Universidade Estadual de Londrina

FERNANDA SOARES RASQUEL DE OLIVEIRA

HESPERIDINA-METIL-CHALCONA INTERAGE COM O RESÍDUO Ser276 DA SUBUNIDADE p65 DO NFκB E INIBE A DOR E A INFLAMAÇÃO EM MODELO DE ARTRITE E A ATIVAÇÃO DE MACRÓFAGOS RAW 264.7 INDUZIDOS POR ZYMOSAN

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Dissertação apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Mestra.

Orientador: Prof. Dr.Waldiceu Apº Verri Jr.

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RESUMO

O zymosan é um polissacarídeo derivado do fungo *Saccharomyces cerevisiae*. A administração deste polissacarídeo na cavidade articular é capaz de induzir artrite em camundongos. A hesperidina-metil-chalcona é um flavonoide do grupo das flavanonas com eficácia terapêutica demonstrada por diversos estudos. Apresenta efeitos analgésicos frente à estímulos inflamatórios, oferece proteção contra radiação UVB, inibe o processo inflamatório e estresse oxidativo. Todavia, seus efeitos sobre a ativação de macrófagos RAW 264.7 e a inflamação articular induzidas pelo zymosan ainda são desconhecidos. Portanto, o presente estudo teve como objetivo avaliar os efeitos e mecanismos de ação do flavonoide hesperidina-metil-chalcona em modelo de artrite induzida por zymosan. Para isso, inicialmente foi realizada uma curva dose-resposta para determinar a dose mais eficaz em reduzir a hiperalgesia mecânica. Em seguida, foram avaliados os efeitos do tratamento com a dose mais eficaz de hesperidina-metil-chalcona sobre o edema articular e recrutamento celular (contagem total e diferencial dos leucócitos, além de análise histopatológica), ensaios colorimétricos de GSH, FRAP, ABTS e NBT como parâmetros de estresse oxidativo. A produção das citocinas IL-33, TNF-α e IL-6 foi determinada por ELISA e expressão de RNAm para $gp91^{phox}$ e HO-1 por RT-qPCR. Ademais, *in vitro*, foi quantificada a produção de IL-33, TNF-α e IL-6, presença de EROs (ensaio DAF2DA para EROs intracelular) e ativação do NFκB. Os resultados obtidos na curva dose-resposta demonstraram que o pré-tratamento com a dose de 100 mg/kg da hesperidina-metil-chalcona apresentou maior eficácia em reduzir a dor inflamatória induzida por zymosan. O tratamento com a dose previamente determinada foi eficaz na redução do edema articular, do recrutamento total e diferencial de leucócitos, reduziu significativamente *in vitro* e *in vivo* os níveis das citocinas pró-inflamatórias IL-33, TNF-α e IL-6 e do infiltrado celular nas análises histopatológicas. Observou-se também aumento na capacidade antioxidante pelos ensaios de GSH, FRAP e ABTS além de diminuir a produção de ânion superóxido pelo ensaio NBT, expressão de $gp91^{phox}$ e aumento na expressão de HO-1. Também foi observada uma redução na geração de EROs totais e na ativação do NFκB, *in vitro*. Além disso, por análise molecular *in silico*, a interação da hesperidina-metil-chalcona com a Ser276 da subunidade p65, um resíduo de aminoácido de interesse para a ativação do NFκB, sugere um dos mecanismos pelo qual esse flavonoide promove inibição do processo inflamatório. Esta é a primeira demonstração da interação direta da hesperidina-metil-chalcona com o NFκB. Dessa forma, esse estudo demonstrou, pré-clinicamente, o potencial analgésico e anti-inflamatório da hesperidina-metil-chalcona em modelo de artrite induzida por zymosan e *in vitro* em macrófagos RAW 264.7.

Palavras-chave: hiperalgesia; flavonoide; antioxidante; nocicepção; in silico.

Oliveira, F. S. R. **Hesperidin methyl chalcone interacts with NFκB Ser276 and inhibits zymosan-induced joint pain and inflammation, and RAW 264.7 macrophage activation**. 2020. 74 f. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2020.

ABSTRACT

Zymosan is a polysaccharide derived from the fungus *Saccharomyces cerevisiae*. Intra-articular injection of this polysaccharide induces inflammatory arthritis in mice. Hesperidin-methyl-chalcone, member of the flavanone group of flavonoids, with therapeutic efficacy demonstrated by several studies. It presents analgesic effects in acute models of pain, protects against UVB radiation, and inhibits the inflammatory process and oxidative stress. However, its effects on RAW 264.7 macrophage activation and zymosan-induced arthritis are still unknown. Therefore, the present study aims to evaluate the effects and mechanisms of action of the flavonoid hesperidin-methyl-chalcone in a zymosan-induced arthritis model. A dose-response curve was initially performed to determine the most effective dose in reducing pain in the arthritis model by testing the mechanical hyperalgesia. The effects of treatment with the best hesperidin-methyl-chalcone dose were evaluated on joint edema and leukocyte recruitment (determination of total and differential leukocyte count, and histopathological analysis). GSH, FRAP, ABTS, and NBT assays were evaluated as parameters of oxidative stress using colorimetric methods. Production of cytokines such as IL-33, TNF-α, and IL-6 were determined by ELISA and mRNA expression of $gp91^{phox}$ and HO-1 by RT-qPCR. Furthermore, *in vitro* production of IL-33, TNF-α and IL-6, presence of intracellular ROS (DAF2DA, total ROS determination) and activation of NFκB was quantified. The results on the dose-response curve demonstrated that the pre-treatment with 100 mg/kg hesperidin-methyl-chalcone dose reduced more effectively the inflammatory pain induced by zymosan. Also, hesperidin-methyl-chalcone treatment was effective in reducing joint edema, as well as reducing leukocyte recruitment, and proinflammatory cytokine levels IL-33, TNF-α, and IL-6 *in vivo* and *in vitro*. An increase in antioxidant capacity was also observed as per GSH, FRAP, and ABTS assays and HO-1 mRNA expression. We also observed reduction in superoxide anion production as observed by lower levels in the NBT assay as well as a decrease in $gp91^{phox}$. *In vitro,* we also observed a reduction in total ROS production and in NFκB activation in macrophages. Furthermore, by performing *in silico* analysis (molecular docking), we found interaction of hesperidin-methyl-chalcone with Ser276 residue of the NFκB p65 subunit. It suggests that hesperidin-methyl-chalcone directly interacts with Ser276 of p65, an amino acid residue of interest for NFκB activation. To our knowledge, this is the first demonstration that hesperidin-methyl-chalcone directly interacts with NFκB, which may contribute to suppress pathological inflammation. Thus, this study demonstrated, pre-clinically, the analgesic and anti-inflammatory potential of hesperidin-methyl-chalcone in a zymosan-induced arthritis model.

Key-words: hyperalgesia; flavonoid; antioxidant; nociception; in silico.

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LISTA DE ABREVIATURAS E SIGLAS

ARE	Elementos De Resposta Antioxidante
CD44	Cluster de diferenciação 44
cGMP/PKG/ATP	GMP cíclico/Proteína Kinase G/Trifosfato de adenosina
COX1/2	Ciclooxigenase-1/2
CREB	Proteína de ligação ao elemento de resposta do AMP cíclico
CXCL1	Ligante para quimiocina CXC 1
CXCR2	Receptor para quimiocina CXC 2
E. coli	*Escherichia coli*
ERNs	Espécies reativas de nitrogênio
EROs	Espécies reativas de oxigênio
ET-1	Endotelina-1
fMLP	Peptídeo Formilado
FoxO3a	Proteína Forkhead Box O3
GSH-Px	Glutationa Peroxidase
HMC	Hesperidina-metil-chalcona
HO-1	Heme Oxigenase-1
ICAM-1	Molécula de Adesão Intracelular 1
IL-1	Interleucina-1
IL-10	Interleucina-10
IL-17	Interleucina-17
IL-1β	Interleucina-1β (Beta)
IL-33	Interleucina-33
IL-6	Interleucina-6
IL-8	Interleucina-8
INFγ	Interferon gamma
iNOS	Óxido nítrico-sintase induzida
LDL	Lipoproteína de baixa densidade
LOX	Lipoxigenase
LPS	Lipopolissacarídeo
LTB4	Leucotrieno B4
Mac-1	Antígeno do macrófago-1

NADPH	Nicotinamida adenina dinucleotídeo fosfato reduzido
Nav1.8	Canal α para sódio dependente de voltagem subunidade 10
NF-κB	Fator nuclear Kappa B
NOX2	NADPH Oxidase 2
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2
PAFs	Fator de Ativação Plaquetária
PAMPs	Padrões moleculares associados a patógenos
PGE_2	Prostaglandina E2
PKA	Proteína Kinase A
PKC	Proteína Kinase C
PRRs	Receptores de reconhecimento de padrão
Rac1	Substrato 1 da toxina botulinica C3 relacionada a Ras
RNAm	RNA mensageiro
SFN	Sulforafano
ST2	Supressor de tumorigênese 2
TGFα	Fator de transformação do crescimento alpha
TLR2/4	Receptor tipo Toll 2/4
Myd88	Proteína de diferenciação de resposta mielóide primária 88
TNFα	Fator de necrose tumoral alpha
TRPA1	Receptor de potencial transitório anquirina 1
TRPV1	Receptor de Potencial Transitório do tipo vanilóide 1
UVB	Raios ultravioletas tipo B

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1 INTRODUÇÃO

1.1 INFLAMAÇÃO

A inflamação é uma resposta defensiva por parte do hospedeiro contra um patógeno ou injúria tecidual (MEDZHITOV, 2008). Essa resposta configura um conjunto de alterações vasculares e celulares que caracterizam os 5 sinais cardinais clássicos inflamatórios: dor, calor, rubor, edema e, quando existe exacerbação dos 4 primeiros sinais, pode ocorrer a perda da função do tecido e/ou órgão acometido (ROCHA E SILVA, 1978).

Uma série de eventos dinamicamente coordenados ocorrem no processo inflamatório. Inicialmente, ocorre a fase vascular, com aumento do calibre vascular e, consequentemente, do fluxo sanguíneo local. A maior parte das modificações vasculares iniciais são causadas por mediadores inflamatórios liberados por células residentes no local da inflamação, tais como histamina, fatores de ativação plaquetária (PAFs, do inglês *Platelet-Activating Factors*), bradicinina, trombina e prostaglandinas. Em seguida, há o aumento na permeabilidade vascular e extravasamento de exsudato que contribui para formação do edema. Em consequência, ocorre a lentificação do fluxo sanguíneo (estase). A evolução do processo de estase altera as condições hemodinâmicas e promove a marginalização leucocitária. Posteriormente, na chamada de fase celular da resposta inflamatória, leucócitos tais como neutrófilos e monócitos transmigram pelo endotélio vascular e migram até o foco inflamatório (ABBAS; KUMAR; FAUSTO, 2005; BOTTARO et al., 1986; MCDONALD et al., 2010; MITTAL et al., 2014; SPECTOR; WILLOUGHBY, 1964; THEOHARIDES et al., 2015b). Especificamente, o recrutamento de neutrófilos depende da característica inicial do estímulo nocivo e segue uma cascata temporal, espacial e hierárquica de mediadores (MCDONALD et al., 2010). Utilizando um modelo de inflamação estéril, foi demonstrado que o recrutamento de neutrófilos é dependente da integrina Antígeno do macrófago-1 (Mac-1) e da molécula de adesão intracelular 1 (ICAM-1). Em contraste, após estímulo com a bactéria *E. coli*, isso ocorre de forma dependente de cluster de diferenciação 44 (CD44) em detrimento de Mac-1, revelando diferentes mecanismos de recrutamento de neutrófilos em modelos de inflamação estéril e não estéril. Além disso, camundongos *knockout* para receptor de

peptídeo formilado (fMLP), mas não para o receptor de quimiocinas CXC 2 (CXCR2), apresentaram redução significativa de neutrófilos no foco necrótico. Isso demonstra que o receptor de fMLP guia de forma precisa os neutrófilos em áreas de necrose, fortificando a ideia de hierarquia de mediadores para o recrutamento de neutrófilos (MCDONALD et al., 2010). Os neutrófilos na circulação normalmente têm uma vida útil curta e rapidamente sofrem apoptose. No entanto, o microambiente inflamatório estimula a expressão de genes relacionados com a longevidade, como FoxO3a (JONSSON; ALLEN; PENG, 2005), além de promover ativação do fator de transcrição nuclear kappa B (NF-κB), o que contribui para produção de mais citocinas pró-inflamatórias e espécies reativas de oxigênio (EROs) e nitrogênio (ERNs) (FATTORI; AMARAL; VERRI, 2016).

Para contrabalancear os efeitos deletérios da exposição prolongada às EROs, o organismo apresenta mecanismos de proteção contra a citotoxicidade dessas moléculas. Entre eles, podemos citar as enzimas superóxido dismutase e glutationa peroxidase (GSH-Px), que são capazes de reduzir superóxido a peróxido de hidrogênio e peróxido de hidrogênio à água, respectivamente (SON et al., 2013). A ativação do fator de transcrição Nrf2, nuclear eritroide 2 relacionado ao fator 2, regula a transcrição de diversos genes com regiões promotoras denominadas Elementos de Resposta Antioxidante (ARE) envolvidos na resposta citoprotetora contra EROS e ERNs (PALL; LEVINE, 2015). Dentre as enzimas resultantes dessa regulação, o Nrf2 ativado promove a síntese da heme oxigenase-1 (HO-1), que confere proteção contra o stress oxidativo (CUADRADO et al., 2018; MA, 2013; PALL; LEVINE, 2015) em tecidos articulares inflamados (ALCARAZ; FERRÁNDIZ, 2019) e, sob determinadas condições, em células do sistema nervoso (NITTI et al., 2018), além de reduzir a migração neutrofílica em modelo de carragenina (FREITAS et al., 2006). É relevante mencionar que há uma comunicação entre o Nrf2 e o NF-κB. Quando a subunidade p65 do NF-κB é fosforilada, há uma competição entre o Nrf2 e p65 pela proteína de ligação ao elemento de resposta do AMPc (CREB) (LIU; QU; SHEN, 2008). Essa competição bloqueia a região promotora ARE, suprimindo, assim, ativação da via de sinalização Nrf2 e, consequentemente, a resposta antioxidante (YU et al., 2011).

O sistema imune inato é capaz de reconhecer uma gama enorme de tipos de patógenos, tais como bactérias, vírus e fungos, através de Receptores de

Reconhecimento de Padrões (PRRs, do inglês *Pattern-Recognition Receptors*), expressos por diversos tipos celulares, incluindo macrófagos, monócitos, células dendríticas e neutrófilos, possibilitando assim uma resolução mais rápida do problema (KAWAI; AKIRA, 2011; TAKEUCHI; AKIRA, 2010). Dentre os PRRs, podemos citar os Receptores tipo Toll (TLRs, do inglês *Toll like receptors*), dos quais a ativação promove a ativação do NF-κB. Como consequência, há um aumento na produção de citocinas pró-inflamatórias, como o fator de necrose tumoral α (TNFα), interleucina (IL)-33, e IL-6 (LIN; KAKKAR; LU, 2016; MEDZHITOV, 2008), além da ativação de sistemas geradores de espécies reativas de oxigênio como a enzima NADPH oxidase 2 (NOX2) e aumento na expressão de $gp91^{phox}$ (ANRATHER; RACCHUMI; IADECOLA, 2006). Em conjunto, esses mediadores contribuem para sensibilização neuronal e dor (LACAGNINA; WATKINS; GRACE, 2018).

As citocinas são proteínas pleiotrópicas capazes de regular a morte celular de tecidos inflamados, modificar a permeabilidade vascular, recrutar células para o foco inflamatório e induzir a produção de proteínas de fase aguda (TAKEUCHI; AKIRA, 2010). Além disso, como demonstrado por Cunha et al. (2005), as citocinas também medeiam a dor inflamatória no modelo da carragenina em camundongos (CUNHA et al., 2005). Estudos em modelo de artrite reumatoide em camundongos salientam a efetividade de terapias anti-TNFα, cujo mecanismo envolve o bloqueio de uma cascata de citocinas passíveis de causar dano tecidual ao hospedeiro (BRENNAN; MCINNES, 2008; FELDMANN; BRENNAN; MAINI, 1996; RANKIN et al., 1995). A citocina IL-33, pertencente à superfamília IL-1, é expressa por diferentes tipos celulares, tais como células epiteliais e endoteliais, e atua como uma alarmina, alertando ao sistema imunológico um dano tecidual presente, proveniente de trauma ou infecção, além de ter papel no processo alérgico pela indução da inflamação de vias aéreas (CAYROL; GIRARD, 2014; LIEW; GIRARD; TURNQUIST, 2016; LOUTEN et al., 2011). Logo, níveis elevados de IL-33 podem ser detectados em casos de determinadas doenças como asma, dermatite atópica, esclerose múltipla e artrite reumatóide (THEOHARIDES et al., 2015b). A IL-6, por sua vez, pode ter propriedades anti- e pró-inflamatórias, dependendo do contexto, e vem se apresentando como um alvo promissor em tratamentos clínicos (HUNTER; JONES, 2015). No caso da artrite inflamatória, por exemplo, níveis elevados de IL-6 tem correlação positiva com danos articulares, além de participar da indução e manutenção do processo autoimune (KIM

et al., 2015).

Como mencionado anteriormente, os PRRs possuem a capacidade de reconhecer e se ligar aos mais variados tipos de moléculas nocivas, formalmente conhecidas como Padrões Moleculares Associados a Patógenos (PAMPs, do inglês *Pathogen-Associated Molecular Patterns*). Exemplos clássicos de PAMPs incluem a flagelina, alguns açúcares e componentes da parede celular de vários microorganismos, tal como o zymosan, um polissacarídeo derivado da parede celular da levedura *Saccharomyces cerevisiae* que tem sido utilizado como estímulo inflamatório por mais de 50 anos (DI CARLO; FIORE, 1958; ZHANG et al., 2002). Este polissacarídeo ativa a via do Receptor tipo Toll 2/Proteína de diferenciação de resposta mielóide primária 88 (TLR2/Myd88) e consequentemente promove ativação do NF-κB (AKIRA; TAKEDA, 2004) em células do sistema imune. Para que esta ativação aconteça, modificações pós-traducionais são necessárias. Um exemplo são as fosforilações em resíduos específicos de subunidades da proteína NFκB, previamente já demonstradas (BAE et al., 2003; NEUMANN; NAUMANN, 2007).

A família de fatores de transcrição NF-κB consiste em um grupo de proteínas, e o complexo mais estudado é o dímero composto pelas subunidades p50 e p65. Modificações pós-traducionais da subunidade p65, especialmente, já foram mais detalhadamente descritas. A p65 contém vários resíduos que demonstraram estar sujeitos a fosforilação e acetilação, como serinas, lisinas e treoninas (JACQUE et al., 2005; JIANG et al., 2003; NEUMANN; NAUMANN, 2007; TAKADA; SINGH; AGGARWAL, 2004). Essas modificações podem regular positivamente ou negativamente a atividade transcricional do NF-κB (SCHMITZ; BAEUERLE, 1991). Especificamente, o resíduo Serina 276 (Ser276) é um dos principais locais de fosforilação da p65, que tem como consequência a ativação do NF-κB e regulação de genes envolvidos na morte celular, produção de citocinas como a IL-6 e IL-8 e expressão de ICAM-1 (JACQUE et al., 2005; OKAZAKI et al., 2003; WILLIAMS et al., 2008). Estudos clínicos e pré-clínicos correlacionam esse mecanismo em contextos de artrite reumatoide (LUO et al., 2009) e promoção de oncogênese (ARUN et al., 2009; PRASAD et al., 2009). Enquanto o TNFα tem sido descrito como um estímulo promotor da fosforilação da Ser276 (REBER et al., 2009; VERMEULEN et al., 2003), tanto a HO-1 quanto a IL-10 são inibidores desse processo (CASTELLUCCI et al., 2015; SELDON et al., 2007).

Entendendo, portanto, que certos processos pós-traducionais podem

ser alvos de interesse terapêutico, tecnologias emergentes que possibilitem a simulação de interações moleculares têm sido aplicadas na pesquisa básica. Uma abordagem para *screening* baseado em estrutura é o docking molecular, metodologia computacional ("*in silico*") que se baseia no ancoramento de pequenas moléculas em sítios de ligação de proteínas-alvo. *In silico* é uma expressão usada para indicar algo ocorrido "em ou através de uma simulação computacional". As técnicas de docking foram desenvolvidas para predizer a melhor orientação e conformação de uma molécula ligante no seu sítio receptor, obtendo um conjunto de conformações do complexo ligante-receptor e classificando-as em ordem de prioridade com base em suas estabilidades energéticas, e delineando relação estrutura-atividade (SAR) (FERREIRA et al., 2015; PINZI; RASTELLI, 2019). Empregando essa técnica, trabalhos previamente publicados correlacionam a inibição ou ativação do NFκB por diferentes compostos (MOHAN et al., 2018; TAREQ HASSAN KHAN et al., 2012; THOMAS; GEORGE; SELVAM, 2019), inclusive por flavonoides (ALAM et al., 2017; K. NAIK; THANGAVEL; ALAM, 2016; NANDEESH et al., 2018; THOMAS; GEORGE; SELVAM, 2019).

Como consequência da ativação do NFκB, mediadores pró-inflamatórios são produzidos, como quimiocinas, citocinas IL-1, TNFα, IL-1β e mediadores lípidicos inflamatórios leucotrieno B4 (LTB_4) e prostaglandina E2 (PGE_2) (GEGOUT et al., 1994; GUERRERO et al., 2008, 2012). A ação direta e indireta destes e de outros mediadores inflamatórios nos neurônios nociceptivos facilitam a despolarização e desencadeiam, consequentemente, a dor inflamatória. De fato, utilizando cultura de neurônios, foi demonstrado previamente que o zymosan aumenta o influxo de cálcio em neurônios positivos para o Receptor de Potencial Transitório do tipo Vanilóide 1 (TRPV1) (KASHEM et al., 2015), ativando diretamente o neurônio para produzir dor.

1.2 DOR

A dor de origem inflamatória é derivada do aumento na excitabilidade do nociceptor (neurônio responsável pela condução do estímulo doloroso) frente a ação de mediadores pró-inflamatórios. Esse fenônomeno é denominado de sensibilização periférica. Atuando em seus respectivos receptores expressos no terminal periférico do nociceptor, há a regulação de cascatas de sinalização, levando

a alterações na atividade neuronal (diminuição do limiar de ativação), bem como no perfil de importantes canais iônicos, como TRPV1, Receptor de potencial transitório anquirina 1 (TRPA1) e o canal α para sódio dependente de voltagem subunidade 10 (Nav1.8) (COOK et al., 2018; PINHO-RIBEIRO; VERRI; CHIU, 2017).

A sensibilização neuronal pode ser induzida por diversos mediadores pró-inflamatórios, tais como as citocinas IL-33, IL-6, TNF-α e IL-1β, prostaglandinas e aminas vasoativas (BINSHTOK et al., 2008; JIN; GEREAU IV, 2006; VERRI et al., 2006). Especificamente, foi demonstratado que no modelo de dor plantar induzido pela carragenina, há uma liberação inicial da alarmina IL-33 seguido pela produção sequencial de TNF-α→IL6→IL-1β→PGE_2; e TNF-α→CXCL1→IL-1β→aminas simpáticas (CUNHA et al., 2005; ZARPELON et al., 2013). Ao se ligarem em seus receptores, esses mediadores ativam proteínas kinases (PKA e PKC, por exemplo) que fosforilam canais de iônicos expressos pelos nociceptores (TRPV1, TRPA1, Nav1.8; por exemplo) (VERRI et al., 2006). Ademais, as citocinas IL-33, IL-6 e TNF-α induzem a ativação de neurônios (JIN; GEREAU IV, 2006; LIU et al., 2016; VERRI et al., 2006). Em modelos pré-clínicos, o papel da IL-6 na dor inflamatória é bem estabelecido (CUNHA et al., 1992; SAINOH et al., 2015; VAZQUEZ et al., 2012; ZHOU et al., 2016). Um trabalho publicado por Vazquez e colaboradores (2012) sugere o papel dessa citocina não somente na sensibilização local, mas também na central, amplificando a hiperalgesia em um modelo de artrite em ratos (VAZQUEZ et al., 2012). A ativação neuronal por citocinas pode acontecer de maneira direta, ativando receptores para citocinas presentes no terminal do nociceptor, ou por ação indireta, regulando a atividade de células não-neuronais que, por sua vez, liberam mediadores que agem no neurônio (COOK et al., 2018).

Além dessas, EROs e ERNs, que também são produzidas no local da inflamação, contribuem para a manifestação da dor (KEEBLE et al., 2009; NDENGELE et al., 2008). Macrófagos são um importante tipo celular capaz de gerar EROs através da ativação da enzima NOX2. De fato, macrófagos de camundongos deficientes para NOX2 apresentam sensibilidade reduzida no modelo de neuropatia *spared nerve injury* (KALLENBORN-GERHARDT et al., 2014). Uma das espécies reativas produzidas pela enzima, o superóxido, autorregula positivamente sua formação, aumentando a expressão do subunidades Rac1 e $gp91^{phox}$ da holoenzima (SALVEMINI et al., 2011). Ademais, o ânion superóxido regula a produção de citocinas

pró-inflamatórias como o TNF-α (YAMACITA-BORIN et al., 2015) e ativação do NF-κB (PINHO-RIBEIRO et al., 2016). Além disso, o ânion superóxido pode ser convertido em peroxinitrito, o qual também está implicado com a ativação do fator de transcrição NF-κB e, por consequência, regulação da produção de mediadores inflamatórios (MATATA; GALIÑANES, 2002). De modo geral, elevação na concentração de superóxido e peroxinitrito está ligada a ativação e/ou indução das enzimas ciclooxigenase-1 e 2 (COX1 e COX2), com subsequente desenvolvimento de sensibilização neuronal periférica e central, intimamente associada com um processo inflamatório (NDENGELE et al., 2008). É importante salientar que a sensibilização central contribui de maneira relevante para a cronificação da dor (ASHMAWI; FREIRE, 2016). Sabendo disso, é crescente o número de estudos demonstrando que a inibição, através de fármacos, da produção de superóxido e peroxinitrito pode prevenir e até reverter doenças comumente associadas à dor inflamatória, dor neuropática e hiperalgesia induzida por opioides (SALVEMINI et al., 2011).

Um dos mecanismos explorados por fármacos e produtos naturais para inibição da dor é através da modulação do Nrf2 (STAURENGO-FERRARI et al., 2019). Ativadores do Nrf2 podem sinergizar com opióides para obter melhores efeitos analgésicos – um exemplo é o Sulforafano (SFN), composto bioativo presente em vegetais, potenciador do efeito analgésico da morfina. Além de aumentar a expressão de receptores μ-opióides a nível espinal, o SFN eleva a expressão de HO-1, explorando o potencial antioxidante do Nrf2 em promover analgesia (REDONDO et al., 2017). Outras exemplos de drogas que modulam o Nrf2 para analgesia incluem Desipramina (ROUMESTAN et al., 2007), Anandamida (LI et al., 2013) e Aspirina (JIAN et al., 2016), além dos flavonóides fisetina (LEE et al., 2011) e baicalina (KIM; MOON; LEE, 2010). De fato, o o efeito analgésico de opioides e canabinóides são potencializados utilizando um indutor de HO-1, o monóxido de carbono (CO). Isso ocorre através da ativação da via dos canais de potássio sensíveis a GMP cíclico/Proteina Kinase G/Trifosfato de adenosina (cGMP/PKG/ATP) (CARCOLÉ et al., 2014), indicando que a estimulação da HO-1 contribui para a analgesia. Este mecanismo é relevante porque foi demonstrado que a morfina ativa essa via para produzir analgesia (CUNHA et al., 2010). Assim moléculas com a capacidade de induzir Nrf2/HO-1 e ativar via dos canais de potássio sensíveis a cGMP/PKG/ATP sem os efeitos colaterais de opióides, se mostram relevantes. Ademais, a HO-1 age também por induzir a produção do íon ferro III (Fe^{3+}), que se liga a ferritina; biliverdina,

que posteriormente se transforma em bilirrubina; e monóxido de carbono, e degradar o heme livre liberado por enzimas envolvidas na geração de EROs (GOZZELINO; JENEY; SOARES, 2010).

Doenças que aprensentam dor crônica como um dos sintomas são uma grande causa de invalidez e, portanto, um problema de saúde pública (GATCHEL et al., 2007; LEE et al., 2015; NEOGI, 2013; PETERS; VLAEYEN; WEBER, 2005). Em pacientes com dores crônicas, a execução de tarefas do cotidiano se tornam um desafio, predispondo ao estresse emocional, ansiedade, sofrimento e depressão (GATCHEL, 2004; GATCHEL et al., 2007). Em indivíduos acometidos por doenças articulares, como alguns tipos de artrite, a dor é o principal sintoma que leva à procura de tratamento médico (MILLS et al., 2019; NEOGI, 2013). Além do alívio sintomático, vários aspectos têm sido considerados no tratamento desses pacientes, como comorbidades, aspectos psicosociais e variações clínicas e bioquímicas individuais (DEVEZA et al., 2017; MILLS et al., 2019). No entanto, ainda se fazem necessárias pesquisas que visem elucidar os mecanismos e busquem tratamentos mais eficazes para melhorar a qualidade de vida desses pacientes.

1.3 ARTRITE

A artrite pode ser definida genericamente como uma desordem músculo-esquelética que afeta as articulações. É mais comum em mulheres e idosos, mas pode afetar todos os sexos, idades e etnias e pode apresentar causas diversas. A importância do estudo e tratamento da artrite se dá pelo fato de que esta é uma doença de alta prevalência e comumente associada a dores contínuas que diminuem a qualidade de vida e podem levar à invalidez do paciente (BEUKELMAN et al., 2017; SPRANGERS et al., 2000), além de apresentar correlação com maiores índices de ansiedade e depressão quando comparado à população em geral (HAWKER et al., 2011; MURPHY et al., 1999; PINCUS et al., 1996).

Os mecanismos envolvidos na artrite estão intimamente relacionados com o dano articular resultante (YOSHIDA et al., 2012). Uma membrana sinovial saudável é relativamente acelular, consistindo de macrófagos dispersos dentro de um estroma e escassos vasos sanguíneos (SIOUTI; ANDREAKOS, 2019). Um dos fatores principais que levam à destruição tecidual local é a infiltração de células inflamatórias, em especial os leucócitos polimorfonucleares na inflamação aguda. A infiltração

dessas células está associada à severidade do quadro clínico, e são comumente predominantes no exsudato de artropatias como a artrite reumatóide (BEZERRA et al., 2007; HARRIS, 1991). Estudos sugerem o envolvimento de citocinas como a IL-1β, TNFα, interferon-gamma (IFN-γ), fator de crescimento transformador alfa (TGFα), IL-33 e óxido nítrico na mobilização dessas células (CANNON et al., 1996; FARRELL et al., 1992; FERRACCIOLI et al., 2010). Um estudo seminal demonstrou que a IL-33 é capaz de induzir dor em modelo de artrite induzida por antígeno (VERRI et al., 2008). O mecanismo proposto se dá através da liberação sequencial de TNF-α→IL-1β→IFN-γ→ endotelina 1 (ET-1)→ PGE_2 induzidas pela IL-33. Ademais, a IL-33 também age como molécula quimioatraente para neutrófilos em modelo de artrite induzida por antígeno (VERRI et al., 2010). Especificamente, o TNF-α induz a expressão do receptor para IL-33 (ST2, do inglês *Suppression Of Tumorigenicity 2*) tornando neutrófilos quimiotratáveis por IL-33. Corroborando, análise de neutrófilos de pacientes com artrite reumatóide tratados apenas com metotrexato (padrão-ouro para tratamento da sinovite) apresentam maior expressão de ST2 em comparação com neutrófilos de pessoas saudáveis ou pacientes em tratamento com metotrexato mais infliximabe (anti-TNFα), indicando a relevância clínica da IL-33 (VERRI et al., 2010). Além disso, segundo Nielsen e colaboradores (2008), altos níveis de citocinas pró-inflamatórias implicam na secreção de enzimas proteolíticas das células do estroma sinovial e de condrócitos, o que tem relação direta com a lesão na cartilagem inflamada (NIELSEN et al., 2008). Apesar de existirem poucos estudos investigando os primeiros estágios da inflamação de membrana sinovial, dados clínicos sugerem que tanto a IL-1β quanto a IL-6 têm papéis fundamentais e portanto são possíveis alvos para tratamento em humanos (FERRACCIOLI et al., 2010; MIMA; NISHIMOTO, 2009). De fato, medicamentos como Anakinra (antagonista de receptor de IL-1) e tocilizumab (anticorpo contra receptor de IL-6) apresentam benefícios na clínica (IKONOMIDIS et al., 2008; KIM et al., 2015).

O polissacarídeo zymosan tem sido amplamente utilizado como estímulo inflamatório em estudos pré-clinicos de artrite, sendo um modelo caracterizado por apresentar sinovite grave e erosiva (GEGOUT et al., 1994; KEYSTONE et al., 1977) associada à hiperalgesia (BUSSMANN et al., 2019; GUAZELLI et al., 2018; GUERRERO et al., 2006, 2008, 2012; ROCHA et al., 1999), além de provocar extravasamento plasmático, aumento dos níveis de óxido nítrico, proliferação de células do revestimento sinovial e degradação da cartilagem (CHAVES

et al., 2011; GEGOUT et al., 1994). Estudos prévios sugerem que a hiperalgesia induzida pelo zymosan está intimamente relacionada com a sinalização pelo receptor TLR2/MyD88 (GUERRERO et al., 2012). Especificamente, camundongos deficientes para TLR2 e MyD88, mas não camundongos da linhagem C3H/HeJ (que possuem uma mutação no TLR4) apresentam diminuição da hiperalgesia mecânica induzida por zymosan, indicando um papel da sinalização TLR2/MyD88, mas não via TLR4 (GUERRERO et al., 2012). Como consequência, há um aumento na produção de TNF-α, IL-1β e CXCL1/KC, que agem de maneira interdependente, além da sensibilização direta de neurônios nociceptivos por prostanóides e aminas simpáticas, resultando em dor articular (GUERRERO et al., 2012). Por outro lado, a citocina IL-17 parece não ter participação no processo de dor induzida por zymosan (PINTO et al., 2010). Estudos *in vitro* com macrófagos derivados da medula óssea demonstraram que a secreção de TNF-α e IL-1β induzida por zymosan é, em parte, dependente de TLR2, corroborando com as evidências *in vivo* do mecanismo de ação do zymosan.

No contexto da artrite, os macrófagos são importantes produtores de citocinas e quimiocinas (TNFα, IL-1β, IL-6 e CCL2, por exemplo) (FELDMANN; BRENNAN; MAINI, 1996; KOCH et al., 1992), são células apresentadoras de antígeno (BURGER; DAYER, 2002), além de secretar enzimas proteolíticas (BARTOK; FIRESTEIN, 2010; TAKAYANAGI et al., 2000) e contribuir para a cronicidade da doença (SIOUTI; ANDREAKOS, 2019). Como um importante regulador do processo inflamatório, estudos demonstram que o NFκB é ativado no núcleo de macrófagos e fibroblastos no tecido sinovial humano em pacientes em estágio inicial e tardio da artrite reumatoide (ASAHARA et al., 1995; MAROK et al., 1996).

Atualmente, o tratamento da artrite reside basicamente no uso de corticosteroides e agentes imunobiológicos que reduzem a dor. Enquanto o uso crônico do primeiro pode causar alterações hormonais, como a síndrome de Cushing iatrogênica (LACROIX et al., 2015), imunobiológicos podem levar à insuficiência cardíaca (SLØRDAL; SPIGSET, 2006). Além disso, tanto corticosteroides quanto agentes imunobiológicos e opióides podem causar imunossupressão (BULLOCK et al., 2019). Por conta dos efeitos adversos, alternativas com potencial terapêutico no tratamento da artrite vêm sido estudadas. Apesar dos resultados promissores, ainda há muito a ser feito a fim de garantir segurança e determinar as medidas mais eficazes para vias de administração, dosagem, minimização de efeitos adversos e também o melhor momento de intervenção. Nesse sentido, tem crescido a atenção dada aos

estudos de compostos naturais, e a aplicação terapêutica de derivados de plantas vem ganhando reconhecimento.

1.4 HESPERIDINA METIL CHALCONA

O crescente interesse no estudo da bioatividade de compostos naturais se deve principalmente ao fato de muitos deles apresentarem várias propriedades benéficas e possuírem baixa toxicidade. Dentre tais compostos, podemos destacar os flavonoides, que são metabólitos secundários encontrados em uma ampla variedade de plantas e/ou de alimentos comumente presentes na nossa dieta, como por exemplo uvas, cerejas, maçãs, groselhas, frutas cítricas, entre outras, e hortaliças como pimenta, tomate, espinafre, cebola, brócolis (BARNES; ANDERSON; PHILLIPSON, 2001). Esses metabólitos secundários são geralmente relacionados à defesa antioxidante contra radiação ultravioleta ou micro-organismos, incluindo bactérias, fungos e vírus (GRAF; MILBURY; BLUMBERG, 2005).

Os flavonoides têm sido objeto de diversos estudos, e muitos já demonstraram que, em modelos animais, os mesmos têm efeitos analgésicos, anti-inflamatórios, antitumorais e antiparasitários. Além destas, o promissor papel dos flavonoides também inclui a inibição da oxidação de lipoproteína de baixa densidade (LDL), proteção contra doenças cardiovasculares e correlação inversamente proporcional ao risco de incidência de demência. Estas propriedades parecem relacionar-se com a ação destas moléculas sobre mediadores celulares como proteínas quinases, enzimas da cascata inflamatória e ação antioxidante. Tais fatos corroboram o potencial analgésico e anti-inflamatório destas moléculas (COMMENGES et al., 2000; EDENHARDER; GRÜNHAGE, 2003; FUHRMAN et al., 1997; HAVSTEEN, 2002; LI et al., 2005; MIDDLETON; KANDASWAMI; THEOHARIDES, 2000). Estudos relatam a habilidade de flavonoides de regular a polarização de macrófagos entre os fenótipos M1/M2 – sendo o M1 considerado pró-inflamatório e M2 anti-inflamatório (KARUPPAGOUNDER et al., 2016; KONG et al., 2019; LU et al., 2018; WANG et al., 2019). O balanço entre os dois fenótipos é importante para a homeostase, uma vez que a polarização extrema para M1 ou M2 é uma característica comum de vários processos patológicos (BOZEC; SOULAT, 2017). Outros estudos relatam que os flavonoides são capazes de inibir a expressão de isoformas das enzimas óxido nítrico sintase, COX e lipooxigenase (LOX),

responsáveis pela produção de óxido nítrico e outros mediadores inflamatórios (TUÑÓN et al., 2009). Parte desse efeito anti-inflamatório se deve à modulação da biossíntese de citocinas que medeiam a adesão de leucócitos circulantes no local de inflamação, fenômeno intimamente relacionado com a inibição do fator de transcrição NF-kB (MANTHEY, 2000), por mecanismos que incluem prevenção de fosforilação de resíduos. Um estudo publicado por Furusawa e colaboradores (2009) demonstrou que o flavonoide licochalcona A suprime significativamente a via de sinalização do LPS através da inibição da fosforilação da Ser276 da subunidade p65 do NF-κB em linhagem de macrófagos RAW 264.7, apresentando efeito anti-inflamatório *in vivo* e *in vitro* (FURUSAWA et al., 2009).

Diferentes características estruturais nas moléculas dos flavonoides acarretam em diferenças em sua bioatividade, performance e metabolismo *in vivo*. A metilação desses compostos parece facilitar a entrada destes na célula e prevenir a degradação, aumentando a biodisponibilidade, além de se tornarem agentes quimiopreventivos mais potentes, quando comparados aos não metilados (KOIRALA et al., 2016; WALLE et al., 2007; WEN; WALLE, 2006). A hesperidina, da subclasse das flavononas, apresenta biodisponibilidade limitada devido à sua baixa solubilidade em água (LI; SCHLUESENER, 2017). A hesperidina-metil-chalcona ($C_{29}H_{36}O_{15}$), ou HMC, composto derivado da hesperidina, metilado e de alta solubilidade, foi descrita como eficaz no tratamento de pacientes com doenças vasculares, quando aliada a outros compostos, tais como a vitamina C e extrato de *Ruscus aculeatus* (JAWIEN et al., 2017). Em um estudo clínico envolvendo 170 pacientes, seis semanas de tratamento com o composto foi capaz de reduzir a dor em 80% e edema em 77% dos indivíduos (NARVÁEZ; TORRES; PEÑALOZA, 2007). Além deste uso, já foi descrito, pré-clinicamente, o efeito analgésico desta molécula frente a alguns estímulos inflamatórios – entre eles carragenina, capsaicina, adjuvante completo de Freund e cristais de urato monossódico (PINHO-RIBEIRO et al., 2015; RUIZ-MIYAZAWA et al., 2018). Ademais, a aplicação tópica desta molécula em camundongos *hairless* promove efeitos protetores contra radiação ultravioleta tipo B (UVB) que incluem reduzir o edema da pele, migração de neutrófilos, produção das citocinas TNF-α, IL-1β, IL-6 e IL-10 e elevar a expressão de RNAm para *Nrf2* e *Ho-1* (MARTINEZ et al., 2016b). Em outros modelos com participação do processo inflamatório e estresse oxidativo – como carcinogênese experimental no colón, toxicidade hepática induzida por acetaminofeno e modelo experimental de artrite reumatoide (AHMAD et al., 2012;

UMAR et al., 2013) – a hesperidina mostrou-se capaz de controlar estes processos, bem como a atividade do NF-kB e da enzima óxido nítrico sintase induzível (iNOS) (AHMAD et al., 2012). Considerando, portanto, as evidências presentes na literatura, o presente trabalho buscou aprimorar a compreensão e elucidar os efeitos benéficos e mecanismos de ação da HMC em um modelo pré-clínico de artrite induzido por zymosan.

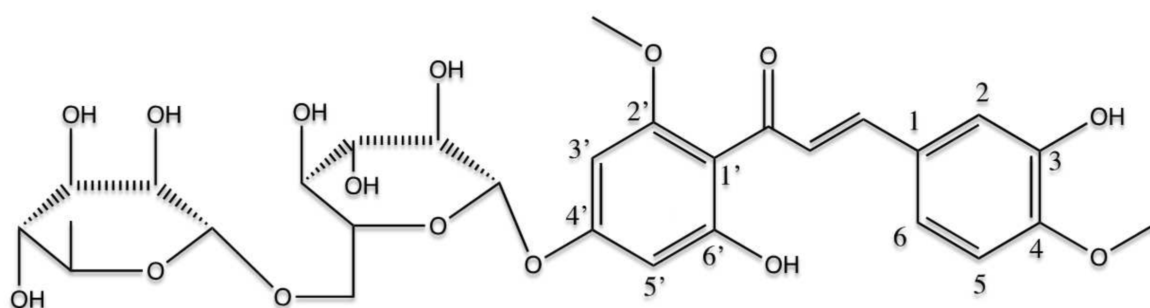

Figura 1. Estrutura da hesperidina-metil-chalcona ($C_{29}H_{36}O_{15}$).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Investigar os efeitos e mecanismos da hesperidina-metil-chalcona em modelo de artrite e a ativação de macrófagos RAW 264.7 induzidos por zymosan, além de analisar possíveis interações moleculares *in silico*.

2.2 OBJETIVOS ESPECÍFICOS

Determinar a dose mais eficaz de hesperidina-metil-chalcona, através de uma curva dose-resposta, sobre a hiperalgesia mecânica utilizando a versão eletrônica dos filamentos de von Frey;

Avaliar o efeito do tratamento com hesperidina-metil-chalcona sobre o edema, utilizando o paquímetro, e recrutamento de leucócitos por contagem total, diferencial e análise histológica;

Avaliar o efeito da hesperidina-metil-chalcona na produção das citocinas IL-33, IL-6 e TNF-α na articulação pelo método de ELISA;

Avaliar o efeito da hesperidina-metil-chalcona sobre o estresse oxidativo (GSH, capacidade antioxidante e produção de ânion superóxido) após estímulo intra-articular com zymosan;

Avaliar, *in vitro*, o efeito da hesperidina-metil-chalcona na produção de citocinas IL-33, IL-6 e TNF-α por ELISA, produção de ROS totais pelo método de DCFDA, e ativação do fator de transcrição NF-κB por imunofluorescência, em macrófagos RAW 264.7;

Avaliar, *in silico*, as interações moleculares da hesperidina-metil-chalcona com a subunidade p65 do fator de transcrição NF-κB (*molecular docking*).

3 ARTIGO

3.1 Os resultados obtidos no período de mestrado e desenvolvidos no Laboratório de Dor, Inflamação, Neuropatia e Câncer (LADINC) da Universidade Estadual de Londrina foram publicados no periódico *Inflammopharmacology* (e seguem formatação da revista), de Qualis A2 na área de Medicina II e fator de impacto 3.83, com o seguinte título:

Hesperidin methyl chalcone interacts with NFκB Ser276 and inhibits zymosan-induced joint pain and inflammation, and RAW 264.7 macrophage activation.

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3.2. Os seguintes trabalhos listados nos anexos foram desenvolvidos e publicados no período de mestrado, porém não fazem parte da dissertação:

Fattori V, Zaninelli TH, Rasquel-Oliveira FS, et al (2020) Specialized pro-resolving lipid mediators: A new class of non-immunosuppressive and non-opioid analgesic drugs. **Pharmacol. Res.**, 151. DOI: 10.1016/j.phrs.2019.104549

Ferraz CR, Carvalho TT, Manchope MF, et al (2020) Therapeutic potential of flavonoids in pain and inflammation: Mechanisms of action, pre-clinical and clinical data, and pharmaceutical development. **Molecules,** 25. DOI: 10.3390/molecules25030762

Fattori V, Rasquel-Oliveira FS, Artero NA, et al (2020) Diosmin Treats Lipopolysaccharide-Induced Inflammatory Pain and Peritonitis by Blocking NF-κB Activation in Mice. **J. Nat. Prod.**, DOI: 10.1021/acs.jnatprod.9b00887

Hesperidin methyl chalcone interacts with NFκB Ser276 and inhibits zymosan-induced joint pain and inflammation, and RAW 264.7 macrophage activation

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Author contributions

TSS and LS-F treated the animals and injected the stimuli. FSR-O and MFM performed behavioural testing. FSR-O, THZ, and VF performed confocal microscopy analysis. FSR-O, MFM, LS-F, TSS, NAA, and SB-G performed experiments. CRF performed molecular docking analysis. FSR-O, MFM, and WAVJ analysed and interpreted data set. WAVJ and RC delineated the study. RC, AdF, and WAVJ received grants and supplied utilized reagents. FSR-O wrote the first draft. FSR-O, VF, and WAVJ revised and modified the manuscript to its final version. All authors read and agreed with the final version of the manuscript.

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Abstract

Arthritis can be defined as a painful musculoskeletal disorder that affects the joints. Hesperidin methyl chalcone (HMC) is a flavonoid with analgesic, anti-inflammatory, and antioxidant effects. However, its effects on a specific cell type and in the zymosan-induced inflammation are unknown. We aimed at evaluating the effects of HMC in a zymosan-induced arthritis model. A dose-response curve of HMC (10, 30, or 100 mg/kg) was performed to determine the most effective analgesic dose after intra-articular zymosan stimuli. Knee joint oedema was determined using a calliper. Leukocyte recruitment was performed by cell counting on knee joint wash as well as histopathological analysis. Oxidative stress was measured by colorimetric assays (GSH, FRAP, ABTS and NBT) and RT-qPCR ($gp91^{phox}$ and HO-1 mRNA expression) performed. *In vitro*, oxidative stress was assessed by DCFDA assay using RAW 264.7 macrophages. Cytokine production was evaluated *in vivo* and *in vitro* by ELISA. *In vitro* NF-κB activation was analysed by immunofluorescence. We observed HMC reduced mechanical hypersensitivity and knee joint oedema, leukocyte recruitment, and pro-inflammatory cytokine levels. We also observed a reduction in zymosan-induced oxidative stress as per increase in total antioxidant capacity and reduction in $gp91^{phox}$ and increase in HO-1 mRNA expression. Accordingly, total ROS production and macrophage NFκB activation was diminished. Molecular docking reveals HMC interacts with p65 at Ser276, a relevant amino acid for NFκB activation. Thus, data presented in this work suggests the analgesic and anti-inflammatory potential of HMC in a zymosan-induced arthritis model, possibly by targeting NFκB activation in macrophages.

Keywords: HMC, knee pain, joint pain, flavonoid, molecular docking, experimental arthritis.

1 Introduction

Arthritis can be broadly defined as a painful musculoskeletal disorder that affects the joints and ultimately, diminishes the quality of life because of disabilities (Sprangers et al. 2000; Beukelman et al. 2017). Current treatments for arthritis rely mainly on methotrexate, corticoids, and immunobiological agents, which often present relevant side effects limiting their use (Laev and Salakhutdinov 2015).

Movement-induced joint pain is one of the main cause of complaint among patients with arthritis and can even worsen disease status (Yoshida et al. 2012). Recruitment of inflammatory cells, especially neutrophils is one of the major elements associated with joint tissue damage due to the production of pro-inflammatory mediators and reactive oxygen species (ROS). In fact, neutrophils are the prevalent cells in the synovial fluid of patients with rheumatic diseases such as rheumatoid arthritis (RA). These cells are also associated with the severity of the clinical condition (Harris 1991; Bezerra et al. 2007). Studies suggest the involvement of ROS and

cytokines such as IL-33, IL-1β, TNFα, and IL-6 in the recruitment of these cells further contributing to pain and inflammation (Harris 1991; Farrell et al. 1992; Cannon et al. 1996; Ferraccioli et al. 2010; Verri et al. 2010). Furthermore, high levels of pro-inflammatory cytokines lead to the release of proteolytic enzymes from the synovial stromal cells and chondrocytes, which is directly related to cartilage degradation (Nielsen et al. 2008). Therefore, new molecules that block the release of these mediators with fewer side effects are needed.

Increasing attention has been paid to flavonoids due to their beneficial properties with low toxicity. Flavonoids are secondary metabolites found in a broad variety of plants and foods commonly present in our daily consumption, such as citrus fruits and vegetables (Barnes et al. 2001; Verri et al. 2012). These secondary metabolites present anti-inflammatory, antioxidative and analgesic activities (Graf et al. 2005). Hesperidin-methyl-chalcone ($C_{29}H_{36}O_{15}$), or HMC, is a highly soluble methylated hesperidin-derived compound. HMC is effective in the treatment of vascular diseases when combined with other compounds such as vitamin C and *Ruscus aculeatus* extract (Jawien et al. 2017; Kakkos et al. 2018). Topical application of HMC in *hairless* mice ameliorates the inflammatory signs of UVB in the skin, such as oedema, neutrophil recruitment, production of the cytokines IL-6, TNFα, IL-10 and IL-1β and increases endogenous defences that protect against oxidative stress (Martinez et al. 2016b). In addition, treatment with HMC reduces inflammatory pain triggered by multiple stimuli, such as carrageenan, monosodium urate crystals (MSU) and capsaicin (Pinho-Ribeiro et al. 2015; Ruiz-Miyazawa et al. 2018). Evidence supports HMC has a safe pre-clinical and clinical profile (Kirtley and Peck 1948; Beltramino et al. 2000; Pinho-Ribeiro et al. 2015). For instance, patients presented no toxic effects upon the consumption of HMC during 90 days (Beltramino et al. 2000). Safety was observed even in the administration of high doses such as 15g daily in humans (Kirtley and Peck 1948). Given these beneficial effects of this flavonoid, our main goal relies on investigating the protective effect of HMC in a model of zymosan-induced arthritis in mice.

2 Materials and Methods

2.1 Animals

In this study, *in vivo* experiments were performed on Swiss mice (20-25g). The number of animals used was the minimum necessary to obtain statistical significance, and their suffering was minimised as much as possible. Animals were randomly divided into standard polypropylene cages, in rooms with regulated temperature (23 ± 2°C) and light-dark cycles (12:12h), as well as free access to water and food. All procedures conducted in this work were approved by Londrina State University Ethics Committee on Animal Research and Welfare (protocol number 5943.2017.85). Animal care and handling methods were in conformity with the guideline of the International Association for Study of Pain (IASP) and the Brazilian Council on Animal Experimentation (CONCEA). Animals were used only in a single experimental group.

2.2 Assessment of articular hyperalgesia

The hypersensitivity of the tibiofemoral joint to mechanical stimulation was assessed using an electronic anesthesiometer. Mice were habituated in cages with wire grid floors for an hour daily, for four days prior the behavioural testing, and an hour before the beginning of measurements. All animals were placed in rooms under controlled illumination and temperature (23 ± 2°C). A large tip probe (4.15 mm^2 area size) was used to apply a perpendicular force in the hind paw, inducing tibiofemoral joint flexion (Guerrero et al. 2006). After a positive response (i.e., articular flexion), the apparatus automatically recorded the intensity of the pressure, in grams. Final response value was obtained by averaging three measurements. Fifteen grams was the maximum pressure applied. The test was performed 1, 3, 5, and 7h after intra-articular injection of zymosan (100µg/10µl, knee joint).

2.3 Histopathological analysis

Tibiofemoral samples obtained from zymosan-stimulated mice treated with HMC or vehicle were collected 7h after intra-articular administration of zymosan and fixed in 10% buffered formaldehyde. After decalcification in 4% ethylenediaminetetraacetic acid solution for 3-4 weeks, the samples were processed,stained with haematoxylin-eosin (HE) for later examination under an optical microscope (Olympus OX31, Olympus, Japan; original magnification, 40), connected to a digital camera (Lumenera Infnity1, Ottawa, Canada). Histopathological score was performed as previously described (Lourenco-Gonzalez et al. 2019). Using the threshold tool on ImageJ 1.44

software (public domain: http://rsb.info.nih.gov/ij/), histological RGB images were analysed using 986357 pixels as the dimension area.

2.4 Knee joint oedema evaluation

Thickness of the tibiofemoral joint was determined with a calliper (Mitutoyo) before (basal), and 1, 3, 5, and 7 hours after the intra-articular injection of zymosan (100μg/10μl, knee joint). Knee joint oedema was calculated by the difference of the measure before (zero time, i.e. basal) and after arthritis induction, in each time point determined.

2.5 *In vivo* leukocyte migration

The tibiofemoral joint wash was obtained 7h after zymosan injection (100μg/10μl, knee joint) for determination of leukocyte recruitment, as described elsewhere (Bussmann et al. 2019). Knee joints were washed with 3.3μL of saline with 1mM EDTA, three times, to estimate leukocyte recruitment. A Neubauer chamber was used for cell counting, mixed with Turk's solution (dilution 1:2). For differential cell counting, Rosenfeld stained slides were used and final values are shown as number of neutrophils or mononuclear cells per articular cavity.

2.6 Total intracellular ROS detection (DCFDA assay)

The H2DCF-DA fluorescent assay was used to detect and quantify intracellular ROS. Briefly, 50 μl of cell suspension were seeded on to Nunc™ Glass Bottom Dishes and incubated for 30 min, under controlled temperature (37°C). Macrophages were pre-treated with HMC (300μM) for 30 min and stimulated with zymosan (300μg/mL) for 5h. Cells were then loaded with 10 μM of H2DCF-DA, incubated for 30 min and washed with Hank's Balanced Salt Solution. Image processing was performed in a Confocal Microscope (TCS SP8, Leica Microsystems). Total intracellular ROS detection was analysed from the mean fluorescence (LAS X Software, Leica Microsystems) and normalised by the number of cells.

2.7 GSH activity assay

Tibiofemoral samples were collected 7h after i.a. injection of vehicle or stimuli and immediately homogenized with Tissue-Tearor (Biospec®) in ethylenediaminetetraacetic acid buffer (4 mL, 0.02 M). Homogenate was treated with

50% trichloroacetic acid. After 15 min and centrifugation of the mixture (1500 g, 15 min, 4°C), 1 mL of supernatant was added to 2 mL of solution containing 0.4 M Tris and 50 µL DTNB. After 5 min, the absorbance (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland) was read at 412 nm. Results are shown as nanomoles of GSH per mg of protein (Bussmann et al. 2019).

2.8 FRAP assay

FRAP assay was performed to verify the ferric reducing properties as an assessment of the capacity of the samples to resist oxidative stress previously described (Navarro et al. 2013). Tibiofemoral samples were obtained 7h after zymosan and homogenized with Tissue-Tearor (Biospec®) in 500 µL of 1.15% KCl buffer. The homogenate was then centrifuged (1500 rpm, 10 min, 4°C) and the supernatant collected. Fifty microliters of the supernatant were added to FRAP reagent solution (150 µL) and incubated at 37°C for 30 min. Absorbance was read at 595 nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). A standard Trolox antioxidant curve (0.02–20 nmol) was used for normalisation of FRAP results obtained (Pinho-Ribeiro et al. 2015).

2.9 ABTS assay

Tibiofemoral samples were collected 7h after zymosan stimuli, homogenized in 500 µL of 1.15% KCl buffer and centrifuged for 10 minutes at 1500rpm, 4°C. A mixture containing 200 µL of ABTS and 10 µL of the sample was added in each well and incubated in room temperature for six minutes. Absorbance was read at 730nm (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland). The radical scavenging capacity in the samples was determined using a standard Trolox curve. Results expressed in ABTS (mmol Trolox equivalent per mg protein) (Pinho-Ribeiro et al. 2015).

2.10 Superoxide anion production assay

Tibiofemoral samples were obtained 7h after stimulus, placed in in KCl buffer and further processed for assessing the superoxide anion production. Samples were mixed with NBT solution (1 mg/mL) and incubated for 60 minutes at 37 °C. The supernatant was discarded. The formazan precipitated was solubilized by 2M KOH and dimethylsulfoxide (DMSO). The optical density was measured at 600nm (Multiskan GO

Microplate, Thermo Fisher Scientific, Vantaa, Finland). The final values are shown as NBT reduction (OD/mg of tissue) (Bussmann et al. 2019).

2.11 RT-qPCR

Tibiofemoral samples were dissected 7h after the zymosan stimulus into 1mL of TRIzol. Total RNA isolation followed manufacturer's directions. Promega GoTaq® 2-Step RT-qPCR System was used for RT-PCR and qPCR. In all preparations, the purity of total RNA was between 1.8 and 2.0 (wavelength absorption ratio 260/280 nm). Specific primers (Applied Biosystems®) utilized were: gp91^{phox}, sense: 5'-AGCTATGAGGTGGTGATGTTAGTGG-3', antisense: 5'-CACAATATTTGTACCAGACAGACTTGAG-3'; Heme-oxygenase-1 (HO-1), sense: 5'-CCCAAAACTGGCCTGTAAAA-3', antisense: 5'-CGTGGTCAGTCAACATGGAT-3'; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense: 5´-CATACCAGGA 5 AATGAGCTTG-3´, antisense: 5´-ATGACATCAAGAAGGTGGTG-3´. Data were normalised by GAPDH mRNA expression.

2.12 Cytokine measurement

In vitro analysis of cytokine levels was performed using the supernatant of RAW 264.7 macrophages culture, collected 5h after stimulus with opsonized zymosan (300µg/ml) (Jang et al. 1999). For *in vivo* measurements, tibiofemoral samples were obtained 7h after i.a. injection (100µg/10µl/ knee joint). IL-33, TNF-α, and IL-6 levels were quantified using ELISA commercial kits (eBioscience, San Diego, CA, USA). Plates were read at 450 nm, using a spectrophotometer (Multiskan GO, Thermo Scientific). Results were expressed as picograms (pg) of each cytokine/mg of tissue or, *in vitro*, pg/mL.

2.13 NFκB activation

A cell suspension of RAW 264.7 macrophages was cultured on circular coverslips in 24-well plates. Each well contained $1,5\times10^5$ cells. Prior to the experiment, cells were incubated overnight at 37 °C, 5% CO_2. Pre-treatment with HMC 300µM was added 30 min before zymosan (300µg/ml). After 5h, cells were fixed with 4% paraformaldehyde for 10 min at 37°C. Then, samples were washed, blocked with BSA 2%, and incubated with primary antibody phospho-NFκB 1:200 (#sc-136,548; Santa Cruz Biotechnology, Dallas, TX, USA) and incubated overnight. After washing, slides were incubated with

secondary antibodies conjugated with Alexa Fluor 488 (#A-110088; Thermo Fisher Scientific, Waltham, MA, USA). DAPI (Thermo Fisher Scientific, 1:1000) was used for nuclear staining. Fluormount (SouthernBiotech) was used for fixation of the coverslips on the top of the slides. A confocal microscope (Leica TCS SP8, Leica, Wetzlar, Germany) generated images with a 40x objective. Images were processed using Leica EL6000 software (Leica, Wetzlar, Germany). The fluorescence intensity of phospho-NFκB of the different groups was quantified in randomly selected fields. In addition, the colocalisation area between the two markers were analysed. Results were expressed as fluorescent intensity (%) and colocalisation area (μm^2), respectively.

2.14 Molecular Docking

Molecular docking of HMC and p65 protein was performed using the AutoDock 4/Vina software (The Scripps Research Institute, CA, USA). Molecular graphics system PyMol was used to visualized docking poses. The p65 protein was downloaded from RCSB protein data bank, (PDB code: 1VKX). Gasteiger charges were applied to the molecule resulting in simplicity and speed of docking, and merging the non-polar hydrogens (Wang et al. 2011). Grid menu was toggled. After loading protein.pdbqt, the map files were selected directly by establishing grid points with 76 x 92 x 70 Å3 dimensions and 0.375 Å centred on (x,y,z) -14.630, 54.821, 63.465 (1VKX). Defined molecular docking with 2,500,000 energy evaluations and 250 runs covering the protein. Predictions was performed three times and the average of the mean binding energies was determined.

2.15 Experimental protocol

Animals were treated with HMC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (10, 30, or 100 mg/kg, diluted in saline) or vehicle, intraperitoneally (i.p.). After 30 minutes, under isoflurane anaesthesia, i.a. injection of zymosan (100μg/10μl/ knee joint) or vehicle (saline) was performed. Mechanical hyperalgesia (measured using electronic von Frey) was evaluated at intervals of 1, 3, 5, 7h after the stimulus. The best analgesic dose of HMC (100 mg/kg) was selected for the next experiments. Knee joint oedema was analysed at 1, 3, 5, and 7h time points after stimulus using a calliper, and joint wash was collected after 7h to assess total and differential leukocyte recruitment. Knee joints were collected 7h after zymosan stimulus for the evaluation of cytokine production IL-33, TNF, and IL-6 (ELISA); expression of gp91^{phox}, and HO-1 (RT-qPCR); total antioxidant ability (GSH, FRAP, and ABTS by colorimetric methods);

superoxide anion production (NBT assay) and histopathological analysis (HE staining). For the *in vitro* analysis, RAW 264.7 macrophages were treated with HMC at 30, 100, or 300μM, 30 minutes before stimulus with zymosan (300μg/ml). Supernatant (for cytokine levels quantification) and cells (for DCFDA and immunofluorescence assays) were collected 5h after the stimulus. The time points chosen for this study and protocols used were previously standardised in our laboratory (Guazelli et al. 2018; Ruiz-Miyazawa et al. 2018; Bussmann et al. 2019).

2.16 Statistical analyses

The results are reported as the mean ± SEM of measurements of six animals per group, per experiment. All data analysis was conducted with GraphPad Prism 6.01 software. All *in vivo* and *in vitro* experiments were performed twice in independent experiments. For two or more groups at multiple timepoints after stimulus administration (mechanical hyperalgesia and knee joint oedema), two-way ANOVA was used. One-way ANOVA was used for experiments with specific time points. ANOVA tests were followed by Tukey's *post-hoc* test. Significant differences were considered for $P < 0.05$.

3 Results

3.1 HMC inhibits joint mechanical hyperalgesia induced by zymosan

Zymosan is a glucan broadly used as stimulus in models of articular pain and inflammation (Guerrero et al. 2008, 2012; Guazelli et al. 2018; Bussmann et al. 2019). Initially, a dose-response curve was performed to determine the analgesic potential of HMC and the most effective dose. The injection of zymosan induced mechanical hyperalgesia, and treatment with 100mg/kg dose significantly reduced the hyperalgesia at all timepoints (Figure 1). Therefore, the 100mg/kg dose was selected for further experiments.

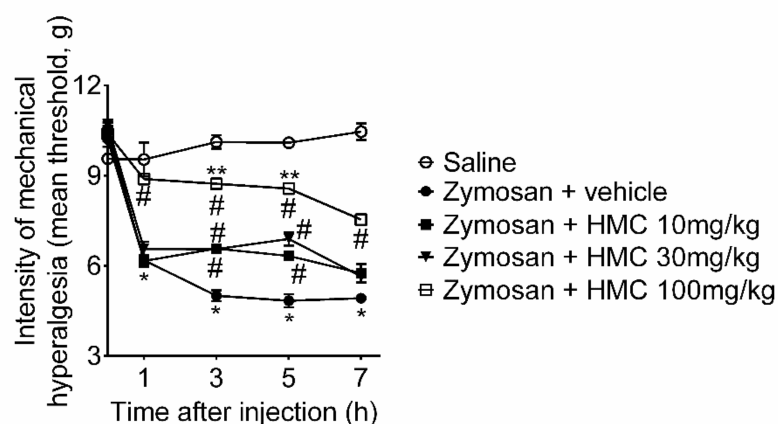

Figure 1. HMC decreases zymosan-induced mechanical hyperalgesia. Mechanical hyperalgesia was measured using electronic von Frey 1, 3, 5, and 7h after stimulus with zymosan. Results are presented as Δmean ± SEM of 6 animals per group. *p<0.05 vs. saline group; #p<0.05 vs. zymosan group; **p<0.05 vs 10 and 30mg/kg (Two-way ANOVA followed by Tukey's t test).

3.2 Treatment with HMC reduces zymosan-induced oedema and leukocyte migration to the tibiofemoral joint

Intra-articular injection with zymosan induces oedema formation, and leukocyte infiltration, which are essential for producing discomfort in rheumatic conditions (Gegout et al. 1994; Chaves et al. 2011). Once the most effective dose was determined (Figure 1), the ability of HMC to reduce zymosan-induced leukocyte recruitment and knee joint oedema was evaluated. To analyse leukocyte recruitment, we first performed haematoxylin and eosin staining of articular samples collected 7h after zymosan injection. Our results show that zymosan increased leukocyte infiltrate score, which was reduced by HMC 100mg/kg treatment (Figure 2). Extending these results, we demonstrated that zymosan significantly induced joint oedema and HMC-treated mice showed reduced knee joint oedema (Figure 3A). We next sought to estimate the number of leukocytes recruited to articular cavity, using the knee joint wash. We observed a greater number of total leukocytes (Figure 3B), mononuclear cells (Figure 3C), and neutrophils (Figure 3D) in the joint wash collected from the zymosan group. On the other hand, treatment with HMC reduced these parameters (Figures 3B-D).

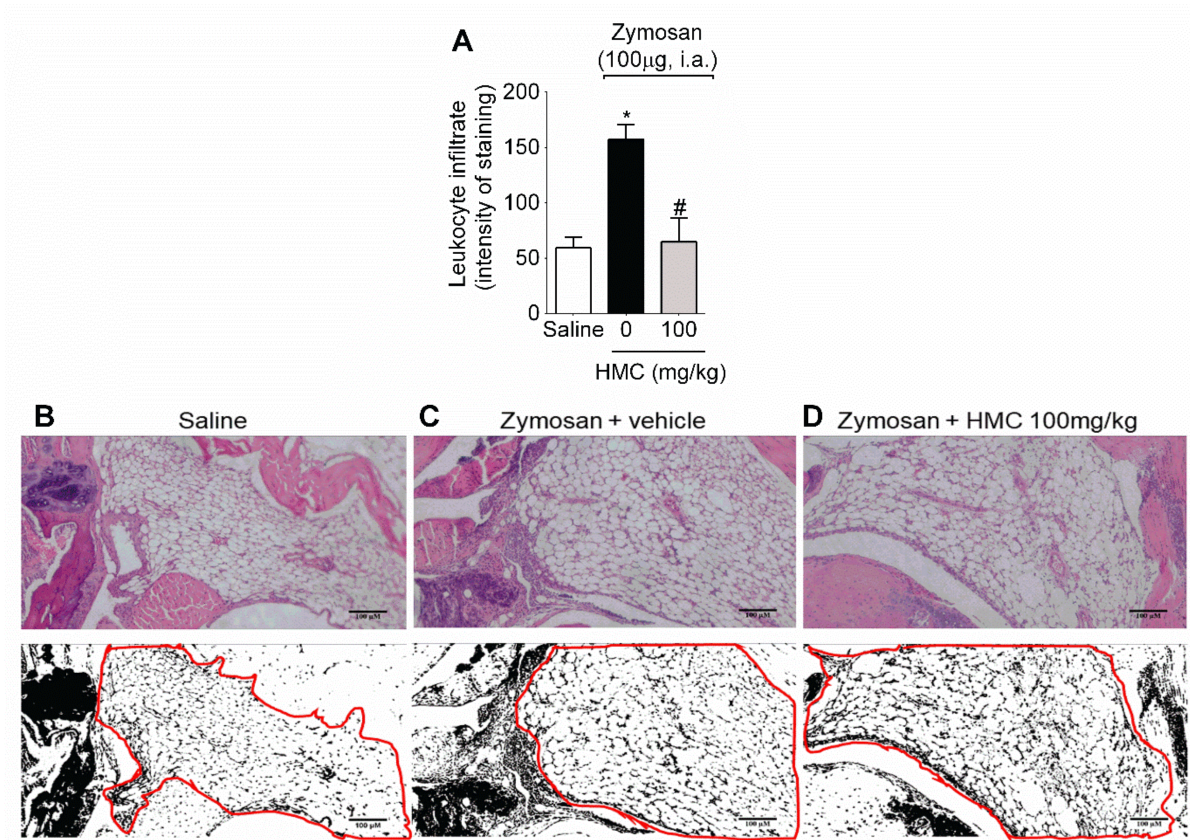

Figure 2. HMC reduces inflammatory infiltrate in histopathological analysis of joint tissue. Tibiofemoral joint samples were collected 7 hours after stimulus with zymosan. Samples, stained with H&E, were analysed with a light microscope (magnification 40x). Total leukocytes infiltrate score is present in (A) and representative histopathological images of each group are presented in (B-D). ImageJ software was used to perform the image analyses (986357 pixels of dimension area, highlighted area in red in B-D). Results are presented as mean ± SEM of 6 animals per group *p<0.05 vs. saline group; #p < 0.05 vs. zymosan group, one-way ANOVA followed by Tukey's t test; mean ± SEM).

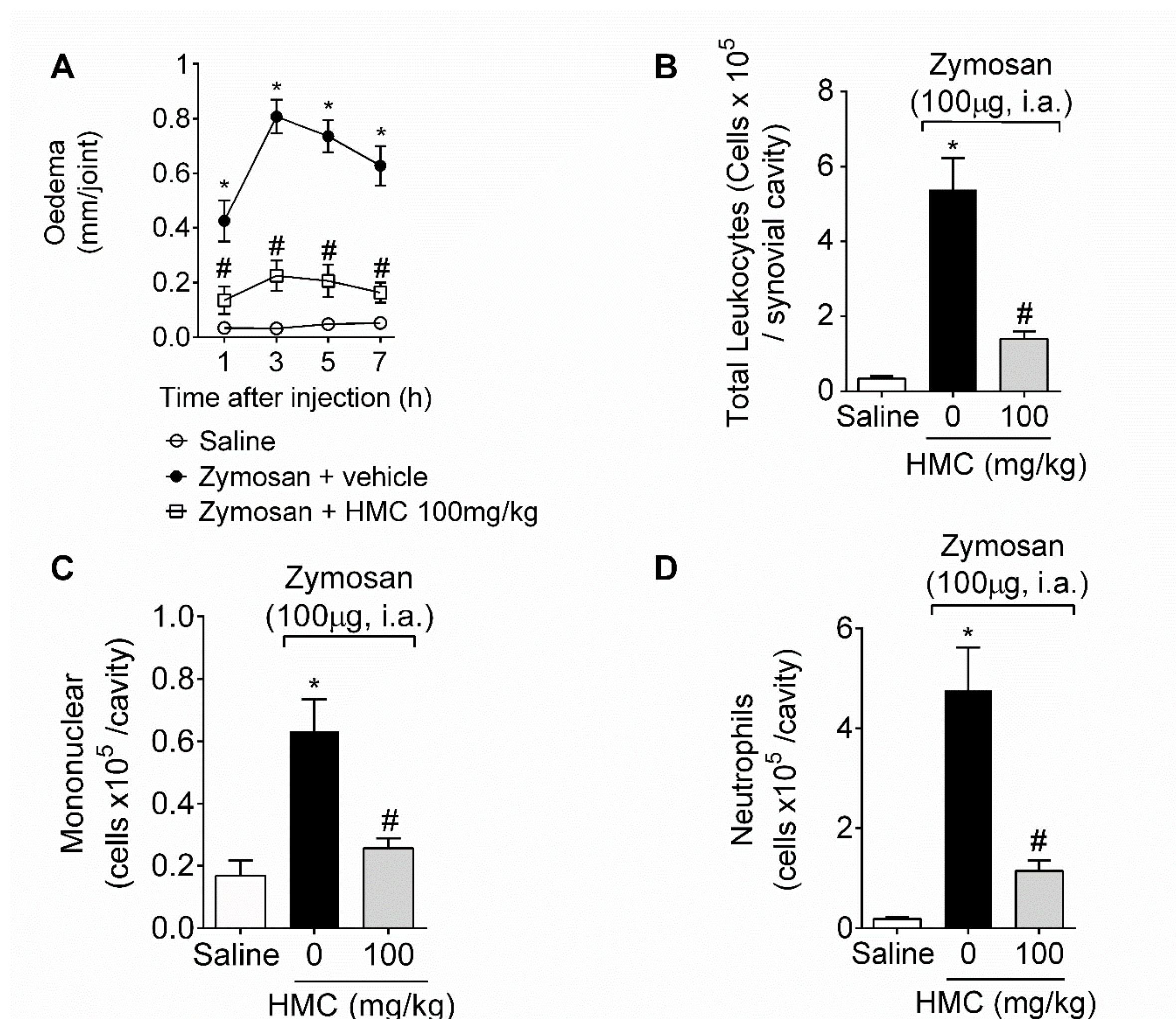

Figure 3. HMC reduces joint oedema and inhibits leukocyte recruitment. Oedema was measured 1, 3, 5, and 7 hours after the stimulus (A) using a calliper. Results are presented as Δmean ± SEM of 6 animals per group. *p<0.05 vs. saline group; #p<0.05 vs. zymosan group (Two-way ANOVA followed by Tukey's t test). Recruitment of total leukocytes (B), mononuclear (C) and polymorphonuclear cells (D) in synovial cavity. Knee joint wash samples were collected 7 hours after the stimuli. Results are presented as mean ± SEM of 6 animals per group. *p<0.05 vs. saline group; #p<0.05 vs. zymosan group (One-way ANOVA followed by Tukey's t test).

3.4 HMC inhibits zymosan-induced oxidative stress *in vivo* and *in vitro*

Superoxide anion, nitric oxide (NO) and other reactive oxygen species are important molecules in the development and maintenance of chronic pathological pain (Salvemini et al. 2011). Given flavonoids, including HMC, are widely known by their antioxidant properties, we next addressed whether HMC reduces zymosan-induced oxidative stress. Using RAW 264.7 macrophages cell culture, we measured ROS production using DCFDA, which when oxidized generates fluorescence product (DCF)

proportional to overall intracellular ROS levels (Figure 4A). A significant decrease in zymosan-induced production of ROS was observed in HMC-treated group (Figure 4B). GSH, FRAP, ABTS, and NBT assays using colorimetric methods, in addition to gp91^{phox} and HO-1 mRNA expression by RT-qPCR were performed to determine antioxidant status *in vivo* (Figure 5). These analyses were performed in the knee joint tissue collected 7 hours after stimulus with zymosan (100µg/10µl). Zymosan stimulus reduced tissue GSH levels and by contrast, mice treated with HMC (100mg/kg) showed a statistically significant increase in GSH levels (Figure 5A). The ability to reduce iron was measured by the FRAP assay. A statistically significant decrease in this antioxidant potential between the zymosan-stimulated group and the saline group was observed. HMC treatment restored this antioxidant potential (Figure 5B). Similar results suggesting the antioxidant potential of HMC can be observed by the ABTS assay (Figure 5C), which measures the ability to scavenge the ABTS$^{\bullet+}$ radical. In the NBT assay (Figure 5D), zymosan group showed increased superoxide anion production, while treatment with HMC significantly decreased these levels. In addition, gp91^{phox} and HO-1 mRNA expression were quantitated by RT-qPCR. In animals stimulated with zymosan, there was a significant increase in gp91^{phox} mRNA expression (Figure 5E), which is consistent with increased superoxide anion production. This increase was significantly inhibited by HMC treatment corroborating data from NBT assay (Figure 5E). Additionally, it was observed that HMC significantly elevated the expression of the antioxidant enzyme HO-1 when compared to zymosan-stimulated group (Figure 5F). Altogether, these results demonstrate that HMC presents *in vivo* and *in vitro* antioxidant capacity.

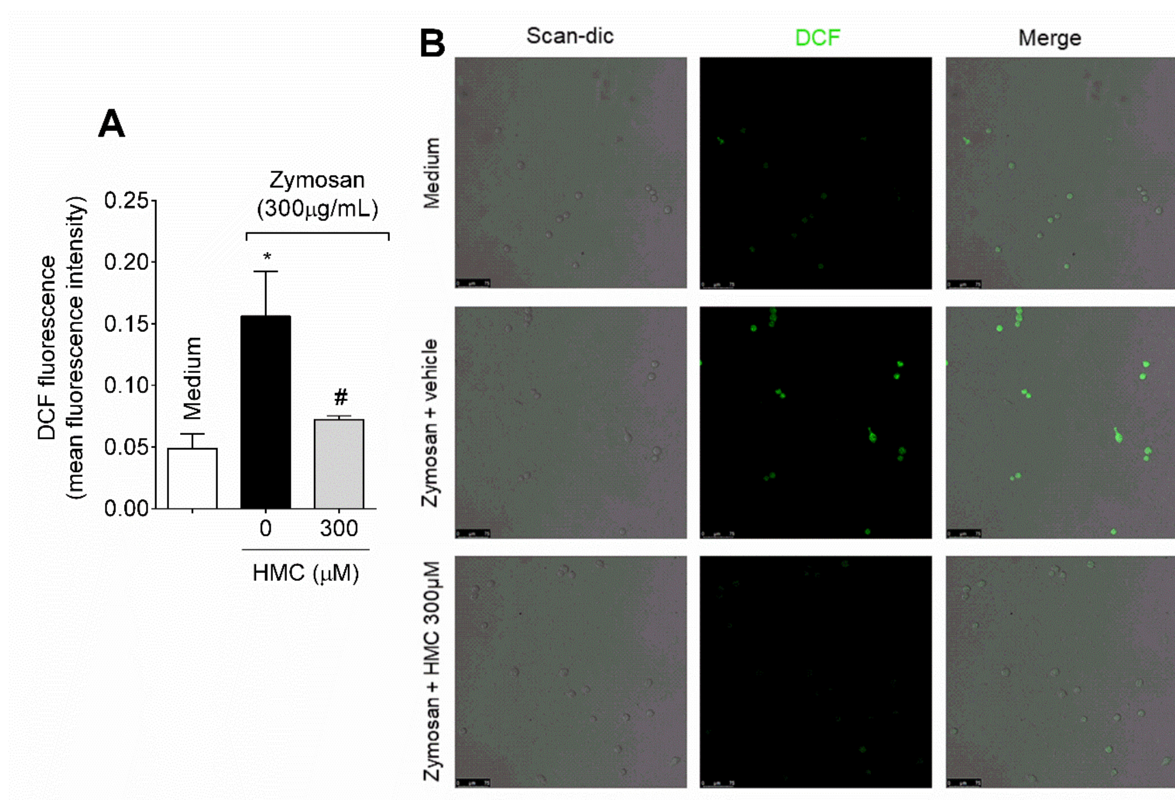

Figure 4. HMC attenuates zymosan-induced ROS generation by RAW 264.7 macrophages. *In vitro* RAW 264.7 macrophages were treated with HMC (300μM) 30 min before stimulus with zymosan (300μg/mL) for 5h. After 30 min incubation of cells with Dihydrofluorescein diacetate (DCF-DA), intracellular ROS levels were analysed using the scan-dic and green channel in a confocal microscope at 40x magnification. DCF fluorescence intensity indicates ROS production, which was quantified (A). Representative images showing DCF fluorescence for negative control, zymosan and HMC groups (B). Results are presented as mean ± SEM of 6 animals per group *$p<0.05$ vs. saline group; #$p<0.05$ vs. with zymosan group (One-way ANOVA followed by Tukey's t test).

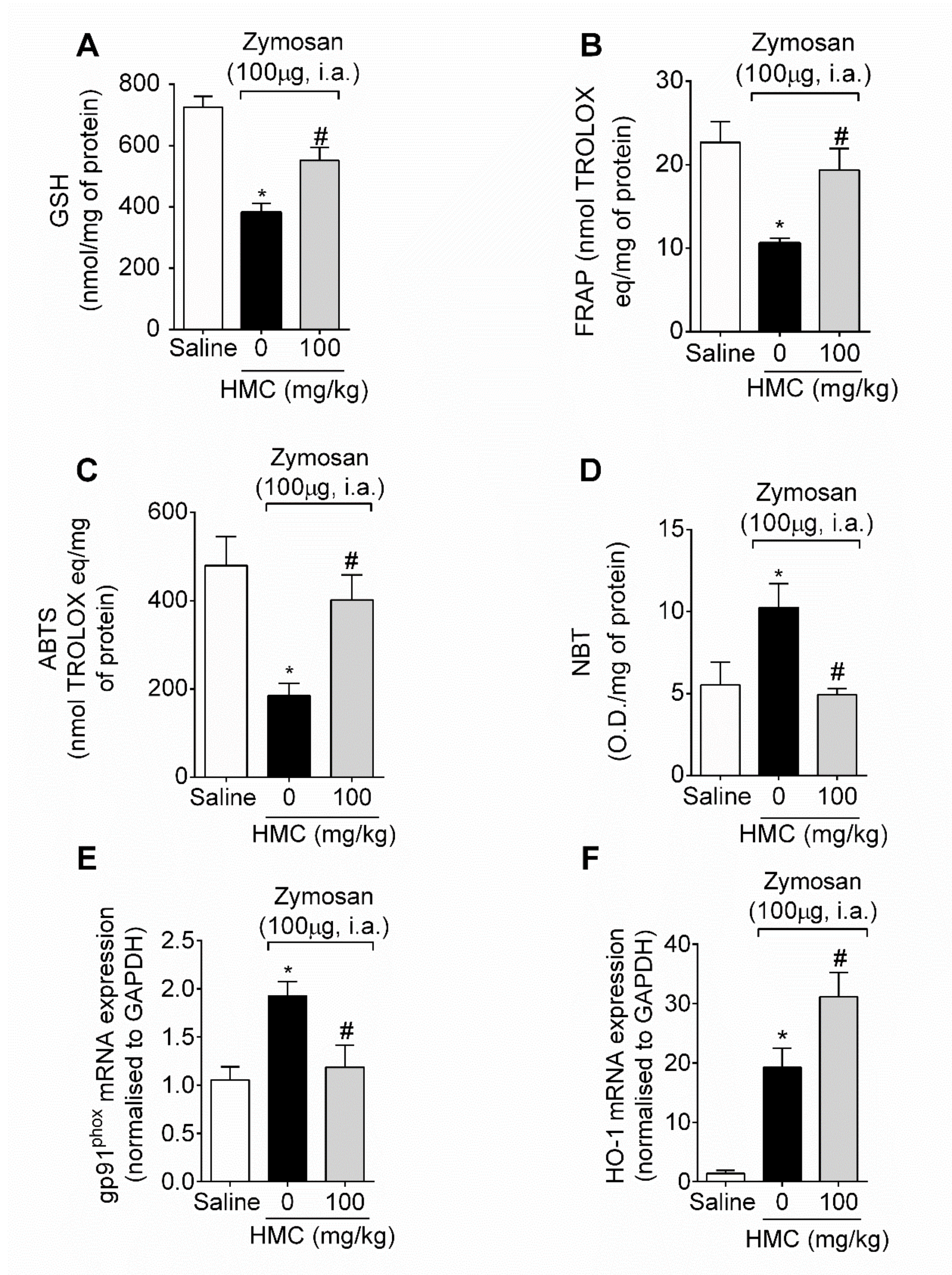

Figure 5. HMC inhibits oxidative stress induced by zymosan in joint samples. Seven hours after zymosan, the antioxidant capacity of HMC was evaluated by GSH (A), FRAP (B), ABTS (C) and NBT (D) assays by colorimetric methods, as well as gp91^{phox} (E) and HO-1 mRNA expression (F) by RT-qPCR). Results are presented as mean ± SEM of 6 animals per group. *$p<0.05$ vs. saline group; #$p < 0.05$ vs. zymosan group, one-way ANOVA followed by Tukey's t test; mean ± SEM).

3.5 HMC reduces IL-33, TNFα, and IL-6 levels *in vivo* and *in vitro*

Blocking the production of cytokines or their interaction with their receptors are recognized as effective analgesic approaches, including in arthritis (Verri et al. 2006). Therefore, the capacity of HMC to reduce peripheral production of pro-inflammatory cytokines IL-33, TNFα, and IL-6 levels were evaluated by ELISA method (Figure 6). Animals stimulated with zymosan (100μg/10μl) showed a significant raise in IL-33 levels (Figure 6A), TNFα (Figure 6B), and IL-6 (Figure 6C). Treatment with HMC significantly decreased these levels in joint tissue when compared to zymosan group. Accordingly, *in vitro* assay using RAW 264.7 macrophages cell line demonstrated that only HMC at 300μM reduced the cytokines IL-33 (Figure 6D), TNFα (Figure 6E), and IL-6 (Figure 6F) levels.

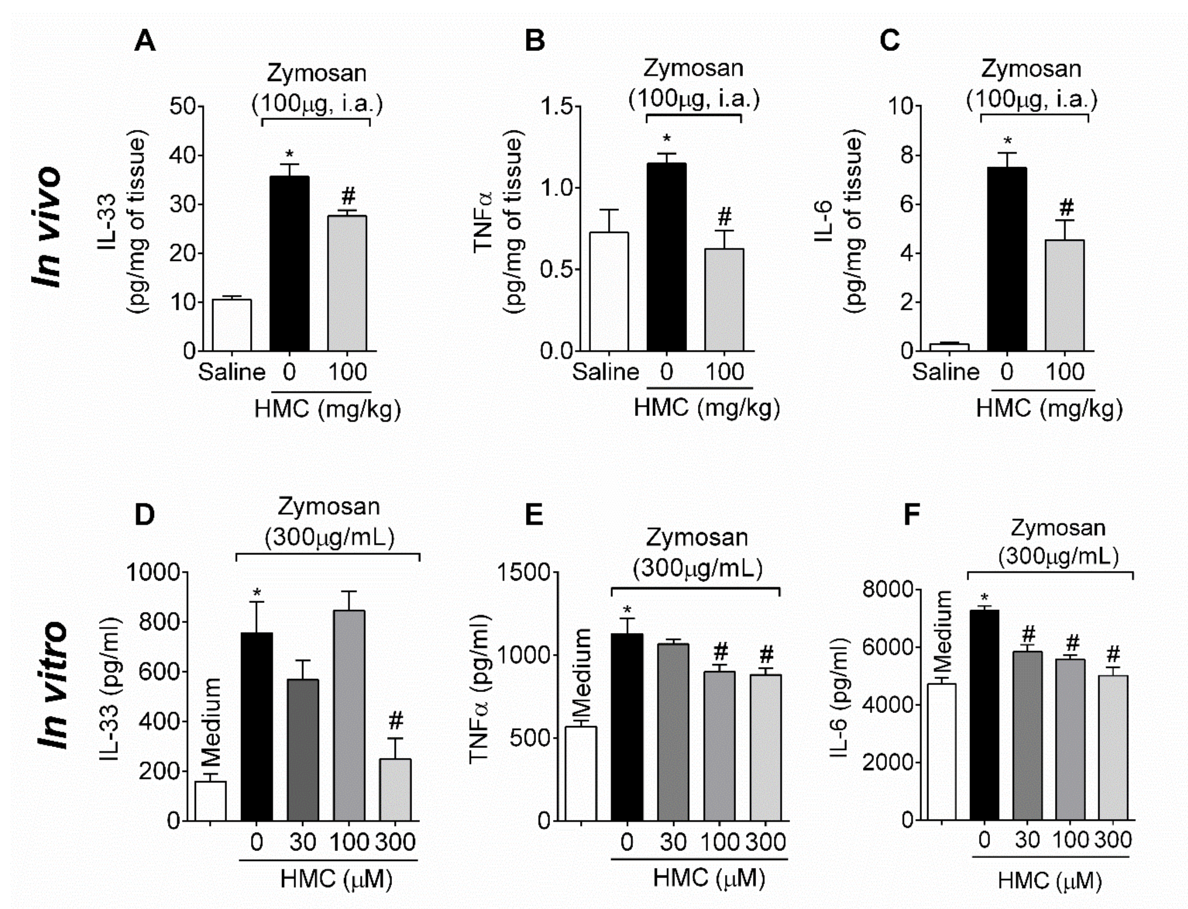

Figure 6. HMC reduces zymosan-induced *in vivo* and *in vitro* cytokine production. Samples of the tibiofemoral joint were obtained 7 hours post-stimuli for determination of in IL-33 (A), TNF-α (B), and IL-6 (C) by ELISA. Moreover, *in vitro* IL-33 (D), TNF-α (E), and IL-6 (F) levels were determined by ELISA, using cell culture of RAW 264.7 macrophages supernatant. Cells were treated with HMC (30-300μM) for 30 min and the supernatant was collected 5h after zymosan stimulus (300μg/mL). Results are presented as mean ± SEM of 6 animals or wells per group. *p<0.05 vs.

saline group; #p<0.05 vs. zymosan group (One-way ANOVA followed by Tukey's test).

3.6 HMC reduces macrophage NFκB activation *in vitro*

Zymosan activates the TLR2/MyD88 pathway and consequently promotes activation of NFκB (Akira and Takeda 2004; Guerrero et al. 2012), which is the main transcription factor involved in cytokine production. Therefore, we next investigated whether treatment with HMC could reduce NFκB activation. Using RAW 264.7 macrophages, we observed that treatment with HMC reduced fluorescence intensity of phosphorylated NFκB p65 subunit when compared to vehicle-treated cells (Figure 7A and C-D). Accordingly, the colocalization area of phosphorylated NFkB p65/DAPI per µm (Figure 7B) further suggests that inhibition of NF-κB activation is, at least, one of the mechanisms by which HMC attenuates zymosan-induced inflammation.

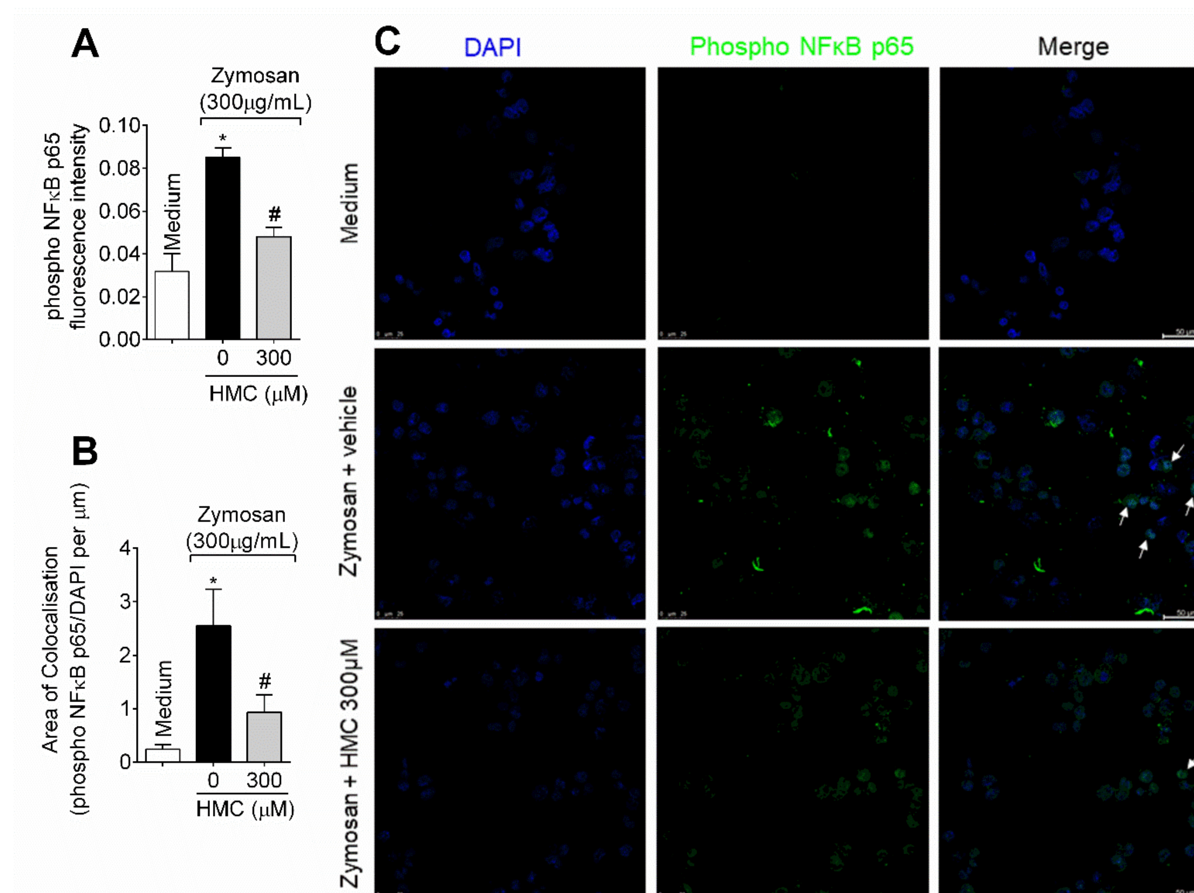

Figure 7. HMC reduces zymosan-induced NF-κB activation. RAW 264.7 macrophages were pre-treated 30 min with HMC (300 µM) before zymosan (300µg/mL) addition. Phospho NF-κB p65 fluorescence intensity (A) and area of

colocalisation with DAPI (B) were analysed 5h after stimulus with zymosan by immunofluorescence in a confocal microscope at 40x magnification. Colocalisation data was calculated using Leica TCS SP8 software and normalised to 84023,99μm dimension area. Arrows indicate representative cells with colocalised fluorescence intensity. Results are presented as mean ± SEM of 6 wells per group. *p<0.05 vs. saline group; #p<0.05 vs. zymosan group, one-way ANOVA followed by Tukey's test.

3.7 HMC directly interact with NFκB p65 protein at Ser276

To advance in terms of mechanisms, we performed *in silico* (molecular docking) analysis to determine whether HMC could directly interact with NFκB p65 protein. Molecular docking shows hydrogen bonds between HMC and four amino acid residues of NF-κB p65 protein, namely Asn155, Asp186, Leu154, and Ser276 (Fig. 8C). Between those, phosphorylation at Ser276 (Fig. 8C, green residue) induces a structural change in p65 protein and increases transcriptional activity and pro-inflammatory gene expression (Jamaluddin et al. 2007), indicating that it is a residue of interest for NF-kB activation. The mean binding energy between HMC and the p65 protein is –7.1±0.153 kcal/mol, indicating that HMC may prevent the p65 phosphorylation and inhibit the nuclear translocation of NF-κB. This *in silico* result corroborates the in vitro data showi HMC inhibits zymosan-induced NF-κB phosphorylation (Fig. 7A) and colocalization of phosphorylated NF-κB and nuclear material (Fig. 7B).

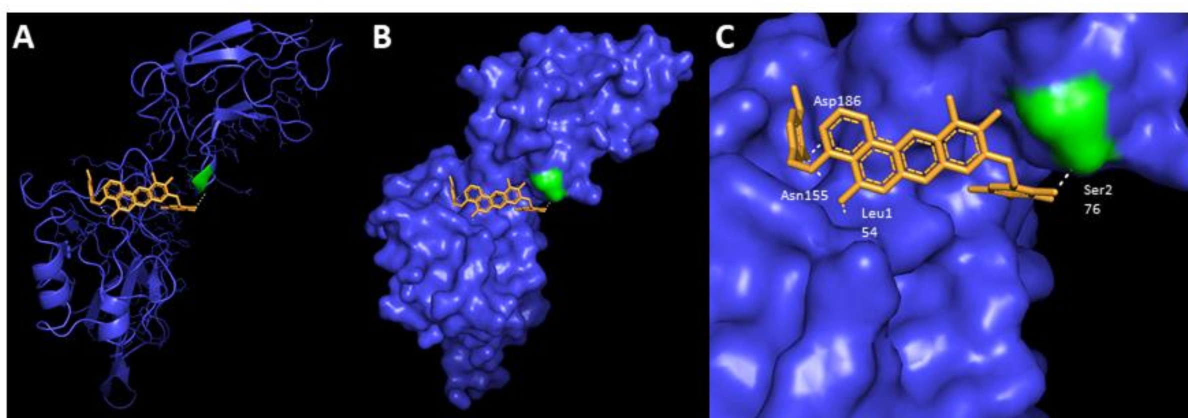

Figure 8. HMC directly interact with NFκB p65 protein at Ser276. HMC molecule is represented in orange. (A) Docking poses p65 protein (PDB code: 1VKX in blue cartoon representation). (B) Docking poses p65 (PDB code: 1VKX in blue surface representation). (C) Docking poses into the phosphorylation sites of the p65 protein (PDB code: 1VKX in blue surface representation). Molecular docking shows hydrogen bonds between HMC and four amino acid residues NF-κB p65 protein, namely Asn155, Asp186, Leu154, and Ser276 (amino acid residue of interest for NF-

kB activation, in green). Hydrogen bonds between HMC and p65 amino acid residues are displayed in white.

4 Discussion and conclusion

Reduction of pain and joint discomfort are the primary goal of treating various forms of arthritis. While effective for a fraction of patients, available treatments have several undesirable effects, and their high cost commonly restrict their applicability as therapeutic approach for rheumatoid arthritis. Therefore, low-toxicity alternatives with minimal adverse effects are necessary. As such, the current study investigated the effects of the flavonoid HMC, which has beneficial properties, including anti-inflammatory effects with no hepatic or gastric injuries, as suggested by other experimental studies (Ahmad et al. 2012; Pinho-Ribeiro et al. 2015; Martinez et al. 2016b) and even clinically (Aguilar Peralta et al. 2007; Jawien et al. 2017). Our study demonstrate an hitherto unknown therapeutic potential of HMC in a model of zymosan-induced arthritis, inhibiting the activation of an important transcription factor, NF-κB, in macrophages. By molecular docking, we predict that HMC directly interacts with Ser276 to reduce NFκB activation. Consequently, we also observed lower levels of NFκB-dependent pro-inflammatory cytokines both *in vivo* and *in vitro*.

Inflammatory stimuli or tissue damage stimulate the activation of a cascade of cytokines, which contributes to inflammatory pain (Verri et al. 2006). More specifically, inflammatory mediators are released and act on neuronal membrane receptors to trigger activation of secondary messengers such as cyclic AMP (cAMP). These intracellular mechanisms are responsible for increasing neuronal excitability, a phenomenon so-called hyperalgesia (Taiwo et al. 1989; Gold et al. 1996; Cunha et al. 1999). Therefore, initially, we investigated whether HMC inhibits the pain-like behaviour caused by i.a. injection of zymosan. Our results show HMC inhibits zymosan-induced mechanical hyperalgesia, corroborating previous data in inflammatory stimuli, such as carrageenan, capsaicin, CFA (Pinho-Ribeiro et al. 2015) and MSU crystals (Ruiz-Miyazawa et al. 2018).

The zymosan-induced arthritis model is constituted by an early phase with pronounced vascular permeability, oedema formation, leukocyte infiltration, and exudation (Penido et al. 2006). Mechanistically, zymosan leads to NF-κB activation through TLR2/MyD88 signalling pathway to generate pro-inflammatory cytokines, neutrophil recruitment, and pain (Guerrero et al. 2012). Recruited neutrophils and

macrophages release eicosanoids, cytokines (such as TNF-α and IL-1β), metalloproteinases, and ROS. The presence of neutrophils and macrophages has been linked with tissue destruction in RA (Bezerra et al. 2007) and inflammatory pain (Cunha et al. 2008; Manchope et al. 2016). Our results show that HMC reduces oedema and leukocyte migration to the inflammatory foci, as demonstrated by the leukocyte count on joint washes and histopathological analysis of synovial infiltrate. These results corroborate others in the literature showing the effect of different flavonoids, such as quercetin, hesperidin, naringenin, and *Daphne genkwa* derivatives, which inhibited leukocyte recruitment and oedema formation (Souto et al. 2011; Verri et al. 2012; Jiang et al. 2014; Ribeiro et al. 2015; Bussmann et al. 2019). A point that reinforces the therapeutic potential of HMC is its known clinical use in combination with vitamin C and *Ruscus aculeatus* extract in the treatment of vascular diseases (Jawien et al. 2017; Kakkos et al. 2018).

It has been commonly reported that peripheral inflammation results in significant production of oxygen radicals locally (Keeble et al. 2009). Endothelial and immune cells produce ROS such as superoxide anion and hydrogen peroxide (H_2O_2) which, in high concentrations, contribute to joint damage and pain (Wang 2004; Frey et al. 2009; Maioli et al. 2015). In fact, patients with arthritis have high levels of ROS and antioxidant therapies reduce pain both in pre-clinical and clinical stages (Abbas and Monireh 2000; Verri et al. 2012). When superoxide anion production overcomes what antioxidant defences can neutralise, the imbalance generated leads to depletion of GSH, a molecule that plays a central role in cell defence against oxidative stress (Huber et al. 2008). Our results demonstrate that HMC treatment effectively restores GSH levels in joint tissue. In line with this, FRAP and ABTS assays show that treatment with HMC increased total antioxidant capacity in knee joint tissue. Concomitantly, using confocal microscopy, we demonstrated that zymosan-induced intracellular ROS production was decreased in RAW 264.7 macrophages after HMC treatment as per DCF assay. Because increase in NADPH oxidase activity and a predominance of pro-oxidant agents have been correlated with cardiovascular problems, brain injuries and progressive joint destruction, we next specifically addressed the effect of HMC on superoxide anion production (Dinauer 1993; Chenevier-Gobeaux et al. 2006; Rabêlo et al. 2010; Dohi et al. 2010). We found HMC decreased gp91^{phox} mRNA expression, a subunit of NADPH oxidase, suggesting that this flavonoid reduces superoxide anion production and corroborates data from the NBT assay. The transcription factor Nrf2

regulates the expression of genes such as heme oxygenase-1 (HMOX-1). HO-1, a protein encoded by the HMOX-1 gene, exert beneficial effects by antioxidant role and controlling excessive inflammation (Calay and Mason 2014). Herein, we observed an increased expression of HO-1 when compared to the stimulus, demonstrating that HMC increased antioxidant defences. Flavonoids can neutralise ROS directly, by electron donation (Pollard et al. 2006; Duarte et al. 2014), or indirectly, through Nrf2 activation (Pallauf et al. 2017). In fact, HMC induces Nrf2 mRNA expression in gout arthritis and UVB irradiation models (Martinez et al. 2016b; Ruiz-Miyazawa et al. 2018). HMC has also the ability to neutralise ABTS and HO radicals, and ferric in cell-free system, indicating that HMC molecule per se presents antioxidant capacity (Martinez et al. 2016a). Therefore, these mechanisms may explain the antioxidant capacity we observed herein and certainly contributed to the analgesic effect observed.

Inflammatory mediators released after the influx of neutrophils and macrophages determine the course of arthritis. Pro-inflammatory cytokines (e.g.,IL-33, TNF-α, and IL-6) are commonly described as players responsible for disease progression and chronicity. In fact, approaches directly affecting cytokine production or their receptor activity, such as etanercept and anakinra, are widely used for the treatment of rheumatic diseases (Verri et al. 2006). In addition to induce immune cell recruitment toward inflammatory foci, cytokines can also modulate metalloproteinases, which are involved in regulating cell-matrix balance. Once unbalanced, the expression of these enzymes can lead to tissue destruction of cartilage (Carlos et al. 2014). For instance, IL-33, TNF-α and IL-6 are present in patients with arthritis. These cytokines promote neutrophil and macrophage recruitment and are linked with disease progression (Ismail Simsek 2011; Kim et al. 2015a; Theoharides et al. 2015b). Consequently, strategies aiming at blocking these cytokines also reduce pain and inflammation (Verri et al. 2008, 2010; Ismail Simsek 2011; Kim et al. 2015b; Theoharides et al. 2015a). Moreover, superoxide anion production is also dependent on TNF-α signalling in mice, indicating a loop between cytokines and ROS (Yamacita-Borin et al. 2015). Our study demonstrated that treatment with HMC inhibits the production of IL-33, TNF-α and IL-6 both in the knee joint and in RAW 264.7 macrophage cell line, corroborating data in another model of pain *in vivo* (Pinho-Ribeiro et al. 2015). Therefore, the reduction of these cytokines represents an relevant mechanism by which HMC reduces zymosan-induced pain and might explain the lower leukocyte recruitment to the articular cavity we observed.

NFκB signalling pathway has a crucial role during inflammation. Activation of NFκB p65 subunit occurs upon an ample range of cellular signals, such as microbial components, antigen receptors, cytokines (TNF-α and IL-1β, for instance), or ROS (Mitchell et al. 2016). Phosphorylation of serine residues in the p65 protein plays a key role in the NFκB activation and DNA-binding activity (Zhong and Suyang 1997; Reber et al. 2009). For instance, the production of ROS is essential for NFκB phosphorylation at Ser276 induced by TNF-α (Jamaluddin et al. 2007). When translocated to the nucleus, NF-κB induces the production of pro-inflammatory cytokines, cyclooxygenase-2, and other inflammatory mediators and enzymes that induce pain and inflammation (Chin 2016). Flavonoids (Tyagi et al. 2009; Lee et al. 2010) and antioxidant compounds (Jamaluddin et al. 2007) can block NFκB phosphorylation and activation at this specific amino acid, indicating that preventing phosphorylation at this site reduces NFκB activation. Hitherto, our group and others have demonstrated that HMC treatment suppresses NF-κB activation *in vivo* (Pinho-Ribeiro et al. 2015; Zhao et al. 2018). Herein, we demonstrated in RAW 264.7 macrophages that HMC diminished the phosphorylated NF-κB p65 subunit staining and co-localisation with the nucleus. Consequently, we also observed reduced levels of the NFκB-dependent cytokines IL-33, TNF-α, and IL-6 in cell culture supernatant. Also, by performing *in silico* analysis, we found interaction bounds between HMC and Ser276 residue in the p65 protein. It suggests that HMC direct interact with Ser276 of p65, an amino acid residue of interest for NFκB activation. To our knowledge, this is the first demonstration that HMC directly interacts with NFκB. Thus, HMC acts, at least in part, by targeting NF-κB activation and therefore suppressing pathological inflammation.

Conflict of interest

Authors declare no conflict of interest.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted: Londrina State University Ethics Committee on Animal

Research and Welfare (approval number 5943.2017.85). Animal care and handling procedures also followed the guideline of the International Association for Study of Pain (IASP) and Brazilian Council on Animal Experimentation (CONCEA).

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4 CONSIDERAÇÕES FINAIS

Em um modelo de artrite induzida por zymosan, o tratamento com a HMC foi capaz de reduzir a inflamação e a dor articular em camundongos. Após o tratamento, foi observada redução na hiperalgesia, na formação de edema, menor migração de leucócitos, diminuição na produção de citocinas pró-inflamatórias e de espécies reativas de oxigênio. Os resultados obtidos sugerem também que os efeitos observados se devem, ao menos em parte, à modulação do NF-κB, como demonstrado por ensaios *in vitro* e *in silico*. Portanto, o presente trabalho demonstra o potencial terapêutico da HMC no tratamento da artrite.

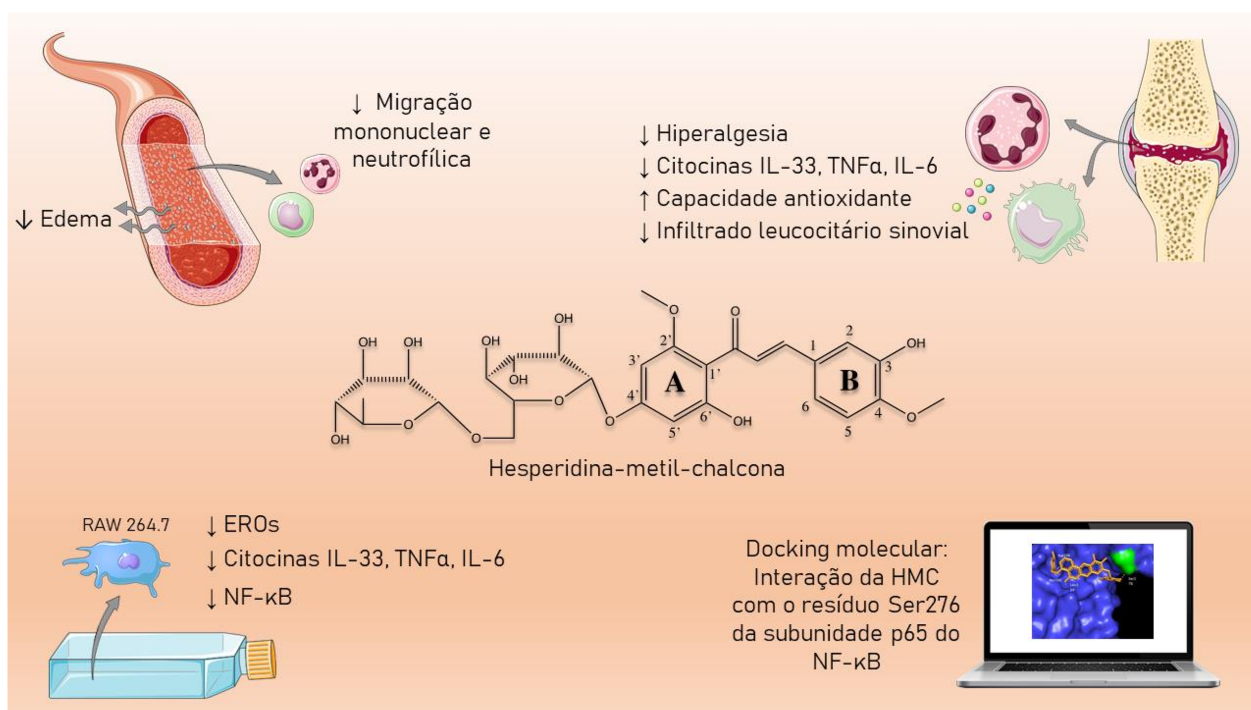

Figura 2. Efeitos e mecanismos anti-inflamatórios da hesperidina-metil-chalcona.

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ANEXOS

ANEXO I

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Inflammopharmacology

ORIGINAL ARTICLE

Hesperidin methyl chalcone interacts with NFκB Ser276 and inhibits zymosan-induced joint pain and inflammation, and RAW 264.7 macrophage activation

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Abstract

Arthritis can be defined as a painful musculoskeletal disorder that affects the joints. Hesperidin methyl chalcone (HMC) is a flavonoid with analgesic, anti-inflammatory, and antioxidant effects. However, its effects on a specific cell type and in the zymosan-induced inflammation are unknown. We aimed at evaluating the effects of HMC in a zymosan-induced arthritis model. A dose–response curve of HMC (10, 30, or 100 mg/kg) was performed to determine the most effective analgesic dose after intra-articular zymosan stimuli. Knee joint oedema was determined using a calliper. Leukocyte recruitment was performed by cell counting on knee joint wash as well as histopathological analysis. Oxidative stress was measured by colorimetric assays (GSH, FRAP, ABTS and NBT) and RT-qPCR ($gp91^{phox}$ and HO-1 mRNA expression) performed. In vitro, oxidative stress was assessed by DCFDA assay using RAW 264.7 macrophages. Cytokine production was evaluated in vivo and in vitro by ELISA. In vitro NF-κB activation was analysed by immunofluorescence. We observed HMC reduced mechanical hypersensitivity and knee joint oedema, leukocyte recruitment, and pro-inflammatory cytokine levels. We also observed a reduction in zymosan-induced oxidative stress as per increase in total antioxidant capacity and reduction in $gp91^{phox}$ and increase in HO-1 mRNA expression. Accordingly, total ROS production and macrophage NFκB activation were diminished. HMC interaction with NFκB p65 at Ser276 was revealed using molecular docking analysis. Thus, data presented in this work suggest the usefulness of HMC as an analgesic and anti-inflammatory in a zymosan-induced arthritis model, possibly by targeting NFκB activation in macrophages.

Keywords HMC · Knee pain · Joint pain · Flavonoid · Molecular docking · Experimental arthritis

Introduction

Arthritis can be broadly defined as a painful musculoskeletal disorder that affects the joints and ultimately diminishes the quality of life because of disabilities (Sprangers et al. 2000; Beukelman et al. 2017). Current treatments for arthritis rely mainly on methotrexate, corticoids, and immunobiological agents, which often present relevant side effects limiting their use (Laev and Salakhutdinov 2015).

Movement-induced joint pain is one of the main causes of complaint among patients with arthritis and can even worsen disease status (Yoshida et al. 2012). Recruitment of inflammatory cells, especially neutrophils is one of the major elements associated with joint tissue damage due to the production of pro-inflammatory mediators and reactive oxygen species (ROS). In fact, neutrophils are prevalent

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ANEXO II

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Review

Specialized pro-resolving lipid mediators: A new class of non-immunosuppressive and non-opioid analgesic drugs

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ABSTRACT

We now appreciate that the mechanism of resolution depends on an active and time-dependent biosynthetic shift from pro-inflammatory to pro-resolution mediators, the so-called specialized pro-resolving lipid mediators (SPMs). These SPMs are biosynthesized from the omega-3 fatty acids arachidonic acid (AA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), or docosahexaenoic acid (DHA). Despite effective for a fraction of patients with rheumatic diseases and neuropathic pain, current analgesic therapies such as biological agents, opioids, corticoids, and gabapentinoids cause unwanted side effects, such as immunosuppression, addiction, or induce analgesic tolerance. A growing body of evidence demonstrates that isolated SPMs show efficacy at very low doses and have been successively used as therapeutic drugs to treat pain and infection in experimental models showing no side effects. Moreover, SPMs work as immunoresolvents and some of them present long-lasting analgesic and anti-inflammatory effects (*i.e.* block pain without immunosuppressive effects). In this review, we focus on how SPMs block pain, infection and neuro-immune interactions and, therefore, emerge as a new class of non-immunosuppressive and non-opioid analgesic drugs.

1. Introduction

Nociceptive pain works as an early warning device to call our attention to potential danger in the environment and injury. Pain generates adaptive learning intimately linked to negative emotions that ultimately protects the individual [1,2]. Therefore, to be effective, nociceptive pain must trigger a physiological response that the host cannot ignore. While unpleasant, it is the most important evolutionary conserved protective sign. Individuals diagnosed with congenital insensitivity to pain often harm themselves and have a reduction of lifespan since they cannot differentiate non-noxious from noxious stimuli. Given the importance of the varied emotional shades for the understanding of pain, the taxonomy committee of the International Association for the Study of Pain (IASP) defines it as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." It is now recognized, for example, that mood, cognitive function, memories, state of humor, attention [3], and the expectations regarding the intensity of the stimulus [4] also shape pain perception.

The contemporary understanding of persistent pathological pain also includes peripheral and spinal cord sensitization of nociceptors and changes in the immune cell phenotypes. While nociceptive pain is triggered by the activation of high threshold nociceptors, in inflammatory pain, a shift from a high threshold to a low threshold type of pain promotes mechanical and thermal hypersensitivity where non-noxious stimuli can now generate pain [5–7]. That hypersensitivity occurs due to sensitization of peripheral nociceptors, known as hyperalgesia (increased pain from a stimulus that normally provokes pain) or allodynia (pain due to a stimulus that does not normally provoke pain) [6]. Interestingly, recent evidence demonstrates that peripheral Schwann cells can also initiate pain behaviors induced by a mechanical stimulus in mice [8]. Optogenetic activation of Sox10-positive cells (Schwann cell marker) shows that these cells help to sense mechanical stimuli, and their activation is sufficient to induce peripheral neuronal firing [8]. During inflammation, however, peripheral innate immune cells such as macrophages, neutrophils, and mast cell release mediators that act on peripheral nerve terminals [7]. Acting on specific receptors, inflammatory mediators such as prostaglandin (PG) E_2, histamine, and cytokines are the main responsible for reducing neuronal threshold and producing peripheral sensitization [6,7,9]. In addition to those

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ANEXO III

Comprovante publicação artigo científico no periódico *Molecules*

Review

Therapeutic Potential of Flavonoids in Pain and Inflammation: Mechanisms of Action, Pre-Clinical and Clinical Data, and Pharmaceutical Development

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Abstract: Pathological pain can be initiated after inflammation and/or peripheral nerve injury. It is a consequence of the pathological functioning of the nervous system rather than only a symptom. In fact, pain is a significant social, health, and economic burden worldwide. Flavonoids are plant derivative compounds easily found in several fruits and vegetables and consumed in the daily food intake. Flavonoids vary in terms of classes, and while structurally unique, they share a basic structure formed by three rings, known as the flavan nucleus. Structural differences can be found in the pattern of substitution in one of these rings. The hydroxyl group (–OH) position in one of the rings determines the mechanisms of action of the flavonoids and reveals a complex multifunctional activity. Flavonoids have been widely used for their antioxidant, analgesic, and anti-inflammatory effects along with safe preclinical and clinical profiles. In this review, we discuss the preclinical and clinical evidence on the analgesic and anti-inflammatory proprieties of flavonoids. We also focus on how the development of formulations containing flavonoids, along with the understanding of their structure-activity relationship, can be harnessed to identify novel flavonoid-based therapies to treat pathological pain and inflammation.

Keywords: clinical trials; natural products; hyperalgesia; allodynia; analgesia; flavonoid; hypersensitivity; inflammation; cytokines; NF-kB

1. Introduction

Inflammatory response induced by micro-organisms or tissue damage trigger the release of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively [1,2]. Tissue-resident immune cells such as macrophages and dendritic cells (DC) recognize these molecules though receptors namely pattern recognition receptors (PRRs). Once activated, these immune cells produce chemoattractant molecules, which are mainly governed by the transcription factor NF-κB [1,2]. The transcription factor NF-κB regulates the expression of inflammatory enzymes, such as COX-2 [3] and pro-inflammatory cytokines [4–6], which makes it one of the most important transcription factors during the inflammatory process and pain. Cytokines and

ANEXO IV

Comprovante publicação artigo científico no periódico *Journal of Natural Products*

JOURNAL OF NATURAL PRODUCTS

pubs.acs.org/jnp Article

Diosmin Treats Lipopolysaccharide-Induced Inflammatory Pain and Peritonitis by Blocking NF-κB Activation in Mice

Victor Fattori, Fernanda S. Rasquel-Oliveira, Nayara A. Artero, Camila R. Ferraz, Sergio M. Borghi, Rubia Casagrande, and Waldiceu A. Verri, Jr.*

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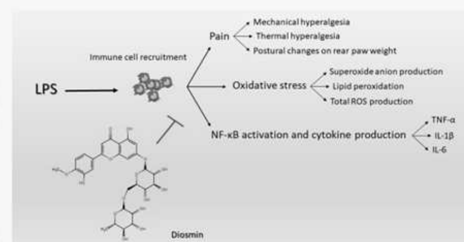
ABSTRACT: Gram-negative bacterial infections induce inflammation and pain. Lipopolysaccharide (LPS) is a pathogen-associated molecular pattern and the major constituent of Gram-negative bacterial cell walls. Diosmin is a citrus flavonoid with antioxidant and anti-inflammatory activities. Here we investigated the efficacy of diosmin in a nonsterile model of inflammatory pain and peritonitis induced by LPS. Diosmin reduced in a dose-dependent manner LPS-induced inflammatory mechanical hyperalgesia, thermal hyperalgesia, and neutrophil recruitment to the paw (myeloperoxidase activity). Diosmin also normalized changes in paw weight distribution assessed by static weight bearing as a nonreflexive method of pain measurement. Moreover, treatment with diosmin inhibited LPS-induced peritonitis as observed by a reduction of leukocyte recruitment and oxidative stress. Diosmin reduced LPS-induced total ROS production (DCFDA assay) and superoxide anion production (NBT assay and NBT-positive cells). We also observed a reduction of LPS-induced oxidative stress and cytokine production (IL-1β, TNF-α, and IL-6) in the paw. Furthermore, we demonstrated that diosmin inhibited LPS-induced NF-κB activation in peritoneal exudate. Thus, we demonstrated, using a model of nonsterile inflammation induced by LPS, that diosmin is a promising molecule for the treatment of inflammation and pain.



Nociceptive pain is an important mechanism preserved during the process of evolution and is essential for the maintenance of body integrity. Inflammatory pain is one of the most common symptoms found in the clinic and represents an important type of pain.[1] Exacerbation of pain occurs due to sensitization of specialized sensory neurons called nociceptors, leading to a state designated as hyperalgesia.[1,2] The peripheral sensitization is mediated by several inflammatory mediators, such as prostaglandin (PG) E_2, histamine, endothelin (ET)-1, and cytokines released by neutrophils and macrophages.[1,2] Clinically, Gram-negative bacterial infections induce inflammation and pain. Lipopolysaccharide (LPS) is a pathogen-associated molecular pattern (PAMP) and the major constituent of Gram-negative bacterial cell walls, which is released in large amounts during infection after the immune system's bactericidal activity.[3] Injection of LPS induces pain dependent on the release of hyperalgesic cytokines such as TNF-α and IL-1β in the paw skin[4] and tibiotarsal joint.[5] LPS-induced release of inflammatory mediators is orchestrated in a cascade of cytokines that occurs in a TRIF-independent and MyD88-dependent manner.[4] In addition to act as members of this cascade that culminate in the release of PGE_2 and sympathetic amines, IL-33,[6] TNF-α,[7] IL-6,[8] and IL-1β[9] can also activate nociceptors and produce pain.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the current choice for the treatment of acute inflammation and pain. While effective, NSAIDs present several side effects and must be used with caution in patients with comorbidities, such as kidney and heart disease.[10−12] Flavonoids and flavonoid-based drugs present safe clinical and preclinical profiles[13−15] and have been extensively used in several experimental models as anti-inflammatory, antioxidant, and analgesic drugs. A 7-year prospective study with 98 469 participants in the US shows that daily intake of flavonoids improves life quality and decreases the incidence of death related to cardiovascular diseases in these participants.[16] It has been also reported that the risk of the development of some chronic diseases such as cerebrovascular disease is reduced under a higher flavonoid intake diet.[17] Diosmin (diosmetin-7-O-rutinoside) is a naturally occurring citrus flavone glycoside that is obtained by dehydrogenation of hesperidin.[18] It works as an antioxidant,

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