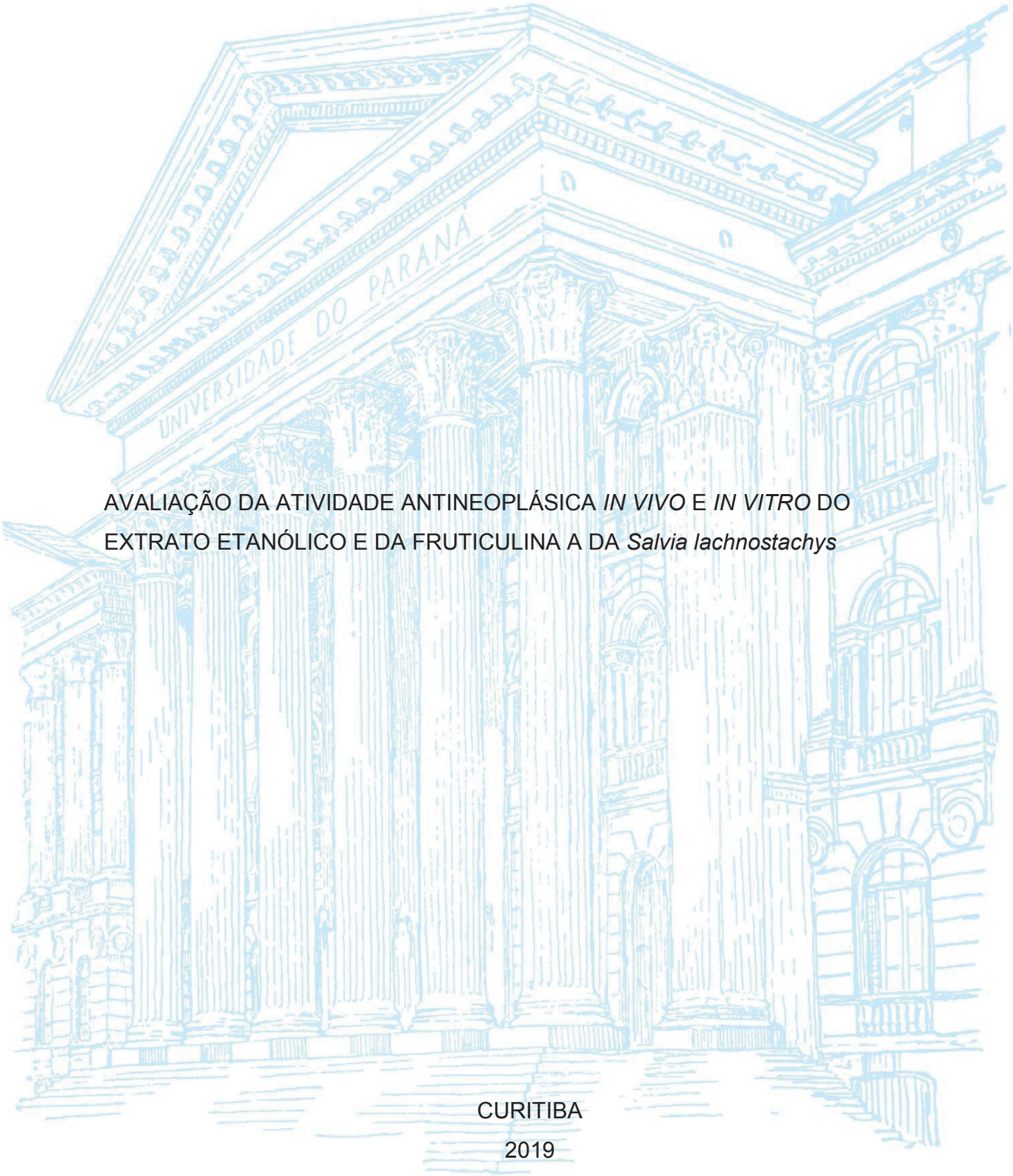
UNIVERSIDADE FEDERAL DO PARANÁ

CLAUDIA RITA CORSO



AVALIAÇÃO DA ATIVIDADE ANTINEOPLÁSICA *IN VIVO* E *IN VITRO* DO EXTRATO ETANÓLICO E DA FRUTICULINA A DA *Salvia lachnostachys*

CURITIBA

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia do Setor de Ciências Biológicas da Universidade Federal do Paraná como requisito para obtenção do título de Doutor em Farmacologia.

Orientadora: Profª Drª Alexandra Acco

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **CLAUDIA RITA CORSO** intitulada: **AVALIAÇÃO DA ATIVIDADE ANTINEOPLÁSICA *IN VIVO* E *IN VITRO* DO EXTRATO ETANÓLICO E DA FRUTICULINA A DA *Salvia lachnostachys***, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

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

CURITIBA, 25 de Março de 2019.

ALEXANDRA ACCO
Presidente da Banca Examinadora (UFPR)

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Avaliador Externo (UFPR)

MARIA FERNANDA DE PAULA WERNER
Avaliador Interno (UFPR)

NOTA EXPLICATIVA

Esta tese é apresentada em formato alternativo – artigos publicados ou submetidos para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos do trabalho e dois artigos científicos abordando os experimentos realizados, com resultados e discussão, além das considerações finais.

Dedicatória

Dedico esta tese a todas as pessoas que estão lutando contra o câncer e aos familiares que lutam junto com os seus entes queridos. Cada experimento realizado neste trabalho foi pensado e analisado com muita atenção e carinho, para proporcionar conhecimentos de alta qualidade para, no futuro, aperfeiçoar o tratamento do câncer e gerar melhor qualidade de vida aos pacientes.

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Muito obrigada!

“Você não pode mudar o vento, mas pode ajustar as velas do barco para chegar onde quer.”

(Confúcio)

RESUMO

Fármacos antineoplásicos representam a terapia de primeira escolha para o câncer, porém com efeitos deletérios para os pacientes. Dessa maneira, pesquisadores vêm estudando novas estratégias para o tratamento do câncer, e os produtos naturais vêm ganhando destaque devido à ampla atividade biológica. Nesse contexto, destaca-se o extrato etanólico da *Salvia lachnostachys* (EES) e seu diterpeno, fruticulina A (fruti), por possuírem ação citotóxica em células tumorais. Assim, o objetivo deste trabalho foi investigar o efeito antitumoral do EES e da fruti em modelo de carcinoma sólido de Ehrlich, além de avaliar o efeito quimiopreventivo do EES *in vivo* e o efeito antitumoral *in vitro* da fruti. Células de Ehrlich foram inoculadas no membro pélvico direito ($2^{x}10^{6}$ células, s.c.) em camundongos Swiss fêmeas. Os animais foram tratados com veículo (10 mL kg^{-1}, v.o.), EES (30 ou 100 mg kg^{-1}, v.o.), ou metotrexato (2,5 mg kg^{-1}, i.p.) por 21 dias (tratamento precoce) ou 14 dias (tratamento tardio) após a inoculação do tumor, ou 10 dias antes da inoculação do tumor e continuado durante 21 dias após a inoculação do tumor (tratamento quimiopreventivo). O tratamento precoce com 100 mg kg^{-1} de EES reduziu o crescimento tumoral, aumentou os níveis de TNF-α e diminuiu a SOD, IL-10 e a expressão de *Ciclina D1* no tumor, e foi observado necrose tumoral. O tratamento com EES por 31 dias diminuiu a atividade hepática e tumoral da SOD, níveis de IL-10 e expressão de *Ciclina D1,* e aumentou GSH, NAG, ROS, LPO, TNF-α e a expressão de *Nrf2*. Nenhuma toxicidade do EES foi observada no ensaio de toxicidade aguda. O tratamento com fruti (3 mg kg^{-1}, v.o.) foi realizado por 21 dias após a inoculação do tumor. A fruti preveniu o desenvolvimento do tumor e reduziu os níveis dos genes *Ciclina D1, Bcl-2* e *Rela tx2*. Além disso, induziu necrose e apoptose no tecido tumoral e aumentou os níveis de NAG e TNF-α, e reduziu os níveis de IL-10 e *Vegf.* Adicionalmente, células MCF-7 e HepG2 foram incubadas com fruti e observou-se redução da proliferação celular, através da redução dos níveis de *CICLINA D1*, e apoptose através da redução de *BCL-2.* Além disso, a fruti diminuiu os níveis do gene *NF-κB* e induziu necroptose pelo aumento de *RIPK1.* Nossos resultados indicam que o EES tem efeito antitumoral inibindo a expressão da *Ciclina D1*, aumentando a inflamação e modulando o sistema antioxidante com tratamento precoce e quimiopreventivo. A fruti também possui efeito antitumoral *in vivo*, observado através da inibição da via do *NF-κB*, reduzindo os níveis de *Ciclina D1* e *Bcl-2. In vitro*, as vias de apoptose e necroptose estão envolvidas na morte celular, enquanto que, *in vivo*, as células morrem por necrose, pelo aumento da inflamação do tumor e redução da angiogênese. Assim, a fruti, por ser um dos compostos majoritários do EES, é responsável por boa parte dos efeitos antitumorais do EES, e tanto o EES quanto a fruti representam opções promissoras para o desenvolvimento de drogas para o tratamento do câncer.

Palavras-chaves: Câncer. *Salvia lachnostahys.* fruticulina A. proliferação. Ciclina D1. NF-κB.

ABSTRACT

Antineoplastic agents represent the first-choice therapy, but with deleterious effects to the patients. Therefore, researchers have been studying new strategies for the treatment of cancer, and natural products have been attracting attention due to the wide biological activity. In this context, the ethanolic extract of *Salvia lachnostachys* (EES) and its diterpene fruticuline A (fruti), stand out due to cytotoxicity activity in cancer cells. Thus, the objective of this work was to investigate the antitumor effect of EES and fruti in a solid Ehrlich carcinoma model, evaluating the chemopreventive effect *in vivo* of EES and the *in vitro* antitumor effect of fruti. Ehrlich cells were inoculated into the right pelvic member ($2^{x}10^{6}$ cells, s.c.) in female Swiss mice. Animals were treated with vehicle (10 mL kg^{-1}, p.o.), EES (30 or 100 mg kg^{-1}, p.o.), or methotrexate (2.5 mg kg^{-1}, i.p.) for 21 days (early treatment) or 14 days (late treatment) after inoculation of the tumor, or 10 days prior to tumor inoculation and continued for 21 days after tumor inoculation (chemopreventive treatment). Early treatment with 100 mg kg^{-1} of EES prevented tumor growth, increased tumor TNF-α levels, and decreased SOD, IL-10 and *Cyclin D1* expression, and tumor necrosis was observed. Treatment with EES for 31 days decreased the liver and tumor activity of SOD, IL-10 levels and *Cyclin D1* expression and increased GSH, NAG, ROS, LPO, TNF-α and the Nrf2 expression. No EES toxicity was observed in the acute toxicity test. Treatment with fruti (3 mg kg^{-1}, p.o.) was performed for 21 days after tumor inoculation. Fruti prevented tumor development and reduced the expression of *the Cyclin D1, Bcl-2 and Rela tx2* genes. In addition, it induced necrosis and apoptosis in tumor tissue and increased levels of NAG and TNF-α, and reduced levels of IL-10 and *Vegf*. In addition, MCF-7 and HepG2 cells were incubated with fruit, resulting in reduction of proliferation, by the reduction of *CYCLIN D1* levels, in apoptosis and *BCL-2* reduction. In addition, fruti decreased *NF-κB* gene levels and induced necroptosis by increasing *RIPK1*. Our results indicate that EES has an antitumor effect by inhibiting *Cyclin D1* expression, increasing inflammation and modulating the antioxidant system with early and chemopreventive treatment. Fruti also has an antitumor effect *in vivo*, indicated through inhibition of the *NF-κB* pathway, reducing levels of *Cyclin D1* and *Bcl-2*. *In vitro*, apoptosis and necroptosis pathways are involved in cell death, whereas, *in vivo*, cells died from necrosis by increased tumor inflammation and reduced angiogenesis. Therefore, fruti, one of the major compounds of EES, is responsible at least in part for the antitumor effects of EES, and both the EES and fruti represent promising options for the development of drugs for the treatment of cancer.

Keywords: Cancer. *Salvia lachnostahys.* fruticuline A. proliferation. Cyclin D1. NF-κB.

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LISTA DE ABREVIATURAS

INTRODUÇÃO

ATP: adenosina trifosfato; Bcl-xL: célula B de linfoma-extra grande; Blc-2: célula-B de linfoma 2; CAT: catalase; COX2: ciclo-oxigenase 2; DNA: ácido desoxirribonucleico; EES: extrato etanólico da *Salvia lachnostachys*; fruti: fruticulina A; GI_{50}: inibição de proliferação de 50%; GSH: glutationa reduzida; GPx: glutationa peroxidase; G6PD: glicose 6-fosfato desidrogenase; HaCat: células de queratinócitos humanos; HeLa: células de cérvix humano; Hepa1c1c7: células de hepatoma murino; HepG2: células de carcinoma hepatocelular humano; HIF1α: fator induzível por hipóxia 1 alfa; ICAM: molécula de adesão intercelular 1; IFN-γ: interferon gama; IL-1: interleucina-1; IL-1β: interleucina-1 beta; IL-6: interleucina-6; IL-8: interleucina 8; IL-10: interleucina-10; iNOS: óxido nítrico sintase induzível; LPO: lipoperoxidação lipídica; MCF-7: células de adenocarcinoma de mama humano; MLKL: domínio quinase de linhagem mista; MMP: metaloproteinases de matriz; NAG: n-acetilglucosamina; NCI-ADR/Res: células de ovário humano resistente a múltiplos fármacos; NCI-H460: câncer de pulmão de células não pequenas; NF-κB: fator nuclear kappa B; Nrf2: fator nuclear eritróide 2 relacionado ao fator 2; OVCAR-03: células de carcinoma ovariano humano; PC-3: células de câncer de próstata humano; PGE_2: prostaglandina E_2; ROS: espécies reativas de oxigênio; RIPK1: receptor de interação serina-treonina de proteína quinase 1; RIPK3: receptor de interação serina-treonina de proteína quinase 3; RNA: ácido ribonucleico; SOD: superóxido dismutase; TNF-α: fator de necrose tumoral-alfa; TNFR1: receptor de TNF-α 1; TNFR2: receptor de TNF-α 2; TRAILR1: receptor 1 de ligante indutor de apoptose relacionado ao TNF-α; TRAILR2: receptor 2 de ligante indutor de apoptose relacionado ao TNF-α; U251: célula de glioblastoma humano; VEGF: fator de crescimento de vasos endoteliais; ZAG: zinco α2-glicoproteína; 786.0: células renais humanas.

ARTIGO CIENTÍFICO 1 - "*Salvia lachnostachys* Benth extract has antitumor and chemopreventive effects against solid Ehrlich carcinoma"

ALT: alanine aminotransaminase; AST: aspartate aminotransferase; ANOVA: analysis of variance; AcOEt; ethyl acetate; CAT: catalase; CC: column chromatography; $CDCl_3$: chloroform; cDNA: complementary deoxyribonucleic acid; CDNB: 1-chloro-2,4-dinitrobenzene; CH_2Cl_2: dichloromethane; CH_3OH-D4: deuterated methanol; CEUA/BIO: ethics committee of animal experimentation/biological; DCFA: 2',7'-dichlorfluoresceindiacetate; DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl; EC_{50}: half maximal efficient concentration; EES: ethanolic extract of *Salvia lachnostachys*; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GSH: reduced glutathione; GST: glutathione *S*-transferase; HT-29: human cancer colon cell line; IL-4: interleukin-4; IL-6: interleukin-6; IL-10: interleukin-10; i.p: intraperitoneal; KCl: potassium chloride; K562: human leukemia cells; LD_{50}: lethal dose 50%; LPO: lipid peroxidation; Me_2CO: acetone; MeOH: methanol; MTX: methotrexate; NaCl: sodium chloride; NAG: n-acetylglucosaminidase; NMR: nuclear magnetic resonance; NO: nitric oxide; *Nrf2*: nuclear factor erythroid 2-related factor 2; Nrf2/Keap1: nuclear factor erythroid 2-related factor 2/kelch like ECH associated protein 1; OECD: organization for economic co-operation and development; PBS: phosphate buffered saline; PCR: polymerase chain reaction; PDA: diode array detector; p.o.: oral route; RT-qPCR: quantitative reverse transcription PCR; s.c.: subcutaneous; SEM: standard error of mean; SOD: superoxide dismutase; TMB: tetramethylbenzidine; TNF-α: tumor necrosis factor-alpha; TLC: thin layer chromatography; Tris-EDTA: Tris ethylenediaminetetraacetic acid; UFPR: federal university of Paraná; UPCB: herbarium of the botanic department of UFPR.

ARTIGO CIENTÍFICO 2 - "Fruticuline A has antineoplastic effect by inhibiting NF-κB pathway *in vitro* and *in vivo*"

ACN: acetonitrile; ALT: alanine aminotransferase; ANOVA: analysis of variance; AST: aspartate aminotransferase; Bcl-2: B-cell lymphoma 2; Bcl-xL: B-cell lymphoma-extra large; CCND1: Cyclin D1; CEUA/BIO: ethics committee of animal experimentation/biological; CO_2: carbon dioxide; COX2: ciclo-oxigenase 2; CyP: Cyclophilin; DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate; DMEM: dulbecco's modified eagle medium; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; DTNB: 5,5'-Dithio-Bis (2-Nitrobenzoic acid); EES: ethanolic extract of *Salvia*

lachnostachys; EDTA: ethylenediaminetetraacetic acid; fruti: fruticuline A; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GR: glutathione reductase; GSH: reduced glutathione; HepG2: liver hepatocellular carcinoma; HIF1α: hypoxia-inducible factor alpha 1; HPLC: high-pressure liquid chromatography; HPRT: hypoxanthine-guanine phosphoribosyltransferase; HT-29: human cancer colon cell line; ICAM: intercellular adhesion molecule 1; IkBa: nuclear factor of kappa B-cells inhibitor alpha; IL-4: interleukin-4; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; iNOS: inducible nitric oxide synthase; JUNK: jun n-terminal kinase; K_2PO_4: potassium phosphate; LD_{50}: lethal dose 50%; MAPK: mitogen-activated protein kinases; MeOH: metanol; MCF-7: human breast adenocarcinoma cell line; MMP: matrix metalloproteinases; MPO: myeloperoxidase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX: methotrexate; NAG: n-acetylglucosaminidase; NADPH: nicotinamide adenine dinucleotide phosphate; $NaHCO_3$: sodium bicarbonate; $Na_2HPO_4.2H_2O$: disodium hydrogenophosphate dihydrate; $NaH_2PO_4.2H_2O$: sodium phosphate dihydrate; $Na_2EDTA.2H_2O$: disodium EDTA dihydrate; Nec-1: necrostatin-1; NCI-ADR/RES: human ovarian-resistant tumor cell line NCI-H460: non-small-cell lung cancer cell line; NF-κB: nuclear factor kappa B cells; NMR: nuclear magnetic resonance; NO: nitric oxide; OVCAR-3: human ovarian cancer cell line; PBS: phosphate buffered saline; PC-3: human prostate cancer cell line; PCR: polymerase chain reaction; PVDF: polyvinylidene difluoride; RIPK1: receptor interacting protein kinase 1; RNA: ribonucleic acid; ROS: reactive oxygen species; RT-qPCR: quantitative reverse transcription PCR; SEC: solid Ehrlich carcinoma; SEM: standard error of mean; SOD: superoxide dismutase; STAT3: signal transducer and activator of transcription 3; TBST: Tris-buffered saline with Tween 20; TNF-α: tumor necrosis factor-alpha; UACC-62: human melanoma cell line; UFPR: federal university of Paraná; U251: human glioblastoma cell line; VEGF: vascular endothelial growth factor; WST-1: water soluble tetrazolium salt 1.

SUMÁRIO

1. INTRODUÇÃO

1.1. DEFINIÇÃO E EPIDEMIOLOGIA DO CÂNCER

O câncer representa um grande conjunto de enfermidades, abrangendo mais de 277 tipos diferentes de doenças cancerígenas, dependendo da origem celular e da região afetada (HASSANPOUR & DEHGHANI, 2017). A palavra câncer vêm do grego "*karkínos*", que significa caranguejo, e foi utilizada pela primeira vez por Hipócrates (460 e 377 a.c.), considerado pai da medicina, que a designou para tumores de mama, que apresentavam vasos sanguíneos inchados ao seu redor, e lembrou-lhe o aspecto de um caranguejo enterrado na areia (LUKONG, 2017; PAPAVRAMIDOU et al., 2010). Os cânceres são neoplasias malignas que têm em comum o crescimento desordenado de células com a capacidade de invadir tecidos e órgãos e que não têm finalidade biológica (INCA, 2018). No entanto, cada tipo de câncer possui características próprias quanto à origem genética e histopatológica, eficiência na proliferação, grau de agressividade e invasividade, e dessa maneira o prognóstico, tratamento e a resposta ao tratamento também serão distintos para cada tipo de câncer e paciente (SAITO et al., 2016).

Devido às altas taxas de mortalidade, o câncer representa um grande problema de saúde pública com alto impacto sócio-econômico (JEMAL et al., 2011). Novos dados publicados pelo projeto Globocan 2018, baseados nos dados da Agência Internacional de Pesquisa de Câncer, sugerem que a carga global de câncer aumentou para 18,1 milhões de casos e 9,6 milhões de mortes entre 2012 e 2018 (BRAY et al., 2018). No Brasil, o INCA estimou cerca de 600 mil casos novos de câncer para o biênio 2018-2019, sendo o câncer de mama com maior incidência em mulheres e o câncer de próstata em homens, sem considerar os tumores de pele não melanoma (INCA, 2018).

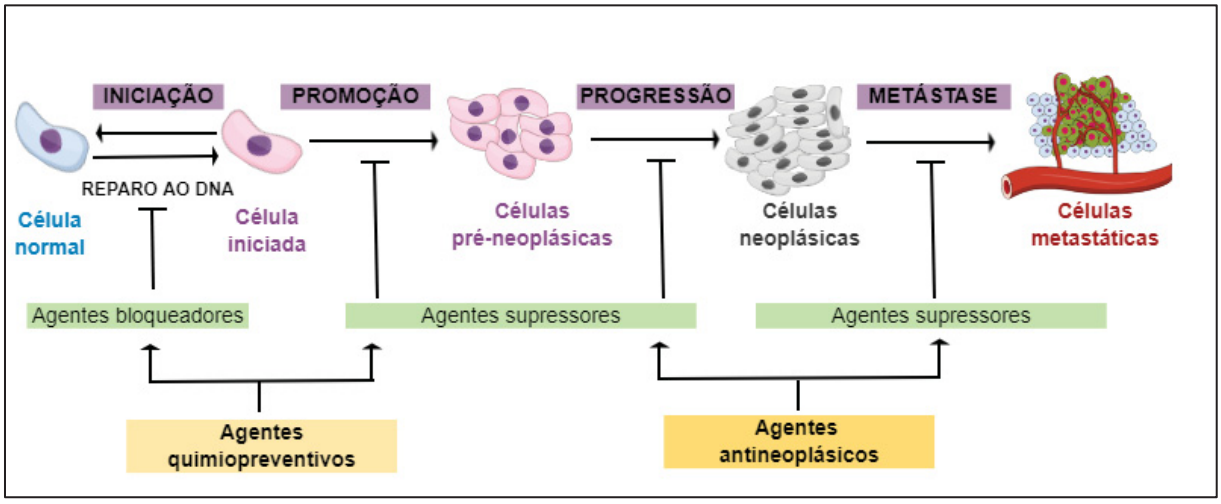
As células neoplásicas (do latim: *neo*= novo; *plasein*= formar) têm a capacidade de se proliferar rapidamente sem diferenciação, propriedade conhecida como auto-renovação (WONGTRAKOONGATE, 2015), responsável pela sua malignidade ao organismo. As causas de neoplasias são variadas, podendo ser por fatores externos ou internos ao organismo, estando ambos inter-relacionados. Os fatores internos são, geralmente, geneticamente pré-determinados, e estão relacionados com a capacidade de defesa do organismo às agressões externas através de genes supressores de tumor ou oncogenes (BURTON & RAI, 2015; HAIGIS & SWEET-CORDERO, 2011). Os fatores externos são responsáveis pelo aumento da incidência do câncer nos países

economicamente desenvolvidos, como resultado da adoção de estilos de vida associados à doença, incluindo o tabagismo (KOYANAGI et al., 2016), sedentarismo, dietas inadequadas (MEHTA & SHIKE, 2014) e exposição em demasia a radiações e ao sol (MANCEBO & WANG, 2014; CALZAVARA-PINTON et al., 2015). Cerca de 80 a 90% dos casos de câncer estão associados aos fatores externos. O envelhecimento também aumenta a susceptibilidade à transformação maligna, o que também aumentou a incidência de câncer em países desenvolvidos, nos quais a expectativa de vida tem aumentado (JEMAL et al., 2011).

1.2. CARCINOGÊNESE

O processo de carcinogênese, ou seja, de formação da neoplasia, em geral acontece lentamente (SIDDIQUI et al., 2015). Esse processo é constituído por três estágios: iniciação, promoção e progressão. A iniciação é uma alteração persistente e hereditária de uma célula por agentes cancerígenos, podendo ser resultante de uma mutação, translocação ou amplificação na célula alvo, sendo um estágio extremamente rápido e geralmente reversível por agentes quimiopreventivos. A promoção, no entanto, envolve a seleção e a proliferação de células iniciadas, levando essas células à pré-malignidade. Essa etapa ocupa a maior parte do período de latência da carcinogênese e pode ser reversível por certos agentes antineoplásicos. E por fim, a progressão, que é devida aos mecanismos genéticos e de sinalização molecular, corresponde ao período entre a pré-malignidade e a neoplasia (câncer), no qual podem ocorrer as metástases, invadindo outros órgãos e tecidos do organismo. Essa etapa também pode ser revertida pelo uso de agentes antineoplásicos (SUN, 1990; TSAO et al., 2004) (Figura 1).

FIGURA 1 - FASES DA CARCINOGÊNESE.



FONTE: modificado de SIDDIQUI et al. 2015.

Vários genes específicos que podem desempenhar um papel importante na carcinogênese foram identificados. Estes incluem os genes supressores de tumor (p. ex. p53), os proto-oncogenes e os genes de reparo de DNA, que codificam proteínas que estão envolvidas no controle do ciclo celular, fazem transdução de sinal e a regulação da transcrição (TSAO et al., 2004). Esses genes garantem que a proliferação celular, apoptose, auto renovação e diferenciação sejam normalmente controlados (WONGTRAKOONGATE, 2015). Entretanto podem ocorrer mutações genéticas e epigenéticas, levando a desregulações na ativação de tais genes, desregulando o ciclo celular e ocasionando proliferação celular descontrolada (RIVENBARK & COLEMAN, 2012). Os proto-oncogenes, quando ativados, estão ligados com a produção de fatores de crescimento, e, portanto com à multiplicação celular, favorecendo o crescimento do tumor (MONTENEGRO, 2008). A metilação do DNA tem sido demonstrada como um dos maiores defeitos no mecanismo de epigenética observado na tumorigênese (CHRUN et al., 2017).

Os vasos sanguíneos e linfáticos são os componentes essenciais que proporcionam nutrientes para o tumor. Os aminoácidos, os lipídios, as lipoproteínas e a glicose podem ser essenciais para a continuação do crescimento do tumor. Vários fatores solúveis do microambiente tumoral podem interagir com órgãos e tecidos induzindo a angiogênese tumoral. Além disso, o requerimento energético do tumor influencia o metabolismo de vários órgãos e tecidos do organismo, podendo levar ao quadro de caquexia (AL-ZOUGHBI et al., 2014), uma síndrome complexa e multifatorial caracterizada pela perda de peso, atrofia muscular, fadiga, fraqueza, perda de apetite e

às vezes acompanhada de anorexia. A caquexia atinge cerca de 60 a 80% dos pacientes com câncer terminal e estima-se que seja a causa de morte de 15 a 20% de todos os pacientes com câncer (AL-ZOUGHBI et al., 2014; TISDALE, 2009). Tem sido relatado que muitos tumores secretam mediadores inflamatórios [p. ex. fator de necrose tumoral alfa (TNF-α), interleucina 6 (IL-6), interleucina 1 (IL-1)] e pró-catabólicos [p. ex. zinco α2-glicoproteína (ZAG)], além de secretar fatores liberados pelo próprio hospedeiro como resposta [p. ex. interferon gama (IFN-γ) e ZAG], que são responsáveis pela promoção de degradação do músculo esquelético e tecido adiposo, e da anorexia e perda de peso, sintomas observados na caquexia (AL-ZOUGHBI et al., 2014). Além disso, foi demonstrado que TNF-α e interleucina-1 beta (IL-1β) circulantes estão relacionadas com a diminuição da gliconeogênese hepática em ratos com tumor de Walker-256, um dos efeitos responsáveis pela caquexia nesse modelo (KELMER-BRACHT et al., 2006).

Um acontecimento chave que inicia o processo angiogênico é a estabilização do fator induzível por hipóxia 1 alfa (HIF1α) no microambiente do tumor, além da IL-1β. Junto com prostaglandina E_2 (PGE_2), a IL-1β regula positivamente os níveis de proteína HIF1α e ativa o fator de crescimento endotelial vascular (VEGF), numa reação que é principalmente mediada pelo fator nuclear kappa B (NF-κB). Esta cascata de ativação do gene ilustra um exemplo importante do papel da inflamação no desenvolvimento de tumores (AL-ZOUGHBI et al., 2014).

O NF-κB representa uma das importantes vias de sinalização devido à indução de transcrição de muitos genes-chave no desenvolvimento do câncer, tais como gene para proliferação (p. ex. Ciclina D1, Ciclina E), apoptose (p. ex. Bcl-2, Bclc-2), angiogênese (p. ex. VEGF, IL-8, HIF1α), adesão celular e metástases [p. ex. molécula de adesão intercelular 1 (ICAM1); metaloproteinases de matriz (MMP); óxido nítrico sintase induzível (iNOS)], sobrevivência (p. ex. Survivin) e inflamação [p. ex. TNF-α, ciclo-oxigenase 2 (COX2), iNOS] (PARK & HONG. 2016). Dados os estudos que mostram a supressão do tumor pela inibição do NF-κB (GUAN et al., 2018; ZAKARIA et al., 2018), esse fator nuclear tornou-se uma estratégia terapêutica atraente para novos compostos antineoplásicos, pois várias investigações revelaram uma importante contribuição do NF-κB para progressão do câncer e sobrevivência (RIEDLINGER et al., 2018).

Dentre os vários fatores que causam o câncer, o estresse oxidativo é um dos mais estudados e mais importantes que favorecem a condição de desenvolvimento e progressão do tumor (RANINGA et al., 2014). O estresse oxidativo é definido como um distúrbio no balanço entre a produção de espécies reativas de oxigênio (ROS) e

antioxidantes como mecanismo de defesa, causando toxicidade ao ambiente celular, danos a proteínas, DNA e lipídeos. As ROS são constantemente geradas em organismos aeróbicos como consequência do metabolismo normal e incluem radicais livres como ânion superóxido, radical hidroxil, óxido nítrico, bem como não-radicais, como o peróxido de hidrogênio. Entre a linha dos antioxidantes que atuam como defesa a esses radicais livres, existem moléculas não enzimáticas como as vitaminas C e E, e a glutationa redutase (GSH), e moléculas enzimáticas como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e a glicose 6-fosfato desidrogenase (G6PD) (DICHI et al., 2014). Vários estudos demonstram que as moléculas antioxidantes estão diminuídas em vários tipos de câncer. Entretanto, no tecido tumoral há aumento das mesmas como um mecanismo de defesa do próprio tumor contra as ROS, facilitando a sua proliferação, sobrevivência e resistência a drogas (RANINGA et al., 2014).

Para manter o balanço fisiológico entre morte e crescimento celular, o processo da apoptose desencadeia um papel muito importante. Este processo complexo é executado através de duas vias principais que participam na ativação de proteases, conhecidas como caspases, que levam à desintegração da cromatina e fragmentação nuclear. No entanto, a desregulação destas vias, muitas vezes contribui para o desenvolvimento do câncer e/ou resistência à terapia do câncer. Muitas células tumorais desenvolvem mecanismos para evitar este programa de morte celular através da manipulação das moléculas anti-apoptóticas ou inativação de componentes pró-apoptóticos (KOFF et al., 2015). As principais proteínas anti-apoptóticas são Bcl-2 e Bcl-X_L, enquanto que a Bax, Bad e Bid são proteínas pró-apoptóticas (PAROLIN & REASON, 2001). O estresse oxidativo também induz apoptose por indução da ativação das caspases, que ativam a família da proteína Bcl-2 e modulam proteínas quinases para induzir morte celular (RANINGA et al., 2014). Também tem sido relatado que o óxido nítrico, mediador e regulador de processos inflamatórios, desempenha um papel importante na tumorigênese. O aumento da expressão de óxido nítrico sintase induzível (iNOS) resulta na inibição da apoptose no tecido tumoral (HUERTA et al., 2006). No entanto, ainda há necessidade de uma melhor compreensão dos mecanismos moleculares que levam à evasão de apoptose em um determinado tipo de câncer, podendo servir de base para novas estratégias para combater a resistência à apoptose e controlar a progressão tumoral (FULDA, 2015).

Outro processo de morte celular associado é a necroptose, também denominado necrose controlada, ou independente da ativação de caspases. Este processo é

desencadeado por diversos receptores, incluindo CD95 (também conhecido como FAS), receptor de TNF1 (TNFR1), TNFR2, receptor 1 de ligante indutor de apoptose relacionado ao TNF (TRAILR1) e TRAILR2. Estes receptores normalmente ativam a maquinaria apoptótica, o qual estimula a ação da proteína quinase de interação ao receptor 1 (RIPK1), proteína quinase de interação ao receptor 3 (RIPK3) e proteína quinase de domínio de linhagem mista (MLKL), resultando na formação do necrossomo (complexo RIPK1-RIPK3-MLKL). O necrossomo diminui a permeabilidade celular aumentando o influxo de Ca^{2+} para o interior da célula, levando à redução de ATP celular, produção de ROS, e, consequentemente, morte celular (OFENGEIM & YUAN, 2013; VANDEBABEELE et al., 2010).

Fica evidente nos parágrafos supracitados que as vias de apoptose, necroptose, proliferação, angiogênese, os componentes do estresse oxidativo, a resposta inflamatória e as várias vias de sinalização molecular são alvos que podem definir a progressão ou o controle da carcinogênese. Por este motivo, essas vias foram investigadas neste projeto de pesquisa, tanto em tecido tumoral de camundongos quanto em células tumorais humanas.

1.3. TRATAMENTO

A quimioterapia é considerada o tratamento padrão no câncer. A quimioterapia age no crescimento celular de diversas maneiras, podendo interromper a série de transformações comentadas nos itens anteriores e direcionar a célula para a morte celular programada. Como causam danos celulares, estes fármacos são chamados de citotóxicos ou citostáticos. Além disso, o tratamento do câncer também pode ser feito através de cirurgia, radioterapia, imunoterapia, (anti)hormonioterapia ou transplante de medula óssea, dependendo do tipo celular envolvido na doença. Em muitos casos, é necessário combinar mais de uma modalidade. A combinação de diferentes quimioterápicos com ação em diferentes fases do ciclo celular aumenta a eficácia em diversos tipos de tumores, diminuindo ou revertendo clones resistentes. A quimioterapia pode ser combinada com outras modalidades de tratamento, exibindo tanto efeitos aditivos quanto sinérgicos à radioterapia, terapias-alvo (p. ex. antihormonioterapia, transplante) e imunoterapia. A quimioterapia tradicional empregada no tratamento do câncer inclui os inibidores de microtúbulos (alcaloides da vinca, taxanos e agentes relacionados), agentes alquilantes

(mostardas nitrogenadas e não nitrogenadas, agentes da platina e antibióticos antitumorais), inibidores da topoisomerase e antimetabólitos (GOMEZ & TORRES, 2017).

Os inibidores de microtúbulos atuam paralisando a mitose na metáfase, interrompendo a divisão celular. Neste grupo estão incluídos os alcalóides da vinca (vincristina, vimblastina e vindesina), taxanos (paclitaxel e docetaxel) e os derivados da podofilotoxina (etoposídeo e teniposídeo) (BLACK & LIVINGSTON, 1990b; WONDER et al. 2016). Os agentes alquilantes são compostos que impedem a replicação através da ligação com o DNA, inibindo a separação dos dois filamentos de DNA. Apesar de efetivos em inúmeras formas de câncer, eles raramente produzem efeito clínico sem a combinação com outros agentes fase-específicos do ciclo celular. Dentre os alquilantes destacam-se a mostarda nitrogenada, mostarda fenil-alanina, ciclofosfamida, bussulfam, nitrosuréias e agentes da platina, como cisplatina e a ifosfamida (BENDER et al., 1978; WONDER et al. 2016). Os antibióticos antitumorais, presentes na classe dos agentes alquilantes, são um grupo de substâncias que apresentam anéis insaturados que permitem a incorporação de excesso de elétrons e, consequentemente, a produção de radicais livres, causando a morte celular. Exemplo destes antibióticos são a mitomicina C, actinomicina D, daunurrobicina e doxorrubicina. Os inibidores da topoisomerase são enzimas nucleares que levam ao bloqueio do passo de ligação do ciclo celular gerando quebra de cadeia simples e dupla de DNA. A introdução dessas rupturas leva subsequentemente à morte celular programada (apoptose). Neste grupo estão as antraciclinas e o campotecano (SINGH et al. 2016). E por fim, os antimetabólitos são uma classe de drogas que inibem a biossíntese dos componentes essenciais do DNA e do RNA, especificamente a síntese do folato ou de purina ou pirimidina, impedindo a multiplicação celular. Dentre os antimetabólitos destacam-se o metotrexato, 5-fluorouracil, 6-mercaptopurina e a pentostatina (BLACK & LIVINGSTON, 1990a; WONDER et al. 2016).

A quimioterapia não é seletiva, portanto afeta todas as células em divisão, produzindo efeitos colaterais, principalmente em tecidos sadios com alta taxa de renovação. Os efeitos adversos mais comuns são: náusea, vômito, perda de apetite, constipação, hemorragias, fadiga, feridas na boca e na garganta (mucosite), mielossupressão, alopecia, neuropatia, perda de equilíbrio, cefaleia, alterações visuais, alterações de memória, toxicidade hepática e renal e problemas sexuais (como alteração na infertilidade e na libido). A janela terapêutica deste tipo de fármaco é estreita e, consequentemente, os pacientes são expostos aos efeitos indesejados do tratamento para atingir uma melhor resposta antitumoral (GOMEZ & TORRES, 2017).

Outra opção terapêutica que vêm sendo muito estudada é a quimioprevenção, definida como o uso sistêmico de agentes químicos naturais ou sintéticos para reverter ou suprimir a progressão de lesões pré-malignas para carcinomas invasores. Os agentes quimiopreventivos devem ser capazes de atuar preferencialmente dentro dos processos de iniciação e promoção da carcinogênese. Uma variedade de fitoconstituintes tem sido explorada pelo seu potencial de prevenir a ocorrência de carcinogênese tanto *in vitro* quanto *in vivo* por meio de diversas abordagens celulares e moleculares (KAUR et al., 2018). Exemplos de compostos naturais quimiopreventivos são a capsaicina, curcumina, licopeno, ácido ursólico e a epigalocatequina-3-galato presente no chá verde (Ko & Moon, 2015). Sabe-se que agentes quimiopreventivos inibem a angiogênese, a produção de ROS e a invasão de tumores primários e, portanto, poderiam ser utilizados para inibir a carcinogênese (SIDDIQUI et al., 2015).

No entanto, mesmo com a descoberta de muitas drogas antineoplásicas nas últimas décadas, a terapia ainda é insatisfatória devido à baixa eficácia, à quimiorresistência e efeitos adversos (JEMAL et al., 2011; Leo, 2008). Uma droga que seja minimamente citotóxica às células normais do organismo tem se tornado foco de pesquisadores, e, neste ponto, o uso de fontes naturais como uma terapia alternativa para o câncer tem ganhado grande destaque para o controle do câncer e da destruição dos mecanismos envolvidos na carcinogênese. Neste sentido, os compostos naturais, principalmente de plantas, representam um grande valor terapêutico, pois têm sido usados na medicina tradicional para muitas doenças há milhares de anos, em especial para o tratamento do câncer. Devido à diversidade estrutural, eles representam uma boa alternativa devido às vastas propriedades biológicas e à alta afinidade dos metabólitos secundários aos receptores biológicos (GINSBURG & DEHARO, 2011). Essa afinidade confere aos produtos naturais perfis de toxicidade relativamente baixos e alta afinidade em células tumorais (GREENLEE, 2012). Quase 80% de todos os medicamentos aprovados pela *Food and Drug Administration* dos Estados Unidos durante as últimas três décadas para terapia do câncer, são produtos naturais *per se* ou são semi-sintéticos derivados de produtos naturais (BISHAYEE & SETHI, 2016). Dessa forma, os produtos naturais são uma excelente fonte de novas estruturas para o desenvolvimento de drogas antineoplásicas.

1.3.1. Salvia lachnostachys e fruticulina A

O gênero *Salvia* L. é o maior na família Lamiaceae, com aproximadamente 1000 espécies encontradas em áreas subtropicais e climas temperados, como América Central e do Sul, Ásia Central/ Mediterrâneo e leste da Ásia (WALKER et al., 2004). Essas espécies possuem cerca de 30 a 150 cm de altura, são geralmente herbáceas e perenes, raramente bienais ou anuais e com flores atraentes em várias cores (TOPÇU, 2006). O nome *Salvia* vem da palavra em latim "*salvare*" que significa curar, devido a várias espécies dessa planta serem utilizadas desde os tempos antigos no tratamento de mais de sessenta doenças, como acne, epilepsia, bronquite, constipação, hemorragias, úlceras e desordens menstruais (PICCINELLI et al., 2014; TOPÇU, 2006). No Brasil, o gênero está representado por mais de 60 espécies de arbustos, subarbustos e ervas distribuídas nas regiões Sul, Sudeste e Centro-Oeste do país (WALKER et al., 2004).

Os principais constituintes de metabólitos secundários de espécies da *Salvia* são terpenóides e flavonóides. As partes aéreas dessas plantas contêm flavonóides, triterpenóides e monoterpenos, particularmente nas flores e folhas, enquanto diterpenos são encontrados principalmente nas raízes. No entanto, algumas espécies de *Salvia* também contêm diterpenos nas partes aéreas e triterpenóides e flavonas nas raízes (TOPÇU, 2006). Diversos estudos farmacológicos vêm indicando o valor terapêutico dos triterpenóides isolados de várias espécies da *Salvia* devido a sua ampla atividade biológica. Dentre essas atividades destacam-se: atividade antimicrobiana, antiviral, antioxidante, anti-inflamatória, hipoglicemiante, quimioprevenção e citotóxica. Uma das mais conhecidas plantas medicinais desse gênero é a *Salvia officinallis* L., nativa da região do Mediterrâneo mas também cultivada no Brasil, com propriedades anti-inflamatórias, antisséptica e gastroproterora (DANIELA, 1993).

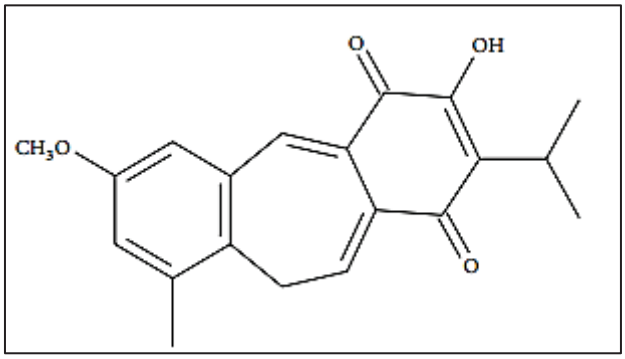
A *Salvia lachnostachys* Benth. (Fig 2) é uma planta herbácea e endêmica no sul do Brasil, mas ainda não há dados de uso na medicina tradicional do país (KASSUYA et al., 2009). Estudos relataram a composição química de óleos essenciais, o isolamento dos triterpenos ácidos ursólico e oleanólico e o diterpeno fruticulina A (fruti), a partir do extrato etanólico das folhas da *Salvia lachnostachys* (EES) (ERBANO et al., 2012; KASSUYA et al., 2009). Recentemente demonstrou-se que ambos, o EES e a fruti (Fig. 3), possuem atividade anti-inflamatória e antihiperalgésica em camundongos (PICCINELLI et al., 2014) e efeito antidepressivo em ratos (SANTOS et al., 2017). Além disso, estudos recentes demonstraram que o EES possui atividade antiartrítica sem causar dano toxicogenético em camundongos (RADAI et al., 2018), e atividade antitumoral *in vitro* contra várias linhagens de células tumorais humanas (OLIVEIRA et al., 2016).

FIGURA 2 - *Salvia lachnostachys* BENTH.

FONTE: MARIA ÉLIDA ALVES STEFANELLO, 2015, acervo pessoal.

Estudos demonstram um valioso valor terapêutico da fruti, como atividade anti-inflamatória e anti-hiperalgésica *in vivo* (BISIO et al., 2008; PICCINELLI et al., 2014; SCHITO et al., 2011), ação quimiopreventiva através da indução da atividade da quinona redutase e da inibição da desacetilação da histona em células de hepatoma murino (Hepa1c1c7) e de cérvix humano (HeLa) (GIACOMELLI et al., 2013) e atividade antitumoral *in vitro* contra as linhagens de células de glioma (U251), mama (MCF-7), ovário resistente a múltiplos fármacos (NCI-ADR/Res), rim (786.0), câncer de pulmão de células não pequenas (NCI-H460), próstata (PC-3), ovário (OVCAR-03) e queratinócitos (HaCat), com valores de inibição de crescimento 50% (GI_{50}) variando entre 3,7 e 7,4 μM (OLIVEIRA et al., 2016). Além disso, a demetil fruticulina A, outro composto presente em espécies como a *Salvia corrugata*, apresentou mecanismos pró-apoptóticos em linhagens tumorais humanas *in vitro*, onde se atribuiu esse efeito à geração de ROS em mitocôndrias (GIANNONI et al., 2010; MONTICONE et al., 2010). No entanto, até o momento, não há estudos sobre a atividade antitumoral de extratos da *Salvia lachnostachys* e de seus compostos isolados em modelo experimental de neoplasia *in vivo,* aspectos que são abordados neste trabalho.

FIGURA 3 - ESTRUTURA MOLECULAR DA FRUTICULINA A.



FONTE: ERBANO et al., 2012.

1.4. MODELO TUMORAL DE EHRLICH

O tumor de Ehrlich é uma neoplasia maligna de origem epitelial, que apareceu pela primeira vez espontaneamente no tecido mamário de camundongas, e foi descoberto por Paul Ehrlich em 1886. Somente em 1905, os pesquisadores Ehrlich e Apolant, usaram-no como um tumor experimental através do transplante de tecidos tumorais entre camundongos por via subcutânea (OZASLAN et al., 2011). Em 1932, os pesquisadores Loewenthal e Jahn obtiveram uma variante especial do carcinoma de Ehrlich e observaram que após a inoculação na cavidade peritoneal de camundongos de uma suspensão dessas células, não só obtiveram tumores na forma sólida, mas também um fluido ascítico que continha um grande número de células neoplásicas, sendo facilmente transmissível a outros animais. A partir disso, os pesquisadores denominaram essa forma tumoral como “tumor ascítico de Ehrlich” (KLEIN, 1950). Desde então, o tumor ascítico de Ehrlich vem sendo amplamente estudado em diferentes propósitos. O tumor de Ehrlich cresce em camundongos na forma ascítica quando inoculado por via intraperitoneal, aumentando a ‘virulência’ tumoral através de passagens repetidas, e quando inoculado por via subcutânea apresenta-se na forma sólida (BAILLIF, 1954).

É um tumor originalmente hiperdiplóide[1] e de rápido crescimento, tem uma elevada capacidade transplantável, não regride, e possui especificidade para camundongos (BAILLIF, 1954; KLEIN, 1950; OZASLAN et al., 2011). O carcinoma de Ehrlich se assemelha a tumores humanos que são sensíveis à quimioterapia, devido ao fato de ser indiferenciado e ter crescimento rápido (OZASLAN et al., 2011). Estudos já demonstraram que após a inoculação subcutânea no membro posterior de camundongos

[1] Célula ou indivíduo com um ou mais cromossomos ou segmentos cromossômicos adicionados.

foi possível observar o desenvolvimento do tumor sólido em 100% dos animais em até 14 dias após a inoculação (ABDIN et al., 2014; BASSIONY et al., 2014) e que podem ser mantidos até 42 dias após a inoculação para avaliações experimentais, uma vez que não denota um tumor agressivo (BASSIONY et al., 2015). Também tem sido relatado crescimento de tumor sólido após inoculação subcutânea na região da cabeça, na região dorsal e nas patas (PEREIRA et al., 2014; MIRANDA-VILELA et al., 2014; MIRANDA-VILELA et al., 2011; NASCIMENTO et al., 2006; PEREIRA et al., 2013).

Pelas características supracitadas, este modelo foi escolhido para avaliar os efeitos antitumorais do EES e da fruti *in vivo*, através de técnicas farmacológicas, bioquímicas e de biologia molecular.

1.5. LINHAGENS CELULARES: MCF-7 E HEPG2

O câncer de mama é a neoplasia mais frequente em mulheres no mundo todo (BRAY et al., 2018). Devido ao seu grande impacto na população, esta doença representa um problema de saúde pública que requer pesquisas adicionais em nível molecular para definir seu prognóstico e tratamento específico. A pesquisa básica é necessária para realizar esta tarefa e isso envolve linhagens celulares (COMŞA et al., 2015). A MCF-7 é uma linhagem celular de câncer de mama (carcinoma) comumente utilizada como fonte de pesquisa há vários anos e por diversos grupos de pesquisa (Lee et al., 2015). As células MCF-7 foram isoladas do derrame pleural de uma mulher de 69 anos de idade com câncer de mama metastático (SOULE et al., 1973), pelo Dr. Soule e seus colegas no *Michigan Cancer Foundation*, de onde deriva seu nome (MCF). É uma célula que contém receptor de estrogênio, progesterona, androgênio e glicocorticoide (HORWITZ et al., 1975). A MCF-7 é uma linhagem não agressiva e não invasiva, normalmente considerada com baixo potencial metastático (COMŞA et al., 2015). Devido a suas características moleculares, é amplamente utilizada como modelo *in vitro* na pesquisa do câncer de origem de carcinoma. Além disso, como o modelo *in vivo* de carcinoma sólido de Ehrlich, utilizado nesse trabalho, é de origem mamária, as células MCF-7 também foram escolhidas para investigação dos efeitos da fruti *in vitro*, para assemelhar-se com o modelo *in vivo*.

O carcinoma hepatocelular, ou hepatocarcinoma, é o terceiro tipo de câncer que mais levou a óbito no mundo todo e o segundo mais incidente em homens em 2018 (BRAY et al., 2018). A hepatocarcinogênese é um sistema complexo que envolve múltiplas vias

moleculares resultando em crescimento desordenado dos hepatócitos, denotando alto grau de malignidade e agressividade (NAKAGAWA & MAEDA, 2012). Dessa maneira, torna-se necessário a pesquisa básica adicional para propor novas opções terapêuticas, bem como estudar os inúmeros mecanismos moleculares envolvidos nessa patologia. As células de carcinoma hepatocelular humano (HepG2) são as mais utilizadas e representam uma opção para esse estudo. A HepG2, descoberta em 1970, é uma linhagem celular imortalizada que consiste em células de carcinoma hepático humano, derivadas do tecido hepático de um adolescente caucasiano de 15 anos de idade que tinha um carcinoma hepatocelular bem diferenciado (Fonte: ATCC). Essas células em cultivo mostram altas taxas de proliferação, morfologia epitelial e expressa uma variedade de funções metabólicas específicas do fígado, como o metabolismo do colesterol e triglicerídeos (ARELLANES-ROBLEDO et al., 2017). Portanto, representa uma ferramenta de estudo para investigar compostos com efeito antitumoral em nível molecular. Deste modo, essa linhagem tumoral também foi escolhida neste trabalho pelas características moleculares e também para investigar a possível ação antitumoral da fruti em outro tipo de câncer, além do câncer de mama.

Dessa maneira, devido às características supracitadas, as células MCF-7 e HepG2 foram escolhidas para avaliar o efeito antitumoral da fruti *in vitro* e investigar os possíveis mecanismos de ação da fruti em células tumorais.

1.6. HIPÓTESE

Com base em dados da literatura do EES e da fruti citados no item 1.3.1, a hipótese deste trabalho é que tanto o extrato etanólico das folhas da *Salvia lachnostachys*, quanto o diterpeno isolado desse extrato, a fruticulina A, têm efeito antitumoral em modelo de neoplasia em camundongos *in vivo*, desencadeado por uma ou mais vias de morte celular. A confirmação dessa hipótese pode representar uma nova opção de terapia e prevenção para o tratamento de câncer sólido, especialmente câncer de mama e de fígado. Para tanto, estudos *in vivo* e *in vitro* foram realizados, aplicando técnicas farmacológicas, bioquímicas e de biologia celular e molecular.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar a atividade antitumoral do EES e da fruti, em modelo de carcinoma sólido de Ehrlich em camundongos, bem como a ação quimiopreventiva do EES, e os possíveis mecanismos de ação envolvidos nesses efeitos. Além disso, avaliar o mecanismo de ação antitumoral *in vitro* da fruti.

2.2. Objetivos específicos

1. Avaliar o efeito antitumoral do EES e da fruti no modelo de Ehrlich através das medidas do tumor e histologia tumoral;
2. Avaliar a ação quimiopreventiva do EES no modelo de Ehrlich;
3. Identificar os mecanismos de ação do EES e da fruti, avaliando marcadores no tecido tumoral de:
    - 3.1. Inflamação: determinar os níveis das citocinas TNF-α, IL-6, IL-10 e IL-4, além dos níveis das enzimas mieloperoxidase (MPO) e n-acetilglucosamina (NAG);
    - 3.2. Morte celular: avaliar apoptose quantificando a expressão gênica ou atividade de proteínas pró- (Caspase 3/7) e anti-apoptóticas (Bcl-2), e necroptose através da quantificação da expressão gênica de RIPK1;
    - 3.3. Angiogênese: através da quantificação da expressão gênica de VEGF e HIF1α;
    - 3.4. Proliferação celular: através da quantificação da expressão gênica e níveis de ciclina D1;
    - 3.5. Estresse oxidativo: através dos níveis ou atividade da GST, GSH, CAT, LPO, SOD e ROS total e da expressão gênica de Nrf2;
4. Avaliar a toxicidade dos tratamentos com EES e fruti:
    - 4.1. Analisar parâmetros de função hepática, renal e metabólica através da bioquímica plasmática;
    - 4.2. Determinar a DL_{50} e a toxicidade aguda com EES;
5. Avaliar o efeito antitumoral *in vitro* da fruti em células MCF-7 e HepG2, bem como os possíveis mecanismos de ação:

5.1. Determinar a viabilidade das células tratadas com fruti;

5.2. Determinar os níveis celulares de ROS e GSH;

5.3. Avaliar o efeito pró-apoptótico;

5.4. Determinar a expressão dos genes Ciclina D1, Bcl-2 e VEGF por qPCR, bem como os níveis proteicos através de Western blot;

5.5. Mensurar os níveis de STAT3 e STAT3 fosforilada através de qPCR e Western blot, respectivamente;

5.6. Mensurar os níveis de pJUNK por Western blot;

5.7. Determinar a expressão gênica de NF-κB;

5.8. Avaliar a necroptose através da expressão do gene RIPK1 em células tratadas com fruti, bem como a ação desse composto sobre a viabilidade celular frente ao co-tratamento com inibidor de RIPK1, Necrostatina-1.

3. ARTIGO CIENTÍFICO 1 - "*Salvia lachnostachys* Benth extract has antitumor and chemopreventive effects against solid Ehrlich carcinoma"

Artigo submetido ao periódico *Molecular Biology Reports*

Salvia lachnostachys Benth extract has antitumor and chemopreventive effects against solid Ehrlich carcinoma

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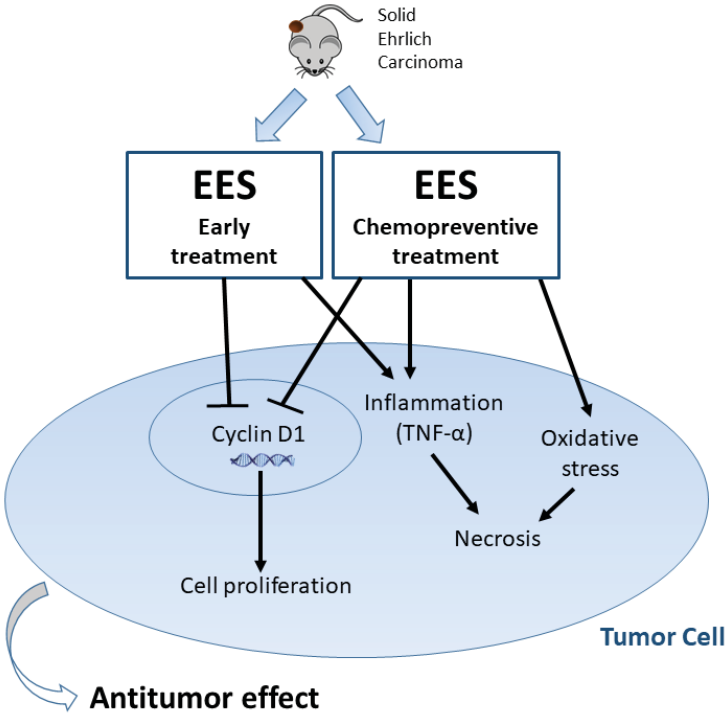
3.1. ABSTRACT

Salvia lachnostachys is an herbaceous plant with anti-inflammatory, analgesic and cytotoxic properties. This study investigated the antitumor effect of an ethanolic extract of *Salvia lachnostachys* leaves (EES) in a solid Ehrlich carcinoma model. Ehrlich cells were inoculated subcutaneously in the right pelvic member (2×10^6 cells) in female Swiss mice. The animals were treated with vehicle (10 mL kg^{-1}, p.o.), EES (30 and 100 mg kg^{-1}, p.o.), or methotrexate (2.5 mg kg^{-1}, i.p.) for 21 days (early treatment) or 14 days (late treatment) after tumor inoculation, or 10 days before tumor inoculation and continued for 21 days after tumor inoculation (chemopreventive treatment). The acute toxicity test was performed according OECD guidelines. Late treatment with EES had no antitumor effect. Early treatment with 100 mg kg^{-1} EES prevented tumor development, increased tumor necrosis factor-α (TNF-α) levels and decreased tumor superoxide dismutase (SOD) activity, interleukin-10 (IL-10) levels and *Cyclin D1* expression, and tumor cell necrosis was observed. Chemopreventive treatment with EES for 10 and 31 days prevented tumor development in the same manner. EES treatment for 31 days decreased hepatic and tumor SOD activity, tumor IL-10 levels and *Cyclin D1* expression and increased tumor reduced glutathione, *N*-acetylglucosaminidase, reactive oxygen species, lipid peroxidation, TNF-α levels and *Nrf2* expression. No toxicity was observed in the acute toxicity assay. In conclusion, EES had an antitumor effect by inhibiting *Cyclin D1* expression and increasing inflammation with early and chemopreventive treatment. Modulation of the antioxidant system also contributed for the antitumor effects of EES.

Keywords: *Salvia lachnostachys*; solid Ehrlich carcinoma; antitumor; *Cyclin D1*; antiproliferative; inflammation.

GRAPHICAL ABSTRACT



3.2. INTRODUCTION

Therapeutic approaches that are currently available for the treatment of solid cancer are mostly unsatisfactory because many patients have cancer recurrence and experience many side effects [1]. Investigations of new compounds as anticancer agents have continued. Natural compounds have substantial structural diversity and often present novel mechanisms of biological activity [2]. Drugs that are minimally cytotoxic to normal cells have become the focus of intense research. The use of natural products as alternative therapies for cancer has gained attention for cancer control and disruption of the mechanisms that are involved in carcinogenesis. Various anticancer agents that are available today are obtained from unmodified natural compounds or are semi-synthetically derived from natural products [2]. Natural products are an excellent sources of new structures for the development of anticancer drugs.

Pharmacological studies have indicated the therapeutic value of several *Salvia* species because of their wide biological activity, including antimicrobial, antiviral, antioxidant, anti-inflammatory, hypoglycemic, antitumor, and cytotoxic effects [3,4]. *Salvia lachnostachys* Benth is an herbaceous plant that is widespread in southern Brazil. It contains essential oils, diterpenes, and triterpenes in its leaves [3]. An ethanolic extract of this plant was described to have anti-inflammatory and antihyperalgesic activity and also exert antitumor activity *in vitro* against several cancer cell lines [5,6]. However, to date, no data are available about its antitumor effects *in vivo*. Thus, the present study investigated the *in vivo* antitumor effects and mechanism of action of an ethanolic extract of *Salvia lachnostachys* leaves (EES) in a solid Ehrlich carcinoma model in mice. Three different treatment protocols were tested. Biomarkers of oxidative stress, inflammation, proliferation, and apoptosis were investigated in tumor tissue. To study the possible systemic toxicity of EES, several blood parameters were analyzed, and an acute oral toxicity test was performed.

3.3. MATERIAL AND METHODS

3.3.1. Chemicals

Bovine serum albumin, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), xylenol orange, Tris ethylenediaminetetraacetic acid (Tris-EDTA),

TRIS-HCl, 2',7'-dichlorfluoresceindiacetate (DCFA), 4-nitrophenyl-*N*-acetyl-*β*-*D*-glucosaminide, *p*-nitrophenol, sodium nitrite, and tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue, 1-chloro-2,4-dinitrobenzene (CDNB), pyrogallol, ethanol, methanol, hexane (mixture of isomers), dichloromethane, ethyl acetate, acetone, ferrous ammonium sulfate, hydrogen peroxide, trichloroacetic acid, formaldehyde, acetic acid, ascorbic acid, *N,N*-dimethylformamide, formaldehyde, hydrogen peroxide, citric acid, sodium acetate, sodium chloride, potassium chloride, sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, dibasic potassium phosphate, hematoxylin, aluminium potassium phosphate, mercuric oxide, eosin, dimethylsulfoxide, sulphanilamide, phosphoric acid, naphthylethylenadiamide, Tween 20, sulfuric acid, Triton X-100, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Vetec (Rio de Janeiro, Brazil). The Bradford Protein Assay was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Cytokines (tumor necrosis factor α [TNF-α], interleukin-4 [IL-4], IL-6, and IL-10) kits were purchases from BD Bioscience (Franklin Lakes, NJ, USA). Aspartate (AST), alanine transaminase (ALT), and urea kits were purchased from Kovalent (São Paulo, Brazil). TriZol and primers were obtained from Invitrogen-ThermoFisher (Waltham, MA, USA). The High Capacity cDNA Reverse Transcription Kit and SYBR Green PCR Master Mix were obtained from Applied Biosystems-ThermoFisher (Waltham, MA, USA).

3.3.2. Plant material

Leaves of *Salvia lachnostachys* Benth were collected in Curitiba, Parana, Brazil (25°30'44.6"S, 49°18'7.13"W) in 2010. The plant was identified by Elide Pereira dos Santos, who deposited a voucher specimen (UPCB 85285) in the Herbarium at Federal University of Parana.

3.3.3. Extraction of EES

Dried and powdered leaves (415.3 g) were extracted at room temperature with hexane to separate polar metabolites, followed by ethanol extraction. The solvents were removed under reduced pressure to give the crude extracts in hexane (11.3 g) and ethanol (EES, 47.6 g), which were used for the biological assays.

A sample of EES (45.0 g) was submitted to silica gel vacuum liquid chromatography (VLC), followed by elution with hexane, hexane: dichloromethane 1:1, dichloromethane, acetone, and methanol. After solvent removal the respective fractions were obtained. The main compounds of the fraction eluted with hexane (1.8 g) were fruticuline A and fruticuline B, as previously reported [6]. The fraction eluted with dichloromethane, (2.07 g) was submitted to silica gel column chromatography (CC) eluted with mixtures of hexane-ethyl acetate in increasing polarity order, to give four subfractions (C_{1-4}). An aliquot (44.5 mg) of subfraction C_2 (385.6 mg) yielded demethylfruticuline A (24.6 mg), and oleanolic acid (2.0 mg), after preparative TLC in hexane-Me_2CO 8:2. Subfraction C_3 (648.5 mg) furnished a mixture of oleanolic and ursolic acids. Subfraction C_4 (449.2 mg) yielded almost pure ursolic acid. An aliquot (1.00 g) of fraction obtained with acetone (22.1 g) was submitted to silica gel CC, eluted with ethyl acetate to give five subfractions (D_{1-5}). Subfraction D_5 (170.1 mg) yielded rosmarinic acid.

HPLC fingerprint analyses of EES were performed on a Waters HPLC apparatus, equipped with 2998 photodiode array detector (PDA). For all analyses, a nucleosil 100-5 C18 column (250 mm x 4.6 mm, 5µm particle size) was used. The mobile phase consisted of H_2O with 0.05% trifluoroacetic acid (A) and methanol (B), applied in a linear gradient from 50:50 (A:B) to 100 (B), over 40 min, followed by isocratic elution with B for 30 min. The flow rate was 0.8 mL min^{-1}, at room temperature. The column effluent was monitored at 211 and 254 nm. Compounds isolated from EES were used as external standards.

3.3.4. DPPH assay

The procedure was the same as described by Blois [7] and Chen et al. [8], with minor modifications. To evaluate the free radical scavenging activity of EES, different concentrations (1, 3, 10, 30, 100, 300, and 1000 µg mL^{-1}) were mixed with DPPH methanolic solution (10 µg mL^{-1}). In the presence of a scavenger molecule, DPPH is reduced, producing a yellow solution. Ascorbic acid (50 µg mL^{-1}) was used as the positive control, and distilled water with 0.1% Tween 20 was used as the negative control. Absorbance was measured at 517 nm using a multi-mode microplate reader (BioTek Synergy HT, BioTek Instruments, Highland Park, VT, USA).

3.3.5. Animals

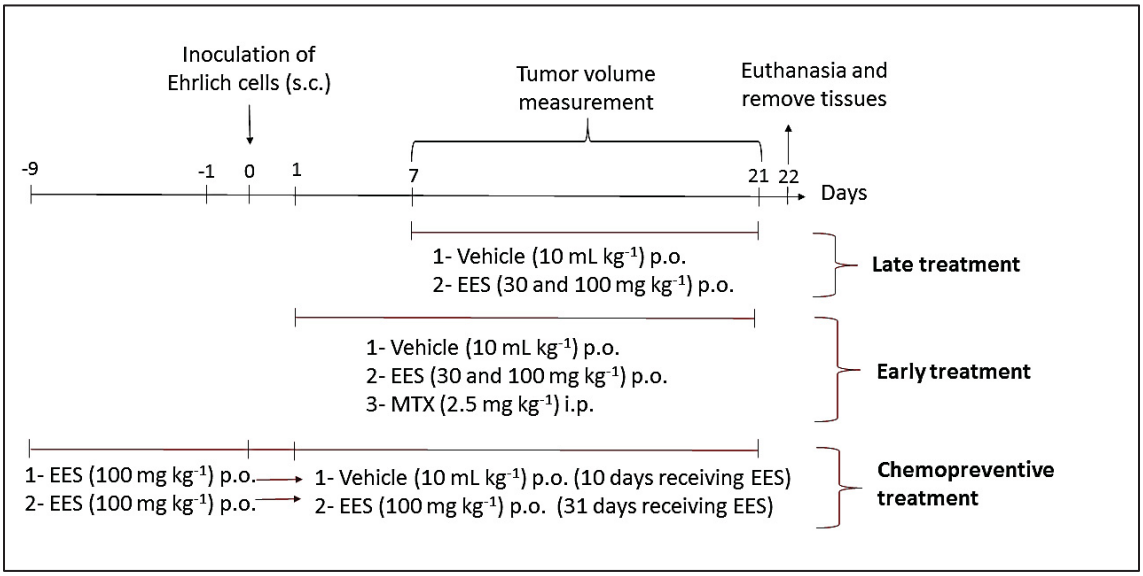
All experiments were approved by the Ethics Committee of Animal Experimentation (CEUA/BIO, UFPR; no. 879). The experiments were conducted using female Swiss mice (*Mus musculus*), weighing 25-30 g. Animals were housed 7 per cage with wood shaving bedding and free access to food and water. Environmental enrichment was used in all cages along experiments to improve the mice welfare, and all animals remain active and with normal mobility throughout the 3 weeks of experiment. The animals were maintained under controlled temperature (22ºC) and a 12 h/12 h light/dark cycle and were randomized to the treatment groups before treatment.

3.3.6. Tumor inoculation and experimental design

Ehrlich cells (2×10^6 cells/animal) were dissolved in 0.2 mL of phosphate-buffered saline (PBS) (16.5 mM phosphate, 137 mM NaCl, and 2.7 mM KCl) and intraperitoneally inoculated in the mice. Six days after inoculation, the animals were euthanized by cervical dislocation. Cells were aseptically collected, and cellular viability was verified using Trypan blue dye in a Neubauer chamber. After three passages, the Ehrlich cells reached ≥ 95% cell viability. Afterward, the cells were subcutaneously inoculated (2×10^6 cells/animal in a volume of 0.2 mL) in the right pelvic member. The animals were orally treated with 10 mL kg^{-1} of vehicle (0.1% Tween 20 and distilled H_2O) as the negative control group or 30 and 100 mg kg^{-1} EES (diluted in 0.1% Tween 20 and distilled H_2O). The positive control group was intraperitoneally treated with 2.5 mg kg^{-1} methotrexate (MTX; diluted in distilled H_2O) every 3 days (i.e., twice weekly) [9]. Three different treatment regimens were employed: (*i*) treatment started 1 day after tumor inoculation and continued for 21 days after tumor inoculation, for a total of 21 days of treatment (early treatment), (*ii*) treatment started 7 days after tumor inoculation and continued for 14 days after tumor inoculation, for a total of 14 days of treatment (late treatment), and (*iii*) treatment started 10 days before tumor inoculation and continued for 21 days after tumor inoculation, for a total of 31 days of treatment (chemopreventive treatment; Fig 1). During the treatments, body weight and tumor volume were assessed daily. The tumor volume was calculated using the standard formula: $V(cm^3) = L\,x\,W^2 x\,0.52$, where *L* is the largest tumor diameter, and *W* is the smallest tumor diameter (in centimeters) [10]. On day 22, the mice were fasted for 16 h and intraperitoneally anesthetized with ketamine (90 mg kg^{-1}) and xylazine (10 mg kg^{-1}) (both from Vetnil Industry and Trade of Veterinary Products, São Paulo, Brazil). Blood was collected from the inferior cava vein for plasma biochemical and hematological analyses.

The liver was subsequently harvested, and the animals were euthanized by puncture of the diaphragm. The tumor and other organs were harvested, weighed, and immediately stored at -80°C for further analysis.

FIGURE 1 - EXPERIMENTAL DESIGN FOR TESTING THE ANTINEOPLASTIC EFFECT OF EES IN MICE THAT BEAR SOLID EHRLICH TUMORS.



LEGEND: EES, ethanolic extract from *Salvia lachnostachys*; MTX, methotrexate; p.o., oral; i.p., intraperitoneal; s.c., subcutaneous.

3.3.7. Oxidative stress parameters

To analyze the redox status of tumor nodules and liver (i.e., the main organ that is involved in xenobiotic biotransformation), the tissues were homogenized in potassium phosphate buffer (pH 6.5, 1:10) and centrifuged at 10000 × *g* at 4°C for 20 min. The homogenate was used to measure GSH [11] and lipid peroxidation (LPO) [12]. The supernatant was used to measure superoxide dismutase (SOD) [13], catalase (CAT) [14], and glutathione *S*-transferase (GST) [15] activity and the levels of reactive oxygen species (ROS) [16] and proteins [17].

3.3.8. Inflammatory parameters

All the inflammatory parameters were measured in tumor tissue. Homogenates were prepared in PBS buffer (pH 7.2) and centrifuged at 9000 × *g* at 4ºC. The supernatant was used to measure nitric oxide (NO) [18] and cytokines (tumor necrosis factor α [TNF-α], interleukin-4 [IL-4], IL-6, and IL-10) according to the manufacturer's instructions (Kits

BD Bioscience). The pellet was homogenized in 0.1% saline-Triton X, and the supernatant was used to measure *N*-acetylglucosaminidase (NAG) [19].

3.3.9. RT-qPCR analysis

The expression of target genes was assessed in tumor samples to evaluate apoptosis, angiogenesis, and proliferation. Total RNA was isolated using TriZol reagent (Invitrogen), and cDNA was performed with 1000 ng RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primers (Invitrogen) were prepared with the following sequences: glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*; forward, 5'-GGTGAAGCAGGCATCT; reverse, 5'-TGTTGAAGTCGCAGGAG), *Nrf2* (forward, 5'-GTGGATCCGCCAGCTACTCCCA; reverse, 5'-TGGGGATATCCAGGGCAAGCGA), *Bcl-2* (forward, 5'-CACTTGCCACTGTAGAGA; reverse, 5'-GCTTCACTGCCTCCTT), *Ccnd1 (Cyclin D1)* (forward, 5'-AGAAGTGCGAAGAGGAG; reverse, 5'-GGATAGAGTTGTCAGTGTAGAT), and *Vegf* (forward, 5'-ACTGGACCCTGGCTTTACTGCT; reverse, 5'-TGATCCGCATGATCTGCATGGTG). The primer concentration was 800 nM for all of the reactions, and cDNA standardization was performed with a 1:5 cDNA dilution. Relative expression levels were calculated using *Gapdh* gene expression as the endogenous control for data normalization.

3.3.10. Histopathology

Tumor tissue was fixed in ALFAC solution (85% ethanol, 10% formaldehyde, and 5% glacial acetic acid) at room temperature for 16 h. Fixed tissue was dehydrated in xylene and ethanol and embedded in paraffin. Samples were sectioned at 5 μm thickness and stained with hematoxylin and eosin. The analyses were performed by a pathologist using optical microscopy with four slides per group of mice. Coagulative necrosis, inflammatory infiltration, apoptosis, and cytological characteristics were evaluated. The histological alterations were standardized in this work and classified by intensity scores: mild (+), moderate (++), and intense (+++). Images were acquired microscopically using an Axio Imager Z2 epifluorescent microscope (Carl Zeiss, Jena, Germany) that was equipped with an automated slide scanner (MetaViewer version 2.0.100, MetaSystems, Altlussheim, Germany).

3.3.11. Hematological and plasma parameters

Blood samples were used to generate complete hemograms using a BC2800-Vet (BioBrasil, São Paulo, Brazil) automated device. Plasma samples were obtained after blood centrifugation at 1.344 × *g* for 5 min. The parameters were analyzed using an automated system (Mindray BS-200, Shenzhen, China). The samples were used to determine urea, creatinine, glucose, total protein, albumin, globulin, ALT, and AST levels using commercial kits (Kovalent, São Gonçalo, Brazil).

3.3.12. Determination of acute toxicity and LD_{50}

Acute toxicity was assessed according to the Organization for Economic Cooperation and Development (OECD) Guidelines for Testing Chemicals [20] in accordance with Normative Resolution No. 17 of August 2014, which provides for a reduction of the number of animals for toxicological tests. Three female mice (18-22 g) were fasted for 4 h prior to the experiment. One hour before treatment, the animals had free access to food. The treatments began with 300 mg kg^{-1} EES (p.o.). As recommended, if no toxicity was observed, the dose was increased to 2000 mg kg^{-1} (p.o.). After administration, the animals were carefully observed for the first 30 min and then periodically for 24 h with special attention during the first 4 h. The animals were further observed daily for the next 14 days. Potential signs that were indicative of toxicity, such as diarrhea, tremors, salivation, convulsions, hypoactivity, ataxia, lethargy, bowing of the tail, bristly hair, lower food and water consumption, and lower body weight, were observed [21]. At the end of the *in vivo* observations, the animals were euthanized by cervical dislocation, and anatomopathological investigations were performed. The LD_{50} was calculated according to the OECD Acute Toxicity Assessment Procedure.

3.3.13. Data analysis

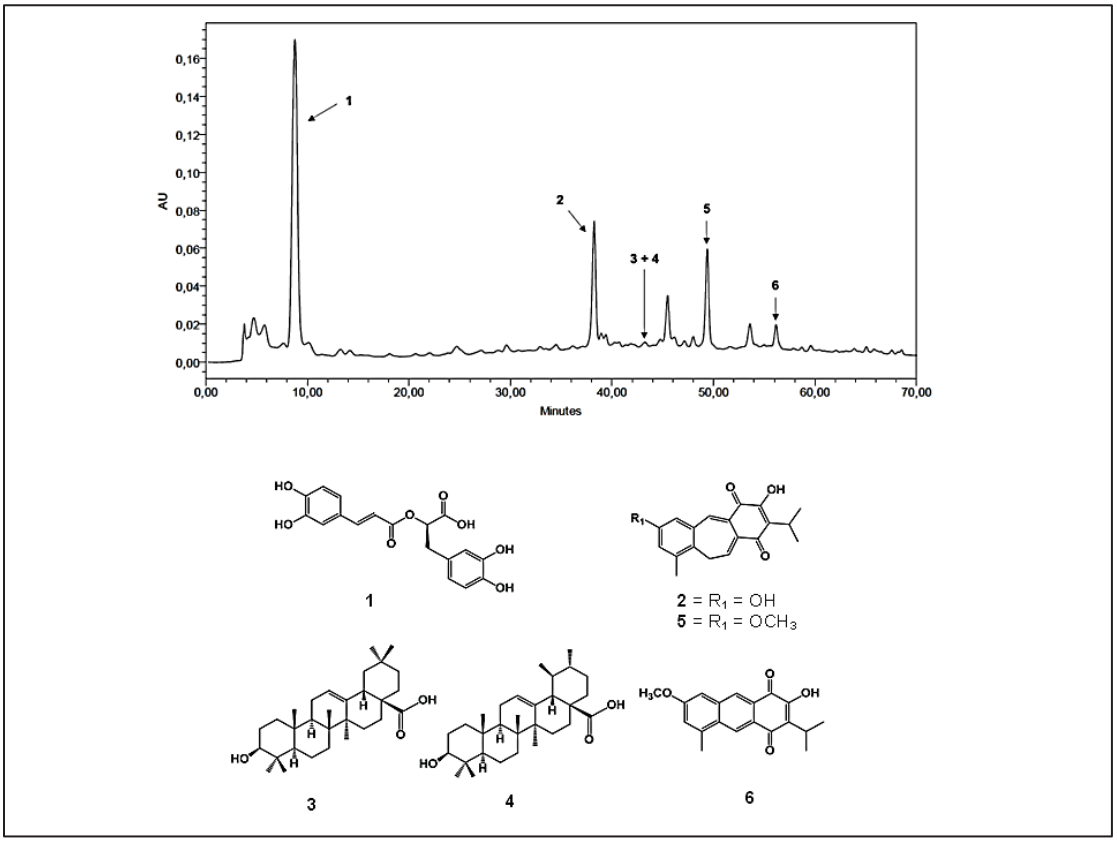
The data are expressed as mean ± standard error of the mean (SEM). The results were analyzed using *t*-tests or one- or two-way analysis of variance (ANOVA), followed by the Newman-Keuls *post hoc* test or Bonferroni's *post hoc* test as appropriate. The results were analyzed using GraphPad Prism 5.0 software. The level of significance was set at 95% ($p < 0.05$).

3.4. RESULTS

3.4.1. Chemical constituents of EES

The diterpenes fruticuline A and fruticuline B, as well as the triterpenes oleanolic acid and ursolic acid, had been previously reported as major constituents in the less polar fractions of EES [3, 6]. In addition, the known compounds demethylfruticuline A [22] and rosmarinic acid [23] were isolated in this work from more polar fractions. The HPLC profile of EES was recorded at 254 nm, and the following compounds were identified: rosmarinic acid (**1**, R_t 8.78 min), demethylfruticuline A (**2**, R_t 38.24 min), oleanolic and ursolic acids (**3** and **4**, co-elution, R_t 43.02), fruticuline A (**5**, R_t 49.39 min), and fruticuline B (**6**, R_t 56.17 min) (Fig 2).

FIGURE 2 - TYPICAL HPLC CHROMATOGRAM OF EES (254 nm), AND STRUCTURES OF THE ISOLATED COMPOUNDS.

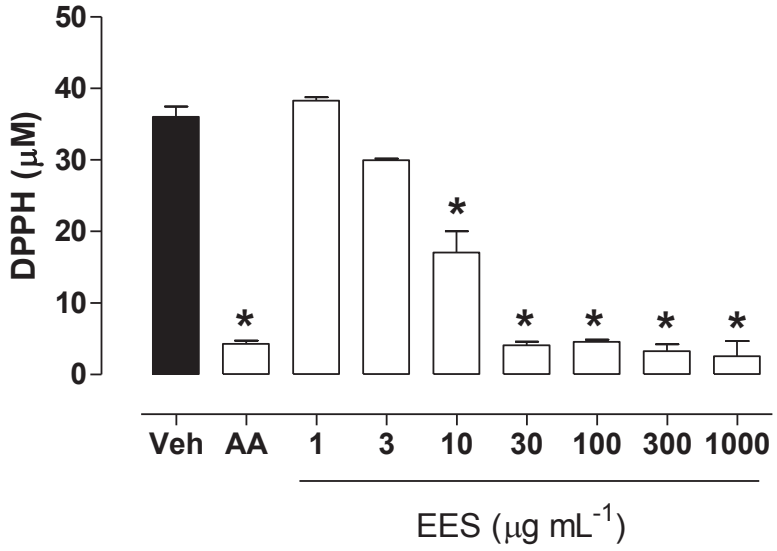


LEGEND: **1**: rosmarinic acid, **2**: demethylfruticuline A, **3**: oleanolic acid, **4**: ursolic acid, **5**: fruticuline A, and **6**: fruticuline B.

3.4.2. Effect of EES in the DPPH assay

EES at 10, 30, 100, 300, and 1000 µg mL^{-1} reduced DPPH radicals, decreasing absorbance by 53%, 89%, 87%, 91%, and 93%, respectively, compared with the vehicle group (Fig 3). Ascorbic acid (50 µg mL^{-1}), which was used as the positive control, reduced absorbance by 88% compared with the vehicle group. The half maximal efficient concentration (EC_{50}) of EES, calculated through the variable slope, was 6.38 µg mL^{-1}.

FIGURE 3 - *IN VITRO* DPPH RADICAL SCAVENGING ACTIVITY OF EES.



LEGEND: Samples were incubated with vehicle (distilled water with 0.1% Tween 20), 50 µg mL^{-1} acid ascorbic (AA), or 1, 3, 10, 30 100, 300, and 1000 µg mL^{-1} EES in the presence of DPPH. The reduction of DPPH levels was detected at 517 nm. The results are expressed as mean ± SEM (n = 3) and analyzed using one-way ANOVA followed by the Newman-Keuls *post hoc* test. *$p < 0.05$, compared with vehicle group.

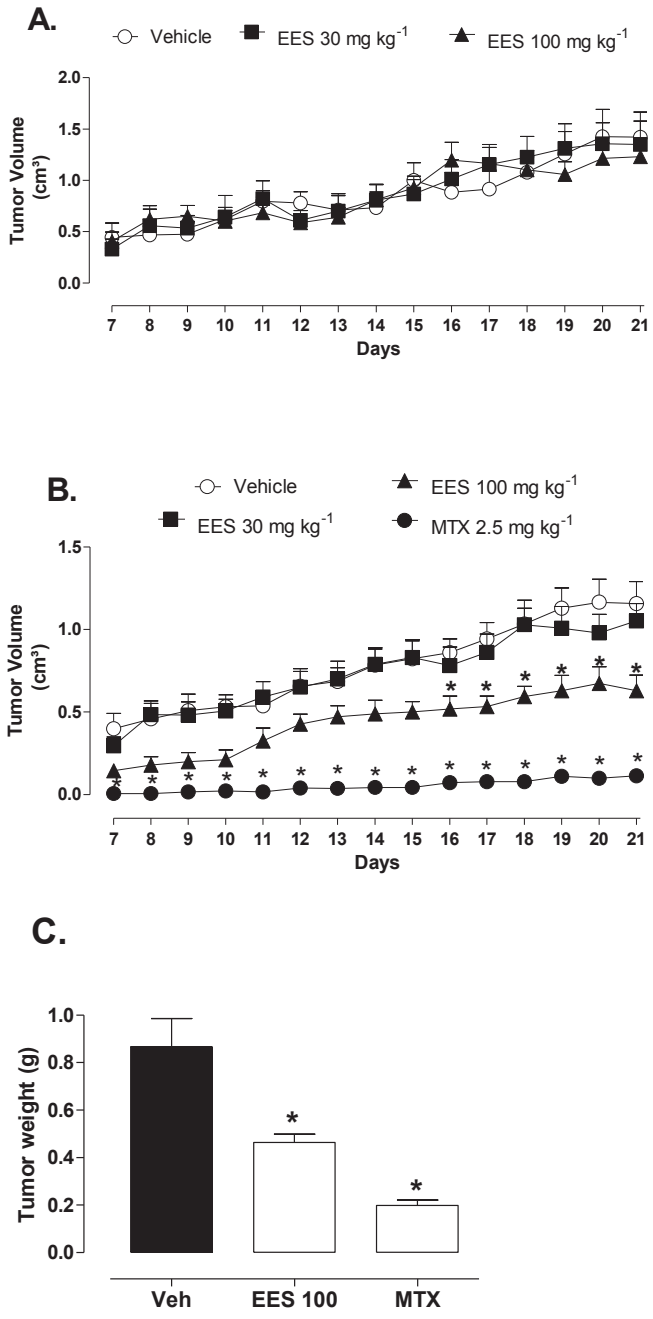
3.4.3. Effects of early and late treatment with EES on Ehrlich carcinoma model

3.4.3.1. Effect of EES on tumor development

Treatment with 30 and 100 mg kg^{-1} EES that started on day 7 after inoculation (late treatment) did not prevent tumor development (Fig 4A). When the treatment began on day 1 after tumor inoculation (early treatment), 100 mg kg^{-1} EES reduced tumor development, mainly from day 10 until day 16 until day 21 (40% and 45%, respectively) compared with the vehicle control group (Fig 4B). EES at a dose of 100 mg kg^{-1} decreased tumor weight by 40% compared with the control group (Fig 4C). The positive control, MTX, prevented tumor development from day 7 until day 21 (99% and 90%, respectively; Fig 4B) and

reduced tumor weight by 75% (Fig 4C) compared with the vehicle group. Based on these results, the subsequent experiments were performed using only the higher dose of EES (100 mg kg^{-1}), which was more effective against tumor development than the lower dose (30 mg kg^{-1}), in the early treatment regimen.

FIGURE 4 - EFFECT OF EARLY AND LATE TREATMENT WITH EES ON SOLID EHRLICH TUMOR VOLUME AND WEIGHT.



LEGEND: (**A**) Animals were orally treated with vehicle (10 mL kg^{-1}), EES (30 or 100 mg kg^{-1}), or MTX (2.5 mg kg^{-1}, i.p.) for 14 days. (**B**) The animals were treated for 21 days. (**C**) The animals were treated for 21

days, and on day 22 the tumor mass was removed and weighed. The results are expressed as mean ± SEM (n = 6-8) and were analyzed using two-way (A, B) or one-way (C) ANOVA followed by the Bonferroni or Newman-Keuls *post hoc* test, respectively. *$p < 0.05$, compared with vehicle group.

3.4.3.2. Effects of EES on oxidative stress and inflammatory parameters in liver and tumor tissue

Ehrlich tumor-bearing mice that were treated with vehicle presented an increase in the LPO rate in liver tissue (108% compared with naive group). Treatment with 100 mg kg^{-1} EES for 21 days after tumor inoculation decreased SOD activity in the liver and tumor tissue (42 and 46%, respectively; Table 1) and increased tumor GSH in 46%. No differences were observed in the other parameters.

In the EES group, a 52% increase in TNF-α levels and 61% decrease in IL-10 levels were observed compared with the vehicle group (Table 1). No differences were observed in NO, IL-4, or IL-6 levels.

TABLE 1 - OXIDATIVE STRESS AND INFLAMMATORY PARAMETERS IN EARLY TREATMENT IN LIVER AND TUMOR TISSUE

(Continued)

Parameters	Experimental groups		
	Naive	Veh	100 mg kg^{-1} EES
Oxidative stress			
Liver tissue			
GSH (μg g $tissue^{-1}$)	661.06 ± 22.28	558.08 ± 62.54	692.05 ± 79.16
SOD (U mg $protein^{-1}$)	201.00 ± 11.41	209.09 ± 5.82	120.09 ± 10.91*
GST (mmol min^{-1} mg $protein^{-1}$)	5.08 ± 0.31	6.11 ± 0.73	5.82 ± 0.21
CAT (nmol min^{-1} mg $protein^{-1}$)	226.04 ± 21.44	264.05 ± 20.44	283.03 ± 19.91
LPO [hydroperoxides] (nmol mg $protein^{-1}$)	0.93 ± 0.19	1.94 ± 0.16 #	1.84 ± 0.17
Tumor tissue			
GSH (μg g $tissue^{-1}$)		170.00 ± 11.99	217.09 ± 32.93*
SOD (U mg $protein^{-1}$)		187.07 ± 14.42	102.03 ± 14.13*
CAT (nmol min^{-1} mg $protein^{-1}$)		7.63 ± 1.02	9.29 ± 0.64
LPO [lipid hydroperoxides] (nmol mg $protein^{-1}$)		0.38 ± 0.03	0.36 ± 0.02
ROS [DCF] (Fluorescence intensity)		84.71 ± 0.91	86.86 ± 1.08

TABLE 1 - OXIDATIVE STRESS AND INFLAMMATORY PARAMETERS IN EARLY TREATMENT IN LIVER AND TUMOR TISSUE

(Conclusion)

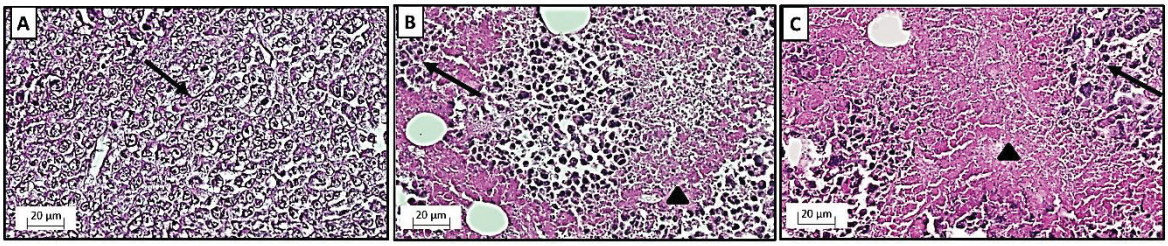
Parameters	**Experimental groups**		
	Naive	**Veh**	**100 mg kg^{-1} EES**
Inflammatory parameters			
Tumor tissue			
NAG (nmol min^{-1} g of tissue^{-1})		20.03 ± 1.30	19.97 ± 1.11
NO [Nitrite levels] (µmol g of tissue^{-1})		9.03 ± 1.36	9.42 ± 1.38
TNF-α (pg mL^{-1})		1470.00 ± 188.00	2238.00 ± 270.00*
IL-4 (pg mL^{-1})		458.07 ± 88.78	570.01 ± 79.82
IL-6 (pg mL^{-1})		65.55 ± 5.13	72.45 ± 23.71
IL-10 (pg mL^{-1})		3232.00 ± 521.00	1267.00 ± 205.06*

LEGEND: Animals were orally treated with vehicle (10 mL kg^{-1} of distilled water; naive and vehicle groups) or 100 mg kg^{-1} EES for 21 days. The results are expressed as mean ± SEM and were analyzed using one-way ANOVA followed by the Newman-Keuls *post hoc* test (n = 7-8) for the liver analysis and t-tests for the tumor analysis. *$p < 0.05$, compared with vehicle group; $^{\#}p < 0.05$, compared with naive group. GSH, reduced glutathione; SOD, superoxide dismutase; GST, glutathione *S*-transferase; CAT, catalase; LPO, lipoperoxidation; DCF, 2',7'-dichlorofluorescein; NAG, N-acetylglucosaminidase; NO, nitrite oxide; TNF-α, Tumor necrosis factor α; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-10, Interleukin-10.

3.4.3.3. Effect of EES treatment on tumor morphology

In the vehicle group, we detected viable Ehrlich cells, a small area of coagulative necrosis, and discrete inflammation, reflected by polymorphonuclear cell infiltration. Treatment with 100 mg kg^{-1} EES for 21 days induced further coagulative necrosis and discrete to high inflammation in tumor tissue. Apoptosis was observed only in the vehicle and 100 mg kg^{-1} EES groups. The MTX group exhibited no apoptosis, presented discrete inflammation, and had more areas of coagulative necrosis compared with the vehicle group (Fig 5).

FIGURE 5 - HISTOLOGY AND QUANTIFICATION OF HISTOLOGICAL ALTERATIONS OF EHRLICH TUMOR TISSUE STAINED BY HEMATOXYLIN AND EOSIN.



Parameters	Experimental groups		
	Vehicle	100 mg kg^{-1} EES	MTX
Apoptosis	+	+	-
Inflammation			
Grade 0 (absent)	+	-	-
Grade I (discrete)	++	++	+++
Grade II (moderate)	-	-	-
Grade III (high)	-	+	-
Coagulative necrosis			
Grade 0 (absent)	-	-	+
Grade I (0 - 20%)	+	-	-
Grade II (21 – 50%)	+	-	-
Grade III (51 – 80%)	+	++	++
Grade IV (81 – 100%)	-	+	-

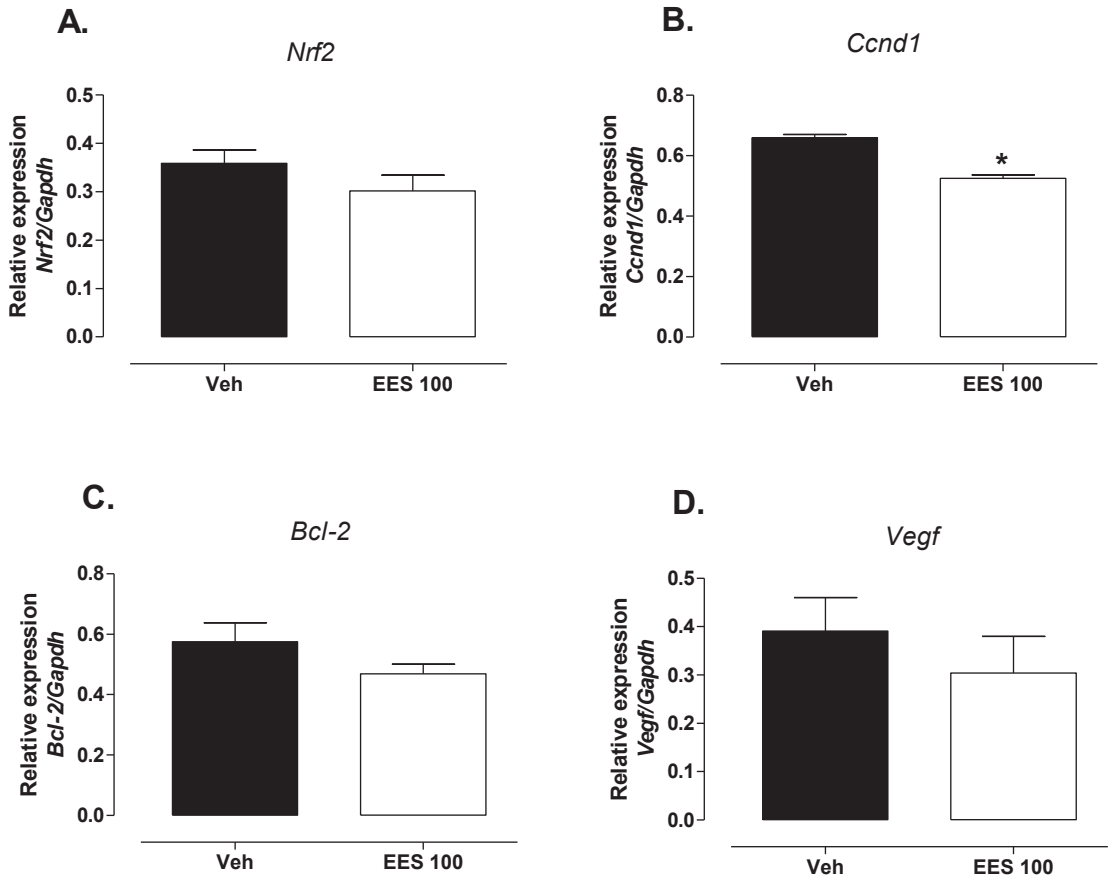
Scores: (-) negative; (+) mild; (++) moderate; (+++) intense.

LEGEND: Animals were orally treated with vehicle (**A**) or 100 mg kg^{-1} EES (**B**) or intraperitoneally treated with 2.5 mg kg^{-1} MTX (**C**) for 21 days. Arrows and arrowheads indicate viable Ehrlich cells and areas of coagulative necrosis, respectively. The samples were observed under an optical microscope at 20× magnification. Scale bar = 20 µm.

3.4.3.4. Effect of EES on tumor gene expression

Treatment with 100 mg kg^{-1} EES significantly decreased *Ccnd1* (*Cyclin D1)* expression in tumor tissue (20%) compared with the vehicle group (Fig 6). No differences were observed in *Nrf2*, *Bcl-2*, or *Vegf* expression.

FIGURE 6 - EFFECT OF EES EARLY TREATMENT ON GENE EXPRESSION IN TUMOR TISSUE.



LEGEND: (**A**) *Nrf2*. (**B**) *Ccnd1*. (**C**) *Bcl-2*. (**D**) *Vegf*. The animals were orally treated with vehicle or 100 mg kg^{-1} EES for 21 days. The results are expressed as mean ± SEM (n = 4) and were analyzed using unpaired t-tests. *$p < 0.05$, compared with vehicle group.

3.4.3.5. Effect of EES on plasma and hematological parameters

Ehrlich tumor-bearing mice exhibited an increase in AST, total protein, and albumin levels and a decrease in hematocrit values compared with the naive group. These alterations were also observed in EES- and MTX-treated animals. Treatment with 100 mg kg^{-1} EES and MTX increased monocyte concentrations compared with the vehicle group. The MTX group exhibited decreases in urea, total protein, globulin, erythrocytes, hemoglobin, and hematocrit compared with the vehicle group (Table 2).

TABLE 2 - PLASMA AND HEMATOLOGICAL PARAMETERS IN HEALTHY AND TUMOR-BEARING MICE.

Parameters	Experimental groups			
	Naive	Vehicle	100 mg kg^{-1} EES	MTX
Glucose (mg dL^{-1})	122.8 ± 12.17	129.5 ± 6.85	132.9 ± 6.85	119.8 ± 24.69
Urea (mg dL^{-1})	54.36 ± 2.31	57.57 ± 1.47	61.37 ± 1.87#	37.97 ± 2.55*, #
AST (U L^{-1})	86.89 ± 8.72	273.6 ± 18.97#	212.2 ± 19.64#	276.9 ± 42.0#
ALT (U L^{-1})	61.55 ± 4.90	64.51 ± 4.10	66.25 ± 6.91	52.57 ± 5.71
Total protein (g dL^{-1})	5.88 ± 0.06	6.61 ± 0.06#	6.65 ± 0.15#	4.38 ± 0.20*, #
Globulin (g dL^{-1})	2.83 ± 0.08	2.87 ± 0.11	2.87 ± 0.18	1.54 ± 0.38*, #
Albumin (g dL^{-1})	3.10 ± 0.07	3.81 ± 0.18#	4.08 ± 0.17#	2.78 ± 0.24#
Leukocytes (× 10^3 μL^{-1}	5.45 ± 0.87	5.17 ± 0.50	6.66 ± 0.52	5.83 ± 1.17
Lymphocytes (× 10^3 μL^{-1})	3.61 ± 0.61	3.76 ± 0.30	3.80 ± 0.19	3.73 ± 0.84
Monocytes (× 10^3 μL^{-1})	0.25 ± 0.06	0.22 ± 0.04	0.35 ± 0.05*, #	0.36 ± 0.07*, #
Erythrocytes (× 10^6 μL^{-1})	9.86 ± 0.11	9.51 ± 0.17	8.80 ± 0.21#	8.35 ± 0.32*, #
Hemoglobin (g dL^{-1})	13.25 ± 0.21	12.59 ± 0.29	12.39 ± 0.16	11.58 ± 0.38*, #
Hematocrit (%)	44.53 ± 1.12	41.38 ± 0.94#	38.36 ± 0.92#	35.13 ± 1.38*, #

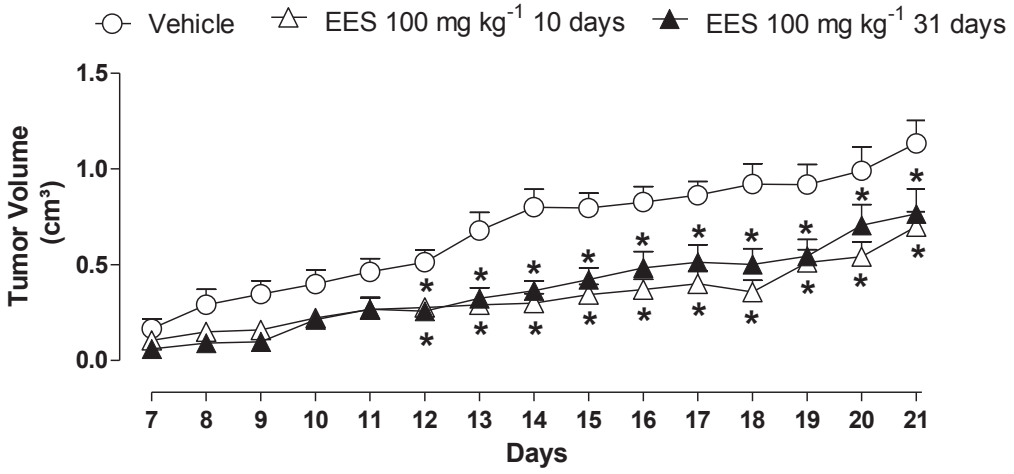
LEGEND: Animals were orally treated with vehicle (10 mL kg^{-1} of distilled water; naive and vehicle groups) or 100 mg kg^{-1} EES, and 2.5 mg kg^{-1} MTX, i.p., every 3 days for 21 days. The results are expressed as mean ± SEM and were analyzed using one-way ANOVA followed by the Newman-Keuls *post hoc* test (n = 7-8) for the liver analysis and *t*-tests for the tumor analysis. *$p < 0.05$, compared with vehicle group; #$p < 0.05$, compared with naive group. AST, aspartate aminotransferase; ALT, alanine aminotransferase; MTX, metothrexate.

3.4.4. Effects of chemopreventive treatment with EES in the Ehrlich carcinoma model

3.4.4.1. Effect of chemopreventive treatment with EES on tumor development

Prophylactic treatment with 100 mg kg^{-1} EES for only 10 days before tumor inoculation prevented tumor development. The statistical significance of this chemopreventive effect was evident from day 12 until day 21 after tumor inoculation compared with the vehicle group. Treatment with 100 mg kg^{-1} EES for 10 days before tumor inoculation and then for 21 days after tumor inoculation (i.e., 31 days of treatment) prevented tumor development (day 12: 53%; day 21: 31%) compared with the vehicle group (Fig 7). Interestingly, no differences in tumor growth were observed between 10 and 31 days of EES treatment in the chemopreventive regimen.

FIGURE 7 - EFFECT OF CHEMOPREVENTIVE TREATMENT WITH 100 MG KG^{-1} EES ON SOLID EHRLICH TUMOR VOLUME.



LEGEND: Animals were orally treated with vehicle (10 mL kg^{-1}) or EES (100 mg kg^{-1}) for 10 days or 31 days. The results are expressed as mean ± SEM (n = 6-8) and were analyzed using two-way ANOVA followed by the Bonferroni *post hoc* test. *p < 0.05, compared with vehicle group.

3.4.4.2. Effects of chemopreventive treatment with EES on oxidative stress and inflammatory parameters in liver and tumor tissue

Treatment with EES for 31 days decreased SOD activity in liver tissue (52%). In tumor tissue, treatment for both 10 and 31 days reduced SOD activity (24 and 14%, respectively) compared with the vehicle group. GSH content increased in both liver tissue (200%) and tumor tissue (181%) in the group that was treated with EES for 31 days. Increases in LPO levels (75%) and ROS levels (58%) in tumor tissue were observed in the group that was treated with EES for 31 days compared with the vehicle group (Table 3).

The group that was treated with EES for 31 days exhibited an increase in NAG activity (36%). Treatment with EES for both 10- and 31-days increased TNF-α levels (123% and 100%, respectively) and decreased IL-10 levels (67% and 65%, respectively) compared with the vehicle group. No differences in NO, IL-4, or IL-6 levels were observed (Table 3).

TABLE 3 - OXIDATIVE STRESS AND INFLAMMATORY PARAMETERS IN CHEMOPREVENTIVE TREATMENT IN LIVER AND TUMOR TISSUE.

Parameters	Experimental groups		
	Vehicle	100 mg kg^{-1} EES	
		10 days	31 days
Oxidative stress			
Liver tissue			
GSH (µg g $tissue^{-1}$)	251.04 ± 56.08	410.00 ± 76.89	754.03 ± 107.1*
SOD (U mg $protein^{-1)}$	608.02 ± 54.13	631.07 ± 22.06	289.04 ± 6.67*
GST (mmol min^{-1} mg $protein^{-1}$)	8.05 ± 0.70	7.73 ± 0.53	8.54 ± 0.52
CAT (nmol min^{-1} mg $protein^{-1}$)	178.07 ± 61.57	96.74 ± 16.04	142.06 ± 28.06
LPO (hydroperoxides) (nmol mg $protein^{-1}$)	2.72 ± 0.17	3.35 ± 0.24	2.84 ± 0.08
Tumor tissue			
GSH (µg g $tissue^{-1}$)	146.07 ± 31.96	215.05± 130.06*	412.08 ± 79.28*
SOD (U mg $protein^{-1}$)	592.07 ± 55.14	452.3± 25.52*	512.04 ± 9.42*
CAT (nmol min^{-1} mg $protein^{-1}$)	13.72 ± 1.55	12.51 ± 1.40	14.50 ± 1.85
LPO (lipid hydroperoxides) (nmol mg $protein^{-1}$)	4.99 ± 0.39	6.25 ± 1.27	8.69 ± 0.88*
ROS (DCF) (fluorescence intensity)	120.00 ± 8.14	109.07 ± 8.48	190.01 ± 34.03*
Inflammatory parameters			
Tumor tissue			
NAG (µmol min^{-1} g of $tissue^{-1}$)	3.61 ± 0.40	4.33 ± 0.14	5.37 ± 0.49*
NO [Nitrite levels] (µmol g of $tissue^{-1}$)	10.43 ± 1.07	10.15 ± 1.68	11.05 ± 1.95
TNF-α (pg mL^{-1})	1633.00 ± 269.06	3638.00 ± 274.09*	3271.00 ± 366.00*
IL-4 (pg mL^{-1})	283.09 ± 37.93	255.00 ± 47.68	377.05 ± 72.58
IL-6 (pg mL^{-1})	102.03 ± 7.34	113.04 ± 31.79	108.07 ± 18.06
IL-10 (pg mL^{-1})	2335.00 ± 609.08	773.02± 51.16*	828.05 ± 189.5*

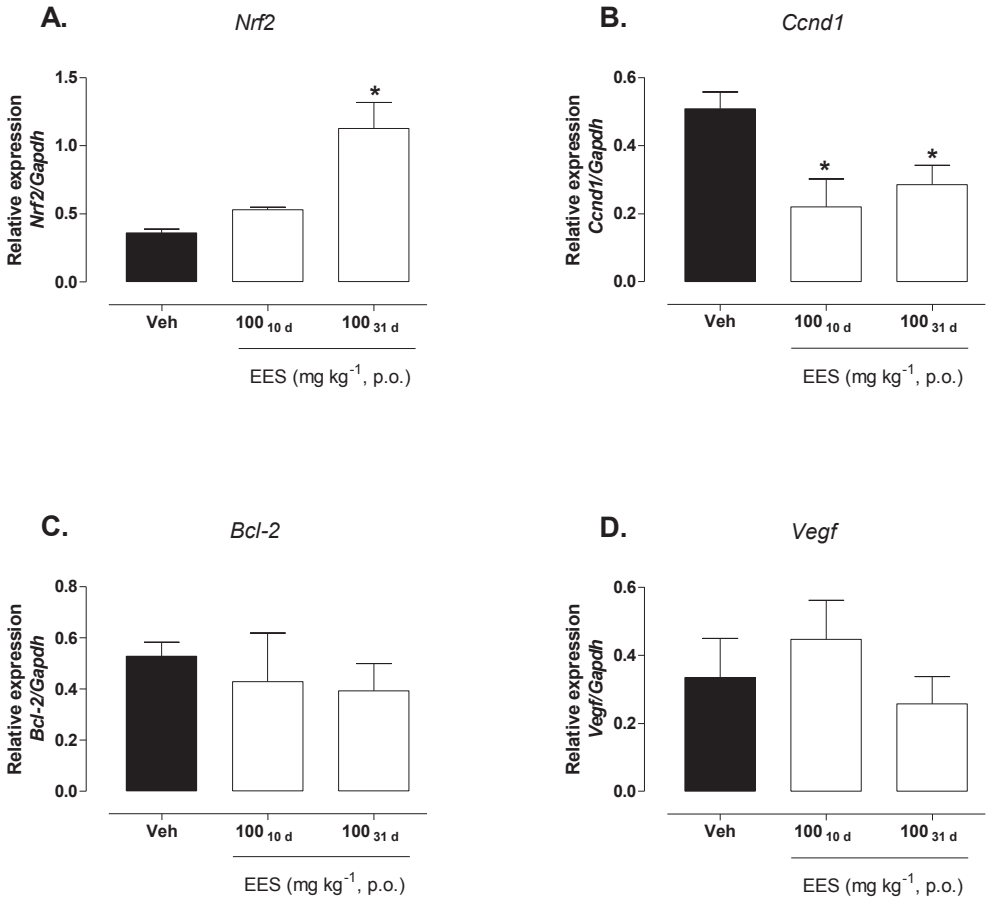
LEGEND: Animals were orally treated with vehicle (10 mL kg^{-1} distilled water) or 100 mg kg^{-1} EES for 10 or 31 days. The results are expressed as mean ± SEM and were analyzed using one-way ANOVA followed by the Newman-Keuls *post hoc* test (n = 5-7). *p < 0.05, compared with vehicle group. GSH, reduced glutathione; SOD, superoxide dismutase; GST, glutathione *S*-transferase; CAT, catalase; LPO, lipoperoxidation; ROS, reactive oxygen species; DCF, 2',7'-dichlorofluorescein. NAG, N-acetylglucosaminidase; NO, nitrite oxide; TNF-α, Tumor necrosis factor α; IL-4, Interleukin 4; IL-6, Interleukin 6; IL-10, Interleukin-10.

3.4.4.3. Effect of chemopreventive treatment with EES on gene expression in tumor tissue

Treatment with EES for 10 and 31 days decreased *Ccnd1* (*Cyclin D1)* gene expression compared with the vehicle group (56% and 43%, respectively). Treatment with

EES for 31 days also increased *Nrf2* expression (221%). No differences were observed in *Bcl-2* or *Vegf* expression among groups (Fig 8).

FIGURE 8 - EFFECT OF EES CHEMOPREVENTIVE TREATMENT ON GENE EXPRESSION IN TUMOR TISSUE.



LEGEND: (**A**) *Nrf2*. (**B**) *Ccnd1*. (**C**) *Bcl-2*. (**D**) *Vegf*. The animals were orally treated with vehicle or 100 mg kg^{-1} EES for 10 or 31 days. The results are expressed as mean ± SEM (n = 4-5) and were analyzed using one-way ANOVA followed by the Newman-Keuls *post hoc* test. *$p < 0.05$, compared with vehicle group.

3.4.4.4. Effect of EES on acute toxicity

No signs of toxicity were observed during the 14 days of the treatment with a single dose of 2000 mg kg^{-1} EES. The LD_{50} was classified as category 5 or unclassified (∞) according to the OECD Acute Toxicity Assessment Procedure [20], indicating that the oral LD_{50} is higher than 2000 mg kg^{-1}.

3.5. DISCUSSION

Salvia species have been described as having essential oils, phenolic compounds, flavonoids, triterpenes, and diterpenes. Several of these compounds have biological properties, such as antiatherosclerotic, cytotoxic, anticancer, antiinflammatory, antimicrobial, and antioxidant effects [24, 25]. Diterpenes are notable because of their relative abundance in *Salvia* and cytotoxic and antitumor activities [26, 27]. Previous studies showed that an ethanolic extract of *S. lachnostachys* (EES) exerted cytotoxic activity against a panel of human cancer cell lines, including U251 (glioma, central nervous system), UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian-resistant), NCI-H460 (lung, non-small-cell), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), and K562 (leukemia). Bioassay-guided fractionation of this extract furnished five diterpenes, of which fruticuline A and isofruticuline A presented significant activity against all the tested cell lines, with the exception of K562 cells [6]. However, no studies of other fractions or antitumor effects *in vivo* have been conducted. Therefore, the present study investigated the chemical constituents of more polar fractions and antitumor activity of EES against solid Ehrlich carcinoma in mice. By HPLC analysis of EES it were identified rosmarinic acid (**1**), demethylfruticuline A (**2**), oleanolic acid (**3**), ursolic acid (**4**), fruticuline A (**5**), and fruticuline B (**6**) in EES (Fig. 2).

The solid Ehrlich carcinoma is a well-characterized model that is widely used for the experimental evaluation of tumor growth and biochemical features [28]. The present study showed that EES had antineoplastic effects against solid Ehrlich tumors in mice using both therapeutic and chemopreventive protocols.

Interestingly, when the treatment with EES began after a palpable tumor formed (day 7 after tumor inoculation in mice; late treatment), no difference in tumor growth was found compared with the control group. However, when the treatment began on day 1 after tumor inoculation (early treatment), EES prevented tumor development. These results indicate that EES is effective as an antitumor agent only with early treatment. Considering this apparent antitumor effect of EES, we speculated that it might also have a chemopreventive effect. Treatment with EES that began 10 days before tumor inoculation was sufficient to prevent tumor development similarly to 31 days of treatment. Immediately after tumor inoculation, the tumor cells create a tumor microenvironment. Therefore, EES appears to disturb this phase, prematurely affecting proliferation and preventing tumor growth. Based on these results, EES may be a potential therapy and chemopreventive agent against solid tumors.

Many cancer-chemopreventive compounds have antioxidant potential. These compounds contain bioactive phytochemicals that can affect proliferation through the activation or repression of multiple signaling cascades, thus playing a vital role in protecting the cells from oxidative stress and having chemopreventive potential [29, 30]. Despite the different protocols that were tested herein, EES appeared to have the same antitumor mechanism. A decrease in *Cyclin D1* expression, an important gene for tumor proliferation and progression [31], was found in tumors of animals that were treated with EES, suggesting that *Cyclin D1* may play a key role in EES's ability to prevent Ehrlich tumor development. *Cyclin D1* overexpression is found in more than 50% of human breast cancers. The increase in *Cyclin D1* expression contributes to the dysregulation of the normal cell cycle that controls tumorigenesis. *Cyclin D1* has been targeted to inhibit breast cancer proliferation. Several clinical trials that tested three inhibitors of cyclin-dependent kinases 4/6 (CDK4/6), such as palbociclib, ribociclib, and abemaciclib, reported positive results in Phase III studies [32, 33 and 34]. The CDKs are a family of serine/threonine kinases that promote the cell cycle. The association between CDK4 and CDK6 plays an important role in the expression of *Cyclin D1* [35]. Notably, Ehrlich tumors that were used in the present study originated from mammary tissue [28]. Therefore, EES may be a promising therapy for solid tumors, mainly those from breast tissues.

With regard to antioxidant activity, we found that EES also had antioxidant effects *in vitro*, reflected by DPPH free radical scavenging activity (Fig 3). We then investigated the antioxidant system in liver and tumor tissues in tumor-bearing mice. Interestingly, EES treatment reduced SOD activity in all the treatment protocols. Superoxide dismutase is responsible for converting anion superoxide into hydrogen peroxide and water, representing one of the first-line enzymes in redox balance against oxidative stress. A reduction of SOD activity suppresses tumor growth and metastasis by increasing anion superoxide and oxidative stress [38]. Thus, the reduction of SOD activity may partially explain the reduction of tumor volume in mice that were treated with EES. Notably, however, upon the initiation of tumorigenesis, there is a shift in cell homeostasis and endogenous antioxidant protection [37]. As a dual effect, the reduction of SOD in healthy animals can lead to tumorigenesis by increasing anion superoxide and oxidative stress [36]. At the initial stage of tumorigenesis, ROS generation induces cellular and DNA damage, mutation, the activation of oncogenic/angiogenic pathways, and aberrant proliferation that promote invasion and metastasis [37]. We measured SOD activity in tumor tissue 21 days after tumor inoculation, a time point at which the Ehrlich tumor was

well-established. Thus, we cannot affirm whether EES modifies SOD activity at the initiation of tumorigenesis, but EES affected SOD activity in later phases of tumor development.

Despite the reduction of SOD activity, no difference in CAT or GPx activity was observed. These enzymes are responsible for converting hydrogen peroxide into water and molecular oxygen [14]. Thus, an imbalance in oxidative status appears to occur with an increase in hydrogen peroxide in tumor tissue. Additionally, treatment with EES for 31 days also led to increases in ROS and LPO levels in tumor tissue. The excessive production of ROS under conditions of oxidative stress can lead to the production of oxidized polyunsaturated fatty acid products, called LPO products, that contribute to several cellular and pathophysiological abnormalities [38], reducing tumor cell viability. The increase in oxidative stress in the tumor microenvironment with chemopreventive treatment can also be responsible for inducing cellular death in tumor tissue, which may at least partially be responsible for the antineoplastic effect of EES.

Curiously, EES increased GSH levels in both liver and tumor tissue after 31 days of treatment, increasing the antioxidant capacity of both tissues. The increase in hepatic GSH levels by chemopreventive treatment with EES may also be responsible for inhibiting tumor development in the Ehrlich carcinoma model. However, elevations of GSH levels in tumor tissue improved antioxidant capacity and the resistance to cellular death by ROS generation in cancer cells [39]. This feature can cause tumorigenic cells to be more resistant to anticancer agents. The increase in hepatic GSH levels can also be a source of GSH in tumor tissue. The bulk of GSH from the liver plays a central role in inter-organ GSH homeostasis [40]. Moreover, the increase in GSH levels in tumor tissue was accompanied by an increase in *Nrf2* gene expression, which also regulates GSH production. Evidence suggests a correlation between high GSH levels and overactivation of the Nrf2/Keap1 pathway in tumor cells, which contributes to the resistance to oxidative stress and tumorigenesis [41]. However, the increase in tumor GSH levels that was observed in the present study does not appear to influence the EES-induced inhibition of tumor growth. The most prominent alterations of biomarkers of oxidative stress were observed with the longest EES treatment time (31 days). Thus, prolonged administration of EES appears to be necessary for modulating the systemic redox balance.

One mechanism that is responsible for cellular death is production of the cytokine TNF-α [42]. TNF-α is mainly produced by activated macrophages, T-lymphocytes, and natural killer cells [43]. We observed an increase in TNF-α levels in tumor tissue and monocytes in blood in tumor-bearing mice that were treated with EES. Furthermore, the

decrease in IL-10 levels in tumor tissue demonstrated a proinflammatory effect of EES treatment. IL-10 inhibits the synthesis of proinflammatory cytokines, such as interferon-γ, IL-2, IL-3, and TNF-α. With a decrease in IL-10 levels, TNF-α levels are not regulated effectively [44]. Therefore, TNF-α levels rise, resulting in inflammation in tumor tissue. The histological analysis of tumor tissue after EES treatment revealed more inflammatory cells and a larger area of necrosis compared with the control group. Inflammation and cell death are intrinsically connected and can positively regulate each other [45]. Additionally, Moneo and colleagues [42] demonstrated that *Cyclin D1* overexpression in cancer cells makes them insensitive to TNF-α treatment and thus resistant to growth arrest. Therefore, the reduction of tumor *Cyclin D1* expression that was observed in the present study may also be correlated with high levels of TNF-α, thus preventing tumor development and inducing cell death with EES treatment.

The hematological and biochemical analyses revealed increases in AST, albumin, and total proteins in the Ehrlich carcinoma model and a slight reduction of hematocrit values. An increase in AST levels was previously reported in Ehrlich-induced tumors in mice [38]. EES treatment did not have apparent cellular toxicity in the kidney, liver, or blood, based on the plasma and hematological parameters and acute toxicity assay (DL_{50}). In addition to the slight reduction of erythrocytes, no difference was observed in hemoglobin levels. In contrast, MTX treatment induced several hematological alterations, including reductions of urea, total protein, globulin, erythrocytes, hemoglobin, and hematocrit. Methotrexate is cytotoxic during the cell cycle. It has toxic effects on rapidly dividing cells that cause many side effects. Long-term and high-dose MTX administration causes anemia, increases plasma proteins, causes cardiac injury, causes kidney damage, and is hepatotoxic in rats and mice [46, 47]. Although we did not observe an increase in ALT or AST levels in Ehrlich tumor-bearing mice after 21 days of treatment with MTX (twice weekly) compared with the control group, we observed a reduction of blood proteins and albumin, which may indicate liver or kidney damages [48]. Thus, treatment with both EES and MTX prevented tumor development in the present study, but EES had an advantage over MTX treatment, namely a lack of toxicity.

In summary, EES exerted an antitumor effect through different mechanisms, including the inhibition of *Cyclin D1* and increase in inflammation in tumor tissue, inhibiting proliferation and inducing cell death. These effects were only observed with early and chemopreventive treatment, thus inhibiting the initiation of tumorigenesis. Furthermore, the EES-induced modulation of the antioxidant system in tumors also contributed to its

antitumor effects. To better elucidate these pathways and the active compounds in EES that are responsible for its antitumor effects, further studies are necessary.

3.6. ACKNOWLEDGEMENTS

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4. ARTIGO CIENTÍFICO 2 – "Fruticuline A exerts antineoplastic effects by inhibiting NF-κB pathway *in vitro* and *in vivo*"

Artigo em preparação para submissão no periódico *Toxicology and applied Pharmacology*

Fruticuline A exerts antineoplastic effects by inhibiting NF-κB pathway *in vitro* and *in vivo*

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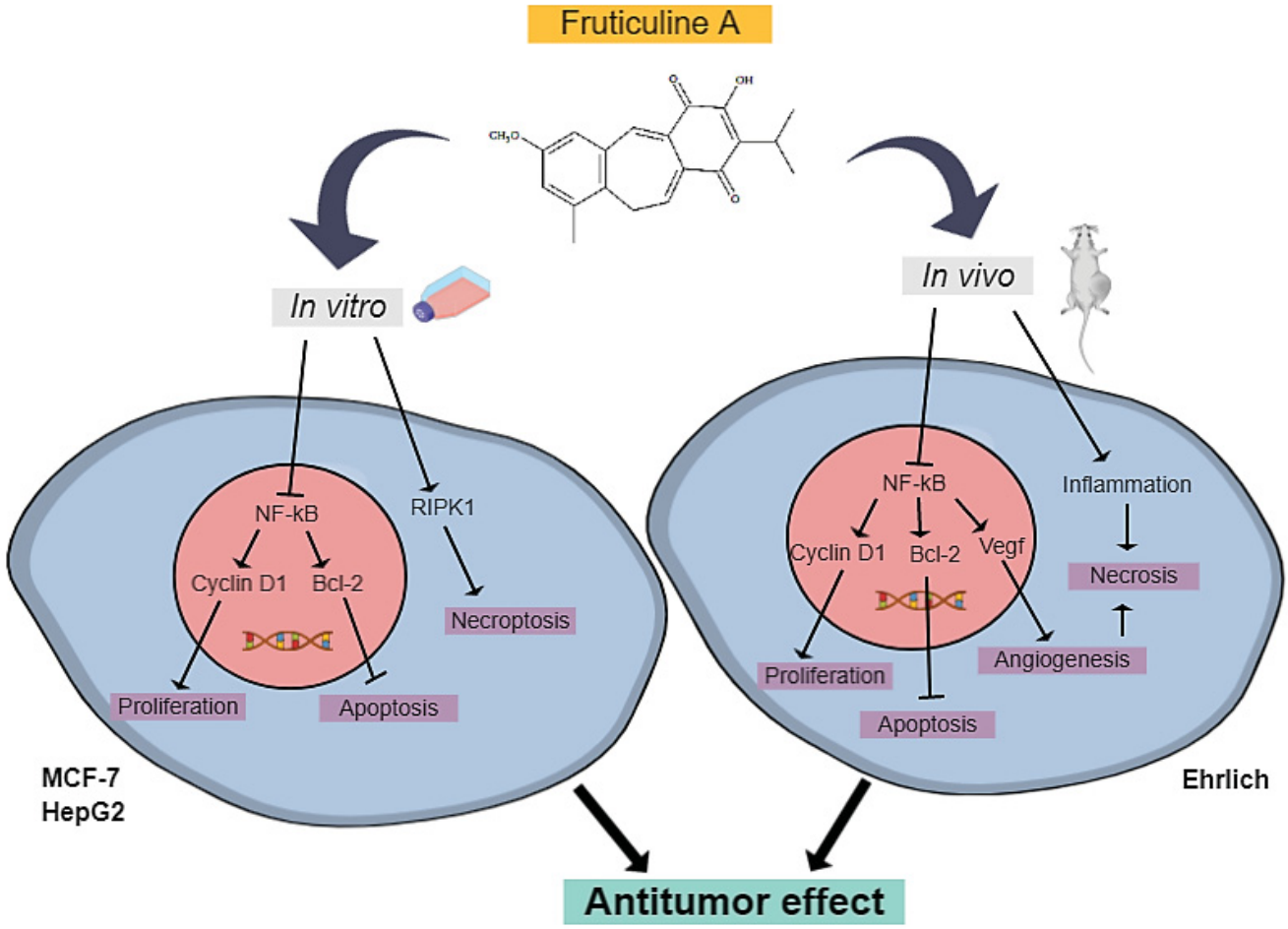
4.1. ABSTRACT

Fruticuline A (fruti) is a diterpene extracted from *Salvia lachnostachys* leaves, which have biological activities. This study aimed to investigate the antitumor effect and correlated mechanisms of fruti in human cancer cell lineages and Solid Ehrlich Carcinoma. MCF-7 and HepG2 cells were incubated with fruti and analyzed for proliferation, metabolism, oxidative stress, angiogenesis, signaling pathways of carcinogenesis and cell death. *In vivo*, the Ehrlich cells were inoculated subcutaneously in the right pelvic member of female Swiss mice. Animals were treated with vehicle, fruti or methotrexate for 21 days. Tumor tissue was analyzed for proliferation, apoptosis, oxidative stress, inflammation, angiogenesis, NF-κB pathway, and cellular death. *In vitro*, fruti reduced MCF-7 and HepG2 proliferation by the reduction of Cyclin D1 levels, also induced apoptosis in HepG2 cells and reduced Bcl-2 gene expression in MCF-7 cells. In addition, fruti decreased NF-κB gene levels in both cells and induced necroptosis by increasing Ripk1 in MCF-7 cells. In mice, fruti prevented tumor development and reduced *Cyclin D1, Bcl-2* and *Rela tx2* gene levels. Furthermore, fruti induced necrosis and apoptosis in tumor tissue and increased NAG and TNF-α levels, and reduced IL-10 and *Vegf* levels. Nevertheless, the *Ripk1* gene was not detected in tumor tissue. Collectively, fruti exerts antitumor effects through the inhibition of the NF-κB pathway, reducing Cyclin D1 and Bcl-2 levels. *In vitro* the apoptosis and necroptosis pathways are involved in the cellular death, whereas *in vivo,* cells undergo necrosis by the increase in tumor inflammation and reduction of angiogenesis. Thus, fruticuline A represents a promise molecule for drug development in cancer treatment.

Keywords: fruticuline A, antitumor, necrosis, necroptosis, inflammation, antiproliferative.

GRAPHICAL ABSTRACT



4.2. INTRODUCTION

Cancer remains the second leading cause of mortality throughout the world. The GLOBOCAN 2018 project estimated 9.3 million of deaths between 2012 and 2018 and 18.1 million of new cases in 2018 worldwide (Bray et al., 2018). Cancer represents an important problem of public health, as the economic impact is significant and is still increasing. In spite of the discovery of many antineoplastic drugs in the last decades, therapy is still vulnerable because of the low efficacy of the chemotherapeutics, chemoresistance and deficiency of knowledge of all mechanisms involved in cancer development (Leo, 2008).

Efforts have been made to better understand the biological basis of cancer progression and the specific pathways that are crucial in carcinogenesis. NF-κB represents one of the important signaling pathway due to its transcription induction of many key-genes in cancer development, such as genes for proliferation [i.e. Cyclin D1 (CCND1), Cyclin E], apoptosis (i.e. Blc-2, Bcl-xL), angiogenesis (i.e. VEGF, IL-8, HIF1α), cell adhesion and metastasis (i.e. ICAM, MMP, iNOS), survival (i.e. Survivin) and inflammation (i.e. TNF-α, COX2, iNOS) (Park and Hong, 2016). Given to the studies showing the tumor-suppressive activity by inhibition of NF-κB (Guan et al., 2018; Zakaria et al., 2018), this nuclear factor became an attractive therapeutic strategy for novel antineoplastic compounds as several studies have revealed an important contribution of NF-κB to cancer progression and survival (Riedlinger et al., 2018). Therefore, the investigations of new candidates for cancer treatment are focusing in identifying molecules that target specific signaling pathways in tumor cells, providing a better therapeutic option for cancer.

The chemotherapeutics-induced side effects limit the treatment and often propitiate a bad quality of life for patients (Vichaya et al., 2015). For this purpose, researches have been focusing on finding a molecule that has its target in tumor cells and is not cytotoxic to normal cells. In this line, natural compounds, mostly from plants, have been used in traditional medicine for many diseases for thousands of years. These compounds represent a good alternative due to its wide biological properties and high affinity to biological receptors (Ginsburg and Deharo, 2011). Almost 80% of all drugs approved for cancer therapy by the US Food and Drug Administration during the last three decades are natural products *per se* or are semi-synthetic products (Bishayee and Sethi, 2016).

A variety of products with anticancer potential can be found in nature, and among them is fruticuline A (fruti). Fruti is a quinone diterpene and one of the majority compounds in the ethanolic extract from leaves of *Salvia lachnostachys* (EES), recently characterized (Oliveira et al., 2016). Previously, our group demonstrated the antitumor effect of EES in an animal model of solid tumor, the Ehrlich tumor in mice (Corso et al., 2019). Furthermore, several biological properties of fruti were already described, such as antinociceptive, anti-inflammatory and antidepressive effects *in vivo* and cytotoxicity against a range of cancer cell lineages *in vitro* (Oliveira et al., 2016; Piccinelli et al., 2014; Santos et al., 2017). However, there are no data about the antitumor effect of fruti *in vivo*. This study investigated the antitumor effect of fruti and its action mechanism, using *in vitro* neoplastic cell lineages and *in vivo* Solid Ehrlich Carcinoma (SEC) of mice. Biomarkers of oxidative stress, inflammation, proliferation, angiogenesis and cellular death were investigated.

4.3. MATERIAL AND METHODS

4.3.1. Isolation of fruticuline A

A detailed procedure of isolation and identification of fruticuline A from *Salvia lachnostachys* was previously reported (Oliveira et al., 2016). Briefly, dried and powdered leaves of *S. lachnostachys* (415.3 g) were extracted with hexane and ethanol, at room temperature. The ethanol extract (EES, 45.0 g) was submitted to silica gel vacuum liquid chromatography and eluted with hexane, hexane: dichloromethane 1:1, pure dichloromethane, dichloromethane: acetone 98: 2, dichloromethane: acetone 95: 5, dichloromethane: acetone 9: 1, dichloromethane: acetone 1: 1 and finally pure acetone, successively. Were collected 71 fractions that were grouped into 14 subfractions. Subfraction 6 revealed to contain mainly impure fruticuline A (458.4 mg) in which was subjected to separation in HLPC (Rt = 8.21 min, methanol-acetonitrile 50:50, isocratic, semipreparative Nucleosil 100-5 C18 column, 250 x 10 mm) to obtain pure fruticuline A (58.8 mg). Fruticuline A was identified by 1D and 2D Nuclear Magnetic Resonance [Bruker Avance 400 spectrometer, using deutered chloroform ($CDCl_3$) a solvent], and compared with previously reported (Bisio et al., 2008).

4.3.2. *In vitro* experiments

4.3.2.1. Culture cells

Human MCF-7, HepG2 and Fibroblasts cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum, 4 mmol L^{-1} of *L*-glutamine and a mixture of antibiotics (5 mg mL^{-1} of streptomycin and 5 mg mL^{-1} penicillin). Cells were incubated at 37ºC in humidified atmosphere containing 5% CO_2. After cells reached ~80% of confluence, cells were harvested and seeded in a 96 well plate or 12 well plate, according to the experimental protocol.

4.3.2.2. WST-1 viability assay

The WST-1 viability assay is a colorimetric assay for the quantification of cell proliferation and viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells producing a colorimetric product, formazan (CELLPRO-Roche, Roche®, Mannheim, Germany). Briefly, 5^x10^3 of MCF-7 cells/well were added in 96 wells plate. Cells were incubated with vehicle (0) or fruti (0.97, 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 µM) dissolved in 0.1% DMSO/DMEM 24 h later. Then, the medium was replaced for a new medium after 24 h cells were incubated with 10 µL of WST-1 for 2 h at 37ºC in humidified atmosphere 5% CO_2. Additionally, fruti was also incubated with Fibroblasts to evaluate the cytotoxicity of fruti in normal cells. Proliferation was measured by spectrophotometer at 450 and 690 nm, after 1 min shaking the plate to equilibrate the intracellular formazan. The results were expressed as relative absorbance (nm) by the subtraction of the absorbances 450 nm and 690 nm. In addition, to evaluate the necroptosis pathway, the same procedure described above was performed, including the incubation of cells with 50 µM necrostatin-1 (Nec-1) (Sigma-Aldrich®, St. Louis, Missouri, EUA), an inhibitor of receptor-associated kinase 1 (RIPK1).

4.3.2.3. Oxidative stress parameter assays

To perform reduced glutathione (GSH) measurement, MCF-7 cells were seeded in 96 wells plate until reaching 80% of confluence. Vehicle (0) or fruti (12.5, 25, 50 and 100 µM) was incubated for 16 and 24 h. After the incubation, cells were scratched in the presence of iced cold 10% perchloric acid and centrifuged for 10 min at 14000 × *g* 4ºC. To

increase the pH to 8, 3.0 M K_2PO_4 was added in the supernatant and samples were immediately frozen in liquid nitrogen. After 30 min samples were thawed and incubated with work solution (DTNB and NADPH) diluted in GSH buffer (0.1 M $Na_2HPO_4.2H_2O$, 0.1M $NaH_2PO_4.2H_2O$ and 0.05 M $Na_2EDTA.2H_2O$) in a 96 wells plate. The reaction was started in the presence of glutathione reductase (GR) and the absorbance was monitored at 412 nm at 37ºC on a Clariostar (BMG LabTech, Cary, USA) analyzer. The results were interpolated with GSH standards and were expressed in nmol GSH/well (Tietze, 1969).

Additionally, to measure total reactive oxygen species (ROS), MCF-7 cells were seeded in a black 96 wells plate. After 24 h cells were briefly washed with DMEM (-) phenol red and supplemented with 1.9 g/L $NaHCO_3$, 5 mM glucose and 0.1% fetal bovine serum. 5 µM DCF-DA was added as stimuli of ROS production following fruti concentrations (25, 50, 75 and 100 µM), dissolved in 0.1% DMSO/DMEM (-) phenol red. The fluorescence was monitored on a Clariostar analyzer at 488/20, 520/20 nm at 37ºC, 5% CO_2. Results were expressed as ROS production rate (AUC/sec) (adapted of Keston and Brandt, 1965).

4.3.2.4. Total RNA isolation and RT-qPCR

Human MCF-7, HepG2 and Fibroblast cells were seeded in 12 wells plate until reaching the confluence. Then the cells were incubated with vehicle or fruti at 10, 25, 50, 75 or 100 µM for 24 h. Total RNA was extracted from cultured cells using Trizol reagent (Sigma-Aldrich®, St. Louis, Missouri, USA) and measured by Nanodrop 100 (Thermo Scientific®, Waltham, Massachusetts, USA). Complementary DNA was synthesized from 2 µg total RNA with an oligo-dT, random hexamers primers and deoxythymidine oligomer, Ribolock RNAse inhibitor and RevertAid reverse transcriptase. Real-time polymerase chain reaction were performed in a Lightcycler apparatus (Roche®, Mannheim, Germany) using the sensiFAST SYBR No-ROX kit (Bioline®, London, UK). Initial fluorescent values were calculated by LinRegPCR3 (version 2013.0; Academic Medical Center, Amsterdam, The Netherlands). Primers sequences were described in Supplementary Table 1. The most stable reference genes were calculated using geNorm (Vandesompele et al., 2002). Transcript levels were normalized to housekeeping genes ratio GAPDH and Cyclophilin (CyP) for MCF-7 and Fibroblasts cells, and the genes ratio HPRT and Actin for HepG2 cells.

4.3.2.5. Western blot

MCF-7 cells were seeded in a 12 wells plate until reaching the confluence. Then the cells were incubated with vehicle (0) or fruti at 10, 25 and 50 µM for 24 h, and harvest in iced cold PBS and homogenized in RIPA buffer freshly supplemented with PhosSTOP phosphatase inhibitor (Roche®, Mannheim, Germany) and 5 mM EDTA-free protease inhibitor cocktail (Roche®, Mannheim, Germany). The samples were centrifuged for 10 min at 4ºC and the supernatant was used for protein measurement by the bicinchoninic acid assay. Forty micrograms of protein were electrophoresed on 8-10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidine difluoride (PVDF) membrane by semidry blotting. The membrane was blocked overnight in 5% nonfat milk/TBST followed of overnight incubation with primary antibody in a cold room at 4ºC. The PVDF membrane was washed 3 times with TBST and incubated with secondary antibody with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G antibody (BioRad®, Califórnia, USA) for 1 hour at room temperature. All antibodies were diluted in 1% nonfat milk/TBST. The lists of antibodies and dilutions used were described in Supplementary Table 2. The bands in the membrane were detected using chemiluminescence reagents (100 mM Tris HCl pH 8.5, 1.25 nM luminol 0.2 mM p-coumarin and freshly added 3 mM H_2O_2) by ImageQuant LAS 4000 (GE Healthcare Life Sciences®, Pittsburgh, USA) and compared with endogenous GAPDH protein.

4.3.2.6. Caspase 3/7 activity assay

As human MCF-7 cells do not express caspase 3 protein (Jänicke, 2009), the caspase 3/7 activity assay was performed seeding HepG2 cells in a 96 wells plate. The cells were incubated with vehicle (0) or fruti at 10, 25, 50, 75, 100 and 125 µM for 16, 24, 48 and 72 h. Caspase 3/7 activity solution was added according to the manufacture instructions (SensoLyte®, AnaSpec EGT Group, Fremont, USA). The fluorescence was monitored at 485-490/520-535 nm on a Clariostar analyzer at 37ºC. Results were expressed as relative fluorescence.

4.3.3. *In vivo* experiments

4.3.3.1. Animals

Female Swiss mices (*Mus musculus,* weighting 25-30g) were housed 7 per cage with free access to food and water and maintained under controlled conditions of humidity, luminosity (12/12 h ligh/dark cicle) and temperature (± 22ºC). All experiments were approved by local Ethics Committee of Animal Experimentation (CEUA/BIO – UFPR; 879) and animals were randomized before the treatments.

4.3.3.2. Ehrlich Carcinoma manipulation and groups of treatment

Ascitic Ehrlich cells ($2^{x}10^{6}$ cells/animal) were maintained by weekly intraperitoneal passage. After cells reach ≥ 98% of viability were inoculated subcutaneously ($2^{x}10^{6}$ cells/animal) in the right pelvic member of mice for SEC development. Animals were orally and daily treated with vehicle (0.1% Tween 20 in distilled water, 10 mg mL^{-1}) or fruti 3 mg kg^{-1} for 21 days. The dose of fruti was based on the yield of the ethanolic extract effect, previously prepared by our group (Corso et al., 2019). Additionally, the positive group was treated with methotrexate (MTX) 2.5 mg kg^{-1} every 3 days by intraperitoneal route during 21 days. The tumor volume was measured from day 7 until day 21 and was calculated according to Mishra et al. (2018) as $V(cm^3) = L\,x\,W^2 x\,0{,}52$, where *L* is the largest tumor diameter and *W* is the smallest tumor diameter (in centimeters). On day 22, the mice were fasted for 16 h and anesthetized with quetamine (90 mg kg^{-1}) and xylazine (10 mg kg^{-1}) (Vetnil Industry and Trade of Veterinary Products LTDA, São Paulo, Brazil) by intraperitoneal route, and blood, tumor and organs were collected for further analysis.

4.3.3.3. Total RNA isolation and RT-qPCR

The expression of target genes for proliferation, apoptosis, necroptosis and angiogenesis was assessed in tumor samples. RNA isolation and complementary DNA synthesis were performed as described in section 4.2.2.4. Primers sequences are described in Supplementary Table 3. Relative expression levels were calculated using *Gapdh* as endogenous control gene.

4.3.3.4. Oxidative stress and inflammatory parameters

To measure oxidative stress parameters, tumor tissue was homogenized in phosphate buffer pH 6.5 (1:10) and centrifuged at 10000 × *g* at 4°C for 20 min. The

homogenate was used to measure reduced glutathione (GSH) (Sedlak and Lindsay, 1968) and the supernatant was used to measure reactive oxygen species (ROS) (adapted of Keston and Brandt, 1965; LeBel et al., 1990).

For inflammatory parameters, tumor tissues were homogenized in PBS and centrifuged at 10000 × *g* at 4°C for 20 min. The supernatant was used to measure NO according to Green et al. (1982) and cytokines (TNF-α, IL-6, IL-10, IL-4) according to the manufacturer's instructions (Kits BD Bioscience®, Califórnia, USA). The pellet was ressuspended in 0.1% Saline-Triton X and centrifugated at 11.000 × *g* at 4ºC for 20 min. The supernatant was used to measure myeloperoxidase (MPO) (Suzuki et al., 1983) and *N*-acetyl-β-D-glucosaminidase (NAG) levels (Bailey, 1988).

4.3.3.5. Tumor histology

Tumor tissue was fixed in ALFAC solution (85% ethanol, 10% formaldehyde, 5% glacial acetic acid) at room temperature for 16 h following the routine dehydration for paraffin embedding. Samples were sectioned at 5 µm thickness and stained with hematoxylin and eosin. Analysis was performed by optical microscopy in 3 or 4 slides per group. The evaluated parameters were apoptosis (Grade 0: absent; Grade I: discrete; Grade II: moderate), inflammatory infiltration (Grade 0: absent; Grade I: discrete; Grade II: moderate; Grade III: high) and coagulative necrosis (Grade 0: absent; Grade I: present in 1-20% of the histological area; Grade II: 21-50%; Grade III: 51-80%; Grade IV: 81-100%). Images were acquired microscopically using Axio Imager Z2 epifluorescence (Carl Zeiss®, Germany), equipped with an automated slide scanner from MetaSystems (MetaViewer version. 2.0.100, Germany).

4.3.3.6. Plasmatic and hematologic parameters

Hematologic parameters were analyzed through complete hemograms using a BC2800-Vet (BioBrasil®, São Paulo, Brazil) automated device. Plasma samples were obtained after blood centrifugation at 1344 × *g* for 5 min. The samples were used to determine creatinine, glucose, total protein, albumin, globulin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, according to commercial kits instructions (Kovalent®, São Gonçalo, Brazil). All parameters were analyzed by an automated system (Mindray BS-200®, Shenzhen, China).

4.3.4. Data analysis

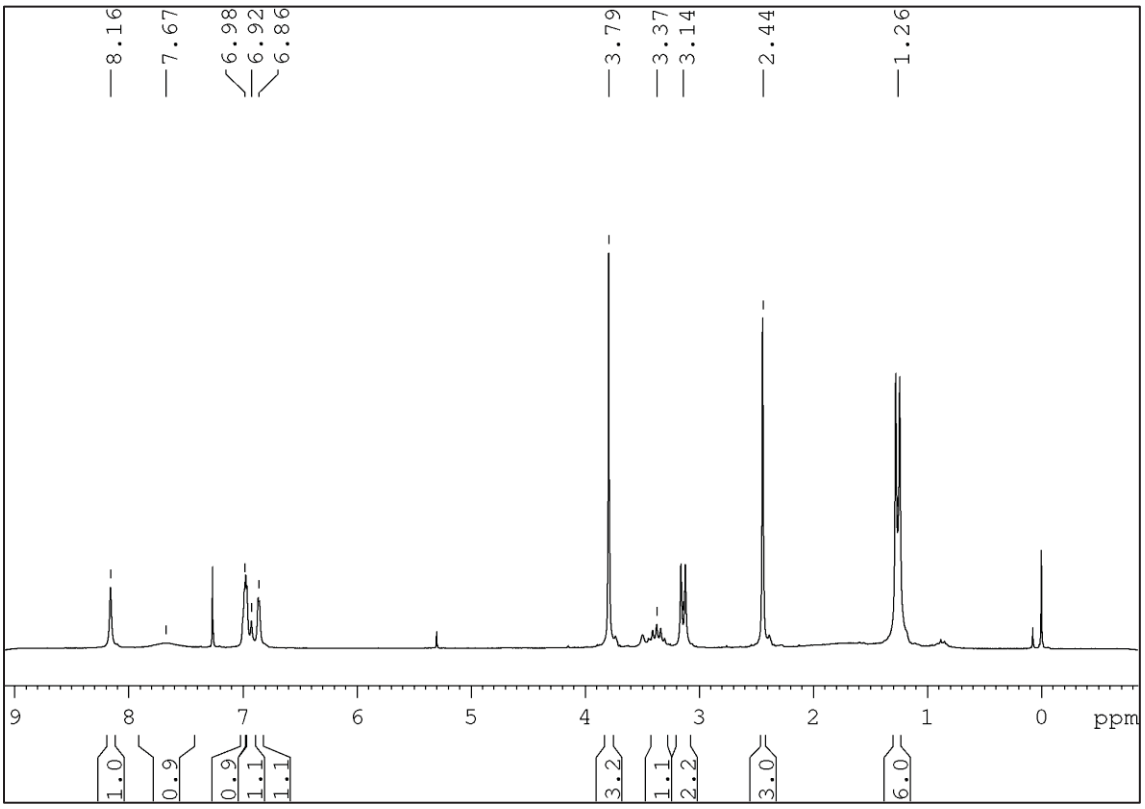
Data were expressed as means ± standard error of mean (S.E.M.). Results were subjected to t-test or one- or two-way analysis of variance (ANOVA), followed by post hoc Newman Keuls test, when applicable. The results were analyzed using GraphPad Prism (v. 5.0). The level of significance was set at 95% ($p<0.05$).

4.4. RESULTS

4.4.1. Isolation and identification of fruticuline A

Pure fruticuline A was obtained by HPLC and analyzed by NMR (^{1}H, HSQC, HMBC). The NMR data were identical with those previously reported (Bisio et al., 2008). Here, the ^{1}H NMR of fruticuline A is provided in order to show the absence of impurities, as aliphatic compounds or other aromatic diterpene (Figure 1). The yield of fruticuline from EES was around 3%.

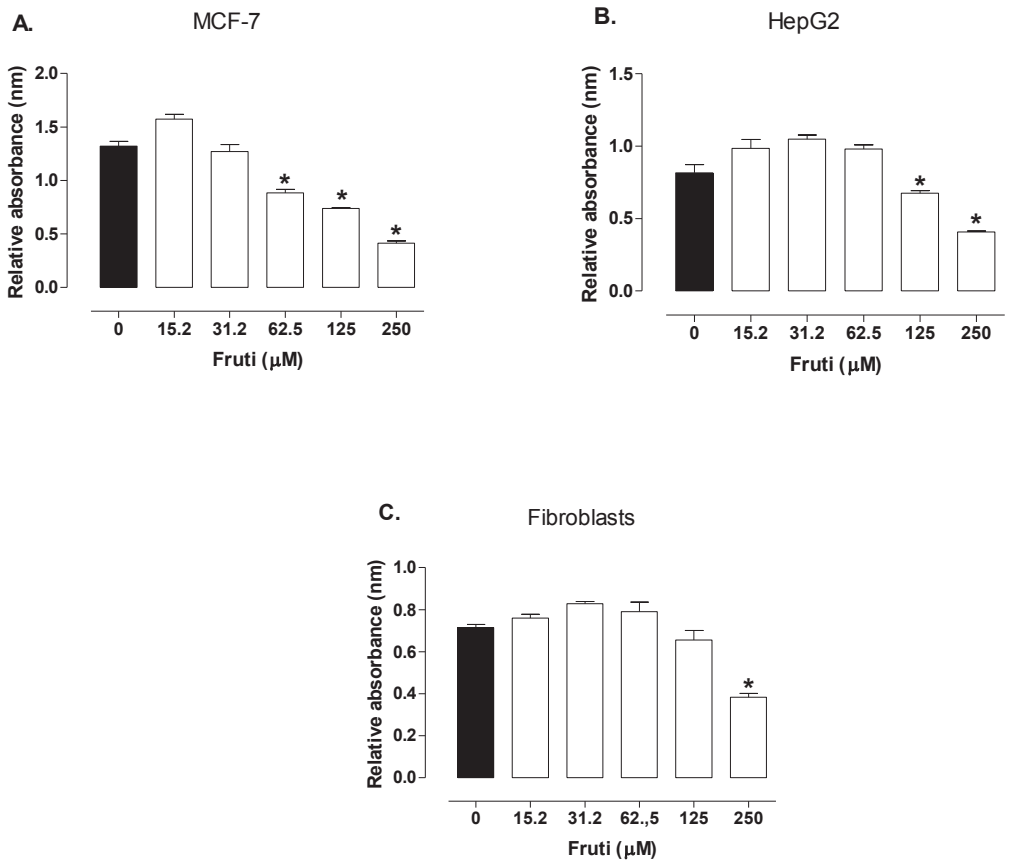
FIGURE 1 – NMR ^{1}H SPECTRUM OF FRUTICULINE A ($CDCl_3$, 400 MHz)



4.4.2. Effect of fruti on proliferation of human fibroblasts and human cancer cells

Fruti at the concentration of 62.5, 125 and 250 μM inhibited MCF-7 proliferation in 33, 44 and 69% respectively, when compared to the vehicle group. In HepG2 the inhibition occurred with Fruti at 125 and 250 μM (17 and 50%, respectively), whereas in Fibroblast, only the concentration of 250 μM was cytotoxic in 46%, compared to vehicle group (Fig. 2). The growth inhibition of 50% (GI_{50}) of the cells was 57.76, 119.4 and 145.3 μM for MCF-7, HepG2 and Fibroblasts, respectively.

FIGURE 2 - CELL VIABILITY IN WST-1 ASSAY.

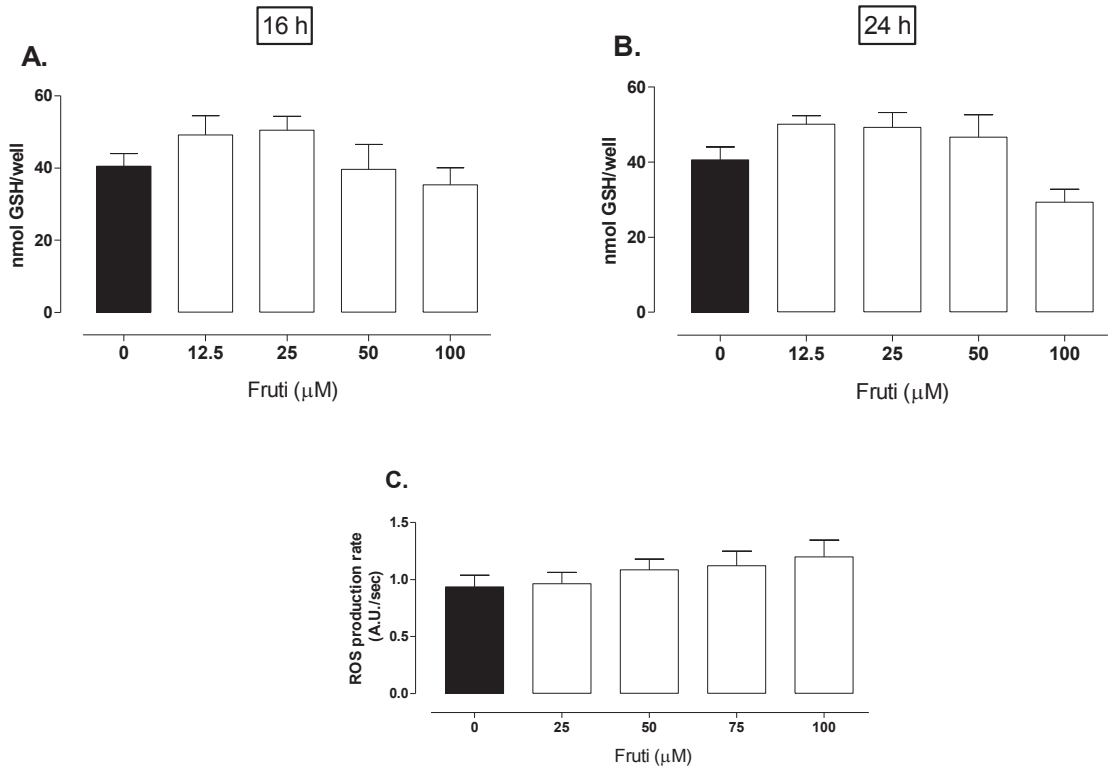


LEGEND: Panel **A:** MCF-7; Panel **B**: HepG2; Panel **C:** Fibroblasts cells. Cells were incubated with vehicle (0) or fruti at 15.2, 31.2, 62.5, 125 and 250 μM for 24 h. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. * $p<0.05$ when compared to vehicle group.

4.4.3. Effect of fruti on oxidative stress in MCF-7 cells

No differences were observed in GSH levels at all time points tested and in ROS levels compared to the vehicle group (Fig. 3).

FIGURE 3 - GSH AND ROS MEASUREMENT IN MCF-7 CELLS.

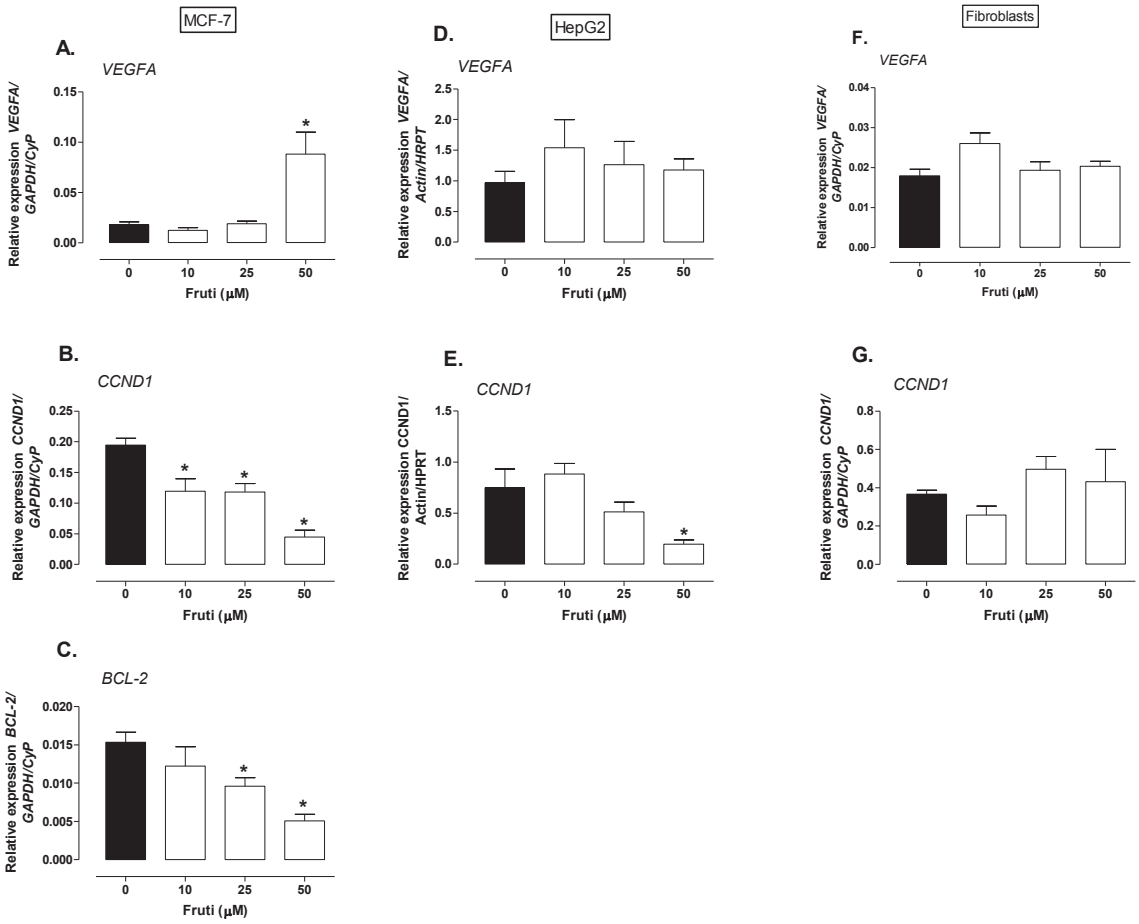


LEGEND: For GSH measurement MCF-7 cells were incubated for 16 (Panel **A**) and 24 (Panel **B**) hours with vehicle (0) or fruti 12.5, 25, 50 and 100 µM. ROS levels (Panel **C**) were conducted with MCF-7 cells pretreated with vehicle (0) or fruti (25, 50, 75 and 100 µM) under the stimuli of DCFH-DA for up to 60 min. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test.

4.4.4. Effect of fruti on *VEGFA*, *CYCLIN D1* and *BCL-2* gene expression in human fibroblasts and cancer cells

The expression of *CYCLIN D1 (CCND1* gene*)* was found decreased in human cancer cells (MCF-7 and HepG2) in the presence of fruti 50 µM when compared to vehicle group (MCF-7: 76%; HepG2: 74%). In addition, fruti at 25 and 50 µM reduced *BLC-2* expression in MCF-7 cells in 36 and 66%, respectively. However, at 50 µM there was an increase in *VEGFA* gene in MCF-7 cells. No differences were found in Fibroblasts (Fig. 4). Furthermore, the expression of TNF-α was not detected in these cell lineages.

FIGURE 4 - GENE EXPRESSION OF *VEGFA*, *CCND1*, AND *BCL-2* IN MCF-7, HEPG2 AND FIBROBLAST CELLS.

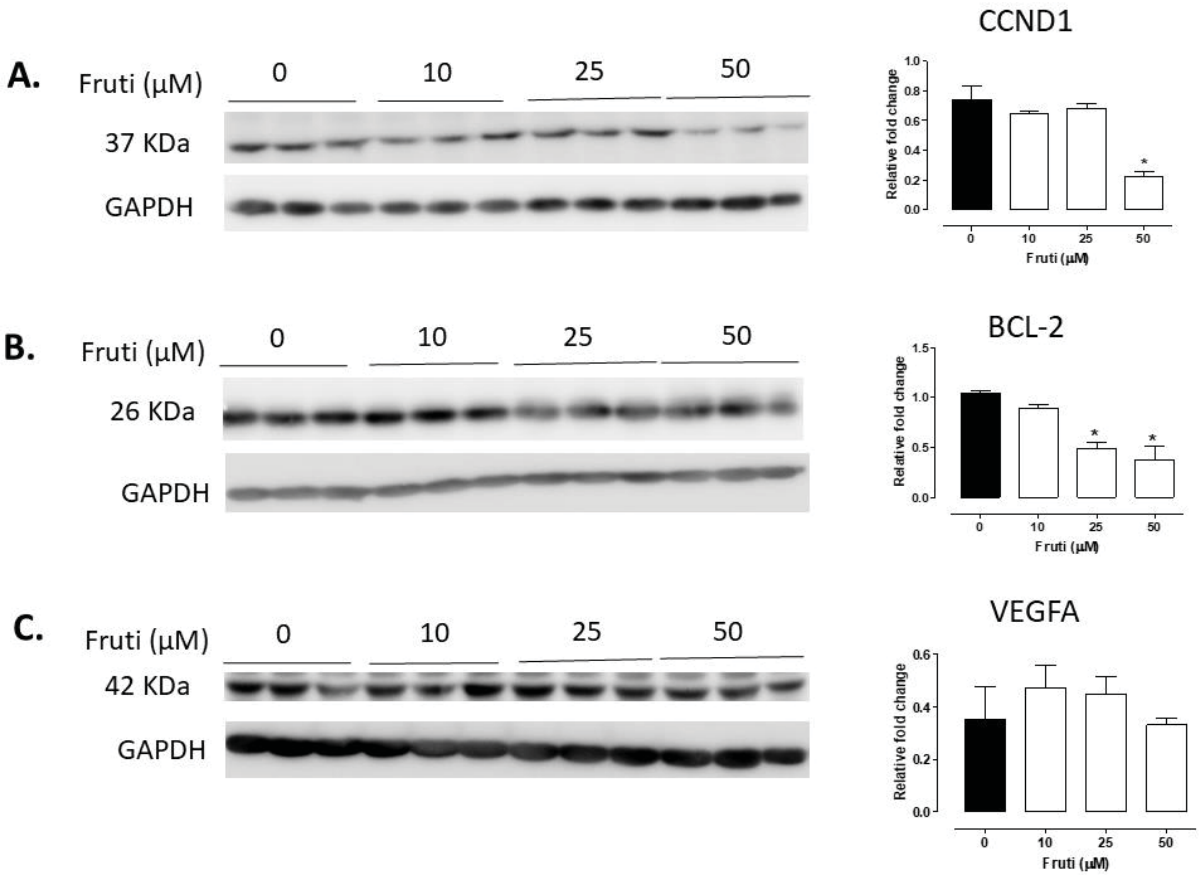


LEGEND: Panel **A**, **B** and **C**: *VEGFA*, *CCND1* and *BLC-2* expression in MCF-7 cells; Panel **D** and **E**: *VEGFA* and *CCND1* expression in HepG2 cells; Panel **F** and **G**: *VEGFA* and *CCND1* in Fibroblasts cells. Cells were incubated with vehicle (0) or fruti 10, 25 and 50 µM for 24 h. Results are expressed as mean ± S.E.M. (n= 4-6) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. * $p<0.05$ when compared to vehicle group.

4.4.5. Effect of fruti on CCND1, BCL-2 and VEGFA proteins in MCF-7 cells

Western blot analysis demonstrated a reduction on CYCLIN (CCND1) protein in MCF-7 cells treated with fruti 50 µM. Furthermore, fruti at 25 and 50 µM decreased BCL-2 levels, and no difference was observed in VEGFA levels (Fig. 5).

FIGURE 5 - PROTEIN LEVELS OF CCND1 (PANEL A), BCL-2 (PANEL B) AND VEGFA (PANEL C) IN MCF-7 CELLS.

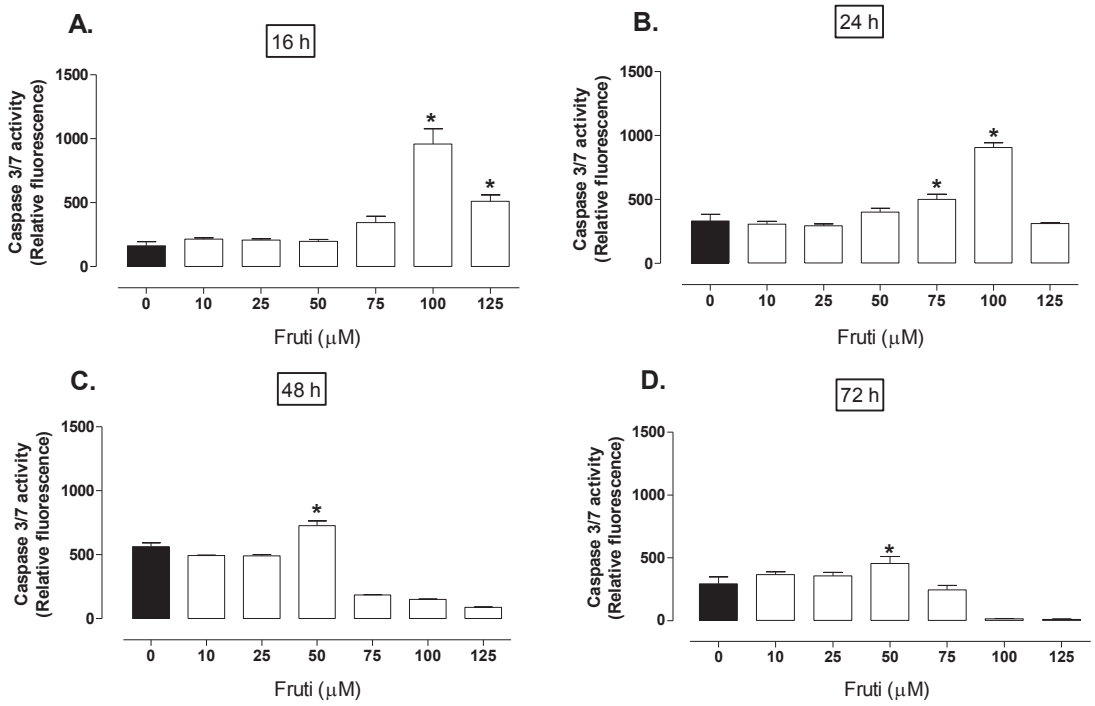


LEGEND: Cells were incubated with vehicle (0) or fruti at 10, 25 and 50 µM for 24 h. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. * $p<0.05$ when compared to vehicle group.

4.4.6. Effect of fruti on Caspase 3/7 activity in HepG2 cells

Lower concentrations of fruti increased caspase 3/7 activity as the incubation time increased. At 16 h and 24 h of incubation 100 µM fruit augmented caspase 3/7 activity in 489 and 172%, respectively. At 48 and 72 h of incubation, 50 µM fruit increased in 29 and 55%, respectively. In addition, at 48 and 72 h some cells incubate with fruti concentrations higher than 100 µM were dead and therefore the caspase 3/7 activity was not detectable (Fig. 6).

FIGURE 6 - CASPASE 3/7 ACTIVITY IN HEPG2 CELLS.

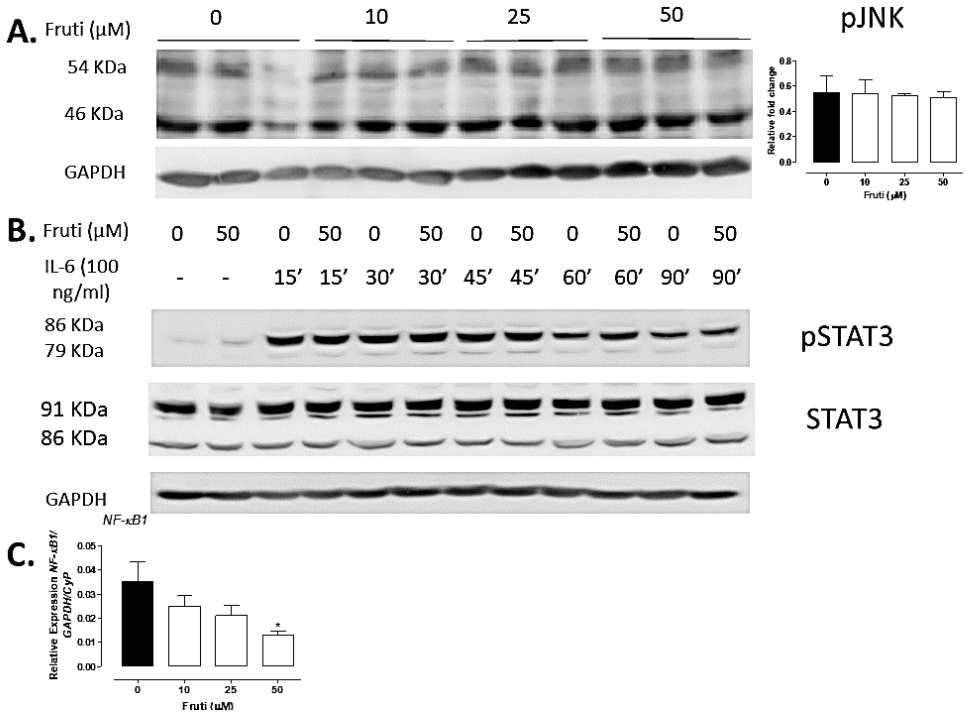


LEGEND: Cells were incubated with vehicle (0) or fruti at 10, 25, 50, 75, 100 and 125 μM for 16 (Panel **A**), 24 (Panel **B**), 48 (Panel **C**) and 72 (Panel **D**) h. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-away ANOVA followed by Newman Keuls post hoc test. * $p<0.05$ when compared to vehicle group.

4.4.7. Effect of fruti on pJUNK and STAT3 proteins and NF-κB1 gene expression in MCF-7 cells

No differences were observed on pJUNK, pSTAT3 and STAT3 levels in cells incubated with fruti (Fig. 7A,B). However, fruti at 50 μM decreased NF-κB1 gene expression (63%) in MCF-7 cells after 24 h of incubation compared to control group (Fig. 7C). Furthermore, fruti at 25 and 50 μM also inhibited NF-κB1 expression in HepG2 cells in 73 and 65%, respectively (Suppl. Fig. S1).

FIGURE 7 - PROTEIN LEVELS OF pJNK (PANEL A), pSTAT3 AND STAT3 (PANEL B) AND *NF-κB1* GENE EXPRESSION (PANEL C) IN MCF-7 CELLS.

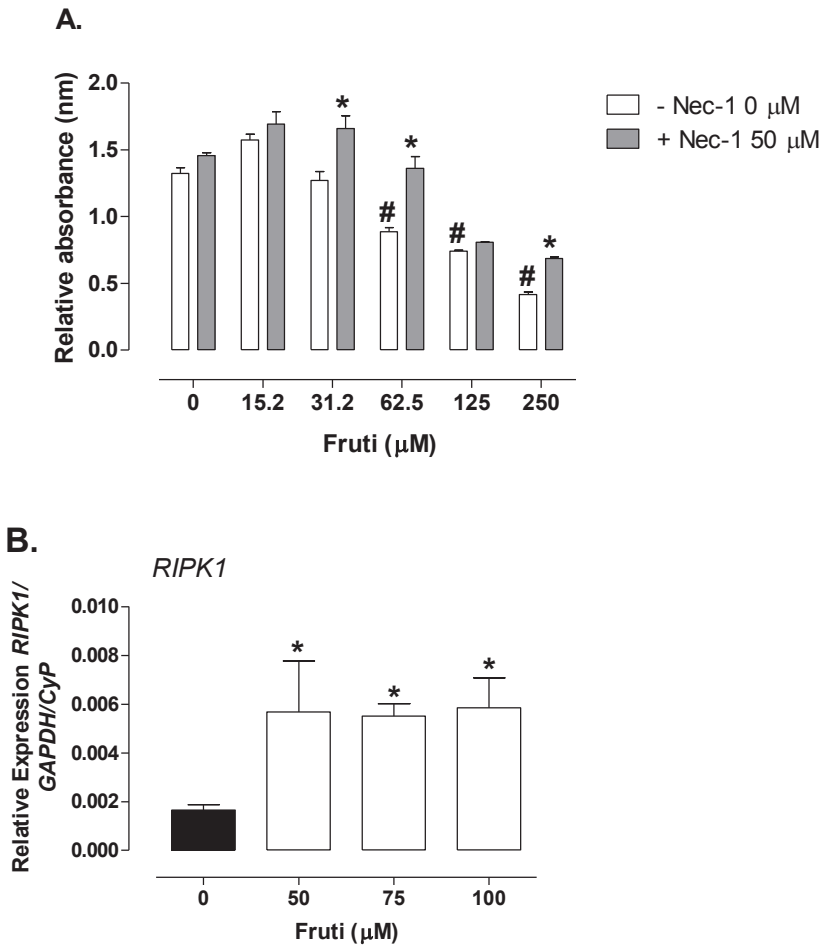


LEGEND: For pJNK and *NF-κB1* analysis, cells were incubated with vehicle (0) or fruti at 10, 25 and 50 μM for 24 h. For pSTAT3 and STAT3 analysis, cells were incubated with vehicle (0) or fruti at 50 μM in the presence or absence of IL-6 (100 ng/ml) for 15 until 90 min. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. * p<0.05 when compared to vehicle group.

4.4.8. Effect of fruti on necroptosis pathway in MCF- cells

Necrostatin-1 (Nec-1) in the presence of fruti at 62.5, 125 and 250 μM after 24h of incubation reversed the inhibition of the MCF-7 cells proliferation (Fig. 8A). The same was observed in HepG2 cells (Suppl. Fig. S2). In addition, fruti at 50, 75 and 100 μM increased *RIPK1* expression compared to the vehicle group (0) (255, 369 and 265%, respectively) (Fig. 7B).

FIGURE 8 - WST-1 VIABILITY ASSAY IN THE PRESENCE AND ABSENCE OF NECROSTATIN 50 µM AND *RIPK1* GENE EXPRESSION IN MCF-7 CELLS.

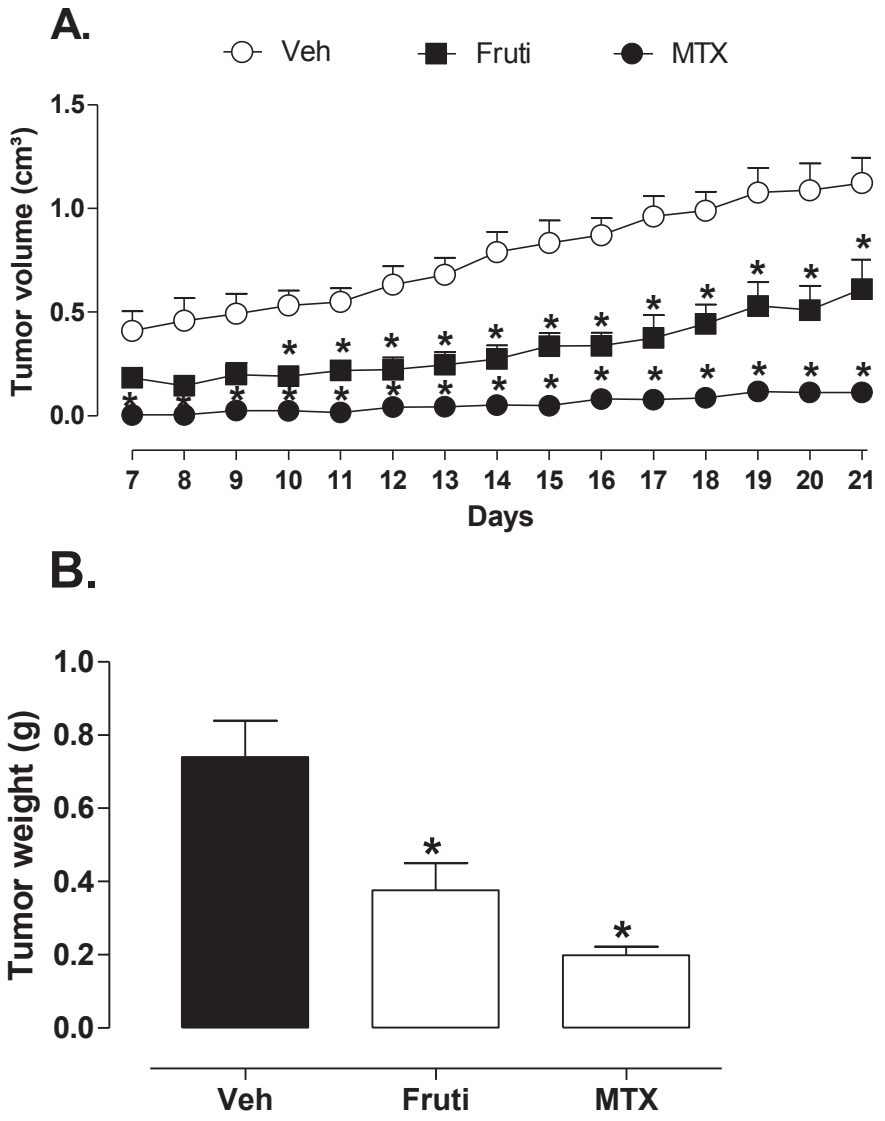


LEGEND: For WST-1 assay, MCF-7 cells were incubated with vehicle (0) or fruti at 15.2, 31.2, 62.5, 125 and 250 µM for 24 h. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. * and # $p<0.05$ when compared to (–) Nec-1 (absence of Necrostatin) and vehicle group (0 µM), respectively. For *RIPK1* analysis, cells were incubated with vehicle (0) or fruti at 50, 75 and 100 µM for 24h. * $p<0.05$ when compared to vehicle group.

4.4.9. Effect of fruti on Ehrlich tumor development *in vivo*

Treatment with fruti 3 mg kg^{-1} prevents tumor development from day 10 until day 21 in 61 and 51%, respectively, when compared to the vehicle group. MTX, used as positive control, also inhibited tumor growth until day 21. In addition, the tumor weight on day 22 was reduced by fruti treatment in 49% (Fig. 9).

FIGURE 9 - EHRLICH TUMOR DEVELOPMENT (A) AND TUMOR WEIGHT (B) IN MICE.

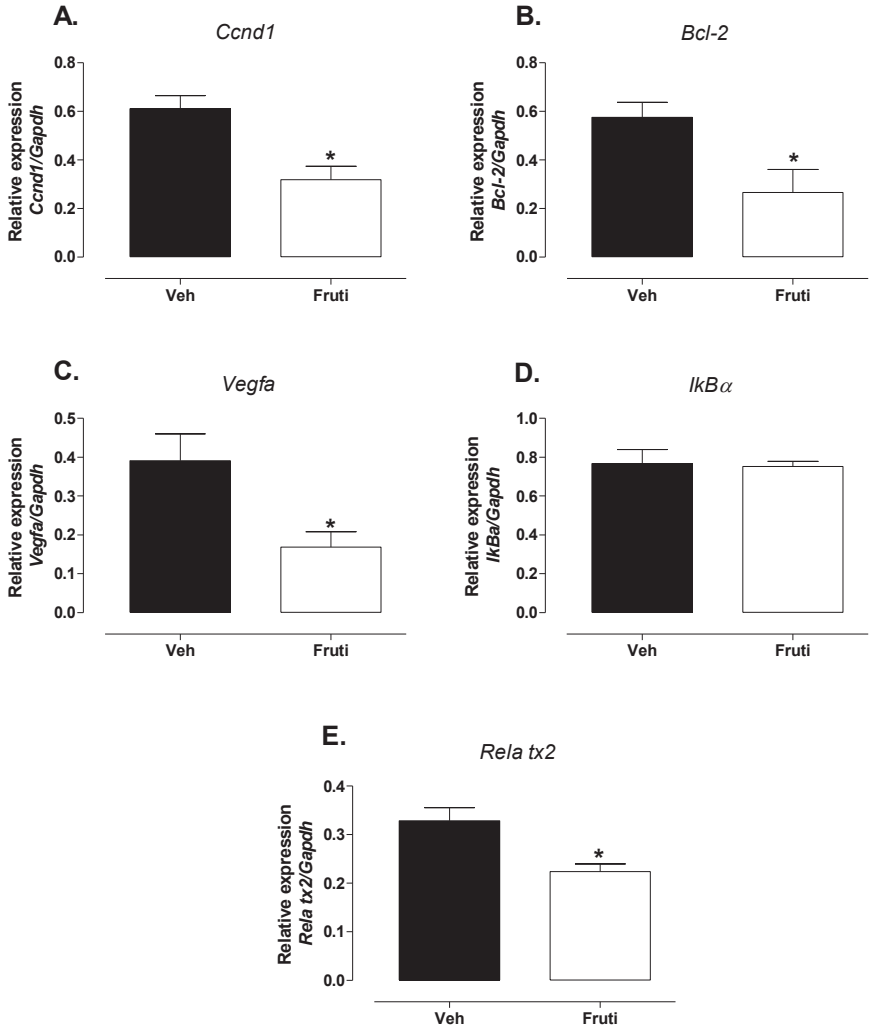


LEGEND: Animals were treated with fruti (3 mg kg^{-1}, p.o.), vehicle (Veh, 0.1% tween 20 in distilled water, 10 mL kg^{-1}, p.o.) or methotrexate (MTX, 2.5 mg/kg, i.p.) for 21 days. Results are expressed as mean ± S.E.M. (n= 6-9) and were analyzed by two-way ANOVA followed by Bonferroni post hoc test (A), one-way ANOVA followed by Newman Keuls post-hoc test (B) and Student t-test (C). * p<0.05 when compared to vehicle group.

4.4.10. Effect of fruti on *Ccnd1, Bcl-2, Vegfa, IkBα* and *Rela tx2* gene expression in tumor tissue

Fruti reduced *Ccnd1 (Cyclin D1)*, *Bcl-2*, *Vegfa* and *Rela tx2* gene expression in 48, 54, 58 and 32%, respectively, when compared to vehicle group, whereas no difference was found on *IkBα* expression (Fig. 10). Nevertheless, the expression of *Ripk1* was not detected in tumor tissue (data not shown).

FIGURE 10 - GENE EXPRESSION OF *CCND1* (PANEL A), *BCL-2* (PANEL B), *VEGFA* (PANEL C), *IkBα* (PANEL D) AND *RELA Tx2* (PANEL E) IN TUMOR TISSUE.



LEGEND: Animals were treated with fruti (3 mg kg^{-1}, p.o.) or vehicle (Veh, 0.1% tween 20 in distilled water, 10 mL kg^{-1}, p.o.) for 21 days. Results are expressed as mean ± S.E.M. (n= 4-5) and were analyzed by Student t-test. * p<0.05 when compared to vehicle group.

4.4.11. Effect of fruti on oxidative stress and inflammatory parameters in tumor tissue

Fruti treatment increased ROS, NAG and TNF-α levels in tumor tissue in 15, 29 and 79%, respectively, and reduced IL-10 in 68%, compared to vehicle group. No differences were observed in the others inflammatory parameters (Table 1).

TABLE 1 - OXIDATIVE STRESS AND INFLAMMATORY PARAMETERS IN EHRLICH TUMOR TISSUE.

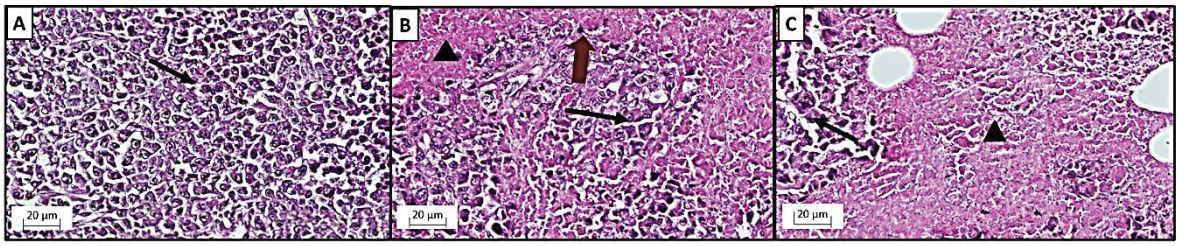
Parameters	Experimental groups	
	Veh	Fruti
GSH (µg g of tissue^{-1})	197.51 ± 14.44	239.01 ± 39.71
ROS [DCF] (Fluorescence intensity)	84.06 ± 1.02	96.69 ± 3.19*
MPO (µmol min^{-1} g of tissue^{-1})	164.07 ± 15.88	145.05 ± 11.16
NAG (µmol min^{-1} g of tissue^{-1})	20.03 ± 1.30	25.89 ± 2.89*
NO (µmol g of tissue^{-1})	8.13 ± 1.18	6.50 ± 0.58
TNF-α (pg mL^{-1})	1525.00 ± 212.05	2735.00 ± 352.09*
IL-4 (pg mL^{-1})	458.07 ± 88.78	456.09 ± 109.01
IL-6 (pg mL^{-1})	65.55 ± 5.13	96.63 ± 23.16
IL-10 (pg ml^{-1})	3232.00± 521.00	1050.00 ± 255.05*

LEGEND: Animals were treated with fruti (3 mg kg^{-1}, p.o.) or vehicle (Veh, 0.1% tween 20 in distilled water, 10 mL kg^{-1}, p.o.) for 21 days. Results are expressed as mean ± S.E.M. (n= 5-7) and were analyzed by Student t-test. * p<0.05 when compared to vehicle group.

4.4.12. Effect of fruti on tumor histology

Tumor tissue of animals treated with vehicle showed Ehrlich viable cells, small area of coagulative necrosis and discrete inflammation observed by polymorfonuclear cells infiltration and discrete apoptosis. Tumor tissue of animals treated with fruti showed more coagulative necrosis area, less Ehrlich viable cells, discrete-high inflammation and discrete-moderate apoptosis in tumor tissue when compared to the vehicle group. Furthermore, MTX group had discrete inflammation and coagulative necrosis, but no apoptosis was observed (Fig. 11).

FIGURE 11 - EHRLICH TUMOR HISTOLOGY.



Parameters	Experimental groups		
	Vehicle	Fruti	MTX
Apoptosis			
Grade 0 (absent)	+	-	+++
Grade I (discrete)	++	++	-
Grade II (moderate)	-	++	-
Inflammation			
Grade 0 (absent)	+	-	-
Grade I (discrete)	++	++	+++
Grade II (moderate)	-	++	-
Grade III (high)	-	-	-
Coagulative necrosis			
Grade 0 (absent)	-	-	+
Grade I (0 - 20%)	+	-	-
Grade II (21 – 50%)	+	++	-
Grade III (51 – 80%)	+	++	++
Grade IV (81 – 100%)	-	-	-

Legend: (-) negative; (+) mild; (++) moderate; (+++) intense

LEGEND: Animals were orally or intraperitoneally treated with vehicle (Panel **A**), fruti 3 mg kg^{-1} (Panel **B**) and MTX 2.5 mg kg^{-1} (Panel **C**) during 21 days. Black Arrows and head arrows indicated Ehrlich viable cells and coagulative necrosis area, respectively. Brown arrow indicates apoptotic cells. Samples were observed in optical microscope at 20 x (scale bar 20 µm).

4.4.13. Effect of fruti on plasmatic and hematologic parameters

As depicted on Supplementary Table 4, fruti treatment induced a slight decreased in ALT and increase in monocytes, compared to vehicle group. MTX treatment reduced total protein, globulin, lymphocytes and hemoglobin levels and increased monocytes compared to vehicle and naïve group (Suppl.Table S4).

4.5. DISCUSSION

Fruticuline A is one of the major constituents of the ethanolic extract of *Salvia lachnostachys* (EES) leaves, which was demonstrated to have antitumor effect against SEC in mice (Corso et al, 2019). Fruti is a diterpene quinone and has some biological properties already described, such as antinociceptive, anti-inflammatory, antidepressive and antimicrobial effects (Piccinelli et al., 2014; Santos et al., 2017; Schito et al., 2011). Cytotoxicity of fruti against a range of human cancer cell lines has also been demonstrated, including U251 (glioma, central nervous system), UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian-resistant), NCI-H460 (lung, non-small-cell), PC-3 (prostate), OVCAR-3 (ovarian) and HT-29 (colon) (Oliveira et al., 2016). However, none of these studies investigated the antitumor effect of fruti and its action mechanism. Thus, the present study was the first one to investigate the antitumor effect of fruti *in vivo* and elucidate to the action mechanism in different tumor cell lineages.

Fruti was capable to inhibit proliferation of tumor cells (MCF-7 and HepG2) indicating antiproliferative effect. As fibroblasts are non-tumor cells that surround tumor tissue, we then investigate whether fruti could inhibit the proliferation of this cell. We observed a reduction on proliferation only at high concentration (250 μM), but none alterations in expression of genes related to proliferation, apoptosis and angiogenesis in these cells. Thus, we hypothesized that instead of damage normal cells, fruti inhibits the proliferation as well as induces cell death only in tumor cells. For this reason, we focused on investigate its antitumor effect and mechanisms only in tumor cells.

Fruti potentially inhibited *CCND1 (CYCLIN D1*) and *BCL-2* gene expression in MCF-7 cells. Cyclin D1 is a protein that regulates proliferation and is found in cytoplasm and nuclei (Alao, 2007), whereas Bcl-2 is an anti-apoptotic protein that regulate apoptosis and determine the response of cancer cells to chemotherapeutics (Timucin et al., 2019). Both genes can be transcriptionally regulated by STAT3 and/or NF-κB, which play an important role in the development of carcinogenesis (Al Zaid Siddiquee and Turkson, 2008; Park and Hong, 2016; Yue and Turkson, 2009). Despite fruti did not change STAT3 levels, it induced a decrease on NF-κB1 gene expression. NF-κB is a family of transcription factors, including RELA*,* RELB*,* NF-κB1*,* NF-κB2*,* and IκBα*,* which are inducible by a variety of stimuli. Protein products of these genes lead to activation of p65, p50 and p52. The nuclear translocation of NF-κB complexes p65/p50 and p52/RelB is triggered by its ubiquitination and proteasomal degradation. Consequently, these complexes regulate the

transcription of genes in the nuclei that mediate a variety of cellular functions such as proliferation (i.e. Cyclin D1, Cyclin E, IL-6), tumor promotion (i.e. VCAM, ICAM, iNOS, COX 2), apoptosis (i.e. Bcl-2, Bcl-xL, Survivin) and inflammation (i.e. TNF-α, COX2, iNOS) (El-Kady et al., 2011; Park and Hong, 2016). Thus, our results suggest the involvement of NF-κB pathway on the regulation of Cyclin D1 and Bcl-2 gene expression induced by fruti. However, a global inhibition of NF-κB is not possible, as it would induce to genetic instability (Riedelinger et al., 2018). In fact, we observed a partial decrease in NF-κB, in order of -63 and -65% in MCF-7 and HepG2 cells, respectively, compared to control group. In addition, it was already described that some diterpenes such as excisanin A and kamebakaurin from *Isodon japonicus*, inhibit NF-κB in inflammatory process (Hwang et al., 2001; Lee et al., 2002). Therefore, it is possible that the antitumor effect of fruti is dependent of NF-κB inhibition.

The mechanism for cellular death in neoplastic cells can be resulted of programmed cellular death (aptoptosis), necrosis or necroptosis. These pathways are induced by different signals, such as DNA damage, oxidative stress, hypoxia and/or ligands of death receptor family (i.e. TNF-α) (Hold and El-Omar, 2008). c-Jun N-terminal kinases (JNK) pathway is one of those involved in the cellular death. JNK forms an important subgroup of the mitogen-activated protein kinases (MAPK) superfamily and are mainly activated by oxidative stress and/or TNF-α, being a key promoter for cell suicide, inducing to apoptosis or necrosis (Sheng and Liu, 2006). The programmed cellular death, on the other hand, has a variety of pathways and proteins that regulate or trigger apoptosis, such as the anti- (i.e. Bcl-2, Bcl-xL) or pro- (i.e. Bax, Bid) apoptotic proteins and caspases effectors (i.e. caspase 3 and 7) (Youle and Strasser, 2008). Herein, TNF-α was not detected in the cells and no difference was observed in pJNK levels. However, the apoptosis was induced by fruti, probably due to the reduction on Bcl-2 levels. In addition to apoptosis, we also observed that fruti enhanced RIPK1 gene expression in the higher concentrations. RIPK1 is the target for necroptosis pathway, a form of regulated necrotic cell death. Nec-1 has been used as a tool to investigate necroptosis pathway due to its ability in inhibit RIPK1 activity (Wegner et al., 2017). In fact, the antiproliferation effect of fruti in MCF-7 cells was reversed in the presence of Nec-1, confirming the involvement of RIPK1 in the cytotoxicity of fruti. Thus, *in vitro* fruti also induced cellular death through the necroptosis pathway.

In vivo, the antitumor effect of fruti was confirmed by the effective prevention of tumor development in animals with SEC. In tumor tissue of animas treated with fruit we

observed the reduction on *Cyclin D1*, *Bcl-2* and *Rela* (a protein from NF-κB pathway), as the same observation in the *in vitro* experiments. However, regarding the VEGF gene the results were different. Reduction on *Vegf* expression was observed with the fruti treatment *in vivo*, whereas *in vitro* only MCF-7 exhibited an increase in *VEGF* expression, in the higher concentration of fruti, but without increase in VEGF protein. The reduction on *Vegf in vivo* can reflect on a reduction of angiogenesis and nutrition to tumor tissue, which induce to cellular death by starvation resulting in coagulative necrosis (Simon et al., 2017). Therefore, beyond the tumor cell death through apoptosis induced by fruti in SEC, it also has anti-angiogenic properties in solid tumors inducing to cell death by coagulative necrosis.

As inflammation is intrinsically correlated with cancer development (Galdiero et al., 2008), we then investigate some cytokines in tumor tissue. Interestingly, there is an increase in tumor TNF-α and decrease in IL-10 levels in animals treated with fruti, showing a pro-inflammatory effect of fruti. The increase in TNF-α levels can be correlated with an increase in blood monocytes, which could be a source for TNF-α production (Takashima et al., 1990). As reviewed by Landskron et al. (2014), several studies have showed the inhibitory effect of IL-10 on NF-κB signaling. Therefore, the reduction on IL-10 observed in our study can explain in part the reduction on *Rela* expression in tumor tissue. In addition to the enhanced inflammatory environment in tumor tissue, we observed a slight increase in ROS levels, which can also be triggered by inflammatory mediators. Indeed, the tumor histology showed more inflammatory cells, coagulative necrotic cells and apoptosis, and less Ehrlich viable cells, in tumor of fruti-treated mice compared to the control group. Thus, the increase in tumor inflammation can also induce tumor cell death or can be a result of the necrosis (Landskron et al., 2014; Rock and Kono, 2008) induced by the fruti**.**

The inflammatory tumor microenvironment can be the explanation for some different results observed in experiments *in vivo* and *in vitro* with fruti, for instance the VEGF and RIPK1 expressions. Despite the increase in RIPK1 *in vitro*, we could not detect *Ripk1* in tumor tissue. Due to the pro-inflammatory effect of fruti we hypothesized that *in vivo* the tumor microenvironment changes the response to antitumor agents, compared to *in vitro* observations. The long last treatment *in vivo* can also change the mechanism for cellular death. Furthermore, the particularity of each tumor cell lineages studied *in vitro* (MCF-7 and HepG2) and *in vivo* (Ehrlich), should also be consider in the interpretation of the results.

Despite the cytotoxic effects of fruit in tumor cells, it did not induce systemic toxicity, observed by the hematological and biochemical parameters. Moreover, none difference among treatments was observed in the body and organs weigh (data not shown). Previously, our group showed that the source of fruti, the *S. lachnostachys* extract, had no toxicity, with a LD_{50} higher than 2000 mg/kg (Corso et al., 2019). On the other hand, MTX, the chemotherapic compound used as positive control, has oral LD_{50} in mice of 146 mg kg^{-1} (ChemIDplus, 1982). MTX treatment in mice bearing Ehrlich tumor produced systemic and hematologic adverse effects. As fruti also had antitumor effect comparing with MTX, this can make fruti a good candidate to treat solid tumors with less toxic effect.

In conclusion, fruticuline A exerts antitumor effects through inhibition of the NF-κB pathway, reducing Cyclin D1 and Bcl-2 levels. The effects depend on both the cell lineages and the experimental conditions. In MCF-7 and HepG2 cells the apoptosis and necroptosis pathways are involved in cell death, whereas in the Ehrlich tumor in mice, due to the long-term treatment and tumor microenvironment, cells undergo necrosis by the increase in tumor inflammation and reduction on angiogenesis. Thus, fruticuline A acts on tumor cells by a combination of two mechanisms and represents a promising molecule for being used directly or in a modified form in cancer therapy, especially against solid mammary and hepatic tumors.

4.6. ACKNOWLEDGMENTS

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4.8. SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE 1 - GENE SEQUENCES OF REAL-TIME QUANTITATIVE PCR PRIMERS FOR HUMAN CELLS.

Target	Forward primer (5'→3')	Reverse primer (5'→3')
VEGFA	ATCTGCATGGTGATGTTGGA	GGGCAGAATCATCACGAAGT
CCND1	CCGTCCATGCGGAAGATC	GAAGACCTCCTCCTCGCACT
BCL-2	GATTGTGGCCTTCTTTGAG	CAAACTGAGCAGAGTCTTC
NF-κB1	TACTCTGGCGCAGAAATTAGGTC	CTGTCTCGGAGCTCGTCTATTTG
RIPK1	GGCATTGAAGAAAAATTTAGGC	TCACAACTGCATTTTCGTTTG
TNF-α	CCTGCTGCACTTTGGAGTGA	GAGGGTTTGCTACAACATGGG
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
CyP	ACGGCGAGCCCTTGG	TTTCTGCTGTCTTTGGGACCT
ACTIN	GAGCACAGAGCCTCGCCTTT	TCATCATCCATGGTGAGCTGG
HPRT	TGACCTTGATTTATTTATTTTGCATACC	CGAGCAAGACGTTCAGTCCT

SUPPLEMENTARY TABLE 2 - LIST OF IMMUNOBLOTTING ANTIBODIES FOR HUMAN CELLS.

Target Protein	Company	Catalog No.	Source	Isotype	Dilution
VEGFA	Santa Cruz	(C-1) sc-7269	mouse	IgG	1:200
CCND1	Santa Cruz	(A-12) sc-8396	mouse	IgG	1:200
BCL-2	Santa Cruz	(100) sc-509	mouse	IgG	1:200
pSTAT3	Cell signaling	9145	rabbit	IgG	1:2000
STAT3	Santa Cruz	(F-2) sc-8019	mouse	IgG	1:200
pJNK	Cell signaling	4668	rabbit	IgG	1:1000
GAPDH	Cell signaling	5174	rabbit	IgG	1:2000

SUPPLEMENTARY TABLE 3 - GENE SEQUENCES OF REAL-TIME QUANTITATIVE PCR PRIMERS FOR MICE TISSUE.

(continua)

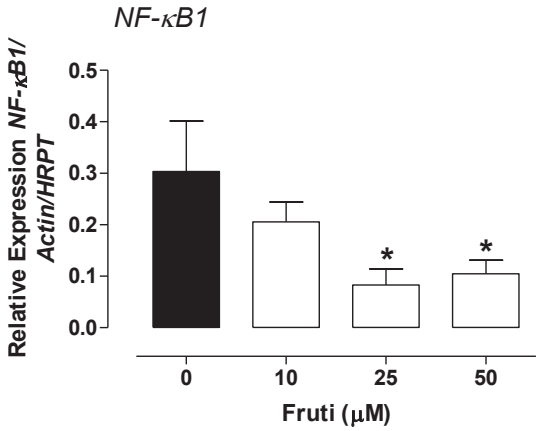
Target	Forward primer (5'→3')	Reverse primer (5'→3')
Vegfa	ACTGGACCCTGGCTTTACTGCT	TGATCCGCATGATCTGCATGGTG
Ccnd1	AGAAGTGCGAAGAGGAG	GGATAGAGTTGTCAGTGTAGAT
Bcl-2	CACTTGCCACTGTAGAGA	GCTTCACTGCCTCCTT
Rela tx2	ACCTGGAGCAAGCCATTAGC	GAGGCGCACTGCATTC
IkBa	GCTACCCGAGAGCGAGGAT	GCCTCCAAACACACAGTCATCAT
Ripk1	TCATCTAGCGGGAGGTTGGA	ATGCCCAGTAGCTTCACCAC
Gapdh	GGTGAAGCAGGCATCT	TGTTGAAGTCGCAGGAG

SUPPLEMENTARY TABLE 4 - PLASMATIC PARAMETERS IN HEALTHY AND TUMOR-BEARING MICE TREATED WITH VEHICHE, FRUTI OR MTX DURING 21 DAYS.

	Experimental groups			
Parameters	**Naive**	**Veh**	**Fruti**	**MTX**
Glucose (mg dL^{-1})	122.08 ± 12.17	133.02 ± 7.11	154.04 ± 15.50	119.08 ± 24.69
Creatinine (mg dL^{-1})	0.68 ± 0.06	0.73 ± 0.05	0.72 ± 0.04	0.63 ± 0.03
AST (U L^{-1})	86.89 ± 8.72	272.02 ± 16.79$^{\#}$	280.09 ± 28.57$^{\#}$	276.09 ± 42.00$^{\#}$
ALT (U L^{-1})	65.02 ± 5.54	65.94 ± 6.03	41.70 ± 2.53$^{*,\#}$	52.57 ± 5.71
Total protein (g dL^{-1})	5.91 ± 0.16	6.56 ± 0.10$^{\#}$	5.81 ± 0.13*	4.30 ± 0.24$^{*,\#}$
Globulin (g dL^{-1})	2.80 ± 0.07	2.76 ± 0.11	3.32 ± 0.15	1.54 ± 0.38$^{*,\#}$
Albumin (g dL^{-1})	3.09 ± 0.08	3.92 ± 0.21$^{\#}$	3.32 ± 0.15*	2.78 ± 0.24*
Leukocytes ($^{x}10^{3}$/µL)	5.64 ± 0.79	5.16 ± 0.44	7.00 ± 0.61	5.83 ± 1.17
Lymphocytes ($^{x}10^{3}$/µL)	3.90 ± 0.60	3.71 ± 0.27	5.37 ± 0.57	3.73 ± 0.84
Monocytes ($^{x}10^{3}$/µL)	0.20 ± 0.04	0.15 ± 0.02	0.28 ± 0.02*	0.36 ± 0.07$^{*,\#}$
Erythrocytes ($^{x}10^{6}$/µL)	9.85 ± 0.10	9.51 ± 0.17	9.79 ± 0.27	8.35 ± 0.32$^{*,\#}$
Hemoglobin (g dL^{-1})	13.28 ± 0.19	12.52 ± 0.27	13.45 ± 0.38	11.58 ± 0.38$^{*,\#}$
Hematocrit (%)	44.58 ± 0.99	41.46 ± 0.83	42.89 ± 1.01	35.13 ± 1.38$^{*,\#}$

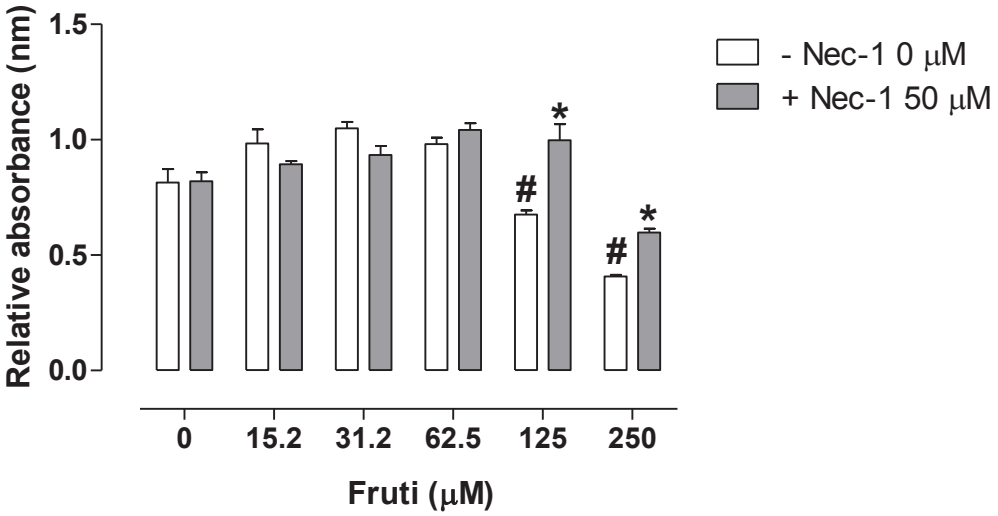
LEGEND: Results were expressed as mean ± S.E.M. and were analyzed by one-way ANOVA followed by Newman-Keuls post hoc test (n=5-8). * and # indicated $p<0.05$ when compared to vehicle and naïve group, respectively.

SUPPLEMENTARY FIGURE S1 - NF-κB1 EXPRESSION IN HEPG2 CELLS.



LEGEND: Cells were incubated with vehicle or fruti at 10, 25 and 50 μM for 24 h. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-away ANOVA followed by Newman Keuls post hoc test. * p<0.05 when compared to vehicle group.

SUPPLEMENTARY FIGURE S2 - WST-1 VIABILITY CELLS ASSAY IN THE PRESENCE AND ABSENCE OF NECROSTATIN 50 μM IN HEPG2 CELLS.



LEGEND: Cells were incubated with vehicle (0) or fruti at 15.2, 31.2, 62.5, 125 and 250 μM for 24 h. Results are expressed as mean ± S.E.M. (n= 3) and were analyzed by one-away ANOVA followed by Newman Keuls post hoc test. * and # indicate p<0.05 when compared to (-) Nec-1 (absence of Necrostatin) and vehicle group (0 μM), respectively.

5. CONSIDERAÇÕES FINAIS

Diante dos resultados obtidos neste trabalho, foi possível caracterizar, através das análises de HPLC, os componentes do EES, bem como o diterpeno fruticulina A como um dos componentes majoritários presentes nesse extrato. A avaliação biológica do EES e da fruti evidenciou seu efeito antitumoral no modelo de carcinoma sólido de Ehrlich em camundongos, através da diminuição da Ciclina D1, aumento da inflamação no tumor e morte celular por necrose *in vivo*. Ainda, ambos os tratamentos não se mostraram tóxicos sistemicamente, podendo representar moléculas potencialmente seguras para uso na medicina humana. Devido aos efeitos similares citados acima da fruti e do EES, pode-se concluir que a fruti, por ser um dos compostos majoritários do EES, é responsável por boa parte dos efeitos antitumorais do EES. Dessa maneira, para aprofundar a investigação, somente a fruti foi testada *in vitro*. A fruti quando isolada apresentou alguns efeitos diferentes do EES, como inibição do NF-κB e morte celular por apoptose *in vivo* e *in vitro*, redução de VEGF *in vivo* e indução de morte celular por necroptose *in vitro*. A diferença de mecanismo de morte celular nos experimentos *in vitro* para o *in vivo* da fruti é devido, provavelmente, ao aumento da inflamação no tumor, diminuição da angiogênese, tempo de tratamento mais prolongado, e à presença do microambiente tumoral, que levam as células à morte celular por necrose. Além disso, as diferenças dos efeitos da fruti em relação ao EES são, possivelmente, devido a uma complexa mistura de componentes bioativos que o EES possui. Deste modo, estudos aprofundados dos outros componentes do EES são necessários. Apesar disso, a fruti, por ser uma molécula isolada e que requer baixas quantidades para obter o efeito terapêutico, representa uma valiosa opção terapêutica para tumores sólidos, principalmente de origem mamária e hepática.

Por fim, a hipótese inicial deste trabalho, relacionada ao efeito antitumoral tanto com o tratamento com EES quanto com a fruti foi confirmada. No entanto, esses dados representam o início de uma longa jornada de investigação sobre os efeitos e mecanismos de ação do EES e da fruti no câncer, que ainda necessitam ser aprofundados. Pesquisas adicionais incluem avaliação em outros modelos de tumor *in vivo* e *in vitro*, investigação de outras vias envolvidas na carcinogênese, estudos farmacocinéticos e toxicológicos crônicos, e avaliação do efeito terapêutico em combinação com fármacos antineoplásicos, com os quais o EES e a fruti podem representar uma terapia coadjuvante na quimioterapia.

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