UNIVERSIDADE FEDERAL DO PARANÁ

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GRACIANNY GOMES MARTINS

Estudo de efeitos antineoplásicos e toxicológicos da *Gochnatia polymorpha* ssp. *floccosa* em ratos com tumor Walker-256

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Profª. Dra. Alexandra Acco

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Ministério da Educação
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Programa de Pós-Graduação em Farmacologia

PARECER

A Comissão Examinadora da Dissertação de Mestrado intitulada "ESTUDO DE EFEITOS ANTINEOPLÁSICOS E TOXICOLÓGICOS DA *Gochnatia polymorpha* ssp. *floccosa* EM RATOS COM TUMOR WALKER-256", de autoria da pós-graduanda GRACIANNY GOMES MARTINS, sob orientação da Prof.ª Dr.ª Alexandra Acco e composta por: Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR); Prof.ª Dr.ª Giseli Klassen (Patologia Básica – UFPR) e Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR), reuniu-se e, de acordo com o Regimento Interno do Programa de Pós-Graduação em Farmacologia, a pós-graduanda foi Aprovada. Para a devida publicação o trabalho deverá sofrer as modificações sugeridas, que serão conferidas por seu orientador. Em Curitiba, 18 de julho de 2014.

Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR)

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ATA DO JULGAMENTO DA 100ª DEFESA DE DISSERTAÇÃO DE MESTRADO

Ao décimo oitavo dia do mês de julho do ano de dois mil e quatorze, às nove horas, no Auditório do Departamento de Farmacologia, Anexo I, Setor de Ciências Biológicas da Universidade Federal do Paraná, reuniu-se a Comissão Examinadora da Dissertação de Mestrado de autoria da pós-graduanda **GRACIANNY GOMES MARTINS**, intitulada "ESTUDO DE EFEITOS ANTINEOPLÁSICOS E TOXICOLÓGICOS DA *Gochnatia polymorpha* ssp. *floccosa* EM RATOS COM TUMOR WALKER-256", sob orientação da Prof.ª Dr.ª Alexandra Acco e composta por: Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR); Prof.ª Dr.ª Giseli Klassen (Patologia Básica – UFPR) e Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR). A Banca Examinadora iniciou os trabalhos. A candidata teve quarenta e cinco minutos para expor oralmente seu trabalho, sendo em seguida arguida durante trinta minutos por cada um dos membros da Banca e tendo trinta minutos para responder a cada uma das arguições. No final da sessão, a Comissão Examinadora emitiu o seguinte parecer: Aprovada. Para a publicação, o trabalho deverá sofrer as modificações sugeridas, que serão conferidas por sua orientadora. Nada mais havendo a tratar, a Presidente deu por encerrada a sessão, da qual foi lavrada a presente ata, que será assinada pela Presidente e pelos demais Membros da Comissão Examinadora em Curitiba, 18 de julho de 2014.

Prof.ª Dr.ª Alexandra Acco (Presidente - Farmacologia - UFPR)

Prof.ª Dr.ª Giseli Klassen (Patologia Básica – UFPR)

Prof.ª Dr.ª Juliana Geremias Chichorro (Farmacologia – UFPR)

Dedico...

À minha querida e amada família, por todo imenso apoio, incentivo, força e muito carinho que recebi durante todos os momentos difíceis longe de vocês.
Pai, por toda a preocupação em me ver bem e pelo imenso amor que recebo de ti...
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RESUMO

Objetivos: Nas últimas décadas, o câncer tornou-se um evidente problema de saúde pública em todo o mundo. A pesquisa com plantas medicinais para fins antineoplásicos vem se tornando cada vez mais consistente, a fim de ampliar as opções de tratamento. Dessa forma, o objetivo deste trabalho foi avaliar a possível atividade antineoplásica e toxicidade da fração diclorometano (F1B), rica em lactonas sesquiterpênicas do subtipo guaionolideos, isolada da *Gochnatia polymorpha* ssp. *flocossa* frente ao modelo de carcinossarcoma Walker-256 em ratos.

Materiais e Métodos: Após a inoculação subcutânea das células Walker-256 no membro pélvico direito de ratos Wistar machos, iniciou-se o tratamento por via oral com F1B na dose de 100 $mg.kg^{-1}$ por 16 dias. A progressão tumoral foi avaliada durante este período. Após o tratamento foram avaliados os seguintes parâmetros: peso tumoral; estresse oxidativo hepático e tumoral (atividade da catalase [Cat], superóxido dismutase [SOD], glutationa-S-transferase [GST], glutationa reduzida [GSH] e peroxidação lipídica); bioquímica plasmática (transaminases ALT e AST, fosfatase alcalina, ureia, creatinina, amilase, proteínas totais e glicemia); parâmetros inflamatórios no tecido tumoral (atividade da mieloperoxidade [MPO], N-acetilglucosaminidase [NAG], dosagem de óxido nítrico e de TNF-α) e histologia hepática e tumoral. A toxicidade da F1B constou da avaliação do peso relativo dos órgãos (fígado, rins, pulmão e baço), além da determinação de sua DL_{50} em camundongos.

Resultados: O tratamento com a F1B produziu uma redução significativa no volume (74%) e no peso tumoral (69%) do carcinossarcoma Walker-256. Não houve diferença nos parâmetros de estresse oxidativo avaliados no tecido tumoral. No entanto, a F1B modificou parâmetros de estresse oxidativo hepático, aumentando os níveis de GSH e restabelecendo a atividade da SOD a valores normais. Ainda, normalizou a glicemia, assim como os níveis de fosfatase alcalina e amilase. Não houve diferença em relação às atividades das enzimas MPO e NAG, bem como nos níveis de óxido nítrico. Porém, houve uma redução nos níveis de TNF-α no tecido tumoral. Além disso, a F1B promoveu aumento da apoptose tumoral avaliada na histologia. Não foram observados sinais clínicos de toxicidade e mortes nos ratos tratados com a F1B. A DL_{50} calculada para camundongos foi de 1209 $mg.kg^{-1}$.

Conclusões: O tratamento com a F1B, isolada da *Gochnatia polymorpha* ssp. *flocossa,* na dose de 100 $mg.kg^{-1}$ mostrou atividade antitumoral contra o carcinossarcoma Walker-256. Este efeito pode estar relacionado, pelo menos em parte, à indução da apoptose e a uma possível inibição na sinalização celular mediada por TNF-α através das lactonas sesquiterpênicas do subtipo guaionolideos, presentes nessa fração.

Palavras-chaves: *Gochnatia polymorpha* ssp. *floccosa*, carcinossarcoma Walker-256, estresse oxidativo, fígado, toxicidade aguda *(DL_{50})*.

ABSTRACT

Aim of the study: In recent decades cancer became an evident public health problem worldwide. A survey of medicinal plants for anticancer purposes is becoming increasingly more consistent with the objective of expanding treatment options. Therefore, the aim of this study was to evaluate the possible antineoplastic activity and toxicity of dichloromethane fraction (F1B), rich in sesquiterpene lactones subtype guaionolide, isolated of *Gochnatia polymorpha* ssp. *flocossa* against the Walker-256 carcinosarcoma in rats.

Materials and Methods: After the subcutaneous inoculation of Walker-256 cells in the right pelvic limb of male Wistar rats, we started orally treatment with F1B at a dose of 100 $mg.kg^{-1}$ for 16 days. The tumor progression was assessed during this period. After the treatment the following parameters were evaluated: tumor weight; oxidative stress in liver and tumor (activity of catalase [Cat], superoxide dismutase [SOD], glutathione-S-transferase [GST], reduced glutathione [GSH] and lipid peroxidation); plasma biochemistry (transaminases ALT and AST, alkaline phosphatase, urea, creatinine, amylase, total protein and glucose); inflammatory parameters in tumor tissue (activity of myeloperoxidase [MPO], N- acetylglucosaminidase [NAG], measurement of nitric oxide and TNF-α), and liver and tumor histology. The toxicity of F1B consisted of assessing the relative weight of the organs (liver, kidney, lung and spleen), and the determination of its LD_{50} in mice.

Results: F1B treatment produced a significant reduction in the tumor volume (74%) and weight (69%) of the Walker-256 carcinosarcoma. There was no difference in oxidative stress parameters evaluated in tumor tissue. However, F1B treatment modified hepatic oxidative stress parameters, increasing the levels of GSH and restoring to normal SOD activity. Additionally, it normalized the plasmatic glucose and the levels of alkaline phosphatase and amylase. There were no differences regarding MPO and NAG activities, as well as the nitric oxide levels. However, there was a reduction in the TNF-α level of tumor. F1B also promoted increased apoptosis in the tumor assessed from tumor histology. No clinical signs of toxicity and death were observed in rats treated with F1B. The LD_{50} calculated for mice was 1209 $mg.kg^{-1}$.

Conclusion: Treatment with F1B, isolated from *Gochnatia polymorpha* ssp. *flocossa,* at the dose of 100 $mg.kg^{-1}$, showed antitumor activity against the Walker-256 carcinosarcoma. This effect may be related, at least in part, to the induction of apoptosis and the inhibited cell signaling mediated by TNF-α through the sesquiterpene lactones, subtype guaionolide, present in this fraction.

Keywords: *Gochnatia polymorpha* ssp. *floccosa*, Walker-256 carcinosarcoma, oxidative stress, liver, acute toxicity (LD_{50}).

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LISTA DE SIGLAS E ABREVIATURAS

ALT – Alanina aminotransferase

ANOVA – Análise de variância

AST – Aspartato aminotransferase

Cat – Catalase

DPPH – 2,2-difenil-1-picrilhidrazil

DL_{50} – Dose letal média

E2F – Fator de transcrição

EGFR – Receptor do fator de crescimento epidérmico

EtOAc - Acetato de etila

EtOH – Etanol

F1B – Fração diclorometano de *Gochnatia polymorpha* ssp. *floccosa*

GSH – Glutationa reduzida

GST – Glutationa-S-transferase

HPLC – Cromatografia líquida de alta eficiência

IARC - Agência Internacional para Pesquisa em Câncer

IFN-γ – Interferon gama

IL-1 – Interleucina 1

IL-6 – Interleucina 6

INCA - Instituto Nacional de Câncer José Alencar Gomes da Silva

MAPK – Proteína quinase ativada por mitógenos

MPO – Mieloperoxidase

NF-κB – Fator de transcrição nuclear kappB

OMS - Organização Mundial da Saúde

PBS – Solução tampão de fosfato

PDGF – Fator de crescimento derivado de plaquetas

PTEN – Homólogo da fosfatase e tensina - gene supressor de tumor

pRB – Proteína do retinoblastoma

RAS – Proteína G monomérica

ROS – Espécies reativas de oxigênio

SLs – Lactonas sesquiterpênicas

SOD – Superóxido dismutase

TGF-β1 – Fator transformador de crescimento beta1

TNF-α – Fator de necrose tumoral alfa

VEGF – Fator de crescimento vascular endotelial

VLC – Cromatografia líquida a vácuo

SUMÁRIO

1. INTRODUÇÃO

1.1 Câncer: Epidemiologia e Terapia

O câncer, também denominado como neoplasia maligna, é uma doença que se caracteriza pela proliferação celular anormal, descontrolada e autônoma, causada por ativação de oncogenes promotores de crescimento celular, inativação de genes supressores de tumor e alterações em genes responsáveis pela síntese de microRNA (CROCE, 2008). As células cancerosas apresentam, em graus variados, uma redução ou ausência de diferenciação celular, capacidade de invasão e comprometimento de tecidos circunjacentes, assim como a capacidade de promover metástases, cuja presença é a principal causa de mortalidade e morbidade na maioria dos cânceres (KUMAR *et al*., 2010; BOGLIOLO; BRASILEIRO, 2011; HANAHAN; WEINBERG, 2011).

Nas últimas décadas o câncer ganhou uma maior dimensão, convertendo-se em um evidente problema de saúde pública mundial. A taxa de mortalidade global por câncer tem aumentado, já que estimativas mundiais do projeto Globocan 2012, da Agência Internacional para Pesquisa em Câncer (IARC) e da Organização Mundial da Saúde (OMS), relataram a ocorrência de 14,1 milhões de casos novos de câncer e um total de 8,2 milhões de mortes por câncer, em todo o mundo, em 2012. No Brasil, a estimativa para o ano de 2014, que será válida também para o ano de 2015, aponta para a ocorrência de aproximadamente 576 mil casos novos de câncer (INCA, 2014). É incontestável que o câncer é hoje, no Brasil, um problema de saúde pública, dada a sua magnitude epidemiológica, social e econômica.

Frente às estimativas sobre a incidência e aos crescentes índices de mortalidade causada por neoplasias em nível global, torna-se necessária a aplicação de medidas preventivas à população em relação aos fatores de risco cancerígeno (uso de tabaco e álcool, hábitos alimentares inadequados, obesidade, agentes infecciosos, radiação ultravioleta e exposições ocupacionais) (INCA, 2014). Além disto, métodos eficazes no aprimoramento de diagnóstico devem ser estimulados, já que uma detecção precoce permite

maior chance de controle da doença. E por fim, um dos fatores mais importante para minimizar os índices de morbidade e mortalidade é a busca por novas opções de tratamento antineoplásico, dirigido essencialmente às células malignas, com o objetivo de promover efeitos menos agressivos ao portador.

Existem três principais abordagens estabelecidas para o tratamento do câncer: excisão cirúrgica, radioterapia e quimioterapia. O valor relativo de cada uma destas abordagens depende do tipo de tumor e o estágio do seu desenvolvimento. A quimioterapia pode ser usada como monoterapia ou como um adjuvante de outras formas de terapia, podendo impedir a recidiva da doença após ressecção cirúrgica de cânceres localizados, como o de mama, colorretal e de pulmão (CHABNER; ROBERTS, 2005; CHABNER, 2012). Os compostos utilizados na quimioterapia de doenças neoplásicas exibem acentuada variação em sua estrutura e no mecanismo de ação, incluindo agentes alquilantes, análogos antimetabólitos do ácido fólico, das pirimidinas e das purinas, produtos naturais, hormônios e antagonistas hormonais, e uma variedade de agentes dirigidos para alvos moleculares específicos (CHABNER; ROBERTS, 2005; HEIDI *et al.*, 2009; CHABNER, 2012). Na atualidade, os agentes quimioterápicos interferem, em sua maioria, na proliferação celular e baseiam-se no ciclo celular rápido e/ou na promoção da apoptose para a sua seletividade relativa contra as células cancerosas (HEIDI *et al*., 2009).

Embora a quimioterapia seja uma importante forma de tratamento em diversos tipos de cânceres, é válido ressaltar os problemas associados aos fármacos antineoplásicos, que apresentam um índice terapêutico estreito e maior potencial de causar efeitos prejudiciais aos indivíduos portadores dessa doença, sendo os principais efeitos colaterais a mielossupressão e imunossupressão, alterações gastrointestinais (náuseas, vômitos, constipação intestinal e diarreia), cardiotoxicidade, hepatotoxicidade, toxicidade pulmonar, neurotoxicidade, disfunção reprodutiva, toxicidade vesical e renal, alterações metabólicas, toxicidade dermatológicas e alopecia (BONASSA, 2005). Estes efeitos adversos ocasionados pelos quimioterápicos relacionam-se ao fato da sua não-especificidade pelas células tumorais, já que os tecidos formados por células de rápida proliferação, como tecido hematopoiético, tecido germinativo, folículo piloso e tecido de revestimento gastrointestinal, por apresentarem semelhança às células neoplásicas em relação a alta atividade mitótica e ciclos

celulares curtos, representam os principais alvos da toxicidade quimioterápica (CLEATOR; PARTON; DOWSETT, 2002). Além disso, a resistência a esses fármacos continua sendo um grande obstáculo ao tratamento bem sucedido do câncer. Essa resistência resulta de uma variedade de alterações farmacocinéticas e moleculares, que podem invalidar os tratamentos mais bem planejados, incluindo absorção e liberação deficientes do fármaco; variabilidade geneticamente determinada no transporte, ativação e depuração do fármaco; e mutações, amplificações ou supressões nos alvos dos fármacos (CHABNER, 2012). Frente a isso, a estratégia para a descoberta de novos fármacos para o tratamento do câncer é a obtenção de agentes que possam atuar de forma efetiva e seletiva em alvos moleculares sobre vias de sinalização, proliferação e a morte celular de tumores (MAHESWARAN *et al*., 2008). Porém, vale ressaltar que apesar do notável sucesso de fármacos dirigidos para alvos moleculares em tipos específicos de câncer, é pouco provável que as novas terapias substituam os agentes citotóxicos, já que ambos continuarão sendo utilizados em combinação (CHABNER; ROBERTS, 2005; CHABNER, 2012).

1.2 Carcinogênese

As células tumorais originam-se de células normais que sofreram alterações no DNA (fatores genéticos) ou em mecanismos que controlam a expressão gênica (fenômenos epigenéticos) em um ou mais locos envolvidos no controle da divisão e da diferenciação celular (HANAHAN; WEINBERG 2000; BOGLIOLO; BRASILEIRO, 2011). A transformação dessas células, decorrente de uma lesão genética não-letal (mutação) pode ser herdada na linhagem germinativa, ou surgir de modo espontâneo, assim como pode ser causada por agentes ambientais, como substâncias químicas, radiação ou vírus (HEIDI *et al*., 2009; KUMAR *et al.*, 2010).

O processo de formação e desenvolvimento de neoplasias, denominado de carcinogênese, é um processo complexo em múltiplas etapas, tanto em nível fenotípico quanto genético, resultante do acúmulo de múltiplas mutações, que culminam no surgimento de clones de células que apresentam a capacidade de se multiplicar de uma forma autônoma, com crescimento

excessivo, além da capacidade de invadir tecidos circunjacentes e formar metástases (BOGLIOLO; BRASILEIRO, 2011). As etapas da carcinogênese são: (1) Iniciação (ou transformação celular) – lesão permanente (mutação) no DNA da célula por exposição aos agentes carcinogênicos físicos, químicos ou biológicos. Esta etapa refere-se a uma mudança no fenótipo de uma célula com controle normal de seu desenvolvimento em outra com crescimento descontrolado, que se torna menos responsiva a fatores que inibem a proliferação celular, a indutores de diferenciação celular ou à apoptose; (2) Promoção – a célula iniciada é estimulada a proliferar, amplificando o clone transformado; e (3) Progressão – o clone transformado prolifera e múltiplas mutações se acumulam, gerando subclones com habilidades variadas de crescimento, invasão, metástase e resistência, tornando o tumor mais agressivo e com maior potencial maligno (HEIDI *et al.*, 2009; KUMAR *et al.*, 2010).

A proliferação autônoma, a senescência replicativa e a insensibilidade a sinais que inibem mitose e a evasão da apoptose, conferem às células neoplásicas a propriedade de imortalidade, possibilitando sua multiplicação excessiva e descontrolada. A autonomia nos sinais de proliferação que essas células neoplásicas adquirem, é resultante de mutações ativadoras de oncogenes, que são os genes que promovem o crescimento celular na ausência de sinais promotores de crescimento normais. Essas mutações são frequentes em genes de fatores de crescimento (p. ex., PDGF), de seus receptores (p. ex., EGFR no carcinoma de mama), de moléculas transdutoras de sinal (p. ex., RAS) e de amplificação em genes que acionam o ciclo celular (p. ex., ciclina D1). A insensibilidade aos sinais inibidores de mitose decorre de: mutação inativadora em genes que codificam moléculas reguladoras da via MAPK (p. ex., *PTEN,* que desfosforila moléculas nessa via), de fatores de transcrição ativadores de genes que controlam o ciclo celular (p. ex., *pRB*, inativador natural de E2F, que ativa a entrada em G1), além de mutação inativadora no gene *p53,* que inativa o complexo ciclina/CDK. A evasão da apoptose resulta da inibição de genes pró-apoptóticos, de hiperexpressão de genes antiapoptóticos ou de inativação do gene *p53*, que promove interrupção do ciclo celular por lesão no DNA e induz a apoptose. As células tumorais apresentam ainda a capacidade de evasão de senescência replicativa, que

ocorre por meio da ativação de telomerase, permitindo a duplicação do DNA. No decorrer dessas alterações o genoma torna-se instável, como resultado do estresse oxidativo durante a duplicação do DNA. Essa instabilidade persistente facilita alterações na regulação genética e epigenética associada à progressão maligna (BOGLIOLO; BRASILEIRO, 2011; HANAHAN; WEINBERG, 2011).

No decorrer desse intenso processo de proliferação e formação de um tumor, as células malignas, assim como as células normais, necessitam de um suprimento sanguíneo para receber nutrientes e oxigênio e para remover produtos de excreção. Portanto, há uma expressiva indução da formação de novos vasos sanguíneos, por processo angiogênico. As células tumorais promovem um aumento na atividade angiogênica por meio da liberação de fatores como o VEGF (fator de crescimento vascular endotelial). A angiogênese apresenta uma correlação com a malignidade tumoral, já que é requerida não somente para o crescimento celular, mas também para o acesso à vasculatura e posterior formação de mestástases. Assim, quanto maior essa atividade, maior é a potência de metastatização do câncer e mais rápida é a sua progressão. Adicionalmente, sabe-se que a modificação na expressão de moléculas de adesão (perda de caderina E) e ativação de genes que favorecem a produção de metaloproteinases, em conjunto com a angiogênese, aumentam a capacidade das células tumorais malignas de invadir tecidos e originar metástases, uma das principais causas de morbidade e mortalidade relacionadas ao câncer (KUMAR *et al.*, 2010; BOGLIOLO; BRASILEIRO, 2011; HANAHAN; WEINBERG, 2011).

Diante de todo esse complexo processo de formação tumoral, vale ressaltar um importante efeito sistêmico presente no paciente portador de câncer, que é a caquexia cancerosa. Esta é uma síndrome complexa e multifatorial caracterizada por intenso consumo generalizado nos tecidos corporais, como muscular e adiposo, com uma perda progressiva de peso, anemia, astenia, balanço nitrogenado negativo, disfunção imune e alterações metabólicas, geralmente associadas à anorexia. É intensificada pelas alterações no metabolismo de nutrientes (carboidratos, proteínas e lipídios) e alterações hormonais, além do aumento das citocinas circulantes, como TNF-α, IL-1, IL-6 e IFN-γ (ARGILES *et al.*, 2009; FEARON *et al.*, 2011). Deste modo,

modelos de estudo que simulam esta condição são aplicados para entender as neoplasias e seus tratamentos.

1.3 Tumor Walker-256: Um modelo de neoplasia

Esta linhagem tumoral tem sido amplamente utilizada em estudos antineoplásicos e de caquexia induzida por tumor, por ser espécie-específica para ratos e facilmente transplantada, além do que, as falhas de inoculação e regressão espontânea são pouco frequentes. (GUAITANI *et al*., 1982; FERNANDES *et al*., 1990). Após a inoculação subcutânea das células Walker-256, o tumor torna-se palpável em aproximadamente quatro a cinco dias, e a partir do 14º dia os animais podem apresentar uma redução no peso corporal, anorexia e alterações metabólicas e eletrolíticas. A massa tumoral formada após esse período pode representar uma fração considerável do peso do animal, e a mortalidade pode ocorrer na maioria dos animais após o 15º da inoculação (VICENTINO *et al*., 2002).

O tumor Walker-256, caracterizado como carcinossarcoma de crescimento rápido, foi identificado pela primeira vez em 1928, por George Walker, nas glândulas mamárias de uma rata gestante (EARLE, 1935). Em 1967, Agostino e Cliffton descreveram a passagem do tumor da forma sólida para a ascítica. Assim, as células tumorais na forma ascítica poderiam, novamente, ser injetadas intraperitonealmente em outros animais e estas mesmas células poderiam, ainda, ser injetadas em diferentes órgãos e tecidos, sempre com o desenvolvimento do tumor sólido (CALDAROLA *et al*., 1968). Dessa forma, o tumor Walker-256 pode se desenvolver tanto na forma sólida quanto ascítica, além de ter a capacidade de disseminação, por via linfática ou hematogênica, dependendo da via de inoculação (CARR *et al*., 1980).

Os mecanismos celulares envolvidos no desenvolvimento do tumor Walker-256 estão relacionados com o aumento na expressão de TNF-α, IL-6, IL-1 e TGF-β1 (VIDO *et al*., 2000; FOLADOR *et al*., 2009). Ainda, alterações decorrentes da formação tumoral estão relacionadas aos processos de metabolismo hepático de lipídeos, aminoácidos e glicose, assim como à

indução de estresse oxidativo e modificações na resposta inflamatória e imunológica (ACCO; BASTOS-PEREIRA; DREIFUSS, 2012). Uma visão geral das principais alterações provocadas pela inoculação das células Walker-256 está esquematizada na figura 1, e um exemplo deste tumor sólido removido de rato está representado na figura 2.

Frente a essas alterações, o tumor Walker-256 tornou-se um modelo bem estabelecido e adequado para os estudos relacionados com a fisiopatologia de tumores sólidos, anorexia e caquexia, bem como para a investigação de novas opções farmacológicas para o tratamento antineoplásico.

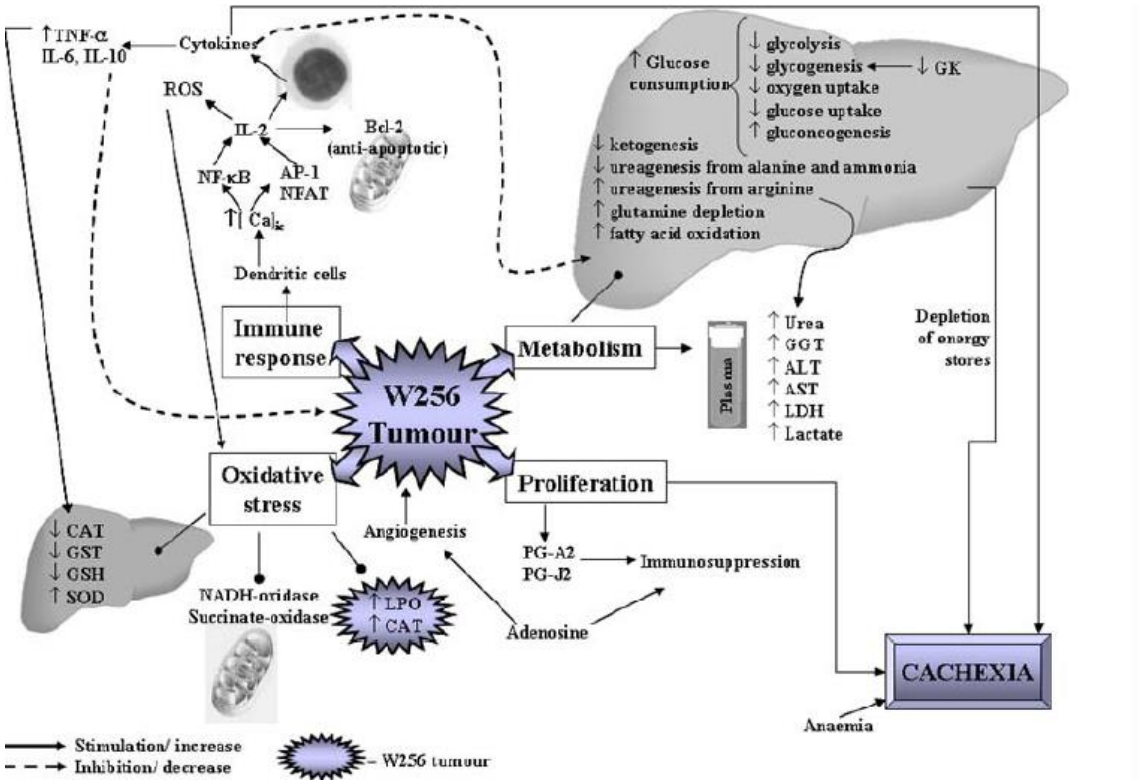

Figura 1. Principais alterações metabólicas e inflamatórias causadas pela inoculação do tumor Walker-256 em ratos, incluindo alterações imunológicas, de proliferação e relacionadas a estresse oxidativo. (ACCO; BASTOS-PEREIRA; DREIFUSS, 2012).

Figura 2. Tumor Walker-256, após 14 dias da inoculação das células tumorais por via subcutânea no membro pélvico de rato Wistar para formação de tumor sólido. Fonte: MARTINS, 2014.

1.4 A pesquisa de novas moléculas de origem vegetal com atividade antineoplásica

Apesar dos avanços científicos no tratamento do câncer, a terapia antineoplásica existente ainda apresenta sérias dificuldades por problemas de resistência, baixa seletividade e toxicidade (HEIDI *et al*., 2009). Frente a isso, novos medicamentos são necessários para tentar contornar esses problemas.

A utilização de plantas com fins medicinais, para tratamento, cura e prevenção de doenças, é uma das mais antigas formas de prática medicinal da humanidade. No mundo ocidental, assistiu-se a um crescente interesse pelo uso das plantas medicinais e dos respectivos extratos na terapêutica, constituindo, em certas circunstâncias, uma ajuda nos cuidados primários de saúde e um complemento terapêutico, compatível com a medicina convencional. A grande diversidade química das plantas faz delas a fonte de escolha para o isolamento de metabólitos farmacologicamente relevantes (BASSO *et al*., 2005). Segundo a Organização Mundial da Saúde (OMS), aproximadamente 25% dos medicamentos utilizados são derivados, direta ou indiretamente de plantas medicinais. No caso de certas classes de produtos farmacêuticos, como medicamentos antitumorais e antimicrobianos, essa porcentagem pode ser maior que 60% (WHO, 2011). Os compostos derivados de plantas têm sido uma fonte de moléculas clinicamente úteis no tratamento do câncer, atuando por diversos mecanismos (CRAGG; NEWMAN, 2005). Muitas moléculas vegetais, seus derivados semi-sintéticos ou seus modelos

moleculares sintetizados são fontes importantes de medicamentos antitumorais, como os alcaloides da vinca – vimblastina, vincristrina e vinorelbina de *Catharanthus roseus;* epipodofilotoxinas – etopósido e tenipósido de *Podophyllum peltatum;* análagos da camptotecina – irinotecana e topotecana de *Camptotheca acuminata;* taxanos – paclitaxel e docetaxel de *Taxus brevifolia*. Estas substâncias de ocorrência natural exercem efeitos citotóxicos potentes e, por isso, conquistaram espaço no arsenal de fármacos antineoplásicos (RANG *et al*., 2007; CHABNER *et al*., 2012).

Nesse contexto, o ramo da pesquisa de moléculas vegetais com propriedades antitumorais ganhou maior dimensão nos últimos anos, sendo uma área de pesquisa de grande interesse na atualidade, com muitos estudos experimentais sendo conduzidos com intuito de obter novas opções terapêuticas para diferentes tipos de cânceres.

1.5 *Gochnatia polymorpha* ssp. *floccosa*

O gênero *Gochnatia* (ou *Moquiniastrum*) pertence à família Asteraceae, que consiste na maior família na filogenia das Angiospermas. As plantas dessa família são utilizadas para uma série de finalidades medicinais, sendo extensivamente pesquisadas quanto a sua composição química e atividade biológica. O gênero *Gochnatia* compreende cerca de 70 espécies, sendo a maioria encontrada no continente americano (México a Argentina) e apenas duas ocorrendo em países asiáticos (FREIRE; KATINAS; SANCHO, 2002). No Brasil são encontradas diversas espécies desse gênero, como a *Gochnatia polymorpha*, presente nos Estados da Bahia, Espírito Santo, Goiás, Minas Gerais, Mato Grosso do Sul, Distrito Federal, São Paulo, Rio de Janeiro, Paraná, Santa Catarina e Rio Grande do Sul.

G. polymorpha (Less) Cabrera (conhecida inicialmente como *Moquinea polymorpha* (Less) DC), é uma árvore de médio porte (figura 3), conhecida no Brasil como cambará, cambará-da-folha-grande ou cambará-do-mato. Na medicina popular, suas folhas, flores e cascas do tronco são utilizadas no preparo de chás para o tratamento de resfriados, infecções e processos inflamatórios no trato respiratório (MORS; RIZZINI; PEREIRA, 2000; BUENO *et*

al., 2005). Vários compostos foram relatados nessa espécie a partir de estudos fitoquímicos, como lactonas sesquiterpênicas, guaianolideos diméricos, diterpenos, triterpenos, flavonóides e cumarinas (BOHMANN *et al*., 1986; SACILOTTO; VICHNEWSKI; HERZ, 1997; MOREIRA *et al*., 2000; CATALAN *et al*., 2003; PIORNEDO *et al*., 2011; SILVA *et al.*, 2011). Atividades anti-inflamatórias e anti-espasmódica foram demonstrados com as folhas e casca do tronco da *G. polymorpha* (MOREIRA *et al*., 2000; PIORNEDO *et al.*, 2011; SCHLEMPER; FREITAS; SCHLEMPER, 2011).

A *G. polymorpha* possui três subespécies: *polymorpha, ceanothifolia* e *floccosa,* sendo esta última, exclusiva do Brasil, amplamente dispersa no estado do Paraná (CATALAN *et al.*, 2003). O extrato e frações de várias partes de *G. polymorpha* ssp. *floccosa* foram avaliados quanto a uma possível atividade antimicrobiana, anti-inflamatória e citotóxica. No estudo de PIORNEDO *et al.* (2011) foi relatada a atividade anti-inflamatória de frações isoladas dessa subespécie, na qual um dos compostos isolados foi identificado como lactona sesquiterpênica – 11, 13-diidrozaluzanina C, responsável pelo menos em parte, por essa ação. Em um recente estudo de STRAPASSON *et al.* (2012) foi demonstrada atividade citotóxica *in vitro* de compostos isolados da casca do tronco de *G. polymorpha* ssp. *floccosa* em linhagens cancerígenas humanas. Os compostos mais ativos foram lactonas diméricas, chamadas 10-desoxigochnatiolideo A e gochnatiolideo A, que inibiram o crescimento de linhagens tumorais de rins, melanoma, ovário e glioma, em uma concentração variando de 0.21 a 1.09 $\mu g.mL^{-1}$. Os estudos de citotoxidade a partir de frações isoladas da casca do tronco de *G. polymorpha* ssp. *floccosa,* demonstram que as lactonas sesquiterpênicas são substâncias fundamentais para promover o efeito citotóxico *in vitro*, o que corrobora com diversos trabalhos que relatam a capacidade antitumoral dessas substâncias através da inibição das respostas inflamatórias, prevenção de metástases e indução de apoptose (ZHANG *et al.*, 2005).

Apesar dos efeitos biológicos relatados a partir da espécie *G. polymorpha* ssp. *floccosa,* ainda são poucos os estudos que demonstram outras atividades biológicas e o potencial terapêutico dos compostos presentes nessa espécie. Além disso, devido sua importante citotoxicidade relatada *in vitro,* em células tumorais humanas, torna-se necessário um estudo para

avaliar a toxicidade das frações e/ou compostos isolados, assim como seu possível efeito em modelos tumorais *in vivo*, ambos aspectos abordados neste trabalho.

Figura 3. *Gochnatia polymorpha,* conhecida popularmente como cambará, cambará-da-folha-grande ou cambará-do-mato. Fonte: http://www.trilhadafloresta.ufpr.br

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a possível atividade antineoplásica e toxicidade da fração diclorometano (F1B), rica em lactonas sesquiterpênicas do subtipo guaionolideos, isolada da *Gochnatia polymorpha* ssp. *flocossa* frente ao modelo de carcinossarcoma Walker-256 em ratos.

2.2 Objetivos específicos:

- Caracterizar e identificar os compostos presentes na fração F1B, isolada a partir da casca do tronco da *Gochnatia polymorpha* ssp. *flocossa;*

- Avaliar o desenvolvimento tumoral em ratos inoculados com as células Walker-256*,* mediante análise da progressão tumoral (volume e peso), bem como a porcentagem de supressão tumoral ao final do tratamento com a fração F1B*;*

- Investigar a natureza do estresse oxidativo e suas implicações no desenvolvimento tumoral e no metabolismo hepático, através de medidas de peroxidação lipídica e glutationa reduzida, e atividades enzimáticas da glutationa-S-transferase, superóxido dismutase e catalase, diante do tratamento com a fração F1B;

- Investigar parâmetros inflamatórios no tecido tumoral através das dosagens das enzimas mieloperoxidase e N-acetilglucosaminidase, assim como verificar os níveis de óxido nítrico, e avaliar o envolvimento do mediador inflamatório TNF-α frente ao tratamento com a fração F1B da *G. polymorpha* ssp. *floccosa*;

- Avaliar parâmetros histológicos no tecido tumoral e investigar o efeito do tratamento com a fração F1B nas células tumorais Walker-256;

- Avaliar a toxicidade da fração F1B, mediante análise de parâmetros hepáticos, renais e pancreáticos, através da bioquímica plasmática; assim como avaliação histológica hepática e verificação do peso relativo (%) de órgãos (fígado, rins, pulmão e baço);
- Determinar a DL_{50} da fração F1B em camundongos.

3. ARTIGO CIENTÍFICO

Sesquiterpene lactones of *Gochnatia polymorpha* ssp. *floccosa* have antineoplastic effects in Walker-256 tumor-bearing rats

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Abstract

Ethnopharmacological relevance: Gochnatia polymorpha ssp. *floccosa* (Asteraceae), known as "Cambará," has been used in folk medicine for the treatment of respiratory diseases, and previous work has shown it is cytotoxic *in vitro* in human cancer cell lines. This study aimed to evaluate the *in vivo* antitumor actions and toxicity of the dichloromethane fraction (F1B) of *G. polymorpha* ssp. *floccosa*, composed of sesquiterpene lactones, against Walker-256 carcinosarcoma in rats.

Material and methods: Male Wistar rats received 100 $mg \cdot kg^{-1}$ F1B per day orally for 16 days after subcutaneous inoculation of Walker-256 cells in the pelvic limb. The tumor progression was monitored, and after treatment, tumor weight, oxidative stress, plasma biochemistry, inflammatory parameters, and histology of tumor and/or liver were evaluated. The toxicity of F1B was analyzed through the relative weight of organs. Additionally, an LD_{50} test was performed in mice.

Results: F1B treatment significantly reduced tumor volume and weight. There was no difference in oxidative stress in tumors after treatment. F1B treatment modified hepatic glutathione and superoxide dismutase, and normalized plasma glucose, alkaline phosphatase, and amylase. F1B did not affect the activity of myeloperoxidase and *N*-acetylglucosaminidase or the nitric oxide levels in tumor tissue. However, F1B decreased the tumor necrosis factor (TNF)-α levels. Additionally, F1B increased apoptosis in the tumor. No clinical signs of toxicity or death were observed in the rats treated with F1B. The LD_{50} calculated for mice was 1209 $mg \cdot kg^{-1}$.

Conclusions: F1B, which is rich in sesquiterpene lactones, showed antitumor activity against Walker-256 carcinosarcoma. This effect may be, at least in part, related to the induction of apoptosis and inhibition of TNF-α signaling.

Keywords: *Gochnatia polymorpha* ssp. *floccosa*, cambará, F1B fraction, Walker-256 cancer, oxidative stress, acute toxicity *(LD_{50})*.

1. Introduction

In recent decades, cancer has become a public health problem worldwide. Cancer, or malignant neoplasm, is characterized by uncontrolled proliferation and spread of abnormal cells, caused by alterations in oncogenes, tumor-suppressor genes, and microRNA genes (Croce, 2008). The malignant characteristics of these cells results in dedifferentiation, increased cell invasion, and metastasis (Kumar et al., 2010). There are three main approaches to treating cancer: surgical excision, irradiation, and chemotherapy. The relative value of each of these approaches depends on the type and stage of the tumor. Importantly, chemotherapy may be used on its own or as an adjuvant to other forms of therapy (Chabner and Roberts, 2005).

The compounds used in neoplastic chemotherapy exhibit marked differences in their structure and mechanism of action, including alkylating agents, antimetabolites, natural products, hormones, and hormone antagonists, in addition to a variety of agents directed at specific molecular targets (Chabner, 2012). Despite the broad variety of antineoplastic drugs currently available, an intense search continues for novel treatment options at the molecular level, including agents that can act on specific signaling pathways, inhibit proliferation and angiogenesis, and promote cell death in tumors. However, despite the importance of discovering novel compounds that act on specific molecular targets, the ability of these drugs to be used in combination with other cytotoxic agents is essential, as this will produce a more beneficial response in patients (Chabner, 2012; Romond et al., 2005; Slamon et al., 2001).

Recently, there has been increasing focus on identifying natural products with antitumor activity. Among these, the guaianolide subtype of sesquiterpene lactones (SLs) have garnered interest, because they have displayed cytotoxic activity against several human tumor cell lines *in vitro* and antitumor activity *in vivo* (Kreuger et al., 2012). SLs may inhibit cancer progression through the inhibition of inflammatory responses, prevention of metastasis and induction of apoptosis (Zhang et al., 2005).

SLs are abundant in *Gochnatia*, a genus of 70 species that is grown from Mexico to Argentina (Katinas et al., 2008). Specifically, *Gochnatia polymorpha*, also known as “Cambará,” is a medium sized tree found in several Brazilian States, as well as

Paraguay, Uruguay, and Argentina. Previous chemical studies have reported that this species contains SLs, dimeric guaianolides, diterpenes, triterpenes, flavonoids, and coumarins (Bohmann et al., 1986; Catalan et al., 2003; Moreira et al., 2000; Piornedo et al., 2011; Silva et al., 2011). The leaves and trunk of *G. polymorpha* have also been shown to have anti-inflammatory and antispasmodic activities (Piornedo et al., 2011; Schlemper et al., 2011). In a recent study by Strapasson et al. (2012), the *in vitro* cytotoxic activity of compounds isolated from the trunk bark of *G. polymorpha* ssp. *floccosa* was demonstrated in human cancer cell lines. The most active compounds, the dimeric lactones 10-desoxygochnatiolide A and gochnatiolide A, inhibited the growth of kidney, skin (melanoma), ovarian and brain (glioma) tumor cell lines at low concentrations (0.21 to 1.09 $\mu g \cdot mL^{-1}$). However, the antineoplastic effects of *G. polymorpha* were not evaluated *in vivo*.

The aim of this study is to evaluate the antineoplastic effect of a fraction obtained from the trunk bark of *Gochnatia polymorpha* ssp. *floccosa*, namely F1B, in rats bearing Walker-256 tumor, a model of solid cancer. To our knowledge, this is the first report of *in vivo* antitumor activity and toxicity evaluation of F1B obtained from this plant.

2. Materials and Methods

2.1 *Plant material*

Gochnatia polymorpha (Less) Cabr. ssp. *floccosa* Cabr. trunk bark was collected in June 2013, in Curitiba, Paraná State, Brazil, and identified by Dr. Armando C. Cervi. A voucher specimen (UPCB 30100) was deposited in the herbarium of the Federal University of Paraná (UFPR), Brazil. F1B of *G. polymorpha* ssp. floccosa was kindly prepared and analyzed by Dr. Maria Élida Stefanello (Department of Chemistry, UFPR). The plant nomenclature was checked with theplantilist.org.

2.2 *Extraction, isolation and chemical analyses*

The extraction and isolation of compounds were carried out as previously reported (Strapasson et al., 2012). Briefly, dried and powdered trunk bark (1.6 kg) was extracted at room temperature with hexane, followed by 95% ethanol (EtOH). The

EtOH extract (65.2 g) was partitioned with dichloromethane, ethyl acetate (EtOAc), and 1-butanol. The dichloromethane fraction (F1, 19.2 g) underwent silica gel vacuum liquid chromatography (VLC), followed by elution with hexane, dichloromethane, EtOAc, and methanol. The fraction eluted with dichloromethane (F1B, 3.6 g) was used in biological assays. The major compounds of this fraction were isolated by successive chromatographic fractionation on silica gel, as previously described (Strapasson et al., 2012). Compounds 1-6 were purified by semi-preparative HPLC and the pure compounds were used to identify the peaks in the HPLC fingerprint of F1B (Figure 1; **1** retention time (R_t) 12.1 min; **2** R_t 18.5 min; **3** R_t 20.1 min; **4** R_t 22.3 min; **5** R_t 24.1 min; **6** R_t 28.6 min).

2.3 *DPPH radical scavenging activity*

The reactivity of F1B with the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described by Chen et al. (1999). DPPH in methanol (250 µL of a 1 mg in 25 mL solution) was combined with the F1B solution (750 µL) in increasing concentrations (1-1000 $\mu g \cdot mL^{-1}$). Changes in absorbance were measured over 1 min. Ascorbic acid (50 $\mu g \cdot mL^{-1}$), a free radical scavenger, was used as positive control, and distilled water was used as a negative control.

2.4 *Animals*

Male Wistar rats weighing 200–250 g were housed in a temperature controlled facility (22 ± 1°C), with a constant 12-h light-dark cycles and free access to standard laboratory food (Nuvital®, Colombo, PR, Brazil) and tap water. All the experimental protocols were approved by the Ethical Committee for Animal Use (CEUA) of Biological Sciences Section of UFPR (authorization number 671).

2.5 *Tumor cells and experimental tumor inoculation*

The maintenance of the Walker-256 cells was performed by weekly intraperitoneal (i.p.) inoculation passages of 10^7 cells/rat, followed by freezing cells at -80 °C (Vicentino et al., 2002). After five to seven days, the ascitic fluid was collected in a solution of ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0, 1:1) and suspended in 1.0 mL of PBS (16.5 mM phosphate, 137 mM NaCl and 2.7 mM KCl).

For implantation in animals, tumor cells were injected subcutaneously (10^7 cells/animal) in the right pelvic limb after verifying viability by the Trypan blue exclusion method in a Neubauer chamber. Tumor cells were kindly donated by Dr. Sandra Coccuzzo Sampaio of Butantã Institute (São Paulo, Brazil).

2.6 *Experimental design*

Rats were treated via oral gavage once a day, for sixteen consecutive days. Treatment began one day following subcutaneous inoculation of tumor cells. The animals were separated in four different groups. The basal group (n = 7), which were not inoculated with tumor cells, and tumor group (n = 10) received vehicle (distilled water containing 2% Tween 80). The basal+F1B group (n = 7) and tumor+F1B (n = 7) groups received 100 $mg \cdot kg^{-1}$ of F1B. The F1B dose was based on previous (unpublished) data from our laboratory, and work from Piornedo et al. (2011). During treatment, animals were weighed, and clinical signs of systemic toxicity were observed. Tumor progression was monitored during the treatment period, and tumor volume was calculated by measuring the tumor diameters, as described by Mizuno et al. (1999), using the formula: $V\ (cm^3) = 4\pi/3 \cdot a^2 \cdot (b/2)$, where *a* is the smallest diameter and *b* is the greater diameter, in cm. The inhibitory effect on the tumor was calculated using the following formula: *Tumor Suppression (%) = (1-T/C)* where *T* is the average volume of the tested group and *C* is the average volume of the control group.

2.7 *Sample collection*

Following F1B treatment, all animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 $mg \cdot kg^{-1}$) and xylazine hydrochoride (10 $mg \cdot kg^{-1}$). Blood samples were obtained from the inferior vena cava for biochemical assays. Liver, kidneys, spleen, lungs, and tumors were removed and weighed in an analytical scale. The weight of each organ was multiplied by 100 and divided by the weight of the animal to obtain the relative organ weight (%). Tumor and liver samples were either immediately stored at −80°C for further analysis, or stored in 4% buffered formalin for histological analysis.

2.8 *Oxidative stress parameters*

For the evaluation of oxidative stress, liver and tumor samples were homogenized in phosphate buffer (pH 6.5), at a 1:10 dilution, and centrifuged at 10,000 ×*g* for 20 min at 4°C. The activity of catalase (Cat), superoxide dismutase (SOD), and glutathione-*S*-transferase (GST) was measured in the supernatant. The rate of lipid peroxidation (LPO) and reduced glutathione (GSH) were also assessed. Results were expressed by the amount of proteins present in homogenates. Protein concentration was determined using the method designed by Bradford (1976).

2.8.1 *Determination of enzyme activity*

Catalase: Cat activity was measured using spectrophotometry, following the addition of exogenous hydrogen peroxide to generate oxygen and water (Aebi, 1984). The reaction was performed in a microplate reader at 240 nm, and enzyme levels were expressed as $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$.

Superoxide-dismutase: The analysis was performed according to the method described by Gao et al. (1998), which is based on the ability of SOD in the tissue (liver or tumor) to inhibit the autoxidation of pyrogallol reagent. Enzyme activity was assessed using a microplate reader at 440 nm, and enzyme activity was expressed as SOD units per milligram of total protein ($U\ SOD \cdot mg \cdot protein^{-1}$).

Glutathione-S-transferase: GST activity was assessed based on the method described by Habig et al. (1974), which analyzes the ability of the enzyme to conjugate the substrate 2,4-dinitrochlorobenzene (DNCB) with glutathione in its reduced form. This results in the formation of a thioether that can be measured by absorbance at 340 nm. Enzyme levels were expressed as $mmol \cdot minute^{-1} \cdot mg\ of\ protein^{-1}$.

2.8.2 *Determination of the lipid peroxidation rate*

The rate of lipid peroxidation was evaluated by ferrous oxidation of xylenol orange (FOX), following a described protocol (Jiang et al., 1991). This technique quantifies the formation of hydroperoxides in the lipid phase during lipid peroxidation. Tumor and liver samples were homogenized in methanol at a ratio of 1:5, and centrifuged at 5000 ×*g* for 5 min at 4°C. The samples were homogenized and incubated for 30 min. Thereafter the FOX-2 reagent was added. The samples were incubated for

30 min at room temperature and then read in a spectrophotometer at 560 nm. The results were expressed in mmol·mg·protein^{-1}.

2.8.3 *Determination of the reduced glutathione levels*

Reduced glutathione (GSH) levels were measured as previously described by Sedlak and Lindsay (1968). Liver and tumor samples were homogenized in phosphate buffer (pH 6.5) at a 1:10 dilution. Subsequently, 250 µL of homogenate were mixed with trichloroacetic acid (200 µL, purity 12.5%). The supernatant was separated by centrifugation at 13,750 ×*g* for 10 min at 4°C. Then, 30 µL of the clear supernatant was mixed with 270 µL of phosphate buffer (0.1 M, pH 8.5) and 5 µL of 5,5'-dithiobis-(2-nitrobenzoic acid) in methanol. Solution absorbance was measured at 415 nm in a microplate reader, using reduced glutathione as external standard.

2.9 *Plasma biochemical assays*

Plasma was obtained after centrifugation of blood at 3000 ×*g* for 10 min. These samples were used for the determination of urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, total serum protein, albumin, amylase, and glucose. These parameters were analyzed by an automatized system (Mindray BS-200), according to the kit manufacturer's instructions (Labtest Diagnóstica™, Lagoa Santa, MG, Brazil).

2.10 *Inflammatory parameters in tumor*

2.10.1 *Tissue extraction and determination of myeloperoxidase and N-acetylglucosaminidase activities*

In order to determine the amount of leucocyte infiltration, tumor samples were weighed, homogenized in 80 mM sodium phosphate buffer (pH 4.7) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The homogenates were centrifuged at 12,000 ×*g* at 4^{o} C for 15 min.

The amount of neutrophils in tumor samples was measured by assaying myeloperoxidase (MPO) activity following the method described by Bradley et al. (1982). MPO activity in the supernatant samples was assayed by measuring the

changes in absorbance (optical density; OD) at 630 nm using tetramethylbenzidine (1.6 mM dissolved in N,N-dimethylformamide) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 30 µL sodium acetate (1.46 M). Results were expressed as the variation in OD.mg of wet tissue^{-1}.

The infiltration of mononuclear cells into the tumor was quantified by measuring the levels of the lysosomal enzyme N-acetylglucosaminidase (NAG) present in high levels in activated macrophages (Sanchez and Moreno, 1999). Samples (100 µL) of the resulting supernatant were incubated for 60 min with 100 µL of p-nitrophenyl-N-acetyl-beta-d-glucosaminide (Sigma®) prepared in citrate/phosphate buffer (0.1 M citric acid, 0.1 M Na_2HPO_4; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of 100 µL of 0.2 M glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 405 nm. Results were expressed as mmol.mg wet tissue^{-1}.

2.10.2 *Measurement of nitric oxide production*

Nitric oxide (NO) is recognized as a mediator and regulator of inflammatory responses (Korhonen et al., 2005), so NO release from tumors was measured by assessing nitrite levels, according to the method described by Green et al. (1982). Tumor samples were incubated for 15 min at 37^{o} C with PBS (500 µL). The incubation medium (100 µL) was mixed with 100 µL of Griess reagent (0.1% *N*-1-naphthylediamine, 1% sulfanilamide in 5% H_3PO_4), and OD was measured at 540 nm. The amount of nitrite in the incubation media was calculated using sodium nitrite (Sigma®) as the standard.

2.10.3 *Measurement of tumor necrosis factor-alpha (TNF-α) level*

Tumors were homogenized in PBS (2 mL/200 mg of the tissue, pH 7.4) and centrifuged at 10,000 *xg* for 40 min. TNF-α was measured in the supernatant (100 µL) using an Immunoassay Kits (R&D Systems®, USA), following the manufacturer's protocol. A 96 well plate was coated with 100 µL of purified anti-mouse TNF-α antibody (1 µg·mL^{-1}) and incubated overnight at 4°C. The following day, a recombinant murine TNF-α standard (3.89–1000 pg·mL^{-1}) and samples were added to the wells, and the plate was incubated overnight at 4°C. Biotinylated anti-mouse TNF-α (100 µL, 50 ng·mL^{-1}) was added to each well. The plates were then incubated for 2 h at room

temperature. Following the removal of the unbound antibody-enzyme reagent, 100 µL of streptavidin–horseradish peroxidase (HRP) solution was added to the wells and incubated for 20 min. Finally, 100 µL of substrate solution containing hydrogen peroxide and *o*-phenylenediamine (OPD; Sigma-Aldrich®) was added to the wells. The enzyme reaction yields a yellow product that turns orange in the presence of 50 µL of the stop solution (H_2SO_4, 1 M), which was added to each well 20 min after starting the reaction. The optical density was determined using a microplate reader at 450 nm. The results were expressed as pg·mg wet tissue^{-1}.

2.11 *Histopathology*

Fragments from the tumors and liver tissue from all experimental animals were fixed in buffered 10% formalin at room temperature. After fixed, samples were dehydrated in ethanol, cleared with xylene and embedded in paraffin. Thereafter, thin sections (4 mm) were processed for histology. Tissues were stained with hematoxylin and eosin, and the resulting slides were submitted for blinded analysis by optic microscopy. The livers were checked for lymphocytic and lymphohistiocytic infiltrates, with or without necrosis. In the tumor tissue the parameters evaluated were: coagulative and suppurative necrosis, apoptosis, tumor infiltration, vascularization plates, vacuolization, and cytological characteristics. The histological alterations were classified by intensity scores: mild (+), moderate (++) and pronounced (+++).

2.12 *Acute toxicity (LD_{50})*

To complement the observation of toxicity in rats treated with F1B, its acute toxicity was accessed through an LD_{50} test in mice, as this value was unknown. Male Swiss mice (26–35 g) were separated in groups (n = 6), and received doses of 50, 500, 1000 and 5000 mg·kg^{-1} of F1B by oral gavage, while one group received an intraperitoneal dose (1000 mg·kg^{-1}) of F1B. These animals were compared with the control group, which received 2% tween 80 solution (vehicle). The animals were monitored for the first 30 min post-administration, and 1, 2, 3, and 4 h after treatment. Behavioral parameters and clinical symptoms observed were recorded according to the methodology described by Almeida et al. (1999). After the first 4 h of

observation, the animals received water and food, and were observed daily for 14 days to record possible alterations or deaths. After this period, the animals were anaesthetized with isoflurane, and liver, kidneys, adrenals, spleen, and lungs of the animals were evaluated macroscopically. The calculation of LD_{50} was performed by the statistical method of Litchfield and Wilcoxon (1949) through the DPCMedLab program.

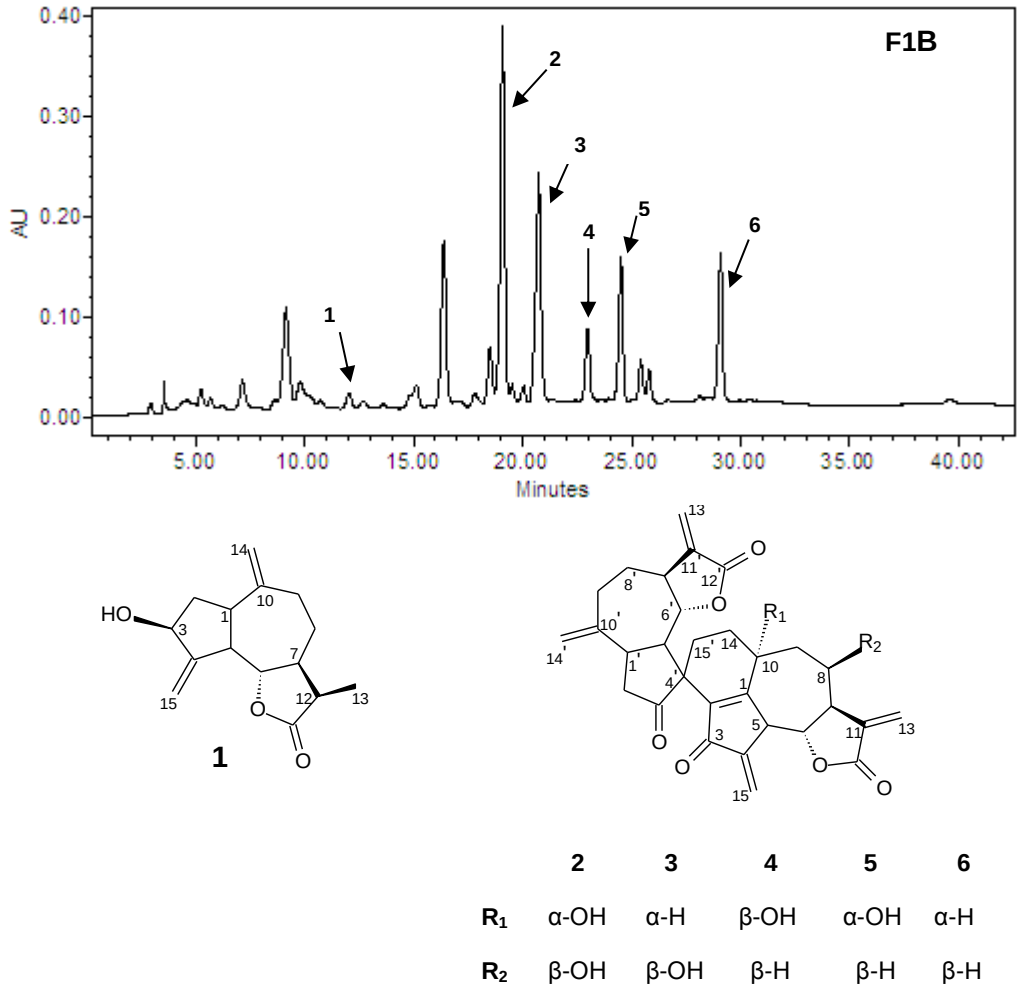
2.13 *Statistical analysis*

The results were expressed as mean ± standard error of the mean (SEM). All data analyzed were submitted to normality testing using the Kolmogorov-Smirnov test. The comparison between two groups was performed using Student's t-test for unpaired data. When three or more groups were compared, we used analysis of variance (ANOVA) followed by Bonferroni test to detect differences between groups. Results were considered significant when $p<0.05$. The program GraphPad Prism® version 5.0 was used for statistical analysis and graphic construction.

3. Results

3.1 *Bio-guided isolation of compounds from G. polymorpha trunk bark*

Isolated compounds from the F1B fraction were identified by analyses of NMR spectra and comparison with literature data (Li et al., 2012; Strapasson et al., 2012). In this way, we identified the guaianolide 11αH-13-dihydrozaluzanin C (1), the dimeric guaianolides 8-hydroxigochnatiolide A (2), 8-hydroxi-10-desoxigochnatiolide A (8-hydroxigochnatiolide C (3), gochnatiolide B (4), gochnatiolide A (5) and, 10-desoxygochnatiolide A (gochnatiolide C, 6). All compounds have previously been reported in *G. polymorpha* (Bohmann et al., 1986; Strapasson et al., 2012).



	2	3	4	5	6
R_1	α-OH	α-H	β-OH	α-OH	α-H
R_2	β-OH	β-OH	β-H	β-H	β-H

Figure 1. Typical HPLC chromatogram of F1B (211 nm) and structures of the isolated compounds F1B. Legend: 11αH-13-dihydrozaluzanin C (1), dimeric guaianolides 8-hydroxigochnatiolide A (2), 8-hydroxi-10-desoxigochnatiolide A (8-hydroxigochnatiolide C, [3]), gochnatiolide B (4), gochnatiolide A (5) and, 10-desoxygochnatiolide A (gochnatiolide C, [6]).

3.2 *DPPH radical scavenging activity of the F1B*

The *in vitro* antioxidant activity of the F1B fraction was evaluated using the free radical DPPH. The F1B fraction displayed antioxidant activity at a concentration of 30 $\mu g \cdot mL^{-1}$, which was sustained up to 1000 $\mu g \cdot mL^{-1}$. These results are shown in Figure 2.

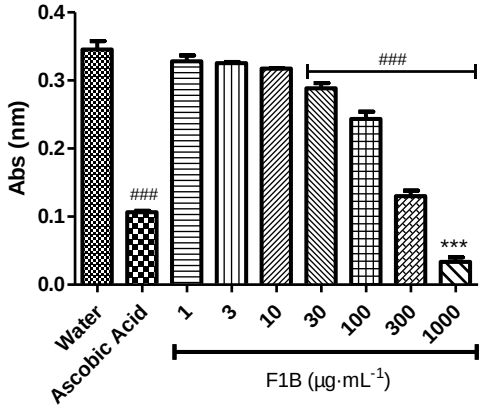


Figure 2. *In vitro* evaluation of free radical scavenging activity of F1B in the range between 1 and 1000 µg·mL^{-1}. Ascorbic acid (50 µg·mL^{-1}) was used as positive control and water as negative control. Symbols: $^{***}p$<0.001 as compared to the ascorbic acid; $^{###}p$<0.001 as compared to the water. Differences between the groups were analyzed by one-way ANOVA followed by Bonferroni test.

3.3 *F1B treatment reduced tumor growth and tumor weight in rats*

Five days after Walker-256 cell inoculation, tumors were palpable and their volumes were measured daily. Treatment with the F1B fraction reduced tumor volume significantly, as compared to the tumor group (17.16 ± 9.58 cm^3 and 64.68 ± 17.02 cm^3, respectively), resulting in a 74% tumor suppression at the end of treatment (Fig. 3A). The tumor weight was reduced 69% after treatment with F1B compared with the tumor group (4.92 ± 2.64 g and 16.52 ± 4.06 g, respectively) (Fig. 3B).

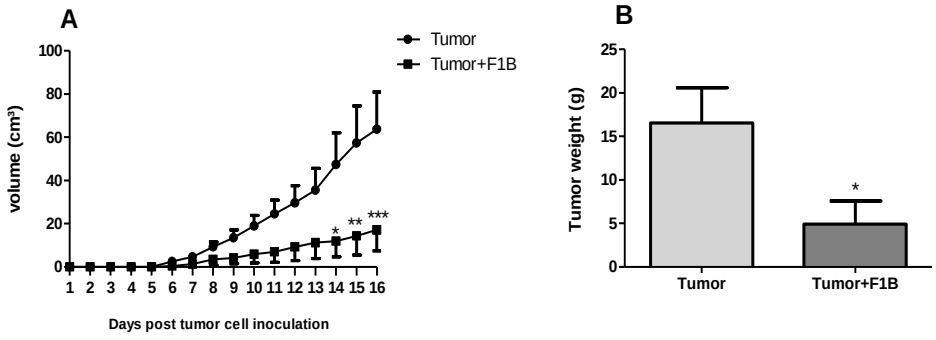


Figure 3. Tumor volume (A) and weight (B) in Walker-256 tumor-bearing rats treated with F1B (Tumor+F1B) or vehicle (Tumor) for 16 days. Each bar represents the mean ± SEM of 7 – 10 rats. Symbols: $^{*}p$<0.05; $^{**}p$< 0.01 and $^{***}p$<0.001 as compared to the tumor group. Two-way ANOVA followed by Bonferroni test (A) and Student t test (B) were used for statistical analysis.

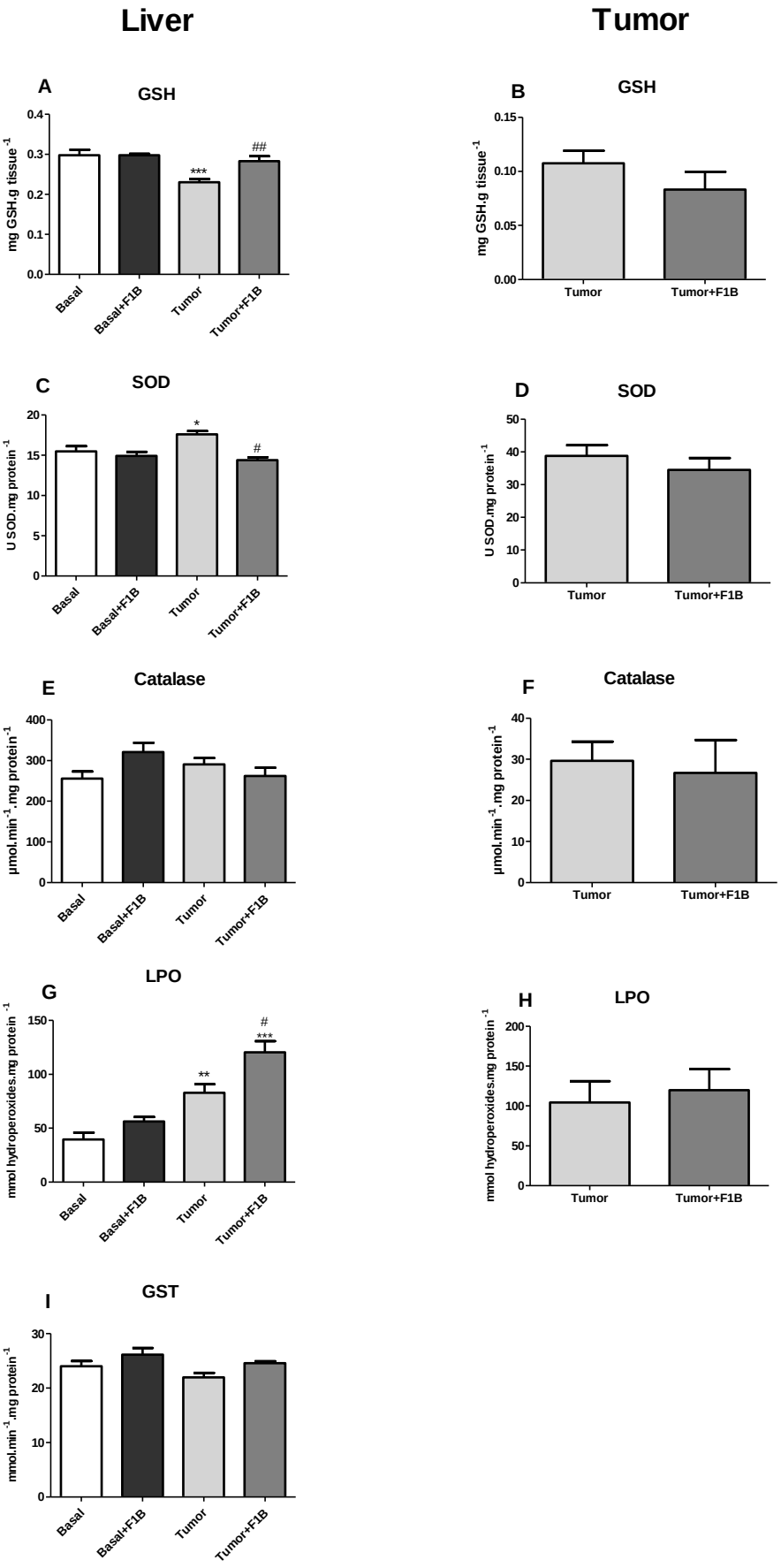
3.4 *Oxidative stress parameters*

3.4.1 *Hepatic oxidative stress*

As tumor growth can enhance reactive oxygen species in the whole body, we next evaluated oxidative stress parameters in the liver, the organ responsible for metabolism and detoxification. The hepatic tissue of the tumor group had GSH levels of 0.23 ± 0.01 mg GSH·g tissue^{-1}, which represents a significant decrease when compared to basal group (0.29 ± 0.01 mg GSH·g tissue^{-1}). In contrast, treatment with F1B increased the GSH levels to 0.28 ± 0.01 mg GSH·g tissue^{-1}, constituting a full reestablishment to basal levels (Fig. 4A). Hepatic SOD activity in the tumor group was 17.58 ± 0.4 U SOD·mg protein^{-1}; however, treatment with F1B reduced these values to basal levels (14.40 ± 0.31 U SOD·mg protein^{-1} and 15.46 ± 0.65 U SOD·mg protein^{-1}, respectively) (Fig. 4C). There was no statistical difference in Cat activity between the groups (Fig. 4E). However, there was an increase in hepatic LPO levels in the tumor+F1B group (120.5 ± 10.19 mmol hydroperoxides·mg protein^{-1}) when compared with tumor group (82.87 ± 7.77 mmol hydroperoxides·mg protein^{-1}).Interestingly, the hepatic LPO levels of the tumor group were markedly increased when compared to basal group (39.68 ± 6.43 mmol hydroperoxides·mg protein^{-1}) (Fig. 4G). No statistically significant differences were found in GST activity between the experimental groups (tumor and tumor+F1B group) (Fig. 4I).

3.4.2 *Tumor oxidative stress*

In tumor tissue, no statistically significant differences were found between tumor and tumor+F1B groups in GSH level, LPO rate, as well as in SOD and Cat activities (Fig. 4B, 4D, 4F, 4H).


Liver
Tumor
A
GSH
mg GSH.g tissue -1
0.4
0.3
0.2
0.1
0.0
***
##
Basal
Basal+F1B
Tumor
Tumor+F1B
B
GSH
mg GSH.g tissue -1
0.15
0.10
0.05
0.00
Tumor
Tumor+F1B
C
SOD
U SOD.mg protein -1
20
15
10
5
0
*
#
Basal
Basal+F1B
Tumor
Tumor+F1B
D
SOD
U SOD.mg protein -1
50
40
30
20
10
0
Tumor
Tumor+F1B
E
Catalase
µmol.min -1.mg protein -1
400
300
200
100
0
Basal
Basal+F1B
Tumor
Tumor+F1B
F
Catalase
µmol.min -1.mg protein -1
40
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Tumor
Tumor+F1B
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mmol hydroperoxides.mg protein -1
150
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Basal
Basal+F1B
Tumor
Tumor+F1B
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LPO
mmol hydroperoxides.mg protein -1
200
150
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Tumor
Tumor+F1B
I
GST
mmol.min -1.mg protein -1
30
20
10
0
Basal
Basal+F1B
Tumor
Tumor+F1B

Figure 4. Main oxidative stress parameters measured in Walker-256 tumor-bearing rats treated with F1B for 16 days (100 mg·kg). GSH activity in liver (A) and tumor (B); SOD activity in liver (C) and tumor (D); Catalase activity in liver (E) and tumor (F), LPO level in liver (G) and tumor (H), and GST in liver (I) of tumor-bearing rats. Symbols: $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$ as compared to the basal group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ as compared to the tumor group. Values represent means ± SEM of n = 7-10 rats. Differences between groups were analyzed by one-way ANOVA followed by Bonferroni test.

3.5 *Biochemical assays*

As expected, the presence of tumors induced several changes in plasma, including a reduction in glucose levels and amylase and alkaline phosphatase activities, as well as an enhancement in AST activity. However, treatment of tumor-bearing rats with F1B returned all these parameters to basal levels, as shown in Table 1. No statistically significant difference was observed in plasma parameters.

Table 1. Plasma parameters of basal or tumor-bearing rats after 16 days of oral treatment with F1B (100 mg·kg) or vehicle.

Parameters	**Experimental groups**			
	Basal	**Basal+F1B**	**Tumor**	**Tumor+F1B**
Glucose ($mg.dL^{-1}$)	130.1 ± 4.4	121 ± 11.8	95.81 ± 5.0*	116.5 ± 9.0
Amlylase ($U.L^{-1}$)	575.6 ± 20.93	687.9 ± 38.58	428.2 ± 33.46*	524.1 ± 38.42
Urea ($mg.dL^{-1}$)	42.13 ± 1.52	40.44 ± 2.86	37.06 ± 1.36	34.70 ± 1.84
Creatinine ($mg.dL^{-1}$)	0.28 ± 0.04	0.34 ± 0.05	0.28 ± 0.02	0.37 ± 0.05
Total protein ($g.dL^{-1}$)	6.08 ± 0.13	6.20 ± 0.10	5.66 ± 0.13	6.36 ± 0.47
Albumin ($g.dL^{-1}$)	3.66 ± 0.06	3.76 ± 0.05	3.37 ± 0.14	3.51 ± 0.10
Globulin ($g.dL^{-1}$)	2.41 ± 0.11	2.44 ± 0.06	2.29 ± 0.07	2.41 ± 0.14
AST ($U.L^{-1}$)	78.71 ± 5.39	72.72 ± 6.48	184.9 ± 36.59*	91.70 ± 10.14$^{\#}$
ALT ($U.L^{-1}$)	39.81 ± 3.08	42.62 ± 7.10	40.07 ± 2.55	38.06 ± 3.84
Alkaline phosphatase ($U.L^{-1}$)	237.1 ± 19.09	250.2 ± 32.85	149.6 ± 9.22**	179.4 ± 19.64$^{\#\#}$

Symbols: $^{*}p<0.05$ and $^{**}p<0.01$ as compared to the basal group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ as compared to the tumor group. Values represent means ± SEM of n = 7-10 animals. Differences between groups were analyzed by one-way ANOVA followed by Bonferroni test.

3.6 *Inflammatory parameters in tumor*

The inflammatory microenvironment of tumors is characterized by the presence of macrophages, neutrophils, eosinophils, and mast cells, all of which produce an assorted array of cytokines and inflammatory mediators (Balkwill and Mantovani,

2001). To evaluate the effect of F1B on inflammation, we analyzed the activities of the enzymes of MPO and NAG, respectively abundant in mononuclear cells and neutrophil granulocytes; as well nitric oxide levels, which play a key role in inflammatory response. The levels of the cytokine TNF-α were also evaluated, as this cytokine contributes to many features of tumor growth and spread (Balkwill, 1994). F1B did not affect MPO (A) and NAG (B) activity, nor did it affect the production of nitric oxide (C). However, treatment with F1B caused a significant reduction (-30%) in TNF-α levels (0.066 ± 0.008 pg·mg wet tissue^{-1}) in tumor tissue when compared to the control (0.095 ± 0.006 pg·mg wet tissue^{-1}), as showed in Figure 5.

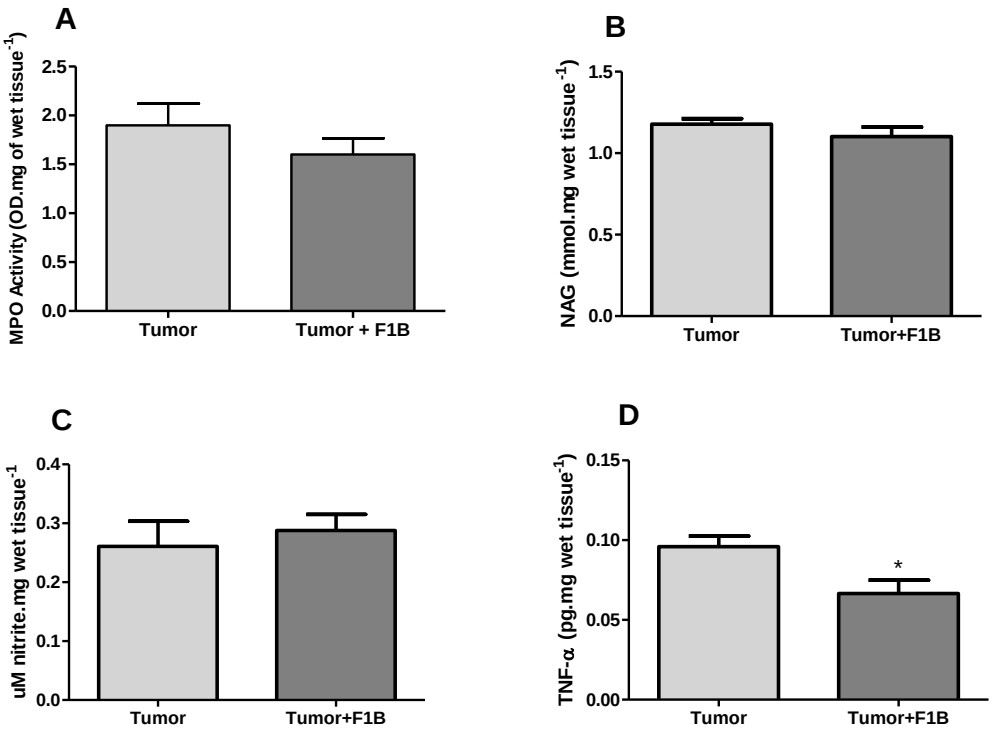


Figure 5. Inflammatory parameters in tumor tissue of Walker-256 tumor-bearing rats treated with F1B (Tumor+F1B) or vehicle (Tumor) for 16 days: MPO (A), NAG (B), nitrite (C) and TNF-α (D). Values represent means ± SEM of n = 7-10 animals. Symbol: *p<0.05. Student t test was used for statistical analysis.

3.7 *Histopathology*

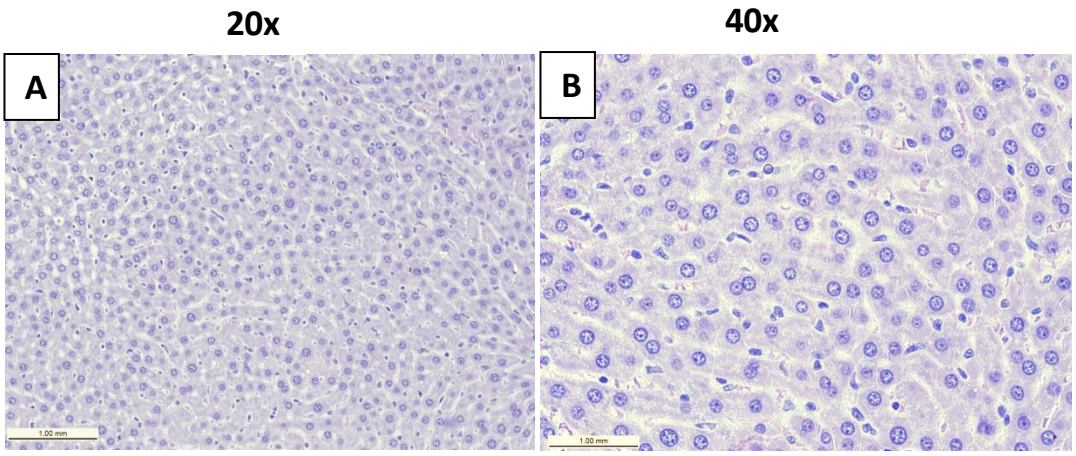
To evaluate possible liver damage induced by the tumor or treatment with F1B, histological analysis of liver samples were performed. The presence of lymphocyte and lymphohistiocytic infiltrates was observed in all groups; however, both alterations

were greater in animals with Walker-256 carcinosarcoma (tumor and tumor+F1B groups). These results are shown in Table 2 and in Figure 6.

Table 2. Histological parameters evaluated in liver tissue of rats after treatment with F1B (100 mg·kg) or vehicle for 16 days.

	Experimental Groups			
Optical Microscopy of Liver	**Basal**	**Basal+F1B**	**Tumor**	**Tumor+F1B**
Lymphocytic infiltrates	+	+	++	++
Lymphocytic infiltrates with necrosis	-	-	+++	+
Lymphohistiocytic infiltrates	+	+	++	++
Lymphohistiocytic infiltrates with necrosis	+	+	++	++

Groups: Basal (n=7) and Tumor (n=10) received vehicle (distilled water containing 2% Tween 80). Basal+F1B (n=7) and Tumor+F1B (n=7) received 100 $mg.kg^{-1}$ of F1B. Scores: mild (+), moderate (++) and pronounced (+++).



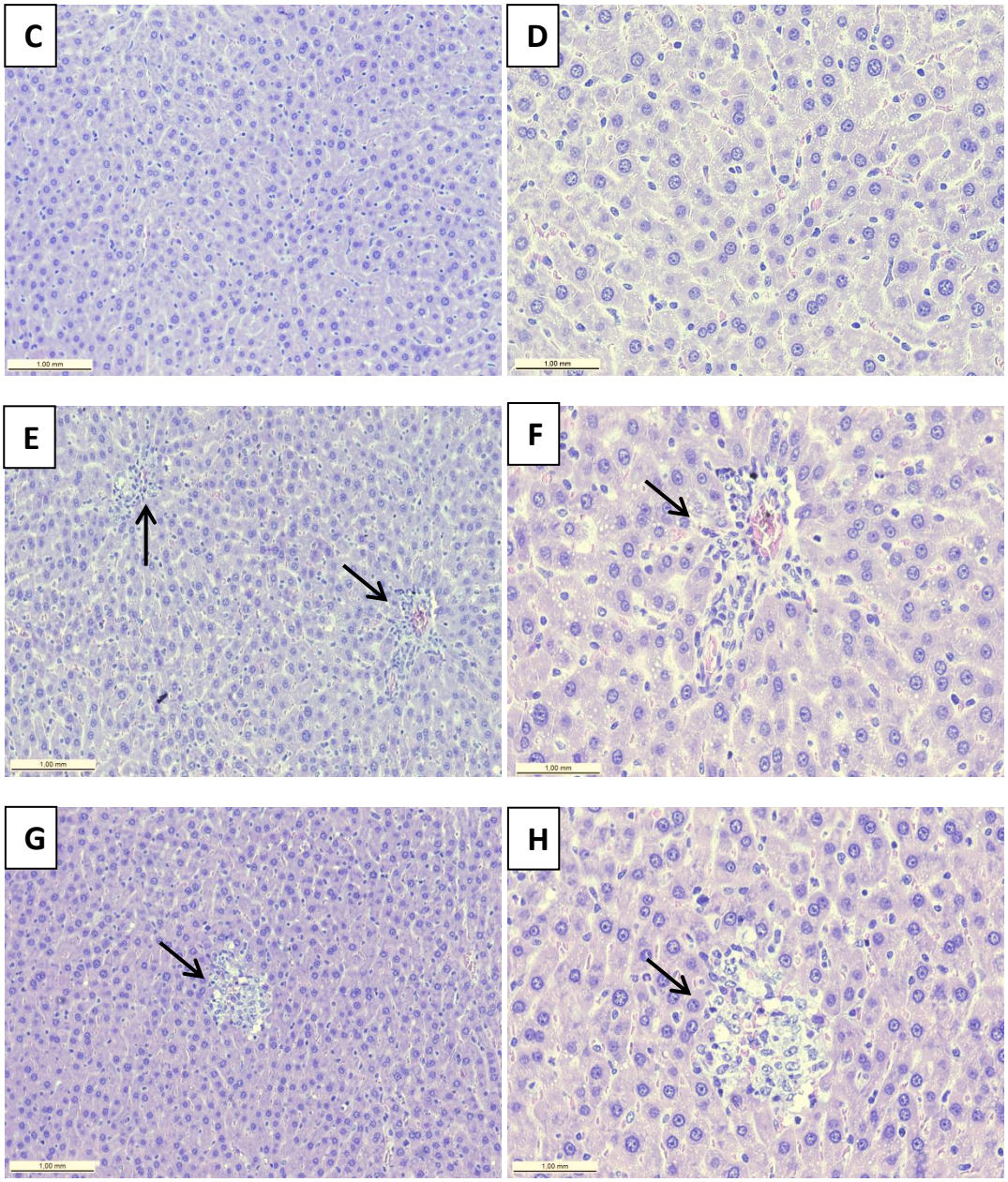


Figure 6. Histological parameters evaluated in liver tissue in rats after treatment with F1B (100 mg·kg) or vehicle for 16 days. (A, B) Basal, (C, D) Basal+F1B, (E, F) Tumor and (G, H) Tumor+F1B. Normal liver is showing in A to D. The presence of lymphocyte and lymphohistiocytic infiltrates was observed in panels E to H (arrows). Scale bar = 1.00 mm.

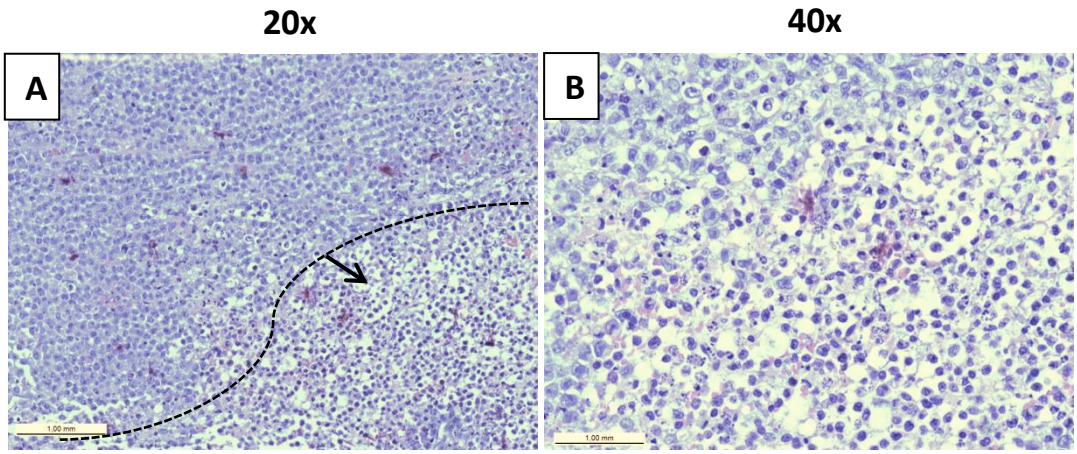
During the histological evaluation levels of coagulative or suppurative necrosis, apoptosis, cytological differences between neoplastic cells of Walker-256 tumor, infiltration capacity, and the formation of vascular plaques and vacuolization were assessed in tumors and F1B-treated tumor bearing rats were observed. The tumor+F1B group had more apoptotic foci and increased vacuolization, as compared to the tumor group. Apoptosis was assessed through the identification of characteristic

morphological features, including peripheral nuclear chromatin condensation and fragmentation of nuclei in strongly basophilic particles (apoptotic bodies). These results are shown in Table 3 and in Figure 7.

Table 3. Histological parameters evaluated in tumor tissue of rats after treatment with F1B (100 mg·kg) or vehicle for 16 days.

	Experimental Groups	
Optical Microscopy of Tumor	**Tumor**	**Tumor+F1B**
Coagulative necrosis	+	+
Suppurative necrosis	-	-
Apoptosis	+	+++
Infiltration	+	+
Vascular plaques	+	+
Vacuolization	+	++

Groups: Tumor (n=10) received vehicle (distilled water containing 2% Tween 80) and Tumor+F1B (n=7) received 100 mg.kg^{-1} of F1B. Scores: mild (+), moderate (++) and pronounced (+++).



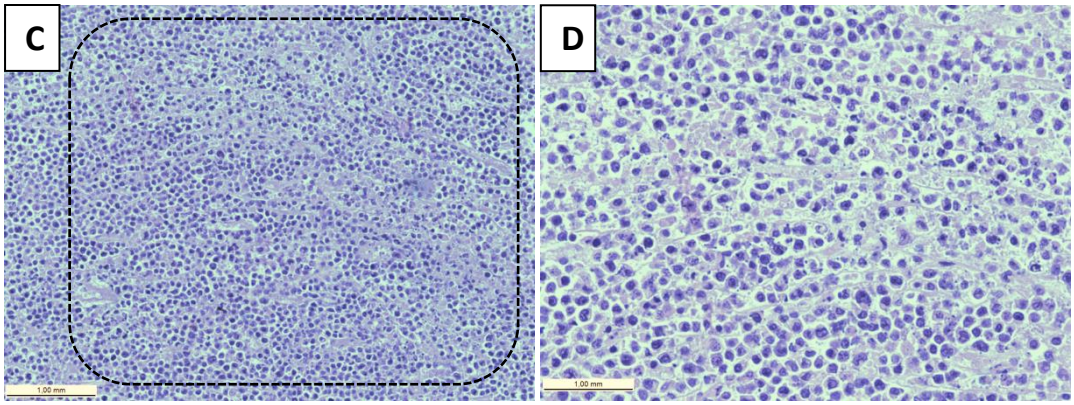


Figure 7. Histological parameters evaluated in tumor tissue (Walker-256 tumor) in rats after treatment with F1B (100 mg·kg) or vehicle for 16 days. (A, B) Tumor and (C, D) Tumor+F1B. Apoptosis are present in panels A and C (---- delimitated area), with extensive apoptotic foci observed in the panel C. Scale bar = 1.00 mm.

3.8 *Toxicity evaluation*

As F1B is being considered as an antineoplastic agent for the treatment of tumor, it was necessary to evaluate its toxicity.

3.8.1 *Clinical signs, body weight and relative organs weight*

No clinical signs of toxicity or death, nor and any variation in body weight among the groups, were observed during the 16 day treatment with F1B (100 $mg \cdot kg^{-1}$) or vehicle (data not shown). Toxicological evaluation of F1B included the calculation of the relative weight (%) of spleen, liver, kidneys and lungs. A significant increase was observed in spleen weight of the tumor group (0.37 ± 0.03%) when compared with basal group (0.26 ± 0.01%). Treatment with F1B prevented the increase in relative weight of spleen (0.28 ± 0.02%) when compared to tumor group, and showed similar values to the basal group.

3.8.2 *Acute toxicity (LD_{50}) in mice*

The administration of F1B at doses of 50, 500 and 1000 $mg \cdot kg^{-1}$ by oral gavage did not cause mortality in mice, whereas oral administration of 5000 $mg \cdot kg^{-1}$ F1B orally and 1000 $mg \cdot kg^{-1}$ by intraperitoneal injection resulted in 100% lethality. The estimated oral LD_{50} of F1B in mice was 1209 $mg \cdot kg^{-1}$, with confidence limits from 1007.6 to 1450.9 $mg \cdot kg^{-1}$.

4. Discussion

The present study demonstrated the *in vivo* antitumor activity of F1B obtained from the trunk bark of *Gochnatia polymorpha* ssp. *floccosa*. The antineoplastic effect against the solid Walker-256 tumor in rats was significant, resulting in an approximately 74% reduction in tumor growth. The *in vivo* result is consistent with previous work that reported the *in vitro* cytotoxic effect of compounds isolated from the bark of *G. polymorpha* ssp. *floccosa* (F1B fraction) in human cancer lines. The most active compounds in F1B were dimeric SLs, called *10-Desoxygochnatiolide* A and *Gochnatiolide A*, which inhibited the growth of kidney, melanoma, ovarian, and glioma tumor cell lines (Strapasson et al., 2012). In our study, this fraction was submitted to consecutive chromatographic fractionation that yielded six known compounds (1-6) among the guaianolide subtype, which were previously characterized in SLs. These compounds, which were also present in the F1B used in the present study (Fig. 1), showed anti-inflammatory activity (Piornedo et al., 2011). Corroborating this data, the levels of the inflammatory cytokine TNF-α were reduced about 30% by treatment with F1B. As studies have confirmed that TNF-α is a key cytokine in the tumor microenvironment that promotes tumor cell proliferation and invasion, the inhibition of TNF-α may play a key role in the mechanism of F1B action (Zhu et al., 2014). High levels of TNF-α can directly contribute to oncogene activation and associated cell signaling pathways through the stimulation of transcription factors and related genes, consequently affecting tumor cell proliferation and activity (Akiyama et al., 2003). Furthermore, TNF-α also promotes tumor cell metastasis (Kim et al., 2009). In addition to inhibiting TNF-α, the F1B fraction also promoted increased areas of apoptotic foci in Walker-256 carcinosarcoma tumors. This result is consistent with previously reported mechanisms of apoptotic induction by SLs (Kreuger et al., 2012).

The dimeric SLs present in the F1B are likely responsible for the antineoplastic action, as they possess known cytotoxic activity *in vitro* (Strapasson et al., 2012). Moreover, several studies have demonstrated the potential of these substances as antineoplastic therapies. According to Zhang et al. (2005) various SLs have been demonstrated to execute their anti-cancer capability via the inhibition of inflammatory

responses and metastasis, as well as the induction of apoptosis. SLs also inhibit NF-κB and MAP kinase activity and induce oxidative stress, followed by G2/M arrest and activation of the tumor suppressor p53, thereby promoting apoptosis (Costantino et al., 2013; Nakshatri et al., 2004). Despite the presence of SLs, the F1B did not induce oxidative stress in Walker-256 tumor tissue. Although the antioxidant activity of F1B was observed *in vitro* (Fig. 2), no significant differences were observed *in vivo* in several tumor antioxidant parameters (Cat, SOD, GST, GSH and LPO) between animals with tumors, and those treated with F1B. However, the response of oxidative stress biomarkers in liver was altered. The tumor group (untreated) showed lower levels of GSH as compared to the basal group or the F1B treated group, demonstrating that treatment with the F1B fraction inhibited hepatic depletion of GSH caused by tumorigenesis. Additionally, treatment of tumor-bearing animals with F1B caused an increase lipid peroxidation rates. This effect can be explained by the induction of apoptosis following endoplasmic reticulum (ER) stress, which involves cytoplasmic calcium elevation, increases in reactive oxygen species (ROS) and the ability to oxidize lipids, which may be one of the cytotoxic effects of SLs (Chadwick et al., 2013; Guzman et al., 2005; Kreuger et al., 2012).

In order to evaluate metabolic alterations and possible cachexia induced by the Walker-256 tumor model (Acco et al., 2012), the body weight was evaluated throughout the treatment. No differences among the groups were observed in relation to this parameter. However, after tumor cell development, glycemia was significantly reduced in tumor bearing rats, which is consistent with previous results (Cassolla et al., 2012; Jumes et al., 2010). Treatment with F1B reversed this alteration (Table 1), as well as reversing the effect of the tumors on AST activity. Enhanced oxidative stress and alterations in the permeability or function of hepatocytes can result from the effect of tumors on the liver. This can result in extravasation of AST to the plasma, likely because AST is localized to the cytosol in these cells (Jagadeesan and Kavitha, 2006). Our data indicate that F1B, at least partially, protected hepatocytes, resulting in decreased levels of plasma AST. This cellular protection may be due the normalized SOD activity and GSH level induced by F1B, both of which are able to reduce the action of free radicals on membranes, proteins and enzymes (DeWeese et al., 2001; Gate et

al., 1999). The histological evaluation of the liver also confirmed that F1B did not induce hepatic alterations.

In accordance with liver protection and the absence of clinical signs or adverse effects, F1B treatment in basal and tumor-bearing rats did not alter the weight of most organs. However, there was an increase in the relative weight of the spleen in untreated animals with tumors. The splenomegaly was expected, as spleen hyperplasia can be caused by neoplasia, as well as by alterations of the reticuloendothelial system or cells of lymphoid lineages, morphological abnormalities of erythrocytes, extra-medullary hematopoiesis, and drugs (Viveros et al., 2013). Although F1B treatment did not change organs macroscopically, the cytotoxic action of F1B was previously demonstrated *in vitro* (Strapasson et al., 2012). In addition, as this work is the first to evaluate the antitumor activity of F1B *in vivo*, the LD_{50} was determined to assess the degree of F1B toxicity and its therapeutic safety. The oral LD_{50} value of F1B in mice, estimated at 1209 $mg \cdot kg^{-1}$, is considered moderately toxic (500–5000 $mg \cdot kg^{-1}$) on a scale of six levels from non-toxic to supertoxic (Lu, 1991). However, the antineoplastic effect of F1B was demonstrated with a dose 12-fold lower (100 $mg \cdot kg^{-1}$) than the LD_{50}.

In conclusion, this study demonstrated the antitumor activity of F1B obtained from *G. polymorpha* ssp. *floccosa* against the carcinosarcoma Walker-256 in rats. Six substances were reported in this fraction, and the dimeric guaianolides 10-desoxygochnatiolide A and gochnatiolide A were likely responsible, at least in part, for the antineoplastic effect. F1B promoted apoptosis induction and probably inhibited cell signaling mediated by the cytokine TNF-α. Further studies are necessary to evaluate the molecular mechanisms by which these compounds exert their effect, in order to promote new antineoplastic therapy options.

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4. CONSIDERAÇÕES FINAIS

Diante dos resultados deste trabalho, conclui-se que:

- A fração diclorometano (F1B) obtida a partir da casca do troco de *Gochnatia polymorpha* ssp. *floccosa* apresentou um importante efeito antitumoral frente ao modelo de carcinossarcoma Walker-256, na dose de 100 $mg \cdot kg^{-1}$.

- Os compostos identificados na fração F1B foram lactonas sesquiterpênicas, do subtipo guaionolideos, responsáveis então pelo efeito antineoplásico. O mecanismo de ação que pode estar envolvido neste efeito é a indução de apoptose das células malignas Walker-256, assim como uma possível inibição na sinalização celular mediada por TNF-α.

- Diante da dose testada, o tratamento com a fração F1B não apresentou alterações nos parâmetros de toxicidade. O efeito antitumoral foi demonstrado com uma dose 12 vezes menor que a DL_{50} calculada (1209 $mg \cdot kg^{-1}$).

- Mais estudos são necessários para se investigar os mecanismos moleculares desses compostos presentes na *Gochnatia polymorpha* ssp. *floccosa*, assim como avaliar a atividade antitumoral de cada um dos compostos isoladamente, frente a outros modelos de indução tumoral.

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