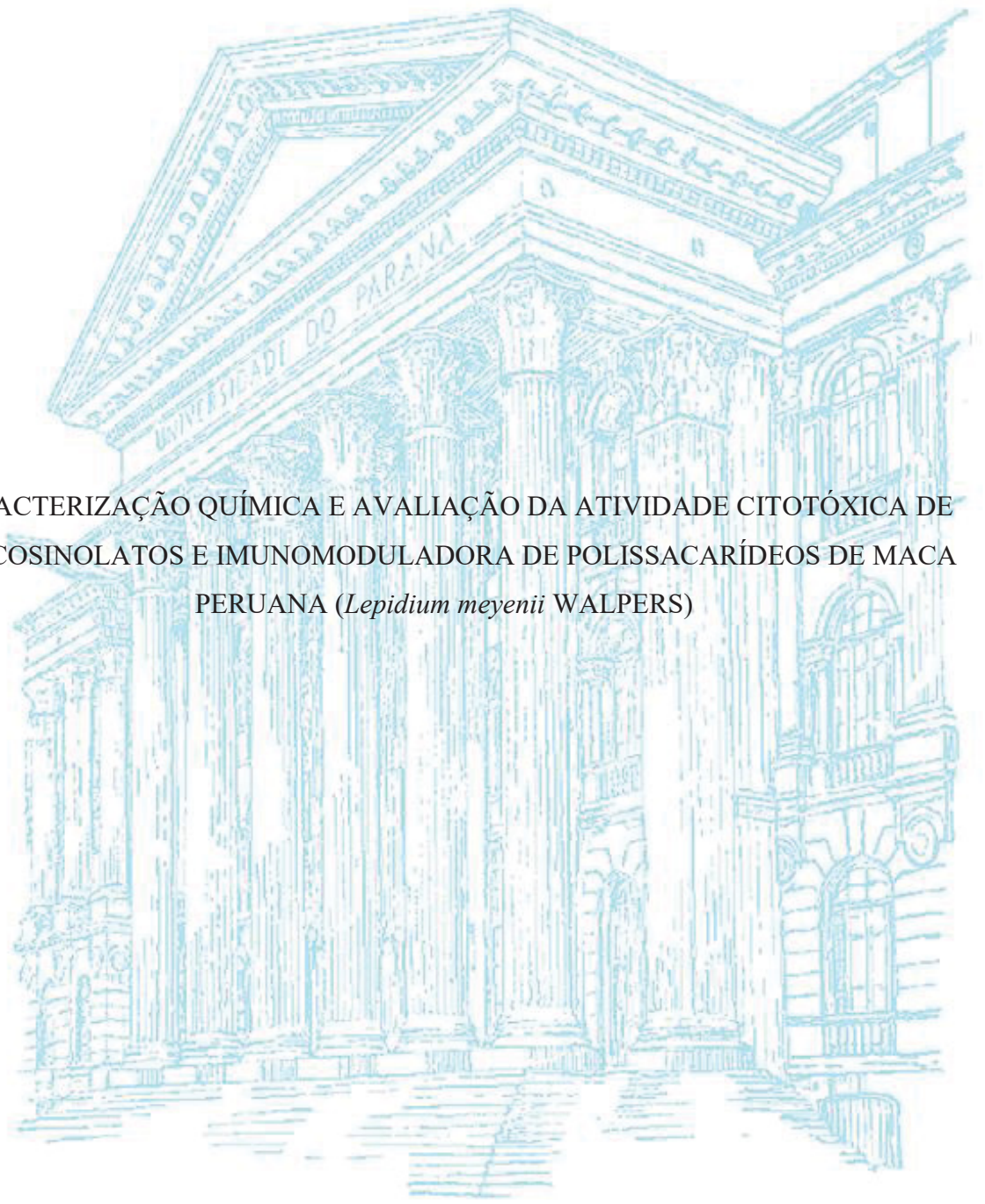
UNIVERSIDADE FEDERAL DO PARANÁ

RAQUELY MOREIRA LENZI



CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DA ATIVIDADE CITOTÓXICA DE GLUCOSINOLATOS E IMUNOMODULADORA DE POLISSACARÍDEOS DE MACA PERUANA (*Lepidium meyenii* WALPERS)

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Ciências (Bioquímica), Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências (Bioquímica).
Orientador: Profa. Dra. Juliana Bello Baron Maurer
Coorientador: Profa. Dra. Carmen Lúcia de Oliveira Petkowicz

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de RAQUELY MOREIRA LENZI, intitulada: **CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DA ATIVIDADE CITOTÓXICA DE GLUCOSINOLATOS E IMUNOMODULADORA DE POLISSACARÍDEOS DE MACA PERUANA (*Lepidium meyenii* WALPERS)**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

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

Curitiba, 27 de setembro de 2019.

JULIANA BELLO BARON MAURER
(Presidente da Banca Examinadora)

THALES RICARDO CIPRIANI (UFPR)

GUSTAVO AMADEU MICKE (UFSC)

SELMA FARIA ZAWADZKI BAGGIO (UFPR)

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Os principais resultados obtidos estão representados na forma de artigos científicos. Este formato está de acordo com as normas do Programa de Pós-Graduação em Ciências - Bioquímica, da Universidade Federal do Paraná. A tese é composta de revisão bibliográfica, objetivos gerais e específicos do trabalho, justificativa, artigos científicos com os experimentos realizados e resultados, conclusões e perspectivas.

À minha família, Daniel, Felipe e Maria Eduarda.

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Quando a gente anda sempre em frente,
não pode mesmo ir longe

O Pequeno Príncipe
Antoine de Saint-Exupéry

RESUMO

Lepidium meyeni Walpers*,* conhecida como maca peruana é um alimento e planta medicinal nativa dos Andes, que tem ganhado destaque mundial em função das propriedades afrodisíacas, energéticas e imunoestimulantes relatadas para essa planta. Entretanto, há um consenso de que os benefícios atribuídos ao consumo da maca não apresentam embasamento científico suficiente. Nesse contexto, o objetivo desse estudo foi obter e caracterizar frações da maca ricas em duas classes de compostos com potencial bioativo: glucosinolatos (GLs) (I) e polissacarídeos (II), a fim de se investigar suas respectivas atividades biológica em ensaios *in vitro*. (I) As frações ricas em glucosinolatos intactos foram obtidas através de extração hidroetanólica e fracionamento através de partição líquido-líquido seguida de cromatografia em sílica gel, e monitoradas por CCD e CLAE. Glucotropaeolina (GTR) e glucolimnantina (GLM) foram identificadas através da combinação de técnicas cromatográficas aliadas à espectrometria de massas (CLAE, MS, LC-MS/MS). O excesso de açúcares livres co-eluídos com os GLs foi removido por extração em fase sólida utilizando mini cartuchos $C_{18,}$ dando origem às frações ricas em GLs: Lm-II (GTR=195,4 µg/mg) e Lm-III (GTR=169,9 µg/mg e GLM = 66,6 µg/mg), as quais foram avaliadas em um ensaio de citotoxicidade (MTT) combinado com a enzima mirosinase (MYR). Ambas frações foram citotóxicas para as linhagens humanas de carcinoma hepatocelular (HepG2/C3A) e adenocarcinoma de cólon (HT-29). Os efeitos citotóxicos foram verificados apenas nos experimentos com frações submetidas à etapa de limpeza do açúcar e quando na presença de MYR. Os valores de IC_{50} obtidos para Lm-II e Lm-III foram de 118,8 e 69,9 µg/mL para HepG2/C3A e 102,6 e 71,5 µg/mL para HT29, respectivamente. Estes resultados sugerem que os produtos de degradação dos GLs pela MYR são os responsáveis pela citotoxicidade. (II) As frações polissacarídicas da maca foram obtidas através da extração sequencial utilizando H_2O 55 ºC (4 h), H_2O 85 ºC (2 h), e NaOH 1 M, 2 M e 4 M utilizando a farinha de maca pré-inativada (EtOH 70%), deslipidificada (tolueno:EtOH) e livre de amido (degradação enzimática com α-amilase e amiloglucosidase). Todas as frações apresentaram Ara, Gal, Man e Glc em sua composição, e adicionalmente as frações de hemiceluloses A e B exibiram também Xyl. As frações MP-Ws-R (MW 298 kDa), MP-HW (PM 2720 kDa) e MP-1Ab (MW 1680 kDa) apresentaram um teor de arabinogalactana-proteínas (AGPs) de 34,3 ± 0,5, 14,6 ± 1,4 e 30,2 ± 1,1%, respectivamente. Considerando as análises químicas e de RMN-HSQC, sugere-se a presença de arabinogalactana tipo II (AG-II) e xiloglucanas nas frações polissacarídicas da maca. Os assinalamentos característicos para AG-II corroboraram com os resultados obtidos em relação ao teor de AGP. Os efeitos exercidos sobre as vias clássica e alternativa do sistema complemento foram avaliados pelo ensaio hemolítico e comparados ao controle de inibição (heparina). Com pré-incubação, os IC_{50} da heparina, MP-Ws-R, MP-HW e MP-1Ab foram de 40; 204,9; 303,8 e 886,9 µg/mL para a via clássica (VC), e 280,2; 224,4; 411,8 e 387 µg/mL para a vi alternativa (VA), respectivamente. Diferente da heparina, nenhuma fração apresentou atividade nos ensaios sem pré-incubação, indicando que as frações MP-Ws-R, MP-HW e MP-1Ab atuam como ativadores da via clássica e da via alternativa do sistema complemento. Estes resultados poderão contribuir para o fortalecimento da maca como um "superalimento" e para o desenho de futuros estudos com foco na abordagem terapêutica.

Palavra chaves: maca peruana, *Lepidium meyenii*, glucosinolatos, mirosinase, isotiocianatos, polissacarídeos, arabinogalactana-proteínas, AGP, MTT, citotoxicidade, HepG2/C3A, HT29, sistema complemento, imunoestimulante, imunomoduladores.

ABSTRACT

Lepidium meyeni Walpers, known as Peruvian maca, is an Andes native crop used as food and for medicinal proposes. Maca has gained worldwide prominence due its related aphrodisiac, energetic, and immunostimulant properties. Nevertheless, the benefits attributed to maca consumption cannot be entirely supported from a scientific standpoint. In this context, the aim of this study was to obtain and characterize maca fractions of (I) glucosinolates (GLs) and (II) polysaccharides, in order to investigate their respective *in vitro* biological activities. (I) Intact GL-enriched fractions were obtained by ethanolic aqueous extraction, followed by liquid-liquid partition and silica gel chromatography. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis were used to monitor the obtainment of maca GL-enriched fractions. After free sugar clean-up step (by solid phase extraction using C_{18} cartridges), Lm-II and Lm-III fractions were obtained. The identification of the GLs was performed with combined chromatographic and mass spectrometry approaches (HPLC, mass spectrometry (MS), liquid chromatography-MS/MS). Glucotropaeolin (GTR) and glucolimnanthin (GLM) were identified on both fractions. These fractions were used on biological assay to evaluate their cytotoxicity on two tumoral human cell lines in absence (-MYR) and presence of myrosinase (+MYR). MYR converts GLs to their products such as isothiocyanates (ITCs). Lm-II (GTR=195.4 µg/mg) and Lm-III (GTR=169.9 µg/mg and GLM = 66.6 µg/mg), were cytotoxic for both, human hepatocellular carcinoma (HepG2/C3A) and colon adenocarcinoma (HT-29) cell lines. Cytotoxicity effects were only observed in the experiments with fractions submitted to free-sugar cleaning step and when MYR was added to culture medium. The IC_{50} values, corresponding to the half-maximal inhibitory concentration of Lm-II and Lm-III (on +MYR assay), were 118.8 and 69.9 µg/mL for HepG2/C3A and 102.6 and 71.5 µg/mL for HT29, respectively. These results suggested that GLs breakdown products formed by MYR can be responsible for the cytotoxic effects. (II) Maca polysaccharide fractions were obtained by sequential extraction performed with H_2O at 55 ºC, H_2O at 85 ºC, 1 M, 2 M and 4 M NaOH, using an inactivated (EtOH 70%), defatted (toluene:EtOH) and starch-free flour (α-amylase e amyloglucosidase). Arabinose, galactose, and glucose were the main monosaccharide constituents in the water-soluble fractions (MP-Ws-R and MP-HW) and in the alkaline fractions (correspondent to hemicelluloses A and B), which presents additionally xylose. MP-Ws-R (MW 298 kDa), MP-HW (MW 2720 kDa) and MP-1Ab (MW 1680 kDa) fractions showed an AGP content of 34.3±0.5, 14.6±1.4 and 30.2±1.1%, respectively. Considering chemical and NMR analysis it can be suggested the presence of arabinogalactan type II (substituted with Rha, 4-*O*-Me-GlcA) and xyloglucans on the maca polysaccharide fractions. The characteristic AG-II assignments corroborated with the results obtained regarding the AGP content. Their modulatory effects on the classical (AP) and alternative (AP) pathways of the complement system (CS) was accessed by the haemolytic fixation test and comparing with heparin (inhibition control). With pre-incubation, the IC_{50} of heparin, MP-Ws-R, MP-HW and MP-1Ab were 40.0, 204.9, 303.8 and 886.9 µg/mL for classical pathway (CP); and 280.2, 224.4, 411.8 and 387 µg/mL for alternative pathway (AP), respectively. Unlike heparin, no fraction showed activity in assays performed without preincubation, indicating that MP-Ws-R, MP-HW and MP-1Ab fractions act as activators of the CP and the AP of the complement system. These results may contribute to strengthening maca as a "superfood" and to the design of future studies focusing on the therapeutic approach.

Palavra chaves: Peruvian maca, *Lepidium meyenii*, glucosinolates, myrosinase, isothiocyanates, polysaccharides, arabinogalactan-protein, AGP, MTT, cytotoxicity, HepG2/C3A, HT29, complement-system, immunostimulant, imunomodulation.

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LISTA DE ABREVIATURAS, SIGLAS, SÍMBOLOS E FÓRMULAS QUÍMICAS

REVISÃO BIOBLIOGÁFICA

δ (delta)	-	Deslocamento químico (ppm)
β-GlcY	-	Reagente β-glucosil Yariv
A4982LM	-	Linhagem celular de carcinoma de rim humano
A549	-	Linhagem celular de adenocarcinoma de pulmão humano
AG	-	Arabinogalactana
AGP(s)	-	Arabinogalactana-proteína(s)
ANVISA	-	Agência Nacional de Vigilância Sanitária
APCI	-	Ionização química a pressão atmosférica *(atmospheric pressure chemical ionization)*
Ara	-	Arabinose
BITC	-	Benzilisotiocianato
C1-C9 a/b	-	Componentes do sistema complemento
CCD	-	Cromatografia em camada delgada
CUMS	-	Modelo experimental - *Chronic unpredictable mild stress*
COLO 302	-	Linhagem celular de adenocarcinoma colorretal
D_2O	-	Água deuterada
DPPH	-	2,2-difenil-1-picrilidrazila
CH_2Cl_2	-	Diclorometano
ESI	-	Ionização por spray de elétrons (*electrospray ionization*)
EtOH	-	Etanol
FAB	-	Bombardeamento por átomos rápidos (f*ast atom bombardment)*
fB	-	Fator B do sistema complemento
fD	-	Fator D do sistema complemento
FDIGROV	-	Linhagem celular de carcinoma de ovário humano
FRAP	-	Método de redução do ferro *(ferric reducing antioxidant power)*
Fru	-	Frutose
Fuc	-	Fucose
Gal	-	Galactose
Gal*p*A	-	Ácido galacturônico *piranosídico*
GL(s)	-	Glucosinolato(s)
GLC	-	Cromatografia líquido-gasosa
Glc	-	Glucose
GPI	-	Glicosilfosfatidilinositol
H_2O	-	Água
HBP	-	Hiperplasia benigna de próstata
HepG2/C3A	-	Linhagem celular de hepatocarcinoma humano
HT29	-	Linhagem celular de adenocarcinoma de cólon humano
Hyp	-	Hidroxiprolina
IC_{50}	-	Inibição da hemólise induzida pelo complemento em 50%

IgM	-	Imunoglobulina M
ITC(s)	-	Isotiocianato(s)
K562	-	Linhagem celular humana de leucemia mieloide crônica
LC	-	Cromatografia líquida
LC-MS/MS	-	Cromatografia líquida acoplada à espectrometria de massas em tandem
LNCap	-	Linhagem de adenocarcinoma de próstata humana
LPS	-	Lipopolissacarídeo bacteriano
m/z	-	Relação massa/carga
MALDI-TOF	-	Ionização e dessorção a laser assistida por matriz e analisador por tempo de vôo *(matrix-assisted laser desorption/ionization-time of flight*)
MAC	-	Complexo de ataque à membrana
Man	-	Manose
MASPs	-	Serina-proteases associadas à lectina sérica ligadora de manose (MBL)
MBL	-	Lectina sérica ligadora de manose
MCF-7	-	Linhagem celular de adenocarcinoma de mama
MDA-MB-231	-	Linhagem celular de carcinoma de mama
MDCK	-	Linhagem celular normal de rim canino (*Madin Darby canine kidney*)
Me	-	Metil (CH_3)
MSH	-	Mistura de soro humano
MRM	-	Monitoramento de reações múltiplas
MS	-	Espectrometria de massas
MS/MS	-	Espectro de fragmentação de massa (Tandem-MS)
NL	-	Perda neutra (*neutral loss*)
OLGA-PH-J/92	-	Linhagem de células neuronais de *Orconectes limosus*
PaCa2	-	Linhagem celular de adenocarcinoma de pâncreas humano
PEITC	-	Fenetil isotiocianato
PC-3	-	Linhagem celular de adenocarcinoma de próstata humano
PIC	-	Cromatografia de pareamento iônico (*paired-ion chromatography*)
pH	-	Potencial hidrogeniônico
Ppm	-	Partes por milhão
RAW264.7	-	Linhagem de macrófagos de camundongo
RMN-^{13}C	-	Espectroscopia de ressonância magnética nuclear de carbono treze
RMN-^{1}H	-	Espectroscopia de ressonância magnética nuclear de hidrogênio
RPLC	-	Cromatografia líquida em fase reversa (*reverse phase liquid chromatography*)
SaF	-	Fração *Sinapsis* sp. (mostarda)
SC	-	Sistema complemento
ShE	-	Eritrócitos de carneiro (*sheep erithrocytes*)
SPE	-	Extração em fase sólida *(Solid phase extraction)*
SFN	-	Sulforafano
TRAP	-	*Total radical – trapping antioxidant parameter assay* (potencial oxidante total)
UM-UC-3	-	Linhagem celular de carcinoma de bexiga humano
UV	-	Ultravioleta

VA	-	Via alternativa do sistema complemento
VC	-	Via clássica do sistema complemento
VL	-	Via das lectinas do sistema complemento
Xyl	-	Xilose

ARTIGO I

HCOOH	-	Formic acid
ANVISA	-	Agência Nacional de Vigilância Sanitária - *National Agency of Sanitary Monitoring*
AqF	-	Aqueous fraction
BITC	-	Benzyl isothiocyanate
BPC	-	base peak chromatogram
dMBITC	-	3,4-dimethoxybenzyl isothiocyanate
ds-GLs	-	Desulphoglucosinolates
EaF	-	Ethyl acetate fraction
GAE	-	Gallic-acid equivalent
GIB	-	Glucoiberin
GLM	-	Glucoliminanthin
GLs	-	Glucosinolates
GTR	-	Glucotropaeolina
Hep-G2/C3A	-	Human hepatocellular carcinoma cell line
HT29	-	Human colon adenocarcinoma cell line
IC_{50}	-	Half-maximal inhibitory concentration
ITCs	-	Isothiocyanates
Lm-I, II, III and IV	-	*Lepidium meyenii* fractions obtained by SPE-C_{18}
LOQ	-	Limit of detection
MBITC	-	3-methoxybenzyl isothiocyanate
MEE	-	Maca ethanolic extract
MEEs	-	Maca ethanolic extract (soluble portion)
MYR	-	Myrosinase
PBS+AA	-	Phosphate buffer saline pH 7,4 containing 1mM ascorbic acid
SaF	-	*Sinapis alba* fraction
S-fractions (S1-S8)	-	Silica fractions (S1-S8)
SIB	-	Sinalbin
SIN	-	Sinigrin
SPE-C_{18}	-	Solid phase extraction

ARTIGO II

AGP	-	Arabinogalactan protein
ANVISA		Agência Nacional de Vigilância Sanitária - National Agency of Sanitary Monitoring
AP	-	Alternative pathway
AP-0	-	Alternative pathway assay without preincubation
AP-30	-	Alternative pathway assay with 30 min preincubation
AP-HB	-	Classical pathway buffer
CP	-	Classical pathway
CP-0	-	Classical pathway assay without preincubation
CP-30	-	Classical pathway assay with 30 min preincubation
CP-HB	-	Classical pathway buffer
CS	-	Complement system
ECM	-	Extracellular matrix
EDTA.Na2	-	Tehylenediamine tetraacetic acid disodium
EGTA	-	Ethylene glycol tetraacetic acid
GA	-	Gum Arabic
HB	-	HEPES/NaCl base buffer
HEPES	-	[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]
HPS	-	Human pooled serum
LP	-	Lectin pathway
MAC	-	Membrane attack complex
MP-1Aa	-	Maca polysaccharide-1M alkaline extraction-hemicellulose a
MP-1Ab	-	Maca polysaccharide-1M alkaline extraction-hemicellulose b
MP-2Aa	-	Maca polysaccharide-2M alkaline extraction-hemicellulose a
MP-2Ab	-	Maca polysaccharide-2M alkaline extraction-hemicellulose b
MP-4Aa	-	Maca polysaccharide-4M alkaline extraction-hemicellulose a
MP-4Ab	-	Maca polysaccharide-4M alkaline extraction-hemicellulose b
MP-HW	-	Maca polysaccharide - hot water extraction
MPW	-	Maca polysaccharide-water extraction
MP-Wp	-	Maca polysaccharide-water extraction-pellet of freeze-thawing process
MP-Ws	-	Maca polysaccharide-water extraction-supernatant of freeze-thawing process
MP-Ws-E	-	Maca polysaccharide-water extraction-supernatant-eluted by ultrafiltration
MP-Ws-R	-	Maca polysaccharide-water extraction-supernatant-retained by ultrafiltration
RaE	-	Rabbit erythrocytes
ShE	-	Sheep erythrocytes
β-GlcY	-	β-glucosyl Yariv reagent

SUMÁRIO

1 INTRODUÇÃO

Embora os termos alimentação e nutrição não sejam sinônimos, representam ações intimamente relacionadas através das quais o homem pode manter seu estado saudável. A escolha, aceitação e consumo de alimentos são profundamente afetados por determinantes psicológicos e socioculturais, entretanto, é de conhecimento comum que a ingestão diária saudável e consciente de micro e macronutrientes desempenha um papel fundamental no bem-estar dos seres humanos (PICCOLELLA et al., 2019). Recentemente, uma onda de conscientização alimentar teve impactos econômicos notórios no Brasil, refletidos em números: a venda de produtos saudáveis aumentou 98% nos últimos seis anos, movimentando 35 bilhões de dólares ao ano no país (EXAME, 2018). De acordo com estudo realizado pela agência *Euromonitor International*, *The Top 10 Consumer Trends for 2017* (As 10 principais tendências de consumo para 2017), essa busca por alimentos naturais é uma tendência mundial.

Em um mercado alimentar globalizado, circula diariamente um grande fluxo de informações relacionando os mais diversos compostos a estilos de vida, dietas, alimentos e nutrientes, muitas vezes com informações equivocadas ou mesmo ilusórias. Os alimentos funcionais são aqueles com alegações de benefícios à saúde as quais devem ser cientificamente embasadas (BIANCO, 2010). Já um nutracêutico, de acordo com a primeira definição do *The foundation for Innovation in Medicine* e cujo fundador, Dr. Stephen De Felice, propôs o termo combinando nutrição com farmacêutica, é um alimento ou parte dele que fornece benefícios médicos ou de saúde, incluindo a prevenção ou tratamento de doenças (PICCOLELLA et al., 2019). Ou seja, um alimento funcional pode conter compostos nutracêuticos. Todavia, ambos os termos estão relacionados à diversidade e riqueza de compostos bioativos, substâncias naturais com potencial de modular um ou mais processos metabólicos, alimentando assim condições de saúde. A maior parte desses compostos são os chamados fitoquímicos, biossintetizados pelos vegetais. Estima-se que existam aproximadamente 400.000 espécies existentes de plantas vasculares na Terra, fonte de centenas de milhares de metabólitos cuja estrutura, função e utilidade foram apenas parcialmente exploradas (WANG et al., 2019). Muitas das drogas vendidas atualmente são modificações sintéticas simples das substâncias obtidas naturalmente (JAMWAL; BHATTACHARYA; PURI, 2018).

Dentre os fitoquímicos, destacam-se algumas proteoglicanas, as arabinogalactanas-proteínas e extensinas, as quais são moléculas do metabolismo primário que adicionalmente estão envolvidas em outros processos da planta como a sinalização celular e defesa; além dos metabólitos secundários, reconhecidos como sendo compostos produzidos para auxiliar na

sobrevivência da planta, mas que não apresentam um papel vital. Exemplos de metabólitos secundários são os compostos fenólicos, terpenos, alcaloides, glucosinolatos, entre outros (NGUEMA-ONA et al., 2014).

Nesse contexto, destaca-se a planta *Lepidium meyenii*, conhecida como maca peruana. É utilizada há mais de 2000 anos pelos nativos como alimento e por suas propriedades terapêuticas, descritas pela medicina popular. Nativa dos Andes Centrais, está adaptada a condições ambientais inóspitas como alta altitude e irradiação solar, frio intenso e terrenos rochosos, onde de acordo com GONZALES (2012) nenhuma outra planta para o sustento do homem poderia ser cultivada. Essa tolerância a condições ambientais extremas é atribuída à sua alta produção de metabólitos secundários (WANG et al., 2007).

Anunciada pela mídia como um superalimento, a popularidade mundial da maca explodiu na última década. De acordo com o site BBC News, o Brasil já é o segundo maior importador de produtos da maca, que em 2018 somaram US$ 862 mil (R$ 3,22 milhões) relativas a um volume de 280.420 kg (com alta de 62% sobre 2017) (MOURA, 2018).

Apesar do grande destaque que tem recebido devido aos promissores efeitos relacionados ao seu consumo, o conhecimento científico a respeito dos princípios ativos da maca ainda é escasso, sendo necessários mais estudos para confirmar os diversos efeitos biológicos relatados pelo conhecimento popular (BEHARRY; HEINRICH, 2018).

Visando contribuir com a ciência e com os conhecimentos sobre essa espécie, foram selecionadas nesse estudo duas classes de compostos bioativos: os glucosinolatos (GLs), os quais foram avaliados quanto seu potencial citotóxico em linhagens de células tumorais, baseando-se em relatos de atividade antitumoral exercida pela maca; e os polissacarídeos, analisados quanto ao seu potencial modulador sobre sistema complemento, relacionando com as atividades imunoestimulantes também atribuídas à essa planta

2 REVISÃO BIBLIOGRÁFICA

2.1 *Lepidium meyenii* Walpers (Maca peruana)

2.1.1 Informações botânicas

A espécie medicinal *Lepidium meyenii* Walpers, conhecida como maca peruana, é usada há séculos como suplemento alimentar e também por suas propriedades terapêuticas descritas pela medicina tradicional (GONZALES, 2012). Pertence à família Brassicaceae e é natural dos Andes Centrais, onde cresce a mais de 4000 m de altura, em uma zona caracterizada por áreas de esterilidade e terrenos rochosos, com alta variação de temperatura ao logo do dia, irradiação solar intensa e ventos fortes. Essa tolerância a condições ambientais extremas é atribuída à sua alta produção de metabólitos secundários (WANG et al., 2007).

A parte comestível da maca é a porção subterrânea (FIGURA 1), que corresponde ao órgão de reserva da planta, definido como uma zona de transição entre o hipocótilo e a raiz (GONZALES, 2012). Nesse trabalho, apenas o termo raiz foi adotado para se referir a esse órgão. Tradicionalmente, após a colheita, as raízes da maca são secas naturalmente, e assim podem ser estocadas por anos (VALERIO; GONZALES, 2005). A principal forma de consumo pela população dos Andes se dá através da fervura das raízes secas em água, mas também é popularmente utilizada na forma de farinha (OCHOA; UGENTE, 2001; GONZALES, 2010).

FIGURA 1 - IMAGENS DE *Lepidium meyenii* WALPERS

FONTE:
https://www.ingredientsnetwork.com/47/product/79/21/65/p792165img_XL.jpg
https://www.exotic-seeds.store/en/home/black-maca-organic-seeds-lepidium-meyenii-aphrodisiac.html
https://www.cultivariable.com/wp-content/uploads/2014/06/maca-plant.jpg planta
https://www.macaguru.com/how-to-grow-maca/
https://www.uol.com.br/vivabem/noticias/redacao/2018/10/18/maca-peruana-beneficios-e-como-consumir.htm
https://www.oxfordvitality.co.uk/maca-lepidium-meyenii-overview
NOTA: A- Raízes de maca nos Andes Peruanos, B - Variedade de cores, C - Colheita da maca, D - Planta, E - Farinha preparada a partir das raízes secas; F - Raízes secas.

2.1.2 Utilização na medicina tradicional e popularização da maca

Além de alimento, a maca é utilizada na medicina tradicional especialmente como afrodisíaco, indicada para a melhora do desejo sexual e da fertilidade em seres humanos e animais domésticos. É também comumente utilizada no tratamento de doenças respiratórias, para regular a secreção hormonal, estimular o metabolismo, reumatismo, problemas relacionados à memória, depressão, anemia, leucemia, AIDS, câncer, alcoolismo, entre outros (WANG et al., 2007).

Nas últimas duas décadas o interesse e demanda pela maca cresceu em todo o mundo. Devido a uma exacerbada campanha de marketing promovida por empresas através da mídia e internet, a planta ganhou destaque como 'superalimento" (Globo Repórter, 2013), símbolo de vitalidade e longevidade, e considerada como "Viagra" ou "*Ginseng* peruano" por suas propriedades afrodisíacas e imunomoduladoras (BEHARRY; HEINRICH, 2018). Dessa forma, a maca se estabeleceu como um dos principais produtos do Peru (SMITH, 2014; BRAND, 2016). Segundo GONZALES (2012), durante o ano de 2010 o Peru arrecadou mais de 6 milhões de dólares com a exportação da maca, um valor 4,36 vezes maior do que em 2001.

A maca é vendida em pó (farinha), pílulas, cápsulas, licor e extratos (GONZALES; GONZALES; GONZALES-CASTAÑEDA, 2009) e está disponível em grandes lojas de varejo, de alimentos saudáveis, produtos naturais e *smoothies* (SMITH, 2014).

Essa recente onda de popularidade levou a produção da maca para além dos Andes peruanos. Na província de Yunnan, na China, o plantio de maca foi implantado visando atender a alta demanda por produtos dessa planta no país, que possui 1,3 bilhões de habitantes e um grande potencial econômico. Entretanto, a mudança radical de localização e dos métodos tradicionais de cultivo para as práticas de produção em massa, incluindo o uso de fertilizantes e pesticidas, podem potencialmente afetar a composição fitoquímica da planta e, assim, a qualidade, segurança e eficácia dos produtos de maca (BEHARRY; HEINRICH, 2018).

Acompanhando essa popularização da maca, o interesse de pesquisadores a respeito dos constituintes químicos da planta também se intensificou nos últimos anos, influenciado não somente pela recente valorização econômica da espécie, mas também pela constante busca por novos bioativos naturais no meio científico (BEHARRY; HEINRICH, 2018).

2.1.3 Composição química

Em relação ao valor nutricional da maca, um estudo realizado por DINI e colaboradores (1994) demonstra que as raízes frescas dessa planta são constituídas de aproximadamente 80% de água e ricas em ferro e cálcio. O pó das raízes secas é composto de 10,2% de proteínas, 59% de carboidratos, 2,2% de lipídeos e 8,5% de fibras (TABELA 1). A maca apresenta um bom conteúdo de compostos insaturados (52,7% do total de ácidos graxos livres) e 18 tipos de aminoácidos, dos quais sete são considerados essenciais Visto que esse conteúdo de aminoácidos é maior do que o encontrado em batatas e cenouras, os autores sugerem a maca como uma importante fonte de alimento, devido ao seu alto valor nutricional.

TABELA 1 - COMPOSIÇÃO QUÍMICA E NUTRICIONAL DA MACA

Composição das raízes secas da *Lepidium meyenii*	
Umidade	10,4 %
Proteínas	10,2 %
Lipídeos	2,2 %
Carboidratos solúveis	59 %
Fibras	8,5 %
Cinzas	4,9 %

FONTE: DINI et al. (1994)

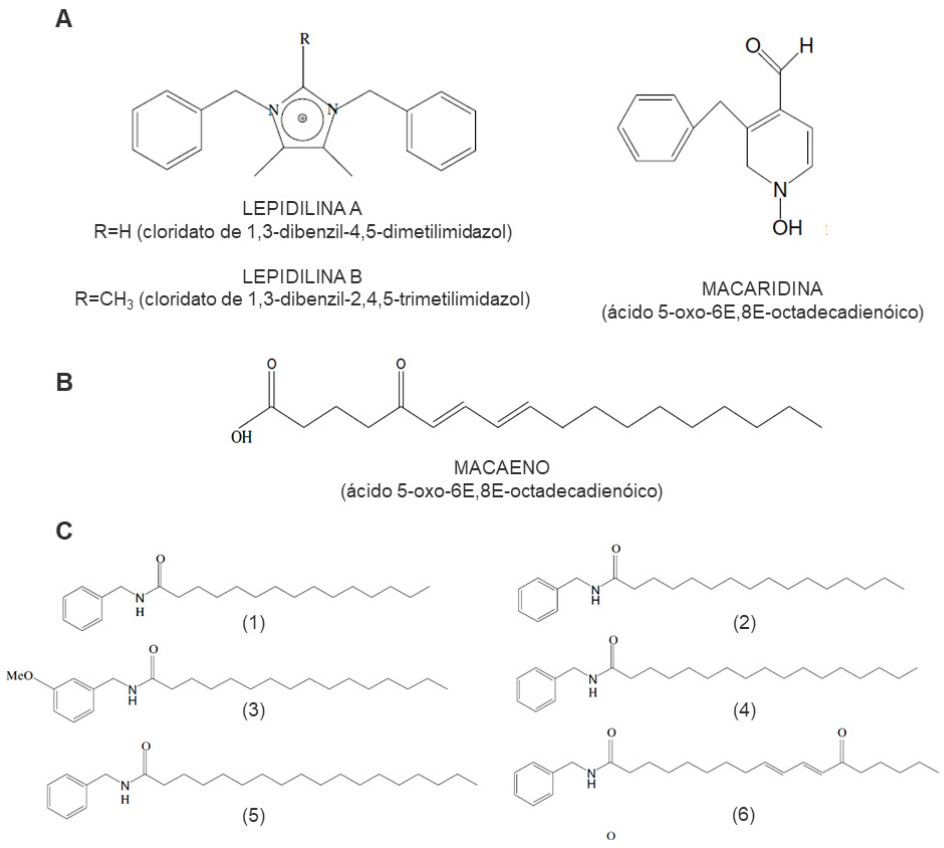
Dentre os compostos majoritários das raízes secas, os carboidratos (60-70%), correspondem em sua maior parte ao amido (23 a 30%) (RONDÁN-SANABRIA; FINARDI-FILHO, 2009), aos demais polissacarídeos (10%) (TANG et al., 2017) e a açúcares livres (23,4% de sacarose, 1,5% de glucose e 4,6% de oligossacarídeos) (VALENTOVÁ et al., 2006).

Em relação aos lipídeos, destaca-se um conjunto de ácidos graxos poli-insaturados e suas respectivas amidas, denominados macaenos e macamidas, os quais são encontrados exclusivamente na maca, além de serem os compostos mais estudados e considerados os marcadores químicos dessa planta (ZHANG; TANG; GONZALES, 2003; ZHENG et al., 2000).

Além desses metabólitos primários, diferentes classes de metabólitos secundários já foram identificadas nas raízes da maca, como os alcaloides lepidilina A, lepidilina B (CUI et al., 2003), macaridina (MUHAMMAD et al., 2002); além de diferentes tipos de glucosinolatos

(CAMPOS et al., 2013; YÁBAR et al., 2011) e polifenóis (BAI et al., 2015; SANDOVAL et al., 2002).

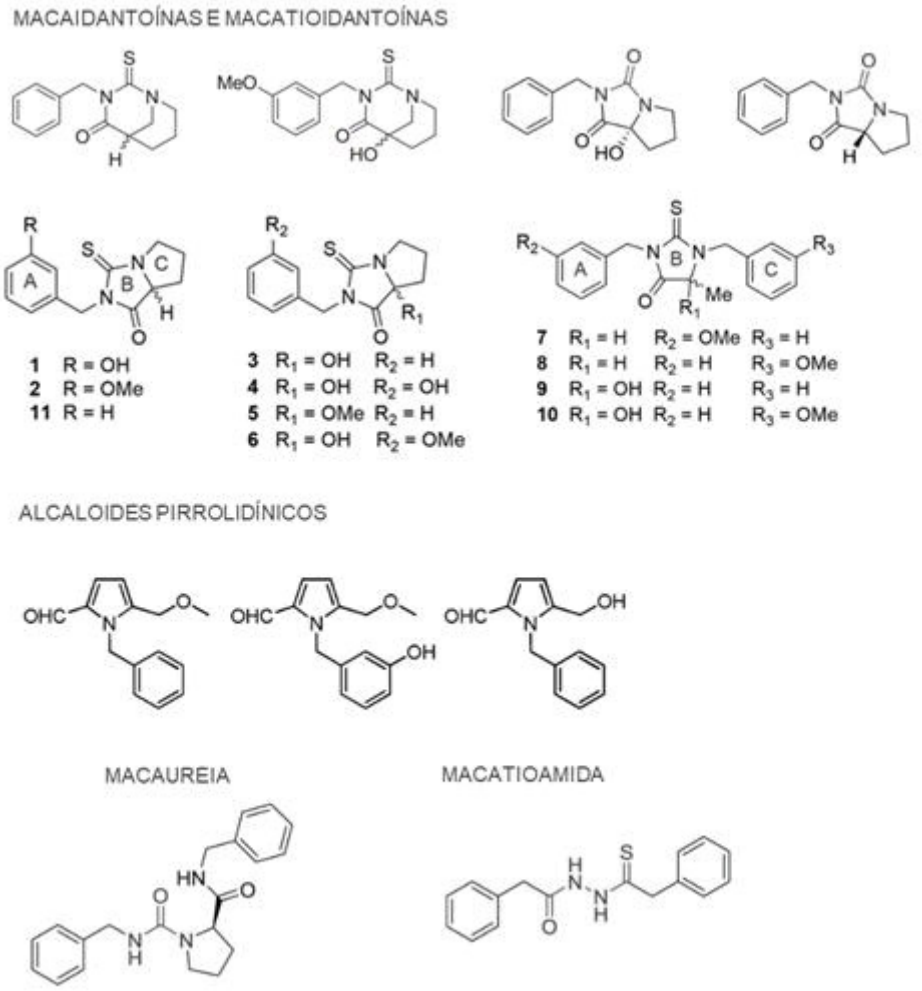
FIGURA 2 - ESTRUTURA QUÍMICA DE COMPOSTOS ENCONTRADOS NA MACA.



NOTA: (A) Alcaloides Lepidilina A, Lepidilina B e Macaridina, (B) Macaeno e (C) Algumas de suas amidas (macamidas); (1) n-benzilpentadecanamida; (2) N-benzilhexadecanamida; (3) N-(3-metoxibenzil) hexadecanamida, (4) N-benzilseptadecanamida, (5) N-benziloctadecanamida, (6) N-benzil-13-oxo-9E,11E-octadecadienamida.
FONTE: WANG et al. (2007).

Nos últimos anos, foram identificados e isolados novos compostos das raízes de maca cultivadas na China, contendo ureia ou tioureia em suas estruturas: as macaidantoínas (TIAN et al., 2018; YU et al., 2017ª, 2017b; ZHOU et al., 2018), as quais são hidantoínas, uma importante classe de alcaloides que contém um resíduo de ureia fundido ao anel cíclico (MARTON et al., 1993); além da macaureia e tiomacamida (TIAN et al., 2018). Foram também identificadas as macapirrolinas (ZHOU et al., 2018), pertencentes à classe de alcaloides pirrolidínicos (por apresentarem um anel pirrol) (TAIZ; ZEIGER, 2006). As estruturas desses compostos estão apresentadas na FIGURA 3.

FIGURA 3 ESTRUTURA QUÍMICA DE COMPOSTOS IDENTIFICADOS EM RAÍZES DE MACA



FONTE: YU et al. (2017); ZHOU et al. (2017), TIAN et al. (2018).

2.1.4 Estudos de farmacologia experimental e clínica

A TABELA 2 apresenta um resumo das atividades biológicas observadas através de ensaios experimentais e clínicos realizados com extratos da maca.

TABELA 2 - ESTUDOS DE INVETIGAÇÃO DE ATIVIDADE BIOLÓGICA UTILIZANDO RAÍZES E /OU EXTRATOS DE RAÍZES DE *Lepidium meyenii*

ATIVIDADE BIOLÓGICA RELATADA	MATERIAL UTILIZADO	ESTUDO/ MODELO EXPERIMENTAL	REFERÊNCIA
Aumento da fertilidade			
Aumento na produção diária de esperma	Extrato aquoso	*In vivo* / ratos (machos)	GONZALES et al. (2006)
Aumento na contagem de espematozóides	Extrato aquoso	*In vivo* / ratos (machos)	GASCO et al. (2007)
Aumento do volume seminal, contagem de esperma e da motilidade dos espermatozóides.	Raiz de maca pulverizada e (gelatinizada)	Clínico / homens adultos	GONZALES et al. (2001)
Aumento no número de gestações	Extrato apolar (penteno) e metanólico	*In vivo* / ratos (fêmeas)	PINO-FIGUEROA; MAHER, (2019)
Influência no comportamento sexual	Raízes secas pulverizadas (pastilhas)	Clínico / homens adultos	GONZALES et al., (2002)
Aumento da libido,	Fração lipídica obtida do extrato metanólico	*In vivo* / ratos e camundongos (machos)	(ZHENG et al., 2000)
melhora na performance e bem-estar sexual	Raízes secas pulverizadas (pastilhas)	Clínico / homens adultos	ZENICO et al. (2009)
Melhora dos sintomas da menopausa	Extrato hidroalcoólico	Clínico / mulheres adultas	MEISSNER et al., 2006)
Melhora dos sintomas da osteoporose	Extrato polar (não especificado)	Clínico / homens e mulheres adultos	MEHTA et al. 2007
Melhora da memória e aprendizado	Extrato aquoso	*In vivo* / camundongos (fêmeas) (ovariectomia)	RUBIO et al. (2011)
Efeito neuroprotetor	Extrato apolar (penteno)	*In vitro* / células neuronais OLGA-PH-J/92)	PINO-FIGUEROA et al. (2010)
		In vivo / ratos (machos)	
Efeito antidepressivo e ansiolítico	Extrato éter de petróleo	In vivo / modelo CUMS em ratos machos	AI et al. (2014)
	Extrato aquoso	*In vivo* / camundongos (ovariectomia)	RUBIO et al., (2006a)(Rubio, Caldas, Dávila, Gasco, & Gonzales, 2006)
Atividade antiproliferativa e antitumoral	Extrato aquoso	*In vivo* / ratos (machos) (redução da HBP induzida)	(Gasco et al., 2007; G. F. Gonzales, Gasco, Malheiros-Pereira, & Gonzales-Castañeda, 2008)GASCO et al. (2007); GONZALES et al. (2008)
Atividade antioxidante	Extrato aquoso	Métodos do DPPH e TRAP	SANDOVAL et al. (2002);
Atividade antiviral	Extrato metanólico	*In vitro* / células MDCK infectadas com vírus humano (Flu-A e Flu-B)	MENDOZA et al. (2014)

NOTA: CUMS, *chronic unpredictable mild stress*; DPPH, 2,2-difenil-1-picrilidrazila; TRAP, *Total radical – trapping antioxidante parameter* (potencial antioxidante total), HBP, Hiperplasia Benigna de Próstata; MDCK, *Madin Darby canine kidney* (linhagem celular normal de rim canino); OLGA-PH-J/92, células neuronais de *Orconectes limosus*
FONTE: O autor (2019).

Através desses estudos (TABELA 2), foi demonstrado que as raízes da maca e seus extratos exercem diferentes efeitos sobre os sistemas biológicos, como por exemplo, aumento

da fertilidade (RUBIO et al., 2006b); melhora do desempenho sexual (GONZALES et al., 2002), dos sintomas da menopausa (LEIVA-REVILLA et al., 2014) e da memória e aprendizado (RUBIO et al., 2011); efeito neuroprotetor (PINO-FIGUEROA; NGUYEN; MAHER, 2010), antidepressivo e ansiolítico (AI et al., 2014; RUBIO et al., 2006a); antiproliferativa e antitumoral (BAI et al., 2015; GASCO et al., 2007; GONZALES et al., 2008), antiosteoporose pós-menopausa (MEHTA et al., 2007), anti-inflamatória (BAI et al., 2015), antioxidante (SANDOVAL et al., 2002), antiviral (MENDONZA et al., 2014) entre outras. Além disso, os extratos de maca não apresentam toxicidade para animais (GASCO et al., 2007; TELLEZ et al., 2002), mas pelo contrário, exercem efeitos citoprotetores no fígado (VALENTOVÁ et al., 2006).

Os princípios ativos responsáveis por cada atividade apresentada pela maca ainda não foram bem estabelecidos (GONZALES, 2012), já que a maior parte desses estudos foram realizados utilizando os extratos da planta, que consistem em uma mistura de compostos.

Em relação aos estudos realizados com compostos isolados ou frações purificadas, CUI e colaboradores (2003) avaliaram o efeito citotóxico dos alcaloides imidazólicos lepidilina A e B, os quais reduziram a viabilidade celular das linhagens de carcinoma humano UM-UC-3 (bexiga), PaCa2 (pâncreas), MDA-MB-231 (mama) e FDIGROV (ovário) com valores de ED_{50} (metade da dose efetiva) de 6,47; 1,38; 1,66 e 5,26 μg/mL, respectivamente. Ambos foram inativos contra linhagens tumorais de pulmão (A549), cólon (HT29), próstata (PC-3) e rim (A4982LM). Adicionalmente, a lepidilina B e uma flavolignana extraída das raízes de maca, a tricina 4′-O-[treo-β-guaiacil-(7″-O-metil)-gliceril] éter, apresentaram citotoxicidade *in vitro* em células de leucemia promielocítica humana (HL60), mas não alteraram a viabilidade celular das linhagens humanas de hepatocarcinoma (HepG2) e adenocarcinoma colorretal (COLO 302). Ambos compostos também inibiram a produção de NO em macrófagos RAW 264.7 ativados com lipopolissacarídeo (LPS).

LIU e colaboradores (2015) verificaram que os esteroides de maca são responsáveis pela prevenção da osteoporose, e ZHENG e colaboradores (2000) sugeriram que a fração lipídica da maca, rica em macamidas, estaria relacionada com a melhora no desempenho sexual. Recentemente, XIA e colaboradores (2018) demonstraram que algumas macamidas isoladas das raízes de maca cultivada na China apresentaram efeitos citotóxicos em linhagem de células de adenocarcinoma de cólon humano (HT-29).

Entretanto, estudos de hiperplasia prostática, função testicular, espermatogênese, fertilidade, humor e memória (CHUNG et al., 2005; GONZALES et al., 2006; 2004; RUBIO et al., 2006b) foram realizados com os extratos aquosos da maca, que de acordo com

MCCOLLOM e colaboradores (2018) contêm apenas traços de macamidas, o que sugere que outros compostos seriam responsáveis por essas atividades. Em uma recente revisão sobre a maca (BEHARRY; HEINRICH, 2018), os autores destacaram que, apesar da crescente popularização da maca, os estudos realizados foram inconclusivos e, devido à falta de dados, as alegações de saúde atribuídas à essa planta não podem ser totalmente apoiadas do ponto de vista científico. Dessa forma, mais pesquisas são necessárias.

Nesse contexto, analisando os componentes presentes nas raízes da maca pouco investigados e com potencial bioativo, destacam-se duas classes de compostos: os glucosinolatos (GLs) e os polissacarídeos. Os GLs, segundo WANG; ZHU (2019), são os metabólitos secundários mais abundantes nos extratos da maca, seguido dos alcaloides. Esses compostos estão relacionados às atividades anticarcinogênicas e antitumorais apresentadas por outras plantas da mesma família da maca (Brassicacea) (FAHEY; ZALCMANN; TALALAY, 2001; ZHANG, 2001). Por sua vez, os polissacarídeos, macromoléculas pertencentes ao metabolismo primário e comumente presentes em extratos aquosos de plantas, também estão relacionados a diferentes atividades biológicas (FERREIRA et al., 2015). Os estudos acerca da estrutura química e potencial biológico dos polissacarídeos da maca foram iniciados apenas nos últimos anos (TANG et al., 2018; WANG et al., 2018; ZHA et al, 2014). Sendo o foco do presente trabalho, essas duas classes de compostos serão detalhadas a seguir.

2.2 GLUCOSINOLATOS

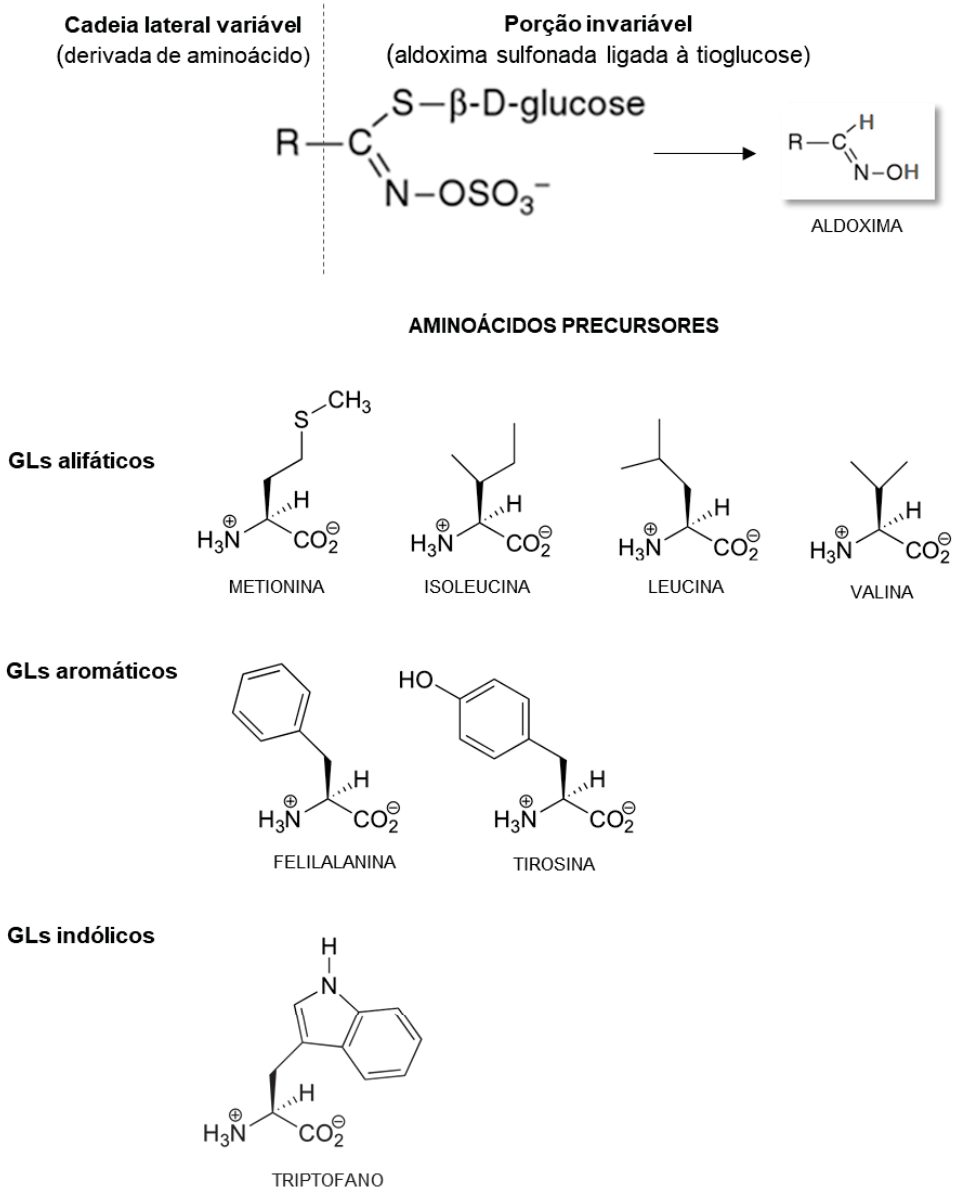
2.2.1 Características químicas e bioatividade

Os glucosinolatos (GLs) são uma classe de metabólitos secundários nitrogenados e sulfatados, que ocorrem principalmente nas plantas pertencentes à família Brassicaceae, mas também são encontrados em espécies de outras famílias, como as Resedaceae, Capparaceae, Moringaceae e Tropaeolaceae. As brássicas (ou crucíferas, como eram antigamente denominadas) compreendem uma grande variedade de hortaliças com grande importância econômica, pois são constituintes da alimentação humana e animal. Brócolis, mostarda, couve, repolho, rabanete e maca peruana são exemplos de crucíferas (REDOVNIKOVIĆ et al., 2008; SARVAN; VERKERK; DEKKER, 2012).

Os GLs são compostos estáveis e altamente solúveis em água. Quimicamente, são β-tioglicosídeos N-hidroxisulfatos e apresentam uma estrutura geral invariável, que compreende um átomo de carbono central de uma aldoxima sulfonada, unido a uma unidade de β-D-glucose

através de um átomo de enxofre (ligação tioéster) e à uma cadeia lateral variável, derivada de aminoácidos (FIGURA 4).

FIGURA 4 - ESTRUTURA QUÍMICA GERAL DOS GLUCOSINOLATOS.



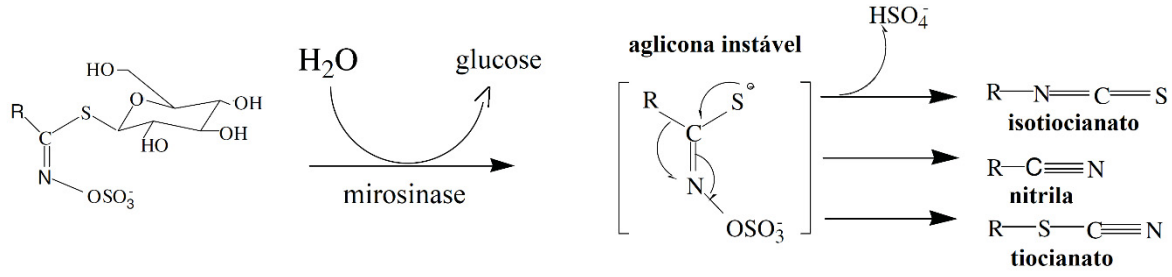
FONTE: O autor (2019)

Esses compostos são divididos em pelo menos três classes, dependendo do aminoácido precursor: glucosinolatos alifáticos, derivados da metionina, isoleucina, leucina ou valina; glucosinolatos aromáticos, derivados da fenilalanina ou tirosina; e glucosinolatos indólicos, derivados do triptofano (ROSA; GOMES, 2002). Geralmente ocorrem na forma sais de sódio ou potássio (WARTON; MATTHIESSEN; SHACKLETON, 2001), e mais de 200 diferentes

estruturas de GLs já foram identificadas em plantas (RAAB; FELDMANN, 2019). Usualmente, os glucosinolatos são conhecidos por nomes triviais, como é o caso do benzilglucosinolato, chamado de glucotropaeolina, e do 2-propenilglucosinolato, conhecido por sinigrina (FAHEY; ZALCMANN, TALALAY, 2001).

Os GLs ocorrem nas plantas, embora fisicamente separados, simultaneamente com a enzima mirosinase, responsável por sua hidrólise. Na Brassica *Arabidopsis thaliana*, células diferenciadas ricas em enxofre (células S), localizadas entre o floema e a endoderme, contêm concentrações extremamente altas de glucosinolatos. Em casos de danos físicos à planta, os tecidos lesionados liberam os GLs, que entram em contato com a mirosinase, localizada em células do parênquima adjacente ao floema (DINKOVA-KOSTOVA, KOSTOV, 2012). Após a clivagem hidrolítica da unidade de β-glucose, o grupamento sulfato é liberado (não enzimaticamente) para formar o tiohidroximato-*O*-sulfonado. Esse intermediário instável (aglucona) então se rearranja para formar isotiocianatos ou outros produtos (tiocianatos, nitrilas, epitionitrilas e oxazolidinas), que agem na defesa da planta como toxinas e repelentes contra herbívoros (THAIZ; ZEIGER, 2009). A natureza química desses produtos depende principalmente da estrutura da cadeia lateral variável, das espécies das plantas onde são encontrados e das condições da reação, como pH, temperatura, presença de íons metálicos e cofatores (BENNETT; MELLON; KROON, 2004). No caso da maca, são convertidos pincipalmente a isotiocianatos (ITCs), tiocianatos e nitrilas (FIGURA 5).

FIGURA 5 - HIDRÓLISE DOS GLUCOSINOLATOS PELA ENZIMA MIROSINASE E OS PRINCIPAIS PRODUTOS FORMADOS NA MACA.



FONTE: WANG et al. (2017)

Importantes componentes da dieta humana, os GLs são considerados por muitos estudiosos os princípios ativos de diversas plantas (FABRE et al., 2007). Os mamíferos não apresentam a enzima mirosinase em seus tecidos. Após a ingestão, os GLs podem ser

parcialmente absorvidos na sua forma intacta através da mucosa gastrointestinal, no entanto, a maior fração é metabolizada no lúmen intestinal. Quando as crucíferas são consumidas sem processamento, a enzima mirosinase presente nestas plantas hidrolisa os glucosinolatos na parte proximal do trato gastrointestinal. Se as crucíferas são cozidas antes do consumo, a enzima é inativada e os glucosinolatos transitam para o cólon, onde os GLs são hidrolisados pela microbiota intestinal, que apresentam a mirosinase. Os ITCs absorvidos são conjugados à glutationa no fígado e excretados na urina como ácido mercaptúrico (N-acetil-S-(N-alquiltiocarbamoil)-L-cisteína) (BARBA et al., 2016; DINKOVA-KOSTOVA; KOSTOV, 2012).

Diferentes trabalhos demonstram que o consumo diário de frutas e vegetais, principalmente crucíferas, reduz a incidência de diversos tipos de câncer (KRISTAL; LAMPE, 2002; VERHOEVEN et al., 1996) e que seus derivados apresentam atividades antiproliferativas e anticarcinogênicas (WANG et al., 2011b; ZHANG, 2001). Grande parte dos estudos se concentram nas atividades exercidas pelos ITCs, que atuam no processo carcinogênico através de diferentes mecanismos: bloqueio de danos ao DNA; indução da parada do ciclo celular; removendo células malignas e células pré-transformadas através da indução da apoptose; e indução de atividades anti-inflamatórias (FAHEY; ZALCMANN; TALALAY, 2001; MI, DI PASQUA; CHUNG, 2011).

A nível celular e molecular, as atividades biológicas derivam das interações diretas e indiretas entre os ITCs e os componentes celulares (WANG et al., 2011b). Portanto, a variação na cadeia lateral da estrutura química que caracteriza cada tipo de GL reflete diretamente na forma e grau de atuação exercida por cada uma dessas moléculas no processo anticarcinogênico. Por exemplo, em um estudo realizado por MI e colaboradores (2007) visando compreender os mecanismos moleculares envolvidos na indução da apoptose exercida por dois diferentes ITCs: o sulforafano (SFN), derivado da glucorafanina (comumente encontrada no brócolis), e o fenetil isotiocianato (PEITC), derivado da gluconasturtina (encontrada no agrião, repolho e acelga), foi observado que, embora o PEITC seja um indutor mais potente da apoptose, é menos eficaz na geração de espécies reativas de oxigênio e danos às células da linhagem de adenocarcinoma de pulmão (A549) quando comparado ao SFN. Buscando relacionar a atividade biológica com as características químicas dos ITCs, LEONI e colaboradores (1997) avaliaram a citotoxicidade de diferentes tipos de GLs sobre a linhagem celular humana de leucemia mieloide crônica (K562) na presença de mirosinase, e os resultados obtidos foram relacionados à lipofilicidade dos derivados ITCs. Quanto mais lipofílico, maior o efeito citotóxico observado. A lipofilicidade dos ITCs está relacionada à capacidade de atravessar a membrana celular e se

ligar às proteínas intracelulares através de interações hidrofóbicas (WANG et al., 2011b; ZHANG, 2001).

Em relação à seletividade, um estudo realizado por WANG e colaboradores (2011b) demonstrou que o benzilisotiocianato (BITC) é capaz de deletar seletivamente a proteína p53 mutante em células cancerígenas.

Portanto, por serem micronutrientes da dieta capazes de modular a atividade das enzimas metabolizadoras envolvidas em importantes processos como biotransformação de drogas e carcinogênese, os GLs e seus derivados são importantes alvo de estudos há pelo menos 30 anos (PSURSKI et al., 2019).

2.2.2 Purificação e identificação dos glucosinolatos

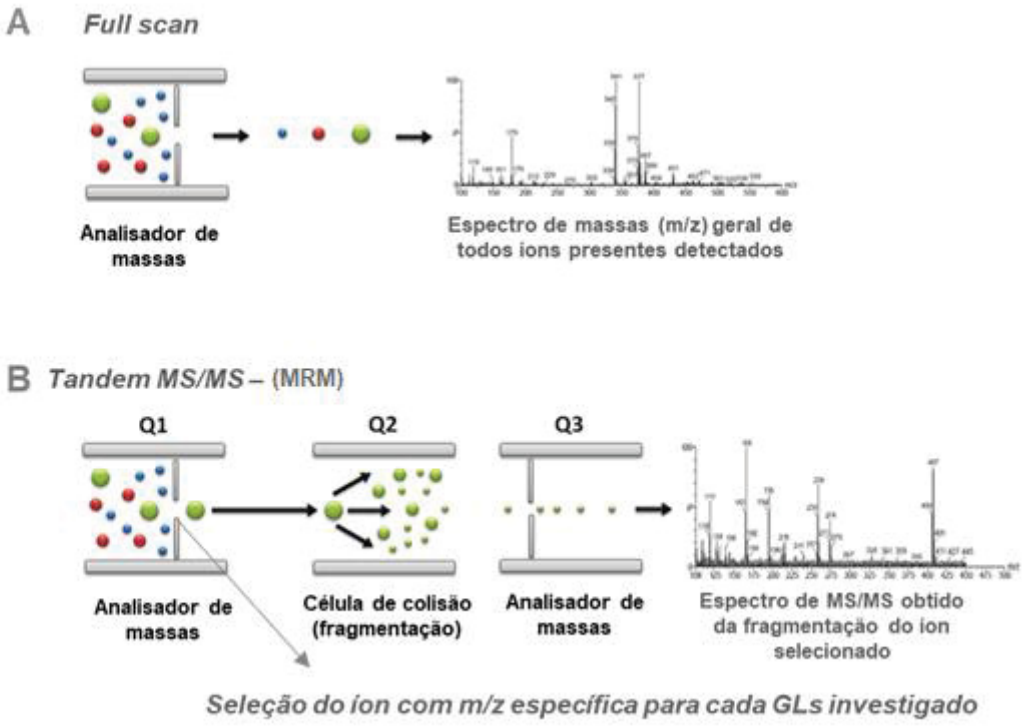
Os primeiros estudos realizados com glucosinolatos datam do século XIX e desde então, muito esforço foi feito para uma eficiente purificação, separação e identificação desses compostos. O grupamento sulfato carregado negativamente dificultava as análises de identificação e separação dos GLs e portanto, não era possível detectá-los em sua forma intacta, apenas após a remoção do grupamento sulfato com o auxílio de sulfatases (dessulfoglucosinolatos). Dessa forma, a atividade biológica desses compostos modificados era comprometida (FAHEY et al., 2001).

Com o passar do tempo, as análises dos glucosinolatos foi aperfeiçoada com o emprego de compostos iônicos carregados positivamente para neutralizar a carga negativa do grupamento sulfato através da cromatografia de pareamento iônico (*paired-ion chromatography*, PIC) (CLARKE, 2010). A PIC é uma estratégia de cromatografia líquida em fase reversa (*reverse phase liquid chromatography*, RPLC) para separação de íons e analitos orgânicos parcialmente ionizados, sem prévio tratamento ou derivatização da amostra. As fases estacionárias e móveis utilizadas são as mesmas que na RPLC, porém a principal característica do PIC é que um reagente de par iônico é adicionado à fase móvel, geralmente um sulfonato ou um sal quaternário de amônio, para alterar o tempo de retenção dos analitos iônicos (STÅHLBERG, 2000). Apesar de permitir a recuperação de uma grande quantidade de glucosinolatos intactos em forma de sal, o excesso do contra-íon hidrofóbico utilizado na eluição dos GLs costuma ser tóxico nos ensaios biológicos, além de inibir a enzima mirosinase.

A detecção de GLs através da espectrometria de massas já foi descrita para a maioria das técnicas de ionização e tipos de analisadores atualmente disponíveis, como por exemplo a

ionização por electrospray (*electrospray ionization,* ESI), bombardeamento por átomos rápidos (*fast atom bombardment,* FAB), ionização e dessorção a laser assistida por matriz e analisador por tempo de vôo (*matrix-assisted laser desorption/ionization - time of flight,* MALDI-TOF), ionização química a pressão atmosférica (*atmospheric pressure chemical ionization*, APCI), utilizando instrumentos de cromatografia líquida (LC) acopladas à espectrometria de massas (LC-MS/MS) do tipo quadrupolo em tandem ou íon-trap. Os padrões de fragmentação adquiridos pela LC-MS/MS e Q-TOF são similares, mas diferem dos dados obtidos através de ion-trap. Uma abordagem muito utilizada para o rastreamento de GLs em extratos de plantas é baseada em espectrometria de massa em equipamentos do tipo triplo quadrupolo por monitoramento de reações múltiplas (MRM) (CLARKE, 2010) (FIGURA 6).

FIGURA 6 - ESQUEMA DA ANÁLISE DOS GLUCOSINOLATOS ATRAVÉS DA ESPECTROMETRIA DE MASSAS



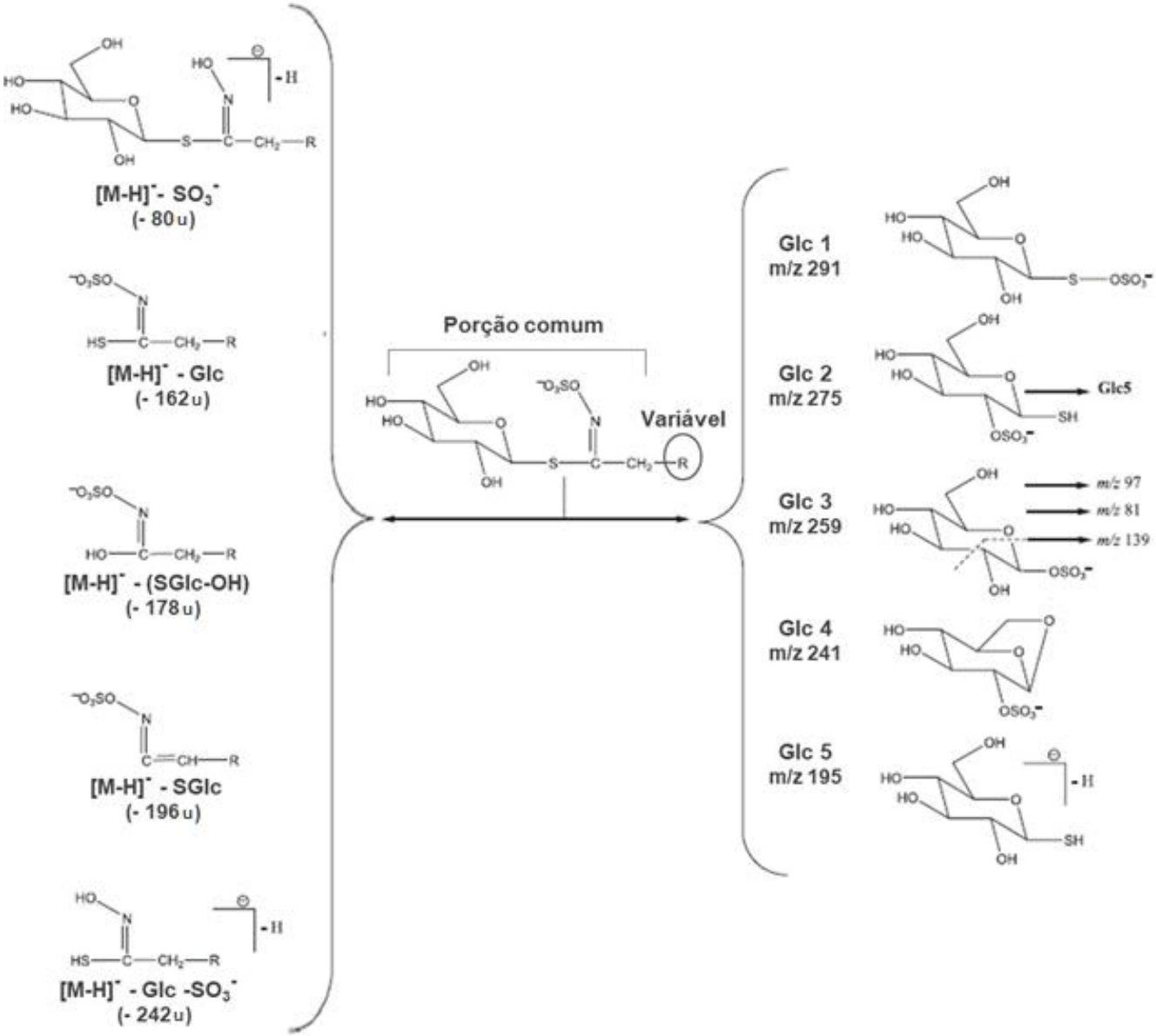
FONTE: Adaptado de DOMON; AEBERSOLD (2006).
NOTA: A - Modo *full scan*. B – Analisador de massas em tandem (MS/MS) – Triplo quadrupolo - Monitoramento de reações múltiplas (MRM). Neste tipo de varredura é monitorada a fragmentação de vários íons precursores, selecionados no triplo quadrupolo Q_1, aos seus correspondentes fragmentos iônicos que atravessam Q_3.

Como todos os glucosinolatos apresentam uma porção estrutural em comum, os fragmentos iônicos gerados dessa parte da molécula são utilizados para indicar a presença dessa classe de compostos em uma determinada amostra. É o caso dos fragmentos iônicos correspondentes às *m/z* 291, 275, 259, 241 e 195, considerados íons diagnósticos e altamente

específicos para GLs. São produtos da unidade de glucose presente em todos os GLs, e derivam da clivagem em ambos os lados da ligação tio éster, como mostrado na FIGURA 7 (lado direito). Os íons de GLs também apresentam em comum perdas neutras de 80 u (SO_3) e íons com *m/z* de 97 [HSO_4^-] e 98 [HSO_3^-] gerados após a clivagem do grupamento sulfato.

Nem todos os fragmentos iônicos da tioglucose estão presentes simultaneamente nos espectros de MS/MS dos GLs, entretanto, o íon de *m/z* 259 geralmente aparece e sua abundância depende da estrutura da cadeia lateral variável do GL. De acordo com FABRE e colaboradores (2007), a presença do íon de *m/z* 97 e um ou mais fragmentos de Glc já é indicativo da ocorrência de GLs em um extrato bruto de planta, por exemplo.

FIGURA 7 - ÍONS DIAGNÓSTICOS COMUNS PROPOSTOS PARA GLUCOSINOLATOS NO ESI-MS/MS MASSAS (MODO NEGATIVO).



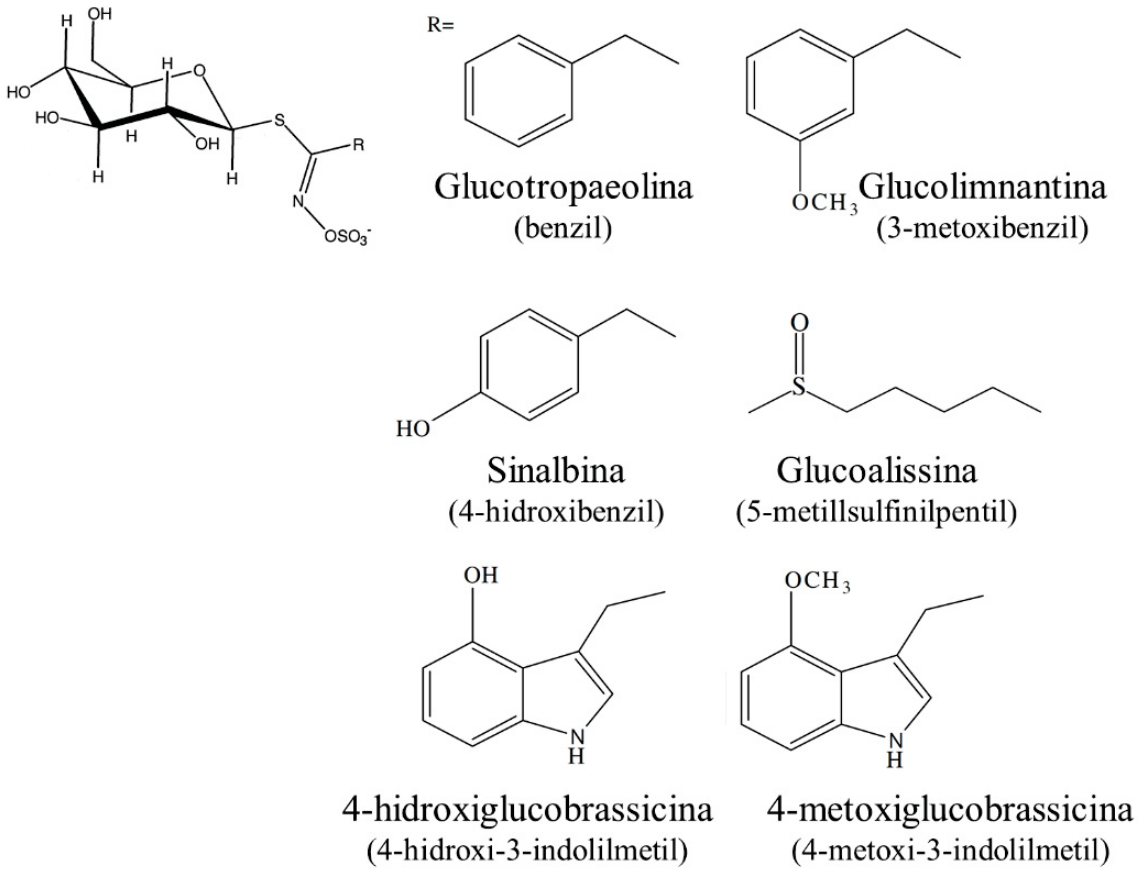
FONTE: FABRE et al (2007).

Além dos íons e perdas neutras relativas à porção comum da estrutura dos GLs, são também gerados íons a partir da cadeia lateral variável, utilizados como diagnósticos para a identificação dos glucosinolatos. Assim como os fragmentos de açúcar, esses produtos são derivados da quebra de ambos os lados do átomo de enxofre presente na molécula dos GLs, e suas estruturas também estão apresentadas na FIGURA 7 (lado esquerdo). Para a glucotropaeolina por exemplo, a perda da glucose (-162 u) vai gerar um tipo de íon [M-H$^-$]- Glc diferente do que seria gerado pela sinigrina, diferindo um do outro apenas na estrutura da cadeia lateral.

2.2.3 Glucosinolatos da maca

Seis diferentes tipos de glucosinolatos já foram identificados na maca, sendo a maioria do tipo aromático (DINI; TENORE; DINI, 2002; JOHNS, 1981; PIACENTE et al., 2002; YÁBAR et al., 2011) (FIGURA 8).

FIGURA 8 - ESTRUTURAS DOS GLUCOSINOLATOS ENCONTRADOS NA MACA



FONTE: Adaptado de WANG et al. (2007)

Cerca de 70-80% do total de GLs correspondem à glucotropaeolina e aproximadamente 20%, à glucolimnantina (CLÉMENT et al., 2010; YÁBAR et al., 2011). A quantidade de GLs presente na maca fresca é aproximadamente 100 vezes maior do que o encontrado em outras crucíferas, como repolho, couve-flor e brócolis (LI; AMMERMANN; QUIRÓS, 2001). Com exceção dos estudos realizados por DINI; TENORE; DINI (2002) e PIACENTE e colaboradores (2002), os glucosinolatos da maca foram identificados na sua forma dessulfatada, ou seja, como dessulfoglucosinolatos.

Como citado anteriormente, não há dados suficientes na literatura que comprovem as atividades exercidas pelos GLs encontrados na maca. Em alguns estudos *in vivo*, GASCO e colaboradores (2007) e GONZALES e colaboradores (2008) verificaram que extratos aquosos e hidroalcoólicos da maca apresentaram capacidade na redução da hiperplasia benigna de próstata induzida em ratos com enantato de testosterona, e que os efeitos estariam relacionados de forma dose-dependente com seu conteúdo de benzilglucosinolatos (glucotropaeolina). Entretanto, em testes *in vitro,* os extratos aquosos da maca não apresentaram efeitos citotóxicos para a linhagem de adenocarcinoma de próstata humana (LNCap) (DÍAZ; CARDENAS; ORIHUELA, 2016). Nesse estudo, os autores sugeriram que a ausência de citotoxicidade observada poderia ser resultado da presença de outros compostos nos extratos (como polifenóis, taninos, saponinas, alcaloides, esteroides ou glicosídeos) os quais estariam inibindo a ação dos glucosinolatos. Entretanto, considerando que as linhagens celulares humanas não apresentam a enzima mirosinase, a conversão dos GLs a seus derivados bioativos não ocorre nas condições do estudo *in vitro.*

2.2.4 Glucosinolatos da mostarda

As mostardas são plantas também pertencentes à família Brassicacea, cujas sementes são amplamente consumidas no mundo como condimento (MARTON; LAVRIC, 2013). *Sinapis alba, Brassica hirta* e *Brassica alba* são sinônimos utilizados para se referir à mostarda branca/amarela, enquanto *Brassica nigra* corresponde à mostarda-negra, e *Brassica juncea* à mostarda-castanha (CLARKE, 2010). Apresentam um alto teor de GLs, cujo perfil nas sementes varia de acordo com a espécie (COOLS; TERRY, 2018; POPOVA; MORRA, 2014). Na *B. juncea*, o principal GL encontrado é a sinigrina, enquanto na *S. alba* é a sinalbina (COOLS; TERRY, 2018).

Uma fração rica em GLs da mostarda foi utilizada nos bioensaios de citotoxicidade celular na ausência e presença de mirosinase (enzima comercial de *Sinapsis alba*; Sigma-

Aldrich, St. Louis, MO, EUA) para fins de comparação com as frações de maca. A extração foi realizada utilizando sementes de mostarda amarela (*Sinapsis* sp.) obtidas no comércio, e a fração obtida através da metodologia descrita por LEE e colaboradores (2006) foi denominada SaF (Fração *Sinapsis* sp.) e quimicamente caracterizada como as demais frações da maca.

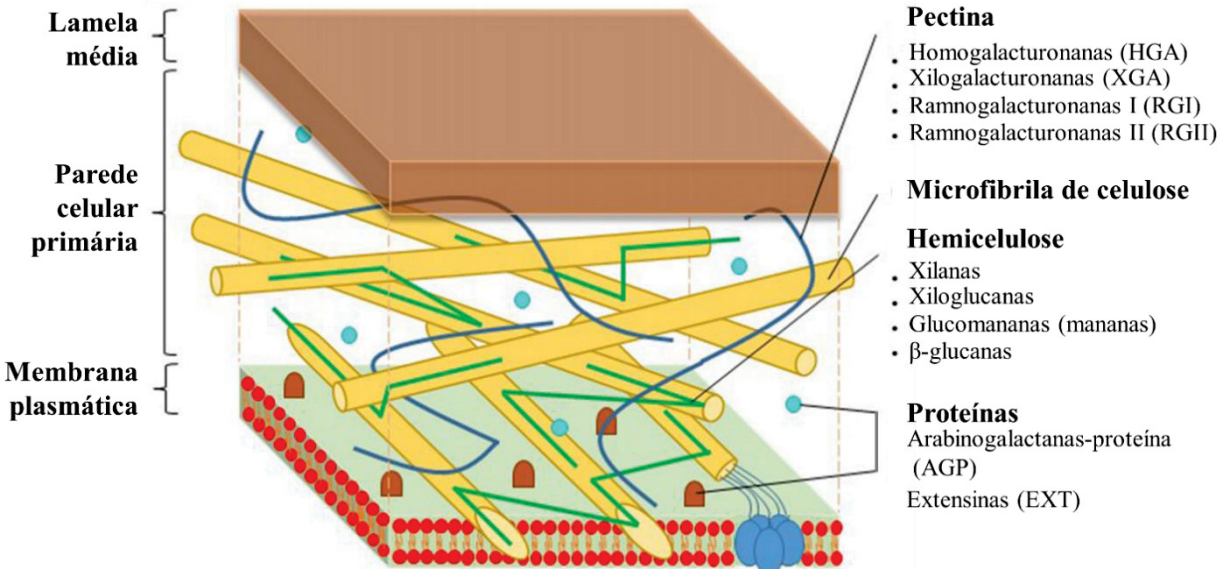
2.3 POLISSACARÍDEOS

A segunda classe de compostos de interesse desse estudo são os polissacarídeos da maca. Os polissacarídeos são polímeros de alta massa molecular e as biomoléculas mais abundantes do planeta. Nas plantas, alguns podem apresentar função de reserva, como por exemplo o amido (principalmente em raízes e sementes), ou estarem associados a uma complexa rede de macromoléculas, a parede celular vegetal, desempenhando funções estruturais de suporte à membrana celular, detectando informações extracelulares e mediando diferentes processos de sinalização (NELSON; COX, 2013; OCHOA-VILLARREAL et al., 2012).

Na maioria dos tipos de células, a parede celular vegetal consiste de três redes de polímeros estruturalmente independentes, mas interligadas (FIGURA 9). As microfibrilas de celulose e hemicelulose são os constituintes da rede primária de polissacarídeos, incorporada em uma segunda rede, uma matriz semelhante a um gel formado por pectinas e outros polissacarídeos. A terceira rede consiste em proteínas estruturais reticuladas e glicoconjugados (como por exemplo, proteoglicanos) ricos em hidroxiprolina (Hyp) (NGUEMA-ONA et al., 2014; PETTOLINO et al. 2012). Dentre essas diferentes classes de polissacarídeos, apenas a celulose é bem definida, consistindo em cadeias de glucanas com ligações do tipo β-(1→4). As pectinas são polissacarídeos altamente heterogêneos, tradicionalmente caracterizados por serem facilmente extraídos com ácido quente ou quelantes e por conter uma grande quantidade de resíduos de ácido galacturônico (Gal*p*A). As hemiceluloses compreendem tradicionalmente os polissacarídeos remanescentes, que podem ser extraídos com tratamento alcalino (FIGURA 9) (OCHOA-VILLARREAL et al., 2012).

.

FIGURA 9 - ESTRUTURA E COMPOSIÇÃO DA PAREDE CELULAR VEGETAL



FONTE: LOIX et al. (2017).
Esquema simplificado da parede celular vegetal. A lamela média forma a interface entre as paredes primárias de células vizinhas. A parede celular primária está localizada externamente à membrana plasmática. Por motivos didáticos, nem todas as interações entre os constituintes da parede celular vegetal estão representadas. Na figura estão indicados exemplos de polissacarídeos pertencentes às diferentes classes (celulose, hemicelulose, pectinas e proteoglicanos).

2.3.1 Polissacarídeos da maca

O primeiro estudo publicado sobre os polissacarídeos da maca foi realizado por ZHA e colaboradores (2014). Os autores analisaram quatro frações contendo os polissacarídeos solúveis dos extratos aquosos das raízes da maca, e verificaram sua capacidade antioxidante *in vitro* de neutralização de radicais livres. Os estudos posteriores foram em sua maioria realizados com a maca cultivada na China. De forma geral, os polissacarídeos da maca são heteropolissacarídeos com pesos moleculares variando de 6,7 a 2213 kDa. constituídos principalmente por arabinose (Ara), glucose (Glc), galactose (Gal) e manose (Man), em diferentes proporções molares. Ensaios *in vivo* e *in vitro* demostraram que essas frações polissacarídicas apresentam atividades antioxidantes, imunoestimulantes, antifadiga e hepatoprotetoras. A TABELA 3 apresenta um resumo dos estudos de caracterização química e ensaios biológicos envolvendo os polissacarídeos extraídos das raízes de maca.

TABELA 3 - COMPOSIÇÃO QUÍMICA E ATIVIDADES BIOLÓGICAS DE FRAÇÕES DE POLISSACARÍDEOS EXTRAÍDAS DE RAÍZES DA MACA

FRAÇÃO	MM (kDa)	% CHO[a]	% AU[b]	COMPOSIÇÃO MONOSSACARÍDICA[c]							ATIVIDADE BIOLÓGICA RELATADA/ MODELO	REFERÊNCIA
				Rha	Ara	Xyl	Man	Gal	Glc	GalA		
LMP-60-90	ND	39-69	ND	+	+	-	-	+	+	-	Antioxidante (*in vitro*) / sequestro dos radicais hidroxila e superóxido/ método do DPPH	ZHA et al. (2014)
MC-1	11.3	97	ND	-	26	-	12	54	8	-	Imunoestimulante (*in vitro*) / RAW 264.7	ZHANG et al. (2016)
MP-21	368	90	39.1	1	5	-	-	5	-	-	Imunoestimulante/ Hepatoprotetora (*in vitro*) / RAW 264.7 e HepG2	WANG et al. (2016)
MPS-1	7.6	93	1.2	-	2	1	-	3	31	-	Antifadiga (*in vivo*) / camundongos (machos)	LI et al. (2017)
MPS-2	6.7	91	26.9	-	1	-	-	1	37	-	Antifadiga (*in vivo*) / camundongos (machos)	LI et al. (2017)
MP	793.5	99.2	+	1	17	-	13	4	30	35	Antifadiga (*in vivo*) / camundongos (machos e fêmeas)	TANG et al. (2017)
MC-2	9.83	ND	ND	-	21	-	4	72	3	-	Imunoestimulante (*in vitro*) / RAW 264.7	ZHANG et al. (2017b)
MP-1	1067.3	92	ND	+	+	+	-	+	+	+	Antioxidante (*in vitro*) / sequestro dos radicais hidroxila e superóxido/ método do DPPH e FRAP Hepatoprotetora (*in vitro*) /HepG2 (*in vivo*) camundongos (fêmeas)	ZHANG et al. (2017a)
MCP	ND	61	ND	2	-	-	-	1	10	-	Antifadiga (*in vivo*) / camundongos (machos)	LI et al. (2018)
MP-1’	467	92	ND	-	2	-	-	1	-	-	Antioxidante (in vitro) / RAW 264.7 - sequestro dos radicais hidroxila e superóxido/ método do DPPH e FRAP, quelação de Fe^{2+}, inibição da peroxidação lipídica e poder de redução. Hepatoprotera (*in vitro*) / HepG2	WANG et al. (2018)
M-PL	2213	90	-	-	2	-	1	3	3	-	Antifadiga (ratos)	TANG et al. (2018)

MM, massa molecular; CHO, carboidratos totais; AU, ácidos urônicos; Rha, ramnose; Ara, arabinose; Xyl, xilose; Man, manose; Gal, galactose; Glc, glucose; GalA, ácido galacturônico; nd, não determinado; +, presente (não quantificado); -, ausente; RAW264.7, linhagem de macrófagos de camundongo; HepG2, linhagem humana de hepatocarcinoma.

a Determinado pelo método do fenol-sulfúrico

b Determinado pelo método do m-hidroxibifenil sulfúrico.

c Determinados por GLC (MC-1, MC-2) GC-MS (MP, MP-21, MCP, MP-1’), HPLC (LMP-60-90) e HPLC-Dionex (MPS-1, MPS-2, MP-1).

FONTE: O autor (2019).

Apesar da semelhança na composição monossacarídica, análises de ressonância magnética nuclear (RMN) das frações MC-1, MC-2, MP e MP-1 (TABELA 4) mostram uma variedade de ligações, com diferentes configurações.

TABELA 4 - PRINCIPAIS TIPOS DE LIGAÇÃO DOS POLISSSACARÍDEOS DE RAÍZES DE MACA DETERMINADAS POR ESPECTROSCOPIA DE RESSONÂNCIA MAGNÉTICA NUCLEAR

FRAÇÃO	RESÍDUO / LIGAÇÃO	REFERÊNCIA
MC-1	α-L-Ara*f*-(1→5), α-L-Man*p*-(1→3), α-L-Man*p*-(1→2,6), α-D-Glc*p*-(1→), α-D-Glc*p*-(1→4), α-D-Glc*p*-(1→6) e β-D-Gal*p*-(1→6)	ZHANG et al. (2016)
MC-2	α-L-Ara*f*-(1→5), α-L-Man*p*-(1→3), α-D-Glc*p*-(1→), α-D-Glc*p*-(1→4), α-D-Glc*p*-(1→6) e β-D-Gal*p*-(1→6)	ZHANG et al. (2017b)
MP	β-D-1,3-Gal*p*A-(1→3), β-D-Glc*p*-(1→3), e α-D-Man*p*-(1→3) - (5:4:1) - ligadas alternadamente na cadeia central	TANG et al. (2017)
MP1	α-D-Ara*f*-(1→3), α-D-Ara*f*-(1→5), a α-D-Ara*f*-(1→), α-D-Ara*f*-(1→2,3,5), β-D-Gal*p*-(1→4)	WANG et al. (2018)
MP-L	α-D-Gal*p*-(1→4), β-D-Gal*p*-(1→4), β-D-Glc*p*-(1→6), α-D-Gal*p*-(1→3), α-D-Man*p*-(1→3,6), α-D-Glc*p*-(1→), β-D-Ara*f*-(1→5)	TANG et al. (2018)

Ara*f*, arabinose furanosídica; Man*p*, manose piranosídica; Gal*p*, galactose piranosídica; Glc*p*, glucose piranosídica; Gal*p*A, ácido galacturônico piranosídico.
FONTE: O autor (2019).

Além dos polissacarídeos das raízes, um estudo realizado por LI e colaboradores (2017b) avaliou uma fração polissacarídica extraída das folhas da maca. Análises de composição monossacarídica indicaram a presença principalmente de Gal, Ara, e menores proporções de Rha, Glc e Man (5,51:4,05:1,15:0,77:0,11).

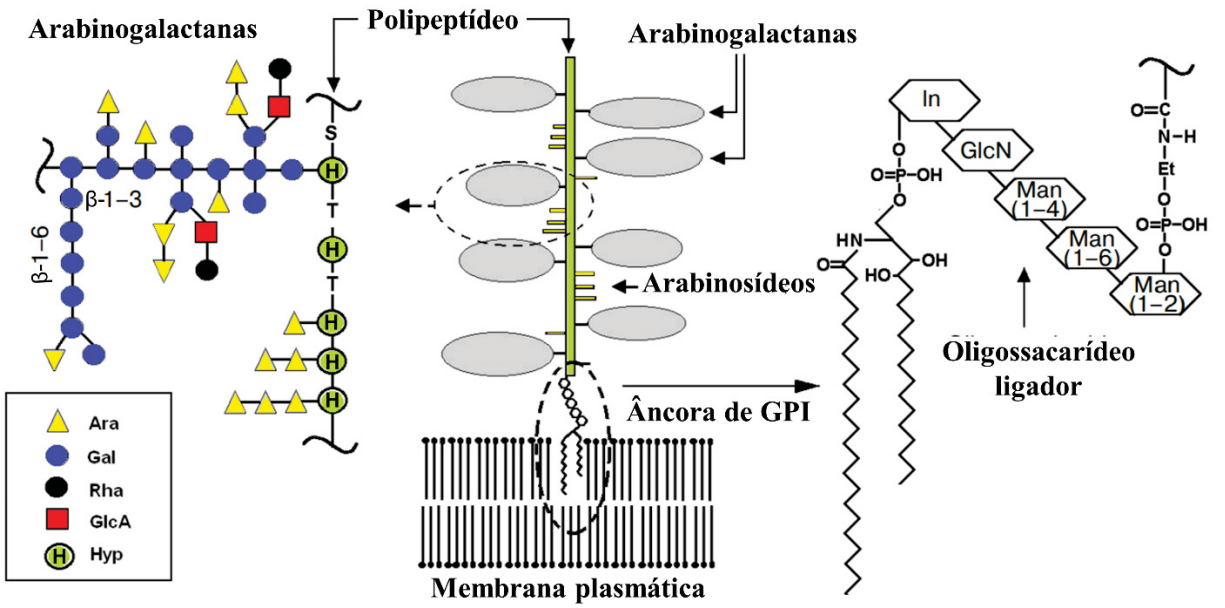
Em um trabalho realizado em nosso grupo de pesquisa por PAREDES (2009), frações polissacarídicas das raízes da maca foram obtidas e caracterizadas. A composição monossacarídica das frações aquosas apresentou altas proporções de glucose, arabinose e ácidos urônicos, além de menores proporções de Gal, Man, Xyl e traços de Rha e Fuc, indicando a presença de glucanas e outros polissacarídeos ácidos. Já as frações alcalinas (obtidas com NaOH 2 M, correspondentes a hemicelulose B), apresentaram altos percentuais de Ara, Gal, Xyl e Glc sugerindo a possível presença de xiloglucanas, arabinanas, galactanas e arabinogalactanas. Essa fração foi fracionada por cromatografia de troca iônica resultando em uma fração neutra e livre de proteína, e uma segunda fração ácida, heterogênea e possivelmente ligada a proteína, com alto conteúdo de Ara e Gal.

A presença de Ara e Gal observada nos polissacarídeos solúveis em água nos estudos citados acima sugerem a presença de arabinogalactanas-proteínas (AGPs) nas raízes de maca.

2.3.2 Arabinogalactana-proteínas

As AGPs são proteoglicanas altamente solúveis em água, encontradas na parede celular de plantas. Consistem em um núcleo proteico de comprimento altamente variável e domínios complexos, ricos em hydroxiprolina (Hyp), uma ou mais cadeias laterais de arabinogalactanas (AG) e frequentemente contém uma âncora lipídica de glicosilfosfatidilinositol (GPI – *glycosylphosphatidylinositol*) (RUMYANTSEVA, 2005). Um esquema da estrutura de uma molécula de AGP está representado na FIGURA 10.

FIGURA 10 - MODELO ESTRUTURAL HIPOTÉTICO DE UMA ARABINOGALACTANA-PROTEÍNA



FONTE: Adaptado de RUMYANTSEVA, (2005).
NOTA: Ara, arabinose; Gal, galactose; Rha, ramnose; GlcA, ácido glucurônico; Man, manose; GlcN, Glucosamina; Hyp, hidroxiprolina; T, treonina, S, serina, GPI, glicosilfosfatidilinositol; Et, etanolamina. Unidades de polissacarídeos (arabinogalactanas tipo II) e arabinooligossacarídeos (arabinosídeos) estão ligadas através do oxigênio da hidroxiprolina ao centro protéico da AGP. A porção carboxi-terminal do polipetídeo de AGP é modificada pela ligação de uma âncora de GPI, consistindo de etanolamina, um oligossacarídeo ligador, e um lipídeo inserido na membrana plasmática.

As AGPs são proteínas *O*-glicosiladas, ou seja, os polissacarídeos e as unidades de oligossacarídeos estão ligados à proteína através do oxigênio da hidroxiprolina. O núcleo

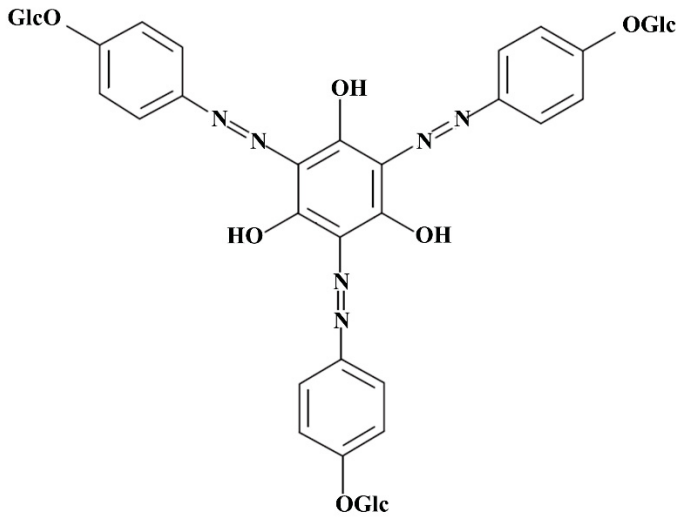
central proteico contribui com 1 a 10% da massa molecular da AGP, que geralmente varia entre 60 a 300 kDa (CLASSEN; BAUMANN; UTERMOEHLEN, 2019; RUMYANTSEVA, 2005).

As cadeias laterais de carboidratos são classificadas como arabinogalactanas do tipo II. Normalmente, consistem em uma cadeia principal contendo unidades β-1,3-D-Gal*p*, ligada ao núcleo proteico, com cadeias laterais formadas por unidades de β-1,6-D-Gal*p,* geralmente substituídas por resíduos de L-Ara*f*. Podem apresentar outros monossacarídeos menos abundantes, como fucose, ramnose, e ácido 4-*O*-metil glucurônico nos terminais não redutores (KNOCH; DILOKPIMOL; GESHI, 2014).

As AGPs estão envolvidas em diversos processos fisiológicos da planta, incluindo crescimento, desenvolvimento, proliferação celular e reprodução. As funções diferem dependendo da espécie, órgão e estágio de desenvolvimento (CLASSEN; BAUMANN; UTERMOEHLEN, 2019). As AGPs ancoradas nas membranas podem ser liberadas na matriz extracelular e atuar como sinalizadores, auxiliando a comunicação com as células vizinhas (SCHULTZ et al., 1998). Além disso, ao lado das extensinas, estão relacionas às respostas das plantas ao estresse biótico e abiótico. Estudos demonstram que as plantas respondem às infecções fúngicas com um aumento na secreção de glicoconjugados ricos em hidroxiprolina (ESQUERRÉ-TUGAYÉ; LAMPORT, 1979). Geralmente, ápices das raízes e exsudatos são ricos em AGPs (NGUEMA-ONA et al., 2014) e alguns estudos sugerem que essas moléculas estariam envolvidas na interação com microrganismos nas raízes, inclusive em associações simbióticas (BERRY et al., 2002).

Uma característica das AGPs é a sua capacidade de se ligar especificamente a um reagente químico sintético, conhecido como reagente β-glucosil Yariv (β-GlcY) (YARIV; LIS; KATCHALSKI, 1967; YARIV; RAPPORT; GRAF, 1962). Os fenilglicosídeos [1,3,5-tri(*p*-glicosiloxifenilazo)-2,4,6-trihidroxibenzeno] são um grupo de compostos químicos inicialmente desenvolvidos como antígenos de carboidratos para a purificação de anticorpos antiglicosídeos e proteínas ligadoras de açúcar. Sua estrutura química está representada na FIGURA 8.

FIGURA 11 – ESTRUTURA QUÍMICA DO REAGENTE β-GLUCOSIL YARIV.



FONTE: CLASSEN; BAUMANN; UTERMOEHLEN (2019).
NOTA: Glc, β-D-Glucose

Tanto o β-glucosil (β-Glc-Yariv) e β-galactosil (β-Gal-Yariv) fenilglicosídeos de Yariv se ligam especificamente às AGPs, enquanto os α-glucosil e α-galactosil Yariv não são capazes de fazer essa interação (KITAZAWA et al., 2013). A utilização do β-GlcY é uma ferramenta útil na purificação e quantificação de AGPs, além de ser empregado em técnicas de microscopia para a detectar AGPs em tecidos vegetais e elucidar o papel dessas moléculas em processos biológicos (SEIFERT; ROBERTS, 2007). O mecanismo de interação entre a AGP e β-GlcY ainda não está completamente esclarecido, entretanto, KITAZAWA e colaboradores (2013) verificaram que o reagente de Yariv se liga à cadeias de galactanas através de ligações β-1,3 com pelo menos 5 resíduos, e que pelo menos 7 resíduos são necessários para a formação de ligações cruzadas e precipitação do complexo.

Estudos relacionados às atividades biológicas das AGPs demonstram que essas moléculas atuam como imunomoduladoras ao promover a proliferação de linfócitos e a produção de IgM em camundongos (CLASSEN et al., 2006; THUDE et al., 2006), e também como moduladoras do sistema complemento (ALBAN et al., 2002; BOVO et al., 2016; YAMASSAKI et al., 2018).

2.4 SISTEMA COMPLEMENTO

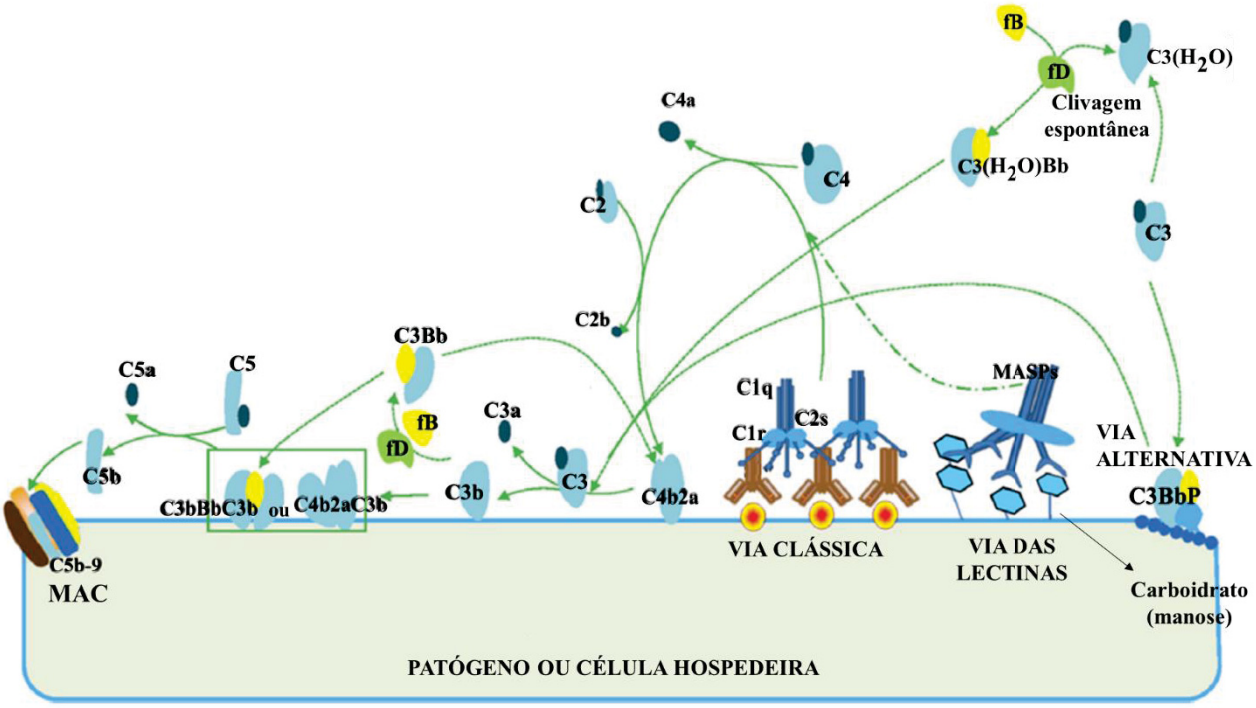
O sistema complemento (SC) consiste em uma rede regulada de proteínas solúveis no plasma ou expressas na membrana celular (LI et al., 2015) e é altamente conservado em todos os vertebrados. Além de desempenhar um papel crítico na imunidade inata e adaptativa e em outros processos biológicos, como regeneração tecidual e crescimento tumoral, o SC é considerado o principal mediador humoral durante o processo inflamatório junto aos anticorpos (SARMA; WARD, 2011).

As três vias de ativação do sistema complemento compreendem as vias clássica (VC), alternativa (VA) e das lectinas (VL), as quais convergem para a ativação de C5b, e finalmente à formação de um complexo de ataque à membrana (MAC) e consequente lise celular (SARMA; WARD, 2011) (FIGURA 12).

A ativação ocorre de forma sequencial e pode ser dividida em quatro principais etapas: iniciação da ativação do complemento, ativação da C3 convertase e amplificação, ativação da C5 convertase e formação do complexo de ataque à membrana. Uma vez ativada, a cascata do complemento gera compostos ativos que são distribuídos a qualquer superfície celular de forma indiscriminada. As primeiras reações enzimáticas que levam à ativação da C3 convertase são rigidamente controladas para assegurar que a ativação e geração de moléculas efetoras ocorram somente quando e no local necessários. Se a ativação da cascata progride, as reações são amplificadas e podem resultar em intensas e potentes funções. Os reguladores de complemento operam em todos os níveis e são categorizados em três classes principais: fase fluida, para a superfície das células hospedeiras e receptores de remoção de complemento de membrana integral. Vários desses reguladores possuem atividades adicionais participando da adesão celular, das interações matriciais extracelulares, ou conectando a cascata do complemento a outras redes fisiológicas importantes, como a cascata de coagulação (ZIPFEL, 2009).

A ativação excessiva, modulação desregulada ou deficiências nas proteínas do complemento levam a uma série de doenças ou condições patológicas relacionadas à imunidade e inflamação (SARMA; WARD, 2011; WANG et al., 2011a).

FIGURA 12 - VISÃO GERAL DAS VIAS DE ATIVAÇÃO DO SISTEMA COMPLEMENTO



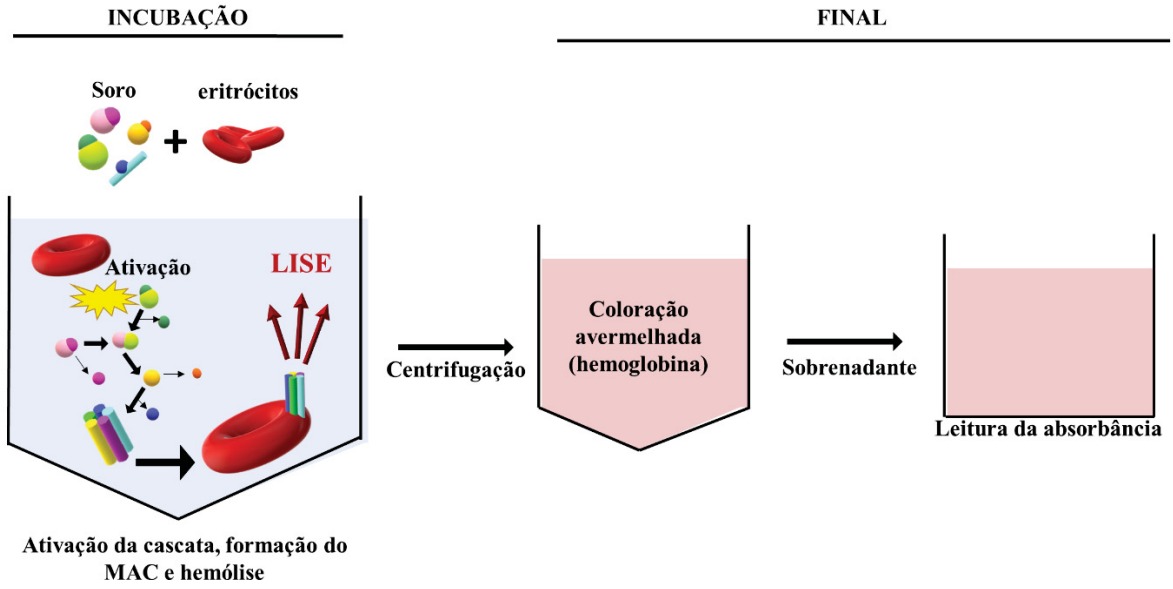
FONTE: Adaptado de LI et al. (2015).
NOTA: Os componentes do complemento são designados pela letra C e por números (C1-C9), por letras (por exemplo, fD: fator D) ou por nomes usuais (por exemplo, fator de restrição homólogo). Após ativação inicial, os diversos componentes interagem em uma cascata altamente regulada. A ativação pode resultar na clivagem de um componente, gerando dois fragmentos: um menor e outro maior, na maioria dos casos denominados de "a" e "b", respectivamente. O fragmento maior se liga ao próximo componente da sequência, dando continuidade à cascata, mas pode tornar-se brevemente inativo caso essa ligação não ocorra. O fragmento menor se difunde a partir do sítio de ativação e, através da ligação a receptores específicos, pode dar início às respostas inflamatórias localizadas. A via clássica é iniciada através da ligação do C1 aos complexos antígeno-anticorpo. A via alternativa é iniciada através da ligação do C3b às superfícies de ativação, tais como paredes celulares bacterianas. A via alternativa também é autoativada de forma contínua e em níveis basais pela clivagem espontânea de C3. A via da lectina é iniciada pela ligação da lectina sérica ligadora de manose (MBL) à carboidratos não-próprios na superfície do patógeno, resultando na ativação de serina-proteases associadas à MBL (MASPs). A via alternativa utiliza alguns componentes (ou fatores) que são únicos, enquanto que as vias clássicas e da lectina possuem componentes comuns após o C1. Todas as três vias geram os mediadores inflamatórios C3a e C5a, as C3 e C5 convertases e o C5 ligado, que é convertido em um complexo de ataque à membrana (MAC) através de uma sequência comum de reações terminais. A hidrólise de C3 é o principal passo na amplificação de todas as vias, gerando grandes quantidades de C3b, que forma parte da C5 convertases. O C3b também pode se difundir da superfície de ativação e se ligar aos complexos imunes ou às superfícies celulares estranhas, onde ele atua como uma opsonina.

Devido à importância do papel desempenhado pelo SC, moléculas com potencial de modulação das vias de ativação do sistema complemento são alvos de estudo. Diversos polissacarídeos desempenham um papel importante tanto na ativação como na inibição do sistema complemento. Os polissacarídeos bacterianos, como o LPS, são potentes ativadores das vias clássica e alternativa, enquanto alguns glicosaminoglicanos GAG, como a heparina, são conhecidos por seu papel inibidor na cascata do complemento (ALBAN et al., 2002).

O ensaio hemolítico do complemento, também conhecido como teste de fixação do complemento, tem sido empregado para avaliar a modulação de polissacarídeos extraídos de

plantas sobre o sistema complemento. Esse ensaio é uma variação do CH_{50}, ou complemento total, empregado nas análises clínicas para testar a capacidade funcional dos componentes do complemento sérico em lisar eritrócitos. O CH_{50} é uma técnica de rastreio para avaliar a ativação da via clássica ou alternativa do complemento, e é sensível à redução, ausência e/ ou inatividade de qualquer componente do caminho. Na via clássica são utilizadas células sanguíneas de carneiro sensibilizadas com anticorpos de coelho antihemácia de carneiro (hemolisina) (ShE). Quando os eritrócitos revestidos com anticorpos são incubados com soro, a via clássica é ativada resultando na hemólise. Se um componente do complemento estiver ausente, o nível de CH_{50} será zero; se um ou mais componentes da via clássica estiverem diminuídos, o CH_{50} será diminuído. Um volume fixo de ShE idealmente sensibilizado é adicionado a cada diluição de soro, e após a incubação, a mistura é centrifugada e o grau de hemólise é quantificado medindo-se a absorbância da hemoglobina liberada no sobrenadante a 405 nm (FIGURA 13) (COSTABILE, 2010; KLERX et al., 1983).

FIGURA 13 - ESQUEMA SIMPLIFICADO DO MECANISMO DO ENSAIO DE CH_{50} OU COMPLEMENTO TOTAL

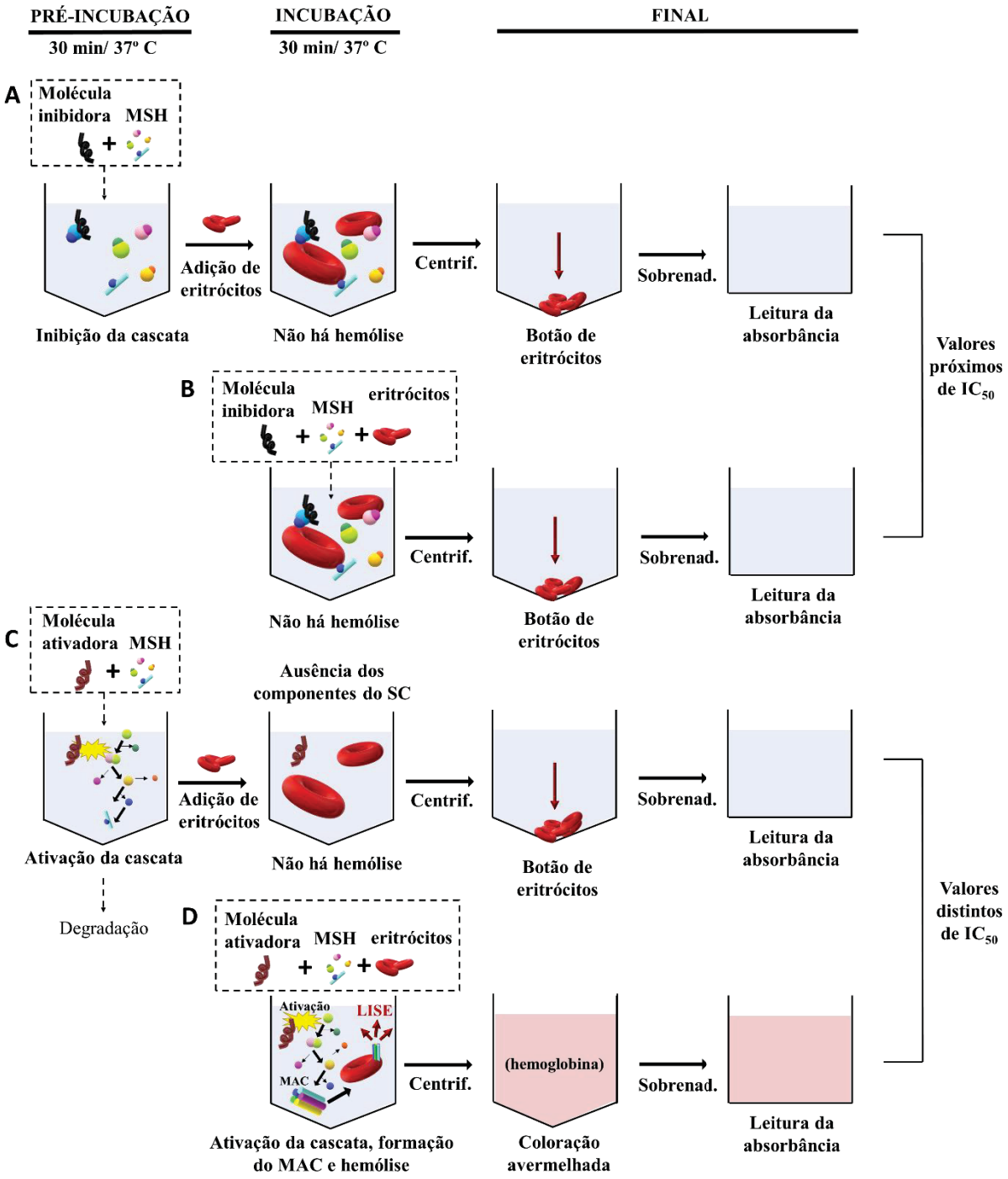


FONTE: O AUTOR (2019)
NOTA: O soro humano contendo os componentes do sistema complemento é incubado *in vitro* com eritrócitos de carneiro sensibilizados com hemolisina, nos ensaios da via clássica, ou com eritrócitos de coelho, via alternativa, em um tampão específico para cada via. O eritrócito é reconhecido pelo SC como uma célula estranha, e a cascata do complemento é ativada, resultando na hemólise. A coloração avermelhada, resultante da liberação da hemoglobina em solução, é proporcional à quantidade de hemólise induzida pelo complemento, e pode ser quantificada através da leitura da absorbância. MAC, complexo de ataque à membrana.

Se um composto-teste (por exemplo, um polissacarídeo), capaz de interferir na atividade hemolítica do complemento, é incubado previamente com o soro (pré-incubação) e

em seguida são adicionados eritrócitos ao meio, o resultado final observado será uma porcentagem reduzida de hemólise (inibição da hemólise) quando comparada com o controle (atividade hemolítica normal do complemento) (FIGURA 14 A e C). Para diferenciar o tipo de interferência provocada pelo composto-teste, é necessária a realização do mesmo ensaio sem a pré-incubação, onde soro, eritrócitos e composto-teste são adicionados simultaneamente ao meio de reação. Caso seja um inibidor, o resultado final será novamente de inibição da hemólise, como no ensaio realizado com pré-incubação (FIGURA 14 B). Entretanto, no caso de uma molécula ativadora, ao final do ensaio sem pré-incubação a hemólise provocada pelo SC será visualizada, o que não ocorre no ensaio com pré-incubação (FIGURA 14 D). Essa diferença metodológica pode ser explicada analisando os mecanismos envolvidos nos processos de inibição e ativação. Para inibir a ativação da via, basta ao composto inibidor se ligar à um componente da cascata e inviabilizar o processo, o que ocorre independente da ordem de adição dos reagentes ao meio. Já para uma molécula ativadora, essa ordem é crucial. Se a mesma entra em contato com os componentes do SC antes da adição dos eritrócitos, a cascata é disparada, mas não há célula disponível para que ocorra a lise. Dessa forma, os componentes do complemento são consumidos e não estarão mais disponíveis no momento de adição dos eritrócitos (FIGURA 14C). No ensaio sem pré-incubação, o efeito ativador da molécula-teste é somado à ativação provocada pelo eritrócito, não alterando a porcentagem final da hemólise. Utilizando diferentes diluições da molécula-teste e concentrações fixas de soro e solução de eritrócitos, o valor de IC_{50}, correspondente à concentração da molécula-teste capaz de inibir 50% da hemólise induzida pelo complemento, pode ser calculado (ALBAN et al., 2002; BOVO et al., 2016; YAMASSAKI et al., 2018).

FIGURA 14 - ESQUEMA DO ENSAIO HEMOLÍTICO: ESTRATÉGIA PARA DIFERENCIAÇÃO ENTRE INIBIDORES E ATIVADORES DO SISTEMA COMPLEMENTO



FONTE: O AUTOR (2019)

NOTA: Padrão observado para moléculas inibidoras do sistema complemento avaliadas em ensaios realizados com (A) e sem (B) a etapa de pré-incubação. Padrão observado para moléculas ativadoras em ensaios com (C) e sem (D) pré-incubação. Dois ensaios simultâneos são realizados para cada molécula-teste: com e sem-pré incubação com uma mistura de soro humano (MSH), fonte de complemento, em microplacas com fundo V. Em seguida, os eritrócitos são adicionados ao meio de reação, e uma nova incubação é realizada. Após centrifugação, o sobrenadante é transferido para uma placa de fundo chato e a leitura da absorbância lida a 405 nm. Quando não ocorre hemólise, os eritrócitos precipitam após a centrifugação formando um botão no fundo V da microplaca. Valores semelhantes de IC_{50} (concentração relativa à 50% de inibição da hemólise provocada pelo complemento) em ambos ensaios, com e sem pré-incubação, são sugestivos de efeito inibidor. Valores diferentes estão relacionados com atividade estimuladora do complemento. MAC, complexo de ataque à membrana.

Considerando a relação entre sistema complemento e inflamação, e o fato de diversos polissacarídeos de plantas serem moduladores do sistema complemento, é possível que os polissacarídeos presentes nos extratos de maca também atuem modulando o SC, e consequentemente, sobre o processo inflamatório, visto que a maca também é indicada para o tratamento de doenças relacionadas à imunidade (BEHARRY; HEINRICH, 2018) e ao processo inflamatório, como o reumatismo (WANG et al., 2007).

Por fim, baseando-se em dados da literatura, a hipótese do presente trabalho é que os glucosinolatos estejam relacionados com a atividade citotóxica apresentada pela maca, e que a os polissacarídeos e glicoconjugados (como AGP) possam ter propriedades relacionadas com as atividades anti-inflamatórias e imunomoduladoras.

3 JUSTIFICATIVA

A maca peruana (*Lepidium meyenii*), uma planta empregada na alimentação, medicina tradicional e fitoterapia, tem despertado o interesse da população e da comunidade científica pelas inúmeras alegações de benefícios à saúde relacionados ao seu consumo (GONZALES et al, 2012). Entretanto, há um consenso de que os benefícios atribuídos ao consumo da maca não apresentam embasamento científico suficiente. Dessa forma, pesquisas a respeito dos constituintes químicos e que busquem elucidar os princípios ativos relacionados aos efeitos biológicos da maca são necessários para se comprovar a eficácia e garantir o consumo seguro dos produtos da maca (BEHARRY; HEINRICH, 2018).

4 OBJETIVOS

4.1 OBJETIVO GERAL

O objetivo geral foi obter e caracterizar frações ricas em glucosinolatos e em polissacarídeos de *Lepidium meyenii* (maca peruana), e investigar *in vitro*, a atividade citotóxica e imunomoduladora destas frações, respectivamente.

4.2 OBJETIVOS ESPECÍFICOS

a) Obter frações ricas em glucosinolatos (GLs) da maca através de extração hidroalcoólica;
b) Quantificar e identificar os GLs presentes nos extratos da maca;
c) Avaliar, *in vitro*, o efeito citotóxico dos glucosinolatos presentes nos extratos de maca em linhagens de células tumorais humanas de hepatocarcinoma (HepG2/C3A) e adenocarcinoma de cólon (HT29), na presença ou ausência da enzima mirosinase;
d) Obter e caracterizar quimicamente frações ricas em polissacarídeos da maca através de extrações aquosas e alcalinas;
e) Quantificar o conteúdo de arabinogalactana-proteínas (AGPs) presentes nos extratos da maca;
f) Avaliar, *in vitro*, a capacidade das frações ricas em AGPs da maca em modular o sistema complemento através das vias clássica e alternativa, pelo ensaio hemolítico do complemento.
g) Contribuir para o melhor conhecimento sobre os componentes químicos e as propriedades farmacológicas da maca.

A presente tese será apresentada na forma de artigo científico, sendo que o artigo 1 terá como objetivo, obter frações ricas em glucosinolatos das raízes da maca e avaliar (*in vitro*) a atividade citotóxica desses compostos e de seus derivados em linhagens de células tumorais humanas de hepatocarcinoma (HepG2/C3A) e adenocarcinoma de cólon (HT29), na presença ou ausência da enzima mirosinase. O artigo 2 envolve o objetivo que foi obter e caracterizar frações da maca ricas em polissacarídeos, investigar a presença de araboinogalactana-proteínas e avaliar sua capacidade de modulação das vias clássica e alternativa, *in vitro,* através do ensaio hemolítico do complemento.

ARTIGO I

Chemical characterization of glucosinolate-enriched fractions from maca (*Lepidium meyenii*) and evaluation of their cytotoxicity on human tumoral cell lines in absence and presence of myrosinase

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Chemical characterization of glucosinolate-enriched fractions from maca (*Lepidium meyenii*) and evaluation of their cytotoxicity on human tumoral cell lines in absence and presence of myrosinase

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Abstract

This study aimed to obtain and chemically characterize intact glucosinolates (GLs) enriched fractions of maca with combined chromatographic and mass spectrometry approaches and to evaluate their cytotoxicity on two tumoral human cell lines in absence (-MYR) and presence of myrosinase (+MYR). MYR converts GLs to their products such as isothiocyanates (ITCs). Maca fractions (Lm-II and Lm-III) rich in GLs, such as glucotropaeolin and glucoliminanthin, were tested on hepatocellular carcinoma (HepG2/C3A) and colon adenocarcinoma (HT-29) cell

lines. Cytotoxic effects were only verified in the experiments with fractions submitted to free-sugar cleaning step and when MYR was added to culture medium. The IC_{50} values, corresponding to the half-maximal inhibitory concentration of Lm-II and Lm-III (on +MYR assay), were 118.8 and 69.9 µg/mL for HepG2/C3A and 102.6 and 71.5 µg/mL for HT29, respectively. These results suggested that the GLs were not cytotoxic at conditions tested, but, compounds (as ITCs) formed by MYR upon GLs can be responsible for cytotoxic effects. High Performance Liquid Chromatography analysis of the tested fractions on bioassay (- MYR and +MYR) was used to monitor the MYR reaction and confirmed the substrate's disappearance. These results will contribute for strengthening of maca as a "superfood" and for design of future *in vivo* studies focus on therapeutic approach using MYR-mediated products of this specie.

Key words: Glucotropaeolin, glucoliminanthin, benzyl glucosinolates, isothiocyanates, Brassicaceae family; HepG2/C3A, HT29.

Chemical compounds studied in this article: glucotropaeolin (PubChem CID: 656498); glucolimnanthin (PubChem CID: 20843359); glucoiberin (PubChem CID: 9548622); sinigrin (PubChem CID: 23682211).

Highlights

- Maca glucosinolate-enriched fractions were characterized by combined analytical techniques.
- Glucotropaeolin and glucolimnanthin were identified and quantified on maca fractions.

- Tumoral cell viability was performed with maca fractions on absence and presence of myrosinase (MYR).
- Results suggested that compounds formed by MYR can be responsible for these effects.
- Free-sugar cleaning step was determinant to access glucosinolate's cytotoxicity mediated to MYR.

1. Introduction

Lepidium meyenii Walpers (maca) is a plant from the Peruvian central Andes belonging to the Brassicaceae family. Maca has been employed as food and as therapeutic product on both traditional and folk medicine (Flores, Walker, Guimarães, Bais & Vivanco, 2003). The edible portion comprises the transition zone between the hypocotyl and the root and constitutes the storage organ of maca (Quirós & Cardenas, 1992). To simplify the terminology, only the term "root" will be used to refer to this organ. Traditionally the fresh or dried maca are boiled in water and consumed as juice (Ochoa, 2001). Most of the maca products commercialized are constituted of maca flour, but also the hydroalcoholic extracts are available as nutraceutical products on the market (Gonzales et al., 2011).

In the last decades, the interest in maca products has globally increased especially after maca have been announced as a "superfood" (Beharry & Heinrich, 2018), due to its applications for the prevention and treatment of several illnesses. Examples of its applications including improvement of sexual performance (Gonzales et al., 2002), increase of fertility (Rubio et al. 2006), regulation of the hormonal secretion (Leiva-Revilla et al., 2014) and anticancer activity (Gasco, Villegas, Yucra, Rubio & Gonzales, 2007; Bai, He, Roller, Lai, Bai & Pan, 2015).

In relation to the chemical compounds, studies have been described that maca presented constituents as carbohydrates (as starch), proteins and lipids (Dini, Tenore & Dini, 2002; Valentová, Buckiová, Křen, Pěknicová, Ulrichová & Šimánek, 2006). It also presented fat acids derivatives (macaenes and macamidas) (Zhao, Muhammad, Dunbar, Mustafa & Khan, 2005) and alkaloids such as lepidilins (Cui, Zheng, He & Zheng, 2003), macahydantoins (Tian et al., 2018) and macapirrolins (exclusively found in maca) (Zhou et al., 2018), as well as, polyphenols (Piacent , Carbone, Plaza, Zampelli & Pizza, 2002; Bai et al., 2015) and glucosinolates (Campos, Chirinosa, Barreto, Noratto & Pedreschi, 2013).

Glucosinolates (GLs) belong to a group of thioglycosides and are the most predominant secondary metabolites in maca (Wang & Zu, 2019). GLs are also present in the *Brassica* vegetables, such as mustard, broccoli, radish, cauliflower, and cabbage and have been associated with health benefits and reduced cancer risk (Verkeck et al., 2009). In maca, six different GLs have been identified, most of them of the aromatic type. About 70-80% of the total maca GLs correspond to glucotropaeolin (GTR) and approximately 20% to glucolimnantinin (GLM) (Campos et al., 2013). The content of GLs present in fresh maca is approximately 100 times higher than that found in other brassicas (Li, Ammermann & Quirós, 2001). Chemically, GLs are very stable water-soluble and relatively non-reactive compounds (Fahey, Zalzmann & Talalay, 2001). Inside the plant cells, they coexist in separated compartments with the enzyme myrosinase (MYR), which is responsible for their hydrolysis when plant tissue is damaged (by insect attack, mastication or fresh vegetables processing) (Fahey, Zalzmann & Talalay, 2001). For example, during the ingestion process, one part of GLs is hydrolyzed by MYR present on plant tissue (released by mastication process) or intestinal microbiota, while another part is absorbed in their intact form through the gastrointestinal mucosa (Dinkova-Kostoglsva & Kostov, 2012; Barba, Nikmaram, Roohinejad, Khelfa, Zhu & Koubaa, 2016). MYR converted GLs to volatile breakdown products, which are responsible for

the characteristic spicy taste and pungency of cruciferous vegetables (Dinkova-Kostoglsva & Kostov, 2012). The chemical nature of the hydrolysis products depends on the structure of the GL side chain, plant species and reaction conditions. At neutral conditions usually results in the formation of isothiocyanates (ITCs) (Redovniković, Glivetić, Delonga & Vorkapić-Furač, 2008), which are known to have anticancer effects (Psurski, Filip-Psurska, Cuprych, Wietrzyk & Oleksyszyn, 2019).

Even though, an increase interest of maca as nutraceutical food have been demonstrated, few studies, especially with combinatory chemical and bioassay approaches, has been published in order to contribute on the understanding of the maca's health benefits (Beharry & Heinrich, 2018). One study regarding the anticancer activity one study showed that both GL-rich aqueous and ethanolic extracts from maca were able to decrease benign prostatic hyperplasia induced by testosterone enanthate (such as increase testosterone levels) in mice, in a dose-dependent manner, related to GTR content (Gonzales et al., 2007). In addition, the cited authors noted that other compounds than GLs could also be responsible for the effects. However, any additional information of chemical characterization of these compounds were mentioned. Another study evaluated the *in vitro* effect of a non-chemical characterized red maca aqueous extract against human prostate cancer cell line (LNCaP) (Díaz, Cardenas & Orihuela, 2016). Despite of this extract demonstrated effects on stimulating androgenic signaling of LNCaP cells, it did not alter the viability neither influenced on the apoptotic activity induced by anticancer drugs (taxol and 2-methoxyestradiol).

This study focused on both chemical and biological aspects of GLs, considered an important maca bioactive component. In addition, the strategy was also to examine which would be the *intact GLs* or the *hydrolysis products of myrosinase* responsible on the cytotoxicity effects against tumoral cells and thereby improve information of the anticancer properties of maca. Thus, the present work aimed to obtain and characterize intact GL-enriched fractions of

maca and to investigate the cytotoxic effects on the human hepatocellular carcinoma cell line (HepG2/C3A) and human colon adenocarcinoma cell line (HT-29) on the absence and presence of myrosinase. A GL-enriched fraction obtained from seed mustard (Brassicaceae Family) was also chemically characterized and used on cytotoxicity bioassay for comparison purposes.

2. Material and methods

2.1 Obtainment of maca GL-enriched fractions

2.1.1 Plant material

Peruvian maca flour (*Lepidium meyenii* Walpers, HEMA "Comércio de Cereais Sítio Cercado"; registration 1.10.746-4 from Brazilian Health Regulatory Agency, ANVISA) was purchase from a local store in Curitiba, Brazil.

2.1.2 Ethanolic extraction and silica gel chromatography

Maca flour (200 g) was previously defatted (3x) with dichloromethane (CH_2Cl_2) (400 mL) for 4 h at 25 ºC. After complete solvent evaporation, the residue (173.67 g) was submitted to an extraction with 70% (v/v) ethanol (EtOH) (400 mL), in water bath at 70 °C for 30 min. The process was repeated, and the supernatants were pooled and evaporated at 40 °C under vacuum. The concentrated crude extract was lyophilized (12.4 g), resuspended in H_2O, mixed (10 min, at 25 ºC), and then centrifugated (1,200 × *g* for 5 min at 10 °C). The supernatant (H_2O soluble portion) was freeze-dried (11.3 g) and labelled as Maca Ethanolic Extract (MEEs).

MEEs (3.9 g) was first submitted to a liquid-liquid partition performed with chloroform:H_2O (1:1, v/v, 100 mL) and ethyl acetate:H_2O (1:1, v/v, 100 mL), three times for each :solvent. In this procedure the chloroform fraction (CF, 156 mg) and ethyl acetate fraction (EaF, 117 mg) were removed and the resulting aqueous phase, labeled as aqueous fraction (AqF, 3.4 g), was solubilized in H_2O (5 mL) and fractionated by silica gel chromatography, using a glass column (40 × 1.5 cm i.d.) filled with 100 g of silica gel 60 (Vetec, Rio de Janeiro, RJ, Brazil). The elution was performed with different organic solvents (increasing polarity) at an average flow rate of 1.5 mL/min: ethyl acetate:EtOH (1:1, v/v, 500 mL); EtOH (500 mL) and EtOH:H_2O (1:1, v/v, 500 mL). The eluates were collected in a total of 300 tubes (5 mL/tube), monitored by thin layer chromatography and pooled by comparison considering the retention factor (R_f) of the bands and staining as described below in item 2.1.3. The first 100 tubes (eluted with ethyl acetate/EtOH) gives rise to the first 6 fractions (S1-S6), the S7 fraction corresponds to the tubes 200-300 (eluted with EtOH) and fraction S8 to the tubes 200-300 (eluted with EtOH:H_2O). The fractions were evaporated at 40 ^oC, followed by freeze-drying. The protocol used for preparation of maca fractions is summarized in **Fig. 1**.

2.1.3 Thin layer chromatography (TLC)

All fractions were analysed by TLC on silica gel plates (60 F254, Merck, Darmstadt, German) with ethyl acetate:acetic acid:*n*-propanol:H_2O (3:1:1:1, v/v) as mobile phase. Compounds were visualized under ultraviolet (UV) light at 254 nm and stained using different spraying reagents: vanilina-sulphuric acid (Wagner & Bladt, 1996) and orcinol-sulphuric acid (Sassaki, Souza, Cipriani & Iacomini, 2008). Identification was performed by comparison (staining band and R_f values) with standards: sinigrin (SIN), caffeine β-sitosterol,

epigallocatechin, quercetin, saponin, glucose (Glc), and fructose (Fru) (Sigma-Aldrich Co., St. Louis, MO, USA), and sucrose (Reagen, Quimibrás, Rio de Janeiro, Brazil).

2.1.4 Solid phase extraction (C_{18})

Since the presence of free sugars was verified by TLC, solid phase extraction (SPE) was used to remove these compounds from the S-fractions. SPE column C_{18} cartridge (Agilent Technologies, Inc., Cary, NC, USA) was preconditioned by passing it through 5 mL of methanol (MeOH), followed by 10 mL of Milli-Q H_2O pH 5.0. Water was used not to wash the fraction, as usual, but to elute sugars and GLs at different times. First, 20 µL of the concentrated fractions prepared in Milli-Q H_2O pH 5.0 (2 g/mL) were loaded on the C_{18} cartridge and slowly eluted with Milli-Q H_2O pH 5.0 (15 mL) in small portions (250 µL/tube) to ensure the separation of sugars from GLs. The remained compounds were eluted from column with MeOH. SPE was monitored by UV absorbance at 233 and 254 nm and through Glc content measurement (Glucose Liquiform kit, Labtest, MG, Brazil) given five SPE-fractions labeled as Lm (*Lepidium meyenii*)-I to III, eluted with H_2O, and Lm-IV, eluted with MeOH.

2.2 Mustard seed GL-enrich fraction

A GL-enriched fraction was obtained from yellow mustard seeds (*Sinapsis* sp., from commercial market) according the methodology described by Lee et al. (2006). The resulted fraction, labelled SaF (*Sinapsis* sp. fraction), was chemically characterized and used on the cell cytotoxicity bioassay in the absence and presence of MYR (commercial enzyme from *Sinapsis alba*; Sigma-Aldrich Co., St. Louis, MO, USA) for comparison purposes with maca fractions.

2.3 Chemical characterization of GL-enriched fractions

Chemical characterization of the fractions was initiated through the combination of approaches by using different techniques of liquid chromatography and mass spectrometry, as described below.

2.3.1 Condition A: High Performance Liquid Chromatography (HPLC) and tandem *Mass Spectrometry (MS/MS)*

An HPLC Agilent-1200 series (Agilent Technologies Inc., Santa Clara, CA, USA), degasser (G1322A), equipped with a quaternary pump (G1311A), manual injector (20 µL loop), thermostatted column compartment (G1316A), an UV-Vis detector (G1365D), a EzChromElite software, a reversed-phase Supelcosil C_{18} column (250 mm×4.6 mm i.d., 5 µm; Supelco, Bellefonte, PA, USA) and a C_{18} guard column was used on this condition. MEEs, AqF, S1 to S8, Lm-II, Lm-III and SaF fractions were dissolved in Milli-Q H_2O, filtered through a 0.22 µm cellulose acetate membranes (Millipore, Billerica, MA, USA) and loaded onto the column. Analysis were performed using a gradient system of 30 mmol/L ammonium acetate (Sigma-Aldrich Co., St. Louis, MO, USA), containing formic acid at pH 5.0 (mobile phase A) and MeOH (mobile phase B) (Tedia, Fairfield, USA), as reported by Lee et al. (2006) with some modifications. The total running time was 35 min, at 25 °C with a flow rate of 0.5 mL/min, with the following gradient: 100% A for 5 min, then increased to 70% A-30% B from 5 to 17 min and kept at 70%A-30% B until the end of running. The eluent was monitored by UV detection at 229, 233, 254, 280 and 300 nm, however, only 233 and 254 nm are presented on the chromatograms.

For quantification of individual GLs, an external nine-point calibration curve in the range of 0.005–1.0 mg/mL using SIN was obtained. Measurements were performed in triplicate. Limit of detection (LOD) and limit of quantification (LOQ) were calculated by means of the signal-to-noise ratio based on the blank standard deviation and parameters of the calibration curve. The quantification was accomplished using the relative response factors for intact GLs (Møller, Plöger & Sørensen, 1985 cited by Buchner, 1987): 1.00 for SIN, 1.09 for glucoiberin (GIB) and 0.88 for glucotropeolin (GTR). Once such data could not be found for intact glucolimnanthin (GLM), the response factor was assumed to be 1.00 (Lewis & Fenwick, 1987).

The tandem mass spectrometry (MS/MS) system employed for analyte identities conformation analyses was a Quattro triple quadrupole (Micromass, Manchester, UK) equipment fitted with a Z-electrospray interface operating on negative ion mode (ESI-). The temperatures of source block and desolvation gas were set at 100°C and 120°C, respectively. Nitrogen was used as both drying (nearly 200 L/h) and nebulizing (nearly 70 L/h) gas, while argon was used as collision gas. The cone voltages employed during the analyses were 80 V and the collision energy ranged from 15 to 25 eV among the analyte's analyses. For identities confirmation, the analyses were carried out in the multiple-reaction monitoring mode. For MEEs and S4 fractions, peaks with higher absorption at 233 nm than at 254 nm, were manually collected and freeze-dried. For MS analysis, the collected peaks were dissolved on 0.5 mL of MeOH and mixed by vortex. Then, 0.5 mL of H_2O with 0.5% (v/v) of formic acid were added. Prepared samples were introduced into the MS/MS system by direct infusion under a flow rate at 10 μL/min.

2.3.2 Condition B - Liquid chromatography coupled with tandem-mass spectrometry (LC-MS/MS)

LC-MS/MS analysis of MEEs, S4 and SaF fractions were performed using the same method described in section 2.3.1. The chromatographic separation was performed in an HPLC system (Shimadzu, Kyoto, Japan) equipped of two pumps (LC-Ad), autoinjector (SIL-30AC), Gemini C_{18} column (250 mm×4.3 mm i.d., 5 μm; Phenomenex Inc., Torrance, CA, USA), column oven (CTO-20A) and an UV-VIS detector (SPD-20A), coupled to an Ultra High-Resolution mass spectrometer (MAXIS-3G, Bruker Daltonics, Bremen, Germany). Mass spectra were recorded in the range at m/z 50–1000 in negative ion modes, the voltage maintained at 4500 V, 2.0 bars ESI nebulizer pressure, 200 °C drying gas temperature and the drying gas flow was 8.0 L/min.

2.3.3 Condition C – *High Performance Liquid Chromatography (HPLC) and Liquid chromatography coupled by tandem-mass spectrometry (LC-MS/MS)*

Lm-II, Lm-III and SaF fractions (1 mg/mL) were analysed in HPLC (equipment described in item 2.3.1) and LC-MS/MS (described below) using the same method described before with the following modifications: volume of injection: 5 μL; autoinjector maintained at 10 ºC; total running time of 90 min at 25 °C; flow rate of 0.2 mL/min; gradient: 100% A for 12 min, then increased to 70% A-30% B from 12 to 42 min and kept at 70%A-30% B until the end of running. The chromatographic separation in LC-MS/MS was performed in Prominence Ultra-Fast Liquid Chromatograph Shimadzu® (Shimadzu, Kyoto, Japan) using an reversed-phase Supelcosil C_{18} column (250 mm×4.6 mm i.d., 5 μm; Supelco, Bellefonte, PA, USA) and a C_{18} guard column coupled to the Bruker® MicroToF-QII Mass Spectrometer (Bruker Daltonics,

Bremen, Germany). Mass spectra were recorded in the range at m/z 50–1000 in negative ion mode, the voltage maintained at 3500 V, 2.0 bars ESI nebulizer pressure, 200 °C drying gas temperature and the drying gas flow was 6.0 L/min.

2.3.4 Nuclear magnetic ressonance (NMR)

For NMR experiments, the samples (1-5 mg) were dissolved in deuterated water (D_2O). NMR spectra (Carbon nuclear magnetic resonance, ^{13}C-NMR, Heteronuclear Single Quantum Coherence, HSQC) were obtained on a BRUKER spectrophotometer, model DRX 400, Avance series (Bruker, Karlsruhe, Germany) at 30 °C, using 2,2,3,3-D_4 -trimethylsilylpropionate (TMSP-d_4, Sigma-Aldrich Co., St. Louis, MO, USA) as internal standard (δ=0).

2.3.5 Total phenolics and sucrose content

The content of total phenolic was determined by the Folin–Ciocalteu method (Singleton, Orthofer & Lamuela-Raventós, 1999) using gallic acid (GA, Sigma-Aldrich Co., St. Louis, MO, USA) as standard and the results were expressed as µg GA equivalent (GAE)/mg fraction. The presence of sucrose was verified by TLC analysis by comparison (staining band and R_f value) of sucrose standard (as conditions described in 2.1.2 section). Qualitatively, sucrose presence was also confirmed after treatment of the fractions with the invertase (5 min, 25 ^oC) with further TLC analysis by comparison of disappearance of the substrate (sucrose band) and appearance of products (Glc and Fru bands).

2.4 Cytotoxicity bioassay

The maca fractions (AqF, Lm-II and Lm-III) and the mustard fraction (SaF) were selected for biological analysis described below.

The human hepatocellular carcinoma (HepG2/C3A) and human colon adenocarcinoma (HT29) cell lines were obtained from Rio de Janeiro Cell Bank. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 1% (m/v) penicillin/streptomycin (Invitrogen, Cat. No. 15240096, Thermo Fisher Scientific Inc., Waltham, MA, USA), and kept in a humidified incubator containing 5% CO_2 at 37 °C. The cells were free-mycoplasma, and under these conditions, the cell viability remained ≥ 90%.

The cytotoxic analysis was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) assay, as described by Mosmann (1983) with some modifications. Aliquots of exponentially HepG2/C3A ($2.5x10^4$ cells/well) and HT29 ($1x10^4$ cells/well) growing cells were put in 96-well plates, and the culture was kept in a humidified incubator containing 5% CO_2 at 37 °C, for 24 h. Solution of the fractions (AqF, Lm-II, Lm-III, and SaF), at 5 mg/mL, were prepared in DMEM and sterilized by filtration through a 0.22 μm cellulose acetate membranes. The cells were exposed in the absence (negative control, -MYR) or in the presence of tested fractions (tested groups, -MYR) at final concentrations of 12.5, 25, 50, 100 and 200 μg/mL, and kept at the same conditions described before, for 48 h. Another identical experiment was prepared and 5 μL of MYR (0.3 units/mL) was added to each well (negative group, +MYR and tested groups, +MYR) (Ibrahim, Kntayya, Ain, Iori, Ioannides & Razis, 2018). The chemotherapeutic agent doxorubicin (Zodiac Produtos Farmacêuticos, Pindamonhangaba, SP,

Brazil) at 1 μM was used as the positive control (for -MYR and +MYR experiments). After 48 h of treatment, the cells were incubated for 4 h with MTT solution (0.5 mg/mL) to form formazan crystals. The cell culture medium was aspirated, and dimethyl sulfoxide was added to dissolve the crystals. The absorbance was measured at 560 nm using a spectrophotometer (Glomax® Instrument, Promega Corporation, Madison, WI, USA). Assays were performed in duplicate and three experiments. Cell viability was expressed as a percentage of negative control (group of cells grown only on DMEM). The morphological analysis was performed using photomicrographs obtained from EVOS™ FL Auto Imaging System (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.5 Monitoring of myrosinase activity and HPLC analysis of maca and mustard fractions tested on bioassay

The myrosinase activity was verified by measuring the disappearance of the substrates (GLs) spectrophotometrically (at 229, 233, 254, 280 nm) (data not shown) and by HPLC analysis of maca and mustard fractions submitted (or not) to myrosinase treatment. Aliquots (100 μL) of each fraction (Lm-II, Lm-III, and SaF) (400 μg/mL) were prepared in phosphate buffer saline (PBS) pH 7,4 containing 1 mM ascorbic acid (AA), added in 96-well UV microplates (Costar model 3635, Corning, NY, USA) and incubated with 8 μL of MYR solution (1 U/mL) or 8 μL of PBS+AA (negative control of activity), for 1 h at 37 °C. The fractions (20 μL) were analyzed by HPLC (Condition A) as described before on the section 2.3.1.

2.6 Statistical analysis

For MTT assay, a two-way ANOVA followed by Tukey post hoc test was performed to determine the effects of different fractions and concentrations in cell viability (CV). Results of

three independent experiments were expressed as mean percentage of CV ± standard deviation (SD). Data were considered significant at $p < 0.05$, $p < 0.01$, or $p < 0.001$ relative to negative control. The half-maximal inhibitory concentration (IC_{50}) values were calculated from a nonlinear regression with variable slope using GraphPad prism software (version 8.1.2).

3. Results and discussion

3.1 Obtainment and preliminary characterization of maca and mustard fractions

The methodology used at the present study was designed to obtain fractions with GLs in their *intact form*, to be chemically characterized and then tested on cytotoxicity bioassay with the presence of MYR (**Fig. 1**).

After the removal of nonpolar components, defatted maca flour was submitted to ethanolic extraction (at 70 ^{o}C) (instead of aqueous extraction) to avoid the co-extraction of high mass compounds present in maca flour, such as starch and other polysaccharides (Antonious, Bomford & Vincelli, 2009; Rondán-Sanabria & Finardi-Filho, 2012; Tang et al., 2017). However, ethanolic extraction can also extracted of other classes of secondary metabolites such as phenolic compounds (Campos et al., 2013). Because of that, the crude ethanolic extract was solubilized in H_2O, and the resulted fraction (MEEs) was subjected to the liquid-liquid partition. By this procedure, most of the compounds visualized on TLC under UV light at 254 nm, indicative of phenolic compound's presence, were retained in EaF (**Table S1**, supplementary material). The AqF, corresponded to the resulting aqueous phase fraction, was then chromatographed on a silica gel column using different solvents for a second separation of the components based on their polarity. Qualitative TLC analysis of silica gel column fractions (S1 to S8) are presented on **Table S1** (supplementary material). By comparison with the respective

standards, presence of sugars can be suggested on the fractions S2 to S4. A free-sugar clean-up step were performed, for that S2 and S3 fractions were submitted to the SPE C_{18}, giving rise to four new fractions: Lm-I, Lm-II, Lm-III (**Fig. S.1**, supplementary material) and Lm-IV. Both free sugars (determined by Glc oxidase kit) than GLs (detected at 233 nm) were eluted with H_2O. Glc was concentrated in the Lm-I, which a yield of 56% (w/w), calculated as dried weight in related to original fraction. GLs were eluted especially in the Lm-II (yield of 4% w/w) and Lm-III (yield of 38%, w/w) fractions. The last fraction Lm-IV fraction with a yield of 2% (w/w) was eluted with MEOH. Lm-IV fraction presented high absorbance at 254 nm (data not shown), compared to others Lm fractions, which can suggest the presence of phenolics compounds, as verified for EaF. EaF and Lm-IV were kept for further additional chemical characterization. The intact GL-enriched fraction of mustard (SaF) was obtained by hot methanolic extraction following clean-up step with Florisil C_{18}. During the fractionation process, the HPLC analysis (condition A) was relevant to monitor the presence of intact GLs (data not shown) since the TLC analysis, despite more feasible than HPLC, presented technical limitation for identification of GLs specially with presence of sugars (such as monosaccharides, disaccharides).

A total of sixteen (16) fractions from maca and one (1) from mustard was obtained on this study. The CF was discarded. The fractions EaF and Lm-IV were only spectrophotometrically characterized by total phenolic contents (**Table 1**). Preliminary characterization of the maca fractions (MEEs, AqF, S1 to S8, Lm-I, Lm-II, Lm-III) and mustard fraction (SaF) suggested the presence of GLs and sugar compounds. The identification of these compounds was performed by using a combined data interpretation of the chemical results obtained of those fractions, as described in the next section.

3.2 Chemical characterization of maca and mustard fractions

The fractions (MEEs, AqF, S1 to S8, Lm-I, Lm-II, Lm-III) were submitted to chemical analysis by using different conditions (A, B or C). The combination of the different conditions was necessary since the concentration of the counterion added to the mobile phase decreases the intensity of the GLs mass fragments, making their detection difficult in the LC-MS / MS analysis.

In the **Fig. 2** is presented the LC-MS/MS analysis (Condition C) of Lm-II, Lm-III and SaF. The peak 1, at 16.0 min on base peak chromatograms (BPC) of Lm-II and Lm-III presented a $[M-H]^-$ ion m/z 341, indicative of a disaccharide formed by two hexoses. This peak was also present, although with very high intensity, on BPC from maca fractions obtained prior to SPE (MEEs, AqF and S-fractions) (data not shown). The peak assignments indicated on tables were based on the MS data results observed on other condition analysis (A and B), comparing to fragmentation pattern reported in the literature (Antonio, Pinheiro, Chaves, Ricardo, Ortuño & Thomas-Oates, 2009). For example, for S4 fraction (at condition B), it was observed a peak at 5.6 min, with $[M-H]^-$ ions m/z 341.1089 and 387.1140, indicating a fragmentation pattern of a disaccharide formed by two hexoses (data not shown). The disintegration of the ion with m/z 387 also produced an ion with m/z 341, corresponding to the loss of formic acid [HCOOH] (-46 *u*), present in the mobile phase employed in this condition, and another $[M-H]^-$ ion m/z 179 [Hex]. The identification of the disaccharide was confirmed by NMR data (HSQC and ^{13}C NMR spectra) (**Fig. S.2**, supplementary material). Correlation signals at δ H 5.40/ δ C 95.1 can be attributed to H-1/C-1 of α-D-Glc*p* and the signal at δ 106.6 can be attributed to C-2 of Fru*f* (Ulrich & Markley, 2013). Considering MS and NMR data the peak 1 was identified as sucrose. The presence of sucrose was also confirmed by TLC analysis of fractions after invertase

treatment (or not); invertase treatment provoked the disappearance of the sucrose band (R_f 0.47) and increased Glc (R_f 0.54) and Fru (R_f 0.50) bands (data not shown).

The peak 2 at R_t 27.2 min on BPC of Lm-II and Lm-III presented a $[M-H]^-$ ion m/z 408 (**Fig. 2**). The HPLC and MS/MS analysis of MEEs, AqF, S2, S3 and S4 (condition A) also demonstrated the presence of compound with a $[M-H]^-$ ion m/z 408 and fragmentation pattern (m/z 328, 274, 259, 241, 195 and 166) (**Fig. S.3**, supplementary material). This compound was the most evident peak on HPLC chromatogram, at Rt 15.1±0.1 min, with higher absorption in 233 nm (data not shown). The MS/MS analysis of peak 2, together with literature data (Fabre, Poinsot, Debrauwer, Vigor & Tulliez, 2007; Shi, Zhao, Sun, Yu & Chen, 2017), is consistent with glucotropaeolin (GTR), a benzylglucosinolate.

The peak 3 at R_t 37.7 min on BPC of Lm-III presented a $[M-H]^-$ ion m/z 438 (**Fig. 2**). The LC-MS/MS analysis of MEEs and S4 (condition B) (data not shown) observed a $[M-H]^-$ value of m/z 438 and the presence of the ion at m/z 96.9608 (HSO_4–), the neutral loss of 79.9571 *u* (SO_3^-) and the ions fragments (m/z 242 and 195) which are, together, indicative for GLs (Fabre, Poinsot, Debrauwer, Vigor & Tulliez, 2007; Popova & Mora; 2014). This result indicated the presence of glucoliminanthin (GLM), a 3-metoxyl-benzylglucosinolate, on peak 3. The analysis of the Lm-II and Lm-III corroborated with the presence of GTR and GLM as main GLs found on maca (Dini et al., 2002; Piacent et al., 2002; Yábar et al., 2011).

Regarding to SaF fraction, only one high intensity peak with R_t 16.0 min was observed on BPC (indicated as peak 1 and 4 on **Fig. 2**). The MS spectra indicated a $[M-H]^-$ ion m/z 341 (indicative of a disaccharide formed by two hexoses) and a $[M-H]^-$ ion m/z 422. Considering MS (m/z 341) and TLC analysis (with invertase) the presence of sucrose is suggested on peak 1. In addition, the analysis of SaF at condition A, presented a HPLC chromatogram (with a peak at R_t 6.5±0.1 min), with greater absorption at 233 nm with a MS/MS spectrum with ion at m/z 422.0241 and at m/z value of 358.6208 (formed by the neutral loss of the CH_3SOH) (data not

shown). This result indicated the presence of glucoiberin (GIB), a 3-methyl-sulfinylpropyl glucosinolate, as GLs constituent of mustard fraction. The peak with R_t 16.0 min represented two co-eluted compounds: sucrose (named peak 1) and GIB (named peak 4). According to literature, each species of mustard seed has a different GL profile, and usually containing sinigrin (SIN) or sinalbin (SIB) as representatives of the most common GL type (Cools & Terry, 2018).

The quantification of GLs presented on the maca fractions (MEEs, AqF, S1 to S8, Lm-I, Lm-II, Lm-III) and mustard fraction (SaF) was performed by HPLC analysis (condition A) using a SIN calibration curve (LOQ: 0.02 µg/mL, LOD: 0.08 µg/mL, R^2 = 0.99) (**Table 1**). In summary, MEEs, AqF, S-fractions obtained prior to SPE C_{18} clean-up were composed of sucrose, with low content (< 20 ug/mg) of GLs. Lm-II fraction presented only GTR (195.4 µg/mg) and Lm-III fraction consists a mixture of GTR (169.9 µg/mg) and GLM (66.6 µg/mg). Both fractions presented a low phenolic content (<20 µg GAE/mg). Lm-IV fraction eluted with MeOH presents a lower concentration of GLM (34.9 µg/mL) when compared to Lm-III, but with a total phenolic content at concentration of 27.8 µg GAE/mg. SaF presented the higher GIB content (238 µg/mg). Relating to sugar content, sucrose presence was identified on Lm-II, Lm-III and SaF fractions.

The presence of free sugars in maca has been reported (Valentová, Buckiová, Křen, Pěknicová, Ulrichová & Šimánek, 2006). According to cited authors, dehydrated powder of maca roots is constituted mainly by 60% carbohydrates (23.4% sucrose, 1.55% Glc and 4.56% of oligosaccharides). However, among other Brassica plants, such as broccoli, cabbage, rucola and radishes, high sucrose content was uncommon (Gupta, Talwar, Jain, Dhawan & Jain, 2003; Jovanovic-Malinovska, Kuzmanova & Winkelhausen, 2014).

Although the SPE-C_{18} was not able to completely separate sucrose from GLs under conditions tested in this work, the Lm-II and Lm-III fractions presented approximately 10 times

more GLs than the S3 silica fraction, and 20 times more than AqF, indicating that this technique was a useful to obtain maca GL-enriched fractions. Due to the chemical characteristics (highly charged and water-soluble), individual separation of GLs is hampered, and much effort has been devoted to the development of methods for efficient isolation and identification of these molecules (Clarke, 2010). In the literature, generally the analyses of these compounds are carried out based on the desulphated GLs derivatives, the desulphoglucosinolates (ds-GLs), obtained after enzymatic removal of the sulphate group, which influenced their biological activity. Another technique commonly used to obtain intact GLs is the paired-ion chromatography, which allow quantitative recovery of glucosinolates salts, but the excess of the hydrophobic counter-ion used to elute the GLs also interferes in the following analyses, since they are very toxic to cell culture and inhibit MYR activity (Prestera, Fahey, Hotlzclaw, Abeygunawardana, Kachinski & Talalay, 1996; Fahey et al., 2001; Clarke, 2010).

3.3 Investigation of cytotoxic effect of GLs fractions in the absence and presence of MYR

The fractions selected for the cytotoxicity bioassays including AqF, Lm-II, Lm-III and SaF. To verify whether the biological effect is exerted by the intact GLs or by their hydrolysis products, the potential cytotoxic effect of these fractions was analysed in the presence or absence of myrosinase (MYR). HepG2/C3A and HT29 cell lines were selected for the bioassay based on their origin and relationship with the organs of the digestive system. The nutraceutical and therapeutics health benefits of bioactive food compounds will be influenced by their digestive fate.

HepG2/C3A (**Fig. 3**) and HT29 (**Fig. 4**) cell lines were cultivated with the fractions (AqF, Lm-II, Lm-III and SaF) at different concentrations in the absence and presence of MYR, in order to determine the cytotoxic activity of the GLs or their hydrolysis products. The

chemotherapeutic drug doxorubicin was used as positive control. Cells treated with only MYR (negative group, +MYR, data not shown) showed similar results to the negative control, indicating that the enzyme alone does not interfere in cell viability.

At the highest concentration (200 µg/mL), the Lm-II and Lm-III fractions reduced the cell viability of the HepG2/C3A cells by 89% and 98%, respectively, while the mustard SaF fraction decreased viability by 55% (**Fig. 3**). The half-maximal inhibitory concentration (IC_{50}) values were 118.3±22.2 µg/mL (Lm-II), 69.8±6.3 µg/mL (Lm-III) and 178.0±14.9 µg/mL (SaF). In HT29 cells, % of cell viability reduction observed at 200 µg/mL were 76%, 81% and 46% for Lm-II, Lm-III and SaF, respectively (**Fig. 4**). The IC_{50} values were 102.6±28 µg/mL (Lm-II), 71.5±32.3 µg/mL (Lm-III) and 215.4±1.8 µg/mL (SaF). For the both cell lines, the morphology aspects of the cells (at 200 µg/mL of Lm-II, Lm-III or SaF) corroborated with the observed results. In the absence of MYR, the cells were similar to the control, with larger number of cells adhered to the plate. In the presence of MYR, apparently there was a general reduction in the number of cells, besides the presence of rounded and suspended cells in the medium, which is indicative of cell death by apoptosis, for Lm-II, Lm-III and SaF (**Fig. S.3** and **S.4**, supplementary material). By comparing the IC_{50} values of maca and mustard fractions, it was observed that maca fractions (GTR and GLM) were more efficient than the mustard fraction (GIB) in reducing the viability of both cell lines. This result could indicate that the type of GLs influence on the cytotoxicity effects, however, additional experiments with structural-activity relationship approach will be necessary to explain the differences among the fractions.

AqF fraction did not presented cytotoxicity effects in the absence or in the presence of MYR, on both cell lines (**Fig. 3** and **Fig. 4**). Similar results were found to MEEs and S4 on HepG2/C3A (without MYR) (data not shown). The absence of activity, on the presence of MYR, founded in the AqF fraction (obtained prior to the SPE-C_{18}) can be justified because of

the high sugar content and, consequently, to a relative low concentration of GLs. Thus, a clean-up step to remove the sugar content was relevant on bioassays, to avoid false-negative results.

Lm-II, Lm-III and SaF showed significant cytotoxic effects at highest concentrations in both cell lines, in a dose-dependent manner only in the presence of MYR. To the best of our knowledge, this is the first time that the cytotoxicity mediated by MYR on maca fractions is observed *in vitro*. Although some studies have been described anticarcinogenic effects of these compounds, there is a lack of information to corroborate with this activity (Gonzales et al., 2007; Gasco et al., 2007; Wang, Wang, McNeil & Harvey, 2007; Díaz et al., 2016; Beharry & Heinrich, 2018; Wang & Zhu, 2019). On the one hand, this result indicated *in vitro* low toxicity of the maca fractions, at conditions tested, as verified by other authors (Valentová et al., 2006).

3.4 Comparison on HPLC analysis of fractions submitted (or not) to the treatment with MYR

The cytotoxicity results founded only on + MYR can suggest that the hydrolysis products of GLs may be responsible for this activity. Thus, a HPLC analysis of Lm-II, Lm-III and SaF submitted (+MYR) or not (-MYR) with myrosinase treatment are presented on **Fig. 5**. On the HPLC chromatogram profile of untreated Lm-II (-MYR) can be observed the presence of a first peak (labelled PBS/AA) corresponds to the ascorbic acid, a second one labelled peak 5 and a third one, labelled peak 2 (**Fig. 5**). The last peak corresponds to GTR, according to the chemical analysis described at 3.2 section. For Lm-III (-MYR) a similar HPLC profile was observed, but an additional peak labelled peak 4 was presented. The peak 4 referred to GLM as identified on section 3.2. Comparing the HPLC chromatogram profiles of Lm-II and Lm-III at -MYR condition with those obtained after myrosinase treatment (+MYR), it can be observed the absence of peaks 2 (GTR) and 3 (GLM), showing, as expected, that these compounds were

MYR substrates (**Fig. 5**). The intensity of peak 5 remained unchanged on both chromatograms. This peak was not identified, however, since its presented higher absorbance on 254 than 233 nm, it can be suggested the presence of a phenolic compound on these maca fractions (**Fig. 5**). As described on Table 2 the Lm-II and Lm-III fractions presented a low phenolic content (<20 μgGAE/mg). The influenced of this compound on the cytotoxicity activity remained to be investigated.

In case of SaF, the HPLC chromatogram profile showed at -MYR condition, the presence of a first peak (labelled PBS/AA) corresponds to the ascorbic acid, a second one labelled peak 4 (**Fig. 5**). This peak corresponds to GIB, according to the chemical analysis previously described at 3.2 section. On the chromatogram of SaF after MYR treatment (+MYR) the peak 4 disappeared, indicating that this GL was also hydrolysed by the enzyme.

The monitoring of myrosinase activity and HPLC analysis of Lm-II, Lm-III and SaF tested on bioassay showed that intact GLs present on these fractions disappeared after MYR treatment (**Fig. 5**). Indirectly, and despite not characterized, it can be inferred that the hydrolysis product formed from GTR, GLM and GIB by MYR activity are responsible to the cytotoxicity activities at conditions tested.

3.5 Relationship of cytotoxicity effects of maca and mustard fractions with anticancer effects of isothiocyanates products based on literature data

MYR converts GLs to their hydrolysis products such as isothiocyanates (ITCs) (Barba et al., 2009). The chemical nature of the ITCs depends on the structure of the GL side chain (e.g. aliphatic, aromatic, indole), as well as, of other factors related to reaction conditions, as temperature and pH (Redovniković, Glivetić, Delonga & Vorkapić-Furač, 2008). It has been described that on experimental culture condition (pH 7.4 at 37 ºC), the hydrolysis products of

MYR formed from GL result mainly in ITCs (Leoni et al., 1997). Thus, in this study, GTR (benzylglucosinolate) and GLM (3-metoxibenzyl glucosinolate) could lead to the formation of benzylisothiocyanate (BITC) and 3-methoxybenzyl isothiocyanate (MBITC), respectively.

The chemical nature of ITCs and their related products will influence the lipophilicity properties of these compounds. Lipophilicity is related with ability of ITCs to pass through cell membranes and is also required for ITCs binding to the proteins inside cell through hydrophobic interactions (Zhang, 2001; Wang et al., 2011). ITCs penetrate the cell membrane freely and bind covalently to target proteins, especially glutathione. This feature allows their rapid intracellular accumulation as conjugates to a very high levels, and the modulation of multiple signalling and metabolic pathways. The anticancer effect observed was directly related to the ITCs intracellular levels (Mi, Pasqua & Chung, 2011).

The analysis of the results using a combinatory chemical and bioassay approach of the present study can corroborated with the higher lipophilicity *versus* higher cytotoxicity relationship. The GLs identified on maca and mustard fractions, GIB (3-methylsulfilpropil glucosinolate), GTR (benzylglucosinolate) and GLM (3-metoxibenzyl glucosinolate) presented crescent lipophilicity properties, i.e, GIB is less lipophilic and GLM are more lipophilic. The bioassay results demonstrated that the fraction Lm-III composed by GTR and GLM (with higher lipophilic properties) presented higher cytotoxicity activity (lower IC_{50}) comparing to the other fractions Lm-II (GTR) and SaF (GIB). In addition, SaF fraction presented the less cytotoxicity activity than Lm-II, which can be related to its less lipophilic aspects.

The molecular mechanisms of ITCs (including BITC and MBITC products) as anticancer agents have been described in the literature. In generally, ITCs act in the initiation, promotion and progression of carcinogenic process through different mechanisms: blocking DNA damage through inhibition of Phase I enzymes (mainly cytochrome p450) and inducing Phase II enzymes, inhibiting cell growth by cell cycle arrest and by apoptosis induction (Zhang, 2001).

Many of these studies have been conducted with BITC, which is one of the most active ITCs due to the lipophilicity of its aromatic ring (Zhang, Tang & Gonzales; 2003; Wang et al., 2011). BITC even acts as an antimetastatic agent in human colon cancer HT29 cells through cell migration and invasion inhibition (Lai et al.; 2010); and it is capable of selectively deplete p53 mutant protein in cancer cells (Wang et al., 2011). Carpenter et al. (2018) observed that MBITC also present anticancer activity. This activity was evaluated on experiments with human primary epidermal keratinocytes and 3D human skin exposed to UVB insult and showed that MBITC present antiphotocarcinogenic and antiphotoaging activities.

Recently, Psurski et al. (2019) propose the 3,4-dimethoxybenzyl ITC (dMBITC) as one of the most potent ITC and a promising anticancer agent. In the study was verified that dMBITC enhanced doxorubicin antitumor potential in a multidrug-resistant colonic cancer cell line (LoVoDX) and attenuates some parameters of doxorubicin toxicity, with clinically relevant doses which can be acquired by a diet based in cruciferous vegetables. The mentioned authors suggested ITCs as potential candidates for employment in anticancer combined treatment to enhance the effectiveness of classical chemotherapeutics.

Unfortunately, the molecular mechanism of action of the hydrolysis products of GLs found on maca and mustard fraction tested on the present study had not been investigated. However, for the first time that cytotoxic effects of maca GLs mediated to MYR were observed *in vitro*. Thus, these results could contribute for design of future *in vitro* and *in vivo* studies focus on anticancer properties by using therapeutic and nutritional approach using MYR-mediated products of this specie.

4. Conclusions

In this study, intact GL-enriched fractions from maca were obtained and characterized by combining different approaches of chromatographic and spectrometric techniques. Lm-II and Lm-III presented the highest GL content (GTR and GLM) among the fractions and were obtained after a sugar cleaning step using SPE-C_{18}, which was crucial for assessing the biological activity of GLs. Their cytotoxic effects on HepG2/C3A and HT29 cells were accessed by MTT assay combined with myrosinase enzyme-mediated hydrolysis of GLs. These fractions were cytotoxic for both tumoral cell lines in a dose-dependent manner, only in the presence of MYR. To the best of our knowledge, this is the first time that cytotoxic effects of maca GLs mediated to MYR were observed *in vitro*.

Due to the importance of GLs which are founded in both ethanolic and aqueous extracts, usual ways of consumption of this plant, these results could permit a range of future studies on the activities of MYR-mediated products, such as *in vitro* investigation of the mechanisms involved in the anticancer process, as well as, *in vivo* studies with animal experimental models or human nutritional-based controlled trial approaches.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure Captions

Fig. 1 Schematic of the methodology protocol used to obtain of maca fractions.

Fig. 2 Identification of compounds in the maca (Lm-II and Lm-III) and mustard (SaF) fractions based on the m/z.

Fig. 3 Cell viability (%) of HepG2/C3A cell line after 48 h of incubation with 6.25–250 μg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the (A) absence (-MYR) or (B) presence (+MYR) of myrosinase, measured using the MTT assay.

Fig. 4 Cell viability (%) of HT29 cell line after 48 h of incubation with 6.25–250 μg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the (A) absence (-MYR) or (B) presence (+MYR) of myrosinase, measured using the MTT assay.

Fig. 5 HPLC chromatograms of maca (Lm-II, Lm-III) and mustard (SaF) fractions before (-MYR) and after (+MYR) myrosinase treatment.

Fig. S.1 Chromatographic profile of SPE extraction using water as eluent, giving rise to the Lm-I to Lm-III fractions (Lm- *Lepidium meyenii*).

Fig. S.2 $^{1}H–^{13}C$ HSQC NMR spectrum of Lm-I in D_2O at 25 °C.

Fig. S.3 Mass spectra (negative ion mode) of peak at Rt 15.1±0.1 min in HPLC chromatogram obtained from maca S4 fraction in condition A.

Fig. S.4 Photomicrograph obtained using the EVOS™ FL Auto Imaging System of HepG2/C3A cells after treatment with 200 μg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the absence (-MYR) or presence (+MYR) of myrosinase fractions (460× magnification).

Fig. S.5 Photomicrograph obtained using the EVOS™ FL Auto Imaging System of HT29 cells after treatment with 200 μg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the absence (-MYR) or presence (+MYR) of myrosinase fractions (460× magnification).

Table 1 - Analysis of glucosinolates, total phenolic and sucrose contents of maca fractions and mustard SaF fraction.

Plant	Fraction names[a]	GLs content (µg/mg) [b]			Total phenolic [c] (µg GAE/mg)	Sucrose content [d]
		GTR	GLM	GIB		
Lepidium meyenii	MEEs	Nd	nd	-	Tr	++
	EaF	Nd	nd	-	48.7±3.6	-
	AqF	8.7	1.3	-	Tr	++
	S3	17.3	1.4	-	Tr	++
	Lm-II	195.4	-	-	Tr	+
	Lm-III	169.9	66.6	-	Tr	+
	Lm-IV	-	34.9	-	27.8±2.2	-
Sinapis sp.	SaF	-	-	238.0	Tr	+

[a] See Material and Methods section for abbreviations. [b] Glucosinolates (GLs) content (GTR, glucotropaeolin; GLM, glucoliminanthin; GIB, glucoiberin) expressed as µg GLs/mg of fraction; determined by HPLC (condition A, for more details see section 2.2.1); nd, not determined. [c] Determined by the Folin–Ciocalteu method expressed as µg gallic-acid equivalent (GAE)/mg of fraction; tr, traces (< 20 µg GAE/mg). [d]Qualitative analysis of sucrose content by using TLC analysis: band intensity: ++ (= or > intensity compared to sucrose standard at 1 mg/mL); + (< intensity compared to sucrose standard at 1 mg/mL); - (not detected). The sucrose presence was also confirmed by disappearance of sucrose band on TLC analysis after invertase treatment (data not shown).

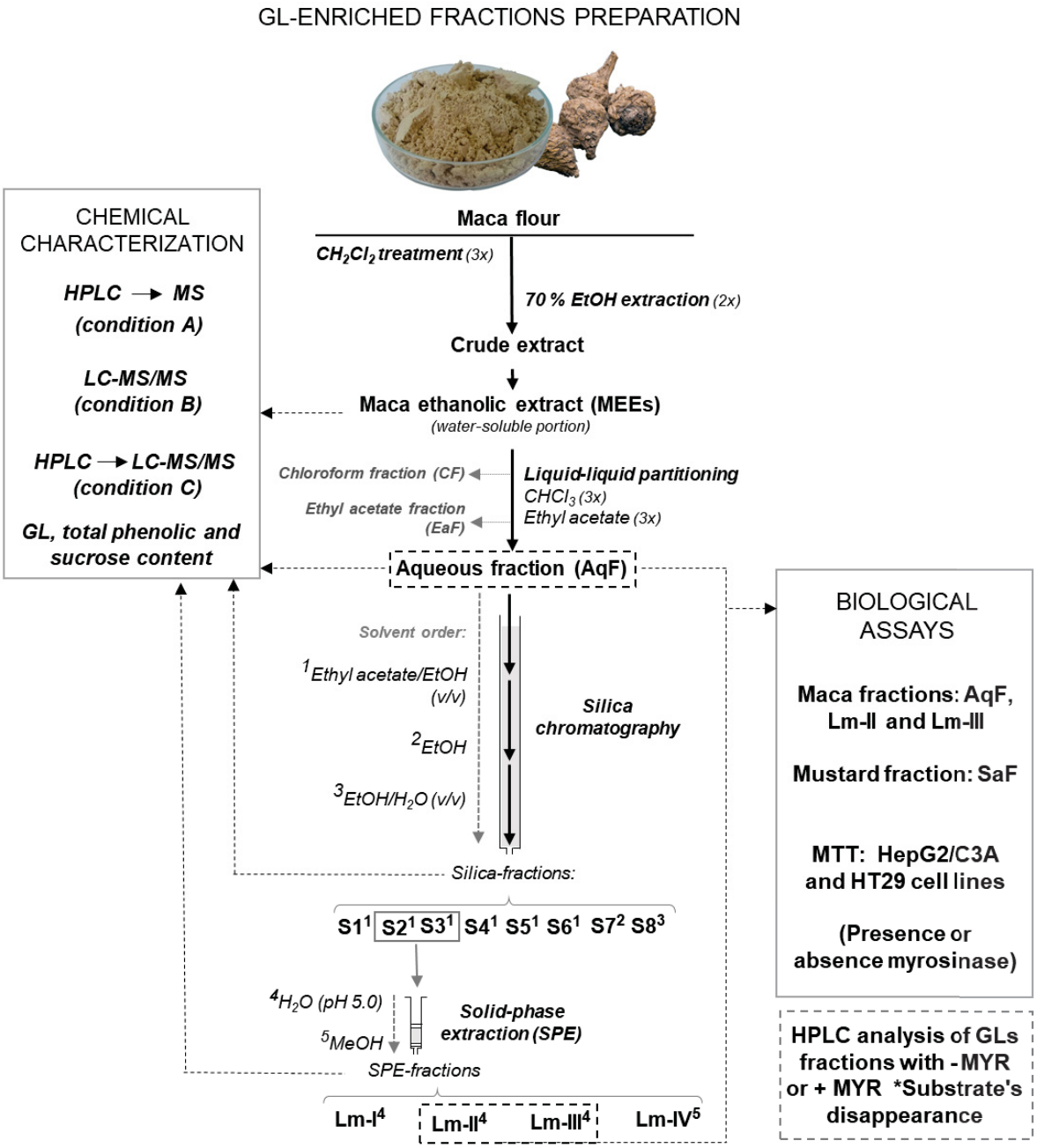
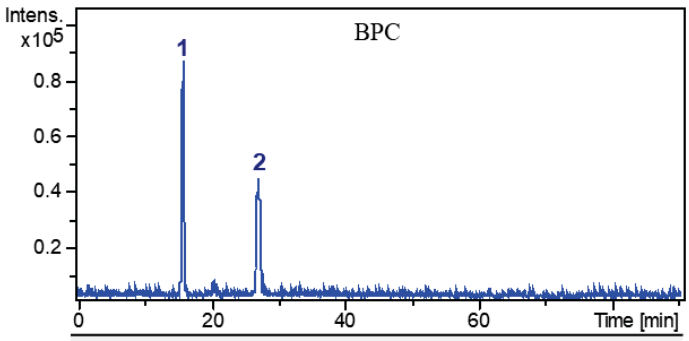
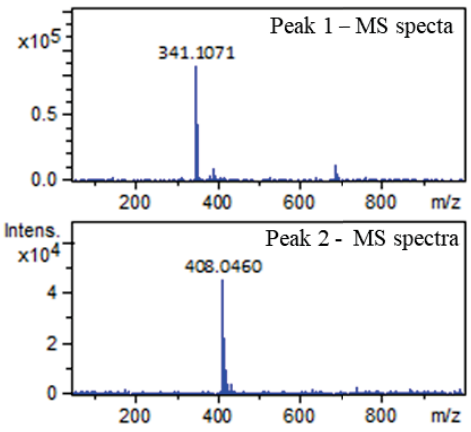


Fig. 1 - Schematic of the methodology protocol used to obtain of maca fractions

The superscript numbers are related to the solvent order used in fractions elution. For more details on the terminology used for the fractions, see section 2.1.2.

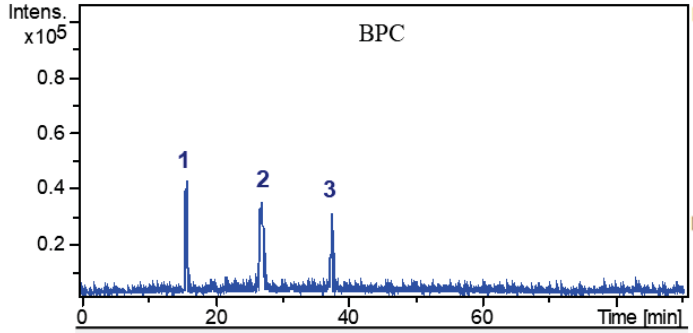
Lm-II

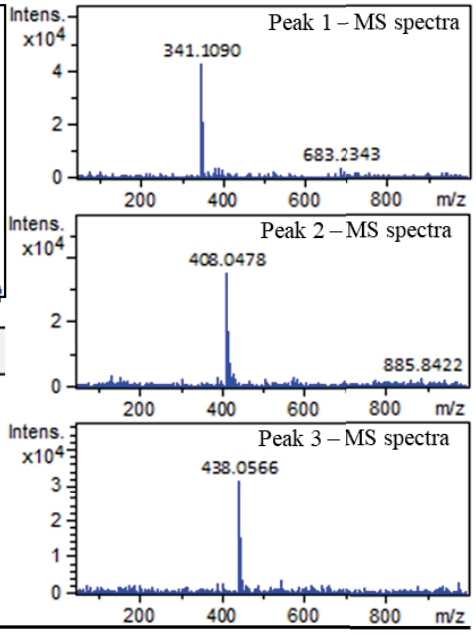




Peak	Rt (min)	[M-H] (m/z)	MS²	Compound
1	16.0	341.1071	179.0552	[Hex-Hex][a]
2	27.2	408.0460	79.9570 96.9599 259.0119	GTR[b]

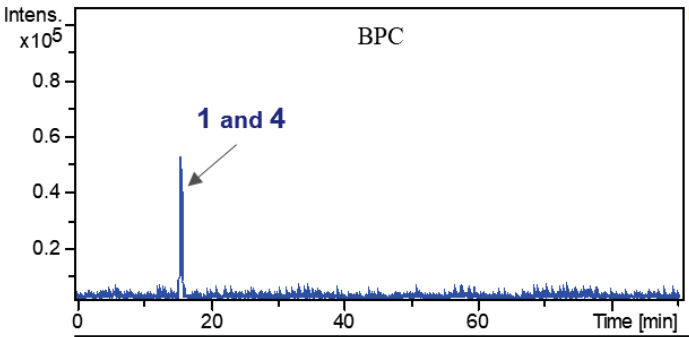
Lm-III

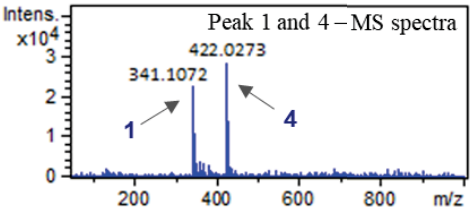




Peak	Rt (min)	[M-H] (m/z)	MS²	Compound
1	16.0	341.1090	179.0533	[Hex-Hex][a]
2	27.2	408.0478	79.9575 96.9600 259.0142	GTR[b]
3	37.7	438.0566	79.9571 96.9608 195.4040	GLM[c]

SaF





Peak	Rt (min)	[M-H] (m/z)	MS²	Compound
1 and 4	16.0	341.1072	nd	[Hex-Hex]
		422.0273	79.9572, 96.9599, 180.0156, 195.9733, 358.6208	GIB[b]

(cont.)

Fig. 2 - Identification of compounds in the maca (Lm-II and Lm-III) and mustard (SaF) fractions based on the m/z.

The base peak chromatogram (BPC) and MS spectra were obtained by condition C. MS^2 fragment ions reported in the respective tables were assignments according to results obtained in condition A and B and literature data ([a] Antonio et al., 2009 and confirmed by NMR, see 3.2 section; [b] Fabre et al., 2007 and Shi et al., 2007; [c] Popova & Mora 2014 and Fabre et al., 2007). GIB, glucoiberin; GLM, glucoliminanthin; GTR, glucotropaeolin; Rt, retention time; nd, not detected; [Hex-Hex], disaccharide composed by two hexoses.

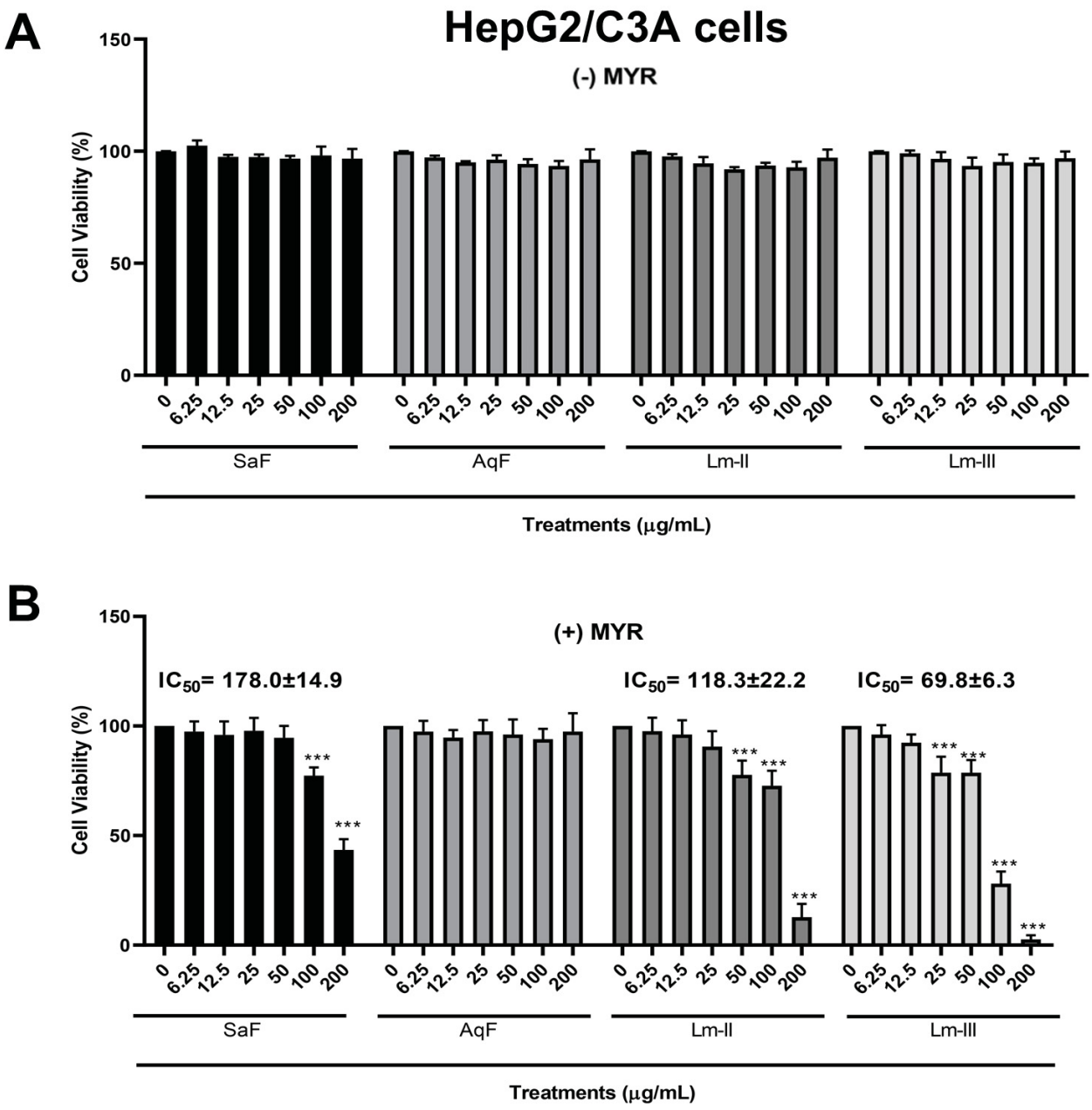


Fig. 3 - Cell viability (%) of HepG2/C3A cell line after 48 h of incubation with 6.25–250 µg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the (A) absence (-MYR) or (B) presence (+MYR) of myrosinase, measured using the MTT assay. Results are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to control using ANOVA followed by Tukey. The IC_{50} values represent the final concentration of the test fraction [µg/mL] that can reduce the cell viability by 50%. When absent, IC_{50} values were not achieved.

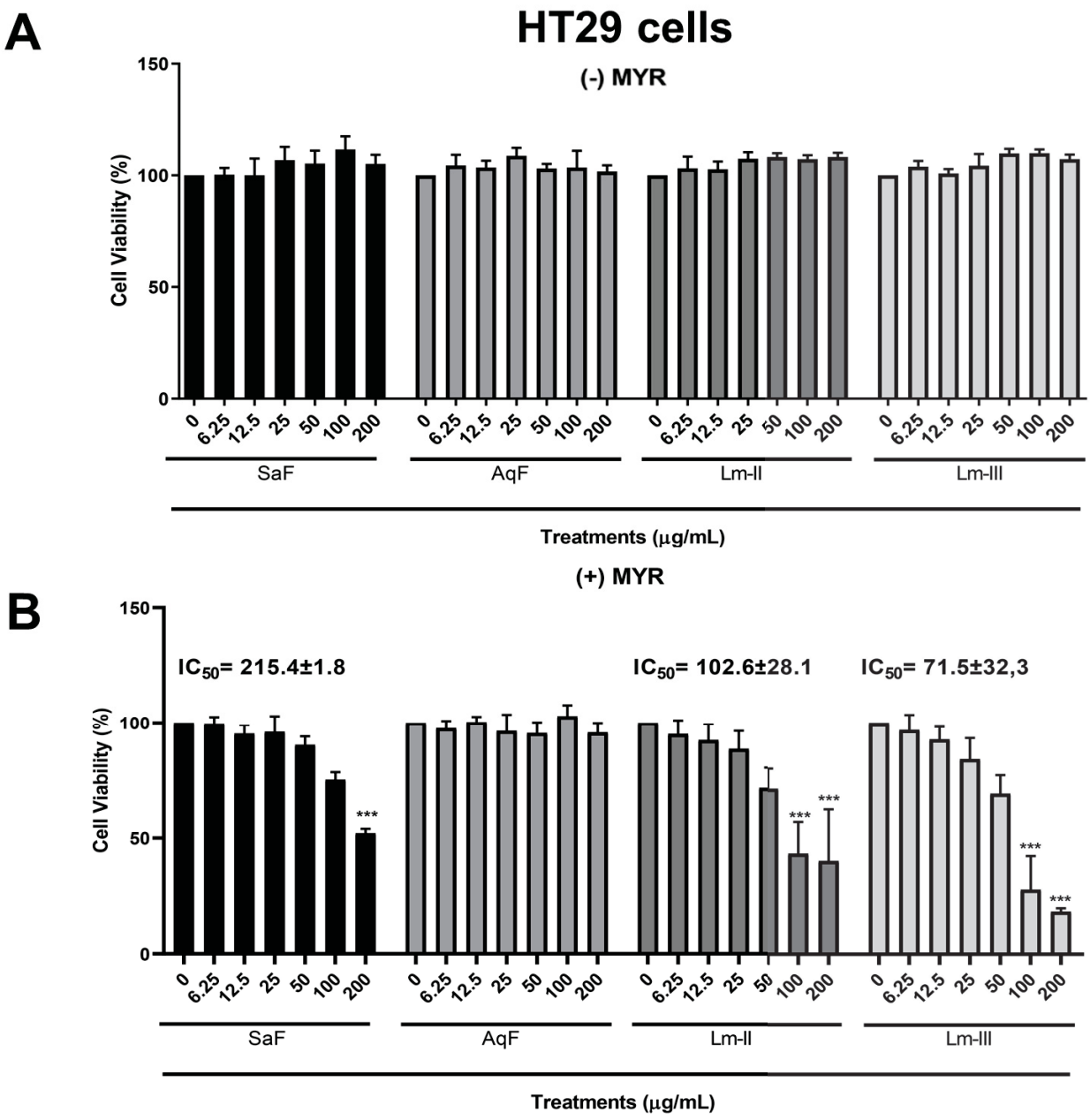


Fig. 4 - Cell viability (%) of HT29 cell line after 48 h of incubation with 6.25–250 µg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the (A) absence (-MYR) or (B) presence (+MYR) of myrosinase, measured using the MTT assay. Results are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to control using ANOVA followed by Tukey. The IC_{50} values represent the final concentration of the test fraction [µg/mL] that can reduce the cell viability by 50%. When absent, IC_{50} values were not achieved.

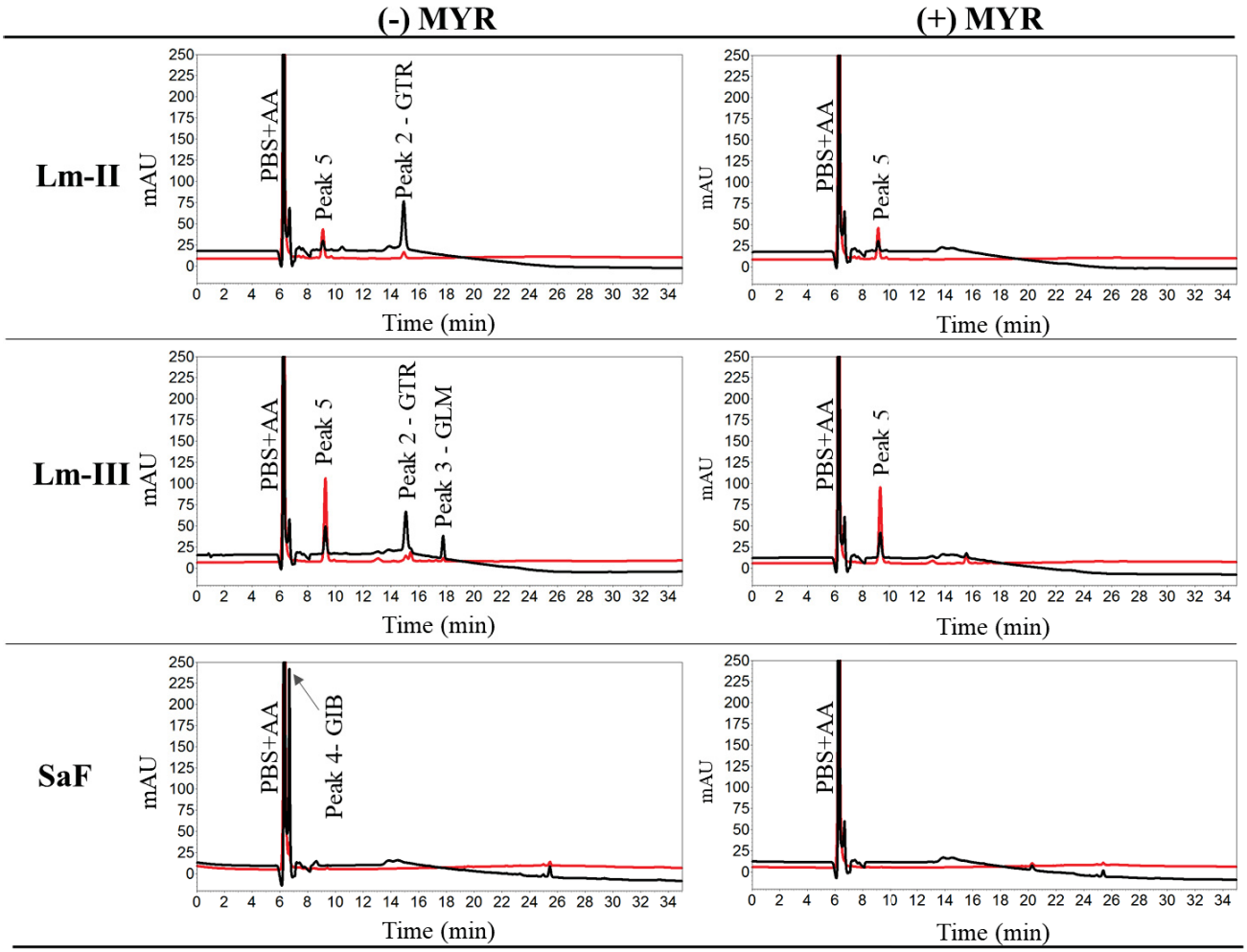


Fig. 5 - HPLC chromatograms of maca (Lm-II, Lm-III) and mustard (SaF) fractions before (-MYR) and after (+MYR) myrosinase treatment.

The HPLC chromatograms were obtained by condition analysis A (see section 2.2.1). Peaks are numbered according to **Fig. 2.** UV detection at 233 nm is represented by a black line, and at 254 nm by a red line. GLs peaks are characterized by higher absorption in 233 nm than in 254 nm. GIB, glucoiberin; GLM, glucoliminanthin; GTR, glucotropaeolin; PBS+AA: phosphate buffer saline pH 7.4 containing 1 mM ascorbic acid.

SUPPLEMENTARY MATERIAL

Table S1 - Qualitative data of chromatographic profile obtained by TLC of the silica fractions (maca) and SaF (mustard)

Retention factor (R_f) values for bands		Maca or mustard fractions												Standards								
		EaF	MEEs	AqF	S1	S2	S3	S4	S5	S6	S7	S8	SaF	1	2	3	4	5	6	7	8	9
UV	*0.82*	+++	-	-	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(254 nm)	*0.68*	+++	-	-	-	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	0.61	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-
Vanillin-sulphuric stain	0.99[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-
	0.99[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
	0.82[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-
	0.67[d]	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	0.64[d]	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	0.54[d]	-	++	++	-	+	++	++	-	-	-	-	-	++	++	-	-	-	-	-	-	-
	0.50[e]	-	++	++	-	-	-	-	-	++	++	-	-	-	-	-	++	-	-	-	-	-
	0.47[d]	-	+++	+++	-	+	++	+++	+++	-	-	-	++	-	-	++	-	-	-	-	-	-
	0.37[d]	-	-	-	-	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-
	0.3[d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++
	0.22[d]	-	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
	0.18[d]	-	++	++	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-

Standards: 1-Sinigrin (GL), 2-Glucose (monosaccharide), 3-Sucrose (disaccharide), 4-Fructose (monosaccharide), 5-Caffeine (alkaloid), 6- β-Sitosterol (sterol), 7-Epigallocatechin (flavanol), 8-Quercetin (flavonol), 9-Saponin (triterpene). Staining color: [a] brown-red, [b] yellow, [c] violet, [d] brown-grey and [e] blue-grey. Band intensity: - not detected; + weak; ++ intermediate and +++ strong. For more details see Material and methods section.

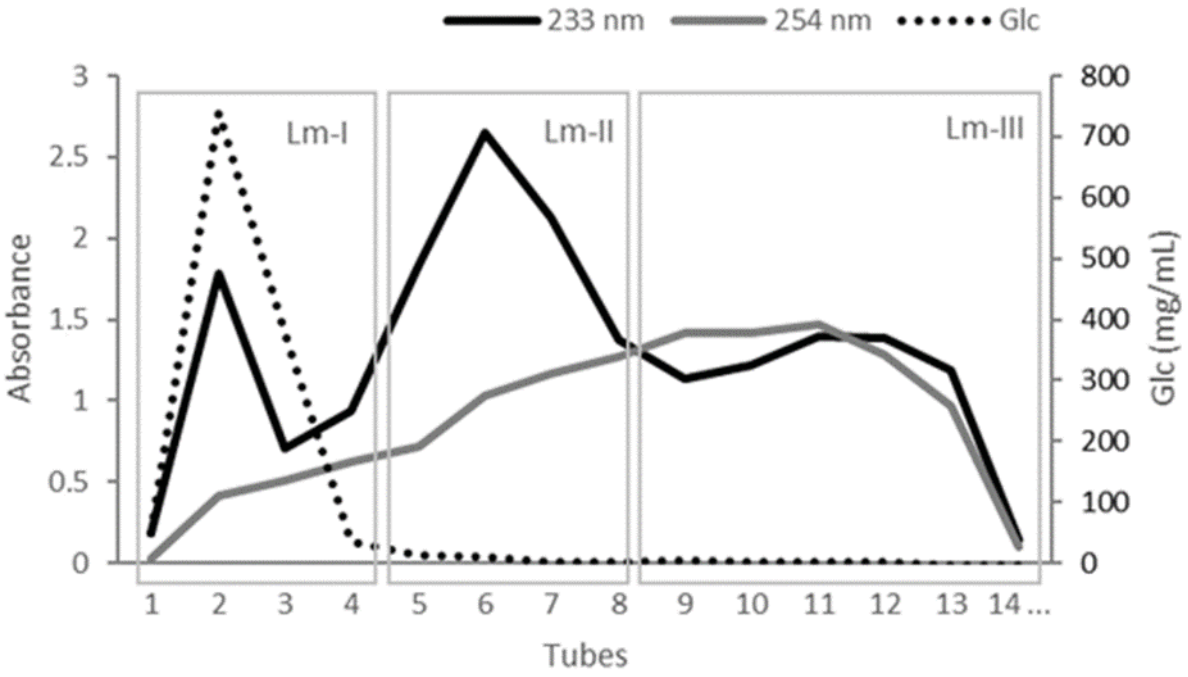


Fig. S.1 - Chromatographic profile of SPE extraction using water as eluent, giving rise to the Lm-I to Lm-III fractions (Lm- *Lepidium meyenii*).

UV absorbance at 233 and 254 was used to monitor GLs and phenolic compounds elution, respectively. Glucose elution was monitored through Glc content measurement using a commercial enzymatic assay kit.

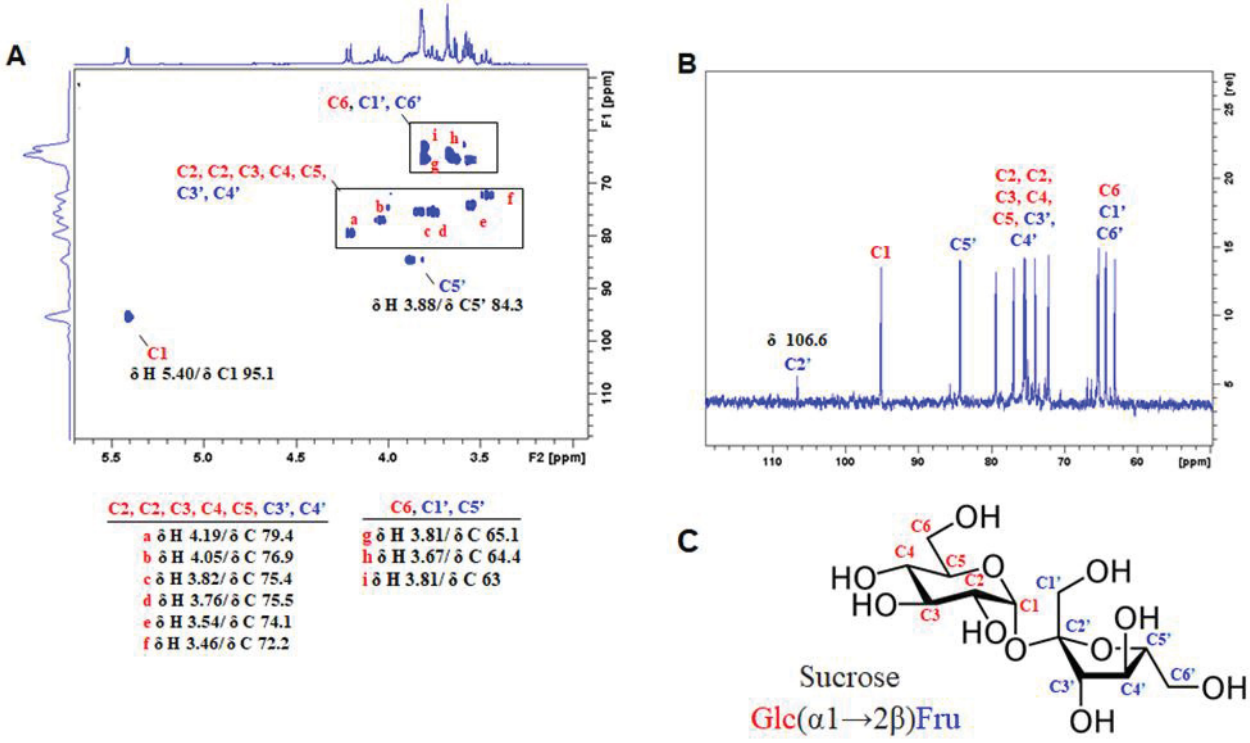


Fig. S.2 - $^{1}H–^{13}C$ HSQC NMR spectrum of Lm-I in D_2O at 30 °C. (A) HSQC spectra, (B) ^{13}C spectra and (C) sucrose structure and C numbers attribution. Glc, glucose; Fru, fructose.

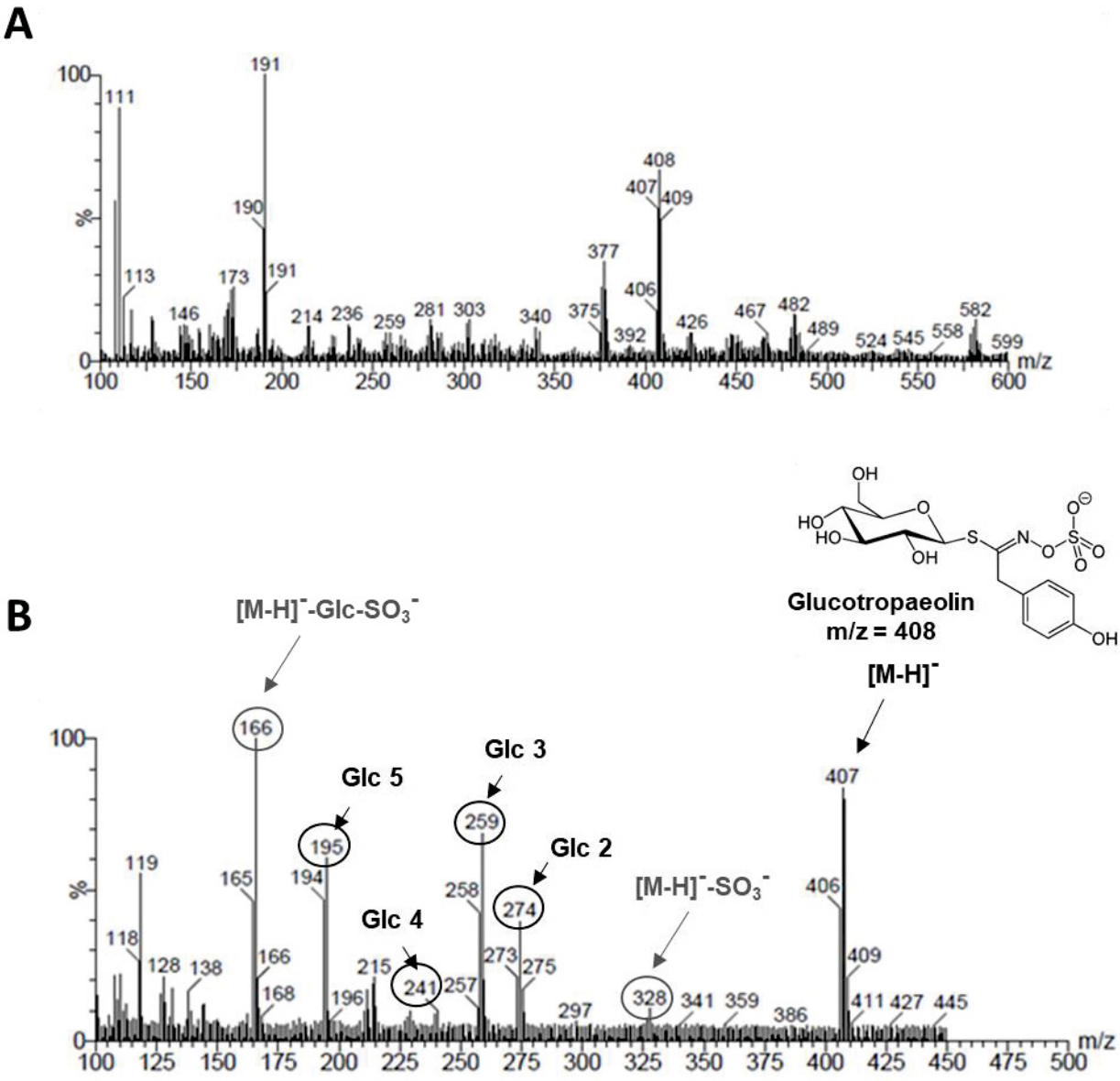


Fig. S.3 - Mass spectra (negative ion mode) of peak at Rt 15.1±0.1 min in HPLC chromatogram obtained from maca S4 fraction in condition A.

(A) MS full scan spectra. (B) MS/MS spectra obtained from $[M-H]^{-1}$ m/z 408, ion fragmentation, marker from glucotropaeolin (GTP). The m/z values circled in black corresponds to GL-specific markers, and in grey, to GTP-specific markers.

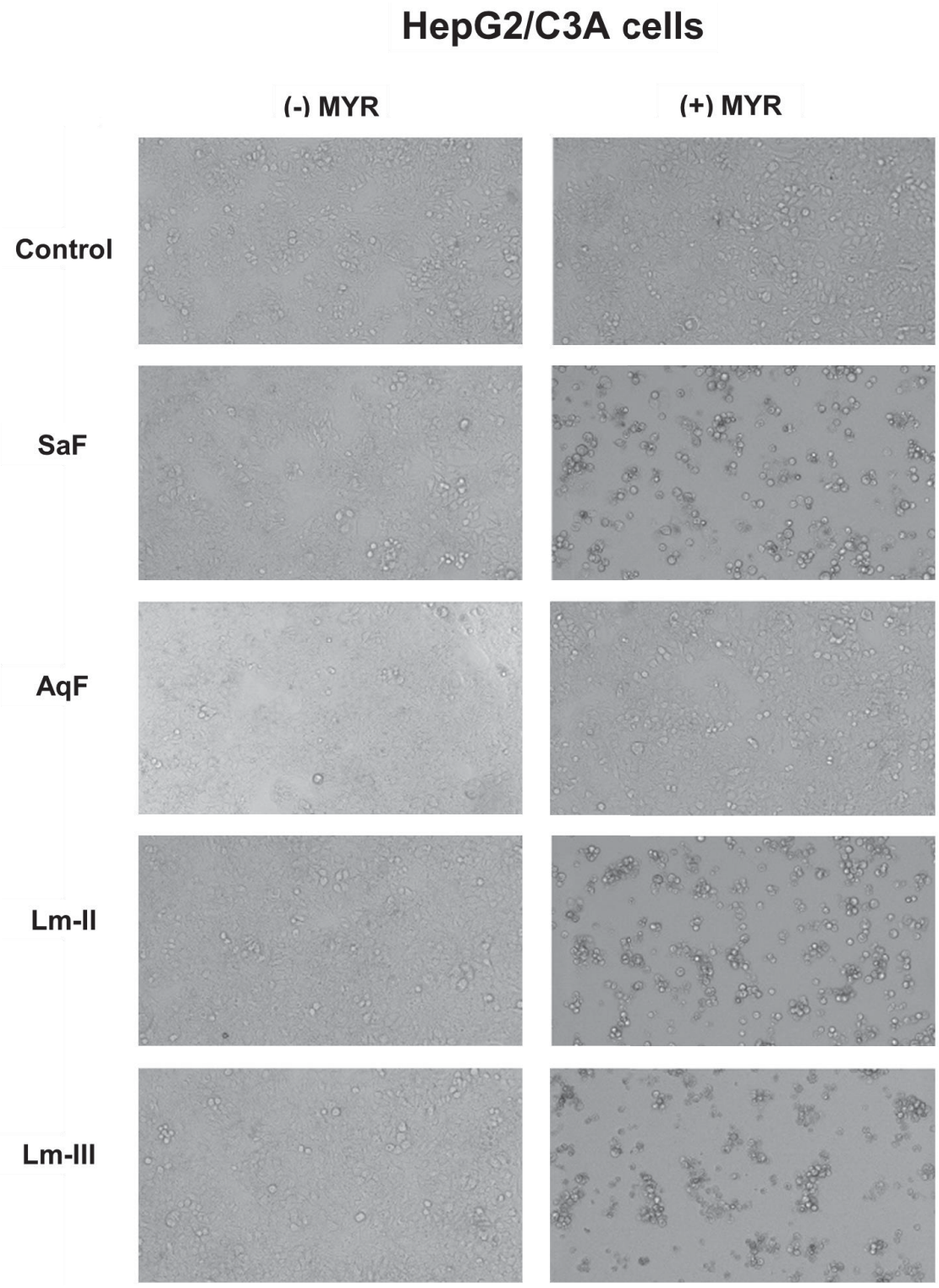


Fig. S.4 - Photomicrograph obtained using the EVOS™ FL Auto Imaging System of HepG2/C3A cells after treatment with 200 µg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the absence (-MYR) or presence (+MYR) of myrosinase fractions (460× magnification).

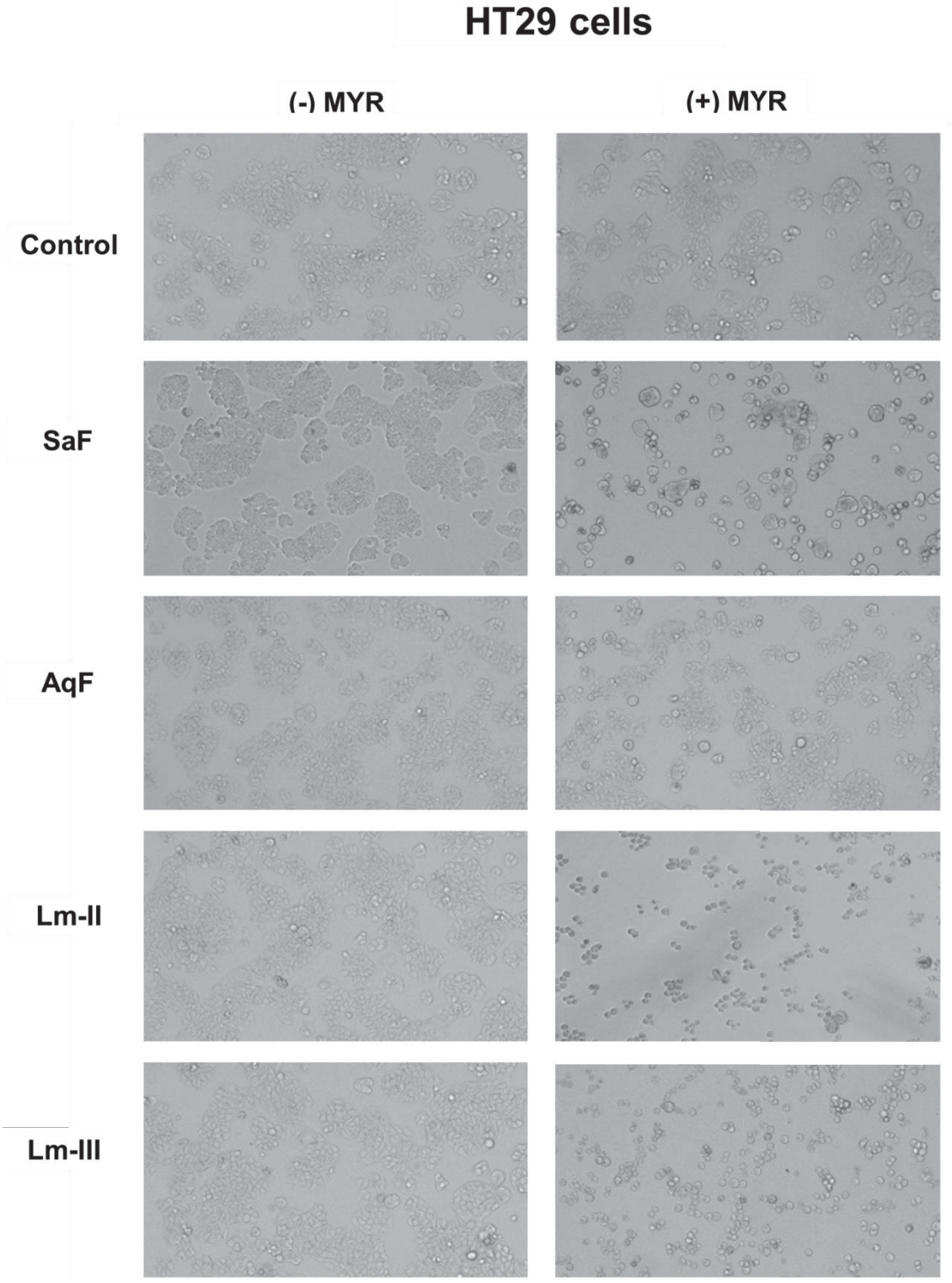


Fig. S.5 - Photomicrograph obtained using the EVOS™ FL Auto Imaging System of HT29 cells after treatment with 200 µg/mL maca (AqF, Lm-II and Lm-III) and mustard (SaF) fractions in the absence (-MYR) or presence (+MYR) of myrosinase fractions (460× magnification).

ARTIGO II

AGP rich fractions of maca roots: chemical characterization and complement fixating properties

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AGP rich fractions of maca roots: chemical characterization and complement fixating properties

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Abstract

Lepidium meyenii Walpers, known as Peruvian Maca, was submitted to enzymatic inactivation and deslipidification. After that, a total of 11 maca polysaccharide fractions were obtained by sequential extraction performed with H_2O at 55 ºC, H_2O at 85 ºC, 1 M, 2 M and 4 M NaOH, after enzymatic starch removal. Arabinose, galactose and glucose were the main monosaccharide constituents in the water-soluble fractions (MP-Ws-R and MP-HW) and in the alkaline fractions (correspondent to hemicelluloses A and B), which presents additionally xylose. MP-Ws-R (MW 298 kDa), MP-HW (MW 2720 kDa) and MP-1Ab (MW 1680 kDa) fractions showed an AGP content of 34.3±0.5, 14.6±1.4 and 30.2±1.1%, respectively. Considering chemical and NMR analysis it can be suggested the presence of arabinogalactan type II (substituted with Rha, 4-*O*-Me-GlcA), and xyloglucans on the maca polysaccharide fractions. MP-Ws-R, MP-HW and MP-1Ab were selected to investigate their complement fixating activity. Their modulatory effects on the classical (CP) and alternative (AP) pathways

of the complement system (CS) was accessed by the haemolytic fixation test and comparing with heparin (inhibition control). With pre-incubation, the IC_{50} of Heparin, MP-Ws-R, MP-HW and MP-1Ab were 40.0, 204.9, 303.8 and 886.9 μg/mL for CP; and 280.2, 224.4, 411.8 and 387 μg/mL for AP, respectively. Without pre-incubation, it is only possible to calculate the heparin IC_{50}. The results indicated that MP-Ws-R, MP-HW and MP-1Ab acts as CS activators in both classical and alternative pathways, since their modulation profile were distinct to that of heparin.

Key words: arabinogalactan-protein, ß-glucosyl Yariv, hemicellulose, immunomodulation.

1. Introduction

Maca *(Lepidium meyenii* Walpers) is a plant originally from the Peruvian Andes and popularly used as food and for medicinal purposes [1]. Traditionally, maca is recognized for its aphrodisiac and energy properties, however, has gained notoriety in the international market since the late 90's due to the claims of indigenous knowledge surrounding improvement of immune system functions, promising to combat leukaemia and AIDS [2].

The dried maca roots are consumed after boiling in water or processed into flour, and consist mainly of carbohydrates (60-70%) [3]. Whereas the edible part of the maca is a storage organ, most of the carbohydrates (23 a 30%) correspond to starch, the typical storage polysaccharide fraction, composed by amylose [linear α-(1→4)-glucan] and amylopectin [ramified α-(1→4) and α-(1→6) glucan] [4,5]. Non-starch maca polysaccharides have been characterized as bioactive compounds with different biological properties as antifatigue [5-7], antioxidative [8] and hepatoprotective effects [9], besides immunoregulatory properties [10,11]. These polysaccharides are structural components of the plant cell wall and have been isolated by aqueous or alkaline extractions. Maca polysaccharides are usually consisting of

arabinose (Ara), glucose (Glc), galactose (Gal) and mannose (Man), with different molar ratio [12]. Previous studies from our research group (unpublished data, [13]) suggested the presence of xyloglucans, arabinans, galactans and arabinogalactans on fractions obtained through aqueous and/or alkaline extraction of Peruvian maca flour. In addition, one polysaccharide fraction mainly composed by Ara and Gal, present positive reaction with β-Glucosyl Yariv reagent (β-Glc Y), indicating the presence of arabinogalactan-proteins. Arabinogalactan proteins (AGPs) are proteoglycans of the plant cell wall extracellular matrix, which are composed by type II arabinogalactan chains (mainly of 3-, 6- and 3,6-linked β-D-Gal*p* residues, substituted with α-L-Ara*f* residues) and a protein moiety (1–10% of the total mass of the molecule) rich in hydroxyproline [14,15]. AGPs have the typically ability to precipitate with β-GlcY, enabling their easy detection and quantification even in crude plant extracts [14]. These molecules have been also characterized as modulators of the classical and alternative pathways of the complement system [16-18].

The complement system (CS) is an oldest and highly conserved immune defence mechanism in all vertebrates [19] responsible for enhance phagocytosis and inflammation, eliminate pathogens through osmotic lysis, removes injured cells and dispose circulating immune complexes [20]. CS is composed by over fifty serum circulating proteins, which forms a complex network with cell surface receptors and regulators to play an instrumental role in immunological and inflammatory processes during homeostasis. Three different pathways, classical (CP), lectin (LP) and alternative (AP), can activate the proteolytic CS cascade through varying in the pattern recognition (antibody-dependent, mannose-binding lectin/ficolins and spontaneous formation of C3b-like molecules, respectively). The three pathways result in the formation of C3 and C5 convertases and culminate in the generation of the opsonin C3b, the potent anaphylatoxins C3a and C5a and the terminal membrane attack complex (MAC) [21]. Complement inhibition or stimulation represents different modulation approaches to this

system, and both are relevant targets for drug development. Inhibition of complement may be useful in cases of organ transplantation and in the use of extracorporeal circuits, reducing the tissue damage caused in these situations, whereas complement activation is important for host defence against microbial pathogens [16].

Thus, the aim of this work was to obtain and chemically characterize polysaccharide fractions from maca root flour, through aqueous and alkaline extractions and to investigate their effect on the modulation of the classical and alternative pathways of the CS *in vitro*.

2. Material and Methods

2.1 Chemicals

Ethylenediamine tetraacetic acid disodium (EDTA.Na_2), ethylene glycol tetraacetic acid (EGTA), gallic acid, gum Arabic (GA), [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Agarose Type C gel was obtained from Calbiochem (La Jolla, CA, USA). Folin-Ciocalteu reagent was obtained from Chromate (São Paulo, SP, Brazil), hemolysin (rabbit anti-sheep erythrocyte antibody) was acquired from Laborclin (Pinhais, PR, Brazil). Water was treated in an ultra-purifier MS 2000 model from Gehaka (São Paulo, Brazil). All other chemicals or standards were commercial products of the highest available purity.

2.2 Plant material and maca polysaccharide-rich fractions preparation

The Peruvian maca (*Lepidium meyenii* Walpers) root flour was commercially acquired in a store in Curitiba, Brazil (HEMA Comércio de Cereais Sítio Cercado, Brazilian Health Regulatory Agency - ANVISA - registration: 1.10.746-4).

Firstly, maca root flour [200 g, with 400 mL of ETOH:H_2O (4:1, v:v) solution] was submitted to enzymatic inactivation, under reflux in condenser for 30 min (**Fig. 1**). After

filtration, the supernatant was discarded. This process was performed three times and at the end, there was a 15% reduction of weight in relation to the initial dry weight. The dried inactivated maca flour was then defatted in a Soxhlet extraction apparatus with toluene: ETOH solution (2:1, v:v) for approximately 12 h (until the toluene: ETOH solution was colourless). The resulting maca (78% of initial material), was dried at room temperature, and kept at -4 ºC.

The inactivated and defatted maca (150 g) was subjected to enzymatic starch removal using 1.47 mL (0.0032 U/mL) of amyloglucosidase (Megazyme, Bray, Ireland), and 750 µL (10 U/mL) of thermostable α-amylase (Sigma-Aldrich Co., St Louis, MO, USA) in aqueous solution (1.5 L). A suspension (100 mg/mL) with 150 g maca in 1.5 L H_2O was prepared and left under mechanic stirring for 30 min at room temperature. After this time, the pH solution was 6.25. The temperature was adjusted to 55 °C and then the enzymes were added. The reaction was kept under gentle agitation for 4 h. The monitoring of the enzymatic reaction was performed by verifying the presence of starch and reducing sugar by iodine test and 3,5-dinitrosalicylic acid (DNS) assay, respectively. The reaction was stopped when the result of starch detection was negative by iodine test. After that, the solution was centrifugated (1.480 x g, for 30 min, at 25°C), resulting *supernatant* and *pellet (residue)*.

The *supernatant* was boiled for 15 min to denature the enzymes (added for starch degradation), which were removed by centrifugation (1.480 x, 4 °C, 30 min) and discarded. The resulting supernatant was concentrated to approximately 100 mL and submitted to precipitation with 20% (w/v) trichloroacetic acid (TCA). This step was to ensure complete removal of the enzymes. After 1h at 4 °C, the solution was centrifuged (1.480 x g, for 30 min, at 4 °C), the supernatant neutralized with concentrated NaOH and dialyzed in a closed system for 7 h (acetate cellulose membrane with size exclusion of 12 - 14 kDa). Finally, 3 x ETOH vol. were added to the dialysed solution in order to precipitate polysaccharide fraction extract during the enzymatic inactivation. After centrifugation, the resulting precipitate was freeze-dried was

named MP-W (maca polysaccharide obtained by extraction with H_2O at 55 ºC). MP-W fraction was submitted to another round of dialysis (acetate cellulose membrane with exclusion size limit of 12 - 14 kDa), in an open system for 20 h, and, then subjected to freeze-thawing process (freeze, -20 °C; thaw, 25 °C, one cycle). After centrifugation (1.480 x g for 30 min at 4 °C) the supernatant was lyophilized, yielding the MP-Ws, referred to soluble fraction obtained by freeze-thawing process; the pellet resulted MP-Wp (referred to the insoluble fraction soluble obtained by freeze-thawing process). MP-Ws was submitted to ultrafiltration under N_2 pressure with regenerated cellulose with exclusion limit of 0.05 μm (Sartorius, Gottingen, German). The process was carried out in a filtration system (model 16249, Sartorius, Gottingen, German), in which 20 mL of MP-Ws (50 mg/mL) was placed. The eluted fraction (MP-Ws-E) and the retained fraction, named MP-Ws-R were lyophilized. MP-Ws-R referred to maca polysaccharide obtained by extraction with H_2O at 55 ºC which was retained on the ultrafiltration step.

The residue (pellet) (obtained after enzymatic starch removal) was washed with H_2O and ETOH and dried at room temperature, resulting in the inactivated, defatted and starch-free maca flour (35% of initial material). The residue was subjected to sequential extractions (aqueous and alkaline). Firstly, the residue (150 g in 1.5 L H_2O) was submitted to extraction at 85 ºC, for 2 h, under mechanical stirring. The material was filtered and centrifuged (1.480 x g for 15 min at 4 °C). The resulting supernatant was submitted to ETOH precipitation (3 x vol.), centrifuged and lyophilized. Resulting the fraction labelled MP-HW, as referred to maca polysaccharide obtained by extraction with H_2O at 85 ºC. The residue was subjected to alkaline extractions with 1 M, 2 M and 4 M of aqueous NaOH (1 L) for 1 h at 25 ºC, under mechanical stirring in the presence of sodium borohydride, for extraction of hemicelluloses [22]. At the end of 1 M alkaline extraction, the solution was centrifugated (2755 x g for 10 min at 4 °C), the residue was destinated for the subsequent alkaline extraction and the supernatant was acidified

with 50% acetic acid until pH 5.0. Additional centrifugation step (2755 x g for 30 min at 4 °C), yield a pellet (correspondent to insoluble polysaccharides at pH 5 or referred as hemicellulose a), which was dialyzed for 24 h, freeze-dried, and called MP-1Aa (referred maca polysaccharide obtained by 1 M alkaline extraction - hemicellulose a). The supernatant (of additional centrifugation) was dialyzed for 24 h and then submitted to ethanolic precipitation (3 x vol.). After centrifugation (2755 x g for 30 min at 4 °C), the pellet was freeze-dried and labelled as MP-1Ab, referred as maca polysaccharide obtained by 1M alkaline extraction - hemicellulose b. The same process was performed for 2 M alkaline extractions, giving MP-2Aa and MP-2Ab, and for 4 M alkaline extraction, giving MP-4Aa and MP-4Ab. The schematic protocol of the isolation of maca polysaccharides is showed in **Fig. 1**.

2.3 Chemical characterization

2.3.1 Determination of the total carbohydrate, uronic acid, protein and phenolic content

Total carbohydrate content was measured by the phenol sulfuric acid micro method adapted from Fox & Robyt using D-glucose (0.4 to 40 μg/mL) as a standard [23]. Uronic acid content was estimated by the improved *m*-hydroxybiphenyl method from Filisetti-Cozzi & Carpita using a calibration curve prepared with glucuronic acid (GlcA) (1 to 100 μg/mL) [24]. Protein content was determined by a colorimetric microassay using bovine albumin (1 to 10 μg/mL) as a standard [25], and the total phenol content was calculated by a micro-assay described by Melo et al. [26], based in the methodology developed by Singleton, Orthofer & Lamuela-Raventós (1999) using a gallic acid (0.2 to 1 mg/mL) standard curve [27].

2.3.2 Quantification of arabinogalactan-protein by single radial gel diffusion assay

AGP contents of the maca polysaccharide fractions were quantified by the single radial diffusion assay described by Van Holst & Clarke [28]. The fractions (MP-W, MP-Wp, MP-Ws, MP-Ws-R, MP-Ws-E, MP-HW, MP-1Aa, MP-1Ab, MP-2Aa, MP-2Ab, MP-4Aa and MP-4Ab) were solubilized in 1% NaCl solution and allowed to diffuse in a humid environment (at room temperature for 18h) into a 1% agarose Type C gel (Calbiochem, La Jolla-CA, USA) containing 0.02 mg/mL of the β-glucosyl Yariv reagent (β-GlcY), which was synthesized as described by Yariv, Rapport and Graf [29].

2.3.3 Monosaccharide composition

The neutral monosaccharide composition was determined using the method reported by Pettolino et al. [30]. Fractions (100 µL, 1 mg/mL) were hydrolyzed with 4 M TFA (100 uL, final concentration of 2 M) at 100 ºC for 1 h. The reduction was performed with 2 M $NaBH_4$ in NH_4OH for 2 h 30 min at room temperature and the acetylation with acetic anhydride for 2 h 30 min at 100 °C. The resulting alditol acetates were analyzed by gas–liquid chromatography (GLC) using a model 5890 S II Hewlett–Packard gas chromatograph at 220 ºC (flame ionization detector and injector temperature, 250 ºC) with a DB-210 capillary column (0.25 mm internal diameter x 30 m), film thickness 0.25 µm, the carrier gas being nitrogen (2.0 mL/min) [31].

2.3.4 High Pressure Size Exclusion Chromatography (HPSEC) combined with Multi-Angle Laser Light Scattering (MALLS) and refractive index (RI) detectors

The fractions homogeneity was determined by HPSEC analysis performed on WATERS equipment (Milford, MA, USA) equipped with 2000, 500, 250, and 120 Ultrahydrogel columns (exclusion limits of 7.10^6, 4.10^5, 8.10^4, 5.10^3, respectively) coupled with Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS), an UV (280 nm) and a refractive index

(RI) detectors; using a flow rate of 0.6 mL/min at 20°C. Fractions (MP-W, MP-Ws, MP-Ws-R, MP-Ws-E, MP-HW, MP-1Aa, MP-1Ab, MP-2Aa, MP-2Ab, MP-4Aa and MP-4Ab) were solubilized (1 mg/mL) in a 0.1 M $NaNO_3$ and NaN_3 (0.2 g/L) solution and filtered through 0.22 μm cellulose acetate membranes (Millipore, Billerica, MA, USA). The molecular mass (MW) was determined by HPSEC-MALLS with the refractive index increment (dn/dc), using the concentrations of 0.2 to 1.0 mg/ml and calculated by Wyatt Technology ASTRA software.

2.3.5 Homonuclear and heteronuclear magnetic resonance (NMR) spectroscopy

NMR spectra (Proton nuclear magnetic resonance, ^{1}H-NMR; Carbon nuclear magnetic resonance, ^{13}C-NMR; Heteronuclear Single Quantum Coherence, HSQC and Heteronuclear Multi-Bond Connectivity, HMBC) of MP-Ws-R, MP-HW, MP-1Ab were obtained on a BRUKER spectrophotometer, model DRX 400, Advance series (Bruker; Karlsruhe, Germany) with a 5 nm inverse probe. The samples (30 mg/mL) were dissolved in deuterated water (D_2O) and chemical shifts expressed in δ (ppm) relative to the external standard acetone (δ 30.2 ppm and δ 2.22 for ^{13}C and ^{1}H, respectively) at 30 °C. For alkaline fractions, NaOH was added to improve solubilization.

2.4 Haemolytic complement assays for the classical and alternative pathway

AGP rich fractions, MP-Ws-R, MP-HW and MP-1Ab, were evaluated for modulation capacity on complement system (CS) through the haemolytic microassay described by Klerx et al. [32] with modifications. Activity differentiation (inhibition or stimulation of the CS) was evaluated according to the adaptations of the technique proposed by Alban et al. [16]. Assays were performed in a HEPES/NaCl base buffer (pH 7.4; 10 mM HEPES, 150 mM NaCl) (HB) supplemented according to the specific requirement for each pathway: 0.11 mM $CaCl_2$ and 0.5

mM $MgCl_2$ (CP-HB) or with 7 mM $MgCl_2$ and 10 mM EGTA (AP-HB) for the classical and the alternative pathways, respectively. Fractions, as well as bovine heparin, used as inhibition control, were prepared first in the HB (5 mg/mL) and then in the pathway-specific buffers by serial dilution to get a final concentration of 1.6 µg/ml to 833 µg/ml.

All experimental protocols involving the procedures for human and animal blood collection were approved by the Committee on Ethics of research involving human subjects (CEP-HC-UFPR 1739.156/2008-08) and of Animal Experimentation (CEUA-BL-UFPR, certificate # 1128), respectively.

2.4.1 Human pooled serum

The human pooled serum (HPS), used as the source of complement components, was obtained from five healthy adult volunteers through blood collection via peripheral venipuncture using vacuum tubes (Vacuette® blood collection system Greiner Bio-One, Frickenhausen, BW, Germany) containing gel separator and silica clot activator. After coagulation at room temperature, the tubes were centrifuged (1440 × *g*, 20 min, 15°C) for serum separation, which were then pooled and maintained at -80 ºC until use. Prior HPS titration was performed for each pathway to find the dilution capable of inducing 50% of the total haemolysis of the erythrocytes. A dilution of 1:60 in CP-HB and 1:4 in AP-HB were used in the CP and AP assays, respectively.

2.4.2 Erythrocyte suspensions

Sheep (ShE) and rabbit (RaE) erythrocytes were used in the CP and AP assays, respectively. Blood collection was performed in the presence of the EDTA.Na_2 anticoagulant. After centrifugation (1440 × *g*, 5 min, 15 °C), the supernatant was removed, and the erythrocytes washed three times with 0.9% (w/v) sodium chloride and once with the specific

buffer for each pathway: CP-HB for ShE and AP-HB for RaE). A cell suspension of ShE was prepared in CP-HB (2.4% v/v) and incubated with an equal volume of diluted haemolysin (1:1500 in CP-HB) for 30 min at 37 °C. The sensitized ShE suspensions (1.2%, v/v) were used for the haemolytic-induced complement assay for CP. For the haemolytic-induced complement assay for AP, RaE suspension (2.4%, v/v) were prepared in AP-HB.

2.4.3 In vitro maca polysaccharide fractions evaluation on the classical and alternative pathways of the complement system (CP and AP assays)

For the CP assays, 40 µL of the sample dilution were preincubated (CP-30) with 100 µL of the HPS dilution in the V-well of microtiter plates for 30 min at 37 °C and subsequently, 100 µL of the ShE suspensions were added. For the CP assays without preincubation (CP-0), the ShE suspensions were immediately pipetted after the fractions and HPS dilutions. Next, the microtiter plates were incubated for 30 min at 37 °C. After microtiter plates centrifugation (1440 × *g*, 5 min, 25°C), the supernatants (100 µL) were transferred to a 96-well flat-bottom microtiter plates and the absorbance measured at 405 nm. AP-30 and AP-0 assays were performed in the same manner using half volumes: 20 µL fraction dilution, 50 µL HPS dilution and 50 µL of RaE suspension.

The following controls were included in each plate used in the assays: (i) erythrocytes cell blank (100 µL ShE + 140 CP-HB or 50 µL RaE + 70 AP-HB for CP and AP assays, respectively), corresponding to basal haemolysis; (ii) 100% complement activity (40 µl CP-HB + 100 µl HPS dilution + 100 µl SHE or 20 µl AP-HB + 50 µl HPS dilution + 50 µl ShE for CP and AP assays, respectively) and (iii) total haemolysis (100 µL ShE + 140 water or 50 µL RaE + 70 water for CP and AP assays, respectively). Due to the yellowish coloration of the low dilution serum (1: 4) used in the AP assays, the serum blank (50 µL HPS dilution + 70 AP-HB)

was also included. The IC_{50} [μg/ml] corresponds to the concentration of the fraction that resulted in 50% of inhibition of sensitized ShE or RaE lysis

2.5 Statistical analysis

The haemolytic complement assays were performed at least two times on alternated days, yielding similar results. The results are expressed as the mean ± standard deviation of triplicates of each tested concentration. One-way analysis of variance (one-way ANOVA) and Tukey's test was performed to determine the interference of different fractions and concentrations in the haemolysis induced by the complement system and data were considered significant at $p < 0.05$. IC_{50} values ($R^2 \geq 0.90$) were calculated from a non-linear with variable slope using GraphPad prism software (version 8.1.2) and corresponds to the concentration inhibiting haemolysis by 50%.

3. Results and discussion

3.1 Obtainment and chemical characterization of maca polysaccharide fractions

Maca root flour was previously prepared for sequential extraction of polysaccharides. At the end of the enzyme inactivation and defat procedure, there was a 22% weight decrease in relation to the dry starting material. However, the most pronounced reduction (61%) occurred after enzymatic starch removal, which as expected, since starch is the major compound in dried maca roots [4,5]. As the conditions used for that purpose (H_2O at 55 ºC) was also favourable for extraction of water-soluble non-starch polysaccharides, after inactivation and removal of enzymes, high mass compounds present in the supernatant were precipitated with ETOH giving rise to MP-W fraction. Subsequent dialysis, freeze-thawing and ultrafiltration were performed

to ensure removal of the remaining mono- and oligosaccharides from starch degradation, resulting in the MP-Ws-R fraction (0.2% of yield) (**Table1**).

The residue from the starch removal process was destined for sequential extraction of maca polysaccharides. A second aqueous extraction, at 80 ^{o}C was performed, resulting in the MP-HW fraction (1.9 % of yield). Then, the alkaline extractions were performed, to obtain the cell wall hemicelluloses. MP-1Aa, MP-2Aa and MP-4Aa fractions correspond to hemicelluloses A, which precipitated after neutralization of the alkaline extracts, while the hemicelluloses B, that remained soluble in the supernatant, were precipitated with ETOH, generating the MP-1Ab, MP-2Ab and MP-4b fractions. The yield of obtained fractions (MP-Ws-R, MP-HW, MP-1Aa, MP-1Ab, MP-2Aa, MP-2Ab, MP-4Aa and MP-4Ab) are shown in **Table 1**. Overall, the maca polysaccharide fractions presents a low yield (0.1-8.2%). The highest yield was observed in the MP-1Aa fraction, corresponding to hemicellulose A.

The neutral monosaccharide analysis showed Ara, Gal and Glc as main monosaccharides present on the polysaccharide fraction extraction with H_2O, as MP-Ws-R and MP-HW. For the alkaline extraction fractions (MP-1Aa, MP-1Ab, MP-2Aa, MP-2Ab, MP-4Aa and MP-4Ab), besides Ara, Gal and Glc; Xyl was also presented as main monosaccharide. The neutral monosaccharide composition of all polysaccharide fractions obtained are shown in **Table 1**.

The results obtained agreed with the literature [5,10,11,12], which suggested arabinogalactans and xyloglucans as main polysaccharides presented in fractions with H_2O and alkali, respectively. According to Caffall & Mohnen [33], hemicelluloses are polymers with β-(1,4)-linked pyranosyl residues, that are solubilized from plant cell wall by alkaline solvents. Compared with aqueous fractions, the maca alkaline fractions presented higher amounts of xylose in their composition, besides Ara, Gal, Man and Glc, monosaccharides typically found in hemicelluloses [30]. Following the extraction order, the fractions obtained with 1M NaOH showed mainly Ara, Gal, Xyl and Glc, suggesting the presence of arabinogalactans and

xyloglucans. Xyloglucan is the most abundant hemicellulose in dicot primary walls making up 21% of angiosperm. [32]. In the MP-2Aa fraction, the high Xyl content suggests the presence of xylans. In subsequent fractions (MP-2Ab, MP-4Aa and MP-4Ab), the balanced content of the major monosaccharides (Glc, Gal, Man, Ara and Xyl) is an indicative of the presence of galactoglucomannans, arabinogalactans and/or xyloglucans.

All polysaccharide fractions (MP-Ws-R, MP-HW, MP-1Aa, MP-1Ab, MP-2Aa, MP-2Ab, MP-4Aa and MP-4Ab) showed a heterogeneous profile through HPSEC-MALLS-RI-UV analysis (**Fig. S.1**) and not significant detection at 280 nm was observed.

Quantitative analysis of AGPs in the fractions was performed by a radial single diffusion on agarose gel containing β-Glc Y (**Fig. 2**). One of the characteristics of AGP is its capacity to complex with β-Glc Y. The formation of the AGP-Yariv complex could be visualized as a characteristic reddish-brown halo which precipitates on agarose gel. The halo diameter is proportional to the AGP content of the sample, allowing its quantification based on analytical calibration of the standard gum arabic (GA) (**Fig. 2**). The fractions MP-Ws-R, MP-HW, and MP-1Ab presented positive reaction with β-Glc Y, suggesting the presence of AGP on these fractions. Thus, considering the presence of AGPs and the water-solubility of MP-Ws-R, MP-HW and MP-1Ab fractions, they were selected for further chemical analysis and biological evaluation on the complement system. The chemical data of MP-Ws-R, MP-HW and MP-1Ab fractions obtained by the colorimetric assays and their AGP content are showed in the **Table 2**.

The water-soluble polysaccharides fractions, MP-Ws-R and MP-HW, presented the highest total carbohydrate content (89.9% and 53.1%, respectively), of which approximately 18% correspond to uronic acids. MP-1Ab was composed by 33.3% of carbohydrate, and with 10% of uronic acid. The three fractions presented a low content of protein and phenolic compounds (<4%). The AGP contents (% calculated in relation to the total carbohydrate content) were 34.3%, 14.6% and 30.2%, respectively, for MP-Ws-R, MP-HW and MP-1Ab. To

the best of our knowledge, no study has reported the quantification and chemical analyses of the AGPs present in maca root extracts.

3.2 NMR analysis of MP-Ws-R, MP-HW, MP-1Ab from maca roots

The structural features of MP-Ws-P, MP-HW and MP-1Ab were investigated by bidimensional HSQC NMR (**Fig. 3**). Despite the complexity and heterogeneity of the AGPs rich-fractions in HPSEC-MALLS, in general, their HSQC analysis showed typical AG type II signals. In the anomeric region of the MP-Ws-R spectra (**Fig. 3A**), signals relative to α-L-Ara*f*-(1→ were observed at δ106.9/5.17, δ106.9/5.14 and δ106.5/5.24, and to →5)-α-L-Ara*f*-(1→ units at δ107.4/5.08. These signals were also observed in MP-HW (**Fig. 3B**) and MP-1Ab (**Fig. 3C**) spectra. Signals at δ104.3/4.62 and 104.5/4.64 in the MP-Ws-R and MP-1AB spectra, respectively, were assigned to β-D-Gal*p*-(1→ units [34], consistent with the central backbone of AGPs, confirmed by the signals at δ83.8/4.07, δ83.9/4.08 and δ84.2/4.08 in the ring region of the MP-Ws-R, MP-HW and MP-1AB spectra, respectively, related to C3 linked of the β-D-Gal*p*-(1→3) [35,36].

Signals related to AG type II were also observed in other regions of the spectra, especially in the MP-Ws-R analysis, as at δ52.9/3.80, attributed to the *–O-CH₃,* demonstrating the presence of methylated uronic acids in this fraction [35, 37-40]; or at δ16-17/1.10-1.35, indicating different chemical environments to C6/H6 of α-L-Rha*p* units [22,34-36]. In the MP-Ws-R ^{13}C-NMR and HMBC spectra (**Fig. S.2**), a signal related to C6/H6 of β-D-Glc*p*A was observed at δ170.7.

Other signals at δ98.4/4.98 and δ99.7/5.38 can be attributed to Glc*p* and/or Man*p* units [41,42], considering the fractions monosaccharide composition. Signals involving the presence of α-D-Man units have recently been reported in polysaccharide obtained from maca [5,43]. The signals at δ92.0/5.41 and δ95.8/4.64 may indicate the presence of C-1 of α-and β-anomers

of Glc*p* reducing units, respectively [41], possibly by the presence of Glc units remaining from starch degradation.

Considering chemical and NMR analysis it can be suggested the presence of arabinogalactan type II (substituted with Rha and 4-*O*-Me-GlcA), xyloglucans and/or glucomannans on the maca polysaccharide fractions.

3.3 Complement fixating properties of MP-Ws-R, MP-HW, MP-1Ab from maca roots

MP-Ws-R, MP-HW and MP-1Ab were analysed, *in vitro*, regarding the modulation capacity on the classical (**Fig. 4**) and alternative pathway (**Fig. 5**) of SC. In the CP-30 assay, all fractions reduced the complement induced haemolysis, similar but less effectively than heparin, as observed by their respective IC_{50} values: 204.9 μg/mL for MP-Ws-R, 303.8 μg/mL for MP-HW and 886.9 μg/mL for MP-1Ab, while the calculated IC_{50} value for heparin was 40 μg/mL. To distinguish whether the fractions exhibited an inhibitory or activating effect on the CS, an identical assay was performed, however without the preincubation step (CP-0). Heparin was used as an inhibition control and showed a similar IC_{50} value both in CP-30 and AP-30, while maca AGP-rich fractions apparently had no effect under these conditions (without preincubation), even at the highest concentrations.

The activity observed in the alternative pathway followed the same pattern: both heparin (IC_{50} value of 260.9 μg/mL) and maca AGP-rich fractions (IC_{50} values: 224.4 μg/mL for MP-Ws-R, 411.8 μg/mL for MP-HW and 387 μg/mL for MP-1Ab) reduced complement-induced haemolysis in preincubation assays (AP-30), but only heparin retained this activity in the assays conducted without preincubation (AP-0) with a IC_{50} value of 280.2 μg/mL).

According to Alban et al. [16], SC activators and inhibitors apparently display the same inhibitory effect of complement-induced haemolysis in preincubation assays, but do it through different mechanisms. If on the one hand, an inhibitor prevents cascade triggering and MAC

formation and, therefore, there is no haemolysis when erythrocytes are subsequently added. On the other hand, an activating compound triggers the cascade and consumes the SC components. Similarly, in the latter situation, there is no haemolysis after the addition of erythrocytes because there are no more SC components available in the reaction medium. However, when reaction components are added to the microplate well at the same time (test compounds, SC components and erythrocytes), as occurs in the assay without pre-incubation, the activating compounds, upon triggering the cascade, will assist the MAC polymerization resulting in erythrocyte haemolysis. In this case, the activating compound activity is masked and apparently does not appear to interfere with complement haemolytic activity, presenting higher IC_{50} values when compared to the preincubation assay. In turn, a real complement inhibitor like heparin retains its activity and displays similar IC_{50} values in both assays.

Thus, the results on the haemolytic complement assays suggest that maca AGP-rich fractions exert a stimulatory effect on the complement system through the classical and alternative pathway.

The highest IC_{50} values observed for all fractions in AP assays was expected and has been previously reported [16-18], since the HPS concentration used is 15 times higher than that used in the CP assays and so, it's may not mean lower activity on this pathway. Higher HPS concentration is required for visualization of the complement-induced haemolysis via the alternative pathway in this kind of assay.

The MP-Ws-R and MP-HW showed the same activation effectiveness in both CP and AP assays, and were as effective as heparin in the AP assays. The lowest activation potential on the SC activity observed for MP-1Ab fraction, evidenced by the statistically significant differences ($p < 0.0001$) in the IC_{50} values (mainly in the CP assays), may be related to its lowest total carbohydrate and AGP content.

AGPs of different plant species have been described as activators of the classical and alternative complement pathways [16,17,44], although some studies have shown AGPs with inhibitory activity [18]. This activation activity seems to be related to the high degree of branching of the AGPs, since the side chains would be involved in the interactions with the complement system components [16,45].

Conclusions

The maca polysaccharide fractions (MP-Ws-R, MP-HW and MP-1Ab) showed results of monosaccharide composition, and NMR analysis suitable to suggest the presence of arabinogalactan type II (AG-II) and xyloglucans on these fractions. Single diffusion with β-Glc Y data demonstrated the presence of arabinogalactan-proteins (AGPs), corroborated with AG-II signals at NMR analysis, on these fractions. To the best of our knowledge, this study is the first to identify arabinogalactan-protein in maca roots. MP-Ws-R, MP-HW and MP-1Ab might be acting as activators of the CS, especially of the AP. This effect could characterize these fractions as potential pro-inflammatory agents. This property may be interesting in activation of the nonspecific immune system, as required for host defense against microbial pathogens or in the process of wound healing. Further purification and chemical characterization are needed to better understand the relationship between AGP structures and other polysaccharides present in the MP-Ws-R, MP-HW and MP-1Ab fractions with the complement system stimulation activity.

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Conflict of interest

The authors have no conflicts of interest to declare.

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Figure Captions

Fig. 1 Schematic of methodology protocol to obtainment maca polysaccharide fractions.

Fig. 2 Ability of maca polysaccharide fractions to bind to β-glucosyl Yariv reagent by single radial diffusion in gel.

Fig. 3 Partial $^{1}H–^{13}C$ HSQC NMR spectrum (anomeric region) of MP-Ws-R (A), MP-HW (B) and MP-1AB (C) in D_2O at 30 °C.

Fig. 4 Effect of MP-Ws-R, MP-HW, MP-1Ab and heparin on complement-induced haemolysis in the classical pathway (CP) of the complement system.

Fig. 5 Effect of MP-Ws-R, MP-HW, MP-1Ab and heparin on complement-induced haemolysis in the alternative pathway (AP) of the complement system.

Fig. S.1 HPSEC-MALLS-RI elution profile of maca polysaccharide fractions.

Fig. S.2 ^{13}C NMR (A) and HMBC (B) spectrum of MP-Ws-R in D_2O at 30 °C.

Table 1 - Yield and neutral monosaccharide composition of maca polysaccharide fractions obtained by sequential extractions.

		Monosaccharide composition (mol%)[b]						
Fractions	Yield (%)[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
MP-Ws-R	0.2	1	1	34	5	11	26	22
MP-HW	1.9	2	Tr	21	4	12	18	43
MP-1Aa	8.2	3	1	26	11	7	26	16
MP-1Ab	0.9	1	1	36	20	4	21	17
MP-2Aa	1.8	1	2	4	77	6	3	7
MP-2Ab	1.7	2	3	17	15	16	21	26
MP-4Aa	1.8	2	2	14	17	18	15	31
MP-4Ab	0.1	2	3	15	8	19	25	28

tr, trace (<1%); For more details on the terminology used for the fractions, see section 2.2.

[a] Calculated as relative percentage related to the weight of initial material (dried inactivated and defatted maca flour).

[b] Determined by GLC analysis as alditol acetates derivatives.

Table 2 - Chemical data of MP-Ws-R, MP-HW, and MP-1Ab from maca roots.

	Fractions		
Analysis	MP-Ws-R	MP-HW	MP-1Ab
Carbohydrate[a] (%)	89.9±0.9	53.1±0.5	33.3±2.2
Protein[b] (%)	Tr	Tr	Tr
Uronic acid[c] (%)	17.8±2.5	17.7±0.6	10.8±2.0
Phenolic[d] (%)	Tr	Tr	Tr
AGP[e] (%)	34.3±0.5	14.6±1.4	30.2±1.1

tr, trace (bellow detection limit). For more details on the terminology used for the fractions, see section 2.2. Values are expressed as relative percentages (mean ± SD).

[a] Expressed as Glc equivalents of dried fraction weight.

[b] Expressed as ovalbumin equivalents of dry fraction weight.

[c] Expressed as GlcA equivalents of the total carbohydrate content.

[d] Expressed as gallic acid equivalents of the dry fraction weight.

[e] Expressed as the percentage of GA equivalents of the total carbohydrate content.

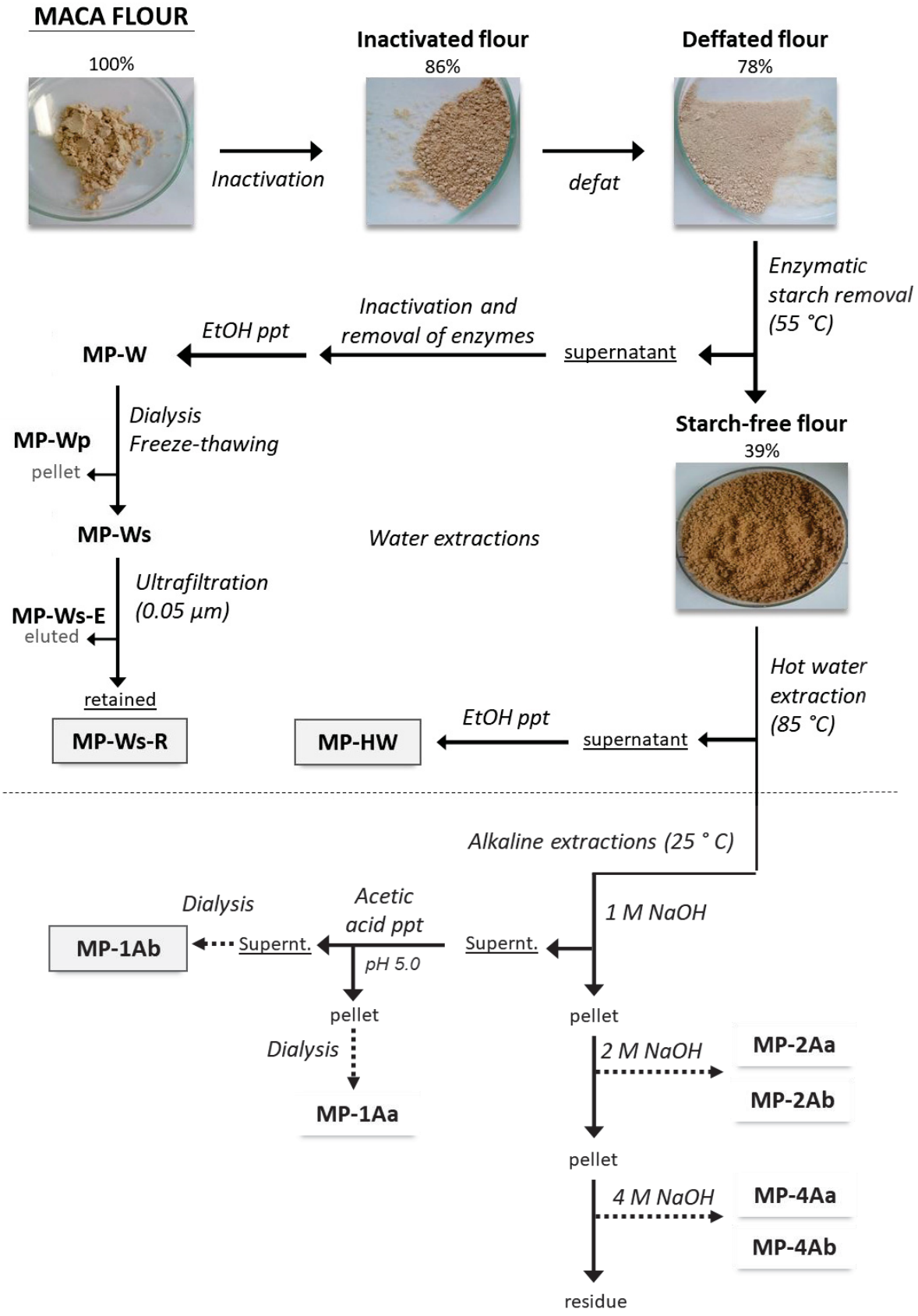

Fig. 1 - Schematic of methodology protocol to obtainment maca polysaccharide fractions. More details on the terminology used for the fractions are founded in Materials and methods section.

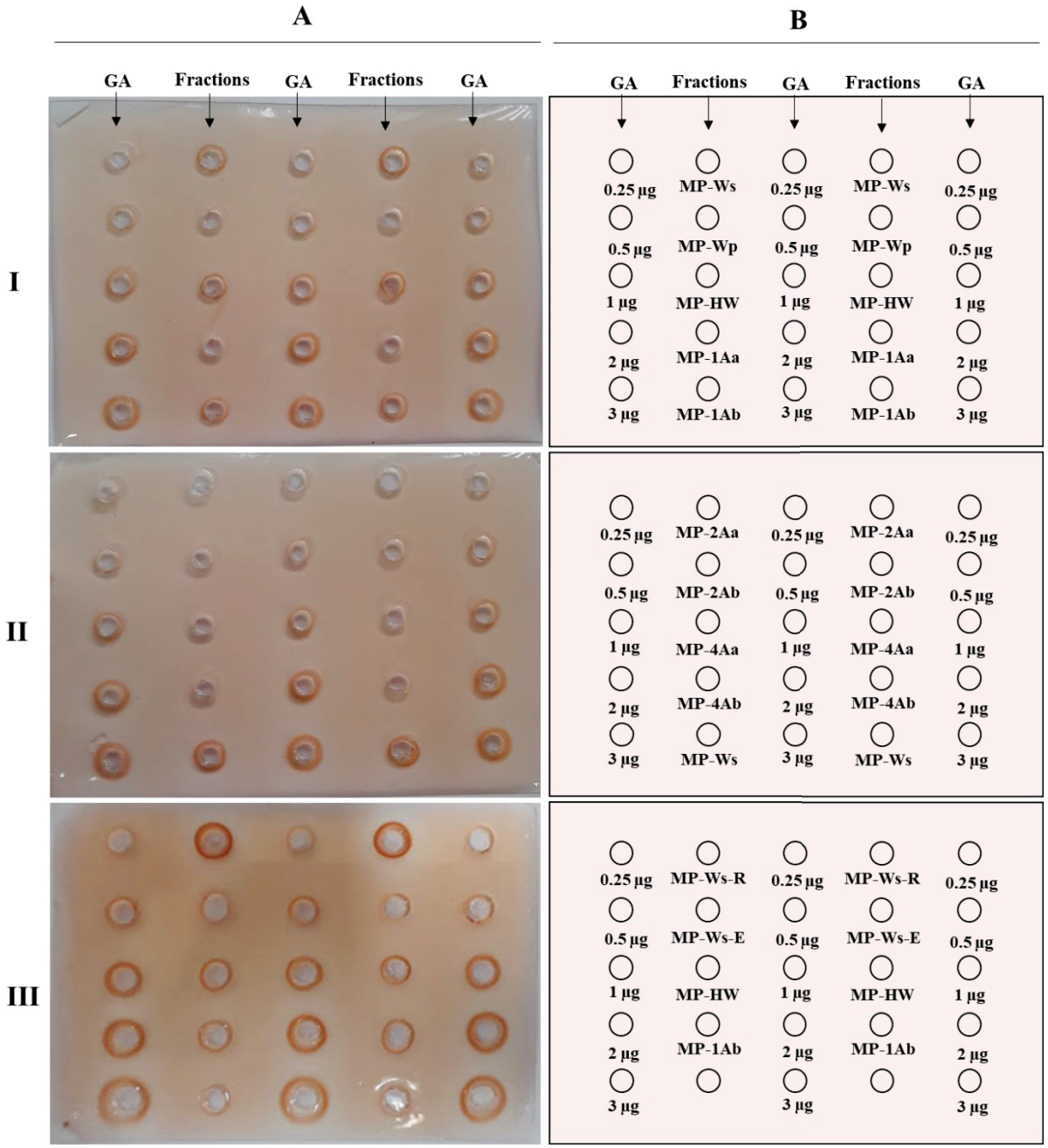

Fig. 2 - Ability of maca polysaccharide fractions to bind to β-glucosyl Yariv reagent by single radial diffusion in gel.

The gum Arabic (GA) was used as standard (0.25 to 3 µg in triplicate). The fractions (10 mg/mL in NaCl 1%) were analysed ate least in duplicate. More details on the terminology used for the fractions are founded in Materials and methods section and Fig. 1. The letters A and B correspond to the photos and layouts, respectively, of plates I, II and III.

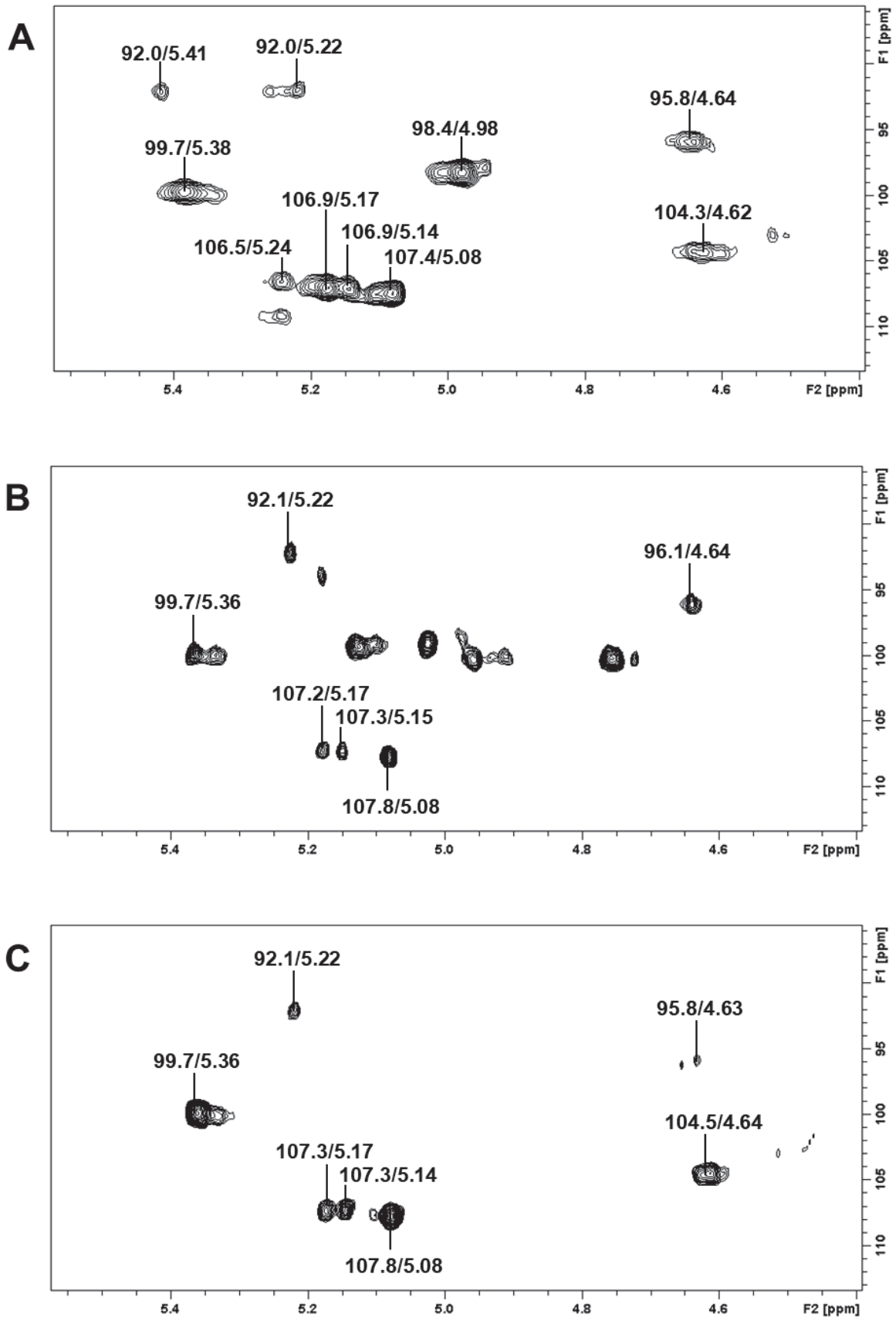

Fig. 3 – Partial ^{1}H–^{13}C HSQC NMR spectrum (anomeric region) of MP-Ws-R (A), MP-HW (B) and MP-1AB (C) in D_2O at 30 °C.

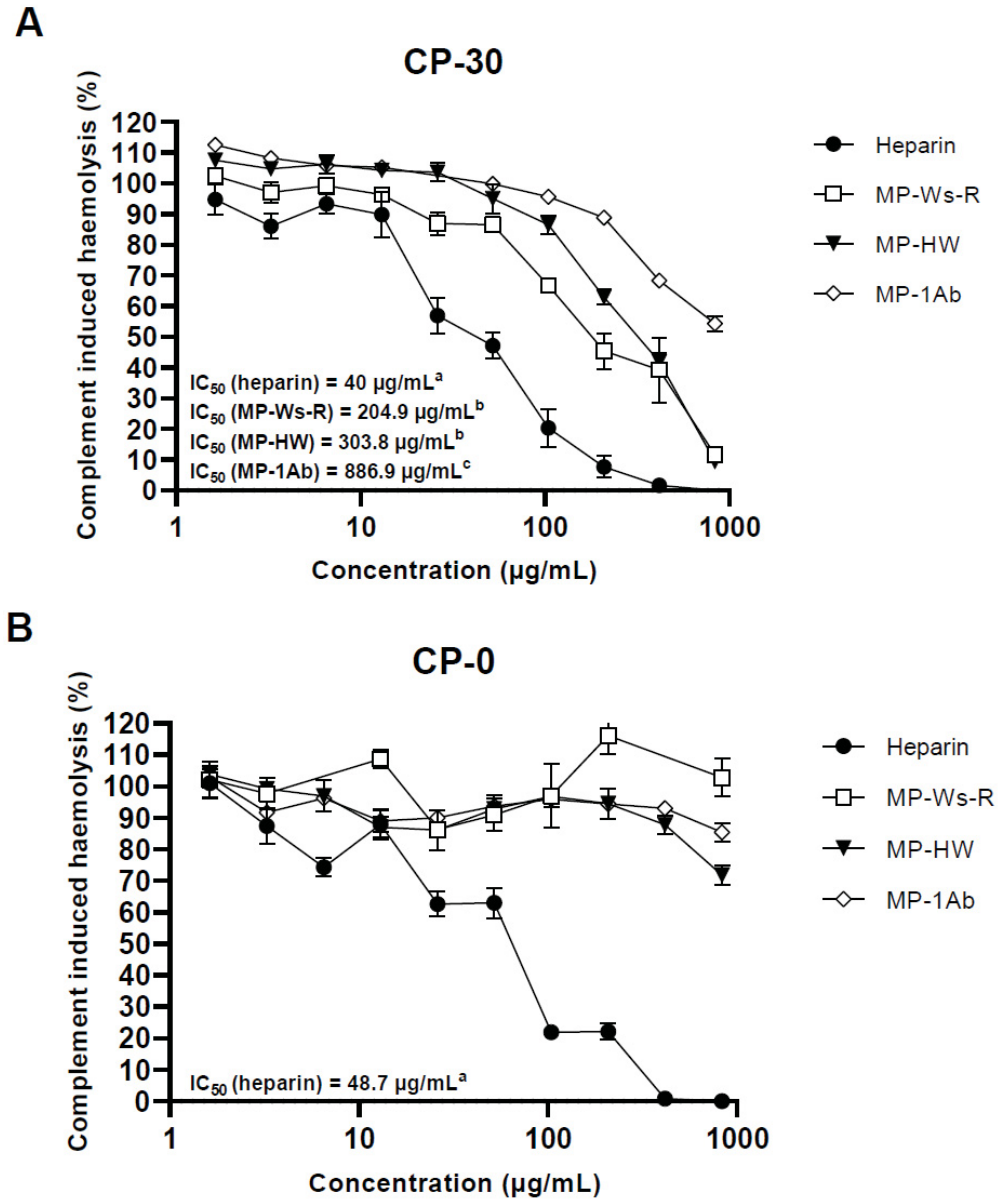

Fig. 4 - Effect of MP-Ws-R, MP-HW, MP-1Ab and heparin on complement-induced haemolysis in the classical pathway (CP) of the complement system.

(A) Assay performed with 30 min preincubation 30 of fractions and HPS (B) Assay performed without preincubation. The values represent means ± SD (n = 3), from two independent experiments. The IC_{50} values correspond the final concentration of the test fraction [µg/mL] capable of reducing the haemolytic activity of HPS by 50%. Statistical analyses of IC_{50} values were performed using all variables at the same time by one-way ANOVA followed by Tukey's post hoc test, with significance represented by different letters ($p < 0.05$).

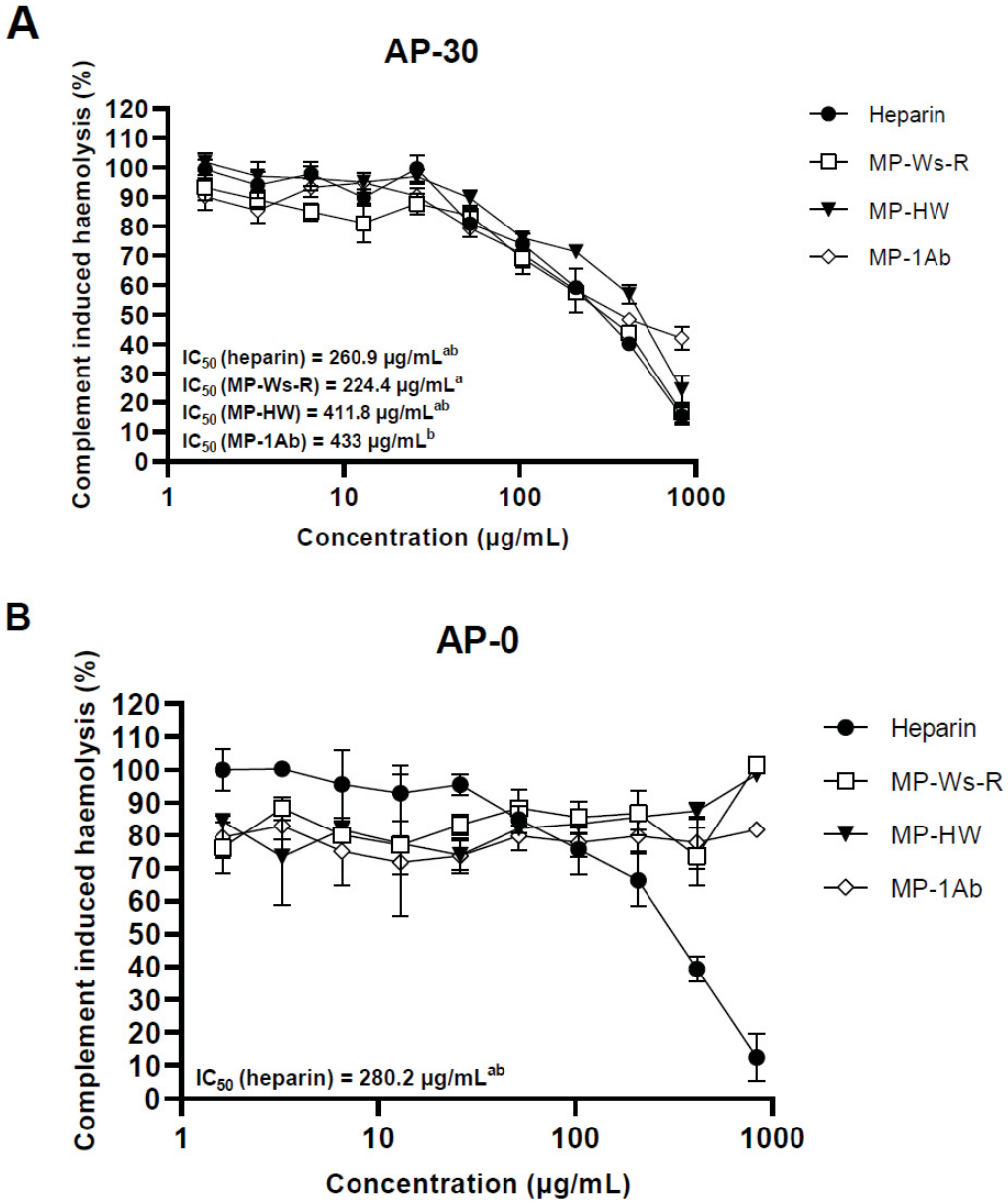

Fig. 5 - Effect of MP-Ws-R, MP-HW, MP-1Ab and heparin on complement-induced haemolysis in the alternative pathway (AP) of the complement system.

(A) Assay performed with 30 min preincubation 30 of fractions and HPS (B) Assay performed without preincubation. The values represent means ± SD (n = 3), from two independent experiments. The IC_{50} values correspond the final concentration of the test fraction [µg/mL] capable of reducing the haemolytic activity of HPS by 50%. Statistical analyses of IC_{50} values were performed using all variables at the same time by one-way ANOVA followed by Tukey's post hoc test, with significance represented by different letters ($p < 0.05$).

SUPPLEMENTARY MATERIAL

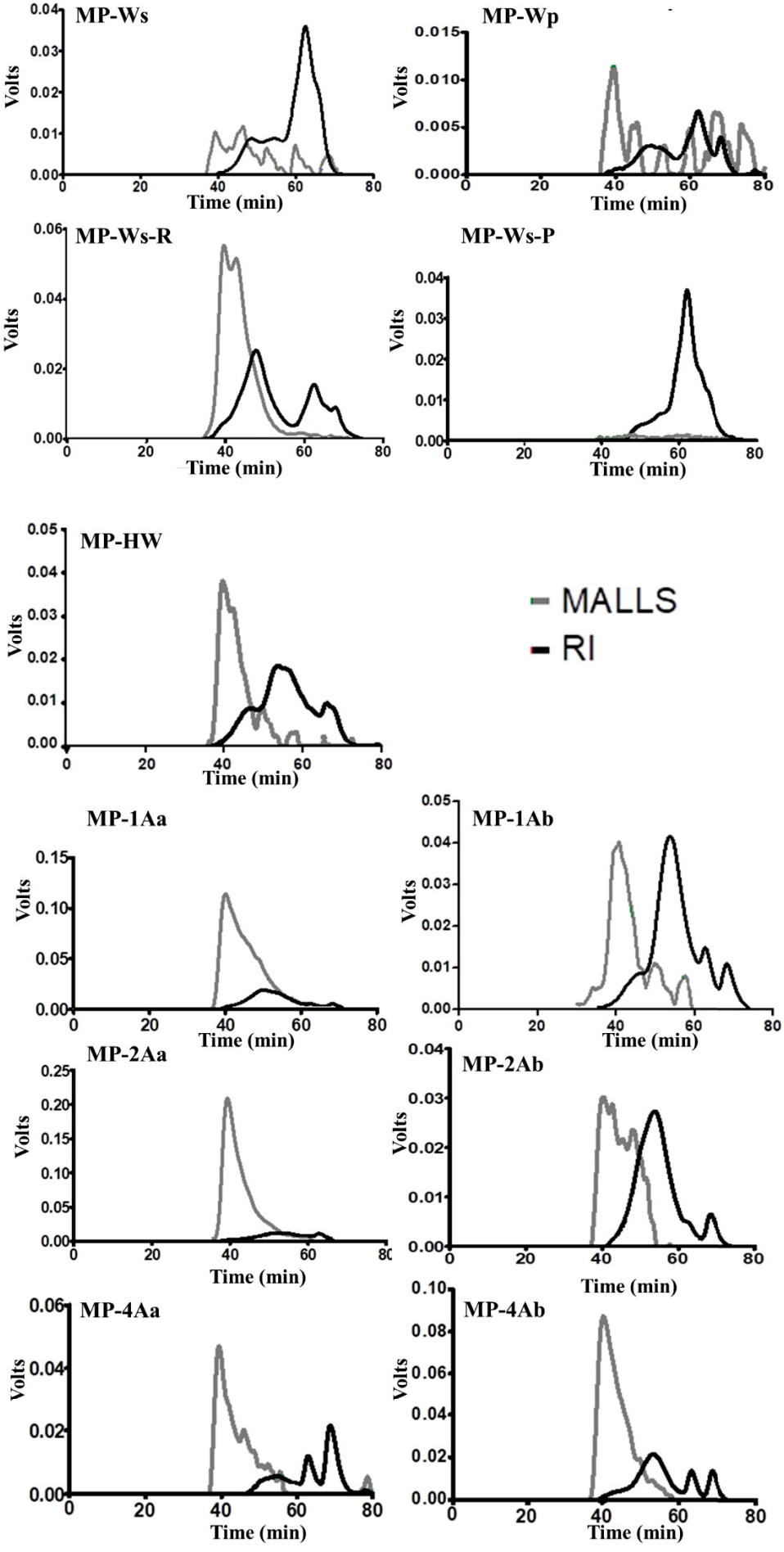
MP-Ws
MP-Wp
MP-Ws-R
MP-Ws-P
MP-HW
MALLS
RI
MP-1Aa
MP-1Ab
MP-2Aa
MP-2Ab
MP-4Aa
MP-4Ab
Volts
Time (min)

Fig. S.1 -HPSEC-MALLS-RI elution profile of maca polysaccharide fractions.

More details on the terminology used for the fractions are founded in Materials and methods section and Fig. 1.

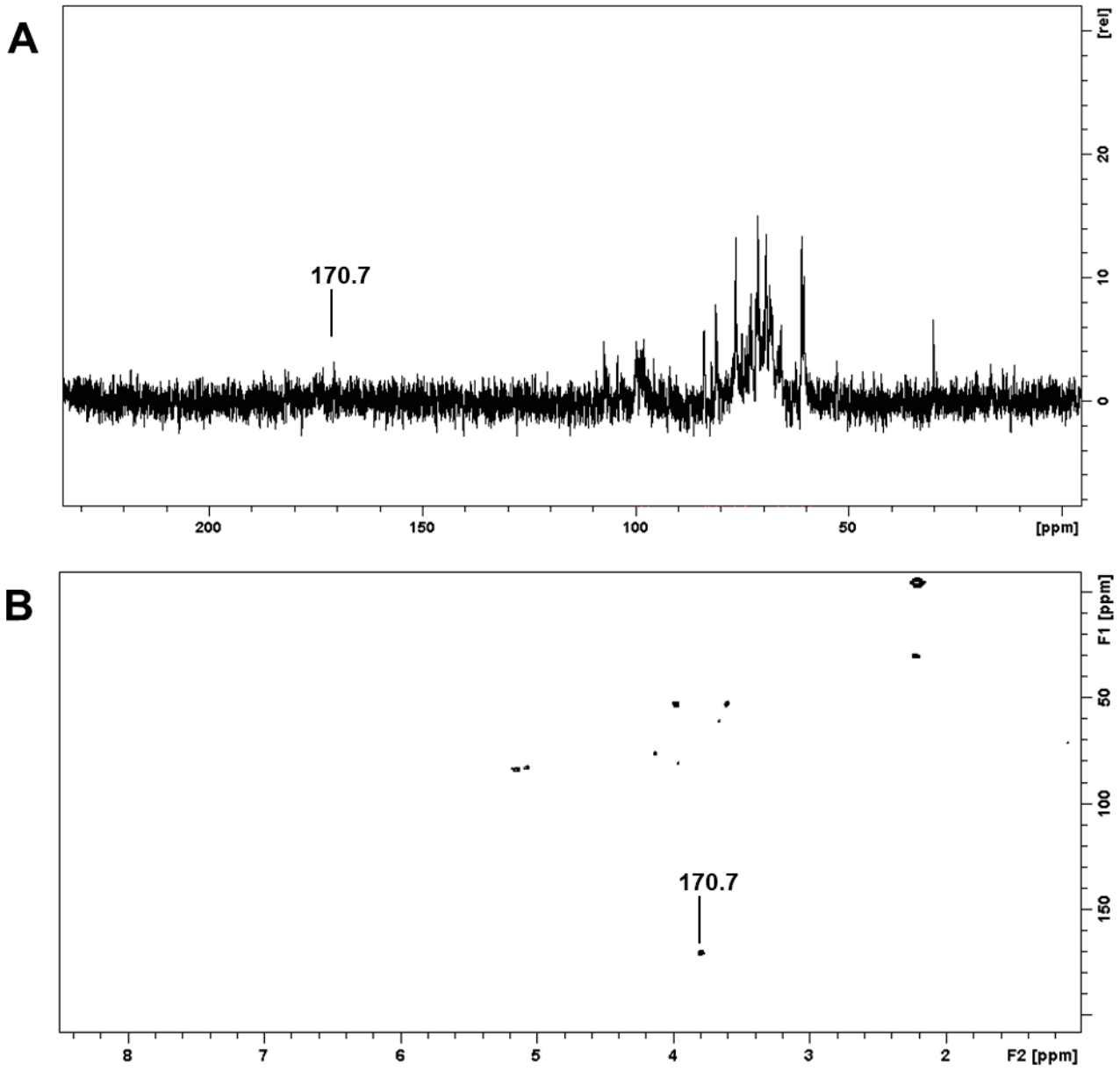

Fig. S.2 - ^{13}C NMR (A) and HMBC (B) spectrum of MP-Ws-R in D_2O at 30 °C.

5 CONCLUSÃO E PERSPECTIVAS

A maca é um material vegetal complexo, que requer estratégias metodológicas complementares (e equipe interdisciplinar) para obtenção/caracterização e avaliação biológica de seus constituintes.

De acordo com os resultados obtidos, é constituída por carboidratos na forma de monossacarídeos, dissacarídeos, polissacarídeos (possivelmente glucanas, arabinogalactanas e xiloglucanas, e glicoconjugados (AGPs) e GLs – glucotropaeolina e glucolimnantina. Compostos fenólicos também estão presentes

Em relação à bioatividade, os produtos de hidrólise dos GLs produzidos pela reação com a mirosinase seriam os responsáveis pelas propriedades citotóxicas, e a lipofilicidade estaria relacionada a uma maior citotoxicidade; nas condições testadas os GLs intactos são inócuos para as linhagens celulares

Esses resultados abrem uma gama de possibilidades de futuros estudos acerca do mecanismo de ação envolvido na citotoxicidade dos derivados dos GLs da maca, que até então não havia sido observada cientificamente. Diferentes abordagens, como por exemplo, determinação dos mecanismos envolvidos na citotoxicidade através da verificação do potencial de indução do apoptose e parada de ciclo celular através de análises citometria de fluxo; de morfologia e da expressão gênica; investigação de atividade citoprotetora exercida pelos glucosinolatos e/ou seus derivados, através de tratamentos associados a um xenobióticos; estudos de combinação dos GLs a quimioterápicos conhecidos e avaliação da capacidade de potencialização do efeito citotóxicos exercidos agentes (por exemplo, doxorrubicina), além de estudos *in vivo* relacionados a aspectos nutricionais e/ou terapêuticos da maca.

A respeito da capacidade de ativação do sistema complemento sugere-se que esta estaria relacionada com a presença de AGPs. Estudos adicionais de purificação e caracterização química das AGPs da maca devem ser realizados, visando compreender e relacionar a atividade biológica relatada com a estrutura química dessas moléculas. Além disso, devido à presença de manose nas frações polissacarídicas da maca, a investigação da modulação sobre a via das lectinas do sistema complemento é pertinente.

Por fim, através desse estudo pode concluir que os extratos aquosos das raízes da maca, principal forma de consumo dessa planta, apresentam compostos precursores de potentes bioativos (GLs), além de polissacarídeos imunomoduladores que contribuem para os efeitos benéficos relacionados ao consumo dessa planta.

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ANEXOS

ANEXO I

HOSPITAL DE CLÍNICAS
UNIVERSIDADE FEDERAL DO PARANÁ
UFPR
EBSERH
HOSPITAIS UNIVERSITÁRIOS FEDERAIS
Ministério da Educação
GOVERNO FEDERAL
BRASIL
PÁTRIA EDUCADORA

EMPRESA BRASILEIRA DE SERVIÇOS HOSPITALARES
COMPLEXO DO HOSPITAL DE CLÍNICAS - UFPR

Curitiba, 01 de setembro de 2016.

Ilmo (a) Sr. (a)
Juliana Bello Baron Maurer
Universidade Federal do Paraná
Neste

Prezada Pesquisadora:

Comunicamos que a **Solicitação para alterações na equipe de pesquisa; inserção de novas espécies vegetais a serem estudas; atualização e inserção de novas metodologias e atualização do cronograma,** datado de 31 de maio de 2016, referente ao Projeto de Pesquisa intitulado: **"ARABINOGALACTANA-PROTEÍNAS E PECTINAS DE PLANTAS MEDICINAIS: CARACTERIZAÇÃO ESTRUTURAL E INVESTIGAÇÃO DE PROPRIEDADES IMUNOMODULADORAS"**, foi analisado e aprovado pelo Comitê de Ética em Pesquisa em Seres Humanos, em 23 de agosto de 2016.

O referido documento atende aos aspectos das Resoluções CNS 466/2012, e sua complementares, sobre Diretrizes e Normas Regulamentadoras de Pesquisa Envolvendo Seres Humanos do Ministério da Saúde.

CAAE: 0187.0.208.000-08
CEP: 1739.156/2008-08

Atenciosamente,

Renato Tambara Filho
Vice Coordenador do Comitê de Ética em Pesquisa
em Seres Humanos do Hospital de Clinicas/UFPR

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ANEXO II

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

Nº 1128

CERTIFICADO

A Comissão de Ética no Uso de Animais do Setor de Ciências Biológicas da Universidade Federal do Paraná (CEUA/BIO – UFPR), instituída pela Resolução Nº 86/11 do Conselho de Ensino Pesquisa e Extensão (CEPE), de 22 de dezembro de 2011, **CERTIFICA** que os procedimentos utilizando animais no projeto de pesquisa abaixo especificado estão de acordo com a Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos (DBCA) estabelecidas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e com as normas internacionais para a experimentação animal.

STATEMENT

The Ethics Committee for Animal Use from the Biological Sciences Section of the Federal University of Paraná (CEUA/BIO – UFPR), established by the Resolution Nº 86/11 of the Teaching Research and Extension Council (CEPE) on December 22nd 2011, **CERTIFIES** that the procedures using animals in the research project specified below are in agreement with the Brazilian Guidelines for Care and Use of Animals for Scientific and Teaching purposes established by the National Council for Control of Animal Experimentation (CONCEA) and with the international guidelines for animal experimentation.

PROCESSO/PROCESS: 23075.215421/2017-11

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

TÍTULO: Arabinogalactana-proteínas e pectinas de plantas medicinais: caracterização estrutural e investigação de propriedades imunomoduladoras.

TITLE: Arabinogalactan-proteins and pectins from medicinal plants: structural characterization and investigation of immunomodulatory properties.

AUTORES/AUTHORS: Juliana Bello Baron Maurer, Marcelo B. Molento, Douglas Luís Vieira, João Carlos Minozzo, Selma Faria Zawadzki-Baggio, Raquely Moreira Lenzi, Pamela Fontana, Hayanna Karla Felipe Santos, Monique Adriani Garcia da Silva.

DEPARTAMENTO/DEPARTMENT: Bioquímica e Biologia Molecular

Profa. Dra. Katya Naliwaiko
Coordenadora da CEUA