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LEYDIANNE LEITE DE SIQUEIRA PATRIOTA

AVALIAÇÃO DAS ATIVIDADES ANTITUMORAL E ANTI-INFLAMATÓRIA DE PROTEÍNAS BIOATIVAS DE ORIGEM VEGETAL

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia da Universidade Federal de Pernambuco, como requisito parcial para a obtenção do título de Doutora em Bioquímica e Fisiologia.

Área de concentração: Bioquímica e Fisiologia.

Orientador: Prof. Dr. Thiago Henrique Napoleão

Coorientadora: Profa. Dra. Rosemairy Luciane Mendes

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Dedico este trabalho àquela que me inspira e fortalece: minha avó Tereza.

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Há uma força motriz mais poderosa que o vapor, a eletricidade e a energia atômica: a vontade.

Albert Einstein

RESUMO

Proteínas bioativas de origem vegetal, tais como lectinas e inibidores de protease, têm sido avaliadas como agentes anti-inflamatórios e antitumorais alternativos complementares às drogas atualmente disponíveis. A presente tese descreve a avaliação das atividades antitumoral e anti-inflamatória *in vivo* da lectina de fronde de *Microgramma vacciniifolia* (MvFL) e do inibidor de tripsina de flores de *Moringa oleifera* (MoFTI) em camundongos fêmeas Swiss. Inicialmente, foi avaliada a citotoxicidade *in vitro* de MvFL e MoFTI para células de sarcoma 180. No ensaio *in vivo*, células de sarcoma 180 foram inoculadas no dorso dos animais e, após 7 dias, os camundongos foram tratados diariamente por 7 dias com MvFL (10 ou 20 mg/kg) ou MoFTI (15 ou 30 mg/kg), por via intraperitoneal. Foram analisados o peso do tumor, a vascularização ao seu redor e aspectos histológicos. A toxicidade dos tratamentos foi avaliada pela determinação de: consumo relativo de água e ração; peso dos órgãos e peso corporal; parâmetros hematológicos e bioquímicos do sangue; e aspectos histopatológicos de fígado, rins e baço. O potencial anti-inflamatório foi estudado usando dois modelos de inflamação aguda. Os camundongos receberam, por via intraperitoneal, MvFL (5 ou 10 mg/kg) ou MoFTI (15 ou 30 mg/kg) 1 hora antes da indução de peritonite ou edema de pata por injeção de carragenina. No modelo de peritonite, o líquido peritoneal foi coletado e avaliado quanto a migração de leucócitos, conteúdo de proteínas e níveis de citocinas e óxido nítrico (NO). O efeito anti-edematogênico foi avaliado medindo o volume da pata durante a primeira (1–2h) e segunda (3–4h) fases da formação do edema. Todos os experimentos foram realizados com controles negativos (veículo) e positivos (metotrexato, dexametasona e indometacina nos ensaios de atividade antitumoral, peritonite e edema de pata, respectivamente). MvFL e MoFTI inibiram o crescimento das células de sarcoma 180 *in vitro*, sendo as concentrações necessárias para inibir 50% da proliferação (CI_{50}) de 20,6 e 67,97 μg/mL, respectivamente. Ambas as proteínas induziram apoptose e necrose das células. Os tratamentos com MvFL causaram uma redução de 89.2–96.8% no peso do tumor, enquanto MoFTI reduziu o tumor em 90.1%–97.9%, sendo observada a necrose do tecido tumoral e a infiltração de leucócitos. MvFL e MoFTI apresentaram ação antiangiogênica, evidenciada pela redução no diâmetro e no número de vasos primários e/ou secundários ao redor do tumor. Nenhuma alteração sugestiva de toxicidade foi observada nos animais tratados com MvFL e MoFTI, uma vez que não foram alterados o peso, consumo de água e ração e nenhuma alteração significativa foi observada nos parâmetros bioquímicos, hematológicos e histológicos investigados. No ensaio de peritonite, MvFL e MoFTI foram capazes de reduzir a infiltração de leucócitos, os níveis de citocinas pró-

inflamatórias (IL-17A, TNF-α e IL-6) e de NO, e o extravasamento de proteínas no fluido peritoneal. Ambas as proteínas inibiram a formação do edema a partir da segunda fase, indicando atuação na concentração de mediadores inflamatórios como citocinas e NO. Em conclusão, MoFTI e MvFL são proteínas bioativas com potencial antitumoral e anti-inflamatório, sem exercer toxicidade de acordo com os parâmetros avaliados, o que estimula mais estudos visando o desenvolvimento de fármacos a partir dessas proteínas.

Palavras-chave: Lectina; Inibidor de protease; *Microgramma vacciniifolia*; *Moringa oleifera*; Sarcoma 180; Carragenina; Peritonite; Edema de pata.

ABSTRACT

Bioactive proteins of plant origin, such as lectins and protease inhibitors, have been evaluated as anti-inflammatory and anti-tumor agents to be complementary alternatives to currently available drugs. The present thesis describes the evaluation of the *in vivo* antitumor and anti-inflammatory activities of the *Microgramma vacciniifolia* frond lectin (MvFL) and the *Moringa oleifera* flower trypsin inhibitor (MoFTI) in Swiss female mice. Initially, the in vitro cytotoxicity of MvFL and MoFTI to sarcoma 180 cells was evaluated. In the *in vivo* trial, sarcoma 180 cells were inoculated on the animals' backs and, after 7 days, the mice were treated daily for 7 days with MvFL (10 or 20 mg/kg) or MoFTI (15 or 30 mg/kg), intraperitoneally. The weight of the tumor, the vascularization around it and histological aspects were analyzed. The toxicity of the treatments was assessed by determining relative consumption of water and food; organ weight and body weight; hematological and biochemical parameters of blood; and histopathological aspects of the liver, kidneys and spleen. The anti-inflammatory potential was studied using two models of acute inflammation. The mice received MvFL (5 or 10 mg/kg) or MoFTI (15 or 30 mg/kg) intraperitoneally 1 hour before induction of peritonitis or paw edema by carrageenan injection. In the peritonitis model, the peritoneal fluid was collected and evaluated for leukocyte migration, protein content and levels of cytokines and nitric oxide (NO). The anti-edematogenic effect was assessed by measuring the volume of the paw during the first (1–2h) and second (3–4h) phases of edema formation. All experiments were performed with negative (vehicle) and positive (methotrexate, dexamethasone and indomethacin in the assays of antitumor activity, peritonitis and paw edema, respectively) controls. MvFL and MoFTI inhibited the growth of sarcoma 180 cells *in vitro*, with the concentrations necessary to inhibit 50% of the proliferation (IC_{50}) of 20.6 and 67.97 μg/mL, respectively. Both proteins induced apoptosis and cell necrosis. MvFL treatments caused a 89.2–96.8% reduction in tumor weight, while MoFTI reduced the tumor in 90.1% –97.9%, with tumor tissue necrosis and leukocyte infiltration being observed. MvFL and MoFTI showed antiangiogenic action, evidenced by the reduction in the diameter and in the number of primary and/or secondary vessels around the tumor. No changes suggestive of toxicity were observed in animals treated with MvFL and MoFTI, once no alterations were found in the weight or water and food consumption and no significant changes were observed in the investigated biochemical, hematological and histological parameters. In the peritonitis assay, MvFL and MoFTI were able to reduce leukocyte infiltration, levels of pro-inflammatory cytokines (IL-17A, TNF-α and IL-

6) and NO, and protein leakage in the peritoneal fluid. Both proteins inhibited the formation of edema from the second phase, indicating an action on the concentration of inflammatory mediators such as cytokines and NO. In conclusion, MoFTI and MvFL are bioactive proteins with anti-tumor and anti-inflammatory potential, without exerting toxicity according to the parameters evaluated, which stimulates further studies aiming at the development of drugs from these proteins.

Keywords: Lectin; Protease inhibitor; *Microgramma vacciniifolia*; *Moringa oleifera*; Sarcoma 180; Carrageenan; Peritonitis; Paw edema.

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SUMÁRIO

1 INTRODUÇÃO

O câncer é uma das principais causas de morbidade e mortalidade no mundo. Segundo a mais recente estimativa mundial do Observatório Global de Câncer (GLOBOCAN), ocorreram aproximadamente 19,3 milhões de novo casos e 10 milhões de mortes foram decorrentes do câncer em 2020 (SUNG, 2021). No Brasil, o Instituto Nacional de Câncer (INCA) estima que para cada ano do triênio 2020-2022 ocorrerão 625 mil novos casos da doença (INCA, 2020). Os quimioterápicos atualmente utilizados apresentam diversos problemas, tais como efeitos colaterais (MAGALHÃES et al., 2020) e a resistência que as células tumorais têm desenvolvido (PAN et al., 2016). Visando minimizar esses impactos, esforços são constantemente realizados na busca de novos agentes terapêuticos para tratamento do câncer (SRIKANTH; CHEN, 2016; GAUTAM et al., 2020).

A inflamação é um processo do sistema imunológico, sendo fortemente regulada e essencial para a sobrevivência (BURINI et al., 2020). Contudo, a desregulação inflamatória está envolvida em alterações metabólicas como obesidade, diabetes, câncer e doenças autoimunes (KISHORE et al., 2019; ZHONG; SHI 2019). Agentes imunomoduladores podem ajustar desbalanços no sistema imunológico, podendo apresentar atividades positivas (imunoestimulação) ou negativas (imunossupressão) sobre funções imunológicas específicas (KUBACKOVA; ZBYTOVSKA; HOLAS, 2020). Considerando que os anti-inflamatórios e imunomoduladores usados hoje para o tratamento de doenças inflamatórias têm alto custo e muitos efeitos colaterais, existe a necessidade de desenvolver opções mais baratas, seguras e potentes (KISHORE et al., 2019).

As lectinas são proteínas que se ligam a carboidratos de forma reversível e específica, sendo capazes de promover a aglutinação de células e precipitar polissacarídeos, glicoproteínas ou glicolipídeos (COELHO et al., 2017). São amplamente encontradas na natureza, sendo isoladas de microrganismos, plantas, invertebrados e vertebrados. Nas plantas, lectinas são encontradas em folhas, entrecascas, raízes, rizomas, bulbos, vagens, sementes, frutos e flores. A interação de lectinas com carboidratos de superfícies celulares pode desencadear diversas respostas intracelulares, o que resulta em uma gama de atividades biológicas, incluindo ações antitumoral, anti-inflamatória e imunomoduladora (CLEMENTE-NAPIMOGA et al., 2019; MISHRA et al., 2019; GAUTAM et al., 2020).

Inibidores de protease são moléculas que bloqueiam ou reduzem a capacidade catalítica de enzimas proteolíticas (NAPOLEÃO et al., 2019). Estes inibidores podem ser peptídicos, sendo encontrados em plantas, animais e microrganismos (CRUZ-SILVA et al., 2016). Essas proteínas atuam controlando a atividade proteolítica endógena e podem interferir com a atividade proteolítica de enzimas em outros organismos (KRISHNAN; MURUGAN, 2017), desempenhando ações anti-inflamatória (RODRIGUES et al., 2019), antitumoral (BONTURI et al., 2018) e imunomodulatória (PATRIOTA et al., 2017).

Conhecida popularmente por "polipódio vacinifólio", "erva-silvina" ou "erva-teresa", *Microgramma vacciniifolia* (Polypodiaceae) é uma planta epífita com diversas propriedades medicinais (PERES et al., 2009; TERCEIRO et al., 2012; NUNES et al., 2020). As frondes de *M. vacciniifolia* possuem uma lectina chamada MvFL (*M. vacciniifolia frond lectin*), com especificidade para fetuína e ovoalbumina, sendo considerada multifuncional por apresentar tanto atividade lectínica quanto inibidora de tripsina. MvFL já teve suas propriedades imunomoduladoras em células mononucleares do sangue periférico humano (PBMCs) demonstradas, sendo capaz de induzir a liberação de citocinas e óxido nítrico (NO) e promovendo a ativação e diferenciação de células T CD8+ (PATRIOTA et al., 2017).

Moringa oleifera, conhecida popularmente como "lírio branco", "quiabo de quina" ou "moringa", é uma planta cujos tecidos têm sido descritos como fontes de compostos com as mais variadas aplicações, inclusive propriedades medicinais para tratar inflamações e tumores (ANWAR et al., 2007; SANTOS et al., 2015; SINGH et al., 2020). As flores de *M. oleifera* contém um inibidor de tripsina chamado MoFTI (*M. oleifera flower trypsin inhibitor*) que apresentou atividades inseticida (PONTUAL et al., 2014) e tripanocida (PONTUAL et al., 2018). MoFTI foi capaz de modular a resposta de PBMCs infectados por *T. cruzi*, sugerindo que esta proteína pode ser capaz de controlar a parasitemia enquanto regula a inflamação (NOVA et al., 2020). Patriota et al. (2021) reportaram que MoFTI induz a liberação de citocinas e NO por esplenócitos de camundongo, concluindo que esses resultados estimulam a avaliação do potencial antitumoral e anti-inflamatório de MoFTI.

Assim, essa tese teve como objetivo avaliar se MvFL e MoFTI apresentam atividades antitumoral e anti-inflamatória. O ensaio de atividade antitumoral foi realizado utilizando camundongos portadores do sarcoma 180, enquanto a atividade anti-inflamatória *in vivo* foi avaliada através dos modelos agudos de peritonite e edema de pata, ambos induzidos por carragenina.

2 OBJETIVOS

2.1 GERAL

Avaliar as atividades antitumoral e anti-inflamatória da lectina de fronde de *M. vacciniifolia* (MvFL) e do inibidor de tripsina de flores de *M. oleifera* (MoFTI).

2.2 ESPECÍFICOS

- Avaliar a citotoxicidade de MvFL e MoFTI *in vitro* contra células de sarcoma 180.
- Determinar a atividade antitumoral de MvFL e MoFTI *in vivo* em camundongos portadores de sarcoma 180 por meio de quantificação da redução da massa tumoral, avaliação da vascularização ao redor o tumor e análise histopatológica do tecido tumoral.
- Determinar a toxicidade dos tratamentos com MvFL e MoFTI nos camundongos portadores de tumores, por meio da avaliação de alterações morfológicas (fígado, rins e baço) e indução de hipertrofia ou hipotrofia em órgãos, e análise de parâmetros hematológicos e bioquímicos.
- Investigar a atividade anti-inflamatória *in vivo* de MvFL e MoFTI em modelos de inflamação aguda (peritonite e edema de pata) induzidos por carragenina em camundongos.
- Avaliar mecanismos envolvidos na atividade anti-inflamatória de MvFL e MoFTI no modelo de peritonite, através da determinação de níveis de citocinas, óxido nítrico e proteínas totais no líquido intraperitoneal.

3 FUNDAMENTAÇÃO TEÓRICA

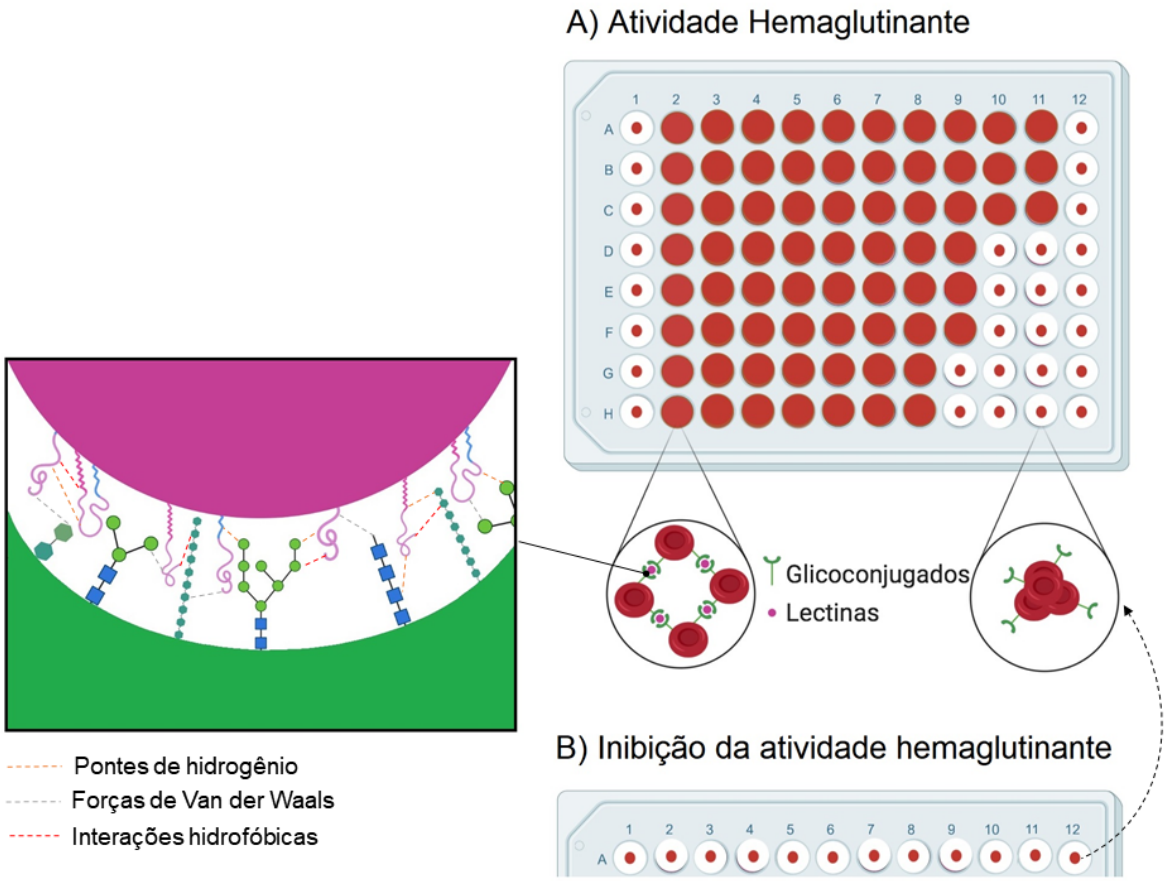
3.1 LECTINAS

As lectinas são proteínas que interagem com carboidratos de forma específica e reversível, sendo amplamente encontradas na natureza (COELHO et al., 2017). Lectinas vêm sendo isoladas de bactérias (VAINAUSKAS et al., 2016; AGARWAL; TRIVEDI; MITRA, 2020), fungos (SINGH; WALIA; KENNEDY, 2019; ZHANG et al., 2019), plantas (AKEV; CANDOKEN; KURUCA, 2020; HIREMATH et al., 2020) e animais (YANG et al., 2018; SATOH; KATO, 2020). Ainda, a engenharia de proteínas traz a possibilidade de obtê-las de forma artificial (HIRABAYASHI; ARAI, 2019). Em todos estes organismos, as lectinas estão envolvidas em diversos processos biológicos, inclusive como parte de mecanismos de defesa (BLEULER-MARTINEZ et al., 2017; NASCIMENTO et al., 2020).

As lectinas possuem pelo menos um domínio não catalítico que é capaz de se ligar a carboidratos simples (monossacarídeos, dissacarídeos), oligossacarídeos complexos (incluindo a porção glicídica de glicoconjugados) e/ou polissacarídeos (COELHO et al., 2017; MADHU et al., 2019; NAPOLEÃO at al., 2019). A interação entre lectinas e carboidratos específicos é reversível e envolve ligações de hidrogênio, forças de Van der Waals e interações hidrofóbicas (PIETRZYK-BRZEZINSKA; BUJACZ, 2020). A capacidade de se ligar a glicoconjugados (glicoproteínas e glicolipídeos) presentes em superfícies celulares e a presença de pelo menos dois sítios de ligação a carboidratos na estrutura de uma lectina confere a ela a propriedade aglutinante (PATRIOTA et al., 2019a).

O ensaio de hemaglutinação (Figura 1), no qual se avalia a capacidade de interação entre a lectina e carboidratos da superfície dos eritrócitos, é o método mais utilizado para detecção de lectinas (PROCÓPIO et al., 2017a; SULTANA; AHMED; ALAM, 2019). Caso não ocorra a aglutinação, tais eritrócitos depositam-se no fundo da placa. Porém, quando uma lectina está presente, se associa com esses açúcares e forma-se uma rede de hemaglutinação (Figura 1A). Uma vez que o aspecto visual da hemaglutinação pode ser similar ao observado em casos de dispersão ou lise dos eritrócitos, deve ser realizado o ensaio de inibição de hemaglutinação (Figura 1B) para confirmar se o fenômeno observado é uma aglutinação promovida por lectinas. Nesse teste, são adicionados carboidratos livres em solução que interagem com as lectinas, ocupando seus sítios de ligação, impedindo que elas se liguem à superfície dos eritrócitos e formem a rede de hemaglutinação (SANTOS et al., 2013).

FIGURA 1 – Ensaios para detecção da presença de lectinas. (A) Ensaio de atividade hemaglutinante. Forma-se uma rede de hemaglutinação devido à interação entre a lectina e glicoconjugados da superfície dos eritrócitos. Essa interação envolve pontes de hidrogênio, forças de Van der Waals e interações hidrofóbicas. (B) Ensaio de inibição da atividade hemaglutinante por carboidratos. Carboidratos livres em solução interagem com as lectinas, ocupando seus sítios de ligação, impedindo que elas se liguem à superfície dos eritrócitos e formem a rede de hemaglutinação.



Fonte: Elaborada pela autora.

A capacidade de ligação a carboidratos de superfícies celulares pode resultar no desencadeamento de respostas intracelulares, o que confere uma gama de propriedades biológicas às lectinas, as quais têm sido exploradas com diversos fins biotecnológicos e biomédicos (DAN; LIU; NG, 2016; COELHO et al., 2017; LIU et al., 2018; MISHRA et al., 2019). Ainda, a significância funcional de carboidratos em doenças, particularmente aqueles associados a proteínas, tem sido elucidada por meio de investigações utilizando lectinas (SILVA, 2018; ABDEL-HAQ, 2019).

Dentre as atividades biológicas descritas para lectinas podemos citar inseticida (NAPOLEÃO et al., 2019; ALVES et al., 2020), antiviral (MAZALOVSKA; KOUIKAN,

2018; AGARWAL; TRIVEDI; MITRA, 2020), antifúngica (SILVA et al., 2018; SILVA et al., 2019), antibacteriana (PROCÓPIO et al, 2017b; CORIOLANO et al., 2020; HIREMATH et al., 2020), antiparasitária (THOMAZELLI et al., 2018), antinociceptiva (PIRES et al., 2019a; RAMOS et al., 2020), antioxidante (LACERDA et al., 2017), imunomoduladora (PROCÓPIO et al., 2018; PATRIOTA et al., 2019b; SANTOS et al., 2020), anti-infecciosa (LIMA et al., 2019), mitogênica (SINGH; KAUR; SINGH, 2015; ZHANG et al., 2019), anti-angiogênica (RAMOS et al., 2019; PETROVIĆ et al., 2020), antitumoral (RAMOS et al., 2019; BHUTIA et al., 2019; PATRIOTA et al., 2019a; GAUTAM et al., 2020), anti-inflamatória (CLEMENTE-NAPIMOGA et al., 2019; PIRES et al., 2019b), gastroprotetora (LACERDA et al., 2017), neuroprotetora (ARAÚJO et al., 2020), nefroprotetora (SANTOS et al., 2019b), entre outras. Adicionalmente, as lectinas têm sido estudadas como ferramentas para cooperar com o diagnóstico e prognóstico de doenças, como o câncer, sendo usadas em sondas histoquímicas devido aos seus mecanismos de reconhecimento diferencial de tecidos ou ainda como ferramentas multifuncionais para marcar e identificar diferentes tipos de células (DÍAZ et al., 2017; LUTSYK et al., 2018; DANG et al., 2020). Além disso, lectinas têm sido usadas como adjuvantes no desenvolvimento de vacinas (SANDER; CORIGLIANO; CLEMENTE, 2019).

Lectinas de plantas podem ser isoladas a partir dos mais variados tecidos e órgãos, tais como flores, folhas, sementes, vagens, bulbos, frutos e raízes (DIAS et al., 2015; REDDY; BANIK; SASIKALA 2016; COELHO et al., 2017). As lectinas de plantas podem ser produzidas constitutivamente ou em resposta ao estresse ambiental e ataque de patógenos (lectinas induzidas). Normalmente, as lectinas produzidas constitutivamente são armazenadas em vacúolos ou no espaço extracelular e estão presentes, principalmente, em tecidos de armazenamento (LANNOO; VAN DAMME, 2010), sendo descritas como relacionadas à defesa de plantas contra patógenos e insetos herbívoros (AL ATALAH; SMAGGHE; VAN DAMME, 2014; NASCIMENTO et al., 2020). Além disso, são envolvidas no transporte de carboidratos em tecidos vegetais (NASCIMENTO et al., 2020). As induzidas, situadas no núcleo e no citoplasma, também estão envolvidas no processo de defesa das plantas (LANNOO; VAN DAMME, 2010, 2014; MACEDO; OLIVEIRA; OLIVEIRA, 2015). Muitos estudos mostraram que as lectinas de plantas exibem uma variedade de atividades biológicas, dentre elas todas as citadas anteriormente.

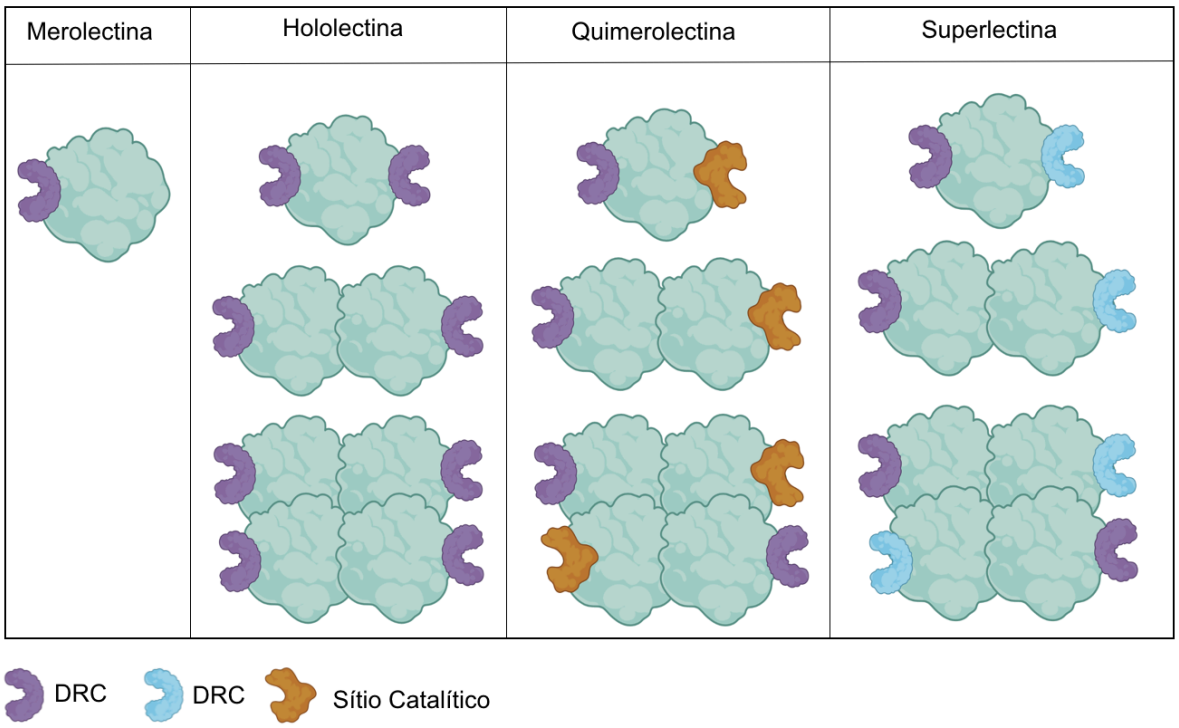
As lectinas de plantas são consideradas um grupo heterogêneo de proteínas em relação às suas propriedades bioquímicas/físico-químicas, relações evolutivas, estrutura molecular e especificidade para carboidratos (NASCIMENTO et al., 2020). Considerando todos esses

aspectos, torna-se difícil a tarefa de classificá-las, mas algumas classificações podem ser encontradas na literatura e são descritas a seguir.

As lectinas de plantas podem ser classificadas com base na sua especificidade de ligação a carboidratos em lectinas ligadoras de glicose/manose, *N*-acetilglicosamina, galactose/*N*-acetilgalactosamina, fucose, ácido siálico, entre outros açúcares (HAMID et al., 2013; MISHRA et al., 2019). Uma outra classificação de lectinas de plantas baseia-se na estrutura e sequência dos domínios que reconhecem carboidratos, sendo agrupadas nas seguintes famílias: (1) aglutininas homólogas a *Agaricus bisporus*, (2) amarantinas, (3) homólogas de quitinase classe V, (4) aglutinina de *Euonymus europaeus*, (5) aglutinina de *Galanthus nivalis*, (6) proteínas com domínio heveínico (motivo estrutural de reconhecimento da quitina), (7) jacalinas, (8) lectinas de leguminosas, (9) lectinas com domínio Lys M (motivo de lisina), (10) aglutinina de *Nicotiana tabacum* e (11) família ricina-B (MACEDO; OLIVEIRA; OLIVEIRA, 2015). Além disso, as lectinas de plantas também podem ser agrupadas em diferentes famílias de acordo com similaridades estruturais e evolução em: amarantinas, lectinas de leguminosas, lectinas de monocotiledôneas, lectinas ligadoras de manose, lectinas ligadoras de quitina, lectinas relacionadas a jacalina, lectinas do floema de Cucurbitaceae e proteínas inativadoras de ribossomos de tipo II (HAMID et al., 2013; MISHRA et al., 2019).

Podem ainda ser categorizadas estruturalmente conforme o número e característica dos domínios que possuem: merolectinas, quando possuem apenas um domínio capaz de ligar a carboidratos e dessa forma não apresentam atividade aglutinante; hololectinas, que possuem dois ou mais domínios ligadores de carboidratos; quimerolectinas, que possuem um domínio que liga a carboidratos e outro que possui uma atividade biológica diversa; e superlectinas, que possuem dois ou mais domínios que reconhecem estruturas diferentes de carboidratos (Figura 2) (MISHRA et al., 2019).

FIGURA 2 – Classificação estrutural das lectinas de plantas. DRC: Domínio de reconhecimento de carboidratos.



Fonte: Elaborada pela autora.

3.2 INIBIDORES DE PROTEASE

As proteases são fatores-chave no funcionamento e viabilidade das células, pois catalisam vários eventos proteolíticos e atuam como intermediários de iniciação de sinais, transmissão e terminação de eventos celulares, estando envolvidas em múltiplos processos, como replicação, transcrição, proliferação celular, diferenciação, apoptose, remodelação da matriz extracelular, digestão de proteínas, processamento de hormônios e peptídeos biologicamente ativos, coagulação sanguínea e resposta inflamatória, entre outros (SHAMSI; PARVEEN; FATIMA, 2016; BOND, 2019). No entanto, essas enzimas também podem ser potencialmente prejudiciais e, portanto, a atividade proteolítica deve ser controlada com precisão (CLEMENTE et al., 2019).

Inibidores de protease são compostos que bloqueiam ou reduzem a capacidade catalítica destas enzimas (NAPOLEÃO et al., 2019). Estes inibidores possuem natureza química distinta podendo ser sintéticos ou, no caso daqueles de origem natural, peptídicos ou metabólitos secundários (PONTUAL et al., 2012; SHAMSI; PARVEEN; FATIMA, 2016). Estudos comprovaram que o uso de inibidores de protease proteicos pode ser mais vantajoso que os

sintéticos, pois são mais seguros e específicos em sua ação (SHAMSI; PARVEEN; FATIMA, 2016). Inibidores de protease proteicos são encontrados em plantas, animais e microrganismos (CRUZ-SILVA et al., 2016).

Os inibidores de protease são armazenados em diversos tecidos de plantas, incluindo sementes, tubérculos, rizoma, frutos, órgãos reprodutores e folhas (MISHRA; REDDY; PRASAD, 2020). Essas proteínas são produzidas constitutivamente ou por indução em tecidos vegetativos e reprodutivos de plantas, estando associadas à defesa contra herbívoros, regulação da atividade proteolítica endógena e regulação da morte celular programada (ZHU-SALZMAN; ZENG, 2015; CLEMENTE et al., 2019). Ainda, esses inibidores podem funcionar como proteínas de armazenamento (CLEMENTE et al., 2019).

Os inibidores de protease proteicos têm demonstrado várias atividades biológicas, interferindo com a atividade proteolítica das proteases-alvo nos organismos (KRISHNAN; MURUGAN, 2017; HELLINGER; GRUBER, 2019). Dessa forma, inibidores de protease proteicos provenientes de plantas vêm sendo purificados, caracterizados e investigados quanto ao potencial terapêutico usando métodos *in vitro* e *in vivo*, tornando-se assim excelentes fontes de pesquisa para fins farmacológicos (RUSTGI et al., 2018; COTABARREN et al., 2020; MISHRA; REDDY; PRASAD, 2020).

Inibidores de protease isolados de plantas apresentam atividades antiviral (MISHRA; REDDY; PRASAD, 2020), antibacteriana (MARTINS et al., 2018), antifúngica (PATRIOTA et al., 2016), antiparasitária (PONTUAL et al., 2018), antioxidante (SHAMSI et al., 2018), anti-inflamatória (BORTOLOZZO et al., 2018; RODRIGUES et al., 2019) antitumoral (SRIKANTH; CHEN, 2016; BONTURI et al., 2018) e imunomoduladora (PATRIOTA et al., 2017). Srikanth & Chen (2016) chamam atenção para a importância da exploração de mais inibidores de protease de plantas (proteicos) e investigação de suas atividades para o tratamento e controle de doenças, devido ao seu enorme potencial na Medicina.

Em relação ao mecanismo de ação, os inibidores de protease proteicos podem ser classificados em competitivos ou não competitivos. Os primeiros atuam competindo com o substrato pelo sítio ativo das proteases, com o qual estabelecem interações estáveis; os não-competitivos, por sua vez ligam-se em locais diferentes do sítio catalítico, resultando em modificações conformacionais na molécula da protease que impossibilitam a realização da catálise (FARADYA & CRAIKA, 2010; LIMA et al., 2019). Ainda, a interação protease-inibidor pode ser classificada como irreversível ou reversível. No primeiro caso, a interação

induz a clivagem de uma ligação peptídica interna no inibidor, desencadeando uma alteração conformacional e o inibidor nunca recupera sua estrutura inicial; na interação reversível o inibidor interage com o sítio ativo da protease de maneira semelhante ao substrato enzimático, e, portanto, o inibidor no complexo é dissociado à sua forma intacta ou modificada (CLEMENTE et al., 2019).

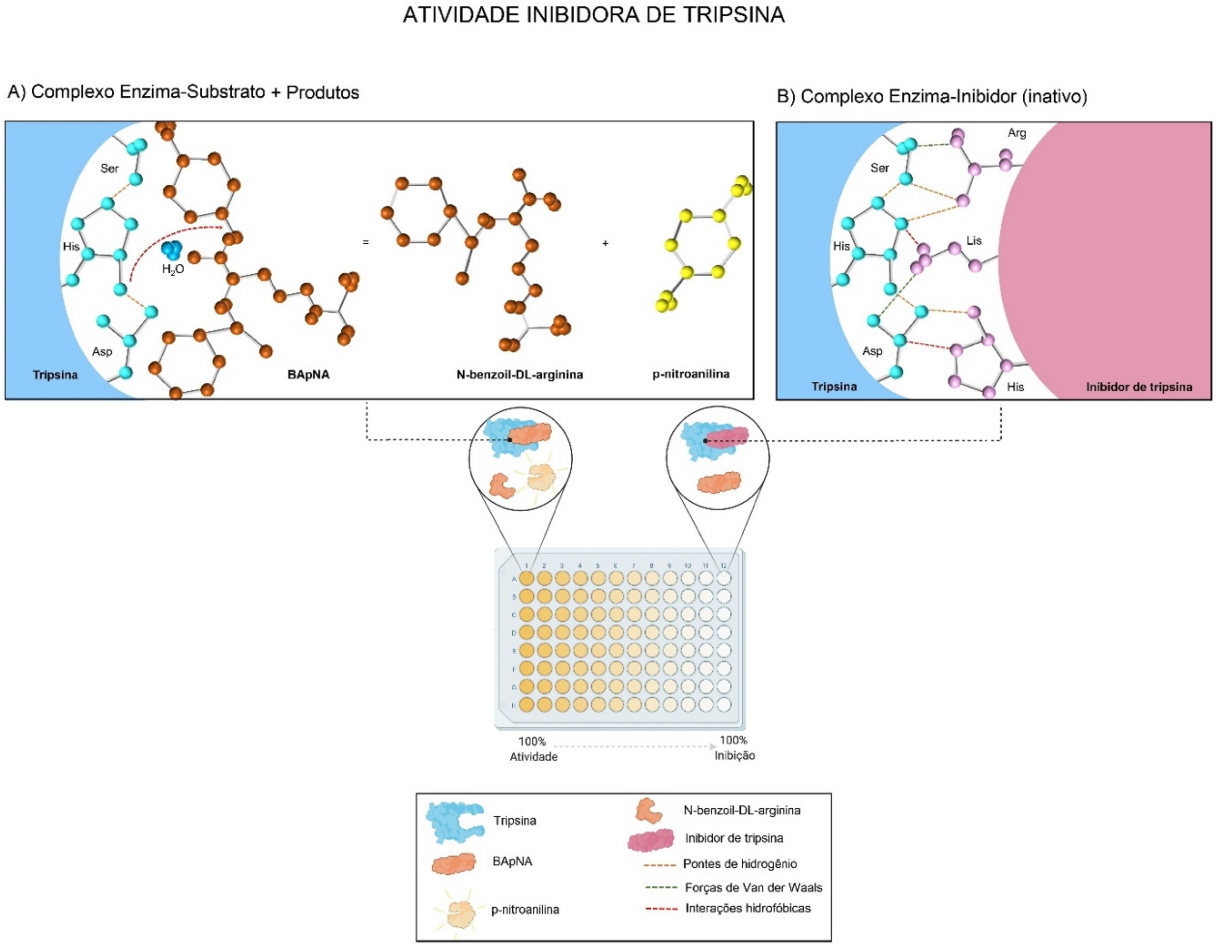
Os inibidores de protease proteicos podem também ser classificados segundo o tipo de protease inibida em: inibidores de serinoproteases, aspártico-proteases, cisteíno-proteases e metaloproteases. Alguns inibidores de protease podem interferir em mais de um tipo de protease. Por exemplo, os da classe das serpinas são geralmente ativos contra as serinoproteases e as cisteíno-proteases (SHAMSI; PARVEEN; FATIMA, 2016; RUSTGI et al., 2018). Os inibidores também são subdivididos em várias famílias baseadas em características estruturais e bioquímicas de homologia entre membros, como sequência de aminoácidos, presença de pontes dissulfeto e localização do sítio ativo (LIMA et al., 2019), sendo descritas cerca de 85 famílias (RAWLINGS et al., 2018). A maioria dos inibidores de protease de plantas são inibidores de serinoproteases, sendo classificados em mais de 20 famílias, a maioria deles pertences às famílias Kunitz e Bowman-Birk, ambos capazes de inibir a tripsina (SOWBAGHYA et al., 2019; COTABARREN et al., 2020).

A tripsina é uma endopeptidase do grupo das serinoproteases amplamente encontrada no sistema digestivo e em outros tecidos de vertebrados e invertebrados, sendo também expressa por microrganismos (ŠLECHTOVÁ et al., 2015; HEGYI; SAHIN-TÓTH, 2017; PILON et al., 2017; ZHAO et al., 2019). A importância desta enzima para esses organismos tem estimulado a busca por inibidores de tripsina para o uso no controle de pragas de interesse econômico, patógenos e como agentes terapêuticos para o tratamento de doenças (PATRIOTA et al., 2016; BENDRE; RAMASAMY; SURESH, 2018; NAPOLEÃO et al., 2019).

A tripsina apresenta, em seu sítio ativo, a tríade catalítica representada pelos resíduos de serina, histidina e aspartato. Esta tríade promove espaço eletrostático favorável para a interação com resíduos de aminoácidos positivamente carregados, como arginina e lisina. Proteínas com atividade inibidora de tripsina podem interagir de forma complementar com o sítio ativo (competitivos) ou com outras regiões da enzima (não competitivos), através de pontes de hidrogênio, interações eletrostáticas e hidrofóbicas (SILVA et al., 2015; BENDRE et al., 2018; WEI et al., 2019). Inibidores de tripsina vêm sendo purificados e caracterizados a partir de plantas (SOWBAGHYA et al., 2019).

A atividade inibidora de tripsina pode ser detectada *in vitro* pela incubação da amostra com a enzima em presença do substrato sintético e cromogênico *N*-benzoil-DL-arginil-ϱ-nitroanilida (BApNA) (Figura 3). A tripsina é capaz de hidrolisar a ligação amida envolvendo o grupo arginil na molécula do BApNA liberando ϱ-nitroanilina no meio reacional (KAKADE; SIMONS; LIENER, 1969). Este composto químico absorve a luz no comprimento de onda correspondente à região do amarelo, podendo ser detectado pela medida da absorbância a 405 nm. A interação entre tripsina e inibidor (complexo inativo) impede a clivagem do BApNA e não ocorre produção de cor (Figura 3) (PONTUAL et al., 2014).

FIGURA 3 – Ensaio de determinação da atividade inibidora de tripsina. (A) Representação da interação entre tripsina e o substrato N-benzoil-DL-arginina-p-nitroanilida (BApNA), a qual resulta na clivagem do BApNA em N-benzoil-DL-arginina e p-nitroanilina. (B) A interação entre tripsina e inibidor forma um complexo inativo devido, por exemplo, à ocupação do sítio ativo.



Fonte: Elaborada pela autora.

3.3 CÂNCER

Segundo a Organização Mundial de Saúde (OMS), câncer (tumores ou neoplasias malignas) é um termo genérico para um grande grupo de doenças que podem afetar qualquer parte do corpo, apresentando como característica definidora o crescimento desordenado de células anormais que podem invadir partes adjacentes do corpo e se espalhar para outros órgãos (metastatizar). Configura-se como uma das principais causas de morbidade e mortalidade no mundo, sendo um grande problema de saúde pública (OMS, 2020).

Em termos estatísticos, a incidência e a mortalidade por câncer vêm aumentando globalmente. Segundo a mais recente estimativa mundial do Observatório Global de Câncer (GLOBOCAN), produzida pela Agência Internacional de Pesquisa em Câncer (IARC), ocorreram aproximadamente 19,3 milhões de novos casos de câncer e 10 milhões de óbitos no ano de 2020. O câncer de mama é o mais incidente no mundo (2,2 milhões), seguido pelo câncer de pulmão (2,2 milhões), próstata (1,4 milhão) e cólon (1,1 milhão). A incidência em homens (10 milhões) representa 52% dos novos casos, sendo um pouco maior que nas mulheres, com 9,2 milhões (48%) de novos casos (SUNG, 2020). Segundo o Instituto Nacional de Câncer (INCA), para o Brasil, a estimativa para cada ano do triênio 2020-2022 aponta que ocorrerão 625 mil novos casos de câncer (INCA, 2020). As estimativas de 2020 dos dez tipos de cânceres mais comuns no Brasil são apresentados na Figura 4.

FIGURA 4 – Distribuição proporcional dos dez tipos de câncer mais incidentes no Brasil estimados para 2020 por sexo, exceto pele não melanoma*

Localização Primária	Casos	%			Localização Primária	Casos	%
Próstata	65.840	29,2%	Homens	Mulheres	Mama feminina	66.280	29,7%
Cólon e reto	20.520	9,1%			Cólon e reto	20.470	9,2%
Traqueia, brônquio e pulmão	17.760	7,9%			Colo do útero	16.590	7,4%
Estômago	13.360	5,9%			Traqueia,brônquio e pulmão	12.440	5,6%
Cavidade oral	11.180	5,0%			Glândula tireoide	11.950	5,4%
Esôfago	8.690	3,9%			Estômago	7.870	3,5%
Bexiga	7.590	3,4%			Ovário	6.650	3,0%
Linfoma não Hodgkin	6.580	2,9%			Corpo do útero	6.540	2,9%
Laringe	6.470	2,9%			Linfoma não Hodgkin	5.450	2,4%
Leucemias	5.920	2,6%			Sistema nervoso central	5.220	2,3%

Fonte: INCA (2020)

Estratégias de prevenção e detecção precoce têm sido estabelecidas para diversos tipos de cânceres com o intuito de diminuir essas estatísticas (SMITH; OEFFINGER, 2020). Os pacientes com câncer podem apresentar manifestações clínicas distintas, e seus sintomas têm

sido descritos como diversos, variando de acordo com o tipo de tumor, estágio da doença e tratamento específico, apesar da presença de alguns sintomas comuns à maioria dos tumores (MAGALHÃES et al., 2020).

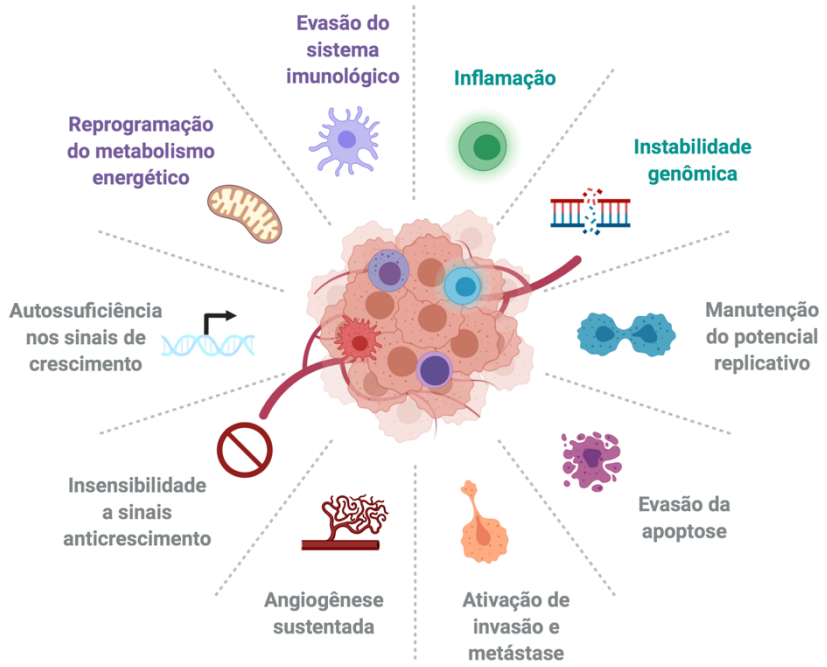
O câncer é uma doença genética que ocorre quando as informações do DNA celular são corrompidas, levando a padrões anormais de expressão de genes. Em geral, os genes associados ao câncer podem ser divididos em dois grupos: oncogenes e genes supressores de tumor (GSTs). Oncogenes são derivados de versões mutantes de genes celulares normais (proto-oncogenes) que controlam a proliferação celular, sobrevivência e invasão/motilidade. Os GSTs são genes celulares normais que funcionam para inibir proliferação e sobrevivência celular. Eles estão frequentemente envolvidos no controle da progressão do ciclo celular e morte celular programada. No câncer, os oncogenes sofrem ativação (ganham função), enquanto GSTs sofrem inativação (perdem função) (NENCLARES; HARRINGTON, 2020).

Algumas características diferenciais das células cancerígenas (*hallmarks* do câncer, Figura 5), que explicam amplamente seu comportamento maligno são: autossuficiência em sinais de crescimento, insensibilidade a sinais anti-crescimento, evasão da apoptose, angiogênese sustentada, manutenção do potencial replicativo e capacidade de invasão/metástase. Mais recentemente, foram adicionadas duas marcas emergentes (reprogramação do metabolismo energético e evasão do sistema imunológico) e duas características habilitadoras (instabilidade genômica e inflamação) (HANAHAN; WEINBERG, 2000, 2011; NENCLARES; HARRINGTON, 2020). A inflamação está intimamente envolvida tanto com a carcinogênese (por falha na resolução de processos inflamatórios) quanto com a manutenção do microambiente tumoral. O câncer é visto como uma ferida que não cicatriza, atraindo assim tipos de células e mecanismos semelhantes aos da cicatrização de feridas e regeneração de tecidos. Assim, a relação entre o câncer e a inflamação tem sido vastamente estudada (FISHBEIN et al., 2020).

O tratamento para o câncer pode envolver cirurgia, radioterapia e quimioterapia (MILLER et al., 2016). Diferentes agentes quimioterapêuticos são usados na terapia do câncer, como etoposídeo, docetaxel (DTX), doxorrubicina (DOX), cisplatina (CP), paclitaxel (PTX) e metotrexato (MTX) (CHOI et al., 2018; KORGAONKAR; YADAV, 2019; ASHRAFIZADEH et al., 2020). A quimioterapia do câncer é baseada no uso de compostos citotóxicos, que normalmente também apresentam efeito em células e tecidos normais, levando a vários e graves efeitos colaterais, degradando a qualidade de vida dos pacientes (BROWN; SANDHU; HERRMANN, 2015; SOUHO et al., 2018; MAGALHÃES et al., 2020). Além disso, a

resistência que as células tumorais têm desenvolvido ao quimioterapêuticos tem reduzido sua eficácia (PAN et al., 2016; RASTEGAR et al., 2018).

FIGURA 5 – Marcas registradas do câncer. Características diferenciais das células cancerígenas que explicam amplamente seu comportamento maligno.



Fonte: Elaborada pela autora.

Nesse sentido, esforços são constantemente realizados na busca de novos agentes terapêuticos para tratamento do câncer (AGARWAL et al., 2019). A descoberta de novos agentes antitumorais derivados de plantas inclui proteínas bioativas como as lectinas e os inibidores de protease (SRIKANTH; CHEN, 2016; GAUTAM et al., 2020), o que pode minimizar o impacto da resistência a drogas e toxicidade, além de promover uma melhor eficácia.

Para o estudo de drogas anti-câncer *in vivo*, vários tipos de tumores transplantáveis têm sido utilizados, dentre eles o sarcoma 180 (tumor de Crocker), que é uma neoplasia maligna de tecido mesenquimal. Essa linhagem celular pode ser transplantada por inoculação subcutânea, intramuscular ou intraperitoneal e cresce rapidamente em 90% a 100% dos animais inoculados. Por ser de fácil obtenção, vários centros de pesquisa têm utilizado esse modelo tumoral para estudos da biologia tumoral e dos efeitos de diversos agentes sobre a patogenia do câncer (CUNHA et al., 2016; GAO et al., 2017; RAMOS et al., 2019).

3.3.1 Lectinas de plantas com atividade antitumoral

A mudança na glicosilação de proteínas e lipídeos da membrana celular é uma característica universal das células tumorais (VARKI et al., 2017). Essas alterações na glicosilação podem resultar tanto em perda quanto em alteração na estrutura dos carboidratos (OLIVEIRA-FERRER et al., 2017). Estas diferenças nos padrões de glicosilação permitem às lectinas distinguir células normais e cancerígenas (TOZAWA-ONO et al., 2017; MAZALOVSKA; KOUOKAM, 2020). Nesse sentido, as lectinas são ferramentas bioquímicas em potencial tanto para diagnóstico quanto para a terapêutica do câncer (LAWANPRASERT et al., 2020).

Diversas lectinas de plantas têm sido descritas quanto a suas propriedades anticâncer *in vivo* e *in vitro* e, em estudos de casos humanos, essas proteínas têm demonstrado que podem funcionar como terapia alternativa (GAUTAM et al., 2020; MAZALOVSKA; KOUOKAM, 2020). Elas são tóxicas para as células cancerígenas através de diferentes mecanismos, os quais são geralmente iniciados através de interações com receptores específicos, tanto glicosilados como não glicosilados, na membrana das células cancerígenas; assim, as lectinas podem ativar cascatas de sinalização ligadas à morte celular e/ou serem internalizadas via endocitose, atingindo diferentes compartimentos e ativando outras cascatas de morte (YAU et al., 2015; BHUTIA et al., 2019). A citotoxicidade de lectinas de plantas para células cancerígenas pode envolver indução de morte celular (apoptose, autofagia, necrose) e/ou inibição do crescimento celular (PATRIOTA et al., 2019a; BHUTIA et al., 2019; LAWANPRASERT et al., 2020).

Resumidamente, a apoptose é a forma mais bem caracterizada da morte celular programada e envolve o desmonte controlado de componentes intracelulares, evitando a inflamação e danos nas células circundantes. É caracterizada por condensação da cromatina, fragmentação nuclear (cariorrexis), encolhimento (picnose), retração pseudópode, formação de corpos apoptóticos e fagocitose por células vizinhas (SCHWARZER; LAURIEN; PASPARAKIS, 2020; NOWAK; EDELSTEIN, 2020). Existem várias vias apoptóticas, as quais podem ser distinguidas pelos adaptadores e caspases iniciadoras envolvidas, mas a maioria dos programas apoptóticos se enquadra nas categorias extrínseca ou intrínseca (GRILO; MANTALARIS, 2019; NOWAK; EDELSTEIN, 2020). A apoptose intrínseca ocorre através da mitocôndria e é iniciada por uma variedade de perturbações microambientais (dano ao DNA; EROs). O passo crítico para a apoptose intrínseca é a permeabilização generalizada e irreversível da membrana mitocondrial externa, que é controlada por membros pró-apoptóticos

(Bax, Bad, Bid) e anti-apoptóticos (Bcl-2, Bcl-x, Bcl-XL) da família de proteínas reguladoras da apoptose (BCL2). Uma vez que ocorre, essa permeabilização promove diretamente a liberação citosólica de fatores apoptogênicos que normalmente residem no espaço intermembrana mitocondrial, entre eles o citocromo c, que se liga ao fator de ativação da peptidase apoptótica 1 (APAF1) para formar o complexo supramolecular conhecido como apoptossoma, responsável pela ativação da caspase iniciadora dessa via, a caspase 9. Por sua vez, apoptose extrínseca é iniciada por perturbações do microambiente extracelular e acionada por receptores de membrana plasmática, incluindo receptores de morte (FAS; TNFR1). Como regra geral, a ligação ao receptor da morte permite que complexos de sinalização operem para regular a ativação da caspase iniciadora, a caspase 8. As vias convergem quando as caspases iniciadoras catalisam a ativação proteolítica das caspases executoras (caspase 3 e 7), que são responsáveis pela demolição celular (GALLUZZI et al., 2018).

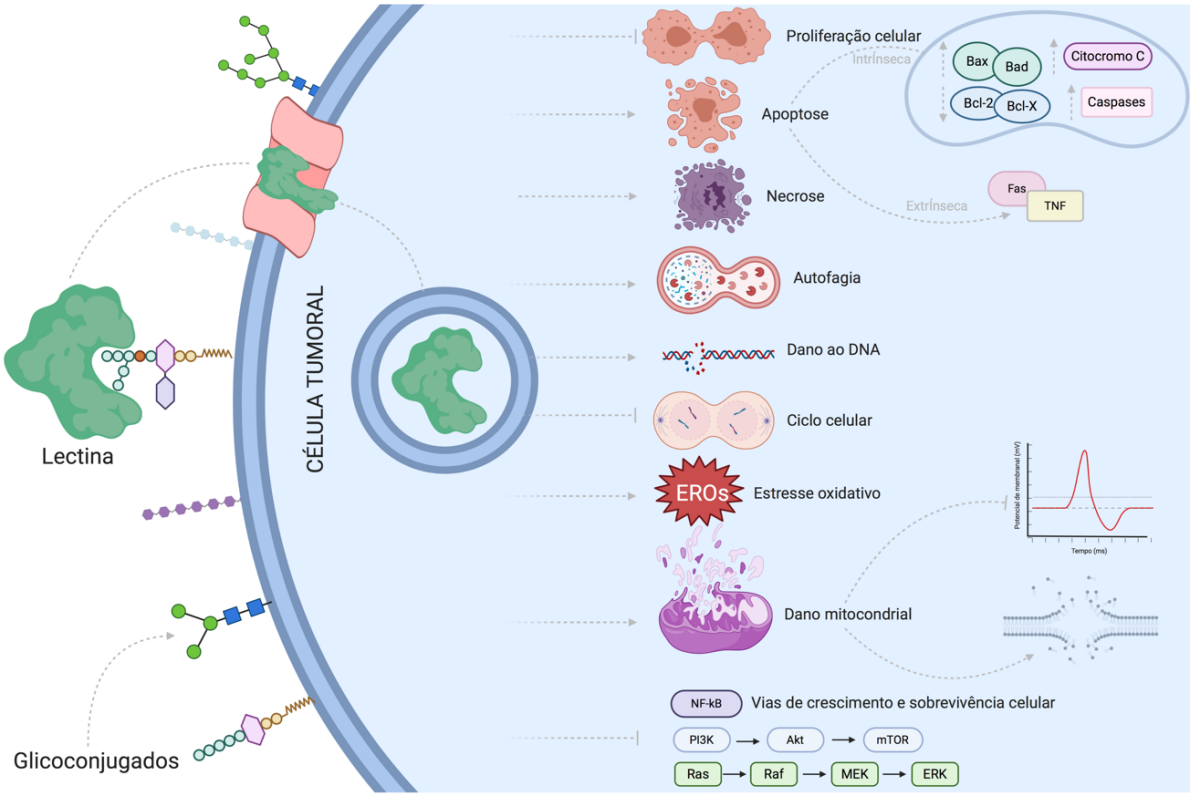
Os mecanismos de evasão de apoptose desenvolvidos pelas células neoplásicas decorrem de mutações que provocam perda da função de proteínas pró-apoptóticas ou aumento da expressão de genes anti-apoptóticos (XIE; LIU; LI, 2020). As lectinas podem reverter essa evasão da apoptose e promoverem a morte celular através da modulação de importantes mecanismos envolvidos na apoptose intrínseca (regulação positiva de proteínas pró-apoptóticas e regulação negativa de proteínas anti-apoptóticas, ativação de caspases e promoção da liberação do citocromo c) e apoptose extrínseca (liberação de receptores de morte como Fas e TNF) (MONTE et al., 2014; SHI et al., 2014; BHUTIA et al., 2019).

A necrose é uma forma de morte celular que se caracteriza pela distensão das organelas com a consequente perda da integridade da membrana celular e a liberação de constituintes intracelulares no espaço extracelular, o que pode levar à inflamação. Após lesão celular aguda, a necrose aparentemente ocorre de forma desordenada. No entanto, estudos recentes mostraram que a necrose também pode ser induzida por estímulos específicos e de forma programada por uma via de sinalização, sendo esse tipo de morte conhecida como necroptose (CHICO; SAGGAU; ADAM, 2017; SCHWARZER; LAURIEN; PASPARAKIS, 2020). As células necróticas apresentam traços característicos, incluindo um citoplasma cada vez mais translúcido, inchaço de organelas citoplasmáticas, condensação da cromatina e um aumento no volume celular que culmina na ruptura mecânica da membrana plasmática. As células necróticas não se fragmentam em corpos discretos, nem seus núcleos, que se acumulam em tecidos necróticos (KUMAR; ABBAS; ASTER, 2018). A necrose tem sido considerada como

um evento passivo resultante de um estímulo extrínseco celular, como a infecção por patógenos (BLÉRIOT; LECUIT, 2016) ou ação de lectinas (SILVA et al., 2014; LUZ et al., 2017).

As lectinas também podem promover a autofagia através da regulação positiva de genes relacionados à autofagia (ATG) e suas respectivas proteínas; interferir no crescimento e sobrevivência celular pelo bloqueio de vias de sinalização como a fosfatidilinositol-3-quinase/ proteína quinase B/ alvo da rapamicina em mamíferos (PI3K/Akt/mTOR), fator nuclear kappa B (NF-κB), quinase regulada por sinal extracelular (ERK); promover o estresse oxidativo e alterar a função mitocondrial (diminuição do potencial de membrana e perda da integridade da membrana); e alterar a progressão do ciclo celular (aumento da p53 e indução de paragem do ciclo celular) (SHI et al., 2014; LUZ et al., 2017; OSTERNE et al., 2017; ASADUZZAMAN et al., 2018). Os mecanismos de ação citotóxica de lectinas de plantas em células cancerígenas estão ilustrados na Figura 6 e exemplos de lectinas com ação antitumoral estão apresentados na Tabela 1.

FIGURA 6 – Mecanismos de ação antitumoral de lectinas de plantas.



Fonte: Elaborada pela autora.

TABELA 1 – Lectinas de plantas com atividade antitumoral e mecanismos envolvidos.

Fonte vegetal	Abreviação	Célula ou modelo tumoral	Mecanismos envolvidos
Abelmoschus esculentus	AEL	MCF-7	Apoptose, aumento da relação Bax/Bcl-2, ativação de caspase 3 e 9
Artocarpus heterophyllus	Jacalin	H1299, MCF-7	Inibição do crescimento celular
Astragalus membranaceus	AML	K562	Apoptose caspase dependente, diminuição de Bcl-2
		HeLa	Inibição do crescimento celular
Bauhinia ungulata	BUL	HT-29	Inibição do crescimento celular
Bauhinia forficata	BfL	MCF-7	Inibição do crescimento celular, interrupção do ciclo celular (fase G2/M), necrose
Benincasa hispida	BhL	A549, HeLa, PANC-1, CFPAC-1, MIA PaCa-2	Inibição do crescimento celular , apoptose com ativação de caspase 9 e 3 em PANC-1, CFPAC-1, MIA PaCa-2
Canavalia brasiliensis	ConBr	MOLT4, HL-60	Apoptose, necrose, despolarização mitocondrial , aumento de ROS
Canavalia virosa	ConV	C6	Inibição do crescimento celular, autofagia, diminuição no potencial de membrana mitocondrial
Calliandra surinamensis	CasuL	K562, T47D	Inibição do crescimento celular
Cratylia mollis	Cramoll	PC-3	Inibição do crescimento celular, perda da inegridade da membrana mitocondrial, aumento de calico citosólico
	rCramoll	PC-3	Inibição do crescimento celular, perda da inegridade da membrana mitocondrial, aumento de calcio citosólico
		Sarcoma 180	Inibição do crescimento tumoral
Datura innoxia	DiL9	A549, HeLa, PANC-1, CFPAC-1, MIA PaCa-2	Inibição do crescimento cellular, apoptose com ativação de caspase 9 e 3 em PANC-1, CFPAC-1, MIA PaCa-2
Dioscorea opposite	-	MCF-7, HepG2, CNE2	Inibição do crescimento celular, apoptose, despolarização mitocondrial
Euphorbia tirucalli	Eutirucallin	HeLa, PC3, MDA-MB-231, MCF-7	Inibição do crescimento celular
		Ehrlich ascites carcinoma	Inibição de crescimento tumoral
Glycine max	SBL	HeLa	Apoptose e autofagia mediada por ROS, ativação de caspases
Lotus corniculatus	LCL	THP-1, HOP62, HCT116	Inibição do crescimento celular, apoptose, despolarização mitocondrial
Moringa oleifera	MOSL	Carcinoma de Ehrlich	Inibição de crescimento tumoral, interrupção do ciclo cellular (fase G2/M), apoptose caspase-3 dependente, inibição da via NFkB de sinalização
	cMoL	B16-F10	Indução de necrose e apoptose com ativação de caspase 3, 8 e 9, aumento de ROS
Morus alba	MLL	MCF-7, HCT15	Apoptose, aumento da atividade de caspase 3
Pinellia ternata	PTL	HeLa, K562	Inibição do crescimento celular
		Sarcoma 180	Inibição do crescimento tumoral com interrupção do ciclo celular (transição G1/S) devido a interrupção de replicação de DNA
Phaseolus vulgaris	PHA	MCF-7, HepG2, CNE1, CNE2	Inibição do crescimento celular
Sophora alopecuroides	SAL	HeLa	Inibição do crescimento celular
Sophora flavescens	SFL	HeLa	Apoptose caspase dependente

A549: adenocarcinoma pulmonar humano; B16F10: células de melanoma murino; CFPAC-1: adenocarcinoma pancreático humano; CNE1: carcinoma nasofaríngeo humano; CNE2: carcinoma nasofaríngeo humano resistente a medicamentos; C6: células de glioma de rato; H1299: carcinoma de pulmão de células não pequenas humanas; HCT116: câncer de cólon humano; HCT15: câncer de cólon humano; HeLa: câncer cervical humano; HepG2: hepatoma humano; HL-60: leucemia promielocítica humana; HOP62: câncer de pulmão humano; HT-29: adenocarcinoma do cólon humano; K562: leucemia mielóide crônica humana; MCF7: carcinoma de mama humano; MDAMB-231: câncer de mama humano; MIA PaCa-2: câncer de pâncreas humano; MOLT4: leucemia linfoblástica aguda humana; PANC 1: carcinoma pancreático humano; PC-3: câncer de próstata humano; T47D: tumor de mama humano; THP-1: leucemia monocítica aguda humana.

Fonte: Traduzida de Patriota et al (2019a)

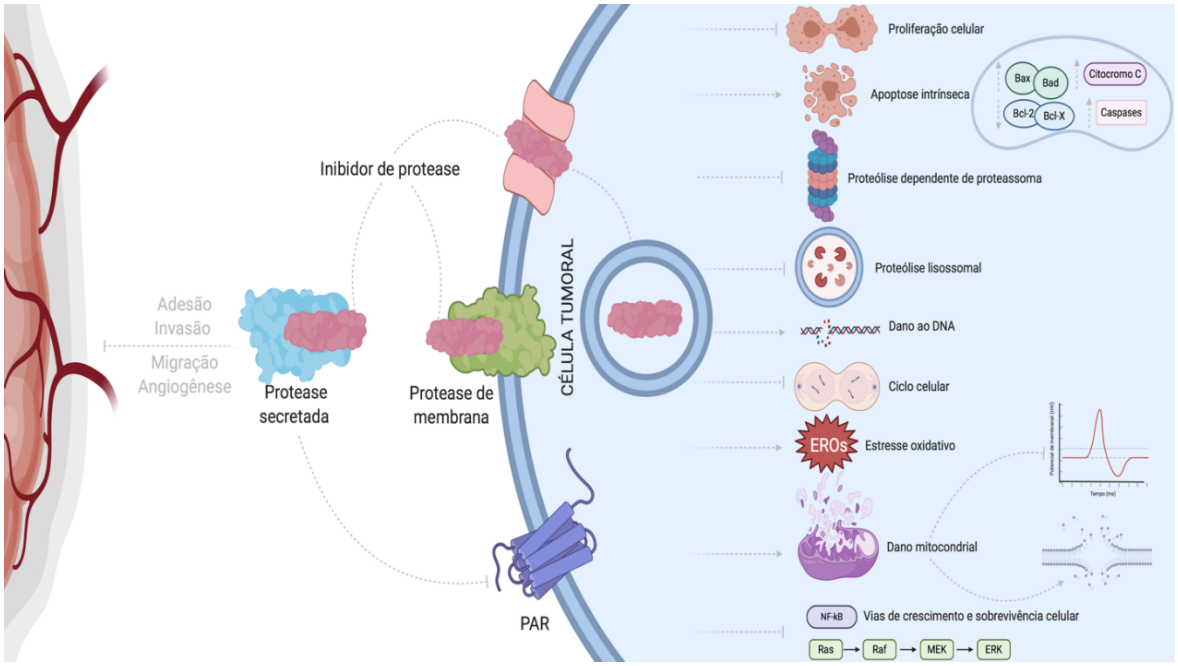
3.3.2 Inibidores de protease de plantas com atividade antitumoral

Os inibidores de protease das plantas são menos estudados que as lectinas quanto à ação antitumoral e as informações disponíveis sobre seus mecanismos antitumorais são consequentemente menores. Porém, os dados atualmente relatados na literatura sinalizam um futuro promissor no estudo dessas proteínas como novos fármacos antitumorais (ZASHEVA; SIMOVA-STOILOVA, 2017; LAPARRA; HAROS, 2019).

Vários tipos de tumores secretam proteases (comprovadamente serino, cisteíno e metaloproteases) que participam de processos relacionados à progressão do tumor, como a proliferação celular, angiogênese, invasão e metástase. A expressão dessas proteases em tecidos normais é baixa, no entanto, é muito aumentada no câncer. Ainda, os tumores são capazes de estimular a expressão de proteases em células vizinhas normais, favorecendo ainda mais o seu crescimento (ZASHEVA; SIMOVA-STOILOVA, 2017; TEIXEIRA; OLIVEIRA; SILVA-LÓPEZ, 2020). Sendo assim, inibidores específicos de proteases alteradas no câncer, podem ser vistos como possíveis agentes anticâncer, uma vez que se ligam nos sítios ativos de proteases tumorais e bloqueiam a progressão do câncer (TEIXEIRA; OLIVEIRA; SILVA-LÓPEZ, 2020). De fato, demonstrou-se que os inibidores de protease exercem efeitos antitumorais nos estágios de iniciação, proliferação e progressão de diferentes tipos de câncer (SRIKANTH; CHEN, 2016), por interferir nas vias proteolíticas ligadas à adesão, invasão e migração de células cancerígenas (TEIXEIRA; OLIVEIRA; SILVA-LÓPEZ, 2020).

Outros mecanismos de ação antitumoral descritos para inibidores de protease de plantas são a indução de morte celular, especialmente por apoptose, que pode envolver regulação positiva de proteínas pró-apoptóticas (Bax e Bak) e regulação negativa de proteínas anti-apoptóticas (Bcl-2 e Bcl-xl) (LI et al., 2009), ativação de caspases (BAI et al., 2015), liberação do citocromo c (FERREIRA et al., 2013), produção de espécies reativas de oxigênio (EROs) (MEHDAD et al., 2016), fragmentação de DNA (PARK; OHBA, 2004), supressão da via da quinase regulada por sinal extracelular (ERK) (CHEN et al., 2005), interrupção do ciclo celular (CLEMENTE et al., 2010) e danos na membrana mitocondrial (MEHDAD et al., 2016). Os mecanismos de ação antitumoral de inibidores de protease de plantas estão ilustrados na Figura 7. Inibidores de protease de plantas estudados quanto à sua atividade antitumoral, as linhagens celulares ou modelos tumorais afetados por eles e os mecanismos de ação estão listados na Tabela 2.

FIGURA 7 – Mecanismos de ação antitumoral de inibidores de protease de plantas.



Fonte: Elaborada pela autora.

TABELA 2 - Inibidores de protease de plantas com atividade antitumoral e mecanismos envolvidos.

Fonte vegetal	Nome	Célula ou modelo tumoral	Mecanismos envolvidos	Referências
Bauhinia bauhinioides e *Bauhinia rufa* (sementes)	rBbKIm	DU145, PC3	Interrupção do ciclo celular, apoptose com ativação de caspases e liberação de citocromo C	(FERREIRA et al., 2013)
Cajanus cajan (sementes)	CCPI	A549	Inibição do crescimento celular	(SHAMSI et al., 2017)
Cicer arietinum (sementes)	----	MDAMB- 231, PC-3, LNCaP	Inibição do crescimento celular	(MAGEE et al., 2012)
Coccinia grandis (folhas)	CGPI	HeLa	Inibição do crescimento celular	(SATHEESH; MURUGAN, 2011)
Crataeva tapia (casca)	CrataBL	U87	Inibiu a invasão e adesão celular	(BONTURI et al., 2019)
Elusine coracana (sementes)	RBI	K562	Inibição do crescimento celular, apoptose	(DUTTA, 2012)
Enterolobium contortisiliquum (sementes)	EcTI	HCT116, HT29, SkBr-3, MCF-7, K562, THP-1	Inibição do crescimento celular; inibição de ativação da proMMP	(NAKAHATA et al., 2011)
		Hs746T, MKN28	Inibiu a invasão e adesão celular	(PAULA et al., 2012)
Fagopyrum sculentum (sementes)	BTI-1 e BTI-2[a]	Jurkat, CCRF-CEM	Apoptose com fragmentação de DNA	(PARK; OHBA, 2004)
	BTI	EC9706, Hep G2, HeLa	Apoptose por regulação positiva de Bax e Bak e regulação negativa de Bcl-2 e Bcl-xl, liberação de citocromo C, perda de potencial de membrana, ativação de caspase 3	(LI et al., 2009)
		H22	Apoptose envolvendo a via mitocondrial e caspase-9	(BAI et al., 2015)

Glycine max (sementes)	*Glycine max* BBI	Tumor colorretal de camundongo	Supressão do tumor	(KENNEDY et al., 2002)
		LNCaP	Supressão da geração de ROS	(SUN et al., 2001)
		Adenocarcinoma de próstata em camundongos	Apoptose por liberação de caspase-3	(TANG et al., 2009)
		MCF-7	Inibição do proteassoma, interrupção do ciclo celular, supressão da atividade da ERK	(CHEN et al., 2005)
		HT29	Inibição do crescimento celular, interrupção do ciclo celular	(CLEMENTE et al., 2010)
Ipomoea batatas (raízes)	----	NB4	Inibição do crescimento celular, interrupção do ciclo celular, acúmulo de p53 e Bax, diminuição de Bcl-2, ativação de caspase 3 e 8	(HUANG et al., 2007)
Lavatera cashmeriana (sementes)	LC-PI I	THP-1, NCIH322, Colo 205, HCT-116, PC-3, MCF-7	Inibição do crescimento celular	(RAKASHANDA et al., 2013a, 2013b)
		A549	Apoptose, interrupção do ciclo celular, efeito antimetastático	(RAKASHANDA et al., 2015)
	LC-PI II	THP-1, NCIH322, Colo 205	Inibição do crescimento celular	(RAKASHANDA et al., 2013a, 2013b)
		A549	Apoptose, interrupção do ciclo celular, efeito antimetastático	(RAKASHANDA et al., 2015)
Lens culinaris (sementes)	LCTI	HT29	Inibição do crescimento celular	(CACCIALUPI et al., 2010)
Macrotyloma axillare (sementes)	DE-3 e DE-4	Neoplasia colorretal	Inibição de vias proteolíticas dependentes de lisossomos e proteassomas	(CARLI et al., 2012)

Medicago scutellata (sementes)	MsTI	MCF-7, HeLa	Aumento da citotoxicidade induzida pela cisplatina e redução da sobrevivência clonogênica	(LANZA et al., 2004)
Peltophorum dubium (sementes)	PDTI	Nb2	Inibição do crescimento celular, apoptose, fragmentação do DNA, hipodiploidia do DNA, ativação da caspase 3	(TRONCOSO; WOLFENSTEIN-TODEL, 2003)
		JURKAT	Apoptose por ativação de caspases	(TRONCOSO et al., 2007)
Pisum sativum (sementes)	TI1B	HT29	Inibição do crescimento celular	(CLEMENTE et al., 2012; CLEMENTE et al., 2014)
Sorghum bicolor (sementes)	XILP	HepG2, MCF-7, SiHa, HT29	Inibição do crescimento celular	(LIN et al., 2013)
Vicia faba (sementes)	FBPI	B16F10	Ação antimetastatogênica	(BANERJI et al., 1998)
		Câncer gástrico de camungongos	Inibição do crescimento celular	(FERNANDES; BANERJI, 1995)
		Carcinogênese cutânea de camundongos	Inibição do crescimento celular	(FERNANDES; BANERJI, 1996)
Vigna unguiculata (sementes)	BTCI	MCF-7	Inibição do crescimento celular, apoptose, dano mitocondrial, produção de EROs	(SOUZA et al., 2014; MEHDAD et al., 2016)

A549: câncer de pulmão humano; B16F10: melanoma de camundongo; Colo 205: câncer de cólon humano; CCRF-CEM: leucemia linfoblástica aguda humana; DU145: câncer de próstata humano; EC9706: câncer de esôfago humano; H22: hepatoma de camundongo; HCT-116: carcinoma colorretal humano; HeLa: câncer cervical humano; HepG2: hepatoma humano; Hs746T: câncer gástrico humano; HT29: adenocarcinoma de colón humano; JURKAT: leucemia humana; K562: leucemia mieloide crônica humana; LNCaP: câncer de próstata humano; MCF-7: carcinoma da mama humano; MDAMB-231: câncer de mama humano; MKN28: câncer gástrico humano; Nb2: linfoma de camundongo; NB4: leucemia promielocítica humana; NCIH322: câncer de pulmão humano; PC-3: câncer de próstata humano; SiHa: carcinoma de útero humano; SkBr-3: adenocarcinoma mamário humano; THP-1: leucemia monocítica aguda huma.

Fonte: Elaborada pela autora.

3.4 INFLAMAÇÃO

3.4.1 Sistema imunológico

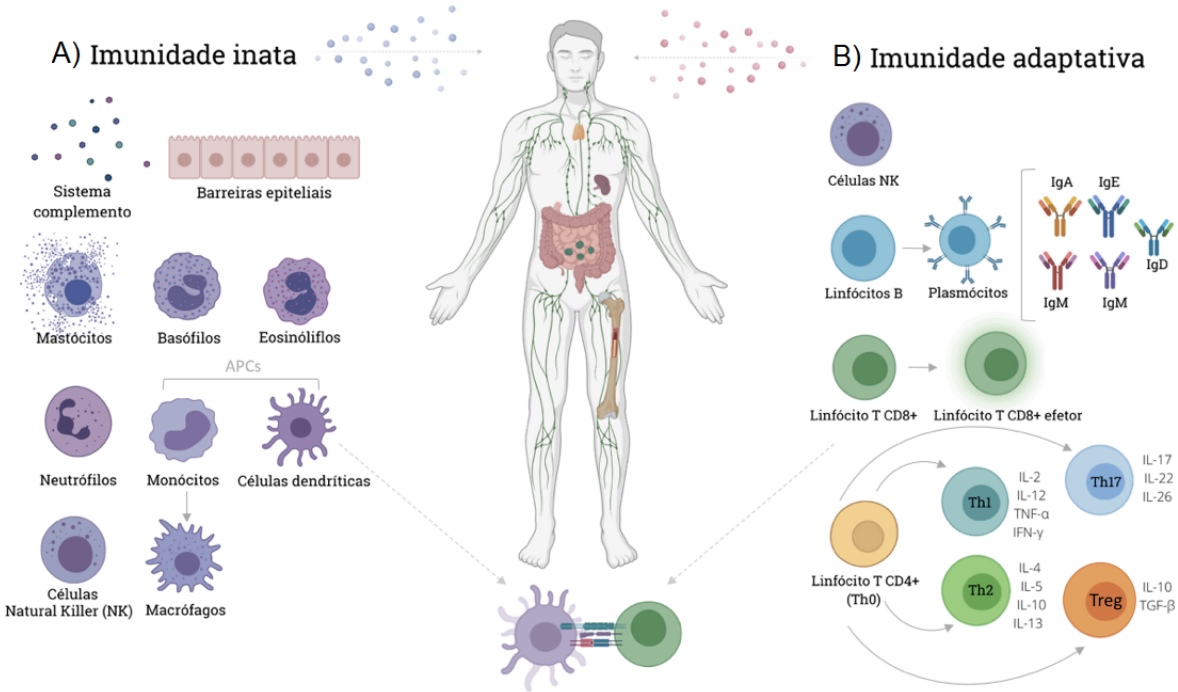
O sistema imunológico é um conjunto de células, tecidos e órgãos que age para eliminar estímulos potencialmente prejudiciais, reconhecendo e respondendo a uma grande variedade de patógenos e lesões. É dividido em sistema imune inato (ou não-específico) e sistema imune adaptativo (ou específico). O sistema inato é uma resposta geral e não se altera com a exposição repetida. Por outro lado, o sistema adaptativo aumenta sua resposta apropriadamente em exposição repetida e exibe especificidade e memória. A integração entre os dois sistemas é mediada por moléculas denominadas citocinas. Cada um dos dois sistemas é constituído por componentes celulares e humorais (proteínas) (ABBAS; LICHTMAN; PILLAI, 2015; ABBAS; LICHTMAN; PILLAI, 2019). Assim, resposta imune envolve a forte cooperação de uma grande variedade de tipos de células, sendo controlada tanto pela interação direta desses diferentes tipos de células (linfóides e mielóides) como pelos produtos de síntese que secretam (imunoglobulinas, mediadores inflamatórios) (GRIGORE, 2017).

As células que pertencem ao sistema imunológico inato estão envolvidas em muitos mecanismos efetores. Mastócitos e basófilos atuam em manifestações alérgicas. Eosinófilos são efetores contra parasitas. Essas três células apresentam maior produção de histamina quando estimuladas pela imunoglobulina E, liberada pelos linfócitos B. Os neutrófilos são as células mais numerosas e importantes da imunidade inata; eles capturam uma variedade de patógenos por fagocitose e os destroem de maneira eficiente em vesículas intracelulares usando enzimas de degradação e outras substâncias antimicrobianas armazenadas em seus grânulos citoplasmáticos. Os macrófagos neutralizam, ingerem e destroem corpos estranhos, além de processar e apresentar antígenos aos linfócitos T. Outra função importante e crucial dos macrófagos é o recrutamento de outras células do sistema imune. As células dendríticas são as mais especializadas na captura e apresentação de antígenos aos linfócitos T. Macrófagos e células dendríticas estão envolvidas no mecanismo de apresentação de antígeno e fagocitose através das proteínas denominadas Complexo Maior de Histocompatibilidade (MHC) tipos I e II. Portanto, essas células também são chamadas de células apresentadoras de antígeno (APCs) (Figura 8A) (PATRIOTA et al., 2019b; ABBAS; LICHTMAN; PILLAI, 2019).

A resposta imune adaptativa começa com a apresentação de antígenos pelas APCs aos linfócitos T citotóxicos (também chamados de linfócitos T CD8+) e linfócitos T helper (Th) (também chamados de linfócitos T CD4+), que reconhecem antígenos através de seus

receptores de membrana. Após isso, esses linfócitos T se diferenciam em linfócitos T efetores, que exercem diferentes funções dentro da resposta imune. Os linfócitos são estimulados por muitos sinais e cada resposta e ativação linfocitária são geradas induzindo um mecanismo efetor rápido de remoção e memória imunológica contra o antígeno (Figura 8B). Os linfócitos T citotóxicos podem destruir células infectadas por vírus e bactérias intracelulares. Quando ativadas, as células Th *näive* ou virgens (Th0) podem se diferenciar em diversos estados de polarização, definidos como células Th1, Th2, Th17 e Treg (reguladoras), dentre outros, que são responsáveis por secretar citocinas que atuam no recrutamento de células do sistema imune que desempenham diferentes funções efetoras. Os linfócitos *natural killers* (NK) atuam tanto na imunidade inata quanto na adaptativa e estão envolvidos na vigilância imune ao câncer (PATRIOTA et al., 2019b; ABBAS; LICHTMAN; PILLAI, 2019).

FIGURA 8 – A integração entre os sistemas imunológicos inato e adaptativo. (A) Componentes da imunidade inata. (B) Componentes da imunidade adaptativa.



Fonte: Elaborada pela autora.

As citocinas são proteínas ou glicoproteínas produzidas por uma grande variedade de células, e sua ação também atinge várias células e tecidos. Essas proteínas formam redes inter-reativas complexas com potencial autócrino, parácrino e endócrino. A pleiotropia funcional (múltiplas propriedades em diferentes células-alvo) e redundância (várias citocinas podem

efetuar as mesmas ações) são características das citocinas, que podem interagir umas com as outras de maneira aditiva, sinérgica ou antagônica, ou ainda pode ocorrer a indução da liberação de uma citocina por outra (KISHORE et al., 2019).

As citocinas constituem um componente-chave do sistema imunológico inato e adaptativo, sendo responsáveis pela comunicação e regulação das diferentes células do sistema imunológico através de várias cascatas de sinalização (PATRA; SHAH; CHOI, 2020). Além do papel na imunidade e inflamação, as citocinas têm funções variadas em todo o organismo, incluindo diferenciação celular, angiogênese, tumorigênese e patogênese microbiana (AIELLO et al., 2020). Assim, elas medeiam numerosas funções biológicas através de receptores específicos de superfície celular expressos pelas células-alvo (KISHORE et al., 2019). Embora as citocinas fossem conhecidas anteriormente como "fatores solúveis", hoje é reconhecido que elas também podem funcionar como proteínas integrais de membrana. Essas proteínas têm uma meia vida muito curta e agem em concentrações picomolares (AIELLO et al., 2020).

Centenas de citocinas já foram reconhecidas (KISHORE et al., 2019), as quais incluem linfocinas, monocinas, quimiocinas, interleucinas (ILs), interferons (IFN), fatores estimuladores de colônias (CSFs) e fatores de crescimento (GFs) (ABBAS; LICHTMAN; PILLAI, 2019). Todas as células do sistema imune, tanto inato quanto adaptativo, secretam, pelo menos, algumas citocinas. Essas citocinas podem ser categorizadas funcionalmente como pró-inflamatórias (ex. IL-1, IL-2, IL-6, IFN-γ, TNF-α, IL-17) ou anti-inflamatórias (ex. IL-4, IL-10, IL-13), como o próprio nome sugere, de acordo com o seu envolvimento na resposta inflamatória (ABBAS; LICHTMAN; PILLAI, 2019).

Na resposta imune adaptativa, moléculas co-estimulatórias e combinações precisas de citocinas promovem a diferenciação de linfócitos Th virgens (Th0) em células efetoras Th1, Th2, Th17, Th22, Th9 ou Treg e polarização de suas respostas imunológicas através de produção de perfis de citocinas e ativação/supressão de células específicas. As citocinas Th1 (ex. IFN-γ, IFN-α, IL-12, TNF-α, TNF-β e IL-2) estão envolvidas na resposta Th1 (resposta imune celular) que é eficaz contra bactérias e vírus intracelulares. As citocinas Th2 (ex. IL-4, IL-5, IL-10, IL-13 e IL-25) fazem parte da resposta Th2 (resposta imune humoral), que desempenha um papel crucial na eliminação de alérgenos e parasitas extracelulares. As citocinas Th17 (ex. IL-17, IL-6, IL-21, IL-22, IL-23, TNF-α) compõem a resposta Th17, que é essencial contra bactérias e fungos extracelulares. Por sua vez, Tregs produzem IL-10, IL-35 e TGF-β, estando envolvidos com a supressão de células T efetoras (VINCZE et al., 2018).

3.4.2 Resposta inflamatória

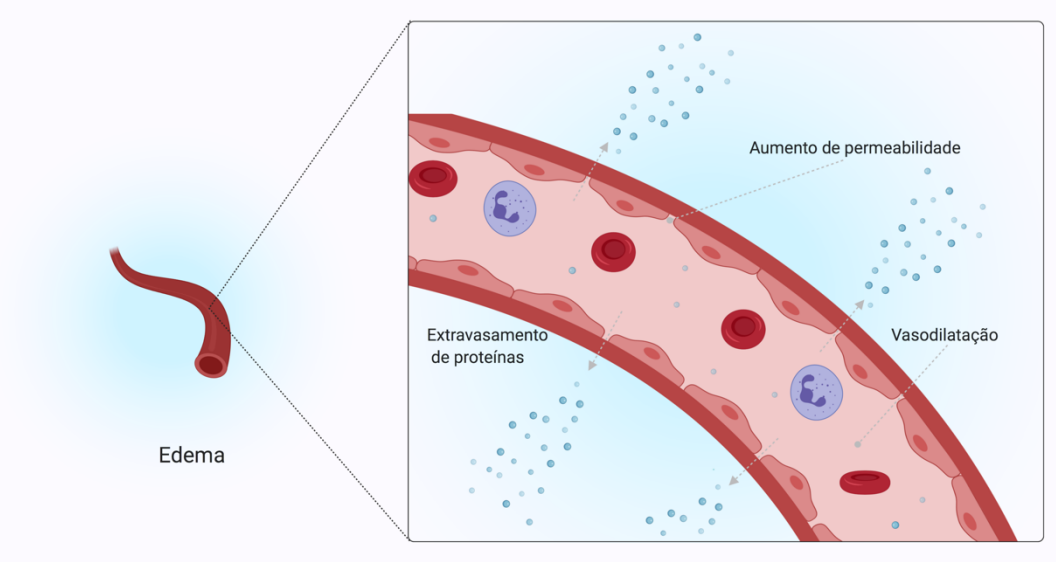
A inflamação é um processo biológico fortemente regulado e essencial para a sobrevivência. Sem a resposta inflamatória, feridas e infecções não curariam, pois, ela é uma tentativa protetora do organismo de iniciar o processo de cicatrização e restaurar a homeostase tecidual (BURINI et al., 2020). Assim, inflamação é o conjunto de mecanismos de reação pelos quais o sistema imunológico do organismo reconhece e responde a estímulos prejudiciais (físicos, químicos ou biológicos), visando minimizar, destruir ou eliminá-los e potencializar a reparação tecidual. Para tanto, a reação inflamatória envolve células imunológicas (mononucleares e polimorfonucleares) e proteínas circulantes (anticorpos, proteínas do complemento, citocinas) (CHEBAIBI et al., 2019; ABBAS; LICHTMAN; PILLAI, 2019). A inflamação pode ocorrer de forma local ou sistêmica e é frequentemente caracterizada, além da dor, por quatro sinais clássicos que são: rubor, calor, inchaço e perda de função (FULLERTON; GILROY, 2016; RONCHETTI; MIGLIORATI; DELFINO, 2017). Levando em consideração a duração e as características patológicas, a inflamação pode ser dividida em duas fases: aguda e crônica (CHEN et al., 2018).

O corpo humano é submetido a insultos constantes que estimulam a resposta inflamatória (SUGIMOTO et al., 2019). A presença desses estímulos prejudiciais é percebida pelas células residentes, principalmente macrófagos, mas também por outros tipos de células, através dos receptores de reconhecimento de padrões (PRRs) que reconhecem os padrões moleculares associados a patógenos (PAMPs) e os padrões moleculares associados a danos (DAMPs). Dentre os PRRs, estão os receptores do tipo *Toll* (TLRs). A ativação dos TLRs ajuda a iniciar vias de sinalização que são essenciais para orquestrar as respostas inflamatórias dessas células, que secretam mediadores inflamatórios (aminas vasoativas; citocinas e outros mediadores) que induzem e regulam a resposta inflamatória (ABBAS; LICHTMAN; PILLAI, 2019; YANG et al., 2020). Por exemplo, a ativação das vias de sinalização das Proteínas Quinases Ativadas por Mitógenos (MAPKs) e fator nuclear kappa B (NF-kB) leva à indução de citocinas pró-inflamatórias, incluindo, entre outras, o fator de necrose tumoral (TNF-α) e a interleucina 1 (IL-1) (PELTZER; WALCZAK, 2019).

A inflamação aguda é a primeira resposta do corpo a esses estímulos prejudiciais, podendo se desenvolver em minutos a horas e tem duração relativamente curta (dias). Possui dois componentes principais: alterações vasculares e eventos celulares. No primeiro caso, mediadores inflamatórios como a histamina e bradicinina, por exemplo, atuam nos pequenos vasos sanguíneos nas proximidades do estímulo, induzindo vasodilatação, que leva ao aumento

do fluxo sanguíneo local e aumenta a permeabilidade microvascular, levando à perda de plasma (exsudato; rico em proteínas plasmáticas) e de fluido para o tecido; essa resposta caracteriza o edema (Figura 9) (KUMAR; ABBAS; ASTER, 2018; ABBAS; LICHTMAN; PILLAI, 2019).

FIGURA 9 – Alterações vasculares e formação de edema na inflamação aguda.

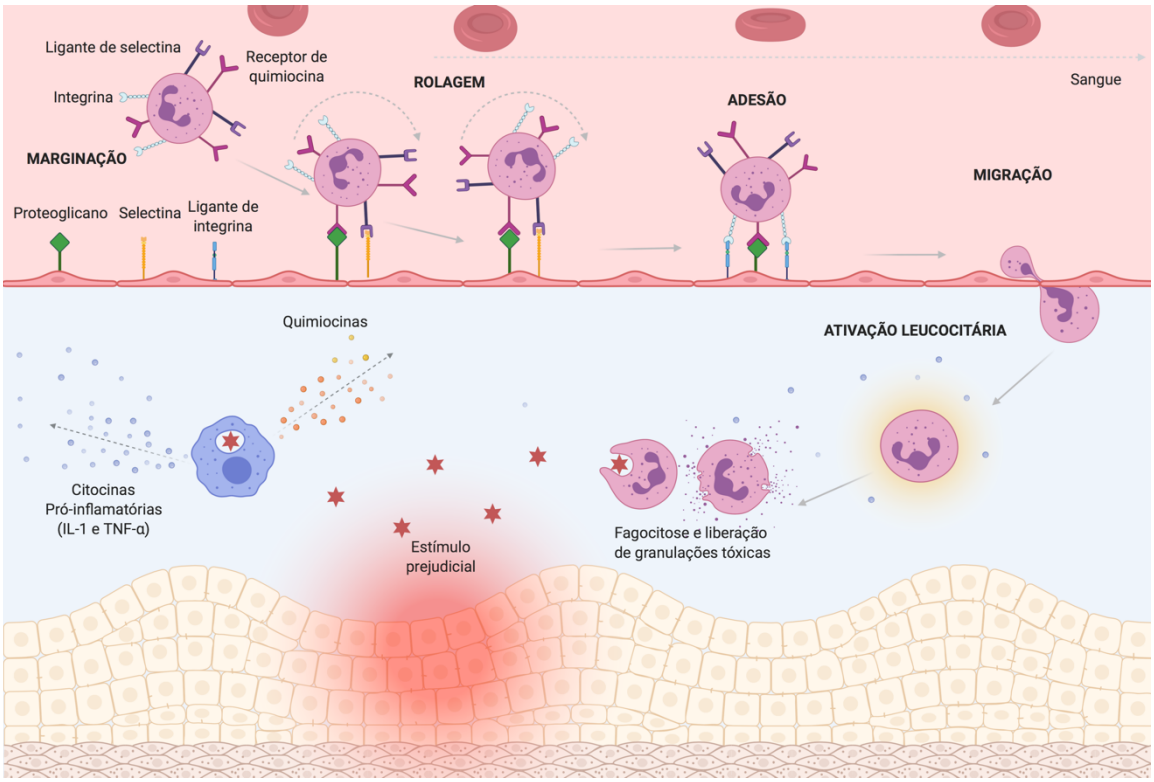


Fonte: Elaborada pela autora.

Essas alterações vasculares provocam estase do fluxo sanguíneo favorecendo a marginação de leucócitos. Assim, eventos celulares ocorrem simultaneamente, como o recrutamento de leucócitos circulantes, predominantemente neutrófilos, para o local de inflamação. Para tanto, citocinas pró-inflamatórias como o TNF-α e a IL-1 são produzidas por células teciduais estimuladas pelo contato com o estímulo prejudicial e ativam células endoteliais, que passam a produzir selectinas, ligantes para integrinas e quimiocinas. As selectinas medeiam a ligação dos leucócitos sanguíneos ao endotélio e a força de cisalhamento do fluxo sanguíneo faz os leucócitos rolarem ao longo da superfície endotelial. As quimiocinas (normalmente ligadas a proteoglicanos) se ligam a receptores nos leucócitos em rolamento, o que resulta em ativação das integrinas do leucócito, promovendo adesão estável dos leucócitos às células endoteliais. Após isso, os leucócitos migram pela parede do vaso, passando pelas junções entre as células endoteliais por diapedese. Ao atingir o sítio inflamatório, os leucócitos são ativados pelo contato direto com o estímulo prejudicial ou pela ação de citocinas secretadas por células da região, passando então a fagocitar e liberar o conteúdo presente nas suas granulações tóxicas, como EROs, espécies reativas de nitrogênio (ERNs), proteases (lisozima, elastase), entre outros, com a finalidade de eliminar o estímulo prejudicial (Figura 10). Além

disso, os leucócitos produzem mediadores que amplificam a reação inflamatória, por recrutamento e ativação de mais leucócitos. O tipo de leucócito emigrante varia com o tempo da resposta inflamatória e com o tipo de estímulo (KUMAR; ABBAS; ASTER, 2018; ABBAS; LICHTMAN; PILLAI, 2019).

FIGURA 10 – Eventos celulares na inflamação aguda: migração leucocitária para o sítio de inflamação.



Fonte: Elaborada pela autora.

Por fim, fibroblastos se proliferam para restabelecer a estrutura do tecido lesado e a inflamação aguda é finalizada com a resolução da reação inflamatória e retorno do tecido lesionado à normalidade ou sua substituição por tecido conjuntivo (KUMAR; ABBAS; ASTER, 2018). É importante que os níveis de mediadores inflamatórios sejam regulados para evitar respostas inflamatórias crônicas; portanto, para a resolução da inflamação aguda é necessário: eliminação do estímulo prejudicial, redução dos sinais pró-inflamatórios e catabolismo dos mediadores e a eferocitose (remoção de células mortas) (SUGIMOTO et al., 2019; FEEHAN; GILROY, 2019).

A persistência da causa da inflamação ou a falha na resposta tecidual pode levar à inflamação crônica, a qual tem duração maior, podendo persistir por longos períodos como resultado do envolvimento constante de respostas imunes inatas e adquiridas, estando

relacionada com presença de linfócitos e macrófagos, sendo estes últimos responsáveis por secretar mediadores inflamatórios que iniciam e mantêm o reparo tecidual, resultando no modelamento da matriz extracelular e culminando com a diminuição da função do tecido (FULLERTON; GILROY, 2016; SUGIMOTO et al., 2019). A desregulação inflamatória está envolvida em várias doenças conhecidas, incluindo diabetes, doenças cardiovasculares, câncer e doenças autoimunes (ZHONG E SHI, 2019; KISHORE et al., 2019; PIOTROWSKI; KULCENTY; SUCHORSKA, 2020).

3.4.3 Mediadores da resposta inflamatória

Os mediadores inflamatórios são substâncias químicas produzidas e liberadas no tecido que sofreu lesão. Essas substâncias atuam nos vasos sanguíneos e nas células inflamatórias, modulando os principais eventos relacionados à inflamação, como a vasodilatação, quimiotaxia e migração, podendo ainda estimular a liberação de outros mediadores. Muitos são os mediadores que participam do processo inflamatório, como por exemplo: aminas vasoativas (histamina e serotonina), peptídeos (bradicinina), e eicosanóides (prostaglandinas, tromboxanos e leucotrienos), EROs, ERNs e citocinas (ABDULKHALEQ et al., 2018; TASNEEM et al., 2019).

Um processo inflamatório é caracterizado por um desequilíbrio entre citocinas anti e pró-inflamatórias. As células Th1 e Th2 desempenham um papel importante na mediação em ambas as respostas, pois sabe-se que citocinas pró-inflamatórias como TNF-α e IFN-γ são produzidas por células Th1, enquanto importantes citocinas anti-inflamatórias como IL-4 e IL-10 pertencem à resposta Th2. Isso gerou interesse no paradigma Th1/Th2 em relação ao desenvolvimento e progressão de várias condições inflamatórias (PATRIOTA et al., 2019b; MAHLANGU et al., 2020).

O TNF-α é uma das mais importantes citocinas pró-inflamatórias, sendo produzida principalmente por macrófagos, mas também por outras células imunes. Afeta vários aspectos da resposta imune e inflamação, dentre eles, fagocitose de macrófagos e recrutamento de leucócitos. Além disso, é uma citocina pleiotrópica que participa de vários processos fisiológicos (sistema nervoso, regulação de coagulação, células endoteliais) (SHARIF et al., 2020).

O IFN-γ é uma citocina pleiotrópica que tem efeitos essenciais na imunidade na inata e adaptativa, sendo sua produção é amplamente restrita a linfócitos T e NK. Sua sinalização é necessária para ativação de macrófagos M1 (pró-inflamatórios), proliferação de linfócitos B,

polarização de células Th0 para Th1 e maturação das células T citotóxicas (ALSPACH; LUSSIER; SCHREIBER, 2019; SILK; MARGOLIN, 2019).

A IL-2 é uma citocina pró-inflamatória produzida principalmente por linfócitos T durante as respostas imunes Th1. A sinalização de IL-2 estimula e controla os diferentes subtipos de linfócitos (células T, B e NK) durante sua proliferação, diferenciação, ativação e resposta imune (SILK; MARGOLIN, 2019).

A IL-4 é produzida por linfócitos T, basófilos, eosinófilos e mastócitos. É típica de resposta Th2, sendo essencial no processo de resposta inflamatória desencadeada por parasitas ou alérgenos. Nos linfócitos T, a IL-4 induz e mantém a diferenciação de células Th0 em Th2; nos linfócitos B, conduz a produção de anticorpos IgE; e nos macrófagos, induzem ativação de macrófagos M2 (anti-inflamatórios), além de expansão de basófilos e eosinófilos, e ativação de mastócitos (JUNTTILA, 2018; HEEB; EGHOLM; BOYMAN, 2020).

A IL-6 é uma citocina multifuncional produzida por macrófagos e outras células imunes, participando ativamente de mecanismos fisiológicos, inflamatórios e imunomoduladores, tais como diferenciação de macrófagos; proliferação, diferenciação e maturação de linfócitos; e síntese e secreção de imunoglobulinas (KANG et al., 2019; KAUR et al., 2020).

A IL-10 é uma citocina imunorreguladora pluripotente produzida por quase todos os tipos de células imunes, possuindo atividade imunossupressora na imunidade inata e adaptativa. A IL-10 atenua a ativação das APCs, suprimindo a expressão do MHC e moléculas co-estimulatórias, e reduz a produção de citocinas pró-inflamatórias. Ainda, promove a diferenciação, sobrevivência e função das células Treg, mas também inibe diretamente a função efetora das células Th1, Th2 e Th17 (ZHANGA; KUCHROO, 2019).

A IL-17 é uma citocina pró-inflamatória produzida predominantemente linfócitos Th17. Sua potente ação inflamatória está principalmente relacionada à sua capacidade de recrutar células imunes e às suas ações sinérgicas com outras citocinas pró-inflamatórias. O papel fisiológico primário da IL-17 é atuar contra agentes patogênicos extracelulares (MCGEACHY; CUA; GAFFEN, 2019; MORALES et al., 2019).

O óxido nítrico (NO) é um radical livre de meia-vida curta sintetizado a partir de L-arginina e oxigênio por uma via catalisada por óxido nítrico sintases (NOS). Na maioria das células animais, são descritos três tipos de NOS: NOS1 (neuronal), NOS2 ou iNOS (induzível) e NOS3 (endotelial). O NO acumulado reage rapidamente com EROs presentes para formar a ERN peroxinitrito (ONOO-), altamente tóxica (GANTNER; LAFOND; BONINI, 2020). O NO é uma molécula ideal para transmissão rápida de informações, permitindo que tipos celulares muito diferentes se comuniquem e coordenem funções complexas dos tecidos de diversos

sistemas (cardíaco, pulmonar, hepático, renal, endócrino). A desregulação da sinalização de NO, no entanto, pode causar processos patológicos em todos esses tecidos (CYR et al., 2020). A NOS2 é considerada a NOS imunológica, pois produz altos níveis de NO em resposta a mediadores inflamatórios e/ou PAMPs, tendo uma função central na regulação das respostas inflamatórias e imunológicas; os macrófagos são as células imunes inatas que mais expressam a iNOS (BOGDAN, 2015; GARCÍA-ORTIZ; SERRADOR, 2018). Dentre os diversos processos, o NO está envolvido na resposta dos macrófagos M1 a agentes patogênicos participando da síntese de ONOO-. Além disso, o NO regula a diferenciação, ativação e função de linfócitos (GARCÍA-ORTIZ; SERRADOR, 2018).

3.4.4 Imunomodulação

Nos últimos 50 anos, observa-se um interesse crescente em compreender os mediadores que causam respostas inflamatórias crônicas visando o tratamento dessas doenças, com o objetivo predominante de inibir a síntese ou ação de mediadores que impulsionam a inflamação usando drogas, incluindo agentes anti-inflamatórios e imunomoduladores (FEEHAN; GILROY, 2019). No entanto, sempre há preocupações de segurança associadas ao uso de anti-inflamatórios não esteroidais (AINEs) (RONCHETTI; MIGLIORATI; DELFINO, 2017; ATKINSON; FUDIN, 2020) e anticitocinas (PATRA; SHAH; CHOI, 2020), por exemplo.

Os conceitos de "imunomodulador" e "anti-inflamatório" geralmente estão fortemente relacionados (GRIGORE, 2017; SHAHBAZ et al., 2019). Contudo, a imunomodulação é um termo genérico que abrange o processo de ajuste do sistema imunológico ao nível desejado (KUBACKOVA; ZBYTOVSKA; HOLAS, 2020). Para tanto, o papel que níveis excessivos ou insuficientes de imunidade desempenham na fisiopatologia do processo de doença ditará a direção da imunomodulação que pode ser benéfica (NADER, 2017). Assim, os imunomoduladores podem apresentar atividades positiva (imunoestimulação) ou negativa (imunossupressão) sobre uma função imunológica específica (NADER, 2017; KUBACKOVA; ZBYTOVSKA; HOLAS, 2020). Por exemplo, o sistema imune é estimulado na vacinação e imunoterapia do câncer ou suprimido para tratar distúrbios autoimunes ou doenças inflamatórias (KUBACKOVA; ZBYTOVSKA; HOLAS, 2020). A imunomodulação pode ser específica ou não-específica. A específica é limitada a um único antígeno, como a vacinação; a não-específica implica em mudanças generalizadas na resposta imunológica, tanto a inata quanto a adaptativa, levando a uma modificação na reatividade do hospedeiro para muitos antígenos diferentes (DHAMA et al., 2015).

Dentre os componentes do sistema imunológico que podem ser modulados estão os mediadores inflamatórios. Alguns dos fármacos mais efetivos desenvolvidos recentemente para tratar doenças imunológicas têm como alvo as citocinas. Embora as citocinas desempenhem funções-chave no sistema imunológico, elas estão implicadas na patogênese de inúmeras doenças inflamatórias, distúrbios autoimunes e cânceres (PATRA; SHAH; CHOI, 2020), podendo imunomoduladores serem usados para estimular/suprimir perfil de citocinas de respostas imunes como Th1/Th2, por exemplo (WEI et al., 2019; KHODABANDEH et al., 2020). Esses agentes também podem ter como alvo citocinas específicas (SILK; MARGOLIN, 2019). A Tabela 3 lista objetivos terapêuticos da estimulação ou supressão de citocinas.

TABELA 3 – Potenciais aplicações terapêuticas da imunomodulação de citocinas.

Citocinas	**Aplicação terapêutica**		**Referências**
	Estimulação/Administração	Supressão/Bloqueio	
TNF-α	Câncer	Câncer; Doenças inflamatórias intestinais (DII); Psoriase; Artrite reumatoide (AR)	SHARIF et al., 2020; LUCENDO et al., 2020; SANZ-MARTÍNEZ et al., 2019; KRISTENSEN et al., 2017
IFN-γ	Doença granulomatosa crônica; Adjuvante de anti-microbianos; Câncer, Infecções virais	Lúpus eritematoso sistêmico (SLE)	KAK; RAZA; TIWARI, 2018; KHODABANDEH et al., 2019; CASTRO et al., 2018; NOH, 2020; BOEDIGHEIMER et al., 2017
IL-2	Câncer; Doenças autoimunes (SLE, AR, esclerose múltipla); Doenças infecciosas	-	MORTARA et al., 2018; VALENCIA et al., 2020
IL-4	Psoriase	Dermatite atópica; Asma	HAHN; GHORESCHI, 2017; PHILIPS; SAMUEL, 2017
IL-6	Co-adjuvante em vacinas	Esclerodermia; AR; DII; câncer	GUO et al., 2017; KANG et al., 2019; KAUR et al., 2020; UNVER; MCALLISTER, 2018
IL-10	Psoríase; AR; DII; Câncer	SLE	WANG et al., 2019; SARAIVA et al., 2020
IL-17	-	Psoríase; Artrite Psoriaca; Espondilite anquilosante; AR	KURSCHUS; MOOS, 2017; MORALES et al., 2019

Fonte: Elaborada pela autora.

Adicionalmente, células imunes também podem ser moduladas (ALMEIDA et al., 2020), tanto aquelas da imunidade inata (macrófagos, células NK, células dendríticas) quanto

da imunidade adaptativa (linfócitos T e B) (CHEN et al., 2020). Imunomoduladores interferem com diversos mecanismos celulares, dentre eles: ativação, proliferação, diferenciação, sobrevivência e funcionalidade (p. ex. capacidade fagocítica) (REIJNDERS et al., 2020). Por exemplo, imunoestimulação de linfócitos T citotóxicos (CD8+) é essencial para a resposta imune do hospedeiro contra infecções virais (XU et al., 2019; QI et al., 2020) e parasitárias (RODRÍGUEZ et al., 2019) e respostas imunológicas anticâncer (VALLE et al., 2020). Por outro lado, sua supressão é necessária para reduzir a rejeição em transplantes (PHILLIPS; CALLAGHAN, 2020).

É crescente o interesse em imunoterapias direcionadas ao câncer, as quais usam várias abordagens para redirecionar ou hiperativar o sistema imunológico para o reconhecimento, restrição e morte de células cancerosas. Essas abordagens incluem neutralização de células imunossupressoras e desencadeamento da imunidade antitumoral como a polarização de macrófagos M1 e células Th1, por exemplo. Por outro lado, as terapias anti-inflamatórias no câncer têm se concentrado na supressão de sinais pró-inflamatórios, como o das citocinas (GRETEN; GRIVENNIKOV, 2019; SHALAPOUR; KARIN, 2019; FISHBEIN et al., 2020).

Considerando que os medicamentos usados hoje para o tratamento de doenças inflamatórias têm alto risco de efeitos colaterais e são caros (especialmente os de uso crônico), existe necessidade de desenvolver opções mais baratas, seguras e mais potentes. Os tecidos vegetais apresentam excelentes fontes de compostos imunomoduladores (CHEN et al., 2020; HARUN et al., 2020) e anti-inflamatórios (TASNEEM et al., 2019). Assim, a descoberta desses agentes em plantas medicinais, menos tóxicos, constitui uma alternativa às estratégias terapêuticas convencionais usadas (GRIGORE, 2017).

3.4.5 Atividades imunomoduladora e anti-inflamatória de lectinas de plantas

Na Imunologia, os glicanos são bem conhecidos por conduzir diversas funções celulares (ZHOU et al., 2018), assim, padrões de glicosilação específicos são requeridos para o funcionamento normal de muitas moléculas e células imunes (JOHANNSSEN; LEPENIES, 2017). Por exemplo, receptores de antígenos em linfócitos T (TCR) e B (BCR), moléculas do MHC, TLRs, citocinas e receptores de citocinas são glicoproteínas (PATRIOTA et al., 2019b). Lectinas de plantas podem ser usadas para desenvolver novas terapias para controlar doenças inflamatórias, pois algumas dessas proteínas têm se mostrado capazes de reconhecer esses padrões de glicosilação e modular o sistema imune inato e adaptativo, promovendo estimulação e/ou supressão em seus componentes (JANDÚ et al., 2017; PEREIRA et al., 2018; MISHRA

et al., 2019; PATRIOTA et al., 2019b). Sendo assim, as lectinas exercem ação imunomoduladora por meio da interação com porções glicídicas da superfície de células imunológicas. Estas interações desencadeiam a transdução de sinais para a produção de determinadas citocinas que induzem respostas imune eficientes (GUPTA et al., 2020). As lectinas podem, por exemplo, interagir com glicanos presentes em diferentes TLRs, tanto intracelulares como extracelulares, estimulando sua ativação e respectiva sinalização celular. Sendo, assim, consideradas agonistas naturais de TLRs (GUPTA et al., 2020).

Lectinas de plantas têm a capacidade de estimular ativação e diferenciação de células do sistema imunológico (BRITO et al., 2017; PATRIOTA et al., 2019b). Além disso, têm sido descritas como fatores mitogênicos, sendo capazes de induzir proliferação em várias células imunes (CARVALHO et al., 2018). Essas proteínas estimulam respostas pró-inflamatórias (WANG et al., 2019) ou anti-inflamatórias (SOUZA et al., 2016), e também, podem exibir induzir perfis Th1 (ZENG et al., 2019) e Th2 (KUMAR; VENKATESH, 2016) de citocinas. Sansone et al. (2016) relatam que o perfil de citocinas induzidas por lectinas pode variar de acordo com o modelo (*in vivo* ou *in vitro*), o tipo de célula imune e a via de administração. Na Tabela 4 estão descritas lectinas isoladas de plantas com atividade imunomoduladora.

Lectinas isoladas de plantas têm apresentado atividade anti-inflamatória em modelos experimentais *in vivo* de peritonite e edema de pata induzidos por carragenina. Lectina de sementes de *Parkia biglobosa* (PBL) reduziu a migração de leucócitos, atuando especificamente nos neutrófilos, e induziu a liberação de citocinas anti-inflamatórias em camundongos (SILVA et al., 2013). Lectina de sementes de *Canavalia boliviana* (Cbol) foi capaz de inibir a migração de neutrófilos e o edema de pata em ratos Wistar (BEZERRA et al., 2014). Lectina de sementes de *Bauhinia bauhinioides* (BBL) reduziu o edema de pata, a migração de leucócitos e a liberação de TNF-α em ratos Wistar (GIRÃO et al., 2015). Lectina isolada de sementes de *Mucuna pruriens* (MPLEC) mostrou atividade anti-edematogênica no edema de pata em camundongos Swiss (LACERDA et al., 2015). A lectina das folhas de *Bauhinia monandra* (BmoLL) reduziu a migração de leucócitos para o peritônio e o edema de pata em camundongos Swiss (CAMPOS et al., 2016).

A lectina de sementes de *Tetracarpidium conophorum* (TcSL) impediu a migração de leucócitos para o peritônio e reduziu o edema de pata em ratos Wistar (OLADOKUN et al., 2019). A lectina de sementes de *Lonchocarpus campestris* (LCaL) inibiu o edema de pata em camundongos Swiss (PIRES et al., 2019a). A lectina de semente de *Lonchocarpus araripensis* (LAL) reduziu a migração de leucócitos e edema de pata em ratos Wistar (PIRES et al., 2019b).

TABELA 4 – Atividade imunomoduladora de lectinas de plantas

Lectina (planta)	Tipo celular	Atividade imunomoduladora
Abrin (*Abrus precatorius*)	Esplenócitos de camundongo	Aumento de IFN-γ e IL-6 (resposta Th1), ativação de linfócitos T
ACA (*Allium cepa*)	Timócitos de camundongo	Aumento de IFN-γ e IL-2 (resposta Th1)
ALL (*Artocarpus lingnanensis*)	Linfócitos T humanos	Secreção de citocinas Th1/Th2, ativação e manutenção de homeostase de linfócitos T
AntinM (*Artocarpus heterophyllus*)	Macrófagos e células dendríticas de camundongo	Aumento de IL-2, IFN-γ e NO (resposta Th1), ativação de macrófagos
	Linfócitos T de camundongos	Efeito mitogênico
ApuL (*Alpinia purpurata*)	Células mononucleares de sangue periférico (PBMCs)	Aumento de IFN-γ, TNF-α e IL-6 (resposta Th1) e IL-17A (resposta Th17), NO e IL-10. Diferenciação e ativação de linfócitos T CD8 e CD4
BanLec (*Musa paradisiaca*)	Esplenócitos de camundongo	IFN-γ, IL-2 e TNF-α (resposta Th1)
	Linfócitos de camundongo	Aumento de IL-4 e IL-10
CFL (*Cratylia argentea*)	Macrófagos peritoneais de camundongo	Aumento de IL-6, IL-12 e IFN-γ
Cramoll (*Cratylia mollis*)	Linfócitos de camundongo	Aumento de IL-1β (pró-inflamatória)
	Esplenócitos de camundongo	Aumento de IFN-γ (resposta Th1), supressão de NO
	Esplenócitos de camundongo	Aumento de IFN-γ (resposta Th1), aumento de NO
	Células de exsudato peritoneal	Aumento de IL-1, IL-6, IFN-γ, TNF-α e NO
	Esplenócitos de camundongo	Aumento de IFN-γ, IL-6, IL- 17A, IL-22 e IL-23
	PBMCs humanos	Aumento de IL-6, IL-17A, IL-22 e IL-23
ConA (*Canavalia ensiformis*)	Esplenócitos de camundongo	Aumento de IFN-γ, IL-6 e NO
	In vivo (camundongos)	Aumento de TNF-α, INF-γ e IL-4 no soro
ConBr (*Canavalia brasiliensis*)	Macrófagos peritoneais de camundongo	Aumento de IL-1 β, TNF- α e IL-6
DLL (*Dolichos lablab*)	Linfócitos humanos e de camundongos	Aumento de IL-2, efeito mitogênico
Jacalin (*Artocarpus integrifolia*)	Macrófagos humanos	Aumento de TNF, IL-6, IL-12 e IL-1β (pró-inflamatória) e IL-10 (anti-inflamatória)
MvFL (*Microgramma vacciniifolia*)	PBMCs humanos	Aumento de TNF-α, IFN-γ, IL-6, IL-10 e NO, diferenciação e ativação de linfócitos T CD8
PHA (*Phaseolus vulgaris*)	Esplenócitos de camundongo	Aumento de IL-17
	Linfócitos humanos	Aumento de IFN-γ, IFN-α, IL-1α, IL-4, IL-8 e TNF-α
PTL (*Pinellia ternata*)	Macrófagos peritoneais de camundongo	Aumento de TNF-α, IL-1β e IL-6 (pró-inflamatória), ativação de macrófagos
VAA (*Viscum album*)	Células dendríticas humanas	Aumento de IFN-γ, IL-4, IL-13 e IL-17, ativação de linfócitos T CD4
VCA-B (*Viscum album* coloratum)	Células dendríticas de camundongo	Aumento de IL-1β, IL-6, IL-12 e TNF-α (resposta Th1), ativação de células dendríticas

Fonte: Traduzida de Patriota et al (2019b)

3.4.6 Atividades imunomoduladora e anti-inflamatória de inibidores de protease de plantas

Especificamente, mais de um terço das proteases humanas são serino-proteases. Em especial, as células imunológicas expressam uma ampla variedade de serino-proteases, que estão amplamente envolvidas nas respostas imunológicas e desempenham um papel importante na regulação da inflamação. Essas são enzimas versáteis, geralmente envolvidas na produção de citocinas pró-inflamatórias e na ativação de células imunes. Assim, sua desregulação durante a inflamação pode ter consequências indesejáveis e promover várias doenças (HEUTINCKA et al., 2010; AMIRI; SOUALMIA, 2017). Essas proteases são liberadas quando mediadores pró-inflamatórios induzem a ativação e degranulação celular (HEUTINCKA et al., 2010; ALMEIDA-REIS et al., 2017).

O microambiente extracelular de tecidos inflamatórios pode ser modulado por serino-proteases através da regulação de receptores específicos da superfície celular, como os TLRs ou receptores ativados por proteinase (PARs). Uma vez ativados por serino-proteases, PARs podem prolongar ou finalizar respostas induzidas por citocinas (HOLLENBERG et al., 2014; RAMACHANDRAN et al., 2016; AMIRI; SOUALMIA, 2017). Por exemplo, quando ativado pela tripsina, o PAR-1 leva à produção de várias citocinas pró-inflamatórias (SHIGETOMI et al., 2010), e o PAR-2 ativa células inflamatórias (SHAMSI et al., 2018).

Ainda, as serinoproteases são elementos-chave da resposta à inflamação devido à sua liberação de leucócitos ativados durante a sua degranulação. As células imunes expressam uma ampla variedade de serino-proteases, como granzimas em linfócitos citotóxicos; elastase, catepsina G e proteinase 3 em neutrófilos; e quimase e triptase em mastócitos. Durante a inflamação, estas proteases são produzidas em grandes quantidades como parte do mecanismo de defesa, mas a sua liberação descontrolada pode causar lesões graves nos tecidos (HEUTINCKA et al., 2010; ALMEIDA-REIS et al., 2017).

Dessa forma, os inibidores de protease podem ter efeitos imunomoduladores e anti-inflamatórios significativos, por exemplo, na redução de citocinas pró-inflamatórias, inibição de ativação celular, e inibição da liberação ou das próprias proteases secretadas durante a inflamação. Assim, um grande esforço tem sido dedicado ao desenvolvimento de inibidores de protease, especialmente de serinoproteases, para uso terapêutico na supressão da resposta inflamatória (AMIRI; SOUALMIA, 2017; HELLINGER; GRUBER, 2019; CLEMENTE et al., 2019; COTABARREN et al., 2020). A atividade imunomoduladora de inibidores de protease de plantas está descrita na Tabela 5.

TABELA 5 – Atividade imunomoduladora de inibidores de protease de plantas

Inibidor de protease (planta)	**Tipo celular**	**Atividade imunomoduladora**	**Referências**
Ancordin (*Anredera cordifolia*)	Macrófagos RAW 264.7	Aumento de NO	CHUANG; LIN; HOU, 2007
BvvTI (*Bauhinia variegata*)	Esplenócitos de camundongos BALB/c	Aumento de IL-1β, IL-2 e TNF-α	FANG et al., 2010a
BBI (*Glycine max*)	Macrófagos RAW 264.7 estimulados por LPS	Redução de TNF-α, IL-1β, IL-6 e aumento de IL-10	LI et al., 2011
		Redução de NO	DIA; BERHOW; GONZALEZ, 2008
	Esplenócitos de camundongo C57BL/6	Aumento de IL-5 e IL-10	TOUIL et al., 2008
KBTI (*Glycine max*)	Esplenócitos de camundongos BALB/c	Aumento de expressão de TNF-α, IL-1β, IL-2 e INF-γ	FANG et al., 2010b
NLTI (*Nephelium lappaceum*)	Macrófagos peritoneais de camundongo BALB/c	Aumento de NO	FANG; NG, 2015
MCoCI (*Momordica cochinchinensis*)	Esplenócitos e linfócitos de camundongo BALB/c	Estímulo de proliferação e diferenciação	TSOI; NG; FONG, 2006
SAPI (*Solanum aculeatissimum*)	Macrófagos RAW 264.7 estimulados por LPS	Redução de NO, TNF-α, IL-1β e IL-6 através de um bloqueio das vias NF-KB e MAPK	KRISHNAN; MURUGAN, 2017

Fonte: Elaborada pela autora.

Os inibidores de protease têm apresentado atividade anti-inflamatória *in vivo*. Inibidores de protease de origem vegetal têm sido avaliados quanto ao efeito anti-inflamatório em modelos de edema. O inibidor de protease isolado das sementes de *Leucaena leucocephala* (LlTI) diminuiu o edema de pata induzido por carragenina ou calor em ratos Wistar (OLIVA et al., 2000). O inibidor de sementes de *Bauhinia bauhinioides* (BbCI) também reduziu o edema de pata induzido por carragenina em ratos Wistar, com forte efeito inibitório na migração de leucócitos nos tecidos inflamados (OLIVEIRA et al., 2010).

Ainda, inibidores de protease de planta têm sido eficazes em outros modelos de inflamação aguda. Por exemplo, o inibidor de tripsina de sementes de *Erythrina velutina* (EvTI) mostrou efeito anti-inflamatório ao reduzir a infiltração de neutrófilos em um modelo experimental de sepse em camundongos Swiss (MACHADO et al., 2013). O BbCI reduziu a migração de neutrófilos para a cavidade pleural em um modelo de pleurisma induzido por carragenina em ratos Wistar albinos (OLIVEIRA et al., 2010). Inibidores de protease nativo e

recombinante de sementes de *Caesalpinia echinata*, CeKI e rCeEI respectivamente, foram capazes de diminuir a infiltração de leucócitos no espaço alveolar em modelo de inflamação pulmonar induzida por LPS em ratos Wistar (CRUZ-SILVA et al., 2016).

Adicionalmente, essas proteínas também têm sido efetivas em modelos de inflamação crônica. O inibidor de tripsina de sementes de *Enterolobium contortisiliquum* (EcTI) atenuou o número de eosinófilos e a inflamação nas vias aéreas em um modelo de asma alérgica induzida por ovalbumina em camundongos BALB/c (RODRIGUES et al., 2019). Além disso, reduziu o número de leucócitos no líquido da lavagem broncoalveolar (LBA) em um modelo de inflamação pulmonar induzida por elastase em camundongos C57Bl/6 (THEODORO-JÚNIOR et al., 2017). Igualmente, BbCI diminuiu o número de leucócitos, incluindo neutrófilos, no LBA e nos septos alveolares em um modelo de enfisema pulmonar induzido por elastase em camundongos C57Bl/6 (ALMEIDA-REIS et al., 2017). Similarmente, o inibidor de calicreína de sementes de *Bauhinia bauhinioides* recombinante (rBbKI) diminuiu o número de neutrófilos, linfócitos e eosinófilos no LBA em um modelo de enfisema pulmonar induzido por elastase em camundongos C57Bl/6 (MARTINS-OLIVERA et al., 2016). EcTI, BbCI e rBbKI atenuaram TNF-α e reduziram o número de células positivas para iNOS nos respectivos modelos de inflamação. Ainda, a lectina de entrecasca *Crataeva tapia* (CrataBL), que é uma glicoproteína bifuncional que exibe atividade de lectina e inibidora de tripsina, foi capaz de controlar a inflamação em um modelo animal de inflamação pulmonar alérgica, reduziu o reduziu o número de eosinófilos, células NFκB positivas e células positivas para iNOS e os níveis de citocinas inflamatórias Th1/Th2/Th17 (BORTOLOZZO et al., 2018).

Além disso, inibidor do tipo Bowman-Birk de *Glycine max* diminuiu a infiltração de células inflamatórias através da barreira hematoencefálica e inflamação no modelo experimental de encefalomielite auto-imune (EAE) de esclerose múltipla em camundongos C57BL/6 (DAI et al., 2011). Ainda, vários estudos relataram que os inibidores de protease diminuem a resposta inflamatória nas doenças inflamatórias intestinais (CLEMENTE; SONNANTE; DOMONEY, 2011).

3.5 AVALIAÇÃO DA TOXICIDADE DE LECTINAS E INIBIDORES DE PROTEASE

Os compostos naturais têm sido frequentemente sugeridos como novas estratégias terapêuticas alternativas, para complementar ou substituir as abordagens existentes da medicina convencional (BONCLER et al., 2017). Porém, muitas pessoas assumem que produtos naturais não são tóxicos e não consideram possíveis efeitos adversos do uso inadequado e da ingestão

irrestrita (KHARCHOUFA et al., 2018). Ao contrário, alguns compostos de plantas têm apresentado toxicidade para os mais diversos órgãos vitais, tais como fígado, rim, coração, entre outros (HUDSON et al., 2018). Além disso, muitos estudos mostram que as plantas medicinais são capazes de produzir uma ampla gama de reações indesejáveis e adversas, como carcinogenicidade, teratogenicidade, condições de risco de vida e até morte (KRISTANC; KREFT, 2016). Dessa forma, requerem investigação e pesquisa adicionais para entender melhor a causa dos efeitos adversos e atenuar e prevenir tais eventos (HUDSON et al., 2018; CHEBAIBI et al., 2019). No entanto, apesar dos esforços para melhorar a descoberta e o desenvolvimento de medicamentos, poucas plantas medicinais foram exploradas e examinadas quanto a ações toxicológicas (KALE; AWODELE; AKINDELE, 2019). Dessa forma, antes que esses novos medicamentos sejam lançados no mercado, eles devem passar por testes rigorosos e longos para provar sua segurança e eficácia, independentemente de terem origem natural ou sintética (HUDSON et al., 2018).

Para lidar com uma diversidade de condições desfavoráveis, as plantas desenvolveram adaptações evolutivas, tais como a elaboração de estratégias sofisticadas de defesa e a síntese de uma grande diversidade de compostos bioativos, alguns dos quais são tóxicos e prejudiciais aos seres humanos (MAAG et al., 2015; HUNTER et al., 2018). Portanto, em virtude de seus constituintes químicos, algumas plantas são potencialmente tóxicas, e seu consumo ou de seus compostos pode causar inclusive envenenamento (CHEBAIBI et al., 2019; MENSAH et al., 2019; SINGH, 2020). Dentre os diferentes compostos tóxicos relatados em plantas estão alguns metabólitos secundários de classes como alcaloides, terpenoides, taninos e flavanóides (BONCLER et al., 2017; GÜNTHARDT et al., 2018). Além disso, existe dentre os metabólitos primários das plantas o já mencionado arsenal de proteínas, tais como lectinas, proteínas inativadoras de ribossomos (RIPs) e inibidores de protease que apresentam uma toxicidade útil para a planta em sua defesa contra insetos herbívoros e patógenos (LANNOO; VAN DAMME, 2014; DANG; VAN DAMME, 2015).

As lectinas foram inicialmente conhecidas por sua toxicidade devido à identificação da ricina, uma lectina tóxica presente no extrato de sementes de *Ricinus communis* (LORD et al., 2003). Lectinas tóxicas para células normais humanas têm sido descritas (FRANZ et al., 2006; ARAÚJO et al., 2013; TAUBENSCHMID et al., 2017). Por outro lado, outras lectinas e inibidores de protease que têm apresentado citotoxicidade para células tumorais ou para células de patógenos também têm se mostrado atóxicos para células normais humanas ou animais nas doses em que apresentam suas bioatividades (PATRIOTA et al., 2016; BATISTA et al., 2017;

PATRIOTA et al 2017; PROCÓPIO et al., 2018; SANTOS et al., 2020). Além disso, estudos de toxicidade *in vivo* confirmaram a eficácia e segurança de lectinas e inibidores de protease nas doses terapêuticas, especialmente em animais (KRISHNAN & MURUGAN, 2016; MAYASA et al., 2016; RAMOS et al., 2019).

A avaliação de toxicidade abrange o estudo dos efeitos adversos dos produtos químicos nos organismos vivos, ou componente subestrutural de um organismo, como a célula (citotoxicidade) ou órgão (organotoxicidade) (MENSAH et al., 2019). A aplicação de métodos eficazes para medir e prever toxicidade é essencial, pois essa é a principal razão para a alta taxa de falha de medicamentos (SUMANTRAN, 2011). Portanto, considerando sua heterogeneidade química e potenciais efeitos multidirecionais, as características toxicológicas de compostos de plantas devem ser apoiadas por análises multiparamétricas nas quais seja possível revelar quaisquer efeitos prejudiciais (BONCLER et al., 2017). Essas análises têm sido feitas em ensaios *in vitro* e *in vivo*.

Uma vez que um fator crítico na Toxicologia é o metabolismo *in vivo*, algumas substâncias que não possuem toxicidade inicialmente podem produzir metabólitos tóxicos após serem expostas a enzimas hepáticas, enquanto outras substâncias que são tóxicas *in vitro* podem deixar de ser. Outros fatores, como a capacidade da substância de penetrar no tecido e a depuração e excreção do produto, não podem ser aferidos pelo uso do modelo celular (MCGAW; ELGORASHI; ELOFF, 2014; MENSAH et al., 2019).

Os estudos de toxicidade *in vivo* podem ser classificados como agudos, subagudos/subcrônicos e crônicos, dependendo da quantidade e duração da administração dos agentes (DENNY & STEWART, 2013). Os estudos toxicológicos agudos investigam os efeitos tóxicos produzidos por uma única exposição de grandes doses a um tóxico com duração não superior a 24 h. Exposição crônica refere-se à administração da droga por um longo período de tempo, geralmente medido em meses ou anos. Períodos de exposição entre aguda e crônica podem ser referidos como subagudos ou subcrônicos, respectivamente (MENSAH et al., 2019). Plantas com toxicidade aguda substancial já são geralmente reconhecidas como perigosas devido aos incidentes históricos de envenenamentos. Por outro lado, a toxicidade subaguda e crônica são mais difíceis de detectar no uso tradicional (KRISTANC & KREFT, 2016).

Além da observação da mortalidade, para avaliar a toxicidade de qualquer novo composto é essencial verificar as mudanças de comportamento, peso, parâmetros bioquímicos, hematológicos e histopatológicos. Dessa forma, é possível aferir o estado geral de funcionamento do organismo e dos órgãos vitais (KALE; AWODELE; AKINDELE, 2019;

KPEMISSI et al., 2020). O estado dos órgãos vitais, especialmente as funções hepática e renal são cruciais (PARÉ et al., 2019).

A capacidade de proteínas de plantas de causar toxicidade tem sido avaliada em modelos experimentais *in vivo*. Toxicidade oral aguda (50–1000 mg/kg) e toxicidade oral subcrônica (500 e 1000 mg/kg) de inibidores de protease dos frutos de *Solanum aculeatissimum* foram avaliados em camundongos Albino e ratos Wistar, respectivamente; não foram observadas alterações sugestivas de toxicidade (KRISHNAN; MURUGAN, 2016). Além disso, inibidores de protease parcialmente purificados de *Glycine max* foram avaliados quanto à toxicidade oral aguda (2000 mg/kg) e toxicidade oral subcrônica (200 mg/kg) em ratos Wistar. A administração subcrônica levou a efeitos antinutricionais nos ratos (MAYASA et al., 2016).

Lectina isolada de sementes de *Tetracarpidium conophorum* foi avaliada quanto a toxicidade aguda oral (500–2500 mg/kg) e intraperitoneal (10–600 mg/kg) em camundongos Wistar albino e não causou toxicidade observável por via oral; no entanto, quando administrado por via intraperitoneal, provocou toxicidade dose-dependente (KUKU et al., 2012).

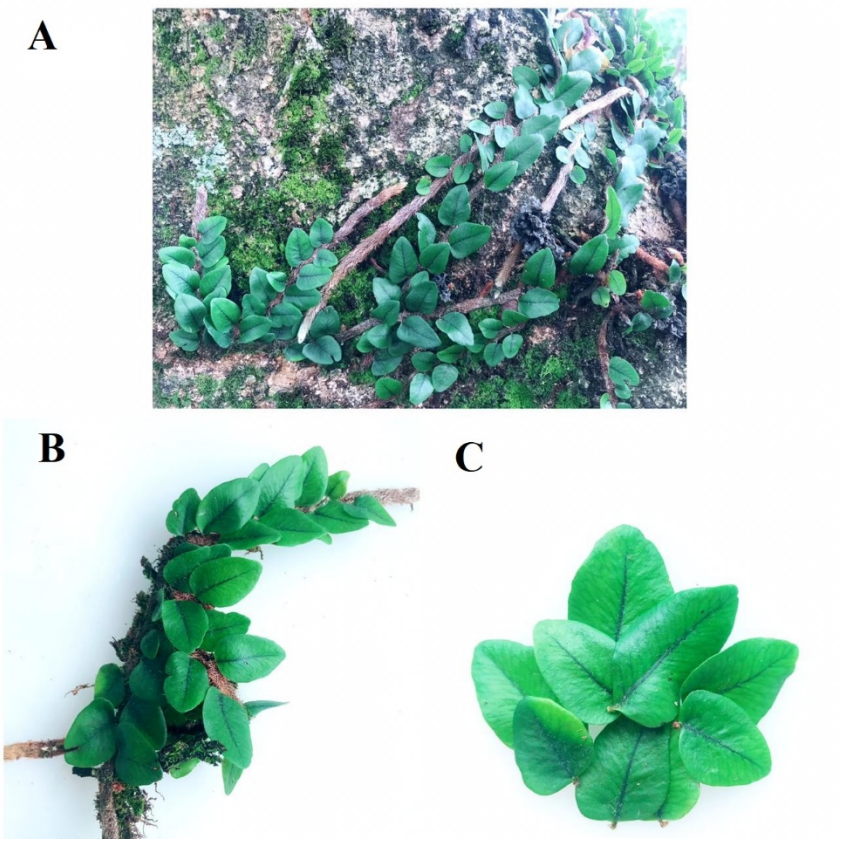
3.6 *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel.

A família Polypodiaceae apresenta aproximadamente 56 gêneros e 1.200 espécies (SMITH et al., 2006), sendo considerada uma das maiores famílias de plantas pteridófitas, constituindo-se principalmente de espécies epífitas (PERES et al., 2009). O gênero *Microgramma* abrange cerca de 30 espécies (TERCEIRO et al., 2012). Dentre as espécies deste grupo de plantas, destaca-se a *Microgramma vacciniifolia*, a qual é encontrada como corticícola e/ou rupícola e facilmente reconhecida por seu caule longo intensamente revestido por escamas e frondes (folhas) dimorfas (as estéreis são ovais e as férteis, lanceoladas) (Figura 11) (PERES et al., 2009; VILLELA et al., 2019). Sua incidência é observada na América Central (Antilhas), América do Sul (Venezuela, Colômbia, Paraguai, Argentina e Brasil) e, eventualmente, na América do Norte (PRADO; HIRAI; SCHWARTSBURD, 2010; GURJAÚ et al., 2011).

Em estudos florísticos realizados no Brasil, *M. vacciniifolia* ocorre em distintas formações vegetacionais (Cerrado, Floresta Atlântica Nordestina e Floresta Atlântica do Sul-Sudeste) e nos estados de Pernambuco, Bahia, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul (PRADO; HIRAI; SCHWARTSBURD, 2010; GURJAÚ et al., 2011). *M. vacciniifolia* encontra-se no interior de florestas úmidas, nas margens de trilhas, nas encostas de matas e também em paredões rochosos, possui uma ampla valência ecológica (capacidade de uma

espécie de povoar meios) para diferentes fatores microclimáticos e é capaz de suportar ambientes com distintos estados de conservação (GONÇALVES et al., 2012).

FIGURA 11 – *Microgramma vacciniifolia*. (A). Hábito da planta. (B) Rizoma e fronde. (C) Frondes.



Fonte: Elaborada pela autora.

A *M. vacciniifolia* é conhecida popularmente por "polipódio vacinifólio", "erva-silvina" ou "erva-teresa", e apresenta propriedades medicinais tais como: adstringente, sudorífera, expectorante, tratamento de cólicas intestinais, diarreias e disenterias, infecções respiratórias, tosse, bronquites, coqueluche e laringite. Sendo, também, empregada no tratamento de hemoptises, em casos de hematúria e em edemas (SANTOS; SYLVESTRE, 2006; AGRA et al., 2008; PERES et al., 2009; TERCEIRO et al., 2012; NUNES et al., 2020).

Metabólitos secundários têm sido identificados tanto em rizomas como em frondes de *M. vacciniifolia*. Estudo fitoquímico do extrato hidroalcoólico do rizoma mostra presença de polifenóis, flavonoides, derivados cinâmicos, terpenos, esteróides, saponinas e açúcares redutores (FERREIRA et al., 2020). Enquanto estudo fitoquímico do extrato hidroalcoólico das frondes apresenta taninos, flavonoides, derivados cinâmicos, terpenos, esteróides, saponinas e

açúcares redutores (SIQUEIRA et al., 2020). Estudo fitoquímico anterior com o extrato etanólico bruto das frondes de *M. vacciniifolia* identificou os esteróides β-sitosterol e hopan-22-ol, a flavona glicosilada 6-metoxiapinenina-7-O-β-D-alopiranosídeo e uma mistura de ésteres graxos. Esse extrato apresentou potencial antioxidante, antimicrobiano e alelopático (PERES et al., 2009). Ainda, a análise histoquímica mostra que os principais compostos encontrados nas frondes são alcalóides, compostos fenólicos, lignina, lipídios e flavonoides (VILLELA et al., 2019).

Albuquerque et al. (2012) isolaram uma lectina do rizoma de *M. vacciniifolia* (MvRL), uma proteína termoestável com capacidade de ligação à quitina, de aproximadamente 17 kDa e dependente de cátions (Ca^{+2} e Mg^{+2}). MvRL apresentou atividade inseticida contra *Nasutitermes corniger* e *Sitophilus zeamais*, atividade antifúngica contra *Fusarium oxysporum* f.sp. *lycopersici*, atividade citotóxica contra células NCI-H292 (carcinoma de pulmão) e atividade moluscicida contra *Biomphalaria glabrata* (ALBUQUERQUE et al., 2012, 2014a, 2014b, 2020; SANTANA et al., 2012). Recentemente, Silva et al. (2020) verificaram a toxicidade aguda do extrato salino do rizoma de *M. vacciniifolia* e fração rica em MvRL em camundongos e não identificaram LD_{50}, apesar da presença de alguns sinais de toxicidade. Além disso, também não foi observada genotoxicidade.

Uma lectina foi isolada da fronde de *M. vacciniifolia* (MvFL), com especificidade de ligação para oligossacarídeos (glicoproteínas fetuína e ovoalbumina). MvFL é uma glicoproteína de massa molecular nativa de 54 kDa, pI 4,52, termoestável, sendo considerada uma proteína multifuncional por apresentar tanto atividade lectínica quanto inibidora de tripsina. MvFL possui propriedades imunomoduladoras em células mononucleares do sangue periférico humano (PBMCs), induzindo a liberação de TNF-α, IFN-γ, IL-6, IL-10 e NO e promovendo a ativação e diferenciação de células T CD8+ (PATRIOTA et al., 2017). Esses resultados estimularam a avaliação das atividades antitumoral e anti-inflamatória de MvFL.

3.7 *Moringa oleifera* Lam.

Moringa oleifera, conhecida popularmente como "lírio branco", "quiabo de quina", "moringa" ou "árvore milagrosa", pertence à família das Moringaceae (LEONE et al., 2015). A moringa é uma planta bastante tolerante e bem adaptada para uma ampla gama de adversidades ambientais, tendo distribuição pan-tropical e se desenvolvendo bem em regiões semiáridas (HASSAN; IBRAHIM, 2013). Apesar de ser nativa da Índia (seu maior produtor comercial) e outros países asiáticos, essa planta tem sido amplamente cultivada nos trópicos de

todo o mundo, como África, América Central e América do Sul, onde tem se adaptado com sucesso (SANTOS et al., 2015; SINGH et al., 2020).

M. oleifera desperta grande interesse devido aos seus extensivos atributos nutricionais e medicinais (SANTOS et al., 2015; SINGH et al., 2020). Todos os seus tecidos têm sido descritos como fontes de compostos primários e secundários com as mais variadas atividades biológicas, dentre elas: atividades antimicrobiana, antioxidante, imunomoduladora, inseticida, hepatoprotetora, hipoglicemiante e neuroprotetora (LEONE et al., 2015; KOU et al., 2018; MA et al., 2019; PADAYACHEE; BAIJNATH, 2019; SINGH et al., 2020), incluindo também atividades anti-inflamatória (ARAÚJO et al., 2013; ALHAKMANI; KUMAR; KHAN, 2013; MINAIYAN et al., 2014; SURESH et al., 2020) e antitumoral (KHALIL et al., 2014; KRISHNAMURTHY et al., 2015; BARHOI et al., 2020).

As flores de *M. oleifera* têm pétalas branco-amareladas desiguais (SINGH et al., 2020) (Figura 12) e são utilizadas para fins medicinais como agente colagogo, diurético e para o tratamento de ascite, diarreia, hipertensão, infecções, resfriados, feridas/úlceras, doenças musculares e cólera (ANWAR et al., 2007; PADAYACHEE; BAIJNATH, 2019). Além disso, são usadas para tratar inflamações e tumores (ANWAR et al., 2007; MAHAJAN; MEHTA, 2009; MEHTA et al., 2011).

FIGURA 12 – Flores de *Moringa oleifera*.

Fonte: https://www.moringa.blog.br/flor-da-moringa/

Extratos das flores de *M. oleifera* mostraram ação anti-inflamatória no método de desnaturação de proteínas *in vitro* (ALHAKMANI; KUMAR; KHAN, 2013) e atividade antitumoral contra linhagem celular de câncer de próstata humana (PC‑3) mediada pela indução de apoptose (JU et al., 2018). Além disso, possuem atividade antioxidante (GIMENIS et al.,

2018), antidiabética (PADAYACHEE; BAIJNATH, 2019), ação moluscicida contra embriões e adultos da espécie *Biomphalaria glabrata* (ROCHA-FILHO et al., 2015) e ação inseticida sobre *Aedes aegypti* (PONTUAL et al., 2012a), Ainda, fração proteica de flores foi ativa contra bactérias gram-negativas e gram-positivas (MOURA et al., 2011) e também apresenta propriedades caseinolítica e coagulante de leite (PONTUAL et al., 2012b).

Além de metabólitos secundários como taninos e flavonoides (GIMENIS et al., 2018; PADAYACHEE; BAIJNATH, 2019), as flores de *M. oleifera* contém um inibidor de tripsina chamado MoFTI (*M. oleifera flower trypsin inhibitor*), que possui massa molecular de 18,2 kDa e apresenta atividade larvicida contra *Aedes aegypti* (PONTUAL et al., 2014) e tripanocida sobre *Trypanosoma cruzi*; nesse último caso, o inibidor apresentou altos índices de seletividade para células do parasita em comparação com macrófagos peritoneais murinos e células Vero (índices de 7,9 e >12, respectivamente) (PONTUAL et al., 2018).

Nova et al. (2020) mostraram que MoFTI não afeta a viabilidade de células mononucleares do sangue periférico humano (PBMCs), bem como foi capaz de modular a resposta de PBMCs infectados por *T. cruzi*, estimulando produção de TNF-α, IFN-γ e NO, bem como da citocina anti-inflamatória IL-10, sugerindo que esta molécula pode ser capaz de controlar a parasitemia enquanto regula a inflamação. Patriota et al. (2021) avaliaram a toxicidade aguda de MoFTI (300 mg/kg, i.p.) e mostraram que esse inibidor não causou morte ou quaisquer efeitos adversos nos camundongos, exceto contorções abdominais em 15-30 min após a administração. O estudo também mostrou que MoFTI não exibiu um efeito hemolítico, nem induziu apoptose ou necrose em esplenócitos de camundongos. Estudo de imunomodulação revelou que MoFTI induz a liberação de TNF-α, IFN-γ, IL-6, IL-10 e NO pelos esplenócitos. Os autores concluíram que esses resultados estimulam a avaliação contínua do potencial antitumoral do MoFTI e seus efeitos em outros alvos imunológicos.

4 RESULTADOS

Os resultados dessa pesquisa são apresentados na forma de artigos.

4.1 ATIVIDADE ANTITUMORAL DE MvFL

Os resultados da avaliação da atividade antitumoral de MvFL em camundongos portadores de sarcoma 180 estão incluídos em artigo publicado no periódico *Phytomedicine Plus*, podendo ser acessado pelo link: https://doi.org/10.1016/j.phyplu.2020.100013. Esse artigo também traz os resultados da avaliação toxicológica dos tratamentos com a lectina.

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Microgramma vacciniifolia frond lectin (MvFL) exhibits antitumor activity against sarcoma 180 in mice

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ABSTRACT

In this study, we evaluated the antitumor activity of a lectin from *Microgramma vacciniifolia* frond (MvFL) in sarcoma 180-bearing mice. First, the *in vitro* cytotoxicity of MvFL on sarcoma 180 cells was evaluated. Tumor cells were inoculated into Swiss female mice, and after 7 days, the animals were treated intraperitoneally with phosphate-buffered saline (PBS, negative control), methotrexate (MTX, positive control, 1.5 mg/kg) or MvFL (10 or 20 mg/kg) for 7 days. We analyzed tumor weight, angiogenesis, relative water and food consumption, body weight, and hematological, biochemical, and histological parameters. MvFL inhibited cell growth (IC_{50}: 20.6 μg/mL) and induced apoptosis and necrosis. Treatment with MvFL led to an 89.2–96.8% reduction in tumor weight. Histopathological analysis of tumors of animals treated with MvFL revealed necrotic points, presence of leukocyte infiltrates, and a smaller number of pleomorphic cells than negative control. MvFL interfered with the angiogenesis around the sarcoma 180 tumors. No changes in water and food consumption or weight gain were observed. There were no important changes in blood hematological and biochemical parameters in lectin-treated animals. The liver, kidneys, and spleen did not show signs of toxicity in all groups. In conclusion, MvFL has strong antitumor potential against sarcoma 180 models, without toxic effects.

1. Introduction

Overall, conventional therapies currently used for the treatment of cancer are overly aggressive, causing various side effects; in addition, the number of reports of tumors resistant to traditional therapies is increasing (Pan et al., 2016). The World Health Organization (2020) defines 'sarcoma' as a heterogeneous group of cancers stemming from mesenchymal tissues, which can be classified into soft-tissue or bone sarcomas and further divided into more than 100 subtypes. The sarcomas account for over 20% of all pediatric solid malignant cancers and around 1% of all adult solid malignant cancers (Burningham et al., 2012). Curative treatments in sarcomas are rare, with 50% of patients eventually relapsing with incurable metastatic disease (Singhi et al., 2018). The development of resistance to conventional chemotherapy is a major cause of treatment failure and tumor recurrence in patients affected by sarcomas (Lin et al., 2020). Therefore, identifying new molecules to treat these tumors is essential for improve the therapeutic and prognosis. Sarcoma 180 (also known as Crocker tumor) is an animal model of tumor universally used for the evaluation of anticancer drugs *in vivo* (Gao et al., 2017).

Lectins are proteins capable of binding specifically and reversibly to carbohydrates. In tumors, aberrant glycosylation patterns can be recognized by lectins, and they can distinguish subtle alterations in the

Abbreviations: ALT, and alanine aminotransferase; AST, aspartate aminotransferase; IC_{50}, concentration of sample that reduces cell viability by 50%; IL, interleukin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MTX, methotrexate; MvFL, *Microgramma vacciniifolia* frond lectin; NO, nitric oxide; PBS, phosphate-buffered saline; RDW, red cell distribution width; SteLL, *Schinus terebinthifolia* leaf lectin; TNF, tumor necrosis factor.

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glycans (Varki et al., 2017). Some lectins have higher affinity for carbohydrates found in cancer cells than for those present in normal cells (Ahmed et al., 2017). This ability of lectins to decipher the glycocode of cancer cells enables their application in cancer therapy (Kaltner et al., 2018).

Lectins have demonstrated an antiproliferative effect against various cancer cell lines *in vitro* and exerted antitumor action on *in vivo* cancer models, including sarcoma 180, which suggest them as promising anticancer agents for future cancer therapy (Mazalovska and Kouokam, 2020). These proteins may act by inducing cell death pathways, such as apoptosis and necrosis (Patriota et al., 2019). Also, lectins can directly affect anticancer immunity through glycan-mediated interactions (Mantuano et al., 2020). Thus, research aspects of developing lectins as anticancer agents can also consider assessments of immunomodulatory effects (Mazalovska and Kouokam, 2020).

Microgramma vacciniifolia (Polypodiaceae) fronds contain a multifunctional protein called MvFL, which exhibits both trypsin inhibitor ability and lectin activity. MvFL is an acidic protein (isoelectric point 4.51) with a molecular mass of 54 kDa with sequence similarities to RNA-binding proteins by tandem mass spectrometry (MS/MS). MvFL was shown to be a glycoprotein whose carbohydrate moiety contains glucose and/or mannose residues since this lectin adsorbed on an affinity matrix containing immobilized concanavalin A. MvFL possess immunomodulatory properties on human lymphocytes, inducing the release of tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), interleukin (IL) 6, IL-10, and nitric oxide (NO). In addition, it promotes the activation and differentiation of T $CD8^+$ cells (Patriota et al., 2017). It has been reported that clonal expansion and activation of T $CD8^+$ lymphocytes can be useful in therapies for diseases such as cancer (Jackson et al., 2014).

The well-recognized anticancer potential of plant lectins, along with previous reports on the immunomodulatory action of MvFL, lays a strong foundation for evaluating the *in vivo* antitumor activity of this lectin. In this study, we investigated the antitumor activity of MvFL in mice bearing the sarcoma 180 tumor. We also evaluate the toxicity of the lectin treatment.

2. Materials and methods

2.1. Isolation of MvFL

Fronds of *M. vacciniifolia* were collected at Recife, Brazil (8°02′56.3″S 34°56′49.8″W), with authorization (number 36,301) from the *Instituto Chico Mendes de Conservação da Biodiversidade*. Access was recorded (A9D147B) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado*. Taxonomic identification was confirmed at the herbarium Dárdano de Andrade Lima (*Instituto Agronômico de Pernambuco*, Recife), where a voucher specimen (number 63,291) was archived. The fronds were washed with tap water and then distilled water. Samples were dried (at 28 °C) for 7 days before being powdered by a blender. Proteins were extracted and MvFL was isolated according to the protocol described by Patriota et al. (2017).

2.2. Protein concentration and hemagglutinating activity

Protein concentration was determined according to Lowry et al. (1951) using a standard curve of bovine serum albumin (31.25–500 μg/mL). The carbohydrate-binding ability of lectin was identified by the hemagglutination assay as described by Patriota et al. (2017) using rabbit erythrocytes fixed with glutaraldehyde (Bing et al., 1967).

2.3. Evaluation of in vitro cytotoxicity to sarcoma 180 cells

Sarcoma 180 cells were collected from the peritoneal cavity of ascitic tumor-bearing mice. Briefly, 2 mL of ascitic fluid were withdrawn from the peritoneal cavity using an insulin syringe, washed with PBS, and centrifuged at 100 *g* for 3 min. The supernatant was discarded, and the cells were resuspended in RPMI 1640 medium with HEPES (Cultilab, Campinas, Brazil), supplemented with 10% (w/v) fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA). Next, cell viability was evaluated as described by Ramos et al. (2019). MvFL or methotrexate (MTX, Sigma-Aldrich) was resuspended in PBS and added to the wells in order to reach final concentrations ranging between 1.56 and 400 μg/mL. PBS was used as a negative control. The plates were incubated for 24 h at 37 °C and the number of viable cells was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation, WI, USA) according to the manufacturer's instructions. After 4 h of incubation, the absorbance at 492 nm was recorded and the percentage of viable cells was calculated by comparing with the negative control. The concentration of sample that reduces cell viability by 50% (IC_{50}) was calculated by non-linear regression analysis using the software GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

2.4. Analysis of cell death

The cells were cultured for 24 h in the absence (control) or the presence of MvFL at 6.25–100 µg/mL. MTX (20 µg/mL) was used as a positive control. After centrifuging (450 *g* at 4 °C for 10 min) and discarding the supernatant, 1.0 mL of PBS was added to the pellet and the cells were resuspended and centrifuged again. The occurrence of apoptosis or necrosis was analyzed using the FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's instructions. The cells were analyzed in a BD Accuri C6 cytometer (BD Biosciences, San José, USA) and 20.000 events were recorded for each sample. The results were analyzed using BD Accuri C6 Software. Cells stained with propidium iodide were considered necrotic while those stained with annexin V were considered to be in apoptosis. Double staining was recorded as necrosis or late apoptosis and double negatives were considered viable cells.

2.5. Evaluation of in vivo antitumor activity

2.5.1. Treatment of animals

The antitumor activity was evaluated using 6–8-week-old Swiss female mice. All the experimental procedures with mice were approved by the Ethics Committee on Animal Use of the *Universidade Federal do Vale do São Francisco* (authorization no. 0001/121218). The housing was maintained at 24 °C with a 12:12 light/dark photoperiod with unlimited water and food. The mice were divided into five groups composed of six animals. The sarcoma 180 cell suspension in PBS (5×10^6 cells/100 μL) was subcutaneously inoculated on the back of each mouse, except those in the Sham group. From the 8th day after inoculation, the treatments were administered intraperitoneally and lasted for 7 days. The Sham and negative control groups received PBS. The positive control group was treated with 1.5 mg/kg methotrexate. One test group was treated with 10 mg/kg MvFL. The other test group received 20 mg/kg body weight (bw). The weight of the animals and their food consumption were measured daily. Relative water and food consumption values were calculated using the following formula: Relative consumption = food consumed (g) or water consumed (mL) / body weight (g) × 100. After the end of treatments, the mice were euthanized using 100 mg/kg ketamine and 10 mg/kg xylazine (i.p.). The tumor, lungs, liver, kidneys, spleen, heart, and stomach of each animal was removed and weighed. The relative weight of each organ was calculated using the formula: Relative weight = organ weight (g) / body weight (g) × 100.

2.5.2. Blood hematological and biochemical analysis

Before euthanasia, the mice were subjected to brachial blood collection into EDTA-treated tubes for the hematological analysis or dry tubes for the biochemical assays (to separate the sera). Global leucometry, counting of erythrocytes, determination of hematocrit, and

measurements of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW) were carried out using the HEMATOCLIN 2.8 VET (Bioclin Belo Horizonte, Brazil). Specific kits for the detection of urea, creatinine, glucose, cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were employed to determine the serum levels of these substances. The protocols provided by the manufacturer were followed precisely (Labtest, Vista Alegre, Brazil).

2.5.3. Evaluation of antiangiogenic activity

To evaluate the vascularization around the tumors, the skin was dissected, and the tissues were photographed. The number and gauge of primary and secondary blood vessels was determined using the vessel width plugin (ImageJ software version 1.48) as described by Liuhanen et al. (2013).

2.5.4. Histopathological analysis

Tumors, livers, kidneys, and spleens were fixed with buffered formalin for 12 h, and then transferred to 70% (v/v) ethanol. The organs were impregnated in paraffin (Tolosa et al., 2003) and sections of 5 μm were cut using a RM 25 RT microtome (Leica Biosystems, Nußloch Germany) and stained with hematoxylin-eosin. The entire slide was read by light microscopy (Lumen Microscope) at progressive increments of magnification (40 ×, 100 × and 400 ×). Images were captured at 40 × (tumor and spleen) or 100 × (liver and kidney) magnification using a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong).

2.6. Statistical analysis

The data are expressed as means of replicates ± standard error, which were calculated using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA, USA). The normality of the samples was analyzed with the Shapiro-Wilk test. Significant differences between treatment groups were analyzed by using one-way ANOVA followed by Tukey's test for parametric data and the Kruskal-Wallis test followed by Dunn's test for non-parametric data, using the same software. Results were considered statistically significant if the confidence level was 95% ($p < 0.05$).

3. Results and discussion

In addition to tumor cells, tumors are composed of a diverse population of leukocytes and other types of infiltrating immune cells, and the interactions between immune and neoplastic cells are crucial in determining the course of tumorigenesis (Coussens and Werb, 2002; Gonzalez et al., 2018). Cancer immunotherapies that engage immune cells to fight against tumors are proving to be powerful weapons in combating cancer. Polli et al. (2016) showed that jacalin, a lectin from the seeds of *Artocarpus integrifolia*, directs macrophages to an antitumor phenotype by exerting pro-inflammatory activity. In the present work, we hypothesized that MvFL, a protein with known immunomodulatory activity, could also have an antitumor effect.

First, we evaluated the *in vitro* cytotoxicity of MvFL to sarcoma 180 cells. MvFL promoted a decrease in the cell viability with an IC_{50} of 20.6 [8.52–32.97] μg/mL. MvFL at 6.25 and 12.5 μg/mL significantly ($p < 0.05$) induced initial apoptosis (Fig. 1A) but there was no increase in the number of cells under necrosis (Fig. 1B) or late apoptosis (Fig. 1C), in comparison with the control. At the highest concentrations of MvFL (25–100 μg/mL), there was an increase in the number of necrotic cells (Fig. 1B) and of those in late apoptosis (Fig. 1C). The binding of lectins to carbohydrates on the tumor cell surface can trigger several intracellular responses leading to apoptosis and cell growth inhibition. Other mechanisms of death can also be induced by plant lectins such as cell cycle arrest, induction of oxidative stress, and the stimulation of autophagy (Patriota et al., 2019).

Therefore, we evaluated the *in vivo* antitumor potential of MvFL in mice transplanted with sarcoma 180 cells. Fig. 2A shows the mean values of tumor weight at the end of the assay, revealing that all treatments reduced tumor weight when compared with the negative control. The reduction values were 96.8 ± 1.64% and 89.2 ± 6.41% in treatments with MvFL at 10 and 20 mg/kg, respectively. The positive control (MTX) caused an 88.9 ± 1.94% reduction in tumor weight. There were no significant differences ($p > 0.05$) in the tumor weights observed between the positive control and either treatment (both MvFL doses).

Other lectins have been reported as antitumor agents against the sarcoma 180 model. These include the recombinant lectin from *Cratylia mollis* seeds at 7 mg/kg (Cunha et al., 2016), the lectin from *Crataeva tapia* bark at 20 mg/kg (Araújo et al., 2011) and the *Schinus terebinthifolia* leaf lectin (SteLL) at 1 and 5 mg/kg (Ramos et al., 2019). The antitumor effect of SteLL was linked to the cytotoxic activity of SteLL through the induction of apoptosis (Ramos et al., 2019).

Histopathological analysis of the tumors from the animals of the negative control group (Fig. 2B) revealed solid tumors with the presence of polygonal cells containing abundant and vesiculated cytoplasm and a pleomorphic nucleus with visible intense mitotic activity. The infiltration of cells in the adipose and muscular tissue was noticeable, being poorly delimited and with no encapsulation. In addition, scarce necrotic spots were found in the tumor center. All treated groups, MTX, MvFL 10 and 20 mg/kg, displayed the presence of a greater number of necrotic points, leukocyte infiltrate and reduced nuclear density. Furthermore, they presented a discrete tumor infiltrate with a nucleus/cytoplasm ratio equivalent to normal cells. In addition, the presence of a smaller number of pleomorphic cells with loose chromatin was observed when compared to the negative control group, which indicates a reduction in mitotic activity (in number and nuclear staining).

Tumor-infiltrating lymphocytes have become widely studied given their vital role in antitumor immunity. Increase in CD8+ cytotoxic T cell infiltrates is associated with improved survival and response to therapy due to the roles of these cells in an immune active environment. Studies characterizing T cell infiltrates in sarcomas have been published and higher CD8+ T cell infiltration has been associated with favorable overall survival (Zhu et al., 2020). It is possible that the immunomodulatory effect of MvFL on T CD8+ cells is related to an antitumor immune response.

We investigated whether MvFL interferes with the angiogenesis around the sarcoma 180 tumors. Regarding vessel gauge, all groups showed a significant ($p < 0.05$) reduction in vessel diameter compared to the negative control (Fig. 3A). No significant differences were observed in the number of primary and secondary vessels around the tumor of the animals treated with MTX (positive control) or MvFL (10 and 20 mg/kg) (Fig. 3B and C). However, animals treated with MvFL at 10 mg/kg had a reduction in the number of secondary vessels (Fig. 3C). The anti-angiogenic effect of lectins might be due to the blocking of the co-receptor binding site for growth factors on endothelial cells, inhibiting its adherence and disrupting the tube formation for angiogenesis (Singh et al., 2016).

In terms of relative water and food consumption (Fig. 4), the animals treated with MTX and MvFL (10 and 20 mg/kg) did not show any reduction ($p > 0.05$) when compared with the Sham and the negative control group animals. There was no weight loss at the end of the assay for any group (Table 1).

The relative weights of animal organs from all groups are provided in Table 2. MvFL at 10 and 20 mg/kg did not cause any increase ($p > 0.05$) in the relative weight of the stomach compared to that of the negative control animals, although at 20 mg/kg there was an increase ($p < 0.05$) in comparison with the Sham group. The MTX group showed increased relative stomach weight and it has been demonstrated that methotrexate causes intestinal mucositis and this, in turn, may delay gastric emptying and gastrointestinal transit in rats (Soares et al., 2011).

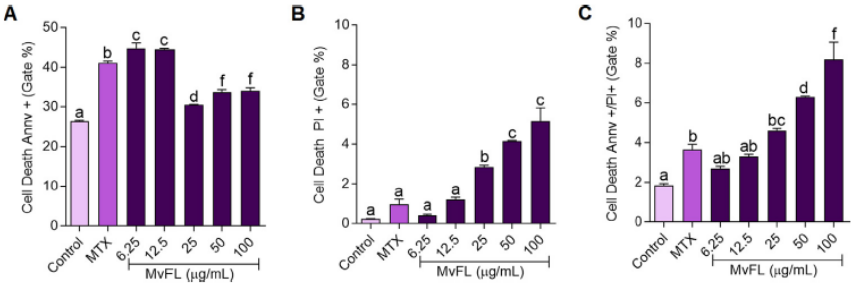

Fig. 1. Investigation of cell death induced by *Microgramma vacciniifolia* frond lectin (6.25–100 µg/mL) in sarcoma 180 cells after incubation for 24 h. The cytotoxic effect was assessed by flow cytometry using annexin V (Ann) and propidium iodide (PI). (A) Ann+/PI– cells were considered apoptotic, (B) Ann–/PI+ cells were regarded as necrotic, and (C) double staining indicated late apoptosis or necrosis. Methotrexate (20 µg/mL) was used as the positive control. Bars represent the mean ± SE of three experiments. Different letters indicate significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

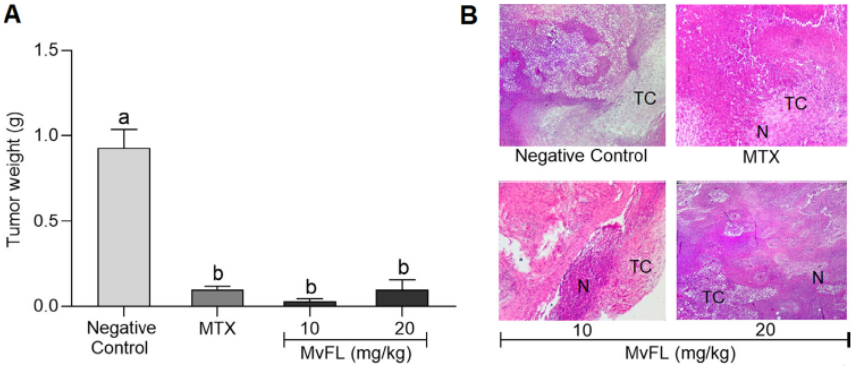

Fig. 2. Evaluation of the *in vivo* antitumor activity of *Microgramma vacciniifolia* frond lectin (MvFL). (A) Weight and (B) histological sections of sarcoma 180 tumors after treatment (7 days) with intraperitoneal PBS (negative control), methotrexate (positive control, at 1.5 mg/kg), or MvFL (10 and 20 mg/kg). Each bar represents the mean ± SE of the weight of the tumor of each animal ($n = 6$ per group). Different letters indicate significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey's test. TC: tumor cells. N: necrosis. MTX: methotrexate. Images were captured at 40 × magnification.

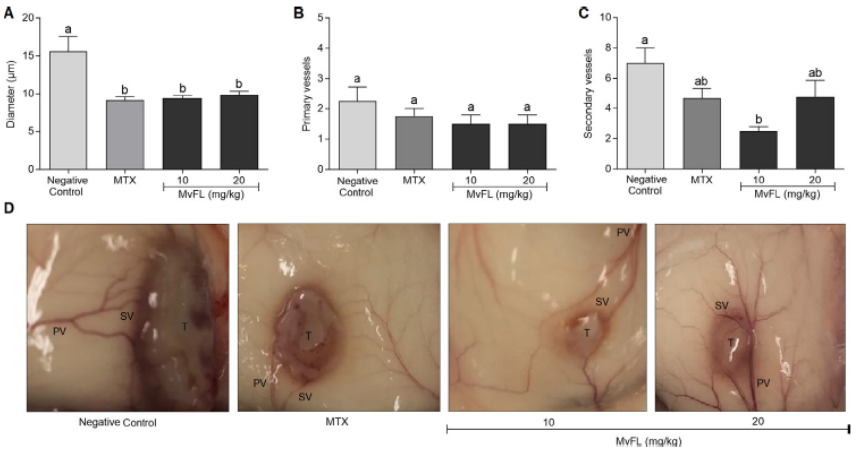

Fig. 3. Evaluation of the vessels around the sarcoma 180 tumors after treatment for 7 days with an intraperitoneal injection of PBS (negative control), methotrexate (MTX, positive control, at 1.5 mg/kg), or *Microgramma vacciniifolia* frond lectin (10 and 20 mg/kg). (A) Gauge of the vessels. (B and C) Number of primary and secondary vessels around the tumors. (D) Images of the back skin of sarcoma 180-bearing animals (PV: primary vessel; SV: secondary vessel; T: tumor). Each bar represents the mean ± SE of the results for six animals. Different letters indicate significant differences ($p < 0.05$) based on one-way ANOVA followed by Tukey's test.

Table 1
Mean of initial and final body weight of healthy animals (Sham group) as well as of sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Microgramma vacciniifolia* frond lectin (MvFL) at 10 and 20 mg/kg.

Groups	Initial body weight (g)	Final body weight (g)	Variation (g)
Sham	25.8 ± 0.16	31.0 ± 0.44	5.16 ± 0.47 a
Negative control	24.8 ± 0.70	30.3 ± 0.49	5.50 ± 0.84 a
MTX	25.6 ± 1.20	29.3 ± 0.55	3.66 ± 0.76 a
MvFL 10 mg/kg	25.1 ± 1.13	28.8 ± 0.60	4.80 ± 1.02 a
MvFL 20 mg/kg	22.3 ± 0.80	28.6 ± 0.33	6.33 ± 0.88 a

Each value corresponds to the mean ± SE of data from six animals. Different letters indicate significant differences ($p < 0.05$) between the groups by Kruskal-Wallis test followed by Dunn's test.

Table 2
Relative weight of organs of healthy animals (Sham group) as well as of sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Microgramma vacciniifolia* frond lectin (MvFL) at 10 and 20 mg/kg.

Organs	Sham	Negative control	MTX	MvFL 10 mg/kg	MvFL 20 mg/kg
Heart	0.40±0.01 ab	0.43 ± 0.01 a	0.41±0.00 ab	0.34 ± 0.01 b	0.40±0.01 ab
Kidney	0.55 ± 0.01a	0.65 ± 0.01 b	0.56 ± 0.01 a	0.56 ± 0.02 a	0.58±0.01 ab
Liver	4.87 ± 0.23 a	5.54 ± 0.30 a	5.20 ± 0.17 a	5.34 ± 0.22 a	5.20 ± 0.15 a
Lung	0.61 ± 0.02 a	0.66 ± 0.04 a	0.60± 0.03 a	0.58 ± 0.04 a	0.68 ± 0.02 a
Spleen	0.61±0.01 ab	0.80 ± 0.06 a	0.54± 0.04 b	0.64±0.06 ab	0.75±0.08 ab
Stomach	1.13± 0.07 a	1.73 ± 0.06 b	2.79± 0.19 c	1.57±0.07 ab	1.97 ± 0.03 b

Each value corresponds to the mean ± SE of data from six animals. For each organ, different letters indicate significant differences ($p < 0.05$) between the groups by one-way ANOVA followed by Tukey's test.

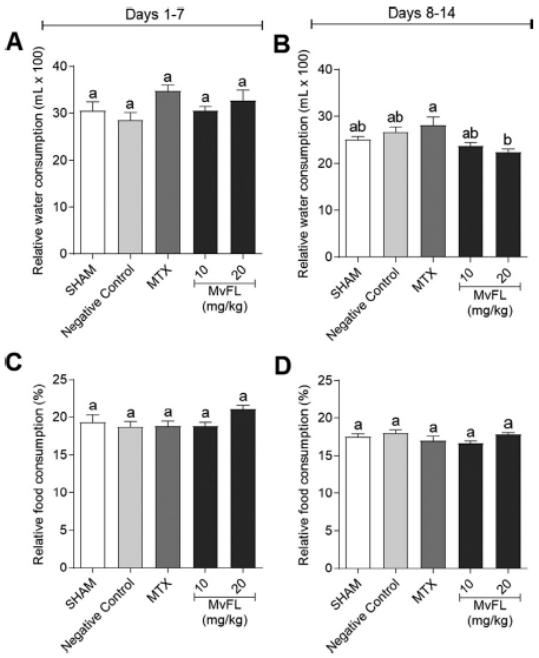

Fig. 4. Relative consumption of water and food by animals after tumor cell inoculation (A and C, days 1–7) and after treatments (B and D, days 8–14) with daily intraperitoneal injection of PBS (negative control), methotrexate (MTX, positive control, 1.5 mg/kg), or *Microgramma vacciniifolia* frond lectin (10 and 20 mg/kg). Each bar represents the mean ± SE of the results for six animals. Different letters indicate significant differences ($p < 0.05$) based on Kruskal-Wallis test followed by Dunn's test.

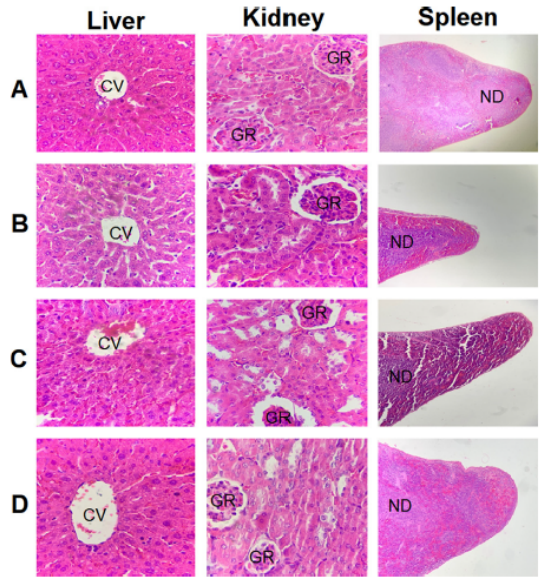

Fig. 5. Histological sections of the liver, kidney and spleen of animals treated with an intraperitoneal injection of PBS (negative control, A), methotrexate at 1.5 mg/kg (B), or *Microgramma vacciniifolia* frond lectin at 10 mg/kg (C) or 20 mg/kg (D). CV: centrilobular vein. GR = glomeruli. ND = nodules. Images were captured at 40 × (spleen) or 100 × (liver and kidney) magnification.

The group of animals treated with MvFL at 10 mg/kg showed a decrease in the relative weight of the heart compared with the negative control group animals, but not when compared with the other groups, including the Sham group.

No significant differences were found regarding the overall leukocyte number in animals treated with MTX and MvFL in comparison with the other groups (Table 3). Although MTX treatment significantly ($p < 0.05$) decreases the hemoglobin, hematocrit, MCV, and RDW values when compared to the negative control group, only the hemoglobin value was different when compared to the Sham group. MvFL at 20 mg/kg, similar to MTX, significantly ($p < 0.05$) decreases the hematocrit value when compared to the negative control but not when compared to the Sham group. All other erythrocyte parameters were normal when compared to the negative control.

There were no significant differences ($p > 0.05$) in the glucose, cholesterol, urea, and creatinine levels in the blood of animals from the different groups (Table 4). Histopathological evaluation of the kidneys of the negative control group (Fig. 5A) showed glomeruli with a homogeneous distribution and morphology preserved in the cortical zone with maintenance of the Bowman space. The distorted proximal and distal tubules were well defined, with no apparent signs of degenerative, inflammatory, or necrotic alterations; also, the medullary layer showed no morpho functional changes. The MTX group (Fig. 5B) possessed preserved cortical and medullary architecture but with a presence of enlarged glomeruli with a reduced Bowman space. The groups treated with MvFL (10 and 20 mg/kg) (Figs. 5C and 5D) presented with necrotic and asymmetric glomerulus and thickening of proximal and distal tubules; however, these alterations did not have an irreversible feature. These results suggest some degree of renal toxicity of MTX and MvFL (10 and 20 mg/kg), although the basic physiological function of the kidneys was not altered within the evaluation period (as indicated by the urea and creatinine levels).

There were no significant differences ($p > 0.05$) in the AST and ALT levels in the blood of animals from the different treatment groups (Table 4). Histopathological evaluation of the liver in the negative control group (Fig. 5A) showed regular strands of hepatocytes, a well-

Table 3
Hematological parameters of healthy animals (Sham group) as well as of sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Microgramma vacciniifolia* frond lectin (MvFL) at 10 mg/kg and 20 mg/kg.

Parameters	Sham	Negative control	MTX	MvFL 10 mg/kg	MvFL 20 mg/kg
Erythrocytes (10^6/mm^3)	9.44 ± 0.42 a	10.0 ± 0.48 a	8.53±0.11 a	8.97 ± 0.27 a	10.1 ± 1.56 a
Hemoglobin (g/dL)	15.8 ± 0.32 a	15.6 ± 0.51 a	13.3 ± 0.21 b	14.1 ± 0.48 a	15.7 ± 0.64 ab
Hematocrit(%)	51.8 ± 2.34 ab	57.7 ± 3.54 a	45.1 ± 0.41b	49.0 ± 1.57 ab	45.4 ± 4.80 b
MCV (fL)	54.9 ± 0.35 ab	57.5 ± 0.92 a	53.7 ± 0.58 b	55.4 ± 0.76 ab	56.1 ± 0.52 ab
MCH (pg)	16.1 ± 0.21 a	14.6 ± 1.14 a	15.7 ± 0.24 a	16.0 ± 0.19 a	16.6 ± 0.11 a
MCHC (g/dL)	29.4 ± 0.30 a	25.6 ± 0.70 a	29.3 ± 0.26 a	29.4 ± 0.22 a	29.5 ± 0.20 a
RDW (%)	13.7 ± 0.10 ab	14.9 ± 0.68 ac	13.0 ± 0.30b	14.2 ± 0.26abc	15.4 ± 0.44 c
Leukocytes (10^3/mm^3)	7.82 ± 0.40 a	8.63 ± 2.22 a	7.37±0.23 a	11.4 ± 0.34 a	9.42 ± 0.77 a
Lymphocytes (%)	81.3 ± 0.80 a	79.8 ± 2.41 a	77.1 ± 2.79 a	82.2 ± 1.17 a	79.6 ± 1.20 a
Monocytes (%)	2.68 ± 0.19 a	4.52 ± 1.20 a	4.00±0.55 a	3.03 ± 0.39 a	3.48 ± 0.41 a
Granulocytes (%)	15.9 ± 0.68 a	16.5 ± 2.14 a	19.2 ± 2.33 a	17.0 ± 1.55 a	17.1 ± 0.91 a

MCV: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. RDW: red cell distribution width. Each value corresponds to the mean ± SE of data from six animals. Different letters indicate significant differences ($p < 0.05$) between the groups by one-way ANOVA followed by Tukey's test.

Table 4
Biochemical parameters of healthy animals (Sham group) as well as of Sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) and *Microgramma vacciniifolia* frond lectin (MvFL) at 10 mg/kg and 20 mg/kg.

Parameters	Sham	Negative control	MTX	MvFL 10 mg/kg	MvFL 20 mg/kg
AST (U/mL)	91.3 ± 6.53 a	106.4 ± 1.41 a	63.2 ± 7.65 a	87.0 ± 17.1 a	72.9 ± 3.12 a
ALT (U/mL)	136.0 ± 15.8 a	159.9 ± 16.1 a	128.8 ± 10.6 a	197.1 ± 22.2 a	138.1 ± 20.8 a
Glucose (mg/dL)	87.5 ± 4.36 a	99.6 ± 14.2 a	117.2 ± 11.9a	145.3 ± 2.9 a	180.6 ± 41.4 a
Cholesterol (mg/dL)	258.1 ± 24.3 a	298.6 ± 46.0 a	202.7 ± 28.4 a	413.5 ± 29.4 a	306.3 ± 56.0 a
Urea (mg/dL)	25.8 ± 1.53 a	29.7 ± 2.76 a	23.7 ± 0.96 a	34.1 ± 4.44 a	33.8 ± 5.88 a
Creatinine (mg/dL)	1.06±0.07 ab	1.22 ± 0.07 b	0.48 ± 0.04 c	0.80 ± 0.05 a	0.94 ± 0.04 a

AST: aspartate aminotransferase. ALT: alanine aminotransferase. Each value corresponds to the mean ± SE of data from six animals. Different letters indicate significant differences ($p < 0.05$) between the groups by one-way ANOVA followed by Tukey's test.

defined sinusoidal space, sometimes with rare red blood cells. The centrilobular veins were in their usual caliber, supported by loose fibrous connective tissue. Small perivascular and intralobular lymphoid plaques were observed, without visible reactive activity. In the group treated with MTX (Fig. 5B), we observed vacuolization, moderate disorganization of the hepatic cords, and some hepatocytes with condensed chromatin. The groups treated with MvFL (10 and 20 mg/kg) (Figs. 5C and 5D) had well-defined hepatic cords around the centrilobular vein. These results are in accordance with the normal levels of liver enzymes, which indicate an absence of damage to this organ.

Histopathological evaluation of the spleen in the negative control group (Fig. 5A) showed a normal architecture with well-delimited red and white pulps of normal dimensions. In the group treated with MTX (Fig. 5B), activation of lymph nodes was observed while normal appearance and morphology was observed in the groups treated with MvFL (10 and 20 mg/kg) (Figs. 5C and 5D).

4. Conclusion

MvFL is a potent antitumor agent that did not cause toxicity in the parameters evaluated. The antitumor effect may be linked to the cytotoxic activity of lectin through the induction of apoptosis and anti-angiogenic activity. The immunomodulatory capacity of the lectin may also be linked to antitumor capabilities. Future studies should clarify the mechanisms involved.

Author contributions

Leydianne L.S. Patriota: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Dalila B.M. Ramos, Yasmym A. Silva, Angela C.L.A. Santos, Maria T.M.F. Araújo, Jéssica S. Brito, Diego J.L Torres:** Data curation, Formal analysis, Investigation. **Alisson M. Oliveira, Diego C.N. Silva:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization. **Virgínia M.B. Lorena:** Funding acquisition, Resources, Methodology, Validation, Visualization. **Patrícia M.G. Paiva:** Conceptualization, Funding acquisition, Resources, Validation, Visualization. **Rosemairy L. Mendes:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Visualization. **Thiago H. Napoleão:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing- review & editing. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.2 ATIVIDADE ANTITUMORAL DE MoFTI

Os resultados da avaliação da atividade antitumoral de MoFTI em camundongos portadores de sarcoma 180 estão incluídos em artigo publicado no periódico *Food and Chemical Toxicology*, podendo ser acessado em: https://doi.org/10.1016/j.fct.2020.111691. Esse artigo também traz os resultados da avaliação toxicológica dos tratamentos com o inibidor.

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Antitumor activity of *Moringa oleifera* (drumstick tree) flower trypsin inhibitor (MoFTI) in sarcoma 180-bearing mice

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ABSTRACT

The plant *Moringa oleifera* is used as food and medicine. *M. oleifera* flowers are source of protein, fiber, and antioxidants, and are used to treat inflammation and tumors. This work evaluated the antitumor activity of the *M. oleifera* flower trypsin inhibitor (MoFTI) in sarcoma 180-bearing mice. Swiss female mice were inoculated with sarcoma 180 cells. Seven days later, the animals were treated intraperitoneally for 1 week with daily doses of PBS (control) or MoFTI (15 or 30 mg/kg). For toxicity assessment, water and food consumption, body and organ weights, histological alterations, and blood hematological and biochemical parameters were measured. Treatment with MoFTI caused pronounced reduction (90.1%–97.9%) in tumor weight. The tumors of treated animals had a reduced number of secondary vessels and lower gauge of the primary vessels compared to the control. No significant changes were observed in water and food consumption or in body and organ weights. Histopathological analysis did not indicate damage to the liver, kidneys, and spleen. In conclusion, MoFTI showed antitumor potential, with no clear evidence of toxicity.

1. Introduction

Protease inhibitors are compounds that interact with proteases, reducing their catalytic activity. In plants, these inhibitors may be polypeptides or secondary metabolites (Pontual et al., 2012; Hellinger and Gruber, 2019). Proteins with protease inhibitor activity are produced by plants for various reasons, for example, to regulate the endogenous proteolytic activity involved in cell death and protein storage, and to provide protection against insects and microorganisms (Bendre et al., 2018; Napoleão et al., 2019).

The biomedical applications of protease inhibitors are varied, as proteases are involved in many processes, such as inflammation, infection, extracellular matrix degradation, blood coagulation, and cancer metastasis (Clemente et al., 2019; Cotabarren et al., 2020). The biological activities reported for these molecules include antimicrobial (Shamsi and Fatima, 2016), anti-inflammatory (Bortolozzo et al., 2018), and antitumor (Zasheva and Simova-Stoilova, 2017) effects.

In tumors, the ratio between proteases and endogenous protease inhibitors is abnormal; this abnormality is usually associated with a decrease in the expression of certain inhibitors. Thus, many researchers have investigated different ways to compensate for the insufficient availability of protease inhibitors by the application of exogenous inhibitors as a means to treat cancer (Storr et al., 2016). Plant-derived protease inhibitors constitute an alternative to synthetic drugs and the

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therapeutic potential of these proteins for the treatment of cancer is currently under investigation (Shamsi et al., 2016; Zasheva and Simova-Stoilova, 2017).

Plant-derived protease inhibitors have been shown to exert anticancer effects through their action against the initiation, proliferation, and progression of different types of tumors (Srikanth and Chen, 2016; Laparra and Haros, 2019). Several protease inhibitors are reported to inhibit the growth in various cancer cell lines (Srikanth and Chen, 2016; Zasheva and Simova-Stoilova, 2017) and the antineoplastic action of protease inhibitors may be related to the induction of cell death, especially apoptosis (Mehdad et al., 2016). In addition, the *in vitro* and *in vivo* toxicities of plant protease inhibitors have been investigated (Mayasa et al., 2016).

A protease inhibitor from *Crataeva tapia* bark (CrataBL) had antitumor activity in prostate cancer cell lines (DU145 and PC3) that was mediated through the induction of apoptosis (Ferreira et al., 2013b), and affected cocultures of mesenchymal stem cells and glioblastoma cells, stimulating the antitumor response (Bonturi et al., 2019). Protease inhibitor from *Glycine* max (soybean) was effective against the MCF-7 breast cancer cell line (Mayasa et al., 2016), induced apoptosis in human prostate carcinoma (LNCaP) cells, and induced prostate adenocarcinoma in SV40T antigen transgenic rats (Tang et al., 2009). In addition, the *G. max* inhibitor reduced the initiation and regularity of dimethylhydrazine-induced colon carcinogenesis in rats (Kennedy et al., 2002).

Nutraceuticals obtained from foods of vegetal origin have been evaluated for the prevention of diseases and as a complement to conventional pharmacological therapy (Durazzo et al., 2020). *Moringa oleifera*, of the Moringaceae family, has multiple uses, including food and medicinal uses; the flowers are considered a good source of protein, dietary fibers, and antioxidant compounds (Santos et al., 2015). In general, several parts of this tree are edible and they are used as a highly nutritive vegetable in many countries (Anwar et al., 2007); this includes the flowers, which are eaten after cooking (Mehta et al., 2011). Thus, *M. oleifera* has a high nutraceutical potential (Kou et al., 2018).

The antitumor potential of compounds from all parts of *M. oleifera* has been reported. Leaf substances have been effective in *in vitro* and *in vivo* assays against several types of cancer (Khor et al., 2018). For example, the *in vivo* anticancer activity of the leaf extract and fraction was determined against Dalton's lymphoma ascites in Swiss albino mice (Krishnamurthy et al., 2015). Leaf extracts exerted *in vivo* antitumor activity against Ehrlich solid tumor (Khalil et al., 2014) and Ehrlich ascites carcinoma (Barhoi et al., 2020). The use of the flowers in folk medicine to treat inflammations and tumors has also been reported (Anwar et al., 2007; Mahajan and Mehta, 2009; Mehta et al., 2011). As a remedy, the aqueous, ethanolic, or hydro-alcoholic extracts of the flowers are used, or an infusion of flowers in hot water (Mahajan and Mehta, 2009; Mehta et al., 2011). The anticancer effect of *M. oleifera* flower extract against human prostate cancer cells was mediated via the induction of apoptosis (Ju et al., 2018).

M. oleifera flowers contain a trypsin inhibitor called MoFTI (*M. oleifera* flower trypsin inhibitor) that has insecticidal (Pontual et al., 2014) and trypanocidal (Pontual et al., 2018) activities. MoFTI was non-toxic to murine macrophages and Vero cells (Pontual et al., 2014, 2018). Given the frequent use of moringa flowers in the diet and reports on their use against tumors, the present work aimed to evaluate the antitumor activity of MoFTI in sarcoma 180-bearing mice. The cytotoxicity of MoFTI to sarcoma 180 cells and the induction of cell death was measured *in vitro*. *In vivo* antitumor assays measured the tumor weight, and the number and gauge of tumor vessels. In addition, the following toxicological parameters were investigated to determine whether MoFTI treatment was harmful to the mice: relative water and food consumption, body and organ weights, histological alterations, and blood hematological and biochemical parameters. The study design is summarized in Fig. 1 and described in detail in the next section.

2. Materials and methods

2.1. Preparation of MoFTI

Flowers of *M. oleifera* Lam. were collected in Recife, Pernambuco, Brazil, with authorization (36301) of the *Instituto Chico Mendes de Conservação da Biodiversidade* from Brazilian Ministry of Environment. A voucher specimen (number 73345) was deposited at the herbarium "Dárdano de Andrade Lima" from the *Instituto Agronômico de Pernambuco* (Recife, Brazil). The access was recorded (A9D147B) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). The fresh flowers were separated from the plant and used for the protein extraction and isolation of MoFTI, as described by Pontual et al. (2014). The isolated inhibitor was dialyzed against distilled water (4 h) and dried by lyophilization (−45 °C, maximum vacuum of 300 μmHg, 24 h) in a LIOTOP L101 freeze-dryer (Liobras, São Carlos, Brazil). The protein concentration (mg/mL) was determined by the method of Lowry et al. (1951). The activity (U/mg) of MoFTI was monitored by using a trypsin inhibitor activity assay, as described by Pontual et al. (2014).

2.2. In vitro assays

2.2.1. Evaluation of toxicity to sarcoma 180 cells

Sarcoma 180 cells were obtained from tumor-bearing animals, as described by Ramos et al. (2019). The sarcoma 180 cells (1×10^5 cells per well) were transferred to 96-well microplates (Kasvi, São José do Pinhais, Brazil) containing RPMI 1640 medium with HEPES (Cultilab Materiais para Cultura de Células, Campinas, Brazil), supplemented with 10% (w/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). MoFTI, resuspended in PBS, was added to the wells at final concentrations between 1.56 and 400 μg/mL. Methotrexate (MTX; Sigma-Aldrich) at 1.56–400 μg/mL was used as the positive control, and PBS was used as the negative control. The plates were homogenized on a shaker (Biomixer, São Paulo, Brazil) and then incubated for 24 h at 37 °C (Oliveira et al., 2019). The number of viable cells was then determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA), containing the active tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), in accordance with the manufacturer's instructions. The percentage of viable cells was calculated relative to the negative control and the concentration (in μg/mL) of the sample that reduced cell viability by 50% (IC_{50}) was calculated by non-linear regression analysis using the software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

2.2.2. Analysis of cell death

The cells were cultured for 24 h in the absence (control) or presence of MoFTI at 6.25–100 μg/mL; 20 μg/mL MTX was used as the positive control. After centrifugation (450 *g*, 4 °C, 10 min), the supernatant was discarded, 1.0 mL PBS was added to the pellet, and the cells were resuspended and centrifuged again. Cell death was then measured using the FITC Annexin V Apoptosis Detection Kit II (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer's instructions. The cells were analyzed in a BD Accuri C6 cytometer (BD Biosciences) and 20,000 events were recorded for each sample. Cells only stained with annexin V or propidium iodide were considered apoptotic or necrotic, respectively. Double staining indicated that the cells were in necrosis or late apoptosis and unstained cells were considered viable cells (Ramos et al., 2019). The results were expressed as the percentage of stained cells in the selected gate.

2.3. In vivo experimental model

2.3.1. Ethical permission and approval

All experimental procedures were approved by the Ethics Committee

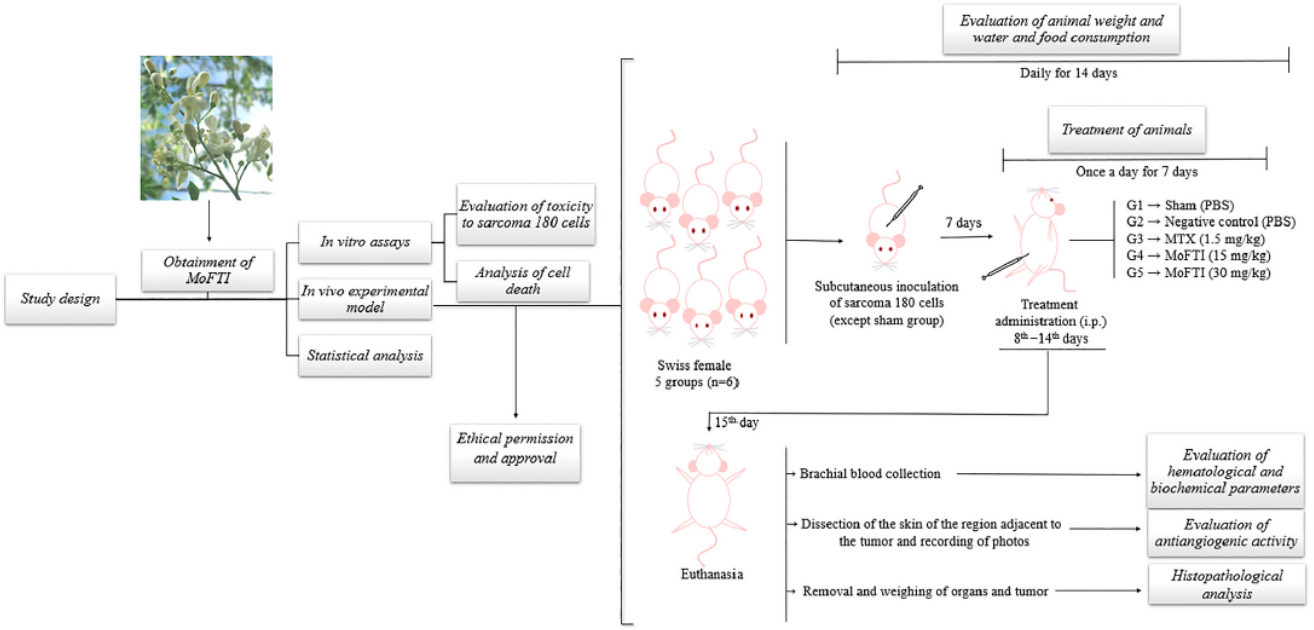

Fig. 1. Graphical representation of the study design.

on Animal Use of the *Universidade Federal do Vale do São Francisco*, UNIVASF (authorization no. 0001/121218). The antitumor activity was evaluated in 6–8-week-old Swiss female mice obtained from the vivarium of UNIVASF, maintained in a controlled environment (24 °C and 12:12 h light/dark photoperiod) and given access to water and food *ad libitum*.

2.3.2. Treatment of animals

The mice were divided into five groups, each composed of six animals. A suspension of sarcoma 180 cells in PBS (5×10^6 cells/100 μL) was subcutaneously inoculated into the back of each mouse, except those belonging to the sham group. The treatments were started on the eighth day after inoculation and lasted for 7 days. The sham and negative control groups received PBS (i.p.) and the positive control group received 1.5 mg/kg MTX (i.p.) The other two groups were treated with 15 or 30 mg/kg MoFTI (i.p.). On the 15th day after tumor cell inoculation, the animals were euthanized by the administration of 100 mg/kg ketamine and 10 mg/kg xylazine (i.p.) (Syntec, Santana de Parnaíba, Brazil). The tumor, lungs, liver, kidneys, spleen, heart, and stomach of each animal were removed and weighed. The relative weight of each organ was calculated from the formula: Relative weight (%) = organ weight (g)/body weight (g) × 100 (Ramos et al., 2019).

2.3.3. Evaluation of antiangiogenic activity

The skin of the region adjacent to the tumor was dissected to evaluate the vascularization around the tumors. The tissues were photographed and analyzed for number and gauge/diameter (μM) of the primary vessel closest to the tumor and the number of primary and secondary vessels. The measurements were performed using the vessel width plugin of ImageJ software (National Institute of Health, Bethesda, MD, USA) version 1.48 (Liuhanen et al., 2013).

2.3.4. Histopathological analysis

The tumor, liver, kidney, and spleen samples were packed into histological cassettes, fixed with buffered formalin and, after 12 h, transferred into a solution of 70% (v/v) ethanol. After the samples were impregnated in paraffin, sections of 5 μm were cut by using a Leica RM 25 RT microtome (Leica Biosystems, Nußloch Germany) and stained with hematoxylin-eosin (Sigma-Aldrich). The entire slide was analyzed using an Eclipse E100 (Nikon, Tokyo, Japan) light microscope at progressive magnification (40 × , 100 × , and 400 ×). Images were captured at 40 × (tumor and spleen) or 100 × (liver and kidney) magnification. The tumor sections were examined to determine the area of tumor cells (Tc; mm^2) through the evaluation of each of the four quadrants by using the area calculator plugin in ImageJ software, version 1.48 (Raghavan and Rao, 2015).

2.3.5. Evaluation of animal weight, and water and food consumption

The weight of the animals and the amount of food consumed were measured daily by using a semi-analytical balance (BEL Engineering, Monza, Italy). Water consumption was also measured daily by using a graduated cylinder (Laborglas, São Paulo, Brazil). Relative water and food consumption was calculated as follows: relative food consumption (%) = food (g) consumed/body weight (g) × 100; relative water consumption (mL/g) = water (mL) consumed/body weight (g).

2.3.6. Evaluation of hematological and biochemical parameters

At the end of the antitumor assay, brachial blood samples (Oliveira et al., 2019) were collected from anesthetized animals into EDTA-treated tubes (Vacuplast, Cral Artigos para Laboratório Ltda, Cotia, Brazil) for hematological analysis and dry tubes (Vacuplast) to separate the serum for the biochemical assays. Global leukometry ($10^3/mm^3$), differential leukometry (%), counting of erythrocytes ($10^6/mm^3$), determination of hematocrit (%), and measurements of the mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; g/dL), and red cell distribution width (RDW; %) were performed by using a HEMATOCLIN 2.8 VET (Bioclin, Belo Horizonte, Brazil). Specific kits for the detection of urea (mg/dL), creatinine (mg/dL), glucose (mg/dL), cholesterol (mg/dL), aspartate aminotransferase (AST; U/mL), and alanine aminotransferase (ALT; U/mL) were employed to determine the serum levels of these substances in accordance with the manufacturer's protocol (Labtest, Vista Alegre, Brazil).

2.4. Statistical analysis

The data were expressed as the mean of replicates ± standard error (SE); these details were calculated using GraphPad Prism, version 5 for

Windows (GraphPad Software, San Diego, CA, USA). The normality of the samples was analyzed with the Shapiro-Wilk test. Significant differences ($p < 0.05$) between treatment groups were analyzed by using one-way ANOVA followed by Tukey's test for parametric data and the Kruskal-Wallis test followed by Dunn's test for non-parametric data, using the same software. In figures and tables, different letters indicate significant differences.

3. Results

MoFTI was cytotoxic to sarcoma 180 cells *in vitro*, with an IC_{50} of 67.97 [61.45–119.8] μg/mL. In the concentration range of 6.25–25 μg/mL, MoFTI significantly ($p < 0.05$) induced apoptosis (Fig. 2A); at the highest concentrations (50 and 100 μg/mL), the cells were in an advanced stage of damage, with a significant increase ($p < 0.05$) in the number of cells in necrosis (Fig. 2B) and late apoptosis (Fig. 2C) compared to the negative control. In comparison with the negative control, MTX induced a significant increase ($p < 0.05$) in apoptosis, but not in necrosis or late apoptosis.

The positive control, MTX, resulted in a significant ($p < 0.05$) reduction of 88.9% ± 1.94% in the tumor mass compared to the control (Fig. 3). At doses of 15 and 30 mg/kg, MoFTI caused significant ($p < 0.05$) reductions in the tumor mass of 97.9% ± 0.45% and 90.1% ± 4.66%, respectively, compared to the control (Fig. 3). No significant difference ($p > 0.05$) in tumor mass was observed between animals treated with MTX and MoFTI, or between the groups treated with 15 or 30 mg/kg MoFTI. MTX showed moderate antiangiogenic activity, with a significant ($p < 0.05$) reduction (44.2% ± 1.89%) in the gauge of the primary vessels closest to the tumor, similar to MoFTI at doses of 15 and 30 mg/kg, which resulted in reductions of 34.4% ± 2.67% and 37.7% ± 4.23%, respectively, compared to the negative control (Fig. 4A). Neither MTX nor MoFTI treatments interfered with the formation of primary vessels (Fig. 4C), but treatment with the higher dose of MoFTI decreased (41.3% ± 3.44%) the number of secondary vessels (Fig. 4D).

Histological analysis of sarcoma 180 tumors from the negative control group (Fig. 5A) revealed solid tumors with the presence of polygonal cells with an abundant, vesiculated cytoplasm and a pleomorphic nucleus with visibly intense mitotic activity; the infiltration of cells in the adipose and muscle tissues with the absence of encapsulation and defined boundaries was shown. The tumors from the MTX group (Fig. 5B) showed leukocyte infiltration and reduced nuclear density. In addition, a few necrotic spots, characterized by pyknosis and nuclear eosinophilia, were observed close to, or in the tumor center. In groups treated with 15 and 30 mg/kg MoFTI (Fig. 5C and D), the presence of leukocyte infiltration was observed, despite the discrete presence of necrotic spots. In addition, a decrease in nuclear density and preserved parenchyma with no infiltration of tumor cells were observed.

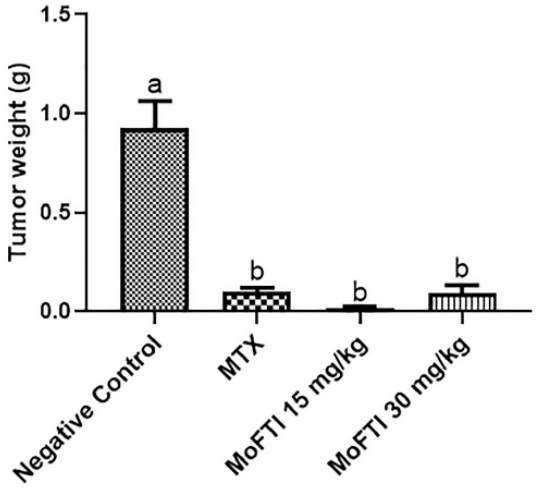

Fig. 3. Evaluation of *in vivo* antitumor activity of the *Moringa oleifera* flower trypsin inhibitor (MoFTI). The weight of sarcoma 180 tumors after treatment for 7 days with intraperitoneal injection of PBS (negative control), methotrexate (MTX, positive control, 1.5 mg/kg) and MoFTI (15 and 30 mg/kg). Each bar represents the mean ± SE of the weight of the tumor in each animal ($n = 6$ per group). Different letters indicate significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

As a visible decrease in tumor mass was observed in the treated groups (MTX and MoFTI), we determined the area of tumor cells in the histological sections (Fig. 6). MTX reduced this area significantly ($p < 0.05$) by 43.3% ± 0.13%, in comparison with the negative control. Similarly, when compared to the negative control, 15 and 30 mg/kg MoFTI promoted significant reductions ($p < 0.05$) in the area of tumor cells, of 62.5% ± 0.08% and 75.6% ± 0.09%, respectively. In addition, MoFTI reduced ($p < 0.05$) the tumor cell area by 35.0% ± 0.16% (15 mg/kg) and 57.7% ± 0.21% (30 mg/kg) compared to the MTX group.

Histopathological evaluation of the livers from mice in the negative control group (Fig. 5A) showed that the tissue had regular strands of hepatocytes, a well-defined sinusoidal space, sometimes filled with rare red blood cells. The centrilobular veins were of their usual caliber, supported by loose fibrous connective tissue. Small perivascular and intralobular lymphoid plaques were observed, with no visible reactive activity. In general, all groups had hepatocytes with polygonal morphology and a granular cytoplasm. In the group treated with MTX (Fig. 5B), preserved architecture was observed, but with the presence of vacuolization and moderate disorganization of the hepatic cords; in

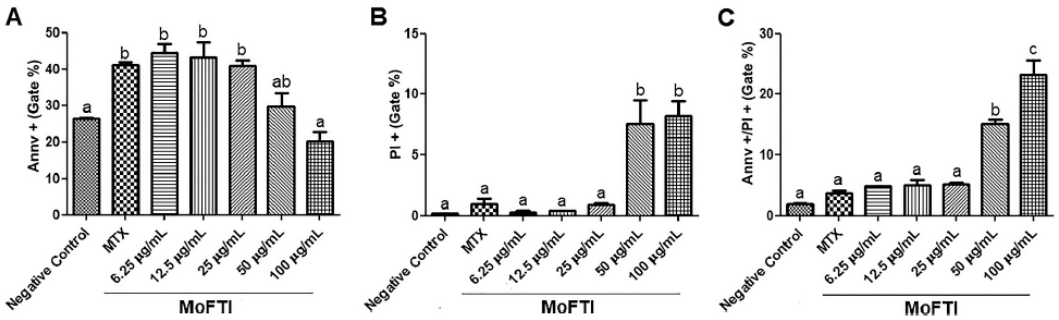

Fig. 2. Examination of cell death induced in sarcoma 180 cells after incubation with MoFTI (6.25–100 μg/mL) for 24 h. Cytotoxicity was assessed by flow cytometry using annexin V (Ann) and propidium iodide (PI). (A) Ann+/PI– cells were considered apoptotic, (B) Ann–/PI + cells were considered necrotic, and (C) double staining was considered to indicate cells in late apoptosis or necrosis. Methotrexate (MTX, 20 μg/mL) was used as the positive control. Each bar represents the mean ± SE of three experiments. Different letters indicate significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

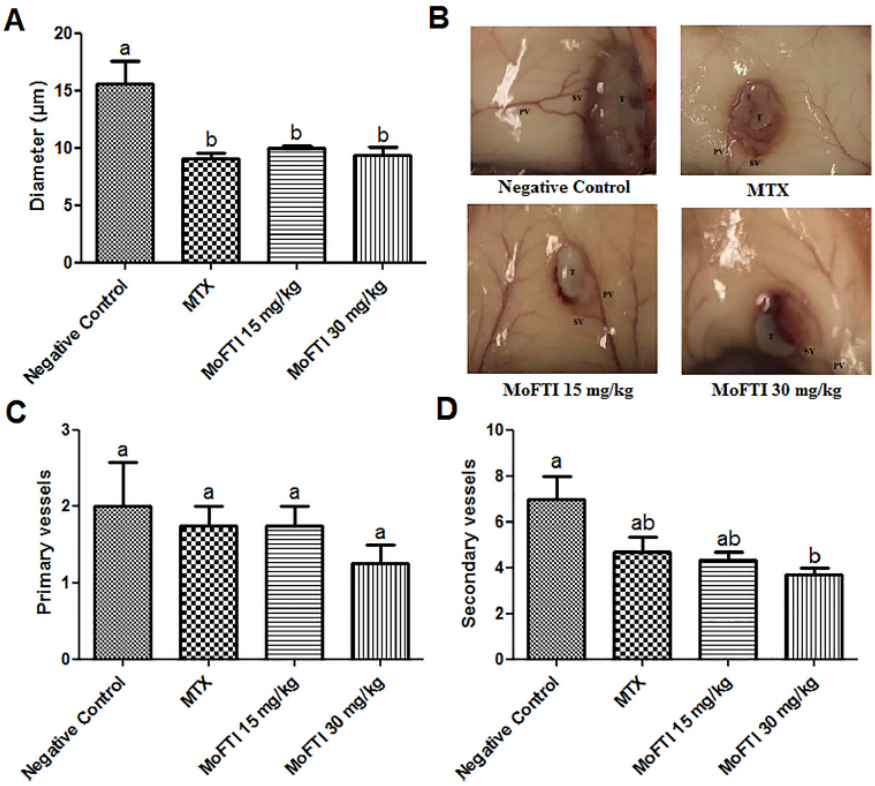

Fig. 4. Evaluation of the vessels nearby to the sarcoma 180 tumors after treatment for 7 days after intraperitoneal injection of PBS (negative control), methotrexate (MTX, positive control, 1.5 mg/kg) and *Moringa oleifera* flower trypsin inhibitor, MoFTI (15 and 30 mg/kg). (A) Gauge of the primary vessel closest to the tumor. (B) Images of the back skin of sarcoma 180-bearing animals (PV: primary vessel; SV: secondary vessel; T: tumor). (C and D) The number of primary and secondary vessels, respectively, around the tumors. Each bar represents the mean ± SE of the results for six animals (n = 6 per group). Different letters indicate significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

addition, some hepatocytes presented condensed chromatin. In the groups treated with MoFTI (15 and 30 mg/kg) (Fig. 5C and D), the tissue had regular hepatocyte cords, a well-defined sinusoid space, and normal-sized centrilobular vein.

Histopathological evaluation of the kidneys from mice in the negative control group (Fig. 5A) revealed glomeruli with homogeneous distribution and morphology preserved in the cortical zone, and maintenance of the Bowman space. The distorted proximal and distal tubules were well defined, with no apparent signs of degenerative, inflammatory, or necrotic alterations; the medullary layer also showed no morphofunctional changes. The MTX group (Fig. 5B) presented enlarged glomeruli with reduced Bowman's space. The kidneys of mice in the groups treated with MoFTI (15 and 30 mg/kg) (Fig. 5C and D) exhibited dispersed cells with condensed chromatin, glomeruli with normal appearance, size, distribution, and morphology, as well as proximal and distal tubules with normal characteristics.

Finally, in the histopathological analysis of the spleen, the animals in the negative control group (Fig. 5A) showed normal architecture, with delimited red and white pulps and with normal dimensions. In contrast, activation of the lymph nodes occurred in the MTX-treated group (Fig. 5B), whereas the groups treated with MoFTI (15 and 30 mg/kg) (Fig. 5C and D) had normal appearance, contours, and morphology.

Neither MTX nor MoFTI significantly altered ($p > 0.05$) the water and food intake (Fig. 7) or weight gain (Table 1). Animals in all groups showed a significant increase ($p < 0.05$) in the stomach weight compared to the healthy group (sham): negative control, 52.8% ± 5.61%; MTX, 146.3% ± 17.4%; MoFTI 15 mg/kg, 43.9% ± 6.76%; and MoFTI 30 mg/kg, 57.4% ± 10.2% (Table 2). However, the increase in MTX was also significantly different ($p < 0.05$) compared to that in the negative control and MoFTI-treated groups, suggesting a delay in gastric emptying. MTX and MoFTI (15 and 30 mg/kg) did not cause any significant changes in the relative weight of the other organs evaluated (Table 2) compared to the sham and negative control groups.

In general, biochemical parameters were not significantly altered ($p > 0.05$) by treatment with MTX or MoFTI compared to the sham and negative control (Table 3), with the exception of creatinine, which was significantly ($p < 0.05$) reduced (60.7% ± 3.61%) in the animals treated with MTX compared to the negative control.

The animals treated with MTX exhibited a significant ($p < 0.05$) decrease in erythrocyte parameters, such as reduced hematocrit (21.8% ± 1.00%), MCV (6.56% ± 1.01%), and RDW (12.6 ± 2.03) when compared to the negative control (Table 4). Conversely, treatment with MoFTI did not alter any erythrocyte or leukocyte parameters compared to the sham and negative control groups (Table 4).

4. Discussion

Proteases have an important role in the normal function of cells, but are also linked to the development of many diseases. There is much evidence to support the role of proteases in cancer transformation, development, metastases, and the epithelial-mesenchymal transition (Zasheva and Simova-Stoilova, 2017; Vasiljeva et al., 2019). Based on previous reports describing the antitumor activity of protease inhibitors, we investigated the antitumor potential of MoFTI in sarcoma 180-bearing mice.

MoFTI was cytotoxic to sarcoma 180 cells through the induction of both apoptosis and necrosis. It has been suggested that the cytotoxicity of protease inhibitors involves primary targets of serine proteases located at secretory vesicles and/or the cell membrane; in addition, protease inhibitors can also be internalized, such as the buckwheat trypsin inhibitor, which enters Hep G2 cells by clathrin-dependent endocytosis (Cui et al., 2013; Zasheva and Simova-Stoilova, 2017). It was previously reported that MoFTI was not cytotoxic to normal mammalian cells, such as murine macrophages and Vero cells, which suggested that this inhibitor has some selectivity (Pontual et al., 2018).

The antitumor activity of protease inhibitors can involve complex

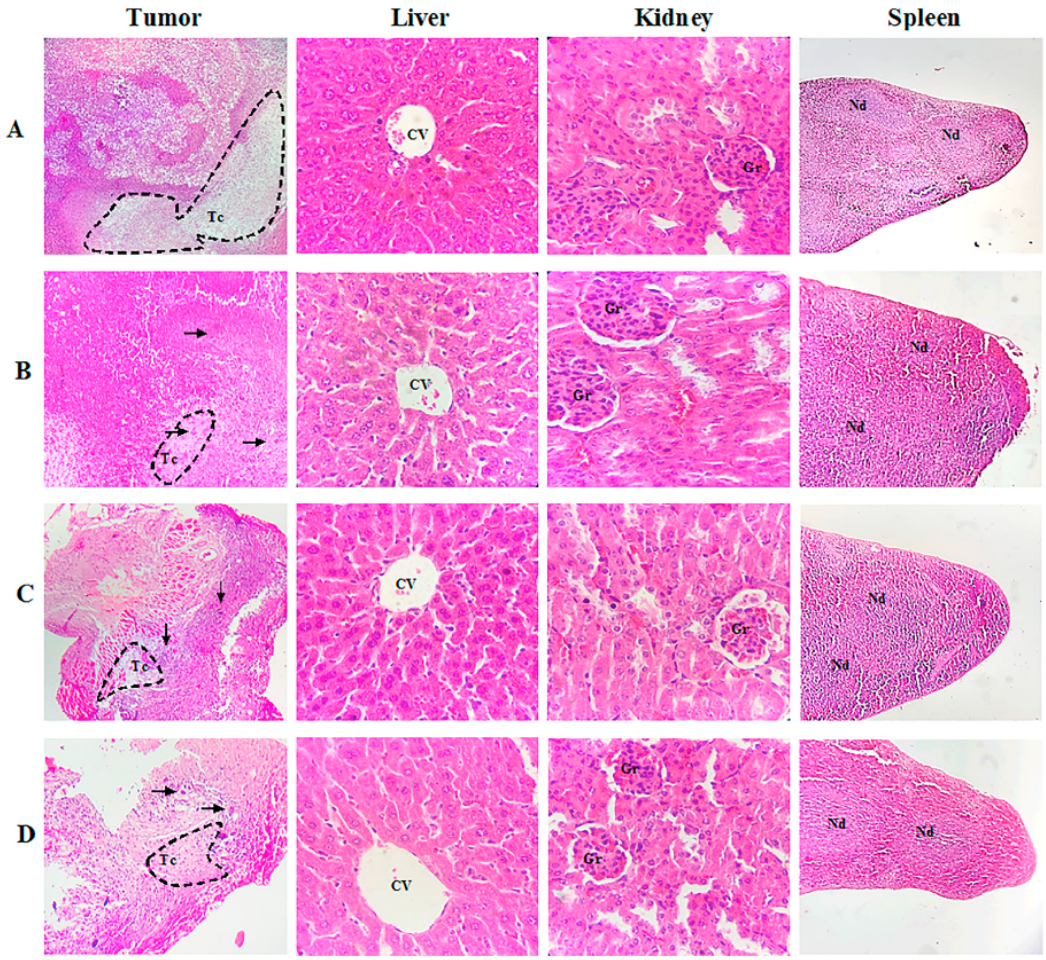

Fig. 5. Histological evaluation of the tumor, liver, kidney, and spleen of the animals treated with intraperitoneal injection of PBS (negative control, A), methotrexate 1.5 mg/kg (B), and *Moringa oleifera* flower trypsin inhibitor (MoFTI) at 15 mg/kg (C) and 30 mg/kg (D). Tc: tumor cells. Dashed lines delimit the Tc area. Arrows: necrosis points. Cv: centrilobular vein. Gr: glomeruli. Nd: nodules. Hematoxylin and eosin staining was used. Magnification: 40 × for the tumor and spleen; 100 × for the liver and kidney.

pathways linked to cell death. The induction of apoptosis by protease inhibitors may involve the upregulation of pro-apoptotic proteins (e.g., Bax and Bak) and the downregulation of anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xl) (Li et al., 2009), caspase activation (Bai et al., 2015), and cytochrome *c* release (Ferreira et al., 2013a). Protease inhibitors can also induce ROS production (Mehdad et al., 2016) and DNA fragmentation (Park et al., 2004), suppress extracellular-signal-regulated kinase (ERK) activity (Chen et al., 2005), disrupt the cell cycle (Clemente et al., 2010), and cause mitochondrial membrane damage (Mehdad et al., 2016).

The growth of sarcoma 180 cells was inhibited by trypsin inhibitors from *Glycine max* and *Acacia confusa* when they were covalently linked to concanavalin A (Con A) or abrin B chain, which acted as vectors to carry these protease inhibitors into the cells (Lin et al., 1989; Chu et al., 1997). Chu et al. (1997) proposed that the antitumor potential of these protease inhibitors resulted from the inhibition of intracellular enzymes, and described the presence of two trypsin-like proteases in the cytosol of sarcoma 180 cells, which appeared to have a physiological role during tumor invasion. As such, it is likely that MoFTI may act on these trypsin-like proteases. More recently, serine-type protease inhibitors (STPIs) from *Chenopodium quinoa*, *Salvia hispanica*, *Avena sativa*, and *Triticum durum* were evaluated for their influence in an *in vivo* hepatocarcinoma (HCC) model and the authors concluded that *C. quinoa* and *S. hispanica* protease inhibitors were able to control the aggressiveness of HCC by altering the innate immunity of the tumor microenvironment (Laparra and Haros, 2019).

MoFTI interfered with angiogenesis in the vicinity of sarcoma 180 tumors. Proteases are involved in many aspects of tumor growth and progression, including proliferation, immune responses, inflammatory cell recruitment, tumor invasion, angiogenesis, and metastasis (Mason and Joyce, 2011; Dudani et al., 2018; Trezza et al., 2020). The protease inhibitors may exert antiangiogenic function through the inhibition of proteolytic cascades involving the inhibition of plasma kallikrein, elastase, and plasmin. The inhibition of these enzymes converges to block the release of growth factors, which then cannot stimulate angiogenesis (Soares et al., 2016).

Necrotic cells and inflammatory infiltrates were the main findings in the histopathological examination of the tumors of animals treated with MTX and MoFTI, similar to those described in other histopathological studies of sarcoma 180 tumors treated with natural products (Ramos et al., 2019; Oliveira et al., 2019). The decrease in the tumor area in the histopathological sections agreed with the results of cytotoxicity analysis and the inhibition of tumor growth induced by MTX and MoFTI.

In addition to determining the antitumor potential of MoFTI, an

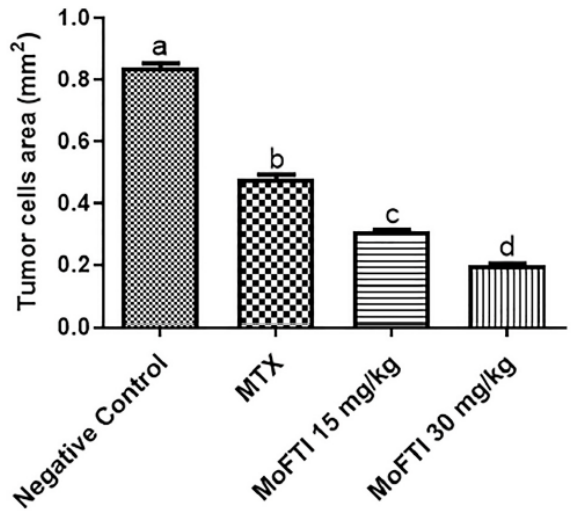

Fig. 6. Quantification of tumor cell area (in mm^2) in the histological sections of tumors of the animals treated with intraperitoneal injection of PBS as the negative control (A), methotrexate 1.5 mg/kg (B), and *Moringa oleifera* flower trypsin inhibitor (MoFTI) at 15 mg/kg (C) or 30 mg/kg (D). Each bar represents the mean ± SE for six replicates (n = 6 per group). Different letters indicate significant differences ($p < 0.05$) by one-way ANOVA followed by Tukey's test.

evaluation of whether the inhibitor caused damage to the mice was performed. To determine this, the consumption of water and food, body weight, relative organ weights, histological alterations and blood biochemical and hematological parameters were measured. In summary, the results highlighted the safety of MoFTI in comparison with MTX. A reduction in food intake is expected in animals treated with MTX as the drug may cause a delay in gastric emptying and gastrointestinal transit in mice (Soares et al., 2011; Ramos et al., 2019). The toxicity of MTX to the stomach is already well described (Soares et al., 2011; Ikeda et al., 2016). The histological, biochemical, and hematological parameters from MoFTI group were not suggestive of toxicity to the treated animals.

5. Conclusion

MoFTI exerted a strong antitumor effects *in vivo* against sarcoma 180 cells, and this antitumor activity may be related to the induction of apoptosis and necrosis in tumor cells, as well as its antiangiogenic activity. There was no evidence of the toxicity of MoFTI to the mice at the doses tested. However, it is important to highlight the need for more toxicological information, such as the determination of acute and chronic toxicity, as the intraperitoneal treatment period used in this work was shorter than 1 week. The results suggest that MoFTI was an

Table 1
Mean of initial and final body weight of healthy animals (Sham group) as well as of sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Moringa oleifera* flower trypsin inhibitor (MoFTI) at 15 and 30 mg/kg.

Groups	Initial body weight (g)	Final body weight (g)	Variation (g)
Sham	25.8 ± 0.16	31.0 ± 0.44	5.16 ± 0.47a
Negative control	24.8 ± 0.70	30.3 ± 0.49	5.50 ± 0.84a
MTX	25.6 ± 1.20	29.3 ± 0.55	3.66 ± 0.76a
MoFTI 15 mg/kg	25.6 ± 0.33	30.5 ± 0.50	4.83 ± 0.47a
MoFTI 30 mg/kg	25.0 ± 0.36	31.0 ± 0.73	6.00 ± 0.51a

Each value corresponds to the mean ± SE of data from six animals (n = 6 per group). Different letters indicate significant differences ($p < 0.05$) between the groups by Kruskal-Wallis test followed by Dunn's test.

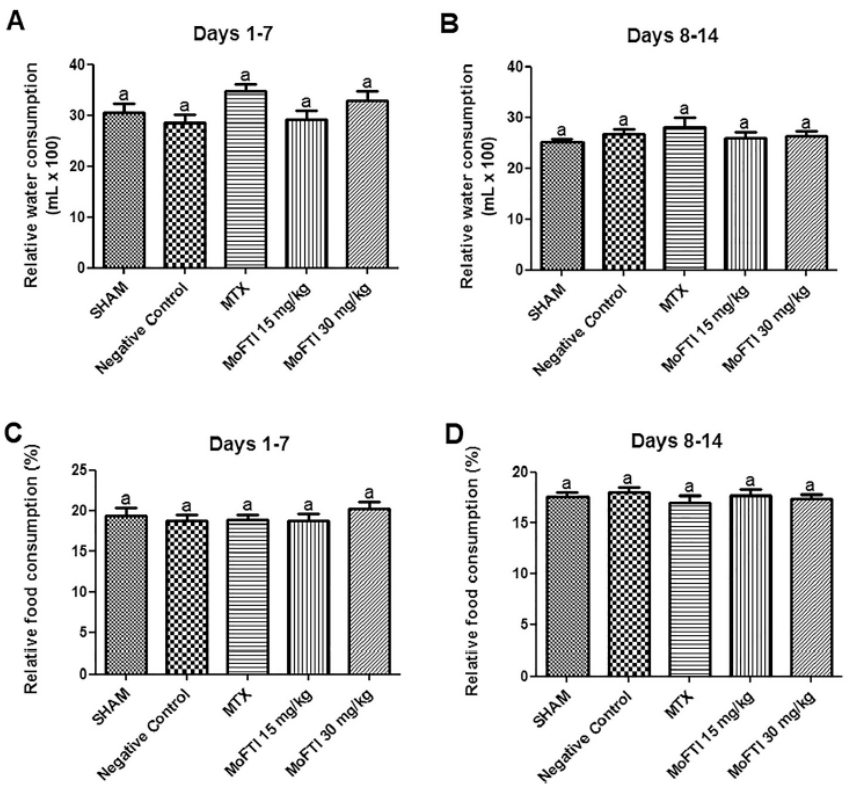

Fig. 7. Relative consumption of water and food by animals after tumor cell inoculation (A and C, Days 1–7) and after treatment (B and D, Days 8–14) with daily intraperitoneal injection of PBS (negative control), MTX (positive control, 1.5 mg/kg) and *Moringa oleifera* flower trypsin inhibitor (MoFTI; 15 and 30 mg/kg). Each bar represents the mean ± SE of the results for six animals (n = 6 per group). Different letters indicate significant differences ($p < 0.05$) by the Kruskal-Wallis test followed by Dunn's test.

Table 2
Relative weight of organs of healthy animals (Sham group) as well as of Sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Moringa oleifera* flower trypsin inhibitor (MoFTI) at 15 and 30 mg/kg.

Organ relative weight (%)	Sham	Negative control	MTX	MoFTI 15 mg/kg	MoFTI 30 mg/kg
Heart*	0.40 ± 0.01 a	0.43 ± 0.01 a	0.41 ± 0.00 a	0.42 ± 0.02 a	0.43 ± 0.02 a
Kidney*	0.55 ± 0.01a	0.65 ± 0.01b	0.56 ± 0.01 a	0.57 ± 0.01 ab	0.59 ± 0.00 ab
Liver*	4.87 ± 0.23 a	5.54 ± 0.30 a	5.20 ± 0.17 a	5.17 ± 0.12 a	4.93 ± 0.12 a
Lung**	0.61 ± 0.02 a	0.66 ± 0.04 a	0.60 ± 0.03 a	0.69 ± 0.01 a	0.62 ± 0.01 a
Spleen**	0.61 ± 0.01 a	0.80 ± 0.06 a	0.54 ± 0.04 a	0.64 ± 0.02 a	0.69 ± 0.04 a
Stomach*	1.13 ± 0.07 a	1.73 ± 0.06b	2.79 ± 0.19 c	1.63 ± 0.07 b	1.78 ± 0.11 b

Each value corresponds to the mean ± SE of data from six animals (n = 6 per group). For each organ, different letters indicate significant differences ($p < 0.05$) between the groups. (*) Parametric data were evaluated by one-way ANOVA followed by Tukey's test. (**) Non-parametric data were assessed by Kruskal-Wallis test followed by Dunn's test.

Table 3
Biochemical parameters of healthy animals (Sham group) as well as of Sarcoma 180 bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Moringa oleifera* flower trypsin inhibitor (MoFTI) at 15 mg/kg and 30 mg/kg.

Parameters	Sham	Negative control	MTX	MoFTI 15 mg/Kg	MoFTI 30 mg/Kg
AST (U/mL)**	91.3 ± 6.53 a	106.4 ± 1.41 a	63.2 ± 7.65 a	100.0 ± 18.2 a	100.0 ± 12.1 a
ALT (U/mL)*	136.0 ± 15.8 a	159.9 ± 16.1 a	128.8 ± 10.6 a	182.2 ± 22.4 a	149.8 ± 9.92 a
Glucose (mg/dL)**	87.5 ± 4.36 a	99.6 ± 14.2 a	117.2 ± 11.9a	144.1 ± 19.2 a	134.7 ± 14.7 a
Cholesterol (mg/dL)*	258.1 ± 24.3 a	298.6 ± 46.0 a	202.7 ± 28.4 a	318.9 ± 87.5 a	305.4 ± 30.3 a
Urea (mg/dL)*	25.8 ± 1.53 a	29.7 ± 2.76 a	23.7 ± 0.96 a	29.5 ± 3.70 a	26.9 ± 0.79 a
Creatinine (mg/dL)**	1.06 ± 0.07 ab	1.22 ± 0.07 a	0.48 ± 0.04 b	1.28 ± 0.05 a	1.08 ± 0.08 ab

AST: aspartate aminotransferase. ALT: alanine aminotransferase. Each value corresponds to the mean ± SE of data from six animals (n = 6 per group). Different letters indicate significant differences ($p < 0.05$) between the groups. (*) Parametric data were evaluated by one-way ANOVA followed by Tukey's test. (**) Non-parametric data were assessed by Kruskal-Wallis test followed by Dunn's test.

active principle involved in the antitumor effect reported for the flowers in folk medicine. In addition, the data support the need for more in-depth studies on the action mechanisms, the activity of MoFTI against other types of tumors, and to determine its therapeutic potential.

CRediT authorship contribution statement

Leydianne Leite de Siqueira Patriota: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Dalila de Brito Marques Ramos:** Conceptualization, Investigation, Methodology. **Angela Caroline Lima Amorim dos Santos:** Investigation. **Yasmym Araújo Silva:** Investigation. **Mariana Gama e Silva:** Investigation. **Diego José Lira Torres:** Investigation. **Thamara Figueiredo Procópio:** Investigation. **Alisson Macário de Oliveira:** Investigation, Formal analysis, Visualization. **Luana Cassandra Breitenbach Barroso Coelho:** Conceptualization, Funding acquisition. **Emmanuel Viana**

Table 4
Hematological parameters of healthy animals (Sham group) as well as of Sarcoma 180-bearing animals treated with PBS (negative control), methotrexate 1.5 mg/kg (MTX) or *Moringa oleifera* flower trypsin inhibitor (MoFTI) at 15 mg/kg and 30 mg/kg.

Parameters	Groups				
	Sham	Negative control	MTX	MoFTI 15 mg/kg	MoFTI 30 mg/kg
Erythrocytes (10^6/mm^3)*	9.44 ± 0.42 a	10.0 ± 0.48 a	8.53 ± 0.11 a	8.94 ± 0.32 a	9.70 ± 0.22 a
Hemoglobin (g/dL)**	15.8 ± 0.32 a	15.6 ± 0.51 a	13.3 ± 0.21 a	13.5 ± 0.44 a	15.9 ± 0.24 a
Hematocrit (%)*	51.8 ± 2.34 ab	57.7 ± 3.54 a	45.1 ± 0.41b	47.0 ± 2.19 ab	54.1 ± 1.36 ab
MCV (fL)**	54.9 ± 0.35 ab	57.5 ± 0.92 a	53.7 ± 0.58 b	55.1 ± 0.59 ab	55.9 ± 0.53 ab
MCH (pg)**	16.1 ± 0.21 a	14.6 ± 1.14 a	15.7 ± 0.24 a	16.3 ± 0.04 a	16.7 ± 0.10 a
MCHC (g/dL)**	29.4 ± 0.30 a	25.6 ± 0.70 a	29.3 ± 0.26 a	29.8 ± 0.18 a	29.9 ± 0.16 a
RDW (%)*	13.7 ± 0.10 ab	14.9 ± 0.68 a	13.0 ± 0.30 b	14.0 ± 0.30 ab	14.4 ± 0.32 ab
Leukocytes (10^3/mm^3)*	7.82 ± 0.40 a	8.63 ± 2.22 a	7.37 ± 0.23 a	8.32 ± 0.76 a	12.1 ± 1,93 a
Lymphocytes (%)*	81.3 ± 0.80 a	79.8 ± 2.41 a	77.1 ± 2.79 a	77.7 ± 0.98 a	80.2 ± 0.46 a
Monocytes (%) **	2.68 ± 0.19 a	4.52 ± 1.20 a	4.00 ± 0.55 a	3.11 ± 0.23 a	2.60 ± 0.06 a
Granulocytes (%)*	15.9 ± 0.68 a	16.5 ± 2.14 a	19.2 ± 2.33 a	19.0 ± 0.92 a	17.2 ± 0.48 a

MCV: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. RDW: red cell distribution width. Each value corresponds to the mean ± SE of data from six animals (n = 6 per group). Different letters indicate significant differences ($p < 0.05$) between the groups. (*) Parametric data were evaluated by one-way ANOVA followed by Tukey's test. (**) Non-parametric data were assessed by Kruskal-Wallis test followed by Dunn's test.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.3 ATIVIDADE ANTI-INFLAMATÓRIA DE MvFL

Os resultados da avaliação da atividade anti-inflamatória de MvFL em camundongos estão incluídos no artigo a seguir, a ser submetido para publicação.

Evaluation of the effects of *Microgramma vacciniifolia* frond lectin (MvFL) on carrageenan-induced peritonitis and paw edema in mice

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Abstract

Anti-inflammatory drugs most used nowadays have a high cost and their prolonged use has been associated with several adverse effects. Thus, the search for new anti-inflammatory agents from plants is increasing. Lectins are carbohydrate-binding proteins that can modulate immune response and release of inflammation mediators. The *Microgramma vacciniifolia* frond lectin (MvFL) was previously reported to be an immunomodulatory agent. This work aimed to evaluate the effects of MvFL on *in vivo* inflammatory status in the carrageenan-induced peritonitis and paw edema, using female Swiss mice. The animals were pretreated intraperitoneally with MvFL (5 and 10 mg/kg). In the peritonitis model, the total and differential migration of leukocytes was evaluated as well as the levels of cytokines, nitric oxide (NO) and total protein in the peritoneal fluid. In the paw edema model, the paw volume was evaluated in the early (from 30 min–2 h) and late (3–4 h) phases of edema formation. MvFL (5 and 10 mg/kg) was capable of reducing neutrophil infiltration, pro-inflammatory cytokines (IL-17A,

TNF-α and IL-6), NO and protein content in the peritoneal fluid. It was also able to inhibit the edema formation in the late phase of the assay. In conclusion, MvFL showed inhibitory effects in *in vivo* acute inflammation, which encouraged future studies exploiting its immunomodulatory ability.

Keywords: plant lectin; cytokines; inflammation; anti-inflammatory agents.

Introduction

Inflammation is a crucial response of the immune system towards injuries, pathogens and toxic compounds (Chen et al., 2018). However, inflammatory dysregulation is involved in several illnesses, including diabetes, cardiovascular diseases, cancer and autoimmune diseases (Zhong and Shi, 2019). Nowadays, the most used anti-inflammatory drugs have a high cost and their prolonged use has been associated with several adverse effects such as adrenal atrophy, osteoporosis, suppression of response to infection or injury, euphoria, cataracts, glaucoma, and peptic ulcers. In this way, the search for new anti-inflammatory agents is increasing, aiming to obtain greater safety and efficacy (Abdulkhaleq et al., 2018).

Lectins are proteins that bind specifically and reversibly to carbohydrates and possess several biotechnological and biomedical applications (Coelho et al., 2017). They have been isolated from the most diverse sources, including plants. Plant lectins have shown strong therapeutic potential (Mishra et al., 2019) and their use has been suggested to combat several pathologies, such as cancer (Patriota et al., 2019a; Gautam et al., 2020); bacterial (Silva et al., 2019a), fungal (Silva et al., 2019b) and viral (Kreis, 2015) infections; and inflammatory conditions (Pires et al., 2019a,b). Lectins can also exert an immunomodulatory action through interaction with glycans present at immune cells surfaces, triggering signal transduction for the modulation of cytokines, for example (Gupta et al., 2020).

Lectins isolated from plants have shown anti-inflammatory activity *in vivo. Canavalia boliviana* seed lectin (Cbol) was able to inhibit carrageenan-induced paw edema and neutrophil migration in a peritonitis model in Wistar rats (Bezerra et al., 2014). The lectin isolated from *Mucuna pruriens* seeds (MPLEC) showed anti-edematogenic properties in the intense edema model caused by carrageenan in Swiss mice (Lacerda et al., 2015). *Bauhinia bauhinioides* seed lectin (BBL) reduced carrageenan-induced paw edema and inhibited leukocyte migration and tumor necrosis factor (TNF-α) release in an experimental model of peritonitis in Wistar rats (Girão et al., 2015). *Tetracarpidium conophorum* seed lectin (TcSL) prevented leukocyte

migration to the peritoneum and reduced carrageenan-induced paw edema in Wistar rats (Oladokun et al., 2019). A seed lectin from *Zizyphus oenoplia* (ZOSL) showed anti-inflammatory activity, preventing anaphylactic shock in Wistar rats (Butle et al., 2016).

Microgramma vacciniifolia frond lectin (MvFL) is a protein with immunomodulatory properties that was able to stimulate *in vitro* the production of TNF-α, interferon-gamma (IFN-γ), interleukin (IL)-6 and nitric oxide (NO) by human peripheral blood mononuclear cells (PBMCs), with a concomitant increase in the release of the regulatory cytokine IL-10. In addition, it promoted the activation and differentiation of T CD8+ cells (Patriota et al., 2017). It has been also showed that MvFL (10 and 20 mg/kg) exhibited antitumor activity against sarcoma 180 in mice, reducing tumor weight in more than 89% and interfering with the angiogenesis around the tumors; in addition, toxic effects were not detected in the animals treated with MvFL at both doses (Patriota et al., 2021).

The previous report on the immunomodulatory activity of MvFL *in vitro* (Patriota et al., 2017) constituted an evidence that this lectin can influence the release of inflammation mediators by immune cells. In the present work, our objective was to evaluate the effects of MvFL on two models of acute inflammation induced by carrageenan in Swiss mice. In the peritonitis model, the total and differential migration of leukocytes were determined as well as the levels of cytokines, NO and total proteins in the peritoneal fluid. In the paw edema model, we evaluated the anti-edematogenic activity of MvFL.

Materials and methods

Purification of MvFL

Fronds of *M. vacciniifolia* were collected at the campus of the *Universidade Federal de Pernambuco* at Recife, Brazil, with authorization (number 36301) from the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio). The access was recorded (A9D147B) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen). Taxonomic identification was confirmed at the herbarium Dárdano de Andrade Lima (*Instituto Agronômico de Pernambuco*, Recife), where a voucher specimen (number 63,291) was archived.

The fronds were washed with tap and distilled water, dried at 28 ºC for 7 days, and then powdered using a blender. Proteins were extracted by homogenization of the powder (10 g) in 0.15 M NaCl (100 mL) for 16 h at 25 ºC using a magnetic stirrer. After filtration through filter

paper and centrifugation (9000 × g, 15 min, 4 ◦ C), it was obtained the frond extract, which was used to isolate MvFL according to the protocol described by Patriota et al. (2017). The purification procedure corresponded to application of the extract onto a Sephadex G-75 (GE Healthcare Life Sciences, Sweden) column (30.0 × 1.5 cm) and subsequent loading of the protein peak with hemagglutinating activity (HA) onto a DEAE-Sephadex A25 (GE Healthcare Life Sciences, Sweden) column (3.0 × 2.0 cm) previously equilibrated with 0.1 M Tris-HCl pH 8.0. MvFL was eluted from the DEAE-Sephadex A25 column with 0.1 M Tris-HCl pH 8.0 containing 1.0 M NaCl.

Protein concentration

Protein concentration was determined according to Lowry et al. (1951) using a standard curve of bovine serum albumin (31.25–500 μg/mL).

Hemagglutinating activity assay (HA)

The carbohydrate-binding ability of lectin was monitored by the hemagglutination assay as described by Patriota et al. (2017) using rabbit erythrocytes treated with glutaraldehyde (Bing et al., 1966). The rabbit erythrocytes were obtained as approved by the Ethics Committee on Animal Experimentation of the *Universidade Federal de Federal de Pernambuco* (process 23076.033782/2015-70). The HA was quantified as the reciprocal value of the highest sample dilution that promoted full agglutination. Specific HA was defined as the ratio between HA and protein concentration (mg/mL).

Animals

The tests were developed at the *Laboratório de Oncologia Experimental* at the *Universidade Federal do Vale do São Francisco* (UNIVASF). Swiss females (*Mus musculus*) from the UNIVASF vivarium, aged between 6 and 8 weeks, were housed in a polypropylene box, at controlled temperature (24 ºC) ans 12-hour light/dark cycles, having *ad libitum* access to water and food. Eight hours before the experiments, the animals were deprived of food but given free access to water. The experimental protocols were approved by the Ethics Committee on Animal Use of UNIVASF under the protocol number 0001/121218.

Carrageenan-induced peritonitis assay

Swiss female mice were divided into four experimental groups of six animals each (n=6). The leukocyte migration was induced by injection of carrageenan (1%, 0.25 mL) into the intraperitoneal cavity 1 h after the administration of MvFL (5 and 10 mg/kg, i.p.), dexamethasone (2 mg/kg, i.p). or vehicle (PBS, i.p.). After 4 h of the stimulus application, the animals were euthanized and the cells were collected from the peritoneal cavity through the application of 3 mL of PBS containing 1 mM EDTA. Immediately, a massage was performed in the abdominal cavity and then the peritoneal fluid was collected with an 3 mL syringe and centrifuged (3.000 rpm for 5 min) at room temperature (Lavor et al., 2018). The supernatant was placed in a tube for subsequent measurement of cytokines, NO and total proteins. The precipitate was used for further total and differential leukocyte counting.

Total and differential leukocyte counting

The peritoneal fluid precipitate was resuspended in 300 µL of PBS-EDTA solution; after homogenization, an aliquot of 10 µL was removed and 200 µL of Turk's solution was added to it for further counting in Neubauer chamber under an optical microscope (Silva et al., 2015). The results were expressed as the number of leukocytes/mL. The leukocyte inhibition (%) was calculated as (1 – T/C) x 100, where T represents leukocyte counts for the treated groups and C represents leukocyte counts for the control (vehicle) (Melo et al, 2011). The differential analysis of leukocytes was performed under an optical microscope, after staining in Giemsa's solution (10%) of smears obtained after centrifuging the peritoneal liquid, being 100 cells counted per slide (Guimarães et al., 2012).

Total protein content

Fluid recovered from the peritoneal cavity of the animals treated with MvFL, dexamethasone or vehicle (PBS) after carrageenan-induced peritonitis were centrifuged (3.000 rpm for 5 min) and the total protein content was quantified in the supernatant according to the method described by Lowry et al. (1951) using a standard bovine serum albumin curve (31.25-500 mg / mL). The results obtained in mg/mL were multiplied by the volume of peritoneal fluid collected (3 mL).

Measurement of cytokine and NO production

Supernatant of peritoneal fluids obtained from animals treated with MvFL, dexamethasone or vehicle (PBS) after carrageenan-induced peritonitis were collected for cytokine quantification using the Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-2, IL-4, IL-6, IL-10, and IL-17A), tumor necrosis factor (TNF), and interferon-gamma (IFN-γ). The assays were performed according to the manufacturer's instructions and data were acquired on the BDAccuri C6 (BD Biosciences). Seven individual cytokine standard curves (0–5000 pg/mL) were generated in each assay. The results were analyzed using the BDAccuri C6 software (BD Biosciences).

Nitrite production was measured by the colorimetric Griess method (Ding et al., 1988). NO concentration was estimated using a standard curve (3,12–400 μmol/mL) and a microplate spectrophotometer (Thermo Fisher Scientific) at 595 nm.

Carrageenan-induced paw edema assay

The carrageenan-induced paw edema model was performed by injecting a volume of 20 μL/animal of 1% carrageenan into the subplantar region in the right hind paw of the mice. The animals were divided into four experimental groups of six mice each (n=6), and treated with MvFL (5 and 10 mg/kg, i.p.), indomethacin (20 mg/kg, i.p.) or vehicle (PBS, i.p.) 1 h before the injection of carrageenan. The volume of the paw was recorded using a plethysmometer (PanLab LE 7500, Spain) by immersing the animals paws up to the lateral malleolus of the heel (Lavor et al., 2018). Measurements were taken before (VA, basal volume) of intraplantar administration of carrageenan and 1, 2, 3 and 4 h after administration (VB) (Silva et al., 2015).

Statistical analysis

The data were expressed as means of replicates ± standard error of the mean (SEM), which were calculated using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences between treatment groups were analyzed using one-way ANOVA followed by Tukey's test using the same software.

Results

MvFL was successfully isolated following the previous established protocol, showing a specific hemagglutinating activity of 10,240, which assures that the carbohydrate-binding ability was working. In the peritonitis assay, the number of total leukocytes in peritoneal cavity (Figure 1A) was significantly ($p < 0.05$) reduced in the treatment with MvFL at 5 mg/kg (leukocyte inhibition of 42.4±2.73 %) when compared with negative control. This effect was similar to that observed for dexamethasone (34.5±1.18 %). At the dose of 10 mg/kg, the MvFL did not reduce significantly ($p > 0.05$) the number of total leukocytes in comparison with negative control. When differential leukocyte evaluation was performed, it was observed that treatments with MvFL at both doses significantly ($p < 0.05$) reduced the percentage of neutrophils in peritoneal fluid (Figure 1B) and increased the percentage of mononucleated cells (Figure 1C), especially lymphocytes, when compared to the negative control. In contrast, dexamethasone treatment increased the proportion of neutrophils and decreased that of mononucleated cells (Figures 1B and 1C).

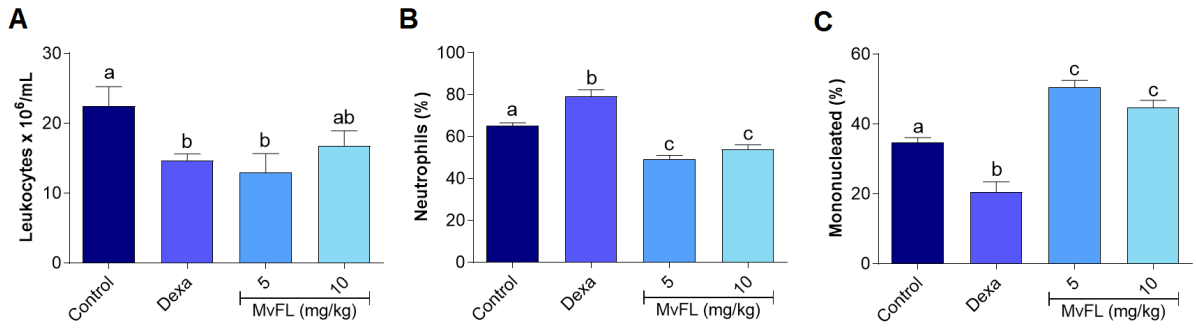

Figure 1. Effects of MvFL (5 and 10 mg/kg) and dexamethasone (2 mg/kg) on leukocyte migration in the peritoneal cavity of mice submitted to carrageenan-induced peritonitis. The graphs show the number of leukocytes per mL (A) and the percentage of neutrophils (B) and mononucleated cells (C) among them. Values are expressed as mean ± standard error of the mean ($n = 6$). Different letters indicated significant ($p < 0.05$) differences between treatments according to ANOVA followed by Tukey's test.

The assessment of cytokine levels (Figure 2) revealed reduced ($p < 0.05$) levels of TNF-α, IL-6, IL-17A and IL-4 in the peritoneal fluid of mice treated with MvFL at both doses (5 and 10 mg/kg) or dexamethasone, when compared to negative control. The levels of IL-10 and IFN-γ did not differ significantly ($p > 0.05$) from the negative control while the IL-2 level was higher

in the group treated with MvFL at 10 mg/kg. Treatments with MvFL (5 and 10 mg/kg) also reduced significantly ($p < 0.05$) the levels of NO (Figure 2H) when compared with negative control, as it was observed for the group treated with dexamethasone.

Plasma extravasation into the stimulated peritoneal cavity was indirectly evaluated by measuring total protein content in the peritoneal fluid. The results indicate that treatments with MvFL (5 and 10 mg/kg) or dexamethasone significantly reduced ($p < 0.05$) the inflammatory exudate (Figure 3) when compared to negative control.

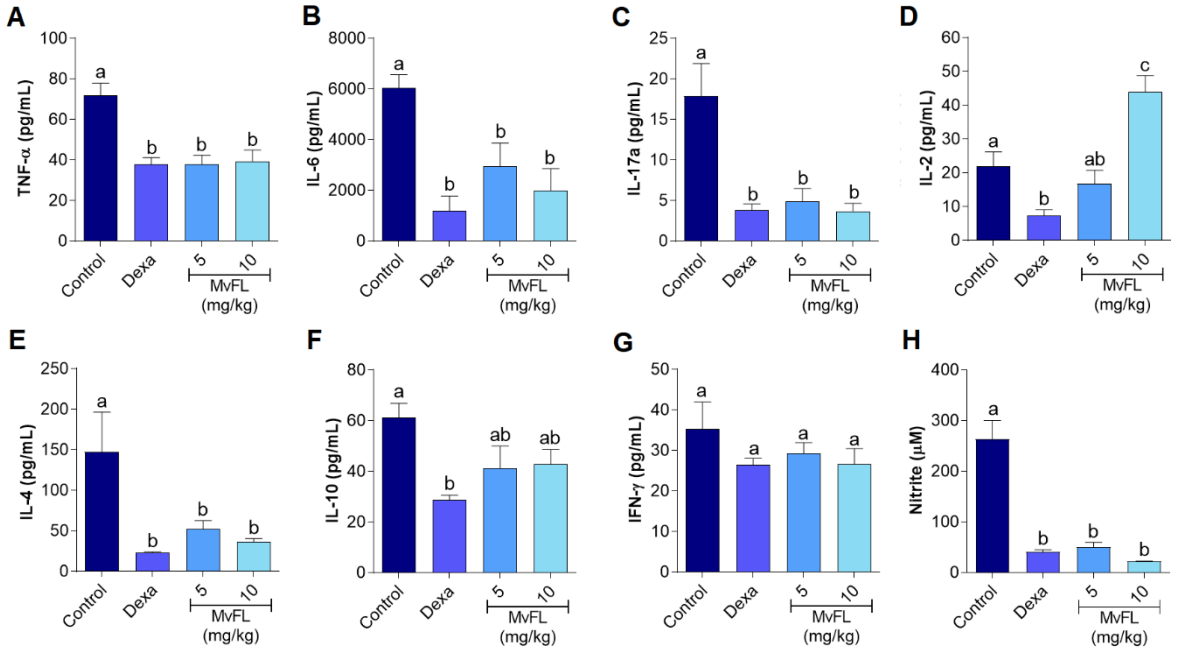

Figure 2. Cytokine and NO levels in the peritoneal fluid of mice submitted to carrageenan-induced peritonitis after pre-treatment with MvFL (5 and 10 mg/kg) or dexamethasone (2 mg/kg). Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicated significant ($p < 0.05$) differences between treatments according to ANOVA followed by Tukey's test.

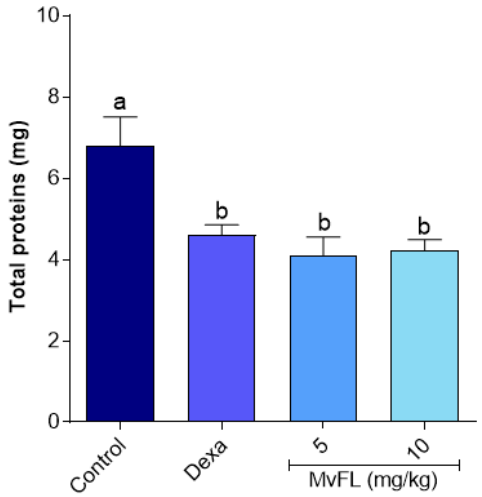

Figure 3. Total protein content in the peritoneal fluid of mice submitted to carrageenan-induced peritonitis after pre-treatment with MvFL (5 and 10 mg/kg) or dexamethasone (2 mg/kg). Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicated significant ($p < 0.05$) differences between treatments according to ANOVA followed by Tukey's test.

The results of the paw edema assay can be seen in Figure 4. The treatments with MvFL (5 and 10 mg/kg) did not inhibit significantly ($p > 0.05$) the formation of the edema in the early phase (1 and 2 h), similarly to the indomethacin-treated group. On the other hand, a significant ($p < 0.05$) edema reduction in groups treated with MvFL at both doses was observed after 3 h and 4 h while the group treated with indomethacin showed significant inhibition only after 4 h.

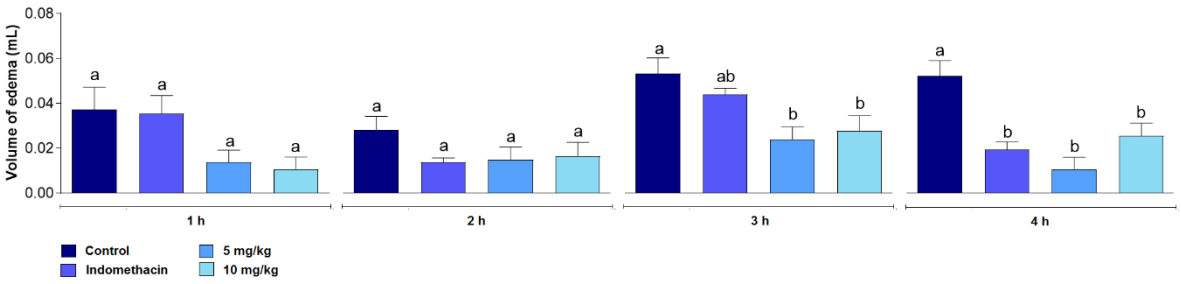

Figure 4. Anti-inflammatory effect of MvFL (5 and 10 mg / kg) and indomethacin (20 mg/kg) on carrageenan-induced paw edema. Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicated significant ($p < 0.05$) differences between treatments according to ANOVA followed by Tukey's test.

Discussion

Modulation of inflammation using compounds from plants is an alternative to conventional therapeutic strategies for numerous diseases (Tasneem et al., 2019). Among the most studied plant compounds, lectins are known as potent immunomodulatory agents, able to act in both innate and adaptive immune system (Jandú et al., 2017; Patriota et al., 2019b) and the anti-inflammatory effects of several plant lectins have been widely reported (Assreuy et al., 1997; Silva et al., 2013; Bezerra et al., 2014; Lacerda et al., 2015; Butle et al., 2016; Pires et al., 2019a,b).

Acute inflammation is the body's first response to harmful stimuli and has two main components: vascular changes and cellular events. The presence of infection or injury is perceived by resident cells, mainly macrophages, but also by other cell types, which secrete molecules (cytokines and other mediators) that induce and regulate the inflammatory response. Carrageenan is a flogistic agent widely employed to induce an acute, non-immune, and highly reproducible inflammation framework in laboratory animals (Oladokun et al., 2019). The peritoneum has been described as a natural hub for leukocytes which rapidly populates the site upon appropriate induction of inflammation; thus, the peritonitis model induced by carrageenan has been widely used (Campos et al., 2016; Pires et al., 2016; Oladokun et al., 2019). The inflammation produced by this model makes it possible to assess the cell migration, participation of cytokines and chemical mediators and the protein extravasation.

In the peritonitis assay, treatments with MvFL at both doses reduced the proportion of neutrophils as well as the levels of TNF-α, IL-6, IL-17A, IL-4, NO and total proteins in the peritoneal fluid. In addition, the total number of leukocytes was lower in the treatment at 5 mg/kg. These results confirm our hypothesis that MvFL could influence the concentration of inflammation mediators released by immune cells, based on the previous findings on the immunomodulatory activity of MvFL *in vitro*. However, the cytokine profile in culture supernatant of MvFL-treated human PBMCs (Patriota et al., 2017) was different from that observed in the peritoneal fluid of MvFL-treated animals. Indeed, the effects of immunomodulatory agents can vary depending on the assay conditions and experimental models. There is a more complex network of interactions between cells and organs in *in vivo* conditions as well as the profile of modulated cytokines depending on the concentration and route of administration (Patriota et al., 2019b). In addition, there was no inflammatory condition in course when the immunomodulatory activity of MvFL was evaluated on PBMCs. Together, the data confirm the interesting immunomodulatory potential of MvFL and reveal that distinct

modulation profiles can be generated *in vitro* or *in vivo* conditions as well as when an inflammatory condition is present or not.

Similar to MvFL, *Lonchocarpus araripensis seed* lectin (LAL) (10 mg/kg) reduced (43 %) leukocyte migration induced by carrageenan in male Wistar rats (Pires et al., 2019a) and the lectin from *Bauhinia monandra* leaves (BmoLL) (15, 30 and 60 mg/kg) significantly reduced (43.5, 54.9, and 60.9%, respectively) the leukocyte migration into the peritoneum of male Swiss mice (Campos et al., 2016). Selectin is an important lectin in leukocyte transmigration into inflammatory sites (Abbas; Lichtman; Pillai, 2019) and the interaction of these cells with an exogenous lectin is a possible factor that can hinder this transmigration (Oladokun et al., 2019).

Neutrophils are recruited at the early hours of inflammation and are usually cleared from inflammatory sites when the early phase of inflammation ends; conversely, lymphocytes are activated and directed to the sites of inflammation at the late phase to mediate chronic inflammatory response (Abbas; Lichtman; Pillai, 2019). As reported above, the treatments with MvFL reduced the percentage of neutrophils and increased the percentage of mononucleated cells, indicating that MvFL seems to favor the lymphocyte response. The *Tetracarpidium conophorum* seed lectin (TcSL) (3, 6 and 12 mg/kg) significantly reduced leukocyte migration into the peritoneum in Wistar rats and a larger percentage of lymphocytes was found in the peritoneal fluid (Oladokun et al., 2019). Similarly, LAL (10 mg/kg) reduced leukocyte migration, rolling and adhesion induced by carrageenan in male Wistar rats (Pires et al., 2019a) and in male Swiss mice, with reduction in the proportion of neutrophils (Pires et al. 2016).

TNF-α is an important mediator of the acute inflammatory response and IL-6 is also important in acute inflammatory responses that have both local and systemic effects. In addition, TNF-α and IL-6 produced at inflammatory sites can enter the blood and be distributed to bone marrow, where they increase the production of neutrophils from the bone marrow progenitors, usually acting in conjunction with colony stimulating factors (Abbas et al., 2015, 2019). The decrease in these cytokines levels in groups treated with MvFL can also be linked to the lower proportion of neutrophils.

IL-17A plays a key role in the acute inflammation and was shown to contribute to the proinflammatory environment by triggering the release of IL-6 and TNF-α. In addition, IL-6 also promotes the differentiation of IL-17-producing T cells (Abbas et al., 2015) and thus, it is understandable that IL-6, TNF-α, and IL-17A levels decreased in the treatments with MvFL. The anti-IL-17A therapy is already a well-established modality of treatment in patients suffering from inflammation (Sud et al., 2018).

Interestingly, IL-2 level in animals treated with MvFL at 10 mg/kg increased when compared to the negative control. IL-2 is the main factor stimulating growth and activation of lymphocytes (Ross and Cantrell, 2018), which were found to be the most abundant cell population that migrated to the inflammatory site after treatment with MvFL. It has been previously described that MvFL is capable of promoting the activation and differentiation of T CD8+ cells (Patriota et al., 2017).

Immunosuppressive cytokines, including IL-4 and IL-10, are typically produced at peripheral sites in a delayed manner to reduce the level of pro-inflammatory cytokine production by activated cells and promote tissue repair (Burmeister and Marriott, 2018). However, treatments with MvFL reduced IL-4 levels and did not affect IL-10 levels. Thus, its anti-inflammatory action seems to be mainly related to reduction in the levels proinflammatory with maintenance of the levels of IL-10 regulatory cytokine.

The modulation of cytokines levels by plant lectins in models of acute inflammation has been described. For example, *Parkia biglobosa* seed lectin (PBL) inducing the release of anti-inflammatory cytokines in a carrageenan-induced peritonitis model (Silva et al., 2013). On the other hand, *Lonchocarpus sericeus* seeds lectin (LSL) decreased leukocyte migration and inflammatory response in male Balb/c mice via inhibition of pro-inflammatory cytokines (Napimoga et al., 2007), similarly to that was detected in the treatments with MvFL.

Inducible Nitric Oxide Synthase (iNOS) is largely expressed after induction by immunologic and inflammatory stimuli and NO has been definitively recognised as one of the key players involved in immunity and inflammation (Lind et al., 2017). Due to its chemical characteristics, NO could rapidly react with other free radicals such as superoxide anion radical ($O_2^{-\bullet}$) to generate the highly reactive oxidant peroxinitrite ($ONOO^-$) and other reactive nitrogen species (RNS), which have been intriguingly associated with many pathological conditions (such as chronic inflammation, autoimmune diseases and cancer), amplifying inflammatory circuits (Predonzani et al., 2015). Thus, the ability of MvFL to strongly reduce NO levels is advantageous in the anti-inflammatory context. The inhibition of NO release can be due to down-regulation of iNOS, since TNF-α induces NO synthesis by activating iNOS (Mansouri et al., 2015).

Proinflammatory mediators such as NO, TNF-α and IL-6 play a major role in the pathogenesis of various inflammatory disorders and serve as potent biomarkers for the assessment of the inflammatory process (Chan et al., 2015; Chen et al., 2018). In addition, the production of these cytokines induces the release of acute-phase response (Abdulkhaleq et al., 2018). Thus, it is highlighted that the ability of MvFL to modulate and reduce all these

mediators can be used therapeutically to control, regulate and limit acute inflammatory processes in order to prevent damages.

During acute inflammation, vasodilation and increased vascular permeability is induced by chemical mediators such as histamine, allowing leukocytes and plasma proteins to enter the extravascular tissues. This causes an increase in the osmotic pressure of the interstitial fluid, leading to a greater efflux of water from the blood to the tissues. The resulting accumulation of protein-rich fluid (exudate) in the extravascular spaces is called edema (Kumar et al., 2018). The carrageenan-induced paw edema is a well-defined model of acute inflammation and has been widely used to evaluate the anti-edematous effect of natural products (Mansouri et al., 2015; Pires et al., 2019a,b). In mice, carrageenan subplantar injection induces a biphasic edema, being then divided into early phase (from 30 min–2 h) and late phase (3–4 h). The early phase of the carrageenan model is mainly increased by vasoactive amines: histamine, serotonin and bradykinin, which increase vascular permeability (Lavor et al., 2018) and are released from mast cells into the surrounding damaged tissues (Karim et al., 2019). The late phase is sustained by prostaglandins, leukotrienes and various cytokines, inclunding IL-6 and TNF-α, produced by tissue macrophages and polymorphnuclear cells (Mansouri et al., 2015; Karim et al., 2019).

Taking into account that MvFL reduced leukocyte migration, pro-inflammatory cytokines, NO and protein leakage in the peritonitis model, we evaluated the anti-edematogenic effect of MvFL in the carrageenan-induced paw edema model. The results suggest that MvFL acts in the second phase of edema development, probably due to the ability to reduce the levels of pro-inflammatory cytokines and chemical mediators like NO, which are important in maintaining this phase of edema.

On the other hand, *Lonchocarpus campestris* seeds lectin (LCaL) (10 mg/kg) inhibited the edema induced by carrageenan in male Swiss mice, mainly in the first phase (Pires et al., 2019b) and TcSL (3 mg/kg, 6 mg/kg, 12 mg/kg) reduced the Wistar rats paw edema, in a dose dependent manner, with the highest dose (12 mg/kg) exhibiting a remarkable minimal edema in the second hour (Oladokun et al., 2019). Otherwise, and similarly to MvFL, LAL (0.1 and 1 mg/kg) reduced the paw edema-time course induced by carrageenan in male Wistar rats, mainly in the second phase (Pires et al., 2019a). In the same way, BmoLL (30 and 60 mg/kg) showed a significant anti-inflammatory activity reducing the paw edema in a dose dependent manner in male Swiss mice, with most significant paw edema reduction being observed after 4 h (Campos et al., 2016).

Conclusion

The present study demonstrated that MvFL when intraperitoneally administered has an *in vivo* anti-inflammatory effect in two models of acute inflammation. MvFL treatment was capable of reducing neutrophil infiltration and decreasing the levels of pro-inflammatory cytokines, NO and in the peritoneal fluid. MvFL is also an anti-edematogenic agent. Hence, the results stimulate more studies on the effects of MvFL on animal models of inflammatory diseases such as asthma.

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4.4 ATIVIDADE ANTI-INFLAMATÓRIA DE MoFTI

Os resultados da avaliação da atividade anti-inflamatória de MoFTI em camundongos estão incluídos no artigo a seguir, que foi submetido para publicação.

The trypsin inhibitor from *Moringa oleifera* flowers (MoFTI) inhibits acute inflammation in mice by reducing cytokine and nitric oxide levels

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Abstract

***Ethnopharmacology relevance*:** The medicinal use of *Moringa oleifera* flowers to treat inflammations, for examples as constituents of tonics, has been reported. Protease inhibitors isolated from plants have been evaluated as potential anti-inflammatory agents and the edible flowers of *Moringa oleifera* contains a trypsin inhibitor called MoFTI.

Aim of the study: In the present work, we evaluated whether MoFTI can be linked to the anti-inflammatory effects reported for *M. oleifera* flowers.

Materials and Methods: Two mouse models of carrageenan-induced acute inflammation (paw edema and peritonitis) were performed. The animals were intraperitoneally administered MoFTI at 15 or 30 mg/kg. The anti-edematogenic effect was assessed by measuring the paw volume during the first and second phases of edema formation. In the peritonitis model, we evaluated the peritoneal fluid for leukocyte migration, total protein content, cytokine levels, and nitric oxide (NO) concentration.

Results: MoFTI reduced paw edema only during the second phase, indicating cytokine modulation. In the peritonitis model, MoFTI reduced leukocyte migration, plasma extravasation (attributed to lower protein content), and the levels of NO and pro-inflammatory (tumor necrosis factor-α, interleukin (IL)-6, and IL-17A) and anti-inflammatory (IL-4 and IL-10) cytokines.

Conclusions: MoFTI promoted an anti-inflammatory activity and its action appears to be mediated via cytokine suppression. Hence, MoFTI is an interesting molecule for evaluation in chronic inflammation models, and as a candidate for the development of novel anti-inflammatory drugs.

Keywords: *Moringa oleifera*; protease inhibitor; acute inflammation; anti-inflammatory agent.

Introduction

Protease functions in processes such as replication, transcription, proliferation, differentiation, signaling cascades, activation, and adhesion are essential for the maintenance of homeostasis and survival of all organisms (Bond et al., 2019). However, if inadequately regulated, the actions of these enzymes can be harmful; thus, proteolytic activities are under a narrow regulatory adjustment, which usually involves the participation of protease inhibitors (Clemente et al., 2019). In some diseases, the homeostasis of proteolytic activity escapes endogenous control, with proteases then considered therapeutic targets. Indeed, protease inhibitors are powerful tools that have gained momentum within the scientific community (Hellinger and Gruber, 2019; Cotabarren et al., 2020).

Inflammation is a physiological response of the immune system that aims to eliminate pathogens, limit tissue damage, and restore homeostasis (Abbas et al., 2019). However, an exacerbated inflammatory condition is a feature of several diseases, including obesity, diabetes,

cancer, asthma, and autoimmune diseases (Zhong and Shi 2019). Proteases have a prominent role in both physiological and pathological inflammatory responses such as pro-inflammatory cytokine production and activation of immune cells; hence, the inhibition of protease activity could control severe inflammation, both acute and chronic (Heutincka et al., 2010; Amiri and Soualmia, 2017). Nonsteroidal and steroidal anti-inflammatory drugs are commonly used for the management of inflammatory conditions; however, these agents are often associated with numerous side effects and a high cost. Therefore, plants used in traditional medicine to treat inflammatory conditions appear to be a viable alternative in the search for safer and effective anti-inflammatory agents (Alhakmani et al., 2013; Abdulkhaleq et al., 2018).

Protease inhibitors are proteins widely distributed in nature, with several isolated from plants and presenting biotechnological and biomedical applications (Rustgi et al., 2018; Hellinger and Gruber, 2019; Cotabarren et al., 2020). Plant-derived protease inhibitors (PPIs) have shown anti-inflammatory activity *in vitro*. Reportedly, a protease inhibitor from *Solanum aculeatissimum* Jacq. (SAPI) inhibits the release of nitrite oxide (NO) and pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6) via mouse macrophages stimulated using lipopolysaccharide (Krishnan and Murugan 2017). The *in vivo* anti-inflammatory effects of PPIs have also been investigated in models of chronic inflammatory lung disease (Martins-Olivera et al., 2016; Almeida-Reis et al., 2017; Theodoro-Júnior et al., 2017; Rodrigues et al., 2019); for example, the trypsin inhibitor from *Crataeva tapia* L. (CrataBL) reportedly controls the hyperresponsiveness of lung inflammation in a model of chronic allergic pulmonary inflammation (Bortolozzo et al., 2018). The Bowman-Birk protease inhibitors from *Glycine max* (L.) Merr. have demonstrated immunoregulatory and anti-inflammatory effects in an experimental autoimmune encephalomyelitis model of multiple sclerosis (Safavi and Rostami, 2012). Additionally, *G. max* inhibitors are known to promote improvements in clinical disease and remission rates in patients with ulcerative colitis (Lichtenstein et al., 2008).

Moringa oleifera Lam. is highly valued owing to its extensive medicinal properties and social importance. The flowers are edible and eaten after cooking, being considered a good source of protein, dietary fibers, and antioxidant compounds (Mehta et al., 2011; Napoleão et al., 2019). It has been recorded the medicinal use of *M. oleifera* flowers aiming to treat inflammations (Anwar et al., 2007; Padayachee and Baijnath, 2020), for example, as tonics (Dadlani, 2018). Alhakmani et al. (2013) reported that *in vitro* anti-inflammatory activity of a *M. oleifera* flower extract could justify the plant use in traditional system of medicine in Oman and other Asian countries. A hot water infusion from flowers was shown to have anti-

inflammatory properties in the carrageenan-induced paw edema (Cáceres et al., 1992). Anti-inflammatory molecules have been detected in seeds (Cretella et al., 2020), flowers (Aliakmon et al., 2013), roots (Cui et al., 2019), and leaves (Oguntibeju et al., 2019) of moringa.

The *M. oleifera* flowers contain a protease inhibitor called MoFTI (*M. oleifera* flower trypsin inhibitor), which is an 18-kDa protein reported to possess insecticidal (Pontual et al., 2014), trypanocidal (Pontual et al., 2018) and antitumor (Patriota et al., 2020) activities. Furthermore, MoFTI has demonstrated immunomodulatory properties in the response of human peripheral blood mononuclear cells towards the infection by *Trypanosoma cruzi* (Nova et al., 2020).

In the present work, we aimed to evaluate whether MoFTI can be linked to the anti-inflammatory effects reported for *M. oleifera* flowers, employing two models of acute inflammation in mice, the paw edema and peritonitis models, both induced using carrageenan. In the paw edema model, we analyzed the anti-edematogenic effect. In the peritonitis model, we evaluated the total and differential migration of leukocytes and measured levels of total proteins, cytokines, and NO in the peritoneal fluid.

Materials and methods

Isolation of MoFTI

Moringa oleifera flowers were collected at the campus of the *Universidade Federal de Pernambuco* (8°02'57.9"S 34°56'47.7"W; Recife, Pernambuco, Brazil), under approval (no. 36301) by the *Instituto Chico Mendes de Conservação da Biodiversidade* from the Brazilian Ministry of Environment and registration (A9D147B) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado*. Plant name has been checked with http://www.theplantlist.org at 29 January 2021. MoFTI was isolated as described by Pontual et al. (2014) following chromatography of an aqueous extract obtained from the flowers using affinity chromatography with trypsin immobilized on agarose. The MoFTI concentration was estimated according to Lowry et al. (1951) and the trypsin inhibitor activity was verified according to Pontual et al. (2014). The purified protein was dialyzed against distilled water (4 h) and dried by lyophilization.

Animals

Female Swiss (*Mus musculus*) mice, 6–8-week-old, were obtained from the bioterium of the *Universidade Federal do Vale do São Francisco* (UNIVASF). The animals were housed at 24ºC with a 12:12 photoperiod, and access to water and food *ad libitum*. Eight hours before commencing the experiment, animals were deprived of food, with free access to water. The experimental procedures described below were approved by the Ethics Committee for Animal Use of the UNIVASF (authorization no. 0001/121218).

Evaluation of anti-inflammatory effect in paw edema model

Experimental groups (n = 6 per group) were treated with intraperitoneal MoFTI (15 and 30 mg/kg), indomethacin (20 mg/kg; positive control), or phosphate-buffered saline (PBS, negative control). One hour after administration, paw edema was induced by injecting 20 µL of 1% (w/v) carrageenan into the subplantar region of the right hind mouse paw. The paw volume was measured immediately before the carrageenan injection (basal volume, V_A) and after 1, 2, 3, and 4 h (V_B) using a LE 7500 plethysmometer (PanLab, Barcelona, Spain), by immersing the paws up to the lateral malleolus of the heel (Lavor et al., 2018). The results are expressed as the inhibition of paw edema, which was calculated using the formula: Inhibition (%) = $[(V_B - V_A) / V_A] \times 100$. Three measurements were performed for each animal at each investigated time point.

Evaluation of anti-inflammatory effect in the peritonitis model

Peritonitis assay

Experimental groups (n = 6 per group) were treated with intraperitoneal MoFTI (15 and 30 mg/kg), dexamethasone (2 mg/kg), or PBS. One hour after administration, inflammation was induced by intraperitoneally administering 250 µL of 1% (w/v) carrageenan into the peritoneal cavity. The animals were sacrificed by cervical dislocation 4 h after treatment administration and the peritoneal cavity was washed with 3 mL of sterile PBS containing 1 mM ethylenediamine tetraacetic acid (EDTA). The abdominal cavity was massaged and peritoneal fluid (3 mL) was collected and centrifuged (2,000 *g*, 5 min). The supernatant was used to measure the total protein content, NO, and cytokine levels following protocols described below.

The precipitate was resuspended in 300 μL of the PBS-EDTA solution, and an aliquot of 10 μL was removed and added to 200 μL of Turk's solution for further total leukocyte counting.

Total and differential leukocyte counting

The total leukocyte count was determined using a Neubauer chamber with an optical microscope. The results are expressed as the number of leukocytes/mL. The percentage of leukocyte inhibition was calculated as: Inhibition (%) = [(1 – *T*) /*C*)] × 100, where *T* represents leukocyte number in the treated group and *C* represents leukocyte number in the control group. Differential analysis of leukocytes was performed under optical microscopy after staining smears (prepared with the precipitate) with Giemsa's solution (10%). One hundred cells were counted per slide and the results are expressed as a percentage of the total number of leukocytes.

Total protein content

The protein concentration in the peritoneal fluid supernatant was estimated according to Lowry et al., (1951) using bovine serum albumin (31.25–500 μg/mL) as the standard.

Measurement of cytokine and NO production

Furthermore, interleukins (IL-2, IL-4, IL-6, IL-10, and IL-17A), TNF-α, and interferon-gamma (IFN-γ) levels were determined in the supernatant. The measurements were performed using the Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Seven individual cytokine standard curves (0–5000 pg/mL) were additionally performed. Data were acquired on the BD Accuri C6 cytometer (BD Biosciences) and the results were analyzed using the BD Accuri C6 software (BD Biosciences).

As an indicator of NO production, the nitrite concentration in the peritoneal fluid supernatant was determined according to the Griess method (Ding et al., 1988). A standard curve of sodium nitrite (3.12–400.00 μmol/mL) was used.

Statistical analysis

Significant differences between treatment groups were analyzed using one-way ANOVA, followed by Tukey's test using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA). The data are expressed as means of replicates ± standard error of the mean. Results were considered statistically significant if the confidence level was 95% ($p < 0.05$).

Results

In the present study, the anti-inflammatory effect of MoFTI was initially evaluated in the carrageenan-induced paw edema model. Figure 1A shows that MoFTI (15 and 30 mg/kg) did not significantly ($p > 0.05$) reduce paw edema during the first phase, corresponding with data collected after 1 and 2 h of carrageenan administration. However, during the second phase (3 and 4 h after administration), edema was significantly reduced ($p < 0.05$) following treatment with both MoFTI doses (15 and 30 mg/kg) when compared with the negative control (Figure 1B). Indomethacin, the positive control, showed an anti-edematogenic effect during the second phase.

In the peritonitis model, MoFTI (30 mg/kg) reduced (38.5%) the leukocyte migration into the peritoneal cavity when compared with the control; the positive control dexamethasone inhibited migration by 34.5% (Figure 2A). In the MoFTI group treated with 15 mg/kg, leukocyte migration was only reduced by 10.1%. In MoFTI treated groups, the highest percentage of migrating leukocytes was composed of neutrophils (Figure 2B), followed by lymphocytes (Figure 2C). However, no significant differences ($p > 0.05$) were observed between the migrating cell populations on comparing MoFTI treatments and negative control. Dexamethasone increased the neutrophil population, decreasing that of mononucleated cells.

Plasma extravasation into the mouse peritoneal cavity was evaluated by measuring the total protein content in the peritoneal fluid. Treatment with both MoFTI doses and dexamethasone significantly ($p < 0.05$) reduced the protein content in the peritoneal fluid when compared with the negative control (Figure 3).

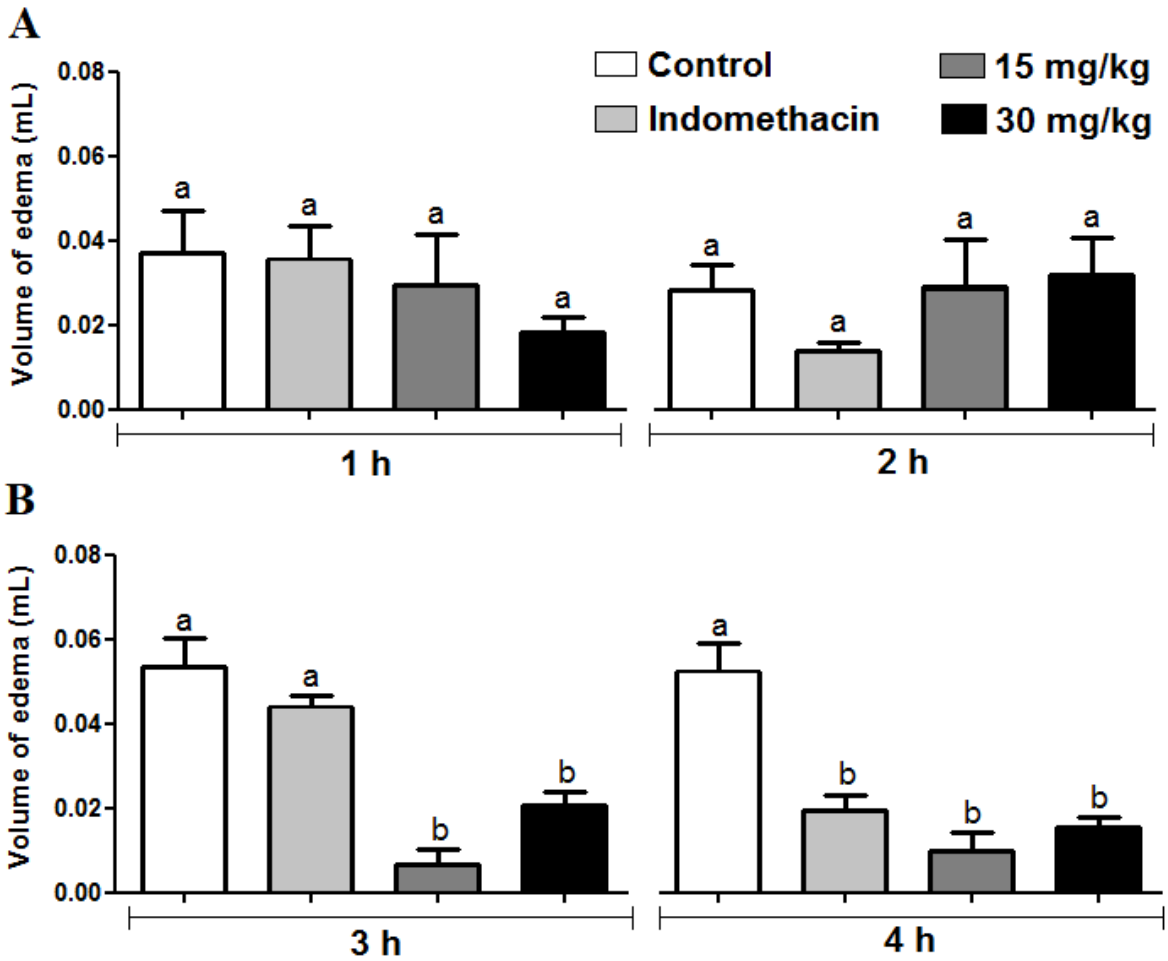

Figure 1. Evaluation of the anti-inflammatory effects of the *Moringa oleifera* flower trypsin inhibitor (MoFTI, 15 and 30 mg/kg) and positive control indomethacin (20 mg/kg) during the first phase (A) and second phase (B) of carrageenan-induced paw edema. The first phase corresponded to the period until 2 h after the administration of the phlogistic agent; second phase results were obtained 3 and 4 h later. Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicate significant differences ($p < 0.05$) when compared with the PBS treated group (control). PBS, phosphate-buffered saline.

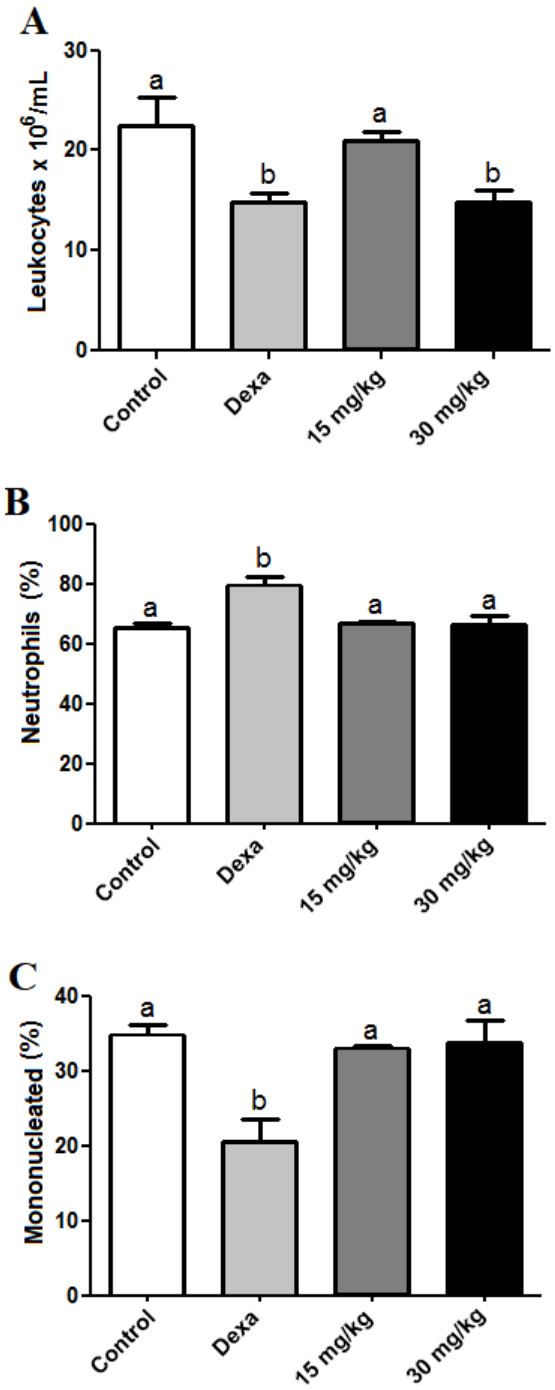

Figure 2. Evaluation of the anti-inflammatory effects of the *Moringa oleifera* flower trypsin inhibitor (MoFTI, 15 and 30 mg/kg) and positive control dexamethasone (2 mg/kg, Dexa) by determining total (A) and differential (B and C) leukocyte counts in the peritoneal cavity of mice presenting carrageenan-induced peritonitis. Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicate significant differences ($p < 0.05$) when compared with the PBS treated group (control). PBS, phosphate-buffered saline.

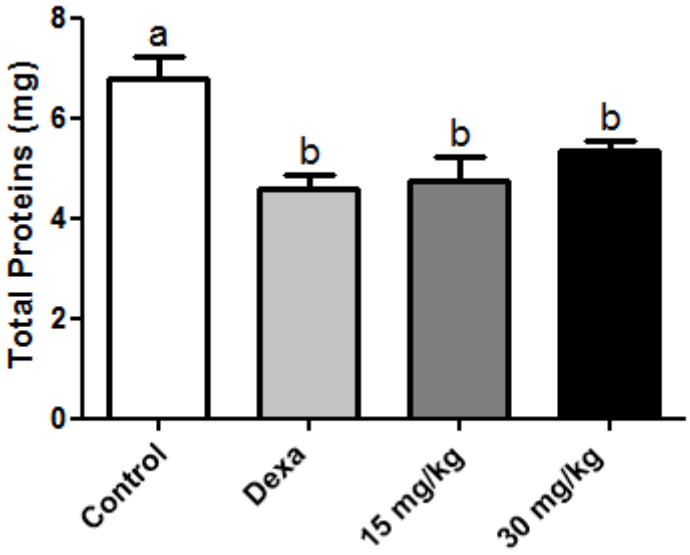

Figure 3. Total protein content in the peritoneal fluid collected from mice presenting carrageenan-induced peritonitis and treated with the *Moringa oleifera* flower trypsin inhibitor (MoFTI, 15 and 30 mg/kg) and positive control dexamethasone (2 mg/kg, Dexa). Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicate significant differences ($p < 0.05$) when compared with the PBS treated group (control). PBS, phosphate-buffered saline.

Regarding cytokine levels, MoFTI (30 mg/kg) significantly ($p < 0.05$) decreased the level of TNF-α (Figure 4A) when compared with the control. Additionally, both MoFTI doses (15 and 30 mg/kg) significantly ($p < 0.05$) reduced the levels of IL-6 (Figure 4B) and IL-17A (Figure 4C), while IL-2 levels remained unaltered (Figure 4D). MoFTI (15 mg/kg) significantly ($p < 0.05$) stimulated the production of IFN-γ when compared with the negative control; however, this result was not observed in the 30 mg/kg MoFTI group (Figure 4E). Finally, MoFTI significantly ($p < 0.05$) decreased the levels of IL-10 (Figure 4F) and IL-4 (Figure 4G) when compared with the control. As expected owing to the suppression of pro-inflammatory cytokine levels, MoFTI (15 and 30 mg/kg) reduced NO levels when compared with the control (Figure 4H). Dexamethasone significantly ($p < 0.05$) decreased the levels of TNF-α, IL-6, IL-17A, IL-2, IL-10, IL-4, and NO when compared with the control (Figure 4).

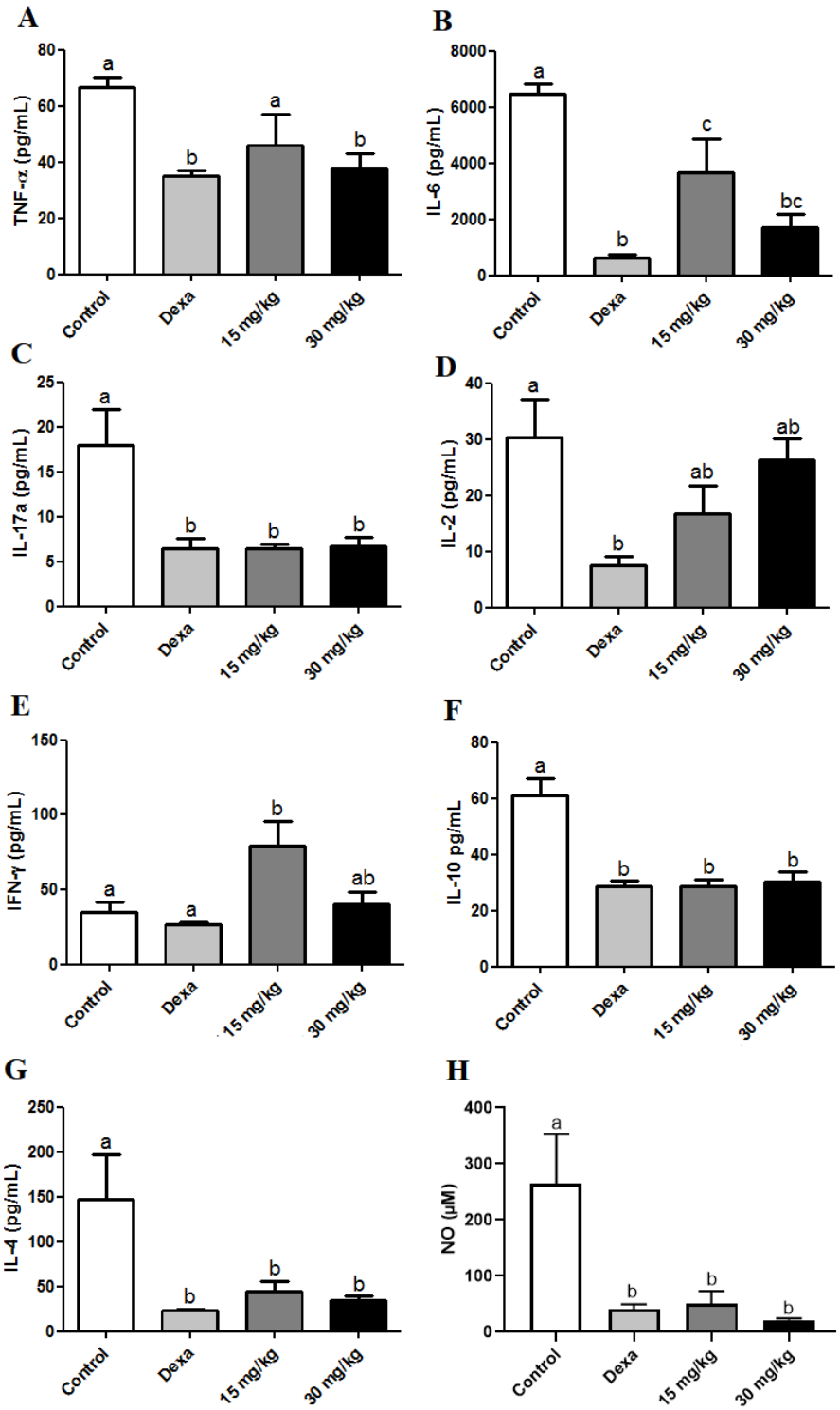

Figure 4. Cytokine and nitric oxide (NO) levels in the peritoneal fluid collected from mice presenting carrageenan-induced peritonitis and treated with the *Moringa oleifera* flower trypsin inhibitor (MoFTI, 15 and 30 mg/kg) and positive control dexamethasone (2 mg/kg, Dexa). Values are expressed as mean ± standard error of the mean (n = 6). Different letters indicate significant differences ($p < 0.05$) when compared with the PBS treated group (control). PBS, phosphate-buffered saline.

Discussion

Proteases are involved in several aspects of inflammation and immune system regulation, processing numerous effector molecules such as cytokines and surface recognition motifs for cell-cell communication in antigen presentation. Reportedly, PPIs are known to target human proteases and modulate their functionality (Hellinger and Gruber, 2019). Accumulated evidence has revealed that protease inhibitors possess both preventive and therapeutic effects on several inflammatory processes (Cotabarren et al., 2020). In the present study, we evaluated the anti-inflammatory activity of the protease inhibitor MoFTI and investigated the possible action mechanisms.

Carrageenan is a widely used phlogistic agent that promotes acute inflammation. Carrageenan-induced inflammation has been typically employed to investigate the pathophysiology of acute inflammation and to evaluate the efficacy of drugs during inflammation. This model is associated with improved edema, leukocyte migration, protein extravasation, as well as cytokine and NO release (Lavor et al., 2018; Oladokun et al., 2019). Thus, we used carrageenan to induce paw edema and peritonitis in mice and then evaluated the anti-inflammatory effects of MoFTI.

In mice, a subplantar carrageenan injection induces a biphasic edematogenic response, divided into the first (from 1 h until 2 h) and second (3–4 h after injection) phases (Lavor et al., 2018; Pires et al., 2019). The first phase is associated with the action of mediators such as vasoactive amines (histamine, for example) that increase vascular permeability (Lavor et al., 2018); therefore, the results indicated that MoFTI did not possess an antihistaminic effect. However, this inhibitor was effective against the second phase, which is maintained by cytokines (Karim et al., 2019). Thus, for MoFTI, the mechanism of action could involve the modulation of cytokine release.

Like MoFTI, other PPIs have been evaluated for anti-inflammatory effects in edema models. The protease inhibitor isolated from *Leucaena leucocephala* (Lam.) de Wit seeds (LlTI), at 5 mg/kg, decreases paw edema induced by carrageenan or heat in male Wistar rats (Oliva et al., 2000). Furthermore, the inhibitor derived from *Bauhinia bauhinioides* (Mart.) J.F.Macbr. seeds (BbCI, 2.5 mg/kg) reduces carrageenan-induced paw edema in male Wistar rats, with a strong inhibitory effect on leukocyte migration (90%) in inflamed tissues (Oliveira et al., 2010). Reportedly, BbCI and the protease inhibitor derived from *Caesalpinia echinata* Lam. seeds (CeEI) prevent acute edematous lung inflammation, inhibiting proteases released by neutrophils, especially elastase (Neuhof et al., 2003; Cruz-Silva, et al., 2013).

Moreover, some *M. oleifera* preparations and compounds have been evaluated against carrageenan-induced paw edema. In rats, an aqueous root extract (750 mg/kg) has demonstrated anti-inflammatory activity by significantly inhibiting the development of edema at 1, 3, and 5 h (Ndiaye et al., 2002). In mice, paw edema was reportedly reduced following treatment with an ethanolic extract prepared from the seeds, as well as with hot water infusions prepared from the leaves, seeds, flowers, roots, and bark (Guevara et al., 1999; Padayachee and Baijnath, 2020).

Additionally, MoFTI demonstrated anti-inflammatory activity in the peritonitis model by reducing leukocyte migration. Several PPIs have been effective in other models of acute inflammation. For example, a trypsin inhibitor derived from the seeds of *Erythrina velutina* Willd. (EvTI) has demonstrated anti-inflammatory effects by reducing neutrophil infiltration (71%) in an experimental model of sepsis in Swiss mice (Machado et al., 2013). In a carrageenan-induced pleurisy model, BbCI (2.5 mg/kg) reportedly reduced neutrophil migration into the pleural cavity by 60% in male Wistar albino rats (Oliveira et al., 2010). Native and recombinant protease inhibitors derived from the seeds of *C. echinata*, CeKI (7.8 and 2.6 mg/kg) and rCeEI (0.84 and 2.6 mg/kg), reduced leukocyte infiltration into the alveolar space in an LPS-induced lung acute inflammation model established in male Wistar rats (Cruz-Silva et al., 2016). PPIs have been widely evaluated in chronic inflammation models, acting on leukocyte migration. For example, CrataBL and the trypsin inhibitor derived from seeds of *Enterolobium contortisiliquum* (Vell.) Morong (EcTI), both at 2 mg/kg, reduce the number of eosinophils, as well as inflammation, in the airways and alveolar septa in an ovalbumin-induced model of chronic allergic pulmonary inflammation in BALB/c mice (Bortolozzo et al., 2018; Rodrigues et al., 2019).

Among leukocytes, polymorphonuclear cells are the first inflammatory cell lines to enter tissues during inflammation (Abbas et al., 2019). Among polymorphonuclear cells, neutrophils compose the major type and are a source of several serine proteases, including elastase, proteinase 3, and cathepsin G (Oliveira et al., 2010; Almeida-Reis et al., 2017). During inflammation, these enzymes can cleave several cellular substrates, and under pathological conditions, they can damage host tissues and play a role in the pathogenesis of inflammatory diseases (Cruz-Silva et al., 2016). Thus, regulation of proteolysis induced by these serine proteases is essential to prevent self-induced damage (Heutincka et al., 2010). Furthermore, neutrophil proteases could mediate the chemotactic action of PMNs, and the inhibition of these enzymes is a key event to reduce PMN migration (Cruz-Silva et al., 2016). As previously described, MoFTI is a serine protease inhibitor known to lower the trypsin-like proteolytic

activity (Pontual et al., 2014). Therefore, the inhibition of leukocyte migration can be attributed to the action of MoFTI on respective serine proteases. Oliveira et al., (2010) have reported that BbCI, a cathepsin G inhibitor, strongly inhibits leukocyte rolling, adhesion, and migration in inflammation models induced by carrageenan.

Moreover, the proteolytic activity of matrix-metalloproteinases (MMP) modulates vascular permeability at the site of inflammation, a factor that may facilitate leukocyte migration (Fingleton, 2017). Reportedly, PPIs can inhibit these MMP (Martins-Olivera et al., 2016; Almeida-Reis et al., 2017; Theodoro-Júnior et al., 2017; Rodrigues et al., 2019). Therefore, MMPs are another possible target of MoFTI to reduce leukocyte migration.

The extravasation and accumulation of plasma proteins at the site of inflammation is another characteristic of acute inflammation, leading to edema (Abdulkhaleq et al., 2018). The data obtained for total protein content in the peritoneal fluid indicated that MoFTI treatment reduced the inflammatory exudate. This finding is consistent with the anti-edematogenic effect of MoFTI in the paw edema model.

Furthermore, serine proteases act as modulators of the immune system by regulating cytokine production. The extracellular microenvironment of inflammatory tissues can be modulated by serine proteases via the regulation of specific cell surface receptors such as toll-like receptors (TLRs) or proteinase-activated receptors (PARs). PAR-1, which can be activated by trypsin, results in the production of several pro-inflammatory cytokines (Shigetomi et al., 2010). Moreover, trypsin-like serine proteases mediate their functions by activating inflammatory cells through PAR2 (Shamsi et al., 2018). Among pro-inflammatory cytokines, MoFTI decreased the levels of TNF, IL-6, and IL-17A, which can be attributable to the interference in trypsin-like activities involved in inflammation. Like MoFTI, EvTI (Machado et al., 2013), EcTI (Theodoro-Júnior et al., 2017), BbCI (Almeida-Reis et al., 2017), and the recombinant *Bauhinia bauhinioides* kallikrein inhibitor (rBbKI) (Martins-Olivera et al., 2016) have revealed suppressive effects on TNF-α, while CrataBL reportedly attenuates IL-17 (Bortolozzo et al., 2018) in the respective inflammation models employed by these authors. The results showed that MoFTI stimulated the production of IFN-γ only when administered at a lower dose (15 mg/kg). Despite its inflammatory activities, contradictory findings have indicated that IFN-γ could be protective in some inflammatory disease models (Shachar et al., 2013).

Additionally, MoFTI decreased the levels of anti-inflammatory cytokines IL-4 and IL-10. Anti-inflammatory cytokines antagonize and limit the release of pro-inflammatory cytokines, acting as protective mediators (Burmeister and Marriott, 2018). Although an increase

in levels of these cytokines could be expected owing to their beneficial effects on inflammation, their suppressive effects on inflammation were possibly not essential, as MoFTI was already demonstrating this role. A similar result was observed with the positive control dexamethasone. Likewise, CrataBL treatment reduces the levels of both pro-inflammatory and anti-inflammatory cytokines (Bortolozzo et al., 2018).

NO is another mediator that participates in the inflammatory processes. Inducible nitric oxide synthase (iNOS) can act in various tissues and organs, and its expression is typically increased by cytokines such as IFN-γ and TNF-α (Abbas et al., 2019). NO produced by iNOS demonstrates a pro-inflammatory role and contributes to the amplification of the inflammatory response. As expected owing to the suppressive effect of MoFTI on pro-inflammatory cytokines, MoFTI treated groups presented low NO levels. The inhibitors EcTI (Theodoro-Júnior et al., 2017; Rodrigues et al., 2019), CrataBL (Bortolozzo et al., 2018), BbCI (Almeida-Reis et al., 2017), and rBbKI (Martins-Olivera et al., 2016) reportedly reduce the number of iNOS-positive cells in the respective animal models of inflammation.

Conclusion

MoFTI promoted an anti-inflammatory activity and its action appears to be mediated via cytokine suppression. This is supported by the fact that MoFTI demonstrated anti-edematogenic effects only during the second phase of the paw edema model, as well as the decreased levels of pro-inflammatory cytokines in the peritoneal fluid. The anti-inflammatory effect of MoFTI in the peritonitis model was accompanied by reduced leukocyte migration, plasma extravasation, and NO levels. Hence, MoFTI is an interesting molecule for evaluation in chronic inflammation models, and as a candidate for the development of novel anti-inflammatory drugs.

Author contributions

Leydianne Leite de Siqueira Patriota: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Dalila de Brito Marques Ramos, Mariana Gama e Silva, Angela Caroline Lima Amorim dos Santos, Yasmym Araújo Silva, Amanda de Oliveira Marinho:** Data curation, Formal analysis, Investigation, Methodology. **Emmanuel Viana Pontual, Luana Cassandra Breitenbach Barroso Coelho, Patrícia Maria Guedes Paiva:**

Conceptualization, Methodology, Funding acquisition, Resources. **Rosemairy Luciane Mendes:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Visualization. **Thiago Henrique Napoleão:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of competing interests

The authors declare no competing interests.

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5 CONCLUSÕES

- A lectina MvFL é um potente agente antitumoral no modelo de sarcoma 180 e não apresentou toxicidade para os animais nas doses testadas. O efeito antitumoral de MvFL pode estar ligado à atividade citotóxica da lectina através da indução de apoptose das células tumorais e também devido a uma atividade antiangiogênica.
- O inibidor de tripsina MoFTI também apresentou forte potencial antitumoral *in vivo* contra o sarcoma 180. A atividade antitumoral de MoFTI pode estar relacionado com indução de apoptose e necrose nas células tumorais e efeito antiangiogênico. Tratamento com MoFTI também não resultou em evidências de toxicidade aos camundongos nas doses testadas.
- A capacidade imunomoduladora de MoFTI e MvFL podem estar ligadas à ação antitumoral e futuros estudos estão sendo elaborados com o objetivo de esclarecer esse envolvimento. Os resultados estimulam estudos da atividade de MoFTI e MvFL contra outros tipos de tumores e determinação de seu potencial terapêutico.
- MvFL e MoFTI apresentam efeitos anti-inflamatório nos dois modelos de inflamação aguda. Ambas as proteínas foram capazes de reduzir a infiltração de leucócitos na cavidade peritoneal, bem como reduzir os níveis de citocinas pró-inflamatórias e NO e o extravasamento de proteínas no líquido peritoneal. Além disso, foram capazes de inibir a formação do edema de pata na fase tardia do processo inflamatório, indicando que atuam sobre os níveis de mediadores como citocinas e NO.
- Os resultados obtidos nesse estudo confirmam o potencial biomédico dessas duas proteínas bioativas isoladas de plantas tanto no câncer quanto na inflamação, estimulando estudos que ampliem o conhecimento sobre os mecanismos envolvidos em seus efeitos.

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