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AVALIAÇÃO TOXICOLÓGICA E ATIVIDADES ANTIBACTERIANA, ANTI-INFLAMATÓRIA, ANTINOCICEPTIVA E ANTITUMORAL DE EXTRATO E FRAÇÃO LECTÍNICA DO RIZOMA DE *Microgramma vacciniifolia*

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutora em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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Dedico essa tese aos meus amados pais Gilberto Amaro (*in memorian*) e Maria Cavalcante (*in memorian*), meus eternos exemplos de fortaleza.

“Levanto os olhos para os montes: de onde me virá auxílio?
Meu auxílio vem do SENHOR, que fez o céu e a terra.
Não deixará teu pé vacilar, aquele que te guarda não dorme.
Não dorme, nem cochila o vigia de Israel.
O SENHOR é o teu guarda, o SENHOR é como sombra que te cobre, e está à tua direita.
De dia o sol não te fará mal nem a lua de noite.
O SENHOR te preservará de todo mal, preservará tua vida.
O SENHOR vai te proteger quando sais e quando entras, desde agora e para sempre.”

(Salmo 121, 1-8)

RESUMO

Microgramma vacciniifolia é uma planta utilizada na medicina tradicional cujo rizoma contêm uma lectina denominada MvRL. Esta tese avaliou a toxicidade aguda, genotoxicidade, efeitos antinociceptivo e anti-inflamatório de extrato salino do rizoma e fração proteica contendo MvRL. Adicionalmente, a fração foi avaliada quanto às atividades antitumoral e antibacteriana. Extrato e na fração foram avaliados quanto à presença de metabólitos secundários por CCD e CLAE, bem como presença de lectina pelo ensaio de atividade hemaglutinante (AH). Toxicidade aguda em camundongos (doses: 1.000, 2.000 e 5000mg/kg *per os*) foi avaliada utilizando parâmetros bioquímicos, hematológicos e histológicos e genotoxicidade *in vivo* (doses 1.000 e 2.000mg/kg) foi determinada pelos ensaios do micronúcleo e cometa em células de sangue periférico e medula óssea. O potencial antinociceptivo do extrato (100 a 400mg/kg *per os*) e fração (25 a 100mg/kg *per os*) foi estudado nos modelos de contorções abdominais induzidas por ácido acético, teste da placa quente e teste da formalina, ao passo que o potencial anti-inflamatório foi avaliado nos modelos de edema de pata, peritonite, bolsão de ar e granuloma. O ensaio antitumoral *in vivo* utilizou camundongos portadores de carcinoma de Ehrlich e o tratamento com a fração foi realizado por VO (100 e 200mg/kg). Foi realizada marcação imunohistoquímica dos tumores para p-53 e ki-67. Atividade antibacteriana da fração foi investigada *in vitro*, através da determinação das CMI e CMB, bem como *in vivo* utilizando larvas de *Tenebrio molitor* infectadas. Foi detectada a presença ácido clorogênico (0,135g%) e derivados cinâmicos (0,271g%) no extrato, enquanto a fração não apresentou nenhum dos metabólitos pesquisados. Extrato e fração não causaram morte dos camundongos no teste de toxicidade aguda; porém, a fração, exceto na dose de 1.000 mg/kg, causou perda de peso e danos no fígado, pulmões e rins, evidenciados histologicamente e/ou bioquimicamente. Ambas as preparações se mostraram seguras do ponto de vista genotóxico e mostraram atividade antinociceptiva, sendo eficazes em diminuir o número de contorções abdominais, aumentar o período de latência para percepção do estímulo da placa quente e reduzir a frequência de lambidas de pata no teste da formalina. Potencial anti-inflamatório foi detectado para extrato e fração, com diminuição da migração leucocitária nos testes de peritonite e bolsão de ar e redução do nível da citocina pro-inflamatória TNF-α no exsudato coletado do bolsão. Ainda, ação anti-inflamatória envolvendo atuação na via da bradicinina foi detectada do teste de edema de pata. A fração apresentou efeito antitumoral frente a carcinoma de

Ehrlich, como demonstrado pela diminuição de peso dos tumores, redução de marcação imunohistoquímica de antígeno de proliferação celular (p-53) e aumento da marcação de antígeno relacionado à supressão tumoral (ki-67). Contudo, a fração apresentou atividade antibacteriana contra *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* e *Staphylococcus aureus* com valores de CMI de 31,2 a 125,0μg/mL e CMB de 2,5 a 200μg/mL. Atividade *in vivo* contra as mesmas bactérias foi detectada no modelo usando *T. molitor*. Em conclusão, as preparações do rizoma de *M. vacciniifolia* apresentam potencial para estudos visando o desenvolvimento de drogas analgésicas, anti-inflamatórias, antibacterianas e antitumorais.

Palavras-chave: *Microgramma vacciniifolia*. Efeito analgésico. Lectina. Ácido Clorogênico. Inflamação. Atividade antimicrobiana Avaliação antitumoral.

ABSTRACT

Microgramma vacciniifolia is a plant used in traditional medicine whose rhizome contains a lectin called MvRL. This thesis evaluated the acute toxicity, genotoxicity, antinociceptive and anti-inflammatory effects of saline rhizome extract and protein fraction containing MvRL. Additionally, the fraction was evaluated for antitumor and antibacterial activities. Extract and fraction were evaluated for the presence of secondary metabolites by CCD and HPLC, as well as the presence of lectin by the hemagglutinating activity test (HA). Acute toxicity in mice (doses: 1,000, 2,000 and 5000mg/kg per os) was assessed using biochemical, hematological and histological parameters and in vivo genotoxicity (doses 1,000 and 2,000mg/kg) was determined by micronucleus and comet assays in cells of peripheral blood and bone marrow. The antinociceptive potential of the extract (100 to 400mg/kg per os) and fraction (25 to 100mg/kg per os) was studied in the models of abdominal contortions induced by acetic acid, hot plate test and formalin test, whereas anti-inflammatory potential was evaluated in the models of paw edema, peritonitis, air pocket and granuloma. The in vivo antitumor assay used mice with Ehrlich carcinoma and treatment with the fraction was performed by VO (100 and 200mg/kg). Immunohistochemical marking of the tumors was performed for p-53 and ki-67. Antibacterial activity of the fraction was investigated in vitro, by determining CMI and CMB, as well as in vivo using infected Tenebrio molitor larvae. The presence of chlorogenic acid (0.135g%) and cinnamic derivatives (0.271g%) was detected in the extract, while the fraction did not present any of the investigated metabolites. Extract and fraction did not cause death of the mice in the acute toxicity test; however, the fraction, except at the dose of 1,000 mg/kg, caused weight loss and damage to the liver, lungs and kidneys, evidenced histologically and / or biochemically. Both preparations proved to be safe from a genotoxic point of view and showed antinociceptive activity, being effective in reducing the number of abdominal contortions, increasing the latency period for perceiving the hot plate stimulus and reducing the frequency of paw licking in the formalin test. Anti-inflammatory potential was detected for extract and fraction, with reduced leukocyte migration in the peritonitis and air pocket tests and reduced level of pro-inflammatory cytokine TNF-α in the exudate collected from the pocket. Also, anti-inflammatory action involving action in the bradykinin pathway was detected in the paw edema test. The fraction showed an antitumor effect against Ehrlich carcinoma, as demonstrated by the reduction in the weight of the tumors, reduction of

immunohistochemical marking of cell proliferation antigen (p-53) and increased antigen marking related to tumor suppression (ki-67). However, the fraction showed antibacterial activity against *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* with CMI values from 31.2 to 125.0μg/mL and CMB from 2.5 to 200μg/mL. In vivo activity against the same bacteria was detected in the model using T. molitor. In conclusion, the preparations of the *M. vacciniifolia* rhizome have the potential for studies aimed at the development of analgesic, anti-inflammatory, antibacterial and anti-tumor drugs.

Keywords: *Microgramma vacciniifolia*. Analgesic effect. Lectin. Chlorogenic acid. Inflammation. Antimicrobial activity. Antitumoral activity.

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1. INTRODUÇÃO

As plantas, devido ao potencial terapêutico já comprovado de muitos fitoquímicos, constituem uma fonte promissora para descoberta de novas drogas que representem novas possibilidades de tratamento, inclusive minimizando os efeitos colaterais (JIA et al., 2016; SILVA et al., 2012; WALKER et al., 2013). Em países subdesenvolvidos e em desenvolvimento, as plantas medicinais representam a primeira escolha por boa parte da população para cura de males; ainda, seu uso é cada vez maior na América do Norte e em vários países da Europa. Baseados em conhecimentos etnofarmacológicos, estudos vêm sendo realizados nos últimos anos tornando crescente o número de medicamentos de origem vegetal bem como de alimentos funcionais (AHMAD et al., 2016; DUTRA et al., 2016; KUO et al., 2018; PEREIRA et al., 2017).

Microgramma vacciniifolia (Langsd. & Fisch) é uma planta epífita pertencente à família Polypodiaceae, a qual dispõe de raízes superficiais que se estendem pelas cascas de arbustos e árvores, absorvendo a matéria orgânica disponível. Quanto ao seu uso medicinal, é indicada para hemorragias, expectorações, disenterias, cólicas intestinais, hidropisia e infecções intestinais (PERES et al., 2009). O rizoma de *M. vacciniifolia* contêm uma lectina (proteína que reconhece carboidratos) denominada MvRL, que apresentou ação citotóxica contra células de carcinoma mucoepidermoide de pulmão (NCI-H292) em concentrações nas quais não afetou a viabilidade de células mononucleares de sangue periférico humano (ALBUQUERQUE et al., 2014). Na literatura, não há estudos sobre os metabólitos secundários encontrados no rizoma dessa planta.

O uso de plantas medicinais não pode ser realizado de forma indiscriminada uma vez que podem causar efeitos tóxicos nas mesmas a curto e longo prazo. Sendo assim, preparações vegetais devem passar por testes a fim de se traçar o perfil de toxicidade, como enfatizado pela *U.S. Food and Drug Administration* (FDA) e a Organização Mundial de Saúde (OMS) (HAZARIKA, 2019; KRAH, DE KRUIJF, RAGNO, 2018; SALEEM et al., 2017). Até o presente momento, não foram encontrados estudos sobre a toxicidade de preparações de *M. vacciniifolia* na literatura.

A dor é considerada um sinal fisiológico que tem como evento principal a transdução do estímulo recebido, seja térmico, físico ou químico, do sistema nervoso periférico para o central. O alívio da dor é de relevância incontestável, uma vez que ela leva os indivíduos a um padecimento aflitivo tanto físico quanto mental (GOLDBERG e

McGEE, 2011). As fontes vegetais têm representado alternativas para tratamento da dor e destas derivaram dois analgésicos bem utilizados na terapêutica atualmente, como o ácido acetilsalicílico (derivado da *Salix alba*) e a morfina (derivada da *Papaver somniferum*). Essa última, bem como outros fármacos opioides, apesar da eficiência em analgesia apresenta desvantagens como efeitos colaterais de constipação, depressão respiratória, dependência, entre outros (BENYAMIN et al., 2008). Neste sentido, surge a necessidade de novas alternativas terapêuticas para alívio da dor com menos efeitos colaterais.

A resposta inflamatória é um condição física de defesa do organismo surgindo, a priori, em decorrência de uma infecção ou lesão. A duração da inflamação varia de acordo com os tecidos afetados e com a extensão e o tipo de dano. O processo se inicia localmente e pode levar a efeitos sistêmicos devido a produção e liberação de citocinas que induzem a produção de prostaglandinas e proteínas de fase aguda, como a proteína reativa C, a qual age no centro nervoso produzindo dor, indisposição e febre (AGARWAL et al., 2019; MEDZHITOV, 2010). Atualmente os anti-inflamatórios não esteroidais (AINES) constituem a opção terapêutica mais frequente em processos inflamatórios, porém a inovação de moléculas nessa área é necessária devido as efeitos indesejáveis dessa classe medicamentosa como hipertensão, toxicidade gástrica e renal, além de osteoporose (ABUBAKAR et al., 2019).

Para 2025 há uma estimativa mundial de 20 milhões de novos casos de câncer e, tratando-se do Brasil, houve uma projeção de 600.000 novos casos entre 2018-2019 (FERLAY et al., 2015). Um fator característico das células tumorais é a heterogeneidade devido à mutação e multiplicação desordenada, o que leva ao insucesso de algumas terapias, para as quais as células desenvolvem resistência. Eventos que acontecem na carcinogênese, como reprogramação metabólica, angiogênese entre outros, têm sido objeto de partida para o estudo de novas terapias, com intuito de driblar os obstáculos dos efeitos adversos das quimioterapias convencionais (XIONG, FENG e CHENG, 2019). O desenvolvimento da massa tumoral estabelece um quadro de base inflamatória e em muitos casos o gerenciamento da dor é indispensável configurando-se em tratamento adjuvante (HAUMANN et al., 2016; Te BOVELDT et al., 2013). Na terapia anticâncer, a falta de especificidade dos agentes antitumorais, bem como a diminuição da competência do sistema imunológico, fomenta a substituição destes por alternativas mais efetivas e sobretudo mais seguras. Já é conhecido o emprego de alguns produtos de

origem natural, como o paclitaxel, que é extraído das cascas da espécie vegetal *Taxus brevifolia* (RIVERA, LOYA e CEBALLOS, 2013).

Um outro problema para a área farmacêutica é a resistência bacteriana aos antibióticos atualmente disponíveis, o que torna a terapia de diversos tipos de infecção um desafio, aumentando os custos de internações, como também a mortalidade associada (FRIERI et al., 2017). O surgimento contínuo de cepas resistentes a múltiplas drogas desperta a busca por novas alternativas terapêuticas (MUNOZ-DAVILA, 2014). Adicionalmente, a eficiência no tratamento de infecções oportunistas, principalmente em pacientes imunocomprometidos, deve acarretar mínimos efeitos indesejáveis (CHOPRA et al., 2016).

Diante do exposto, a presente tese descreve a avaliação de extrato salino de rizoma de *M. vacciniifolia* e fração enriquecida na lectina MvRL quanto à toxicidade aguda, genotoxicidade e potencial para desenvolvimento futuro de drogas com atividades antinociceptiva, anti-inflamatória, antibacteriana e antitumoral.

1.1. OBJETIVOS

1.1.1 Geral

Avaliar a toxicidade aguda, genotoxicidade e atividades antinociceptiva, anti-inflamatória, antibacteriana e antitumoral de extrato salino e fração enriquecida em lectina obtidos do rizoma de *M. vacciniifolia.*

1.1.2 Específicos

- Investigar metabólitos secundários presentes no extrato salino e na fração enriquecida em lectina do rizoma de *M. vacciniifolia.*
- Avaliar a toxicidade aguda do extrato e fração em camundongos utilizando parâmetros comportamentais, bioquímicos, hematológicos, histológicos e estresse oxidativo.
- Avaliar a genotoxicidade do extrato e fração em camundongos.
- Investigar o potencial antinociceptivo do extrato e fração em modelos de dor inflamatória e não-inflamatória.
- Avaliar a atividade anti-inflamatória *in vivo* do extrato e fração.

- Investigar a atividade antibacteriana da fração *in vitro* e *in vivo* utilizando larvas de *Tenebrio molitor*.
- Avaliar a atividade antitumoral *in vivo* da fração em camundongos portadores de carcinoma de Ehrlich.

2. FUNDAMENTAÇÃO TEÓRICA

2.1 Avaliação de toxicidade

A utilização de plantas medicinais objetivando a cura de doenças data dos primórdios e correlaciona-se com a sobrevivência da humanidade. O conhecimento das atividades terapêuticas das plantas atravessa gerações e fomenta o interesse pela elucidação da presença e concentração dos metabólitos ativos responsáveis pelos efeitos. Cerca de 25% dos medicamentos prescritos são derivados de espécies vegetais e a pesquisa de novas moléculas é crescente (NEWMAN e CRAGG, 2016; SCAPINELLO et al., 2019; WINK, 2016). Porém, os próprios metabólitos de ações terapêuticas, bem como outros compostos presentes nas preparações vegetais, podem apresentar toxicidade, principalmente a ativação de vias oxidativas e de dano celular (EJEH et al., 2019; MOREIRA et al., 2014).

Apesar do desenvolvimento e progresso no sistema de saúde em geral, em torno de 60% da população depende do uso de plantas medicinais para a melhora de diversas injúrias à saúde, devido a fatores como inclinação cultural, alto custo de medicamentos sintéticos e situação econômica restrita (EZURUIKE, PRIETO, 2014; MIRI et al., 2015). Além do baixo custo, a população faz uso de plantas medicinais devido à fácil disponibilidade. Porém, muitas vezes essa utilização é feita sem o suporte de conhecimento científico que comprove eficácia e segurança e o aumento global do consumo dessas substâncias justifica a preocupação e os esforços relacionados à determinação da segurança do uso (CARNEIRO et al., 2014; SILVA et al., 2014; WU et al., 2018).

No âmbito das drogas vegetais, outro ponto que inspira preocupação é a administração concomitante com drogas convencionais devido a potenciais interações farmacológicas, nas quais pode-se verificar inibição ou indução de enzimas ou proteínas transportadoras responsáveis pela metabolização (MOREIRA et al., 2014). As pesquisas sobre segurança de drogas vegetais têm revelado certa toxicidade que, além de natureza

toxicocinética, pode ser inerente aos diversos princípios ativos, superdosagem, uso a longo prazo, entre outros fatores (ABBAS, EJIOFOR e YAKUBU, 2018; CHANDA et al., 2015).

Ainda que possam ser realizados testes *in vitro*, as agências normatizadoras exigem testes *in vivo*, nos quais sinais clínicos dos animais são monitorados e podem auxiliar na tradução de efeitos tóxicos (KIFAYATULLAH et al., 2015). Com efeito, a investigação e comprovação das propriedades farmacológicas de vários fitoquímicos fomenta o desenvolvimento de mais pesquisas para avaliar os possíveis riscos e/ou finalidades terapêuticas (GARCÍA-RODRÍGUEZ et al., 2014; OMOKHUA et al., 2016).

O teste de toxicidade aguda em animais é empregado de forma rotineira para traçar o perfil de segurança de compostos através da avaliação de mudanças comportamentais bruscas e de mortalidade (FAROOQ et al., 2019). Além de morte e de variações de peso, sinais clínicos como alterações comportamentais, na respiração e na atividade somatomotora também podem ser considerados indicadores de toxicidade. Órgãos como fígado, rins e baço são frequentemente afetados por substâncias tóxicas, sendo então importante analisá-los quanto aos aspectos histológicos, bem como através de marcadores hematológicos e bioquímicos (HAZARIKA, 2019).

Os estudos *in vivo* de toxicidade aguda são normatizados pelo protocolo 423 da Organização para Cooperação e Desenvolvimento Econômico (OECD), que deve ser aplicado para qualquer candidato à fármaco de uso humano. Seu desenvolvimento almeja estimar uma dose mínima que possa causar letalidade em roedores como ilustra a Figura 1, sendo mais comumente utilizadas as vias oral e intraperitoneal (CHANDA et al., 2015).

Tratando-se de pesquisa e desenvolvimento de novas drogas, os testes de carcinogenicidade são os mais longos e de alto custo dentro daqueles enquadrados como pré-clínicos, e podem ser dispensados quando os estudos de genotoxicidade a curto prazo revelam resultados satisfatórios (MOREIRA et al., 2014).

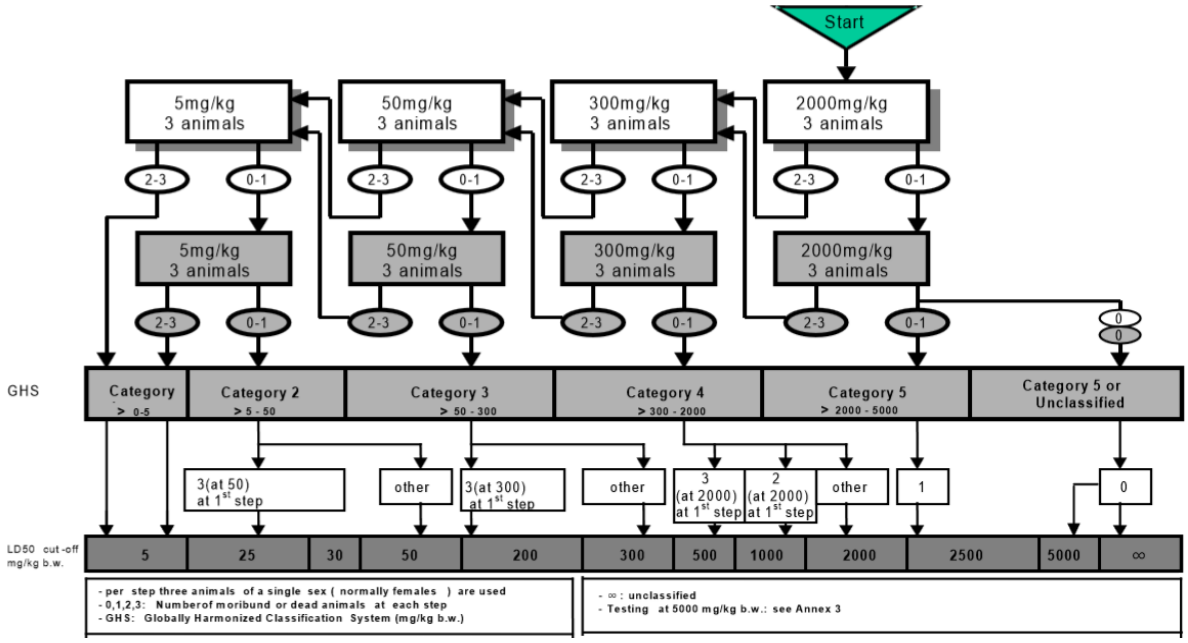
2.2 Dor e atividade antinociceptiva

A Associação Internacional para o Estudo da Dor (IASP) define esta como um processo sensorial interligado ao desenvolvimento de um dano tecidual. Mesmo que seja um mecanismo de resposta fisiológica à quebra da homeostase, há a necessidade de gerenciamento quando a dor evolui para patológica, sendo o seu tratamento necessário em várias situações. Esse tratamento constitui ainda um desafio devido aos efeitos

adversos potenciais dos fármacos analgésicos atuais (CORTES-ALTAMIRANO et al., 2018; GONZÁLEZ-TRUJANO et al., 2019; LOESER e TREEDE, 2008).

Fundamentalmente desenvolvida em quatro passos (transdução, transmissão, percepção e modulação), a nocicepção inicia-se de um estímulo ou injúria a um tecido, seja de ordem mecânica, térmica ou química. A partir deste, mediadores inflamatórios como serotonina, prostaglandinas, leucotrienos, bradicinina, entre outros, são liberados atuando tanto na diminuição do limiar de estimulação como aumentando a resposta dos nociceptores (BRUNTON et al., 2018). Logo, através desta sensibilização periférica ocorre transdução desse sinal para o corno dorsal da medula espinhal e ascendência através do quadrante ventral da medula até chegar ao tálamo e ao tronco cerebral. O sistema tálamo-cortical é responsabilizado por estimular a resposta consciente à dor, emitindo assim sinais, agora descendentes, para fins modulatórios com a liberação de serotonina, norepinefrina e opioides endógenos (HOWARD e BRANT, 2019; SCHAIBLE, EBERSBERGER, 2011).

Figura 1- Avaliação de toxicidade aguda de acordo com o guia da Organização para Cooperação e Desenvolvimento Econômico (OECD).



O protocolo orienta a administração de uma dose inicial de 2000 mg/kg para um grupo de três animais. Caso haja morte, orienta redução de dose, a fim de se obter o valor da dose letal para 50% dos animais (DL_{50}) e categorizar o potencial tóxico.

Fonte: OECD (2001).

Quando caracterizada no padrão agudo, a algesia é condicionada em um recorte temporal limitado, considerada ainda como fisiológica, e que alerta sobre uma lesão. Caso esta persista, ocorrem modificações tanto nos tecidos como no sistema nervoso central e periférico resultando em prolongamento da dor. Seu manejo imediato resguarda o organismo de sofrer processos fisiopatológicos que levam à dor crônica, a qual já não tem um propósito útil, afetando o bem-estar emocional e físico do indivíduo (BURNS e MULLEN, 2015). Nesta perspectiva, os analgésicos são utilizados no papel de adjuvantes na terapia da causa da dor ou quando o estímulo nocivo que a provoca não pode ser removido, resultando em alívio do sintoma doloroso sem provocar alterações de consciência (HOSSAIN et al., 2015).

A dor é tida como um problema de saúde pública, uma vez que, 1 a cada 5 adultos são acometidos levando a problemas em seu bem estar geral, relações interpessoais, produtividade, entre outros. Atualmente os anti-inflamatórios não esteroidais (AINEs) são os mais prescritos tanto para reverter sintomas de algesia bem como inflamação devido à sua ação de prevenção de formação das prostaglandinas por inibição da cicloxigenases COX 1 e COX 2. Porém, quando há o uso prolongado, alguns efeitos colaterais são observados, principalmente gastrointestinais, como sangramentos e até ulcerações (DÉCIGA-CAMPOS, MATA e RIVERO-CRUZ, 2017; YAP e GOH, 2015).

Outra arma terapêutica utilizada para analgesia são os fármacos enquadrados como opioides que, apesar de eficazes, estão associados a riscos de abuso e dependência e dificuldades de eleger e monitorar a posologia, vias de administração e duração da terapia (CHEUNG et al., 2017; ROGERS et al., 2013; VOLKOW, BENVENISTE e MCLELLAN, 2018). Enquanto os opioides são capazes de inibir a dor neurogênica e inflamatória (fases 1 e 2), os AINES são capazes de inibir apenas a dor inflamatória (FLORENTINO et al., 2016).

As propriedades de analgesia de compostos podem ser verificadas *in vivo* através do teste da formalina, no qual o animal responde a uma dor moderada e persistente decorrente de um dano tecidual, diferente dos testes de estímulos mecânicos ou térmicos. Este modelo experimental se assemelha mais às condições clínicas reais, com o animal experimentando as fases 1 e 2 da dor; logo, é frequentemente utilizado na triagem de atividades antinociceptiva e antitérmica de protótipos terapêuticos (MASOCHA, KOMBIAN e EDAFIOGHO, 2016).

O processo nociceptivo de ordem neurogênica, experimentado no teste da formalina, é caracterizado pela estimulação direta das fibras sensoriais do tipo C, enquanto a dor inflamatória causa a liberação de bradicinina, prostaglandinas e serotonina nos tecidos periféricos em resposta a alterações funcionais no corno dorsal da medula (REYNOSO et al., 2013). Além deste, pode-se citar tanto os testes de estimulação física, como o teste da placa quente, no qual avalia-se o limiar de sensibilidade que causa dor aguda; e os testes de estimulação química, como o de contorções induzidas por ácido acético, no qual mensura-se a resposta à estimulação de longa duração por injúria às vísceras do animal desencadeando dor de origem inflamatória (BURNS e MULLEN, 2015; MOGIL et al. 1998; ZHANG et al., 2018b).

Comum a diversos tipos de patologias, as reações dolorosas são frequentemente e culturalmente tratadas com drogas vegetais (ADAMS JR. e WANG, 2015; ANTUNES e MONICO, 2015; BRUM et al., 2016). Dentro os metabólitos secundários, a responsabilidade por propriedades analgésicas é atribuída em larga escala aos alcaloides (QUINTANS et al., 2014).

2.3 Inflamação

A inflamação é um processo fisiopatológico presente em várias doenças, desde infecções até artrite e doenças cardiovasculares (VYSAKH et al., 2018). Compreende uma resposta dos tecidos vasculares a lesões nos tecidos adjacentes, na intenção de eliminação do estímulo nocivo e preparação da área lesada para o possível reparo, salvo casos de cronicidade, no qual o próprio processo inflamatório provoca danos teciduais (CHEN et al., 2018; SREEJA et al., 2018).

O processo inflamatório é desencadeado a partir do metabolismo do ácido araquidônico, ácido graxo de membrana, o qual é liberado a partir de um estímulo nocivo à célula. Nesta via de sinalização temos o protagonismo das prostaglandinas que são originadas a partir da conversão do ácido araquidônico pelas cicloxigenases (KATANIC et al., 2018; RICCIOTTI e FITZGERALD, 2011). Este estímulo nocivo ou dano tecidual de diversas origens, além de ativar a cascata de inflamação propriamente dita, desencadeia diversas respostas imunológicas, dentre elas a exposição prolongada a agentes pró-oxidantes no tecido gerando acumulação destes, além de ativar a angiogênese. Os receptores ativados pelo proliferador de peroxissomos (PPRA), que estão implicados com a expressão gênica da óxido nítrico sintase, cicloxigenases e fator

de crescimento endotelial vascular, entre outros, são sensibilizados pelos mediadores inflamatórios (CASTRO et al., 2014; FERGUSON, 2010). O processo inflamatório sofre regulação do sistema imune, o qual também estimula a secreção de mediadores da inflamação pelas células, bem como ocorre efetiva participação do estresse oxidativo na fisiopatologia desse evento (LUGRIN et al., 2014).

Existem diversos mediadores químicos envolvidos nesse processo e que são liberados no plasma, como óxido nítrico, histamina, serotonina, fator de necrose tumoral (TNF-α) e interleucinas (THEOHARIDES et al., 2012). Pode-se ainda diferenciar dois fenótipos da inflamação: a aguda, que se instala por um período curto de até 4 dias e na qual são observadas alterações funcionais, e a crônica, a qual é desencadeada por alterações estruturais resultando por vezes em necrose tecidual, devido à persistência do estímulo nocivo por semanas, meses ou até anos (FREIRE e VAN DYKE, 2013). A inflamação crônica é mais silenciosa do que a aguda, em termos dos sinais passivos como edema, vermelhidão, febre e dor, porém pode-se observar, por exemplo, altos índices de interleucinas, proteína C reativa (PCR), interferon, TNF-α e fator de crescimento β (TGF-β) (RUPARELIA et al., 2017).

Na redução da inflamação, são utilizados comumente no tratamento os AINEs, que detêm efeito modulador das cicloxigenases. A COX-1, constitutiva e presente em condições normais em variados tipos celulares, é responsável, entre outros, pela manutenção das condições de homeostase gástrica e renal. Já a COX-2 tem produção induzida por fatores pró-inflamatórios, com produção máxima em condições de inflamação. A fim de driblar os efeitos colaterais produzidos pelos inibidores da COX-1, foram desenvolvidos inibidores da COX-2, porém já foram demonstrados efeitos colaterais como retenção de água, sódio e potássio (KLAMPFER, 2011; RICCIOTTI e FITZGERALD, 2011; VYSAKH et al., 2018).

O edema de pata induzido por carragenina é frequentemente utilizado, devido sua reprodutibilidade, para avaliar tanto efeitos anti-edematogênicos como anti-inflamatórios de produtos naturais (QUEIROZ et al., 2010). Logo após a injeção subcutânea, se desenvolvem os sinais cardinais da inflamação, que são dor, rubor, calor, edema e perda de função (YI et al., 2010). A fase inicial corresponde ao edema não fagocítico, que perdura de 60 a 90 min, com participação de mediadores como bradicinina, serotonina e histamina, agindo na permeabilidade vascular. A fase secundária corresponde a aumento da formação do edema, que pode permanecer até 5 h, na qual participam mediadores

como prostaglandinas e óxido nítrico, os quais são produzidos e liberados no próprio sítio de inflamação (ZHU et al., 2011).

A peritonite é um modelo de inflamação lenta e prolongada, que permite avaliar tanto a secreção de exsudato quanto a migração leucocitária, como também a participação de diversos mediadores, como óxido nítrico, prostaglandinas, TNF-α e interleucinas (KUMAWAT et al., 2012). Já o método do granuloma induzido por sedimento de algodão é direcionado para avaliar a fase crônica na inflamação, incluindo características de angiogênese, exsudação, proliferação de fibroblastos e infiltração de monócitos (YADAV et al., 2011). No teste do granuloma, o peso úmido do algodão está ligado ao transudato inflamatório, ao passo que o peso seco correlaciona-se com a proliferação de tecido granulomatoso; logo, este modelo pode avaliar os componentes transudativos e proliferativos da inflamação crônica, caracterizada por degeneração e fibrose tecidual (MISHA et al., 2011). Substâncias com potencial anti-inflamatório agem diminuindo o tamanho do granuloma formado devido à diminuição da infiltração de granulócitos e consequentemente da geração de fibras colágenas (KUMAWAT et al, 2012).

Culturalmente, diversas preparações de plantas medicinais são utilizadas na terapia tradicional para doenças inflamatórias, sendo a ação anti-inflamatória atribuída a fitoquímicos como compostos fenólicos em geral (MENA et al., 2014), além de terpenoides, flavonoides, saponinas, entre outros (KUMAWAT et al., 2012).

2.4 Bactérias patogênicas

As bactérias *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* e *Enterobacter* (inseridas no conjunto conhecido como SKAPE) e *Escherichia coli* estão entre as cepas bacterianas clínicas mais frequentemente associadas ao desenvolvimento de resistência a terapia antimicrobiana (ZHONG et al., 2019), relacionando-se com o aumento dos custos do tratamento, além de morbi e mortalidade (SILVA e DOMINGUES, 2016). Configurando uma ameaça consistente à saúde humana, a resistência de microrganismos aos antimicrobianos tem aumentado globalmente (MOLINA e CISNEROS, 2015). A falta de higienização e controle de infecções e regulamentações ineficientes do uso de antimicrobianos estão entre os motivos associados à essa crescente resistência (ZELLWEGER et al., 2017). Devido a correlação entre o uso dos antibióticos e a diminuição da sensibilidade de cepas microbianas (DYAR et al., 2015), tem sido destacadas a restrição na utilização e acesso

aos mesmos (DAVEY et al., 2017) e a necessidade de desenvolvimento de moléculas antimicrobianas inovadoras OMS, 2017). É relatado que os compostos naturais têm atividades frente várias cepas de bactérias patogênicas e podem atuar como moduladores da resistência bacteriana (CHEN, ZHAO, MENG e YU, 2017; KATRI et al., 2016).

Staphylococcus aureus está associada a diversos tipos de infecções, desde dérmicas leves até bacteremia, sendo um microrganismo altamente adaptável a situações adversas no ambiente, como privação de nutrientes (GONCHEVA et al., 2019). A forma comensal nas narinas pode ser facilmente disseminada no ambiente, oferecendo uma fonte de infecção que se agrava quando se trata de pessoas em estado imunocomprometido (MOSCHOU et al., 2019).

Klebsiella pneumoniae é reconhecida devido a seus múltiplos mecanismos de resistência, como também pelo fato de ser um microrganismo reconhecidamente oportunista e líder de infecções hospitalares. Pode estar relacionada com casos de menigite, pneumonia, infecções de feridas, septicemia, abscessos, como também está relacionada a intoxicações alimentares e diarreia (DAVIS et al., 2015).

Acinetobacter baumannii detêm habilidade de sobreviver por semanas e até meses em locais tanto úmidos como secos (CARVALHEIRA et al., 2017), além de possuir ampla distribuição em ambientes naturais, sociais e nosocomiais, sendo sua propagação facilitada pela fato de povoar a pele humana e mucosas (YING et al., 2015). Atualmente, tem sido associada com infecções derivadas dos cuidados com a saúde e com alta capacidade em adquirir determinantes de resistência (BELMONTE et al., 2014).

Pseudomonas aeruginosa trata-se de um bacilo não fermentativo e Gram-negativo (CAI et al., 2018), sendo o segundo agente mais correlacionado com pneumonias. Vários mecanismos tornam cepas dessa espécie resistentes a β-lactâmicos (CASTANHEIRA et al., 2014). Também pode estar correlaciona a infecções de pele, urinárias e bacteremias (TORRENS et al., 2016). Está associada a altas taxas de mortalidade, maior tempo de internação, como também maiores elevaçãodos custos de tratamento (WI et al., 2017).

Escherichia coli é uma bactéria habitante da microbiota gastrointestinal humana, mas cepas virulentas causam as mais diversas infecções intestinais e extra-intestinais (AHUMADA-SANTOS et al., 2019). Está entre as bactérias uropatogênicas mais comuns presentes e adquiridas na comunidade e em ambientes nosocomiais (TERLIZZI, GRIBAUDO e MAFFEI, 2017). Estudos recentes demonstram um aumento expressivo da multirresistência de bactérias Gram negativas, centre elas a *E. coli* (CONCIA et al., 2017).

2.5 Câncer

A incidência de diversos tipos de câncer tem aumentado devido tanto às alterações ambientais como também no estilo de vida da população, a qual está constantemente é exposta a fatores predisponentes como substâncias químicas, fumaça de cigarro, radiação solar, entre outros. O câncer se desenvolve através de um dano genético que evolui a partir de mutações espontâneas, as quais podem ser influenciadas por questões de ordem hormonal e metabólicas (DIAS et al., 2018; SHARMA, SINGH, GOYAL, 2016).

O câncer tem distribuição global mesmo em países desenvolvidos (GUN et al., 2019; RAHIB et al., 2014). Dentre os tipos mais incidentes, excluindo o câncer de pele não-melanoma, destacam-se os de pulmão, mama, próstata e colorretal, os quais superam em número a soma de todos os outros tipos de câncer, de forma que a atenção de agências normatizadoras e da indústria farmacêutica direciona-se para esses. Ademais, um aumento substancial está projetado entre os anos de 2020 e 2030, fato este sobretudo atribuído ao aumento no número de adultos com 65 anos ou mais. Para 2030, são previstos como maior causa de morte os cânceres de pulmão, fígado e pâncreas para homens e de pulmão, mama e pâncreas para mulheres (RAHIB et al., 2014).

O câncer se instala a partir dos estágios de iniciação, promoção e progressão, fomentados por fatores que facilitam esse processo, conhecidos como fatores cancerígenos, que podem ser divididos em iniciadores ou promotores. Considera-se que, embora haja diversos tipos de cânceres, estes têm características comuns, dentre as quais a mais marcante seria a instabilidade genômica, a qual induz diversas e sequenciais mutações. Somada a esta existe a reconhecida presença de uma base inflamatória no tecido, a qual é desencadeada pela ação de mediadores e de células imunológicas (CHEN et al., 2018; MA et al., 2010; NEGRINI, GORGOULIS e HALAZONETIS, 2010).

Ocorre também uma reprogramação metabólica dessas células tumorais, já que deve existir uma adaptação a um microambiente de hipóxia, carência de nutrientes e substâncias pró-oxidativas. Logo, as alterações metabólicas do câncer tem recebido atenção e pode ser peça-chave para alcançar a inibição do desenvolvimento da massa tumoral. A reprogramação gera alteração no nível de metabólitos que são cofatores e até mesmo substratos para enzimas epigenéticas, que quando transferidas ao núcleo gera a produção de oncometabólitos, que quando se acumulam levam à progressão do câncer. Embora o metabolismo da glicose e da glutamina tenha sido foco nos últimos anos, há

grande utilização de aminoácidos sulfurados, ácidos graxos essenciais e vitaminas pelas células tumorais (PAVLOVA e THOMPSON, 2016; THAKUR e CHEN, 2019; WARD e THOMPSON, 2012). Além disso, verifica-se o aumento da angiogênese, que também tem sido ponto de estudo para novas terapias, uma vez que novos vasos são necessários para dar suporte ao volume de replicação que existe na massa tumoral. Por fim, como fenótipo característico, existe a capacidade de evasão à ação do sistema imunológico (HANAHAN e WEINBERG, 2011).

Apesar dos avanços nas terapias antineoplásicas, muitas falhas são detectadas no tratamento quimioterápico de vários pacientes devido a efeitos colaterais e multirresistência das células tumorais, e assim o câncer ainda constitui a segunda causa de mortalidade mais incidente mundialmente (AGHAJANPOUR et al., 2017; SALHI et al., 2018). As propriedades anticancerígenas das espécies vegetais chamam atenção considerando que quimioterápicos promissores utilizados na terapêutica atualmente derivaram de vegetais, como por exemplo o paclitaxel (SCHEFER et al., 2017). Fitoquímicos nos quais são verificados propriedades anti-inflamatórias e antiangiogênicas podem ser experimentados com finalidade antitumoral (GUO, CALLAWAY e TING, 2015; MICHEL et al., 2015).

Dentre os tumores experimentais mais comuns destaca-se o Carcinoma de Erlich, que surgiu como um câncer espontâneo na mama de um rato fêmea (TASKIN, 2002). É caracterizado por ser um tumor indiferenciado (o que o torna sensível a diversas quimioterapias), ser detentor de 100% de malignidade, hiper diploide e de alta proliferação (OZASLAN et al., 2011). Tais características servem de subsídio para que essa linhagem seja utilizada para experimentações com produtos derivados de vegetais (SALEM et al., 2016). Quando injetado intraperitonealmente, desenvolve-se de forma ascítica; por sua vez, se implantado por via subcutânea é desenvolvida a forma sólida (ZEYBEK, 1996).

2.6 Compostos bioativos de origem vegetal

As plantas medicinais e tratamentos tradicionais despertam atenção do meio científico, devido às múltiplas finalidades para as quais são usadas, que compreendem desde o aumento da imunidade até a prevenção de doenças (SEN e CHAKRABORTY, 2011). Os compostos vegetais têm emergido como fonte direta de novas moléculas ou ainda como inspiração para a otimização estrutural e criação de novas moléculas (ALI et

al., 2014). Tal relevância se reflete no fato de que cerca de 70% dos medicamentos sintéticos foram desenhados a partir de compostos de plantas (PATWARDHAN, 2014). Por exemplo, etoposídeo, camptotecinina e paclitaxel, atualmente utilizadas na terapia oncológica, são moléculas com origem na ampla gama de fitoquímicos naturais (ALI et al., 2014).

As substâncias fitoquímicas podem ser classificadas como metabólitos primários ou secundários. Os compostos envolvidos no metabolismo primário possuem uma distribuição universal nas plantas, sendo o caso de aminoácidos, nucleotídeos, lipídios, carboidratos e clorofila. Estão diretamente envolvidos nos processos de crescimento, desenvolvimento e reprodução da planta. A defesa contra patógenos requer aporte do metabolismo das espécies vegetais, sobretudo demanda alta carga energética, a qual é suprida principalmente pelos metabólitos primários (ROJAS et al., 2014). Além disso tais metabólitos participam na cadeia de sinalização de moléculas que agem contra diversos patógenos (KACHROO e ROBIN, 2013). Estudos revelam a participação de proteínas, por exemplo, no combate à invasão de bactérias, fungos e vírus nos tecidos vegetais (WANG, WANG e GUAN, 2012).

Os metabólitos secundários compreendem vários compostos de baixo peso molecular, os quais conferem vantagens para as respostas adaptativas bióticas e abióticas das plantas (MANDAL, CHAKRABORTY e DEY, 2010). Sua síntese se processa devido a presença de estímulos, como por exemplo, defesa contra herbívoros, outras espécies vegetais, vírus, bactérias, radiação UV, entre outros, além de conferir cor e aroma dos produtos derivados do vegetal (HELENO et al., 2015).

2.6.1 Compostos fenólicos e derivados cinâmicos

Os compostos fenólicos são metabólitos contendo um ou mais grupos hidroxila ligados a um anel aromático e derivam dos aminoácidos L-fenilalanina e L-tirosina, através da via do chiquimato (KUMAR e PRUTHI, 2014). Eles podem ser simples ou polimerizados e classificados em ácidos fenólicos, taninos, flavonoides, estilbenos, flavonoides e lignanas, de acordo com o esqueleto da cadeia carbonada (BALASUNDRAM, SUNDRAM e SAMMAN, 2006; GIADA, 2013). São quase que onipresentes nas espécies vegetais e têm despertado interesse para o desenvolvimento de novos produtos farmacêuticos, principalmente devido aso seus potenciais antioxidantes, podendo ser utilizados no tratamento de doenças de ordem cardiovascular, diabetes, entre

outras (BHUYAN e BASU, 2017). Além disso, são relatadas atividades anti-inflamatória (MARTINS, BARROS e FERREIRA, 2016), antimicrobiana e antitumoral (AHMED et al., 2016). Os ácidos fenólicos (aqueles que apresentam um grupo carboxílico) possuem potencial antioxidante mais elevado do que conhecidas vitaminas (KHODDAMI, WILKES e ROBERTS, 2013).

A luteolina, representante dos compostos fenólicos, apresenta efeitos anti-inflamatórios devido à atuação na diminuição da atividade da enzima óxido nítrico sintetase e da liberação de interleucinas IL-1β e IL-6 (WANG et al., 2017). Outro exemplo de composto fenólico, o ácido gálico, é capaz de reduzir a formação de espécies reativas de oxigênio, bem como inibir produção de prostaglandinas (LIN et al 2015). Estudos apontam que os compostos fenólicos apresentam eficiência na redução da glicemia por promoverem a inibição de enzimas como α-glicosidases e α-amilase (HANHINEVA et al., 2010).

Os compostos fenólicos podem exibir propriedades antimicrobianas e atuar como preservantes de alimentos a fim de prolongar a vida útil destes (LLORENT-MARTINEZ et al., 2017). A atividade inibitória de microrganismos está ligada a posição e número de substituintes no anel benzênico, bem como ao comprimento da cadeia carbonada: compostos com cadeias carbonadas maiores apresentam maior eficiência como antimicrobiano (CUEVA et al., 2010; MERKL et al., 2010). Os compostos fenólicos já foram correlacionados com a inibição do crescimento de microrganismos virulentos e com alta taxa de resistência (LIMA et al., 2019). Extrato da *Cryptotaenia japonica* Hassk, rico em composto fenólicos como luteolina e ácidos gálico, cafeico e clorogênico demonstrou efeito antimicrobiano contra *Staphylococcus aureus* e *Escherichia coli* (LU et al., 2018).

Evidências apontam que uma dieta rica em polifenóis é preventiva no desenvolvimento de alguns tipos de câncer (ZHOU et al., 2016). Os polifenóis podem agir modulando as proteínas que impulsionam a apoptose de células cancerígenas, sendo a promoção de apoptose uma das estratégias mais bem-sucedidas nas intervenções quimioterápicas (FANTINI et al., 2015; KUMASAKA et al., 2013). Estudos apontam que o ácido hidroxibenzoico e seus derivados apresentam potencial para prevenção e tratamento do câncer (KUMAR et al., 2017), atuando na inibição da iniciação tumoral, seja bloqueando a formação de moléculas genotóxicas, inibindo enzimas envolvidas em mutações (SŁOCZYNSKA et al., 2014), ou ainda regulando a formação de adutos de DNA (KOU et al., 2013). O ácido clorogênico demonstrou significantes resultados frente

a células de câncer de estômago e cólon (SHAO, ZHANG, CHEN e SUN, 2015) e células cancerígenas hepáticas (SISWANTO, OGURO e IMAOKA, 2017).

Encontrado largamente em tecidos vegetais e presença frequente em aromas e essenciais vegetais, o ácido cinâmico é um derivado dos ácidos fenólicos, composto de um anel fenil substituído por um grupo ácido acrílico (RODRIGUES et al., 2019). Geralmente ocorre na forma geométrica trans e é precursor das auxinas, hormônios vegetais que atuam no crescimento e diferenciação celular, sendo intermediários na produção de moléculas como os estilbenos e estirenos (SHARMA et al., 2011). Os ácidos cinâmicos são tidos como intermediários da síntese dos polifenóis, uma vez que são produtos da associação entre o ácido chiquimato e a fenilalanina (DEWIK, 2009). Aos ácidos cinâmicos, como também seus derivados ésteres, são atribuídos potenciais anti-carcinogênico (ZHU et al., 2016), anti-inflamatório (TAOFIQ et al., 2016), antimicrobiano (LAVERTY et al., 2015), antioxidante (CHAVARRIA et al., 2015), além de atuação na angiogênese, neuroproteção e proteção vascular (ZHANG et al., 2018a).

Os ácidos cinâmicos apresentam ação anti-inflamatória por inibirem a expressão gênica da enzima óxido nítrico sintetase e induzirem enzimas antioxidantes como superóxido dismutase e glutationa peroxidase, conforme verificado em ensaio com cultura de células (LIAO et al., 2012). Em avaliação *in vitro*, utilizando suspensão de células, um derivado semissintético do ácido cinâmico promoveu diminuição de níveis de óxido nítrico e expressão da óxido nítrico sintetase, através da inibição da ativação das quinase c-Jun N-terminal (JNK) (KWON et al., 2012). Em estudo utilizando o 2'-hidroxicinamaldeído, verificou-se redução dos níveis de óxido nítrico em células RAW 264.7, através da redução da ativação do fator nuclear kappa B (NF-kB) (RAO e GAN, 2014). Derivados de ácidos hidroxicinâmicos são apontados como moduladores de migração de neutrófilos humanos, sendo a este também relacionadas ações anti-inflamatória e antioxidante (SILVA et al., 2016a).

Potencial antimicrobiano é relatado aos derivados cinâmicos frente espécies Gram-positivas e Gram negativas, como por exemplo *E. coli* (YE et al., 2013), *Staphylococcus* spp. (FRIEDMAN, 2017), *Salmonella* spp. (CALO et al., 2015), *Pseudomonas* spp. (VASCONCELOS, CRODA e SIMIONATTO, 2018), *Streptococcus pyogenes* (SILVA et al., 2016a), entre outras. Compostos sintetizados a partir do cinamaldeído mostraram-se eficazes frente cepas de *E. coli* e *Staphylococcus* spp. (WANG et al., 2016). Para o derivado α-bromocinamaldeído é relatada atividade antibacteriana frente a *E. coli* (SHEN et al., 2017).

Artepilina C, um derivado de ácido cinâmico, apresentou citotoxicidade acentuada pela indução da autofagia em células de câncer prostático (ENDO et al., 2018). Os ácidos cinâmicos e derivados exibiram citotoxicidade contra células de câncer da cavidade oral (KIM et al., 2010) e câncer de cólon (LEE et al., 2013), bem como células tumorais de pulmão, melanoma, próstata e glioblastoma (HUNKE et al., 2018). Atividade anticancerígena de derivados cinâmicos, através da indução da apoptose com posterior ruptura do citoesqueleto, foi verificada frente a linhagem tumoral de melanoma humano (NIERO e MACHADO-SANTELLI, 2013).

2.6.2 Lectinas

As lectinas são proteínas com a capacidade de se ligarem específica e reversivelmente a carboidratos. Nas plantas, estão envolvidas em funções de transporte e armazenamento de carboidratos, proteção contra patógenos, reconhecimento celular, entre outras (NASCIMENTO et al., 2019). A participação das lectinas nesses processos está interligada ao reconhecimento e interação entre células (FEIZI e HALTIWANGER, 2015). Considera-se um grupo heterogêneo de proteínas em relação às propriedades físico-químicas, bioquímicas e atividades biológicas, além de localização intracelular, especificidade e período de síntese no tecido vegetal (WIEDERSCHAIN, 2013). A especificidade de ligação das lectinas pode ser variável, podendo reconhecer desde monossacarídeos até carboidratos pertencentes a biomoléculas complexas, como os glicolipídios (GABIUS et al., 2011).

É bem conhecida a alta precisão das lectinas na identificação de porções de açúcar (IORDACHE et al., 2011), de forma que podem atuar em eventos como reconhecimento e dissociação celular, organização de glicoproteínas (HIMANSHA e SARATHI, 2012), mediação de respostas imunes e adesão de microrganismos às células hospedeiras (LU et al., 2014). É justamente essa interação entre lectinas e glicoconjugados que estimula pesquisas por seus efeitos biológicos, como potencial antimicrobiano (OLIVEIRA et al., 2015), antitumoral (FU et al., 2011), antinociceptivo (BARI et al., 2016) e anti-inflamatório (NASCIMENTO et al., 2019).

A lectina isolada da alga *Caulerpa cupressoides* apresentou consideráveis efeitos antinociceptivo e anti-inflamatório via sinalização do TNF-α e inibição da COX-2 (RIVANOR et al., 2018). Efeito antinociceptivo, através da diminuição de tempo de lambida de pata dos camundongos no modelo experimental da formalina, como também

efeito anti-inflamatório, pela diminuição do volume de edema da pata induzido por carragenina, foram constatados em camundongos tratados com a lectina isolada das sementes de *Tetracarpidium conophorum* (OLADOKUN et al., 2019). Lectinas isoladas da família Fabaceae foram efetivas na redução da migração de neutrófilos no modelo inflamatório da peritonite, exibindo potencial anti-inflamatório (NASCIMENTO et al., 2018). A lectina de *Centrolobium tomentosum* foi eficaz como agente anti-inflamatória de forma dose dependente em modelo de peritonite em camundongos (ALMEIDA et al., 2016). A lectina isolada de *Lonchocarpus araripensis* promoveu inibição do edema de pata em camundongos com efeito na diminuição da permeabiliade vascular (PIRES et al., 2016).

As lectinas podem agir inibindo a multiplicação de microrganismos através da aglutinação, imobilização e indução da morte (LAGARDA-DIAZ, GUZMAN-PARTIDA e VAZQUEZ-MORENO, 2017). Essas propriedades podem estar relacionadas com a ligação das lectinas com componentes glicídicos das membranas celulares como peptideoglicanos, ácidos teicoicos e lipopolissacarídeos (COELHO et al., 2018). Lectinas ligantes de manose podem neutralizar uma ampla gama de microrganismos induzindo neutralização, fagocitose e aglutinação (SILVA et al., 2019). A ligação das lectinas às membrana microbianas podem provocar aumento da permeabilidade da célula bacteriana (MOURA et al., 2015).

A lectina isolada do líquen *Cladonia verticillaris* mostrou atividades bacteriostática e bactericida contra *S. aureus, E. coli* e *K. pneumoniae* (RAMOS et al., 2014). Lectina isolada das sementes de *Moringa oleifera* apresentou atividade antibacteriana contra *Bacillus* sp., *Bacillus cereus*, *Bacillus pumillus*, *Bacillus megaterium*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Micrococcus* sp., *Micrococcus luteus Pseudomonas* sp., *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, *Serratia* sp. e *Serratia marcescens*, promovendo aglutinação e extravasamento do conteúdo das células bacterianas (MOURA et al., 2015; CORIOLANO et al., 2019). A lectina proveniente da *Canavalia ensiformis* inibiu a formação do biofilme de bactérias como *Escherichia coli* e *Listeria monocytogenes* (JIN, LEE e HONG, 2018). Lectina isolada da sarcotesta de *Punica granatum* provocou alterações estruturais em cepas de *S. aureus* sensíveis e resistentes à meticilina, exibido efeito anti-agregante e antibiofilme (SILVA et al., 2019). A lectina extraída dos folíololos de *Calliandra surinamensis*, embora tenha apenas exibido efeito bacteriostático, foi eficaz em reduzir a formação de biofilme por cepas de *Staphylococcus saprophyticcus* e *S. aureus* (não resistente e

oxacilina-resistente) (PROCÓPIO et al., 2017). Foi detectado efeito sinérgico na associação entre a lectina derivada de *Dioclea violacea* e gentamicina na inibição da multiplicação de cepas de *S. aureus* e *E. coli* (SANTOS et al., 2019). Lectina isolada das inflorescências da *Alpinia purpurata* exibiu efeito sinérgico quando em associação com oxacilina e ceftazidima frente a cepas de *S. aureus* oxacilina-resistente e *P. aeruginosa* multirresistente, respectivamente (FERREIRA et al., 2018).

Estudos também mostram que lectinas podem ativar a via da apoptose em células cancerígenas através da interação com receptores de membrana (NEUMANN et al., 2014; TATSUTA et al., 2014). A lectina derivada da *Arachis hypogaea* mostrou efeitos consideráveis em experimentação *in vitro* utilizando a linhagens de células de carcinoma de ovário (AVICHEZER e ARNON, 1996). A lectina da *Sophora flavescens* exerceu, tanto *in vitro* como *in vivo*, efeitos antitumorais frente a células MCF-7 de carcinoma de mama humano (SHI et al., 2014). A lectina da *Euphorbia tiruca* mostrou efeito citotóxico para linhagem de células de câncer de próstata (PALHARINI et al., 2017). Em estudo com a lectina extraída de *Pisum sativum* foi detectada inibição do crescimento tumoral em camudongos portadores de carcinoma de Ehrlich ascítico, provocando parada do ciclo celular na fase G2/M (KABIR et al., 2013). No mesmo modelo, a lectina do fruto da *Artocarpus integrifolia* administrada no dose de 200 µg por via intraperitoneal, em camundongos, -inibiu o crescimento do carcinoma de Ehrlich ascítico em 83% (AHMED, CHATTERJEE e DEBNATH, 1988).

2.7. *Microgramma vacciniifolia*

A familia Polypodiaceae está entre uma das maiores famílias de pteridófitas abrangendo cerca de 1500 espécies, a maioria delas epífitas (SMITH et al., 2006; SUNDUE et al., 2014). É tida como cosmopolita, embora a maior diversidade esteja concentrada nas regiões tropicais e subtropicais (GELORINI et al., 2011). O epifitismo pode ser terrestre ou rupícola e as espécies epífitas apresentam frequentemente caule dorsiventral, dispondo de fileira de frondes férteis ou não, adjacentes a filopódios curtos. O gênero *Microgramma* apresenta lâminas inteiras e soros com paráfises e engloba cerca de 30 espécies no neotrópico, que se estende desde a América Central (incluindo o sul do México, Califórnia, Flórida e todas ilhas do Caribe) até toda a América do Sul (PRADO, HIRAI, SCHWARTSBURD, 2010; SALINO et al., 2008). O referido gênero pertence ao clã das gramíneas (BAURET et al., 2017).

Os bosques úmidos promovem o desenvolvimento de pteridófitas do gênero *Microgramma* (MOURELLE, PRIETO e GARCÍA-RODRÍGUEZ, 2017), mas a urbanização não intimida o crescimento das plantas do gênero, que é diretamente correlacionado com a umidade das cascas das espécies arbóreas que as suportam (BECKER, LINDEN, e SCHMITT, 2017). A diferenciação do gênero *Microgramma* se faz pela apresentação de frondes dimorfas ou amorfas, escamas no caule peltadas, paráfises escamosas e nervuras anastomosadas. Dentre as diversas espécies, *Microgramma squamulosa* (Kaulf.) de la Sota é a mais relatada na literatura, apresentando alta concentração de manose e glicomananas na parede celular (SINGH, SINGH e ARYA, 2018) e sendo descrita dentre as epífitas que mais fazem interações com espécies arbóreas (CEBALLOS, CHACOFF e MALIZIA, 2016).

Várias espécies do gênero são descritas quanto a suas propriedades medicinais. Estudos etnofarmacológicos desenvolvidos no sul do Brasil apontam o uso como antidiabético de todas as partes da *M. squamulosa* na forma de chá (TROJAN-RODRIGUES et al., 2012). Também foi relatada a utilização dessa planta no tratamento de úlceras, sendo tal efeito correlacionado com a presença de taninos (SUFFREDINI, BACCHI e SERTIE, 1999), além do uso no tratamento de dores menstruais (MOHANTY e TRIPATHY, 2011). A administração do chá das raízes das espécies *Microgramma lycopodioides* (L.) Copel. e *Microgramma percussa (*Cav.) de la Sota foi relatada no tratamento de picada de cobra (GIOVANNINI e HOWES, 2017; GIRÓN et al., 1991). Já a *Microgramma percussa* (Cav.) de la Sota é relatada como vermífugo (BREITBACH et al., 2013) e *Microgramma nitida* (J. Sm.) A. R. Sm. é usada no tratamento de distúrbios gastrointestinais (ALONSO-CASTRO Et al., 2011; ANKLI et al., 2002).

A espécie *Microgramma vacciniifolia* (Figura 1) apresenta escamas no caule que podem apresentar margens inteiras ou denticuladas de coloração castanho-claro, com base alargada estreitando-se em um ápice filiforme (SUFFREDINI, BACCHI e SERTIE, 1999). Possui frondes dimorfas, sendo que as estéreis têm tamanho maior que as férteis, com base cuneada e ápice arredondado a levemente obtuso; os soros estão medianamente localizados entre a margem e a costa da lâmina, dispostos em uma vênula livre única; além disso, verifica-se escamas aracnoides na lâmina. Erva-silvina, erva-tereza, erva-de-lagarto, cipó-cabeludo e cipó-peludo são alguns dos nomes pelos quais é conhecida popularmente (PRADO, HIRAI, SCHWARTSBURD, 2010; SANTOS E SYLVESTRE, 2006). Autores citam aplicações da *M. vacciniifolia* como diurético, sendo utilizadas todas as partes da planta principalmente na forma de infusão. As folhas da planta são

também utilizadas na terapia de doenças do trato gênito-urinário (BOLSON et al., 2015). Relata-se que seus rizomas são utilizados na medicina popular para tratar diarreias, demonstrando também eficiência terapêutica em infecções no trato intestinal e respiratório (BARROS et al., 1997). Somado a estas propriedades, destaca-se ainda atividade adstringente, sendo também recomendada para hemorragias (AGRA et al., 2008), expectorações, cólicas intestinais (SANTOS e SYLVESTRE, 2006) e hidropisia (PERES et al., 2009).

Uma lectina (proteína que se liga a carboidratos) denominada MvRL foi isolada a partir do rizoma da *M. vacciniifolia* (ALBUQUERQUE et al., 2012). MvRL apresentou atividade antifúngica frente a *Fusarium oxysporum* f. sp. *lycopersici* (ALBUQUERQUE et al., 2014a), atividade inseticida contra cupins da espécie *Nasutitermes corniger* (ALBUQUERQUE et al., 2012) e atividade moluscicida contra *Biomphalaria glabrata* (ALBUQUERQUE et al., 2014b). Ainda, MvRL apresentou ação citotóxica contra células de carcinoma mucoepidermoide de pulmão (NCI-H292) em concentrações na qual não afetou a viabilidade de células mononucleares de sangue periférico humano (ALBUQUERQUE et al., 2014b).

Santana et al. (2012) demonstraram que uma fração enriquecida em MvRL pode ser obtida a partir do tratamento do extrato salino do rizoma com sulfato de amônio a 60% de saturação. Essa fração se mostrou homogênea quanto à presença de proteínas, apresentando somente 1 banda polipeptídica de aproximadamente 17 kDa, em eletroforese em gel de poliacrilamida em condições desnaturantes, que corresponde a MvRL.

Figura 2- *Microgramma vacciniifolia*. Disposição sobre o tronco de uma árvore (A), Rizoma e folhas segregados (B).

A

B

3. RESULTADOS

Os resultados deste trabalho são apresentados na forma de artigos.

3.1 – Artigo 1: Toxicity assessment of saline extract and lectin-rich fraction from *Microgramma vacciniifolia* rhizome

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Toxicity assessment of saline extract and lectin-rich fraction from *Microgramma vacciniifolia* rhizome

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ABSTRACT

Microgramma vacciniifolia is broadly used in folk medicine but safety information is unavailable. Therefore, we evaluated the toxicity of a saline extract and a lectin-rich fraction of *M. vacciniifolia* rhizome. The extract showed hemolytic activity on mice erythrocytes at 1000 μg/mL, whereas the fraction promoted hemolysis (8.57–26.15%) at all tested concentrations (10–1000 μg/mL). Acute toxicity test in mice indicated an LD_{50} of >5000 mg/kg. Hematological alterations and increased serum alkaline phosphatase level were observed in the treated animals. Transaminases and urea levels increased in the groups treated with the extract or fraction at 5000 mg/kg. Leukocyte infiltration was observed in the liver of extract-treated animals and in the liver and lungs of mice treated with the fraction. The kidneys of animals treated with the fraction at 5000 mg/kg presented hydropic degeneration. The extract and fraction did not induce oxidative stress in the liver and did not show genotoxicity, as examined by micronucleus and comet assays. In conclusion, the preparations were not lethal to mice but caused some signs of toxicity, mainly the fraction. The results indicated the need to evaluate the toxicity of *M. vacciniifolia* rhizome in other models and in chronic assays.

1. Introduction

It is estimated that 80% of the world population treat primary health problems using medicinal plants and their derivatives, mainly owing to the low cost and easy access (Xiang et al., 2016). However, the use of medicinal plants is often not supported by a scientific evidence, and its potential harmful effects have not been studied (Kharchoufa et al., 2018). Adverse effects that may be correlated with the short term use of plant-derived products include dermatological and gastrointestinal disorders, and those related to long-term use of plant-derived medications include reproductive, hepatic, and renal dysfunctions (Moreira et al., 2014). In addition, some plant preparations can be dangerous, especially in the presence of comorbidities and when used concomitantly with other therapies (Villas Boas et al., 2018). Increasing use of herbal products worldwide has raised the need for integration between traditional and conventional medicines; thus, ethnopharmacological studies have been performed to provide scientific evidence of the medicinal properties of these herbal products, elucidate the main phytochemicals involved in their bioactivities, and ensure their safe use (Thelingwani and Masimirembwa, 2014; Dragos and Gilca, 2018).

Microgramma vacciniifolia (Langsd. & Fisch.) Copel. (Polypodiaceae, Pteridophyta) is an epiphytic plant commonly used to treat diarrhea, intestinal and respiratory infections, hemorrhages, and hydrops, and as well as to promote expectoration (Barros and Andrade, 1997; Santos and Sylvestre, 2006; Agra et al., 2008; Peres et al., 2009). *M. vacciniifolia* rhizomes contain a lectin (carbohydrate-binding protein) called MvRL, which is isolated through protein extraction in saline solution, ammonium sulphate fractionation (60% saturation), and chromatography on a chitin column (Albuquerque et al., 2012). Santana et al. (2012) showed that the fraction obtained with ammonium sulphate is rich in MvRL.

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MvRL showed antifungal activity on *Fusarium oxysporum* f.sp. *lycopersici* (Albuquerque et al., 2014a), insecticidal activity on *Nasutitermes corniger* (Albuquerque et al., 2012), and embryotoxic effect on *Biomphalaria glabrata* (Albuquerque et al., 2014b). This lectin also showed cytotoxic action against lung mucoepidermoid carcinoma (NCI–H292) cells at concentrations that did not affect the viability of human peripheral blood mononuclear cells (Albuquerque et al., 2014b). The authors recommended future evaluation of the potential use of MvRL as a chemotherapeutic drug.

Because safety data on the use of preparations from *M. vacciniifolia* rhizome are scarce, it is necessary to evaluate their toxicity before investigating their putative pharmacological properties. In this sense, the present study aimed to evaluate a saline extract (the starting material to isolate MvRL) and a MvRL-rich fraction of *M. vacciniifolia* rhizome for cytotoxicity to CHO–K1 cells *in vitro* and for acute toxicity and genotoxicity to mice in vivo.

2. Materials and methods

2.1. Plant material

Rhizomes of *M. vacciniifolia* were collected in September 2016 at the campus of the *Universidade Federal de Pernambuco* (UFPE) in Recife (08°03′07″ S, 34°56′59″ W), Brazil. Taxonomic identification was performed, and a voucher specimen (no. 63,291) was deposited at herbarium Dárdano de Andrade Lima of the *Instituto Agronômico de Pernambuco* (Recife). The access was recorded (A347889) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen).

2.2. Preparation of saline extract and MvRL-rich fraction

Saline extract and MvRL-rich fraction were obtained as previously described (Albuquerque et al., 2012; Santana et al., 2012). The rhizomes were washed with distilled water and allowed to dry for 7 days at 28 °C and for an additional 3 days in an oven at 35 °C. Next, the dried rhizomes were powdered using a knife mill. The powder (100 g) was extracted with 0.15 M NaCl (1 L) for 12 h at 28 °C under magnetic stirring. The suspension was subsequently filtered and centrifuged (9000×*g*, 15 min, 28 °C), and the supernatant was collected as the saline extract. To obtain a MvRL-rich fraction, the extract was treated with ammonium sulphate at 60% saturation (Green and Hughes, 1955) for 4 h and then centrifuged (9000×*g*, 15 min, 28 °C). The resulting precipitate was then resuspended in 0.15 M NaCl and dialyzed with distilled water for 6 h (two liquid changes). The saline extract was also submitted to dialysis for removal of salt. The dialyzed extract and fraction were dried by lyophilization.

2.3. Protein concentration and hemagglutinating activity

Protein concentration was determined according to a method described by Lowry et al. (1951) using a standard curve of bovine serum albumin (31.25–500 μg/mL). Hemagglutinating activity (HA) was assayed according to a method described by Albuquerque et al. (2012) using a suspension (2.5%, v/v) of human O-type erythrocytes in 0.15 M NaCl. HA was defined as the reciprocal value of the highest dilution of sample that promoted full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between HA and protein concentration (mg/mL).

2.4. Phytochemical screening

Thin-layer chromatography (TLC) was used to detect the presence of alkaloids, coumarins, cinnamic acid derivatives, flavonoids, tannins, terpenes/steroids, and sugars in the extract and MvRL-rich fraction. Plates were developed in a high-performance thin-layer chromatography (HPTLC) system (CAMAG, Switzerland) consisting of a Linomat V sample applicator equipped with a 100 μL syringe (Hamilton, Switzerland) connected to compressed air, and the winCATS® software (CAMAG) was used to process the data. Silica gel plates (60-F_{254}; Merck, Germany) were placed in a twin through vertical glass chamber (10 × 10 cm; CAMAG), and the plates were developed using the mobile phase required for each compound class (Markhan, 1982; Abreu, 2000; Wagner and Bladt, 2001). The saturation time for the mobile phase was 30 min at 25 ± 2 °C. After development, the plates were derivatized by spraying with the reagents specific to each class compound. For phenolic compounds, the plates were visualized under 254 or 365 nm ultraviolet light. the Images were acquired using a MultiDoc-It™ Imaging System (Model 125, USA) with the UVP software (Fisher Scientific International, Inc., Hampton, NH, USA) and a Canon camera Rebel T3, EOS 1100 D (Tokyo, Japan).

2.5. High-performance liquid chromatography (HPLC) analyses

The dried saline extract or MvRL-rich fraction (10 mg) was diluted in 5 mL of 50% (v/v) ethanol to obtain a stock solution. Next, a 5-mL aliquot of this solution was diluted to 10 mL with ultrapure water (Purelab® Classic UV; ELGA LabWater, USA), and the sample was filtered on PVDF membranes (0.45 μm). Chlorogenic acid solution (100 μg/mL) in ethanol was also prepared for use as standard.

HPLC analyses was performed on an HPLC Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a photodiode array detector (DAD; Thermo Fisher Scientific) and equipped with a binary pump (HPG-3x00 R S; Thermo Fisher Scientific), degasser, and autosampler with a 20-μL loop (ACC-3000; Thermo Fisher Scientific). The wavelength was set at 350 nm based on the results of TLC assay. Chromatographic separations were performed at 26 °C with a Phenomenex® C18 (250 mm × 4.6 mm d.i., 5 μm) column equipped with pre-column (4 mm × 3.9 m C18; Phenomenex®). The mobile phase consisted of ultrapure water (A) and methanol (B), both acidified with 0.05% trifluoroacetic acid, and the flow was 0.6 mL/min. A gradient program was applied as follows: 0–10 min, 30–45% B; 10–15 min, 45–60% B; 15–25 min, 60–90% B; 25–30 min, 90-30% B. Data were analyzed after triplicate injection and processed using the Chromeleon 6.8 software (Dionex; Thermo Fisher Scientific).

Chlorogenic acid content was calculated according to the following equation:

$$\frac{Sc \times SA}{Sa \times m} \times DF \times 100$$

Sc = standard concentration (g/mL); *Sa* = standard area; *SA* = sample area; *DF* = dilution factor; *m* = sample weight.

2.6. Cytotoxicity assays

2.6.1. Evaluation of cytotoxicity to Chinese hamster ovary cells (CHO–K1)

CHO–K1 cells were obtained from the *Banco de Células do Rio de Janeiro* of the *Universidade Federal do Rio de Janeiro* and *Associação Técnico-Científica Paul Ehrlich* (Duque de Caxias, Brazil). After thawing, the cells were kept at 37 °C, 5% CO_2, and 90% humidity in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), all purchased from Sigma-Aldrich (St. Louis, MO, USA). The average cell cycle time was 12 h under these conditions, and the cells were used in the bioassays after the fourth passage. Prior to each experiment, cell viability was determined using the trypan blue dye exclusion test. Cells were used only when the viability was higher than 95%.

Cytotoxicity was evaluated through the Alamar Blue assay with slight modifications (Nakayama et al., 1997). CHO–K1 cells were plated (1×10^5 cells/mL) into 96-well microplates containing 200 μL of DMEM and then incubated for 16 h (37 °C, 5% CO_2). Afterwards, the cells were treated with the extract or MvRL-rich fraction (3.125–400 μg/mL, in a

culture medium). In the negative control group, cells were treated with only the culture medium. Doxorubicin (3.125–400 μg/mL) was used as a positive control. The treatments were removed after 24 or 72 h of incubation, and 200 μL of 10% (w/v) Alamar Blue (resazurin) were added to each well, which was then further incubated for 6 h. The conversion of resazurin to resorufin by the cells was evaluated by measuring the absorbance at 540 nm (oxidized state) and 620 nm (reduced state) in a microplate-reader spectrophotometer (Multiskan EX; Thermo Scientific, USA). The concentration required to reduce cell viability to 50% (IC_{50}) was determined.

2.6.2. Hemolytic assay

The assay was performed as described by Jimenez et al. (2003) in 96-well microplates using a 2% (v/v) suspension of mice erythrocytes in a 0.85% NaCl containing 10 mM $CaCl_2$. Next, the saline extract or MvRL-rich fraction at different concentrations (10, 50, 100, 250, 500, or 1000 μg/mL) were added to 2 mL of the erythrocyte suspension. The saline solution was used for the negative control (0% hemolysis) and Triton X-100 for the positive control (100% hemolysis). The samples were incubated for 1 h under constant stirring (100 rpm) at 22 ± 2 °C. Afterwards, they were centrifuged at 2500×*g* for 5 min, and the absorbance at 540 nm was measured (Rangel et al., 1997). All experiments were performed in triplicate.

2.7. Animals

Female Swiss mice (*Mus musculus*), 50 days old and weighing 30–35 g, were obtained from the vivarium of the *Laboratório de Imunopatologia Keizo Asami* of UFPE. The animals were used in the tests after 1 week of adaptation in the experimental laboratories located at the *Departamento de Bioquímica* of UFPE or the *Centro de Biotecnologia* of the *Universidade Federal da Paraíba* (João Pessoa, Brazil). The mice were housed at a temperature of 22 °C, with 12:12 photoperiod and *ad libitum* access to food (Purina; Nestlé Brasil Ltda., Brazil) and water. The Ethics Committee on Animal Experimentation of UFPE approved all the experiments (process number 23076.042699/2016–72).

2.8. Acute toxicity evaluation

2.8.1. Treatments

Acute toxicity was assessed following the protocol 423 of the Organization for Economic Cooperation and Development (2001). The animals (n = 3 per group) were treated with the extract or MvRL-rich fraction (dissolved in 0.9% NaCl) by gavage at a single dose of 2000 or 5000 mg/kg body weight (b.w.). In the control group, the animals received saline solution. After administration, the animals were individually observed within the first 4 h and then monitored daily for weight and intake of water and food. At the end of the observation period (day 14), the animals were anesthetized (80 mg/kg ketamine and 20 mg/kg xylazine, i.p.), and blood samples were collected for hematological and biochemical analyses. Next, the mice were euthanized by cervical dislocation, and the liver, kidney, heart, lung, stomach, and spleen were collected and weighed. Organ weight was expressed as relative weight (g/10 g b.w.).

2.8.2. Hematological and biochemical analyses

Blood samples from each animal were divided into two tubes, one containing EDTA for hematological analysis the other containing a separating gel for biochemical analyses. Samples for hematological analysis were evaluated for erythrocyte count, leukocyte count, hemoglobin count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), using an ABX-PENTRA-60 automated hematology analyzer (Horiba, Japan). Samples for biochemical analysis were centrifuged for 10 min at 3500 rpm to obtain serum for analyses of urea, creatinine, transaminases, and phosphatase alkaline using specific kits (Labtest Diagnóstica, Lagoa Santa, Brazil) and a COBAS Mira Plus analyzer (Roche Diagnostics Systems, Basel, Switzerland).

2.8.3. Histological analysis

After the organs were weighed, portions of the liver, kidney, spleen, and lung were immediately fixed in buffered formalin (10%, v/v), dehydrated through a graded ethanol series (70–100%), diaphanized in xylol, and embedded in paraffin. Histological slices (5 μm-thick) were stained with hematoxylin-eosin and mounted using cover slips with Entellan resin (Merck) (Kierman, 2008). The samples were observed using a Motic BA200 microscope coupled to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong). The organs were sectioned at 5 μm in thickness and stained with hematoxylin and eosin for general examination of tissue architecture. The sections were viewed under an optical Motic BA200 microscope, and photomicrographs were obtained using a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong).

2.8.4. Oxidative stress evaluation

Homogenates of the liver (500 μg) were prepared in 50 mM Tris-HCl pH 7.4 containing 1 mM EDTA, 1 mM sodium orthovanadate, and 2 mM phenylmethanesulfonyl fluoride. After centrifugation at 2500×*g* for 10 min at 4 °C, the supernatants were collected, and the total protein content was determined as described by Bradford (1976).

Lipid peroxidation was evaluated by estimating the level of thiobarbituric acid-reactive substances (TBARS) according to a method by Ohkawa et al. (1979). Briefly, liver homogenate (100 μL) was incubated for 4 h 30% (w/v) trichloroacetic acid (10 μL), and 10 mM Tris-HCl pH 7.4 (390 μL). The mixture was centrifuged at 2500×*g* for 10 min, and the supernatant was transferred to another tube and then mixed with 0.8% (v/v) thiobarbituric acid (10 μL). The mixture was boiled in a water bath for 30 min, and after cooling, the absorbance of the organic phase was measured at 535 nm. The results are expressed as nmol of malondialdehyde (MDA) per mg of protein.

To measure catalase (CAT) activity, liver homogenate (80 μL) was added to 1 mL of 0.8 M potassium phosphate pH 7.4, and the suspension was centrifuged at 2500×*g* for 15 min. A solution of hydrogen peroxide (0.059 M) in this potassium phosphate buffer was used as substrate. In a quartz cuvette, 0.1 mL of the supernatant was mixed with 1 mL of the hydrogen peroxide solution and 1.9 mL of distilled water. Enzyme activity was measured by monitoring the change in absorbance at 240 nm between the first and sixth minutes (Beers and Sizer, 1952). One unit of CAT activity was defined as the amount of enzyme required to convert 1 μmol of H_2O_2 per minute to H_2O. Tissue CAT enzymatic activity is expressed as mU/mg protein.

To measure the total tissue superoxide dismutase (SOD) activity, liver homogenate (80 μL) was first incubated with 880 μL of 0.05% (w/v) sodium carbonate pH 10.2 containing 0.1 mM EDTA in a water bath at 37 °C. The reaction was developed by the addition of 20 μL of 30 mM epinephrine in 0.05% (v/v) acetic acid. SOD activity was measured by evaluating the kinetics of inhibition of epinephrine autoxidation according to the absorbance at 480 nm. One unit of SOD activity was defined as the amount of protein required to inhibit the autoxidation of 1 μmol of epinephrine per minute. Tissue SOD enzymatic activity is expressed as U/mg protein.

2.9. Genotoxicity evaluation

2.9.1. Treatments

The mice were divided into experimental groups of 10 animals each. The extract and MvRL-rich fraction were dissolved in saline solution (0.9% NaCl) and administered by gavage at a single dose of 0.1 mL at 1000 or 2000 mg/kg b.w. The negative control group received only saline solution, whereas the positive control group received an intraperitoneal injection of cyclophosphamide (CPA) at 80 mg/kg b.w.

2.9.2. Micronucleus test

The assay was performed following the protocols described by Hayashi et al. (1990) and Maistro (2014). Five animals from each group were sacrificed at 24 h after treatment, whereas another five animals were sacrificed after 48 h. Bone marrow was collected from the femur using 2 mL of saline and then centrifuged (3000×*g*) for 7 min. For each animal, two 10 μL of bone marrow was placed on slides previously stained with acridine orange, and the presence of micronuclei was assessed under the fluorescence microscope Olympus BX 50 (Olympus Corporation, Tokyo, Japan). For each sample, a total of 2000 polychromatic erythrocytes were counted, and the analyses were performed blindly by a single person. The number of micronucleated polychromatic erythrocytes (MNPCE) was determined.

2.9.3. Comet assay

The comet assay was performed according to Burlinson et al. (2007) with both bone marrow and peripheral blood. Tail vein peripheral blood samples were obtained from five mice at 4 and 24 h after the treatments. Cells were counted using a hemocytometer, and cell viability was determined by trypan blue dye exclusion. The experiment was performed only if the number of viable cells was greater than 90%. After electrophoresis, the slides were quickly rinsed in distilled water, covered with 30 mL of ethidium bromide staining solution (20 mg/mL), and cover-slipped. The material was immediately evaluated at 400 × magnification using the fluorescence microscope Olympus B×50 with a 515–560 nm excitation filter and a 590 nm barrier filter. Only individual nucleoids were scored. The extent of DNA damage was assessed by examining at least 100 randomly selected and non-overlapping cells per animal in a blind analysis. Cells were visually scored according to tail size according to the following four classes: 0, no tail; 1, tail shorter than the head (nucleus) diameter; 2, tail length measuring 1–2 times the head diameter; 3, tail length measuring more than twice the head diameter (Hartmann and Speit, 1997). The total score for 200 comets was obtained by multiplying the number of cells in each class by the damage class, and then ranged from 0 (all undamaged) to 300 (all damaged).

2.10. Statistical analysis

The results are expressed as mean ± standard deviation (SD) or mean ± standard error of mean (SEM). One-way analyses of variance (ANOVA) followed by Bonferroni's (acute toxicity) or Dunnett's (other assays) post-test was used to determine statistical significance between groups with the GraphPad Prism Software 5.0 (GraphPad Software, La Jolla, CA, USA). A p value < 0.05 was considered significant. The IC_{50} in the cytotoxicity assay was calculated by nonlinear regression using the Graph Pad Prism software.

3. Results and discussion

Microgramma vacciniifolia has been widely used in folk medicine for several purposes; however, information of its safety is unavailable. In the present study, we evaluated the toxicity of a saline extract from *M. vacciniifolia* rhizome and a fraction from it enriched in the lectin MvRL, which was previously reported to exert several biological activities.

TLC analysis revealed only the presence of cinnamic acid derivatives

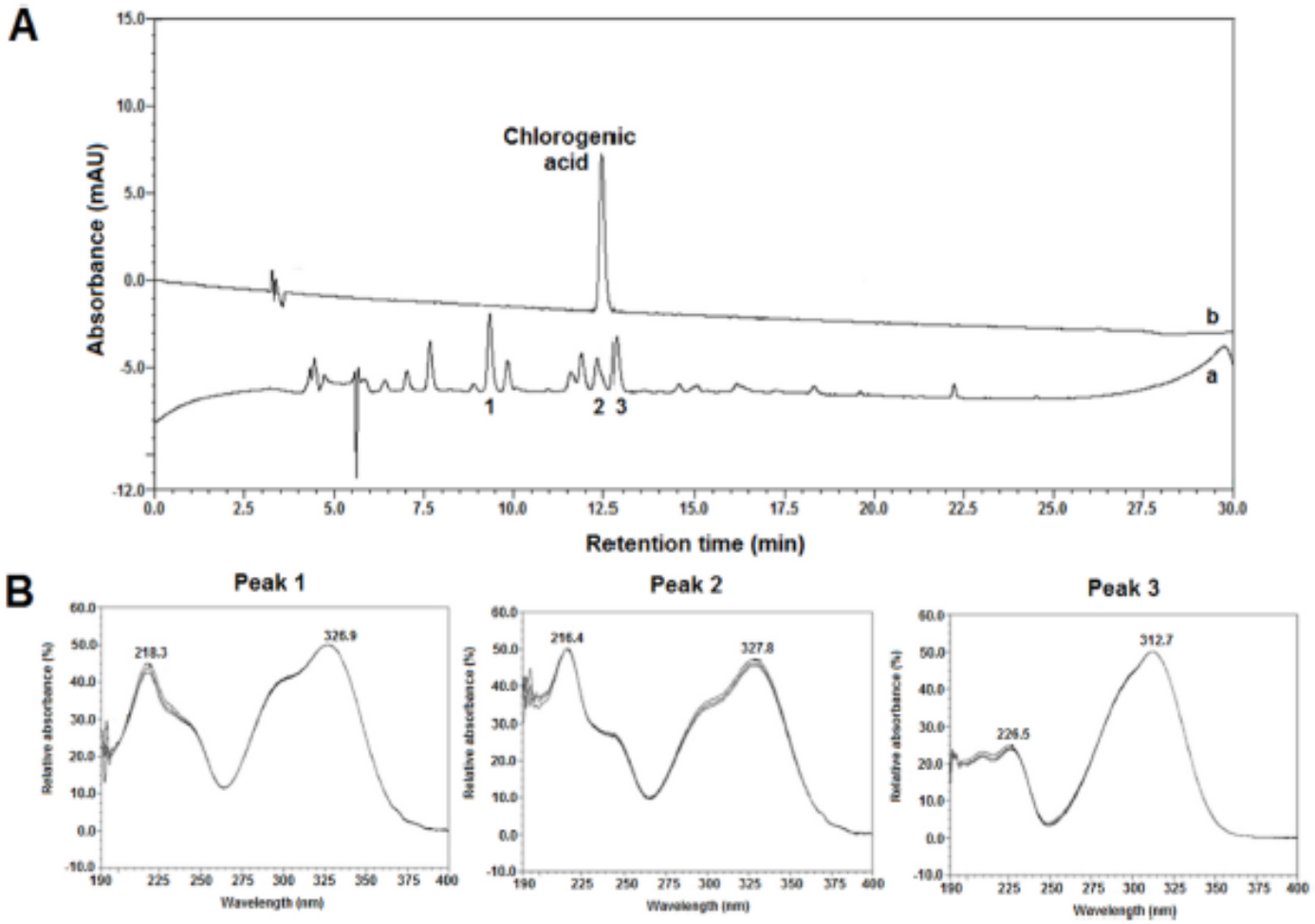

Fig. 1. High-performance liquid chromatography (HPLC) profile of saline extract of *Microgramma vacciniifolia* rhizome. (A) Chromatograms at 350 nm of the extract (a) and the standard chlorogenic acid (b). (B) UV spectra of the three main peaks detected in the extract.

in the extract. Fig. 1A shows the HPLC profile of the saline extract of *M. vacciniifolia* rhizome (chromatogram a). Among the peaks observed, three were identified. One of these peaks was observed at 12.5 min (peak 2), which corresponded to the same retention time of the standard chlorogenic acid (chromatogram b). Two other peaks present in the chromatographic profile (peaks 1 and 3) were found to correspond to cinnamic acid derivatives, according to their ultraviolet spectra (Fig. 1B). The calculated content was 0.135 ± 0.0001 g% for chlorogenic acid and 0.271 ± 0.0001 g% for cinnamic acid derivatives.

Chlorogenic acid, which is widely distributed in plant species, belongs to the group of polyphenols and participates as an intermediate in lignin synthesis (Fu et al., 2017). It is found in a range of plant foods, with coffee as a main source of this compound in human diet. Anti-inflammatory (Hwang et al., 2014; Meinhart et al., 2019), antibacterial (Peng et al., 2015), antiviral (Butiuk et al., 2016), anti-obesity (Flanagan et al., 2014), antioxidant (Hwang et al., 2017), antidiabetic (Oboh et al., 2015), antihypertensive, and hypocholesterolemic (Wan et al., 2013) activities have been described for this compound. In addition, studies have reported the antitumor effectiveness of chlorogenic acid, mainly against colon and stomach cancers (Shao et al., 2015) as well as its inhibitory effect on the development of liver cancer cells (Siswanto et al., 2017; Yan et al., 2017).

Cinnamic derivatives have been correlated with antidepressant, sedative, neuroprotective (Gunia-Krzyżak et al., 2015), anti-inflammatory (Rao and Gan et al., 2014), antimicrobial (Guzman, 2014), antioxidant (Sova, 2012), antitumor (De et al., 2011), and antimalarial (Pérez et al., 2013) effects. Studies have shown a close relationship between cinnamic derivatives and modulation of the central and peripheral nervous systems, in which they participate in important interactions with GABAergic opioids and glutaminergic receptors as well as voltage-dependent K + ATPase channels, among others (Gunia-Krzyżak et al., 2015).

None of the evaluated secondary metabolite classes was detected by TLC in the MvRL-rich fraction, although the fraction had a brownish color. The fraction was also subjected to HPLC analysis, but no peaks were detected in the chromatogram. The saline extract showed a protein concentration of 5.37 mg/mL and specific HA of 11.9, whereas the MvRL-rich fraction contained 14.21 mg/mL of protein and presented specific HA of 144.1. These data indicated the presence of lectins in both preparations and confirmed that the fraction was richer in the lectin MvRL than the extract (purification factor: 12.1).

Different cells and organisms may be used in toxicological evaluations. For cytotoxicity assays, a range of mammalian cells, such as Chinese hamster ovary cells 6, have been used as models for examining cytotoxicity to human cells (Guo et al., 2020). The extract and MvRL-rich fraction did not affect the viability of CHO–K1 after 24 and 72 h of treatment, indicating low cytotoxicity with IC_{50} > 200 μg/mL (Froelich et al., 2007). Doxorubicin (positive control) showed cytotoxicity after 72 h of incubation, with an IC_{50} of 0.28 ± 0.02 μg/mL.

The extract was only able to promote hemolysis at the concentration of 1000 μg/mL, compared with the negative control (Fig. 2A). On the contrary, the MvRL-rich fraction promoted hemolysis at all tested concentrations, with the degree ranging from 8.57% to 26.15% (Fig. 2B). The interaction of compounds present in the extract and fraction with the erythrocyte membrane may result in pore formation through which hemoglobin can be released. The data suggested that the hemolytic activity of the *M. vacciniifolia* preparations studied here can be attributed to the presence of MvRL or other compounds that were more concentrated in the fraction than in the extract. Lectins are known for their hemagglutinating activity, but some of them are described as not only able to agglutinate blood but also to cause erythrocyte lysis (Mancheño et al., 2010). For example, Unno et al. (2014) reported that the lectin CEL-III from sea cucumber (*Cucumaria echinata*) possesses two domains that bind to erythrocyte-surface carbohydrates and another domain that forms transmembrane pores, leading to cell lysis or death. Volodymyr and Stoika (2012) showed that lectins of different mushroom species have varying degrees of hemolytic activity: those from *Amanita phalloides* and *Amanita virosa* presented higher hemolytic activity than hemagglutinating activity, whereas those from *Mycena pura* and *Laetiporus sulphureus* showed higher hemagglutinating activity than hemolytic activity.

In the acute toxicity test, no death was observed among mice treated orally with the extract or fraction, either at 2000 or 5000 mg/kg. Thus, a LD_{50} > 5000 mg/kg was estimated, indicating that these substances were nontoxic according to the OECD protocol. In addition, no behavioral changes were observed in the group treated with the extract; in contrast, the animals that received the fraction showed prostration in the first 15 min, irritability and agitation for 15–30 min, and absence of urination in the interval of 15–30 min. However, these changes were no longer observed after 30 min.

Table 1 shows that there was a significant decrease in water and food intake as well as weight gain in the animals treated with the extract at 5000 mg/kg and with the fraction at 2000 and 5000 mg/kg. The decrease in animal weight gain was subsidized by the decrease in food and water intake (Chindo et al., 2012). This reduction in weight gain may be considered as indicative of mild intoxication and may have resulted from abnormalities in macronutrient metabolism or even mineral regulation (Ezeonwumelu et al., 2011; Piao et al., 2013). Madingou et al. (2016) pointed out that the effects of toxic substances may lead to decreased appetite and, consequently, decreased food intake.

Table 2 shows some differences in hematological parameters between the treatment group and the control group: hematocrit, hemoglobin, MCV, and MCH increased in animals that received both doses of the extract and MvRL-rich fraction, suggesting a possible effect on

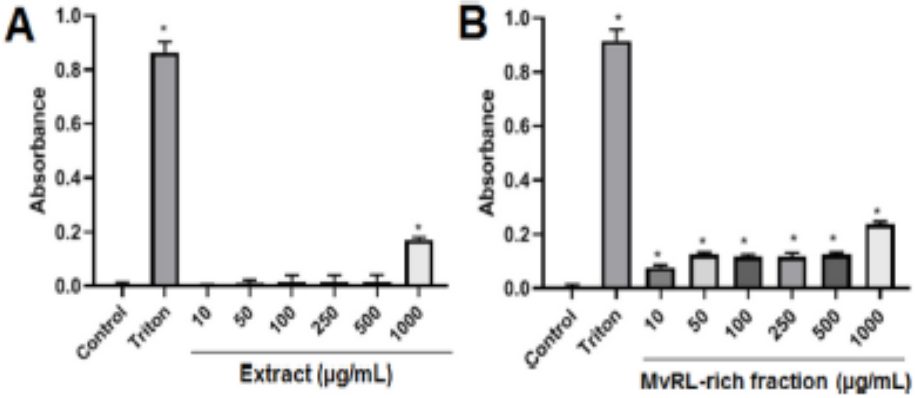

Fig. 2. Evaluation of the hemolytic activity of a saline extract (A) and a MvRL-rich fraction (B) of *Microgramma vacciniifolia* rhizome on human erythrocytes. The results are expressed as mean ± SD. (*) indicates significant differences between the treatment and negative control groups ($p < 0.05$). Triton X-100 was used as a positive control (100% hemolysis).

Table 1
Evaluation of food and water consumption as well as weight gain in animals from control group and treated with the saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome.

Parameter	Control	Extract		MvRL-rich fraction	
		2000 mg/kg	5000 mg/kg	2000 mg/kg	5000 mg/kg
Water consumption (mL)	31.05 ± 0.94	30.97 ± 0.46	26.44 ± 0.76[a]	28.57 ± 0.62[a]	27.77 ± 0.82[a]
Food consumption (g)	14.85 ± 0.21	14.92 ± 0.31	11.12 ± 0.52[a]	12.18 ± 0.21[a]	9.09 ± 0.89[a]
Weight gain (g)	6.01 ± 0.55	6.00 ± 0.32	4.98 ± 0.11[a]	4.12 ± 0.50[a]	2.72 ± 0.09[a]

[a] Significantly different in comparison with control according to one-way ANOVA followed by Bonferroni's test. Values represent the mean ± SEM (n = 3/group).

Table 2
Hematological parameters of animals from control group and treated with the saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome.

Parameter	Control	Extract		MvRL-rich fraction	
		2000 mg/kg	5000 mg/kg	2000 mg/kg	5000 mg/kg
Erythrocytes (10^6/mm^3)	6.09 ± 0.41	6.01 ± 0.66	5.94 ± 0.23	5.98 ± 0.52	5.57 ± 0.48
Hematocrit (%)	39.23 ± 0.61	40.03 ± 0.32*	48.56 ± 0.22*	53.33 ± 0.21*	52.33 ± 0.42*
Hemoglobin (%)	15.90 ± 0.12	16.02 ± 0.28*	17.43 ± 0.26*	17.73 ± 0.24*	16.70 ± 0.32*
MCV (%)	65.47 ± 0.58	89.03 ± 0.14*	91.76 ± 0.37*	93.07 ± 0.19*	89.80 ± 0.45*
MCH (%)	26.51 ± 0.82	29.08 ± 0.73*	31.48 ± 0.92*	30.26 ± 0.88*	30.47 ± 0.62*
MCHC (%)	39.56 ± 0.97	39.11 ± 0.27	40.02 ± 0.45	33.13 ± 0.64*	38.77 ± 1.01*
Leukocytes (10^3/mm^3)	8.75 ± 0.13	8.15 ± 0.50	8.00 ± 0.37	8.15 ± 0.50	8.00 ± 0.37
Segmented (%)	55.67 ± 0.16	48.10 ± 0.78*	50.21 ± 1.34*	43.10 ± 1.38*	44.01 ± 1.21*
Eosinophils (%)	1.33 ± 0.21	1.77 ± 0.25	1.32 ± 0.19	1.67 ± 0.21	1.33 ± 0.21
Lymphocytes (%)	39.33 ± 1.72	55.21 ± 1.42*	48.02 ± 0.71*	54.33 ± 1.57*	47.09 ± 0.92*
Monocytes (%)	1.33 ± 0.21	1.35 ± 0.32	1.36 ± 0.29	1.33 ± 0.21	1.67 ± 0.21

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. Values represent the mean ± SEM (n = 3/group). (*) Significantly different in comparison with control according to one-way ANOVA followed by Bonferroni's test.

erythropoiesis (Wu et al., 2018). Moreover, MCHC decreased in the animals treated with the fraction. Liver damage can lead to cholesterol esterification deficiency, resulting in excess of membranes in erythrocytes and consequent macrocytosis (Lorenzi, 2006). In addition, substances that affect DNA biosynthesis, such as antibiotics, antimetabolites, and antivirals, have been reported to cause macrocytosis (Arslan and Altundag, 2012). There was a significant decrease in segmented leukocytes and an increase in lymphocytes in the animals treated with the extract and fraction at both doses. The recruitment of lymphocytes may be due to the activation of the immune system as a defense mechanism of the organism (Fisher et al., 2008).

Biochemical analysis results (Table 3) showed that alkaline phosphatase level increased in the animals treated with both doses of the extract and fraction, whereas transaminase and urea levels significantly increased only in the groups treated with the extract or fraction at 5000 mg/kg dose. These changes in transaminase and alkaline phosphatase levels suggested liver damage, as these enzymes are recognized markers

Table 3
Blood biochemical parameters of animals from control group and treated with the saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome.

Parameter	Control	Extract		MvRL-rich fraction	
		2000 mg/kg	5000 mg/kg	2000 mg/kg	5000 mg/kg
ALT (U/L)	59.10 ± 0.89	58.93 ± 0.67	77.06 ± 2.11*	60.53 ± 0.02	65.03 ± 4.73*
AST (U/L)	97.83 ± 1.31	99.99 ± 1.54	115.54 ± 2.24*	101.97 ± 2.01	119.93 ± 4.11*
Alkaline phosphatase (IU/L)	17.47 ± 0.29	17.59 ± 1.01*	24.73 ± 2.65*	28.37 ± 2.52*	24.73 ± 2.65*
Urea (mg/dL)	49.25 ± 0.63	48.96 ± 1.32	55.26 ± 1.59*	49.07 ± 2.53	58.13 ± 2.05*
Creatinine (mg/dL)	0.29 ± 0.02	0.28 ± 0.49	0.36 ± 0.10*	0.21 ± 0.09	0.24 ± 0.06

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. Values represent the mean ± SEM (n = 3/group). (*) indicates significant difference ($p < 0.05$) in comparison with control.

of liver injury (Rafiae et al., 2017). Increased creatinine level was observed only in the animals treated with the highest dose of the extract; this marker is correlated with renal function, and its level is often altered owing to clearance of excess substances (Traesel et al., 2014). The high diversity of compounds present in the extract may be linked to this increase.

Relative weights of the organs remained unchanged in the four groups of animals treated with *M. vacciniifolia* rhizome preparations, compared to those in the control (Table 4). Histopathological evaluation results supported the alterations found in the hematological and biochemical analyses. The animals that received the saline extract (Fig. 3) showed slight leukocyte infiltration around the centrilobular vein in their liver. The lungs and spleen presented preserved architecture; moreover, though the urea and creatinine levels increased, the kidneys presented preserved architecture, indicating that acute tissue damage could not be observed histologically.

In the animals that received the MvRL-rich fraction (Fig. 3), at both doses, considerable leukocytes infiltration was observed in the liver around the centrilobular vein as well as in the lungs surrounding the bronchioles and in the pulmonary parenchyma. Liver and lung infiltration may be related to the pathogenesis of lesions due to exposure to chemical substances (Kolios et al., 2006). Regarding the kidneys, the animals treated with the fraction at 5000 mg/kg presented hydropic degeneration with small glomeruli. Both groups showed large lymph nodes in the spleen. However, these findings did not induce any physiological changes; therefore, the general condition of the animals was not

Table 4
Weights of the organs of animals from control group and treated with the saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome.

Parameter	Control	Extract		MvRL-rich fraction	
		2000 mg/kg	5000 mg/kg	2000 mg/kg	5000 mg/kg
Heart (g)	0.18 ± 0.00	0.18 ± 0.01	0.18 ± 0.00	0.17 ± 0.01	0.19 ± 0.01
Lung (g)	0.24 ± 0.00	0.26 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02
Liver (g)	2.11 ± 0.10	2.05 ± 0.05	2.09 ± 0.09	2.01 ± 0.05	2.03 ± 0.05
Kidney (g)	0.23 ± 0.01	0.25 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.22 ± 0.02
Spleen (g)	0.33 ± 0.03	0.29 ± 0.041	0.31 ± 0.04	0.27 ± 0.04	0.28 ± 0.04

No significant differences were found in comparison with control according to one-way ANOVA followed by Bonferroni's test.

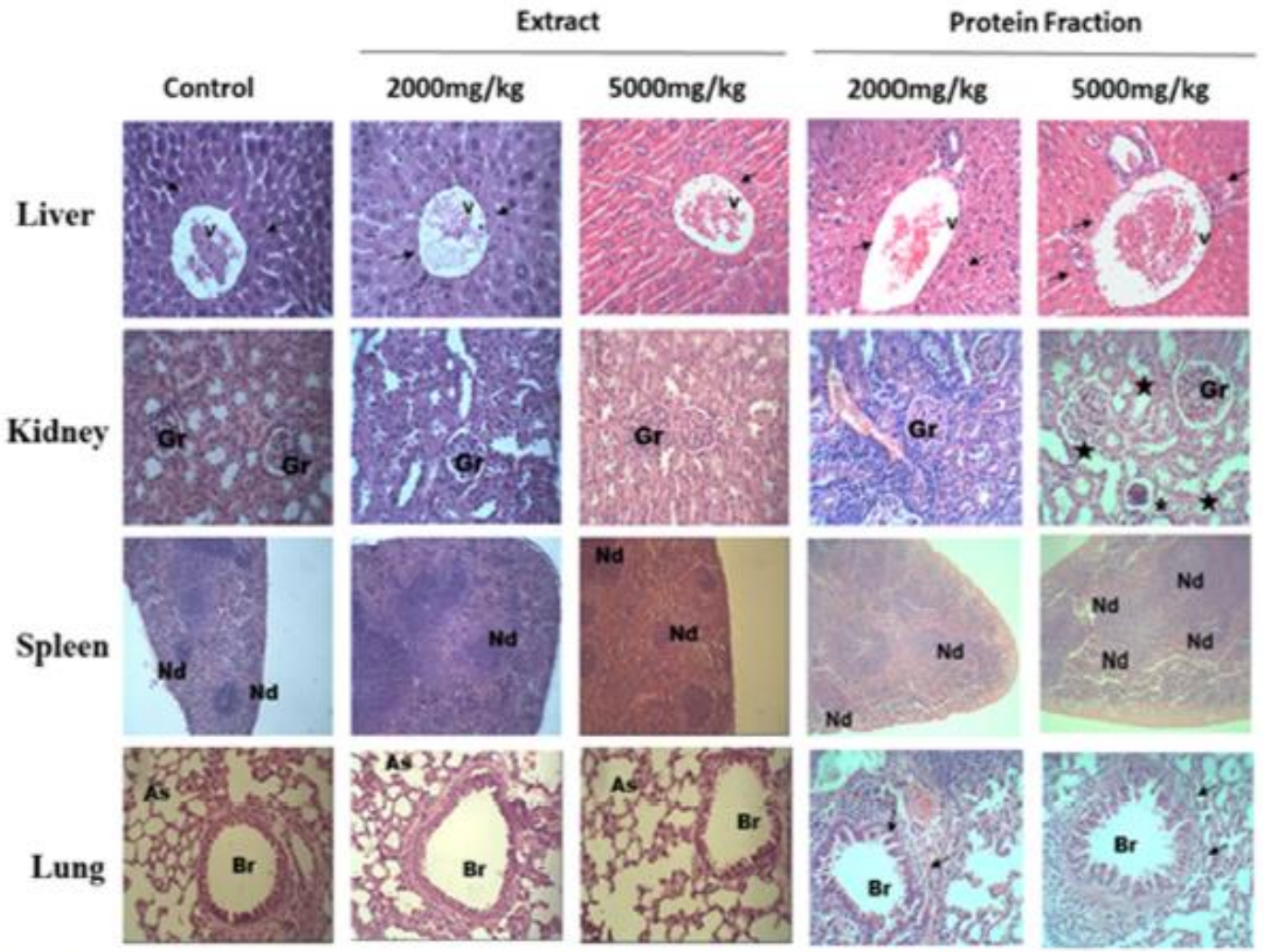

Fig. 3. Representative photomicrographs of the organs of animals treated with a saline extract and a MvRL-rich fraction of *M. vacciniifolia* rhizome at a single dose of 2000 or 5000 mg/kg. **Liver:** the centrilobular vein (v) is observed in all images. In the control group, the hepatocyte bundles are preserved and ordered. Mice who received doses of the saline extract presented slight leukocyte infiltration (short arrows), whereas mice who received doses of the MvRL-rich fraction presented accentuated leukocyte infiltration (short arrows). **Kidneys:** Renal glomeruli (Gr) and contorted tubules without alterations are visible in the control and treated groups, except for the group that received the MvRL-rich fraction at 5000 mg/kg, which presented degeneration (stars). **Spleen:** The lymph nodes (Nd) are well-defined in the control and treated groups. The spleens of mice who received the MvRL-rich fraction showed considerable activation of lymph nodes. **Lungs:** The bronchiole (Br) and alveolar sac (As) were observed, with preserved architecture in the control and in mice treated with the extract and fraction. The alveolar duct can also be observed in one image. The mice that received the MvRL-rich fraction showed leukocyte infiltration (short arrows) around the bronchioles (br) and lung parenchyma.

impaired, which corroborated with the high LD_{50}.

In addition, we evaluated the oxidative profile of the liver of mice treated with the saline extract or MvRL-rich fraction. The liver is a primary source of xenobiotic metabolism, making it a target organ for oxidative damage (Chen et al., 2014). The saline extract and MvRL-rich fraction did not induce any significant ($p > 0.05$) changes in MDA, SOD, and CAT levels in mouse liver (Table 5), indicating the absence of oxidative stress induction.

Micronucleus assay results showed that treatment with the extract and the fraction at both doses (1000 and 2000 mg/kg) did not significantly increase the number of MNPCE, compared with the negative control (Table 6). Genotoxicity was also assessed by the comet assay (Table 7), and the results showed no significant differences in peripheral blood cells or bone marrow cells between the mice treated with the preparations and those treated with the negative control. The positive control, cyclophosphamide, induced genetic damage in both micronucleus and comet assays. Furthermore, neither the extract nor the fraction affected the production of new erythroblasts, as visualized through the ratio between polychromatic and normochromatic cells (PCE/NCE), indicating that these substances did not affect bone marrow and its cell

Table 5
Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels in liver of mice from control group and treated with the saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome.

Parameter	Control	Extract		Fraction	
		2000 mg/kg	5000 mg/kg	2000 mg/kg	5000 mg/kg
MDA (nM/mg of protein)	4.94 ± 0.38	4.42 ± 0.26	4.56 ± 0.37	4.37 ± 0.31	4.35 ± 0.26
SOD (U/mg of protein)	9.96 ± 0.06	9.89 ± 0.62	9.81 ± 0.34	9.88 ± 0.34	9.82 ± 0.22
CAT (nM/mg of protein)	0.33 ± 0.03	0.36 ± 0.02	0.35 ± 0.02	0.36 ± 0.01	0.35 ± 0.02

Values represent the mean ± SEM (n = 3/group). No significant differences were found in comparison with control according to one-way ANOVA followed by Bonferroni's test.

Table 6
Evaluation of in vivo genotoxicity of saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome by determination of the number of micronucleated polychromatic erythrocytes (MNPCE) from bone marrow of mice.

Treatments	Time sample	Number of MNPCE per Animal					MNPCE (mean ± SD)	PCE/NCE (mean ± SD)
		M1	M2	M3	M4	M5		
Negative control	24 h	0	1	0	1	1	0.60 ± 0.18	1.16 ± 0.12
	48 h	0	0	1	1	0	0.40 ± 0.12	1.21 ± 0.13
Saline extract								
1000 mg/kg	24 h	1	1	1	0	0	0.60 ± 0.21	1.21 ± 0.12
	48 h	1	1	0	1	0	0.60 ± 0.23	1.17 ± 0.14
2000 mg/kg	24 h	1	1	0	0	1	0.60 ± 0.22	1.14 ± 0.12
	48 h	1	1	0	0	1	0.60 ± 0.23	1.14 ± 0.10
MvRL-rich fraction								
1000 mg/kg	24 h	1	1	1	1	0	0.80 ± 0.21	1.21 ± 0.12
	48 h	1	1	1	1	0	0.80 ± 0.23	1.17 ± 0.14
2000 mg/kg	24 h	0	1	1	1	1	0.80 ± 0.22	1.14 ± 0.12
	48 h	1	0	1	1	1	0.80 ± 0.23	1.14 ± 0.10
CPA 80 mg/kg	24 h	17	24	22	21	24	21.6 ± 2.50*	1.15 ± 0.16
	48 h	20	18	21	24	22	21.0 ± 1.80*	1.17 ± 0.15

Table 7
Evaluation of in vivo genotoxicity of saline extract and MvRL-rich fraction from *Microgramma vacciniifolia* rhizome by comet assay in mice peripheral blood and bone marrow: mean frequency of damaged cells, average distribution per class and average damage score.

Sample	Collection time	Treatments	Frequency[a]	Damage class				Score
				0	1	2	3	
Peripheral blood	4 h	*Negative control*	1.18 ± 0.10	98.82 ± 0.50	1.04 ± 0.22	0.14 ± 0.40	0.00 ± 0.00	1.50 ± 0.56
		Saline extract						
		2000 mg/kg	2.31 ± 0.17	97.69 ± 0.47	2.01 ± 0.17	0.30 ± 0.01	0.00 ± 0.00	2.87 ± 0.47
		5000 mg/kg	4.20 ± 0.14	95.80 ± 0.34	4.01 ± 0.21	0.19 ± 0.04	0.00 ± 0.00	4.58 ± 0.03
		MvRL-rich fraction						
		2000 mg/kg	2.31 ± 0.27	98.33 ± 0.21	2.01 ± 0.27	0.30 ± 0.10	0.00 ± 0.00	2.07 ± 0.47
		5000 mg/kg	2.23 ± 0.14	97.77 ± 0.11	2.23 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.01 ± 0.03
		CPA	50.92 ± 3.09*	49.08 ± 3.27*	35.17 ± 4.18*	14.42 ± 1.12*	1.33 ± 0.58*	61.49 ± 2.41*
	24 h	*Negative control*	2.01 ± 0.26	97.99 ± 0.19	1.28 ± 0.31	0.73 ± 0.12	0.00 ± 0.00	1.99 ± 0.11
		Saline extract						
		2000 mg/kg	1.65 ± 0.22	98.35 ± 0.23	1.65 ± 0.16	0.00 ± 0.00	0.00 ± 0.00	1.62 ± 0.16
		5000 mg/kg	1.26 ± 0.15	98.74 ± 0.37	1.06 ± 0.13	0.20 ± 0.51	0.00 ± 0.00	1.13 ± 0.86
		MvRL-rich fraction						
		2000 mg/kg	1.99 ± 0.16	98.01 ± 0.30	1.99 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	0.97 ± 0.16
		5000 mg/kg	1.55 ± 0.75	98.45 ± 0.42	1.55 ± 0.35	0.00 ± 0.00	0.00 ± 0.00	1.03 ± 0.86
		CPA	80.34 ± 2.49*	19.65 ± 2.17*	70.48 ± 2.49*	7.81 ± 1.07*	2.05 ± 0.61*	88.37 ± 4.04*
Bone marrow	24 h	*Negative control*	1.43 ± 0.03	98.57 ± 0.19	1.43 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	1.09 ± 0.32
		Saline extract						
		1000 mg/kg	1.55 ± 0.98	98.45 ± 0.52	1.55 ± 0.18	0.00 ± 0.00	0.00 ± 0.00	1.83 ± 0.28
		2000 mg/kg	1.32 ± 0.78	98.68 ± 0.40	1.32 ± 0.22	0.00 ± 0.00	0.00 ± 0.00	1.08 ± 0.44
		MvRL-rich fraction						
		2000 mg/kg	1.39 ± 0.98	98.61 ± 0.28	1.39 ± 0.98	0.00 ± 0.00	0.00 ± 0.00	1.23 ± 0.98
		5000 mg/kg	1.59 ± 0.78	98.41 ± 0.33	1.59 ± 1.78	0.00 ± 0.00	0.00 ± 0.00	1.08 ± 1.78
		CPA	71.88 ± 3.55*	28.11 ± 4.38*	47.72 ± 4.36*	21.09 ± 2.20*	3.07 ± 1.02*	80.25 ± 5.02*

The extract and fraction were administered by gavage. The positive control cyclophosphamide (CPA) was administered intraperitoneally. (*) Significantly different from the negative control ($p < 0.001$). [a]Total number of damaged cells (class 1 + 2 + 3).

production (Kim et al., 2011).

4. Conclusion

A saline extract and lectin-rich fraction from *M. vacciniifolia* rhizome were not lethal to mice when administered at a single dose of 2000 or 5000 mg/kg. However, some signs of toxicity, such as weight loss and damages in the liver, lungs and kidneys, were observed, mainly in mice that received the fraction. However, no damage to mouse genetic material was detected. The results indicated the need to evaluate the toxicity of *M. vacciniifolia* rhizome preparations in other models and in chronic effects assays.

CRediT authorship contribution statement

Gabriela Cavalcante da Silva: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Alisson Macário de Oliveira:** Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization. **Janaina Carla Barbosa Machado:** Data curation, Formal analysis, Investigation, Methodology. **Magda Rhayanny Assunção Ferreira:** Data curation, Formal analysis, Investigation, Methodology. **Paloma Lys de Medeiros:** Data curation, Formal analysis, Investigation, Methodology. **Luiz Alberto Lira Soares:** Data curation, Formal analysis, Funding acquisition, Resources. **Ivone Antônia de Souza:** Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Visualization. **Patrícia Maria Guedes Paiva:** Conceptualization, Funding acquisition, Methodology, Resources, Software, Visualization, Writing - review & editing. **Thiago Henrique Napoleão:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

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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3.2 – Artigo 2: Antinociceptive and anti-inflammatory effects of saline extract and lectin-rich fraction from *Microgramma vacciniifolia* rhizome in mice

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Abstract

Pain and inflammation are physiological signs with a protective role, but if the underlying cause is not assessed and treated, they can have strong functional impacts and reduce life quality. Previous studies have characterized a saline extract from *Microgramma vacciniifolia* rhizome and its lectin(MvRL)-rich fraction as of low acute toxicity. In the present work, we evaluated these preparations for acute toxicity (1,000 mg/kg) and antinociceptive and anti-inflammatory activities (100–400 mg/kg for the extract and 25–50 mg/kg for the fraction). There were no death nor behavioral, hematological, biochemical and histological disturbances in the animals in the acute toxicity assay. Both the extract and fraction increased the latency time for nociception in the hot plate assay, decreased the writhings induced by acetic acid, and also promoted analgesic effects in phases 1 and 2 of the formalin test. In this last, it was also determined that the antinociceptive mechanism involves interaction with opioid receptors and K^+ ATPase channels. The extract and fraction decreased carrageenan-induced paw edema in 46.15% and 77.22%, respectively, at the highest doses tested; in addition, the fraction showed to act on the bradykinin pathway. The ability to decrease leukocyte migration after treatment was also verified in the peritonitis and air-pouch models. There was still a decrease in the levels of TNF-α and an increase in IL-10 in the exudates collected from air pouches. In the evaluation of chronic inflammation using the granuloma model, both extract and fraction studied were effective in inhibiting the formation of granulomatous tissue. In conclusion, the substances studied in this paper can be used in the development of new therapeutic options for pain and inflammatory processes.

Keywords: antinociception; anti-inflammatory; pteridophyte; acute toxicity.

Introduction

The inflammation is a defensive response of our body to a tissue injury caused by several factors, such as infection, toxins, radiation, burns, caustic substances, immune reactions, etc. Inflammation can also be cause and/or consequence of a wide range of human health disorders such as cancer, autoimmune diseases, gastrointestinal and respiratory disorders (Zygmunt et al., 2014). It comprises cellular and vascular events, including fluid leakage and release of chemotaxis mediators (Silva and Macedo, 2011; Buckley et al., 2015).

According to the International Association for the Study of Pain, the pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage". In physiological circumstances, the pain has a protective role by warning for damages to body's integrity and unchaining protective behavioral responses (May et al., 2017). However, the management of the pain associated with several pathologies (e.g. cancer, bone problems, arthritis, and spinal problems) is crucial to minimize the loss of life quality, becoming one of the main factors in the therapy (Goldberg and McGee, 2011; Radulović et al., 2015).

Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are used in the management of inflammation but their chronic use has been associated with cardiovascular, gastrointestinal, and renal toxicities (NSAIDs) and hypertension, hyperglycemia, osteoporosis, and growth arrest (steroidal drugs) (Patil et al., 2019). Among the therapeutic arsenal against nociceptive pain, there are the NSAIDs and opioids while antidepressants, anesthetics, higher dose opioids and anticonvulsants are used for neuropathic pain management (Dalacorte et al., 2011). However, a disadvantage of some widely used analgesics is the incidence of side effects, including gastrointestinal and renal damage, as well as bronchospasm and cardiac abnormality (Sofidiya et al., 2014). In addition, opioid drugs have been associated with tolerance, physical dependence, constipation, respiratory depression, and nausea (Benyamin et al., 2008). These are obstacles for better adherence and treatment effectiveness (González-Trujano et al., 2017), which stimulates the search for new anti-inflammatory and analgesic agents.

Medicinal plant preparations represent a source of new chemicals with these effects (Tasneem et al., 2019; Wu et al., 2019).

Microgramma vacciniifolia is an epiphyte plant belonging to the Polypodiaceae family. Its rhizomes are used in folk medicine to treat diarrhea and respiratory infections (Barros et al., 1997). The rhizome contains a lectin (carbohydrate-binding protein) called MvRL, which is isolated through protein extraction in saline solution, ammonium sulphate fractionation (60% saturation), and chromatography on chitin column (Albuquerque et al., 2012). MvRL showed cytotoxic action against lung mucoepidermoid carcinoma (NCI-H292) cells at concentrations that did not affect the viability of human peripheral blood mononuclear cells (Albuquerque et al., 2014).

Santana et al. (2012) showed that the fraction obtained with ammonium sulphate from the saline extract of *M. vacciniifolia* rhizome is a preparation rich in MvRL, with high protein homogeneity. Previous study evaluating the acute toxicity to mice of saline extract and this MvRL-rich fraction at 2,000 and 5,000 mg/kg showed that no death occurred but some signals of toxicity were observed such as increase of serum transaminases and urea levels and presence of, leukocyte infiltrates in the liver and lungs (Silva et al., to be submitted). Thus, in the present work, we first evaluated the acute toxicity of saline extract and MvRL-rich fraction at 1,000 mg/kg and then choose safe doses to evaluate their antinociceptive and anti-inflammatory activities using different models.

Material and methods

Plant material

Rhizomes of *M. vacciniifolia* were collected in September 2016 at the campus of the *Universidade Federal de Pernambuco* (UFPE) in Recife (08° 03' 07" S, 34° 56' 59" W), Pernambuco, Brazil. Taxonomic identification was performed and a voucher specimen (no. 63,291) is deposited at the herbarium Dárdano de Andrade Lima Herbarium of the *Instituto Agronômico de Pernambuco* (Recife). The access was recorded (A347889) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen).

Preparation of saline extract and MvRL-rich fraction

The saline extract and MvRL-rich fraction were obtained as previously described (Albuquerque et al., 2012; Santana et al., 2012). The rhizomes were washed with distilled water and put to dry for 7 days at 28 ºC and additional 3 days in an oven at 35 ºC. Next, the dried rhizomes were powdered using a knife mill. The powder (100 g) was submitted to extraction in 0.15 M NaCl (1 L) for 12 h at 28 ºC under magnetic stirring. After this period, the suspension was filtered and centrifuged (9,000 *g*, 15 min, 28 ºC) and the supernatant corresponded to the saline extract. The MvRL-rich fraction was obtained after treatment of the extract with ammonium sulfate at 60% saturation (Green and Hughes, 1955) for 4 h, followed by centrifugation (9,000 *g*, 15 min, 28 ºC), collection of the precipitate by resuspending it in 0.15 M NaCl, and dialysis against distilled water for 6 h (two liquid changes). The saline extract was also submitted to dialysis for removal of salt. The dialyzed extract and fraction were dried by lyophilization.

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). Hemagglutinating activity (HA) was performed according to Albuquerque et al. (2012) using a suspension (2.5%, v/v) of human O-type erythrocytes in 0.15 M NaCl. The HA was defined as the reciprocal value of the highest dilution of sample that promoted full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between the HA and protein concentration (mg/mL).

Animals

Male and female Swiss mice (*Mus musculus*), 50-day-old and weighing 30±2 g, were obtained from the vivarium of the *Laboratório de Imunopatologia Keizo Asami* (LIKA) of the UFPE. The animals were used in the tests after one week of adaptation in the experimental laboratory at the *Departamento de Bioquímica* of the UFPE. The mice were housed at a temperature of 22 °C, 12:12 light/dark cycle and *ad libitum* access to food (Purina, Nestlé Brasil Ltda., Brazil) and water. The Ethics Committee on Animal Use of UFPE approved all the experiments (process number 23076.042699/2016-72).

Acute toxicity evaluation

Acute toxicity was assessed following the protocol 425 of the Organization for Cooperation and Development (2001). The female mice (n = 3 per group) were treated with the saline extract or the MvRL-rich fraction (dissolved in 0.9% NaCl) by gavage in a single dose of 1,000 mg/kg body weight (b.w.). In the control, animals received saline solution. After administration, the animals were individually observed within the first 4 h and then monitored daily for weight and intake of water and food. At the end of the observation period (day 14), the animals were anesthetized (80 mg/kg ketamine and 20 mg/kg xylazine, i.p.) and blood samples were collected by cardiac puncture for hematological and biochemical analysis.

The blood samples from each animal were divided into two tubes, one with EDTA for hematological analysis [erythrocytes, leukocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV) mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)] using an ABX-PENTRA-80 automated hematology analyzer (Horiba, Japan)] and other containing a separating gel, which was centrifuged for 10 min at 3500 rpm to obtain serum for the biochemical analyses of total protein, albumin, aminotransferases, gamma-glutamyl transferase, urea, creatinine, alkaline phosphatase, total cholesterol and triglycerides using specific kits (Labtest Diagnóstica, Lagoa Santa, Brazil) and a COBAS Mira Plus analyzer (Roche Diagnostics Systems, Basel, Switzerland).

Liver, kidney, spleen and lungs were also collected and immediately fixed in buffered formalin (10%, v/v), dehydrated through a graded ethanol series (70–100%), diaphanized in xylol, and embedded in paraffin. Histological slices (5 μm) were stained with hematoxylin-eosin and mounted using cover slips with Entellan resin (Merck, Germany) (Kiernan, 2008). The samples were observed using a Motic BA200 microscope coupled to a Moticam 1000 1.3 MP digital camera (Motic Incorporation Ltd., Causeway Bay, Hong Kong). The organs were sectioned at 5 microns and stained with hematoxylin and eosin for general examination of tissue architecture. The sections were viewed under optical microscope and photomicrographs obtained using a camera attached to the computer.

Antinociceptive activity

Acetic acid-induced writhing test

Groups of eight male animals each were separated and received saline extract (100, 200 or 400 mg/kg *per os*), MvRL-rich fraction (25, 50 or 100 mg/kg *per os*), morphine (10 mg/kg i.p), indomethacin (20 mg/kg i.p.) or vehicle (0.9%, w/v, NaCl *per os*; control). After 1 h, nociception was induced by intraperitoneal injection of 0.85% (v/v) acetic acid (0.1 mL/10 g b.w.) in order to induce contractions of the abdominal musculature and/or stretching of the hind limbs. The mice were placed in clear polyethylene cages, where they were observed and the writhing number was recorded in the period of 5-15 min after acetic acid injection.

Hot plate

Male animals were pre-selected 24 h before the experiments and those with latency time greater than 20 s were not used in the study. The mice (n = 6 per group) were treated with vehicle (0.9% NaCl *per os*), saline extract (100, 200 or 400 mg/kg *per os*), MvRL-rich fraction (25, 50 or 100 mg/kg *per os*) or morphine (10 mg/kg i.p.). After 30, 60, 90 and 120 min of treatment, the animals were placed in a hot plate apparatus (Insight Equipamentos, Ribeirão Preto, Brazil) maintained at 55±0.5 °C and the latency time for a discomfort reaction (pawing or jumping) was registered. A cut-off time of 20 s was chosen to prevent tissue injury.

Formalin test

Groups of eight male animals each received saline extract (100, 200 or 400 mg/kg *per os*), MvRL-rich fraction (25, 50 or 100 mg/kg *per os*), morphine (10 mg/kg i.p.), indomethacin (20 mg/kg i.p.) or vehicle (0.9% NaCl *per os*). After 60 min, 20 μL of 2.5% (v/v) formalin in saline was injected into the subplantar region of the right hind paw of each animal. The time spent by the mouse licking its paw was recorded during the first 5 min after formalin injection (first phase: neurogenic pain), as well as 15–30 min after the injection (second phase: inflammatory pain) (Hunskaar and Hole, 1987).

To evaluate mechanisms involved in the antinociceptive activity, groups were treated with the non-selective opioid receptor antagonist naloxone (20 mg/kg, i.p.) or glibenclamide 30 min before administration of extract (400 mg/kg *per os*), MvRL-rich

fraction (100 mg/kg *per os*) or morphine (10 mg/kg, i.p.). Other groups were pre-treated with glibenclamide (10 mg/kg, i.p.) 30 min before the treatment in order to verify the possible contribution of ATP-sensitive K^+ channels.

Anti-inflammatory activity

Carrageenan-induced paw edema

A baseline measurement of the hind paw volume of the mice was measured by plethysmometry. Subsequently, the male animals (n = 6 per group) were pre-treated orally with 0.9% (w/v) NaCl (control), dexamethasone (10 mg/kg i.p., positive control), saline extract (100, 200 or 400 mg/kg, b.w *per os*) or MvRL-rich fraction (25, 50 or 100 mg/kg b.w *per os*). After 1 h, the animals received 1% (w/v) carrageenan (20 μL/paw) in the right hind paw and vehicle in the left hind paw. The volume of the hind paws of each animal were recorded after 1, 2, 3 and 4 h of the carrageenan injection (Lapa, 2007). Results were expressed using the formula:

$$\%\ \text{reduction} = \frac{(\text{Final paw volume} - \text{Initial paw volume})}{\text{Initial paw volume}} X\ 100$$

Histamin or bradykinin-induced paw edema

Male animals (n = 6 per group) were treated with saline extract (400 mg/kg *per os*), MvRL-rich fraction (100 mg/kg *per os*) or the reference drug diphenhydramine (DPH, 20 mg/kg/i.p). The initial paw volume was measured for all animals using the plethysmometer. After 30 min of administration, inflammation was induced by the administration of 0.1 mL of the phlogistic agent (histamine or bradykinin at 1%, w/v) to the right hind foot plantar region. Paw volume was measured at 30-min intervals for up to 3 h. The volume of the hind paws of each animal were recorded and the change in paw volume was recorded.

Carrageenan-induced peritonitis

Male animals (n = 6 per group) were treated *per os* with 0.9% NaCl (control), dexamethasone (10 mg/kg/i.p), saline extract (100, 200 or 400 mg/kg, *per os*) or MvRL-rich fraction (25, 50 or 100 mg/kg). After 1 h of the treatment, the animals received an intraperitoneal injection of 1% (w/v) carrageenan. After 4 h, the animals were euthanized, and 3 mL of heparinized PBS were injected into the peritoneal cavity. Leukocyte count in peritoneal lavage was performed counted using the automated hematology analyzer.

Carrageenan-induced air pouch

Male mice were separated into the following groups (n = 6 per group): negative control (0.9% NaCl *per os*), positive control (indomethacin 10 mg/kg i.p), saline extract (100, 200 and 400 mg/kg *per os*) or MvRL-rich fraction (25, 50 and 100 mg/kg *per os*). Each group received a subcutaneous injection of sterile air (3 mL) twice, with an interval of 3 days between each one. On the seventh day, 1% (w/v) carrageenan was injected in the air pouch and, after 1 h, the animals received the respective treatment. The exudates in the air pouches were collected 6 h after treatment, and the numbers of leukocytes were counted using an automated hematology analyzer. The exudates were also used for determination of the levels of TNF-α e IL-10 cytokines levels using commercial Elisa kit (eBioscience) following the manufacturer's recommendations. Colorimetric reaction readings were taken at 450 nm in a microplate reader (Infinite M200, Teacn, Männedorf, Switzerland).

Granuloma assay

For this test, the mice were anesthetized (60 mg/kg ketamine and 6 mg/kg xylazine i.p.) and four cotton pellets weighing 10 mg each were implanted in the dorsal region. The groups (n = 6 per group) were divided into: negative control (0.9% NaCl *per os*), positive control (indomethacin 10 mg/kg i.p.), saline extract (100, 200 and 400 mg/kg *per os*) and MvRL-rich fraction (25, 50 and 100 mg/kg *per os*), treated daily for 10 days. On the tenth day, the animals were sacrificed and the cotton balls removed and oven dried at 37 ºC for 24 h. Subsequently, the cotton granules were homogenized and protein content was measured by the biuret method and the absorbance at 550 nm was recorded (Swingle and Shideman, 1972).

Statistical analyses

The data obtained were analyzed using the GraphPad Prism® version 6.0 (GraphPad Software, La Jolla, CA, USA) and expressed as mean values with standard deviation (±SD) or standard error of the mean (SEM). Statistically significant differences were calculated using one-way analysis of variance (ANOVA) followed by Bonferroni test. Values were considered significantly different at $p < 0.05$ and $p < 0.01$.

Results and discussion

Silva et al. (submitted), using the same samples employed in the present work, reported the presence of cinnamic acid derivatives (0.271±0.0001 g%), including a chlorogenic acid content of 0.135±0.0001 g%, in the saline extract of *M. vacciniifolia* rhizome; the MvRL-rich fraction did not contain secondary metabolites and is a preparation richer in the lectin than the extract (purification factor: 12.1). At doses of 2000 and 5000 mg/kg/vo, although not lethal, some signs of toxicity were observed, such as weight loss and damage to some organs such as the liver, lungs, kidneys; which arouses interest in verifying the presence of these signs of toxicity at lower doses close to the possible therapeutic doses.

No deaths or any other signs (motor and/or sensory alterations) of toxicity were observed in animals treated with the saline extract and MvRL-rich fraction at 1,000 mg/kg. No significant differences in water or food intake were observed between treated groups and control as well as no changes in the weight of the animals (Table 1). In addition, none of the hematological (Table 2) and biochemical (Table 3) parameters showed significant differences when control and treated groups were compared. Histological structures of the liver, spleen, kidneys, lungs were also found to be preserved (Figure 1). And both preparations from *M. vacciniifolia* rhizome were classified as safe (OECD, 2001) with LD_{50} > 1,000 mg/kg. In comparison with the previous study with these samples at 2,000 and 5,000 mg/kg (Silva et al., submitted), the toxic signs previously observed were not detected when testing the lower dose of 1,000 mg/kg. Thus, the doses chosen for evaluation of antinociceptive and anti-inflammatory activities were below 1,000 mg/kg.

In the acetic acid induced abdominal writhing test, it was observed a reduction in the number of writhings in mice treated with the saline extract and the MvRL-rich

fraction, at all tested doses, in comparison with the control (Figure 2), indicating a pain suppressing effect. The writhing test can evaluate both neurogenic and inflammatory pain since acetic acid activates chemosensitive nociceptors as well as irritates visceral surfaces triggering vasodilation and release of prostaglandins, serotonin, histamine, nitric oxide and bradykinin (Dash et al., 2013; Trevisan et al., 2014). Generally, drugs that reduce writhing-related pain also inhibit prostaglandin synthesis in peripheral tissues, such as the non-steroidal anti-inflammatory drugs (NSAIDs) (Sarwar et al., 2014).

In the hot plate test, which evaluates sensitivity to physical stimulation, the treatment with the extract at the highest dose (400 mg/kg) increased the latency time since 30 min post-administration; from 90 min, the three doses promoted increase of the latency period (Figure 3A). Regarding the MvRL-rich fraction, the dose of 100 mg/kg was able to decrease mice sensitivity since 30 min while the other doses showed significant results only after 90 and 120 min (Figure 3B). At the highest doses, both extract and fraction showed results close to that found for the standard drug, morphine compared to control group. The nociception in the hot plate test is due to direct thermal stimulation of the nociceptors, triggering supraspinatus transmission; the act of the animal licking its paw and jumping indicates spinal and supraspinatus responses (Ishola et al., 2014). This is an experimental model for centrally acting antinociceptive drugs, suggesting that the saline extract and the MvRL-rich fraction under study have a central action.

The neurogenic pain in the phase 1 (0-5 min) of the formalin test is triggered by the high concentration of substance P in the central nervous system while the inflammatory pain in phase 2 (15-30 min) is due to the release of mediators and medullary sensitization (Begnami et al., 2018). Extract and MvRL-rich fraction, at all doses, promoted analgesia in the first phase while antinociceptive effect in the second phase was detected only for treatments with the extract at 400 mg/kg and the fraction at 50 and 100 mg/kg (Figure 4). The effects of the extract (400 mg/kg) and fraction (100 mg/kg) in first phase were reversed by naloxone (Figure 4), an opioid antagonist, indicating an action via opioid receptors, which are present at peripheral nerve endings (Adeyemi et al., 2018; Begnami et al., 2018). However, the antinociceptive effects on the second phase were not reversed by naloxone. The analgesia provided by the extract and MvRL-rich fraction in both phases was reversed by blockage of K^+ ATPase channels by the drug glibenclamide (Figure 4). The opening of the K^+ ATPase channels plays an important role in excitable cells, causing K^+ ions outflow by hyperpolarizing the cells, preventing the consequent

depolarization and impulse transmission. Cinnamic derivatives, metabolites found in the extract under study, have already been shown to act as modulators of voltage dependent K^+ ATPase channels (Gunia-Krzyżak et al., 2015).

Drugs that act centrally as opioids, mainly represented by morphine, can inhibit both neurogenic (central) and inflammatory (peripheral) pain, unlike NSAIDs, represented by indomethacin, which can only suppress inflammatory pain (Florentino et al, 2016). According to the data obtained, the saline extract and MvRL-rich fraction can inhibit the central and peripheral pathways of pain presenting important analgesic action by blocking chemically and thermally triggered nociceptive processes.

The inflammatory process is a research highlight because of its relationship with most human and animal diseases, involving a considerable cascade of cell migration, plasma leakage, activation of enzymes and mediators, and tissue damage and repair (Hossain et al, 2012). The carrageenan is a red algae polysaccharide with intense inflammatory and edematogenic activity, which produces, within 4 h of administration, an edema that can last for up to 96 h (Silva et al, 2015). Anti-inflammatory effect of saline extract and MvRL-rich fraction on carrageenan-induced edema in the hind paws was evaluated and the results are presented in Table 4. A gradual increase in paw edema volume of the carrageenan-treated control group was noted. In the groups treated with saline extract and MvRL-rich fraction at all doses, there was a significant reduction in the paw edema volume in comparison with control. After 4 h, the saline extract at 400 mg/kg promoted 46.15% inhibition and MvRL-rich fraction at 100 mg/kg reduced the edema by 71.22%. The reference drug dexamethasone caused a reduction of 39.31%.

Histamine is a biogenic amine with short action that promotes increase in blood flow and vasodilation linked to the activation of both H1 type receptors, present in tissues, as well as H4 type located in hematopoietic cells (Bently, 2015). When histamine was used as phlogistic agent instead of carrageenan, the paw edema increased similarly in the control and the treatments with the extract and fraction (Table 5). These results indicate that the anti-inflammatory effects observed in the carrageenan-induced paw edema model are not correlated with participation in the histamine-induced inflammation pathway. On the other hand, the MvRL-rich fraction seems to exert anti-inflammatory action via inhibition of the bradykinin pathway since from 60 min the bradykinin-induced paw edema showed a lower increase than in control group (Table 4). Bradykinin is a peptide produced in plasma from kallidin and bradykinin type B2 receptors are related to the acute inflammation pattern; once sensitized, they activate phospholipase A2 and thus increase

the synthesis of prostaglandins and prostacyclins (Blaes and Girolami, 2013). The MvRL-rich fraction may have inhibited bradykinin action on its receptors and consequently prostaglandin production.

The peritonitis is a model that assesses acute inflammation allowing the quantification of leukocyte migration (mainly neutrophils and macrophages) for intraperitoneal cavity due to chemotaxis mediated mainly of leukotrienes and interleukins (Syam et al, 2014). In the peritonitis model, there was a significant decrease in leukocyte and neutrophil migration in the groups treated with saline extract and fraction, in comparison with the untreated control (Table 6). The highest doses of the saline extract (40 mg/kg) and fraction (100 mg/kg) promoted 71.04% and 75.79% inhibition of leukocyte migration, respectively, while indomethacin (10 mg/kg) caused 71.41% inhibition. For neutrophils, the extract (400 mg/kg), fraction (100 mg/kg) and indomethacin (10 mg/kg) inhibited the migration in 70.60%, 72.69% and 67.73%, respectively. These results corroborate with those of the second phase of the formalin test since they confirm the effect of the substances tested in the inflammatory pathway.

Similar results were found in the air pouch model, with a decreased of leukocyte migration promoted by both extract and fraction (Table 7). Inhibition percentages of 44.14% and 68.05% were detected for the highest doses of the extract (400 mg/ kg) and MvRL-rich fraction (100 mg/kg), respectively, while indomethacin (10 mg/kg) promoted 59.52% inhibition. Cell migration involves the participation of adhesion molecules such as E, P and L-selectin that undergo activation by cytokines such as TNF-α and IL-1β (Patel and Murugananthan, 2012). Figure 5 illustrates the measurement of cytokine concentrations in the exudate from the air pouch. For the treatments with the extract, only the highest dose (400 mg/kg) led to a decrease of TNF-α concentration and increase of circulating IL-10 levels. For MvRL-rich fraction, TNF-α decrease and IL-10 increase was observed in treatments at all doses tested (25, 50 and 100 mg/kg). TNF-α activates the production of nitric oxide, an important vasodilator, and is the earliest mediator in the inflammatory response by activating other cytokines, as well as in the expression of adhesion molecules, glucocorticoids, and cellular apoptosis; thus, decreased synthesis of this cytokine reduces leukocyte migration (Oliveira et al, 2011; Mota et al, 2018). The IL-10 is an anti-inflammatory cytokine that downregulates the expression of Th1 cytokines (Oliveira et al, 2011). Therefore, the decrease in leukocyte migration observed in the present study, mainly promoted by MvRL-rich fraction, is supported by this finding of decreased TNF-α and increased IL-10 observed.

The granuloma assay allows to evaluate chronic inflammation through the analysis of transudative and exudative components. This method is characterized by the accumulation of proliferative cells and proteins participating in the process and the dry weight of the cotton pellets correlates with the extent of granulomatous tissue, composed by the infiltration of granulocytes, collagen fibers and mucopolysaccharides (Azam et al, 2015). Treatments with both saline extract and MvRL-rich fraction resulted in significant decrease in the dry mass of cotton pellets, indicating inhibition of the formation of granulomatous tissue (Table 8). The total protein content (which can be represented by collagen and inflammation-acting protein molecules such as cytokines, chemotactic proteins and others) also decreased in the treatments with the extract and fraction (Table 8). Thus, the preparations of *M. vacciniifolia* rhizome would be effective in chronic inflammatory condition, as supported by their efficacy in inhibiting the number of fibroblasts and proteins and reduce the synthesis of collagen during granuloma tissue formation.

The antinociceptive and anti-inflammatory properties of the saline extract from *M. vacciniifolia* may be due to the presence of cinnamic derivatives and chlorogenic acid previously demonstrated (Silva et al., to be submitted), which were also reported as anti-inflammatory and analgesic agents (Rao and Gan, 2014; Meinhart et al., 2019). Studies with chlorogenic acid-rich plant species have shown the ability to decrease carrageenan-induced paw edema in rats (Shi et al. 2013). Antinociceptive and anti-inflammatory activities have been also described for lectins. For example, the *Lonchocarpus campestris* lectin showed antinociceptive effect in the formalin model as well as reduced carrageenan-induced hypernociception (Pires et al, 2019); the lectin isolated from *Tetracarpidium conophorum* seeds showed antinociceptive effect by decreasing mouse paw licking time as well as anti-inflammatory effect by decreasing carrageenan-induced paw edema volume and decreasing leukocyte migration in the peritonitis model (Oladokun et al., 2019). Lectin derived from *Caulerpa cupressoides* showed considerable antinociceptive and anti-inflammatory effects via TNF-α signaling and COX-2 inhibition (Rivanor et al, 2018). Anti-inflammatory action was also reported for lectin isolated from *Platypodium elegans* seeds, which reduced paw edema (Araripe et al., 2017). The lectin from *Parkia platycephala* inhibited 74% of the writhes induced by acetic acid in mice, at a concentration of 1 mg/ mL, as well as decreased leukocyte migration (Bari et al., 2016). Lectin derived from *Bryothamnion triquetrum* decreased carrageenan and dextran-

induced paw edema, as well as decreased leukocyte migration and the levels of inflammatory cytokines in the peritonitis model (Fontenelle et al., 2018).

Conclusion

Saline extract and MvRL-rich fraction from *M. vacciniifolia*, at doses considered safe, showed antinociceptive and anti-inflammatory activities in mice. Both preparations presented were able to inhibit peripheral and central pain and were effective against thermally and chemically induced inflammation, being able to reduce cell migration and release of proinflammatory cytokine. The fraction was also able to interfere with the bradykinin signaling pathway. The results indicate that both samples have potential for further studies aiming to develop of new analgesic and anti-inflammatory formulations.

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Figure captions

Figure 1. Photomicrographs of the liver, spleen, kidney and lungs of mice from the control group and treated with the saline extract or MvRL-rich fraction (1,000 mg/kg b.w.) from *M. vacciniifolia* rhizome. **Livers**: the centrilobular vein (cv) is seen in all images. The hepatocyte bundles (arrow heads) are preserved and ordered. **Spleen:** The lymph nodes (Nd) are well-defined in the control and treated groups. **Kidneys:** Renal glomeruli (Gr) and contorted tubules without alterations are visible in control and treated groups. **Lungs:** bronchiole (Br) and alveolar sac (As) can be visualized, with preserved architecture. Hematoxylin-eosin staining was used. Magnification: 400 ×.

Figure 2. Antinociceptive effect of saline extract (A) and MvRL-rich fraction (B) from *M. vacciniifolia* rhizome (oral administration) and the reference drugs indomethacin (20 mg/kg i.p.) and morphine (10 mg/kg i.p.) in the acetic acid induced writhing assay. Control group received only vehicle (0.9% NaCl). The bars represent the mean numbers of writhing ± SD. (***) indicates significant difference ($p < 0.01$) in the number of writhings versus the control.

Figure 3. Antinociceptive effect of saline extract (A) and MvRL-rich fraction (B) from *M. vacciniifolia* rhizome (oral administration) and the reference drug morphine (10 mg/kg i.p.) in the hot plate model. Control group received only vehicle (0.9% NaCl). The animals were evaluated 30, 60, 90 and 120 min after treatments. The bars represent the mean writhing ± SD. (***) indicates significant difference ($p < 0.01$) versus the control.

Figure 4. Antinociceptive effect of saline extract (A) and MvRL-rich fraction (B) from *M. vacciniifolia* rhizome (oral administration) and the reference drugs, indomethacin (20 mg/kg,
i.p.) and morphine (10 mg/kg, i.p.), on both phases of the formalin assay. Control group received only vehicle (0.9% NaCl). The involvement of opioid receptors in the antinociceptive effect was evaluated by administering naloxone (Nal) 20 mg/kg i.p. to the mice 30 min before the administration of the extract or morphine. The involvement of K^+ ATPase channels was also evaluated by administering glibenclamide (Glib) 10 mg/kg i.p. to the mice 30 min before the administration of the extract or morphine. The bars represent the mean writhing ± SD. (*) indicates significant difference ($p < 0.01$) versus the control.

Figure 5. Levels of cytokine TNF-α and IL-10 in exudates collected from animals submitted to the carrageenan-induced air pouch test and treated with saline extract or MvRL-rich fraction from *M. vacciniifolia* rhizome (oral administration) or the reference drug indomethacin (20 mg/kg, i.p.). Control group received only vehicle (0.9% NaCl). The bars represent the mean writhing ± SD. (*) indicates significant difference ($p < 0.01$) versus the control.

Figure 1

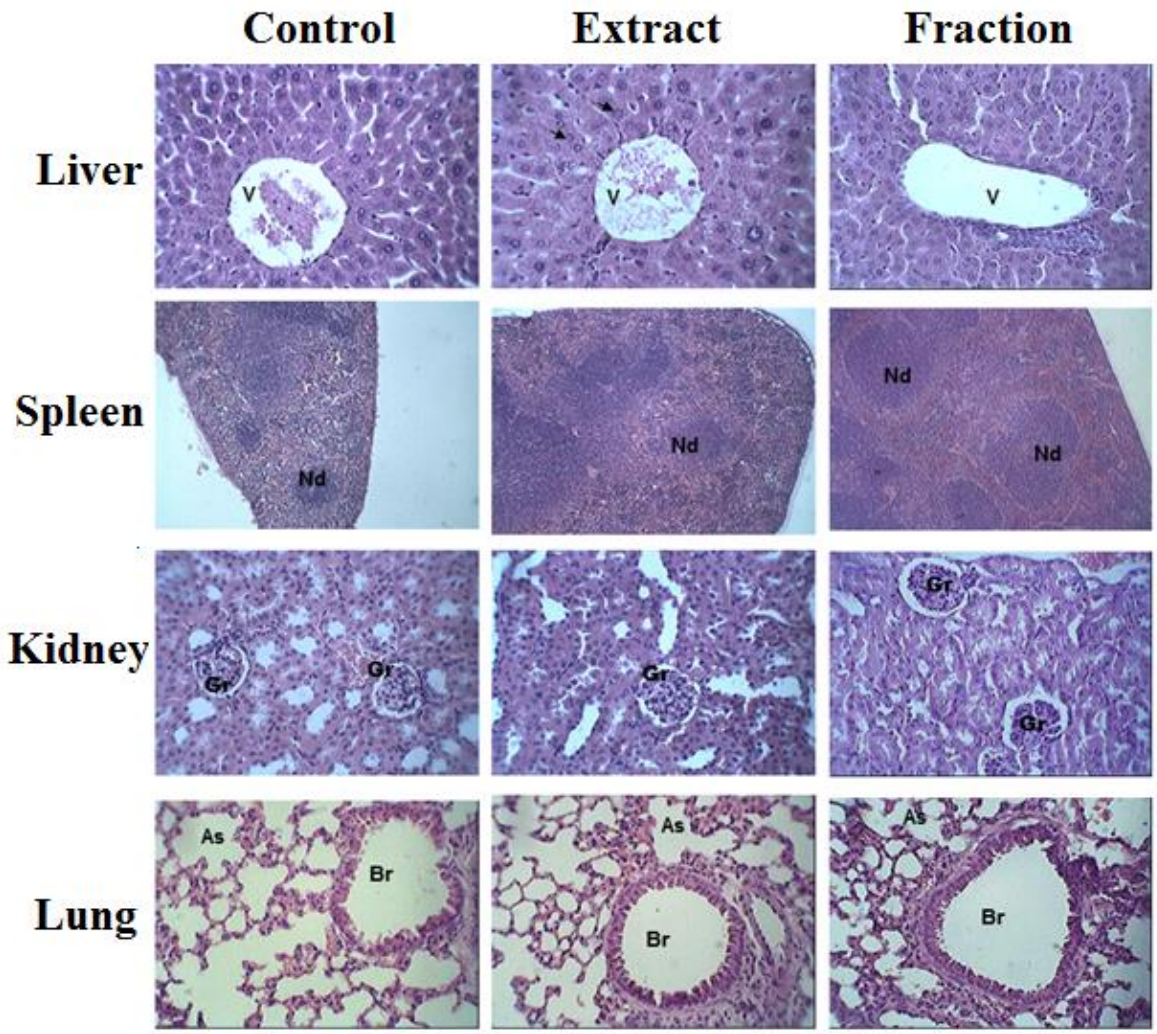
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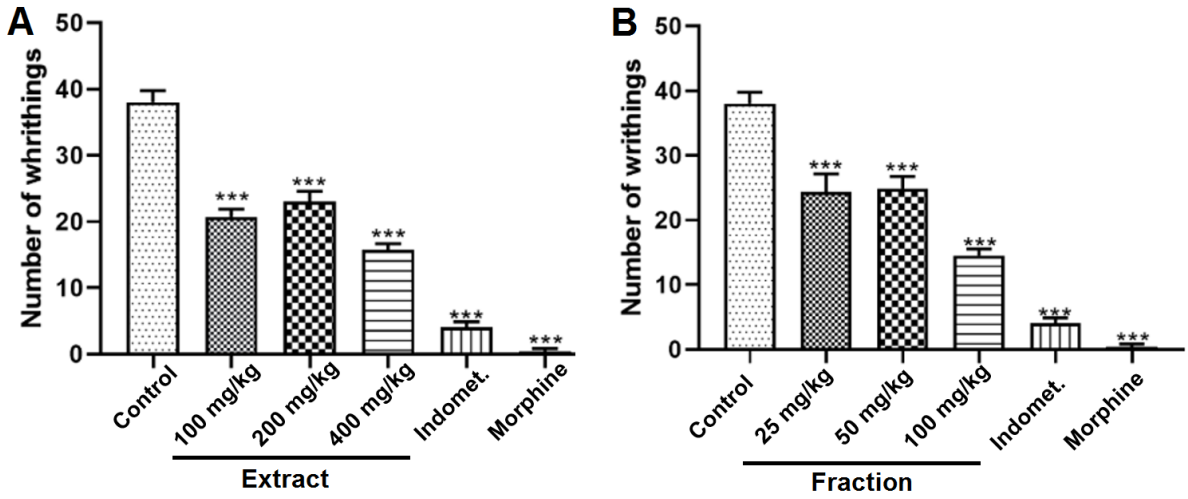
Figure 2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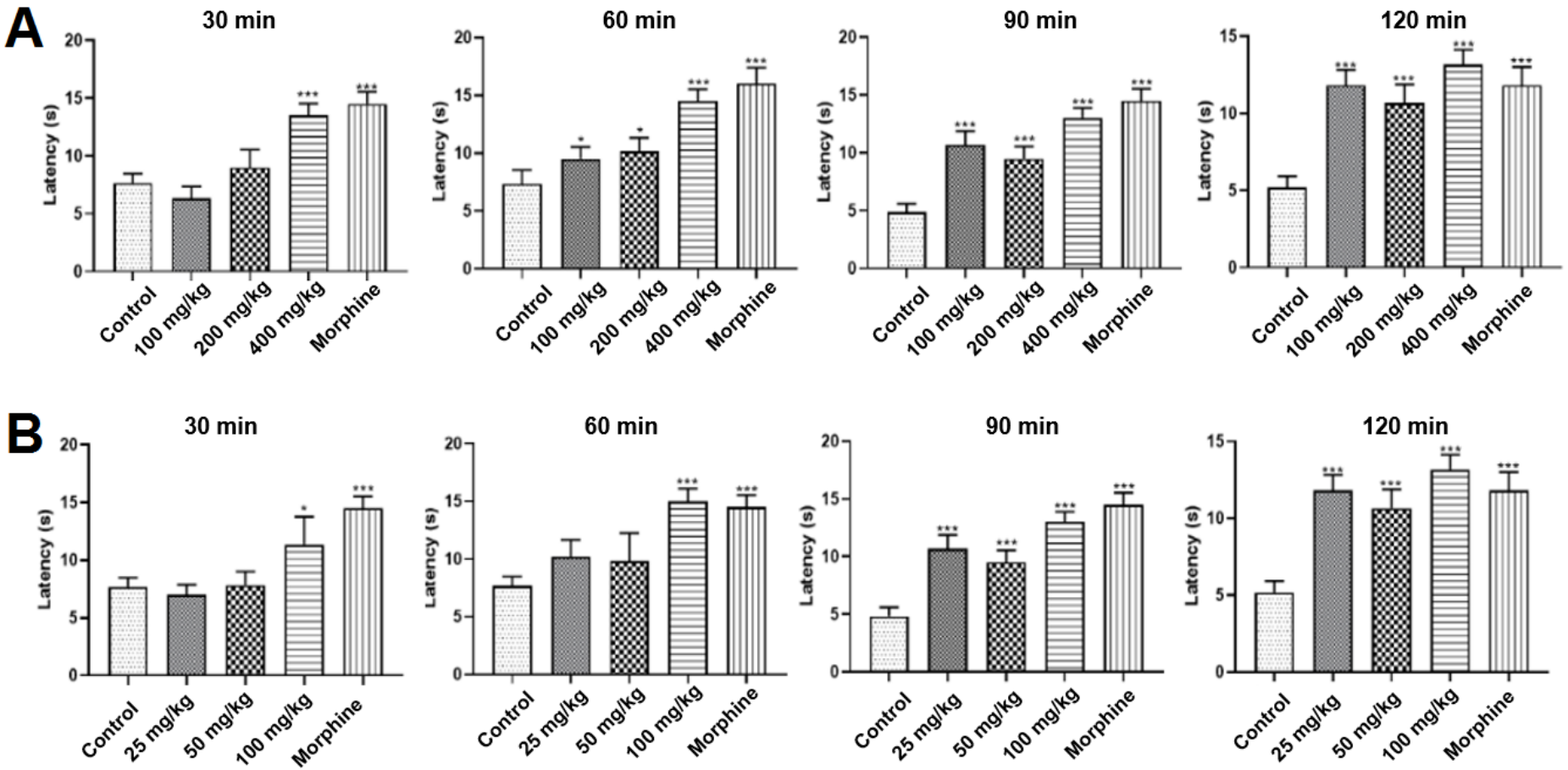
Morphine
Fraction

Figure 3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Morphine

Figure 4

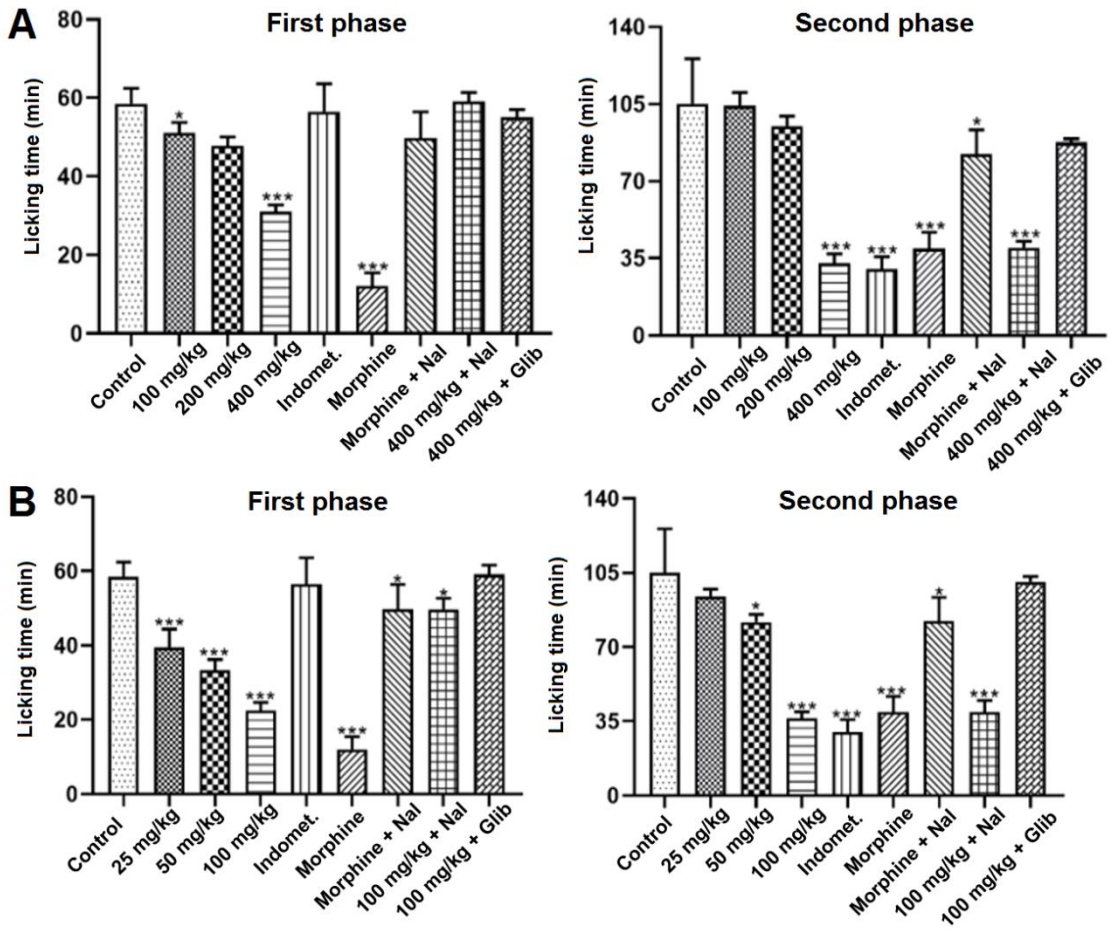

Figure 5

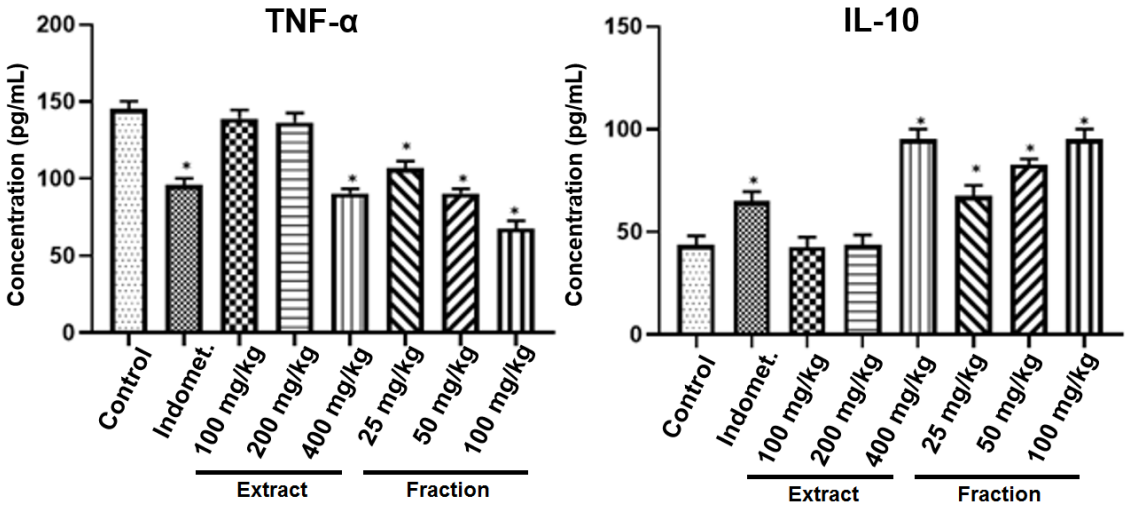

Table 1. Evaluation of food and water consumption, and weight gain in control and groups treated *per os* with saline extract and MvRL-rich fraction from *M. vacciniifolia* at 1,000 mg/kg b.w. for 14 days.

Parameter	Control	Extract	Fraction
Water consumed (mL)	22.17±0.55	22.32±0.44	22.02±0.38
Food consumed (g)	10.22±0.12	10.11±0.09	10.01±0.10
Weight gain (g)	3.15±0.06	3.16±0.09	3.15±0.10

No significant differences were found by analysis of variance (ANOVA) followed by Bonferroni's test. Control group received only vehicle (0.9% NaCl) n=3.

Table 2. Hematological parameters of mice from control and groups treated *per os* with saline extract and MvRL-rich fraction from *M. vacciniifolia* at 1,000 mg/kg b.w. for 14 days.

Parameter	Treatments		
	Control	Extract	Fraction
Erythrocytes (10^6/mm^3)	6.24±0.12	6.22±0.20	6.21±0.15
Hematocrit (%)	30.44±0.26	30.27±0.32	30.55±0.27
Hemoglobin (g/dL)	12.22±0.09	12.21±0.08	12.22±0.10
MCV (fL)	41.00±0.22	40.39±0.09	41.108±0.11
MCH (pg)	14.35±0.09	14.20±0.19	14.28±0.14
MCHC (%)	30.28±0.10	30.29±0.13	30.30±0.12
Leukocytes (10^3/mm^3)	7.65±0.30	7.64±0.26	7.60±0.22
Segmented (%)	68.94±0.14	69.01±0.31	69.12±0.32
Lymphocytes (%)	25.32±0.16	25.30±0.15	25.28±0.19
Monocytes (%)	4.64±0.06	4.67±0.09	4.60±0.08

MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. No significant differences were found by analysis of variance (ANOVA) followed by Bonferroni's test. Control group received only vehicle (0.9% NaCl) n=3..

Table 3. Biochemical parameters of blood from mice of control and groups treated *per os* with saline extract and MvRL-rich fraction from *M. vacciniifolia* at 1,000 mg/kg b.w. for 14 days.

Parameter	Treatments		
	Control	Extract	Fraction
Albumin (g/dL)	1.85±0.12	1.87±0.13	1.84±0.10
ALT (U/L)	78.35±0.36	78.27±0.28	78.05±0.43
AST (U/L)	99.09±0.32	99.14±0.40	99.25±0.25
Total protein (g/dL)	5.22±0.08	5.25±0.10	5.24±0.15
Alkaline phosphatase (IU/L)	9.10±0.06	9.09±0.08	9.06±0.09
GGT (U/L)	8.65±0.27	8.63±0.17	8.64±0.20
Urea (mg/dL)	29.11±0.08	29.09±0.06	29.02±0.10
Creatinine (mg/dL)	0.32±0.02	0.32±0.01	0.32±0.04
Total cholesterol (mg/dL)	107.50±1.09	108.22±1.02	108.45±1.22
Triglycerides (mg/dL)	109.23±1.11	108.05±1.30	109.34±1.18

ALT: alanine aminotransferase. AST: aspartate aminotransferase. GGT: gamma-glutamyl transferase. No significant differences were found by analysis of variance (ANOVA) followed by Bonferroni's test. Control group received only vehicle (0.9% NaCl) n=3..

Table 4. Evaluation of the anti-inflammatory activity of saline extract and MvRL-rich fraction *M. vacciniifolia* on carrageenan-induced edema in the hind paw of mice.

Treatment	Dose (mg/kg)	Period after administration				Reduction (%) after 4 h
		1 h	2 h	3 h	4 h	
		Change in paw volume				
Control	-	0.382±0.04	0.355±0.04	0.345±0.03	0.351±0.02	-
Dexamethasone	10	0.305±0.02*	0.270±0.03*	0.255±0.05*	0.213±0.01*	39.31
Saline extract	100	0.311±0.04*	0.268±0.02*	0.224±0.02*	0.217±0.04*	38.17
	200	0.306±0.01*	0.257±0.02*	0.214±0.02*	0.209±0.03*	40.45
	400	0.299±0.02*	0.245±0.02*	0.197±0.01*	0.189±0.02*	46.15
MvRL-rich fraction	25	0.293±0.02*	0.247±0.01*	0.202±0.03*	0.144±0.02*	58.97
	50	0.280±0.03*	0.235±0.03*	0.191±0.01*	0.124±0.05*	64.67
	100	0.227±0.03*	0.216±0.02*	0.179±0.03*	0.101±0.03*	71.22

Values are represented as mean ± SD. (*) indicates significant difference ($p < 0.05$) from control. Control group received only vehicle (0.9% NaCl).

Table 5. Evaluation of the anti-inflammatory activity of saline extract and MvRL-rich fraction *M. vacciniifolia* on edema in the hind paw of mice induced by histamine or bradykinin.

Groups	Change in paw volume (%)					
	Period after administration (min)					
	30	**60**	**90**	**120**	**150**	**180**
Histamine-induced edema						
Control	10.17±2.01	17.25±1.33	22.09±2.45	23.19±2.24	16.12±3.04	11.85±2.65
DPH	6.84±0.52*	7.31±1.21*	6.57±1.78*	5.28±1.89*	5.33±1.12*	4.47±0.35*
Extract (400 mg/kg)	13.02±3.22	18.12±2.67	21.34±2.44	24.03±3.22	18.17±3.31	12.89±2.89
Fraction (100 mg/kg)	12.56±2.34	18.00±2.99	22.11±3.67	23.11±3.04	17.38±2.78	13.19±2.66
Bradykinin-induced edema						
Control	9.14±1.12	15.89±1.45	21.76±1.78	22.03±2.01	17.33±1.86	13.64±2.01
Extract (400 mg/kg)	13.55±3.21	16.95±2.55	20.87±2.39	19.06±2.12	16.12±3.04	11.85±2.65
Fraction (100 mg/kg)	12.39±2.67	13.65±2.05*	15.77±2.92*	16.03±1.97*	13.06±1.66*	9.75±1.87*

Values are represented as mean ± SD. (*) indicates significant difference ($p < 0.05$) from control. Control group received only vehicle (0.9% NaCl).

Table 6. Effect of saline extract and MvRL-rich fraction from *M. vacciniifolia* rhizome on leukocyte and neutrophil migration in peritoneal lavage from carrageenan-induced inflammation in mice.

Groups	Dose (mg/kg)	Leukocytes		Neutrophils	
		Number (10^5/mL)	Reduction (%)	Number (10^5/mL)	Reduction (%)
Control	-	8.22±0.21		5.92±0.03	
Indomethacin	10	2.35±0.17*	71.41	1.91±0.05*	67.73
Saline extract	100	2.79±0.14*	66.05	2.38±0.05*	59.79
	200	2.65±0.11*	67.76	2.01±0.05*	66.04
	400	2.38±0.09*	71.04	1.74±0.05*	70.60
Fraction	25	2.34±0.08*	71.53	2.01±0.03*	66.04
	50	2.18±0.11*	73.47	1.87±0.05*	68.41
	100	1.99±0.14*	75.79	1.64±0.05*	72.29

Values are represented as mean ± SD. (*) indicates significant difference ($p < 0.05$) from control. Control group received only vehicle (0.9% NaCl).

Table 7. Effect of saline extract and MvRL-rich fraction from *M. vacciniifolia* rhizome on lymphocytes migration in exudate from carrageenan-induced air pouch in mice.

Groups	Dose (mg/kg)	Cells (10^3 cells/mm^3)	Reduction (%)
Control	-	12.55±0.06	-
Indomethacin	10	5.08±0.10*	59.52
Saline extract	100	8.79±0.12*	29.96
	200	7.65±0.19*	39.04
	400	7.01±0.09*	44.14
Fraction	25	5.49±0.15*	56.25
	50	4.22±0.07*	66.37
	100	4.01±0.14*	68.05

Values are represented as mean ± S.E.M. (*) indicates significant difference ($p < 0.05$) from control. Control group received only vehicle (0.9% NaCl).

Table 8. Effect of saline extract and MvRL-rich fraction from *M. vacciniifolia* rhizome on the formation of granuloma in mice.

Parameter	Control	Dexamethasone	**Saline extract (mg/kg)**			**MvRL-rich fraction (mg/kg)**		
			100	200	400	25	50	100
Mass of pellets (g)	0.19±0.01	0.08±0.01*	0.14±0.01*	0.15±0.02*	0.14±0.02*	0.12±0.01**	0.10±0.01**	0.07±0.01**
Protein (A550)	0.082±0.02	0.045±0.02*	0.072±0.02*	0.074±0.02*	0.070±0.02*	0.069±0.02**	0.053±0.02**	0.038±0.02**

The values represent the arithmetic mean ± S.E.M.). Mass of the pellets. A550: absorbance at 550 nm. (*) Statistically different from the negative control ($p < 0.05$). (**) Statistically different from the negative control ($p < 0.01$).

3.3 – Artigo 3: Antibacterial and antitumor activities of lectin-rich preparation from *Microgramma vacciniifolia* rhizome

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Abstract

The rhizome of *Microgramma vacciniifolia* contains a lectin (carbohydrate-binding protein) called MvRL. Studies demonstrated that a MvRL-rich fraction did not show *in vivo* genotoxicity and acute toxicity in mice. This study aimed to evaluate the MvRL-rich fraction from *M. vacciniifolia* rhizome for antibacterial activity *in vitro* and *in vivo* as well as antitumor effect *in vivo* using the Ehrlich carcinoma model in mice. The fraction showed antibacterial activity against *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* with minimal inhibitory concentrations ranging from 31.2 to 125.0 μg/mL and minimal bactericidal concentrations from 62.5 to 200 μg/mL. The fraction was also effective *in vivo* against infection caused by these bacteria on *Tenebrio molitor* larvae considering the parameters evaluated. In regard to the antitumor activity, the treatments of Ehrlich carcinoma-bearing mice with the fraction at 100 and 200 mg/kg *per os* resulted in 62.58% and 75.43% of tumor inhibition, respectively. Increased expression of p53 and decreased expression of Ki-67 in the tumors were detected. In conclusion, the MvRL-rich fraction showed *in vivo* antibacterial and antitumor activities and thus can be considered as an alternative of natural origin for the development of candidates for therapy.

Keywords: pteridophyte; antibacterial, antitumor; lectin.

Introduction

Morbidity and mortality are still closely correlated with the development of infections (Quiles et al., 2015). Pathogens of the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) are mainly correlated with nosocomial infections due to their ability to proliferate in the hospital environment, which is attributed to the production of accessory metabolites that give them greater aptitude for survival (Tiwari, Meena and Tiwari, 2018). In addition, the potential to form biofilms and the development of resistance are also correlated to these pathogens, which increases the difficulty of treatment (Bales et al., 2013). Multidrug bacterial resistance has become a challenge in the therapy of diverse types of infections, increasing the costs of hospitalization as well as the associated mortality rate (Frieri et al., 2017). The spread of resistant strains is influenced by several factors and the related genes can be transmitted to pathogens or gut microbiota through contaminated food or the environment (Robinson et al., 2016). The emergence of multidrug-resistant strains awakens researchers in the search for new therapeutic alternatives (Munoz-Davila, 2014). The diversity of phytochemicals with antimicrobial properties put the plants in evidence for drug development (Turner et al., 2019).

The cancer remains the second leading cause of death worldwide (Martel et al., 2020). Despite the current availability of various chemotherapeutic substances, the therapies sometimes fail due to tumor cell resistance as well as complications due to side effects (Shan et al., 2016). Tumor cell multidrug resistance causes relapses and metastases, which complicate the prognosis (Wu et al., 2019). Thus, it is essential the search for new molecules with antineoplastic properties and less toxicity; in this scenario, natural products have arisen interest of many research groups (Aghajanpour et al., 2017; Salhi et al., 2018).

Microgramma vacciniifolia is an epiphytic plant that belongs to Polypodiaceae family, cited in the literature for its astringent activities and recommendations to treat hemorrhages, expectorations, dysenteries, intestinal cramps and hydrops (Agra et al., 2008; Santos and Sylvestre, 2006). The rhizome of *M. vacciniifolia* contains a lectin called MvRL. Lectins are carbohydrate-binding proteins that are broadly found in plants and possess several biological activities, including antimicrobial (Coelho et al., 2018) and anticancer (Patriota et al., 2019) properties. MvRL showed antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici*

(Albuquerque et al., 2014a), insecticidal activity against *Nasutitermes corniger* (Albuquerque et al., 2012) and cytotoxic action against lung mucoepidermoid carcinoma (NCI-H292) cells (Albuquerque et al., 2014b).

Studies demonstrated that a MvRL-rich fraction (obtained by fractionation of saline extract from the rhizomes with ammonium sulphate) did not show *in vivo* genotoxicity and did not cause behavioral, hematological, and histopathological alterations in mice when evaluated for acute toxicity at a dose of 1,000 mg/kg b.w. (Silva et al., to be submitted). In addition, MvRL-rich fraction promoted central and peripheral analgesia as well as anti-inflammatory effects by interfering with bradykinin via and inhibiting inflammatory cell migration (Silva et al., to be submitted).

This study aimed to evaluate the MvRL-rich fraction from *M. vacciniifolia* rhizome for antibacterial activity *in vitro* and *in vivo* as well as antitumor effect *in vivo* using the Ehrlich carcinoma model in mice.

Materials and methods

Plant material

Rhizomes of *M. vacciniifolia* were collected in September 2016 at the campus of the *Universidade Federal de Pernambuco* (UFPE) in Recife (08°03'07" S, 34°56'59" W), Brazil. Taxonomic identification was performed and a voucher specimen (no. 63,291) is deposited at the herbarium Dárdano de Andrade Lima of the *Instituto Agronômico de Pernambuco* (Recife). The access was recorded (A347889) in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen).

Preparation of MvRL-rich fraction

MvRL-rich fraction was obtained as previously described (Albuquerque et al., 2012; Santana et al., 2012). The rhizomes were washed with distilled water and put to dry for 7 days at 28 ºC and additional 3 days in an oven at 35 ºC. Next, the dried rhizomes were powdered using a knife mill. The powder (100 g) was submitted to extraction in 0.15 M NaCl (1 L) for 12 h at 28 ºC under magnetic stirring. After this period, the suspension was filtered and centrifuged (9,000 *g*, 15 min, 28 ºC) and the supernatant corresponded to the saline extract. The MvRL-rich fraction was obtained after treatment of this extract with ammonium sulfate at 60%

saturation (Green & Hughes, 1955) for 4 h, followed by centrifugation (9,000 *g*, 15 min, 28 ºC), collection of the precipitate by resuspending it in 0.15 M NaCl, and dialysis against distilled water for 6 h (two liquid changes). The dialyzed fraction was dried by lyophilization.

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). Hemagglutinating activity (HA) was performed according to Albuquerque et al. (2012) using a suspension (2.5%, v/v) of human O-type erythrocytes in 0.15 M NaCl. The HA was defined as the reciprocal value of the highest dilution of sample that promoted full agglutination of erythrocytes. Specific hemagglutinating activity was defined as the ratio between the HA and protein concentration (mg/mL).

Evaluation of antibacterial activity

Determination of minimal inhibitory (MIC) and bactericidal (MBC) concentrations

The minimal inhibitory concentration (MIC) of the fraction was determined according to Işcan e al. (2002) in 96-well culture plate, with some modifications. The microorganisms tested were *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). The cultures were grown in Mueller Hinton Broth and final cell concentrations were adjusted to 5×10^5 colony-forming units (CFU)/mL according to the McFarland scale. The culture medium (170 μL) was added to each well and the fraction (dissolved in 10%, v/v, DMSO) was serially two-fold diluted to achieve concentrations in the range 7.8– 1,000 μg/mL. Next, 10 μL of the microorganism culture was added. The plates were incubated at 37 °C for 24 h and a resazurin solution (0.01%, w/v) was used to evaluate bacterial growth, which was indicated by color changing from blue to pink. The lowest concentration at which no color change occurred was recorded as the MIC. For MBC determination, aliquots (10 μL) of the wells (before resazurin addition) were transferred to Mueller-Hinton Agar plates and incubated for 24 h at 37 °C. The MBC was determined as the lowest concentration of protein fraction capable of preventing bacterial growth. Two independent experiments were performed in triplicate.

Time-kill analysis

To evaluate the kinetics of microbial death, bacterial suspensions (10^5 CFU/mL) were treated with the fraction at concentrations of ½ MIC and MIC, incubated at 37 °C for 24 h, and then seeded in a plate with Muller Hinton Agar medium. A growth control with no test sample was also performed. The growth was accompanied by visual counting of colonies at time zero and after 1, 2, 4, 8, 12 and 24 h. Two independent experiments were performed in triplicate and time-kill curves were constructed by plotting log CFU/mL versus time (Bendali et al. 2008).

In vivo evaluation of antibacterial activity

In vivo antibacterial activity of MvRL-rich fraction against the same isolated described above was evaluated using the infection model in *Tenebrio molitor* larvae. The larvae were randomly assigned to groups (n = 10 per group) and infected by injecting 10 μL of bacterial suspension (1×10^5 CFU/larva) into the last left proleg. After 2 h incubation at 37 °C, the larvae received into the last left proleg a single dose of 10 μL of the fraction at MIC or 2×MIC and the assays were incubated again at 37 °C. Infected larvae inoculated with the vehicle (0.1%, v/v, DMSO) were used as positive control while vehicle-treated uninfected larvae corresponded to negative control. The fraction was also inoculated in uninfected larvae to verify the occurrence of any mortality related to the sample. Mortality rates of each group were recorded daily for 5 days.

Antitumor activity

Animals

Female Swiss mice (*Mus musculus*), 50 day-old and weighing 30–35 g, were obtained from the vivarium of the *Laboratório de Farmacologia e Cancerologia Experimental* from the *Departamento de Antibióticos* of UFPE. The mice were housed at the same laboratory at a temperature of 22 °C, with 12:12 photoperiod and *ad libitum* access to food (Purina, Nestlé Brasil Ltda., Brazil) and water. The Ethics Committee on Animal Experimentation of UFPE approved all the experiments (process number 23076.042699/2016-72).

Tumor transplantation and treatments

For the transplantation of Ehrlich carcinoma, the tumor cells were removed from an 8-day-old donor animal by aspiration of the ascitic fluid and introduction (25×10^6 cells/mL) into the recipient animals subcutaneously in the subaxillary region. After 48 h of implantation, the animals were divided into groups (n = 5 per group) and treated orally for 7 days with distilled water (negative control) or with MvRL-rich fraction at 100 or 200 mg/kg. On the 8th day, the animals were weighed, anesthetized (80 mg/kg ketamine and 20 mg/kg xylazine, i.p.) and blood samples were collected by cardiac puncture for hematological analysis. Then, the mice were euthanized by cervical dislocation and tumors were collected and had their weight determined. The percentage of tumor reduction was then calculated (Koniyama and Funayama, 1992).

Hematological analyses

The blood samples from each animal were allocated into EDTA-containing tubes for hematological analysis [erythrocytes, leukocytes, hemoglobin, mean corpuscular volume (MCV) mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)] using an ABX-PENTRA-80 Automated Hematology Analyzer (Germany).

Immunohistochemical analysis

The tumor specimens collected were embedded in paraffin and sections of 4 μm were cut. Next, the samples were deparaffinized in xylol and rehydrated. Then, antigen retrieval in 10 mM citrate buffer pH 6.0 was performed at 125 °C in a Pascal pressure chamber (DakoCytomation Colorado, Inc., Fort Collins, CO, USA). The slides were cooled to room temperature and endogenous peroxidase activity was blocked by immersion for 7.5 min in a peroxidase blocking solution. After cooling, they were incubated overnight at 8 °C with monoclonal antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) at a 1:500 dilution against the proteins studied (p53 and Ki-67). The slides were then washed with phosphate buffered saline (PBS), incubated with a magnification solution (DAKO EnVision), washed in buffer again, and incubated in chromogen diaminobenzidine solution. After washing with water, the slides were counterstained in hematoxylin, dehydrated and mounted. Qualitative reactivity analysis was performed by examining the nucleus and cytoplasm of the cells (Ramos-Vara and Beissenherz, 2000).

Statistical analysis

The data were expressed as means of replicates ± standard deviation (SD) or standard error (SE) values, which were calculated using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences ($p < 0.05$) between treatment groups were analyzed using one-way ANOVA followed by Tukey's test using the GraphPad Prism software. Kaplan-Meier survival curves of *T. molitor* larvae were submitted to log-rank test the same software.

Results and discussion

Natural products of plant origin have been the focus of studies aiming to find alternatives to fight the spread of microbial resistance (Liu et al., 2017). Lectins are reported as antimicrobial agents by acting through different mechanisms and exerting several effects such as growth inhibition, damage to cell integrity and inhibition of biofilm formation (Procópio et al., 2017; Coelho et al., 2018).

It was previously reported that the MvRL-rich fraction did not contain secondary metabolites of the classes alkaloids, coumarins, cinnamic acid derivatives, flavonoids, tannins, and terpenes/steroids (Silva et al., to be submitted). The saline extract from *M. vacciniifolia* showed a protein concentration of 5.37 mg/mL and specific HA of 11.9 while the MvRL-rich fraction contained 14.21 mg/mL of protein and presented specific HA of 144.1. These data confirms the fraction as a preparation rich in the lectin MvRL.

The MvRL-rich fraction was effective against the growth all bacteria tested with MIC values ranged from 31.2 to 125.0 μg/mL and bactericidal effect was also detected, with MBC ranging from 62.5 to 250.0 μg/mL (Table 1). The *S. aureus* isolate was the most sensitive to the fraction and the MBC/MIC ratio for all bacteria was 2 or 4, indicating that the fraction is a bactericidal drug (Levison, 2004). The time-kill curves of bacteria treated with the fraction can be seen in Figure 1. A reduction in the growth was observed already in the treatments at ½ MIC, mainly in the first 12 h; in some cases, a little growth recovery after 24 h. In the results obtained for the MIC treatments, bacterial growth was strongly inhibited in the first 12 h and the bacteriostatic effect remained even after 24-h incubation.

Lectin isolated from *Alpinia purpurata* inflorescence showed MIC of 200 μg/mL against non-resistant *S. aureus* strains but did not affect the viability of *P. aeruginosa* strains up to the concentration of 400 μg/mL (Ferreira et al., 2018). The lichen-derived lectin *Cladonia*

verticillaris showed MIC of 229.9, 7.18 and 114.9 µg/mL for *S. aureus, E. coli* and *K. pneumoniae* (Ramos et al., 2014). Lectin from *Moringa oleifera* seeds (WSMoL) presented a MIC of 250.0, 31.2, 7.8 µg/mL for *E. coli*, *K. pneumoniae* and *S. aureus*, respectively (Ferreira et al, 2011; Coriolano et al., 2019). The lectin isolated from *Punica granatum* fruit presented MIC of 6.25 µg/mL against *S. aureus* (Silva et al, 2016). The results show that the MIC values found for MvRL-rich fraction and within the range found in evaluations with other isolated lectin preparations.

Invertebrate hosts, such as *Drosophila melanogaster, Caenorhabditis elegans*, *Galleria mellonella*, and *Tenebrio molitor* have been used in studies on microbial infection due to some advantages like easy commercialization and cultivation in the laboratory (Fuchs et al., 2010; Merkx-Jacques et al., 2013). In addition, body temperature of *T. molitor* larvae (25-37ºC) coincides with the human body temperature at which pathogens of medical importance develop, for example (Li et al., 2013). Once the MvRL-rich fraction showed good results in the *in vitro* antimicrobial assay, we evaluated its *in vivo* antimicrobial potential using *T. molitor* larvae as bacterial host . The results (Figure 2) showed that the infected larvae treated with the fraction had a longer life span than infected untreated larvae, revealing that the fraction also showed antibacterial activity *in vivo*.

Interaction of lectins with glycoconjugates may provoke internalization and induction of cellular responses across the surface of cells (Loh et al, 2017). The binding of lectins to cell wall components of Gram-positive and negative bacteria such as teichoic acids and lipopolysaccharides may trigger changes in permeability and nutrient uptake or induce oxidative stress induction (Procópio et al., 2017; Coelho et al., 2018). Other cellular responses may include inhibition of protein or nucleic acid synthesis as well as inhibition of synthesis or plasma membrane functionality (Rezaei et al., 2012).

Medicinal plants have been studied as source of new bioactives against tumor cells and some drugs currently used in cancer chemotherapy are plant derivatives, such as paclitaxel (Wang et al., 2016). The reduction of side effects underlies the significant search in recent years for phytochemicals with antitumor potential, which may have anticancer properties through several mechanisms, but the most frequently correlated is the induction of apoptosis (Cragg and Newman, 2013; Kabeer et al., 2019). It was previous reported that MvRL exerted cytotoxic action on cancer cells without being toxic to human peripheral blood mononuclear cells (Albuquerque et al., 2014b). In the present study we evaluated the tumor inhibition properties of MvRL-rich fraction against Ehrlich mammary carcinoma in mice. Cells of this strain have high proliferation and may develop ascitic or solid tumor, which are characterized as

undifferentiated, hyperdiploid and rapidly proliferating tumor with a predilection to metastasize to the lungs, liver, pancreas, kidneys and bones (Chen and Watkins, 1970; Ozaslan et al., 2011; Mishra et al., 2018).

Treatment with MvRL-rich fraction at the two doses tested (100 and 200 mg/kg) significantly decreased the weight of Erlich carcinoma in comparison with untreated control (Figure 3). The treatments with 100 and 200 mg/kg promoted 62.58% and 75.43% of tumor inhibition, respectively. Antiproliferative effects on tumor cells *in vivo* have been reported to other lectins; for example, a lectin from the seeds of *Moringa oleifera* (called MOSL) reduced the Ehrlich carcinoma in 55% when administered to mice at a daily dose of 4 mg/kg i.p. (Asaduzzaman et al, 2018). The *Kaempferia rotunda* lectin, at a dose of 2.5 mg/kg/day i.p., achieved a tumor growth inhibition percentage of 67% of Erlich carcinoma in mice (Kabir et al, 2011).

Anemia with decreased hemoglobin and hematocrit levels are often observed in chemotherapy patients as indicative of damage to hematopoietic cells (Gaspar et al., 2015; Chopra et al., 2016). In this sense, we investigated whether MvRL-rich fraction would have interfered with hematological parameters in the tumor-bearing animals. Table 2 shows that the animals treated with the fraction presented an improvement in the red series in comparison with the untreated group. Treatment with the lectin from *Trichosanthes cucumerina* seeds (1 mg/kg/day i.p.) led to a 62% reduction in tumor growth and increase in red blood cells and hemoglobin content in animals with Ehrlich carcinoma (Kabir et al. , 2012), likewise the lectin from *Momordica charantia* seeds (8 mg/kg/day i.p.), which promoted 75% inhibition of tumor proliferation and increased red blood cell and hemoglobin rates (Kabir et al, 2015).

Other common side effects of anticancer drugs are the damages to immune system cells, being leukopenia and lymphocytopenia common findings in patients during the chemotherapy (Liu et al., 2013). In the present study, an increase in leukocyte rates was observed in animals treated with the fraction in comparison with the untreated group (Table 2). Immunomodulation is one of the promising correlated effects in anticancer therapies, especially with phagocyte activation (Kou et al., 2016). These data stimulate future studies on the immunomodulatory potential of the MvRL-rich fraction.

Inhibition of tumor growth by MvRL-rich fraction was also evaluated by immunohistochemical staining (Figure 4). The behavior of neoplasms can be monitored by the expression of tumor suppression/pro apoptotic antigens such as p53, which acts by preventing the transition of the cell cycle from phase G1 to S (Ferriz-Martinez et al, 2010). This protein is considered key to cycle arrest since, after damage to the cells DNA, it triggers the release of

Bax, which in turn emits apoptotic signals to mitochondria (Yu and Zhang, 2008). The treatments with the fraction led to a noticeable increase in p53 antigen staining compared to control group. Increased p53 expression has also been reported in Ehrlich carcinoma cells treated with the lectin from the *Geodorum densiflorum* rhizome (Kabir et al., 2019).

The Ki-67 is expressed at the end of G1 phase and remains expressed in subsequent phases of the cell cycle, which in turn points to tumor proliferation (Dowsett et al, 2011). The treatments with MvRL-rich fraction resulted in decrease of the Ki-67 antigen marking. The Ki-67 mediates the mobility of chromosomes and their connection as the mitotic spindle, preventing collapse right after the opening of the nucleus (Cuylen et al., 2016). In addition, a role is also established in mRNA transcription during protein production in ribosomes (Rahmanzadeh et al., 2007). The hydroalcoholic extract of *Justicia spicigera* showed antiproliferative effect on prostate cancer cells by reducing the expression of Ki-67, decreasing the number of cells in a proliferative state, and suggesting regulation of the cell cycle in the interphase (Fernández-Pomares et al., 2018).

Conclusion

According to the findings presented MvRL-rich fraction showed *in vivo* antibacterial and antitumor activities and thus can be considered as an alternative of natural origin for the development of candidates for therapy.

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Figure captions

Figure 1. Time-kill curves of bacteria of medical importance treated or not with the MvRL-rich fraction from *Microgramma vacciniifolia* rhizome. Each point represents the mean ± SD of two independent experiments performed in triplicate.

Figure 2. Effects of the MvRL-rich fraction from *Microgramma vacciniifolia* rhizome on infection with bacteria of medical importance using *Tenebrio molitor* larvae as model. The Kaplan-Meier curves of uninfected insects treated with vehicle (0.2% DMSO) as well as infected insects treated with vehicle or the fraction (MIC and 2 × MIC) can be seen. Survival

log-rank test indicate a significant ($p < 0.05$) increase in the survival time in the fraction-treated larvae in comparison with infected untreated larvae.

Figure 3. Weight of tumors after treatment of animals with Ehrlich carcinoma for 7 days with distilled water (negative control) or MvRL-rich fraction at 100 or 200 mg/kg *per os*. Each bar represents the mean± SE of the weight of the tumor of each animal. (*) indicates significant differences ($p < 0.05$) between treatments and control.

Figure 4. Immunohistochemical labeling for p53 and Ki-67 of tumors from animals with Ehrlich carcinoma for 7 days with distilled water (negative control) or MvRL-rich fraction at 100 or 200 mg/kg *per os.* The scale bars corresponds to 100 µm.

Figure 1

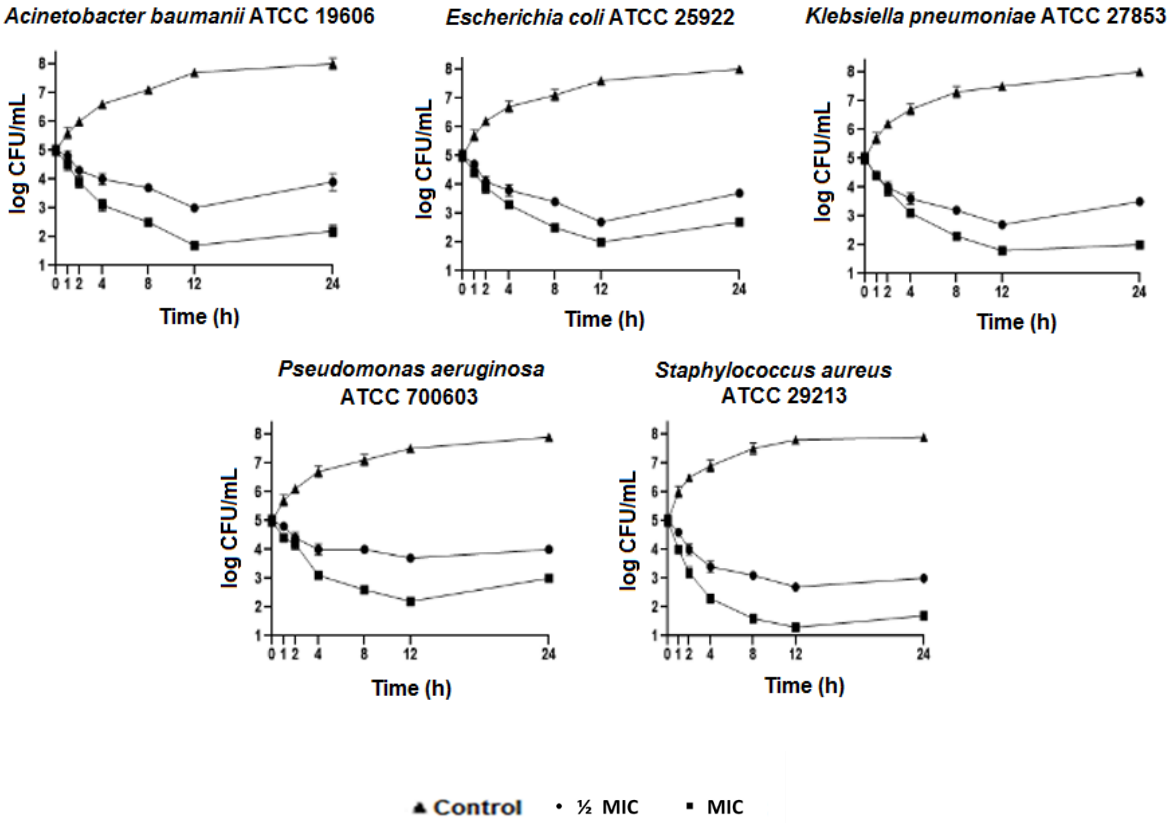

Figure 2

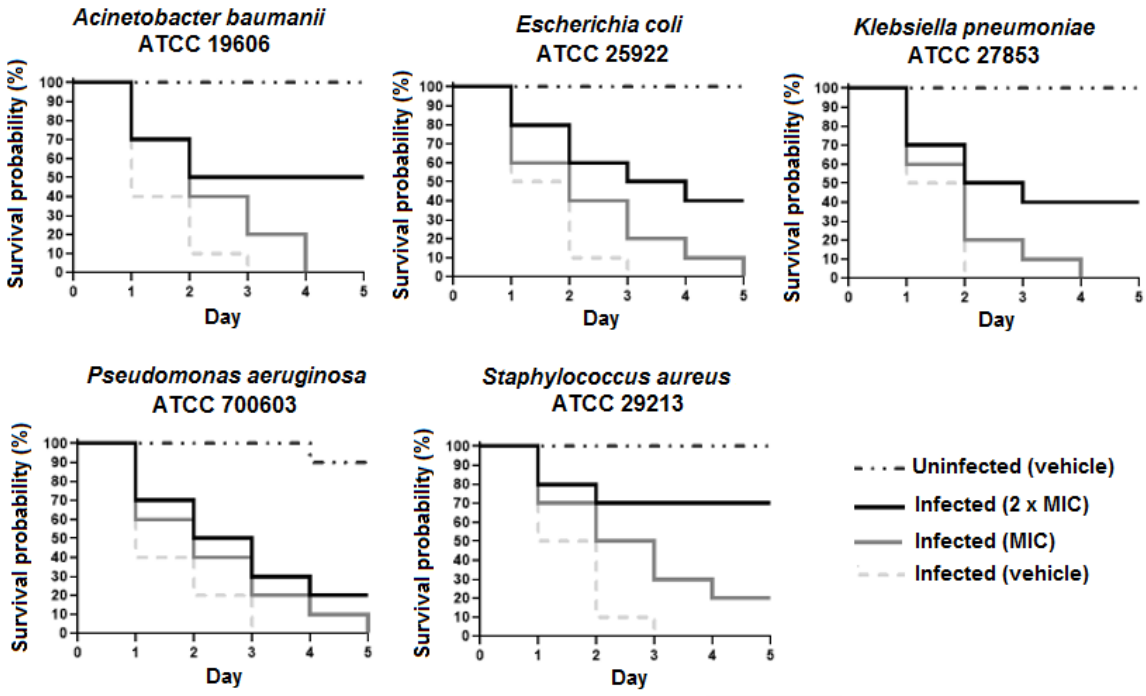

Figure 3

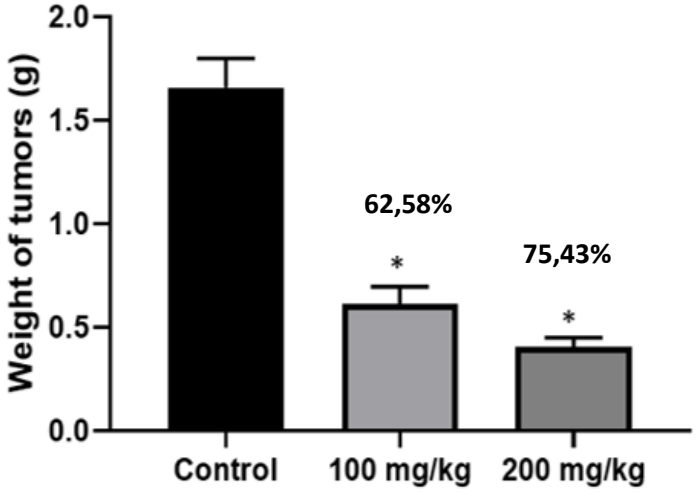

Figure 4

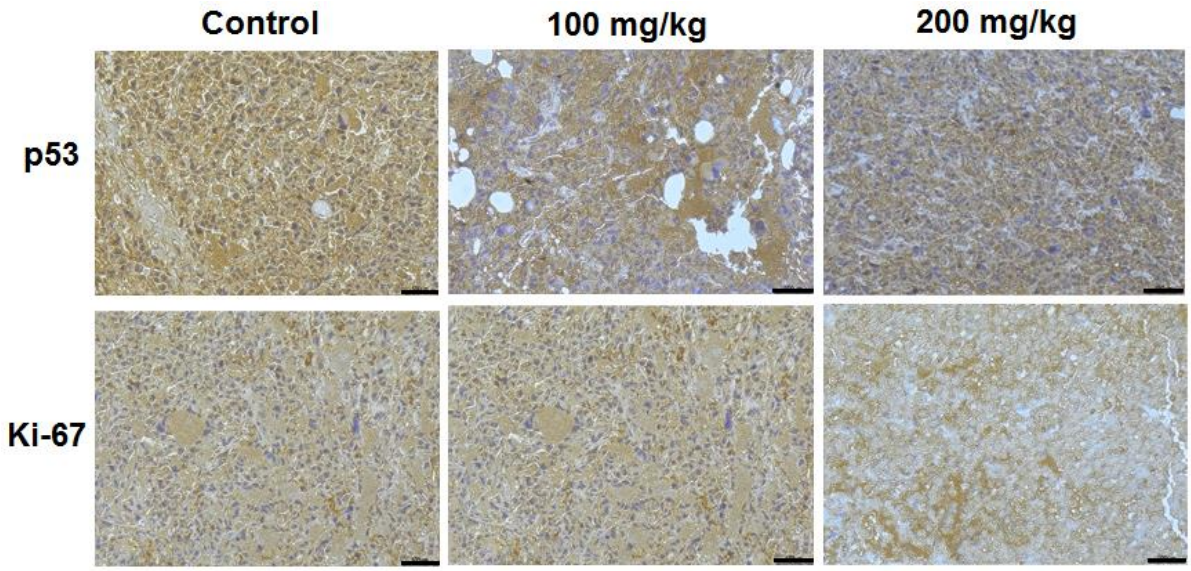

Table 1. Minimal inhibitory (MIC) and bactericidal (MBC) concentrations of the MvRL-rich fraction against bacterial species.

Bacteria	Concentration (μg/mL)		MBC/MIC ratio
	MIC	MBC	
Acinetobacter baumannii (ATCC – 19606)	62.5	250.0	4.0
Escherichia coli (ATCC - 25922)	125.0	250.0	2.0
Klebsiella pneumoniae (ATCC - 700603)	125.0	250.0	2.0
Pseudomonas aeruginosa (ATCC- 27853)	62.5	125.0	4.0
Staphylococcus aureus (ATCC - 29213)	31.2	62.5	2.0

Table 2. Hematological parameters of animals with Ehrlich carcinoma treated for 7 days with distilled water (negative control) or MvRL-rich fraction at 100 or 200 mg/kg *per os*.

Parameters	Treatments		
	Control	MvRL-rich fraction	
		100 mg/kg	200 mg/kg
Erythrocytes (10^6/mm^3)	7.90 ± 0.20	8.98 ± 0.52*	9.07 ± 0.48*
Hematocrit (%)	38.30 ± 0.32	41.90 ± 0.21*	42.65 ± 0.42*
Hemoglobin (g/dL)	11.63 ± 0.08	12.83 ± 0.33*	13.09 ± 0.52*
MCV (fL)	48.43 ± 0.37	46.83 ± 0.59*	45.65 ± 0.74*
MCH (pg)	14.77 ± 0.41	15.00 ± 0.66	15.35 ± 0.51*
MCHC (%)	30.47 ± 0.25	32.10 ± 0.43*	32.06 ± 0.41*
Leukocytes (10^3/mm^3)	7.07 ± 0.19	10.03 ± 0.90*	11.97 ± 0.77*

MCV: mean corpuscular volume. MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration.

4. CONCLUSÕES

- Extrato salino e fração proteica rica em MvRL, obtidos do rizoma de *M. vacciniifolia* foram avaliados quanto à toxicidade por diferentes ensaios, sendo detectada atividade hemolítica moderada para a fração, mas nenhum efeito citotóxico sobre células de ovários de hamster. Com relação à toxicidade aguda para camundongos, extrato salino e fração, nas doses de 1.000, 2.000 ou 5.000 mg/kg *per os*, não provocaram morte de camundongos. Porém a fração, exceto na dose de 1.000 mg/kg, demonstrou alguns sinais de toxicidade, como perda de peso e danos no fígado, pulmões e rins, evidenciados histologicamente e bioquimicamente. Por fim, extrato e fração se mostraram seguros do ponto de vista genotóxico quando testados nas doses 1.000 e 2.000 mg/kg *per os*.
- Em doses consideradas seguras, as duas preparações derivadas do rizoma de *M. vacciniifolia* mostraram atividade antinociceptiva, atuando tanto na via central quanto na via periférica da dor.
- Extrato e fração também apresentaram propriedades anti-inflamatórias, atuando na diminuição da migração de células inflamatórias e na redução dos níveis de citocinas pro-inflamatórias. A fração mostrou atuar na via da bradicinina.
- A fração apresentou atividade antibacteriana frente a cepas padrão de patógenos, tanto *in vitro* quanto *in vivo*, em modelo de infeção em *T. molitor*.
- A fração também se mostrou detentora de potencial antitumoral frente a carcinoma de Ehrlich, como demonstrado pela diminuição de peso dos tumores de grupos tratados, redução de marcação imunohistoquímica de antígenos de proliferação celular e aumento da marcação de antígenos relacionados a supressão tumoral.

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ANEXOS

ANEXO A – Autorização do comitê de ética

Universidade Federal de Pernambuco
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Av. Prof. Nelson Chaves, s/n
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Recife, 18 de julho de 2017

Ofício nº 59/17

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Thiago Henrique Napoleão**
Departamento de Bioquímica
Centro de Biociencias
Universidade Federal de Pernambuco
Processo nº **23076.042699/2016-72**

Certificamos que a proposta intitulada **"avaliação toxicológica e de atividade antitumoral da fração proteica e da lectina do rizoma de microgramma vacciniifolia."** registrada com o **nº 23076.042699/2016-72** sob a responsabilidade de **Prof.ª Thiago Henrique Napoleão** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 07/06/2017.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	26/09/2016
Espécie/ linhagem/raça	Camundongo heterogênico/Swiss *Mus musculus*
Nº de animais	250
Peso/Idade	30-40g / 60 dias
Sexo	Macho e fêmea
Origem	Laboratório de Imunopatologia Keizo Asami (LIKA) UFPE

Atenciosamente,

Prof. Sebastião R. F. Silva
Vice-Presidente CEUA/UFPE
SIAPE 2345891