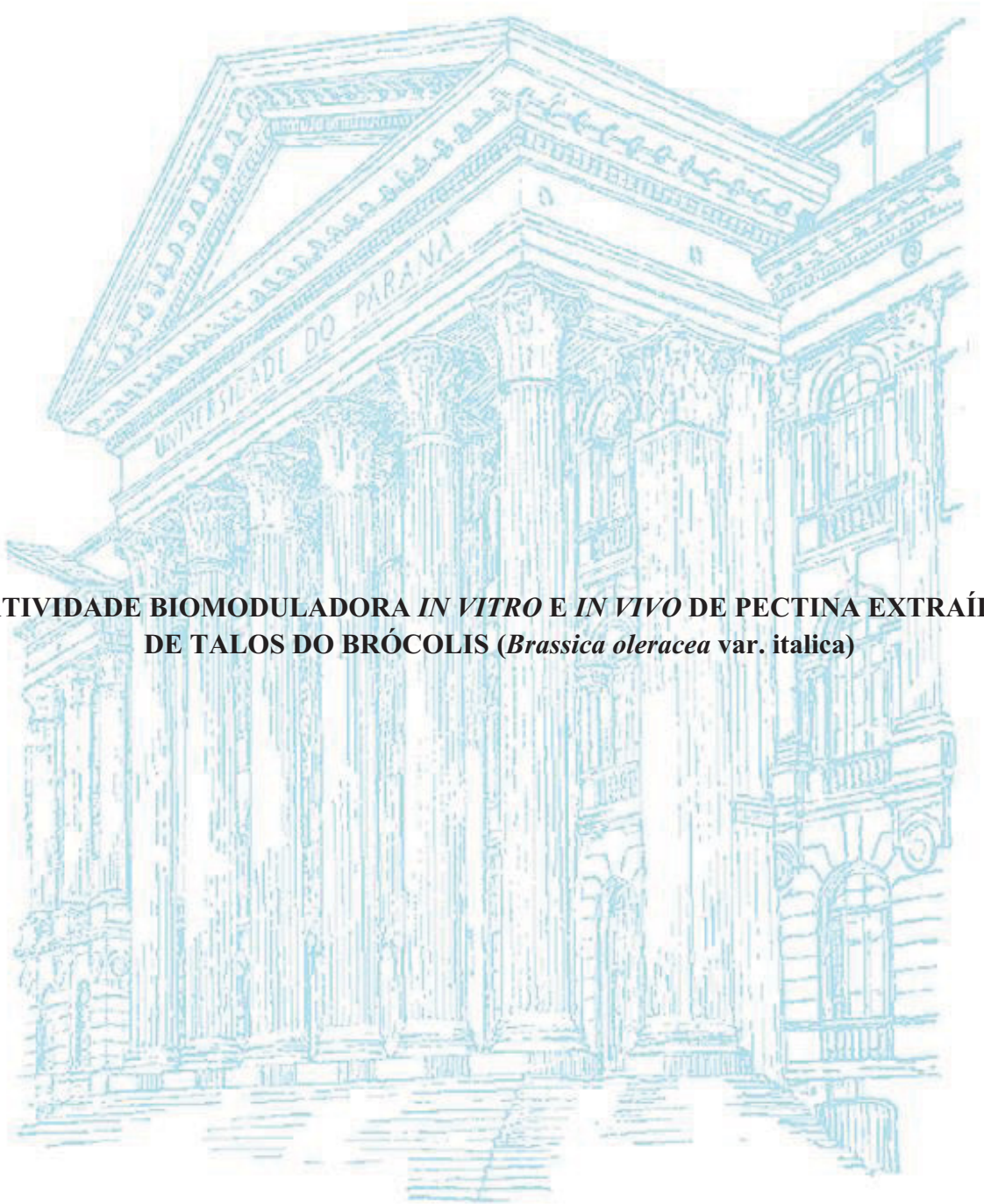
UNIVERSIDADE FEDERAL DO PARANÁ

BIANCA BUSATO



ATIVIDADE BIOMODULADORA *IN VITRO* E *IN VIVO* DE PECTINA EXTRAÍDA DE TALOS DO BRÓCOLIS (*Brassica oleracea* var. italica)

CURITIBA

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ATIVIDADE BIOMODULADORA *IN VITRO* E *IN VIVO* DE PECTINA EXTRAÍDA DE TALOS DO BRÓCOLIS (*Brassica oleracea* var. italica)

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Ciências – Bioquímica, no Curso de Pós-graduação em Ciências – Bioquímica do Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientadora: Profa. Dra. Guilhermina Rodrigues Noleto
Co-orientadora: Profa. Dra. Carmen Lucia 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 dissertação de Mestrado de **BIANCA BUSATO** intitulada: **Atividade biomoduladora *in vitro* e *in vivo* de pectina extraída de talos do brócolis (*Brassica oleracea* var. italica)**, 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 mestre 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, 03 de Julho de 2018.

GUILHERMINA RODRIGUES NOLETO
Presidente da Banca Examinadora (UFPR)

FABÍOLA IAGHER
Avaliador Externo (UFPR)

GLAUCIA REGINA MARTINEZ
Avaliador Interno (UFPR)

A minha avó Tereza,
meu exemplo de determinação e fé.

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“Os planos fracassam por falta de
conselho, mas são bem-sucedidos
quando há muitos conselheiros.”
Provérbios 15:22

RESUMO

Vários estudos têm demonstrado os efeitos imunomoduladores e antitumorais de pectinas, com destaque para as pectinas modificadas (MP), cuja atividade biológica é favorecida pela modificação estrutural. A pectina extraída de *Brassica oleracea* var. italica (FB) apresenta baixo peso molecular e galactose como o principal açúcar neutro, características que podem torná-la mais facilmente absorvida pelo sistema gastrointenstinal, acessando assim a circulação sanguínea e levando a respostas biomoduladoras. No presente estudo, FB foi avaliada quanto a sua atividade imunomoduladora e citotóxica para células de hepatocarcinoma humano (HepG2). FB (750 μg/ml) reduziu a viabilidade de macrófagos peritoneais de camundongos em ~28% com 48h de incubação. A atividade fagocítica destas células após incubação por 48h com FB (500 μg/ml) aumentou em 30% e 35% nos ensaios com leveduras e com vermelho neutro, respectivamente. O número de macrófagos ativados avaliados por análise morfológica após incubação com FB 250 μg/ml e 500 μg/ml aumentou em 40% e 68%, respectivamente. Adicionalmente, aumento de 120% no número de macrófagos peritoneais ativados foi observado em camundongos após 5 dias de administração oral (200 mg/kg) da pectina. FB (200 mg/kg) injetada na cavidade peritoneal induziu o recrutamento de células para este local após 24 horas, alcançando ~ 490% e um aumento de 251% no número destas células com perfil ativado em relação ao controle. No sobrenadante de macrófagos de animais (normais) incubados durante 48 h com FB (250 e 500μg/ml) e no fluido peritoneal de camundongos tratados com a pectina (200 mg/kg por 5 dias) não houve aumento na produção de óxido nítrico e interleucinas IL-1β e IL-12 em relação ao controle, avaliados pelos métodos de Griess e ELISA, respectivamente. Entretanto, após tratamento *in vivo*, houve aumento na produção de IL-10 por estas células. A proliferação de linfócitos totais isolados de baço de camundongos tratados oralmente com FB (200mg/kg) por 5 dias chegou a 51% em relação ao controle sem tratamento. Ao serem plaqueados e incubados com concanavalina A (ConA) por 72h, houve um aumento de 24,4% no número de células em relação ao controle. O sobrenadante da cultura de linfócito de camundongos tratados oralmente com FB estimulou a proliferação de células da medula óssea, representando um aumento de 46,1% no número de células em comparação com o grupo controle. Este resultado indica que FB foi capaz de promover proliferação celular mediada por linfócitos. Adicionalmente, FB reduziu a viabilidade de células HepG2 após incubação com diferentes concentrações por 48 e 72 horas. Ensaio preliminar para apoptose mostrou que incubação de HepG2 com FB a 250 e 500 μg/ml por 48 horas resultou em aumento na proporção de células fixadas com anexina V e PI em comparação com os controles não tratados. Estes resultados sugerem que FB administrada oralmente pode levar a respostas anti-inflamatórias e antitumorais.

Palavras-chave: *Brassica oleracea* var. italica. Pectina. Imunomodulação. Macrófagos. Linfócitos.

ABSTRACT

Many studies have been reported the immunomodulatory and antitumor effects of pectins, highlighting modified pectins (MPs), whose biological activity is favored by structural modification. Pectin extracted from *Brassica oleracea* var. italica (FB) presents low molecular mass and galactose as the main neutral sugar, which could be more easily absorbed by the microfold cells in the gastrointestinal tract, access the blood and trigger biomodulatory responses. In the present study, FB was evaluated for its immunomodulatory activity and cytotoxicity to hepatocellular carcinoma cells (HepG2). FB (750 μg/ml) reduced the viability of peritoneal macrophages of mice by ~ 28% after 48h of incubation. In the same conditions, FB (500 μg/ml) increased the phagocytic activity of macrophages by 30% e 35% in the assays with yeast as phagocytic particles and neutral red, respectively. Morphological analysis revealed an increase of 40% and 68% in the number of activated macrophages in the presence of FB 250 μg/ml and 500 μg/ml, respectively. FB (200 mg/kg) by oral administration enhanced 120% the number of activated macrophages after 5 days of treatment. When the same dose was injected intraperitoneally, an increase of 490% in the peritoneum cell number was observed. The analysis of these cells revealed an increase of 251% in the number of cells with activated profile, in respect to control. In the supernatant of macrophages from normal mice incubated with FB (250 and 500μg/ml) for 48h, and in the peritoneal fluid from mice after treatment with FB (200mg/kg), no alterations were detected in the nitric oxide and interleukins (IL-1β and IL-12) production, in respect to control. However, after *in vivo* treatment, an increase in IL-10 production by these cells was observed. The proliferation of spleen lymphocytes from mice orally treated with FB (200mg/kg) for 5 days reached 51% in respect to control. These cells were then plated in the presence of concanavalin A for 72 h, and an increase of 24,4% in the cell number was observed. The supernatant of lymphocytes from mice orally treated with FB (200mg/kg) promoted the proliferation of bone marrow cells, representing an increase of 46.1% in respect to control (untreated). This result suggests that FB was able to stimulate the lymphocyte-mediated bone marrow cell proliferation. A preliminary assay for apoptosis showed that HepG2 cells incubated with FB at 250 and 500 μg/ml for 48 h had an increase in the proportion of annexin V/PI-staining cells in comparison with untreated controls. These findings showed that oral administration of FB might trigger anti-inflammatory and antitumoral responses.

Keywords: *Brassica oleracea* var. italica. Pectin. Immunomodulation. Macrophages. Lymphocytes.

LISTA DE FIGURAS

REVISÃO DE LITERATURA

ARTIGO CIENTÍFICO

LISTA DE ABREVIATURAS E SIGLAS

BRM – *Biological response modifiers* (modificadores de resposta biológica)
ConA – *Concanavalin A* (concanavalina A)
CRD – *Carbohydrate Recognition Domain* (região de reconhecimento de carboidrato)
DMEM – *Dulbecco's Modified Eagle's Medium*
DMSO – Dimetilsulfóxido
ELISA – Ensaio de enzima ligada ao imunoabsorvente
FB – "Fração β" de pectina obtida dos talos de brócolis (*Brassica oleracea* var. italica) por extração com ácido nítrico fervente por 30 minutos
FBS – *Fetal bovine serum* (soro fetal bovino)
FITC – Isotiocianato de fluoresceína
Gal – Galactose
Gal-3 – Galectina-3
GalA – Ácido galacturônico
GALT – *Gut Associated Lymphoid Tissue* (tecido linfoide associado ao intestino).
GM-CSF – *Granulocyte-Macrophage Colony-Stimulating Factor* (fator estimulador de colônias de granulócitos e macrófagos)
HBSS – *Hanks' Balanced Salt Solution*
HEPES – N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid
HG – Homogalacturonana
IFN – Interferon
IFN-γ – Interferon-γ
IgA – Imunoglobulina A
IL – Interleucina
IL-1 – Interleucina-1
IL-1α – Interleucina-1α
IL-1β – Interleucina-1β
IL-3 – Interleucina-3
IL-4 – Interleucina-4
IL-5 – Interleucina-5
IL-6 – Interleucina-6
IL-10 – Interleucina-10
IL10-R – Receptor de interleucina-10

IL-12 – Interleucina-12

IL-13 – Interleucina-13

IL-23 – Interleucina-23

LPS – Lipopolissacarídeo

MCP – *Modified Citrus Pectin* (pectina modificada de *Citrus*)

MP – *Modified pectin* (pectina modificada)

MTT – Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio

NK – Células *Natural Killers*

NO – *Nitric Oxide* (óxido nítrico)

PAMP – *Pathogen-Associated Molecular Pattern* (padrão molecular associado a patógenos)

PBMC – *Peripheral Blood Mononuclear Cells* (células mononucleares de sangue periférico)

PBS – *Phosphate Buffered Saline* (tampão fosfato-salino)

PRR – *Pattern Recognition Receptor* (receptor de reconhecimento de padrão)

RG-I – Ramnogalacturonana I

RG-II – Ramnogalacturonana II

Rha – Ramnose

ROS – *Reactive Oxygen Species* (espécies reativas de oxigênio)

RNS – *Reactive Nitrogen Species* (espécies reativas de nitrogênio)

RPMI – Meio Roswell Park Memorial Institute

STAT – *Signal Transducer and Activator of Transcription* (transdutor de sinais e ativador de transcrição)

TGF – *Transforming Growth Factor* (fator de transformação do crescimento)

TGF-β – *Transforming Growth Factor-β* (fator de transformação do crescimento-*β*)

Th1 – Célula T *helper* 1

Th2 – Célula T *helper* 2

TLR – *Toll-Like Receptor* (receptor do tipo "*toll-like*")

TNF – *Tumor Necrosis Factor* (fator de necrose tumoral)

TNF-α – *Tumor Necrosis Factor-α* (fator de necrose tumoral-*α*)

XG – Xilogalacturonana

SUMÁRIO

1 INTRODUÇÃO

Os polissacarídeos estão amplamente distribuídos na natureza e podem ser obtidos de diferentes fontes (LEUNG et al., 2006). O progresso em relação aos métodos de extração, análises, modificação química e melhor compreensão dos efeitos biológicos, têm ampliado substancialmente as possibilidades de aplicação destes biopolímeros (YU et al., 2018b).

Polissacarídeos obtidos de plantas não causam efeitos colaterais significantes, propriedade que os coloca como potenciais agentes terapêuticos em diversas áreas da medicina (SCHEPETKIN; QUINN, 2006). Muitas plantas medicinais contêm polissacarídeos, como β-glucanas, galactanas e pectinas, que apresentam efeitos imunomoduladores e antitumorais (CHAN; CHAN; SZE, 2009; GRØNHAUG et al., 2011; MAXWELL et al., 2012; POPOV et al., 2014; GEORGIEV et al., 2017; ZHANG; XU; ZHANG, 2015).

Dentre as diversas classes de polissacarídeos advindos de plantas, grande importância tem sido dada às pectinas em relação a seu potencial biomodulador. Pectinas são uma família de polissacarídeos ácidos com estrutura complexa presentes na parede celular de plantas (MAXWELL et al., 2012). As diferenças estruturais das pectinas influenciam diretamente em suas funções, como a capacidade de modular o sistema imune e a ação antitumoral (POPOV; OVODOV, 2013).

A maioria dos estudos com pectinas bioativas foi realizado com pectinas modificadas de fonte cítrica (MCPs) (LECLERE et al., 2016; RAMACHANDRAN et al., 2017; OGUTU et al., 2018). As MCPs são modificadas quimicamente para produzir moléculas de menor massa molecular e que apresentam alto conteúdo de galactose, as quais poderiam ser absorvidas pelo organismo quando consumidas oralmente. O mesmo mecanismo de absorção seria semelhante ao proposto para β-glucanas, o qual utiliza o mecanismo de internalização de antígenos das células do tecido linfoide associado ao intestino (GALT) para explicar sua translocação até a corrente sanguínea. Após atingirem a circulação, as MCPs poderiam executar as ações biológicas a elas atribuídas (CHAN; CHAN; SZE, 2009; BI et al., 2018).

A modulação do sistema imune por polissacarídeos, incluindo pectinas, pode ocorrer de diversas maneiras, como por exemplo, a ligação desses biopolímeros a receptores de membrana, como dectina-1, receptor de manose e receptor "*toll like*" em macrófagos, ativando-os e desencadeando assim uma resposta imune (MANTOVANI; SICA, 2010; SCHEPETKIN; QUINN, 2006). A presença destas moléculas no organismo também pode levar à ativação de linfócitos, evidenciada por sua proliferação e secreção de citocinas (ZHANG et al., 2012; PANDA et al., 2015; LI et al., 2018a). A ativação do sistema imune *per si* pode contribuir para

o efeito antitumoral ou combater previamente micro-organismos. A presença de fragmentos de pectina no organismo poderia exercer efeito tóxico direto em células tumorais, por serem potencias ligantes de regiões de reconhecimento de carboidrato (CRD) presentes na proteína pró-metastática Galectina-3 (Gal-3), expressa em diversos tipos tumorais. A inibição de Gal-3 reduz a progressão do tumor (KIM et al., 1999; NANGIA-MAKKER et al., 2007; SATHISHA et al., 2007; ZHANG; XU; ZHANG, 2015).

As células de carcinoma hepatocelular expressam altos níveis de Gal-3, além de receptores de asialoglicoproteínas (ASGP-Rs), que reconhecem glicoproteínas e outras moléculas com ligantes galactosídeos, o que pode facilitar o acesso da pectina ao interior da célula e desencadear o efeito tóxico (ZHANG; XU; ZHANG, 2015). Vários estudos mostram efeitos inibitórios de pectinas em tumores de fígado (LIU et al.; 2008; STRAUBE et al., 2013; ZHANG; LIU; GAO, 2010, ZHANG; XU; ZHANG, 2015). Xu e colaboradores (2015) mostraram que um polissacarídeo péctico isolado do brócolis inibiu a proliferação de células HepG2.

Embora a maioria das pectinas modificadas seja obtida de fonte cítrica (MCPs), outras fontes vegetais, em conjunto com procedimentos de extração alternativos, podem fornecer pectinas bioativas, como a pectina extraída de *Brassica oleracea* var. italica (FB) (PETKOWICZ; WILLIAMS, 2017), que possui naturalmente baixa massa molecular e galactose como principal açúcar neutro e, portanto, assemelha-se a MCPs, o que a torna um potencial ligante para alvos do sistema imune e de células tumorais, como descrito. Assim, no presente estudo, foi investigada a atividade imunomoduladora e citotóxica em células tumorais da pectina dos talos do brócolis.

1.1 JUSTIFICATIVA

A busca por compostos de origem natural que possam exercer efeitos biomoduladores, visando aplicação na terapêutica da imunomodulação e tratamento do câncer, é uma área de intensas pesquisas. Todo esse interesse se deve, em parte, ao fato de muitas biomoléculas, como as pectinas, apresentarem baixa toxicidade para o organismo e capacidade de potencializar o sistema imune, além de serem citotóxicas para células tumorais (ZHANG; XU; ZHANG, 2015).

Considerando a semelhança estrutural entre a pectina extraída *Brassica oleracea* var. italica (FB) e pectinas modificadas; a redução da proliferação de células HepG2 por um polissacarídeo péctico isolado do brócolis (XU et al., 2015); bem como a importância deste vegetal na prevenção e tratamento do câncer, por conter o anticarcinógeno sulforanfano (LI et

al., 2016) torna-se promissor investigar um possível efeito de FB quanto à atividade biomoduladora *in vivo* e *in vitro*. No presente estudo, para os efeitos relacionados à imunomodulação, foram utilizados camundongos Swiss, enquanto que para a citotoxicidade em células tumorais foram utilizadas células de hepatocarcinoma humano (HepG2), por possuirem características como alta expressão de Gal-3 (JIANG et al., 2014) e receptores de asialoglicoproteinas (ASGP-Rs) (LIU et al., 2008; STRAUBE et al., 2013; ZHANG; XU; ZHANG, 2015), o que favoreceria a ação da pectina e poderia aumentar a possibilidade de efeito citotóxico.

1.2 OBJETIVOS

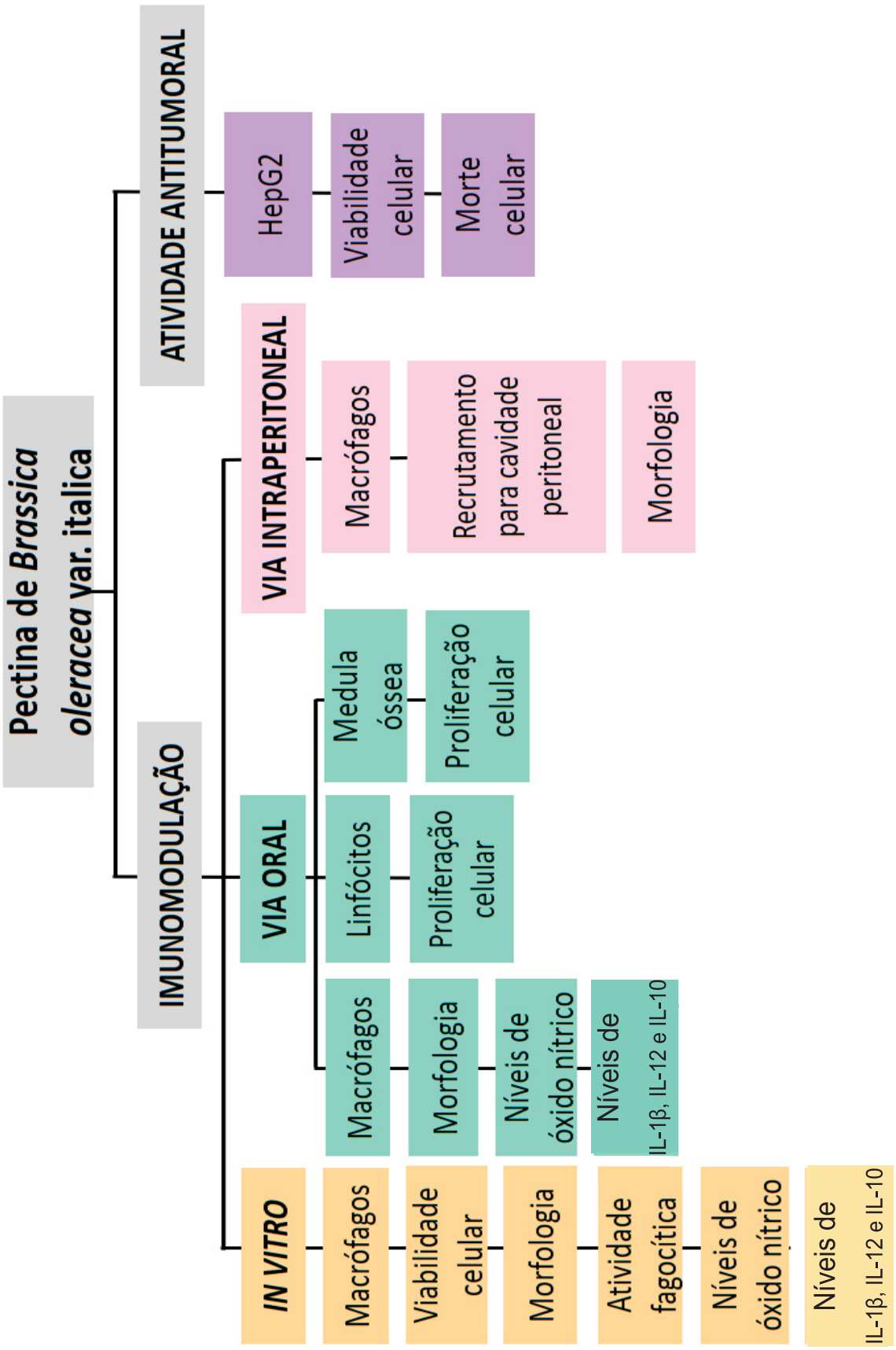
1.2.1 Objetivo geral

Avaliar a pectina isolada de *Brassica oleracea* var. italica quanto à atividade imunomoduladora e citotóxica para células tumorais.

1.2.2 Objetivos específicos

- Avaliar o potencial imunomodulador do polímero, verificando:
    - A modulação de macrófagos peritoneais de camundongos por meio da análise de morfologia, atividade fagocítica, produção de óxido nítrico e de interleucinas após incubação com a pectina;
    - O efeito da pectina em macrófagos peritoneais por meio da análise de morfologia e da produção de óxido nítrico e interleucinas em camundongos tratados por administração oral com pectina;
    - A capacidade da pectina em recrutar macrófagos para a cavidade peritoneal de camundongos tratados com a pectina via intraperitoneal e de elicitar essas células;
    - O perfil proliferativo de linfócitos incubados com a pectina, e de linfócitos de camundongos tratados oralmente com a pectina;
    - A proliferação de células da medula óssea após incubação com o sobrenadante de linfócitos de camundongos tratados oralmente com a pectina.
- Avaliar o efeito da pectina em células de hepatocarcinoma humano (HepG2), verificando a viabilidade celular e apoptose por citometria de fluxo.

2 ESTRATÉGIA EXPERIMENTAL



3 REVISÃO DE LITERATURA

3.1 ESTRUTURA E ATIVIDADE BIOLÓGICA DE PECTINAS

Polissacarídeos são polímeros macromoleculares que podem ser obtidos de plantas, micro-organismos, algas, animais e fungos. Nos últimos anos, têm atraído especial atenção em relação à variedade de atividades farmacológicas, como a antitumoral e a imunomoduladora (YU et al, 2018b).

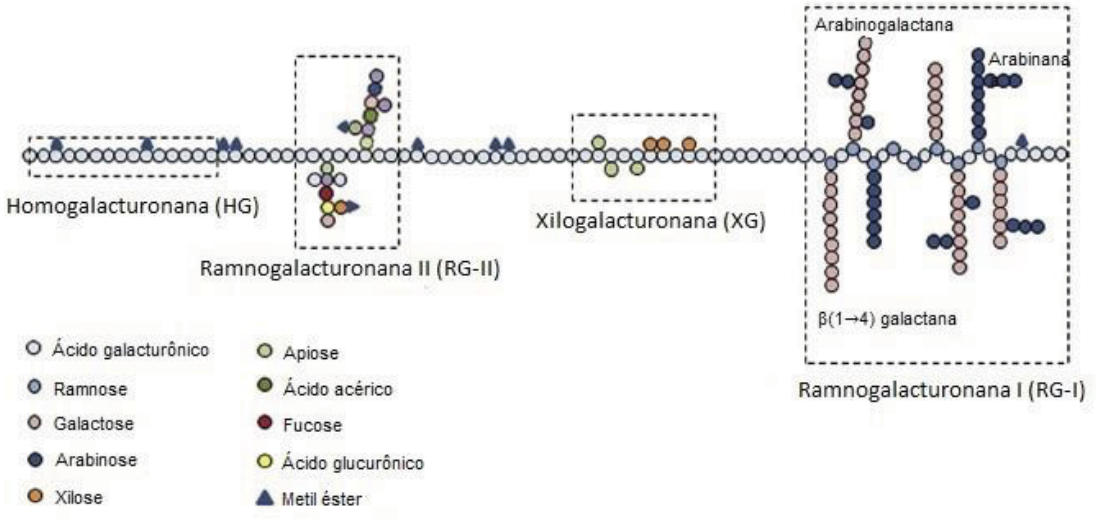
As plantas são ótimas fontes de obtenção de polissacarídeos, os quais podem ser extraídos de seus exsudatos, sementes, frutos, tubérculos e cascas (CUNHA; PAULA; FEITOSA, 2009). A maior parte dos polissacarídeos derivados de plantas apresenta baixa toxicidade, evidenciada pela não detecção de efeitos colaterais significativos. Sendo assim, são candidatos ideais para atuação terapêutica em diversas doenças (SCHEPETKIN; QUINN, 2006). Dentre as várias classes de polissacarídeos de plantas estudadas por sua aplicação biológica, grande importância tem sido dada às pectinas.

Os cientistas franceses Braconnot e Payen foram os primeiros a identificar as pectinas, em 1825. Desde então, a atividade fisiológica desses polissacarídeos tem recebido atenção. Os seres humanos o consomem tanto em alimentos quanto em medicamentos, sendo seu consumo diário médio de 1 a 7 g, o que equivale de 10 a 100 mg/kg de peso corporal (RIDLEY; O'NEILL; MOHNEN, 2001). Além disso, pectinas extraídas de plantas podem ser utilizadas pela indústria alimentícia como agentes geleificantes e estabilizantes em diversas preparações. Dentre as ações conhecidas na saúde humana, sabe-se que, no trato gastrointestinal, estas mesmas propriedades da pectina podem ser responsáveis por inúmeros benefícios, como prevenção e controle de diabetes, melhora do metabolismo lipídico, prevenção e tratamento de aterosclerose, acidente vascular cerebral e doença cardíaca coronária, dentre outros (LATTIMER; HAUB, 2010).

Pectinas são polissacarídeos ácidos que possuem uma complexa estrutura, e estão presentes na parede celular de vegetais superiores, geralmente associadas a outros componentes, como glicoproteínas estruturais e xiloglucanas (RIDLEY; O'NEILL; MOHNEN, 2001). As pectinas criam uma matriz que se liga às microfibrilas de celulose, e estão envolvidas no transporte de íons, retenção de água, além de influenciarem no crescimento de plantas e desempenharem um importante papel defensivo contra fitopatógenos (POPOV; OVODOV, 2013).

Quimicamente, pectinas são uma família de heteropolissacarídeos complexos que envolvem até 17 monossacarídeos distintos e 20 tipos de ligações glicosídicas. Apesar de sua heterogeneidade, as pectinas possuem como característica comum a presença predominante de ácido galacturônico (GalA), presentes em quatro estruturas principais (Figura 1): homogalacturonana (HG), ramnogalacturonana-I (RG-I), ramnogalacturonana-II (RG-II) e xilogalacturonana (XG). HG é a região linear da pectina, composta por unidades de α-D-ácido galacturônico (GalA) unidos por ligação do tipo α(1—4), nos quais alguns dos grupos carboxila são metil-esterificados, ou, dependendo da planta, também parcialmente O-acetilados nos carbonos 2 ou 3. RG-I é a principal região ramificada da pectina, composta por um esqueleto com a repetição do dissacarídeo [-4)-α-D-GalA-(1,2)- α- L-Rha-(1]$_n$, altamente ramificado com cadeias de açúcares neutros, como galactose e arabinose, formando as arabinanas, galactanas e arabinogalactanas. Já a RG-II é uma região ramificada que ocorre com menor frequência. Estruturalmente, difere de RG-I por possuir uma cadeia principal de HG, a qual apresenta cadeias laterais contendo diferentes monossacarídeos. Há ainda a ocorrência eventual de outras estruturas, como XG, que consiste de HG substituídas por unidades de β-D-xilose (RIDLEY; O'NEILL; MOHNEN, 2001; MAXWELL et al., 2012).

FIGURA 1 – REPRESENTAÇÃO ESQUEMÁTICA DA ESTRUTURA DE PECTINAS



FONTE: adaptada de Maxwell et al. (2012).
NOTA: Composição e estrutura sugerida para homogalacturanana, ramnogalacturonana-I, ramnogalacturonana-II e xiloglucana. Nesse modelo, a região RG-I é considerada ligada à região HG, que é parcialmente metil-esterificada e também está ligada às regiões RG-II e XG. Cadeiras laterais neutras de RG-I são galactanas, arabinanas e arabinogalactanas.

As diferenças estruturais das pectinas influenciam diretamente em sua atividade biológica, como a habilidade em modular o sistema imune e a ação antitumoral. Na revisão de Popov e Ovodov (2013), há uma descrição geral relacionando a estrutura de pectinas com os efeitos biológicos. Os estudos mostram que as diferentes regiões da estrutura do biopolímero apresentam efeitos variados. A atividade imunossupressora, por exemplo, pode ser desencadeada pelas cadeias lineares com alto conteúdo de ácido galacturônico, e fragmentos de galacturonanas podem exercer ação anti-inflamatória. Por outro lado, os segmentos ramificados da pectina, principalmente a região RG-I, parecem ser responsáveis por sua atividade imunoestimuladora (POPOV; OVODOV, 2013). Já em relação à atividade antitumoral, sabe-se que HG de baixa massa molecular e a fração RG-I de pectinas podem também ser responsáveis pela indução de apoptose de células de câncer, além de uma ação antitumoral indireta, ocasionada pela imunoestimulação (MAXWELL et al., 2016; YU et al., 2018b).

Nesse contexto, muitos estudos têm evidenciado o papel imunomodulador e antitumoral de pectinas. Suh e colaboradores (2013) trataram camundongos com um polissacarídeo péctico de *Citrus unshiu* a 2 g/kg por 21 dias, e observaram que o sobrenadante das placas de Peyer retiradas desses animais foi capaz de induzir a proliferação de células de medula óssea. Kim e colaboradores (2017) verificaram que um polissacarídeo rico em arabinoxilana e ramnogalacturonana-I induziu a produção de IgA e citocinas relacionadas, como TGF-β1, IL-6 e GM-CSF, por linfócitos T de camundongos, após tratamento via oral por 20 dias com 250 mg/kg do polímero. Panda e colaboradores (2015), extraíram um polissacarídeo péctico dos frutos verdes de *Momordica charantia* (karela) e observaram um aumento na produção de óxido nítrico em macrófagos da linhagem RAW264.7 tratados com o polímero, o qual também foi capaz de estimular a proliferação de esplenócitos e timócitos.

Em relação à atividade antitumoral, Jayaram, Kappor e Dharmesh (2015) observaram o efeito antitumoral e antimetastático de pectina extraída do milho (*Zea mays* L.). Nesse estudo, a pectina foi capaz de inibir a proliferação de células de melanoma da linhagem B16F10, alcançando uma redução da viabilidade destas células de 60% na concentração de 20 μg/ml. No mesmo estudo, as células B16F10 foram inoculadas em camundongos e, após tratamento com a pectina e depois de 21 dias da inoculação, verificou-se que o polissacarídeo foi capaz de inibir a metástase no pulmão desses animais. Já Lin e colaboradores (2016) verificaram que a viabilidade celular de linhagens de câncer pancreático (BxPC-3 e PANC-1) foi reduzida em cerca de 70% e 50%, respectivamente, quando incubadas por 72 h com pectina extraída de flores de *Lonicera japonica.*

Embora não se tenha clareza de todos os eventos que envolvem a absorção e ação de pectinas, é consenso que, *in vivo*, os mecanismos antitumorais das pectinas ingeridas pela dieta ou por administração terapêutica via oral estão relacionados a sua atividade probiótica e ativação do sistema imunológico (ZHANG; XU; ZHANG, 2015). Mais informações sobre os possíveis mecanismos de ação antitumoral de pectinas estão descritas no item 2.4.3.

3.2 MECANISMO DE ABSORÇÃO DE PECTINAS PELO TRATO GASTROINTESTINAL

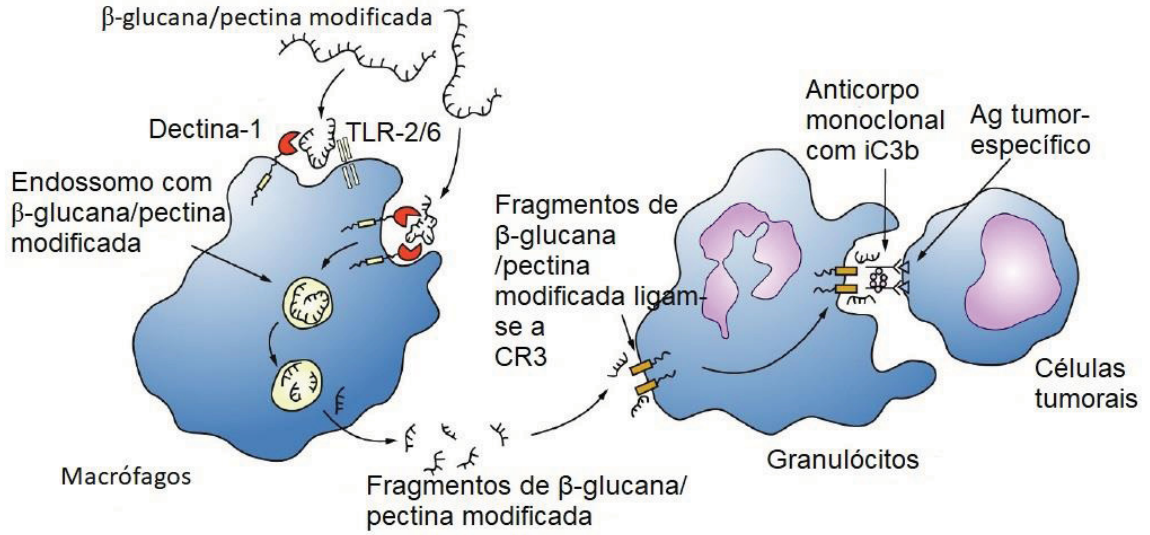
A demonstração da atividade biomoduladora de pectinas administradas via oral (SUH et al., 2013; KIM et al., 2017) colocou em cheque evidências anteriores de que estes biopolímeros não poderiam ser absorvidos pelo trato gastrointestinal (TGI), uma vez que tal fato impossibilitaria os resultados encontrados. Muitos desses estudos ocorreram com pectinas modificadas (MP), em especial as de fonte cítrica, conhecidas como *modified citrus pectins* (MCPs) (GLINSKY; RAZ, 2009). As pectinas modificadas podem ser obtidas de pectinas comerciais por meio de alterações no pH e na temperatura, que causam a quebra das cadeias da pectina e originam fragmentos de baixa massa molecular e baixo grau de esterificação, que poderiam então ser mais facilmente absorvidos pelo TGI, seguindo o mesmo modelo proposto para β-glucanas (MAXWELL et al., 2012).

Considerando que humanos possuem em seu TGI um sistema imune especializado para reconhecer organismos e moléculas não-próprias – chamado de tecido linfoide associado ao intestino (GALT) – foi sugerido um mecanismo de internalização de polissacarídeos, como β-glucanas, através do GALT (CHAN; CHAN; SZE, 2009). Resumidamente, o GALT é constituído de tecidos linfoides organizados, como mesentério, gânglios linfáticos e placas de Peyer, além de linfócitos difusos (FORCHIELLI, WALKER, 2005). Cerca de 10% das células epiteliais associadas aos folículos são *microfold cells*, ou células M. Estas células são especializadas em realizar a fagocitose e transcitose de macromoléculas, bem como qualquer micro-organismo ou antígeno particulado, através do epitélio intestinal, até entrarem em contato com linfócitos e fagócitos mononucleares que captam estas substâncias e/ou moléculas após interação com receptores específicos na membrana plasmática (MABBOT et al., 2013).

No modelo proposto para as β-glucanas, estes biopolímeros, após internalizados nos macrófagos, seriam fragmentados em moléculas menores e, através do sistema do retículo endotelial, liberados na corrente sanguínea (Figura 2), possibilitando assim, que estas moléculas modulem o sistema imune ou atuem diretamente em células tumorais (MAXWELL et al., 2012; CHAN; CHAN; SZE, 2009; BI et al., 2018). O mecanismo sugerido para β-glucanas também é

aceito para pectinas. Até o presente, não há outro modelo mais consistente que explique os resultados de imunomodulação observados pela administração oral de polissacarídeos.

FIGURA 2 – CAPTAÇÃO DE β-GLUCANAS/PECTINAS MODIFICADAS POR CÉLULAS DO SISTEMA IMUNE E AÇÕES SUBSEQUENTES



FONTE: adaptada de Chan; Chan; Sze (2009).
NOTA: Polissacarídeos que são absorvidos no trato gastrointestinal interagem com células do sistema imune, como macrófagos, por meio de receptores específicos, como Dectina-1 ou TLR-2/6, são engolfados por estas células, fragmentados e liberados na corrente sanguínea, onde podem exercer as atividades biomoduladoras aferidas a eles. No exemplo, os fragmentos estimulam um granulócito a reconhecer e eliminar uma célula tumoral. CR3 – Complement receptor 3; iC3b – Inactivated complement 3b; TLR-2/6 – Toll Like Receptors 2/6.

3.3 PECTINA DE *Brassica oleracea* var. italica

A espécie *Brassica oleracea* var. italica, conhecida vulgarmente como brócolis, é pertencente à família *Brassicaceae*, da qual faz parte o gênero *Brassica*, o qual compreende vegetais como repolho, couve, nabo e abóbora. Brócolis são vegetais muito populares e com alto valor nutritivo e, por isso, têm atraído grande interesse nos últimos anos. Um de seus componentes mais estudados é o sulforafano, um isotiocianato que é capaz de reduzir o risco de desenvolvimento de muitos tipos de câncer, atuando por meio da parada do ciclo celular e induzindo à apoptose (LI et al., 2016; YU et al., 2018a).

Apesar da maioria dos estudos se concentrar em torno da pectina de fontes cítricas e de suas formas modificadas, outros vegetais também podem ser fontes de pectinas com potencial bioativo. As plantas da espécie *Brassica oleracea* também podem ser estudadas quanto a seu potencial para extração de MPs. Em estudos preliminares, Samuelsen et al. (2006)

isolaram pectinas de três variações de *Brassica oleracea*: repolho, couve e couve-vermelha, que apresentaram estruturas semelhantes. Foi realizado teste de fixação de complemento e constatou-se que todos os polímeros foram capazes de induzir hemólise, sendo as frações com maior quantidade de cadeias laterais ligadas a galactose por meio de ligações do tipo (1→6) e 1→3,6) as mais bioativas. Um polissacarídeo péctico (BPCa) isolado do brócolis, constituído de Ara, Gal, Rha, e GalA, reduziu a proliferação das células HepG2 (XU et al., 2015).

A pectina extraída de talos do brócolis por Petkovicz e Williams (2017) possui naturalmente baixa massa molar (72.218 g/mol), de acordo com a análise de espalhamento de luz, 60% de GalA, 7% de Rha e Gal como principal açúcar neutro (17%), determinado por métodos cromatográficos, características que a fazem estruturalmente similar a pectinas modificadas. Seu grau de metil esterificação é de 56%, além de 1,1% de acetil e 8,1% de proteína. Por meio de espectroscopia de RMN-13C, foram estudadas as estruturas químicas da pectina, que mostrou sinais característicos de homogalacturonana, ramnogalacturonana-I e galactana. A similaridade da pectina de brócolis com a de pectinas modificadas pode indicar que é possível a sua internalização pelas células do GALT e, por consequência, desencadear atividades imunomoduladoras e antitumorais *in vivo*.

3.4 ALVOS DE AÇÃO DE PECTINAS

3.4.1 Macrófagos

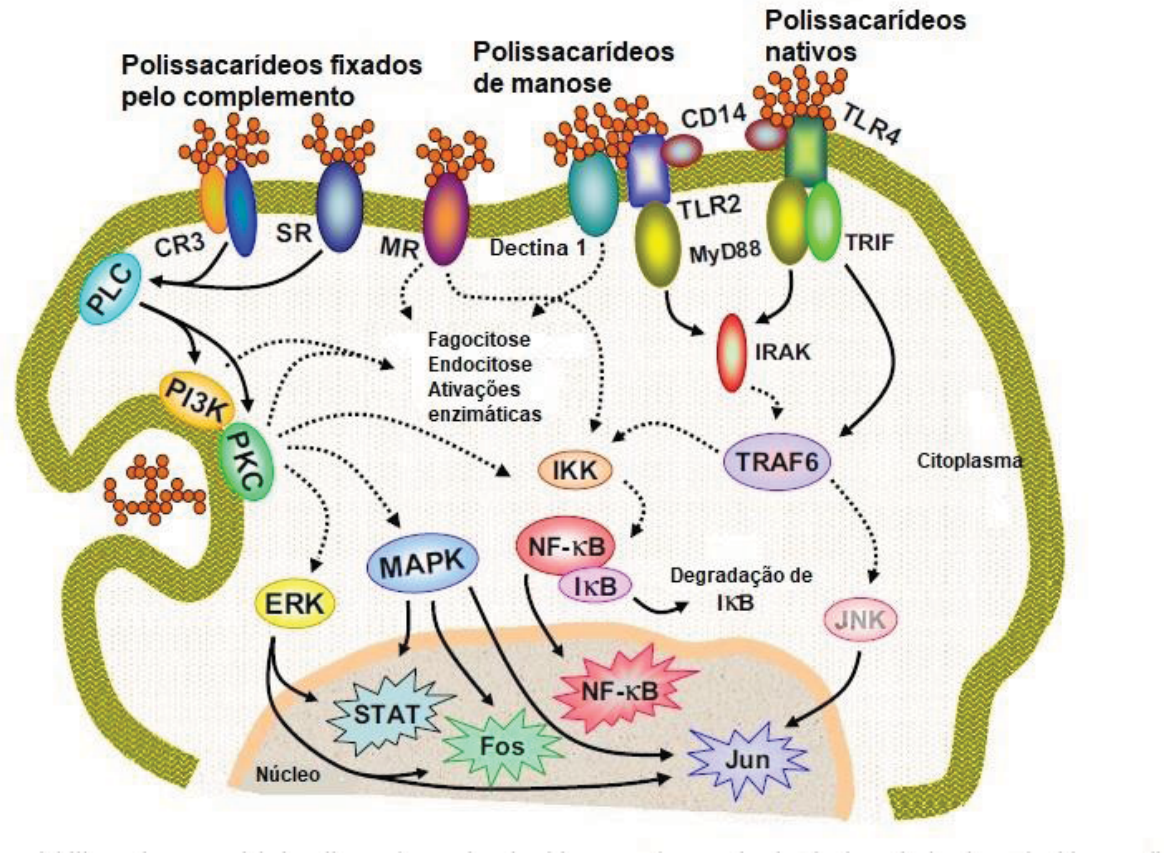
O sistema imunológico humano é complexo e envolve muitos tipos celulares com papéis distintos, porém por vezes sobrepostos. Os fagócitos, como neutrófilos, monócitos e macrófagos, desempenham um papel chave na resposta imune, porque são as primeiras células a responder à invasão por organismos patogênicos (SCHEPETKIN; QUINN, 2006). Os macrófagos estão amplamente distribuídos em todos os organismos multicelulares e podem alterar seu fenótipo, dependendo do microambiente, devido à sua ação reguladora da homeostase local e sistêmica, por meio de diversos receptores de membrana e moléculas secretadas (SHAPOURI-MOGHADDAM et al., 2018). Estas células desempenham funções no crescimento, diferenciação e angiogênese, além de reconhecerem micróbios, partículas e imunógenos e iniciarem a regulação de respostas inflamatórias e imunológicas (TOEWS, 2002). Com base nessa plasticidade, estas células podem ser classificadas em macrófagos M1 (ativados pela via clássica) e M2 (ativados pela via alternativa).

O fenótipo M1 é resultado da ação das citocinas IFN-γ e TNF-α produzidas por células T (Th1), ou por reconhecimento de padrões moleculares associados a patógenos (PAMPs), como lipopolissacarídeos (LPS) bacterianos, por meio de receptores de reconhecimento padrão (PRR). Esses macrófagos secretam altos níveis de citocinas pró-inflamatórias, como TNF-α, IL-1β, IL-6, IL-12 e IL-23, mediadores, como espécies reativas de oxigênio (ROS) e nitrogênio (RNS), além de aumentar a expressão da enzima ciclo-oxigenase-2 (COX-2). Todas estas moléculas estão envolvidas nas atividades microbicidas e citotóxicas (TOEWS, 2002; MANTOVANI; SICA, 2010; MARTINEZ; GORDON, 2014; SHAPOURI-MOGHADDAM et al., 2018).

Já os macrófagos M2 são polarizados pelas citocinas IL-4 e IL-13 produzidas por linfócitos T (Th2), e exibem um perfil anti-inflamatório, caracterizado pela baixa produção de IL-12 e alta produção de IL-10 e TGF-β (TOEWS, 2002). Diante da habilidade dos macrófagos em alterar seu fenótipo para executar funções relacionadas à imunoestimulação ou imunossupressão, estas células têm sido alvo de estudos para investigar a ação de polissacarídeos modificadores de resposta biológica, com o objetivo de aplicação farmacológica. Estudos relacionados à atividade imunomoduladora de polissacarídeos consideram que estes polímeros, por sua composição estrutural, poderiam ligar-se a receptores de membrana de macrófagos, ou então interagir diretamente com a membrana plasmática (Figura 3). Dentre os receptores-alvo conhecidos pela interação com polissacarídeos ou glicoproteínas, destacam-se os receptores "*toll like"* (TLRs), receptor *scavenger*, dectina-1 e receptor de manose (MANTOVANI; SICA, 2010; SCHEPETKIN; QUINN, 2006).

A ligação de polissacarídeos a receptores em macrófagos pode desencadear a ativação de vias de sinalização intracelular, que culminam na produção de espécies reativas de oxigênio e nitrogênio, secreção de citocinas pró ou anti-inflamatórias, além do processo de fagocitose, que é a principal linha de defesa de macrófagos contra agentes estranhos ao organismo (HIRAYAMA; IIDA; NAKASE, 2018). Diante do descrito, os efeitos de polissacarídeos, incluindo a classe das pectinas, em macrófagos, podem modular estas células para diferentes respostas, que vão desde ativá-los para a fagocitose e aumentar sua função na imunidade até a ativação para secreção de mediadores para atividades microbicida e antitumoral. Como exemplo da ativação das vias de sinalização intracelulares, Kumalasari e colaboradores (2014) verificaram que uma fibra dietética extraída de um tubérculo (*Pachyrhizus erosus* (L.) urbano) com características pécticas foi capaz de estimular macrófagos para a produção de NO, IL-6 e TNF-α. Os autores também verificaram a ativação das vias de sinalização JNK, p38, ERK e NF-κB.

FIGURA 3 – MECANISMOS DE RECONHECIMENTO DE POLISSACARÍDEOS POR MACRÓFAGOS E VIAS DE SINALIZAÇÃO INTRACELULAR



FONTE: adaptada de Schepetkin e Quinn (2006).
NOTA: Mecanismo de reconhecimento de ligantes através da membrana plasmática ou receptores (receptor para complemento 3, receptor "*scavenger*", receptor de manose, dectina-1; e receptores "*toll-like*") desencadeando ativação, fosforilação ou desfosforilação de enzimas em cascata, como proteína quinase C, fosfolipase C, fosfoinositol-3-quinase, proteínas quinase ativadas por mitógeno, fatores de transcrição, como STAT, NF-κB, Jun, Fos, os quais resultam na ativação ou repressão da transcrição de diferentes genes.

3.4.2 Linfócitos

Os linfócitos, assim como os macrófagos, são importantes componentes do sistema imunológico. Originados no baço, timo e medula óssea, estas células são classificadas como linfócitos T, linfócitos B e células "*natural killers*" (NK), que são linfócitos diferenciados. Os linfócitos T e B atuam como mediadores da imunidade humoral e celular devido a sua habilidade de reconhecer e responder especificamente a antígenos. Enquanto os linfócitos B são produtores de anticorpos, os linfócitos T participam da imunidade celular de diversas maneiras, como por exemplo, secretando citocinas e/ou destruindo células infectadas pela ação de células T citotóxicas (ABBAS; LICHTMAN; PILLAI, 2012; BI et al., 2018; CHEN; HUANG, 2018). A comunicação entre as diferentes células do sistema imune, por meio da produção de

citocinas/interleucinas, é complexa e não faz parte desta revisão detalhar seus mecanismos moleculares.

Alguns estudos mostram que certos polissacarídeos atuam em linfócitos induzindo sua proliferação e estimulando a secreção de citocinas. Panda e colaboradores (2015) mostraram que uma pectina extraída de frutos verdes de *Momordica charantia* (Karela) estimulou a proliferação de linfócitos do baço. Em condições similares, Bi e colaboradores (2015) observaram aumento na proliferação de linfócitos do baço e produção de IL-12 com polissacarídeo extraído de *Cordyceps militaris*. Aumento na proliferação de linfócitos do baço e secreção de IL-2 e IFN-γ foram observados após tratamento de camundongos com 120 mg/ml de um polissacarídeo extraído de *Grifola frondosa* (LI et al., 2018a). Tais estudos demonstram a capacidade de polissacarídeos de fontes diversas em estimular o sistema imune por meio do aumento da proliferação de linfócitos e da secreção de interleucinas por estas células *in vitro* e *in vivo*.

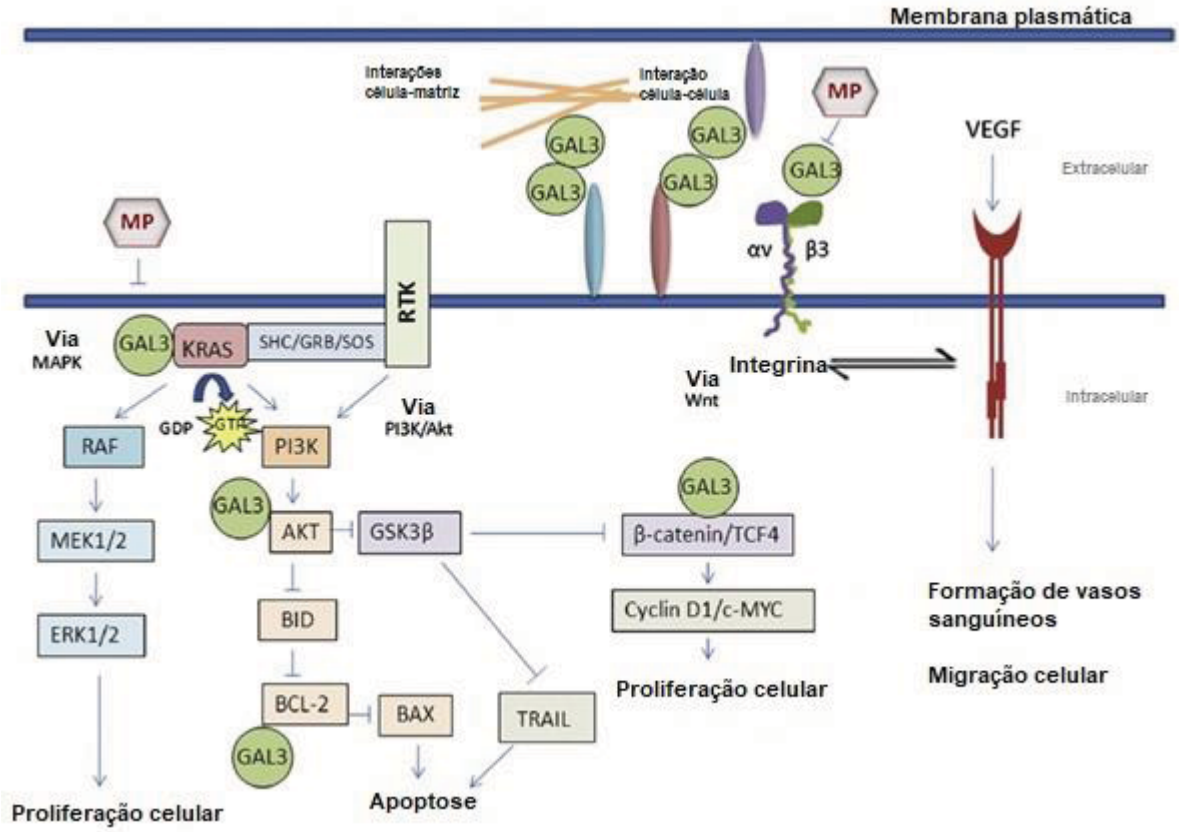
3.4.3 Galectina-3

Nas revisões de Maxwell e colaboradores (2012) e Zhang, Xu e Zhang (2015), estão agrupados vários estudos com pectinas modificadas (MP) abordando seu potencial bioativo, com destaque para absorção pelo trato gastrointestinal e atividade antitumorigênica, por meio da inibição da proteína pró-metastática galectina 3 (Gal-3). O modelo de mecanismo de absorção para as β-glucanas/pectinas (item 2.2), propõe que os fragmentos dos biopolímeros liberados na corrente sanguínea podem agir diretamente em células tumorais (CHAN; CHAN; SZE, 2009). Em estudos com animais, os efeitos das pectinas modificadas (MPs) em tumores e metástases indicam que as MP são efetivamente absorvidas. Esses fragmentos são potenciais ligantes para a Gal-3, expressa em muitos tipos de células tumorais, bloqueando sua atividade.

Gal-3 é uma proteína de aproximadamente 30kDa, constituída por uma região amino-terminal com sítio de fosforilação de serina-6, importante para seu direcionamento dentro da célula; uma sequência de aminoácidos similar ao colágeno; e uma região carboxi-terminal com um domínio de ligação a carboidratos (CRD) com alta afinidade por resíduos de β-galactose presentes em diversos carboidratos e glicoconjugados (MAXWELL et al., 2016). Sua localização no espaço extracelular, na membrana plasmática, no citoplasma e até mesmo no núcleo, lhe garante grande flexibilidade para regular vários processos biológicos, como crescimento celular, adesão, diferenciação, progressão de tumores, resistência à apoptose, angiogênese e metástase (KIM et al., 1999; NANGIA-MAKKER et al., 2007). Tendo em vista

o seu papel na carcinogênese, Gal-3 tem sido objeto de estudos com pectinas, que são seus potenciais ligantes e que poderiam vir a interromper os efeitos desta galectina, principalmente na proliferação celular e apoptose (MAXWELL et al., 2012).

FIGURA 4 –VISÃO GERAL DAS VIAS DE SINALIZAÇÃO CELULAR ASSOCIADAS COM GAL-3



FONTE: adaptada de Yang et al. (1996) apud Maxwell et al. (2012).
NOTA: Pectina modificada (MP) liga-se e inibe GAL3. Ainda não é conhecido se os fragmentos de MP podem ser pequenos o suficiente para entrar na célula, ou se permanecem externos. No espaço extracelular, GAL-3 pode se ligar a glicoconjugados na superfície celular para aumentar a adesão célula-célula e formar interações com a matriz extracelular. Ela pode também se ligar a integrinas, causando uma aglomeração que pode subsequentemente ativar a sinalização de VEGF, que leva à angiogênese. No meio intracelular, GAL-3 liga-se e ativa RAS, que por sua vez ativa RAF e PI3K. Via MAPK: RAF fosforila MEK1 e 2, que fosforilam ERK1 e 2. ERKs exerce um importante papel no ciclo celular, na apoptose, na diferenciação e na migração celular. Via PI3K/AKT: PI3K ativa proteínas que se ligam a AKT. AKT inibe proteínas indutoras de apoptose (BAX, BID, TRAIL) e a proteína inibidora de proliferação GSK3b. GAL3 pode se ligar e ativar AKT, levando à inibição da apoptose e ao aumento da proliferação celular. Via Wnt: inibição de GSK3b permite que β-catenina entre no núcleo e se ligue a TCF4. GAL3 pode se ligar diretamente a GSK3b e β-catenina, levando à regulação positiva das proteínas relacionadas com a proliferação celular ciclina D1 e cMYC. BCL-2: GAL3 se liga a BCL-2 na membrana mitochondrial, interagindo ou imitando a proteínas inibidora de apoptose.

O papel da Gal-3 na carcinogênese ainda não está totalmente compreendido, mas vários estudos sugerem que ela seja capaz de inibir a anoikis (apoptose induzida por perda de ancoragem) e parada do ciclo celular na fase G1 tardia. Este efeito ocorre devido à regulação negativa de ciclinas E e A, e positiva das proteínas inibitórias p21 e p27 (KIM et al., 1999). Uma pectina modificada de fonte cítrica (MCP), apresentando baixa massa molar e maior

conteúdo de galactose que a forma nativa, regulou negativamente as proteínas ciclinas B e cdc2, levando ao acúmulo das células cancerígenas na fase G2/M, promovendo a indução de apoptose (GLINSKY; RAZ, 2009).O efeito antimetastático da pectina por suas propriedades antiadesivas foi avaliado em camundongos, pela injeção intravenosa de MCP e células tumorais de carcinoma de mama humano (MDA-MB-435) e carcinoma de próstata humano (DU-145) marcadas com fluorescência. Após 3 h, a pectina reduziu em > 90% a formação de metástases (GLINSKII et al., 2005). Em relação às propriedades angiogênicas de Gal-3, Nangia-Makker e colaboradores (2000) observaram *in vitro* que a pectina bloqueou a quimiotaxia de células endoteliais de veia umbilical humana por Gal-3 *in vitro*, inibindo completamente sua motilidade e organização. Em células de carcinoma hepatocelular (HCC), foi verificado que Gal-3 estimula a angiogênese e é um marcador de mau prognóstico (JIANG et al., 2014).

O hepatocarcinoma humano é um cancer de figado reconhecido por ser de dificil tratamento, apresentando, portanto, alto índice de mortalidade (FORNER; REIG; BRUIX, 2018). Os medicamentos disponíveis (sorafenibe e rigorafenibe) (BRUIX; QIN; MERLE, 2017; LLOVET; RICCI; MAZZAFERRO, 2008) geralmente não levam à cura do paciente, apenas promovem uma melhora paliativa e, mesmo assim, desencadeiam graves efeitos colaterias. Desta forma, a busca por compostos eficazes e com reduzidos efeitos colaterais é de necessidade urgente. As células de hepatocarcinoma humano (HepG2) expressam altos níveis de Gal-3 (JIANG et al., 2014). Estas células também expressam receptores de asialoglicoproteinas (ASGP-Rs), cuja função é transportar glicoproteínas ricas em galactosídeos para dentro das células. Sugere-se que estes receptores poderiam transportar MPs para dentro de células do fígado e inibir tumores (LIU et al., 2008; STRAUBE et al., 2013; ZHANG; XU; ZHANG, 2015). Caso os ASGP-Rs sejam alvos de ligação da pectina de *Brassica oleracea* var. italica, o polímero poderia ser internalizado e aumentaria a possibilidade de interação com a Gal-3 intracelular, podendo resultar em comprometimento da viabilidade celular.

4 ARTIGO CIENTÍFICO

Essa dissertação foi escrita conforme a Norma Interna 12/2013 do Programa de Pós-Graduação em Ciências – Bioquímica da Universidade Federal do Paraná (UFPR), a qual prevê no Art. 1º que as dissertações devem ser apresentadas segundo as Normas de Apresentação de Documentos Científicos publicadas pela editora da UFPR ou em formato de artigo científico. O artigo científico apresentado neste trabalho será submetido para a revista científica Carbohydrate Polymers.

Title

Pectin from *Brassica oleracea* var. italica triggers immunomodulating effects *in vivo* and *in vitro* and cytotoxicity in HepG2 cells

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Abstract

The immunomodulatory and antitumor effects of pectins have been reported. The pectin extracted from broccoli stalks (*Brassica oleracea* var. italica) (FB) had low molecular mass and galactose as the main neutral sugar. FB was evaluated for its immunomodulatory ability and cytotoxicity for tumor cells. FB (500 μg/ml) for 48h increased the number of activated macrophages by 39% and phagocytic activity by 30%. FB (200 mg/kg) intraperitoneally increased the number of peritoneal macrophages in mice at 490% after 24h and elicited them. By oral administration, FB (200mg/kg) stimulated lymphocytes from spleen and bone marrow proliferation. FB induced IL-10 production and did not induce nitric oxide and interleukins IL-1β and IL-12 production by macrophages. In addition, FB reduced the viability of HepG2 cells, probably by apoptosis. Our findings show that FB triggers immune response and cytotoxic effect to tumor cells and put it as a target for new immunomodulator and antitumor drugs.

Keywords: *Brassica oleracea* var. italica; pectin; immunomodulatory effects, macrophages, lymphocytes, HepG2 cells.

1. Introduction

Plants are abundant sources of polysaccharides, since they are present in the cell wall of all land plants (Carpita & McCann, 2000). Pectins are polysaccharides found in plant cell walls and commercially used in many areas, as food and pharmaceutical industries (Wicker et al., 2014). In addition, these biopolymers are widely investigated to application in medicine due to their immunomodulating and antitumoral potential (Zhang, Xu & Zhang, 2015).

The structure of pectins is complex and it can vary according with its source and extraction procedure. They are classified into two main types according to similar features, being the most common the homogalacturonan (HG), a homopolymer constituted mainly of methyl-esterified (1→4) linked α-D-GalA*p*. The other one is the highly branched rhamnogalacturonan-I (RG-I). The RG-I backbone is composed of [→4)-α-DGalA*p*-(1→2)-α-L-Rha*p*(1→] repeated disaccharide. Sides chains of arabinans, galactans and arabinogalactans are found attached to the rhamnose units. Besides these, other structural regions named xylogalacturonan (XG) and rahmnogalacturonan II (RG-II) may occur (Maxwell, Belshaw, Waldron & Morris, 2012).

The biological activity of polysaccharides depends on their molecular and structural features, such as size, linkage types, conformation, degree and pattern of branching and presence of charge (Ji et al., 2017). In this sense, modifications in the structure of polysaccharides usually affect their biological properties. To increase the effect and bioavailability, the pectin molecules can be degraded by different procedures to obtain fractions with low molecular mass, increased galactan content and low degree of esterification (DE), which are named as modified pectins (MPs) (Krall & McFeeters, 1998, Morris, Gromer, Kirby, Bongaerts, & Gunning, 2011, Zhang, Xu & Zhang, 2015).

The structural complexity of pectins could impair its biological activity, due to the low solubility. However, this affirmative is not sustained by observations that dietary MPs exert antitumor effects and activate the immune system (Zhang, Xu & Zhang, 2015). Then, somehow, these molecules can achieve the bloodstream. It has been demonstrated that MPs can be absorbed by the human body and display antitumor effects and activate the immune system (Morris, Belshaw, Waldron & Maxwell, 2013, Zhang, Xu & Zhang, 2015).

These MPs possess physicochemical and pharmaceutical properties that are similar to β-glucans. They could be bio-absorbed by intestinal epithelial cells, GALT (gut-associated lymphoid tissue) and M cells, subsequently internalized by macrophages, fragmented,

transported and released into blood as proposed to β-glucans. Thus, in recent years, the majority of studies on bioactive pectins are performed with modified pectins, including modified citrus pectins (MCPs) (Maxwell, Belshaw, Waldron & Morris, 2012).

Many evidences suggest that the antitumor effects exhibited by MPs are triggered by its action on immune system and/or by inhibition of the galectin-3 (Gal-3) (Maxwell, Belshaw, Waldron & Morris, 2012). This galectin is highly expressed in many cancers and protects cancer cells from apoptosis impairing the antitumor treatments (Gao et al., 2013; Jiang et al., 2014). Gal-3 has a conserved carbohydrate recognition domain (CRD) that specifically binds to β-galactosides (Dumic, Dabelic & Flögel, 2006), chemical units generally present in pectins. Gal-3 is important in biological processes, such as proliferation, differentiation, and apoptosis, thus has implications in several diseases, including cancer (Liu et al., 2005; Argüeso et al., 2009).

The anticancer activity by several pectins, including the modified pectins (MPs), has been widely investigated in many cancer cells lines by reduction of cell proliferation, migration, adhesion, and induction of apoptosis. All these events are related with action of Gal-3 (Gao et al., 2013, Leclere et al., 2016, Maxwell et al., 2016, Zhang, Xu & Zhang, 2015, Huang et al., 2018). In respect to hepatocellular carcinoma, Gal-3 is associated with a poor prognosis (Jiang et al., 2014). The treatment of hepatocellular carcinoma is very restrict, not effective and triggers serious side effects (Balogh et al., 2016). Pectins obtained from natural sources have low toxicity and can bind to Gal-3 and hepatic cells by asialoglycoprotein receptors (ASGP-Rs) (Zhang; Xu & Zhang, 2015), which motivate their investigation, with the intention of discovering new anti-cancer drugs.

Brassica oleracea var. italica, also known as broccoli, is a vegetable widely consumed and provide critical components of a healthy diet, such as vitamins, minerals and fiber. This vegetable is studied for its content of various nutritional phytochemicals, and are known by its use on “epigenetic diets”, due to the presence of sulforaphane, an isothiocyanate largely studied by its capacity to reduce the risk of developing many common cancers, through cell cycle arrest and induction of apoptosis (Hubbard et al., 2017; Li, Buckhaults, Cui & Tollesfbol, 2016). In a previous study, Xu et al. (2015) reported that a polysaccharide rich in arabinose, extracted from broccoli with water at 80 ºC, inhibited carcinoma cell lines proliferation, including HepG2 cells.

Petkowicz and Williams (2017) extracted pectin from broccoli stalks using boiling 0.1M nitric acid. The pectin, named as FB, had low molecular mass and galactose was the main neutral sugar, making the polymer structurally similar to modified pectins (MP). Considering the structural similarity of FB with MPs and the possibility of internalization of this polymer by gastrointestinal tract and interaction with Gal-3, the aim of this study was to investigate the effects of FB on immune cells of mice and the cytotoxic effect on hepatocellular carcinoma cells (HepG2).

2. Experimental

2.1. Material

Lipopolysaccharide from *Escherichia coli* (LPS), 3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), hematoxilin, naphthylethylene-diamine, N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES), Dulbecco's modified eagle medium (DMEM), annexin V-FITC apoptosis detection kit, cisplatin, polygalacturonate and sulfanilamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Penicilin, gentamicin, streptomycin, fetal bovine serum and RPMI 1640 medium were obtained from Gibco. Tissue culture materials were provided by Techno Plastic Products (Schweiz, Saxonia, Germany). ELISA kits for mouse interleukin 1β (IL-1β), and interleukin 12 (IL-12), were obtained from eBioScience®. Dimethyl Sulfoxide (DMSO), sulphuric acid, phenol, calcium acetate, formaldehyde, ethanol, acetic acid, sodium nitrite and eosin were obtained from Merck (Darmstadt, Hesse, Germany). Bouin solution was made with 75% picric acid (Nuclear Química), 20% formaldehyde (Merck®) and 5% glacial acetic acid (Baker®).

2.2. Pectin

The pectin was previously isolated from broccoli stalk and characterized by one of us (Petkowicz; Williams, 2017). The polysaccharide, named FB, was obtained by extraction with boiling 0.1 M nitric acid for 30 min. FB had a low molecular mass (72,218 g/mol) and was composed of homogalacturonan chains (~ 62%) with high degree of esterification (56.2%) and rhamnogalacturonan regions (~ 32 %) substituted by β-1,4-D-galactans chains. Solutions for biological assays were prepared by dissolving FB (1,8 mg/ml) in phosphate buffer saline (PBS) solution at 50 ºC for 24h. The solution was filtered through a 0.45 μm pore size membrane (Millipore®), followed by filtration through a sterile membrane of 0.22 μm pore size (Millipore®) and then frozen (-20 ºC). Total carbohydrate content was determined (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) before and after the filtration to correct any loss during the filtration process.

2.3. Cell culture

Hepatocellular carcinoma cells HepG2 were obtained from ATCC. Cells were maintained in DMEM high glucose, supplemented with 10% of fetal bovine serum and antibiotics (penicillin G 100 UI/ml and streptomycin 100 µg/ml). HepG2 cells were grown in cell culture flasks at 37º C, 5% CO_2 under controlled humidity.

2.4. Animals

Female albino swiss mice (6-8 week-old) were housed at 22 ºC ± 1 under a 12/12h light–dark cycle and had free access to a standard laboratory diet (Purine®) and water *ad libidum*. All recommendations of the Brazilian National Law (n. 6.638, November 5, 1979) for scientific management of animals were respected. In addition, animal handling procedures for scientific research were approved by the Animal Ethics Committee of UFPR number 1069.

For *in vivo* assays, mice were treated for 5 days with FB at 200 mg/kg using oral gavage feeding needles; or treated for 24 h with FB at 200 mg/kg by intraperitoneal injection. Control groups were treated with PBS.

2.5. Peritoneal macrophage isolation

Peritoneal macrophages were obtained according to standard protocol described elsewhere (Noleto et al., 2002). Briefly, after euthanize of mice by inhaled anesthetic, 10 ml ice-cold sterile phosphate-buffered saline solution (HBSS) were infusing in their peritoneal cavity. The exudate collected was centrifuged at 300 × g, at 4 ºC for 20 min and the pellet was resuspended with Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% fetal bovine serum (FBS), 50 µg/ml gentamicin, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were counted and plated in an appropriate density according to each experiment. After 2 hours at 37 ºC under 5% CO_2 for adhesion, the non-adherent cells were removed by washing once with HBSS at 37 ºC. For *in vitro* evaluations, to the adherent macrophages, medium containing pectin was added and incubated in the same conditions varying the time according to each experimental protocol.

For isolation of peritoneal macrophages from *in vivo* experiments, the peritoneal exudate was obtained by infusion of only 3 ml of HBSS in their peritoneum. After centrifugation, the peritoneal fluid and cells were analyzed as described.

2.6. Cell viability

Peritoneal exudate cells (2×10^5 cells/well) were incubated in 96-well plates for adhesion as described. Medium (control) or pectin (100, 250, 500 and 750 µg/ml) was added to the adherent macrophages. After 48h, the cell viability was evaluated using the 3-[4.5-dimethylthiazol-2-yl]-2.5 diphenyltetrazolium bromide (MTT) method as described by Mosmann (1983) and Noleto et al. (2002).

HepG2 cells were seeded in 96-well plates at a density of 2×10^4 cells/well and incubated 24h to adhere. Then, the cells were incubated with pectin at various concentrations (100, 250, 500 and 750 µg/ml) for 24, 48 and 72h at 37 ºC under 5% CO_2. Cell viability was evaluated using the MTT assay (Mosmann, 1983; Noleto et al., 2002).

2.7. Phagocytic activity

2.7.1. Neutral red assay

The phagocytic activity by neutral red assay was performed according to Weeks, Keisler & Myrvik (1987) with some modifications. Peritoneal exudate cells (4×10^5 cells/well) were incubated in 96-well plates for adhesion as described. Medium (negative control), pectin (250 and 500 µg/ml) or LPS (100 ng/ml; positive control) was added to the adherent macrophages. After 48h, 10 µl of neutral red solution (neutral red 0.02 g in 1ml of DMSO) was added to each well, and the plate was incubated for 30 min. Next, the plate was centrifuged at 300 × g for 5 min, the supernatant was removed, the cells were washed with PBS and 100 µl of Baker fixative solution (2 g sodium chloride, 1g calcium acetate, 4 ml formaldehyde and 93 ml deionized water) was added. After 30 min, the solution was removed and 100 µl of mixture (1 ml acetic acid and 49 ml ethanol) were added following incubation for 30 min. The absorbance was measured at 550 nm.

2.7.2. Phagocytic activity using yeasts as phagocytizing particles

Peritoneal exudate cells (2×10^5 cells/well) on glass coverslips in a 24-well plate were incubated at 37 ºC under 5% CO_2 in the presence or absence of pectin (250 and 500 µg/ml) or LPS (100 ng/ml) as positive control. After 48h, the cell preparations were washed twice with HBSS and the yeast (ratio of macrophages/yeasts 1:5) was added, followed by incubation for

1 h. Then, non-fagocytated yeasts were removed by washing 2 times with HBSS. The coverslips were fixated with Bouin solution for 5 min, stained with Giemsa for 30 min and mounted with Entellan®. The preparations were examined in an optical microscopy, in order to obtain the ratio of phagocytated yeasts/macrophages (Amorim, Vriesmann, Petkowicz, Martinez & Noleto, 2016).

2.8. Macrophage activation by morphological analysis

For *in vitro* assay, adherent macrophages from peritoneal exudate cells (2×10^5 cells/well) on glass coverslips contained in a 24-well plate were incubated in absence or presence of pectin (250 and 500 µg/ml) or LPS (100 ng/ml) as positive control. After 48h at 37 ºC under 5% CO_2, the glass coverslips were washed twice with HBSS pH 7.4, fixated for 5 min in Bouin solution, stained with hematoxylin for 1 minute and with eosin for 30 s, and dehydrated in successive solutions of pure acetone, acetone: xylene (2:1, 1:1 and 1:2) and pure xylene. The slides were then mounted with Entellan® and examined microscopically. At the same time, after 2h for adhesion, macrophages from the peritoneal exudate (2×10^5 cells/well) of mice orally treated with pectin were directly processed for optical microscopy as described in section 2.7.2. The number of activated and resident macrophages was counted in all groups (Amorim, Vriesmann, Petkowicz, Martinez & Noleto, 2016).

2.9. Nitric oxide production

The nitric oxide production was indirectly assessed by measuring nitrite in the culture medium using the Griess reaction with modifications, as previously described (Keller, Wechsler, Leist & Van der Meide, 1990). For *in vitro* assay, peritoneal exudate cells (5×10^5 cells/well) were incubated in 96-well plates in the presence or absense of pectin (250 and 500 µg/ml) or LPS (100 ng/ml) as positive control for 48h. After that, 100 µl of supernatants were mixed with an equal volume of Griess reagent and incubated at 25 ºC for 10 min. At the same time, 100 µl of the supernatants isolated from the peritoneal exudate of mice orally treated with pectin were also used to NO quantification. In both cases, absorbance was measured at 550 nm in microplate reader (Epoch®). The nitrite concentration was calculated from a sodium nitrite standard curve. The results are expressed as µmol.

2.10. Quantification of IL-1β, IL-12 and IL-10

For *in vivo* experiments, mice were orally treated as previously described. After 24 h of the last dose, mice were euthanized and its peritoneal cells exudate were collected (in a restricted volume – 3 ml of HBSS – to not dilute the cytokines), centrifuged and stored at -80 ºC until use. For *in vitro* experiments, peritoneal exudate cells (4×10^5 cells/well) were incubated in a 96-well plate in absence or presence of pectin (250 and 500 μg/ml) or LPS (100 ng/ml) for 48 h. Supernatant were then collected, centrifuged and stored at -80º C until use.

Interleukin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol. Values were compared to a standard curve for each interleukin.

2.11. Recruitment of cells to peritoneal cavity

In order to measure the pectin's recruitment or eliciting activity of macrophages, mice (5 animals/group) received a single intraperitoneal dose of pectin (200 mg/kg), according to protocol previously reported by our group (Moretão, Zampronio, Gorin, Iacomini & Oliveira, 2004). In the control group, animals were inoculated with sterile PBS. After 24 h, the peritoneal macrophages were obtained as described in section 2.5. The number of cells in the intraperitoneal fluid from each mouse was determined in a Neubauer hemocytometer and compared with the control group. To measure the activation of these cells by morphological analysis, 2×10^5 cells/well on glass coverslips in a 24-well plate were added and incubated 2h at 37 ºC under 5% CO_2. The glass coverslips were then processed for optical microscopy as described in section 2.7.2. Resident and activated macrophages were counted and compared with control group.

2.12. Lymphocyte isolation

Lymphocyte from non-treated and pectin treated (200 mg/kg for 5 days) mice spleen were obtained as described by Lin et al. (2016a). Briefly, the spleen was aseptically removed from each mouse and placed in a sterile petri dish containing cold RPMI 1640 medium. With a sterile wire mesh, the spleens were gently disrupted. The resulting single-cell suspensions were centrifuged at 300 × g and 4 ºC for 5 min. Next, red blood cells were lysed using 1 ml of ammonium-chloride-potassium (ACK) lysis buffer for 1 minute, and 20 ml of PBS. The cells

were centrifuged and this procedure was repeated until complete lysis of red blood cells. The pellets were resuspended with RPMI 5% FBS and counted with trypan blue.

2.13. Lymphocyte proliferation

For *in vitro* assay, lymphocytes from non-treated mice were plated in 96-well plate (3×10^5 cells/well) and incubated in absence or presence of pectin (250 and 500 µg/ml) and 1 µg/ml concanavalin A (ConA) for 72h at 37 ºC under 5% CO_2. After 72 h, the cell proliferation was determined by counting in a Neubauer chamber.

For *in vivo* assay, cells of each spleen from mice treated with pectin were counted separately to compare the number of cells of the control group with those of the treated group. These cells were plated in 96-well plate (3×10^5 cells/well) and incubated with 1 µg/ml of ConA for 72h under the same conditions. The cell proliferation was measured by counting as described above. Additionally, lymphocytes supernatant resulting of *in vivo* assay were used to stimulate bone marrow cells.

2.14. Bone marrow cells proliferation

The femur was excised from non-treated mice in sterile conditions as described by Grønhaug et al. (2011) with modifications. The epiphysis was removed and the bone marrow cells were flushed out using RPMI 1640 5% FBS medium and a 23-gauge needle. The suspension was filtered using a sterile wire mesh and centrifuged at 300 × g, at 4 ºC for 5 min. The cells were resuspended in RPMI 1640 5% FBS, counted, plated at 5×10^5 cells/well and incubated for 48h. After that, cell plate was centrifuged at 300 × g and 4 ºC for 10 min, the supernatant was discarded and the culture supernatant from lymphocytes from mice previously treated with pectin were added to each well. After 72h, cell plate was centrifuged as described above and cell proliferation was measured using the MTT assay.

2.15. HepG2 apoptosis by annexin V/PI flow cytometer

Cells were seeded in 12-well plates at a density of 2×10^5 cells/well and cultured with pectin at 250 and 500 µg/ml for 48 h. Cisplatin 20M were used as positive control. Adherent cells were first trypsinized and washed once with serum-containing medium, at 300 × g for 2 min. Cells were then resuspended in 100 µl binding buffer and stained with 5 µl of annexin

V-FITC and 5 µl of propidium iodide for 10 min in the dark at room temperature. Next, 400 µl of binding buffer was added and the cells were then read by a flow cytometer.

2.16. Statistical analysis

The results were submitted to analysis of variance (ANOVA) test, followed by Tukey's test for average comparison, using GraphPad Prism® statistic program. Experimental values were expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD) of at least two independent experiments in duplicate or triplicate. Significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Effects of FB on viability, morphology and phagocytic activity of peritoneal macrophages

Considering that the regulation of immunological surveillance may lead to possibly cures for diseases, and that some natural polysaccharides, such as pectins, have been described as biological response modifiers (BRMs), the studies of the effects of pectin on modulation of immune system are of great interest (Popov & Ovodov, 2013; Wang et al., 2015).

In order to determine the non-toxic concentration of the pectin FB for adherent peritoneal macrophages *in vitro*, the cells were incubated with FB at different concentrations for 48h and the cell viability was evaluated. As shown in Fig. 1, FB did not affect the viability of macrophages at concentrations 100, 250 and 500 µg/ml. However, at concentration 750 µg/ml, FB was considered slightly toxic, decreasing cell viability by ~28% in respect to untreated macrophages (control). In a previous study, we have shown that a modified pectin extracted from *Theobroma cacao* reduced the macrophage viability by ~80% in the concentration of 400 µg/ml, at the same incubation time (Amorim, Vriesmann, Petkowicz, Martinez & Noleto, 2016). Nascimento et al. (2017) observed that concentrations up to 300 µg/ml of native and modified sweet pepper pectins were non-toxic to macrophage THP-1 cells after 24h of incubation. Razali, Ismail, Abidin & Shuib (2014) found that the SN-ppF3 fraction of a polysaccharide extracted from *Solanum migrum* stalks, constituted by Rha and Gal, probably originated from pectic polysaccharides, presented increase in cytotoxicity in RAW264.7 cells by 28% at 100 µg/ml after 72h. Lastly, it is important to consider the cell line, since the cytotoxicity also is influenced by the type of cell, besides the polysaccharide effect. In addition, it was performed at 24h, while the others for 48h. Thus, to subsequent *in vitro* evaluations, the concentrations of 250 and 500 µg/ml of the polymer were employed.

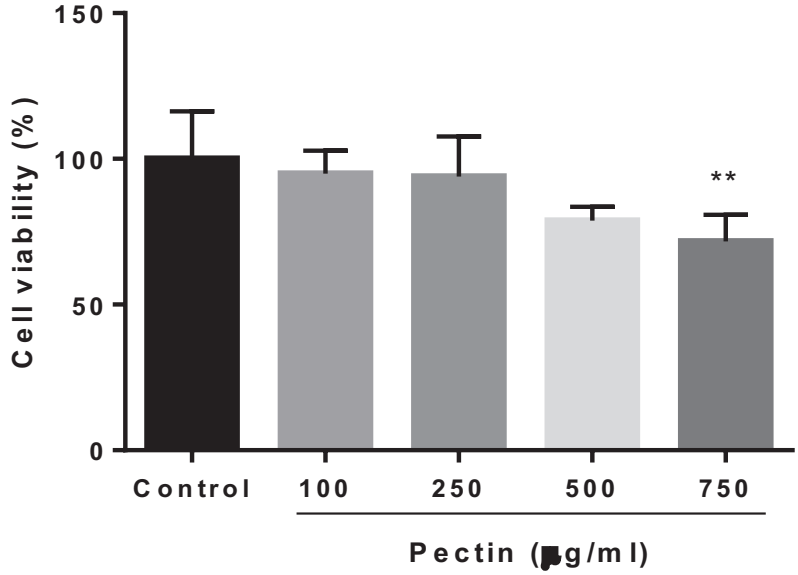

Fig. 1. Effect of pectin on viability of peritoneal macrophages. Culture medium was used as negative control, corresponding to 100% viability. The results are expressed as mean ± SD (n = 2, each experiment in triplicate), ANOVA $p < 0.05$ significant difference from control group. ** $p < 0.01$.

It is widely known that macrophages in contact with substances such as pathogen-associated molecular patterns or certain polysaccharides became activated (Leung et al., 2006). This activated phenotype can be visualized in different ways, as changes in their morphology and products secretion (Van Furth, 1988, Ross & Auger, 2002). The main morphological alterations include considerable increase in the cell size and the nucleus size, presence of binucleated cells, besides numerous hair-like membrane protrusions or filopodia (Xu et al., 2015). Whereas macrophage modulation can be observed by alterations in its morphology, the effects of FB on adherent macrophages were investigated. Considering the characteristics described, the activated and resident macrophages were counted using an optical microscope. Fig. 2(A–D) shows representative images of resident and activated macrophages in absence or presence of FB *in vitro*. The results of the evaluation of macrophage morphology are depicted in Fig. 2E. In the control group ~23% of macrophages were in activated state, while in the presence of FB at 250 and 500 μg/ml the activation of these cells reached ~ 33 and 40%, respectively, representing an increase of ~ 40 and 68% compared to the control. Concerning to the macrophages from mice orally treated with FB (200 mg/kg), the number of macrophages with morphologically activated profile reached 15%, while only 7% was observed in the control group, representing an increase of ~ 120% (Fig. 3A–C).

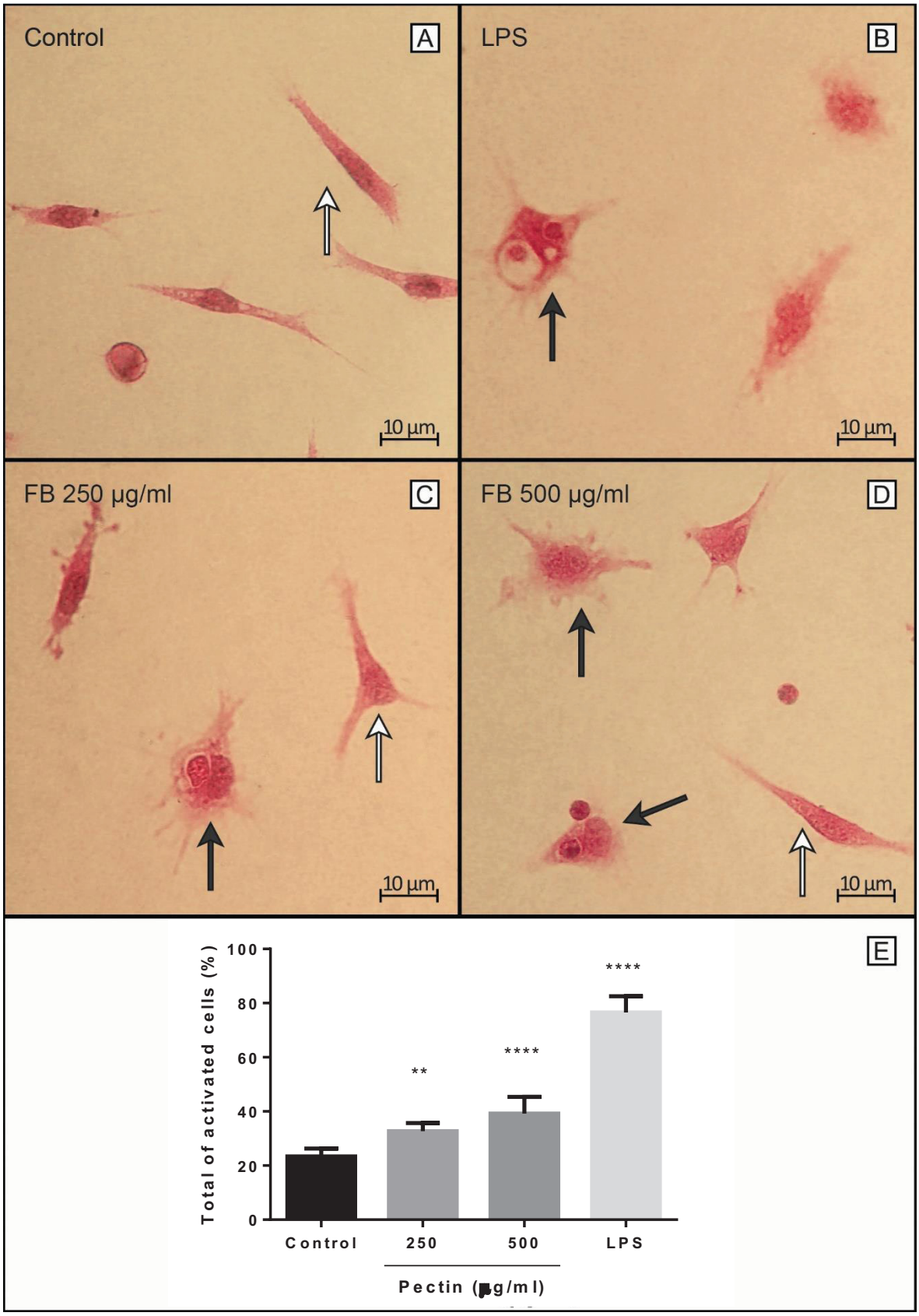

Fig. 2. Representative micrographs (1000x) of mice peritoneal macrophages incubated with medium (A), LPS (B) or pectin FB at 250 µg/ml (C) and 500 µg/ml (D) Figure 2E show the percentage of activated macrophages in relation to total counted macrophages. Black arrows show macrophages with activated features and empty arrows show macrophages with resident features. Results are expressed as mean ± SD (n = 2, each experiment in triplicate), ANOVA $p < 0.05$ significant difference from the control (medium) group. **$p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$.

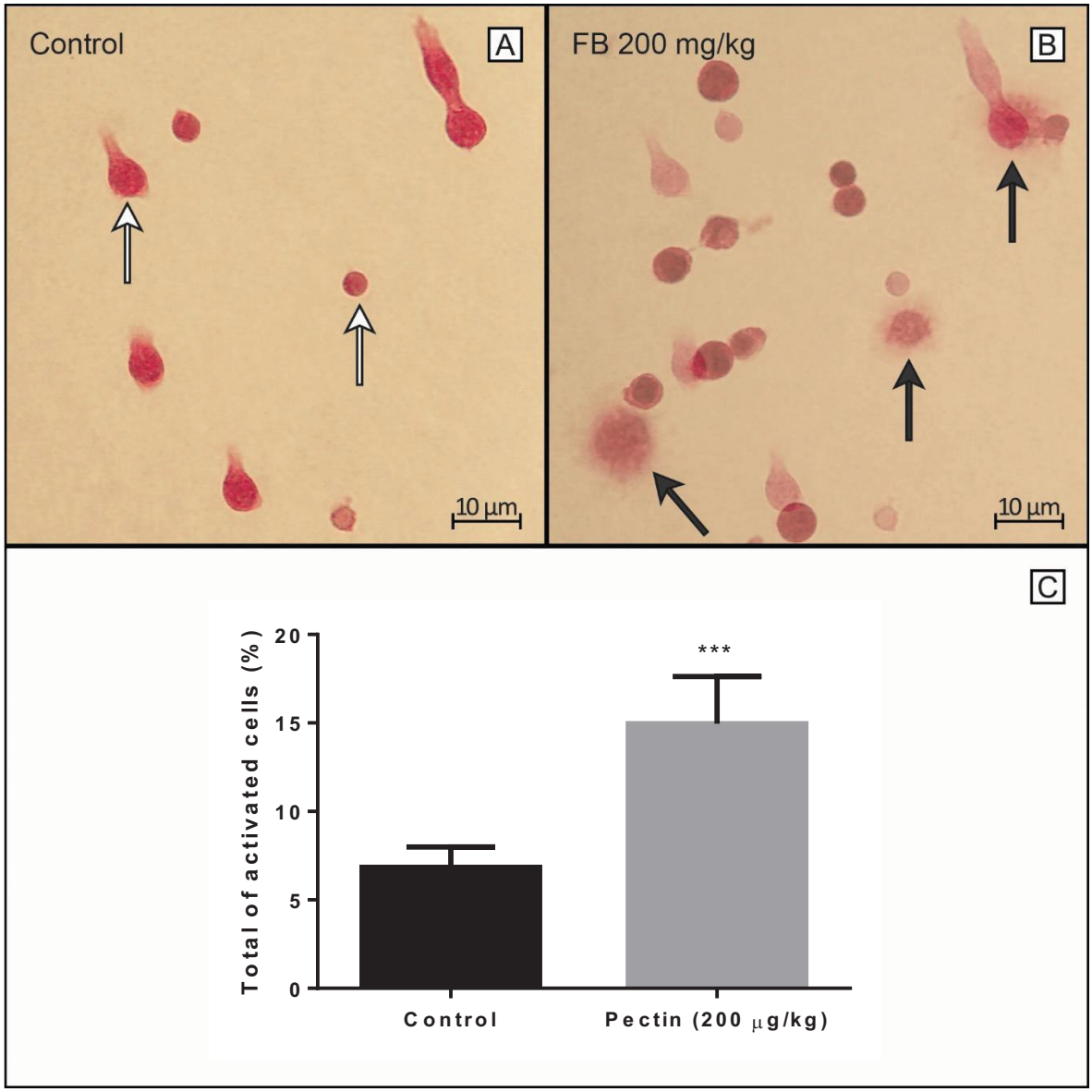

Fig. 3. Representative micrographs (1000x) of peritoneal macrophages from mice orally treated with sterile PBS (A) or pectin FB at 200 mg/kg (B). Figure C show the percentage of activated macrophages in relation to total counted macrophages. Black arrows show macrophages with activated features and empty arrows show macrophages with resident features. Results are expressed as mean ± SD (n = 2, each experiment in triplicate), ANOVA $p < 0.05$ significant difference from the control (medium) group. *** $p < 0.001$.

These results are close to those reported for a modified pectin from *Theobroma cacao* (MOP) (Amorim, Vriesmann, Petkowicz, Martinez & Noleto 2016). MOP (200 µg/ml) increased the number of activated macrophages at ~80% in similar conditions, while in the present study the increase was of ~40% with 250 µg/ml of FB. Other studies have shown activation of macrophages by pectin and derivatives through changes in the morphology. A pectin from the pulp of fruit *Spondias cytherea* at 50 µg/ml increased by 20% the number of mice peritoneal macrophages with activated morphology (Iacomini et al., 2005). Moretão, Buchi, Gori, Iacomini & Oliveira (2003) observed activation of ~50% of the macrophages when

incubated with an arabinogalactan from *Anadenanthera colubrina* at 300 μg/ml. Cui, Wang, Wang, Li & Zhang (2015) found increase in size and an irregular shape of RAW264.7 macrophages after treatment with a polysaccharide extracted from *Pleurotus nebrodensis* at 20 and 40 μg/ml for 48 h. These findings suggest that polysaccharides, including pectins, are able to induce significant change in morphology of macrophages and could represent their potential as modulators of these cells. In the present study, FB exhibited this effect both *in vitro* and *in vivo,* put it as an attractive polymer to be investigated by oral administration.

Macrophages have an exceptional phagocytic ability, which is important to maintain homeostasis and immunity (Gordon, 2016). The activity of polysaccharides is widely investigated by means of phagocytosis pathway, since it is generally triggered by ligands of dectin-1, mannose and *toll like* receptors, which are the main targets of the recognition of polysaccharides (Schepetkin & Quinn, 2006). In the present study, the activation of macrophages to phagocytosis by FB (250 and 500 μg/ml) was investigated by two different methods. Firstly, it was determined from the ratio of phagocytized yeasts/macrophages incubated in the presence or absence of FB (Fig. 4A). At 500 μg/ml, the pectin augmented the macrophage phagocytic activity by ~30% in respect to control group. Using neutral red reagent, pectin increases 28 and 35% the phagocytic activity of mice peritoneal macrophages compared to the control group, at 250 and 500 μg/ml, respectively (Fig. 4B). In contrast, modified pectin from *Theobroma cacao* did not promote increase in the phagocytosis of yeast by mice peritoneal macrophages in the same conditions (Amorim, Vriesmann, Petkowicz, Martinez & Noleto 2016). Recently, Ren et al. (2017) reported that a polysaccharide from *Hericium erinaceus* and its hydroxyethylated form were able to significantly stimulate phagocytosis in RAW264.7 cells, both at 100 and 200 μg/ml. Peritoneal macrophages from mice orally treated with homogalacturonan from *Hippophe rhamnoides* L. berry at 50, 100 and 200 mg/kg had the phagocytic activity increased by 45, 57 and 63%, respectively (Wang et al., 2015). These results suggest that, as other polysaccharides, FB could lead macrophages to recognize and kill microorganisms or tumor cells.

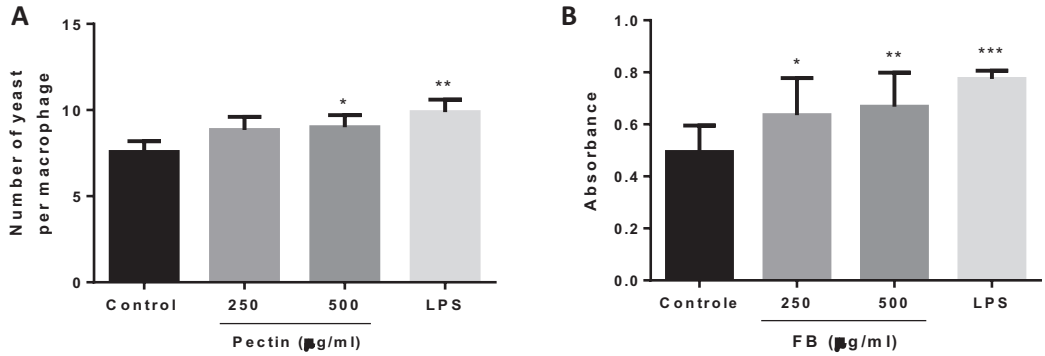

Fig. 4. Effect of pectin on phagocytic activity of peritoneal macrophages. A) Ratio of phagocytized yeasts/macrophages. B) Neutral red assay. Results are expressed as mean ± SD (n = 2, each experiment in duplicate), ANOVA $p < 0.05$ significant difference from the control (medium) group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.2. Effect of FB on recruitment of cells to peritoneal cavity and eliciting activity

To evaluate the effect of FB on recruitment of cells to peritoneal cavity of mice, the polymer was injected intraperitoneally and, 24h later, the total number of peritoneal exudate cells was determined. Fig. 5C shows that FB promoted an increase of 490% in peritoneal cells number in respect to control group (treated with sterile PBS). Cells recruited to peritoneal cavity by some irritant compounds presenting an "activated" phenotype, and are called "elicited macrophages" (Pavlou, Wang, Xu & Chen, 2017). Next, recruited cells were processed to optical microscopy and the number of activated and resident cells was counted (Fig. 5D). The number of cells with activated profile was 251% higher than that in the control group. An arabinogalactan from the gum of *Anadenanthera colubrina* increased ~88% the cell number into peritoneal cavity of mice after 24h of the peritoneal injection (Moretão, Zampronio, Gorin, Iacomini & Oliveira, 2004). In the same conditions, xyloglucans from seeds are also able to recruit cells to peritoneal cavity, reaching 576% to xyloglucan from *Hymenaea courbaril* (XGJ) (Rosário et al., 2008). A pectic polysaccharide isolated from fruit of *F. limonia* (linn.) increase macrophage number on peritoneal cavity by 56% at 100 µg/ml after 24 h (Saima, Das, Sarkar & Sur, 2000). These results showed that FB potentially induce monocyte migration into the peritoneum and become them elicited.

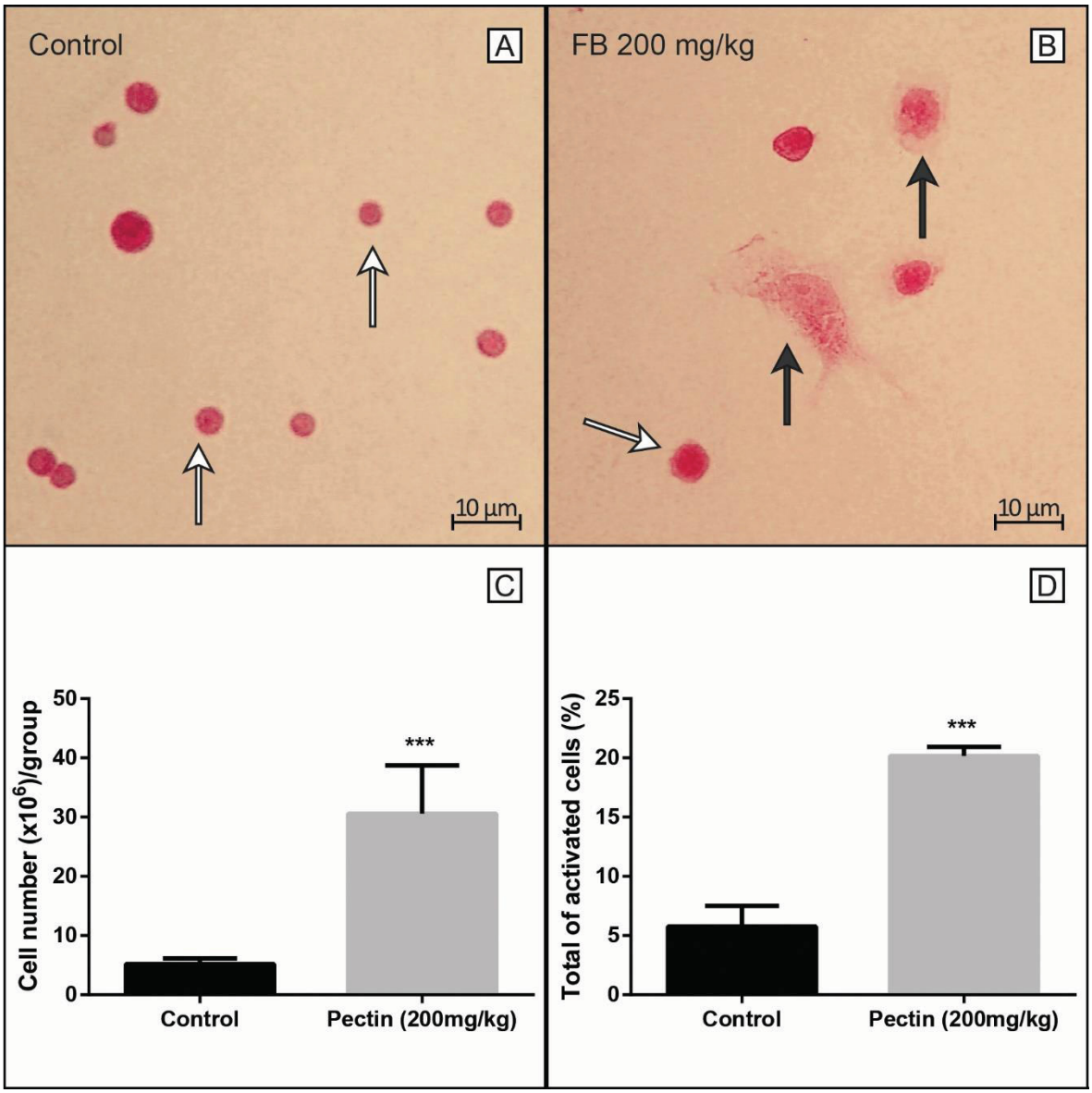

Fig. 5. Effect of intraperitoneal administration of pectin on recruitment of cells to peritoneal cavity and eliciting activity. Number of peritoneal macrophages (C) and total of activated peritoneal macrophages (D) following 24h of sterile PBS (control) and 200 mg/kg pectin administration. A) Representative micrograph of control group and B) representative micrograph of treated group (1000x). Results are expressed as mean ± SD (n = 5, each experiment in triplicate), Anova $p < 0.05$ significant difference from the control group. *** $p < 0.001$.

3.3. Effect of FB on nitric oxide, IL-1β, lL-12 and IL-10 production by macrophages *in vivo* and *in vitro*

Nitric oxide is a key inflammatory mediator secreted by macrophages, and acts as an intracellular messenger molecule for mediation of different biological functions. The activation of its pathway triggers microbicidal and anti-tumor effects (Xie et al., 2017) and increased phagocytosis by macrophages (Chen & Huang, 2018). In the present study, FB did not stimulate NO production by macrophages *in vitro* in the tested conditions (Fig. 6A). In mice orally treated

with pectin, the NO production in the peritoneal fluid was not affected as well (Fig. 6B). Amorim, Vriesmann, Petkowicz, Martinez & Noleto (2016) found different results for three fractions of pectin. A commercial homogalacturonan and native pectin from *Theobroma cacao* did not stimulated the NO production. In contrast, the modified pectin from *Theobroma cacao* potentially increased the NO releasing. Differently from FB, the modified pectin from *Theobroma cacao* had a low degree of methyl esterification and 9.4% of acetylation. Wang et al. (2015) found an increase in NO production by macrophages treated with a homogalacturonan from *Hippophae rhamnoides* L. berry. Ho et al. (2015) used native and partially hydrolyzed pectins to investigate the structure *versus* immunomodulating activity relationship of pectins from berries of *Sambucus nigra*. Partial acid hydrolysis and enzymatic hydrolysis were used to produce different structural patterns. The authors observed that removal of arabinose and reduction in the side chain of galactose 1→3,6 linked resulted in lower NO production. They also found that RG-I regions with side chains of arabinogalactans type I and II had higher NO production than the native pectin. These observations corroborate with the hypothesis that the activation of macrophages to production of NO is not ability common to all pectin preparations and make clear the structure-activity relationship.

Cytokines are small-molecule proteins secreted by immune cells, including macrophages. In general, these mediators are classified in pro-inflammatory (e.g. IL-1β, IL-6, IL-12, TNF-α) and anti-inflammatory (e.g. IL-1ra, IL-10) and play an important regulatory role in immunity (Chen & Huang, 2018). The effects of FB on the production of pro-inflammatory IL-1β and IL-12 interleukins and anti-inflammatory IL-10 interleukin were determined. As shown in figures 6C and 6E, peritoneal macrophages incubated with FB did not increase the IL-1β or IL-12 production. In the peritoneal fluid from mice orally treated with pectin, the production of these interleukins was also not modified in respect to control (Fig. 6D and 6F). On the other hand, FB increased IL-10 production in the peritoneal fluid from mice orally treated with the polymer (Fig. 6G and 6H). This may indicate that FB exerts an anti-inflammatory effect when orally administered.

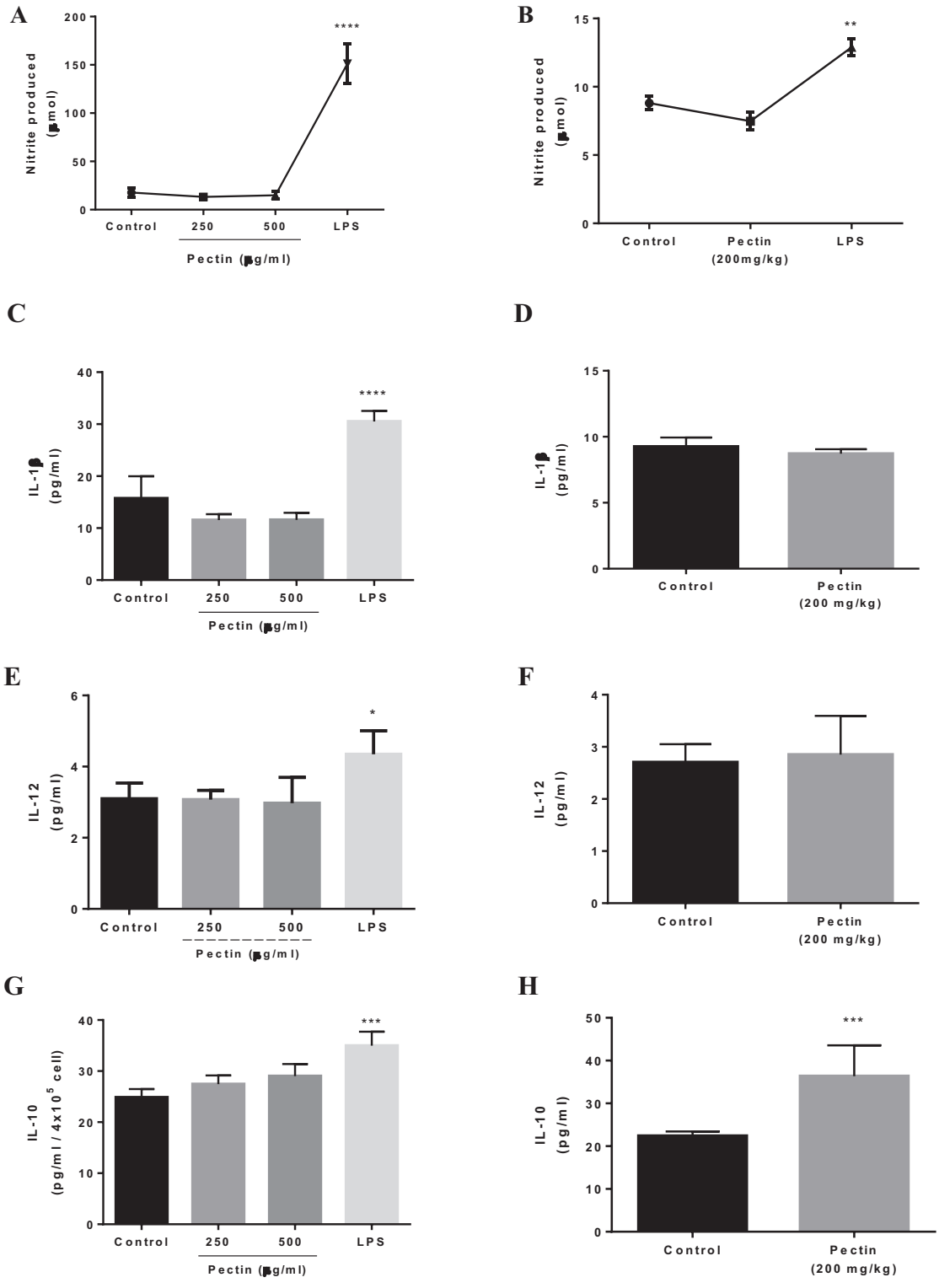

Fig. 6. Effect of pectin FB on nitric oxide, IL-1β, IL-12 and IL-10 production by peritoneal macrophages. A) NO production by macrophages incubated with pectin, B) NO production by macrophages from orally treated mice, C) IL-1β production by macrophages incubated with pectin, D) IL-1β production by macrophages from orally treated mice, E) IL-12 production by macrophages incubated with pectin, F) IL-12 production by macrophages from orally treated mice, G) IL-10 production by macrophages incubated with pectin, H) IL-10 production by macrophages from orally treated mice. Results are expressed as mean ± SD (n = 2 (in vitro) and n = 5 (in vivo), each experiment in duplicate), ANOVA $p < 0.05$ significant difference from the control group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Different profiles in the production of cytokines by macrophages or other interleukin producing cells in contact with pectins have been reported. Salman, Bergman, Dialdetti, Orlin & Bessler (2008) observed different responses of peripheral blood mononuclear cells (PBMC) in presence of citrus pectin (CP) depending on the degree of methyl-esterification. The CP with degree of methyl-esterification of 60%, similar to FB (56%), inhibited the pro-inflammatory IL-1β and did not affect TNF-α and IL-6 levels. On the other hand, the releasing of anti-inflammatory IL-1 and IL-10 interleukins was increased. Similarly, Amorim, Vriesmann, Petkowicz, Martinez & Noleto (2016) observed an increase on IL-10 production and no effect on IL-12 and TNF-α production by a pectin fraction (OP) of *Theobroma cacao* with high degree of methyl-esterification. Interestingly, a fraction of the same pectin with low degree of methyl-esterification (20,8%) presented the opposite effect, increasing IL-12 and TNF-α secretion by macrophages.

Nascimento et al. (2017) studied the capacity of sweet pepper pectins on cytokine secretion and found out that native sweet pepper pectin could induce TNF-α, IL-1β and IL-10 secretion by THP-1 cells at 300 μg/ml. The polymer was submitted to partial acid hydrolysis with HCl, resulting in a polymer composed of homogalacturonan with some rhamnogalacturonan insertions. This modified sweet pepper pectin presented lower TNF-α and IL-10 secretion than native form, but was more effective to increase IL-1β secretion at the same concentration, proving that structural modifications may lead to different cell surface receptor interactions and activation of other pathways.

These studies corroborate the propositions that the degree of esterification interferes in the immunomodulatory activity of pectins.

3.4. Effect of FB on lymphocyte proliferation and lymphocyte-mediated bone marrow cell-proliferation

Another way to assess the capacity of pectin to act as BRM is evaluating its effect on lymphocyte proliferation. Lymphocytes are important cellular components of the body's immune function (Chen & Huang, 2018). The lymphocyte proliferation is important to the activation cascade of cellular and humoral immune responses. It results in an increase in cytokine release, which is probably the pathway for the antitumor activity of the polysaccharides isolated from medicinal plants (Wang et al., 2015).

In the present study, lymphocytes from normal mice spleen were incubated with concanavalin A (ConA), a mitogen inductor, in absence or presence of FB for 72h. Figure 7A

shows that incubation with FB did not enhance lymphocyte proliferation in respect to control (ConA). On the other hand, the lymphocyte proliferation of spleen from mice orally treated with FB (200 mg/kg) increased by ~51% compared to control group (Fig. 7B). This effect was maintained when these cells were incubated with ConA for 72h (Fig. 7C). An increase of 24,4% on lymphocyte number was observed for FB treatment, compared to control group. This result indicate that, somehow, the oral treatment with FB was capable induce lymphocyte proliferation and ability these cells to be more sensitive to ConA.

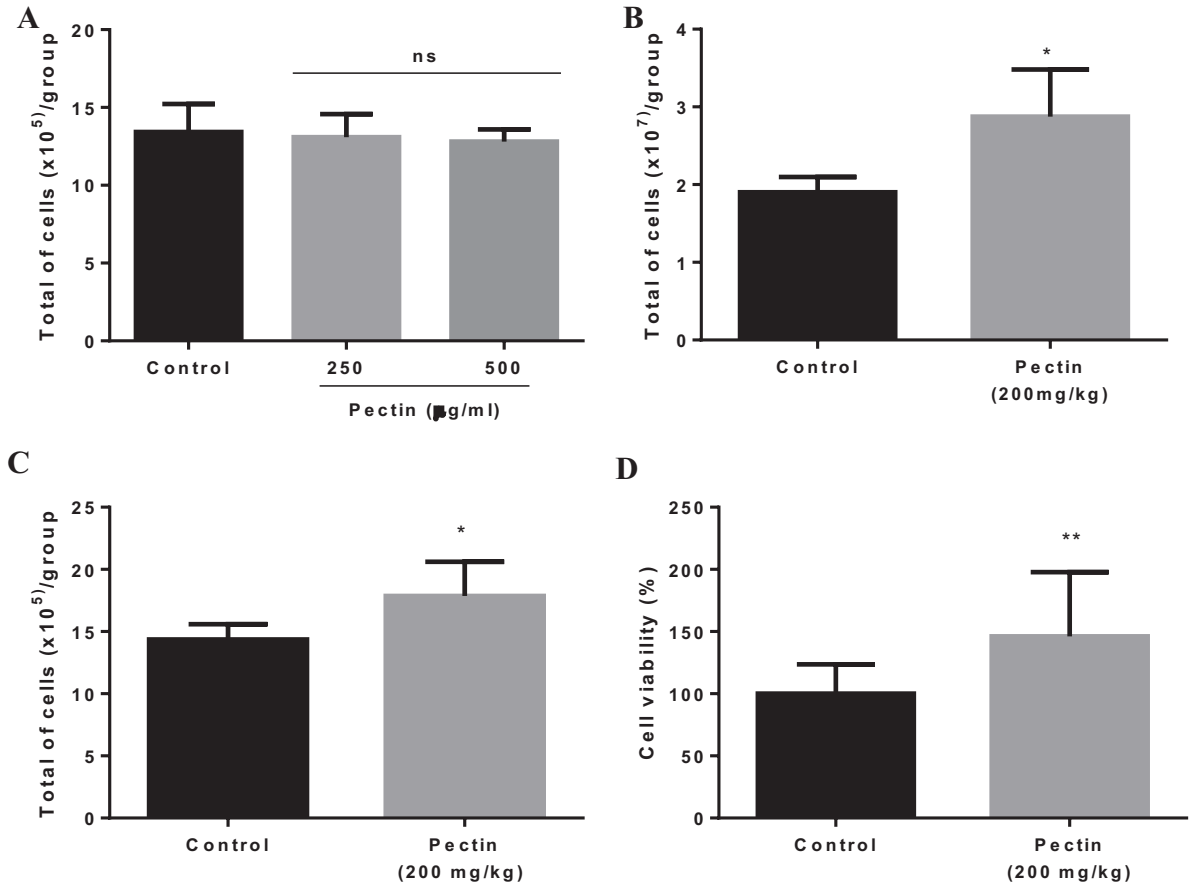

Fig. 7. Effect of pectin on lymphocyte proliferation and lymphocyte-mediated bone marrow cell-proliferation. A) Total of lymphocytes following incubation with ConA and pectin at 250 and 500 µg/ml for 72h; B) Total lymphocytes from spleen of orally treated mice; C) Total lymphocytes from orally treated mice following incubation with ConA for 72h; D) Cell viability of bone marrow cells incubated for 72h with culture supernatant of lymphocytes obtained from orally treated mice. Results were expressed as mean ± SD (n = 5, each experiment in triplicate), ANOVA $p < 0.05$ significant difference from the control group (ConA). * $p < 0.05$ ** $p < 0.01$.

A pectic polysaccharide from the green fruits of *Momordica charantia* (karela) was also able to induce lymphocyte proliferation of splenocytes from normal mice (Panda et al., 2015). Recently, Li et al. (2018b) found that native and partially hydrolyzed pectin without arabinose from *Nelumbo nucifera* did not stimulate the lymphocyte proliferation. In contrast, it

was reported that native and partially hydrolyzed pectin from *Panax Ginseng* induced the proliferation of splenocytes (Li et al., 2018a; Zhang et al., 2012). The authors suggest that these varied profiles could be related to structural differences, such as molecular mass, acetylation and the presence of many side chains (Li et al., 2018a). Since ConA generally stimulates T-lymphocyte immunity, the results of the present study showed that FB might have immunoregulatory activity to T-lymphocytes, although its activity to B lymphocytes remains to be enlightened.

The next assay evaluated the lymphocyte-mediated bone marrow cell-proliferation. Bone marrow total cells were incubated for 72h with culture supernatant of lymphocytes obtained from mice orally treated. Pectin showed enhancing activity on lymphocyte-mediated bone marrow cell-proliferation, representing an increase of 46,1% in respect to control (Fig. 7D). Based on this result, it can be said that the bone marrow cell-proliferation might occurred due cytokines present in the supernatant of lymphocytes, such as GM-CSF. Using the same premise, the proliferation of bone marrow cells cultured in the resulting culture supernatant from Peyer's patch cells with a β-D-(1→4)-galactan-containing side chain in RG-I regions of pectic polysaccharide from *Biophytum petersianum* Klotzsch at 20 μg/ml for 4 days was significantly enhanced in a dose-dependent manner. The authors suggest that the IL-6 production in cultured supernatant of Peyer's patch cells contribute to stimulation of bone marrow cell proliferation in the tested conditions (Grønhaug et al., 2011). Suh et al. (2013) utilized the culture supernatant of Peyer's patch cells from mice orally treated for 3 weeks with 200 mg/kg of a fraction of the pectic polysaccharide from peel of *Citrus unshiu* to stimulate bone marrow cells for 5 days and found an increase in proliferation to 1.76-fold compared to the control. Peyer's patch cells are mainly composed of T/B cells, and T cells are known as a source of hematopoietic growth factors and cytokines (Suh et al., 2013). Considering this fact, oral administration of pectic polysaccharides may contribute to activation of T cells and the secretion of hematopoietic factors, which lead to bone marrow cell proliferation.

3.5. Effect of pectin on HepG2 cell viability and apoptosis

Since modified pectins are potential targets to HepG2 cells aiming antitumoral effects (Leclere et al., 2016; Xu et al., 2015; Lin et al., 2016b), we speculate if FB could have antitumor applications. Firstly, the cytotoxic effect of FB on HepG2 cells was evaluated by the MTT method. As shown in figure 8, treatment with FB resulted in decrease in the cell viability only after 48 h of incubation, with significant effects at the concentrations 250, 500 and 750 μg/ml.

After 72 h, treatment with FB at all concentrations (100, 250, 500 and 750 µg/ml) decreased HepG2 viability by ~55, ~63, ~78 and ~80%, respectively, compared to control. These results suggest the cytotoxic effect of FB in a time and concentration-dependent manner.

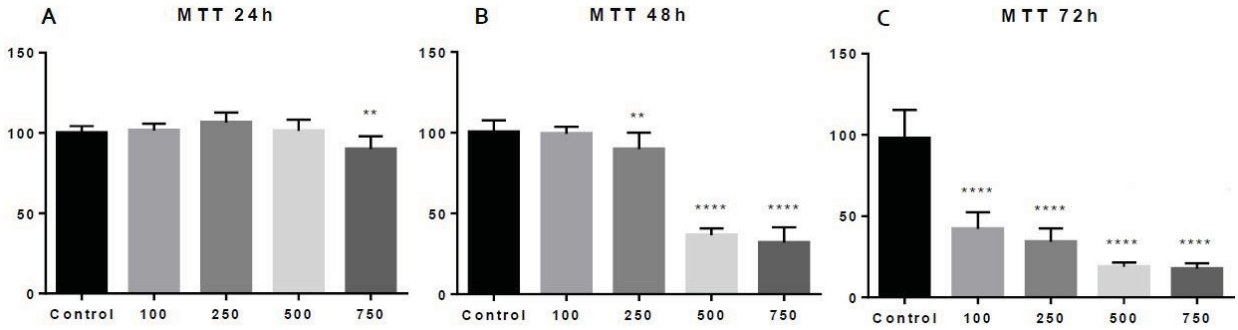

Fig. 8. Cytotoxic effect of FB at different concentrations on HepG2 cells after A) 24, B) 48 and C) 72 h. Results are expressed as mean ± SD (n = 2, each experiment in triplicate), ANOVA $p < 0.05$ significant difference from the control group. ** $p < 0.01$ and **** $p < 0.0001$

A polysaccharide rich in arabinose, extracted from broccoli with water, at concentration of 2 mg/ml, reduced the viability of HepG2 cells by ~80%, after 96 h of incubation (Xu et al., 2015). In the present study, FB promoted similar effect at concentration 2.7 times lower for 72 h. FB was also more potent than a citrus pectin, which at 3 mg/ml reduced the viability of HepG2 cells by ~90% after 48h (Leclere et al., 2016). In the present study, FB reduced by ~70% HepG2 viability, in concentration four times lower (0.750 mg/ml) at the same time of incubation (Fig. 8B). Chai & Zao (2016) found that 3 fractions of the pectin extracted from *Viscum coloratum* (Kom.) *Nakai* significantly inhibit the proliferation of HepG2 cells in a dose dependent manner (20, 40, 60, 80 and 100 µg/ml for 24 h). Lin et al. (2016b) showed that a pectin extracted from *Lonicera japonica* flowers was not cytotoxic to normal liver cells. On the other hand, the pectin (1 mg/ml) reduced the viability of pancreatic cancer cells (BxPC-3 and PANC-1) by 66.7% and 52.1%, respectively, after incubation of 72h. In general, these findings show that pectins are effective to kill tumor cells and exhibit specificity, favoring its use as anti-cancer drugs with low side effects.

In order to investigate the cytotoxic effects of FB on HepG2 cells, a preliminar assay for the detection of apoptosis by annexin V/PI method was performed. As shown in Fig. 9, HepG2 cells incubated with FB at 250 and 500 µg/ml for 48 h had an apparent increase in the proportion of annexin V/PI-staining cells in comparison with the untreated controls.

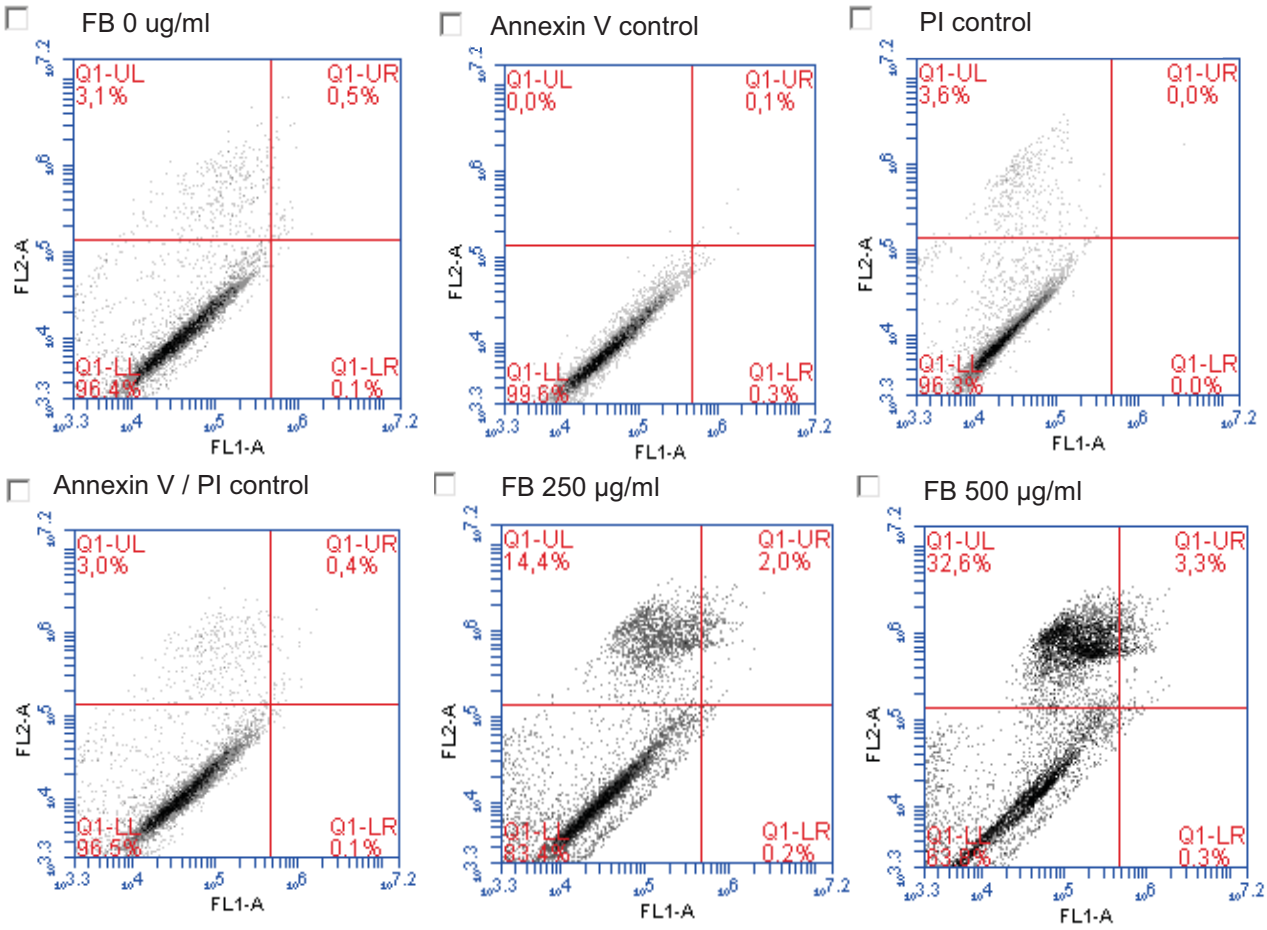

Fig. 9. Representative figures of flow cytometric analysis of apoptosis in HepG2 cell line after treatment with the pectin FB (250 and 500 µg/ml). The lower left quadrant represents viable cells (Annexin/PI negative). The right lower quadrant represents cells in the recent stage of apoptosis (Annexin positive and PI negative). The upper right quadrant represents cells in the late stage of apoptosis or dead (Annexin/PI positive). The upper left quadrant represents dead cells (Annexin negative and PI positive).

Leclere et al. (2016) found an apoptosis-like cell death of HepG2 cells treated with 3 mg/ml heat-modified citrus pectin for 24 h. The cells were analyzed by phase-contrast microscopy. HepG2 cells showed caspase-3 and -7 activations, an presented a displayed shrunken nuclei. In contrast, the same unmodified pectin at 3 mg/ml did not affect cell morphology. Huang et al. (2018) found that a water-soluble polysaccharide from *Agrimonia pilosa* induced apoptosis on U-2 OS cells in a dose-dependent manner (25, 50 and 100 µg/ml for 24 h). Similar pattern was found by Zeng et al. (2017) for a polysaccharide from *Laminaria japonica*. The results indicated that the polymer induced HONE-1 cell apoptosis, which was mainly the late apoptosis, at 20, 80 and 320 µg/ml for 72 h, in a dose-deppedent manner. These findings demonstrate that polysaccharides, including pectins, may have antitumor activity, indicated by cytotoxic and apoptotic effects.

4. Conclusion

The present study showed that pectin from *Brassica oleracea* var. italica (FB) was effective to modulate immune cells, evidenced by an expressing increase in the number of macrophages morphologically activated, increase the phagocytic activity and IL-10 production in these cells, as well as the ability to recruit blood monocytes to peritoneum of mice and eliciting them. In addition, when orally administered in mice, FB induced the proliferation of lymphocytes, and the supernatant of these cells induced the proliferation of bone marrow cells of normal mice. Finally, FB showed cytotoxic effect on HepG2 cells and, in a preliminary annexin V/PI assay, it triggered apoptosis. These results put the pectin from *Brassica oleracea* var. italica as a potential target to investigation for immunological and antitumoral applications. Although the interaction of FB with Gal-3 and ASGP-Rs has not been evaluated, they may be involved in the observed effects. Investigations to clarify the mechanism of action of FB is subject of future research.

Acknowledgements

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5 CONCLUSÃO

O presente estudo mostrou que a pectina de *Brassica oleracea* var. italica (FB) foi efetiva em modular células do sistema imune, evidenciado por um expressivo aumento no número de macrófagos morfologicamente ativados, aumento na capacidade fagocítica destas células, aumento na produção de IL-10 destas células, assim como a habilidade de FB em recrutar monócitos para a cavidade peritoneal e elicitá-los. Adicionalmente, quando FB foi administrada oralmente em camundongos, houve aumento da proliferação de linfócitos e, além disso, quando o sobrenadante destes linfócitos foi incubado com células da medula óssea de camundongos não tratados, observou-se aumento no número destas células. Finalmente, FB mostrou efeito citotóxico em células HepG2 e, em um ensaio preliminar de apoptose com anexina V/PI, FB levou estas células à apoptose. Esses resultados colocam a pectina de *Brassica oleracea* var. italica como alvo potencial de investigações para aplicação em terapias imunomoduladoras e antitumorais.

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