UNIVERSIDADE FEDERAL DO PARANÁ

ELIANA REZENDE ADAMI

EFEITOS DE POLISSACARÍDEOS EXTRAÍDOS DO FRUTO DO *Capsicum annuum* (PIMENTÃO) EM MODELOS *IN VIVO* E *IN VITRO* DE TUMOR MAMÁRIO

CURITIBA

2019

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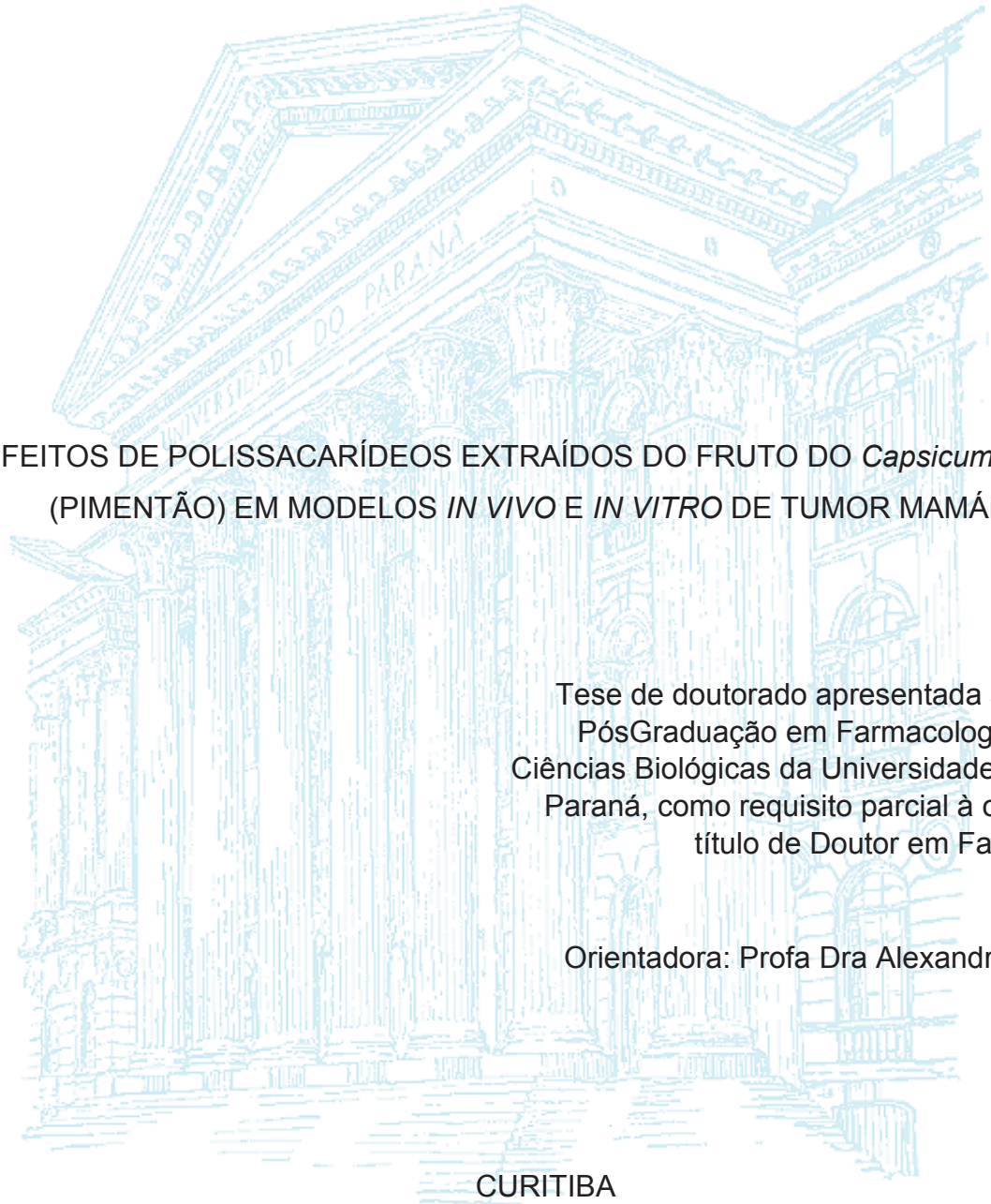

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Orientadora: Profa Dra Alexandra Acco.

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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 **ELIANA REZENDE ADAMI** intitulada: **EFEITOS DE POLISSACARÍDEOS EXTRAÍDOS DO FRUTO DO *Capsicum annuum* (PIMENTÃO) EM MODELOS *IN VIVO* E *IN VITRO* DE TUMOR MAMÁRIO**, 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, 27 de Junho de 2019.

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Esta tese é apresentada em formato alternativo – artigos 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, dois artigos científicos e considerações finais. Os artigos foram formatados de acordo com as normas propostas por periódicos internacionais.

“Estou entre aqueles que acham que a ciência tem uma grande beleza.”

Marie Curie

Dedico essa conquista ao papai,
Múcio Vasconcelos Adami e
à mamãe, Lêda Rezende Adami,
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a tudo que fizeram por mim,
tudo que sou hoje devo a vocês,
obrigada pelo amor incondicional!
Amo vocês além da vi

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RESUMO

O câncer de mama representa um problema de saúde pública, pois é o mais incidente entre as mulheres. Dentre as modalidades de tratamento está a quimioterapia, realizada com fármacos citotóxicos que causam vários efeitos colaterais. Nesse contexto, buscam-se novos agentes anticancerígenos com menos efeitos adversos. Este estudo investigou o efeito antitumoral de polissacarídeos extraídos do pimentão (*Capsicum annuum* - CAP, via oral) em camundongos portadores do tumor de Ehrlich em 3 protocolos *in vivo*: ***(a)*** Convencional (21 dias), ***(b)*** Tratamento longo (LT, 31 dias) e ***(c)*** Associado com metotrexato (CAP v.o. + MTX i.p., 21 dias). O CAP também foi testado *in vitro* em células de tumor mamário humano (MCF-7, MDA-MB-231 e MDA-MB-436). Investigou-se o mecanismo de ação do CAP através de biomarcadores tumorais de estresse oxidativo, inflamação, angiogênese, apoptose e ciclo celular. Os resultados do ***(a)*** protocolo convencional mostraram que o CAP, nas três doses testadas (50, 100 e 150 mg kg^{-1}, v.o.) foi capaz de reduzir o volume do tumor em 28%, 40% e 54%, respectivamente, enquanto o controle positivo (Metotrexato – MTX 2,5 mg kg^{-1}, via i.p., a cada 72 h) reduziu o volume do tumor em 85%. Assim, a dose de 100 mg kg^{-1} de CAP foi escolhida para as demais análises. Nos parâmetros inflamatórios tumorais, o tratamento com CAP aumentou os níveis de IL-6, mas não alterou MPO, NAG, Nitrito, TNF-α, IL-10 e IL-4. O CAP não alterou no tecido tumoral a expressão de genes relacionados à apoptose (*Bcl-2*, *Bax* e *Caspase-8*) e proliferação celular (*Ciclina D1*), mas reduziu a expressão de *Vegf* em 40% quando comparado com o veículo, o que foi confirmado pela redução da área de vasos nos cortes histológicos do tumor, mesmo sem reduzir o número total de vasos. O CAP não alterou parâmetros de estresse oxidativo no tumor, tampouco apresentou efeitos antioxidantes *in vitro* no teste do DPPH, demonstrando que seu mecanismo de ação não envolve esta via. Os resultados do protocolo ***(b)*** LT, no qual o tratamento teve início 10 dias antes da inoculação das células tumorais de Ehrlich, persistindo até 21 dias após, corroboraram com o protocolo ***(a)***. O CAP provocou redução do desenvolvimento tumoral em 91%, além de aumentar IL-6 (85%) e MPO (37%) tumoral, reduzir IL-10 (95%) e IL-4 (94%), e reduzir a expressão gênica de *Vegf* (55%) e a área vascular (47%) no tumor. No protocolo ***(c)*** foi realizado o tratamento do CAP associado ao MTX por 21 dias, o que reduziu ainda mais o desenvolvimento tumoral (95% de inibição), bem como elevou os níveis tumorais de IL-6 (702%) e TNF-α (390%), e diminuiu Nitrito (62%), IL-10 (57%), IL-4 (85%), a expressão de *Vegf* (43%) e a área vascular (48%) nos tumores. O CAP não evidenciou toxicidade através de parâmetros bioquímicos e hematológicos, exceto redução de leucócitos no protocolo ***(c)***. Paralelamente, *in vitro* o CAP reduziu colônias celulares nas três linhagens tumorais humanas (MCF-7, MDA-MB-231 e MDA-MB-436), mas não reduziu a viabilidade da linhagem mamária não-tumoral HB4a. De acordo com dados *in vivo*, o CAP também reduziu a expressão gênica de VEGF em células MCF-7 e MDA-MB-436, mas não em células MDA-MB-231, considerada uma linhagem triplo-negativa e muito agressiva. *In vitro*, a associação CAP+MTX diminuiu a viabilidade das células MDA-MB-231 e MDA-MB-436 de modo mais expressivo quando comparada com os dois compostos isoladamente. Portanto, o CAP apresenta efeito antineoplásico contra células tumorais mamárias, pois foi capaz de reduzir o volume tumoral nos três protocolos testados *in vivo*, além de reduzir a viabilidade de células tumorais humanas de glândula mamária. Seu mecanismo antineoplásico parece depender da regulação da inflamação e da angiogênese, desencadeando necrose em células tumorais. O CAP se apresenta promissor como terapia adjuvante no tratamento de tumores mamários, tanto isolado quanto combinado com quimioterapia. Estudos em outros modelos e linhagens celulares, abordando aspectos farmacocinéticos e toxicológicos, e interação com diferentes fármacos quimioterápicos, devem ser continuados.

Palavras-chave: Câncer de mama. Polissacarídeos. *Capsicum annuum*. Adenocarcinoma mamário de Ehrlich. Inflamação. Angiogênese.

ABSTRACT

Breast cancer represents a public health problem, as it is the most incident in women. The chemotherapy is used in the treatment, performed with cytotoxic drugs that cause several side effects. In this context, new anticancer agents with fewer adverse effects are sought. This study investigated the antitumor effect of polysaccharides extracted from sweet green pepper (*Capsicum annuum* – CAP, *per os*) in mice with Ehrlich tumor in 3 protocols *in vivo*: ***(a)*** Conventional (21 days), ***(b***) Long treatment (LT, 31 days) and ***(c)*** Associated with methotrexate (CAP p.o. + MTX i.p., 21 days). CAP was also tested *in vitro* in human mammary tumor cells (MCF-7, MDA-MB-231 and MDA-MB-436). We investigated the mechanism of action of CAP through tumor biomarkers of oxidative stress, inflammation, angiogenesis, apoptosis and cell cycle. The results of the ***(a)*** conventional protocol showed that CA P, at the three doses tested (50, 100 and 150 mg kg^{-1}) was able to reduce the tumor volume by 28%, 40% and 54%, respectively, while the positive control (Methotrexate - MTX 2.5 mg kg^{-1}, via i.p., every 72 h) reduced tumor volume by 85%. Thus, the 100 mg kg^{-1} dose of CAP was chosen for the next analyzes. About the inflammatory parameters in tumor, CAP treatment increased IL-6 levels, but did not alter MPO, NAG, Nitrite, TNF-α, IL-10 and IL-4 level. CAP did not alter the expression of apoptosis-related genes (*Bcl-2*, *Bax* and *Caspase-8*) and cell proliferation (*Cyclin D1*) in the tumor tissue, but reduced the expression of *Vegf* by 40% when compared to the vehicle, which was confirmed by the reduction of vessel area in the histological sections of the tumor, even without reducing the total number of vessels. CAP did not alter oxidative stress parameters in the tumor, nor did it show antioxidant effects *in vitro* in the DPPH test, demonstrating that its mechanism of action does not involve oxidative stress. The results of protocol ***(b)*** LT, which started 10 days prior to inoculation of Ehrlich tumor cells and persisted for up to 21 days thereafter, corroborated withthose of protocol ***(a)***. CAP in ***(b)*** also reduced the tumor development by 91%, increased tumor levels of IL-6 (85%) and MPO (37%), reduced IL-10 (95%) and IL-4 (94%), and reduced the gene expression of *Vegf* (55%) and the vascular area (47%) in the tumor. In protocol ***(c)*** CAP + MTX treatment was performed for 21 days, which reduced the tumor development (95% inhibition), as well as elevated tumor levels of IL-6 (702%) and TNF-α (390%), and decreased levels of Nitrite (62%), IL-10 (57%), IL-4 (85%), the *Vegf* gene expression (43%) and the vascular area (48%) in tumors. CAP did not show toxicity through biochemical and hematological parameters, except a leukocyte reduction in protocol ***(c)***. In parallel, CAP reduced cell colonies in three human tumor lineages (MCF-7, MDA-MB-231 and MDA-MB-436), but did not reduce the viability of the non-tumor mammary HB4a cells. According to *in vivo* data, CAP also reduced VEGF gene expression in MCF-7 and MDA-MB-436 cells, but not in MDA-MB-231 cells, considered a triple-negative and very aggressive cell lineage. *In vitro*, the CAP + MTX association decreased the viability of the MDA-MB-231 and MDA-MB-436 cells more significantly when compared to both compounds isolated. Therefore, CAP has an antineoplastic effect against mammary tumor cells, since it was able to reduce the tumor volume in three protocols tested *in vivo*. In addition, CAP reduced the viability of human mammary gland tumor cells. Its antineoplastic mechanism seems to depend on the regulation of inflammation and angiogenesis, triggering necrosis in tumor cells. CAP is promising as adjuvant therapy in the treatment of breast tumors, both isolated and combined with chemotherapy. Studies in other tumor models and cell lineages, addressing pharmacokinetic and toxicological aspects, and interaction with different chemotherapeutic drugs, should be continued.

Key words: Breast cancer. Polysaccharide. *Capsicum annuum*. Ehrlich breast adenocarcinoma. Inflammation. Angiogenesis.

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LISTA DE SIGLAS E ABREVIATURAS

ALT: Alanina aminotransferase

ANOVA: Análise estatística de variância

ANWS: fração solúvel em água a frio

FAL: Fosfatase alcalina

ARE: Antagonista de receptores de estrogênio

AST: Aspartato aminotransferase

ATCC: American Type Culture Collection

ATP: Adenosina trifosfato

Bax: Bcl-2 associada à proteína X

Bcl-2: Proteína 2 de células B de linfoma

BRCA: Gene do câncer de mama

CA: *Capsicum annuum*

CAP: Polissacarídeos do *Capsicum annuum*

CAT: Catalase

CEUA: Comissão de Ética no Uso de Animais

cDNA: Ácido desoxirribonucleico complementar

DNA: Ácido desoxirribonucleico

DL_{50}: Dose letal 50%

DMSO: Dimetilsulfóxido

DPPH: 2,2–Difenil–1–picril–hidrazila

DTNB: Ácido 5,5-ditiobis(2-nitrobenzóico)

EDTA: ácido etilenodiamino tetracético

ER: Receptores de estrogênio

ER (-): Receptores de estrogênio negativo

EROs: Espécies reativas de oxigênio

FBS: Soro fetal bovino

Gapdh: Gliceraldeíde-3-fosfato desidrogenase

GSH: Glutationa reduzida

HB: Hemoglobina

Ht: Hematócrito

HE: Hematoxilina e eosina

HER2: Receptor 2 do fator de crescimento epidérmico humano

HER2 (-): Receptor 2 do fator de crescimento epidérmico humano negativo

H_2O_2: Peróxido de hidrogênio

IA: Inibidores de aromatase

IFN-Y: Interferon Y

IL-1β: Interleucina 1 beta

IL-4: Interleucina 4

IL-6: Interleucina 6

IL-10: Interleucina 10

INCA: Instituto Nacional do câncer

I.P: Intraperitoneal

I.V.:Intravenoso

LPO: Peroxidação lipídica

K_2HPO_4: Fosfato dipotássico

KH_2PO_4: Fosfato monopotássico

LNS: Linfonodo sentinela

MPO: Mieloperoxidase

mRNA: Ácido ribonucleico mensageiro

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

MTX: Metotrexato

MRE: modulador de receptor de estrogêneo

NADPH: Nicotinamida adenina dinucleótido fosfato

NAG: N-acetilglicosaminidase

NK: Células Natural killer

NaOAc: Acetato de sódio

NO: Óxido nítrico

Nos2: Óxido nítrico sintase 2

$O2^{\bullet -}$: Ânion superóxido

OH•: Radical hidroxila

OMS: Organização Mundial da Saúde

PCR: Reação em cadeia polimerase

p53: Proteína 53

PBS: Salina tamponada com fosfato

pH: Potencial hidrogeniônico

PR: Receptores de progesterona

PR (-): Receptores de progesterona negativo

RBC: Glóbulos vermelhos

RNA: Ácido ribonucleico

RO•: Radical alcoxil

ROO•: Radical peroxil

ROOH•: Radical hidroperoxil

rpm: Rotação por minuto

Rplp0: Proteína lateral ribossômica subunidade P0

RT-qPCR: Reação em cadeia da polimerase quantitativa em tempo real

S.C: Subcutâneo

SEM: Erro padrão da média

SOD: Superóxido dismutase

TE: Tumor de Ehrlich

TMB: Tetrametilbenzidina

TMSP: Ácido 3-trimetilsilil-2H_4-propiônico

TNBC: Câncer de mama triplo negativo

TNF-α: Fator de necrose tumoral alfa

VCM: Volume Corpuscular Médio

VEGF: Fator de crescimento endotelial vascular

V.O.: Via oral

ZAG: zinco α2-glicoproteína

SUMÁRIO

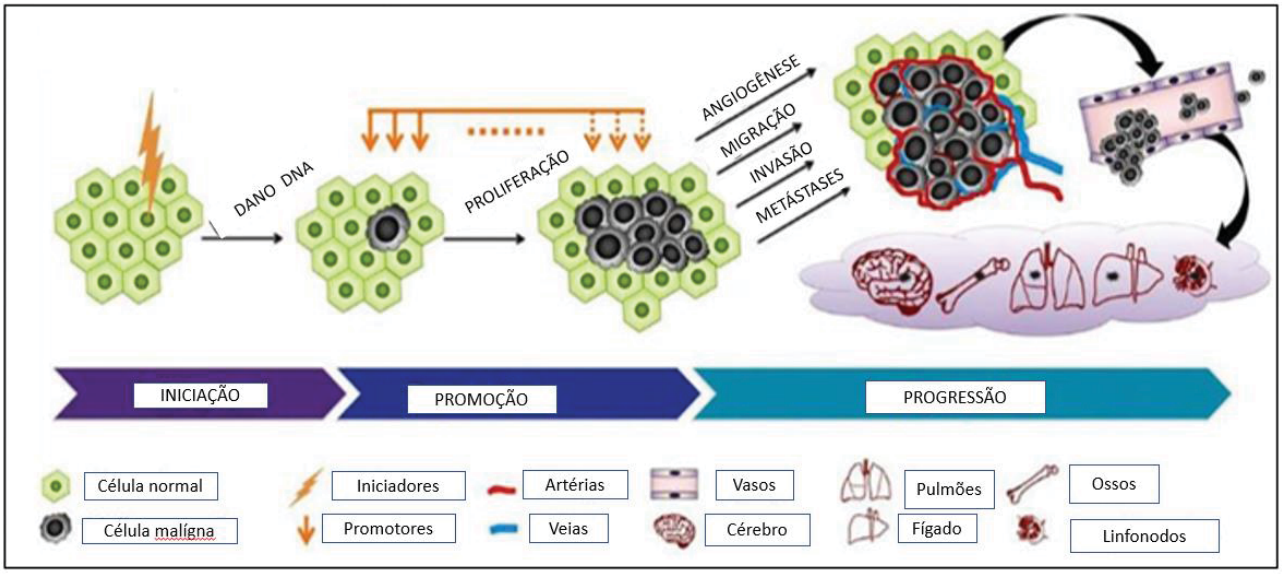
1. INTRODUÇÃO

1.1 CÂNCER

O câncer pode iniciar-se de forma espontânea ou ser provocado pela ação de agentes carcinogênicos (químicos, físicos ou biológicos) que podem ser endógenos ou exógenos. Os agentes endógenos (enzimas, processos patológicos e células) são, via de regra, geneticamente pré-determinados, e estão relacionados com a defesa do organismo às agressões externas (INCA, 2018). Os agentes exógenos (poluentes, gases atmosféricos e radiação) são responsáveis pelo aumento da incidência do câncer nos países economicamente desenvolvidos, como resultado da adoção de estilos de vida pouco saudáveis, incluindo o tabagismo, falta de atividade física, dietas inadequadas e exposição excessiva ao sol. Cerca de 80 a 90% dos casos de câncer estão associados com fatores externos (INCA, 2018).

O envelhecimento também aumenta a susceptibilidade à transformação maligna, o que contribuiu para aumentar a incidência de câncer em países desenvolvidos (JEMAL et al., 2011; INCA, 2018). Independente da causa, ocorrem alterações mutagênicas e não-mutagênicas ou epigenéticas nas células, sendo que o processo de carcinogênese (Figura 1) geralmente ocorre de forma lenta (LIU et al., 2015; INCA, 2018).

Figura 1 – Etapas da Carcinogêse



Fonte: Adaptado de LIU et al., 2015.

Durante o processo de carcinogênese ocorrem alterações moleculares muito dinâmicas que controlam a transformação das células normais em tumorais, sendo as mais comuns as dez características mostradas na Figura 2, essas alterações auxiliam no desenvolvimento dos cânceres acelerando o processo de carcinogênese, pois favorecerão o crescimento e manutenção do tumor. Portanto, esses fatores constituem alvos terapêuticos, no sentido de impedir o desenvolvimento dos cânceres (HANAHAN; WEINBERG, 2011).

Figura 2 – Alterações Moleculares Comuns nos Cânceres

Fonte: Adaptado de HANAHAN e WEINBERG (2011).

Alguns genes desempenham um papel importante como regulatórios da carcinogênese, garantindo que a proliferação celular, apoptose, auto-renovação e diferenciação sejam normalmente controladas (WONGTRAKOONGATE, 2015; SAFA, 2019). Dentre eles, os proto-oncogenes são considerados dominantes, por serem capazes de transformar as células mesmo na presença de seu alelo normal; e os genes supressores tumorais como o p53, que são considerados recessivos, pois ambos os alelos normais precisam ser lesados para ocorrer a transformação. Os proto-oncogenes, quando ativados, estão ligados com a produção de fatores de crescimento, e, portanto com a multiplicação celular, favorecendo o crescimento do tumor (MONTENEGRO & FRANCO, 2008). Exemplos de genes que podem se comportar tanto como proto-oncogenes quanto como supressores tumorais são os reguladores da apoptose. Um exemplo que pode ser evidenciado é o de alterações herdadas do gene supressor do tumor encontradas em algumas síndromes cancerígenas hereditárias causando certos tipos de câncer, em determinadas famílias. É importante ressaltar que a maioria das mutações de genes supressores do tumor é adquirida, não herdada. Como exemplo

podem ser citadas as anormalidades do gene TP53 (que codifica a proteína p53) que foram encontradas em mais de metade dos cânceres humanos (INCA, 2018).

As células tumorais, assim como as demais células do organismo, precisam do oxigênio e dos nutrientes carregados pelo sangue e linfa para sobreviverem e se multiplicarem. Quando as células do tumor necessitam de sangue extra, elas secretam substâncias que estimulam o desenvolvimento de novos vasos, esse processo é denominado de angiogênese. A principal dessas substâncias é o fator de crescimento endotelial vascular (VEGF). Ao chegar à parede do vaso sanguíneo mais próximo, as moléculas de VEGF estimulam novos vasos a brotarem do existente e crescerem em direção às células que emitiram o sinal (BOARETO et al., 2015). Assim, fármacos anti-angiogênese parecem ser um alvo terapêutico efetivo em cânceres, a exemplo do bevacizumabe.

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; WEST et al., 2019). A caquexia é uma síndrome complexa e multifatorial caracterizada pela perda de peso, atrofia muscular, fadiga, fraqueza, perda de apetite e as vezes acompanhada de anorexia. Atinge cerca de 60 a 80% dos pacientes com câncer terminal e se estima que é a causa de morte de 15 a 20% de todos os pacientes com câncer (AL-ZOUGHBI et al., 2014; WEST et al., 2019). Estudos relatam que muitos tumores secretam mediadores inflamatórios como o fator de necrose tumoral alfa (TNF-α), interleucina 6 (IL-6), intereleucina 1 (IL-1), e pró-catabólicos como o zinco α2-glicoproteína (ZAG), além de fatores liberados sistemicamente em resposta ao tumor, como o interferon gama (IFN-γ). Este fatores estão ligados à degradação do músculo esquelético e tecido adiposo, anorexia e perda de peso, sintomas observados na caquexia (AL-ZOUGHBI et al., 2014; WEST et al., 2019). A resposta inflamatória no microambiente tumoral tem sido alvo de muitas pesquisas, pois pode regular o desenvolvimento de tumores através de seus vários mediadores e células inflamatórias.

Paralelamente, dentre os vários fatores que regulam o desenvolvimento tumoral, o estresse oxidativo se destaca (RANINGA et al., 2014; GLASAUER, CHANDEL, 2014). O estresse oxidativo é definido como um distúrbio no balanço entre a produção de espécies

reativas de oxigênio (ROS) e nitrogênio (RNS) e antioxidantes como mecanismos de defesa, causando toxicidade ao ambiente celular, danos a proteínas, DNA e lipídeos. As ROS e RNS 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. Os antioxidantes, que atuam como defesa a esses radicais livres, são moléculas não enzimáticas como as vitaminas C e E, e moléculas enzimáticas como a superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa reduzida (GSH) e a glicose 6-fosfato desidrogenase (G6PD) (DICHI et al., 2014). Estudos demonstram que as moléculas antioxidantes estão diminuídas sistemicamente em vários tipos de câncer. Entretanto, no tecido tumoral há aumento das mesmas como um mecanismo de defesa do próprio tumor contra as ROS, favorecendo a sua proliferação, sobrevivência e resistência a drogas (RANINGA et al., 2014). Assim, este sistema se constitui de vários alvos farmacológicos para impedir o crescimento tumoral.

O estresse oxidativo também é capaz de induzir apoptose, por ativação das caspases que ativam a família da proteína Bcl-2 e modulam proteínas quinases para induzir morte celular (RANINGA et al., 2014). Para manter o balanço fisiológico entre morte e crescimento celular, o processo da apoptose desencadeia um papel muito importante. 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 (envolvidos na morte celular) (KOFF et al., 2015). As principais proteínas anti-apoptóticas são Bcl-2 e Bcl-XL, enquanto que a Bax, Bad e Bid são proteínas pró-apoptóticas (PAROLIN & REASON, 2001). No entanto, 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 de tratamento para combater a resistência à apoptose e controlar a progressão tumoral (FULDA, 2015).

1.1.1 Câncer de mama

O câncer de mama é o segundo tipo de câncer mais frequente no mundo, perdendo somente para o câncer de pele não-melanoma. Esse tipo de câncer representa 23% de todos os cânceres em mulheres e 14% das mortes para essa doença (BRAY et al., 2018; JUNQUEIRA e CHAMMAS, 2018; SIEGEL; MILLER; JEMAL, 2018; WORLD HEALTH ORGANIZATION, 2018). Na população mundial, a sobrevida média após cinco anos do diagnóstico é de 61%, variando de 80% ou mais na América do Norte, Suécia e Japão, cerca de 60% em países de renda média e abaixo de 40% em países de baixa renda (COLEMAN et al., 2008; INCA, 2014; INCA, 2018; JUNQUEIRA e CHAMMAS, 2018). Dados revelam que este câncer é responsável por aproximadamente 25% dos casos novos a cada ano (WORLD HEALTH ORGANIZATION, 2018). No Brasil é o mais comum entre as mulheres. Foram estimados para 2018/2019 em torno de 59.700 novos casos de câncer de mama em mulheres (Figura 3), e destas, aproximadamente 24% irão a óbito devido a esse câncer, sendo que aproximadamente 15.403 destas mulheres irão a óbito em até cinco anos após o diagnóstico (INCA, 2018).

Figura 3 - Distribuição proporcional dos tipos de câncer mais incidentes estimados em mulheres para 2019, exceto pele não melanoma

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	Localização primária	Casos	%
Mulheres 	Mama Feminina	59.700	29,5%
	Cólon e Reto	18.980	9,4%
	Colo do Útero	16.370	8,1%
	Traqueia, Brônquio e Pulmão	12.530	6,2%
	Glândula Tireoide	8.040	4,0%
	Estômago	7.750	3,8%
	Corpo do Útero	6.600	3,3%
	Ovário	6.150	3,0%
	Sistema Nervoso Central	5.510	2,7%
	Leucemias	4.860	2,4%

Fonte: INCA (2018). Estimativa feminina dos dez tipos de câncer mais incidentes na população brasileira, desconsiderando os tipos de câncer de pele não melanoma, prevista para os anos de 2018/2019.

O câncer de mama é uma doença heterogênea de natureza complexa, que pode se manifestar por meio de múltiplas formas de apresentação clínica e histopatológica, bem como por diferenças na pré- e pós-menopausa, pelos diferentes graus de agressividade tumoral e pelo potencial metastático (PIRES e DELGADO, 2004; EISENBERG e KAIFMAN, 2001; INCA, 2018).

Dados na literatura mostram que fatores relacionados à idade e vida reprodutiva da mulher, tais como menarca precoce, nuliparidade, primeira gestação acima dos 30 anos, menopausa tardia, uso prolongado de anticoncepcional, entre outros, aumentam os riscos de câncer de mama. Outros fatores como obesidade, a grande ingestão de gordura animal, o sedentarismo, o tabagismo e a reposição hormonal podem aumentar as chances de se desenvolver câncer de mama. Além desses, a exposição à radiação ionizante, mesmo em baixas doses, também é considerada um fator de risco, particularmente durante a puberdade (CARVALHO, 2005; INCA, 2014; INCA, 2018). A *American Cancer Society* acrescenta ainda que entre os riscos de câncer de mama estão os fatores genéticos, os quais contribuem com cerca de 5% a 10%, principalmente ao

envolver mutações nos genes *breast cancer 1* (*BRCA1)* e *breast cancer 2* (*BRCA2*), história familial história pessoal de câncer de mama, raça, etnia e tecido mamário denso (NATIONAL CENTER FOR HEALTH STATISTICS, 2013).

Os sinais e sintomas do câncer de mama podem variar entre as mulheres, mas as alterações mais comuns são nódulo único, irregular, endurecido e indolor. Outros sinais são o edema cutâneo, deixando a pele semelhante à 'casca de laranja', além de retração cutânea, dor, inversão do mamilo, hiperemia, descamação ou ulceração do mamilo e secreção papilar, especialmente quando é unilateral. Pode ocorrer secreção sanguinolenta ou serosa pelos mamilos e aparecer linfonodos palpáveis na axila (INCA, 2015; INCA, 2018).

O autoexame de mama e a mamografia são procedimentos utilizados para o diagnóstico precoce desse tipo de câncer, sendo a mamografia o método mais sensível para a detecção deste câncer em estágio pré-invasivo. O câncer de mama é considerado uma modalidade de bom prognóstico quando identificado em estágios iniciais e tratado convenientemente. Não obstante, suas taxas de mortalidade continuam elevadas no Brasil, infelizmente a maioria dos casos (60%) é diagnosticada em estágios avançados. Por isso, o número de mastectomias realizadas no Brasil é considerado alto (MAKLUF et al., 2006; ADAMI et al., 2015). Em tais condições, observa-se uma diminuição das chances de sobrevida, comprometimento dos resultados do tratamento e, consequentemente, redução na qualidade de vida das pacientes (INCA, 2016). Sendo assim, o diagnóstico precoce é especialmente importante, pois possibilita terapias mais efetivas, menos agressivas e contribui para a redução do estágio de apresentação da doença (INCA, 2018; BRASIL, 2013; WHO, 2007). Desta maneira, o principal fator que dificulta o seu tratamento é o estágio avançado em que a doença é descoberta.

O câncer de mama é, portanto, uma preocupação da saúde pública. É necessário compreender todos os processos envolvidos na doença, para que se possa contribuir para uma prevenção mais ampla e um tratamento eficaz, com menos efeitos colaterais, melhorando assim a qualidade de vida das pacientes.

1.1.2 Tratamentos

Em relação ao câncer de mama, a cirurgia foi o primeiro tratamento que efetivamente alterou o curso da doença, e até hoje é um dos principais métodos utilizados, tanto com finalidade diagnóstica, preventiva, curativa ou mesmo paliativa. Adicionalmente, quimioterapia, radioterapia, hormonioterapia, e, mais recentemente, imunoterapia, vem sendo utilizadas.

A terapia atualmente é direcionada de acordo com o tipo de câncer, estadiamento da doença e as condições clínicas das pacientes, buscando um tratamento mais eficaz (LIMA et al., 2014) que minimize a mutilação e os danos emocionais causados às mulheres submetidas à mastectomia, melhorando a qualidade de vida, além de diminuir a morbidade (MACMILLIAN e MCCULLEY, 2016). Assim, a quimioterapia com drogas citotóxicas é a terapia mais utilizada para impedir a proliferação celular. Dentre os agentes antineoplásicos disponíveis estão os antimetabólitos, alquilantes, antibióticos antitumorais, inibidores mitóticos e inibidores da topoisomerase, dentre outros. A Tabela 1 evidencia alguns fármacos, seus mecanismos, indicações e efeitos adversos.

Tabela 1. Exemplos de fármacos quimioterápicos

Classe Farmacológica	Exemplo de Droga	Mecanismo de Ação	Indicações	Principais Efeitos Adversos
Antimetabólito	Metotrexato	Antifolato inibidor da síntese de DNA	Câncer de cabeça, pescoço e mama	Mielossupressão, mucosite e diarreia
Antimetabólito	Mercaptopurina	Metabólitos que inibem a síntese de DNA e RNA	Leucemia linfoblástica aguda	Mielossupressão e toxicidade gastrointestinal
Agente Alquilante	Ciclofosfamida	Ligação cruzada de DNA e bloqueio da síntese e função do DNA	Câncer de mama e ovário	Urotoxicidade, alopecia, mielossupressão, náuseas e vômitos
Agente Alquilante	Isofosfamida	Inibição da síntese e função do DNA por metabólitos que formam ligações cruzadas	Câncer testicular e sarcomas	Urotoxicidade e mielossupressão
Agente alquilante	Compostos de Platina	Interfere nos processos de transcrição e replicação celular no	Câncer avançado de pulmão de células não	Ototoxicidade

		DNA	pequenas	
Antibiótico Antitumoral	Doxorrubicina	Inibição da síntese e função do DNA por intercalar com pares de bases	Câncer de mama e linfoma não Hodgkin	Mielossupressão, cardiotoxicidade e alopecia
Antibiótico Antitumoral	Mitomicina C	Inibição da síntese e função do DNA por alquilação do DNA e RNA	Câncer gástrico e pancreático	Mielossupressão e mucosite
Inibidores mitóticos	Vincristina	Inibição da polimerização da tubulina e lise de microtúbulos na mitose	Leucemia linfoblástica aguda, neuroblastoma e linfoma de Hodgkin	Neurotoxicidade e mielossupressão
Inibidores mitóticos	Vimblastina	Inibição da polimerização da tubulina e lise de microtúbulos na mitose	Tumor de células germinativas e linfoma de Hodgkin e não Hodgkin	Mielossupressão e sintomas gastrointestinais
Inibidores mitóticos	Taxanos (paclitaxel e docetaxel	Estabilização dos microtúbulos da mitose, impedindo sua desagregação e levando à morte celular	Carcinoma mamário e de pulmão	Neutropenia e mucosite
Inibidores da topoisomerase	Topotecano	Inibição de topoisomerase II e bloqueio do desenrolamento do DNA	Carcinoma metastático de ovário	Alopecia e mielossupressão
Inibidores da topoisomerase	Etoposídeo	Inibição de topoisomerase I e bloqueio do desenrolamento do DNA	Tumores de células germinativas e de células não pequenas pulmonares	Alopecia, mielossupressão e segunda neoplasia
Antimetabólito/análogo de pirimidina	Fluorouracil (5-FU)	Interfere na síntese proteica, inibe a timidilato sintetase e atua diminuindo replicação do DNA. Agente específico de fase do ciclo celular (fase S)	Câncer gástrico metastático, colorretal e mama	Alopecia; erupção maculopapular; prurido; fotossensibilidade, diarreia; anorexia; náusea; vômito; estomatite.

Adaptado de SAITO, LANA, MEDRANO E CHAMMAS, 2016.

A ação dos antimetabólitos é atuar impedindo a multiplicação e a função das células normais, através da inibição da biossíntese dos componentes essenciais do DNA e RNA (WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018). Dentre os agentes antimetabólicos, se destaca o metotrexato (MTX), que estruturalmente, é um análogo do ácido fólico, que atua inibindo de maneira competitiva a atividade da enzima diidrofolato-

redutase, sendo considerado quimioterápico específico da fase S (síntese) do ciclo celular. Sua ação é mais marcante sobre populações celulares em fase de crescimento exponencial, o que explica o efeito seletivo sobre células tumorais e a ação sobre tecidos em proliferação (FRIEDMAN et al., 2018). O MTX foi utilizado como controle positivo nos experimentos *in vivo* e *in vitro* do presente trabalho, pois as linhagens empregadas são sensíveis a este fármaco.

Ainda, os agentes alquilantes se ligam ao DNA impedindo sua replicação. Na clínica costuma-se associar esses agentes a outros que atuam no ciclo celular (WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018). Os antibióticos antitumorais provocam inibição da síntese dos ácidos nucleicos ou de proteínas, através do aumento de produção de ROS, que provocam morte celular. Já os inibidores mitóticos atuam interrompendo a divisão celular ao impedir que ocorra a mitose na metáfase (WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018), enquanto que os inibidores da topoisomerase interferem na transcrição e replicação do DNA ao inibir as enzimas isomerases, impedindo assim, sua ação sobre o DNA (WORDING, PERISSINOTTI E MARINI, 2016; INCA, 2018).

A quimioterapia convencional disponível, com os antimetabólitos, agentes alquilantes, antibiótico antitumoral, inibidores mitóticos, inibidores da topisomerse e outros, apresenta uma resposta antitumoral satisfatória para alguns tipos de câncer, mas esses quimioterápicos além de atuarem nas células neoplásicas, são extremamente tóxicos às células normais do organismo, por isso apresentam diversos efeitos colaterais, como dor, náuseas, vômito, hemorragia, fadiga, mielossupressão, alopecia, disfunção cognitiva, ganho de peso, palidez, menopausa induzida, disfunção sexual e até infertilidade (INCA 2018). Devido a isso, estudos relacionados à imunoterapia têm avançado, principalmente como componente da terapia adjuvante multimodalidade. O uso de anticorpos monoclonais específicos, que atuam promovendo uma modulação do sistema imune ou direcionando para alvos específicos, vem ganhando espaço na terapia. Entre os fármacos dessa classe, estão disponíveis o Trastuzumabe e Pertuzumabe, anticorpos monoclonais humanizados que atuam seletivamente no domínio extracelular da proteína do receptor-2 (*HER-2*); Bevacizumabe, um inibidor do VEGF; e Ipilimumab, um anticorpo monoclonal totalmente humanizado, anti-CTLA-4 (proteína T-linfócito-

associada citotóxico 4 ou CD152), que impede a regulação negativa da ativação dos linfócitos T, o que em última análise, leva à potencialização da ativação linfocitária e, consequentemente, à diminuição da tolerância aos antígenos associados ao melanoma (XU et al., 2019) , impedindo que sejam ativados, reduzindo o estímulo que mantém as células tumorais ativas (BRUNTON et al., 2011; NETO et al., 2013; SAITO, LANA, MEDRANO E CHAMMAS, 2016).

Apesar do advento dos anticorpos monoclonais a hormonioterapia com moduladores do receptor de estrogênio (MRE) é o tratamento adjuvante mais estabelecido do câncer de mama. O tamoxifeno pode ser utilizado em pacientes com câncer de mama hormonal positivo na pré- e pós-menopausa e atua ligando-se ao receptor tumoral para estradiol, competindo, assim, com esse hormônio. Outros representantes são o raloxifeno e o arzoxifeno. Além dos MRE, também podem ser empregados os inibidores da aromatase (IA) e os antagonistas do receptor de estrogênio (ARE). Os IA, como o anastrozol, exemestano e letrozol, atuam inibindo a enzima aromatase, responsável por converter os hormônios estrona e testosterona em estrogênio no tecido mamário, o que resulta na diminuição da ação proliferativa desencadeada pelo estrogênio. Os ARE agem seletivamente sobre o receptor de estrogênio, promovendo a sua degradação e impedindo a resposta proliferativa do estrogênio. O mais utilizado é o fulvestranto, em pacientes com câncer de mama metastático avançado, que é resistente às terapias com IA ou MRE (WU et al., 2015).

Apesar da possibilidade de evitar o desenvolvimento do câncer, a hormonioterapia leva ao desenvolvimento de inúmeros efeitos colaterais como insônia, diarreia, artrites, fogachos, suor, calor, alterações vaginais e pólipos no endométrio, alterando a qualidade de vida das pacientes. Além disso, estudos mostram que o tratamento prolongado com o tamoxifeno predispõe a um alto risco para desenvolvimento de câncer do endométrio, trombose e embolia pulmonar (DECENSI et al., 2013; FISHER et al., 1998).

Mesmo com as diversas abordagens terapêuticas para o câncer de mama, ainda os desafios continuam devido à falta de opção para aquelas pacientes com resistência à terapia sistêmica, efeitos adversos e o alto custo dos tratamentos. Estudos vêm sendo realizados na busca de novos fármacos e terapias mais eficazes, que apresentem menos

efeitos colaterais e que melhorem os resultados do tratamento do câncer de mama a curto, médio e a longo prazo (VOROBIOF, 2016; JUNQUEIRA e CHAMMAS, 2018; INCA 2018). Alguns polissacarídeos se apresentam como possíveis fármacos, isolados ou em combinação, no tratamento de neoplasias. Por conseguinte, estas moléculas foram abordadas como principal objeto de estudo neste trabalho.

1.2 Polissacarídeos

Os polissacarídeos são um grupo estruturalmente heterogêneo de compostos neutros ou ácidos, formados por longas cadeias contendo centenas ou milhares de unidades de monossacarídeos (LEHNINGER et al., 2002, LEHNINGER, 2006). Há vários tipos de polissacarídeos, tanto em organismos animais como vegetais. As frutas particularmente apresentam uma variedade de compostos polisacarídicos, como os pécticos,que compreendem um grupo heterogêneo de polissacarídeos. As pectinas são moléculas complexas que possuem como principais constituintes ácido galacturônico, ramnose, arabinose e galactose, sendo a principal característica do grupo a presença de unidades de ácido D-galacturônico (GalpA) em sua estrutura. Estes polímeros são os mais solúveis entre os polissacarídeos que constituem a parede celular vegetal, podendo ser facilmente extraídos com água quente ou quelantes de cálcio (ASPINALL, 1980; BRETT e WALDRON, 1990).

Na indústria alimentícia, as pectinas são muito utilizadas como geleificantes e estabilizantes de produtos alimentícios. Pectinases especificas são utilizadas como suplemento de alimentos infantis (LANG et al., 2000), na produção de purês, iogurtes e pudins, e no processo de fermentação de café e chá, pois aceleram estes processos melhorando a qualidade do produto final (UENOJO; PASTORE, 2007). Os produtos de degradação das pectinases são classificados como prebióticos, podendo ser usados como promotores de saúde em nutrição humana e animal, por estimularem seletivamente o crescimento e/ou a atividade de espécies de bactérias residentes no cólon intestinal (LANG et al., 2000).

Terapeuticamente as pectinas podem reduzir os níveis de colesterol total e LDL no sangue, quando consumidos pelo menos 6 g ao dia, apresentando um efeito protetor contra a aterosclerose (THAKUR et al., 1997). São indicadas para o tratamento de desordens gastrointestinais, para diminuir a absorção de glicose da alimentação e impedir a absorção de substâncias tóxicas pelo organismo, como metais pesados e microrganismos tóxicos (THAKUR et al., 1997). Estudos mostram que a pectina isolada do maracujá amarelo *(Passiflora edulis flavicarpa)* apresenta atitividade antitumoral *in vivo* e *in vitro* e apresentou baixa toxicidade para células saudáveis (SILVA et al., 2012). Outros relatam o possível efeito das pectinas e seus produtos de degradação no combate ao câncer de colon (OLANO-MARTIN et al., 2003). Entretanto, tais estudos ainda são incipientes e não esclarecem os mecanismos e o real potencial desses polissacarídeos como fármacos antitumorais.

No geral, os polissacarídeos pécticos e não-pécticos atuam aumentando ou potencializando os mecanismos de defesa do hospedeiro e surgem como uma possível alternativa para inibir o crescimento tumoral. A função imune decresce progressivamente com o crescimento persistente do tumor, ficando clara a imunodeficiência de pacientes com tumores no seu estágio final (HADDEN, 2003). Além disso os polissacarídeos têm atraído maior atenção nos campos nutricionais e médicos por causa de seus vários benefícios para a saúde (VARGHESE et al., 2017). Diversos polissacarídeos naturais que foram isolados de algas, cogumelos, plantas (frutos, folhas, raízes e caules) e animais têm efeitos imunomoduladores potentes (FAN et al., 2018), antioxidantes e antitumorais, sem efeitos colaterais expressivos (SONG et al., 2008).

Os polissacarídeos isolados a partir do fungo *Coriolus versicolor* exibiram um marcante efeito antitumoral contra tumores alogênicos como Sarcoma 180 e carcinoma de Ehrlich em camundongos, quando administrados tanto por via oral quanto intraperitoneal (DOU et al., 2019). O homo-polissacarídeo isolado de sementes da *Kottukonam de Mangifera*, demonstrou citotoxicidade seletiva contra células cancerígenas tanto *in vitro* quanto em modelos de camundongos murinos, devido ao potencial imunoestimulatório. O galactoxiloglucano, isolado das sementes de *Tamarindus indica*, demonstrou ser um agente anticâncer e imunomodulador eficaz (VARGHESE et al., 2019). Nosso grupo de pesquisa previamente demonstrou o efeito antitumoral *in vivo* de

polissacarídeos e oligossacarídeos extraídos de vinho tinto carbenet franc (STIPP et al., 2017; TURIN-OLIVEIRA et al., 2019) e do jambo (TAMIELLO et al., 2018). Porém, como a diversidade de polissacarídeos na natureza é enorme, muitos vegetais ainda não foram estudados quanto a sua composição polissacarídica e seus efeitos biológicos. Neste contexto, uma fração de polissacarídeos extraídos do pimentão verde (*Capsicum annuum)* foi neste trabalho avaliada, uma vez que é um fruto amplamente conhecido devido sua utilização na dieta humana e na culinária.

As *Capsicum* são plantas solanáceas nativas do México, América Central e do Sul. São por vezes agrupadas juntamente com outras pimentas pouco pungentes sob a designação de pimentas doces. Produzem frutos com diferentes cores, sendo as mais conhecidas o verde, o amarelo e o vermelho. Porém, existem outras variedades bastante exóticas, como o branco, roxo, azulado, preto e laranja. Possuem grande relevância como fonte de alimentos, de alcalóides esteroidais e de compostos fenólicos (IMRAN et al., 2019).

Fruto de alto valor nutricional, o pimentão é utilizado em diversas preparações culinárias, mas existem relatos de seu uso na medicina popular, principalmente como antinociceptivo e anti-inflamatório (BOHS, 1989; DO NASCIMENTO et al., 2017). Dentre os componentes químicos presentes no pimentão verde, encontra-se elevado conteúdo de vitamina C (ácido ascórbico), β-carotenos e outros pigmentos carotenóides (licopeno e zeaxantina) e polifenóis (DO NASCIMENTO et al., 2017). Quanto aos polissacarídeos do pimentão, que são basicamente do tipo pectinas. As pectinas de modo geral podem exibir um grande número de propriedades terapêuticas benéficas, tais como anti-inflamatórias, analgésicas, antitumorais, imunomoduladoras, antioxidantes e anticolesterêmicas (COOPER et al., 2011; KOLLMANNSBERGER et al., 2011; STARKENMANN et al., 2011). Neste contexto, o presente estudo avaliou a atividade antitumoral *in vivo e in vitro* dos polissacarídeos do pimentão verde em modelo de tumor de Ehrlich em camundongos e em linhagens de células de tumor humano mamário do tipo MB-MDA-231, MB-MDA-436 e MCF-7.

1.3 Modelos e linhagens celulares de tumor mamário

1.3.1 *In vivo*: tumor de Ehrlich (TE)

O tumor de Ehrlich é uma neoplasia maligna de origem epitelial, que apareceu pela primeira vez espontaneamente no tecido mamário de camundongos fêmeas, foi descoberto por Paul Ehrlich em 1886. Nos anos seguintes usaram-no como um tumor experimental através do transplante de tecidos tumorais por via subcutânea de camundongo para camundongo (OZASLAN et al., 2011), ou como tumor ascítico de Ehrlich, através de um fluído ascítico que continha um grande número de células neoplásicas. Estas células aumentam a "virulência" através de passagens repetidas no peritôneo, porém quando inoculadas por via subcutâneo o líquido ascítico produz uma massa tumoral sólida (BAILLIF, 1954).

O carcinoma de Ehrlich se assemelha a tumores humanos que são sensíveis à quimioterapia, devido ao fato de ser indiferenciado e ter uma taxa de crescimento rápido (OZASLAN et al., 2011). Estudos demonstraram que após a inoculação subcutânea no membro pélvico de camundongos é possível observar o desenvolvimento do tumor sólido em 100% dos animais em até 14 dias (ABDIN et al., 2014; BASSIONY et al., 2014) ou 6 dias (TURIN-OLIVEIRA et al., 2019; CORSO et al., 2019) após a inoculação. Também tem sido relatado crescimento de tumor sólido após inoculação subcutânea na região da cabeça, na região dorsal e na pata (DE FATIMA PEREIRA et al., 2014; MIRANDA-VILELA et al., 2014; MIRANDA-VILELA et al., 2011; NASCIMENTO et al., 2006; PEREIRA et al., 2013).

Após a inoculação de células tumorais de Ehrlich, essas células sofrem alterações morfológicas e metabólicas como diminuição do número de mitocôndrias, diminuição de síntese de DNA e RNA, perda de nucleotídeos intracelulares, declínio da concentração de ATP, diminuição da síntese de proteínas, e aumento dos triglicerídeos, ésteres de colesterol e ácidos graxos livres (OZASLAN et al., 2011). No tecido tumoral também tem sido demonstrado que há aumento da concentração de TNF-α, diminuição da concentração da catalase e glutationa redutase e aumento da concentração de

malondialdeído (SEGURA et al., 2001). Assim, o TE é um bom modelo para estudar a patogenia e a terapia de tumores sólidos de humanos.

1.3.2 *In vitro*: linhagens tumorais humanas

Foram utilizadas neste estudo diferentes linhagens celulares de câncer de mama, entre elas: MCF-7 linhagem do tipo luminal B (ER positivo, PR positivo e *HER2* positivo), as linhagens triplo negativas do tipo basal MDA-MB-231 e MDA-MB-436 (NEVE et al., 2009); e HB4a, uma linhagem celular luminal de mama normal imortalizada e que expressa níveis basais do receptor *HER-2* (STAMPS et al., 1994).

As células MCF-7 originalmente foram obtidas a partir do derrame pleural de uma mulher caucasiana de 69 anos que apresentava um adenocarcinoma da mama. Essas células apresentam características do epitélio mamário diferenciado, incluindo síntese de estradiol. As células expressam os ER de tipo selvagem e variante, bem como o PR (NEVE et al., 2009).

As células MDA-MB-231 são uma linhagem epitelial de câncer de mama humana que foi estabelecida através de derrame pleural de uma mulher caucasiana de 51 anos com adenocarcinoma mamário metastático. São células de linhagem mamária triplo-negativo altamente agressivas, invasivas e pouco diferenciadas, uma vez que não expressam ER e nem PR, e são negativas para o *HER2*, sendo inicialmente classificada como uma linhagem celular de câncer de mama basal (CHAVEZ et al., 2010). Semelhante a outras linhas de células cancerosas invasivas, a invasividade das células MDA-MB-231 é mediada por degradação proteolítica da matriz extracelular (CHAVEZ et al., 2010).

As células MDA-MB-436 também foram isoladas de efusões pleurais de uma paciente caucasiana com adenocarcinoma mamário metastático. Possuem a mesma origem e são semelhantes às MDA-MB-231, pois não expressam receptores (ER negativo, PR negativo e *HER2* negativo), mas são menos agressivas (CHAVEZ et al., 2010; MARIA, 2014).

2. JUSTIFICATIVA

A alta incidência do câncer de mama é alarmante, pois estimativas mostram que uma a cada oito mulheres desenvolverá este câncer durante a vida (SMITH et al., 2018), o que reforça a necessidade de contínuos estudos sobre sua patogênese, carcinogênese e tratamentos eficazes. Investigações *in vivo* e *in vitro* são formas efetivas de obter resultados confiáveis e relativamente rápidos acerca de novas terapias, incluindo as que possam ser obtidas de fontes naturais.

Os produtos naturais estão sendo amplamente estudados tanto na prevenção como no tratamento de neoplasias, podendo ser associados a uma droga já existente, melhorando a eficácia de terapias e diminuindo os efeitos colaterais. Alguns compostos bioativos de espécies vegetais já foram aprovados como quimioterápicos, a exemplo dos alcaloides da vinca (*Vinca rosea*), vincristina e vimblastina, e o paclitaxel, originário da *Taxus brevifolia*, todos fármacos amplamente utilizados em quimioterapias. Em ensaios pré-clínicos realizados por nosso grupo de pesquisa extratos de plantas e bioativos isolados também demonstraram atividades antineoplásicas, a exemplo da *Uncaria tomentosa* (unha-de-gato) (DREIFUSS et al., 2010, 2013), *Moquiniastrum polymorphum* (cambará) (MARTINS et al., 2015) e *Salvia lachnostachys* (CORSO et al., 2019). Do mesmo modo, polissacarídeos de diversas fontes, como do jambo (TAMIELLO et al., 2018) e do vinho tinto (STIPP et al., 2017) demonstraram efeitos antineoplásicos em modelos de tumores mamários *in vivo.* Porém, ainda não se tem nenhum estudo mostrando especificamente a ação antitumoral dos polissacarídeos extraídos do pimentão verde.

Diante do exposto o presente trabalho avaliou o efeito antitumoral dos polissacarídeos do pimentão em vários regimes posológicos em camundongos: ***(a)*** tratamento convencional, ***(b)*** tratamento prolongado e ***(c)*** em associação com o metotrexato, a fim de avaliar seu efeito antitumoral, mecanismos de ação, toxicidade e potencial como ajuvante de terapia em modelos de tumor mamário. Para tanto, foram utilizadas técnicas farmacológicas, bioquímicas, de biologia celular e molecular em modelo de tumor ectópico *in vivo* e linhagens celulares *in vitro.*

A hipótese deste trabalho é que os polissacarídeos do pimentão verde (CAP) apresentam efeito antitumoral no modelo de Ehrlich *in vivo* e em células cancerígenas de linhagem mamária humana *in vitro*, podendo atuar como adjuvante de quimioterapias.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar a atividade antitumoral *in vivo* e *in vitro* dos polissacarídeos do *Capsicum annuum* em modelo de carcinoma sólido de Ehrlich em camundongos e em células cancerígenas de origem mamária, bem como os possíveis mecanismos de ação envolvidos.

3.2. Objetivos específicos

1- Avaliar o crescimento tumoral em camundongos tratados com CAP nas doses de 50, 100 e 150 mg kg^{-1} durante 21 dias (tratamento convencional);

2- Avaliar o crescimento tumoral e a toxicidade do CAP em camundongos tratados com o CAP em regime prolongado (31 dias);

3- Avaliar o crescimento tumoral em camundongos tratados com o CAP em combinação com metotrexato (MTX);

4- Investigar os possíveis mecanismos de ação do CAP, nas vias que envolvem:

4.1) mediadores inflamatórios;

4.2) estresse oxidativo (*in vivo* no tumor e fígado, e *in vitro* pelo teste do DPPH);

4.3) apoptose;

4.4) angiogênese;

5- Avaliar os efeitos adversos do tratamento do CAP nos diferentes regimes posológicos, através de indicadores bioquímicos plasmáticos e hemograma;

6- Identificar alterações histológicas no tecido tumoral dos animais tratados nos diferentes regimes de tratamento;

7- Avaliar a atividade antineoplásica do CAP em três tipos de células cancerígenas humanas de origem mamária, denominadas MCF-7, MDA-MB-231 e MDA-MB-436.

4. ARTIGOS CIENTÍFICOS

4.1 ARTIGO 1: publicado na *Carbohydrate Polymers*, 2018.

Antineoplastic effect of pectic polysaccharides from green sweet pepper (*Capsicum annuum*) on mammary tumor cells *in vivo* and *in vitro*

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ABSTRACT

The present study investigated the antineoplastic effects of pectic polysaccharides that were extracted from green sweet pepper (*Capsicum annuum* [CAP]) in the Ehrlich carcinoma in mice and in human mammary tumor lineages. After the subcutaneous inoculation of 2×10^6 Ehrlich tumor cells, Female Swiss mice received 50, 100, or 150 mg/kg CAP or vehicle orally once daily or methotrexate (2.5 mg/kg, i.p., every 5 days) for 21 days. CAP dose-dependently reduced Ehrlich tumor growth. It also reduced the viability of MCF-7, MDA-MB-231, and MDA-MB-436 human mammary cell lineages. Treatment with CAP reduced the gene expression of vascular endothelial growth factor *in vivo* and *in vitro*, reduced vessel areas of the tumors, and induced necrosis in Ehrlich solid tumors. CAP treatment significantly increased Interleukin-6 in tumors. The antineoplastic effect of CAP appears to depend on the regulation of inflammation and angiogenesis. Further studies are encouraged to better understand the CAP potential for the treatment of breast tumors.

Keywords: Ehrlich solid tumor; pectic polysaccharide; green sweet pepper; VEGF; mammary tumor cells; interleukin-6.

1 INTRODUCTION

Cancer is a heterogeneous disease, the incidence and prevalence of which continue to rise. It is a public health problem with high mortality rates. Cancer cells acquire unique capabilities that most healthy cells do not possess. For example, cancer cells become resistant to growth-inhibitory signals, proliferate without dependence on growth-stimulatory factors, replicate without limit, evade apoptosis, and acquire invasive and angiogenic properties (Hanahan & Weinberg, 2000).

Cancer is initiated and progresses by multiple genetic alterations and aberrant signaling pathways. The identification of molecular targets that are involved in the steps of tumor development will provide opportunities to establish promising strategies to combat cancer. Antineoplastic drugs are effective, but they cause several side effects. Therefore, it is necessary to discover new drugs with fewer side effects and the ability to increase patient survival and quality of life.

Polysaccharides can be found in nature with great structural diversity. They are considered a novel source of natural compounds for drug discovery. Polysaccharides have drawn greater attention in the nutritional and medical fields because of their various health benefits (Sharon & Lis, 1993; Varghese, Joseph, S.R., B.S., & Sreelekha, 2017). Several natural polysaccharides that have been isolated from algae, mushrooms, plants (fruits, leaves, roots, and stems), and animals have potent immunomodulatory (Fan et al., 2018), antioxidant, and antitumor effects with no side effects (Song et al., 2008; Zhu, Chen, & Lin, 2007). The anti-metastatic and anti-angiogenic nature of polysaccharides further enhances their potential for cancer treatment (Bao et al., 2016; Liu, Kuang, Wu, Jin, & Sun, 2016). Angiogenesis is the physiological or pathological process by which new blood vessels originate from preexisting vessels (Carmeliet, 2005; Rui, Pan, Shao, & Xu, 2017). Angiogenesis does not initiate malignancy but can promote tumor progression and metastasis. Intensive efforts have been made to develop therapeutic strategies to inhibit angiogenesis in cancer over the past decades (Carmeliet, 2005).

Recently, a fraction that contained pectic polysaccharides from green sweet pepper (*Capsicum annuum* L. cv Magali [CAP]) was isolated and characterized (do Nascimento, Iacomini, & Cordeiro, 2017). Notwithstanding some of the aforementioned characteristics of polysaccharides, no studies have reported the antitumoral activity of polysaccharides that are directly extracted from green sweet pepper. Thus, our hypothesis was that CAP exerts an antineoplastic effect. The aim of the present study was to evaluate the *in vivo* and *in vitro* antineoplastic activity of the previously characterized green sweet pepper pectic polysaccharides in Ehrlich tumor-bearing mice and lineages of human mammary cancer cells, respectively. The possible mechanisms of action of CAP were also investigated with regard to angiogenesis, apoptosis, oxidative stress, and inflammation. The results demonstrated that the most pronounced effects of CAP were on the angiogenic and inflammatory process.

2 MATERIAL AND METHODS

2.1 Chemicals

Bovine serum albumin, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), glutathione reductase, NADPH, xylenol orange, K_2HPO_4, KH_2PO_4, 1M Tris, 5 mM ethylenediaminetetraacetic acid (EDTA), TRIS HCl, sodium nitrite, tetramethylbenzidine (TMB), dimethylsulfoxide (DMSO), and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB), pyrogallol, absolute ethanol and methanol, ferrous ammonium sulfate, hydrogen peroxide, trichloroacetic acid, formaldehyde, sodium azide, acetic acid, ascorbic acid, diethyl ether, *N,N*-dimethylformamide, formaldehyde, hydrogen peroxide, magnesium chloride, sodium acetate, sodium carbonate, sucrose, trichloroacetic acid, 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). Aspartate (AST), alanine transaminase (ALT), and alkaline phosphatase (AP) kits were purchased from Kovalent (São Paulo, Brazil). Tumor necrosis factor α (TNF-α), Interleukin-4 (IL-4), IL-6, and IL-10 kits were obtained from BD Biosciences (Franklin Lakes, NJ, USA). 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). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Gibco-ThermoFisher (Waltham, MA, USA). Glutamine (Invitrogen, Grand Island, NE, USA), garamycin (Santisa, Bauru, Brazil), crystal violet (Dinamica, Diadema, Brazil), and pure distilled water were used for the eluent preparation.

2.2 Isolation of CAP

Fresh green sweet pepper fruits (*Capsicum annum* L. cv Magali) were purchased from the organic sector of the municipal market in Curitiba, Paraná, Brazil. The CAP fraction that contained pectic polysaccharides was isolated and characterized by Nascimento, Iacomini, & Cordeiro (2017), who described it as an *annum* cold-water-soluble fraction (ANWS). Briefly, fruits without seeds were freeze-dried and defatted with chloroform:methanol (1:1). Polysaccharides were extracted from the residue with water at 100^{o}C for 2 h (× 6.1 L each) and precipitated from the extract with ethanol (3 vol). CAP

was then obtained by freeze-thaw treatment (cold-water soluble fraction). The fraction was composed mainly of uronic acids (67%), with minor amounts of rhamnose (1.6%), arabinose (6.4%), xylose (0.3%), galactose (6.7%), and glucose (4.4%) and consisted of a highly methoxylated homogalacturonan (degrees of methyl esterification and acetylation of 85% and 5%, respectively), together with type I arabinogalactan anchored to rhamnogalacturonan.

The protein content of CAP was determined using the method of Bradford (1976). A calibration curve of bovine serum albumin was generated, and the results are expressed as g of protein.100 g^{-1} of sample. Total phenolic compounds were determined using the Folin-Ciocalteu method, adapted to microplates. Twenty microliters of the CAP fraction at 10 mg ml^{-1} was placed in each well of a microplate, and 100 µl of Folin-Ciocalteu reagent was added. After 5 min in the dark, 75 µl of 7.5% sodium carbonate solution was added. The microplate was then stirred and left to stand for 40 min in the dark. Absorbance was then read at 740 nm using a spectrophotometer (Singleton & Rossi, 1965). A calibration curve of gallic acid at concentrations of 20-120 µg/ml was generated, and the results are expressed as gallic acid equivalents (g of GAE/100 g of sample dry weight).

2.3 Animal model, Ehrlich tumor inoculation, and experimental design

Ehrlich carcinoma is a transplantable model of solid cancer. Female Swiss mice, weighing 20-30 g, were obtained from the vivarium of the Federal University of Paraná (Curitiba, Brazil). The animals remained under controlled room temperature (22°C ± 1°C) and a 12 h/12 h light/dark cycle with free access to food and water. All of the experimental protocols were approved by the institutional Ethical Committee for Animal Care (CEUA; authorization no. 984).

The maintenance of Ehrlich cells was performed by weekly passages of intraperitoneal (i.p.) injections of 2×10^6 cells/mice, which were previously kept frozen at -80°C. The cells were collected from the peritoneum in 1 ml of phosphate-buffered saline (PBS; 16.5 mM phosphate, 137 mM NaCl, and 2.7 mM KCl), pH 7.4, and a solution of 0.5 M EDTA (pH 8.0). After three or four passages, cell viability was > 98%, determined by the trypan blue dye exclusion method in a Neubauer chamber (de Fátima Pereira, da Costa, Magalhães Santos, Pinto, & Rodrigues Da Silva, 2014; El-Sisi, Sokar, Salem, & Abu

Risha, 2015). The tumor cells were then injected subcutaneously (s.c.; 2×10^6 cells) in the right hindlimb of the mice (Abdin, Soliman, & Saied, 2014; Bassiony, Sabet, El-Din, Mohamed, & El-Ghor, 2014). A palpable solid tumor mass developed within 7 days.

The animals were divided into six equal groups (*n* = 7-9/group): (*i*) naive (no tumor) and treated with vehicle (distilled water), (*ii*) tumor-bearing and treated with vehicle, (*iii*) tumor-bearing and treated with 50 mg/kg CAP, (*iv*) tumor-bearing and treated with 100 mg/kg CAP, (*v*) tumor-bearing and treated with 150 mg/kg CAP, and (*vi*) tumor-bearing and treated with 2.5 mg/kg methotrexate (MTX), i.p. (positive control group). The mice were treated with CAP or vehicle by oral gavage based on previous studies (Ma et al., 2017; Raso, Pacilio, & Carlo, 2002) from day 1 after cell inoculation until day 21. Methotrexate was dissolved in distilled water and then administered i.p. every 5 days (on days 1, 5, 9, 13, and 21) according to the experimental design (Figure 1). Additionally, another group of (*vii*) non-tumor-bearing mice was treated with 100 mg/kg CAP (naive + CAP100), serving as a control to assess the possibly toxicity of 21 days of oral CAP treatment.

The tumor was measured daily after day 7 until day 21, and the tumor volume was calculated using the following formula: $V\ (cm^3) = 4\pi/3.a^2.(b/2)$, where *a* is the smallest tumor diameter, and *b* is the largest tumor diameter (in centimeters). Likewise, the tumor inhibition rate was calculated using the following formula: $Tumor\ suppression\ (\%) = (1-T/C)$, where *T* is the tumor volume in the tested group, and *C* is the volume in the control group on the last experimental day (Mizuno, Minato, Ito, Kawade, & Terai, 1999). During the experiment, body weights were recorded daily. Tumor weight was also recorded at the end of the experiment.

After 21 days of treatment, the animals were fasted for 12 h with free access to water and anesthetized with an intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg) for biological material collection. Blood was collected from the inferior cava vein for subsequent hematological and plasma biochemical analysis. The tumor and liver were then harvested, weighed, fragmented for histological analysis, and partially frozen (-80^{o}C) for the subsequent evaluation of oxidative stress and inflammatory

parameters and gene expression. The spleen, lungs, and kidneys were also harvested and weighed.

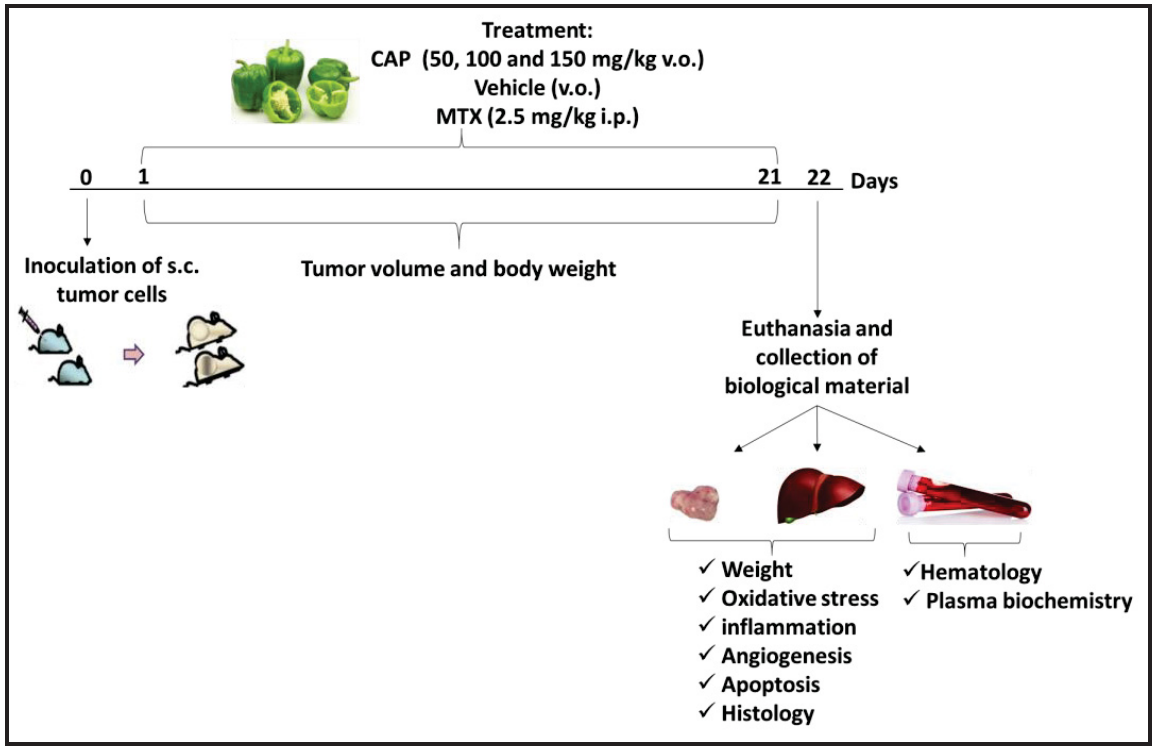


Figure 1. **Experimental design in mice inoculated with Ehrlich cells and treated according to the groups described in section 2.3.** CAP, *Capsicum annuum* pectic polysaccharides; s.c., subcutaneous; v.o., oral; i.p., intraperitoneal; MTX, methotrexate.

2.4 Hematological and biochemical assays

At the end of treatment, blood was collected in heparinized syringes for biochemical and hematological analysis. The measurements included red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), leukocyte count (white blood cells [WBCs]), differential leukocyte count, platelet count, and red cell distribution width (RDW). The blood samples were centrifuged at 3400 × *g* for 10 min, and the plasma was used for the analysis of ALT,

AST, and AP using commercial kits with an automated device (Mindray BS-200, Shenzhen, China).

2.5 Determination of tumor and hepatic oxidative stress parameters

Tumor and liver samples were homogenized in 0.1 M potassium phosphate buffer (pH 6.5), and the pure homogenate was used to determine GSH levels. Afterward, the remaining homogenates were centrifuged at 9000 × *g* for 20 min at 4°C, and the supernatant was diluted 1:10 in phosphate buffer to determine the other parameters.

For the measurement of tumor and hepatic GSH levels, the samples were subjected to the method that was described by Sedlak and Lindsay (1968), the reaction of which relies on the ability of glutathione *S*-transferase (GST) to cSunonjugate the substrate 2,4-dinitrochlorobenzene (DNCB) with GSH, monitored by an increase of absorbance at 340 nm. Superoxide dismutase (SOD) was measured according to the method of Gao (Gao, Yuan, Zhao, & Gao, 1998), which is based on the ability of this enzyme to inhibit pirogallol autoxidation at 440 nm. Catalase (Cat) was measured according to Aebi (1984), the reaction of which is based on the conversion of hydrogen peroxide to water and oxygen and spectrophotometrically measured at 240 nm. Lipoperoxidation (LPO) rates were measured according to Jiang et al. (1991). Finally, GST activity was analyzed only in liver homogenates according to the method of Habig et al. (1974). All of these assays were measured in a 96-well microplate reader (Synergy HT, Biotek, VT, USA).

Most of the results of the oxidative stress parameters are expressed as the amount of proteins that were present in the homogenates. The tissue protein concentration was determined spectrophotometrically using the method of Bradford (1976) in a microplate reader (Synergy HT, Biotek, VT, USA) at 595 nm.

2.6 *In vitro* determination of CAP free radical scavenging activity

The scavenging activity of different concentrations of CAP (1, 3, 10, 30, 100, and 300 μg/ml) against the stable free radical 2,2 diphenyl-1-picrylhydrazyl (DPPH) was determined. This method was adapted from Chen et al. (2004). Briefly, CAP was mixed

with DPPH methanolic solution (10 µg/ml), and absorbance was immediately read at 517 nm in a microplate reader (Synergy HT, Biotek, VT, USA). Ascorbic acid (50 µg/ml) and distilled water were used as positive and negative controls, respectively.

2.7 Evaluation of inflammatory parameters in tumor tissue

2.7.1 Determination of nitrite levels

Samples of 0.1 g of tumor tissue were homogenized with PBS (pH 7.4) and then centrifuged at 9000 × *g* at 4°C for 20 min. The supernatant was separated for nitric oxide (NO) and cytokine measurements. Nitrite levels, an indirect measure of NO, were measured at 540 nm using Griess solution (0.1% *N*-1-naphthyl-tilediamine and 1% sulfanilamide in 5% H_3PO_4) according to the method of Green et al. (1982). The amount of nitrite in the incubation medium was calculated using sodium nitrite as the standard.

2.7.2 Quantification of cytokines

Cytokines levels were measured in the supernatant of the homogenized tumor tissue, prepared the same way as for the determination of nitrite levels. TNF-α, IL-4, IL-6, and IL-10 concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) according to the manufacturer's instructions.

2.7.3 Determination of myeloperoxidase and N-acetylglucosaminidase

The pellets from the centrifuged tumor homogenates were resuspended and homogenized using 1.0 ml of saline 0.1% Triton X-100 and centrifuged at 11000 × *g* at 4°C for 10 min. The supernatants were then used to determine myeloperoxidase (MPO) and *N*-acetylglucosaminidase (NAG) levels, which indicate neutrophil and macrophage (mononuclear cell) migration, respectively.

The method of Bradley et al. (1982) was used for readings of absorbance of MPO at 620 nm. The reaction was initiated by adding 18.4 mM tetramethylbenzidine (TMB) diluted in 8% dimethylformamide in water, followed by incubation for 3 min at 37°C. The reaction

was stopped by adding sodium acetate (NaOAc) immersed in ice. The measurement of NAG levels was performed according to Sánchez & Moreno (1999), in which the hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine (substrate) in *N*-acetyl-β-D-glucosamine releases *p*-nitrofen, the absorbance of which was measured at 405 nm. Both parameters were measured using a microplate reader (Synergy HT, Biotek, VT, USA).

2.8 Histopathological analysis

Fragments of tumor and liver tissue were fixed in ALFAC medium (840 ml of 85% alcohol, 50 ml of glacial acetic acid, and 100 ml of formaldehyde concentrate) at room temperature for 12 h. After fixation, the samples were dehydrated in ethanol, cleared in xylene, and then embedded in paraffin. Tissue slices (5 μm) were stained with hematoxylin and eosin (HE) and then subjected to blind analysis by optical microscopy.

The following histological parameters were observed in tumor slices: necrosis, apoptosis, inflammation, and cytological features. The following classification was used for tumor lesions: 0 (lesions within < 5% of tissue), I (lesions within 5-25% of tissue), II (lesions within 26-50% of tissue), III (lesions within 51-75% of tissue), and IV (lesions within > 75% of tissue (Alves de Souza et al., 2017). In liver slices, the analysis included inflammatory infiltration, necrosis, apoptosis, and hepatocellular degeneration.

The number and area of vessels of the tumor were morphometrically analyzed. Images of tumor slides were captured using an Olympus DP72 camera that was attached to an Olympus BX51 microscope and then analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). For vessel quantification, images of 15 random fields per group that were stained with HE were captured at 200× magnification. The vessels of each field were summed. The vascular area was considered the sum of the vessel area divided by the number of vessels in each field.

2.9 RT-qPCR of Ehrlich tumors

The expression of genes that are related to apoptosis and angiogenesis was assessed in tumor samples from the vehicle and 100 mg/kg CAP groups. First, RNA was isolated using TriZol reagent, and complementary DNA (cDNA) synthesis was performed from 1.0 µg of this RNA using the High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using 1x SYBR Green PCR Master Mix and 800 nM of each primer in a volume of 25 µl in StepOne Plus equipment (Applied Biosystems). The samples were diluted 1:5 for all of the reactions. In all of the analyses, the *Rplpo* and *Gapdh* genes were used as housekeeper controls. The sequences of specific primers that were used for amplification were the following: Bcl-2-associated protein (*Bax*; forward, 5'-GCCTCCTCTCCTACTTC; reverse, 5'-CCTCAGCCCATCTTCTT), B-cell lymphoma 2 (*Bcl-2*; forward, 5'-CACTTGCCACTGTAGAGA; reverse, 5'-GCTTCACTGCCTCCTT), *caspase 8* (forward, 5'-CCAGGAAAAGATTTGTGTCTA; reverse, 5'-GGCCTTCCTGAGTACTGTCACCTG), *cyclin D1* (forward, 5'-AGAAGTGCGAAGAGGAG; reverse, 5'-GGATAGAGTTGTCAGTGTAGAT), vascular endothelial growth factor (*Vegf*; forward, 5'-ACTGGACCCTGGCTTTACTGCT; reverse, 5'-TGATCCGCATGATCTGCATGGTG), *Gapdh* (forward, 5'-GGTGAAGCAGGCATCT; reverse, 5'-TGTTGAAGTCGCAGGAG), and *Rplpo* (forward, 5'-CGACCTGGAAGTCCAACTAC; reverse, 5'-ACTTGCTGCATCTGCTTG). The Ct values were subjected to ΔΔCt analysis. The final data are expressed as relative expression using *Gapdh* as the control gene.

2.10 *In vitro* clonogenic assay of breast tumor cells

Ehrlich tumor cells were originally from the mammary gland of mice. We also tested the effect of CAP in human cell lineages from this gland. The human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-436 were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, and 40 mg/ml garamycin. MCF-7 cells were supplemented with 0.01 mg/ml human recombinant insulin. After confluence in culture, 1×10^3 cells/ml were seeded in a six-well cell culture plate and treated with 0.1

mg/ml CAP (do Nascimento et al., 2017) for 24 h. After that, the medium was removed, and the cells were kept in fresh medium for 9 days until the control achieved 50 cells per colony. The medium was removed, and the cells were fixed in 1% formalin and stained with 1% crystal violet in methanol. The plate was air dried, and colonies were macroscopically counted (Franken, Rodermond, Stap, Haveman, & van Bree, 2006; Munshi, Hobbs, & Meyn, 2005).

2.11 MTT assay of normal breast cells and breast tumor cells

To evaluate the cytotoxicity of CAP in normal breast cells (immortalized HB4a cells) and tumor breast cells (MCF-7, MDA-MB-231, and MDA-MB-436 cells), the cell lineages were cultured. Viability was tested using the MTT assay. The sensitivity of breast cell lines to CAP was evaluated at different concentrations (0.025-0.4 mg/ml). A total of 5×10^3 cells were distributed in a 96-well plate and exposed or not to CAP treatment for 48 h. Viable cells were quantified using the MTT assay (Riss et al., 2013). The IC_{50} was calculated using GraphPad Prism 6.0 software.

2.12 RT-qPCR of breast tumor cells

The human breast cancer cell lines MCF-7, MDA-MB-231, and MDA-MB-436 were cultured as described above (section 2.10) and treated with 0.1 mg/ml CAP or vehicle (PBS) for 24 h. RNA was then extracted, and cDNA synthesis was performed as described above (section 2.9). The cDNA was diluted 1:2, and the primers of VEGF (forward, 5'-CCAGCAGAAAGAGGAAAGAGGTAG; reverse, 5'-CCCCAAAAGCAGGTCACTCAC) were prepared at 600 nM. RT-qPCR was performed, and the gene values are shown as relative expression using human GAPDH (forward, 5'-CTGCACCACCAACTGCTTA; reverse, 5'-CATGACGGCAGGTCAGGTC) as the control.

2.13 Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) and were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test with GraphPad Prism 6.0 software. Tumor volume curves were analyzed using two-way ANOVA followed of Bonferroni's *post hoc* test. For comparisons between means of two groups, Student's *t*-test was used. Values of $p < 0.05$ were considered statistically significant.

3 RESULTS

3.1 CAP treatment reduced Ehrlich tumor development

The tumor was visible 7 days after Ehrlich cell inoculation; thus, the measurement of tumor volume began on day 7. All of the groups that were treated with CAP exhibited a significant and dose-dependent reduction of tumor volume (Figure 2A). On the last day of treatment, tumor suppression was 28%, 40%, and 54% in the 50, 100, and 150 mg/kg CAP groups, respectively, and 85% in the 2.5 mg/kg MTX group compared with the vehicle group. These differences were statistically significant beginning on day 11 of treatment until the last day of treatment. Treatment with CAP also reduced tumor weight compared with the vehicle group (Fig. 2B). The tumor in the MTX group developed less than in the other groups (Fig. 2A, B).

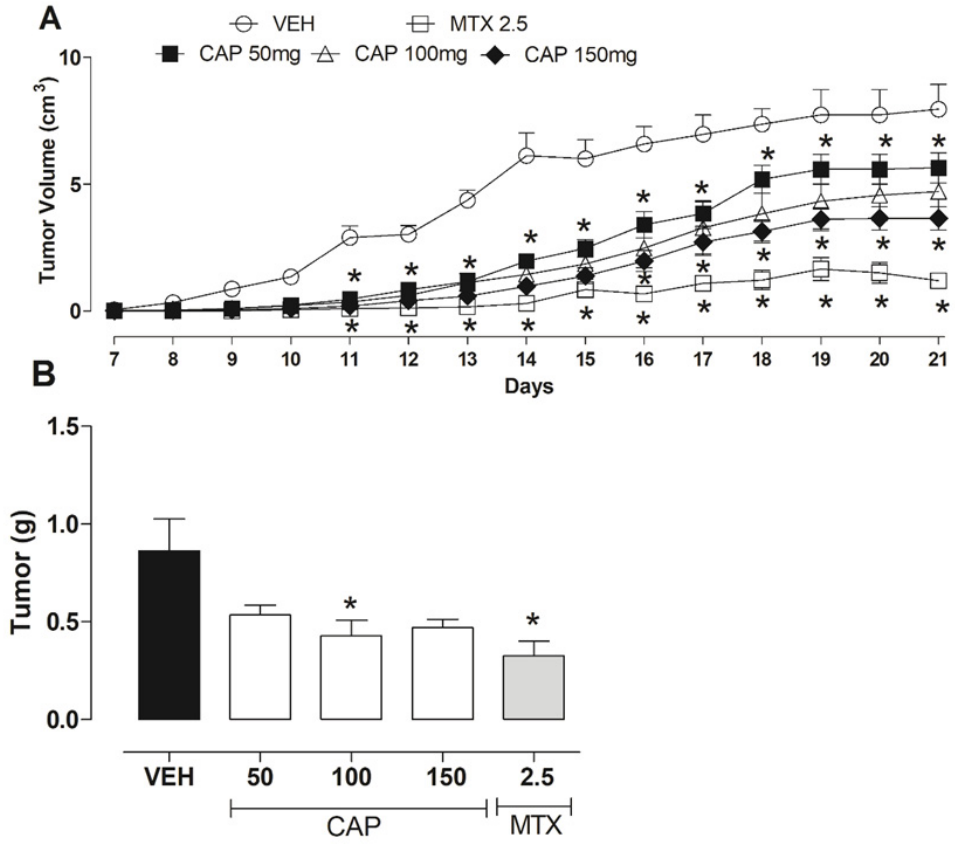


Figure 2. Effect of CAP treatment on Ehrlich solid tumor volume (A) and weight (B). The mice were orally treated with vehicle (VEH), CAP (50, 100, and 150 mg/kg), or MTX (2.5 mg/kg, i.p.) for 21 days. The results are expressed as mean ± SEM (*n* = 7-9/group) and compared using two-way (A) or one-way (B) ANOVA followed by Bonferroni´s *post hoc* test. CAP, *Capsicum annuum* polysaccharides; MTX, methotrexate. **p* < 0.05, compared with vehicle group.

3.2 Effect of CAP treatment on hematological and biochemical parameters

Blood parameters were evaluated to determine the effects of CAP on organ function and blood cells. The results are shown in Table 1. Total WBC count and the percentage of lymphocytes and monocytes were decreased by the presence of the tumor in the vehicle group compared with the naive group. Treatment with all doses of CAP completely recovered WBC counts and lymphocyte values and partially restored monocyte counts. All

of the tumor groups presented a higher percentage of granulocytes compared with the naive group. The other hematological indices, including RBCs, hemoglobin, hematocrit, RDW, and platelets, were not significantly different among groups (data not shown).

The presence of the tumor increased plasma AST levels and decreased AP levels, and ALT rates did not change. Both 100 and 150 mg/kg CAP increased plasma ALT levels with a greater increase in AST levels. Plasma AP levels were recovered to naive levels only with MTX treatment and not with CAP treatment.

Non-tumor-bearing mice that were treated with 100 mg/kg CAP (naive + CAP100 group) exhibited slight alterations of hematological parameters, but the values of these parameters were within the range of reference values for Swiss mice (Santos et al., 2016; Supplementary Table S1). CAP increased ALT and AST levels in non-tumor-bearing mice similarly to tumor-bearing mice (Supplementary Table S1). However, no alterations of body weight gain or the relative weight of the liver, lungs, kidneys, or spleen were observed in these mice (Supplementary Figure S1). No mortality was observed in any of the groups that were treated with CAP (i.e., tumor-bearing and treated with 50, 100, or 150 mg/kg CAP and non-tumor-bearing and treated with 100 mg/kg CAP).

Table 1. Hematological and biochemical parameters in healthy (naive) and tumor-bearing mice.

	Experimental Group					
Parameter	**Naïve**	**Vehicle**	**50 mg/kg CAP**	**100 mg/kg CAP**	**150 mg/kg CAP**	**2.5 mg/kg MTX**
WBC ($\times 10^3 \cdot \mu l^{-1}$)	7.18 ± 1.53	4.06 ± 0.76$^{\#}$	7.66 ± 1.84	5.98 ± 0.73	9.57 ± 1.25	8.10 ± 0.88
Lymphocyte (%)	82.10 ± 0.43	58.68 ± 4.80$^{\#}$	73.21 ± 4.80	74.73 ± 1.86	75.83 ± 3.46	67.45 ± 31.73
Monocyte (%)	2.38 ± 1.15	0.23 ± 0.04$^{\#}$	0.51 ± 0.16$^{\#}$	0.31 ± 0.06$^{\#}$	0.67 ± 0.14$^{\#}$	1.70 ± 0.60
Granulocyte (%)	15.52 ± 0.41	23.95 ± 4.81	37.10 ± 3.94$^{\#}$	30.50 ± 3.08	37.19 ± 3.88$^{\#}$	27.00 ± 2.51
ALT ($U \cdot L^{-1}$)	44.88 ± 7.36	50.00 ± 13.30	44.41 ± 11.15	85.13 ± 8.54$^{\#}$	69.40 ± 8.99$^{\#}$	47.30 ± 6.41
AST ($U \cdot L^{-1}$)	74.84 ± 10.64	136.90 ± 5.93$^{\#}$	218.1 ± 29.48$^{\#}$	293.6 ± 60.00$^{\#*}$	264.7± 24.68$^{\#*}$	233.8 ± 19.86$^{\#}$
AP ($U \cdot L^{-1}$)	66.38 ± 5.67	21.60 ± 4.06$^{\#}$	25.41 ± 5.01$^{\#}$	35.70 ± 9.73	43.00 ± 8.00	50.09 ± 7.88

Animals without tumors (naive) or with tumors were treated for 21 days with vehicle, 50, 100 and 150 mg/kg *Capsicum annuum* polysaccharides (CAP; v.o.), or 2.5 mg/kg methotrexate (MTX; i.p.). The results are expressed as mean ± SEM (n = 6-9). The statistical analyses were performed using one-way ANOVA followed by Bonferroni´s *post hoc* test. WBC, white blood cells; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase. $^{*}p < 0.05$, compared with vehicle group; $^{\#}p < 0.05$, compared with naive group.

3.3 CAP treatment slightly modified oxidative stress parameters

Tumor growth can trigger oxidative stress in the whole body. We evaluated biomarkers of oxidative stress in tumor tissue and the liver, the organ that is responsible for metabolism and detoxification. Treatment with CAP (50, 100, and 150 mg/kg) significantly increased GSH levels in the tumor by 138%, 118%, and 146%, respectively, compared with the vehicle group. Treatment with CAP did not alter SOD activity or LPO rates in the tumors (Table 2).

Tumor development also caused alterations of hepatic oxidative stress parameters compared with the naive group, manifested by a significant increase (65%) in SOD activity. Additionally, a decrease in GSH levels (-52%) and increase in LPO rate (77%) were found compared with the naive group. Both higher doses of CAP recovered hepatic GSH levels to those of the naive group but did not influence the other parameters. Interestingly, MTX treatment only slightly influenced biomarkers of oxidative stress (Table 2).

Table 2. Effect of CAP treatment on tumor and hepatic oxidative stress biomarkers in Ehrlich tumor-bearing mice.

	Experimental Group					
Biomarker	**Naïve**	**Vehicle**	**50 mg/kg CAP**	**100 mg/kg CAP**	**150 mg/kg CAP**	**2.5 mg/kg MTX**
GSH Tumor		116.30 ± 12.62	276.2 ± 39.97*	253.90 ± 29.80*	285.90 ± 49.98*	134.30 ± 12.70
GSH Liver	1259.10 ± 0.10	593.90 ± 101.60	545.6 ± 132.70	1054.00 ± 72.48*	1251.00 ± 67.92*	880.20 ± 86.65
SOD Tumor		199.9 ± 9.5	223.5 ± 15.3	171.3 ± 13.9	254.1 ± 14.6	238.10 ± 32.0
SOD Liver	133.43 ± 0.92	219.6 ± 26.5#	223.50 ± 15.3	171.3 ± 0.84	254.1 ± 14.6	281.3 ± 44.0
LPO Tumor		8.21 ± 0.95	8.83 ± 0.72	7.29 ± 0.67	8.79 ± 0.73	8.42 ± 0.71
LPO Liver	2.63 ± 0.29*	4.66 ± 0.78	8.31 ± 2.69#	5.41 ± 0.46	5.27 ± 0.49	3.15 ± 0.21
GST Liver	10.5 ± 0.68	8.88 ± 0.17	8.74 ± 0.76	9.19 ± 0.90	9.82 ± 0.65	9.96 ± 1.06
Cat Liver	195.36 ± 21.80	328.30 ± 39.56	330.70 ± 38.34	453.40 ± 69.04#	310.00 ± 53.48	238.40 ± 35.35

Animals without tumors (naive) or with tumors were treated for 21 days with vehicle (VEH), *Capsicum annuum* polysaccharides (CAP; 50, 100, and 150 mg/kg), or methotrexate (MTX; 2.5 mg/kg, i.p.). The results are expressed as mean ± SEM (n = 6-9/group). Comparisons were performed using one-way ANOVA followed by Bonferroni's *post hoc* test. GSH, reduced glutathione (μg GSH·g of tissue^{-1}); SOD, superoxide dismutase (U SOD·mg of protein^{-1}); LPO, lipoperoxidation (nmol hydroperoxides·min^{-1}·mg of protein^{-1}); GST, glutathione S-transferase (mmol·min^{-1}·mg of protein^{-1}); Cat, catalase (nmol·min^{-1}·mg of protein^{-1}); *$p < 0.05$, compared with vehicle group; #$p < 0.05$, compared with naive group.

3.4 CAP does not have *in vitro* antioxidant activity

Consistent with the discrete effects of CAP on biomarkers of oxidative stress *in vivo*, direct scavenging activity of CAP against the DPPH radical was not observed (Figure 3).

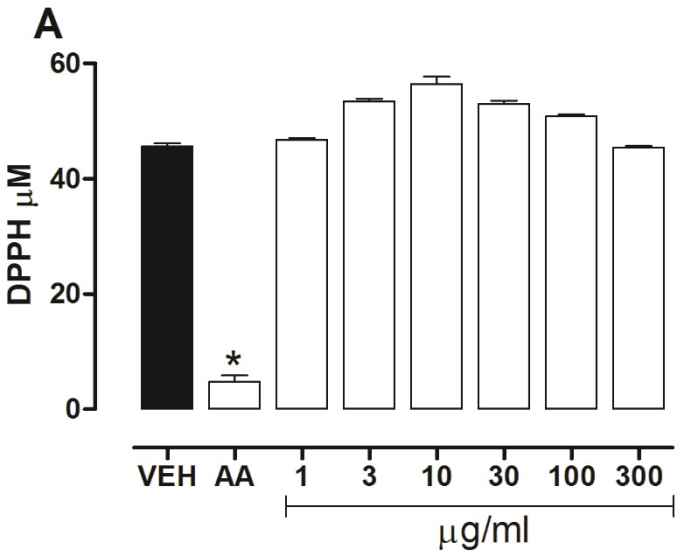
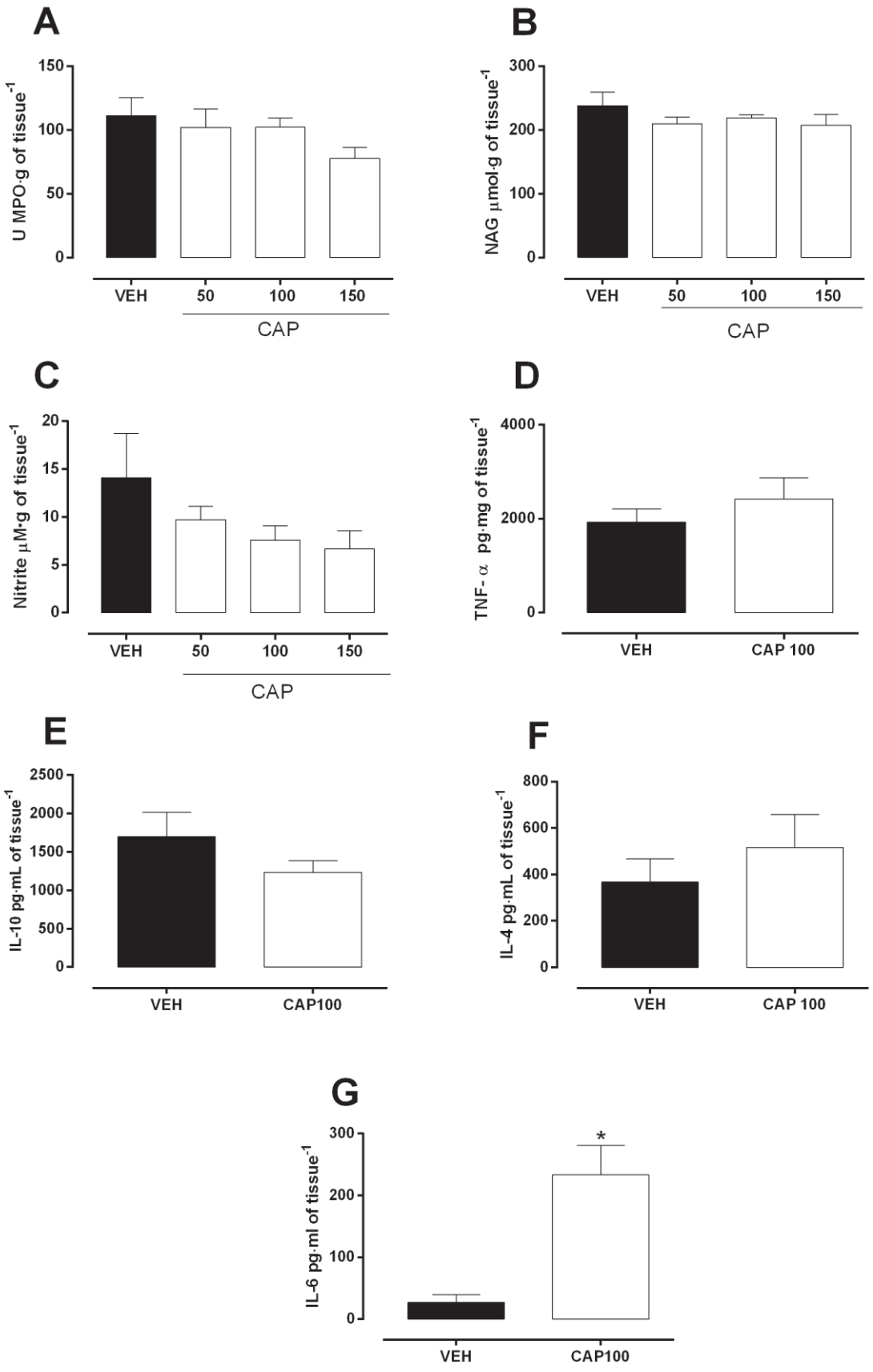


Figure 3. Evaluation of scavenging potential of several concentrations of CAP (1-300 μg/ml) in the DPPH test. Ascorbic acid (AA) and distilled water (VEH) were the positive and negative controls, respectively. The results are expressed as the mean ± SEM of experiments that were performed in triplicate. Comparisons were performed using one-way ANOVA followed by Bonferroni´s *post hoc* test. *$p < 0.05$, compared with VEH group.

3.5 CAP treatment increased IL-6 levels but no other inflammatory parameters in tumor tissue

The enzymatic activity of MPO (Figure 4A) and NAG (Figure 4B) in tumor tissue was not significantly different among groups. Tumor levels of NO decreased in all of the CAP groups compared with the vehicle group, but these differences were not statistically significant (Figure 4C). The cytokines TNF-α, IL-4, and IL-10 (Figure 4D-F) were not significantly different among groups, but tumor IL-6 levels in CAP-treated tissue were 8.6-fold higher than in the vehicle group (Figure 4G). The MTX group presented the smallest tumor size, and the amount of tumor tissue that was collected from this group limited the detection of some parameters. For this reason, inflammatory parameters were not assessed in tumors in the MTX group.


A
U MPO·g of tissue-1
150
100
50
0
VEH
50
100
150
CAP
B
NAG µmol·g of tissue-1
300
200
100
0
VEH
50
100
150
CAP
C
Nitrite µM·g of tissue-1
20
15
10
5
0
VEH
50
100
150
CAP
D
TNF- α pg·mg of tissue-1
4000
2000
0
VEH
CAP 100
E
IL-10 pg·mL of tissue-1
2500
2000
1500
1000
500
0
VEH
CAP100
F
IL-4 pg·mL of tissue-1
800
600
400
200
0
VEH
CAP 100
G
IL-6 pg·ml of tissue-1
300
200
100
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VEH
CAP100

Figure 4. Inflammatory parameters in tumor tissue in mice that were treated orally with vehicle (VEH) or *Capsicum annuum* pectic polysaccharides (CAP; 50, 100, and 150 mg/kg) for 21 days. (A) Myeloperoxidase. (B) *N*-acetylglucosaminidase. (C) Nitrite. (D) TNF-α. (E) IL-10. (F) IL-4. (G) IL-6. The results are expressed as mean ± SEM (*n* = 5-8/group). The statistical analyses were performed using one-way ANOVA followed by Bonferroni´s *post hoc* test (A-C) or Student's *t*-test (D-G). **p* < 0.05, compared with VEH group.

3.6 CAP induced necrosis and reduced the vessel area in tumor tissue but not in liver tissue

Tumors in the control and CAP groups had a high degree of coagulation necrosis, which was central, focal to multifocal (Figure 5A-D), and classified with increasing intensities of I, II, III, IV, and IV in the vehicle group, 50, 100, and 150 mg/kg CAP groups, and MTX group, respectively. All of the groups presented mild (+) mononuclear infiltrate in peripheral regions adjacent to the capsule (predominantly lymphocytes), fewer macrophages and plasmocytes, and rare polymorphonuclear cells (neutrophils). Although the number of vessels in tumor tissue was similar among both groups VEH and CAP100, the vessel area was significantly reduced by CAP (Figure 5E, F). Slices of the liver showed preserved tissue, without relevant alterations in any of the groups (data not shown).

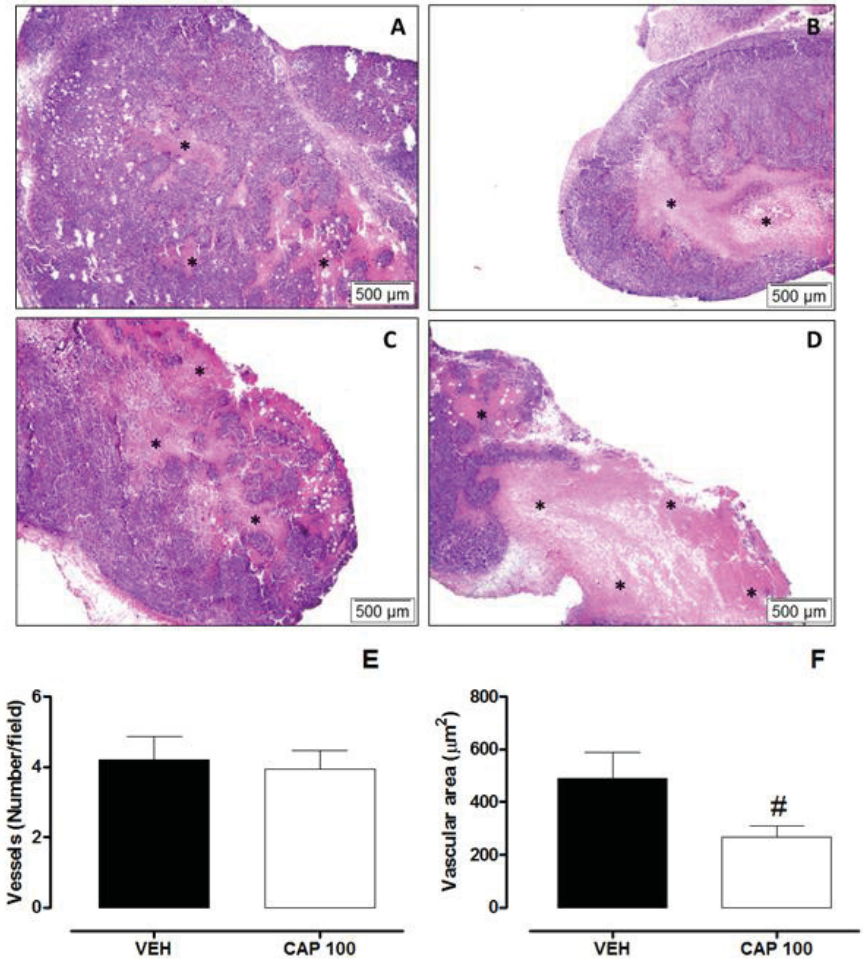

Figure 5. Representative slices of Ehrlich tumors in mice treated with (A) vehicle or (B-D) *Capsicum annuum* polysaccharides (CAP; 50, 100, or 150 mg/kg) and (E) number and (F) area of tumor vessels. The slices were stained with HE, indicating progressively a higher degree of necrosis (*). The results in (E) and (F) are expressed as mean ± SEM (*n* = 15 images/group). The statistical analyses were performed using Student's *t*-test. $^{\#}p < 0.05$, compared with VEH group.

3.7 CAP altered VEGF gene expression in Ehrlich tumors

Consistent with the histological observations, the vehicle and 100 mg/kg CAP groups did not present differences in the expression of genes that are related to apoptosis (*Bcl-2*, *Bax*, and *caspase-8*) or the expression of a gene that is related to cell cycle progression

(c*yclin D*). However, the mRNA expression of *Vegf* in tumor tissue in the 100 mg/kg CAP group was reduced by 41% compared with the vehicle group (Fig. 6).

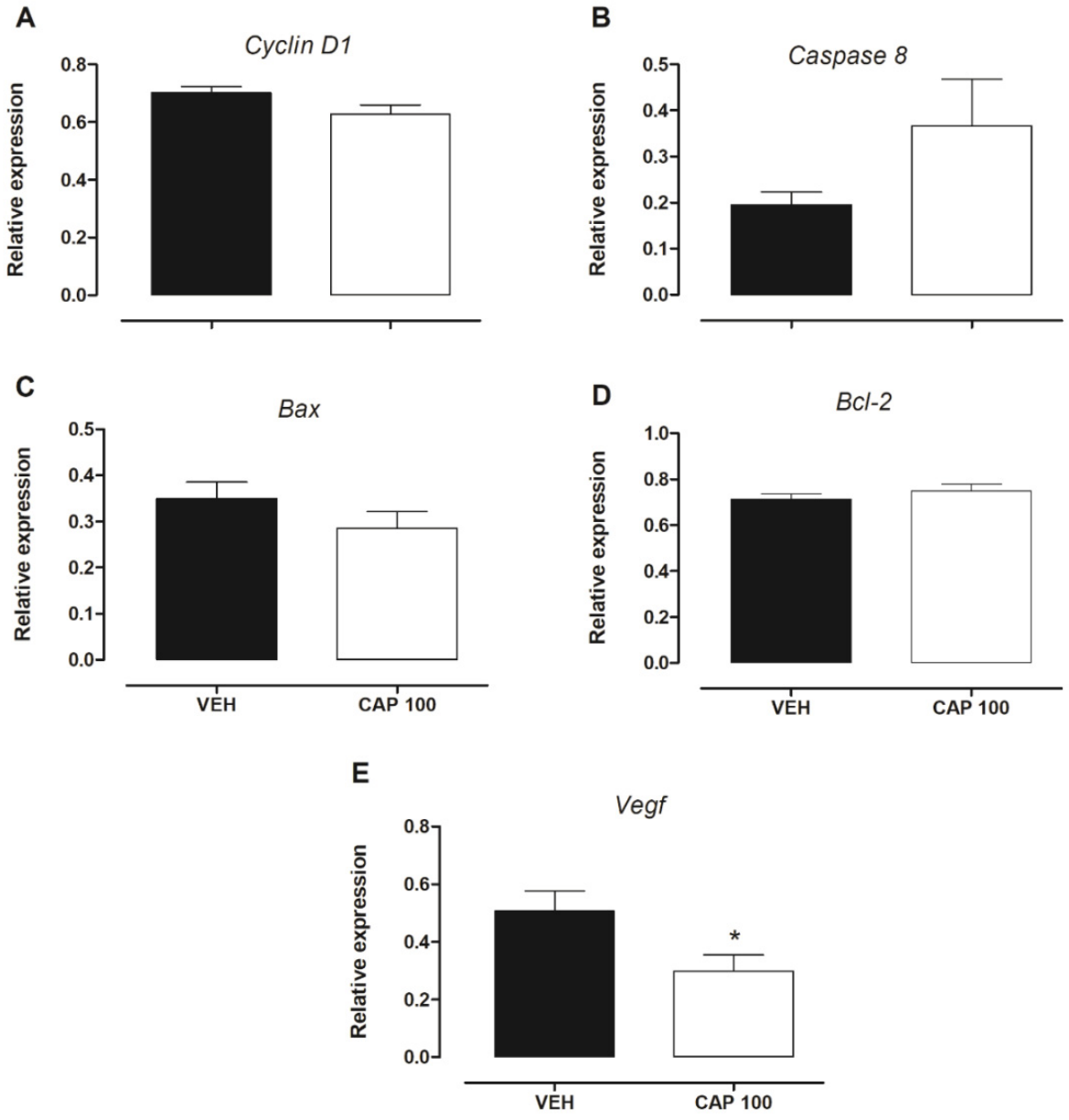

Figure 6. Gene expression of (A) *cyclin D1*, (B) *caspase-8*, (C) *Bax*, (D) *Bcl-2*, and (E) *Vegf* in tumor tissue from mice that were treated orally with vehicle (VEH) or CAP (100 mg/kg) for 21 days. The results are expressed as mean ± SEM (*n* = 5-6/group) and represent expression relative to the *Gapdh* reference gene. The data were analyzed using one-way ANOVA followed by Bonferroni´s *post hoc* test. **p* < 0.05, compared with vehicle group.

3.8 CAP inhibited mammary tumor cell proliferation and viability

Cancer cells acquire the ability to rapidly multiply. Considering the antineoplastic effect of CAP against Ehrlich tumors in mice, the effect of CAP on colony formation was tested in human mammary cancer cell lineages. CAP concentration-dependently reduced the proliferative capacity of MCF-7, MDA-MB-231, and MDA-MB-436 cancer cells in the clonogenic test (Figure 7). Considering the three lineages together, the average inhibition of proliferation was ~26% for 0.1 mg/ml CAP.

Cell viability was tested using the MTT method. After 48 h of CAP incubation, the normal HB4a cell line exhibited a ~15% reduction of viability, as expected. The MCF-7 and MDA-MB-436 tumor cell lines exhibited 27% and 31% reductions of viability, respectively (Figure 8A, C, D). Interestingly, the MDA-MB-231 tumor cell line was less sensitive to CAP, exhibiting a ~10% reduction of viability (Figure 8B). The IC_{50} for the MCF-7 and MDA-MB-231 tumor cell lines was 0.71 mg/ml (r^2 = 0.93) and 2.27 mg/ml (r^2 = 0.84), respectively.

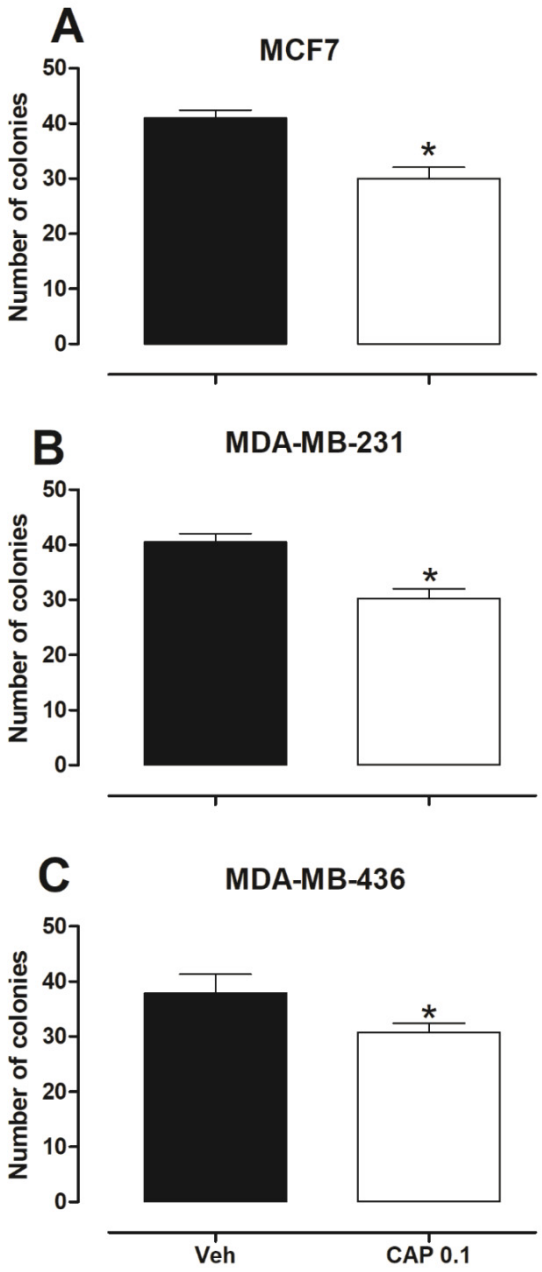

Figure 7. Colony formation of mammary cancer cell lineages after treatment with vehicle (VEH) or CAP (0.1 mg/ml) for 24 h. (A) MCF-7. (B) MDA-MB-231. (C) MDA-MB-436. The cells were cultured as described in the Material and Methods. The results are expressed as mean ± SEM (n = 3). The data were analyzed using one-way ANOVA followed by Bonferroni´s *post hoc* test. *$p < 0.05$, compared with vehicle group.

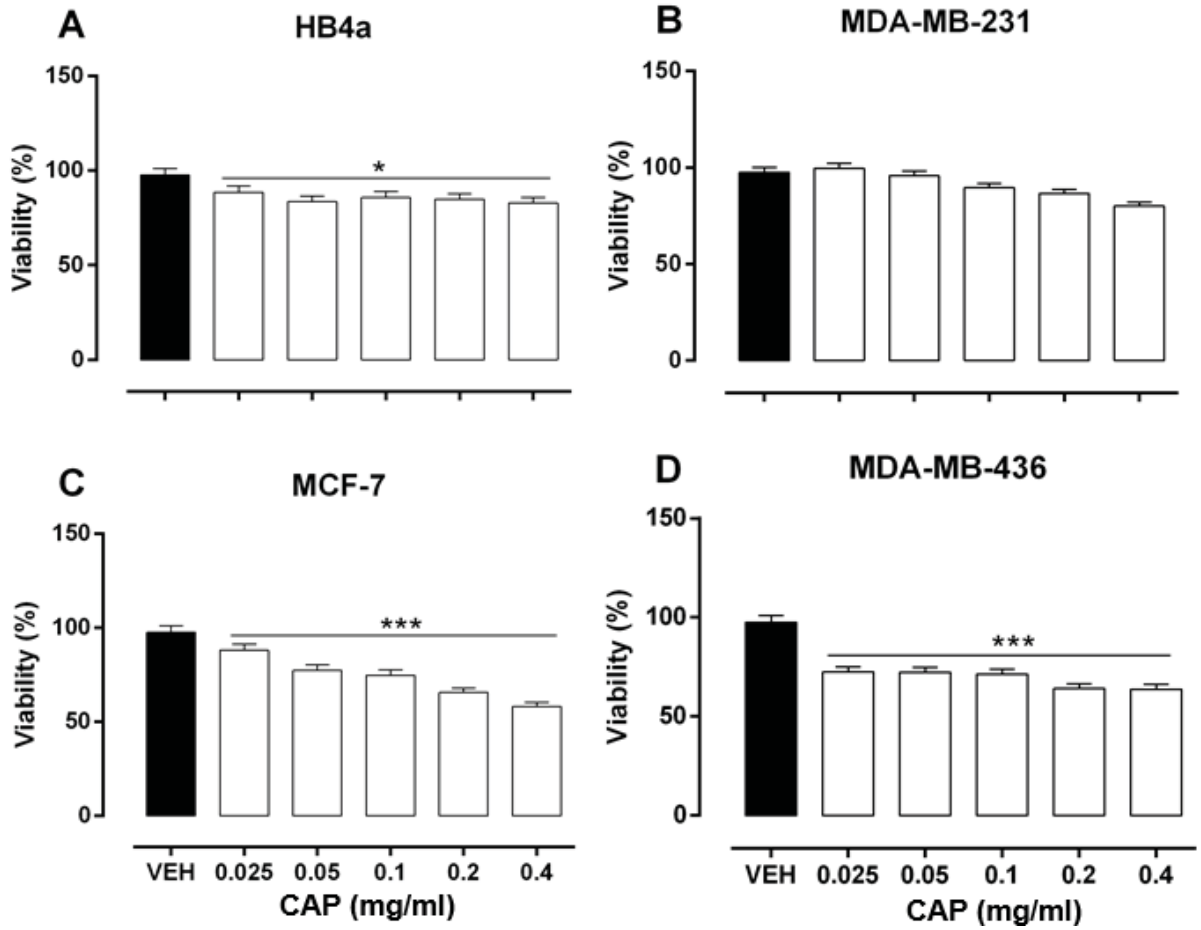

Figure 8. Viability of breast cancer cell lines after treatment with CAP (0.025-0.4 mg/ml) for 48 h. (A) HB4a cells. (B) MDA-MB-231 cells. (C) MCF-7 cells. (D) MDA-MB-436 cells. The cells were cultured as described in the Material and Methods and evaluated using the MTT assay. The results are expressed as mean ± SEM (*n* = 3). The data were analyzed using one-way ANOVA followed by Bonferroni's *post hoc* test. **p* < 0.05, ****p* < 0.001, compared with vehicle group.

3.9 CAP inhibited VEGF expression in mammary tumor cells

CAP reduced the gene expression of *Vegf* in Ehrlich tumor tissue. Its influence on VEGF expression in human breast cancer cells was then evaluated. Consistent with the *in vivo* results, CAP inhibited the gene expression of VEGF in MCF-7 (-24%) and MDA-MB-436 (-39%) cells but not in MDA-MB-231 cells (Fig. 9).

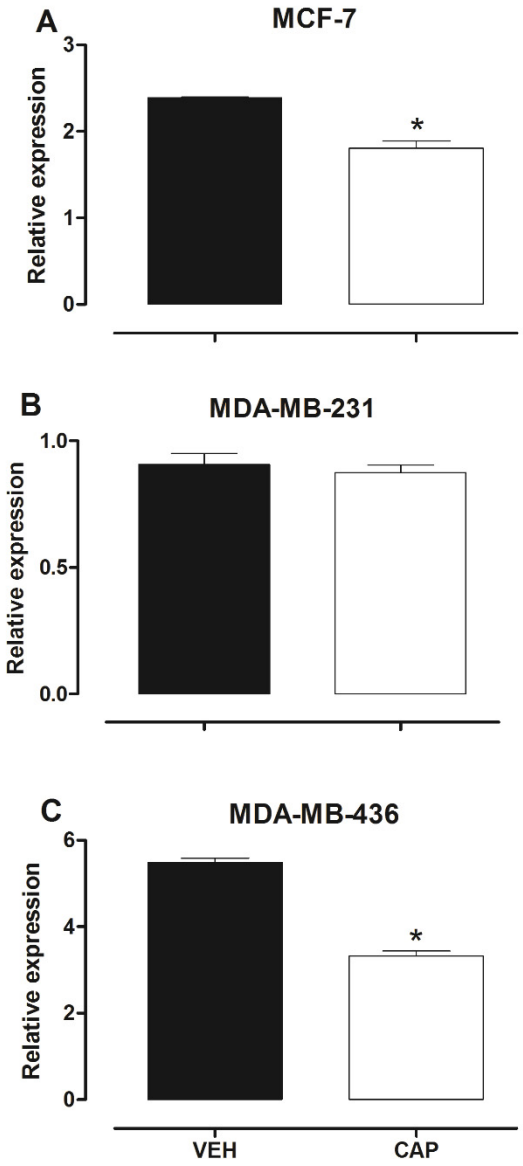

Figure 9. Gene expression of VEGF in mammary cancer cell lineages after treatment with vehicle (VEH) or CAP (0.1 mg/ml) for 24 h. (A) MCF-7. (B) MDA-MB-231. (C) MDA-MB-436. The results are expressed as mean ± SEM (*n* = 3). The statistical analyses were performed using Student's *t*-test. *$p < 0.05$, compared with vehicle group.

4 DISCUSSION

The present results demonstrated the antineoplastic effects of pectic polysaccharides that were extracted from green sweet pepper (CAP) both *in vivo* and *in vitro*. To investigate this

effect, Ehrlich tumors, which are a malignant neoplasm of epithelial tissue in mice, were used. Ehrlich tumors have a mammary origin; therefore, CAP was also tested in human breast cancer cells, namely MCF-7, MDA-MB-231, and MDA-MB-436 lineages. CAP reduced Ehrlich tumor growth *in vivo* at all doses tested and reduced the proliferation of cells *in vitro* at both tested concentrations. Previous studies reported the antitumor activity of polysaccharides from different sources, such as polysaccharides from *Punica granatum* that inhibited tumor metastasis of B16F10 melanoma cells in mice (Varghese et al., 2017) and *Coriolus versicolor* fungus that exerted a marked antitumor effect against Sarcoma 180 and Ehrlich carcinoma in mice (Kobayashi, Rassenti, Meisenholder, Carson, & Kipps, 1993). Our group previously reported the antitumor effects of polysaccharides from *Agaricus brasiliensis* mushroom (Jumes et al., 2010) and cabernet franc red wine (Stipp et al., 2017) in rats with Walker-256 tumors. The present study investigated the antineoplastic activity of polysaccharides that were isolated from green sweet pepper fruit.

To explore the effects of CAP on the tumor microenvironment, inflammation, oxidative stress, apoptosis, and angiogenesis were investigated. Oxidative stress was first evaluated. The overproduction of reactive oxygen species causes oxidative stress, resulting in mitochondrial apoptosis and cellular dysfunction. However, cancer cells regulate the redox system differently, causing the overexpression of antioxidant enzymes to ensure cell survival. Therefore, the antioxidant system is a target for antineoplastic drugs. In the present study, SOD activity and LPO levels in Ehrlich tumors were unaffected by CAP treatment, whereas the tumor and hepatic levels of GSH increased (Table 2). GSH is one of the main antioxidants in cells. The increase in tumor levels of GSH in all of the CAP-treated groups could contribute to controlling LPO levels in the tumor microenvironment to protect tumor cells against oxidative damage. CAP did not have antioxidant activity *per se* when reacting *in vitro* with the radical DPPH (Figure 3). In contrast, polysaccharides from *Zizyphus jujuba* exerted antioxidant effects against the DPPH radical but at much higher concentrations (maximum of 5000 μg/ml; (Zhang et al., 2017a) than in the present study for CAP (maximum 1000 μg/ml). Altogether, these data indicate that CAP does not influence regulation of the redox system in tumor cells, thus indicating that the redox system does not contribute to its antineoplastic effect. Notably, in healthy tissue, such as the liver, the increase in GSH levels that was observed herein at

higher doses of CAP (100 and 150 mg/kg) may represent a beneficial effect because the liver is the main metabolism-associated organ and is often subjected to metabolic injury. High hepatic levels of GSH may help in the detoxification process and cellular protection.

Another pathway that we investigated that may be related to CAP-induced cell death is apoptosis. Apoptosis is regulated by multiple genes at the cellular level, including cleaved-caspase 8, Bcl-2, and Bax. Caspase 8 is an effector that initiates cell degradation in the final stages of apoptosis. The pro-apoptotic protein Bax and survival-promoting protein Bcl-2 are members of the Bcl-2 family that plays a key role in regulating intrinsic apoptotic signaling (Bhattacharjee et al., 2008; Guo et al., 2014; Zarnescu, Brehar, Chivu, & Ciurea, 2008). The gene expression of *Caspase 8*, *Bcl-2*, and *Bax* in tumor tissue was unaffected by CAP treatment (Fig. 6), indicating that these polysaccharides likely do not regulate the apoptosis process, at least in Ehrlich cells. These results were corroborated by the histological analyses, which suggested the occurrence of necrosis rather than apoptosis in Ehrlich tumors in mice that were treated with CAP. In contrast, *Angelica sinensis* polysaccharides were previously reported to promote the apoptosis of a human glioblastoma cell line (U251). The apoptosis suppressor protein Bcl-2 was downregulated, and the expression of pro-apoptotic proteins Bax and cleaved-caspase 3 increased (Zhang et al., 2017b). Additionally, the lower expression of cyclins was found (Zhang et al., 2017b), which also differed from our data because *Cyclin D1* expression was unaltered by CAP treatment. Other studies demonstrated that nostoglycan, a polysaccharide from cultured *Nostoc sphaeroides* colonies, induced the apoptosis of human lung adenocarcinoma A549 cells via caspase 3 activation (Li et al., 2018). Importantly, these data from distinct polysaccharides were obtained using different cell lineages *in vitro*, whereas we investigated apoptosis in Ehrlich tumors *in vivo* under different experimental conditions.

The inflammatory process in tumor tissue was also analyzed. The levels of NAG, MPO, NO, TNF-α, IL-4, and IL-10 levels were unaffected by CAP treatment, whereas IL-6 levels increased (Figure 4). Distinct results were previously observed when THP-1 macrophages were treated with the same concentration of CAP (0.1 mg/ml), increasing the levels of TNF-α and IL-10 (do Nascimento et al., 2017). These discrepant results may be explained by the distinct experiment protocols that were used (i.e., cytokines were

measured in THP-1 and Ehrlich tumor cells *in vitro* after 18 h of CAP treatment and *in vivo* after 21 days of CAP treatment, respectively). Thus, the time-point of the inflammatory process that was analyzed herein was different from the previous study (do Nascimento et al., 2017). Distinct time-points for cytokine production during 13 days of Ehrlich tumor development were previously reported (Gentile, Queiroz-Hazarbassanov, Massoco, & Fecchio, 2015).

Diverse polysaccharides can differentially influence parameters of inflammation. For example, a polysaccharide extract from *Zizyphus jujuba* that contained mannose, rhamnose, galactose, galacturonic acid, glucose, and arabinose reduced the synthesis of IL-1β and enhanced the synthesis of TNF-α in THP-1 cells (Zhang et al., 2017A). The same elevation of tumor TNF-α and reduction of tumor NAG, MPO, and NO were found in Walker-256 tumor-bearing rats that were treated with polysaccharides that were extracted from red wine, consisting of arabinogalactans, mannans, and pectins (Stipp et al., 2017). Marine exopolysaccharides that were derived from *Crypthecodinium cohnii* exerted various effects on cytokine production in RAW 264.7 cells, with increases or decreases that were concentration-dependent (Ma et al., 2017). In the present study, CAP was composed mainly of uronic acids (67%), with minor amounts of rhamnose (1.6%), arabinose (6.4%), xylose (0.3%), galactose (6.7%), and glucose (4.4%), with relatively low amounts of protein (1%) and phenolic compounds (0.5 g GAE/100 g). Additionally, CAP consisted of a highly methoxylated homogalacturonan (the degrees of methyl esterification and acetylation were 85% and 5%, respectively), together with type I arabinogalactan anchored to rhamnogalacturonan (do Nascimento et al., 2017). Thus, different structures of polysaccharides can have distinct cellular effects, including on cytokine production in tumor and normal cells. This composition induced an elevation of IL-6 levels in the Ehrlich tumor microenvironment.

The role of IL-6 in tumor development and its influence on mammary cancer cells have been studied (Dethlefsen, Højfeldt, & Hojman, 2013; Fisher, Appenheimer, & Evans, 2014; Sanguinetti, Santini, Bonafè, Taffurelli, & Avenia, 2015). Elevated plasma levels of IL-6 have been related to invasiveness and poor prognosis, whereas an increase in intramural IL-6 can trigger tumor cell death, including the death of human breast cancer cells (for review, see Dethlefsen, Højfeldt, & Hojman, 2013; and Fisher, Appenheimer, &

Evans, 2014). Our data from Ehrlich tumors in mice that were treated with CAP corroborate this relationship because high tumor levels of IL-6 were correlated with a reduction of tumor development in this group. Additionally, IL-6 has been shown to directly stimulate angiogenesis. In contrast to VEGF, however, IL-6 stimulated defective vessels (Gopinathan et al., 2016). Angiogenesis is a prerequisite for cancer progression. It is a complex process that involves degradation of the extracellular matrix and the proliferation, migration, and morphological differentiation of endothelial cells to form vessels. Many factors control this process, such as growth factors and cytokines, but VEGF has been a particular focus of research because of its key role in angiogenesis (Podar, Fan, Schimming, & Jaeger, 2012). In the present study, CAP reduced the vessel area of Ehrlich tumors (Fig. 5) and downregulated *Vegf* gene expression in Ehrlich tumor-bearing mice (Figure 6E) and in MCF-7 and MDA-MB-436 human breast cancer cell lines (Figure 9A, C). Importantly, all these cell lineages have a mammary origin, indicating that green sweet pepper polysaccharides may have the potential to treat breast cancer.

In addition to downregulating VEGF in mammary cells, CAP reduced the proliferation of three human breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-436) in the clonogenic assay. CAP also reduced the viability of MCF-7 and MDA-MB-436 cells but had less effect on the MDA-MB-231 and HB4a breast cell lines. MDA-MB-231 cells are a highly aggressive, invasive, and poorly differentiated triple-negative breast cancer (TNBC) cell line that lacks estrogen receptors (ERs), progesterone receptors, and human epidermal growth factor receptor 2. MDA-MB-436 is an infiltrating ductal carcinoma cell line that is hormone-independent. MCF-7 cells express substantial levels of ERs and progesterone receptors, mimicking the majority of invasive human breast cancers that express ERs (Lee, Oesterreich, & Davidson, 2015) with low metastatic potential (Comşa, Cîmpean, & Raica, 2015; Shirazi, 2011). CAP reduced the proliferation of all of these breast cancer cell lineages and was less effective against normal HB4a breast cells. As many as 40-50% of ER+ tumors fail to respond to endocrine therapy and eventually recur as aggressive and metastatic cancers (Dunnwald, Rossing, & Li, 2007), with a poor prognosis at the time of treatment. The present results suggest that CAP may be a therapeutic candidate.

Importantly, no visible adverse effects were observed in animals that were treated with CAP, which were able to maintain physiological conditions during the 21 days of the experiment (Supplementary Table S1). Treatment with CAP did not affect body weight or relative organ weight (Supplementary Figure S1). The higher percentage of blood granulocytes that was observed in tumor-bearing mice is likely related to the tumor rather than to CAP treatment. The elevated plasma levels of AST likely did not derive from hepatocytes because the histopathological analysis of the liver did not reveal any such alterations. This enzyme is also present in the heart, skeletal muscle, kidneys, brain, and red blood cells, but these tissues were not evaluated in the present study.

In conclusion, the present results demonstrated the antineoplastic effect of pectic polysaccharides from green sweet pepper against tumor cells that originate from the mammary gland, both *in vivo* and *in vitro*. The antineoplastic mechanism of action of CAP appears to depend on the regulation of IL-6 and VEGF expression, triggering necrosis in tumor cells. Further studies that test different posologies, times of CAP treatment, and other tumor models and lineages are encouraged to evaluate the beneficial effects of polysaccharides that are present in green sweet pepper fruit.

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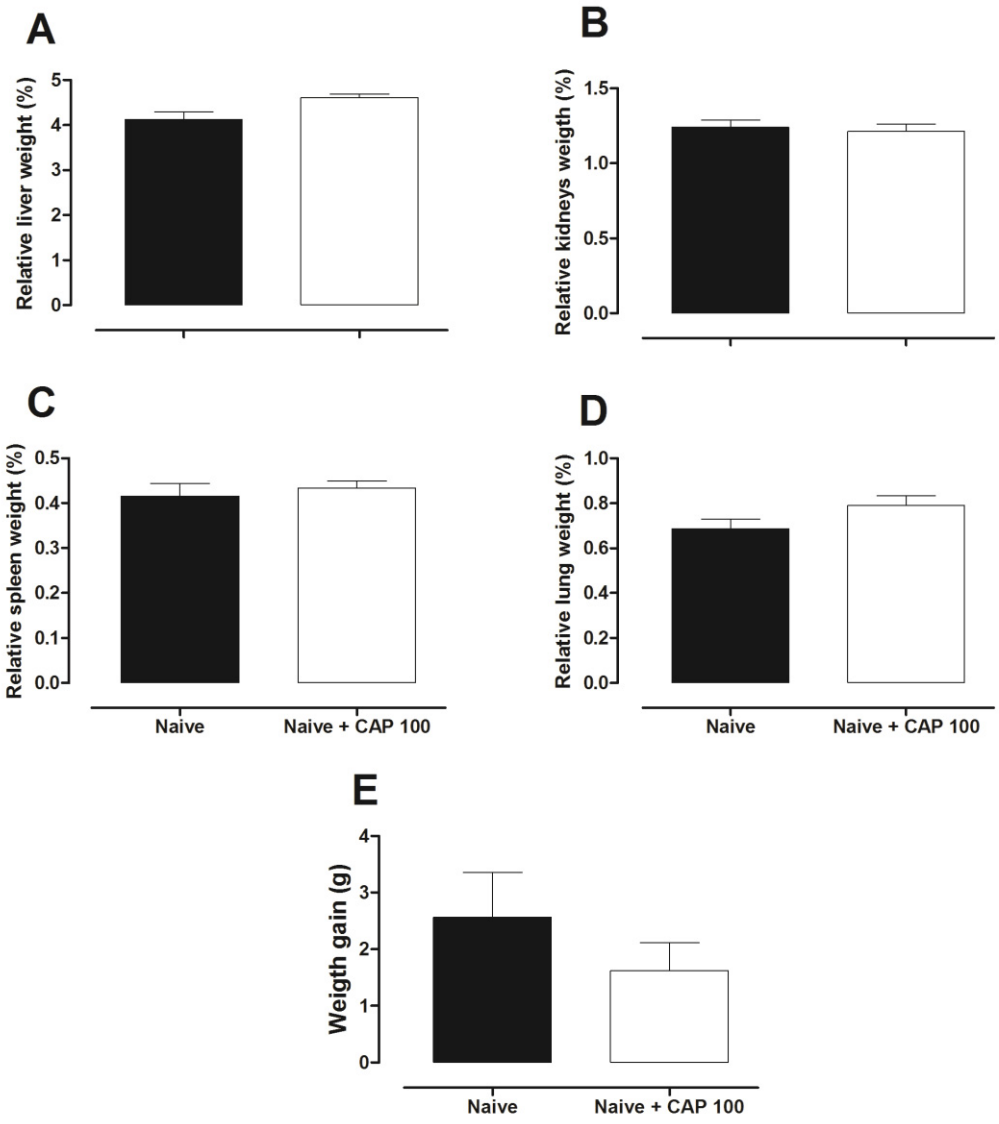
SUPPLEMENTRAY MATERIAL



Supplementary Figure S1. Relative organs weight and body weight in healthy mice treated with vehicle or CAP. A, liver; B, kidneys; C, spleen; D, lungs; E, body weight gain. Animals without tumors were orally treated with vehicle (Naive) or 100 mg/kg CAP (Naive + CAP 100) for 21 days. The results are expressed as mean ± SEM (n = 7-9/group) and compared using Student's t-test. CAP, *Capsicum annuum* polysaccharides.

Supplementary Table S1. Hematological and biochemical parameters in healthy mice treated with vehicle or CAP.

Parameter	Naïve	Naïve+CAP100	Reference value[1]
WBC ($\times 10^3 \cdot \mu l^{-1}$)	7.18 ± 1.53	6.90 ± 0.67	1.9 – 7.0
Lymphocytes (%)	82.10 ± 0.43	79.97 ± 1.06	74 – 90
Monocytes %()	2.38 ± 1.15	3.20 ± 0.26	0 – 5.0
Granulocytes (%)	15.52 ± 0.41	16.38 ± 1.05	
RBC ($\times 10^6 \cdot \mu l^{-1}$)	9.43 ± 0.12	8.29 ± 0.26*	5.2 – 10.4
Hemoglobin ($g \cdot dL^{-1}$)	13.44 ± 0.35	11.70 ± 0.31*	11.1 – 14.8
Hematrocrit (%)	41.32 ± 0.44	35.90 ± 0.99*	32.1 – 46.5
Platelets ($\times 10^3 \cdot \mu l^{-1}$)	432.40 ± 83.22	465.70 ± 17.56	315 - 758
RDW (%)	15.36 ± 0.30	15.85 ± 0.17	
ALT ($U \cdot L^{-1}$)	44.88 ± 7.36	162.9 ± 76.30	
AST ($U \cdot L^{-1}$)	78.84 ± 10.64	309.90 ± 82.27*	
AP ($U \cdot L^{-1}$)	66.38 ± 5.67	67.64 ± 6.36	
Glucose ($mg \cdot dL^{-1}$)	160.10 ± 40.15	126.40 ± 8.66	130 - 210

Animals without tumors were orally treated with vehicle (Naïve) or 100 mg/kg CAP (Naïve + CAP 100) for 21 days. The results are expressed as mean ± SEM (n = 7-9/group) and compared using Student's t-test. CAP, *Capsicum annuum* polysaccharides; WBC, white blood cells; RBC, red blood cells; RDW, red cell distribution width; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AP, alkaline phosphatase. *$p < 0.05$, compared with Naïve group; [1]Santos EW, et al. Hematological and biochemical reference values for C57BL/6, Swiss Webster and BALB/c mice. *Braz. J. Vet. Res. Anim. Sci*. 53(2):138-145, 2016.

4.2 ARTIGO 2: a ser submetido

Pectic polysaccharides from green sweet pepper increase the antineoplastic effect of methotrexate on mammary tumor cells

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ABSTRACT

This study investigated the antineoplastic efficacy and toxicity of long treatment (LT) with polysaccharides from sweet green pepper (*Capsicum annuum*, CAP), and the concomitant therapy of CAP and methotrexate (MTX) on mammary tumor cells *in vivo* and *in vitro*. Ehrlich tumor cells were inoculated subcutaneous in the right pelvic member ($2x10^6$ cells/mice) of female Swiss mice. For LT (31 days) the animals were treated with vehicle or CAP (100 mg kg^{-1}, p.o.) for 10 days before tumor inoculation followed by the next 21 days. For associated protocol, 100 mg kg^{-1} CAP (v.o.) + MTX 1 mg kg^{-1} (i.p.), MTX (1.0 or 2.5 mg kg^{-1} i.p.), or vehicle were administered during 21 days after the tumor inoculation. Both protocols with CAP, isolated or associated with MTX, and MTX alone reduced the tumor development. However, the higher tumor growth inhibition (95%) was reached with the combined treatment. Both CAP alone and CAP+MTX treatments reduced the gene expression of tumor *Vegf*, the tumor vessels area, and the interleukin (IL)-4 and IL-10; and increased IL-6 levels and degree of necrosis in tumor tissue. Additionally, CAP + MTX increased de TNF-α level in tumor. Slight modifications in biochemical and hematological parameters were observed with the combination of CAP + MTX. Additionally, CAP+MTX reduced the viability of human mammary tumor lineages MDA-MB-231 and MDA-MB-436 in culture. Thus, CAP presented antineoplastic effects *in vivo* and *in vitro* against mammary tumor cells, possibly by modulates the inflammation and angiogenesis. The combined effects of CAP with a lower dose of MTX expressively reduced the tumor growth without toxicity. CAP can be a prospect therapy for breast cancer, in order to reduce the toxicity of chemotherapy.

Keywords: Solid Ehrlich carcinoma, polysaccharides, *Capsicum annuum*, *Vegf*, breast cancer.

1. Introduction

Breast cancer is the major cause of morbidity among women population worldwide and is the main cause of cancer death in more than 100 countries. In 2018, 2.1 million women received the diagnosis of breast cancer, accounting for almost 1 in 4 cancer cases in women (Bray et al., 2018). The high incidence of breast cancer in last years is not only associated with hereditary and genetic factors, but also as a consequence of higher prevalence of known risk factors related to menstruation, reproduction, exogenous hormones intake, inadequate dietary and physical characteristics (Alwan et al., 2019). The breast cancer treatment involves several modalities, such as partial or total mastectomy, radiotherapy, (anti)hormone therapy, immunotherapy and chemotherapy, frequently combined. The antineoplastic drugs available are cytotoxic to cancer cells, but unluckily also attack normal cells leading to many side effects. For this reason, there is need for developing new drugs that have high efficacy and less side effects. In this context, polysaccharides appear as new potential drug, since they present numerous biological properties. Studies have shown that polysaccharides have biological activities such as antioxidant, immunomodulatory and antitumor (Sharon & Lis, 1993; Varghese et al., 2017; Fan et al., 2018; Adami et al., 2018; Song et al., 2008; Zhu et al., 2007). However, a great amount of polysaccharides from vegetables are still not characterized and, consequently, their biological effects and medical properties are not known.

The polysaccharides are found in foods, fruits and vegetables and have properties to prevent the development of many types of cancer (Li et al., 2018). Pharmacological studies previously conducted by our group showed that polysaccharides of sweet green pepper (*Capsicum annuum,* CAP) have antitumor and cytotoxic activity *in vivo* and *in vitro* against mammary tumor cells (Adami et al., 2018). Beyond polysaccharides are natural

compounds with diverse biological interactions and minimal toxicity to humans (Fathi et al., 2018), it can also exhibit synergistic effect when combined with chemotherapeutic drugs for cancer treatment (Pang et al., 2018; Uzoigwe & Sauter, 2012). Thus, the hypothesis of this study is that CAP can improve the efficacy of chemotherapy currently used in cancer, with less toxicity. Therefore, firstly we evaluated the toxicity of CAP in a long protocol of treatment (31 days) in mice inoculated with solid Ehrlich tumor. Posteriorly, the association of CAP and methotrexate (MTX) *in vivo* and *in vitro* was tested. MTX was chosen since it has been used in adjuvant therapy of breast cancer (Gandhi et al., 2015) and is known to induce toxicity such as in renal, hepatic and cardiac system (Patel et al., 2014; Abdel-daim et al., 2017). Additionally, the molecular mechanisms involved in CAP mechanism of action were also investigated in tumor tissue.

2. Material and Methods

2.1 Chemicals

Bovine serum albumin, N-acetylglucosaminidase, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium nitrite, p-nitrophenol and tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue, N,N-dimethylformamide, sodium phosphate, monobasic potassium phosphate, dibasic sodium phosphate, dibasic potassium phosphate, hematoxilin, eosin, sulphanilamide were obtained from Vetec (Rio de Janeiro, Brazil), phosphoric acid, dymethilsulfoxide, naphthylethylenadiamide, Triton X-100, sodium acetate, sodium carbonate, sucrose, trichloroacetic acid, 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). Aspartate

(AST), alanine transaminase (ALT) and alkaline phosphatase (AP) kits were purchased from Kovalent (São Paulo, Brazil). TriZol and primers were obtained from Invitrogen-ThermoFisher (Waltham, MA, USA). The SYBR Green PCR Master Mix and the High Capacity cDNA Reverse Transcription Kit were obtained from Applied Biosystems-ThermoFisher (Waltham, MA, USA). RPMI 1640 (Gibco), penicillin/ streptomycin, 5-Aza-2'-deoxycytidine (5Azadc) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Isolation of CAP

The fraction that contained the pectic polysaccharides (CAP) was previously characterized by Do Nascimento, Iacomini, & Cordeiro (2017). In brief, the *annum* cold-water-soluble fraction was isolated from fresh green sweet pepper fruits (*Capsicum annuum* L. cv Magali) by a consecutive process of hydrophobic compounds removal, hot water extraction, centrifugation, dialysis and freeze-thawing. The fraction characterized was composed by a highly methoxylated and acetylated homogalacturonan (degrees of methyl esterification and acetylation of 85% and 5%, respectively) together with a type I arabinogalactan linked to a type I rhamnogalacturonan, with an average molecular weight (Mw) of 3.67×10^5 g mol^{-1}.

2.3 Animals

All experimental protocols were previously approved by local Ethics Committee of Animal Experimentation (CEUA/BIO – UFPR; n. 984). Experiments were conducted using female Swiss mice (*Mus musculus*) weighing 25-30 g, housed in groups of 7 per cage with bedding consisting of wood shaving, and food and water *ad libitum*. Animals were

maintained under standard conditions of environment (temperature of 22ºC with a 12 h light/dark cycle) and were randomized before treatment. Environmental enrichment was used in the cages along all experimental protocols. All experimental protocols were in accordance with the international laboratory animal care guide to avoid animal suffer (NRC 2011).

2.4 Tumor inoculation and Experimental design

Ehrlich cells ($2x10^6$ cells/animal) dissolved in 0.2 mL of PBS buffer (16.5 mM phosphate, 137 mM NaCl and 2.7 mM KCl) were inoculated through intraperitoneal injection in mice. Six to ten days after the inoculation, animals were euthanized, cells were aseptically collected and the viability of cells was verified through the tripan blue dye method using Neubauer chamber. After 3 passages the Ehrlich cells reached viability ≥ 98%. The tumor cells were inoculated through subcutaneous route ($2x10^6$ cells/animal, 0.2 mL) in the right pelvic member of mice to induce solid Ehrlich tumor. The experiments were divided in two protocols (Figure 1), as described below:

(i) *Long Treatment* (LT): animals were orally and daily treated 10 days before tumor inoculation following 21 days after tumor inoculation (total treatment of 31 days) and were divided in two groups: (1) vehicle (distilled water, 10 mL kg^{-1}) ; (2) CAP (100 mg kg^{-1}).

(ii) *Associated Treatment*: animals were treated during 21 days after tumor inoculation and were divided in five groups: (1) vehicle (distilled water; 10 mL kg^{-1}, p.o., daily); (2) MTX 1 (1 mg kg^{-1}, i.p. twice a week); (3) MTX 2.5 (2.5 mg kg^{-1}, i.p., twice a week); (4) CAP + MTX (CAP 100 mg kg^{-1} p.o. daily plus MTX 1 mg kg^{-1}, i.p., twice a week); (5) naive (no tumor, distilled water; 10 mL kg^{-1}).

During the treatment, tumor volume and body weight were assessed daily. The tumor volume (in cm³) was calculated using the following equation: $Vol = L\ x\ W^2 x\ 0.52$, as previously described by Misha et al. (2018), where *W* is the smallest tumor diameter and *L* is the largest tumor diameter (in centimeters). On day 22 after tumor inoculation, mice were fasted for 12 h and anesthetized with cetamine (90 mg kg^{-1}) and xylazine (3 mg kg^{-1}) (both from Vetnil Industry and Trade of Veterinary Products LTDA, Sao Paulo, Brazil) by intraperitoneal route. Under anesthesia, the peritoneal cavity was open and the inferior cava vein was assessed to collect blood samples. Subsequently, the animals were euthanized trough puncture of diaphragm. Lastly, tumor mass was carefully removed, weighted, and a fragment was immediately immerse in liquid nitrogen to further stored at -80ºC to perform analyses, while another portion was stored in ALFAC medium for histological studies.

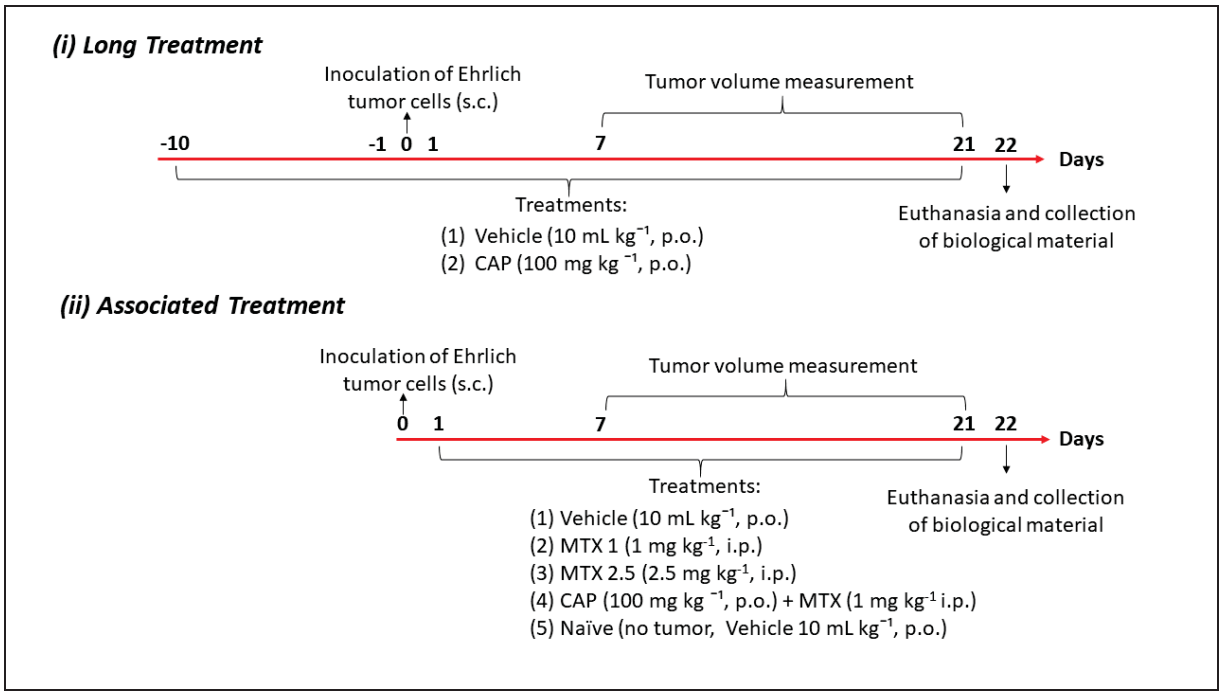


Figure 1. Experimental design for testing the antineoplastic effect of CAP in two protocols in solid Ehrlich tumor-bearing mice. ***(i)*** Long Treatment with CAP and ***(ii)*** Associated treatment

of CAP and MTX. CAP: *Capsicum annuum* polysaccharides; MTX: Methotrexate; p.o.: oral route; i.p.: intraperitoneal route; s.c.: subcutaneous route.

2.5 Inflammatory parameters

All the inflammatory parameters were measured in tumor tissue. Homogenates were prepared in PBS buffer (pH 7.2) and centrifuged in 9000 x *g* at 4ºC. The supernatant was used to measure nitric oxide (NO) (Green et al., 1982) and cytokines levels (TNF-α, IL-6, IL-10 and IL-4), determined by enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) following the manufacturer's instructions. In addition, the tissue pellet was homogenized in 0.1% Saline-Triton X centrifuged at 11000 x *g* for 20 min at 4ºC and the supernatant was used for measurement of N-acetyl-β-D-glucosaminidase (NAG) (Bailey, 1988) and myeloperoxidase (MPO) (Suzuki et al., 1983).

2.6 Histopathology

After euthanasia, tumor samples were immediately immersed in ALFAC solution (85% ethanol, 10% formaldehyde, 5% glacial acetic acid) at room temperature for 16 hr. Subsequently, tumor tissue was processed according routine protocol of dehydration using increased alcohol concentration, and xylene to embedding samples in paraffin. Samples were sectioned in 5 μm and stained with hematoxylin and eosin and analyzed in optical microscope by a pathologist. The parameters analyzed were inflammatory cells, necrosis area and apoptotic cells. The histological alterations were classified in mild (+), moderate (++) or (+++) intense. In addition, the number and area of vessels in Ehrlich tumor were quantified. Fifteen fields at 200x magnification were captured by an Olympus system

composed by camera (DP72) and microscope (BX51) and analyzed using ImageJ software (NIH, Bethesda, MD, USA). The vessels area of each field was calculated by the formula: $Vascular\ area = \frac{\sum vessels\ area}{number\ of\ vessels}$

2.7 RT-qPCR of Ehrlich tumors

The expression of mRNA of vascular endothelial growth factor (*Vegf*), a gene related to angiogenesis, was assessed in tumor samples of groups: (1) Vehicle and (2) CAP from the Long Treatment, and (4) CAP + MTX from the associated protocol. First, RNA was isolated using TriZol reagent, and complementary DNA (cDNA) synthesis was performed from 1.0 µg of this RNA using the High Capacity cDNA Reverse Transcription kit following the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using 1x SYBR Green PCR Master Mix and 800 nM of primers in a volume of 25 µl and StepOne Plus equipment (Applied Biosystems). The samples were diluted 1:5 for the reactions. The sequences of specific primers that were used for amplification are the following: *Vegf*, forward 5'-ACTGGACCCTGGCTTTACTGCT, reverse 5'-TGATCCGCATGATCTGCATGGTG); and *Rplp0,* forward 5'-CGACCTGGAAGTCCAACTAC, reverse 5'-ACTTGCTGCATCTGCTTG. Ribosomal protein lateral stalk subunit P0 (*Rplp0*) was used as endogenous control gene to show the relative expression (Machado et al., 2015).

2.8 Hematological and plasmatic parameters

At the end of treatment, blood samples were collected in heparinized syringes for hematological and biochemical analysis. The measurements included hemoglobin (Hb),

hematocrit (Ht), red blood cell (RBC) count, leukocyte count (white blood cells [WBCs]), differential leukocyte count. Subsequently, blood samples were centrifugated at 1344 *x g* for 5 min to obtain plasma, which was used for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AF) levels, by an automated system (Myndray BS-200), following commercial kits instructions (Kovalent, São Gonçalo, Brazil).

2.9 Viability of human breast tumor cells *in vitro*

To evaluate the cytotoxicity of CAP in association of MTX, 5×10^3 breast human tumor cells, namely MDA-MB-231 and MDA-MB-436, were cultured in a 96-well plate in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, and 40 mg mL^{-1} garamycin. CAP (0.025 mg mL^{-1}) was diluted in the culture medium and added to cells for 24 h, followed by removal of medium and addition of 0.1 to 0.8 mM of MTX. After 48 h of MTX into the culture the viable cells were quantified using the MTT assay, as previously described (Riss et al., 2013). This *in vitro* experiment was performed in triplicate.

2.10 Data analysis

All the results were analyzed using GraphPad Prism (v. 5.0) and expressed as mean ± standard error of mean (S.E.M.). Comparisons between two paired groups were subject to Student's *t*-test. For more than two groups or two variables one-way or two-way analysis of variance (ANOVA), followed by post hoc Newman Keuls test or Bonferroni's, respectively, were applied. Results were considered significant when $p \leq 0.05$ (95%).

3. Results

3.1 Effects of Long Treatment with CAP

3.1.1 Long treatment with CAP reduced the Ehrlich tumor development

Long Treatment (LT) with CAP during 31 days prevented the tumor development in mice from day 12 (74%) until day 21 (91%) after tumor inoculation, compared to vehicle group. In addition, tumor weight was also reduced by CAP in 40% compared to vehicle group (Figure 2A-C).

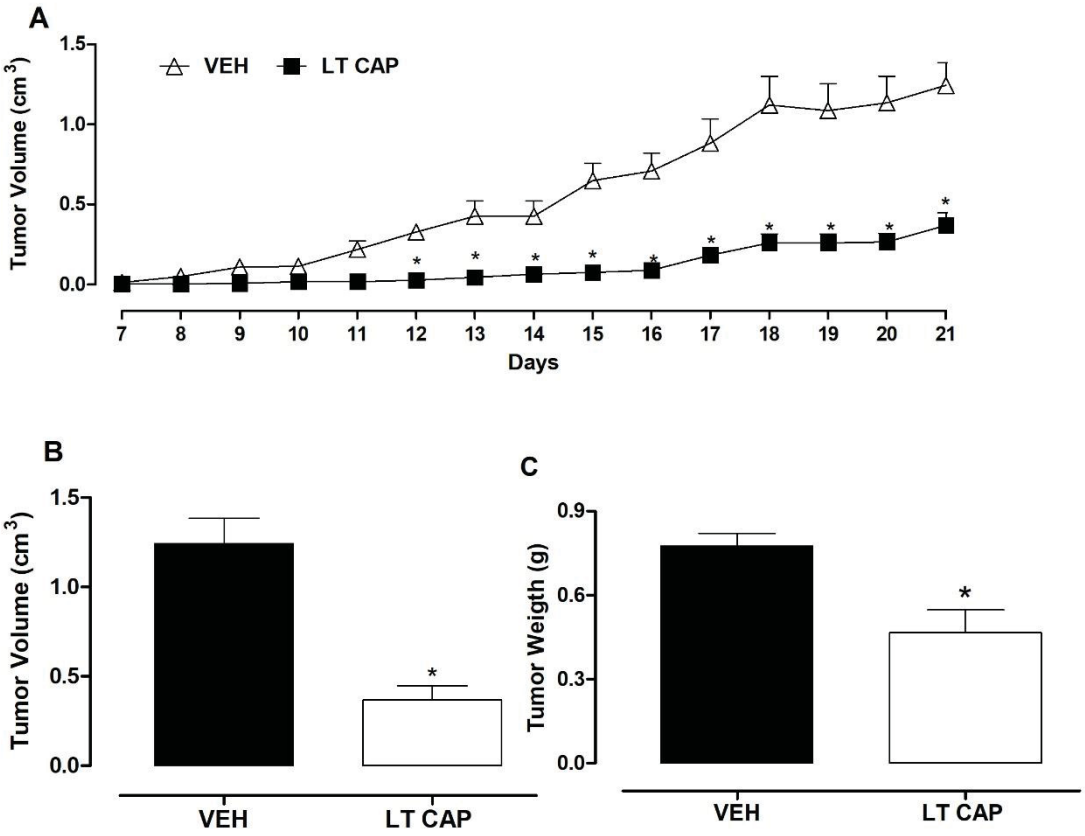


Figure 2. Antitumor effect of CAP in Long Treatment. Panel A: Ehrlich tumor volume (cm^3). Panel B: Ehrlich tumor volume (cm^3) in the last day of treatment. Panel C: Ehrlich tumor mass (g) in the last day of treatment. Mice were orally treated with vehicle (10 mL kg^{-1}) or CAP (100 mg kg^{-1}) for 31 days. The results are expressed as mean ± S.E.M. Data were analyzed by two-way ANOVA followed by Bonferroni's post hoc test (Panel A) or by Student's *t*-test (Panel B-C). *$p \leq 0.05$ compared with vehicle group.

3.1.2 Long Treatment with CAP modulated the inflammation in Ehrlich tumor

The treatment with CAP during 31 days increased tumor MPO activity (37%), and IL-6 (85%), and decreased IL-10 and IL-4 (95% and 94%, respectively) levels when compared to vehicle group. No differences were observed in NAG, nitrite and TNF-α tumor levels (Figure 3).

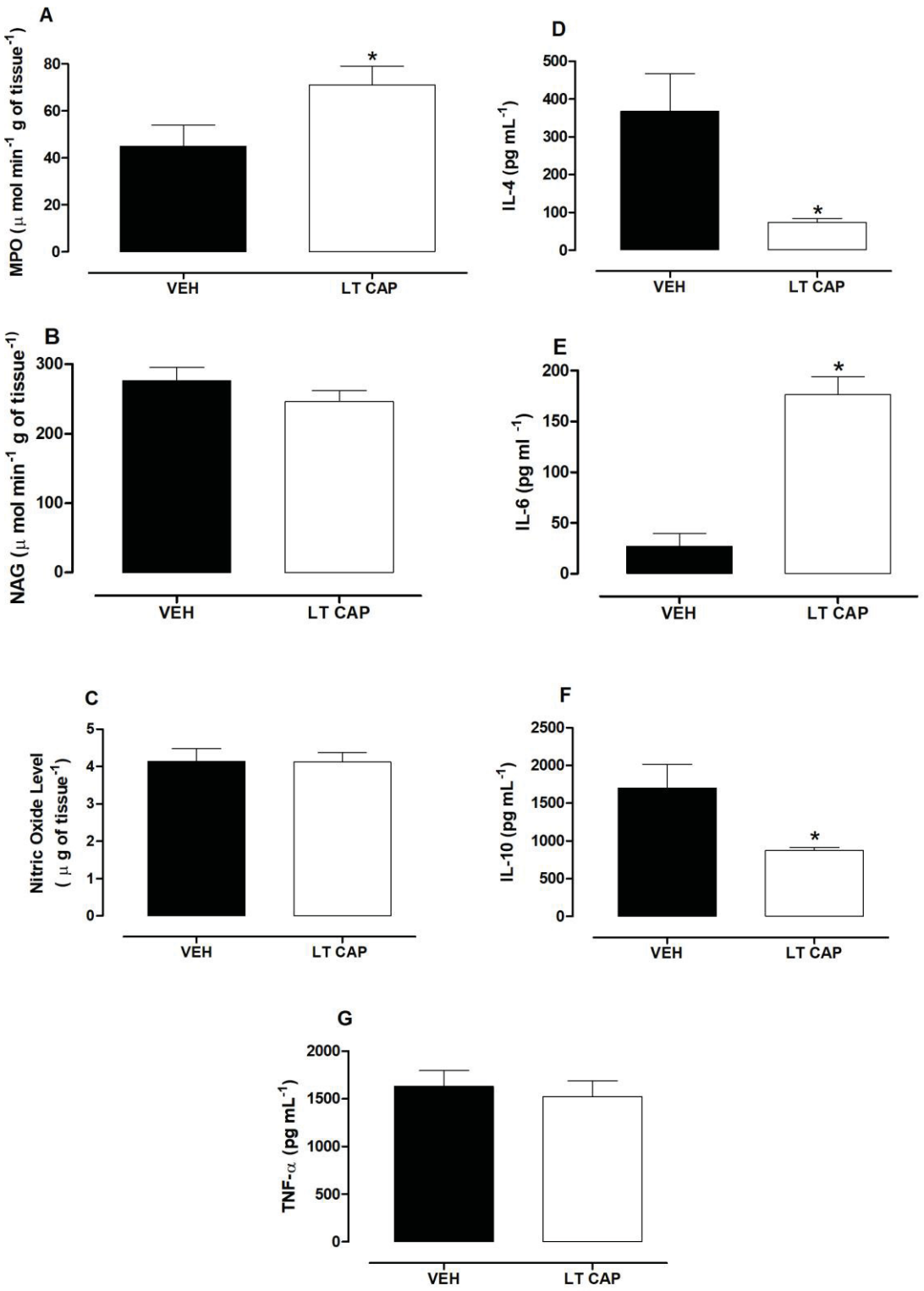


Figure 3. Effect of Long Treatment with CAP on inflammatory parameters in tumor tissue.

Panel A: myeloperoxidase (MPO). Panel B: N-acetylglucosaminidase (NAG). Panel C: Nitrite oxide

(NO). Panel D: Interleukin (IL)-4. Panel E: Interleukin (IL)-6. Panel F: Interleukin (IL)-10. Panel G: Tumor necrosis factor-alpha (TNF-α) tumor. Animals were orally treated with vehicle or CAP 100 mg kg^{-1} during 31 days. Results were expressed as mean ± S.E.M. and were analyzed by Student's *t*-test (n= 7-8). * $p < 0.05$, compared to vehicle group.

3.1.3 Long Treatment with CAP induced necrosis and vessels reduction in tumor tissue

In vehicle group it was detected Ehrlich viable cells, discrete inflammation, and discrete to moderate area of coagulative necrosis. LT with 100 mg kg^{-1} CAP during 31 days induced moderate coagulative necrosis and mild to moderate inflammation in tumor tissue (Figure 4A-C). Apoptosis was not observed in both groups. The number of vessel in tumor tissue was the same in both treatment (Fig. 4E). However, the vascular area reduced 47% in CAP treated-mice (Figure 4D). Considering these results the gene expression of tumor *Vegf* was measured. The relative expression of *Vegf* reduced significantly (55%) in CAP treatment compared with vehicle group (Figure 4F).

A

Parameters	Experimental groups	
	Vehicle	LT CAP
Inflammation - Grade I (discrete)	+	-
Grade II (moderate)	-	++
Coagulative necrosis - Grade I (0 – 20 %)	+	-
Grade II (21 – 50%)	++	++

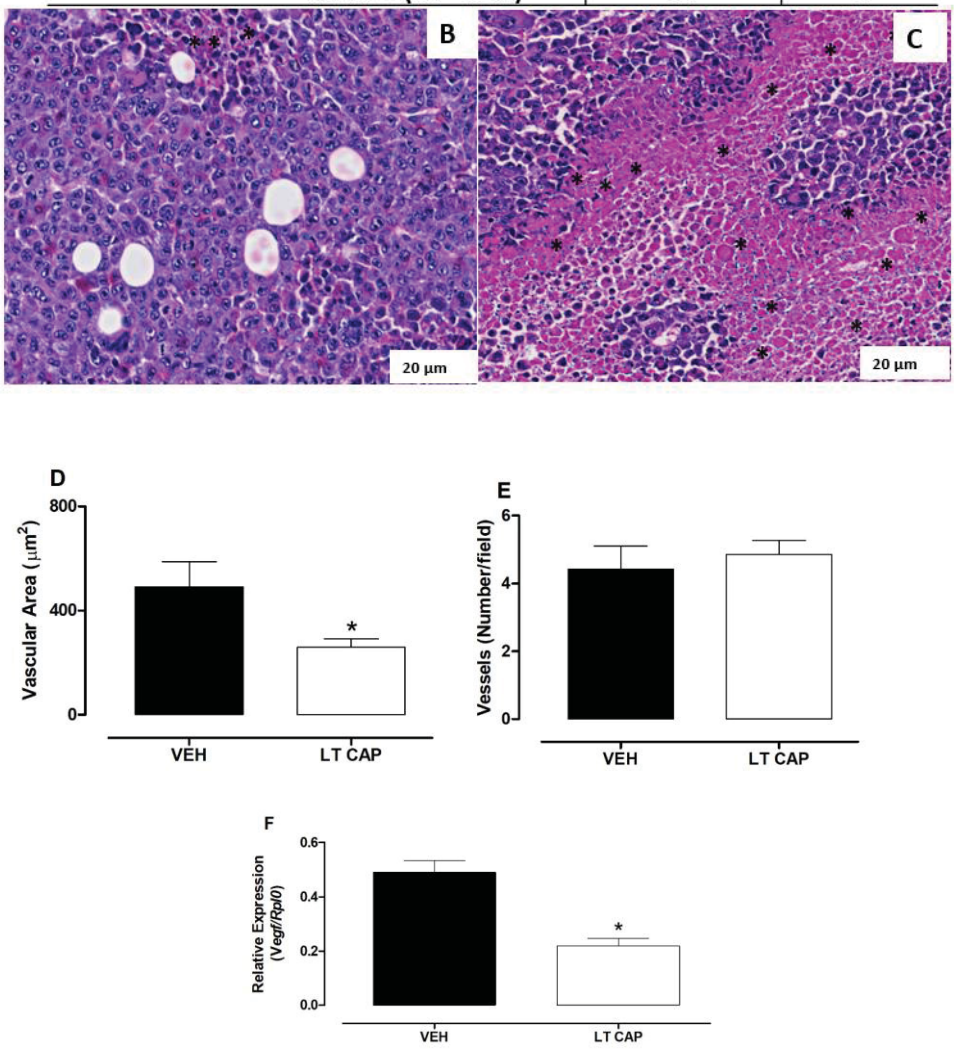


Figure 4. Histology of Ehrlich tumor tissue stained by hematoxilin/ eosin and tumor vessels analysis. Animals were orally treated with vehicle and LT CAP during 31 days. Table A: quantification of histological alterations on Ehrlich tumor of mice after Long Treatment (LT) with CAP or vehicle. Scores: (-) negative; (+) mild; (++) moderate. Panel B: Vehicle group. Panel C: LT CAP 100 mg kg^{-1}. Panel D: tumor vascular area. Panel E: number of tumor vessels. Panel F: tumor

Vegf expression. In B and C * indicates degree of necrosis. The results in D and E are expressed as mean ± S.E.M. (n=15 images/group). The statistical analyses were performed using Student's *t*-test. * $p < 0.05$, compared with VEH group. Slices of tumor were observed in optical microscope at 20 x (scale bar 20 μm).

3.1.4 Long Treatment with CAP slightly changed plasmatic and hematologic parameters

Ehrlich tumor-bearing mice (Vehicle group) showed a decrease in white blood cells, lymphocytes, monocytes, hemoglobin, hematocrit and alkaline phosphatase (AP), and increase in AST when compared to naïve group. Most of these parameters did not change with LT CAP, indicating that these alterations are induced by the tumor. However, AP returned to levels similar to naïve group after LT CAP (Table 1). After 31 days of CAP treatment there was no additional systemic toxicity.

Table 1. Plasmatic and hematological parameters in healthy (naïve) and tumor-bearing mice.

Parameter	Naïve	VEH	LT CAP	CAP + MTX	MTX 2.5
WBC × 10^3/μl	7.18 ± 1.53	4.06 ± 0.76$^{\#}$	4.61 ± 0.81	2.40 ± 0.32$^{*\#}$	9.65 ± 0,65 *
Lymphocyte %	82.10 ± 0.43	58.68 ± 4.80$^{\#}$	64.49 ± 2.93$^{\#}$	57.98 ± 6.28$^{\#}$	63.01 ± 0.45
Monocyte %	2.38 ± 1.15	5.96 ± 1.264$^{\#}$	5.01 ± 0.73$^{\#}$	5.60 ± 0.64	5.98 ± 1.32 #
Granulocyte %	15.52 ± 0.41	23.95 ± 4.81	30.50 ± 2.36	43.50 ± 8.29	29.80 ± 2.72
RBC x 10^6/mm^3	9.43 ± 0.12	8.42 ± 0.43	6.93 ± 0.74$^{\#}$	7.16 ± 0.38$^{\#}$	7.93 ± 0.29
Hemoglobin g/dl	13.44 ± 0.35	11.16 ± 0.47$^{\#}$	9.27 ± 1.09$^{\#}$	9.45 ± 0.59$^{\#}$	11.48 ± 0.33
Hematocrit %	41.32 ± 0.44	35.04 ± 1.52	29.00 ± 3.05$^{\#}$	31.73 ± 1.96$^{\#}$	35.13 ± 1.37
ALT U/L	44.88 ± 7.36	71.50 ± 22.34	62.40 ± 6.33	50.19 ± 3.19	32.13 ± 8.64*
AST U/L	78.84 ± 10.64	325.5 ± 41.5$^{\#}$	356.60 ± 22.37$^{\#}$	220.40 ± 14.39$^{*\#}$	233.80 ± 19.86$^{*\#}$
AP U/L	66.38 ± 5.67	21.65 ± 4.06$^{\#}$	55.60 ± 6.86	74.54 ± 6.69	60.36 ± 8.45

Animals were orally treated with vehicle (Naïve and VEH, distilled water, 10 mL kg^{-1}) and LT CAP during 31 days, or CAP + MTX for 21 days. The results were expressed as mean ± S.E.M. and were analyzed by one-way ANOVA followed by Newman-Keuls post hoc (n= 7-8). * and # indicated $p < 0.05$ when compared to vehicle and naïve group, respectively. WBC: White blood cells (Leukocytes), AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, AP: Alkaline phosphatase.

3.2 Effects of Associated Treatment of CAP with MTX *in vivo*

3.2.1 CAP + MTX improved the growth inhibition of Ehrlich tumor

Considering that LT with CAP presented antitumor effect without evident toxicity, we tested this effect in association with MTX, an established chemotherapeutic drug. The figure 5 shows the Ehrlich tumor development along 21 days of treatments. The figure 5A-D shows that isolated treatments with CAP and MTX 2.5 reduced the tumor volume and weight, while MTX 1 had lower effect. However, the drugs combination (CAP + MTX) produced the lowest tumor volume, reaching 95% inhibition at day 21 of treatment when compared with vehicle group (Figure 5A-C).

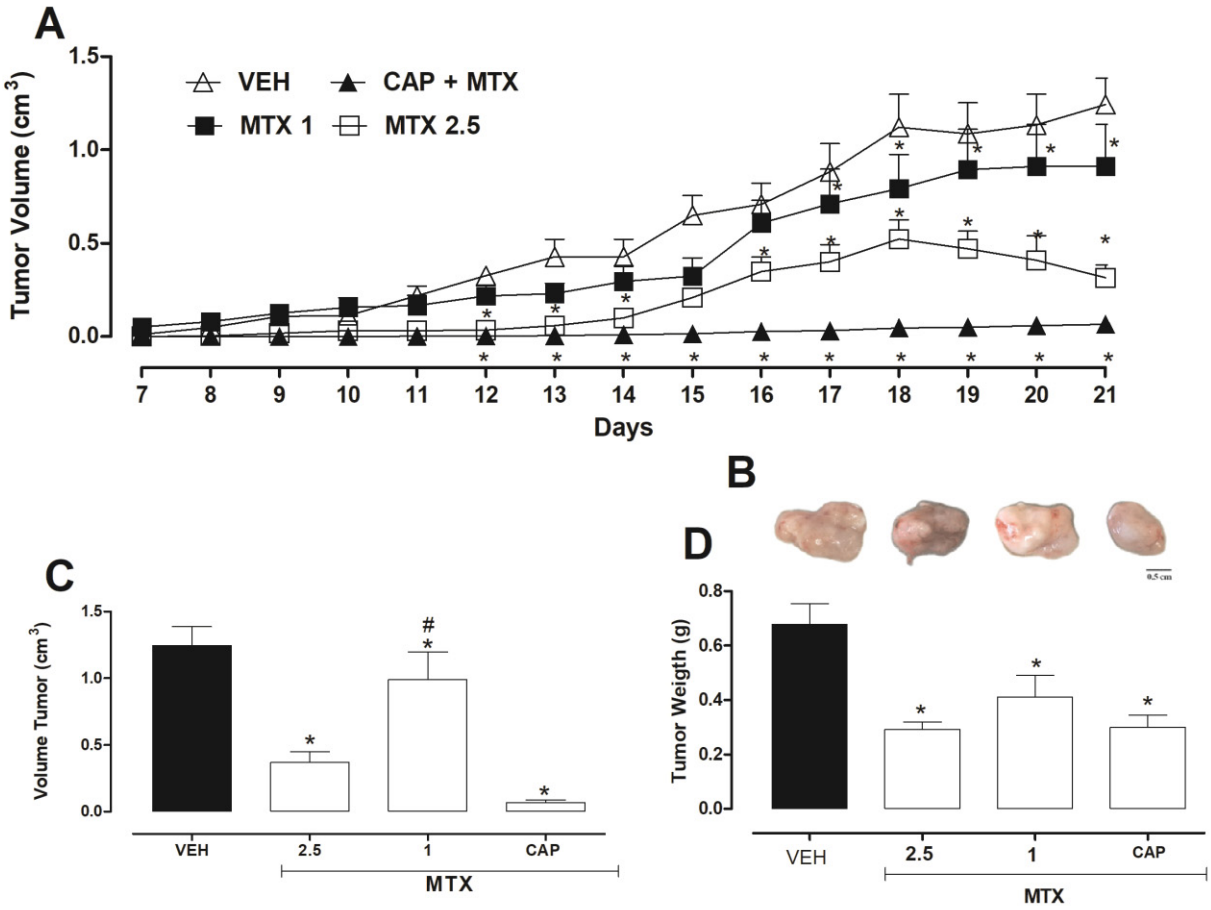


Figure 5. Effect of CAP + MTX combination in Ehrlich tumor development. Panel A: tumor volume (cm^3) along the treatment. Panel B: representative tumor mass of each group. Panel C: tumor volume (cm^3) in the last day of treatment. Panel D: tumor mass (g) in the last day of treatment. Mice were orally treated with vehicle or CAP 100 mg kg^{-1} (daily), and intraperioneally with MTX 1 or 2.5 mg kg^{-1} twice a week during 21 days. The associated treatment were performed with 1 mg kg^{-1} MTX. The results are expressed as mean ± S.E.M. The data were analyzed using one-way (Panel C-D) or two-way (Panel A) ANOVA followed by Bonferroni's post hoc test. *p < 0.05, compared with vehicle group, # p < 0.05, compared with MTX 2.5 group.

3.2.2 CAP + MTX modulated inflammation in tumor tissue

Consistently with the results of LT with CAP upon inflammatory parameters (Figure 3) the association of CAP + MTX modulated inflammatory biomarkers in Ehrlich tumor tissue. This treatment decreased nitrite (-62%), IL-10 (-85%) and IL-4 (-85%) levels, and increased IL-6 and TNF-α levels in 7-fold and 4-fold, respectively, when compared to vehicle group. No differences were observed in MPO and NAG activity (Figure 6).

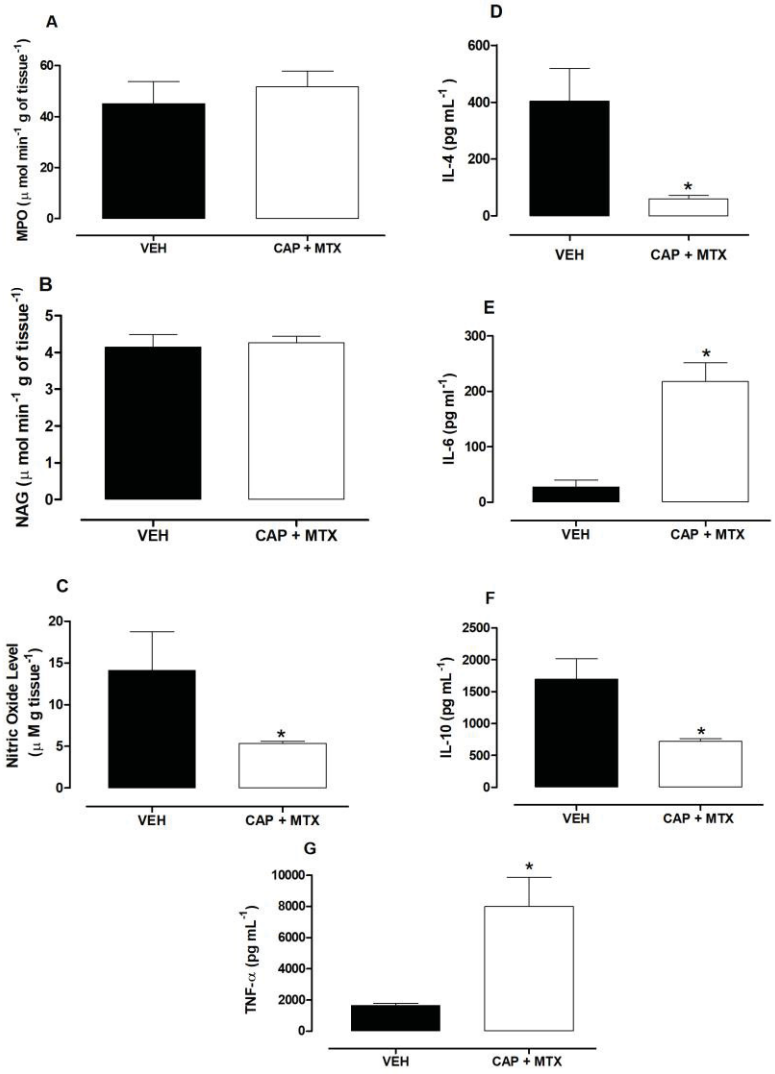


Figure 6. Effect of CAP + MTX treatment on inflammatory parameters in tumor tissue. Panel A: Myeloperoxidase (MPO). Panel B: N-acetylglucosaminidase (NAG). Panel C: Nitrite oxide (NO).

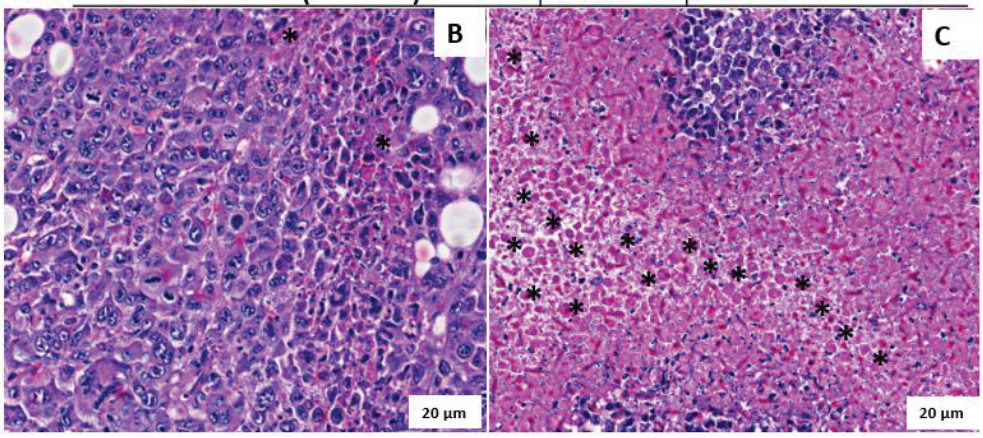
Panel D: Interleukin-4 (IL-4). Panel E: Interleukin-6 (IL-6). Panel F: Interleukin-10 (IL-10). Panel G: Tumor necrosis factor-α (TNF-α). Animals were orally treated with vehicle or CAP 100 mg kg^{-1} (daily), and intraperioneally with MTX 1 mg kg^{-1} twice a week during 21 days. Results were expressed as mean ± S.E.M. and were analyzed by Student's *t*-test (n= 7-8). * $p < 0.05$ when compared to vehicle group.

3.2.3 CAP + MTX increased the necrosis and reduced the angiogenesis of tumor

The vehicle group showed peritumoral inflammation, few areas of coagulative necrosis and higher amount of Ehrlich viable cells in tumor, whereas the combined treatment of CAP + MTX induced intense coagulative necrosis and discrete inflammation in tumor tissue, but no apoptosis (Figure 7). Furthermore, the associated treatment induced a reduction in vascular area (48%) and gene expression of *Vegf* (43%) in tumor tissue despite the number of vessels was simular among groups (Figure 7D-F).

A

Parameters	Experimental groups	
	Vehicle	CAP + MTX
Inflammation - Grade I (discrete)	+	+
Coagulative necrosis		
Grade I (0 - 20%)	+	-
Grade II (21 – 50%)	++	-
Grade III (51 – 80%)	-	+++



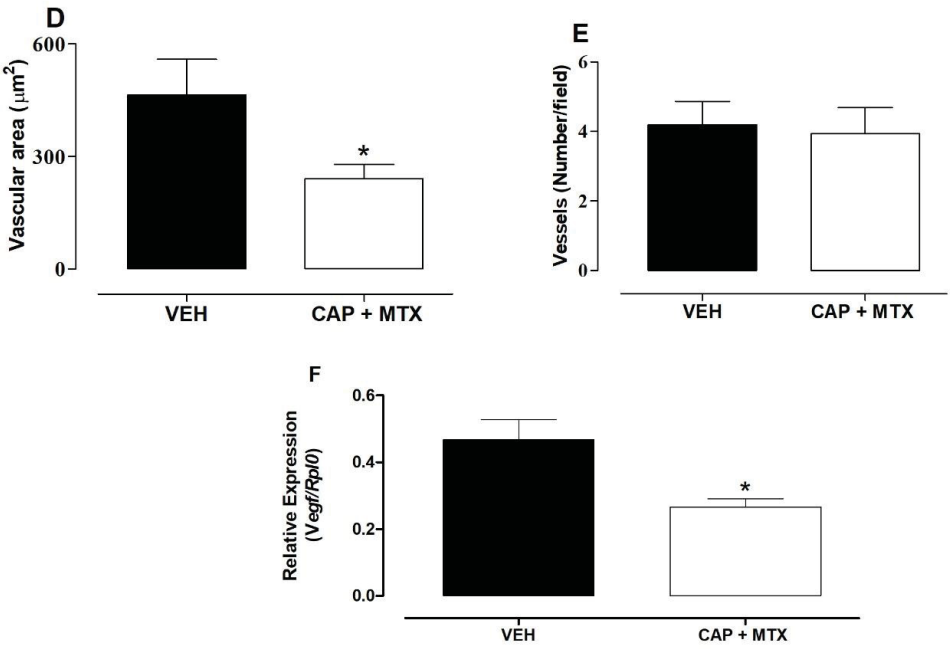


Figure 7. Effect of association treatment in histological parameters of Ehrlich tumor tissue stained by hematoxilin/ eosin, tumor vessels morphometry and *Vegf* relative expression. Animals were orally treated with vehicle and 100 mg kg^{-1} CAP associated with MTX 1 mg kg^{-1} twice

a week during 21 days. Table A: quantification of histological alterations. Scores: (-) negative; (+) mild; (++) moderate; (+++) intense. Panel B: tumor tissue of vehicle. Panel C: tumor tissue of CAP + MTX group Panel D: tumor vascular area. Panel E: number of tumor vessels. Panel F: tumor *Vegf* expression. In B and C, * indicates degree of necrosis. The results in D and E are expressed as mean ± S.E.M. (n=15 images/group). The statistical analyses were performed using Student's *t*-test. * $p < 0.05$, compared with VEH group. Slices of tumor were observed in optical microscope at 20 x (scale bar 20 μm).

3.2.4 CAP + MTX slightly changed plasmatic and hematologic parameters

MTX alone increased the global leukocytes and monocytes, and reduced plasmatic ALT. The association treatment also reduced leukocytes, compared to vehicle group. The reduction of hemoblogin and hemotocrit levels with associated protocol indicated an alteration induced by the tumor, compared with naïve group. Besides the plasmatic AST and ALT levels were reduced compared with vehicle group, most of the hematologic parameters did not change with CAP + MTX treatment (Table 1).

3.3 CAP + MTX reduced the viability of mammary human tumor cells *in vitro*

In order to evaluate if CAP could improve the antineoplastic effect of MTX also upon human mammary tumor cells, the MDA-MB-231 and MDA-MB-436 lineages were cultured with CAP (24 h) followed by the MTX (48 h). The association reduced the viability of both cell lineages in comparison with cells incubated only with MTX (Figure 8A-B). However, MDA-MB-436 cells were more sensitive, since at the intermediated concentration of the drugs combination (0.025 mg mL^{-1} CAP + 0.4 mM MTX) the cell viability was reduced to ~20% (Figure 8B).

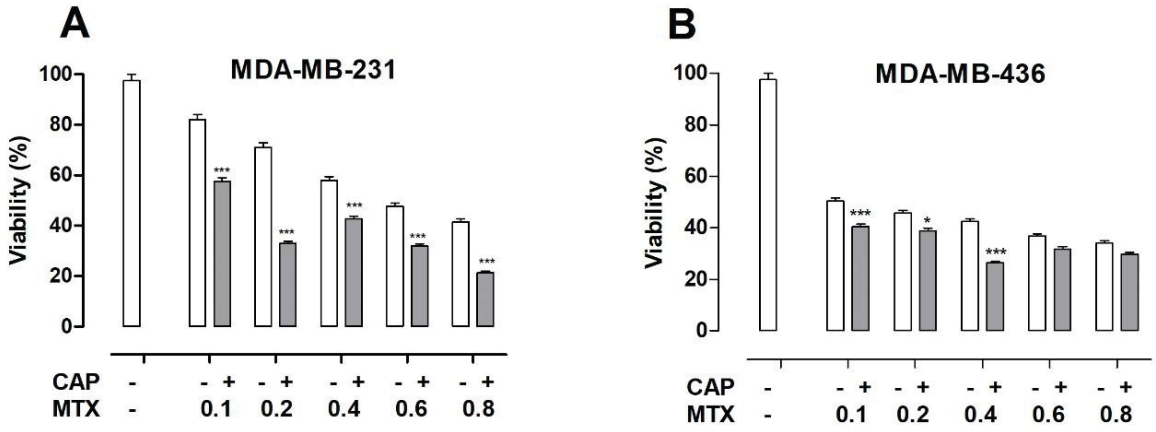


Figure 8. Effect of CAP + MTX in human mammary tumor cells. Viability of MDA-MB-231 cells (Panel A) and MDA-MB-436 cells (Panel B) after treatment with CAP (0.025 mg mL^{-1}) for 24 h and MTX in different concentrations (0.1 - 0.8 mM) for 48 h. The results are expressed as mean ± S.E.M. The data were analyzed using two-way ANOVA followed by Bonferroni's post hoc test. * $p < 0.05$, *** $p < 0.001$, compared with MTX treatment in the same condition.

4. Discussion

The present results demonstrated the antineoplastic effects of pectic polysaccharides from sweet green pepper (*Capsicum annuum*, CAP) in long treatment and associated with methotrexate. We succeed in demonstrate the effect of CAP for reducing the tumor growth without inducing toxicity in a prolonged administration (31 days), as well as the synergic cytotoxic effect of CAP and MTX *in vivo* and *in vitro*. The synergic effect was consistently showed in different mammary tumor cells, from human (MDA-MB-231 and MDA-MB-436 lineages) and murine (Ehrlich lineage) origin. The present results corroborate those previously published by our group showing the inhibitory effects of CAP in these lineages and MCF-7 cells (Adami et al., 2018).

Following previous indication that CAP modulates inflammation and angiogenesis in tumor cells (Adami et al., 2018), these pathways were herein investigated. LT along 31 days with CAP induced more immuno-regulatory alterations than that previously observed with 21 days treatment (Adami et al., 2018). LT with CAP increased the tumor levels of IL-6, consistently with prior data (Adami et al., 2018), but also reduced the levels of anti-inflammatory cytokines IL-10 and IL-4. Our data partially agree with those of Ghoneum and Agrawal (2011), who found an increase in the production of IL-6 and IL-10 by dendritic cells in response to polysaccharide from arabinoxylan rice bran. These results are contrary those of Wang and colleagues (2014), whose studies revealed that polysaccharides from the roots of *Salvia miltiorrhiza* elevated the serum levels of IL-4 and IL-10 and inhibited serum IL-6 levels in gastric tumor-bearing rats. An important difference among our study in comparison with from Wang et al. (2014) is the material used for cytokines measurement. We measured cytokines in tumor tissue, while these authors measured in serum. The function of inflammatory mediators in each compartment can be distinct. For instance, elevated plasma levels of IL-6 have been related to invasiveness and cancer poor prognosis, whereas intramural high IL-6 expression can trigger tumor cell death (Dethlefsen et al., 2013; Fisher et al., 2014) and was associated with good prognostic of breast cancer, i.e. lower tumor size (Ahmad et al., 2018). According to Ahmad et al. (2018), the relation of IL-10 and IL-6 in breast cancer, altering cancer cell motility and metastatic ability, still requires further investigation. In our study the CAP treatment, isolated or associated with MTX, elevated the tumor levels of IL-6, but reduced the levels of IL-4. This result is interesting since increased expression of IL-4 and IL-4 receptor (IL-4Rα) has been reported in several cancer types, including in breast, ovarian, colon, lung, and thyroid (Kim et al., 2016; Venmar et al., 2014; Koller et al., 2010; Todaro et al., 2008).

IL-4 also promotes tumor growth (Li, Chen and Qin, 2009) and metastasis (Ruffell, Affara and Coussens, 2012). The inflammation, when induced locally, may lead to the elimination of a local tumor, but this response appears to be detrimental in patients with cancer (Oft, 2014). In this context, our results clearly point CAP as a modulator of inflammation in Ehrlich tumor microenvironment, what certainly contributes to its antitumor actions.

Interestingly, CAP + MTX, but not CAP isolated, increased TNF-α level in tumor. This effect can correlate with the high degree of necrosis observed in the tumor tissue from CAP + MTX group. TNF-α is known as an inductor of necroptosis (Liu et al., 2016), which histologically is observed as necrosis. Necroptosis has been considered as a therapeutic strategy against cancer, mainly for tumor cells that are resistant to apoptosis (Su et al., 2016). It was already reported that polysaccharides extracted from cabernet franc wine induced TNF-α production and, consequentely, activated the necroptosis pathway in tumor cells (Stipp et al., 2017). This death pathway is a possible action deflagrated by the combined treatment of CAP + MTX in Ehrlich cells, contributing for the smallest tumor development (95% inhibition) observed in this group (Figure 5A).

Inflammation and angiogenesis are closed regulated in tumor tissues. The presence of cytokines might influence, positively or negatively, the tumor progression and angiogenesis. The progression of tumors in chronically inflamed tissue is a consequence of increased cell proliferation signaling and tumor-promoting microenvironment with increase in angiogenesis (Oft, 2014). Herein, CAP regulated the inflammation in tumor tissue and also down-regulated the angiogenesis. Both protocols of treatment with CAP, in LT or in association with MTX, down-regulated the expression of *Vegf* and reduced the vascular area of tumor tissue. These results are consistent with previous data reporting the anti-angiogenic effect of CAP administered in 21 days-therapy against Ehrlich tumor

(Adami et al., 2018). Angiogenesis is not only necessary for the growth of cancer, but also for tumor metastasis (Yao et al., 2018; Grothey and Galanis, 2009). Anti-angiogenesis appears to be a validated effective therapeutic target for the design of anti-breast cancer agents (Wu et al., 2017). Bevacizumab, a humanized monoclonal antibody against VEGF, has been tested in several clinical trials in patients with breast cancer (Ex. NCT00369655 and NCT00109239). Thus, CAP could be a candidate to adjuvant therapy of breast cancer in women, since it reduced the VEGF expression, the angiogenesis and sensitized mammary tumor cells to MTX effects, suggesting an adjuvant or chemopreventive effect. Natural polysaccharides are recognized as adjuvant drugs during chemotherapy and radiotherapy, allowing reduction in doses of cytotoxic drugs and radiation (Pang et al., 2018). It was reported that the fucoida, a sulfated polysaccharide present in brown seaweed, is a suitable chemopreventive candidate agent, tested in HT-29 colon cancer cells (Kim and Nam, 2018); while polysaccharide extracted from the roots of *Polygala tenuifolia* can be a powerful chemopreventive agent for the patients with ovarian cancer, especially at advanced stage (Yao et al., 2018).

In this context, two important aspects may be stressed in the present study: *(a)* CAP combined with lower dose of MTX (1 mg kg^{-1}) inhibited *in vivo* tumor growth more than MTX alone in higher dose (2.5 mg kg^{-1}); and *(b)* CAP inhibited the development of three mammary tumor lineages, specifically Ehrlich cells in mice, human MDA-MB-231 cells, and human MDA-MB-436 cells. Both human mammary tumor cells lineages were sensitive to CAP + MTX, mainly MDA-MB-436, an invasive ductal carcinoma that represents a triple-negative breast cancer. These results suggest that CAP functions as a natural adjuvant for immunoregulation in cancer and can be used in strategies against mammary cancer. Current approved polysaccharide products such as from *Lentinan* (LNT), *Achyranthes*

bidentata, *Astragalus*, *Polyporus* and *Coriolus versciclor* (He et al., 2011), and β-glucans (Vetvicka et al., 2019) have been recognized as immune adjuvants for treatment of human malignant tumor (He et al., 2011). Currently, there are 177 clinical trials registered (NIH – USA) with β-glucans, mostly in cancer, combining this polysaccharide with monoclonal antibodies (Vetvicka et al., 2019). Among those products, LNT is the most popular for combined therapy due to its effectiveness of immunoregulation and its low economic cost (Pang et al., 2018). Oka et al. (1992) reported that intraabdominal or intrapleural injection of 4 mg LTN reduced malignant ascites and pleural effusion in patients with gastric carcinoma without observed adverse reaction. Guo et al. (2012) reported that *Astragalus* polysaccharide improved quality of life and decreased adverse effects of vinorelbine and cisplatin. In this line, CAP has potential to be a new source of commercial product, since it demonstrates effectiveness and safety, alone and in combination with MTX. However, two points that need more investigation is the reduction of WBC observed after the CAP + MTX treatment (Table 1), and the effects of CAP in combination with other antineoplastic drugs, both features not covered by the present study.

In summary, polysaccharides from sweet green pepper, isolated or in association with low dose of methotrexate, can be a promise cancer therapy, with effectiveness and low incidence of side effects. The long treatment with CAP showed its potential to be used as adjuvant of chemotherapy against breast tumors, because CAP sensitized mammary tumor cells to MTX effects. In both protocols of CAP administration the antineoplastic mechanisms appear to depend of the inflammation and angiogenesis modulation.

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8 CONSIDERAÇÕES FINAIS

Este estudo demostrou o efeito antineoplásico de polissacarídeos pécticos extraídos de pimentão verde contra células tumorais que se originam da glândula mamária, *in vivo* e *in vitro*. Nos três protocolos testados em camundongos o CAP foi capaz de reduzir o desenvolvimento tumoral, apresentou aumento de IL-6, e diminuição de *Vegf* e da área dos vasos, apontando para um mecanismo antineoplásico dependente da regulação inflamatória e da angiogênese. Interessantemente, o envolvimento na angiogênese não está relacionado ao número total de vasos tumorais, e sim ao tamanho reduzido dos vasos. Este efeito pode ser decorrente também da elevação da IL-6, que já foi demonstrada como indutora da formação de vasos defeituosos. Os dados *in vitro* com as linhagens tumorais mamárias humanas, especialmente MCF-7 e MDA-MB-436, também evidenciam redução de VEGF após adição de CAP ao cultivo celular, reforçando a regulação da angiogênese desencadeada por estes polissacarídeos.

A regulação da inflamação pelo tratamento *in vivo* com CAP foi claramente demonstrada, embora nem todos os parâmetros avaliados estivessem alterados nos três protocolos estudados. A regulação deste processo parece ser tempo-dependente, pois mais alterações foram observadas em parâmetros inflamatórios com o tratamento mais prolongado (LT, 31 dias), em comparação ao tratamento convencional (21 dias) com CAP. Por exemplo, a atividade de MPO e os níveis de IL-10 e IL-4 no tumor não se alteraram com 21 dias de tratamento, mas sim com 31 dias (LT). Ainda, apenas no protocolo de tratamento combinado (CAP+MTX) o nível de TNF-α no tecido tumoral foi elevado. Neste aspecto, uma limitação do presente estudo é que os biomarcadores de inflamação foram mensurados apenas no tumor, e não no plasma, então não se pode inferir que a regulação destes processo está ocorrendo também sistemicamente. Estudos com diferentes tempos de tratamento são necessários para avaliar a regulação da inflamação e as consequências disto para o organismo como um todo.

Os eventos regulatórios do desenvolvimento celular que foram alterados pelo CAP, induziram necrose no tumor, mas não apoptose, conforme observado na histologia. A ausência de apoptose também foi comprovada pela expressão inalterada de genes

associados com esta via de morte celular, particularmente *Bcl-2*, *Bax* e *Caspase-8*, após o tratamento convencional com CAP *(a)*. Diante destes resultados, tais genes não foram mensurados nos demais protocolos *in vivo* [*(b)* LT e *(c)* Associação CAP + MTX] e *in vitro*.

A ação sinérgica do CAP com MTX, demonstrada *in vivo* e *in vitro*, abre perspectivas para avaliar sua ação em combinação com outros quimioterápicos indicados para a terapia de tumores mamários, como paclitaxel, cisplatina e carboplatina. Esta possibilidade pode reduzir a dose dos quimioterápicos, sabidamente citotóxicos e indutores de muitos efeitos adversos. O CAP, por outro lado, induziu apenas redução de leucócitos no protocolo de associação com MTX, e não alterou os demais parâmetros bioquímicos e hematológicos, indicando baixa toxicidade. No entanto, outros estudos bioquímicos e toxicológicos precisam ser conduzidos, para avaliar, por exemplo, a dose letal (DL_{50}) do CAP, que não é conhecida.

Em conclusão, o CAP se apresenta promissor como terapia adjuvante no tratamento de tumores sólidos, especialmente de tumores mamários. Estudos em diferentes modelos e linhagens celulares, abordando outras posologias e parâmetros farmacocinéticos, farmacodinâmicos e toxicológicos, ainda são necessários.

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