CÁSSIA CALIXTO DE CAMPOS

# EFEITO DE POLIFENÓIS EM MODELOS EXPERIMENTAIS DE DOR

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Dissertação de Doutorado apresentada ao Departamento de Ciências Patológicas da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de DOUTOR em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior

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Dedico este trabalho aos meus pais
que sempre me deram suporte e força
para esta conquista!

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## RESUMO

O processo inflamatório e seus sinais clínicos como a dor são essenciais para a sobrevivência do homem atuando como um mecanismo de alerta e defesa, porém sua exacerbação pode ser prejudicial e tem sido um biomarcador de doenças, inclusive o câncer. Os tratamentos para o processo inflamatório apresentam diversos efeitos adversos. Diante deste fato, os compostos fenólicos, metabólitos secundários de plantas, têm sido foco de estudos por apresentar vários efeitos biológicos. Assim, neste trabalho foi investigado o papel dos polifenóis: ácido vanílico, quercetina e naringenina em diferentes modelos experimentais de dor. Em todos os trabalhos foram utilizados camundongos Swiss machos ou fêmeas. No primeiro trabalho, foi avaliado o efeito do tratamento agudo com o ácido vanílico em diferentes modelos de dor inflamatória induzidas por ácido acético, PBQ, formalina, CFA e carragenina. O efeito crônico do tratamento também foi avaliado no modelo do CFA crônico. Os tratamentos agudo e crônico foram capazes de reduzir os parâmetros comportamentais (contorções, tempo de lambida, *flinches*, hiperalgesia mecânica e edema). Os efeitos do ácido vanílico foram avaliados na pata e se apresentaram, anti-inflamátorio: por reduzir o recrutamento de neutrófilos e macrófagos, reduzir a produção de citocinas (IL-1β, TNFα e IL-33) e inibir a ativação do NFκB; e antioxidante por reduzir o estresse oxidativo (métodos de FRAP, ABTS, GSH e TBARS). O tratamento não apresentou efeitos gastro ou hepatotóxicos. No segundo trabalho foi avaliado o efeito da quercetina no modelo de dor no câncer induzida pelas células tumorais de Ehrlich. Camundongos receberam as células tumorais ($1x10^6$ ou $1x10^7$células/pata) e foram tratados agudo ou cronicamente. O tratamento foi capaz de reduzir os parâmetros comportamentais induzidos pelo tumor (hiperalgesia mecânica, térmica e dor espontânea). Os efeitos do tratamento foram avaliados na pata e na medula espinal. A quercetina se apresentou anti-inflamatória por reduzir o recrutamento de neutrófilos e produção de citocinas (IL-1β e TNFα), antioxidante (FRAP, ABTS e GSH) e analgésica por ativar mecanismos opióides dependentes, os quais potencializam o efeito da quercetina em menores doses. Não houve alteração no crescimento tumoral, nem alterações histológicas na pata dos animais tratados. No terceiro trabalho, foi avaliado o efeito da naringenina no modelo de dor no câncer. Camundongos receberam o tumor e foram tratados com a naringenina agudo ou cronicamente (1x ou 2x ao dia). Os tratamentos reduziram os parâmetros comportamentais de hiperalgesia mecânica, térmica e dor espontânea. A espessura da pata foi reduzida pelo tratamento crônico (2x ao dia), contudo seu efeito foi apenas em reduzir o recrutamento de células imunes e não a proliferação tumoral. Os efeitos da naringenina foram avaliados na pata e medula, e se apresentaram anti-inflamatórios por reduzir recrutamento de neutrófilos e macrófagos, e reduzir mRNA para citocinas (*Tnfα* e *pró-Il-1β*), antioxidantes por reduzir o estresse oxidativo (FRAP, ABTS e GSH) e induzir aumento de mRNA do *Nrf2* e *HO-1*, e analgésico por inibir ativação de células da glia. O tratamento não causou gastro ou hepatotoxidade. Concluindo, este trabalho demonstra o efeito anti-inflamatório, antioxidante e analgésico dos polifenóis em modelos de dor inflamatória

e dor no câncer.

**Palavras-chave:** Ácido vanílico. Quercetina. Naringenina. Dor inflamatória e dor no câncer.

CALIXTO-CAMPOS, Cássia. **Effect of polyphenols in experimental models of pain.** 2017, 126 p. Thesis (Doctorate in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2017.

## ABSTRACT

The inflammatory process and its clinical signs as pain are essential for man's survival, but it exacerbation can be harmful and has been considered as biomarker of diseases, including cancer. Treatments for the inflammatory process can presents malefic effects. Phenolic compounds are secondary metabolites of plants and has been focus of many studies for it several beneficial effects. Thus, in this study, were investigated the role of polyphenols compounds: vanillic acid, quercetin and naringenin in different pain models. All experiments were performed using male Swiss mice. In the first study, were evaluated the effect of pre-treatment with vanillic acid in different inflammatory pain-models induced by acetic acid, PBQ, formalin, CFA and carrageenan, and in post-treatment of CFA chronic model-pain. The pre and post-treatment reduced the pain behaviors (contortions, licking time, flinches, mechanical hyperalgesia and paw edema). The anti-inflammatory effect were involved in reducing the cytokines production (IL-1β, TNFα and IL-33) and inhibit the NFκB activation, and antioxidant for reducing the oxidative stress (FRAP, ABTS, GSH and TBARS methods). The treatment did not present gastro or liver damage. In the second study, were evaluated the effect of quercetin in cancer pain model induced by Ehrlich tumor cells. Mice received tumor cells ($1x10^6$ or $1x10^7$cells/paw) and were treated with quercetin in acute or chronic model. Treatment reduced the mechanical and thermal hyperalgesia, and pain-like behavior. The effects were evaluated on paw skin and spinal cord. Quercetin presents anti-inflammatory effect for reducing the neutrophil recruitment and cytokine production (IL-1β and TNFα), antioxidant (FRAP, ABTS and GSH) and analgesic for activate opioid mechanisms dependent, which potentiate the effect of quercetin in smaller doses. There was no inhibit the tumor growth or histological changes by treatment. In third study, we evaluated the effect of naringenin in cancer pain model. Mice received tumor and were treated for acute or chronic (1x or 2x per day) models. The treatment reduced the parameters of mechanical and thermal hyperalgesia, and pain-like behavior. Paw thickness was reduced only twice daily treatment, however its effect on reducing the immune cells recruitment and not the tumor cells proliferation. The effects of naringenin were evaluated in the paw skin and spinal cord. Anti-inflammatory effect were observed for reduced the neutrophil and macrophages recruitment and reduce the mRNA expression for cytokines (*Tnfα* and *pro-Il-1β*); antioxidants (FRAP, ABTS and GSH) and for increase the antioxidants factor *Nrf2* and *HO-1*; and analgesic for inhibit glia cells activation. The treatment not presents toxic effect. In conclusion, this study presents the anti-inflammatory, antioxidant and analgesic effect of polyphenols in models of inflammatory and cancer pain.

**Key words:** Vanillic acid. Quercetin. Naringenin. Inflammatory pain. Cancer pain.

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| ABTS | Ácido 2,2-azino-bis-(3-etilbenzotiazolina)-6-sulfônico |
| AC | Adenilato ciclase |
| AMPc | Adenosina monofosfato cíclico |
| ARE | Gene de elementos de respostas antioxidantes |
| ASIC3 | Canais iônicos ácidos-sensíveis-3 |
| AP-1 | Fator de transcrição ativador de proteína-1 |
| ATF-2 | Fator ativador de transcrição-2 |
| ATP | Adenosina trifosfato |
| B1/B2 | Receptor da bradicinina |
| BK | Bradicinina |
| Ca+ | Cálcio |
| CFA | Adjuvante completo de Freund |
| Cg | Carragenina |
| COX-2 | Cicloxigenase-2 |
| DAMPs | Padrões moleculares associados ao dano |
| DNA | Ácido desoxirribonucleico |
| DAG | Diacilglicerol |
| EO | Estresse oxidativo |
| EROs | Espécies reativas de oxigênio |
| ERNs | Espécies reativas de nitrogênio |
| ET-1 | Endotelina-1 |
| $ET_AR$ | Receptor da ET-1 |
| EP | Receptor de prostaglandina |
| FRAP | Poder antioxidante redutor do Ferro |
| GRD | Gânglio da raiz dorsal |
| GSH | Glutationa reduzida |
| GST | Glutationa S transferase |
| GMPc | Monofosfato cíclico de guanosina |
| HO-1 | Heme oxigenase-1 |
| IASP | Associação Internacional do Estudo da Dor |
| IL | Interleucina |

| | | |
|---|---|---|
| 1 | IL-RI | Receptor da IL-1 tipo 1 |
| 2 | IL-RII | Receptor da IL-1 tipo 2 |
| 3 | INCA | Instituto Nacional do Câncer |
| 4 | iNOS | Óxido nítrico sintase induzida |
| 5 | IP3 | Trifosfato inositol |
| 6 | Keap1 | *Kelch-like* associado a proteína1 |
| 7 | KV | Canais potássio voltagem dependente |
| 8 | LPS | Lipopolissacarídeo |
| 9 | MAP quinases | Proteínas quinases ativadas por mitógeno |
| 10 | MPO | Mieloperoxidase |
| 11 | mRNA | RNA mensageiro |
| 12 | NADPH | Fosfato de dinucleotídeo de nicotinamida e adenina |
| 13 | NAV | Canais de Sódio |
| 14 | NAG | N-acetylglucosaminidase |
| 15 | NFκB | Fator nuclear Kappa-B |
| 16 | NGF | Fator de crescimento de nervo |
| 17 | Nrf2 | Fator nuclear eritróide 2 |
| 18 | NO | Óxido nítrico |
| 19 | PAMPs | Padrões moleculares associados a patógenos |
| 20 | PBQ | Fenil-p-benzoquinona |
| 21 | PG | Prostaglandina |
| 22 | PGE2 | Prostaglandina E2 |
| 23 | PIP2 | Difosfato inositol |
| 24 | PKA | Proteína quinase A |
| 25 | PKC | Proteína quinase C |
| 26 | PKG | Proteína quinase G |
| 27 | $P_2X_3$ | Receptor purinérgico |
| 28 | RNA | Ácido ribonucleico |
| 29 | STAT3 | Transdutor de sinais e ativador de transcrição-3 |
| 30 | SNC | Sistema nervoso central |
| 31 | TBARS | Substâncias reativas ao ácido tiobarbitúrico |
| 32 | TLR | Receptores Toll-Like |
| 33 | TH1 | Resposta imune T helper 1 |
| 34 | TH2 | Resposta imune T helper 2 |

| | |
|---|---|
| TNF-α | Fator de necrose tumoral-α |
| TNFR | Receptor do TNF |
| TRP | Receptor potencial transiente |
| TRPV | Receptor potencial transiente vanilóide |
| TRPA | Receptor potencial transiente *ankyrin* |
| UV | Radiação ultravioleta |

**SUMÁRIO**

# 1 INTRODUÇÃO

A dor é uma experiência sensorial muito importante para a sobrevivência humana, pois normalmente está associada a um dano real ou em potencial, podendo ser de origem fisiológica ou patológica. A dor fisiológica induz uma resposta protetora ao nosso organismo, com o intuito de evitar uma lesão ou uma injúria tecidual, já a dor patológica, normalmente está associada a um evento prejudicial, desencadeado a partir do reconhecimento de um agente nocivo, endógeno ou exógeno, pelo organismo e pela tentativa de combatê-lo.

O reconhecimento do agente nocivo permite modificações vasculares e celulares, que levam ao desenvolvimento e instalação do processo inflamatório. O processo inflamatório é reconhecido pelo aparecimento dos seus sinais cardinais: calor, rubor, tumor e dor. Sendo o mecanismo de defesa mais importante do nosso organismo. Contudo, sua exacerbação é maléfica, pois gera danos em tecidos saudáveis que acarretam prejuízo ao indivíduo. A exacerbação do processo inflamatório normalmente está associada a persistência do agente nocivo ou a respostas inflamatórias inadequadas, como no caso das doenças auto-imunes ou do desenvolvimento do câncer.

Os anti-inflamatórios (esteroidais e/ou não esteroidas), bem como os analgésicos, são amplamente utilizados, sendo estes medicamentos os mais procurados nas farmácias e drogarias. Entretanto, sua utilização descompensada pode ser maléfica, pois estes apresentam muitos efeitos adversos que podem gerar prejuízos à saúde do indivíduo, como úlceras gástricas, hemorragias, problemas renais, cardíacos, ente outros.

Desta maneira, muitos estudos têm focado atualmente na pesquisa de tratamentos naturais para o combate do processo inflamatório. Estes estudos tem demonstrado o efeito biológico benéfico de um classe de compostos em especial, os polifenóis. Estes são reconhecidos por apresentarem baixos efeitos tóxicos e adversos, e serem de fácil acesso por estarem presentes nos alimentos, frutas e vegetais utilizados no nosso cotidiano. Muitos estudos têm demonstrado que a estrutura química dos polifenóis é responsável por seus efeitos no combate e até na prevenção de várias doenças. Assim, esta tese apresenta resultados de estudos *in vivo,* que demonstraram o efeito dos polifenóis: ácido vanílico, quercetina e naringenina em modelos de dor inflamatória e dor no câncer.

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 Inflamação

A inflamação é uma reação complexa que envolve respostas vasculares e celulares desencadeadas por agentes exógenos e endógenos nocivos, na tentativa de destruir, diluir e isolar tais agentes. Seu objetivo é de limitar uma infecção, eliminar o agente nocivo, evitar danos celulares, eliminar células necróticas, limpar o local da resposta inflamatória e iniciar o reparo tecidual (KUMAR et al., 2008).

O processo inflamatório é uma resposta crucial para sobrevivência do homem, pois quase sempre está associado a um evento prejudicial (LAWRENCE; WILLOUGHBY; GILROY, 2002). No entando, quando não há resolução do processo inflamatório, quando a inflamação é muito intensa ou quando a inflamação é inadequada ao organismo, como nas doenças autoimunes, ocorre sua cronificação. A cronificação do processo inflamatório leva a uma resposta exacerbada responsável por muitos prejuízos ao indivíduo (ABBAS; LICHTMAN; PILLAI, 2011).

Desde a antiguidade, o processo inflamatório pode ser definido pelo aparecimento dos sinais cardinais: calor, rubor, tumor, dor e perda da função (sinal recentemente apresentado) (BRASILEIRO, 2011). Para que o processo inflamatório seja instalado é necessário que ocorram as modificações vasculares e celulares, as quais são responsáveis pelo aparecimento dos clássicos sinais cardinais. O calor e o rubor, por exemplo, ocorrem pelas alterações vasculares e o edema pelas alterações celulares e vasculares (KUMAR et al., 2008). Já a dor, ocorre pelas alterações celulares e pelos mediadores liberados destas células, como prostaglandinas, neuropeptídios e citocinas que são capazes de ativar as fibras nociceptivas (PINHO-RIBEIRO; VERRI; CHIU, 2017; VERRI et al., 2006).

O processo inflamatório tem início a partir do reconhecimento dos agentes injuriantes pelas células residentes do tecido periférico, este reconhecimento ocorre via padrões moleculares associados aos patógenos (PAMPs) ou padrões moleculares associados aos danos (DAMPs) (ABBAS; LICHTMAN; PILLAI, 2011). Os principais agentes exógenos responsáveis por induzir o processo

inflamatório são os microrganismos, como bactérias, vírus, fungos e parasitas (KUMAR et al., 2008).

O reconhecimento dos PAMPs presentes nos agentes infecciosos exógenos, por exemplo, ocorrem por uma série de receptores relacionados a resposta imune inata, os receptores do tipo toll-like receptor (TLR). Aproximadamente 10 famílias de receptores do tipo TLR foram identificadas em humanos e pelo menos 13 em camundongos. Todos os receptores possuem como estrutura básica um domínio amino terminal, caracterizado por ricas repetições de leucina e um domínio TIR carboxi-terminal, qual interage com moléculas adaptadoras intracelularmente. Cada tipo de receptor TLR reconhece padrões específicos dos patógenos (LIM; STAUDT, 2013).

Após a ligação do PAMP ao TLR ocorre ativação do domínio TIR citoplasmático, que recruta moléculas adaptadoras como MyD88, TIRAP, TRAM e TRIF. As moléculas adaptadoras geram respostas intracelulares diferentes, ou seja dependendo da molécula adaptadora recrutada, ocorre a ativação de quinases e fatores de transcrições diferentes (LIM; STAUDT, 2013).

A desregulação dos TLRs tem sido associada a algumas doenças, bem como a deficiência nas moléculas adaptadores tem sido relacionada com as infecções recorrentes (CASANOVA; ABEL; QUINTANA-MURCI, 2011), demonstrando que esta resposta é extremamente importante para a saúde do indivíduo. Contudo, a manipulação dos TLRs (ZHAO; LEE; HWANG, 2011), a inibição das vias de sinalização (LEE et al., 2000) e inibição da ativação de fatores de transcrição (HAYDEN; GHOSH, 2004) também tem sido associada a possíveis terapias para o controle do processo inflamatório quando exacerbado.

Um dos principais fatores de transcrição ativado pela via do receptor TLR é o fator de transcrição nuclear kappa-B (NFkB). O NFkB tem um importante papel durante a reposta imune inata e ganhou muita atenção nos recentes estudos (KIM et al., 2011; NENNIG; SCHANK, 2017; PINHO-RIBEIRO et al., 2016), principalmente por estimular a produção de mediadores inflamatórios como citocinas que propiciam a exacerbação da inflamação (KHANSARI; SHAKIBA; MAHMOUDI, 2009). Em condições basais o NFkB no citosol da célula está bloqueado por uma proteína inibidora conhecida como inibidor do NFkB, o IkB. O IkB quinase (IKK) é um complexo que consiste de duas subunidades catalíticas (IKKα e IKKβ) e uma subunidade reguladora (NEMO). Quando ativado, o IKK fosforila o IkB

(GERONSAKIS et al., 2014) e leva a degradação proteossômica e liberação do dímero NFkB. O NFkB ativado desencadeia a expressão dos mediadores inflamatórios, incluindo citocinas pró-inflamatória como o fator de necrose tumoral-alfa (TNFα) e interleucina (IL)-1β, quimiocinas e moléculas de adesão celular. Outro aspecto importante é a existência de um circuito de realimentação positiva do processo inflamatório via NFkB, uma vez que as próprias citocinas inflamatórias como TNFα e IL-1β ao se ligarem em seus receptores (TNFR e IL-1R, respectivamente) estimulam a ativação do fator NFkB, que leva a produção de mais citocinas, amplificando o processo inflamatório (NENNING; SCHANK, 2017).

O aumento dos mediadores químicos estabelecem a inflamação gerando as alterações vasculares e celulares. Essas alterações permitem a chegada de proteínas do plasma e leucócitos ao foco inflamatório (KUMAR et al., 2008). Serotonina, óxido nítrico (NO), histamina e prostaglandina (PG) agem nas células endoteliais promovendo vasodilatação e alteração da permeabilidade vascular, que permitem o extravasamento das proteínas plasmáticas levando ao quadro de exsudato inflamatório local. Em decorrência dos eventos vasculares e a lentificação do fluxo sanguíneo, ocorrem os eventos celulares em que os leucócitos são marginizados no vaso sanguíneo, ativados e migrados para o foco inflamatório pela presença das citocinas TNFα, IL-1β e dos agentes quimiotáticos (KUMAR et al., 2008).

O TNFα é uma citocina pró-inflamatória com peso molecular de 17 kDa, que foi previamente chamada de caquexina e identificada por causar necrose de tumores *in vivo* (CARSWELL et al., 1975; TRACEY et al., 1988). Hoje é reconhecida como uma potente citocina inflamatória, que é rapidamente produzida em grandes quantidades por uma variedade de células, principalmente macrófagos (durante ativação clássica - M1), linfócitos T CD4+, neutrófilos, células *natural killers*, mastócitos, células endoteliais, cardiomiócitos, adipócitos, fibroblastos e neurônios em resposta a estímulos inflamatórios como, por exemplo, infecções bacterianas (MACINTYRE; REID; MCKENZIE, 1995; SWARDFAGER et al., 2010; VERRI et al., 2006; WAJANT et al., 2003).

Além do processo de recrutamento celular (SCHAFERS et al., 2002), esta citocina participa do processo hiperalgésico em modelos inflamatórios (CUNHA et al., 2005; PINHO-RIBEIRO; VERRI; CHIU, 2017; VERRI et al., 2006) e nas doenças inflamatórias como artrite reumatoide (MCLNNES; SCHETT et al., 2011,

VERRI et al. 2010), aterosclerose (KUSTERS et al., 2014), colite (LAWRANCE, 2015), neuropatia (LEUNG; CAHIL, 2010) e no câncer (CONSTANTIN et al., 2008; PATEL et al., 2015). O TNFα é uma das primeiras citocinas liberadas em decorrência de diversos estímulos, como carragenina, ácido acético, adjuvante completo de Freund (CFA) e formalina (segunda fase) (WOOLF et al., 1997; CHICHORRO; LORENZETTI, ZAMPRONIO, 2004; VERRI et al., 2006; CUNHA et al., 2007), e inicia a cascata de ativação de várias outras citocinas e fatores de crescimento. O TNFα também modula a atividade de múltiplos canais de íons, incluindo canais receptor de potencial (TRP) vanilóide tipo 1 (TRPV1), canais de sódio ($Na^{+}$), cálcio ($Ca^{++}$) e potássio ($K^{+}$), agindo diretamente sobre o neurônio nociceptor aumentando sua excitabilidade (PINHO-RIBEIRO; VERRI; CHIU, 2017).

Existem dois tipos de receptores para o TNFα: TNFR1 ou receptor do tipo 1 e o TNFR2, do tipo 2. A principal diferença entre os tipos de receptores esta na atividade biológica desempenhada a partir de sua ativação. O TNFR1 primeiramente foi descrito por estar envolvido ao processo inflamatório, promovendo a migração celular, inflamação, dor e apoptose. Por outro lado o TNFR2 parecia estar envolvido na mediação da morte celular e necrose (VERRI et al., 2006). Contudo, o estudo destes receptores a partir de animais *knockout* demostrou que ambos, TNFR1 e TNFR2, podem participar do processo inflamatório e da dor, embora o TNFR1 desempenhe um papel predominante na mediação da dor inflamatória por mecanismos periféricos e centrais (ZHANG et al., 2011).

A sinalização celular induzida pela ligação do TNFα com TNFR1 leva a uma série de respostas, incluindo ativação do fator de transcrição NFκB, sinalização via proteínas quinases ativadas por mitógeno (MAP Quinases) ou induzindo sinalização de morte celular (apoptose) (GAUR; AGGARWAL, 2003; LEE et al., 2004; TAKADA; AGGARWAL, 2004; WAJANT et al., 2003; ZHOU et al., 2006; ZHOU; CONNELL; MACEWAN, 2007).

Evidências vem demonstrando que a modulação do TNFα pode ser uma importante terapia para a dor. Infliximab (anticorpo quimérico anti-TNFα), etanercept (forma dimérica solúvel do receptor p75 do TNF) e adalimumab (anticorpo monoclonal totalmente humano anti-TNFα) já vem sendo utilizados em tratamentos clínicos para doenças como psoríase e artrite reumatoide (HARAOUI, 2005; MORELAND, 1999; TOBIN; KIRBY, 2005).

A citocina IL-1 também é considera uma citocina pró-inflamatória e hiperalgésica (FERREIRA et al., 1988). Ela foi primeiramente descrita como uma proteína que induz febre, sendo chamada de pirógeno leucocítico humano (DINARELLO; RENFER; WOLFF, 1977). É constituída de 2 proteínas principais, a IL-1α e lL-1β. Até o momento, existem 11 membros da família da IL-1. A IL-1α e IL-1β humanas são compostas de estruturas homotriméricas, com peso molecular de 17 kDa e 16 kDa, respectivamente (DINARELLO, 2009).

A IL-1α e lL-1β exercem efeitos similares pela ligação ao receptor tipo 1 da IL-1 (IL-1RI). Elas também podem se ligar ao receptor tipo 2 (IL-1RII), que age como um receptor *decoy* para a IL-1 e não está envolvido com transdução de sinais. Após sua ligação ao receptor de membrana IL-1RI, ocorre a aproximação intracelular de um co-receptor que permite o recrutamento das moléculas adaptadoras como MyD88, resultando na ativação de vias de sinalização do NFκB e MAP quinases como ERK 1/2, JNK e p38, que induzem a expressão dos genes alvo pró-inflamatórios, como cicloxigensase-2 (COX-2), óxido nítrico-sintetase induzida (iNOS), IκBα, lL-1α, lL-1β e TLR2 (MASSARO et al., 2006; SAKAI et al., 2004; WEBER et al., 2010). Contudo a IL-1β tem tido maior interesse clínico em processos inflamatórios.

Um ponto importante desta citocinas é que ao ser produzida a IL-1β é traduzida primeiramente como pró-IL-1β, não apresentando atividade biológica nesta forma, até que seja clivada pela protease intracelular enzima conversora da IL-1β, também conhecida como caspase-1, que faz parte de um complexo de proteínas intracelulares chamado de *inflammasome* (DINARELLO et al., 2011). Após sua maturação a IL-1β produzida é transportada para o exterior da célula onde age localmente ou entra na corrente sanguínea, podendo agir de forma endócrina (DINARELLO et al., 1994).

São muitas as funções biológicas da IL-1β, podendo agir como mediador inflamatório em modelos pulmonares (RABOLLI et al., 2014), coronarianos (LEE et al., 2012), artrite reumatoide (VERRI et al., 2010) e no câncer (PATEL et al., 2015). Sua administração intraplantar (FERREIRA et al., 1988) e intraperitoneal (WATKINS et al., 1994) também resulta em prolongada dor e inflamação.

Desta maneira, é possível o entendimento de que a modulação da IL-1β também poderia ser um potente alvo para o controle da dor inflamatória (VERRI et al., 2006). De fato, a inibição da IL-1 tem demonstrado esse papel

benéfico em modelos de artrite reumatoide, pericardite, dor muscular e colite (DINARELLO, 2011; BORGHI et al., 2014; BACK; HANSSON, 2015; POUILLON; BOSSUYT; PEYRIN-BIROULET, 2017).

A IL-33 é um citocina da família da IL-1, que fica armazenada na forma de pró-IL33 com 30 kDa em humanos. Estudos iniciais *in vitro* sugeriram que da mesma forma que a IL-1, a IL-33 seria ativada pela quebra a partir da caspase-1 (SCHMITZ et al., 2005).

Citocinas de padrão TH1 tem sido considerada indutora de hipernocicepção, enquanto citocinas TH2 inibem a hipernocicepção (VERRI et al., 2010). Contudo, está citocina foge desta regra, podendo ser considerada uma citocina pleiotrópica, pois mesmo considera TH2 pode induzir mecanismos pró-nociceptivos. São evidenciados altos níveis desta citocinas em modelos experimentais de inflamação e sua inibição apresenta um papel analgésico e anti-inflamatório potente (MAGRO et al., 2013; VERRI et al., 2010; ZARPELON et al., 2013, 2016).

O papel biológico desta citocina desempenhado quando ocorre sua ligação no receptor ST2. Este receptor pode se apresentar como um receptor ancorado a membrana (ST2L), ou sua variante solúvel sST2 (SCHMITZ et al., 2005). A sinalização IL-33/ST2 é regulada por vários mecanismos, um dos mais estudados envolve a capacidade do sST2 solúvel atuar como um receptor *decoy*, sequestrando a IL-33 e bloqueando a resposta pró-inflamatória induzida por esta citocina *in vitro* e *in vivo.* A ativação do receptor ST2L que leva a ativação de vias de sinalização pelo recrutamento de MyD88, quinase associada a IL-RI 4 (IRAK4) e TRAF6 (HAYAKAWA et al., 2007).

Desta maneira, podemos considerar que as citocinas tem demonstrado importante papel para o desenvolvimento da inflamação (de OLIVEIRA et al., 2011; VERRI et al., 2006) e a redução dos níveis das citocinas estão relacionado com a redução da hiperalgesia induzida por estímulo inflamatório como carragenina (BORGHI et al., 2013; ZARPELON et al., 2013), redução da dor neuropática em modelo de constrição de nervo ciático (LONGHI-BALBINOT et al., 2016), redução da inflamação na artrite reumatoide (VERRI et al., 2010), no lúpus eritematoso sistêmico (CLARK et al., 2013) e na psoríase (COIMBRA et al., 2012).

Além das citocinas, o estresse oxidativo (EO) desempenha um importante papel no processo inflamatório. O estresse é gerado a partir da produção

de componentes tóxicos conhecidos como espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) formados durante o processo de fagocitose por neutrófilos e macrófagos em busca de eliminar o agente patogênico (mieloperoxidase, MPO), também podem ser gerados durante o processo fisiológico de respiração celular nas mitocôndrias (cadeia respiratória), pela xantina oxidases, NADPH oxidases (NOX) e a radiação (BABIOR, 2004). Dentre estes mecanismos, o mais importante é a ativação da enzima nicotinamida adenina dinucleótideo fosfato (NADPH) oxidase durante o processo da fagocitose, que catalisa a transformação do oxigênio em ânion superóxido (BABIOR, 2004), esta ERO pode levar a produção de outras EROs e ERNs, como radical hidroxil, peróxido de hidrogênio e peroxinitrito, que destroem os microrganismos ainda no fagolisossomo (ESPINOSA-DIEZ et al., 2015).

Existe em contrapartida sistemas antioxidantes enzimáticos e não enzimáticos dentro das células, como a superóxido dismutase, glutationa redutase, catalase, glutationa reduzida (GSH), bilirrubina e ácido úrico tentam manter o balanço entre a produção de EROs e as defesas antioxidantes, enquanto houver um balanço entre ambos o organismo combate a instalação do EO (ESPINOSA-DIEZ et al., 2015; LIMÓN-PACHECO; GONSEBATT, 2009).

Contudo, as EROs quando produzidas em grande quantidades ou quando as defesas antioxidantes não forem suficientes para cambater o excesso de EROs, há a instalação do EO. O EO pode apresentar grande potencial lesivo ao organismo, mediando lesões celulares e teciduais, intensificando a resposta inflamatória e seus sintomas clínicos, como a dor (MAIOLI et al., 2015; SALVEMINI et al., 2011).

A intensificação da dor ocorre pelo fato de que o ânion superóxido e o peroxinitrito ativam fatores de transcrição como a proteína ativadora 1 1 (AP-1), NFκB, além de MAP quinases, as quais culminam na indução da expressão de COX-2, aumentando assim a produção de prostanóides, o que promove a sensibilização dos nociceptores (LITTLE; DOYLE; SALVEMINI, 2012).

Além disto, o aumento da resposta inflamatória ocorre devido a produção amplificada de outros mediadores inflamatórios, como as citocinas TNFα, IL-33 e endotelina-1 (ET-1). O TNFα por sua vez ativa a NADPH oxidase e aumentam a produção das EROs (KILPATRICK et al., 2010), como o ânion superóxido. Em resposta, o ânion superóxido ativa o fator de trancrição NFκB que induz a produção de mais citocinas e mais mediadores pró-inflamatórios,

retroalimentando positivamente esta via e amplificando o processo inflamatório (WANG et al., 2006; WANG; DASHWOOD, 2011, YAMACITA-BORIN et al., 2015). O ânion também pode induzir a montagem do inflamassoma necessário para a ativação da capase-1 que leva a clivagem e da ativação da citocina pró IL-1β em IL-1β (VERRI et al., 2012)

Ademais, a produção de espécies reativas de oxigênio pelo complexo da NADPH influencia a migração dos polimorfonucleares, auxiliando-os no direcionamento ao sítio da inflamação durante a quimiotaxia (HATTORI et al., 2010).

As moléculas antioxidantes são capazes de reagir com as EROs doando elétrons para sua estabilização. A Glutationa (GSH), por exemplo é considerada uma das moléculas mais abundante entre os antioxidantes endógenos, sendo capaz de doar um ou dois eletróns para estabilidar as EROs (ESPINOSA-DIEZ et al., 2015). O reconhecimento do estresse oxidativo também pode levar a transcrição de fatores antioxidantes como fator nuclear eritróide 2 (Nrf2). O fator Nrf2 esta associado a uma proteína inibitória a Keap1. Na presença de EO ocorre o desligamento da Keap1 deixando o Nrf2 livre para se translocar ao núcleo e desempenhar sua atividade transcripcional em genes de elementos de respostas antioxidantes (ARE) (ESPINOSA-DIEZ et al., 2015).

O sistema hemeoxigenase (HO) é um sistema enzimpatico também ativado duante o EO e esta envolvido na degradação do grupamento heme. O heme é um complexo ferro-protoporfirina com papel essenciais nos sistemas biológicos. É um grupo essencial de enzimas com funções como armazenamento e transporte de oxigênio (hemoglobina e mioglobina), transporte de elétrons e geração de energia (NADPH oxidase, guanilil ciclase e família do citocromo P450); e ativação de sistemas enzimáticos como catalase, peroxidase, óxido nítrico sintase (NOS) e ciclooxigenase (KUMAR, 2005). Foi demonstrado em condições patológicas que o excesso de heme livre apresenta efeitos prejudiciais, como danos celulares e teciduais, e geração de EROS (ARRUDA et al., 2004; TRACZ; ALAM NATH 2007; MUNÕZ-SÁNCHEZ; CHÁNEZ-CÁRDENAS 2014).

O sisteme HO regula os níveis de hemeproteína e protege as células dos efeitos deletérios do heme livre, catalizando a conversão de heme á pigmentos biliares (biliverdina), liberação de ferro ferroso livre ($Fe2^{+}$), monóxido de carbono (CO), sendo que os produtos gerados a partir da catalização apresentam importante papel fisiológico (RYTER, ALAM, CHOI, 2006; MUNÕZ-SÁNCHEZ; CHÁNEZ-

CÁRDENAS 2014).

A HO-1 possui um amplo espectro de indutores, incluindo o grupamento heme, metais de transição, $H_2O_2$, β-amilóide, dopamina, citocinas, prostaglandinas, endotoxinas, e compostos vasoativos, choque térmico, radiação, hipóxia e hiperoxia. Por outro lado a HO-2 é considerada uma isoforma constitutiva (DENNERY, 2001; RYTER, ALAM, CHOI, 2006; MUNÕZ-SÁNCHEZ; CHÁNEZ-CÁRDENAS 2014).

Desta maneira, é possivel entender que muitos mecanismos são ativados para alcançar o objetivo do processo inflamatório. Assim, se a inflamação aguda cumprir seu papel em conter o agente lesivo, ações de reparo começam a sobressair sobre as ações inflamatórias. Já que o processo de reparo inicia-se tão cedo quanto o processo inflamatório. Contudo, se não ocorrer a eliminição do agente agressor a inflamação não será cessada e passa de aguda para crônica, dependendo do tempo de duração deste processo e os tipos de células predominantes no foco inflamatório, sendo que a inflamação crônica e exacerbada é a principal responsável por vários prejuizos ao indivíduo (ABBAS; LICHTMAN; PILLAI, 2011; BRASILEIRO, 2011).

Muito se tem estudado para estabelecer e aprimorar a fisiopatologia do processo inflamatório. Cada estímulo inflamatório, seja por patógenos ou as próprias doenças, emitem respostas inflamatórias com padrões específicos. O estudo destes padrões é necessário para o entendimento das doenças e também para descoberta de tratamentos mais direcionados, o que justifica a continuidade de linhas de pesquisa nesta área.

## 2.2 Dor Inflamatória

A dor é um sinal de risco e alerta do organismo para o indivíduo sobre um possível dano em potencial (FERREIRA et al., 2009), sendo definida pela Associação Internacional do Estudo da Dor (IASP) como “uma experiência sensorial e emocional desagradável associada a um dano tecidual real ou potencial, ou descrita em termos de tal dano” (IASP, 2017). Este é um importante mecanismo do organismo para alertar a injúria, exercendo proteção e sendo, desta forma, imprescindível para manutenção da vida humana, porém quando em excesso pode

tomar diferentes proporções, tornando-se um agravante na qualidade de vida dos indivíduos (PINHO-RIBEIRO; VERRI; CHIU, 2017).

A dor pode ser dividida em dois tipos: a primeira informa quando estímulos ambientais podem causar danos a integridade física do corpo e a segunda informa a existência de disfunções orgânicas no corpo, como ocorrem nos processos inflamatórios e/ou neuropáticos (FERREIRA, et al., 2009). Estes estímulos são reconhecidos por nociceptores, neurônios sensoriais, que fisiologicamente são ativados apenas por estímulos de alto limiar, ou seja, aqueles estímulos potencialmente lesivos ao organismo. Estes nociceptores podem ser classificados em: mecanotérmicos que respondem a pressão mecânica intensa ou a variações térmicas intensas (quentes> 45ºC ou frias < 5ºC) e são compostos por fibras do tipo Aδ-β. Existem também os nociceptores polimodais que respondem a estímulos nocivos mecânicos, térmicos ou químicos. E dentre os nociceptores, também existem uma classe de nociceptores considerados silenciosos, que são ativados por estímulos químicos gerados no processo inflamatório, sendo compostos por fibras do tipo C (FERREIRA, et al., 2009; FEIN et al., 2011).

As fibras do tipo Aδ-β são mielinizadas e as fibras do tipo C não são mielinizadas, consideradas silenciosas e polimodais, dentre elas, apenas as fibras δ e C estão relacionadas ao processo inflamatório e conduzem respostas nociceptivas rápidas ou lentas (12 a 30 m/s ou 0,5 a 2 m/s, respectivamente). Além do tempo de resposta gerado diferentemente pelas fibras, o tipo de sensação dolorosa também é diferente, sendo que a fibras δ gera uma resposta de dor acentuada em picada e a fibra C gera sensação dolorosa fraca em queimação (FEIN et al., 2011).

Os nociceptores apresentam vários receptores em sua membrana que podem ser ativados por mediadores moleculares, químicos, lipídicos, proteases e fatores de crescimento, sendo que sua ativação, bem como o aumento desses componentes no microambiente inflamatório culminam na modificação e remodelação desses receptores neuronais, bem como no limiar de excitabilidade do nociceptor (PINHO-RIBEIRO; VERRI; CHIU, 2017).

Dentro do processo inflamatório, a dor é um dos sinais cardinais mais importantes, resultante do reconhecimento do patógeno e produção de mediadores químicos para combatê-lo (VERRI et al., 2006). No que se refere à dor inflamatória, os mediadores inflamatórios liberados durante a resposta imune inata podem ser divididos em dois grupos: os mediadores hiperalgésicos intermediários e

mediadores hiperalgésicos finais (FERREIRA, et al., 2009; PINHO-RIBEIRO; VERRI; CHIU, 2017).

Os mediadores hiperalgésicos intermediários são liberados no início e durante a inflamação, sendo responsáveis pela liberação em cascata de outros mediadores. Dentre eles podemos destacar as citocinas como TNFα e IL-1β (CUNHA et al., 1992; FERREIRA et al., 1988). O TNFα, por exemplo, induz a secreção de IL-1β pelas células em resposta inflamatórios que culmina na produção de prostaglandinas (PGs), um mediador hiperalgésico final, por outro lado o TNFα também estimula liberação de quimiocinas que leva a liberação de aminas simpáticas, também um mediador hiperalgésico final (FERREIRA, et al., 2009). Contudo, tem sido demonstrado o papel dos mediadores intermediários se comportando como mediadores finais por ativar diretamente os receptores dos neurônios, como o caso do TNFα se ligando ao receptor TNRF1 que ativa via da MAP quinase p38 mediando a fosforilação dos Nav 1.8 e 1.9, alterando a excitabilidade neuronal por mecanismos dependentes do receptor (TRPV1) (JIM; GEREAU, 2006), e também por ativar a mobilização de $Ca^{++}$ (POLLOCK et al., 2002). A IL-1 também pode ativar seu receptor neuronal IL-1R1 e aumentar a expressão de TRPV1 (EBBINGHAUS, et al., 2012).

Já os mediadores hiperalgésicos finais, PGs, prostaciclinas, aminas simpáticas, leucotrienos, histamina, serotonina, bradicinina e endotelina (ET-1) interagem com seus receptores específicos nos neurônios promovendo modificações moleculares responsáveis por sua sensibilização (FERREIRA, et al., 2009; PINHO-RIBEIRO; VERRI; CHIU, 2017).

Dentre os mediadores inflamatórios que podem causar essas modificações, podemos destacar principalmente as PGs e as aminas simpáticas, esses mediadores hiperalgésicos finais interagem com seus receptores na membrana neuronal (EP e β1/β2, respectivamente) e ativam vias de sinalização intracelular que geram potencial de ação no neurônio (KATSUNG, 2014). A expressão destes receptores estão associadas as fibras do tipo C, ou seja, aos nociceptores silenciosos que são ativados durante o processo inflamatório (FERREIRA et al., 2009).

Os receptores EP colcoar qual receptor e os efeitos inversos e β1/β2 estão acoplados a proteína G (q ou s, respectivamente) e são considerados receptores metabotrópicos por não estarem diretamente ligados a canais iônicos ou

seja, eles precisam ativar vias de sinalização intracelular para gerar sua respectiva resposta. A ativação do receptor acoplado a proteína Gs ativa a via da adenilato cíclica (AC) que leva a produção do primeiro mensageiro, adenosina 3´, 5´ monofosfato cíclico (AMPc) a partir da adenosina trifosfato (ATP). O aumento de concentração no citosol do AMPc ativa proteínas quinases dependente de AMPc, PKA e PKC, que estão envolvidas na fosforilação/fechamento/abertura de canais Nav1.8, Kv ou TRPV1. A PKA, por exemplo, fosforila Nav1.8 e Kv, facilitando o disparo de atividade elétrica da membrana neuronal (FERREIRA, et al., 2009; KATSUNG, 2014). A bradicinina liberada durante a inflamação ativa seu receptor nos nociceptores induzindo sinalização via PKA, que potencializa a abertura dos receptores TRPA1 (HANS; SCHMIDT; STRICHARTZ, 2009; PINHO-RIBEIRO; VERRI; CHIU, 2017).

A ativação das proteínas Gq, por outro lado, libera uma unidade catalítica capaz de decompor o trifosfato inositol (IP3) em difosfato inositol (PIP2) e diacilglicerol (DAG), cujo aumento de concentração no citosol estimula a PKC, levando à fosforilação e ao fechamento de canais Kv e abertura de canais tipo TRPV1 e Nav1.8, facilitando também o disparo de atividade elétrica da membrana neuronal (FERREIRA, et al., 2009; KATSUNG, 2014).

A via das MAP quinases p38 também tem sido envolvida na dor inflamatória por ativar fatores de transcrição como ATF-2 e por induzir produção de PGs e citocinas (JI et al., 2009). O aumento dos mediadores químicos durante o processo inflamatório e sua ativação nos receptores neuronais, principalmente dos nociceptores silenciosos, geram modificações no potencial de repouso do neurônio nociceptivo, qual diminui o limiar de ativação da membrana neuronal, facilitando assim seu disparo por estímulos que anteriormente não eram dolorosos e que após essas modificações passam a ser (FERREIRA, et al., 2009; PINHO-RIBEIRO; VERRI; CHIU, 2017).

A continuidade da resposta nociceptiva consiste na condução da informação dolorosa pelas fibras aferentes primárias periféricas (neurônios de primeira ordem) até o gânglio da raiz dorsal (GRD), o qual entra na medula espinal pelo corno dorsal levando a comunicação sináptica por meio de neurotransmissores, como aminoácido excitatório glutamato ou substância P, com os neurônios de segunda ordem nas lâminas de Rexed. Grande parte dos neurônios nociceptores associados à inflamação terminam nas lâminas mais superficiais (camadas I e II –

área conhecida como substância gelatinosa) no GRD. Este local pode ser dividido em até 6 lâminas sendo que as fibras não nociceptivas formam sinapses em lâminas mais profundas. O estímulo sensorial pode então ser levado para distintas áreas do sistema nervoso central (SNC), onde a informação dolorosa será processada. A experiência da dor é percebida no córtex e a informação é consequentemente reenvida para a medula (via descendentes) (FERREIRA et al., 2009; KUNER, 2010; PINHO-RIBEIRO; VERRI; CHIU, 2017).

Na prática experimental podemos visualizar estes sinais dolorosos em respostas comportamentais e denominadas de alodinia ou dor espontânea (estímulo normalmente não doloroso que passa a desenvolver dor) e hiperalgesia (resposta exacerbada a um estímulo previamente doloroso) (VERRI et al., 2006).

## 2. 3 Do no Câncer

O câncer é uma desordem genética causada por mutações no ácido desoxirribonucleico (DNA) que podem ser adquiridas espontaneamente, induzidas por agressões do ambiente ou herdadas, sendo que a grande maioria das mutações surgem espontaneamente como consequência de danos químicos, físicos ou biológicos ao DNA, que resultam em alterações funcionais de genes cruciais (BERTRAM, 2001). Ou seja, o acúmulo dessas mutações permite que a célula cancerígena adquira vantagens em relação as células normais como: autossuficiência nos sinais de crescimento; ausência de resposta aos sinais inibidores de crescimento; falta de controle proliferativo; evasão da morte celular; potencial replicativo ilimitado; desenvolvimento de angiogênese; capacidade de invadir tecidos locais e disseminar-se para locais distantes; reprogramação das vias metabólicas e capacidade de escapar do sistema imune (KUMAR et al., 2008).

As mutações geradas nas células cancerígenas alteram os dois mecanismos básicos de manutenção do número de células normais no nosso organismo, que são os proto-oncogenes e os genes supressores tumorais. Para cada célula que entra em divisão celular uma célula deve entrar em apoptose, assim o número de células no nosso corpo será sempre constante. Qualquer fator que altere esse equilíbrio entre a divisão e a morte celular pode levar ao desenvolvimento de uma proliferação celular descontrolada, alterando o número

total de células em um órgão ou tecido específico, que futuramente será detectável como uma massa tumoral. A perda do controle da divisão celular ocorre quando os genes proto-oncogenes e supressores tumorais perdem suas funções, desta maneira, os proto-oncogenes passam a ser oncogenes e promovem o crescimento celular exacerbado, enquanto os genes supressores perdem sua capacidade de inibir o processo de divisão celular (BERTRAM, 2001).

O processo pelo qual uma célula normal se transforma em tumoral é bem conhecido e pode ser dividido em três etapas: 1) Iniciação: o processo pelo qual célula normal adquire mutações no genoma e passa a ser uma célula iniciada, resultante de processos endógenos por erros na replicação do DNA, instabilidade química intrínseca das bases de DNA, a partir do ataque de radicais livres gerados durante o metabolismo, exposição a agentes exógenos como radiação ionizante, radiação UV, carcinógenos químicos, físicos ou biológicos. Se o dano do DNA não for reparado, essas mutações genéticas levarão ao desenvolvimento de uma célula mutada. As células assim modificadas são denominadas células transformadas e perdem suas características originais se tornando células atípicas (BRASILEIRO, 2011; SNUSTAD; SIMMONS, 2001); 2) Promoção: para que ocorra a formação da massa tumoral, é necessário que as células mutadas entrem em processo de promoção, este processo permite a expansão clonal da célula iniciada, transmitindo assim suas mutações para outras células, originando os nódulos, pólipos ou papilomas; 3) Progressão: o estágio de progressão tumoral é caracterizado pela transformação das células pré-neoplásicas em tumores malignos com capacidade de invadir tecidos e formar metástases (CONTRAN, 2000; JAKÓBISIAK al., 2003).

De fato, o Instituto Nacional do Câncer (INCA, 2017) define o câncer como um conjunto de mais de 100 doenças que têm em comum o crescimento desordenado das células. As causas do desenvolvimento do câncer pode ser dividida em externas, internas, ou inter-relacionadas. As causas externas referem-se ao meio ambiente e aos hábitos ou costumes próprios dos indivíduos que podem levar ao desenvolvimento das mutações no DNA, já as causas internas são na maioria das vezes, geneticamente pré-determinadas e estão ligadas à capacidade do organismo de se defender das agressões externas (SNUSTAD; SIMMONS, 2001) e as inter-relacionadas, relação das duas causas.

Em termos de nomenclatura, neoplasia refere a um novo crescimento, e muitas vezes a neoplasia é referenciada como tumor, qual pode estar

associado ao edema causado principalmente pelo processo inflamatório no microambiente tumoral. Ainda, o neoplasma ou tumor pode ser dividido em dois tipos, benigno e maligno. Os tumores benignos são aqueles em que suas características micro e macroscópicas são consideradas relativamente inocentes, normalmente localizado e encapsulado por um tecido fibroso. Já os tumores malignos referem-se a neoplasia com capacidade de destruir estruturas adjacentes e disseminar-se para locais distantes (metástases), sendo chamados de câncer. Ambos, possuem dois componentes básicos: o parênquima constituído das células neoplásicas em proliferação constante; e o estroma constituído de tecido conectivo, vasos sanguíneos que dão suporte e sustentação a massa tumoral, e células inflamatórias (KUMAR et al., 2008).

O câncer é uma doença crônico-degenerativa considerada um problema de saúde pública (INCA, 2017). A confirmação da doença pode influenciar diretamente o estilo e qualidade de vida do indivíduo (MACHADO; SAWADA, 2008). O diagnóstico quando realizado precocemente é importante para o incentivo da luta contra esta doença, porém pode causar danos devastadores para as famílias de ordem psíquica, social e econômica (SILVA, 2004). No último levantamento estatístico do INCA (2016), estimava-se que 596.070 pessoas, entre homens e mulheres, teriam câncer em 2016. Tendo uma maior prevalência no câncer de próstata e de mama. O câncer de mama é o tipo de câncer mais comum entre as mulheres no mundo e no Brasil, depois do de pele não melanoma, respondendo por cerca de 28% dos casos novos a cada ano. Este tipo de câncer é relativamente raro antes dos 35 anos e acima desta idade sua incidência cresce progressivamente, especialmente após os 50 anos. Estima-se que aproximadamente 57.960 novos casos de câncer de mama acontecerão em 2016 (INCA, 2017). Apesar da idade avançada ser considerada um fator de risco, os casos de câncer de mama em pacientes jovens chama atenção especialmente por estar associado a um pior prognóstico quando comparado com outras faixas etárias (SANTOS et al. 2012).

O tumor de Ehrlich é descrito como um adenocarcinoma espontâneo de glândula mamária de camundongos fêmeas descoberto por Paul Ehrlich em 1896 e desenvolvido inicialmente sob a forma sólida, qual pode ser convertido para a forma ascitica, quando inoculado no peritônio ou cavidades. Contudo, este tipo de tumor pode retornar ao seu estado sólido quando inoculado em tecidos. O tumor tem sido utilizado e descrito em vários modelos experimentais animais (BALAMURUGAN

et al., 2009; KAMETANI et al., 2007). Recentemente nosso laboratório padronizou um modelo para o estudo da dor no câncer a partir da inoculação das células tumorais de Ehrlich na pata de camundongos (CALIXTO-CAMPOS et al., 2013), o qual vem servindo como modelo para o estudo deste tipo de dor, dos mecanismos fisiopatológicos envolvidos na dor e na busca de terapias mais efetivas para o tratamento da dor no câncer, uma vez que as terapias utilizadas podem não desenvolver analgesia adequada aos pacientes.

O estudo da dor no câncer é extremamente importante, pois este tipo de dor é considerado um grave problema para os pacientes com câncer, responsável principalmente pela diminuição da qualidade de vida destas pessoas. O quadro destes indivíduos normalmente é de indisposição, e a não erradicação da dor agrava a situação podendo levar a ansiedade, depressão e hostilidade, prejudicando todas as outras tarefas cotidianas do paciente (FERREIRA et al., 2009). A etiologia da dor no câncer não é totalmente compreendida, acredita-se que este tipo de dor possa ser gerada por inúmeros fatores, desde efeitos colaterais e tóxicos da quimioterapia ou radioterapia agressiva, sintomas agudo ou crônico após cirurgia de remoção do tumor, processo inflamatório gerado no microambiente tumoral ou pela compressão de nervo dependendo da localidade da massa tumoral (SCHIMIDT, 2014). É importante destacar que um alto grau de dor antes da remoção cirúrgica do tumor correlaciona-se com um alto grau de dor após a remoção cirúrgica, indicando que os mecanismos envolvidos na dor do câncer induzem plasticidade neuronal do sistema nociceptivo a longo prazo e até mesmo irreversível que resulta em dor crônica (CHEVILLE; TCHOU, 2007). Em alguns casos a dor é o primeiro sinal para descobrimento do câncer e acredita-se que a maioria das pessoas que possuem esta doença irão experimentar este tipo de dor, de maneira moderada a grave, em algum momento do curso da doença (MANTYH, 2010).

A apresentação clínica da dor no câncer parece depender de três fatores: o tipo histológico do câncer; a localização primária da massa tumoral; e a localização metastática. Por exemplo, um paciente com câncer de mama metastizado para a coluna vertebral desenvolverá sintomas muito diferente de um paciente com câncer oral, pois nem todos os pacientes com câncer de mama possuem nódulos dolorosos, contudo podem passar a sentir dor quando há metastização para os ossos, ou seja, sua localização secundária. Por outro lado, no

câncer oral, o paciente pode apresentar dor durante a função oral fisiológica em estágios mais iniciais do câncer (SCHIMIDT et al., 2010).

Antigamente rotulava-se a dor no câncer como uma dor associada à inflamação, contudo atualmente estudos pré-clínicos tem demonstrado que tumores dolorosos podem não apresentar processo inflamatório (SCHMIDT, 2014), reforçando esta hipótese temos modelos experimentais que induzem dor no câncer não modulável por drogas anti-inflamatórias (CALIXTO-CAMPOS et al., 2013). Isto demonstra a amplitude da fisiopatologia da dor no câncer, embora exista também o câncer com processo inflamatório. Logo, fica difícil entender o tipo de dor envolvida no câncer, no caso da dor associada ao processo inflamatório é plausível o entendimento que seja devido ao aumento dos mediadores químicos produzidos no ambiente tumoral, já a dor associada a neuropatia é possível que seja oriunda do crescimento rápido do tumor e compressão das fibras nociceptivas periféricas que levam a lesões e injúria nos nervos (MANTYH et al., 2002). O que mais se tem entendido atualmente é o envolvimento do sistema nervoso central neste tipo de dor, pois independente dos mecanismos inflamatórios e/ou neuropáticos há um conjunto de alterações neuroquímicas na medula espinhal que parecem ser primordiais para a instalação deste tipo de dor (SCHMIDT, 2014).

No microambiente tumoral com presença de processo inflamatório nota-se várias células imunes incluindo, macrófagos, neutrófilos, mastócitos, células dendríticas, células *natural killer,* linfócitos T e B, além da células tumorais e seu estroma circundante (que consiste em fibroblastos, células endoteliais e mesenquimais) quais se comunicam na tentativa de modular o processo inflamatório (GRIVENNIKOV; GRETEN; KARIN, 2010). Vários estudos demonstraram que os mediadores produzidos no microambiente tumoral podem ser produzidos pelo próprio tumor, bem como pelas células imunes adjacentes (SCHIMIDT et al., 2010), sendo os macrófagos uma das principais células relacionadas à amplificação do processo inflamatório e também associado ao mal prognóstico desta doença (GRIVENNIKOV; GRETEN; KARIN, 2010).

Dentre os mecanismos inflamatórios envolvidos na dor no câncer, tem sido demonstrado a presença de vários mediadores como citocinas e quimiocinas (GALDIERO, 2013; GRIVENNIKOV; GRETEN; KARIN, 2010; KRESS, 2010), ET-1 (WACNICK et al., 2001), PGs (SABINO et al., 2002), fator de crescimento de nervo (NGF) (YE et al., 2011), bradicininas (BK) (FUJITA et al.,

2010) e ATP (XIA et al., 2014). Estes mediadores são capazes de sensibilizar os nociceptores assim como na dor inflamatória, por se ligarem em receptores específicos na membrana dos nociceptores levando a sensibilização e aumento da excitabilidade neuronal (KRESS, 2010; SCHIMIDT et al., 2014; VERRI et al., 2006). A transmissão da dor no câncer pode ocorrer via receptores TRPV, que são amplamente expressos nos nociceptores e capazes de detectar estímulos como calor, acidose, prótons extracelulares e metabolitos lipídicos (MANTHY, 2002). O microambiente tumoral é considerado um ambiente acidificado qual pode ativar estes receptores, bem como canais iónicos de detecção de ácido-3 (ASIC3) (SCHIMIDT et al., 2010). Ainda, no câncer, devido ao mecanismo de neuroplasticidade pode haver o aumento da expressão dos canais de TRPV nos nociceptores (HIRONAKA et al., 2014), o que aumenta a facilitação da despolarização neuronal (GHILARDI, 2005; SHINODA, 2008).

Outros receptores também estão envolvidos na ativação neuronal da dor no câncer, como os receptores purinérgicos ($P_2X_3$) que podem ser ativados por ATP liberados pelas células, bem como receptores específicos de mediadores químicos como receptor da ET-1 ($ET_AR$), EP, receptor tirosina quinase (TrkA) que reconhece NFG e receptor de BK (B1 e B2). A ativação destes receptores levam assim como na dor inflamatória, o aumento da excitabilidade do neurônio, induzindo fosforilação de canais de Nav1.8 e 1.9, e diminuição do limiar de excitabilidade neuronal (MANTHY, et al., 2002).

É bem sabido que um estimulo periférico induz ativação espinal, devido a uma reorganização neuroquímica nos segmentos da medula que recebem as fibras nociceptivas que foram estimuladas na periferia, como resultado há o aumento dos níveis do neurotransmissor excitatório Glutamato e toxicidade concomitante no sistema nervoso central (MANTHY et al., 2012). A ativação do SNC tem um importante papel no desenvolvimento da dor crônica, normalmente gerada por uma mal adaptação resultante da neuroplasticidade e da permanência do estímulo prejudicial (COSTIGAN et al., 2009; JI et al., 2009). A neuroplasticidade da medula espinhal também é caracterizada pelo aumento da expressão de substância P e do seu receptor (SCHIMIDT, 2014). Assim, é plausível o envolvimento da dor dependente da ativação e remodelação da medula espinal, bem como das células da glia no câncer, mesmo quando o tumor é inoculado ou originado perifericamente. De fato, a injeção periférica de células tumorais na pata de camundongos tem

demonstrado a ativação espinal avaliada pela ativação de astrócitos e micróglia (YANG et al., 2015; ZHANG et al., 2005) os quais poderiam ser responsáveis pela produção de citocinas como TNFα e IL-1β neste local (CALIXTO-CAMPOS et al., 2015; OLIVEIRA et al., 2014). No câncer, a estimulação espinal além de levar a liberação de citocinas (CALIXTO-CAMPOS et al., 2015), também pode ativar o fator de transcrição NFκB (XU et al., 2014), MAP quinases (GAO et al., 2009) e gerar estresse oxidativo na medula espinal (CALIXTO-CAMPOS et al., 2015), os quais amplificam o processo inflamatório e os sinais de dor.

A produção de citocinas no microambiente tumoral a partir das células imunes podem ser resultado da ativação do NFkB, STAT3 e AP-1, e os mediadores gerados a partir destes fatores estimulam muitas vezes a proliferação celular e a sobrevivência celular responsável pelo mecanismo de promoção tumoral. O próprio TNFα pode induzir a ativação do NFkB e AP-1 (GRIVENNIKOV; GRETEN; KAREN, 2010). Clinicamente, pacientes diagnosticado com câncer de mama, câncer de próstata ou mieloma múltiplo apresentam elevados níveis de IL-1β, sendo que os pacientes com altas concentrações de IL-1β apresentam aumento de quatro vezes na probabilidade de sofrer metástase devido aos efeitos proliferativos diretos, ativação do processo inflamatório e sinalização de angiogênese induzidos por este mediador (LIU et al., 2013; OLIVEIRA et al., 2014). Ademais, citocinas como TNFα também foram detectadas em células de pacientes com câncer de cabeça e pescoço (OLIVEIRA et al., 2014). O TNFα também participa da destruição óssea, um mecanismo importante para a dor no câncer (VENDRELL et al., 2015) e seus altos níveis séricos no soro dos pacientes estão associados com maior desenvolvimento de dor (OLIVEIRA et al., 2014). Desta maneira, a inibição das citocinas pode ser considerada uma importante ferramenta terapêutica para a dor no câncer.

As EROs também tem tido importante papel no desenvolvimento da inflamação e da dor (MAIOLI et al., 2015), sendo seu papel no câncer ainda pouco compreendido. A própria proliferação tumoral exacerbada pode levar ao desenvolvimento das EROs (NASHED; BALENJO; SINGH, 2014), as células imunes locais também podem liberar EROs que amplificaria o processo inflamatório local e seus sinais clínicos (LARCO; WUERTZ; FURCHT, 2004), por outro lado os quimioterápicos agem por aumentar os níveis de EROs intracelular e levar a morte das células cancerígenas (THAKUR et al., 2008).

Apesar de todos os avanços no estudo da dor no câncer, os diferentes tipos de câncer, as várias localidades do desenvolvimento da massa tumoral e a falta de entendimento dos mecanismos que desenvolvem e mantém a dor no câncer dificulta a produção de medicamentos efetivos. Por isto, elucidar e compreender os mecanismos que envolvem a dor nos diferentes tipos de câncer é essencial para intervenção adequada da dor nos pacientes, assim como para a busca de novas e melhores terapias (CALIXTO-CAMPOS et al., 2013, SCHIMIDT et al., 2010).

## 2.4 Polifenóis

As plantas produzem diversos componentes orgânicos divididos em metabólitos primários e secundários. Os metabólitos primários possuem função estrutural, plástica e de armazenamento de energia. Já os metabólitos secundários, não possuem relação com crescimento e desenvolvimento da planta (TAIZ; ZEIGER, 2006), mas sim com mecanismos de adaptação e sobrevivência (SOUZA FILHO; ALVES, 2002).

Dentre os compostos do metabolismo secundário podemos destacar os fenólicos ou polifenóis, caracterizados por um ou mais núcleos aromáticos ou anéis fenólicos contendo substituintes hidroxilados e/ou seus derivados funcionais (ésteres, metoxilas, glicosídeos) (HARBORNE, 1994) (Figura 1). Os polifenóis podem ser divididos em várias classes de acordo com o número de anéis fenólicos apresentados em sua estrutura (MARTIN; BOLLING et al., 2015). As principais classes dos polifenóis são os flavonóides, ácidos fenólicos, estilbenos, ácidos cinâmicos, cumarinas, ácidos benzóicos, taninos e ligninas (FANTINI et al., 2015; HOUNSOME et al., 2008).

Esses compostos são amplamente encontrados em alimentos como brócolis, couve-flor, couve de bruxelas, nabos, couve manteiga, mostarda, aspargos, espinafre, alface, frutas, chá, vinho, chocolate, café, entre muitos outros, podendo ser considerados como componentes importantes da dieta humana (HOUNSOME et al., 2008). Estes conferem muito sabor, odor e coloração aos diversos vegetais, sendo um atrativos dos alimentos que os produzem.

Os compostos fenólicos podem ser divididos em: A) Fenólicos simples ou ácidos fenólicos: os quais podem ser derivados do ácido hidroxicinâmico ou do ácido hidroxibenzoico. B) Flavonoides: Podem ser classificados em sete grupos: •Flavonas: apigenina encontrada em frutas cítricas e aipo; •Flavanonas (ou di-hidroflavonas): naringenina, naringena e hesperidina encontradas em frutas cítricas; •Flavonols: quercetina, canferol e miricetina encontradas em chá, cebola, maçã, brócolis e pequenas frutas ou frutas vermelhas; •Flavanonols (di-hidroflavonol): taxifolina encontrada em frutas; •Isoflavonas: genisteina, daidazina encontradas em leguminosas como a soja e os feijões; •Flavanols ou catequinas: epicatequina, epigalocatequina galato encontradas em chás como o chá verde e o chá preto; •Antocianidinas: cianidina, delfinidina, malvidina, pelargonidina e peonidina encontradas em frutas de coloração escura, frutas vermelhas ou pequenas frutas. C) Estilbenos: O resveratrol é o representante mais conhecido, encontrado em uvas, suco de uva e vinho. D) Lignanas: encontradas em linho e gergelin. E) Taninos: Encontrados geralmente em cascas de frutas e sementes (VIZZOTO; KROLOW; WEBER, 2010).

Atualmente os polifenóis tem sido foco de vários estudos, os quais tem demonstrado seus efeitos anti-inflamatórios, antioxidantes, antimicrobianos e antitumorais (MARTIN; BOLLING, 20157). Estas propriedades são responsáveis por seu papel na prevenção e tratamento de várias doenças (FALLER; FIALHO, 2009).

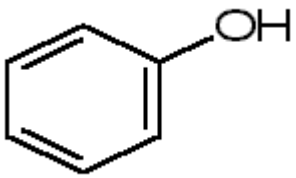


**Figura 1:** Estrutura química básica do fenól simples: Presença de um grupo hidroxila ligado diretamente ao anel benzênico

### *2.4.1 Ácido Vanílico*

O ácido vanílico (Figura 2) é uma forma oxidada da vanilina (LESAGE-MEESSEN et al., 1996) e pertencente aos compostos polifenóis, na classe dos ácidos benzoicos, podendo ser encontrado em grandes quantidades na raiz da *Angelica sinensis*, assim como em várias frutas e plantas, sendo amplamente utilizado como um agente aromatizante (KIM et al., 2011).

Este composto se mostrou eficaz na redução de colite ulcerativa induzida por dextran sulfato de sódio (KIM et al. 2010), apresentou efeito cardioprotetor (KUMAR; PRAHALATHAN; RAJA, 2014; R; RAJAKUMAR; DHANASEKAR, 2011), hepatoprotetor (ITOH et al., 2009), além de modular a resposta imunológica através da diminuição da produção de IL-6, TNFα e NFκB em modelo de inflamação induzida por lipopolissacarídeo (LPS) (KIM et al., 2011).

Ademais, o ácido vanílico apresenta efeitos antioxidante (TAI; SAWANO; YAZAMA, 2011), analgésico (MORUCCI et al., 2012), antibacteriano (RAI; MAURYA, 1996) e também antitumoral (TSUDA et al., 1994).

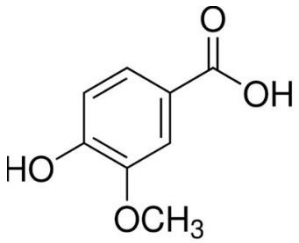


**Figura 2:** Estrutura química do ácido vanílico

### *2.4.2 Flavonoides*

Os flavonoides são compostos polifenóis biossintetizados pelas plantas e precursores de vários grupos de substâncias como aminoácidos, terpenóides, ácidos graxos, dentre outros. Eles participam de importantes funções no crescimento, desenvolvimento e na defesa dos vegetais contra o ataque de patógenos (DIXON; HARRISON, 1990), podendo ser encontrado nas plantas, concentrados nas sementes, frutos, cascas, raízes, folhas e flores (FELDMANN, 2001). As principais fontes de flavonoides incluem frutas como uvas, cerejas, maçã,

groselhas, frutas cítricas, entre outras, e hortaliças como pimenta, tomate, espinafre, cebola, brócolis, dentre outras (BARNES; ANDERSON; PHILLIPSON, 2001).

Sua estrutura básica consiste em um núcleo fundamental, constituído de quinze átomos de carbono arranjados em três anéis (C6-C3-C6), sendo dois anéis fenólicos substituídos (A e B) e um pirano (cadeia heterocíclica C) acoplado ao anel A (DI CARLO et al., 1999) (Figura 3).

A diversidade dos flavonoides pode ser atribuida as variações estruturais no seu esqueleto básico, que podem ser a nível de oxidação ou promovidas por reações de alquilação, glicolisação ou oligomerização (TAHARA, 2007). Desta forma os flavonoides podem ser encontrados como agliconas, glicosídeos, metilados ou acilados (HAVSTEEN, 2002; VEITCH; GRAYER, 2008). Já modificações no anel central destas substâncias dão origem a subclasses distintas: chalconas, flavononas, flavononóis, flavonas, flavonóis, isoflavonas, flavan-3-ols e antocianidinas (BOOTS; HAENEN; BAST, 2008; VEITECH; GRAYER, 2008).

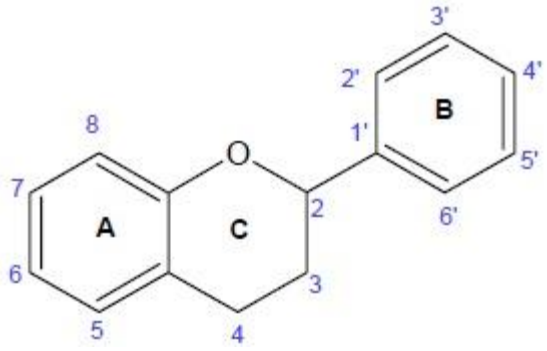


**Figura 3:** Estrutura química básica dos flavonoides

As funções biológicas dos flavonoides ainda não são totalmente compreendidas. No entanto, os flavonoides têm demonstrado várias propriedades benéficas *in vivo* e *in vitro*, que podem estar relacionadas ao seu potente efeito antioxidante em neutralizar as EROs (VERRI et al., 2012). Tem sido bem estabelecido o papel das EROs em induzir dor inflamatória, doenças inflamatórias e até mesmo o câncer (MAIOLI et al., 2015; NASHED; BALENJO; SALVEMINI et al., 2011).

Os flavonoides não apresentam apenas um mecanismo de ação, mas sim diversos mecanismos, sendo que o mesmo flavonoide pode apresentar diferentes efeitos de acordo com o modelo experimental estudado ou doença

estudada. Esta variação dificulta a seleção de apenas um flavonoide para o tratamento das doenças, por isso o estudo destes compostos são extremamente necessários.

Ademais, os flavonoides são conhecidos por apresentarem baixa toxicidade, estão amplamente distribuídos nos alimentos do nosso cotidiano e possuem fácil acesso comercial, o que facilita a fabricação de formulações terapêuticas que poderiam ser utilizadas adicionalmente aos tratamentos convencionais ou até mesmo para substituírem alguns tratamentos utilizados atualmente na clínica para dor.

#### *2.4.2.1 Quercetina*

A quercetina é um flavonoide pertencente a classe dos flavonóis, podendo ser encontrada em uma variedade de alimentos, incluindo maçãs, alcaparras, uvas, cebolas, chá, tomates, em sementes, nozes, flores, cascas e folhas (Figura 4). A quercetina também é encontrada em plantas medicinais, incluindo *Ginkgo biloba*, *Hypericum perforatum* e *Sambucus canadensis* (LI et al., 2016). Vários trabalhos tem demonstrado seu efeito anti-inflamátorio, analgésico, antioxidante e antitumoral (CASAGRANDE et al., 2006; GUAZELLI et al., 2013; SOUTO et al., 2011; VALÉRIO et al., 2009).

Em relação aos efeitos anti-inflamatórios, a quercetina se demonstrou eficaz em reduzir o recrutamento celular induzido por diferentes quimiocinas *in vivo* (SOUTO et al., 2011), reduzir a inflamação intestinal induzida por ácido acético (GUAZELLI et al., 2013), reduzir a periodontite (NAPIMOGA et al., 2013), reduzir a ativação do NFκB (INDRA et al., 2013), reduzir produção de citocinas pró-inflamatórias em macrófagos estimuladas com LPS (MANJEET; GHOSH, 1999) e inibir a expressão de COX-2 (KIM et al., 1998).

Os efeitos antioxidantes também foram observados em vários modelos experimentais (CASAGRANDE et al., 2006; VALÉRIO et al., 2009; MAIOLI et al., 2015), assim como seu efeito analgésico (ANJANEYULU; CHOPRA, 2003; MAIOLI et al., 2015). Um efeito importante da quercetina é a possível indução da liberação de opióides endógenos no modelo de neuropatia diabética induzida por estreptozotocina (ANJANEYULU; CHOPRA, 2003). A quercetina também parece ter

efeitos antitumorais por inibir a hiperproliferação e displasia de células tumorais e reduzindo o tumor de colón induzido em camundongos (DESCHNER et al., 1993; SO et al., 1996).

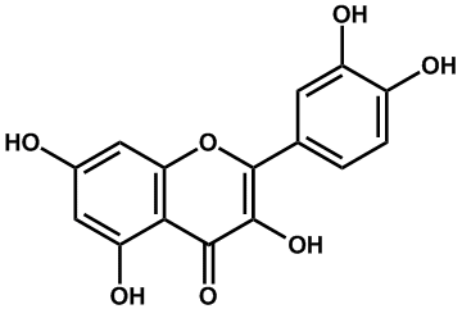


**Figura 4:** Estrutura química da quercetina

*2.4.2.2 Naringenina*

A naringenina é um flavonoide abundante na natureza presente em frutas cítricas como a toranja, pomelo, tomate, grãos e vegetais, pertence à classe das flavononas (Figura 5). Vários trabalhos têm demonstrado sua potente atividade anti-inflamatória, analgésica, antioxidante e antitumoral (BIRT; HENDRICH; WANG, 2001; FRANCIS; SHETTY; BHATTACHARYA, 1989; SO et al., 1996).

Em relação aos efeitos anti-inflamatórios a naringenina reduziu a migração de eosinófilos no fluído pulmonar, diminui o infiltrado de células inflamatórias e citocinas TH2 em modelo de asma por ovalbumina (IWAMURA et al., 2010), inibiu a ativação do NFkB em macrófagos e micróglia *in vitro* (PARK; KIM; CHOI, 2012), inibiu a produção de $PGE_2$ induzida por LPS em cultura de macrófagos (BIRT; HENDRICH; WANG, 2001), inibiu o edema de pele induzido pela irradiação UVB (MARTINEZ et al., 2015), inibiu a produção de citocinas e ativação do NFkB pela carragenina *in vivo* (PINHO-RIBEIRO et al., 2016), inibiu produção de citocinas pró-inflamatórias, reduziu expressão de ET-1, COX-2 e diminui atividade da mieloperoxidase induzidas pelo $KO_2$ (MANCHOPE et al., 2016), reduziu a disponibilidade de ácido araquidônico e seus produtos lipídicos (JAYARAMAN et al., 2012; LÄTTIG et al., 2007), e inibiu a ativação de $PI_3K$, MAP quinases e também a expressão de iNOS (HAMALAINEN et al., 2007; YANG et al., 2011).

Em relação as atividades analgésicas, o tratamento com a

naringenina, em diferentes modelos de dor inflamatória (MANCHOPE et al., 2016; PINHO-RIBEIRO et al., 2016) e neuropática (KAULASKAR et al., 2012; HU; ZHAO, 2014), se mostrou eficaz e parece envolver mecanismos analgésicos pela ativação da via de sinalização analgésica NO/GMPc/PKG/ATP dependente (NO/GMPc (guanosina monofosfato cíclica), PKG (proteína quinase G), canais de potássio sensíveis ao ATP (adenosina trifosfato) (MANCHOPE et al., 2016).

Sua atividade antioxidante também foi demonstrada pela avaliação do consumo do GSH e avaliação da capacidade antioxidante (MANCHOPE et al., 2016; MARTINEZ et al., 2015; PINHO-RIBEIRO et al., 2016), e por ativar via de sinalização de fatores de genes antioxidantes Nrf2 (LOU et al., 2014; RAMPRASATH et al., 2014). Ademais, o efeito da naringenina também foi observado em ativar a via das caspases-3, marcador de indução de apoptose (ZIELINSKA-PRZYJEMSKA et al., 2008), bem como inibir a proliferação de carcinoma mamário humano *in vitro* (SO et al., 1996).

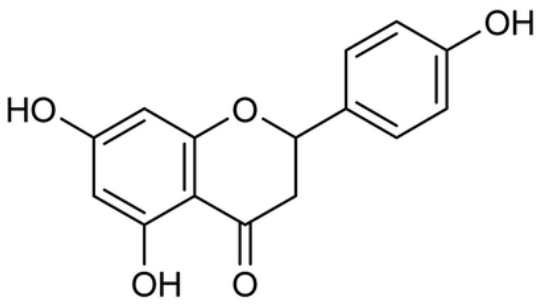


**Figura 5:** Estrutura química da naringenina

Desta maneira, é possível observar que os polifenóis tem mostrado vários efeitos benéficos (anti-inflamatórios, antioxidantes e analgésicos). Contudo, o estudo destes compostos ainda se faz necessário, uma vez que existem poucos relatos na literatura do seu efeito em modelos de dor no câncer. É importante também o entendimento de que cada modelo experimental estudado ou cada composto polifenólico escolhido para estudo, apresentam particularidades. Assim, seus estudos permitem melhor entendimento dos mecanismos de ação destes compostos, bem como dos mecanismos fisiopatológicos envolvidos na dor inflamatório e na dor no câncer.

# 3 OBJETIVO

## 3.1 Objetivo geral

Este trabalho objetivou avaliar o efeito de diferentes compostos polifenóis: ácido vanílico, quercetina e naringenina, em modelos experimentais de dor infamatória e dor no câncer.

## 3.2 Objetivo específico

a) Avaliar o efeito do tratamento agudo com o ácido vanílico em diferentes modelos de dor inflamatória induzidas por ácido acético, PBQ, formalina, CFA e carragenina (Cg), mediante avaliação dos parâmetros de contorções abdominais, tempo de lambida, *flinches*, hiperalgesia mecânica e edema;

b) Avaliar o efeito crônico do tratamento com o ácido vanílico no modelo do CFA crônico nos parâmetros de hiperalgesia mecânica e edema;

c) Avaliar a atividade da mieloperoxidase (MPO) e n-acetylglucosaminidase (NAG) pelo tratamento com ácido vanílico nos modelos inflamatórios agudo (Cg) e crônico (CFA);

d) Avaliar possíveis danos estomacais pelo MPO e hepáticos pelos marcadores AST e ALT após o tratamento com o ácido vanílico;

e) Avaliar o efeito antioxidante do tratamento com o ácido vanílico pelos métodos de FRAP, ABTS, GSH e TBARS;

f) Avaliar o efeito anti-inflamatório do tratamento com o ácido vanílico pela dosagem de citocinas IL-1β, TNF-α e IL-33 e pela avaliação da ativação do fator de transcrição NFκB;

g) Avaliar o efeito do tratamento com a quercetina no modelo de dor no câncer intraplantar pelos parâmetros de hiperalgesia mecânica, térmica e dor espontânea;

h) Avaliar o efeito do tratamento com a quercetina no crescimento tumoral ou/aumento da espessura da pata dos animais e nas alterações histológicas;

i) Avaliar o efeito anti-inflamatória do tratamento com a quercetina na atividade da MPO e na produção de citocinas (IL-1β e TNFα);

j) Avaliar o efeito antioxidante do tratamento com a quercetina nos métodos do FRAP, ABTS e GSH;

k) Avaliar o efeito do tratamento com a quercetina nos mecanismos da via dos opióides e em todos os parâmetros supracitados;

l) Avaliar o efeito do tratamento com a naringenina no modelo de dor no câncer intraplantar pelos parâmetros de hiperalgesia mecânica, térmica e dor espontânea;

m) Avaliar o efeito do tratamento com a naringenina no aumento da espessura da pata, proliferação tumoral no modelo de tumor ascítica, recrutamento celular ascítica e atividade da MPO e NAG ascítica;

n) Avaliar os possíveis danos estomacais pelo MPO e hepáticos pelos marcadores AST e ALT após o tratamento com a naringenina;

o) Avaliar o efeito antioxidante do tratamento com a naringenina pelos métodos de FRAP, ABTS e GSH e pela expressão de mRNA do fator de transcrição *Nrf2* e *Ho-1*;

p) Avaliar o efeito anti-inflamatória do tratamento com a naringenina na atividade da MPO e NAG, expressão de mRNA das citocinas *Tnfα* e *Il-1β* e da expressão do mRNA de marcadores das células da glia *Gfap* e *Iba-1.*

# 4 DESENVOLVIMENTO

Esta tese foi desenvolvida no Laboratório de Dor, Inflamação, Neuropatia e Câncer e está constituída por 3 artigos científicos, sendo 2 artigos já publicados: **Vanillic Acid Inhibits Inflammatory Pain by Inhibiting Neutrophil Recruitment, Oxidative Stress, Cytokine Production, and NFκB Activation in Mice**. Calixto-Campos C, Carvalho TT, Hohmann MS, Pinho-Ribeiro FA, Fattori V, Manchope MF, Zarpelon AC, Baracat MM, Georgetti SR, Casagrande R, Verri WA Jr. Artigo publicado no periódico Journal Natural Products, 78 (8):1799-808, 2015, doi: 10.1021/acs.jnatprod.5b00246 e **Quercetin reduces Ehrlich tumor-induced cancer pain in mice**. Calixto-Campos C, Corrêa MP, Carvalho TT, Zarpelon AC, Hohmann MS, Rossaneis AC, Coelho-Silva L, Pavanelli WR, Pinge-Filho P, Crespigio J, Bernardy CC, Casagrande R, Verri WA Jr. Artigo publicado no periódico Analytical Cellular Pathology, 285708, 2015. doi: 10.1155/2015/285708, e 1 artigo redigido: **Naringenin inhibits Ehrlich tumor cells-induced cancer pain: Role of leukocyte recruitment, oxidative stress, cytokine production, Nrf2 and HO-1 expression, and glia cells.** Calixto-Campos C, Fattori V, Carvalho TT, Manchope MF, Ferraz CR, Coelho-Silva L, Borghi SM, Pinho-Ribeiro FA, Casagrande R, Verri WA Jr. á ser submetido no período Inflammation Research.

1 **4.1 Artigo 1**

JOURNAL OF NATURAL PRODUCTS

Article

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# Vanillic Acid Inhibits Inflammatory Pain by Inhibiting Neutrophil Recruitment, Oxidative Stress, Cytokine Production, and NFκB Activation in Mice

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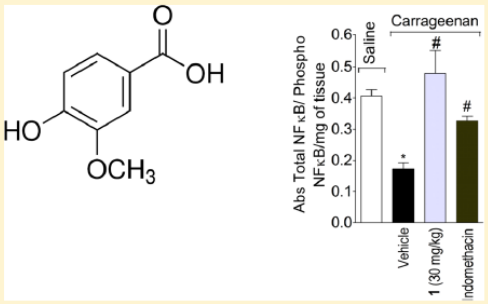
**ABSTRACT:** Vanillic acid (**1**) is a flavoring agent found in edible plants and fruits. It is an oxidized form of vanillin. Phenolic compounds form a substantial part of plant foods used as antioxidants with beneficial biological activities. These compounds have received considerable attention because of their role in preventing human diseases. Especially, **1** presents antibacterial, antimicrobial, and chemopreventive effects. However, the mechanisms by which **1** exerts its anti-inflammatory effects *in vivo* are incompletely understood. Thus, the effect of **1** was evaluated in murine models of inflammatory pain. Treatment with **1** inhibited the overt pain-like behavior induced by acetic acid, phenyl-*p*-benzoquinone, the second phase of the formalin test, and complete Freund's adjuvant (CFA). Treatment with **1** also inhibited carrageenan- and CFA-induced mechanical hyperalgesia, paw edema, myeloperoxidase activity, and *N*-acetyl-β-D-glucosaminidase activity. The anti-inflammatory mechanisms of **1** involved the inhibition of oxidative stress, pro-inflammatory cytokine production, and NFκB activation in the carrageenan model. The present study demonstrated **1** presents analgesic and anti-inflammatory effects in a wide range of murine inflammation models, and its mechanisms of action involves antioxidant effects and NFκB-related inhibition of pro-inflammatory cytokine production.




Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids.[1] Vanillic acid (**1**) (4-hydroxy-3-methoxybenzoic acid) is a phenolic derivative of edible plants and fruits such as in the trunk bark of *Amburana cearensis* A.C. Smith (Fabaceae) (popularly known as "cumaru", "amburana", or "amburana-de-cheiro"),[2] in the roots of *Angelica sinensis* (Oliv.) Diels (family Umbellaceae), commonly known as Dong quai or "female ginseng",[3,4] largely used in traditional Chinese medicine,[5] in the aerial parts of *Scrophularia sambucifolia* (L.) subsp. *sambucifolia* (Scrophulariaceae),[6] in the leaf extract of *Ginkgo biloba*, and in the leaf extract of *Poliomintha longiflora*, Mexican oregano.[7] It is an oxidized form of vanillin used as a flavoring agent,[4] and it is also an intermediate in the production of vanillin from ferulic acid.[4]

The antioxidant activity of **1** seems to be an important action.[8] The *in vitro* antioxidant mechanisms of **1** include free radical scavenging activity, reducing power, and inhibition of lipid peroxidation.[9] Furthermore, **1** reduced lipid peroxidation products and significantly restored enzymatic antioxidants and nonenzymatic antioxidants in the plasma of hypertensive rats.[10] Additional actions of **1** include antimicrobial properties, as demonstrated by the *in vitro* inhibition of the growth of species and strains of *Listeria* spp.,[11] and a chemopreventive effect in hepatocarcinogenesis induced by 2-amino-3-methylimidazo-[4,5-*f*]quinoline in rats.[12]

Regarding inflammation and pain, oral treatment (50 mg/kg) with **1** reduces the time spent licking the paw in both phases of the formalin test.[2] On the other hand, **1** reduced only the second phase of formalin-induced paw licking when intraperitoneal treatment (100 mg/kg) was used,[13] indicating that pharmacokinetic issues modulate its activities. Compound **1** also inhibited the acetic acid-induced writhing response at doses of 3−30 mg/kg,[13] carrageenan-induced paw edema in mice, and carrageenan-induced recruitment of neutrophils to the peritoneal cavity of rats.[2]

The nociceptive and inflammatory responses are related to pro-inflammatory mediators such as cytokines (e.g., TNF-α, IL-1β, and IL-6), which are produced by the activation of transcription factors including NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells). NFκB performs a crucial role in the expression of many genes involved in

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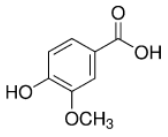
immune and inflammatory responses. NFκB is a member of the Rel family of transcription factors as a heterodimer composed of p50 and p65 subunits or homodimers.[4,14] Indeed, **1** decreased the activation of NFκB and production of TNF-α and IL-6 induced by lipopolysaccharide (LPS) *in vitro*.[4] Moreover, inflammation induces cyclooxygenase (COX) expression that generates prostaglandins (PGs), such as $PGE_2$, which is involved in paw edema and pain.[4] Compound **1** inhibited LPS-induced COX-2 expression and $PGE_2$ production by mouse peritoneal macrophages *in vitro*.[4]

Although many studies have shown the role of **1** in varied *in vitro* models and some *in vivo* models as reported above, the *in vivo* mechanisms involved in the analgesic and anti-inflammatory effects of **1** are incompletely understood. In this sense, the effect and mechanisms of **1** were investigated in murine models of inflammation and inflammatory pain.




**1**

## RESULTS AND DISCUSSION

**Vanillic Acid (1) Inhibits Acetic Acid- and Phenyl-*p*-benzoquinone (PBQ)-Induced Writhing Response.** In the first series of experiments, the antinociceptive effect of **1** was assessed by acetic acid- and PBQ-induced overt pain-like behavior tests (Figure 1). These tests are widely used for screening and determination of the mechanisms of action of novel drugs.[15] Mice received intraperitoneal (ip) treatment with **1** (3−30 mg/kg; 5% Tween 80 diluted in saline) 1 h before ip stimulus with acetic acid (0.8% v/v, diluted in saline, 10 mL/kg) (Figure 1A). The doses of 10 and 30 mg/kg but not of 3 mg/kg of **1** significantly inhibited the writhing response induced by acetic acid (Figure 1A). The antinociceptive effect of the dose of 30 mg/kg of **1** was significantly different compared to the other doses (Figure 1A). This result is in agreement with a previous study showing the antinociceptive effect of **1** in acetic acid-induced writhing although with gender differences.[13] Compound **1** achieved maximal antinociception at 3 mg/kg in female Swiss mice[13] and at 30 mg/kg in male Swiss mice (present data). At first view this would indicate females might respond better to **1**. However, the degree of antinociception was higher in males than in females. The dose of 30 mg/kg ip of **1** was selected to determine the influence of time of pretreatment in its efficacy. Mice were treated with compound **1** at 15, 30, 60, or 120 min before ip stimulus with acetic acid 0.8% (Figure 1B). The time points 60 and 120 min of treatment, but not 15 or 30 min, significantly inhibited the writhing response (Figure 1B). Furthermore, the antinociceptive effect of **1** treatment at 60 min before stimulus was significantly greater than 30 min; thus, 60 min of pretreatment was selected for the next experiments. Other studies also used 60 min of pretreatment time.[2,13] The antinociceptive efficacy of **1** was also tested by treatment via other routes of administration. Mice were treated with **1** (3−30 mg/kg, 1 h; 5% Tween 80 diluted in saline) by subcutaneous (sc) (Figure 1C) or per oral (po) (Figure 1D) routes, and the acetic acid-induced writhing response was assessed. All doses of **1** by sc (Figure 1C) and the doses of 10 and 30 mg/kg by po treatment

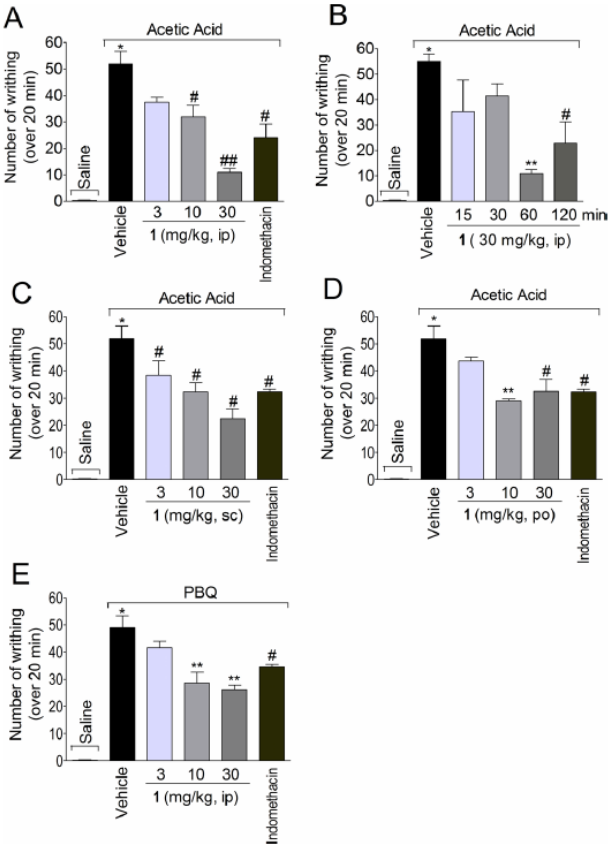



**Figure 1.** Vanillic acid (**1**) inhibits the writhing response induced by acetic acid and phenyl-*p*-benzoquinone (PBQ). Mice were treated with **1** (3−30 mg/kg, intraperitoneal [ip], 1 h, 5% Tween 80 in saline) or vehicle (ip, 1 h) before ip stimulus with acetic acid (0.8%, v/v in saline, 10 mL/kg) (A). The effect of varying the time of pretreatment with **1** at the dose of 30 mg/kg ip was tested at indicated time points (B). Treatment with **1** (3−30 mg/kg, 5% Tween 80 in saline) was also performed subcutaneously (sc) and per orally (po) before acetic acid stimulus (C and D, respectively). The effect of **1** (3−30 mg/kg, ip, 1 h, 5% Tween 80 in saline) on the PBQ-induced writhing response (1890 μg/kg, 2% DMSO in saline, 200 μL) was assessed (E). Treatment with indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as the control (A−E). The cumulative number of abdominal contortions (writhing score) was evaluated over 20 min. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group, $^{\#}p < 0.05$ comparing treatments with the vehicle group, $^{**}p < 0.05$ compared to the vehicle and to the time of 30 min or with the dose of 3 mg/kg of **1**, and $^{\#\#}p < 0.05$ compared to the vehicle group and to the doses of 3 and 10 mg/kg of **1** (one-way ANOVA followed by Bonferroni's multiple comparison test)].

reduced the number of abdominal contortions (Figure 1D). In a subsequent experiment, mice received **1** (3−30 mg/kg, ip, 1 h; 5% Tween 80 diluted in saline) and were stimulated with PBQ (diluted in 2% DMSO and saline, 1890 μg/kg), and the number of writhing events was evaluated. It was observed that **1** at 10 and 30 mg/kg inhibited PBQ-induced writhing (Figure 1E). There was no significant difference between doses of 10 and 30 mg/kg, but both doses presented a statistically significant effect compared to the dose of 3 mg/kg. The mechanisms of nociception involved in the acetic acid and PBQ models present similarities including the production of



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nociceptive mediators such as interleukin (IL)-33, tumor necrosis factor $\alpha$ (TNF-$\alpha$), IL-1$\beta$, prostanoids, and spinal activation of mitogen-activated protein (MAP) kinases.[15,16] On the other hand, each model also has singular mechanisms. For instance, the PBQ- but not the acetic acid-induced writhing response depends on cytokines such as IL-18 and interferon $\gamma$ (IFN-$\gamma$) and endothelin-1.[17] This is particularly important when there is inhibition in one model but not in the other; then the singular mechanisms of that model should be searched, which was not the case of the present study. The treatment with indomethacin (cyclooxygenase inhibitor, 5 mg/kg, 40 min, ip, diluted in Tris/HCl buffer) before ip stimulus with acetic acid or PBQ inhibited the writhing response (Figure 1A, C, D, and E). Thus, we can suggest that **1** could inhibit one or more of the nociceptive mechanisms shared by acetic acid and PBQ.

**Vanillic Acid (1) Inhibits Formalin- and Complete Freund's Adjuvant (CFA)-Induced Paw Flinch and Time Spent Licking the Paw.** Mice received **1** (3 and 30 mg/kg, ip; 5% Tween 80 diluted in saline) 1 h before intraplantar (ipl) injection of formalin 1.5% (25 $\mu$L) (Figure 2A and B) or CFA (10 $\mu$L) (Figure 2C and D). Compound **1** inhibited only the second phase of the formalin test (Figure 2A and B). The doses 3 and 30 mg/kg of **1** inhibited the formalin test regarding the number of paw flinches (Figure 2A). The time spent licking the paw was inhibited only by 30 mg/kg of compound **1** (Figure 2B). This finding is in agreement with a previous study showing **1** inhibited the second phase of the test,[13] but others also found that **1** inhibits both phases of the licking time of the formalin test.[2] The first phase (0−5 min) of the formalin test depends on neurotransmitter release such as serotonin and bradykinin, molecules from resident cells such as histamine,[18] and activation of transient receptor potential ankyrin 1 (TRPA1) expressed by neurons.[19] In the second phase (10−30 min), also known as the inflammatory phase, there is involvement of inflammatory mediators, for example, cytokines (IL-1$\beta$, TNF-$\alpha$, and IL-33) and prostaglandins, that are produced in response to the formalin stimulus.[16,18] Because the inhibition of the second phase of the formalin test was replicated by more than one laboratory (present data),[13] it is likely that the most prominent effect of **1** over formalin-induced nociception results in the inhibition of that phase only, demonstrating the importance of data presented in Figure 2A and B. In this context, the antinociceptive effect of **1** may be associated with the reduction of cytokines and prostaglandins, which mediate the second phase of the formalin test. Furthermore, previous evidence demonstrated **1** inhibits formalin-induced paw licking, and the present data show inhibition of paw flinching and licking. Paw flinching behavior depends on peripheral and spinal nociceptive processing, while paw licking has the addition of supraspinal nociceptive structures.[20,21] The present data advance by showing **1** may affect the three levels (peripheral, spinal, and supraspinal) of nociceptive processing involved in the formalin test. In CFA (10 $\mu$L/ipl)-induced overt pain-like behavior, mice were treated as in Figure 2A and B, and paw flinching (Figure 2C) and time spent licking the paw were assessed (Figure 2D). The dose of 30 mg/kg of **1** but not 3 mg/kg inhibited CFA-induced flinches (Figure 2C) and time spent licking (Figure 2D) induced by CFA (10 $\mu$L/ipl). The CFA-induced inflammation also depends on the production of IL-1$\beta$, TNF-$\alpha$, and $PGE_2$, which sensitizes the nociceptor.[18] Treatment with the control drug indomethacin (as in Figure 1) inhibited paw flinching and licking in the second phase of the formalin test and CFA inflammation (Figure 2). Thus, it is plausible that the antinociceptive mechanism of **1** may depend on the reduction of inflammatory mediator production.

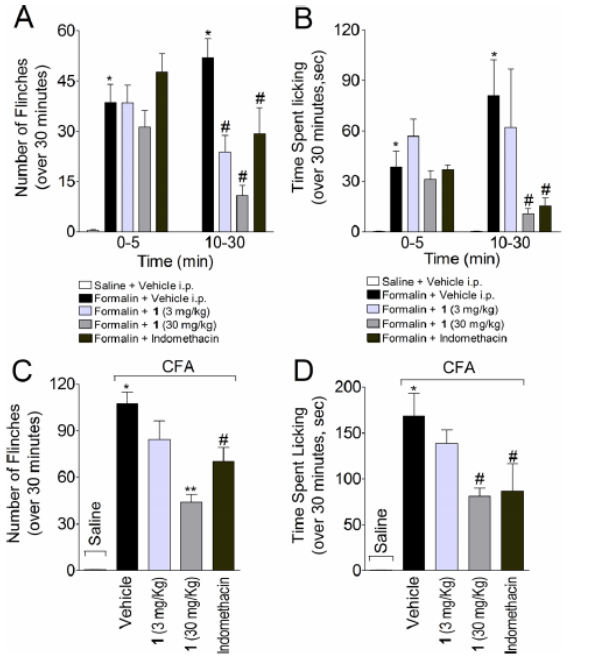


**Figure 2.** Vanillic acid (**1**) inhibits formalin- and complete Freund's adjuvant (CFA)-induced paw flinches and time spent licking the paw. Mice were treated with **1** (3 or 30 mg/kg, 5% Tween 80 in saline, ip) or vehicle (ip, 1 h) before intraplantar (ipl) injection of formalin (1.5%, v/v in saline, 25 $\mu$L) (A and B) or CFA (10 $\mu$L/ipl) (C and D). The total number of flinches (A and C) and the time spent licking the paw (B and D) were evaluated during 30 min in the formalin and CFA tests. Treatment with indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as a control (A−D). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group, $^{\#}p < 0.05$ comparing treatments with the vehicle group, and $^{**}p < 0.05$ compared to the vehicle group and with the dose of 3 mg/kg of **1** (one-way ANOVA followed by Bonferroni's multiple comparison test)].

**Vanillic Acid (1) Inhibits Carrageenan-Induced Mechanical Hyperalgesia, Paw Edema, and Neutrophil and Macrophage Recruitment.** Mice were treated with **1** (3−30 mg/kg, ip) 1 h before ipl injection of carrageenan (300 $\mu$g/paw). Mechanical hyperalgesia (Figure 3A) and paw edema (Figure 3B) were evaluated 1−5 h after stimulus. The dose of 3 mg/kg of **1** did not alter carrageenan-induced mechanical hyperalgesia (Figure 3A) (1−5 h), but reduced paw edema at 5 h (Figure 3B). The doses of 10 and 30 mg/kg inhibited mechanical hyperalgesia between 1 and 5 h (Figure 3A). Carrageenan-induced paw edema was inhibited by 10 mg/kg of **1** only at 5 h (Figure 3B), while 30 mg/kg of **1** inhibited paw edema at 1−5 h (Figure 2B). These results suggest the potential therapeutic applicability of **1** in acute inflammatory pain conditions. Next we investigated whether the analgesic effect of **1** would be related to inhibition of neutrophil and/or macrophage recruitment. Tissue neutrophil (Figure 3C) and macrophage (Figure 3D) recruitment was assessed in the mice paw skin tissue by determining the myeloperoxidase (MPO) and *N*-acetyl-$\beta$-D-glucosaminidase (NAG) activity, respectively.[22] No effect was observed with the doses of 3 and 10



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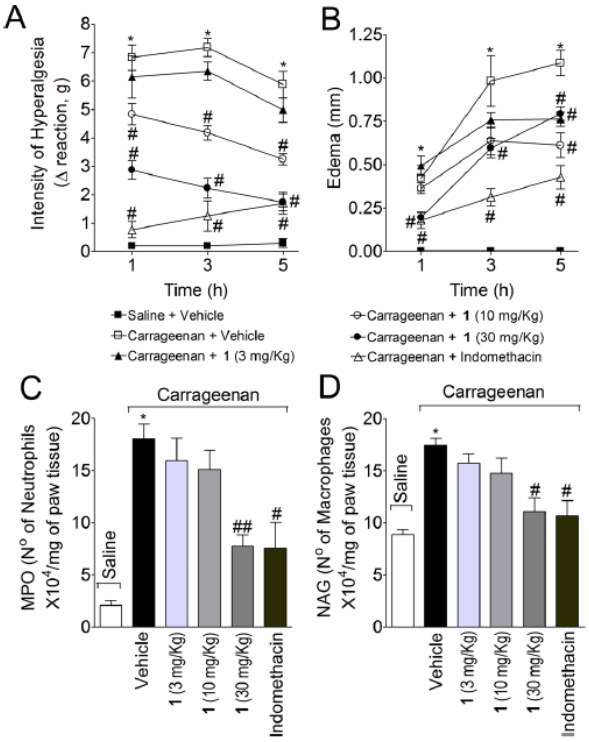


**Figure 3.** Vanillic acid (**1**) inhibits carrageenan-induced mechanical hyperalgesia, paw edema, and neutrophil and macrophage recruitment. Mice were treated with **1** (3−30 mg/kg, 5% Tween 80 in saline, ip, 1 h) or vehicle (ip, 1 h) before carrageenan (300 $\mu$g, ipl, 25 $\mu$L) or saline ipl injection followed by evaluation of mechanical hyperalgesia (A), paw edema (B), and recruitment of neutrophils (C, myeloperoxidase [MPO] activity) and macrophages (D, *N*-acetyl-$\beta$-D-glucosaminidase [NAG] activity). The intensity of mechanical hyperalgesia and paw edema was measured for 1−5 h by an electronic pressure meter and an analog caliper, respectively. At the 5th hour paw skin samples were collected for MPO and NAG activity assays. Treatment with indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as a control (A−D). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group, $^{\#}p < 0.05$ comparing treatments with the vehicle group, and $^{\#\#}p < 0.05$ compared to the vehicle and to the doses of 3 and 10 mg/kg of **1** (one-way ANOVA followed by Bonferroni's multiple comparison test)].

mg/kg of **1** in both tests. The dose of 30 mg/kg of **1** inhibited neutrophil (Figure 3C) and macrophage (Figure 3D) recruitment to the paw skin tissue. The control drug indomethacin inhibited carrageenan-induced mechanical hyperalgesia, paw edema, and neutrophil and macrophage recruitment (Figure 3). Inhibition of leukocyte recruitment is a consistent effect of **1** since it also inhibited carrageenan-induced neutrophil recruitment to the rat peritoneal cavity.[2] Recruited leukocytes play an essential role in inflammatory pain by further producing nociceptive mediators.[23] Thus, reducing leukocyte recruitment to the paw skin may constitute a contributing analgesic and anti-inflammatory mechanism of **1**.

**Vanillic Acid (1) Inhibits CFA-Induced Mechanical Hyperalgesia, Paw Edema, and Neutrophil and Macrophage Recruitment.** In order to further investigate the therapeutic applicability of **1**, the antinociceptive and anti-inflammatory effects of **1** were tested in the CFA-induced inflammatory pain model. Mice received an ipl injection of CFA (10 $\mu$L/ipl) and after 24 h started to receive daily treatment with **1** (3, 10, and 30 mg/kg, ip). Mechanical hyperalgesia (Figure 4A) and paw edema (Figure 4B) were assessed daily.

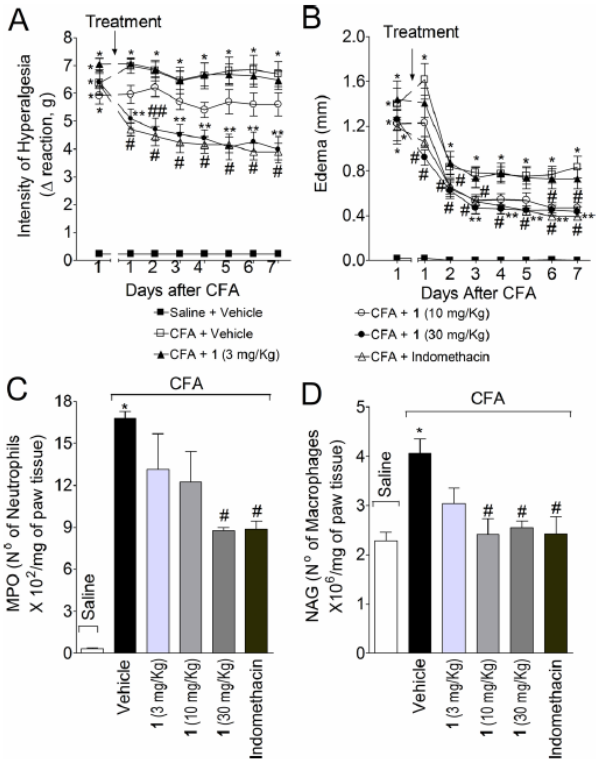


**Figure 4.** Vanillic acid (**1**) inhibits CFA-induced persistent inflammatory mechanical hyperalgesia, paw edema, and neutrophil and macrophage recruitment. Mice were post-treated with **1** (3−30 mg/kg, 5% Tween 80 in saline, ip) or vehicle (ip) daily, for 7 days, starting 24 h after CFA (10 $\mu$L/ipl) stimulus. Mechanical hyperalgesia (A) and paw edema (B) were assessed at indicated time points, and on the 7th day after the beginning of the treatment, paw skin samples were collected for myeloperoxidase [MPO] activity (C) and *N*-acetyl-$\beta$-D-glucosaminidase [NAG] (D) activity assays. Treatment with indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as a control (A−D). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group, $^{\#}p < 0.05$ comparing treatments with the vehicle group, $^{**}p < 0.05$ compared to the vehicle group and with the dose of 3 mg/kg of 1, and $^{\#\#}p < 0.05$ compared to the vehicle group and to the doses of 3 and 10 mg/kg of **1** (one-way ANOVA followed by Bonferroni's multiple comparison test)].

The doses of 3 and 10 mg/kg of compound **1** did not alter CFA-induced mechanical hyperalgesia (Figure 4A). Furthermore, the dose of 30 mg/kg of **1** inhibited CFA-induced mechanical hyperalgesia (Figure 4A) at all time points evaluated. Regarding CFA-induced paw edema (Figure 4B), the dose of 3 mg/kg of **1** did not alter the response. The dose of 10 mg/kg inhibited paw edema only at days 2, 3, 6, and 7, and the dose 30 mg/kg inhibited paw edema at all time points after treatment. On the last day (day 7), mice were euthanized after all measurements of hyperalgesia and edema for paw skin sample collection and determination of MPO (Figure 4C) and NAG (Figure 4D) activity assays. Compound **1** at the dose of 30 mg/kg, but not 3 and 10 mg/kg, inhibited CFA-induced



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neutrophil recruitment (MPO activity) (Figure 4C). CFA-induced recruitment of macrophages (NAG activity) (Figure 4D) was inhibited by 10 and 30 mg/kg of **1**. The control drug indomethacin (2.5 mg/kg, ip, 7 days of treatment) inhibited CFA-induced mechanical hyperalgesia, paw edema, and MPO and NAG activities (Figure 4). These results demonstrated that **1** is also effective in treating established inflammatory pain, edema, and leukocyte recruitment (Figure 4). In addition to cellular recruitment, oxidative stress[24,25] and cytokine production[18,26] are important mechanisms of carrageenan- and CFA-induced pain and inflammation.

**Vanillic Acid (1) Does Not Induce Liver or Stomach Lesions.** Nonsteroidal anti-inflammatory drugs are a widely clinically used class of anti-inflammatory and analgesic drugs. However, they may induce liver toxicity and/or stomach lesions.[27,28] Therefore, it is important to determine whether a novel molecule with potential therapeutic use as an anti-inflammatory and analgesic also induces similar side effects to currently used drugs. To compare the effect of compound **1** with indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), mice were treated daily with **1** (30 mg/kg, ip, 5% Tween 80 diluted in saline) or indomethacin (positive control, 2.5 mg/kg, ip, diluted in Tris/HCl buffer) for 7 days, which is a common time period of anti-inflammatory treatment.[29] The dose of **1** was selected based on results presented in Figures 1−4. No significant difference was observed in the plasmatic levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Figure 5) between **1** and vehicle-treated mice. On the other hand, indomethacin, an NSAID, induced a significant increase in AST and ALT levels. AST and ALT levels in the plasma are widely used to assess liver damage.[22] These data demonstrated that treatment with **1** for 7 days does not induce liver damage, while the positive control of indomethacin induced a significant increase of these enzymes. Nonsteroidal anti-inflammatory drugs that are nonselective inhibitors of COX-1 induce gastric lesions, observed as increased MPO activity in stomach tissue samples.[27] Using the same protocol, we found the vehicle and compound **1** did not alter MPO activity, while indomethacin induced a significant increase in MPO activity in stomach samples, indicating tissue lesions (Figure 5). Therefore, treatment with **1** for 7 days does not induce liver toxicity or stomach damage and seems to be a safe compound in a common time of treatment for anti-inflammatory drugs. It has been demonstrated that **1** inhibits COX-2 expression in Dextran Sulfate Sodium-induced colitis[3] and LPS-induced COX-2 expression and prostaglandin $E_2$ production by macrophages.[4] Therefore, it is likely that the mechanism of action of **1** depends on inhibiting COX-2 expression rather than inhibiting COX activity, and as a result, it does not induce gastric damage.

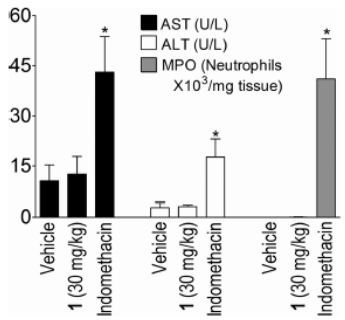


**Figure 5.** Vanillic acid (**1**) does not induce liver damage or stomach lesions. Mice were treated daily with **1** (30 mg/kg, 5% Tween 80 in saline, ip), indomethacin (2.5 mg/kg, in Tris/HCl buffer, pH 8.0, ip), or vehicle (saline or Tris/HCl buffer, ip) for 7 days. Plasma and stomach samples were collected. Liver and stomach damage was assessed by determining plasmatic levels of AST and ALT or MPO activity, respectively. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$*p < 0.05$ compared with the vehicle group (one-way ANOVA followed by Bonferroni's multiple comparison test)].

**Vanillic Acid (1) Prevents Carrageenan-Induced Oxidative Stress.** Oxidative imbalance plays an important role in inflammatory pain.[25,30] In fact, antioxidants inhibit carrageenan-induced hyperalgesia.[25,31] In this sense, the effect of **1** on carrageenan-induced oxidative stress was assessed by the antioxidant capacity and lipid peroxidation in the plantar tissue. Mice were treated with **1** (3 and 30 mg/kg, ip, 5% Tween 80 diluted in saline) 1 h before ipl injection of carrageenan (300 $\mu$g/paw), samples of cutaneous plantar tissue were collected after 3 h, and the antioxidant capacity was assessed by the ferric reducing potential (FRAP) assay (Figure 6A), ability to scavenge 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) assay (Figure 6B), endogenous reduced glutathione (GSH) levels (Figure 6C), and lipid peroxidation by determining thiobarbituric acid reactive substance (TBARS) (Figure 6D). Compound **1** at the dose of 30 mg/kg, but not at the dose of 3 mg/kg, prevented carrageenan-induced reduction of FRAP (Figure 6A), the ability to scavenge ABTS (Figure 6B), and GSH levels (Figure 6C) in the plantar tissue induced by carrageenan. Reduced antioxidant capacity can result in oxidative stress.[32] Thus, treatment with 30 mg/kg of **1** can inhibit carrageenan-induced oxidative stress by preventing the depletion in the antioxidant capacity. Corroborating our findings, **1** prevented the depletion of GSH and enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione reductase and the increased lipid peroxidation in a model of cardiotoxicity in rats.[1] *In vitro* studies have also demonstrated that **1** reduces oxygen radical absorbance capacity[33] and scavenges DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS,[33] superoxide, and hydroxyl radicals.[1] The hydroxyl radical is an initiator of lipid peroxidation,[34] a major outcome of free radical-mediated injury to tissues and one of the most important results of oxidative stress. In fact, the quantification of the end products (TBARS) of this event is commonly used to assess oxidative stress in tissues.[35] Compound **1** at the dose of 30 mg/kg, but not 3 mg/kg, also inhibited carrageenan-induced lipid peroxidation (Figure 6D). Studies have shown that the reduction of oxidative stress is an antinociceptive mechanism,[25,30,31] indicating the prevention of oxidative stress as an important analgesic mechanism of **1** in carrageenan-induced inflammatory pain. Indomethacin inhibited the oxidative stress, as observed in the FRAP (Figure 6A), GSH (Figure 6C), and TBARS (Figure 6D) assays without affecting the ABTS assay (Figure 6B). Recruited leukocytes contribute to oxidative stress and hyperalgesia because activated leukocytes are sources of prostaglandins, leukotriene $B_4$, and reactive oxygen species (ROS) known to have nociceptor-sensitizing properties, resulting in inflammatory pain.[36−39] Accordingly, we observed that **1** reduced carrageenan-induced mechanical hyperalgesia (Figure 3A), MPO activity (Figure 3C), NAG activity (Figure 3D), and oxidative stress (Figure 6). Thus, these data support the notion that inhibiting leukocyte recruitment to inflammatory foci



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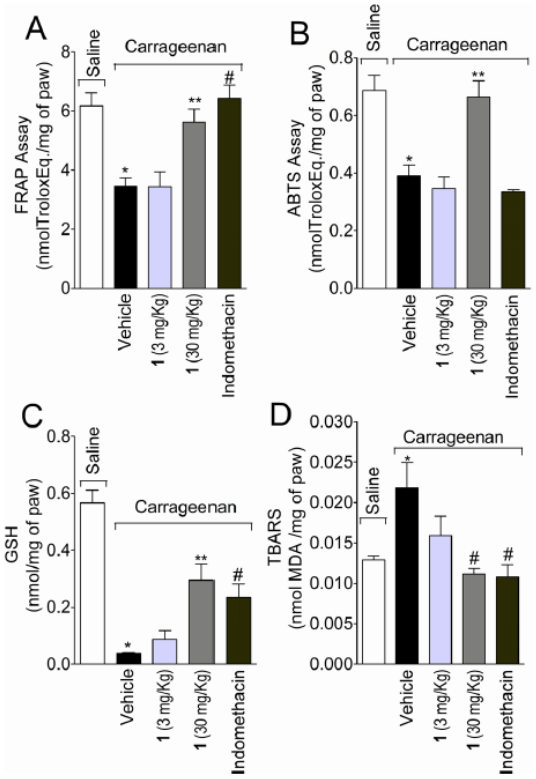


**Figure 6.** Vanillic acid (**1**) inhibits carrageenan-induced oxidative stress. Mice were treated with **1** (3 or 30 mg/kg, 5% Tween 80 in saline, ip, 1 h) or vehicle (ip, 1 h) before carrageenan (300 $\mu$g/paw) or saline (25 $\mu$L) ipl injection. Paw skin samples were collected 3 h after stimulus, and oxidative stress was assessed by determining ferric reducing antioxidant power (FRAP assay) (A), free radical scavenging ability (ABTS assay) (B), GSH levels (C), and lipid peroxidation (TBARS assay) (D). Treatment with indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as a control (A–D). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group, $^{\#}p < 0.05$ comparing treatments with the vehicle group, and $^{**}p < 0.05$ compared to the vehicle group and to the dose of 3 mg/kg of **1** (one-way ANOVA followed by Bonferroni's multiple comparison test)].

contributes to the inhibition of oxidative stress by treatment with **1**. On the other hand, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase-derived ROS have an important role as signaling molecules in the directionality of neutrophils toward the inflammatory foci, and targeting ROS reduces the chemotaxis of neutrophils.[40] Therefore, it is also likely that inhibition of oxidative stress by **1** reduces the migration of leukocytes to the inflammatory foci, resulting in diminished participation of recruited leukocytes in amplifying inflammatory pain.

**Vanillic Acid (1) Inhibits Carrageenan-Induced Pro-inflammatory Cytokine (IL-1$\beta$, TNF-$\alpha$, and IL-33) Production.** The effect of **1** in cytokine production was evaluated. Mice were treated with **1** (3 and 30 mg/kg, ip, 5% Tween 80 diluted in saline) 1 h before ipl injection of carrageenan (300 $\mu$g/paw), and samples of cutaneous plantar tissue were collected 3 h after stimulus injection for cytokine (IL-1$\beta$, TNF-$\alpha$, and IL-33) level determination (Figure 7). Pro-hyperalgesic cytokines, including IL-1$\beta$, TNF-$\alpha$, and IL-33,

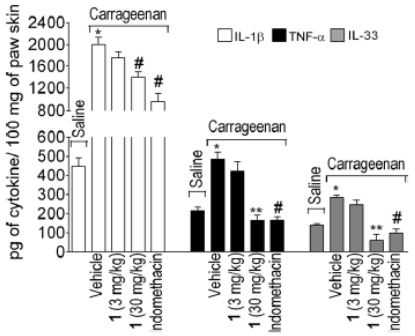


**Figure 7.** Vanillic acid (**1**) inhibits carrageenan-induced cytokine (IL-1$\beta$, TNF-$\alpha$, and IL-33) production. Mice were treated with **1** (3 or 30 mg/kg, 5% Tween 80 in saline, ip, 1 h) or vehicle (ip, 1 h) before carrageenan (300 $\mu$g/paw) or saline (25 $\mu$L) ipl injection. Paw skin samples were collected 3 h after stimulus, and IL-1$\beta$, TNF-$\alpha$, and IL-33 levels were determined by enzyme-linked immunosorbent assay (ELISA). Treatment with indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as a control. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group, $^{\#}p < 0.05$ comparing treatments with the vehicle group, and $^{**}p < 0.05$ compared to the vehicle group and with the dose of 3 mg/kg of **1** (one-way ANOVA followed by Bonferroni's multiple comparison test)].

induce inflammatory pain by stimulating the production of molecules such as $PGE_2$ and/or sympathetic amines that sensitize nociceptors, resulting in hyperalgesia.[26,41−43] In this sense, it is important to determine whether **1** affects cytokine production. In agreement with the behavioral data, the dose of 30 mg/kg of **1** inhibited carrageenan-induced production of IL-1$\beta$, TNF-$\alpha$, and IL-33 and also presented significant statistical differences compared to the dose of 3 mg/kg on TNF-$\alpha$ and IL-33 levels (Figure 7). The dose of 3 mg/kg of **1** did not alter cytokine production (Figure 7). In agreement, **1** inhibited TNF-$\alpha$, IL-6, and $PGE_2$ production by lipopolysaccharide-stimulated macrophages *in vitro*.[4] Compound **1** may also selectively affect IL-1$\beta$ maturation since it inhibited LPS-induced caspase-1 expression *in vitro* by macrophages.[4] LPS-induced hyperalgesia depends on hyperalgesic cytokine production similarly to carrageenan and other models of inflammation,[44] suggesting modulating cytokine production as a potentially general mechanism of action of **1**. Furthermore, cytokines are involved in oxidative stress. For instance, TNF-$\alpha$ and IL-1$\beta$ can activate NADPH oxidase, inducing the production of superoxide anion and starting the generation of ROS.[30,45] On the other hand, oxidative stress induces the production of cytokines.[14] Therefore, there is an interdependent relationship between cytokines and oxidative stress, which is significantly dependent on the transcription factor NF$\kappa$B, since it can be activated by cytokines and oxidative stress, resulting in the further production of cytokines and enzymes responsible for the production of ROS.[14] Treatment with the control drug indomethacin (5 mg/kg, ip, 40 min) also inhibited IL-1$\beta$, TNF-$\alpha$, and IL-33 production (Figure 7). In addition to the inhibition of COX-1 activity, indomethacin also inhibits NF$\kappa$B activation depending on the dose, an effect that explains the inhibition of cytokine production.[46]

**Vanillic Acid Suppresses Carrageenan-Induced Activation of NF$\kappa$B.** The effect of **1** on the activation of NF$\kappa$B was evaluated. Mice received **1** (30 mg/kg, ip, 5% Tween 80 diluted



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in saline) 1 h before ipl injection of carrageenan (300 $\mu$g/paw), and samples of the cutaneous plantar tissues were collected 3 h after stimulus injection. Carrageenan-induced activation of NF$\kappa$B was observed by a decrease in the ratio of total NF$\kappa$B/phosphorylated NF$\kappa$B, while treatment with **1** as well as the control drug indomethacin (5 mg/kg, ip, diluted in Tris/HCl buffer, 40 min before stimulus) inhibited carrageenan-induced activation of NF$\kappa$B (Figure 8). The suppression of NF$\kappa$B

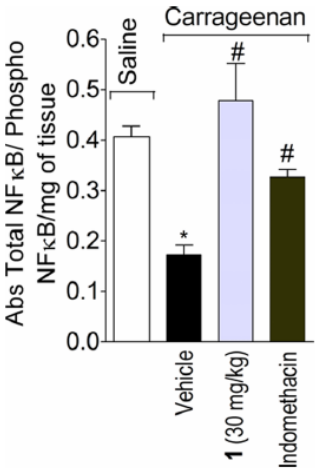


**Figure 8.** Vanillic acid (**1**) inhibits carrageenan-induced NF$\kappa$B activation. Mice were treated with **1** (30 mg/kg, 5% Tween 80 in saline, ip, 1 h) or vehicle (ip, 1 h) before carrageenan (300 $\mu$g/paw) or saline (25 $\mu$L) ipl injection. Paw skin samples were collected 3 h after the stimulus, and NF$\kappa$B activation was determined by ELISA. Indomethacin (5 mg/kg, ip, 40 min, in Tris/HCl buffer, pH 80) was used as the control drug. Results are shown by the ratio total NF$\kappa$B/phospho NF$\kappa$B p65 subunit and presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ comparing the vehicle group with the saline group and $^{\#}p < 0.05$ comparing treatments with the vehicle group (one-way ANOVA followed by Bonferroni's multiple comparison test)].

activation has been previously linked with anti-inflammatory effects since NF$\kappa$B is the major transcription factor involved in the production of pro-inflammatory cytokines and the expression of a variety of enzymes involved in inflammatory diseases such as NADPH oxidase, which is responsible for superoxide anion production.[47] Compound **1** inhibited LPS-induced phosphorylation of I$\kappa$B (cytoplasmatic NF$\kappa$B inhibitor) in the cytoplasm extract and NF$\kappa$B p65 subunit expression in the nuclear extract of macrophages *in vitro* as determined by Western blot.[4] The present data further advance the effect of **1** over NF$\kappa$B activation by demonstrating *in vivo* by quantitative assay (ELISA) that it inhibits carrageenan-induced NF$\kappa$B activation in experimental conditions related to mechanical hyperalgesia, paw edema, and leukocyte recruitment (MPO and NAG activity assays).

In conclusion, the present study has demonstrated the analgesic and anti-inflammatory effects of vanillic acid (**1**) and that such effects are dependent on targeting leukocyte recruitment, oxidative stress, cytokine production, and NF$\kappa$B activation. Importantly, in a 7-day treatment regimen that inhibited CFA-induced inflammation and pain, **1** did not induce gastric lesions or liver damage, further corroborating its usefulness. Therefore, the present data showed that vanillic acid (**1**) deserves to be further investigated in preclinical and clinical studies as an anti-inflammatory and analgesic molecule.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** During the experiments, mice received intraperitoneal (3, 10, or 30 mg/kg) treatment with vanillic acid (**1**) or vehicle (5% Tween 80 diluted in saline) 1 h before or 24 h after inflammatory stimuli injection as indicated. In all experiments, indomethacin (2.5 or 5 mg/kg, ip, diluted in Tris/HCL buffer) was used as reference standard drug. The doses of inflammatory stimuli were determined previously in our laboratory in pilot studies and are based on previous work.[15,25,26,29,41] The writhing response was evaluated during 20 min after ip injection of acetic acid (0.8%) or phenyl-*p*-benzoquinone (1890 $\mu$g/kg). The paw flinching and licking nociceptive responses were quantified during 30 min after formalin 1.5% (25 $\mu$L/ipl) or CFA (10 $\mu$L/ipl) injection. Mechanical hyperalgesia was evaluated 1−5 h after carrageenan (300 $\mu$g/ipl) or during 7 days after CFA (10 $\mu$L/ipl) stimulus. All inflammatory stimuli induced only ipsilateral (in the paw in which the stimulus was injected) mechanical hyperalgesia. FRAP, ABTS, GSH, TBARS, IL-1$\beta$, TNF$\alpha$, IL-33, and NF$\kappa$B levels were evaluated 3 h after carrageenan (300 $\mu$g/paw) injection. MPO or NAG activities were evaluated 5 h or 7 days after carrageenan or CFA administration, respectively. The plasma concentration of AST and ALT and stomach MPO activity were determined after 7 days of treatment. Different individuals prepared the solutions to be injected, performed the injections, and performed the measurements.

**Test Compounds.** The compounds used in this study were saline (NaCl 0.9%; Fresenius Kabi Brasil Ltda Aquiraz, CE, Brazil), 5% Tween 80, 2% DMSO, vanillic acid (**1**; 97% purity from Sigma-Aldrich, St. Louis, MO, USA), complete Freund's adjuvant, phenyl-*p*-benzoquinone (Sigma-Aldrich), carrageenan (Santa Cruz Biotechnology, Santa Cruz, CA, USA), acetic acid and formaldehyde (Mallinckrodt Baker, S.A., Mexico, Mexico City), and indomethacin (Prodome, Campinas, SP, Brazil).

**Animals.** Male Swiss mice (25−30 g), from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21 °C. All behavioral testing was performed between 9 A.M. and 5 P.M. in a temperature-controlled room. Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number no. 10716.2013.53). Every effort was made to minimize the number of animals used and their suffering.

**Nociception Tests. Writhing Response Tests.** The PBQ- and acetic acid-induced writhing models were performed as previously described.[17] PBQ (DMSO 2% diluted in saline, 1890 $\mu$g/kg), acetic acid (0.8% v/v, diluted in saline, 10 mL/kg), or vehicle was injected into the peritoneal cavities of mice. Each mouse was placed in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs. The intensity of the writhing response was expressed as the cumulative writhing score over 20 min.

**Formalin Test.** The number of paw flinches and the time spent licking the paws were determined between 0 and 30 min after intraplantar injection of 25 $\mu$L of formalin 1.5%, as previously described.[48] The period was divided into intervals of 5 min and clearly demonstrated the presence of the first and second phases, which are characteristic of the method.[48] Results were obtained for both the first (0−5 min) and second (10−30 min) phases.

**Complete Freund's Adjuvant-Induced Overt Pain-like Behavior.** The number of paw flinches and time spent licking the stimulated paw were determined between 0 and 30 min after ipl injection of 10 $\mu$L of CFA. Results were expressed by the total number of flinches and licks performed in 30 min.[48]



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**Carrageenan- and CFA-Induced Mechanical Hyperalgesia.** In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 15−30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 $mm^2$ polypropylene tip.[49] The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements 1, 3, or 5 h after stimulus of carrageenan (300 μg/paw) or measurements in 1−7 days after CFA stimulus (10 μL/paw). Withdrawal threshold was 9.7 ± 0.09 g (mean ± SEM; $n$ = 72).

**Carrageenan- and CFA-Induced Paw Edema.** The paw edema was determined before and at indicated time points after the injection of carrageenan or CFA using an analog caliper. Paw edema was presented as Δ mm.[29]

**Myeloperoxidase and *N*-Acetyl-β-D-glucosaminidase Activity.** The neutrophil recruitment to the paw skin and stomach tissue and macrophage recruitment to the paw skin was evaluated by the MPO and NAG colorimetric assays, respectively, as previously described.[22,48] Briefly, mice were terminally anesthetized, and the paw skin samples were collected in 400 μL of 50 mM $K_2HPO_4$ buffer (pH 6.0) containing 0.5% HTAB and then homogenized in ice-cold Tissue-Tearor (Biospec). After that, homogenates were centrifuged (16100$g$, 2 min, 4 °C), and the supernatants were collected. For the MPO assay, aliquots of 30 μL of supernatant were placed in a 96-well plate and mixed with 200 μL of 50 mM $K_2HPO_4$ buffer (pH 6.0), containing 0.0167% *ortho*-dianisidine dihydrochloride and 0.05% $H_2O_2$. The absorbance was determined after 5 min at 450 nm (Multiskan GO microplate spectrophotometer, ThermoScientific, Vantaa, Finland). The MPO activity of samples was compared to a standard curve of neutrophils and presented as MPO activity. For NAG activity, 20 μL of supernatant was obtained as described for the MPO activity assay and added to a 96-well plate, followed by the addition of 80 μL of 50 mM phosphate buffer, pH 6.0. The reaction was initiated by adding 2.24 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide. Then, the plate was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 100 μL of 0.2 M glycine buffer, pH 10.6. The enzymatic activity was determined at 400 nm (Multiskan GO microplate spectrophotometer, ThermoScientific, Vantaa, Finland). NAG activity of samples was compared to a standard curve of macrophages and presented as NAG activity.

**Hepatotoxicity.** Plasma concentrations of AST and ALT were used as indicators of hepatotoxicity. These assays were performed using a diagnostic kit from Labtest (Lagoa Santa, Minas Gerais, Brazil).[22]

**ABTS and FRAP Assays.** The ability of samples to resist oxidative damage was determined as free radical scavenging ability (ABTS assay) and ferric reducing ability (FRAP assay). The tests were adapted to a 96-well microplate format from previously described assays.[31] Plantar tissue samples were collected 3 h after ipl stimulus with carrageenan (300 μg/paw) and immediately homogenized with 500 μL of 1.15% KCl. The homogenates were centrifuged (10 min × 200$g$ × 4 °C), and the supernatants were used in both assays. For the ABTS assay, ABTS solution was diluted with phosphate buffer saline (PBS) pH 7.4 to an absorbance of 0.80 at 730 nm. Then 200 μL of diluted ABTS solution was mixed with 20 μL of sample in each well. After 6 min of incubation at 25 °C, the absorbance was measured at 730 nm. For the FRAP assay, 50 μL of supernatant was mixed with 150 μL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm. The results of ABTS and FRAP assays were equated against a Trolox standard curve (0.02−20 nmol). The results of both assays are expressed as nmol of Trolox equivalents per mg of tissue, which is the amount of Trolox (nmol) with an equivalent antioxidant potential to 1 mg of the tissue under investigation.

**Reduced Glutathione Assay.** Paw skin samples were collected 3 h after ipl stimulus with carrageenan (300 μg/paw) to determine GSH levels (pool of four paw samples) using a spectrophotometric method. Samples were homogenized (IKA T10) in 4 mL of 0.02 M EDTA. Homogenates (2.5 mL) were treated with 2 mL of Milli Q $H_2O$ plus 0.5 mL of 50% trichloroacetic acid. After 15 min, the homogenates were centrifuged at 1500$g$ for 15 min, and 1 mL from the supernatant was added to 2 mL of a solution containing Tris 0.4 M (pH 8.9) plus 50 μL of DTNB. After 5 min, the measurements were performed in 412 nm against a blank control [UV−vis spectrophotometer (UV-1650, Shimadzu)]. The results are presented as nmol of GSH per mg of tissue.[31]

**Lipid Peroxidation.** Paw skin samples were collected 3 h after ipl stimulus with carrageenan (300 μg/paw) to determine lipid peroxidation by determining TBARS levels using a method previously described. For this assay, 10% TCA was added to the homogenate, and the mixture was centrifuged (1000$g$, 3 min, 4 °C) to precipitate proteins. The protein-free supernatant was then separated and mixed with TBA (0.67%). The mixture was kept in a water bath (15 min, 100 °C). Malondialdehyde (MDA), an intermediate product of lipid peroxidation, was determined by the difference between absorbances at 535 and 572 nm using a microplate spectrophotometer reader. The results are presented as nmol of MDA per mg of tissue.[50]

**Cytokine Measurement.** Paw skin samples were collected 3 h after ipl stimulus with carrageenan (300 μg/paw). The samples were homogenized in 500 μL of buffer containing protease inhibitors, and IL-1β, TNF-α, and IL-33 levels were determined as described previously by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results are expressed as picograms (pg) of cytokine/100 mg of tissue.[51]

**NFκB Activity.** Plantar tissue samples were collected 3 h after ipl stimulus with carrageenan (300 μg/paw). The assessment of total and phosphorylated NFκB production was performed by ELISA kits as specified by the manufacturer. The samples were removed and homogenized in lysis buffer and were centrifuged (3000 rpm/10 min, 4 °C), and the supernatant was used to assess the levels of total and phosphorylated NFκB by ELISA following the manufacturer's instructions. The results were obtained by comparing the optical density of samples and the samples' weight.[52]

**Statistical Analysis.** Results are presented as means ± SEM of measurements made on six mice in each group per experiment and are representative of two separate experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Bonferroni's multiple comparison test was performed on each occasion. On the other hand, when the hyperalgesic responses were measured once after the administration or enforcement of the stimuli, the differences between responses were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test. Statistical differences were considered to be significant at $p < 0.05$.

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### Notes

The authors declare no competing financial interest.



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## 4.2 Artigo 2

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*Research Article*

# Quercetin Reduces Ehrlich Tumor-Induced Cancer Pain in Mice

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Cancer pain directly affects the patient's quality of life. We have previously demonstrated that the subcutaneous administration of the mammary adenocarcinoma known as Ehrlich tumor induces pain in mice. Several studies have shown that the flavonoid quercetin presents important biological effects, including anti-inflammatory, antioxidant, analgesic, and antitumor activity. Therefore, the analgesic effect and mechanisms of quercetin were evaluated in Ehrlich tumor-induced cancer pain in mice. Intraperitoneal (i.p.) treatments with quercetin reduced Ehrlich tumor-induced mechanical and thermal hyperalgesia, but not paw thickness or histological alterations, indicating an analgesic effect without affecting tumor growth. Regarding the analgesic mechanisms of quercetin, it inhibited the production of hyperalgesic cytokines IL-1$\beta$ and TNF$\alpha$ and decreased neutrophil recruitment (myeloperoxidase activity) and oxidative stress. Naloxone (opioid receptor antagonist) inhibited quercetin analgesia without interfering with neutrophil recruitment, cytokine production, and oxidative stress. Importantly, cotreatment with morphine and quercetin at doses that were ineffective as single treatment reduced the nociceptive responses. Concluding, quercetin reduces the Ehrlich tumor-induced cancer pain by reducing the production of hyperalgesic cytokines, neutrophil recruitment, and oxidative stress as well as by activating an opioid-dependent analgesic pathway and potentiation of morphine analgesia. Thus, quercetin treatment seems a suitable therapeutic approach for cancer pain that merits further investigation.

## 1. Introduction

Approximately 50% of all cancer patients have pain [1] in early-state cancer or advanced cancer [1–4]. Cancer patients may present hyperalgesia, allodynia, and spontaneous pain, which account for poor life quality [5]. Cancer pain is a severe clinical health problem for these patients and currently the treatment for this pain is inadequate enhancing this problem [6]. In fact, at least half patients with cancer pain have received inadequate analgesic therapy [7]. One explanation for inadequate analgesic prescription could be a failure to identify pain mechanisms [2].

Several studies have demonstrated the participation of varied pathways and mediators involved in cancer pain development, such as cytokines [8–10], spinal glial activation [11–14], transient receptor potential vanilloid receptor 1 (TRPV1), acid-sensing ion channels (ASICs), bradykinin, adenosine triphosphate (ATP), endothelin [15], reactive oxygen species [16], and intracellular signaling pathway such as mitogen-activated protein kinases p38 [17] and JNK [18]. Cancer pain mechanisms are also dependent on the cancer type implicating that some slight variations in the mechanisms or role of a certain pathway may be greater depending on cancer type. Therefore, cancer pain is a complex condition and as

already mentioned its control might also depend on adequate pharmacological tools. Opioids are effective clinically used analgesics in cancer pain; however, they have many side effects that increase with the dose of opioid and, in addition to tolerance, the dose regimen increases with the tumor growth [19]. Thus, it is important to find novel therapeutic approaches to reduce cancer pain and/or improve current clinical therapies.

Flavonoids such as quercetin present low toxicity [20], which together with its antinociceptive effect in models of inflammation [21] and neuropathic pain [22] suggests its usefulness as an analgesic drug. Moreover, cancer pain might present components of inflammatory pain related to the inflammatory response against the tumor cells and neuropathic pain related to neuronal damage and nerve compression. It has been demonstrated in models of inflammation that the mechanisms of quercetin are related to inhibition of oxidative stress and cytokine production [23, 24]. In models of diabetic neuropathic pain, quercetin induces an analgesic effect amenable by opioid receptor antagonist [22]. In fact, inhibition of oxidative stress, cytokine production, and opioid receptor-dependent effects seem to be major mechanisms of quercetin since they were also observed in models such as colitis [25], neuropathy [26], hepatic fibrosis [27], periodontitis-induced bone resorption [28], and allergic inflammation [29].

In the present study, the analgesic activity and mechanisms of quercetin were investigated in Ehrlich tumor-induced cancer pain in mice [30]. This is a model of murine mammary adenocarcinoma-induced pain presenting features like those of preoperative breast cancer with spontaneous pain and pain upon examination (pressure of the lump, hyperalgesia) [30–32] with the benefit of development in standard Swiss mice. Furthermore, Ehrlich tumor induces bone/cartilage destruction indicating the possible involvement of a bone pain component in its nociceptive mechanisms [30].

## 2. Material and Methods

*2.1. General Experimental Procedures.* The measurement of basal responses to mechanical and thermal stimuli and paw thickness was performed at day 0. Afterwards, mice received intraplantar (i.pl.) injection of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$). Ehrlich's tumor cells are cultivated *in vivo*, by passages in the peritoneum of Swiss mice in ascitic form. Ten days after the intraperitoneal (i.p.) injection of 0.2 mL of ascitic peritoneal fluid containing Ehrlich tumor cells in mice, the ascitic fluid of tumor cells was collected and washed in phosphate-buffered saline (PBS, pH 7.4) followed by centrifugation (200 g, 10 min) three times. The cell viability was determined by 0.5% trypan blue exclusion method in Neubauer chamber. Ehrlich tumor cells were suspended to the final concentrations of $1 \times 10^6$ or $1 \times 10^7$ in 25 $\mu$L of saline and injected into the subcutaneous tissue of mice, which passes from ascitic form to solid form [30]. Mice received the Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ in 25 $\mu$L of saline) and received the acute treatment with quercetin (10–100 mg/kg, i.p.) or vehicle (2% DMSO in saline) on the 8th day after injection of the cells, and mechanical and thermal hyperalgesia and paw thickness were determined after 1, 3, 5, and 7 h. For chronic treatment, mice were treated with quercetin (10–100 mg/kg, i.p) 10 min after Ehrlich tumor cells injection followed by daily treatment. Mechanical and thermal hyperalgesia and paw thickness were evaluated on days 2, 4, 6, 8, 10, and 12 after the injection of $1 \times 10^6$ cells and 3 h after treatment with quercetin. A control group received saline (25 $\mu$L/paw, vehicle of Ehrlich tumor cells) and quercetin (100 mg/kg, i.p) treatment. On the 12th day of the injection of tumor cells, 3 h after the daily treatment with quercetin (100 mg/kg i.p., both tumor and saline group) or vehicle, paw samples were collected for histological analysis and microscopic observation. Paw skin and spinal cord samples were collected to determine myeloperoxidase (MPO) activity, interleukin-1$\beta$ (IL-1$\beta$), and tumor necrosis factor $\alpha$ (TNF$\alpha$) concentration by ELISA, FRAP, ABTS, and GSH levels. In another set of experiments, mice received $1 \times 10^7$ Ehrlich tumor cells or saline and were treated with quercetin (100 mg/kg, i.p.) or vehicle starting 10 min after Ehrlich tumor cells injection and followed by daily treatment during 8 days. On the 8th day, 3 h after treatment, the overt pain-like behavior was assessed. In other experiments, mice received Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$) and were treated with quercetin (100 mg/kg, i.p.) or vehicle daily during 8 days; on the 8th day, mice received the treatment with naloxone (1 mg/kg i.p.) (an opioid receptor antagonist) followed by evaluation of mechanical and thermal hyperalgesia, paw thickness, overt pain, and collection of spinal cord and paw skin and samples for evaluation of myeloperoxidase (MPO) activity (only paw skin), IL-1$\beta$ and TNF$\alpha$ concentration, FRAP, ABTS, and GSH levels. Lastly, we assessed the effect of cotreatment with quercetin (10 mg/kg, i.p.) and morphine (1 mg/kg, i.p.) (at doses that were not effectively analgesic as single treatment) over Ehrlich tumor-induced ($1 \times 10^6$ or $1 \times 10^7$ cells) mechanical hyperalgesia, thermal hyperalgesia, paw thickness, and overt pain-like behavior. Time points of the analyzed parameters were standardized in our laboratory [30].

*2.2. Test Compound.* The compounds used in this study were PBS pH 7.4, saline (NaCl 0.9%, Fresenius Kabi Brasil Ltda., Aquiraz, CE, Brazil), Tween, and DMSO 2%, and quercetin at 95% purity was purchased from Acros Organics (Fair Lawn, NJ, USA).

*2.3. Ehrlich Tumor Cells.* Peritoneal ascitic fluid of mice that received Ehrlich tumor cells i.p. was collected and injected in other mice. Ten days after the injection of ascitic fluid containing Ehrlich tumor cells, the ascitic fluid was collected for experiments. Ehrlich tumor cells were developed by Paul Ehrlich in 1896 and described as a spontaneous breast adenocarcinoma of female mice. It was originally developed as an ascitic form but can be converted to solid form when inoculated into tissues. Injection of Ehrlich tumor cells in the paw induces mechanical hyperalgesia, thermal hyperalgesia,

increase of paw thickness, and overt pain-like behavior [30].

*2.4. Animals.* Male Swiss mice (25–30 g), from the Universidade Estadual de Londrina, Londrina, Parana, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12 : 12 h and kept at 21°C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (13279.2011.76). Every effort was made to minimize the number of animals used and their suffering.

*2.5. Mechanical Hyperalgesia.* Mechanical hyperalgesia was evaluated as previously reported [30]. In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer, Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 $mm^2$ polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the mean measurements at 1, 3, 5, and 7 h after acute treatment on the 8th day after injection of the Ehrlich tumor cells or 3 h after each daily treatment with quercetin in the chronic protocol on days 2, 4, 6, 8, 10, and 12 after injection of the cells from the zero-time mean measurements.

*2.6. Thermal Hyperalgesia.* Mice were placed in a 10 cm-wide glass cylinder on a hot plate (IITC Life Science, Inc., Woodland Hills, CA, United States) maintained at 55°C. Two control latencies of at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 10–15 s. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 20 s to avoid tissue damage [30]. The results are expressed as thermal threshold.

*2.7. Paw Thickness or Tumor Growth.* Paw thickness was determined before and at indicated time points (at 48 h intervals) after the injection of Ehrlich tumor cells using an analog caliper. Paw thickness/tumor growth was presented as Δ mm [30].

*2.8. Overt Pain-Like Behavior Evaluation.* Mice received 1 × $10^7$ cells/paw in 25 μL and were placed in clear glass compartments at room temperature. After an acclimation period of 10 min, mice were observed during 10 min, and the cumulative number of flinches was determined [30].

*2.9. Histopathological Analyses.* Twelve days after the injection of the Ehrlich tumor cells, mice were euthanized and the paw was removed and decalcified in EDTA solution during 35 days. Samples were embedded in paraffin, sectioned into 5 μm section, and stained with hematoxylin and eosin for light microscopic analysis [30].

*2.10. Myeloperoxidase (MPO) Activity.* Neutrophil recruitment to the paw skin was evaluated by the MPO kinetic-colorimetric assay [25]. Paw skin samples were collected in 50 mM $K_2PO_4$ buffer (pH 6.0) containing 0.5% HTAB and were homogenized using a Polytron (PT3100). After the homogenates were centrifuged at 16.100 g for 2 min, the resulting supernatant was assayed for MPO activity at 450 nm (Multiskan GO Microplate Spectrophotometer) with three readings within 1 min. The MPO activity of the samples was compared with a standard curve of neutrophils. The results were presented as the MPO activity (number of neutrophils × $10^5$/mg of tissue).

*2.11. Cytokine Measurement.* Mice spinal cord (L4–L6) and paw skin samples were collected and homogenized in 500 μL of buffer containing protease inhibitors, and IL-1β and TNFα levels were determined as described previously by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results were expressed as picograms (pg) of cytokine/mg of spinal cord or paw skin. As a control, the concentrations of these cytokines were determined in animals injected with saline and treated with vehicle [25].

*2.12. Antioxidants Tests.* Spinal cord and paw skin tissue samples were collected and immediately homogenized with 500 μL of 1.15% KCl. Samples were centrifuged (10 min, 0.2 g, and 4°C) and the total antioxidant capacity was determined by the FRAP (ferric reducing ability potential) and ABTS (ability to scavenge ABTS radical) assays [25]. For FRAP assay, 50 μL of supernatant was mixed with 150 μL of deionized water and 1.5 mL of FRAP reagent freshly prepared. The reaction mixture was incubated at 37°C for 30 min and absorbance was measured at 595 nm. For ABTS assay, ABTS solution was diluted with phosphate buffer saline pH 7.4 (PBS) to an absorbance of 0.80 at 730 nm. Then, 1.0 mL of diluted ABTS solution was mixed with 20 μL of supernatant. After 6 min, the absorbance was measured at 730 nm. The results were equated against a Trolox standard curve (1.5–30 μmol/L, final concentrations). The results were expressed as Trolox equivalents per gram of spinal cord or paw skin in both assays. For GSH measurement, spinal cord and paw skin samples were collected and maintained at −80°C for at least 48 h. Samples were homogenized with 200 μL of 0.02 M EDTA. The homogenate was mixed with 25 μL of 50% trichloroacetic acid and was homogenized three times during 15 min. The mixture was centrifuged (15 min, 1.5 g, and 4°C). The supernatant was added to 200 μL of 0.2 M TRIS buffer, pH 8.2, and 10 μL of 0.01 M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve with GSH was performed. The results are expressed as GSH per mg

of protein of spinal cord or paw skin [25]. For protein determination, 60 $\mu$L of supernatant was mixed with 60 $\mu$L of copper reagent freshly prepared. After 10 min, 180 $\mu$L of Folin solution was added. The resulting solution was incubated at 50°C for 10 min. The absorbance was measured at 660 nm and the results equated to a standard curve of bovine serum albumin [33].

*2.13. Statistical Analysis.* Results are presented as means ± SEM of measurements made on six mice in each group per experiment and are representative of two independent experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's $t$-test was performed on each occasion. Statistical differences were considered to be significant at $p < 0.05$.

## 3. Results and Discussion

*3.1. Quercetin Inhibits Pain-Like Behavior and Neutrophil Recruitment Induced by Ehrlich Tumor Cells.* Ehrlich tumor cells induced significant mechanical hyperalgesia starting at the 4th day up to the 12th day and thermal hyperalgesia starting at the 2nd day up to the 12th day confirming previous standardization [30]. The acute analgesic effect of quercetin (10–100 mg/kg, i.p. 2% DMSO diluted in saline) was assessed on the 8th day after injection of the Ehrlich tumor cells at 1, 3, 5, and 7 h after treatment. Quercetin (100 mg/kg, i.p.) treatment significantly reduced the mechanical and thermal hyperalgesia at 3 and 5 h after treatment (Figures 1(a) and 1(b), resp.) but did not alter the paw thickness (Figure 1(c)). The chronic posttreatment with quercetin (10–100 mg/kg, 2% DMSO diluted in saline) significantly reduced the mechanical hyperalgesia from days 6 to 12 (Figure 2(a)) and thermal hyperalgesia between 4 and 12 days (Figure 2(b)) in a dose-dependent manner. The inhibition of Ehrlich tumor-induced mechanical and thermal hyperalgesia was not accompanied by alteration of paw thickness, indicating that quercetin did not affect tumor growth (Figure 2(c)). The treatment with quercetin (100 mg/kg, i.p) of mice that received Ehrlich tumor vehicle (saline) did not alter the basal mechanical or thermal hyperalgesia, or paw thickness (Figures 2(a)–2(c)) indicating that quercetin did not present *per se* effects.

In the present model, Ehrlich tumor cells induced overt pain-like behavior, such as paw flinching, at the dose of $1 \times 10^7$ cells with peak of response at the 8th day after injection [30]. At this time point, the daily treatment with quercetin also inhibited Ehrlich tumor-induced paw flinching (Figure 2(d)) with significant analgesic effect with the dose of 100 mg/kg of quercetin over 10 and 30 mg/kg. There was no effect on mice that received Ehrlich tumor vehicle (saline) plus quercetin treatment (100 mg/kg, i.p.). Considering the results of Figures 1 and 2, the dose of 100 mg/kg of quercetin was selected for the next experiments. Corroborating the present data, quercetin also inhibited mechanical hyperalgesia, thermal hyperalgesia, and overt pain-like behavior induced by varied stimuli in other models of inflammatory and neuropathic pain [21, 22, 34, 35], and the dose of 100 mg/kg of quercetin was also selected [21, 22, 25, 36].

In agreement with the results of Figure 2(c), hematoxylin/eosin staining of paw samples revealed no histological differences between mice with tumor treated with quercetin and vehicle control group. Mice that received saline in the paw and were treated with the vehicle of quercetin (Figure 3(a)) or quercetin (100 mg/kg i.p.) (Figure 3(b)) showed normal tissue. The arrows show the presence of epidermis, dermis, skeletal muscle fibers, and intact bone and cartilage. On the other hand, mice that received Ehrlich tumor cells and were treated with the vehicle of quercetin (Figures 3(c) and 3(e)) or with quercetin (100 mg/kg i.p) (Figures 3(d) and 3(f)) presented cartilage destruction, tissue necrosis, and intense tumor proliferation. This could be seen as a drawback data in the sense that quercetin does not inhibit Ehrlich tumor cells growth and, therefore, quercetin does not present an antitumor effect at this analgesic dose. On the other hand, the positive side is that quercetin exerts an analgesic effect without affecting tumor growth; thus, it is suitable for treatment of cancer pain and does not promote tumor growth. Nevertheless, some studies have shown the antitumor effect of quercetin. For instance, treatment with quercetin induced apoptosis and/or inhibited the growth of human breast carcinoma MCF-7 cells [37], K562 human chronic myeloid leukemia, Molt-4 acute T-lymphocytic leukemia, Raji Burkitt lymphoma [38], nasopharyngeal carcinoma cells [39], and other cancer cell lines [40, 41]. Dose of treatment, *in vivo* versus *in vitro* contexts, and cancer cell lines are some possible explanations for this divergent data. Nevertheless, it is possible that higher doses of quercetin would present antitumor effect with improved analgesia since it would present an intrinsic analgesic effect plus reduction of tumor and the immune response against the tumor.

There is evidence that ascitic Ehrlich tumor induces the recruitment to the peritoneal cavity of mice of cellular populations consistent with dendritic cells, monocytes, and neutrophils [42]. In the present study, it was observed that Ehrlich tumor injection in the paw induces an increase of myeloperoxidase (MPO) activity and daily treatment with quercetin (100 mg/kg, i.p.) inhibited this increase of MPO activity (Figure 4). The saline group treated with quercetin (100 mg/kg, i.p) did not present alteration of MPO activity compared to quercetin vehicle. MPO is an important enzyme of neutrophil microbicidal activity and is used as a marker of inflammation and neutrophil recruitment [43]. The inhibition of neutrophil recruitment or activation is an analgesic mechanism since recruited neutrophils contribute to hyperalgesia by further producing nociceptive molecules [43]. Therefore, inhibiting neutrophil recruitment might be accounting for the analgesic effect of quercetin. In addition to neutrophils, macrophages express MPO, suggesting that the inhibition of MPO activity by quercetin treatment could also involve the reduction of macrophage counts. This is consistent with the demonstration that Ehrlich tumor cells induce the recruitment of monocytes, which

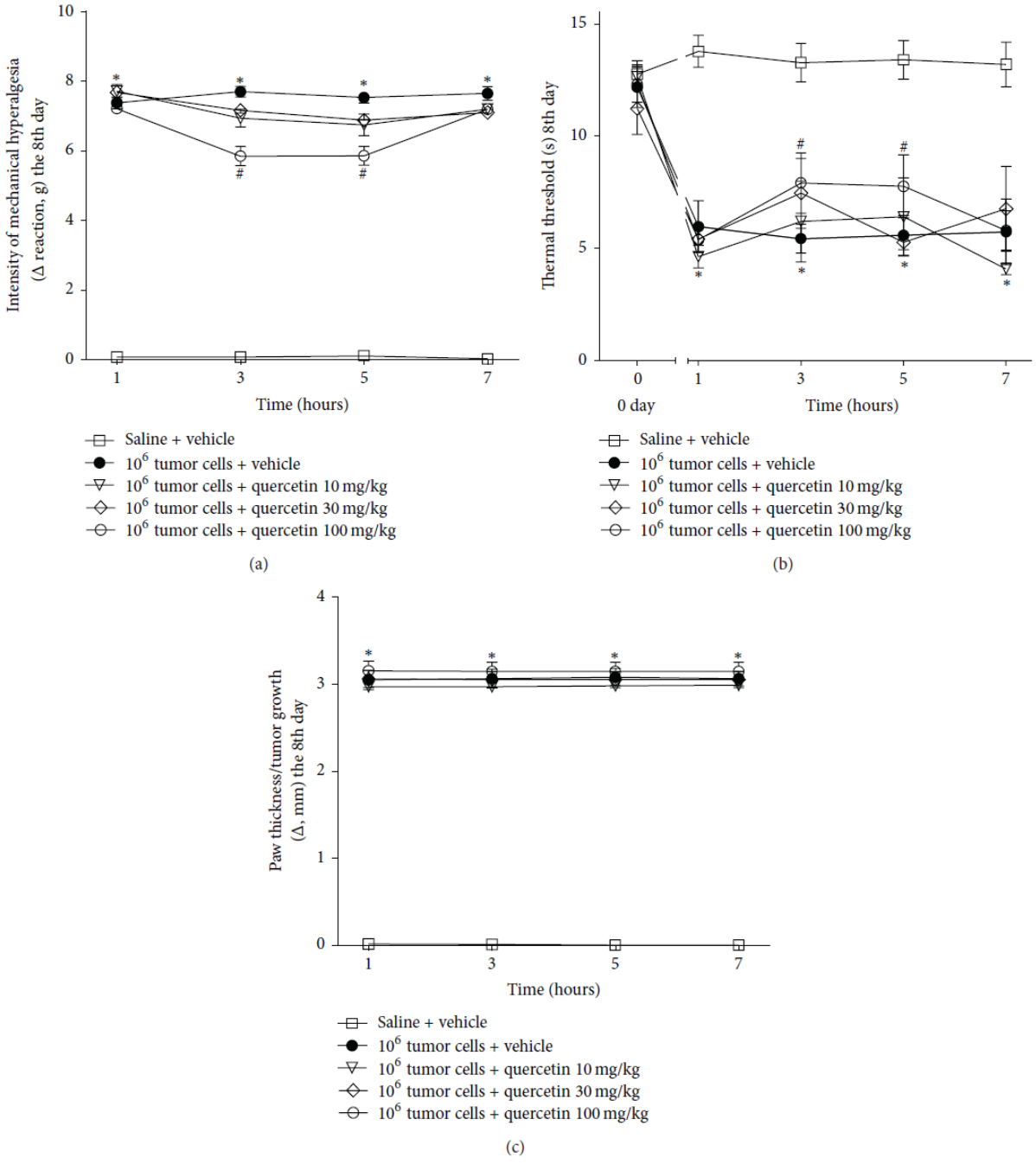


FIGURE 1: Acute treatment with quercetin inhibits Ehrlich tumor-induced pain-like behavior in mice. Mice received the intraplantar (i.pl.) administration of Ehrlich tumor cells $1 \times 10^6$ (a–c), and in the 8th day after injection, the tumor cells mice received the acute treatment with quercetin (10, 30, and 100 mg/kg i.p.). Mechanical hyperalgesia (a), thermal hyperalgesia (b), and paw thickness (c) were accessed at 1, 3, 5, and 7 hours after the treatment. Data are presented as means ± SEM of six mice per group per experiment and are representative of two separated experiments: $^{*}p < 0.05$ compared to the saline group and $^{\#}p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

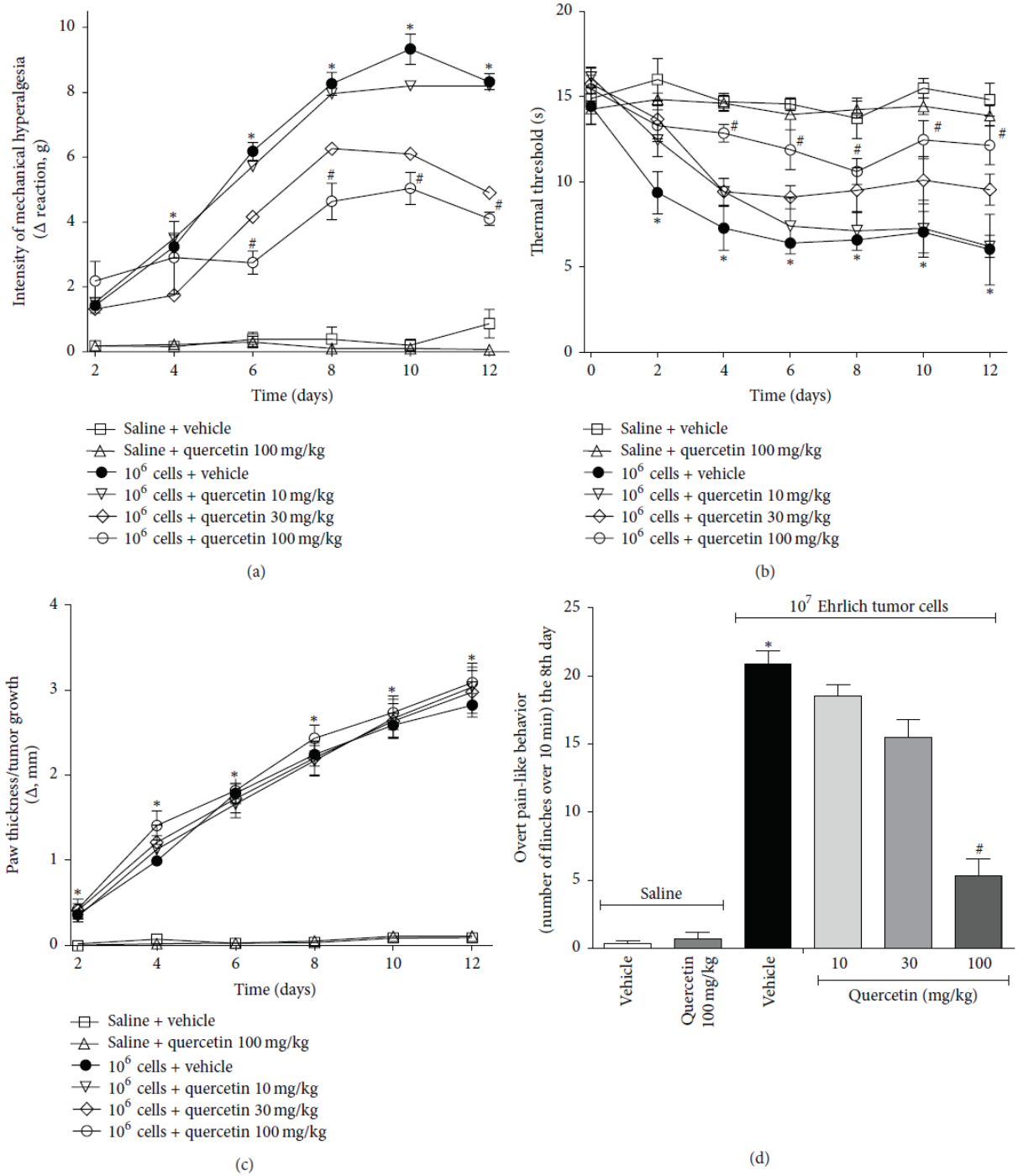


FIGURE 2: The chronic treatment with quercetin inhibits in a dose-dependent manner Ehrlich tumor-induced pain-like behavior in mice. Mice received the intraplantar (i.pl.) administration of Ehrlich tumor cells ($1 \times 10^6$ (a–c) or $1 \times 10^7$ (d)) and were treated daily with quercetin (10, 30, and 100 mg/kg i.p.) during 12 days (a–c) or 8 days (d) starting 10 min after tumor injection. The control group of Ehrlich tumor vehicle was saline and saline plus quercetin group was a control of possible *per se* effects of quercetin. Mechanical hyperalgesia (a), thermal hyperalgesia (b), paw thickness (c), and overt pain-like behavior (d) were evaluated 3 h after the treatment. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^{*}p < 0.05$ compared to the saline group and $^{\#}p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

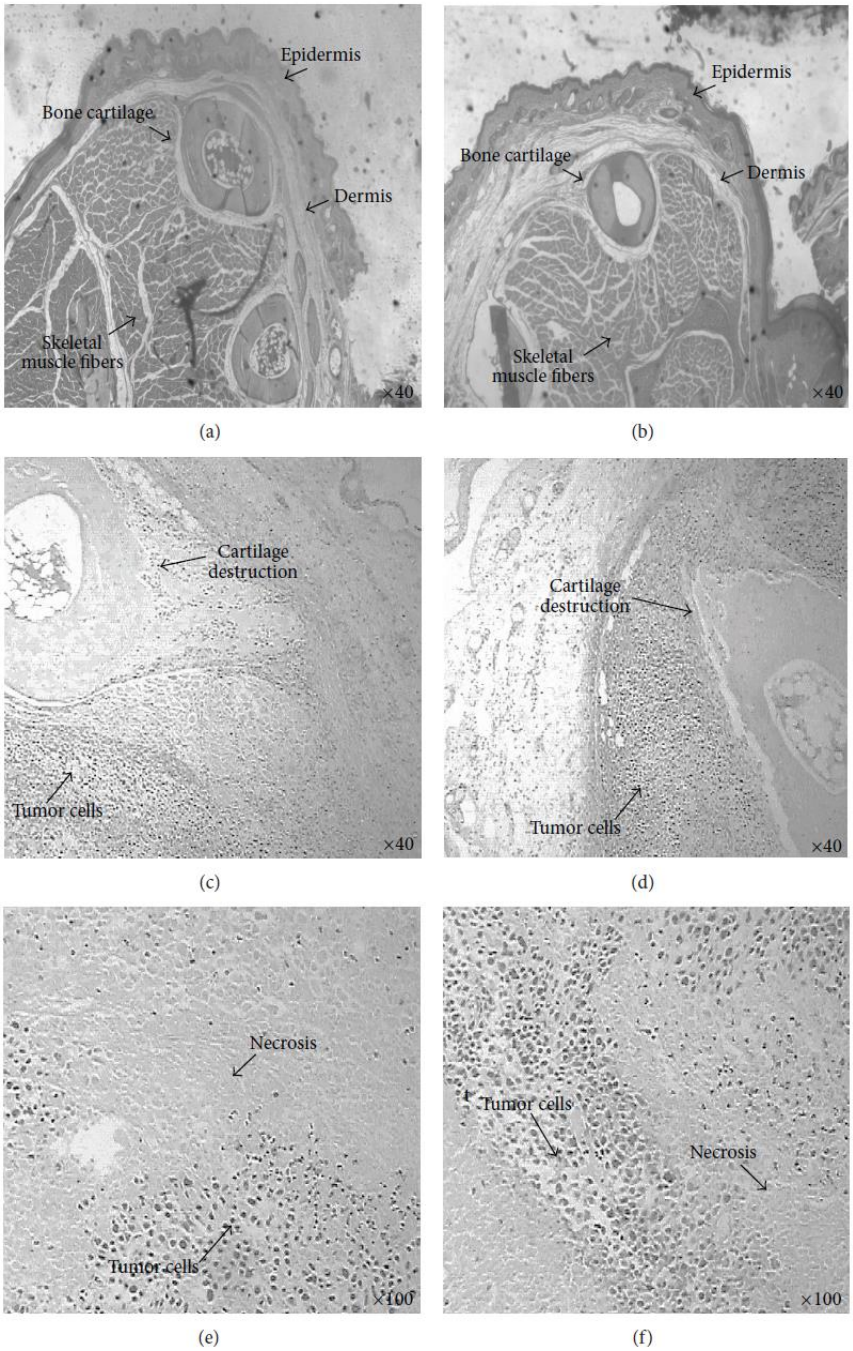


FIGURE 3: Quercetin does not alter Ehrlich tumor-induced histological modifications. Mice received saline (25 $\mu$L) or Ehrlich tumor cells ($1 \times 10^6$/25 $\mu$L) and were treated i.p. with quercetin (100 mg/kg, 2% DMSO diluted in saline) or vehicle (2% DMSO) 10 min after the i.pl. injection. The treatment continued daily during 12 days. In the 12th day, mice were euthanized and the paw was collected for histological analysis performed with hematoxylin/eosin staining. Panel (a) shows the histology of saline i.pl. plus quercetin vehicle group, (b) saline i.pl. plus quercetin (100 mg/kg i.p.), (c and e) tumor animal treated with vehicle, and (d and f) tumor animal treated with quercetin (100 mg/kg i.p.). Arrows indicate intact bone cartilage, presence of skeletal muscle fibers, dermis and epidermis: (a-b) bone/cartilage destruction (c-d), tissue necrosis (e-f), and presence of tumor cells (c–f).

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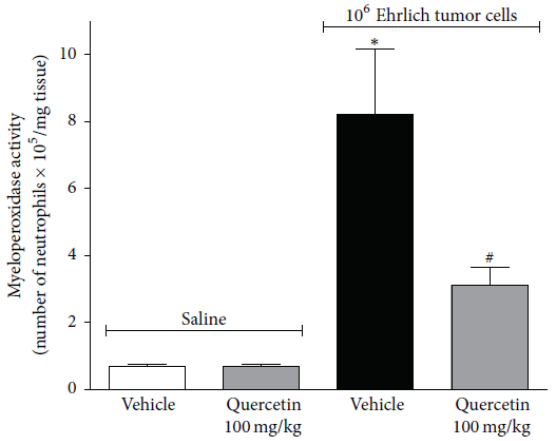


FIGURE 4: Quercetin inhibits neutrophil recruitment induced by Ehrlich tumor cells. Mice were treated i.p. with quercetin (100 mg/kg, 2% DMSO diluted in saline) or vehicle (2% DMSO) 10 min after the injection of Ehrlich tumor cell ($1 \times 10^6$/25 $\mu$L) or saline (25 $\mu$L). The neutrophil recruitment was evaluated in samples of paw skin collected after 12 days of treatment using the myeloperoxidase (MPO) activity assay. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^*p < 0.05$ compared to the saline group and $^\#p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

could differentiate into macrophages [42]. Treatment with quercetin also inhibits MPO activity *in vitro* [44] and also the neutrophil recruitment *in vivo* and neutrophil chemotaxis *in vitro* induced by chemokines, fMLP (formyl-methionyl-leucyl-phenylalanine) and leukotriene $B_4$ [40]. Therefore, quercetin is able to inhibit the MPO enzyme as well as the recruitment of cells expressing MPO. In addition to inhibiting the chemotactic effects of cytokines, peptides, and lipid mediators, quercetin also inhibits the production of such molecules. Thus, in the next set of experiments, whether quercetin would inhibit the production of cytokines with hyperalgesic and chemotactic functions such as IL-1$\beta$ and TNF$\alpha$ was investigated [45].

*3.2. Quercetin Inhibits IL-1β and TNFα Production Induced by Ehrlich Tumor Cells in the Spinal Cord and Paw Skin.* Mice received daily treatment during 12 days with quercetin (100 mg/kg, i.p.) starting 10 min after the injection of saline or Ehrlich tumor ($1 \times 10^6$, i.pl.) injection as described at Figure 2, and samples were collected in the 12th day (Figure 5). Ehrlich tumor cells induced significant production of IL-1$\beta$ in the spinal cord (Figure 5(a)) and in the paw skin (Figure 5(b)). TNF$\alpha$ levels were also increased in spinal cord (Figure 5(c)) and paw skin (Figure 5(d)). Quercetin treatment inhibited Ehrlich tumor-induced IL-1$\beta$ and TNF$\alpha$ production in the spinal cord and paw skin (Figure 5). The daily treatment with quercetin (100 mg/kg i.p.) in mice that received i.pl. control saline did not alter the production of cytokines compared to naive group. Cytokines including IL-1$\beta$ and TNF$\alpha$ have spinal cord and peripheral nociceptive roles as observed in inflammation and neuropathic and cancer models. Therefore, inhibiting IL-1$\beta$ and/or TNF$\alpha$ production or action at the central spinal cord or peripheral levels is a promising analgesic approach [45]. In fact, the intrathecal treatment with IL-ra (an IL-1 receptor antagonist) inhibited the hyperalgesia induced by AT-3.1 prostate cancer cells into the tibia of rats [46] and systemic treatment with anakinra (an IL-1 receptor antagonist) reduced the hyperalgesia induced by osteosarcoma model of bone cancer pain [47]. The i.pl. injection of lung carcinoma cells induces hyperalgesia in mice accompanied by high peripheral production of IL-1$\beta$ and TNF$\alpha$, and the treatment with etanercept (a TNF-neutralizing soluble receptor) prevented the development of heat hyperalgesia. Furthermore, TNF-induced cancer-related heat hyperalgesia through nociceptor sensitization is linked to upregulation of transient receptor potential vanilloid 1 (TRPV1) [8]. Similarly, etanercept also reduced the mechanical hyperalgesia in a bone cancer model [13, 48]. The nociception triggered by IL-1$\beta$ and TNF$\alpha$ presents peripheral and central spinal cord mechanisms. For instance, TNF$\alpha$ triggers acute inflammatory hyperalgesia by inducing IL-1$\beta$ production, which in turn induces prostaglandin $E_2$ production. Prostaglandin $E_2$ is ultimately responsible for sensitization of nociceptive neurons [45]. After the first inflammatory stimulus, there is a condition named hyperalgesic priming representing prolonged inflammation in which TNFR1 expression is induced in nociceptive neurons and, therefore, TNF$\alpha$ can exert a direct sensitizing effect [49]. *In vitro*, dorsal root ganglia (DRG) neurons express TNFR1 receptors possibly due to the collection procedure of the DRG, which resembles axotomy. In this condition, TNF$\alpha$ induces p38 mitogen-activated protein (MAP) kinase activation that phosphorylates tetrodotoxin-resistant sodium

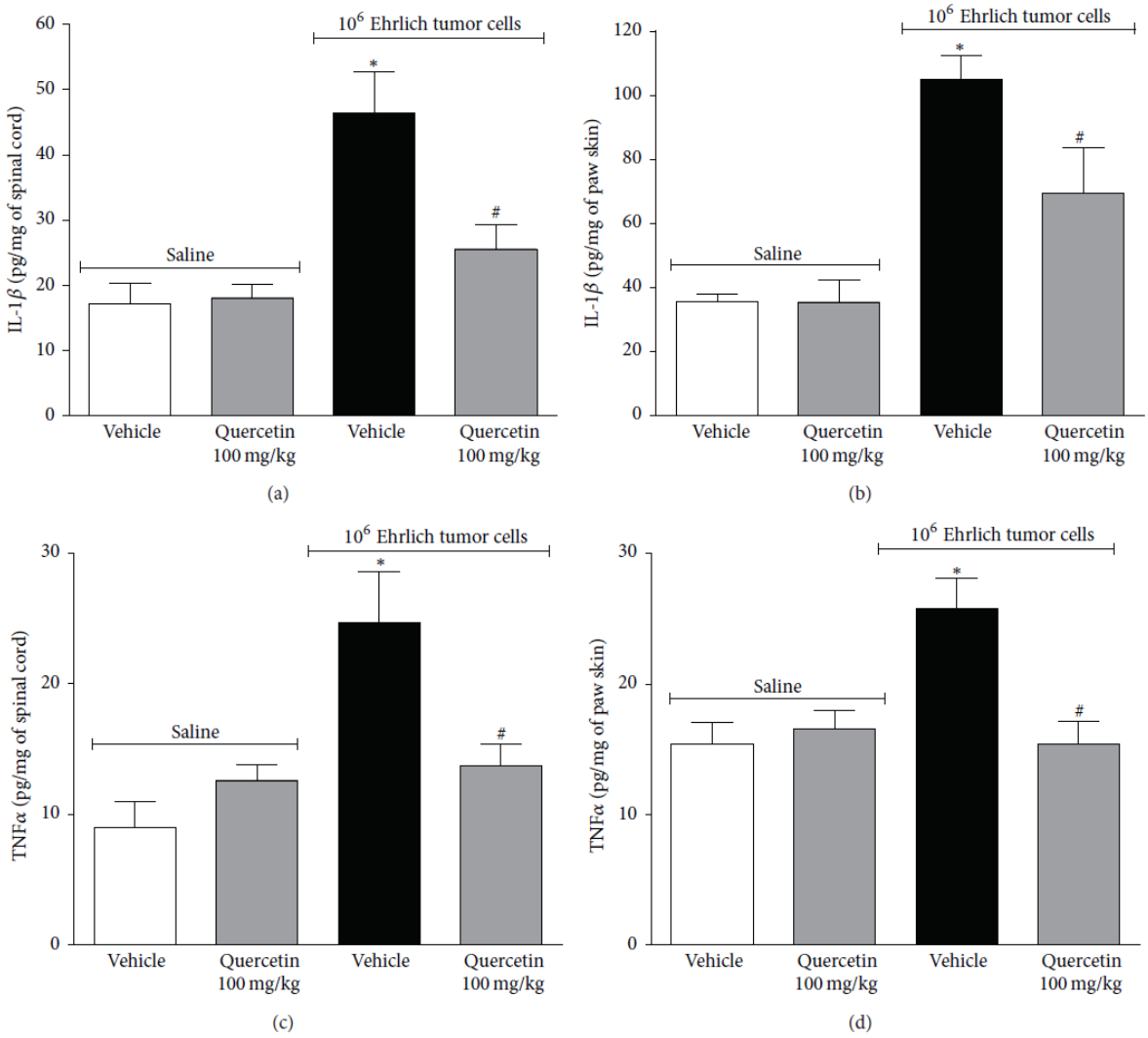


FIGURE 5: Quercetin inhibits IL-1$\beta$ and TNF$\alpha$ production induced by Ehrlich tumor cells in the spinal cord and paw skin. Mice were treated i.p. with quercetin (100 mg/kg, 2% DMSO diluted in saline) or vehicle (2% DMSO) 10 min after the injection of Ehrlich tumor cell ($1 \times 10^6/25\,\mu$L) or saline (25 $\mu$L). The treatment continued daily. In the 12th day after injection, the Ehrlich tumor cells, the spinal cord, and the paw skin samples were collected for cytokine measurement. IL-1$\beta$ in spinal cord (a) or paw skin (b) and TNF$\alpha$ in spinal cord (c) or paw skin (d) were determined by ELISA. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^*p < 0.05$ compared to the saline group and $^\#p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

channels resulting in neuronal depolarization [50]. TNFR1 and TNFR2 also participate in the spinal cord activation of astrocytes and pain [13]. In cancer, the inhibition of p38-MAPK signaling pathway attenuates breast cancer-induced bone pain [17]. TNFR2 also plays a pronounced role in lung carcinoma cells-induced heat hyperalgesia [8]. Therefore, cytokines such as IL-1$\beta$ and TNF$\alpha$ are involved in the neuronal activation at peripheral sites, DRG, and spinal cord in varied painful conditions and targeting these cytokines is one of the efficient analgesic approaches in cancer pain.

*3.3. Quercetin Inhibits the Oxidative Stress Induced by Ehrlich Tumor Cells.* There is close relation between cytokines and oxidative stress in pain induction. IL-1$\beta$ and TNF$\alpha$ can activate nicotinamide adenine dinucleotide phosphate-(NADPH-) oxidase, resulting in the production of superoxide anion. In turn, superoxide anion activates nuclear factor kappa B (NF$\kappa$B) and enhances cytokine production [23, 51, 52]. In this sense, the effect of quercetin on Ehrlich tumor-induced oxidative stress was accessed by the total antioxidant capacity depletion in the spinal cord and paw skin using

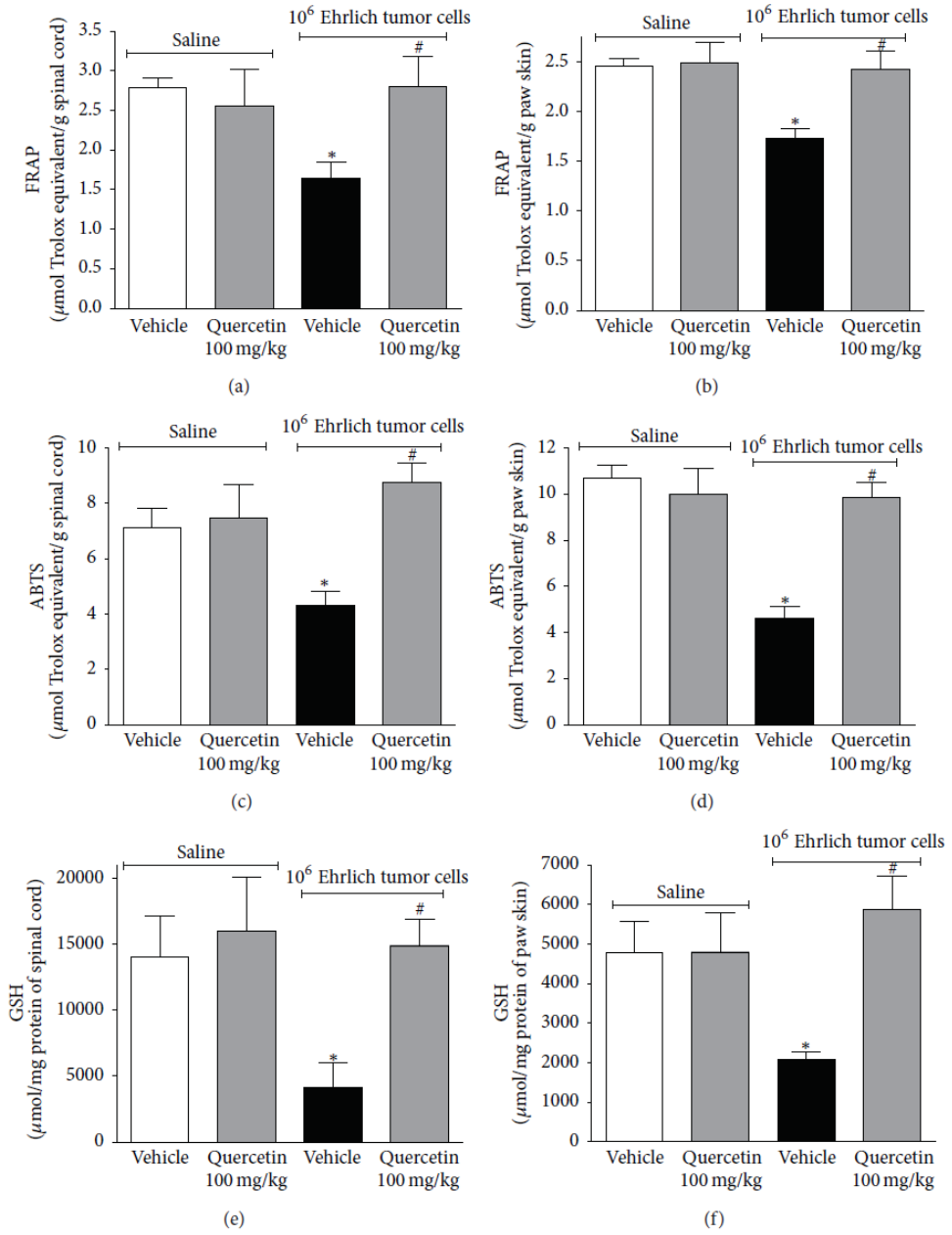


FIGURE 6: Quercetin inhibits the oxidative stress induced by Ehrlich tumor cells. Mice were treated with quercetin (100 mg/kg, i.p.) or vehicle 10 min after the injection of Ehrlich tumor cells ($1 \times 10^6/25\,\mu$L) or saline. The treatment continued daily during 12 days, and, in the 12th day, 3 h after the treatment, samples of spinal cord and paw skin were collected for the oxidative stress assays. The FRAP and ABTS ability of spinal cord ((a) and (c), resp.) and paw skin ((b) and (d), resp.) tissues and GSH levels in spinal cord (e) and paw skin (f) were accessed. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^{*}p < 0.05$ compared to the saline group and $^{\#}p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

the ability to ferric reducing potential (FRAP) assay, scavenge 2,2′-azinobis-(3-ethylbenzothiazoline 6-sulfonic acid radical) (ABTS) assay, and reduced glutathione (GSH) levels. Mice were divided and treated as in Figure 2 and samples were collected in the 12th day. Ehrlich tumor cells induced oxidative stress (Figure 6). The quercetin treatment showed a significant increase in FRAP at both the spinal cord (Figure 6(a)) and paw skin (Figure 6(b)) and ABTS in the spinal cord (Figure 6(c)) and paw skin (Figure 6(d)). It is known that quercetin is an antioxidant flavonoid and its

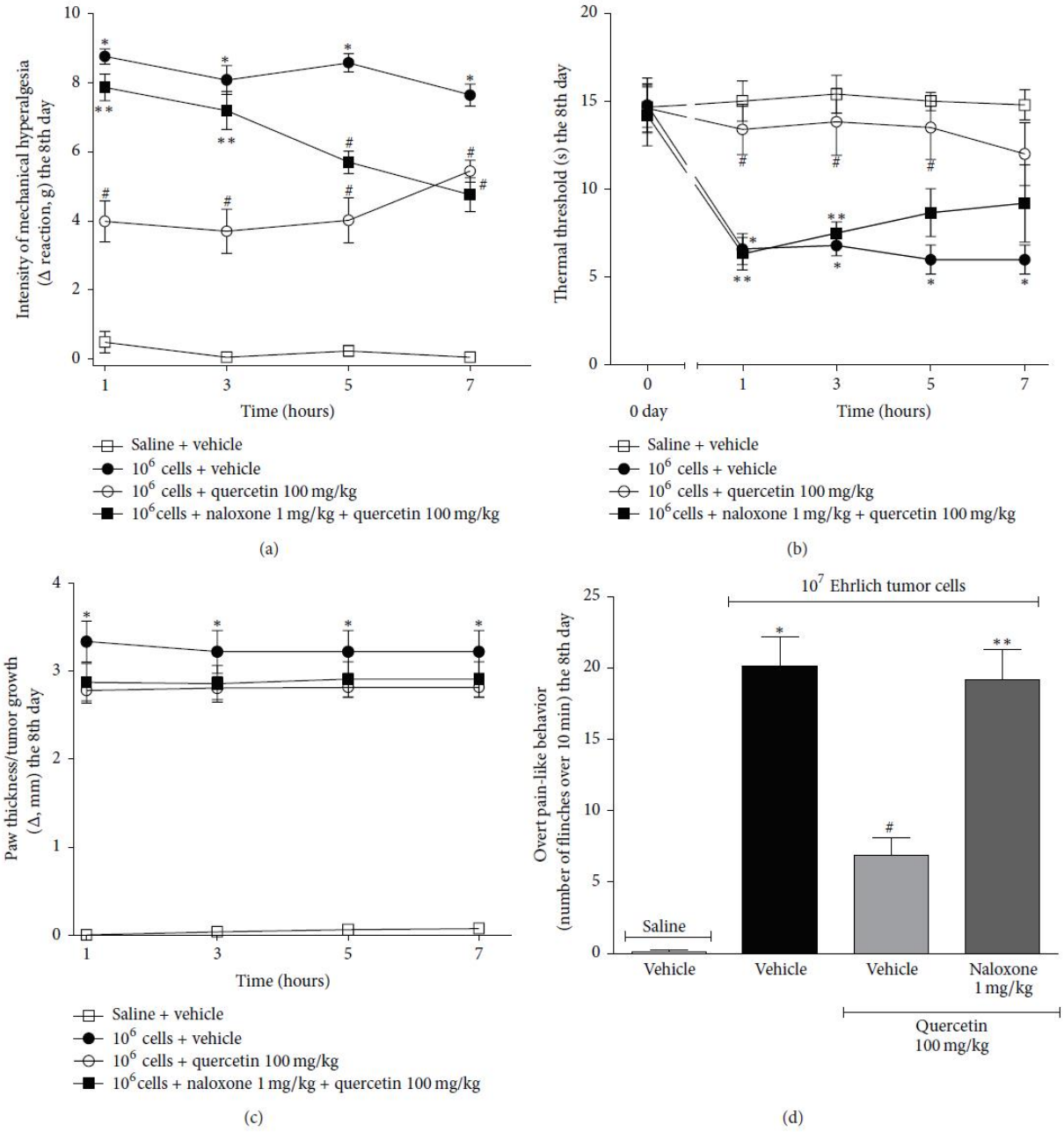


FIGURE 7: The opioid receptor antagonist, naloxone, inhibits quercetin analgesia in the Ehrlich tumor-induced pain model. Mice were treated with quercetin (100 mg/kg, i.p., starting 10 min after tumor administration) during 8 days after the injection of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ cells/25 $\mu$L) or saline and, in the 8th day, one group of mice that received quercetin was also treated with naloxone (1 mg/kg i.p. diluted in saline) 1 h before the treatment with quercetin. The evaluation of mechanical hyperalgesia (a), thermal hyperalgesia (b), and paw thickness (c) was performed 1, 3, 5, and 7 h after the treatments, and the overt pain-like behavior (d) was evaluated 1 h after the treatment. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^{*}p < 0.05$ compared to the saline group, $^{\#}p < 0.05$ compared to the tumor group, and $^{**}p < 0.05$ compared to the quercetin group. One-way ANOVA followed by Tukey's test.

effects could be explained by the presence of structural antioxidant chemical groups [53]. However, there is no antioxidant structural relationship of flavonoids and the inhibition of intracellular signaling pathways such as mitogen-activated protein kinases [23]. Therefore, the presence of structural antioxidant chemical groups does not fully explain the activities of quercetin.

In cancer and during chemotherapy treatment, there is increased production of reactive species [54], which can result in antioxidant depletion and, consequently, oxidative stress. The main consequence of rapid cellular division in cancer is the increase of the metabolic by products, such as excessive production of reactive oxygen species (ROS) [55]. Decreased levels of GSH have been reported in patients with breast cancer [56, 57]. The increased oxidative stress gives rise to inflammation, which could further aggravates the pain [57]. In this sense, quercetin may present an important applicability in reducing cancer-induced oxidative stress. It is noteworthy that the inhibition of peripheral oxidative stress observed may also be attributed to the reduction in neutrophil recruitment by quercetin (Figure 4), because activated neutrophils are important sources of reactive oxygen and nitrogen species in the tissue. Quercetin also inhibited Ehrlich tumor-induced GSH depletion in the spinal cord (Figure 6(e)) and paw skin (Figure 6(f)). This is in agreement with previous studies demonstrating that quercetin presents beneficial effects through antioxidant activities in other experimental models such as colitis [25] and inflammatory pain [21]. It has been suggested that the prevention of GSH depletion may be an important analgesic mechanism [58]. GSH can reduce reactive species and is an important molecule of the endogenous antioxidant system. In this sense, the preservation of GSH levels by quercetin may also prevent total antioxidant capacity depletion and oxidative stress [54]. Therefore, the antinociceptive activity of quercetin could also be associated with the inhibition of oxidative stress in this model.

*3.4. Quercetin Analgesia, but Not the Anti-Inflammatory Effect, Depends on Endogenous Opioids.* Mice were treated with quercetin as in Figure 2 daily during 8 days. In the 8th day, one group was treated with naloxone (an opioid receptor antagonist, 1 mg/kg, diluted in saline, i.p.) 1 h before the treatment with quercetin (Figure 7) and mechanical hyperalgesia, thermal hyperalgesia, and paw thickness were assessed after 1, 3, 5, and 7 h (Figures 7(a)–7(c)). Quercetin significantly reduced Ehrlich tumor-induced mechanical and thermal hyperalgesia at all time points. The analgesic effect of quercetin was inhibited by naloxone at 1 and 3 h after treatment (Figures 7(a) and 7(b)). As observed in Figure 2, quercetin did not affect paw thickness and naloxone did not alter this absence of effect of quercetin over Ehrlich tumor growth (Figure 7(c)). The same treatment regimen was performed on mice that receive $1 \times 10^7$ Ehrlich tumor cells to induce paw flinching. In the 8th day, 1 h after treatment with quercetin, Ehrlich tumor cell-induced paw flinches were evaluated. Quercetin significantly decreased Ehrlich tumor-induced paw flinches and treatment with naloxone inhibited

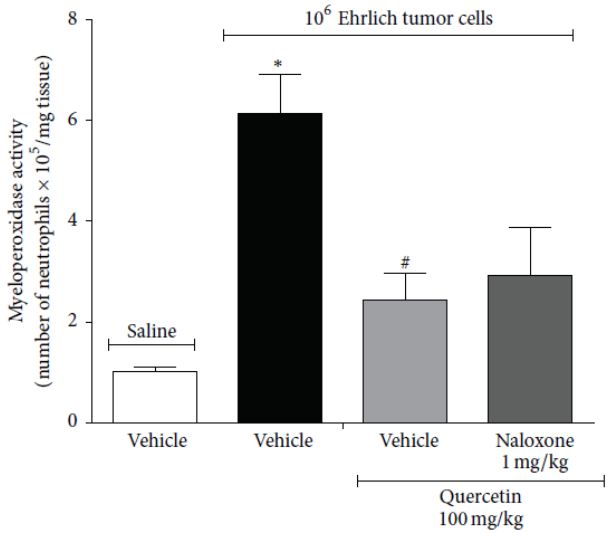


FIGURE 8: Naloxone did not reverse the effect of quercetin in reducing Ehrlich tumor cells-induced neutrophil recruitment. Mice were treated with quercetin (100 mg/kg, i.p., starting 10 min after tumor administration) during 8 days after the injection of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ cells/25 $\mu$L) or saline and, in the 8th day, one group of mice that received quercetin was also treated with naloxone (1 mg/kg i.p. diluted in saline) or its vehicle 1 h before the treatment with quercetin. The neutrophil recruitment was evaluated in samples of paw skin collected after 3 h of the treatment with quercetin by the myeloperoxidase (MPO) activity assay. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^*p < 0.05$ compared to the saline group and $^\#p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

the analgesic effect of quercetin (Figure 7(d)). The dose of naloxone was selected in previous studies [30]. These results indicate that the analgesic effect of quercetin in Ehrlich tumor-induced pain depends on opioid mechanisms. In agreement with our study, the analgesic effect of quercetin in a model of streptozotocin-induced diabetic neuropathic pain [22] and lipopolysaccharide-induced hyperalgesia [59] also depends on opioid mechanisms and is reversible by treatment with naloxone. On the other hand, using the same protocol as for Figure 7, we observed that naloxone did not alter the quercetin inhibition of Ehrlich tumor cells-induced MPO activity in the paw skin (Figure 8). Furthermore, following the same protocol of Figure 7, the effect of naloxone on quercetin inhibition of Ehrlich tumor cells-induced spinal cord and paw skin production of IL-1$\beta$ (Figures 9(a) and 9(b)), TNF$\alpha$ (Figures 9(c) and 9(d)), FRAP, ABTS, and GSH (Figures 10(a)–10(f)) were determined. The treatment with naloxone did not alter the anti-inflammatory and antioxidant effects of quercetin (Figures 9 and 10). The anti-inflammatory effect of opioids has already been described. For instance, kappa-opioid agonist exerts anti-inflammatory actions by reduction of adhesion molecule expression, inhibition of cell trafficking, and TNF release and expression [60]. Our data

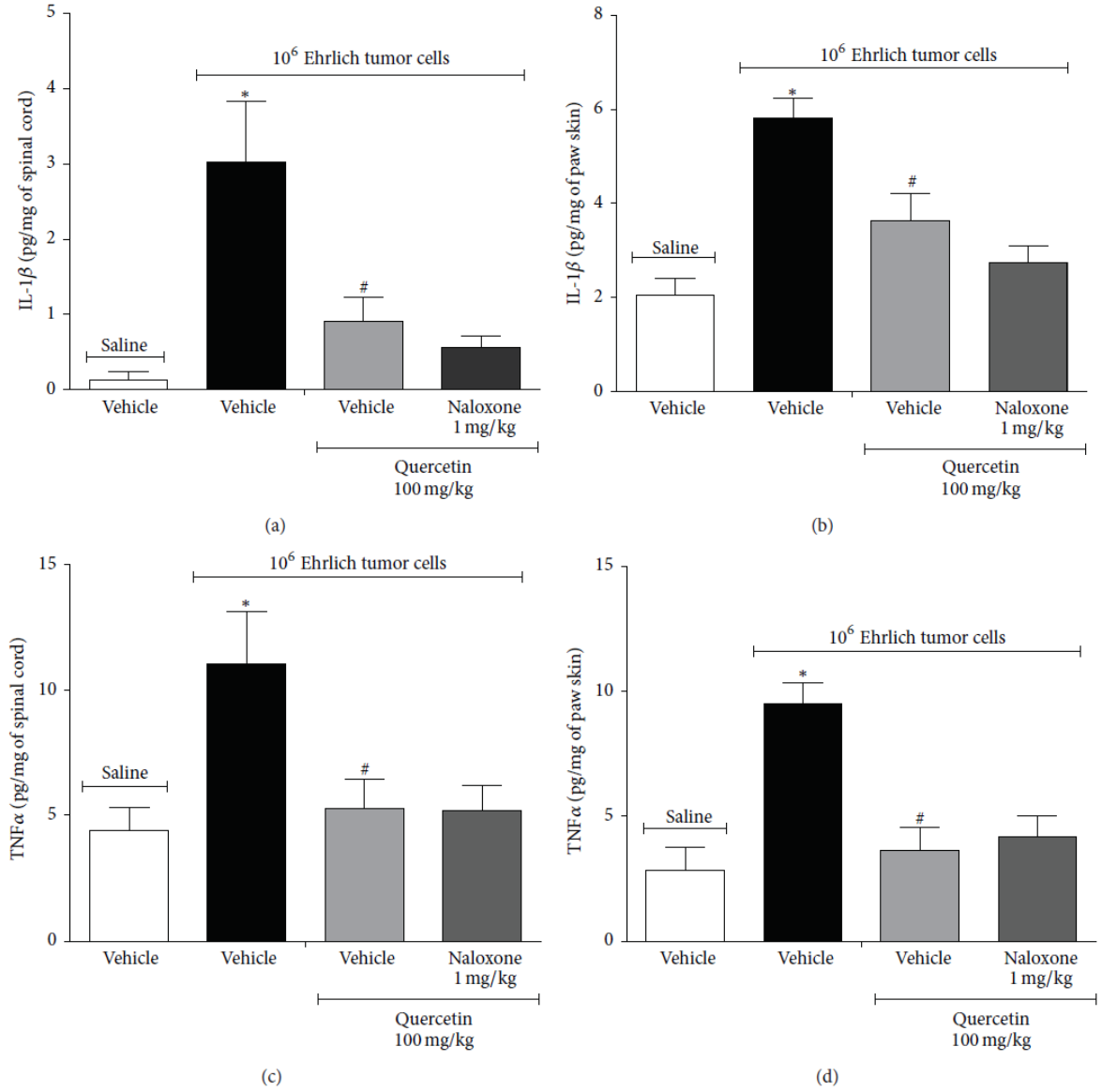


FIGURE 9: Naloxone did not reverse the effect of quercetin in reducing Ehrlich tumor cells-induced cytokine production. Mice were treated with quercetin (100 mg/kg, i.p., starting 10 min after tumor administration) during 8 days after the injection of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ cells/25 $\mu$L) or saline and, in the 8th day, one group of mice that received quercetin was also treated with naloxone (1 mg/kg i.p. diluted in saline) or its vehicle 1 h before the treatment with quercetin. IL-1$\beta$ concentration in spinal cord (a) or paw skin (b) and TNF$\alpha$ concentration in spinal cord (c) or paw skin (d) were determined by ELISA 3 h after the treatment with quercetin. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^*p < 0.05$ compared to the saline group and $^\#p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

suggest that the analgesic effect of quercetin in Ehrlich tumor-induced cancer pain is dependent on endogenous opioid mechanisms; however, these opioid-dependent mechanisms are not responsible for the anti-inflammatory and antioxidant actions of quercetin observed as reduction of MPO activity, cytokine production, and oxidative stress in the current protocol.

*3.5. Combined Treatment with Quercetin and Morphine at Doses That Are Ineffective as Single Treatment Reduces Ehrlich Tumor-Induced Pain-Like Responses.* Mice were treated with quercetin (10 mg/kg i.p., a dose without significant analgesic effect *per se*, Figure 2) 10 min after administration of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ cells, i.pl.). Mice were treated daily during 8 days. In the 8th day, mice were treated with

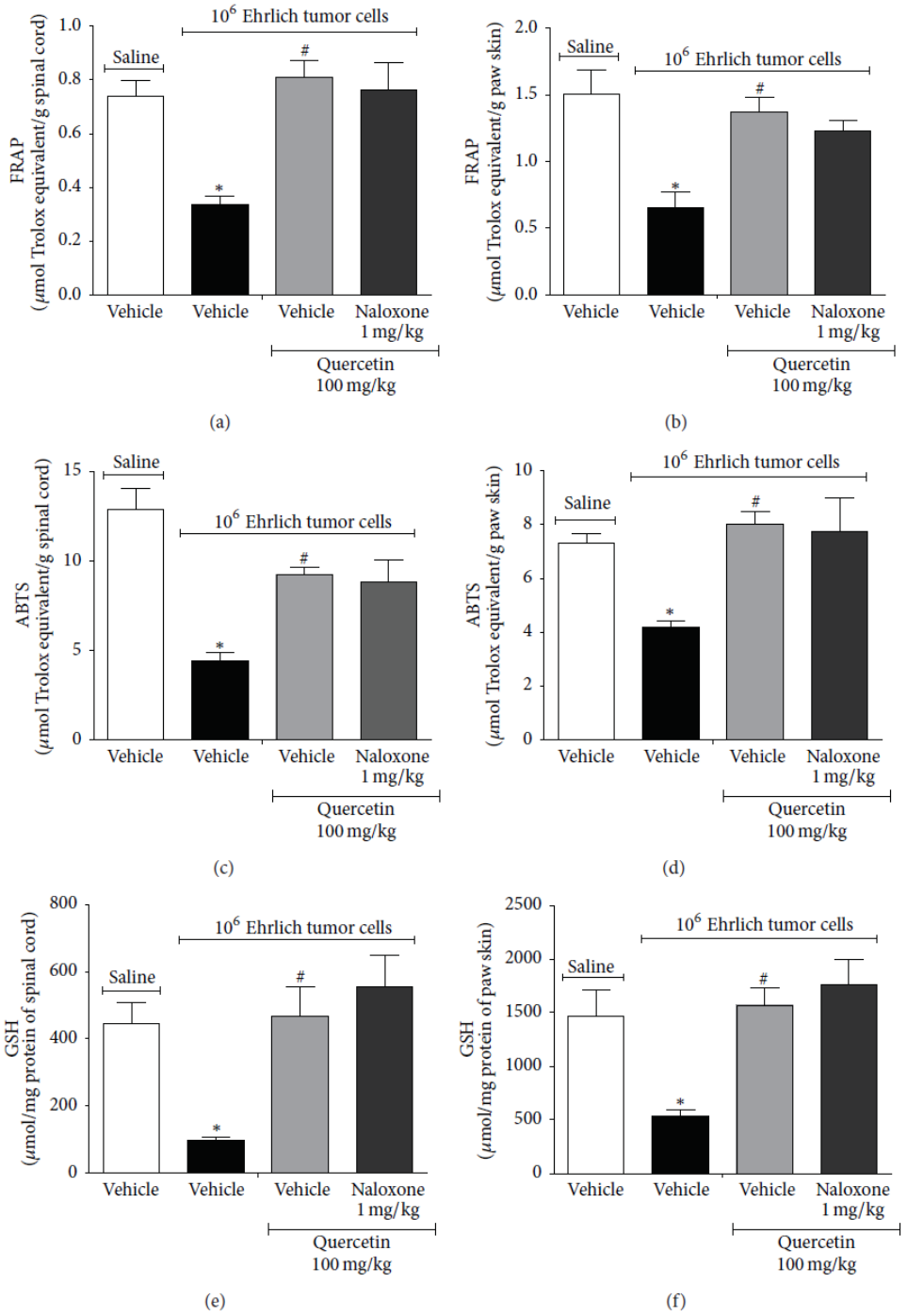


FIGURE 10: Naloxone did not reverse the effect of quercetin in reducing Ehrlich tumor cells-induced oxidative stress. Mice were treated with quercetin (100 mg/kg, i.p., starting 10 min after tumor administration) during 8 days after the injection of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ cells/25 $\mu$L) or saline and, in the 8th day, one group of mice that received quercetin was also treated with naloxone (1 mg/kg i.p. diluted in saline) or its vehicle 1 h before the treatment with quercetin. Three hours after the treatment with quercetin, samples of spinal cord and paw skin were collected for the oxidative stress assays. The FRAP and ABTS ability of spinal cord ((a) and (c), resp.) and paw skin ((b) and (d), resp.) tissues and GSH levels in the spinal cord (e) and paw skin (f) were accessed. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^{*}p < 0.05$ compared to the saline group and $^{\#}p < 0.05$ compared to the tumor group. One-way ANOVA followed by Tukey's test.

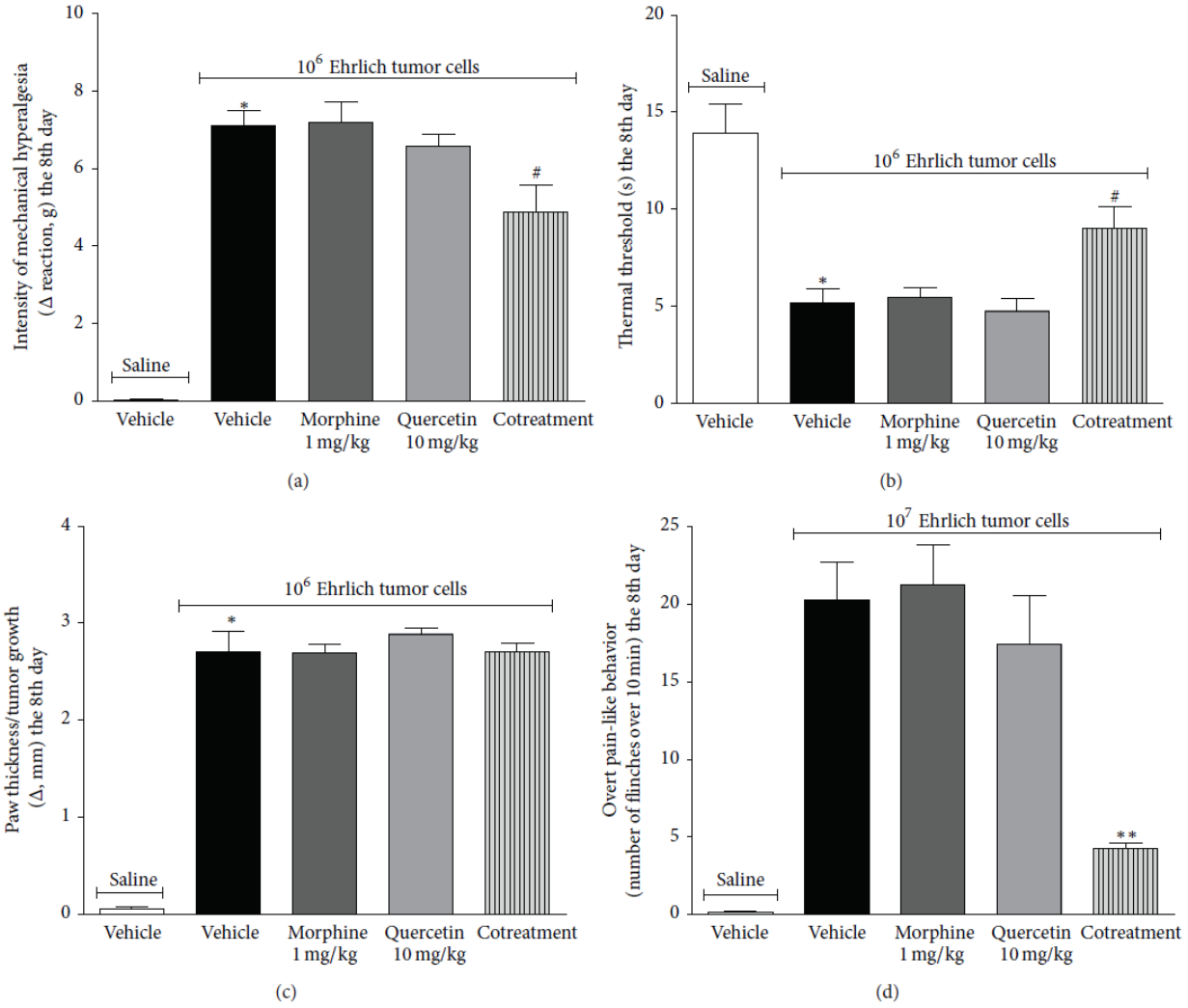


FIGURE 11: Combined treatment with quercetin and morphine at doses that are ineffective as single treatment reduces Ehrlich tumor-induced pain-like responses. Mice were treated with quercetin (10 mg/kg i.p., a dose without significant analgesic effect *per se*), before the injection of Ehrlich tumor cells ($1 \times 10^6$ or $1 \times 10^7$ cells, i.pl.). Mice were treated daily during 8 days and, in the 8th day, mice were treated with quercetin and after 2 h and 15 min received morphine (1 mg/kg i.p., a dose without significant analgesic effect *per se*). Mechanical (a) and thermal hyperalgesia (b), paw thickness (c), and overt pain-like behavior (d) were evaluated 3 h after the last quercetin treatment. Data are presented as means ± SEM of six mice per group per experiment and representative of two separated experiments: $^{*}p < 0.05$ compared to the saline group, $^{\#}p < 0.05$ compared to the tumor group, and $^{**}p < 0.05$ compared to the quercetin 10 mg/kg and morphine 1 mg/kg. One-way ANOVA followed by Tukey's test.

morphine (1 mg/kg i.p., a dose without significant analgesic effect *per se*) 2 h and 15 min after quercetin administration. Mechanical hyperalgesia, thermal hyperalgesia, paw thickness ($1 \times 10^6$ Ehrlich tumor cells), and paw flinching ($1 \times 10^7$ Ehrlich tumor cells) were assessed 45 min after morphine treatment or 3 h after quercetin treatment (Figures 11(a)–11(d)). Ehrlich tumor-induced mechanical and thermal hyperalgesia were not reduced by treatment with quercetin (10 mg/kg, i.p.) or morphine (1 mg/kg, i.p.) alone. However, the cotreatment with quercetin and morphine significantly reduced the mechanical (Figure 11(a)) and thermal hyperalgesia (Figure 11(b)). Ehrlich tumor-induced increase in the paw thickness was not altered by quercetin, morphine, or cotreatment with both molecules (Figure 11(c)). Finally, Ehrlich tumor-induced paw flinches were also reduced by cotreatment with quercetin and morphine, but not by quercetin or morphine alone (Figure 11(d)). These results suggest a synergic analgesic effect of quercetin and morphine over Ehrlich tumor-induced pain. Moreover, this synergy was more evident in the overt pain-like response, which clearly

showed a potentiation of analgesia (Figure 11(d)). Therefore, these results on synergy or even potentiation of analgesia by cotreatment with quercetin and morphine at doses without analgesic effect as single treatment are important in the sense that indicates possible reduction of morphine dosage by combination with quercetin treatment to control cancer pain.

Evidence supports a synergy/potentiation between quercetin and opioids/morphine in other models, indicating that this effect should be addressed. For instance, quercetin reduces the morphine tolerance [61], reduces naloxone-precipitated withdrawal contracture of the acute morphine-dependent guinea-pig ileum [62], and exhibits morphine-like inhibition of acetylcholine release in the coaxially stimulated ileum [63]. Therefore, the opioid-related actions of quercetin are consistent in varied systems and may contribute to reduce morphine dosage ([22, 61] and present data) as well as morphine tolerance [62]. Mechanistically, quercetin inhibits morphine tolerance by inhibiting nitric oxide synthase activity [61]. Therefore, it is likely that quercetin potentiates opioid activity indirectly by inhibiting mechanisms that would limit opioid effects and not by inducing opioid release or binding to and activating opioid receptors, which explain a synergic/potentiating effect of quercetin and morphine.

In addition to the analgesic effects, opioids also present anti-inflammatory actions *in vitro* and *in vivo* [64, 65]. The present results suggest that quercetin inhibits Ehrlich tumor cells-induced pain by two independent mechanisms: (a) an opioid-related analgesic mechanism and (b) an anti-inflammatory/antioxidant mechanism. The opioid-related mechanism might present central analgesic effects since per oral treatment with quercetin inhibited diabetic neuropathic pain in mice in the tail-immersion in warm water test, which evaluates the involvement of central nociceptive responses, in a naloxone sensitive manner [22]. The anti-inflammatory/antioxidant mechanism of quercetin is related to the inhibition of proinflammatory signaling pathways and intrinsic structural antioxidant chemical groups of quercetin [23].

In conclusion, the present study demonstrates that quercetin inhibits Ehrlich tumor-induced pain by mechanisms targeting peripheral and spinal cord oxidative stress and hyperalgesic cytokine production as well as inducing an opioid-related analgesic mechanism, resulting in potentiation of morphine analgesia. The analgesic dose of quercetin did not alter tumor growth demonstrating; therefore, its analgesia does not depend on reducing tumor mass.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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## 4.3 Artigo 3

Naringenin inhibits Ehrlich tumor cells-induced cancer pain: Role of leukocyte recruitment, oxidative stress, cytokine production, Nrf2 and HO-1 expression, and glial cells.

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**Abstract**

*Objective:* Evaluate the effect of naringenin in Ehrlich tumor cells-induced pain

*Treatment:* Naringenin 16.7, 50, or 150 mg/kg per oral

*Methods:* Mice received i.pl. or i.p. injection of Ehrlich tumor cells. Acute treatment with naringenin (16.7, 50, or 150 mg/kg, at the $8^{th}$ day post-tumor) or chronic treatment (150 mg/kg, once or twice daily during 10 or 12 days). Mechanical hyperalgesia was evaluated by electronic anesthesiometer; thermal hiperalgesia by hot plate; leukocyte recruitment MPO and NAG by colorimetric method; number of total leukocytes and tumor cells in Neubauer chamber; oxidative stress using colorimetric methods for FRAP, ABTS, and GSH; and RT-qPCR for Nrf2, HO-1; cytokines Tnfα, Il-1β; and glial cells activation**.** Data were analyzed by ANOVA followed by Tukey's ($p < 0.05$).

*Results:* Naringenin reduced the cancer pain by reducing neutrophil and macrophage recruitment to the paw and by inhibiting the oxidative stress and cytokine production in paw skin and spinal cord tissues. The antioxidant mechanism of naringenin could be related to the modulation of Nrf2 and HO-1 in the spinal cord and the anti-inflammatory by inhibition of Tnfα, Il-1β, and glial cells. The treatment did not induce toxicity and had no effect in growth tumor.

*Conclusions:* Naringenin inhibits cancer pain by inhibiting cellular recruitment, oxidative stress and cytokine production.

**Keywords:** Naringenin · Ehrlich tumor cells · cancer pain · cytokines · oxidative stress

## Introduction

Cancer pain represents a challenge in health care. Ninety percent of individuals with advanced cancer have moderate to severe pain that can appear in the initial or intermediary stages of the disease [1]. In addition to cancer-induced pain, human patients also experience pain caused by chemotherapies used for treat the cancer [2]. The mechanism of cancer pain involves a complex process that contributes to morphological, neurochemical and physiological changes in both peripheral and central nervous systems and conducts to a neuropathic and inflammatory condition [3]. Although these conditions are not fully understood, one plausible neuropathic mechanism is the occurrence of degeneration or injury of the nerve fibers [4]. The degeneration of myelinated fibers increases pro-inflammatory cytokines [5] which are involved in pain [6]. Moreover, the treatment itself for cancer and the use of chemotherapy drugs such as paclitaxel [7] often causes neuropathic pain in patients, which results in greater suffering. Inflammation is recognized as a critical component for tumor progression, being considered as a hallmark of cancer [8] and an inductor of tumorigeneses [9]. In the tumor microenvironment, there is the presence of various components including stromal cells, growing blood vessels factors [10], nerve growth factor [11], immune cells recruited [12], inflammatory mediators such as cytokines [13-15], endothelin-1 (ET-1) [16], bradykinin (BK) [17], and prostaglandin [18] that are extremely important to cancer pain development.

Stromal and tumor cells produce chemokines and growth factors that are responsible, at least in part, for the recruitment of macrophages and neutrophils to the tumor microenvironment. Both cells recruited are involved in cancer progression [19,20] as well as produced mediators that sensitize the nociceptor neurons [8, 21, 22]. An increasing in the level of cytokines has been found in patients with several types of cancer and chemotherapy also induces the release of pro-inflammatory mediators. Paclitaxel, for example, mimic the effects of lipopolysaccharide (LPS) by activating Toll-like receptor 4 (TLR4) and release TNFα and IL-1β [14].

The consequence and the persistence of cancer pain cause physical and psychological suffering, which limit the life quality of the patients. Current regimens for management of cancer pain includes the use of opioids, tricyclic antidepressants, and certain anticonvulsants, which often produce undesirable side effects [23, 24]. Thus, the management of chronic pain in cancer pain it is still a great challenge for

the clinicians, which makes necessary the study of the cancer pain mechanisms and novel analgesic therapies [25].

Flavonoids are polyphenolic compounds found in human diet [26]. Naringenin (4',5,7-tryhidroxy-flavonone) is a flavonoid which belongs to the flavanone class and it is found in citrus fruits including, lemon, orange, tangerine and grapefruit [27]. Some studies have demonstrated the beneficial effect of naringenin in inhibiting the nociceptive responses in models of pain induced by inflammatory stimuli such as phenyl-*p*-benzoquinone, acetic acid, formalin, complete Freund's adjuvant, capsaicin, carrageenan [28], superoxide anion [29], LPS [30], neuropathic pain [31, 32]. Moreover, naringenin reduces UVB-induced inflammation and skin edema in a model of [33], and DNA-damage induced by radiation [34]. The mechanism involved in these effects include the inhibition of nuclear factor kappa B (NFκB) activation [28], inhibition of mitogen activated protein kinases (MAPK) [35], inhibition of oxidative stress [33], inhibition of cytokine production [36], increasing the nuclear factor erythroid-derived-like 2 (Nrf2) and heme oxygenase-1 (HO-1), and activation of the analgesic NO−cGMP−PKG−ATP sensitive potassium channel (KATP) signaling pathway [29]. On cancer, naringenin demonstrated anti-cancer properties by inhibition of colon cancer cells, melanoma cells, breast cancer, stomach cancer, liver cancer and cervix cancer proliferation *in vitro* [37-39]. Also, naringenin induced apoptotic cell death in prostate cancer cell *in vitro* [40], reduced metastases in breast carcinoma 4T1 line-induce *in vitro* [41] and inhibit subcutaneous tumor growth sarcoma S-180-induced *in vivo* [38]. However, there is no data investigating the effect of naringenin in a model of cancer pain. Thus, in this work, we aimed to evaluate the antinociceptive effect of naringenin in Ehrlich tumor cells-induced pain.

## Materials and Methods

### Animals

Male Swiss mice (25-30 g), from the Universidade Estadual de Londrina, Londrina, Parana, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and kept at 21$^{o}$C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were in

accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 8482.2014.86).

**Test compound**

The compounds used in this study were naringenin (purity 98%; Santa Cruz Biotechnology), and saline (NaCl 0.9%; Fresenius Kabi Brasil Ltda. Aquiraz, CE, Brazil) and phosphate-buffered saline (PBS, pH 7.4).

**Experimental protocols**

The measurement of basal responses to mechanical and thermal stimuli and paw thickness were performed at day 0. Afterwards, mice received intraplantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$ or $1x10^7$). Ehrlich tumor cells are cultivated *in vivo*, by passages in the peritoneum of Swiss mice in ascitic form. Ten days after the intraperitoneal (i.p.) injection of Ehrlich tumor cells, the ascitic fluid of tumor cells were collected and washed in PBS (pH 7.4) followed by centrifugation (200 *g*, 10 min) three times. The cell viability was determined by 0.5% trypan blue exclusion method in Neubauer chamber. Ehrlich tumor cells were suspended to the final concentrations of $1x10^6$ or $1x10^7$ in 25 µL of saline and injected into the subcutaneous tissue/ intra-plantar (i.pl.) of mice, which passes from ascitic form to solid form [42]. On the $8^{th}$ day after mice received the Ehrlich tumor cells ($1x10^6$ in 25 µL of saline), they received the acute post-treatment with naringenin (16.7, 50 and 150 mg/kg, per oral (p.o.) diluted in saline) or vehicle (saline) and the mechanical and thermal hyperalgesia, and paw thickness were determined on 1, 3, 5, and 7 hours after treatment. For chronic post-treatment, mice were treated once a day with naringenin (150 mg/kg, p.o.) for 12 days, starting 10 min after Ehrlich tumor cells ($1x10^6$ or $1x10^7$) injection. Mechanical and thermal hyperalgesia and paw thickness were evaluated on days 2, 4, 6, 8, 10 and 12 after stimulation with $1x10^6$ cells, 3 hours after treatment with naringenin. The overt pain-like behavior ($1x10^7$ cells) was evaluated on the $8^{th}$ day, 3 hours after the treatment. Naringenin effect was also evaluated with a twice daily treatment protocol. Mechanical and thermal hyperalgesia and paw thickness were evaluated on days 2, 4, 6, 8, 10 and 12 ($1x10^6$ cells) and overt pain-like behavior ($1x10^7$ cells) was evaluated on the $8^{th}$ day in the same time as descripted above. On the $12^{th}$ day after injection, the paw skin tissue was

collected for the evaluation of myeloperoxidase (MPO) and N-Acetyl-β-D-Glucosaminidase (NAG) activity in both treatment protocols. For evaluation of the ascitic tumor proliferation, mice received Ehrlich tumor cells $1x10^6$ (200 µL, i.p.) and were treated twice daily for 10 days with naringenin (150 mg/kg p.o.). On the $10^{th}$ day, 3 hours after the treatment mice were euthanized and the ascitic liquid collect and used to determine the total volume and the concentration of tumor cells, as well as to determine the number of total and differential leukocytes, and MPO and NAG activity. The plasma concentration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and stomach MPO activity were determined on the $12^{th}$ day. In the same protocol, the paw skin and spinal cord samples of mice were collected for evaluation of oxidative stress for: ferric reducing ability (FRAP), free radical scavenging ability (ABTS) and reduced glutathione levels (GSH), as well as RT-qPCR for nuclear factor erythroid-derived-like 2 (Nrf2) and heme oxygenase-1 (HO-1); inflammatory response by RT-qPCR for cytokine (*TNFα* and *IL-1β*), and for glial cells (glial fibrillary acidic protein (*GFAP)* and ionized calcium-binding adapter molecule-1 *(Iba-1)).*

**Mechanical hyperalgesia**

Mechanical hyperalgesia was evaluated as previously reported [42]. In a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15-30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 $mm^2$ polypropylene tip. The investigator is trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. Mice received $1x10^6$ Ehrlich tumor cells i.pl. and the results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements 1, 3, 5 and 7 hours after acute treatment on the $8^{th}$ day after injection the Ehrlich tumor cells; 3 hours after once a day treatment; or 3 hours in the first treatment for twice daily protocol on 2, 4, 6, 8, 10 and 12 days after the injection of the cells.

**Thermal hyperalgesia.**

Mice were placed in a 10 cm-wide glass cylinder on a hot plate (Insight, Ribeirao Preto, SP, Brazil) maintained at 55°C. Two control latencies of at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 10–12.5 s. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 15 s to avoid tissue damage [42]. The results are expressed as thermal threshold and the evaluation was done in the same time points presented in mechanical hyperalgesia.

**Paw thickness or tumor growth**

Paw thickness was determined before and at indicated time points in mechanical hyperalgesia after the injection of Ehrlich tumor cells using an analog caliper. Paw thickness/tumor growth was presented as Δ mm [42].

**Overt pain-like behavior evaluation**

Mice received $1x10^7$ cells i.pl. in 25 µL, and were placed in clear glass compartments at room temperature. After an acclimatization period of 10 min, mice were observed during 10 min, and the cumulative number of flinches was determined [42].

**Myeloperoxidase (MPO) and n-Acetyl-β-D-Glucosaminidase (NAG) activity**

The neutrophil recruitment to the paw skin and stomach tissue, and ascitic liquid and the macrophages recruitment to paw skin tissue or ascitic liquid were evaluated by the MPO and NAG colorimetric assays, respectively, as previously described [43]. Briefly, mice received the Ehrlich tumor cells and were treated once or twice daily with naringenin (150 mg/kg, p.o.) for 12 days. On the $12^{th}$ day, 3 hours after the treatment, mice were euthanized and the paw skin samples were collected in 400 µL of 50 mM K2HPO4 buffer (pH 6.0) containing 0.5% HTAB and then homogenized in ice-cold Tissue-Tearor (Biospec). After that, homogenates were centrifuged (16,100*g*, 2 min, 4 °C) and the supernatants were collected. For the MPO assay, aliquots of 30 µL of supernatant were placed in a 96-well plate and mixed with 200 µL of 50 mM K2HPO4 buffer (pH 6.0), containing 0.0167% ortho-dianisidine dihydrochloride and 0.05% $H_2O_2$. The absorbance was determined after 5 min at 450 nm (Multiskan GO microplate spectrophotometer, ThermoScientific, Vantaa, Finland).

The MPO activity of samples was compared to a standard curve of neutrophils and presented as MPO activity. For NAG assay, 20 µL of supernatant was obtained as described for the MPO activity assay and added to a 96-well plate, followed by the addition of 80 µL of 50 mM phosphate buffer, pH 6.0. The reaction was initiated by adding 2.24 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide. Then, the plate was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 100 µL of 0.2 M glycine buffer, pH 10.6. The enzymatic activity was determined at 400 nm (Multiskan GO microplate spectrophotometer, ThermoScientific, Vantaa, Finland). NAG activity of samples was compared to a standard curve of macrophages and presented as NAG activity.

**Hepatotoxicity**

Mice received the Ehrlich tumor cells and were treated twice daily with naringenin (150 mg/kg, p.o.) for 12 days. On the $12^{th}$ day, 3 hours after the treatment mice were euthanized, and the plasma levels of AST and ALT were used as indicators of hepatotoxicity. These assays were performed using a diagnostic kit from Labtest® (Lagoa Santa, Minas Gerais, Brazil) [43].

**Ascitic tumor proliferation**

Mice received Ehrlich tumor cells $1x10^6$ in 200µL i.p. and were treated twice daily with naringenin (150 mg/kg p.o.) for 10 days. On the $10^{th}$ day, 3 hours after the treatment mice were euthanized and the ascitic liquid collected. The total ascitic volume in mL was determined with graduated test tube and the concentration of Ehrlich tumor cells was determined with a Neubauer chamber [44].

**Determination of leukocytes in ascitic liquid**

Mice received Ehrlich tumor cells $1x10^6$ (200µL i.p.) and were treated twice daily with naringenin (150 mg/kg p.o.) for 10 days. On the $10^{th}$ day, 3 hours after the treatment mice were euthanized and the ascitic liquid collected to evaluate total and differential leukocyte counts. Total cell counts were performed in Neubauer chamber using Turk solution, and differential cells counts (100 cells per slide) were performed in slides stained with panoptic kit (Laborclin Produtos para Laboratórios Ltda., Pinhais, PR, Brazil) under light microscope. Results were expressed as total leukocytes, polymorphonuclear and mononuclear cells [45].

**Ferric reducing ability (FRAP) and free radical scavenging ability (ABTS) Assays**

The ability of samples to resist oxidative damage was determined by FRAP and ABTS assay. The tests were adapted to a 96-well microplate format from previously described assays [46]. Mice were terminally anaesthetized and paw skin and spinal cord samples were collected on the 12$^{th}$ day after stimulus and homogenized on Tissue-Tearor (Biospec) with 500 µL of 1.15% KCl. The homogenates were centrifuged (10 min × 200*g* × 4 °C) and the supernatants were used in both assays. For the ABTS assay, ABTS solution was diluted with phosphate buffer saline (PBS) pH 7.4 to an absorbance of 0.80 at 730 nm. Then, 200 µL of diluted ABTS solution was mixed with 20 µL of sample in each well. After 6 min of incubation at 25 °C, the absorbance was measured at 730 nm. For the FRAP assay, 50 µL of supernatant was mixed with 150 µL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37 °C for 30 min and the absorbance was measured at 595 nm. The results FRAP and ABTS assays were equated against a Trolox standard curve (0.02−20 nmol). The results of both assays are expressed as nmol of Trolox equivalents per mg of paw skin or spinal cord, which is the amount of Trolox (nmol) with an equivalent antioxidant potential to 1 mg of the tissue under investigation.

**Reduced Glutathione Assay (GSH)**

Paw skin and spinal cord samples of mice were collected on the 12$^{th}$ day after stimulus and GSH levels were determined using a spectrophotometric method. Samples were maintained at -80ºC for at least 48 h after collection. Samples were homogenized with Tissue-Tearor with 200 µL of 0.02M EDTA. The homogenate was mixed with 25 µL of 50% trichloroacetic acid and was homogenized three times during 15 min. The mixture was centrifuged (15 min, 1.5 *g,* 4ºC). The supernatant was added to 200 µL of 0.2 M TRIS buffer, pH 8.2, and 10 µL of 0.01M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve with GSH was performed. The results are expressed as GSH per mg of paw skin or spinal cord [47].

**Real-time PCR and quantitative PCR**

Paw skin and spinal cord tissues were collected on the 12$^{th}$ day, 3 hours after the treatment and homogenized in Trizol® reagent. Total mRNA was isolated

according to manufacturer's directions. RNA purity was confirmed by the 260/280 ratio. RT-PCR and quantitative PCR were performed using GoTaq® 2-Step RT-qPCR System (Promega) following the manufacturer's directions on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The following primer sequences were used: *Nrf2:* sense 5´-TCACACGAGATGAGCTTAGGGCAA-3´, antisense 5´–TACAGTTCTGGGCGGCG ACTTTAT-3´; *HO-1*: sense 5´-CCCAAAACTGGCCTGTAAAA-3´, antisense 5´-CGTGGTCAGTCAACATGGAT-3´; *Tnfα*: sense 5'-TCTCATCAGTTCTATGGCCC-3', antisense 5'-GGGAGTAGACAAGGTACAAC-3'; *pro-Il-1β*: sense 5'-GAAATGCCACCTTTTGACAGTG-3', antisense 5'-TGGATGCTCTCATCAGGACAG-3'; *Gfap*: sense 5'-GGCGCTCAATGCTGGCTTCA-3', antisense 5'-TCTGCCTCCAGCCTCAGGTT-3'; *Iba-1*: sense 5'-ATGGAGTTTGATCTGAATGGAAAT-3', antisense 5'-TCAGGGCAGCTCGGAGATAGCTTT-3'; *β-actin,* sense: 5´-AGCTGCGTTTTACACCCTTT-3´, antisense: 5´- AAGCCATGCCAATGTTGTCT-3´. The expression of *β-actin* mRNA was used as a reference gene to normalize data.

**Statistical Analysis**

Results were presented as means ± SEM of measurements made on six mice in each group per experiment and are representative of two independent experiments. Two-way analysis of variance (ANOVA) followed by Tukey's post hoc was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment interaction. Differences between responses were evaluated by one-way ANOVA followed by Tukey's post hoc for data of single time point. Statistical differences were considered significant when $p < 0.05$.

## Results

### Single dose of post-treatment with naringenin inhibits the Ehrlich tumor cells-induced pain like-behavior in a dose and time depend manner

Mice received Ehrlich tumor cells $1x10^6$ in 25µL i.pl, and were treated with naringenin in doses (16.7, 50, or 150 mg/kg, p.o. diluted in saline) or vehicle on the $8^{th}$ day after tumor cells injection. Ehrlich tumor cells injection induced significant mechanical hyperalgesia on the $8^{th}$ day as previous standardization [42]. The mechanical and thermal hyperalgesia and paw thickness were assessed at 1, 3, 5 and 7 hours after treatment for (Fig. 1). The treatment with naringenin 150 mg/kg p.o. inhibited the mechanical hyperalgesia at 1 and 3 h after the treatment (Fig. 1a). The intensity of thermal hyperalgesia was inhibited 3 h after the treatment (Fig. 1b) at doses 50 and 150 mg/kg p.o. of naringenin. Naringenin did not reduce the paw thickness (Fig. 1c). Based on fact that only naringenin at 150 mg/kg reduced thermal hyperalgesia, this dose was chosen for the next experiments.

### Chronic post-treatment with naringenin inhibits Ehrlich tumor cells-induced mechanical and thermal hyperalgesia, paw thickness, neutrophil and macrophage recruitment and overt pain-like behavior.

In the next protocol, mice received Ehrlich tumor cells $1x10^6$ i.pl. after 10 min and received the treatment with naringenin (150 mg/kg p.o. diluted in saline, once a day during 12 days). Mechanical and thermal hyperalgesia and paw thickness were evaluated at indicated time points (Fig. 2). Naringenin (150 mg/kg p.o.) significantly reduced the mechanical hyperalgesia from days 4-10 (Fig. 2a and b). The inhibition of Ehrlich tumor cells-induced mechanical and thermal hyperalgesia was accompanied by alteration of paw thickness (Fig. 2c). On the $12^{th}$ day after stimulus mice were euthanatized and the paw skin tissue was collect to determine MPO and NAG activity. Once a day treatment inhibited the MPO and NAG activity (Fig. 2d and e), respectively. Moreover, treatment with naringenin at 150 mg/kg reduced Ehrlich tumor cells-induced overt pain-like behavior (Fig. 2f). As the once daily treatment protocol exhibit a mild reduction of the behavioral evaluated parameters, we next wonder whether twice daily treatment regimen could produce a more prominent reduction. The twice daily treatment significantly reduced the mechanical hyperalgesia from day 2-12 (Fig. 3a) demonstrating that naringenin effects stated

earlier. Thermal hyperalgesia was also reduced from day 6-12 (Fig. 3b). The inhibition of Ehrlich tumor-induced mechanical and thermal hyperalgesia was accompanied by reduction of paw thickness from day 4-12, two days earlier when compared with once daily protocol (Fig. 3c). The MPO and NAG activity were inhibited with twice daily treatment with naringenin (Fig. 3d and e, respectively). Further, the twice daily treatment regimen reduced Ehrlich tumor cells-induced overt pain-like behavior (Fig. 3f). As the behavioral parameters were better reduced with the twice daily treatment regimen, this protocol was chosen for the next experiments.

**Naringenin reduces Ehrlich tumor cells-induced leukocytes recruitment without affecting the tumor proliferation**

Given that chronic treatment with naringenin reduced Ehrlich tumor cells-induced edema, we next wonder whether this inhibition was related to leukocyte recruitment and/or cytotoxic activity over tumor cells. Mice received the Ehrlich tumor cells (200 µL of $1x10^6$ cells, i.p.) and were treated with naringenin (150 mg/kg, p.o.) twice daily per 10 days. On the $10^{th}$ day, mice were euthanized and the ascitic liquid was collect. Naringenin did not inhibit the tumor cells proliferation from the evaluation of ascitic tumor volume (Fig. 4a) and cell tumor count (Fig. 4b). On the other hand, naringenin treatment (150 mg/kg, p.o., twice daily) inhibited the inflammatory cells migration as observed with reduction in total leukocytes (Fig. 5a), polymorphonuclear (Fig. 5b), and mononuclear cells (Fig. 5d). The MPO and NAG activity also were inhibited by the naringenin treatment (Fig. 5c and e, respectively), confirming the data demonstrated in the Fig. 2d, 2e and Fig. 3d, 3e. These data show that the reduction on paw edema observed by naringenin is related to the inhibition of leukocyte recruitment rather than an anti-tumor effect.

**Naringenin inhibits Ehrlich tumor cells-induced oxidative stress**

Mice received Ehrlich tumor cells $1x10^6$ i.pl. and were treated with naringenin as in Fig. 3. On the $12^{th}$ day, mice were euthanized and the paw skin and spinal cord tissues were collected 3 hours after treatment. Ehrlich tumor cells induced oxidative stress in both hind paw skin and spinal cord by decreasing the total antioxidant capacity (FRAP and ABTS assays) and depletion of GSH levels (Figure 6). Treatment with naringenin was able to prevent this depletion in both hind paw skin

and spinal cord tissues (Fig 6a-d). GSH depletion was also prevented in paw skin (Fig. 6e) and spinal cord (Fig. 6f) tissues. These data shown that naringenin possesses peripheral and central antioxidant effect in this model of cancer pain.

**Naringenin modulates differently Ehrlich tumor cells-induced peripheral and spinal cord the mRNA expression of Nrf2 and HO-1**

Mice received Ehrlich tumor cells $1x10^6$ i.pl. and were treated with naringenin as in Fig. 3. On the $12^{th}$ day, mice were euthanized and the paw skin and spinal cord tissues were collected 3 hour after treatment. Ehrlich tumor cells increased the mRNA expression of Nrf2 and HO-1 in paw skin of mice which were reduced by naringenin treatment (Fig 7a and c). On the other hand, in the spinal cord, naringenin increased the mRNA expression of both Nrf2 and HO-1 (Fig. 7b and d).

**Naringenin inhibits peripheral and spinal cord Ehrlich tumor cells-induced pro-inflammatory cytokines (TNFα and IL-1β)**

The effect of naringenin in mRNA cytokine expression was then evaluated. Mice received Ehrlich tumor cells and were treated as in Fig. 3. On the $12^{th}$ day, mice were euthanized and the paw skin and spinal cord tissues were collected 3h after the treatment for the determination of mRNA expression of *Tnfα* and *Il-1β* (Fig. 8). Ehrlich tumor cells induced an increased peripheral and spinal cord mRNA expression of *Tnfα* (Fig 8a and b) and *Il-1β* (Fig. 8c and d). Importantly, naringenin inhibited peripheral and spinal cord mRNA expression of both *Tnfα* and *Il-1β,* demonstrating that the analgesic mechanism of naringenin could involve peripheral and central anti-inflammatory effects.

**Naringenin inhibits Ehrlich tumor cells-induced astrocytes and microglia activation**

Mice received Ehrlich tumor cells $1x10^6$ i.pl. and were treated with naringenin as in Fig. 3. On the $12^{th}$ day, mice were euthanized and the spinal cord tissue was collected 3h after treatment. The mRNA expression of astrocytes and microglia was evaluated (Fig. 9). Ehrlich tumor cells induced glial cells activation by the increase in mRNA expression of *Gfap* and *Iba-1* (Fig. 9). Importantly, treatment with naringenin inhibited astrocytes (Fig. 9a) and microglia (Fig. 9b) activation, demonstrating that naringenin might modulate Ehrlich tumor cells-induced glial cells activation.

## Discussion

Cancer pain is a complex problem that involves many mechanisms including those related to neuropathic and inflammatory response [3]. These mechanisms can be related to the tumor growth, type of tumor cells, localization of tumor mass and aggressive treatment, which affect the life quality of patients [50]. Herein, we observed that naringenin reduced Ehrlich tumor cells-induced chronic pain by inhibiting leukocyte recruitment, peripheral and spinal cord pro-inflammatory cytokines, peripheral and spinal cord oxidative stress, and glial cells activation. In this model, intra-plantar injection of Ehrlich tumor cells induces chronic hyperalgesia and overt pain-like behaviors [42]. This is relevant given that patients with chronic pain, more often present spontaneous ongoing or intermittent pain, that is pain arising in the absence of any peripheral stimulus, and therefore, an experimental model of cancer pain that induces spontaneous pain it is likely a more relevant readout for human pain.

In cancer, the inflammation begins as a low-grade response without any manifestations of acute reaction [51] and the main cells involved in this response are neutrophils, macrophages, and lymphocytes [9, 20]. Increased neutrophil accounts in patients with advanced stage cancer are associated to poor prognosis for disease [52].

Compounds as flavonoids are widely known for its anti-inflammatory and antioxidant properties [26, 51]. In fact, naringenin showed anti-inflammatory effects in several models [28-30, 32, 53-56] through mechanisms such as the inhibition of leukocyte recruitment [29, 30, 33], $PGE_2$ production in activated macrophages [55], reduction of Chlamydia trachomatis-induced TLR2 and TLR4 and thereby downstream p38-MAPK pathway in infected macrophages [56], and inhibition of carrageenan-induced NF-kB activation [28].

Herein, we observed that naringenin reduced paw edema, which leads to questioning whether or not the treatment with naringenin has anti-tumor effect in Ehrlich tumor-model, as in other studies naringenin presents this effect [37-41]. To investigate this hypothesis more thoroughly, we performed the ascitic model in the peritoneal cavity [44] and showed that the total ascitic volume and the number of tumor cells per mL were not altered by the treatment for 10 days in mice, as compared to the control tumor group. Thus, the reduction in paw thickness is related to the inhibition of leukocyte migration. This difference in response might be related

to type of tumor cells studied, for example, in our study we used a mammary adenocarcinoma from female mice and other studies used various types of malignance human tumor cells [37-41], as well as the most studies has been performed *in vitro* assays. The doses and treatment scheme used also were different since we used 150 mg/kg during 10-12 days, Zhang et al., [41] used naringenin treatment with 200 mg/kg for 30 days *in vivo* and observed the reduction of proliferation and metastases cancer-induced in different tumor line cells (4T1) inoculated in a different tissue. Kanno et al. [38] used a subcutaneous model of tumor growth sarcoma S-180 and observed that treatment with naringenin reduced the weight of excised tumor. However, the doses (30, 100 or 300 mg/kg once a day for 5 days p.o.), the analysis methods to evaluate the tumor growth and also the cell line used in Kanno's study were different, which could justify the different results.

As neutrophils and macrophages produce nerve growth factor (NGF), pro-inflammatory cytokines, and reactive oxygen species (ROS) that can activate and sensitize nociceptive neurons [9, 57], promoting the persistence of the inflammatory process in cancer [58], the leukocyte recruitment inhibition to the tumor microenvironment may contribute for the reduction in the inflammatory process and, consequently, can decrease pain [59].

Neutrophil and macrophage recruitment to the tumor microenvironment can contribute to the increase in oxidative stress and pro-inflammatory cytokines production [60, 61]. Once ROS possesses anti-tumor effects due to DNA damage-induced [20, 61], ROS have been described as possible therapy for treatment and prevention of cancer. Of note, chemotherapy increases ROS production to induce the death of tumor cell [62], which seems to be involved in the induction of apoptosis by the regulation of the phosphorylation and ubiquitination of pro-apoptotic proteins [63]. Regarding cancer pain, pronociceptive actions of ROS might be related to the change in the response to glutamate by primary afferent neurons and the release of glutamate itself as an algogenic substance by cancer cells [64]. Moreover, ROS are also implicated in the activation of NF-κB [65] and neutrophil recruitment [66]. Therefore, in terms of pain, molecules that inhibit oxidative stress are likely to be highly attractive. In this study, we demonstrated that the peripheral stimulation with tumor cells in the paw skin tissue induces peripheral and spinal cord oxidative stress, which were reduced by naringenin treatment. Flavonoids are known for their antioxidant effect [26, 51] and naringenin possesses antioxidant activity [28, 29, 53,

67-69]. In fact, *in vitro* data showed that naringenin presents scavenger ability at 40 μM and molecular docking also demonstrated that naringenin reduces NADPH oxidase activation by inhibiting PKC-mediated $p47^{phox}$ phosphorylation through interaction with Gly-253 and Leu-251 amino acid residues [70]. Other studies have shown that naringenin induce an increase in ROS to increase apoptosis in tumor cells [40]. Our data showed that the Ehrlich tumor cells increased the mRNA expression of Nrf2 and HO-1 in the paw skin, which might be related to a protective mechanism triggered by cancer cells. However, despite this attempt, it is likely that the peripheral oxidative stress was already well-established in the tumor group (as observed by reduction in FRAP, ABTS and GSH levels) and the increase in Nrf2 mRNA expression was not enough to counteract local oxidative stress. Importantly, naringenin reduced Ehrlich tumor-induced peripheral oxidative stress, which might account for its analgesic effect. Of note, in the spinal cord, naringenin increased Nrf2 and HO-1 mRNA expression, suggesting that naringenin not only reduces ROS but also increases antioxidant defense. Moreover, this increase is relevant given that the downstream Nrf2 target, HO-1 can activate the cGMP/PKG/ATP-sensitive potassium channel pathway leading to analgesia [29].

Besides ROS in the microenvironment of tumor, cytokines also are major players in cancer pain physiopathology. Therefore, cytokine-targeting therapies are related to the inhibition of chronic pain [71-73]. Cytokines are release by leukocytes recruited in microenvironment and by tumor cells in response to ROS, for instance. Peripheral pro-inflammatory cytokines such as TNFα and IL-1β are increased in Ehrlich tumor cells-induced pain model [73]. At the inflammatory foci, TNFα stimulates immune cells, which have the potential to produce nociceptive agents that interact with primary afferent nociceptors [3]. Moreover, TNFα can also activate nociceptor neuron as these cells express TNFR [74]. In addition, inflammatory cytokines have been suggested as biomarkers in breast cancer patients [75] and increased TNFα levels have been associated in patients with cancer pain [72]. Naringenin inhibited the mRNA expression of TNFα in the Ehrlich tumor cells-induced pain in both, paw skin and spinal cord. In fact, our data corroborates others showing that naringenin inhibits TNFα [28, 33, 76, 77]. IL-1β levels were also increased in Ehrlich tumor cells-induced pain in both, paw skin and spinal cord tissues, and the production and secretion of IL-1β is associated with pain in pathological conditions like tumor growth [78]. For instance, patients diagnosed with breast cancer, prostate

cancer and multiple myeloma present elevated levels of IL-1β and higher serum concentrations of IL-1β are related with malignancies where there is a low survival rate from time of diagnosis and bad prognosis [79, 80]. The treatment with naringenin reduces the tumor cells-induced release of IL-1β. In other models of pain and inflammation, naringenin showed the same effect [28, 33]. Naringenin, as other flavonoids, can inhibit NF-κB activation [30, 77], and thereby this inhibition might account for the inhibition of pro-inflammatory cytokines.

Chronic pain is a maladaptive pain, resulting from the development of neural plasticity in the peripheral nervous system (peripheral sensitization) and central nervous system (central sensitization) [81]. Upon nerve injury, nociceptor neurons release molecules such as ATP, CCL2, and fractalkine which can activate both astrocytes and microglia [3]. In turn, astrocytes and microglia when activated also produce cytokines as TNFα and IL-1β, which are crucial in the spinal cord processing of pain [72, 82]. Glial cells-derived mediators lead to cancer pain models by inducing spinal cord plasticity by overexpression of nociceptive mediators and receptors and electrophysiological changes in spinal cord [3]. Thus, the modulation of glial cells activation might represent a promising strategy for analgesia in cancer pain as well. In this work, naringenin treatment reduced Ehrlich tumor cells-induced glia cells activation and pro-inflammatory cytokines TNFα and IL-1β. In fact, naringenin inhibits LPS/IFN-γ-induced glial cells activation as well inhibit the TNFα production and neuronal injury *in vitro* [83]. The inhibition of TNFα and IL-1β is relevant given that both TNFα and IL-1β can enhance the amplitude of AMPA- and glutamate-induced excitatory currents, while only IL-1β reduces GABA and glycine-induced inhibitory transmission in nociceptor neurons at spinal cord, and thereby contributing to an enhanced pain state [84].

In conclusion, we found that naringenin reduced Ehrlich tumor cells-induced cancer pain by reducing paw thickness and leukocyte recruitment (neutrophils and macrophages) to the paw skin tissue, also by inhibiting the oxidative stress and inflammation in paw skin and spinal cord tissues. Of note, naringenin treatment presented no effect in Ehrlich tumor cells proliferation. At the spinal cord, naringenin treatment increased mRNA expression of both Nrf2 and HO-1, which might contribute to the analgesic effect herein observed. Further, naringenin treatment reduced the peripheral and spinal cord mRNA expression of the pro-inflammatory cytokines TNFα and IL-1β, and inhibited the activation of glial cells. Importantly, naringenin treatment

did not induce liver toxicity or stomach lesions. To our knowledge, this is the first work demonstrating the analgesic effect of naringenin in a murine model of cancer pain.

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## Figures and legends

### Figure 1

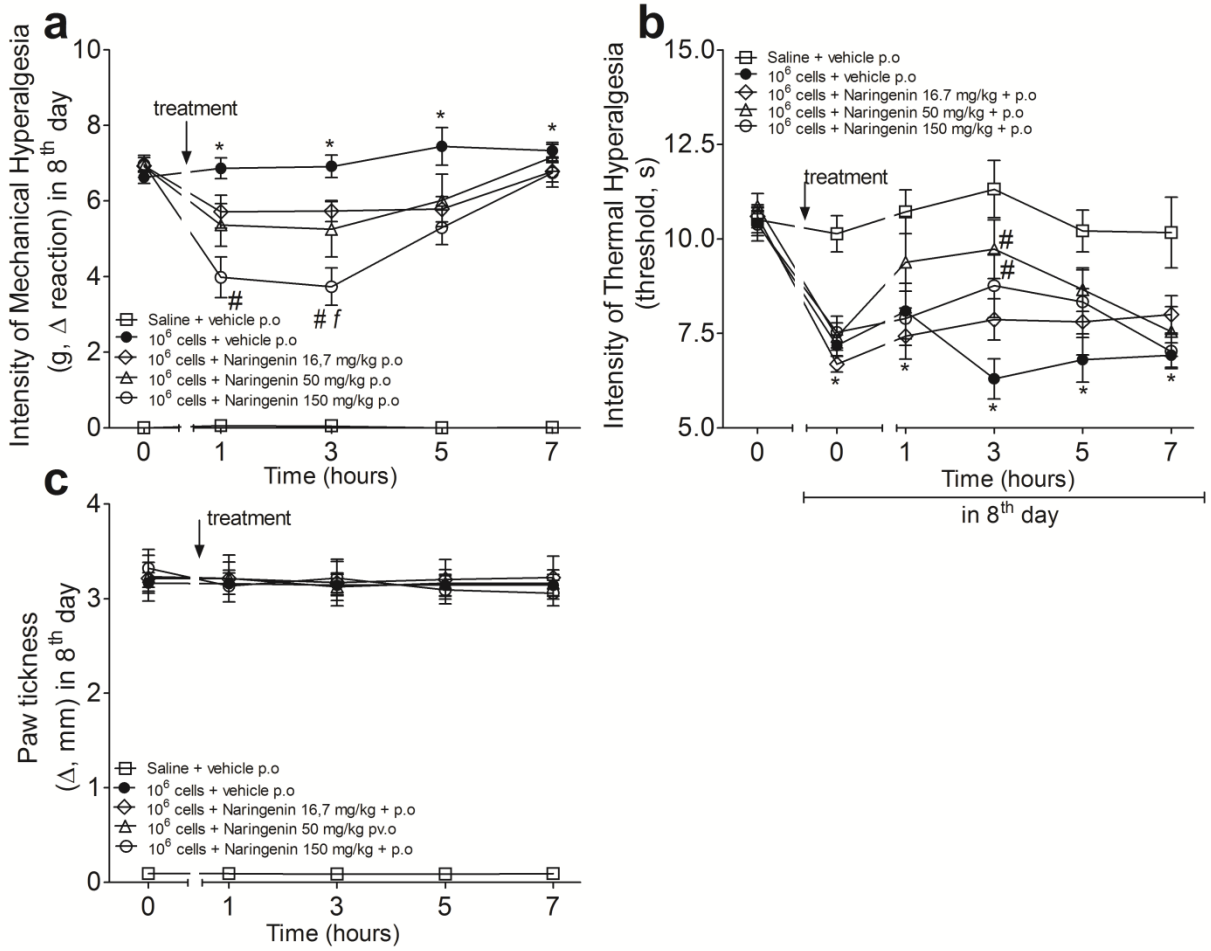


**Fig. 1: Acute treatment with naringenin inhibits pain-like behavior induced by Ehrlich tumor cells.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and, on the 8th day, were treated with naringenin (16.7; 50 or 150 mg/kg diluted in saline) or vehicle (saline) per oral (p.o.). Mechanical hyperalgesia (a), thermal hyperalgesia (b) and paw thickness (c) were evaluated 1, 3, 5, and 7 hours after treatment. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). *p < 0.05 vs saline group, # p < 0.05 vs tumor group, #f p < 0.05 vs tumor group + 16.7 mg/kg of naringenin. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 2**

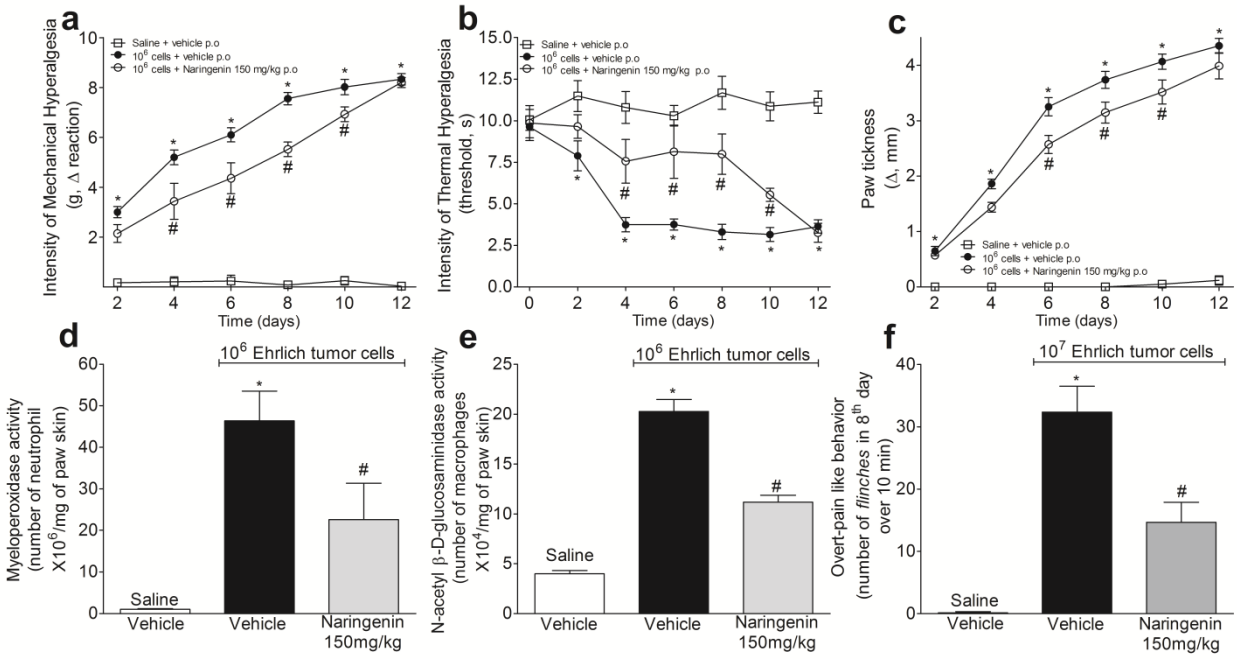


**Fig. 2: Chronic treatment with naringenin once a day inhibits pain-like behavior induced by Ehrlich tumor cells.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Mice were treated once a day during 12 days; mechanical hyperalgesia (a), thermal hyperalgesia (b), and paw thickness (c) were evaluated 3 hours after treatment on days 2, 4, 6, 8, 10, and 12 after the injection of the Ehrlich tumor cells. On the $12^{th}$ day after stimulation, paw skin tissue was collected for the evaluation of myeloperoxidase (MPO) (d) and N-Acetyl-β-D-Glucosaminidase (NAG) (e) activity. For the evaluation of overt pain-like behavior, mice received Ehrlich tumor cells ($1x10^7$/25 µL) or saline (25 µL) and were treated p.o. with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) 10 min after the injection of the stimulus. The treatment was performed once a day during 8 days and the number of *flinches* was evaluated for 10 min (f). Results are expressed as mean ± SEM ($n$ = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 3**

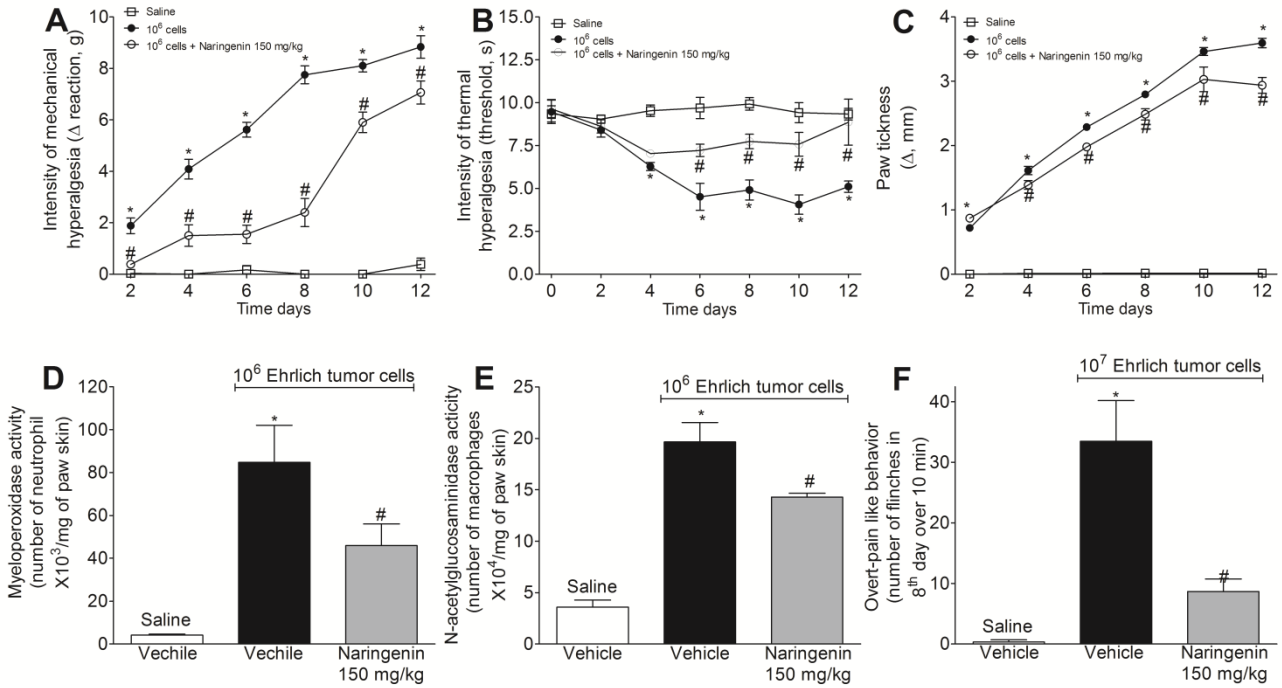


**Fig. 3: Chronic treatment with naringenin twice a day inhibits pain-like behavior induced by Ehrlich tumor cells.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Mice were treated twice a day during 12 days; mechanical hyperalgesia (a), thermal hyperalgesia (b), and paw thickness (c) were evaluated 3 hours after treatment on days 2, 4, 6, 8, 10, and 12 after the injection of the Ehrlich tumor cells. On the 12$^{th}$ day after injection, paw skin tissue was collected for the evaluation of myeloperoxidase (MPO) (d) and N-Acetyl-β-D-Glucosaminidase (NAG) (e) activity. For the evaluation of overt pain-like behavior, mice received Ehrlich tumor cells ($1x10^7$/25 µL) or saline (25 µL) and were treated p.o. with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) 10 min after the injection of the stimulus. The treatment was performed twice a day during 8 days, and the number of *flinches* was evaluated for 10 min (f). Results are expressed as mean ± SEM ($n$ = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 4**

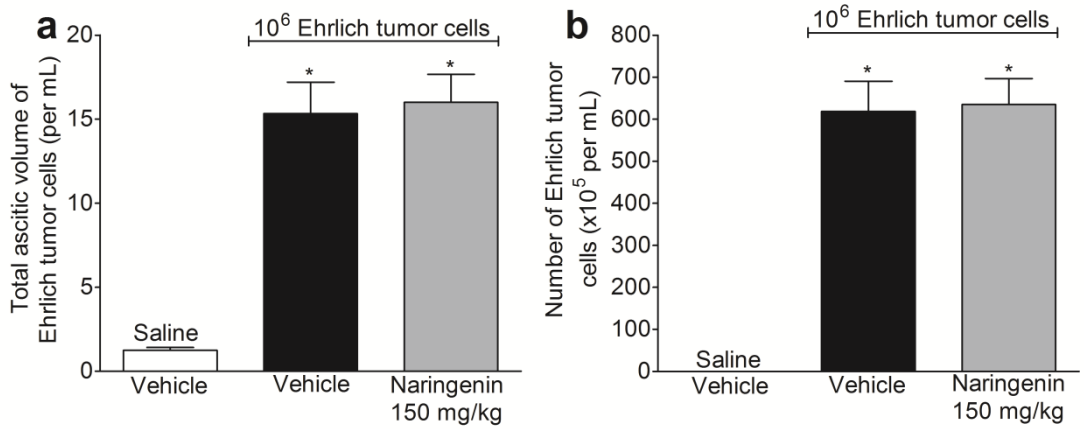


**Fig. 4: Naringenin does not inhibit the volume or number of tumor cells in the ascitic Ehrlich tumor model.** Mice received intraperitoneal (i.p.) injection of Ehrlich tumor cells ($1x10^6$/200 µL) or saline (200 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 10 days. On the $10^{th}$ day after injection, the ascitic liquid of mice was collected, the volume was measured in a graduated test tube (a) and 1 mL of this ascitic liquid was used to count the number of tumor cells (b) in a Neubauer chamber. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 5**

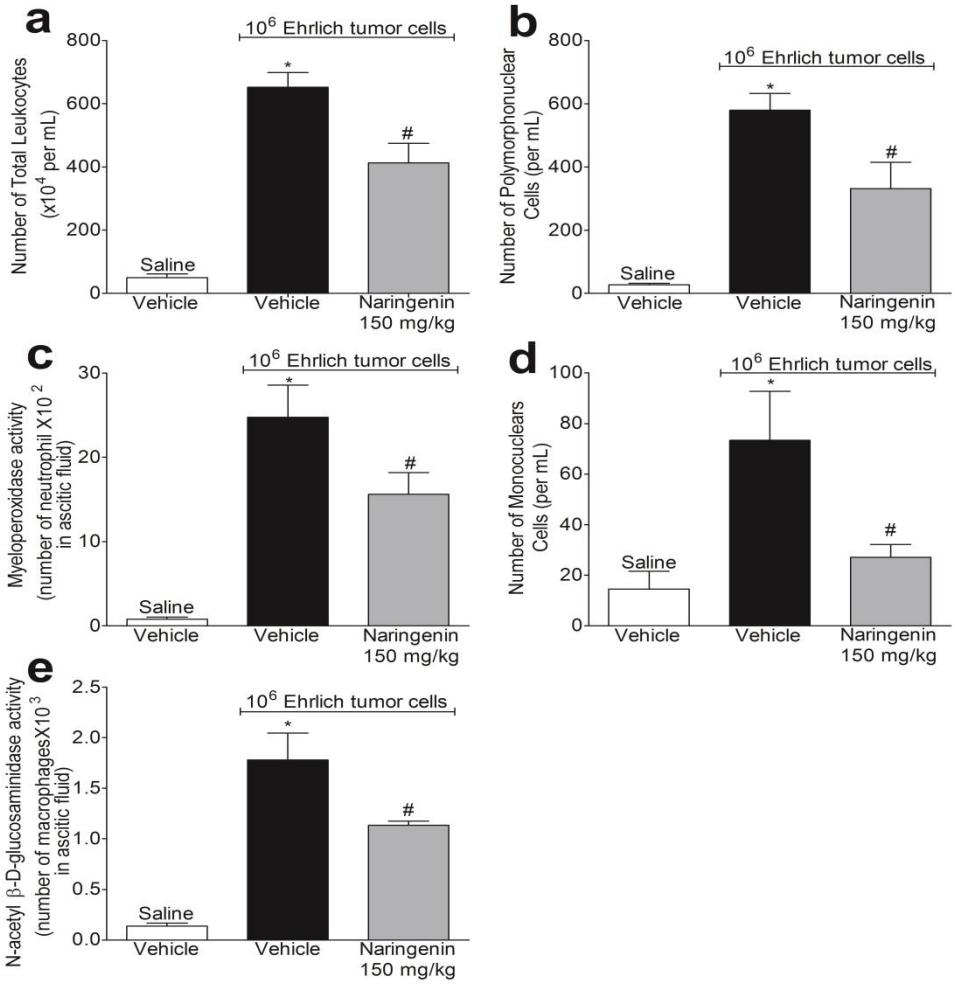


**Fig. 5: Naringenin inhibits leukocyte recruitment in ascitic Ehrlich tumor model.** Mice received intraperitoneal (i.p.) injection of Ehrlich tumor cells (1x10$^6$/200 µL) or saline (200 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 10 days. On the 10$^{th}$ day after injection, the ascitic liquid of mice were collected and 1 mL was used to count the number of total leukocytes (a) in a Neubauer chamber and polymorphonuclear (b) and monocuclear (d) cells using hematoxycilin and eosin stain. Myeloperoxidase (MPO) (c) and N-Acetyl-β-D-Glucosaminidase (NAG) (e) activity were also evaluated in the ascitic liquid. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 6**

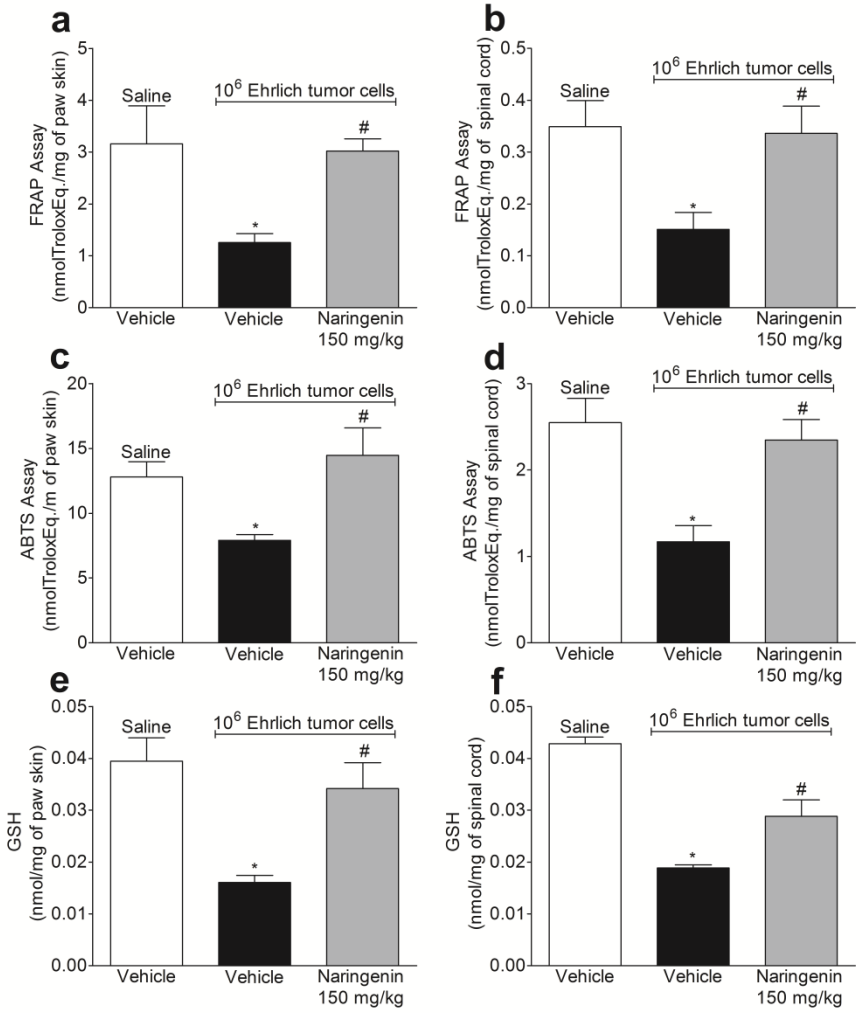


**Fig. 6: Naringenin inhibits the oxidative stress induced by Ehrlich tumor cells.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 12 days. On the $12^{th}$ day after injection, mice were euthanized and the paw skin (a, c, e) and spinal cord (b, d, f) were collected to evaluate the antioxidant capacity by FRAP (a, b) and ABTS methods (c, d) and measurements of reduced glutathione (GSH) levels (e, f). Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 7**

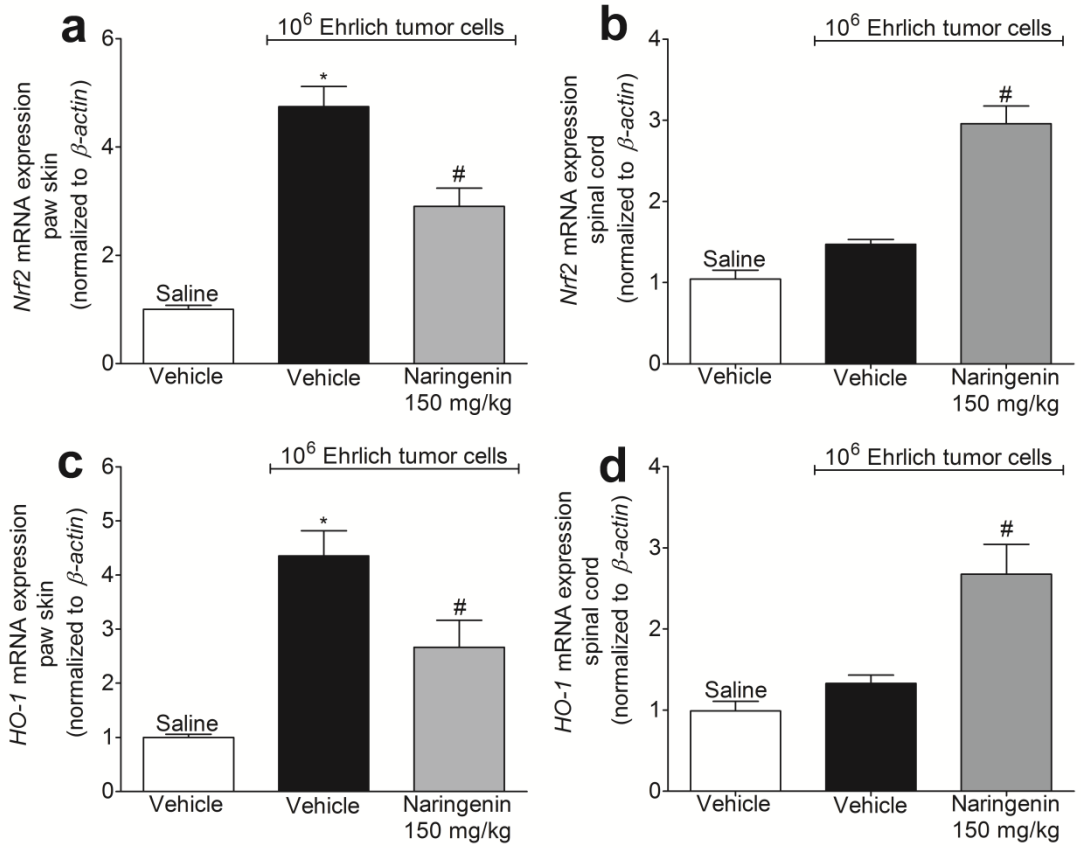


**Fig. 7: Naringenin modulates *Nrf2* and *HO-1* mRNA expression induced by Ehrlich tumor cells in the paw skin and spinal cord.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 12 days. On the $12^{th}$ day after injection, mice were euthanized and the paw skin (a, c) and spinal cord (b, d) were collected and the mRNA expression for *Nrf2* factor (a, b) and *HO-1* (c, d) were measured. *β-actin* was used as a reference gene to normalize mRNA expression. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 8**

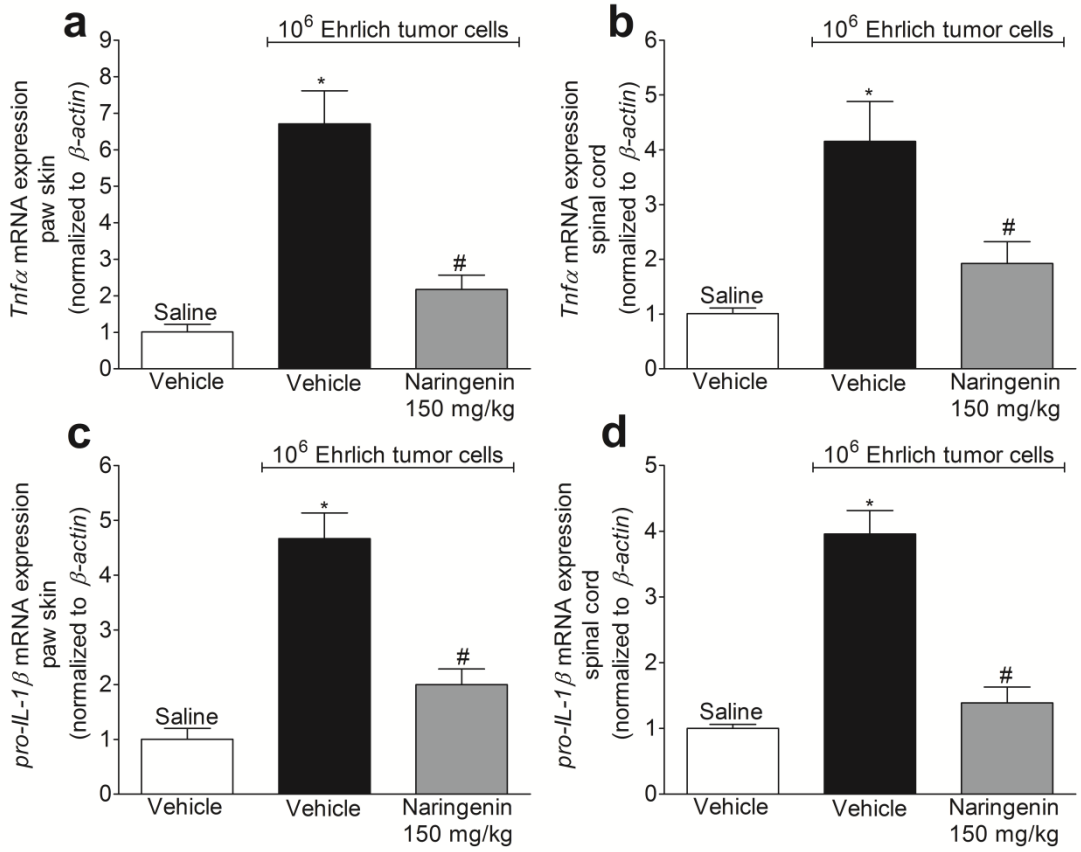


**Fig. 8: Naringenin inhibits cytokine mRNA expression induced by Ehrlich tumor cells.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells (1x$10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 12 days. On the 12$^{th}$ day after injection, mice were euthanized and the paw skin (a, c) and spinal cord (b, d) were collected and the mRNA expression for *TNFα* (a, b) and *IL-1β* (c, d) were measure. *β-actin* was used as a reference gene to normalize data mRNA expression. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 9**

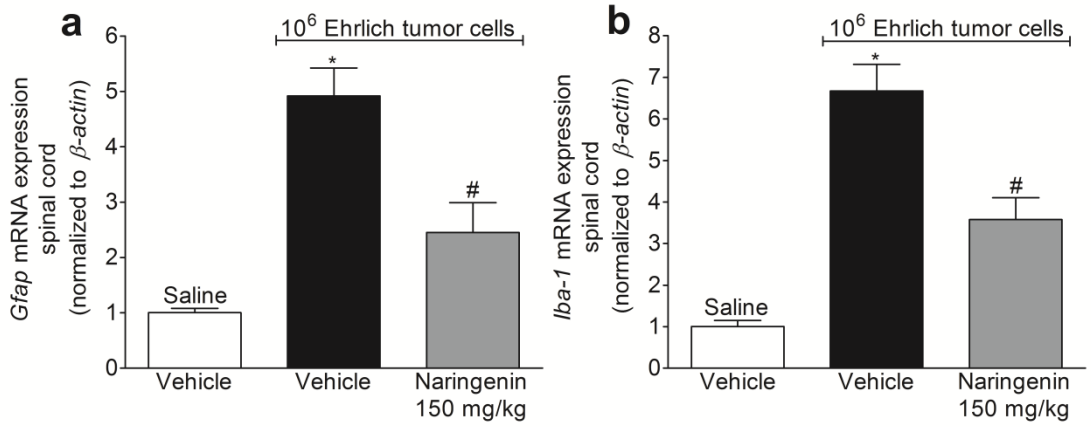


**Fig. 9: Naringenin inhibits the activation of glia cells induced by Ehrlich tumor cells.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 12 days. On the 12$^{th}$ day after injection, mice were euthanized, the spinal cord was collected and astrocytes (*GFAP*) (a) and microglia (*Iba-1*) (b) mRNA expression were evaluated. *β-actin* was used as a reference gene to normalize mRNA expression. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). * $p < 0.05$ vs saline group, # $p < 0.05$ vs tumor group. One-way ANOVA followed by Tukey's multiple comparison test.

**Figure 10**

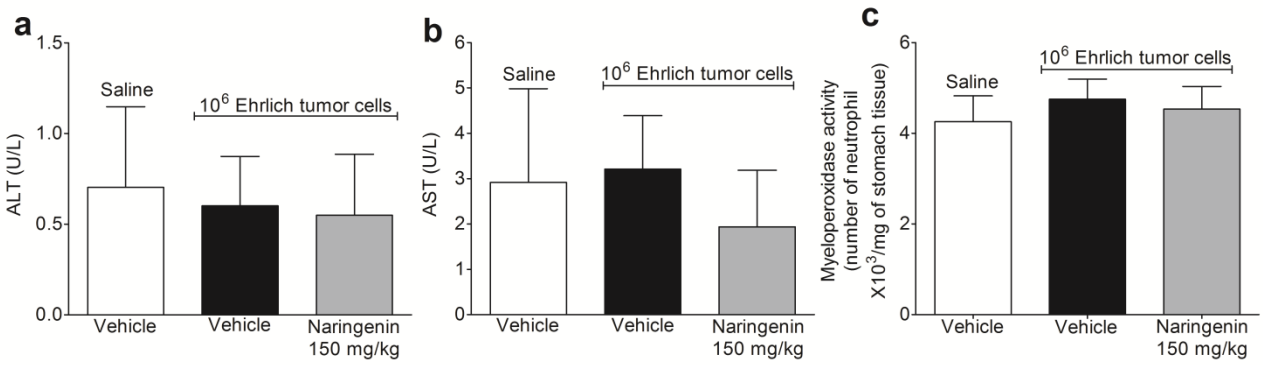


**Fig. 10: Naringenin does not induce liver toxicity or stomach lesions.** Mice received intra-plantar (i.pl.) injection of Ehrlich tumor cells ($1x10^6$/25 µL) or saline (25 µL) and after 10 min were treated with naringenin (150 mg/kg; diluted in saline) or vehicle (saline) per oral (p.o.). Treatment was performed twice a day during 12 days. On the $12^{th}$ day after injection, mice were euthanized and 1 mL of blood was collected to assess aspartate aminotransferase (AST) (a) and alanine aminotransferase (ALT) (b) levels and the stomach (c) was collected for Myeloperoxidase (MPO) activity. Results are expressed as mean ± SEM (*n* = 6 per group, representative of two separate experiments). One-way ANOVA followed by Tukey's multiple comparison test.

## 5 CONCLUSÃO

Em conclusão, o presente estudo demonstrou os efeitos benéficos dos polifenóis: ácido vanílico, quercetina e naringenina em diferentes modelos de dor inflamatória e dor no câncer. Os resultados obtidos demonstram que os efeitos atribuídos a estes compostos estão relacionados a sua ação anti-inflamatória por reduzir a migração de neutrófilos e macrófagos, reduzir produção de citocinas pró-inflamatórias e inibir a ativação do fator nuclear NFκB; sua ação antioxidante também foi observada nos diferentes modelos por aumentar a capacidade antioxidantes dos animais tratados e por evitar o consumo do sistema antioxidante endógeno. O efeito analgésico dos compostos também foram observados, sendo plausível o entendimento de que este efeito possa estar relacionado ao potencial anti-inflamatório e antioxidante dos polifenóis, contudo observamos em especial, a quercetina, se mostrando eficaz em modular mecanismos opióides dependentes, reforçando desta maneira, que cada modelo estudado e cada composto estudado, podem apresentar suas particularidades. Assim, podemos sugerir que os polifenóis poderiam ser considerados uma nova ferramenta farmacológica para o tratamento da dor inflamatória e da dor no câncer, sendo capaz de modular não apenas o processo inflamatório local, mas também as possíveis alterações a nível espinal decorrente dos estímulos periféricos. Contudo, estudos pré-clínicos ainda devem ser realizados para esta afirmação.

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