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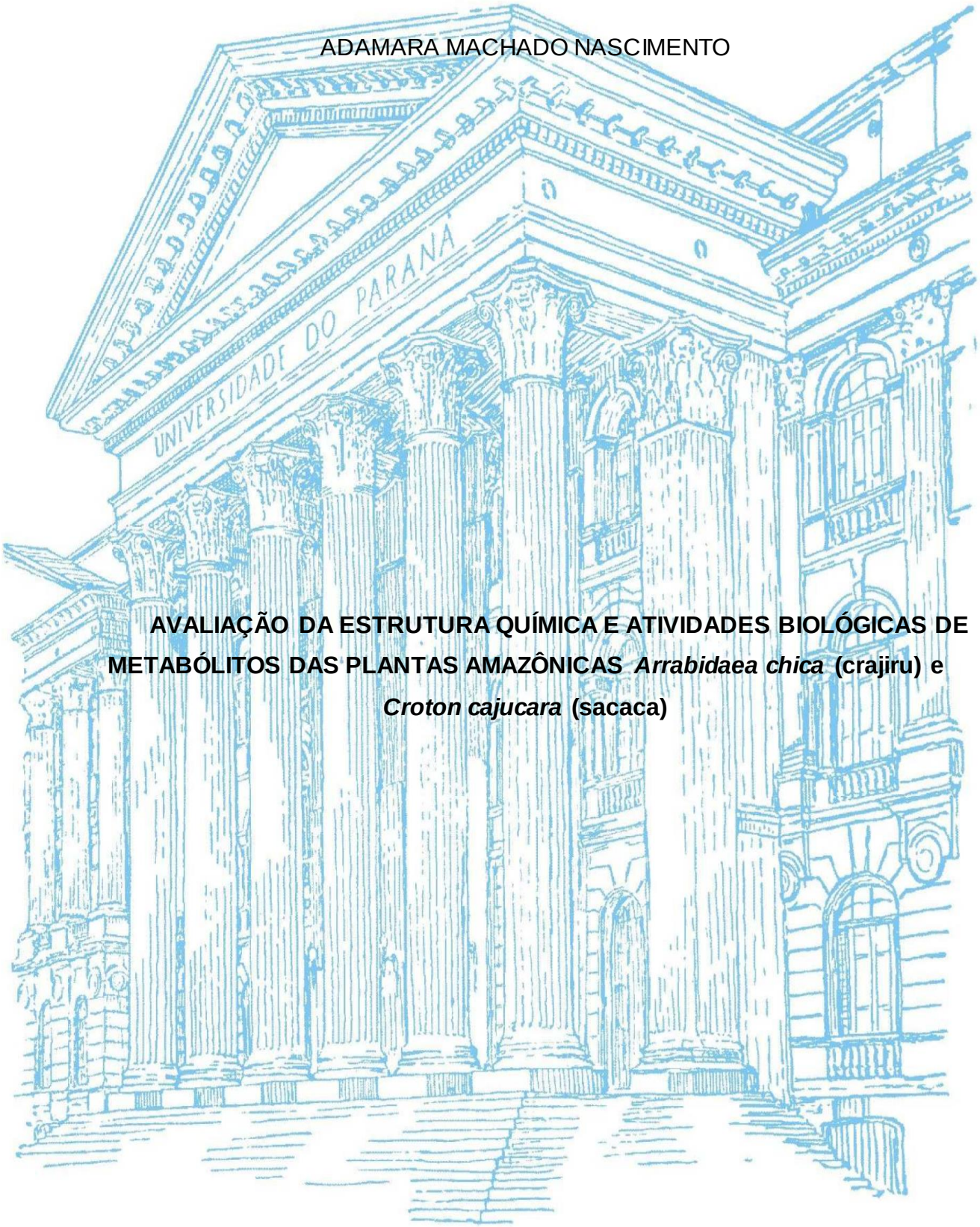
ADAMARA MACHADO NASCIMENTO




# AVALIAÇÃO DA ESTRUTURA QUÍMICA E ATIVIDADES BIOLÓGICAS DE METABÓLITOS DAS PLANTAS AMAZÔNICAS *Arrabidaea chica* (crajiru) e *Croton cajucara* (sacaca)

CURITIBA

2017

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# AVALIAÇÃO DA ESTRUTURA QUÍMICA E ATIVIDADES BIOLÓGICAS DE METABÓLITOS DAS PLANTAS AMAZÔNICAS *Arrabidaea chica* (crajiru) e *Croton cajucara* (sacaca)

Tese apresentada ao Programa de Pós-Graduação em Ciências–Bioquímica, do Departamento de Bioquímica e Biologia Molecular, do Setor de Ciências Biológicas, da Universidade Federal do Paraná, como requisito parcial à obtenção do grau de Doutor em Ciências-Bioquímica.

Orientador: Prof. Dr. Thales R. Cipriani

Coorientadores: Prof. Dr. Marcello Iacomini
Dr. Lauro Mera de Souza

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# TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ADAMARA MACHADO NASCIMENTO** intitulada: **Avaliação da estrutura química e atividades biológicas de metabólitos das plantas amazônicas *Arrabidaea chica* (crajiru) e *Croton cajucara* (sacaca)**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO.

CURITIBA, 27 de Janeiro de 2017.

THALES RICARDO CIPRIANI
Presidente da Banca Examinadora (UFPR)

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Avaliador Externo (UFPR)

Dedico este trabalho, com amor, à minha família, que me incentiva a continuar sempre e é o meu apoio em todos os momentos.

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*“Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável.”*

(Galileu Galilei)

## NOTA EXPLICATIVA

Esta tese está apresentada no formato alternativo, de acordo com as normas estabelecidas no Programa de Pós-Graduação em Ciências–Bioquímica, da Universidade Federal do Paraná. Assim, foram inseridos um artigo publicado e dois artigos a serem submetidos, os quais foram originados no decorrer do doutoramento. Desta forma, este trabalho apresenta-se com a seguinte estrutura básica: introdução, justificativa, revisão bibliográfica, objetivo geral e objetivos específicos, os artigos científicos produzidos (contendo os experimentos realizados, resultados, discussões e conclusões) e as conclusões gerais.

## RESUMO

*Arrabidaea chica* V. (crajiru) é comum da região Norte do Brasil, onde é utilizada na forma de chá e extratos hidroalcoólicos ou alcoólicos. Este trabalho apresenta a caracterização de um polissacarídeo (AC25R) obtido das folhas de *A. chica* por extração aquosa. AC25R apresentou os monossacarídeos neutros Gal, Ara, Glc, Man, Rha e 3-*O*-Me-Gal numa razão molar de 11:10.6:10:3.1:2.7:1.0, além de 6.1 g% de GlcA e $M_w$ de 49.690 g/mol. Análises de metilação e RMN indicaram que AC25R é uma arabinogalactana do tipo II (AG II), constituída por uma cadeia principal de unidades de β-D-Gal*p* 3-O-ligadas, substituída em O-6 por cadeias laterais de β-D-Gal*p* 6-O-ligadas, as quais são substituídas em O-3 por cadeias de α-L-Ara*f* 2-, 3- e 5-O-ligadas. Além disso, 3-O-Me-Gal*p* e terminais não-redutores de α-D-Glc*p* fazem parte da estrutura do polímero. AC25R apresentou efeito imunoestimulatório sobre macrófagos THP-1 ao induzir a produção das citocinas pró-inflamatórias TNF-α, IL-1β e anti-inflamatória IL-10. *Croton cajucara* B. (sacaca) é uma árvore amazônica e suas cascas e folhas são usadas na forma de chás e extratos para tratar uma variedade de problemas, incluindo úlcera gástrica e inflamação. A fração bruta contendo polissacarídeos, obtida por extração aquosa das folhas da planta, foi capaz de promover gastroproteção em ratos. Um fracionamento bioguiado foi realizado para isolar a fração polissacarídica ativa e após os processos de gelo e degelo, ultrafiltração e diálises sequencias a fração 25R foi obtida. Ela apresentou Glc, Gal, Rha, Ara, GalA and Man em uma razão molar de 7:5:5:3:1:1 e $M_w$ de 42.840 g/mol. Análise de metilação e RMN indicaram que 25R é uma fração complexa contendo ramnogalaturonana do tipo I, arabinana, arabinogalactana do tipo I, arabinogalactana do tipo II e ramnana. 25R apresentou efeito gastroprotetor, preservando muco e GSH no estômago. Além disso, um método de análise por LC-MS de compostos de baixa massa molar presentes no extrato aquoso das folhas de *C. cajucara* foi desenvolvido. A presença de diferentes compostos fenólicos foi verificada. Estes compostos foram fracionados de acordo com suas polaridades e as frações foram testadas para atividade anti-inflamatória usando um modelo de edema de pata em camundongos, mostrando resultados positivos.



**Palavras-chave**: *Arrabidaea chica* / *Croton cajucara* / Polissacarídeos / Compostos de baixa massa molar / Efeito gastroprotetor / Inflamação / Efeito imunoestimulatório

## ABSTRACT

*Arrabidaea chica* V. (crajiru) is common of the North Region of Brazil, where is used as tea and hydroalcoholic or alcoholic extracts. This work presents the characterization of a polysaccharide (AC25R) from *A. chica* leaves obtained by aqueous extraction. AC25R presented the neutral monosaccharides Gal, Ara, Glc, Man, Rha, and 3-*O*-Me-Gal in a 11:10.6:10:3.1:2.7:1.0 molar ratio, besides 6.1 g% of GlcA and Mw 49.690 g/mol. Methylation analysis and NMR spectroscopy indicated that AC25R is a type II arabinogalactan (AG II), constituted for a main chain β-D-Gal*p* 3-O-linked units, O-6-substituted by β-D-Galp 6-O-linked side chains, which are substituted at O-3 position by α-L-Ara*f* 2-, 3-, and 5-O-linked. Moreover, 3-*O*-Me-Gal*p* and non-reducing end units of α-D-Glc*p* are part of the structure of the polymer. AC25R presented immunostimulatory effect on THP-1 macrophages by inducing the production of inflammatory cytokines TNF-α, IL-1β and anti-inflammatory IL-10. *Croton cajucara* B. (sacaca) is an amazon tree and its stem bark and leaves are used as tea and extracts for treat of a wide variety of problems, including ulcer gastric and inflammation. The crude fraction containing polysaccharides, obtained by an aqueous extraction from leaves of plant, was able to promote gastroprotection in rats. A guided fractionation was performed to isolate the active polysaccharide fraction and after freezing-thawing, ultrafiltration and sequential dialysis the fraction 25R was obtained. It presented Glc, Gal, Rha, Ara, GalA and Man in a 7:5:5:3:1:1 molar ratio approximately, and $M_w$ of 42,840 g/mol. Methylation analysis and NMR spectroscopy indicated that 25R is a complex fraction, containing type I rhamnogalacturonan, arabinan, type I arabinogalactan, type II arabinogalactan and rhamnan. 25R showed gastroprotective effect, preserving mucus and GSH in the stomach. Furthermore, an LC-MS analysis of the low molar compounds present in ethanolic supernatant from leaves of *C. cajucara* was developed. The presence of diferent phenolic compounds was verified. These compounds were fractioned according to their polaritities and assayed for anti-inflammatory activity using a model of paw edema in mice, showing positive results.



**Keywords:** *Arrabidaea chica* / *Croton cajucara* / Polysaccharides / Low molar compounds / Gastroprotective effect / Inflammation / Immunostimulatory effect

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### ARTIGO 1



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## LISTA DE ABREVIATURAS E SIGLAS E TERMOS

### Compostos químicos

$Ac_2O$ - Acetic anhydride (Anidrido acético)

AceA - Ácido Acérico (3-C-carboxi-5-deoxi-L-xilose)

AG I - arabinogalactana do tipo I

AG II - arabinogalactana do tipo II

Api - Apiose (3-C-carboxi-5-deox-L-xilose)

Ara*f* - Arbinose furanosídica

$CHCl_3$ - Chloroform (Clorofórmio)

DCTN - *trans*-dehidrocrotonina

Dha - Ácido 3-deoxi-lyxo-heptulosárico

DMSO - Dimethyl sulfoxide (dimetilsulfóxido)

DPPH - 2,2-Difenil-1-picril-hidrazila

EDTA - Ethylenediamino tetraacetic acid

EtOH - Ethanol (etanol)

Gal*p* - Pyranosidic galactose (Galactose piranosídica)

Gal*p*A - Pyranosidic Galacturonic acid (ácido galacturônico piranosídico)

Glcp - Pyranosidic Glucose (glucose piranosídica)

GSH - Reduced glutathione (Glutationa reduzida)

$H_2SO_4$ - Sulfuric acid (Ácido sulfurico)

$HCO_2H$ - Formic acid (ácido fórmico)

HDL - High Density Lipoprotein (Lipoproteína de alta densidade)

HGA - Homogalacturonan (homogalacturonana)

IL-10 - Interleukin – 10 (Interleucina – 10)

IL-1β - Interleukin – 1β (Interleucina – 1β)

KdoA - Ácido 2-keto-3-deoxi-D-mano-octulosonico

LDL - Low Density Lipoprotein (Lipoproteína de baixa densidade)

LPS - Lipopolysaccharide (lipopolissacarídeo)

MeI - Iodomethane (Iodometano)

MeOH - Methanol (metanol)

$NaBD_4$ - Deuterated sodium borohydride (Borohidrete de sódio deuterado)

$NaBH_4$ - Sodium Borohydride (Borohidreto de sódio)

NaOH - Sodium Hydroxide (Hidróxido de sódio)
NF-kB - Nuclear factor-kappa B (Fator nuclear kappa B)
NK - Natural Killer
PBS - Phophate buffered saline (Tampão fosfato-salino)
PMA - Phorbol 12-myristate 13-acetate
PrOH - Propanol
RG I - ramnogalacturonana do tipo I
RG II - ramnogalacturonana do tipo II
Rha - Ramnose
RPMI - Roswell Park Memorial Institute Medium
TFA - Trifluoroacetic acid (Ácido fluoroacético)
THP-1 -
TNF-α - Fator de necrose tumoral-α

**Amostras, extratos e frações**

25E - Fração eluída em membrane de 25 kDa (diálise), obtida de CC50E
25R - Fração retida em membrana de 25 kDa (diálise), obtida de CC50E
*A. chica* - *Arrabiadaea chica*
AC100E - Fração eluída em membrane de 100 kDa (diálise), obtida de ACS
AC100R - Fração retida em membrane de 100 kDa (diálise), obtida de ACS
AC25E - Fração eluída em membrane de 25 kDa (diálise), obtida de AC50E
AC25R - Fração retida em membrane de 25 kDa (diálise), obtida de AC50E
AC50E - Fração eluída em membrane de 50 kDa (diálise), obtida de AC100E
AC50R - Fração retida em membrane de 50 kDa (diálise), obtida de AC100E
ACI - Fração insolúvel em água (após gelo/degelo), obtida de ACPE
ACPE - Fração bruta contendo polissacarídeos do extrato aquoso de *A. chica*
ACS - Fração solúvel em água (após gelo/degelo), obtida de ACPE
AQ - Aqueous fraction (Fração aquosa, obtida de ESF)
BU - Butanol fraction (Fração butanólica, obtida de ESF)
*C. cajucara* - *Croton cajucara*
CC100E - Fração eluída em membrana de 100 kDa (ultrafiltração), obtida de CCS
CC100R - Fração retida em membrana de 100 kDa (ultrafiltração), obtida de CCS
CC50E - Fração eluída em membrana de 50 kDa (diálise), obtida de CC100E
CC50R - Fração retida em membrana de 50 kDa (diálise), obtida de CC100E

CCP - Fração bruta contendo polissacarídeos obtida do extrato aquoso das folhas de *C. cajucara*

CCS - Fração solúvel em água, obtida de CCP

CR - Carboxyl-reduced

EA - Ethyl acetate fraction (Fração acetato de etila, obtida de ESF)

ESF - Ethanol Soluble Fraction (Fração solúvel em etanol obtida por decocto de folhas de *C. cajucara*)

**Técnicas e termos associados as técnicas de análise**

a.m.u. - atomic mass units (unidades de massa atômica)

GC-MS - Gas chromatography-mass spectrometry (cromatografia gasosa acoplada à espectrometria de massas)

HPSEC - High Performance High Exclusion Chromatography (cromatografia de exclusão estérica de alta performance)

HSQC - Heteronuclear single quantum coherence

kDa - kilo-Dáltons

LC-MS - Liquid chromatography-mass spectrometry (cromatografia líquida acoplada a espectrometria de massas)

*m/z* - mass to charge ratio (relação massa/carga)

MALLS - Multiangle laser light scattering

MS - Mass spectrometry (espectrometria de massas)

$M_w$ - Massa molecular relativa

NMR - Nuclear Magnetic Ressonance (Ressonância magnética nuclear)

PDA - Photodiode array detector

RI - Differential refractometer

TLC - Thin layer chromatograph (cromatografia de camada delgada)

UHPLC - Ultra-high performance liquid chromatography

UV - Ultravioleta

**Outros termos e siglas**

i.p. - intraperitoneal

*M. circinelloides* - *Mucor circinelloides*

*M. smegmatis* - *Mycobacterium smegmatis*

*M. tuberculosis* - *Mycobacterium tuberculosis*

OMS - Organização Mundial de Saúde

*R. oryzae* - *Rhyzopus oryzae*

RENISUS - Relação Nacional de Plantas Medicinais de Interesse ao SUS

*S. aureus*. - *Staphylococcus aureus*

SUS - Sistema Único de Saúde

**SUMÁRIO**

# 1 INTRODUÇÃO

As plantas medicinais *Arrabidaea chica* Verlot. e *Croton cajucara* Benth. são utilizadas por diferentes comunidades na Região Amazônica para o tratamento de distúrbios de saúde, incluindo alterações relacionadas a processos inflamatórios agudos e crônicos e úlceras pépticas.

A planta *A. chica* (crajiru), é encontrada em toda a região tropical da América, sendo bastante comum na Amazônia. As folhas de *A. chica* são empregadas na medicina popular para cicatrização de feridas, tratamento de processos inflamatórios, cólica intestinal e anemias. Há inegável valor farmacológico em seu uso medicinal uma vez que a planta consta entre as 71 espécies selecionadas na Relação Nacional de Plantas Medicinais de Interesse ao SUS (RENISUS). Além disso, as folhas são usadas como fonte de pigmentos aplicados sobre a pele, roupas e utensílios domésticos por algumas etnias indígenas. Estudos fitoquímicos das folhas de *A. chica* mostraram a presença de diferentes compostos fenólicos, e os principais são as desoxiantocianidinas, pigmentos que conferem coloração vermelha-alaranjada aos extratos obtidos das folhas.

*C. cajucara* é uma árvore nativa da Amazônia, conhecida popularmente como sacaca. A casca do caule e suas folhas são usadas na forma de chás para o tratamento de diferentes distúrbios de saúde. No uso popular, o chá das folhas, é empregado para inúmeras finalidades, dentre as quais: antiúlcera gástrica, antidiabética, anti-inflamatória e antinociceptiva. Alguns terpenóides desta planta já foram caracterizados quimicamente, sendo o diterpenóide clerodano *trans*-dehidrocrotonina (DCTN) o mais estudado. Ele é obtido da casca do caule da árvore, e a ele tem-se atribuído as principais atividades biológicas relatadas para a planta. De suas folhas foram caracterizados alguns compostos químicos dentre os quais óleos essenciais e um terpeno chamado cajucarinolide aos quais são atribuídos várias atividades biológicas da planta.

Extratos de plantas são, usualmente, misturas complexas que contêm várias classes diferentes de moléculas e constituem um grande desafio para a química de produtos naturais (MACIEL *et al*., 2006). As plantas utilizadas na medicina popular necessitam da elucidação de seus componentes químicos aliada ao estudo de suas atividades biológicas, com o intuito da comprovação científica de eficácia frente aos usos populares observados.

Polissacarídeos de vegetais são metabólitos primários aos quais têm sido relacionados diversas atividades biológicas, dentre as quais anti-inflamatória, imunoestimulante, imunomoduladora, antiúlcera gástrica, antiviral, antitumoral, anticomplemento, anticoagulante e hipoglicemiante. Os metabólitos secundários, compostos de baixa massa molar, apresentam estruturas diversas e complexas e são produzidos pelas plantas lhes conferindo funções adaptativas e de defesa. Usualmente são atribuídas aos metabólitos secundários de plantas as atividades medicinais que plantas apresentam quando consumidas pela população.

Considerando o interesse científico na elucidação de componentes químicos de plantas com propriedades terapêuticas, o objetivo do presente trabalho foi o isolamento, a caracterização estrutural e a avaliação de atividades biológicas (antiúlcera gástrica e/ou anti-inflamatória e/ou efeito imunomodulador) de polissacarídeos e/ou metabólitos secundários presentes em extratos aquosos de folhas das plantas *A. chica* e *C. cajucara*.

## 2 JUSTIFICATIVA

A Organização Mundial de Saúde afirma que várias populações de países em vias de desenvolvimento seguem utilizando a medicina tradicional como resultado de circunstâncias históricas e crenças culturais, sendo que esta tem sido observada também como medicina alternativa e complementar em países já desenvolvidos (OMS, 2002). O uso de plantas medicinais está inserido na medicina tradicional uma vez que é um costume observado ao longo da história humana e sempre constituiu uma fonte importante de princípios ativos para a descoberta de novos fármacos (PHILLIPSON, 2001; GURIB-FAKIM, 2006).

O Brasil tem uma das maiores biodiversidades do mundo com diferentes biomas que refletem a sua riqueza em flora e fauna. Ademais, a diversidade étnica e cultural da população traz proeminente interesse em pesquisas etnobotânicas, etnofarmacognósticas e etnofarmacológicas as quais, recuperam as informações populares de plantas. Tais pesquisas utilizam estudos dirigidos a observação de plantas com fins medicinais consumidas por diferentes comunidades, buscando informações junto a seus usuários combinadas a estudos químicos, farmacognósticos e farmacológicos (PATZLAFF; PEIXOTO, 2009).

A região amazônica tem uma grande diversidade biológica e social, sendo considerada uma das maiores biodiversidades em flora e fauna do mundo além de ter em seu território representação de diferentes tipos de comunidades regionais como ribeirinhos, indígenas de diferentes etnias, seringueiros e mais recentemente comunidades daimistas. Aliado a isso, muitas plantas medicinais da flora nativa são consumidas com pouca ou nenhuma comprovação de suas propriedades farmacológicas. O difícil acesso aos medicamentos constantes nas políticas públicas de saúde brasileiras incentiva a utilização de plantas para fins terapêuticos como uma alternativa de tratamento aos mais diferentes tipos de alterações de saúde.

Os polissacarídeos de plantas podem ser obtidos juntamente com metabólitos secundários no processo de extração aquosa. As plantas *A. chica* e *C. cajucara* não possuem trabalhos prévios com enfoque caracterização e/ou avaliação de atividades biológicas relacionadas aos polissacarídeos de suas folhas. Embora existam alguns estudos avaliando estruturalmente metabólitos secundários da planta *C. cajucara*, a maioria desses estudos abrange extratos provenientes da casca do caule da planta. Há poucos relatos sobre estudos de extratos aquosos obtidos das folhas desta planta,

sendo que estas são consumidas principalmente na forma de chá. Além de óleos essenciais e outras substâncias obtidas por extração com hexano (um solvente apolar) pouco é conhecido a respeito de metabólitos de baixa massa molar que podem estar participando das atividades biológicas destacadas para esta planta.

Portanto, este trabalho tem o seu valor científico por buscar a elucidação de componentes químicos (polissacarídeos e compostos de baixa massa molar) e a comprovação de algumas atividades biológicas observadas tradicionalmente pelo uso empírico das plantas estudadas.

# 3 REVISÃO BIBLIOGRÁFICA

## 3.1 PLANTAS MEDICINAIS

O uso de plantas para fins terapêuticos tem sido observado ao longo da história das civilizações. As plantas medicinais são usadas em todas as regiões do Brasil, no preparo de remédios para os mais variados fins de promoção de saúde. Segundo o Programa Nacional de Plantas Medicinais e Fitoterápicos do Ministério da Saúde do Brasil (2009) o país é reconhecido no mundo inteiro por sua diversidade biológica e social no qual várias comunidades com perfis, crenças e costumes distintos se sobressaem em conhecimentos populares relacionados, inclusive, ao uso de plantas. Os saberes e práticas relacionados ao uso de plantas medicinais em cada uma das regiões do Brasil denotam, além dos costumes e valores, os recursos naturais disponíveis em cada comunidade e em cada região (MELLO, 1980; ELISABETSKY; WANNMACHER, 1993). A floresta amazônica é reconhecida em todo o mundo por sua elevada biodiversidade em flora e fauna e ao longo dos anos o interesse da ciência e de indústrias cosméticas e farmacêuticas na pesquisa de substâncias ativas provenientes de produtos naturais daquela região tem aumentado (MIRANDA; MATTOS, 1992; DUTRA *et al*., 2016).

Diferentes classes de compostos químicos são isoladas de plantas medicinais, aumentando o interesse de pesquisadores e indústrias na identificação de metabólitos com fins terapêuticos. Muitos artigos científicos têm focado na avaliação de propriedades farmacológicas de extratos brutos provenientes de plantas (PRANDO *et al*., 2015; VERDAM *et al*., 2015) e alguns no isolamento, elucidação estrutural e avaliação do mecanismo de ação de moléculas provenientes desses extratos (ADEKELAN *et al*., 2008; XU *et al*., 2015; DUTRA *et al*., 2016). Extratos brutos de origem vegetal consistem em uma mistura de diferentes metabólitos (primários e secundários) que podem ser úteis na terapêutica executando ações em vários alvos ao mesmo tempo. As diferentes moléculas presentes em um extrato vegetal podem atuar de modo sinérgico, acentuando o efeito principal observado por um metabólito, através da interação de diferentes moléculas com vários tipos de receptores ou alvos (VERDAM *et al*., 2015). A interação molecular que pode ser observada pela administração de extratos vegetais, além de atuar de forma sinérgica, pode ser benéfica por limitar ou evitar efeitos colaterais ou adversos quando comparados a

atuação de um único fármaco sobre um determinado receptor ou alvo (KOEBERLE; WERZ, 2014). Ademais, muitos metabólitos isolados de plantas medicinais, quando administrados em organismos vivos são capazes de produzir efeitos biológicos. Salicilato, curcumina e resveratrol são alguns exemplos de princípios ativos isolados de plantas com poderosas atividades terapêuticas.

Uma vez que plantas medicinais são facilmente encontradas em feiras livres, mercados populares ou cultivadas em hortas caseiras, não há um controle criterioso acerca de seus usos. Considerando o fácil acesso e muitas vezes o uso irrestrito desse tipo de material para finalidades terapêuticas, levantamentos etnofarmacobotânicos têm surgido como uma maneira de orientar a pesquisa científica para o conhecimento de moléculas e/ou extratos de plantas relacionados com seus efeitos biológicos e usos populares (COELHO-FERREIRA, 2009; BIESKI *et a*l., 2015; CERCATO, *et al*., 2015; KFFURI *et al*., 2016; PEDROLLO *et al*., 2016; SAMOISY; MAHOMOODALLY, 2016). Apesar do uso de plantas medicinais ser um hábito comum, o estudo científico não consegue abranger a maioria das plantas consumidas. Os metabólitos de plantas utilizadas na medicina popular despertam o interesse científico, uma vez que a elucidação química de componentes de plantas medicinais, aliada ao estudo das atividades biológicas, vem comprovar a eficácia dos usos populares, podendo, ao final de um longo processo resultar em um novo fármaco. Ao longo da história, diferentes moléculas têm sido isoladas e confirmadas como sendo responsáveis pela atividade medicinal principal atribuída a diferentes tipos de plantas (DECORTE, 2016).

## 3.2 *Arrabidaeae chica* VERLOT.

### 3.2.1 Aspectos botânicos de *A. chica*

A família Bignoniaceae, que possui 120 gêneros e 650 espécies distribuídos na América Tropical e África, é constituída por plantas que apresentam grande relevância por seus constituintes bioativos (TAKEMURA *et al*., 1995; ZORN *et al*., 2001; RAHMATULLAH *et al*., 2010). O gênero Arrabidaeae ocorre na América tropical, desde o sul do México até a região central do Brasil (TAKEMURA, 1993). A planta *A.*

*chica*, que apresenta *Friedericia chica* (Bonpl.) L. G. Lohmann como sinonímia botânica, é uma espécie comum na Amazônia brasileira, integrante da Bignoniaceae.

A planta é nativa da Amazônia, e cresce nas matas tropicais, sendo descrita como um arbusto, com atributos ornamentais, medindo 2,5 m de altura, aproximadamente, com folhas medindo de 8 a 13 cm de comprimento (QUEIROZ *et al*., 2008; CARTAGENES *et al.*, 2010). Uma imagem de um ramo terminal com três folhas de *A. chica* está mostrado na Figura 1. As flores desta planta são de cor rósea ou violácea, medindo cerca de 18 a 20 cm de comprimento (LORENZI e MATOS, 2002). Uma imagem das flores de *A. chica* está apresentada na Figura 2. A Figura 3 mostra um exemplar doméstico da planta.

**FIGURA 1** – Folhas de *Arrabidaea chica* Verlot.
(Fonte: A Autora, 2014)

**FIGURA 2** – Flores de *Arrabidaea chica* Verlot.

(Fonte: Tropicos.org. Missouri Botanical Garden. Acessado em: 04 Março 2017 disponível em: http://www.tropicos.org/Name/3700601)

**FIGURA 3 –** *Arrabidaea chica* Verlot.

(Fonte: A Autora, 2014)

### 3.2.2 Usos populares de *A. chica*

A espécie é conhecida na Região Norte do Brasil como crajirú, principalmente nos Estados do Acre e Amazonas; pariri no Estado do Pará; e cipó-cruz, cipó-pau, carajuru, capiranga, grajiru, carajura e puca panga em outros estados brasileiros. Embora comum na Região Amazônica, a planta pode ser encontrada em todo o Brasil e em outros países da América do Sul e da África (VON POSER *et al.*, 2000). A população latino-americana faz uso de *A. chica* para diferentes fins terapêuticos e também como planta ornamental (CARTAGENES *et al*., 2010).

Na medicina tradicional amazônica *A. chica* tem sido utilizada como cicatrizante de feridas localizadas sobre a pele ou mucosas, em casos de inflamações da pele como psoríase, disfunções sanguíneas como hemorragia e anemia, e também como antimicrobiana (BARBOSA *et al*., 2008; SILVA, 2002; JORGE *et al*., 2008; CARTAGENES *et al.*, 2010). Segundo Queiroz *et al*. (2008) a planta é popularmente utilizada para inflamações do útero e dos ovários, conjuntivite, enterocolites e também em casos de diabetes. O uso das folhas também é observado para o tratamento de cólica intestinal, diarreia e anemia, sendo consumidas na forma de infusão, decocto ou macerado que podem ser administrados por via oral ou aplicadas diretamente em áreas inflamadas da mucosa ou da pele (BEHRENS; TELLIS; CHAGAS, 2012). A planta consta entre as 71 espécies vegetais da Relação Nacional de Plantas Medicinais de Interesse ao SUS – RENISUS onde estão incluídas plantas usadas na medicina popular que apresentam potencial para avançar na cadeia produtiva e gerar produtos de interesse para a saúde (BRASIL, 2009). Além disso, o extrato de suas folhas é usado na confecção de sabonetes cremosos com indicações para o tratamento de acnes, antimicrobiano e antifúngico (CARTAGENES *et al.*, 2010).

Alguns grupos étnicos indígenas da Região Amazônica utilizam tinturas ricas em pigmentos obtidas a partir das folhas da planta, sobre a pele em rituais religiosos, como protetor solar da pele e repelente de insetos (BARBOSA *et al.*, 2008). O pigmento avermelhado obtido das folhas da planta também é utilizado para pintura de utensílios, roupas e artesanatos, principalemente pelos povos indígenas.

### 3.2.3 Composição química de *A. chica*

Em análises fitoquímicas realizadas com diferentes extratos de *A. chica* foram detectadas a presença de diversas classes de substâncias químicas, dentre as quais compostos fenólicos (flavonoides), esteróides e triterpenos (TAKEMURA, 1993). Em uma tintura obtida de folhas da planta foram detectados açúcares redutores, alcalóides, antocianidinas, antraquinonas, esteroides, triterpenoides, fenóis, flavonóis, flavanonas, saponinas e taninos (ALVES *et al*., 2010).

Os principais constituintes químicos relatados para *A. chica* são da classe das antocianidinas, sendo as duas principais as 3-desoxiantocianidinas: 6,7,4'-trihidroxi-5-metoxi-flavilium, conhecida como carajurona (Figura 2A) e 6,7-dihidroxi-5,4'-dimetoxi-flavilium, conhecida como carajurina (Figura 2B), ambos pigmentos da classe dos flavonoides obtidos das folhas e responsáveis pela coloração vermelha-alaranjada característico de seus extratos (CHAPMAN; PERKIN; ROBINSON, 1927; ZORN *et al*., 2001; DEVIA *et al*., 2002). Os pigmentos 3-desoxiantocianidinas são relativamente raros em plantas embora biossinteticamente sejam mais simples que outras antocianidinas (HARBORNE, 1977). As antocianidinas são moléculas agliconas obtidas a partir de antocianinas após a perda de açúcar por hidrólise ácida (TAFFARELLO, 2008). A presença de antocianidinas foi confirmada também nas flores da planta (SCOGIN, 1980). Além da carajurina e da carajurona caracterizadas por Chapmam *et al*. (1927), outras desoxiantocianidinas obtidas das folhas foram caracterizadas: 6,7,3'-trihidroxi-5,4'-dimetoxiflavilium e 6,7,3',4'-tetrahidroxi-5-metoxiflavilium (ZORN *et al*., 2001); 6,7,3',4'-tetrahidróxi-5-metóxiflavilium e 6,7,4'-trihidroxi-5-metoxoflavilium (DEVIA *et al*., 2002). Esses achados corroboram com a hipótese levantada por Harborne (1977) de que a 6-hidroxilação é um padrão estrutural comum para flavonoides desta espécie.

Um extrato etanólico, obtido das folhas de *A. chica* por maceração, passou por partição líquido-líquido sequencial com *n*-hexano, acetato de etila e metanol e a fração acetato de etila foi submetida a uma coluna cromatográfica, que resultou em diferentes frações de onde puderam ser isolados os flavonóides: vicenina-2 e kaempferol. Na fração *n*-hexano foi isolado o flavonóide 4'-hidroxi-3,7-dimetoxiflavona (BARBOSA *et al*., 2008). De um outro extrato etanólico de suas folhas, a partir de uma fração solúvel em diclorometano, foi detectado o flavonóide luteolina (AMARAL *et al*., 2006). Uma flavona chamada carajuflavona (6,7,3',4'-tetrahidroxi-5-metóxiflavona) foi

isolada e caracterizada por análise espectroscópica de folhas de *A. chica* f. *cuprea*, que é uma variedade desta planta de ocorrência restrita aos Estados do Sul do Brasil (TAKEMURA *et al*., 1995).

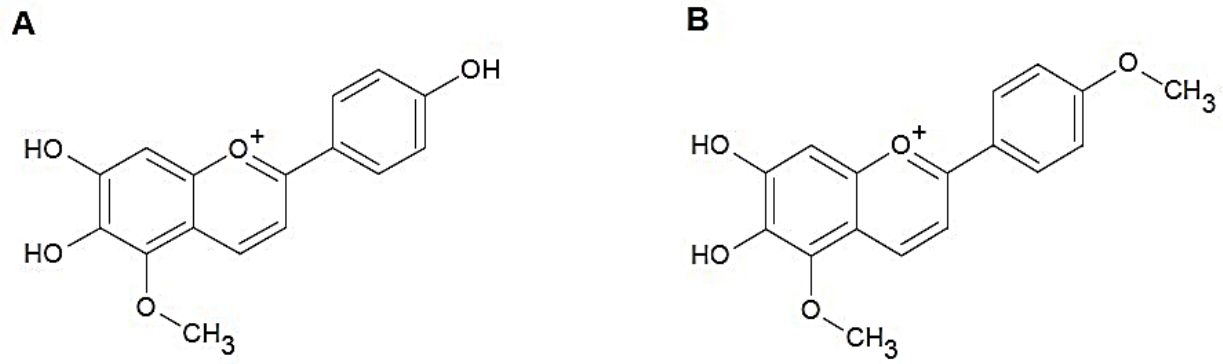


**FIGURA 4** – Metabólitos obtidos das folhas de *A. chica*.

A - 6,7,4'-trihidroxy-5-metoxi-flavilium – carajurona; B - 6,7-dihidroxi-5,4'-dimetoxi-flavilium – carajurina
FONTE: Modificada de CHAPMAN; PERKIN; ROBINSON (1927) com a permissão de Royal Society of Chemistry © 1927.

### 3.2.4 Atividades biológicas descritas para *A. chica*

A família Bignoniaceae apresenta um notável número de compostos bioativos os quais já demonstraram várias atividades farmacológicas. Estudos prévios com *A. chica* demostraram propriedades medicinais relacionadas a efeitos anti-inflamatórios. Um extrato apolar obtido das folhas da planta, contendo antocianidinas e a desoxiantocianidina carajurina inibiram o fator de transcrição NF-κB, um mediador central da resposta imune humana que regula a transcrição de genes relacionados com a codificação de diferentes moléculas inflamatórias (citocinas e enzimas), podendo atuar na modulação de processos inflamatórios (ZORN *et al*., 2001). Um extrato metanólico bruto das folhas demonstrou atividade cicatrizante relacionada ao estímulo de fibroblastos e síntese de colágeno tanto *in vitro* quanto *in vivo*, além de moderada atividade antioxidante (JORGE *et al*., 2008). O mesmo extrato também mostrou eficiência, através de aplicação tópica, na cicatrização de lesão em tendão, onde foi observado o estímulo da síntese de colágeno (ARO *et al*., 2013a). Além disso, a aplicação tópica do extrato metanólico bruto das folhas melhorou a organização de fibras de colágeno (ARO *et al*., 2013b).

Um extrato aquoso obtido das folhas apresentou atividade anti-inflamatória sobre o edema induzido por venenos de serpentes dos gêneros *Brothrops* e *Crotalus* em

camundongos, sugerindo a presença de princípios ativos anti-inflamatórios hidrofílicos (OLIVEIRA *et al*., 2008). Além disso, a planta demonstrou outras atividades biológicas: efeito tripanocida em *Trypanosoma cruzi*, protozoário causador da doença de chagas, e inibição total do crescimento do fungo *Trichophyton mentagrophytes*, agente etiológico de micoses de unhas e de pele (BARBOSA *et al*., 2008).

## *3.3 Croton cajucara* BENTH.

### 3.3.1 Aspectos botânicos de *C. Cajucara*

O gênero *Croton* pertence à ordem Euphorbiales da subfamília Crotonoideae e é um dos gêneros mais numerosos da família Euphorbiaceae (TIEPPO, 2007). Croton possui cerca de 1300 espécies, incluindo plantas arbóreas, arbustivas e herbáceas (GOVAERTS; FRODIN; RADCLIFFE, 2000). *Croton cajucara* Benth. é uma árvore que cresce na floresta amazônica, onde é popularmente conhecida como sacaca ou marassacaca. A planta pode alcançar de 6 a 10 m de altura, com copa estreita e casca aromática e pulverulenta (TIEPPO, 2007), folhas medindo entre 7 a 16 cm de comprimento (LORENZI; MATOS, 2002) e flores de cor amarelada. Uma exsicata da planta é mostrada na Figura 5 e uma imagem da árvore cultivada em jardim, na Figura 6.

### 3.3.2 Usos populares de *C. cajucara*

Informações etnobotânicas indicam que as folhas e cascas do caule são usadas na medicina popular para o tratamento de problemas de saúde que incluem úlcera gástrica, inflamação, desordens hepáticas e renais, febre e malária e ainda para a diminuição do nível de colesterol sanguíneo (MARTINS, 1989; DI STASI; HIRUMA; SANTOS, 1994; CARVALHO *et al*., 1996; MELO; JUSTO; DURAN, 2004; SALATINO; NEGRI, 2007). Além disso, a planta é usada para o tratamento de diabetes, diarreias e problemas estomacais. No Pará existe a comercialização de cápsulas contendo o pó da casca da árvore com indicação para tratar várias alterações de saúde, incluindo controle de peso (MACIEL *et al*., 2000; MACIEL *et al.*, 2002; CAMPOS *et al*., 2002). A planta consta entre as 71 espécies vegetais da Relação Nacional de Plantas Medicinais de Interesse ao SUS – RENISUS (BRASIL, 2009). Para o consumo das

folhas da planta é comum o preparo de chá por infusão ou decocto (MACIEL *et al*., 2000).


**FIGURA 5 –** Exsicata da planta *Croton cajucara* Benth.

(Fonte: Herbário Virtual Reflora. Jardim botânico do Rio de Janeiro. Disponível online em: <http://reflora.jbrj.gov.br>. Acessado em: 05 Março 2017. Link para a imagem da exsicata: <http://reflora.jbrj.gov.br/reflora/geral/ExibeFiguraFSIUC/ExibeFiguraFSIUC.do?idFigura=16828670>

**FIGURA 6** – *Croton cajucara* Benth.
(Fonte: Acervo pessoal da Autora, 2015)

### 3.3.3 Composição química da casca do caule e das folhas de *C. cajucara*

Estudos utilizando a casca do caule de *C. cajucara* relatam a planta como uma importante fonte de diferentes terpenos. O principal terpeno já isolado a partir deste material é do tipo clerodano chamado *trans*-dehidrocrotonina (*t*-DCTN) mostrado na Figura 4A (ITOKAWA *et al*., 1999). Vários outros terpenos já foram caracterizados do caule da planta, dentre os quais *trans*-crotonina e ácido acetil aleuritórico, cuja concentração varia ao longo da vida da planta (MACIEL *et al*., 2000). O único terpeno que está presente tanto casca do caule e também nas folhas da planta é o terpeno chamado cajucarinolide (FIGURA 4B) isolado por cromatografia em coluna de um extrato hexânico das folhas da planta (MACIEL *et al*., 2000).

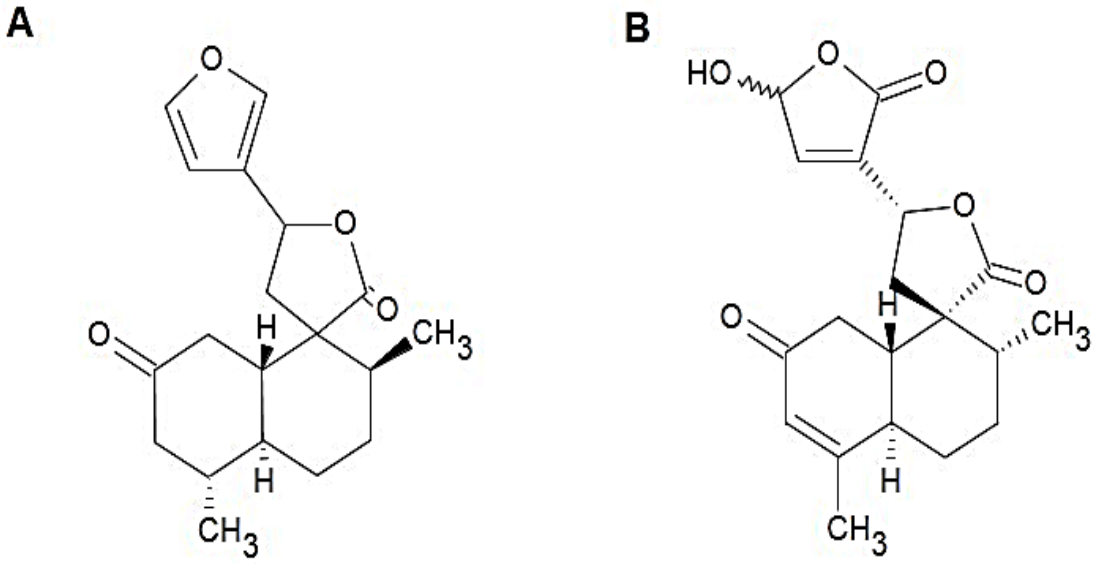


**FIGURA 7** – Diterpenos obtidos da casca do caule de *C. cajucara*.
(A) *trans*-dehidrocrotonina; (B) cajucarinolide
FONTE: ITOKAWA *et al*. (1999) com a permissão de Elsevier Copyright © 1999.

Em prévias investigações da composição química das folhas foi demonstrada a presença de óleos essenciais: linalool e 7-hidroxicalameneno (FIGURA 5A-B). Os óleos essenciais observados nas folhas também estão presentes nas cascas do caule da planta (BIGHETTI *et al*., 1999; PAULA *et al*., 2008). Dois flavonoides, kaempferol 3,4',7-trimetil-éter e kaempferol 3,7-dimetil-éter (FIGURA 5C-D), foram detectados em um extrato apolar das folhas da planta, os quais apresentaram conteúdo constante em plantas de diferentes idades e sem variações em decorrência da estação do ano sob as condições de estudo realizadas por Maciel *et al*. (2000). Compostos químicos da classe dos esteroides, os quais se constituíram de uma mistura de β-sitosterol, estigmasterol e sitoesterol-3-*O*-β-glicosídeo (FIGURA 5E-G), foram identificados em todas as partes da planta (raiz, caule, ramos e folhas) e seu conteúdo foi variável conforme a idade da planta e a estação do ano (MACIEL *et al*., 2000).

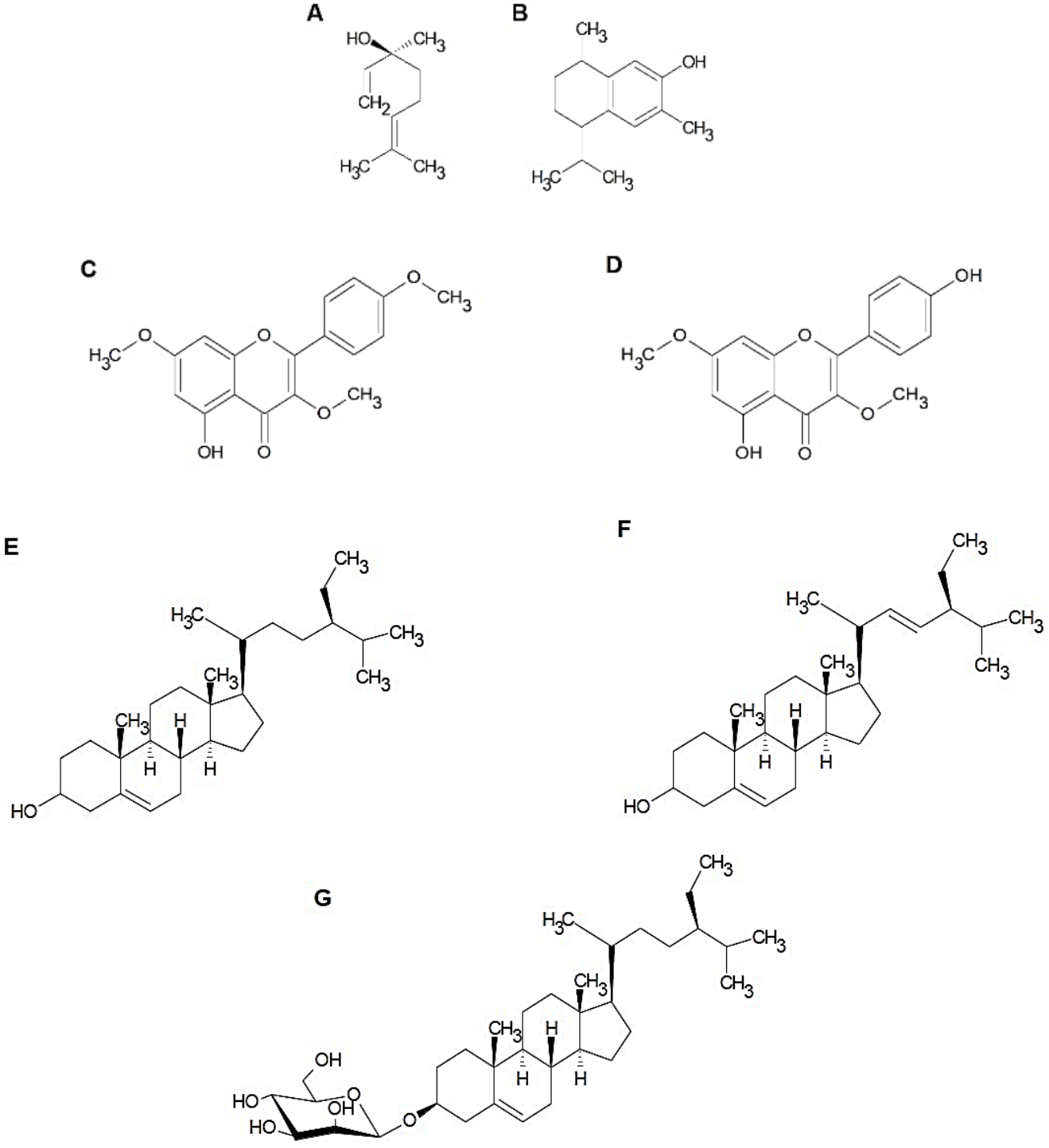


**FIGURA 8** – Metabólitos das folhas de *C. cajucara*.

(A) linalool; (B) 7-hidroxicalameneno; (C) kaempferol 3,4',7-trimetil-éter; (D) kaempferol 3,7-dimetil-éter; (E) β-sitosterol; (F) estigmasterol; (G) sitosterol-3-*O*-β-glicosídeo
(B) FONTE: (MACIEL *et al*. (2000) com a permissão de Elsevier Copyright © 2000.

### 3.3.4 Atividades biológicas descritas para *C.cajucara*

Estudos farmacológicos desenvolvidos com terpenos, óleos essenciais e diferentes extratos provenientes do caule e das folhas da planta demonstram correlação do uso popular com a presença de moléculas biologicamente ativas (FARIAS *et al*., 1997; BIGHETTI *et al*., 1999; HIRUMA-LIMA *et al*., 2000; GRASSI-KASSISSE *et al*., 2003; MACIEL *et al*., 2000; RODRIGUES *et al*., 2012).

#### 3.3.4.1 Estudos desenvolvidos com extratos obtidos da casca do caule

Diferentes ensaios utilizando extratos aquosos obtidos do caule da planta têm demonstrado atividades biológicas. O efeito antioxidante de um extrato aquoso foi verificado *in vivo* em situações onde havia um estresse oxidativo pré-existente. Por outro lado, em situações onde não havia o estresse oxidativo o uso continuado da planta atuou como um pró-oxidante (RODRIGUES *et al*., 2012). Assim, a planta demontrou capacidade de modular a atividade oxidativa. Além disso, em um ensaio *in vivo*, um extrato aquoso demonstrou capacidade de reduzir o ganho de peso e aumentar a sensibilidade da resposta lipolítica para isoprenalina e adrenalina por parte dos adipócitos em animais tratados, quando comparados a ratos do grupo controle (não tratados), dando suporte para o uso popular da casca da planta em tratamentos para a perda de peso (GRASSI-KASSISSE *et al*., 2003).

Estudos farmacológicos com os terpenos *trans*-DCTN, *trans*-crotonina e ácido acetil aleuritórico mostraram correlação do uso popular no controle de hiperlipidemias e patologias associadas (MACIEL *et al*., 2000; MACIEL *et al*., 2002). O diterpeno *trans*-DCTN, isolado da casca do caule, vem demonstrando as correlações mais significativas com as indicações terapêuticas preconizadas pelo uso popular (MACIEL *et al*., 2007). Significante efeito hipoglicêmico de *t*-DCTN foi observado em ratos com diabetes induzida por alloxan (FARIAS *et al*., 1997). A molécula também demonstrou efeito hipotensivo e de bradicardia em estudos com ratos, sendo apresentada como um potencial agente terapêutico em condições crônicas de hipertensão (SILVA *et al*., 2005). O diterpeno *t*-DCTN também mostrou atividade antiúlcera gástrica em diferentes modelos de úlcera em ratos (SOUZA-BRITO *et al*., 1998).

Óleos essenciais da planta demonstraram atividade anti-inflamatória e analgésica em roedores, corroborando com o uso da casca da planta na medicina

popular no tratamento de processos inflamatórios (BIGHETTI *et al*., 1999). Os óleos essenciais administrados por via oral também demonstram boa atividade no tratamento de úlcera gástrica e na proteção da mucosa gástrica com eficácia proveniente da habilidade em estimular a síntese local de muco e a produção de prostaglandina pela mucosa e sem demonstrar efeitos tóxicos (HIRUMA-LIMA *et al*., 1999; HIRUMA-LIMA *et al*., 2000).

3.3.4.2 Estudos desenvolvidos com extratos obtidos das folhas

Extratos provenientes das folhas de *C. cajucara* foram testados frente a diferentes atividades biológicas relacionadas ao uso popular da planta. Extratos brutos (hexânico, clorofórmico e metanólico) das folhas foram capazes de promover um significante efeito antinociceptivo em ratos (CAMPOS et al., 2002). Além disso, extratos hidroalcoólicos foram capazes de reduzir o nível de colesterol total, LDL-colesterol e triglicerídeos, além de elevar no nível de HDL-colesterol no soro de ratos tratados quando comparados ao grupo controle (FARIAS *et al*., 1996). Outro estudo demonstrou capacidade de um extrato aquoso em atuar como antioxidante frente ao radical livre DPPH (TIEPPO *et al*., 2006).

A presença do diterpeno cajucarinolide nas folhas de *C. cajucara* pode indicar capacidade das folhas em inibir o processo inflamatório, uma vez que esta molécula já demonstrou atividade anti-inflamatória e foi capaz de inibir a fosfolipase-A2 do veneno de abelha *in vitro* (ICHIARA *et al*., 1992).

O óleo essencial de *C. cajucara*, presente em suas folhas e caule, mostrou significante efeito gastroprotetor baseado na habilidade do mesmo em estimular a síntese do muco gástrico e a produção de prostaglandinas pela mucosa gástrica (HIRUMA-LIMA *et al*., 2002). Óleos essenciais da planta também mostraram eficiência no tratamento de úlcera gástrica crônica (PAULA *et al*., 2008).

Uma fração contendo óleos essenciais, rica em linalool, das folhas da planta mostrou ser um potente agente contra *Leishmania amazonensis* (ROSA *et al*., 2003). Além disso, em testes para verificar a atividade antimicrobiana, frações constituídas de óleos essenciais, ricas em linalool mostraram capacidade em inibir o crescimento de *Candida albicans*, *Lactobacillus casei*, *Porphyromonas gengivalis*, *Staphylococcus aureus* e *Streptococcus mutans* que são microorganismos envolvidos em doenças da cavidade oral (ALVIANO *et al*., 2005). Um outro óleo essencial, presente nas folhas

da planta, o 7-hidroxicalameneno, foi capaz de inibir o crescimento do protozoário *Leishmania chagasi* sem aparentemente interferir com as células mamíferas (RODRIGUES *et al*., 2013). Diferentes amostras, contendo elevada concentração do óleo essencial 7-hidroxicalameneno foram eficientes contra *S. aureus* resistentes a meticilina, *M. smegmatis*, *M. tuberculosis*, *M. circinelloides* e *R. oryzae*, demonstrando que o óleo essencial apresenta potencial atividade antibacteriana e antifúngica (AZEVEDO *et al*., 2013).

## 3.4 POLISSACARÍDEOS DE PLANTAS

De acordo com sua função, polissacarídeos de plantas podem ser classificados em polissacarídeos de reserva ou estruturais. Dentre os polissacarídeos de reserva podem ser citados o amido e as frutanas, os quais são os mais abundantes em plantas. Os polissacarídeos estruturais participam da composição da parede celular dos vegetais, sendo componentes majoritários, podendo estar associados a outros componentes químicos como proteínas e substâncias aromáticas (HELDT, 1997). Os polissacarídeos estruturais dos vegetais são considerados os compostos mais abundantes da Terra (REID, 1997).

### 3.4.1 Polissacarídeos da parede celular de vegetais

A parede celular de vegetais consiste em um envoltório com um complexo grau de estruturação, que pode ter variada composição química em decorrência da função do tecido e da espécie de planta (PETTOLINO *et al*., 2012). Este envoltório celular se constitui em um importante componente para a vida da célula uma vez que desempenha diferentes funções relacionadas à determinação da forma celular, à textura do tecido e à morfologia do vegetal, sendo capaz de conferir força mecânica e rigidez (REID, 1997). Além disso, a parede celular está envolvida na adesão celular, participa na comunicação célula-célula, controla o crescimento e participa da proteção celular (BRETT; WALDRON, 1990).

A parede celular é um compartimento dinâmico que sofre mudanças ao longo da vida da célula vegetal. A parede celular primária é responsável por envolver células em expansão em tecidos jovens e é não-lignificada, enquanto a parede celular

secundária confere resistência mecânica após o crescimento celular ter cessado em tecidos mais maduros e apresenta lignina. Polissacarídeos compõem aproximadamente 90% do peso seco da parede celular primária e aproximadamente 60% da parede celular secundária (PETTOLINO *et al*., 2012). A água pode estar presente compondo aproximadamente 60% (p/v) da parede celular primária, estando essencialmente ausente da parede celular secundária. Os polissacarídeos da parede são constituídos por três tipos de redes de polímeros estruturais independentes, mas interconectados: microfibrilas de celulose revestidas por polissacarídeos não-celulósicos aderentes (dentre os quais xilanas, xiloglucanas, glucuronoarabinogalactanas ou glucomananas são os mais abundantes), uma matriz tipo gel constituída por pectinas e proteínas estruturais (WHISTLER, 1970; PETTOLINO *et al*., 2012).

A parede celular primária é composta predominantemente por um arranjo complexo de polissacarídeos que compõem aproximadamente 90% desta parede e proteínas que aparecem compondo 10% (DOBLIN; PETTOLINO; BACIC, 2010). Em todos os tipos celulares as microfibrilas de celulose estão embebidas em uma matriz na forma de gel composta por polissacarídeos não celulósicos e glicoproteínas, intimamente associados uns aos outros (POPPER, 2008; DOBLIN; PETTOLINO; BACIC, 2010). A composição química dos polissacarídeos da parede celular primária de dicotiledôneas é de, aproximadamente, 25-40% celulose, 15-25% hemiceluloses, 15-40% pectinas, além de 5-10% de proteínas e traços de componentes fenólicos (AVIGAD; DEY, 1997). Na parede celular secundária polímeros de pectinas e proteínas estão em pouca quantidade ou ausentes enquanto se apresentam ricas em lignina, sendo sua composição de polissacarídeos aproximada de 40-45% celulose, 15-35% hemiceluloses, traços de pectinas além de 15-30% de lignina (AVIGAD; DEY, 1997; PETTOLINO *et al*., 2012).

Os polissacarídeos que compõem a parede celular de vegetais podem ser classificados de acordo com o modo de extração para obtê-los. As pectinas são extraídas por soluções aquosas e oxalato de amônio ou EDTA capazes de quelar o cálcio ou outros cátions metálicos divalentes presentes entre grupamentos carboxílicos carregados negativamente. Hemiceluloses requerem o uso de soluções alcalinas para serem removidas e a celulose é obtida dos resíduos das extrações aquosas e alcalinas (AVIGAD; DEY, 1997).

As pectinas são polissacarídeos complexos que contem unidades de ácido galacturônico em sua composição, sendo ricas em ramnose, arabinose e galactose. São constituídos por três classes de polímeros: homogalacturonanas (HGs), ramnogalacturonas do tipo I (RGs I) e ramnogalacturonanas do tipo II (RGs II). Polímeros de arabinose (arabinanas), galactose (galactanas) e arabinogalactanas são frequentemente encontrados em frações pécticas como cadeias laterais nas RG I (ASPINALL, 1980; McNEIL *et al*., 1984; KOMALAVIVAS; MORT, 1989; BRETT; WALDRON, 1990). Além de HGs, RGs I e RGs II, xilogalacturonanas e apiogalacturonanas são considerados polissacarídeos pécticos primários (POPOV; OVODOV, 2013).

As homogalacturonanas se referem a polissacarídeos lineares (não substituídos) constituídos exclusivamente por unidades de ácido α-D-Gal*p*A, unidos por ligações do tipo (1→4), podendo algumas unidades apresentar grupamentos carboxílicos metil-esterificados, (O´NEILL; ALBERSHEIM; DARVILL, 1990). Os resíduos de GalA podem ser parcialmente metil-esterificados e acetil-esterificados nas posições O-2 e/ou O-3, dependendo da origem da planta (YAPO, 2011). A presença de meti-éster e acetil-éster em pectinas é importante para o comportamento geleificante deste tipo de polímero (YAPO, 2011). Homogalacturonanas lineares nas quais mais que 50% das unidades de α-D-GalA*p* são metil-esterificadas são chamadas convencionalmente de metil-esterificadas HGs (YAPO, 2011).

As RG I são heteropolímeros que geralmente aparecem altamente ramificados e apresentam uma cadeia principal composta pela repetição do grupo dissacarídico [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→], parcialmente substituído em O-4 de algumas unidades de L-Rha*p* (O'NEILL; ALBERSHEIM; DARVILL, 1990). Mais raramente, a posição O-3 dos resíduos de L-Rha*p* podem aparecer substituídas (YAPO, 2011). Além disso, a presença de grupamentos O-acetil substituindo os C-2 e/ou C-3 das unidades de ácido α-D-GalA*p* também é um achado comum em RG I (LEROUGE *et al*., 1993). Acetilação unusual na posição O-3 de resíduos de L-Rha*p* já foi relatada da parede celular do quiabo (SENGKHAMPARN *et al*., 2009). Unidades de Gal*p* tem sido verificados ligadas a posições O-3 e O-4 de resíduos de Rha em RG-I de um extrato aquoso da mucilagem de linhaça e também de pectinas extraídas do quiabo (MURALIKRISHNA; SALIMATH; THARANATHAN, 1987; NARAN; CHEN; CARPITA, 2008; SENGKHAMPARN *et al*., 2009; YAPO, 2011). As RG I costumam ser altamente heterogêneas e além dos resíduos de α-D-Gal*p*A e α-L-Rha*p*, resíduos

de α-L-Ara*f* e β-D-Gal*p* são achados comuns (YAPO, 2011). As substituições podem ocorrer por um único resíduo neutro ou por cadeias laterais poliméricas de arabinanas, galactanas, arabinogalactanas do tipo I (AG I) e arabinogalactanas do tipo II (AG II) ligadas a posição O-4 de resíduos de L-Rha*p*.

As rhamnogalacturonanas do tipo II (RG II) são galacturonanas constituídas por uma cadeia principal formada por unidades de α-D-Gal*p*A unidos por ligação do tipo 1→4, metil-esterificadas em C-6 dos resíduos de GalA. As RG II são altamente conservadas e apresentam substituições em C-2 por quatro tipos cadeias laterais oligossacarídicas (O'NEILL; ALBERSHEIM; DARVILL, 1990). Esse tipo de polímero é formado por 11 a 12 diferentes monossacarídeos com aproximadamente 28 a 36 resíduos de açucares individuais, interconectados por mais de 20 diferentes tipos de ligações glicosídicas resultando em macromoléculas altamente complexas (YAPO, 2011). As cadeias laterais de RG II são formadas por oligossacarídeos que apresentam monossacarídeos raramente observados em substâncias naturais, que são apiose (Api, 3-C-hidroximetil-β-D-eritrose), 2-*O*-metil-fucose, 2-*O*-metil-xilose, ácido acérico (AceA, 3-C-carboxi-5-deoxi-L-xilose), Ácido 2-keto-3-deoxi-D-mano-octulosonico (KdoA), Ácido 3-deoxi-lyxo-heptulosárico (Dha) e α-L-Gal*p* (CARPITA; GIBEAUT, 1993; REID, 1997; YAPO, 2011). Resíduos de AceA podem ser mono-O-acetilados ou di-O-acetildos em O-3, 2-O-Me-Fuc na posição O-3 e/ou O-4 e Rha pode ser metilada em O-3 (MATSUNAGA *et al*., 2004; YAPO, 2011).

As arabinogalactanas (AGs) estão presentes na parede celular de vegetais superiores e podem aparecer associadas ou como substituintes de RG I (STEPHEN, 1983). AGs já foram isoladas de folhas, caules, raízes, flores, sementes, gomas ou exsudatos de vegetais e costumam apresentar estruturas complexas (FINCHER; STONE; CLARKE, 1983; MENESTRINA *et al.*, 1998; MELLINGER *et al*., 2005). Arabinogalactanas são classificadas, conforme as diferenças das ligações químicas envolvidas na formação da cadeia principal, em arabinogalactanas do tipo I (AG I) e arabinogalactanas do tipo II (AG II). As AG I com cadeia principal formadas por unidades de β-D-galactose (1→4) ligadas, geralmente contem poucas ramificações, mas podem aparecer ramificadas em O-3 por unidades de arabinose e costumam ser associadas às RG I (ASPINAL, 1993; CARPITA; GIBEAUT, 1993). As AG II costumam apresentar maior complexidade do que as AG I, apresentando elevado grau de ramificação, sendo constituídas por cadeias de (1→3) e (1→6) β-D-galactose conectadas umas às outras por pontos de ramificação em O-3 e O-6, podendo

apresentar unidades de arabinose em algumas posições O-3 e O-6 (CARPITA; GIBEAUT, 1993).

Um modelo estrutural de parede celular primária de plantas dicotiledôneas foi proposto por Carpita e Gibeaut (1993). Neste tipo de parede, chamado parede do tipo I, microfibrilas de celulose apresentam-se entrelaçadas a xiloglucanas e esta estrutura encontra-se embutida em uma matriz de polissacarídeos pécticos: ácido poligalacturônico e rhamnogalacturonanas (FIGURA 6). Ademais, uma figura esquemática da estrutura de pectinas é demonstrada na Figura 7.

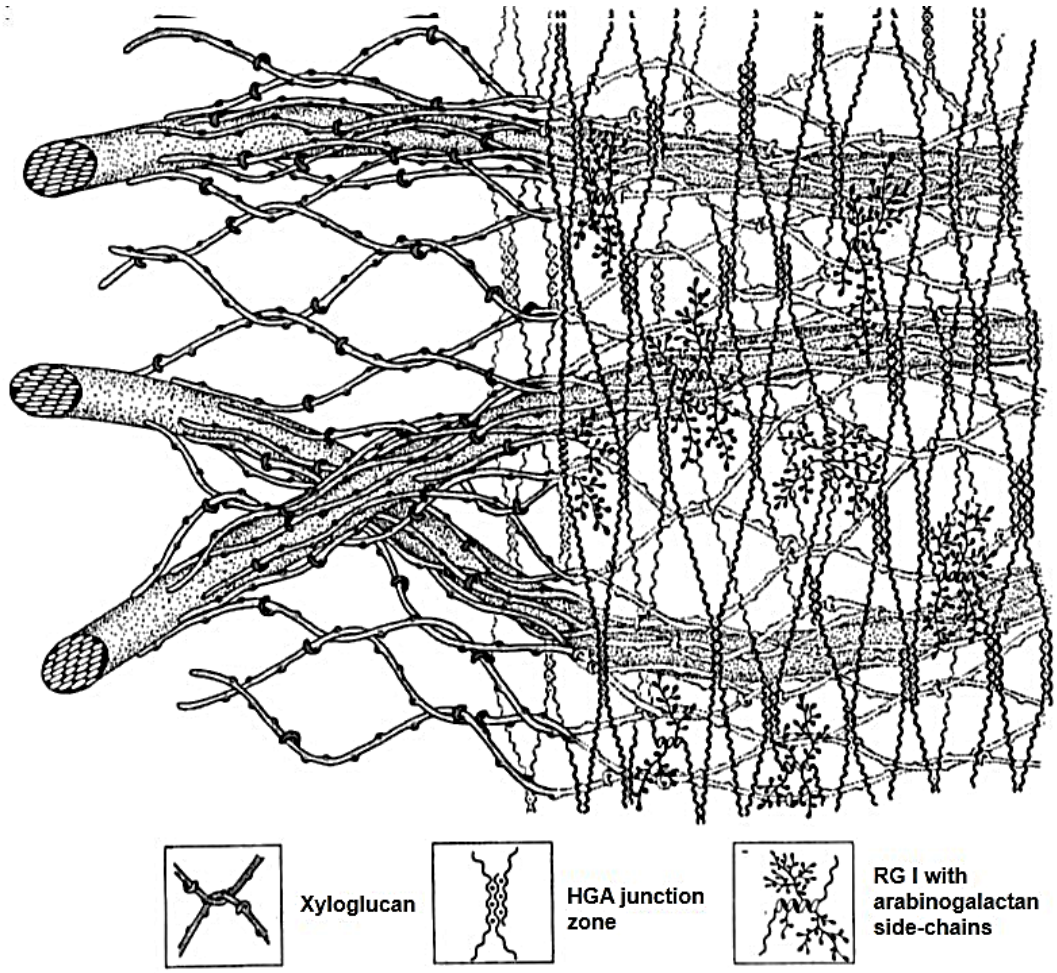


**FIGURA 9** – Modelo estrutural da parede celular primária de dicotiledôneas
FONTE: Modificada de CARPITA; GIBEAUT (1999) com a permissão de John Wiley and Sons © 1999.

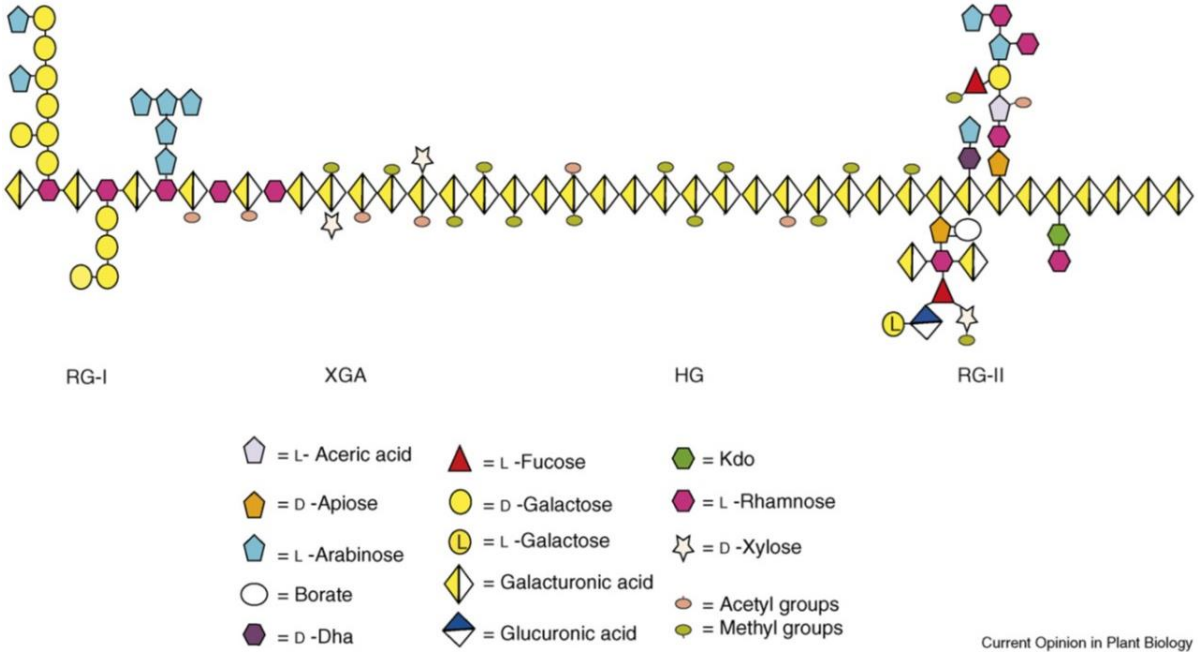


**FIGURA 10** – Estrutura esquemática de pectina
RG-I – Rhamnogalacturonana do tipo I; XGA – xilogalacturonana; HG – Homogalacturonana; RG-II – Rhamnogalacturonana do tipo II
FONTE: MOHNEN (2008) com a permissão de Elsevier Copyright © 2008.

### 3.4.2 Atividades biológicas de polissacarídeos de plantas

Polissacarídeos são considerados uma importante classe de metabólitos primários, aparecendo como um dos principais ingredientes ativos de plantas medicinais, responsáveis por várias atividades farmacológicas, além de serem relativamente não-tóxicos e, portanto, sem efeitos colaterais significantes (LIU; WILLFOR; XU, 2015). Diferentes atividades biológicas são atribuídas a polissacarídeos de origem vegetal, dentre as quais, antioxidante (YAN et al., 2010; DALONSO; PETKOWICZ, 2012), antiviral (LI; PENG, 2013; OLIVEIRA et al., 2013), imunomoduladora e imunoestimulatória (CAPEK et al., 2003; CAPEK; HRÍBOLOVÁ, 2004; SIMAS-TOSIN *et al*., 2012; LIU *et al*., 2015), anti-inflamatória (LIAO; LIN, 2012), anticomplemento (YAMADA *et al*., 1985), antitussígena (KARDOSOVÁ et al., 1997), antifadiga (WANG *et al*., 2010) e antiúlcera gástrica (SRIVASTAVA; ULSHVESHTHA, 1989; YAMADA, 1991; SUN; MATSUMOTO; YAMADA, 1992; YAMADA *et al*., 1994; NERGARD *et al*., 2005; CIPRIANI *et al*., 2006).

### 3.4.2.1 Atividade antiúlcera gástrica

A atividade antiúlcera gástrica tem sido atribuída a vários polissacarídeos de origem vegetal. Ela foi verificada pela administração oral de polissacarídeos pécticos obtidos por extração aquosa, contendo altos teores de ácido galacturônico, da planta *Panax ginseng* (SUN; MATSUMOTO; YAMADA, 1992). De um extrato aquoso obtido das raízes de *Bupleurum falcatum* foi isolado outro polissacarídeo péctico com elevado teor de ácido galacturônico e com presença, em menores proporções, dos monossacarídeos neutros arabinose, ramnose e galactose, o qual apresentou potente atividade gasroprotetora em ratos (YAMADA, 1991). Uma fração gastroprotetora, consistindo de uma RG I apresentando elevado teor de ácido galacturônico metil-esterificado e ramificações de AG II, foi obtida de um extrato aquoso de *Acmella olearacea* (NASCIMENTO *et al*., 2013).

Uma AG II isolada do chá das folhas de *Maytenus ilicifolia* demonstrou efeito gastroprotetor em modelos de lesão aguda induzida por etanol em ratos (CIPRIANI *et al*., 2006). AG I obtida do farelo de soja também demonstrou efeito gastroprotetor em ratos (CIPRIANI *et al*., 2008). Além disso, uma arabinana isoladas das sementes de *Chenopodium quinoa* demonstrou atividade gastroprotetora (CORDEIRO *et al*., 2012).

Diferentes mecanismos gastroprotetores podem ser atribuídos a polissacarídeos, estando relacionados à formação de uma camada de proteção local na mucosa gástrica, à inibição da secreção de suco gástrico, ao aumento da produção de muco, ou ao sequestro de radicais livres (YAMADA, 1994; MATSUMOTO; MORIGUCHI; YAMADA, 1993; CIPRIANI *et al*., 2006).

### 3.4.2.2 Atividades imunomoduladora, imunoestimulatória e anti-inflamatória

Polissacarídeos podem interagir direta ou indiretamente sobre o sistema imune, desencadeando alguns eventos celulares e/ou moleculares que podem levar à sua ativação (FERREIRA *et al*., 2015). Polissacarídeos de origem vegetal são capazes de atuar na resposta imune inata especialmente por sua ação sobre os macrófagos, os quais têm sido considerados por um longo tempo um dos principais alvos destes polímeros. Os macrófagos são células do Sistema Monocítico Fagocitário que desempenham papéis fundamentais na resposta imune celular. Essas células são originadas dos monócitos do sangue que se diferenciam nos tecidos e, dependendo

do tecido no qual residem, apresentam características morfológicas distintas. A ativação de macrófagos no tecido se dá pelo reconhecimento de estímulos nocivos ao organismo através de receptores específicos presentes na membrana desta células, o que desencadeia reações que podem resultar na resposta inflamatória.

A resposta inflamatória é constituída por uma cascata de eventos altamente regulados no qual os macrófagos executam um papel central. Os macrófagos atuam na iniciação do processo inflamatório e na imunomodulação, que consiste na produção de mediadores inflamatórios que coordenam respostas em diferentes tecidos e órgãos (FUJIWARA; KOBAYASHI, 2005). Polissacarídeos testados na resposta imune inata demonstram capacidade em ativar macrófagos, estimulando a fagocitose e o aumento da produção de mediadores inflamatórios (LIAO; LIN, 2012). Macrófagos ativados podem apresentar aumento da produção de citocinas pró-inflamatórias, dentre as quais interleucina-1β (IL-1β) e fator de necrose tumoral-alfa (TNF-α), que são capazes de modular a atividade de uma variedade de células, causando mudanças metabólicas durante infecção ou injúria tecidual (ADEREN; ULEVITCH, 2000; FUJIWARA; KOBAYASHI, 2005). Ademais, a ativação de macrófagos por determinadas vias do sistema imune pode ocasionar aumento na produção de interleucina-10 (IL-10), uma citocina anti-inflamatória, que é capaz de inibir a produção de outras citocinas inflamatórias por monócitos e macrófagos (KASHIWAGI; SUKUKI, 1992). Neste contexto, vários extratos e polissacarídeos de plantas que apresentam atividade sobre macrófagos tem demonstrado capacidade de regular a produção destas e de outras citocinas (SCHEPETKIN; QUINN, 2006).

Há inúmeros relatos de pectinas, bem como outros polissacarídeos presentes nas cadeias laterais destes polímeros ácidos (arabinanas, galactanas e arabinogalactanas), com capacidade de atuar sobre o sistema imune (PAULSEN, 2001; SCHEPETKIN; QUINN, 2006). As diferenças estruturais destes polissacarídeos estão relacionadas com a sua atuação sobre o sistema imune, embora não haja um consenso a respeito do mecanismo pelo qual diferenças estruturais finas desencadeiam respostas distintas. Um exemplo da atuação de pectinas, está relacionado ao teor de resíduos de GalA determinando sua reatividade sobre o sistema imune. Pectinas com alto conteúdo de resíduos de GalA exibem atividade imunossupressora; fragmentos de pectinas obtidas por hidrólise ácida mostram ação anti-inflamatória; diferenças estruturais na cadeia péctica como a presença de ramificações, grau de metil-esterificação e peso molecular influenciam a habilidade de

pectinas inibirem a atividade funcional de leucócitos (POPOV; OVODOV, 2013). Regiões mais ramificadas de pectinas contendo RG I como cadeia principal, com cadeias laterais de monossacarídeos neutros, apresentam capacidade potencializada em atuar sobre o sistema complemento (PAULSEN, 2001).

Arabinogalactanas de diferentes plantas já demonstraram capacidade de ativar e modular a resposta do sistema imune (SCHEPETKIN; QUINN, 2006). Muitas arabinogalactanas relatadas com atividade biológica são AG II (PAULSEN, 2002). Uma AG II extraída da raiz de *Angelica acutiloba* foi relatada por mostrar atividade sobre o sistema complemento (YAMADA et al., 1985; PAULSEN, 2001). As folhas da planta *Plantago major* são usadas na cicatrização de feridas, e através do estudo de seus polissacarídeos foi possível a caracterização de uma AG II com alto grau de ramificação e capaz de atuar sobre o sistema complemento (PAULSEN, 2002). Polissacarídeos contendo AG II, além de porções pécticas, das plantas *Arnica montana* e *Plantago major*, foram capazes de executar um efeito pronunciado sobre o sistema complemento e estimular macrófagos a aumentarem a secreção de TNF-α (PUHLMANN; ZENK; WAGNER, 1991; SAMUELSEN *et al*., 1995; PAULSEN, 2001). Polissacarídeos contendo 55% de arabinose e galactose em sua composição, isolado de *Chlorella pyrenoidosa*, mostrou ser um potente ativador de monócitos humanos pelo aumento nos níveis de RNAm das citocinas IL-1β e de TNF-α (PUGH *et al*., 2001). AG II ligada a RG I, obtida da planta *Glinus oppositifolius*, mostrou propriedades imunomoduladoras por sua atividade sobre o sistema complemento e imunoestimulante, com habilidade para induzir a proliferação de células B, aumentar a secreção de IL-1β por macrófagos e aumentar RNAm para IFN-γ em células NK (INNGJERDINGEN *et al*., 2007).

Não há um consenso acerca do mecanismo pelo qual polissacarídeos conseguem atuar sobre o sitema imune, seja sobre células ou mesmo sobre moléculas, como é o caso dos fatores do sistema complemento, mas existem algumas características que são compartilhadas por estes polímeros. Geralmente, apresentam uma cadeia principal com muitos pontos de ramificação e com cadeias laterais também ramificadas, como no caso de RG I ramificadas por AG II. O tamanho molecular é outro fator que influencia na atividade (PAULSEN, 2002; INNGJERDINGEN *et al*., 2007). A relação estrutura-atividade foi estudada pela comparação da atuação de uma AG II péctica nativa, com frações obtidas após degradação enzimática e hidrólises parciais, sobre o sistema complemento e sobre o

sistema imune intestinal. Os resultados mostraram a importância de porções ramificadas de AG II sobre a capacidade imunomoduladora do polissacarídeo (INNGJERDINGEN *et al*., 2007). Além disso, em AG II covalentemente ligada a polímeros pécticos, a remoção de regiões lineares, compostas por unidade de GalA*p* (1→4) ligadas, resultou em aumento da atividade imunoestimulatória, enquanto a remoção de regiões ramificadas de AG II (pontos de ramificação de galactose e arabinose terminal) diminuiu esta atividade (TOGOLA *et al*., 2008; NERGARD *et al*., 2005; FERREIRA *et al*., 2015).

## 3.5 METABÓLITOS SECUNDÁRIOS DE PLANTAS

Compostos do metabolismo secundário não apresentam uma função vital ao vegetal sendo, portanto, considerados não essenciais para a vida do organismo. Os produtos desse metabolismo não apresentam uma distribuição universal, mas conferem as plantas vantagens para a sobrevivência e para a perpetuação da espécie no ecossistema facilitando sua interação ao ambiente (SIMÕES *et al*., 2003; LOPEZ-BUCIO *et al*., 2006). Nesse grupo de moléculas encontram-se substâncias cuja produção é limitada a determinados organismos, com bioquímica e metabolismo específicos e únicos (SIMOES *et al*., 2003).

Produtos do metabolismo secundário podem desempenhar papéis importantes para o vegetal, dentre os quais, atrativo de polinizadores, defesa contra herbívoros e patógenos, defesa química contra predadores maiores (LOPEZ-BUCIO *et al*., 2006). Além disso, a concentração de vários metabólitos secundários pode ser alterada em condições de estresse causados por mudanças de temperatura, disponibilidade de água, exposição à radiações UV e deficiência de nutrientes. As condições do ambiente podem impactar a via metabólica responsável pela produção e o aumento de alguns metabólitos pode levar a sobrevivência da planta (RAMAKRISHNA; RAVISHANKAR, 2011).

Os produtos do metabolismo secundário de plantas são, frequentemente, apresentados na literatura como os principais responsáveis por efeitos terapêuticos que a planta apresenta. Estas moléculas possuem constituintes químicos diversos e por vezes são marcadores para a classificação taxonômica desses vegetais, uma vez

que determinadas famílias, gêneros ou espécies produzem moléculas que lhes são características (GOBBO-NETO; LOPES, 2007).

Embora uma molécula seja classificada como sendo do metabolismo primário ou secundário as reações bioquímicas as quais originam um determinado metabólito não ocorrem de maneira independente em uma determinada planta (SIMÕES *et al*., 2003). Um grande número dos constituintes secundários são produzidos via rotas metabólicas vegetais básicas que levam a produção de um ou poucos metabólitos-chave, dos quais numerosos derivados são formados por reações enzimáticas simples, na maioria dos casos (HARTMANN, 1996). A origem dos metabólitos secundários pode ser resumida à partir do metabolismo da glucose por dois intermediários principais, o ácido chiquimico e o acetato. A figura 10 permite a visualização esquemática da origem dos metabólitos secundários.

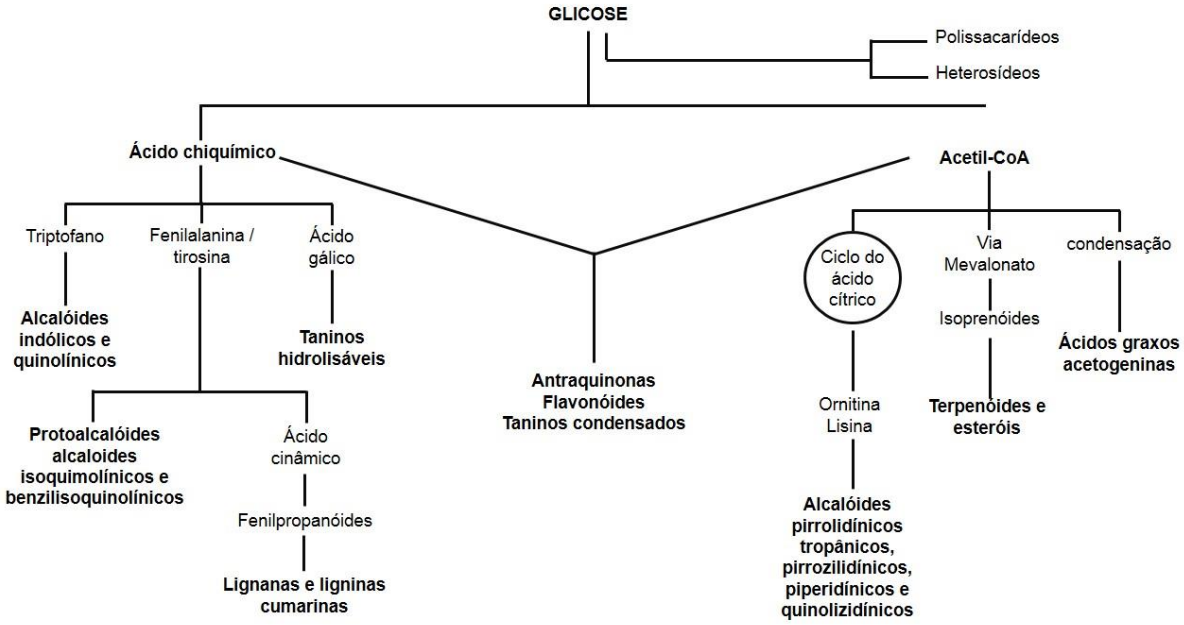


**FIGURA 11** – Ciclo biossintético dos metabólitos secundários.
(Fonte: SIMÕES *et al*., 2003, p. 411)

### 3.5.1 Compostos fenólicos

Compostos fenólicos são uma classe de moléculas na qual estão incluídas uma grande diversidade de estruturas, simples e complexas, que possuem pelo menos um anel aromático no qual pelo menos um hidrogênio é substituído por um grupamento hidroxila (SIMÕES *et al*., 2003). Os compostos fenólicos provenientes do

metabolismo secundário de vegetais são amplamente consumidos na alimentação humana sendo encontrados em folhas, frutos, flores e raízes podendo conferir aos vegetais características específicas como odor, sabor e cor. Ademais, dada esta última característica, podem apresentar valor econômico pela utilização como flavorizantes e corantes de alimentos e bebidas. O papel de compostos fenólicos, provenientes de plantas na saúde humana requer atenção, uma vez que já foram demonstradas em vários trabalhos a capacidade dessas moléculas em executar diversos efeitos terapêuticos.

Considerando a alta diversidade de compostos fenólicos, existem diferentes maneiras para classificá-los, seja pelo tipo estrutural ou origem biossintética. Um tipo de classificação para os fenólicos pode ser feita considerando o tipo de esqueleto principal (básico) da estrutura química. Conforme esta classificação as classes de compostos fenólicos são representadas por: fenóis simples e benzoquinonas, ácidos fenólicos, acetofenonas e ácidos fenil acéticos, fenilpropanóides (ácidos cinâmicos e compostos análogos, fenilpropenos, cumarinas, isocumarinas e cromonas), naftoquinonas, xantonas, estilbenos e antraquinonas, flavonóides e isoflavonóides, lignanas, diflavonóides, melaninas vegetais, ligninas, taninos hidrolisáveis, taninos condensados (SIMÕES *et al*., 2003). Breves descrições sobre flavonóides, taninos condesados e derivados de ácidos hidroxicinamicos serão abordadas, dada a relevância destas classes de metabólitos no presente trabalho.

Flavonóides são polifenóis biossintetizados a partir da via dos fenilpropanóides relativamente abundantes em plantas. A presença de flavonóides como constituinte da dieta humana é garantida pelo consumo de frutas e vegetais, e também de bebidas como vinho, café, chá e cerveja (DI CARLO; MASCOLO; IZZO, 1999). As moléculas, representantes desta classe, apresentam em sua maioria, 15 átomos de carbono em seu núcleo fundamental. O núcleo fundamental dos flavonóides é constituído de duas fenilas ligadas por três carbonos entre elas, e em compostos formados por três ciclos, cada ciclo é denominado núcleo A, B e C com os átomos de carbono recebendo números ordinários para os núcleos A e C e números ordinários procedidos por uma linha para o núcleo B, como mostrado esquematicamente na Figura 11 (SIMÕES *et al*., 2003). As agliconas dos flavonóides são subdivididas em flavona, flavonol, flavanona flavanol (FIGURA 12).

Estruturalmente a variação entre os tipos de flavonoides se deve em parte as suas substituições que podem ser hidroxilação, metoxilação, fenilação, acetilação,

sulfatação ou glicosilação (HARBORNE; WILLIAMS, 2000; STOBIECK, 2000; SOUZA *et al*., 2008). Um grande número de flavonóides naturais ocorre conjugado com açucares, podendo ser *O*-glicosilados ou *C*-glicosilados quando a ligação se dá por intermédio de uma hidroxila ou por um átomo de carbono da glicona diretamente ligado ao um carbono da aglicona, respectivamente. Alguns grupos OH de flavonóides são usualmente glicosilados: em flavonas, flavanonas e isoflavonas, o grupo OH ligado ao C-7 e em flavonols e flavanol os grupos OH ligados aos C-3 e C-7 (PRASAIN WANG; BARNES, 2004).

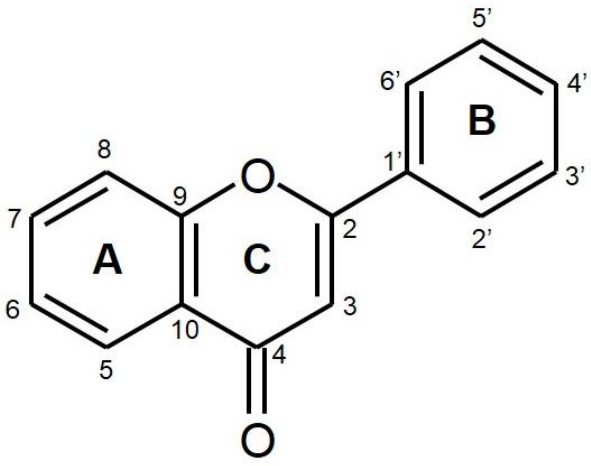


**FIGURA 12** – Esquema descrevendo o núcleo dos flavonóides.
(Fonte: SIMÕES *et al*., 2003, p. 579)

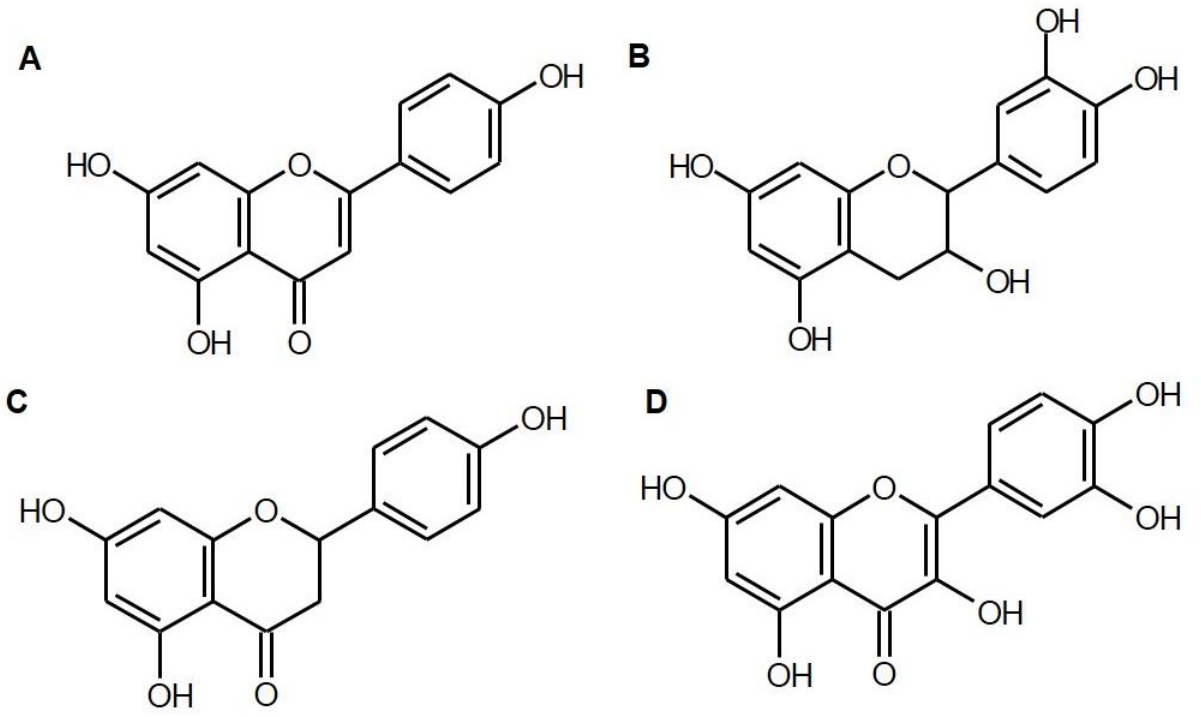


**FIGURA 13** – Tipos de agliconas observadas em flavonóides.
A – Flavona; B – Flavanol; C – Flavanona; D – Flavonol.
FONTE: Modificada de PRASAIN WANG; BARNES (2004) com a permissão de Elsevier Copyright © 2004.

Diferentes estudos atribuem a flavonóides várias atividades biológicas incluindo anti-inflamatória (KIM *et al*., 2010), antioxidante (CHEN; HO, 1997), anti-espasmódica (BEIL; BIRKHOLZ; SEWING, 1995), anti-ulcera (MATA *et al*., 1997); antimicrobiana, antitumoral (RAUHA *et al*., 2000; PARK *et al*., 2007; SIMIRGIOTIS *et al*., 2013). Flavonóides de plantas tem demonstrado atividade anti-inflamatoria *in vitro* e *in vivo*. Embora o mecanismo de atuação de vários tipos de flavonóides não seja completamente elucidado, flavonóides provenientes de diferentes fontes vegetais foram estudados por seus efeitos sobre a resposta inflamatória aguda, induzida por carragenana em camundongos e demonstraram capacidade de inibir o metabolismo do ácido araquidônico (FERRÁNDIZ; ALCARAZ, 1991). Os resultados sugerem que a inbição do metabolismo do ácido araquidônico pode ser um dos mecanismos pelo qual os flavonóides desempenham atividade anti-inflamatória (FERRÁNDIZ; ALCARAZ, 1991). Neste contexto, um mecanismo de ação importante é a inibição de eicosanoides que geram enzimas, incluindo a fosfolipase $A_2$, ciclooxigenase e lipoxigenases, dessa forma diminuindo as concentrações de prostanóides e leucotrienos (KIM *et al*., 2004). Ademais, flavonóides já foram relatados com

capacidade de modular a expressão de genes relacionados a produção de enzimas e citocinas pró-inflamatórias (KIM *et al*., 2004).

Os taninos condensados são oligômeros e polímeros de flavan-3-ols, também chamados de proantocianidinas (FULCRAND et *al*.,1999). As proantocianidinas (PAs) consistem de uma sequencia de compostos fenólicos construídos por unidades monoméricas únicas, as quais podem ser, afzelequina, epiafzelequina, catequina, epicatequina, galocatequina e epigalacatequina (LI; DEINZER, 2007). Além disso, as unidades monoméricas de flavan-3-ols algumas vezes são esterificadas com ácido gálico para formar 3-*O*-galato (SOUZA *et al*., 2008). PAs do tipo B são formadas por oligômeros ou polímeros (os quais podem exibir diferentes graus de polimerização) de flavan-3-ols ligados por C4→8 principalmente ou algumas vezes C4→6 e quando a ligação éter é formada entre C2→7 os compostos são denominados PAs do tipo A (LI; DEINZER, 2007). A Figura 13 mostra um exemplo de PAs do tipo B formado por três unidades (tetrâmero) de (epi)catequina.

Plantas medicinais ricas em taninos são empregadas para tratar diferentes tipos de problemas de saúde dentre os quais diarreia, hipertensão arterial, reumatismo, hemorragia, feridas, queimaduras, problemas estomacais, renais e do sistema urinário além de processos inflamatórios em geral (SIMÕES *et al*., 2003)

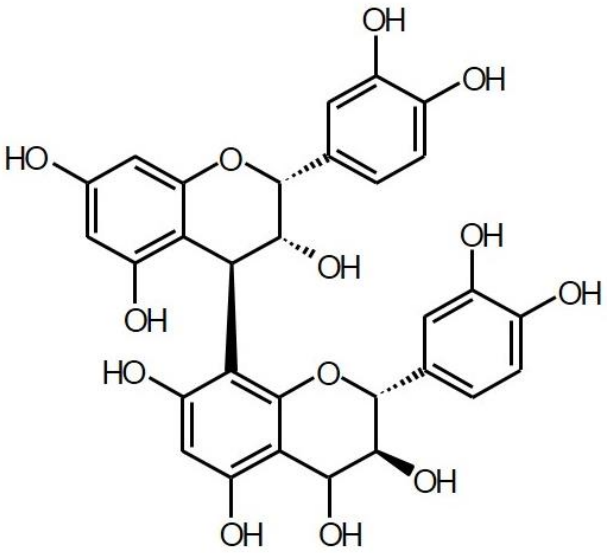


**FIGURA 14** – Proantocianidina dimérica
(epicatequina-4→8)-catequina (Fonte: SIMÕES *et al*., 2003, p. 628)

Em contraste com polifenóis, o conhecimento acerca de ácidos hidroxicinâmicos e seus derivados é limitado embora eles sejam amplamente distribuídos em vegetais. Estruturalmente os ácidos cinâmicos possuem nove átomos de carbono ($C_6$-$C_3$) predominantemente na forma hidroxilada (FRESCO *et al*., 2006). Em plantas os derivados de ácidos hidroxicinâmicos são originados dos aminoácidos fenilalanina e tirosina pela via biosintetica do chiquimato (EL-SEEDI *et al*., 2012). Os ácidos cinâmicos ocorrem nas formas livre ou conjugada, usualmente como ésteres de ácidos orgânicos e/ou glicosídeos ou ligados a proteínas ou a outros polímeros da parede celular (CHEN; HO, 1997).

Os compostos de ácidos hidroxicinâmicos são quase exclusivamente derivados dos ácidos *p*-cumárico, cafeico e ferrúlico, enquanto os derivados do ácido sináptico são comparativamente mais raros (HERMANN *et al*., 1989). Na Figura 14 estão esquematizados os ácidos hidroxicinâmicos mais comuns.

Os derivados de ácidos hidroxicinâmicos tem sido observados na dieta humana desempenhando um importante papel devido a sua abundância e diversidade (EL-SEEDI *et al*., 2012). Eles demonstram relevância por possuir algumas atividades biológicas, dentre as quais, antioxidante, anti-inflamatória, tratamento de obesidade e anti-câncer (CHEN e HO, 1997; CHEEL *et al*., 2005; FRESCO *et al*., 2006; LIN; HARNLY, 2007; EL-SEEDI *et al*., 2012; ROCHA; MONTEIRO; TOLEDO, 2012; ALAM *et al*., 2016).

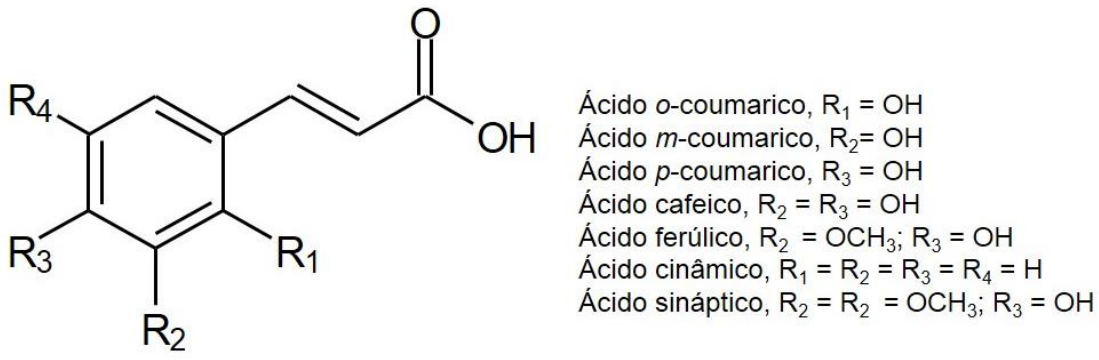


**FIGURA 15** – Estruturas de ácidos hidroxicinâmicos.

FONTE: Modificada de EL-SEEDI *et al*. (2012) com a permissão de American Chemical Society © 2012.

# 4 OBJETIVOS

## 4.1 OBJETIVO GERAL

Este estudo apresentou como objetivo extrair, isolar e caracterizar estruturalmente polissacarídeos e metabólitos secundários presentes nas folhas das plantas: *Arrabidaea chica* Verlot., e *Croton cajucara* Benth., bem como a avaliar as atividades gastroprotetora e anti-inflamatória *in vivo*, e efeito imunoestimulatório *in vitro*.

## 4.2 OBJETIVOS ESPECÍFICOS

- Obter e caracterizar polissacarídeos de extratos aquosos das folhas de *A. chica* e *C. cajucara*;
- Testar a atividade gastroprotetora *in vivo*, de frações obtidas a partir de um extrato aquoso de *C. cajucara*;
- Testar o efeito imunomodulador de polissacarídeos provenientes de extrato aquoso de *A. chica*, em macrófagos humanos da linhagem THP-1;
- Isolar frações e identificar compostos de baixa massa molar de um extrato aquoso de *C. cajucara*;
- Verificar atividade anti-inflamatória, utilizando o modelo de edema de pata induzido por carregenana em camundongos, dos compostos de baixa massa molar obtidos por extração aquosa das folhas de *C. cajucara*.

## 5 ARTIGOS CIENTÍFICOS

ARTIGO 1 – Polysaccharide from aqueous extract of *Arrabidaea chica* (crajiru): chemical structure and immunostumulatory activity

ARTIGO 2 – Gastroprote tive effect and chemical characterization od a polysaccharide from leaves of *Croton cajucara*

ARTIGO 3 – Phytochemical analysis and anti-inflammatory evaluation of compounds form aqeuous extract of *Croton cajucara* Benth.

# ARTIGO 1

(Artigo ainda não enviado para publicação)

**Polysaccharide from aqueous extract of *Arrabidaea chica* (crajiru): chemical structure and imunistimulatory activity**

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ABSTRACT

An unusual type II arabinogalactan was obtained from leaves of *Arrabidaea chica* Verlot., popularly know as crajiru, by hot aqueous extraction followed by freezing-thawing process and sequential dialysis at membranes with different cut-off. The fraction retained on dialysis at 25 kDa cut-off membrane (AC25R) showed a single peak on HPSEC, had a $M_w$ of 49,690 g/mol, and showed a monosaccharide composition of galactose, arabinose, glucose, mannose, rhamnose and 3-O-Me-Galactose in a 11:10.6:10:3.1:2.7:1.0 molar ratio and 6.1 g% of glucuronic acid. Methylation analysis and NMR spectroscopy indicated that AC25R is composed mainly of a type II arabinogalactan containing unusual non-reducing ends of glucose and 3-*O*-Me-Gal. This polysaccharide showed immunostimulatory effect on THP-1 macrophages by the increase of inflammatory cytokines TNF-α, IL-1β e IL-10 indicating that it could be related to biological activities of the plant.

**Keywords**: *Arrabidaea chica* V.; Polysaccharide; Type II arabinogalactan; Immunostimulatory effect

## 1 Introduction

*Arrabidaea chica* Verlot. (Bignoniaceae) is found in Amazon, where is popularly known as *crajiru*. Infusion of its leaves is used in popular medicine to healing wounds and inflammation of the skin and mucous membranes, psoriasis and infections [1,2]. It is also used to treat intestinal pain, diarrhea and anemia [3]. The infusion of *A. chica* can be administered orally or directly on inflamed areas. Some indigenous ethnic groups of the Amazon Region of Brazil have used extracts from *A. chica* leaves for paint their bodies in religious rituals, as natural sunscreen and insect repellent [4]. This plant appears among the 71 species in the National List of Medicinal Plants of Interest to Health Unique System of Brazil (RENISUS) that includes plant species with potential to generate health interest products. Moreover, the glycolic extract from its leaves is used in the manufacture of creamy soaps with antiseptic and anti-inflammatory indications [2].

Previous studies with *A. chica* have shown medicinal properties related to its anti-inflammatory effect. The main chemical constituents reported for its ethanolic extract are the 3-desoxianthocianidins carajurin and carajuron, both pigments of the flavonoids chemical class [5]. Other desoxianthocianidins of its leaves have been characterized and to this phenolic compound class is attributed the anti-inflammatory and wound healing activities of the plant [6,7]. A crude methanol extract from its leaves showed healing activity related to stimulation of fibroblasts and collagen synthesis *in vivo* and *in vitro* [2]. This same extract healed tendon lesions via topical application and apparently improved the organization of collagen fibers [8,9]. An aqueous extract also showed anti-inflammatory activity on edema induced by snake venoms of *Brothrops* and *Crotalus* in mice [10]. Moreover, the plant has already demonstrated a significant trypanocidal effect on *Trypanosoma cruzi*, and total inhibition of *Trichophyton mentagrophytes* fungal growth [4].

Since the leaves of *A. chica* are used as an infusion, other chemical constituents including polysaccharides can be present in their aqueous extracts and may participate in the reported biological activities. Polysaccharides are readily obtained by hot aqueous extraction and some activities from different plants have been attributed to them, including immunomodulatory activity [11-16]. Polysaccharides are able to initiate activation of leucocytes, to stimulate the phagocytosis and production of inflammatory mediators [16].

The inflammatory response is constituted by a cascade of events highly regulated in which macrophages perform important roles. A key function of macrophages is the initiation of inflammation and immunomodulation that consist in inflammatory mediators and cytokines production, which coordinate additional response of different tissue or organs of the host [17]. Each cytokine produced by an activated macrophage exert a distinct activity with effects extended over the cells [18]. The macrophages activation results to inflammatory effects for release pro-inflammatory cytokines such as interleukine-1 (IL-1) and tumor necrosis factor-α (TNF-α) that are endogenous pyrogens, responsible by modulating activities in a variety of immune cells type causing countless metabolic changes during infection or tissue injury [17,19]. The inhibitory cytokine interleukine-10 (IL-10) shows ability to inhibit the production of inflammatory cytokines by monocytes and macrophages [20]. LPS stimulation result initially in the synthesis of inflammatory cytokines, including TNF-α and IL-1, and later to the synthesis of IL-10 that down-regulates the inflammatory cytokines production and itself production [19]. Previous reports showed that TNF-α is enable to induce IL-10 production and IL-10 is enable do suppress TNF-α. Probably these cytokines display an auto-regulatory feedback response [21].

Considering the popular use of the infusion of *A. chica* leaves and the need to know its active molecules, this study had the purpose of to isolate an aqueous soluble polysaccharide from this plant, characterize it structurally and evaluate its immunostimulatory effect on THP-1 macrophages**.**

## 2 Materials and methods

### 2.1 *Plant material*

Leaves of the plant *Arrabidaea chica* (Bonpl.) Verlot. were collected in the State of Acre (Amazon Region), in July 2014. The plant was identified by Bianchini R. S. Ph.D (Botanical Institute, Secretary of the Environment, São Paulo, Brazil) and it was compared with the existing voucher SP 430336. The leaves were dried at 60 °C for two days, before the extraction process.

## 2.2 *Extraction and fractionation of polysaccharides*

Dried leaves of *A. chica* (170 g) were extracted with hot water (1.7 L) under conditions of reflux for 3 h (x 3). The aqueous extract was obtained by filtration, evaporated to a small volume, and treated with EtOH (x 3 vol.). The resulting precipitate was recovered by centrifugation (10,000 rpm for 25 min), dialyzed (6-8 kDa cut-off membrane) for three days and freeze-dried, to give the crude polysaccharide fraction (2.4 g). It was dissolved in water (240 mL) at room temperature, and was submitted to freeze-thawing procedure until no more precipitate appeared. The soluble fraction was submitted to sequential dialysis using 100, 50 and 25 kDa cut-off membranes. The fraction retained on the 25 kDa cut-off membrane (AC25R) presented homogeneous elution profile on HPSEC and was chemically characterized and submitted to biological assays.

## 2.3 *Monosaccharide analysis*

Each fraction (2 mg) was hydrolyzed with 1 M TFA (1 mL) at 100 °C for 16 h, the solution was then evaporated, and the residue dissolved in water (1 mL). The resulting monosaccharide mixture was examined by silica-gel 60 thin layer chromatography (TLC; Merck), the plates being developed with *n*-propanol:water (7:3, v/v) and stained with orcinol-sulfuric acid [22]. The hydrolyzate was also treated with $NaBH_4$ (2 mg) overnight. HOAc was added, the solution evaporated to dryness and the resulting boric acid removed as trimethyl borate by co-evaporation with MeOH. Acetylation was carried out with $Ac_2O$-pyridine (1:1 v/v, 0.6 mL) at room temperature overnight, and the resulting alditol acetates were extracted with $CHCl_3$. These were analyzed by GC-MS (Varian Saturn 2000R - 3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225 column (30 m x 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C/min, with He as the carrier gas. Components were identified by their typical retention times and electron impact spectra. The monosaccharide composition was given as mol%.

The uronic acid content of polysaccharides was quantified using the colorimetric *m*-hydroxybiphenyl method [23].

## 2.4 *Methylations analysis*

AC25R (10 mg) was per-*O*-methylated using powdered NaOH in DMSO-MeI, according to the method of Ciucanu and Kerek (1984) [24]. The product was hydrolyzed with 45% aqueous $HCO_2H$ (0.5 mL) at 100 °C for 18 h. The acid was evaporated and the resulting mixture of *O*-methyl aldoses dissolved in water (1 mL) and submitting to reduction with $NaBD_4$ (2 mg) overnight. The solution was neutralized with HOAc (0.5 mL), evaporated to dryness, and the boric acid from reduction procedure was removed as trimethyl borate by co-evaporation with MeOH. The acetylation was then carried out as described for monosaccharide analysis to give a mixture of partially *O*-methylated alditol acetates. These were analyzed by GC-MS using the same conditions as described for alditol acetates, except for the final temperature hold at 215 °C. They were identified according to section 2.3 by comparison to partially *O*-methylated alditol acetates from standard monosaccarides [25]. The composition of *O*-methylated alditol acetates was given as mol% [26].

## 2.5 *HPSEC analysis*

Homogeneity and average molar mass ($M_w$) of AC25R were determined by high-performance size-exclusion chromatography (HPSEC) coupled to a Waters 2410 differential refractometer (RI), DSP-F Wyatt Technology multi-angle laser light scattering (MALLS). The analysis was performed in a Waters chromatograph equipped with four gel permeation Ultrahydrogel columns in series, with exclusion sizes of 7 x $10^6$, 4 x $10^5$, 8 x $10^4$, and 5 x $10^3$ Da. The eluent was 0.1 M aqueous $NaNO_2$ containing 200 ppm $NaN_3$ at 0.6 mL/min. The fraction was previously filtered through a membrane (0.22 μm) and injected (100 μL loop) at a concentration of 1 mg/mL. The specific refractive index increment (*dn/dc*) was determined using the same equipment describe above, but the columns were uncoupled. AC25R fraction at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL, previously filtered (membrane of 0.22 μm), was injected (500 μL loop) and analyzed at 25 °C using the RI detector. The results were processed with software provided by the manufacturer (Wyatt Technologies ASTRA software, version 4.70.07).

## 2.6 *NMR spectroscopy*

HSQC-DEPT spectrum was obtained using a 400 MHz Bruker Avance III spectrometer, equipped with a 5 mm inverse probe. The AC25R fraction was dissolved in $D_2O$ (500 µL) at 60 mg/mL and the experiments were performed at 70 °C. Chemical shifts ($\delta$) were expressed in ppm relative to acetone, at $\delta$ 30.2/2.22 ($^{13}C/^{1}H$) and the NMR signals assigned on the basis of literature data.

## 2.7 *Biological activity assay of AC25R on macrophages*

### 2.7.1 *Cell culture and macrophage differentiation*

The biological assays were performed using human monocytic cell line THP-1. The monocytes were maintained in RPMI 1640 culture medium (Sigma Aldrich, cat. R8758) supplemented with 10% heat-inactivated fetal calf serum sterile A (Gibco, cat. 161010-159), streptomycin (100 µg/mL) and penicillin (100 UI/mL), in a humidified incubator (37 °C, 5% $CO_2$) and it was renewed twice a week. The cells were multiplied until a density of $2{,}0x10^5$ cells/mL and were used to experiment at the maximum passage of 10.

The differentiation to macrophage happened by treatment of monocytes THP-1 with 5 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 h. The cells were maintained in a 96-wells tissue culture plate (containing a volume 200 of µL/well) for viability assay or in a 24-wells tissue culture plate (containing a volume of 1 mL/well) for cytokine quantification. After differentiation, adherent macrophages to surface enabled the removal of medium containing PMA and the wells were washed with sterile PBS (phosphate buffered saline). Free PMA culture medium was replaced and cells were incubated under appropriate conditions (37 °C in 5% $CO_2$), for 24 h.

### 2.7.2 *Cell viability assay*

The cell viability assay was performed using THP-1 macrophages ($2{,}0x10^5$ cells/well) in a 96-well tissue culture plate previously incubated for adhesion as described above. Adherent macrophages were incubated under appropriate

conditions, for 24 h in the presence of sterile PBS (50 µL) or different concentrations of AC25R (2, 10, 50 and 250 µg/mL) in quintuplicate. AC25R fraction was solubilized in ultra-pure water (Sigma-Aldrich) at different concentrations and sterilized by filtration in a sterile membrane (0.22 µm pore size). Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay as described by Reilly (1998) [27].

### 2.7.3 *IL1β, IL10 and TNF-α quantification*

Adherent THP-1 macrophages ($2{,}0x10^5$ cells/well) were incubated in a 24-wells tissue culture plate in fresh medium containing AC25R at different concentrations (2, 5, 10 µg/mL), or sterile PBS (50 µL) as negative control or lipopolysaccharide (LPS, 100 ηg/mL) as pro-inflammatory control. After 18 h of incubation, the supernatants were separated and stored at -80 °C until the cytokines assays were performed. The concentration of interleukin 1β (IL-1β), interleukin 10 (IL-10) and tumor necrosis factor (TNF)-α were determined in the supernatants using Elisa Ready-Set-go kits specific for human cytokines, according to the manufacturer's instructions (assay kits e-Bioscience, nº. 88-7346, 887010, 88-7106).

### 2.7.4 *Statistical analysis*

The data obtained were express as mean ± standard error of the mean (SEM). The statistical significance of differences between the groups was determined using one-way analysis of variance (ANOVA) followed by Newman Keuls test. Calculations were performed using GraphPad Prism version 6 (GraphPad Prism Software, San Diego, USA). In all cases, a $p < 0.05$ was considered statistically significant.

## 3 Results and discussion

### 3.1 *Isolation of the polysaccharide fraction*

Dried leaves of *A. chica* (170 g) were extracted with water under reflux (1.7 L) and then, extract was treated with excess EtOH to provide a precipitate containing

crude polysaccharides (2.4 g). This fraction was submitted to freezing-thawing process, and the soluble component was fractioned by sequential dialysis at 100, 50 and 25 kDa cut-off membranes (Fig. 1). All fractions displayed heterogeneous elution profiles except the fraction retained in the 25 kDa cut-off membrane (AC25R) which presented a homogeneous elution profile on HPSEC-MALLS/RI analysis, with $M_W$ of 49.690 g/mol (*dn/dc* = 0.207) (Fig. 2).

### 3.2 *Structural analysis of AC25R*

GC-MS analysis of alditol acetates from AC25R demonstrated that the polysaccharide is composed of galactose (28.7 g%), arabinose (27.5 g%), glucose (26.1 g%), mannose (8.1 g%), rhamnose (7.0 g%) and an unusual 3-*O*-Me-galactose (2.6 g%) as neutral monosaccharides, besides of uronic acid (6.1 %) which were colorimetrically quantified. TLC analysis of the AC25R indicated that only glucuronic acid was present (Fig. 3).

Methylation analysis of AC25R (Table 1) showed that galactose is present as 3,6-, 3- and 6-linked units or terminals, according to the methylated derivatives 2,4-$Me_2$-Gal (12.1%), 2,4,6-$Me_3$-Gal (5.5%), 2,3,4-$Me_3$-Gal (13.5%) and 2,3,4,6-$Me_4$-Gal (5.7%). These glycosidic linkages were observed in the HSQC correlation map (Fig. 4), which showed chemical shifts at δ 103.1/4.49 and δ 103.1/4.50 of C1/H1 by *β*-D-Gal*p* units. The correlations at *δ* 80.0/3.72 and 69.2/4.06 (DEPT-inverted) are typical of C3/H3 and C6/H6 substituted of *β*-D-Gal*p* units [28]. Moreover, the correlation at δ 56.4/3.46 was attributed to the methyl group of the 3-*O*-Me-Gal observed in the monosaccharide composition analysis. The presence of 3-*O*-Me-Gal was already observed for polysaccharides of roots, twigs, and leaves of other plants [29-31]. From the *Salvia officinalis* was isolated a α-L-arabino-3,6-D-galactan containing 3-*O*-Me-Gal units at side chain [12]. 3-, 6- and 3,6-linked Gal*p* units are present in type II arabinogalactans. This class of polysaccharide is formed by 3-linked β-Gal*p* units in the main chain, which are frequently branched at *O*-6 position by side chains of 6-linked β-Gal*p* units. These side chains frequently are substituted at *O*-3 by galactosyl or arabinosyl residues [32]. In AC25R the ratio of 3-linked to 3,6-linked Gal*p* residues (1:2.2) is indicative of a highly branched polymer.

The arabinosyl units of AC25R appeared exclusively as Ara*f*, which were 2,5-, 3-, 5-, 2-linked or terminals, according to the 3-Me-Ara (10.9%), 2,5-$Me_2$-Ara (6.6%), 2,3- $Me_2$-Ara (5.5%), 3,5-$Me_2$-Ara (2.3%) and 2,3,5-$Me_3$-Ara (6.6%) methylated derivatives. On the HSQC correlation map, typical correlations of C1/H1 of non-reducing end units of α-L-Ara*f* can be observed at δ 109.0/5.25, 107.7/5.38 [33]. The correlations at δ 107.2/5.21 and 107.5/5.08 were attributed to C1/H1 of 5- and 2,5-linked α-L-Ara*f* units, respectively (Fig. 4) [34-36]. Moreover, the correlation at δ 87.2/4.25, 84.0/4.09 and 66.6/3.86 (DEPT-inverted) are from C2/H2, C3/H3 and substituted C5/H5 of Ara*f* units [36-37]. Terminals of arabinose and/or arabinan chains frequently are found branching type II arabinogalactans [38].

The glucosyl units of AC25R were mainly terminals, according to the presence of the 2,3,4,6-$Me_4$-Glc*p* (16.0%) methylated derivative. Although unusual, glucosyl residues on the terminal position can appear in type II arabinogalactans [39-41]. Moreover, the 4- (6.2%) and 4,6-linked (1.9%) Glc*p* unit of AC25R is indicative of starch in the fraction. On HSQC, the correlation of C1/H1 of α-D-Glc*p* was observed at δ 99.8/5.01 (C1/H1) [42].

GlcA*p* residues, which were observed in the polysaccharide isolate from *A. chica*, have already been found as terminal units in type II arabinogalactans from other plants [43,44]. A low proportion of Rha*p* terminal units were found in AC25R (2,3,4-$Me_3$-Rha, 3.1%, Table 2) and the correlations at δ 96.3/5.12 and 16.9/1.17 (Fig. 4) can be attributed to C1/H1 and C6/H6 of them [45].

### 3.3 *Biological activity of AC25R fraction on THP-1 macrophages*

Several immunomodulatory herbal extracts and compounds including polysaccharides have shown the capacity to increase cytokine production by macrophages [14, 46]. Thus, in this work were performed assays to evaluate the capacity of AC25R fraction from *A. chica* leaves to stimulate the production of the pro-inflammatory cytokines TNF-α, IL-1β, and the anti-inflammatory IL-10 in THP-1 macrophages. In order to determine the non-toxic concentration of the fraction, adherent THP-1 macrophages were incubated with different concentrations of AC25R (2, 10, 50 and 250 µg/mL). After 24 h the cell viability was verified by MTT assay, which showed that AC25R fraction did not have cytotoxic effect in the lower dosages, however, in the higher dose (50 and 250 µg/mL) it decreased the cell viability to 78.9%

and 70% respectively (Fig. 4). Thus, since AC25R was cytotoxic at the higher concentrations tested, the subsequent biological assays were performed with concentrations of 2, 5 and 10 µg/mL.

The assays to evaluate the capacity of AC25R fraction to increase production of the cytokines by THP-1 macrophages were compared to PBS (negative control) and LPS (positive control) was chosenbecause it is an inflammatory stimulus responsible for increase the levels of cytokine by THP-1 macrophages. The time chosen to the assay was 18 h since THP-1 macrophages present the maximal increase of cytokines secretion induced by LPS in this time [47]. As expected, production of TNF-α, IL-1β and IL-10 increased significantly in macrophages treated with LPS (Fig. 5). Treatment with AC25R also stimulate the production of all three cytokines by macrophages, and its effect was in a concentration-dependent manner (Fig. 5). The level of TNF-α induced by 10 µg/mL of AC25R fraction presented an increase by approximately fifteen-fold when compared to negative control group (PBS). At 2 µg/mL and 5 µg/mL of AC25R, the TNF-α production was five and ten-fold higher than the negative control group, respectively (Fig. 5A). The level observed of IL-1β production show also increase in a concentration-dependent manner. Although at the lower doses (2 and 5 µg/mL) no significant difference was observed between untreated (PBS) and treated (AC25R fraction), the THP-1 macrophages treated with 10 µg/mL of AC25R presented increase significant of the level of IL-1β production (Fig. 5B).

The level of IL-10, an anti-inflammatory cytokine, was increased by AC25R fraction when compared to negative control group (C) in a concentration-dependent manner similar with the data showed to levels of TNF-α and IL-1β (Fig. 5C). The concentrations of 2, 5 and 10 µg/mL increased IL-10 production again, by approximately two, three and four times respectively and LPS (100 ηg/mL) promoted an increase by four times approximattely, when compared to negative control group (PBS). These preliminary results indicate that AC25R is able to induce up-regulation of inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10) cytokines in THP-1 macrophages, and they are in agreement with previous reports, confirming the ability of plant polysaccharides to enhace macrophages function, including to increase the cytokines secretion [12,14,48, 49]. Plant polysaccharide have already demonstrate the ability to interact on the immune system, triggering some cellular and/or molecular events that may lead to its activation [50]. These polymers are able to act on the innate

immune response and especially on macrophages to induce secretion of inflammatory cytokines, which can influence the wound healing and infections [50, 51].

Extracts of the medicinal plant *A. chica* are widely used for the treatment of inflammatory disorders. Previous reports demonstrated that the wound healing effect of crude extracts from this plant can be related to initial activation of macrophages, since these cells participate from the beginning to the end of the of inflammatory response, producing inflammatory mediators, which activate cellular migration to the injury site, removing foreign bodies and directing tissue development [1,8]. In the present work is showed an initial evidence of immunostimulatory effect of a polysaccharide fraction (AC25R) from an aqueous extract of *A. chica* leaves. AC25R was able to activate THP-1 macrophage to release cytokines, indicating that it is able to act on the immune response. TNF-α and IL-1β are among the first cytokines that appear in an inflammation response. Sequentially, IL-10 production is stimulates, which down-regulate TNF-α and IL-1β prodution [21]. Although different polysaccharides from plants have demonstrated the capacity of interact with the immune system, their differences on primary structure, size and level of branched influence on the way which they act and consequently the level and the expected response [52]. Arabinogalactans from different plants have already demonstrated ability to activate and modulate the immune system response [14]. Type II arabinogalactans from the different genus of plants are described by their enable to influence the immune system and activate macrophages, enhancing production of cytokines related to inflammatory response [52, 53]. Moreover, the presence of 3-*O*-Me-Gal residues in arabinogalactans, which is a characteristic of AC25R, has already been described as component of type II arabinogalactan enable to exert immunomodulatory activity [12]. Type II arabinogalactan from different plants showed increased macrophages activity, promoving characteristics morphological alterations [54, 55]. The leaves of the *Plantago major*, a plant used in wound healing, contain a type II arabinogalactan with a high degree of branching and capable of acting on the complement system [52]. An another type II arabinogalactan from *Chlorella pyrenoidosa* was found to be a potent activator of human monocytes by increasing the mRNA levels of IL-1β and TNF-α cytokines [56]. Type II arabinogalactan bound to rhamnogalacturonan type I as a minor component, obtained from the *Glinus oppositifolius*, showed immunomodulatory properties with ability to promove cell B

proliferation, increase the secretion of IL- 1β by macrophages and increase mRNA of IFN-γ in NK cells [53].

## 4 Conclusions

For the first time a polysaccharide fraction (AC25R) of an aqueous extract from *A. chica* leaves was chemically characterized and subjected to an immustimulatory activity assay. The chemical data demonstrate that it consists mainly of type II arabinogalactan containing unusual 3-*O*-Me-galactose residues, besides of glucose at the terminal position. AC25R showed prominent imunostimulatory effect *in vitro* on THP-1 macrophages through increased production of inflammatory cytokines. Considering the pharmacological value of *A. chica* further investigations are necessary to describe the pathway and mechanisms of this activation of macrophages.

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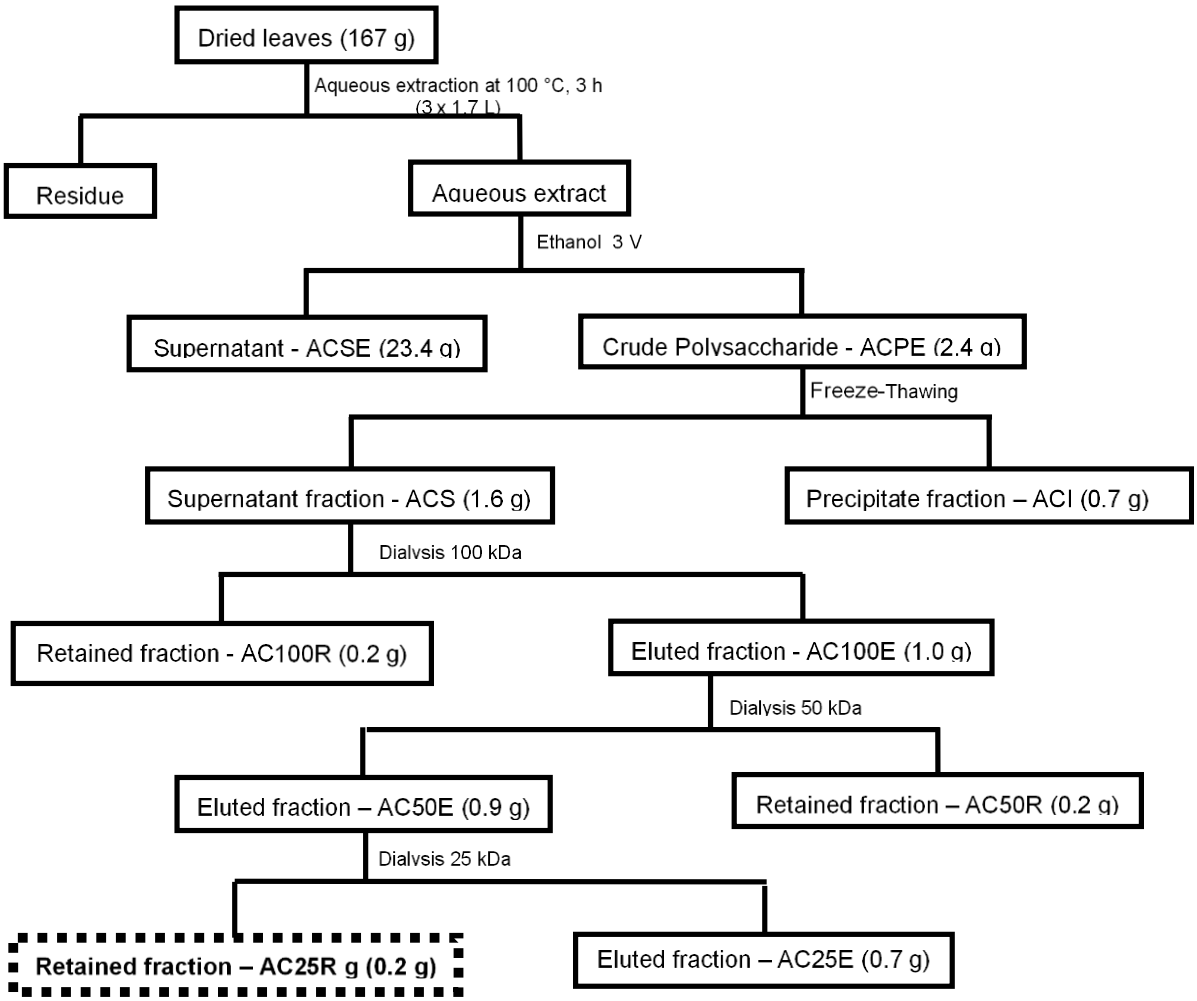


**FIGURE 1** - Scheme of extraction and fractionation of polysaccharides from *A. chica* leaves.

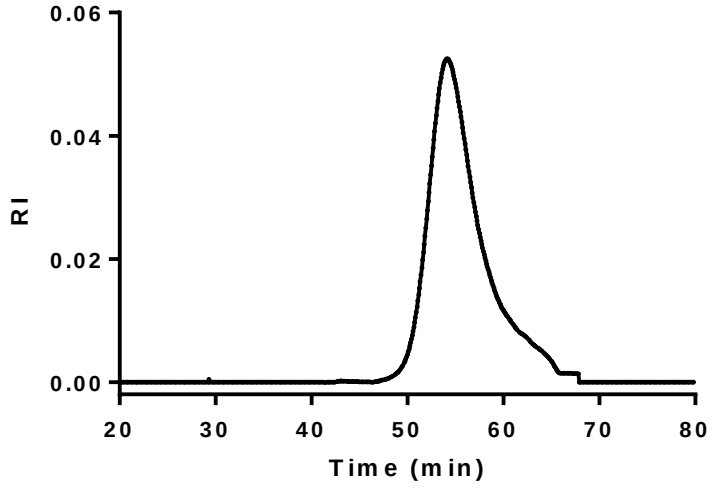


**FIGURE 2** – Elution profile of AC25R on HPSEC

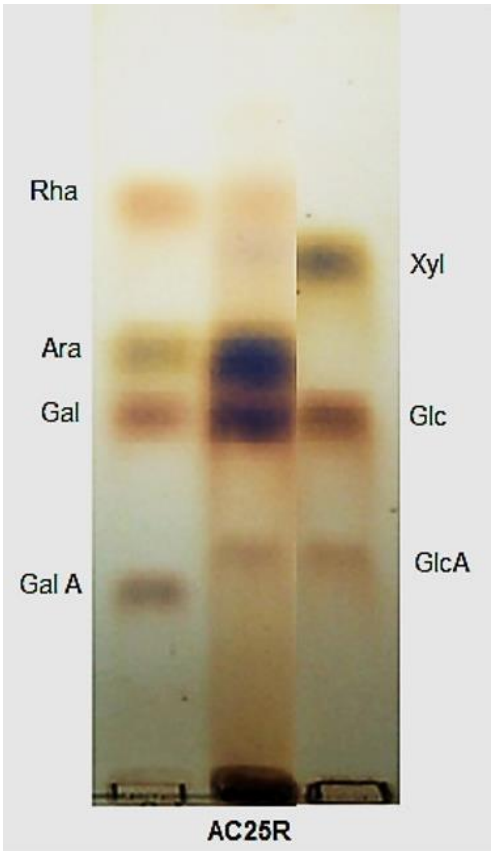


**FIGURE 3** – Thin Layer Chromatograph of AC25R (Rha: Rhamnose; Ara: Arabinose; Gal: Galactose; GalA: Galacuronic Acid; Xyl: Xylose; Glc: Glucose; GlcA: Glucuronic acid)

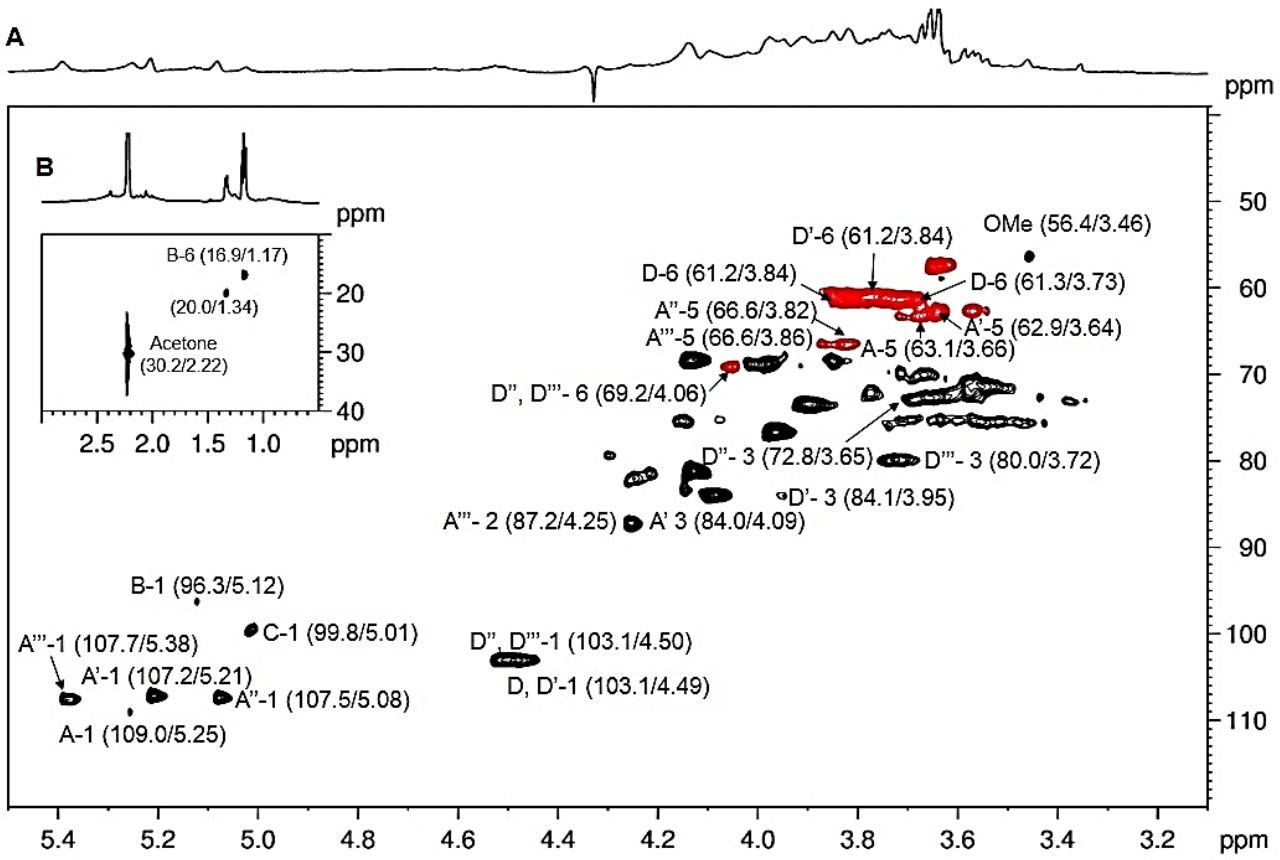


**FIGURE 4** - HSQC correlation map of AC25R. Solvent $D_2O$ at 70 °C; numerical values are in δ ppm. A (non-reducing end α-L-Ara*f*), A' (5-linked α-L-Ara*f*), A''(2,5-linked α-L-Ara*f*), A''' (non-reducing end α-L-Ara*f*), B (α-L-Rha*p*), C (α-D-Glc*p*), D (non-reducing end β-D-Gal*p*),D' (3-linked β-D-Gal*p*),D'' (6-linked β-D-Gal*p*), D''' (3,6-linked β-D-Gal*p*). The letters are followed by the carbon number of the monosaccharide unit.

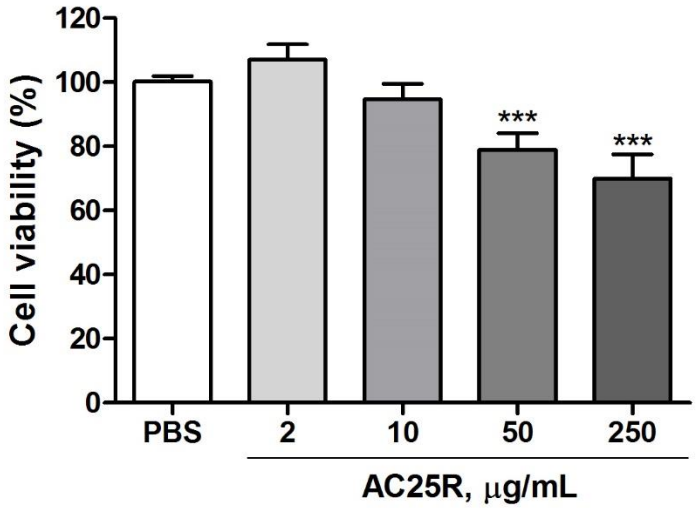


**FIGURE 5** - Influence of AC25R on THP-1 macrophages viability determined by MTT method expressed as percentage. Cells were incubated for 14 h with PBS (negative control) or different concentrations of AC25R in 5% $CO_2$ at 37 °C. The results were expressed as mean ± SEM (n=5). Asterisks (*) represents statistical significant difference from the negative control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, determined by Newman Keuls test.

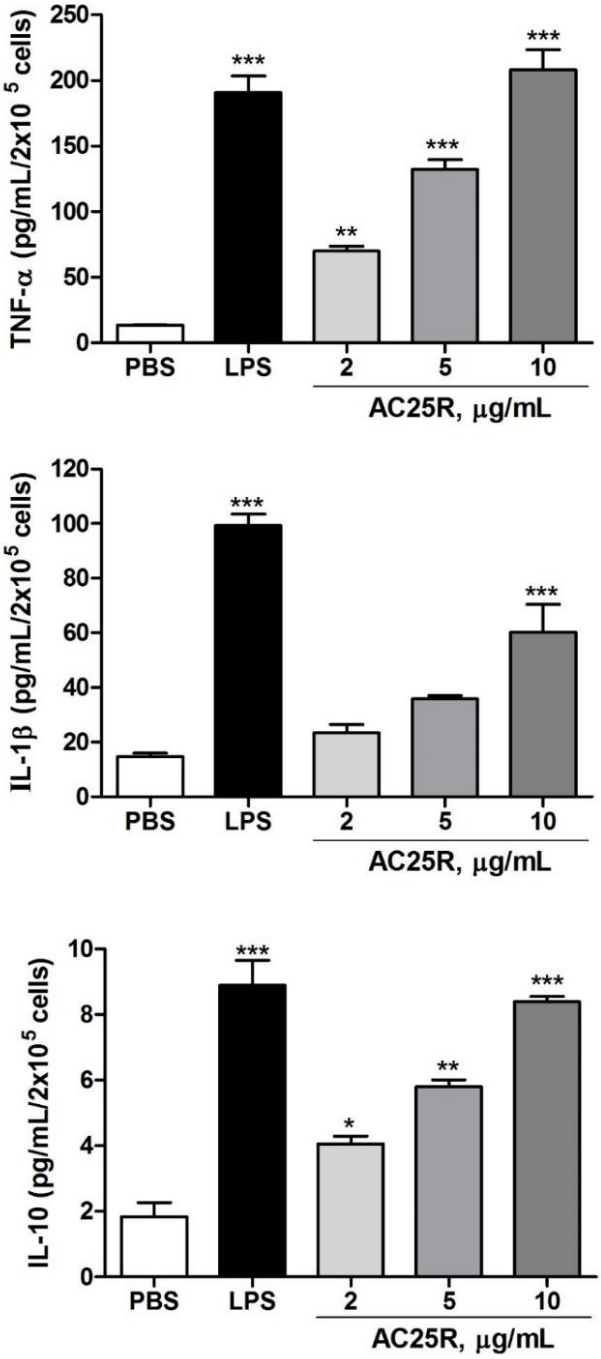


**FIGURE 6** - Effect of AC25R on TNF-α, IL-1β and IL-10 secretion in THP-1 macrophages. Cells were incubated for 18 h with PBS (negative control), LPS (positive control), or different concentrations of AC25R in 5% $CO_2$ at 37 °C. The concentration of cytokines were determined in the cell-free supernatants using Elisa Ready-Set-go kits specific for human cytokines, according to the manufacturer's instructions. The results were expressed as mean ± SEM for two independent experiments in quadruplicate. Asterisks (*) represents statistical significant difference from the negativ control group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), determined by Newman Keuls test.

**Table 1** – Profile of partially O-methylated alditol acetates of AC25R obtained on methylation analysis.

| *O*-Me-Alditol acetate | Structure | $t_R$ | AC25R mol % |
|---|---|---|---|
| 2,3,5-$Me_3$-Ara | Ara*f*-(1→ | 0.771 | 6.6 |
| 2,3,4-$Me_3$-Rha | Rha*p*-(1→ | 0.834 | 3.1 |
| 3,5-$Me_2$-Ara | →2)-Ara*f*-(1→ | 0.927 | 2.3 |
| 3,4-$Me_2$-Rha | →2)-Rha*p*-(1→ | 0.948 | 1.6 |
| 2,5-$Me_2$-Ara | →3)-Ara*f*-(1→ | 0.958 | 6.6 |
| 2,3,4,6-$Me_4$-Glc | Glc*p*-(1→ | 1.000 | 16.0 |
| 2,3-$Me_2$-Ara | →5)-Ara*f*-(1→ | 1.032 | 5.5 |
| 2,3,4,6-$Me_4$-Gal | Gal*p*-(1→ | 1.063 | 5.7 |
| 3-Me-Rha | →2,4)-Rha*p*-(1→ | 1.245 | 0.8 |
| 2,4,6-$Me_3$-Gal | →3)-Gal*p*-(1→ | 1.297 | 5.5 |
| 2,3,6-$Me_3$-Man | →4)-Man*p*-(1→ | 1.353 | 1.7 |
| 3-Me-Ara | →2,5)-Ara*f*-(1→ | 1.366 | 10.9 |
| 2,3,6-$Me_3$-Glc | →4)-Glc*p*-(1→ | 1.441 | 6.2 |
| 2,3,4-$Me_3$Gal | →6)-Gal*p*-(1→ | 1.643 | 13.5 |
| 2,3-$Me_2$-Glc | →4,6)-Glc*p*-(1→ | 2.119 | 1.9 |
| 2,4-$Me_2$-Gal | →3,6)-Gal*p*-(1→ | 2.326 | 12.1 |

$t_R$ = relative retention time to 2,3,4,6-tetra-*O*-metilglucitol acetate; The uronic acid content of AC25R was 6.1%, according to the spectrophotometric method of Filisetti-Cozzi e Carpita (1991).

## ARTIGO 2

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**Gastroprotective effect and chemical characterization of a polysaccharide from leaves of *Croton cajucara***

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ABSTRACT

*Croton cajucara* Benth. is a tree from the Amazon Forest, where it is known as sacaca. Its leaves and barks are used in medicinal preparations to treat different diseases, including gastric ulcers. The crude polysaccharide fraction (CCP), obtained from the hot aqueous extract of *C. cajucara* leaves, was able to promote gastroprotection on an ethanol induced gastric ulcer model. Therefore, a bioguided fractionation was performed to isolate the active polysaccharide fraction. After freezing-thawing, ultrafiltration and dialyses at 100, 50, and 25 kDa cut-off membranes, fraction 25R was obtained. It contained glucose, galactose, rhamnose, arabinose, galacturonic acid and mannose in a 7:5:5:3:1:1 molar ratio approximately, and had a Mw of 42,840 g/mol. Methylation analysis and NMR spectroscopy indicated that 25R is a very complex

polysaccharide fraction containing type I rhamnogalacturonan, arabinan, type I arabinogalactan, type II arabinogalactan, rhamnan, starch and mannan. It was able to reduce ethanol-induced gastric ulcers in rats, through preservation of mucus and GSH levels.

## 1 Introduction

The use of plants for therapeutic purposes has been observed throughout the history of civilizations. Brazil has considerable biodiversity in flora and local populations consume plants as remedy to treat different diseases, especially in regions where people have less access to conventional manufactured medicines.

Peptic ulcer is a gastrointestinal disorder that consists in an excoriated area of the gastric or duodenal mucosa by action of the gastric juice. Natural products including extracts derived from plants have traditionally been used for the prevention and treatment of gastric ulcer [1,2]. Some chemical compounds, isolated or present in crude extracts from plants, have shown antiulcer activity [3]. Several studies have revealed the effectiveness of plant polysaccharides as gastroprotective agents, acting by different mechanisms, which depend on their chemical structure [4-9]. In aqueous extracts like teas, the presence of polysaccharides can be detectable, and thus they can contribute for the biological activities observed for plants.

The plant *Croton cajucara* Benth. is a tree native from the Amazon Forest, where it is popularly known as *sacaca*. Currently, ethnopharmacological information indicates that its leaves and barks are used in folk medicine to treat different health disorders, including gastric ulcers, inflammation, hepatic disorders, diabetes, fever and malaria [10-13]. Its leaves and barks are used to prepare teas by infusion or decoction [14].

Studies with *C. cajucara* barks show the chemical characterization of some terpenes. The main terpene is a clerodane-type-diterpene called *trans*-dehydrocrotonin (*t*-DCTN). Several studies suggest that the anti-inflammatory, antinociceptive, hypoglycemic, antiulcer, antispasmodic, antitumor and antiestrogen activities of the plant are related to the *t*-DCTN [14,15]. In *C. cajucara* leaves, *t*-DCTN was not yet detected [14,16,17]. A clerodane-type diterpene known as cajucarinolide was also extracted from barks of the plant and showed anti-inflammatory activity [18]. Phytochemical studies with apolar extracts of *C. cajucara* leaves revealed the

presence of cajucarinolide, steroids - β-sitosterol, stigmasterol, sitosterol-3-*O*-β-glucoside, and flavonoids containing kaempferol as aglycone moiety [14]. The leaves also contain essential oils – linalool and 7-hydroxycalamenene, which showed antimicrobial and antileishmanial activities [19,20].

Considering the popular use of *C. cajucara* leaves teas to treat gastric ulcers and that polysaccharides can act as gastroprotective agents, the aim of this investigation was to verify if polysaccharides could be related with this biological property of the plant. They were obtained from hot aqueous extraction and submitted to a bioguided fractionation using the ethanol-induced gastric ulcer model in rats.

## 2 Materials and methods

### 2.1 *Plant material*

Leaves of *Croton cajucara* Benth. were collected in the Amazon Region (Cruzeiro do Sul, State of Acre, Brazil), in February 2014. The plant was identified by Inês Cordeiro PhD (Botanic Institute of São Paulo, São Paulo, Brazil), and it was compared with the existing voucher SP 319378.

### 2.2 *Extraction and fractionation of the polysaccharides*

Dried and milled leaves (226 g) were extracted with water (2.3 L) under conditions of reflux for 3 h (x 3). The aqueous extracts were obtained by filtration, combined, evaporated to a small volume (300 mL), and added to cold EtOH (x 3 vol.). The resulting precipitate was recovered by centrifugation (8,000 rpm for 15 min), dissolved in water, dialyzed at a 6-8 kDa cut-off membrane and freeze-dried, to give the crude polysaccharide fraction (9.5 g). This fraction was dissolved in water (950 mL) at room temperature, and was submitted to freeze-thawing until no more precipitate appeared. The soluble portion was submitted to ultrafiltration at a 100 kDa cut-off membrane, yielding a retained and an eluted fraction. The latter was dialyzed at a 50 kDa cut-off membrane and then the eluted portion was dialyzed at a 25 kDa cut-off membrane (Fig. 1).

### 2.3 *Uronic acids analysis*

The uronic acid found in CCP and 25R was identified by thin layer chromatography (TLC). Each fraction (1 mg) was hydrolyzed with 1 M TFA (1 mL) at 100 °C for 16 h, the acidic solution was then evaporated, and the residue dissolved in water (1 mL). The resulting monosaccharide mixture was examined by silica-gel 60 TLC (Merck), the plates being developed with *n*-PrOH:$H_2O$ (7:3, v/v) and stained with orcinol-$H_2SO_4$ at 100 °C [21].

The uronic acid found in CCP and 25R was quantified using the colorimetric *m*-hydroxybiphenyl method [22].

### 2.4 *Carboxyl-reduction and methylation analysis*

25R (10 mg) was per-*O*-methylated according to the method of Ciucanu and Kerek, using powdered NaOH in DMSO-MeI [23]. The product was hydrolyzed with 45% aqueous $HCO_2H$ (1 mL) at 100 °C for 20 h. The acidic solution was evaporated, the mixture of partially *O*-methylated monosaccharides was dissolved in water (1 mL) and treated with $NaBD_4$ (2 mg). After 18 h, concentrated HOAc (0.5 mL) was added, the solution evaporated to dryness and the resulting boric acid removed as trimethyl borate by co-evaporation with MeOH. Then, acetylation was carried out with $Ac_2O$-pyridine (1:1 v/v, 0.6 mL) at room temperature for 18 h, and the resulting *O*-methylated alditol acetates were extracted with $CHCl_3$. These were analyzed by GC-MS (Varian Saturn 2000R–3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225 column (30 m x 0.25 mm i.d.) programmed from 50 to 210 °C at 40 °C/min, with He as carrier gas. They were identified by their typical retention times and electron impact spectra, compared to partially *O*-methylated alditol acetates prepared from standard monosaccharides (Sigma-Aldrich) according to Sassaki *et al*. (2005) [24]. The results were given as mol%, calculated according to Pettolino *et al.* (2012) [25].

An aliquot of 25R (20 mg) was carboxyl-reduced by the carbodiimide method, using $NaBH_4$ as the reducing agent [26]. After dialysis at a 6-8 kDa cut-off membrane, the material was freeze-dried and submitted to another cycle of carboxyl-reduction.

The carboxyl-reduced polysaccharide (25R-CR) was then submitted to the methylation protocol and derivatization to partially *O*-methylated alditol acetates.

## 2.5 *HPSEC analysis*

Homogeneity and average molar mass ($M_w$) of 25R were determined by high-performance size exclusion chromatography (HPSEC) according to Reed (1995) [27]. The analysis were performed at 25 °C on a Waters chromatograph equipped with four Ultrahydrogel columns connected in series (2000, 500, 250, 120; with size exclusion of 7 x $10^6$ Da, 4 x $10^5$ Da, 8 x $10^4$ Da and 5 x $10^3$ Da; $V_t$ = 41 mL and $V_0$ = 24 mL), coupled to a Waters 2410 differential refractometer (RI) detector and a DSP-F Wyatt Technology multiangle laser light scattering (MALLS) detector. The eluent was 0.1 M aqueous $NaNO_2$ containing 0.5 g/L $NaN_3$ at 0.6 mL/min. The sample at a concentration of 1 mg/mL, prepared with the eluent solution, was filtered through a membrane (0.22 μm) and then injected (100 μL loop). The specific refractive index increment (*dn/dc*) was determined using the same equipment with the columns uncoupled. The sample at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL, previously filtered through a membrane (0.22 μm), was injected (500 μL loop) and analyzed at 25 °C using the RI detector only. The eluent was the same described above. The results were obtained using the Wyatt Technology ASTRA software, version 4.70.07.

## 2.6 *NMR analysis*

The HSQC-DEPT spectrum was obtained in a 400 MHz spectrometer (Bruker, Avance III), with a 5 mm inverse probe. The sample (25R) was dissolved in $D_2O$ at 20 mg/mL and analyzed at 30 °C. Chemical shifts (δ) were expressed in ppm relative to the internal standard acetone (30.2/2.22, $^{13}C/^{1}H$). NMR signals were assigned on the basis of literature data.

### 2.7 *Animals*

Wistar female rats (~200 g) were housed 5 per cage with wood shaving bedding with free access to pelleted food (Nuvilab CR-1, Quimtia S/A, Brazil) and tap water, maintained in a controlled-temperature and luminosity environment (22 ± 2 °C, 12 h light/dark cycle). The experiment protocols were previously approved by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO – UFPR; approval number 837) and were performed according to the Guide for the Care and Use of Laboratory Animals (8th edition, National Research Council, 2011).

### 2.8 *Pharmacological evaluation*

#### 2.8.1 *Ethanol-induced gastric ulcer model*

The ethanol-induced gastric ulcer model [28] was used to investigate the gastroprotective activity of the polysaccharides fractions. Rats (n = 5-6) fasted for 24 h with free access to water were orally pretreated with crude polysaccharide (CCP, 1, 3 and 10 mg/kg), CCS (1.3 mg/kg), CC100R (0.2 mg/kg), CC100E (0.7 mg/kg), CC50R (0.2 mg/kg), 25E (0.2 mg/kg), 25R (0.2 mg/kg, v.o. or 0.02 mg/kg i.p.), Omeprazole (0.40 mg/kg), Sucralfate (S, 100 mg/kg) or water (Control (C), 1 mL/kg), 1 h for gavage or 30 min for i.p. treatments before oral administration of ethanol P.A. (1 mL/animal). After 1 h of ethanol administration, rats were sacrificed by thiopental overdose (100 mg/kg, i.p.) followed by cervical dislocation. The stomachs were removed, opened along the greater curvature, cleaned with cold saline, stretched and photographed for the evaluation of the area of hemorrhagic lesions ($mm^2$), measured using the program Image Tool 3.0®.

#### 2.8.2 *Determination of gastric mucus and glutathione content*

Stomachs from the experiments evaluating the gastroprotection promoted by 25R (0.2 mg/kg, v.o. or 0.02 mg/kg, i.p.) were divided into two parts to investigate the underlying mechanism involved in its effects. For mucus analysis, one half of the glandular segment was weighed and incubated in a 0.1% Alcian Blue solution for 2 h

at room temperature. Then, the stomachs were washed twice with 0.25 M sucrose for 15 and 45 min, and the gastric wall mucus complexed with Alcian Blue was extracted with 0.5 M magnesium chloride solution for 2 h. The dye extract was mixed with an equal volume of diethyl ether, centrifuged at 1300 × g for 10 min and the absorbance of the supernatant was spectrophotometrically measured at 598 nm. Mucus amounts were calculated using a standard curve of Alcian Blue (6.25–100 µg) and the results were expressed in µg of Alcian Blue/g of tissue [29].

Measurement of the glutathione (GSH) levels in the other half of stomachs was performed according to the method described by Sedlak & Lindsay [30]. The glandular segments were weighed and homogenized with 0.2 M potassium phosphate buffer, pH 6.5. Trichloroacetic acid (12.5%) was mixed with the homogenates, vigorously vortexed and centrifuged for 15 min at 900 × g. A 0.4 M Tris–HCl buffer (pH 8.9) and 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were mixed with the supernatant aliquots. Absorbance was spectrophotometrically measured at 415 nm, the values were interpolated into a standard curve of GSH (0.375–3 µg) and the results were expressed as µg of GSH/g of tissue.

### 2.9 *Statistical analysis*

Data were expressed as mean ± standard error of the mean (SEM) with 5–9 rats per group. The statistical significance of differences between the groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The ED50 values (effective dose capable of inhibiting the gastric lesions formation by 50% relative to the control value) were determined by nonlinear regression analysis and reported as geometric mean. Calculations were performed using GraphPad Prism version 5 (GraphPad Prism Software, San Diego, USA). In all cases, a $P < 0.05$ was considered to be significant

## 3 Results and discussion

### 3.1 *Bioguided isolation of the gastroprotective polysaccharide fraction*

*C. cajucara* leaves teas are popularly used for treatment of gastric ulcer. Moreover, polysaccharides, which are present in aqueous extracts like teas, have showed gastroprotective action and could contribute for this biological activity of *C. cajucara*.

The crude polysaccharide fraction (CCP; 4.2 g%), obtained from the hot aqueous extract of *C. cajucara* leaves, was able to promote gastroprotection on an ethanol induced gastric ulcer model. Gastric lesions produced by ethanol (1 mL/animal, v.o.) were identified as diffused and marked hemorrhagic areas with 266.9 ± 50.06 $mm^2$ in the stomach of the ulcerated vehicle control group (C). The positive control, omeprazole (40 mg/kg, v.o.), promoted 74 ± 8% of gastric protection, when compared to the vehicle control group. The oral treatment with CCP at 3 and 10 mg/kg significantly reduced the gastric lesion in 64 ± 9% and 79 ± 4%, with an ED50 of 4.06 mg/kg (Fig. 2). A bioguided fractionation was performed to isolate the active polysaccharide fraction. The fractionation process is showed on Fig. 1. The theoretical ED50 of each resulting polysaccharide fraction was calculated taking into account its yielding and the ED50 value of CCP.

CCP yielded a soluble fraction after the freezing-thawing process (CCS; 1.3 g%), which reduced the ethanol-induced gastric lesions in 74 ± 6% at a dose of 1.25 mg/kg (v.o.), when compared to the control group (C: 275.5 ± 28.8 $mm^2$) (Fig. 3). CCS was submitted to ultrafiltration at a 100 kDa cut-off membrane, yielding the polysaccharide fractions CC100R (0.2 g%) and CC100E (0.7 g%). The latter reduced the ethanol-induced gastric lesions in 77 ± 8% at a dose of 0.7 mg/kg (v.o.), whereas CC100R (0.2 mg/kg) did not promote gastric protection (Fig. 3). CC100E was then sequentially fractionated by dialyses at 50 and 25 kDa cut-off membranes, yielding the polysaccharide fractions CC50R (0.2 g%), 25R (0.2 g%) and 25E (0.2 g%).

Oral treatment with 25R (0.2 mg/kg) reduced the ethanol-induced gastric lesions in 80 ± 2%. However, fractions CC50R (0.2 mg/kg) and 25E (0.2 mg/kg) did not promote gastric protection (Fig. 3). Based on the relative abundances and theoretical ED50, 25R was 20 times more potent than the precursor extract (CCP).

It is well known that gastric ulcers can be treated with classical drugs, such as $H^+K^+$ATPase inhibitor (omeprazole), $H_2$R antagonist (ranitidine) or barrier agents (sucralfate) [31]. However, there are several natural products that are also effective and safe in the antiulcer therapy [6, 32, 33]. The use of antiulcer polysaccharides as therapeutic strategies could reduce the incidence of side effects observed with

commercial drugs, leading people to choose natural medicines. The antiulcer effect obtained with 25R resembles those observed for both positive controls, since omeprazole (40 mg/kg, v.o.) and sucralfate (100 mg/kg, v.o.) significantly reduced the ethanol-induced gastric lesion in 81 ± 4% and 98 ± 1%, respectively (Fig. 4A).

Interestingly, when administered by intraperitoneal route to avoid the first pass effect, 25R (0.02 mg/kg) significantly decreased the gastric mucosal damage induced by the necrotizing agent in 70 ± 4%, when compared to the vehicle control group (Fig. 4A).

It is well known that, in the stomach, the penetration of the necrotizing agent ethanol is responsible for the depletion of the protective factors of the mucosa, through decreasing mucus and bicarbonate and non-proteic sulphydrilic groups such as reduced glutathione (GSH), promoting membrane damage, erosion and then ulcer formation [34]. Thus, we evaluated the mucus and GSH levels in the gastroprotective effect of 25R.

The hemorrhagic lesion caused by oral ethanol administration was allied to the destruction of the mucus barrier (464.9 ± 58.65 µg of Alcian Blue/g of tissue). Interestingly, animals pretreated with omeprazole showed preserved gastric mucus levels (1744 ± 58.65 µg of Alcian Blue/g of tissue) while sucralfate did not preserve mucus levels (598.5 ± 75.01 µg of Alcian Blue/g of tissue), when compared to the ethanol control group. Even if sucralfate may locally exert the cytoprotective action against ethanol induced ulcer, mainly by coating the gastric mucosa, this mechanism is not enough to maintain mucus levels. However, both oral and intraperitoneal treatment of animals with 25R (0.2 mg/kg and 0.02 mg/kg, respectively) maintained the mucus content in 1535 ± 130.3 and 2093 ± 416.6 µg of Alcian Blue/g, of tissue, respectively, when compared to the ethanol control group (Fig. 4B). The mucus constitutes the first line of mucosal protection against acid secretion due to formation of a viscous, elastic, adherent and transparent gel [35,36]. Another important cytoprotective mechanism against gastric lesion formation is GSH, which acts as an antioxidant, providing cellular protection against oxidative damage [37]. Besides decreasing gastric mucus, the administration of ethanol also decreased the GSH levels to 407.3 ± 13.4 µg/g of tissue, whereas omeprazole and sucralfate appears to replenish the GSH levels to 897.2 ± 69.4 and 816.7± 30.8 µg/g of tissue, respectively. Moreover, ethanol-induced reduction of GSH levels was markedly prevented by oral (0.2 mg/kg) or intraperitoneal (0.02 mg/kg) pretreatment with 25R (749 ± 53.6 and 869.2 ± 91.2

µg/g of tissue, respectively) (Fig. 4C). In summary, our data reveals that 25R promotes a gastroprotective effect independently of forming a cytoprotective barrier as sucralfate, since by both administration routes (oral and intraperitoneal) it decreased ethanol-induced gastric ulcer and preserved mucus and GSH levels.

### 3.2 *Structural analysis of 25R*

The polysaccharide fraction that had the most potent gastroprotective effect was 25R. It derived from fractionation of CCP, which contained glucose (40.3%), galactose (15.3%), arabinose (11.5%), rhamnose (7.0%), manose (4.5%), xylose (1.9%) and fucose (1.7%) as neutral monosaccharides, and 17.8% of uronic acid. The activity observed for CCP and intermediate fractions CCS and CC100E can be explained by the presence of 25R within them.

On HPSEC analysis, using RI detection, 25R showed an elution profile containing a single peak (Fig. 5), with $M_w$ of 42.840 g/mol *(dn/dc* = 0.225). It contained glucose (31.1%), galactose (24.4%), rhamnose (20.4%), arabinose (13.7%), and manose (4.5%) as neutral monosaccharides, as well as 5.9% of uronic acids. TLC analysis of the monosaccharide mixture obtained by acid hydrolysis of 25R indicated the presence of galacturonic acid.

The result of the methylation analysis showed the presence of several *O*-methylated alditol acetate derivatives (Table 1), indicating that 25R is a complex polysaccharide fraction. *O*-methylated alditol acetate derivatives of acid monosaccharides are less volatile and resistant to analysis by GC-MS. Thus, to determine the substitution profile of GalA residues, 25R was submitted to carboxyl-reduction prior to methylation analysis, resulting in 25R-CR. This process transformed GalA in Gal. Analysis of 25R and 25R-CR showed the presence of 1.2% and 7.1% of the 2,3,6-$Me_3$-Gal derivative respectively, indicating that GalA residues are 4-linked and corresponding to 5.9% of the total monosaccharides. Moreover, the presence of 3,4-$Me_2$-Rha (3.7%) and 3-Me-Rha (3.3%), which corresponded to 2- and 2,4-linked Rha*p* residues respectively, strongly suggests that this fraction contains a type I rhamnogalacturonan (RGI). The ratio between 2- and 2,4-linked Rha*p* residues indicates that half of Rha*p* are branched. RGI are formed by sequences of alternating

4-linked *α*-D-Gal*p*A and 2-linked α-L-Rha*p* units, frequently branched at *O*-4 of Rha*p* by side chains of arabinans, galactans, or arabinogalactans [25,38-42].

Methylation analysis (Table 1) indicated that the RGI of the 25R fraction has side chains of arabinan, type I arabinogalactan and type II arabinogalactan (AGII). The presence of alditol acetates of 2,4,6-$Me_3$-Gal (5.6%), 2,4-$Me_2$-Gal (3.6%) and 2,3,4-$Me_3$-Gal (2.7%) are from 3-, 3,6- and 6-linked Gal*p* units respectively, which are from AGII [25,41,42]. On the other hand, alditol acetates of 2,3,6-$Me_3$-Gal (1.2%) and 2,6-$Me_2$-Gal (3.0%) are from 4- and 3,4-linked Gal*p* units respectively, of AGI. In relation to the alditol acetates of 2,3-$Me_2$-Ara (4.1%), 3,5-$Me_2$-Ara (2.9%), 2,5-$Me_2$-Ara (2.2%) and 3-Me-Ara (2.2%), they are from 5-, 2-, 3- and 2,5-linked Ara*f* units respectively, which can arise from arabinan, AGI and AGII [25,42].

As described above, Rha units are commonly present in RGI. In this case, Rha*p* units are 2- and 2,4-linked, and the relation Rha:GalA is not bigger than one. However, the monosaccharide composition analysis of the 25R fraction showed 20.4% of Rha and only 5.9% of GalA, and its methylation analysis revealed the uncommon alditol acetates of 2,4-$Me_2$-Rha (5.2%) and 4-Me-Rha (2.3%), which are from 3- and 2,3-linked Rha*p* units. Thus, these results indicate the presence of a rhamnan, which could be interspersed in the RGI. A RGI with more Rha than GalA, and with 2,3-linked *α*-L-Rha*p* residues, was detected in the gum from flaxseed hulls [43].

According to the methylation analysis, starch can also be present in 25R, since 4- and 4,6-linked Glc*p* units were observed (8.7 and 2.3% respectively). Moreover, 2- and 4-linked Man*p* units (3.4 and 1.1% respectively) suggest the presence of mannan.

The $^{1}H/^{13}C$ HSQC-DEPT of 25R (Fig. 6) showed correlations at δ 99.8/5.13 and and 16.6/1.27 of C1/H1 and C6/H6 of α-L-Rhap units respectively, and at δ 78.6/4.48 of C4/H4 of 4-linked α-D-Gal*p*A units [38, 43-47]. Correlations at δ 103.1/4.52 and 102.9/4.51 characteristic of C1/H1 and at δ 80.0/3.74, 75.6/4.15 and 66.2/3.92, of C3/H3, C4/H4 and C/6H6 respectively of 3-, 4- and 6-linked *β*-D-Gal*p* units were also observed [44, 48-52]. The correlations of C1/H1 of α-L-Ara*f* units appeared at δ 109.0/5.26, 107.7/5.38, 107.5/5.08 and 107.5/5.21 [49]. The $^{1}H/^{13}C$ correlations described above are in accordance with the methylation analysis, which indicated the presence of rhamnan and RGI with side chains of arabinan, AGI and AGII in 25R. The correlation observed at δ 99.2/5.02 was attributed to C1/H1 of α-D-Glc*p* units from starch, and that at 100.8/4.81 to C1/H1 of *β*-D-Man*p* from mannan [53, 54].

## 4 Conclusion

Though secondary metabolites are frequently associated with properties of medicinal plants, polysaccharides can contribute for some biological activities. In this study we showed that polysaccharides from *C. cajucara* leaves are related to the gastroprotective activity of the plant. A complex polysaccharide fraction containing type I rhamnogalacturonan, arabinan, type I arabinogalactan, type II arabinogalactan, rhamnan, starch and mannan decreased ethanol-induced gastric ulcer, preserving mucus and GSH levels.

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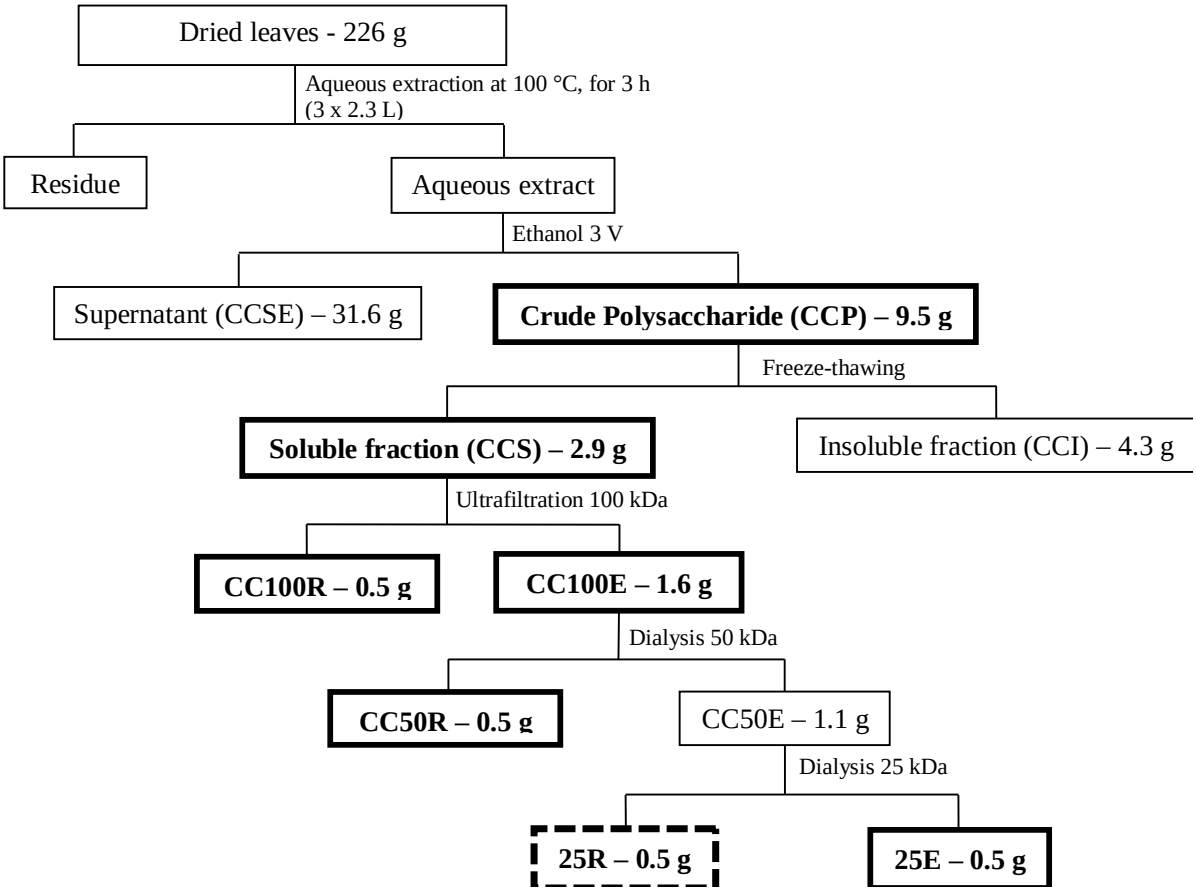


**Figure 1** - Scheme of extraction and isolation of 25R fraction of leaves from *Croton cajucara* B. The fractions in bold were evaluated for gastroprotective activity

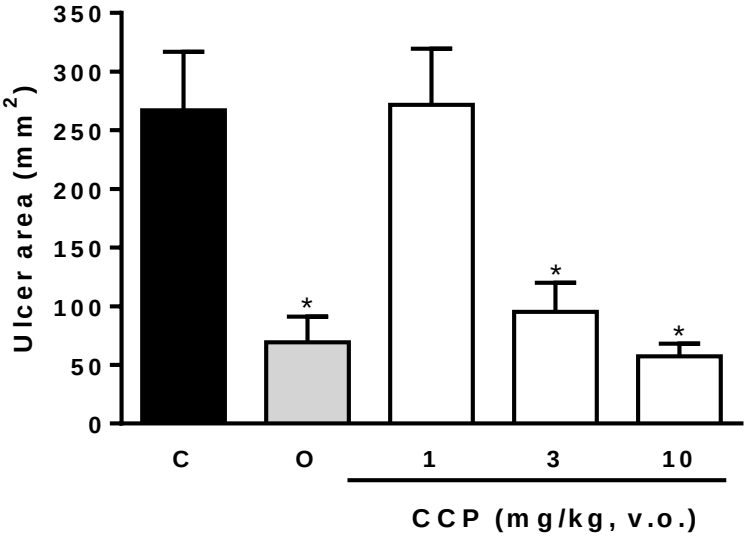


**Figure 2** - Effect of crude polysaccharide (CCP) on acute gastric lesion induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 mL/kg), Omeprazole (O: 40 mg/kg), CCP (1, 3 and 10 mg/kg) 1 h before administration of ethanol P.A. (1 mL/animal). The results are expressed as mean ± S.E.M. (n = 5-6). ANOVA followed by Bonferroni's test. **P < 0.05* when compared to the control group (C).

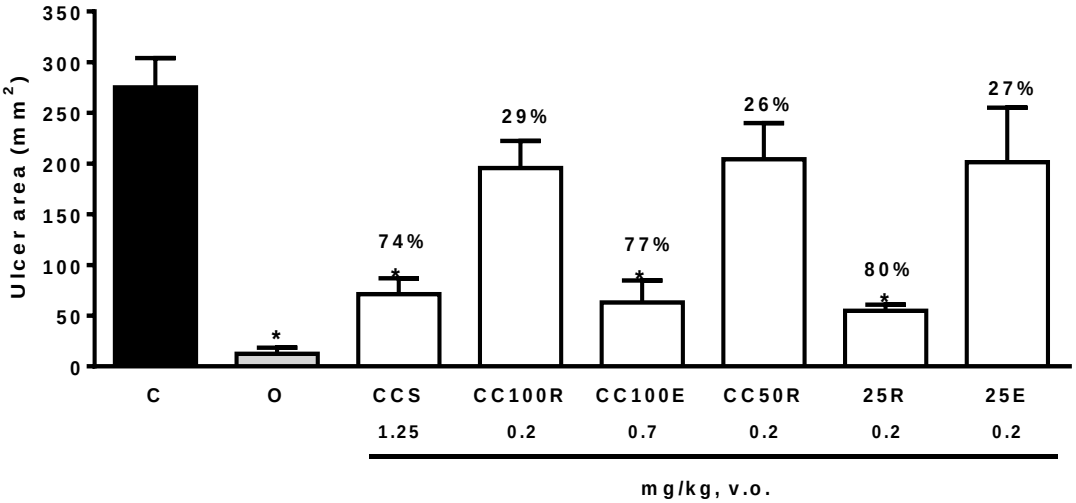


**Figure 3** - Effect of CCS, CC100R, CC100E, CC50R, 25R and 25E polysaccharide fractions on acute gastric lesions induced by ethanol P.A. in rats. The animals were orally treated with vehicle (C: water, 1 mL/kg), omeprazole (O: 40 mg/kg), CCS (1.25 mg/kg), CC100R (0.2 mg/kg), CCS100E (0.7 mg/kg), CCS50R (0.2 mg/kg), 25R (0.2 mg/kg) and 25E (0.2 mg/kg) 1 h before oral administration of ethanol P.A. (1 mL/animal). The results are expressed as mean ± S.E.M. (n = 5-6). ANOVA followed by Bonferroni's test. **P < 0.05* when compared to the control group (C).

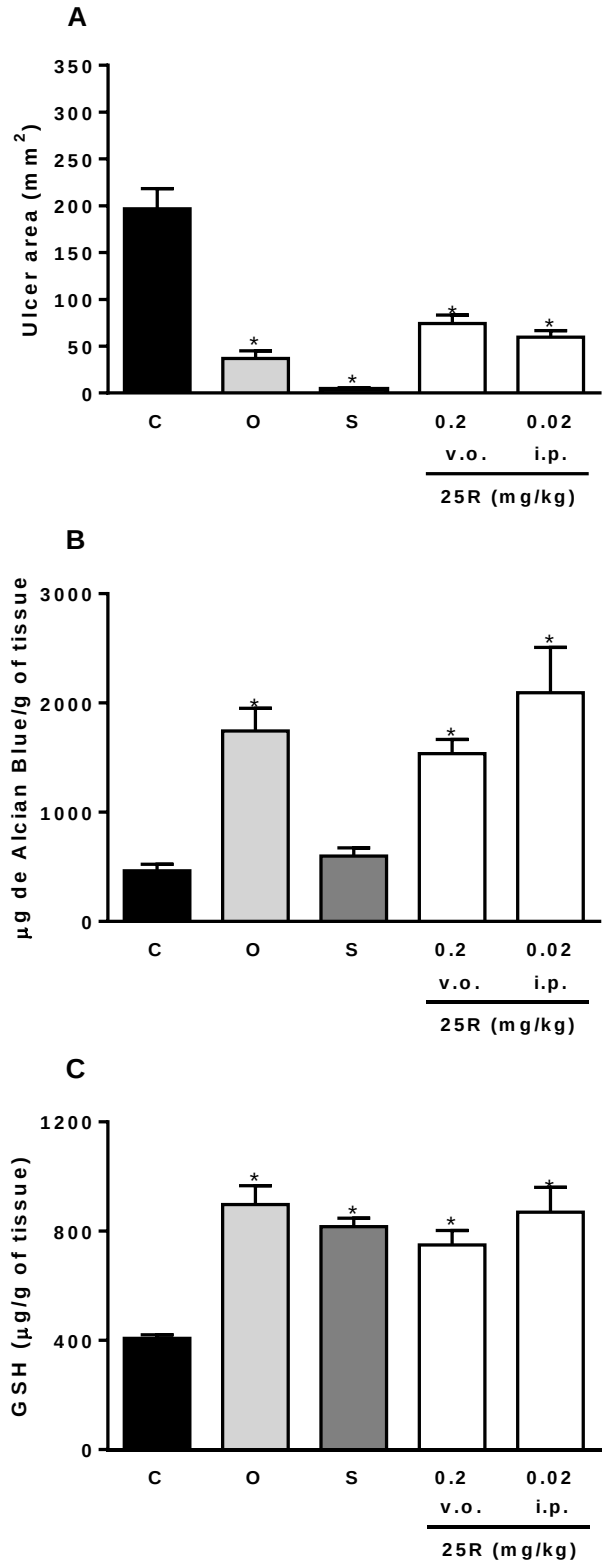


**Figure 4 -** Effect of 25R on acute gastric lesions induced by ethanol P.A. in rats (Panel A) on mucus (Panel B) and GSH (Panel C) levels. The animals were orally treated with vehicle (C: water, 1 mL/kg), omeprazole (O: 40 mg/kg), sucralfate (100 mg/kg) or 25R (0.2, mg/kg, v.o. and 0.02 mg/kg, i.p.). The results are expressed as mean ± S.E.M. (n = 6-9). ANOVA followed by Bonferroni's test. *$P < 0.05$ when compared to ulcerated group (C).

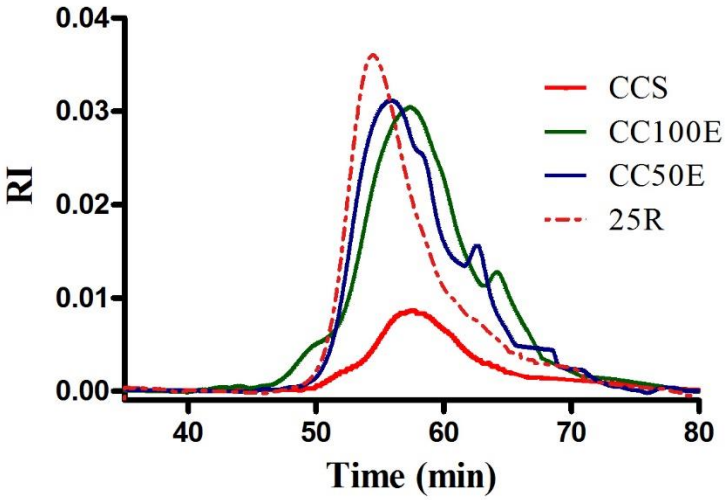


**Figure 5** - Elution profiles of CCS, CC100E, CC50E, 25R and 25E on HPSEC.

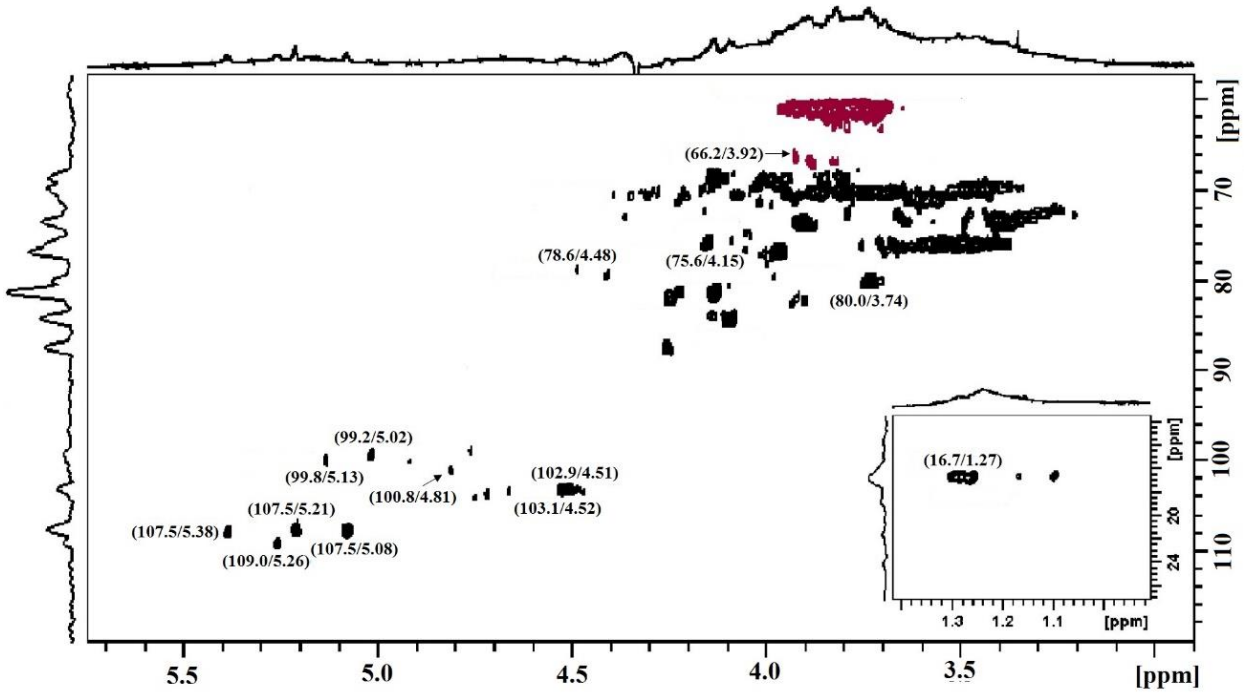


**Figure 6** - . $^{1}H/^{13}C$ HSQC NMR spectra of 25R. Solvent $D_2O$ at 30 °C; numerical values are in δ ppm.

**Table 1.** Profile of partially *O*-methylated alditol acetates of carboxy-reduced 25R (25R-CR) obtained on methylation analysis.

| *O*-Me-Alditol acetate | Structure | mol% | |
|---|---|---|---|
| | | 25R-CR | Total |
| 2,3,5-$Me_3$-Ara | Ara*f*-(1→ | 2.4 | |
| 2,3-$Me_2$-Ara | →5)-Ara*f*-(1→ | 4.1 | |
| 3,5-$Me_2$-Ara | →2)-Ara*f*-(1→ | 2.9 | 13.7 |
| 2,5-$Me_2$-Ara | →3)-Ara*f*-(1→ | 2.2 | |
| 3-Me-Ara | →2,5)-Ara*f*-(1→ | 2.2 | |
| 2,3,4-$Me_3$-Rha | Rha*p*-(1→ | 5.9 | |
| 2,4-$Me_2$-Rha | →3)-Rha*p*-(1→ | 5.2 | |
| 3,4-$Me_2$-Rha | →2)-Rha*p*-(1→ | 3.7 | 20.4 |
| 4-Me-Rha | →2,3)-Rha*p*-(1→ | 2.3 | |
| 3-Me-Rha | →2,4)-Rha*p*-(1→ | 3.3 | |
| 2,3,4,6-$Me_4$-Gal | Gal*p*-(1→ | 6.3 | |
| 2,4,6-$Me_3$-Gal | →3)-Gal*p*-(1→ | 5.6 | |
| 2,3,6-$Me_3$Gal[a] | →4)-Gal*p*-(1→ | 1.2 | |
| 2,3,4-$Me_3$-Gal | →6)-Gal*p*-(1→ | 2.7 | 24.4 |
| 2,6- $Me_2$-Gal | →3,4)-Gal*p*-(1→ | 3.0 | |
| 2,4-$Me_2$-Gal | →3,6)-Gal*p*-(1→ | 3.6 | |
| 2-$Me_2$-Gal | →3,4,6)-Gal*p*-(1→ | 2.0 | |
| 2,3,6-$Me_3$Gal[b] | →4)-Gal*p*A-(1→ | 5.9 | 5.9 |
| 3,4,6-$Me_3$-Man | →2)-Man*p*-(1→ | 3.4 | 4.5 |
| 2,3,6-$Me_3$-Man | →4)-Man*p*-(1→ | 1.1 | |
| 2,3,4,6-$Me_4$-Glc | Glc*p*-(1→ | 10.2 | |
| 2,4,6-$Me_3$-Glc | →3)-Glc*p*-(1→ | 5.6 | |
| 2,3,6-$Me_3$-Glc | →4)-Glc*p*-(1→ | 8.7 | 31.1 |
| 2,3-$Me_2$-Glc | →4,6)-Glc*p*-(1→ | 2.3 | |
| 3,4-$Me_2$-Glc | →2,6)-Glc*p*-(1→ | 4.3 | |

[a] Obtained from the sample not carboxyl-reduced.

[b] Obtained from the carboxyl-reduction of GalA.

# ARTIGO 3

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**Phytochemical analysis and anti-inflammatory evaluation of compounds from an aqueous extract of *Croton cajucara* Benth.**

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ABSTRACT

*Croton cajucara* Benth. is a medicinal plant popularly used in the Brazilian Amazonia, where it is known as *sacaca*, being consumed as tea, decoction or infusion, from leaves and stem bark. From decoction of leaves, a comprehensive phytochemical analysis was developed by liquid chromatography-mass spectrometry. Many unreported compounds were identified, such as *O*-glycosides containing kaempferol and quercetin as aglycone moiety, flavonoid-*C*-glycosylated, tannins and cinnamic acid derivatives. These compounds were fractioned by polarity and assayed for their anti-

inflammatory activity, using a model of mice edema, induced by an intraplantar injection of carrageenan. All fractions exhibited anti-inflammatory properties.

**Keywords**: *Croton cajucara*; flavonoids; anti-inflammatory activity; LC-MS.

## 1 Introduction

The genus Croton, a member of the Euphorbiaceae family, is found mainly in tropical regions worldwide [1]. Species of this genus are used in folk medicine for different treatments, such as infections, inflammation, cancer, fever, diabetes, hypertension [2]. *Croton cajucara* Benth., poularly known as “sacaca” or “marassacaca”, is a tree widely distributed in Brazilian Amazonia. The local population uses its leaves and stem bark, mainly as infusion or decoction, to treat several health complications including inflammation, hepatic disorders, stomachache, gastritis, gastrointestinal symptoms, bellyache, diabetes, liver disorders, fever, jaundice and, also, malaria [3].

Some previous investigations relate to the medicinal properties of *C. cajucara* to the terpenes, mainly to the *trans*-dehydrocrotonin, a furan diterpene present in its stem bark [4,5]. Few compounds from leaves were identified in previous works, including the essential oils linalool and 7-hydroxycalamenene, the diterpene cajucarinolide, along with the steroids β-sitosterol, stigmasterol, sitosterol-3-*O*-β-glucoside and the flavonoids kaempferol 3,4’,7-trimethyl ether and 3,7-dimethyl-ether [6]. Some therapeutic properties from leaves, as leishmanicidal, anti-inflammatory and antimicrobial, are attributed to presence of essential oils [7-9]. Moreover, gastroprotective property was observed from a polysaccharide-rich fraction from its leaves [10].

Phenolic compounds are important metabolites from plants being widely ingested in the human feeding since they are found in leaves, fruits, and roots. Their role in the human health deserves attention because they can display chemoprotective effects. Flavonoids are an important class of phenolics, appearing as free aglycones, but also as *O*- and *C*-glycosides or attached to other chemical groups as acetyl or sulfate [11-13]. They also appear as proanthocyanidins or condensed tannins, exhibiting a different degree of polymerization and linkages [14,15]. Flavonoids are known for exhibit different biological activities including anti-inflammatory, antioxidant

and antimicrobial [16-18]. The hydroxycinnamic acids and derivatives also occur in many species of plants, with important antioxidant, anti-inflammatory and anticancer activities [14,19-22].

Considering the importance of *C. cajucara* in the folk medicine, the goal of the present investigation was the development of a comprehensive phytochemical investigation of leaves from *C. cajucara*. Furthermore, considering the popular uses of its leaves as decoction, the fractions obtained from this aqueous extraction were assayed for the acute anti-inflammatory activity, using carrageenan-induced paw edema in mice.

## 2 Materials and methods

### 2.1 *Plant material*

The leaves from *C. cajucara* were collected in the Amazon Region (Cruzeiro do Sul, State of Acre, Brazil), in February 2014. The botanical identification was performed by Inês Cordeiro PhD (Botanic Institute of São Paulo, São Paulo, Brazil) and it was compared to the existing voucher number SP319378, deposited at the Herbarium of the Botanic Institute of São Paulo. The leaves were dried at 60 °C, for two days and, then, submitted to the extraction process.

### 2.2 *Extraction*

The aqueous extraction was performed by decoction from dried leaves of *C. cajucara* (100 g) using water (1 L) heated to boiling for 15 to 20 minutes. The decoction was filtered and evaporated to a small volume, then added to EtOH (x 3 vol.). The ethanol soluble fraction (ESF), containing the lower molecular compounds (11.4 g), was evaporated under reduced pressure, lyophilized and stored at -20 $^{o}$C until the subsequent fractionating.

### 2.3 *Liquid/liquid fractionation*

ESF (1.0 g) was fractionated by polarity by liquid/liquid partition in $H_2O$ (100 mL), followed by the addition of ethyl acetate (100 mL). The organic layer was removed and process was repeated thrice. The remaining aqueous layer was similarly

fractionated with *n*-butanol, resulting in three fractions: ethyl acetate (EA), *n*-butanol (BU) and aqueous (AQ). The fractions were dried and stored at -20 ºC.

2.4 *Thin layer chromatography* (TLC)

The fractions ESF, AQ, BU and EA were dissolved in MeOH:$H_2O$ (1:1, v/v) at 2 mg/mL and analyzed by TLC, using silica-gel 60 G plate (Merck). The chromatography was developed in EtOAc:$H_2O$:HOAc:$HCO_2H$ (9:2.3:1:1, v/v) and stained with orcinol:$H_2SO_4$ reagent, at 100 °C, for glycosides [23].

2.5 *Ultra-high performance liquid chromatography (UHPLC)*

Liquid chromatography analysis was performed using an Acquity-UPLC™ system (Waters, Miliford, MA), incorporating a binary solvent pump, sample manager and column oven. The column used was a C18 HSS T3 with 100 mm x 2.1 mm i.d. and 1.7 µm particle size (Waters). The fractions obtained from ESF were prepared in MeOH-$H_2O$ (1:1 v/v) at 2 mg/mL. The chromatography was developed with the column temperature at 60 ºC, using ultra-pure water and acetonitrile, both containing 0.1 % formic acid (v/v), with a linear gradient of acetonitrile from 0 to 20 % in 7 minutes and then to 70 % at 13 minutes, returning to the initial condition in 13.50 minutes at flow rate of 400 µL/minute. The column was re-equilibrated by 2 min. The volume of injection of each fraction was 3 µL and the detection was performed with a photodiode array detector (PDA, 200 – 400 nm) and high resolution mass spectrometry (*m/z* 100-2000).

2.6 *Mass Spectroscopy (MS)*

The mass spectrometry was acquired on an electrospray ionization mass spectrometry (ESI-MS) LTQ-Orbitrap-XL (Thermo-Scientific) operating in the negative/positive ionization at atmospheric pressure ionization (API). The source temperature was 350 ºC and the dessolvation was aid by a nitrogen flow at 35 arbitrary units (a.u.) on sheath gas and 10 a.u. on auxiliary gas. The set up for detection of negative ions was: electrospray at 3.5 kV, capillary at -20 V and tube lens at -100 V. For positive ions: electrospray at 4.5 kV, capillary at 40 V and tube lens at 120 V. The fragmentations were obtained by collision-induced dissociation (CID) with a normalized

energy 25%. The instrument calibration was performed with a calibration solution (100 to 2000 *m/z*) and resolution was set at 15,000 FWHM in LC-MS mode. Acquisition was obtained in total ion current (TIC) mode, with *m/z* 100-2000.

### 2.7 *Monosaccharide and aglycone analysis*

2 mg of each fraction obtained from liquid-liquid partition were hydrolyzed with 1 M TFA (1 mL) at 100 °C for 8 h. The solutions were evaporated, and the residue dissolved in $H_2O$ (1 mL) and fractionated with *n*-butanol (1 mL). The layers were separated and dried under $N_2$ stream, and the organic layer was reserved to LC-MS analysis for aglycone identification, in same conditions described above. The monosaccharides in the aqueous layer, were reduced with $NaBH_4$ (2 mg) and the resulting alditols were acetylated, overnight, with $Ac_2O$-pyridine (1:1 v/v, 0.5 mL) at room temperature [24,25]. The alditol acetates were recovered in $CHCl_3$, and washed with water to remove the reagents. The analysis was developed by GC-MS (Varian Saturn 4000, Ion Trap Detector), using a DB1-MS column (30 m x 0.25 mm i.d.) programmed from 100 to 220 °C at 5 °C/min and 220 to 280 °C at 18 °C/min, with He as carrier gas at 1mL/min. The monosaccharides were identified by their typical retention times and electron ionization spectra (70 eV).

### 2.8 *Methylation analysis*

The fractions BU and EA (5 mg) were per-*O*-methylated according to the method of Ciucanu and Kerek [26], using powdered NaOH in DMSO-MeI. The product was hydrolyzed with 45% aqueous formic acid (1 mL) at 100 °C for 17 h. The resulting partially *O*-methylated monosaccharides were evaporated, dissolved in water (0.5 mL), reduced with $NaBD_4$ and acetylated as described above, to give a mixture of partially *O*-methylated alditol acetates. These were analyzed by GC-MS using the same conditions as described for alditol acetates. They were identified by their typical retention times and electron ionization (70 eV) spectra [27].

## 2.9 *Oxidation of glucuronic and galacturonic acids*

Glucuronic and galacturonic acids (1 mg) were each dissolved in water (50 µL), then added 100 µL of 0.1 N iodine-potassium iodide, followed by additions of 0.1 N NaOH (200 µL), held for 15 minutes at room temperature. The solutions were acidified with 1 M HCl in MeOH (100 µL) and evaporated [28, 29].

These oxidation products (glucaric and galactaric acids) and the AQ fraction (2 mg) were converted to methyl esters by treatment with 0.5 M HCl in MeOH (200 µL) for 1 h at 80 °C, followed by evaporation and acetylation, as described above. The resulting derivatives were analyzed by GC-MS using the same conditions as described above.

## 2.10 *NMR spectroscopy*

NMR spectra were obtained in a Bruker III HD spectrometer equipped with a 5 mm inverse probe (QXI) with Z-gradient, operating at 14.1 Tesla (600 MHz for $^{1}$H). All experiments were performed at 30 °C using methanol-$d_4$ as solvent. $^{1}$H spectrum was obtained using a spectral width (SW) of 10.6541 ppm (6393.862 Hz), time domain size (TD) of 64 k, acquisition time (AQ) of 5.12 s and high power pulse (P1) of 11.0 µsec with relaxation delay of 1 s. Presaturation of residual HDO was carried out with the pulse program *zgpr*, with the same parameters as above, except the relaxation delay that was 4 s.

2D $^{1}$H-$^{13}$C, multiplicity-edited HSQC experiments were obtained using the heteronuclear correlation via double inept transfer with decoupling during acquisition, using sensitivity improvement, trim pulses in inept transfer, multiplicity editing during selection step and shaped pulses for all 180° pulses on the $^{13}$C chanel (hsqcedetgpsisp 2.2 on Bruker spectrometers). $^{1}$H-$^{13}$C HMBC, heteronuclear correlation via zero and double quantum coherence optimized on long range couplings was acquired with the hmbcgplpndqf pulse sequence. Chemical shifts (δ) are given in ppm relative to methanol-$d_4$ at $\delta$ 49.1/3.31. NMR signals were assigned on the basis of data from $^{1}$H-$^{13}$C multiplicity-edited HSQC, HMBC and literature data.

### 2.11 *Animals*

Anti-inflammatory experiments were performed using adult male Swiss mice (20-30 g), maintained in a controlled temperature and luminosity environment (22 ± 2 °C, 12 h light/dark cycle). Mice were housed 10 per cage with wood shaving bedding (no environment enrichment) with free access to pelleted food (Nuvilab CR-1, Quimtia S/A, Brazil) and tap water, acclimated to the laboratory environmental conditions for at least 1 h before testing. The experiment protocols were performed upon approval by the Committee of Animal Experimentation of the Federal University of Paraná (CEUA/BIO – UFPR, approval number 657) and were carried out in accordance to the "Principles of Laboratory Animal Care" (NIH Publication 85-23, revised 1985) and ethical guidelines for investigations of experimental pain in conscious animals [30].

### 2.12 *Carrageenan-induced paw edema*

The acute anti-inflammatory effects of *C. cajucara* on edema formation caused by the intraplantar injection of carrageenan in mice was analyzed according the method previously reported by Erthal *et al*. [31]. Mice were treated intraperitoneally with vehicle (C: saline, 1 ml/kg), Dexamethasone (D: 0.5 mg/kg), *C. cajucara* fraction (ESF - 30 – 300 mg/kg, AQ - 76 mg/kg, EA - 8 mg/kg or BUT - 16 mg/kg) 30 min before the intraplantar (i.pl.) injection of carrageenan (300 µg/paw, 20 µl), which were administered into the right hind paw. The degree of inflammation evidenced by paw edema was measured using a digital micrometer (Great, MT-045B, China) before the induction of edema (B: basal measurement) and 3 h after the injection of carrageenan. The magnitude of the carrageenan-induced paw edema was determined as the difference between the thickness before and after the injection into the paw (mm) [31].

### 2.13 *Statistical analysis*

The results were expressed as the mean ± SEM with 5 animals per group. The statistical significance of differences between groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni’s test using Graph-Pad software

(GraphPad software, San Diego, CA, USA). Differences were considered to be significant when $P < 0.05$.

## 3 Results and discussion

### 3.1 *Phytochemical investigation*

The low molecular weight compounds from leaves of *C. cajucara* were obtained by decoction followed by ethanol precipitation. The ethanol soluble fraction (ESF – 11.4 g%) was examined by ultra-high performance liquid chromatography with photodiode array detector and mass spectrometry (UHPLC-PDA-MS) in the negative and positive ionization modes. The ESF chromatogram showed high complexity, with the main components being identified as phenolics, such as hydroxycinnamic acids, free flavan-3-ols, condensed tannins, flavonol-glycosides, and flavone-glycosides, giving characteristics ultraviolet (UV) and mass spectrometry (MS) profile. ESF was also investigated by thin layer chromatography (TLC), stained by orcinol reagent, evidencing the presence of free monosaccharides and sucrose. These free glycans would hinder the analysis of the glycosides, thus ESF was submitted to liquid/liquid fractionation with ethyl acetate, to give the EA-fraction, (7.8 g%), followed by *n*-butanol, yielding the BU-fraction (16.0 g%), and the remaining and aqueous fraction (AQ, 74.6 g%).

#### 3.1.1 *Analysis of AQ fraction*

The AQ fraction retained the most polar compounds from ESF, as observed by the presence of monosaccharides and sucrose by TLC (data not showed). On LC-MS analysis in the negative ion mode (Fig. 1A) the major peak in this fraction appeared at 3.29 minutes (peak 4), with an UV-spectrum profile consistent with hydroxycinnamic acids, with $\lambda_{max}$ at 325 nm [32-34]. With mass spectrometry the ion at *m/z* 355.0673 $[M-H]^-$ was detected, producing fragments at *m/z* 209.030, 191.020. Whereas the fragment at *m/z* 209.030 was consistent with the entire aldaric acid $[M-H]^-$, produced by the loss of a *p*-coumaroyl residue (-146.037 atomic mass units - a.m.u.), the fragment at *m/z* 191.020 was consistent with dehydration of aldaric acid. The peak 6

($t_R$ 3.71 min) had similar $MS^1$, $MS^2$ and UV profile, consistent with an isomer of compound/peak 4 (Fig. 1B).

The peak 9 appeared with the negative ion at *m/z* 385.0775 $[M-H]^-$ and UV at $\lambda_{max}$ 325 nm. The fragments were observed at *m/z* 355.067 (loss of a methanal from methoxyl group, -30.0105 a.m.u.), and those at *m/z* 209.030, 191.020 from aldaric acid (Fig. 1C), being consistent with the structure of feruloyl-aldaric acid [35]. The presence of these hydroxycinnamic acids was confirmed by GC-MS analysis of derivatized components, compared with authentic standards of ferulic acid and *p*-coumaric acid, similarly derivatized. Glucaric and galactaric acids (obtained from oxidation of glucuronic and galacturonic acids) were also analyzed by GC-MS as acetylated methyl ester derivatives, confirming the presence of galactaric acid in the AQ fraction. Thus, the peaks 4 and 6 were identified as isomers of *p*-coumaroyl-galactaric acids and peak 9 as feruloyl-galactaric acid, being previously described only in other plants [32,35,36,37]. Some compounds found in the AQ-fraction were more concentrated in the BU-fraction, such as the tetraglycosides and will be described below.

### 3.1.2 *Analysis of BU fraction*

Although some compounds were retained in both, ethyl acetate and butanol fractions, as occurred with flavonol di-*O*-glycosides, the butanol fraction concentrated the higher polar flavonol-glycosides. Flavonols with different degree of glycosylation, ranging from di-*O*- to tetra-*O*-glycosides, were found in this fraction (Fig. 2A). These glycosyl moieties were accessed by hydrolysis/reduction/acetylation and GC-MS analysis, revealing only glucose (87 %) and rhamnose (13 %). The flavonols were confirmed as quercetin ($t_R$ 9.73 min.) and kaempferol ($t_R$ 10.37 min) by LC-MS, compared with authentic standards. The interglycosidic linkages were obtained by methylation/GC-MS analysis. Rhamnose (Rha*p*) appeared exclusively as non-reducing ends (10.0 %), but glucose (Glc*p*) was non-reducing end units (55 %), 6-*O*- (13 %), 2-*O*- (3 %), and 2,6-di-*O*-substituted (19 %).

Flavonol tri-*O*-glycosides were observed in this fraction and, although they were eluted after the tetra-*O*-glycosides, they will be firstly described in order to ease further structural insights. Since the negative fragments from flavonol-*O*-glycosides were poorly informative, the discussion will be done from protonated and lithiated ions. The

peak 26 showed the ion at *m/z* and 757.2185 $[M+H]^+$ and fragments at *m/z* 611.161, loss of rhamnosyl residue, *m/z* 465.103, loss of second rhamnosyl residue and *m/z* 303.050 loss of glucosyl residue (Fig. 3A, Table 1). These results indicated the structure of a Rha*p*-(1→6)-[Rha*p*-(1→2)]-Glc*p*-(1→3)-Quercetin, supported by interglycosidic linkage analysis. The peak 29 was detected at *m/z* 741.2238 $[M+H]^+$ and gave rise to fragments at *m/z* 595.166 $[M+H -(Rha)]^+$, 449.108 $[M+H -(Rha)_2]^+$ and 287.055 $[M+H -(Glc), -(Rha)_2]^+$ indicate a structure similar to the peak 26, but having kaempferol as aglycone.

Considering that flavonoids exhibit a much diversified glycosylation pattern, with mono- and/or oligosaccharides attached in single site or in 2 different sites of same molecule. In addition, the fragments from protonated ions usually do not provide information to differentiate multiple glycans moieties from single glycan moiety, the peaks 26 and 29 were obtained as lithium adducts and fragmented. The lithium (as other alkaline cations) complex with better with carbohydrate moieties producing fragments from entire oligosaccharides [13]. Thus, the fragment-ion at *m/z* 461.184 confirms the presence of a trisaccharide attached to flavonols in a single site. These results were very similar to that found in *Maytenus ilicifolia* [13, 38], thus to confirm the similarities, the fractions from *C. cajucara* were compared with an extract of leaves of *M. ilicifolia*, and the chromatography from these triglycosides exhibited same Rt values, indicating the glycosylation site on flavonols at 3-*O* position.

The peak 23 with $MS^1$ at *m/z* 773.2136 $[M+H]^+$, with fragments at *m/z* 627.156, from a rhamnosyl residue loss, *m/z* 611.161 from glucosyl residue loss, with those at *m/z* 465.103 and 303.049 suggesting the structure of Glc*p*-(1→6)-[Rha*p*-(1→2)]-Glc*p*-(1→)-Quer or Glc*p*-(1→2)-[Rha*p*-(1→6)]-Glc*p*-(1→)-Quer. This triglycoside is important since it can be a precursor of the main flavonols that appeared as tetraglycosides.

Flavonoids tetra-*O*-glycosides appeared at *m/z* 919.2721 $[M+H]^+$ (peak 25) with $MS^2$ at *m/z* 773.213 $[M+H -(Rha)]^+$, *m/z* 757.219 $[M+H -(Glc)]^+$, *m/z* 611.162 $[M+H -(Glc), -(Rha)]^+$, *m/z* 465.103 $[M+H -(Glc), -(Rha)_2]^+$, and *m/z* 303.051 from quercetin ion. The peak 28 with $MS^1$ at *m/z* 903.2764 $[M+H]^+$ and fragments at *m/z* 757.219 $[M+H -(Rha)]^+$, *m/z* 741.224 $[M+H -(Glc)]^+$, *m/z* 595.166 $[M+H -(Glc), -(Rha)]^+$, *m/z* 449.108 $[M+H -(Glc), -(Rha)_2]^+$ and *m/z* 287.055 from kaempferol ion. These fragmentation profiles suggest similar structures, with different aglycone moieties. In order to confirm the glycan moieties as tetrasaccharides, these compounds were analyzed as lithium

adducts [*m/z* 925.2956 (peak 25) and 909.2910 (peak 28)] and fragmented, producing the main fragment-ion at *m/z* 623.241 after losing the aglycone, confirming the presence of the tetrasaccharide, formed by 2 units of glucose and 2 units of rhamnose (Fig. 2 B-C).

The structure of tetraglycosides deserved better attention since the fragments at *m/z* 757.219 (from peak 25) and *m/z* 741.224 (from peak 28) indicate the loss of glucosyl residue, supposedly linked to a rhamnose unit. However, the methylation/GC-MS analysis indicated rhamnose exclusively as terminal units. Thus, the fragmentation pathway seems to undergo an internal residue loss mechanism, as described in other glycosides and oligosaccharides [39,40]. In these tetraglycosides, the rearrangement involves the migration of one the terminal rhamnosyl producing a new linkage in a different site, thus the internal glucosyl residue could be removed, producing the fragment with NL of 162.05 a.m.u., typical from the loss of hexosyl residues (Fig. 2B). Another rhamnosyl group can also migrate to aglycone with the loss of a second glucosyl moiety, producing the fragments at *m/z* 595.167 (from *m/z* 919.2721) and 579.174 (from *m/z* 903.2764). The linkage of rhamnosyl unit to aglycone could also be evidenced by the presence of the fragment at *m/z* 449.109 and *m/z* 433.113, from peak 25 (*m/z* 919.2721) and peak 28 (*m/z* 903.2764), respectively (Fig. 2 C-D). Therefore, based on MS/MS data and methylation analysis, the putative structure for peaks 25 and 28 is Rha*p*-(1→2 or 6)-Glc*p*-(1→2 or 6)-[Rha*p*-(1→2 or 6)]-Glc*p*-(1→)-Aglycone (Quercetin or Kaempferol).

Another abundant peak from the BU fraction (peak 22) appeared at *m/z* 593.1513 $[M-H]^-$, being an isomer from kaempferol-3-*O*-rutinoside (peak 34). However, this ion showed the $MS^2$ spectrum quite different from kaempferol-3-*O*-rutinoside, with main fragment-ions at *m/z* 503.118, 383.077, 353.066 obtained in the negative ionization mode. The repetitive neutral losses of 90 a.m.u. and 120 a.m.u. are characteristic from flavone *C*-glycosides [18,41-43].

Since the monosaccharides from *C*-glycoside are not removed with common acid hydrolysis, the BU fraction was submitted to a methanolysis (MeOH-HCl). The resistant *C*-glycosides were recovered in the *n*-butanol fraction, whereas free monosaccharides (those from *O*-glycosides) were retained in water. The LC-MS analysis of this methanolized fraction confirmed the presence of *C*-glycoside (peak 22), which was submitted to NMR analysis.

The two-dimensional $^{1}H$-$^{13}C$-NMR experiment (multiplicity-edited gHSQC) showed main chemical shifts of glucosyl residues at δ 75.5/5.02, 72.9/4.11, 79.7/3.54, 70.6/3.57, 82.7/3.48 and 62.5/4.52 (phase-inverted) from C1/H1, C2/H2, C3/H3, C4/H4, C5/H5, and C6/$H_2$6. In comparison with *O*-glycosides, the anomeric signals of glucosyl appeared at upfield δ 75.5/5.02, characteristic of *C*-glycosides (Fig. 3A). The aglycone signals appeared at δ 103.7/6.64, 130.0/7.98, 129.9/7.89 and 117.0/6.94 consistent with the C3/H3, C2'/H2', C6'/H6' and C5'/H5', characteristic of apigenin (Fig. 3B) [44-47]. The long-range coupling experiment (HMBC) showed coupling signals between H1 of Glc*p* (δ 5.02) units and C9 (δ 156.3), and C7 (δ 161.5) from the aglycone, confirming the position of Glc*p* units at C8 and C6 positions (Fig. 3C). Thus, considering MS/MS and NMR analysis the ion at *m/z* 593.1514 (peak 22) was consistent with apigenin-6,8-di-*C*-β-D-glucopyranoside (vicenin-2) [48,49].

A compound observed in the chromatogram of BU fraction, at *m/z* 518.2027 $[M-H]^-$ (peak 36) was not identified. However, it has ions typical of nitrogenated compounds, such as alkaloids. Other compounds not identified and their MS/MS profile are summarized in table 1.

### 2.1.3 *Analysis of EA fraction*

Flavan-3-ols, condensed tannins, and some flavonol glycosides were concentrated in the ethyl acetate fraction, as observed in the LC-MS analysis (Fig. 4A). For these flavonoids, the negative ionization was better than positive, thus the negative ions will drive the discussion.

Free flavan-3-ols appeared at *m/z* 289.0709 $[M-H]^-$ (peaks 10 and 16) characteristic of catechin and epicatechin, confirmed by comparison with authentic standards. Catechin, afzelechin, and gallocatechin, or their isomers of "epi"-series, are common structures in the condensed tannins producing many isomers that could be differentiated by $MS^2$ [15]. However, even though many isomers being detected for the condensed tannins examined in *C. cajucara*, no evidence for the presence of afzelechin, gallocatechin or their epi-isomers were obtained.

Many peaks with same mass spectrometry profile were observed in the chromatogram, indicating the presence of many isomers, observed as dimers, at *m/z* 577.1337 $[M-H]^-$ composed by two units of catechin or epicatechin [(epi)Cat-(epi)Cat],

indicative of B-type proanthocyanidin, supported by the presence of fragments at *m/z* 425.086, 289.070/287.054 (Fig. 4B, Table 1). Similarly, many peaks for the trimers appeared at *m/z* 865.1958 $[M-H]^-$ with very similar fragment-ions at *m/z* 577.124 and 287.055/289.069, without any evidence for the presence of afzelechin, gallocatechin. So, to explain the presence of many peaks at *m/z* 577.133 and 865.196 (Fig. 4A - Table 1), we suggest that these tannins are formed by different combinations of catechin and epicatechin, with different linkages within him, that can occur between the carbons at 4→6 or 4→8 positions, allowing the formation of several of isomers.

Glycosylated flavonoids were also found in the EA fraction and similarly to BU fraction, only glucose (75%) and rhamnose (25%) were found, along with quercetin ($t_R$ 9.72 min.) and kaempferol ($t_R$ 10.36 min). The interglycosidic linkages analysis indicated non-reducing ends of Glc*p* (73%) and Rha*p* (14%), and glucose units substituted at 6-*O*- (8%), and 2-*O*- (5%).

Quercetin-*O*-glucoside at *m/z* 463.0875 $[M-H]^-$ (peak 33) with the main $MS^2$ fragments appeared as regular ion at *m/z* 301.034 $[M-H]^-$ and radical ion, at *m/z* 300.026 $[M-H]^{\bullet-}$, characteristic of quercetin. The peak 35, with $MS^1$ at *m/z* 447.0936 $[M-H]^-$ and $MS^2$ at *m/z* 300.027 $[M-H]^{\bullet-}$ and 301.035 $[M-H]^-$ (NL of 146.058 a.m.u.) consistent with quercetin-*O*-rhamnoside (Table 1). Moreover, the peak 37 with $MS^1$ at *m/z* 431.0979 $[M-H]^-$ and main fragment at *m/z* 285.040 (NL 146.057 a.m.u.) was characteristic of kaempferol-*O*-rhamnoside (Table 1).

The peak 31, also observed at *m/z* 431.0979 $[M-H]^-$, had a different fragmentation profile, appearing at *m/z* 341.065 and 311.055. The NL of 90 and 120 a.m.u. typical of flavone *C*-glycoside, suggesting a structure similar to the apigenin-*C*-glucoside. The peak 32 had similar MS profile, suggesting the presence of vitexin and isovitexin, respectively, based on chromatographic elution [50].

Flavonol di-*O*-glycosides also appear in this fraction, with $MS^1$ at *m/z* 611.1608 $[M+H]^+$ and the $MS^2$ fragments at *m/z* 465.104, 303.050 consistent with an NL of a rhamnosyl residue (146.0568 a.m.u.) followed by glucosyl residue (162.0540 a.m.u.) with quercetin as aglycone, consistent with rutin (Table 1). The peak 34 with a positive $MS^1$ ion at *m/z* 595.1660 $[M+H]^+$ with main fragments *m/z* 449.108 and 287.055, being consistent with kaempferol-rutinoside, since, on the methylation analysis, the glucose units have appeared mainly as 6-*O*-substituted.

The peak 38, that appeared in this fraction in low abundance, showed the negative ion in $MS^1$ analysis at *m/z* 593.1302 $[M-H]^-$. This compound can easily be

mistaken with kaempferol-rutinoside or another isomer since this ion is only slightly different from the ion of peak 34 (*m/z* 593.1517 $[M-H]^-$). However, this difference in the $MS^1$ and the fragments at *m/z* 447.093, 307.082, 285.040 (Table 1) indicated a different structure. If one hand the fragment at *m/z* 447.093 and 285.040 were consistent with kaempferol-glucoside, on the other hand, the fragment at *m/z* 307.082 is not common from flavonol diglycosides. Moreover, the examination of the UV spectra indicates a different profile from those of flavonols (free or glycosides), with absorbance at λ 260 nm and 315 nm. The compound/peak 38 was assigned as kaempferol-3-*O*-(*p*-coumaroyl)-glucoside that matches all requirements of the UV and MS data observed. Thus, the fragment at *m/z* 307.082 was consistent with a *p*-coumaroyl-glucosyl residue [52]. Furthermore, although this compound had never been described in *C. cajucara*, it was already found in other species from Croton genus [52,53].

## 2.2 *Anti-inflammatory evaluation*

Several studies report the anti-inflammatory activity of plants extracts to the phenolic compounds [17,54,55]. Decoctions of leaves of *C. cajucara* are widely used as anti-inflammatory in folk medicine on Amazonia. In order to investigate its potential anti-inflammatory properties, the fractions from ESF were assayed on of the edema paw edema induced by carrageenan.

As expected, the carrageenan increased the paw thickness in the control group (C: 1.22 mm ± 0.12 mm). On the other hand, when the ESF was administered in the animals (30, 100 and 300 mg/kg, i.p.), the higher doses significantly inhibited the paw edema in 59 and 47% when compared to the control group (Fig. 5A). Dexamethasone (D: 0.5 mg/kg, i.p.), the positive control of the test, also reduced the paw edema by 57% when compared to the control group.

Interestingly, all the fractions from liquid partition exerted anti-inflammatory effects, with doses corresponding to their yields from the original ESF. Thus, AQ (76 mg/kg, i.p.), BU (16 mg/kg, i.p.) and EA fraction (8 mg/kg, i.p.) were able to inhibit the paw edema in 68%, 68 % and 55 %, respectively, when compared to the control group (C: 1.16 ± 0.10 mm) (Figure 5B). The positive control dexamethasone (D: 0.5 mg/kg, i.p.) reduced the paw edema by 58% when compared to the control group (Fig. 5B).

All fractions evaluated (AQ, BU, and EA) and ESF fraction contained different types of phenolic compounds including many different flavonoids. Flavonoids are important low-weight molecular compounds abundant an aqueous extract from plants responsible for exerting countless biological effects in human healthy including anti-inflammatory property. Moreover, flavonoids seem to affect acute inflammatory reactions for different mechanisms including inhibition of chemical mediators of inflammation [56,57].

## 3 Conclusions

The number of compounds identified in *C. cajucara* leaves was increased with the identification of several phenolic compounds, including catechins, proanthocyanidins, flavonol *O*- and *C*-glycosides and cinnamic acid derivatives. The approach employed to the elucidation of the compounds from *C. cajucara* proved to be very important, since it combines liquid/liquid fractionation, that eliminates free oligosaccharides that could, otherwise, interfere with the final results causing misinterpretations, with a thorough mass spectrometry investigation, including GC-MS analysis of glycan units. With these combined results, many glycosides were identified without the need of laborious purification. Fractionation is also important to reduce the complexity of samples to the pharmacological investigation. However, all fractions from *C. cajucara* assayed had positive effects on carrageenan-induced paw edema. This could be a result of the presence, in all fractions, of many phenolic compounds, that are extensively associated to many biological/pharmacological activities from plants. Thus, this result is in agreement with some the popular uses of *C. cajucara*, revealing its potential therapeutics.

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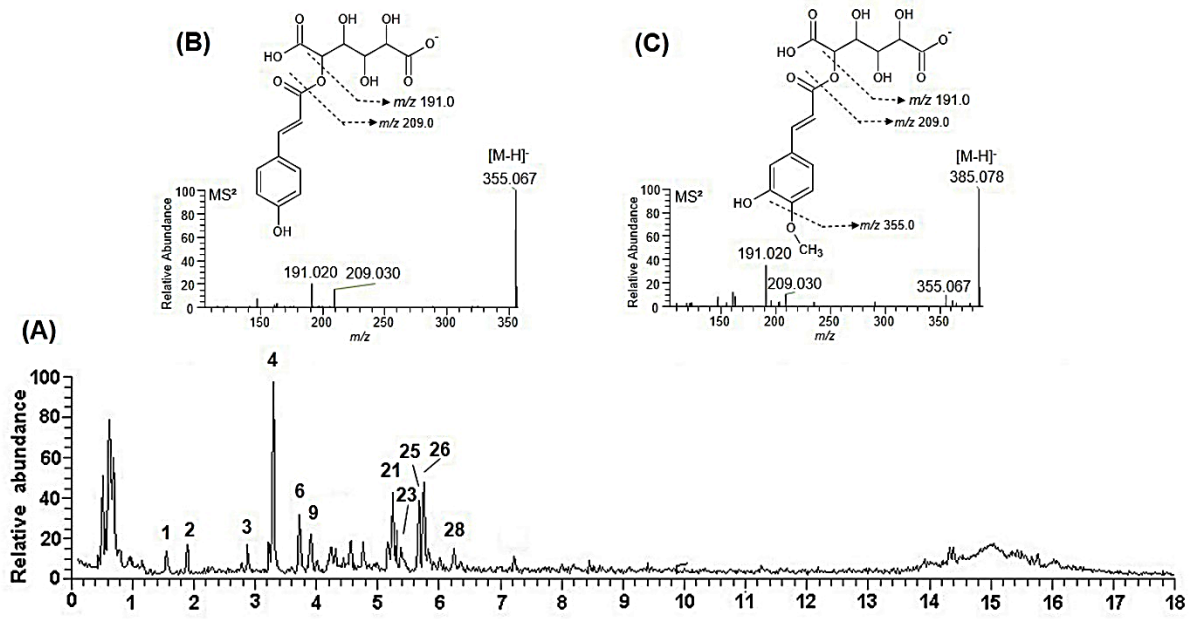


**Figure 1.** UHPLC chromatogram of AQ fraction (A). CID-fragmentation profile with a negative ionization mode of *p*-coumaroyl-galactaric acids with $MS^1$ 355.0673 correspondents to peaks 4 and 6 (B); CID-fragmentation profile with a negative ionization mode of feruloyl-galactaric acid with $MS^1$ 385.0775 correspondents to peak 9 (C).

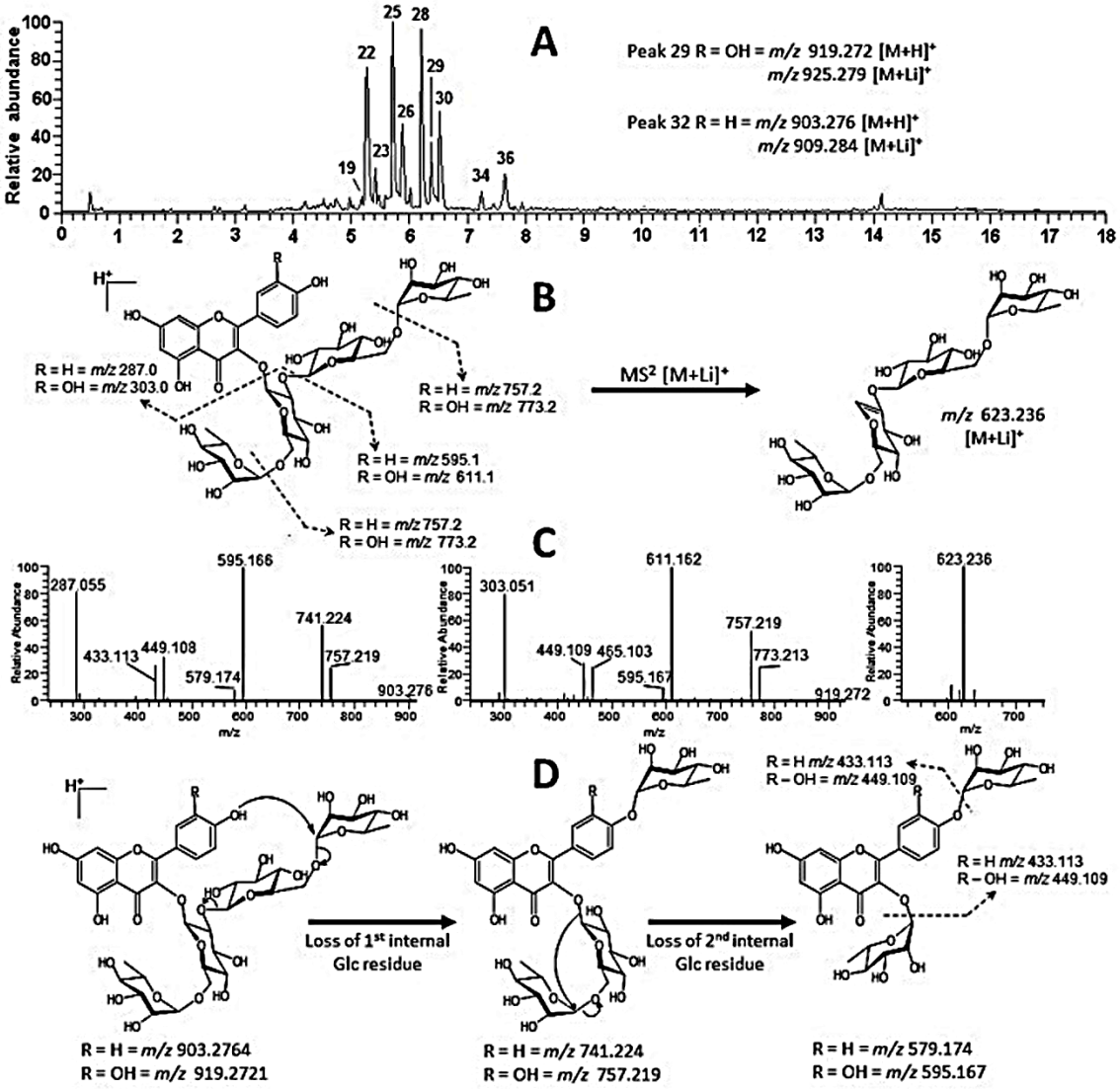


**Figure 2.** UHPLC chromatogram of BU fraction (A). This fraction retained mainly the flavonol glycosides, such as the tetraglycosides that were identified based on MS2 profile of protonated [M+H]+ and lithiated [M+Li]+ 690 ions (B and C). Tentative assignment of additional fragmentation pathway, via internal residue loss mechanism was used to explain the losses of glucosyl residues (D).

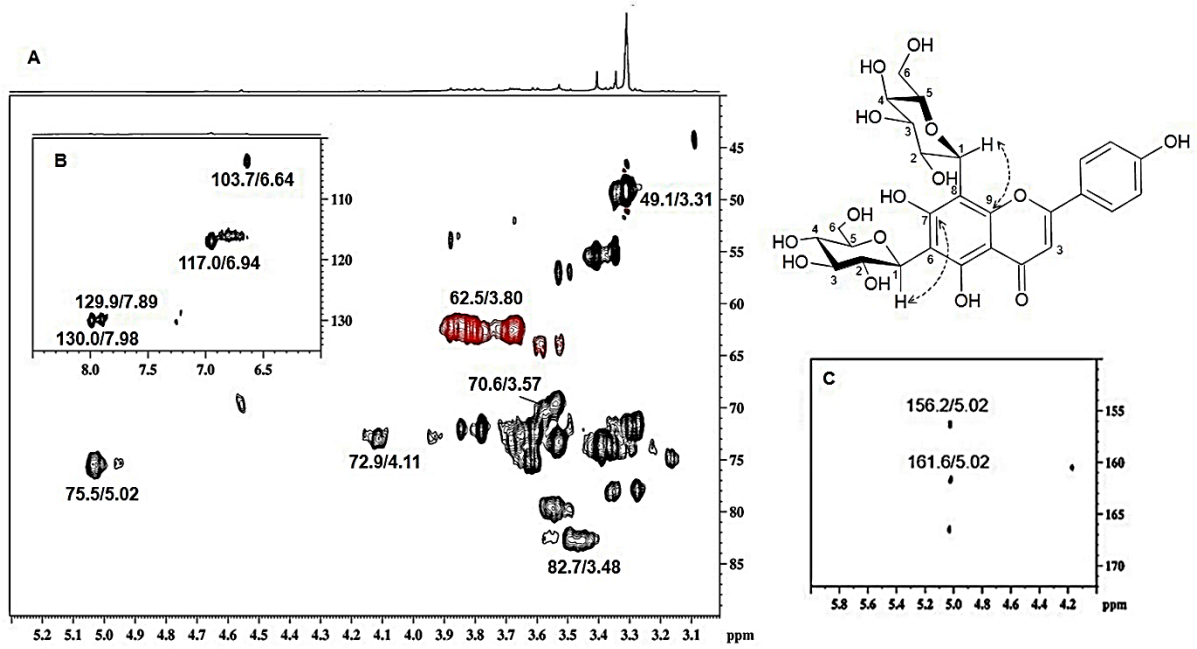


**Figure 3.** 2D $^{1}H$-$^{13}C$-NMR (multiplicity-edited gHSQC) of BU fraction (A and B); 2D $^{1}H$-$^{13}C$-NMR long-range coupling HMBC (C); The experiments were performed with the BU fraction after methanolysis procedure. Solvent Methanol-$d_4$ at 30 °C; numerical values are in $\delta$ ppm.

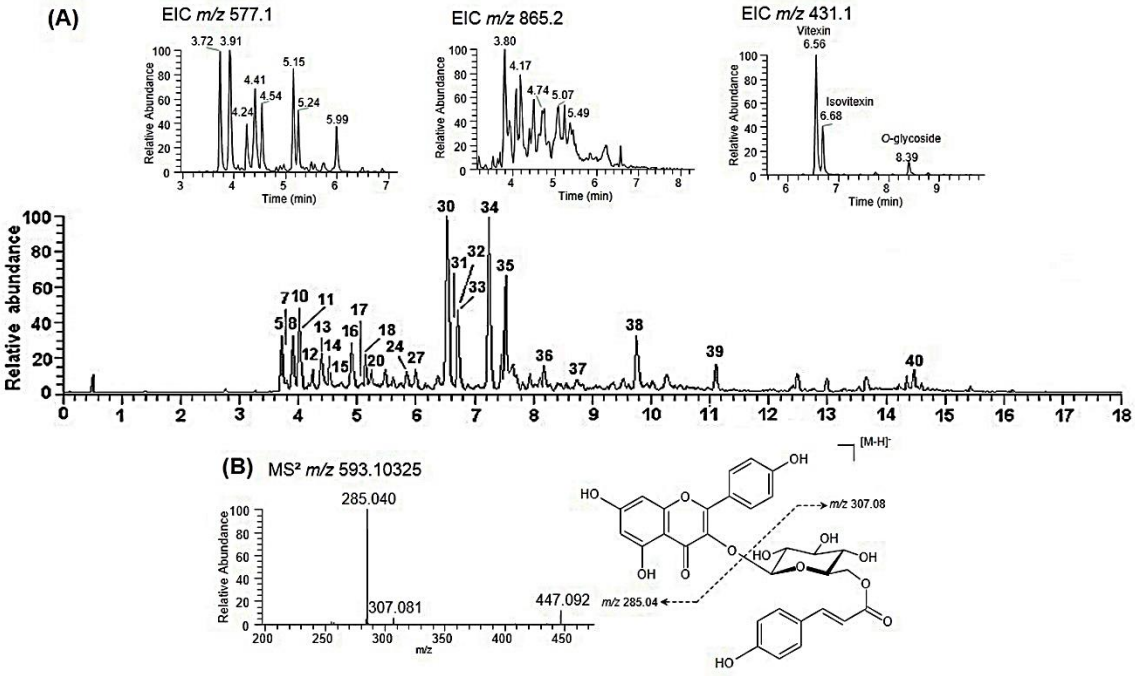


**Figure 4.** UHPLC chromatogram of EA fraction (A); Extracted ion chromatogram (EIC) of (epi)cat dimer at *m/z* 577.1337 (B); Extracted ion chromatogram (EIC) of (epi)cat trimer at *m/z* 865.1964 (C); Extracted ion chromatogram (EIC) of vitexin, isovitexin and (epi)cat dimer at *m/z* 431.0979 (D); CID-fragmentation profile with a negative ionization mode of kaempferol-3-*O*-(*p*-coumaroyl)-glucoside at $MS^1$ 593.13025, peak 45 (E).

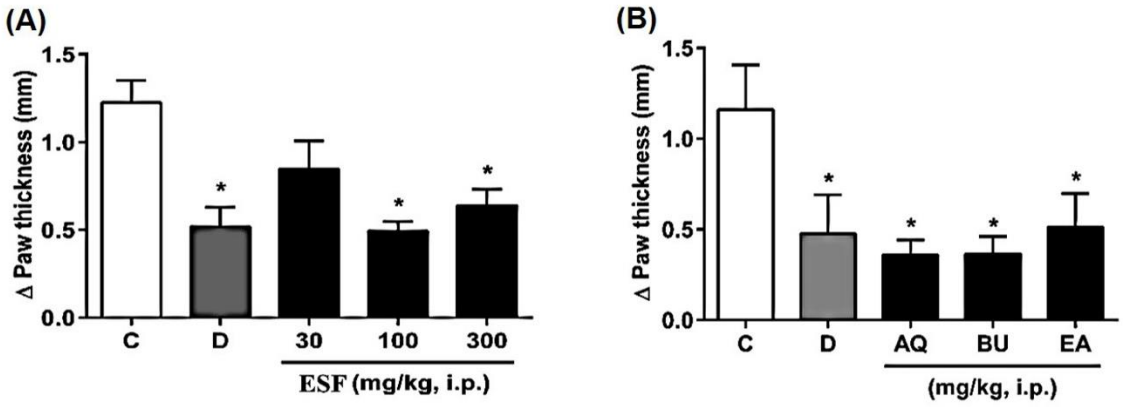


**Fig. 5** Effect of ESF (30, 100, 300 mg/kg, i.p.) on paw edema induced by carrageenan in mice (A). Effect of AQ (76 mg/kg, i.p.), BU (16 mg/kg, i.p.) and EA (8 mg/kg, i.p.) fractions on paw edema induced by carrageenan in mice (B). The results are expressed mean ± SEM (n = 5). Positive control dexamethasone (D: 0.5 mg/kg, i.p.) and negative control (C: saline, 1ml/kg). Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test. Differences from control group (*$p < 0.05$).

**TABLE. 1** - Analysis of low molecular compounds from ethanol supernatant (ESF) and fractions partitioned (AQ, BU, and EA) from decoction of leaves *C. cajucara*, by UHPLC-MS of the compounds on negative mode $[M-H]^-$, positive mode $[M+H]^+$ and lithiated positive mode $[M+Li]^+$.

| Peak | $t_R^*$ | $MS^1$ | $MS^2$ | Tentative structures | Fraction |
|---|---|---|---|---|---|
| 1 | 1.57 | 669.2028[a] | 653.615, 597.640, 553.798, 367.128, 307.894, 234.529, 210.162 | n.i. | AQ |
| 2 | 1.89 | 513.1430[a] | 492.659, 470.007, 365.436, 351.087, 306.879, 180.579, 160.494 | n.i. | AQ |
| 3 | 2.88 | 315.072[a] | | n.i. | AQ |
| 4 | 3.29 | 355.0673[a] | 209.030, 191.020 | *p*-coumaroyl-galactaric acid | AQ |
| 5 | 3.72 | 577.1337[a] | 559.123, 451.101, 425.086, 407.076, 299.055, 289.070 | [(epi)Cat-(epi)Cat] | EA |
| 6 | 3.71 | 355.0676[a] | 209.030, 191.020 | *p*-coumaroyl-galactaric acid | AQ |
| 7 | 3.80 | 865.1958[a] | 739.165, 695.132, 577.129, 287.052 | [(epi)Cat-(epi)Cat-(epi)Cat] | EA |
| 8 | 3.91 | 577.1338[a] | 559.123, 451.102, 425.086, 407.075, 299.055, 289.070 287.055, | [(epi)Cat-(epi)Cat] | EA |
| 9 | 3.91 | 385.0775[a] | 355.067, 209.030, 191.019 | feruloyl-galactaric acid | AQ |
| 10 | 4.02 | 289.0709[a] | 245.0816, 198.5092 | Catechin | EA |
| 11 | 4.17 | 865.1964[a] | 739.172, 695.138, 619.146, 577.124, 483.186, 289.073 | [(epi)Cat-(epi)Cat-(epi)Cat] | EA |
| 12 | 4.24 | 577.1334[a] | 559.123, 451.104, 425.086, 407.076, 289.072 | [(epi)Cat-(epi)Cat] | EA |
| 13 | 4.41 | 577.1330[a] | 559.124, 451.102, 425.086, 407.075, 299.057, 289.070, 287.055 | [(epi)Cat-(epi)Cat] | EA |
| 14 | 4.54 | 577.1326[a] | 559.119, 451.102, 425.085, 407.075, 299.056, 289.069, 287.054 | [(epi)Cat-(epi)Cat] | EA |
| 15 | 4.74 | 865.1957[a] | 739.165, 695.132, 577.129, 287.052 | [(epi)Cat-(epi)Cat-(epi)Cat] | EA |
| 16 | 4.91 | 289.0708[a] | 245.0821, 187.0838 | Epicatechin | EA |
| 17 | 5.07 | 865.1952[a] | 577.134, 543.250, 451.167, 289.070, 287.167 | [(epi)Cat-(epi)Cat-(epi)Cat] | EA |

| | | | | | |
|---|---|---|---|---|---|
| 18 | 5.15 | 577.1325[a] | 559.122, 467.096, 451.101, 425.084, 331.081, 299.056, 289.071 | [(epi)Cat-(epi)Cat] | EA |
| 19 | 5.18 | 757.2177[b] | 739.205, 611.164, 595.167, 463.670, 449.108, 287.055 | Glc*p*-(1→2/6)-[Rha*p*-(1→2/6)]-Glc*p*-(1→)-Kaemp | BU |
| 20 | 5.24 | 577.1329[a] | 559.122, 467.098, 451.102, 425.088, 331.080, 289.071, 245.044 | [(epi)Cat-(epi)Cat] | EA |
| 21 | 5.25 | 916.2503[a] | 690.856, 626.979, 554.707, 414.102 | n.i. | AQ |
| 22 | 5.27 | 593.1513[a] | 575.139, 503.118, 473.108, 383.077, 353.066 | 6,8-bis-*C*-glucosyl-apigenin (Vicenin-2) | BU, AQ |
| 23 | 5.41 | 773.2136[b] | 627.156, 611.161, 465.103, 449.108, 303.050 | Glc*p*-(1→2/6)-[Rha*p*-(1→2/6)]-Glc*p*-(1→)-Quer | BU |
| 24 | 5.49 | 865.1950[a] | 735.155, 599.115, 577.132, 453.118,289.069 | [(epi)Cat-(epi)Cat-(epi)Cat] | EA |
| 25 | 5.72 | 919.2721[b] | 773.213, 757.219, 611.162, 595.167, 465.103, 449.109, 303.051 | Rha*p*-(1→2/6)-Glc*p*-(1→2/6)-[Rha*p*-(1→2/6)]-Glc*p*-(1→)-Quer | BU, AQ |
| | | 925.2956[c] | 623.241; 477.182; 315.128 | | |
| 26 | 5.86 | 757.2185[b] | 611.161, 595.168, 465.103, 449.108, 303.050 | Rha*p*-(1→6)-[Rha*p*-(1→2)]-Glc*p*-(1→3)-Quer | BU |
| | | 763.2325[c] | 461.184, 315.126 | | |
| 27 | 5.99 | 577.1334[a] | 559.123, 451.101, 425.088, 407.084, 289.071, 245.045 | [(epi)Cat-(epi)Cat] | EA |
| 28 | 6.21 | 903.2764[b] | 757.219, 741.224, 595.166, 449.108, 433.113, 287.055 | Rha*p*-(1→2/6)-Glc*p*-(1→2/6)-[Rha*p*-(1→2/6)]-Glc*p*-(1→)-Kaemp | BU, AQ |
| | | 909.2910[c] | 623.241; 605.230; 477.182; 315,128 | | |
| 29 | 6.38 | 741.2238[b] | 595.166, 579.171, 449.108, 433.113, 287.055 | Rha*p*-(1→6)-[Rha*p*-(1→2)]-Glc*p*-(1→x)-Kaemp | EA, BU |
| | | 747.2085[c] | 601.174; 461.184; 443.173; 315.126 | | |
| 30 | 6.53 | 611.1608[b] | 465.104, 303.050 | Rha*p*-(1→6)-Glc*p*-(1→3)-Querc (Rutin) | EA, BU |
| | | 617.1774[c] | 599.135, 471.111, 315.126 | | |
| 31 | 6.56 | 431.0979[a] | 413.087, 341.065, 311.055 | Flavone-*C*-glucoside (Vitexin) | EA |
| 32 | 6.68 | 431.0979[a] | 413.087, 341.066, 311.055 | Flavone-*C*-glucoside (Isovitexin) | EA |
| 33 | 6.72 | 463.0875[a] | 301.034, 300.026 | Quercetin-*O*-glucoside | EA |

| | | | | | |
|---|---|---|---|---|---|
| | | 465.1027[b] | 303.0946 | | |
| 34 | 7.25 | 595.1660[b] | 449.108, 287.055 | Rha*p*-(1→6)-Glc*p*-(1→)-Kaemp | EA, BU |
| | | 601.1787[c] | 315.126 | | |
| 35 | 7.53 | 447.0936[a] | 301.035, 300.027 | Quercetin-*O*-rhamnoside | EA |
| | | 449.1082[b] | 303.050 | | |
| 36 | 7.64 | 518.2027[a] | 356.149 | n.i. | BU |
| | | 520.2182[b] | 358.166, 299.128, 220.097 | | |
| 37 | 8.39 | 431.0979[a] | 285.040 | Kaempferol-*O*-rhamnoside | EA |
| 38 | 9.75 | 593.13025[a] | 447.093, 307.082, 285.040 | Kaempferol-3-*O*-(*p*-coumaroyl)-glucoside | EA |
| 39 | 11.10 | 691.2756[b] | 345.132 | n.i. | EA |
| 40 | 14.17 | 315.1595[b] | 269.1540, 251.1434 | n.i. | EA |

[*] $t_R$ = retention time; Glc*p*: glucopyranosyl; Rha*p*: rhamnopyranosyl; Quer: quercetin; Kaemp: kaempferol

[a] $[M-H]^-$, [b] $[M+H]^+$, [c] $[M+Li]^+$, n.i. = not indentified

# 6 CONCLUSÕES GERAIS

Após análise dos resultados obtidos neste trabalho, pode-se concluir que:

- A partir de uma extração aquosa das folhas de *A. chica* foi obtida uma fração polissacarídica (AC25R) constituída principalmente por uma arabinogalactana do tipo II contendo uma cadeia principal de unidades de β-D-Gal*p* (1→3)-ligadas, substituída em O-6 por cadeias laterais de β-D-Gal*p* (1→6)-ligadas. As cadeias laterais são substituídas em O-3 por terminais não redutores de α-D-Glc*p* principalmente. Terminais não redutores de β-D-Gal*p*, α-L-Ara*f* e α-L-Rha*p* também estão presentes em menores proporções. Este polissacarídeo mostrou-se homogêneo na análise por HPSEC, com $M_w$ de 49.690 g/mol.

- A avaliação do efeito imunomodulador de AC25R em macrófagos THP-1 demonstrou que ele é capaz de aumentar a atividade de macrófagos de maneira dose-dependente, uma vez que aumentou significativamente a produção das citocinas TNF-α, IL-1β e IL-10.

- O processo de fracionamento bioguiado por atividade gastroprotetora do precipitado etanólico da extração aquosa de folhas de *C. cajucara* gerou uma fração (25R) que deteve um significante efeito gastroprotetor, se mostrando capaz de diminuir a úlcera gástrica induzida por etanol em ratos, preservando o muco e os níveis de GSH. 25R mostrou-se uma complexa fração polissacarídica constituída por ramnogalacturonana do tipo I, com uma cadeia principal de unidades de α-D-GalA*p* (1→4)-ligadas, intercalada com unidades de α-L-Rha*p* (1→2)-ligadas, substituídas em O-4 por cadeias laterais de arabinana, arabinogalactana do tipo I e arabinogalactana do tipo II. A presença de unidades de ramnose 2-*O* e 2,3-di-*O*-ligadas, aliada ao elevado teor de α-L-Rha*p* em contraste ao teor de α-D-GalA*p* indicaram a presença de uma ramnan que pode intercalar a RG I. A presença de amido e manana em pequena proporção também foram detectados nesta fração.

- A análise fitoquímica dos compostos de baixa massa molar de um extrato aquoso de *C. cajucara* demonstrou a presença de diferentes compostos fenólicos identificados pela primeira vez para extratos da planta. Estes compostos foram

fracionados por polaridade sendo possível a determinação da estrutura de flavonóis *O*-glicosilados contendo kaempferol e quercetina como agliconas, flavonoides *C*-glicosilados, taninos e derivados de ácidos cinâmicos. As frações contendo estes compostos químicos exibiram propriedade anti-inflamatória quando submetidas a ensaios de atividade usando um modelo de edema de pata induzido por injeção intraplantar de carragenana em camundongos.

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**ANEXO 1**

SECRETARIA DO MEIO AMBIENTE
**INSTITUTO DE BOTÂNICA**

Caixa Postal 68041
04045-972 São Paulo, SP - Brasil

LAUDO DE IDENTIFICAÇÃO

Para: Adamara Machado Nascimento
Doutoranda em Bioquímica. Laboratório de Química de Carboidratos - E1
Departamento de Bioquímica e Biologia Molecular
Centro Politécnico - Universidade Federal do Paraná

As plantas que nos foram submetidas à identificação, tratam-se de:

Amostra 1

***Fridericia chica* (Bonpl.) L.G.Lohmann - Bignoniaceae**
[tem como sinônimo: *Arrabidaea chica* (Bonpl.) Verl.]
Identificador: R.S. Bianchini
Nome popular: Crajirú. Outros nomes populares: cipó-cruz, chica, cajuru, cipó-pau, paripari, pariri, oajuru, carajuru, puca panga.
Local de coleta: Rua da Paraíba, 043, Bairro da Escola Técnica.
Data da coleta: 01/07/2015
Material examinado: Ramo com folhas secas.
Material comparado com: *R.T. Shirasuna & O. Yano 970*, 19/02/2008 (SP 430336)

Amostra 2

***Croton cajucara* Benth. - Euphorbiaceae**
Identificador: Inês Cordeiro
Nome popular: sacaca. Outro nome popular: marassacaca
Local de coleta: Estrada da Maloca, 551, Bairro da Cohab, Cruzeiro do Sul, Acre.
Data da coleta: 01/07/2015
Material examinado: Ramo com folhas secas.
Árvore de 4-6 m alt.
Material comparado com: *W. Rodrigues s.n.*, 25/09/1995 (SP 319378)

Amostra 3

***Justicia calycina* (Nees) V.A.W.Graham - Acanthaceae**
[tem como sinônimo: *Justicia acuminatissima* (Miq.) Bremek.]
Identificador: Cíntia Kameyama
Nome popular: paratudo
Local de coleta: Rua do Pará, Bairro do Cruzeiro, Cruzeiro do Sul, Acre.
Data da coleta: 03/07/2015
Material examinado: Ramos secos com folhas. Flores rosa.
Arbusto 1,7-1,8 m alt.
Material depositado no Herbário SP, número 474031

Dra. Rosângela Simão Bianchini
São Paulo, 09 de setembro de 2015

Av. Miguel Estéfano, 3687 - Água Funda
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**Publication:** International Journal of Biological Macromolecules

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| **Article type** | Full length article |

**Abstract**

Croton cajucara Benth. is a medicinal plant popularly used in the Brazilian Amazonia, where it is known as sacaca, being consumed as tea, decoction or infusion, from leaves and stem bark. From decoction of leaves, a comprehensive phytochemical analysis was developed by liquid chromatography-mass spectrometry. Many unreported compounds were identified, such as O-glycosides containing kaempferol and quercetin as aglycone moiety, flavonoid-C-glycosylated, tannins, and cinnamic acid derivatives. These compounds were fractioned by polarity and assayed for their anti-inflammatory activity, using a model of mice edema, induced by an intraplantar injection of carrageenan. All fractions exhibited anti-inflammatory properties.

| | |
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| **Keywords** | Croton cajucara; flavonoids; anti-inflammatory activity; LC-MS. |
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| **Corresponding Author** | Lauro Souza |
| **Corresponding Author's Institution** | Research Institute "Pequeno Príncipe" |
| **Order of Authors** | Adamara Machado Nascimento, Daniele Maria-Ferreira, Fernando Dal Lin, Alexandre Kimura, Arquimedes Santana-Filho, Maria Fernanda Werner, Marcello Iacomini, Guilherme Sassaki, Thales Cipriani, Lauro Souza |
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