KENJI WILLIAM RUIZ MIYAZAWA

EFEITOS ANTI-INFLAMATÓRIO, ANTIOXIDANTE, ANALGÉSICO E MECANISMOS DE AÇÃO DOS FLAVONOIDES TRANS-CHALCONA, HESPERIDINA METIL-CHALCONA E QUERCETINA EM MODELO DE ARTRITE GOTOSA EM CAMUNDONGOS

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Orientador: Prof. Dr. Waldiceu A. Verri Jr.

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Londrina, 10 de Abril de 2017.

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RUIZ-MIYAZAWA, Kenji William. **Efeitos anti-inflamatório, antioxidante, analgésico e mecanismos de ação dos flavonoides Trans-Chalcona, Hesperidina Metil-Chalcona e Quercetina em modelo de artrite gotosa em camundongos**. 2017. 140 p. Tese de Doutorado (Patologia Experimental) – Universidade Estadual de Londrina. Londrina-Pr, 2017.

RESUMO

Artrite gotosa é uma doença inflamatória que acomete articulações e caracteriza-se pela deposição de cristais de ácido úrico (MSU) intra e periarticular. Os sinais mais comuns são o edema articular e o aumento da sensibilidade à dor nas articulações (hiperalgesia). Uma nova abordagem terapêutica para o tratamento da doença é a inclusão de compostos químicos naturais presentes em plantas como os flavonoides. Trans-chalcona (TC), Hesperidina metil-chalcona (HMC) e Quercetina (QC) são flavonoides, presentes principalmente em vegetais e frutas, que apresentam diversas propriedades biológicas capazes de modular processos inflamatórios por meio de suas atividades antioxidantes e anti-inflamatórias e, além disso, atuam sobre fatores de transcrição, como Nrf2 e NFκB. Desta forma, o objetivo do presente trabalho foi avaliar os efeitos e mecanismos de ação dos flavonoides TC e HMC, além de avaliar se os efeitos apresentados pela QC eram dependentes de receptores opioides no modelo de artrite gotosa induzida por MSU em camundongos. O estudo foi aprovado pelo comitê de ética animal da Universidade Estadual de Londrina (CEUA-UEL- processo nº 13279.2011.76 e nº 14600.2013.73). A artrite gotosa foi induzida pela injeção intra-articular (100 µg/10 µL) de MSU e foram avaliados a hiperalgesia mecânica, edema articular, migração leucocitária, histologia articular (H/E), estresse oxidativo, produção de citocinas, ativação do NFκB e expressão de RNAm do inflamassoma NLRP3 (qPCR) e, *in vitro,* a produção de IL-1β madura no sobrenadantes de macrófagos derivados da medula (BMDMs). Inicialmente foi realizada uma dose reposta dos flavonoides TC (3, 10 e 30 mg/kg), HMC (3, 10 e 30 mg/kg) por via oral e QC (10, 30 e 100 mg/kg) por via subcutânea para determinar a dose mais eficaz. Além disso, foi realizado uma dose resposta da naloxona, um inibidor não seletivo de receptores opioide nas doses de (0.3, 1 e 3 mg/kg) por via intraperitoneal, juntamente ao modelo de gota e quercetina, para avaliar se os efeitos da quercetina eram dependentes de receptores opioides. Desta forma as doses selecionadas foram TC (30 mg/kg), HMC (30 mg/kg), QC (100 mg/kg) e naloxona (3 mg/kg). O tratamento com esses três flavonoides foram capazes de reduzir a hiperalgesia mecânica e o edema induzidos por MSU. Além disso, quando avaliada a migração leucocitária, foi possível observar que os flavonoides diminuíram o recrutamento de leucócitos para a cavidade e tecido articular, reduzindo assim a sinovite. O tratamento com os flavonoides foram capazes de proteger contra o estresse oxidativo induzido pela injecção i.a. de MSU. A quercetina foi capaz de promover a restauração dos niveis de GSH, aumento do Frap, ABTS e redução de anions superóxidos (NBT), além disso, os tratamentos com TC e HMC também reduziram a produção de óxido nítrico (NO) na articulação. Os tratamentos com os flavonoides foram capazes de reduzir a expressão do RNAm de $gp91^{phox}$ ao mesmo tempo que induziram a ativação da via Nrf2/HO-1 conferindo à essas moléculas propriedades antioxidantes. A produção de citocinas pro-inflamatórias como IL-1β e TNFα foram reduzidas pelo tratamento com QC, ademais

IL-6 foi reduzida pelo tratamento com TC e HMC. Em relação ás citocinas anti-inflamatórias os dados demonstram que os níveis de IL-10 foram reduzidos pelo tratamento HMC e não alterados no tratamento com TC, além disso, os tratamentos com TC e HMC foram capazes de aumentar os níveis de TGF-β na articulação. O estímulo com MSU foi capaz de ativar vias de sinalizações inflamatórias ativados pelo NFκB; os flavonoides, por sua vez, inibiram a ativação, contribuindo com a redução de citocinas pro-inflamatórias. Quando avaliada a expressão dos RNAm das moléculas do inflamassoma NLRP3, ASC, Pro-caspase e Pro-IL1β todos os flavonoides avaliados inibiram sua expressão de maneira significativa. Entretanto, no modelo *in vivo*, os efeitos promovidos pelo tratamento com a quercetina na dose de 100 mg/kg, s.c., foram revertidos de maneira siginificativa pelo pre-tratamento com a naloxona na dose de 3 mg/kg, i.p. em todos os ensaios realizados. *In vitro*, foi avaliado os níveis de IL-1β madura no sobrenadante de BMDMs co-estimuladas com LPS (sinal 1) e MSU (sinal 2). Esta estimulação levou ao aumento nos níveis de IL-1β, sendo que o tratamento com quercetina (30 µM) inibiu a produção de IL-1β madura no sobrenadante de BMDMs, contudo, o seu efeito foi revertido pela naloxona na concentração de (20 µM). Entretanto o tratamento com HMC em nenhuma das concentrações testadas foram capazes de inibir a sua produção de maneira significativa, logo o efeito da HMC não é depentende da inibição da ativação do inflamassoma, mas sim pela inibição da ativação do NFκB. Assim, os dados do presente trabalho sugerem que os tratamentos com os flavonoides TC e HMC exibem efeito analgésico, anti-inflamatório e antioxidante que podem estar associados com a inibição do NFκB no modelo *in vivo*, assim como a quercetina atuou sobre a inibição tanto de NFκB quanto de Inflamassoma NLRP3 no modelo *in vivo e in vitro*, sendo os seus efeitos dependentes da participação dos receptores opioide no modelo de artrite gotosa induzida por MSU.

Palavras chaves: Trans-Chalcona. Hesperidina metil chalcona. Quercetina. Inflamassoma NLRP3. Estresse oxidative. Hiperalgesia.

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ABSTRACT

Gout is an inflammatory disease that affect articular joint and it is characterized by acute burning arthritis with local hyperalgesia and pain caused by monosodium urate (MSU) crystals accumulated into the affected joint. A novel therapeutic approach for the treatment of the disease is the addition of natural compounds present in plants such as flavonoids. Trans-Chalcone (TC), Hesperidin Methyl Chalcone (HMC) and Quercetin (QC) are flavonoids present mainly in vegetable and fruits and shown several biologic proprieties able to reduces the inflammatory process due present antioxidant capacity and anti-inflammatory effect, in addition, this flavonoids can also act on transcription factors (e.g. Nrf2 and NFκB). Thus, the aim of the present study was investigate the mechanism and effects of TC, HMC and QC flavonoids, In addition to evaluating whether the effects applied by QC were dependent on non-model opioid receptors of MSU-induced gout arthritis in mice. This study was approved by Animal Ethics Committee of the State University of Londrina (CEUA-UEL-process nº 13279.2011.76 and nº. 14600.2013.73). Gout was induced by MSU injection (100 µg/10 µL, i.a.) and *in vivo* model the mechanical hyperalgesia and edema and leucocyte migration and histological analysis (H/E) and oxidative stress and cytokines productions and NFκB activation and mRNA expression of NLRP3 (qPCR) were evaluated and *in vitro*, IL-1β production was mensuared in supernatants of marrow-derived macrophages (BMDMs). In first line of experiments, was made a dose-response of all flavonoids TC (3, 10 and 30 mg/kg), HMC (3, 10 and 30 mg/kg) by per oral route and QC (10, 30 e 100 mg/kg) by subcutaneous route to determine the effective dose. In addition, a concentration response of naloxone (0.3, 1 and 3 mg/kg, i.p.) a non-selective opioid receptor inhibitor was performed to assess whether the effects of quercetin were dependent on opioid receptors. Thus, the dose of TC (30 mg/kg), HMC (30 mg/kg), QC (100 mg/kg) and Naloxone (3 mg/kg) were selected to next experiments. In the treatment schedule, all flavonoids were able to reduce the mechanical hyperalgesia and edema induced by MSU injection. In addition, the leucocyte migration was inhibits by the treatments with flavonoids in the articular cavity. The inflammatory infiltration into the articular tissue was reduced by treatment of flavonoids (TC, HMC and QC) reducing the synovitis in the focus of lesion. All flavonoids were able to protect against oxidative stress induced by MSU. Quercetin treatment was able to promote a restoration of GSH levels, increase of Frap, ABTS and reduced of NBT, in addition, the treatments with TC and HMC decreasing nitric oxide (NO) in articular joint. All molecules were able to reduce the mRNA expression of gp91phox and increase the Nrf2 and HO-1 expression conferring an antioxidant property to these compounds. The pro-inflammatory cytokines IL-1β and TNFα was decrease by the treatment with QC, furthermore IL-6 was reduces by TC and HMC treatment. However the anti-inflammtory cytokines such as IL-10 levels was reduced by HMC treatment and not alteraded by TC treatment, however the TC and HMC treatment were able to

increase TGF-β levels in articular joint. Stimuli with MSU crystals were able to activate inflammatory signaling pathways such as NFκB and the flavonoids TC, HMC and QC treatment were able to inhibit this pathway, promoting the pro-inflammatory cytokines reduction. When assessed the mRNA expression of inflammassome NLRP3 molecules (NLRP3, ASC, Pro-caspase and Pro-IL1β), all flavonoids were able to reduce in a significant manner the expression of this molecules. However, *in vivo* model, the effects promoted by treatment with quercetin (100 mg/kg, s.c.), were significantly reversed by pretreatment with naloxone (3 mg/kg, i.p.) in all the tests performed *In vitro*, IL-1β levels in supernatant macrophages (BMDMs) induced with LPS (first signal) and with MSU (second signal) promotes an increasing of IL-1β levels; quercetin treatment (30 μM) induced an inhibition of the IL-1β levels of BMDMs supernatant, however, quercetin effect was reversed by pre-treatment with naloxone (20 μM), increasing the IL-1β levels. HMC treatment was not able to inhibit the IL-1β production. Thus, our data suggest that treatment with flavonoids exhibits an analgesic, anti-inflammatory and antioxidant effects. HMC is not dependet on inhibiting inflammasome activation, but rather on inhibiting NFκB activation, in addition, TC and QC were able to inhibiting inflammasome NLRP3, which the quercetin effect was reversed by naloxone treatment, so, quercetin effects might be associated with the participation of opioid receptors in gouty arthritis model induced by MSU.

Keywords: Trans-Chalcone. Hesperidin methyl chalcone. Quecetin. NLRP3 inflammasome. Hyperalgesia.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ABTS	2,2’ azinobis (3-etilbenzotiazolina-6-ácido sulfônico)
ANOVA	Análise de variância
CFA	Adjuvante completo de Freund’s
DTNB	Ácido 5,5’-ditio-bis-(2-nitrobenzóico)
EDTA	ácido etilenodiamino tetra-acético
ELISA	Ensaio Imunoenzimático
ERO	Espécie reativa de oxigênio
Fe^{2+}	Ferro
$FeCl_3.6H_2O$	Cloreto férrico hexahidratado
FRAP	Poder antioxidante de redução férrica
GSH	Glutationa reduzida
HCl	Ácido clorídrico
HMC	Hesperidina Metil Chalcona
HO-1	Hemeoxigenase-1
HTAB	Hexadecyltrimethylammonium bromide
IASP	Associação Internacional para o Estudo da Dor
IL-1	Interleucina 1
IL-1β	Interleucina 1 Beta
IL-8	Interleucina 8
IL-10	Interleucina 10
i.a	Intra-articular
KOH	Hidróxido de potássio
MDA	Malondialdeído
MMPs	Metaloproteinases da matriz
MPO	enzima mieloperoxidase
MSU	Cristais de urato monossódico
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NOX_2	Nonphagocytic NADPH Oxidase isoforma do tipo 2
NaOH	Hidróxido de sódio
NBT	*Nitroblue tetrazolium*
NF-κB	Fator nuclear Kappa B

NGN	Naringenina
Nrf2	Fator nuclear eritróide 2 relacionado ao fator 2
NaCl	Cloreto de sódio
PBS	Tampão fosfato salino
PCR	Reação em cadeia da polimerase
PGD_2	Prostaglandina D_2
PGE_2	Prostaglandina E_2
pg	picogramas
p.o	per oral
RNAm	Ácido ribonucleico mensageiro
TGF-β	Fator de transformação do crescimento *beta*
TNF	Fator de Necrose Tumoral
TNFα	Fator de Necrose Tumoral Alfa
TPTZ	2,4,6 tripiridil-S-triazina
Tris	Hidroximetil aminometano
v.o	via oral

SUMÁRIO

1 INTRODUÇÃO

1.1 Patogenia da artrite gotosa aguda

A artrite gotosa é uma das artrites inflamatórias que acomete cerca de 1 a 2% da população mundial (RICHETTE; BARDIN, 2010). Evidências epidemiológicas sugerem que em países desenvolvidos essa doença apresenta uma maior prevalência em homens de meia idade, idosos e mulheres na pós-menopausa, devido à queda no nível de estrógenos (que são uricosúricos), aumentando a uricemia (CASSETTA; GOREVIC, 2004; HAK; CHOI, 2008; KUO; TSAI; LIOU, 2008).

Alguns autores relatam aumento da prevalência da doença em indivíduos que não levam um estilo de vida saudável e que fazem usos de medicamentos como diuréticos, aspirina e álcool com frequência (VANITALLIE, 2010).

Diversos fatores de riscos estão relacionados ao desenvolvimento da artrite gotosa, dentre eles, o elevado consumo de urato de sódio e/ou purinas presentes em carnes vermelhas, miúdos (coração, fígado e rins), enlatados e frutos do mar, associado a uma falha na eliminação ou uma superprodução dos ácidos úricos pelo organismo, caracterizam-se grandes fatores de risco que predispõem o individuo ao desenvolvimento da artrite gotosa. Quando tal equilíbrio não é mantido e os níveis de ácido úrico no sangue ultrapassa 7.0 mg/dL em homens e 6.0 mg/dL em mulheres esses indivíduos são considerados hiperuricemicos (ROTT; AGUDELO, 2003).

Nestes casos, o excesso de urato se depositam na articulação ou em tecidos peri-articulares podendo ligar-se a um cátion sódio formando o urato monossódico, este por sua vez é mais solúvel que o urato livre, porem seu limite de solubilidade é de 7 mg/dL a 37ºC, logo, concentrações elevadas deste sal assim como a diminuição do limite de solubilidade do mesmo, favorecem a formação e precipitação destes cristais nas articulações de pacientes, promovendo assim, o desenvolvimento da doença (PASCUAL et al., 2015). Além disso, baixas temperaturas promovem uma diminuição na solubilidade desses cristais levando a sua precipitação, principalmente em regiões distais como dedos dos pés, dedos das mãos, cotovelos, orelhas e joelhos (ARBER et al., 1994; ELLIOT; CROSS; FLEMING, 2009; NEOGI et al., 2014).

Assim, a artrite gotosa é uma doença caracterizada por uma intensa e dolorosa reação inflamatória em resposta a deposição de cristais de urato monossódico (MSU) nas junções articulares e peri-articulares, principalmente de pacientes que apresentam elevados níveis de ácido úrico (hiperuricemia), provocando desta forma, uma intensa reação inflamatória com consequente dor articular intensa (GHAEMI-OSKOUIE; SHI, 2011; JIN et al., 2014).

O ácido úrico é formado a partir da ação da enzima xantina oxidase sobre as purinas adenosina e guanina, sendo este produzido em quantidades elevadas sobre a lesão celular. A liberação desses ácidos úricos provenientes das células lesadas formam os cristais de MSU por meio da ligação de anticorpos específicos para ácido úrico (BANNASCH et al., 2008).

Assim, a literatura relata que os cristais de MSU podem ser reconhecidos como um adjuvante endógeno e por sinais pro-inflamatórios análogos chamados de padrão moleculares associado ao perigo (DAMP) por meio das células fagocíticas inata como células dendriticas, macrófagos e neutrófilos. Logo, esses padrões análogos podem desencadear uma resposta imune inflamatória sistêmica na ausência de um patógeno ou agente infeccioso (SHI; EVANS; ROCK, 2003).

Desta forma, diversos autores demonstraram que o processo inicial da resposta inflamatória aos cristais de MSU ocorre quando os macrófagos residentes no do espaço articular reconhecem os cristais de MSU por meio de receptores de reconhecimento de antígenos como receptores do tipo Toll (*Toll Like-receptor* (TLR) 2/4 e CD 14 que ativam a via MyD88/TRIF, culminando na ativação do fator de transcrição nuclear kappa B (NF-κB) (LIU-BRYAN et al., 2005).

Além disso, por meio da fagocitose e distúrbios citoplasmáticos, levando a formação de proteínas do complexo multiproteico inflamassoma NLRP3, resultando na ativação de caspase-1 e processamento e secreção de IL-1β madura, além de induzir a formação de diversas espécies reativas de oxigênio (ERO) (PUNZI et al., 2012; SHI; MUCSI; NG, 2010).

A citocinas IL-1β juntamente com outras citocinas pro-inflamatórias como TNF-α, IL-6 e IL-8 são responsáveis por promoverem um aumento no influxo de neutrófilos para o foco da lesão aumentando desta forma a resposta imune local (LIU-BRYAN et al., 2005).

O aumento do recrutamento de neutrófilo para a articulação possui um papel muito importante no desenvolvimento da inflamação articular,

principalmente por meio da liberação extracelular de diversos mediadores inflamatórios, como ERO, enzimas proteolíticas, citocinas, quimiocinas e prostaglandina E_2 (PGE_2) o qual está relacionada com o progresso de degradação e danos articular (KODITHUWAKKU et al., 2013; SABINA; NAGAR; RASOOL, 2011).

Estudos têm demonstrando que a diminuição de macrófagos é capaz de diminuir o infiltrado de neutrófilos na articulação inflamada, desta maneira diminuindo a produção de citocinas inflamatórias, principalmente IL-1β. Especula-se que a inibição da formação desses mediadores inflamatórios e/ou de vias de sinalizações inflamatórias como NF-κB ou inflmassoma NLRP3 em macrófagos poderiam ser uma potencial abordagem terapêutica para o tratamento da artrite gotosa induzidas por cristais de ácido úrico (MARTIN; WALTON; HARPER, 2009).

1.1.1 Inflamassoma NLRP3 e inflamação por cristais de MSU

O inflamassoma é caracterizado por um complexo multiprotéico citoplasmático de elevado peso molecular composto de uma proteína da família NLRP (ou NALP), uma proteína adaptadora ASC e uma caspase inflamatória (BUSSO; SO, 2010), que apresenta atividade enzimática de ativação da citocina pro-inflamatória IL-1β (MARTINON; BURNS; TSCHOPP, 2002), sendo essa atividade dependente do recrutamento de caspase inflamatórias ao complexo ativo do inflamassoma (MARTINON; TSCHOPP, 2004; NADIRI; WOLINSKI; SALEH, 2006).

Uma das principais caspases abordada na patogênese da artrite gotosa é a capase-1, que tem como principais substratos a produção de citocinas, como por exemplo, IL-1β e IL-18, que são mediadores inflamatórios e participam do processo inflamatório, entretanto necessitam de um processamento para poderem se tornar ativa (MARTINON, 2010).

A produção da IL-1β, ocorre por meio de três etapas distintas (BURNS; MARTINON; TSCHOPP, 2003). Inicialmente, após estimulação, ocorre a formação do inflamassoma que procede por meio de uma mudança conformacional que permite a ligação do NLRP com o ASC por meio do domínio pirina. A molécula ASC atua como uma proteína adaptadora que se liga à pro-caspase-1 por meio do domínio de recrutamento da caspase. Por fim, o complexo NLRP3 é formado constituindo uma plataforma de ativação da caspase-1 por clivagem proteolítica,

desta forma, ativando-a. Em seguida ocorre à clivagem da pro-IL-1β em IL-1β permitindo assim a sua secreção (MARTINON, 2010; TSCHOPP; SCHRODER, 2010).

Desta forma, de uma maneira resumida, a ativação do inflamassoma ocorre por meio de dois sinais, inicialmente através da ativação do fator de transcrição NFκB responsável, pela produção da pro-IL-1β, e o segundo sinal decorrente de um distúrbio intracelular (ERO, Catepsina B e influxo de potássio) responsável pela ativação do complexo proteico do inflamassoma, que por final resultará na ativação das citocinas pro-inflamatória IL-1β responsável por desencadear toda a resposta inflamatória assim como o quadro de inflamação aguda de algumas doenças como a artrite gotosa.

1.1.2 Atividades antioxidantes via Nrf2 e heme oxigenasse 1

Doenças inflamatórias como a artrite gotosa apresentam fisiopatologia complexa, no entanto, por ser decorrente de uma intensa reação inflamatória, sabe-se da participação de formação de radicais livres induzindo o estresse oxidativo (Shi et al. 2010; Punzi et al. 2012).

A formação de espécies reativas de oxigênio (ERO) pode promover danos celulares. Desta forma, o organismo pode promover o equilíbrio metabólico do oxigênio no meio intracelular via enzimas antioxidantes e por sistemas celulares não enzimáticos (Vani et al. 2013). A produção dessas enzimas podem ocorrer por meio da ativação da via de sinalização antioxidante como o fator eritróide nuclear 2 relacionado ao fator 2 (Nrf2)/ elemento de resposta antioxidante (Lee et al. 2015; Xia et al. 2015).

Desta forma, Nrf2 (*nuclear factor erythroid 2 (NF-E2)-related transcription factor*) é um fator de transcrição bZip (*basic leucine-zipper*) e membro da família de proteínas reguladoras *Cap'n'Collar* (CNC) que esta envolvido na resposta celular a oxidantes e eletrófilos pela ligação a um elemento potenciador na região promotora dos genes de proteção celular, chamado *antioxidant response element* ou *electrophile response element* (ARE ou EpRE) (Moi et al. 1994; Itoh et al., 1997).

A regulação de Nrf2 ocorre via Keap1 resultando numa ativação da expressão de genes regulados pelo ARE (Niture et al., 2010). No entanto, alguns estudos têm avaliado mecanismos de regulação do Nrf2 independentes da Keap1. Estes mecanismos englobam a regulação do Nrf2 pela sua interação com novas proteínas e regulação epigenética da expressão Nrf2 e Keap1 (Copple, 2012).

A importância do Nrf2 na resposta celular ao stress oxidativo é validada por estudos que utilizam camundongos *knockout* para Nrf2 ($Nrf2^{-/-}$), em que estes apresentam maior sensibilidade a xenobióticos e também em estudos de doenças inflamatórias crônicas que envolvem stress oxidativos (Baird and Dinkova-Kostova, 2011, Kensler et al., 2007; 2007, Jaiswal, 2010).

A ativação da via Nrf2/ARE regula a expressão enzimas desintoxicantes de fase II incluindo NADPH, NAD (P) H quinona oxidoredutase 1, glutationa peroxidase, heme oxigenase-1 (HO-1) e genes antioxidantes que protegem as células de várias lesões (Braun et al., 2002; Chen et al., 2006; Ahmed et al. 2016). A ativação desta via permite também o reconhecimento, reparação e eliminação de proteínas danificadas, inibe a inflamação mediada por citocinas e a autofagia, ativa proteínas que regulam a expressão de receptores e outros fatores de transcrição e de crescimento (Brigelius-Flohe and Flohe, 2011).

Enzimas de fase II de desintoxicação, também conhecidas como, enzimas antioxidantes, são proteínas que inibem a produção de ERO, eliminam radicais livres e, consequentemente, diminuem a apoptose induzida pelo estresse oxidativo. As principais enzimas são heme oxigenasse -1 (HO-1), NAD (P) H: oxidorredutase quinina 1 (NQO1) e gama-glutamilcisteína sintase (gama-GCS) (Vani et al. 2013).

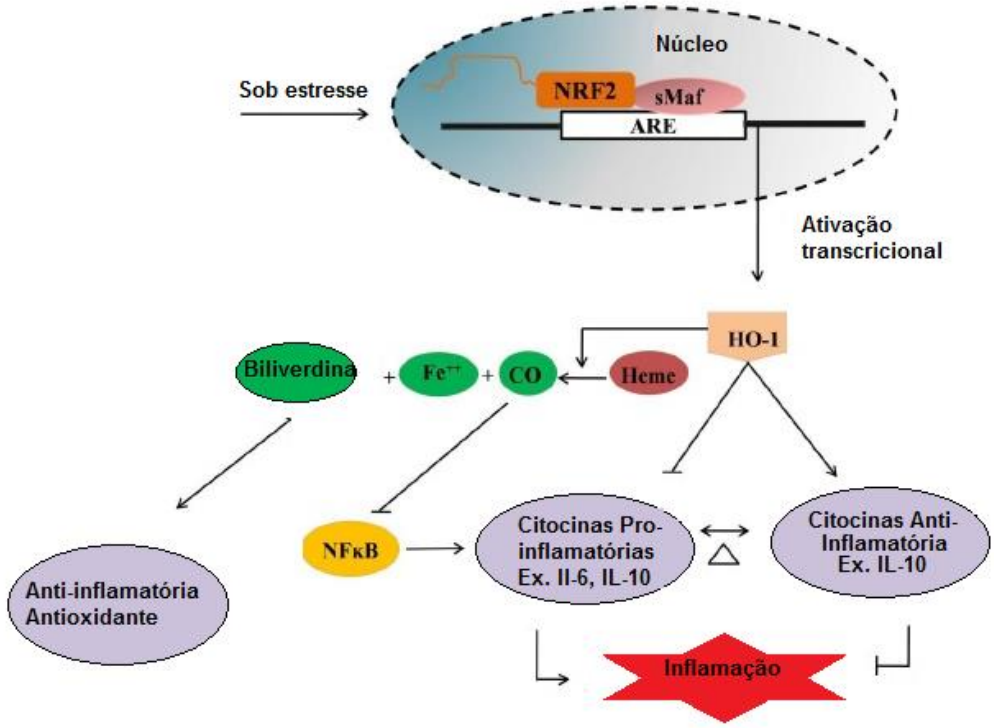
Heme oxigenase-1 é uma enzima de isoforma induzível cujo RNA mensageiro (RNAm) é transcrito a partir de um gene de resposta ao estresse oxidativo, sendo regulada por vários estímulos fisiológicos e exógenos. É responsável pela manutenção da homeostase (*redox)* e executa papel essencial na proteção contra o estresse oxidativo (Prestera et al.,1995; Wunder; Potter, 2003).

Durante o estresse oxidativo, Nrf2 livre transloca-se para o núcleo, onde se dimeriza com membros da pequena família Maf e se liga a ARE tais como HO-1. A HO-1 regulado catalisa a heme em monóxido de carbono (CO), bilirrubina e ferro livre. A geração de bilirrubina em quantidades equimolares confere proteção antioxidante, bem como a formação de ferritina, que inativa o ferro na cascata de

lesão oxidativa (HILL-KAPTURCZAK; CHANG; AGARWAL, 2002), além disso, o CO atua como um inibidor da via NF-κB que consequentemente diminui a produção de citocinas pro-inflamatórios e óxido nítrico (Ahmed et al. 2016; Kim et al. 2013c).

Além disso, HO-1 inibe diretamente citocinas pró-inflamatórias, bem como induz a ativação das citocinas anti-inflamatórias promovendo o balanço do processo inflamatório (Figura 1) (Ahmed et al. 2016) e estudos demonstraram que o efeito da HO-1 sobre a interação entre leucócitos e o endotélio vascular *in vivo* foi capaz de reduzir a adesão de leucócitos ao endotélio após estímulos pró-oxidantes, e a administração de biliverdina, mas não de CO (HAYASHI et al., 1999). Logo, é importante salientar que estes mecanismos antioxidantes citados ocorrem de maneira endógena. No entanto, existem compostos naturais, muitas vezes consumidos na dieta diária, que podem apresentar efeitos similares. Estudos demonstraram que flavonoides apresentam atividades anti-inflamatórias e antioxidante via ativação de Nrf2 (Huang et al., 2013; Mansuri et al., 2014).

Figura 1 – Via Nrf2/HO-1. Adaptado de Ahmed et al. 2016.



1.1.3 Efeitos analgésicos, anti-inflamatórios e antioxidantes relacionados ao sistema opióide

Os receptores opioides pertencem à classe dos receptores acoplados a proteína G da família das radopsinas e são responsáveis pela modulação de atividades biológicas através da interação com proteínas G heterotriméricas. Estes receptores representam a classe mais abundante de receptores nas superfícies celulares, sendo alvos de aproximadamente 1/3 das drogas aprovadas para comercialização, considerando que o sistema opioide é essencial para modulação de comportamentos relacionados à dor e anti-nocicepção (SHANG; FILIZOLA, 2015; SPAMPINATO, 2015; TUOMINEN et al., 2011).

Estes receptores podem ser encontrados em diferentes órgãos e tecidos como cérebro, medula espinal, trato gastrointestinal, entre outros, representando uma linha de pesquisa extremamente ativa na comunidade científica pelo seu papel determinante no estudo e controle da dor. Os três principais subtipos de receptores opióides são μ (ROM), κ (ROK) e δ (ROD), correspondendo respectivamente aos genes OPRM1, OPRK1 e OPKD1. Estes receptores são caracterizados por sete domínios transmembrânicos, com três alças extracelulares, três alças intracelulares, uma porção N-terminal extracelular e outra porção C-terminal intracelular (SHANG; FILIZOLA, 2015; SPAMPINATO, 2015).

Os receptores opioides são ativados por peptídeos endógenos como as endorfinas, encefalinas e dinorfinas, mas também por alcaloides naturais e moléculas semissintéticas e sintéticas (SHANG; FILIZOLA, 2015). Um quarto subtipo de receptor opioide denominado receptor NOP que corresponde ao gene ORL1 possui aproximadamente 60% de similaridade estrutural com os receptores opioides “clássicos”, no entanto, possuem pouco ou nenhuma afinidade pelos peptídeos opioides endógenos ou moléculas como a morfina, ao contrário, tendo como ligante um neuropeptídeo descrito como nociceptina (BUTOUR et al., 1997).

As funções biológicas descritas para os opioides incluem efeitos analgésicos, anti-inflamatórios e antioxidantes, embora também existam evidências de que os opióides possam induzir dor em condições clínicas e experimentais, um fenômeno reconhecido como hiperalgesia-induzida por opióides, que está

relacionada com a tolerância a esta classe de moléculas (BRYANT et al., 2009; CHANG; CHEN; MAO, 2007; LITTLE et al., 2013).

Os efeitos analgésicos característicos do sistema opioide são atribuídos a mecanismos distintos, que são a inibição da enzima adenilato ciclase que leva a redução dos níveis intracelulares de AMPc e consequentemente redução da produção de PGE_2, ativação da via de sinalização analgésica PI3Kγ/AKT/nNOS/NO/K_{ATP}, e inibindo a produção de citocinas e recrutamento de leucócitos. Estes efeitos levam a hiperpolarização dos neurônios nociceptivos periféricos, redução da liberação de neurotransmissores e da excitabilidade neuronal, culminando na redução da hiperalgesia (CLARK et al., 2007; CUNHA et al., 2010; MARTIN et al., 2010; WANG et al., 2005).

Diversos modelos experimentais demonstraram os efeitos anti-inflamatórios do sistema opioide na dor inflamatória/inflamação (CUNHA et al., 2010, 2012; GARLICKI et al., 2006; MARTIN et al., 2010; WENK; BREDERSON; HONDA, 2006). A morfina, por exemplo, um agonista opioide é capaz de induzir analgesia periférica através da ativação da via PI3Kγ/AKT/nNOS/NO/K_{ATP}, provavelmente via receptores periféricos opioides κ inibindo assim a hiperalgesia inflamatória (CAI et al., 2014; CUNHA et al., 2010, 2012; FRANCHI et al., 2012).

Quanto aos efeitos antioxidantes relacionados ao sistema opiode, demonstrou-se que os receptores opioides tem papel importante no controle do dano oxidativo, por serem capazes de inibir a ação de potentes oxidantes como o peroxido de hidrogênio, e também por inibir a peroxidação lipídica e aumentos na concentração de nitritos (CUNHA et al., 2010; SAYASITH; SIROIS; LUSSIER, 2014; SAZONOVA et al., 2016; WU et al., 2015; YIN et al., 2015). A participação de opióides endógenos e seus receptores como substâncias anti-nociceptivas em modelos experimentais de dor articular foi recentemente descrita em articulações temporomadibular e do joelho (GARLICKI et al., 2006; MACEDO et al., 2016a, 2016b; MOON et al., 2016).

Por fim, compostos naturais chamados de flavonoides tem a capacidade de induzir efeitos terapêuticos sobre a dor via ativação do sistema opioide (ANJANEYULU; CHOPRA, 2003; CHEN et al., 2015; DEMIR ÖZKAY; CAN, 2013; DONATO et al., 2014; FILHO et al., 2013; HARA et al., 2014; ZHU et al., 2016). Estes dados representam grandes avanços na busca por novos compostos que possam substituir opioides sintéticos como a meperidina no tratamento da dor

aguda e crônica, para que possam ser utilizados como meios terapêuticos com menos efeitos colaterais e riscos para os pacientes, buscando evitar desta forma o potencial para abuso e vício, prescrições de superdosagens com consequente tolerância anti-nociceptiva e outros efeitos adversos como hiperalgesia induzida por opioides, dependência física, depressão respiratória e constipação.

1.2 Tratamento

O tratamento da artrite gotosa possui três abordagens principais, inicialmente baseadas em um melhor estilo de vida que visa o controle da ingesta de alimentos ricos em ácido úrico, utilização de fármacos que diminuem a uricemia e utilização de anti-inflamatórios para diminuir o quadro inflamatório de artrite gotosa (CHOI et al., 2004a, 2004b).

As aboradagens atuais incluem os anti-inflamatórios não esteroidais (AINES), glicocorticoides e colchicinas (RIDER; JORDAN, 2010). A utilização dos AINES é para o alivio dos sintomas do processo inflamatório, um dos AINES mais utilizados para o tratamento da artrite gotosa é o ibuprofeno, caracterizado por ser um inibor das enzimas ciclo-oxigenases (COXs), atuando sobre a inibição de prostaglandinas inflamatórias como a PGE_2 (SCHLESINGER, 2014b). Contudo, estes fármacos apresentam elevados efeitos colaterais como, por exemplo, perda de função renal, retenção de líquidos, gastro e hepatopatia e função cognitiva prejudicada (GONZALEZ; MILLER; AGUDELO, 1994).

As prostaglandinas estão envolvidas em diversos processos, tanto fisiológicas como patológicos: vasodilatação ou vasoconstrição (FOUDI et al., 2017), contração ou relaxamento da musculatura brônquica ou uterina (KUTYREV et al., 2017), hipotensão (NAKANO et al., 2016), ovulação (TANG et al., 2017), aumento do fluxo sanguíneo renal (SRIVASTAVA et al., 2014), proteção da mucosa gástrica (com aumento da secreção do muco protetor), resposta imunológica e hiperalgesia (VIEIRA et al., 2016), dentre outras funções. Todavia, o fármaco colchicina, mesmo sendo um dos principais medicamentos utilizados para o tratamento da gota, apresenta severos efeitos gastrointestinais adversos e elevadas taxas de toxicidade que aumentam em pacientes idosos, como doenças hepáticas ou renais (AGUDELO; WISE, 1998).

Outra abordagem terapêutica para o tratamento da artrite gotosa é a utilização de glicocorticoides que apresentam uma eficácia significativa principalmente em pacientes com doenças renais crônicas (NEOGI, 2011; TERKELTAUB, 2003). Uns dos principais glicocorticoide utilizados para o tratamento da artrite gotosa é a prednisona, o qual atua bloqueando a liberação do ácido araquidônico por meio das liporcotina que por sua vez atua inibindo a Fosfolipase A2, desta forma culminando na inibição da produção tanto de prostaglandinas quanto de leucotrienos (Jurgens et al. 2013), sendo indicado para tratamento de curto prazo, uma vez que a utilização dos AINES pode ser contraindicada.

As utilizações de fármacos que atuam regulando os níveis de ácido úrico tornam-se importante abordagem terapêutica, uma vez que diminuem sua produção (drogas uricostáticas) ou que aumentem a sua excreção renal (drogas uricosúrias, como por exemplo, a probenicida) (TERKELTAUB, 2016). O alupurinol é um dos fármacos mais utilizados com capacidade de diminuir a produção de ácidos úricos no sangue, atua inibindo a enzima xantina oxidase, apresentando baixos efeitos colaterais (ANZAI et al., 2008; TERKELTAUB, 2003).

Entretanto, este fármaco apresenta algumas limitações, como o desenvolvimento de hipersensibilidade em torno de 2% dos pacientes (BECKER et al., 2005) e em cerca de 20% ocorre intolerância ou refratariedade para a droga (ZHANG et al., 2006). Porem no ano de 2009, a agência *Food and drug Administration* (FDA) aprovou a utilização de um novo inibidor da xantina oxidase, febuxostate, o qual apresenta efeitos colaterais menores quando comparados ao alopurinol (NEOGI, 2011).

Os fármacos uricosúricos apresentam como função a prevenção da reabsorção do ácido úrico pelos rins, o que favorece a sua excreção. Os principais alvos terapêuticos que apresentam essa função são as drogas probenecida e benzbromarone (ENOMOTO et al., 2002). No entanto, esses medicamentos são menos utilizados quando comparados aos inibidores da xantina oxidase, sendo estes contraindicados na presença de algumas doenças como nefrolitíase (REINDERS et al., 2009).

Por fim, um dos tratamentos que tem apresentado maior eficácia é com o uso de agentes anti-citocinas. Isto porque, a participação da citocina IL-1 na patogênese da artrite gotosa tem sido bem abordada, desta forma novos fármacos anti-IL-1 que atuam sobre essa citocina tem sido investigado (CRONSTEIN;

SUNKUREDDI, 2013; NEOGI, 2010; SCHLESINGER, 2014a; SO et al., 2007, 2010; TERKELTAUB et al., 2009).

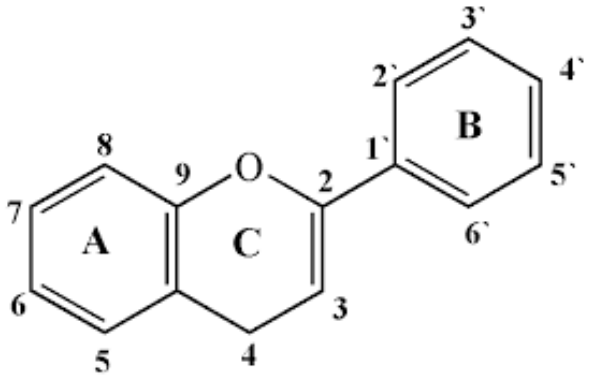
Essa nova abordagem terapêutica visa à inibição da produção desta citocina, de forma a diminuir os quadros de gota aguda. Logo, os fármacos inibidores da ação da citocina IL-1, como anakira um antagonista de receptor IL-1ra, rilonacept e canakinumab têm sido utilizados no âmbito de combater a patogênese desta doença, sendo utilizadas com sucesso tanto para o tratamento quanto prevenção da artrite gotosa (DUMUSC; SO, 2015; FABREGUET; SO, 2012; SO et al., 2007, 2010).

A terapia com imunobiológicos, por sua vez, apresentam alto custo, prejudicando sua viabilidade (KEITH; GILLILAND, 2007). Assim, há necessidade do desenvolvimento de novas abordagens terapêuticas para o tratamento da gota com objetivo de reduzir os efeitos colaterais e o custo do tratamento.

1.3 Flavonoides

Os flavonoides constituem um grupo de compostos fenólicos sintetizados em plantas. Estes compostos podem ser divididos em várias classes de acordo com suas características estruturais (BÖHM et al., 1998). Estas classes são constituídas por flavonas, flavononas, isoflavonas, flavonóis, flavolonolois flavan-3-ois, antocianidinas, chalconas e auronas (BRAVO, 1998; LAGOA et al., 2011), e apresentam mais de dez mil compostos descritos, entretanto, que ainda merecem ser investigados detalhadamente (JIMENEZ et al., 2015). As estruturas básicas dos flavonoides são formadas por 15 átomos de carbono arranjados em três anéis (C6-C3-C6), denominados A, B e C (Figura 2). As diferentes classes de flavonoides diferem no grau de oxidação e padrão de substituição do anel central (anel C), enquanto os compostos da mesma classe diferem no padrão de substituição dos anéis A e B (BÖHM et al., 1998; SADIK; SIES; SCHEWE, 2003).

Figura 2. Estrutura química dos Flavonóides



Os flavonoides são componentes importantes da dieta humana e podem ser encontrados em vegetais, frutas, café, chás e vinho tinto (JIMENEZ et al., 2015; SADIK; SIES; SCHEWE, 2003). Os diferentes tipos de flavonoides podem ser atribuídos às variações estruturais no seu esqueleto básico, os quais podem ocorrer no nível de oxidação ou promovidas por reações de alquilação, glicosilação ou oligomerização (TAHARA, 2007). Desta forma, os flavonoides podem ser encontrados como agliconas, glicosídeos, metilados e/ou acilados (HAVSTEEN, 2002; VEITCH; GRAYER, 2008). Logo, as formas glicosiladas por sua vez sofrem hidrólise para que sejam absorvidos e se tornem biologicamente ativas, desempenhando suas propriedades (VERRI et al., 2012).

Desta forma, a estrutura química dos flavonoides apresentarem um baixo potencial redox e uma alta probabilidade em transferir elétrons nas reações, sugerido assim, o uso potencial destas substâncias como agentes terapêuticos (BÖHM et al., 1998), conferindo aos flavonoides um grande potencial antioxidante, onde suas proprieades biológicas são relacionadas com a sua capacidade em diminuir o estresse oxidativo devido a sua capacidade de neutralizar as espécies reativas de oxigênio celulares e mitocondriais (Sadik et al. 2003; Sánchez et al. 2006).

As características estruturais dos flavonoides são requisitos determinantes para as suas propriedades biológicas, que estão relacionadas à ligação dupla 2,3 em conjugação com a função 4-oxo e a presença de substituinte hidroxila (OH) nos carbonos 5, 7 e 3 e no anel B nos carbonos 3’ e 4’ (grupo catecol) que está relacionado com a capacidade dos flavonoides em doarem elétrons aos radicais livres, permitindo a estabilidade estrutural desta molécula, portanto,

neutralizando radicais livres (BURDA; OLESZEK, 2001; HEIM; TAGLIAFERRO; BOBILYA, 2002; LEMAŃSKA et al., 2001; SUGIHARA et al., 1999).

Os flavonoides cabem assegurar propriedades antioxidante, anti-inflamatória, analgésica, anticancerígena e antimicrobiana (BÖHM et al., 1998; DUTHIE; CROZIER, 2000; IWATA et al., 1995; KIM et al., 2013; LIN; SHIH, 2014; PIETTA, 2000; VERRI et al., 2012; YANG et al., 2011). Há evidências que demonstram que os flavonoides são capazes de inativar fatores de transcrição nucleares como NF-κB, que está relacionado como um dos principais mecanismos responsáveis pela ação anti-inflamatória dos compostos, entretanto, estudos demonstraram que alguns flavonoides como a Baicalina, são capazes de atuar de maneira independente do NF-κB, ligando-se a quimiocinas como IL-8, MIP-1β e MCP-2 e limitando sua atividade biológica (GUO; BRUNO, 2015; LI et al., 2000).

Desta forma, os flavonoides por apresentarem propriedades antioxidantes, atuando na neutralização direta de espécies reativas de oxigênio (GEORGE et al., 2016), quelação de metais (CASAGRANDE et al., 2006), aumento de antioxidante endógenos (BARCELOS et al., 2011; PENG et al., 2015), ativação de genes que codificam enzimas antioxidantes (KUMAR et al., 2011) e inibição de oxidases (CAMPANINI et al., 2013), também podem reduzir a resposta inflamatória, por exemplo, por reduzir a produção de diferentes citocinas e a atividade de enzimas como MPO e MMP-9 (BAI et al., 2014; CHU et al., 2016; KOOSHA et al., 2016; LIN; SHIH, 2014).

Os flavonoides podem reduzir a produção de citocinas por inibir a sinalização do NFκB e/ou a atividade de NADPH oxidase (isoforma NOX_2) (BUI et al., 2014; CAMPANINI et al., 2013; DOU et al., 2013; PINHO-RIBEIRO et al., 2016a, 2016b). Outros estudos relatam que flavonoides podem inibir MAP quinases como ERK, p38 e ou JNK e, consequentemente, reduzir a produção de mediadores inflamatórios e espécies reativas de oxigênio (LIU et al., 2016; TSAI et al., 2014; YANG et al., 2011).

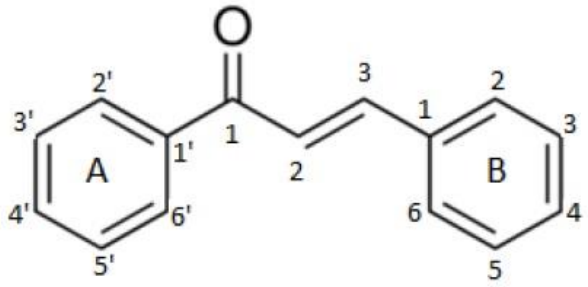
Desta forma, os efeitos dos flavonoides podem variar de acordo com a concentração utilizada e estrutura conformacional dos flavonoides, que por sua vez pode atuar inibindo diferentes vias de sinalização intracelular, assim como interferir na ação de diferentes biomoléculas que participam do estresse oxidativo e inflamação. Entretanto, nem todos os flavonoides apresentam os mesmo efeitos biológicos, logo, faz-se necessário novis estudos *in vivo* e *in vitro* que abordem

diferentes classes de flavonoides em diferentes modelos experimentais a fim de compreender melhor cada molécula.

1.3.1 Trans-chalcona

As chalconas, conhecidas quimicamente como 1,3-difenil-2-propen-1-ona (Figura 3) são precursores intermediários na biossíntese dos flavonoides, presentes de maneira significativa em frutas, vegetais e plantas de uso terapêutico (DUTHIE; CROZIER, 2000; NOWAKOWSKA, 2007; YADAV et al., 2011). A forma trans do composto chalcona é considerada a mais estável termodinamicamente, sendo desta forma, o isômero mais abundante nas plantas (HIJOVA, 2006).

Figura 3. Representação esquemática da estrutura do flavonoide Trans-Chalcona.



As chalconas apresentam uma estrutura química conhecida como cetona α-β-insaturadas, com presença da ligação dupla 2,3 em conjugação com a função 1-oxo conferindo características importantes para a suas atividades biológicas (HIJOVA, 2006; THAKKAR; VON; BOLLIGER, 2012).

Entre as propriedades biológica conferida a estrutura cetonas α-β-insaturada é possível citar propriedades anticancerígena (IWATA et al., 1995; LAHSASNI; AL KORBI; ALJABER, 2014), anti-inflamatórias (SINGH et al., 2016; YAMAZAKI; KAWANO; NAGASHIMA, 2012) e antioxidantes (KARKHANEH et al., 2016; SIKANDER et al., 2011; YADAV et al., 2011). Caso haja uma redução da dupla ligação das chalconas ocorre indução de perda parcial ou total dos seus

efeitos biológicos (FUNAKOSHI-TAGO et al., 2010; SINGH; ANAND; KUMAR, 2014).

Diversos autores relataram os efeitos biológicos da Trans-Chalcona em diversos modelos experimentais. Por exemplo, o tratamento com trans-chalcona foi capaz de reduzir de maneira significativa o estresse oxidativo induzido por modelo de aterosclerose em camundongos, elevando assim os níveis de enzimas antioxidantes (KARKHANEH et al., 2016). Outros autores evidenciaram que o tratamento com trans-chalcona foi capaz de inibir a atividade de enzima como mileoperoxidase (MPO), tanto como a expressão de TNFα e IL-6 além de inibir a translocação do NFκB para o núcleo no modelo de colite induzida por ácido trinitrobenzeno sulfônico em ratos (PARK et al., 2012).

Estudos também demonstram que a trans-chalcona promove efeito hepatoprotetor em modelo de injuria hepática induzida por CCL_4 em ratos reduzindo os níveis de enzimas hepáticas como AST, ALT e ALP, inibindo produção de citocinas pro-inflamatórias como TNFα, e promovendo efeito antioxidante sobre a produção de GSH, e reduzindo os níveis de peroxidação lipídica em fígado de ratos (SINGH et al., 2016).

A trans-chalcona também foi capaz de inibir a neovascularização da retina por meio da inibição da expressão do fator de crescimento do endotélio vascular (VEGF) e moléculas de adesão intercelular, como o (ICAM-1). Neste trabalho, estes efeitos foram relacionados à inibição da ativação dos fatores de transcrição NFκB e STAT3 (LAMOKE et al., 2011).

Desta forma, a conformação da trans-chalcona em cetona α,β-insaturada foi atribuída como responsável pelos efeitos anti-inflamatórios, pelo fato de ser capaz de inibir a produção de mediadores inflamatório como TNFα e NO em macrófagos estimulados com lipopolissacarideo (LPS) (YAMAZAKI; KAWANO; NAGASHIMA, 2012). Além disso, a conformação também é responsável por ativar vias antioxidantes como Nrf2/ARE e consequentemente indução de enzimas antioxidantes e de detoxificação em cultura de células (FORESTI et al., 2005; KUMAR et al., 2011).

Perante esses dados, o flavonoide trans-chalcona, por apresentar mecanismos capazes de inibir processos inflamatórios e estresse oxidativos, torna-se um grande candidato para combater doenças como artrite gotosa, considerando

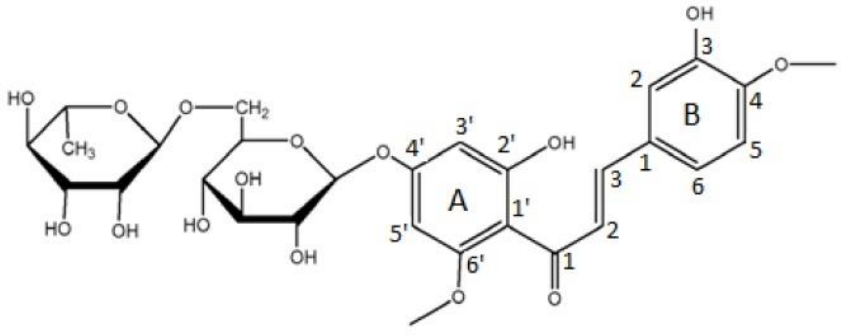
que a patogênese desta doença envolve a ativação de vias de sinalizações como NFκB, e geração de espécies reativas de oxigênio.

1.3.2 Hesperidina Metil-chalcona

A hesperidina é um flavonoide pertencente a classes das flavanonas (Figura 4) encontrada em grandes quantidades em frutas cítricas. Desta forma, frutas e seus sucos são a maior fonte de flavanonas na dieta humana (JUSTESEN; KNUTHSEN; LETH, 1997). A hesperidina apresenta propriedades antioxidantes e anti-inflamatórias (GALATI et al., 1994; PARHIZ et al., 2015), contudo apresenta uma baixa absorção intestinal de maneira semelhante aos outros flavonoides.

Assim, o processo de metilação dos flavonoides tem como objetivo aumentar a estabilidade metabólica, biodisponibilidade oral e distribuição tecidual, além da inibição da proliferação de células cancerígenas (WALLE, 2007). A metilação da flavanona hesperidina em condições alcalinas promove a formação da hesperidina metil-chalcona, uma chalcona que apresenta melhor resistência metabólica e distribuição quando comparadas à flavanona hesperidina (GIL-IZQUIERDO et al., 2001).

Figura 4. Representação esquemática da estrutura do flavonoide Hesperidina Metil Chalcona.



Diversos trabalhos demonstram as propriedades biológicas relacionadas a hesperidina metil chalcona.Tais como atividade vasoprotetora que são encontrada em medicamentos usados para tratamento de insuficiência venosa

crônica como Cyclo 3 Fort®, melhorando a qualidade de vida desses pacientes (ALLAERT et al., 2011; BELTRAMINO; PENENORY; BUCETA, 2000).

Outros estudos demonstraram que o tratamento prolongado com a hesperidina metil chalcona em associação com outros fármacos como ácido ascórbico ou *Ruscus aculeatus* durante 12 semanas são capazes de reduzir os sintomas clínicos e a insuficiência venosa crônica (ALLAERT, 2016; GUEX et al., 2009, 2010).

Em estudos mais recentes, pesquisadores demonstraram diversos outros efeitos relacionados à hesperidina metil-chalcona, como efeitos antioxidantes e anti-inflamatórios em diferentes modelos experimentais. Em modelo de inflamação e estresse oxidativo induzido por radiação UVB em camundongos, hesperidina metil chalcona foi capaz de reduzir de maneira significativa a produção de mediadores inflamatórios como IL-1β, TNFα, IL-6 e IFN-γ, e promover atividade antioxidante como na inibição da depleção de moléculas antioxidante, e também a redução da expressão de genes envolvidos no estresse oxidativo como $gp91^{phox}$ (MARTINEZ et al., 2015).

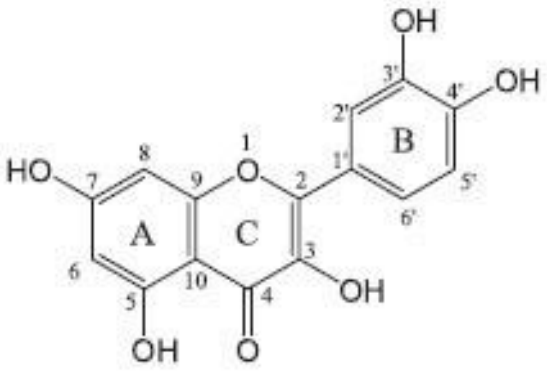
A hesperidina metil chalcona também atua sobre vias de sinalização como a do NFκB, inibindo-as. Desta forma, diminuindo a produção de mediadores inflamatórios como IL-1β, TNFα e IL-6, e promovendo um perfil antioxidante em diferentes modelos de dor inflamatória, como a da carragenina e Adjuvante completo de Freund (PINHO-RIBEIRO et al., 2015).

De maneira importante, o tratamento prolongado com a hesperidina metil chalcona em humanos mostrou-se segura em pacientes que receberam 150 mg por um período de 90 dias (BELTRAMINO; PENENORY; BUCETA, 2000). Ademais adordagens com elevadas doses de até 15 g por dia (KIRTLEY; PECK, 1948), não demonstraram efeitos citotóxicos *in vitro (KIM; LEE; MOON, 2013)*. Em modelos animais, o tratamento por 7 dias por via intraperitoneal também não apresentou atividades gastro ou hepatotóxica, demonstrado por manutenção dos níveis das enzimas AST e ALT no fígado e MPO no estômago (PINHO-RIBEIRO et al., 2015).

1.3.3 Quercetina

A quercetina é um flavonoide conhecido quimicamente por 3,3',4',5,7 – pentahidroxiflavona (Figura 5). É o principal flavonoide presente na dieta humana, com seu consumo diário podendo variar entre 50 a 500 mg/dia (DESCHNER et al., 1991). Logo, 95% do total dos flavonóides ingeridos podem ser encontrados principalmente em frutas e vegetais como maçã, cebola e brócolis (KIDD, 2009; KIM; LEE; JEON, 2015; NIJVELDT et al., 2001).

Figura 5. Representação esquemática da estrutura do flavonoide Quercetina.



O flavonóide quercetina é um antioxidante geralmente encontrado nos alimentos na forma glicosilada, às vezes, como β-glicosidase. A natureza da glicosilação é conhecida por influenciar a eficiência de sua absorção, desta forma, tornando os efeitos antioxidantes da quercetina mais eficazes (CRESPY et al., 1999). Este efeito pode ser devido a suas propriedades em sequestrar radicais livres, assim como quelar íons metálicos promovendo proteção aos tecidos contra os radicais livres e consequentemente peroxidação lipídica. Nesta atividade antioxidante a atuação ocorre sobre o radical hidroxil (•OH) e o ânion superóxido ($•O2^-$), que são espécies altamente reativas envolvidas na peroxidação lipídica (CHOI et al., 2002).

Diversos outros mecanismos antioxidantes têm sido propostos para os flavonoides, que podem atuar diminuindo a formação de espécies reativas do

oxigênio pela inibição do sistema enzimático responsável pela geração de radicais livres (ciclooxigenase, lipoxigenase ou xantina oxidase), quelação de íons metálicos que podem iniciar a produção de radicais hidroxil pela Reação de Fenton ou Harber-Weis, sequestro de radicais livres, regulação positiva ou proteção das defesas antioxidantes por induzir a fase II de enzimas como glutationa transferase que aumenta a excreção de espécies oxidadas ou indução de enzimas antioxidantes como a metalotioneína que é uma proteína queladora de metais, com propriedades antioxidantes (MIDDLETON; KANDASWAMI; THEOHARIDES, 2000; PIETTA, 2000).

Além das propriedades antioxidantes outros efeitos anti-inflamatórios têm sido conferidos a esta molécula. Diversos autores têm demonstrado que a quercetina pode atuar inibindo enzimas como fosfolipase A2, responsável pela hidrólise de fosfolipídeos presentes nas membranas celulares, com a liberação do ácido araquidônico (LEE; MATTELIANO; MIDDLETON, 1982). A quercetina tambem atua sobre enzimas como ciclooxigenase e a lipoxigenase, impedindo a formação das prostaglandinas e leucotrienos, diminuindo assim os processos inflamatórios (SOBOTTKA et al., 2000).

Estudos prévios têm demonstrado que a quercetina pode atuar sobre diversas vias de sinalização inflamatórias, diminuindo a atividade de fatores de transcrições como NFκB (CHEN et al., 2005) consequentemente diminuindo a produção de mediadores inflamatórios como as citocinas IL-1β e TNFα (VALÉRIO et al., 2009). Tambem foi evidênciado à inibição da ativação do inflamassoma NLRP3 em diversos modelos como de injuria medular e lesões hepaticas (CAI et al., 2016; ZHANG et al., 2015).

Assim, de acordo como essas informações, ambos os mecanismos mencionados acima fazem parte na patogênese da artrite gotosa induzida por cristais de urato monossódico, responsável pelo desenvolvimento de todo o processo inflamatório assim como o quadro doloroso característicos desta doença. Frente a esses dados, a quercetina torna-se uma potente abordagem terapêutica para o tratamento da artrite gotosa.

2 OBJETIVOS

2.1 Objetivo Geral

O presente estudo teve como objetivo investigar os efeitos anti-inflamatórios, antioxidantes e analgésicos dos flavonoides Trans-Chalcona (TC), Hesperidina Metil Chalcona (HMC) e Quercetina (QC) em modelo de artrite gotosa induzida pela injeção intra-articular de cristais de urato monossódico (MSU) em camundongos Swiss além de investigar se os efeitos da quercetina eram dependentes de receptores opioides *in vivo*. O efeito *in vitro* da QC e HMC foi investigado em macrófagos derivados da medula (BMDMs).

2.2 Objetivos Específicos

2.2.1 Flavonoides Trans-Chalcona e Hepseridina Metil Chalcona em modelo de artrite gotosa induzida por cristais de ácido úrico (MSU) em camundongos Swiss avaliando:

- Hiperalgesia mecânica e edema por meio de versão eletrônica de von Frey e paquímetro, respectivamente;
- Migração leucocitária na cavidade articular por meio de contagem em câmara de neubauer;
- Infiltrado leucocitário no tecido articular (sinovite) por meio de corte histológico com coloração por H/E;
-Atividade antioxidante (GSH, FRAP, ABTS, NBT) e produção de NO por reações colorimétricas;
-Expressão de RNAm para moléculas (gp91phox, Nrf2 e HO-1) envolvidas no mecanismo atividade antioxidativo por RT-qPCR;
-Produção de citocinas pró-inflamatórias como IL-1β, TNFα e IL-6, e anti-inflamatórias como IL-10 e TGF-β por ELISA;
-Ativação do fator de transcrição NFκB por ELISA;
-Expressão de RNAm para moléculas do inflamassoma (NLRP3, ASC, Pro-caspase 1e Pro-IL-1β) por RT-qPCR;

2.2.2 Quercetina em modelo *in vivo* de artrite gotosa induzida por cristais de urato monossódico (MSU) em camundongos Swiss avaliando:

-Hiperalgesia mecânica por meio de versão eletrônica de von Frey;

-Migração leucocitária na cavidade articular por meio de contagem em câmara de neubauer;

- Infiltrado leucocitário no tecido articular (sinovite) por meio de corte histológico com coloração por H/E;

-Produção de citocinas pró-inflamatórias TNFα e IL-1β por ELISA;

-Ativação do fator de transcrição NFκB por ELISA;

-Expressão de RNAm para moléculas do inflamassoma (NLRP3, ASC, Pro-caspase 1e Pro-IL-1β) por RT-qPCR;

-Atividade antioxidante (GSH, FRAP, ABTS, NBT) por reações colorimétricas e RNAm para gp91phox por RT-qPCR;

-Expressão de RNAm para moléculas envolvidas na atividade antioxidante (Nrf2 e HO-1)por RT-qPCR;

- Atividade anti-inflamatória, antioxidante e analgésica depentendes de receptores opioides por meio do tratamento com naloxona.

2.2.3 Hesperidina Metil Chalcona e Quercetina em modelo *in vitro* de inflamação em macrófagos derivados da medula (BMDMs) com cristais de urato monossódico (MSU) avaliando:

-Níveis de IL-1β madura em sobrenadante de macrófagos derivado da medula (BMDMs) por ELISA;

-O efeito da quercetina era dependente de receptores opioides por meio do tratamento com naloxona.

3 PRODUÇÃO CIENTÍFICA

ARTIGO 1- Trans-Chalcone inhibits gout arthritis by targeting NLRP3 Inflammasome and NFκB activation in mice

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Trans-Chalcone inhibits gout arthritis by targeting NLRP3 Inflammasome and NFκB activation in mice

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Table of Contents Graphics

Trans-Chalcone attenuates gouty arthritis by targeting NLRP3 inflammasome and NFκB activation in mice

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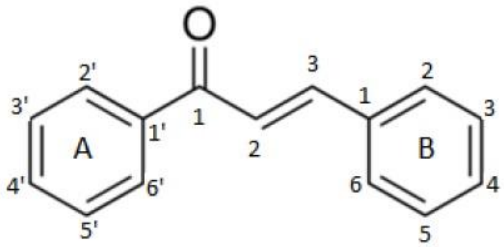
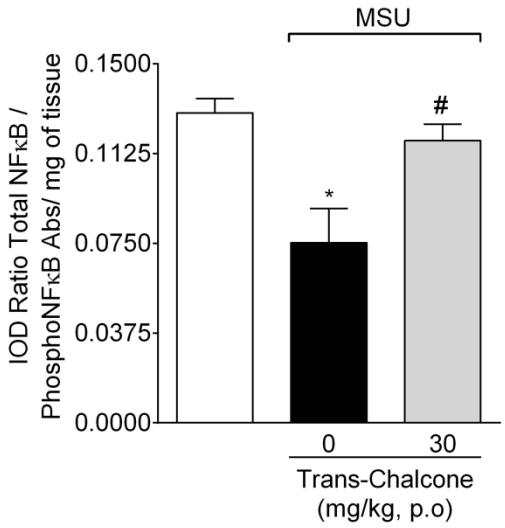


trans-chalcone



ABSTRACT

Trans-chalcone (1,3-diphenyl-2-propen-1-one) is a flavonoid precursor presenting biological activities such as anti-inflammatory proprieties. Gout arthritis is characterized by intense inflammatory response triggered by monosodium urate crystal (MSU), provoking a painful inflammatory disease leading to reduced life quality of patients. The aim of this work was to evaluate the protective effects of trans-chalcone in gout arthritis model. Mice were treated with trans-chalcone (3, 10 and 30 mg/kg, per orally) or vehicle (saline) 1h before MSU (100 μg/kg) intra-articular administration. Trans-chalcone inhibited MSU-induced joint mechanical hyperalgesia, edema and leukocyte recruitment (total leukocytes, mononuclear cells and neutrophils) in a dose-dependent manner. Trans-chalcone also inhibited MSU-induced synovitis. The anti-inflammatory and analgesic effects of trans-chalcone were related to inhibition of MSU-induced IL-1β, TNF-α and IL-6 production, maintaining IL-10 levels and increase TGF-β production, enhancement of antioxidant parameters (ferric reducing ability, scavenge of free radical ABTS, maintained GSH levels), inhibition of oxidative stress (superoxide anion production and nitric oxide) and nitric oxide production (NO). Furthermore, trans-chalcone inhibited MSU-induced NF-κB activation, and $gp91^{phox}$, NLRP3, ASC, pro-caspase-1 and pro-IL-1β mRNA expression as well as enhanced Nrf2 and HO-1 mRNA expression. Concluding, trans-chalcone inhibited MSU-induced inflammation and pain targeting inflammasome system, cytokine production and oxidative stress by inhibiting the activation of the central inflammatory transcription factor NF-κB. Therefore, trans-chalcone is a promising molecule for the treatment of gout arthritis that merits further pre-clinical and clinical investigation.

Gout arthritis is an inflammatory disease caused by high levels of uric acid in the blood with deposition and crystallization of monosodium urate (MSU) in the articular and periarticular

tissues. MSU crystals triggers an intense inflammatory response with the participation of synoviocytes, mast cells, neutrophils, and monocytes/macrophages provoking severe, painful and disabling disease in the articular joint reducing patient´s quality of life.[1,2]

MSU crystals are capable of triggering the maturation of IL-1β. MSU crystals induce the rupture of lysosome releasing cytochalasin, which induces the assembling of a cytosolic complex called nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3 (NLRP3) inflammasome. This complex involves a receptor NLRP3, apoptosis-associated speck-like protein (ASC) that links NLRP3 to pro-caspase-1. Upon agonist binding, there is the assembling of the complex and activation fo pro-caspase-1 that cleaves pro-IL-1β into mature IL-1β.[3] In agreement with the importance of this inflammasome-dependent mechanism in gout arthritis, targeting IL-1β is a novel therapeutic strategy for gout arthritis.[4] In fact, treatment with IL-1 receptor antagonist reduces gout arthritis-induced pain and inflammation.

The inflammasome activation is also called signal 2. Signal 1 is related to the activation of the transcription factor NFκB, which contributes to enhancing the machinery to signal 2.[5] Furthermore, phagocytosis of MSU crystals induces reactive oxygen species (ROS) production such as hydrogen peroxide (H_2O_2) and superoxide radicals ($O2^{-\bullet}$). ROS can both activate signal 1 and signal 2 supporting their importance in gout arthritis by regulating NFκB and NLRP3 inflammasome activation.[6–8]

Flavonoids are natural compounds and represent a major subset of polyphenols widely distributed in vegetables, fruits, and herbs.[9,10] In general, evidence supports that flavonoids present varied biological activity such as antioxidant, anti-allergic, antitumoral, antimicrobial, anti-inflammatory and analgesic.[10,11] Trans-chalcone (1,3-diphenyl-2-propen-1-one) is the precursor of flavonoids in plants.[12] Trans-chalcone has been previously shown to promote vascular homeostasis by modulating the expression of different cytokines involved in acute

inflammatory responses.[13,14] Other studies with trans-chalcone reported the antioxidant activity by delaying the consumption of GSH and/or other cellular antioxidants in hepatocellular carcinoma (HepG2) Cells.[15] Trans-chalcone also has anti-inflammatory effects. Trans-chalcone inhibited carbon tetrachloride-induced liver inflammatory lesion by reducing TNFα production in rats.[16] Furthermore, trans-chalcone inhibited NFκB activation in a model of ischemic retinopathy in retinal neovascularization (RNV). NFκB is an important transcription factor in the regulation of pro-inflammatory genes.[14]

Considering the recent evidence on the biological activities of trans-chalcone, the effect and mechanisms of trans-chalcone in MSU-induced gout arthritis were investigated.

Results and Discussion

Trans-chalcone inhibits MSU-induced mechanical hyperalgesia and edema in a dose-dependent manner. The first set of experiments addressed whether trans-chalcone would inhibit MSU-induced knee joint mechanical hyperalgesia and edema (Figure 1). Mice were treated with trans-chalcone (3, 10 or 30 mg/kg/, p.o) 1h before MSU (100 µg/10 µL) i.a injection. Mechanical hyperalgesia (Figure. 1A) and edema (Figure 1B) were evaluated at indicated time points. MSU administration induced significant mechanical hyperalgesia, which was not affected by 3 mg/kg of trans-chalcone. On the other hand, 10 mg/kg of trans-chalcone inhibited MSU-induced mechanical hyperalgesia between 5-15 h (up to 20%) with significant statistical differences compared to the lower dose of trans-chalcone. The dose of 30 mg/kg of trans-chalcone inhibited MSU-induced mechanical hyperalgesia between 3-15 h (up to 36%), with significant statistical differences compared to the lower doses of trans-chalcone (Figure 1A). Trans-chalcone also inhibited MSU-induced knee joint edema at the dose of 30 mg/kg (61%) between 5-15 h. The dose of 10 mg/kg of trans-chalcone inhibited MSU-induced edema only at 5 h, and 3 mg/kg of trans-chalcone presented no significant

effect on MSU-induced edema (Figure 1B). Supporting the present data, other chalcones from Allyl isothiocyanate (mustard oil) induce TRPA1 desensitization, resulting in analgesic effect.[17] Flavonoids such as quercetin, naringenin and hesperidin methyl-chalcone present analgesic effects in other models of inflammation.[18–23] Flavonoids such as cardamonin, hesperidin methyl-chalcone, other chalcones (1,3-diarylprop-2-en-1-one) and licochalcone A also inhibit edema induced by stimuli such as carrageenan[24], complete Freund´s adjuvant[20], formalin[25], arachidonic acid (AA) and 12-O-tetradecanoylphorbol 13-acetate (TPA).[26] Thus, to our knowledge, this is the first study demonstrating that trans-chalcone inhibits MSU-induced knee joint mechanical hyperalgesia and edema.

Trans-chalcone inhibits MSU-induced Leukocytes Recruitment. Intense recruitment of inflammatory cells into the joint cavity is a physiopathological characteristic of gout arthritis.[27] Thus, the effect of trans-chalcone on MSU-induced leukocyte recruitment was evaluated. Mice were treated with trans-chalcone (3, 10 and 30 mg/kg, p.o.) 1h before MSU i.a injection. Total leukocyte (Figure 2A), neutrophils (Figure 2B) and mononuclear cells (Figure 2C) counts were determined 15 h after stimulus injection. MSU -induced total leukocytes, neutrophils and mononuclear cells recruitment to the knee joint cavity were not affected by 3 and 10 mg/kg of trans-chalcone except by 10 mg/kg that inhibits mononuclear cells recruitment. Nevertheless, the dose of 30 mg/kg of trans-chalcone inhibited MSU-induced total leukocytes, neutrophils and mononuclear cells recruitment (up to 79, 80 and 74%, respectively). Therefore, the dose of 30 mg/kg of trans-chalcone and the 15 h time point were selected for the next experiments. The pathophysiology of gout arthritis involved the activation of tissue resident macrophage-like synoviocytes that produce IL-1β resulting in the recruitment of leukocytes to the joint cavity.[28–30] MSU crystals are toxic to neutrophils, which release lysosomal contents enhancing tissue destruction and inflammation.[31,32] Neutrophils

also produce ROS further enhancing signals 1 and 2 to increase inflammatory cells activation.[33] Mononuclear cells including monocytes are activated to macrophages and amplify IL-1β production.[34,35] Neutrophils also contribute to hyperalgesia, thus, reducing neutrophil recruitment might be a contributing mechanism for trans-chalcone analgesic effect.

Trans-chalcone reduces MSU-induced synovitis. Mice were treated with trans-chalcone (30 mg/kg) (Figure 3C) 1h before MSU (100 μg/10 μL) i.a. stimulus, and after 15 h the articular joint samples were collected for histological analysis. Knee joint slices were stained with hematoxylin and eosin (magnification A-C, ×400). MSU induced knee joint synovitis characterized by increase of number of leukocytes into the synovial tissue (Figure 3B), mainly neutrophils when compared with control (Figure 3A), which are responsible to provoke an intense inflammatory due promote an inflammatory mediators productions leading to synovitis profile. Trans-chalcone treatment inhibited by 80% MSU-induced synovitis (Figure 3D). Therefore, in addition to marked reduction of clinical signs of gout arthritis (pain and edema), trans-chalcone treatment also inhibited MSU-induced histological pathological alterations.

Trans-chalcone inhibits MSU-induced oxidative stress. Free radicals belonging to reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by activated neutrophils and macrophages in the knee joint cavity. These ROS and RNS contribute to tissue damage and boosting of inflammation. Therefore, oxidative stress is a crucial feature of gout arthritis accounting to disease progression. Flavonoids are recognized by their antioxidant effect, therefore, it was reasonable to investigate whether trans-chalcone would affect MSU-induced inflammatory oxidative stress. Knee joint samples were collected 15 h after MSU stimulus in mice treated with trans-chalcone, and analysed for reduced

gluthathione (GSH) levels, ferric reducing ability (FRAP), ability to scavenge the cationic free radical ABTS (ABTS), superoxide anion production (nitroblue tetrazolium reduction, NBT) and NO levels by the Griess method (Figure 4). Trans-chalcone inhibited MSU-induced reduction of GSH levels (Figure 4A), FRAP (Ferric Reducing Ability (86%) (FRAP assay; Figure 4B), free radical scavenging ability (45%) (ABTS assay; Figure 4C), nitroblue tetrazolium reduction ability (100%) (NBT assay; Figure 4D) and Nitric Oxide (100%) (NO assay; Figure 4E). Therefore, trans-chalcone treatment inhibited MSU-induced decrease of antioxidant defenses (GSH, FRAP and ABTS) as well as inhibited the enhancement of ROS and RNS oxidative stress including superoxide anion and nitric oxide production, respectively. However, it is important to highlight that trans-chalcone does not present the common antioxidant chemical groups of flavonoids.

Antioxidant capacity of phenolic compounds is determined by their structure, in particular by hydroxyl which can donate electrons and as a result supports the relocation around the aromatic system. Another important structural determinant is the antioxidant capacity of flavonoids attributed to hydroxyl C4 and C3, which act to increase the antioxidant potential.[36] Therefore, the current chemical understanding of flavonoid structure-antioxidant relationship does not suggest trans-chalcone as an antioxidant. Thus, the present data is not totally expected and suggests that the antioxidant effect of trans-chalcone can be a result of anti-inflammatory actions.

Trans-Chalcone inhibits MSU-induced gp91^{phox}, Nrf2 and HO-1 mRNA expression. One of the main sources of superoxide anion during inflammation is NADPH oxidase present in infiltrating phagocytes such as neutrophils and macrophages.[37] Superoxide anion is an important mediator of inflammation since induces pain, leukocyte recruitment, edema, and depending on its levels tissue lesion via damaging DNA, RNA, lipid membranes and

proteins.[22,38–41] Treatment with trans-chalcone inhibited MSU-induced gp91^{phox} mRNA expression (Figure 5A), which is a subunit of NADPH oxidase. Thus, it is reasonable that trans-chalcone inhibition of MSU-induced superoxide anion production depends at least in part in reducing NADPH oxidase components mRNA expression. Furthermore, MSU did not induce significant alteration of Nrf2 (Figure 5B) and HO-1(Figure 5C) mRNA expression 15 h after its injection. However, trans-chalcone treatment induced a significant increase of mRNA expression of Nrf2 and its downstream target HO-1. These results reinforce the concept that trans-chalcone is not solely an antioxidant molecule due to chemical groups, but rather that trans-chalcone has *in vivo* activity to induce the mRNA expression of the transcription factor Nrf2, which induces the expression of for instance HO-1 and GSH resulting in antioxidant effects. Further, trans-chalcone inhibited gp91^{phox} mRNA expression, which also accounts to reduce ROS production.

Trans-chalconce modulates MSU-induced cytokine production. Trans-chalcone inhibited MSU-induced production of pro-inflammatory cytokines IL-1β (28%) (Figure 6A), TNFα (100%) (Figure 6B) and IL-6 (47%) (Figure 6C), did not alter IL-10 levels (Figure 6D) and induced TGF-β (100%) (Figure 6E) production. The interaction of MSU crystals with immune phagocyte cells such as synovial cells, macrophages, and infiltrating leukocytes promote a secretion of various inflammatory mediators such IL-1β[34], TNF-α[42] and IL-6[43] leading to tissue damage.[44]

IL-1β is the pivotal inflammatory mediator responsible to regulate cell proliferation, differentiation, and apoptosis in gout arthritis, in turn, induces the expression of a wide range of pro-inflammatory cytokines and chemokine, which acts on influx of neutrophils into the synovium, a hallmark of gouty arthritis.[45] Furthermore, MSU crystals stimulate the secretion of TNF-α and IL-6 by synovial cells, monocyte-macrophages, and neutrophils, which promote

an intense inflammation.[46] TNFα is reported to promote an increase of adhesion of molecules expression such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) which increase the recruitment of leukocytes into to synovial tissue[42,47], in addition induces a pro-inflammatory activation, maturation, and increased monocyte to macrophage transformation[42] and together with IL-6 cytokine, inflammatory response are amplified[43,48], in turn, neutrophil influx results in further phagocytosis of MSU crystals and the endurance of IL-1β release that trigger membranolysis, generation of oxygen free radical, prostaglandin E2 (PGE2), leukotriene and release of lysosomal enzymes, resulting in inflammation and tissue damage.[49] The TGF-β cytokine present a pivotal role in the resolution of gout arthritis, this anti-inflammatory cytokines is a powerful inhibitor of inflammation[50], is involved in the resolution of gouty inflammation in experiments, in which administration of recombinant TGF-β attenuated MSU crystal-induced inflammation in an in vivo air-pouch model, in addition TGF-β significantly inhibits leukocyte infiltration and suppresses monocyte pro-inflammatory cytokine release in response to MSU crystals, endothelial cell activation in response to monocyte-derived cytokines, and macrophage release of TNF-α.[51]

Moreover, MSU presence induce a oligomerization and dysfunctional activity of NLRP3 inflammasome macromolecules[52], resulting in hyperactivity of caspase-1, in turn, this process causes increased secretion of several inflammatory cytokines triggering a cascade of pro-inflammatory mediators by nuclear factor NFκB activation, leading to endothelial activation and leukocyte recruitment and promoting the gout arthritis development.[35]

Trans-chalcone inhibits MSU-induced NF-κB activation. ROS such as superoxide anion can induce NFκB activation, although this effect does not occur with all ROS in all cells. In an inverse pathway, NFκB activation induces the production of enzymes and cytokines that

contribute to ROS production such as superoxide anion. These enzymes include NADPH oxidase, cyclooxygenase and 5-lipoxigenase, and cytokines such as TNFα and IL-1β. Considering that trans-chalcone inhibited MSU-induced gp91^{phox} mRNA expression and pro-inflammatory cytokine production, it is reasonable to investigate whether this flavonoid inhibited MSU-induced NFκB activation. In fact, trans-chalcone inhibited MSU-induced NFκB activation as observed by a re-establishement of total NFκB/ phosphorylated NFκB ratio by trans-chalcone treatment (Figure 7). In agreement with the present data, trans-chalcone inhibited LPS[14,53] and trinitrobenzenesulfonic acid colitis-induced NFkB activation[54], which are models involving bacteria components and bacteria-dependent inflammation contrasting with the MSU gout arthritis that is not driven by infectious agents. Trans-chalcone also inhibited cancer cell proliferation by targeting NFκB.[55] Thus, the NFκB activation inhibition by trans-chalcone is consistent independently if the inflammatory stimulus is infectious, sterile or neoplasic.

Trans-chalcone inhibits MSU-induced NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β mRNA expression. The activation of NLRP3 inflammasome is a crucial physiopathological mechanism of gout arthritis.[35] Considering that trans-chalcone inhibited IL-1β production and NFκB activation, it is possible that it could affect inflammasome components expression. Trans-chalcone inhibited MSU-induced mRNA expression of NLRP3 (Figure 8A), ASC (Figure 8B), Pro-caspase-1 (Figure 8C) and Pro-IL-1β (Figure 8D) as demonstrated by qPCR. Therefore, although these results do not show a direct effect of trans-chalcone over MSU-induced NLRP3 inflammasome activation, they are still important since demonstrate a clear down regulation of mRNA expression of a crucial macromolecular platform components and reduction of IL-1β production-related to NFκB inhibition.

In conclusion, trans-chalcone inhibited gout arthritis inflammation and pain by reducing cytokine production and oxidative stress. The activity of trans-chalcone was dependent on inducing antioxidant responses and not solely on inhibiting oxidative stress. Importantly, trans-chalcone inhibited MSU-induced NFκB activation, which resulted in the reduction of pro-inflammatory cytokine production as well as reduced the mRNA expression of NLRP3 inflammasome platform. Therefore, trans-chalcone is a conceivable therapeutic approach for the treatment of gout arthritis that deserves further pre-clinical and clinical investigation.

EXPERIMENTAL SECTION

Experimental procedures. Mice were treated with trans-chalcone (3, 10 and 30 mg/kg, p.o or vehicle (saline) 1h before stimulus with MSU (100 µg/10 µL, i.a.), and hyperalgesia was evaluated 1, 3, 5, 7 and 15 h after inflammatory stimulus administration. The dose of 30 mg/kg, p.o. of trans-chalcone was chosen for subsequent experiments. Mechanical hyperalgesia and edema was evaluated at the indicated time points. In another sets of experiments designed to determine the mechanism of action of trans-chalcone, mice were treated with 30mg/kg, p.o of trans-chalcone and stimulated with MSU (100 µg/10 µL, i.a. injection) and the leukocytes migration, cytokines production (IL-1β, TNF-α, IL-6, IL-10 and TGF-β levels), stress oxidative (reduced glutathione (GSH), nitroblue tetrazolium (NBT) reduction, Nitric Oxide (NO), (ABTS [2,2′-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay, ferric reducing (FRAP assay), NF-κB activation (Total and phosphorylated p65), histological analysis and Quantitavive (q)PCR were measured 15h after MSU stimulus.

Animals. Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water with a light/dark cycle of 12/12 h at a constant temperature of

21ºC. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled (21 °C) room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 14600.2013.73). All efforts were made to minimize animal suffering and to reduce the number of animals used.

MSU crystal preparation. MSU crystals were prepared according to the method described previously.[56] In brief, 800mg of monosodium urate was dissolved in 155ml boiling water containing 5ml 1N NaOH. After the pH was adjusted to 7.2, the solution was cooled gradually by stirring at room temperature. The crystals were collected by centrifugation at 3,000 g for 2 min at 4◦C. The crystals were evaporated and sterilized by heating at 180◦C for 2 hours and stored in a sterile environment until use.

Induction of articular joint inflammation. Joint inflammation was induced by administration of MSU (100 μg/10 μL, i.a.) and injected into the right articular joint of mice that were mildly anaesthetized. Control animals received an intraarticular injection of 10 μL sterile saline.

Electronic pressure-meter test. Experiments were performed as described previously. [57] Similar to cutaneous hypernociception measurement, an increasing perpendicular force was applied to the central area of the plantar surface, inducing the flexion of the tibiotarsal joint. A nonnociceptive tip probe with area size of 4.15 mm^2 was used. The results were expressed as the flexion-elicited withdrawal threshold (in grams).

Edema test. The volume of the joint was measured with a caliper (Mitutoyo, Suzano, SP, Brazil) before (zero time) the intra-articular stimulus with MSU, and after the administration at the indicated times on figures. The amount of articular swelling was determined for each

mouse and the difference between the times indicated on figures and the zero time. The edema value was expressed as edema/mm.

Total and differential cell counts. The articular joint was exposed by surgical incision and washed three times with 3.33 µL phosphate-buffered saline (PBS) that contained ethylenediaminetetraacetic acid (EDTA) and was diluted to a final volume of 50 µL with PBS/EDTA to determine total cell counts. The total number of leukocytes, diluted in Turk's solution, was determined in a Neubauer chamber under a light microscope. The results are expressed as the number of leukocytes per cavity.

Histopathological analysis. Mice were sacrificed 15 hours after MSU injection. The articular joint of mice were removed, fixed with 10% paraformaldehydein PBS, and then decalcified for 10 days with EDTA and embedded in paraffin for histological analysis. The paraffin sections were stained with hematoxylin and eosin for conventional morphological evaluation. Dimension used for the analysis was 738 x 927 pixels for analysis (Field) and the arrow indicate representative infiltrate inflammatory cells counted.

GSH levels measurement. Samples of articular joint were collected and maintained at -80 ºC for at least 48 h. The sample was homogenized with 200 µL of 0.02 M EDTA. The homogenate was mixed with 25 µL of trichloroacetic acid 50% and was homogenized three times over 15 min. The mixture was centrifuged (15 min x 1500 g x 4 ºC). The supernatant was added to 200 µL of 0.2 M TRIS buffer, pH 8.2, and 10 µL of 0.01M DTNB. After 5 min, the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific) against a blank reagent with no supernatant. A standard GSH curve was formed. The results are expressed as GSH per mg of protein.[58]

ABTS and FRAP assays. The ability of samples to resist oxidative damage was determined by their free radical scavenging (ABTS [2,2'-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay) and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate format as previously described.[59] Articular tissue samples were collected 15h after MSU i.a injection (100 μg/10μL) and homogenized immediately in ice-cold KCl buffer (500 μL, 1.15% w/v). The homogenates were centrifuged (200 g × 10 min × 4 °C), and the supernatants were used in both assays. Diluted ABTS solution (200 μL) was mixed with 10 μL of sample in each well. After 6 min of incubation at 25 °C, the absorbance was measured at 730 nm. For FRAP assay, the supernatants (10 μL) were mixed with the freshly prepared FRAP reagent (150 μL). The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm (Multiskan GO Thermo Scientific). The results of ABTS and FRAP assays were equated against a standard Trolox curve (0.02 – 20 nmol).

Superoxide anion production. The measurement of superoxide anion production in tissue homogenates (10 mg/mL in 1.15% KCl) was performed using the nitroblue tetrazolium (NBT) assay adapted to a microplate as described previously.[60] The NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). The tissue weight was used for data normalization.

Nitrite production. Samples from articular joint were collected 15 h after MSU injection, homogenized in 500 μL of saline, and nitrite (NO2-) concentration was determined by the Griess reaction as an indicator of nitric oxide (NO) production.[61] Briefly, 100 μL of the homogenate was incubated with 100 μL of Griess reagent for 5 min at 25 °C, and NO2- concentration was determined by measuring the optical density at 550 nM (Multiskan GO, Thermo Scientific) in reference to a standard curve of NaNO2 solution. Results are expressed as μmol of NO2- per mg of tissue.

Quantitative (q)PCR. Quantitative Polymerase Chain Reaction (qPCR). qPCR was performed as previously described.[62] Samples were homogenized in Trizol reagent, and total RNA was extracted by using the SV Total RNA Isolation System (Promega). All reactions were performed in triplicate using the following cycling conditions: 50° C for 2 min, 95°C for 2 min, followed by 40 cycles of 95° C for 15 s and 60° C for 30 s. qPCR was performed in a LightCycler Nano Instrument (Roche, Mississauga, ON, USA) sequence detection system by using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, USA). The mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were Gapdh forward: CAT ACC AGG AAA TGA GCT TG, reverse: ATG ACA TCA AGA AGG TGG TG; Nrf2, forward: TCA CAC GAG ATG AGC TTA GGG CAA, reverse: TAC AGT TCT GGG CGG CGA CTT TAT; $gp91^{phox}$, forward: AGC TAT GAG GTG GTG ATG TTA GTG G, reverse: CAC AAT ATT TGT ACC AGA CAG ACT TGA G; Nlrp3, forward: AGC TAT GAG GTG GTG ATG TTA GTG G, reverse: CAC AAT ATT TGT ACC AGA CAG ACT TGA G; HO-1, forward: CCC AAA ACT GGC CTG TAA AA, reverse: CGT GGT CAG TCA ACA TGG AT; Pro-caspase-1: forward: TGG TCT TGT GAC TTG GAG GA, reverse: TGG CTT CTT ATT GGC ACG AT; Pro-IL-1β, forward: GAA ATG CCA CCT TTT GAC AGT G, reverse: TGG ATG CTC TCA TCA GGA CAG; ASC, forward: ATG GGG CGG GCA CGA GAT G, reverse: GCT CTG CTC CAG GTC CAT CAC. The SYBR green PCR Master Mix was used according to the manufacturer's instructions.

Cytokine measurement. The articular joint samples were homogenized in 500 µL of buffer containing protease inhibitors (1 mM Phenylmethanesulfonyl fluoride, Sigma Aldrich). IL-1β, TNF-α, IL-6, TGF-β and IL-10 levels being determined as previously described by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits.[58]

The results are expressed as picograms (pg) of cytokine/100 mg of tissue.

NF-κB activation. The articular joint samples were collected and homogenized in ice-cold lysis buffer (Cell Signaling). The homogenates were centrifuged (14000 rpm × 10 min × 4 °C), with the supernatants used to assess the levels of phosphorylated and total NF-κB p65 subunit by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer's directions. The results represent the sample ratio (total p65/phospho-p65) measured at 450 nm (Multiskan GO Thermo Scientific).

Data analysis. Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 5.0). Results are presented as means +/- SEM of measurements made on 6 mice per group per experiment and are representative of two independent experiments. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time, and time versus treatment interaction. One-way ANOVA followed by Tukey's test was performed for each time-point. $P < 0.05$ was considered significant.

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Figures

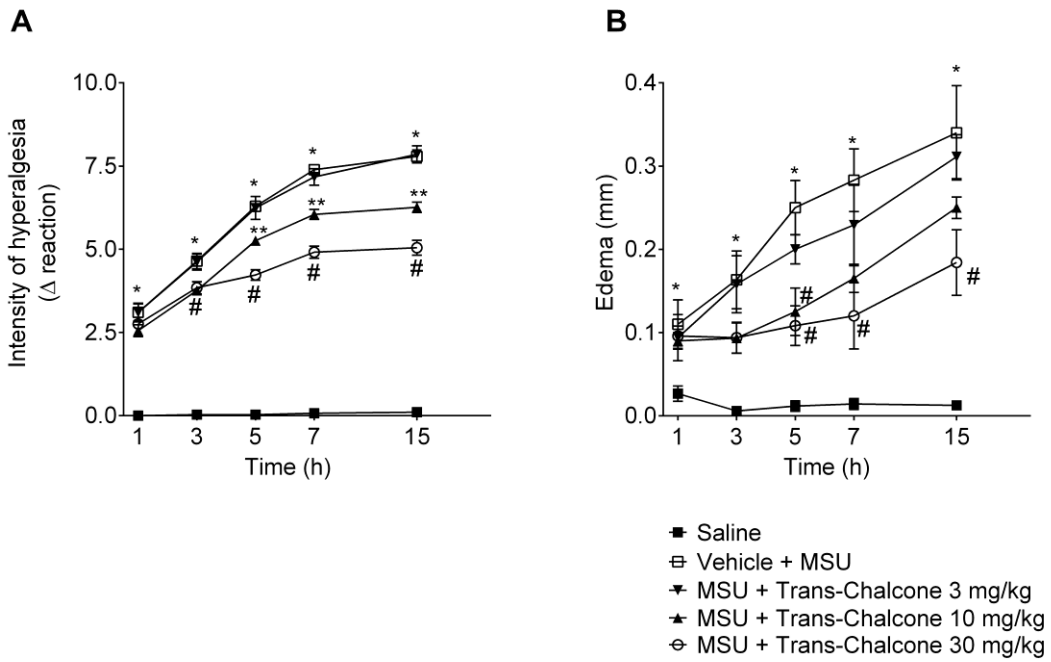

Figure 1. Trans-Chalcone inhibited gouty arthritis-induced by monosodium urate crystals. Mice were treated with trans-chalcone (3, 10 or 30 mg/kg, p.o.) or vehicle (saline) 1 h before MSU (100 μg/10 μL) i.a injection. Mechanical Hyperalgesia (A) and Edema (B) were assessed at indicated time points after MSU administration using an electronic pressure and pachymeter, respectively. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated

experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group, $^{**}P < 0,05$ compared to the dose of 3 mg/kg of trans-chalcone. $^{\#\#}P < 0,05$ compared to the dose of 30 mg/kg of trans-chalcone. ANOVA followed by Tukey's test.

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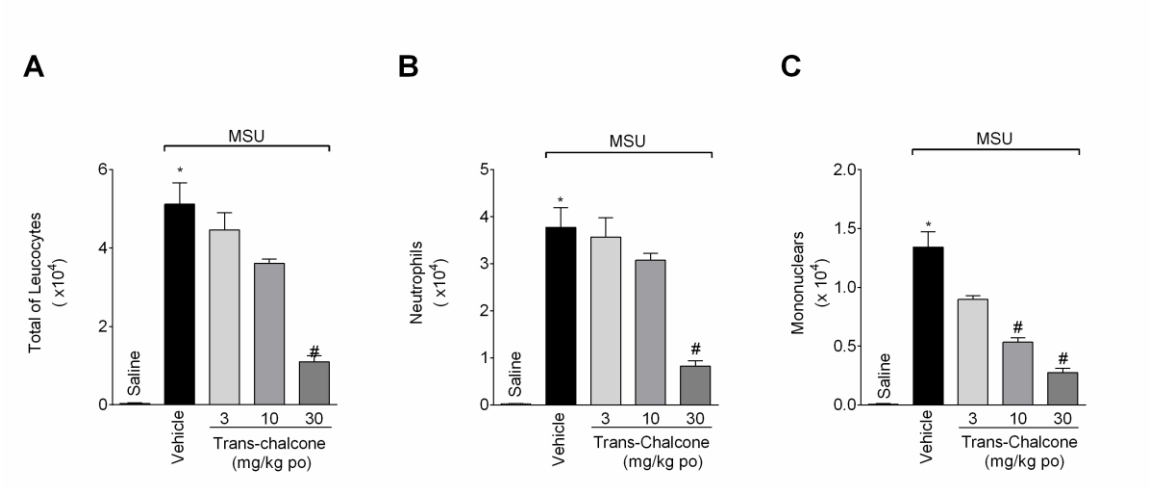

Figure 2. Trans-Chalcone inhibits Leukocyte Migration- induced by monosodium urate crystals. Mice were treated with trans-chalcone (3, 10 or 30 mg/kg, p.o.) or vehicle (saline) 1 h before MSU (100 µg/10 µL) i.a injection. The Total of Leukocytes (A), Neutrophils (B) and Mononuclear (C) was assessed 15 hours after administration of monosodium urate crystals (100 µg/10 µL i.a). Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

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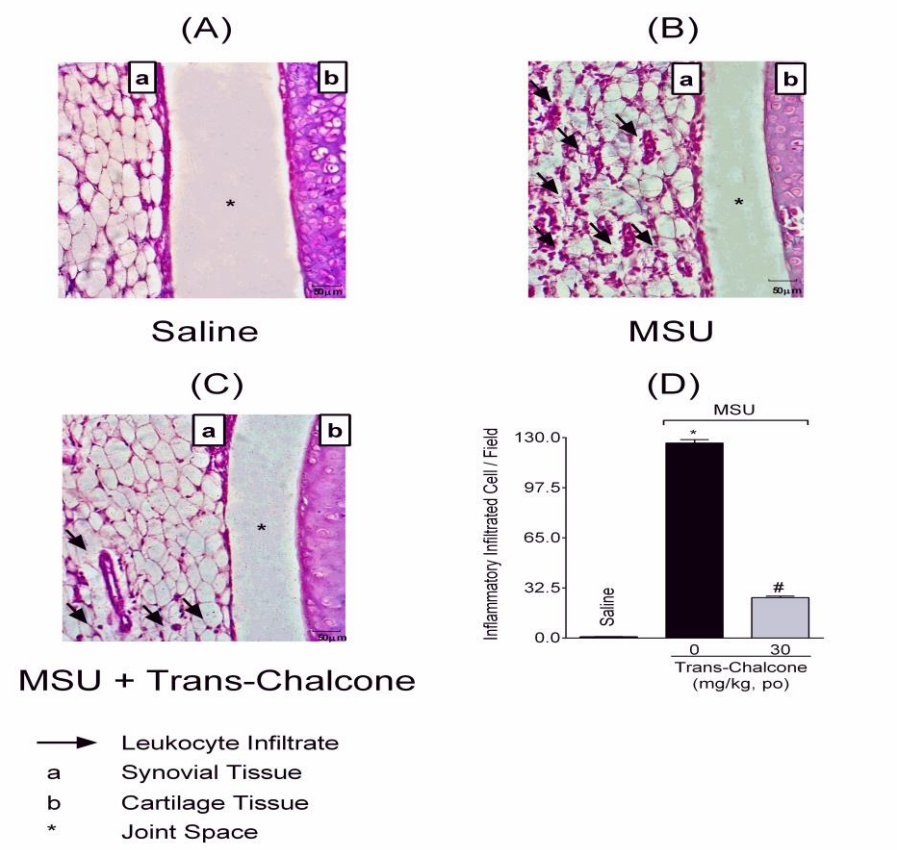

Figure 3. Effects of Trans-chalcone on synovial tissue histopathology of gouty arthritis in mice. Mice were treated with trans-chalcone (30 mg/kg, p.o.) or vehicle 60 min before i.a injection of MSU (100 μg/10 μL), and after 15 h articular joints samples were collected for the histological analysis. Control (A), MSU (B), MSU and Trans-Chalcone (C) and inflammatory infiltrated cells/field (D). All sections were stained with Hematoxylin and Eosin (magnification A-C, ×400) and the figure is representative of all experiment. Dimension used was 738 x 927 pixels for analysis (Field). Scale Barrs: 50μm. The arrow indicates representative infiltrate inflammatory cells counted. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

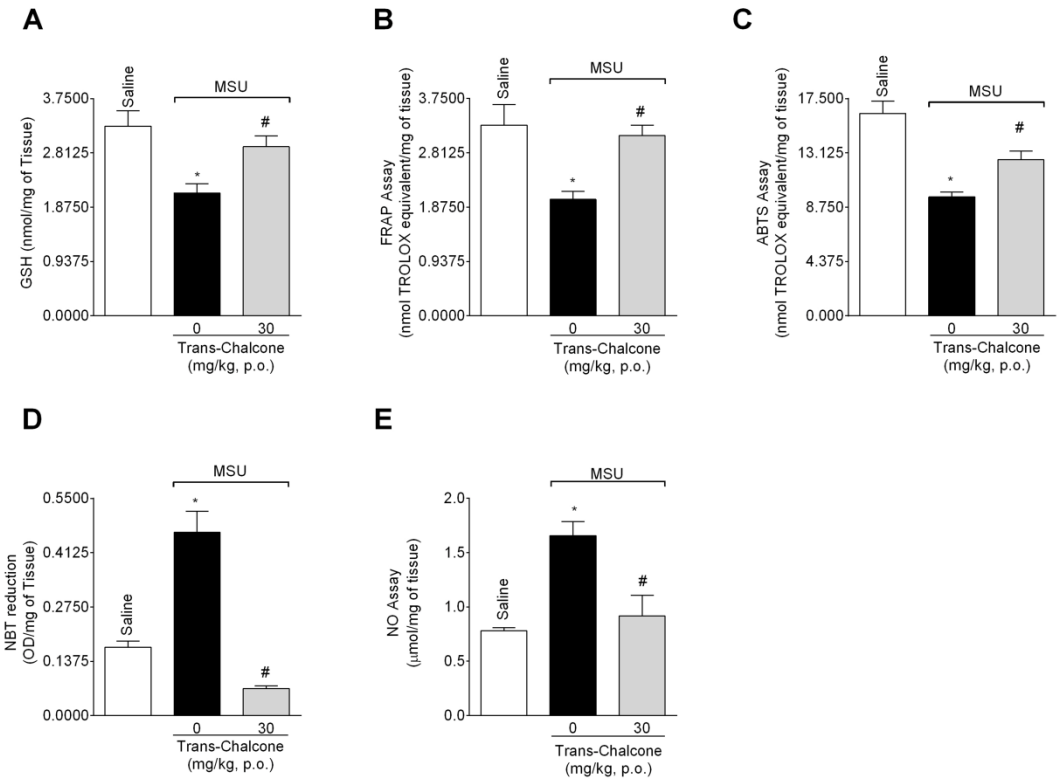

Figure 4. Trans-Chalcone reduced monosodium urate crystal (MSU)-induced decrease of GSH level and increase antioxidative capacity in FRAP, ABTS, NBT and Nitric Oxide (NO) assays. Mice were treated with trans-chalcone (30 mg/kg, p.o.) or vehicle 60 min before i.a injection of MSU (100 µg/10 µL), and after 15 h articular joints samples were collected for GSH level measurement (A), FRAP (Ferric Reducing Ability) (B), ABTS (Free Radical Scavenging Ability) (C), NBT (Nitroblue Tetrazolium Reduction) (D) and NO (Nitric Oxide) (E) assays. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

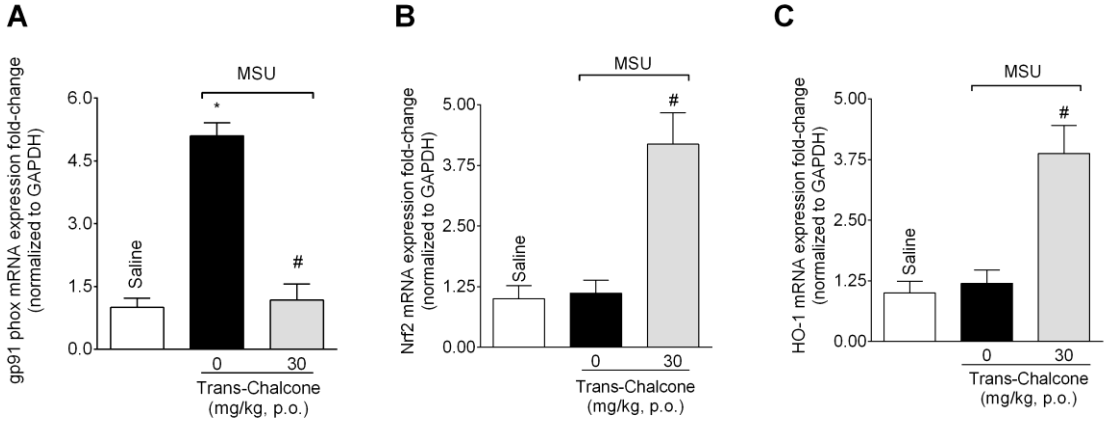

Figure 5. Trans-Chalcone inhibited gp91^{phox}, Nrf2 and HO-1 mRNA expression. The animals were treated with trans-chalcone (30 mg/kg, p.o.) or vehicle 1 h before i.a. injection of MSU crystals, and after 15 h articular joints samples were collected in (TRIzol®Reagent) for the isolation of total RNA/DNA from cells and tissues to measure gp91^{phox} (A), Nrf2 (B) and HO-1 (C). Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

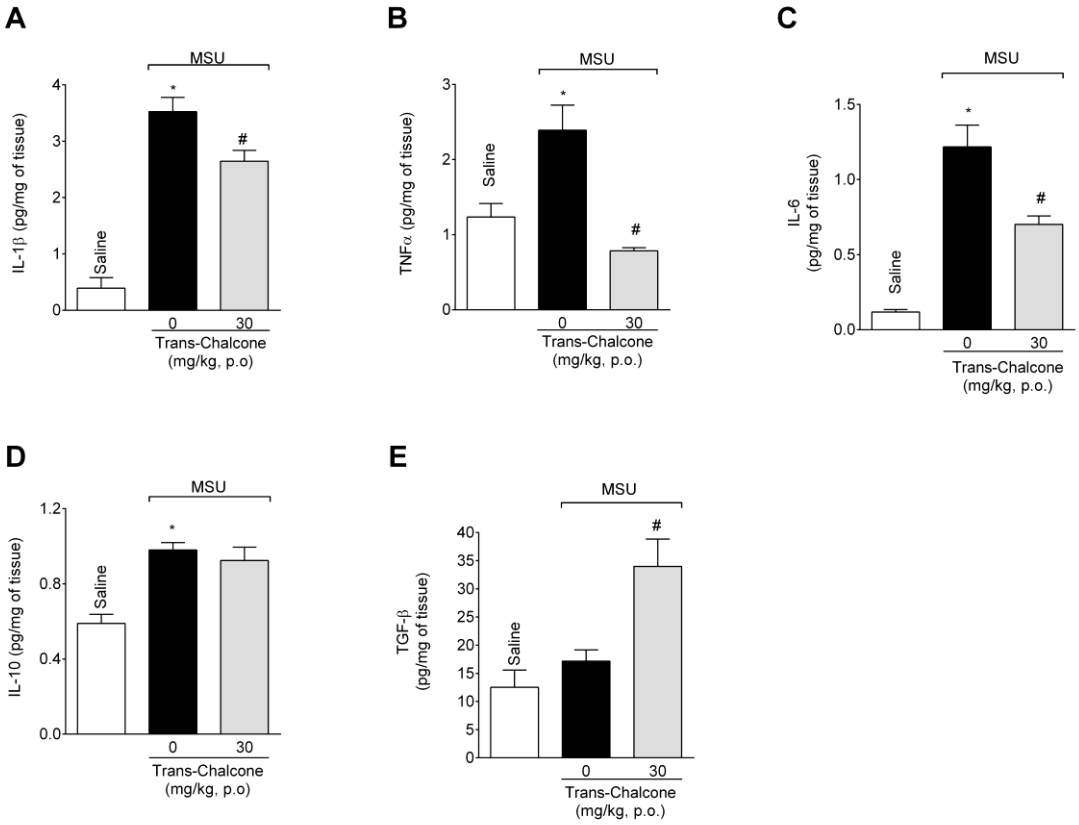

Figure 6. Trans-Chalcone inhibited MSU-induced IL-1β, TNFα, IL-6 and maintained IL-10 and increase TGF-β production. Mice were treated with trans-chalcone (30 mg/kg, p.o.) or vehicle (saline) 1 h before MSU (100 μg/10 μL) i.a injection. After additional 15 h, articular joint samples were collected for the determination of IL-1β (A), TNFα (B), IL-6 (C), IL-10 (D) and TGF-β (E) production. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

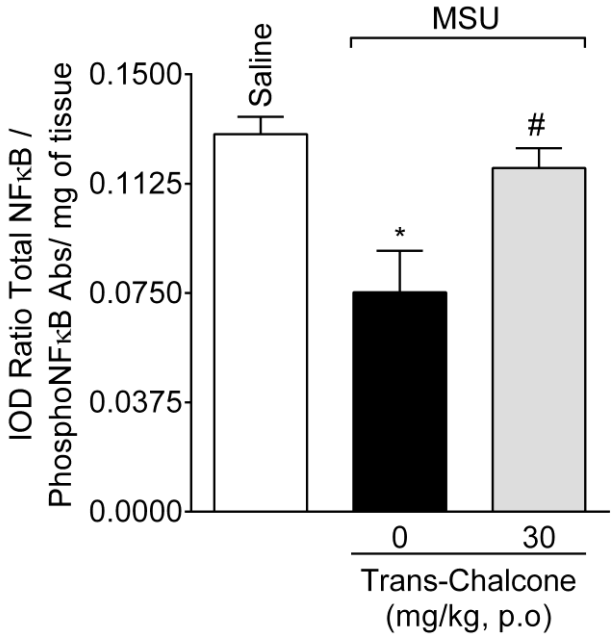

Figure 7. Trans-chalcone inhibited Monosodium Urate Crystal-induced NFκB activation. Mice were treated with trans-chalcone (30 mg/kg, p.o.) or vehicle 1 h before i.a injection of MSU, and after 15 h articular joints samples were collected in lysis buffer for measure the NF-κB activation. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

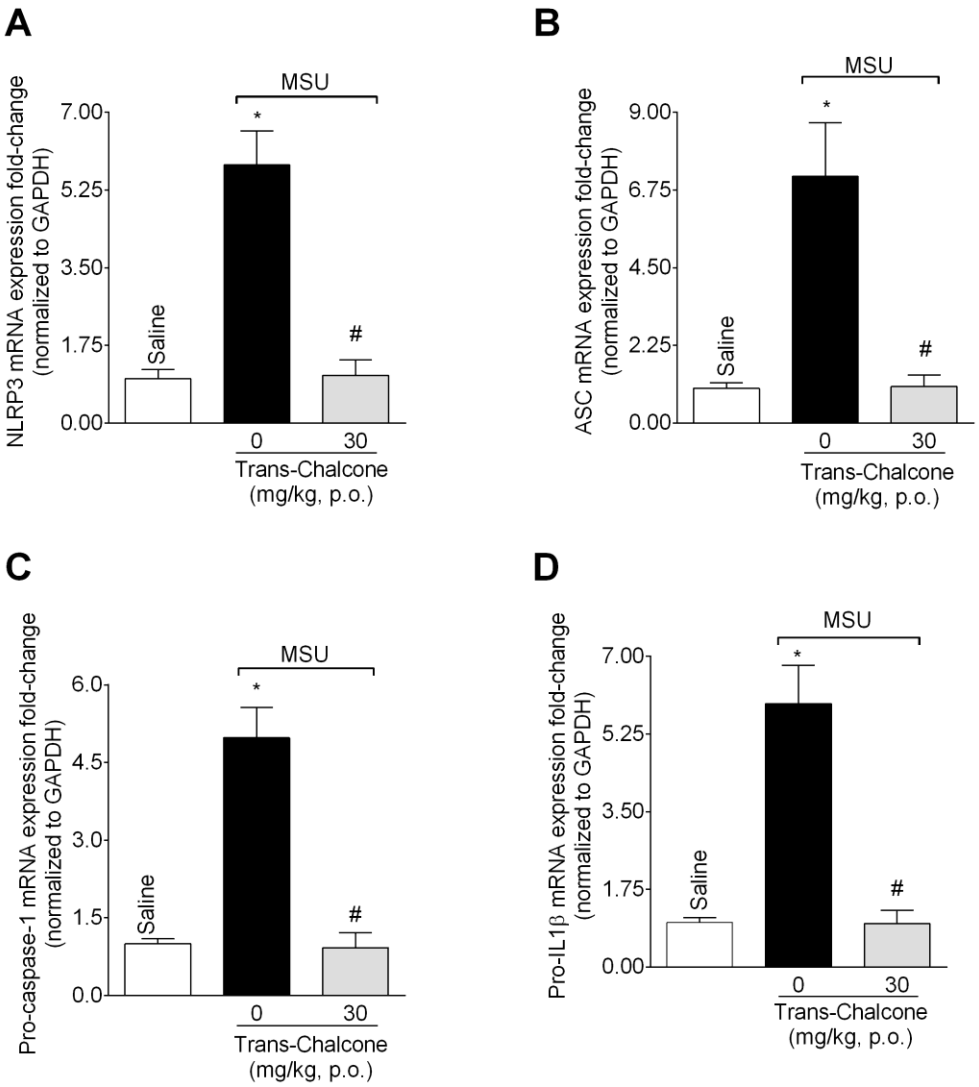

Figure 8. Trans-Chalcone inhibited NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β expression. The animals were treated with trans-chalcone (30 mg/kg, p.o.) or vehicle 1 h before i.a. injection of MSU crystals, and after 15 h articular joints samples were collected in (TRIzol®Reagent) for the isolation of total RNA/DNA from cells and tissues to measure NLRP3 (A), ASC (B), Pro-caspase-1 (C) and Pro-IL-1β (D). Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

ARTIGO 2 – Hesperidin Methyl-Chalcone suppresses experimental gout arthritis in mice by inhibiting NFκB activation

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Research paper

Hesperidin Methyl-Chalcone suppresses experimental gout arthritis in mice by inhibiting NFκB activation

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Abstract

Gout arthritis is a painful inflammatory disease dependent on the deposition of monosodium urate (MSU) crystals in joints and peri-articular tissues. Hesperidin methyl-chalcone (HMC) is a bi-flavonoid which presents antioxidant and anti-inflammatory properties through inhibition of NF-κB activation and NLRP3 inflammasome. We investigated the effect and mechanisms of HMC in mechanical hyperalgesia, oedema, leukocyte recruitment, histopathology analysis (H/E), oxidative stress (Reduced gluthathione [GSH], Ferric-Reducing Ability Potential [FRAP], Free-Radical Scavenging Ability [ABTS], Nitrobluetetrazolium [NBT] and nitric oxide [NO], mRNA expression by qPCR (gp91phox, Nrf2, HO-1, NLRP3, ASC, Pro-caspase-1 and pro-IL-1β), cytokines levels and NFκB activation (ELISA) induced by intra-articular of MSU in mice. We found that HMC reduces in

a dose-dependent manner MSU-induced mechanical hyperalgesia, oedema and leukocyte recruitment. HMC also inhibited MSU-induce synovitis, oxidative stress and pro-inflammatory cytokine production. HMC inhibited MSU-induced NFκB activation and mRNA expression of gp91phox, NLRP3 inflammasome components (NLRP3, ASC, Pro-caspase-1 and pro-IL-1β) as well as induced TGFβ production and Nrf2 and HO-1 mRNA expression. *In vitro* HMC did not inhibit IL-1β secretion by LPS-primed macropahges challenged with MSU crystals. Thus, pivotal role of HMC effect is probably by inhibiting NFκB activation route, which corroborated with no effect on IL-1β levels on macrophages cells supernatant in inflammasome activation assay. Therefore, HMC represents a possible pharmacological approach for the treatment of gout arthritis-induced pain and inflammation.

Keywords: Gouty arthritis; Cytokines; NLRP3 inflammasome; NFκB; oxidative stress

1. Introduction

Gout arthritis is a painful arthritic disease characterized by acute inflammation triggered by monosodium urate (MSU) crystals in the articular and peri-articular tissues [1–3]. There is an intense inflammatory response and immune cell activation due to the MSU crystals-induced cytokine production by tissue resident cells such as chondrocytes and macrophage-like and fibroblast-like synoviocytes as well as recruited cells including monocytes and neutrophils. Evidence indicates that MSU crystals are phagocyted depending on CD14 and Toll-like receptor (TLR) 4, which triggers the production of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-8, IL-6 and monocyte chemotactic factors [4,5]. Nevertheless, the deficiency on varied TLRs does not affect MSU-induced peritonitis in mice, which suggests that MSU-induced inflammation is TLR-independent [6]. MSU crystals are potent activators of the so-called signal 2 or NLRP3 inflammasome activation to induce caspase-1-depentent processing of pro-IL-1β into active IL-1β. In turn, IL-1β induces inflammation as well as further activates signal 1 or NFκB. This is a central mechanism in the development of gout arthritis [7].

Hesperidin (3,5,7-trihydroxy flavanone 7-rhamnoglucoside) is a flavonoid belonging to the class of flavanones, which is found in vegetables and fruits such as oranges and grape fruit, especially in citrus fruits [8]. In general, flavonoids present several biological properties such as antioxidant, anti-allergic, antitumoral, antimicrobial, anti-inflammatory and analgesic effects [9,10].

Several studies demonstrated that hesperidin reduces oxidative stress and inflammation [11,12]. However, hesperidin presents poor water solubility and absorption in intestine compared with other flavonoids, which is an explanation for limited effects. Methylation under alkaline conditions produces hesperidin methyl chalcone (HMC) that presents greater bioavailability, metabolic stability and tissue distribution than hesperidin [13,14].

A wide range of pharmacological activities has been shown for HMC. For instance, HMC induces vasodilation, vascular permeability and stabilizes prostaglandin E [15–17]. In fact, HMC has similar vasoprotective activity as other drugs used to treat vascular disorders such as Cyclo 3 Fort and Cirkan [18,19]. Several pro-inflammatory cytokines induce vascular disorders that lead to blood stasis disturbance and endothelial cell permeability changes that triggers inflammation and chronic venous insufficiency [20,21] leading to redness, heat, edema and pain reducing the quality of life in patients [22,23]. Recently our group showed that HMC presents analgesic proprieties by inhibiting inflammation and oxidative stress in the

mouse models of inflammatory pain induced by acetic acid, phenyl-p-benzoquinone, formalin and carrageenan. In agreement with the anti-inflammatory and analgesic effects of HMC, this bi-flavonoid inhibited carrageenan-induced oxidative stress, cytokine production and NFκB activation [24]. Thus, highlighting the therapeutic potential of HMC to treat inflammatory diseases.

Considering the anti-inflammatory and analgesic activity of HMC in vascular disorders and models of inflammation, the aim of this study was to investigate the effect and mechanisms of HMC in gout arthritis.

2. Methods

2.1. Animals

Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water with a light/dark cycle of 12/12h at a constant temperature of 21ºC. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled (21 °C) room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 14600.2013.73). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Materials

The following materials were obtained from the sources indicated: NaCl 0.9% (Fresenius Kabi Brasil Ltda. Aquiraz, CE, Brazil), HMC (Santa Cruz Biotechnology, Santa Cruz, CA, USA); enzyme-linked immunosorbent assay (ELISA) Ready-SET-Go! (eBioscience, San Diego, CA, USA) and PathScan® (Cell Signaling, Beverly, MA, USA) kits; 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), and Nitroblue Tetrazolium (NBT) (Sigma–Aldrich, St. Louis, MO, USA).

2.3. MSU crystal preparation

MSU crystals were prepared according to the method described previously [25]. In brief, 800mg of monosodium urate was dissolved in 155ml boiling water containing 5ml 1N NaOH. After the pH was adjusted to 7.2, the solution was cooled gradually by stirring at room temperature. The crystals were collected by centrifugation at 3,000 g for 2 min at 4◦C. The

crystals were evaporated and sterilized by heating at 180°C for 2 hours and stored in a sterile environment until use.

2.4. Induction of articular joint inflammation

Joint inflammation was induced by administration of MSU (100 μg/10μL, i.a.) and injected into the right articular joint of mice that were mildly anaesthetized. Control animals received an intra-articular injection of 10 μL sterile saline.

2.5. Electronic pressure-meter test

Experiments were performed as described previously [26]. Similar to cutaneous hypernociception measurement, an increasing perpendicular force was applied to the central area of the plantar surface, inducing the flexion of the tibiotarsal joint. A nonnociceptive tip probe with area size of 4.15 mm2 was used. The results were expressed as the flexion-elicited withdrawal threshold (in grams).

2.6. Oedema

The volume of the joint was measured with a caliper (Mitutoyo, Suzano, SP, Brazil) before (zero time) the intra-articular stimulus with MSU, and after the administration at the indicated times on figures. The amount of articular swelling was determined for each mouse and the difference between the times indicated on figures and the zero time. The oedema value was expressed as oedema/mm.

2.7. Total and differential cell counts

The Knee joint was exposed by surgical incision and washed three times with 5 μL of phosphate-buffered saline (PBS) that contained ethylenediaminetetraacetic acid (EDTA) and was diluted to a final volume of 50 μL with PBS/EDTA to determine total cell counts. The total number of leukocytes, diluted in Turk's solution, was determined in a Neubauer chamber under a light microscope. The results are expressed as the number of leukocytes per cavity.

2.8. Histopathological analysis

Mice were euthanized 15 hours after MSU injection. The knee joint of mice were removed, fixed with 10% paraformaldehydein PBS, and then decalcified for 10 days with EDTA and embedded in paraffin for histological analysis. The paraffin sections were stained with hematoxylin and eosin for conventional morphological evaluation. Dimension used for the

analysis was 544 x 582 pixels for analysis (Field) and the arrow indicate representative infiltrate inflammatory cells counted.

2.9. GSH levels measurement

Samples of Knee joint were collected and maintained at -80 ºC for at least 48 h. The sample was homogenized with 200 µL of 0.02 M EDTA. The homogenate was mixed with 25 µL of trichloroacetic acid 50% and was homogenized three times over 15 min. The mixture was centrifuged (15 min x 1500 g x 4 ºC). The supernatant was added to 200 µL of 0.2 M TRIS buffer, pH 8.2, and 10 µL of 0.01M DTNB. After 5 min, the absorbance was measured at 412 nm (Multiskan GO, Thermo Scientific) against a blank reagent with no supernatant. A standard GSH curve was formed. The results are expressed as GSH per mg of protein [27].

2.10. ABTS and FRAP assays

The ability of samples to resist oxidative damage was determined by their free radical scavenging (ABTS [2,2'-Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay) and ferric reducing (FRAP assay) properties. The tests were adapted to a 96-well microplate format as previously described [28]. Articular tissue samples were collected 15h after MSU i.a injection (100 µg/10 µL) and homogenized immediately in ice-cold KCl buffer (500 µL, 1.15% w/v). The homogenates were centrifuged (200 g × 10 min × 4 °C), and the supernatants were used in both assays. Diluted ABTS solution (200 µL) was mixed with 10 µL of sample in each well. After 6 min of incubation at 25 °C, the absorbance was measured at 730 nm. For FRAP assay, the supernatants (10 µL) were mixed with the freshly prepared FRAP reagent (150 µL). The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm (Multiskan GO Thermo Scientific). The results of ABTS and FRAP assays were equated against a standard Trolox curve (0.02 – 20 nmol).

2.11. Superoxide anion production

The measurement of superoxide anion production in tissue homogenates (10 mg/mL in 1.15% KCl) was performed using the nitroblue tetrazolium (NBT) assay adapted to a microplate as described previously [29]. The NBT reduction was measured at 600 nm (Multiskan GO, Thermo Scientific). The tissue weight was used for data normalization.

2.12. Nitrite production

Samples from articular joint were collected 15 h after MSU injection, homogenized in 500 μL of saline, and nitrite (NO2-) concentration was determined by the Griess reaction as an indicator of nitric oxide (NO) production [30]. Briefly, 100 μL of the homogenate was incubated with 100 μL of Griess reagent for 5 min at 25 °C, and NO2- concentration was determined by measuring the optical density at 550 nM (Multiskan GO, Thermo Scientific) in reference to a standard curve of NaNO2 solution. Results are expressed as μmol of NO2- per mg of tissue.

2.13. Quantitative (q)PCR

Quantitative Polymerase Chain Reaction (qPCR). qPCR was performed as previously described (Verri et al., 2008). Samples were homogenized in Trizol reagent, and total RNA was extracted by using the SV Total RNA Isolation System (Promega). All reactions were performed in triplicate using the following cycling conditions: 50° C for 2 min, 95°C for 2 min, followed by 40 cycles of 95° C for 15 s and 60° C for 30 s. qPCR was performed in a LightCycler Nano Instrument (Roche, Mississauga, ON, USA) sequence detection system by using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, USA). The mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were Gapdh forward: CAT ACC AGG AAA TGA GCT TG, reverse: ATG ACA TCA AGA AGG TGG TG; Nrf2, forward: TCA CAC GAG ATG AGC TTA GGG CAA, reverse: TAC AGT TCT GGG CGG CGA CTT TAT; $gp91^{phox}$, forward: AGC TAT GAG GTG GTG ATG TTA GTG G, reverse: CAC AAT ATT TGT ACC AGA CAG ACT TGA G; Nlrp3, forward: AGC TAT GAG GTG GTG ATG TTA GTG G, reverse: CAC AAT ATT TGT ACC AGA CAG ACT TGA G; HO-1, forward: CCC AAA ACT GGC CTG TAA AA, reverse: CGT GGT CAG TCA ACA TGG AT; Pro-caspase-1: forward: TGG TCT TGT GAC TTG GAG GA, reverse: TGG CTT CTT ATT GGC ACG AT; Pro-IL-1β, forward: GAA ATG CCA CCT TTT GAC AGT G, reverse: TGG ATG CTC TCA TCA GGA CAG; ASC, forward: ATG GGG CGG GCA CGA GAT G, reverse: GCT CTG CTC CAG GTC CAT CAC. The SYBR green PCR Master Mix was used according to the manufacturer's instructions.

2.14. Cytokine measurement

The Knee joint samples were homogenized in 500 μL of buffer containing protease inhibitors (1 mM Phenylmethanesulfonyl fluoride, Sigma Aldrich). IL-1β, TNF-α, IL-6, IL-10 and

TGF-β levels being determined as previously described by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits [27]. The results are expressed as picograms (pg) of cytokine/100 mg of tissue.

2.15. NF-κB activity

The knee joint samples were collected and homogenized in ice-cold lysis buffer (Cell Signaling). The homogenates were centrifuged (14000 rpm × 10 min × 4 °C), with the supernatants used to assess the levels of phosphorylated and total NF-κB p65 subunit by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer's directions. The results represent the sample ratio (total p65/phospho-p65) measured at 450 nm (Multiskan GO Thermo Scientific).

2.16. Preparation of bone marrow-derived macrophages (BMDMs) and inflammasome activation assay

Femora and tibiae of mice C57BL/6 mice (8 weeks old) were aspirated with RPMI1640 media. Bone marrow cells were cultured in RPMI 1640 medium containing 10% FBS and 15% L929 cell conditioned medium. BMDM were harvested at day 7 and plated at the density of 1,5 x 10^5 cells/well in 96-well plate. BMDM were stimulated with 500 ng/mL E. coli LPS (Santa Cruz Biotechnology) and 3 h later treated with 450 µg/mL of MSU to stimulate NLRP3 inflammasome activation as described previously (Martinon et al. 2006). BMDM were treated with HMC (30; 100 or 300 µM), or Quercetin (30 µM), 30 min before MSU stimulation. Supernatants were collected 5 h after MSU stimulation and IL-1β concentration quantitated by ELISA

2.17. Experimental procedures

Mice were treated with Hesperidin Methly-Chalcone (HMC; 3, 10, 30 and 100 mg/kg, p.o.) or vehicle (saline) 1h before stimulus with MSU (100 µg/10µL, i.a.), and mechanical hyperalgesia and oedema were evaluated 1, 3, 5, 7 and 15 h after inflammatory stimulus administration. Leukocyte migration was evalualted 15 h after MSU injection. The dose of 30 mg/kg, p.o. of HMC was chosen for subsequent experiments. In another sets of experiments designed to determine the mechanism of action of HMC, mice were treated with 30mg/kg, p.o. of HMC and stimulated with MSU (100 µg/10 µL, i.a. injection) and cytokines production (IL-1β, TNF-α, IL-6, IL-10 and TGF-β levels), oxidative stress (reduced glutathione [GSH], nitroblue tetrazolium [NBT] reduction, Nitric Oxide [NO], ABTS [2,2′-

Azinobis-3-ethylbenzothiazoline 6-sulfonic acid] assay, ferric reducing [FRAP assay]), NF-κB activation (Total and phosphorylated p65), histological analysis and Quantitavive (q)PCR were performed in samples collected 15h after MSU stimulus. *In vitro*, macrophages BMDMs were performed in 96 wells plate, LPS (500 ng/mL)-primed BMDM was incubated during 3 hours, in addition, HMC (30-300 µM) or Quercetin (30µM) treatment performed during 30 minutes before stimulation with MSU crystals (450 µg/mL) and incubated during 5 hours. IL-1β levels in the culture supernatant were measured.

3. Data analyses

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 5.0). Results are presented as means +/- SEM of measurements made on 6 mice per group per experiment and are representative of two independent experiments. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time, and time versus treatment interaction. One-way ANOVA followed by Tukey's test was performed for each time-point. $P < 0.05$ was considered significant.

4. Results

4.1. Hesperidin Methyl-Chalcone (HMC) inhibits MSU-induced knee joint mechanical hyperalgesia and oedema.

Mice were treated with HMC (3, 10 or 30 mg/kg/saline, p.o.) 1 h before MSU (100 µg/10 µL) i.a. injection, and mechanical hyperalgesia (Fig. 1A) and oedema (Fig. 1B) were evaluated at indicated times points. Monosodium urate crystals induced significant mechanical hyperalgesia, which was not affected by 3 mg/kg of HMC. Furthermore, 10 mg/kg of HMC significantly inhibited MSU-induced mechanical hyperalgesia between 7-15 h with significant differences with the lower dose of HMC. The dose of 30 mg/kg of HMC inhibited MSU-induced mechanical hyperalgesia between 5-15 h (up to 40%), with significant difference compared to the positive MSU control group. In the same treatment schedule, HMC also inhibited MSU-induced oedema at the doses of 30 mg/kg (58%). On the other hand, the dose 10 mg/kg of HMC significantly inhibited MSU-induced oedema between 7-15 h compared with the lower dose of HMC (Fig. 1B).

4.2. HMC inhibits MSU-induced leukocyte recruitment to the knee joint.

Mice were treated with HMC (3, 10 or 30 mg/kg, p.o.) 1 h before MSU (100 µg/10 µL) i.a. injection. Fifteen hours after MSU stimulus, samples of knee joint exudate were collected to determine the number of total leukocytes (Fig. 2A), neutrophils (Fig. 2B) and mononuclear (Fig. 2C) cells. MSU-induced significant increase of total leukocytes, neutrophil and mononuclear cellular counts in the knee joint exudate (Fig. 2). HMC presented the same profile of inhibition of MSU-induced total leukocytes, neutrophil and mononuclear cellular counts in the knee joint exudate (Fig. 2) in which the dose of 3 mg/kg of HMC presented no effect, 10 mg/kg induced a tendency of reduction that was not significant, and the doses of 30 mg/kg induced significant inhibition (57%-total leukocytes (Fig. 2A), 56%-neutrophils (Fig. 2B), 49%-mononuclear (Fig. 2C)). Considering the results of Fig. 1 and Fig. 2, the dose of HMC of 30 mg/kg was selected for the following experiments.

4.3. HMC reduces MSU-induced knee joint synovitis

Mice were treated with HMC (30 mg/kg, p.o.) 1 h before MSU (100 µg/10 µL) i.a. injection, and after 15 h the knee joint samples were collected for histological analysis (Fig. 3). All sections were stained with Hematoxylin and Eosin (magnification A-C, ×400). Saline group presented regular histological appearance (Fig. 3A). In turn, MSU induced significant joint synovitis with the marked increase of inflammatory cells (Fig 3B), which was decrease by the treatment with HMC by 77% (Fig. 3C) as observed by the inflammatory score (Fig. 3D).

4.4. HMC inhibits MSU-induced depletion of endogenous antioxidant and oxidative stress in the knee joint.

Mice were treated with HMC (30 mg/kg/saline, p.o.) 1 h before MSU (100 µg/10 µL) i.a. injection, and knee joint samples were collected after additional 15 h for GSH (reduced gluthathione), FRAP (ferric reducing ability potential), ABTS (free-radical scavenging ability), NBT (nitroblutetrazolium reduction by superocide anion) and Griess (nitric oxide; NO) assays (Fig. 4). MSU induced significant decrease of antioxidant defense in the knee joint, which was inhibited by HMC treatment as observed in the GSH (88%, Fig. 4A), FRAP (100%, Fig. 4B), ABTS (100%, Fig. 4C), NBT (100%, Fig. 4D) and NO (100%, Fig. 4E) assays.

4.5. HMC inhibits MSU modulation of gp91^{phox}, Nfr2 and HO-1 mRNA expression in the knee joint.

Mice were treated with HMC (30 mg/kg, p.o.) 1 h before MSU (100 µg/10 µL) i.a. injection, knee joint samples were collected after 15 h for processing to evaluate gp91^{phox}, Nrf2 and HO-1 mRNA expression by qPCR (Fig. 5). MSU induced a significant increase of gp91^{phox} mRNA expression, which was inhibited by (82%) (Fig. 5A). MSU did not alter knee joint Nrf2 and HO-1 mRNA expression at this time point, however, HMC treatment enhanced Nrf2 and HO-1 mRNA expression by 4.5 and 6 fold, respectively (Fig. 5B and 5C, respectively).

4.6. HMC modulates MSU-induced cytokine production in the knee joint.

Mice were treated with HMC (30 mg/kg/saline, p.o.) 1 h before i.a. injection of MSU (100 µg/10 µL), and knee joint samples were collected after 15 h to determine the levels of IL-1β, TNFα, IL-6, IL-10 and TGF-β by ELISA (Fig. 6). MSU injection induced significant production of pro-inflammatory cytokines (IL-1β, TNFα and IL-6), which was inhibited by HMC treatment (40, 100 and 42%, respectively). HMC also inhibited the MSU-induced production of the anti-inflammatory cytokine IL-10 (Fig. 6D). Nevertheless, HMC induced a 2 fold increase of MSU-induced TGF-β production, which is also an anti-inflammatory cytokine in gout arthritis (Fig. 6E).

4.7. HMC inhibits MSU-induced NF-κB activation in the knee joint.

Mice were treated with HMC (30 mg/kg/saline, p.o.) 1 h before i.a. injection of MSU (100 µg/10 µL), and knee joint samples were collected after 15 h to determine the ratio of total NFκB p65 / phosphorylated NFκB p65. MSU-induced a significant decrease of the total NFκB/phosphorylated NFκB ratio indicating NFκB activation, which was inhibited by HMC treatment (83%) (Fig. 7).

4.8. HMC inhibits MSU-induced increase of inflammasome components (NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β) mRNA expression in the knee joint.

Mice were treated with HMC (30mg/kg, p.o.) 1 h before MSU (100 µg/10 µL) i.a. injection, knee joint samples were collected after 15 h for processing to evaluate NLRP3, ASC, pro-caspase-1 and pro-IL-1β mRNA expression by qPCR. MSU stimulus increased NLRP3, ASC, pro-caspase-1 and pro-IL-1β mRNA expression compared to saline control group (Fig. 8A, 8B, 8C and 8D, respectively). In turn, HMC treatment inhibited MSU-

induced NLRP3, ASC, pro-caspase-1 and pro-IL-1β mRNA expression by 87, 90, 89 and 89%, respectively (Fig. 8A, 8B, 8C and 8D, respectively).

4.9 HMC not inhibits IL-1β levels in BMDMs macrophages supernatant in gouty arthritis.

LPS priming (signal 1) followed by MSU stimulation (signal 2) induced an increase of IL-1β levels in BMDM, which was not reduced by HMC treatment (Fig. 9). However, isolated stimuli from macrophages derived from bone marrow with LPS or MSU were not able to promote increased of IL-1β levels in the supernatant of macrophages (BMDMs) (data not shown). In turn, quercetin at the concentration of 30 µM was able to prevented inflammasome activation by MSU crystals decreasing IL-1β levels in BMDMs supernatant in order to morphine at the concentration of 20 µM. However, naloxone treatment was able to reversed d the quercetin effect at concentration 20 µM.

5. Discussion and Conclusion

The gout arthritis affects generally 2-6 fold men compared to women [31]. Gout arthritis incidence is directly related to increasing age, thus, affecting mainly middle-aged or older patients, reaching a plateau after 70 years old men. In women, gout is more commonly observed after menopause due to the reduction in estrogen levels, an important hormone in the elimination of uric acid by the kidneys [32]. The current treatments for gout arthritis include anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids (CS) and colchicine [33], which act by inhibiting cyclooxigenase, NFκB and toll-like receptor signaling. These drugs cause many side effects such as gastrointestinal ulceration, hormonal imbalance, bleeding and renal dysfunction, which supports the need of developing novel strategies [34]. Gout arthritis pathology depends on two intracellular molecular signals. Signal 1 induces NFκB activation, which induces the expression of inflammasome components. Signal 2 induces the activation of inflammasome resulting in the secretion of mature and active IL-1β. In this sense, drugs targeting signal 1 and/or signal 2 could be useful in the treatment of gout arthritis. In fact, corticoids inhibit signal 1, but present side effects due to their endogenous role in controlling lipid metabolism; and anakinra (IL-1ra) inhibits IL-1β effect, but this biological therapy is expensive. The present data shows that hesperdin methyl-chalcone (HMC) inhibits gout arthritis-induced inflammation and pain by targeting signal 1.

Evidence demonstrates that hesperdin methyl-chalcone (HMC) improves vascular tonus and inhibits symptoms of vascular diseases such as acute hemorrhoid and chronic venous disease [22,23]. HMC also inhibits the inflammation induced by ultraviolet B irradiation, acetic acid and phenyl-p-benzoquinone and capsaicin, carrageenan and Complete Freund's Adjuvant (CFA) and formalin stimuli [24,35]. However, it remained to be investigated whether HMC would affect gout arthritis.

Per oral treatment with HMC inhibited MSU-induced mechanical hyperalgesia, joint oedema and leukocyte recruitment in a dose-dependent manner. Thus, HMC consistently inhibited MSU clinical inflammatory signs as well as reduced joint synovitis that is an important parameter indicating reduction of tissue lesion. Recruited leukocytes such as macrophages and neutrophils represent a hallmark of acute gout arthritis. These cells contribute to the pathogenesis of gout arthritis by [36–39], inducing the release of variety of mediators including reactive oxygen species, proteolytic enzymes and pro-inflammatory cytokines (IL-1β and TNFα). The inflammatory mediators, oxidative stress and proteolytic enzimes induce cartilage degradation and joint damage [40–42]. Therefore, the HMC inhibition of leukocyte infiltration, joint edema, pain and synovitis demonstrates the therapeutic potential of this biflavonoid.

MSU crystals induce the rupture of lysosomal membrane of phagocytes provoking the release of lysosomal enzymes. Activated NADPH oxidase in phagocytes produces superoxide anion generating various ROS that target proteins, lipids and nucleic acids [43–45]. Nitric oxide is also produced and contributes to arthritis by reacting with superoxide anion generating peroxinitrite that causes tissue lesion [46]. Similarly, synovial tissue from gouty arthritis patients exhibits an increase of nitric oxide production that was upregulated in response to *in vitro* MSU stimulation of monocytes/macrophages [47]. The excessive production of ROS causes an imbalance leading to depletion of GSH levels, oxidation of cysteine groups, and maladaptive activation of the NF-κB and NLRP3 inflammasome pathway [48–50]. Cytokines and oxidative stress contribute edema [51–53], leukocyte recruitment [54,55], further pro-inflammatory cytokines production and pain [56–58]. HMC inhibited MSU-induced oxidative stress and re-established the levels of endogenous antioxidants and tissue antioxidant activity. HMC induced Nrf2 mRNA expression, a transcription factor known to induce HO-1 and GSH lining up with the results of enhanced HO-1 mRNA expression and GSH levels by HMC treatment. Further, HMC inhibited MSU-induced $gp91^{phox}$ mRNA expression and superoxide anion production, a component of NADPH oxidase and its product, respectively. Therefore, in addition to the structural

antioxidant chemical properties [9,59], it is likely that HMC up-regulates endogenous antioxidant and anti-inflammatory mechanisms.

The pro-inflammatory cytokines present a pivotal role in gout arthritis. HMC inhibited MSU-induced production of IL-1β, TNFα, IL-6 and IL-10. IL-1β, TNFα and IL-6 are pro-inflammatory cytokines. These cytokines have been linked to the activation of NADPH oxidase and the production of superoxide anion, NO and other ROS [60–62]. Therefore, these data corroborate the oxidative stress results. Furthermore, these cytokines induce pain, edema, leukocyte recruitment and oxidative stress [40,63]. IL-10 is an anti-inflammatory cytokine that is co-produced with pro-inflammatory cytokines limiting the production and activity of the pro-inflammatory cytokines [64]. Flavonoids have been shown to induce the increase of IL-10 production, which explains in part the anti-inflammatory actions of these molecules [65]. HMC did not induce an increase of IL-10, but rather reduced the production of this anti-inflammatory cytokine. It is likely that as HMC inhibited the production of pro-inflammatory cytokines, IL-10 release was also reduced since its limiting actions over inflammatory were not necessary [24,35,66]. MSU-induced knee joint inflammation was also accompanied by an increase of TGF-β production. HMC treatment enhanced MSU-induced TGF-β production. TGF-β is associated with an increase in the ability of the differentiating monocyte/macrophages to phagocyte apoptotic neutrophils, a process strongly linked with neutrophil clearance and resolution of acute inflammation [67–69]. Therefore, the HMC increase of MSU-induced TGF-β production was a beneficial anti-inflammatory and pro-resolution effect.

NF-κB and NLRP3 inflammasome pathways have pivotal roles in inflammation [6,7,70,71]. NF-kB activation induces the expression of cytokines and enzymes that produce ROS. Cytokines and ROS, in turn, activate NFκB, and this reciprocal circle amplifies the inflammatory response [72,73]. HMC inhibited MSU-induced NFκB activation in the knee joint. This result support that HMC targets an essential transcription factor in the inflammatory response and explains the wide HMC effect in reducing MSU-induced knee joint inflammation. The activation of NFκB is seen as signal 1 in gout arthritis since it induces the expression of NLRP3 inflammasome (NLRP3, ASC, pro-caspase-1 and pro-IL-1β). The NLRP3 inflammasome was shown to be an essential mechanism of gout arthritis and is named signal 2. Macrophages phagocyte MSU crystals, and as other crystals, they damage the lysosome releasing cathepsin G. In turn, cathepsin activates the NLR receptor NLRP3 resulting in caspase-1 activation that cleaves pro-IL-1β into IL-1β. Then, IL-1β induces

inflammation. The present study shows that HMC inhibited NFκB activation *in vivo*, which corroborates the inhibition of oxidative stress and cytokine production data. Together, the inhibition of NFκB, oxidative stress and cytokine production explain the beneficial effects of HMC in gout arthritis-induced knee joint inflammation and pain. The inhibition of NFκB activation is also in agreement with the HMC reduction of gout arthritis-induced mRNA expression of inflammasome components. This result is important since demonstrates that HMC reduces the up-regulation of an inflammatory platform that is an essential mechanism of gout arthritis disease development.

We have recently demonstrated that the flavonoid quercetin inhibits ASC oligomerization resulting in the inhibition of inflammasome activation and reduction of inflammasome-dependent inflammation in a model of vasculitis [74]. Therefore, we reason that it is rational to evaluate whether HMC would also inhibit inflammasome activation. To this end, macrophages were primed with LPS (signal 1) and stimulated with MSU crystals (signal 2). This is a suitable system to evaluate MSU activation of inflammasome [7,74]. Quercetin inhibited MSU-induced IL-1β secretion, which further corroborates the suitability of this system to evaluate inflammasome activation. However, HMC did not affect MSU-induced inflammasome-dependent IL-1β secretion. Therefore, inhibiting MSU-induced NLRP3 activation is not a mechanism of action of HMC. These data also reveal that not all flavonoids inhibit inflammasome activation indicating the importance on further studies addressing structure-relationship of flavonoids and inflammasomes.

Concluding, to our knowledge, this is the first study demonstrating that HMC inhibits MSU-induced knee joint inflammation and pain. The mechanism of action of HMC is not dependent on inhibiting inflammasome activation, but rather on inhibiting NFκB activation, and as a consequence, HMC inhibits the production of ROS and cytokines, explaining its anti-inflammatory and analgesic effects.

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Conflict of interest

The authors declare no conflict of interest.

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Figures and figures legends

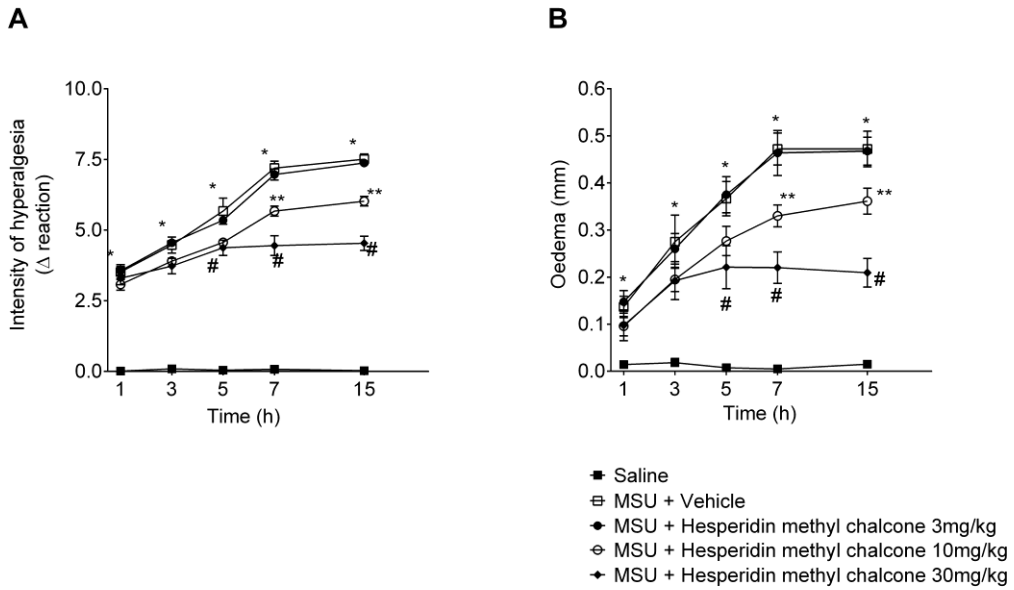

Fig. 1. Hesperidin Methyl-Chalcone inhibited gouty arthritis-induced by monosodium urate crystals. Mice were treated with Hesperidin Methyl-Chalcone (3, 10 or 30 mg/kg/saline, p.o.) or vehicle (saline) 1 h before MSU (100 μg/10 μL) i.a injection. Mechanical Hyperalgesia (A) and Edema (B) were assessed at 1-15 h after MSU administration using an electronic pressure and pachymeter, respectively. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group, $^{**}P < 0,05$ compared to the dose of 3 mg/kg of HMC. ANOVA followed by Tukey's test.

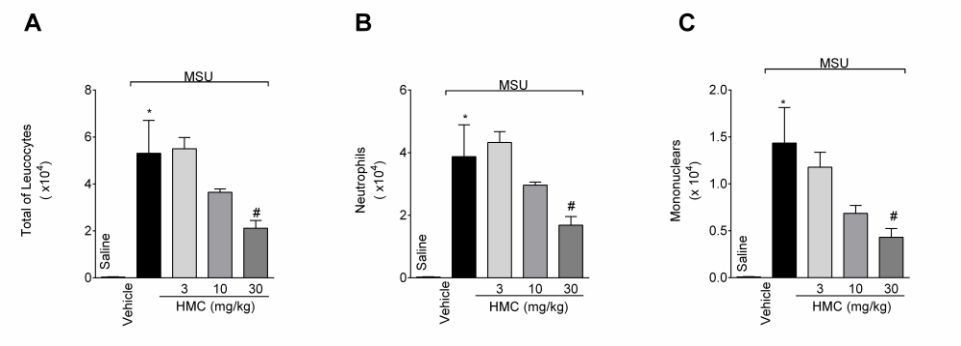

Fig. 2. Hesperidin Methyl-Chalcone inhibits Leukocyte Migration - induced by monosodium urate crystals. Mice were treated with Hesperidin Methyl-Chalcone (3, 10 or 30 mg/kg/saline, p.o.) or vehicle (saline) 1 h before MSU (100 µg/10 µL) i.a. injection. The Total of Leukocytes (A), Neutrophils (B) and Mononuclear (C) were assessed 15 hours after administration of monosodium urate crystals (100 µg/10 µL i.a.). Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

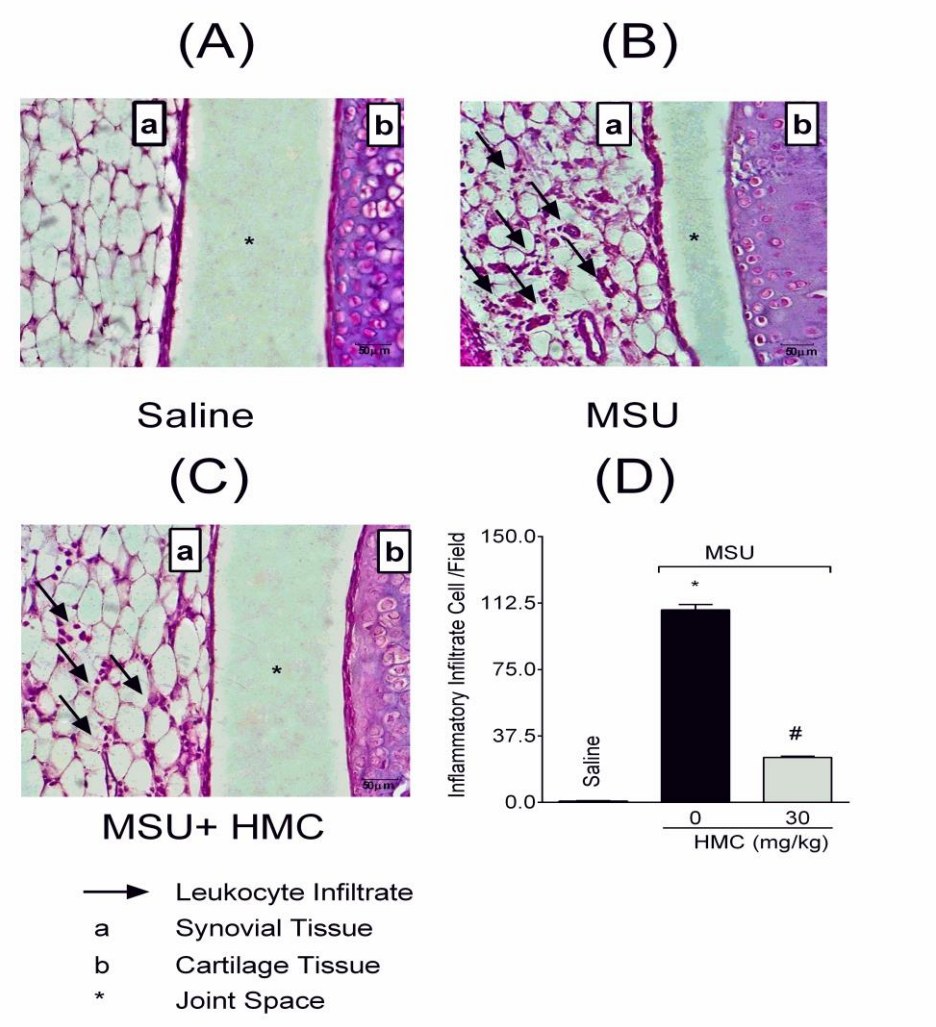

Fig. 3. **Effects of Hesperidin Methyl-Chalcone on synovial tissue histopathology of gouty arthritis in mice**. Mice were treated with Hesperidin Methyl-Chalcone (30 mg/kg/saline, p.o.) or vehicle 60 min before i.a injection of MSU (100 µg/10 µL), and after 15 h knee joints samples were collected to the histological analysis. Control (A), MSU (B), MSU and HMC (C) and Inflammatory infiltrate cells/field (D). All sections were stained with Hematoxylin and Eosin (magnification A-C, ×400) and the figure is representative of all experiment. Dimension used was 544 x 582 pixels for analysis (Field). Scale Barrs: 50µm. The arrow indicates representative infiltrate inflammatory cells counted. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

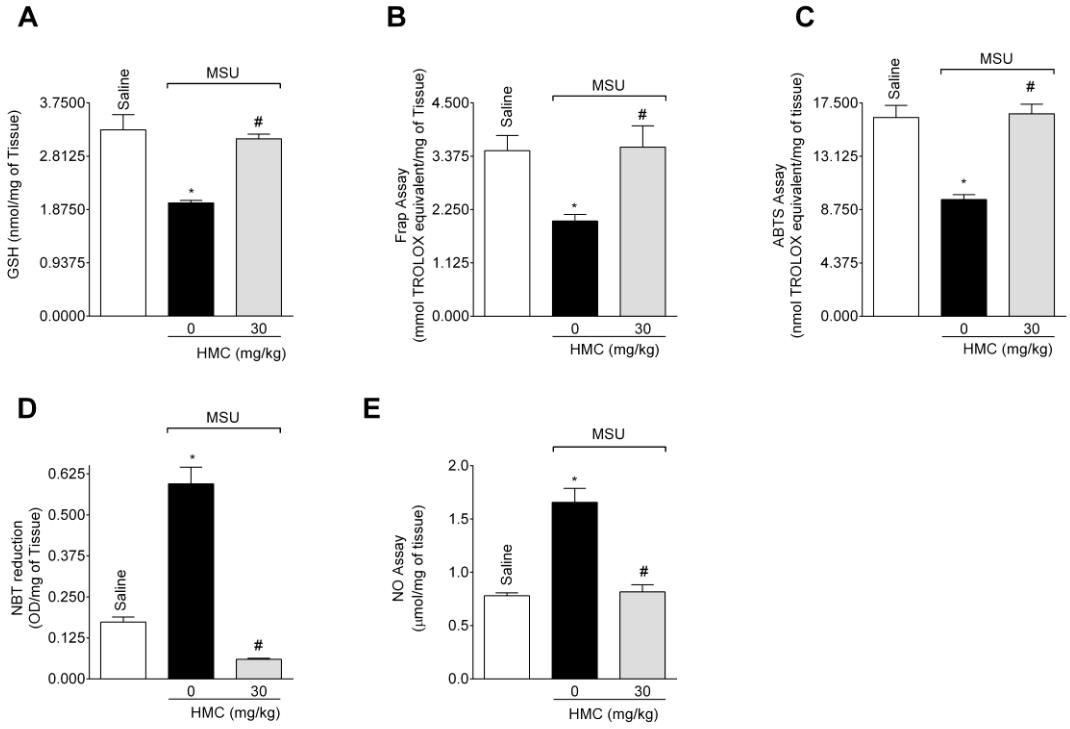

Fig. 4. Hesperidin Methyl-Chalcone prevented the Monosodium Urate Crystal (MSU)-induced reduction of GSH level and increase antioxidant capacity in FRAP, ABTS, NBT and NO assays. Mice were treated with hesperidin methyl-chalcone (30 mg/kg/saline, p.o.) or vehicle 60 min before i.a. injection of MSU (100 µg/10 µL), and after 15 h knee joints samples were collected for GSH level measurement (A), FRAP (Ferric Reducing Ability) (B), ABTS (Free Radical Scavenging Ability) (C), NBT (Nitroblue Tetrazolium Reduction) (D) and Nitric Oxide (E) assays. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

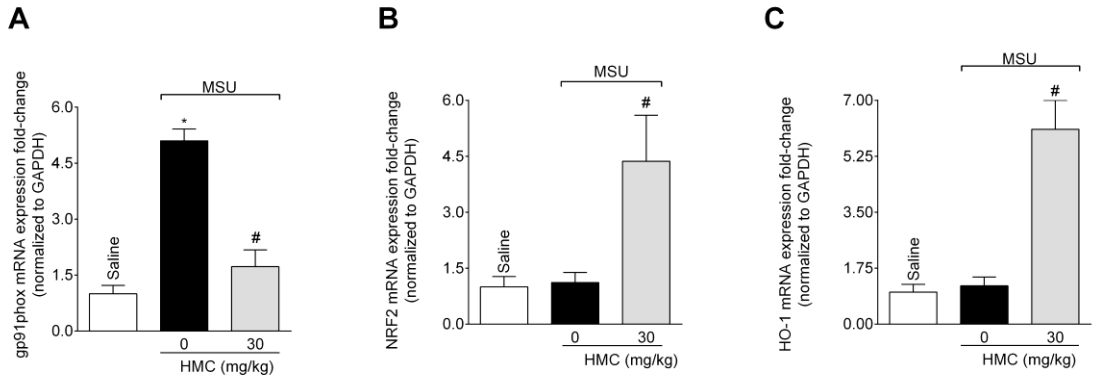

Fig. 5. Hesperidin Methyl-Chalcone inhibited gp91^{phox} and increase Nrf2 and HO-1, expression. The animals were treated with HMC (30 mg/kg/saline, p.o.) or vehicle 1 h before i.a. injection of MSU crystals, and after 15 h knee joints samples were collected in (TRIzol®Reagent) for the isolation of total RNA/DNA from cells and tissues to measure gp91^{phox} (A), Nrf2 (B) and HO-1 (C). GAPDH was a reference gene to normalize mRNA expression data. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. *$P < 0.05$ compared to saline group, and #$P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

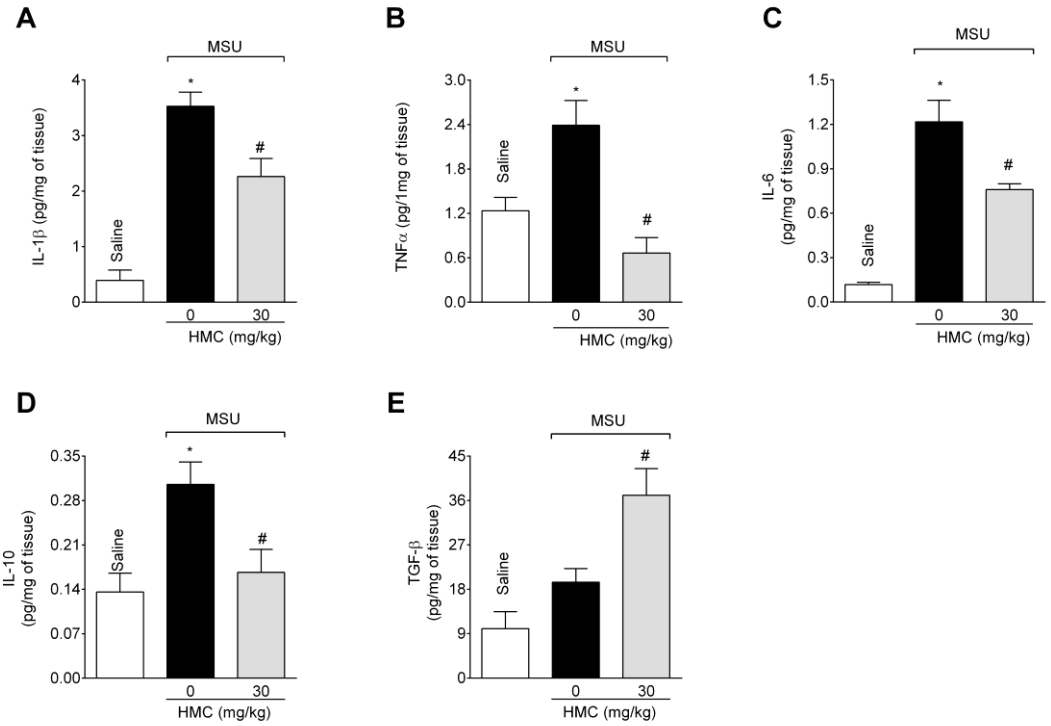

Fig. 6. Hesperidin Methyl-Chalcone inhibited MSU-induced IL-1β, TNFα, IL-6 and IL-10 and TGF-β increase production. Mice were treated with Hesperidin Methyl-Chalcone (30 mg/kg/saline, p.o.) or vehicle (saline) 1 h before MSU (100 μg/10 μL) i.a injection. After additional 15 h, articular joint samples were collected for the determination of IL-1β (A), TNFα (B), IL-6 (C), IL-10 (D) and TGF-β (E) production (Panels A, B, C, D and E, respectively). Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

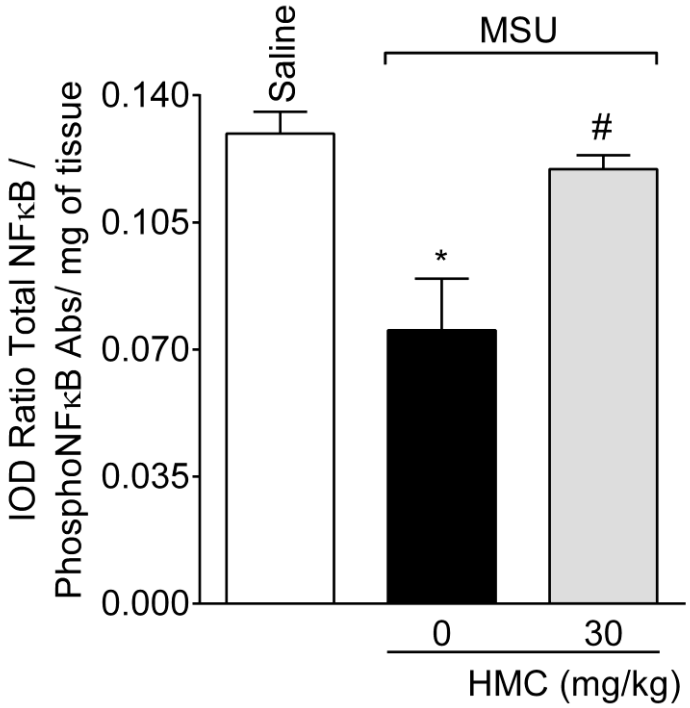

Fig. 7. Hesperidin Methyl-Chalcone inhibited Monosodium Urate Crystal-induced NFκB activation. The mice with gouty arthritis were treated with Hesperidin Methyl-Chalcone (30 mg/kg/saline, p.o.) or vehicle 1 h before i.a. injection of MSU, and after 15 h knee joints samples were collected in lysis buffer for measure the NF-κB activation. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

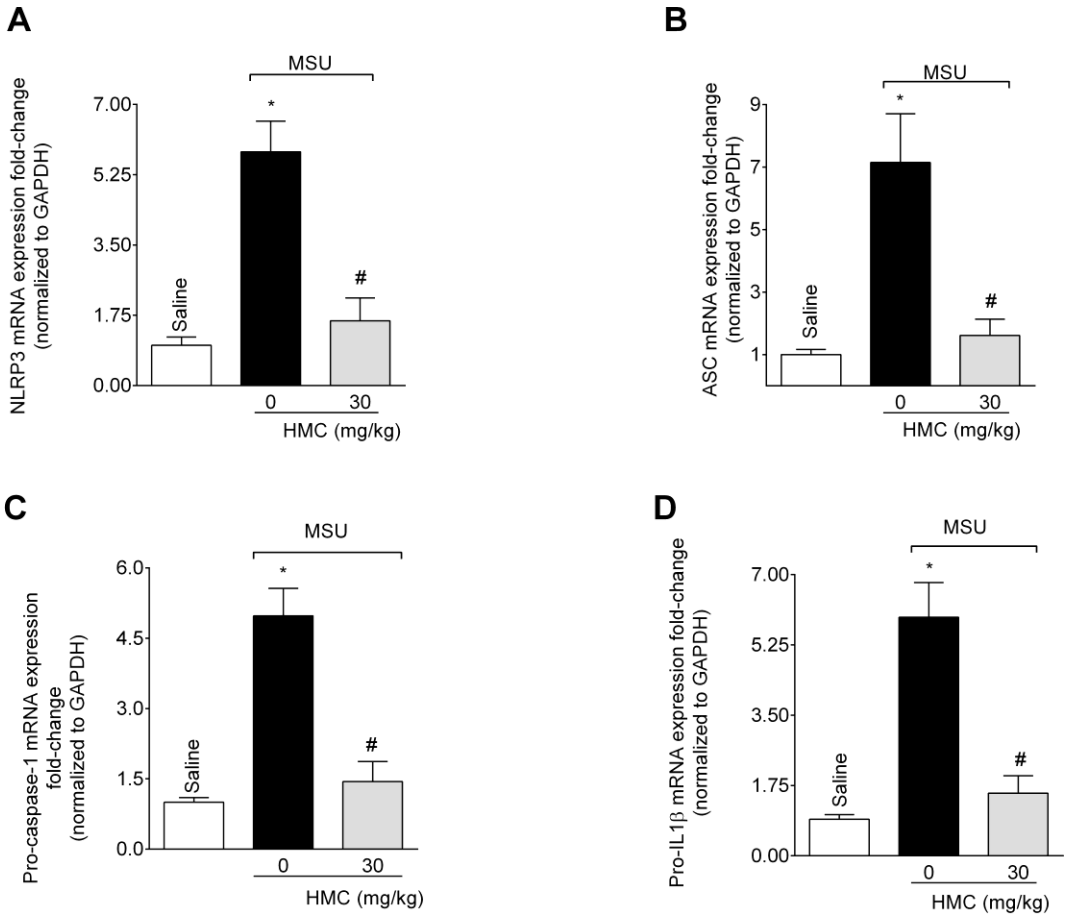

Fig. 8. Hesperidin Methyl-Chalcone inhibited NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β expression. The animals were treated with HMC (30 mg/kg/saline, p.o.) or vehicle 1 h before i.a. injection of MSU crystals, and after 15 h knee joints samples were collected in (TRIzol®Reagent) for the isolation of total RNA/DNA from cells and tissues to measure NLRP3 (A), ASC (B), Pro-caspase-1 (C) and Pro-IL-1β (D). GAPDH was a reference gene to normalize mRNA expression data. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. $^{*}P < 0.05$ compared to saline group, and $^{\#}P < 0.05$ compared to MSU + vehicle group. ANOVA followed by Tukey's test.

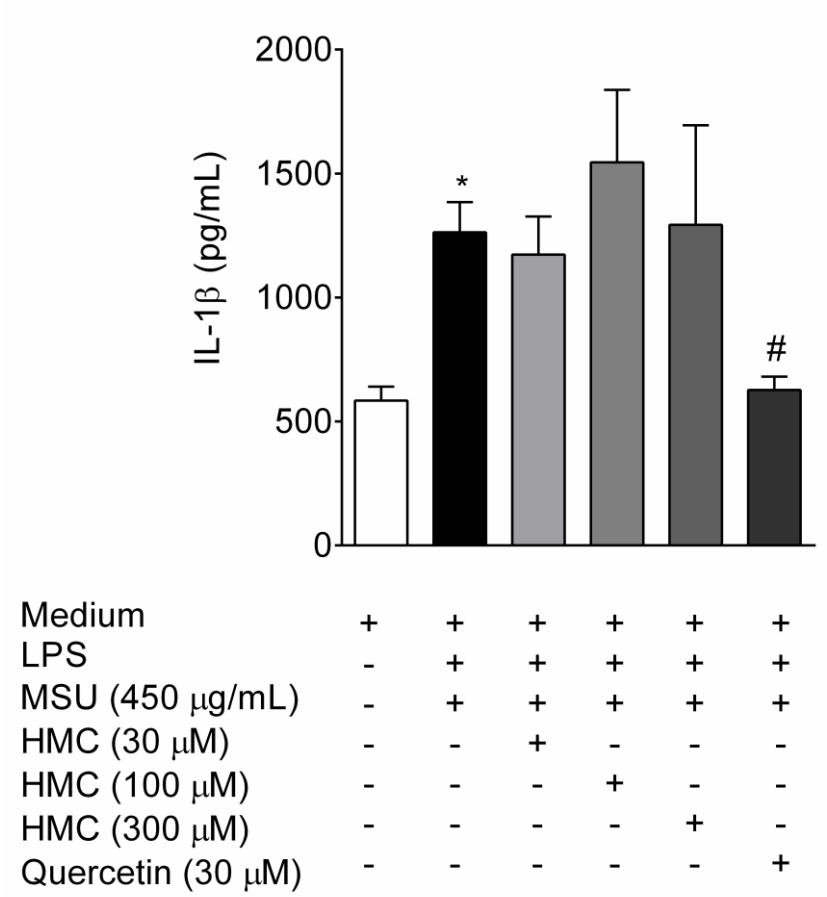

Fig. 9. Hesperdin Methyl-Chalcone not inhibits MSU-induced IL-1β release by supernatant of BMDMs macrophages. LPS (500 ng/ml)-primed BMDM were treated with HMC (30-300μM) or quercetin (30μM) 30 minutes before stimulation with MSU crystals (450 μg/mL) during 5 hours. IL-1β concentration in the culture supernatant was measured by ELISA. Results are means ± SEM are representative of or more independent experiments. *P < 0.05 compared to saline group, #P < 0.05 compared to MSU group. ANOVA followed by Tukey's t test.

ARTIGO 3- Quercetin inhibits gout arthritis in mice: induction of an opioid-dependent regulation of inflammasome

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Quercetin inhibits gout arthritis in mice: induction of an opioid-dependent regulation of inflammasome

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Abstract We investigated the anti-inflammatory and analgesic effects of quercetin in monosodium urate crystals (MSU)-induced gout arthritis, and the sensitivity of quercetin effects to naloxone, an opioid receptor antagonist. Mice were treated with quercetin, and mechanical hyperalgesia was assessed at 1-24h after MSU injection. *In vivo*, leukocyte recruitment, cytokine levels, oxidative stress, NFκB activation and $gp91^{phox}$ and inflammasome components (NLRP3, ASC, Pro-capase-1 and Pro-IL-1β) mRNA expression by qPCR were determined in the knee joints at 24h after MSU injection. Inflammasome activation was determined, *in vitro*, in lipopolysaccharide-primed macrophages challenged with MSU. Quercetin inhibited MSU-induced mechanical hyperalgesia, leukocyte recruitment, TNFα and IL-1β production, superoxide anion production, inflammasome activation, decrease of antioxidants levels, NFκB activation and inflammasome components mRNA expression. Naloxone pre-treatment prevented all the inhibitory effects of quercetin over MSU-induced gout arthritis. These results demonstrate that quercetin exerts analgesic and anti-inflammatory effect in the MSU-induced arthritis in a naloxone-sensitive manner.

Keywords: monosodium urate crystals; gouty arthritis; quercetin; NLRP3; inflammasome; cytokines; inflammatory pain.

INTRODUCTION

Gout is an arthropathy characterized by intense inflammation and pain induced by monosodium urate (MSU) crystal deposition into the articular joint and surrounding tissues (Khanna et al. 2012; McGettrick and O'Neill 2013). The interaction of MSU crystals with phagocytes such as macrophages and infiltrating leukocytes induces the secretion of various inflammatory mediators, consequently triggering a typical inflammatory response and increased pain sensitivity, provoking hyperalgesia (Verri et al. 2006; Martin et al. 2009; Zarpelon et al. 2013). It is well known that MSU crystals undergo phagocytosis followed by rupture of the phagolysosome and activation of NLRP3 inflammasome, which culminates in the release of the active form of IL-1β. It has also been reported that patients with gout arthritis show a significant response to IL-1 inhibition, indicating the crucial role of IL-1β in driving intense inflammatory pathology (Lu et al. 2014). Further, IL-1β activates synoviocyte-like macrophages promoting the release of additional pro-inflammatory cytokines such IL-8, IL-17 and TNFα (Martinon et al. 2006; Lu et al. 2014). Inflammatory cytokines such as IL-1β and TNFα activate the transcription factor NF-κB resulting in production of chemotactic molecules including IL-8 to induce neutrophil chemoattraction (Martinon et al. 2006; Neuschäfer-Rube et al. 2013; Dhanasekar et al. 2015).

The current therapeutic approaches against gouty arthritis are given mainly by the treatment of inflammation and the control of hyperuricemia. For instance, allopurinol acts by inhibiting hepatic xanthine oxidase enzyme (XOD) activity then reducing serum uric acid levels. However, allopurinol is not able to control the acute gout attacks (Dubchak and Falasca 2010), and also can induce several side effects such as fever, skin rashes, allergic reactions, hepatitis and nephropathy (Umamaheswari et al. 2009). Thus, non-steroidal anti-inflammatory (NSAIDs) drugs (e.g. Indomethacin) and alkaloid drugs (e.g. Colchicine) are frequently used as the first-line approach therapies for treatment of acute gout inflammation. Nevertheless, these classes of drugs induce severe side effects such as gastrointestinal and renal toxicity, and gastrointestinal bleeding (Cronstein and Terkeltaub 2006). Immunobiological therapies targeting IL-1β are also useful in the treatment of gout arthritis attack, but the high cost reduces its wide clinical use (Dubchak and Falasca 2010). Thus, the research on novel drugs to treat gout arthritis has been a growing focus of interest (Sabina et al. 2011; de Souza et al. 2012; Rukdee et al. 2015; Zhao and Huang 2015; Xu et al. 2016). Therapeutic natural compounds are a growing field since current therapies have relayed on natural compounds to develop efficacious treatments such as morphine, capsaicin, salicylate, and menthol (Julius 2013).

Flavonoids are multi-target molecules that inhibit inflammation and oxidative stress (Pinho-Ribeiro et al. 2015; Martinez et al. 2015a; Martinez et al. 2015b; Sun et al. 2016; Kim and Park 2016; Manchope et al. 2016; Liu et al. 2016). Evidence supports the safety of flavonoids due to low or no toxic effect contrasting to non-steroidal anti-inflammatory drugs (Okamoto 2005; Pinho-Ribeiro et al. 2016). Quercetin, a bioflavonoid presenting low toxicity (Okamoto 2005) and several biological properties such as antioxidant and anti-inflammatory (Valério et al. 2009; Wang et al. 2012; Guazelli et al. 2013; Sun et al. 2015a) has been a focus of varied studies on pain and inflammation (Anjaneyulu and Chopra 2003a; Valério et al. 2009; Souto et al. 2011; Calixto-Campos et al. 2015). Quercetin acts in an opioid-dependent manner inducing analgesia in diabetic- and cancer-induced pain (Anjaneyulu and Chopra 2003a; Calixto-Campos et al. 2015). Quercetin inhibits MSU-induced knee joint edema, histological leukocyte infiltration, IL-1β, TNFα, prostaglandin E2 and nitric oxide production, cyclooxygenase-2 expression and lipid peroxidation. Furthermore, quercetin treatment reverted

MSU-induced reduction of superoxide dismutase, catalase and glutathione peroxidase activities in rats (Huang et al. 2012). However, it remains to be determined the molecular mechanisms of gout arthritis physiopathology that are targeted by quercetin.

This study aimed to investigate the effect of quercetin treatment in experimental gout arthritis induced by intra-articular injection of MSU assessing its analgesic, anti-inflammatory and antioxidant effects, including the NFκB and NLRP3 inflammasome pathways. Hence, we evaluated the contribution of endogenous opioid in the effect of quercetin.

MATERIAL AND METHODS

Animals

The experiments were performed with male Swiss mice (20–25 g, Universidade Estadual de Londrina, Londrina, PR, Brazil) housed in standard clear plastic cages (5-6 per cage) with free access to food and water. All testing was performed between 9:00 am and 5:00 pm in a temperature-controlled room. Animal care and handling procedures were performed with the approval of the Ethics Committee of the Universidade Estadual de Londrina (process number 13279.2011.76) and followed the International Association for the Study of Pain guidelines as described by Zimmermann (1983). All efforts were made to minimize the number of animals used and their suffering.

Drugs and Reagents

The following materials were obtained from the sources indicated: Quercetin at 95% purity from Acros (Pittsburg, PA); MSU and naloxone were from Sigma-Aldrich (St Louis, MO); Boric acid was from Promega, and uricase/fasturtec was from Sanofi-Synthelabo. ELISA kits to determine murine TNF-α and IL-1β were obtained from eBioscience (San Diego, CA).

MSU crystal preparation

MSU crystals were prepared according to the method described previously (Nishimura et al. 1997). In brief, 800 mg of monosodium urate was dissolved in 155 mL of boiling water containing 5 mL of 1 N NaOH. After the pH was adjusted to 7,2, and the solution was gradually cooled by stirring at room temperature. The crystals were collected by centrifugation at 3,000 *g* for 2 min at 4◦C. The crystals were evaporated and sterilized by heating at 180◦C for 2 hours and stored in a sterile environment until use.

Experimental protocols

In the first set of experiments, mice were treated with quercetin (10-100 mg/kg, 20% Tween 80, s.c., 30 min) or vehicle (20% tween 80 in saline) before the MSU injection (100 μg/10 μL per knee joint) to determine the dose-response of quercetin (Fig. 1a). The intensity of mechanical hyperalgesia was measured 1-24 h after MSU injection by the electronic pressure-meter test. To investigate the participation of endogenous opioids in the analgesic and anti-inflammatory effects of quercetin, mice were pre-treated with naloxone (0.3-3 mg/kg, i.p., 1 h) before quercetin treatment (100 mg/kg, s.c.) or vehicle (saline). The inflammatory parameters were evaluated at 24 h after injection of the MSU crystals. After 24 h, the articular cavities were washed with

saline/EDTA solution for leukocytes counts and differential cell counts determination. The inflammatory cell infiltrate (histopathological analysis), cytokines, NFκB activation, inflammasome components mRNA expression (NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β), antioxidant capacity (GSH, FRAP and ABTS assay), superoxide anion production (NBT reduction), $gp91^{phox}$, Nrf2 and HO-1 mRNA expression by qPCR were determined in the articular tissue. *In vitro* (Fig. 1b), bone marrow-derived macrophages (BMDMs) were cultured in 96-well plates. The activation of BMDMs first signal was induced by LPS (500 ng/mL) stimulation during 3 hours. The participation of endogenous opioid in quercetin effect was assessed using naloxone (0.2-20 µM) treatment of BMDMs 1 h before quercetin treatment. In addition, quercetin (0.3-30 µM) and morphine (0.2-20 µM) treatments were performed 30 minutes before stimulation with MSU crystals (450 µg/mL - second signal) and incubated during 5 hours. ELISA was used to measure the IL-1β levels in the culture supernatant.

Electronic pressure-meter test (Mechanical hyperalgesia)

Evaluation of mechanical hyperalgesia was performed as described previously (Guerrero et al. 2006) using an electronic pressure meter (INSIGHT Instruments). The flexion withdrawal threshold was used to in behavioral responses associated with pain. Results are expressed in grams force (g).

Leukocyte total and differential counts

To determine the leukocyte recruitment to the articular cavities, joint cavities were washed 3 times with 3,3 µL of PBS containing 1 mM EDTA. The total numbers of leukocytes were determined in Neubauer chamber diluted in Turk's solution. Differential cell counts were determined in cytocentrifuge Rosenfeld stained slices (Cytospin 4; Shandon, Pittsburg, PA). Total and differential cell counts were performed with a light microscope and the results were expressed as the number (mean ± SEM) of total of leukocytes or neutrophils $x10^3$ per cavity (Verri et al. 2010).

Histopathological analysis

Mice were sacrificed 24 hours after MSU injection. The articular joint of mice were removed, fixed with 10% paraformaldehydein PBS, and then decalcified for 10 days with EDTA and embedded in paraffin for histological analysis. The paraffin sections were stained with hematoxylin and eosin for conventional morphological evaluation. Dimension used for the analysis was 616 x 662 pixels for analysis (Field) and magnification ×400 and the arrow indicate representative infiltrate inflammatory cells counted.

Cytokine production

At 24 h after the i.a. injection of MSU, animals were terminally anaesthetized, and the samples of knee joints were removed and homogenized in 500 µL of the appropriate buffer containing protease inhibitors. The TNF-α and IL-1β levels were determined as described previously (Verri et al. 2010) by enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions (eBioscience). The results were expressed as picograms (pg) of each cytokine per 100 milligrams of tissue weight. As a negative control, the concentrations of these cytokines were determined in mice injected with saline.

NF-κB activation

The articular joint samples were collected and homogenized in ice-cold lysis buffer (Cell Signaling). The homogenates were centrifuged (14000 rpm × 10 min × 4 °C), with the supernatants used to assess the levels of phosphorylated and total NF-κB p65 subunit by ELISA using PathScan® kits (Cell Signaling) according to the manufacturer's directions. The results represent the sample ratio (total p65/phospho-p65) measured at 450 nm (Multiskan GO Thermo Scientific).

Quantitative Polymerase Chain Reaction (qPCR)

The knee joint samples were collected at 24 h after the i.a. injection of MSU, animals were terminally anaesthetized and homogenized in 500 µL of Trizol reagent and centrifuged (12000 rcf x 15 min x 4 °C), and total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations and extracted by using the SV Total RNA Isolation System (Promega) (Verri et al. 2008). All reactions were performed in triplicate using the following cycling conditions: 50° C for 2 min, 95°C for 2 min, followed by 40 cycles of 95° C for 15 s and 60° C for 30 s. qPCR was performed in a LightCycler Nano Instrument (Roche, Mississauga, ON, USA) sequence detection system by using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, USA). The mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used were Gapdh forward: CAT ACC AGG AAA TGA GCT TG, reverse: ATG ACA TCA AGA AGG TGG TG; Nrf2, forward: TCA CAC GAG ATG AGC TTA GGG CAA, reverse: TAC AGT TCT GGG CGG CGA CTT TAT; Gp91phox, forward: AGC TAT GAG GTG GTG ATG TTA GTG G, reverse: CAC AAT ATT TGT ACC AGA CAG ACT TGA G; Nlrp3, forward: AGC TAT GAG GTG GTG ATG TTA GTG G, reverse: CAC AAT ATT TGT ACC AGA CAG ACT TGA G; HO-1, forward: CCC AAA ACT GGC CTG TAA AA, reverse: CGT GGT CAG TCA ACA TGG AT; Pro-caspase-1: forward: TGG TCT TGT GAC TTG GAG GA, reverse: TGG CTT CTT ATT GGC ACG AT; Pro-IL-1β, forward: GAA ATG CCA CCT TTT GAC AGT G, reverse: TGG ATG CTC TCA TCA GGA CAG; ASC, forward: ATG GGG CGG GCA CGA GAT G, reverse: GCT CTG CTC CAG GTC CAT CAC. The SYBR green PCR Master Mix was used according to the manufacturer's instructions.

Reduced glutathione (GSH) measurement

At 24 h after the i.a. injection of MSU, animals were terminally anaesthetized and the samples of knee joints were removed and maintained at -80°C for at least 48 h. The samples were homogenized with 200 µL of 0.02 M EDTA. The homogenate was mixed with 25 µL of 50 % trichloroacetic acid and was homogenized three times during 15 min. The mixture was centrifuged (15 min×1,500×g×4 °C). The supernatant was added to 200 µL of 0.2 M TRIS buffer, pH 8.2 and 10 µL of 0.01 M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve was performed with standard GSH. The results are expressed as GSH per milligram of tissue (Sedlak and Lindsay 1968; Borghi et al. 2013; Staurengo-Ferrari et al. 2014).

Nitrobluetetrazolium (NBT) test

The quantitation of O_2^- production in tissue homogenate (10 mg/mL in 1.15% KCl) was performed using the nitroblue tetrazolium test (NBT). Briefly, 50 µL of homogenate tissue was incubated with 100 µL of NBT (1 mg/mL) in 96-well plates at 37°C for 1 h. The supernatant was then removed and the reduced formazan solubilized by adding 120 µL of 2 M KOH and 120 µL of DMSO. The NBT reduction was measured using a Multiscan spectrophotometer at 600 nm. The results were expressed as optical density per mg of tissue.

Ferric-Reducing Ability Potential (FRAP) and Free-Radical Scavenging Ability (ABTS) Assays.

The samples of knee joints were collected, immediately homogenized with 500 µL of 1.15% KCl, and centrifuged (10 min × 200g × 4 °C). The ability of the sample to resist oxidative damage was determined as ferric-reducing ability using the FRAP assay and as free-radical scavenging ability using the ABTS assay (Borghi et al. 2013). For the FRAP assay, 50 µL of supernatant was mixed with 150 µL of deionized water and 1.5 mL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm. For the ABTS assay, the ABTS solution was diluted with phosphate buffer saline at pH 7.4 to an absorbance of 0.80 at 730 nm. Then, 1.0 mL of diluted ABTS solution was mixed with 20 µL of supernatant. After 6 min, the absorbance was measured at 730 nm. The results were equated against a Trolox standard curve (1.5−30 µmol/L, final concentrations). The results are expressed as Trolox equivalents per milligram of tissue weight in both assays.

Preparation of bone marrow-derived macrophages (BMDMs) and inflammasome activation assay

Femora and tibiae of mice C57BL/6 mice (8 weeks old) were aspirated with RPMI1640 media. Bone marrow cells were cultured in RPMI 1640 medium containing 10% FBS and 15% L929 cell conditioned medium. BMDM were harvested at day 7 and plated at the density of 1,5 x 10^5 cells/well in 96-well plate. BMDM were stimulated with 500 ng/mL *E. coli* LPS (Santa Cruz Biotechnology) and 3 h later treated with 450 µg/mL of MSU to stimulate NLRP3 inflammasome activation as described previously (Martinon et al. 2006). BMDM were treated with quercetin (0.3; 3 or 30 µM), or morphine (0.2; 2 or 20 µM), 30 min before MSU stimulation. Naloxone (0.2; 2 or 20 µM) was added 1 h prior to quercetin treatment (Khabbazi et al. 2015). Supernatants were collected 5 h after MSU stimulation and IL-1β concentration quantitated by ELISA

Data analyses

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 5.0). Results are presented as means +/- SEM of measurements made on 6 mice per group per experiment and are representative of two independent experiments. Two-way ANOVA was used to compare the groups and doses at all times when the parameters were measured at different times after the stimulus injection. The analyzed factors were treatments, time, and time versus treatment interaction. One-way ANOVA followed by Tukey's test was performed for each time-point. $P < 0.05$ was considered significant.

RESULTS

Quercetin inhibits MSU-induced mechanical hyperalgesia and leukocyte recruitment in gout arthritis. Quercetin decreased the parameters of mechanical hyperalgesia and leukocyte recruitment, however, the dose of 10 mg/kg of quercetin affect MSU-induced mechanical hyperalgesia only at 1 h after MSU injection. On the other hand, the dose of 30 mg/kg inhibited at 1, 5, 7 and 24 h, and the dose of 100 mg/kg inhibited all points evaluated of mechanical hyperalgesia (Fig. 2a). Only dose of 100 mg/kg of quercetin inhibited the MSU-induced recruitment of total of leukocytes and neutrophils to the knee joint (Fig. 2b-c). Based on the results of figure 2, the dose of 100 mg/kg of quercetin was selected for next experiments (Fig. 2a-b).

Quercetin inhibits MSU-induced mechanical hyperalgesia and leukocyte recruitment in a naloxone-sensitive manner. The dose of 0.3 mg/kg of naloxone was ineffective in inhibiting the antinociceptive effect of quercetin in mechanical hyperalgesia test. On the other hand, the doses of 1 and 3 mg/kg of naloxone prevented since the 1st h the antinociceptive effect of quercetin (Fig. 3a) in MSU-induced mechanical hyperalgesia. The pre-treatment with naloxone also reversed the quercetin inhibition of MSU-induced recruitment of total leukocytes (Fig. 3b) and neutrophils (Fig. 3c) only at the dose of 3 mg/kg. Thus, the quercetin inhibition of MSU-induced mechanical hyperalgesia and leukocyte recruitment depends on activation of opioid receptors.

Quercetin reduces the synovitis in articular joint-induced by gouty arthritis in a naloxone-sensitive manner. Quercetin inhibits MSU-induced synovitis in articular joint, decreasing the inflammatory infiltrate cells (Fig. 4 a-f). In addition, quercetin effect was reversed by pre-treatment with naloxone at dose 3 mg/kg in a sensitive manner (Fig. 4f). The treatment with naloxone did not present effect per se.

Quercetin inhibits MSU-induced TNF-α and IL-1β production in a naloxone-sensitive manner. Quercetin inhibited MSU-induced production of TNF-α (Fig. 5a) and IL-1β (Fig. 5b). In turn, naloxone pre-treatment prevented quercetin inhibition of MSU-induced TNF-α and IL-1β production in the knee joints at 24 h (Fig. 5a-b). The treatment with naloxone did not present effect per se.

Quercetin inhibits MSU-induced NFκB activation in a naloxone-sensitive manner. Quercetin inhibited MSU-induced NFκB activation as observed by an increase of total NFκB/phosphoNFκB ratio. In turn, naloxone treatment prevented quercetin inhibition of MSU-induced NFκB activation (Fig. 6). The treatment with naloxone did not present effect per se.

Quercetin inhibits MSU-induced NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β mRNA expression in a naloxone-sensitive manner. Quercetin inhibited MSU-induced mRNA expression of the inflammasome components NLRP3, ASC, pro-caspase-1 and pro-IL-1β (Fig. 7a-d) in the knee joints at 24 h post-stimulus injection as determined by qPCR. On the other hand, naloxone treatment prevented quercetin inhibition of MSU-induced mRNA expression of inflammasome components.

Quercetin inhibits MSU-induced decrease of antioxidant capacity, superoxide anion production and gp91phox mRNA expression in a naloxone-sensitive manner. Quercetin inhibited MSU-induced reduction of

antioxidant defenses as observed by a maintenance of GSH levels (Fig. 8a), ferric-reducing ability potential (FRAP assay – Fig. 8b), free radical scavenging ability (ABTS assay – Fig. 8c). Quercetin also inhibited MSU-induced NBT reduction (superoxide production – Fig. 8d) assessment and gp91phox mRNA expression (Fig. 8e) in the knee joints. In turn, quercetin effects were inhibited by naloxone treatment (Fig. 8a-e). The treatment with naloxone did not present effect per se.

Quercetin induces Nrf2 and HO-1 mRNA expression in MSU-induced arthritis in a naloxone-sensitive manner. MSU stimulus did not alter Nrf2 (Fig. 9a) or HO-1 (Fig. 9b) mRNA expression in the knee joints. On the other hand, quercetin induced significant increase of Nrf2 (Fig. 9a) or HO-1 (Fig. 9b) mRNA expression, which was inhibited by naloxone treatment.

Quercetin inhibits MSU-induced IL-1β levels in BMDMs supernatant in gouty arthritis in a naloxone-sensitive manner *in vitro*. LPS priming (first signal) followed by MSU stimulation (second signal) induced an increase of IL-1β levels in BMDM, which was inhibited by quercetin in a dose dependent manner (Fig. 10a). Quercetin at the concentration of 30 μM prevented inflammasome activation by MSU crystals decreasing IL-1β levels in BMDMs supernatant. In a similar way, morphine treatment also inhibited NLRP3 activating when stimulated with MSU crystals at the concentration of 20 μM (Fig. 10c). In turn, NLPR3 inflammasome stimulation in the presence of naloxone at concentration (20 μM) reversed the quercetin effect (Fig. 10b).

Discussion

Monosodium urate (MSU) crystals activate phagocytes in the joints leading to the development of inflammatory response due to activation of NF-κB and NLRP3 inflammasome. These signaling pathways promote, for instance, the production of IL-1β resulting in the influx of neutrophils into the affected articular space. In fact, neutrophil recruitment is a major feature of acute gout arthritis provoking synovial membrane damage and release of lysosomal enzymes, elastase, oxygen-derived free radicals, chemotactic factors, and pro-inflammatory cytokines, which lead to enhanced inflammatory response and pain (Desaulniers et al. 2001; Cronstein and Terkeltaub 2006; Huang et al. 2012). The present data show that MSU-induced gout arthritis promotes mechanical hyperalgesia, leukocyte recruitment, TNF-α and IL-1β cytokine production as well as NFκB activation, up regulation of NLRP3, ASC, Pro-caspase-1 and pro-IL-1β mRNA expression and oxidative stress. All these MSU-induced physiopathological alterations in the joints were inhibited by the flavonoid quercetin in a naloxone-sensitive manner.

Quercetin presents analgesic effect, decreasing the pain threshold and leukocyte recruitment in varied pain models such as Ehrlich tumor-induced cancer pain (Calixto-Campos et al. 2015), streptozotocin-induced diabetic neuropathic pain (Anjaneyulu and Chopra 2003a), superoxide anion-induced pain (Maioli et al. 2015), lipopolysaccharide (LPS)-induced hyperalgesia (Anjaneyulu and Chopra 2003b), and carrageenan-induced hyperalgesia (Valério et al. 2009) and leukocyte recruitment (Souto et al. 2011). Therefore, the analgesic effect of quercetin is consistent among varied models. However, it was not known, to our knowledge, whether quercetin would have analgesic effect in the gout arthritis model. Quercetin treatment inhibited MSU-induced mechanical hyperalgesia at all time-points concomitantly with a reduction of leukocyte recruitment in a dose-dependent manner. Despite the description that recruited neutrophils contribute to inflammatory hyperalgesia

(Cunha et al. 2008), the inhibition of hyperalgesia and neutrophil recruitment lined up with the dose of quercetin of 100 mg/kg while the dose of 30 mg/kg inhibited only the MSU-induced hyperalgesia, which suggests that the inhibition of neutrophil recruitment and hyperalgesia are not fully interdependent. There is evidence that quercetin inhibits MSU-induced edema and leukocyte recruitment by inhibiting cytokine production and oxidative stress (Huang et al. 2012).

Previous studies identified that the analgesic effect of quercetin depends on endogenous opioids and therefore, is reversed by the opioid receptor antagonist naloxone (Anjaneyulu and Chopra 2003a; Calixto-Campos et al. 2015; Maioli et al. 2015). However, it is unknown if this is the mechanism by which quercetin inhibits MSU-induced inflammation. In the present study, naloxone reversed in a dose-dependent manner the quercetin reduction of MSU-induced mechanical hyperalgesia and leukocyte recruitment. Opioids can act by three different mechanisms. First, opioids inhibit neuronal adenilate cyclase resulting in the reduction of PGE_2-induced activation of adenilate cyclase and hyperalgesia; second, opioids act on peripheral nociceptor neurons activating the phosphatidylinositol 3-kinase (PI3K)g/protein kinase B (AKT)/neuronal nitric oxide synthase (nNOS)/ nitric oxide (NO)/cyclic guanosine monophosphate-dependent protein kinase (PKG)/ATP-sensitive potassium channel (KATP) signaling inducing hyperpolarization of nociceptors (Cunha et al. 2010); and third, opioids inhibit cytokine production and the consequent hyperalgesia and neutrophil recruitment (Wang et al. 2005; Clark et al. 2007; Martin et al. 2010).

In experimental conditions related to opioid tolerance, opioids can enhance pain through increased TLR4 signaling and cytokine production (Ellis et al. 2016; Liang et al. 2016). On the other hand, in regular clinical conditions, opioids induce analgesia and inhibit inflammation. For instance, the κ-opioid receptor agonist U50,488H inhibited myocardial ischemia and reperfusion injury-induced TLR4 expression, NFκB activation, myeloperoxidase (MPO) activity, and TNF-α production (Cai et al. 2014). The μ opioid receptor agonist morphine reduces TLR4 expression by macrophages. In turn, the μ opioid receptor antagonist naltrexone increases macrophage TLR4 expression, which indicates endogenous role of μ opioid receptors in regulating TLR4 expression (Franchi et al. 2012). Thus, opioid receptor activation has been related to consistent anti-inflammatory effects.

The anti-inflammatory and analgesic effects of quercetin depend on inhibiting NFκB activation (Chen et al. 2005; Vicentini et al. 2011; Borghi et al. 2016), which corroborates the quercetin inhibition of MSU-induced cytokine production in rats and mice (Huang et al. 2012). Nevertheless, our data expand these findings demonstrating that quercetin inhibition of NFκB activation is naloxone-sensitive and is an underlying mechanism by which quercetin reduced MSU-induced inflammation and pain. As a result of NFκB inhibition, quercetin inhibited MSU-induced NLRP3, ASC, pro-caspase-1, and pro-IL-1β mRNA expression.

In BMDM primed with LPS, the challenge with MSU induces IL-1β release in the supernatant, which was inhibited by quercetin in a naloxone-sensitive manner. The *in vitro* system used in the present study allows to determine whether after signal 1 activation with LPS, a test drug can inhibit signal 2 that results in IL-1β maturation and release in the supernatant of cell culture (Martinon et al. 2006). The present data shows that quercetin inhibits both signal 1 and 2, which explains the major anti-inflammatory and analgesic effect of quercetin in MSU-induced arthritis. Quercetin also inhibited NLRP3 inflammasome components expression and decreased IL-1β production in models of hepatic inflammation (Zhang et al. 2015), carrageenan-induced paw inflammation (Valério et al. 2009), renal injury (Hu et al. 2012) and spinal cord injury (Zhou et al. 2010; Jiang et

al. 2016) although these studies did not investigate whether quercetin would be able to inhibit signal 2 as performed in the present work. In agreement with the present data, it has been recently demonstrated that quercetin inhibits ATP, nigericin, alum (NLRP3 activators) and double strand DNA (AIM2 activator)-induced inflammasome activation by diminishing ASC oligomerization (Domiciano et al. 2017).

Reactive oxygen species (ROS) contribute to acute and chronic inflammation (Rasool and Varalakshmi 2006). MSU-induced arthritis promoted oxidative stress observed as decrease of GSH levels, FRAP and ABTS activities, and increase of NBT reduction (superoxide anion production) and $gp91^{phox}$ mRNA expression. Quercetin inhibited MSU-induced oxidative stress in a naloxone-sensitive manner. Quercetin has inherent antioxidant chemical groups that can explain its antioxidant effects *in vitro* and *in vivo* (Verri et al. 2012). This flavonoid inhibits ROS such as hydrogen peroxide (H_2O_2) (Boumaza et al. 2016) and decreases $gp91^{phox}$ gene expression (Sun et al. 2015b; Borghi et al. 2016) in models of inflammation, and also attenuates lipid peroxidation and increases antioxidant enzymes in gout arthritis models in rats (Huang et al. 2012). Importantly, quercetin acting on oxidative stress sensitive signaling pathways induces Nrf2 and HO-1 gene expression (Yao et al. 2007; Liu et al. 2015) enhancing the expression of multiple antioxidant enzymes such as SOD and GSH, thus, decreasing oxidative stress (Jung and Kwak 2010; Bryan et al. 2013), which contributes to the resolution of oxidative stress damages. The sensitivity to naloxone treatment suggests that the antioxidant effect of quercetin is not solely dependent on its antioxidant chemical groups in the MSU-induced arthritis. In fact, quercetin treatment induced an increase of Nrf2 and its downstream target HO-1 mRNA expression. Nrf2 also has an effect in the increase of GSH levels. Moreover, naloxone inhibited quercetin induction of Nrf2 and HO-1 mRNA expression. Thus, in addition to the quercetin antioxidant effects dependent on its chemical groups, triggering an endogenous opioid system might account to the antioxidant effect of quercetin in reducing the MSU-induced inflammasome activation and arthritis.

The opioid receptors have been reported to present crucial role in oxidative stress in several models by acting on inhibition of H_2O_2 *in vitro* (Sazonova et al. 2016), inducing gastric protection (Ribeiro et al. 2016) and reducing NSAID-induced oxidative stress (Yin et al. 2015). Opioid receptor agonists have potent antioxidant effects by inhibiting the generation of ROS such as inducible nitric oxide synthase (iNOS) by modulation of sigma-1 receptor (σ1R) in lipopolysaccharide (LPS)-stimulated BV2 microglia (Wu et al. 2015). In line with the current findings and contribution of the opioid system to quercetin effects, quercetin inhibits interstitial cells of Cajal (ICCs) pacemaker activities via opioid receptors in cultured murine ICCs and decreases oxidative stress (Gim et al. 2015). Other flavonoids have been reported to reduce oxidative stress by acting on opioid receptors such as kappa and delta-opioid receptors (Meotti 2005; Donato et al. 2014; Rosales et al. 2014).

The δ-opioid receptor-mediated the increase in Nrf2 translocation promoting a cytoprotection against hypoxic injury *in vitro*, reducing the oxidative molecules and oxidative damage of cells (Cao et al. 2015). Activation of other opioid receptors such as σ1R protects against cellular oxidative stress and activates antioxidant responsive elements (ARE), increases the levels of the antioxidant protein peroxiredoxin 6 (Prdx6), endoplasmic reticulum (ER) chaperone BiP (GRP78), NAD(P)H quinone oxidoreductase 1 (NQO1) and superoxide dismutase 1 (SOD1) mRNA expression in COS cells (Pal et al. 2012). Thusly, it is well established that antioxidant response element (ARE) activation triggers the transcription of genes involved in antioxidant defense by a mechanism involving the Nrf2 and KEAP1 pathway (Muthusamy et al. 2012; Gan and Johnson 2014). In models of retinal disease, the σ1R increased Nrf2 mRNA expression and decrease oxidative stress in

Müller glia cells, thereby demonstrating a major contribution of σ1R as a mediator of the key antioxidant pathway Nrf2/Keap1 (Wang et al. 2015) and endoplasmic reticulum (ER) stress genes (BiP/GRP78, Atf6, Atf4, Ire1α) (Ha et al. 2014).

It is noteworthy to mention that in the Ehrlich tumor-induced pain model in mice, quercetin inhibited tumor-induced hyperalgesia, paw skin myeloperoxidase activity (an indirect marker of neutrophil and macrophage recruitment), oxidative stress and cytokine production. However, naloxone treatment reversed only the analgesic effect of quercetin (Calixto-Campos et al. 2015). A likely explanation for this lack of effect of naloxone contrasting with the present data is that quercetin treatment was performed during eight days whilst naloxone treatment was performed only at the 8th day post-tumor inoculation (Calixto-Campos et al. 2015), and in the present study, both quercetin and naloxone were administrated once. Furthermore, the dose of naloxone used in the Ehrlich tumor model was three fold lower than in the present study. These differences in treatment protocols (number of treatments and doses) in addition to the differences in the physiopathology of the experimental models (tumor-induced pain *versus* innate immune response-induced pain) and time-course of disease might explain the contrasting results.

In conclusion, the present data demonstrate that quercetin inhibits MSU-induced gout arthritis diminishing mechanical hyperalgesia and leukocyte recruitment. These anti-inflammatory and analgesic effects of quercetin were accompanied by inhibition of NFκB activation, which is a likely explanation for the reduction of TNF-α and IL-1β production as well as inflammasome components and $gp91^{phox}$ mRNA expression. Furthermore, quercetin inhibited MSU-induced IL-1β release by LPS-primed macrophages, which suggests that this flavonoid inhibits NRLP3 inflammasome activation. Quercetin also induced Nrf2 and HO-1 mRNA expression that contributed to the antioxidant effect. The analgesic and anti-inflammatory effects of quercetin were sensitive to naloxone treatment, suggesting that quercetin induces endogenous opioid-dependent mechanisms regulating NFκB and Nrf2 transcription factors-dependent pathways as well as inflammasome activation. In fact, morphine was also capable of inhibiting MSU-induced IL-1β release by LPS-primed macrophages. Therefore, quercetin deserves further investigation as a potential treatment for acute gout arthritis.

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Conflict of interest

The authors declare no conflict of interest.

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1 **Figure Captions**

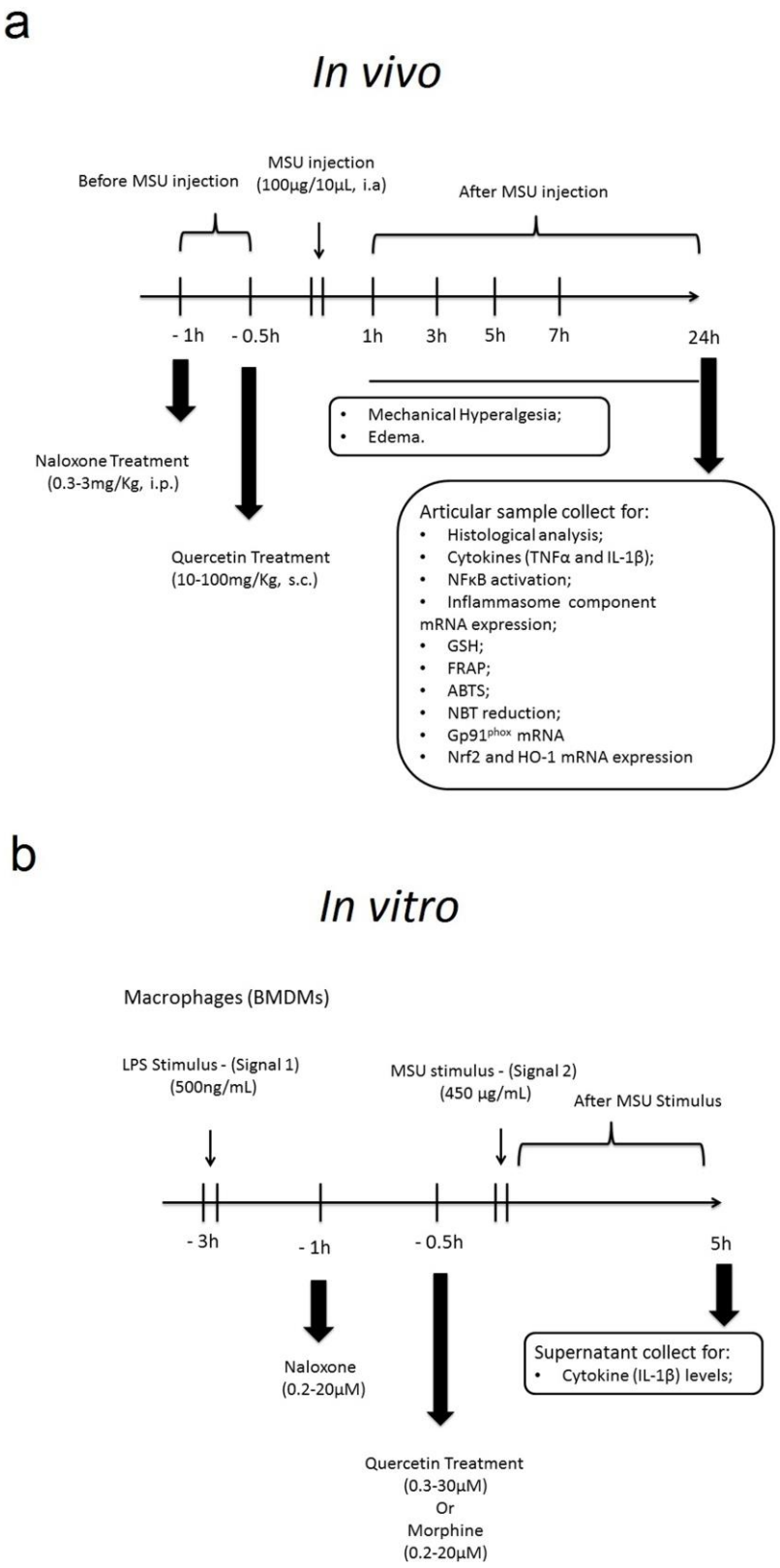

Fig. 1 Schematic experimental protocols. . *In vivo* (a): Mice were anesthetized (10 mg/kg ketamine:xylazine injected intraperitoneally [i.p.]) and were injected with MSU crystals (100 µg/10 µL) into the tibio-femoral knee joint. In the first set of experiments, mice were treated with quercetin (10-100 mg/kg, s.c. [Subcutaneous], 30 min) or vehicle (20% tween 80 in saline) before MSU injection. The intensity of mechanical hyperalgesia was measured 1-24 h after MSU injection by the electronic pressure-meter test. To determine the participation of endogenous opioid on quercetin analgesic and anti-inflammatory effect, mice were pre-treated with naloxone (0.3-3 mg/kg, i.p., 1 h) before quercetin treatment (100 mg/kg, s.c.) or vehicle. The inflammation parameters were evaluated at 24h after injection of the MSU crystals. After 24 h, the articular cavities were washed with saline/EDTA solution for leukocytes counts and differential determination. Periarticular tissues were removed from the joints for evaluation of synovitis in articular tissue, cytokines, NFκB activation, inflammasome components mRNA expression (NLRP3, ASC, Pro-caspase-1 and Pro-IL-1β), evaluation of antioxidant capacity (GSH, FRAP and ABTS assay), superoxide anion production (NBT reduction), $Gp91^{phox}$, Nrf2 and HO-1 by qPCR. *In vitro* (b): The macrophages BMDMs were performed in 96 wells plate, LPS (500 ng/mL)-primed BMDM was incubated during 3 hours, after that naloxone (0.2-20 µM) incubated with BDMNs cells 1 hours before quercetin treatment, in addition, quercetin (0.3-30 µM) or morphine (0.2-20 µM) treatment performed during 30 minutes before stimulation with MSU crystals (450 µg/mL) and incubated during 5 hours. IL-1β levels in the culture supernatant were measured.

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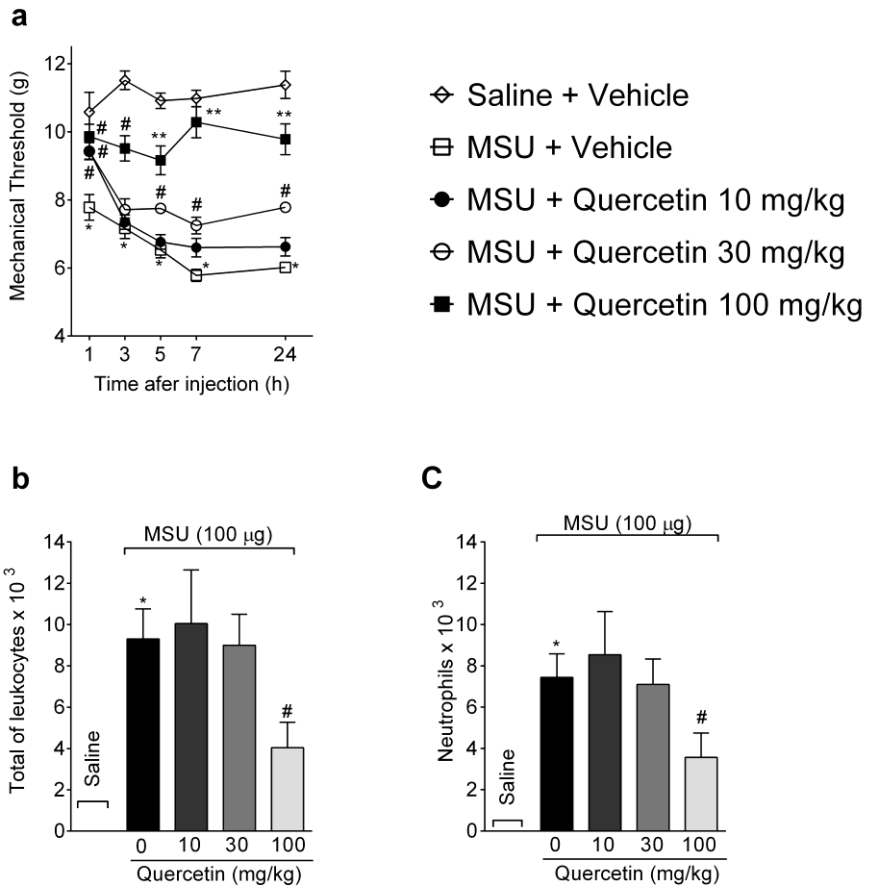

Fig. 2 Quercetin dose-dependently reduces monosodium urate crystal (MSU)-induced mechanical hyperalgesia and leukocytes migration. Treatment with quercetin (10, 30 and 100 mg/kg, s.c.) or vehicle (Tween 80 plus saline) was performed 30 minutes before stimulus with MSU (100 μg/10μL, intra-articular [i.a.]). The mechanical hyperalgesia (a) was evaluated 1-24 h after stimulus injection with an electronic pressure meter test. The total of leukocytes (b) and neutrophils (c) counts were determined using Newbauer chamber and Rosenfelt stained slices 24 h after MSU injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared with MSU + vehicle group and ***P* < 0.05 compared with 10 mg/kg of Quercetin. ANOVA followed by Tukey's test.

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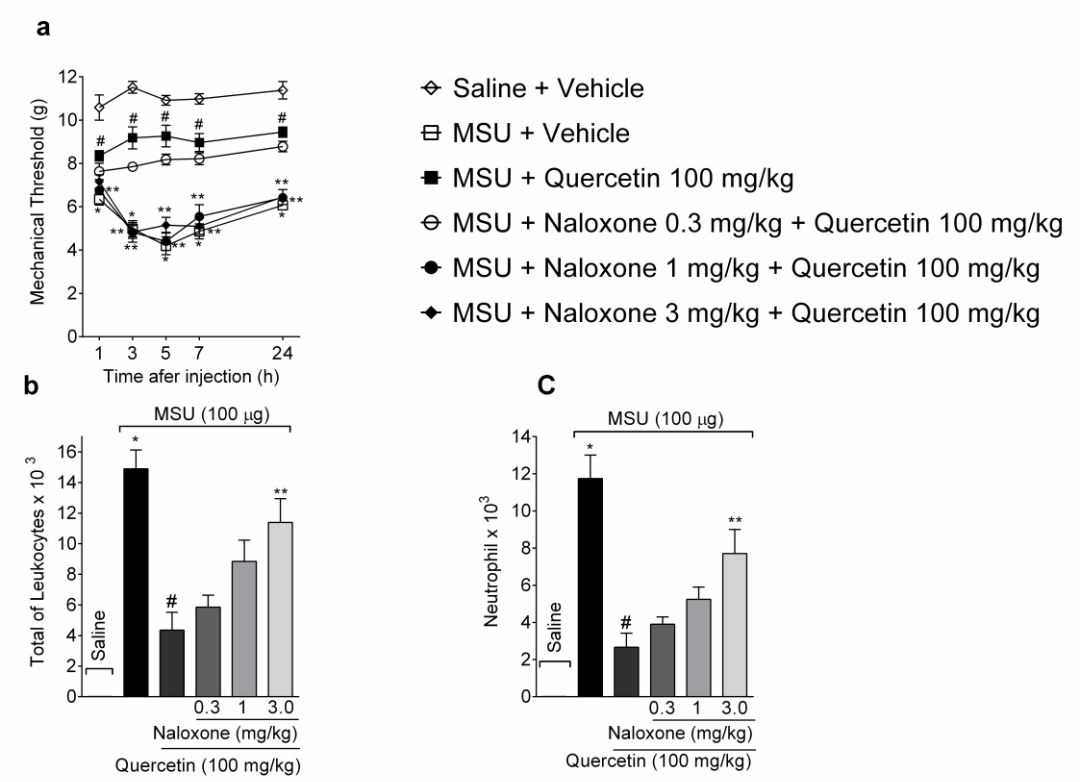

Fig. 3 Quercetin inhibited MSU-induced mechanical hyperalgesia and leukocyte recruitment in an opioid-dependent manner. Treatment with naloxone (0.3-3 mg/kg/saline, i.p.) was performed 1h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 min, mice received MSU injection (100 μg/10 μL, i.a.). The mechanical hyperalgesia (a) was evaluated 1-24 h after stimulus injection with an electronic pressure meter test. The total of leukocytes (b) and neutrophils (c) counts were determined using Newbauer chamber and Rosenfelt stained slices 24 h after MSU injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared to MSU, ***P* < 0.05 compared to quercetin group. ANOVA followed by Tukey's test.

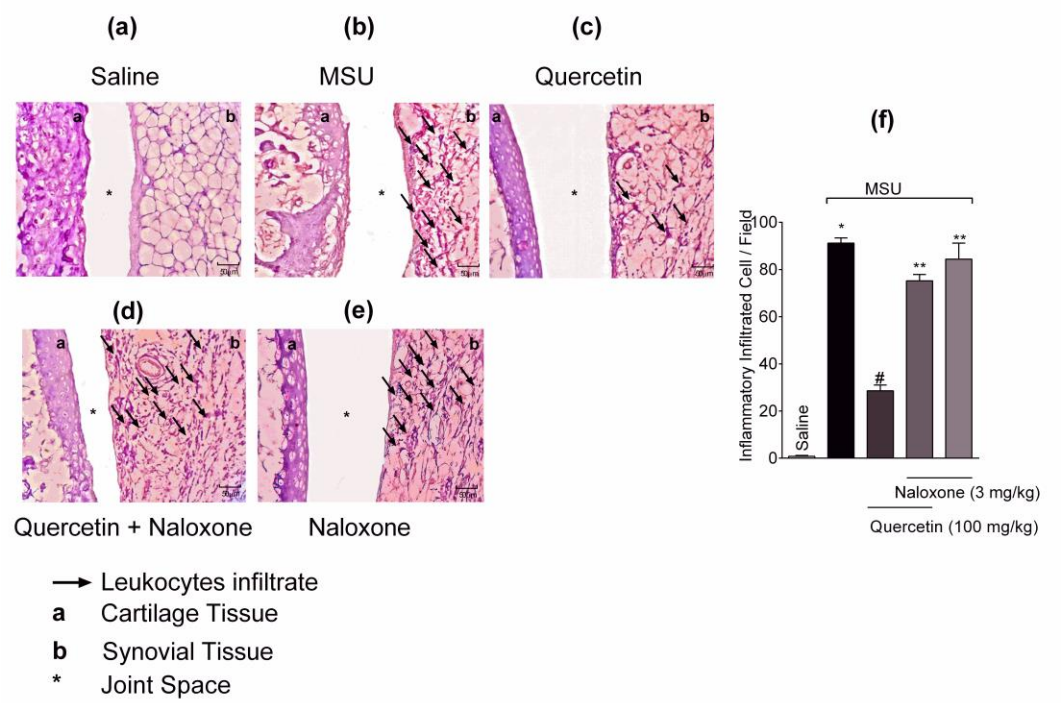

Fig. 4 Quercetin inhibits inflammatory infiltrate cells on synovial tissue in an opioid-dependent manner. Treatment with naloxone (3 mg/kg, i.p.) was performed 1 h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 minutes, mice received MSU injection (100 µg/10 µL, i.a.). The joint samples were collected at 24 hours after MSU injection. Control (a), MSU (b), MSU and quercetin (c), MSU and quercetin and naloxone (d) and MSU and naloxone (e) and inflammatory infiltrate cells analysis/fields (f). All sections were stained with Hematoxylin and Eosin (magnification a-f, ×400) and the figure is representative of all experiment. Dimension used was 616 x 662 pixels for analysis (Field). Scale Barrs: 50 µm. The arrow indicates representative infiltrate inflammatory cells counted. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared to MSU group and ***P* < 0.05 compared with Quercetin group. ANOVA followed by Tukey's t test.

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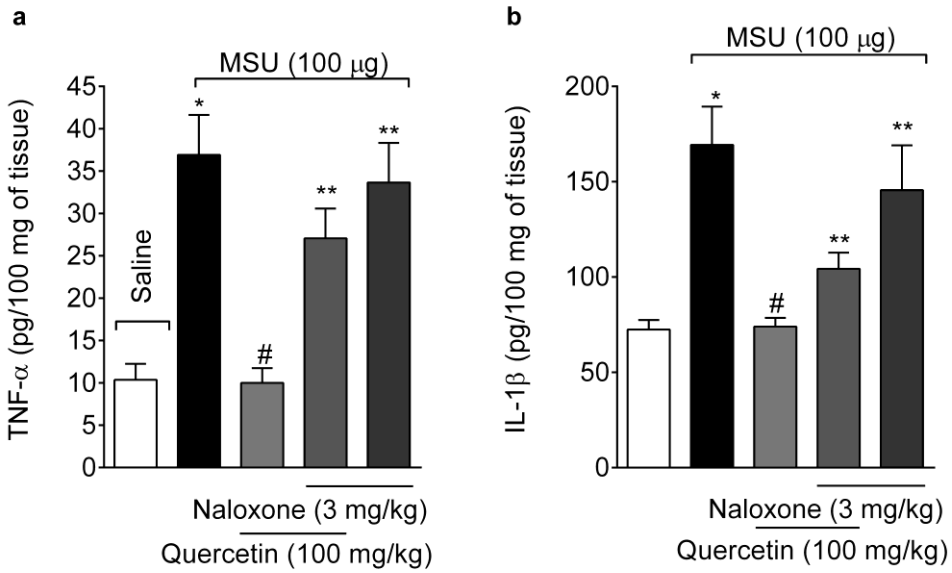

Fig. 5 Quercetin inhibited MSU-induced TNF-α and IL-1β production in joint in an opioid-dependent manner. Treatment with naloxone (3 mg/kg, i.p.) was performed 1 h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 minutes, mice received MSU injection (100 μg/10 μL, i.a.). The joint samples were collected at 24 hours after MSU injection, and TNF-α (a) and IL-1β (b) levels were determined by ELISA method. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared to MSU group and ***P* < 0.05 compared with Quercetin group. ANOVA followed by Tukey's t test.

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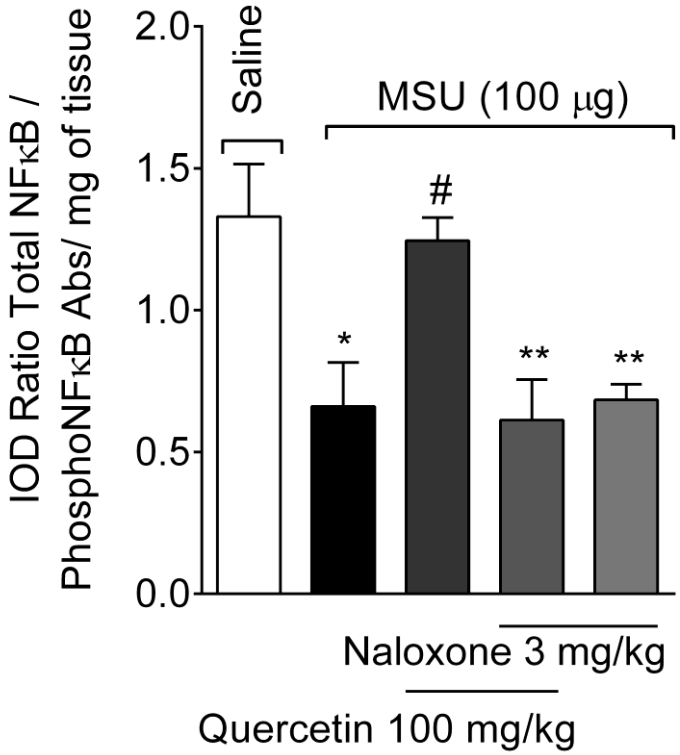

Fig. 6 Pre-treatment with naloxone reversed quercetin inhibition of MSU-induced NFκB activation. Treatment with naloxone (3 mg/kg, i.p.) was performed 1 h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 minutes, mice received MSU injection (100 μg/10μL, i.a.). The joint samples were collected at 24 hours after MSU injection, and total NFκB/phospho NFB ratio (activation) was determined by ELISA method. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared to MSU group and ***P* < 0.05 compared with Quercetin group. ANOVA followed by Tukey's t test.

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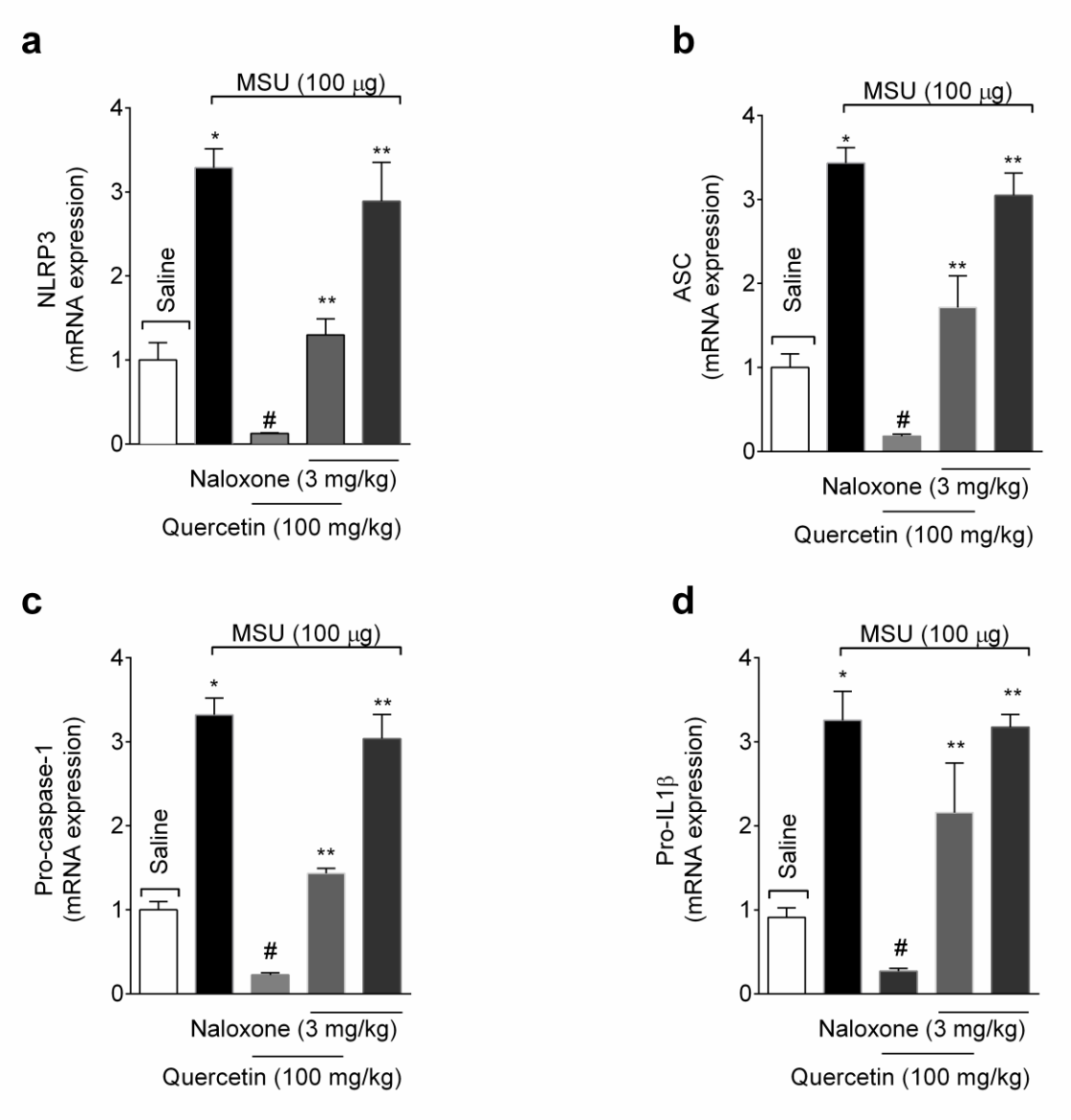

Fig. 7 Naloxone treatment reversed quercetin inhibition of MSU-induced NLRP3 inflammasome components mRNA expresion. Treatment with naloxone (3 mg/kg/saline, i.p.) was performed 1h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 minutes, mice received MSU injection (100 μg/10μL). The NLRP3 (a), ASC (b), Pro-caspase-1 (c) and Pro-IL-1β (d) mRNA expression were evaluated 24 h after MSU injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared to MSU group and ***P* < 0.05 compared with Quercetin group. ANOVA followed by Tukey's t test.

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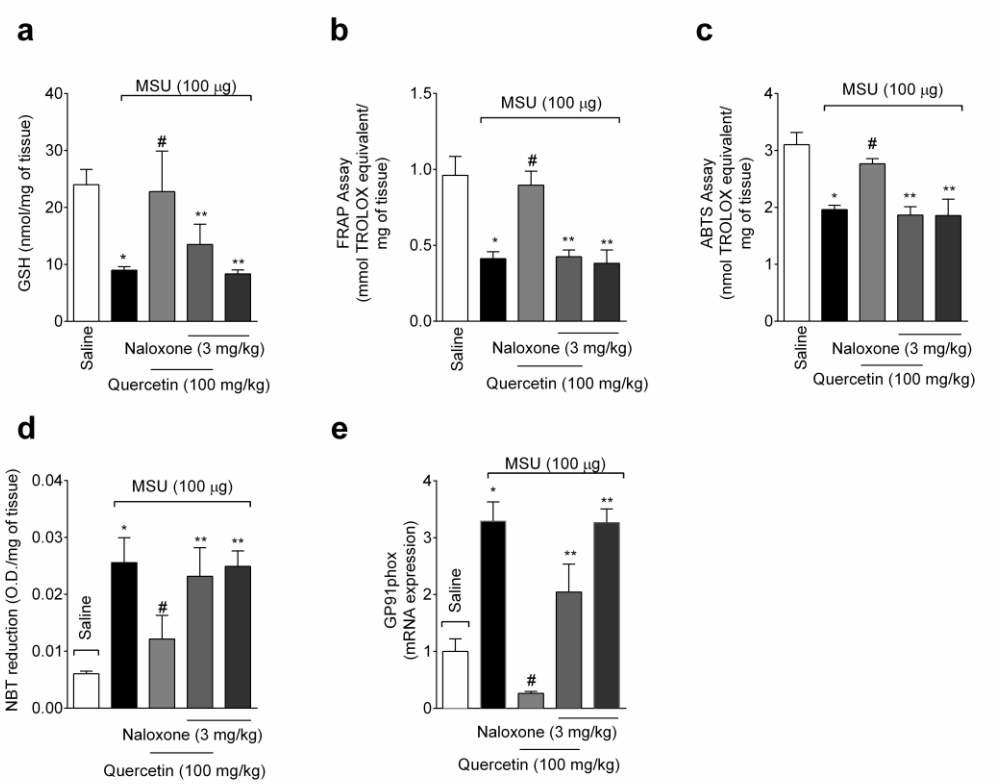

Fig. 8 Quercetin inhibited MSU-induced the decrease of antioxidant capacity, and superoxide production and gp91phox mRNA expression in an opioid-dependent manner. Treatment with naloxone (3 mg/kg/saline, i.p.) was performed 1h before quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 minutes, mice received MSU injection (100 μg/10μL). The GSH (a), FRAP (b), ABTS (c), NBT (d) and gp91phox mRNA expression (e) were evaluated 24 h after MSU injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group; #*P* < 0.05 compared to MSU group and ***P* < 0.05 compared with Quercetin group. ANOVA followed by Tukey's t test.

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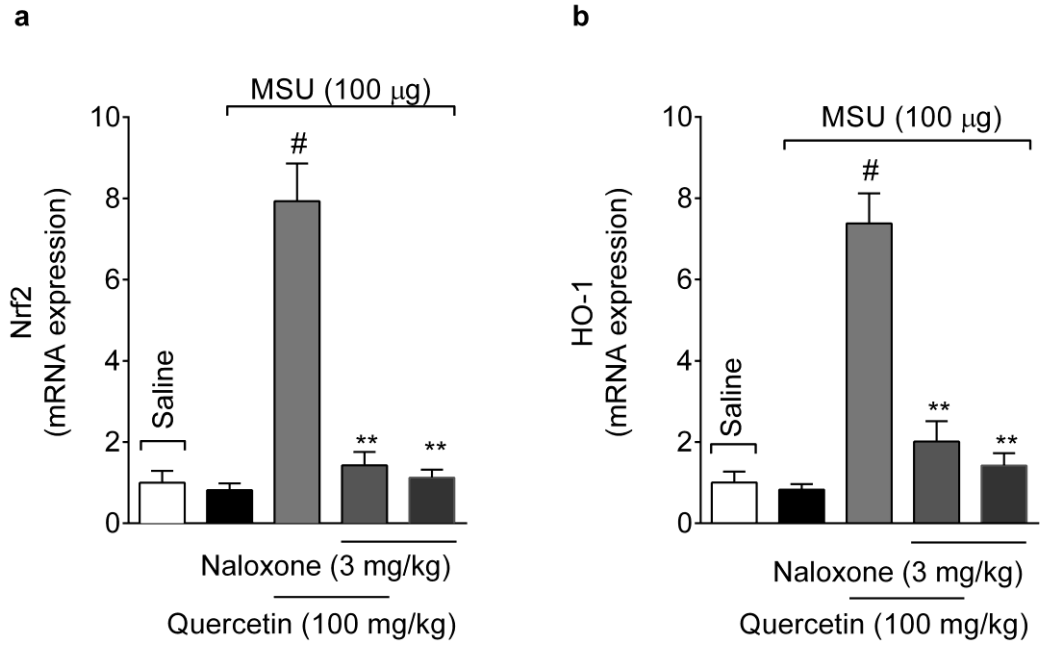


Fig. 9 Quercetin inhibited MSU-induced Nrf2 and HO-1 mRNA expression in a naloxone-sensitive manner. Treatment with naloxone (3 mg/kg/saline, i.p.) was performed 1h before Quercetin (100 mg/kg, s.c.) or vehicle (Tween 80 plus saline), and after additional 30 minutes, mice received MSU injection (100 μg/10μL). The Nrf2 (a) and HO-1 (b) mRNA expression were evaluated 24 h after MSU injection. Results are presented as means ± SEM of 6 mice per group per experiment, and are representative of 2 separated experiments. **P* < 0.05 compared to saline group, #*P* < 0.05 compared to MSU group, ***P* < 0.05 compared with Quercetin group. ANOVA followed by Tukey's t test.

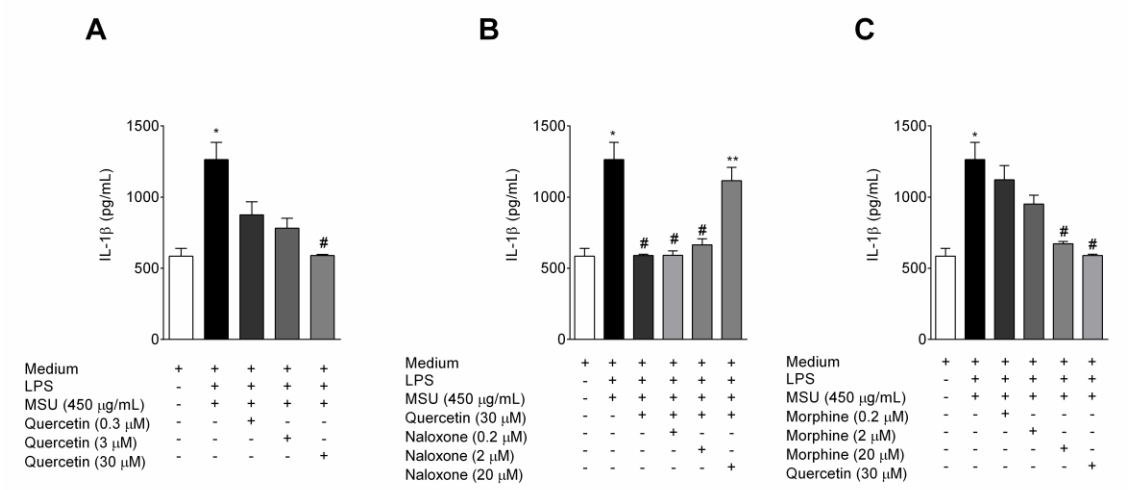


Fig. 10. **Quercetin inhibits MSU-induced IL-1β release by macrophages in a naloxone-sensitive manner.** LPS (500 ng/ml)-primed BMDM were treated with quercetin 30 minutes before stimulation with MSU crystals (450 μg/mL) during 5 hours. IL-1β concentration in the culture supernatant was measured by ELISA. (a) Concentration response of quercetin, (b) Concentration response of naloxone added 1 h before quercetin treatment and (c) Concentration response of morphine. Results are means ± SEM are representative of or more independent experiments. *P < 0.05 compared to saline group, #P < 0.05 compared to MSU group, **P < 0.05 compared with quercetin group. ANOVA followed by Tukey's t test.

4 CONCLUSÃO

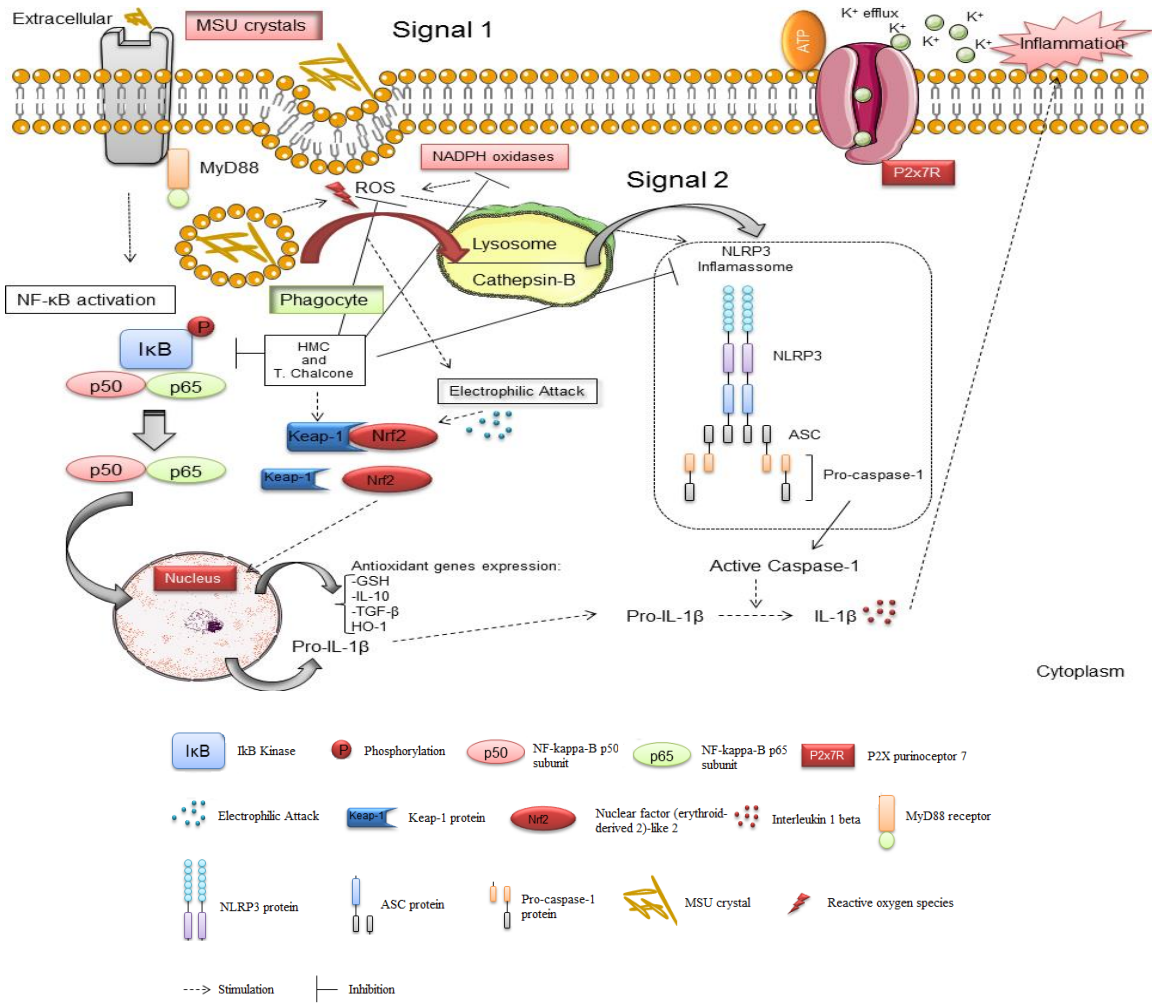
Os resultados apresentados pelo tratamento com os flavonoides se mostraram promissores para combater a artrite gotosa. O tratamento via oral com o precursor dos flavonoides Trans-Chalcona (TC) no modelo de artrite gotosa induzida por cristais de urato monossodico (MSU), foi capaz de inibir o perfil inflamatório induzido pelos cristais de MSU. A Trans-Chalcona promoveu uma analgesia inibindo a hiperalgesia mecânica assim como o edema na articulação. Além disso, reduziu o infiltrado leucocitário diminuindo o recrutamento celular e o processo inflamatório local (sinovite). Apresentou um perfil antioxidante melhorando a atividade e capacidade antioxidante da molécula sobre ERO induzuido pelos cristais de MSU. Atuou sobre a inibição de citocinas pro-inflamatórias, além de promover o aumento da citocina TGF-β. Atuou inibindo vias de sinalizações como NFκB e expressão de RNAm de moléculas do inflamassoma NLRP3 (Figura 6).

A Hesperidina Metil-Chalcona (HMC), foi capaz de promover um perfil analgésico reduzindo a hiperalgesia mecânica. Atuou inibindo o edema articular, além de reduzir o infiltrado leucocitário e o processo inflamatório (sinovite). Apresentou um perfil antioxidante no modelo *in vivo,* atuando sobre moléculas antioxidantes, além de aumentar a expressão de RNAm de vias antioxidantes. Atuou na inibição da produção de citocinas pro-inflamatórias, além de aumentar níveis da citocinas anti-inflamatória (TGF-β). Além disso, foi capaz de inibir a sinalizações de NFκB, além da expressão de RNAm de moléculas do inflamassoma NLRP3 (Figura 6). No modelo *in vitro*, HMC não reduziu os níveis de IL-1β avaliado no sobrenadante de macrófagos (BMDMs). Logo o seu efeito não é dependente da ativação do inflamassoma, mas sim, pela inibição da ativação de NFκB.

Todavia, a quercetina (QC) foi capaz de inibir de maneira dose dependente a hiperalgesia mecânica e a migração leucocitária. Quando tratados com o inibidor não seletivo de receptor opioide (naloxona), o efeito da quercetina foi revertido promovendo um aumento na hiperalgesia e no infiltrado leucocitário na articulação. Quando avaliado a sinovite articular, QC inibiu o infiltrado leucocitário. Além disso, a QC inibiu a produção de citocinas pro-inflamatórias, além de inibir a ativação de NFκB, sendo estes paramentros, revertidos pelo tratamento como o inibidor de receptores opioides. Esse flavonoide foi capaz de reduzir a expressão de RNAm de moléculas do inflamassoma NLRP3, assim como inibir o estresse oxidativo apresentando um perfil antioxidante aumentando a capacidade antioxidante, além de atuar sobre a expressão de RNAm de vias antioxidantes, entretanto esse perfis foram revertido pelo tratamento com o naloxona. No modelo *in vitro,* os macrófagos derivados da medula que foram estimulados com LPS (sinal 1) e MSU (sinal 2) promoveram um aumento nos níveis de IL-1β no sobrenadante de macrófagos (BMDMs). Quercetina na concentração de 30 µM inibiu a sua produção de maneira semelhante ao tratamento com morfina (20 µM), sendo os níveis de IL-1β revertido pela naloxona (20 µM). Assim, o efeito anti-inflamatório e antioxidante apresentado

pela quercetina provavelmente pode estar envolvido com a participação dos receptores opioide no modelo de artrite gotosa induzida por MSU.

Figura 6. Mecanismo *in vivo* dos Flavonoides Trans-Chalcona e Hesperidina Metil-Chalcona no modelo de artrite gotosa induzida por cristais de MSU. Fonte: autor



Desta forma, todas as moléculas avaliadas apresentaram um perfil anti-inflamatório, antioxidante e analgésico, atuando sobre vias de sinalizações e moléculas induzidas pelos cristais de urato monossódico (MSU) na articulação de camundongos swiss. Logo, esses favonoides demonstraram ser uma abordagem terapêutica promissora para o tratamento da artrite gotosa induzida por MSU.

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