al.*, 2010) and *Cedrela odorata* gum (RINCÓN *et al.*, 2009).

The Cox and Merz rule was used to compare experimental data obtained under continuous and oscillatory flow conditions. This rule is an empirical relationship expressed by superposing the apparent viscosity (η_a) to the complex viscosity (η^*) at equal values of shear rate ($\dot{\gamma}$) and frequency (ω), and can be used to obtain information on the interactions of the chains (COX e MERZ, 1958).The aqueous STW-A dispersions at 3, 5 and 8% followed Cox and Merz generalization, once η_a and η^* are overlapping the applied shear rate and frequency ranges (Fig. 4A). This behavior is typically found in fluids with a homogeneous structure such as dispersions of random-coil polysaccharides (SILVA e RAO, 2006). However, in the

presence of sucrose, STW-A dispersions did not follow the Cox and Merz rule (Fig. 4B). Values of complex viscosity (η^*) higher than those of the steady apparent viscosity (η_a) were observed for STW-A at 1% in the presence of sucrose over the tested range of shear rates and frequencies. In addition, STW-A at 2 and 3% with sucrose presented a different behavior with $\eta^* < \eta_a$ at low shear rate and frequency values, and with $\eta^* > \eta_a$ at high shear rate and frequency values. According to SILVA e RAO (2006), the departure of the Cox and Merz rule is observed in two situations (a) where $\eta^* > \eta_a$ occurs with HM pectin dispersions in the presence of sugars and also with some other molecules that associate to form delicate weak gelled networks, and (b) where $\eta^* < \eta_a$ could be attributed to aggregation phenomena of pectin in solution. This deviation ($\eta^* > \eta_a$) was also reported for okra pectin, at 4 -10% and pH 6, at low shear rate (KONTOGIORGOS *et al.*, 2012).

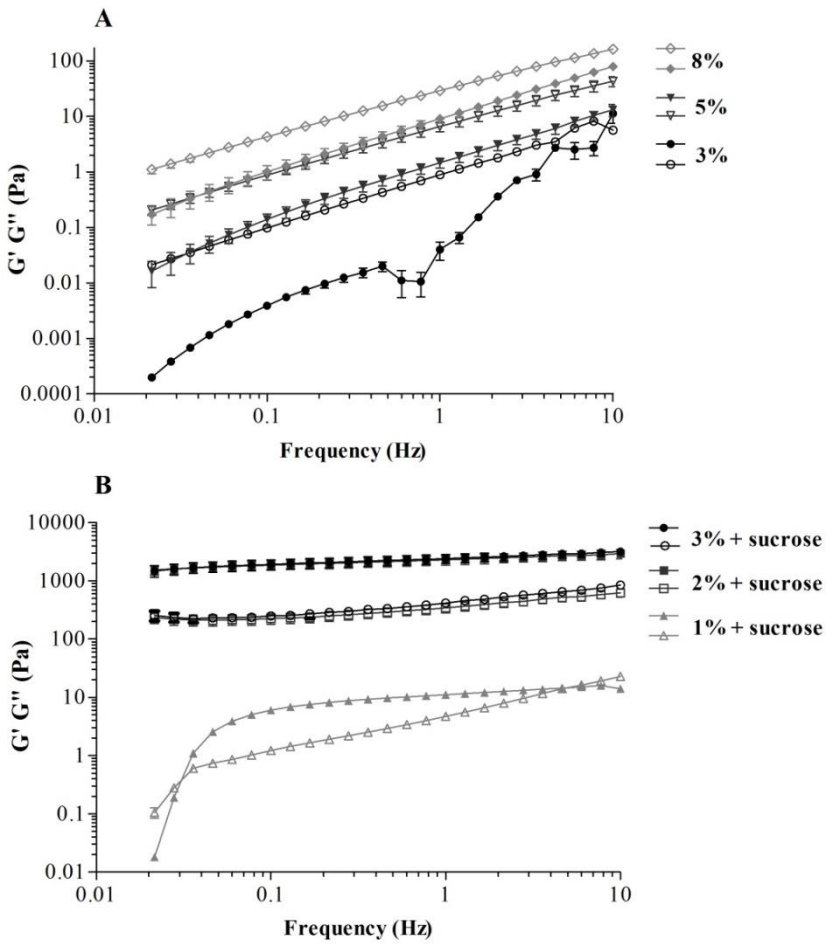

Figure 3 - Frequency sweeps at 20˚C of fraction STW-A from tamarillo pulp. Elastic modulus (G′) is represented with full symbols while viscous modulus (G″) with open symbols. Fixed stress at: 0.05, 0.3 and 1.0 Pa for STW-A at 3, 5 and 8%, respectively (A); and 0.1, 0.1, and 1.0 Pa for STW-A at 1, 2, and 3% with sucrose at 50% (w/w), at pH 3, respectively (B).

Table 2 - Variation of elastic modulus values (G′) and G′/G″ ratios of STW-A samples over the frequencies 0.1, 1 and 10 Hz

Samples	Frequency (Hz)					
	0.1		1		10	
	G′ (Pa)±SEM	G′\G″	G′ (Pa)±SEM	G′\G″	G′ (Pa)±SEM	G′\G″
1% + sucrose	6.007±0.133[a]	4.92	11.147±0.106[a]	2.37	14.120±0.880[a]	0.62
2% + sucrose	1866.7±347.0[b]	8.29	2295.1±423.9[b]	6.69	2933.9±545.6[b]	4.70
3% + sucrose	1915.1±384.5[b]	7.67	2412.5±426.7[b]	5.79	3184.8±436.9[b]	3.77

[a] and [b] represents statistically different mean values ($p < 0.05$) at the same frequency (0.1, 1, or 10Hz). Analyses were performed at 20°C.

In order to evaluate the effect of the temperature on the rheological properties of STW-A at 2 and 3% with sucrose, these gels were submitted to temperature sweeps from 5 to 80°C and from 80 to 5°C, at 2°C/min (Fig. 5). During heating and cooling, G′ values were higher than G″ for both gel samples characterizing their thermostability. This thermostability within the temperature range is compatible with the solid character of the gels and it was also observed for gels formed by HM pectin from cupuassu pulp (VRIESMANN *et al.*, 2010). During heating, the values of G′ and G″ remained broadly constant in both samples. However, during cooling, the gel with 3% of pectin showed an increase in the G′ values, a behavior which had not been observed in the gel with 2% of pectin. Since water evaporation was avoided, this behavior probably occurred due to new intra- and inter-molecular interactions stabilized by nonpermanent cross-links of gel networks that had been formed after the heating of gel with 3% of STW-A. An increase in the moduli was also observed during cooling of gels formed by aqueous dispersions of LM pectins (DM = 37%) at 1, 2 and 3% (STRÖM *et al.*, 2014). Accordingly, DA SILVA e GONÇALVES (1994) also found that values of G′ were dependent on temperature variation.

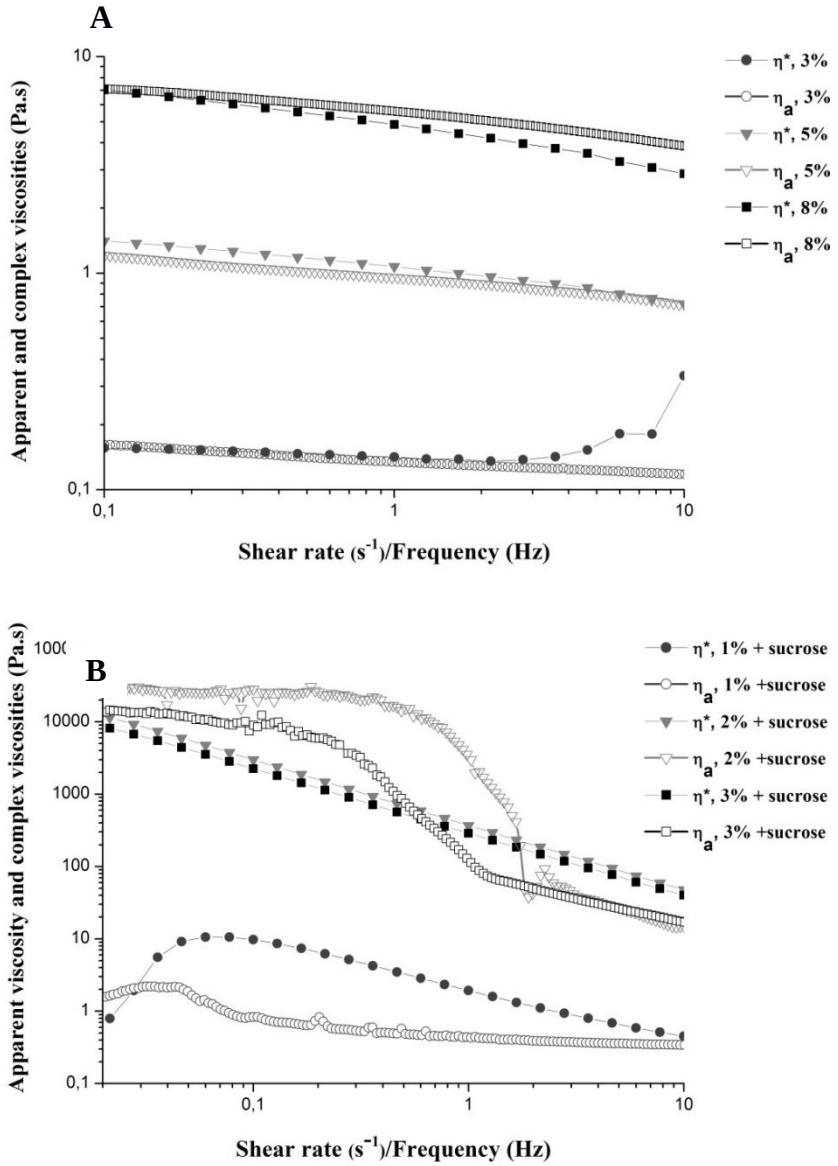

Figure 4 - Apparent viscosity (η_a) and complex viscosity (η^*) as a function of shear rate (γ) and frequency (ω). Aqueous STW-A dispersion from tamarillo pulp at 3, 5 and 8% (A) and aqueous STW-A dispersion at 1, 2 and 3% with sucrose at 50% (w/w), at pH 3 (B).

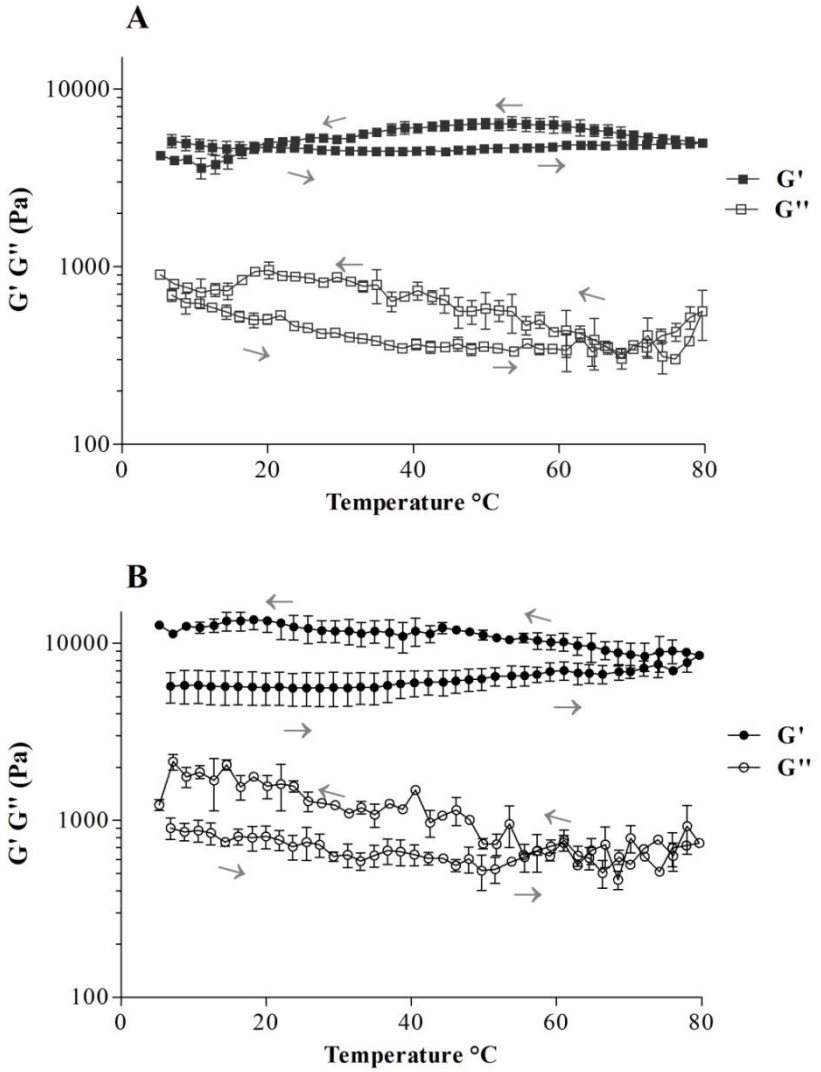

Figure 5 - Elastic moduli (G′, full symbols) and viscous moduli (G″, open symbols) as a function of temperature for heating and cooling of STW-A samples at 2% (A), and 3% (B), containing sucrose at 50% (w/w), at 1 Hz and 1 Pa.

4 Conclusions

Rheological analyses of fraction STW-A from tamarillo pulp, which were majoritarily composed of an HM pectin, showed that the viscosity of the dispersions was influenced by the concentration of STW-A and by the presence of sucrose. All samples showed shear-thinning behaviors, although it was more evident when STW-A (at 2 and 3% w/w) was prepared in the presence of sucrose. STW-A (at 3, 5, and 8%) in water showed a liquid-like behavior. A change in the viscoelastic sample profile was observed when sucrose (50% w/w) was added to

STW-A at 2 and 3% but not with 1% where strong gel-like behaviors were observed. These gels were both thermostable and did not show significative strength differences. Considering the use of the tamarillo fruit in different food preparations, the study of the rheological properties of their pectic fraction can add commercial value to the fruit and promote further application for its pectin as a food additive.

Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgments

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ARTIGO III

Arabinoxylan from mucilage of tomatoes (*Solanum lycopersicum* L.): structure and antinociceptive effect in mouse models

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Arabinoxylan from mucilage of tomatoes (*Solanum lycopersicum* L.): structure and antinociceptive effect in mice models

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ABSTRACT

Tomato is a known functional food due to its content of bioactive compounds. Herein, polysaccharides were extracted from mucilage of tomatoes, and a purified fraction (PTOK) was analyzed by sugar composition, methylation, and NMR spectroscopy analysis. The results showed the presence of an arabinoxylan, having (1→4)-linked β-D-Xyl*p* units in the main chain, which carried a low proportion of branching (~5.6%), at *O*-2 and *O*-3 position, with side chains constituted by single Ara*f* or Xyl*p* units. Intraperitoneal administration of the arabinoxylan in mice significantly reduced the number of abdominal constrictions induced by 0.6% acetic acid and the inflammatory phase of nociception induced by 2.5% formalin, indicating that it had an antinociceptive effect on inflammatory pain models, amplifying the biological role displayed by arabinoxylans in the diet. Furthermore, this study reports the presence of an arabinoxylan in a dicotyledon plant, and also it is the first study of polysaccharides from mucilage of tomatoes.

Keywords: Tomatoes mucilage, *Solanum lycopersicum L.*, arabinoxylan, antinociceptive activity.

1. Introduction

Tomato (*Solanum lycopersicum*) is one of the most important crops present in the human diet. Tomatoes are good sources of potassium, folate, vitamins C, E, and A, and flavonoids (rutin and naringerin). Among its bioactive compounds the carotenoid lycopene, which supplies >85% of the total lycopene consumed in the diet, stands out, and it is often assumed to be responsible for the positive health effects of tomato. The strong antioxidant effect of the lycopene is correlated with prevention of prostate cancer and chronic degenerative and cardiovascular diseases in epidemiological studies in which tomato consumption is increased (CANENE-ADAMS *et al.*, 2005; HOLZAPFEL *et al.*, 2013).

Furthermore, tomatoes are an important source of dietary fiber with about 1 g/100 g of fresh weight (CLAYE *et al.*, 1996) and about 18 g/100 g of dry weight (ENGLYST e HUDSON, 1996). The concept of dietary fiber is complex and can be summarized as nondigestible carbohydrates that pass into large bowel, where they can be partially or completely fermented by microbiota (ENGLYST *et al.*, 2007; JONES, 2013). Dietary fibers act in all processes of the gut such as digestion, absorption, gastrointestinal motility and its control, gastrointestinal immunity, and prebiotic effects, which as result may have an impact on cardiovascular/systemic health, immune function, weight management, colonic health, and the levels of blood total cholesterol and postprandial glucose (BROWNLEE, 2011).

In addition, several other biological properties for plant polysaccharides, both in vitro and in vivo experiments, also have attracted attention, such as antioxidant (PRISTOV *et al.*, 2011; WU *et al.*, 2014b), immunomodulatory (DING *et al.*, 2012; BAO *et al.*, 2013; CAPEK e MATULOVA, 2013), anticomplement (XU e CHEN, 2007; TOGOLA *et al.*, 2008), gastroprotective (NASCIMENTO *et al.*, 2013; CANTU-JUNGLES *et al.*, 2014; MALAFAIA *et al.*, 2015),and anti-inflammatory activities (POPOV *et al.*, 2011; PEREIRA *et al.*, 2012). These properties are correlated with structural parameters such as monosaccharide composition, type and configuration of the glycosidic linkage, size and frequency of branched points, and molecular mass of the polysaccharides (LIU *et al.*, 2015). The polysaccharide structural features display wide ranges in abundance and occurrence according to taxonomy and vegetal tissue (LEE *et al.*, 2011). For instance, xylans are hemicellulosic polysaccharides constituted by a β-(1→4)-linked D-xylopyranosyl polymer as the backbone. In the Poaceae family (monocotyledon), which includes grasses and cereals (e.g., wheat, rye, corn, barley, oat, rice, and sorghum), neutral heteroxylans represent the main xylan component in the endosperm, where the backbone is heavily substituted with L-arabinofuranosyl (Ara*f*) residues

through α-(1→2) and/or α-(1→3) linkages and are known as arabinoxylans. Ara*f* substituents can be further esterified with *p*-coumaric or ferulic acids esters at the *O*-3 position. On the other hand, in dicotyledon plants, such as tomato, the main substituents of xylan backbone are α-glucuronic acid and/or 4-*O*-methylglucuronic acid and are termed glucuronoxylans. Ara*f* could be also present in these acidic xylans in small amounts (BURTON *et al.*, 2010; SCHELLER e ULVSKOV, 2010). Considering the importance of plant foods as a source of polysaccharides and because their biological activities are directly related to their structure, in this work, we extracted the hemicellulosic polysaccharides from mucilage of ripe tomato fruits. An arabinoxylan was characterized and its antinociceptive activity was evaluated using models of pain in mice.

2. Materials and Methods

2.1 Plant material

Ripe fruits of tomato (*Solanum lycopersicum L.*), variety Santa Clara, were purchased at municipal market of Curitiba, State of Paraná, Brazil.

2.2. Extraction and purification of polysaccharides

Seeds surrounded by mucilage were manually separated from pulp (pericarp) of tomato fruits (1.6 kg). The mucilage was separated from the seeds with the aid of a sieve. The mucilage obtained (226 g) was freeze-dried and defatted with chloroform/methanol (1:1) by sequential extraction through a Soxhlet apparatus to remove lipids, pigments, and other hydrophobic material. The polysaccharides were first extracted from the residue with water at 100 °C under reflux for 2 h (four times, 1 L each).

The aqueous extracts were obtained by centrifugation (12000g, 20 min at 10 °C), joined, and concentrated below 60 °C under reduced pressure. The polysaccharides were precipitated with EtOH (3 vol). The resulting precipitate was dialyzed and freeze-dried, to give fraction TOW (2.6%). To obtain the hemicellulosic polysaccharides, the remaining residue of aqueous extraction was then submitted to extraction with aqueous 10% KOH at 100 °C under reflux for 2 h (four times, 1 L each). The alkaline extracts were neutralized with

acetic acid, dialyzed for 48 h with tap water using a 12−14 kDa cut-off membrane, concentrated under reduced pressure, and freeze-dried, originating fraction TOK (6.6%).

A freeze−thaw treatment was applied in fraction TOK to give coldwater-soluble (STOK, 5.0% yield) and insoluble (PTOK, 1.6% yield) fractions (Figure 1). In this procedure, the sample was dissolved in water, frozen, and then thawed at room temperature, and the fractions were recovered by centrifugation. The freeze−thaw treatment was repeated until no more precipitate appeared.

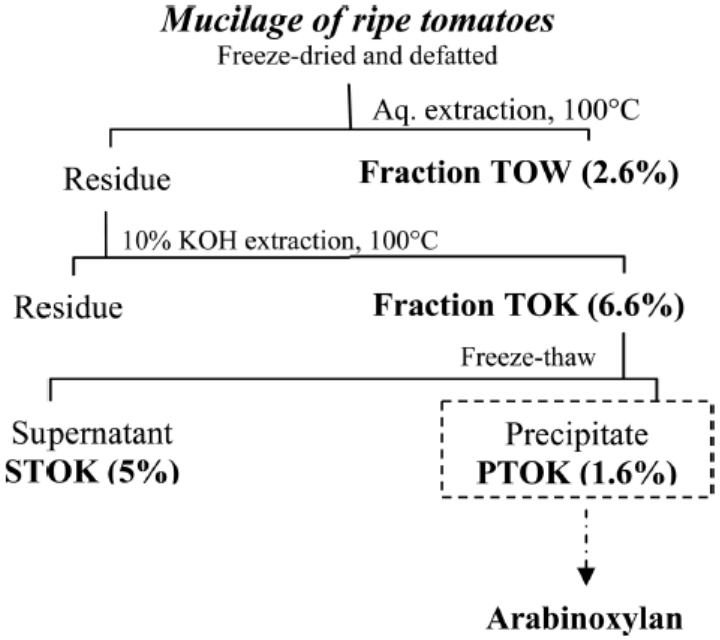

Figure 1 - Scheme of extraction and purification of arabinoxylan from mucilage of ripe tomatoes. The yields were expressed as percent based on the weight of dried and defatted mucilage of tomatoes that was submitted to extraction (7.6 g).

The yields of polysaccharide fractions were expressed as percent based on the weight of dried and defatted tomato mucilage that was submitted to extraction (7.6 g), whereas the moisture and nonpolar compounds were expressed as percent based on the weight of wet tomato mucilage (226 g).

2.3. Sugar composition

Neutral monosaccharide components of the polysaccharide fractions (2 mg) and their ratio were determined by hydrolysis with 2 M TFA (1 mL) for 8 h at 100 °C. The

hydrolysates were converted to alditol acetates by successive $NaBH_4$ reduction, followed by acetylation with Ac_2O/pyridine (1:1, v/v, 1 mL) at 100 °C for 30 min. The resulting alditol acetates were analyzed by GC-MS using a Varian gas chromatograph and mass spectrometer, model Saturn 2000R, with He as carrier gas. A DB-225 capillary column (30 m × 0.25 mm i.d.), held at 50 °C during injection for 1 min, then programmed at 40 °C/min to 220 °C, and held at this constant temperature for 19.75 min, was used for the quantitative analysis. The alditol acetates were identified by their typical electron impact breakdown profiles and retention times.

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

2.4 Methylation analysis of polysaccharide

The purified polysaccharide PTOK (4 mg) was O-methylated according to the method of CIUCANU e KEREK (1984), using powdered NaOH in Me_2SO/MeI. The per-*O*-methylated derivatives were then submitted to methanolysis in 3% HCl/MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5 M, 12 h, at 100 °C) and neutralization with $BaCO_3$. The resulting mixture of partially O-methylated monosaccharides was successively reduced with $NaBD_4$ and acetylated with Ac_2O/pyridine. The products (partially *O*-methylated alditol acetates) were analyzed by GC-MS. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection for 1 min, then programmed at 40 °C/min to 210 °C, and held at this temperature for 31 min, was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (SASSAKI *et al.*, 2005a).

2.5 Nuclear magnetic resonance (NMR) spectroscopy

$^{13}C\{^1H\}$ NMR and HSQC spectra were obtained with a Bruker AVANCE III 400 NMR, operating at 9.5 T, observing 13C at 100.61 MHz and 1H at 400.13 MHz. Analyses were performed using a 5 mm multinuclear inverse detection probe with z-gradient, at 70 °C. The sample was acquired in Me_2SO-d_6 with chemical shifts expressed as δ (ppm), using the resonances of CH_3 (δ 39.7) and ^1H (δ 2.60) groups of Me_2SO- d_6 as internal references. All pulse programs were supplied by Bruker.

2.6 Animals

Experiments were conducted using female Swiss mice (25−35 g), provided by the Federal University of Paraná colony. Animals were kept under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2 °C) with food and water provided *ad libitum*. Animals were acclimatized to the laboratory for at least 12 h before testing and were used only once for experiments. The mice were placed individually into glass cylinders over 30 min before the beginning of the experiment. All of the experiments were performed after approval of the respective protocols by the Committee of Animal Experimentation of Federal University of Paraná (CEUA/BIO − UFPR; approval no. 832). The study was conducted in accordance with the Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985) and with the ethical guidelines for investigations of experimental pain in conscious animals. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.7 Abdominal constriction induced by acetic acid

The animals were pretreated with vehicle (saline, 10 mL/kg) or PTOK fraction (0.1, 1, and 10 mg/kg) by intraperitoneal (ip) route, 30 min before the ip injection of 0.6% aqueous acetic acid (0.45 mL/mouse, made up in saline). The numbers of abdominal constrictions were cumulatively counted over a period of 20 min (RODRIGUES *et al.*, 2012).

2.8 Nociception induced by formalin

Animals were intraperitoneally treated with vehicle (saline, 10 mL/kg, ip) or PTOK fraction (1, 3, and 10 mg/kg). After 30 min, mice received 20 μL of a 2.5% formalin solution (0.92% formaldehyde, in saline) via an intraplantar injection in the ventral surface of the right hind paw. Animals were observed from 0 to 5 min (early phase) and from 15 to 30 min (late phase), and the time that they spent licking the injected paw was considered as indicative of nociception (RODRIGUES *et al.*, 2012).

2.9 Statistical analysis

Data were expressed as means ± standard error of mean (SEM) with five or six animals per group. Comparisons between experimental and control groups were performed by one-way analysis of variance (ANOVA) followed by Newman−Keul's test. P values <0.05 were considered as indicative of significance.

3. Results and discussion

3.1 Isolation and structural characterization of the arabinoxylan

The mucilage from ripe tomatoes was freeze-dried and then defatted with chloroform/methanol (1:1) in Sohxlet apparatus, yielding moisture and nonpolar compound contents of 95 and 0.6%, respectively. The defatted residue was then submitted to successive extraction with water and 10% aqueous KOH, both at 100 °C, and the extracted polysaccharides (fractions TOW and TOK, respectively) recovered by EtOH precipitation and dialysis, respectively (Figure 1).

The fraction TOK, on monosaccharide analysis, revealed the presence of rhamnose (4.5%), arabinose (23.4%), xylose (53.1%), mannose (12.1%), galactose (2.4%), and glucose (4.6%). This fraction was submitted to freeze−thaw treatment, giving rise to supernatant STOK and precipitate PTOK. Monosaccharide analysis of STOK showed arabinose (10.9%), xylose (12.1%), mannose (35.6%), galactose (14.0%), glucose (24.0%), and uronic acids (3.4%). Otherwise, PTOK revealed only arabinose (4.0%) and xylose (96.0%), indicating the presence of an arabinoxylan in this fraction.

Methylation analysis of PTOK is reported in Table 1, and it is in agreement with monosaccharide composition. The main observed derivative was 2,3-Me2-Xyl-ol acetate, which corresponded to (1→4)-linked Xyl*p* residues, indicating a (1→4)-linked xylan as main chain. A low proportion of branching (~5.6%) could be observed, at either *O*-2 or *O*-3, due to the presence of 3-Me-Xyl-ol and 2-Me-Xyl-ol, respectively. The Ara*f* units were present only as terminal units, together with small amounts of Xyl*p* units.

NMR spectral data (^{13}C and HSQC) of the arabinoxylan were in good agreement with the results of the linkage analysis. Thus, its 13C NMR spectrum is dominated by five signals of the (1→4)-linked β-D-Xyl*p* units of the main chain, with resonances at δ 101.7 (C-1), 75.5

(C-4), 74.0 (C-3), 72.6 (C-2), and 63.2 (C-5) (Figure 2A). Their coupled hydrogens were seen in the HSQC experiment, at δ 4.40, 3.65, 3.40, 3.19 and 4.01/3.33, respectively (Figure 2B). Signals of low intensity are also present at δ 107.2/5.43 (C-1/H-1), 85.9/4.10 (C-4/H-4), 80.5/3.95 (C-2/H-2), 77.8/3.76 (C-3/H-3), 61.8/3.60, and 3.53 (C-5/H-5, H-5′) and could be assigned to terminal Ara*f* units (Figure 2). These assignments are in agreement with published literature data (EBRINGEROVÁ *et al.*, 1990; EBRINGEROVÁ *et al.*, 1995)

Table 1- Linkage types based on analysis of partially O-methylated alditol acetates obtained from tomato mucilage (*Solanum lycopersicum*).

Partially *O*-methylalated aditol acetate	PTOK[a]	Linkage type[b]
2,3,5-Me_3-Ara[c]	3.5	Ara*f*-(1→
2,3,4-Me_3-Xyl	1.8	Xyl*p*-(1→
2,3-Me_2-Xyl	89.3	→4)-Xyl*p*-(1→
2-Me-Xyl/3-Me-Xyl [d]	5.3	→3,4)-Xyl*p*-(1→/→2,4)-Xyl*p*-(1→

[a] Percentage of peak of *O*-Methylarabinitol acetates relative to total area, determined by GC-MS. [b] Based on derived *O*-methylalditolacetates. [c] 2,3,5-Me_3-Ara = 2,3,5-tri-O-methylalditol acetates, etc. [d] The ratio of 2-Me-xylitol acetate and 3-Me-xylitol estimated by their specific fragmentation patterns in GC-MS was 2.7: 1.

With regard to the polysaccharides from ripe tomato fruits, pectins and the hemicellulosic polymers xyloglucans and galactoglucomanns are undoubtedly the main polysaccharides present in the pulp (SEYMOUR *et al.*, 1990; LAHAYE *et al.*, 2012; ASSOR *et al.*, 2013).Although quantitatively in small amounts, xylans have also been reported, but as acid xylans (glucuroxylans) and mainly complexed with pectins or glucomannans (SEYMOUR *et al.*, 1990; QUÉMÉNER *et al.*, 2007; PRAKASH *et al.*, 2012; ASSOR *et al.*, 2013).With regard to chemical characterization of arabinoxylans in dicotyledon plants, there are only reports in the literature about arabinoxylans present in the leaves and bark of tropical Litsea species (HERATH *et al.*, 1990; WIMALASIRI e KUMAR, 1995; DAS *et al.*, 2013).

Thus, this study reports the presence of an arabinoxylan in another dicotyledon plant, and also it is the first study that describes the chemical structure of a polysaccharide extracted from mucilage of tomatoes.

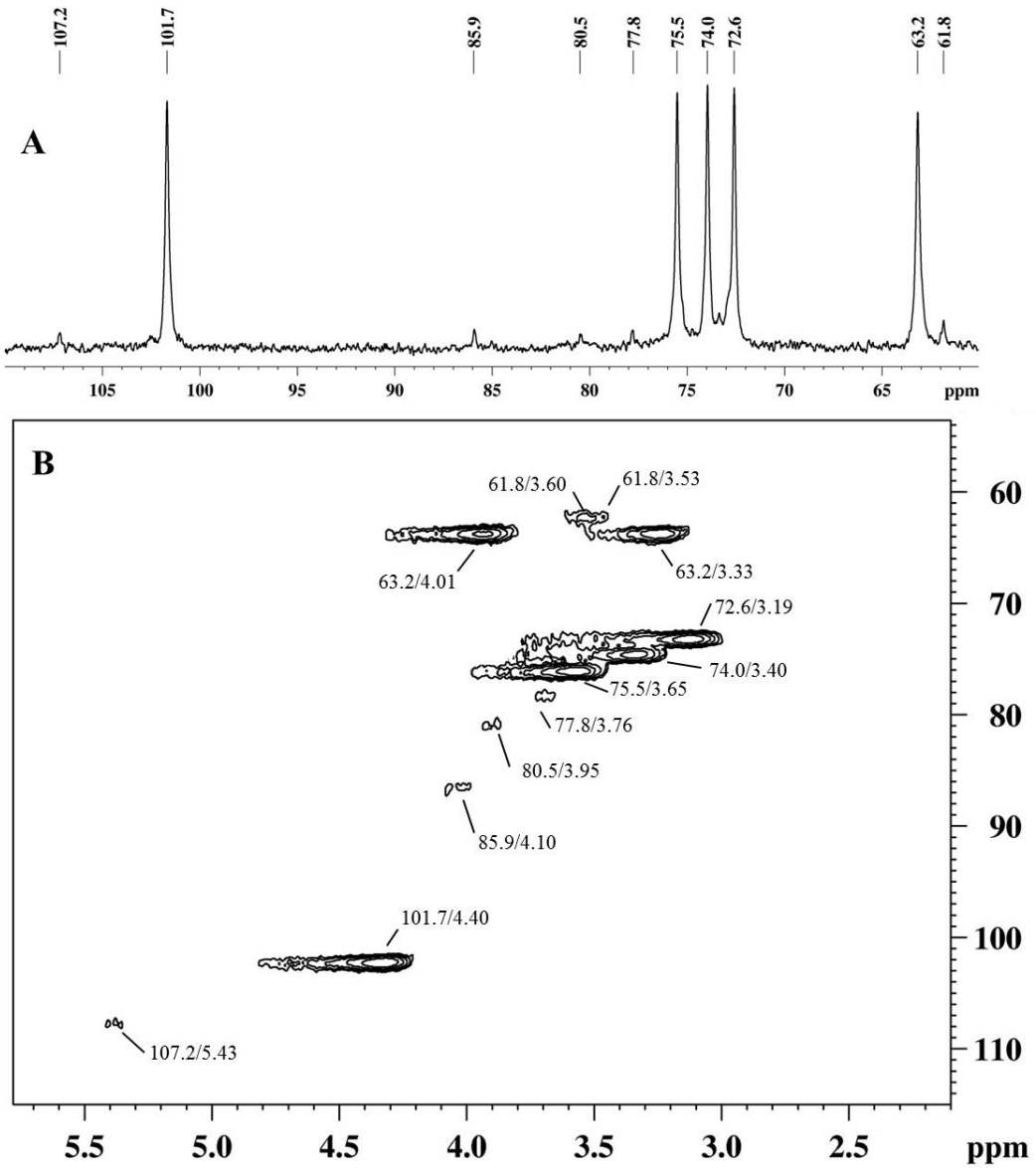

Figure 2 - ^{13}C NMR spectrum (A) and 2D $^{1}H-^{13}C$ HSQC correlation map showing some carbon and hydrogen assignments (B) of fraction PTOK obtained from mucilage of ripe tomatoes. Sample was dissolved in Me_2SO-d_6, and data were collected at a probe temperature of 70 °C.

3.2 Antinociceptive activity of PTOK

Our results showed that intraperitoneal administration of fraction PTOK at 1 and 10 mg/kg produced dose-related inhibition of the abdominal constrictions induced by acetic acid, a screening model for assessment of antinociceptive and anti-inflammatory properties of new

agents because of its simplicity and sensitivity (Figure 3). In this model PTOK displayed a mean ID_{50} of 2.60 mg/kg (95% confidence limits 0.90−7.48) and at 10 mg/kg inhibited the number of writhes by 84 ± 8%. To confirm the effect of PTOK on inflammatory pain, we performed the formalin test. This model is constituted by two distinct phases: neurogenic pain (early phase), which results from the direct irritating effect on nociceptors, and inflammatory pain (late phase), mediated by a combination of peripheral input and spinal cord sensitization (TJØLSEN *et al.*, 1992). However, the inhibition of the inflammatory phase of formalin-induced nociceptive response occurred only when PTOK was administered at 10 mg/kg (inhibition of 90 ± 5%) (Figure 4). These findings suggested that the arabinoxylan present in the tomato mucilage promotes antinociceptive effects through anti-inflammatory mechanisms. Both models were also used by DO NASCIMENTO *et al.* (2013) to evaluate the antinociceptive activity of an arabinoglucuronoxylan from tamarillo pulp. This complex acid heteroxylan showed a mean ID_{50} of 0.47 mg/kg (95% confidence limits 0.18−1.19) and at 10 mg/kg inhibited the number of abdominal writhes induced by acetic acid by 78 ± 6%. In the formalin test, this heteroxylan inhibited the inflammatory phase and displayed a mean ID_{50} of 0.09 mg/kg (0.03−0.23) and a maximal inhibition of 80 ± 6% at 1 mg/kg. Structurally, this heteroxylan fraction contained glucuronic acid and a higher degree of branching (~20%) compared to neutral PTOK fraction (~5.6%) evaluated in this work, indicating that differences in the fine structure of side chains of xylan contribute to biological response. It is important to note that differences in the structures of xylans, such as a higher content of arabinose or acid monosaccharides, were also related to more potent biological activities, such as those observed for antiulcer (CIPRIANI *et al.*, 2008), immunological (EBRINGEROVÁ *et al.*, 1995), anticomplementary (SAMUELSEN *et al.*, 1999), and antitussive (NOSAL'OVA *et al.*, 2000) activities.

Different biological activities have already been attributed to the arabinoxylan biomolecules. Dietary fiber effects are well documented for arabinoxylans, particular from the Poaceae family (SEDLMEYER, 2011; LIU *et al.*, 2015). Their poly- and oligosaccharides have attracted interest as prebiotics (MENDIS e SIMSEK, 2014; SINGH *et al.*, 2015). Effects on innate and acquired immune response in mice (CAO *et al.*, 2011) and anti-inflammatory (OGAWA *et al.*, 2005), antioxidant (RAO e MURALIKRISHNA, 2006), and anticomplementary activities (HROMÁDKOVÁ *et al.*, 2013) were also attributed to arabinoxylans as well as antitumor activity probably due to immunostimulatory properties (AKHTAR *et al.*, 2012). Finally, the arabinoxylan extracted from green leaves of a

dicotyledon *Litsea glutinosa* was also biologically active, showing splenocyte, thymocyte, and macrophage activations (DAS *et al.*, 2013).

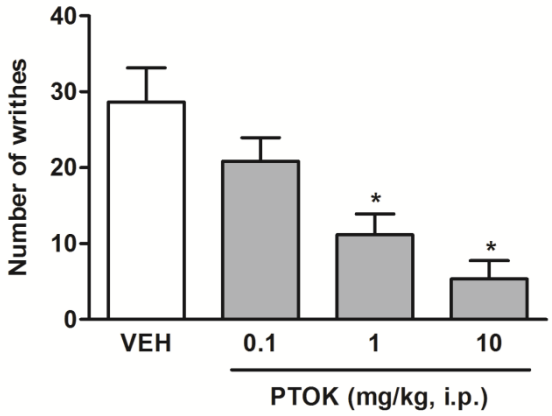

Figure 3 - Effect of intraperitoneal administration of PTOK on abdominal constriction induced by 0.6% acetic acid in mice. The animals were treated with vehicle (VEH, 10 mL/kg, ip) or PTOK (0.1, 1, or 10 mg/kg, ip). Data are expressed as means ± SEM (n = 6−8), followed by post hoc Newman−Keul's test. Differences from vehicle group (∗, $p < 0.05$).

Therefore, fraction PTOK isolated from mucilage of tomatoes was composed of an arabinoxylan, which is an unusual polysaccharide for dicotyledons. Furthermore, this fraction significantly reduced the number of abdominal writhes induced by 0.6% acetic acid and inhibited the inflammatory phase of formalin-induced nociceptive response. These results amplify the role of biological activities displayed by arabinoxylans, as molecules with analgesic effects, probably through an anti-inflammatory mechanism. However, more extensive study is necessary to identify the specific mechanism(s) behind this inhibition of pain.

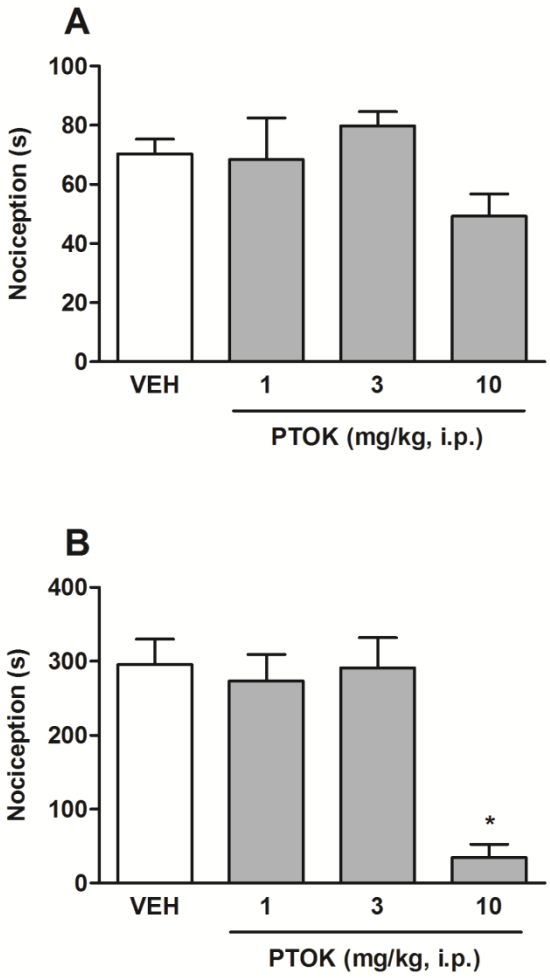

Figure 4 - Effect of intraperitoneal administration of PTOK on neurogenic phase (A) and inflammatory phase (B) of nociception induced by 2.5% formalin in mice. The animals were treated with vehicle (VEH, 10 mL/kg, ip) or PTOK (1, 3, or 10 mg/kg, ip). Data are expressed as means ± SEM (n = 6 - 8), and statistical comparison was performed using one-way ANOVA followed by post hoc Newma-Keul's test. Differences from vehicle group (∗, $p < 0.05$).

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Notes

The authors declare no competing financial interest.

Abbreviations used

2,3,5-Me_3-Ara-ol, 2,3,5-tri-O-methylarabinitolacetate; Ac_2O, acetic anhydride; Ara*f*, arabinofuranosyl; $BaCO_3$, barium carbonate; EtOH, ethanol; HCl, hydrochloric acid; H_2SO_4, sulfuric acid; HSQC, heteronuclear single-quantum coherence; KOH, potassium hydroxide; $NaBD_4$, sodium borodeuteride; $NaBH_4$, sodium borohydride; MeI, methyl iodide; MeOH, methanol; Me_2SO, dimethyl sulfoxide; Me_2SO-d_6, deuterated dimethyl sulfoxide; NMR, nuclear magnetic resonance; NaOH, sodium hydroxide; ppm, parts per million; TFA, trifluoroacetic acid; Xyl*p*, xylopyranosyl

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ARTIGO IV

New findings on green sweet pepper (*Capsicum annuum*) pectins: rhamnogalacturonan and type I and II arabinogalactans

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DO NASCIMENTO, G. E.; IACOMINI, M.; CORDEIRO, L. M. C. New findings on green sweet pepper (*Capsicum annuum*) pectins: rhamnogalacturonan and type I and II arabinogalactans. **Carbohydrate Polymers,** v. 171, p. 292-299, 2017

New findings on green sweet pepper (*Capsicum annuum*) pectins: rhamnogalacturonan and type I and II arabinogalactans

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ABSTRACT

Polysaccharides were extracted from sweet pepper (*Capsicum annuum*) with hot water and named ANW (9% yield). Starch was precipitated by freeze-thaw treatment, while pectic polysaccharides (8% yield) remained soluble and consisted of GalA (67.0%), Rha (1.6%), Ara (6.4%), Xyl (0.3%), Gal (6.7%) and Glc (4.4%). A highly methoxylated homogalacturonan (HG, degree of methylesterification of 85% and degree of acetylation of 5%), and type I and type II arabinogalactans (AG-I and AG-II) were observed in NMR analyses. These were fractionated with Fehling's solution to give HG (5.5% yield) and AG fractions (0.6% yield). AG-I and AG-II were further separated by ultrafiltration. AG-II (0.2% yield) consisted of Ara (17.1%), Gal (36.0%), Rha (5.6%) and GalA (12.0%), had a molecular weight of 5.3 x 10^4 g/mol and methylation and $^1H/^{13}C$ HSQC-DEPT-NMR analyses showed that it was anchored in type I rhamnogalacturonan. This is the first study that reports the presence of AG-I and AG-II in sweet pepper fruits.

Keywords: sweet pepper fruits; homogalacturonan; type I arabinogalactan; type II arabinogalactan; type I rhamnogalacturonan; pectins.

1. Introduction

Sweet (bell) peppers are fruits known worldwide for their importance in human diet as a food or as a condiment. The species *Capsicum annuum* L (Solanaceae) refers not only to sweet peppers, but also to wax peppers, cayenne peppers, chili peppers, and jalapeno peppers. Sweet peppers are known as important sources of vitamin C (ascorbic acid), β-carotene, other carotenoid pigments (such as lycopene and zeaxanthin), phytochemicals and polyphenols (CHUN *et al.*, 2005; HALLMANN e REMBIAŁKOWSKA, 2012). With respect to the macronutrient content, carbohydrate is the main macronutrient present in green sweet peppers with 4.9 g and a total dietary fiber of 2.6 g per 100 g of wet weight (TACO, 2011). The monosaccharide analysis of non-starch polysaccharides has shown a high uronic acid content which has been associated with large amounts of pectins in sweet pepper fruits (LOPEZ-HERNANDEZ *et al.*, 1996; PAIK *et al.*, 2003; VILLANUEVA-SUÁREZ *et al.*, 2003). A pectic polysaccharide named capsicuman was recently characterized by POPOV *et al.* (2011). It was extracted from fresh sweet pepper using a simulated gastric medium (saline solution containing hydrochloric acid at pH 1.5 and pepsin at 37 ºC for 4 h) and was shown to consist of D-galacturonic acid (GalA, 74.0%), rhamnose (Rha, 1.6%), arabinose (Ara, 2.6%) and galactose (Gal, 2.4%) residues. The study revealed that an acetylated and methyl esterified homogalacturonan (HG) was the core of the capsicuman. This polysaccharide showed immunomodulatory activity by decreasing TNF-α and increasing IL-10 secretion in LPS-stimulated whole blood in mice besides improving the survival of mice that were subjected to a lethal dose of LPS.

In this study the chemical characterization of *C. annuum* polysaccharides was performed. In addition to capsicuman, we report the presence of type I arabinogalactan anchored in type I rhamnogalacturonan, as well as the isolation and detailed structural characterization of a type II arabinogalactan.

2. Materials and methods

2.1 Plant material

Fresh green sweet pepper fruits (*C. annuum* L. cv Magali) were purchased from the organic sector of the municipal market in Curitiba, Paraná, Brazil, in April 2014 and September 2016.

2.2 Polysaccharide extraction and fractionation

Seeds were removed manually with the aid of a knife and then the deseeded fruit with skin (1.7 kg) was freeze-dried and milled (136 g). The dried powder was submitted to extraction through a Soxlet apparatus with chloroform/methanol (1:1) to remove pigments and hydrophobic materials (LUQUE De CASTRO E PRIEGO-CAPOTE, 2010).

The residue from Soxlet extraction was submitted to extraction with water at 100 ºC under reflux for 2 h (six times, 1 L each). The extracts were pooled, concentrated and the polysaccharides were precipitated with cold EtOH (3:1 v/v.) and collected by centrifugation (12000 x *g*, 20 min at 10 °C). The insoluble material was dissolved in water, dialyzed against tap water (12-14 kDa cut-off Spectra-Por® membrane) for 24 hours to remove the remaining low-molecular weight compounds, and freeze-dried to give aqueous polysaccharide fraction, ANW (12.2 g). As a first purification step, ANW was submitted to freeze-thawing cycles (GORIN e IACOMINI, 1984). For this ANW was redissolved in water, frozen, and the thawed at room temperature. Cold-water-soluble (ANWS) and insoluble (ANWP) fractions were obtained by centrifugation (12000 x *g*, 20 min at 10 °C) (Fig. 1). The freeze-thawing process was repeated for both fractions until no more precipitated appeared in ANWS and the ANWP supernatant was clear.

The ANWS fraction was treated with Fehling's solutions according to the method described by JONES e STOODLEY (1965). The insoluble polysaccharide-copper complex (ANWS-PF fraction) and a soluble fraction (ANWS-SF fraction) were separated by centrifugation (12000 x *g*, 20 min at 10 °C), neutralized with acetic acid (HOAc), dialyzed (12-14 kDa cut-off Spectra-Por® membrane) against tap water and deionized with a cation

exchange resin (Fig. 1). The fraction ANWS-SF was further submitted to ultrafiltration through membrane with 50 kDa cut-off (PLHK04710-Ultracel, Millipore) (Fig. 1).

The yields of polysaccharide fractions were expressed as percent based on the weight of dried sweet pepper (136 g), whereas the moisture and nonpolar content were expressed as percent based on wet weight (1.7 kg).

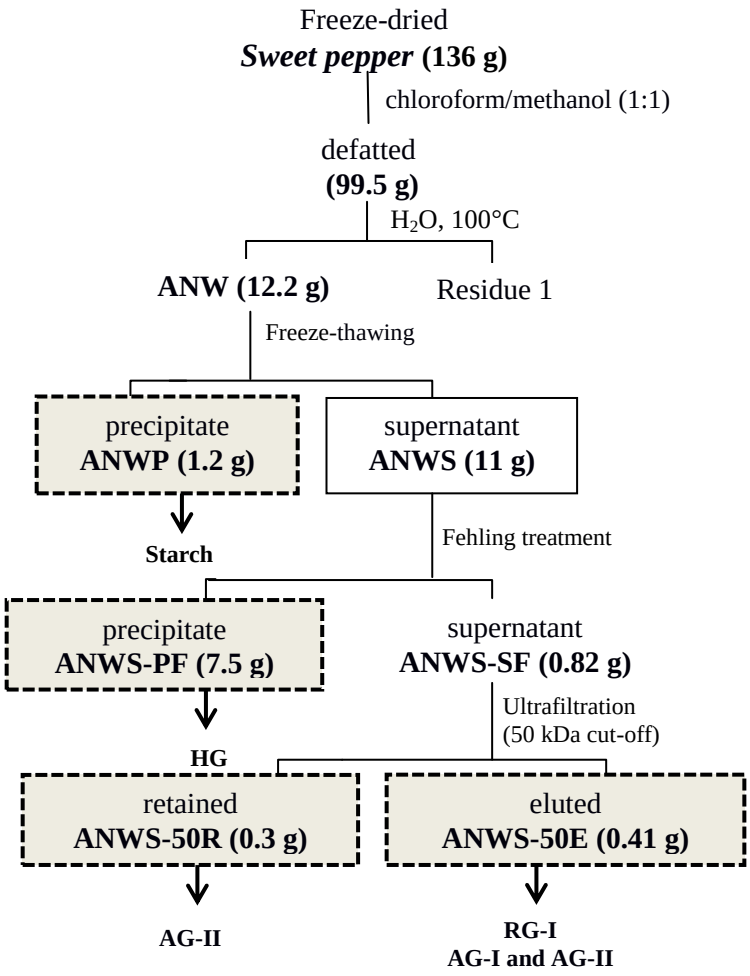


Figure 1. Scheme of extraction and fractionation of water extracted polysaccharides from green sweet pepper fruits *(Capsicum annuum.)*. AG-I = type I arabinogalactan, AG-II = type II arabinogalactan, HG = homogalacturonan, RG-I = type I rhamanogalacturonan.

2.4. Sugar composition

Neutral monosaccharide components of the polysaccharides (1 mg) and their ratios were determined following hydrolysis with 2 M TFA (1 ml, for 8h at 100°C). The acid was

then evaporated, and the residue dissolved in water (1 mL). The hydrolyzate were converted to alditol acetates by treatment with $NaBH_4$ (1 mg), followed by acetylation with acetic anhydride-pyridine (1:1 v/v, 1 ml) at 100°C for 30 min, and the resulting alditol acetates were extracted with chloroform. The alditol acetate analysis was carried out using a Varian gas chromatograph and mass spectrometer, model Saturn 2000R, with He as carrier gas. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50°C during injection for 1 min, then programmed at 40°C/min to 220°C and held at this constant temperature for 19.75 min was used for the quantitative analysis.

The alditol acetates were identified by their retention times and typical electron impact breakdown profiles compared with standards. The results were given as weight percentages taking in account the coefficients from the detector response (York, Darvill, McNeil, & Stevenson, 1985) and the content of total sugar, which was determined according to DUBOIS et al. (1956), using the relative concentration of the monosaccharides present in the fraction as calibration curve.

Uronic acid contents were determined spectrofotometrically using the *m*-hydroxybiphenyl method (FILISETTI-COZZI e CARPITA, 1991), using galacturonic acid as standard.

2.5. Carboxyl-redution of polyssacarides

Fraction ANWS-50R was carboxyl-reduced by the carbodiimide method (TAYLOR e CONRAD, 1972), using sodium borohydride as the reducing agent, giving products with the COOH groups of its uronic acid residues reduced to CH_2OH.

2.6 Methylation analysis of polysaccharide

Prior to glycosyl linkage analysis, the carboxyl-reduced fraction ANWS-50R was *O*-methylated according to the method of CIUCANU and KEREK (1984), using powdered NaOH in DMSO-MeI. The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (at 80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 16 h, at 100°C). The resulting mixtures of *O*-methyl aldoses were neutralized with $BaCO_3$, reduced with $NaBD_4$ and acetylation as described above for sugar composition. The products

(partially *O*-methylated alditol acetates) were analyzed by GC-MS. A capillary column (25 m x 0.25 mm i.d.) of VF-5, programmed at 10 ºC/min to 280 ºC and held at this temperature for 35 min was used for separation. They were identified by their typical electron impact breakdown profiles and retention times (SASSAKI *et al.*, 2005a; SASSAKI *et al.*, 2005b)

2.7 HPSEC analysis and determination of the molecular weight of polysaccharides

The homogeneity and relative molecular weight of soluble polysaccharides were determined by high performance size exclusion chromatography (HPSEC), at 25 ºC using a Waters 2410 differential refractometer as detection equipment. Four columns were used in series, with exclusion sizes of 7 x 10^6 Da (Ultrahydrogel 2000, Waters), 4 x 10^5 Da (Ultrahydrogel 500, Waters), 8 x 10^4 Da (Ultrahydrogel 250, Waters) and 5 x 10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 µg/mL aq. NaN_3 at 0.6 mL/min. The samples were dissolved in the eluent at a concentration of 1 mg/mL, filtered through a membrane (0.22 µm, Millex-GV, Millipore®) and injected (250 µl loop). The results were processed with software ASTRA provided by the manufacturer (Wyatt Technologies). The relative molecular weight of ANWS and ANWS-50R was calculated based on a calibration curve using standard dextrans (4.87 x 10^5 g/mol, 2.66 x 10^5 g/mol, 1.24 x 10^5 g/mol, 7.22 x 10^4 g/mol, 4.02 x 10^4 g/mol, 17.2 x 10^4 g/mol and 9.4 x 10^3 g/mol, from Sigma).

2.8 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C, ^{1}H, 2D $^{1}H/^{13}C$ HSQC and $^{1}H/^{13}C$ HSQC-DEPT NMR spectra were obtained with a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{13}C at 100.61 MHz and ^{1}H at 400.13 MHz. Analyses were performed with a 5 mm multinuclear inverse detection probe with z-gradient. For ^{13}C-NMR spectrum, the ANWP fraction was acquired in dimethyl sulfoxide-d_6 at 70 °C, with chemical shifts expressed as δ ppm, using the resonances of CH_3 groups (δ 39.7) from dimethyl sulfoxide-d_6 as internal reference. For ^{13}C-NMR and ^{1}H and 2D-NMR spectra of other fractions, samples were acquired in deuterium oxide, at 50 °C or 70 °C, using the resonances of CH_3 groups (δ 30.2/ 2.22) from acetone as internal reference.

For ^{1}H NMR spectroscopy, used to determine the degree of methyl esterification (DM) and acetylation (DA) of ANWS fraction, and for $^{1}H–^{13}C$ HSQC-NMR experiments, the fractions were deuterium-exchanged three times by freeze-drying with D_2O solutions, finally dissolved in D_2O and transferred into a 5-mm NMR tube. The ^{1}H NMR spectra were acquired with 256 scans, at pD 5.0. The values of DM and DA were calculated by hydrogen integration according GRASDALEN *et al.* (1988) and NGUYEN *et al.* (2011), respectively. All pulse programs were supplied by Bruker.

3. Results and discussion

Polysaccharides were extracted from defatted sweet pepper fruits with boiling water, giving fraction ANW (9% yield). It was submitted to freeze-thawing treatment, giving cold-water insoluble ANWP (0.9% yield) and soluble ANWS (8% yield) fractions (Fig. 1). The first, ANWP was composed mainly of glucose (70.0% of sugars) (Table 1) and its ^{13}C NMR spectrum showed the presence of starch, with six amylose signals at δ 100.1 (C-1), δ 72.1 (C-2), δ 73.3 (C-3), δ 78.9 (C-4), δ 71.7 (C-5) and δ 60.7 (C-6) (Fig.2).

Table 1. Monosaccharide composition of obtained fractions from green sweet pepper fruits *(Capsicum annuum.)*.

Fractions	**Monossaccharide (weight %)**						
	Rha	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**GalA**
ANWP	-	7.6	2.0	2.6	6.9	59.9	6.5
ANWS	1.6	6.4	0.3	-	6.7	4.4	67.0
ANWS-SF	2.6	19.3	5.8	0.9	26.5	2.1	17.6
ANWS-PF	-	-	-	-	-	-	83.4
ANWS-50E	2.3	17.8	8.9	0.9	20.2	2.8	20.6
ANWS-50R	5.6	17.1	-		36.0	-	12.0

Rha (rhamnose), Ara (arabinose), Xyl (xylose), Man (mannose), Gal (galactose), Glc (glucose), GalA (galacturonic acid).

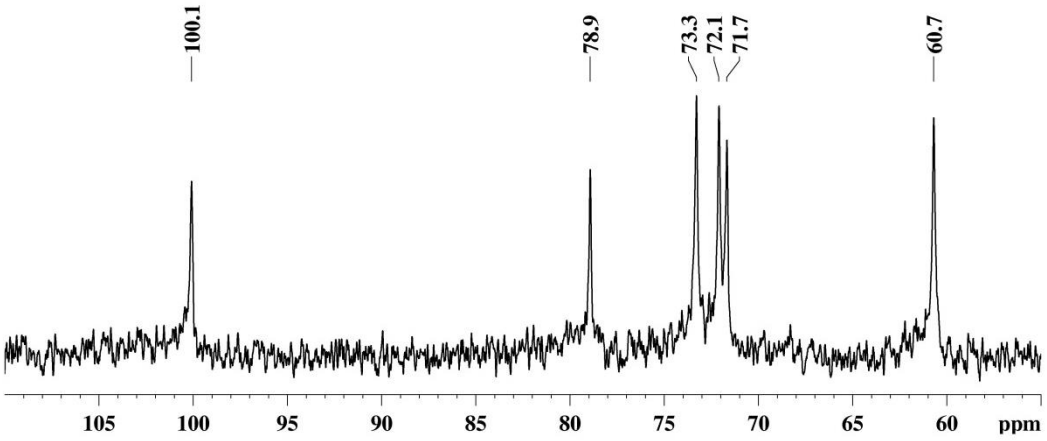


Figure 2. ^{13}C-NMR spectrum of ANWP fraction. Sample was dissolved in dimethyl sulfoxide-d_6 and data collected at probe temperature of 70ºC.

The soluble fraction, ANWS, was composed mainly of uronic acids (67%), with minor amounts of rhamnose, arabinose, xylose, galactose and glucose (Table 1), suggesting the presence of mostly pectin. The homogeneity of ANWS was determined by size exclusion chromatography analysis (HPSEC), which showed the presence of only one peak (Fig. 3), but asymmetrical, suggesting the presence of different populations with an average molecular weight (Mw) of 3.67 x 10^5 g/mol.

In order to compare our data with that of POPOV *et al.* (2011) for capsicuman, we calculated the degree of branching, size of side chains and linearity. The ratio of Rha/GalA indicates the contribution of rhamnogalacturonan (RG) domain to pectin population, the ratio of (Ara + Gal)/Rha shows the extent of RG branching and the ratio of GalA/(sum of neutral sugars) reflects the linearity of pectin backbone (RENARD e GINIES, 2009; HOUBEN *et al.*, 2011; CHRISTIAENS *et al.*, 2015). The obtained ratios were 0.024, 8.2 and 3.4 for ANWS and 0.021, 3.1 and 9.4 for capsicuman, respectively. These ratios demonstrate that the pectin isolated herein by hot water extraction has same proportion of RG domain with a greater RG side chains (2.6 fold) and lower backbone linearity (2.7 fold) than capsicuman isolated by simulated gastric medium. These differences could be due to the acid hydrolysis susceptibility of the glycosidic linkages of neutral sugars that compose the pectin side chains (WHISTLER e CORBETT, 1955; GOLOVCHENKO *et al.*, 2012; WANG *et al.*, 2016). Capsicuman may have had some of the side chain units removed during the simulated gastric medium

extraction employed by POPOV *et al.* (2011), which consisted of HCl (1.27 g/L at pH 1.5) and pepsin at 37 ºC for 4 h. An intense partial degradation, with removal of Ara and smaller amounts of Gal, Xyl and Man from side chains and also cleavages of the main chain of an arabinogalactan from *Phyllanthus niruri* was observed by MELLINGER *et al.* (2008) after treatment with human gastric fluids or aq. HCl solution at pH 2 for 3h at 37 ºC. ZHANG *et al.* (2003) verified that under acidic conditions of the human stomach (HCl pH 1.0–3.0, at 37°C) up to 10% of the L-arabinose was released from side chains of corn arabinoxylan, larch arabinogalactan and banana peel hemicellulose B.

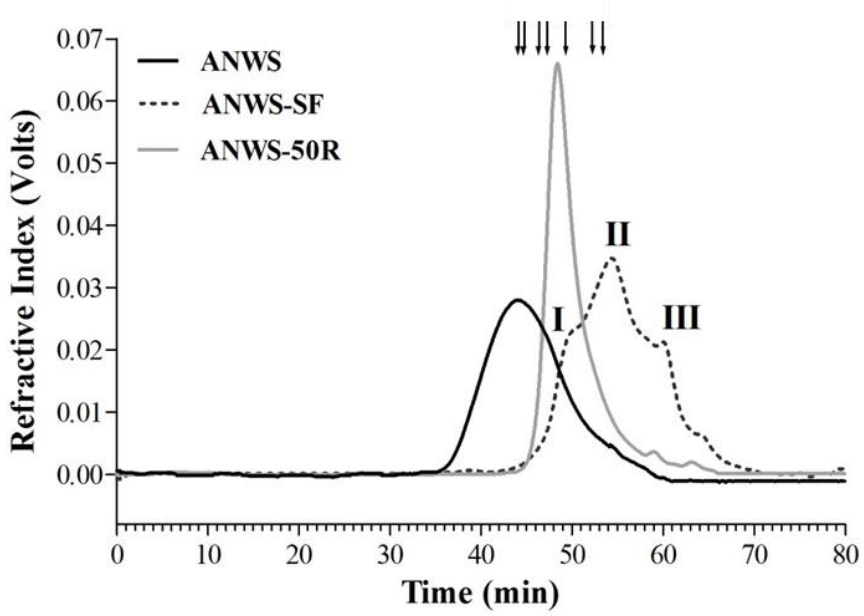


Figure 3. HPSEC elution profiles of fractions ANWS, ANWS-SF and ANWS-50R obtained from green sweet pepper fruits *(Capsicum annuum.)*. Refractive index detector. Peak retention times of dextran standards of molecular weight 4.87 x 10^5 g/mol, 2.66 x 10^5 g/mol, 1.24 x 10^5 g/mol, 7.22 x 10^4 g/mol, 4.02 x 10^4 g/mol, 17.2 x 10^4 g/mol and 9.4 x 10^3 g/mol, respectively, are marked with arrows (left to right).

The main NMR signals observed in in the ANWS spectrum arose from a HG (Fig. 4), as previously observed by POPOV *et al.* (2011). The $^1H/^{13}C$ HSQC signals at δ 100.1/4.96 and δ 70.7/5.05 corresponded to C-1/H-1 and C-5/H-5 of methylesterified units of α-D-Gal*p*A, respectively, while the signals at δ 99.4/5.13 and δ 71.4/4.69 corresponded to C-1/H-1 and C-5/H-5 of α-D-Gal*p*A unesterified units. The remaining assignments of D-Gal*p*A ring carbons and hydrogens were seen at δ 78.6/4.45 (*O*-substituted C-4/H-4), δ 68.2/3.98 (C-3/H-3) and δ

68.2/3.73 (C-2/H-2). Moreover, its ^{13}C-NMR spectrum (not shown) showed signals at δ 170.5 and δ 174.7 attributed to carboxyl groups (C-6) of esterified and unesterified α-D-GalpA units, respectively. Signals of methyl and acetyl groups linked to α-D-GalpA units appeared at δ 52.8/3.81 and δ 20.3/2.09, respectively. Due to the presence of esterified α-D-GalpA units, the degree of methyl esterification (DM) and degree of acetylation (DA) were determined by ^{1}H NMR spectroscopy, giving values of 85% and 5%, respectively. On the other side, POPOV *et al.* (2011) determined a DM value of only 50% for capsicuman, extracted using gastric extraction. Interestingly, simulated gastrointestinal digestion of kiwifruit pulp was shown to result in a reduction of the DM of pectin (CARNACHAN *et al*., 2011).

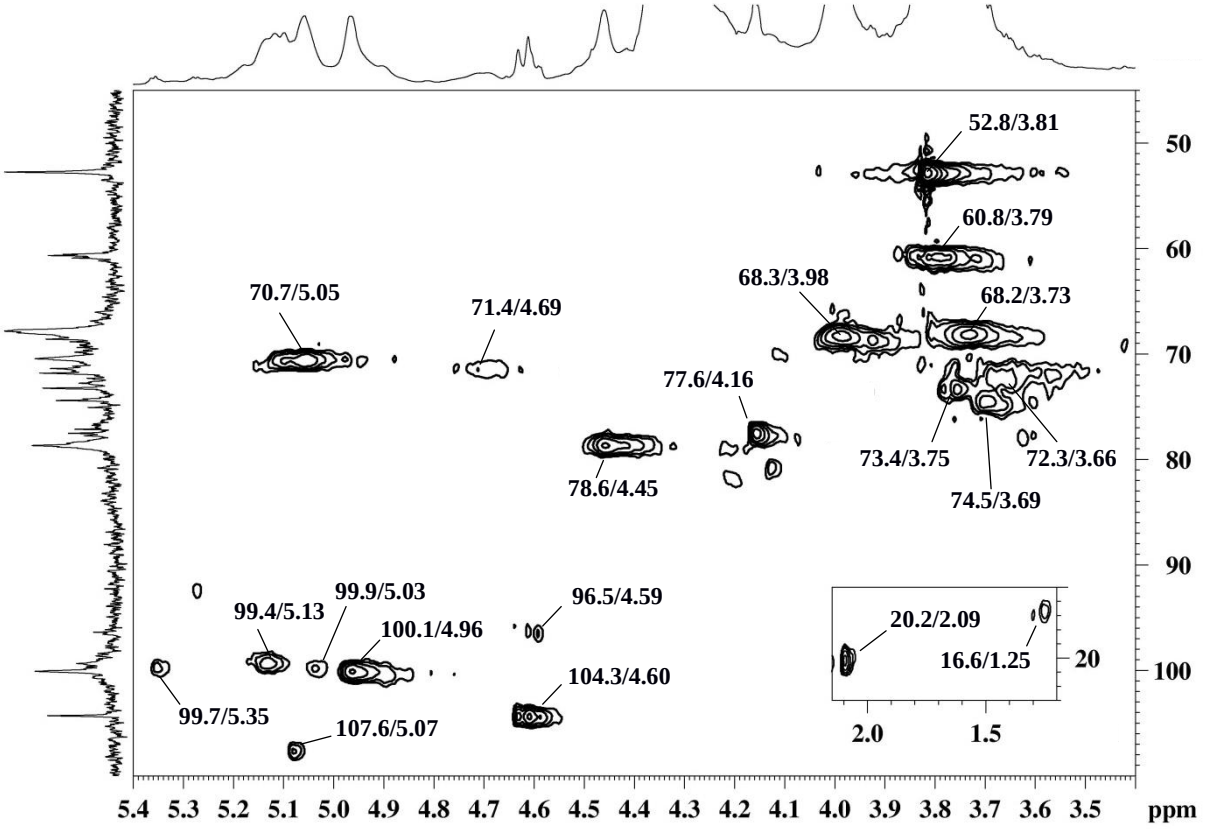
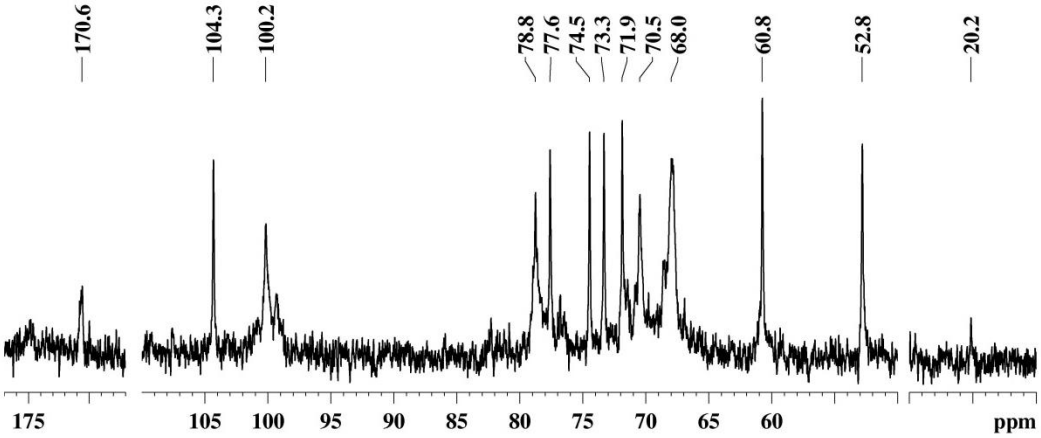


Figure 4. ^{1}H/^{13}C HSQC correlation map of ANWS fraction. Sample was dissolved in deuterium oxide and data collected at probe temperature of 70 ºC.

Besides the presence of HG signals, ANWS also had ^{13}C-NMR and ^{1}H/^{13}C HSQC signals that indicated the presence of a type I arabinogalactan (AG-I) in sweet pepper fruits.

The signal at δ 107.6/5.07 (C-1/H-1) can be attributed to α-L-Ara*f* units and those at δ 104.3/4.60 (C-1/H-1), δ 72.3/3.66 (C-2/H-2), δ 73.4/3.75 (C-3/H-3), δ 77.6/4.16 (*O*-substituted C-4/H-4), δ 74.5/3.69 (C-5/H-5) and δ 60.8/3.79 (C-6/H-6) can be assigned to (1→4)-linked- β-D-Gal*p* units of the AG-I main chain. The signal at 99.7/5.35 and the upfield signal at δ 16.6/1.25 were attributed to anomeric and to the CH_3 groups, respectively, of α-L-Rha*p* units from a type I rhamnogalacturonan (RG-I) portion where the AG-I is probably attached. A comparison of NMR spectrum of ANWS extracted in 2014 (Fig. 4) with that extracted in 2016 (Supplementary Figure 1) showed the presence of the same pectic polysaccharides, and thus, we performed the purification only in the former fraction.



Supplementary Figure 1. ^{13}C-NMR spectrum of ANWS (collected in September 2016). Sample was dissolved in deuterium oxide and data collected at probe temperature of 50ºC.

It is widely accepted in the literature that different structural elements of pectins (HG, RG-I, RG-II and/or xylogalacturonan) form a polysaccharide complex covalently inter-linked. However, the way they are positioned relative to one another in such a macromolecular pectin-complex is still under discussion. Traditional models show HG regions intercalating neutral sugar-branched RG-I, while others indicated an exclusive RG-I backbone where the HG and xylogalacturonan constitute the side chains, together with arabinan, AG-I and/or AG-II (VORAGEN *et al.*, 1995; VINCKEN *et al.*, 2003; YAPO, 2011). In our research group, however, we have observed that the HG can be found free from the other pectic polysaccharides and can be separated by precipitation with Fehling's solution (CANTU-JUNGLES *et al.*, 2014; DO NASCIMENTO *et al.*, 2015; DO NASCIMENTO *et al.*, 2016b;

LEIVAS *et al.*, 2016). Therefore, ANWS fraction was treated with Fehling's solution and, as expected, the HG formed a complex with Cu^{2+} and precipitated (fraction PF-ANWS, 5.5% yield), while the AG-I-RG-I macromolecule remained soluble (fraction SF-ANWS, 0.6% yield). Interestingly, in terms of yield, 68.2% of ANWS corresponded to homogalacturonan (fraction PF-ANWS), which is in agreement with the uronic acid content observed in ANWS (67%, Table 1). Moreover, the presence of HG in PF-ANWS was confirmed by monosaccharide analysis, which showed only uronic acids, while SF-ANWS showed arabinose and galactose in higher amounts than ANWS, probably from AG-I (Table 1). The six typical NMR signals of HG were seen in PF-ANWS ^{13}C-NMR spectrum (Fig. 5A) at δ 98.9 (C-1), δ 68.4 (C-2), δ 69.0 (C-3), δ 78.0 (*O*-substituted C-4), δ 71.4 (C-5) and 175.3 (C-6). A de-esterification occurred due to the employed alkaline pH in the Fehling treatment and the signal at δ 52.8 attributed to methyl carbons of esterified carbonyls in α-D-Gal*p*A units previously seen in ANWS spectrum disappeared, while the C-1 and C-6 signals shifted to δ 98.9 and δ 175.3, respectively.

Regarding ANWS-SF fraction, its ^{13}C-NMR DEPT 135 spectrum (Fig. 5B) showed intense signals at δ 104.4 (C-1), δ 77.7 (*O*-substituted C-4), δ 74.5 (C-5), δ 73.4 (C-3), δ 71.9 (C-2) and δ 60.7 (inverted C-6) that corresponded to 4-*O*-linked-β-D-Gal*p* units from AG-I main chain. Moreover, another anomeric signal at δ 103.2 was also observed and together with an inverted signal at δ 69.5 indicate the presence of 6-*O*-linked-β-D-Gal*p* units, probably from AG-II. Signals of AG side chains were observed at δ 109.1 and δ 107.6 from C-1 and that at δ 67.0 from substituted C-5 of α-L-Ara*f* units. Signals from RG-I backbone could be seen at δ 98.3 and δ 97.7, assigned respectively to C-1 of →2)-Rha*p*-(1→ and →4)-Gal*p*A-(1→ units, and that at δ 16.6 relative to CH_3 of α-L-Rha*p* units (DO NASCIMENTO *et al.*, 2015; LEIVAS *et al.*, 2016).

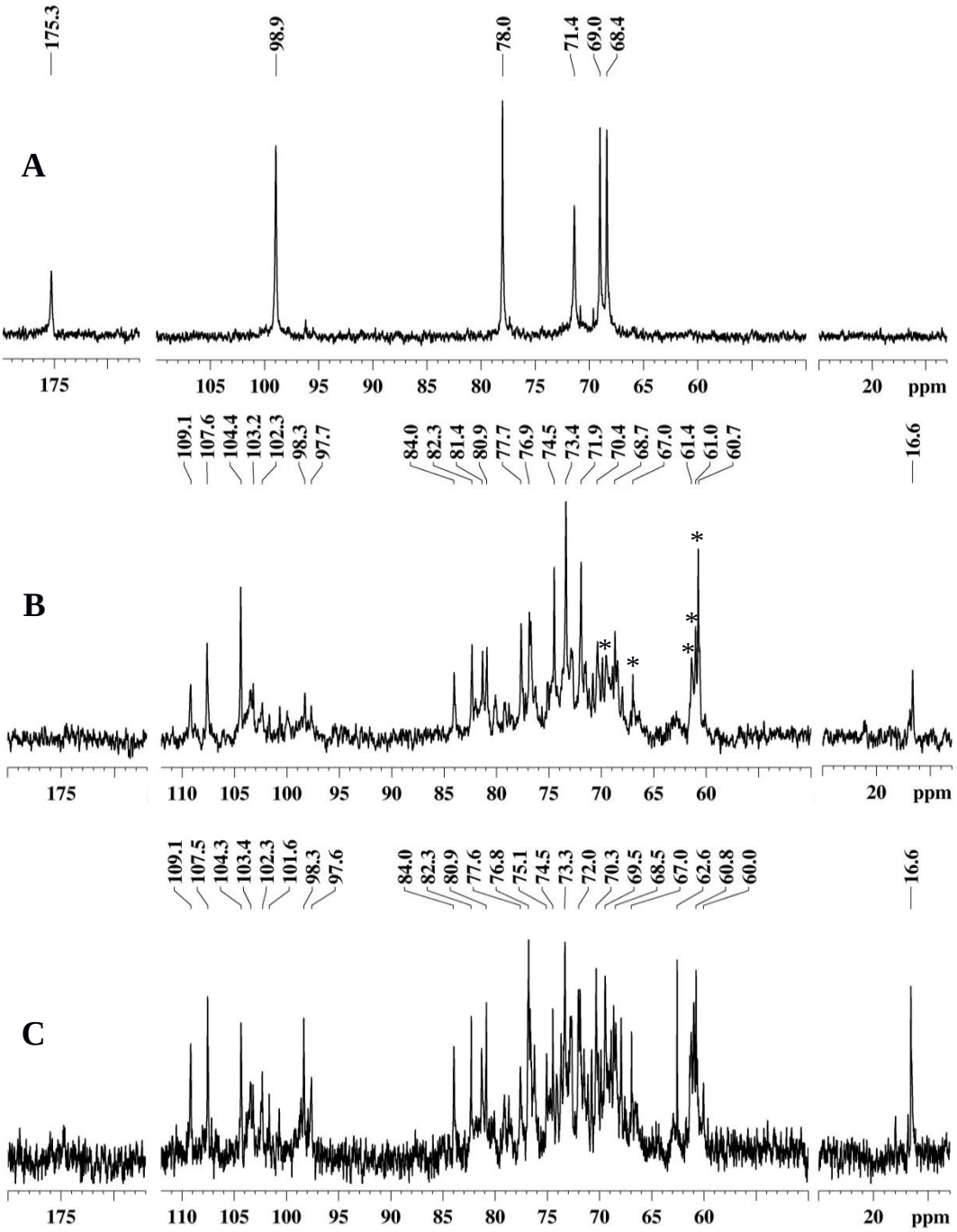


Figure 5. ^{13}C-NMR spectra of fractions: PF-ANWS (A), SF-ANWS with inverted signals in DEPT 135 experiment marked with asterisk (B) and ANWS-50E (C). Samples were dissolved in deuterium oxide and data collected at probe temperature of 50 ºC.

As observed on HPSEC analysis, SF-ANWS showed a heterogeneous elution profile, with three peaks (I, II, III) (Fig. 3). Therefore, it was further fractionated by ultrafiltration through 50 kDa cut-off membrane. The retained material (ANWS-50R, 0.2% yield),

corresponding to peak I, was largely homogeneous and had a Mw of 5.3 x 10^4 g/mol (Fig. 3). The filtrate from the ultrafiltration (ANWS-50E, 0.3% yield) was heterogeneous and contained mostly peaks II and III from ANWS-SF (data not shown).

The ANWS-50E fraction, on monosaccharide analysis, showed mainly arabinose, galactose, xylose and uronic acids (Table 1). Its ^{13}C-NMR (Fig. 5C) spectrum suggested the presence of AG-I, AG-II and RG-I similar to that previously observed for ANWS-SF (Fig. 5B). On the other hand, the purified ANWS-50R was composed of rhamnose, arabinose, galactose and uronic acids (Table 1) and the presence of type II arabinogalactan was evidenced by methylation analysis (Table 2). The methylated derivatives 2,3,4-Me_3-Gal-ol-acetate, 2,4,6-Me_3-Gal-ol-acetate and 2,4- Me_2-Gal-ol-acetate arose from the typical AG-II galactan main chain formed by 6-*O*-, 3-*O*- and 3,6-*O*-linked Gal*p* units, respectively. Unusual derivatives, such as 2,3-Me_2-Gal-ol-acetate and 2-Me-Gal-ol-acetate, from 4,6-*O*- and 3,4,6-*O*-linked Gal*p* units were also observed. This latter has already been reported for AG-II isolated from various sources (PONDER e RICHARDS, 1997; KANG *et al.*, 2011; CORRÊA-FERREIRA *et al.*, 2014; CARLOTTO *et al.*, 2016), while the first has also been found in some AG-I (IACOMINI *et al.*, 2005; CANTU-JUNGLES *et al.*, 2014; DO NASCIMENTO *et al.*, 2015; LEIVAS *et al.*, 2015). However, the presence of both methylated derivatives has also been described for AG-II from *Angelica acutiloba* roots (ZHANG *et al.*, 1996; LIM *et al.*, 2016). The occurrence of structural variations within different arabinogalactans was pointed out by some authors. HINZ *et al.* (2005) and TANAKA *et al.* (2010) found 3-*O*-linked Gal*p* units interspersing the (1→4)-linked galactan chain of AG-I from potato, onions and gum exuded from *Cereus peruvianus*, while XU *et al.* (2010) isolated from *Platycodon grandiflorum* roots an AG with 4-*O*- and 6-*O*-linked Gal*p* units in the backbone.

Among the side chains, 5-*O*-, 3-*O*- and 3,5-*O*-linked Ara*f* units were observed in ANWS-50R. The terminal units were formed by Ara*f*, Gal*p*, carboxyl-reduced Glc*p*A and Rha*p*. These are often present in AG-II and may be attached directly to the galactan core or via arabinofuranosyl residues (GASPAR *et al.*, 2001). Finally, the methylated derivatives 2,3,6-Me_3-Gal-ol-acetate, 3,4-Me_2-Rha-ol-acetate and 3-Me-Rha-ol-acetate arose from carboxyreduced 4-*O*-linked Gal*p*A, 2-*O*-linked Rha*p* and 2,4-*O*-linked Rha*p* units, while 2,3,6-Me_3-Gal-ol-acetate probably comes from carboxyl-reduced 4-*O*-linked Gal*p*A units, which are structural elements of type I rhamnogalacturonan.

These results were corroborated by ANWS-50R NMR analysis (Fig. 6). The $^{1}H/^{13}C$ HSQC- DEPT showed anomeric signals at δ 103.2/4.47, assigned to 6-*O*- and 3,6-*O*-linked β-D-Gal*p* units and at δ 103.7/4.70 and δ 104.2/4.63 attributed to internal 3-*O*-linked β-D-Gal*p* and terminal β-D-Gal*p* linked to neighboring 3-*O*-linked β-D-Gal*p* units, respectively. In addition, inverted C-6 signals of these units can be seen at δ 69.4/4.04-3.91, corresponding to substituted C-6/H-6 of 3,6 and 6-*O*-linked β-D-Gal*p* units and that at δ 61.2/3.82-3.72 corresponding to nonsubstituted C-6/H-6 of terminal and 3-*O*-linked β-D-Gal*p* units. The substituted C-3/H-3 of 3-*O*- and 3,6-*O*-linked β-D-Gal*p* were observed at δ 81.9/3.85 and 80.1/3.73, respectively. Regarding Ara*f* units, the intense anomeric signal at δ 109.0/5.25 was attributed to overlapped terminal and 3-*O*-linked α-L-Ara*f* units, while 5- and 3,5-*O*-linked α-L-Ara*f* units can be observed at δ 107.5 /5.08. Inverted substituted C-5/H-5 from 5-*O*- and 3,5-*O*-linked α-L-Ara*f* units appeared at δ 66.9/3.88-3.80, while those unsubstituted from terminal and 3-*O*-linked α-L-Ara*f* were observed at δ 62.6/3.63-3.56. The RG-I core was demonstrated by anomeric signals at δ 97.8/5.04 and δ 98.3/5.29 which corresponded to α-D-Gal*p*A and α-L-Rha*p* units, respectively. Signals at δ 16.5/1.25 and at δ 16.7/1.31 were attributed to C-6/H-6 of 2-*O*- and 2,4-*O*-linked α-L-Rha*p* units, respectively. Finally, the signal at δ 100.7/4.79 could be assigned to terminal α-L-Rha*p* present in the AG-II side chains. All the assignments are in agreement with published literature data (BRECKER *et al.*, 2005; TAN *et al.*, 2010; MAKAROVA *et al.*, 2016; SHAKHMATOV *et al.*, 2016; YANG *et al.*, 2016; YUAN *et al.*, 2016).

4. Conclusion

Taken together, our results demonstrated that, in addition to homogalacturonan (POPOV *et al.*, 2011), the aqueous extract of sweet bell pepper was composed of approximately 7% of type I and type II arabinogalactans anchored in type I rhamnogalacturonan. This is the first study that reports the presence of all these structures in sweet pepper fruits. Moreover, a type II arabinogalactan was purified and its detailed structural characterization was performed. These polysaccharide structural data may be helpful in structure–function relationship studies.

Table 2. Linkage types based on analysis of partially *O*-methyl alditol acetates of purified ANWS-50R from sweet pepper fruits (*C. annuum*).

Partially *O*-methylalditol acetate	ANWS-50R mol%[b]	Linkage type[c]
Arabinose		
2,3,5-Me_3-Ara[a]	13.8	Ara*f*-(1→
2,5-Me_2-Ara	1.3	→3)-Ara*f*-(1→
2,3-Me_2-Ara	8.8	→5)-Ara*f*-(1→
2–Me-Ara	1.2	→3,5)-Ara*f*-(1→
Galactose		
2,3,4,6-Me_4-Gal	11.7	Gal*p*-(1→
2,3,4-Me_3-Gal	15.4	→6)-Gal*p*-(1→
2,4,6-Me_3-Gal	3.9	→3)-Gal*p*-(1→
2,3,6-Me_3-Gal	4.2[d]	→4)-Gal*p*-(1→
2,3- Me_2-Gal	5.2	→4,6)-Gal*p*-(1→
2,4- Me_2-Gal	16.5	→3,6)-Gal*p*-(1→
2- Me-Gal	0.5	→3,4,6)-Gal*p*-(1→
Glucose		
2,3,4,6-Me_4-Glc	5.0[d]	Glc*p*-(1→
Rhamnose		
3,4,6-Me_3-Rha	4.0	Rha*p*-(1→
3,4- Me_2-Rha	5.3	→2)-Rha*p*-(1→
3-Me-Rha	3.2	→2,4)-Rha*p*-(1→

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc. [b] According PETTOLINO et al. (2012). Samples were carboxy-reduced by the carbodiimide method (TAYLOR E CONRAD, 1972), prior to methylation analysis. [c] Based on derived *O*-methylalditol acetates. [d] Percentage of carboxy-reduced uronic acids (12%).

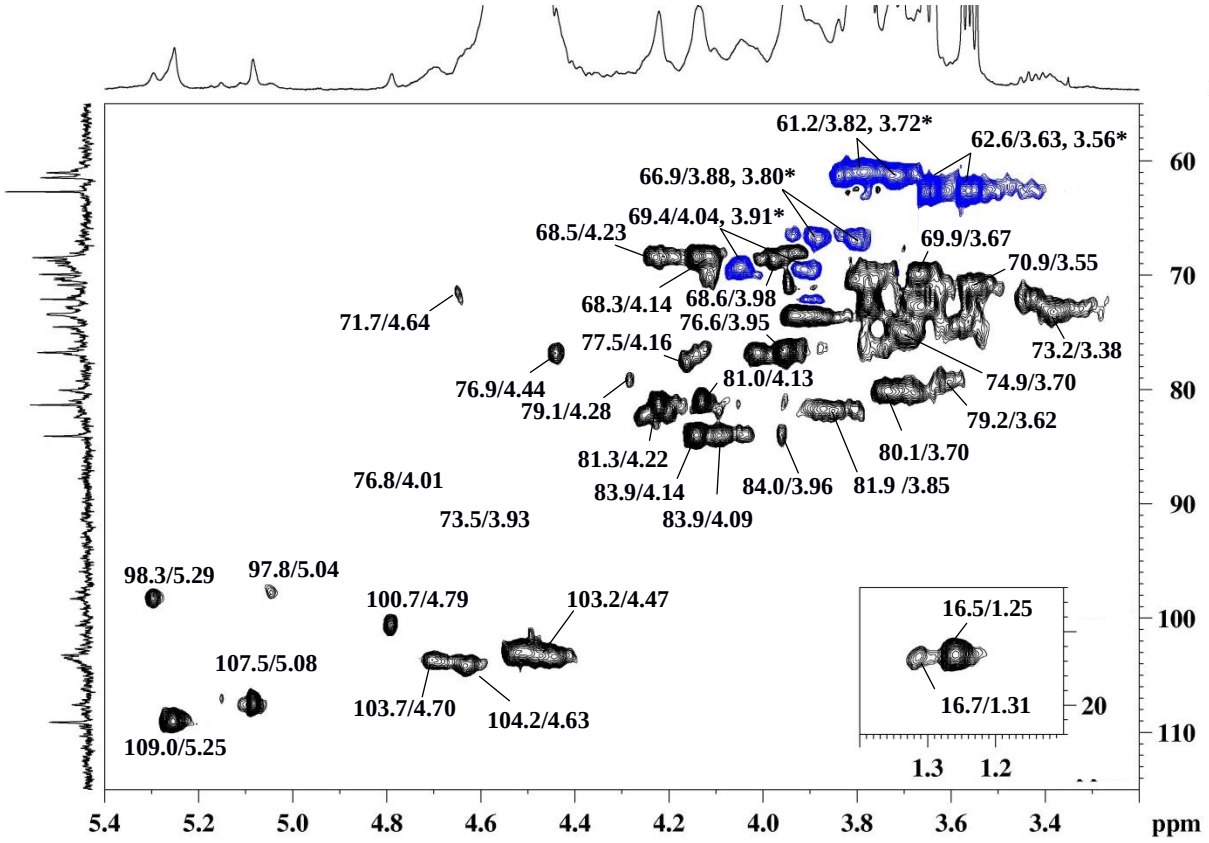


Figure 6. $^{1}H/^{13}C$ HSQC-DEPT correlation map of ANWS-50R fraction. Sample was dissolved in deuterium oxide and data collected at probe temperature of 70 ºC. Inverted signals in DEPT experiment are marked with an asterisk (*).

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ARTIGO V

The influence of sweet pepper pectin structural characteristics on cytokine secretion by THP-1 macrophages

The influence of sweet pepper pectin structural characteristics on cytokine secretion by THP-1 macrophages

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ABSTRACT

Pectins can modulate the biological responses interacting directly with immune cells. The observed responses can strongly be affected by polysaccharide structural features. We analyzed the intrinsic activation capacity of native (ANWS) and modified (ANWS-M) sweet pepper pectin on cytokine secretion by THP-1 cells as well as compare their effects in the presence of LPS. ANWS-M was obtained by partial acid hydrolysis which promoted the removal of ANWS side chains as well as the reduction of its molecular weight and degree of methyl esterification (DM). The results showed that both fractions had no effect on THP-1 viability. ANWS at 300 μg/mL increased TNF-α, IL-1β and IL-10 secretion by THP-1 macrophages. However, in the presence of LPS, ANWS can attenuate the inflammatory response by reducing the production of the pro-inflammatory cytokines TNF-α and IL-1β and increasing the anti-inflammatory cytokine IL-10, as well as decreasing the TNF-α/IL-10 and IL-1β/ IL-10 ratios. The structural modifications caused by acid hydrolysis affected the intrinsic activation capacity of ANWS to modulate the cytokine secretion. These results indicate that DM, molecular weight and presence of side chains are important structural features of pectins involved in the modulation of cytokine secretion by THP-1 macrophages.

Keywords: sweet pepper pectin, THP-1 macrophages, cytokine secretion, immunomodulatory activity, structure-function relationship.

1 Introduction

High dietary fiber intake is part of heath dietary recommendations and is related to reduction in systemic inflammation (AJANI *et al.*, 2004), property that has been attributed mainly to microbiota-dependent effects, especially relative of short-chain fatty acids generate by their fermentation (ANDOH *et al.*, 1999; MEIJER *et al.*, 2010; LANDBERG, 2012). However, some studies have demonstrated dietary fibers are able to cross the small intestinal epithelium and can be found in blood and Peyer's patches where interacting directly with immune cells (WISMAR *et al.*, 2010; MCDOLE *et al.*, 2012; COURTS, 2013; SUH *et al.*, 2013). Potential mechanisms which dietary fibers contact with immune allowing the modulation of biological responses after oral intake are very well detailed by VOS *et al.* (2007).

Pectins, a component of soluble dietary fibers, are acidic polysaccharides with significant chemical heterogeneity, which determinate diverse biological activities from stimulation to suppression of the immune response (SCHEPETKIN *et al.*, 2008; POPOV e OVODOV, 2013). Linear domains formed by homogalacturonans and branched rhamnogalacturonan regions ramified mainly by arabinogalactans are the most frequent reported pectins, although their detailed structural characteristics vary depending on the source and type of extraction (BURTON et al., 2010).

Here, we investigated the intrinsic activation capacity of a pectic fraction isolated from sweet pepper fruits to induce cytokine secretion by THP-1 macrophages and to modulate the LPS pro-inflammatory response. The polysaccharide fraction was constituted by high methyl esterified homogalacturonan associated with arabinogalactans anchored in rhamnogalacturonan. In addition, in order to study structure–function relationship we introduced structural modifications in the tested pectin and compared its biological effects with native fraction.

2. Materials and methods

2.1 Isolation of pectic polysaccharides

Fresh green sweet pepper fruits (*Capsicum annuum* L. cv Magali) were purchased from the organic sector of the municipal market in Curitiba, Paraná, Brazil. Fruits without seeds were freeze-dried and defatted with chloroform–methanol (1:1). Polysaccharides were extracted from the residue with water at 100 ºC for 2 h (×6, 1 L each) and precipitated from the extract with EtOH (3 vol). Freeze–thaw treatment was applied to give cold-water soluble fraction (having native sweet pepper pectin named ANWS). It was then characterized by monosaccharide analysis, degree of methyl and acetyl esterification, homogeneity, molecular weight (high performance size exclusion chromatography - HPSEC) and NMR analyses (DO NASCIMENTO *et al.*, 2016b).

2.2 Partial acid hydrolysis

In order to remove the side chains, ANWS was submitted to partial acid hydrolysis with HCl 0.1 M, at 90 °C for 16 hours. It was then submitted to dialysis (6-8 kDa cut-off dialysis tubes) against distilled water (for 48 h) and the retained material (ANWS-M, modified pectin) was freeze-dried. For structural characterization the sugar composition, homogeneity, molecular weight and NMR analyses were also performed (DO NASCIMENTO *et al.*, 2016b).

2.3 Detection of LPS contamination

As a control of LPS contamination, ANWS and ANWS-M were submitted to methanolysis, acetylated and analyzed by GC-MS as previously described in detail by DE SANTANA-FILHO *et al*. (2012). The detection was based on 3-hydroxy fatty acids methyl esters as specific chemical markers for LPS and the quantification using a standard curve obtained with endotoxin from Escherichia coli O111:B4 (Sigma).

2.4 Cell culture and macrophage differentiation

THP-1 (human monocytic cell line) cells were grown in RPMI 1640 culture medium (Sigma–Aldrich, cat. R8758) supplemented with 10% heat-inactivated fetal calf serum Sterile A (Gibco, cat. 161010-159), 100 µg/mL streptomycin and 100 U/mL penicillin (Sigma–Aldrich), in a humidified incubator at 37 °C in 5% CO_2. The medium was renewed twice a week. The cells were grown to a density of 1-8x10^5 cells/mL and used in the experiments at maximum passage of 10.

The mature macrophage-like state was induced by treating THP-1 monocytes (2x10^5 cells/mL) for 48 h with 5 ng/mL (~8 nM) phorbol 12-myristate 13-acetate (PMA; Sigma–Aldrich). This concentration was chosen following PARK *et al.* (2007), which stated that it is enough to induce stable differentiation of monocytes cells to macrophages without undesirable upregulation of pro-inflammatory genes and activated phenotype, with good response to a secondary weak stimulus. The cells were cultured in 96-well culture plates with 200 µl cell suspension/well for citotoxity assessment or in 24-wells culture plates with 1 mL cell suspension/well for cytokine measurement. After differentiation, cells are adhered to the surface allowing removal of the culture medium with PMA, then the wells were washed twice with sterile PBS (phosphate buffered saline) and fresh medium, free of PMA, was replaced. Then, the THP-1 macrophages (differentiated cells) were incubated for 24 h at 5% CO_2, at 37 ºC, for resting.

2.5 Citotoxicity of ANWS and ANWS-M on THP-1 macrophages

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure the cytotoxic activity of ANWS or ANWS-M fractions. The assay is based on the reduction of MTT salt by mitochondrial dehydrogenase to purple formazan product, which reflects the normal functioning of mitochondria and hence the metabolic rate of cells (REILLY *et al.*, 1998). After differentiation of THP-1 cell into macrophages and resting, the medium was removed and replaced by fresh medium containing the fractions at 10, 30, 100 and 300 µg/mL, or sterile PBS (50 µL) in quintuplicate and then, incubated for 24 h in a humidified 5% CO_2 atmosphere at 37 ºC. After the incubation period, 20 µL of MTT solution (5 mg/mL) was added to each well. After 3 h of incubation, the media were removed and

formazan crystals were solubilized in DMSO (100 μL/well). The absorbance was measure at a wavelength of 595 nm.

2.6. Effect of ANWS and ANWS-M on TNF-α, IL-1β and IL-10 secretion by THP-1 macrophages

After differentiation and resting THP-1 macrophages were exposed to some treatments with (a) fresh medium containing ANWS (at 10, 30, 100 and 300 μg/mL) or ANWS-M (at 300 μg/mL) or sterile PBS (negative control, 50 μL) or lipopolysaccharide (LPS; at 100 ng/mL, pro-inflammatory control) and (b) fresh medium containing ANWS (at 300 μg/mL) plus LPS (100 ng/mL) or ANWS-M (at 300 μg/mL) plus LPS (100 ng/mL). The cells were then incubated in humidified 5% CO_2 atmosphere at 37 ºC for 18 h. This exposure time was chosen since CHANPUT *et al.* (2010) observed that the maximal cytokines secretion upon LPS-induced inflammation occurred at this time point. At the end of the incubation period the cell-free supernatants were collected and stored at - 80 °C for measurements of cytokines secretion. The concentration of pro-inflammatory (TNF-α, IL-1β) and anti-inflammatory (IL-10) cytokines was quantified by Elisa Ready-Set-go kits specific for human cytokines (eBioscience kits, cat. n^{o}. 88-7346, 88-7010 and 88-7106, respectively), according to the manufacturer's instructions. Results are presented as secretion index (SI), which is the concentration of the cytokine in the medium containing the treatments divided by the concentration of the cytokine in medium containing PBS.

2.7. Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA). As a post hoc test the Newman-Keuls Multiple Comparison test was used. The threshold of statistical significance was $p < 0.05$. The graphs were drawn and the statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Structural characterization of ANWS and ANWS-M fractions

ANWS fraction was composed mainly of uronic acids (67%) with minor amounts of galactose (6.7%), arabinose (6.4%), rhamnose (1.6%), xylose (0.3%) and glucose (4.4%). The ^{13}C NMR spectrum of ANWS (Fig. 1A) presented typical signals of a methyl esterified homogalacturonan (HG). The signals at δ 100.1 (C-1), δ 67.8 (C-2 and C-3 overlapped), δ 78.7 (*O*-substituted C-4), δ 70.5 (C-5), δ 174.7 (C-6) were attributed to methyl esterified α-D-Gal*p*A units, while those at δ 99.4 (C-1), δ 67.8 (C-2 and C-3 overlapped), δ 78.7 (*O*-substituted C-4), δ 71.4 (C-5), δ 170.5 (C-6) were assigned to α-D-Gal*p*A unesterified units. Signals of methyl and acetyl groups linked to α-D-GalpA units appeared at δ 52.8 and δ 20.3, respectively, and thus the degree of methyl esterification (DM) and degree of acetylation (DA) were determined by ^{1}H NMR spectroscopy, giving values of 85% and 5%, respectively. Besides the HG signals, ANWS also presented signals at δ 104.3 (C-1), δ 71.8 (C-2), δ 73.2 (C-3), δ 77.5 (*O*- substituted C-4), δ 74.4 (C-5) and δ 60.7 (C-6) that can be assigned to (1→4)-linked-β-D-Gal*p* units, probably from a type I arabinogalactan (AG-I) main chain. The signals of α-L-Ara*f* units were not visible in the ANWS ^{13}C NMR spectrum, but on ^{1}H/^{13}C HSQC appeared at δ 107.6/5.07 (C-1/H-1, data not shown). An upfield signal at δ 16.6/1.25 was also observed in the ^{1}H/^{13}C HSQC and was attributed to the CH_3 groups of α-L-Rha*p* units from a type I rhamnogalacturonan (RG-I) portion where the AG-I is probably attached. Thus, ANWS consist mainly of highly methoxylated homogalacturonan, together with type I arabinogalactan anchored in rhamnogalacturonan.

In order to remove the side chains, ANWS was submitted to partial acid hydrolysis, giving rise to a modified fraction (ANWS-M), which presented uronic acids (91%) and rhamnose (9 %) in the sugar composition analysis. Accordingly, comparative analysis of its^{13}C NMR spectrum with that of ANWS (Fig. 1A and B) showed the disappearance of the signals attributed to the (1→4)-linked-β-D-Gal*p* units of galactan core. Some de-esterification occurred as could be observed by the decrease of the signals at δ 52.8 (-COO-$\underline{C}H_3$) and at δ 100.1 (C-1 of methyl esterified α-D-GalpA units) and by the increase of the signal at δ 99.5 (C-1 of unesterified α-D-GalpA units), giving a DM of 17% by ^{1}H NMR spetroscopy. The signal of acetyl group at δ 20.2 also disappeared. The CH_3-signal of Rha*p* units at δ 16.6/1.25

were observable only in the $^{1}H/^{13}C$ HSQC (data not shown). As expected, the structural analysis showed the removal of the pectin side chains by the partial acid hydrolysis and that ANWS-M consisted mainly of a linear polysaccharide formed by a low methyl esterified homogalacturonan. Due to the presence of rhamnose units, a small portion of the type I rhamnogalacturonan core remained in the fraction.

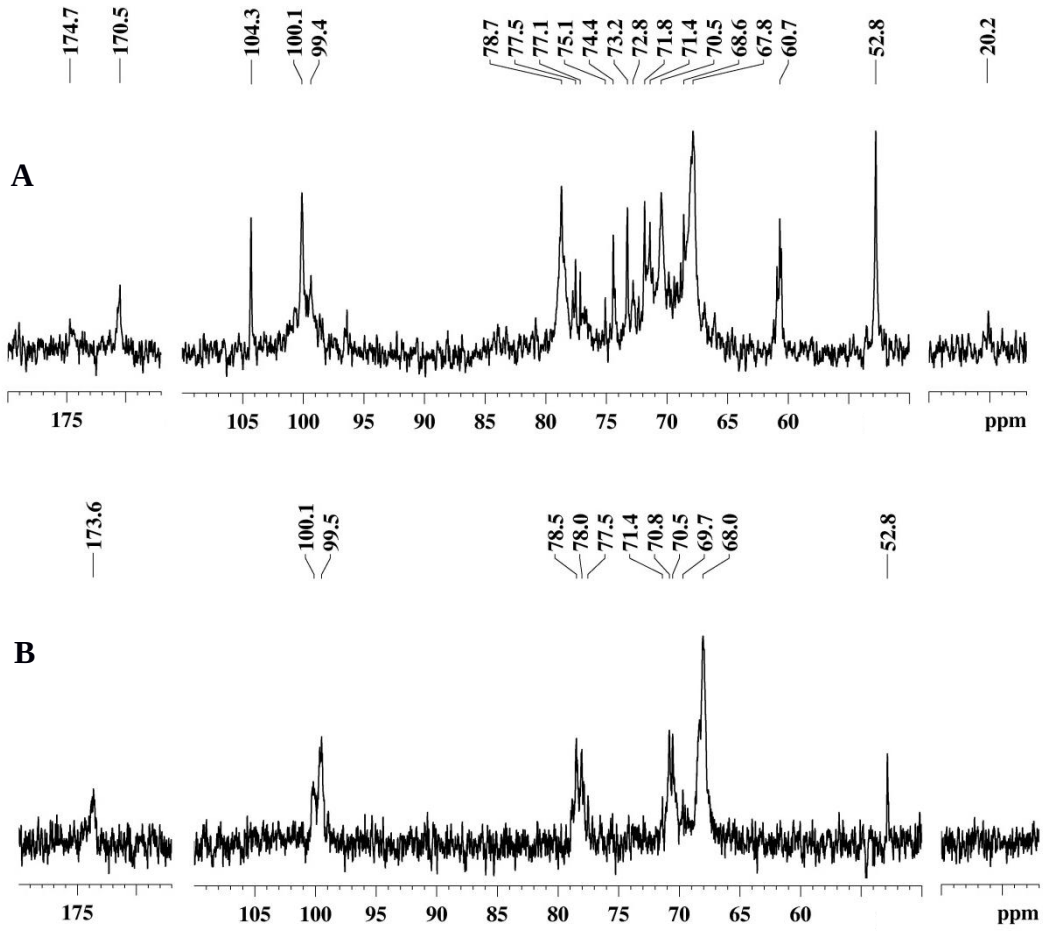

Figure 1. ^{13}C-NMR spectra of fractions ANWS (A) and ANWS-M (B). Samples were dissolved in deuterium oxide and data collected at probe temperature of 50 ºC.

When analyzed by size exclusion chromatography (HPSEC) (Fig. 2), native ANWS showed a single poly disperse peak, with an average molecular weight (Mw) of 367 kDa. The treatment with HCl generated a polymer that eluted as a narrow peak, with lower Mw (36 kDa).

The ANWS was negative for LPS contamination, while ANWS-M showed a LPS content of 4.0 ng/mg of dry weight. Considering that 300 μg/mL of this fraction was added to the cells, only 1.2 ng/mL of LPS was present as contaminant, which is too low to interfere in the results.

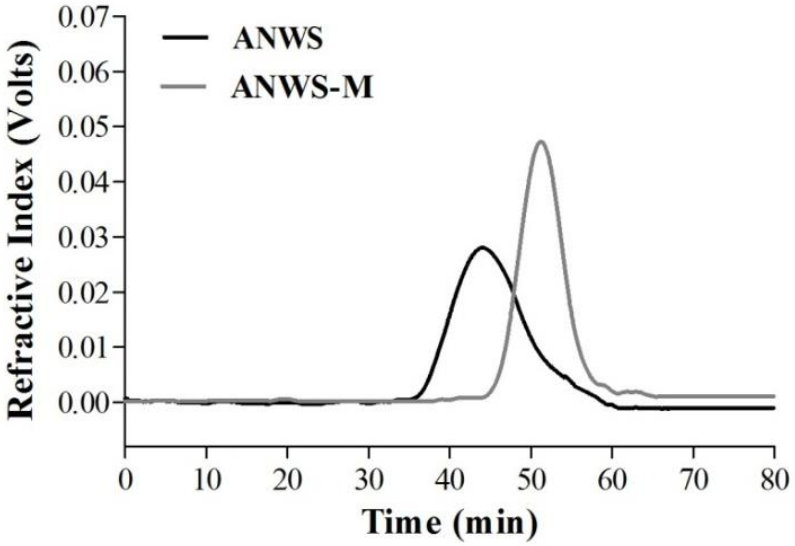

Figure 2. HPSEC elution profiles of fractions ANWS and ANWS-M. Refractive index detector.

3.2. ANWS and ANWS-M did not affect viability of the THP-1 macrophages

When incubated with THP-1 macrophages for 24 h, neither native ANWS nor ANWS-M fraction at 10, 30, 100 and 300 μg/mL showed cytotoxic effects (Fig. 3). Instead, an apparent increase (not statistically significant) in the cell viability, mainly for ANWS could be observed. This probably be due to the activation of macrophages, since when these cells are activated, an increase in their metabolism may occur, which could be evidenced by MTT assay (WU *et al.*, 2014a; AMORIM *et al.*, 2016; LV *et al.*, 2016).

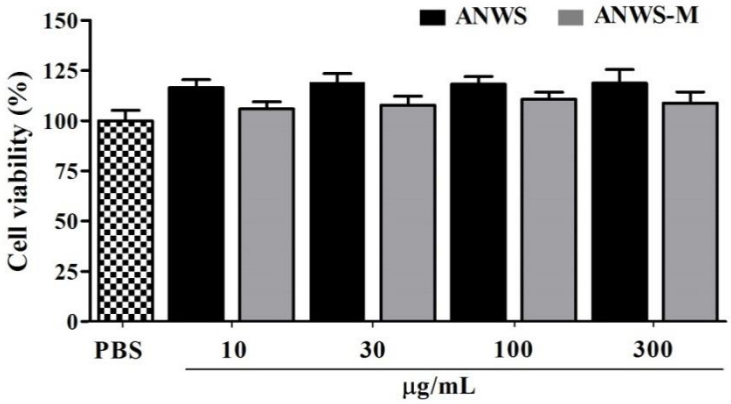

Figure 3. Effects of ANWS and ANWS-M on the viability of THP-1 macrophages. THP-1 macrophages were exposed for 24 h with pectins at the indicated concentrations. Cell viability was determined by MTT assay. Culture medium with PBS was used as negative control, corresponding to 100% viability. Data represent mean ± SEM (n = 2, each experiment in quintuplicate).

3.3. ANWS increased TNF-α and IL-1β secretion at 300 µg/mL and IL-10 at all tested doses

The intrinsic activation capacity of ANWS on THP-1 macrophage cytokine (TNF-α, IL-1β and IL-10) secretion is shown in Fig. 4. The basal level (PBS - negative control) of both TNF-α and IL-1β secretion by THP-1 macrophages were approximately 12 to 16 pg/mL. When stimulated with LPS (100 ng/mL, positive control), THP-1 macrophages increased the release of these cytokines around 15 and 7-fold, respectively. Macrophages treated with ANWS at 10, 30 and 100 µg/mL presented similar levels of these cytokines when compared to the negative control. On the other hand, when treated with ANWS at 300 µg/mL, the increase of TNF-α and IL-1β secretion by THP-1 macrophages became evident (almost 14.4-fold and 4-fold, respectively), and for TNF-α was statistically equivalent to LPS – induced secretion (Fig. 4A and B). Regarding the production of the anti-inflammatory IL-10 cytokine, an increase was observed for LPS and ANWS at all tested concentrations compared with negative control. ANWS at 300 µg/mL induced the highest IL-10 macrophage secretion (6.0 fold), higher than that LPS stimulated one (4.8 fold).

3.4 ANWS alters the cytokine secretion induced by LPS

THP-1 macrophages were cultured with simultaneous addition of ANWS at 300 μg/mL and LPS (100 ng/mL). Interestingly, the TNF-α and IL-1β secretion decreased when compared with LPS alone (Fig. 5). When compared with ANWS stimulus alone, the secretion induced by ANWS plus LPS was lower for TNF-α and higher for IL-1β. On the other side, ANWS plus LPS increased the secretion of IL-10 when compared to both LPS or ANWS alone. Then, we calculated the ratio between pro- (TNF-α and IL-1β) and anti-inflammatory (IL-10) cytokines produced by LPS and ANWS with LPS stimuli. The TNF-α/IL-10 ratios were 3.4 and 1.0, while for IL-1β/ IL-10 ratios the obtained values were 1.6 and 0.6, respectively (Fig. 6).

3.5 The production of cytokines is altered by removal of ANWS side chains

To evaluate the effect of side chains removal, the acid hydrolyzed fraction ANWS-M (at 300 μg/mL) was also tested, alone and with simultaneous addition of LPS. Our results showed that ANWS-M induced the lowest TNF-α secretion, as that observed for PBS control. The IL-1β and IL-10 production was similar to LPS treated cells. When compared to native ANWS, TNF-α and IL-10 secretion reduced, while IL-1β secretion increased (Fig. 5).

The simultaneous addition of LPS to ANWS-M treated THP-1 macrophages had no difference on cytokine production when compared with ANWS-M alone. Nevertheless, when compared with native ANWS in the presence of LPS, ANWS-M induced lower TNF-α and IL-10 secretion and similar IL-1β secretion.

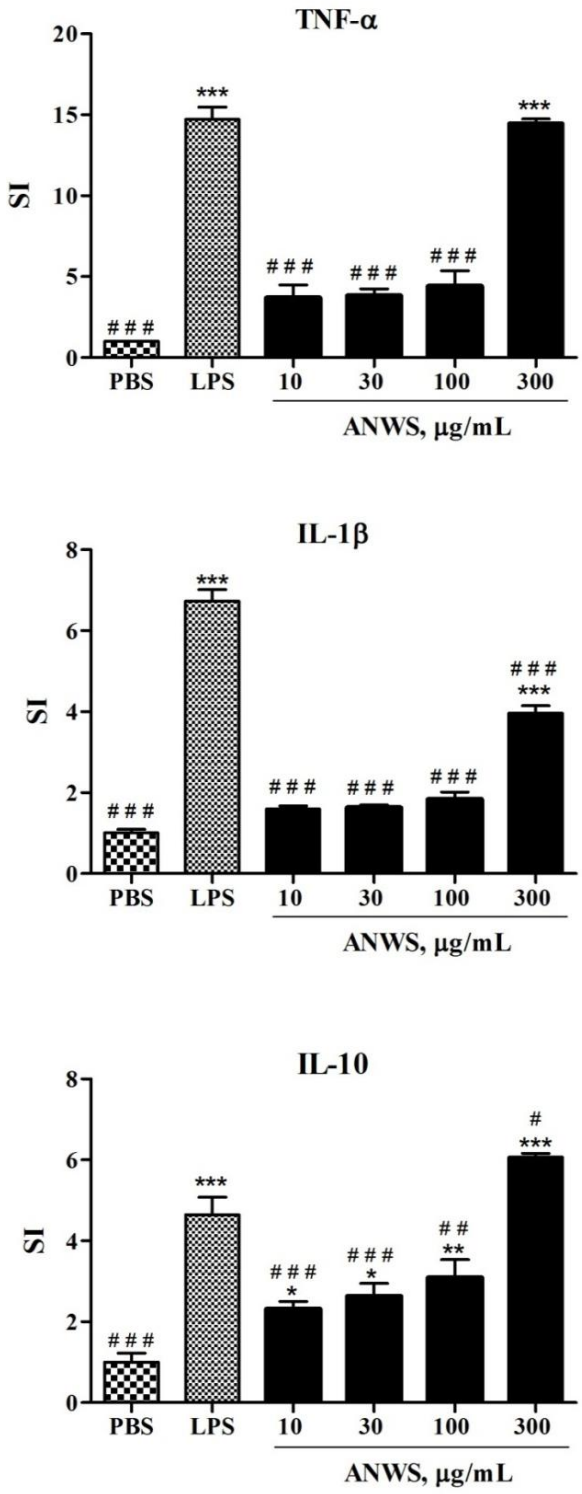

Figure 4. The intrinsic activation capacity of ANWS to induce TNF-α, IL-1β and IL-10 secretion by THP-1 macrophages. The cells were incubated for 18 h with indicated concentrations of ANWS or positive control (LPS, 100 ng/mL) or negative control (PBS).The results are expressed as means of secretion index (SI) ± SEM for two independent experiments in quadruplicate. Asterisks (*) represents statistically significant difference from the negative control-PBS (* $p < 0.05$, *** $p < 0.001$). Hash marks (#) represents statistically significant difference from the positive control-LPS (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$).

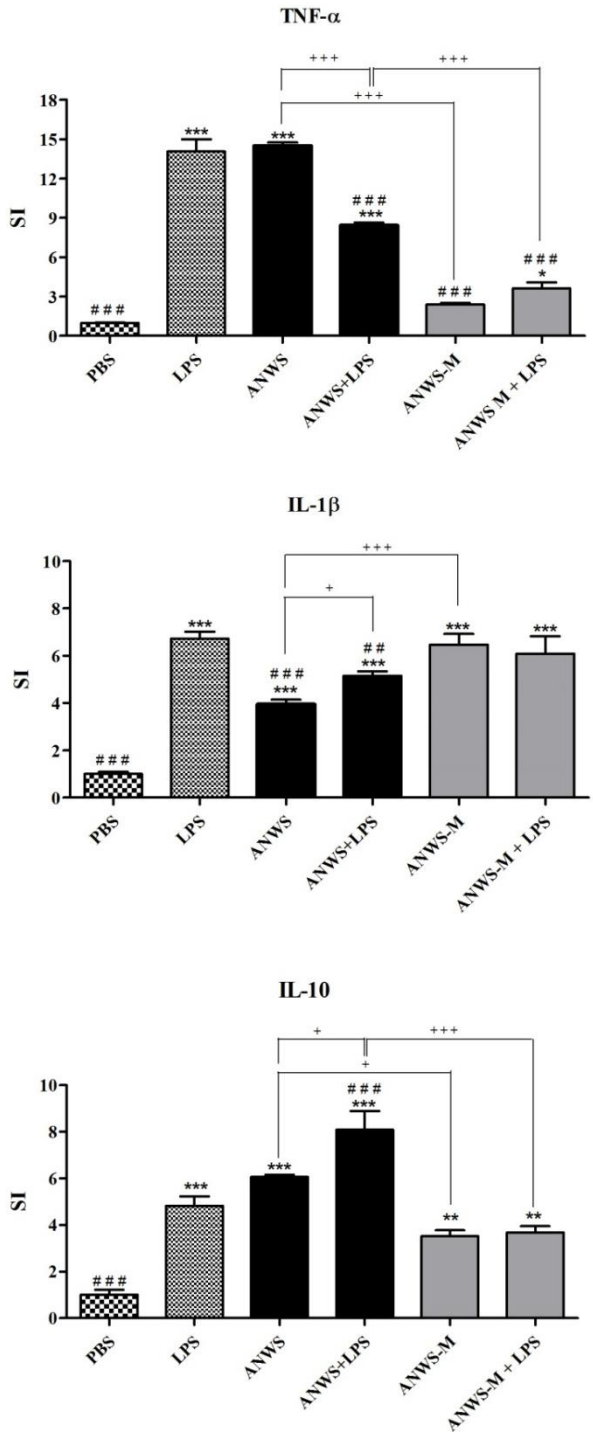

Figure 5. The intrinsic activation capacity of ANWS-M to induce TNF-α, IL-1β and IL-10 secretion by THP-1 macrophages and the effects of ANWS (300 µg/mL) and ANWS-M (300 µg/mL) on modulation of the LPS-pro-inflammatory response. The cells were incubated for 18 h with treatments or positive control (LPS, 100 ng/mL) or negative control (PBS). The results are expressed as means of secretion index (SI) ± SEM for two independent experiments in quadruplicate. Asterisks (*) represents statistically significant difference from the negative control-PBS (* $p < 0.05$, *** $p < 0.001$). Hash marks (#) represents statistically significant difference from the positive control-LPS (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). Symbol (+) represents statistically significant difference between the treatments (+ $p < 0.05$, + + $p < 0.01$, + + + $p < 0.001$).

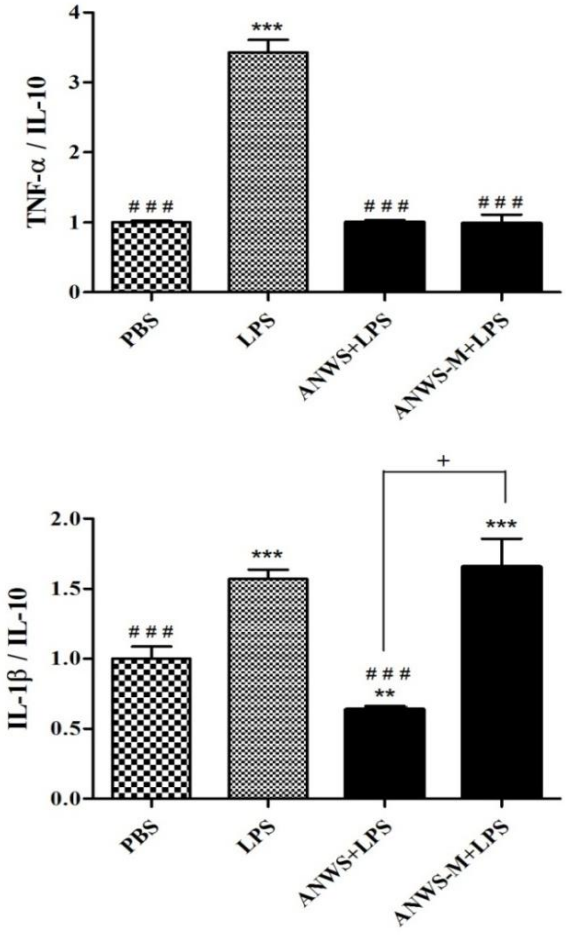

Figure 6. Effects of ANWS or ANWS-M on TNF-α/IL-10 and IL-1β/IL-10 ratios. The ratio was calculated dividing the SI of the pro-inflammatory cytokines by the SI of IL-10. The cells were incubated for 18 h with treatments (ANWS or ANWS-M plus LPS, 300 μg/mL) or positive control (LPS, 100 ng/mL) or negative control (PBS). Asterisks (*) represents statistically significant difference from the negative control-PBS (* $p < 0.05$, *** $p < 0.001$). Hash marks (#) represents statistically significant difference from the positive control- LPS (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). Symbol (+) represents statistically significant difference between the treatments (+ $p < 0.05$).

4 Discussion

In order to investigate the structure–function relationship of pectins on immune system modulation, we studied the cytokine secretion by THP-1 macrophages induced by native and modified pectins from sweet pepper fruits. The native pectin was composed of a highly methoxylated homogalacturonan, together with type I arabinogalactan anchored in rhamnogalacturonan. This was submitted to partial acid hydrolysis with HCl, which caused

the loss of its side chains, giving a linear polymer (ANWS-M) formed mainly by homogalacturonan with some rhamnogalacturonan insertions.

THP-1 cells were chosen because they are widespread used in *in vitro* studies due to their capacity to acquire phenotypic and functional characteristics that closely resemble those of primary human macrophages (CHANPUT *et al.*, 2014). The results showed herein that both fractions were non-cytotoxic to THP-1 cells at all tested concentrations. We studied the intrinsic activation capacity of sweet pepper pectins on cytokine secretion and found that native ANWS pectin could induce TNF-α, IL-1β and IL-10 secretion by THP-1 cells, but only at the highest employed concentration (300 μg/mL).FREYSDOTTIR *et al.* (2016) observed that a pectic polysaccharide from *Achillea millefolium* did not stimulate cytokine secretion by THP-1 monocytes (except to IL-1ra), but the highest evaluated dose was 100 μg/mL. However, at 200 μg/mL a partially deacetylated and de-esterified pectin from cacao (*Theobroma cacao*) pod husks increased substantially the levels of TNF-α, IL-12 and IL-10 cytokines in relation to untreated mice peritoneal macrophages. This may demonstrate that concentration is an important factor that should be evaluated in cellular stimulation studies (AMORIM *et al.*, 2016).

We also evaluated the effect of ANWS side chains removal and observed that it affected the ANWS induced cytokine secretion by THP-1 macrophages. The hydrolyzed polymer ANWS-M at 300 μg/mL induced lower TNF-α and IL-10 secretion than ANWS, but was more effective to increase IL-1β secretion. It can be observed in the literature that structural modifications can induce different cell surface receptor interactions and consequently differences on activation of downstream pathways. As for example, the presence of side chains in the *Dendrobium nobile* stem pectin was reported to be important for induction of T- and B-lymphocyte proliferation (WANG *et al.*, 2010). Moreover, ZHANG *et al.* (2012) verified that the type II arabinogalactan side chains are essential for stimulating nitric oxide secretion and lymphocyte proliferation, while ramified region of *Bupleurum falcatum* pectin could be responsible for the enhanced B cells cytokine secretion (GUO *et al.*, 2000) and mitogenic activity (SAKURAI *et al.*, 1999). Indeed, according to a recent review, the interaction between polysaccharides and Toll-like receptor (TLR)-4/MD-2 should occur through receptor-polysaccharide complex formation, where the polysaccharide must be present at a suitable concentration, and branched polysaccharides could induce poly-TLR4/MD-2 dimerization more easily than linear ones (ZHANG *et al.*, 2016). VOGT *et al.* (2016) have also demonstrated that the activation of TLR receptors is influenced by the DM

of pectins. They observed that the higher the DM, the higher the TLR-mediated NF-κB/AP-1 activation in THP-1 cells and this is important mainly for TLR2 activation. In addition, CHEN *et al.* (2006) observed that the DM of pectins is important for the expression of inducible nitric oxide synthase and cyclooxygenase-2 in LPS-activated peritoneal macrophages, where the high DM pectins were the most active ones.

The differences on THP-1 macrophages stimulation observed herein could be related to the DM of tested pectins, since we observed different cytokine secretion profile when the THP-1 were treated with the high methoxylated pectin (ANWS) or with ANWS-M, which suffered partial de-esterification. In contrast, the de-esterification of cacao (*T. cacao*) pod husk pectin lead to greater intrinsic capacity to stimulate peritoneal macrophages to secrete TNF-α, IL-12 and IL-10 than the native polymer (AMORIM *et al.*, 2016).

Other structural features that may be important in the pectin immunomodulatory activity are the molecular weight of the polymer. VOGT *et al.* (2016) observed that reduction of the lemon pectin length resulted in loss of TLR activation while SUH *et al.* (2013) verified that the Peyer's patch-mediated intestinal immune system modulating activity of enzimatically digested *Citrus unshiu* pectic polysaccharide potently decreased. We observed in this study a reduction in the molecular weight of hydrolized ANWS, which was followed by a decreased TNF-α and IL-10 secretion.

Studies into the immunomodulatory effects of pectins often fail to evaluated the intrinsic effects of the different pectic structures and usually compare their effect in the presence of a pro-inflammatory agent, such as LPS. As observed above, at the highest employed concentration (300 μg/mL), native ANWS pectin induced the TNF-α, IL-1β and IL-10 secretion. On the other side, our results demonstrated that ANWS at 300 μg/mL may attenuate the inflammatory response induced by LPS by reducing the production of the pro-inflammatory cytokines TNF-α and IL-1β and increasing that of anti-inflammatory cytokine IL-10. ANWS also led to a decrease in the TNF-α/IL-10 and IL-1β/ IL-10 ratios, indicating an anti-inflammatory effect of ANWS at this concentration. Compared with LPS, the hydrolyzed ANWS-M promoted changes only in the TNF-α level, also decreasing the TNF-α/IL-10 ratio. These findings resemble those obtained by SALMAN *et al.* (2008) for cytokine production induced by citrus pectin on human peripheral blood mononuclear cells. They verified that a high methoxylated citrus pectin (DM of 60 and 90) induced a dose-dependent inhibition of the pro-inflammatory cytokine IL-1β secretion as well as an increased secretion of the anti-

inflammatory cytokine IL-10 in LPS stimulated cells. When testing a low methoxylated pectin (DM of 30) they observed that it did not affect the secretion of IL-1β at all tested concentrations (0.25-1.0 mg/mL) and decreased that of IL-10 only at doses higher than 500 μg/mL. Differently, at all three esterification degrees tested, they had not found differences on TNF-α secretion, while we observed a decreased secretion of this cytokine for both ANWS and ANWS-M. The authors stated that citrus pectin exerts an immunomodulatory response in human peripheral blood mononuclear cells and that is dependent on its structure, particularly on the DM.

It is worth not regarding sweet pepper pectins that POPOV *et al.* (2011) reported the *in vivo* anti-inflammatory activity of pectic polysaccharides isolated from fresh sweet pepper by extraction using a simulated gastric medium (HCl at pH 1.5 and pepsin at 37 ºC for 4 h). After oral administration to mice, the polysaccharides caused a decreased TNF-α release and an increased production of IL-10 in LPS-stimulated whole blood. This pectin also improved the survival of mice that were subjected to a lethal dose of LPS.

In summary, both native ANWS and modified ANWS-M at the highest concentration (300 μg/mL) had the intrinsic activation capacity to modulate the TNF-α, IL-1β and IL-10 secretion by THP-1 macrophages, but the chemical differences of these pectins promote distinct effect on stimulation. However, in the presence of LPS, the anti-inflammatory effect of ANWS could be observed, by reducing the production of pro-inflammatory and increasing that of anti-inflammatory cytokines, as well as decreasing the TNF-α/IL-10 and IL-1β/ IL-10 ratios. Thus, ANWS is not purely pro-inflammatory, but could be used as an immunomodulator. Moreover, the structure modification of ANWS by acid hydrolysis caused differences on the responses.

Finally, our results corroborate with previous reports where structural differences within pectin subtypes can induce different effects on immune cells and from our results it can be concluded that DM, molecular weight and presence of side chains can be important structural features that can influence the immunomodulatory activity of pectins.

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Conflict of Interest Statement

All authors declare no conflicts of interest.

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CONCLUSÕES

Ao longo dessa tese foram abordados os seguintes aspectos: 1) avaliação da estrutura química de polissacarídeos presentes nas mucilagens dos frutos do tomate (*Solanum lycopersicum*) e do tamarillo (*S. betaceum*), assim como dos frutos do pimentão (*Capsicum annuum*) obtidos por extrações aquosas e alcalinas; 2) avaliação do comportamento reológico da principal fração péctica da polpa do tamarillo; 3) avaliação do efeito imunomodulador de uma arabinoxilana presente na mucilagem do tomate em modelos *in vivo* e 4) avaliação do efeito imunomodulador da fração péctica do pimentão em modelo *in vitro*.

As seguintes conclusões podem ser propostas:

- A mucilagem do tamarillo apresentou homogalacturonanas altamente metil-esterificadas contendo inserções de ramnogalacturonanas do tipo I com cadeias laterais constituídas principalmente por arabinogalactanas do tipo I; além de uma (1→5)-α-L-arabinana e uma heteroxilana. Comparativamente com os polissacarídeos da polpa, os polissacarídeos da mucilagem diferiram no rendimento, comprimento das cadeias laterais das pectinas e no grau de ramificação das xilanas.
- O estudo do comportamento reológico da fração péctica presente na polpa do tamarillo mostrou que em água, as soluções com diferentes concentrações da pectina (3, 5 e 8%) apresentaram pouca viscosidade aparente, porém positivamente afetadas pelo aumento da concentração. Essas soluções apresentaram comportamentos pseudoplástico, semelhante a líquidos, e obedeceram à regra de Cox-Merz, podendo ser descritos pelo modelo de Ostwald-de Waele. Em condições que favorecem a formação de gel (ou seja, presença de sacarose 50% e pH 3), a solução com apenas 1% da pectina apresentou comportamento de uma solução concentrada. Enquanto que o aumento para 2 e 3% de pectina, as soluções apresentam comportamento de gel, com as curvas de fluxo melhor descritas pelo modelo de Hershel-Bulkley. Esses géis também apresentaram termoestabilidade a variações de temperatura entre 5 a 80 °C.
- Em relação aos polissacarídeos da mucilagem do tomate, foi purificada uma fração contendo uma arabinoxilana, formada por uma cadeia principal de β-D-Xyl*p* (1→4)-ligada, pouco ramificada, nas posições *O*-2 e *O*-3, por unidades terminais de Ara*f* ou Xyl*p*. Esta fração apresentou efeito antinociceptivo através de mecanismos anti-inflamatórios, quando

avaliada em teste de contorção abdominal induzida por ácido acético e teste da formalina em camundongos.

- A principal fração péctica do pimentão também foi caracterizada como sendo constituída principalmente por uma homogalacturonana altamente metil-esterificada, e aproximadamente 31% de arabinogalactanas do tipo I e II ancoradas por ramnogalacturonana tipo I. Em adição, uma arabinogalactana do tipo II foi purificada e uma detalhada caracterização estrutural foi realizada.
- Por fim, esta fração péctica do pimentão apresentou efeito imunomodulador sobre células THP-1 diferenciadas em macrófagos. A fração foi capaz de estimular a secreção das citocinas TNF-α, IL-1β e IL-10 na dose de 300 μg/mL. Enquanto que na presença de LPS, a fração reduziu os níveis de secreção de TNF-α e de IL-1β, e aumentou o nível de secreção de IL-10, além de reduzir as razões TNF-α/IL-10 e IL-1β/ IL-10. A modificação da estrutura química da pectina através de hidrólise ácida parcial demonstrou que o grau de metil-esterificação, peso molecular e presença de cadeias laterais podem ser importantes características estruturais para a modulação da secreção das citocinas testadas pelas células THP-1 diferenciadas em macrófagos.

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ANEXOS

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